Mimicking microbial interactions under nitrate-reducing conditions in an anoxic bioreactor: enrichment of novel Nitrospirae bacteria distantly related to *Thermodesulfovibrio*

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Summary

Microorganisms are main drivers of the sulfur, nitrogen and carbon biogeochemical cycles. These elemental cycles are interconnected by the activity of different guilds in sediments or wastewater treatment systems. Here, we investigated a nitrate-reducing microbial community in a laboratory-scale bioreactor model that closely mimicked estuary or brackish sediment conditions. The bioreactor simultaneously consumed sulfide, methane and ammonium at the expense of nitrate. Ammonium oxidation occurred solely by the activity of anammox bacteria identified as *Candidatus Scalindua brodae* and *Ca. Kuenenia stuttgartiensis*. Fifty-three percent of methane oxidation was catalyzed by archaea affiliated to *Ca. Methanoperedens* and 47% by *Ca. Methyloirabilis* bacteria. Sulfide oxidation was mainly shared between two proteobacterial groups. Interestingly, competition for nitrate did not lead to exclusion of one particular group. Metagenomic analysis showed that the most abundant taxonomic group was distantly related to *Thermodesulfovibrio* sp. (87–89%

16S rRNA gene identity, 52–54% average amino acid identity), representing a new family within the Nitrospirae phylum. A high quality draft genome of the new species was recovered, and analysis showed high metabolic versatility. Related microbial groups are found in diverse environments with sulfur, nitrogen and methane cycling, indicating that these novel Nitrospirae bacteria might contribute to biogeochemical cycling in natural habitats.

Introduction

The biogeochemical nitrogen, carbon and sulfur cycles are interdependent on each other, particularly through the activity of microorganisms exchanging metabolites belonging to different cycles (Falkowski *et al.*, 2008). In estuary or coastal anoxic sediments, reactive nitrogen compounds such as ammonium and nitrate discharged from agriculture can be present along with sulfide from the marine environment and methane from deeper methanogenic layers (Egger *et al.*, 2015). Therefore, these ecosystems provide substrates for chemolithotrophic microorganisms involved in the nitrogen, carbon and sulfur cycles. Microorganisms that are capable of performing either partial or full denitrification can convert nitrate or nitrite to molecular nitrogen (N_2), thereby contributing to nitrogen loss from the ecosystem. Anammox bacteria are capable of N_2 production by the anaerobic oxidation of ammonium (NH_4^+) with nitrite (NO_2^-) (van de Graaf *et al.*, 1996). Nitrite is usually not supplied by agricultural discharge but produced during incomplete denitrification. In an estuary, coastal or brackish anoxic ecosystem, sulfide-dependent denitrification may release nitrite (Dong *et al.*, 2000) or reduce nitrate completely to dinitrogen gas (Sorokin *et al.*, 2008). Autotrophic sulfide-dependent denitrifiers have been found to supply nitrite to anammox bacteria in wastewater treatment plants (Mulder *et al.*, 1995; van de Graaf *et al.*, 1996), and this interaction might also occur in natural environments, e.g., marine oxygen minimum zones (Canfield *et al.*, 2010). Indeed, when the interaction of anammox

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bacteria with autotrophic sulfide-dependent denitrifiers was studied in enrichment cultures mimicking marine oxygen minimum zones, it was found that the latter group provided nitrite for the anammox reaction (Russ *et al.*, 2014; Toro and Cervantes, 2016). Besides sulfide, methane can also be oxidized during partial denitrification. Such activity has been demonstrated for the recently discovered nitrate-reducing anaerobic methanotrophic archaea tentatively named *Candidatus* Methanoperedens nitroreducens (Raghoebarsing *et al.*, 2006; Haroon *et al.*, 2013). Furthermore, bacterial anaerobic methanotrophs belonging to the NC10 clade tentatively named *Ca. Methyloirabilis oxyfera* are capable of nitrite-dependent methane oxidation (Ettwig *et al.*, 2010). They have been shown to co-exist with *Ca. M. nitroreducens* where they compete for methane but cooperate for nitrite removal (Haroon *et al.*, 2013). On the other hand, *Ca. M. oxyfera* competes with anammox bacteria for their common substrate nitrite (Luesken *et al.*, 2011; Shi *et al.*, 2013). Under high electron donor to nitrogen ratios, nitrate may not be denitrified but instead be reduced to ammonium by a process termed dissimilatory nitrate reduction to ammonium (DNRA) (Kraft *et al.*, 2011; van den Berg *et al.*, 2015).

Investigating such interactions between nitrogen-, sulfur- and methane-cycling microorganisms is relevant not only to understand natural ecosystems where denitrification is coupled to sulfide and methane oxidation but also to further develop the treatment of moderately saline wastewater produced by the pickling industry, in landfill leachate or through new technologies using sea water for toilet flushing (Xiao and Roberts, 2010).

In this study, we investigated the interactions of chemolithoautotrophic microorganisms involved in the methane, nitrogen and sulfur biogeochemical cycles in an anoxic laboratory-scale bioreactor that mimicked estuary, coastal or brackish sediment nutrient conditions. We monitored the composition of the microbial community as well as their substrate turnover in the bioreactor over a period of 382 days with physiological assays, fluorescence *in situ* hybridization imaging and metagenome sequencing. We observed that cross-feeding of nitrite and competition for nitrate and methane occurred between anammox bacteria, autotrophic sulfide-dependent denitrifiers and nitrate- and nitrite-dependent methanotrophs respectively. Surprisingly, we discovered that the most abundant member of the microbial community, comprising more than 24% of total metagenome reads, was a previously uncultured microorganism distantly related to *Thermodesulfovibrio* species (87–89% 16S rRNA gene identity), representing a new family within the Nitrospirae phylum. A high quality draft genome was assembled and encoded complete respiratory pathways for oxygen, nitrate (DNRA and denitrification to N₂O), sulfate, dimethylsulfoxide and trimethylamine N-oxide, alongside the putative ability to use a wide range

of electron donors, i.e., formate, lactate, sulfur, acetate, pyruvate, hydrogen and carbohydrates. Its environmental distribution indicates that this bacterial group is involved in nutrient turnover in a wide range of nitrogen and sulfur cycling ecosystems.

Results

A denitrifying, methane-oxidizing and sulfide-oxidizing microbial community enriched in a laboratory-scale bioreactor system

A laboratory-scale bioreactor system was inoculated with biomass from a marine enrichment culture containing sulfide-dependent autotrophic denitrifiers and anammox bacteria (Russ *et al.*, 2014) and mixed with biomass from a freshwater methane-oxidizing denitrification culture (Ettwig *et al.*, 2016). The reactor received ammonium, nitrate, methane and sulfide in a 1% NaCl mineral medium and was operated for 382 days. The reactor received 3 mmol nitrate and 1.4 mmol ammonium per day of which 2.3 and 1.2 mmol were consumed respectively. Furthermore, the daily added 0.29 mmol sulfide was almost completely consumed by the microbial community as the residual amount of sulfide in the bioreactor was less than 0.3 µmol. Methane oxidation rates could not be determined in the continuous bioreactor as the consumption was relatively low compared to the added methane flux. The methane oxidation rates were therefore determined in the bioreactor under batch mode. No nitrite was added to the bioreactor so that nitrite-dependent microorganisms had to completely rely on the partial conversion of nitrate to nitrite by other microorganisms in the community. The apparent amount of nitrite in the bioreactor was less than 3 µmol, indicating a near complete conversion of produced nitrite. Ammonium was consumed through the anammox process, which is the only known pathway to anaerobically oxidize ammonium. As a control, samples retrieved from the bioreactor were tested for aerobic ammonium oxidation activity in a respiratory chamber; however, no activity could be observed (data not shown). Therefore, all ammonium was assumed to be converted by anammox bacteria leading to average ammonium and nitrite consumptions rates of 1.2 and 1.5 mmol in 24 h in the 1.5 l working volume of the bioreactor respectively. Additionally, nitrate is a by-product of the anammox metabolism (Strous *et al.*, 1998) and thus about 0.3 mmol of nitrate would be recycled back into the system. Considering the additional nitrate produced by anammox, the net average nitrate consumption rate was 2.6 and not 2.3 mmol in 24 h. Eventually, the remainder of the produced nitrite would be consumed by nitrite-dependent methanotrophs.

