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ORIGINAL PAPER

Inhibitory effects of free ammonia on Anammox bacteria

Cigdem Kalkan Aktan · Kozet Yapsakli · Bulent Mertoglu

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Abstract Anammox bacteria can effectively treat high ammonia and nitrite concentrations under anoxic environments. However, the presence of high ammonia and nitrite concentrations may cause free ammonia and nitrous acid inhibition at high pH and temperature environments. In this study, the inhibitory effect of free ammonia on Anammox bacteria was investigated in a lab-scale upflow fixed-bed reactor with Kaldnes biofilm carriers. Results of continuous operation showed that inhibition was not observed in the Anammox reactor when the free ammonia concentration gradually increased up to 150 mg/L. However, Anammox activity suddenly dropped to 10 % when the free ammonia concentration reached to 190 mg/L. Nevertheless, high influent ammonia and nitrite concentrations up to 1,500 and 500 mg/L, respectively, did not noticeably inhibit the Anammox activity. Gradually decreasing Anammox activity was also supported by fluorescent in situ hybridization (FISH) analysis. FISH and 16S rRNA gene analysis results revealed that main Anammox organisms were phylogenetically related to *Candidatus Kuenenia stuttgartiensis*, *Candidatus Jettenia asiatica* and *Candidatus Brocadia anammoxidans*.

Keywords Free ammonia · Inhibition · Kaldnes rings · Anammox · Molecular tools

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Introduction

The removal of ammonia in wastewater is an important part of advanced treatment and has a relatively high oxygen demand, which increases the wastewater treatment plant operational cost significantly. A new microbial process for nitrogen removal, first discovered in a denitrifying pilot plant reactor in Delft (Mulder et al. 1995), called anoxic ammonium oxidation (Anammox), involves oxidation of ammonium to dinitrogen gas, with nitrite as the electron acceptor under anoxic conditions (Jetten et al. 1998). The Anammox process would significantly decrease oxygen demand and sludge production rate without the addition of external organic matter, as compared to the conventional nitrification–denitrification process (van Dongen et al. 2001; Abma et al. 2007). However, the Anammox process needs special attention because of the requirement of satisfactory and stable partial nitritation as pretreatment before the Anammox and the long start-up period due to slow growth rates of

Anammox bacteria (Jetten and van de Vossenberg 2005; Egli et al. 2001; van Dongen et al. 2001).

Partial nitritation is generally required to obtain the appropriate ratio of nitrite/ammonium in the mixture to be used as an influent for the Anammox process. In this process, conversion of nitrite to nitrate can be prevented by controlling pH, dissolved oxygen, temperature, HCO_3^-/NH_4–N ratio, and hydraulic and sludge retention time (Hellinga et al. 1998; Mata-Alvarez et al. 2007; Liu and Liang 2008). However, in full-scale studies, it may be difficult to obtain stable nitritation efficiency because of variable influent nitrogen and organic matter loads and different environmental conditions (van Kempen et al. 2001; van der Star et al. 2007). Biodegradable organic compounds were found to affect the Anammox process performance, but the exact inhibitory levels still remain unclear (Angelidaki et al. 2009). On the other hand, the nitrite/ammonium ratio in the influent of the Anammox process must be controlled to prevent ammonia or nitrite accumulation which may cause free ammonia or free nitrous acid inhibition in the reactor. Nitrite inhibition is widely studied for the Anammox bacteria (Strous et al. 1999). However, there are only limited studies about the inhibition caused by free ammonia in literature. The fact that free ammonia rather than ammonium ion is inhibitory to bacteria is due to the ability of ammonia to easily penetrate through the cell membrane and to dissolve in lipid (Speece 1996; Gallert and Winter 1997). The uncharged and lipid soluble ammonia molecule causes proton imbalance and it ends up with a specific enzyme inhibition (Sprott and Patel 1986).

The inhibitory effects of free ammonia, as far as is known, influence mainly methanogenesis step for anaerobic treatment (Calli et al. 2005; Sung and Liu 2003) and nitritation (i.e. oxidation of ammonia to nitrite) or nitritation step for nitrification (i.e. oxidation of nitrite to nitrate) (Anthonisen et al. 1976; Villaverde et al. 2000; Kim et al. 2006; Bae and Park 2009). The effect of free ammonia on ammonia and nitrite oxidizing bacteria is well known. Nitrite oxidizers are known to be more sensitive to free ammonia in the range of 0.1–1.0 mg/L while ammonia oxidizers are inhibited in the range of 10–150 mg/L (Anthonisen et al. 1976; Villaverde et al. 2000; Kim et al. 2006; Bae and Park 2009). In addition, recent studies report the inhibition by free ammonia on the growth of some microalgae (Ergas et al. 2011) and the electricity generation in microbial fuel cells (MFC) (Kim et al. 2010). Anammox bacteria, like methanogens, are mesophilic and slowly growing bacteria, and are known to be easily influenced from environmental conditions. In addition to this, the optimum temperature for both microorganisms is mesophilic conditions; therefore, operation of units at higher temperatures makes them especially prone to free ammonia inhibition. Up to date only few studies focused on the free ammonia inhibition in the Anammox process, however, further studies in literature are needed to clarify to which extent the Anammox bacteria are resistant to free ammonia inhibition. The purpose of this study is, therefore, to evaluate the effect of free ammonia on Anammox bacteria and to understand how the culture responds to increasing free ammonia concentrations along the height of the reactor which is packed with Kaldnes rings. The molecular fingerprinting and long term variation of Anammox culture was monitored and evaluated by the use of fluorescence in situ hybridization (FISH) and phylogenetic analysis.

