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# Hydroxylamine metabolism of *Ca.* Kuenenia stuttgartiensis

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ABSTRACT

Hydroxylamine is a key intermediate in several biological reactions of the global nitrogen cycle. However, the role of hydroxylamine in anammox is still not fully understood. In this work, the impact of hydroxylamine (also in combination with other substrates) on the metabolism of a planktonic enrichment culture of the anammox species *Ca.* Kuenenia stuttgartiensis was studied. Anammox bacteria were observed to produce ammonium both from hydroxylamine and hydrazine, and hydroxylamine was consumed simultaneously with nitrite. Hydrazine accumulation - signature for the presence of anammox bacteria - strongly depended on the available substrates, being higher with ammonium and lower with nitrite. Furthermore, the results presented here indicate that hydrazine accumulation is not the result of the inhibition of hydrazine dehydrogenase, as commonly assumed, but the product of hydroxylamine disproportionation. All kinetic parameters for the identified reactions were estimated by mathematical modelling. Moreover, the simultaneous consumption and growth on ammonium, nitrite and hydroxylamine of anammox bacteria was demonstrated, this was accompanied by a reduction in the nitrate production. Ultimately, this study advances the fundamental understanding of the metabolic versatility of anammox bacteria, and highlights the potential role played by metabolic intermediates (i.e. hydroxylamine, hydrazine) in shaping natural and engineered microbial communities.



## 1. Introduction

Anaerobic ammonium oxidizing bacteria (anammox) were first reported in the 90s in a wastewater treatment plant (Mulder et al., 1995). Anammox bacteria autotrophically oxidize ammonium to dinitrogen gas with nitrite as electron-acceptor (Jetten et al., 1998). Before their discovery, even if predicted thermodynamically, ammonium activation in absence of oxygen had never been identified in nature (Broda, 1977). Since then, significant efforts focused on understanding the central metabolism of anammox bacteria (e.g. (Kartal et al., 2011; Oshiki et al., 2016; Strous et al., 1998; Van De Graaf et al., 1997)).

Initially, hydroxylamine was hypothesized to be an obligate intermediate of anammox catabolism, and hydrazine was shown to accumulate when hydroxylamine was added in anammox cultures (Van De Graaf et al., 1997). More recently, NO was proposed to be the actual intermediate in the catabolic pathway (Kartal et al., 2011). The current working hypothesis for the anammox metabolism involves three reactions. First, nitrite is converted to NO (Eq. (1)) via a nitric reductase (Nir) enzyme. Then, NO is used to activate $NH_4^+$ and form the N-N bond needed to produce hydrazine ($N_2H_4$) (Eq. (2)) catalysed by hydrazine synthase (HZS). Finally hydrazine is further converted to dinitrogen gas (Eq. (3)) by hydrazine dehydrogenase (HDH).

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

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \quad (2)$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad (3)$$

However, even with NO as the central intermediate, hydroxylamine seems to play a key, yet elusive role in anammox metabolism. For example, *Ca.* Brocadia spp. strains do not encode for a Nir enzyme (Oshiki et al., 2015). Thus, either *Ca.* Brocadia spp. have hydroxylamine as central intermediate as proposed by Oshiki and coworkers (Oshiki et al., 2016), or another enzyme (like kustc0458) rather than Nir is converting nitrite to NO (Hu et al., 2019). Moreover, different studies showed that one of the most highly expressed enzyme in anammox bacteria is the hydroxy-

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lamine oxidase (HOX; kustc1601), which is proposed to convert hydroxylamine to NO (Hu et al., 2019; Kartal et al., 2011). The reason for anammox bacteria to invest energy to express HOX at such high levels has been hypothesized to be the possibility to reuse any hydroxylamine leaking from HZS (Dietl et al., 2015; Kartal and Keltjens, 2016). Briefly, the actual reaction mechanisms of HZS is proposed to involve NO transformation to hydroxylamine, and ammonium and hydroxylamine reacting to form hydrazine (Dietl et al., 2015; Kartal and Keltjens, 2016). Overall, the role of hydroxylamine in anammox metabolism remains poorly understood.

In the environment where anammox bacteria thrive, free hydroxylamine has been measured ((Hu et al., 2017; Liu et al., 2017; Poot et al., 2016; Soler-Jofra et al., 2018; Stüven et al., 1992; Su et al., 2019; Terada et al., 2017; Yang and Alleman, 1992; Yu and Chandran, 2010; Yu et al., 2010; Yu et al., 2018)). For instance, hydroxylamine has been shown to transiently accumulate in concentrations ranging from 0.006-1 mg-N/L in different ammonium oxidizing bacteria (AOB) pure cultures (Liu et al., 2017; Stüven et al., 1992; Yu and Chandran, 2010; Yu et al., 2010; Yu et al., 2018) or mixed consortia (Hu et al., 2017; Poot et al., 2016; Soler-Jofra et al., 2018; Su et al., 2019; Terada et al., 2017; Yang and Alleman, 1992). Thus, anammox exposure to external hydroxylamine cannot be ruled out, in particular in biofilm systems, where hydroxylamine can be produced by a nitrifying population and can reach higher concentrations than in the bulk liquid (Sabba et al., 2015). In these systems, hydroxylamine can be produced in the external oxic layers where AOB are present, diffuse through the biofilm and reach the anoxic (anammox bacteria) layers (Sabba et al., 2015).

Batch tests with hydroxylamine addition are generally used to study the short term effects of hydroxylamine on anammox bacteria primarily to (i) demonstrate anammox activity (Egli et al., 2001; Jetten et al., 1998), and (ii) study the "boosting" effect of intermediates on anammox activity (Hu et al., 2011; Zekker et al., 2012). However, to the best of our knowledge, the effect of different substrate combinations on anammox hydroxylamine consumption has not been dedicatedly studied. The only in depth study on the effect of hydroxylamine on anammox metabolism used ammonium as sole co-substrate (van der Star et al., 2008b).

In the present work, the impact of different combinations of substrates together with hydroxylamine on a planktonic anammox *Ca.* Kuenenia stuttgartiensis culture in batch tests was studied. Also, the long-term impacts of hydroxylamine addition were studied, and the effects on stoichiometry and microbial composition were quantified. Finally, a thermodynamic and modelling approach was developed to estimate the key kinetic parameters, and further understand the anammox metabolism with hydroxylamine as (co-)substrate.

## 2. Materials and methods

### 2.1. Batch test, preparation and procedure

Biomass was collected from a 10L MBR highly enriched in planktonic *Ca.* Kuenenia stuttgartiensis (79±4 % as estimated by 16S rRNA gene-based amplicon sequencing analysis) (see Supplementary Information (SI)) and centrifuged for 15 minutes at 4200 rpm at room temperature. Cells were re-suspended in $N_2$-sparged mineral medium to the desired biomass concentration. The mineral medium had the same composition as described in SI, but without ammonium or nitrite and supplemented with 1g/L $NaHCO_3$. The pH and optical density at 660nm ($OD_{660}$) were measured before aliquoting 50mL of cell suspension among 112 mL serum bottles. The optical density was correlated with gVSS/L (Fig. S1). Biomass used in negative controls was boiled for 5 minutes before aliquoting. The bottles were sealed with rubber stoppers, and anoxic conditions were achieved by sparging (ca. 1 minute) and vacuuming (ca. 3 minutes) with Argon three times per bottle. Bottles were placed on a shaker (Incubator Hood TH30, Edmund Bühler GmbH, Bodelshausen, Germany) at 30°C and 170 rpm. The pH was not adjusted, but remained between 8.0 and 8.5, within the optimal range for anammox bacteria (Kartal et al., 2012). Bottles were incubated overnight with stoichiometric concentrations of ammonium and nitrite to ensure activity.

After overnight incubation, the experiment was performed by adding a pulse of ammonium, nitrite, hydroxylamine, and/or hydrazine from anoxic stock solutions to reach the desired initial concentration in each batch test (see Table 1). Sampling was done over time depending on the biomass activity by removing 4 mL of cell suspension with a syringe. Samples were centrifuged for 3-5 minutes (4200 rpm, 4°C), and the supernatant was kept for further analysis of the dissolved nitrogen compounds (see SI). Samples used for hydrazine (Watt and Chrisp, 1952) and hydroxylamine (Frear and Burrell, 1955) determination were treated with 200 µL of a 0.1 g/mL sulfamic acid solution to remove the dissolved nitrite. Nitrite was previously shown to interfere in the hydroxylamine determination (Soler-Jofra et al., 2016). In the present study, nitrite was also shown to interfere the hydrazine measurement (see Fig. S2). Nitrogen consumption/production rates calculations are described in SI. Microbial community dynamics were not followed during the batch tests, but they would not be expected to shift significantly as no significant changes were observed during long term hydroxylamine feeding in a continuous reactor (see Section 3.2).