Methane conversion rates were determined in batch mode of the bioreactor. During this test, no sulfide was added to the bioreactor so nitrate reduction would be

exclusively dependent on anaerobic methane oxidizers. Heterotrophic denitrification was not tested as dead biomass would be the only organic carbon source for this process and, therefore, this activity was expected to be negligible. Reactor batch operation was started with 10% methane in the headspace, 10.95 mmol nitrate and 483 μ mol ammonium. After 24 h of continuous batch operation, methane in the reactor headspace had decreased to 6.5% and nitrate to 7.65 mmol (Fig. 1A and B), equalling a total consumption of 1.16 mmol methane and 2.2 mmol nitrate. Ammonium was already depleted after 12 h (Fig. 1C), after which a low amount of nitrite (12 μ mol) accumulated in the bioreactor.

Fluorescence in situ hybridization and metagenomics analysis of the biomass

Molecular characterization of the microbial community in the reactor was performed through fluorescence *in situ* hybridization (FISH) on day 63 and day 258 and by metagenome sequencing on day 258. After 63 days, FISH analysis showed the presence of the nitrate- and nitrite-dependent anaerobic methane oxidizers *Ca. M. nitroreducens* and *Ca. M. oxyfera* (Fig. 2A). Furthermore, anammox bacteria as well as *Sedimenticola* sp., a gammaproteobacterial sulfide-dependent denitrifier, were detected (Fig. 2B). After 258 days, *Ca. M. nitroreducens*, *Ca. M. oxyfera* (Fig. 2C) and anammox bacteria could still be detected (Fig. 2F), whereas *Sedimenticola* sp. had disappeared (data not shown). Neither *Sedimenticola*-specific nor gammaproteobacterial probes resulted in a positive FISH signal. Instead, the betaproteobacterium *Thiobacillus* sp. (Fig. 2D) and other betaproteobacteria (Fig. 2E) had become abundant in the bioreactor.

Microbial community composition was examined through 16S rRNA gene analyses, uploading metagenome reads in the SILVA database project tool (Fig. 3A) and also by assembly and binning. This yielded 50 bins (Fig. 3B), including seven high quality bins to which over 50% of all sequencing data mapped. In line with the FISH results, we were able to assign genome bins to *Ca. M. nitroreducens*, *Ca. M. oxyfera* and the anammox bacteria *Ca. Kuenenia stuttgartiensis* and *Ca. Scalindua brodae*. These genome bins contained key genes for archaeal anaerobic methanotrophy, bacterial methanotrophy and the anammox reaction (Supporting Information Table S2) which have been described in detail elsewhere (Ettwig *et al.*, 2010; van de Vossenberg *et al.*, 2013; Arshad *et al.*, 2015). It is interesting to note that *Ca. M. nitroreducens* only made up 2% of the microbial community according to 16S rRNA gene analyses (Fig. 3A), which contrasts the higher abundances indicated in the FISH micrographs (Fig. 2C). This may be due to DNA extraction biases that select against these archaea (Morono *et al.*, 2009; Luo *et al.*, 2014). Additionally, we identified three genome bins that showed the potential for sulfide-dependent denitrification: two closely related to *Sulfuricella denitrificans* and one affiliated within the alphaproteobacterial family Phyllobacteriaceae. The most abundant putative sulfur oxidizer had a 98.53% complete draft genome with 0.87% contamination and a S3 protein (RpsC) 82.6% similar to the one in *S. denitrificans* skB26 (no 16S rRNA gene present). This genome revealed the presence of the complete sulfide oxidation and nitrate reduction pathways (Supporting Information Table S3). In this bin, we found the potential for sulfide conversion to elemental sulfur (S^0) or sulfite either by sulfide quinone reductase (Sqr) or the dissimilatory sulfite reductase complex (encoded by *dsrAB*) respectively. Sulfite can be further oxidized to adenosine

Fig. 1. Whole reactor batch activity assay. A. Methane oxidation recorded over a period of 24 h. B. Consumption of nitrate during the batch experiment. C. Anammox activity established through consumption of ammonium and nitrite. The samples were collected every 4 h and measured in triplicates. Error bars represent standard deviation. The error bars in (A) and (B) are masked by the size of the data point.

Fig. 2. Fluorescence *in situ* hybridization micrographs of the reactor biomass after 63 days (A and B) and 258 days (C–F). A. *Candidatus M. nitroreducens* visible in red (D-arch641-Cy3) and *Ca. M. oxyfera* in green (D-bact193-FLUOS). B. Anammox bacteria visible in blue (Amx0820-Cy5), *Sedimenticola* sp. in green (GAM781-FLUOS) and *Ca. M. oxyfera* in red (D-bact193-Cy3). C. *Candidatus M. nitroreducens* visible in green (D-arch641-FLUOS) and *Ca. M. oxyfera* in red (D-bact193-Cy3). D. *Thiobacillus* spp. visible in green (Betthio1001-FLUOS) and general bacteria visible in blue (Eub338 I-III-Cy5). E. Betaproteobacteria visible in red (Bet42-Cy3), *Ca. M. nitroreducens* in green (D-arch641-FLUOS) and most bacteria labelled blue (Eub338 I-III-Cy5). F. *Anammox* sp. visible in red (AMX368-Cy3). The scale bar represents 20 μm .

5'-phosphate (APS) by APS reductase (Apr) and eventually to sulfate by 5'-triphosphate sulfurylase (Sat). The genome also encoded the Sox enzyme system used for thiosulfate oxidation. Furthermore, genes encoding a complete denitrification pathway were present (Supporting Information Table S3). Interestingly, a membrane bound NarGHI nitrate reductase most closely related to *Thiohalomonas* sp., *Thiohalomonas denitrificans* and *Methylotenera mobilis*, respectively, in addition to a periplasmic NapAB related to that from *S. denitrificans* sKB26 and *Aspergillus oryzae*, respectively, were identified. A cytochrome *cd₁* nitrite reductase (NirS) and nitrous oxide reductase (NosZ), along with the nitric oxide reductase NorBC, were also detected. Furthermore, this bin encoded the ammonium-forming nitrite reductase NirBD, which was most closely related to the corresponding subunits in *Thiobacillus* sp. and *Thauera phenylacetica*. In addition to that, we recovered a high quality genome bin (98.8% complete with 0.73% contamination) phylogenetically placed within the Xanthomonadaceae family (93% RpsC amino acid sequence similarity to *Dyella koreensis*). This organism was the third most abundant phylotype in the bioreactor (8.85% of the reads) and was likely involved in nitrogen cycling given the anoxic conditions in the bioreactor. An incomplete denitrification pathway to N_2O was identified based on the presence of a nitrate

transporter, *narGHI*, *nirKS* and *norBC*. We hypothesize that this organism may be involved in sulfide oxidation based on the presence of genes encoding a sulfide quinone oxidoreductase and two rhodanese-related sulfur transferases. No sulfur oxygenase reductase, ferredoxins, soluble HdrABC-type heterodisulfide reductases or molybdopterin oxidoreductases were encoded so it is unclear what the final product of sulfide oxidation may be. Under oxic condition, this microorganism might use aerobic respiration as the draft genome encoded the aerobic electron transport chain including NADH dehydrogenase (*nuoA-M*), succinate dehydrogenase (*sdhABCD*), cytochrome *bc₁* complex (ISP, cyt *b* and cyt *c₁*) and both *aa₃*- and *cbb₃*-type cytochrome *c* oxidases (*cyoE* and *coxABCD* and subunits I, II, III and IV respectively). All genes in the central carbon metabolism for glycolysis, Entner-Doudoroff pathway, pentose phosphate pathway, citrate cycle and glyoxylate shunt were identified.

The most abundant community member in the bioreactor was a metabolically versatile novel species representing a new family within the Nitrospirae

Surprisingly, the most abundant taxonomic group according to the metagenome data was related to previously uncultured microorganisms within the Nitrospirae distantly

Fig. 3. Metagenome analysis of the bioreactor after 258 days.

A. Percent of metagenome reads that mapped to 16S rRNA genes. (a–d) 16S rRNA gene reads belonging to Phycisphaerae, *Ca. M. nitroreducens*, Deltaproteobacteria and Brocadiales were 1%, 2%, 2% and 4% respectively. (e–g) 16S rRNA reads associated with moderately abundant bacteria corresponded to Alphaproteobacteria (6%), Betaproteobacteria (6%) and Gammaproteobacteria (7%). (h) A total of 16% of the reads matched the *Ca. M. oxyfera* 16S rRNA, the second most abundant microorganism. (i) A total of 17% of the 16S rRNA reads belonged to the microorganisms related to Bacteroidetes, Acidobacteria, Chloroflexi and Gemmatimonadetes. (j) 16S rRNA reads belonging to class Nitrospirae.