Materials and methods

Anammox seed sludge

The seed sludge for the lab-scale reactor was taken from an ongoing Anammox reactor which served as a biofilter system filled with zeolite particles. The reactor was inoculated with approximately 100 mL of Anammox culture containing ~200 mg/L mixed liquor suspended solids. The zeolite biofilter reactor previously used seed sludge taken from a leachate treatment plant in Essen/Germany and enriched for over 1 year.

Experimental set up

A plexiglass column was designed with an inner diameter of 90 mm, a height of 600 mm and a volume of 2.35 L with a water bath (Fig. 1). The column was filled with Type K1 Kaldnes rings as packing material for the Anammox bacteria. The nominal length and diameter of the rings were 7.2 and 9.1 mm, respectively. Kaldnes rings were supplied from AnoxKaldnes Company (Norway). The carriers are made of polyethylene (PEHD) with a density of 0.95 g/cm^3. Bacteria are protected from shear stress since the

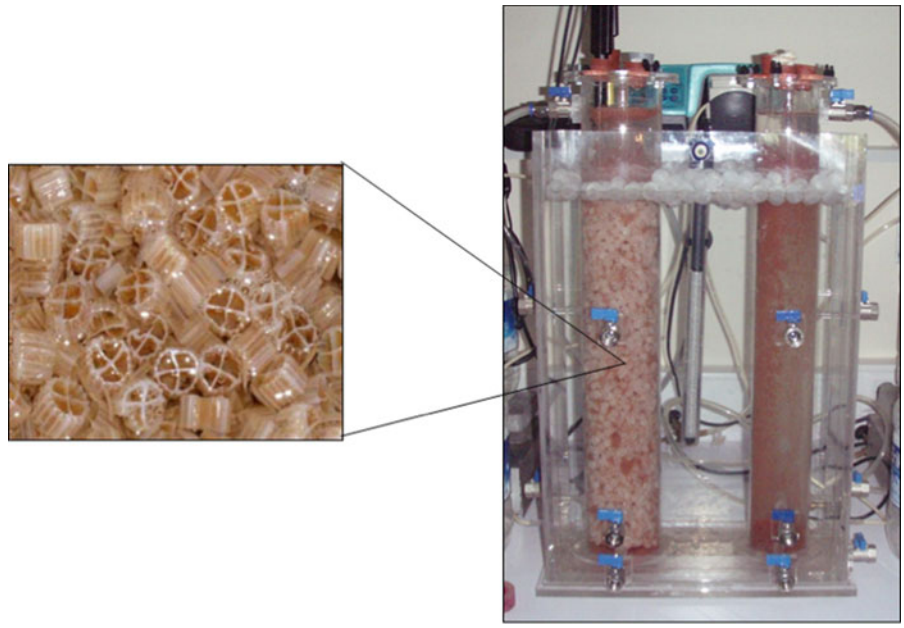

Fig. 1 Lab-scale continuous flow Anammox reactor setup (only left hand side reactor was used in the scope of the study)

biomass is mainly growing on the protective inside surface of the carriers. Although this material is frequently used for the moving bed biofilm reactors, the reactor was fully packed (100 % fill) with Kaldnes rings for the maximization of area for biomass growth.

The dissolved oxygen concentration of the feeding solution was reduced by continuous sparging with nitrogen gas. The biofilter system was operated at 34 °C using a water bath around the reactor in order to maintain a constant reactor temperature. Average flow rate of the system was 1,700 mL/day, and hydraulic retention time in the system was about 17 h. In addition to this, a mixture of 90 % N_2 + 10 % CO_2 gas was supplied from the bottom of the column to maintain the anoxic conditions in the reactor. The reactor was operated in the upflow mode and CO_2 present in the supplied gas was sufficient to maintain inorganic carbon requirement of Anammox bacteria. The dissolved oxygen concentration in the reactor was continuously measured and recorded by a HACH HQ 40d Multimeter, which is dropped to the solution from the top of the reactor. Dissolved oxygen concentration at any time of the reactor operation was below 0.1 mg/L. A synthetic medium was prepared as a feed solution, as described by Egli et al. (2001). In order to provide anoxic conditions and prevent decaying of Anammox sludge, nitrate was added to feed solution at a concentration of 25 mg/L. At the start up period ammonia and nitrite concentrations of feed solution was kept low, and then as the bacteria grow, the concentrations were increased gradually. Ammonium, nitrite and nitrate nitrogen concentrations were determined colorimetrically according to standard methods (APHA 2005).

