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Review

Hydroxylamine and the nitrogen cycle: A review

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ABSTRACT

Aerobic ammonium oxidizing bacteria were first isolated more than 100 years ago and hydroxylamine is known to be an intermediate. The enzymatic steps involving hydroxylamine conversion to nitrite are still under discussion. For a long time it was assumed that hydroxylamine was directly converted to nitrite by a hydroxylamine oxidoreductase. Recent enzymatic evidences suggest that the actual product of hydroxylamine conversion is NO and a third, yet unknown, enzyme further converts NO to nitrite. More recently, ammonium oxidizing archaea and complete ammonium oxidizing bacteria were isolated and identified. Still the central nitrogen metabolism of these microorganisms presents to researchers the same puzzle: how hydroxylamine is transformed to nitrite. Nitrogen losses in the form of NO and N_2O have been identified in all three types of aerobic ammonium oxidizing microorganisms and hydroxylamine is known to play a significant role in the formation. Yet, the pathways and the factors promoting the greenhouse gas emissions are to be fully characterized. Hydroxylamine also plays a yet poorly understood role on anaerobic ammonium oxidizing bacteria and is known to inhibit nitrite oxidizing bacteria. In this review, the role of this elusive intermediate in the metabolism of different key players of the nitrogen cycle is discussed, as well as the putative importance of hydroxylamine as a key nitrogen metabolite for microbial interactions within microbial communities and engineered systems. Overall, for the first time putting together the acquired knowledge about hydroxylamine and the nitrogen cycle over the years in a review, setting potential hypothesis and highlighting possible next steps for research.

1. Hydroxylamine in the nitrogen cycle

Nitrogen is one of the essential elements on Earth, widely present in the environment, as well as, in living organisms. The biggest reservoir of nitrogen on Earth is dinitrogen gas (N_2), but it is too inert for most of living organisms to be directly incorporated into cellular structures (Kuypers et al., 2018). Microorganisms involved in the nitrogen cycle have different enzymes that catalyse all sorts of nitrogen conversions. For example, nitrogen fixers are able to transform dinitrogen gas to ammonium, which makes nitrogen bioavailable to other microorganisms and other forms of life. Traditionally, nitrogen cycle conversions have been classified in: assimilation, ammonification, nitrification, denitrification, anaerobic ammonium oxidation (anammox) and nitrogen fixation (see some of them in Fig. 1A). However, new microbial conversions have been discovered, expanding the traditional processes (Fig. 1B).

Microbial conversions are responsible for the major fraction of the nitrogen fluxes between different nitrogen reservoirs on Earth (Kuypers et al., 2018). Since the discovery of the Haber-Bosch process, which transforms dinitrogen gas to ammonium to produce fertilizers, human intervention generated a large anthropogenic nitrogen flux on Earth, hampering the natural equilibrium. Eutrophication of waters and increased nitrous oxide emissions are typical examples of human generated problems related to the nitrogen cycle. To mitigate such problems, engineered systems like biological wastewater treatment plants (WWTPs) are used, where ammonium dissolved in water is transformed to dinitrogen gas using different nitrogen microbial conversions (Van Loosdrecht and Jetten, 1998). Thus, detailed knowledge of the nitrogen cycle microorganisms is crucial to further understand both natural and anthropogenic nitrogen fluxes and avoid possible environmental problems.

Hydroxylamine is an inorganic highly reactive compound that is intermediate or side metabolite in different nitrogen cycle microorganisms (Fig. 1B & C). Hydroxylamine impacts NO and N_2O emissions by aerobic ammonium oxidizers microorganisms. The impact

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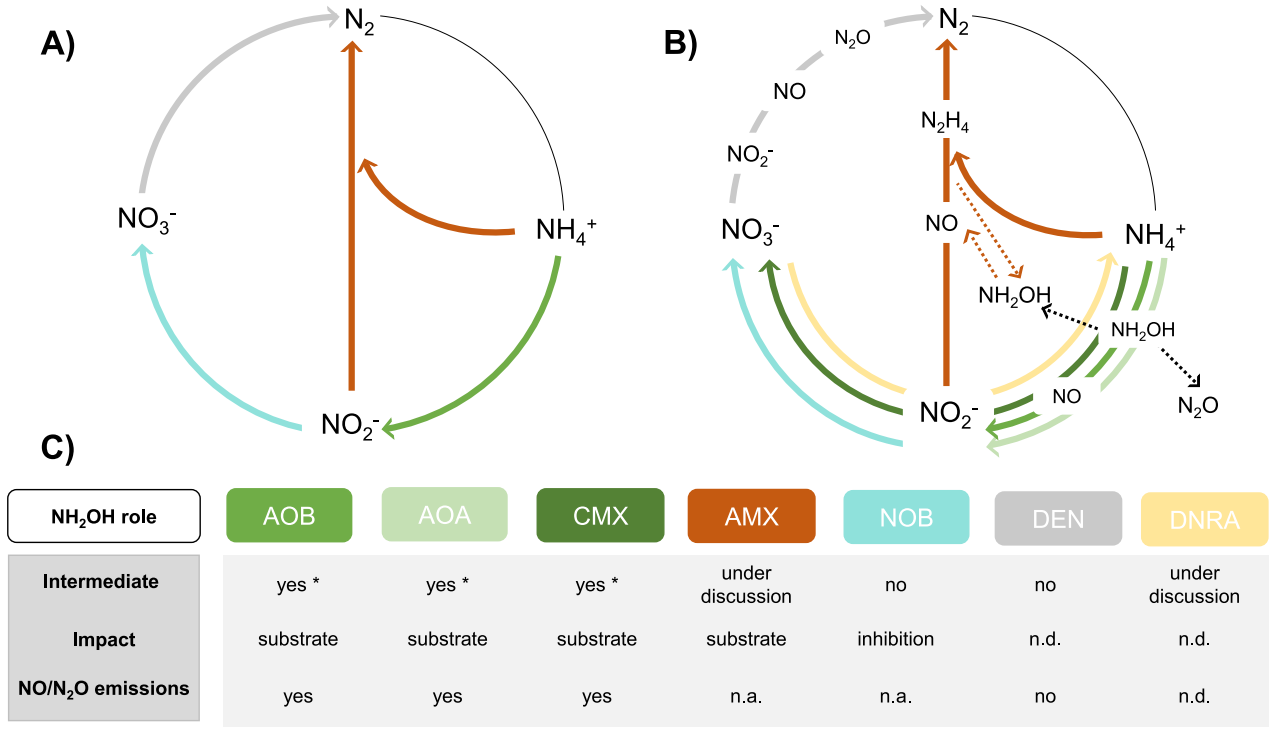

NH_2OH role	AOB	AOA	CMX	AMX	NOB	DEN	DNRA
Intermediate	yes *	yes *	yes *	under discussion	no	no	under discussion
Impact	substrate	substrate	substrate	substrate	inhibition	n.d.	n.d.
NO/N_2O emissions	yes	yes	yes	n.a.	n.a.	no	n.d.

Fig. 1. Impact of hydroxylamine in the microorganisms of the nitrogen cycle involved in wastewater treatment. A) Simplified version of the nitrogen cycle, where only major substrates and products are represented, B) Nitrogen cycle conversions, where intermediates are depicted and the role of hydroxylamine highlighted, C) Simplified summary of the known roles of hydroxylamine in different microorganisms of the nitrogen cycle. Point style arrows indicate putative pathways or hydroxylamine interactions. * enzymes involved in the conversions are under discussion, n.d. – not determined, n.a. – not applicable, AOB – Ammonium oxidizing bacteria, NOB – nitrite oxidizing bacteria, AMX – anammox, CMX – comammox, DEN – anoxic heterotroph denitrifiers, DNRA – dissimilatory nitrate reduction to ammonium.

of this compound in other microorganisms of the nitrogen cycle is little studied. To the best of our knowledge this review is the first to target the current knowledge about the role of hydroxylamine in the different communities of the nitrogen cycle. The review aims to assess the current state of the art on the role of hydroxylamine in the conversions by the various microbial groups participating in the nitrogen cycle, with a special focus on wastewater treatment processes. The level of understanding and research related to hydroxylamine and the microorganisms transforming nitrogen is not the same for each microbial process in the nitrogen cycle. Aerobic and anaerobic ammonium oxidizing microorganisms are presented (Sections 2 to 5). As they are known to harbour hydroxylamine conversion capacity in their genomic inventory. A wide range of studies investigated the role of hydroxylamine on ammonium oxidation bacteria and anaerobic ammonium oxidizing bacteria (Sections 2 and 5, respectively). Less information is available regarding the role of hydroxylamine in more recently discovered microorganisms such as ammonium oxidizing archaea or comammox (Sections 3 and 4, respectively). Nitrite oxidizing bacteria are not able to transform hydroxylamine, but the inhibition of nitrite oxidizers by hydroxylamine might be of relevance when shaping nitrogen cycle communities, this is analysed in Section 6. The impact of hydroxylamine on other nitrogen cycle microorganisms, such as denitrifiers or dissimilatory nitrate reducers to ammonium (DNRA) organisms has been hardly investigated, therefore only a brief analysis was included in Section 7. In this section, also other microorganisms that might be relevant for wastewater treatment are mentioned. Finally, the bottlenecks for hydroxylamine measurement, its role as a putative interaction compound in microbial nitrogen cycling communities and research challenges regarding this compound are discussed in the last sections (Sections 8 to 10).

2. Ammonium oxidizing bacteria (AOB)

Ammonia oxidizing bacteria (AOB) are able to transform ammonium to nitrite with oxygen as electron acceptor. These microorganisms are aerobic chemolithoautotrophic bacteria comprised in the beta and gamma subdivision of proteobacteria (Teske et al., 1994).

