Unraveling nitrogen, sulfur and carbon metabolic pathways and microbial community transcriptional responses to substrate deprivation and toxicity stresses in a bioreactor mimicking anoxic brackish coastal sediment conditions

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Abstract

Microbial communities are key drivers of carbon, sulfur and nitrogen cycling in coastal ecosystems, where they are subjected to dynamic shifts in substrate availability and exposure to toxic compounds. However, how these shifts affect microbial interactions and function is poorly understood. Unraveling such microbial community responses is key to understand their environmental distribution and resilience under current and future disturbances. Here, we used metagenomics and metatranscriptomics to investigate microbial community structure and transcriptional responses to prolonged ammonium deprivation and sulfide and nitric oxide toxicity stresses in a controlled bioreactor system mimicking coastal sediment conditions. *Candidatus Nitrobium versatile*, identified in this study as a sulfide-oxidizing denitrifier, became a rare community member upon ammonium removal. The methanotroph *Ca. Methanoperedens nitroreducens* showed remarkable resilience to both experimental conditions, dominating transcriptional activity of dissimilatory nitrate reduction to ammonium (DNRA). After the ammonium removal experiment, a novel methanotroph species that we have named *Ca. Methyloirabilis tolerans* outcompeted *Ca. Methyloirabilis lanthanidiphila* and the anaerobic ammonium oxidizer (anammox) *Ca. Kuenenia stuttgartiensis* outcompeted *Ca. Scalindua rubra*. At the end of the sulfide and nitric oxide experiment, a gammaproteobacterium affiliated to the family *Thiohalobacteraceae* was enriched and dominated transcriptional activity of sulfide:quinone oxidoreductase. Our results indicate that some community members could be more resilient to stresses than others in coastal ecosystems, leading to dynamic microbial community shifts and novel

functional states. Methane and sulfide oxidation could be ecosystem functions preserved across the investigated disturbances, while differing nitrogen cycling pathways might be favored in response to stresses.

Importance

Coastal ecosystems are primary zones of biogeochemical cycling, processing inputs of nutrients both generated *in situ* and derived from land runoff. Microbial communities that inhabit coastal sediments perform these biogeochemical reactions, but microbial responses to dynamic, periodic substrate deprivation and exposure to toxic compounds remain elusive. In this study, we sought to address this knowledge gap in a controlled bioreactor system, unraveling microbial metabolic pathways and monitoring microbial responses to stresses that might occur in coastal sediments. We identified key microbial players and shifts in their abundance and transcriptional activity. Our results indicated that methanotrophs were particularly resilient to stresses, sulfide oxidizers differed in resiliency but the community maintained sulfide oxidation function across stresses, and that anaerobic ammonium oxidizing (anammox) bacteria were sensitive to substrate deprivation but could restore activity once favorable conditions were reestablished. These insights will help to understand and predict coastal ecosystem responses to future disturbances.

Introduction

Microorganisms drive and link the biogeochemical cycles of carbon, nitrogen and sulfur by a variety of redox reactions (1). Anthropogenic nutrient inputs from land into the ocean constitute a major impact on marine ecosystems, altering seawater and sediment biogeochemistry and leading to increased primary production that can result in toxic algal blooms and oxygen depletion (2). Such impacts, combined with ocean warming and consequent seawater stratification and deoxygenation, can further stimulate the production of methane and nitrous oxide, potent greenhouse gases, as well as sulfide and nitric oxide, toxic products of sulfate reduction and denitrification (2–5). In coastal sediments, ammonium and nitrate can be introduced via agricultural runoff, while sulfide, nitrogen oxides, methane, and ammonium are generated *in situ* via sulfate reduction, partial denitrification, methanogenesis, and organic matter decomposition, respectively (6). Therefore, characterizing microbial communities, interactions and reactions performed by microorganisms that couple methane, nitrogen and sulfur cycling is fundamental for understanding

57 biogeochemical cycling and linked greenhouse gas emissions in dynamic coastal ecosystems impacted by
58 anthropogenic activity.

59

60 Modeling efforts suggest links between future environmental changes, biogeochemical cycles and ecosystem
61 functions (7, 8). However, given that most microorganisms are widespread and functionally redundant, they
62 are frequently treated as a “black box” in models - preventing the effective modeling of their reactions and
63 responses. Additionally, recent efforts with laboratory cultures and engineered systems have obtained
64 insights about impacts of substrate availability changes on environmentally and economically relevant
65 microbial communities (9–14). Yet, few studies have examined microbial community responses to prolonged
66 periods of substrate scarcity or environmental stresses in controlled systems (15, 16). However, these are
67 highly relevant disturbances in coastal ecosystems, where, for instance, nitrogen limitation is a major control
68 on eutrophication (17) and sulfide toxicity can lead to mortality of marine life (18). Such studies are needed
69 to unravel key microbial interdependencies, competitive interactions and functional shifts, as well as to
70 comprehend their environmental distribution and resilience under current and future disturbances.

71

72 Carbon-, nitrogen- and sulfur-cycling microbial communities harbor potential for biotechnological
73 applications such as the improvement of wastewater treatment systems. For instance, Deng *et al.* proposed
74 that sulfide-driven partial denitrification could be coupled to anaerobic ammonium oxidation (anammox) in
75 future applications, given that rapid oxidation of sulfide to elemental sulfur can prevent toxicity and
76 inhibition of anammox activity (13). On the other hand, sulfide addition in a controlled aerated bioreactor
77 setting promoted undesirable production of nitrous oxide and nitrite via partial denitrification and
78 dissimilatory nitrite reduction to ammonium (DNRA), respectively (14). These observations indicate that
79 further studies are necessary to understand complex microbial community interactions and to evaluate how
80 they can be best employed in biotechnological applications.

81

82 In this research project, we investigated transcriptional stress responses of a complex microbial community
83 enriched in an anoxic laboratory-scale bioreactor mimicking dynamic, brackish sediment conditions, where
84 periodic ammonium deprivation, and sulfide and nitric oxide (NO) toxicity stresses, the chosen stressors in

this study, might occur. The culture performed sulfide, ammonium and methane oxidation at the expense of nitrate via sulfide-oxidizing denitrifiers, anammox bacteria, and nitrite/nitrate-dependent anaerobic methane oxidizers (19). This study's aims were (1) to understand the effect of periodic ammonium removal on dissimilatory nitrite reduction to ammonium (DNRA) as a source of ammonium for anammox activity, as well as on general microbial community structure and transcriptional activity, (2) to unravel potential metabolic reactions utilized by key community members, and (3) to characterize microbial community structure and transcriptional responses to prolonged sulfide and NO toxicity stresses while attempting to enrich sulfide-oxidizing denitrifiers.

Materials and methods

Bioreactor operation under regular and experimental conditions

A previously described complex co-culture of sulfide- and methane-oxidizing denitrifiers and anammox bacteria (19) was subjected to a 10-week substrate starvation (ammonium removal) experiment, and later, after regular operation conditions were re-established, to a 7-week sulfide and nitric oxide stress experiment (Figure 1). Briefly, under regular operation, the reactor culture was kept under anoxic conditions and fed daily with 0.67 mmol of sulfide, 1.4 mmol of ammonium, 3 mmol of nitrate, and 488 mmol of methane. The medium contained a solution of salts, trace elements, minerals and vitamins as previously described (19), and the pH was kept at 7.1. Under experimental operation, the following conditions were modified. During the ammonium removal experiment, the ammonium concentration in the medium was gradually decreased from 7 to 0 mM over one month. Two more weeks passed without ammonium addition to the reactor in order to ensure that no residual ammonium was present, and the first biomass sample for metatranscriptomic sequencing (T1) was collected and stored at -80°C. After 10 weeks more without ammonium, another biomass sample for metatranscriptomic sequencing was collected (T2) and ammonium was re-introduced into the medium.

