Chemosphere 308 (2022) 136232

Contents lists available at ScienceDirect

Chemosphere

journal homepage: www.elsevier.com/locate/chemosphere

Response of mixed community anammox biomass against sulfide, nitrite and recalcitrant carbon in terms of inhibition coefficients and functional gene expressions

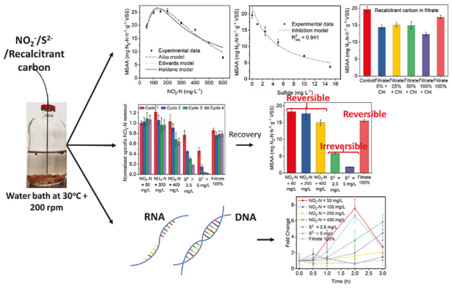
Soklida Hong [a], Haydée De Clippeleir [b], Ramesh Goel [a,*]

[a] *Civil and Environmental Engineering Department, University of Utah, 110 S Central Campus Drive, Salt Lake City, UT, 84112, United States*
[b] *DC Water, 5000 Overlook Ave. SW, Washington, DC, 20032, United States*

HIGHLIGHTS

- Nitrite caused substrate inhibition with an inhibition coeff. K_{I,NO_2^-} of 324 mgN/L.
- Nitrite inhibition was reversible up to 400 mg N/L.
- Sulfide irreversibly inhibited (toxic) anammox activity with $K_{I,S^{2-}}$ of 4.39 mg/L.
- Recalcitrant carbon had minimal effects on anammox activity.
- *HzsB* expression correlated with sulfide inhibition but not with nitrite inhibition.

GRAPHICAL ABSTRACT



ARTICLE INFO

Handling Editor: Dr Y Yeomin Yoon

Keywords:
Anammox
Inhibition
Sulfide
Nitrite
Recalcitrant carbon
Thermal hydrolysis process
Hzs gene

ABSTRACT

Anaerobic ammonium oxidation (anammox) has evolved as a carbon and energy-efficient nitrogen management bioprocess. However, factors such as inhibitory chemicals still challenge the easy operation of this powerful bioprocess. This research systematically evaluated the inhibition kinetics of sulfide, nitrite, and recalcitrant carbon under a genomic framework. The inhibition at the substrate and genetic levels of sulfide, nitrite and recalcitrant carbon on anammox activity was studied using batch tests. Nitrite inhibition of anammox followed substrate inhibition and was best described by the Aiba model with an inhibition coefficient K_{I,NO_2^-} of 324.04 mg N/L. Hydrazine synthase (*hzsB*) gene (anammox biomarker) expression was increased over time when incubated with nitrite up to 400 mg N/L. However, despite having the highest specific nitrite removal (SNR), the expression of *hzsB* at 100 and 200 mg N/L of nitrite was more muted than in most other samples with lower SNRs. Sulfide severely inhibited anammox activities. The inhibition was fitted with a Monod-based model with a $K_{I,S^{2-}}$ of 4.39 mg S/L. At a sulfide concentration of 5 mg/L, the *hzsB* expression decreased throughout the experiment from its original value at he beginning. Recalcitrant carbon of filtrate from thermal hydrolysis process pretreated anaerobic digester had a minimal effect on maximum specific anammox activity (MSAA), and thus the value of the inhibition coefficient could not be calculated. At the same time, its *hzsB* expression profile was similar to that in the control. Resiliency and recovery tests indicated that the inhibition of nitrite (up to 400 mg N/L) and recalcitrant carbon (in 100% filtrate) were reversible. About 32% of MSAA was recovered after repeated

---

* Corresponding author.
E-mail addresses: soklida.hong@utah.edu (S. Hong), Haydee.DeClippeleir@dcwater.com (H. De Clippeleir), ram.goel@utah.edu (R. Goel).

https://doi.org/10.1016/j.chemosphere.2022.136232
Received 18 July 2022; Received in revised form 23 August 2022; Accepted 24 August 2022

exposures to sulfide at 2.5 mg/L, while at 5 mg/L, the inhibition was irreversible. Findings from this study will be helpful for the successful design and implementation of anammox in full-scale applications.

1. Introduction

Anaerobic ammonium oxidation (anammox) has emerged as an efficient process for inorganic nitrogen management in liquid waste streams (Cao et al., 2017; Li et al., 2018). Compared to the conventional nitrification-denitrification process, the anammox process effectively removes inorganic nitrogen with less aeration and no organic carbon addition, has a smaller footprint, potentially causes less sludge handling costs, and has a lesser production of nitrous oxide (N_2O), a potent greenhouse gas (Azari et al., 2017; Cao et al., 2017; Du et al., 2019; Kamp et al., 2019; Kosgey et al., 2020; Laureni et al., 2015; Wen et al., 2020).

The anammox process has been employed as a sidestream application mainly to treat filtrate/centrate from conventional anaerobic digester (AD) and thermal hydrolysis process pre-treated AD (THP-AD) (Daigger et al., 2011; Driessen et al., 2020; Driessen and Hendrickx, 2021; Izadi et al., 2021; Joss et al., 2009; Kotay et al., 2013; Lackner et al., 2014; Lemaire and Christensson, 2021; van Dongen et al., 2001; Wang et al., 2022). However, mainstream applications of anammox are also on the rise to manage ammonium nitrogen in municipal wastewater treatment plants (Cao et al., 2017; Kamp et al., 2019; Li et al., 2018; Lotti et al., 2015; Ma et al., 2020; Wett et al., 2015). Nevertheless, sidestream applications of anammox are common with several full-scale applications in Europe, as well as some pilot and a few full-scale installations in the United States (Azari et al., 2017; Bhattacharjee et al., 2017; Gonzalez-Gil et al., 2015; Gonzalez-Martinez et al., 2015; Klaus et al., 2017; Lackner et al., 2014; Lawson et al., 2017; Nsenga Kumwimba et al., 2020; Trinh et al., 2021; Yang et al., 2021).

Anammox bacteria are known for their slow growth, and thus, process manipulations have been incorporated to tackle challenges associated with the slow growth of anammox bacteria (Hu et al., 2010). Furthermore, anammox activities are sensitive to external toxic substances like metals and sulfides in the feedstocks (Bi et al., 2014; Jin et al., 2013a; Zhang et al., 2016). Nitrite toxicity through substrate inhibition has also been well demonstrated in anammox bacteria (Carvajal-Arroyo et al., 2014; Dapena-Mora et al., 2007; De Prá et al., 2016; Fernández et al., 2012; Jin et al., 2012; Kimura et al., 2010; Li et al., 2016; Lotti et al., 2012; Puyol et al., 2014). With the rise of innovations in anaerobic digester technologies, such as using thermal hydrolysis (THP) as a pretreatment, anaerobic effluents are more complex (containing recalcitrant carbon) than those coming out of the conventional digestion process (Barber, 2016). However, the response of anammox bacteria to these more complex effluents is not fully understood.

Sulfide is commonly found in anaerobic effluent with concentrations of 0.03–25 mg/L (Rodríguez-Gómez et al., 2005) and raw sewage ranging from 0 to 4.3 mg/L (Liang et al., 2019). Sulfide inhibition has also been reported on anammox processes, though with conflicting findings (Jin et al., 2013a). Van De Graaf et al. (1996) found that sulfide, when supplied as pulsation at 64 mg/L, enhanced anammox activity by promoting ammonium uptake. Later studies have proven otherwise, though without an agreement on the threshold dose of sulfide inhibition. Dapena-Mora et al. (2007) reported that sulfide negatively affected anammox with 80% inhibition at 32 mg/L. Similar findings were also reported by Carvajal-Arroyo et al. (2013) in a granular enrichment culture of anammox. However, Jin et al. (2013a) reported a relatively high concentration of sulfide inhibition with a 50% reduction of anammox activity at 264 mg/L. Jin et al. (2013b) concluded that sulfide was inhibitory rather than toxic to anammox, meaning the inhibition was reversible, contrasting a conclusion made by Carvajal-Arroyo et al. (2013). Additionally, these studies did not look at sulfide toxicity to anammox bacteria at the genetic level, and the information on the inhibition coefficient for sulfide toxicity to anammox is seldom reported.

Nitrite is both a substrate and electron acceptor in anammox biochemistry. However, in excess, nitrite has been shown to be toxic to anammox bacteria (Jin et al., 2012). A wide range of nitrite inhibition thresholds of anammox has been documented. Depending on studies, elevated nitrite concentrations ranging from 100 to 400 mg N/L have been shown to cause a 50% reduction of anammox activity, while the reversibility of its toxicity has remained unsettled (Carvajal-Arroyo et al., 2013; Kimura et al., 2010; Lotti et al., 2012; Strous et al., 1999). In a survey by Lackner et al. (2014), nitrite build-up in full-scale partial nitritation/anammox plants was common, with 50% of them encountering the problem. Much like in the case of sulfide toxicity, findings about nitrite toxicity have been conflicting and evidence at the gene expression level is not common.

THP is employed as a pretreatment to accelerate biomass hydrolysis towards efficient digestion of sludge to generate more methane gas (Schieder et al., 2000). However, THP could cause more recalcitrant carbon to solubilize and eventually escape through the AD process. This recalcitrant carbon could potentially inhibit anammox bacteria and pose challenges for sidestream treatment of such anaerobic effluents. Recalcitrant carbon concentration up to 5000 mg/L as chemical oxygen demand (COD) was reported in THP-AD filtrates (Zhang et al., 2016). It has been reported that high concentrations of organic carbon in THP-AD filtrate severely impacted anammox activity (Zhang et al., 2018).

While many studies have investigated the inhibition of anammox by nitrite, sulfide, and recalcitrant carbon, most of them focused mainly on anammox's engineering side rather than the underlying genetic mechanisms. The information on the inhibition coefficients for these chemicals on anammox is also unclear. Most of the previous efforts were independent and employed different anammox enrichment or pure cultures to study these inhibitory responses. Wang et al. (2016) studied the expression of anammox functional genes in response to nitrite shock while using a highly enriched culture of *Candidatus Kuenenia stuttgartiensis*. However, different anammox species may use different routes with different intermediates or possess other enzymes in the anammox reaction (Kartal and Keltjens, 2016; Oshiki et al., 2016a). This diversity suggests that different anammox species may respond differently to different stress. Therefore, there is a need to simultaneously study the effect of common inhibitors frequently found in anammox processes using the same enrichment culture.

The main objectives of this study were to examine the effects of nitrite, sulfide, and recalcitrant carbon; (1) on maximum specific anammox activity (MSAA), (2) to determine inhibition coefficients, (3) to study whether the inhibitory effects are reversible or irreversible, and (4) to study genetic responses of anammox bacteria as gene expression levels. In this study, a mixed culture of enriched anammox consortium fed with real AD filtrate collected from a local municipal wastewater treatment plant was used to study the inhibitory effects of nitrite, sulfide, and recalcitrant carbon on anammox engineering performance and gene expressions. Batch experiments were conducted and anammox biomass was taken from a stable two-stage anammox reactor operational in Dr. Ramesh Goel's Lab (Dept. of Civil & Environmental Engineering, University of Utah) and reported elsewhere (Kotay et al., 2013; Lawson et al., 2017). Findings from this study will improve our understanding of the inhibition of anammox by NO_2^-, S^{2-} and recalcitrant carbon at the genetic level and allow a systematic comparison of the effects of these inhibitors on the same anammox bacteria consortia. These understandings will help for troubleshooting as well as improving the efficiency of the anammox reactor. Moreover, the value of the inhibition coefficients will assist the anammox process's design and robustness for pilot and full-scale applications.

