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ORIGINAL PAPER

Accelerating effect of hydroxylamine and hydrazine on nitrogen removal rate in moving bed biofilm reactor

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Abstract In biological nitrogen removal, application of the autotrophic anammox process is gaining ground worldwide. Although this field has been widely researched in last years, some aspects as the accelerating effect of putative intermediates (mainly N_2H_4 and NH_2OH) need more specific investigation. In the current study, experiments in a moving bed biofilm reactor (MBBR) and batch tests were performed to evaluate the optimum concentrations of anammox process intermediates that accelerate the autotrophic nitrogen removal and mitigate a decrease in the anammox bacteria activity using anammox (anaerobic ammonium oxidation) biomass enriched on ring-shaped biofilm carriers. Anammox biomass was previously grown on blank biofilm carriers for 450 days at moderate temperature 26.0 (±0.5) °C by using sludge reject water as seeding material. FISH analysis revealed that anammox microorganisms were located in clusters in the biofilm. With addition of 1.27 and 1.31 mg N L^{-1} of each NH_2OH and N_2H_4, respectively, into the MBBR total nitrogen (TN) removal efficiency was rapidly restored after inhibitions by NO_2^-. Various combinations of N_2H_4, NH_2OH, NH_4^+, and NO_2^- were used as batch substrates. The highest total nitrogen (TN) removal rate with the optimum N_2H_4 concentration (4.38 mg N L^{-1}) present in these batches was 5.43 mg N g^{-1} TSS h^{-1}, whereas equimolar concentrations of N_2H_4 and NH_2OH added together showed lower TN removal rates. Intermediates could be applied in practice to contribute to the recovery of inhibition-damaged wastewater treatment facilities using anammox technology.

Keywords Anammox · Biofilm · Reject water · Moving bed biofilm reactor · Biofilm carriers

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Introduction

Nitrogenous wastewaters with a low biodegradable C to N ratio (COD/TN ratio ≤3) are generated in several domestic and industrial waste management processes (Vlaeminck et al. 2012). Domestic examples include sewage sludge reject water (Jeanningros et al. 2010), urine (Udert et al. 2008), black water digestate (Vlaeminck et al. 2009), and pre-treated sewage (Verstraete and Vlaeminck 2011) and industrial examples include various chemical and food processing industries (Desloover et al. 2011) (pretreated) manure (Bernet and Beline 2009) and landfilling (López et al. 2008). These streams have high treatment

costs when conventional nitrification–denitrification methods are applied. Novel short-cut biological nitrogen removal methods, such as combined partial nitritation-anoxic ammonium oxidation (anammox), save about 60 % of the aeration, 90 % of the sludge handling and transport, and 100 % of the organic carbon addition (Vlaeminck et al. 2012). Overall costs can therefore be cut down by 30–40 % when applying partial nitritation-anammox compared to conventional nitrification–denitrification on wastewaters with a low C to N ratio (Fux and Siegrist 2004).

In practice, the autotrophic nitrogen removal can be performed either in one or two stages, in the latter case nitritation phase and anammox phase being spatially separated (Vlaeminck et al. 2012). This study focussed on a separate anammox stage using a MBBR as this particular bioreactor type enables easy biomass retention and has low construction costs when applied in a full-scale wastewater treatment facility Ødegaard 2006). The anammox process is sensitive to several inhibiting factors (including NO_2^-, dissolved oxygen, and free ammonia). NH_4^+ concentrations of about 1,000 mg N L^{-1} and NO_2^- concentrations of only 100 mg N L^{-1} were inhibitory to the anammox bacteria (Strous et al. 1999). HCO_3^- concentrations exceeding 1,500 mg L^{-1} have been reported to be inhibiting to the anammox bacteria as well, whereas the mechanism of inhibition is unclear and possibly independent from pH (Dexiang et al. 2008). Literature provides conflicting evidences about nitrite inhibition and post-inhibition recovery times (Lotti et al. 2012; Wett 2007). Overcoming NO_2^- inhibition depends on biomass maturation as well as duration of nitrite exposure time (Lotti et al. 2012). Mature biomass could short-termly tolerate nitrite concentrations around 400 mg NO_2^-–N (Lotti et al. 2012; Fernández et al. 2012). Acceleration of post-inhibition recovery of the anammox process is a matter of great interest.

Optimal concentrations of intermediate metabolites of the anammox process (hydrazine (N_2H_4), hydroxylamine (NH_2OH), and nitric oxide (NO)) could promote TN removal (Bettazzi et al. 2010; Strous et al. 1999; Hu et al. 2011) and facilitate recovery from inhibition within reasonably short time. Several models describing the metabolic pathways involving putative intermediates have been proposed in the literature (Kuenen and Jetten 2001; Strous et al. 2006; Van der Star et al. 2008; Hu et al. 2011). These metabolic pathways are summarized on Fig. 1.

In most of the studies NH_2OH has provoked N_2H_4 production (Hu et al. 2011; Egli et al. 2001; van der Star et al. 2008). However, some reports bring forth that in the production of N_2H_4 either NH_2OH or nitric oxide (NO) or both of them could be anammox intermediates (Strous et al. 2006). Addition of trace amounts of NH_2OH or N_2H_4 separately promoted recovery from the NO_2^- inhibition in a SBR system (Strous et al. 1999). However, according to Bettazzi et al. (2010) adding combinations of N_2H_4 and NH_2OH together could be even more beneficial for recovering from NO_2^- inhibition and for achievement of the maximum NO_2^- removal rate of the anammox process. According to Hu et al. (2011), in experiments performed with anammox granules, raising the ratio of NH_2OH to NO_2^- from 1:1 to 2:1 increased the NO_2^- utilization rate.

To our knowledge there are few reports (except Schalk et al. 1998) on the effects of intermediates on the anammox microorganisms enriched on biofilm carriers, especially on MBBR configuration. In our study the anammox biofilm process was studied in detail with broader range of quantities of intermediates added. The aims of this study were to evaluate the role of the effects of various concentrations of NH_2OH and N_2H_4 on the acceleration of the total nitrogen (TN) removal rate and to find out the optimum concentrations of intermediates, which bring along the highest TN removal rate. Overcoming of inhibition caused by NO_2^- was studied by addition of optimum amounts of intermediates.

