Good servant, bad master: sulfide influence on partial nitritation of sewage

V. Kouba, E. Proksova, H. Wiesinger, D. Vejmelkova and J. Bartacek

ABSTRACT

When applying partial nitritation (PN) to anaerobically pre-treated sewage, ammonium oxidizing bacteria (AOB) and nitrite oxidizing bacteria (NOB) will be exposed to dissolved sulfide and methane. Both sulfide and methane may inhibit nitrification. To gain knowledge necessary for sustaining PN under these conditions, we exposed an AOB enrichment and a mixed nitrifying culture to dissolved sulfide and methane. In the mixed nitrifying culture, sulfide selectively inhibited NOB activity ($K_{I,AOB1} = 150$ mg-S L^{-1}, $K_{I,NOB} = 10$ mg-S L^{-1}) which shows that sulfide may help establish PN. The AOB enrichment showed similar $K_{I,AOB2}$ (130 mg-S L^{-1}), but nitritation activity lagged longer than the time necessary to remove sulfide from the liquid. This demonstrates that feeding of sulfide into established PN should be avoided. Methane inhibition of AOB enrichment was assessed in batch assays with 10 mg-CH_4 L^{-1}. As compared to control without methane, AOB enrichment activity was identical. Up to 51% of methane was converted to methanol, thus reducing the greenhouse gas emissions.

Key words | ammonium oxidizing bacteria (AOB), FISH analyses of nitrifying biomass, main stream nitritation, nitrite oxidizing bacteria, NOB inhibition, sulfide toxicity

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INTRODUCTION

Compared to conventional activated sludge process, the combination of an anaerobic membrane bioreactor (AnMBR) and partial nitritation-anammox (PN/A) has the potential to be a far more resource-efficient way of removing organic carbon and nitrogen from sewage (Kartal *et al.* 2010). However, the implementation of such a concept involves overcoming various challenges, the main one of which is the need to suppress undesirable nitrite oxidizing bacteria (NOB) in a partial nitritation (PN) reactor (Cao *et al.* 2017). The current strategies for suppressing NOB in a PN reactor are mostly based on kinetic selection, and all have specific limitations (Isanta *et al.* 2015; Lotti *et al.* 2015; Laureni *et al.* 2016; Seuntjens *et al.* 2016; Kouba *et al.* 2017). Although these strategies could be augmented by selective NOB inhibition (e.g. sodium azide, sodium chlorate, etc.), this approach is generally not preferred because feeding in of an inhibitory compound increases both process costs and operational complexity. But, one promising selective NOB inhibitor is already present in AnMBR effluent: sulfide (typically 31 ± 41 mg-S L^{-1} (Delgado Vela *et al.* 2015)).

Sulfide is intrinsically present in AnMBR, because sulfate in municipal wastewater undergoes reduction in anaerobic conditions. Sulfide is thought to inhibit both AOB and NOB via interactions with their enzymes containing metals (i.e. copper) and precipitation of metal co-factors of these enzymes (Juliette *et al.* 1993; McCarty 1999; Bejarano Ortiz *et al.* 2013).

To the best of our knowledge, sulfide has only been used to establish PN in one study (Erguder *et al.* 2008), where a single sulfide dose of 45 or 80 mg-S L^{-1} accumulated 75 and 100% of nitrite, respectively, while an ammonium oxidation efficiency above 83% was maintained at low loading rates. The study used a high initial concentration of total ammonium nitrogen (TAN) (700 mg-N L^{-1}), and the pH peaks caused by the addition of sulfide were not adjusted. This meant that the NOB were exposed to several hundreds milligrams of free ammonia (NH_3), an order of magnitude higher than the inhibitory threshold (Anthonisen *et al.* 1976). However, while such a combination of sulfide and free ammonia is relevant for nitrogen-rich reject water, it is of little use for diluted sewage.

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A few studies have reported findings that suggest that sulfide has potential for NOB inhibition in anaerobically pre-treated sewage. Unfortunately, the only study that used a relevant initial pH (8.0 ± 0.4 as compared to suitable pH of 8.0–8.5 (Delgado Vela *et al.* 2015)), also used a high temperature (30 °C), thereby inducing H_2S solubility and dissociation, both of which are irrelevant to anaerobically pre-treated sewage (10–20 °C) ($IC_{50,AOB}$ = 2.5 ± 0.3 mg-S L^{-1}, $IC_{50,NOB}$ = 1.2 ± 0.2 mg-S L^{-1} (Bejarano Ortiz *et al.* 2013)). In addition, the actual initial pH in those experiments was significantly higher (8.8 ± 0.3) than authors originally stated due to the dosing of sulfide, further inhibiting both AOB and NOB (Grunditz & Dalhammar 2001). Studies by Beristain-Cardoso *et al.* (2010) and Bejarano-Ortiz *et al.* (2015) also used high temperature (26 and 30 °C, respectively), in addition to lower pH (7.3 ± 0.3 and 7.0 ± 0.5), which reduced the ratio of free hydrogen sulfide to the less toxic, ionized, form. And Zhou *et al.* (2014) elevated the effect of sulfide inhibition by pre-exposing the culture to sulfide in anaerobic conditions. However, none of these studies worked with real anaerobically pre-treated sewage, which contains reduced amounts of the metal (i.e. copper) co-factors of nitrifying enzymes due to their precipitation in anaerobic conditions.

While the studies above imply the potential of sulfide for NOB inhibition in anaerobically pre-treated sewage, there is a major obstacle to be overcome: sulfide can inhibit not only undesirable NOB, but also desirable ammonium oxidizing bacteria (AOB) (Beristain-Cardoso *et al.* 2010; Bejarano Ortiz *et al.* 2013; Bejarano-Ortiz *et al.* 2015). Bejarano-Ortiz *et al.* (2015) reported a 50% reduction in activity (IC50) for nitrite (1.2 ± 0.2 mg-S L^{-1}) and ammonium oxidation (2.5 ± 0.3 mg-S L^{-1}). In fact, just 1 mg-S L^{-1} of sulfide was able to negatively affect the activity of un-adapted NOB, and even that of un-adapted AOB.

Reactors with established PN will contain AOB enrichment and only limited amounts of NOB and heterotrophs, which is a much less diverse culture than the mixed nitrifying sludge present during PN start-up. Such a less diverse culture has less chance of including the functional elements necessary to enable its adaptation to various stress elements (Cardinale *et al.* 2006). Therefore, we hypothesize that AOB enrichment may be more susceptible to sulfide inhibition than a mixed nitrifying culture. Although this issue is critical for determining the sulfide concentration that will enable NOB inhibition without damaging the PN process, no study has directly assessed the inhibition of AOB enrichment by sulfide.

In addition to sulfide, AnMBR effluent contains dissolved methane (typically 23 ± 13 mg L^{-1} (Delgado Vela *et al.* 2015)), and its potential inhibitory effects on AOB enrichment are largely unexplored. AOB co-oxidize methane with ammonium with an enzyme ammonium monooxygenase (Chain *et al.* 2003). However, the methane oxidation consumes electrons, inducing co-substrate inhibition of AOB (Taher & Chandran 2013).