2. Materials and methods

2.1. Enriched anammox biomass

Biomass in granular forms, with a diameter ranging from 1 to 5 mm, was collected from a bench-top two-stage anammox reactor operational in Dr. Goel's laboratory in the Department of Civil and Environmental Engineering at the University of Utah. The reactor has been fed with effluent from a bench-top partial nitritation reactor, which receives real anaerobic digester filtrate from a local municipal wastewater treatment plant, Central Valley Water Reclamation Facility (CVWRF, Salt Lake City, UT). The details of this reactor can be found elsewhere in our earlier publications (Kotay et al., 2013; Lawson et al., 2017). The granular biomass was collected from the reactor in a sieve (opening size of 0.425 mm) and washed first with distilled water, then with the feed medium solution. The wet biomass was weighted to a serum bottle at 700 mg/25 mL of medium solution (section 2.2) to obtain consistent volatile suspended solids (VSS) concentrations at ~ 1 g/L. Anammox activities have been shown to be independent of the biomass concentrations ranging from 0.2 to 4 g VSS/L (Lotti et al., 2012). From batch to batch, biomass was taken from the reactor at the exact same point of the cycle, where ammonium, nitrite, and nitrate concentrations in the reactor were also measured. Microbial community characteristics of the biomass were analyzed using QIIME2 software based on the V4 gene region sequencing of the 16 S rRNA gene (Bolyen et al., 2019; Kozich et al., 2013).

2.2. Medium and stock solutions preparation

The synthetic medium was used in all experiments except in experiments with recalcitrant carbon. The medium solution used in the experiment contained (in 1 L): $(NH_4)_2SO_4 = 944$ mg; $KH_2PO_4 = 27.2$ mg; $NaHCO_3 = 840$ mg; $MgSO_4{\cdot}7H_2O = 58.6$ mg; $CaCl_2{\cdot}2H_2O = 73.5$ mg; and 1.25 mL of each Trace element solution 1 and 2 (Van De Graaf et al., 1996). The pH of the medium solution was adjusted to 7.5 by either 1 N H_2SO_4 or 1 N NaOH. To evaluate the effect of recalcitrant carbon on anammox activity, different proportions of filtrate from THP-AD (DC Water, Washington DC) mixed with the synthetic medium were used. Ammonium in THP-AD filtrate was stripped out by increasing the pH to 9.5 using NaOH, which was then brought back to 7.5 by 10 N H_2SO_4. A stock solution of NO_2–N was prepared from $NaNO_2$ at a 10 g N/L concentration and stored in an amber bottle at 4 °C. Sulfide stock solution at 3200 mg/L was prepared per the Standard Methods (APHA, 2005). In brief, $Na_2S{\cdot}9H_2O$ crystals were collected on a plastic spoon. The crystals were then rinsed with deionized (DI) water (pre-purged with N_2) to remove surface contaminants (polysulfides, polythionates, and sulfate) and blotted with a clean tissue repeatedly until clear crystals were obtained. The clear crystals were then quickly weighed and dissolved in degassed DI water. The sulfide stock solution was stored in an air-tight vial at room temperature.

2.3. Maximum specific anammox activity (MSAA) tests

In a 60-mL serum bottle, 25 mL of the medium solution/filtrate (for recalcitrant carbon inhibition tests) and 700 mg of biomass were added. The bottle was sealed with an air-tight PTFE-silicone septum. The bottle was then purged with a gas mixture of N_2/CO_2 (95%/5%) for 10 min to remove dissolved oxygen before transferring to a water bath shaker operated at 200 rpm and 30 °C for another 10 min. After reaching equilibrium, the build-up pressure in the bottle resulting from the rising temperature was released using a needle with a tubing whose other end was soaked in water. Then, nitrite or nitrite/sulfide stock solution was injected at the desired final concentration. Two mL of the sample was withdrawn for chemical analysis. The pressure was immediately measured using a manometer (3461CC, Traceable, Texas, USA). Pressure values were recorded every 10–15 min for 2–3 h. At the end of the experiment, the solution was thoroughly mixed, and 10 mL of the solution was filtered for VSS analysis and the filtrate was used for chemical analysis. Several controls were also run, including abiotic control and assays without ammonium or nitrite. A curve of N_2 produced over time was constructed from the built-up pressure using the ideal gas law, and the maximum slope of the curve was the N_2 production rate. MSAA was calculated by dividing the N_2 production rate by the mass of VSS (Dapena-Mora et al., 2007). Another similar batch test was conducted to verify the results of MSAA from N_2 gas measuring. In this test, nitrite and ammonium were measured, and MSAA was determined by the nitrite consumption rates instead. All experiments were conducted in triplicate.

2.4. Gene expression tests

These experiments were carried out in parallel to the MSAA tests. A 60-mL medium solution/filtrate in a 125-mL serum bottle was used, and NO_2^--N and NH_4^+-N concentrations were monitored for anammox activity. 10-mL of the well-mixed solution was filtered for VSS analysis at the beginning of the experiment. Instead of recording pressure, samples were taken every 0.5–1 h for the gene expression tests for 3 h. About 5 mL of sample, including biomass, was collected at each sampling interval using a needle and a syringe. After leaving it idle in the syringe for a minute, the settled biomass was transferred to a microfuge tube with glass beads and then 600 μL of the RNA lysis buffer was added. The remaining supernatant was filtered through a 0.22 μm pore-size membrane for chemical analysis. The biomass was homogenized using a bead beater (Omni International, GA). RNA extraction was performed immediately or the next day by storing the lysate at 4 °C overnight. All experiments were conducted in triplicate.

2.5. Resiliency and recovery tests

In these tests, 35 mL of medium/filtrate and 1 g of washed biomass were placed in 60-mL serum bottles. VSS was analyzed at the beginning of the experiments using 10 mL of the solution. The bottles were then sealed and purged with N_2/CO_2 (95%/5%) for 10 min. Then, nitrite or nitrite/sulfide stock solution was injected at the desired final concentration. Two mL of the sample was withdrawn for chemical analysis. The bottles were then placed in a water bath shaker operated at 200 rpm and 30 °C for 1 h 55 min. The bottles were left idle for 5 min after which the supernatant was withdrawn entirely and filtered for chemical analysis without removing the biomass. The settled biomass was washed with 5 mL of a fresh medium twice. A new cycle was started by adding 25 mL of the medium/filtrate and proceeded as mentioned above. After 4 cycles, the biomass was subjected to a fifth cycle for a prolonged time of 12 h, where all other variables remained constant. In cases when toxicity was recorded during the fifth cycle, the biomass was suspended in 40 mL of a fresh medium with NO_2^--N at ~20 mg N/L for up to 48 h for recovery. During recovery, nitrite concentration was checked periodically and added when it became less than 5 mg N/L. MSAA was tested on the recovered biomass with the procedure as described in section 2.3. All tests were conducted in triplicate.

2.6. RNA and DNA extraction and quantitative RT-qPCR

Total RNA was extracted using Quick-RNA Kits (Zymo Research, CA). Genomic DNA was removed by an on-column DNase set included in the kits. The quantity and quality of RNA were checked using NanoDrop (ThermoFisher, MA). Reverse transcription for cDNA synthesis was conducted using the High-Capacity cDNA Reverse Transcription Kit (ThermoFisher, MA) by adding 1000 μg RNA for each sample. Trace DNA contamination in the RNA samples was evaluated by running negative reverse transcription control (NRTC). The average difference in C_T (threshold cycle) value between cDNA and NRTC was about 10 cycles, suggesting that DNA contamination was negligible. DNA was isolated using Qiagen PowerSoil Kits per the manufacturer's protocols.

Quantitative reverse transcription RT-PCR was done to quantify anammox 16 S rRNA gene (DNA) and *hzsB* genes (cDNA) using different primer sets, as listed in Supplementary Table S1. Each reaction mixture (20 μL) contained 1 μL of template, 10 μL of SYBR Green master mix (ThermoFisher Scientific, USA), 1 μL of each primer (forward and reverse, 10 μM), and the remainder (7 μL) was nuclease-free water. The qPCR conditions for each reaction were applied according to the references listed in Table S1 (Tsushima et al., 2007; Wang et al., 2012). Plasmid DNA with a known concentration was used to construct calibration curves with five data points ($R^2 > 0.98$). Gene expression was calculated using the ratio of copies of *hzsB* mRNA (cDNA) to copies of the 16 S rRNA gene and normalized to the results of time 0. qPCR analysis was carried out in triplicate on QuantStudio3 (ThermoFisher Scientific, USA).

2.7. Inhibition models and kinetic coefficients

2.7.1. Nitrite inhibition

Nitrite inhibits anammox activity through substrate inhibition. In this study, commonly used mathematical models, including Haldane, Eq. (1) (Haldane, 1965); Edwards Eq. (2) (Edwards, 1970); and Aiba Eq. (3) (Aiba et al., 1968) were used to fit the experimental data of the nitrite inhibition from Section 2.3. The best model selection was based on evaluating the goodness-of-fit statistics and the Akaike Information Criterion (AIC) scores (Akaike, 1974). Firstly, the *P* value was assessed. Generally, when $P < 0.05$, it can be concluded that the independent variable can be used to predict the dependent variable. Secondly, adjusted R^2 (R^2_{adj}), *F* value, and AIC scores were considered to choose the best models. A larger R^2_{adj} (close to 1) indicates that the model fits better with the empirical data, whereas a large *F* value (>1) implies that the independent variables contribute to the prediction of the dependent variable. Lower AIC means a lower relative amount of information lost by the model, resulting in a better model.

$$MSAA = \frac{MSAA_{max} S}{K_S + S + \frac{S^2}{K_{I,NO_2^-}}} \tag{1}$$

$$MSAA = MSAA_{max}\left(\exp\left(-\frac{S}{K_{I,NO_2^-}}\right) - \exp\left(-\frac{S}{K_S}\right)\right) \tag{2}$$

$$MSAA = \frac{MSAA_{max} S}{K_S + S}\exp\left(-\frac{S}{K_{I,NO_2^-}}\right) \tag{3}$$

where *MSAA* is the empirical MSAA (mg N_2–N/h·g VSS) values in response to different NO_2^--N concentrations, $MSAA_{max}$ is the maximum theoretical MSAA (mg N_2–N/h·g VSS), *S* is the NO_2^--N concentration (mg N/L), K_S is the half-saturation coefficient (mg N/L), and K_{I,NO_2^-} is the inhibition coefficient for nitrite (mg N/L).

