Short and long term continuous hydroxylamine feeding in a granular sludge partial nitritation reactor

Soler-Jofra, Aina; Schmidtchen, Lisbeth; Olmo, Lluc; van Loosdrecht, Mark C.M.; Pérez, Julio

DOI
10.1016/j.watres.2021.117945

Publication date
2022

Document Version
Final published version

Published in
Water Research

Water Research 209 (2022) 117945

Contents lists available at ScienceDirect

Water Research

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

Short and long term continuous hydroxylamine feeding in a granular sludge partial nitritation reactor

Aina Soler-Jofra [a], Lisbeth Schmidtchen [a], Lluc Olmo [b], Mark C.M. van Loosdrecht [a,*], Julio Pérez [b]

[a] Department of Biotechnology, Faculty of Applied Sciences, Delft University of Technology, Van der Maasweg 9, Delft 2629 HZ, the Netherlands
[b] Department of Chemical, Biological and Environmental Engineering, Universitat Autonoma de Barcelona, Cerdanyola del Valles, Spain

ARTICLE INFO

Keywords:
Ammonium
Nitrite
Hydroxylamine
Ammonium oxidizing bacteria

ABSTRACT

Hydroxylamine is a nitrogen intermediate of ammonium oxidizing bacteria (AOB) that can transiently accumulate during nitrification. The impact of hydroxylamine on aerobic ammonium oxidations is still obscure. In the present study the short and long term impact of hydroxylamine on partial nitritation granular sludge was investigated. Dissolved oxygen was the governing factor determining the hydroxylamine impact in short term studies with continuous hydroxylamine feeding. Continuous short term hydroxylamine feeding together with low dissolved oxygen resulted in higher hydroxylamine accumulation, higher N_2O production and decreased or maintained ammonium consumption. Instead, high dissolved oxygen reduced hydroxylamine accumulation and N_2O production and increased ammonium consumption. Long term continuous hydroxylamine feeding reduced ammonium consumption rate while the constant nitrite production rate indicated that dosed hydroxylamine was mainly transformed to nitrite. This indicates that hydroxylamine was preferred over ammonium as substrate. *Nitrosomonas* sp. was shown to be predominant during continuous hydroxylamine feeding while the side community shifted.

1. Introduction

Aerobic ammonium oxidizing bacteria (AOB) have been studied for over a century. These autotrophic organisms are able to transform ammonium to nitrite aerobically. Hydroxylamine was postulated as intermediate in ammonium oxidation by Lees and coworkers (Lees, 1952). Thus, according to the current AOB central nitrogen metabolism ammonium is transformed to hydroxylamine by the enzyme ammonium monooxygenase (AMO) (see Eq. (1)). The transformation of ammonium by AMO requires oxygen and two electrons. Then produced hydroxylamine is subsequently transformed to nitrite (see Eq. (2)) by hydroxylamine oxidoreductase (HAO). It has also been proposed that HAO only catalyses the transformation of hydroxylamine to NO (see Eq. (3)), while NO is further transformed to NO_2^- chemically or by a yet unidentified enzyme (Eq. (4)) (Caranto and Lancaster, 2017; Hooper and Terry, 1979). Independently of the intermediate steps, the conversion of hydroxylamine to nitrite results in 4 electron being produced. It is generally accepted that two of these electrons are funneled back to AMO, while two are dedicated to energy generation within the terminal electron acceptor (Arp and Stein, 2003; Simon and Klotz, 2013; Stein, 2011). Where, oxygen generally acts as terminal electron acceptor (Eq. (5)).

$$NH_3 + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (1)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (2)$$

$$NH_2OH \rightarrow NO + 3H^+ + 3e^- \quad (3)$$

$$NO + H_2O \rightarrow NO_2^- + 2H^+ + 1e^- \quad (4)$$

$$\frac{1}{2}O_2 + 2H^+ + 2e^- \rightarrow H_2O \quad (5)$$

Two N_2O production pathways have been described in AOB. When there is not sufficient terminal electron acceptor (anoxic conditions), nitrite can act as terminal electron acceptor while generating N_2O. The use of nitrite as electron acceptor is the so-called nitrifier denitrification pathway (Stein, 2011). The anoxic conversion of hydroxylamine to N_2O

* Corresponding author.
E-mail address: M.C.M.vanLoosdrecht@tudelft.nl (M.C.M. van Loosdrecht).

https://doi.org/10.1016/j.watres.2021.117945
Received 14 July 2021; Received in revised form 25 October 2021; Accepted 5 December 2021
Available online 7 December 2021

has been recently proposed to be mediated by the cytP460 of HAO (Caranto et al., 2016). Contrarily, the hydroxylamine oxidation pathway producing N_2O is generally associated with high oxygen concentrations leading to hydroxylamine partial oxidation to NO or N_2O (Stein, 2011; Yu et al., 2010).

Hydroxylamine is seldom measured or experimentally evaluated in wastewater treatment related research. Only few studies have reported transient hydroxylamine accumulation events with concentrations ranging from 0.003 up to 4.3 mg-N/L (Hu et al., 2017; Liu et al., 2017; Soler-Jofra et al., 2016; Stüven et al., 1992; Su et al., 2019b; Yu et al., 2010, 2018). Generally, reported data are associated with SBR operations (Hu et al., 2017; Su et al., 2019b), batch tests (Liu et al., 2017; Stüven et al., 1992) or switching conditions from aerobic/anoxic environments (Yu and Chandran, 2010; Yu et al., 2018). The mechanisms leading to such transient hydroxylamine accumulations are not fully understood.

Different studies have investigated the impact of hydroxylamine on AOB communities, with contradictory results. For instance, some studies proposed the use of hydroxylamine to avoid complete nitrification (considered as nitrite being further oxidized to nitrate by nitrite oxidizing bacteria) and achieved a stable partial nitritation operation (Wang et al., 2016, 2015; Kindaichi et al., 2004; Noophan et al., 2004). Contrarily, Harper and co-workers showed that hydroxylamine addition boosted ammonium consumption in the short term, but negatively impacted conversion on the long term resulting into a disaggregation of microcolonies (Harper et al., 2009). The scattering or morphological changes were also observed in Kindiachi et al. (2004). Most of these studies used a pulse like feeding strategy of hydroxylamine, with generally high hydroxylamine concentrations. High concentrations of hydroxylamine are not usual in natural environments and might have caused inhibitory effects, as it is a highly reactive compound. Instead, using a rate-limiting continuous feeding would limit the potential toxicity of hydroxylamine and would mimic putative environmental conditions of hydroxylamine exposure due to transient accumulation. The only study that supplied rate-limitng concentrations of hydroxylamine was by de Bruijn et al. (1995) with a planktonic chemostat culture of *Nitrosomonas europaea*. In this study hydroxylamine led to a higher biomass yield as compared to growth on ammonium only. They also observed a soluble nitrogen loss that increased with increasing hydroxylamine load, as NO and/or N_2O was formed (de Bruijn et al., 1995).

Additionally, mathematical models developed to describe NO and N_2O emissions from nitrifying sludge included the description of the hydroxylamine oxidation by declaring hydroxylamine concentration as state variable (Ni and Yuan, 2015). Most of the modeling calibrations to determine the kinetic parameters related to hydroxylamine oxidation were performed by fitting other measured nitrogen species, DO and/or N_2O (Law et al., 2012; Ni et al., 2014, 2011), but without direct measurements of hydroxylamine concentration. The exception is the study by Domingo-Felez et al. (2017), that included hydroxylamine concentration measured data in order to estimate the parameters of hydroxylamine oxidation step and N_2O production (Domingo-Félez et al., 2017).

A more comprehensive understanding of the hydroxylamine metabolism by AOB is needed since its metabolism has a complex kinetic regulation and it is involved in a wide range of engineered systems. Factors leading to hydroxylamine accumulation and its impact on N_2O emissions are missing. Not only, hydroxylamine affects N_2O emissions, but also it is known to inhibit NOB. Hydroxylamine pulse addition has been proposed as a strategy to avoid NOB proliferation in partial nitritation systems (Wang et al., 2016, 2015; Kindaichi et al., 2004). Still, the mechanisms leading to NOB inhibition by hydroxylamine or information on how hydroxylamine impacts typical nitrification side communities (i.e. anammox, denitrification) is scarce. Overall, from an engineering point of view understanding hydroxylamine metabolism in typical biofilm systems used in wastewater treatment (i.e. activated sludge or emerging granular sludge technologies), where different microbial communities are clustered together, could help to design more efficient wastewater treatment plants and minimize N_2O emissions (Sabba et al., 2015). In this work we focus on granular sludge as an emerging technology for wastewater treatment, but insights obtained equally apply for biofilm and activated sludge systems. The main objective of the present study was to investigate how rate-limiting feeding of hydroxylamine, similar to a putative exposure in natural environments, can impact aerobic ammonium oxidizing conversions; both on short term (i.e. pulse feed exposure of AOB to hydroxylamine) as on long term (i.e. continuous exposure to hydroxylamine). Short term exposure tests were performed by comparing batch tests with ammonium to fed-batch tests performed with an ammonium pulse and continuous hydroxylamine feeding performed at different dissolved oxygen tensions. Furthermore, long term hydroxylamine feeding was performed in an aerobic granular sludge reactor performing partial nitritation to study the impact on the metabolism and microbial community dynamics. The results were analyzed using a simplified kinetic model that helped to identify the rate limiting steps depending on the substrate conditions and it was also used to explain the hydroxylamine accumulation dynamics. Finally, the apparent kinetic parameters related to the hydroxylamine oxidation were roughly estimated.

2. Materials and methods

2.1. Reactor set up and operation

Two different airlift reactors were operated in this study: R1 with a 5 L working volume and R2 with a 2.6 L working volume (Figs. S2, S4 and S6). Inoculum characteristics and synthetic medium are described in SI. Two different reactors were used, as R1 showed biomass attachment in the settler from day 180 (Fig. S2). To solve this problem, R2 was used, which had a different settler geometry.

The pH and the dissolved oxygen (DO) concentrations were measured using online sensors, but they were not controlled. Air flow was controlled with a rotameter (Aalborg, Denmark) in both R1 and R2. Air flow rate and influent rate were adjusted, when needed, to maintain partial nitritation (see Figs. S2, S4 and S6A). During R2 operation with hydroxylamine loading, air flow was maintained at 6 L/h, air flow for R1 can be found in Fig. S2A. Between days 140 and 240 of operation of R2 (Figs. S4 and S6), the reactor operation was maintained but not fully characterized due to impossibility to go often to the laboratory.

N_2O in the liquid phase was measured when needed and when possible using a Clark type sensor (Unisense,Denmark) located in the settler of the reactor.

Temperature was controlled with an external water jacket and subsequent external cryostat at ca. 22 °C, when needed during R1 operation (Fig. S2B). Temperature in R2 was only controlled during the start-up at ca. 25 °C for ca. 100 days (Fig. S4B). However, during the hydroxylamine loading experiments, temperature was not controlled but remained rather stable at ca. 22 °C (Fig. S6B).

Periodically, samples were withdrawn from the reactor for further analysis of dissolved nitrogen compounds concentrations, biomass concentration, average size distribution, settling velocity tests, FISH and 16 s analysis.