To investigate this, we assessed sulfide inhibition of AOB enrichment and of a mixed nitrifying culture (AOB + NOB) in batch assays. The substrate was real sewage pre-treated in a laboratory-scale AnMBR (Hejnic *et al.* 2016). Our results show a dramatic difference in the susceptibility to sulfide of the mixed nitrifying culture and of AOB enrichment, and, thus, should help not only in the selective inhibition of NOB at PN start-up, but also in preventing sulfide from damaging an established PN reactor. We also performed batch assays to evaluate the inhibition of AOB enrichment at dissolved methane. At 10 mg L^{-1} of methane, no inhibition of ammonium oxidation was observed.

MATERIALS AND METHODS

Inoculum

Mixed nitrifying culture

A mixed nitrifying culture was obtained from a central wastewater treatment plant (WWTP) in Prague. This activated sludge plant is operated as simultaneous nitrification-denitrification with aerated regeneration tank receiving reject water from anaerobic digestion. The whole range of sulfide concentrations was tested with the same sludge sample. These results were further complemented by additional experiments with samples of new sludge per each batch assay (time span of 8 months).

AOB enrichment for sulfide inhibition

The nitrifying sludge was enriched with AOB using a lab-scale sequencing batch reactor (SBR). The SBR was fed with media consisting of 450 mg L^{-1} of TAN, 4 g L^{-1} of $NaHCO_3$, 10 mg L^{-1} of $CaCl_2.2H_2O$, 50 mg L^{-1} of KH_2PO_4, 300 mg L^{-1} of NaCl, 7.5 mg L^{-1} of $FeSO_4.7H_2O$ and a mix of micronutrients (Hockenbury & Grady 1977; Tappe *et al.* 1996). Tap water was used for dilution (parameters of tap water are displayed in Table S1, available with the online version of this paper). The SBR had an

effective volume of 40 L, operated at 22–28 °C and solids retention time of 19 days. The SBR was equipped with an optical dissolved oxygen sensor (Hamilton VisiFerm DO Arc 120, USA), connected to a cRIO control module running LabView (National Instruments, USA). Compressed air was supplied through a glass diffuser situated at the bottom of the reactor. To avoid limitation of AOB by lack of dissolved oxygen, DO was kept at 3.0 ± 0.1 mg L^{-1} using a PID control system adjusting the air supply based on the actual oxygen consumption rate. The automatically controlled cycle consisted of settling (30 min), decanting (6 min), introducing influent (6 min), and aerobic phase. Aerobic phase was stopped at 5% of the maximum value of oxygen uptake rate as measured during the cycle. The samples of enriched biomass were obtained 242–248 days after the inoculation of the reactor.

Assessment of methane inhibition in AOB enrichment

The AOB enrichment were kept in fully mixed PN SBR with working volume 0.9 L. The oxygen was supplied the same way as in the pilot-scale SBR for AOB enrichment. The aerobic phase was stopped at 20% of the maximum value as measured during the cycle. The temperature of the SBR was set to 12 °C using a thermostat F250 (Julabo, Germany). SRT was kept at 9 days. The SBR cycle consisted of effluent decanting (4 min), feeding influent (3 min), aerobic phase (automatic), dosing of $FeCl_3$, flocculation (10 min) and sedimentation (1.5 h). To ensure excess ammonium in effluent, aerobic phase of SBR cycle was stopped when oxygen consumption decreased to 20% of the maximum within the current cycle. The dosing of $FeCl_3$ (10 mg L^{-1}) was applied to restrict biomass washout with the effluent, thus keeping the target SRT of 9 days. The reactor was fed with anaerobically pre-treated sewage identical to the wastewater used in the batch assays. The tests were performed 77–85 days after inoculation.

Wastewater

The wastewater was the effluent from a laboratory-scale anaerobic membrane bioreactor (AnMBR) treating sewage obtained from Central WWTP in Prague before screening. The detailed operation of the AnMBR was described by Hejnic *et al.* (2016). To avoid increasing sulfide and methane concentrations during the test, the wastewater was flushed with air for 2–20 min depending on the sulfide concentration in the AnMBR effluent.

The most important characteristics of the wastewater were: 52 ± 11 mg L^{-1} of TAN, 40 ± 12 mg L^{-1} of soluble COD, and 7 ± 2 mg L^{-1} of phosphate phosphorus. Average influent alkalinity was 430 mg L^{-1} of $CaCO_3$. In comparison, wastewater after pre-treatment in AnMBR typically contains: 36 ± 17 mg L^{-1} of ammonium, 99 ± 46 mg L^{-1} of soluble COD (excluding sulfide), 6 ± 7 mg L^{-1} of phosphate, 31 ± 42 mg L^{-1} of sulfide and 23 ± 13 mg L^{-1} of dissolved methane (Delgado Vela *et al.* 2015).

Tests of sulfide inhibition

The batch assays with sulfide were performed in fully mixed vessels with an effective volume of 0.7 L. The temperature was kept between 15.0 ± 0.5 °C. First, 550 mL of wastewater were poured into the vessel. Simultaneously, hydrate of Na_2S (38% of H_2O) was grinded to fine particles to accelerate its dissolution. After the temperature of wastewater fell below 15 °C, settled sludge and Na_2S were introduced to the reactor in immediate succession (resulting sulfide concentrations of 13–178 mg-S L^{-1}). After the addition of sulfide, pH value increased up to 9.5. Then, pH value was manually reduced to 7.9–8.0 in less than 20 s using HCl (1M). For up to 1 h, pH value was steadily increasing, so 2–4 times, we manually reduced it back to 7.9–8.0. During the test, dissolved oxygen concentration was kept at 3–9 mg L^{-1}. The sludge volatile suspended solids (VSS) concentrations were 1.32 ± 0.17 and 0.77 ± 0.15 g L^{-1} for mixed nitrifying sludge and AOB enrichment, respectively. The sludge concentration of mixed nitrifying culture including additional experiments was 1.29 ± 0.40 g L^{-1}. The development of sulfide concentration during batch assays was determined separately from assessing changes in microbial activity with the same set-up.

Tests of methane inhibition

Before the actual batch assay with methane, a control experiment was performed by assessing the development of nitrogen species in the aerobic phase of the SBR cycle using regular AnMBR effluent rid of methane. Subsequently, AnMBR effluent was saturated for 1 h by gaseous methane in 0.7 L hermetically closed vessel with several needle inlets for methane gas and one outlet. After obtaining the sample for measuring the exact methane concentration (10 mg L^{-1}), 0.3 L of saturated liquid was carefully poured into the reactor vessel instead of regular influent. Then, the development of nitrogen species and methanol with methane was assessed. The biomass concentration during tests was 0.46–0.49 g L^{-1} of VSS.

