https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Biogeosciences Discussions

Cryptic role of tetrathionate in the sulfur cycle: A study from Arabian Sea sediments

Subhrangshu Mandal[1], Sabyasachi Bhattacharya[1], Chayan Roy[1], Moidu Jameela Rameez[1], Jagannath Sarkar[1], Tarunendu Mapder[2], Svetlana Fernandes[3], Aditya Peketi[3], Aninda Mazumdar[3,*] and Wriddhiman Ghosh[1,*]

[1] Department of Microbiology, Bose Institute, P-1/12 CIT Scheme VIIM, Kolkata 700054, India.
[2] ARC CoE for Mathematical and Statistical Frontiers, School of Mathematical Sciences, Queensland University of Technology, Brisbane, QLD 4000, Australia.
[3] CSIR-National Institute of Oceanography, Dona Paula, Goa 403004, India.

*** Correspondence emails:** wriman@jcbose.ac.in / maninda@nio.org
Running Title: Tetrathionate metabolism in marine sediments

KEYWORDS: sulfur cycle, tetrathionate, marine oxygen minimum zone, sediment biogeochemistry

ABSTRACT To explore the potential role of tetrathionate in the sulfur cycle of marine sediments, population ecology of microorganisms capable of metabolizing this polythionate was revealed at 15-30 cm resolution along two, ~3-m-long, cores collected from 530 and 580 meters below the sea level, off India's west coast, within the oxygen minimum zone (OMZ) of the Arabian Sea. Metagenome analysis along the two sediment-cores revealed widespread occurrence of genes involved in microbial formation, oxidation, and reduction of tetrathionate; high diversity and relative-abundance was also detected for bacteria that are known to render these metabolisms *in vitro*. Results of slurry-incubation of the sediment-samples in thiosulfate- or tetrathionate-containing microbial growth media, data obtained via pure-culture isolation, and finally metatranscriptome analyses, corroborated the *in situ* functionality of tetrathionate-forming, oxidizing, and reducing microorganisms. Geochemical analyses revealed the presence of up to 11.1 µM thiosulfate along the two cores, except a few sample-sites near the sediment-surface. Thiosulfate oxidation by chemolithotrophic bacteria prevalent *in situ* is the apparent source of tetrathionate in this ecosystem. However, potential abiotic origin of the polythionate can neither be ruled out nor confirmed from the geochemical information currently available for this territory. Tetrathionate, in turn, can be either oxidized to sulfate (via oxidation by the chemolithotrophs present) or reduced back to thiosulfate (via respiration by native bacteria). Up to 2.01 mM sulfide present in the sediment-cores may also reduce tetrathionate abiotically to thiosulfate and elemental sulfur. As tetrathionate was not detected *in situ*, high microbiological and geochemical reactivity of this polythionate was hypothesized to be instrumental in its cryptic status as a central sulfur cycle intermediate.

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

1 Introduction

Phylogenetically diverse microorganisms oxidize or reduce different sulfur species to meet their bioenergetic requirements, and in doing so play profound roles in biogeochemical sulfur cycling in nature (Baumgartner et al., 2006; Ghosh and Dam, 2009; Wasmund et al., 2017). Within the marine realm, microbial processes of sulfur cycle are` central to benthic biogeochemistry as they are linked to the *in situ* transformations, sequestrations, and fluxes of carbon, nitrogen and iron. There have been extensive studies of benthic/sedimentary sulfur cycle across the global ocean (Jørgensen, 1990; Jørgensen and Bak 1991; Rudnicki et al., 2001; Tostevin et al., 2014), and focus has typically been on geomicrobial transformations of the two end-members sulfate and sulfide (Holmkvist et al., 2011; Jørgensen et al., 2019), and also thiosulfate which constitutes a key junction in the network of sulfur species transformation in marine sediments (Jørgensen, 1990; Jørgensen and Bak 1991; Thamdrup et al., 1994). However, tetrathionate or other polythionates are rarely investigated for their potential role(s) in marine sedimentary sulfur cycle, presumably because these sulfur species are not abundant in these environments.

Overall paucity of polythionates in natural environments is largely attributable to their high reactivity under biotic (Kanao et al., 2007; Ghosh and Dam, 2009; Boden et al., 2010; Pyne et al., 2017, 2018) as well as abiotic (Schippers et al., 1999; Schippers and Jørgensen, 2001) conditions. Cryptic nature of these sulfur species notwithstanding, several bacteria capable of producing and/or utilizing tetrathionate for bioenergetic purposes have been isolated from different terrestrial and aquatic (both fresh-water and marine) habitats (Kaprálek, 1972; Oltmann and Stouthamer, 1975; Barrett and Clark, 1987; Price-Carter et al., 2001; Sorokin, 2003; Ghosh et al., 2005; Ghosh and Roy, 2006, 2007). At the same time, several enzymes catalyzing redox reactions involving tetrathionate have been characterized in taxonomically diverse microorganisms. For instance, thiosulfate dehydrogenase (TsdA), widespread in photo- or chemolithotrophic bacteria, is attributed to the formation of tetrathionate from thiosulfate (Hensen et al., 2006; Denkmann et al., 2012; Brito et al., 2015, Pyne et al., 2018; Rameez et al., 2019). In several other archaeal and bacterial chemolithotroph, thiosulfate to tetrathionate conversion is mediated by thiosulfate:quinone oxidoreductase (TQO or DoxDA; see Muller et al., 2004; Rzhepishevska et al., 2007; Quatrini et al., 2009; Kikumoto et al., 2013). Chemolithotrophic oxidation of tetrathionate to sulfate, on the other hand, is rendered either (i) by the pyrroloquinoline quinone (PQQ)-binding tetrathionate hydrolase (TetH), as described in *Acidithiobacillus* species (De Jong et al.,1997; Kanao et al., 2007; Rzhepishevska et al., 2007; van Zyl et al., 2008; Kanao et al., 2013), or (ii) via coupling with glutathione (to form glutathione:sulfodisulfane and sulfite) by the action of another PQQ-binding protein called thiol