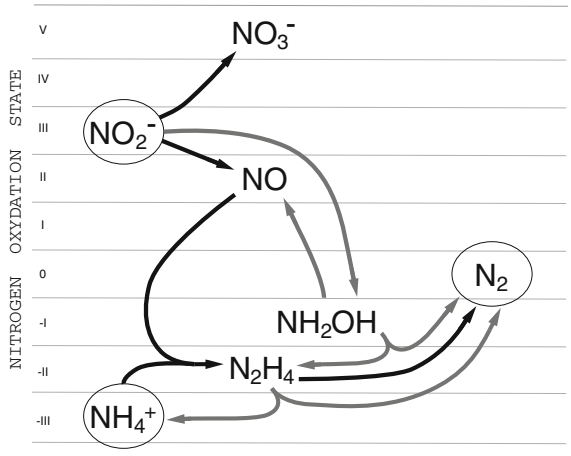

Fig. 1 A compendious scheme describing the metabolism of anammox intermediates. *Black arrows*, the main anammox pathway described by Kartal et al. (2010). Alternative pathways, describing the involvement of NH_2OH are depicted in *gray* (Kuenen and Jetten 2001; Strous et al. 2006; Van der Star et al. 2008 and Hu et al. 2011)

Materials and methods

Anammox enrichment in moving bed biofilm reactor (MBBR)

A plexiglass reactor with a 20 L liquid volume, equipped with a water jacket was used for the enrichment of anammox microorganisms at a constant temperature (26.0 ± 0.5 °C) maintained by an Assistant 3180 (Assistant, Germany) water bath thermostat. Anammox biofilm was allowed to develop onto the surface of blank carriers made of polyethylene (Bioflow 9, Aquamyc (RVT Process Equipment GmbH) Germany) by creating continuous flow-through conditions of diluted anaerobic tank reject water (NH_4^+ source) taken from the Tallinn wastewater treatment plant (WWTP) and the synthetically added NO_2^-. The anammox process had been carried out for 450 days (efficient anammox process evolved in about 4 months) before the biomass for batch experiments were taken.

Batch assays

A set of batch assays were conducted to study the effect of various combinations of N_2H_4 and NH_2OH concentrations on the anammox TN removal rate. An Assistant 3180 (Assistant, Germany) water bath thermostat maintained the temperature at 25 (±0.5 °C) (Fig. 2).

Duplicate/triplicate batch tests were performed using 200 biofilm carriers containing a mature anammox biofilm, with the concentration of the total suspended solids (TSS) of the biofilm 2,212 mg L^{-1}. Before using in experiments, the biocarriers were washed with tap water for 4–5 times. As sources for nitrogen and inorganic carbon, NH_4Cl, $NaNO_2$, and $NaHCO_3$ were dissolved in demineralised water. 3 mL of an acidic solution of microelements in addition to 3 mL of an alkaline solution of microelements were dispensed into the substrate of batch experiments along with 40 mL solution of macroelements made according to Zhang et al. (2009). NH_2OH and N_2H_4 were added in the form of hydroxylamine hydrochloride ($NH_2OH \times HCl$) and hydrazine sulfate ($N_2H_4 \times H_2SO_4$). Stock solutions TN concentration of around 45–85 mg N L^{-1} was prepared for the tests. Negative control measurements with no biomass added into the substrate were also performed.

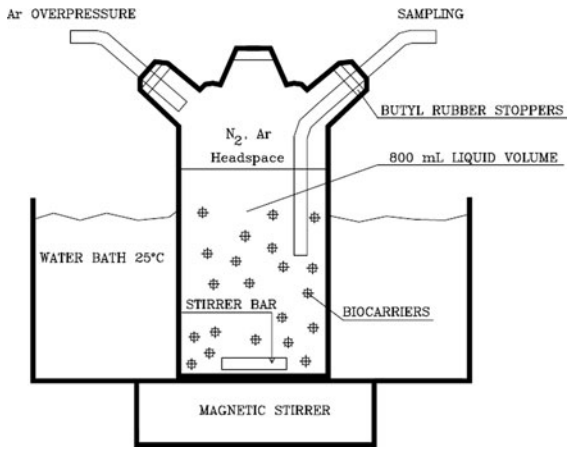

Fig. 2 Scheme of 800 mL liquid volume batch reactor

Before the start of the reaction, the liquid phase of the batch reactors was flushed with N_2 or Ar for about 15 min to eliminate oxygen from the liquid and gas phase, also favouring biomass acclimatization with the substrate. Then the batch reactors were sealed with butyl rubber stoppers. Sampling was performed with the aid of overpressure of N_2 or Ar created at one end of the three-necked reactor. The batch reactor was stirred by a magnetic bar at around 200 rpm. The pH value was maintained consistently at 8.0–8.5 by a HCO_3^- buffer system formed. Concentrations of nitrogen species were monitored every 2 h.

Linear regressions of substrates concentrations changes in time were derived in order to determine the conversion rates of TN and other substrates. The conversion rate per test was determined as the maximum rate, excluding the value obtained when substrate was depleted. The linear correlation coefficients (R^2) were higher than 0.9 in all cases. To calculate the biomass-specific conversion rate (mg N g^{-1} TSS h^{-1}), the maximum volumetric conversion rates were divided by added biomass concentration of 2.212 g TSS L^{-1}. Data and statistical analyses were performed by the MS Excel 2010 Analysis ToolPak. Homogeneity of group variances and the difference between group means were checked using the *F*-test and the two-way *t*- test, respectively. The level of significance was set at $\alpha < 0.05$.

