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Interactions between anaerobic ammonium and sulfur-oxidizing bacteria in a laboratory scale model system

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Summary

Fixed nitrogen is released by anaerobic ammonium oxidation (anammox) and/or denitrification from (marine) ecosystems. Nitrite, the terminal electron acceptor of the anammox process, occurs in nature at very low concentrations and is produced via (micro)aerobic oxidation of ammonium or nitrate reduction. The coupling of sulfide-dependent denitrification to anammox is particularly interesting because besides hydrogen, sulfide is the most important reductant at the chemocline of anoxic marine basins and is abundant within sediments. Although at μM concentrations, sulfide may be toxic and inhibiting anammox activity, a denitrifying microorganism could convert sulfide and nitrate at sufficiently high rates to allow anammox bacteria to stay active despite an influx of sulfide. To test this hypothesis, a laboratory scale model system containing a co-culture of anammox bacteria and the autotrophic denitrifier *Sulfurimonas denitrificans* DSM1251 was started. Complementary techniques revealed that the gammaproteobacterial *Sedimenticola* sp. took over the intended role of *Su. denitrificans*. A stable coculture of anammox bacteria and *Sedimenticola* sp. consumed sulfide, nitrate, ammonium and CO_2. Anammox bacteria contributed 65–75% to the nitrogen loss from the reactor. The cooperation between anammox and sulfide-dependent denitrification may play a significant role in environments where sulfur cycling is active and where actual sulfide concentrations stay below μM range.

Introduction

The suboxic regions in the oceans, such as continental shelf sediments, areas of limited circulation (i.e. fjords or basins) and oxygen minimum zones (OMZs) are major sinks for fixed nitrogen in nature (Falkowski, 1997; Gruber and Galloway, 2008). Denitrification and anaerobic ammonium oxidation (anammox) are the two main pathways that use inorganic nitrogen and release dinitrogen gas (N_2) (Lam and Kuypers, 2011). Anammox bacteria derive their energy for growth from the reduction of nitrite (NO_2^-), combining it with ammonium (NH_4^+) into N_2, thereby constituting a sink for fixed nitrogen. Their contribution to nitrogen loss from several anoxic marine systems has been estimated to be more than 50% (Dalsgaard *et al*., 2003; Kuypers *et al*., 2003; Arrigo, 2005; Lam *et al*., 2009). Ammonium and nitrite are intermediate products in several biogeochemical processes, and they are constantly and rapidly turned over at oxic/anoxic interfaces via ammonium oxidation and nitrite oxidation in the presence of O_2 (Füssel *et al*., 2012) or by denitrification and/or anammox in the absence of O_2 (Francis *et al*., 2005; Lam and Kuypers, 2011; Zehr and Kudela, 2011). This means that nitrogen-transforming microorganisms have to compete with and are dependent on each other for the supply of their substrates.

It was estimated that ammonium-oxidizing archaea (AOA) could supply substantial amounts of nitrite (33%) in the suboxic waters at the oxycline in the Peruvian OMZ (Francis *et al*., 2005; Lam *et al*., 2009). This was also tested in a laboratory-scale model system, which showed that AOA could indeed provide nitrite for anammox bacteria under oxygen limitation (Yan *et al*., 2012). An alternative source of nitrite for anammox is nitrate reduction to nitrite. The peak in anammox cell numbers and their high activity at zones of nitrate decrease already points to a co-occurrence within the same niche (Kuypers *et al*., 2005; Hamersley *et al*., 2007; Galán *et al*., 2009). The fact that nitrate is indeed a major source of nitrite for anaerobic ammonium oxidation was shown by labelling experiments in continental shelf sediments as well as in the Benguela and Chile OMZs, where massive losses of nitrogen by coupling partial denitrification to anammox were observed (Thamdrup and Dalsgaard, 2002; Kuypers *et al*., 2005;

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Canfield *et al*., 2010). Ammonium and nitrite may also be produced through dissimilatory nitrate reduction to ammonium (DNRA). Recent studies highlighted the importance of this relatively overlooked process that could contribute significantly to the cycling of nitrogen in benthic systems, at hydrothermal vent sites, but also open-waters systems (Preisler *et al*., 2007; Lam *et al*., 2009; Dong *et al*., 2011; Kamp *et al*., 2011). The potential coupling of DNRA and anammox would result in the production of double-labelled $^{15}N^{15}N$ by anammox bacteria, thereby interfering with traditional isotope-pairing experiments, in which the production of $^{30}N_2$ from ^{15}N nitrate is used as a proxy for denitrification (Kartal *et al*., 2007).

Nitrate reduction via denitrification and DNRA require the presence of a suitable electron donor, which in the case of heterotrophic denitrification is organic carbon. There are also several sulfur-oxidizing bacteria that are able to reduce nitrate using inorganic, reduced sulfur compounds (i.e. sulfide, thiosulfate, etc.) as electron donors (reviewed in Shao *et al*., 2010). This so-called autotrophic denitrification is performed by several representatives of the Alpha-, Beta-, Gamma- and Epsilonproteobacteria. Indications of the importance of this process were observed in the stratified water column of the Black Sea (Vetriani *et al*., 2003), the Baltic Sea (Labrenz *et al*., 2005; Glaubitz *et al*., 2009), the upwelling systems off the coast of Chile (Canfield *et al*., 2010) and Namibia (Lavik *et al*., 2009), where especially representatives of the Gamma- and Epsilonproteobacteria seemed to play an important role in dark carbon fixation and sulfide detoxification. The exact mechanism favouring either anammox or denitrification is still unknown. However, as autotrophic denitrification uses sulfide as electron donor and there are indications that anammox activity is inhibited by μM levels of sulfide, such an interaction is unlikely to occur in sulfidic waters (Dalsgaard *et al*., 2003; Jensen *et al*., 2008; Jin *et al*., 2013).