### 2.2. Batch tests, thermodynamic analysis

Based on the batch test data, the thermodynamics of the putative reactions involved in hydroxylamine consumption were studied (see Table 2). Reactions 3, 6, 7, 10 and 11 were originally postulated by van der Star (van der Star et al., 2008b). Half reactions were derived based on N, O, H and charge balances (see example in SI, based on (Kleerebezem and Van Loosdrecht, 2010)). The standard Gibbs energy change ($\Delta G^o_R$) of each reaction in Table 2 was calculated with the standard Gibbs energy of formation ($G^\circ_f$) of each compound (Table S1 and Eq. (S1)). $\Delta G^o_R$ was corrected with the measured concentration of each compound at each batch test time point to obtain the actual Gibbs energy change of reaction ($\Delta G^1_R$) evolution during the batch tests (Eq. (S2) and (S3)). The aim was to detect any possible thermodynamics limitations to explain the hydrazine accumulation behaviour or the possibility to have NO as intermediate in reactions involving hydroxylamine (the approach, calculations and results are detailed in SI).

### 2.3. Batch tests, kinetic parameters determination

A kinetic model (Table S2 and S3) was proposed and adapted from van der Star (van der Star et al., 2008b) to obtain the kinetic parameters of the reactions involved in the batch tests. The model was implemented in Matlab R2018b and aimed to minimize the error between the experimental data and the set of differential equations proposed for each compound. As the experiments were performed in batch, the set of differential equations were equal to the results of a matrix multiplication between the proposed stoichiometric matrix and the process rate matrix (Table S2 and S3, respectively). A step wise approach to obtain the kinetic constants from more simple to more complex batch tests was followed (Fig. S3). This was combined with the use of different objective functions (Eqs. (S4)–(S7)) to assess if the solution of the optimization performed was independent of the objective function used (full description of the procedure is detailed in SI).

**Table 1**
Batch test conditions and specific rates measured with (if applicable) and without hydroxylamine present. Two duplicates ($n$=2) were performed per condition tested. *– This rate corresponds to hydrazine consumption with hydroxylamine present.

| Batch | Initial concentrations | | | | Specific rates with $NH_2OH$ | | | Specific rates without $NH_2OH$ | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | $NH_4^+$ | $NO_2^-$ | $NH_2OH$ | $N_2H_4$ | $qNH_4^+{}_{,NH_2OH}$ | $qNO_2^-{}_{,NH_2OH}$ | $qNH_2OH_{,NH_2OH}$ | $qNH_4^+$ | $qNO_2^-$ | $qN_2H_4$ production | $qN_2H_4$ consumption |
| | mg-N/L | | | | mg-N/gVSS/h | | | mg-N/gVSS/h | | | |
| 1 | 33.4 ± 0.1 | 16.94 ± 0.02 | - | - | - | - | - | -46 ± 2 | -57 ± 3 | - | - |
| 2 | - | - | 22.8 ± 0.1 | - | 18 ± 1 | - | -76 ± 1 | 2.9 ± 0.3 | - | 16 ± 1 | -4.3 ± 0.3 |
| 3 | - | - | 6.8 ± 0.1 | - | 9.2 ± 0.6 | - | -47 ± 2 | 1.2 ± 0.4 | - | 7 ± 1 | -1.9 ± 0.1 |
| 4 | 1.64 ± 0.03 | - | 7.7 ± 0.2 | - | 13 ± 2 | - | -51 ± 3 | 2.78 ± 0.04 | - | 11 ± 1 | -3.6 ± 0.1 |
| 5 | 2.4 ± 0.3 | - | 7.3 ± 0.1 | - | 12.3 ± 0.5 | - | -47 ± 1 | 1.8 ± 0.2 | - | 5 ± 1 | -2.3 ± 0.1 |
| 6 | 29 ± 2 | - | 21.9 ± 0.8 | - | 17 ± 2 | - | -75 ± 1 | 3.7 ± 0.4 | - | 32 ± 2 | -10 ± 1 |
| 7 | 1.1 ± 0.7 | - | | 4.3 ± 0.1 | - | - | - | 3.8 ± 0.1 | - | - | -6.3 ± 0.1 |
| 8 | - | 19.9 ± 0.3 | 20.1 ± 0.7 | - | 6.0 ± 0.8 | -7.4 ± 0.9 | -42 ± 6 | -9 ± 1 | -7.4 ± 0.9 | 5.9 ± 0.6 | -3 ± 1 |
| 9 | - | 6.89 ± 0.04 | 7.2 ± 0.6 | - | 4.7 ± 0.4 | -11.0 ± 0.2 | -37 ± 2 | -5.3 ± 0.9 | -11.0 ± 0.2 | 2.1 ± 0.2 | -0.62 ± 0.02 |
| 10 | 84 ± 2 | 19 ± 3 | 30 ± 1 | - | -26 ± 4 | -22 ± 2 | -113 ± 4 | -19 ± 7 | -31 ± 2 | 15 ± 5 | -26 |
| 11 | 52 ± 4 | 18.8 ± 0.1 | 8.7 ± 0.4 | - | -31.5 ± 0.9 | -17 ± 1 | -39 ± 2 | -26 | -33 ± 1 | 29 ± 2 | -9.8 ± 0.4 |
| 12 | 36 ± 4 | 10.79 ± 0.01 | 4.8 ± 0.1 | - | -42 ± 2 | -18.9 ± 0.4 | -30.9 ± 0.2 | -8.7 ± 0.4 | -30.8 ± 0.3 | 33 ± 1 | -6.6 ± 0.6 |
| 13 | 1.5 ± 0.3 | - | 8.09 ± 0.01 | 4.90 ± 0.01 | 33 ± 3 | -24 ± 2 * | -60 ± 4 | 4.0 ± 0.1 | - | - | -6.4 ± 0.2 |

**Table 2**
Reactions involved in hydroxylamine and hydrazine disproportionation. $e^-$ refers to electron. $\Delta G^0{}_R$ refers to standard Gibbs free energy calculated at 25°C, 1atm and 1M in kJ/mol referred to mol of hydroxylamine, (*) mol $NH_4^+$, (**) mol $N_2H_4$ or (***) mol of NO reacting. Reactions 3, 6, 7 10 and 11 as proposed in the model of van der Star (van der Star et al., 2008b). Half reactions were derived based on N, O, H and charge balances as in (Kleerebezem and Van Loosdrecht, 2010).

| Number | Name | Comments | Reaction | $\Delta G^0{}_R$ (kJ/mol) |
|---|---|---|---|---|
| Hydroxylamine disproportionation | | | | |
| 1 | Hydrazine production ($e^-$ acceptor) | Half reaction | $NH_2OH + H^+ + e^- \rightarrow 0.5N_2H_4 + H_2O$ | -149.9 |
| 2 | Hydrazine production ($e^-$ donor) | Half reaction | $NH_4^+ \rightarrow 0.5N_2H_4 + 2H^+ + e^-$ | 143.3(*) |
| 3 | **Hydrazine production in hydroxylamine disproportionation** | (sum of 1 & 2) | $\boldsymbol{NH_4^+ + NH_2OH \rightarrow N_2H_4 + H_2O + H^+}$ | -6.6 |
| 4 | Hydrazine consumption ($e^-$ acceptor) | Half reaction | $NH_2OH + 3H^+ + 2e^- \rightarrow NH_4^+ + H_2O$ | -293.2 |
| 5 | Hydrazine consumption ($e^-$ donor) | Half reaction | $N_2H_4 \rightarrow N_2 + 4H^+ + 4e^-$ | -127.8(**) |
| 6 | **Hydrazine consumption in hydroxylamine disproportionation** | (sum of 2*4 & 5) | $\boldsymbol{2NH_2OH + N_2H_4 + 2H^+ \rightarrow 2NH_4^+ + N_2 + 2H_2O}$ | -357.1 |
| 7 | **Hydroxylamine disproportionation** | (sum of 3 & 6) | $\boldsymbol{3\ NH_2OH + H^+ \rightarrow NH_4^+ + N_2 + 3\ H_2O}$ | -240.3 |
| Hydrazine disproportionation | | | | |
| 8 | Hydrazine disproportionation ($e^-$ acceptor) | Half reaction | $N_2H_4 + 4H^+ + 2e^- \rightarrow 2\ NH_4^+$ | -286.6(**) |
| 9 | Hydrazine disproportionation ($e^-$ donor) | Half reaction | $N_2H_4 \rightarrow 4H^+ + 4e^- + N_2$ | -127.8 (**) |
| 10 | **Hydrazine disproportionation** | (sum of 2*8 & 9) | $\boldsymbol{3\ N_2H_4 + 4\ H^+ \rightarrow 4\ NH_4^+ + N_2}$ | -233.6 (**) |
| 11 | Hydrazine consumption | (option 2 for hydrazine consumption) | $N_2H_4 + H^+ + H_2O \rightarrow NH_4^+ + NH_2OH$ | 6.6 (**) |
| Hydroxylamine disproportionation via NO, modified reactions involving hydroxylamine | | | | |
| 12 | Hydrazine production via NO ($e^-$ donor) | Half reaction | $NO + 4H^+ + 4e^- \rightarrow 0.5N_2H_4 + H_2O$ | -259.9(***) |
| 13 | Hydrazine production via NO ($e^-$ acceptor) | Half reaction | $NH_2OH \rightarrow NO + 3H^+ + 3e^-$ | 110 |
| 14 | **Overall reaction hydrazine production ($e^-$ acceptor)** | (Sum of 11 &12), same as 1 | $\boldsymbol{NH_2OH + H^+ + e^- \rightarrow 0.5N_2H_4 + H_2O}$ | -149.9 |
| 15 | Hydrazine consumption via NO ($e^-$ donor) | Half reaction | $NO + 6H^+ + 5e^- \rightarrow NH_4^+ + H_2O$ | -403.2(***) |
| 16 | Hydrazine consumption via NO ($e^-$ acceptor) | Half reaction | $NH_2OH \rightarrow NO + 3H^+ + 3e^-$ | 110 |
| 17 | **Overall reaction hydrazine consumption ($e^-$ acceptor)** | (Sum of 14 & 15), same as 4 | $\boldsymbol{NH_2OH + 3H^+ + 2e^- \rightarrow NH_4^+ + H_2O}$ | -293.2 |