2.7.2. Sulfide and recalcitrant carbon inhibition

A Monod-based model (Eq. (4)) was used to describe the inhibition of anammox by sulfide and recalcitrant carbon. Preliminary analyses showed that Edwards, Haldane and Aiba models were not a good fit for sulfide and recalcitrant carbon inhibitions.

$$MSAA = MSAA_{max}\left(\frac{S}{S + K_S}\right)\left(\frac{K_{I,S^{2-}}}{I + K_{I,S^{2-}}}\right) \tag{4}$$

where *MSAA* is the empirical MSAA (mg N_2–N/h·g VSS) values in response to different sulfide/recalcitrant carbon concentrations, $MSAA_{max}$ is the maximum theoretical MSAA (mg N_2–N/h·g VSS), *S* is the NO_2^--N concentration (mg N/L), K_S is the half-saturation coefficient (mg N/L), $K_{I,S^{2-}}$ is the inhibition coefficient (mg/L), and *I* is the concentration of sulfide/recalcitrant carbon (mg/L). Note that for sulfide and recalcitrant carbon tests, NO_2^--N was fixed in all tests at 50 mg N/L. Therefore, *S* was constant (50 mg N/L), whereas $MSAA_{max}$ and K_S were obtained from the nitrite inhibition fitting.

2.8. Other analytical methods

NO_2^--N and NO_3^--N were analyzed via an ion chromatographic method (883 Basic IC Plus, Metrohm), while NH_4–N and sulfide were measured using HACH kits (HACH, USA). The concentrations of total and volatile suspended solids were analyzed per the Standard Methods (APHA, 2005). Dissolved organic carbon (DOC) was quantified with a Shimatzu TOC analyzer (TOC-V CSN).

One-way ANOVA with Tukey's test was used to compare the difference between the data points. A *P* value < 0.05 was considered significant. Statistical analyses and model fittings were performed using OriginPro, Version 2021b (OriginLab Corporation, Northampton, MA, USA).

Specific nitrite removal (SNR, mg N/g VSS) was calculated using Eq. (5).

$$SNR = \frac{NO_2^- \text{-} N_0 - NO_2^- \text{-} N_t}{VSS} \tag{5}$$

where NO_2^--N_0 is the concentration of NO_2^--N at time 0 (mg N/L), NO_2^--N_t is the concentration of NO_2^--N at time t, and VSS is the biomass concentration (g/L).

3. Results and discussion

3.1. Biomass microbial taxonomy

Microbial compositions of the biomass collected from the serum bottle are shown in Fig. 1 at the phylum and genus levels. The phylum of Planctomycetes, to which all known anammox lineages belong (Kartal et al., 2013), was found as the most abundant phylum comprising 46.7% of the total bacterial population. At the genus level, *Candidatus Brocadia* was the most abundant (44.6%), along with substantial populations of heterotrophic bacteria *Ignavibacterium* (13.5%) and heterotrophic denitrifiers *Denitratisoma* (4.7%). *C. Brocadia* has been frequently reported as the most abundant anammox genera enriched in full-scale anammox reactors (Gonzalez-Martinez et al., 2015; Speth et al., 2016). Additionally, due to the use of the actual filtrate (through partial nitritation pretreatment), the microbial characteristics of the biomass were similar to what are usually found in full-scale two-stage anammox reactors (Gonzalez-Gil et al., 2015). This resemblance suggests that the biomass used in this study was a good representation of the microbial community found in full-scale anammox reactors.

3.2. MSAA and hzsB gene expression for nitrite inhibition

In this study, NH_4^+-N was fixed at 200 mg/L in all tests unless otherwise stated. Ammonium was supplied in excess, ensuring that it did not become a limiting substrate. All tests were conducted with an initial pH of 7.5. Around this pH level, studies have reported that a total ammonium concentration of 200 mg N/L had a negligible inhibitory effect on anammox activity (Carvajal-Arroyo et al., 2013; Dapena-Mora et al., 2007; Jin et al., 2012; Strous et al., 1999).

MSAA based on specific N_2 production rates was determined for NO_2^--N concentrations ranging from 50 to 600 mg N/L (Fig. 2). At an initial nitrite concentration of 50 mg N/L, MSAA was 19.62 mg N_2–N/h·g VSS. In their study of nitrite inhibition in anammox, Lotti et al. (2012) also reported a similar MSAA value of 19.08 mg N_2–N/h·g VSS with an initial nitrite concentration of 50 mg N/L. MSAA increased with increasing nitrite concentrations from 50 to 200 mg N/L, at which the highest MSAA of 25.39 mg N_2–N/h·g VSS was recorded, significantly higher than the MSAA value at 50 mg N/L. At NO_2^--N above 200 mg N/L, MSAA decreased as the nitrite concentration increased. At 400 mg N/L

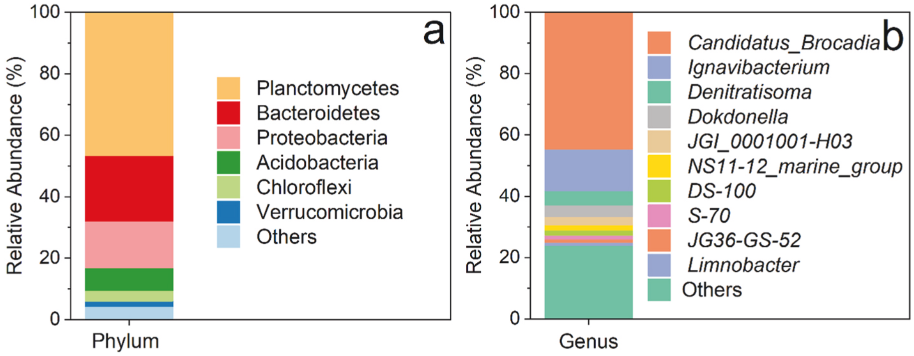


Fig. 1. Microbial community compositions of the enrichment anammox biomass at the (a) phylum and (b) genus level. Others are unassigned, unknown, and taxa with small relative abundance.

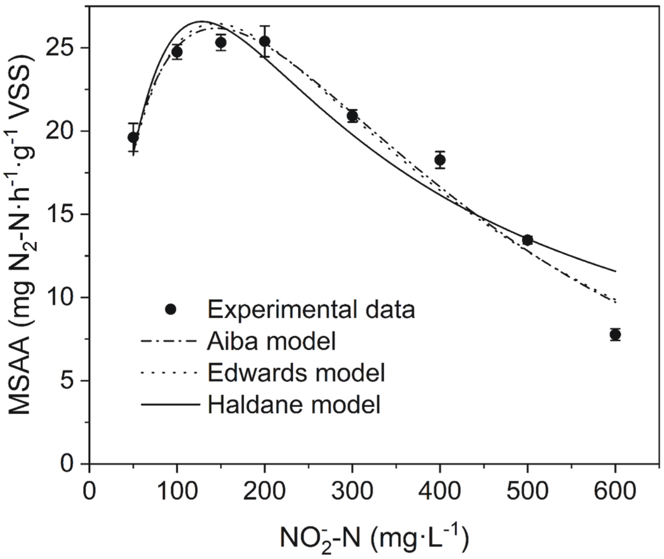


Fig. 2. Maximum specific anammox activity (MSAA) at different initial NO_2^--N concentrations. All samples were tested in the synthetic medium with an initial NH_4^+-N concentration of 200 mg N/L.

of NO_2^--N, MSAA dropped to 18.25 N_2–N/h·g VSS below that of NO_2^--N at 50 mg N/L. At the highest tested NO_2^--N concentration of 600 mg N/L, MSAA dropped by 70% from its highest value at 200 mg N/L. In another batch experiment with initial NO_2^--N concentrations at 50, 100, and 200 mg N/L, similar values and trend (Supplementary Table S2) of MSAA based on nitrite consumption rates were also obtained. These results verified that the N_2-based MSAA accurately calculated MSAA (results not included). This inhibition pattern suggests that nitrite inhibition in anammox processes followed the typical substrate inhibition of an enzymatic reaction where the kinetics rose to a maximum before descending as the substrate concentration increased. Wang et al. (2016), while studying the expression of anammox functional genes in response to nitrite shock, also noted that specific anammox activity was highest at 200 mg N/L of nitrite. A similar trend of nitrite inhibition was also shown in a study by Dapena-Mora et al. (2007), with the highest MSAA recorded at 140 mg N/L. Besides anammox bacteria, nitrite has been reported to induce substrate inhibition in nitrite-oxidizing and denitrifying microorganisms (Carrera et al., 2004; Glass et al., 1997).

Table 1 summarizes the fitting results of the three models and their statistical criteria. Firstly, all coefficients (Coeff) predicted by Aiba and Edwards models had a P value < 0.05, while all coefficients from the Haldane model had a P value > 0.05. In this case, the Haldane model was not valid for describing nitrite inhibition of anammox. Secondly, the Aiba model had a higher R^2_{adj} and F value and a lower AIC score than those of the Edwards model. Thus, the Aiba model was chosen to describe the nitrite inhibition of anammox. It should be noted that the Aiba model (Eq. (3)) is just the Monod equation in the event of no inhibition, where K_I is infinite, making the exponential equal to 1.

Table 1
Summarized results and statistical criteria of the inhibition models.

Model		Coeff	P	R^2_{adj}	F	AIC
Aiba	$MSAA_{max}$	73.9	0.007	0.958	680	21.38
	K_S	116.69	0.041			
	K_{I,NO_2^-}	324.03	0.0005			
Edwards	$MSAA_{max}$	46.58	0.002	0.944	504	23.77
	K_S	68.27	0.003			
	K_{I,NO_2^-}	386.91	0.002			
Haldane	$MSAA_{max}$	*253.01*	*0.665*	0.875	223	30.24
	K_S	*546.28*	*0.699*			
	K_{I,NO_2^-}	*30.09*	*0.688*			

Coefficients values in *italics* do not pass the *P criterion* (<0.05).

The Aiba model predicted an $MSAA_{max}$ of 73.9 mg N_2–N/h·g VSS. The ratio between the observed maximum MSAA (25.39 mg N_2–N/h·g VSS) and the theoretical $MSAA_{max}$ was 0.34. This relatively small ratio value suggests that increasing nitrite concentration from 50 mg N/L resulted in a gradual increase in nitrite inhibition. The threshold concentration of nitrite inhibition was 145 mg N/L (Aiba model's maximum point), at which higher NO_2^--N concentrations would cause a decrease instead of an increase in MSAA.

The half-saturation coefficient K_S was found to be 116.69 mg N/L. Literature values of K_S for nitrite have a wide range, from as low as 0.003 to as high as 56.4 mg N/L (De Prá et al., 2016; Oshiki et al., 2016b; Zhang and Okabe, 2020). The enormous difference in the reported values of K_S is mainly due to the use of different inhibition models as well as different anammox genera and the forms and sizes of the anammox biomass in those studies. In fact, the K_S value in this study predicted by the Edwards model was 68.27 mg N/L, similar to the value (56.4 mg N/L) reported by De Prá et al. (2016), who also used the Edwards model. Generally, it has been found that anammox bacteria in the genus of *C. Brocadia* tend to have a higher half-saturation coefficient K_S for nitrite than other genera (Oshiki et al., 2016b; Zhang and Okabe, 2020). Aggregates of anammox biomass into granules also induce mass transfer resistance, resulting in a higher K_S value. Lotti et al. (2014) found that K_S increased exponentially with increasing aggregate sizes up to 700 μm. Therefore, the high K_S value found in this study could be due to the abundance of anammox in the genus of *C. Brocadia* and the relatively large granules of the biomass (1–5 mm in diameter in this case, as determined by scanning electron microscopy).