Nevertheless, anammox bacteria were discovered in a denitrifying fluidized bed reactor, where anaerobic ammonium removal was dependent on nitrate with sulfide and/or organic compounds being the major electron donors in the system (Mulder *et al*., 1995). This already indicated that sulfide-based partial denitrification was probably feeding anammox with nitrite. Furthermore, anammox activity was stimulated when sulfide was present accompanied with a transient accumulation of nitrite, probably also the result of partial, autotrophic denitrification (van de Graaf *et al*., 1996).

Such an interaction of autotrophic sulfide oxidizing denitrifiers and anammox bacteria could also occur in nature. Indeed, the discovery of a cryptic sulfur cycle within the OMZ of Chile in the absence of measureable concentrations of sulfide revealed the possibility for nitrate reduction to nitrite coupled to the oxidation of sulfide to supply anammox with considerable amounts of nitrite in these environments (Canfield *et al*., 2010). Because of the high turnover of sulfide to sulfate and accordingly nitrate to nitrite by an active community of autotrophic denitrifiers, the actual sulfide concentration would be negligible, and the intermediate nitrite produced could be consumed by anammox.

In this study, we investigated whether anammox activity could be linked to autotrophic denitrification. This hypothesis was tested under laboratory conditions by using a co-culture of anammox bacteria and the known autotrophic denitrifier *Sulfurimonas denitrificans* DSM 1251 (Timmer-Ten Hoor, 1975) that was supplied with only nitrate and ammonium, but not nitrite under a continuous influx of sodium sulfide. We hypothesized that denitrification would convert sulfide and nitrate, thereby allowing anammox to stay active despite an influx of sulfide and supply them with substrate (NO_2^-) under conditions of partial denitrification.

Results and discussion

Sulfide toxicity in anammox bacteria

The sensitivity of anammox bacteria enriched from wastewater treatment plants towards short-time hydrogen sulfide exposure has been described in the literature several times concluding that the half maximal inhibitory concentration (IC50) is in the low mM range (van de Graaf *et al*., 1996; Dapena-Mora *et al*., 2007; Jin *et al*., 2013). In the environment, these critical inhibitory concentrations seem to be even lower with low μM concentrations completely inhibiting anammox activity in sediments (Jensen *et al*., 2008). This would also explain the absence of anammox from sulfidic waters as has been reported in several studies (Dalsgaard *et al*., 2005; Lam *et al*., 2007). We tested the nitrite-reducing capacity of the free-living planktonic anammox cells in the presence of increasing sulfide concentrations to determine the IC50 value of the culture we later on used to inoculate the reactor system. Short-term exposure (2 h) had a strong effect on anammox activity (Fig. 1) resulting in an IC50 as low as 10 (± 4) μM. This is significantly lower than previously described inhibitory concentrations of enrichment cultures. Several reasons might explain these results: Previous experiments were done with aggregated biomass from wastewater treatment plants. It is known that biofilm formation allows for a much higher resistance to toxic agents which might explain the higher maximal inhibitory concentration of granules compared with planktonic cells (Costerton *et al*., 1987). Although the exact molecular mechanism of sulfide toxicity is not resolved yet, it has been shown that H_2S reversibly inhibits cytochromes and other haem-containing proteins (reviewed in Kabil and Banerjee, 2010). Thus, the high sensitivity of anammox to

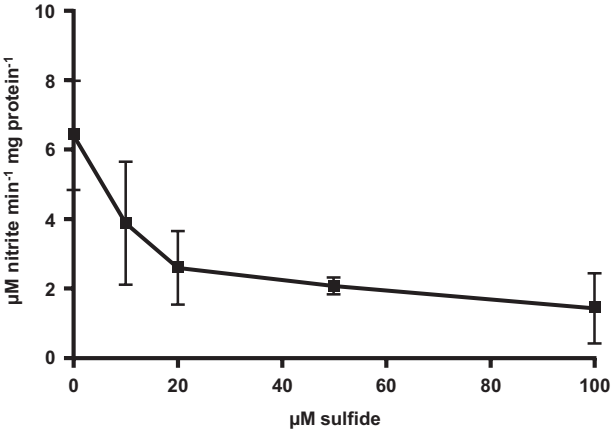

Fig. 1. Nitrite-reducing activity of anammox planktonic cells in the presence of different concentrations of Na_2S (± standard error of four replicates).

sulfide is probably due to fact that anammox bacteria harbour an exceptionally high number of haem-containing proteins (Strous *et al*., 2006; Kartal *et al*., 2011). Although it has been observed that the inhibitory effect of H_2S on other haem-containing proteins such as the mitochondrial cytochrome oxidase is reversible (Petersen, 1977; Cooper and Brown, 2008), no increase in nitrite-reducing activity could be observed when we continued the experiment for up to 6 h. This is despite the fact that sulfide could no longer be detected in incubations with the lowest original sulfide concentrations. Additionally, we performed a continuous culture experiment to study the long-term effects of sulfide exposure on our anammox culture. Sulfide was initially added at a loading rate of ~ 70 µM day^{-1} and instantly resulted in a drop in anammox activity that was evident from the accumulation of nitrite. Further, a significant drop in optical density (OD_{600}) from 0.32 to 0.18 was observed within 7 days, which pointed to cell lysis (data not shown).

