Interactions of anaerobic ammonium oxidizers and sulfide-oxidizing bacteria in a substrate-limited model system mimicking the marine environment

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Abstract

In nature anaerobic ammonium oxidation (anammox) and denitrification processes convert fixed nitrogen to gaseous nitrogen compounds, which are then released to the atmosphere. While anammox bacteria produce N_2 from ammonium and nitrite, in the denitrification process nitrate and nitrite are converted to N_2 and the greenhouse gas nitrous oxide (N_2O). Furthermore, nitrite needed by the anammox bacteria can be supplied by nitrate reduction to nitrite. Consequently, the interplay between nitrogen-transforming microorganisms control the amount of harmless N_2 or the greenhouse gas N_2O released to the atmosphere. Therefore, it is important to understand the interactions of these microorganisms in the natural environment, where dynamic conditions result in fluctuating substrate concentrations. Here, we studied the interactions between the sulfide-oxidizing denitrifier *Sedimenticola selenatireducens* and the anammox bacterium *Scalindua brodae* in a bioreactor mimicking the marine environment by creating sulfide, ammonium and nitrate limitation in distinct operational phases. Through a microbial interaction, *Se. selenatireducens* reduced nitrate to nitrite, which together with the supplied ammonium was converted to N_2 by *Sc. Brodae*. Using comparative transcriptomics, we determined that *Sc. Brodae* and *Se. selenatireducens* had significant responses to ammonium and nitrate limitation, respectively, indicating that the activities of these microorganisms are regulated by different nitrogen compounds.

Introduction

Loss of fixed nitrogen from the environment proceeds via two distinct processes: denitrification and anaerobic ammonium oxidation (anammox) (Kuypers *et al.*, 2018). Microorganisms that perform these processes require suitable electron donors and oxidized nitrogen species (i.e. nitrate or nitrite) to ultimately produce gaseous nitrogenous end-products: N_2 for anammox and predominantly N_2O and N_2 for denitrification. Denitrification starts with the reduction of nitrate (NO_3^-) to nitrite (NO_2^-) followed by its stepwise reduction to dinitrogen gas ($NO_2^- \rightarrow NO \rightarrow N_2O \rightarrow N_2$), where any of the intermediate products can also be released to the environment. N-oxide-reducing microorganisms use either an organic (organotrophic) or an inorganic (lithotrophic) compound as electron donor. In the anammox process NO_2^- is first reduced to NO, which is then combined with ammonium (NH_4^+) to form hydrazine (N_2H_4), which is further oxidized to form N_2 (Kartal *et al.*, 2011). NO_2^-, the electron acceptor in the anammox pathway, occurs at low concentrations in nature. This implies that anaerobic ammonium-oxidizing bacteria are dependent on other nitrogen transformations for the supply of the bulk of their electron acceptor nitrite, which can be produced either via nitrate reduction or aerobic oxidation of ammonium. Both processes have been shown to indirectly contribute to nitrogen release by supplying significant amounts of NO_2^- to drive the anammox process in both manmade and natural ecosystems such as the oxygen minimum zones (OMZ) in the ocean (Kuypers *et al.*, 2005, Lam *et al.*, 2007, Lam *et al.*, 2009, Lam & Kuypers, 2011). In the OMZs, under oxygen limitation, aerobic ammonia-oxidizing archaea and microorganisms that reduce nitrate provide anammox bacteria with nitrite depending on the availability of suitable substrates (Lam *et al.*, 2007, Lam *et al.*, 2009, Lam & Kuypers, 2011). At times, sulfide produced in the sulfidic zones underlying the OMZ diffuses into the OMZ, thereby fueling nitrate reduction to nitrite and in turn anaerobic ammonium oxidation (Lavik *et al.*, 2009, Schunck *et al.*, 2013, Callbeck *et al.*, 2018, Kuypers *et al.*, 2018).

Laboratory-scale model systems in continuous bioreactors mimicking the environment have been used to achieve a better understanding of how the anammox process and other nitrogen-transforming processes interact. In such systems, it was shown that ammonia-oxidizing archaea and/or bacteria could indeed provide nitrite for anammox bacteria under oxygen limitation (Sliekers *et al.*, 2002, Yan *et al.*, 2012). Furthermore, a coculture of a sulfide-oxidizing nitrate reducer closely related to *Sedimenticola selenatireducens*, and the anammox bacterium *Scalindua brodae* was used to demonstrate that when supplied with ammonium, nitrate and sulfide, sulfide-dependent nitrate reduction produces nitrite to support anammox activity even though sulfide appears to be toxic to anammox bacteria (Russ *et al.*, 2014). In this bioreactor, *Se. selenatireducens* oxidized sulfide to sulfate, and kept sulfide concentration below detection limit while reducing nitrate to nitrite. The produced nitrite and the supplied ammonium were converted to N_2 by the anammox bacteria.

Under these conditions, *Se. selenatireducens* was electron donor (sulfide) limited and the anammox bacteria were electron acceptor (nitrite) limited. In the natural environment substrate fluxes can vary greatly, at many instances they become limiting, and microorganisms have to respond to these changes by regulating their metabolic activity. Mimicking the natural environment in a continuous bioreactor, including the changes that can occur in substrate fluxes, is a powerful approach that can be used to unravel how microorganisms regulate their metabolisms in response to fluctuations in substrate fluxes (Russ *et al.*, 2014, Arshad *et al.*, 2017).

