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 *Sulfurimonas 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 oxygen minimum zone (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 labeling 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; 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 labeled $^{15}\text{N}^{15}\text{N}$ by anammox bacteria, thereby interfering with traditional isotope pairing experiments, in which the production of $^{30}\text{N}_2$ 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

90 detoxification. The exact mechanism favoring either anammox or denitrification is still unknown.
91 However, as autotrophic denitrification uses sulfide as electron donor and there are indications that
92 anammox activity is inhibited by μM levels of sulfide, such an interaction is unlikely to occur in
93 sulfidic waters (Dalsgaard *et al.*, 2003; Jensen *et al.*, 2008; Jin *et al.*, 2013).

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

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

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

117 **Results and Discussion**

118 *Sulfide toxicity in anammox bacteria*

119 The sensitivity of anammox bacteria enriched from wastewater treatment plants towards short time
120 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.*, 1995; 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 $10 (\pm 4) \mu\text{M}$. 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 to planktonic cells (Costerton *et al.*, 1987). Although the exact molecular mechanism of sulfide toxicity is not resolved yet, it has been shown that H₂S reversibly inhibits cytochromes and other heme-containing proteins (reviewed in Kabil and Banerjee, 2010). Thus the high sensitivity of anammox to sulfide is probably due to fact that anammox bacteria harbor an exceptionally high number of heme-containing proteins (Strous *et al.*, 2006; Kartal 2011). Although it has been observed that the inhibitory effect of H₂S on other heme-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 $\sim 70 \mu\text{M} \cdot \text{d}^{-1}$ and instantly resulted in a drop in anammox activity which was evident from the accumulation of nitrite. Further, a significant drop in optical density (OD₆₀₀) 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

Due to 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 *Sulfurimonas denitrificans* DSM 1251 (Timmer-Ten Hoor, 1975) and anammox bacteria by introducing the anammox bacteria to a stable culture of sulfur oxidizers. *S. denitrificans* was precultured anaerobically in an adapted anammox medium using sulfide and nitrate as substrates. The initial addition of sulfide ($70 \mu\text{M}\cdot\text{d}^{-1}$) lead to the visible formation of elemental sulfur. The supply of excess nitrate concentrations caused a transient nitrite accumulation ($10\text{-}13 \mu\text{M}$) as a result of incomplete denitrification (Fig. 2). When the culture was stably consuming all supplied sulfide (effluent $[\text{HS}^-] < 2\mu\text{M}$) anammox biomass was added at the ratio of one part anammox and three parts *S. denitrificans* according to OD_{600} values (day 0). As an immediate result, the nitrite concentration in the effluent decreased below the detection limit ($5 \mu\text{M}$). At day 27, the influent sulfide load was increased ($267 \mu\text{M}\cdot\text{d}^{-1}$) 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 ($\sim 5 \mu\text{M}$), 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 $66 \pm 2\%$ 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 lead to a minor nitrite accumulation ($\sim 6 \mu\text{M}$) 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 ($\sim 180 \mu\text{M}$). When the feed was adapted to the stable operation phase, the reactor gradually recovered.

Assessment of the community composition by shotgun metagenomic sequencing, FISH and PCR

The initial composition of anammox biomass in the reactor consisted for about 50% of *Kuenenia stuttgartiensis* and 50% *Scalindua profunda* which had been added to the running reactor of autotrophic denitrifiers in a ratio of 1:3. To confirm the presence of *Sulfurimonas denitrificans* 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 *Sulfurimonas 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*. 43.9% of all reads 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 *Sulfurimonas 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&D). To trace the source of these *Sedimenticola* sp. and their diversity, DNA was isolated from the original DSMZ 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 *Sedimenticola 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 *Sedimenticola selenatireducens*, we assembled all mapped reads into 580 contigs and analyzed the assembled genome for key genes of sulfur oxidation and denitrification. The *Sedimenticola* sp. present in the reactor harbored a range of genes involved in the oxidation of reduced, inorganic sulfur compounds, which could facilitate the complete oxidation of sulfide to sulfate (Supplementary Table 2). 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 reductase (*apr*) oxidizing sulfite to adenosine 5'-phosphosulfate (APS) and

adenosine 5'-triphosphate sulfurylase (*sat*) oxidizing APS to sulfate. Several other sulfur-oxidizing bacteria also harbor 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 which 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 *cd₁* nitrite reductase (*nirS*) and nitrous oxide reductase (*nosZ*)] were also detected in the genome assembly of *Sedimenticola* sp. (Supplementary Table 2). 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 *S. selenatireducens* DSM 17993 seems to encode even 2 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% (Supplementary Table 2).