Co-cultivation of anammox and sulfide oxidizers

Because of the fact that our anammox cultures were very sensitive to low sulfide concentrations, instead of stimulating the growth of sulfide oxidizers that could be present in the anammox culture, we decided to start a co-culture of *Su. denitrificans* DSM 1251 (Timmer-Ten Hoor, 1975) and anammox bacteria by introducing the anammox bacteria to a stable culture of sulfur oxidizers. *Su. denitrificans* was precultured anaerobically in an adapted anammox medium using sulfide and nitrate as substrates. The initial addition of sulfide (70 µM day^{-1}) lead to the visible formation of elemental sulfur. The supply of excess nitrate concentrations caused a transient nitrite accumulation (10–13 µM) as a result of incomplete denitrification (Fig. 2). When the culture was stably consuming all supplied sulfide [effluent (HS^-) < 2 µM) anammox biomass was added at the ratio of one part anammox and three parts *Su. denitrificans* according to OD_{600} values (day 0). As an immediate result, the nitrite concentration in the effluent decreased below the detection limit (5 µM). At day 27, the influent sulfide load was increased (267 µM day^{-1}) leading to an increase in the consumption of ammonium and nitrate, while sulfide was still below the detection limit. When nitrate became limiting (day 40), low concentrations of nitrite could be measured (~ 5 µM), possibly caused by the inhibition of anammox activity by residual sulfide (not determined) that accumulated because nitrate concentrations were insufficient to consume it all. To facilitate a stable operation, the reactor was run under sulfide limitation by adding excess nitrate. During this phase, the reactor consumed 1.27 mol NO_3^- and 0.83 mol NH_4^+ per mol of HS^-. Based on the assumption that anammox bacteria were responsible for all ammonium conversion, anammox bacteria were estimated to contribute 66 ± 2% to the nitrogen loss from the reactor system. Afterwards, the reactor was alternatively limited in nitrate or ammonium (experimental phase) to allow quantification of the activity of the two guilds of microorganisms and their relative contribution to the nitrogen fluxes with stable isotopes. Short-term nitrate limitation led to a minor nitrite accumulation (~ 6 µM) that was consumed as soon as the nitrate influent was restored. Periods of combined nitrate and ammonium starvation (day 253) caused sulfide (not determined colorimetrically) and nitrite accumulation (~ 180 µM). When the feed was adapted to the stable operation phase, the reactor gradually recovered.

Assessment of the community composition by shotgun metagenomic sequencing, fluorescence in situ *hybridization (FISH) and polymerase chain reaction (PCR)*

The initial composition of anammox biomass in the reactor consisted for about 50% of *Kuenenia stuttgartiensis* and 50% *Scalindua profunda* that had been added to the running reactor of autotrophic denitrifiers in a ratio of 1:3. To confirm the presence of *Su. dentrificans* and both anammox genera as the dominant players in the reactor, we sequenced the metagenome of the whole reactor during the stable operation phase (at day 133). Surprisingly, only 3502 of 3 847 959 reads (0.09%) could be mapped to *Su. denitrificans*, indicating that this autotrophic denitrifier was lost from the reactor even though the reactor was still oxidizing sulfide. Further analysis of the metagenome showed that a Gammaproteobacterium was dominant in the reactor, which possibly took over the role of *S. denitrificans*. Of all reads, 43.9% could be mapped to the genome of

Sedimenticola selenatireducens DSM 17993 (Narasingarao and Häggblom, 2006) with a similarity fraction of 0.9. This bacterium was originally isolated as a selenate reducer but was also able of anaerobic respiration of acetate and pyruvate with nitrate or nitrite. Using the 16S rRNA gene consensus sequence of *Sedimenticola* sp., specific probes and primers were designed to target these organisms and determine when the shift from *Su. denitrificans* to *Sedimenticola* sp. had taken place. The initial sample taken for FISH analysis after addition of the anammox biomass (day 0) could not conclusively show the presence of *Sedimenticola* sp. (Fig. 3A). Nevertheless, these microorganisms established themselves quickly and were the most abundant bacteria within the reactor system within 133 days (Fig. 3C). *Sedimenticola* sp. remained the dominant sulfide oxidizer throughout the course of the experiment accounting for more than 70% of the bacterial biomass (Fig. 3C and D). To trace the source of these *Sedimenticola* sp. and their diversity, DNA was isolated from the original DSMZ (German Collection of Microorganisms and Cell Cultures) biomass, the reactor biomass from just before anammox addition, the anammox inoculum and the co-culture after 133 days. *Sedimenticola* sp. was detected in all samples, and all sequenced clones had a similarity of > 98% with *Se. selenatireducens* on 16S rRNA gene level. The DSMZ original culture as well as our anammox inoculum did also contain *Sedimenticola*-like sequences.

To determine whether the *Sedimenticola* sp. in our reactor system had the genetic potential to perform autotrophic denitrification, which had not been described for *Se. selenatireducens*, we assembled all mapped reads into 580 contigs and analysed the assembled genome for key genes of sulfur oxidation and denitrification. The *Sedimenticola* sp. present in the reactor harboured a range of genes involved in the oxidation of reduced, inorganic sulfur compounds, which could facilitate the complete oxidation of sulfide to sulfate (Supporting Information Table S2). Sulfide can be converted to elemental sulfur (S^0) by sulfide dehydrogenase (*fccAB*). The dissimilatory sulfite reductase (*dsr*) complex allows the conversion of S^0 to sulfite, which can in turn be oxidized directly to sulfate by a sulfite oxidase or in a two-step process including adenosine 5′-phosphosulfate (APS) reductase (*apr*) oxidizing sulfite to APS and adeno-