The involved regulation can be determined at the molecular level by observing the differential transcription of key genes involved in catabolic and anabolic biochemical reactions in the cell. Here, we determined the changes in gene expression of anammox bacteria and the denitrifier *Se. selenatireducens* in a model system mimicking environmental conditions (Russ *et al.*, 2014). By comparing the sulfide-limited baseline condition to ammonium and nitrate limitation in distinct experiments, we observed that *Se. selenatireducens* responded significantly to nitrate

limitation, whereas for the *Sc. brodae* the effect of ammonium limitation was the most prominent.

Materials and Methods

Reactor operation

A bioreactor containing a co-culture of *Scalindua brodae* and the sulfur-oxidizing *Sedimenticola selenatireducens* was operated in this study as described by Russ et al. 2014 (Russ *et al.*, 2014). When the reactor was operated under the control conditions the influent contained 9 mM of nitrate and 7 mM of ammonium corresponding to a load of 240 µM NO_3^- $\cdot d^{-1}$ and 187 µM $NH_4^+ \cdot d^{-1}$. Sodium sulfide was added separately from an anaerobic 6 mM stock solution at a flow rate of 90-100 ml$\cdot d^{-1}$ (540-600 µM $Na_2S \cdot d^{-1}$), and was limiting throughout the experiments. The concentrations of ammonium and nitrate were adjusted depending on the experimental conditions. For the ammonium-limiting condition, ammonium concentration was lowered from 7 mM to 1 mM keeping the influent medium flowrate constant. After ammonium limitation was achieved, cells were harvested for (meta)transcriptome analysis, and the influent ammonium concentration was restored to 7 mM. In a separate experiment, nitrate concentration was lowered from 9 mM to 1 mM keeping the influent medium flowrate constant. After the nitrate concentration in the reactor was below the detection limit (< 200 nM), cells were harvested for (meta)transcriptomic analyses. Ammonium and nitrate limitation experiments were performed twice to obtain a biological replicate.

RNA isolation and library preparation

RNA was extracted from 12 ml biomass using the TRIzol® Reagent (Life Technologies, USA) following the manufacturer's instructions. RNA concentrations were determined spectrophotometrically using NanoDrop (Thermo Scientific). 10 µg total RNA was used in the MICROBExpress™ kit (Life Technologies, USA) to enrich bacterial mRNAs by specifically removing 16S and 23S rRNA. The sample was eluted in 25 µl nuclease free water. An

additional cleaning step was performed to clean the sample from 5S rRNA and tRNA using the MEGAclear™ Kit (Life Technologies, USA). 24 µl of sample from the previous purification step was used and the RNA quantity was checked on the NanoDrop and 2100 Bioanalyzer (Agilent Technologies, USA) yielding 170 to 220 ng mRNA per sample. Further library preparation for IonTorrent sequencing was performed using the Ion Total RNA-Seq Kit v2 for whole transcriptome libraries (Life technologies, USA) according to the manufacturer's instructions, incubating the sample with RNAse III for 7 min. 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, USA).

Data analysis

Reads were length and quality score trimmed (>50 bp, 0.05) with CLC genomics workbench 11 (Qiagen) resulting in datasets that were submitted to the European Nucleotide Archive (ENA) (accession numbers ERS3140201 to ERS3140209). After trimming, reads were mapped to the genomes of *Sc. brodae* (Speth *et al.*, 2015) and *Se. selenatireducens* DSM 17993 using the STAR alignment software 2.7.0 adjusted to analysis of small genomes as is described in the software manual (Dobin *et al.*, 2013). Gene expression counts were obtained with htseq-count version 0.11.1 and analyzed with DESeq2 version 1.14.1 (Love *et al.*, 2014) in R comparing the different ammonium- and nitrate-limiting conditions to the sulfide-limiting baseline condition according to the manual.

Analytical methods

Nitrite was measured colorimetrically at 540 nm after a 5 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% ortho-phthaldialdehyde as has been described previously (Taylor *et al.*, 1974). Nitrate was measured by converting nitrite and nitrate in the samples to nitric oxide in a saturated solution of VCl_3 in 1M HCl at 95 °C. NO was then

measured by the nitric oxide analyzer NOA280i (GE Analytical Instruments, USA) according to the manufacturer's instructions.

Results and Discussion

In natural and manmade environments, anaerobic ammonium-oxidizing (anammox) bacteria largely depend on the activity of ammonia-oxidizing or nitrate-reducing microorganisms for the supply of their substrate nitrite (Lavik *et al.*, 2009, Kartal *et al.*, 2010). Reduction of nitrate to nitrite can be achieved by a myriad of nitrogen-transforming microorganisms that utilize organic and/or inorganic electron donors such as sulfide (Lavik *et al.*, 2009, Canfield *et al.*, 2010, Schunck *et al.*, 2013, Callbeck *et al.*, 2018, Kuypers *et al.*, 2018). For example, sulfide-rich waters can intrude into the oxygen minimum zones (OMZ) of the ocean, and support sulfide-dependent nitrate reduction to nitrite, which in turn fuels anaerobic ammonium oxidation (Lavik *et al.*, 2009, Canfield *et al.*, 2010, Schunck *et al.*, 2013, Callbeck *et al.*, 2018). Such an interaction was recreated earlier in a continuous bioreactor, where a coculture of anammox bacteria and a sulfide-dependent nitrate-reducing *Se. selenatireducens* were supplied with ammonium, nitrate and sulfide (Russ *et al.*, 2014). Here, *Se. selenatireducens* reduces nitrate to nitrite while oxidizing sulfide, which is toxic to anammox bacteria. Through the activity of *Se. selenatireducens* sulfide is kept at limiting low micromolar concentrations (<5 μM) so that anammox bacteria are not inhibited. The anammox bacteria convert the produced nitrite and the supplied ammonium, via nitric oxide (NO) and hydrazine, to N_2 and nitrate (Russ *et al.*, 2014). Under these growth conditions, anammox bacteria are nitrite limited. Here, we investigated how these two clades of microorganisms respond to ammonium or nitrate limitation, which occurs under the dynamic conditions of the natural environment (Kuypers *et al.*, 2018).