In preparation for the next experiment, the reactor was moved into a laminar flow hood. Methane flow was stopped at the time of reactor moving and reintroduced after 15 days. Once regular operation conditions and

113 substrate consumption were reestablished, the sulfide and NO toxicity stress experiment began. Initially, the
 114 NO concentration in the reactor headspace was gradually increased to 1-5% for 10 weeks so that the
 115 community could adapt. Then, NO concentrations were increased to ~10-13% and all substrates except
 116 sulfide were removed from the reactor, resulting that only sulfide, NO and medium were provided to the
 117 reactor for the following 7 weeks. A biomass sample for metatranscriptomic sequencing was collected before
 118 any NO was added to the reactor (T4) and after the 17 weeks (T5). Originally, the experiment was planned to
 119 be conducted in 20 weeks, being that during the 10 last weeks the reactor would be maintained under only
 120 sulfide and NO. However, covid-19 lockdown in the Netherlands resulted in restricted access to the
 121 laboratory and the timing had to be changed. Additional metatranscriptomics samples in between
 122 experiments for were included in this study: T0, approximately 2 months before the ammonium removal
 123 experiment, and T3, 6 months after the ammonium removal experiment and 11 months before the sulfide and
 124 NO stress experiment (Figure 1).

125

126

127 **Figure 1.** Timeline of experiments. Zero indicates when the bioreactor was stably maintained; 1, when the
 128 ammonium removal experiment happened; 2, after the ammonium removal experiment and re-stabilization
 129 period; 3; reactor placement under a fume hood in preparation for the next experiment; 4, preparatory period
 130 for the nitric oxide and sulfide toxicity stresses experiment; 5, nitric oxide and sulfide toxicity stresses
 131 experiment. Samples for metagenomics (G0-G5), metatranscriptomics (T0-T5) and RT-qPCR (R0-R1 and
 132 R3-R4) were collected as indicated across experimental time points and in between them. The dashed line in
 133 the ammonium removal experiment indicates that removal was conducted stepwise (in mM: 7 → 4.5 → 3.5
 134 → 2.5 → 0). Exact dates and experiment durations are provided in Supplemental Table 1.

135

136 During regular and experimental conditions, the reactor was checked daily for general parameters including
 137 pH with an Applisense electrode (Applikon, Delft, The Netherlands) and nitrate and nitrite concentrations

with MQuant™ colorimetric test strips (Merck, Darmstadt, Germany). During experimental conditions, nitrate and nitrite were also measured with the Griess assay (Griess, 1879) as previously described (19), and ammonium was measured fluorometrically after reaction with 10% ortho-phthalaldehyde as previously described (20). NO in headspace samples was measured with a Sievers Nitric Oxide analyzer (NOA280i; GE Power Water & Process Technologies, Boulder, CO), and sulfide in liquid samples was measured with the methylene blue assay (21) (HACH, Loveland, CO, USA). Sulfate was determined via the barium precipitation method using the Sulfate Assay Kit following manufacturer's instructions (Sigma Aldrich, Saint Louis, MI, USA). Sample measurements were carried out in technical triplicates.

Nucleic acid extractions and sequencing

Biomass samples for metagenomic sequencing were collected during regular and experimental periods (Figure 1) and stored at -20°C. All DNA extractions were performed using the DNAeasy Power Soil Kit (Qiagen, Hilden, Germany) following manufacturer's instructions with two modifications: bead beating was performed in a TissueLyzer (Qiagen, Hilden, Germany) for 10 minutes and autoclaved MilliQ water was used instead of the kit's buffer in the last elution step. RNA extractions were initially performed (for T1 and T2) using the RiboPure Bacteria Kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. During the stress experiment (T4 and T5), this method no longer resulted in sufficient extracted RNA for sequencing, potentially due to extensive extracellular polymeric substance production, and thus the method was changed to RNAeasy Power Soil Kit (Qiagen, Hilden, Germany), which was also used for two additional samples (T0 and T3). All RNA samples were treated with DNAase I at 37°C for 30 min from the RiboPure Bacteria Kit (Thermo Fisher Scientific, Waltham, MA, USA). DNA and RNA concentrations were measured with a Qubit 2.0 fluorometer using the dsDNA and RNA HS Kits (Thermo Fisher Scientific, Waltham, MA, USA). DNA and RNA quality were determined using a NanoDrop Spectrophotometer ND-1000 (Isogen Life Science, Utrecht, The Netherlands) and a Bioanalyzer 2100 (Agilent, Santa Clara, CA, USA), respectively.

Metagenomic libraries were prepared using the Nextera XT Kit (Illumina, San Diego, California, USA) following manufacturer's instructions. Enzymatic tagmentation was performed with 1 ng DNA, followed by

the incorporation of indexed adapters and amplification of the library. Amplified DNA libraries were then purified, and their quality and concentration were determined as aforementioned. Libraries were sequenced on an Illumina MiSeq platform (San Diego, California, USA) using the MiSeq Reagent Kit v3 (San Diego, California, USA), generating 300 bp paired-end reads. RNA samples of T1 and T2 were rRNA-depleted with MICROBexpress Kit (ThermoFisher, Waltham, USA) and MEGAclear kit (Ambion, Life Technologies, Carlsbad, CA USA). Subsequently, 0.1 - 4 µg of RNA from T1-T3 were used to construct strand specific RNA-Seq libraries. Non-rRNA in RNA-Seq libraries were enriched by selective priming during the first strand cDNA synthesis reaction, as well as in the final library construction steps using TruSeq Stranded mRNA sample preparation guide (Illumina proprietary catalog RS-122-9004DOC). RNA from these samples was sequenced with an Illumina MiSeq platform (Illumina, CA, USA), generating 151 bp single-end reads. Metatranscriptomic libraries of T0, T4 and T5 were constructed using TruSeq stranded mRNA library Kit (Illumina, San Diego, California, USA). These RNA-Seq libraries were sequenced with an Illumina NovaSeq 6000 platform (San Diego, California, USA), generating 151 bp paired-end reads (San Diego, California, USA). All metatranscriptomes were triplicate RNA extractions and sequencing.

Metagenomic and metatranscriptomic analyses

Metagenomic data were analyzed as it follows. Read quality was accessed with FASTQC v0.11.8 before and after quality-trimming, adapter removal and contaminant filtering, performed with BBDuk (BBTools v38.75). Trimmed reads were co-assembled *de novo* using MEGAHIT v1.2.911 (22) and mapped to assembled contigs using BBDuk (BBTools v38.75) (23). Sequence mapping files were handled and converted using SAMtools v1.10. Contigs at least 1000 bp-long were used for binning with CONCOCT v1.1.0 (24), MaxBin2 v2.2.7 (25), and MetaBAT2 v2.1512 (26). Resulting metagenome-assembled genomes (MAGs) were dereplicated with DAS Tool v1.1.213 (27) and taxonomically classified with GTDB-Tk v1.3.0 (28) release 9514. MAG completeness and contamination was estimated with CheckM v1.1.2 (29).

MAGs were annotated with DRAM v1.0 (30) with default options, except -min_contig_size 1000 bp, and genes of interest were searched in annotation files as well as via BLASTP and HMM analyses. Only high and medium quality MAGs (>50% complete and < 10% contaminated) were included in genome-centric

analyses, and the entire dataset (binned and unbinned contigs) was considered in gene-centric analyses. For phylogenetic trees, sequences were aligned with muscle v3.8.31 (31), alignment columns were stripped with trimAl v1.4.rev22 (32) using the option -gappypout, and trees were built with FastTree v2.1.10 (33) or UBCG v3.0 (34) and visualized with iTOL v6 (35). For calculating average amino acid identity (AAI) between selected MAGs, genomes were gene-called with Prodigal v2.6.3 (36), and amino acid fasta files were used as input to the Kostas Lab online tool (<http://enve-omics.ce.gatech.edu/g-matrix/index>, (37)). The Genome-to-Genome Distance Calculator v3.0 tool was used online (<https://ggdc.dsmz.de/ggdc.php#>, (38)). Heat maps were constructed with the package heatmap.2 on RStudio v1.3.959, R v4.0.4.

Metatranscriptomic reads were quality trimmed with Sickle v1.33 (39) using the using the sickle se (single end) or pe (paired-end) options for sanger sequencing (-t sanger). Trimmed transcripts were mapped against the annotated metagenome with Bowtie2 (40) (bowtie2 -D 10 -R 2 -N 1 -L 22 -i S,0,2.50 -q -a -p 30), allowing only one mismatch. Index stats files were imported into RStudio to calculate Transcripts per Million (TPM) values according to the formula $TPM = (\text{number of reads} / (\text{gene length}/10^3)) / 10^6$, which were used as unit of gene transcription. Aware of differing extraction and sequencing methods for T1 and T2, these two data points have only been used in separate analyses. TPM values were used for bubble plot generation with the packages ggplot on RStudio. All figures were edited on Adobe Illustrator CC v22.1.