2.2. Short term hydroxylamine feeding: batch and fed-batch tests

To assess the short term impact of hydroxylamine in AOB, a first ammonium pulse was dosed to granular sludge performing partial nitritation. Once ammonium was totally consumed, a fed-batch test was performed with a pulse ammonium and continuous rate-limiting hydroxylamine feeding. Thus, the ammonium consumption by AOB could be studied with and without hydroxylamine feeding. Granular sludge from two reactors (R1 and R2, see SI for further details) performing partial nitritation was used. The rationale behind using a continuous addition strategy for the hydroxylamine feeding was to mimic what

could happen in natural environments where hydroxylamine can transiently accumulate in the bulk liquid (Liu et al., 2017; Stüven et al., 1992; Su et al., 2019b; Yu et al., 2018). Instead of using pulse like additions with high initial hydroxylamine concentrations, as it has been previously done (Chandran and Smets, 2008; Wang et al., 2015). The batch tests performed with biomass from R1 were performed at different days of operation and also targeting different hydroxylamine loading rates. Consequently, different dissolved oxygen concentrations were reached at each test (Table 1). A targeted study of the DO impact was performed with biomass from R2 and in 4 consecutive days to perform batch and fed-batch tests 6 to 9 in Table 1. When these tests were performed N_2O was also measured as there was a N_2O probe available. In between experimental days the R2 biomass was aerated and left with an ammonium pulse, to avoid as much as possible starvation. Thus, the next morning the biomass was washed with free nitrogen medium and the procedure for the tests started.

To perform the tests 1 L of R1 or R2 biomass was withdrawn from the reactor and washed twice with nitrogen free synthetic medium. R1 biomass was resuspended in 1 L of free nitrogen medium and transferred to a 1.2 L vessel to perform the batch/fed-batch tests. The final volume of the vessel used with biomass from R2 was 0.8 L, as 1 L vessel was used. A magnetic stirrer was used for agitation. Tests were performed at 25 °C controlling the temperature with an external water bath. pH was controlled at pH 8 ± 0.075 with a pH sensor (WTW-Sentix 81), and acid (1 M HCl) or base (1 M NaOH) addition with a microburete. An O_2 sensor (WTW-Cellox 325) and an N_2O sensor (Unisense) were used to monitor dissolved oxygen and dissolved nitrous oxide online. Compressed air or nitrogen (when needed) were sparged using a stone diffuser at a flow rate controlled with a mass flow controller (Bronckhorst F201-C).

After stabilization of all online signals (DO, pH) once pH control was started, the oxygen gas transfer coefficient (k_La) was estimated for each batch tests by stopping the air flow (or sparging with nitrogen) followed by air sparging again (see SI). Afterwards, the batch tests (B) were started by a pulse addition of ammonium to the targeted initial concentrations (Table 1). Once the batch was finalized, the fed-batch test was started by combining a pulse of ammonium and fed-batch (FB) addition of hydroxylamine at the desired load with a programable microburete. A cometabolization (COM in Table 1) test of hydroxylamine and ammonium was performed by pulse addition of both substrates to assess putative hydroxylamine inhibition.

Liquid samples (3,4 mL) were withdrawn over time, filtered and stored in the fridge for further analysis of nitrogen compounds. For hydroxylamine determination, 1.8 mL of sample were mixed with 0.2 mL of sulfamic acid at 5 g/mL. Biomass concentration during the test was analyzed at the end. See Physiochemical analysis section for further details. Rate calculations, N_2O emissions factor and other calculations related to the batch tests are described in the SI.

2.3. Long term hydroxylamine continuous feeding

Hydroxylamine long term dosing was performed in R2 from days 254 to 372 by supplying the usual synthetic medium (see SI) and adding an extra dosing pump for hydroxylamine addition. Hydroxylamine addition was performed separately from the original medium to avoid any type of reaction prior to reaching the reactor. Hydroxylamine addition flow rate was maintained constant at 1.17 ± 0.04 L/d, while hydroxylamine influent concentration was increased in each phase, in order to increase the hydroxylamine loading rate. Hydroxylamine solution concentrations used were 57 ± 12, 84 ± 6, 135 ± 7 and 132 ± 8 mg-N/L for Phase I, II, III and deterioration, respectively. Samples were withdrawn from the reactor for further analysis of dissolved nitrogen compounds concentrations, biomass concentration, average size distribution, settling velocity tests, FISH and 16 s analysis.

2.4. Determination of hydroxylamine kinetic parameters from experimental data

The continuous airlift reactor data weres used to estimate the apparent maximum hydroxylamine consumption rate and the half-saturation coefficient for hydroxylamine. Residual hydroxylamine concentration measured during the continuous reactor operation against total AOB consumption ($r_{NH_2OH,\ total} = r_{NH_4^+} + r_{NH_2OH}$) was fit to a Monod equation (Eq. (6)) using Sigma Plot. Total AOB consumption was used as assumed that all ammonium being transformed was being converted via hydroxylamine to nitrite. Thus, contributing to the total hydroxylamine consumption rate of the system.

Table 1
Batch/fed-batch tests conditions performed with biomass from R1 or R2. A batch (B) tests with a pulse of ammonium (initial NH_4^+) was followed with a fed-batch tests (FB) with a pulse addition of ammonium (initial NH_4^+) and continuous hydroxylamine feeding (NH_2OH load). Temperature was 25 °C and pH set point of 8 leading to an averaged pH of 8.0 ± 0.1. DO - dissolved oxygen reached during the stable phase, Max. – maximum. Hydroxylamine consumption rate (qNH_2OH) accounts for the difference between the accumulated hydroxylamine and the loading rate and does not take into account the amount of hydroxylamine transformed during ammonium oxidation (qNH_4^+). $\Delta qNH_4^+{}_{,FB/B}$ stands for the difference on ammonium consumption rate between the fed-batch and the batch test. Calculations are described in SI. * Initial hydroxylamine concentration (mg-N/L).

Test	Biomass source & day	Type of test	Air flow (mL/min)	DO (mg-O_2/L)	Initial NH_4^+ (mg-N/L)	NH_2OH load (mg-N/gVSS/h)	qNH_4^+ (mg-N/gVSS/h)	qNH_2OH (mg-N/gVSS/h)	Max. NH_2OH (mg-N/L)	$\Delta qNH_4^+{}_{,FB/B}$ (%)
1	R1, 99d	B	200	2.1 ± 0.2	19.7		69.2 ± 0.1			
1	R1, 99d	FB	200	2.0 ± 0.1	18.2	4.6	64.4 ± 0.1	4.6	0.03	−7
2	R1, 129d	B	200	2.6 ± 0.1	19		58.9 ± 0.1			
2	R1, 129d	FB	200	2.6 ± 0.1	18.3	9.0	58.0 ± 0.1	8.59 ± 0.02	0.16	−1
3	R1, 144d	B	200	1.2 ± 0.1	20.6		45.9 ± 0.1			
3	R1, 144d	FB	200	1.0 ± 0.5	21.1	14.2	46.3 ± 0.3	10.87 ± 0.04	1.38	1
4	R1, 164d	B	200	1.5 ± 0.1	18.6		38.4 ± 0.1			
4	R1, 164d	FB	200	1.3 ± 0.1	21.6	6.2	26.4 ± 0.1	5.62 ± 0.02	0.18	−31
5	R1, 211d	B	200	3.7 ± 0.2	15.1		25.1 ± 0.2			
5	R1, 211d	FB	200	3.6 ± 0.2	19.7	6.0	29.9 ± 0.2	4.8 ± 0.1	2.74	19
6	R2, 98d	B	400	5.8 ± 0.2	20.8	0	82.4 ± 0.4			
6	R2, 98d	FB	400	4.5 ± 0.2	19.7	16.2	93.3 ± 0.4	13.61 ± 0.03	0.38	13
7	R2, 98d	B	100	3.7 ± 0.3	20.1	0.0	89.8 ± 0.2			
7	R2, 98d	FB	100	3.4 ± 0.3	18.2	13.4	92.7 ± 0.1	13.03 ± 0.03	0.62	3
8	R2, 98d	B	10	0.8 ± 0.2	18.9	0.0	31.7 ± 0.1			
8	R2, 98d	FB	10	0.8 ± 0.2	18.1	13.8	30.5 ± 0.1	12.57 ± 0.04	1.42	−4
9	R2, 98d	B	40	1.9 ± 0.1	19.5	0.0	61.7 ± 0.1			
9	R2, 98d	FB	40	1.9 ± 0.1	20.0	13.6	64.7 ± 0.1	10.08 ± 0.04	1.08	5
9	R2, 98d	COM	40	2.7 ± 0.4	16.2	7*	56.7 ± 0.1	18.40 ± 0.08		

$$r_{NH_2OH,total} = \frac{r^{max}_{NH_2OH}\, C_{NH_2OH}}{K_{NH_2OH} + C_{NH_2OH}} \quad (6)$$

2.5. Physicochemical analysis and microbial community analysis

Nitrogen compounds were analyzed in liquid samples after filtration (0.22 μm). Nitrite and nitrate were analyzed by ionic chromatography using ICS-2000 Integrated Reagent-Free IC system (DIONEX Corporation, USA). Ammonium was measured using a gas selective electrode (GSE) (AMTAX sc, Hach Lange, Germany). When needed, ammonium, nitrite or nitrate were measured using Hach Lange kits (LCK303, LCK342 and LCK 339, respectively, Hach Lange, Germany).

Hydroxylamine was measured spectrophotometrically after pretreatment with sulfamic acid as in Frear and Burrell (1955), Soler-Jofra et al. (2016). Briefly, 1 mL of 0.05 M phosphate buffer, 1 mL of sample (diluted, if applicable), 0.8 mL of demineralized water, 0.2 mL of a 12wt% trichloroacetic acid solution, and 1 mL of a 1% 8-quinolinol solution were added to Pyrex tubes. After mixing, 1 mL of a 1 M Na_2CO_3 solution was also added. After mixing, the tube's content was boiled in a water bath (100 °C) for 1 min. After, tubes were cooled down for 15 min at room temperature. Afterwards, absorbence at 705 nm were measured the absorbence of the solution was. Blank was performed by substituting the 1 mL of sample for 1 mL of mili-Q water.

For biomass concentration determination total suspended solids (TSS) and volatile suspended solids (VSS) were determined according to standard methods (Apha, 2012). To assess the settleability of the granular sludge, the Sludge Volume Index (SVI) of 1 L of biomass was determined at 5 min and 30 min in a graduated cylinder. Volume size distribution and average particle size was determined with a laser diffraction analysis system (Malvern Mastersizer Series 2600, Malvern instruments Ltd., UK) (see Figs. S2, S4 and S6). Biomass was harvested periodically for microbial community analysis by FISH and 16S rRNA sequencing as described in SI.

3. Results

3.1. Batch/Fed-batch tests as a tool to assess the impact of hydroxylamine in partial nitritation aerobic granular sludge

3.1.1. Dissolved oxygen regulates ammonium and hydroxylamine consumption as well as N_2O production

Granular sludge from two different reactors was used for batch (B) tests with an ammonium pulse followed with fed-batch (FB) tests with the same ammonium pulse but with hydroxylamine continuous feeding (see Fig. 1).