### *2.4. Reactor operation - continuous long-term study*

One litre of biomass from the same 10L MBR used in batch tests and described in SI was used as inoculum for a 2L MBR reactor (Fig. S4). The HRT was kept at 2.3±0.2 d during the whole operation using a custom-made ultrafiltration membrane unit (Van Der Star et al., 2008a). An SRT of 8.7±1.0 d was maintained throughout the operation period by withdrawing the desired reactor content per day. Temperature was controlled at 30°C with an external jacket, and the reactor was stirred at 170rpm. The reactor pH was controlled at 7.04±0.04 with a 53g/L $NaHCO_3$ solution.

When hydroxylamine was continuously fed (Phase II, days 38-54 in Table S4), two bottles were used to avoid hydroxylamine reaction with any of the mineral medium components. The total volumetric mineral medium loads were maintained by correcting media preparation.

Samples were collected daily from the effluent line and centrifuged, supernatant was collected for further analysis of the dissolved nitrogen compounds (see SI) and treated with sulfamic acid if needed. The biomass pellets were kept at -80°C. A fresh sample from the reactor was used to monitor daily optical density. During reactor operation volatile suspended solids (VSS) were measured periodically, and the specific correlation between $OD_{660}$ and VSS was used for calculations (Fig. S5). DNA samples from days 23, 33, 38, 40, 45, 50 and 55 were analysed at Novogen for 16S rRNA amplicon sequencing (see SI).

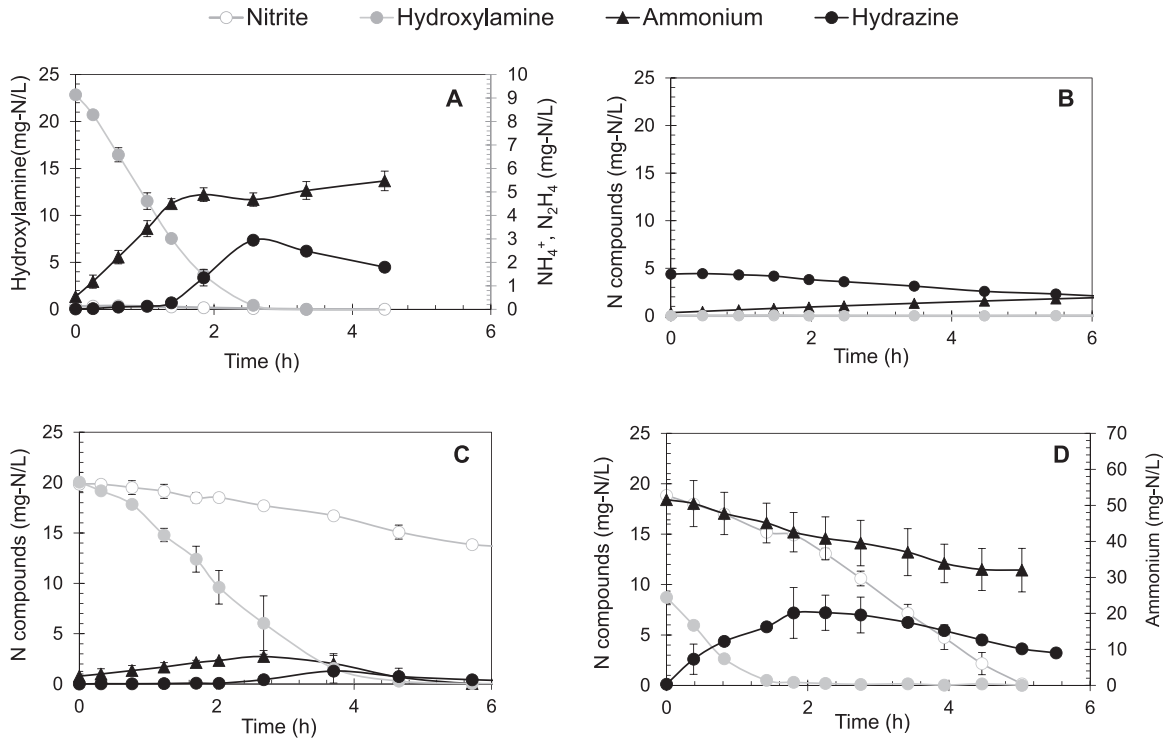


**Fig. 1.** Dynamics of nitrogen compounds during anammox batch tests with different combinations of substrates: (A) hydroxylamine (Test 2 in Table 1) (note that ammonium and hydrazine are represented on the right axis), (B) hydrazine (Test 7 in Table 1),(C) hydroxylamine and nitrite (Test 8 in Table 1), (D) hydroxylamine, nitrite and ammonium (Test 11 in Table 1) (note that ammonium is represented on the right axis). Error bars represent the standard deviation between duplicates.

## 3. Results & discussion

### *3.1. Short term anammox metabolism with hydroxylamine as substrate*

Anammox bacteria primarily convert ammonium and nitrite to dinitrogen gas, yet are also capable to metabolize other substrates, such as hydroxylamine, hydrazine and organic carbon (Jetten et al., 1998). In biofilm systems, hydroxylamine can leak from AOB communities (Liu et al., 2017; Su et al., 2019; Yang and Alleman, 1992), diffuse through the biofilm (Sabba et al., 2015), and reach anammox bacteria in anoxic layers. Batch tests to evaluate the capacity of anammox bacteria to metabolize hydroxylamine were performed by supplying hydroxylamine together with different combination of substrates to a *Ca.* Kuenenia stuttgartiensis enrichment. The aim was to investigate if different combination of substrates impacted the conversion dynamics of the nitrogen species. Hydroxylamine concentrations used in batch are higher than those to which anammox bacteria might be exposed in nature (i.e. values of 0.006-1 mg-N/L hydroxylamine have been reported in different nitrification systems (Liu et al., 2017; Su et al., 2019; Yang and Alleman, 1992), among others), but were needed to be able to investigate the conversions.

Trends in nitrogen compounds consumption and production were independent of the initial concentrations (Table 1). Therefore, only tests with higher initial concentrations are discussed here (Fig. 1), unless differently stated (Fig. S6). The impact of substrate combinations on hydroxylamine metabolism will be discussed in the present section, whereas the impact on hydrazine accumulation will be discussed further in the next section. Positive controls to assess anammox activity with ammonium and nitrite (Fig. S7A) were included. Data from the positive controls indicated that once nitrite was consumed, no significant changes in the ammonium concentrations were detected. Denitrifying activity was ruled out by providing nitrite as substrate (Fig. S7D) to the anammox enrichment culture. Abiotic controls with boiled biomass and all the substrates used in the batch test were performed and did not show significant activity compared to the biological rates; for example an increase of ca. 0.1 mg-N/L of ammonium was detected in the abiotic control after ca. 7h (Fig. S7C and E) compared to 5 mg-N/L of ammonium produced in 2h in biological tests (Fig. 1A).