AOB catabolism consists of the conversion of ammonium to nitrite as electron donor and oxygen as electron acceptor as in Eq. (1). In AOB this reaction is divided in two steps. First, the enzyme ammonia monooxygenase (AMO) catalyses the oxidation of NH_4^+ to NH_2OH (Eq. (2)). Afterwards, hydroxylamine is further converted to NO_2^- by hydroxylamine oxidoreductase (HAO) (Eq. (3)) (see Fig. 2A). Two of the electrons generated in this last step are used by AMO to catalyse the first reaction, whereas the rest is invested in energy generation (Yu and Chandran, 2010).

$$NH_4^+ + 1.5\ O_2 \rightarrow NO_2^- + 2H^+ + H_2O \quad (1)$$

$$NH_4^+ + O_2 + 2H^+ + 2e^- \rightarrow NH_2OH + H_2O \quad (2)$$

$$NH_2OH + H_2O \rightarrow NO_2^- + 5H^+ + 4e^- \quad (3)$$

Recently, it was suggested that the product of HAO is NO instead of nitrite, and that NO is disproportionated abiotically or by an unknown enzyme to nitrite (Caranto and Lancaster, 2017). Thus, first ammonium is oxidized to hydroxylamine by AMO (Eq. (2)), hydroxylamine to NO by HAO (Eq. (4)) and NO to nitrite (Eq. (5)) by an uncharacterized enzyme. A proteomic comparative study has recently suggested that nitrosocyanin (NcyA) was highly expressed in 3 different AOB strains, and was proposed as this third missing enzyme (Stein, 2019) (see Fig. 2B). Nevertheless, the activity of HAO

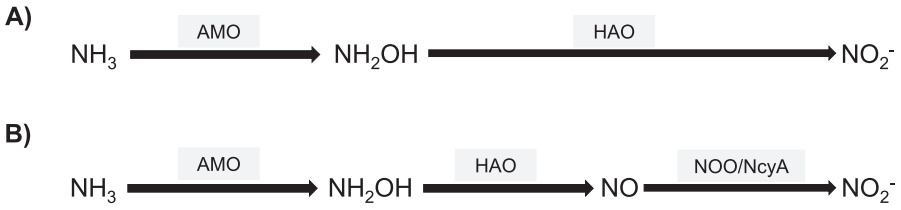

Fig. 2. Proposed enzymes for ammonium conversion by ammonium oxidizing bacteria (AOB). A) Traditional pathway, where hydroxylamine is directly converted to nitrite. B) Alternatively, hydroxylamine is first transformed to NO and further oxidized to nitrite by a yet not fully characterized enzyme. AMO – Ammonium monooxygenase, HAO – Hydroxylamine oxidoreductase, NOO - Nitric oxide reductase, NcyA – Nitrososcyanin. Sources - Lancaster et al., 2018; Stein 2019.

Table 1.
Hydroxylamine build-up by ammonium oxidizing bacteria (a) recalculated from free hydroxylamine equilibria (b) converted from mM (c) converted from µM, (d) *Nitrosovibrio* alone did not accumulate hydroxylamine, PN – partial nitritation, DO – dissolved oxygen, Max. – Maximum, In. – Initial, SBR –sequential batch reactor.

Type of biomass	Type of test	Temperature (°C)	pH	DO (mg/L)	In. NH_4^+ (mg-N/L)	Max. NH_2OH (mg-N/L)	Reference
Nitrosomonas europaea	Batch	30	7.7	n.d.	7 and 28 (b)	0.006 and 0.011 (c)	(Liu et al., 2017)
Nitrosospira multiformis	Batch	30	7.7	n.d.	7 and 28 (b)	0.013 0.031 (c)	(Liu et al., 2017)
Nitrosomonas europaea	Batch	28	7.8	n.d.	140	0.003–0.015 (c)	(Stüven et al., 1992)
Nitrosovibrio & *Nitrobacter*(d)	Batch	28	7.8	7.4.	140	0.006–0.024	(Stüven et al., 1992)
Nitrosomonas europaea	Recover from anoxia	n.d.	6.8–7.4	0.5	230	0.3	(Yu and Chandran 2010)
Nitrosomonas europaea	Recover from anoxia	n.d.	6.8–7.4	1.5	230	0.4	(Yu and Chandran 2010)
Nitrosomonas europaea	Recover from anoxia	n.d.	6.8–7.4	3	230	0.35	(Yu and Chandran 2010)
Nitrosomonas europaea	Recover from anoxia	21	7.5		30	0.2	(Yu et al., 2018)
Nitrifying culture	Batch test	28	7.0–8.5	0.5–6	200–500	0.3–4.3 (a)	(Yang and Alleman 1992)
PN granular airlift	Load increase	20	7.7	3.5	2 to 22	0.06	(Poot et al., 2016)
PN sludge SBR	Two different loads	30	8	0.4–0.5	50 to 70	0.06	(Hu et al., 2017)
PN sludge SBR	Different pH set point	20–26	6.5, 7, 7.5, 8 and 8.5	0.7 ± 0.1	74±39	0.1–0.05	(Su et al., 2019b)

producing NO has only been shown *in-vitro*, thus further confirmation of this pathway in-vivo is needed. In-vivo metabolic studies in combination with transcriptomics/proteomics studies could shed light into another putative enzyme being able to convert hydroxylamine to nitrite.

$$NH_2OH \rightarrow NO + 3H^+ + 3e^- \quad (4)$$

$$NO + H_2O \rightarrow NO_2^- + 2H^+ + 1e^- \quad (5)$$

Beyond hydroxylamine as an intermediate in AOB metabolism, other putative roles of hydroxylamine are known. Hydroxylamine has been shown to transiently accumulate in AOB planktonic or mixed cultures, which might lead to interactions with other nitrogen communities. The short and long term impact of hydroxylamine has been tested in AOB without reaching definitive conclusions. Finally, it is known to be a precursor to N_2O emissions. All these roles are discussed in depth in the following sections.

2.1. Hydroxylamine transient accumulation events

Since hydroxylamine first mention (Mumford, 1914) as intermediate and identification by Lees (Lees, 1952), it has been shown that it can transiently accumulate in the bulk liquid during cultivation at concentrations from 0.003 up to 4.3 mg-N/L (Table 1). These accumulations were reported in a wide variety of nitrification systems and operation conditions. For example, when performing batch tests with axenic cultures (Liu et al., 2017; Stüven et al., 1992) or nitrifying cultures (Yang and Alleman, 1992). When AOB axenic chemostat cultures were switched from anoxic to aerobic conditions (Yu and Chandran, 2010; Yu et al., 2018) or when partial nitrifying reactors were operated in sequencing batch mode (Hu et al., 2017; Su et al., 2019b). Also hydroxylamine accumulated when a change of reactor load was imposed to a continuous partial nitrification airlift reactor leading to an increase of ammonium accumulation from 2 to 25 mg-N/L (Poot et al., 2016). Hu et al., also observed hydroxylamine accumulation in a continuous reactor when imposing a change of load, but higher load did not correlate with a change of the hydroxylamine accumulation peak (Hu et al., 2017).

Overall, all the mentioned experiments (Table 1) showed that switching AOB cells from a resting state (i.e. without/low ammonium or oxygen) to an active state (i.e. with ammonium or oxygen) triggered hydroxylamine accumulation. Indicating that hydroxylamine accumulation depended on the unbalanced coupling between the production and consumption of hydroxylamine by the designated enzymes. Thus, if AMO produces hydroxylamine faster than can be converted by HAO and other consumption reactions (or the recently proposed, still unknown enzyme (Caranto and Lancaster, 2017)) are able to consume hydroxylamine, a metabolic imbalance is created, leading to the observed hydroxylamine accumulation.

Different studies point out that hydroxylamine accumulation can also be strain dependant. For instance, *Nitrosomonas europaea* and *Nitrosospira multiformis* accumulated hydroxylamine up to 11 and 31 µg-N/L, respectively (Liu et al., 2017). In contrast, other AOB strains such as *Nitrosomonas nitrosa Nm90* and *Nitrosomonas communis* did not show hydroxylamine accumulation in batch tests with the same initial ammonium concentrations (Liu et al., 2017). The authors attributed the differences observed for hydroxylamine accumulation levels to differences in the ammonia consumption rates. For example, *N. multiformis* showed the highest ammonium consumption rate also resulting in the highest hydroxylamine accumulation (Liu et al., 2017). Nevertheless, *N. communis* had the fastest ammonium uptake rate but no hydroxylamine accumulation was detected, which might be due to a more efficient hydroxylamine conversion to nitrite by HAO (Liu et al., 2017).

Another study indicating that hydroxylamine accumulation is strain dependant and might also depend on the surrounding nitrogen community was performed by Stüven and coworkers (Stüven et al., 1992). For instance, *Nitrosovibrio* did not accumulate hydroxylamine when it was cultured alone, and yet hydroxylamine accumulation (5.6–23.8 µg-N/L) occurred when it was cultured to-

gether with the NOB *Nitrobacter* (Stüven et al., 1992). Contrarily, in the same set of experiments *Nitrosomonas europaea* showed hydroxylamine accumulation (2.8–15.4 µg-N/L) when it was cultured alone, but in co-culture with *Nitrobacter* lower hydroxylamine levels where reported (5.6 to less than 2.8 µg-N/L) (Stüven et al., 1992). This is the only study that point towards a possible impact of side communities to the hydroxylamine accumulation behaviour of AOB strains. The differential behaviour related to hydroxylamine accumulation when AOB was cultured alone or together with NOB, might be due to a possible competition strategy to avoid NOB growth in the co-culture or to promote it to avoid product inhibition. Thus far, the impact of side communities on hydroxylamine accumulation has been little studied and it is still not fully understood.

The unbalance between hydroxylamine production and consumption leading to hydroxylamine accumulation might have different explanations: i) the turnover of HAO (or the enzyme responsible of hydroxylamine consumption) is smaller than the capacity of AMO to produce hydroxylamine. Genetic differences between different species in the hydroxylamine production and consumption enzymes could explain the different hydroxylamine accumulation dynamics observed between species. ii) pH can be another factor that might cause hydroxylamine build up. As pH has an impact on both the dissociation of ammonium/hydroxylamine, as well as it has a strong impact on the enzymes rates (Su et al., 2019b). For instance ammonium consumption is strongly impacted by acidification, while hydroxylamine oxidation is barely affected (Frijlink et al., 1992).This could be of importance in biofilm like systems, where strong pH gradients can occur, and pH is more acidic in the internal part of the granule (De Beer et al., 1993; Gieseke et al., 2006; Poot et al., 2016; Schreiber et al., 2009; Uemura et al., 2011; Winkler et al., 2011). Future studies might shed more light on the difference in balancing the production and consumption of hydroxylamine in different AOB. For example, comparative transcriptomic/proteomic between AOB strains, studies focused on enzymatic activities and affinities comparison, or research focused on characterizing external factors promoting hydroxylamine accumulation.