In our bioreactor, under the baseline sulfide-limiting conditions ammonium, nitrate and sulfide were supplied and there was a remainder of nitrate (5.1 mM and 1.7 mM at time of first and

second sampling, respectively) and ammonium (30 μM and 2.7 mM at time of first and second sampling, respectively); NO_2^- and sulfide were below detection limit (<5 μM) (Figure 1A). In two distinct time periods, the co-culture was subjected to nitrate or ammonium limitation. The substrate limitation experiments were performed twice to achieve real biological replicates. The microbial community did not change throughout the two experimental phases.

Under the first ammonium-limiting conditions ammonium and sulfide concentrations in the bioreactor were below detection limit (10 and 5 μM, respectively), and there was excess nitrate (3.7 mM). Due to ammonium limitation, the activity of the anammox bacteria was restricted, which resulted in nitrite accumulation in the reactor up to 191 μM (Figure 1A). Nitrite accumulation suggested that under sulfide (electron donor) limitation, *Se. selenatireducens* favored nitrate reduction to nitrite rather than performing complete reduction of nitrate to N_2.

Under the first nitrate-limiting conditions nitrite and nitrate were below detection limit (200 nM) and there was a residual ammonium concentration of 25 μM (Figure 1B). After the first sets of experiments were completed, the bioreactor was returned to the sulfide-limited baseline conditions where both nitrate and ammonium were in excess, and after a stabilization period, nitrate and ammonium limiting conditions were recreated separately to have biological replicates for the tested conditions. In the second experimentation period when the reactor was ammonium limited, ammonium concentration was below detection limit (<10 μM), there was 1.2 mM nitrate in the reactor and nitrite accumulated to 88 μM (Figure 1B). Under the second nitrate limitation period nitrate, nitrite and sulfide concentrations were below detection limit whereas the ammonium concentration in the reactor was 2.95 mM (Figure 1B).

The bioreactor was sampled for transcriptome analysis after 1-2 days of exposure to either ammonium or nitrate limitation. Transcripts derived from the ammonium or nitrate limitation conditions were compared to the sulfide-limiting baseline condition where nitrate and ammonium were in excess. The molecular responses of the two key players in the bioreactor to differential short-term substrate limitation was examined in two biological replicates (Figure

2AB). Genes that were considered for further analysis showed a differential expression by at least 4-fold and their adjusted P-value (padj value) was below 0.1 between the sulfide-limiting baseline condition and either nitrate or ammonium limited condition thereby making data interpretation more robust. The changes in gene expression in *Sc. brodae* were more prominent under ammonium-limiting conditions (Figure 2A). 213 genes were either up- or downregulated significantly, whereas under nitrate limitation significant changes only occurred in the expression of 5 genes. The latter was not surprising as *Sc. brodae* was limited in nitrite both under the sulfide-limited baseline condition and nitrate-limiting condition. *Se. selenatireducens* differentially expressed 261 genes under nitrate limitation, whereas ammonium limitation resulted in the up- or downregulation of 14 genes. As nitrate serves as an electron acceptor in sulfide oxidation the effect of nitrate limitation on differential gene expression in *Se. selenatireducens* (Figure 2B) is stronger.

Se. selenatireducens uses ammonium only as a nitrogen source, whereas the anammox bacteria use it as an electron donor and nitrogen source. The catabolic reactions of anammox bacteria take place in a membrane-bound intracellular compartment called the anammoxosome (van Teeseling *et al.*, 2013), where all the key enzymes of these microorganisms are located (de Almeida *et al.*, 2016). In order to reach the catabolic machinery, ammonium and nitrite have to be transported through two sets of membranes, that surround the cytoplasm and the anammoxosome. To this end, anammox bacteria encode an array of transporters. The genome of the *Sc. brodae* in our bioreactor encodes 7 different ammonium transport proteins (scabro_02011, scabro_02017, scabro_02143, scabro_02145, scabro_02151, scabro_02194, scabro_02195)(Speth *et al.*, 2015). When ammonium was limiting, the transcription of scabro_02143, scabro_02145, scabro_02147 and scabro_02151 were upregulated, 20, 11, 101 and 4-fold, respectively (Figure 3). Interestingly, the two P-II regulatory proteins encoded in the vicinity of these ammonium transporters, scabro_02144 and scabro_02148, were also upregulated 25 and 73-fold, respectively (Figure 3). It could be speculated that in *S. brodae*

cells, each membrane, cytoplasmic and anammoxosome, contains one regulatory protein and two ammonia transporters to facilitate the efficient delivery of ammonium to hydrazine synthase located in the anammoxosome(de Almeida *et al.*, 2015, Pfluger *et al.*, 2018).