Free ammonia inhibition assessment using port sampling analysis

The concentration of free ammonia nitrogen (NH_3–N) was calculated by using the following formula (Heinrichs et al. 1990):

$$NH_3 - N = \frac{\text{Total ammonia nitrogen}}{1 + 10^{(pK_a - pH)}} \quad (1)$$

$$pK_a = 0.09018 \frac{2729.92}{T + 278.15} \quad (2)$$

where pK_a is the dissociation constant for ammonium ion, and T is temperature in °C.

Port sampling experiments were daily carried out (starting from day 85) in order to examine the NH_4–N, NO_2–N and pH profiles and analyze the extent of inhibition that Anammox bacteria exposed along the height of the reactor. The ports were located in the

following order: Lower port is located at the bottom, middle port is located at 30 cm above the bottom and upper port is located at 60 cm above the bottom. The feeding solution entered the set-up at the same level as the bottom port however it is located on the other side of the reactor (Fig. 1).

DNA extraction and PCR amplification

Samples were taken from the bottom and middle ports of the reactor in order to monitor the microbial speciation at different operational periods of reactor on days 108, 184, 358 and 371. Nucleic acid extraction was performed using Bio 101 FastDNA® SPIN kit (Q-BIOgene) according to the manufacturer's instructions. Samples were agitated in a 2-mL screw-capped microcentrifuge tube using a FastPrep-24 bead-beating system (Bio 101) set at maximum speed for 45 s. The amount and purity of DNA was estimated using Quant-iT™ PicoGreen® dsDNA Reagent Kit (Molecular Probes) according to the manufacturer's protocol with a few modifications. Stock PicoGreen reagent was diluted 1/80 instead of 1/200 in TE buffer, and 10 μL of the prepared solution were mixed with 10 μL of extracted DNA in the LightCycler glass capillaries. Fluorescence was measured on the LightCycler Instrument (Roche, Mannheim, Germany). For calibration, a serial dilution of lambda DNA standard ranging from 50 to 2000 ng/mL was prepared with TE buffer. After DNA extraction, partial 16S rDNA fragments were amplified for cloning experiments with Anammox primers, Pla46f (GGA TTA GGC ATG CAA GTC) (Neef et al. 1998) and Amx667r (ACC AGA AGT TCC ACT CTC) (van Kempen et al. 2001; van der Star et al. 2007). A reverse primer Amx667R was used to amplify part of 16S rDNA genes of the Anammox cluster containing 621 base pairs. The PCR amplification reaction was performed in a Progene thermocycler (Techne, Cambridge, UK) and the following program was used: pre-denaturation (95 °C, 5 min), 35 cycles of denaturation (95 °C, 60 s), annealing (56 °C, 90 s), elongation (72 °C, 90 s) and post elongation (72 °C, 10 min). The reactions were subsequently cooled to 4 °C. Amplifications were performed in accordance with the manufacturer's recommendations by using 0.5 μM each primer, 1.5 U of *Taq* DNA polymerase (MBI Fermentas), 1 × PCR buffer, 1.5 mM $MgCl_2$, 1.25 mM of each dNTP. The template DNA was diluted 10 times to dilute possible PCR-inhibiting compounds and obtain suitable PCR amplicons.

FISH analysis

The sludge samples were fixed overnight in 4 % paraformaldehyde/phosphate-buffered saline (PBS) at 4 °C. Fixed cells were spotted on gelatin-coated multiwell glass slides and allowed to dry in a sterile hood. Hybridizations were performed at 46 °C for 2 h, according to (Mertoglu et al. 2005). After hybridization, unbound oligonucleotides were removed by rinsing with washing buffer containing the same components of the hybridization buffer except for the probes. For detection of all DNA, samples were additionally stained with DAPI. Vectashield (Vector Laboratories) was used to prevent photobleaching. The slides were examined with a Leica DM-LB fluorescent microscope, and digital images of the slides were captured with a Leica DC350F digital camera. Oligonucleotide main probes used for FISH analyses, the sequences and optimal formamide concentrations in the hybridization buffers are displayed in Table 1.

Table 1 Oligonucleotide probes used for the detection of Anammox organisms (Schmid et al. 2005)

Probe	Specificity	Sequence (5′–3′)	Formamide (%)
Planc 046	*Planctomycetales*	GACTTGCATGCCTAATCC	25
Amx368	All Anammox organisms	CCTTTCGGGCATTGCGAA	15
Amx820	*Ca. Brocadia anammoxidans*	AAAACCCCTCTACTTAGTGCCC	40
	Ca. Kuenenia stuttgartiensis		
BS820	*Ca. Scalindua wagneri*	TAATTCCCTCTACTTAGTGCCC	40
	Ca. Scalindua sorokinii		
Kst1275	*Ca. Kuenenia stuttgartiensis*	TCGGCTTTATAGGTTTCGCA	25

Cloning and sequencing analysis

The phylogenetic analysis of Anammox bacteria was started with the purification of PCR products of partial 16S rRNA genes using the QIAquick PCR purification kit (Qiagen) prior to cloning. Then, the purified amplicons were cloned in EZ competent cells using the QIAGEN PCR Cloning plus Kit (Qiagen) with ampicillin selection and blue/white screening following the manufacturer's protocol. White colonies were picked up from each cloned sample and reamplified. Before DNA sequencing, the plasmids of selected transformants were purified using the fermentas PCR purification kit. DNA sequences were analyzed in Iontek Laboratories (Istanbul, Turkey). Afterwards, with the derived partial 16S rRNA sequences from the clones, a similarity search was performed in the GenBank database using the BLAST search program of the National Center for Biotechnology Information sequence search service (http://www.ncbi.nlm.nih.gov). Sequences were analyzed using Chromas-Pro software (Technelysium Pty Ltd., Eden Prairie, MN) and aligned by the multiple alignment Clustal W2 program. Phylogenetic trees were constructed with the neighbor-joining method using molecular evolutionary genetics analysis package (MEGA version 2.1). The robustness of the phylogeny was tested by bootstrap analysis with 1,000 iterations.