The Aiba model predicted the nitrite inhibition coefficient K_{I,NO_2^-} to be 324.03 mg N/L. De Prá et al. (2016) have reported a K_{I,NO_2^-} value of 525.34 mg N/L for nitrite in anammox granular sludge. Other studies have also reported different values of K_{I,NO_2^-} ranging from 105 to 224 mg N/L (Zhang et al., 2017). The differences in reported K_{I,NO_2^-} values could be due to different niches of anammox species. Like K_S, K_{I,NO_2^-} has also been found to be species-dependent (Oshiki et al., 2016b; Zhang et al., 2017; Zhang and Okabe, 2020). The sizes of the granules may have played an essential role in the varying K_{I,NO_2^-} values due to the differences in mass transfer. Therefore, another batch experiment was conducted to measure the MSAA of broken anammox granules to test the effect of the granule size on K_S and K_{I,NO_2^-}. Intact anammox granules in a sealed bottle were homogenized by passing 5 times through an 18-gauge needle (0.838 mm in inner diameter) attached to a syringe. The MSAA values of the broken granules at varying nitrite concentrations (50, 100, 200 and 600 mg N/L) are shown in Supplementary Table S3. The broken granules had the highest MSAA at the initial nitrite concentration of 50 mg N/L, despite at 200 mg N/L for the intact granules. This result suggests that the smaller granules had a lower substrate saturation concentration and therefore a lower K_S than the bigger granules. However, at 600 mg N/L, nitrite exerted more inhibition on the anammox activity in the broken granules with an MSAA of 3.66 mg N_2–N/h·g VSS compared to 7.77 mg N_2–N/h·g VSS for the intact granules. This means that the smaller granules were more sensitive to high nitrite concentration than their bigger granule counterparts, or in other words, had a lower K_{I,NO_2^-}. As expected, lower K_S and K_{I,NO_2^-} values were predicted from the Aiba model for the broken granules with K_S at 58.09 mg N/L and K_{I,NO_2^-} of 239.51 mg N/L. It should be noted that these values are only used to point out the drop in K_S and K_{I,NO_2^-} in the smaller granules. For a more accurate prediction of the coefficients, more data points are needed. These findings have practical implications. Although bigger granules have slower activities than smaller granules at nitrite concentrations usually maintained in full-scale anammox reactors, they provide better protection for anammox bacteria from high nitrite concentrations and potentially from other inhibitors such as O_2 during reactor failures. Depending on the operation conditions, a balance between small and big granules should be maintained for high anammox activities and reactor stability.

Hzs (hydrazine synthase enzyme), which catalyzes the production of the intermediate hydrazine, is unique and found in all five known genera of anammox, while its function is irreplaceable by other enzymes (Harhangi et al., 2012; Kuypers et al., 2018; Zhou et al., 2017). A parallel batch experiment to the MSAA tests was conducted to investigate the expression of the *hzsB* gene. Fig. 3a shows specific nitrite removals (SNRs) of samples throughout the experiment. It should be noted that anammox activities in this test were in line with the results of MSAA (section 3.2) and repeated (section 3.5) tests. For example, samples with initial nitrite at 100 and 200 mg N/L had the highest nitrite removal efficiency in all these tests. These results suggest consistency of anammox activities across all of the experiments in this study. Fig. 3b depicts the expression of the *hzsB* gene in response to stress over time. At 50 mg/L of NO_2–N, the *hzsB* gene expression increased with the decrease of nitrite (Fig. 3b) in the first 2 h. After that, when nitrite was exhausted, *hzsB* expression also decreased in the next hour. The expression increased almost 8 folds higher ($P < 0.05$) at its peak at 2 h than the initial value at time 0. With nitrite at 400 mg N/L, the expression of *hzsB* gradually increased from time 0–3 h with 5.86 folds ($P < 0.05$) even though, as found in the earlier MSAA test, nitrite was inhibitory at this concentration. Interestingly, there were only slight increases in *hzsB* expression from time 0–3 h in samples with nitrite at 100 (1.47, $P < 0.05$) and 200 mg N/L (2.06, $P < 0.05$), despite having the highest SNR. It is surprising that at the highest SNRs, the expression of *hzsB* was more muted when compared to samples with lower SNRs. Wang et al. (2016) also reported a similar trend for *hzsA*, *nirS*, and *hdh* gene expression in anammox bacteria after exposing them to different nitrite concentrations. These results suggest that the increase in the observed anammox activity did not necessarily imply increasing *hzsB* expression. Reed et al. (2010) suggested that substrate inhibition might have diverse and essential biological functions. For example, they hypothesized that the substrate inhibition of phosphofructokinase (one of the enzymes in ATP production) by ATP is to ensure that resources are not devoted to producing ATP when it is plentiful. This hypothesis may be relevant to the phenomenon observed here at the mRNA level. At initial nitrite concentrations of 100 and 200 mg N/L, Hzs already operated at its highest rate (from the MSAA experiment), while simultaneously, energy may have been overproduced (from high and constant SNRs for the whole 3 h), and thus anammox cells were in no hurry to produce more Hzs, resulting in lower expression of the gene. The cells may have diverted the surplus energy for use in other necessary cellular processes. In fact, by using a primer set specifically targeting the anammox 16 S rRNA gene (Tsushima et al., 2007; Yang et al., 2020), an extremely high expression of the gene at 3 h (~190 folds higher than at time 0, data is not shown) was found, where only slight changes were observed in between for the biomass exposed to an initial nitrite concentration of 200 mg N/L. rRNAs are essential for protein synthesis and cell growth, and thus, bacterial cells have to maintain a high abundance of rRNA corresponding to their growth (Bremer and Dennis, 2008; Otto et al., 2019). The highly increased expression of the 16 S rRNA gene may suggest that the anammox cells were preparing for cell growth. These findings are interesting and should be further investigated to see whether 16 S rRNA expression is more relevant to nitrite inhibition as well as to advance our understanding of the biology of anammox bacteria. Again, high expression of 16 S rRNA after exposure to nitrite at 200 mg N/L suggest that nitrite was not toxic to anammox bacteria at this concentration. At 400 mg N/L of nitrite, a higher expression of *hzsB* at 3 h compared to those at nitrite concentrations of 100 and 200 mg N/L could be from the anammox bacteria trying to compensate for the loss of the enzyme activity from the inhibition.

3.3. MSAA and hzsB expression for sulfide inhibition

MSAA was also tested with sulfide at 0–15 mg S/L, whereas nitrite

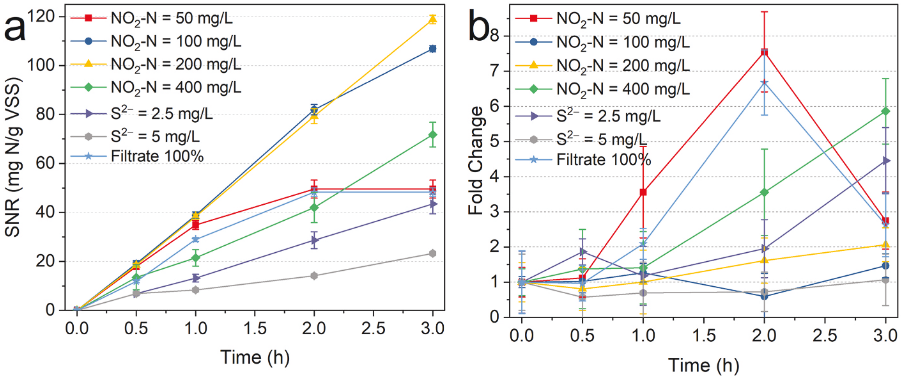


Fig. 3. (a) Specific nitrite removal (SNR) and (b) the expression of *hzsB* in response to different nitrite and sulfide concentrations and THP-AD filtrate. Fold change in each sample was calculated by normalizing *hzsB* mRNA with 16 S rRNA gene and time 0. For nitrite samples, the initial nitrite concentration is denoted in their name. For sulfide samples (sulfide concentration is denoted in their name) and Filtrate 100%, the initial nitrite concentration was 50 mg N/L. Nitrite samples and sulfide samples were tested with the synthetic medium. Filtrate 100% was tested with 100% THP-AD filtrate. All tests were carried out with an initial NH_4^+-N concentration of 200 mg/L.

was fixed at 50 mg N/L for all tests. This relatively low concentration of NO_2^--N was chosen to minimize any possibility of a co-inhibition of nitrite, which could potentially happen at higher concentrations. The presence of sulfide had a pronounced inhibitory effect on anammox activity. As shown in Fig. 4a, MSAA markedly dropped as sulfide concentration increased from 0 to 15 mg S/L. At 15 mg S/L of sulfide, anammox activity was almost completely inhibited, as MSAA dropped 80% compared to the control (without sulfide). These findings were similar to those previously reported by Dapena-Mora et al. (2007) and Carvajal-Arroyo et al. (2013). Sulfide also has been found as a potent inhibitor of nitrification and denitrification (Bejarano-Ortiz et al., 2015; Caffrey et al., 2019; Joye and Hollibaugh, 1995; Liang et al., 2020; Sørensen et al., 1980). The information on sulfide inhibition mechanisms on nitrifying and denitrifying microbial communities is limited and incomplete. It has been hypothesized that the sulfide inhibition in nitrifying and denitrifying bacteria works by sulfide binding to the heme iron of the cytochrome enzyme disrupting the enzyme activity (Bejarano Ortiz et al., 2013). The anammox activity relies almost exclusively on a wide range of cytochrome-type enzymes, such as NirS (nitrite reductase), Hzs, and Hdh (hydrazine hydrogenase) (Kartal and Keltjens, 2016; Kuypers et al., 2018), can be severely inhibited by sulfide binding to the heme iron of said enzymes. This could also explain the immediate inhibition of sulfide on anammox activity, as observed in these tests. Sulfide inhibition was fitted using a Monod-based model (Eq. (4)), with the $MSAA_{max}$ and K_S values being supplied from the Aiba model in the nitrite inhibition. The model fitted well to the experimental data with an R^2_{adj} of 0.947. The inhibition coefficient $K_{I,S^{2-}}$ of sulfide was found to be 4.39 mg S/L. This value was relatively low compared to the K_{I,NO_2^-} of nitrite (324.04 mg N/L, section 3.2), indicating that sulfide was a strong inhibitor to anammox.

The expression of *hzsB* responding to sulfide inhibition (Fig. 3b) at 2.5 mg/L increased throughout the test with a rise of 4.46 folds ($P < 0.05$) at 3 h compared to the value at time 0. At 5 mg/L of sulfide, despite gradually removing nitrite, the expression of *hzsB* in the sample was subdued throughout the test. In fact, this was the only sample in which the expression of *hzsB* dropped below the initial value at time 0 and stayed so for the remainder of the test. This suppression of *hzsB* expression suggests sulfide at 5 mg/L was toxic to anammox bacteria.