The apparent affinity of *S. brodea* for ammonium was estimated to be 200 nM under the growth conditions applied in this study (Yan, 2012). This leaves *Se. selenatireducens* with quite a challenge to scavenge ammonium for assimilation, when ammonium is limiting in the bioreactor. Indeed, under ammonium limitation, not many of the key genes of *Se. selenatireducens* were significantly affected, but the transcription of both its genes encoding *amtB* ammonium transporters (WP_029134270 and WP_029133680) were upregulated (6- and 58-fold, respectively) (Figure 4). Furthermore, as in the case with *Sc. brodae*, also the transcription of the P-II type regulatory protein (WP_029133681) was upregulated (39-fold) as a response to decreasing ammonium concentration in the bioreactor (Figure 4).

Following its transport into the cell, ammonia can be assimilated via two main pathways. One of these is a low-affinity system, where glutamate dehydrogenase (GDH) produces glutamate by the reductive amination of 2-oxoglutarate. The other one is a high-affinity system (GS-GOGAT), where glutamine synthase first produces glutamine, which is in turn converted to glutamate via the transfer of the amide group of glutamine to 2-oxoglutarate by glutamate synthase (Yan, 2007, Gunka & Commichau, 2012). *Sc. brodae* favored the high-affinity system for ammonium assimilation as the GDH (scabro_01753) was hardly expressed under all conditions. Although the high-affinity GS-GOGAT system was expressed under all conditions, both components were upregulated when ammonium was limiting. The putative glutamine synthase of *Sc. brodae* (scabro_00522) was upregulated by 4-fold and the large and small subunits (scabro_02334 and scabro_02335) of glutamate synthase were upregulated by 3 and 4-fold, respectively, albeit these values are below our Log_2-fold cut-off value of 2. In *Se. selenatireducens* the GDH pathway was not transcribed in high levels, suggesting that also for this microorganism the GS-GOGAT was the preferred ammonium assimilation pathway. Under

ammonium limitation, glutamine synthase (WP_029132173) representing the first step in the GS-GOGAT system was up-regulated 7-fold (Figure 4). *Se. selenatireducens* might also use nitrite as an additional N-source under ammonium limitation by reducing nitrite, which transiently accumulated in the bioreactor, to ammonia by the assimilatory nitrite reductase (*nirB*, WP_029134437), which was upregulated 13-fold (Figure 4). Conversely, the catalytic subunit A (*napA*) of the dissimilatory nitrate reductase was the only protein significantly downregulated (5-fold). Albeit below our cutoff value, also one of the subunits of the dissimilatory nitrite reductase (WP_029134679) was also downregulated (2-fold) (Figure 4). It is conceivable that under ammonium limitation the *Se. selenatireducens* regulated the transcription of its dissimilatory and assimilatory nitrite/nitrate reduction pathways, thereby regulating the use of these compounds as N-source or electron acceptors.

The transient accumulation of nitrite was also reflected in the transcription of nitrite transporters by *Sc. brodae*, which encodes four transporters that belong to the formate/nitrite transporter family that are likely to be involved in nitrite translocation(van de Vossenberg *et al.*, 2013). One of these share the highest similarity to *nirC* (scabro_03681) and the other two with "formate transport systems" *focA* (scabro_03528 and scabro_03529). NirC and FocA proteins are not selective for nitrite and formate, respectively; both transporters can translocate both anions, but usually with lower affinity than their dedicated substrates (Lü et al., 2012). The *nirC* gene (scabro_03681) was the highest transcribed FNT family transporter under nitrite-limited control conditions, suggesting that scabro_03681 could be a dedicated high-affinity nitrite transporter. In line with this observation, when nitrite accumulated in the bioreactor under ammonium-limiting conditions, scabro_03681 was 6-fold downregulated. Scabro_03683, which encodes a histidine kinase like protein, was 12-fold downregulated, indicating that scabro_03683 could be the sensory protein controlling the transcription of the scabro_03681 nitrite transporter. Furthermore, two other genes in this gene cluster, scabro_03682 and scabro_03684 were downregulated 12 and 6-fold, respectively (Figure 3). Although

scabro_03682 and scabro_03684 were annotated as "hypothetical proteins", they had conserved domains implicated in signal transduction. It is conceivable that scabro_03681-84 gene cluster was responsible for detection of nitrite and the regulation of its transport into the cells. Under control conditions nitrite was not detectable in the bioreactor, therefore nitrite uptake most likely required a high-affinity transporter. However, when ammonium was limiting, nitrite accumulated in the reactor, possibly requiring a transport system with a lower affinity for nitrite. Under ammonium limitation, the *focA*-like genes, scabro_03528 and scabro_03529, were upregulated 8-fold and 6-fold, respectively (Figure 3). These two proteins could potentially fulfill the role of low-affinity nitrite transporters. Alternatively, nitrite transport into the cell might be restricted in general, as the shortage of ammonium would slow down downstream anammox reactions. This would suggest a first line regulation of metabolism, preventing high intracellular nitrite concentration. Still, it should be considered that nitrite, just like ammonium, has to pass the cytoplasmic as well as the anammoxosome membrane, each of which require specific transporters.

In the anammoxosome nitrite and ammonium are transformed to N_2 and nitrate via the concerted activity of five key proteins (Kartal *et al.*, 2011, de Almeida *et al.*, 2016). Here, a cytochrome cd_1 nitrite reductase (encoded by *nirS*) reduces nitrite to NO, which is condensed into hydrazine via hydrazine synthase (HZS) by the input of ammonium and three electrons (Kartal *et al.*, 2011, Dietl *et al.*, 2015). It has been proposed that hydrazine synthase loses its enzyme-bound intermediate, hydroxylamine, which is oxidized back to NO by a hydroxylamine oxidoreductase (HOX) (Maalcke *et al.*, 2014, Dietl *et al.*, 2015). The oxidation of hydrazine by hydrazine dehydrogenase (HDH) releases four low-potential electrons (Maalcke *et al.*, 2016), which are used for hydrazine synthesis and cell carbon fixation (Kartal *et al.*, 2013, Hu *et al.*, 2019)). Finally, a nitrite oxidoreductase (NXR) oxidizes nitrite to nitrate, delivering electrons necessary for nitrite reduction to NO (Kartal *et al.*, 2011, Hu *et al.*, 2019).

