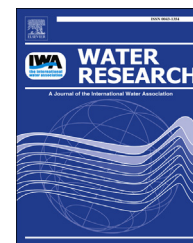


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Starved anammox cells are less resistant to NO_2^- inhibition



José M. Carvajal-Arroyo^{*,1}, Daniel Puyol¹, Guangbin Li¹,
Andrew Swartwout¹, Reyes Sierra-Álvarez¹, Jim A. Field¹

Department of Chemical and Environmental Engineering, University of Arizona, P.O. Box 210011, Tucson, AZ, USA

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ABSTRACT

Anaerobic ammonium oxidizing (anammox) bacteria are inhibited by their terminal electron acceptor, nitrite. Serious nitrite inhibition of the anammox bacteria occurs if the exposure coincides with the absence of the electron donating substrate, ammonium and $\text{pH} < 7.2$. Starvation of biomass occurs during underloading of bioreactors or biomass storage. This work investigated the effect of starvation on the sensitivity of anammox bacteria to nitrite exposure. Batch activity tests were carried out evaluating the response of anammox biomass subjected to different levels of starvation upon exposure to nitrite in the presence and absence of ammonium (active- and resting-cells, respectively). The response of the bacteria was evaluated by measuring the specific anammox activity and the evolution of the ATP content in the biomass over time. The 50% inhibitory concentrations of nitrite in starved- and fresh-resting-cells was 7 mg N L^{-1} and 52 mg N L^{-1} , respectively. By contrast, only moderate nitrite inhibition occurred to starved anammox biomass when exposed to nitrite and ammonium simultaneously. Maximum ATP levels were observed in fresh cells. The ATP content in starved resting cells peaked 2–3 h after addition of NO_2^- . The response was hindered in cells starved for long periods. These findings agreed with a bioreactor study in which underloading of anammox biomass ($0.10 \text{ g N L}^{-1} \text{ d}^{-1}$) decreased its tolerance to a nitrite (only) exposure ($101 \text{ mg NO}_2^- \text{ N L}^{-1}$) and completely disrupted the N removal capacity of the biomass. A similar accumulation of $108 \text{ mg NO}_2^- \text{ N L}^{-1}$ after operation at $0.95 \text{ g N L}^{-1} \text{ d}^{-1}$ did not cause observable inhibition of the bacteria. The results taken as a whole demonstrate that starved anammox biomass is highly sensitive to nitrite toxicity. An explanation is proposed based on energy requirements to translocate nitrite in the cell.

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1. Introduction

The anammox bacteria catalyze the oxidation of ammonium (NH_4^+) using nitrite (NO_2^-) as electron acceptor, generating N_2 gas as major final product. After its discovery in the early

1990s, the anammox process has been applied to the treatment of NH_4^+ rich wastewaters. Due to the chemolithoautotrophic nature of the process and that elemental oxygen (O_2) is not needed, anammox technology is advantageous over conventional nitrification – denitrification systems, which are costly and energy intensive. Anammox cells

* Corresponding author. Tel.: +1 520 621 6457; fax: +1 520 621 6048.

E-mail address: jcarvaja@email.arizona.edu (J.M. Carvajal-Arroyo).

¹ All the authors contributed equally to the article.

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have a complex internal organization, with three lipid membranes that divide the cell in several compartments. The central organelle, called anammoxosome, houses the enzymes responsible for the anammox catabolism (Kartal et al., 2011). As a result of their catabolism, anammox bacteria generate a transmembrane proton gradient between both sides of the anammoxosome membrane, which is used for synthesis of ATP (van der Star et al., 2010). Furthermore the anammox bacteria accumulate glycogen and polyhydroxyalkanoates, which are polymers for energy storage that the anammox bacteria may use for cell maintenance during periods of starvation (van Niftrik et al., 2008).

One of the most intriguing aspects of anammox bacteria is their potential to be inhibited by one of their substrates, NO_2^- . Although the literature reporting NO_2^- inhibition of anammox bacteria is abundant, the mechanism by which it occurs is not known and there is a wide divergence on the threshold levels of NO_2^- that cause complete inhibition ranging from 100 to 750 mg N L⁻¹ (Kimura et al., 2010; Strous et al., 1999). The physiological status of the cells may affect the sensitivity of anammox bacteria to NO_2^- (Lotti et al., 2012; Scaglione et al., 2012). Recently we have shown that resting cells (exposed just to NO_2^- without NH_4^+) are more sensitive to NO_2^- than metabolically active cells (simultaneously exposed to NO_2^- and NH_4^+) (Carvajal-Arroyo et al., 2014a). Furthermore, the toxic effect caused by NO_2^- to resting cells, is most obvious when the pH is in the lower range of the pH optima (6.8–7.2) (Carvajal-Arroyo et al., 2014b).