#### *3.1.1. Ammonium production occurs from both hydroxylamine and hydrazine*

When hydroxylamine was added as the only substrate (Batches 2-5, Table 1), hydrazine did accumulate, as expected from previous studies (Van De Graaf et al., 1997; Van Der Star et al., 2010). However, two distinct ammonium production events occurred (Fig. 1A and Fig. S6). The first ammonium production started as soon as hydroxylamine was added (ca. 0-2.4h in Fig. 1A, see also Fig. S6). When the hydroxylamine concentration became low, hydrazine started to accumulate (around 1.6h in Fig. 1A, after ca. 15-25min in Fig. S6, with lower initial hydroxylamine concentration). Once hydroxylamine was depleted, a second ammonium production period started, correlating with the decrease of hydrazine (from ca. 2.4h onwards in Fig. 1A, see also Fig. S6). Thus, ammonium was produced both with hydroxylamine and hydrazine (Fig. 1A), apparently, with preference for utilization of hydroxylamine over hydrazine (Fig. S7B).

The initial production of ammonium from hydroxylamine is in accordance with the hydroxylamine disproportionation reactions (reaction 7, Table 2) proposed by van de Star et al. (van der Star et al., 2008b). Hydroxylamine disproportionation occurs via two intermediate reactions (reaction 3 and 6, in Table 2). The imbalance of reactions 3 and 6 resulted in the observed hydrazine accumulation (see Section 3.1.4 for further details). The experimental stoichiometric ratio of consumed hydroxylamine per ammonium produced was 3.8 mol/mol, which is quite close to the theoretically expected of 3 for hydroxylamine disproportionation (reaction 7 in Table 2).

The second ammonium production from hydrazine was not observed by van der Star et al.(van der Star et al., 2008b), most likely obscured by the high initial ammonium concentration used in the tests (2-8 mM, 28-112 mg-N/L). The consumption of hydrazine was proposed to follow two possible reactions (reaction 10 or 11, Table 2) (van der Star et al., 2008b). In the present study, once hydroxylamine was totally consumed, ca. 1.4 mM of ammonium were produced per mM of hydrazine consumed, close to the 1.3 theoretical ratio of hydrazine disproportionation via reaction 10 in Table 1 (Fig. 1A). Furthermore, hydrazine disproportionation via reaction 10 was confirmed by performing a batch test with hydrazine as the only substrate (Fig. 1B, batch 7 in Table 1). 1.2 mM mole of ammonium were produced per mM of hydrazine, which is in agreement with the proposed stoichiometry of hydrazine disproportionation (reaction 10, Table 2) producing 1.3 moles of ammonium per mol of hydrazine. Similar stoichiometries for ammonium produced per hydrazine consumed were previously shown in a batch test performed with only hydrazine present, but no mechanisms were proposed (Van De Graaf et al., 1997).

Based on the experimental stoichiometry, the production of ammonium from hydrazine is most likely to result from hydrazine disproportionation (as in reaction 10, Table 2, see also Fig. 4, and Section 3.1.8 for a discussion on putative enzymes involved).

The occurrence of hydrazine disproportionation throughout the batch test contributed to the ammonium production when hydroxylamine is still present. Multiple confirmations were obtained from the experimental data sets and the mathematical model. For instance, when hydroxylamine and hydrazine were provided together as substrates both were consumed simultaneously (batch 13 in Table 1 and Fig. S7B). Hydroxylamine was initially consumed c.a. 2.5 times faster than hydrazine (60 ± 4 and 24±2 mg-N/gVSS/h, respectively) and a transient slow down on hydrazine consumption could be observed . Furthermore, the proposed mathematical model with the parameters obtained (see Section 3.1.9 for further details) was used to compute the rates of reaction 3, 6 and 7 during a batch tests with hydroxylamine as substrate. The simulations indicate that hydrazine disproportionation is occurring throughout the batch test, even if at one order of magnitude lower rate than hydroxylamine disproportionation (see Fig. S16).

Overall, two ammonium production events were observed in the same batch test with only hydroxylamine as substrate (test 2 to 4 in Table 1). When hydroxylamine is present, hydroxylamine disproportionation to ammonium and dinitrogen gas via reaction 7 is the dominant process (Table 2) with hydrazine disproportionation taking place at one order of magnitude lower rate (see Fig. S16). Once hydroxylamine is consumed, the accumulated hydrazine is disproportionated to ammonium and dinitrogen gas via reaction 10 (Table 2).

### 3.1.2. Ammonium produced from hydroxylamine and hydrazine is used to consume nitrite

To further analyse the ammonium production capacity of anammox bacteria from either hydroxylamine or hydrazine, batch tests 8 and 9 (Table 1) were performed. Hydroxylamine and nitrite were dosed simultaneously to assess if the ammonium production capacity from hydroxylamine and hydrazine could support nitrite consumption. As expected, the ammonium produced from hydroxylamine was used to consume nitrite (Fig. 1C). A slight transient accumulation of hydrazine and ammonium was measured. Nitrite consumption stopped as soon as all the ammonium produced from hydroxylamine and hydrazine was consumed (Fig. 1C or Fig. S7F). Furthermore, the ca. 20 mg-N/L hydroxylamine consumed via the disproportionation reaction (reaction 7, Table 1), would lead to ca. 6.6 mg-N/L $NH_4^+$ which could be consumed via normal anammox metabolism. If we assume a stoichiometry close to that reported by Lotti et al. (Lotti et al., 2014) of 1.146 mol $NO_2^-$ mol $NH_4^+$, a maximum consumption of 7.6 mg-N/L nitrite could be converted, which is close to the experimentally observed of 6.4 mg-N/L of nitrite consumed. These observations, together with the fact that in the rest of tests (except tests 10-12 in Table 1, that also had nitrite) ammonium accumulated instead of being consumed, confirmed that the substrate consumed together with nitrite was ammonium. The transient accumulation of ammonium with nitrite was shown by Hu and coworkers (Hu et al., 2011), however no hydrazine accumulation was described in those tests. Also the hydroxylamine conversion slowed down with time, maybe due to the higher concentrations used in their tests.

These results further expand the metabolic versatility of anammox bacteria: if hydroxylamine is present with nitrite only (*i.e.* without $NH_4^+$), anammox bacteria can generate ammonium from hydroxylamine and consume nitrite via the canonical anammox conversion. These results show a potential role of hydroxylamine in *Ca.* Kuenenia stuttgartinesis metabolism, in contrast to the prior unique implication of hydroxylamine in *Ca.* Brocadia sinica (Oshiki et al., 2016). This situation might occur in partial nitritation/anammox (PN/A) systems when ammonium is fully depleted while residual, low concentrations of hydroxylamine might be still present, e.g. in biofilms due to gradients between microcolonies.

### 3.1.3. Hydroxylamine is consumed simultaneously with nitrite, but faster

Batch tests were performed to assess the impact of hydroxylamine when both nitrite and ammonium were present (batches 10, 11, 12, Table 1). This substrates combination is likely to occur in PN/A biofilms, although hydroxylamine concentrations might be lower than those used here. Hydroxylamine was consumed simultaneously with nitrite (Fig. 1D). The specific hydroxylamine consumption rate ($qNH_2OH$) was 1.6 to 5 times higher than that of nitrite ($qNO_2^-$), depending on the initial hydroxylamine concentration. As soon as all hydroxylamine was depleted, a 50% increase of nitrite consumption rate was measured (Table 1). Interestingly, even after hydroxylamine depletion, nitrite consumption rates remained lower than the maximal rate measured in positive controls, performed with nitrite and ammonium, usual anammox substrates (batch 1, Table 1). Thus, these results suggest that hydroxylamine is consumed simultaneously with nitrite, but faster than nitrite. Hydroxylamine presence together with nitrite and ammonium had a putative toxic or partially irreversible effect on nitrite consumption rates, as when hydroxylamine was fully consumed nitrite consumption rates did not reach the levels of the positive controls.

Within the range of concentrations tested, the hydroxylamine consumption rate linearly depended on the initial hydroxylamine concentration (Fig. 2A), and was not affected by the combination of available substrates. The linear consumption of hydroxylamine observed is consistent with previously reported measurements with ammonium only (van der Star et al., 2008b). In biological systems, Monod-like kinetics are usually observed for substrates consumption. For Monod kinetics to be linear, the substrate concentration has to be smaller than the half saturation coefficient. Thus, in the present study, the hydroxylamine half saturation constant would then be unexpectedly high for suspended cells (ca. > 22 mg-N-$NH_2OH$/L, Fig. 2A), as for example the half saturation constant for nitrite in a similar culture was 35 µg-N/L (Lotti et al., 2014). Another putative explanation for the observed linearity is the passive transport of hydroxylamine over the membrane. Hydroxylamine has a pKa of 5.9 at 25°C (Haynes, 2014), thus it is mostly unprotonated under the tested conditions (pH 8 and 30°C in the batch tests). As a result, passive diffusion through the membrane, strictly depending on the difference between the bulk and cell concentration of the substrate, is likely (Alberts et al., 2007).