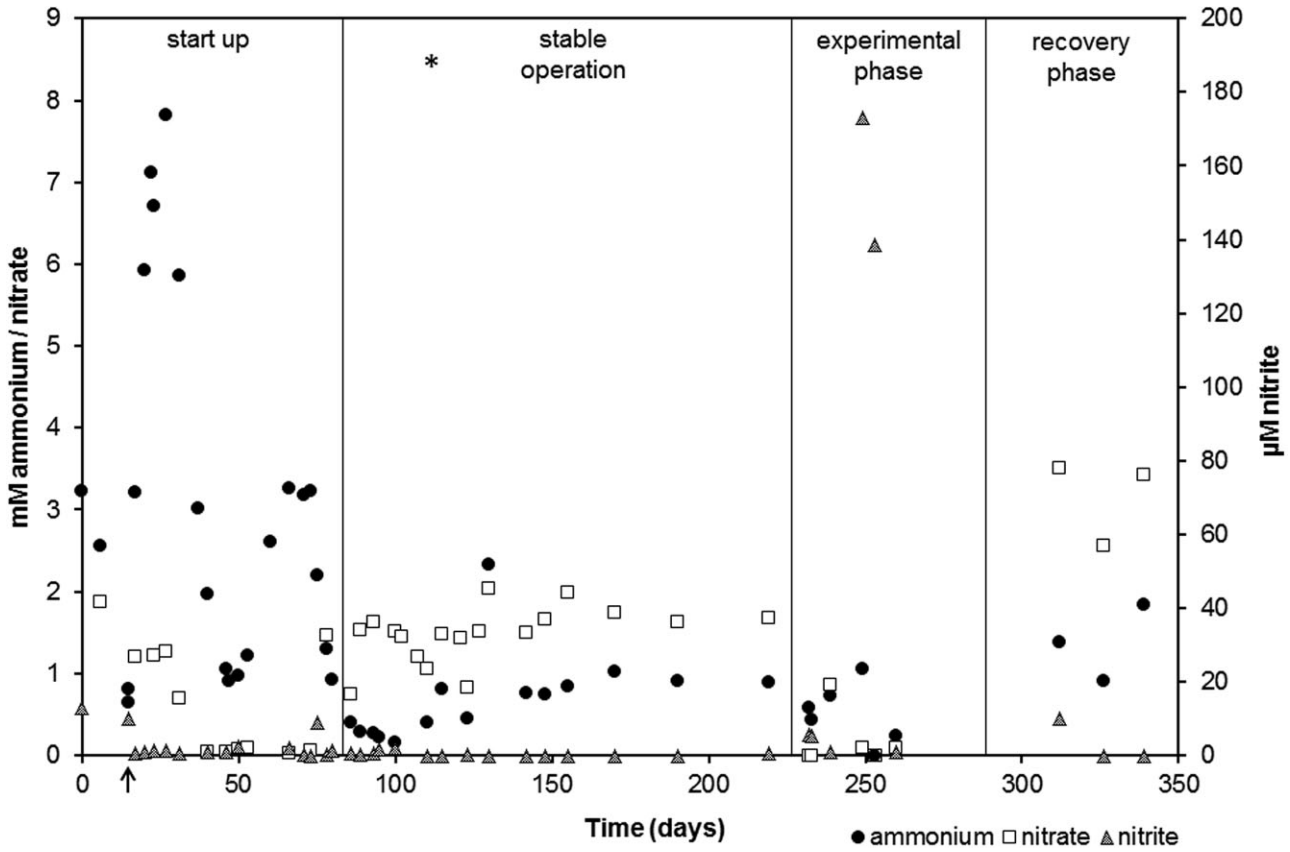

Fig. 2. Effluent concentrations of ammonium, nitrate and nitrite of the reactor system hosting the co-culture during different stages in the experiment. The arrow (↑) indicates the day of anammox addition, asterisk (*) the day of sampling for metagenomic analysis. Sporadic sulfide measurements indicated concentrations below the detection limit.

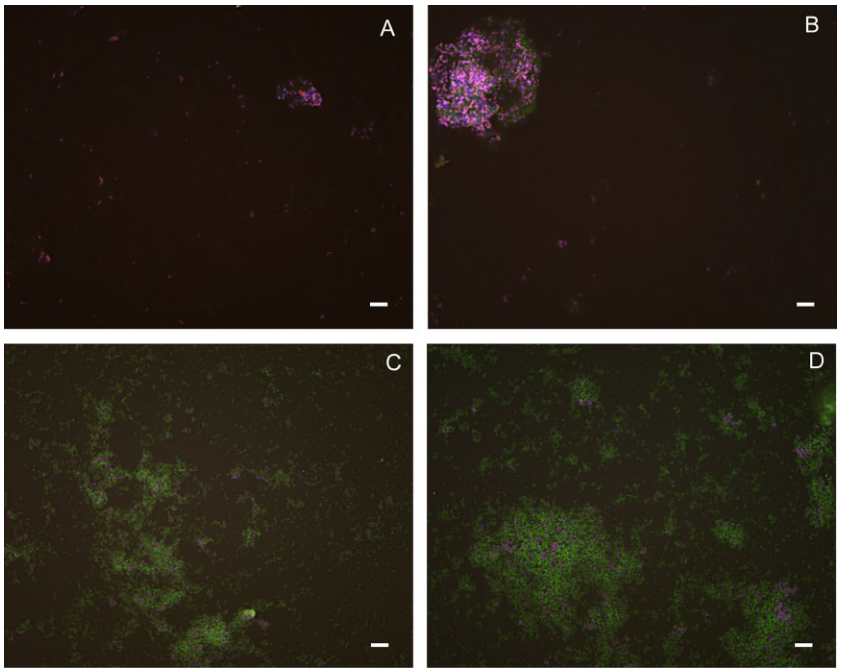

Fig. 3. FISH of the reactor system over time, using Kst1240 in Cy3, AMX820 in Cy5 and Gam781 in FLUOS. *Kuenenia stuttgartiensis* is visible in pink due to double binding of Kst1240 and AMX820, other anammox bacteria colour blue and *Sedimenticola* sp. is visible in green. A shows the day of anammox addition (day 0); B, C and D show the community after 25, 133 and 204 days respectively. The scale bar represents 5 μM.

sine 5′-triphosphate sulfurylase (*sat*) oxidizing APS to sulfate. Several other sulfur-oxidizing bacteria also harbour both sulfite-oxidizing pathways granting them a very adaptable dissimilatory sulfur metabolism (Kappler and Dahl, 2001). The genome of the *Sedimenticola* strain also encoded the Sox pathway (*soxABXYZ*) for the oxidation of thiosulfate that might be an important intermediate in areas with a low sulfide to oxygen ratio or in sediments due to the oxidation of free and iron-bound sulfides (Chen and Morris, 1972; Moses *et al*., 1987; Schippers and Sand, 1999). Furthermore, genes encoding for an almost complete denitrification pathway [a membrane-bound (*nar*) and a periplasmic (*nap*) nitrate reductase, a cytochrome cd_1 nitrite reductase (*nirS*) and nitrous oxide reductase (*nosZ*)] were also detected in the genome assembly of *Sedimenticola* sp. (Supporting Information Table S2). The catalytic subunit of the nitric oxide reductase (*norB*) could not be retrieved from the metagenome, but several nitric oxide reductase activation proteins (*norDEQ*) were found. That and the fact that *Se. selenatireducens* DSM 17993 seems to encode even two copies of *norB* lead to the assumption that the gene must also be present in our strain. For almost all key functional genes, the similarity with the type strain on nucleotide level was more than 95% (Supporting Information Table S2).