Comparing the ammonium consumption rate results of tests with only ammonium and the tests where hydroxylamine was also continuously fed, the impact of a low (limiting) hydroxylamine concentration in the medium on the ammonium uptake rate (qNH_4^+ in Table 1) could be assessed. Depending on the dissolved oxygen concentration during the test two different trends where observed: (i) at low DO to middle DO range (0.8 to 3.6 mg-O_2/L, being tests 1, 2, 3, 4, 8 and 9 in Table 1), hydroxylamine dosing resulted in a slight increase (1–5%) in the ammonium uptake rate (tests 3, 7 and 9 in Table 1) or a 1–31% decrease in the ammonium uptake rate (tests 1, 2, 4 and 8 in Table 1). (ii) At a high DO concentration (3.6 to 5.8 mg-O_2/L, test 5 and 6, in Table 1), the ammonium uptake rate increased when hydroxylamine was also dosed to the system (13 and 19% increase, respectively). Notice, that an increased ammonium consumption at high DO was observed with tests performed with biomass from two different reactors (R1 and R2). Total nitrogen oxidation activity (i.e., including N-ammonium and N-hydroxylamine) was generally increased (see Fig. 1).

Overall, with granular sludge from both R1 and R2, DO concentration was the variable impacting the most the metabolism of ammonium and hydroxylamine consumption. The effect of DO concentration can be observed specially with tests 6–9 (Fig. 1B and Table 1). Dissolved oxygen (DO) strongly affected ammonium consumption rates as it ranged from ca. 30 to 93 mg-N/gVSS/h) depending on the DO. Instead, hydroxylamine consumption rate was slightly influenced being rather constant around 10–13 mg-N/gVSS/h (Table 1 and Fig. 1B).

Interestingly, DO not only impacted consumption rates, but also

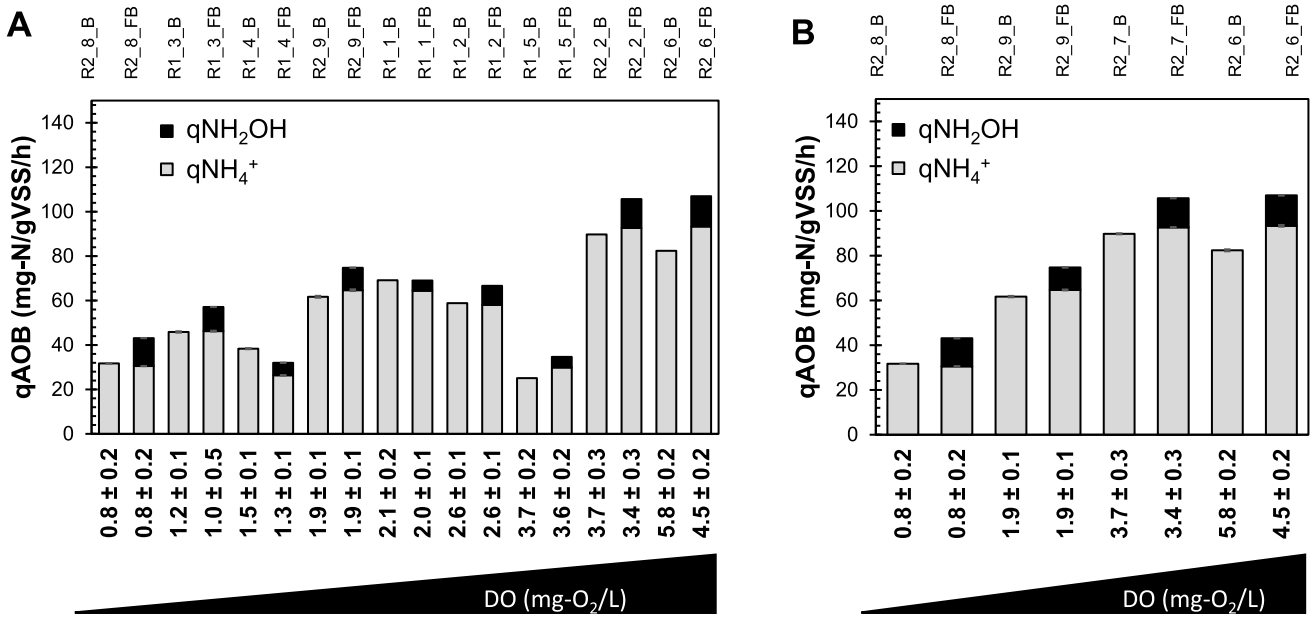


Fig. 1. Tests results performed with biomass from either inoculation period 1 (R1) or 2 (R2) followed by the number that corresponds with test conditions in Table 1. All respirometer tests consisted of an initial batch test (B) with only ammonium followed by a fed-batch (FB) with ammonium batch addition combined with hydroxylamine fed-batch. 9A) Comparison of all tests performed batch and fed-batch ammonium oxidation activity specific activity (qAOB), it is the addition of ammonium oxidation specific activity (qNH_4^+) and hydroxylamine oxidation specific activity (qNH_2OH) sorted by increasing conditions of oxygen. (B) Comparison of batch and fed-batch tests performed with biomass from R2, sorted in increasing dissolved oxygen concentrations.

hydroxylamine accumulation and N_2O emissions (Fig. 2), only when hydroxylamine was dosed continuously during the fed-bath tests. Lower DO led to higher hydroxylamine accumulation, as hydroxylamine accumulation went from 0.35 mg-N/L to 1.4 mg-N/L when oxygen went down from 4.5 to 0.8 mg-O_2/L (Fig. 2A). Furthermore, lower DO also resulted in higher N_2O emissions factor. The N_2O emission factor went from 1.3 to 3% at DO 1.8–5 mg-O_2/L up to ca. 21% at DO of 0.8 mg-O_2/L (Fig. 2B) when hydroxylamine was continuously added in the tests. Consequently, lower DO resulted in higher hydroxylamine accumulation and higher N_2O emissions.

As just mentioned, in some fed-batch tests hydroxylamine was accumulating up to relatively high concentrations (>1 mg-N/L see Table 1). To specifically investigate the effect of elevated hydroxylamine concentrations the co-metabolization of hydroxylamine and ammonium was evaluated in tests were a pulse of both hydroxylamine and ammonium where added after a nitrification batch test without hydroxylamine addition. The initial hydroxylamine concentration was 7 mg-N/L, for test 9 (see Table 1). Thus, hydroxylamine concentrations were higher during the co-metabolization experiments than those observed in the tests with continuous hydroxylamine addition. The ammonium oxidation rate was 61 mg-N/gVSS/h during the batch with only ammonium and 56 mg-N/gVSS/h when hydroxylamine and ammonium were both present.

Summarizing, at low DO hydroxylamine addition resulted in decreased or slightly increased ammonium consumption rate, hydroxylamine accumulation and N_2O production, whereas at high DO, hydroxylamine addition increased ammonium consumption with less hydroxylamine accumulating and less N_2O produced (Figs. 1 and 2).

3.2. Long term impact of hydroxylamine feeding to a continuous flow partial nitritation reactor

3.2.1. Hydroxylamine is transformed to nitrite and reduces ammonium consumption

A stable operating continuous flow granular sludge nitritation reactor was subjected to adding hydroxylamine at various loading rates. The impact on the nitrogen conversion processes and the microbial population dynamics were assessed.

The hydroxylamine load to the granular sludge airlift reactor was gradually increased during 3 operational phases (Phase I, II and III in Figs. S6 and S8). The air flow was maintained stable during the whole operation, the DO gradually increased from 3.6 ± 0.2 mg-O_2/L without hydroxylamine addition to 4.3 ± 0.4, 4.4 ± 0.9 and 5.2 ± 0.5 mg-O_2/L in phase I, II and III, respectively (see Figs. S6B and S8B). The pH did not vary significantly during the operation (Figs. S6B and S8C). Granule size and particle size distribution was stable over time (Fig. S7) as well as biomass concentration during Phase I, II and III (Figs. S6C and S8A).

Interestingly, ammonium consumption rate decreased with increasing hydroxylamine loading rate (see Fig. 3A). Nitrite production remained the same with or without hydroxylamine. The decreased ammonium conversion and constant nitrite production indicates that the hydroxylamine conversion to nitrite compensated the decreased ammonium conversion. The stoichiometric ratio calculated from the produced nitrite over total nitrogen consumed, including NH_2OH and ammonium consumption, was close to the theoretical value of 1 (Fig. 3D). A maximum of ca. 2–7% nitrogen was unaccounted in the nitrogen balance during the whole reactor operation, which can be due to accumulated measurement error combined with N_2O production. For instance, occasional N_2O was detected in the liquid phase, specially at the beginning of hydroxylamine feeding during Phase I, but decreased after few hours (see Fig. S9A). Occasionally, N_2O peaks were detected in the liquid (<1 mg-N/L), but not in a consistent and continuous way (see Fig. S9B). More N_2O measurements could not be performed during the rest of the phases due to technical problems with the sensor.

3.2.2. Microbial community dynamics

The microbial population dynamics during the airlift reactor operation without and with hydroxylamine feeding was investigated using FISH and 16S rRNA sequencing. FISH performed in pottered biomass indicated that the majority of the population was formed by AOB. Over time, a slight increase in NOB population and a decrease of the EUB signal was observed in the slides for pottered sludge samples (Fig. S10). When observing cryosectioned granules a very low number of NOB could be observed (Fig. S11). The relative abundance of AOB *Nitrosomonas* sp. in the 16S rRNA analysis was stable at 42 ± 6% of the OTU's over the full operational period (**Fig. 4**). Which corresponded with the generally distributed hybridization of Nso190 probe in the FISH. Three different OTUs were identified as *Nitrosomonas*. One of them *Nitrosomonas sp.* 2 in Fig. 4 significantly increased from the beginning of the hydroxylamine feeding from 5 ± 2% to 13 ± 3% in days 102–283 and days 296–332, respectively. The *Nitrosomonas* OUT's could not be conclusively allocated to a cluster (see Table S3 for the raw sequences). Interestingly, no NOB OTU's were detected with 16S-RNA analysis in accordance with FISH performed on cryosectioned granules and with the low nitrate production observed during cultivation. Still, contradicting partially the FISH results for pottered biomass samples, where NOB signal, although at a low intensity, was detected in some of the samples.

The side population, as observed by 16S rRNA sequencing, showed a considerable change when hydroxylamine was fed. For instance,

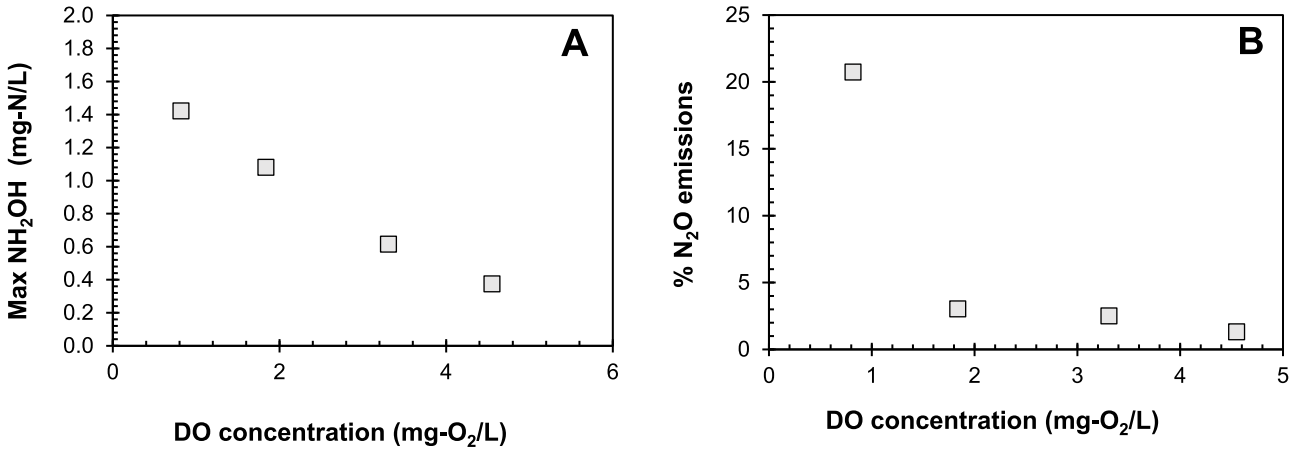


Fig. 2. Nitrification tests with ammonium present and continuous hydroxylamine feeding with granular sludge (biomass from reactor R2). Impact of dissolved oxygen concentration on: (A) Maximum accumulated hydroxylamine concentration (total amount added was between 7.4 to 3.5 mg-N/L, depending on the duration of the test) and (B) N_2O emissions as percentage of the total N_2O emitted over the total nitrogen consumed (ammonium + hydroxylamine).