The exact reasons causing the disappearance of *Sulfurimonas 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 *Sulfurimonas 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 μ M (Bruckner *et al.*, 2012; Grote *et al.*, 2012). The *Sedimenticola* strain could therefore have outcompeted *Sulfurimonas 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; Kartal *et al.*, 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. 3A-D). According to the mapping results, the anammox population in the bioreactor consisted for about 27% of *Scalindua profunda* and about 73% of *Kuenenia 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 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 N₂ 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, NO₃⁻ and NH₄⁺. When ¹⁵NH₄⁺ and ¹⁴NO₃⁻ were used as substrates, anammox activity would result in the production of ²⁹N₂, whereas denitrification would lead to the production of ²⁸N₂. On the other hand, when ¹⁴NH₄⁺ and ¹⁵NO₃⁻ were used as substrates, denitrification and anammox would produce ³⁰N₂ and ²⁹N₂, respectively. Upon the addition of sulfide, ¹⁵NH₄⁺ and ¹⁴NO₃⁻ to the reactor, ²⁹N₂ was produced with a rate of 11.3 ± 1.3 μmol·h⁻¹ (Fig. 4). When ¹⁴NH₄⁺ and ¹⁵NO₃⁻ were supplied, both processes were active simultaneously as ²⁹N₂ and ³⁰N₂ concentrations increased with rates of 6.4 μmol·h⁻¹ and 3.3 μmol·h⁻¹, respectively (Fig. 5). The production of NO and N₂O as intermediates was not detected at any time. Anammox activity was lower when the labeling experiment was conducted with ¹⁵NO₃⁻. This might be due to two factors: The initial starvation of the reactor for nitrate before the addition of ¹⁵NO₃⁻ resulted in sulfide accumulation, which has a negative effect on anammox activity therefore rendering their activity slightly lower. Further, ammonium starvation before the addition of ¹⁵NH₄⁺ 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 labeled 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 labeling 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 $^{15}\text{NO}_3^-$ 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 $210 \pm 15 \mu\text{M}$ 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 ($<10 \mu\text{M}$). A slightly lower anammox activity was observed in incubations with $^{15}\text{NH}_4^+$ compared to those with $^{15}\text{NO}_3^-$ (Fig. 6). We assume that this might be the result of an overestimation of anammox activity in case of $^{15}\text{NO}_3^-$ -labeling, as there might be a small pool of unlabeled nitrate present. This unlabeled nitrate might be stored internally and could be used to as an electron acceptor for denitrification thereby contributing to the $^{29}\text{N}_2$ pool. The increase of $^{29}\text{N}_2$ also in the absence of sulfide and the production of small amounts of $^{29}\text{N}_2$ when both substrates were labeled also indicated a possible internal nitrate storage (Fig. 6). In both reactor and batch labeling 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 $^{29}\text{N}_2$ gas was produced in the presence of $^{15}\text{NO}_3^-$, indicating that nitrate was first converted to $^{15}\text{NO}_2^-$ before it was combined with ammonium to form $^{29}\text{N}_2$ (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 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 μ M (Wakeham *et al.* 2012, Fuchsmann *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 such an anammox activity 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 N₂ production after a major inflow event of oxygenated North Sea water that resulted in a sulfide free zone (Hanning *et al.*, 2007). Anammox bacteria might enter dormancy in times of unfavorable conditions, but regain their activity quickly as soon as conditions improve. Alternatively, they might switch to a different metabolism (Strous *et al.*, 2006, Kartal *et al.*, 2007, 2008).

In marine sediments the sulfur-fueled 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 N₂O reduction in denitrifying cells, a scenario with a significantly lower release of NO and the green gas N₂O 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 N₂, 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 N₂O 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 fueled 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

Fluorescence In Situ Hybridization

Biomass was fixed in a 4% w/v paraformaldehyde solution for 1-3h on ice. After washing cells were stored at $-20\text{ }^{\circ}\text{C}$ in a 50% v/v 1 X PBS and 50% v/v ethanol solution. Probe sequences and optimal formamide concentrations for hybridization are listed in Supplementary Table 1. Staining with DAPI 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 $94\text{ }^{\circ}\text{C}$ for 2 min and continued with a standard amplification program of 35 cycles (30 sec $94\text{ }^{\circ}\text{C}$; 40 sec $55\text{ }^{\circ}\text{C}$; 1 min $72\text{ }^{\circ}\text{C}$). The final elongation step was done at $72\text{ }^{\circ}\text{C}$ for 10 min.

Next generation sequencing

DNA was extracted from 24 ml co-culture biomass using the PowerSoil® DNA isolation kit (MO BIO, USA) following the protocol. DNA quality was checked by agarose gel electrophoresis (1%) with ethidium bromide. Concentrations were determined spectrometrically using the

NanoDrop (Thermo Scientific). 1 µg genomic DNA was sheared for 7 minutes using the Ion Xpress™ Plus Fragment Library Kit (Life technologies, USA) following the manufacturer's instructions. Further library preparation was performed using the Ion Plus Fragment Library Kit (Life technologies, USA) following manufacturer's instructions. Size selection of the library was performed using an E-gel 2% agarose gel (Life technologies, USA) resulting in a mean fragment length of 339 bp. Emulsion PCR was done using the Onetouch 200bp kit and sequencing was performed on an IonTorrent PGM using the Ion PGM 200bp sequencing kit and an Ion 318 chip (Life technologies, USA), resulting in 4,349,930 reads of average length 167 bp.

Analysis of reads was performed using CLC genomics workbench 6.0.2 (CLCbio, Denmark). The reads were length and quality score trimmed (>50bp, 0.05) resulting in a dataset of 3,847,959 reads. 43.8% of all reads could be mapped to the genome of *Sedimenticola 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 server for annotation (Aziz *et al.* 2008).