B. Microbial abundances based on percent of mapped metagenome reads highlights the enrichment of a new Nitrospirae organism (darker grey in contrast with others in light grey). Percent of metagenome reads (assembled into contigs) that mapped to reconstructed microbial genomes (bins) are shown. While 83.1% of reads mapped to bins, 16.9% of the reads mapped to unbinned contigs. The number of bins (“# bins”) in each category is indicated at the x-axis, while coverage (“x”), combined for each category, is displayed on top of each bar.

related to *Thermodesulfovibrio* sp. A high quality draft genome was obtained for this species through metagenome binning (100% complete, predicted contamination level of 4.98% based on a single-copy maker gene analysis (Parks *et al.*, 2015)). Total genome coverage was 242x, amounting to 24% of total binned reads in the bioreactor metagenome. The genome contained some common features with *Thermodesulfovibrio* sp. (potential for sulfate reduction, electron donors hydrogen, formate and acetate) but also harbored new metabolic capabilities indicating a more versatile lifestyle than cultured *Thermodesulfovibrio* sp. and other Nitrospirae (Fig. 4 and Supporting Information Table S4). We found that the genome encoded a full sulfate reduction pathway (Sat, AprAB, reductive DsrAB) as well as an enzyme system catalysing DNRA (NirBD, NrfAH) and a complete aerobic respiratory chain (NADH dehydrogenase, succinate dehydrogenase, cytochrome *bc*₁ complex, cytochrome *aa*₃ oxidase). Partial denitrification was also encoded (nitrate permease, NarGHI, NapAB, NorBC). Divergent NirK/NirS might be present: two ORFs (Node 192, gene 26 and Node 268, gene 29) were ~24–28% identical in amino acid sequence to reference NirK/NirS sequences. It has been suggested previously that divergent NirK proteins sharing only 10% sequence identity with reference proteins might still reduce nitrite to

nitric oxide (Helen *et al.*, 2016). However, experimental data are needed to confirm this hypothesis. Furthermore, the genome encoded the anaerobic respiratory enzymes trimethylamine *N*-oxide (TMAO) and dimethylsulfoxide (DMSO) reductase. As electron donors, the small organic acids acetate (presence of genes encoding acetyl-CoA synthetase), formate (genes encoding formate dehydrogenase FdoG/FdfH), pyruvate (seven copies of pyruvate water dikinase and pyruvate: ferredoxin oxidoreductase) and lactate (lactate dehydrogenase) might be used. We also identified 5 alcohol dehydrogenases and an aldehyde: ferredoxin oxidoreductase. Several hydrogenase gene clusters (*hyf* and *hyb*, including maturation system *hyp*) suggest the possibility of hydrogen uptake or production. We did not find any indication for butyrate and propionate oxidation. Furthermore, we detected the genomic potential that these novel Nitrospirae bacteria are able to use simple carbohydrates based on a complete glycolysis pathway with the exception of aldolase, as well as a complete pentose phosphate pathway. Other potential electron donors are methyl groups. We found several subunits of different methyl transferases for methanol, mono-, di- and trimethyl amine related to enzymes found in sulfate reducers, acetogens and methanogens. Even though neither methanol or methylamine dehydrogenases nor a complete methyl

Fig. 4. Metabolic reconstruction of Nitrospirae phylum related bacteria based on a genome obtained from metagenomics data.

The main respiratory and central carbon metabolism pathways are represented with focus on potential electron donors and acceptors. We have identified the electron transport chain for oxygen (SDH: succinate dehydrogenase/fumarate reductase; NuoA-N: NADH dehydrogenase; Q: quinone [quinone biosynthesis pathway]; Cyt *bc*₁: Complex III; C: cytochrome *c*; Cyt *c*, *aa*₃: *aa*₃-type terminal oxidase), nitrate (Per: nitrate permease; NarGHI: membrane-bound nitrate reductase; NirBD: cytoplasmic ammonium-forming nitrite reductase; NapAB: periplasmic nitrate reductase; NirAH: membrane-bound ammonium-forming nitrite reductase; NirKS: nitric oxide-forming nitrite reductase; NorBC: nitric oxide reductase) and sulfate [Sat: sulfate adenylyltransferase; AprAB: adenosine 5'-phosphosulfate (APS) reductase; DsrAB: dissimilatory sulfite reductase]. Candidate electron donors (printed in bold) include hydrogen, formate, acetate, alcohols, lactate, pyruvate and glucose. AAs: amino acids; ACS: acetyl-coenzyme A synthetase; ADH: alcohol dehydrogenase; AFOR: aldehyde:ferredoxin oxidoreductase; ALDH: aldehyde dehydrogenase; FDH: formate dehydrogenase; LDH: lactate dehydrogenase; M/D/TMA: mono-, di- and trimethylamines; NiFe Hyd: nickel-iron hydrogenases; PDH: pyruvate dehydrogenase; PEP: phosphoenolpyruvate; PFOR: pyruvate:ferredoxin oxidoreductase; PK: pyruvate phosphate dikinase; PW: pyruvate water dikinase; TCA: tricarboxylic acid cycle.

transferase operon was found, the abundance of individual subunits implicated in a methylotrophic lifestyle indicates that potentially methyl groups may be used as electron donors. The genome also encoded an (incomplete) tricarboxylic acid (TCA) cycle missing only the succinyl-CoA synthetase. No evidence was found for a reductive TCA cycle (no ATP-citrate lyase). Unlike *Thermodesulfovibrio* sp., the here presented draft genome encoded a complete Wood-Ljungdahl pathway for autotrophic carbon fixation.

Discussion

Competition and collaboration in the bioreactor reveal several major players in substrate turnover

Competition for substrates and collaboration for providing intermediates or removal of toxic substances are key drivers for the shaping of microbial communities. We investigated the community dynamics and physiological activity of a microbial community under brackish conditions (1% NaCl) enriched in a laboratory-scale bioreactor supplied with mineral medium and nitrate, sulfide, ammonium and methane. After 1 year of operation, a stable microbial community in the bioreactor metabolized all substrates and it was possible to attribute tentative removal rates to different physiological groups (Supporting Information Fig. S1). Nitrogen loss in the form of N₂ was due to the activity of

anammox bacteria (Eq. 1), sulfide-dependent denitrifiers (Eq. 2) and the combined activity of nitrate- and nitrite-dependent anaerobic methanotrophs (Eq. 3):

Metagenome sequencing indicated that, about 27% from the total anammox-associated reads belonged to *Ca. K. stuttgartiensis* while remaining 73% could be recovered in the genome bin belonging to *Ca. S. brodae*. Anammox bacteria produced 1.22 mmol N₂ and the nitrite-dependent methanotrophs 0.86 mmol. For the sulfide-dependent denitrifiers, a maximal amount of 0.23 mmol N₂ could be produced if all nitrate was reduced to N₂; probably some nitrate was reduced to nitrite (or ammonium) thus diminishing the amount of N₂ emitted by this physiological group. In total, this would amount to a maximum total nitrogen loss of 2.31 mmol N₂, of which 53% were produced by anammox bacteria. The nitrite-dependent methane oxidizers *Ca. M. oxyfera* contributed to 37% of the total nitrogen loss, whereas the sulfide-dependent denitrifiers only contributed to about 10% of the nitrogen loss if all nitrate was reduced to N₂. A previous study investigating the nitrogen loss in a

bioreactor model that did not contain methane concluded that anammox accounted for 65–75% and sulfide-dependent denitrifiers for 25–35% of the nitrogen loss (Russ *et al.*, 2014). Our results clearly demonstrate that when methane is included as electron donor, methane-dependent denitrifiers are competitive and will start to contribute substantially to the overall nitrogen loss. Although the sulfide-dependent denitrifiers only cause a maximum of 10% of the N₂ loss, they contribute significantly (31%) in providing nitrite for anammox bacteria and nitrite-dependent methanotrophs *Ca. M. oxyfera*. The remaining 69% of nitrite is produced by the nitrate-dependent methanotrophs *Ca. M. nitroreducens*. Methane was oxidized to almost equal parts by *Ca. M. oxyfera* (53%) and *Ca. M. nitroreducens* (47%) as was evident from the methane oxidation rates under sulfide depletion in the batch reactor assays. Under these conditions, nitrate is reduced to nitrite exclusively by *Ca. M. nitroreducens* with stoichiometric methane oxidation according to the following equation:

Candidatus M. oxyfera is responsible for the remainder of the methane oxidation rate and nitrite reduction to N₂ according to the following equation:

Besides competition and collaboration for substrates, the microbial community in the bioreactor was dependent on the removal of intermediates that could have a toxic effect. It has been noted previously that anammox bacteria are sensitive to sulfide stress (Russ *et al.*, 2014) with a low IC₅₀ of 10 µM. Therefore, anammox bacteria are dependent on the activity of sulfide-oxidizing microorganisms to keep the sulfide concentration below 1 µM. On the other hand, it seemed that sulfide stimulated the growth of *Ca. M. nitroreducens*, presumably through the maintenance of a low redox potential and strictly anoxic conditions, as these methanotrophs rely on enzymes that are readily inactivated by oxygen.