Reverse-transcription quantitative polymerase chain reaction (RT-qPCR)

Selected RNA samples were used for RT-qPCR (Figure 1) in order to confirm patterns emerging from metatranscriptomic analyses. Three primer pairs were selected to quantify transcription of genes of interest: hydrazine synthase subunit A, with *hzsA*-F (5'-WTCGGRTATCARTATGTAG-3') and *hzsA*-R (5'-AAATGGYGAATCATARTGGC-3'), adapted from previously published primers (41); particulate methane monooxygenase subunit A, with *pmoA*-F (5'-SCGRGTRMAGCCSGGTGAGA-3') and *pmoA*-R (5'-YGATGGYCCMGGYACMGAGT-3'), designed for this study; and methyl-coenzyme M reductase subunit A, with *mcrA*-F (5'-AAAGTGCGGAGCAGCAATCACC-3') and *mcrA*-R (5'-TCGTCCCATTCTGCTGCATTGC-3') (42). Bacterial and archaeal 16S rRNA gene-targeting primers 16S-F (5'-AACTYAAAKGAATTGRCGG') and 16S-R (5'-ACGGGCGGTGWGTRC-3') were used as

housekeeping genes for data normalization, adapted from previously published primers (43). For each sample, 50 ng of RNA was used for the reverse transcription reaction using the RevertAid H Minus First Strand cDNA Synthesis Kit (Thermo Fisher Scientific, Waltham, MA, USA) following manufacturer's instructions. Each qPCR reaction consisted of 12 µL SYBR Green FastMix (QuantaBio, MA, USA), 1 µL forward and 1 µL reverse primer (10 µM working solutions), 10 µL DEPC-treated water (Thermo Fisher Scientific, Waltham, MA, USA) and 1 µL template single stranded cDNA in a total volume of 25 µL. The qPCR program consisted of the following steps: initial denaturation (94 °C for 5 min), denaturation (94°C for 30 sec), annealing (temperature as below for 30 sec), elongation (72°C for 30 sec), and melting curve (50-95°C, 0.5°C increase per 5 sec). Denaturation, annealing and elongation steps were repeated for 40 cycles. Annealing temperatures for *hzaA*, *pmaA*, *mcrA* and general 16S rRNA gene primers were 55°C, 62°C, 62°C and 55°C, respectively. qPCR performed directly on RNA samples discarded DNA contamination given that all Ct values were above the threshold of 30 cycles. RT-qPCR units were expressed in $2^{-\Delta\Delta CT}$ by subtracting, first, normalizer with target functional gene CT values, and second, R0 and R3 from R1 and R4 CT values, respectively. Replicates were discarded if CT values above 30.

Data Availability

Metagenomic trimmed reads, metatranscriptomic trimmed reads, metagenome-assembled genomes and unbinned contigs have been deposited on NCBI under BioProject number PRJNA758578.

Results

Diverse microbial community members cycled methane, nitrate, nitrite, ammonium, and sulfide

During the ammonium removal experiment, the bioreactor received the same daily quantities of nitrate (3 mmol), methane (488 mmol) and sulfide (0.67 mmol) as previously described (19). A comprehensive investigation of daily substrate consumption was carried out in the aforementioned study (corresponding the zero time point in Figure 1), which showed that sulfide was completely consumed with a nitrate removal rate of 2.6 mmol per day. The residual nitrate concentrations fluctuated around 4 mM, while no nitrite could be detected in the bioreactor (19). Ammonium was removed from the medium (T1), and nitrate, nitrite and

250 ammonium substrate consumption was monitored for a period of 12 days prior to T2 sample collection (10
251 weeks without ammonium). Nitrate had a residual concentration of 3 mM, while ammonium, nitrite and
252 sulfide concentrations remained below the detection limit. The overall nitrate and nitrite concentrations were
253 consistent with the previously determined concentrations.

254

255 During the sulfide and nitric oxide (NO) toxicity stress experiment, methane, nitrate and ammonium were
256 not provided to the reactor. While sulfide concentrations remained below detection limit for the entire
257 experiment, indicating continuous complete sulfide removal, nitrite accumulated to 870 μ M during the
258 second week of the bioreactor receiving only sulfide and nitric oxide, and remained between 100-400 μ M
259 during the remainder of the experiment. This indicated that the community removed residual nitrate via
260 denitrification and DNRA for several weeks. Biomass decay was evident from the visual increase in reactor
261 turbidity and, to a degree, change of granule color from red to black, until the end of the experiment.

262

263 The composition of the bioreactor microbial community was investigated via a time-series of metagenomic
264 sequencing. In a previous study with this bioreactor, community members included several proteobacteria,
265 *Ca. Kuenenia stuttgartiensis*, *Ca. Scalindua brodae*, *Ca. Methanoperedens nitroreducens*, *Ca.*
266 *Methyloirabilis* species, and a novel bacterium within the Nitrospirota phylum, *Ca. Nitrobium versatile*,
267 potentially linking sulfur and nitrogen cycling (19, 44–46). Co-assembly of eight samples resulted in 30 high
268 (>90% complete, <5% contaminated) and 29 medium (>50% complete, <10% contaminated) quality
269 metagenome-assembled genomes (MAGs) (Figure 2). We will first introduce the most dominant members
270 and, in the next section, we will discuss their change in abundance over time. The only archaeon detected in
271 the bioreactor was *Ca. Methanoperedens nitroreducens* (MAG 36), a nitrate-reducing anaerobic
272 methanotroph. Bacteria were much more diverse and represented by five MAGs affiliated to candidate phyla
273 (AABM5-125-24, ARS69, FEN-1099, GWC2-55-46, OLB16), two to *Acidobacteriota*, one to
274 *Actinobacteriota*, one to *Armatimonadota*, eleven to *Bacteroidota*, six to *Chloroflexota*, one to
275 *Cyanobacteria*, two to *Methyloirabilota*, two to *Myxococcota*, one to *Nitrospirota*, one to *Omnitrophota*,
276 five to *Planctomycetota*, and twenty to *Proteobacteria*.

277

278 Two of the *Planctomycetota* MAGs were the anammox bacteria *Candidatus* Kuenenia stuttgartiensis (MAG
279 32) and *Candidatus* Scalindua rubra (MAG 55). The one *Nitrospirota* MAG 39 was *Candidatus* Nitrobium
280 versatile, which we further characterized in this study. The two MAGs affiliated to *Methylomirabilota* were
281 classified by GTDB-Tk as *Candidatus* Methylomirabilis oxyfera (MAG 37) and *Candidatus*
282 Methylomirabilis sp002634395 (MAG 38). However, phylogenetic and average amino acid identity (AAI)
283 analyses revealed that the first MAG represented a species of *Candidatus* Methylomirabilis lanthanidiphila
284 (100% AAI to the type genome (47)), and the latter was a novel species sharing only 81% AAI to *Candidatus*
285 Methylomirabilis limnetica (Supplemental Figure 1 and 2). The genome-to-genome distance between MAG
286 38 and *Ca. M. limnetica* ranged from 0.15-0.59, with a probability of DNA-DNA hybridization > 70%
287 ranging from 0-0.08%, supporting AAI results. We have named this novel species *Candidatus*
288 Methylomirabilis tolerans due to its persistence over the sulfide and NO stress experiment (Figure 3).
289 Genome analyses indicated that *Candidatus* Methylomirabilis tolerans had a similar metabolic potential for
290 nitric oxide dismutation coupled to methane oxidation via intracellular oxygen generation as previously
291 described, sharing several other characteristics with *Ca. Methylomirabilis lanthanidiphila* (Table 1). Finally,
292 several previously identified and novel putative sulfide oxidizers were identified based on key gene analyses
293 (Figure 4 and 5): MAG 58 Sulfuricellaceae, MAG 10 Andersenellaceae, MAG 23 Dokdonella, MAG 40
294 Notoacmeibacter, MAG 50 Rhodocyclaceae 1, MAG 52 Rhodocyclaceae 3, MAG 57 Sulfuricella
295 denitrificans, MAG 56 Sulfuricella, MAG 64 Thiobacillus, MAG 65 Thiohalobacteraceae, and MAG 63
296 Thermomonas.
297

Figure 2. Up-to-date bacterial core gene (UBCG) tree of 92 concatenated genes extracted from high and medium quality MAGs in this study. Genome completeness (in yellow) and contamination (in purple) values in percent are indicated to the right. MAGs in bold were selected from detailed characterization due to large shifts in abundance across experiments.