The exact reasons causing the disappearance of *Su. denitrificans* remain elusive, but there seems to be a physiological difference between gamma- and epsilonproteobacterial sulfur oxidizers. *Sulfurimonas denitrificans* has been described to perform the complete reduction of nitrate to dinitrogen gas (Timmer-Ten Hoor, 1975), and also its close relative *Su. gotlandica*, which plays an important role at the redoxcline in the Baltic Sea, did accumulate neither nitrite nor zero-valent sulfur and could grow on sulfide only if the concentration was below 10 μM (Bruckner *et al*., 2012; Grote *et al*., 2012). The *Sedimenticola* strain could therefore have outcompeted *Su. denitrificans* by using sulfide as electron donor. Also, the ability to conserve enough energy from partial denitrification might have been of importance upon the addition of anammox bacteria, as anammox bacteria have a very high affinity for nitrite and might outcompete a complete denitrifier (Strous *et al*., 1999; Kartal *et al*., 2007; 2008).

Metagenome analysis and FISH also pointed to a shift within the anammox population. Approximately 10% of all reads could be mapped to anammox genomes (similarity fraction 0.85), although FISH revealed that actual anammox numbers were much higher (> 25%) (Fig. 3). According to the mapping results, the anammox population in the bioreactor consisted for about 27% of *Sc. profunda* and about 73% of *K. stuttgartiensis*. The simultaneous use of a *K. stuttgartiensis*-specific probe (Kst1240) and the general anammox probe (AMX820) in FISH revealed similar results, showing a clear dominance of *K. stuttgartiensis* after 133 days despite the marine settings of the reactor system (Fig. 3C). Although we used an artificial medium for the enrichment of marine anammox bacteria, salt and nitrite limitation did obviously not select against *K. stuttgartiensis*. It is known that freshwater anammox bacteria grow in salt concentrations up to 30 g l^{-1} (Kartal *et al*., 2006; Liu *et al*., 2009), but still the genus *Scalindua* sp. seems to dominate natural marine

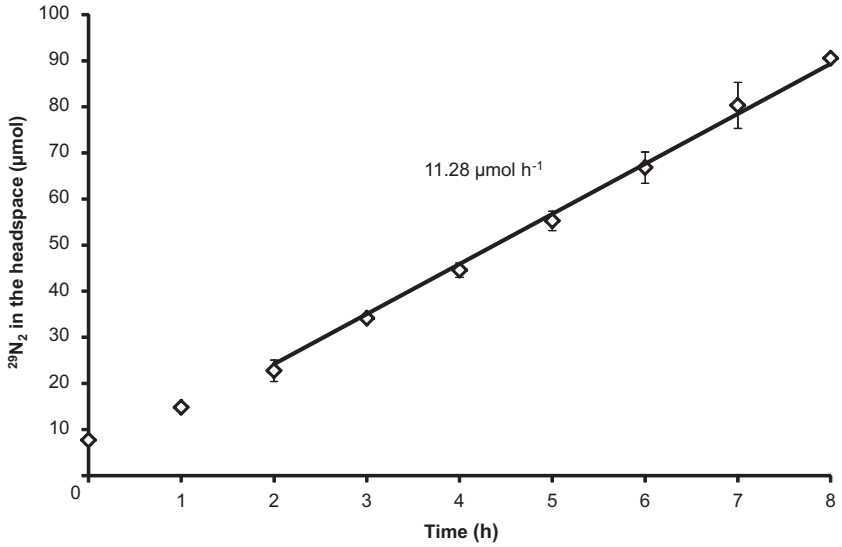

Fig. 4. $^{29}N_2$ production of the reactor system from $^{15}NH_4^+$ and $^{14}NO_3^-$ during 8 h of operation as a fed batch (headspace volume = 1.5 l).

environments (Penton *et al*., 2006; Schmid *et al*., 2007; Woebken *et al*., 2008) raising the question what the determining selective factor is between freshwater and marine anammox genera.

Contribution to N_2 *production in the bioreactor*

The interaction between autotrophic denitrification and anammox and the relative contribution of these processes to the nitrogen loss from the bioreactor was monitored with differential addition of stable isotopes. All experiments were performed with sulfide, NO_3^- and NH_4^+. When $^{15}NH_4^+$ and $^{14}NO_3^-$ were used as substrates, anammox activity would result in the production of $^{29}N_2$, whereas denitrification would lead to the production of $^{28}N_2$. On the other hand, when $^{14}NH_4^+$ and $^{15}NO_3^-$ were used as substrates, denitrification and anammox would produce $^{30}N_2$ and $^{29}N_2$ respectively. Upon the addition of sulfide, $^{15}NH_4^+$ and $^{14}NO_3^-$ to the reactor, $^{29}N_2$ was produced with a rate of 11.3 ± 1.3 µmol h^{-1} (Fig. 4). When $^{14}NH_4^+$ and $^{15}NO_3^-$ were supplied, both processes were active simultaneously as $^{29}N_2$ and $^{30}N_2$ concentrations increased with rates of 6.4 µmol h^{-1} and 3.3 µmol h^{-1} respectively (Fig. 5). The production of NO and N_2O as intermediates was not detected at any time. Anammox activity was lower when the labelling experiment was conducted with $^{15}NO_3^-$. This might be due to two factors: The initial starvation of the reactor for nitrate before the addition of $^{15}NO_3^-$ resulted in sulfide accumulation, which has a negative effect on anammox activity therefore rendering their activity slightly