Analytical methods

NH_4^+–N, NO_2^-–N, NO_3^-–N, and HCO_3^- were analyzed according to Greenberg et al. (1992). TSS

concentrations of the biofilm were measured as differences in the weights of biocarriers with and without the biomass (removed by the chromic acid solution). DO was measured by a portable oxygen meter (Marvet Junior MJ2000, Estonia) and pH by a portable pH meter (Evikon, Estonia). N_2H_4 concentration (measuring range 0–0.26 mg N L^{-1}) was detected by the Dr. Lange method (based on a method described by (Watt and Chrisp 1952)) at 458 nm by using Hydraver 2 reagent, and NO_2^- interference was eliminated by 0.5 % sulfamic acid (George et al. 2008). NH_2OH concentrations were measured by a spectrophotometer Lange DR 200 at 705 nm according to Frear and Burrell (1955).

Amplification of polymerase chain reaction (PCR) and denaturing gradient gel electrophoresis (DGGE)

Detailed information about microbial characterization is discussed in (Zekker et al. 2012). Anammox microorganisms were determined via PCR with a wide-range primer set Eub27f/Eub1492r (Lane 1991) in the first PCR round, and in the second PCR round by a *Planctomycetes*-specific primer Pla46f (Neef et al. 1998) coupled with an anammox-specific primer Amx368r (Sąnchez-Melsió et al. 2009). Nitrite oxidizing bacteria were determined with the *Nitrospira* specific primer set NSR1113f/NSR1264r (Dionisi et al. 2002).

Fluorescence in-situ hybridization (FISH)

Fluorescent in-situ hybridization (FISH) was performed to detect anammox bacteria. Biomass was harvested from the MBBR, fixed in a 4 % paraformaldehyde solution and FISH was performed according to Amann et al. (1990). The probe Amx820 with Cy3 label was used at 35 % formamide to target the anammox genera "*Candidatus Brocadia* and *Kuenenia*" (Schmid et al. 2001). The samples were counterstained with the DNA stain 4',6-diamidino-2-phenylindole (DAPI). Images were acquired on a Carl Zeiss Axioskop 2 Plus epifluorescence microscope (Jena, Germany) equipped with differential interference contrast (DIC), and scales were added using ImageJ freeware.

Results and discussion

Moving bed biofilm reactor (MBBR)

MBBR operation

The MBBR system was operated with continuously increasing TN loading rates, which brought along the maximum TN removal rate of 1,000 g N m^{-3} d^{-1} (Fig. 3) with a satisfactory (around 85 %) TN removal efficiency. The average consumption ratio of NO_2^-/NH_4^+ and the ratio of produced NO_3^- to NH_4^+ consumed were 1.14 and 0.26, respectively.

When the TN loading rate was increased above 1,100 g N m^{-3} d^{-1}, inhibition episodes of the process occurred, as can be seen from the high effluent NO_2^- concentrations (over 100 mg N L^{-1}). As a recovery strategy, the TN loading rate was decreased twice around day 330, which restored the process in 1 week. As a second strategy (from day 500 onwards), anammox intermediates were added into the reactor at 1.31 and 1.27 mg N L^{-1} for N_2H_4 and NH_2OH, respectively. The system restored from inhibition in 1 day after intermediate dosing (Fig. 3). The main benefit of the second strategy applied was no need for decreasing the TN loading rate for maintaining the efficient process; decreasing the influent load in a full-scale wastewater treatment plant would also be rather difficult.

Spiked additions (about once a week) of anammox intermediates into the reactor enabled to sustain the TN loading rate of about 500 g N m^{-3} d^{-1} with TN removal efficiency of over 90 % (days 550–572). N_2H_4 and NH_2OH were consumed rapidly as after

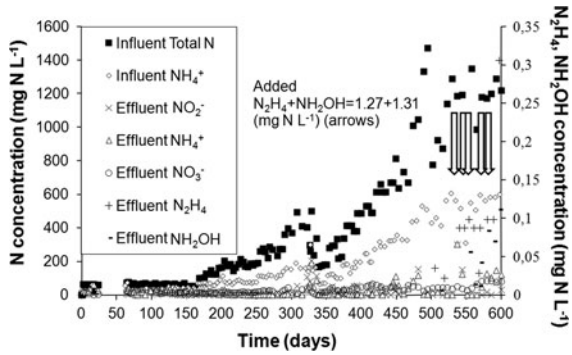

Fig. 3 Changes in nitrogen species (including intermediate species) concentrations in time, injections of intermediates during inhibiting episodes

12 h the effluent concentrations were in the range 0.04–0.13 mg N L^{-1} and 0.17–0.42 mg N L^{-1}, respectively. Without dosing of the intermediates TN removal efficiency stayed in a lower range 70–80 %.

In order to restore the activities of the inhibition-damaged hydrazine oxidizing enzyme and hydroxylamine oxidoreductase (Kuenen and Jetten 2001), a combination of two intermediates instead of N_2H_4 alone would be more beneficial. The two-step conversion of intermediates (NH_2OH to N_2H_4 and N_2H_4 to N_2) would sustain them longer in the process, enabling short and effective overcoming from the inhibition.

According to Bettazzi et al. (2010), spiked injections of NH_2OH (6.36 mg N L^{-1} in total) gave a 20 % permanent recovery from the complete NO_2^- inhibition. Our experiments showed that after the system had been inhibited, addition of NH_2OH and N_2H_4 resulted in an increase of TN removal efficiency of around 20 %.

MBBR microbial characterization

The microbial community present in the anammox biofilm was described by PCR-DGGE and the phylogenetic relations were evaluated for the detected bacteria as described previously (Zekker et al. 2012).

Concerning anammox, 323 bp DNA sequences belonging to uncultured *Planctomycetales bacterium clone P4* were detected, with a 99 % sequence similarity to the anammox species "*Candidatus Brocadia* fulgida" (Zekker et al. 2012). Feeding with ammonium and nitrite and inorganic carbon source provision can lead to the enrichment of *Candidatus Brocadia fulgida* (Kartal et al. 2008), also similar to clone *P4*. *P4* is not often occurring, its presence has been described only by Quan et al. (2008).