First enrichment of novel Nitrospirae bacteria distantly related to Thermodesulfovibrio that are frequently found in environments with sulfur, nitrogen and methane cycling

The most abundant microorganism according to metagenome sequencing in the present study was organisms only distantly related to *Thermodesulfovibrio* (87–89% 16S rRNA gene identity) as the closest isolated representative and constituted about 24% of the microbial community based on the number of reads mapped to the draft genome. The metabolic reconstruction of this genome

revealed the genomic potential for the use of a wide range of electron donors, i.e., hydrogen, pyruvate, lactate, acetate and formate, as well as a complete pathway for oxygen, nitrate, sulfate, DMSO and TMAO respiration. Also, it harbored a complete Wood–Ljungdahl pathway for autotrophic CO₂ fixation or acetate oxidation. This microorganism can therefore be viewed as versatile and probably facultative anaerobic, capable of either an organoheterotrophic or chemolithoautotrophic lifestyle. In the current bioreactor system, only ammonium, sulfide and methane were provided as electron donors. The Nitrospirae related genome encodes neither ammonium nor methane activating enzymes indicating that it probably does not metabolize these substrates. Sulfide might be used by the reverse reaction of the sulfate reduction pathway; however, when we analyzed the phylogenetic relationship of the relevant DsrA protein encoded by these organisms, we noted that it falls into the sulfate-reducing and not the sulfide-oxidizing cluster, making it unlikely that these bacteria are able to oxidize sulfide with DsrAB. It has however recently been reported that a reductive-type DsrAB might be able to work in the oxidative direction (Thorup *et al.*, 2017), but this result has not yet been biochemically validated. We did not find a sulfide quinone oxidoreductase that could complement this activity, making it unlikely that sulfide is used as an electron donor. It has recently been observed that sulfur oxidation or disproportionation can be coupled to DNRA (Mardanov *et al.*, 2016; Slobodkina *et al.*, 2017), but the genes involved are not yet clear. Provided that sulfur compounds more oxidized than sulfide are excreted by the proteobacterial sulfide oxidizers, these might be substrates for the Nitrospirae-related bacteria. Other compounds, e.g., hydrogen and methanol, may be intermediary products of methane oxidation that are potentially released into the medium, alongside electron donors that become available from the turnover of biomass. It is, however, difficult to imagine how leakage products could lead to such a high abundance of this taxonomic group in the bioreactor, even if DNA extraction biases are taken into consideration.

When we analyzed the nitrogen cycling metabolic potential of these bacteria, it was interesting to note that both redundancies and omissions in well-described pathways occurred. Nitrate could clearly be reduced to nitrite by either NarGHI or NapAB type nitrate reductases. We found NrfA which is the catalytically active subunit of the nitrite: ammonium oxidoreductase enabling DNRA, together with its membrane anchor NrfH (Simon *et al.*, 2003). Interestingly, it was not clear if the genome lacked genes encoding the nitrite reductases NirS and NirK. The genome encoded a nitric oxide reductase (NorBC) for the reduction of NO to N₂O. The NO reductase encoded by *nosZ* was missing in the genome. The presence of millimolar quantities of nitrate in the bioreactor makes it unlikely that sulfate

Fig. 5. Phylogenetic tree of sequences closely related to the novel *Nitrospirae* family bacterium enriched in this study.

The 100 best BLAST hits (nucleotide similarity > 90%) to the 16S rRNA gene from the *Nitrospirae* family bacterium were retrieved. Representative sequences from 40 different studies were selected along with 20 isolate reference sequences from the NCBI and SILVA databases. All sequences affiliate within the phylum *Nitrospirae*, except the outgroup *Nitrospina gracilis* from the phylum *Nitrospirae*. The sequence similarity between the 16S rRNA gene of the novel *Nitrospirae* family bacterium and environmental sequences putatively clustering in a new family ranged from 91% to 95.5%.

reduction was occurring, as nitrate respiration will thermodynamically outcompete sulfate respiration. However, it has been observed that microorganisms do not always abide to this rule (Dalsgaard and Bak, 1994; Canfield *et al.*, 2010; Chen *et al.*, 2017), and simultaneous nitrate and sulfate reduction cannot be excluded. In such a case, a cryptic sulfur cycle might be happening. Cryptic sulfur cycling has been previously found in marine oxygen minimum zones (Canfield *et al.*, 2010) as well as terrestrial peat soils (Hausmann *et al.*, 2016), freshwater sediments (Hansel *et al.*, 2015) and sub-surface coal wells (Glombitza *et al.*, 2016). Another possibility is that the *Nitrospirae*-related bacteria disproportionate sulfur compounds, eliminating the need for a designated electron donor. Unfortunately, the disproportionation of sulfur compounds is highly complex and not yet fully understood and the involved genes are unknown (Finster *et al.*, 1998;

Frederiksen and Finster, 2003; Hardisty *et al.*, 2013). Future research will target the isolation of these interesting microorganisms to study their physiology and possible role in the bioreactor.

Additionally, taxonomic groups related to the *Nitrospirae* bacteria found in this bioreactor occur in a wide range of environments that are implicated in sulfur, nitrogen and methane cycling. *Thermodesulfovibrio* spp. are the closest cultured representatives of the here presented bacterial group with 87–89% rRNA gene identity (Fig. 5). The phylogenetic tree topology was corroborated by a phylogenetic tree of 17 concatenated ribosomal protein sequences (Supporting Information Fig. S2) and an average amino acid identity (AAI) value of 52–53% (Supporting Information Table S5). Taking these values together, this novel *Nitrospirae* microorganism presumably belongs to a new family within the phylum *Nitrospirae*. We propose the

following naming: *Candidatus Nitroblum versatile* gen. nov., sp. nov. Ni.tro'bi.um. L. n. nitrogenium nitrogen; Gr. n. bios life. neut. n. Nitroblum a living entity metabolizing nitrogen compounds. *Candidatus N. versatile* sp. nov. (ver.sa'ti.le L. adj. with a versatile metabolism). At the moment, all members of this phylum are classified as genera within the family Nitrospiraceae. Our data show that there are probably several distinct families to be defined within the family Nitrospiraceae. Therefore, the proposal of a new family within the phylum Nitrospirae would require a revision of the complete taxonomy within this order, even though our 16S rRNA gene identities and AAI values support the presence of a new family. A recent preprint article detected related microorganisms distantly related to *Thermodesulfobivibrio* sp. in rice paddy microcosms that were amended with gypsum ($\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$) and based on a metagenomics assembled genome tentatively named *Ca. Sulfobium mesophilum* (Zecchin *et al.*, 2017). As this genome sequence is not yet publically available the exact phylogenetic affiliation needs to be resolved in the future. We suggest to direct future efforts at resolving the taxonomic ambiguity within the phylum Nitrospirae.