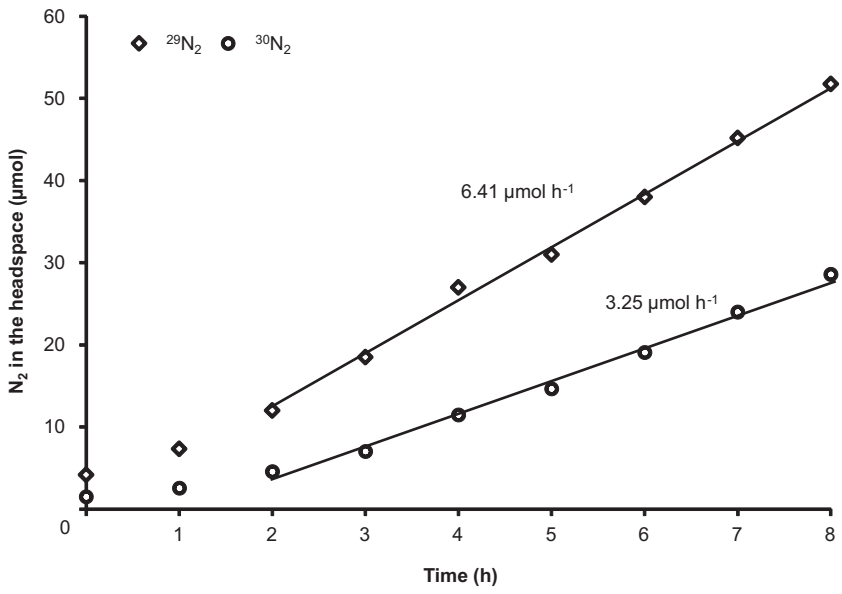

Fig. 5. $^{29}N_2$ and $^{30}N_2$ production of the reactor system from $^{14}NH_4^+$ and $^{15}NO_3^-$ during 8 h of operation as a fed batch (headspace volume = 1.5 l).

lower. Further, ammonium starvation before the addition of $^{15}NH_4^+$ generally resulted in residual nitrate in the reactor. As there was a higher demand for nitrate than for ammonium because both processes require it as a substrate, the actual nitrate availability was higher when labelled ammonium was added, which could lead to a higher anammox activity.

Batch activity assays

To test the activity of the co-culture under more controlled conditions, batch activity assays were performed with differential labelling ammonium and nitrate and in the presence and absence of sulfide as electron donor. To avoid disturbance of the signal by heterotrophic denitrification that could use nitrate with degradation products as electron donors, the duration of the experiment was limited to 24 h. A total of 150 μM sulfide was added in intervals of at least 2 h during this period.

The interaction between anammox and autotrophic denitrification was strongly dependent on sulfide as there was no net production of dinitrogen gas in the absence of sulfide (Fig. 6). We performed five biological replicate incubations. One of which consumed up to 150 μM nitrate explaining the relatively high standard error with $^{15}NO_3^-$ in the absence of sulfide, but there was a significantly lower nitrate consumption and N_2 production without sulfide. This showed that anammox bacteria depended on the conversion of nitrate to nitrite by autotrophic denitrification and under these conditions did not directly metabolize nitrate to produce NO or dinitrogen gas themselves. When sulfide was added, the culture consumed 210 ± 15 μM nitrate and transiently produced 25–30 μM of nitrite during the first 24 h (data not shown). In the absence of sulfide, the nitrite produced was much lower (< 10 μM). A slightly lower anammox activity was observed in incubations with $^{15}NH_4^+$ compared with those with $^{15}NO_3^-$ (Fig. 6). We assume that this might be the result of an overestimation of anammox activity in case of $^{15}NO_3^-$ labelling, as there might be a small pool of unlabelled nitrate present. This unlabelled nitrate might be stored internally and could be used to as an electron acceptor for denitrification, thereby contributing to the $^{29}N_2$ pool. The increase of $^{29}N_2$ also in the absence of sulfide and the production of small amounts of $^{29}N_2$ when both substrates were labelled also indicated a possible internal nitrate storage (Fig. 6). In both reactor and batch labelling experiments, contribution of anammox to the nitrogen loss was in the same range (65–75%).

Environmental significance of partial autotrophic denitrification coupled to anammox

In marine environments, an interaction between denitrification and anammox was previously shown in continental shelf sediments where $^{29}N_2$ gas was produced in the presence of $^{15}NO_3^-$, indicating that nitrate was first converted to $^{15}NO_2^-$ before it was combined with ammonium to form $^{29}N_2$ (Dalsgaard *et al.*, 2003). Additional evidence on the possible importance of this process in marine environments originated from a metagenomics and transcriptomics study in the OMZ off of the coast of Chile: Anammox and gammaproteobacterial sulfur-oxidizers of the so-called SUP05 subgroup seemed to be present in the same layer of the water column (Canfield *et al.*, 2010; Stewart *et al.*, 2012). Although it is generally