Assessment of inhibition

The activity of AOB and NOB was determined as the removal rate of total ammonium and production rate of nitrate, respectively. Because, in control experiments with mixed nitrifying sludge, NOB were limited by low concentration of nitrite, actual inhibition of NOB by sulfide was likely more efficient. The rates were described by Michaelis-Menten kinetics extended with non-competitive inhibition model (Equation (1)). In this equation, r_S is the specific substrate uptake rate (mg-N g-VSS^{-1} d^{-1}), where S refers to a specific substrate (mg-N gVSS^{-1} d^{-1}), $r_{S,max}$ is the maximum specific substrate uptake rate, [S] the substrate concentration (mg L^{-1}) and K_S is the affinity constant. The K_S of AOB enrichment used in this study was 14 mg-N L^{-1}. The detailed method for the determination of the K_S of AOB enrichment is described in the supplemental information (available with the online version of this paper). In mixed nitrifying culture, the K_S of AOB (*Nitrosomonas oligotropha*) and NOB (*Nitrospira*) of 0.48 mg-N L^{-1} and 0.9 mg-N L^{-1} were adopted from Park & Noguera (2007) and Blackburne *et al.* (2007), respectively.

As described further, the presence of sulfide reduced the $r_{S,max}$ significantly. Therefore, the Monod equation was extended with non-competitive inhibition model based on Monod kinetics (Equation (1)), where K_I is the inhibition constant (sulfide in this case), [i] is the concentration of inhibitor, X is the concentration of biomass, and r'_{max} is the modified maximum substrate uptake rate. Relative activity was obtained as the ratio between the specific activity in the control and in the sample exposed to sulfide (Equation (2)). When fitting the experimental data with Equation (2), concentration of substrates (ammonia and nitrite) was considered non-limiting, i.e. $[S] >> K_S$. These relative activities were used for the approximation of K_I using the least square method. The results of methane inhibition tests were evaluated identically.

$$r_S = \frac{d[S]}{dt}\frac{1}{X} = r_{S,max}\ \frac{[S]}{[S]+K_S}\ \frac{K_i}{K_i+[i]} \tag{1}$$

$$\frac{r_S}{r_{S,max}} = \frac{K_i}{K_i+[i]} \tag{2}$$

Analytical methods

The regular sampling of batch vessels was carried out to determine TAN as the sum of N-NH_3 and N-NH_4^+, N-NO_3^-, N-NO_2^- and sulfide. The sampling of suspended solids and VSS was performed once during the test. All spectrophotometric and gravimetric analyses were performed according to APHA (2005). Temperature and the value of pH was analyzed by SENTRON SI400 probe. DO was determined by the HAMILTON VisiFerm DO 120 probe connected to a real-time controller Compact RIO run by LabVIEW software (National Instruments, US). CH_4 was determined by conventional salting out method according to Daelman *et al.* (2012) using a gas chromatograph (GC) with thermal conductivity detector (TCD).

Free ammonia concentration was calculated using the Equation (3) by Ford *et al.* (1980) c_{FA} refers to the concentration of free ammonia, and c_{TAN} refers to concentration of N-NH_4^+ and N-NH_3, $^\circ C$ refers to the temperature.

$$c_{FA} = \frac{17}{14}\cdot\frac{c_{TAN}\cdot 10^{pH}}{exp(6334/(273+^\circ C))+10^{pH}} \tag{3}$$

Fluorescence *in situ* hybridization

To characterize the microbial cultures used in this study, fluorescence *in situ* hybridization (FISH) was used. Fixation and hybridization was done according to Nielsen *et al.* (2009). The signal of the probes was assessed semi-quantitatively, which means it was divided into three categories: '+' states for positive signal of loose cells or up to several clusters per sample analyzed (in one well on a slide, two wells assessed in parallel), '++' states for larger clusters appearing throughout the well on a slide and '+++' states for large compact clusters representing dominant biomass. The samples were examined using epifluorescence microscope Olympus BX51 under 400× magnification. All probes used in this study, together with formamide concentration and the results for AOB enrichments and mixed nitrifying culture, are summarized in Table 1. Sequences of the probes and their competitors are listed in probeBase (Greuter *et al.* 2015) except for Ntoga122 (Lücker *et al.* 2015).

FISH analysis of mixed nitrifying culture

FISH of mixed nitrifying sludge identified among AOB mainly *Nitrosomonas oligotropha* lineage (probe Cluster6a192) and some halophilic and halotolerant *Nitrosomonas* (probe NEU). As for NOB, only *Nitrospira*-like organisms were present (probe mix of Ntspa662 + Ntspa712). Furthermore, some filamentous bacteria belonging to Chloroflexi and genus *Paracoccus* were detected (Table 1).

Table 1 | FISH results of probes specific for ammonium, nitrite- and sulfur-oxidizing bacteria (Greuter *et al.* 2015; Lücker *et al.* 2015)

Probe	Specificity	FA [%]	Functional group	Mixed nitrifying culture	AOB enrichment S	AOB enrichment M
Nso190 + Nso1225	Betaproteobacterial AOB	35	AOB	++	+++	+++
NEU	Most halophilic and halotolerant *Nitrosomonas* spp.	35		+	+++	+++
Cluster6a192	*Nitrosomonas oligotropha* lineage	35		++	–	+
Ntspa662 + Ntspa712	Phylum Nitrospirae	35	NOB	++	–	–
NIT3	*Nitrobacter* spp.	40		–	–	+
Ntoga122	Genus *Nitrotoga*	40		–	–	+
TBD121	*Thiobacillus denitrificans* and *T. thioparus*	20	SOB	–	–	nd
TBD1419	*Thiobacillus denitrificans*	50		–	–	nd
TMD131	*Thiomicrospira denitrificans*	35		–	–	nd
PAR651	Genus *Paracoccus*	40	potential SOB	++	–	nd
CFX1223 + GNSB-941	Phylum Chloroflexi	35		++	+	nd

AOB enrichment 'S' and 'M' indicates inhibition tests of sulfide and methane. some members of phylum Chloroflexi and genus *Paracoccus* are able to oxidize sulfur. The signal of the probes was assessed semi-quantitatively, where '+' states for positive signal of loose cells or up to several clusters per sample analyzed (one well on a slide), '++' states for larger clusters appearing throughout the well on a slide and '+++' states for large compact clusters representing dominant biomass. S = sulfide inhibition tests, M = methane inhibition tests.

FISH analysis of AOB enrichment for sulfide inhibition

According to FISH analysis, the biomass contained AOB, specifically halophilic and halotolerant *Nitrosomonas* (probes NEU and mix of Nso190 + Nso1225). NOB were not identified (probes NIT3, Ntspa662 + Ntspa712 and Ntoga122). In addition to nitrifying bacteria, few filaments belonging to Chloroflexi were detected (Table 1).

FISH analysis of AOB enrichment for methane inhibition

The biomass used for methane inhibition was dominated by AOB, specifically large and compact clusters of halophilic and halotolerant *Nitrosomonas* (probes NEU and Nso190 + Nso1225) and very small amount of oligotrophic *Nitrosomonas* (probe Cluster6a). Considering NOB, only a few small clusters of *Nitrobacter* and *Nitrotoga* were identified (probes NIT3, Ntoga122). The probes targeting sulfur oxidizing bacteria (SOB) were not used for this biomass (Table 1).