Batch activity assays for IC50 determination

Active anammox biomass (50% *Scalindua profunda*, 50% *Kuenenia stuttgartiensis*) planktonic cells were harvested from a membrane reactor. 10 ml 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 5mm butyl rubber stoppers and made anoxic by alternatively applying underpressure and flushing with Argon several times, ultimately maintaining an overpressure of 1 bar on the bottles. Incubation was done at room temperature and shaken at 200 rpm for 2 h, during which the decrease in nitrite and ammonium was followed.

Analytical methods

Nitrite was measured colorimetrically 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% naphthylethylene

diaminedihydrochloride. Ammonium was measured at 420 nm on a Cary Eclipse Fluorescence Spectrophotometer after reaction with 10% ortho-phthaldialdehyde 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 μ l sample into an HP Agilent 1050 series auto sampler using a sodium hydroxide solution at a flow of 1.5 ml $\cdot$ 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 4x250mm Ionpac AS11-HC (Dionex, UK) 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 *Sulfurimonas 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 $\cdot$ l⁻¹ and added to the reactor with a flow of 150-200 ml $\cdot$ d⁻¹. The pH was controlled at 7.1 with a sterile 100 g $\cdot$ l⁻¹ KHCO₃ solution. Sodium sulfide was added separately from an anaerobic 6 mM stock with a flow of 90-100 ml $\cdot$ d⁻¹. The reactor was operated under anaerobic conditions by constant flushing with Arg/CO₂ (10 ml $\cdot$ min⁻¹). Sulfide was limiting with influent nitrate and ammonium concentrations of 8 mM NO₃⁻ and 5 mM NH₄⁺ during stable operation. During the limitation experiments NO₃⁻ or NH₄⁺ concentrations were lowered until either NO₃⁻ or NH₄⁺ was below the detection limit. Afterwards influent nitrate and ammonium concentrations were either 2 mM ¹⁵NO₃⁻ and 4 mM ¹⁴NH₄⁺ under nitrate-limiting conditions or 4 mM ¹⁴NO₃⁻ and 2 mM ¹⁵NH₄⁺ under nitrate-limiting conditions, corresponding to a total of ~ 125 μ M labelled substrate to the reactor system in 8 h. The reactor was sealed and the Arg/CO₂ 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 rpm (10 min, 4 °C) and washed once before resuspending it in fresh medium (pH 7.1) supplemented with 10 mM KHCO₃. Total concentrations of 1 mM ¹⁴NO₃⁻ or ¹⁵NO₃⁻ and ¹⁴NH₄⁺ or ¹⁵NH₄⁺ were added. Anaerobic conditions were obtained by alternately applying vacuum and flushing with Arg/CO₂. An overpressure of 1 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 rpm at 20 °C.

For whole reactor activity assays the reactor was starved in either nitrate or ammonium before feeding either 2 mM $^{15}\text{NO}_3^-$ and $^{14}\text{NH}_4^+$ or $^{14}\text{NO}_3^-$ and $^{15}\text{NH}_4^+$ via the influent at the rates described above. To avoid washout of labeled gas compounds the reactor was operated as batch during 8 h of measurement. The production of $^{28}\text{N}_2$, $^{29}\text{N}_2$ and $^{30}\text{N}_2$ 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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References

- Arrigo, K.R. (2005) Marine microorganisms and global nutrient cycles. *Nature* **437**: 349-355
- Aziz, R.K., Bartels, D., Best, A.A., DeJongh, M., Disz, T., Edwards, R.A., *et al.* (2008) The RAST Server: Rapid Annotations using Subsystems Technology. *BMC Genomics* **9**: 75
- Bruckner, C.G., Mammitzsch, K., Jost, G., Wendt, J., Labrenz, M., and Jürgens, K. (2012) Chemolithoautotrophic denitrification of epsilonproteobacteria in marine pelagic redox gradients. *Environ Microbiol* **15**: 1505-1513.
- Campbell, B.J., Summers Engel, A., Porter, M.L. and Takai, K. (2006) The versatile ε-Proteobacteria: Key players in sulphidic habitats. *Nature Rev Microbiol* **4**: 458-468.
- Canfield, D.E., Stewart, F.J., Thamdrup, B., De Brabandere, L., Dalsgaard, T., Delong, E.F. *et al.* (2010) A Cryptic Sulfur Cycle in Oxygen-Minimum-Zone Waters off the Chilean Coast. *Science* **330**: 1375-1378.
- Chen, K.Y., and Morris, J.C. (1972) Kinetics of oxidation of aqueous sulfide by oxygen. *Environ Sci Technol* **6**: 529-537.

460 Cooper, C., and Brown, G. (2008) The inhibition of mitochondrial cytochrome oxidase by the
 461 gases carbon monoxide, nitric oxide, hydrogen cyanide and hydrogen sulfide: chemical
 462 mechanism and physiological significance. *J Bioenerg Biomembr* **40**: 533-539.

463 Costerton, J.W., Cheng, K.J., Geesey, G.G., Ladd, T.I., Nickel, J.C., Dasgupta, M., and Marrie,
 464 T.J. (1987) Bacterial Biofilms in Nature and Disease. *Annu Rev Microbiol* **41**: 435-464.