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

dehydrotransferase (ThdT), followed by the oxidation of glutathione:sulfodisulfane via iterative actions of sulfate thiol esterase (SoxB) and *c*-type cytochrome containing sulfur dehydrogenase (SoxCD), as reported in *Advenella kashmirensis* (Pyne et al., 2017, 2018). On the reductive half, typical tetrathionate-reducing bacteria such as *Salmonella*, *Citrobacter* and *Proteus* transform tetrathionate to thiosulfate by tetrathionate reductase (TtrABC) followed by the conversion of thiosulfate to sulfide by thiosulfate reductase (PhsAB and PsrA) (Oltmann et al., 1975; Barrett and Clark, 1987; Hensel et al., 1999; Stoffels et al., 2011).

Here we use approaches of molecular microbiology to investigate the potential involvement of tetrathionate in the sulfur cycle of sediments underlying the perennial oxygen minimum zone (OMZ) of the Arabian Sea, off the west coast of India. Community structures and functions of tetrathionate-forming, oxidizing, and reducing, microorganisms were revealed by metagenome analyses and slurry incubation experiments along two ~3-m-long sediment-cores collected from 530 and 580 meters below the sea level (mbsl); pore-water chemistry was analyzed using ion chromatography. *In situ* activity of tetrathionate-metabolizers was tested by pure-culture isolation and metatranscriptome analysis for the deepest sample-site, within the sulfate-methane transition zone, of one of the two cores. Correspondence was also explored between the *de novo* sequenced genomes of the isolates and the metatranscriptome or the metagenomes sequenced. The microbial ecology delineated in this way was considered in the context of the *in situ* geochemistry to infer implications for the sedimentary sulfur cycle.

2 Materials and methods

2.1 Study site and, sample collection and storage

During a comprehensive exploration of the sediment biogeochemistry of eastern Arabian Sea OMZ (ASOMZ), onboard RV Sindhu Sankalp (SSK42), a number of gravity cores were collected from water-depths spanning 225 and 1275 mbsl, covering the entire thickness of the eastern ASOMZ (Fernandes et al., 2018). Of these, SSK42/5 and SSK42/6, on which the present study is based, were collected from 580 mbsl (16°49.88' N, 71°58.55' E) and 530 mbsl (16°50.03' N, 71°59.50' E) water-depths respectively (Fernandes et al., 2018), i.e. the approximate center of the vertical expanse of the ASOMZ off the west coast of India. Both the cores were ~3-m-long and 12 cm in diameter; their onboard sampling was carried out at 15 to 30 cm intervals, as described previously, under constant shower of high-purity N_2 to minimize exposure of the native microflora to aerial O_2 and avoid aerial oxidation of the H_2S, Fe^{2+} and other reduced chemicals present in the sediments (Fernandes et al., 2018).

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

In order to protect the sediment-samples from aerial oxidation only one ~30-cm-long C-shaped part of PVC core-liner was removed at a time, as shown in Fig. S1. The 30 cm length exposed at a time for sampling was constantly and closely showered with high-purity N_2 emitted from multiple nozzles fitted to multiple nitrogen-generators. Immediately after the C-shaped longitudinal part of the PVC core-liner was cut open, top one cm of the exposed surface was scraped off along the core-circumference, using sterile scalpels, to eliminate potential contaminations from the core-liners' inner-surfaces and/or sea-waters through which the cores had passed (Fernandes et al., 2018). Subsequently, to sample a particular sediment-depth of the core for microbiological studies, an approximately 5-mm-thick sediment-slice (spanning equally on either side of the core-height marking) was scooped out with a sterile scalpel and put into a sterile polypropylene bottle. For every sediment-depth, two such sample-replicates or slices were collected for duplicate metagenome (plus other metaomics) analyses (these were designated as sample-replicates 1 and 2, see Tables S1 and S2); a third replicate was taken for all culture-dependent studies. The head-space of every sample-containing bottle was flushed with high-pure N_2, following which it was sealed with Parafilm (Bemis Company Inc., Neenah, USA) and placed immediately under refrigeration. Sample-replicates meant for culture-independent and culture-dependent studies were stored at −20°C and 4°C respectively. From the laboratory onboard SSK42, *en route* to that at Bose Institute, and over subsequent preservation of samples, these temperatures were maintained all along.

For onboard extraction of pore-waters, samples from a particular sediment-depth were taken out by inserting sterile 50 ml cut off syringes deep inside the core cross-section, multiple times along the circumference on the exposed 'C half'. The samples were immediately collected in sterile 50 ml centrifuge tubes. All these operations were carried out under focused streams of high-pure N_2. The tubes were centrifuged at 4700 × g for 15 minutes at 4°C, and the supernatants collected were syringe-filtered through 0.22 µm cellulose acetate membranes. Aliquots for different chemical analyses were dispensed to individual glass vials containing sodium azide that arrests microbial activity; only the vials meant for precipitating dissolved sulfide (ΣHS^-) from the aliquots [in the form of cadmium sulfide (CdS)] contained cadmium nitrate [$Cd(NO_3)_2$] instead of sodium azide. All the vials were crimped immediately after N_2 flushing, and stored at 4°C until further analysis.