RESULTS AND DISCUSSION

Sulfide inhibition assays

The inhibition effect of sulfide on AOB and NOB was assessed in the context of a PN reactor treating sewage after anaerobic pre-treatment. As reviewed by Delgado Vela *et al.* (2015), anaerobically pre-treated sewage typically contains 2–92 mg-S L^{-1} (average 31 mg-S L^{-1}). This range was expanded to account for peak sulfide concentrations up to 178 mg-S L^{-1}. Generally, the activity of AOB and NOB correlated inversely with the concentration of sulfide (Figure 1).

Mixed nitrifying culture

To simulate the start-up of PN or the proliferation of heterotrophs and NOB, first set of experiments used mixed nitrifying culture from a sewage treatment plant (Figure 2(a) and 2(b)). Using a single sample of mixed nitrifying culture, exposure to sulfide to 22–178 mg-S L^{-1} reduced the activity of AOB to 60–70% of the activity of the control. The resulting inhibition of AOB by sulfide was characterized by a $K_{i,AOB1}$ of 150 mg-S L^{-1}. The activity of NOB was reduced to 0–28%, and respective $K_{i,NOB}$ was 10 mg-S L^{-1}.

Additional assays used mixed nitrifying culture obtained from the WWTP over the course of 8 months (Figure S2, available with the online version of this paper). There, AOB activity was reduced to 60% (average) in the whole interval of sulfide concentrations (13–178 mg-S L^{-1}). NOB activity was reduced to 5–27% (average) with the resulting $K_{i,NOB}$ of 10 mg-S L^{-1}, which is identical to the assays using single sample of sludge.

The development of nitrogen species during batch assays is shown in Figure 3. In the assay with 156 mg-S L^{-1}, the total nitrogen concentration sharply decreased

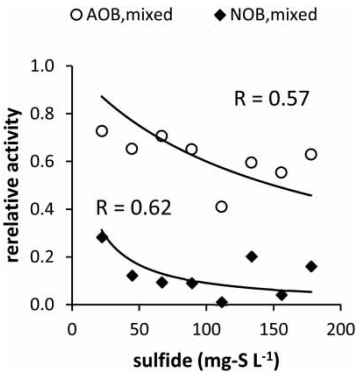

Figure 1 | Relative activity ($r_S/r_{S,max}$) of AOB and NOB in the mixed nitrifying culture at sulfide concentrations 22–178 mg-S L^{-1}. The experimental data were fitted with Equation (2) using the least square method ($K_{i,AOB1} = 150$ mg-S L^{-1}, $K_{i,NOB} = 10$ mg-S L^{-1}, $r_{AOB} = 0.57$, $r_{NOB} = 0.62$).

during the first 30 min, which we attribute to the stripping of free ammonia (FA). The reason is that the reduction of total nitrogen correlates with the reduction of TAN, at a time when pH value was quickly reduced from 9.5 to 8.0. In these conditions, FA constituted 3–57% of TAN (Ford *et al.* 1980) with FA concentration of 2–44 mg L^{-1}. This explains the losses of nitrogen at the beginning of the assay. Lower concentrations of sulfide induced less significant fluctuations of pH which reduced the losses of nitrogen. The median initial concentration of nitrite nitrogen was 1.1 mg L^{-1}, but this increased immediately as a result of the activity of AOB.

AOB enrichment

The impact of sulfide on AOB in a PN reactor (Figure 4) was assessed using AOB enrichment (Figure 2(c)). Using sulfide concentrations 13–134 mg-S L^{-1}, the inhibition of AOB was characterized by a $K_{i,AOB2}$ of 130 mg-S L^{-1}. Contrary to the assays with mixed nitrifying culture, we noticed a lag phase before the oxidation of ammonium to nitrite started (0.8–1.5 h for 36–134 mg-S L^{-1}, Figure 5). The duration of the lag phase seems to partially correspond with the time necessary to remove the sulfide from the supernatant (Figure 5) which was attributed to chemical oxidation and/or stripping (Figure S3, available with the online version of this paper). The reason is that the removal rates of sulfide from both AOB enrichment and mixed nitrifying culture corresponded to the time necessary to remove sulfide from raw wastewater by aeration without the presence of bacteria (Figure S3). In the first 60 min of the assays (Figure 5), total nitrogen was reduced along with the TAN. Similar to the assays with the mixed nitrifying culture, this reduction of total nitrogen can be explained by the stripping of FA.

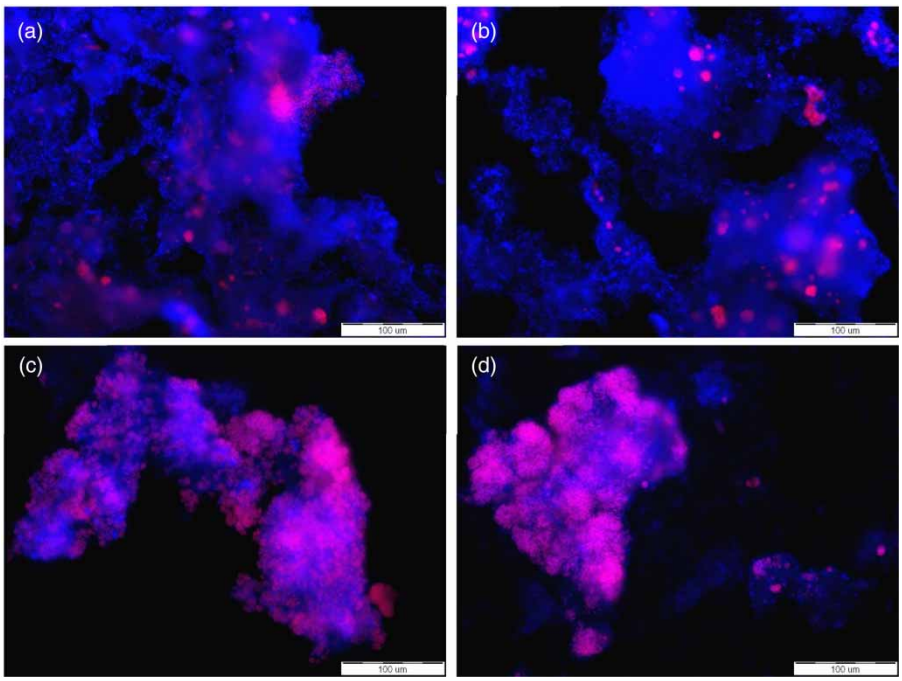

Figure 2 | FISH signal of Cy3-labeled probes (pink) and DAPI stain (blue). (a) Mixed nitrifying culture, probes Nso190 + Nso1225. (b) Mixed nitrifying culture, probes Ntspa662 + Ntspa712. (c) AOB enrichment for sulfide inhibition tests, probes Nso190 + Nso1225. (d) AOB enrichment for methane inhibition tests, probes Nso190 + Nso1225. The magnification of 400× was used for all the pictures. Please refer to the online version of this paper to see this figure in colour: http://dx.doi.org/10.2166/wst.2017.490.