Table 1. Comparison of functional genes and pathways in selected *Ca. Methyloirabilis* species from this and other studies. Abbreviations are as follows: PMO, particulate methane monooxygenase; Mxa, calcium-dependent methanol dehydrogenase; Xox, lanthanide-dependent methanol dehydrogenase; H₄F,

307 tetrahydrofolate pathway; H₄MPT, tetrahydromethanopterin pathway; FDH, formate dehydrogenase; Nxr,
 308 membrane-bound nitrate reductase/ nitrite oxidoreductase phylogenetically closest to nitrite-oxidizing
 309 enzyme sequences; Nap; periplasmic nitrate reductase; NirS, cytochrome *cd₁*-containing nitrite reductase;
 310 NirBD, cytoplasmic ammonium-forming nitrite reductase; NOD, nitric oxide dismutase; qNOR, quinone-
 311 oxidizing electrogenic nitric oxide reductase; sNOR, cytochrome *c*-oxidizing electrogenic nitric oxide
 312 reductase; gNOR quinone-oxidizing non-electrogenic nitric oxide reductase; COX, cytochrome *c* oxidase
 313 complex; Hao; hydroxylamine oxidoreductase; NI, not identified.

Reaction	<i>Ca. M. oxyfera</i> (47)	<i>Ca. M. lanthanidiphila</i> (48)	<i>Ca. M. tolerans</i> (this study)	<i>Ca. M. limnetica</i> (49)
Carbon metabolism				
Carbon fixation	Calvin cycle	Calvin cycle	Calvin cycle	Calvin cycle
Methane oxidation	PMO	PMO	PMO	PMO
Methanol oxidation	Mxa, Xox	Xox	Xox	Xox
Formaldehyde oxidation	H ₄ F, H ₄ MPT	H ₄ F, H ₄ MPT	H ₄ F, H ₄ MPT	H ₄ F, H ₄ MPT
Formate oxidation	FDH	FDH	FDH	FDH
Nitrogen metabolism				
Nitrate reduction/ nitrite oxidation	Nxr, Nap	Nxr, Nap	Nxr, Nap	Nap
Nitrite reduction to nitric oxide	NirS	NirS	NirS	NirS
Nitrite reduction to ammonium	NirBD fusion	NirBD fusion	NirBD fusion	NI

Nitric oxide dismutation	NOD	NOD	NOD	NOD
Nitric oxide reduction	qNOR, sNOR, gNOR	qNOR, sNOR, gNOR	qNOR, sNOR, gNOR	qNOR
Nitrous oxide reduction	NI	NI	NI	NI
Oxygen reduction	COX	COX	COX	COX
Hydroxylamine oxidation	Hao-like	Hao-like	Hao-like	-
Others				
<i>bc_L</i> complex	Canonical	Min. 2 non- canonical	Min. 2 non- canonical	Min. 2 non- canonical
Gas vesicle	NI	NI	NI	Yes (<i>gvpA</i> and associated proteins)

314

315

316 **MAG coverages revealed shifts in microbial community structure during experimental conditions**

317 Normalized genome coverage for each MAG was used as a proxy for organism abundances across time
318 points. Under regular operation conditions (G0), the most abundant microorganism in the reactor was MAG
319 39 *Ca. Nitrobium versatile*, with genome coverage of approximately 245x (Figure 3). MAG 37 *Ca.*
320 *Methylomirabilis lanthanidiphila* had normalized genome coverage of 89x, MAG 60 *Thermoanaerobaculum* 1
321 of 26x, MAG 57 *Sulfuricella denitrificans* of 26x, and MAG 55 *Scalindua rubra* of 25x. However, large
322 shifts in microbial community structure occurred after ammonium was removed from the medium and
323 subsequently re-introduced (G1). MAG 36 *Methanoperedens nitroreducens*, which had previously had a
324 normalized genome coverage of 5x, increased to 278x, and MAG 38 *Methylomirabilis tolerans*, which had a
325 coverage of 19x before, increased to 84x. Other abundant genomes at G1 were MAG 44 *Plantomycetes* 1
326 (50x), MAG 22 *Desulfobacterota* (39x), MAG 32 *Kuenenia stuttgartiensis* (37x), and MAG 56 *Sulfuricella*

(37x). These genomes remained the most abundant in the bioreactor during the recovery period (G2), with MAG 36 *Methanoperedens nitroreducens* reaching 366x coverage, and MAG 38 *Methylomirabilis tolerans*, 110x, indicating that the community reached another distinct stable structure. During the preparatory phase for the sulfide and NO toxicity experiment (G3), MAG 38 *Methylomirabilis tolerans* became dominant (292x), followed by MAG 36 *Methanoperedens nitroreducens* (126x), MAG 60 *Thermoanaerobaculia* 1 (71x), MAG 44 *Plantomycetes* 1 (33x), and MAG 32 *Kuenenia stuttgartiensis* (18x). These MAGs remained, in this order, the most abundant genomes in the reactor until the sulfide and NO toxicity experiment started (G4). Finally, at the end of this experiment (G5), shifts in community structure were detected: MAG 36 *Methanoperedens nitroreducens* returned as the dominant genome (207x), followed by MAG 38 *Methylomirabilis tolerans* (198x), MAG 65 *Thiohalobacteraceae* (52x), MAG 44 *Plantomycetes* 1 (36x), MAG 60 *Thermoanaerobaculia* 1 (29x), and MAG 32 *Kuenenia stuttgartiensis* (19x).

338

339

340 **Figure 3.** Heat map displaying normalized genome coverages of most abundant MAGs across experimental
341 conditions. Abbreviations are as follows: B, before; A, after; R, recovery; pre, preparatory phase.
342 Experiments are indicated in bold on top. MAGs are ordered based on normalized genome coverage values
343 and abundance change patterns.

344

345 **Metatranscriptomic analyses reveal active metabolic pathways in key microbial community members** 346 **cycling methane, nitrogen and sulfur**

347 In order to investigate transcriptional responses of the bioreactor microbial community to experimental
348 conditions and to unravel microbial pathways likely utilized for metabolic activity in the reactor, we
349 conducted a time series of metatranscriptomic sequencing, from which we calculated transcript per million
350 (TPM) values as unit of gene transcription. We had particular interest in microorganisms that achieved
351 highest abundances or persisted across experimental time points, and selected several key species for in-
352 depth metabolic reconstruction: two key putative sulfur-oxidizing microorganisms (MAG 39 Nitrobium
353 versatile and MAG 65 Thiohalobacteraceae), two key methane oxidizers (MAG 36 Methanoperedens
354 nitroreducens and MAG 38 Methyloirabialis tolerans), and two key nitrogen-cycling microorganisms - the
355 ammonium oxidizer *Ca. Kuenenia stuttgartiensis* (MAG 32) and a putative denitrifying acidobacterium
356 (MAG 60 Thermoanaerobaculia 1).

357

358 The *Nitrospirota* MAG 39 Nitrobium versatile, which was the most abundant microbial community member
359 based on genome coverage before the ammonium removal experiment (G0, Figure 3), was estimated to be a
360 100% complete genome with 5% contamination (Figure 2). Based on metatranscriptomic analyses, we infer
361 that the most likely metabolism performed by this organism was sulfide oxidation coupled to denitrification
362 (Figure 4). This organism was severely affected by the ammonium removal experiment, as indicated by
363 genome coverage (Figure 4). TPM values suggest that *Ca. Nitrobium versatile* became a rare community
364 member but remained transcriptionally active across all time points. When the reactor was primed for the
365 sulfide and NO toxicity experiment (T4), transcripts for 161 genes were detected, including *norC* and *phsA*,
366 while by the end of the experiment (T5), for 130 genes (Supplemental Table 2).