2.2 Analytical method

Sulfide and sulfate concentrations in the pore-water samples were determined and reported previously by Fernandes et al., (2018). Concentration of dissolved thiosulfate in the pore-water

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

samples was determined by ion chromatography using an Eco IC (Metrohm AG, Herisau, Switzerland) equipped with a conductivity detector (Metrohm, IC detector 1.850.9010). Chemical suppression was used for this purpose, while separation was carried using a Metrosep A Supp5 - 250/4.0 anion exchange column (Metrohm AG). A mixed solution of 1.0 mM sodium hydrogen carbonate and 3.2 mM sodium carbonate was used as the eluent; 100 mM sulfuric acid was used as the regenerant; flow rate was 0.7 mL min^{-1}, and injection volume 100 µL. Prior to analysis, pore-water samples were diluted 1000-fold with de-ionized water (Siemens, < 0.06 µS) and passed through 0.22 µm hydrophilic polyvinylidene fluoride membrane filters (Merck Life Science Private Limited, Bengaluru, India). Analytical grade thiosulfate IC Standard (Sigma Aldrich, St. Louis, USA) was used to prepare the calibration curve for quantification of this anion. Three different concentrations of thiosulfate, 0.5 µM, 5 µM and 20 µM, were measured for the construction of calibration curve by plotting peak height against concentration. Based on triplicate analyses of the standards, deviations from actual concentrations were found to be less than 2.5%.

Concentration of tetrathionate in the pore-water samples was measured by cyanolytic method (Kelly and Wood, 1994), where tetrathionate reacts with cyanide to form thiocyanate according to the reaction $S_4O_6^{2-} + 3CN^- + H_2O \leftrightarrow SCN^- + S_2O_3^{2-} + SO_4^{2-} + 2HCN$, and the thiocyanate ($SCN^-$) formed is quantified spectrophotometrically in terms of the absorbance of ferric thiocyanate (ε = 5030 M^{-1} cm^{-1}, λ = 460 nm), which is formed by reacting the thiocyanate ion with ferric nitrate. Each reaction was carried out at a final volume of 5 ml. 0.1 ml of the pore-water sample was added to 0.8 ml of cyanolytic buffer (a mixture of 50 ml of 0.2 M NaH_2PO_4 and 39 ml of 0.2 M NaOH; pH 7.4) and the volume was made up to 2.0 ml. The mixture was then chilled on ice for 20 minutes and 1 ml of 0.1 M chilled potassium cyanide was added and mixed rapidly and incubated on ice for another 20 minutes. Finally, 0.6 ml of ferric nitrate reagent [30.3 g $Fe(NO_3)_3.9H_2O$ in 21.7 ml of 72% perchloric acid, made up to 50 ml with distilled water] was added with continuous agitation and allowed to warm to room temperature until the white precipitate (if any) re-dissolved. Volume was made up to 5 ml and optical density of the colour due to ferric thiocyanate was measured at 460 nm by a spectrophotometer. Tetrathionate in the different samples was quantified from a standard curve prepared using three different concentrations (0.2 µM, 0.4 µM and 0.8 µM) of analytical grade tetrathionate (Sigma Aldrich, St. Louis, USA). Based on triplicate analyses of these standards, deviations from actual concentrations were found to be less than 5%.

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

2.3 Extraction of total DNA/RNA from sediment-samples/pure-culture isolates

Total community DNA was extracted from the sediment-samples using PowerSoil DNA Isolation Kit (MoBio, Carlsbad, USA), as per the manufacturer's protocol. Microgram-level of DNA was obtained from each batch of preparatory reaction that started with 0.5 g sediment-sample. Genomic DNA of pure culture isolates was extracted using HiPurA Bacterial Genomic DNA Purification Kit (Himedia Laboratories, Mumbai, India), following manufacturer's instructions. Quality of metagenomic/genomic DNA samples was checked by electrophoresis and considered to be of high quality when no degradation signs were apparent. DNA quantity was determined using Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific, Waltham, USA).

Total community RNA was extracted from the sediment-sample collected from 275 centimeters below the sea-floor (cmbsf) of SSK42/6, using RNA PowerSoil Total RNA Isolation Kit (MoBio), as per manufacturer's protocol. Nanogram-level total RNA was obtained after pooling the products of 15 individual preparatory reactions, each carried out using 2 g sediment-sample. All the individual RNA preparations were subjected to DNase digestion by RNase free DNase I (Thermo Fisher Scientific) and purified using RNeasy MinElute Cleanup Kit (Qiagen, Hilden, Germany); their concentrations were measured using Quant-iT RiboGreen RNA Assay Kit (Thermo Fisher Scientific). Integrity of RNA (RIN) within the individual preparations was determined on a TapeStation RNA ScreenTape electrophoretic system (Agilent Technologies, Santa Clara, USA) and only high-quality preparations having RIN value > 7.0 were added to the RNA pool that were subsequently used for sequencing library construction.

2.4 Metagenome (total community DNA) / genome sequencing

The duplicate set of total community DNA (metagenomes) extracted for each sediment-depth explored along SSK42/5 and SSK42/6 were shotgun sequenced individually on an Ion Proton sequencing platform (Thermo Fisher Scientific) using 200 nucleotide read-chemistry, as described previously (Ghosh et al., 2015). Complete lists of sedimentary communities investigated along SSK42/5 and SSK42/6are given in Tables S1 and S2 respectively, together with the accession numbers of the metagenomic sequence datasets.

1 µg DNA from each metagenome-sample was taken for deep shotgun sequencing by the Ion Proton platform using 200 bp read chemistry on a PI V2 Chip. Sequencing libraries were constructed using Ion Plus Fragment Library Kit (Thermo Fisher Scientific), following the manufacturer's Ion Plus gDNA Library Preparation User Guide. The Proton library was generated using 1 µg of genomic DNA which was fragmented to approximately 200 base pairs by the Covaris S2 system (Covaris, Inc., Woburn, USA) and purified with 1.8X Agencourt Ampure XP

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Beads (Beckman Coulter, Brea, USA). Fragmentation was followed by end-repair, blunt-end ligation of the Ion Xpress Barcode and Ion P1 adaptors, and nick translation.