FISH analyses confirmed the presence of anammox microorganisms in the biofilm of MBBR belonging to "*Candidatus Brocadia* and *Kuenenia*" (Fig. 4). Dense clusters of anammox bacteria were detected, and the anammox cells were abundantly present. Although anammox cells have been described to grow in clusters mostly on the inner surface of biofilm support material (Tal et al. 2006), anammox cells seemed to be located throughout the biofilm in our case. Breakage of the clusters during the operation of an anammox system may be responsible for lower production of anammox intermediates, reducing the biomass growth (Bettazzi et al. 2010). Additionally, the activity of clustered anammox cells might benefit from quorum sensing molecules (De Clippeleir et al. 2011). Thus, it is of great importance to sustain dense anammox bacteria clusters.

Besides anammox bacteria, aerobic nitrogen oxidizing organisms were retrieved. One of them was closely related to *Nitrosomonas europaea* (84 %) (Zekker et al. 2012), an aerobic ammonium-oxidizing bacteria which is commonly in oxygen-limited reactors relying on partial nitritation (Vlaeminck et al. 2010). Note that the genes of this bacterium can encode for incomplete denitrification (NO_2^- and NO reduction; Chain et al. 2003), which might have played a minor role in the MBBR. The biofilm also harbored the nitrite oxidizing bacteria "*Candidatus Nitrospira* defluvii" and uncultured *Nitrospira sp. clone S1-62* (Zekker et al. 2012). Interestingly, *Nitrospira* could thrive under the very low oxygen conditions in the anammox MBBR, in congruence with the observations of Park and Noguera (2008) and Off et al. (2010). However, since the observed nitrate production approached the stoichiometric value of anammox, the contribution of nitrite oxidizing bacteria in the reactor must have been minimal.

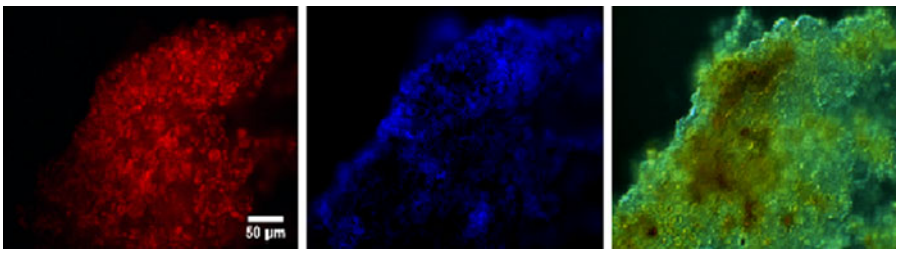

Fig. 4 Representative micrograph set of the MBBR anammox biofilm measured after 556 days of reactor start-up, with a FISH staining displaying anammox bacteria with Cy3-labeled Amx820 (*left*), a DAPI staining displaying all cells (*middle*) and a DIC image (*right*)

Effect of NH_2OH/N_2H_4 addition in anammox batch tests

Batch assays were performed to determine nitrogen conversion rates by the anammox process by different amounts and combinations of anammox intermediates (NH_2OH and N_2H_4). Based on the substrates used in batch assays the experiments performed could be categorized into such main groups as (Table 1).

NH_4^+ and NO_2^- as substrates

Conversions rates of nitrogen species without added intermediates are displayed in Fig. 5. TN removal rate of 4.26 mg N g^{-1} TSS h^{-1} was measured without intermediates addition. The ratios of the produced NO_3^- to the consumed NH_4^+ were 0.11/1 and the consumed NO_2^- to the consumed NH_4^+ were 1.35/1. The difference with the expected anammox stoichiometric ratios, i.e. 0.26 and 1.32, respectively, indicate activity of denitrifying bacteria (present in reject water, see below) reducing NO_2^- and NO_3^-. Production of N_2H_4 during the experiment was not recorded as it was consumed simultaneously with its production.

NH_4^+ and NH_2OH as substrates

Tests were performed with and without biomass to confirm the NH_2OH decomposition and N_2H_4 formation biologically by anammox microorganisms, not by an abiotic chemical reaction (Fig. 6a, b). Areal substrate removal rates are implied to evaluate the rates in tests without biomass added.

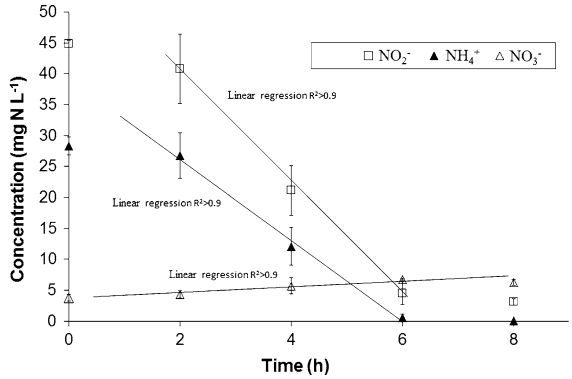

Fig. 5 Time courses of nitrogen species concentrations during anammox batch experiments. Linear regressions and regression coefficients used for calculating nitrogen removal rates are shown

A reference batch experiment which involved substrate (NH_4^+ and NH_2OH at concentrations of 50 and 16.6 mg N L^{-1}, respectively) without anammox biomass indicated a low NH_4^+ removal rate, 0.733 mg N m^{-2} h^{-1} with a moderate NH_2OH reduction rate of 1.38 mg N m^{-2} h^{-1} and a low N_2H_4 production rate of 0.01 mg N m^{-2} h^{-1}. Whereas with the biocarriers the NH_4^+, NH_2OH removal rates reached 3.24 and 5.06 mg N m^{-2} h^{-1} (1.46 mg N g^{-1} TSS h^{-1}), respectively, and the N_2H_4 production rate of 0.25 mg N g^{-1} TSS h^{-1} was measured. Without biomass N_2H_4 was present in a low concentration at the end of experiment (below 0.04 mg N L^{-1}) (Fig. 6b). Instead, in experiments with biomass the N_2H_4 concentration increased from 0.04 to 3.35 mg N L^{-1} (Fig. 6b) showing that NH_2OH was converted into N_2H_4 biologically by anammox bacteria. The ratio of NH_2OH converted into N_2H_4 generated was about 1/0.16, only slightly lower