2.2. Effect of hydroxylamine dosing in ammonium oxidizing bacteria

Several studies have investigated the effect of externally added hydroxylamine on ammonium oxidizing bacteria (Table 2). Since hydroxylamine first discovery as intermediate of nitrification, one of the initial questions was if hydroxylamine could be used for growth. Two parallel studies have shown that different species of AOB are able to use hydroxylamine mixotrophically together with ammonium for growth (Böttcher and Koops, 1994; de Bruijn et al., 1995). Both studies (Böttcher and Koops, 1994; de Bruijn et al., 1995) reported a higher experimental growth yield than the theoretically expected, when using a mixture of hydroxylamine and ammonium as substrate. So far, it is not fully understood why and how hydroxylamine boosts the growth. In addition, to our knowledge it is still not shown if AOB can grow on hydroxylamine as single substrate.

Other studies have focused on the short term effect of externally added hydroxylamine to ammonium oxidation using batch tests or respirometry tests. When providing ammonium to an AOB batch culture, usually there is a lag period, or so called acceleration phase, which is the time that the culture needs to switch from a slower ammonium consumption to maximum consumption rate (Chandran and Smets, 2008). Different studies showed that externally added hydroxylamine accelerated this initial ammonium uptake rate (Chandran and Smets, 2008; de Bruijn et al., 1995; Harper et al., 2009; Kindaichi et al., 2004). The externally added hydroxylamine to a biofilm system led to disaggregation from microcolonies to scattered cells (Harper et al., 2009; Kindaichi et al., 2004). Based on these results two different hypotheses on why hydroxylamine accelerates the ammonium uptake rate were proposed (Chandran and Smets, 2008; Harper et al., 2009; Kindaichi et al., 2004): i) Hydroxylamine impacts the cell morphology, scattering the cells and having a higher cell area available, so an increased mass transfer, leading to higher ammonium uptakes, ii) Electrons obtained in the hydroxylamine transformation to nitrite are recirculated to AMO enzyme, which triggers its activity increasing ammonium consumption.

Conventional nitrogen removal in WWTPs was traditionally performed by combination of nitrification and denitrification processes. Since the discovery of anammox, the possibility to combine partial nitrification with anammox has been intensively studied. Partial nitrification-anammox technologies offer the opportunity to lower WWTPs costs. However, one of the bottlenecks for its implementation is the stable operation of partial nitrification and efficient repression of NOB (Cao et al., 2017). Hydroxylamine has been used to recover partial nitrification in reactors where nitrate started to accumulate (Wang et al., 2016; Wang et al., 2015; Xu et al., 2012). In these studies hydroxylamine dosing combined with proper reactor operation triggered the stabilization of partial nitrification. In both studies, the activity of AOB was not hampered, whereas NOB were inhibited as nitrate stopped accumulating in the reactor (Wang et al., 2016; Wang et al., 2015; Xu et al., 2012) . A rapid start-up of partial nitrification reactors was also achieved by hydroxylamine addition. For example, intermittent dosing of hydroxylamine in up-flow biofilm reactor (Okabe et al., 2011) or SBR (Li et al., 2019a; Li et al., 2019b) helped to speeding up the start-up of a partial nitritation process. A stable partial nitritation was maintained only if after stopping hydroxylamine dosage, a proper reactor control was implemented (Li et al., 2019a).

Overall, hydroxylamine showed to be efficient in inhibiting nitrite oxidation to nitrate (see next section), not damaging and even promoting AOB activity in most of the studies (Böttcher and Koops, 1994; de Bruijn et al., 1995; Li et al., 2019a; Li et al., 2019b; Wang et al., 2016; Wang et al., 2015; Xu et al., 2012). However, there are other studies that claim that long term exposure to hydroxylamine hampered AOB activity (Harper et al., 2009) and even an inhibition model for AOB has been proposed (Wan et al., 2016). In addition, the observed negative effect of hydroxylamine on mixed cultures biofilm like structures (Harper et al., 2009; Kindaichi et al., 2004) will not be desirable in certain operational modes, which rely on biofilm systems (i.e. granule, biofilm carriers).

Most of the “long term” hydroxylamine studies have been performed using a pulse feeding strategy, which leads to initially high hydroxylamine concentrations (Table 2). Furthermore, hydroxylamine feeding was mostly added temporarily for a start-up period or to promote partial nitritation. These exposures to sudden high hydroxylamine concentrations are not likely to occur in natural environments. Thus far, the only study with continuous and limiting hydroxylamine concentration was performed by de Bruijn et al. (1995) with *Nitrosomonas europaea*. Using hydroxylamine limiting conditions can help to understand the mechanisms (i.e. over or under regulation of genes/proteins) by which hydroxylamine is promoting or hampering AOB activity in nitrification environments, without the potential inhibitive effects of hydroxylamine.

2.3. N_2O production from hydroxylamine

In addition to the regular ammonia oxidation metabolism, two possible pathways have been proposed in order to explain the nitrogen loss in form of nitric or nitrous oxide gasses during nitritation: i) Nitrifier denitrification, which involves the reduction of NO_2^- to NO and N_2O by a nitrite reductase (Nir) and nitric oxide

Table 2
Short term and long-term effect of hydroxylamine addition to AOB pure cultures or nitrification systems. n.d. - not determined, SBR-sequential batch reactor, RDRs – rotating disk reactor.*-assumed that the abiotic tests were performed at the same temperature than the biological cultivations. ** 10 mg/L NH_2OH converted to mg-N/L.

Type of biomass	Type of reactor/test	Temperature (°C)	pH	NH_4^+ (mg-N/L)	NH_2OH (mg-N/L)	Type of addition	Effect	Reference
Nitrosomonas europaea	Chemostat	30	8	280	19.6–145.6	Continuous	Growth mixotrophically on ammonium and hydroxylamine (0.34 g/mg-N-NH_2OH)	(de Bruijn et al. 1995)
Nitrosomonas europaea ATCC29578	Shake flask	n.d	7.8	56	28	Fed-batch	Growth mixotrophically on ammonium and hydroxylamine (36 μg protein/mg-N-NH_2OH/L)	(Böttcher and Koops 1994)
Nitrosococcus oceanus Nc.1	Shake flask,	n.d	7.8	56	28	Fed-batch	Growth mixotrophically on ammonium and hydroxylamine (20 μg protein/mg-N-NH_2OH/L)	(Böttcher and Koops 1994)
Nitrosomonas nitrosa Nm 90	Shake flask	n.d	7.8	56	28	Fed-batch	Growth mixotrophically on ammonium and hydroxylamine (30 μg protein/ mg-N-NH_2OH/L)	(Böttcher and Koops 1994)
Nitrosomonas europaea ATCC19718	Flasks, planktonic	25	8.3	n.a.	1.4	Fed-batch	Starvation of cells decreased NH_2OH activity but not ammonium.	(Wilhelm et al., 1998)
Nitrifying culture	RDRs, biofilm	20	7.6 ± 0.2	50.4	3.5	Continuous	Partial nitrification, inhibition NOB, Higher ammonium consumption, dense clusters to single scattered cells	(Kindaichi et al., 2004)
Nitrifying enrichment	Respirometric tests (from a 2 L SBR)	25	7.5	3–12	n.d.	Batch tests	Study of acceleration phase. Hydroxylamine shortened acceleration phase.	(Chandran and Smets 2008)
Full nitrification aggregates	Batch tests	23*	7.5–8	150	15	Batch	Hydroxylamine addition increased ammonium uptake rate. Impact on the aggregate size	(Harper et al., 2009)
Full nitrification aggregates	Fed-batch reactors	23*	7.5–8.8	200	10,20,40	Pulse feeding	AOB and NOB inhibition, decrease of the aggregate size	(Harper et al., 2009)
Star-up PN	Up-flow biofilm PN	35	7.8 ± 0.1	Gradually increased.	3.5	Added continuously	Achieved PN during start-up	(Okabe et al., 2011)
Full nitrifying culture to PN	SBR	25	7.8–8.2	100	2.0 **	Pulse feeding every 2 days	Full nitrification switched to partial nitritation in one week	(Xu et al., 2012)
Nitrifying enrichment	Respirometric tests (biomass from a 4 L SBR)	25	7.5	8	1–3	Batch test	Model including self- inhibition of hydroxylamine in AOB	(Wan et al., 2016)
PN/AMX	SBR	33	7.9 ± 0.2	1750–221	5,10,20	Pulse feed	Inhibition of NOB, but population recovered when dosing was stopped	(Wang et al., 2016; Wang et al., 2015)
Start-up PN	SBR	19.5–28.2	6.7–7.9	70	5	Pulse feeding start each cycle	Both AOB activity and NOB affected. *Nitrospira* more inhibited than *Nitrobacter*	(Li et al., 2019b)
Star-up PN	SBR	25±1	n.d.	70.5 ± 6	4.5	Pulse feeding every 24h	Both AOB activity and NOB affected. *Nitrospira* more inhibited than *Nitrobacter*	(Li et al., 2019a)