3.4. MSAA and hzsB expression for recalcitrant carbon inhibition

Organic contents in the form of recalcitrant carbon in filtrate from THP-AD tend to be much higher than from conventional AD processes (Barber, 2016). A high concentration of recalcitrant carbon and its biological inertness could induce toxicity with a sustained effect in anammox bacteria (Gu et al., 2018). The impact of recalcitrant carbon on anammox activity was studied using THP-AD filtrate obtained from DC Water (Washington DC, USA). In these tests, the initial NH_4^+-N in the filtrate was lowered to 200 mg N/L through stripping, and nitrite was fixed at 50 mg N/L. After ammonium stripping, the organic carbon in the filtrate was 582 mg/L as DOC (for detailed filtrate characteristics, refer to Supplementary Table S4). In the presence of organic carbon in the filtrate, denitrification may occur, interfering with N_2 gas quantification from anammox activity. Chloramphenicol (Chl) was reported as an effective denitrification inhibitor (Brooks et al., 1992). Chloramphenicol was added to the samples denoted with a +Chl in Fig. 4b. The addition of chloramphenicol caused a decrease in MSAA to 14.42 N_2/h·g VSS in the synthetic medium (0% Filtrate + Chl) from 19.62 N_2–N/h·g VSS in the control (synthetic medium without chloramphenicol), or about a 26.5% reduction (Fig. 4b). The results suggest that chloramphenicol also affected MSAA, similar to the results found by Phanwilai et al. (2020). Within the samples with chloramphenicol, recalcitrant carbon in filtrate at 25 and 50% had no effect on MSAA when compared to filtrate at 0%, while filtrate at 100% saw a drop of MSAA of 14.6%. An experiment with 100% filtrate without chloramphenicol was also tested. Compared to the control, there was a 12% ($P < 0.05$) drop in MSAA for the filtrate without chloramphenicol, similar to the 14.6% drop within the sample that did contain chloramphenicol. DOC concentrations (data not shown) were relatively stable throughout the test, suggesting that denitrification was negligible. Besides recalcitrant carbon a slight reduction in MSSA observed in the filtrate could also be attributed to high osmotic pressure exerted by high dissolved solids content in the filtrate (Chen et al., 2014; Dapena-Mora et al., 2010; Kartal et al., 2006; Lin et al., 2021). Due to its minimal inhibition on anammox activity, an inhibition model was not fitted for recalcitrant carbon. Our results contrast with a previous report, which found that recalcitrant carbon in filtrate drastically impaired anammox activity (Zhang et al., 2018).

The expression profile of *hzsB* with 100% Filtrate (Fig. 3b), albeit lower in increased intensity, was similar to that of the sample with NO_2–N at 50 mg/L. This similarity confirms the minimal impact of recalcitrant carbon in 100% Filtrate on the anammox activity found in the MSAA tests. The low inhibition observed in this study could be from the high abundance of anammox bacteria in the biomass and its large granular forms. The large granules could have protected the anammox bacteria from toxic compounds due to the constraint of mass transfer within the granule, as found earlier in the case of nitrite (see section 3.2). However, further research should be done to confirm this hypothesis.

3.5. Resiliency and recovery test

In the previous MSAA tests, the effects of shock exposure to nitrite, sulfide, and recalcitrant carbon were examined. However, repeated exposure to the stressors may have had a different effect on anammox activity. The resiliency of anammox activity against nitrite, sulfide and recalcitrant carbon was vigorously assessed. First, anammox bacteria

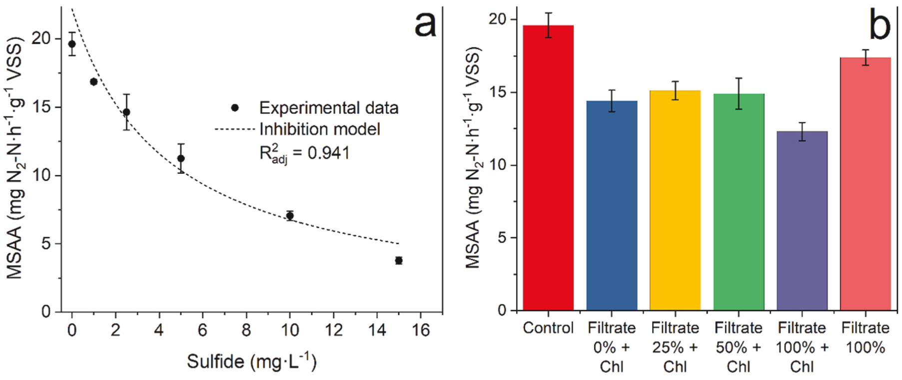


Fig. 4. MSAA at different concentrations of (a) sulfide and (b) filtrate. Chi is chloramphenicol at 300 mg/L. Control is the synthetic medium. Synthetic medium was used to dilute the THP filtrate. All samples were tested with an initial NH_4^+-N and $N0_2^-$ N concentration of 200 and 50 mg N/L, respectively.

were subjected to repeated and prolonged exposure to the inhibitors. Then the biomass was tested for its recoverability after the exposures. Fig. 5a shows the effects of repeated (2 h for each cycle) exposure to nitrite, sulfide, and recalcitrant carbon on anammox activity. At an initial nitrite concentration of 50 mg N/L, slight improvements, though statistically insignificant, in specific nitrite removal (SNR) were observed in the successive cycles. At a nitrite concentration of 200 mg N/L, SNR of the first cycle was about 121% compared to SNR of the first cycle of nitrite at 50 mg N/L. These results are in line with the results of MSAA found in the previous section, where nitrite at 200 mg N/L had a higher MSAA than at 50 mg N/L. When repeatedly exposed to nitrite at 200 mg N/L, anammox activity decreased at the second (13%) and third (8.7%) cycles and leveled out at the fourth cycle compared to its immediate prior cycle. However, none of these drops in SNR were significant compared to the first cycle's SNR and that of nitrite at 50 mg N/L. At 400 mg N/L, SNR decreased 12% ($P > 0.05$) at the second cycle, 34% ($P < 0.05$) at the third cycle and 38% ($P < 0.05$) at the fourth cycle compared with the SNR of the first cycle. Due to the substantial amount of nitrite remaining at the end of each cycle for samples with an initial nitrite concentration of 400 mg N/L, nitrite inhibition could have been carried over to the next cycle. This may be the reason for the reduction of SNR in successive cycles.

Similar to the MSAA results, repeated exposure to sulfide severely inhibited anammox activity. A relatively constant and significant drop of SNR after each cycle was recorded, with an average of 38.7% and 66% or a total decrease of SNR of 77% and 96% from the first to the fourth cycle for sulfide at 2.5 and 5 mg S/L respectively. These findings indicate that repeated exposure, even for a total of 8 h to sulfide at 5 mg S/L, was lethal to anammox activity.

For filtrate at 100%, the first cycle's SNR was about 14% less than that of the sample with nitrite at 50 mg N/L. Again, this difference in the first cycle's SNR of the two samples agreed with earlier MSAA results. There was an 11% ($P > 0.05$) reduction of SNR in the second cycle, compared with the first cycle's SNR, in the sample with 100% filtrate. However, in the third and fourth cycles, SNR kept increasing slightly compared to the SNR of the second cycle. This finding suggests that anammox activity did not wear out by repeatedly being subjected to recalcitrant carbon, even at a concentration of 582 mg/L as DOC.

After the fourth cycle, the same biomass was prepared for the next cycle, a prolonged exposure test with 12 h contact time. Results (Fig. 5b) show that there was complete removal of nitrite in samples with nitrite at 50 and 200 mg N/L and Filtrate at 100%. In the sample with NO_2^--N = 400 mg/L, there was about a 28.3% (111.7 mg N/L) nitrite removal. Samples with sulfide at 2.5 and 5 mg S/L saw nitrite removal of 64.3% (34 mg N/L) and 30.4% (15.9 mg N/L). Finally, after the extended exposure test, the biomass was washed with the medium and incubated with 40 mL of medium with nitrite at about 20 mg N/L for a recovery period of 6 h. For the biomass previously exposed to sulfide at 2.5 and 5 mg/L where strong inhibition was observed in the repeated and prolonged exposure tests, a longer recovery time of 48 h was applied. During this recovery time, nitrite in the samples was checked periodically and added if lower than 5 mg N/L. After the recovery period, the biomass was prepared for a manometric test in the same manner as mentioned in Section 2.3. The synthetic medium with a nitrite concentration of 50 mg N/L was used for all samples in this test. Fig. 6 depicts the recovered MSAA of each sample. The recovered MSAA of the sample previously exposed to nitrite at 50 mg N/L (control) was 18.26 N_2–N/h·g VSS, similar to the MSAA of the fresh biomass (19.62 N_2–N/h·g VSS, section 3.2). The sample previously exposed to 200 mg N/L had a comparable MSAA, while samples previously exposed to nitrite at 400 mg N/L and Filtrate at 100% saw lower recovery at 82% ($P < 0.05$) and 85% ($P < 0.05$), respectively, compared to the control. The anammox activity could be recovered almost entirely after prolonged exposure to nitrite up to 400 mg N/L, confirming previous reports by Kimura et al. (2010) and Lotti et al. (2012). The high recoverability of activity suggests that nitrite (up to 400 mg N/L) and THP-AD filtrate inhibitions were reversible. Only partial anammox activity could be recovered after 48 h of recovery time in biomass exposed to S^{2-} at 2.5 mg/L, with a recovered MSAA of 31.6% ($P < 0.05$) compared to the control. Sample $S^{2-} = 5$ had an even muted response where only 14.7% ($P < 0.05$) of the activity was recovered, suggesting that sulfide inhibition was

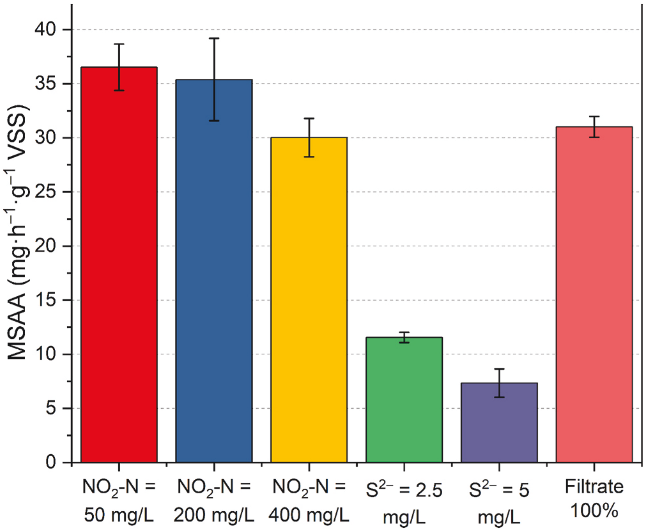


Fig. 6. MSAA of biomass recovered from the repeated and prolonged exposure tests. Despite the sample name, all samples were tested with the same sample matrix using the synthetic medium with an initial NH_4^+ -N and $N0_2^-$ N concentration of 200 and 50 mg N/L respectively.