Physiological characteristics of the closely related and cultured *Thermodesulfobivibrio* spp. are the ability to perform dissimilatory sulfate, sulfite and thiosulfate reduction with a limited set up electron donors, i.e., hydrogen, formate, pyruvate and lactate (Henry *et al.*, 1994; Haouari *et al.*, 2008; Sekiguchi *et al.*, 2008; Frank *et al.*, 2016). All of the current isolated *Thermodesulfobivibrio* sp. are thermophiles whereas the enriched Nitrospirae-related bacteria grew at ambient temperature. Konno and colleagues detected *Thermodesulfobivibrio*-like sequences (91–92% 16S rRNA gene identity) in freshwater aquifers in the terrestrial subsurface which is a very oligotrophic ecosystem (Konno *et al.*, 2013). Lau and colleagues (2014) found *Thermodesulfobivibrio*-like metagenome sequences in the South African continental crust under mesophilic conditions where biogeochemical cycling of nitrogen, sulfur and methane had been shown previously. Baker and colleagues (2015) investigated the genomic potential of a microbial community in estuary sediments. They were able to reconstruct environmental genomes harboring the potential to contribute to the carbon, nitrogen and sulfur cycle. One of their environmental genomes was distantly related to *Thermodesulfobivibrio*. That genome harbored the metabolic potential for some fermentative pathways, hydrogen production or consumption and dissimilatory sulfate reduction. Interestingly, a recent amplicon sequencing survey of river sediments impacted by freshwater mussels discovered the co-occurrence of anammox bacteria, nitrate- and nitrite-dependent methanotrophs as well as *Thermodesulfobivibrio*-like sequences (Black *et al.*, 2017). Their survey did not target sulfide-dependent denitrifiers, but proteobacteria were abundantly present and could potentially be involved

in nitrate-dependent sulfide oxidation. Especially these two studies by Baker and colleagues (2015; 2017) describe ecosystems that mirror the enrichment conditions in the here presented bioreactor: the interlinkage of sulfur, nitrogen and methane cycling leading to the enrichment of known and new players in these processes.

Furthermore, we performed a database survey targeting environmental 16S rRNA gene sequences (Fig. 5) related to those found in our novel Nitrospirae family bacterial genome. We found sequences that shared 91–95.5% identity to the one identified here in 40 different studies. Most notably, half of them originated from freshwater sediments (wetlands, rivers, lakes, reservoirs) and soils (peat, fen, paddies). Interestingly, 21% of environmental sequences were recovered from bioreactors or sediment slurries for the degradation of diverse compounds, while 14% of these sequences derived from the deep terrestrial subsurface. The ubiquitous distribution of members in this new Nitrospirae family highlights the potential role of such organisms in the biogeochemical cycles in diverse environments relevant for methane, sulfur and nitrogen cycling.

Experimental procedures

Enrichment and reactor operation

A 2.5 l bioreactor (Applikon, Delft, The Netherlands) with a working volume of 1.5 l was used for cultivation of the enrichment culture for a period of 382 days. It was operated at room temperature. The inoculum consisted of biomass from a marine enrichment culture containing sulfide-dependent autotrophic denitrifiers and anammox bacteria (Russ *et al.*, 2014) and biomass from a freshwater methane-oxidizing denitrification culture (Ettwig *et al.*, 2016). The reactor was operated at 150 rpm with a stirrer that contained two standard six-blade turbines. The flow of methane gas to the reactor was kept at 7.5 ml min^{-1} using a mass flow controller (Brooks Instrument, Ede, The Netherlands). Additionally, the bioreactor was constantly flushed with Ar/CO_2 (95:5) to ensure anoxic conditions. The pH of the reactor liquid was monitored with a pH electrode (Applisens; Applikon, Delft, The Netherlands) and was maintained at 7.1 with $100 \text{ g l}^{-1} \text{ KHCO}_3$ solution. The pH pump was controlled by an ADI 1010 biocontroller (Applikon). The mineral medium per litre contained 10 g coral pro salt (Red Sea), 7 mM NH_4Cl , 15 mM NaNO_3 , 0.6 ml anammox specific trace element solution (van de Graaf *et al.*, 1996) ($15 \text{ g l}^{-1} \text{ EDTA}$, $0.43 \text{ g l}^{-1} \text{ ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $0.24 \text{ g l}^{-1} \text{ CoCl}_2 \cdot 6\text{H}_2\text{O}$, $0.99 \text{ g l}^{-1} \text{ MnCl}_2 \cdot 4\text{H}_2\text{O}$, $0.25 \text{ g l}^{-1} \text{ CuSO}_4 \cdot 5\text{H}_2\text{O}$, $0.22 \text{ g l}^{-1} \text{ Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $0.2 \text{ g l}^{-1} \text{ NiCl}_2 \cdot 6\text{H}_2\text{O}$, $0.067 \text{ g l}^{-1} \text{ SeO}_2$, $0.014 \text{ g l}^{-1} \text{ H}_3\text{BO}_3$, $0.05 \text{ g l}^{-1} \text{ Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$), 0.6 ml FeSO_4 , 0.5 ml $100 \text{ g l}^{-1} \text{ K}_2\text{HPO}_4$, 1.25 ml (288 mg l^{-1}) MgSO_4 , 1.25 ml (192 mg l^{-1}) CaCl_2 and 1 ml trace element solution for DAMO microorganisms ($0.5 \text{ g l}^{-1} \text{ ZnSO}_4 \cdot 7\text{H}_2\text{O}$, $0.12 \text{ g l}^{-1} \text{ CoCl}_2 \cdot 6\text{H}_2\text{O}$, $2 \text{ g l}^{-1} \text{ CuSO}_4$, $0.2 \text{ g l}^{-1} \text{ NiCl}_2 \cdot 6\text{H}_2\text{O}$, $0.014 \text{ g l}^{-1} \text{ H}_3\text{BO}_3$, $0.3 \text{ g l}^{-1} \text{ MnCl}_2 \cdot 4\text{H}_2\text{O}$, $0.04 \text{ g l}^{-1} \text{ Na}_2\text{WO}_4 \cdot 2\text{H}_2\text{O}$, $0.2 \text{ g l}^{-1} \text{ Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$, $0.02 \text{ g l}^{-1} \text{ SeO}_2$, $0.8 \text{ g l}^{-1} \text{ CeCl}_2$). The 6.7 mM sulfide solution was provided as separate anaerobic medium with a flow rate 100 ml day^{-1} . The nitrate and nitrite concentrations in the bioreactor were measured daily

with MQuant™ colorimetric test strips (Merck, Darmstadt, Germany). Sulfide concentration was measured through acidification with 0.5 M HCl and injecting the gas samples to a gas chromatograph (7890B GC systems, Agilent Technologies, Santa Clara, CA). The GC was equipped with a Carboxen BHT-100 column (60–80 mesh) and flame photometric detector (FPD). The injection and detection temperature was 200°C, and the oven temperature was 80°C.

Whole culture batch activity assays

To measure methane consumption, medium and gas supplies were stopped and headspace methane concentration decreased to ca. 10% by flushing with Ar/CO₂ (95:5). The methane concentration was then measured every 4 h for a period of 2 h. At each sampling time, headspace gas samples of 100 µl were withdrawn with a gas tight glass syringe (Hamilton, Switzerland) and immediately measured through a HP 5890 gas chromatograph equipped with a Porapak Q column (80/100 mesh) and flame ionization detector (Hewlett Packard, Palo Alto, CA). The injection and detection temperature was 150°C, and the oven temperature was 120°C. Final methane concentrations were calculated through calibration gas and self-made standards. Additionally, 2 ml liquid sample was centrifuged and supernatant stored at –20°C for determination of nitrogenous compounds. Nitrite was measured colorimetrically at 540 nm after a 15 min-reaction of 1 ml sample (0.05–0.5 mM nitrite) with 1 ml 1% sulfanilic acid in 1 M HCl and 1 ml 0.1% naphthylethylene diaminedihydrochloride (Griess, 1879). Ammonium was measured at 420 nm on a 96-well fluorescence spectrophotometer after reaction with 10% ortho-phthalaldehyde as described previously (Taylor *et al.*, 1974). Nitrate was measured with a Sievers Nitric Oxide analyzer (NOA280i; GE Power Water & Process Technologies, Boulder, CO). The sample measurements were carried out in duplicates.