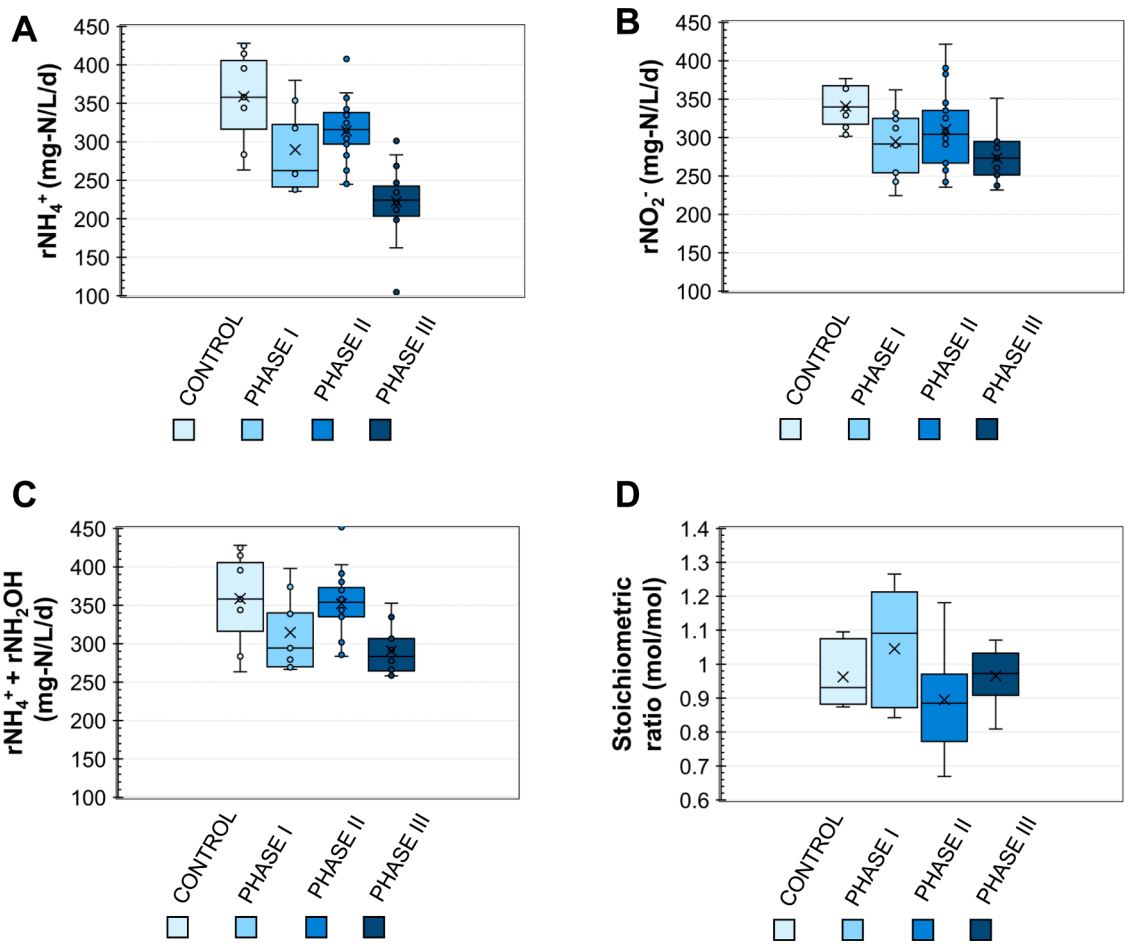


Fig. 3. Airlift reactor conversion rates and stoichiometric ratios for different hydroxylamine loading rates. A) Ammonium consumption rate, B) Nitrite production rate, C) Sum of ammonium and hydroxylamine consumption rate, D) Stoichiometric ratio of nitrogen produced over nitrogen consumed taking into hydroxylamine consumption.

Acidovorax went from ca. 6 ± 1% in the control phase up to ca. 25 ± 3% in Phase I and II. However, in Phase III it decreased again up to 14 ± 2% and the side population switched to Unclassified microorganisms from the family *Xanthomonadaceae, Thermomonas* and *Brevundimonas*.

3.2.3. Experimental assessment of apparent hydroxylamine consumption kinetics

In the present work, during the continuous feeding of hydroxylamine and ammonium to the continuous flow granular sludge airlift reactor, residual hydroxylamine was measured daily for each hydroxylamine loading rate. Taking into account that total hydroxylamine consumption by the reactor is derived from the inflow hydroxylamine as well as from the ammonium oxidation (as hydroxylamine is an intermediate step). The apparent half-saturation coefficient and apparent maximum consumption rate for the granular sludge system was estimated using curve fitting of the data to a typical Monod consumption kinetic model (Fig. 5). Maximum hydroxylamine consumption was estimated to be 359 ± 16 mg-N/L/d (46 ± 22 mg-N/gVSS/h) and hydroxylamine half-saturation coefficient was estimated as 0.015 ± 0.006 mg-N/L.

4. Discussion

4.1. Dissolved oxygen governs hydroxylamine accumulation and N_2O production

The DO concentration was identified as the main factor governing hydroxylamine accumulation and N_2O production in a nitrification process (Fig. 2). Additionally, the ammonium consumption rate was impacted by hydroxylamine dosage, depending on the DO concentration (Figs. 1 and 2). In order to be able to explain the observed impact of hydroxylamine addition on ammonium oxidation, the kinetics and factors governing ammonium oxidation are crucial (see Fig. 6). Three main reactions are responsible for the observed ammonia oxidation rates: (i) ammonium transformation to hydroxylamine (mediated by AMO), (ii) Hydroxylamine oxidation to nitrite (mediated by HAO), and (iii) the electron transport chain (E.T.C), these reactions are all linked by the reduced (Mred) or oxidized forms of the electron carriers (Mox) (see Fig. 6).

When both ammonium and oxygen concentrations are high, their corresponding Monod terms will tend towards one (see r_{NH_3} and r_{O_2} in Fig. 6), resulting in the simplified Eqs. (16) and (17). The availability of Mred will depend on the hydroxylamine consumption rate (r_{NH_2OH}). Consequently, $r_{NH_{2OH}}$ will determine the process velocity. In such conditions, hydroxylamine is not likely to accumulate as sufficiently Mox should be available and hydroxylamine consumption will determine both r_{NH_3} and r_{O_2} by providing them with Mred. Consequently, r_{max} of each reaction and the affinities for Mred will govern the r_{NH_3} and r_{O_2} kinetic rates (see Eqs. (7) and (8)).

$$r_{NH_3} = r_{max,NH_3} \cdot \frac{C_{Mred}}{C_{Mred} + K_{Mred,NH_3}} \quad (7)$$

$$r_{O_2} = r_{\max,O_2} \cdot \frac{C_{Mred}}{C_{Mred} + K_{Mred,ETC}} \quad (8)$$

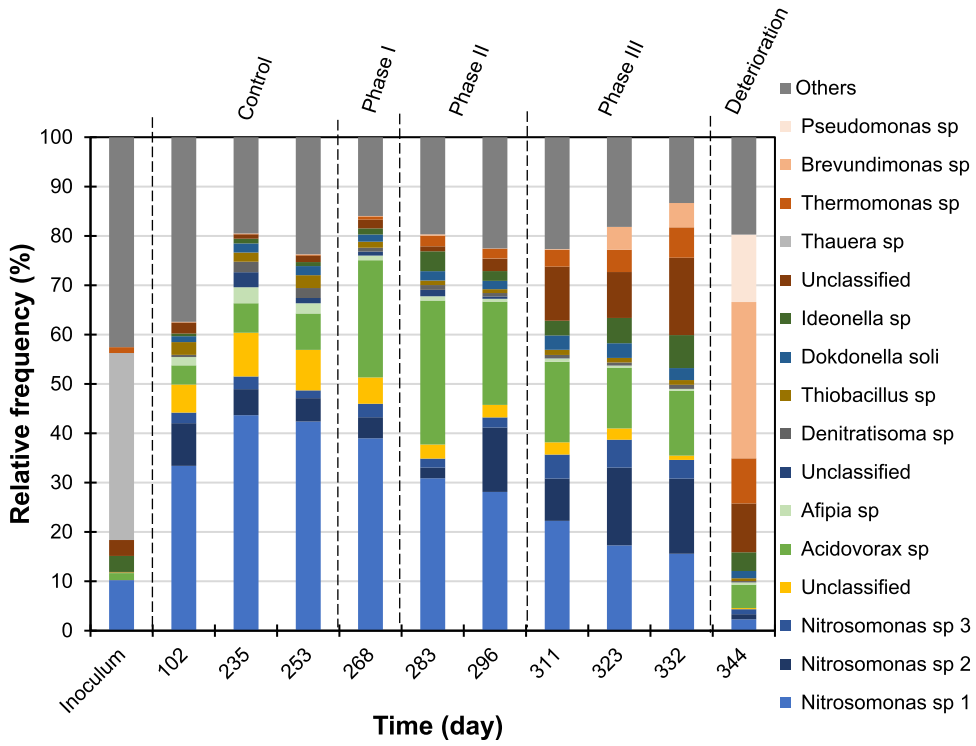


Fig. 4. Microbial relative frequency based on 16S rRNA sequencing of the predominant OTUs during different days of the airlift reactor operation.

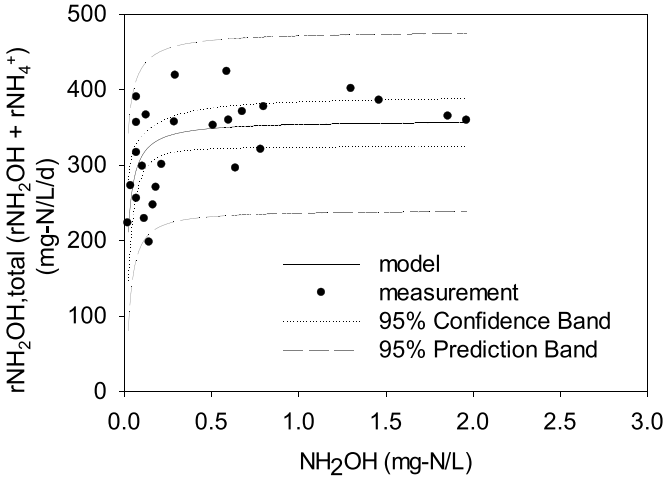


Fig. 5. Estimations of apparent kinetic parameters for ammonium oxidation by fitting Eq. (15) to the data from effluent hydroxylamine residual concentration over total ammonium oxidation consumption (hydroxylamine and ammonium) during continuous long term feeding.