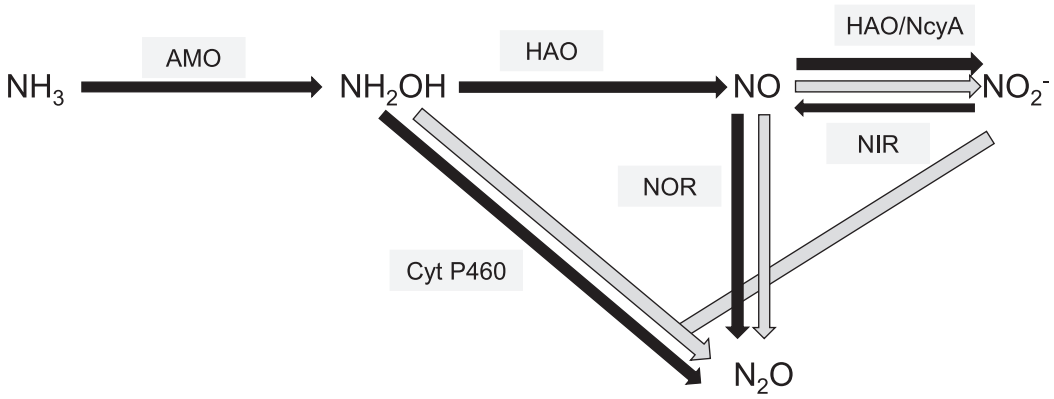

Fig. 3. Putative N_2O emission pathways in AOB. Black arrows represent biologically mediated pathways, grey arrows represent abiotic conversions. AMO – ammonium monooxygenase, HAO – hydroxylamine oxidoreductase, NcyA - Nitrososcyanin, Cyt P460 – Cytochrome P460, NIR – nitrite reductase, NOR – NO reductase. Sources: Caranto et al., 2016; Soler-Jofra et al., 2018; Stein 2019; Terada et al., 2017.

reductase (Nor) respectively. It has been suggested that this pathway is predominant at low oxygen concentrations (Kozlowski et al., 2014). ii) Hydroxylamine oxidation that involves the oxidation of NH_2OH to NO by HAO. Then, NO can be further converted to N_2O by Nor. N_2O production through this pathway is thought to be favoured at higher O_2 concentrations (Hooper and Terry, 1979; Klotz and Stein, 2011; Kozlowski et al., 2014) (Fig. 3).

However, recent studies have highlighted the occurrence and contribution of other pathways to the total N_2O emissions (Caranto et al., 2016; Soler-Jofra et al., 2016; Terada et al., 2017). Most of these new proposed pathways have hydroxylamine as substrate. Briefly, Caranto et al., showed that the cytochrome P_{460} of HAO can directly produce N_2O from hydroxylamine (Caranto et al., 2016).

Besides biological conversion, hydroxylamine has been shown to react chemically by either disproportionation or with medium components such as Fe, Mn or HNO_2 resulting in N_2O formation [see (Heil et al., 2015; Schreiber et al., 2012) for detailed reactions]. Two different studies showed independently the occurrence of a chemical reaction between nitrite and hydroxylamine at conditions relevant for wastewater treatment (Harper et al., 2015; Soler-Jofra et al., 2016). Furthermore, the chemical N_2O production rate by the reaction of hydroxylamine and nitrite (or the protonated form, nitrous acid) was comparable to the biological N_2O production rates (Soler-Jofra et al., 2018; Terada et al., 2017). Contradicting results were presented by Su and co-workers (Su et al., 2019a), proposing that abiotic reactions would only be relevant at acidic pH. All mentioned studies (Soler-Jofra et al., 2018; Soler-Jofra et al., 2016; Su et al., 2019a; Terada et al., 2017) were performed with different medium compositions, and the impact of different compounds to the putative final reported rates is yet unknown. For instance, performing the same test with demineralized water or synthetic medium increased the hydroxylamine disproportionation by 2 to 22 fold (Su et al., 2019a). However, abiotic tests containing both free nitrous acid and hydroxylamine were only performed with demineralized water at neutral pH (Su et al., 2019a). At pH 8 (when free nitrous acid concentration is really low) the reaction rate increased by at least one order of magnitude when using medium instead of demineralized water (Su et al., 2019a). Thus, from our point of view it is unclear if pH and/or medium compounds are both important when determining the chemical reaction rate. Consequently, we suggest that the kinetic characterization of hydroxylamine abiotic reactions, and the impact of different environmental conditions (i.e. trace elements concentrations, iron and others) is of relevance to understand this process properly. Even more importantly, the impact of a real wastewater matrix into such reactions is yet to be studied.

From an engineering point of view, N_2O mitigation strategies in wastewater treatment have already been implemented, even if the actual conversions behind the emissions are not fully understood (Kampschreur et al., 2008). To improve these strategies, a more in-depth understanding of the pathways contributing to the total N_2O emissions is needed. This would help to identify the factors promoting N_2O emissions and to include this knowledge in the design of wastewater treatment process, instead of applying mitigation strategies after operation started. The challenge is to develop a methodology that allows to identify the pathways contribution to the total N_2O emissions, as well as studying the factors impacting them. The large number of compounds and microbial groups involved combined with potential chemical conversions makes this a difficult task. For example, implementing a comprehensive approach including a combination of ^{15}N tracer studies, natural isotope signatures, modelling and transcriptomics/proteomics might be needed to be able to fully differentiate between pathways (Duan et al., 2017).

Overall, hydroxylamine has been shown for years to be a promotor of N_2O emissions, which has a 300-fold larger warming potential than that of CO_2 . Thus, further understanding the factors promoting N_2O emissions from hydroxylamine, will help in the design of mitigation strategies.

3. Ammonium oxidizing archaea (AOA)

Ammonium oxidizing archaea (AOA) were firstly identified using genomic tools, as the detected *amo* gene was corresponding with an archaeon scaffold (Treusch et al., 2005; Venter et al., 2004). Later, the first isolation of an AOA microorganisms demonstrated its abilities to oxidize ammonium to nitrite (Könneke et al., 2005). AOA might play an important role in nitrification in environments such as the oceans and soils, where substrates are usually found at low concentrations and AOA high ammonium affinities allow its survival (Stahl and de la Torre, 2012; Wuchter et al., 2006). Also nitrifying drinking water filters are often reported to contain AOA (Erguder et al., 2009; Kasuga et al., 2010; Van der Wielen et al., 2009). Due to their phylogenetic differences with other archaea, AOA were proposed to be classified inside a new phylum in the archaea domain; Thaumarchaeota (Brochier-Armanet et al., 2008).

The first pure culture AOA was obtained 12 years ago (Könneke et al., 2005), as their bacterial counterparts, its central nitrogen metabolism is still under discussion. For instance, AMO enzyme is conserved in all known AOA, but no HAO homologues have been identified (Hatzenpichler, 2012; Lancaster et al., 2018;

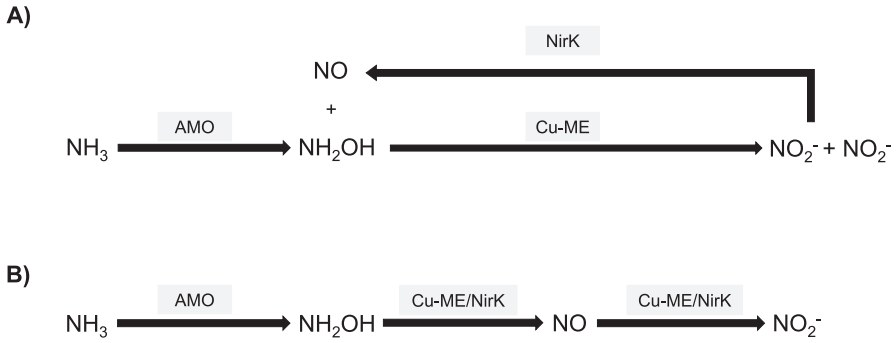

Fig. 4. - Proposed enzymes for ammonium conversion by ammonium oxidizing archea (AOA). No homologues of hydroxylamine oxidoreductase (HAO) are present in AOA, thus alternative pathways are proposed A) A cooper-based enzymatic complex (Cu-ME) is able to transform NO and hydroxylamine to form two nitrite molecules, one of this nitrite molecules is transformed back to NO by nitrite reductase (NirK) B) Alternatively, hydroxylamine is first transformed to NO and further oxidized to nitrite by a yet not fully characterized enzyme that could be either Cu-ME or NirK. Sources - Lancaster et al., 2018; Stein 2019.

Stein, 2019; Vajrala et al., 2013). Thus, the conversion of ammonium to hydroxylamine (Eq. (2)) has been proposed to be conserved and catalysed by the archaeal AMO enzyme. The further conversion of hydroxylamine to nitrite is under consideration (as it occurs with AOB and comammox). The fact that no HAO homologues have been detected in the AOA genome led to two possible central nitrogen metabolic models (Stein, 2019): i) a copper complex uses NO and NH_2OH to form two molecules of nitrite, NirK enzyme is involved in the transformation of nitrite to NO (Fig. 4A), ii) two enzymes consecutively oxidize NH_2OH to NO and NO to nitrite, proposed to be mediated by NirK or an uncharacterized copper complex (Fig. 4B).

To the yet not fully resolved hydroxylamine to nitrite conversion pathway, it must be added the lack of cytochrome-c type proteins usually performing the electron transportation in AOB respiration. Instead, a copper based electron transport system has been postulated, as a high number of protein copper domains have been identified in the genome (Stahl and de la Torre, 2012; Walker et al., 2010).

Regarding NO and N_2O emissions AOA are not capable to perform nitrifier denitrification (Kozlowski et al., 2016b; Stieglmeier et al., 2014), as no N_2O was formed with limited oxygen supply. Isotopic signature also suggested that the nitrogen found in N_2O comes from both ammonium and nitrite (Stieglmeier et al., 2014).Thus, N_2O production was linked to ammonium conversion, and proposed that hydroxylamine or N-intermediates abiotically react leading to N_2O (Kozlowski et al., 2016a; Stieglmeier et al., 2014).

Due to its relatively recent identification few experiments used or measured hydroxylamine in AOA cultures. So far, externally added hydroxylamine has been used to demonstrate its role as intermediate and postulate that hydroxylamine oxidation is coupled to ATP generation in *Nitrosopumilus maritimus* (Vajrala et al., 2013). In addition, externally added hydroxylamine concentrations of 14 m-N/L showed to completely inhibit *N. maritimus* (Vajrala et al., 2013), which indicates a higher sensitivity to hydroxylamine exposure of AOA to that observed in AOB (Table 2). Nevertheless, to our knowledge no other studies of batch or continuous exposure to externally added hydroxylamine of other AOA strains have been reported, yet. Thus, a differential behaviour of AOA strains to hydroxylamine exposure remains to be investigated.