Metagenome sequencing and analysis

On day 258, 150 ml biomass for genomic DNA extraction was sampled from the bioreactor, homogenized in a glass homogenizer to disrupt granules, and DNA was extracted with two different extraction methods in triplicate, the FastDNA Spin Kit (MP Biomedicals, Santa Ana CA) and the cetyltrimethylammoniumbromide (CTAB) method (Zhou *et al.*, 1996). DNA was quantified with the Qubit Fluorometer (Thermo Fisher Scientific, Waltham, MA). DNA (1 ng) from both extraction methods was used for MiSeq library preparation. The genomic DNA was sheared and adapters were ligated in the same step. The Illumina Nextera® XT Library Prep Kit was used according to the manufacturer's instructions (Illumina, San Diego, CA). The library was normalized to 4 nM and sequencing was performed with an Illumina MiSeq (Illumina) using the 300 paired-end sequencing protocol. Quality-trimming, adapter removal and contaminant-filtering of paired-end sequencing reads was performed using BBDOCK (BBTOOLS version 37.17) (Bushnell, 2015). The Fast Spin DNA extraction kit and CTAB method yielded 10.8 and 7.8 million trimmed reads > 150 bp respectively. Reads obtained from both DNA extractions were co-assembled with metaSPAdes v3.10.1 (Nurk *et al.*, 2017)

using default settings. MetaSPAdes iteratively assembled the metagenome using kmer sizes 21, 33, 55, 77, 99 and 127. Reads were mapped back to the metagenome for each extraction separately using Burrows-Wheeler Aligner 0.7.15 (Li and Durbin, 2010) (BWA), employing the “mem” algorithm. The generated sequence mapping files were handled and converted as needed using SAMtools 2.1 (Li *et al.*, 2009). Metagenome binning was performed employing five different binning algorithms: BinSanity v0.2.5.9 (Graham *et al.*, 2017), COCACOLA (Lu *et al.*, 2016), CONCOCT (Alneberg *et al.*, 2014), MaxBin 2.0 2.2.3 (Wu *et al.*, 2016) and MetaBAT 2 2.10.2 (Kang *et al.*, 2015). The five resulting bin sets were supplied to DAS Tool 1.0 (C.M.K. Sieber, A.J. Probst, A. Sharrar, B.C. Thomas, M. Hess, S.G. Tringe and J.F. Banfield, unpublished) for consensus binning to obtain the final optimized bins. The quality of the consensus bins was assessed using CheckM 1.0.7 (Parks *et al.*, 2015). From ~ 18 million trimmed, quality controlled metagenome reads, 93.9% were assembled into contigs, 82.8% mapped to contigs and 78% mapped to binned contigs. Genes were called and annotated as previously described by (Wrighton *et al.*, 2012) with a pipeline available online (https://github.com/TheWrightonLab/metagenome_annotation). Briefly, genes were called with Prodigal (Hyatt *et al.*, 2010) and annotated based on forward and reverse blast hits to amino acid sequences in UniRef90 (<http://www.uniprot.org/help/uniref>) and KEGG (<http://www.genome.jp/kegg/>). Thresholds for reciprocal best blast matches were a minimum 300 bit score and, for one-way blast matches, a minimum 60 bit score. Motifs were also analysed using InterproScan. Complete or partial 16S rRNA genes in bins were identified with SSU-Align (Nawrocki, 2009). The 16S rRNA gene of the novel Nitrospirae phylum bacterium was completed by two cycles of stringent mapping and elongation of the contigs obtained after assembly of all 16S rRNA gene reads extracted from the metagenome. The metagenome data are available at NCBI under accession number (PRJNA397647). For the biogeography, the complete 16S rRNA gene in the Nitrospirae bin was blasted on NCBI (<https://www.ncbi.nlm.nih.gov/>). The first 100 best hits were further analysed for similarity, sample type and country of origin. For phylogenetic trees, sequences (nucleotide or amino acid, as indicated in each figure) were aligned with MUSCLE (Edgar, 2004). The biogeography 16S rRNA gene tree was built on an alignment containing 1,651 positions using FastTree (Price *et al.*, 2010) with 100 bootstraps. For the concatenated ribosomal protein tree, 17 ribosomal proteins (L1, L2, L3, L4, L5, L6, L14, L15, L16, L19, L20, L24, S3, S8, S11, S13 and S19) were identified using hmmsearch (-E 0.00001) (Johnson *et al.*, 2010) and independently aligned. Alignment columns with 95% gaps were stripped and then concatenated in Geneious v. 9.0.5 (Kearse *et al.*, 2012), generating an alignment with 3,304 positions. This tree was built using RAxML v. 8.2.9 with 100 bootstraps under the LG model of evolution on a pipeline available online at <https://github.com/TheWrightonLab/Protpipeline> as previously described (Solden *et al.*, 2017). Trees were visualized with iTOL (Letunic and Bork, 2016). Reference sequences in these trees were retrieved from NCBI or SILVA (<https://www.arb-silva.de/>). Plots were made in R (R Development Core Team, 2017), and all figures were edited in Adobe Illustrator version 16.0.0 (Adobe Systems Inc., San Jose, CA). Average

amino acid identity (AAI) values were calculated using the Kostas lab tool available at <http://enve-omics.ce.gatech.edu/g-matrix/>.

Fluorescence *in situ* hybridization (FISH)

Biomass samples were taken after 63 and 258 days and prepared as previously described (Russ *et al.*, 2014). FISH was performed as described by Amann *et al.* (1990) using a hybridization buffer containing either 30% or 20% (vol/vol) formamide. Specifications and the details of the probes used in this study are provided in Supporting Information Table S1. For image acquisition, a Zeiss Axioplan 2 epifluorescence microscope equipped with a CCD camera was used together with the Axiovision software package (Zeiss, Oberkochen, Germany). Vectashield mounting fluid with DAPI (4,6-diamidino-2-phenylindole) was used on all samples to stain all DNA.

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References

- Alneberg, J., Bjarnason, B.S., de Bruijn, I., Schirmer, M., Quick, J., Ijaz, U.Z., *et al.* (2014) Binning metagenomic contigs by coverage and composition. *Nat Methods* **11**: 1144–1146.
- Amann, R.L., Binder, B.J., Olson, R.J., Chisholm, S.W., Devereux, R., and Stahl, D.A. (1990) Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Appl Environ Microbiol* **56**: 1919–1925.
- Arshad, A., Speth, D.R., de Graaf, R.M., Op den Camp, H.J.M., Jetten, M.S.M., and Welte, C.U. (2015) A metagenomics-based metabolic model of nitrate-dependent anaerobic oxidation of methane by *Methanoperedens*-like archaea. *Front Microbiol* **6**: 1423.
- Baker, B.J., Lazar, C.S., Teske, A.P., and Dick, G.J. (2015) Genomic resolution of linkages in carbon, nitrogen, and sulfur cycling among widespread estuary sediment bacteria. *Microbiome* **3**: 14.
- van den Berg, E.M., van Dongen, U., Abbas, B., and van Loosdrecht, M.C. (2015) Enrichment of DNRA bacteria in a continuous culture. *ISME J* **9**: 2153–2161.
- Black, E.M., Chiment, M.S., and Just, C.L. (2017) Effect of freshwater mussels on the vertical distribution of anaerobic ammonia oxidizers and other nitrogen-transforming microorganisms in upper Mississippi river sediment. *PeerJ* **5**: e3536.
- Bushnell, B. (2015) *BBMap* – Bushnell B. URL sourceforge.net/projects/bbmap/.
- Canfield, D.E., Glazer, A.N., and Falkowski, P.G. (2010) The evolution and future of earth's nitrogen cycle. *Science* **330**: 192–196.
- 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, J., Hanke, A., Tegetmeyer, H.E., Kattelmann, I., Sharma, R., Hamann, E., *et al.* (2017) Impacts of chemical gradients on microbial community structure. *ISME J* **11**: 920–931.
- Dalsgaard, F., and Bak, T. (1994) Nitrate reduction in a sulfate-reducing bacterium, *Desulfovibrio desulfuricans*, isolated from rice paddy soil. *Appl Environ Microbiol* **60**: 291–297.
- Dong, L.F., Thornton, D.C.O., Nedwell, D.B., and Underwood, G.J.C. (2000) Denitrification in sediments of the river Colne Estuary, England. *Mar Ecol Prog Ser* **203**: 109–122.
- Edgar, R.C. (2004) MUSCLE: multiple sequence alignment with high accuracy and high throughput. *Nucleic Acids Res* **32**: 1792–1797.
- Egger, M., Rasigraf, O., Sapart, C.J., Jilbert, T., Jetten, M.S.M., Rckmann, T., *et al.* (2015) Iron-mediated anaerobic oxidation of methane in brackish coastal sediments. *Environ Sci Technol* **49**: 277–283.
- Ettwig, K.F., Butler, M.K., Le Paslier, D., Pelletier, E., Mangenot, S., Kuypers, M.M.M., *et al.* (2010) Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. *Nature* **464**: 543–548.
- Ettwig, K.F., Zhu, B., Speth, D., Keltjens, J.T., Jetten, M.S.M., and Kartal, B. (2016) Archaea catalyze iron-dependent anaerobic oxidation of methane. *Proc Natl Acad Sci USA* **113**: 12792–12796.
- Falkowski, P.G., Fenchel, T., and Delong, E.F. (2008) The microbial engines that drive earth's biogeochemical cycles. *Science* **320**: 1034–1039.
- Finstler, K., Liesack, W., and Thamdrup, B. (1998) Elemental sulfur and thiosulfate disproportionation by *Desulfocapsa sulfoexigens* sp. nov., a new anaerobic bacterium isolated from marine surface sediment. *Appl Environ Microbiol* **64**: 119–125.
- Frank, Y.A., Kadnikov, V.V., Lukina, A.P., Banks, D., Beletsky, A.V., Mardanov, A.V., *et al.* (2016) Characterization and genome analysis of the first facultatively alkaliphilic *Thermodesulfovibrio* isolated from the deep terrestrial subsurface. *Front Microbiol* **7**: 2000.
- Frederiksen, T.-M., and Finster, K. (2003) Sulfite-oxidoreductase is involved in the oxidation of sulfite in *Desulfocapsa sulfoexigens* during disproportionation of thiosulfate and elemental sulfur. *Biodegradation* **14**: 189–198.
- Glombitza, C., Adhikari, R.R., Riedinger, N., Gilhooly, W.P., Hinrichs, K.-U., and Inagaki, F. (2016) Microbial sulfate reduction potential in coal-bearing sediments down to ~2.5 km below the seafloor off Shimokita Peninsula, Japan. *Front Microbiol* **7**: 1576.
- van de Graaf, A.A., de Bruijn, P., Robertson, L.A., Jetten, M.S.M., and Kuenen, J.G. (1996) Autotrophic growth of anaerobic ammonium-oxidizing micro-organisms in a fluidized bed reactor. *Microbiology* **142**: 2187–2196.