Post-ligation, size selection was done using E-Gel Size-Select 2% Agarose gels (Thermo Fisher Scientific) with 300 bp target size. Final PCR was performed using platinum PCR SuperMix High Fidelity and Library Amplification Primer Mix (Thermo Fisher Scientific), for 5 cycles of amplification. The resulting library was purified using 1.2X AMPure XP reagent (Beckman Coulter) and the concentration determined with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific); size distribution was done with Agilent 2100 Bioanalyzer high-sensitivity DNA kit (Agilent Technologies). Libraries were pooled in equimolar concentrations and used for template preparation.

Library templates for sequencing were prepared using OneTouch 2 protocols and reagents (Thermo Fisher Scientific). Library fragments were clonally amplified onto ion sphere particles (ISPs) through emulsion PCR and then enriched for template-positive ISPs. Proton emulsion PCR reactions utilized the Ion PI Template OT2 200 Kit v3 (Thermo Fisher Scientific). Following recovery, enrichment was completed by selectively binding the ISPs containing amplified library fragments to streptavidin coated magnetic beads, removing empty ISPs through washing steps, and denaturing the library strands to allow for collection of the template-positive ISPs. For all reactions, these steps were accomplished using the ES module of the Ion OneTouch 2. The selected ISPs were loaded on PI V2 Chip and sequenced with the Ion PI 200 Sequencing Kit (Thermo Fisher Scientific) using the 500 flow (125 cycle) run format.

Whole genomes of isolated bacterial strains were sequenced on an Ion S5 platform (Thermo Fisher Scientific) using 400 nucleotide read-chemistry on a 530 or 520 Chip. Libraries were constructed by the Ion Xpress Plus Fragment library kit (Thermo Fisher Scientific) using 100 ng genomic DNA from each isolate. In this procedure genomic DNA samples were fragmented using Ion Shear Plus Reagents (Thermo Fisher Scientific). The fragmented libraries were purified using 1.8X Agencourt Ampure XP Beads (Beckman Coulter), and subjected to barcode-adapter ligation and nick repair. Adapter-ligated and nick-repaired libraries were purified again by 1X Agencourt Ampure XP Beads (Beckman Coulter).

Size selection of the libraries was done using E-Gel Size-Select 2% Agarose gels (Thermo Fisher Scientific) with 480 bp target size. Final PCR was performed using platinum SuperMix High Fidelity PCR system and Library Amplification Primer Mix (both from Thermo Fisher Scientific), for 8 cycles of amplification. The resulting libraries were purified using 1x Agencourt AMPure XP reagent (Beckman Coulter). Concentrations of the purified libraries were determined

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

with Qubit dsDNA HS Assay Kit (Thermo Fisher Scientific). Libraries were then pooled in equimolar concentrations and used for template preparation.

The library template to be used for sequencing was prepared using Ion OneTouch 2 reagents (Thermo Fisher Scientific). Library fragments were clonally amplified onto ion sphere particles (ISPs) through emulsion PCR and then enriched for template-positive ISPs. Following emulsion PCR, enrichment was completed by selectively binding the ISP-containing amplified library fragments to streptavidin-coated magnetic beads, removing empty ISPs through washing steps, and denaturing the library strands to allow for collection of the template-positive ISPs using the Ion OneTouch ES instrument (Thermo Fisher Scientific). The selected ISPs were loaded on a 530 or 520 Chip and sequencing was performed with the Ion S5 Sequencing Kit (Thermo Fisher Scientific) using the 850-flow run format.

2.5 Metatranscriptome (community mRNA) sequencing

The pooled total RNA preparations were selectively converted to a library of template molecules using TruSeq Stranded mRNA and Total RNA kit (Illumina Inc., San Diego, USA). Depletion of rRNAs was carried out using the Ribo-Zero Gold system (Illumina Inc.), which is an integral part of the kit used for preparing the library. The rRNA-depleted RNA pool, which was expected to contain only the total mRNA, was fragmented into small pieces using divalent cations under elevated temperature. The cleaved RNA fragments were copied into first strand cDNAs using reverse transcriptase and random primers. This was followed by second strand cDNA synthesis using DNA Polymerase I and RNase H. cDNA fragments were then subjected to end-repair, addition of single 'A' bases, adaptor ligation, purification and enrichment with PCR to create the final library, which was sequenced on a HiSeq4000 platform (Illumina Inc.) using paired end, 2 × 150 nucleotide, sequencing by synthesis read-chemistry with dual indexing workflows. Furthermore, in order to extract and eliminate any rRNA read that may have remained in the raw metatranscriptomic sequence dataset, the 26,579,343 read-pairs available in all were mapped onto SILVA large subunit as well as small subunit rRNA gene sequence database (Quast et al., 2012), using the short read aligner Bowtie2 v.2.3.4.3 (Langmead and Salzberg, 2012) in default local (sensitive) alignment mode. This identified ~0.3% reads as ascribable to rRNAs, thereby leaving 26,496,769 read-pairs in the final dataset used for downstream analyses.

2.6 *De novo* assembly and annotation of genomes/metagenomes/metatranscriptome

High quality reads (Phred score cut-off 20) from the duplicate metagenomic sequence datasets available for each sediment-community were co-assembled using Megahit v1.2.x (Li et al., 2015)

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

with the kmer lengths of 21, 29, 39, 59, 79, 99, 119 and 141. Contigs of > 100 bp length were searched using MetaGeneMark (Zhu et al., 2010) for genes encoding peptides having lengths of > 30 amino acids. For the genomes of pure-culture isolates, high quality reads (Phred score cut-off 20) were assembled using SPAdes 3.13.0 (Nurk et al., 2013), with kmer lengths of 21, 33, 55, 77, 99 and 127, and minimum coverage cut-off 35X. The whole genome sequences were deposited to the GenBank and annotated using Prokaryotic Genome Annotation Pipeline (PGAP located at https://www.ncbi.nlm.nih.gov/genome/annotation_prok/) of National Center for Biotechnology Information (NCBI), Bethesda, MD, USA. Total metagenomic or metatranscriptomic reads available for the different sediment-communities were mapped onto the individual genome sequences or the manually-curated gene-catalogs obtained from the individual genomes, using Bowtie2 v.2.3.4.3 (Langmead and Salzberg, 2012) in default local (sensitive) alignment mode. The rRNA-sequence-free metatranscriptomic dataset was assembled using the python script rnaspades.py, available within SPAdes 3.13.0 (Nurk et al., 2013), with default parameters. Genes encoding continuous stretches of minimum 30 amino acids were predicted in contigs longer than 100 bp using Prodigal v2.6.3 (Hyatt et al., 2010).