465 Dalsgaard, T., Thamdrup, B., and Canfield, D.E. (2005) Anaerobic ammonium oxidation
 466 (anammox) in the marine environment. *Res Microbiol* **156**: 457-464.

467 Dalsgaard, T., Canfield, D., Petersen, J., Thamdrup, B., and Acuña-González, J. (2003) N₂
 468 production by the anammox reaction in the anoxic water column of Golfo Dulce, Costa Rica.
 469 *Nature* **422**: 606-608.

470 Dalsgaard, T., De Brabandere, L., and Hall, P.O.J. (2013) Denitrification in the water column of
 471 the central Baltic Sea. *Geochim Cosmochim Acta* **106**: 247-260

472 Dapena-Mora, A., Fernández, I., Campos, J.L., Mosquera-Corral, A., Méndez, R., and Jetten,
 473 M.S.M. (2007) Evaluation of activity and inhibition effects on Anammox process by batch
 474 tests based on the nitrogen gas production. *Enzyme Microb Technol* **40**: 859-865.

475 Dong, L.F., Naqasima Sobey, M., Smith, C.J., Rusmana, I., Phillips, W., Stott, A. *et al.* (2011)
 476 Dissimilatory reduction of nitrate to ammonium, not denitrification or anammox, dominates
 477 benthic nitrate reduction in tropical estuaries. *Limnol Oceanogr* **56**: 279.

478 Falkowski, P.G. (1997) Evolution of the nitrogen cycle and its influence on the biological
 479 sequestration of CO₂ in the ocean. *Nature* **387**: 272-274.

480 Fonselius, S., Dyrssen, D., and Yhlen, B. (2007) Determination of hydrogen sulphide. In *Methods*
 481 *of Seawater Analysis*: Wiley-VCH Verlag GmbH, Weinheim, Germany pp. 91-100.

482 Francis, C., Roberts, K., Beman, J., Santoro, A., and Oakley, B. (2005) Ubiquity and diversity of
 483 ammonia-oxidizing archaea in water columns and sediments of the ocean. *Proc Natl Acad Sci*
 484 *USA* **102**: 14683-14688.

485 Fuchsman, C.A., Staley, J.T., Oakley, B.B., Kirkpatrick, J.B., Murray, J.W. (2012) Free-living and
 486 aggregate-associated Planctomycetes in the Black Sea. *FEMS Microbiol Ecol* **80**: 402-416.

487 Füssel, J., Lam, P., Lavik, G., Jensen, M.M., Holtappels, M., Günter, M., and Kuypers M.M.M.
 488 (2012) Nitrite oxidation in the Namibian oxygen minimum zone. *ISME J* **6**: 1200-1209.

489 Galán, A., Molina, V., Thamdrup, B., Woebken, D., Lavik, G., Kuypers, M.M.M., and Ulloa, O.
490 (2009) Anammox bacteria and the anaerobic oxidation of ammonium in the oxygen minimum
491 zone off northern Chile. *Deep-Sea Res Pt II* **56**: 1021-1031.

492 Glaubitz, S., Lueders, T., Abraham, W.R., Jost, G., Jürgens, K., and Labrenz, M. (2009) ¹³C-
493 isotope analyses reveal that chemolithoautotrophic Gamma- and Epsilonproteobacteria feed
494 a microbial food web in a pelagic redoxcline of the central Baltic Sea. *Environ Microbiol* **11**:
495 326-337.

496 Grote, J., Schott, T., Bruckner, C.G., Glöckner, F.O., Jost, G., Teeling, H. *et al.* (2012) Genome
497 and physiology of a model Epsilonproteobacterium responsible for sulfide detoxification in
498 marine oxygen depletion zones. *Proc Natl Acad Sci USA* **109**: 506.

499 Gruber, N., and Galloway, J.N. (2008) An Earth-system perspective of the global nitrogen cycle.
500 *Nature* **451**: 293-296.

501 Hamersley, M.R., Lavik, G., Woebken, D., Rattray, J.E., Lam, P., Hopmans, E.C. *et al.* (2007)
502 Anaerobic ammonium oxidation in the Peruvian oxygen minimum zone. *Limnol Oceanogr*
503 **52**: 923.

504 Hannig, M., Lavik, G., Kuypers, M.M.M., Woebken, D., Martens-Habbena, W., Jürgens, K.
505 (2007) Shift from denitrification to anammox after inflow events in the central Baltic Sea.
506 *Limnol Oceanogr* **52**: 1336–1345

507 Jannasch, H.W., Nelson, D.C., and Wirsén, C.O. (1989) Massive natural occurrence of unusually
508 large bacteria (*Beggiatoa* sp.) at a hydrothermal deep-sea vent site. *Nature* **342**: 834 - 836

509 Jensen, M.M., Kuypers, M.M.M., Lavik, G., and Thamdrup, B. (2008) Rates and regulation of
510 anaerobic ammonium oxidation and denitrification in the Black Sea. *Limnol Oceanogr* **53**:
511 23.

512 Jin, R.-C., Yang, G.-F., Zhang, Q.-Q., Ma, C., Yu, J.-J., and Xing, B.-S. (2013) The effect of
513 sulfide inhibition on the ANAMMOX process. *Water Res* **47**: 1459-1469.