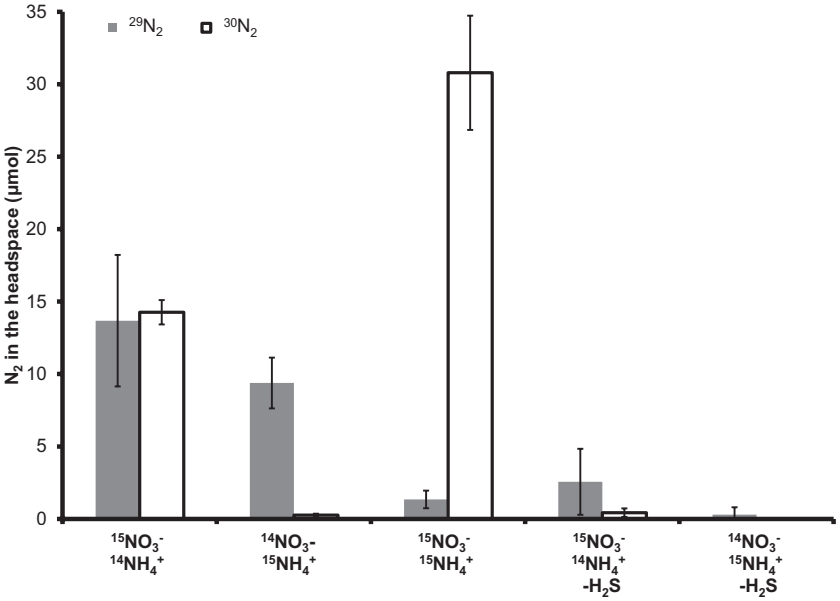

Fig. 6. $^{29}N_2$ and $^{30}N_2$ production in batch activity assays using co-culture biomass and differentially labelled substrates.

stated that anammox is absent from sulfidic waters (Dalsgaard *et al*., 2005; Lam *et al*., 2007), recent studies describe the presence of certain phylotypes of the marine *Scalindua* sp. in the lower suboxic zone of the Black Sea where sulfide concentrations were up to 10 μM (Wakeham *et al*., 2007; Fuchsman *et al*., 2012; Kirkpatrick *et al*., 2012). This could mean that there was a certain divergence in the adaptation towards sulfide. A very adaptive metabolism responding quickly to a changing environment might be one of the attributes determining the success of *Scalindua* phylotypes in marine habitats with fluctuating conditions. An example of this was described in the Baltic Sea; although anammox activity could not be shown in the presence of measurable sulfide, these microorganisms contributed significantly to the total N_2 production after a major inflow event of oxygenated North Sea water that resulted in a sulfide-free zone (Hannig *et al*., 2007). Anammox bacteria might enter dormancy in times of unfavourable conditions but regain their activity quickly as soon as conditions improve (Jones and Lennon, 2010). Alternatively, they might switch to a different metabolism (Strous *et al*., 2006; Kartal *et al*., 2007; 2008).

In marine sediments, the sulfur-fuelled nitrogen cycle is an important process (Schulz *et al*., 1999; Campbell *et al*., 2006). In the deep sea sediments of the Guaymas Basin, anammox bacteria were detected within sulfidic sediments (Russ *et al*., 2013). Their numbers were highest at the sediment interface where sulfide concentrations were lowest, but still, functional genes of sulfide oxidizers could be retrieved from the same depth, again showing that these two organisms could potentially share the same niche. Also in freshwater ecosystems, anammox and autotrophic denitrifiers occur in the same water layer. Although direct exchange of intermediates has not been determined, both processes were actively taking place (Wenk *et al*., 2013).

From the applied point of view, such a coupling might offer interesting opportunities for the treatment of ammonium-rich, sulfidic wastewater. As sulfide leads to partial inhibition of NO reduction and a strong inhibition of N_2O reduction in denitrifying cells, a scenario with a significantly lower release of NO and the green gas N_2O from such systems would be possible (Sørensen *et al*., 1980; Schönharting *et al*., 1998; Dalsgaard *et al*., 2013). In our experiments, we never saw the release of any other gas than N_2 because anammox bacteria have a very high affinity for nitrite so most of it has been consumed by anammox before it can be transformed into NO or N_2O by denitrification (Strous *et al*., 1999). The system would however be sensitive to high sulfide loads or ammonium limitation.

The complex interactions of the nitrogen cycle bacteria is an interesting example of how complex the determination of fluxes can be, with many different players and transformations to consider. Using a laboratory scale model system, we showed that the anammox bacteria could indeed be fuelled by nitrate reduction at the constant influx of sulfide and ammonium. We believe that this interaction between anammox and denitrification (autotrophic and also heterotrophic) could play an important role in the environment, especially in zones of cryptic sulfur cycling where sulfide is at sub μM level.

Experimental procedures

FISH

Biomass was fixed in a 4% w/v paraformaldehyde solution for 1–3 h on ice. After washing cells were stored at −20°C in a 50% v/v 1 × PBS and 50% v/v ethanol solution. Probe sequences and optimal formamide concentrations for hybridization are listed in Supporting Information Table S1. Staining with 4,6-diamidino-2-phenylindole was included as a positive control.

DNA isolation and 16S PCR

Genomic DNA was isolated from different samples using the PowerSoil® DNA Isolation Kit (MO BIO Laboratories, Inc.) according to the manufacturer's protocol.

All PCR amplifications were performed in a total volume of 25 μl using 12.5 μl PerfeCTa® SYBR® Green FastMix (Quanta), 0.4 μM forward/reverse primer (Sed784F-5′-GCCAAGATGCTCCCAACAAC and Sed1249R-5′-AGCCTGATCCAGCAATACCG) 2 μl of template and 10.5 μl of DEPC-treated H_2O. Amplification was initiated with a denaturation step at 94°C for 2 min and continued with a standard amplification program of 35 cycles (30 s 94°C; 40 s 55°C; 1 min 72°C). The final elongation step was done at 72°C for 10 min.

Next-generation sequencing

DNA was extracted from 24 ml co-culture biomass using the PowerSoil DNA isolation kit (MO BIO) following the protocol. DNA quality was checked by agarose gel electrophoresis (1%) with ethidium bromide. Concentrations were determined spectrometrically using the NanoDrop (Thermo Scientific). One microgram of genomic DNA was sheared for 7 min using the Ion Xpress™ Plus Fragment Library Kit (Life Technologies) following the manufacturer's instructions. Further library preparation was performed using the Ion Plus Fragment Library Kit (Life Technologies) following manufacturer's instructions. Size selection of the library was performed using an E-gel 2% agarose gel (Life Technologies) resulting in a mean fragment length of 339 bp. Emulsion PCR was done using the Onetouch 200 bp kit, and sequencing was performed on an IonTorrent PGM using the Ion PGM 200 bp sequencing kit and an Ion 318 chip (Life Technologies), resulting in 4 349 930 reads of average length 167 bp.