Ultimately, the presented results further expand the known metabolic versatility of anammox bacteria. Hydroxylamine and hy-

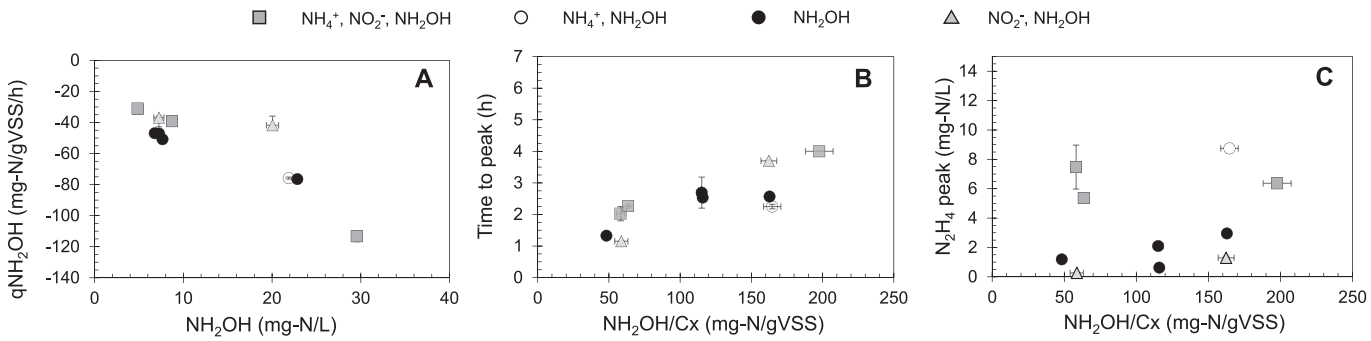


**Fig. 2.** Impact of initial $NH_2OH$ concentration [$NH_2OH$ (mg-N/L) in A] or specific initial hydroxylamine concentration [$NH_2OH/C_x$ (mg-N/gVSS) in B and C] to: (A) initial specific hydroxylamine rate [$qNH_2OH$ (mg-N/gVSS/L)], (B) time to reach the hydrazine peak [Time to peak (h)] and (C) maximum measured hydrazine concentration [$N_2H_4$ peak (mg-N/L)]. Cx stands for biomass concentration (gVSS/L). Batch tests were performed with different combination of substrates: (i) $NH_4^+$, $NO_2^-$, $NH_2OH$ (squares), (ii) $NH_4^+$, $NH_2OH$ (empty circles), (iii) $NH_2OH$ (filled circles), (iv) $NO_2^-$, $NH_2OH$ (triangles). Linear dependencies between parameters are shown in A and B, independently of the combination of substrates used. Batch tests were ammonium was present (squares and empty circle) had higher $N_2H_4$ peak than when no ammonium was present (filled circles and triangles). Notice the Y axes are different in each figure and X axis scale is different in Fig. 2A. Error bars represent the standard deviation between biological duplicates.

drazine disproportionation were proven to occur simultaneously and, if available, hydroxylamine was shown to be consumed simultaneously and faster than nitrite as substrate.

### 3.1.4. Hydroxylamine disproportionation controls hydrazine accumulation

To date, hydrazine accumulation has only been reported in the presence of hydroxylamine (e.g. (Jetten et al., 1998; Kartal et al., 2011; Van De Graaf et al., 1997)). However, hydrazine is not usually analysed in anammox systems, thus its actual concentration is unknown. To understand the mechanisms underlying hydrazine turnover, hydrazine concentration was measured during all batch tests (Table 1). Hydrazine transiently accumulated when hydroxylamine was added as substrate (Batches 2-6 and 8-12 in Table 1), and the time of the hydrazine peak depended on the initial specific hydroxylamine concentration (mg-N/gVSS, Fig. 2B), consistently with van der Star (van der Star et al., 2008b).

Hydrazine transient accumulation can be explained with hydrazine disproportionation (reaction 7, Table 2). The disproportionation is the addition of two reactions (reactions 3 and 6, Table 2). Hydrazine starts accumulating when hydroxylamine concentration is low (ca. <5 mg-N/L in Fig. 1A). Hydroxylamine concentration affects both hydrazine production (reaction 3, Table 2) and hydrazine consumption (reaction 6, Table 2), but two moles of hydroxylamine are needed for hydrazine consumption as opposed to one mole needed for its production. Thus, a lower concentration of hydroxylamine decreases the hydrazine consumption rate more than the hydrazine production rate, leading to transient hydrazine accumulation. This was further supported by the mathematical model (Fig. S16): the hydrazine consumption rate (via reaction 6 in Table 2) is always lower than the hydrazine production rate (via reaction 3 in Table 2), resulting in hydrazine accumulation.

Originally, the accumulation of hydrazine upon addition of hydroxylamine was ascribed to the inhibition of HDH, the enzyme responsible for the conversion of hydrazine to dinitrogen gas. This hypothesis was based on the observed *in vitro* inhibition of HDH by NO and hydroxylamine (Maalcke et al., 2016; Van De Graaf et al., 1997). However, our results show that HDH inhibition cannot be the explanation of hydrazine accumulation, as hydrazine did not accumulate directly after hydroxylamine addition at high initial hydroxylamine concentrations (ca. 20 mg-N/L Fig. 1A). Instead, the hydrazine peak occurred after the consumption of more than ca. 15 mg-N/L of hydroxylamine, when hydroxylamine concentration was <5 mg-N/L. This delay was also observed by van der Star et al. (van der Star et al., 2008b), who initially proposed that HDH inhibition could not be the cause for hydrazine accumulation. This is contradictory with the results of Maalcke et al. (Maalcke et al., 2016) that showed that HDH was inhibited by NO and hydroxylamine in vitro. Similarly, Hu and co-workers did not observe any sign of HDH inhibition when NO was fed continuously to an anammox culture (Hu et al., 2019). Overall, the HDH inhibition by hydroxylamine (and NO) demonstrated *in vitro* (Maalcke et al., 2016) was not observed to occur *in vivo*, meaning another process underlies the hydrazine accumulation. Instead, the imbalance of the hydrazine production rate and consumption rate (reaction 3 and 6 in Table 1) during hydroxylamine disproportionation is the proposed cause of hydrazine accumulation.

### 3.1.5. Hydrazine accumulation depends on the available substrates

The wide range of combinations of added substrates used in this research showed that hydrazine accumulation is strongly impacted by the used substrate combination (Fig. 2C). Specifically, the accumulation of hydrazine was higher when ammonium was present, while the presence of nitrite resulted in lower accumulations (Fig. 2C). Previous research on hydrazine accumulation used only ammonium and hydroxylamine as substrate (van der Star et al., 2008b).

This can also be explained by the imbalance of reactions 3 and 6 during hydroxylamine disproportionation . For the hydrazine production during hydroxylamine disproportionation, ammonium is consumed (reaction 3, Table 2), thus higher concentration of ammonium would favour hydrazine production. Contrarily, hydrazine consumption results in ammonium production (reaction 6, Table 2), thus the presence of ammonium would result in this reaction being less thermodynamically favourable (see Section 3.1.6).

On the other hand, nitrite decreased the hydrazine peak (Fig. 2C). When nitrite and hydroxylamine are present as the only substrates, nitrite is consumed with the ammonium that is being produced from either hydroxylamine or hydrazine disproportionation (Fig. 1C). Thus, nitrite presence decreases the final ammonium concentration, favouring hydrazine consumption (reaction 6, Table 2). When both ammonium and nitrite were present together with hydroxylamine (batches 10,11 and 12, Fig. 1D), ammonium dominated the possible effect of nitrite, leading to high hydrazine accumulations.