514 Kabil, O., and Banerjee, R. (2010) Redox Biochemistry of Hydrogen Sulfide. *J Biol Chem* **285**:
515 21903-21907.

516 Kamp, A., de Beer, D., Nitsch, J.L., Lavik, G., and Stief, P. (2011) Diatoms respire nitrate to
517 survive dark and anoxic conditions. *PNAS* **108**: 5649-5654.

518 Kappler, U., and Dahl, C. (2001) Enzymology and molecular biology of prokaryotic sulfite
519 oxidation. *FEMS Microbiol Lett* **203**: 1-9.

520 Kartal, B., Koleva, M., Arsov, R., van der Star, W., Jetten, M.S.M., and Strous, M. (2006)
 521 Adaptation of a freshwater anammox population to high salinity wastewater. *J Biotechnol*
 522 **126**: 546-553.

523 Kartal, B., Rattray, J., Van Niftrik, L.A., Van De Vossenberg, J., Schmid, M.C., Webb, R.I., *et al.*
 524 (2007) *Candidatus* ‘Anammoxoglobus propionicus’ a new propionate oxidizing species of
 525 anaerobic ammonium oxidizing bacteria. *Syst Appl Microbiol* **30**: 39–49.

526 Kartal, B., Van Niftrik, L., Rattray, J., Van De Vossenberg, J.L.C.M., Schmid, M.C., Sinninghe
 527 Damsté, J. *et al.* (2008) *Candidatus* ‘Brocadia fulgida’: an autofluorescent anaerobic
 528 ammonium oxidizing bacterium. *FEMS Microbiol Ecol* **63**: 46-55.

529 Kirkpatrick, J., Fuchsman, C., Yakushev, E., Staley, J., and Murray, J. (2012) Concurrent activity
 530 of anammox and denitrifying bacteria in the Black Sea. *Front Microbiol* **3**: 256.

531 Kuypers, M.M.M., Lavik, G., Woebken, D., Schmid, M., Fuchs, B., Amann, R. *et al.* (2005)
 532 Massive nitrogen loss from the Benguela upwelling system through anaerobic ammonium
 533 oxidation. *Proc Natl Acad Sci USA* **102**: 6478-6483.

534 Kuypers, M.M.M., Sliemers, A., Lavik, G., Schmid, M., Jørgensen, B., Kuenen, J. *et al.* (2003)
 535 Anaerobic ammonium oxidation by anammox bacteria in the Black Sea. *Nature* **422**: 608-
 536 611.

537 Labrenz, M., Jost, G., Pohl, C., Beckmann, S., Martens-Habben, W., and Jürgens, K. (2005)
 538 Impact of different in vitro electron donor/acceptor conditions on potential
 539 chemolithoautotrophic communities from marine pelagic redoxclines. *Appl Environ*
 540 *Microbiol* **71**: 6664-6672.

541 Lam, P., and Kuypers, M.M.M. (2011) Microbial Nitrogen Cycling Processes in Oxygen
 542 Minimum Zones. In *Ann Rev Mar Sci*. Carlson, C.A., and Giovannoni, S.J. (eds). Palo Alto:
 543 Annual Reviews, pp. 317-345.

544 Lam, P., Jensen, M.M., Lavik, G., McGinnis, D.F., Müller, B., Schubert, C.J. *et al.* (2007) Linking
 545 crenarchaeal and bacterial nitrification to anammox in the Black Sea. *Proc Natl Acad Sci USA*
 546 **104**: 7104-7109.

547 Lam, P., Lavik, G., Jensen, M., van de Vossenberg, J., Schmid, M., Woebken, D. *et al.* (2009)
 548 Revising the nitrogen cycle in the Peruvian oxygen minimum zone. *Proc Natl Acad Sci USA*
 549 **106**: 4752-4757.

550 Lavik, G., Stührmann, T., Brüchert, V., Van der Plas, A., Mohrholz, V., Lam, P. *et al.* (2009)
 551 Detoxification of sulphidic African shelf waters by blooming chemolithotrophs. *Nature* **457**:
 552 581-584.

553 Liu, C., Yamamoto, T., Nishiyama, T., Fujii, T., and Furukawa, K. (2009) Effect of salt
 554 concentration in anammox treatment using non-woven biomass carrier. *J Biosci Bioeng* **107**:
 555 519-523.

556 Moses, C.O., Kirk Nordstrom, D., Herman, J.S., and Mills, A.L. (1987) Aqueous pyrite oxidation
 557 by dissolved oxygen and by ferric iron. *Geochim Cosmochim Acta* **51**: 1561-1571.

558 Mulder, A., van de Graaf, A.A., Robertson, L.A., and Kuenen, J.G. (1995) Anaerobic ammonium
 559 oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiol Ecol* **16**: 177-
 560 184.

561 Narasingarao, P., and Häggblom, M.M. (2006) *Sedimenticola selenatireducens*, gen. nov., sp.
 562 nov., an anaerobic selenate-respiring bacterium isolated from estuarine sediment. *Syst Appl*
 563 *Microbiol* **29**: 382-388.