Analysis of reads was performed using CLC genomics workbench 6.0.2 (CLCbio). The reads were length- and

quality-score-trimmed (> 50 bp, 0.05) resulting in a data set of 3 847 959 reads. Of all reads, 43.8% could be mapped to the genome of *Se. selenatireducens* DSM 17993 with mismatch penalty 2, In/Del penalties 3 and 90% identity over 50% of the read length. The subset was extracted and a *de novo* assembly included in the program mapping the reads back to the contigs (Length fraction 0.5, similarity fraction 0.8) was performed using the default settings. This resulted in 580 contigs. These contigs were submitted to the RAST sever for annotation (Aziz *et al*., 2008).

Batch activity assays for IC50 determination

Active anammox biomass (50% *Sc. profunda*, 50% *K. stuttgartiensis)* planktonic cells were harvested from a membrane reactor. Ten millilitres cell biomass were added to a 30 ml serum bottle, and a total concentration of 2 mM ammonium chloride and 2 mM sodium nitrite were added as substrate. Sodium sulfide was added in concentrations of 5, 10, 20, 50 and 100 μM from a 100 mM anaerobic stock solution. The bottles were sealed with 5 mm butyl rubber stoppers and made anoxic by alternatively applying underpressure and flushing with Argon several times, ultimately maintaining an overpressure of one bar on the bottles. Incubation was done at room temperature and shaken at 200 r.p.m. for 2 h, during which the decrease in nitrite and ammonium was followed.

Analytical methods

Nitrite was measured colorimetically at 540 nm after a 15 min reaction of 1 ml sample (0.1–0.5 mM nitrite) with 1 ml 1% sulfanilic acid in 1 M HCl and 1 ml 0.1% naphtylethylene diaminedihydrochloride. Ammonium was measured at 420 nm on a Cary Eclipse Fluorescence Spectrophotometer after reaction with 10% orthophthaldialdehyde as has been described previously (Taylor *et al*., 1974). Sulfide concentrations were determined by detection of hydrogen sulfide as methylene blue at 460 nm (Fonselius *et al*., 2007). Nitrate was measured by injection of 10 μl sample into an HP Agilent 1050 series autosampler using a sodium hydroxide solution at a flow of 1.5 ml min^{-1} as the liquid phase. The anions were eluted via an isocratic method with 30 mM NaOH in 6 min and separated on a 4 × 250 mm Ionpac AS11-HC (Dionex) column. The UV/Vis absorbance spectrum of nitrate was measured at 220 nm.

Reactor operation

A fermentor (working volume 1.5 l) was used to gradually adapt *Su. denitrificans* DSM 1251 to higher salt concentrations (2.5%) and sulfide as electron donor. Medium generally used to cultivate marine anammox bacteria (van de Vossenberg *et al*., 2008) was adjusted to a salt content of 37.5 g l^{-1} and added to the reactor with a flow of 150–200 ml day^{-1}. The pH was controlled at 7.1 with a sterile 100 g l^{-1} $KHCO_3$ solution. Sodium sulfide was added separately from an anaerobic 6 mM stock with a flow of 90–100 ml day^{-1}. The reactor was operated under anaerobic conditions by constant flushing with Arg/CO_2 (10 ml min^{-1}). Sulfide was limiting with influent nitrate and ammonium concentrations of 8 mM NO_3^- and 5 mM NH_4^+ during stable operation. During the limitation experiments NO_3^- or NH_4^+ concentrations were lowered until either NO_3^- or NH_4^+ was below the detection limit. Afterwards influent nitrate and ammonium concentrations were either 2 mM $^{15}NO_3^-$ and 4 mM $^{14}NH_4^+$ under nitrate-limiting conditions or 4 mM $^{14}NO_3^-$ and 2 mM $^{15}NH_4^+$ under nitrate-limiting conditions, corresponding to a total of ~125 μM labelled substrate to the reactor system in 8 h. The reactor was sealed and the Arg/CO_2 was switched off. Gas samples were taken from a sampling port every hour.

Activity assays

For batch activity assays, biomass was harvest from the co-culture by centrifugation at 4000 r.p.m. (10 min, 4°C) and washed once before re-suspending it in fresh medium (pH 7.1) supplemented with 10 mM $KHCO_3$. Total concentrations of 1 mM $^{14}NO_3^-$ or $^{15}NO_3^-$, and $^{14}NH_4^+$ or $^{15}NH_4^+$ were added. Anaerobic conditions were obtained by alternately applying vacuum and flushing with Arg/CO_2. An overpressure of one bar was maintained. Sodium sulfide was added in portions of 20 μM (to avoid a toxicity effect) to a total concentration of 150 μM. Incubations were shaken with 150 r.p.m. at 20°C.

For whole reactor activity assays, the reactor was starved in either nitrate of ammonium before feeding either 2 mM $^{15}NO_3^-$ and $^{14}NH_4$, or $^{14}NO_3^-$ and $^{15}NH_4^+$ via the influent at the rates described earlier. To avoid washout of labelled gas compounds, the reactor was operated as batch during 8 h of measurement. The production of $^{28}N_2$, $^{29}N_2$ and $^{30}N_2$ was followed by gas chromatography coupled to mass spectrometry on an Agilent 6890/5975c MSD. For the quantification of N_2 standard curves, a commercially available gas mixture was used containing 1% N_2. Nitrate, ammonium and nitrite were determined as described earlier.

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Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Description of FISH probes used in this study.
Table S2. Genes of *Sedimenticola* sp. involved in nitrate reduction and sulfide oxidation.