Sc. brodae highly expresses cytochrome cd_1 nitrite reductase encoded by *nirS* (scabro_03875)(van de Vossenberg *et al.*, 2013, Speth *et al.*, 2015) . In addition, these species encode two hydroxylamine oxidoreductase (HAO)-like octaheme proteins (scabro_03687 and scabro_03890), which have been suggested to reduce nitrite to NO based on sequence analyses (Kartal *et al.*, 2013). Interestingly, unlike nitrite transporters, the proteins implicated in nitrite reduction were not downregulated except scabro_03890, which was downregulated 3-fold, which was below our cutoff value. Conversely, the catalytic subunits of the nitrite:nitrate oxidoreductase was down-regulated under ammonium limitation. *nxrB* (scabro_01072) and *nxrC* (scabro_01071) were downregulated 5 and 4-fold, respectively; whereas *nxrA* (scabro_01076) was also downregulated, but the downregulation factor was below our cutoff value (2-fold).

Anammox bacteria activate ammonium using nitric oxide through the activity of hydrazine synthase (HZS)(Kartal *et al.*, 2011, Dietl *et al.*, 2015). Instead of the three subunits (αβγ) observed in other anammox bacteria, in *Scalindua* species HZS is composed of two subunits as the genes encoding β and γ subunit of this enzyme are fused (scabro_01046) and scabro_01598 encodes for the α subunit of HZS (van de Vossenberg *et al.*, 2013, Speth *et al.*, 2015, Kartal & Keltjens, 2016). Similar to its high expression in *K. stuttgartiensis* (Kartal *et al.*, 2011), HZS was also highly expressed in *Scalindua* (van de Vossenberg *et al.*, 2013). In our bioreactor genes encoding HZS (scabro_01046 and scabro_01598) were highly transcribed under all experimental conditions. Still, short-term ammonium limitation triggered a 7-fold upregulation of *hzsA* (scabro_01598) and a 6-fold *hzsBC* (scabro_01046) (Figure 3). The ortholog of HOX (scabro_01597) that recycles hydroxylamine back to NO (Maalcke *et al.*, 2014), was highly transcribed in all conditions, and its transcription did not show any significant change. Hydrazine synthesis was suggested to be the rate-limiting step in the overall anammox reaction under the sulfide-limited baseline conditions (Kartal *et al.*, 2011). Under ammonium limitation this bottleneck might become even tighter as the enzyme was not only limited by its own

reaction speed, but also by the limited supply of ammonium due to the unfavorable ammonium to nitrite ratio; furthermore, it would have to compete with the GOGAT ammonium assimilation machinery. This might result in an increased transcription of the HZS enzymatic subunits to increase the chance of converting ammonium as efficiently as possible. Once hydrazine is synthesized, it is oxidized to N_2 by hydrazine dehydrogenase (*hdh*) (Maalcke *et al.*, 2016). The electrons released from this reaction reaction are used for catabolic and anabolic reactions(Kartal *et al.*, 2013, Hu *et al.*, 2019) . Under ammonium limitation, the transcription of *hdh* (scabro_01590) was slightly downregulated at 2-fold (Figure 3). Although it is below our cutoff value, this down-regulation is most likely due to the decreasing flux of hydrazine through the upstream catabolic reactions.

The low-potential electrons derived from hydrazine oxidation are used for hydrazine synthesis and energy conservation. Anammox bacteria encode several Rieske/cytochrome *b* complexes similar to quinol:cytochrome c oxidoreductase (complex III), containing a Rieske-type iron-sulfur protein, a membrane-bound heme b subunit and a c-type heme subunit(Kartal *et al.*, 2013). These complexes are suggested to link the electron released from hydrazine oxidation to the reduction of the Q pool, and thereby generate a proton motive force, which is in turn used by an ATP synthase to generate ATP (Kartal & Keltjens, 2016). Under ammonium limitation, the genes in *Sc. brodae* that encode the Rieske/cyt*b* proteins (scabro_03884-88) homologous to the highest expressed Rieske/cyt*b* gene cluster in *K. stuttgartiensis* (kuste4569-74), and the associated HAO-like protein (scabro_03890) were slightly downregulated (below 4-fold)(Kartal *et al.*, 2013, de Almeida *et al.*, 2016). Out of the genes cluster that encodes the highest transcribed proton-translocating ATP synthase (scabro_01306-11), only the alpha (scabro_01308) and the gamma (scabro_01310) subunits were significantly downregulated (5 and 4-fold, respectively) (Figure 3).

The generated ATP is used to derive anabolic reactions such as CO_2 fixation into biomass. In anammox bacteria this is achieved via the Wood-Ljungdahl pathway(Schouten *et al.*, 2004,

Strous *et al.*, 2006, van de Vossenberg *et al.*, 2013), where CO_2 is first reduced to CO and consequently to acetyl-CoA catalyzed by a bifunctional cluster of the two enzymes: CO dehydrogenase and acetyl-CoA synthase (CODH/ACS). The CO_2-fixing CODH/ACS complex in *Sc. brodae* was encoded by scabro_03343-49. This cluster included one of the catalytic subunits (*acsB*) as well as *acsCFD* and a putative ferredoxin-like protein belonging to the same cluster. Generally, the carbon fixation pathway was affected by ammonium limitation, possibly as a response by the anammox bacteria to the scarce availability of their energy source, ammonium. Genes in this cluster were downregulated ranging from insignificant 2-fold change to one of the genes that encode a ferredoxin (scabro_03347fold) to 10-fold change for CODH/ACS synthase alpha subunit (scabro_03343) (Figure 3).