Hydroxylamine transient accumulation has been shown to occur in AOA cultures, also pointing towards a differential strain behaviour towards hydroxylamine accumulation (Liu et al., 2017). For example, *N. gragensis* only released hydroxylamine (4.6 μg-N/L) when incubated with 28 mg-N/L ammonium, but not with 7 mg-N/L. *N. uzonensis* produced hydroxylamine with both ammonium initial concentrations of 7 and 28 mg-N/L, and reached higher concentrations (4.8 μg-N/L) when incubated with the higher ammonium concentrations. Contrarily, *N. viennensis* and *Ca.* N. sp. Nd2 did not produce hydroxylamine. Thus, the hydroxylamine accumulation strain dependency observed in AOB seems to also be a differential strain trait of AOA.

Overall, the recent identification and isolation of AOA presents still unresolved questions, such as the central nitrogen metabolism, further characterization of the NO/N_2O emissions or the differential strain response to hydroxylamine accumulation and exposure.

4. Complete ammonium oxidizing bacteria (COMAMMOX)

Since nitrification first discovery, it was always thought that ammonium oxidation to nitrate involved a two-step microbial conversion, involving AOB and nitrite oxidizing bacteria (NOB). Complete ammonium oxidation to nitrate by a single microorganism was predicted thermodynamically possible (Costa et al., 2006), but overlooked for years until two independent studies demonstrated its existence (Daims et al., 2015; van Kessel et al., 2015).

Complete ammonium oxidating (comammox) bacteria were first identified in two parallel studies demonstrating that they have all the cell machinery to oxidize ammonium to nitrite and further to nitrate (Daims et al., 2015; van Kessel et al., 2015). Mainly two reasons prevented comammox identification for so many years; i) AOB dedicated qPCR primers targeting *amo* gene were not covering the comammox *amoA gene*, due to only ca. 60% amino-acid identity, ii) 16S rRNA sequencing does not allow to distinguish comammox from NOB. Thus, comammox were usually misclassified as canonical NOB (Lawson and Lücker, 2018).

The current running hypothesis for the central metabolism of comammox is postulated to involve AMO, HAO and NXR enzymes, as copies of all the genes encoding for these enzymes have been found in the genome (Daims et al., 2015; van Kessel et al., 2015). Thus, ammonium is first transformed to hydroxylamine by AMO, hydroxylamine is further oxidized to nitrite by HAO and finally nitrite is converted to nitrate by NXR (Fig. 5A). As well as for AOB and AOA, the occurrence of a third intermediate step involving HAO converting NH_2OH only to NO and a further conversion of NO to nitrite is under discussion (Kits et al., 2019) (Fig. 5B). As discussed previously, NcyA has been hypothesised to be a lacking third enzyme for AOB. However, yet no NcyA encoding gene has been found in the available comammox genomic data (Camejo et al., 2017; Kits et al., 2019; Palomo et al., 2018). Thus the hypothesis of comammox encoding a NO oxidoreductase in the genome has not been confirmed yet.

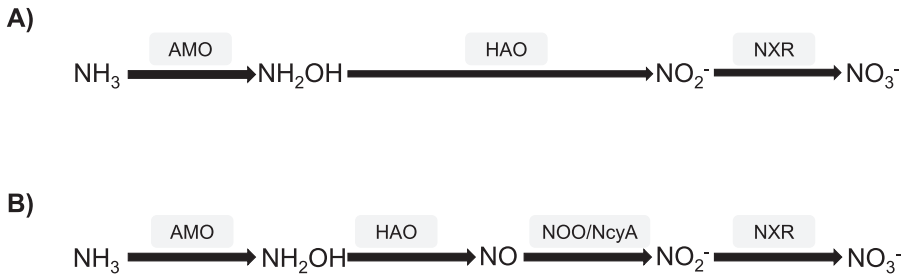

Fig. 5. Proposed enzymes for ammonium conversion by comammox. A) Traditional pathway, where hydroxylamine is directly converted to nitrite followed by the conversion to nitrate. B) Alternatively, hydroxylamine is first transformed to NO and further oxidized to nitrite by a yet not fully characterized enzyme. AMO – Ammonium monooxygenase, HAO – Hydroxylamine oxidoreductase, NOO - Nitric oxide oxireductase, NcyA – Nitrososcyanin, NXR – nitrite oxidoreductase. Sources - Lancaster et al., 2018; Stein, 2019.

The first kinetic analysis of the first isolate *Nitrospira inopinata* (Kits et al., 2017) showed a low ammonium half-saturation coefficient (high affinity for ammonium) and a high growth yield (compared to that of AOB or AOA). This is in agreement with the theoretical higher growth yield prediction (Costa et al., 2006) as well as the comammox distribution in the environment (Lawson and Lücker, 2018). As comammox has been detected at substrate depleted zones (Lawson and Lücker, 2018), thus low ammonium affinity constant and high growth yield allows them to thrive in such minimal environments.

Due to its recent discovery little is known about the role of hydroxylamine besides being involved as intermediate in the central metabolism. Regarding hydroxylamine transient accumulation, Liu and co-workers showed hydroxylamine accumulation in batch like experiments up to 6 μg-N/L, even calculated hydroxylamine accumulation could have been up to 25 to 132 μg-N/L, depending on the initial ammonium concentration used (Liu et al., 2017). A recent study also postulated towards abiotically formed N_2O from hydroxylamine as the main comammox emission source (Kits et al., 2019).

Overall, due to its novelty and recent discovery there is plenty of room for research to further understand the hydroxylamine role in comammox metabolism. Among others, the characterization of hydroxylamine build up in cultures, the impact of externally added hydroxylamine in the metabolism or the involvement of hydroxylamine in N_2O emissions.

5. Anaerobic ammonium oxidizing bacteria (ANAMMOX)

Ammonium conversion without oxygen, even predicted thermodynamically favourable (Broda, 1977), was thought inexistent until the discovery of anammox (Mulder et al., 1995). Anammox bacteria are able to transform ammonium and nitrite to dinitrogen gas autotrophically and anoxically (Jetten et al., 1998). The central metabolism of anammox has been a hot topic of research since its discovery ((Hu et al., 2019; Kartal et al., 2011; Oshiki et al., 2016; Strous et al., 1998; Van De Graaf et al., 1997), among others).

Hydroxylamine was initially hypothesized to be an intermediate of the anammox conversion, as when it was added to anammox cultures, hydrazine accumulated (Jetten et al., 1998; Van De Graaf et al., 1997). Since then, hydroxylamine addition/hydrazine accumulation experiments have been used as characteristic activity tests to demonstrate anammox activity in enrichment cultures (Egli et al., 2001; Jetten et al., 1998, among others). Later, NO was proposed to be intermediate of the central nitrogen metabolism instead of hydroxylamine (Kartal et al., 2011). The current hypothesis for the anammox nitrogen metabolism consists of three reactions. First, a nitrite oxidase (Nir) enzyme converts nitrite to NO (Eq. (6)). Then, NO reacts with NH_4^+ and form hydrazine (N_2H_4) (Eq. (7)) catalysed by hydrazine synthase (HZS). Finally, hydrazine dehydrogenase (HDS) further converts hydrazine to dinitrogen gas (Eq. (8)) (Fig. 6).

$$NO_2^- + 2H^+ + e^- \rightarrow NO + H_2O \quad (6)$$

$$NO + NH_4^+ + 2H^+ + 3e^- \rightarrow N_2H_4 + H_2O \quad (7)$$

$$N_2H_4 \rightarrow N_2 + 4H^+ + 4e^- \quad (8)$$

5.1. The yet unknown role of hydroxylamine in anammox bacteria

Hydroxylamine role in the anammox metabolism is still not fully understood. For instance, not all anammox strains (i.e. *Ca.* Brocadia spp.) encode the Nir enzyme (Oshiki et al., 2015). Consequently, either another enzyme, like the one encoded in the gene *kustc0458*, is doing the job (Hu et al., 2019), or hydroxylamine is involved in the pathway (Oshiki et al., 2016).

Another surprising and characteristic trait is that hydroxylamine oxidase (HOX), which converts hydroxylamine to NO, is one of the most highly expressed enzymes in anammox (Hu et al., 2019; Kartal et al., 2011). Thus, anammox is investing energy and nutrients on keeping a high HOX protein content in the cell, which is puzzling, if hydroxylamine does not have an important role in the metabolism (Fig. 6).

The only hypothesis to explain this high HOX expression, is that HZS enzyme can leak hydroxylamine and HOX is able to transform any leaking of hydroxylamine back to NO (Dietl et al., 2015; Kartal and Keltjens, 2016). Precisely, it is proposed that in HZS catalytic centre, NO is actually transformed to hydroxylamine, and hydroxylamine is reacting with ammonium to form hydrazine (Dietl et al., 2015; Kartal and Keltjens, 2016). Another explanation for this high HOX expression is that hydroxylamine might be important in anammox like environments. Overall, the role of hydroxylamine in the anammox metabolism remains as yet poorly understood.