Overall, we showed that hydrazine accumulation occurs in the presence of hydroxylamine, and the accumulation is promoted by ammonium and reduced by nitrite. Elucidating the role and occurrence of hydrazine accumulation in biological systems requires further full-scale experimental confirmation as measurements of hy-

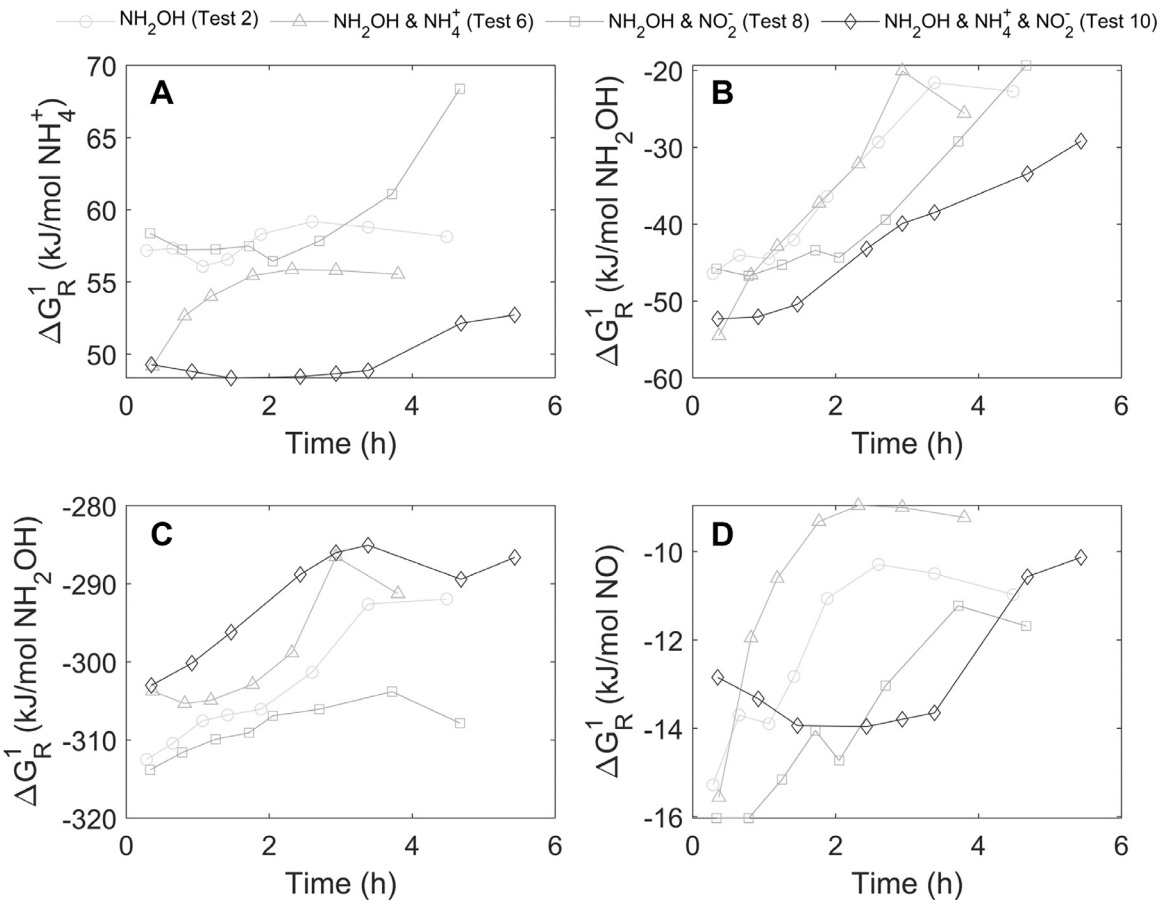


**Fig. 3.** Thermodynamics of selected reactions during different batch tests (Test 2, 6, 8 and 10 of Table 1) (A) ammonium to hydrazine conversion electron donor (reaction 2 in Table 2) is the only thermodynamically positive conversion, (B) hydrazine production in hydroxylamine disproportionation (reaction 3 in Table 2), (C) hydrazine consumption in hydroxylamine disproportionation (reaction 6 in Table 2), (D) NO conversion to hydrazine (reaction 12 in Table 2).

drazine during reactor operation remain rare. Hydrazine accumulation or leakage by anammox would be unfavourable from an energetic point of view, as hydrazine transformation to $N_2$ is one of the anammox electron sources (see Eq. (3)). However, leakage of hydrazine can be a potential advantage for anammox against other direct competitors, as hydrazine is toxic to other microbes of the nitrogen cycle, such as NOB (Yao et al., 2013). Further discussion on the thermodynamics of hydrazine accumulation and the putative enzymes involved can be found in Sections 3.1.6–3.1.8.

*3.1.6. Thermodynamics cannot explain hydrazine accumulation*

To identify any possible thermodynamic limitation underlying the observed hydrazine accumulation, a thermodynamic analysis of the reactions involved (Table 2) was performed along concentration profiles during batch tests (Figs. S8 and S9).

All reactions involved in hydrazine and hydroxylamine disproportionation were thermodynamically favourable, as they had a negative Gibbs energy change of reaction ($\Delta G^0_R$), with the exception of the half reaction of ammonium conversion to hydrazine (reaction 2, Table 2). Even after the correction for actual batch conditions, hydrazine production from ammonium had a positive actual Gibbs energy change of reaction ($\Delta G^1_R$) (Fig. 3A). Even if one of the half reactions was not thermodynamically favourable (reaction 2), the overall hydrazine production (reaction 3) had negative actual Gibbs free energy. However, the hydrazine production step (reaction 3; Table 2), was close to the equilibrium, and batch conditions strongly impacted its overall $\Delta G^1_R$ (Fig. 3B). Thus, hydrazine production thermodynamics heavily depend on batch test conditions (Fig. 3A and B). Conversely, the reactions involved in hydrazine consumption were strongly favourable (Fig. 3C). Consequently, the thermodynamic analysis does not explain the observed accumulation of hydrazine. A kinetic limitation or and enzymatic/biological bottleneck impacting hydrazine consumption might be the explanation, as also discussed in the previous section and as shown by the mathematical model (Fig. S16).

Impact of batch conditions, namely pH, temperature, ammonium and hydroxylamine concentration, on the potential hydrazine conversions were also investigated (Fig. S11). Higher hydroxylamine and ammonium concentration (Fig. S11A and B) resulted in a more favourable hydrazine production, in agreement with the experimental results (Fig. 2C). Higher pH was also shown to make hydrazine production more thermodynamically favourable (Fig. S11E), whereas temperature had little impact over the tested range (Fig. S11D). In this perspective, it is worth noting that the anammoxosome - where hydrazine is being produced - is more acidic than the cytoplasm (Van Der Star et al., 2010; van Niftrik et al., 2004), thus making hydrazine production less thermodynamically favourable.

*3.1.7. Thermodynamics suggest that NO is an unlikely intermediate in hydroxylamine turnover*

The feasibility and potential occurrence of NO as intermediate in the reactions involving hydroxylamine (reactions 12-17; Table 2, Fig. S10) was analysed based on thermodynamic characteristics of the conversions. Hydroxylamine is hypothesized to be first transformed to NO, and then NO is transformed to the final product. The estimated $\Delta G^1_R$ for the transformation of NO to hydrazine during batch tests was close to equilibrium, *i.e.* could potentially become

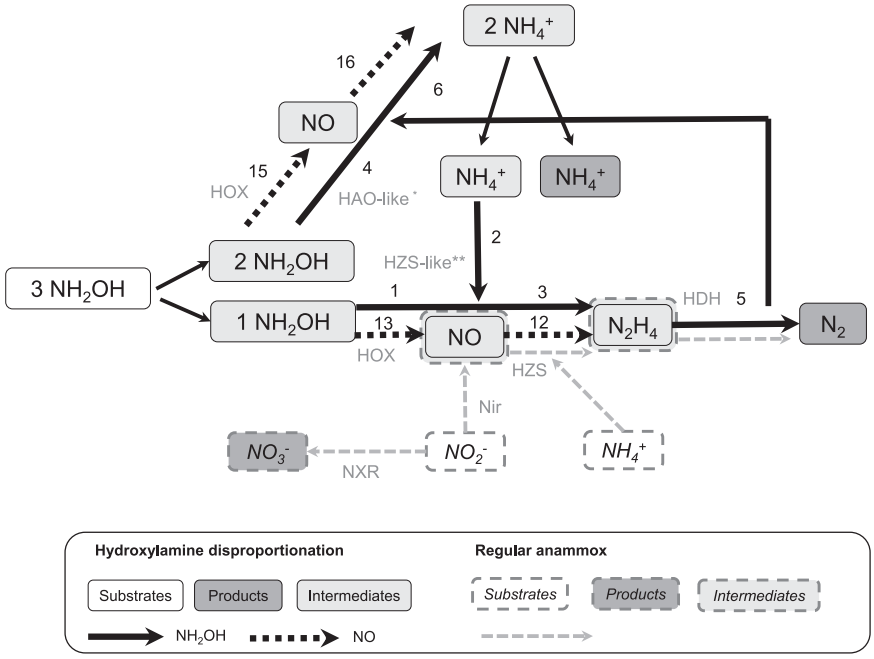


**Fig. 4.** Putative enzymes involved in hydroxylamine disproportionation conversions depending on the assumed intermediates: Black lines are conversion where hydroxylamine is directly transformed to hydrazine or ammonium, black dashed lines are reactions where NO might be an intermediate. Numbers correspond to reactions in Table 2, where corresponding Gibbs free energy values can be found. Regular anammox metabolism is represented with grey dashed boxes and arrows. Notice that NO and $N_2H_4$ have both solid and dashed line boxes. *Anammox genome encodes more than 10 HAO-like proteins, which the function of some of them is still unknown, **HZS has been proposed to have hydroxylamine as inner intermediate (Dietl et al., 2015), or in *Ca.* Brocadia it has been shown to transform hydroxylamine and ammonium to hydrazine (Oshiki et al., 2016; Oshiki et al., 2015). Thus, we hypothesise that there is an HZS-like protein able to transform ammonium and hydroxylamine to dinitrogen gas from the more than 10 HAO-like proteins encoded in anammox genome.

unfavourable (positive) depending on the conditions (Fig. 3D; reaction 12, Table 2). As a result, considering the previously discussed close to equilibrium reaction 3 (i.e. hydroxylamine to NO; Table 2), the transformation of hydroxylamine via NO would have two intermediate steps close to thermodynamic equilibrium. Form a thermodynamic point of view the forward conversion is possible, but highly depending on the exact batch test conditions. The experimental evidence on the potential role of NO as intermediate in the hydroxylamine conversion would still be needed.