- Graham, E.D., Heidelberg, J.F., and Tully, B.J. (2017) BinSanity: unsupervised clustering of environmental microbial assemblies using coverage and affinity propagation. *PeerJ* **5**: e3035.
- Griess, P. (1879) Bemerkungen zu der Abhandlung der HH. Weselsky und Benedikt "Ueber einige Azoverbindungen". *Ber Dtsch Chem Ges* **12**: 426–428.
- Hansel, C.M., Lentini, C.J., Tang, Y., Johnston, D.T., Wankel, S.D., and Jardine, P.M. (2015) Dominance of sulfur-fueled iron oxide reduction in low-sulfate freshwater sediments. *ISME J* **9**: 2400–2412.
- Haouari, O., Fardeau, M.-L., Cayol, J.-L., Casiot, C., Elbaz-Poulitchet, F., Hamdi, M., et al. (2008) *Desulfotomaculum hydrothermale* sp. nov., a thermophilic sulfate-reducing bacterium isolated from a terrestrial Tunisian hot spring. *Int J Syst Evol Microbiol* **58**: 2529–2535.
- Hardisty, D.S., Olyphant, G.A., Bell, J.B., Johnson, A.P., and Pratt, L.M. (2013) Acidophilic sulfur disproportionation. *Geochim Cosmochim Acta* **113**: 136–151.
- Haroon, M.F., Hu, S., Shi, Y., Imelfort, M., Keller, J., Hugenholtz, P., et al. (2013) Anaerobic oxidation of methane coupled to nitrate reduction in a novel archaeal lineage. *Nature* **500**: 567–570.
- Hausmann, B., Knorr, K.-H., Schreck, K., Tringe, S.G., Glavina del Rio, T., Loy, A., et al. (2016) Consortia of low-abundance bacteria drive sulfate reduction-dependent degradation of fermentation products in peat soil microcosms. *ISME J* **10**: 2365–2375.
- Helen, D., Kim, H., Tytgat, B., and Anne, W. (2016) Highly diverse nirK genes comprise two major clades that harbour ammonium-producing denitrifiers. *BMC Genomics* **17**: 155.
- Henry, E.A., Devereux, R., Maki, J.S., Gilmour, C.C., Woese, C.R., Mandelco, L., et al. (1994) Characterization of a new thermophilic sulfate-reducing bacterium. *Arch Microbiol* **161**: 62–69.
- Hyatt, D., Chen, G.-L., LoCascio, P.F., Land, M.L., Larimer, F.W., and Hauser, L.J. (2010) Prodigal: prokaryotic gene recognition and translation initiation site identification. *BMC Bioinformatics* **11**: 119.
- Johnson, L.S., Eddy, S.R., and Portugaly, E. (2010) Hidden Markov model speed heuristic and iterative HMM search procedure. *BMC Bioinformatics* **11**: 431.
- Kang, D.D., Froula, J., Egan, R., and Wang, Z. (2015) MetaBAT, an efficient tool for accurately reconstructing single genomes from complex microbial communities. *PeerJ* **3**: e1165.
- Kearse, M., Moir, R., Wilson, A., Stones-Havas, S., Cheung, M., Sturrock, S., et al. (2012) Geneious Basic: an integrated and extendable desktop software platform for the organization and analysis of sequence data. *Bioinformatics* **28**: 1647–1649.
- Konno, U., Kouduka, M., Komatsu, D.D., Ishii, K., Fukuda, A., Tsunogai, U., et al. (2013) Novel microbial populations in deep granitic groundwater from grimsel test site, Switzerland. *Microb Ecol* **65**: 626–637.
- Kraft, B., Strous, M., and Tegetmeyer, H.E. (2011) Microbial nitrate respiration – genes, enzymes and environmental distribution. *J Biotechnol* **155**: 104–117.
- Lau, M.C.Y., Cameron, C., Magnabosco, C., Brown, C.T., Schilkey, F., Grim, S., et al. (2014) Phylogeny and phylogeography of functional genes shared among seven terrestrial subsurface metagenomes reveal N-cycling and microbial evolutionary relationships. *Front Microbiol* **5**: 531.
- Letunic, I., and Bork, P. (2016) Interactive tree of life (iTOL) v3: an online tool for the display and annotation of phylogenetic and other trees. *Nucleic Acids Res* **44**: W242–W245.
- Li, H., and Durbin, R. (2010) Fast and accurate long-read alignment with Burrows–Wheeler transform. *Bioinformatics* **26**: 589–595.
- Li, H., Handsaker, B., Wysoker, A., Fennell, T., Ruan, J., Homer, N., et al. (2009) The sequence alignment/map format and SAMtools. *Bioinformatics* **25**: 2078–2079.
- Lu, Y.Y., Chen, T., Fuhrman, J.A., and Sun, F. (2016) COCA-COLA: binning metagenomic contigs using sequence COMposition, read CoverAge, CO-alignment and paired-end read LinkAge. *Bioinformatics* **33**: 791–798.
- Luesken, F.A., Sanchez, J., van Alen, T.A., Sanabria, J., Op den Camp, H.J.M., Jetten, M.S.M., et al. (2011) Simultaneous nitrite-dependent anaerobic methane and ammonium oxidation processes. *Appl Environ Microbiol* **77**: 6802–6807.
- Luo, C., Rodriguez-R, L.M., Johnston, E.R., Wu, L., Cheng, L., Xue, K., et al. (2014) Soil microbial community Responses to a decade of warming as revealed by comparative metagenomics. *Appl Environ Microbiol* **80**: 1777–1786.
- Mardanov, A.V., Beletsky, A.V., Kadnikov, V.V., Slobodkin, A.I., and Ravin, N.V. (2016) Genome analysis of *Thermosulfurimonas dismutans*, the first thermophilic sulfur-disproportionating bacterium of the phylum Thermodesulfobacteria. *Front Microbiol* **7**: 950.
- Morono, Y., Terada, T., Masui, N., and Inagaki, F. (2009) Discriminative detection and enumeration of microbial life in marine subsurface sediments. *ISME J* **3**: 503–511.
- Mulder, A., Graaf, A.A., Robertson, L.A., and Kuenen, J.G. (1995) Anaerobic ammonium oxidation discovered in a denitrifying fluidized bed reactor. *FEMS Microbiol Ecol* **16**: 177–184.
- Nawrocki, E.P. (2009) Structural RNA homology search and alignment using covariance models. PhD Thesis. St. Louis, MO: Washington University, Computational Biology.
- Nurk, S., Meleshko, D., Korobeynikov, A., and Pevzner, P.A. (2017) metaSPAdes: a new versatile metagenomic assembler. *Genome Res* **27**: 824–834.
- Parks, D.H., Imelfort, M., Skennerton, C.T., Hugenholtz, P., and Tyson, G.W. (2015) CheckM: assessing the quality of microbial genomes recovered from isolates, single cells, and metagenomes. *Genome Res* **25**: 1043–1055.
- Price, M.N., Dehal, P.S., Arkin, A.P., and Poon, A.F.Y. (2010) FastTree 2 – approximately maximum-likelihood trees for large alignments. *PLoS One* **5**: e9490.
- R Development Core Team. (2017) *R: A Language and Environment for Statistical Computing*. Vienna, Austria: R Foundation for Statistical Computing.
- Raghoebarsing, A.A., Pol, A., van de Pas-Schoonen, K.T., Smolders, A.J.P., Ettwig, K.F., Rijpstra, W.I.C., et al. (2006) A microbial consortium couples anaerobic methane oxidation to denitrification. *Nature* **440**: 918–921.
- Russ, L., Speth, D.R., Jetten, M.S.M., Op den Camp, H.J.M., and Kartal, B. (2014) Interactions between anaerobic ammonium and sulfur-oxidizing bacteria in a laboratory scale model system. *Environ Microbiol* **16**: 3487–3498.
- Sekiguchi, Y., Muramatsu, M., Imachi, H., Narihiro, T., Ohashi, A., Harada, H., et al. (2008) *Thermodesulfobrevibrio*