Externally added hydroxylamine in form of batch tests had different outcomes Table 3: i) When hydroxylamine was added, accumulation of hydrazine occurs, which has been used to demonstrate anammox activity (Egli et al., 2001; Jetten et al., 1998), ii) Hydroxylamine has been shown to “boost” the anammox activity (Hu et al., 2011; Zekker et al., 2012), iii) Hydroxylamine addition allowed to characterize anammox hydroxylamine metabolism (Van De Graaf et al., 1997; van der Star et al., 2008). Hydroxylamine anammox metabolism occurs via disproportionation to ammonium and dinitrogen gas (Eq. (9)). However during this disproportionation hydrazine accumulation occurs. The accumulation is due to an imbalance between the two reactions involved in hydroxylamine disproportionation that produce (Eq. (10)) and consumes hydrazine (Eq. (11)), respectively (van der Star et al., 2008).

$$3\ NH_2OH + H^+ \rightarrow NH_4^+ + N_2 + 3\ H_2O \quad (9)$$

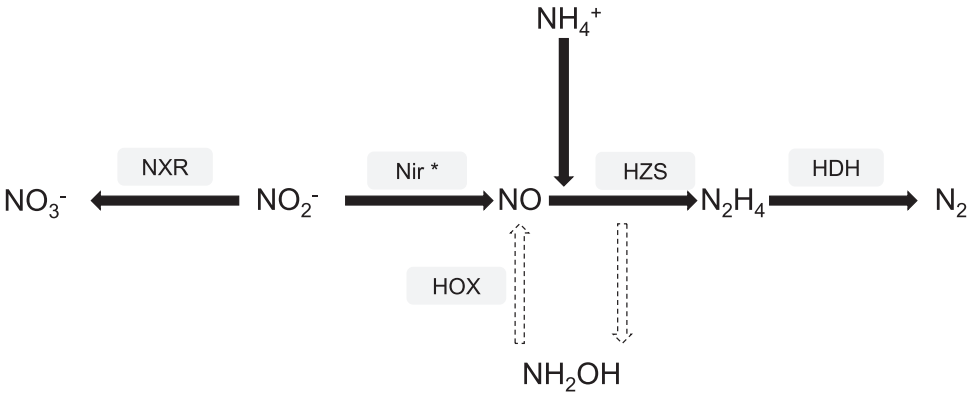

Fig. 6. Central nitrogen metabolism of anaerobic ammonium oxidazing bacteria (anammox). * Nir is depicted here as the enzyme responsible to convert nitrite to NO, this might vary for *Ca.* Brocadia strain (Oshiki et al., 2016). Also a proposed enzyme for this conversion is the one encoded in the gene *kustc0458* (Hu et al., 2019). NXR – nitrite/nitrate oxidoreductase, Nir- nitrite reductase, HZS- hydrazine synthase, HDH – hydrazine dehydrogenase, HOX- hydroxylamine oxidase. Sources:Kartal and Keltjens, 2016.

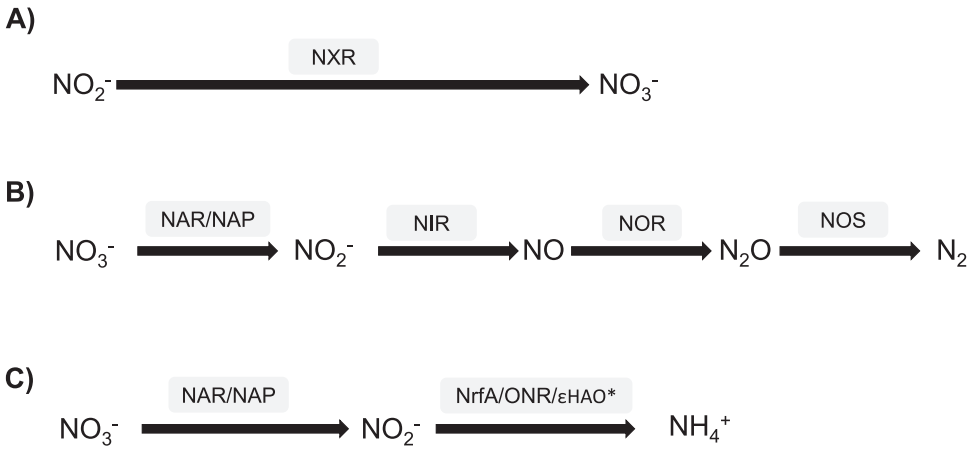

Fig. 7. Central nitrogen metabolism of: A) Nitrite oxidizing bacteria (NOB), B) Anaerobic heterotrophic denitrifiers (DEN), C) Dissimilatory nitrate reduction to ammonium (DNRA). NXR – nitrite oxidoreductase, NAR/NAP – nitrate reductase, NIR – nitrite reductase, NOR – NO reductase, NOS – N_2O reductase, NrfA- ammonia forming nitrite reductase, ONR- nitrite reductase,εHAO- Epsilonproteobacterial hydroxylamine oxidoreductase.

$$NH_4^+ + NH_2OH \rightarrow N_2H_4 + H_2O + H^+ \quad (10)$$

$$2NH_2OH + N_2H_4 + 2H^+ \rightarrow 2NH_4^+ + N_2 + 2H_2O \quad (11)$$

Co-metabolisation of other substrates with hydroxylamine impacts its metabolism (Soler-Jofra et al., 2020). Continuous and limiting addition of hydroxylamine to anammox showed to decrease the stoichiometric nitrate needed for growth and reported no negative impact on the anammox community (Soler-Jofra et al., 2020). Thus, anammox could use hydroxylamine and survive in environments where it is present.

6. Nitrite oxidizing bacteria (NOB)

Nitrite oxidizing bacteria perform the second step of nitrification catalysing the conversion of nitrite to nitrate with oxygen as electron acceptor. Up to date, 7 genera have been described belonging to 6 different phyla in α, β, γ Proteobacteria (Daims et al., 2016).

NOB couple the nitrite oxidation to nitrate as electron donor (Eq. (12)) with oxygen respiration as electron acceptor (Eq. (13)), resulting in NOB central nitrogen catabolism (Eq. (14)). Nitrite oxidation to nitrate is catalysed by nitrite oxidoreductase (NXR), a membrane-bound enzyme that was first isolated and characterized in *Nitrobacter* by Meincke et al. (1992) (Fig. 7A).

$$NO_2^- + H_2O \rightarrow NO_3^- + 2H^+ + 2e^- \quad (12)$$

$$0.5O_2 + 2H^+ + 2e^- \rightarrow 2H_2O \quad (13)$$

$$NO_2^- + 0.5\ O_2 \rightarrow NO_3^- \quad (14)$$

6.1. Hydroxylamine inhibits NOB

Hydroxylamine is not an intermediate in NOB metabolism, but NOB communities usually are found close to AOB, which are able to leak hydroxylamine (Table 1). Hydroxylamine concentrations from 0.2 to 20 mg-N/L have been reported as a potent inhibitor of NOB (Table 4). For instance, Castignetti and Gunner (1982) reported inhibition of *Nitrobacter agilis* by hydroxylamine at concentration of 5 mg NH_2OH–N/L. Stuven et al. (1992) also reported hydroxylamine inhibition of *Nitrobacter* at concentrations of 1 mg NH_2OH-N/L. Later, Hao and Chen (1994) demonstrated hydroxylamine inhibition in NOB by measuring nitrite build-up in complete nitrification submerged filters by the addition of 2.5–5 mg-N/L. Moreover, more than 30 days were needed to recover regular operation (Hao and Chen, 1994). Concentration of less than 0.2 mg N–NH_2OH /L were reported to inhibit NOB by Blackburne and coworkers (Blackburne et al., 2004).

Table 3
Summary of hydroxylamine consumption experiments performed with anammox biomass. a) Units in µmol-N/vial. n.d. not determined, n.a. – not applicable, *Ca.* – Candidatus, Temp. –Temperature, HRT – Hydraulic retention time, CSTR – continuous stirred tank, MBR – membrane bioreactor. * Experiments performed in the optimal range for anammox growth.

Anammox specie	Type of sludge	Reactor conditions	Temp. (°C)	pH	Initial NH_4^+ (mg-N/L)	Initial NO_2^- (mg-N/L)	Initial NH_2OH (mg-N/L)	NH_2OH consumption rate, q_{NH2OH} (mg-N/gVSS/h)	N_2H_4 peak (mg-N/L)	N_2H_4 consumption rate, q_{N2H4} (mg-N/gVSS/h)	Reference
n.d.	Flocs	Batch tests	30	7	112	112	42	n.d.	14.0	n.d.	(Van De Graaf et al., 1997)
Ca. Brocadia anammoxidans & stuttgarteniss	Flocs	Schott flasks	37	7	11.2	0	39.2	n.d.	8.7	n.d.	(Egli et al., 2001)
Ca. Kuenenia stuttgartensis	Granular sludge	CSTR, batch tests	37	*	28	0	7–140	4.3 – 12.7	0.4–2.7	0.2 – 1.0	(van der Star et al., 2008)
Ca. Brocadia fulgida	Granular sludge	SBR, batch tests	37	*	42	0	56	n.d.	2.7	n.d.	(van der Star et al., 2008)
n.d.	Granular sludge	16 L reactor, batch tests	35	7.5	43.4	0	39.2	2.9	10.1	0.9	(Hu et al., 2011)
n.d.	Granular sludge	16 L reactor, batch tests	35	7.5	35	30.8	0	5.6	n.a.	n.a.	(Hu et al., 2011)
Ca. Brocadia fulgida	Biofilm carriers	MBR, batch tests	25	8–8.5	84	0	14	1.5	3.4	n.d.	(Zekker et al., 2012)
Ca. Brocadia sinica	Planktonic cells	MBR, batch tests	37	7.6	0	0	21	n.d.	0.1 (a)	n.d.	(Oshiki et al., 2016)
Ca. Kuenenia stuttgartiensis	Planktonic cells	Batch tests	30	8–8.5	0	0	22.4	75.6	2.6	4.2	(Soler-Jofra et al., 2020)
Ca. Kuenenia stuttgartiensis	Planktonic cells	Batch tests	30	8–8.5	0	19.6	19.6	42	1.3	2.8	(Soler-Jofra et al., 2020)
Ca. Kuenenia stuttgartiensis	Planktonic cells	Batch tests	30	8–8.5	84	19.6	29.4	112	7.2	25.8	(Soler-Jofra et al., 2020)

Another indication that supports strong NOB inhibition by hydroxylamine is the general trend observed that full nitrification fails, accumulating nitrite, when hydroxylamine is added to the system (Harper et al., 2009; Wang et al., 2015; Xu et al., 2012). As discussed before, hydroxylamine external addition in nitrification systems is used to inhibit NOB either to obtain a stable partial nitrification operation or to achieve a rapid start-up of a partial nitrification reactor (Li et al., 2019a; Li et al., 2019b; Wang et al., 2016; Wang et al., 2015).