Results and discussion

Column operation

The continuous-flow system was operated for ~375 days under gradually increased ammonia loadings in order to test the effect of free ammonia on the reactor performance. In the first period, from days 0 to 115, both ammonia and nitrite concentrations were increased to obtain an enriched Anammox culture. Inoculation of an active culture enabled high removal efficiencies to be observed immediately after start. Hence, feed concentrations which are prepared according to 1:1 ratio (NH_4–N/NO_2–N) were gradually increased from 100 to 500 mg/L. Over 90 % of the inlet nitrogen load (ammonia and nitrite) into the Anammox reactor was removed, and the sludge production was negligible. After this period, the feed NH_4–N/NO_2–N ratio was increased in a stepwise manner in order to maintain higher free ammonia concentrations within the reactor. The operating conditions are summarized in Table 2.

The stoichiometric nitrite-N to ammonium-N ratio of 1.32:1 is generally accepted by researchers (Strous et al. 1998). In our study, the average removal ratio was found to be 1.11:1 (±0.02 % according to 95 % confidence interval) which shows a variation from the reported value. In the literature, this ratio varies between 1.04 (Bettazzi et al. 2010) and 1.50 (Strous et al. 1997). Kartal et al. (2007) reported that this stoichiometric ratio also can change in different stressed conditions. The nitrite to ammonium nitrogen consumption ratio in our case lies within the interval proposed earlier by researchers. This ratio did not change when the reactor was operated at increasing influent ammonia/nitrite ratios. However, a higher ammonia concentration was observed in the effluent when the reactor was exposed to higher NH_4–N/NO_2–N ratios (i.e. going from a ratio of 1.00 to 3.56).

Since ammonia is fed in excess, Anammox activity should be evaluated using the nitrite removal efficiency. Figure 2 depicts influent nitrite nitrogen concentrations and corresponding removal efficiencies during 375 days of reactor operation. At all NH_4–N loadings, 95 % (±0.28 % according to 95 % confidence interval) of the influent nitrite was removed except when inhibition was observed during the last 21 days of operation. The results showed that Anammox bacteria can effectively treat high ammonia and nitrite concentrations under an anoxic environment without any inhibition. The high influent ammonia concentration (up to 1,500 mg/L) did not affect the nitrite removal efficiency; however, a sharp decrease in the nitrite removal was experienced above this load. High ammonia concentrations within the reactor may cause free ammonia inhibition at high pH and temperature environments. Because of the high temperature necessity for proper nitrogen removal, Anammox bacteria may be exposed to high free ammonia concentrations. This will be discussed in more detail in "Free ammonia levels" section. Free ammonia inhibition is governed by the concentration of free ammonia within the reactor rather than the free ammonia in the influent stream. In completely stirred tank reactors substrate concentration in tank is uniform and equal to effluent concentration. This means that a single mixed liquor sample obtained from homogenous solution can represent the free ammonia concentration within the reactor. On the other

Table 2 Influent ammonia and nitrite concentrations

Application period, day	Influent ammonia concentration, mg/L	Influent nitrite concentration, mg/L	Influent NH_4–N/NO_2–N ratio	Estimated free ammonia concentration in the effluent[a]
0–7	99.8 (±6.72)[b]	100.5 (±3.77)	0.99	0.9
8–33	195.4 (±45.6)	205.3 (±45.0)	0.95	1
34–50	303.1 (±47.3)	313.1 (±47.8)	0.97	2
51–79	395.3 (±68.3)	399.2 (±57.5)	0.99	3
80–117	508.7 (±68.4)	488.3 (±56.0)	1.04	7
118–140	555.5 (±10.1)	446.8 (±10.6)	1.24	14
141–154	606.8 (±19.3)	449.1 (±12.3)	1.35	19
155–166	661.4 (±14.4)	441.7 (±31.8)	1.50	25
167–184	703.8 (±20.8)	447.6 (±28.3)	1.57	28
185–201	747.2 (±17.3)	447.9 (±29.5)	1.67	32
202–217	808.8 (±16.5)	453.3 (±13.7)	1.78	38
218–247	846.2 (±19.9)	457.6 (±20.8)	1.85	41
248–257	902.0 (±11.0)	446.3 (±19.8)	2.02	47
258–271	988.1 (±17.5)	450.8 (±5.75)	2.19	55
272–285	1121 (±33.3)	452.6 (±25.2)	2.48	67
286–307	1204 (±26.1)	456.3 (±14.2)	2.64	75
308–323	1297.3 (±18.6)	450.0 (±7.54)	2.88	84
324–338	1404.5 (±19.8)	442.4 (±10.7)	3.17	95
339–359	1516.0 (±36.5)	456.9 (±31.0)	3.32	104
360–375	1589.6 (±34.3)	456.6 (±15.9)	3.48	111