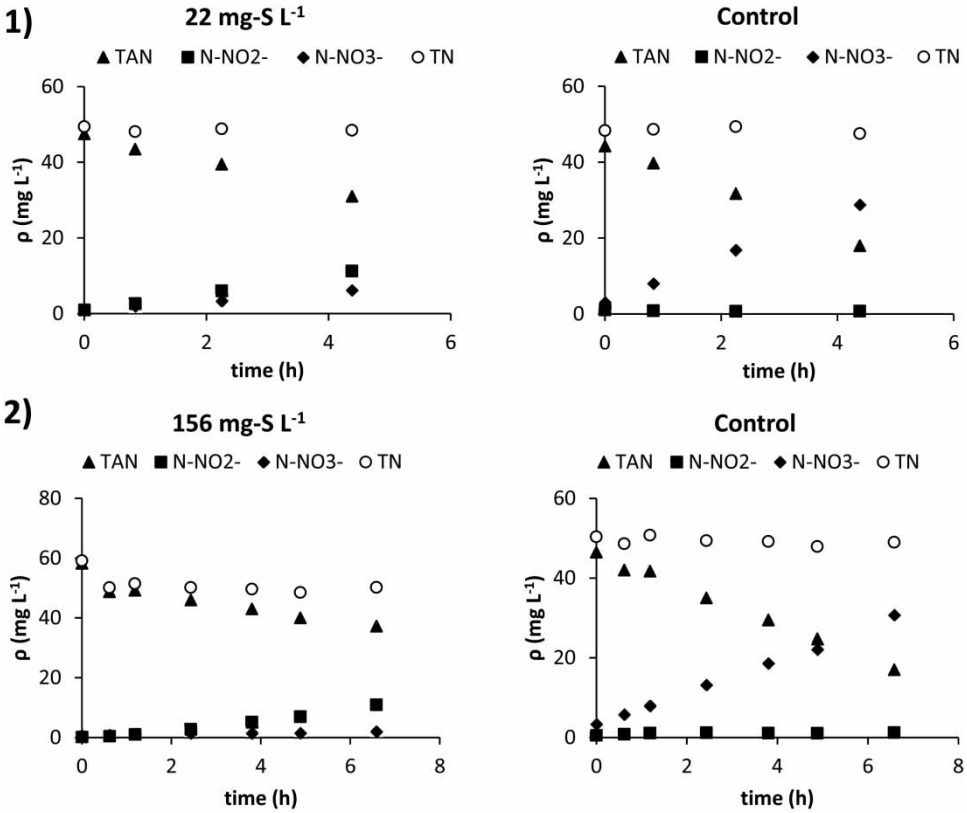

Figure 3 | The development of nitrogen species in batch assays using mixed nitrifying culture exposed to sulfide concentrations of 45 and 156 mg-S L^{-1} (1) and (2) and respective control experiments without sulfide.

The tests for Figures 1 and 4 were done with one biomass sample and biomass from consistently operated cultivation reactor, respectively. This explains consistent inhibition pattern. In contrast, Figure S2 combines several samples of nitrifying sludge from WWTP over a period of 8 months. Over this period, biomass characteristics such as AOB species and/or adsorbed Fe content could change, thus affecting the inhibition.

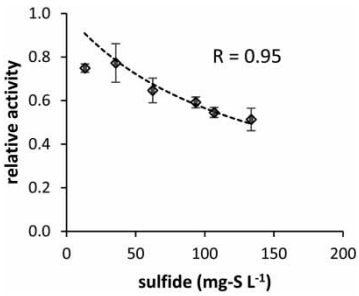

Figure 4 | Relative activity ($r_S/r_{S,max}$) of AOB enrichment (AOB2) exposed to sulfide concentrations of 13–134 mg-S L^{-1}. The experimental data were fitted with Equation (2) using the least square method ($K_{i,AOB2}$=130 mg-S L^{-1}, r_{AOB} =0.95).

The impact of sulfide inhibition on PN

Our results show that sulfide concentrations typical for anaerobically pre-treated sewage selectively inhibit NOB. Specifically, the average sulfide concentration in AnMBR effluents as shown by Delgado Vela *et al.* (2015) (31 mg-S L^{-1}) reduced the activity of NOB and AOB to 20% and 70%, respectively. In comparison to other studies (Erguder *et al.* 2008; Beristain-Cardoso *et al.* 2010; Bejarano-Ortiz *et al.* 2015), we report significantly less efficient inhibition of both AOB and NOB. Mixed nitrifying culture in our study was characterized by a $K_{i,AOB1}$ of 150 mg-S L^{-1} and a $K_{i,NOB}$ of 10 mg-S L^{-1} and AOB enrichment had $K_{i,AOB2}$ 130 mg-S L^{-1}, while Beristain-Cardoso *et al.* (2010) reported $IC_{50,AOB}$ of 13 mg-S L^{-1}. Bejarano-Ortiz *et al.* (2015) showed even lower $K_{i,AOB}$ of 2.5 mg-S L^{-1} and mixed NOB inhibition with $K_{i,NOB}$ of 0.21 mg-S L^{-1} (competitive inhibition) and 1.0 mg-S L^{-1} (non-competitive inhibition). We propose that the difference between our results and these literature data was mainly caused by higher initial pH used in this study. Bejarano-Ortiz *et al.* (2013) also reports more efficient sulfide inhibition ($K_{i,AOB}$ of

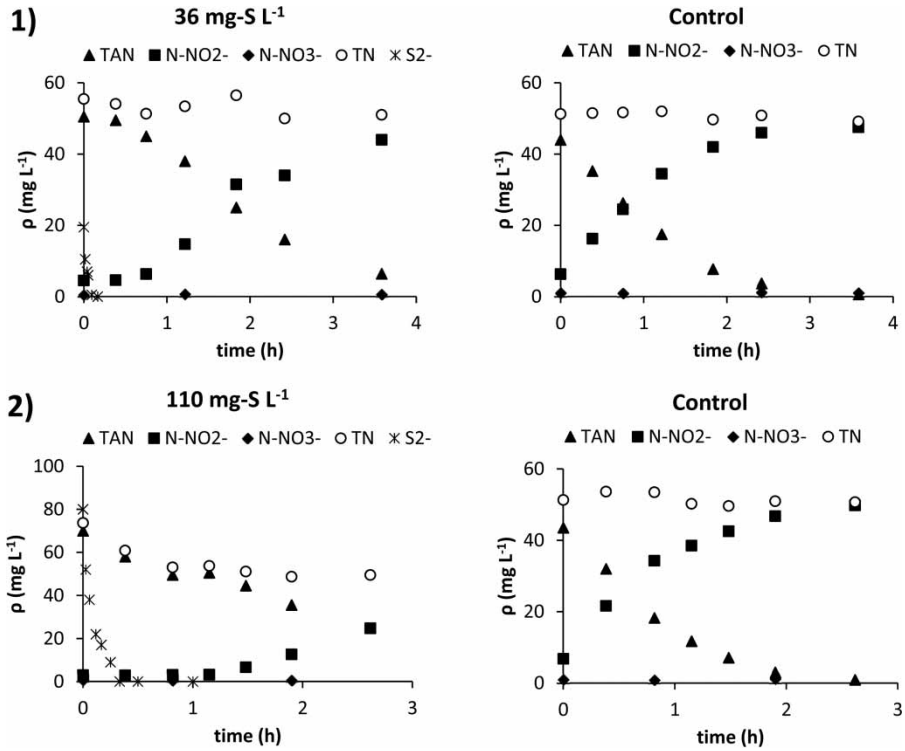

Figure 5 | The development of nitrogen species and sulfide in batch assays using AOB enrichment exposed to sulfide concentrations of 36 and 134 mg-S L^{-1} and respective control experiments without sulfide.