Ultimately, it should also be noted that a better characterization of hydroxylamine standard Gibbs energy of formation ($G°_{f,NH_2OH}$) value is needed for a definitive thermodynamic study. In literature, different values for $G°_f$ of hydroxylamine can be found (Table S1), which profoundly impact the obtained results (Fig. S12).

### *3.1.8. Hydroxylamine and hydrazine disproportionation can be explained by known enzymatic anammox conversions*

We hypothesize that disproportionation of hydroxylamine and hydrazine might be catalysed by multiple enzymes, most of which are already characterized in anammox bacteria. For instance, hydrazine production (reaction 3 in Table 2) in hydroxylamine disproportionation could be catalysed by different combination of enzymes, either with NO as intermediate or not, in hydroxylamine conversions: i) $NH_2OH$ is first transformed to NO (reaction 12, 13 and overall 14) by HOX. Then, NO and $NH_4^+$ could be reduced to hydrazine (reaction 2) by HZS (see Fig. 4). ii) Alternatively, anammox bacteria encode more than 10 anammox HAO-like proteins in the genome, the function of some of them is still unknown. Thus, it could be that one of the HAO-like proteins in *Ca.* Kuenenia could perform a similar conversion as the HZS in *Ca.* Brocadia (Oshiki et al., 2016). Thus, the Brocadia-like HZS transforms directly hydroxylamine and ammonium to hydrazine, and HDH funnels hydrazine into dinitrogen gas (reaction 1,2 leading to the overall reaction 3) (see Fig. 4).

Hydrazine consumption (reaction 6) in hydroxylamine disproportionation could be a combination of a HAO-like protein reducing $NH_2OH$ to ammonium (reaction 4) and the known activity of HDH (reaction 5) (see Fig. 4).

Hydrazine disproportionation (reaction 10) would need HDH (reaction 9) and a dedicated enzyme to produce ammonium from hydrazine (reaction 8). An enzyme catalysing this last conversion, has not been described yet in anammox.

From the overall enzymatic conversions proposed, ammonium production from hydroxylamine and hydrazine have not been shown in anammox bacteria. However, ammonium producing activity from hydroxylamine has been hypothesized to exist based on metagenomic data from the anammox bacterium *Ca.* Scalindua profunda (van de Vossenberg et al., 2013). Furthermore, hydroxylamine conversion to ammonium has been shown in ammonium oxidizing bacteria *Nitrosomonas* (Kostera et al., 2008) and enzymatic activity found in the dissimilatory nitrate reducing bacteria *Nautilia profundicola* (Hanson et al., 2013), it is hypothesized that there is an HAO-like enzyme with an ammonium producing activity from hydroxylamine. Nevertheless, to further confirm this hypothesis, transcriptomics and proteomics data would be valuable.

### *3.1.9. Kinetic modelling supports the potential impact of hydroxylamine on nitrite metabolism*

The estimation of the kinetic parameters for the discussed reactions is the prerequisite for their inclusion in mathematical models (Henze et al., 2000). Experimental parameter determination requires highly precise measurements methods, and usually kinetic models are applied (van Loosdrecht et al., 2016). The fact that hydroxylamine is consumed via two simultaneous reactions (reactions 3 & 6, Table 2), and the need of both of them to describe hydrazine accumulation, makes the experimental determination of parameters impossible. Instead, a step-wise modelling approach using different optimization functions was used to assess if the

**Table 3**
Kinetic parameters determined with the kinetic model. When standard deviation is given, average between the results obtained with different objective functions was performed. When no standard deviation is given, the value selected is the one that resulted in a smaller error between the model and experimental data.

| Substrates | $k_1$ mmol/gVSS/h | $K_{1,NH_2OH}$ mM | $K_{1,NH_4^+}$ mM | $k_2$ mmol/gVSS/h | $K_{2,NH_2OH}$ mM | $K_{2,N_2H_4}$ mM | $k_3$ mmol/gVSS/h | $K_{3,N_2H_4}$ mM | $k_4$ mmol/gVSS/h | $K_{4,NH_4^+}$ mM | $K_{4,NO_2^-}$ mM |
|---|---|---|---|---|---|---|---|---|---|---|---|
| $N_2H_4$ | | | | | | | 0.285±0.007 | 0.030±0.003 | | | |
| $NH_2OH$ | 2.8 | 0.16 | 0.057 | 2.8 | 0.0027 | 0.56 | | | | | |
| $NO_2^- + NH_4^+$ | | | | | | | | | 4.5±0.1 | 0.78±0.01 | 0.03±0.04 |
| $NO_2^- + NH_4^+ + NH_2OH$ | | | | | | | | | 2.2 | 1.6 | 0.004 |

available data set allows for parameter determination (see SI). To this end, the kinetic model proposed by van der Star (van der Star et al., 2008b) was adapted to take into account the impact of ammonium (Table S3). The anammox stoichiometry was also introduced in the set of reactions to model the impact of hydroxylamine addition with ammonium and nitrite (Table S3).

Two independent optimizations were performed to determine the kinetic parameters of hydrazine consumption ($k_3$ and $K_{3,N_2H_4}$) and anammox ($k_4$, $K_{4,NO_2^-}$, and $K_{4,NH_4^+}$). Independently of the used optimization function, a single set of parameters was obtained (Fig. S13A and D, and S14A and D). However, when hydroxylamine was the only substrate, the values obtained for the remaining kinetic parameters ($k_1$, $K_{1,NH_2OH}$, $K_{1,NH_4^+}$, $k_2$, $K_{2,NH_2OH}$, $K_{2,N_2H_4}$) depended on the objective function used (Figs. S13B and S14B). The set of parameter values ($k_1$, $K_{1,NH_2OH}$, $K_{1,NH_4+}$, $k_2$, $K_{2,NH_2OH}$, $K_{2,N_2H_4}$) resulting in the lowest sum of squared errors was selected (Table 3), and used in subsequent optimizations.

Next, parameters obtained with control ($k_4$, $K_{4,NO_2^-}$, and $K_{4,NH_4^+}$), hydrazine ($k_3$ and $K_{3,N_2H_4}$) and hydroxylamine tests ($k_1$, $K_{1,NH_2OH}$, $K_{1,NH_4+}$, $k_2$, $K_{2,NH_2OH}$, $K_{2,N_2H_4}$) were used to simulate tests where ammonium, nitrite and hydroxylamine were simultaneously provided. Without any extra optimization step, the parameters previously obtained were not able to describe the experimental data. Specifically, the depletion of nitrite was predicted to be faster by the model (Fig. S15A), suggesting a direct impact of hydroxylamine on the regular anammox metabolism of nitrite consumption. Consequently, parameters affecting the nitrite consumption ($k_4$, $K_{4,NO_2^-}$, and $K_{4,NH_4^+}$) were optimized using the experimental data when hydroxylamine was present, while the other parameters were kept constant (Figs. S13C, S14C and S15B). The optimization resulted in ca. a 50% decrease in the specific maximum anammox rate constant ($k_4$), and 50% increase in the nitrite half saturation coefficient ($K_{4,NO_2^-}$). Consistently with the experimental results, these observations further support the strong impact of hydroxylamine on the nitrite consumption by anammox bacteria.

In literature, the only available set of parameters for the reactions involving hydroxylamine (van der Star et al., 2008b) estimated a maximum rate constant (k) one order of magnitude smaller than the ones reported in the present study (Table 3). This could be explained by the use of granular biomass (van der Star et al., 2008b) instead of planktonic culture as done here. Part of the biomass in the granules could be inactive leading to an apparent (slower) rate. Moreover, differences in the average growth rates between the two systems could also contribute to the differences in maximum rate constants.