[a] Expected values for a temperature of 34 °C and pH 8. Free ammonia concentrations were estimated using a NO_2–N/NH_4–N consumption ratio of 1.11

[b] Standard deviation

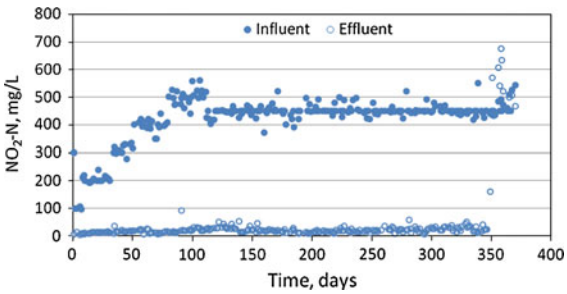

Fig. 2 Influent and effluent nitrite concentrations along the operational period

hand, in systems where a substrate gradient along the length of the reactor exists, it is necessary to conduct port sampling experiments in order to correctly evaluate the inhibition that the bacteria are actually exposed.

Ammonia, nitrite and pH profiles along the height of columns

The port sampling experiments carried out daily enabled examining the gradient profile within the reactor. In the lab-scale reactor packed with Kaldnes rings, a concentration gradient in terms of NH_4–N and NO_2–N from bottom to top existed along the length (Fig. 3). Nitrite removal is coupled to ammonia removal at a stoichiometric ratio of 1.11:1 (data not shown). The results showed that during the first 180 days of column operation, an immediate ammonia and nitrite removal took place as soon as the influent wastewater entered the column. Nearly complete nitrite removal was observed in the lower port as a result of both biodegradation and dilution effect. As the ammonia concentration in feed increased in a stepwise manner, untreated ammonia concentration probably led to increase in free ammonia levels within the reactor which in turn decreased the nitrite removal efficiency at the lower port at later days of operation. There was a shift in the active biodegradation zone due to the increase in free ammonia levels. However, the overall nitrite removal efficiency of the system was still high despite high free ammonia concentrations at lower regions. At later days of operation, upper parts were mainly responsible for biodegradation. Figure 3

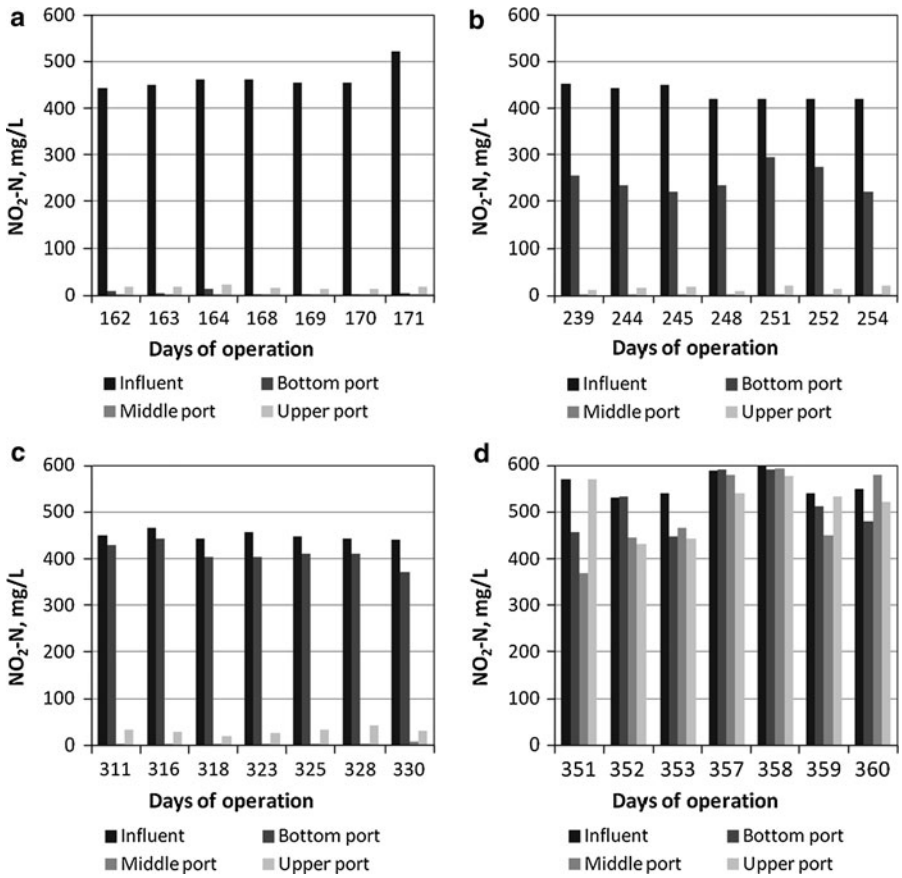

Fig. 3 Nitrite profiles at various time intervals of column operation **a** between 0 and 171 days; **b** between 239 and 259 days; **c** between 311 and 330 days; **d** between 351 and 361 days

clearly shows the progress in the increase in nitrite concentration at different sampling points. At the last 21 days of operation, the active zone disappeared which resulted in the deterioration in the removal efficiency. Evaluation of the nitrite concentration data at different points led to the conclusion that the local inhibition that a specific zone is experiencing may not represent the inhibition of the whole system. This is especially true in plug flow-like systems where there is a gradient of substrate.