367

368 A previously unidentified sulfide:quinone oxidoreductase *sqr* gene in *Ca. Nitrobium versatile* was one of the
 369 mostly highly transcribed functional genes (TPM = 0.2 ± 0.05) of this organism in a thriving period (T0,
 370 Supplemental Table 2), along with cytochrome *c*-oxidizing nitric oxide reductase genes *norBC* (respectively,
 371 TPM = 1.9 ± 0.7 and 2.8 ± 1.2). Sulfide could thus be oxidized to elemental sulfur, which might be the
 372 substrate for a dissimilatory sulfite reductase (*dsrABC*) operating in the oxidative direction, despite the two
 373 *dsrA* copies in the genome being phylogenetically related to reductive *dsrA* genes (Supplemental Figure 3)
 374 and the presence and transcription of a *dsrD* gene. Interestingly, both *dsrABC* copies in the genome were
 375 transcribed, one approximately 3 times more than the other (Supplemental Table 2, average TPM 0.3-0.4 vs
 376 0.1). Sulfite could be oxidized to sulfate via transcribed adenosine-phosphosulfate (APS) reductase (*aprAB*)
 377 and sulfate adenylyltransferase (*sat*) or via a sulfite:cytochrome *c* oxidoreductase (*sorAB*). Although we did
 378 not provide reduced sulfur compounds to the reactor other than sulfide, two genes encoding putative sulfide-
 379 generating enzymes were identified and transcribed: a sulfhydrogenase polysulfide reductase (*hydB* subunit
 380 beta only, TPM = 0.1 ± 0.06), and a thiosulfate reductase / polysulfide reductase (chain A only, *phsA*, present
 381 twice in the genome, TPMs = 0.09 ± 0.05 and 0.016 ± 0.018).

382
 383 Interestingly, in MAG 39 *Nitrobium versatile*, a nickel-dependent hydrogenase- (*hyd-2*) and maturation
 384 protein-encoding genes were also transcribed at lower levels, indicating that hydrogen could be oxidized.
 385 Several other functional genes were transcribed at low levels: membrane-bound and periplasmic nitrate
 386 reductase genes (*narGHI* and *napA*), ammonium-forming cytochrome *c*₅₅₂-nitrite reductase (*nrfA*), and
 387 anaerobic dimethyl sulfoxide reductase (*dmsABC*). Although previously identified (19), *napB* and *nirBD*
 388 were absent in the genome, and *nrfH* was present in the genome but no transcription was detected. All genes
 389 encoding subunits of electron transport chain proteins were transcribed (Figure 4). Two genes encoding
 390 complexes with homology *caa*₃-type low affinity cytochrome *c* oxidase proteins in *Acidobacteria* were
 391 identified: (i) *ctaCFED*, which was followed by a downstream *sco* assembly protein-encoding gene and a
 392 cytochrome *c*₆ (homolog to *petJ*, K08906), which we have putatively denominated *ctaX*, for it could be part
 393 of the complex, and (ii) *sco* followed by a downstream *ctaDEFC*. All these subunits were transcribed, except
 394 for *ctaF* of the latter complex. A Rieske *bc*₁ complex was encoded by *petBCD*, which was preceded by a
 395 cytochrome *c* protein-encoding gene immediately upstream. Two other copies of *petBC* were present in the

396 genome. All these subunits were transcribed except for the first *petB* (Supplemental Table 4). Finally, the
397 MAG 39 *Ca. Nitrobium versatile* had two ammonium transporter-encoding genes (*amt*), of which only the
398 second was transcribed, solely in T0 (Supplemental Table 4).

399

400 The gammaproteobacterial MAG 65 Thiohalobacteraceae, representing an organism that seemed enriched
401 over the sulfide and NO toxicity experiment (Figure 3), was estimated to be a 98.1% complete genome with
402 0.3% contamination (Figure 2). Based on metatranscriptomic analyses, we infer that the most likely
403 metabolism performed by this organism was sulfide oxidation coupled to denitrification (Figure 4). A
404 sulfide:quinone oxidoreductase-encoding *sqr* gene was among the functional genes with highest transcription
405 by the end of the sulfide and NO toxicity experiment (T5; TPM = 0.43 ± 0.36 ; Supplemental Table 2,
406 Supplemental Table 4). Also highly transcribed were the genes *dsrA* (TPM = 1.07 ± 0.78), *dsrB* (TPM =
407 0.95 ± 0.5), *dsrC* (TPM = 4.3 ± 1.1), as well as additional *dsrABC* copies. Sulfite oxidation to sulfate could
408 proceed via proteins encoded by *sorA*, *soeABC* (quinone-sulfite dehydrogenase), as well as *aprAB* and *sat*,
409 which were all transcribed. Sulfur oxidation system *soxBZYX* genes for thiosulfate oxidation and a
410 thiocyanate dehydrogenase *tcdh* gene were transcribed, indicating that these substrates could also be
411 oxidized, although they were not provided to the reactor. No *soxC* gene was detected in the genome. A
412 cytochrome *cd1*-nitrite reductase *nirS* gene was highly transcribed (TPM = 1.74 ± 0.78), along with all other
413 genes in the denitrification pathway, including a copper-containing nitrite reductase *nirK* gene. Ribulose-
414 biphosphate carboxylase *cbbSL* genes were among the most highly transcribed in the genome, and all genes
415 encoding subunits of the electron transport chain were transcribed.

416

417 MAG 36 (*Ca. Methanoperedens nitroreducens*) was 99.4% complete with 4.6% contamination, and had all
418 genes in the reverse methanogenesis pathway (Figure 4, Supplemental Table 4) as well as potential for
419 carbon fixation via the Wood–Ljungdahl pathway with carbon monoxide dehydrogenase/ acetyl-CoA
420 synthase (CODH/ACS)-encoding genes and acetate production or assimilation via an acetyl-CoA synthetase-
421 encoding *acss* gene. All these genes were transcribed by the end of the sulfide and NO toxicity experiment
422 (T5), as well as electron transport chain-encoding genes (Figure 4). The succinate dehydrogenase *sdhC*
423 subunit was not present in the genome. Remarkably, the genome had genes encoding two copies of the

membrane-bound nitrate reductase (*narGHI*), an ammonium-forming nitrite reductase (*nrfAH*), a non-electrogenic cytochrome *c*-oxidizing (cNOR) nitric oxide reductase (*norBC*), and an electrogenic quinone-oxidizing (qNOR) nitric oxide reductase (*norZ*). Methyl-coenzyme M reductase genes *mcrBDGA* had TPM values around ~200-400 before the sulfide and NO toxicity experiment (T4), and ~20-40 after (T5, Supplemental Table 2), indicating that, although *Ca. Methanoperedens nitroreducens* was still abundant by the end of the experiment (Figure 3), it suffered a degree of inhibition. Three hypothetical genes had highest transcription at T5 and were upregulated relative to T4 (Supplemental Table 2).

A second key methane oxidizer was represented by MAG 38 (*Ca. Methyloirabilis tolerans*), 94.5% complete with 2% contamination. This genome had all genes for carbon fixation via the Calvin cycle and for the methane oxidation via particulate methane monooxygenase (*pmoABC*) coupled to nitric oxide dismutation, with four putative *nod* genes present and transcribed. Additionally, nitrate, nitrite and nitric oxide reductase-encoding genes were present and transcribed: *narGHI*, ammonium-forming cytoplasmic NADH-nitrite reductase *nirBD* genes, electrogenic cytochrome *c*-oxidizing (sNOR) nitric oxide reductase-encoding *norSY* genes, non-electrogenic quinone-oxidizing (gNOR) nitric oxide reductase-encoding *norGH* genes, and *norZ*. All four genes encoding the two subunits of the nitric oxide dismutases NOD-1 and NOD-2 had some of the highest transcription (TPM ~7-60) when the community was being primed for the sulfide and NO toxicity experiment, receiving ~1-5% external NO in the reactor along with methane, ammonium and sulfide (T4), as well as *pmoABC* (TPM ~6-22) and lanthanide-dependent methanol dehydrogenase-encoding *xoxF* gene (TPM = 5.5± 3.5) (Supplemental Table 2). Although *Ca. Methyloirabilis tolerans* was still abundant after the sulfide and NO toxicity experiment (Figure 3), TPM values for all aforementioned functional genes decreased 10-100 fold from T4 to T5, indicating that the microorganism suffered a degree of inhibition (Supplemental Table 2).