On the contrary, if ammonium concentration is high and dissolved oxygen concentration is low, the conversion of hydroxylamine (r_{NH_2OH}) will be limited by the turnover of Mox once Mred is used during ammonium consumption or the oxygen electron consumption in the terminal electron acceptor chain. In such conditions, the ammonium Monod term or r_{NH_3} will not play an important role as it will approach 1 (see r_{NH3} in Fig. 6). As oxygen concentrations are low, oxygen affinities and maximum rates will now play an important role and small differences in these values will determine if r_{NH3} or r_{O_2} is the rate limiting process (see Eqs. (9) and (10)). Thus, hydroxylamine (and putatively other intermediates) will tend to accumulate, if the Mox needed for the reaction is not regenerated fast enough. As a strategy to avoid NH_2OH (and other intermediates) accumulation, Mred can be used to convert nitrite or NO generated from hydroxylamine to N_2O and generating Mox, as it does not depend on oxygen availability.

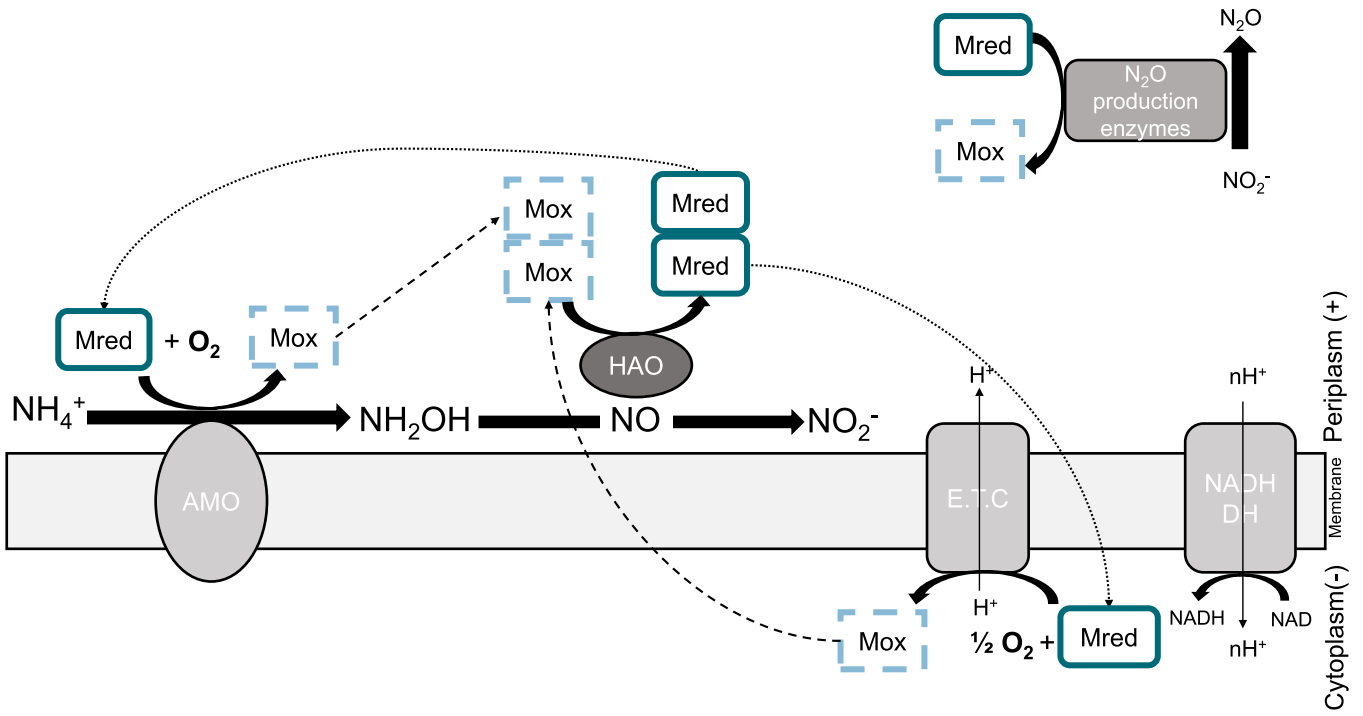
$$r_{NH_3} = r_{\max,NH_3} \frac{C_{O_2}}{C_{O_2} + K_{O_2,NH_3}} \cdot \frac{C_{Mred}}{C_{Mred} + K_{Mred,NH_3}} \quad (9)$$

$$r_{O_2} = r_{\max,O_2} \frac{C_{O_2}}{C_{O_2} + K_{O_2}} \cdot \frac{C_{Mred}}{C_{Mred} + K_{Mred,ETC}} \quad (10)$$

Overall, ammonium oxidation is highly regulated by the availability and turnover of Mred and Mox. The affinities for substrates and the difference of maximum rates between the AMO, HAO and the E.T.C corresponding reactions might impact the process regulation (see Eqs. (15) to (9)). Depending on the ammonium and oxygen consumption parameters, hydroxylamine accumulation will be most likely at high ammonium concentrations and limiting oxygen concentrations.

The kinetic explanation is in agreement with the experimental observations reported here. For instance, in the fed-batch tests where hydroxylamine was continuously dosed and performed at lower DO and high initial ammonium concentrations (batches 8 and 9 in Table 1 and Fig. 2A) hydroxylamine accumulated the most. More N_2O was produced at lower DO concentrations (Fig. 2B), as a strategy to use the available Mred to generate Mox without consuming oxygen. When DO was higher, less hydroxylamine accumulated and less N_2O was produced (Fig. 2). Overall, the impact of DO on hydroxylamine accumulation shows that somehow the limiting step for the process is the Mox production (either resulting from ammonium consumption or oxygen consumption in E.T.C.).

The kinetic analysis proposed here, also easily fits with observed transient accumulation events of hydroxylamine reported in literature (without external addition of hydroxylamine). For instance, hydroxylamine has been transiently detected when switching from anoxic to aerobic conditions, in planktonic chemostat cultures of *Nitrosomonas eruopaea* (Yu et al., 2010, 2017, 2018). At such conditions, the oxidized form of the electron carriers will be limiting at the beginning of the aeration phase, limiting the availability of Mox for hydroxylamine



PROCESS	ENZYMES	SUBSTRATES	PRODUCTS	RATES
1. Ammonium oxidation	AMO	NH_4^+ O_2 Mred	NH_2OH Mox	$r_{NH_3} = r_{max,NH_3} \frac{C_{O_2}}{C_{O_2}+K_{O_2,NH_3}} \cdot \frac{C_{NH_3}}{C_{NH_3}+K_{NH_3}} \cdot \frac{C_{Mred}}{C_{Mred}+K_{Mred,NH_3}}$
2. Hydroxylamine oxidation	HAO	NH_2OH 2 Mox	NO_2^- (via NO) 2 Mred	$r_{NH_2OH} = r_{max,NH_2OH} \cdot \frac{C_{NH_2OH}}{C_{NH_2OH}+K_{NH_2OH}} \cdot \frac{C_{Mox}}{C_{Mox}+K_{Mox,NH_2OH}}$
3. Electron terminal acceptor	E.T.C	1/2 O_2 Mred	Mox	$r_{O_2} = r_{max,O_2} \frac{C_{O_2}}{C_{O_2}+K_{O_2,}} \cdot \frac{C_{Mred}}{C_{Mred}+K_{Mred,O_2}}$
4. Nitrifier denitrification	N_2O production enzymes	NO_2^- Mred	1/2 N_2O Mox	$r_{N_2O,1} = r_{max,N_2O,2} \frac{C_{NO_2^-}}{C_{NO_2^-}+K_{NO_2^-,N_2O}} \cdot \frac{C_{Mred}}{C_{Mred}+K_{Mred,2,N2O}}$

Fig. 6. Schematic model of ammonium oxidation. Electron transport has been simplified by using Mred and Mox as the reduced and oxidized forms of the electron carriers, respectively. AMO – Ammonium monooxygenase, HAO – hydroxylamine oxidoreductase, E.T.C – Electron transport chain. Notice that the location of the catalytic site of AMO is not fully defined. Thus, it could also be located in the periplasm. Also as hydroxylamine oxidation can occur without ammonium, thus two Mox equivalents might come from the E.T.C. The figure was created based on the adaptation and combination of different sources (i.e. Ni et al., 2011, Stein, 2011, Yu et al., 2010).

consumption. It makes it likely that hydroxylamine transiently accumulates until sufficient Mox is generated. Indeed N_2O peaks were detected just after the switch from anoxic to aerobic conditions in nitrification cultures (Yu et al., 2010, 2017, 2018). Consequently, N_2O was generated at the beginning of the transition to aerobic conditions to use the available Mred and convert it to Mox to generate sufficient Mox for hydroxylamine conversion. Similarly, in SBR processes with mixed cultures transient hydroxylamine production right after the start of the feeding phase has been detected (Su et al., 2019b). A step increase in ammonium loading rate also showed to generate transient accumulation events in continuous systems (Hu et al., 2017; Poot et al., 2016). When a step-increase in ammonium load occurs, the system will tend to be more (kinetically) oxygen limited.

Finally, when performing batch tests with only ammonium, hydroxylamine has also been shown to accumulate (Liu et al., 2017; Stüven et al., 1992). The initial high concentration of ammonium allowing a fast conversion can easily lead to oxygen uptake limitations and higher levels of Mred and accumulation of some hydroxylamine.

Liu et al. (2017) measured higher hydroxylamine concentration peaks with higher initial ammonium concentrations. The higher the ammonium concentration the more likely it will be kinetically limited by oxygen. Liu et al. (2017) also observed different hydroxylamine accumulation pattern in different AOB/AOA and comammox strains. This would fit with the high dependency of hydroxylamine accumulation on the affinities and maximum rates of AMO and E.T.C. conversions. Thus, depending on the affinities or maximum velocities of the different AOB/AOA/comammox strains they will be able to maintain a sufficiently high Mox supply or reducing ammonium consumption to avoid hydroxylamine accumulation.