2.5 mg-S L^{-1} and $K_{i,NOB}$ of 1.2 mg-S L^{-1}). But, the actual initial pH following the addition of sulfide was 8.8 ± 0.3. Such high pH can inhibit both AOB and NOB, with more severe effect on NOB. In the literature, pH of 9 reduced the activity of AOB and NOB to 60% and 20%, respectively (Grunditz & Dalhammar 2001). In addition, Bejarano-Ortiz *et al.* (2013) used high temperature (30 °C), which alters H_2S gas solubility and dissociation and makes the conditions irrelevant to anaerobically pre-treated sewage (10–20 °C).

Focusing on anaerobically pre-treated sewage, we observed the maximum value of pH 7.9–8.0, while Bejarano-Ortiz *et al.* (2015) and Beristain-Cardoso *et al.* (2010) used initial pH values 7.5 ± 0.2 and 7.3 ± 0.3, respectively. Higher pH in this study shifted the dissociation equilibria of $H_2S/HS^-/S^{2-}$ from H_2S towards HS^-. In the literature, sulfide inhibition is often associated with non-dissociated H_2S, because H_2S can diffuse through cell membranes (Tursman & Cork 1989). H_2S subsequently denaturizes native proteins through the formation of sulfide and disulfide cross-links between polypeptide chains (Conn *et al.* 1987) and interferes with various coenzyme sulfide linkages (Vogels *et al.* 1988). Thus, higher initial pH induced less efficient inhibition. In the case that the AnMBR effluent will have lower pH, sulfide will induce a stronger inhibitory effect.

Higher pH also leads to higher FA concentrations. Directly after the addition of sulfide, pH increased to 9.5 for a few seconds, which induced FA concentrations up to 44 mg L^{-1} (77 mg L^{-1} of TAN, 15 °C). But after less than 20 s, pH was reduced to 7.9–8.0 manually with 1 M solution of HCl, which minimized the possible inhibition of AOB and NOB by FA. In this study (pH 8, 77 mg L^{-1} of TAN, 15 °C), the maximum FA was 2.6 mg L^{-1}. Although the original study by Anthonisen *et al.* (1976) reported inhibition threshold for NOB of 0.08–0.8 mg-N-NH_3 L^{-1}, subsequent studies reported diverse results. For instance, Simm *et al.* (2006) determined that *Nitrospira* (the only NOB detected in this study), both in mixed and pure cultures, was not inhibited by up to 10 mg L^{-1} of FA. Also for *Nitrospira*, Blackburne *et al.* (2007) determined a much lower inhibitory threshold of 0.04–0.08 mg-N-NH_3 L^{-1}. This difference can possibly be attributed to the adaptation of NOB culture or strain variability.

Comparing sulfide toxicity to AOB in the enrichment and mixed nitrifying culture

The $K_{I,AOB}$ for AOB enrichment after lag phase and mixed nitrifying culture was 130 and 150 mg-S L^{-1}, respectively.

Although this difference between $K_{I,AOB}$ is minor, the existence of the lag phase in the activity of AOB enrichment after the exposure to sulfide suggests that mixed nitrifying culture was less sensitive to sulfide than the AOB enrichment.

The greater sensitivity of the enrichment as compared to mixed culture may be due to the different composition of the AOB community. AOB in the enrichment were halotolerant and halophilic *Nitrosomonas*, while AOB in the mixed nitrifying culture were mainly of *Nitrosomonas oligotropha* lineage.

FISH analysis of mixed nitrifying culture identified the presence of a few filaments of phylum Chloroflexi and genus *Paracoccus*. Some of the members of these groups are SOB (Friedrich *et al.* 2005; Ghosh & Dam 2009). As compared to AOB enrichment, sulfide removal rate in mixed nitrifying culture containing suspected SOB was lower (Figure S4, available with the online version of this paper). Therefore, in mixed nitrifying culture, the presence of suspected SOB did not result in less efficient exposure of AOB to sulfide.

Implications for full-scale PN reactors

Future main-stream PN/A systems are likely to be operated in two separate stages in order to achieve higher total nitrogen removal rates (Isanta *et al.* 2015). These higher nitrogen removal rates cannot be achieved in single-stage PN/A, as AOB activity is limited by the lack of oxygen in the single-stage reactors (Lotti *et al.* 2015; Kouba *et al.* 2016; Seuntjens *et al.* 2016). Consequently, the reactors receiving municipal wastewater after anaerobic pre-treatment with sulfide will likely contain AOB enrichment. In this study, the oxidation of ammonia by AOB enrichment did not start in the presence of sulfide in the medium. Moreover, AOB activity remained reduced even after sulfide was removed from the liquid medium. And, if AOB are exposed to sulfide in anoxic conditions, the inhibition of AOB will be even more severe (Zhou *et al.* 2014). Therefore, it is necessary to remove sulfide prior to the oxidation of ammonium by AOB enrichment.

Importantly, current PN reactors suffer from the occasional proliferation of NOB (Lackner *et al.* 2014), particularly at cold temperatures favoring NOB over AOB (Hellinga *et al.* 1998). Our results suggest that the concentrations of sulfide common for anaerobically pre-treated sewage may efficiently inhibit NOB, or at least provide additional selection pressure for the growth of AOB. This may enhance currently used strategies for PN in the main stream at WWTP as described in detail by Lotti *et al.* (2015), Isanta *et al.* (2015), Wett *et al.* (2015), Kouba *et al.* (2017), or Blackburne *et al.* (2008). For the long-term stability of PN, a research on continuous exposure of AOB and NOB to sulfide in relevant conditions needs to be conducted to assess possible adaptation mechanisms.

In all experiments, the inhibitory effect remained even after all sulfide disappeared from the solution (Figures 3 and 5). Since many systems used for PN are operated in SBR, the conditions of the batch assays in this paper are relevant for the potential full-scale installations.

In addition to inhibition of AOB and NOB, sulfide may also slow down phosphate uptake rate by polyphosphate accumulating organisms (Rubio-Rincón *et al.* 2017).