Gene-catalogs obtained after *de novo* assembly of the individual metagenomes, or the solitary metatranscriptome, were functionally annotated by searching against EggNOG v5.0 database (http://eggnog5.embl.de/download/eggnog_5.0/) with EggNOG-mapper (Huerta-Cepas et al., 2016) (http://beta-eggnogdb.embl.de/#/app/emapper) using HMMER algorithms. Putative protein sequence catalogs obtained for the individual genomes via PGAP annotation were re-annotated by searching against EggNOG v5.0 database with EggNOG-mapper using HMMER algorithm. Genes encoding proteins involved in tetrathionate formation, oxidation and reduction were identified within the annotated genomes by their orthology numbers designated in *Kyoto Encyclopedia of Genes and Genomes* (KEGG; Kanehisa et al., 2016).

2.7 Direct taxonomic annotation of raw metagenomic reads

Raw (unassembled) reads contained in the duplicate metagenomic sequence datasets obtained for each sediment-community were directly annotated for their taxonomic affiliation by separately searching against the NCBI non-redundant (*nr*) protein sequence database, using the Organism Abundance tool of MG-RAST 3.6 (Meyer et al., 2008). The two independent values obtained in this way for the relative abundances of genera within a community were averaged and used for comparisons between communities. In these analyses, percentage allocation of reads over various genera was taken as a direct measure of the relative abundance (prevalence) of those genera within the community (Ghosh et al., 2015). Within MG-RAST, sequences were trimmed to

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

contain no more than 5 successive bases with phred score < 15. To classify reads using Organism Abundance tool, Best Hit Classification algorithm was followed [BlastX search with minimum 45 nucleotides (15 amino acids) alignment and ≥ 60% identity, and maximum *e*-value allowed $1e^{-5}$].

2.8 Slurry culture experiments

Ability of the different sedimentary microbial communities to form tetrathionate (from thiosulfate), oxidize tetrathionate (to sulfate) or reduce tetrathionate (to thiosulfate and/or sulfide) was tested via slurry incubation in culture media supplemented with thiosulfate or tetrathionate. For each experiment testing the formation or oxidation of tetrathionate, 10% (w/v) sediment-sample was suspended in artificial sea water (ASW) supplemented with thiosulfate (T) or tetrathionate (Tr), i.e. ASWT or ASWTr broth medium (Alam et al., 2013), respectively; the culture flask was incubated aerobically at 15°C on a rotary shaker (150 rpm). For each anaerobic experiment testing tetrathionate reduction, 10% (w/v) sediment-sample was suspended in tetrathionate-supplemented Rappaport Vassiliadis (RVTr) medium (Vassiliadis, 1983) that was already made O_2-free by addition of sodium thioglycolate. Addition of sediment-samples to sterile O_2-free RVTr media (contained in a screw-capped bottles) and subsequent incubation of the culture bottles were all carried out inside a Whitley H35 Hypoxystation (Don Whitley Scientific, West Yorkshire, UK) preset at 75% humidity, 15°C temperature and 0% partial pressure of O_2, using the gas mixture N_2:H_2:CO_2 = 80:10:10 (v/v/v). The ASWT or ASWTr medium (both having pH 7.5) contained ASW supplemented with $Na_2S_2O_3.5H_2O$ (10 mM) or $K_2S_4O_6$ (5 mM) respectively (the two sulfur compounds were added to the media separately after filter-sterilization) (Alam et al., 2013). ASW contained the following L^{-1} distilled water: 25.1 g NaCl, 1 g $(NH_4)_2SO_4$, 1.5 g $MgSO_4$, $7H_2O$, 0.3 g $CaCl_2.2H_2O$, 0.2 g $NaHCO_3$, 2.4 g Tris, 1 mL trace element solution and 0.5 g K_2HPO_4 (added after autoclaving separately). 1 L trace element solution (pH 6.0), in turn, contained 50 g EDTA, 22 g $ZnSO_4.7H_2O$, 5.06 g $MnCl_2$, 4.99 g $FeSO_4$, 1.1 g $(NH_4)_6 MoO_{26}.4H_2O$, 1.57 g $CuSO_4$ and 1.61 g $CoCl_2.6H_2O$. RVTr medium (pH 5.4) contained the following L^{-1} of distilled water: 4.5 g soya peptone, 8.0 g NaCl, 0.4 g K_2HPO_4, 0.6 g KH_2PO_4, 29.0 g $MnCl_2$, 0.036 g Malachite green, 10 mM $K_2S_4O_6$ (added separately after filter sterilization) and 0.5 g sodium thioglycolate and 0.1 mg resazurin (to indicate presence of any O_2).

Concentrations of the various redox species of sulfur were estimated in the slurry cultures at every 6 hr interval of incubation. End-point (final) concentrations of the different sulfur species in a given medium were recorded when no further changes were detected in their concentrations over three consecutive estimations; rate of a particular sulfur species conversion was calculated

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

by dividing the mM S substrate finally transformed (this was always in equivalence with the mM S product generated) by the time taken to reach that final concentration. Concentrations of thiosulfate, tetrathionate and sulfate in the media were measured by iodometric titration, cyanolysis and gravimetric sulfate precipitation method respectively at different time intervals (Alam et al., 2013). Possible presence of dissolved sulfides was checked by precipitating as CdS by the addition of 2 M $Cd(NO_3)_2$, followed by spectroscopic estimation as described previously (Cline, 1969).