564 Penton, C.R., Devol, A.H., and Tiedje, J.M. (2006) Molecular evidence for the broad distribution
 565 of anaerobic ammonium-oxidizing bacteria in freshwater and marine sediments. *Appl Environ*
 566 *Microbiol* **72**: 6829-6832.

567 Petersen, L.C. (1977) The effect of inhibitors on the oxygen kinetics of cytochrome c oxidase.
 568 *Biochim Biophys Acta* **460**: 299-307.

569 Preisler, A., de Beer, D., Lichtschlag, A., Lavik, G., Boetius, A., and Jorgensen, B.B. (2007)
 570 Biological and chemical sulfide oxidation in a Beggiatoa inhabited marine sediment. *ISME J*
 571 **1**: 341-353.

572 Russ, L., Kartal, B., Op den Camp, H.J.M., Sollai, M., Le Bruchec, J., Caprais, J.-C. *et al.* (2013)
 573 Presence and diversity of anammox bacteria in cold hydrocarbon-rich seeps and hydrothermal
 574 vent sediments of the Guaymas Basin. *Front Microbiol* **4**: doi: 10.3389/fmicb.2013.00219.

575 Schippers, A., and Sand, W. (1999) Bacterial Leaching of Metal Sulfides Proceeds by Two Indirect
 576 Mechanisms via Thiosulfate or via Polysulfides and Sulfur. *Appl Environ Microbiol* **65**: 319-
 577 321.

578 Schmid, M.C., Risgaard-Petersen, N., Van De Vossenberg, J., Kuypers, M.M.M., Lavik, G.,
 579 Petersen, J. *et al.* (2007) Anaerobic ammonium-oxidizing bacteria in marine environments:
 580 widespread occurrence but low diversity. *Environ Microbiol* **9**: 1476-1484.

581 Schönharting, B., Rehner, R., Metzger, J.W., Krauth, K., Rizzi, M. (1998) Release of nitrous oxide
 582 (N_2O) from denitrifying activated sludge caused by H_2S -containing wastewater:
 583 Quantification and application of a new mathematical model. *Water Sci Technol* **38**: 237-246.
 584 Schulz, H.N., Brinkhoff, T., Ferdelman, T.G., Hernández Mariné, M., Teske, A., and Jørgensen,
 585 B. B. (1999) Dense Populations of a Giant Sulfur Bacterium in Namibian Shelf Sediments.
 586 *Science* **284**: 493-495.
 587 Shao, M.-F., Zhang, T., and Fang, H. (2010) Sulfur-driven autotrophic denitrification: diversity,
 588 biochemistry, and engineering applications. *Appl Microbiol Biotechnol* **88**: 1027-1042.
 589 Sørensen, J, Tiedje, J.M., Firestone, R.B. (1980) Inhibition by sulfide of nitric and nitrous oxide
 590 reduction by denitrifying *Pseudomonas fluorescens*. *Appl Environ Microbiol* **39**: 105-108.
 591 Stewart, F.J., Ulloa, O., and DeLong, E.F. (2012) Microbial metatranscriptomics in a permanent
 592 marine oxygen minimum zone. *Environ Microbiol* **14**: 23-40.
 593 Stewart, V., Lu, Y., and Darwin, A.J. (2002) Periplasmic Nitrate Reductase (NapABC Enzyme)
 594 Supports Anaerobic Respiration by *Escherichia coli* K-12. *J Bacteriol* **184**: 1314-1323.
 595 Strous, M., Kuenen, J.G., and Jetten, M.S.M. (1999) Key Physiology of Anaerobic Ammonium
 596 Oxidation. *Appl Environ Microbiol* **65**: 3248-3250
 597 Strous, M., Pelletier, E., Mangenot, S., Rattei, T., Lehner, A., Taylor, M.W. *et al.* (2006)
 598 Deciphering the evolution and metabolism of an anammox bacterium from a community
 599 genome. *Nature* **440**: 790-794.
 600 Taylor, S., Ninjoor, V., Dowd, D.M., and Tappel, A.L. (1974) Cathepsin B2 measurement by
 601 sensitive fluorometric ammonia analysis. *Anal Biochem* **60**: 153-162.
 602 Thamdrup, B., and Dalsgaard, T. (2002) Production of N_2 through Anaerobic Ammonium
 603 Oxidation Coupled to Nitrate Reduction in Marine Sediments. *Appl Environ Microbiol* **68**:
 604 1312-1318.
 605 Timmer-Ten Hoor, A. (1975) A new type of thiosulphate-oxidizing, nitrate-reducing
 606 microorganism: *Thiomicrospira denitrificans* sp. nov. *Neth J Sea Res* **9**: 344-350.
 607 van de Graaf, A.A., de Bruijn, P., Robertson, L.A., Jetten, M.S.M., and Kuenen, J.G. (1996)
 608 Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed
 609 reactor. *Microbiology* **142**: 2187-2196.