The response of *Se. selenatireducens* to ammonium limitation did not result in the down-regulation of many of its key genes with changes not exceeding the 4-fold cutoff value in both sets of experiments. In contrast, nitrate limitation had a significant effect on the transcription of the key genes of *Se. selenatireducens* as it is an organism that is able to reduce nitrate all the way to N_2, *Se. selenatireducens* encodes all the necessary N-oxide reductases. For the first step, nitrate reduction to nitrite, *Se. selenatireducens* possesses both a membrane-bound (*nar*) and a periplasmic (*nap*) nitrate reductase gene cluster. The differential regulation of these two systems in this bacterium is unknown, but in other microorganisms, different nitrate concentrations can favor one nitrate reductase over the other (Wang et al., 1999; Stewart et al., 2002).

Under NO_3^- limitation the genes encoding the α (WP_029132616), β (WP_029132615), γ (WP_029132613) and δ (WP_029132614) subunits of the membrane-bound nitrate reductase (NAR) were downregulated 10, 11, 9 and 4-fold, respectively (Figure 4). Furthermore, the nitrate antiporter (WP_029132617) encoded in the same gene cluster was downregulated 6-fold. Another nitrate transporter from the NarK/NasA family (WP_084609827) was also

downregulated (3-fold) albeit it was below the cutoff value. The periplasmic nitrate reductase (NAP) encoded by *Se. selenatireducens* was not significantly regulated.

In *E. coli* the use of nitrate as terminal electron acceptor and the expression of the nitrate reductase gene is closely coupled to formate dehydrogenase (Wang & Gunsalus, 2003). Under anaerobic conditions formate dehydrogenase oxidizes formate to CO_2 in the periplasm and transfers the electrons via the quinone pool to proteins for the reduction of respiratory substrates. Under denitrifying conditions, the electrons are shuttled to nitrate reductase to subsequently reduce nitrate in the cytoplasm thereby establishing a proton gradient. In *Se. selenatireducens* an N-type formate dehydrogenase (FDH) was encoded in the gene cluster WP_029133272-WP_029133276. The gene was highly transcribed under the sulfide-limiting baseline conditions. From the *fdh* gene cluster the α subunit (WP_029133274) was 8-fold downregulated, and a gene encoding 4Fe-4S iron-sulfur dicluster protein (WP_029133273) was 4-fold downregulated (Figure 4). This was in line with the previously observed regulation pattern as all subunits of the complex were significantly under nitrate-limiting conditions, similar to the downregulation of the *nar* cluster.

The transcription of the cd_1-type nitrite reductase (*nirS*, WP_029134679), catalyzing the second step in denitrification was downregulated 5-fold (Figure 4). The last steps in denitrification involve the reduction NO to N_2O by nitric oxide reductase (*norB*) followed by the reduction of N_2O to N_2 by nitrous oxide reductase (*nosZ*). The transcription of these genes did not change significantly under nitrate limitation (Figure 4)). Previously it was shown that the majority of nitrate was reduced to nitrite by the *Se. selenatireducens* and that only around 35-25% of the produced N_2 was formed through further reduction of nitrite, and apparently nitrate limitation did not affect the transcription of the downstream genes (Russ et al., 2014).

Limitation of nitrate meant that the availability of a suitable electron acceptor for *Se. selenatireducens* was scarce. This also resulted in the down-regulation of many genes involved in the oxidation of its electron donor sulfide (Figure 4). All the key genes involved in the sulfur

metabolism were down-regulated except sulfate adenylyltransferase (WP_029134400*, sat,* 6-fold), which carries out the last step in sulfide oxidation, the conversion of adenylyl sulfate to sulfate. The transcription of the *soxA* (WP_029132144)*, soxX* (WP_084609708)*, soxY* (WP_029132147) and *soxZ* (WP_029132148) genes from the SOX system were 8, 11, 6, 6fold downregulated, respectively (Figure 4). Furthermore, the gene encoding the sulfurtransferase protein (WP_029132146) was 15-fold downregulated. Sulfide dehydrogenase (*fcc*, WP_029133266) and adenylylsulfate reductase (*apr*, WP_029133846) were more than 7 and 5-fold downregulated, respectively (Figure 4).

Similar to *Sc. brodae*, also in *Se. selenatireducens* the decrease in the availability of the substrates resulted in the downregulation of many genes involved in energy conservation. Due to substrate limitation, α (WP_029132215), β (WP_029132213), γ (WP_029132216), δ (WP_029132214) and ε (WP_029132218) subunits of F_OF_1 ATP synthase were downregulated 6, 4, 7, 7 and 4-fold, respectively (Figure 4). The NADH-ubiquinone oxidoreductase subunit J (WP_029135006) of complex I was downregulated (5-fold), pyruvate dehydrogenase (WP_037374574) from the the TCA cycle was downregulated 7-fold (Figure 4). On the other hand, the highest transcribed gene encoding (WP_029132057) the ribulose bisphosphate carboxylase (Rubisco) catalyzing the first step in the CO_2 fixation via the Calvin-Benson-Bassham (CBB) cycle was upregulated 7-fold (Figure 4). Put together, the *Se. selenatireducens* cells were apparently regulating their genes to make the most out of the available electron acceptor that they could import and reduce.