- aggregans* sp. nov. and *Thermodesulfobrio thiophilus* sp. nov., anaerobic, thermophilic, sulfate-reducing bacteria isolated from thermophilic methanogenic sludge, and emended description of the genus *Thermodesulfobrio*. *Int J Syst Evol Microbiol* **58**: 2541–2548.
- Shi, Y., Hu, S., Lou, J., Lu, P., Keller, J., and Yuan, Z. (2013) Nitrogen removal from wastewater by coupling anammox and methane-dependent denitrification in a membrane bio-film reactor. *Environ Sci Technol* **47**: 11577–11583.
- Simon, J., Sanger, M., Schuster, S.C., and Gross, R. (2003) Electron transport to periplasmic nitrate reductase (NapA) of *Wolinella succinogenes* is independent of a NapC protein. *Mol Microbiol* **49**: 69–79.
- Slobodkina, G.B., Mardanov, A.V., Ravin, N.V., Frolova, A.A., Chernyh, N.A., Bonch-Osmolovskaya, E.A., *et al.* (2017) Respiratory ammonification of nitrate coupled to anaerobic oxidation of elemental sulfur in deep-sea autotrophic thermophilic bacteria. *Front Microbiol* **8**: 87.
- Solden, L.M., Hoyt, D.W., Collins, W.B., Plank, J.E., Daly, R.A., Hildebrand, E., *et al.* (2017) New roles in hemicellulosic sugar fermentation for the uncultivated Bacteroidetes family BS11. *ISME J* **11**: 691–703.
- Sorokin, D.Y., Tourova, T.P., Galinski, E.A., Muyzer, G., and Kuenen, J.G. (2008) *Thiohalorhabdus denitrificans* gen. nov., sp. nov., an extremely halophilic, sulfur-oxidizing, deep-lineage gammaproteobacterium from hypersaline habitats. *Int J Syst Evol Microbiol* **58**: 2890–2897.
- Strous, M., Heijnen, J.J., Kuenen, J.G., and Jetten, M.S.M. (1998) The sequencing batch reactor as a powerful tool for the study of slowly growing anaerobic ammonium-oxidizing microorganisms. *Appl Microbiol Biotechnol* **50**: 589–596.
- Taylor, S., Ninjoor, V., Dowd, D.M., and Tappel, A.L. (1974) Cathepsin B2 measurement by sensitive fluorometric ammonia analysis. *Anal Biochem* **60**: 153–162.
- Thorup, C., Schramm, A., Findlay, A.J., Finster, K.W., Schreiber, L., and Newman, D.K. (2017) Disguised as a sulfate reducer: growth of the Deltaproteobacterium *Desulfurivibrio alkaliphilus* by sulfide oxidation with nitrate. *mBio* **8**: e00671–17.
- Toro, E.E.R., and Cervantes, F.J. (2016) Coupling between anammox and autotrophic denitrification for simultaneous removal of ammonium and sulfide by enriched marine sediments. *Biodegradation* **27**: 107–118.
- van de Vossenberg, J., Woebken, D., Maalcke, W.J., Wessels, H.J.C.T., Dutilh, B.E., Kartal, B., *et al.* (2013) The metagenome of the marine anammox bacterium “*Candidatus Scalindua profunda*” illustrates the versatility of this globally important nitrogen cycle bacterium. *Environ Microbiol* **15**: 1275–1289.
- Wrighton, K.C., Thomas, B.C., Sharon, I., Miller, C.S., Castelle, C.J., VerBerkmoes, N.C., *et al.* (2012) Fermentation, hydrogen, and sulfur metabolism in multiple uncultivated bacterial phyla. *Science* **337**: 1661–1665.
- Wu, Y.-W., Simmons, B.A., and Singer, S.W. (2016) MaxBin 2.0: an automated binning algorithm to recover genomes from multiple metagenomic datasets. *Bioinformatics* **32**: 605–607.
- Xiao, Y., and Roberts, D.J. (2010) A review of anaerobic treatment of saline wastewater. *Environ Technol* **31**: 1025–1043.
- Zecchin, S., Mueller, R.C., Seifert, J., Stingl, U., Anantharaman, K., van Bergen, M., *et al.* (2017) Rice paddy Nitrospirae encode and express genes related to sulfate respiration: proposal of the new genus *Candidatus Sulfobium*. *bioRxiv* doi: 10.1101/196774.
- Zhou, J., Bruns, M.A., and Tiedje, J.M. (1996) DNA recovery from soils of diverse composition. *Appl Environ Microbiol* **62**: 316–322.

Supporting information

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

Fig. S1. Schematic overview of the contribution of individual microorganisms to elemental cycling in the bioreactor. Please see the main text for explanation and details.

Fig. S2. Maximum Likelihood phylogenetic tree using 17 concatenated ribosomal protein sequences. The topology was the same as found in the 16S rRNA gene tree.

Table S1. Overview of the FISH probes used to target specific microbial groups

Table S2. Comparison of genome bins obtained from the laboratory-scale bioreactor fed with ammonium, sulfide, nitrate and methane with previously published genome sequences identified by the locus identifier and gene annotation. MO, MN, A1 and A3: respective genome bin; NODE: contig number; length: contig length; cov: contig coverage; last underscore followed by digit: gene number.

Table S3. Analysis of a reconstructed genome (bin das_tool.binsanity.asm_contigs_metaspades_gt1500-bin_1-refined_1.fa or pSox1_ANNOTATED_contigs_1000.genes.fna) with metabolic potential for sulfur oxidation and nitrate reduction. This genome is 98.53% complete with 0.87% of contamination as estimated by analysis of single copy marker genes. The S3 ribosomal protein was 82.6% similar to the one in *Sulfuricella denitrificans* skB26. The genome coverage was 28 times, with 1.98% of assembled reads mapping to this bin, making it the 7th most abundant organism. pSox1: putative sulfur oxidizer number 1; NODE: contig number; length: contig length; cov: contig coverage; last underscore followed by digit: gene number; % identity: amino acid percent similarity.

Table S4. Analysis of a reconstructed genome (das_tool.binsanity.asm_contigs_metaspades_gt1500-bin_18.fa or Nitrospirae_ANNOTATED_contigs_1000.genes.fna) with metabolic potential for oxygen reduction, sulfate reduction and nitrate reduction to nitrous oxide or ammonium. This organism was enriched and became the dominant microbial member in the bioreactor, representing a new group in the Nitrospirae phylum. Proteins of interest are highlighted in this table. NODE: contig number; length: contig length; cov: contig coverage; last underscore followed by digit: gene number.

Table S5. Average amino acid identity (AAI) table of the novel *Ca. Nitrobium versatile* and its twenty closest representatives with available genome sequences. *Nitrospina gracilis* was used as an outgroup.