- van de Vossenberg, J., Rattray, J.E., Geerts, W., Kartal, B., Van Niftrik, L., Van Donselaar, E.G. *et al.* (2008) Enrichment and characterization of marine anammox bacteria associated with global nitrogen gas production. *Environ Microbiol* **10**: 3120-3129.
- Vetriani, C., Tran, H.V., and Kerkhof, L.J. (2003) Fingerprinting Microbial Assemblages from the Oxic/Anoxic Chemocline of the Black Sea. *Appl Environ Microbiol* **69**: 6481-6488.
- Wakeham, S.G., Amann, R., Freeman, K.H., Hopmans, E.C., Barker Jørgensen, B., Putnam, I.F., *et al.* (2007) Microbial ecology of the stratified water column of the Black Sea as revealed by a comprehensive biomarker study. *Org Geochem* **38**: 2070-2097
- Wang, H., Tseng, C.-P., and Gunsalus, R.P. (1999) The *napF* and *narG* Nitrate Reductase Operons in *Escherichia coli* Are Differentially Expressed in Response to Submicromolar Concentrations of Nitrate but Not Nitrite. *J Bacteriol* **181**: 5303-5308.
- Wenk, C.B., Blees, J., Zopfi, J., Veronesi, M., Bourbonnais, A., Schubert, C.J. *et al.* (2013) Anaerobic ammonium oxidation (anammox) bacteria and sulfide-dependent denitrifiers coexist in the water column of a meromictic south-alpine lake. *Limnol Oceanogr* **58**: 1-12.
- Woebken, D., Lam, P., Kuypers, M.M.M., Naqvi, S., Kartal, B., Strous, M. *et al.* (2008) A microdiversity study of anammox bacteria reveals a novel Candidatus *Scalindua* phylotype in marine oxygen minimum zones. *Environ Microbiol* **10**: 3106-3119.
- Yan, J., Haaijer, S.C.M., Op den Camp, H.J.M., van Niftrik, L., Stahl, D.A., Könneke, M. (2012) Mimicking the oxygen minimum zones: stimulating interaction of aerobic archaeal and anaerobic bacterial ammonia oxidizers in a laboratory-scale model system. *Environ Microbiol* **14**: 3146-3158.
- Zehr, J.P., and Kudela, R.M. (2011) Nitrogen Cycle of the Open Ocean: From Genes to Ecosystems. *Ann Rev Mar Sci* **3**: 197-225.

Figure legends

Fig. 1: Nitrite-reducing activity of anammox planktonic cells in the presence of different concentrations of Na_2S ($\pm$ S.E. of 4 replicates).

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 ($\uparrow$) 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: Fluorescent in situ hybridization of the reactor system over time, using Kst1240 in Cy3, AMX820 in Cy5 and Gam781 in FLUOS. *K. stuttgartiensis* is visible in pink due to double binding of Kst1240 and AMX820, other anammox bacteria color 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 .

Fig. 4: $^{29}\text{N}_2$ production of the reactor system from $^{15}\text{NH}_4^+$ and $^{14}\text{NO}_3^-$ during 8 h of operation as a fed batch (headspace volume = 1.5 l).

Fig. 5: $^{29}\text{N}_2$ and $^{30}\text{N}_2$ production of the reactor system from $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$ during 8 h of operation as a fed batch (headspace volume = 1.5 l).

Fig. 6: $^{29}\text{N}_2$ and $^{30}\text{N}_2$ production in batch activity assays using co-culture biomass and differentially labeled substrates.

Supplementary Table 1: Description of FISH probes used in this study

Name	Target organism	Sequence (5'- 3')	Labeling dye	Formamide	Reference
Kst1240	<i>Kuenenia stuttgartiensis</i>	CTTAGCATCCCTTTGTACCGACC	Cy3	20-30%	This study
AMX820	All anammox	AAAACCCCTCTACTTAGTGCCC	Cy5	20-30%	Schmid et al. 2001
Gam781	<i>Sedimenticola</i> -like	AGAGCCAAGATGCTCCCAAC	FLUOS	20-40%	This study
Gam784	<i>Sedimenticola</i> -like	GCCAAGATGCTCCCAACAAC	FLUOS	20-40%	This study

Supplementary Table 2. Genes of *Sedimenticola* sp. involved in nitrate reduction and sulfide oxidation