Here, we used a coculture of the anammox bacterium *Sc. brodae* and a sulfide-dependent nitrate reducer *S. selenatireducens.* to study the effect of short-term ammonium and nitrate limitation on the transcription of genes encoding key metabolic enzymes. Our results indicated that *Sc. brodae* responded to ammonium limitation with significant differential expression of its key genes, whereas *Se. selenatireducens* had a more pronounced response to nitrate limitation. It

should be noted that transcriptional regulation and protein expression do not necessarily respond in the same fashion to environmental changes, and neither can always be directly linked to the presence of an observed metabolic activity. Nevertheless, transcriptional regulation of key genes could be used as an indication of the metabolic responses of microorganisms to changes in environmental factors, which can be prevalent in both natural and manmade ecosystems.

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Figure 1

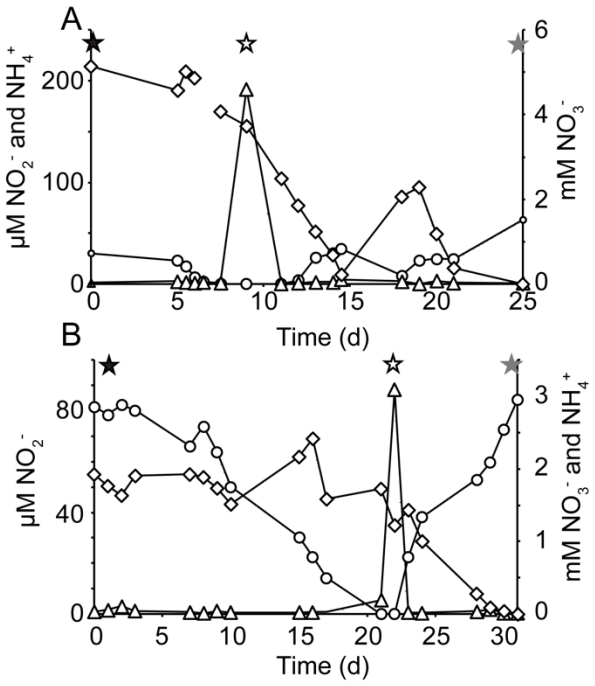

Figure 1. Nutrient concentrations in the bioreactor in the two experimental phases. Panel A and B depict experimental phase 1, and phase 2, respectively. Circles, diamonds and triangles indicate ammonium, nitrate and nitrite, respectively. Filled black, open black and filled gray stars indicate control, ammonium-limited, and nitrate-limited conditions, respectively.

Figure 2

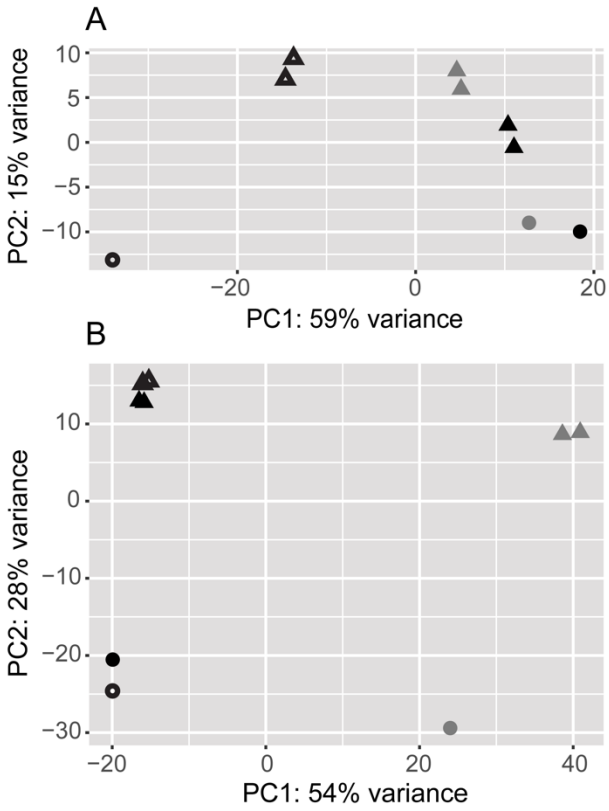

Figure 2. Principal component analysis plot showing sample-to-sample distances between the sulfide limited baseline (filled black), ammonium limited (empty black) and nitrate limited (filled gray) conditions for *Sc. brodae* (A) and *Se. selenatireducens* (B). Filled circles and filled triangles depict experimental phase 1 and phase 2, respectively. The PCA plot is based on the differential expression analysis with the R package DEseq2 (Love et al., 2014).