In the present study, during the batches (B) with only ammonium as substrate, no hydroxylamine accumulation was observed independent of the DO concentration imposed. However, increased hydroxylamine accumulation was observed with decreasing oxygen concentration during fed-batch tests (Fig. 2). Thus, it could be expected that in inner

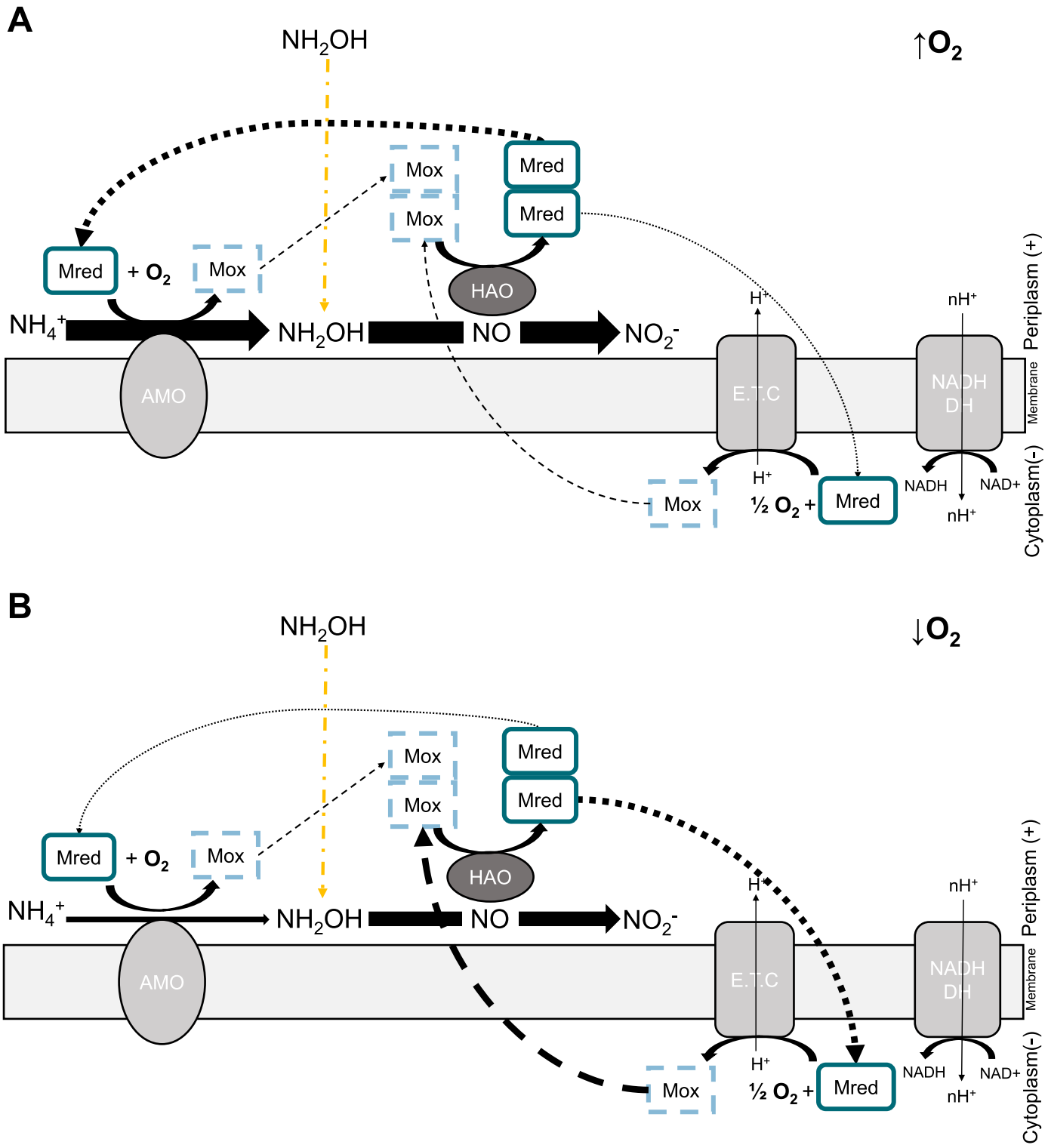


Fig. 7. Redistribution of Mox/Mred leads to higher or lower ammonium consumption when external hydroxylamine is provided depending on the DO available on the system: (A) High dissolved oxygen concentration, (B) Low dissolved oxygen concentration.

zones of the biofilm hydroxylamine could accumulate, as DO is lower. However, once hydroxylamine diffuses to outer layers of the biofilm, with higher DO concentrations it might be consumed there and not seen in the bulk liquid. Consequently, the granular structure of our sludge might have masked putative transient accumulation of hydroxylamine during batches with only ammonium. Thus, hydroxylamine could be transformed before reaching the bulk liquid (as also discussed in Poot et al., 2016).

Overall, it should be highlighted that when having conditions with low oxygen and high ammonium concentrations, the ammonium oxidation or the consumption of oxygen in electron transport chain could be limiting the hydroxylamine conversion step. Thus, favoring hydroxylamine accumulation if the oxidized electron mediators are not provided fast enough.

4.2. Ammonium consumption is regulated based on hydroxylamine and electron donor availability

The DO concentration not only triggered hydroxylamine accumulation, but also had a direct impact on the ammonium consumption rate. In the batch tests with continuous hydroxylamine feeding, ammonium consumption was increased when hydroxylamine was present at high DO (batches 5 and 6 in Fig. 1 and Table 1). However, if DO was low ammonium consumption was maintained or slightly reduced when hydroxylamine was fed (Fig. 1, Table 1). This can be explained because at high DO, hydroxylamine conversion will be less limited by Mox (Fig. 6). When hydroxylamine is added extra, the available Mred will increase which can boost the ammonium consumption (see Fig. 7A).

In previous studies, the impact of hydroxylamine was only investigated during the so called "acceleration phase", but never during continuous exposure. The "acceleration phase" is related to the delay observed on reaching the maximum ammonium consumption capacity when adding an ammonium pulse to a nitrification system (Chandran and Smets, 2008; Guisasola et al., 2006). The hypothesis was that the level of reduced electron mediator was initially limiting the ammonium oxidation (Guisasola et al., 2006). Adding hydroxylamine in form of a pulse to an aerated system, before the addition of ammonium, reduced dramatically the acceleration phase (Chandran and Smets, 2008). It was hypothesized that hydroxylamine would increase the level of reduced electron mediator, allowing ammonium to proceed at maximal rate from the start of ammonium addition (Chandran and Smets, 2008). However, our study did not focus on the initial ammonium consumption rate as in (Chandran and Smets, 2008; Guisasola et al., 2006), but rather on the ammonium consumption rate once stable DO was reached. Thus indicating that extra hydroxylamine can increase ammonium consumption when sufficient oxygen is available.

When DO was low and hydroxylamine was provided, ammonium consumption was either maintained, slightly increased or reduced and hydroxylamine accumulated resulting in high N_2O formation in the fed-batch tests (Figs. 1 and 2). When hydroxylamine was fed continuously to the airlift reactor at different loading rates, ammonium oxidation was also reduced and DO increased gradually, indicating a decrease on the oxygen uptake rate (Figs. 3A and SI). During the long term feeding of hydroxylamine, the DO might have been rate limiting especially in the inner parts of the granules. The conditions observed during the continuous feeding might have been comparable to the middle DO conditions of the fed-batch test experiments: low amounts of hydroxylamine accumulated and a low N_2O production was observed while ammonium consumption decreased with increasing hydroxylamine loading rates (Fig. 3A). The range of DO triggering higher ammonium consumption rates might be impacted by other conditions. Continuous long term operation was performed at lower pH and temperature (ca. 7.6 and 22 °C) than the batch tests (pH 8 and 30 °C). At lower temperature and lower pH the ammonium consumption rate is decreased (Jubany et al., 2008). Consequently, maximum ammonium consumption rate will be lower and extra hydroxylamine dosing might have a different impact as limiting DO ranges will differ.

The reduction of ammonium consumption when hydroxylamine was provided and DO was kinetically limiting might be explained as follows: from a kinetic point of view other process competing for similar substrates (oxygen and Mred) are relatively faster than ammonium consumption, resulting in the reduced ammonium oxidation rate. Alternatively, when supplying extra hydroxylamine, more Mred is generated with higher DO. Thus, the affinity for Mred is less critical. If DO is not sufficiently high, the extra generated Mred is not redistributed to increase ammonium consumption. Oxygen will then limit the turnover of Mred to Mox, not providing the Mox needed for hydroxylamine conversion.

Having a slightly faster oxygen consumption in the E.T.C than for ammonium consumption when oxygen is limiting makes sense as a strategy to avoid as much as possible intermediates accumulation (NH_2OH or NO) under oxygen limiting conditions. This is what was actually observed in the continuous airlift reactor, where ammonium consumption was reduced and dissolved oxygen in the system increased with increasing hydroxylamine loading rates (see Figs. 3A and SI). It should be highlighted that even ammonium consumption was reduced, total nitrite production was not (Fig. 3). Indicating that hydroxylamine was mainly being transformed to nitrite (Fig. 3).

With the observed increased bulk DO concentration over time, it would have been expected for AOB to use such extra DO to consume more ammonium. However, ammonium consumption rate was reduced. Suggesting that the E.T.C. rate was the main rate-governing factor in the process, as if more electrons were derived from NH_2OH there was less demand to derive electrons from full ammonium oxidation to nitrite. Consequently, the use of electrons from hydroxylamine conversion reduced both the ammonium and oxygen consumption. Overall, small differences between the parameters will efficiently regulate the whole process. As oxygen consumption during ammonium consumption needs to be generally faster than the E.T.C, otherwise, ammonium consumption would not take place. Understanding the coupling between each process would allow to further understand the central nitrogen metabolism regulation.

N_2O formation was detected both in the batch tests and during the continuous airlift reactor feeding (Figs. 2 and S9). Specifically, higher N_2O emissions were detected during the batch tests with hydroxylamine feeding at low DO concentration (Fig. 2). At the conditions at which the tests were performed (pH 8 and 25 °C and low nitritre concentrations), the abiotic reaction of hydroxylamine and free nitrous acid is not likely (Soler-Jofra et al., 2016; Su et al., 2019a). The traditional biochemical pathway resulting in N_2O emissions when oxygen concentration is low, is called nitrifier denitrification (Stein, 2011). Nitrite is reduced to N_2O to compensate limiting amounts of oxygen as terminal electron acceptor. However, in our experiments hydroxylamine accumulation might be more direct related to N_2O formation. Recently a biochemical route from hydroxylamine to N_2O was proposed to be mediated by cytP460 (Caranto et al., 2016) in the absence of oxygen. At the low DO in granular sludge systems the deeper layers in the granules will be deprived of oxygen. To further confirm if either nitrite or hydroxylamine was being consumed to form N_2O, ^{15}N or/and site preference studies will be necessary. Furthermore, an initial high N_2O production was observed at the beginning of the hydroxylamine long term dosing in the airlift reactor (Fig. S9). This initial high N_2O production might have been a transient metabolic solution in order to provide sufficient Mred to consume the extra hydroxylamine dosed and avoid its accumulation. Most likely, DO was initially insufficiently high to cope with the extra dosing of hydroxylamine, using N_2O production as a solution. Targeted experiments to understand NO/N_2O production with rate-limiting hydroxylamine feeding and how the metabolism rearranges from a transcriptomic/proteomic point of view would be crucial to understand such complex behaviors.

Overall, when oxygen is high, the hydroxylamine oxidation step is in charge on regulating the whole AOB metabolism. At these conditions, if

external hydroxylamine is available, ammonium consumption is boosted by the extra formation of Mred. It is interesting to realize that the 'maximal' ammonium oxidation rate can be increased in the presence of externally added hydroxylamine. When oxygen is limiting, the preferential use of oxygen as terminal electron acceptor will reduce the ammonium oxidation rate, preventing accumulation of nitrification intermediates. Indirectly, hydroxylamine oxidation will be also regulated by the oxygen use in the E.T.C., if not sufficient Mox is provided hydroxylamine will accumulate. Consequently, the differences between the ammonium oxidation rate and the turnover of terminal acceptor will determine the putative accumulation of hydroxylamine.

4.3. More hydroxylamine rate-limiting continuous feeding studies are needed to understand the long term impact of hydroxylamine on ammonium oxidation

In the continuous airlift reactor hydroxylamine was efficiently transformed to nitrite, combined with a reduced ammonium oxidation. However, after ca. 3 months of operation with hydroxylamine feeding, the reactor operation deteriorated quite quickly (see Fig. S6). Other indications of deterioration are the decreased EUB signal observed with FISH over time, and the appearance of a mainly oligotrophic side-population (Figs. 4, S10 and S11). Different possibilities might explain this deterioration. At the working conditions (pH 7.7) hydroxylamine is mainly unprotonated (see Fig. S11B), thus it can diffuse over the cell membrane. This gives an uncoupling effect and results in extra energy demand reducing the growth of bacteria. Another possibility was that the cell could not cope with the reduced ammonium consumption, increased E.T.C. velocity and growth at the same time.