2.9 Enrichment, isolation and characterization of bacterial strains

Isolation of sulfur chemolithotrophs from the 275 cmbsf sediment-sample of SSK42/6 was carried out in mineral salt-thiosulfate-yeast extract (MSTY), ASWT and ASWTY media. While the ASWTY (pH 7.5) medium was an yeast extract (500 mg L^{-1}) supplemented derivative of ASWT, MSTY (pH 7.0) contained modified basal and mineral salts (MS) solution supplemented with 20 mM $Na_2S_2O_3.5H_2O$ and 500 mg L^{-1} yeast extract (Ghosh and Roy, 2006). MS, in turn, contained the following L^{-1} distilled water: 1 g NH_4Cl, 4 g K_2HPO_4, 1.5 g KH_2PO_4, 0.5 g $MgSO_4.7H_2O$ and 5.0 mL trace metals solution (Vishniac and Santer, 1957). Three portions of the 275-cmbsf sediment-sample of SSK42/6 were added (5% w/v) individually to MSTY, ASWT and ASWTY and broths, and incubated aerobically at 15°C until phenol red indicator present in the media turned yellow (apparently due to production of sulfuric acid from thiosulfate). Post yellowing, individual enrichment slurries were kept undisturbed for 1 h to allow sediment particles to settle down; 10 mL cell suspension from each flask was then centrifuged at 6000 *g* for 10 min and the pellet re-suspended in 1 mL of the corresponding medium, serially diluted, and spread onto agar plates of the corresponding medium, and incubated at 15°C. Morphologically distinct colonies were picked up and dilution-streaked until all colonies in individual plates looked similar; representative colonies from such pure-culture plates were taken as strains and maintained in their respective isolation-medium. Only *Methylophaga*, though isolated in ASWT, was maintained in ASW supplemented with 0.3% (v/v) methanol (ASWM; this medium had a pH of 7.5) because its growth in ASWT waned after six straight sub-cultures. Chemolithotrophic abilities of the new isolates to oxidize thiosulfate to tetrathionate and/or tetrathionate to sulfate were tested in MSTY, MSTrY, ASWT, ASWTr, ASWTY, ASWTrY, ASWTM and ASWTrM media. MSTrY (pH 7.0) contained MS solution supplemented with 10 mM $K_2S_4O_6$ and 500 mg L^{-1} yeast extract. ASWTrY (pH 7.5) was an yeast extract (500 mg L^{-1}) supplemented derivative of ASWTr. ASWTM (pH 7.5) and ASWTrM (pH 7.5) were thiosulfate (10 mM $Na_2S_2O_3.5H_2O$) and tetrathionate (10 mM

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

$K_2S_4O_6$) supplemented variant of ASWM respectively. Concentrations of dissolved thiosulfate, tetrathionate and sulfate in the spent media were measured as described above.

Tetrathionate-reducing bacterial strains were isolated from the 275 cmbsf sediment-sample of SSK42/6 in RVTr medium (Vassiliadis, 1983) under strictly anaerobic condition. 2.5 g sediment-sample was added to 45 mL RVTr broth that was contained, and already autoclaved, in a screw-capped bottle. Sediment addition to the medium, and subsequent incubation of the screw-capped bottles at 15°C for one month, were all carried out inside the Whitley H35 Hypoxystation preset to zero O_2 as stated above. After one month, still inside the Hypoxystation, 1 mL of the sediment- RVTr slurry was serially diluted and spread onto RVTr agar plates and incubated at 15°C. After growth appeared in the RVTr agar plates, they were taken out, repeatedly dilution streaked on to fresh plates, and then incubated aerobically until all colonies in the individual plates looked similar. Representative colonies from such pure-culture plates were taken as strains and maintained aerobically in Luria broth medium. Tetrathionate-reducing abilities of the new isolates were tested by growing them for 30 days in RVTr broth, inside the H35 Hypoxystation, as described above. Concentrations of dissolved thiosulfate, tetrathionate and sulfide in the spent RVTr medium were also measured as described above.

Genomic DNA extracted from the individual isolates was used as template for PCR amplification of 16S rRNA genes with the Bacteria-specific universal primer-pair 27f and 1492r (Gerhardt, 1994). 16S rRNA gene sequences were determined from the PCR products using a 3500xL Genetic Analyzer automated DNA sequencer (Thermo Fisher Scientific). The 16S rRNA gene sequence of each strain was compared against sequences available in the GenBank/EMBL/DDBJ databases, using BLASTN; strains were finally classified down to the lowest identifiable taxonomic category on the basis of their 16S rRNA gene sequence similarities with the closest, validly-published species having standing in nomenclature (http://www.bacterio.net/; see also Euzéby, 1997; Parte, 2013).

3 Results

3.1 Tetrathionate-forming/oxidizing/reducing microorganisms and genes corresponding to such processes are abundant along SSK42/5 and SSK42/6

The duplicate metagenomic sequence datasets obtained for each of the 25 distinct sediment-samples explored along SSK42/5 and SSK42/6 were co-assembled and annotated individually. 23 out of the 25 contig-collections obtained in this way were found to contain genes for tetrathionate formation (Table S3), while all 25 encompassed genes for tetrathionate oxidation

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

(Table S4). Furthermore, 24 out of the 25 contig-collections contained genes for tetrathionate reduction (Table S5). The tetrathionate-formation-related genes identified included those encoding for the different subunits of the thiosulfate dehydrogenases TsdA (Denkmann et al., 2012; Pyne et al., 2018) and DoxDA (Quatrini et al., 2009), which catalyze the oxidation of thiosulfate to tetrathionate in taxonomically diverse bacteria and archaea. While the genes identified for tetrathionate oxidation encoded the sulfate thiol esterase SoxB and the sulfur dehydrogenase SoxC (Lahiri et al., 2006; Pyne et al., 2018), those detected for tetrathionate reduction encoded subunits of tetrathionate (TtrABC) and thiosulfate reductases (PhsAB and PsrA) (Barrett and Clark, 1987; Stoffels et al., 2011).