### 3.2. Long term continuous exposure to hydroxylamine reduces the $NO_3^-$ production

The long term effects of continuous exposure of anammox bacteria to hydroxylamine had not been studied yet. To this end, a planktonic culture of *Ca.* Kuenenia stuttgartiensis was operated in continuous mode for more than 54 days (Fig. 5A, Fig. S17 and Table S4). After the initial 20 days of stabilization, two operational phases can be distinguished: i) Phase I with ammonium and nitrite (days 20-37, Table S4), ii) Phase II with ammonium, nitrite and hydroxylamine (days 38-54, Table S4). To mimic the expected low hydroxylamine accumulation by AOB in PN/A processes, a small hydroxylamine load (ca. 26 mg-N/L/d) compared to the nitrite load (ca. 478 mg-N/L/d; Table S4) was chosen.

The addition of hydroxylamine did not impact the microbial community composition as revealed by 16S rRNA amplicon sequencing (Fig. 5B). The relative abundance of *Ca.* Kuenenia stuttgartiensis remained stable at 79±4 % during 2 complete SRTs with hydroxylamine feeding. The genus *Ignavibacterium* represented the most abundant side population during the whole operation. These results indicate that simultaneous consumption of hydroxylamine, ammonium and nitrite does not impact anammox bacteria, and might even represent a competitive advantage in biofilm PN/A systems against canonical NOB, often reported to be inhibited by hydroxylamine (Blackburne et al., 2004; Blackburne et al., 2008; Castignetti and Gunner, 1982; Hao and Chen, 1994; Noophan et al., 2004; Wang et al., 2015).

During the whole experiment, hydrazine and hydroxylamine concentrations remained below detection limits (Fig. S17C). As soon as hydroxylamine was fed, a statistically significant decrease ($p \leq 0.001$, Mann-Whitney Rank Sum Test) in the nitrate production to ammonium consumption ratio was observed, from 0.24 ± 0.03 (Phase I) to 0.17±0.01 mol-$NO_3^-$/mol-$NH_4^+$ (Phase II) (Table S4 and Fig. 5A). Thus, hydroxylamine reduced the formation of nitrate in the anammox conversion. It is noteworthy that the response was immediate without any visible adaptation effect. Based on the anammox biochemistry presented in this study, hydroxylamine can be metabolized via two pathways: i) $NH_2OH$ is converted to NO and then further to $N_2$ via the conventional anammox metabolism in Eqs. (1)–(3), or ii) $NH_2OH$ is transformed via hydroxylamine disproportionation reactions, forming $NH_4^+$ and $N_2$ as in the batch tests (reaction 7, Table 2). From nitrogen mass balances only, the hydroxylamine pathway could not be resolved. Given the low hydroxylamine load compared to nitrite, hydroxylamine disproportionation would have resulted in ca. 8 mg-N/L/d of extra ammonium. Such low concentrations would be masked by the high residual ammonium concentration in the reactor. Nevertheless, in a study from van De Graaf and colleagues (Van De Graaf et al., 1997) the addition of $^{15}NH_2OH$ together with unlabelled ammonium and nitrite lead to $^{30}N_2$ production, most likely resulting from the reaction of $^{15}NH_4^+$ - produced from hydroxylamine - and $^{15}NH_2OH$ itself. Thus, based on the latter study and the batch tests performed here, hydroxylamine disproportionation is likely to be the dominant pathway.

Recently, anammox bacteria were also shown to grow on only NO and ammonium, with no nitrate production (Hu et al., 2019). These results challenged the common assumption that nitrite oxidation to nitrate is needed to provide the electrons to reduce $CO_2$ for biomass synthesis. Instead, the new hypothesis proposed that the high energy electrons produced during hydrazine conversion to $N_2$ are more likely those used in anabolism (Hu et al., 2019). Similarly, in the present study a clear decrease in nitrate production was observed with continuous hydroxylamine feeding. Independent of the pathway, hydroxylamine conversion releases extra

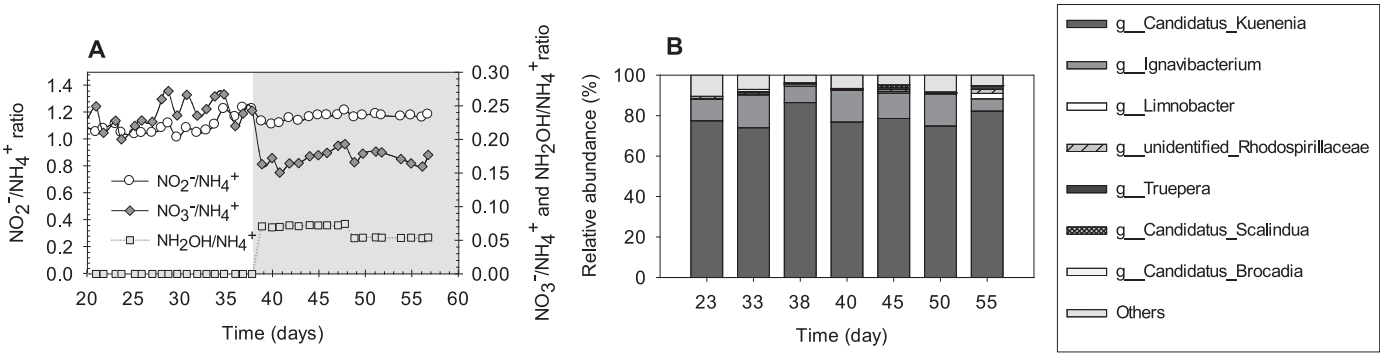


**Fig. 5.** Reactor operation dynamics: (A) stochiometric ratios during reactor operation without (white background) and with hydroxylamine load (grey area). (B) Microbial relative abundance based on 16S rRNA amplicon sequencing of reactor samples at different operational days.

high energy content electrons reducing the need for nitrite oxidation to nitrate.

This study further extends our knowledge of the metabolic versatility of anammox bacteria, demonstrating the simultaneous consumption of ammonium, nitrite and hydroxylamine by anammox bacteria. Furthermore, the reactions involved in hydroxylamine metabolism were further characterized using batch tests. This characterization resulted in the following findings: (i) ammonium can be produced from either hydroxylamine or hydrazine; and (ii) the co-metabolization of other substrates impacts hydrazine accumulation. In addition, hydrazine accumulation was analysed from a kinetics and thermodynamics point of view, further confirming that hydrazine accumulation is governed by the reactions involved in hydroxylamine disproportionation, rather than inhibition of hydrazine dehydrogenase.

Overall, this work highlights the huge metabolic versatility of anammox bacteria. This unique ability to use a broad range of substrates represents a clear competitive advantage likely underlying the ability of anammox to thrive in different environments. For example, hydroxylamine is known to be toxic for NOB, directly competing with anammox for nitrite (Castignetti and Gunner, 1982; Stüven et al., 1992; Yang and Alleman, 1992). From an engineering point of view, this kind of competitive advantage can be useful in systems like partial nitritation anammox, where NOB are not desired. For example, hydroxylamine external addition has been tested to obtain a successful partial nitritation anammox process in laboratory conditions (Wang et al., 2015).

Finally, the present work sets the basis to further understand hydroxylamine metabolism by anammox bacteria. Next steps could be directed to further confirm the pathway followed by hydroxylamine in continuous operation with either $^{15}N$ labelling or increased loads. Doing similar experiments with other anammox bacteria species (i.e. *Ca.* Brocadia) would also help to assess putative differences between anammox species metabolism. Comparative transcriptomic or proteomic data would provide more information in the enzymes involved in the process. Overall, intermediates of the nitrogen cycle are overlooked and not studied in depth, and could be a source for not recognized conversions or interactions in microbial communities. Understanding such interactions is crucial for improving its implementation as technology.

## 4. Conclusions

The combination of batch tests, continuous feeding, thermodynamics analysis and modelling allowed to elucidate more details about the hydroxylamine metabolism of anammox bacteria,

- Ammonium can be produced from both hydroxylamine and hydrazine. If only nitrite and hydroxylamine are available, ammonium can be produced from hydroxylamine and used for nitrite consumption.
- When hydroxylamine, ammonium and nitrite are present together in anammox batch tests, hydroxylamine and nitrite are consumed simultaneously, with hydroxylamine consumption being faster than nitrite consumption.
- Hydrazine accumulation only occurs when hydroxylamine is present and seems to be due to a biological imbalance rather than a thermodynamic limitation or enzymatic inhibition. The extent of hydrazine accumulation depends on the combination of substrates provided, i.e. promoted by ammonium and reduced by nitrite.
- Anammox bacteria can grow simultaneously with ammonium, nitrite and hydroxylamine, reducing the nitrate production.
- Anammox microbial population is not impacted by long term feeding of hydroxylamine.
- Hydroxylamine, if available in the environment, might play an important yet overlooked role in the metabolism of *Ca.* Kuenenia stuttgartiensis.

## 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.

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## Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.watres.2020.116188.

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