Previous studies with hydroxylamine were mainly performed by pulse like addition strategies (Harper et al., 2009; Okabe et al., 2011; Wan et al., 2016; Wang et al., 2015). With these type of feedings a negative impact of hydroxylamine was reported by Harper and co-workers on the long run (Harper et al., 2009), while other studies used hydroxylamine as a strategy to promote partial nitrification over full nitrification (Wan et al., 2016; Wang et al., 2015).

The only comparable study in terms of feeding strategies was performed by de Bruijn et al. (1995). A higher growth yield was reported when feeding mixotrophically ammonium and hydroxylamine than when feeding only with ammonium. Ammonium oxidation was not deteriorated in de Brujin study, indicating that *Nitrosomonas europaea* could grow mixotrophically with hydroxylamine. This would fit with the fact that *Nitrosomonas* was the predominant AOB in the present study (Fig. 4) and that the biomass concentration was maintained during the different hydroxylamine feeding (Figs. S8A and 6C). De Bruijn et al. (1995) also observed a bigger nitrogen imbalance than observed in the present study. Their experiments were performed with planktonic cells and conditions (i.e. 30% DO saturation, pH 8 and 30 °C) that are deviating from those in the present study (i.e. granular sludge, 22.3 ± 1.2 °C, pH 7.4 ± 0.1, DO from 3.6 ± 0.2 to 5.2 ± 0.5 mg-O_2/L, see Figure S8). One putative explanation for the higher nitrogen imbalance observed by de Bruijn is that at higher pH and temperature ammonium consumption is promoted (see Fig. S12). Thus, if on top of that, an intermediate like hydroxylamine is fed, it is more likely that hydroxylamine accumulates; promoting a bigger nitrogen imbalance and the formation of N_2O emissions, as seen in the experiments of de Bruijn et al. (1995). It would be interesting to have a better characterization of NO/N_2O emissions, as due to technical problems N_2O was not followed during the whole operation with hydroxylamine.

Overall, to assess the possible long term impact of hydroxylamine feeding, transcriptomics and proteomics will be crucial to give more insight on the impact of hydroxylamine in nitrification. More studies will be needed with limiting hydroxylamine feeding to reach definitive conclusions.

4.4. Importance of the hydroxylamine oxidation step in modeling N_2O emissions

The hydroxylamine accumulation and the NO or N_2O production as a function of the oxygen concentration will be very sensitive to the kinetic parameters (half-saturation coefficient and maximum rate) governing each process step in a mathematical model. The importance of parameter estimations in model applications is crucial for a good model use and process understanding (Petersen et al., 2003; Sin et al., 2005). The widely used Activated Sludge Models (ASM) did not include intermediate processes, such as nitrite or hydroxylamine (Henze et al., 2000). This was justified by the main model purpose, estimating the effluent concentrations. The concentrations of intermediates of ammonium conversion to nitrate have a marginal impact on the nitrogen mass balance of a municipal wastewater treatment plant. In recent years the ASM have been extended for nitrite because of the interest in partial nitrification/anammox process (Hao and van Loosdrecht, 2004). With the growing interest in greenhouse gas emissions also other intermediates in nitrogen conversion processes (NO and N_2O) became state variables in activated sludge models (Ni and Yuan, 2015). Parameters related to the hydroxylamine oxidation step in nitrification (r_{NH_2OH} in Fig. 6), such as hydroxylamine half-saturation coefficient (K_{NH_2OH}) or maximum hydroxylamine oxidation rate (μ_{NH_2OH}, k_{NH_2OH} or r_{max,NH_3} in d^{-1} or mg-N/gVSS/h depending on the nomenclature and notation used) are usually not evaluated from dedicated experiments. Even, more importantly, the oxygen affinity of the E.T.C. during hydroxylamine oxidation as single substrate has rarely been determined. Hydroxylamine conversion related parameters have usually been obtained from multivariable optimization of a large set of parameters through ammonium, nitrite, DO and/or N_2O data fitting (Law et al., 2012; Ni et al., 2011). Recently, Domingo-Felez et al. (2017), presented a calibration methodology to obtain such parameters which takes into account hydroxylamine measurement data and dedicated respirometry experiments. There are hardly or no good hydroxylamine conversion data with mixed cultures and only few experimental data with hydroxylamine conversion by pure cultures (de Bruijn et al. 1995; Frijlink et al., 1992; Stein et al., 1997). Furthermore, in view of the DO impact on ammonium and hydroxylamine oxidation rates as well as on the N_2O formation (see Figs. 1 and 2). Kinetic parameter determinations will be really sensible to oxygen concentrations. For instance to be able to determine maximum ammonium consumption rates one should be aware to avoid or reduce as much as possible oxygen limitations.

With the hydroxylamine continuous addition experiments performed in the present study an apparent maximum hydroxylamine conversion coefficient and apparent affinity constant were estimated (Fig. 5). It should be noted that the parameters presented here are all apparent and performed with granular sludge. Thus, they might differ depending on the biofilm type, structure and microbial composition.

Apparent kinetic parameters are commonly used in ASM modeling. Still, obtaining intrinsic parameters would be desirable. However, to obtain intrinsic parameters from floc or biofilm systems is not trivial, as several parameters impact the determination from calculations (van den Berg et al., 2020). For example, substrate diffusivities, granule size, granule porosity and density, etc. Even if all the parameters could be determined accurately microcolony size and distribution within the granule structure will impact the parameter estimation (see Picioreanu et al., 2016). Consequently, planktonic pure cultures will be needed for obtaining intrinsic kinetic parameters. The experimental set-up used here and in de Bruijn et al. (1995), continuous rate-limiting feeding of hydroxylamine, could be used for hydroxylamine kinetic parameters determination in planktonic cultures.

Also the inclusion of NO measurements would allow for a better understanding of the role of this other intermediate in nitrification (Hao and van Loosdrecht, 2004; Kampschreur et al., 2008a, 2008b). NO measurements during hydroxylamine feeding might also be of interest to see if this compound transiently accumulates or is emitted when

hydroxylamine is added.

Overall, if hydroxylamine is introduced as a state variable in a model, its kinetic apparent parameters are to be calibrated accordingly, which is not commonly performed.

4.5. Implication of the findings and future challenges

In the present study the effect of DO on hydroxylamine by an AOB enriched granular sludge biomass was studied. One important aspect that was not investigated is the pH effect. The dissociation of nitrogen compounds has in general a strong impact on nitrification processes (Anthonisen, 1976). Hydroxylamine is a weak base (pKa value 5.94 at 25 °C (Haynes, 2014)) and will therefore change from NH_3OH^+ to NH_2OH in the physiological pH range (see Fig. S11B). In the simplified model proposed (Fig. 6), pH can have several effects on the different reactions involved in ammonium oxidation: (i) the equilibrium between protonated and unprotonated form of ammonium and hydroxylamine might impact its diffusion through the membrane, (ii) Frijlink et al. (1992) showed that ammonium oxidation was highly impacted by pH whereas hydroxylamine oxidation was not, reducing ammonium oxidation with lower pH (iii) The difference in proton concentration between the periplasm and cytoplasm will impact the E.T.C. and potentially also the energy generation step. Moreover, the intrinsic impact of pH on substrates and each conversion step rates, pH gradients usually exist in biofilm systems and granule systems. Thus, different consumption rates might be expected depending on the location in the biofilm. This overall complexity of influence of pH on hydroxylamine conversion warrants a more detailed investigation in the future.

The impact of intermediates (NO and hydroxylamine) on nitrous oxide production during nitrification can only be understood when the detailed biochemistry of these conversion has been revealed (Soler-Jofra et al., 2020; Stein, 2011). Proteomics and transcriptomics techniques have emerged and strongly developed in recent years. Transcriptomics and proteomic studies in similar systems as the one studied here might help to reveal the enzymes involved in N_2O productions or the putative enzyme involved in NO conversion to nitrite (if there is one). As if forcing hydroxylamine conversion, one would expect that the enzymes related to its transformation to be upregulated. As also discussed in the present study, the combination of rate-limiting hydroxylamine feeding with ^{15}N and site preference techniques could help also to elucidate and further understand the fate of hydroxylamine in such systems depending on the conditions. For example, helping to reveal if it is nitrite or hydroxylamine the one being transformed to N_2O at low dissolved oxygen concentrations.

Finally, it should be highlighted that most of the wastewater treatment facilities operate at low DO concentrations, to minimise aeration energy and costs. Furthermore, fluctuations in ammonium concentrations are usually occurring (Chen et al., 2020). These form the perfect conditions for hydroxylamine accumulation, according to what has been discussed in the present work and the transient hydroxylamine accumulations events observed in literature (Liu et al., 2017; Stüven et al., 1992; Su et al., 2019b). The transient accumulation that is seen in the bulk liquid is the result of consumption and production processes. Inside flocs and biofilms conditions also vary and DO is low. Thus, accumulation of hydroxylamine in the inner parts of the biofilms is likely, even due to the reactivity of hydroxylamine it cannot be measured in the bulk liquid. Consequently, the understanding of how small quantities of hydroxylamine might impact common side communities of AOB, such as NOB, anammox, DNRA, denitrifiers or neighboring cells of AOB is important. More interestingly, the contribution of hydroxylamine to produce N_2O anoxically has still to be further investigated. Highlighting the importance of understanding intermediate steps to be able to understand the process as a whole.

5. Conclusions

The main objective of the present study was to further understand how rate-limiting hydroxylamine feeding, similar to a putative exposure in natural environments, could impact aerobic ammonium oxidizing metabolism. By means of different experiments involving hydroxylamine continuous rate-limiting feeding with aerobic granular sludge performing partial nitritation the following conclusions were reached:

- Hydroxylamine short term continuous rate-limiting feeding triggered N_2O emissions in comparison with the batch tests with only ammonium as substrate, where no N_2O was detected. DO combined with high ammonium concentrations kinetically governed hydroxylamine accumulation and N_2O emissions. An increasing trend was observed for both hydroxylamine accumulation and N_2O emissions with lower DO.
- DO also determines the impact of hydroxylamine in ammonium consumption. When DO is sufficiently high, ammonium consumption can be increased, as more reducing equivalents are generated from supplemented hydroxylamine oxidation. On the contrary, when DO is low, ammonium consumption will be reduced.
- Long term hydroxylamine feeding reduced ammonium consumption, while nitrite production was maintained. Dissolved oxygen increased with increasing loading rate and those extra electrons generated from hydroxylamine were allocated in the electron transport chain instead of ammonium oxidation.
- *Nitrosomonas* remained rather dominant during long term airlift reactor operation with hydroxylamine continuous feeding

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.

Acknowledgments

This research was financially supported by the SIAM Gravitation Grant 024.002.002, the Netherlands Organization for Scientific Research and by the Dutch Technology Foundation (STW - Simon Stevin Meester 2013) and by the Spanish Ministerio de Economía, Industria y Competitividad (MINECO), Agencia Estatal de Investigación (AEI) and Fondo Europeo de Desarrollo Regional (FEDER, EU), CTQ2017-82404-R.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2021.117945.

References

Apha, A. (2012) WEF. (2012). Standard methods for the examination of water and wastewater 22.

Anthonisen, A.C., Loehr, R. C., Prakasam, T. B. S., & Srinath, E. G, 1976. Inhibition of nitrification by ammonia and nitrous acid. ournal (Water Pollution Control Federation) 835–852.