Pathway	Gene number	Contig	Gene	% <i>S. selenatireducens</i> (blastn)	Function
Cytoplasmic dissimilatory nitrate reduction	2415	396	<i>narG1</i>	91	Respiratory nitrate reductase alpha chain
	2985	54	<i>narG2</i>	98	
	2986	54	<i>narH</i>	97	
	2988	54	<i>narJ</i>	98	
	2989	54	<i>narI</i>	95	
Periplasmic dissimilatory nitrate reduction	1643	278	<i>napA</i>	90	periplasmic nitrate reductase, large subunit
	2257	370	<i>napH1</i>	97	Polyferredoxin
	3272	69	<i>napH2</i>	96	
	2258	370	<i>napB</i>	94	Nitrate reductase cytochrome c550-type subunit
	2662	454	<i>napA</i>	97	Periplasmic nitrate reductase precursor
	2663	454	<i>napF</i>	94	Ferredoxin-type protein
	2259	370	<i>napC1</i>	97	Cytochrome c-type protein
	2882	515	<i>napC2</i>	100	
	470	149	<i>napC3</i>	96	Ferredoxin-type protein
	3271	69	<i>napG</i>	98	
Dissimilatory nitrite reduction to NO	469	149	<i>nirS</i>	97	cytochrome cd1 nitrite reductase
	473	149	<i>nirC</i>	96	cytochrome c55X precursor
	474	149	<i>nirF</i>	98	heme d1 biosynthesis protein
	475	149	<i>nirD</i>	98	heme d1 biosynthesis protein
	476	149	<i>nirL</i>	97	heme d1 biosynthesis protein
	477	149	<i>nirG</i>	97	heme d1 biosynthesis protein
	732	177	<i>nirE</i>	99	uroporphyrinogen-III methyltransferase
	2480	405	<i>nirJ1</i>	94	heme d1 biosynthesis protein
	2694	458	<i>nirJ2</i>	97	
	2481	405	<i>nirN1</i>	94	cytochrome cd1 nitrite reductase associated cytochrome
	2693	458	<i>nirN2</i>	97	
Nitric oxide reduction	390	14	<i>norE1</i>	96	nitric oxide activation protein
	552	158	<i>norE2</i>		
	554	158	<i>norQ</i>	98	nitric oxide activation protein
	556	158	<i>norD</i>	98	nitric oxide activation protein
Nitrous oxide reduction	84	69	<i>nosD</i>	99	nitrous oxide reductase maturation protein
	3263	69	<i>nosZ</i>	96	nitrous oxide reductase
	3270	69	<i>nosD</i>	98	nitrous oxide reductase maturation protein
	3276	69	<i>nosY</i>	97	nitrous oxide reductase maturation trans-membrane protein
Sulfide oxidation	2723	469	<i>sqr</i>	97	Sulfide-quinone reductase
	2388	390	<i>fccA1</i>	97	Cytochrome subunit of sulfide dehydrogenase precursor
	560	158	<i>fccA2</i>	98	
	2389	390	<i>fccB1</i>	98	Sulfide dehydrogenase flavoprotein chain
	561	158	<i>fccB2</i>	98	
Thiosulfate oxidation	3121	59	<i>soxB</i>	91	Sulfur oxidation protein
	3123	59	<i>soxZ1</i>	95	Sulfur oxidation protein
	1571	266	<i>soxZ2</i>	95	
	3124	59	<i>soxY1</i>	94	Sulfur oxidation protein
	1570	266	<i>soxY2</i>	93	
	3127	59	<i>soxA1</i>	95	sulfur oxidation protein
	1569	266	<i>soxA2</i>	94	
	3128	59	<i>soxX1</i>	97	Sulfur oxidation protein
	1568	266	<i>soxX2</i>	92	

	862	190	<i>soxH</i>	98	SoxH protein, homolog
	1000	204	<i>soxW1</i>	99	
	387	139	<i>soxW2</i>	95	thioredoxin SoxW
Sulfite reduction/oxidation	1228		<i>dsrK1</i>		
	3644	23	<i>dsrK2</i>	96	Sulfite reduction-associated complex DsrMKJOP protein DsrK
	3645		<i>dsrK3</i>		
	1229	23	<i>dsrM</i>	96	Sulfite reduction-associated complex DsrMKJOP protein
	1230		<i>dsrC1</i>		
	1239	23	<i>dsrC2</i>	99	tRNA 2-thiouridine synthesizing protein E
	2049		<i>dsrC3</i>		
	1231	23	<i>dsrH1</i>	97	
	3541	90	<i>dsrH2</i>	78	tRNA 5-methylaminomethyl-2-thiouridine synthase TusB
	1232	23	<i>dsrF1</i>	98	
	3542	90	<i>dsrF2</i>	84	tRNA 5-methylaminomethyl-2-thiouridine synthase TusC
	1233	23	<i>dsrE1</i>	99	
	3543	90	<i>dsrE2</i>	88	tRNA 5-methylaminomethyl-2-thiouridine synthase TusD
	1234	23	<i>dsrB1</i>	99	
	3544	90	<i>dsrB2</i>	89	dissimilatory sulfite reductase beta subunit
	1235	23	<i>dsrA1</i>	98	
	3545	90	<i>dsrA2</i>	86	dissimilatory sulfite reductase alpha subunit
	3604	95	<i>dsrL</i>	96	Glutamate synthase-like, clustered with sulfite reductase
	3605	95	<i>dsrJ</i>	99	Sulfite reduction-associated complex multiheme protein
	3606	95	<i>dsrO</i>	95	Sulfite reduction-associated complex iron-sulfur protein
	3607	95	<i>dsrP</i>	96	Sulfite reduction-associated complex protein DsrP
	3608	95	<i>dsrN</i>	98	Cobyrinic acid A,C-diamide synthase
	3609	95	<i>dsrR</i>	99	IscA-like protein
APS reduction/oxidation	2854		<i>aprA1</i>	93	
	2855	508	<i>aprA2</i>	93	Adenylylsulfate reductase alpha-subunit (EC 1.8.99.2)
	2856	509	<i>aprA3</i>	89	
	2870	512	<i>aprB1</i>		
	2871	513	<i>aprB2</i>		Adenylylsulfate reductase beta-subunit
	1434	251	<i>aprM</i>	90	Adenylylsulfate reductase membrane anchor
Sulfur oxidation	3632	97	<i>sat</i>	99	Sulfate adenylyltransferase, dissimilatory-type