pH had a different trend along the height (Fig. 4). Despite the variations of pH in the feed due to varying concentrations ammonia, a slight decrease in pH upon entrance to the reactor was observed. A further pH decrease in the middle port was attributed to the dissolution of CO_2 gas along the height. However, much higher pH values were recorded near the air–liquid interphase at the upper port. The reason for this observation was probably due to stripping of acidic carbondioxide from the surface and thus acting to raise the pH level. A significant pH increase in the effluents of Anammox reactor were also reported by Tang et al. (2009) and Liu et al. (2008). The increase in pH was attributed to H^+ consumption according to Anammox reaction stoichiometry. The same mechanism may play a role in our case; however, it may not be the dominant mechanism due to the fact that pH values in the effluent continued to increase during the last days of column operation even when the removal efficiencies were very low or column was experiencing complete inhibition.

Free ammonia levels

A higher ammonia supply than in necessary stoichiometric proportions resulted in a buildup of ammonia within the reactor which turn out to increase the free ammonia levels (Fig. 5). Figure 5 indicates that free ammonia at upper port is always higher than that in lower and middle ports despite lower ammonia concentrations. This is due to higher pH values measured at the air–water interphase (Fig. 4). High

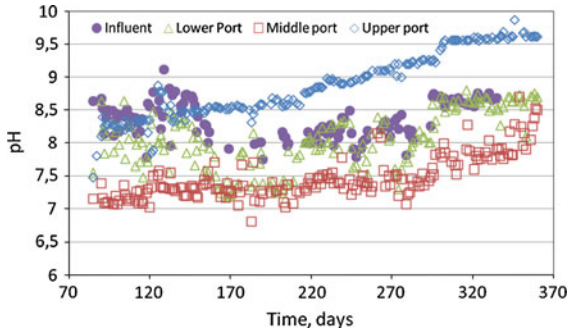

Fig. 4 pH profiles through the height of the reactor during the experimental study

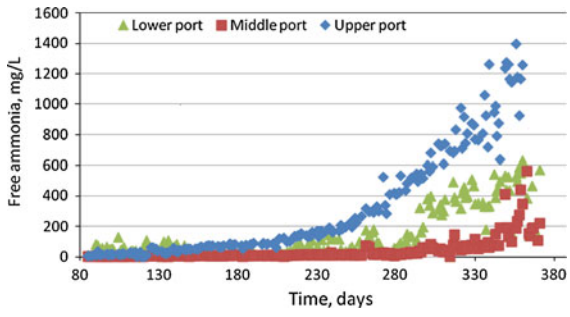

Fig. 5 Free ammonia levels through the height of the reactor during the experimental study

pH values up to 10 were observed at the upper port when ammonia concentrations reached 1,500 mg/L in the influent. Examination of Eq. 1 indicated that free ammonia concentration was much more dependent on pH and temperature than ammonia concentration. Free ammonia concentrations in the upper port ranged between 3 and 1,400 mg/L throughout the experimental study. However, it is considered that a free ammonia level as high as 1,400 mg/L did not cause an important deterioration on the overall reactor performance, since this region represents the situation at the exit of reactor.

pH profile at the lower port resembled to that in feed solution and free ammonia levels were generally less than 10 mg/L until 170th day of reactor operation. These free ammonia levels were coupled to very high nitrite removal efficiencies in the lower port. Continuous increase in free ammonia up to 70 mg/L until day 250 ended up with a nitrite removal efficiency of 47 %. There was a rapid increase in free ammonia levels between days 250 and 330 and nitrite removal efficiencies were correspondingly low (15 % ± 2.0 according to 95 % confidence interval). Average free ammonia levels in this period were 220 mg/L (±8.1 according to 95 % confidence interval) in the lower port. From this point on, nitrite removal is not observed and free ammonia levels increased up to 600 mg/L levels. The free ammonia profile in the middle port was quite different than in lower port. During the first 345 days of reactor operation, nitrite concentrations in the middle port was always less than 5 mg/L despite continuous increase in free ammonia. Anammox reaction was not adversely affected by free ammonia concentration up to 150 mg/L. The inhibitory effect of free ammonia was observed after 345 days when the free ammonia concentration increased to 190 mg/L which caused the nitrite removal efficiencies to suddenly drop to 10 %. After this period, a further increase in free ammonia concentrations up to 500 mg/L resulted in complete loss of efficiency with no recovery.