Table 1 Overview of the substrates and putative intermediates added to the anammox batch tests

Test	NH_4^+ (mg N L^{-1})	NO_2^- (mg N L^{-1})	NH_2OH (mg N L^{-1})	N_2H_4 (mg N L^{-1})	Biomass	Figure
1	28	45	–	–	Yes	5
2	86	0.2	14.3	0.03	Yes	6a
	48	–	16.5	–	No	6b
3	28	45	–	0.44, 0.88, 3.5, 4.4, 10.9, 21.9	Yes	7
	–	–	–	4.4	Yes	–
4	28	45	10.6, 21.21	–	Yes	–
5	28	45	1.7, 2.12, 5.3	1.8, 2.2, 5.5	Yes	8
	28	45	–	3.5, 4.4, 10.9	Yes	8

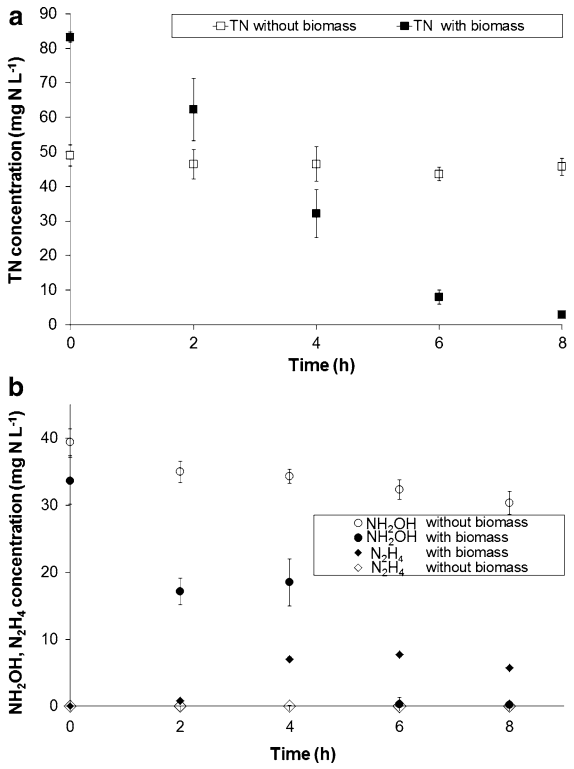

Fig. 6 Time courses of TN removal (**a**), and NH_2OH and N_2H_4 (**b**) in batch anammox activity tests with and without biomass supplied with NH_4^+ and NH_2OH

from that achieved by Hu et al. (2011) in their research with concentrations of NH_2OH (39.2 mg N L^{-1}) and NH_4^+ (38.1 mg N L^{-1}) the ratio of NH_2OH converted into the N_2H_4 generated was 1/0.151 (±0.009). The average and maximum conversion rates of NH_2OH achieved by Hu et al. (2011) were higher than those achieved in our study, 2.9 and 7.85 mg N g^{-1} VSS h^{-1}, similarly to us over 95 % NH_2OH was utilized within the 7 h of cultivation. The maximum N_2H_4 production rate achieved by these researchers was 0.77 mg N g^{-1} VSS h^{-1}.

NH_4^+, NO_2^-, and N_2H_4 as substrates

To determine the effect of the amount of added N_2H_4 on the TN removal rate, incubations with N_2H_4, NO_2^-, and NH_4^+ were performed. The TN removal rate achieved with N_2H_4 concentration of 4.38 mg N L^{-1} was 5.43 mg N g^{-1} TSS h^{-1}, about 20 % higher in comparison with the experiment into which no intermediate was added (Fig. 7). A significant difference in the replicates maximum TN removal rates with and without addition of intermediates (two- tailed *t*- test: $p = 0.013$) was noted.

With high N_2H_4 concentrations (21.9 mg N L^{-1}) added, the rate was 4.88 mg N g^{-1} TSS h^{-1}, lower than with 4.38 mg N L^{-1} of N_2H_4 addition. N_2H_4 removal rates were 0.44 and 1.71 mg N g^{-1} TSS h^{-1}, respectively. The N_2H_4 concentration of 21.9 mg N L^{-1} did not promote nitrogen removal because instead of consuming NH_4^+ and NO_2^- mainly N_2H_4 was utilized, forming NH_4^+ from N_2H_4 as assumed in Fig. 1. R2 subunit of ribonucleotide reductase from Escherichia coli, was shown to catalyze conversion of hydrazine into dinitrogen gas and ammonium (Han et al. 1996). In batch anammox cultures with hydrazine it was observed that 3 mol N_2H_4 was converted to 4 mol NH_4^+. Whether one enzyme is responsible for the conversion of hydrazine into dinitrogen gas and ammonium or two different enzymes are involved needs to be investigated. It is possible that an enzyme similar to that observed in the R2 subunit or an enzyme similar to that of HAO from N. Europaea is presenting hydrazine conversion in anammox cell (Schalk et al. 1998).

The nitrogen removal rate was promoted most with the optimum amount of N_2H_4 added, also probably due to the intensification of anammox enzymatic reactions.

Similarly to Bettazzi et al. (2010), addition of anammox metabolites at concentrations higher than optimal (total of around 1.75 mg N L^{-1}) did not improve the nitrogen removal rate in batch assays.

However, the batch experiments performed at 30 °C containing 1.5 and 0.5 g VSS L^{-1} of anammox

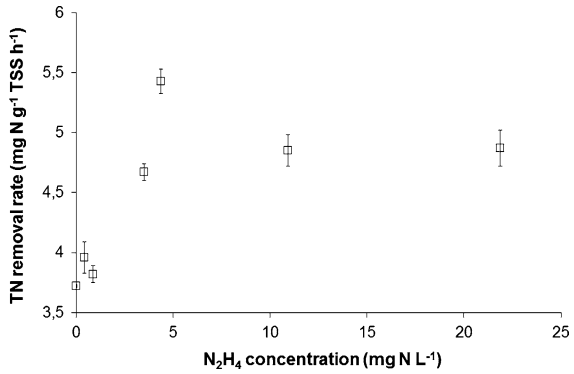

Fig. 7 Dependence of TN removal rates on different injected N_2H_4 concentrations

biomass (Schalk et al. 1998) with a concentration of N_2H_4 (around 43.75 mg N L^{-1}) coupled with NO_2^- (56 mg N L^{-1}) have shown a rapid conversion of N_2H_4 and NO_2^- with high conversion rates of 10.56 and 3.53 mg N g^{-1} VSS h^{-1}, respectively.