Concurrent with the above findings, direct taxonomic annotation of the raw (unassembled) metagenomic sequence datasets revealed that considerable proportions of the reads obtained from the individual sediment-depths of SSK42/5 and SSK42/6 were ascribable to bacterial genera whose members are known to render tetrathionate formation, oxidation or reduction. In that way, 1.3-4.36% and 3-7.8% of metagenomic reads obtained from the individual sample-sites of SSK42/5 (Fig. 1A) and SSK42/6 (Fig. 1B) were ascribable to the genera *Pseudomonas* and *Halomonas*, majority of marine strains of which are known to form tetrathionate from the oxidation of thiosulfate under aerobic or anaerobic condition (Tuttle, 1980; Mason and Kelly 1988; Sorokin et al., 1999; 2003). 0.1-1.5 and 0.4-6.4% of metagenomic reads obtained from the individual sample-sites of SSK42/5 (Fig. 1A) and SSK42/6 (Fig. 1B) were ascribable to the genera *Acidithiobacillus*, *Halothiobacillus* and *Thiomicrospira*, all members of which oxidize tetrathionate chemolithotrophically (Hedrich and Johnson, 2013; Watsuji et al., 2016; Boden et al., 2017). 0.1-0.3 and 0.2-0.4% of metagenomic reads obtained from the individual sample-sites of SSK42/5 (Fig. 1A) and SSK42/6 (Fig. 1B) were ascribable to the genera *Citrobacter*, *Proteus* and *Salmonella*, all members of which respire by reducing tetrathionate to thiosulfate and/or sulfide (Kaprálek, 1972; Barrett and Clark, 1987; Price-Carter et al., 2001; Stoffels et al., 2011).

3.2 Synchronized population-fluctuation of different tetrathionate-metabolizing types, along SSK42/5 and SSK42/6

Analyses based on the direct taxonomic annotation of the unassembled metagenomic data from discrete sediment-depths of SSK42/5 revealed that the relative abundances of reads ascribed to the genera of tetrathionate-forming, oxidizing, and reducing bacteria fluctuate synchronously along the sediment-surface to core-bottom trajectory (Fig. 1A). Corroboratively, pair-wise Pearson correlation coefficients (CC) as well as Spearman rank correlation coefficients (RCC) between the prevalence of the three metabolic-types are also significantly high in SSK42/5 (Fig.

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

1A; Table S6), which indicate the existence of strong syntrophic interdependence between the three tetrathionate-metabolizing types in this sediment-horizon. Relative abundances of metagenomic reads ascribed to the genera of tetrathionate-forming, oxidizing, and reducing bacteria also fluctuate more or less synchronously along SSK42/6, excepting the region between 250 and 275 cmbsf (Fig. 1B), which is the sulfate-methane transition zone (SMTZ) of this sediment-horizon (notably, SMTZ in SSK42/5 laid below the 280 cmbsf sediment-depth explored in this core; see Fernandes et al., 2018 for the methane profiles of all the SSK42 cores). While lack of synchrony in the lower end of SSK42/6 apparently resulted in the lower correlation values obtained for this core (Table S7) as compared to those obtained for SSK42/5, it seems quite plausible that the changes in geochemistry and community architecture associated with the shallowing of SMTZ in SSK42/6 impacted the population ecology of tetrathionate-metabolizing microorganisms in this region. Sedimentation rate, age-depth profile and other geochemical features of the two cores separated by a distance of only one kilometer are otherwise largely comparable (Bhattacharya et al., 2019).Consistent prevalence of reads ascribed to the thiosulfate-to-tetrathionate-converting bacterial genera *Halomonas* and *Pseudomonas* in the metagenomes extracted from the different sample-sites of SSK42/5 and SSK42/6 (Figs. 1A and 1B) indicated that tetrathionate could be bioavailable in the chemical milieu of this sediment-horizon (notably, pure-culture strains belonging to these two genera were also isolated from the 275 cmbsf sample of SSK42/6; see section 3.3 below, and also Fig. 2). Apart from these two, several such genera were also found to be well represented in the metagenomes of SSK42/5 and SSK42/6, some members of which are known to produce tetrathionate as a free intermediate during the oxidation of thiosulfate to sulfate and release the same to the extra-cellular milieu (Tables S8 and S9). These organisms, affiliated to the genera *Acidithiobacillus*, *Advenella*, *Halothiobacillus*, *Paracoccus*, *Pusillimonas* and *Thiomicrospira*, can well increase tetrathionate availability in the ASOMZ sediments, even as they themselves are potential users of the tetrathionate produced (Ghosh et al., 2005; Hedrich and Johnson, 2013; Watsuji et al., 2016; Boden et al., 2017; Rameez et al., 2019). Tables S8 and S9 show the percentages of metagenomic reads that were found to be ascribed to these genera in the different sediment-samples of SSK42/5 and SSK42/6 respectively.

Tetrathionate can be oxidized *in situ* as a potential energy and electron source by members of the obligately chemolithotrophic genera *Acidithiobacillus*, *Halothiobacillus* and *Thiomicrospira* that were detected via direct taxonomic annotation of the unassembled metagenomic data (Figs. 1A and 1B) and/or isolated as pure cultures from the 275 cmbsf sample of SSK42/6 (Fig. 2). In addition, several such genera were detected (via direct annotation of

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

metagenomic reads) along SSK42/5 and SSK42/6, some chemolithotrophic members of which are known to oxidize tetrathionate to sulfate. These organisms, affiliated to the genera *Advenella*, *Bosea*, *Burkholderia*, *Campylobacter*, *Hydrogenovibrio*, *Pandoraea, Pusillimonas, Pseudaminobacter, Sulfurivirga*, *Thiohalorhabdus,* and *Thiobacillus* may contribute to further tetrathionate depletion from the sediments (Tables S10 and S11 show the relevant references, and the metagenomic read percentages ascribed to these genera along SSK42/5 and SSK42/6 respectively).