Effect of methane on ammonium oxidation

The effect of dissolved methane in anaerobically pre-treated sewage on the ammonium oxidation rate in AOB enrichment was determined at 12 °C (Figure 2(d)). As compared to the control, the influent dissolved CH_4 of 10 mg L^{-1} induced 98 ± 12% activity of AOB. In the PN reactor, methane is partially oxidized to methanol by AOB via the enzyme ammonium monooxygenase. This process may compete with ammonium oxidation (Taher & Chandran 2013). During the methane inhibition assays, the maximum methanol concentration of 10.3 mg L^{-1} was identified, which has only limited potential to inhibit AOB (Taher & Chandran 2013). In comparison, Taher & Chandran (2013) reported that 7-h exposure to methanol induced noncompetitive inhibition of ammonium monooxygenase with a $K_{I,CH3OH}$ of 41.9 mg L^{-1}. To assess the actual conversion of methane to methanol, one needs to consider also the subsequent stripping of methanol and its further oxidation via AOB and heterotrophic microorganisms. This is important when assessing the reduced emissions of methane, which is 56× as potent greenhouse gas as CO_2 over the period of 20 years (Houghton 1996). Calculating with 10.3 mg L^{-1} of methanol detected in the liquid upon the termination of the assay with 10 mg L^{-1} of dissolved CH_4 in the reactor, methane emissions were reduced by up to 52%. The concentrations of formaldehyde were below the detection limit in all assays.

In sum, the inhibition of AOB by methane in anaerobic effluent was marginal. And, the biological oxidation of methane in PN reactor can significantly reduce the emissions of dissolved methane from anaerobic effluent. A comprehensive assessment of dissolved methane emissions from reject water was performed by Pijuan *et al.* (2013) and needs to be expanded by studies on methane emissions from cold anaerobically pre-treated sewage.

CONCLUSION

To the best of our knowledge, this study is the first to show that sulfide concentrations relevant for anaerobically pretreated municipal wastewater will selectively inhibit NOB in PN reactor ($K_{i,NOB} = 10$ mg-S L^{-1}), while preserving AOB activity. This was shown with mixed nitrifying culture, where *Nitrospira* was the dominant NOB species. In batch assays under conditions relevant to anaerobic effluent, we show that sulfide may inhibit AOB in enrichment culture significantly more than in mixed nitrifying culture, as demonstrated by the lag phase in ammonium oxidation. Although the inhibitory effect for additional NOB strains and the adaptability of AOB and NOB to sulfide needs to be assessed in long term, this work will enable PN in previously challenging conditions such as extremely low temperatures. In addition, we show that methane in anaerobic effluent will be partially converted to CH_3OH (up to 51%), reducing the greenhouse gas emissions of PN/A.

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REFERENCES

Anthonisen, A. C., Loehr, R. C., Prakasam, T. B. S. & Srinath, E. G. 1976 Inhibition of nitrification by ammonia and nitrous acid. *Journal (Water Pollution Control Federation)* **48**, 835–852.

APHA 2005 *Standard Methods for the Examination of Water and Wastewater*. American Public Health Association, Washington, DC, USA.

Bejarano Ortiz, D. I., Thalasso, F., Cuervo López, F. D. M. & Texier, A. C. 2013 Inhibitory effect of sulfide on the nitrifying respiratory process. *Journal of Chemical Technology and Biotechnology* **88**, 1344–1349.

Bejarano-Ortiz, D. I., Huerta-Ochoa, S., Thalasso, F., De María Cuervo-López, F. & Texier, A.-C. 2015 Kinetic constants for biological ammonium and nitrite oxidation processes under sulfide inhibition. *Applied Biochemistry and Biotechnology* **177**, 1665–1675.

Beristain-Cardoso, R., Gómez, J. & Méndez-Pampín, R. 2010 The behavior of nitrifying sludge in presence of sulfur compounds using a floating biofilm reactor. *Bioresource Technology* **101**, 8593–8598.

Blackburne, R., Vadivelu, V. M., Yuan, Z. & Keller, J. 2007 Kinetic characterisation of an enriched *Nitrospira* culture with comparison to *Nitrobacter*. *Water Research* **41**, 3033–3042.

Blackburne, R., Yuan, Z. & Keller, J. 2008 Demonstration of nitrogen removal via nitrite in a sequencing batch reactor treating domestic wastewater. *Water Research* **42**, 2166–2176.

Cao, Y., van Loosdrecht, M. C. M. & Daigger, G. T. 2017 Mainstream partial nitritation–anammox in municipal wastewater treatment: status, bottlenecks, and further studies. *Applied Microbiology and Biotechnology* **101**, 1365–1383.

Cardinale, B. J., Srivastava, D. S., Duffy, J. E., Wright, J. P., Downing, A. L., Sankaran, M. & Jouseau, C. 2006 Effects of biodiversity on the functioning of trophic groups and ecosystems. *Nature* **443**, 989–992.

Chain, P., Lamerdin, J., Larimer, F., Regala, W., Lao, V., Land, M., Hauser, L., Hooper, A., Klotz, M. & Norton, J. 2003 Complete genome sequence of the ammonia-oxidizing bacterium and obligate chemolithoautotroph *Nitrosomonas europaea*. *Journal of Bacteriology* **185**, 2759–2773.

Conn, E. E., Stumpf, P. K., Bruening, G. & Doi, R. H. 1987 *Outlines of Biochemistry*. John Wiley & Sons, Hoboken, NJ, USA.

Daelman, M. R., Van Voorthuizen, E. M., Van Dongen, U. G., Volcke, E. I. & Van Loosdrecht, M. C. 2012 Methane emission during municipal wastewater treatment. *Water Research* **46**, 3657–3670.

Delgado Vela, J., Stadler, L. B., Martin, K. J., Raskin, L., Bott, C. B. & Love, N. G. 2015 Prospects for biological nitrogen removal from anaerobic effluents during mainstream wastewater treatment. *Environmental Science & Technology Letters* **2**, 234–244.

Erguder, T. H., Boon, N., Vlaeminck, S. E. & Verstraete, W. 2008 Partial nitrification achieved by pulse sulfide doses in a sequential batch reactor. *Environmental Science & Technology* **42**, 8715–8720.

Ford, D. L., Churchwell, R. L. & Kachtick, J. W. 1980 Comprehensive analysis of nitrification of chemical-processing wastewaters. *Journal of the Water Pollution Control Federation* **52**, 2726–2746.

Friedrich, C. G., Bardischewsky, F., Rother, D., Quentmeier, A. & Fischer, J. 2005 Prokaryotic sulfur oxidation. *Current Opinion in Microbiology* **8**, 253–259.

Ghosh, W. & Dam, B. 2009 Biochemistry and molecular biology of lithotrophic sulfur oxidation by taxonomically and ecologically diverse bacteria and archaea. *FEMS Microbiology Reviews* **33**, 999–1043.

Greuter, D., Loy, A., Horn, M. & Rattei, T. 2015 Probebase – an online resource for rRNA-targeted oligonucleotide probes and primers: new features 2016. *Nucleic Acids Research* **44** (D1).

