The role of the NO_2^-:NH_4^+ ratio and the nitrogen loading rate on the stability of ANAMMOX bioreactors

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Abstract

The potential inhibitory effect of nitrite on anammox bacteria remains one of the difficulties in the application of the anammox process. Neither the mechanism by which it occurs nor the conditions under which nitrite affects the performance of continuous bioreactors, have been elucidated. The performance of three upflow anammox reactors fed with synthetic wastewaters containing the same NO_2^- concentration but different molar NO_2^-:NH_4^+ ratios was investigated. The bioreactors were operated for 183 days at anammox loading rates ranging from 0.28 to 2.32 g N L^{-1} d^{-1}, depending on the reactor and experimental stage. Periodic assessment of the maximum specific anammox activity of the sludge in the reactors confirmed a moderate increase in the activity of biomass from the reactor operated with 10% excess NH_4^+ (NO_2^-:NH_4^+= 1.20). In contrast, a sharp decrease of the microbial activity (85% reduction) was observed in the two reactors operated under ammonium-limited conditions (NO_2^-/NH_4^+= 1.90 or 2.64) after 91 days of operation, coinciding with the period when the lowest loading rate was maintained. During this period, the levels of nitrite present in the effluent of both reactors (< 110 mg NO_2^- L^{-1}) were considerably lower than reported 50% inhibitory concentrations (608 mg NO_2^- L^{-1}). The activity of the biomass in both reactors improved gradually as the loading rate was progressively increased 6-7 fold. The observed decrease in anammox activity is likely due to extended reactor underloading, which causes microbial stress and could increase the susceptibility of anammox bacteria to residual nitrite concentrations. These results suggest that relatively low nitrite concentrations, resulting from sustained imbalance in the nitritation step prior to the anammox process or other causes, could be detrimental for the performance of anammox bioreactors operated under conditions of nitrogen underloading.

Keywords

Inhibition; toxicity; nitrous acid; anammox activity; nitrogen removal

INTRODUCTION

The anaerobic oxidation of ammonium using nitrite as electron acceptor (Anammox) is a novel biological process for the treatment of nitrogen rich wastewaters. Discovered in the early 90s, the Anammox process has gained interest during the last years as it offers substantial advantages over conventional nitrification-denitrification processes when wastewaters have a low C/N ratio. In these cases, cost- and energy-savings are realized since oxygen requirements can be reduced up to 60% and additional electron donor for denitrification is not needed.

Anammox is catalyzed by chemolithoautotrophic bacteria (Strous *et al.* 1999a) belonging to five genera: *Brocadia, Kuenenia, Anammoxoglobus, Jettenia* and *Scalindua* of the phylum *Planctomycetes* (Harhangi *et al.* 2011). Unlike other prokaryotes, anammox bacteria have a complex internal compartmentalization (van Niftrik *et al.* 2004), which is probably one of the causes for their slow growth and low cell yield (Strous *et al.* 1998):

$$NH_4^+ + 1.32NO_2^- + 0.066HCO_3^- + 0.13H^+ \rightarrow 1.02N_2 + 0.26NO_3^- + 0.066CH_2O_{0.5}N_{0.15} + 2.03H_2O \quad (1)$$

The long doubling time of anammox bacteria leads to extended periods for reactor start-up (Tsushima *et al.* 2007) and long recovery times following eventual toxic shocks that kill off the biomass. Several studies have revealed inhibition of anammox microorganisms due to compounds commonly found in wastewaters such as sulphide (Dapena-Mora *et al.* 2007), dissolved oxygen

(Egli *et al.* 2001), free ammonia (Jaroszynski *et al.* 2011), and nitrite (Strous *et al.* 1999b). Among these compounds, nitrite, a substrate of anammox bacteria, has been reported to cause anammox inhibition in both continuous bioreactors (van der Star *et al.* 2007) and batch bioassays (Dapena-Mora *et al.* 2007; Lotti *et al.* 2012). Understanding the factors affecting the sensitivity of anammox microorganisms to nitrite toxicity is essential for improving the applicability of the anammox process. The objective of this work was to investigate the effect of the NO_2^-:NH_4^+ ratio, at different nitrogen loading rates, on the performance of anammox bioreactors. NO_2^-:NH_4^+ ratios can diverge from design values due to events such as imbalances in the nitritation step prior to the anammox process.