The ratio of TN-fed/added anammox metabolites can be of crucial importance in optimizing concentrations of intermediates bringing along the highest TN removal rate. Bettazzi et al. (2010) received the most suitable ratio of TN fed/added metabolites to be around 40, which brought along the maximum NO_2^- removal rates. In our case, the value of the optimal ratio of TN-fed/added metabolites was around 10 with very similar initial NO_2^- concentrations to those used by Bettazzi et al. (2010). In our case higher concentrations of metabolites were needed as compared to Bettazzi et al. (2010) to gain the highest TN removal rates due to a slower diffusion of substrates into the biofilm.

In case of NH_4^+ and NO_2^- being added to N_2H_4 concentration of 4.38 mg N L^{-1}, the rate of the N_2H_4 reduction was significantly higher (30 %) than with N_2H_4 concentration of 4.38 mg N L^{-1} used solely. Ensuing from free energies brought forth by Hu et al. (2011), Schalk et al. (1998), and Bettazzi et al. (2010) N_2H_4 is oxidized with NO_2^- rather than disproportionated by reason of being thermodynamically more favorable. The difference in free energies between reactions containing NO_2^- and in these containing only N_2H_4 was 42 % (Schalk et al. 1998), while the N_2H_4 reduction was 30 % higher in the test containing NH_4^+, NO_2^-, and N_2H_4 than in the test containing only N_2H_4.

An average NH_4^+ production rate determined with 4.38 mg N $L^{-1}$of N_2H_4 being solely added into the reaction mixture was 0.22 mg N g^{-1} TSS h^{-1} per 1 mol of N_2H_4, 1.63 mol of NH_4^+ were formed, an amount slightly higher than assumed by Schalk et al. (1998). An average N_2H_4 conversion rate reported by Hu et al. (2011) was 0.45 mg N g^{-1} VSS h^{-1}, being about one-third of the value reported by Schalk et al. (1998). During N_2H_4 conversion ammonium was produced, displaying a linear relationship with time during 33 h with an average rate of 0.71 mg mg N g^{-1} VSS h^{-1} (Hu et al. 2011), which was much higher than that observed by van der Star et al. (2008), but was about half of which was reported by Schalk et al. (1998). The maximum N_2H_4 consumption rate and the maximum ammonium production rates were 1.13 and 1.01 mg N g^{-1} VSS h^{-1}, respectively. When using N_2H_4 at a concentration of 43.75 mg N L^{-1}, Schalk et al. (1998) calculated the N_2H_4 conversion and the NH_4^+ formation rates to be 1.36 and 1.51 mg N g^{-1} VSS h^{-1}, respectively.

NH_4^+, NO_2^-, and NH_2OH as substrates

For lower concentrations NH_2OH (0–4.38 mg N L^{-1}) coupled with NH_4^+ and NO_2^-, NH_2OH was converted into N_2H_4 and simultaneously consumed. The maximum N_2H_4 production rate with the NH_2OH concentration of 10.6 mg N L^{-1} was 0.51 mg N g^{-1} TSS h^{-1}.

By using the NH_2OH concentration of 21.21 mg N L^{-1}, the formation of N_2H_4 was 0.23 mg N g^{-1} TSS h^{-1}, twice lower than achieved in the experiment with the NH_2OH concentration of 10.6 mg N L^{-1}.

Van der Star et al. (2008) detected that after the formation of N_2H_4 during the anammox process (taking 3 h), it was disproportionated rapidly (taking around 1 h) when the initial NH_2OH concentration of 55.15 mg N L^{-1} was used. Also, with higher initial NH_2OH concentrations (140 mg N L^{-1}) the added maximum (peak) amount of N_2H_4 (1.4 mg N L^{-1}) accumulated in the system. According to another study (Egli et al. 2001) the highest produced N_2H_4 concentration detected in the batches was 4.38 mg N L^{-1} when the initial NH_2OH concentration was 36.82 mg N L^{-1}. In our case with a lower initial concentration of 21.21 mg N L^{-1} of NH_2OH, 0.53 mg N L^{-1} of N_2H_4 was produced. The results achieved in our case and those by Van der Star et al. (2008) and by Hu et al. (2011) varied because of different substrate concentrations and a lower temperature (25 °C) used in our batches as compared with 35–36 °C used by the referred authors.

NH_4^+, NO_2^-, NH_2OH, and N_2H_4 as substrates

Concentrations of N_2H_4 of 3.45, 4.38, and 10.94 mg N L^{-1} added to NO_2^- and NH_4^+ brought along 4, 10 and 20 % higher TN removal rates, respectively, than in case of equimolar amounts of N_2H_4 + NH_2OH, NO_2^-, and NH_4^+ used showing weaker benefits of NH_2OH addition in accelerating TN removal in batches as compared with N_2H_4 addition (Fig. 8).

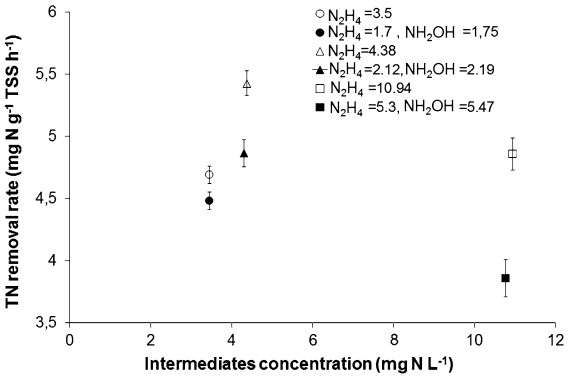

Fig. 8 TN removal rate with different N_2H_4 and NH_2OH + N_2H_4 concentration combinations

Earlier reports by Bettazzi et al. (2010) have shown a positive effect of using N_2H_4 and NH_2OH together on overcoming NO_2^- inhibition. When inhibiting factors (NO_2^-–N) were applied at concentrations with minimal inhibiting effect on anammox bacteria better TN removal rates were achieved by using N_2H_4 for anammox process accelerations.