Comparison of results leads to the fact that the inhibition levels were variable at different ports of the reactor. The lower port was observed not to withstand as high free ammonia concentrations as the middle port. There may be several plausible explanations that might account for this difference. Firstly, acclimation may be an important factor in the sensitivity to free ammonia inhibition. The bacterial culture in the middle port may get accustomed to high free ammonia. The level of inhibition was evaluated on the basis of nitrite removal efficiency and hence, high nitrite concentration in the reactor was attributed to free ammonia inhibition. However, high nitrite levels at this port may not be due to free ammonia inhibition alone but may also be due to insufficient detention time necessary for removal. Water sample taken from the lower port is actually at the same level as the influent pipe. High free ammonia levels observed at lower port probably decreased the substrate utilization rate which in turn caused insufficient detention time to the bacteria to process substrate at high loads. The middle port, on the other hand, accommodates enough retention with lower free ammonia levels. The Pearson's product momentum correlation coefficient (rp) for the relationship was used for linear estimation of the strength and direction of the correlation. High free ammonia levels hindered nitrite removal efficiencies. The overall Pearson coefficient determined at 95 % confidence interval (0.84) indicated a moderate correlation between the free ammonia levels and nitrite

removal efficiency in the middle port. This relation was slightly weaker for the case of lower port (0.79) which means that both free ammonia and detention time played a role in the effluent nitrite concentrations.

There are a few studies focused on free ammonia inhibition in Anammox bacteria. Fernandez et al. (2008) observed a total loss of Anammox efficiency when free ammonia concentration reached values between 35 and 40 mg/L in a continuous lab-scale SBR reactor inoculated with biofilm biomass. The authors reported that free ammonia concentrations up to 20 mg/L had no effect on the process stability. Waki et al. (2007) presumed that free ammonia concentrations of 13–90 mg/L could adversely affect the performance of the Anammox process. Tang et al. (2009) resulted that effluent ammonia and nitrite nitrogen concentrations significantly increased when free ammonia concentration reached 64–73 mg/L. Jung et al. (2007) investigated the effects of dissolved oxygen, free ammonia, and nitrite concentrations on Anammox activity during the startup period. It was concluded that free ammonia below 2 mg/L and nitrite nitrogen below 35 mg/L did not inhibit cultivated Anammox cells in the continuous bioreactor. Yang et al. (2011) indicated that there also existed a minimum threshold concentration of free ammonia (2.56 mg/L) below which an inhibition was observed. Our results are not similar to previously reported values; a much higher free ammonia concentration (>150 mg/L) disturbed the stability of Anammox process. Stepwise increment of free ammonia, the reactor type or the active Anammox bacteria responsible for treatment may play a role in this observation. Therefore, it is important to determine the active species which are tolerant to high free ammonia concentrations. FISH and cloning experiments were performed for this purpose.

Molecular studies

As a result of the FISH experiment, it was found that currently known Anammox genera *Candidatus Brocadia,* Kuenenia and *Scalindua* organisms were present and active in the reactor. High-density clusters were detected with Amx820 oligonucleotide probe labeled with Cy3 on days 108 and 184 (Fig. 6). When the free ammonia concentration was increased to 190 mg/L on day 358, Anammox bacteria were rarely detected in the granules. It seems that the inhibition by free ammonia selectively suppressed the growth of Anammox bacteria and partially washed them out of the reactor. On day 371, Anammox bacteria were not detected by FISH due to the inhibitory effect of free ammonia. In literature, sensitive and robust Anammox