MATERIALS AND METHODS

Continuous-flow bioreactors

Three upflow bioreactors (450 mL) were inoculated with granular anammox sludge (3.8 g VSS L^{-1}) and fed with mineral medium (Sun *et al.* 2011) supplied with different concentrations of NO_2^- and NH_4^+, depending on the experimental period. The reactors were operated at hydraulic retention time of 5.3 h in a dark incubator at 30ºC. During the first 36 days, the reactors were operated with a stoichiometric excess of NH_4^+ (10%) to prevent accumulation of NO_2^- and ensure the same initial steady-state conditions. After this initial period, the molar NO_2^-:NH_4^+ ratio in the feed of two of the reactors was modified, by decreasing the concentration of NH_4^+, to 1.90 (30% excess of NO_2^-) and 2.64 (50% excess of NO_2^-) for R2 and R3, respectively, in order to ensure accumulation of nitrite in the treated effluent. After day 95, the N load was increased stepwise in all reactors by increasing the influent concentration of both NO_2^- and NH_4^+ while keeping constant their respective NO_2^-:NH_4^+ ratios. The pH of the effluent from the reactors was 7.3.

Batch experiments

Batch activity tests were carried out periodically with biomass sampled from the reactors. Glass vials (25 mL) were supplied with culture medium (15 mL), spiked with NH_4^+ (2.7 mM) and NO_2^- (3.6 mM), and inoculated with granular sludge from the reactors (700 mg VSS L^{-1}). The flasks were sealed with rubber stoppers, and both the liquid and headspace were flushed with He/CO_2 (80/20, v/v) to achieve anaerobic conditions and maintain a pH value of 7.2. The flasks were incubated in an orbital shaker (10 rpm) placed in a dark climate-controlled room at 30±2ºC. Liquid samples were collected at the beginning and at the end of each test for analysis of NH_4^+, NO_2^- and NO_3^-. Headspace samples were periodically analysed for N_2 gas content. The maximum specific anammox activity (SAA) was calculated from the ratio of the maximum rate of N_2-N generation and the amount of microbial biomass (measured as volatile suspended solids, VSS) in each assay as follows: $SAA = \Delta N_2 (\Delta t \cdot VSS)^{-1}$ (mg N mg^{-1} VSS d^{-1}).

Analytical methods

NH_4^+, NO_2^- and NO_3^- in liquid samples and N_2 gas content in the headspace were analysed as previously described (Sun *et al.* 2011). Other determinations (pH, VSS) were performed following the procedures described in the Standard Methods (APHA 2005).

RESULTS AND DISCUSSION

The reactors (R1, R2 and R3) were operated for 183 days. Complete removal of NO_2^- was achieved in the three reactors 25 days after inoculation, and NO_2^- elimination remained over 90% until day 36. NH_4^+ was fed in 10% excess, and accumulation of 5-6 mg NH_4^+ L^{-1} was observed during this period. After day 36, the concentration of NH_4^+ in the feed of R2 and R3 was decreased, resulting in influent NO_2^-:NH_4^+ molar ratios of 1.90 and 2.64, respectively. NO_2^- concentration built up as a consequence of the deficit of NH_4^+, resulting in effluent NO_2^- concentrations ranging 41-65 mg L^{-1} for R2, and 73-109 mg L^{-1} for R3. NH_4^+ removal in the reactors R2 and R3 was almost 100%.

These conditions remained constant until day 95, after which the nitrogen loading rate (NLR) of all reactors was increased stepwise while keeping constant the NO_2^-:NH_4^+ ratio. As a consequence, the NO_2^- concentration in the effluent of R2 and R3 increased according to the increment in NLR applied (Fig. 1A). Reactor 1 showed excellent nitrogen removal until day 169, after which NH_4^+ started to accumulate (results not shown), suggesting reactor overloading. After day 169, NH_4^+ accumulation also appeared in R2, reaching a maximum concentration of 85 mg L^{-1} on day 183. NH_4^+ removal in R3 was almost 100% during the whole experiment.