The available reports on NO_2^- inhibition of anammox bacteria are based on batch experiments which utilize biomass from nursing reactors, or observations made on continuous bioreactors, but little is known about the sensitivity of anammox bacteria to NO_2^- after being subjected to starving conditions. Starvation can occur to anammox bacteria in underloaded bioreactors or during storage of sludge. Due to the slow growth of anammox bacteria, new bioreactors are usually started up with enriched biomass from other wastewater treatment plants (Joss et al., 2009; Vlaeminck et al., 2012; Wett, 2007), and the biomass is often stored, remaining inactive during weeks or months. Although studies have been carried out to optimize storage conditions (Vlaeminck et al., 2007), there is no reports on the effect of the starvation on the tolerance of the bacteria to NO_2^- .

In this work we evaluated the inhibitory effect of NO_2^- on an anammox enrichment culture subjected to different degrees of starvation. The impact of NO_2^- toxicity and starvation were evaluated by studying the specific anammox activity of fresh and starved biomass after treatments of NO_2^- exposure, as well as the evolution of the ATP content of the biomass during such exposure events. Furthermore, we explored the resilience of continuous anammox bioreactors to events of NO_2^- accumulation, operated under different nitrogen loading rates (NLR).

2. Materials and methods

2.1. Batch bioassays

Anammox granular sludge was used in all the experiments. The inoculum, previously characterized, had a $\text{NO}_2^-:\text{NH}_4^+$

consumption ratio of 1.31 ± 0.06 (Puyol et al., 2013), and was dominated by the genus *Brocadia* (Carvajal-Arroyo et al., 2013). The average size of the Anammox granules was 2.4 ± 0.6 mm. This value was calculated by image analysis of a photograph of a granular sludge sample using the software ImageJ. Batch activity tests were performed in duplicate and incubated in an orbital shaker (160 rpm) in a dark climate controlled room at 30 ± 2 °C. The serum flasks (160 mL) were supplied with 100 mL basal mineral medium (Sun et al., 2011) and inoculated with 0.71 g VSS L⁻¹ of anammox granules. The basal medium was buffered with NaHCO_3 (4 g L⁻¹) unless otherwise indicated. Subsequently the serum flasks were sealed with rubber stoppers and aluminum crimp seals. In bicarbonate buffered experiments, the liquid and headspace were flushed with a gaseous mixture of He/ CO_2 (80/20, v/v), leading to a pH ranging 7.1–7.3. When HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) (5.95 g L⁻¹) was used, the pH of the medium was adjusted to 7.2 with a concentrated solution of NaOH, and the liquid and the headspace of the serum flasks were flushed with He gas. Incubations were carried out in an orbital shaker (160 rpm) in a dark, climate controlled room at 30 ± 2 °C.

In experiments with “fresh biomass” the inoculum was withdrawn from the nursing reactor immediately before the preparation of the experiments. In experiments performed with “starved” biomass, the bottles were incubated as described above, in absence of N sources for a defined period of time (starvation period). After the starvation period, the biomass was decanted, washed and replenished with buffered fresh mineral medium. Then the flasks were sealed and purged with He/ CO_2 as previously described.

The substrates were added by injection of concentrated solutions of NaNO_2 and NH_4HCO_3 . In pre-exposure experiments, the bottles were supplemented with NO_2^- , and incubated for a “pre-exposure period” of 24 h (resting cells) prior to addition of the NH_4^+ . In simultaneous exposure experiments (metabolically active cells), both substrates were fed together to the concentration desired in each experiment.

2.2. Continuous bioreactors

Three laboratory-scale upflow anammox reactors (500 mL) were operated in parallel. Each reactor was inoculated with 1.43 g VSS L⁻¹ of anammox granular sludge and incubated in a dark climate controlled room at 30 ± 2 °C. The reactors were fed with a basal mineral medium (described above), and operated always at a hydraulic retention time of 0.25 d. The feeding media contained 4 g L⁻¹ of NaHCO_3 , and was flushed with He/ CO_2 (80/20; v/v) to make it anaerobic and provide a pH of 7.2–7.3. The reactors were operated for 136 days. NO_2^- and NH_4^+ were fed to the reactors at a molar ratio of 1.2 ($\text{NO}_2^-:\text{NH}_4^+$). The NLR of the reactors was varied in four different stages, 0.95 g N L⁻¹ d⁻¹ (0–42 d), 0.20 g N L⁻¹ d⁻¹ (43–82 d), 0.10 g N L⁻¹ d⁻¹ (83–119 d) and again 0.95 g N L⁻¹ d⁻¹ (120–136 d). Three times during the operation of the reactors (days 29, 78 and 120), the feeding of either NH_4^+ and NO_2^- , or both was interrupted for 48 h. During these interruption events, R1 was fed with medium containing no N compounds; the feed of R2 contained just 129 mg NO_2^- N L⁻¹, but no NH_4^+ , and the R3 did not receive any NO_2^- , and the concentration of NH_4^+ in the feeding was 108 mg N L⁻¹.

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