However, the mechanism of hydroxylamine inhibition in NOB is still unknown (i.e. if it is affecting the gene expression, interfering with cell compounds). Some studies refer to a reversible inhibition, as far as full nitrification is restored with time after hydroxylamine exposure (Li et al., 2019b; Wang et al., 2015; Xu et al., 2012). The only hypothetical theory is that the un-protonated form of hydroxylamine can diffuse through the membrane and affect the gene expression (Yang and Alleman, 1992). Specifically, in a later study Wang and co-workers reported a decrease in *nxrA*, a gene related with NOB (Wang et al., 2016). Another interesting observation is that *Nitrospira* and *Nitrobacter* might be differentially inhibited by hydroxylamine, impacting more *Nitrospira* than *Nitrobacter* (Li et al., 2019b)

In full nitrification and partial nitrification processes, AOB and NOB populations are usually clustered together. Thus, understanding further the impact of hydroxylamine on NOB activity is vital, as AOB have been shown to transiently accumulate this compound. Addition of hydroxylamine has already been shown to promote a partial nitritation system over full nitrification (Wang et al., 2015; Xu et al., 2012). Further understanding of this process (hydroxylamine accumulation and effect on NOB) could contribute to avoid NOB proliferation in partial nitritation anammox systems and further understand microbial community interactions.

7. Other wastewater treatment related microorganisms and their interactions with hydroxylamine

AOB, AOA, anammox and comammox are recognized consumers of hydroxylamine, and hydroxylamine metabolism has been dedicatedly studied, but still unknowns remain. The inhibition of nitrite oxidizing bacteria by hydroxylamine has been dedicatedly studied. Particularly, with the growing interest of implementing partial nitritation. Conversely, heterotrophic denitrifying bacteria play a crucial role in engineered systems such as wastewater treatment plants (WWTPs) transforming nitrate to dinitrogen gas through nitrite, NO and N_2O (see Fig. 7B). There is only very limited information on the impact of hydroxylamine on denitrifying bacteria. A recent study showed that nitrite accumulation was favoured when dosing hydroxylamine to a complete denitrification reactor (Zhang et al., 2020). Batch tests with hydroxylamine dosing from (2–21 mg-N/L) were also performed. Nitrate consumption seemed to be promoted at low hydroxylamine concentrations dosages (2–8 mg-N/L), whereas nearly any nitrate consumption was observed during the first 20 min. when hydroxylamine concentrations were higher than 14 mg-N/L. Interestingly, nitrite accumulation was always higher when hydroxylamine was added in the batch tests. Nitrate reductase (NAR) and nitrite reductase (NIR) activity were also measured after hydroxylamine exposure, showing a grater increase in NAR activity than NIR for increasing hydroxylamine doses. The difference in enzymatic activities might explain the nitrite accumulation . This was in agreement with the gene expression, as napA expressions was up to 2.76-fold increased when hydroxylamine was dosed (Zhang et al., 2020). Nevertheless, the impact of hydroxylamine on the subsequent steps: NO and N_2O conversions was not investigated. Also the fact that pH 9 was used in this study, might have impacted the results, as hydroxylamine is mainly unprotonated. Thus, it is able to diffuse through the mem-

Table 4
Summary of literature reporting NOB inhibition by hydroxylamine (a) converted from mg-NH_2OH/L, * referred as free hydroxylamine, OUR – oxygen uptake rate, n.d.-not determined, SBR –sequential batch reactor, FBBR-Fed-batch bioreactor, PN – Partial Nitritation.

Type of biomass	NH_2OH concentration (mg-N/L)	Type of inhibition	Comments	References
Nitrobacter agilis	5	Irreversible	No activity was detected when the culture was transferred to fresh medium	(Castignetti and Gunner, 1982)
Nitrobacter vulgaris	1	n.d.	No nitrate formation	(Stüven et al., 1992)
Full nitrification culture	0.3–4.3*	n.d.	Deterioration of full nitrification, nitrite transient accumulation	(Yang and Alleman, 1992)
Full nitrification culture (submerged fixed film)	1 - 5	Reversible	Deterioration of full nitrification, nitrite accumulation. 30 days recovery.	(Hao and Chen, 1994)
NOB enrichment (SBR)	0.2- 3	n.d.	Hydroxylamine decreased OUR	(Blackburne et al., 2004)
Full nitrification culture (FBBR)	10–40	n.d.	Deterioration of full nitrification, nitrite accumulation	(Harper et al., 2009)
Partial nitritation/anammox	4.2–8.5 (a)	Reversible	Decreased nitrate accumulation by NOB	(Wang et al., 2015)
Full nitrification culture	1–3	Non-competitive	Decreased OUR (Ki=3.233±0.093 mmol-N/L)	(Wan et al., 2016)
Start- up PN (SBR)	1.9 (a)	n.d.	*Nitrospira* more inhibited than *Nitrobacter*	(Li et al., 2019a)
Start- up PN (SBR)	2.1 (a)	Reversible	*Nitrospira* more inhibited than *Nitrobacter*, nitrate production recovered	(Li et al., 2019b)

branes. Overall, more studies confirming the observed trends by (Zhang et al., 2020) would be needed, specially performed at more usual pH (7–8) for wastewater treatment.

Hydroxylamine usage capacity of dissimilatory nitrate reducers to ammonia (DNRA) (see Fig. 7C) has been hypothesised based on the hydroxylamine detoxification capacity of some of their enzymes (ONR or εHAO) (Haase et al., 2017; Simon and Klotz, 2013) and hydroxylamine has been proposed as intermediate for *Nautilia profundicola* (Hanson et al., 2013). For both denitrifiers and DNRA bacteria few studies are available and no conclusions can be drawn regarding the impact of hydroxylamine.

Finally, it is worth mentioning other microorganisms that are able to use hydroxylamine and that might be involved in wastewater treatment engineered processes, even not considered directly as part of the nitrogen cycle. For instance, heterotrophic aerobic bacteria with nitrification or/and denitrification activity. The pathway for nitrification encoded in these microorganisms includes hydroxylamine as intermediate (Stein, 2011). For example, *Photobacteriu* sp. (Liu et al., 2019)., *Alcaligenes faecalis* (Joo et al., 2005; Sorokin, 1989; Sorokin and Dubinina, 1986), *Pseudomonas* (Jetten et al., 1997) or *Enterobacter* (Padhi et al., 2017). Methanotrophs are also well known to have hydroxylamine oxidation capacity, which is involved in nitrous and nitric oxide production by those microorganisms (Campbell et al., 2011; Stein and Klotz, 2011; Versantvoort et al., 2020).

Overall, hydroxylamine oxidation capacity is widespread within microorganisms of the nitrogen cycle and others involved in wastewater treatment, whereas it is also known to inhibit some of them. To achieve a comprehensive picture of its role when shaping microbial communities, further investigation on this compound in relation to the diverse N-cycle conversion is needed.

8. Hydroxylamine presence might shape microbial communities and biofilms dynamics

All of the microorganisms discussed in the present review are known to be found close together in the natural environment (i.e. ocean, soils) and engineered systems where they typically grow in biofilms or aggregates (i.e. WWTPs) (Kuypers et al., 2018). Thus, microbial interactions between different communities occur, and usually microbes rely on these interactions to get their substrate or to avoid product inhibition (i.e. AOB/NOB interactions).

In engineered systems, such as WWTPs, the interaction between AOB and NOB and denitrifiers have been conventionally used to remove nitrogen from wastewater. More recently, other players such as anammox, comammox or AOA have been added to the already complex community interactions. In these kind of engineered systems microorganisms are usually found forming aggregates either as activated sludge, granules or attached biofilms. As we have seen in this review and according to literature, AOB, AOA and comammox can leak hydroxylamine, and it can be related to fluctuations of substrate/oxygen. In WWTPs substrate fluctuations are usual, but also within biofilm systems strong gradients occur. Thus, fluctuations of substrate/oxygen can be enhanced by the biofilm structure (i.e. some cells that have been under starvation, receive substrates when there is an increase in the bulk liquid concentration). Consequently, hydroxylamine build up can be enhanced within a biofilm system. This fact has already been proposed by mathematical simulations (Sabba et al., 2015).

Another important factor that can impact hydroxylamine accumulation and usage by microorganisms is pH. In a biofilm a pH gradient is generated with a more acidic pH in the inner core of the granule (De Beer et al., 1993; Poot et al., 2016; Schreiber et al., 2009; Uemura et al., 2011; Winkler et al., 2011). Acidic pH has been shown to strongly impact ammonium oxidation by *Nitrosomonas*, whereas hydroxylamine oxidation was barely affected (Frijlink et al., 1992). This fact might favour hydroxylamine usage over ammonium by AOB in inner layers of a biofilm system.

Overall, hydroxylamine build up due to aerobic ammonium oxidazing microorganisms can have an impact in other microbial communities such as anammox, NOB, denitrifiers or the same neighbour clusters of aerobic ammonia oxidizers. Furthermore, hydroxylamine can also trigger N_2O emissions, as it has been discussed. Thus, hydroxylamine might have a yet not fully understood role when shaping microbial communities.

9. Hydroxylamine measurement: the bottleneck?

In wastewater treatment related research hydroxylamine measurements are almost absent. This is due to two factors: i) hydroxylamine available measurement techniques are laborious and really time consuming, ii) being usually an intermediate, the method should be sensitive enough for the expected low concentrations. Nevertheless, the fact that hydroxylamine is mutagenic and toxic compound for humans, microorganisms and animals has brought the need of its adequate quantitative measurement in different fields (Kolasa and Wardencki, 1974).