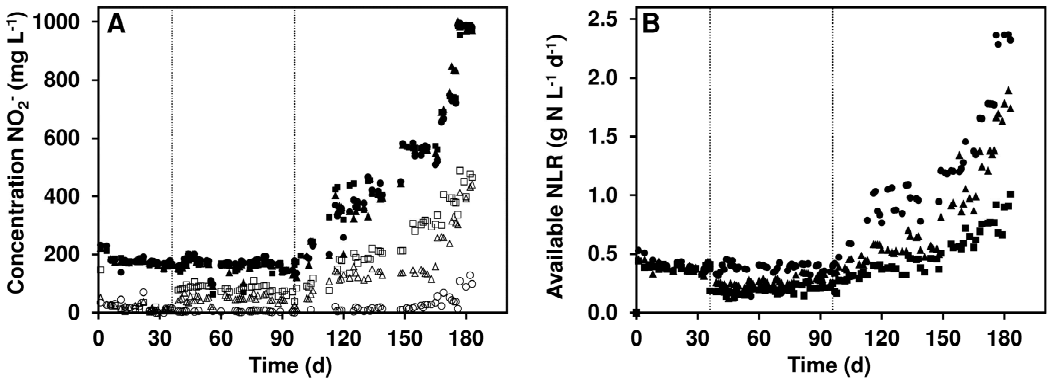

Figure 1. (**A**) Nitrite concentration in the influent (closed symbols) and effluent (open symbols), and (**B**) available anammox-nitrogen loading rate for reactor 1 (circles), reactor 2 (triangles), and reactor 3 (squares) at different times of reactor operation.

Activity tests were carried out periodically to evaluate the effect of the NLR and NO_2^-:NH_4^+ ratio on the "health" of the biomass. The maximum specific anammox activity (SAA) of the inoculum was 0.20 mg N mg VSS^{-1} d^{-1}. The SAA of the R1 biomass was 0.23 mg N mg VSS^{-1} d^{-1} on day 91, and the activity increased slightly to 0.29 mg N mg VSS^{-1} d^{-1} by the end of the experiment, which is consistent with enrichment of anammox bacteria. Activity tests performed on day 91 showed a dramatic activity decrease for the biomass in R2 and R3, coinciding with the period of lowest NLR (Fig. 2). The gradual increase in NLR had a positive effect on these reactors. Activity tests performed on day 183 demonstrated an almost complete recovery of the anammox activity in R2 (0.17 mg N mg VSS^{-1} d^{-1}) when compared to the initial SAA, whereas the activity of the R3 biomass increased to 0.11 mg N mg VSS^{-1} d^{-1}.

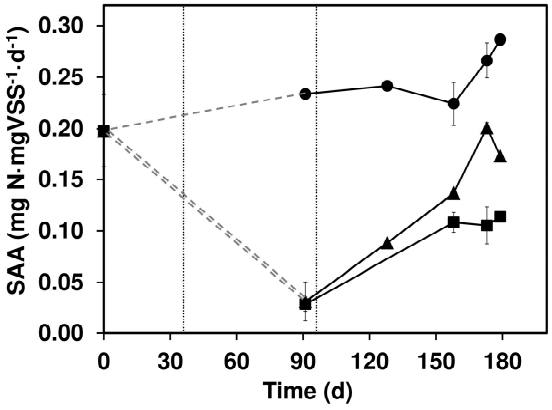

Figure 2. Specific anammox activity (SAA) of the biomass in reactor 1 (●), reactor 2 (▲), and reactor 3 (■) at different times of the experiment.

The sharp drop observed in the anammox activity of the biomass in R2 and R3 on day 91 cannot be attributed solely to the presence of residual nitrite in the effluent. The concentrations of nitrite in the effluent of R2 and R3 during the period of interest (day 36-91) ranged from 41-65 and 73-109

mg L^{-1}, respectively (Fig. 1A). These values are considerably lower compared to the 50% inhibitory concentration (608 mg NO_2^- L^{-1}) recently reported for nitrite in assays with the inoculum used in these experiments (Carvajal-Arroyo, In press). The activity decrease might be related to the low available anammox-nitrogen loading rate (AA-NLR, *i.e.*, total N loading available to anammox bacteria based on the limiting substrate) maintained in both reactors. Low AA-NLR values could cause microbial "starvation" which would limit the capacity of the biomass to grow and keep up with cell maintenance. Interestingly, ongoing experiments indicate that the inhibitory effect of nitrite on anammox bacteria is exacerbated by starvation (results not shown). Based on this hypothesis, the gradual improvement in the anammox activity of the R2 and R3 biomass after day 91 (Fig. 2), even in the presence of increasing NO_2^- concentrations (up to 440 and 488 mg NO_2^- L^{-1} for R2 and R3, respectively), could be attributed to the substantial increase of the AA-NLR in both reactors which prevented further biomass starvation (Fig. 1B).

These results suggest that relatively low nitrite concentrations, resulting from sustained imbalance in the nitritation step prior to the anammox process or other causes, could be detrimental for the performance of anammox bioreactors operated under conditions of nitrogen underloading.

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