Grunditz, C. & Dalhammar, G. 2001 Development of nitrification inhibition assays using pure cultures of *Nitrosomonas* and *Nitrobacter*. *Water Research* **35**, 433–440.

Hejnic, J., Dolejs, P., Kouba, V., Prudilova, A., Widiayuningrum, P. & Bartacek, J. 2016 Anaerobic treatment of wastewater in colder climates using UASB reactor and anaerobic membrane bioreactor. *Environmental Engineering Science* **33** (11).

Hellinga, C., Schellen, A. A. J. C., Mulder, J. W., Van Loosdrecht, M. C. M. & Heijnen, J. J. 1998 The SHARON process: an innovative method for nitrogen removal from ammonium-rich waste water. *Water Science & Technology* **37**, 135–142.

Hockenbury, M. R. & Grady Jr, C. P. L. 1977 Inhibition of nitrification: effects of selected organic compounds. *Journal of the Water Pollution Control Federation* **49**, 768–777.

Houghton, J. T. 1996 *The Science of Climate Change: Summary for Policymakers and Technical Summary of the Working Group I Report; Part of the Working Group I Contribution to the Second Assessment Report of the Intergovernmental Panel on Climate Change*, Cambridge University Press, Cambridge, UK.

Isanta, E., Reino, C., Carrera, J. & Pérez, J. 2015 Stable partial nitritation for low-strength wastewater at low temperature in an aerobic granular reactor. *Water Research* **80**, 149–158.

Juliette, L. Y., Hyman, M. R. & Arp, D. J. 1993 Inhibition of ammonia oxidation in *Nitrosomonas europaea* by sulfur compounds: thioethers are oxidized to sulfoxides by ammonia monooxygenase. *Applied and Environmental Microbiology* **59**, 3718–3727.

Kartal, B., Kuenen, J. G. & Van Loosdrecht, M. C. M. 2010 Sewage treatment with anammox. *Science* **328**, 702–703.

Kouba, V., Widiayuningrum, P., Chovancova, L., Jenicek, P. & Bartacek, J. 2016 Applicability of one-stage partial nitritation and anammox in MBBR for anaerobically pre-treated municipal wastewater. *Journal of Industrial Microbiology & Biotechnology* **43**, 965–975.

Kouba, V., Vejmelková, D., Proksova, E., Wiesinger, H., Concha, M., Dolejs, P., Dolejs, P., Hejnic, J., Jenicek, P. & Bartacek, J. 2017 High-rate partial nitritation of municipal wastewater after psychrophilic anaerobic pre-treatment. *Environmental Science & Technology* **51** (19), 11029–11038. doi 10.1021/acs.est.7b02078.

Lackner, S., Gilbert, E. M., Vlaeminck, S. E., Joss, A., Horn, H. & Van Loosdrecht, M. C. M. 2014 Full-scale partial nitritation/anammox experiences – an application survey. *Water Research* **55**, 292–303.

Laureni, M., Falås, P., Robin, O., Wick, A., Weissbrodt, D. G., Nielsen, J. L., Ternes, T. A., Morgenroth, E. & Joss, A. 2016 Mainstream partial nitritation and anammox: long-term process stability and effluent quality at low temperatures. *Water Research* **101**, 628–639.

Lotti, T., Kleerebezem, R., Hu, Z., Kartal, B., De Kreuk, M. K., Van Erp Taalman Kip, C., Kruit, J., Hendrickx, T. L. G. & Van Loosdrecht, M. C. M. 2015 Pilot-scale evaluation of anammox-based mainstream nitrogen removal from municipal wastewater. *Environmental Technology* **36**, 1167–1177.

Lücker, S., Schwarz, J., Gruber-Dorninger, C., Spieck, E., Wagner, M. & Daims, H. 2015 *Nitrotoga*-like bacteria are previously unrecognized key nitrite oxidizers in full-scale wastewater treatment plants. *The ISME Journal* **9**, 708–720.

Mccarty, G. 1999 Modes of action of nitrification inhibitors. *Biology and Fertility of Soils* **29**, 1–9.

Nielsen, P. H., Daims, H. & Lemmer, H. 2009 *FISH Handbook for Biological Wastewater Treatment: Identification and Quantification of Microorganisms in Activated Sludge and Biofilms by FISH*. IWA Publishing, London, UK.

Park, H. D. & Noguera, D. R. 2007 Characterization of two ammonia-oxidizing bacteria isolated from reactors operated with low dissolved oxygen concentrations. *Journal of Applied Microbiology* **102**, 1401–1417.

Pijuan, M., Torà, J., Rodríguez-Caballero, A., César, E., Carrera, J. & Pérez, J. 2013 Effect of process parameters and operational mode on nitrous oxide emissions from a nitritation reactor treating reject wastewater. *Water Research* **49**, 23–33.

Rubio-Rincón, F. J., Lopez-Vazquez, C. M., Welles, L., Van Loosdrecht, M. C. M. & Brdjanovic, D. 2017 Sulphide effects on the physiology of *Candidatus* Accumulibacter phosphatis type I. *Applied Microbiology and Biotechnology* **101**, 1661–1672.

Seuntjens, D., Bundervoet, B., Mollen, H., De Mulder, C., Wypkema, E., Verliefde, A., Nopens, I., Colsen, J. & Vlaeminck, S. 2016 Energy efficient treatment of A-stage effluent: pilot-scale experiences with shortcut nitrogen removal. *Water Science & Technology* **73**, 2150–2158.

Simm, R., Mavinic, D. & Ramey, W. 2006 A targeted study on possible free ammonia inhibition of Nitrospira. *Journal of Environmental Engineering and Science* **5**, 365–376.

Taher, E. & Chandran, K. 2013 High-rate, high-yield production of methanol by ammonia-oxidizing bacteria. *Environmental Science & Technology* **47**, 3167–3173.

Tappe, W., Tomaschewski, C., Rittershaus, S. & Groeneweg, J. 1996 Cultivation of nitrifying bacteria in the retentostat, a simple fermenter with internal biomass retention. *FEMS Microbiology Ecology* **19**, 47–52.

Tursman, J. & Cork, D. 1989 Influence of sulfate and sulfate-reducing bacteria on anaerobic digestion technology. *Advances in Biotechnological Processes* **12**, 273–285.

Vogels, G. D., Keltjens, J. T. & Van Der Drift, C. 1988 Biochemistry of methane production. *Biology of Anaerobic Microorganisms* 707–770.

Wett, B., Podmirseg, S., Gómez-Brandón, M., Hell, M., Nyhuis, G., Bott, C. & Murthy, S. 2015 Expanding DEMON sidestream deammonification technology towards mainstream application. *Water Environment Research* **87**, 2084–2089.

Zhou, Z., Xing, C., An, Y., Hu, D., Qiao, W. & Wang, L. 2014 Inhibitory effects of sulfide on nitrifying biomass in the anaerobic–anoxic–aerobic wastewater treatment process. *Journal of Chemical Technology and Biotechnology* **89**, 214–219.

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