MAG 60 *Thermoanaerobaculia* 1, 88.3% complete with 1.5% contamination, had highest normalized genome coverage when the reactor was being primed for the sulfide and NO toxicity experiment (G4, Figure 3). Based on metatranscriptomic analyses, the most likely metabolism performed by this organism was heterotrophic denitrification (Figure 4). A nitrous oxide reductase *nosZ* gene was among the functional genes

452 with highest transcription (T4, TPM = 0.21 ± 0.17), followed by *narGHI* and *nrfAH*. Interestingly,
453 hydrogenase-2 and low affinity cytochrome *c* oxidase-encoding *ctaCDEF* genes were also transcribed, as
454 well as all electron transport chain-encoding genes, including two RNF complexes (Supplemental Table 2
455 and 4).

456

457 Finally, *Ca. Kuenenia stuttgartiensis*, represented by MAG 32, 95.6% complete with 0.6% contamination,
458 was one of two microorganisms that persisted throughout all regular and experimental conditions (Figure 3).
459 Interestingly, the transcription of hydrazine synthase *hzsABC* genes decreased 18-27x during the ammonium
460 removal experiment (T1 to T2), and 2-5x during the sulfide and NO toxicity experiment (T4 to T5),
461 suggesting that substrate deprivation was a strong stressor. MAG 32 had all genes encoding substrate
462 oxidation and electron transport chain proteins in the anammox pathway, as well carbon fixation via the
463 Wood-Ljungdahl pathway, which were transcribed at all time points (Figure 4). The second microorganism
464 that persisted throughout all regular and experimental conditions was represented by MAG 44, 93.1%
465 complete with 3.4% contamination, a poorly classified *Planctomycetes* genome. Based on metagenomic and
466 transcriptomic analyses, the most likely metabolism performed by this organism was heterotrophic
467 denitrification via *narGHI* and *nirS* (Supplemental Table 2). While normalized genome coverage of this
468 MAG varied only between 16-50x across regular and experimental conditions, summed TPM values for all
469 genes transcribed in each time point were highest in the preparatory phase for the sulfide and NO toxicity
470 experiment, as well as before and after the experiment (T3-5; TPM = ~168-361) in comparison to previous
471 time points (T0-2; TPM = ~33-43; Supplemental Table 2).

MAG 36 Ca. Methanoperedens nitroreducens

MAGs 38 Ca. Methyloirabilis tolerans

MAG 39 Ca. Nitrobium versatile

MAG 65 Thiohalobacteraceae

MAG 60 Thermoanaerobaculia

MAG 32 Ca. Kuenenia stuttgartiensis

473 **Figure 4.** Metabolic reconstruction of six genomes of interest. Only genes with TPM > 0 in
474 metatranscriptomic analyses are shown in selected time points in which the highest number of genes were
475 transcribed (T5 for MAGs 36, 60 and 65, T4 for MAG 38, and T0 for MAGs 32 and 39). Gene abbreviations,
476 loci and annotations are provided in Supplemental Table 4. The gene *nrfH* was absent in MAG 39 *Ca.*
477 *Nitrobium versatile* (in grey) but it was previously identified in the first MAG representing this organism
478 (19). In MAG 32 *Ca. Kuenenia stuttgartiensis*, the internal compartment represents the anammoxosome.

479

480 **Microbial community transcriptional responses to substrate removal and toxicity stresses provide** 481 **insights into community dynamics and resiliency**

482 Gene-centric analyses aimed to elucidate the transcriptional responses of the reactor community to two
483 experimental conditions. The first, ammonium removal from the medium while methane, sulfide and nitrate
484 were still provided to the reactor, tested the strength of microbial interactions for the supply of ammonium
485 via dissimilatory nitrate reduction to ammonium (DNRA) to community members - in particular, to
486 anammox bacteria. The second, sulfide and nitric oxide addition while no other substrates were provided to
487 the reactor, aimed to enrich sulfide-oxidizing denitrifiers and to test the limits of microbial community
488 resiliency to these stresses.

489

490 A major response to ammonium removal was a general inhibition of methane, ammonium and sulfide
491 oxidizers, as indicated by downregulation of their key genes *mcrA*, *pmoA*, *hzsA*, *sqr*, *soxB*, *sorA*, and *dsrABC*
492 (Figure 5). RT-qPCR of *mcrA*, *pmoA*, and *hzsA* confirmed these trends (Supplemental Table 3). Not
493 surprisingly, anammox bacteria were particularly affected, with an, approximately, 18-fold decrease in *hzsA*
494 transcription. On the other hand, nitrogen fixation *nifDHK* genes, ammonium transporter *amt* genes, and the
495 ammonium-forming nitrite reductase *nrfA* gene were upregulated, likely to compensate for the ammonium
496 limitation. *Ca. Methanoperedens nitroreducens* accounted for 97.6% of *nrfA* transcription and 48.2% of *amt*
497 transcription 10 weeks after ammonium was completely removed from the medium (T2). Other taxa that
498 shared a large proportion of *amt* transcription in T2 were *Ca. Methyloirabilis tolerans* (14.5%), *Ca.*
499 *Methyloirabilis lanthanidiphila* (9.6%), and *Ca. Kuenenia stuttgartiensis* (3.2%). The *hzsA* gene of *Ca.*
500 *Kuenenia stuttgartiensis* had at least 20x higher transcription than *hzsA* of *Ca. Scalindua rubra* across all time

501 points, although *Ca. Scalindua rubra* had higher genome coverage before experiments were conducted
502 (Figure 3). Finally, minor changes included the upregulation of the periplasmic nitrate reductase *napA* gene,
503 cytoplasmic ammonium-forming nitrite reductase *nirB* gene, quinone-sulfite dehydrogenase *soeA* gene, and
504 quinone-thiosulfate dehydrogenase *doxD* gene.

505

506 A major response to the sulfide and NO toxicity experiment was upregulation of all sulfur oxidation genes
507 (*sqr*, *soxB*, *sorA*, *soeA*, and *dsrABC*) (Figure 5). MAG 65 Thiohalobacteraceae accounted for 67% of *sqr*
508 transcription by the end of the experiment (T5). Interestingly, the transcription of nitric oxide reductase *norB*
509 genes decreased 32 times. However, *norB* TPM values were the highest among all nitrogen cycle marker
510 genes across all time points in this study except T5 (average TPM = 5.22), in which *hzsA* TPM values were
511 higher (average TPM = 7). At T5, *Ca. Methyloirabilis tolerans* accounted for 43% of *norB* transcription at
512 T5, which included NOD-1 (18%), NOD-2 (19%), and qNOR (6%)-encoding genes, followed by *Ca.*
513 *Methanoperedens nitroreducens* (21%, with the qNOR-encoding gene accounting for 13% and cNOR-
514 encoding gene for 8%), *Ca. Methyloirabilis lanthanidiphila* (10%) and MAG 65 Thiohalobacteraceae
515 (9.6%). The transcription of several other genes encoding proteins involved in denitrification (*narG*, *napA*,
516 *nirS*, and *nirB*) was downregulated. However, *nirK* had a 12-fold increase in transcription, and *nosZ* had a
517 minor increase. Methane oxidation marker genes were downregulated, as indicated by a 19-fold decrease in
518 *mcrA* transcription and 21-fold decrease in *pmoA* transcription. Transcription of the anammox marker gene
519 *hzsA* decreased 2.7 fold. RT-qPCR of *mcrA*, *pmoA*, and *hzsA* confirmed these trends (Supplemental table 3).
520 Carbon fixation via CODH/ACS was downregulated, but CO₂ fixation via ribulose-bisphosphate
521 carboxylase-oxygenase was upregulated.

522

Figure 5. Transcriptional responses to ammonium removal and sulfide and NO toxicity experiments. Log₂ fold change, as well as up- or downregulation of genes encoding proteins involved in carbon (C), sulfur (S) and nitrogen (N) cycling, are provide to the right, while taxonomic contributions to selected gene transcription are provided to the left. Under *norB*, subunits of nitric oxide dismutases are also included. Abbreviations are as follows: B, before; A, after.