In future studies the effect of NO as one of intermediates of the anammox reaction on the kinetics of nitrogen removal in biofilm systems should be studied in detail. Also, the effect of the addition of NO on overcoming the effect of inhibiting substances should be studied.

Conclusion

The effect of putative anammox intermediates was examined on anammox biofilms in a MBBR system and in batch experiments. Earlier detected *Anammox bacterium clone P4* was closely (99 %) related to *"Candidatus Brocadia* fulgida", in congruence with the FISH detected *"Candidatus Brocadia* fulgida and *Kuenenia*" strains.

In MBBR with the aid of addition of hydrazine and hydroxylamine it was possible to recover from overload shock in 1 day as opposed to 1 week by traditional methods.

Batch experiments confirmed qualities of anammox containing biofilm to produce hydrazine from hydroxylamine and to consume it biologically. The optimum amount of anammox intermediate N_2H_4 increasing TN removal rate in batch experiments was 4.38 mg N L^{-1}. Hydrazine disproportionation was significantly slower in batch incubation without nitrite and ammonium, fitting perfectly with previous findings.

All short-term batch experiments showed better effect of using N_2H_4 as compared with using equimolar concentrations of N_2H_4 and NH_2OH. With N_2H_4 addition up to 20 % higher TN removal rates were registered than in case of equimolar amounts of N_2H_4 + NH_2OH addition.

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References

Amann RI, Krumholz L, Stahl DA (1990) Fluorescent-oligonucleotide probing of whole cells for determinative, phylogenetic, and environmental studies in microbiology. J Bacteriol 172:762–770

Bernet N, Beline F (2009) Challenges and innovations on biological treatment of livestock effluents. Bioresour Technol 100:5431–5436

Bettazzi E, Caffaz S, Vannini C, Lubello C (2010) Nitrite inhibition and intermediates effects on anammox bacteria: a batch-scale experimental study. Process Biochem 45:573–580

Chain P, Lamerdin J, Larimer F, Regala W, Lao V, Land M, Hauser L, Hooper A, Klotz M, Norton J (2003) Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. J Bacteriol 185:2759–2773

De Clippeleir H, Defoirdt T, Vanhaecke L, Vlaeminck SE, Carballa M, Verstraete W, Boon N (2011) Long-chain acylhomoserine lactones increase the anoxic ammonium oxidation rate in an OLAND biofilm. Appl Microbiol Biotech 90:1511–1519

Desloover J, De Clippeleir H, Boeckx P, Du Laing G, Colsen J, Verstraete W, Vlaeminck SE (2011) Floc-based sequential partial nitritation and anammox at full scale with contrasting N_2O emissions. Water Res 45:2811–2821

Dexiang L, Xiaoming L, Qi Y, Guangming Z, Liang G, Xiu Y (2008) Effect of inorganic carbon on anaerobic ammonium oxidation enriched in sequencing batch reactor. J Environ Sci 20:940–944

Dionisi HM, Layton AC, Harms G, Gregory IR, Robinson KG, Sayler GS (2002) Quantification of *Nitrosomonas oligotropha*-like ammonia-oxidizing bacteria and *Nitrospira* spp. from full-scale wastewater treatment plants by competitive PCR. Appl Environ Microbiol 68:245–253

Egli K, Fanger U, Alvarez PJJ, Siegrist H, van derMeer JR, Zehnder AJB (2001) Enrichment and characterization of anammox bacterium from a rotating biological contactor treating ammonium-rich leachate. Arch Microbiol 175: 198–207

Fernández I, Dosta J, Fajardo C, Campos JL, Mosquera-Corral A, Méndez R (2012) Short- and long-term effects of ammonium and nitrite on the anammox process. J Environ Manage 95:170–174

Frear DS, Burrell RC (1955) Spectrophotometric method for determining hydroxylamine reductase activity in higher plants. Anal Chem 27:1664

Fux C, Siegrist H (2004) Nitrogen removal from sludge digester liquids by nitrification/denitrification or partial nitritation/anammox: environmental and economical considerations. Water Sci Technol 50:19–26

George M, Nagaraja KS, Balasubramanian N (2008) Spectrophotometric determination of hydrazine. Talanta 75:27–31

Greenberg A, Clesceri L, Eaton A (1992) Standard methods for the examination of water and wastewater, 18th edn. American Public Health Association Publications, Washington

Han JY, Swarts JC, Sykes AG (1996) Kinetic studies on the hydrazine and phenylhydrazine reductions of the Escherichia coli R2 subunit of ribonucleotide reductase. Inorg Chem 35:4629–4634

Hu A, Zheng P, Mahmood Q, Zhang L, Shen L, Ding S (2011) Characteristics of nitrogenous substrate conversion by anammox enrichment. Biores Technol 102:536–542

Jeanningros Y, Vlaeminck SE, Kaldate A, Verstraete W, Graveleau L (2010) Fast start-up of a pilot-scale deammonification sequencing batch reactor from an activated sludge inoculum. Water Sci Technol 61:1393–1400

Kartal B, van Niftrik L, Rattray J, de Vossenberg J, Schmid MC, Damste JSS, Jetten MSM, Strous M (2008) Candidatus '*Brocadia fulgida*': an autofluorescent anaerobic ammonium oxidizing bacterium. FEMS Microbiol Ecol 63(1): 46–55

Kartal B, Tan NCG, Van de Biezen E, Kampschreur MJ, Van Loosdrecht MCM, Jetten MSM (2010) Effect of nitric oxide on anammox bacteria. Appl Environ Microbiol 76:6304–6306