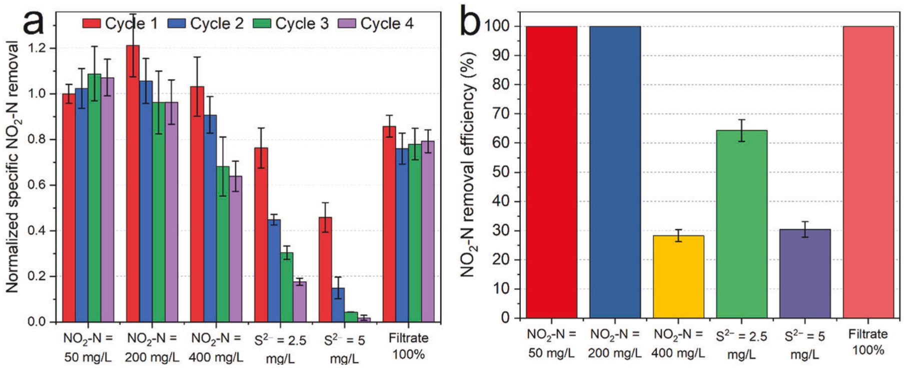


Fig. 5. (a) Normalized SNR after 2 h reaction in each cycle. All data was normalized with the SNR value of Cycle 1 of sample $N\,0_2^-$- N = 50 mg/L which was 45.34 mg N/g VSS. (b) Nitrite removal efficiency of biomass from repeated exposures after a 12 h reaction. Sample matrices are mentioned in Fig. 3 caption.

irreversible on the anammox activity at this concentration. This irreversible inhibition means sulfide at a concentration as low as 2.5 mg/L was toxic to anammox bacteria, potentially causing cell death.

These findings suggest that anammox bacteria were tolerant to nitrite up to 50 mg N/L for repeated and prolonged exposures, while their activities could be fully recovered after repeated and prolonged exposures to nitrite up to 200 mg N/L. Recalcitrant carbon in filtrate at 100% caused slight and reversible inhibition on anammox activity. However, prolonged exposure to sulfide, even at a concentration as low as 2.5 mg/L, was severely detrimental to anammox activities, causing irreversible inhibition potentially due to cell death.

4. Conclusions

In this study, exhaustive experiments – MSAA, gene expression, repeated and prolonged exposure tests, and recovery tests – were tested to examine the inhibitory effects of common inhibitors – nitrite, sulfide, and recalcitrant carbon – on anammox activity. Results were conclusive that nitrite inhibition exhibited a typical substrate inhibition, and its inhibition on anammox was reversible up to 400 mg N/L. Sulfide was an acute inhibitor of anammox activities, and its inhibition was irreversible (toxic) after prolonged exposure to as low as 5 mg S/L. Recalcitrant carbon did not have any significant effect on anammox activity. Inhibition coefficients for nitrite and sulfide were determined that would be useful for reactor design in pilot-scale and full-scale applications. The expression of *hzsB* over time during the exposure to nitrite, sulfide, and recalcitrant carbon could help understand the underlying mechanisms of these inhibitions on anammox. Furthermore, the usage of biomass with the microbial community closely resembled that of full-scale anammox reactors, making the findings in this study more applicable to engineering applications of anammox processes.

Credit author statement

SH performed all experiments and prepared the first draft of the manuscript. HDC helped with manuscript review, advice on data analysis and modeling. RKG supervised the whole study, reviewed the manuscript and finalized it for its final submission. RKG is the corresponding author.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

Acknowledgements

This research was supported by funding from the National Science Foundation GOALI Program (NSF Grant # 1903922) and the Global Change and Sustainability Center (GCSC) at the University of Utah, Salt Lake City, Utah. The views expressed in this manuscript are those of authors and not necessarily reflect any views from funding agencies. We appreciate reviewers for their time and useful comments.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.chemosphere.2022.136232.

References

Aiba, S., Shoda, M., Nagatani, M., 1968. Kinetics of product inhibition in alcohol fermentation. Biotechnol. Bioeng. 10, 845–864. https://doi.org/10.1002/bit.260100610.

Akaike, H., 1974. A new look at the statistical model identification. IEEE Trans. Automat. Control 19, 716–723. https://doi.org/10.1109/TAC.1974.1100705.

Azari, M., Walter, U., Rekers, V., Gu, J.D., Denecke, M., 2017. More than a decade of experience of landfill leachate treatment with a full-scale anammox plant combining activated sludge and activated carbon biofilm. Chemosphere 174, 117–126. https://doi.org/10.1016/j.chemosphere.2017.01.123.

Barber, W.P.F., 2016. Thermal hydrolysis for sewage treatment: a critical review. Water Res. 104, 53–74. https://doi.org/10.1016/j.watres.2016.07.069.

Bejarano-Ortiz, D.I., Huerta-Ochoa, S., Thalasso, F., Cuervo-López, F. de M., Texier, A.C., 2015. Kinetic constants for biological ammonium and nitrite oxidation processes under sulfide inhibition. Appl. Biochem. Biotechnol. 177, 1665–1675. https://doi.org/10.1007/s12010-015-1844-3.

Bejarano Ortiz, D.I., Thalasso, F., Cuervo López, F. de M., Texier, A.-C., 2013. Inhibitory effect of sulfide on the nitrifying respiratory process. J. Chem. Technol. Biotechnol. 88, 1344–1349. https://doi.org/10.1002/jctb.3982.

Bhattacharjee, A.S., Wu, S., Lawson, C.E., Jetten, M.S.M., Kapoor, V., Domingo, J.W.S., Mcmahon, K.D., Noguera, D.R., Goel, R., 2017. Whole-Community Metagenomics in Two Different Anammox Configurations: Process Performance and Community Structure. https://doi.org/10.1021/acs.est.6b05855.

Bi, Z., Qiao, S., Zhou, J., Tang, X., Cheng, Y., 2014. Inhibition and recovery of Anammox biomass subjected to short-term exposure of Cd, Ag, Hg and Pb. Chem. Eng. J. 244, 89–96. https://doi.org/10.1016/j.cej.2014.01.062.

Bolyen, E., Rideout, J.R., Dillon, M.R., Bokulich, N.A., Abnet, C.C., Al-Ghalith, G.A., Alexander, H., Alm, E.J., Arumugam, M., Asnicar, F., Bai, Y., Bisanz, J.E., Bittinger, K., Brejnrod, A., Brislawn, C.J., Brown, C.T., Callahan, B.J., Caraballo-Rodríguez, A.M., Chase, J., Cope, E.K., Da Silva, R., Diener, C., Dorrestein, P.C., Douglas, G.M., Durall, D.M., Duvallet, C., Edwardson, C.F., Ernst, M., Estaki, M., Fouquier, J., Gauglitz, J.M., Gibbons, S.M., Gibson, D.L., Gonzalez, A., Gorlick, K., Guo, J., Hillmann, B., Holmes, S., Holste, H., Huttenhower, C., Huttley, G.A., Janssen, S., Jarmusch, A.K., Jiang, L., Kaehler, B.D., Kang, K. Bin, Keefe, C.R., Keim, P., Kelley, S.T., Knights, D., Koester, I., Kosciolek, T., Kreps, J., Langille, M.G.I., Lee, J., Ley, R., Liu, Y.X., Loftfield, E., Lozupone, C., Maher, M., Marotz, C., Martin, B.D., McDonald, D., McIver, L.J., Melnik, A.V., Metcalf, J.L., Morgan, S.C., Morton, J.T., Naimey, A.T., Navas-Molina, J.A., Nothias, L.F., Orchanian, S.B., Pearson, T., Peoples, S.L., Petras, D., Preuss, M.L., Pruesse, E., Rasmussen, L.B., Rivers, A., Robeson, M.S., Rosenthal, P., Segata, N., Shaffer, M., Shiffer, A., Sinha, R., Song, S.J., Spear, J.R., Swafford, A.D., Thompson, L.R., Torres, P.J., Trinh, P., Tripathi, A., Turnbaugh, P.J., Ul-Hasan, S., van der Hooft, J.J.J., Vargas, F., Vázquez-Baeza, Y., Vogtmann, E., von Hippel, M., Walters, W., Wan, Y., Wang, M., Warren, J., Weber, K.C., Williamson, C.H.D., Willis, A.D., Xu, Z.Z., Zaneveld, J.R., Zhang, Y., Zhu, Q., Knight, R., Caporaso, J.G., 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. Nat. Biotechnol. 37, 852–857. https://doi.org/10.1038/s41587-019-0209-9.

Bremer, H., Dennis, P.P., 2008. Modulation of chemical composition and other parameters of the cell at different exponential growth rates. EcoSal Plus 3. https://doi.org/10.1128/ecosal.5.2.3.

Brooks, M.H., Smith, ', And, R.L., Macalady2, D.L., 1992. Inhibition of Existing Denitrification Enzyme Activity by Chloramphenicol Downloaded from. APPLIED AND ENVIRONMENTAL MICROBIOLOGY.

Caffrey, J.M., Bonaglia, S., Conley, D.J., 2019. Short exposure to oxygen and sulfide alter nitrification, denitrification, and DNRA activity in seasonally hypoxic estuarine sediments. FEMS Microbiol. Lett. 366, 1–10. https://doi.org/10.1093/femsle/fny288.

Cao, Y., van Loosdrecht, M.C.M., Daigger, G.T., 2017. Mainstream partial nitritation–anammox in municipal wastewater treatment: status, bottlenecks, and further studies. Appl. Microbiol. Biotechnol. 101, 1365–1383. https://doi.org/10.1007/s00253-016-8058-7.

Carrera, J., Jubany, I., Carvallo, L., Chamy, R., Lafuente, J., 2004. Kinetic models for nitrification inhibition by ammonium and nitrite in a suspended and an immobilised biomass systems. Process Biochem. 39, 1159–1165. https://doi.org/10.1016/S0032-9592(03)00214-0.

Carvajal-Arroyo, J.M., Puyol, D., Li, G., Lucero-Acuña, A., Sierra-Álvarez, R., Field, J.A., 2014. Pre-exposure to nitrite in the absence of ammonium strongly inhibits anammox. Water Res. 48, 52–60. https://doi.org/10.1016/j.watres.2013.09.015.

Carvajal-Arroyo, J.M., Sun, W., Sierra-Alvarez, R., Field, J.A., 2013. Inhibition of anaerobic ammonium oxidizing (anammox) enrichment cultures by substrates, metabolites and common wastewater constituents. Chemosphere 91, 22–27. https://doi.org/10.1016/j.chemosphere.2012.11.025.

Chen, H., Ma, C., Ji, Y.X., Ni, W.M., Jin, R.C., 2014. Evaluation of the efficacy and regulation measures of the anammox process under salty conditions. Separ. Purif. Technol. 132, 584–592. https://doi.org/10.1016/j.seppur.2014.06.012.