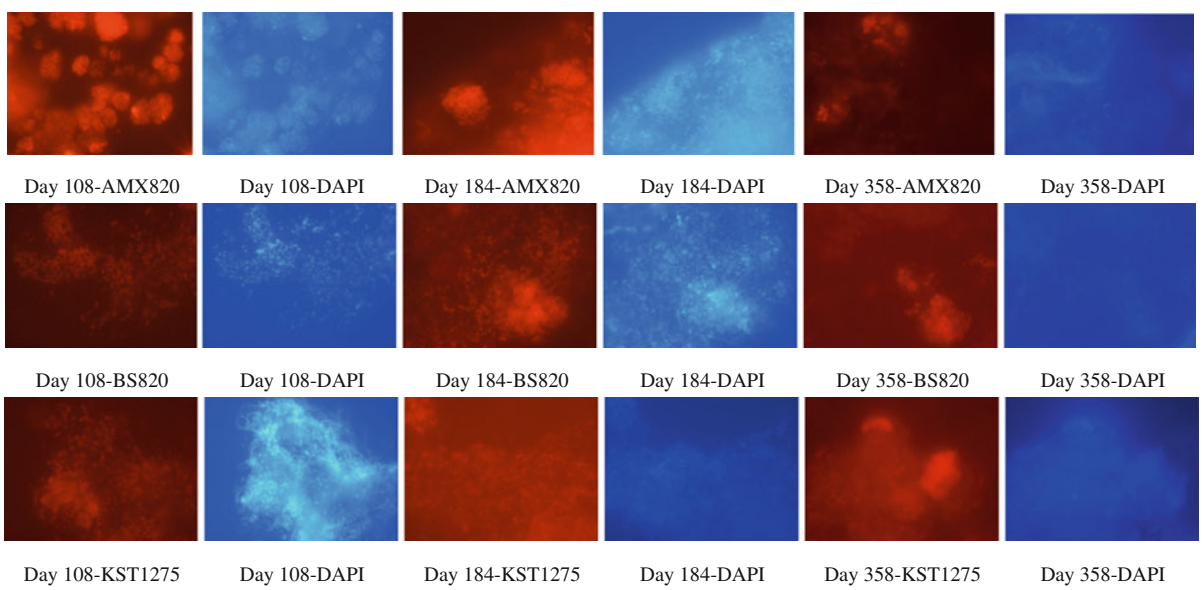

Fig. 6 In situ identification of Anammox bacteria. All microorganisms stained with DAPI (*blue*). Sludge samples hybridized with Amx820, BS820 and Kst1275 oligonucleotide probes (labeled with Cy3-*red*). DAPI and Cy3 couples represent the same fields of the microscopic view

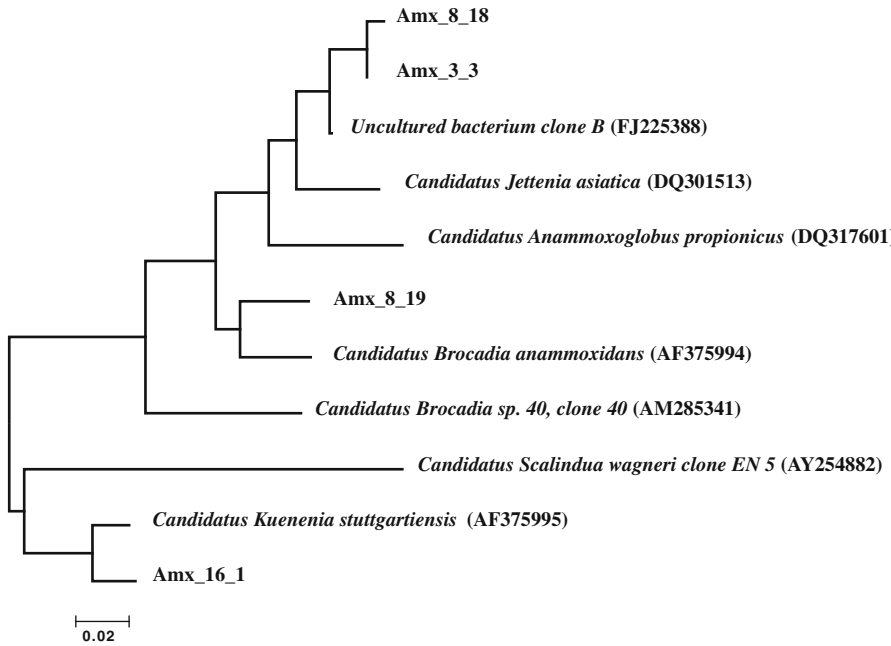

Fig. 7 Neighbor-joining phylogenetic tree of Anammox sequences generated from 16S rDNA bacterial gene. The significance of each branch is indicated by bootstrap values (1,000 replicates). The *scale bar* represents 0.02 inferred substitutions/nucleotide position

species under high free ammonia concentrations were not investigated in detail. Gaul et al. (2005) suggested that *Brocadia* cells were more susceptible to nitrite inhibition, and therefore were not dominant in reactor at high nitrite levels. van der Star et al. (2008) hypothesized that *Kuenenia* is an affinity (*K*) strategist, and *Brocadia* is a growth rate (*r*) strategist. Similar to these study, Tokutomi et al. (2011) operated Anammox reactor with excess nitrite concentration and concluded that *Kuenenia* cells have higher affinity for substrates. In our study, at higher ammonia and nitrite concentrations both *Kuenenia* and *Brocadia* species were present in the reactor. When the Anammox bacteria exposed to high free ammonia concentration after day 358, whole species disappeared simultaneously. In addition to FISH assays, Anammox diversity was investigated by cloning and DNA sequencing analysis on days 108, 184 and 358. For each sample, twenty clones were picked up and screened by restriction analysis before being DNA sequenced. A phylogenetic tree was constructed from the genetic distances determined by the neighbor-joining method (Fig. 7). The results revealed that *Kuenenia stuttgartiensis* genome fragment KUST_E which is previously identified from the microbial community of a laboratory bioreactor (Strous et al. 2006) was the most frequently observed species in all samples (53 of the 60 clones). This sequence is related to *Candidatus Kuenenia stuttgartiensis* and showed greater than 99 % similarity. Five clones were related to uncultured bacterium clone B (100 %). This phylotype was closely related to *Candidatus Jettenia asiatica*. Two clones were identified as *Candidatus Brocadia anammoxidans* and showed 96 % similarity.

Conclusions

Ammonia inhibition in Anammox systems should be associated with unionized free ammonia concentration rather than total ammonia concentration. The presence of a concentration gradient in upflow reactor systems led to different free ammonia exposure levels along the height of the reactor. The results of our study showed that the maximum free ammonia level that the Anammox bacteria can tolerate was 150 mg/L at the middle port of reactor upon acclimation. Future studies are needed to dampen the effects of free ammonia in Anammox reactor by the testing cation antagonism effects.

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