Kuenen JG, Jetten MSM (2001) Extraordinary anaerobic ammonium-oxidizing bacteria. Asm News 67:456–463

Lane DJ (1991) 16/23S rRNA sequencing. In: Stackenbrandt E, Goodfellow M (eds) Nucleic acid techniques in bacterial systematics. Wiley, Chichester, pp 177–204

López H, Puig S, Ganigué R, Ruscalleda M, Balaguer MD, Colprim J (2008) Start-up and enrichment of a granular anammox SBR to treat high nitrogen load wastewaters. J Chem Technol Biotechnol 83:233–241

Lotti T, van der Star WRL, Kleerebezem R, Lubello C, van Loosdrecht MCM (2012) The effect of nitrite inhibition on the anammox process. Water Res. doi:10.1016/j.watres.2012.02.011

Neef A, Amann RI, Schlesner H, Schleifer KH (1998) Monitoring a widespread bacterial group: in situ detection of planctomycetes with 16S rRNA-targeted probes. Microbiology 144:3257–3266

Ødegaard H (2006) Innovations in wastewater treatment: the moving bed biofilm process. Water Sci Technol 53:17–33

Off S, Alawi M, Spieck E (2010) Enrichment and physiological characterization of a novel Nitrospira-like bacterium obtained from a marine sponge. Appl Environ Microbiol 76:4640–4646

Park HD, Noguera DR (2008) Nitrospira community composition in nitrifying reactors operated with two different dissolved oxygen levels. J Microbiol Biotechnol 18: 1470–1474

Quan ZX, Rhee SK, Zuo JE, Yang Y, Bae JW, Park JR, Lee ST, Park YH (2008) Diversity of ammonium-oxidizing bacteria in a granular sludge anaerobic ammonium-oxidizing (anammox) reactor. Environ Microbiol 10:3130–3139

Sąnchez-Melsió A, Cịliz J, Balaguer MD, Colprim J, Vila X (2009) Development of batch-culture enrichment coupled to molecular detection for screening of natural and man-made environments in search of anammox bacteria for N-removal bioreactors systems. Chemosphere 75:169–179

Schalk J, Oustad H, Kuenen JG, Jetten MSM (1998) The anaerobic oxidation of hydrazine: a novel reaction in microbial nitrogen metabolism. FEMS Microbiol Lett 158:61–67

Schmid M, Schmitz-Esser S, Jetten M, Wagner M (2001) 16S–23S rDNA intergenic spacer and 23S rDNA of anaerobic ammonium-oxidizing bacteria: implications for phylogeny and in situ detection. Environ Microbiol 3:450–459

Strous M, Kuenen JG, Jetten MSM (1999) Key physiology of anaerobic ammonium oxidation. Appl Environ Microbiol 65:3248–3250

Strous M, Pelletier E, Mangenot S, Rattei T, Lehner A, Taylor MW, Horn M, Daims H, Bartol-Mavel D, Wincker P, Barbe V, Fonknechten N, Vallenet D, Segurens B, Schenowitz-Truong C, Médigue C, Collingro A, Snel B, Dutilh BE, Op den Camp HJ, van der Drift C, Cirpus I, van de Pas-Schoonen KT, Harhangi HR, van Niftrik L, Schmid M, Keltjens J, van de Vossenberg J, Kartal B, Meier H, Frishman D, Huynen MA, Mewes HW, Weissenbach J, Jetten MS, Wagner M, Le Paslier D (2006) Deciphering the evolution and metabolism of an anammox bacterium from a community genome. Nature 440:790–794

Tal Y, Watts JEM, Schreier HJ (2006) Anaerobic ammonium-oxidizing (anammox) bacteria and associated activity in fixed-film biofilters of a marine recirculating aquaculture system. Appl Environ Microbiol 72:2896–2904

Udert KM, Kind E, Teunissen M, Jenni S, Larsen TA (2008) Effect of heterotrophic growth on nitritation/anammox in a single sequencing batch reactor. Water Sci Technol 58:277–284

Van der Star W, van de Graaf MJ, Kartal B, Picioreanu C, Jetten MJM, van Loosdrecht MCM (2008) Response of anaerobic ammonium oxidizing bacteria to hydroxylamine. Appl Environ Microbiol 74:4417–4426

Verstraete W, Vlaeminck SE (2011) ZeroWasteWater: short-cycling of wastewater resources for sustainable cities of the future. Int J Sustain Dev World Ecol 18(3):253–264. doi: 10.1080/13504509.2011.570804

Vlaeminck SE, Terada A, Smets BF, Van der Linden D, Boon N, Verstraete W, Carballa M (2009) Nitrogen removal from digested black water by one-stage partial nitritation and anammox. Environ Sci Technol 43:5035–5041

Vlaeminck SE, De Clippeleir H, Verstraete W (2012) Microbial resource management of one-stage partial nitritation/

anammox. Microb Biotechnol. doi:10.1111/j.1751-7915.2012.00341.x

Vlaeminck SE, Terada A, Smets BF, De Clippeleir H, Schaubroeck T, Bolca S, Verstraete W (2010) Aggregate size and architecture determine microbial activity balance for one-stage partial nitritation and anammox. Appl Environ Microbiol 76:900–909

Watt GW, Chrisp JD (1952) A spectrophotometric method for the determination of hydrazine. Anal Chem 24:2006–2008

Wett B (2007) Development and implementation of a robust deammonification process. Water Sci Technol 56:81–88

Zekker I, Rikmann E, Tenno T, Lemmiksoo V, Menert A, Loorits L, Vabamäe P, Tomingas M, Tenno T (2012) Anammox enrichment from reject water on blank biofilm carriers and carriers containing nitrifying biomass: operation of two moving bed biofilm reactors (MBBR). Biodegradation. doi:10.1007/s10532-011-9532-7

Zhang L, Zheng P, He YH, Jin RC (2009) Performance of sulfate-dependent anaerobic ammonium oxidation. Sci China Ser B: Chem 52:86–92