Daigger, G.T., Sanjines, P., Pallansch, K., Sizemore, J., Wett, B., 2011. Implementation of a full-scale anammox-based facility to treat an anaerobic digestion sidestream at the alexandria sanitation authority water resource facility. Water Pract. 6 https://doi.org/10.2166/wpt.2011.033. Technol.

Dapena-Mora, A., Fernández, I., Campos, J.L., Mosquera-Corral, A., Méndez, R., Jetten, M.S.M., 2007. Evaluation of activity and inhibition effects on Anammox process by batch tests based on the nitrogen gas production. Enzym. Microb. Technol. 40, 859–865. https://doi.org/10.1016/j.enzmictec.2006.06.018.

Dapena-Mora, A., Vázquez-Padín, J.R., Campos, J.L., Mosquera-Corral, A., Jetten, M.S.M., Méndez, R., 2010. Monitoring the stability of an Anammox reactor under high salinity conditions. Biochem. Eng. J. 51, 167–171. https://doi.org/10.1016/j.bej.2010.06.014.

De Prá, M.C., Kunz, A., Bortoli, M., Scussiato, L.A., Coldebella, A., Vanotti, M., Soares, H. M., 2016. Kinetic models for nitrogen inhibition in ANAMMOX and nitrification process on deammonification system at room temperature. Bioresour. Technol. 202, 33–41. https://doi.org/10.1016/j.biortech.2015.11.048.

Driessen, W., Hendrickx, T., 2021. Two decades of experience with the granular sludge-based ANAMMOX® process treating municipal and industrial effluents. Process 9 (9), 1207. https://doi.org/10.3390/PR9071207.

Driessen, W., Van Veldhoven, J.T.A., Janssen, M.P.M., Van Loosdrecht, M.C.M., 2020. Treatment of sidestream dewatering liquors from thermally hydrolised and anaerobically digested biosolids. Water Pract. Technol. 15, 142–150. https://doi.org/10.2166/WPT.2020.007.

Du, R., Peng, Y., Ji, J., Shi, L., Gao, R., Li, X., 2019. Partial denitrification providing nitrite: opportunities of extending application for anammox. Environ. Int. 131, 105001. https://doi.org/10.1016/j.envint.2019.105001.

Edwards, V.H., 1970. The influence of high substrate concentrations on microbial kinetics. Biotechnol. Bioeng. 12, 679–712. https://doi.org/10.1002/bit.260120504.

Fernández, I., Dosta, J., Fajardo, C., Campos, J.L., Mosquera-Corral, A., Méndez, R., 2012. Short- and long-term effects of ammonium and nitrite on the Anammox process. J. Environ. Manag. 95, S170–S174. https://doi.org/10.1016/j.jenvman.2010.10.044.

Glass, C., Silverstein, J., Oh, J., 1997. Inhibition of Denitrification in Activated Sludge by Nitrite, Water Environment Research. https://doi.org/10.2175/106143097x125803.

Gonzalez-Gil, G., Sougrat, R., Behzad, A.R., Lens, P.N.L., Saikaly, P.E., 2015. Microbial community composition and ultrastructure of granules from a full-scale Anammox reactor. Microb. Ecol. 70, 118–131. https://doi.org/10.1007/s00248-014-0546-7.

Gonzalez-Martinez, A., Osorio, F., Morillo, J.A., Rodriguez-Sanchez, A., Gonzalez-Lopez, J., Abbas, B.A., van Loosdrecht, M.C.M., 2015. Comparison of bacterial diversity in full scale anammox bioreactors operated under different conditions. Biotechnol. Prog. 31, 1464–1472. https://doi.org/10.1002/btpr.2151.

Gu, Z., Li, Y., Yang, Y., Xia, S., Hermanowicz, S.W., Alvarez-Cohen, L., 2018. Inhibition of anammox by sludge thermal hydrolysis and metagenomic insights. Bioresour. Technol. 270, 46–54. https://doi.org/10.1016/j.biortech.2018.08.132.

Haldane, J.B.S., 1965. Enzymes. MIT Press, Cambridge.

Harhangi, H.R., Le Roy, M., van Alen, T., Hu, B., lan, Groen J., Kartal, B., Tringe, S.G., Quan, Z.X., Jetten, M.S.M., Op den Camp, H.J.M., 2012. Hydrazine synthase, a unique phylomarker with which to study the presence and biodiversity of anammox bacteria. Appl. Environ. Microbiol. 78, 752–758. https://doi.org/10.1128/AEM.07113-11.

Hu, B. lan, Zheng, P., Tang, C. jian, Chen, J., van der, wei, Biezen, E., Zhang, L., Ni, B. jie, Jetten, M.S.M., Yan, J., Yu, H.Q., Kartal, B., 2010. Identification and quantification of anammox bacteria in eight nitrogen removal reactors. Water Res. 44, 5014–5020. https://doi.org/10.1016/j.watres.2010.07.021.

Izadi, Parin, Izadi, Parnian, Eldyasti, A., 2021. Towards mainstream deammonification: comprehensive review on potential mainstream applications and developed sidestream technologies. J. Environ. Manag. 279, 111615 https://doi.org/10.1016/J.JENVMAN.2020.111615.

Jin, R.C., Yang, G.F., Yu, J.J., Zheng, P., 2012. The inhibition of the Anammox process: a review. Chem. Eng. J. 197, 67–79. https://doi.org/10.1016/j.cej.2012.05.014.

Jin, R.C., Yang, G.F., Zhang, Q.Q., Ma, C., Yu, J.J., Xing, B.S., 2013a. The effect of sulfide inhibition on the ANAMMOX process. Water Res. 47, 1459–1469. https://doi.org/10.1016/j.watres.2012.12.018.

Jin, R.C., Zhang, Q.Q., Yang, G.F., Xing, B.S., Ji, Y.X., Chen, H., 2013b. Evaluating the recovery performance of the ANAMMOX process following inhibition by phenol and sulfide. Bioresour. Technol. 142, 162–170. https://doi.org/10.1016/j.biortech.2013.05.022.

Joss, A., Salzgeber, D., Eugster, J., König, R., Rottermann, K., Burger, S., Fabijan, P., Leumann, S., Mohn, J., Siegrist, H.R., 2009. Full-scale nitrogen removal from digester liquid with partial nitritation and anammox in one SBR. Environ. Sci. Technol. 43, 5301–5306. https://doi.org/10.1021/es900107w.

Joye, S.B., Hollibaugh, J.T., 1995. Influence of sulfide inhibition of nitrification on nitrogen regeneration in sediments. Science 80–, 623–625. https://doi.org/10.1126/science.270.5236.623.

Kamp, A., Ottosen, L.D.M., Thøgersen, N.B., Revsbech, N.P., Thamdrup, B., Andersen, M. H., 2019. Anammox and partial nitritation in the mainstream of a wastewater treatment plant in a temperate region (Denmark). Water Sci. Technol. 79, 1397–1405. https://doi.org/10.2166/wst.2019.141.

Kartal, B., De Almeida, N.M., Maalcke, W.J., Op den Camp, H.J.M., Jetten, M.S.M., Keltjens, J.T., 2013. How to make a living from anaerobic ammonium oxidation. FEMS Microbiol. Rev. 37, 428–461. https://doi.org/10.1111/1574-6976.12014.

Kartal, B., Keltjens, J.T., 2016. Anammox biochemistry: a tale of heme c proteins. Trends Biochem. Sci. 41, 998–1011. https://doi.org/10.1016/j.tibs.2016.08.015.

Kartal, B., Koleva, M., Arsov, R., van der Star, W., Jetten, M.S.M., Strous, M., 2006. Adaptation of a freshwater anammox population to high salinity wastewater. J. Biotechnol. 126, 546–553. https://doi.org/10.1016/j.jbiotec.2006.05.012.

Kimura, Y., Isaka, K., Kazama, F., Sumino, T., 2010. Effects of nitrite inhibition on anaerobic ammonium oxidation. Appl. Microbiol. Biotechnol. 86, 359–365. https://doi.org/10.1007/s00253-009-2359-z.

Klaus, S., Baumler, R., Rutherford, B., Thesing, G., Zhao, H., Bott, C., 2017. Startup of a partial nitritation-anammox MBBR and the implementation of pH-based aeration control. Water Environ. Res. 89, 500–508. https://doi.org/10.2175/106143017X14902968254476.

Kosgey, K., Chandran, K., Gokal, J., Kiambi, S.L., Bux, F., Kumari, S., 2020. Critical analysis of biomass retention strategies in mainstream and sidestream ANAMMOX-mediated nitrogen removal systems. Environ. Sci. Technol. 55, 9–24. https://doi.org/10.1021/ACS.EST.0C00276.

Kotay, S.M., Mansell, B.L., Hogsett, M., Pei, H., Goel, R., 2013. Anaerobic ammonia oxidation (ANAMMOX) for side-stream treatment of anaerobic digester filtrate process performance and microbiology. Biotechnol. Bioeng. 110, 1180–1192. https://doi.org/10.1002/bit.24767.

Kozich, J.J., Westcott, S.L., Baxter, N.T., Highlander, S.K., Schloss, P.D., 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the miseq illumina sequencing platform. Appl. Environ. Microbiol. 79, 5112–5120. https://doi.org/10.1128/AEM.01043-13.

Kuypers, M.M.M., Marchant, H.K., Kartal, B., 2018. The microbial nitrogen-cycling network. Nat. Rev. Microbiol. 16, 263–276. https://doi.org/10.1038/nrmicro.2018.9.

Lackner, S., Gilbert, E.M., Vlaeminck, S.E., Joss, A., Horn, H., van Loosdrecht, M.C.M., 2014. Full-scale partial nitritation/anammox experiences - an application survey. Water Res. 55, 292–303. https://doi.org/10.1016/j.watres.2014.02.032.

Laureni, M., Weissbrodt, D.G., Szivák, I., Robin, O., Nielsen, J.L., Morgenroth, E., Joss, A., 2015. Activity and growth of anammox biomass on aerobically pre-treated municipal wastewater. Water Res. 80, 325–336. https://doi.org/10.1016/j.watres.2015.04.026.

Lawson, C.E., Wu, S., Bhattacharjee, A.S., Hamilton, J.J., McMahon, K.D., Goel, R., Noguera, D.R., 2017. Metabolic network analysis reveals microbial community interactions in anammox granules. Nat. Commun. 8, 1–12. https://doi.org/10.1038/ncomms15416.

Lemaire, R., Christensson, M., 2021. Lessons learned from 10 years of anita mox for sidestream treatment. Processes 9, 863. https://doi.org/10.3390/pr9050863.

Li, G., Vilcherrez, D., Carvajal-Arroyo, J.M., Sierra-Alvarez, R., Field, J.A., 2016. Exogenous nitrate attenuates nitrite toxicity to anaerobic ammonium oxidizing (anammox) bacteria. Chemosphere 144, 2360–2367. https://doi.org/10.1016/j.chemosphere.2015.11.013.

Li, X., Klaus, S., Bott, C., He, Z., 2018. Status, challenges, and perspectives of mainstream nitritation-anammox for wastewater treatment. Water Environ. Res. 90, 634–649. https://doi.org/10.2175/106143017x15131012153112.