Discussion

In this study, we conducted metagenomic and metatranscriptomic analyses in order to unravel metabolic

532 pathways, microbial dynamics, and responses to stresses in a bioreactor community mimicking anoxic,
 533 brackish coastal sediment conditions. Microbial interactions and resiliency were tested by removal and
 534 addition of key substrates. Removal of ammonium from the medium had major effects on microbial activity
 535 and community structure: *Ca. Methanoperedens nitroreducens* dominated dissimilatory nitrate reduction to
 536 ammonium (DNRA) activity (Figure 5), and, after ammonium was reintroduced in the medium, became the
 537 most abundant community member (Figure 3). *Ca. Kuenenia stuttgartiensis* replaced *Ca. Scalindua rubra* as
 538 the most abundant anammox bacteria, several sulfide oxidizers replaced *Ca. Nitrobium versatile*, and *Ca.*
 539 *Methylomirabilis tolerans* dominated over *Ca. Methylomirabilis lanthanidiphila* (Figure 3 and 5). These
 540 results suggest strong microbial cooperation between *Ca. Methanoperedens nitroreducens* and *Ca.*
 541 *Methylomirabilis* species for the exchange of nitrite under the abundance of methane. Before the ammonium
 542 removal experiment, TPM values indicate that *Ca. Methanoperedens nitroreducens* reduced nitrate to nitrite,
 543 which was reduced to nitric oxide mostly by *Ca. Methylomirabilis lanthanidiphila*. Nitric oxide was then
 544 shared among three main organisms: *Ca. Methylomirabilis lanthanidiphila*, *Ca. Methylomirabilis tolerans*,
 545 and *Ca. Nitrobium versatile*. However, under ammonium limitation, *Ca. Methanoperedens nitroreducens*
 546 further reduced nitrite to ammonium via DNRA, and this had a cascade effect that changed microbial
 547 community structure, resulting that *Ca. Methylomirabilis tolerans* dominated and *Ca. Methylomirabilis*
 548 *lanthanidiphila* and *Ca. Nitrobium versatile* concomitantly decreased in abundance and activity. These results
 549 suggest that *Ca. Methylomirabilis tolerans* was a more competitive organism in scavenging nitric oxide.
 550 However, the mechanisms remain to be elucidated. Both *Ca. Methylomirabilis* species encoded lanthanide-
 551 dependent methanol dehydrogenases and could be enriched in the bioreactor due to cerium being provided as
 552 part of the medium (47, 48, 50).

553
 554 Anammox bacteria were more strongly limited by ammonium removal than *Ca. Methylomirabilis* species,
 555 presumably due to the dual competition for both ammonium and nitrite. Although *Ca. Methylomirabilis*
 556 organisms have a lower affinity for nitrite ($K_s = 7 \mu\text{M}$; *Ca. M. oxyfera* and *Ca. M. lanthanidiphila* (50)) than
 557 anammox bacteria ($K_s = 0.2\text{-}3 \mu\text{M}$; *Ca. K. stuttgartiensis* and *Ca. Scalindua* sp. (51)), additional competition
 558 for ammonium could have caused anammox to be less fit. Yet, *Ca. K. stuttgartiensis* ($K_s < 5 \mu\text{M}$ for
 559 ammonium (52)) seemed to better withstand ammonium starvation than *Ca. Scalindua rubra* ($K_s = 3 \mu\text{M}$ for

560 ammonium in several *Ca. Scalindua* species (51)), indicating that *Ca. K. stuttgartiensis*' affinity for
 561 ammonium could be higher than that of *Ca. Scalindua*. *Ca. Nitrobium versatile*, on the other hand, seemed to
 562 rely on nitric oxide for sulfide oxidation, decreasing in abundance as *Ca. Methanoperedens nitroreducens*
 563 ($K_s = 2.1 \pm 0.4 \text{ mg N L}^{-1}$ for nitrate (53)) and *Ca. Methyloirabilis tolerans* increased (Figure 3). All three
 564 organisms had potential to generate ammonium (Figure 4), but *Ca. Methanoperedens nitroreducens*
 565 dominated DNRA activity (Figure 5). Therefore, we conclude that DNRA from *Ca. M. nitroreducens* alone
 566 could not sustain anammox activity under ammonium, nitrite and organic carbon (except methane)
 567 limitation, favoring *Ca. Methyloirabilis tolerans*, which likely outcompeted *Ca. N. versatile* and anammox
 568 for nitrite and nitric oxide. These results are contrasting to estuary ecosystems in which anammox activity
 569 could be sustained by DNRA, positively correlating to sulfide and sediment organic carbon content (54).
 570 High sediment organic carbon to nitrate ratio and ferrous iron availability have been reported to favor DNRA
 571 over denitrification in estuary ecosystems (55). Having methane as the only organic carbon source (at
 572 saturation) and a high nitrate load (3 mmol/day) could have thus modulated DNRA activity to become
 573 insufficient to sustain anammox and changed microbial community structure.

574
 575 We also tested microbial community resiliency to sulfide and NO toxicity while attempting to enrich sulfide-
 576 oxidizing nitric oxide reducers. We found genomic potential and transcriptional evidence for this metabolism
 577 in *Ca. Nitrobium versatile*, the dominant microorganism in the bioreactor community before the ammonium
 578 removal experiment (Figure 3). However, *Ca. Nitrobium versatile* became a rare community member after
 579 this experiment and could no longer be enriched. Instead, MAG 65 Thiohalobacteraceae, likely performing
 580 sulfide oxidation coupled to denitrification, increased in abundance (Figure 3, 4 and 5). The sulfide and NO
 581 toxicity experiment revealed unusual resiliency of *Ca. M. nitroreducens* and *Ca. M. tolerans*, which persisted
 582 as the most abundant community members (Figure 3) even though methane and nitrate were no longer
 583 provided to the reactor for the 7 weeks of the experiment, consistent with the downregulation of *mcrA* and
 584 *pmoA* (Figure 5, Supplemental Table 3). Given that the hydraulic retention time of the reactor was 5 days, it
 585 is unlikely that our sequencing results and the dominance of *Ca. M. nitroreducens* and *Ca. M. tolerans* simply
 586 reflect older decaying biomass.

587

MAG 65 Thiohalobacteraceae, MAG 44 Plantomycetes 1, MAG 60 Thermoanaerobaculia 1, and MAG 32 *Ca. Kuenenia stuttgartiensis* followed as the most abundant community members at the end of the experiment. While the first three could make use of sulfide or organic carbon from decaying biomass as electron donors and residual nitrate or nitrite and nitric oxide as electron acceptors, *Ca. K. stuttgartiensis* could have used ammonium generated from decaying biomass and nitrite from residual nitrate reduction or nitric oxide (56). Of these four MAGs, only MAG 65 Thiohalobacteraceae increased in abundance and had both *nirK* and *norB* transcription increased during the sulfide and NO toxicity experiment (Figure 5, Supplemental Table 2), while the other three suffered a degree of inhibition inferred from both decreases in genome coverage (Figure 3) and marker gene transcriptional activity (Figure 5, Supplemental Table 2). Given that sulfide remained below detection limit (0.15 μ M) during all time points, we infer that this inhibition is attributed to nitrite (~100-400 μ M) and nitric oxide (~10-13% of headspace). While nitrite and nitric oxide were likely toxic for several community members, MAG 65 Thiohalobacteraceae seemed to have taken advantage of these compounds as terminal electron acceptors.

MAG 65 Thiohalobacteraceae had similar metabolic potential as the gammaproteobacterium *Thiohalobacter thyonaticus*, which has sulfur oxidation genes encoding FccAB, DsrABC, AprAB, Sat, SoeABC, and SoxXABYZ, as well as thiocyanate dehydrogenase and carbon fixation via the Calvin cycle (57). Additionally, MAG 65 had three *sqr* copies and two *sorA* copies. Interestingly, MAG 39 *Ca. N. versatile* also had a sulfur oxidation pathway that included *sqr*, *dsrABC*, *sorA* or *aprAB* and *sat*. Umezawa *et al.* (2020) described the YTD gene cluster composed of genes *yedE*-like, *tusA*, *dsrE*-like, *chp-1* and *chp-2*, which encoded proteins for sulfur disproportionation in *Nitrospirota* (44). As in this previous study, we also detected an impartial YTD gene cluster in *Ca. N. versatile* (MAG 39), indicating that it might lack sulfur disproportionation potential via this cluster, contrasting to the *Nitrospirota* microorganism species 45J (44), which shares 64% average amino acid identity (AAI) to *Ca. N. versatile*. The lack of a complete YTD gene cluster is also described in the *Nitrospirota* microorganism *Candidatus* *Sulfobium mesophilum* (44, 46), which has *sqr* and *dsrABCD* as well as *napAB*, and *nrfAH*, and shares 57% average amino acid identity to *Ca. N. versatile*. These AAI values indicate that *Ca. N. versatile* and species 45J are likely part of the same *Ca. Nitrobium* genus but distinct species, and that together with *Ca. Sulfobium mesophilum* they form a

616 family-level taxonomic group (58). Umezawa *et al.* (2021) isolated a sulfur-disproportionating
617 microorganism that was named *Dissulfurispira thermophila*, a strain belonging to species 45J (45), therefore
618 matching the genus *Ca. Nitrobium* described by our group in 2017 (19).