Arp, D.J., Stein, L.Y, 2003. Metabolism of inorganic N compounds by ammonia-oxidizing bacteria. Crit. Rev. Biochem. Mol. Biol. 38 (6), 471–495.

Caranto, J.D., Lancaster, K.M, 2017. Nitric oxide is an obligate bacterial nitrification intermediate produced by hydroxylamine oxidoreductase. Proc. Natl. Acad. Sci. 114 (31), 8217–8222.

Caranto, J.D., Vilbert, A.C., Lancaster, K.M, 2016. Nitrosomonas europaea cytochrome P460 is a direct link between nitrification and nitrous oxide emission. Proc. Natl. Acad. Sci. 113 (51), 14704–14709.

Chandran, K., Smets, B.F, 2008. Biokinetic characterization of the acceleration phase in autotrophic ammonia oxidation. Water Environ. Res. 80 (8), 732–739.

Chen, G.H., van Loosdrecht, M.C., Ekama, G.A., Brdjanovic, D, 2020. Biological Wastewater Treatment: Principles, Modeling and Design. IWA Publishing.

de Bruijn, P., Van De Graaf, A.A., Jetten, M.S., Robertson, L.A., Kuenen, J.G, 1995. Growth of nitrosomonas europaea on hydroxylamine. FEMS Microbiol. Lett. 125 (2–3), 179–184.
Domingo-Félez, C., Calderó-Pascual, M., Sin, G., Plósz, B.G., Smets, B.F, 2017. Calibration of the comprehensive NDHA-N2O dynamics model for nitrifier-enriched biomass using targeted respirometric assays. Water Res. 126, 29–39.
Frear, D., Burrell, R, 1955. Spectrophotometric method for determining hydroxylamine reductase activity in higher plants. Anal. Chem. 27 (10), 1664–1665.
Frijlink, M.J., Abee, T., Laanbroek, H.J., de Boer, W., Konings, W.N, 1992. The bioenergetics of ammonia and hydroxylamine oxidation in Nitrosomonas europaea at acid and alkaline pH. Arch. Microbiol. 157 (2), 194–199.
Guisasola, A., Chandran, K., Smets, B.F., Baeza, J., Carrera, J., Lafuente, J., 2006. Observation and mathematical description of the acceleration phenomenon in batch respirograms associated with ammonium oxidation. Water Sci. Technol. 54 (8), 181–188.
Hao, X.D., van Loosdrecht, M, 2004. Model-based evaluation of COD influence on a partial nitrification-Anammox biofilm (CANON) process. Water Sci. Technol. 49 (11–12), 83–90.
Harper, W.F., Terada, A., Poly, F., Le Roux, X., Kristensen, K., Mazher, M., Smets, B.F, 2009. The effect of hydroxylamine on the activity and aggregate structure of autotrophic nitrifying bioreactor cultures. Biotechnol. Bioeng. 102 (3), 714–724.
Haynes, W.M, 2014. CRC Handbook of Chemistry and Physics. CRC press.
Henze, M., Gujer, W., Mino, T., Van Loosdrecht, M, 2000. Activated Sludge Models ASM1, ASM2, ASM2d and ASM3. IWA publishing.
Hooper, A.B., Terry, K, 1979. Hydroxylamine oxidoreductase of nitrosomonas: production of nitric oxide from hydroxylamine. Biochim. et Biophys. Acta (BBA) Bioenerg. 571 (1), 12–20.
Hu, B., Ye, J., Zhao, J., Ding, X., Yang, L., Tian, X, 2017. Characteristics of N_2O production and hydroxylamine variation in short-cut nitrification SBR process. Water Sci. Technol., wst2017496
Jubany, I., Carrera, J., Lafuente, J., Baeza, J.A, 2008. Start-up of a nitrification system with automatic control to treat highly concentrated ammonium wastewater: experimental results and modeling. Chem. Eng. J. 144 (3), 407–419.
Kampschreur, M.J., Tan, N.C., Kleerebezem, R., Picioreanu, C., Jetten, M.S., Loosdrecht, M.C.V, 2008a. Effect of dynamic process conditions on nitrogen oxides emission from a nitrifying culture. Environ. Sci. Technol. 42 (2), 429–435.
Kampschreur, M.J., van der Star, W.R., Wielders, H.A., Mulder, J.W., Jetten, M.S., van Loosdrecht, M.C, 2008b. Dynamics of nitric oxide and nitrous oxide emission during full-scale reject water treatment. Water Res. 42 (3), 812–826.
Kindaichi, T., Okabe, S., Satoh, H., Watanabe, Y, 2004. Effects of hydroxylamine on microbial community structure and function of autotrophic nitrifying biofilms determined by *in situ* hybridization and the use of microelectrodes. Water Sci. Technol. 49 (11–12), 61–68.
Law, Y., Ni, B.J., Lant, P., Yuan, Z, 2012. N2O production rate of an enriched ammonia-oxidising bacteria culture exponentially correlates to its ammonia oxidation rate. Water Res. 46 (10), 3409–3419.
Lees, H, 1952. Hydroxylamine as an intermediate in nitrification. Nature 169 (4291).
Liu, S., Han, P., Hink, L., Prosser, J.I., Wagner, M., Brüggemann, N, 2017. Abiotic conversion of extracellular NH_2OH contributes to N_2O emission during ammonia oxidation. Environ. Sci. Technol. 51 (22), 13122–13132.
Ni, B.J., Peng, L., Law, Y., Guo, J., Yuan, Z, 2014. Modeling of nitrous oxide production by autotrophic ammonia-oxidizing bacteria with multiple production pathways. Environ. Sci. Technol. 48 (7), 3916–3924.
Ni, B.J., Ruscalleda, M., Pellicer-Nacher, C., Smets, B.F, 2011. Modeling nitrous oxide production during biological nitrogen removal via nitrification and denitrification: extensions to the general ASM models. Environ. Sci. Technol. 45 (18), 7768–7776.
Ni, B.J., Yuan, Z, 2015. Recent advances in mathematical modeling of nitrous oxides emissions from wastewater treatment processes. Water Res. 87, 336–346.
Noophan, P., Figueroa, L.A., Munakata-Marr, J, 2004. Nitrite oxidation inhibition by hydroxylamine: experimental and model evaluation. Water Sci. Technol. 50 (6), 295–304.
Okabe, S., Oshiki, M., Takahashi, Y., Satoh, H, 2011. Development of long-term stable partial nitrification and subsequent anammox process. Bioresour. Technol. 102 (13), 6801–6807.
Petersen, B., Gernaey, K., Henze, M., Vanrolleghem, P, 2003. Calibration of activated sludge models: a critical review of experimental designs. Biotechnology for the Environment: Wastewater Treatment and Modeling, Waste Gas Handling, pp. 101–186.
Picioreanu, C., Pérez, J., van Loosdrecht, M.C, 2016. Impact of cell cluster size on apparent half-saturation coefficients for oxygen in nitrifying sludge and biofilms. Water Res. 106, 371–382.
Poot, V., Hoekstra, M., Geleijnse, M.A., van Loosdrecht, M.C., Pérez, J, 2016. Effects of the residual ammonium concentration on NOB repression during partial nitritation with granular sludge. Water Res. 106, 518–530.
Sabba, F., Picioreanu, C., Pérez, J., Nerenberg, R, 2015. Hydroxylamine diffusion can enhance N_2O emissions in nitrifying biofilms: a modeling study. Environ. Sci. Technol. 49 (3), 1486–1494.
Simon, J., Klotz, M.G, 2013. Diversity and evolution of bioenergetic systems involved in microbial nitrogen compound transformations. Biochim. Biophys. Acta (BBA) Bioenergetics 1827 (2), 114–135.
Sin, G., Van Hulle, S.W., De Pauw, D.J., Van Griensven, A., Vanrolleghem, P.A, 2005. A critical comparison of systematic calibration protocols for activated sludge models: a SWOT analysis. Water Res. 39 (12), 2459–2474.
Soler-Jofra, A., Pérez, J., van Loosdrecht, M.C, 2020. Hydroxylamine and the nitrogen cycle: a review. Water Res., 116723
Soler-Jofra, A., Stevens, B., Hoekstra, M., Picioreanu, C., Sorokin, D., van Loosdrecht, M. C., Pérez, J., 2016. Importance of abiotic hydroxylamine conversion on nitrous oxide emissions during nitritation of reject water. Chem. Eng. J. 287, 720–726.
Stein, L.Y, 2011. Surveying N_2O-producing pathways in bacteria. Meth. Enzymol. 486, 131.
Stein, L.Y., Arp, D.J., Hyman, M.R, 1997. Regulation of the synthesis and activity of ammonia monooxygenase in nitrosomonas europaea by altering pH To affect NH (inf3) availability. Appl. Environ. Microbiol. 63 (11), 4588–4592.
Stüven, R., Vollmer, M., Bock, E, 1992. The impact of organic matter on nitric oxide formation by nitrosomonas europaea. Arch. Microbiol. 158 (6), 439–443.
Su, Q., Domingo-Félez, C., Jensen, M.M., Smets, B.F, 2019a. Abiotic nitrous oxide (N_2O) production is strongly pH dependent, but contributes little to overall N_2O emissions in biological nitrogen removal systems. Environ. Sci. Technol. 53 (7), 3508–3516.
Su, Q., Domingo-Félez, C., Zhang, Z., Blum, J.M., Jensen, M.M., Smets, B.F, 2019b. The effect of pH on N2O production in intermittently-fed nitritation reactors. Water Res.
van den Berg, L., Kirkland, C.M., Seymour, J.D., Codd, S.L., van Loosdrecht, M.C., de Kreuk, M.K, 2020. Heterogeneous diffusion in aerobic granular sludge. Biotechnol. Bioeng. 117 (12), 3809–3819.
Wan, X., Xiao, P., Zhang, D., Lu, P., Yao, Z., He, Q, 2016. The kinetics for ammonium and nitrite oxidation under the effect of hydroxylamine. Water Sci. Technol. 73 (5), 1067–1073.
Wang, Y., Wang, H., Zhang, J., Yao, L., Wei, Y, 2016. Deciphering the evolution of the functional genes and microbial community of the combined partial nitritation-anammox process with nitrate build-up and its *in situ* restoration. RSC Adv. 6 (113), 111702–111712.
Wang, Y., Wang, Y., Wei, Y., Chen, M, 2015. In-situ restoring nitrogen removal for the combined partial nitritation-anammox process deteriorated by nitrate build-up. Biochem. Eng. J. 98, 127–136.
Yu, R., Chandran, K, 2010. Strategies of nitrosomonas Europaea 19718 to counter low dissolved oxygen and high nitrite concentrations. BMC Microbiol. 10 (1), 70.
Yu, R., Kampschreur, M.J., Loosdrecht, M.C.V., Chandran, K, 2010. Mechanisms and specific directionality of autotrophic nitrous oxide and nitric oxide generation during transient anoxia. Environ. Sci. Technol. 44 (4), 1313–1319.
Yu, R., Perez-Garcia, O., Lu, H., Chandran, K, 2017. Nitrosomonas europaea adaptation to anoxic-oxic cycling: insight from transcription analysis, proteomics and metabolic network modeling. Sci. Total Environ. (accepted).
Yu, R., Perez-Garcia, O., Lu, H., Chandran, K, 2018. Nitrosomonas europaea adaptation to anoxic-oxic cycling: insights from transcription analysis, proteomics and metabolic network modeling. Sci. Total Environ. 615, 1566–1573.