Figure 3

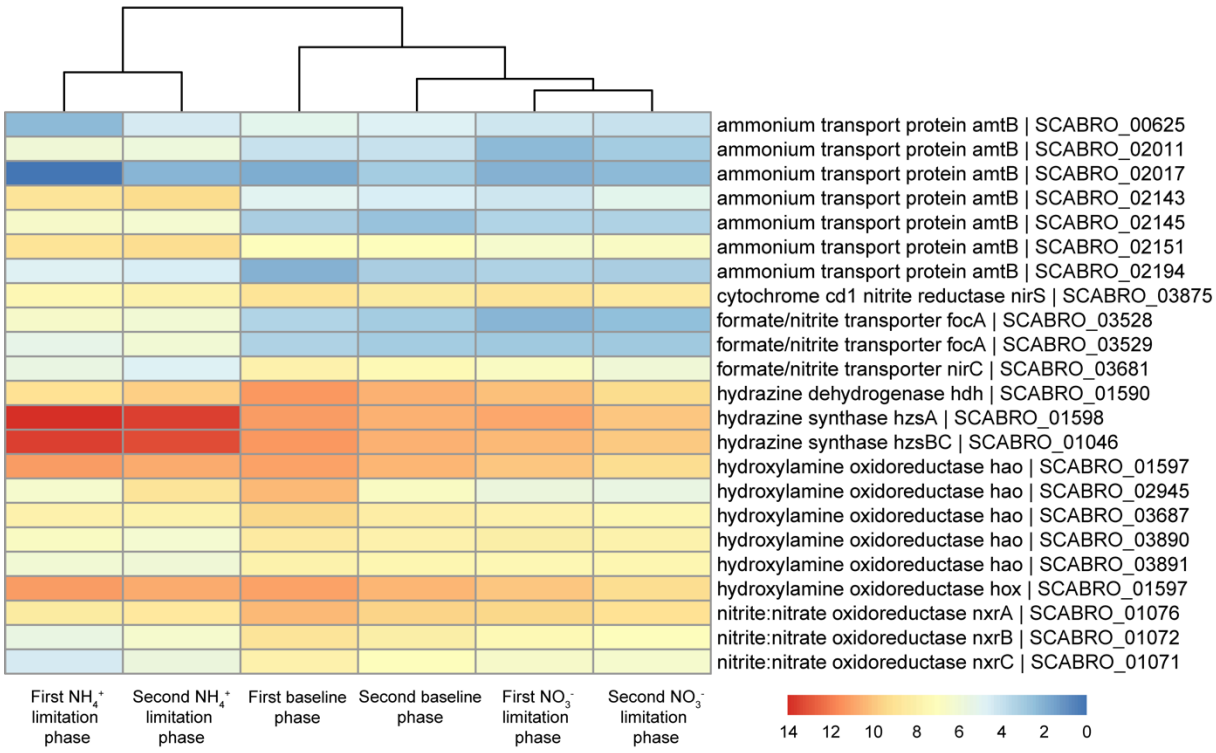

Figure 3. Differential transcription of genes involved in key metabolism of *Scalindua brodae* under 3 different experimental conditions. Heatmap shows $\log_2(n+1)$ for normalized counts (generated by DEseq2). Column clustering is based on Euclidean distance.

Figure 4

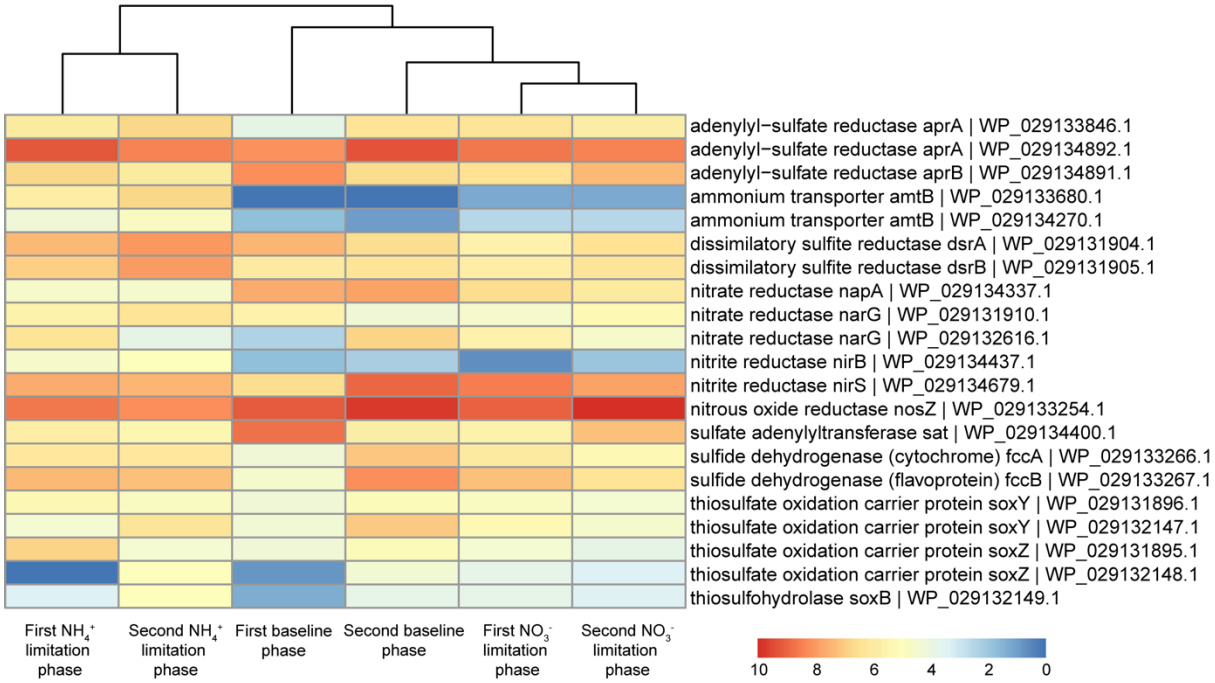

Figure 4. Differential transcription of genes involved in key metabolism of *Sedimenticola selenatireducens* under 3 different experimental conditions. Heatmap shows $\log_2(n+1)$ for normalized counts (generated by DEseq2). Column clustering is based on Euclidean distance.