Liang, Z., Sun, J., Chau, H.K., Leong, E.I.M., Wu, D., Chen, G., Jiang, F., 2019. Experimental and modelling evaluations of sulfide formation in a mega-sized deep tunnel sewer system and implications for sewer management. Environ. Int. 131, 105011 https://doi.org/10.1016/j.envint.2019.105011.

Liang, Z., Sun, J., Zhan, C., Wu, S., Zhang, L., Jiang, F., 2020. Effects of sulfide on mixotrophic denitrification by: thauera -dominated denitrifying sludge. Environ. Sci. Water Res. Technol. 6, 1186–1195. https://doi.org/10.1039/c9ew01014a.

Lin, L., Pratt, S., Crick, O., Xia, J., Duan, H., Ye, L., 2021. Salinity effect on freshwater Anammox bacteria: ionic stress and ion composition. Water Res. 188, 116432 https://doi.org/10.1016/j.watres.2020.116432.

Lotti, T., Kleerebezem, R., Hu, Z., Kartal, B., De Kreuk, M.K., Van Erp Taalman Kip, C., Kruit, J., Hendrickx, T.L.G., Van Loosdrecht, M.C.M., 2015. Pilot-scale evaluation of anammox-based mainstream nitrogen removal from municipal wastewater. Environ. Technol. 36, 1167–1177. https://doi.org/10.1080/09593330.2014.982722.

Lotti, T., Kleerebezem, R., Lubello, C., van Loosdrecht, M.C.M., 2014. Physiological and kinetic characterization of a suspended cell anammox culture. Water Res. 60, 1–14. https://doi.org/10.1016/j.watres.2014.04.017.

Lotti, T., van der Star, W.R.L., Kleerebezem, R., Lubello, C., van Loosdrecht, M.C.M., 2012. The effect of nitrite inhibition on the anammox process. Water Res. 46, 2559–2569. https://doi.org/10.1016/j.watres.2012.02.011.

Ma, W., Li, G., Huang, B., Jin, R., 2020. Advances and challenges of mainstream nitrogen removal from municipal wastewater with anammox-based processes. Water Environ. Res. 92 https://doi.org/10.1002/wer.1342.

Nsenga Kumwimba, M., Lotti, T., Şenel, E., Li, X., Suanon, F., 2020. Anammox-based processes: how far have we come and what work remains? A review by bibliometric analysis. Chemosphere 5, 263–276. https://doi.org/10.1016/j.chemosphere.2019.124627.

Oshiki, M., Ali, M., Shinyako-Hata, K., Satoh, H., Okabe, S., 2016a. Hydroxylamine-dependent anaerobic ammonium oxidation (anammox) by "Candidatus Brocadia sinica. Environ. Microbiol. 18, 3133–3143. https://doi.org/10.1111/1462-2920.13355.

Oshiki, M., Satoh, H., Okabe, S., 2016b. Ecology and physiology of anaerobic ammonium oxidizing bacteria. Environ. Microbiol. 18, 2784–2796. https://doi.org/10.1111/1462-2920.13134.

Otto, M., Wynands, B., Drepper, T., Jaeger, K.E., Thies, S., Loeschcke, A., Blank, L.M., Wierckx, N., 2019. Targeting 16S rDNA for stable recombinant gene expression in Pseudomonas. ACS Synth. Biol. 8, 1901–1912. https://doi.org/10.1021/acssynbio.9b00195.

Phanwilai, S., Piyavorasakul, S., Noophan, P., Lek), Daniels, K.D., Snyder, S.A., 2020. Inhibition of anaerobic ammonium oxidation (anammox) bacteria by addition of high and low concentrations of chloramphenicol and comparison of attached- and suspended-growth. Chemosphere 238, 124570. https://doi.org/10.1016/j.chemosphere.2019.124570.

Puyol, D., Carvajal-Arroyo, J.M., Sierra-Alvarez, R., Field, J.A., 2014. Nitrite (not free nitrous acid) is the main inhibitor of the anammox process at common pH conditions. Biotechnol. Lett. 36, 547–551. https://doi.org/10.1007/s10529-013-1397-x.

Reed, M.C., Lieb, A., Nijhout, H.F., 2010. The biological significance of substrate inhibition: a mechanism with diverse functions. Bioessays 32, 422–429. https://doi.org/10.1002/bies.200900167.

Rodríguez-Gómez, L.E., Delgado, S., Álvarez, M., Elmaleh, S., 2005. Inhibition of sulfide generation in a reclaimed wastewater pipe by nitrate dosage and denitrification kinetics. Water Environ. Res. 77, 193–198. https://doi.org/10.2175/106143005x41762.

Schieder, D., Schneider, R., Bischof, F., 2000. Thermal hydrolysis (TDH) as a pretreatment method for the digestion of organic waste. Water Sci. Technol. 41, 181–187. https://doi.org/10.2166/wst.2000.0070.

Sørensen, J., Tiedje, J.M., Firestone, R.B., 1980. Inhibition by sulfide of nitric and nitrous oxide reduction by denitrifying Pseudomonas fluorescens. Appl. Environ. Microbiol. 39.

Speth, D.R., In'T Zandt, M.H., Guerrero-Cruz, S., Dutilh, B.E., Jetten, M.S.M.M., In 't Zandt, M.H., Guerrero-Cruz, S., Dutilh, B.E., Jetten, M.S.M.M., In'T Zandt, M.H., Guerrero-Cruz, S., Dutilh, B.E., Jetten, M.S.M.M., 2016. Genome-based microbial ecology of anammox granules in a full-scale wastewater treatment system. Nat. Commun. 7, 1–10. https://doi.org/10.1038/ncomms11172.

Strous, M., Kuenen, J.G., Jetten, M.S.M., 1999. Key physiology of anaerobic ammonium oxidation. Appl. Environ. Microbiol. 65, 3248–3250. https://doi.org/10.1128/aem.65.7.3248-3250.1999.

Trinh, H.P., Lee, S.H., Jeong, G., Yoon, H., Park, H.D., 2021. Recent developments of the mainstream anammox processes: challenges and opportunities. J. Environ. Chem. Eng. 9 https://doi.org/10.1016/j.jece.2021.105583.

Tsushima, I., Kindaichi, T., Okabe, S., 2007. Quantification of anaerobic ammonium-oxidizing bacteria in enrichment cultures by real-time PCR. Water Res. 41, 785–794. https://doi.org/10.1016/j.watres.2006.11.024.

Van De Graaf, A.A., De Bruijn, P., Robertson, L.A., Jetten, M.S.M., Kuenen, J.G., 1996. Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. Microbiology 142, 2187–2196. https://doi.org/10.1099/13500872-142-8-2187.

van Dongen, U., Jetten, M., van Loosdrecht, M., 2001. The SHARON ®-Anammox ® Process for Treatment of Ammonium Rich Wastewater.

Wang, Y., Ma, X., Zhou, S., Lin, X., Ma, B., Park, H.-D.D., Yan, Y., 2016. Expression of the nirS, hzsA, and hdh genes in response to nitrite shock and recovery in Candidatus Kuenenia stuttgartiensis. Environ. Sci. Technol. 50, 6940–6947. https://doi.org/10.1021/acs.est.6b00546.

Wang, Y., Zhu, G., Harhangi, H.R., Zhu, B., Jetten, M.S.M., Yin, C., Op den Camp, H.J.M., 2012. Co-occurrence and distribution of nitrite-dependent anaerobic ammonium and methane-oxidizing bacteria in a paddy soil. FEMS Microbiol. Lett. 336, 79–88. https://doi.org/10.1111/j.1574-6968.2012.02654.x.

Wang, Z., Zheng, M., Duan, H., Yuan, Z., Hu, S., 2022. A 20-year journey of partial nitritation and anammox (PN/A): from sidestream toward mainstream. Cite Environ. Sci. Technol. 2022, 7531. https://doi.org/10.1021/acs.est.1c06107.

Wen, R., Jin, Y., Zhang, W., 2020. Application of the anammox in China—a review. Int. J. Environ. Res. Publ. Health 17, 1090. https://doi.org/10.3390/ijerph17031090.

Wett, B., Podmirseg, S.M., Gómez-Brandón, M., Hell, M., Nyhuis, G., Bott, C., Murthy, S., 2015. Expanding DEMON sidestream deammonification technology towards mainstream application. Water Environ. Res. 87, 2084–2089. https://doi.org/10.2175/106143015x14362865227319.

Yang, S., Peng, Y., Zhang, S., Han, X., Li, J., Zhang, L., 2021. Carrier type induces anammox biofilm structure and the nitrogen removal pathway: demonstration in a full-scale partial nitritation/anammox process. Bioresour. Technol. 334 https://doi.org/10.1016/j.biortech.2021.125249.

Yang, Y., Li, M., Li, H., Li, X.Y., Lin, J.G., Denecke, M., Gu, J.D., 2020. Specific and effective detection of anammox bacteria using PCR primers targeting the 16S rRNA gene and functional genes. Sci. Total Environ. 734, 139387 https://doi.org/10.1016/j.scitotenv.2020.139387.

Zhang, Q., De Clippeleir, H., Su, C., Al-Omari, A., Wett, B., Vlaeminck, S.E., Murthy, S., 2016. Deammonification for digester supernatant pre-treated with thermal hydrolysis: overcoming inhibition through process optimization. Appl. Microbiol. Biotechnol. 100, 5595–5606. https://doi.org/10.1007/s00253-016-7368-0.

Zhang, L., Narita, Y., Gao, L., Ali, M., Oshiki, M., Okabe, S., 2017. Maximum specific growth rate of anammox bacteria revisited. Water Res. 116, 296–303. https://doi.org/10.1016/j.watres.2017.03.027.

Zhang, L., Okabe, S., 2020. Ecological niche differentiation among anammox bacteria. Water Res. 171, 115468 https://doi.org/10.1016/j.watres.2020.115468.

Zhang, Q., Vlaeminck, S.E., DeBarbadillo, C., Su, C., Al-Omari, A., Wett, B., Pümpel, T., Shaw, A., Chandran, K., Murthy, S., De Clippeleir, H., 2018. Supernatant organics from anaerobic digestion after thermal hydrolysis cause direct and/or diffusional activity loss for nitritation and anammox. Water Res. 143, 270–281. https://doi.org/10.1016/j.watres.2018.06.037.

Zhang, Z.Z., Zhang, Q.Q., Xu, J.J., Deng, R., Ji, Z.Q., Wu, Y.H., Jin, R.C., 2016. Evaluation of the inhibitory effects of heavy metals on anammox activity: a batch test study. Bioresour. Technol. 200, 208–216. https://doi.org/10.1016/j.biortech.2015.10.035.

Zhou, Z., Chen, J., Meng, H., Dvornyk, V., Gu, J.D., 2017. New PCR primers targeting hydrazine synthase and cytochrome c biogenesis proteins in anammox bacteria. Appl. Microbiol. Biotechnol. 101, 1267–1287. https://doi.org/10.1007/s00253-016-8013-7.