Focusing on the measurements of hydroxylamine in water samples there are mainly two extended techniques used (Fig. 8): i) Spectrophotometric technique, based on the production of indooxine from the reaction of 8-quinolinol with hydroxylamine in presence of carbonate and ethyl alcohol, which develops a green colour (Frear and Burrell, 1955), ii) Gas chromatography (GC) based method, which relays on the measurement of N_2O formed during

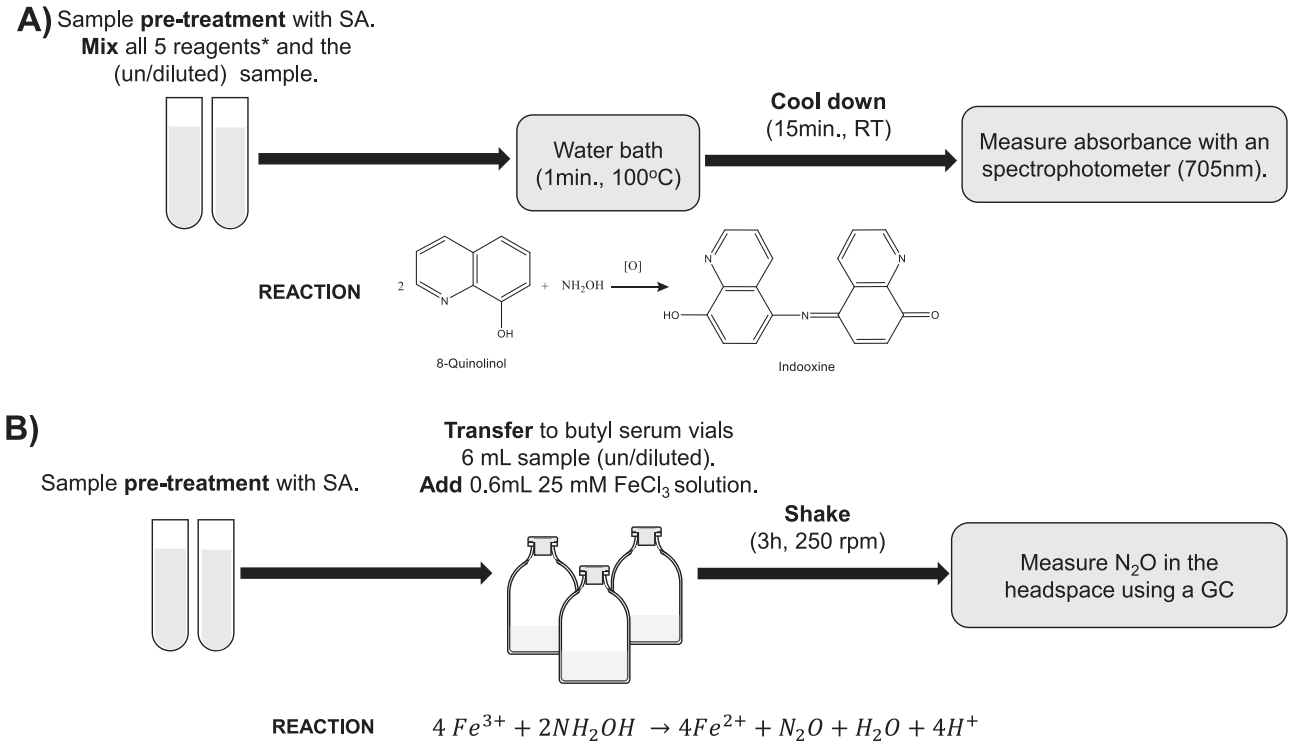

Fig. 8. Hydroxylamine measurement techniques for hydroxylamine concentration determination in water: A) Spectrophotometric based method, B) Gas chromatography (GC) based method. Based on techniques described by (Frear and Burrell 1955; Liu et al., 2014) * Reagents used are phosphate buffer solution, mili-Q water, trichloroacetic acid solution, 8-quinolinol and carbonate solutions (see (Frear and Burrell, 1955) for exact concentrations). SA states for sulfamic acid. RT states for room temperature.

the transformation of hydroxylamine to N_2O catalysed by Fe^{3+} in sealed vials (Butler and Gordon, 1986; Liu et al., 2014).

Both techniques have their advantages and disadvantages. Briefly, the spectrophotometric based technique can be easily implemented, as it is a reactive based methodology which needs of general present laboratory equipment (spectrophotometer, water bath and pyrex tubes). The use of a fume hood is necessary due to the toxicity of the chemicals used and it is a quite laborious method. The GC based method, has a lower detection range (ca. >0.001 mg-N/L) than that of the spectrophotometric method (ca. >0.035 mg-N/L), which might be useful for applications were concentrations of hydroxylamine are actually low. However, the need for a GC with an N_2O detection method, and ideally an autosampler, might limit its implementation in many laboratories. Also a good determination of the N_2O already present in the sample is crucial for an accurate hydroxylamine quantification (Liu et al., 2014).

Both methods are known to be impacted by interferences like pH and salinity (Butler and Gordon, 1986). These interferences impact a lot the N_2O recovery from hydroxylamine in the GC method, thus a good pre-treatment depending on the sample is needed (Liu et al., 2014).

Independently of the method used, pre-treatment of samples and rapid analysis is generally extensive, due to the high reactivity of hydroxylamine. For example, addition of sulfamic acid to the sample is used in both measurement techniques (Liu et al., 2014; Soler-Jofra et al., 2016) with two purposes: i) acidify the sample to stabilize hydroxylamine, and ii) remove nitrite from the sample, which has been shown to react with hydroxylamine and interferes in both methods (Liu et al., 2014; Soler-Jofra et al., 2016). Nevertheless, time from sample collection to its analysis is still crucial, long time storage is not possible.

The complexity of hydroxylamine measurement is one of the main limitations for understanding the role of this compound in the nitrogen cycle and N_2O emissions. Thus, developing of commercial available hydroxylamine sensors might be crucial for future research. Some preliminary results on the development of a hydroxylamine sensor have been reported ((Foroughi et al., 2014; Zhang et al., 2010), among others). However, to our knowledge, there are no initiatives to have such sensors commercially available. If such sensors would become available, as happened for NO and N_2O sensors, or an easier measurement method technique is developed it would boost hydroxylamine related research and our understanding of the nitrogen cycle.

10. Conclusions and future outlook

Here the current understanding of the role of hydroxylamine in the nitrogen cycle, with special focus on the microbial communities involved in wastewater treatment has been presented. Hydroxylamine conversion is widespread within different nitrogen cycle microorganisms, whereas some are negatively impacted by it. We have highlighted that there are plenty of questions and unknowns about the role of hydroxylamine in the nitrogen cycle. The key gap of knowledges are summarized as follows:

- Clarification on how hydroxylamine is converted to nitrite by AOA, AOB and comammox is needed. So far, there are biocatalytic evidences that HAO transforms hydroxylamine to NO in AOB. AOA do not harbour HAO in their genome, thus a different transformation of hydroxylamine has been proposed. Comammox pathway for this conversion is still to be mapped. Overall, it will be interesting to assess if aerobic ammonia oxidizers have evolved differently on how to deal with the conversion of hydroxylamine to nitrite.
- Transient accumulation of hydroxylamine is usually linked to a switch from low to maximum activity (i.e., anoxic/aerobic cycles, batch tests, SBR reactors). The turnover of the differential

enzymes involved in the transformation might be crucial. In addition, it seems to be a strain dependant trait.
- pH might have a crucial role on hydroxylamine usage. First, because it affects the equilibrium between the protonated and unprotonated hydroxylamine form. Secondly, because ammonium oxidation rate is highly impacted by pH, whereas hydroxylamine oxidation to nitrite is not. Thus, pH being a potential contributor to hydroxylamine accumulation.
- Transient hydroxylamine accumulations seems also to be strain dependant in aerobic ammonium oxidizers, as well as it differs between AOB, AOA and comammox. Differences on enzymatic level are hypothesised to the responsible for such observations.
- Hydroxylamine is involved in N_2O emissions in AOA, AOB and comammox. Factors promoting N_2O emissions from hydroxylamine are still to be fully understood.
- Anammox is known to be able to use hydroxylamine as substrate. It is also proposed to be intermediate in *Ca.* Brocadia, whereas NO is proposed to be the intermediate in *Ca.* Kuenenia stuttgartiensis. Nevertheless, in *Ca.* Kuenenia stuttgartinesis, there is a high overexpression of HOX, an enzyme hypothesised to transform the leaking hydroxylamine from HZS to NO. Thus, the role of hydroxylamine in anammox is yet to be understood.
- pH also impacts the equilibrium between the unprotonated (free hydroxylamine) and protonated form of hydroxylamine. Thus, free hydroxylamine has the capacity to diffuse through the bacterial membranes. This is hypothesised to be the cause of NOB inhibition by hydroxylamine. However, it is yet to be demonstrated. A similar inhibition mechanism might impact denitrifiers leading to nitrite accumulation.

Overall, there are a wide range of topics to be investigated regarding hydroxylamine and the nitrogen cycle. Below we provide some crucial points and recommendations for future research, that will broaden our understanding of hydroxylamine:

- Developing an easy implemented hydroxylamine measurement technique would totally facilitate hydroxylamine related research. Either the use of sensors or an improved measuring technique that is not extremely labour intensive, would lead to widespread hydroxylamine measurements in nitrogen related research.
- Developing an integrated research approach including a combination of transcriptomics/proteomics, enzymology and ^{15}N tracer studies could help to further understand the mechanisms involved in hydroxylamine conversion and transient accumulation. Such an integrated approach will be crucial to map hydroxylamine conversion pathways as well as studying such conversions in microbial communities.
- Understanding hydroxylamine enzymatic conversions will help also on understanding NO/N_2O production pathways. Thus, helping on the design of mitigation strategies.

To conclude, the main focus of nitrogen cycle research has usually been the substrates and end products of the microbial conversions. More recently, due to the urge to reduce greenhouse gas emissions, intermediates such as NO and N_2O, started to be extensively studied. The complexity of intermediate reactions and the lack of easily implemented techniques and methods for the usual low concentration measurements, results in a hard topic of study. Nevertheless the central role of hydroxylamine as intermediate in the nitrogen cycle and its relation to N_2O formation urges more attention for this compound in future research projects.

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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