619

620 *Ca. Sulfobium mesophilum* and *Ca. N. versatile* both have *dsrA* genes that affiliate with bacterial-type
621 reductive sequences, and a *dsrD* gene, suggested as a potential marker for sulfate reduction, absent in sulfur
622 oxidizers that utilize the reverse Dsr pathway (59–61). However, our metatranscriptomic results suggest that
623 *Ca. N. versatile* was performing sulfide oxidation. Therefore, it seems that *Ca. N. versatile*, similarly to the
624 deltaproteobacterium *Desulfurivibrio alkaliphilus*, was yet another example of a microorganism disguised as
625 a sulfate reducer (62). Interestingly, *Ca. Sulfobium mesophilum* and *Desulfurivibrio alkaliphilus* could
626 couple sulfide oxidation to DNRA, a potential also present in MAG 39, with transcripts to *nrfA* detected in
627 our study (T0; TPM = 0.030±0.031). Given that *norBC* had significantly higher transcriptional detection than
628 *nrfA* and other denitrification genes (T0, TPM *norB* = 1.9±0.7, TPM *norC* = 2.8±1.1), it is more likely that
629 *Ca. N. versatile* coupled sulfide oxidation to nitric oxide reduction. Given that *Ca. N. versatile* could not be
630 enriched under sulfide and NO, we hypothesize that MAG 65 Thiohalobacteraceae was a more competitive
631 microorganism. However, the mechanism and substrate affinities remain to be elucidated.

632

633 In our study, methanotrophs could withstand prolonged periods of ammonium, methane and nitrate
634 deprivation as well as exposure to elevated concentrations of nitrite and nitric oxide. During these
635 disturbances, *Ca. Methanoperedens nitroreducens* and *Ca. Methyloirabilis tolerans* did not thrive, as
636 indicated by downregulation of *mcrA* and *pmoA*, but tolerated stresses and persisted as abundant community
637 members. These results suggest that methane oxidation could be a relatively stable community function in
638 coastal ecosystems under the stresses investigated in this study. Interestingly, the *Ca. Methanoperedens*
639 *nitroreducens* genome (MAG 36) encoded nitric oxide reductases (Figure 4), which had not been described
640 before in this organism. The qNOR-encoding gene, present in the same contig as *mcr*, *nar* and *nrf* genes, had
641 increased transcriptional activity by the end of the sulfide and NO stress experiment (Figure 5). Future
642 studies should further evaluate lateral transfer of *nor* genes in *Methanoperendaceaea*, which seem prone to
643 acquire novel metabolic traits via later gene transfer events (63), potentially via recently described borgs

644 (64), as well as metabolic flexibility of *Ca. Methanoperedens nitroreducens* under methane deprivation.
645 Hypothetical genes in *Ca. Methanoperedens nitroreducens* identified in this study with high transcription and
646 upregulation in response to stresses could be targets for these future investigations.

647

648 Sulfide oxidizers had differential resiliency to the stresses investigated in this study, dynamically changing in
649 abundance and transcriptional activity across regular and experimental conditions. However, sulfide was
650 completely removed at all time points, likely due to functional redundancy in the microbial community,
651 indicating that sulfide oxidation could also be a relatively stable community function in coastal ecosystems
652 under the investigated stresses. Finally, in our study, denitrification was a dominant nitrogen-cycling
653 pathway, as previously suggested in coastal sediments of the Bothnian Sea (65) - particularly, the nitric oxide
654 reduction step, as indicated by *norB* TPM values (Supplemental Table 2). Anammox activity, as indicated by
655 *hzsA* TPM values, also had a significant contribution to nitrogen cycling, and dominated at the end of the
656 sulfide and nitric oxide stress experiment (T5). This community function was highly impacted by ammonium
657 deprivation, but could be restored when favorable conditions were reestablished. On the other hand, DNRA,
658 as indicated by *nrfA* TPM values, was a minor nitrogen-cycling pathway, but under ammonium deprivation it
659 became more significant. Therefore, anammox and DNRA activity might similarly oscillate in coastal
660 ecosystems under the stresses investigated in this study.

661

662 In conclusion, this study contributed to the elucidation of metabolic pathways for carbon, sulfur and nitrogen
663 cycling in a bioreactor community mimicking anoxic, brackish coastal sediment conditions, as well as shifts
664 in microbial abundance and transcriptional activity in response to prolonged substrate deprivation and
665 exposure to toxic compounds. These results will help understanding complex microbial interactions and
666 functions in dynamic coastal ecosystems, and should be considered into future modelling efforts that aim to
667 predict coastal ecosystem responses to environmental change.

668

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675

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868 Supplemental Files

869

870 **Supplemental Figure 1.** Phylogenetic tree of NC10 phylum-affiliated genomes retrieved from NCBI
 871 (accession numbers are indicated), from Nie et al upon request (12), and from this study (MAG 37 and 38).
 872 Ninety-two genes were extracted with UBCG, concatenated and aligned with FastTree. The *candidatus*
 873 genus *Methylomirabilis* is highlighted in orange. Genomes in bold were used for average amino acid identity
 874 analyses in Supplemental Figure 2.

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877 **Supplemental Figure 2.** Average amino acid identity (AAI) matrix highlighting the *candidatus* genus

878 *Methyloirabilis* (yellow) and some genomes of the same species (red).

879

Supplemental Figure 3. Phylogenetic tree of DsrA sequences from this study (in bold) and reference sequences (indicated by NCBI accession numbers). Sequences putatively assigned to the sulfur reduction direction are highlighted in blue, and to the oxidative direction are highlighted in yellow.

Supplemental Table 1. Specific dates, duration (left) and numbering (right) of the ammonium removal and toxicity stresses experiments. Right columns- specific biomass collection dates and numbering for metagenomics (G), metatranscriptomics (T), and RT-qPCR (R) before/after substrate removal and stresses. (dd.mm.yyyy).

Experiment date/ duration	metaG (G)		metaT (T)		RT-qPCR (R)	
22.6.2016 - 11.03.2017 ~ 1 year	0 No changes – stable condition					
	02.01.2017	G0*	01.02.2017	T0	01.02.2017	R0
12.03.2017-04.04.2017 ~ 7 weeks	1 Ammonium removal (7-0mM)					

18.04.2017-20.06.2017 ~ 10 weeks			18.04.2017	T1		
			20.06.2017	T2	01.08.2017	R1
29.10.2017-01.10.2019 ~ 2 years	2 Ammonium restored (7mM) – stable condition					
	07.11.2018	G1*				
	19.02.2019	G2	17.12.2018	T3		
01.10.2019-14.10.2019 ~ 15 days	3 Methane saturation removed					
	25.11.2019	G3	26.11.2019	T4	26.11.2019	R3
24.11.2019-02.02.2020 ~ 10 weeks	4 Methane saturation restored and external nitric oxide supply (~1-5%)					
	06.02.2020	G4				
06.02.2020-02.04.2020 ~ 7 weeks	5 Only sulfide (6.7mM) and further increased external nitric oxide supply (~10-13%)					
	02.04.2020	G5	02.04.2020	T5	02.04.2020	R4

*DNA extractions (DNAe) not included in analysis but used for assembly improvement (total of 8 metagenomes)

G0.1: using CTAB, alternative DNAe method to Power Soil

G1.1: DNAe from bioreactor wall to improve assembly

Supplemental Table 2. Transcript per million (TPM) values for each gene and genome in this study (excel spreadsheet).

Supplemental Table 3. RT-qPCR R0-R1 and R3-R3 results calculated in $2^{-\Delta\Delta CT}$ values for selected functional genes using three biological and technical duplicates per time point (R).

$2^{-(-\Delta\Delta CT)}$	<i>hzsA</i>	<i>pmoA</i>	<i>mcrA</i>
R0-R1	0.00	1.24	1.15
R3-R4	0.02	0.00	0.00

Supplemental Table 4. Gene annotations and loci for MAGs included in Figure 4 (excel spreadsheet).