Tetrathionate in the ASOMZ sediments can also be utilized as a respiratory substrate by bacteria such as *Citrobacter*, *Proteus* and *Salmonella,* which were detected by direct annotation of metagenomic reads (Figs. 1A and 1B) and all members of which are known to reduce tetrathionate. In addition, strains of *Enterobacter* such as those isolated as pure cultures from 275 cmbsf of SSK42/6 (Fig. 2H) can add to the *in situ* reduction of tetrathionate to thiosulfate or sulfide. Furthermore, several such genera were also detected along SSK42/5 and SSK42/6 (via direct annotation of metagenomic reads), some members of which are known to respire via reduction of tetrathionate in the absence of O_2; these included *Alteromonas, Alcaligenes, Desulfotomaculum, Desulfovibrio, Edwardsiella, Morganella, Pasteurella, Providencia, Serratia* and *Shewanella* (Tables S12 and S13 show the relevant references, and the metagenomic read percentages ascribed to these genera along SSK42/5 and SSK42/6 respectively).

3.3 The tetrathionate-forming/oxidizing microorganisms of the ASOMZ sediments are alive and active *in situ*

Aerobic slurry incubation of the sediment-samples of SSK42/5 and SSK42/6 in thiosulfate-containing artificial sea water (ASWT) medium resulted in either the formation of tetrathionate with no further oxidation of this polythionate, or the formation of tetrathionate followed by oxidation of the latter to sulfate, or no transformation at all. These findings, in conjunction with the results of pure culture isolation, illustrated that the sulfur-chemolithotrophic microorganisms present in this sediment-horizon are alive *in situ* and possess distinct pathways for oxidizing thiosulfate. For SSK42/5, ASWT-incubation of the 0, 15, 90 and 160 cmbsf samples resulted in the formation of tetrathionate as the sole and final product of thiosulfate oxidation, which happened *in vitro* at a rate of 6.45-17.72 μmol S day^{-1} g $sediment^{-1}$ (Fig. 3A, Table S14). In contrast, ASWT-incubation of the 45, 60 and 295 cmbsf samples of SSK42/5 resulted in the initial formation of tetrathionate from thiosulfate at a rate of 1.11-6.45 μmol S day^{-1} g $sediment^{-1}$ (notably, no sulfate was produced during this period of incubation); subsequently, the accumulated tetrathionate was converted to sulfate at a rate of 5.86-13.75 μmol S day^{-1} g

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

sediment^{-1} (Fig. 3A, Table S15). Microbial communities present in the remaining five sediment-samples of SSK42/5 did not convert any thiosulfate out of the 20 mM S supplied (in the ASWT medium) to any higher oxidation state of sulfur. For SSK42/6, ASWT-incubation of the 120, 175 and 275 cmbsf samples resulted in the formation of tetrathionate as the sole and final product of thiosulfate oxidation, which happened *in vitro* at a rate of 17.2-29.71 µmol S day^{-1} g sediment^{-1} (Fig. 3B, Table S16). In contrast, ASWT-incubation of the 2, 30 and 45 cmbsf samples of SSK42/6 resulted in the initial formation of tetrathionate from thiosulfate at a rate of 21.05-33.68 µmol S day^{-1} g sediment^{-1} (notably, no sulfate was produced during this period of incubation); subsequently, the accumulated tetrathionate was converted to sulfate at a rate of 24-54 µmol S day^{-1} g sediment^{-1} (Fig. 3B, Table S17). Microbial communities of the remaining seven sediment-samples of SSK42/6 did not transform any part of the thiosulfate supplied to any higher oxidation state of sulfur.

Slurry incubation of a number of sediment-samples from SSK42/5 and SSK42/6 in ASWTr medium resulted in the oxidation of tetrathionate to sulfate. These data, together with the results of pure-culture isolation, indicated that the tetrathionate-oxidizing sulfur-chemolithotrophs present in the different sediment-samples were alive *in situ*. Of the individual microbial communities present in the different sediment-samples of SSK42/5, those from 0, 15, 45, 90, 120, 160 and 295 cmbsf oxidized tetrathionate at a rate of 2.5-23.5 µmol S day^{-1} g sediment^{-1} (Fig. 3C, Table S18). Slurry incubation of samples from the remaining five sediment-depths of SSK42/5 did not result in any oxidation of tetrathionate. For SSK42/6, ASWTr-incubation of the 2, 30, 45, 60, 75 and 90 cmbsf sediment-samples resulted in the oxidation of tetrathionate to sulfate at an identical rate (approximately 140 µmol S day^{-1} g^{-1} sediment). While ASWTr-incubation of the samples from 120, 135, 160 and 175 cmbsf resulted in tetrathionate oxidation at a common rate of approximately 40 µmol S day^{-1} g sediment^{-1}, the same for the 220, 250 and 275 cmbsf samples led to tetrathionate oxidation at a rate of approximately 75 µmol S day^{-1} g sediment^{-1} (Fig. 3D, Table S19).

In the above experiments it was remarkable that the individual communities present within the sediment-depth-zones spanning 2-90 cmbsf, 120-175 cmbsf or 220-275 cmbsf of SSK42/6 exhibited mutually identical rates of tetrathionate oxidation *in vitro*, despite having dissimilar composition/abundance of chemolithotrophic taxa. This could be explained as follows. When a natural sample is incubated in selective culture media (such as ASWTr) certain specific microbial species present in the sample often outgrow all metabolic competitors by virtue of higher substrate affinity and culture-condition suitability. Consequently, the growth/substrate-utilization phenotype(s) manifested by such enriched consortia are actually contributed to by the selected

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

few rather than the entire community of metabolic equivalents present in the sample (Roy et al., 2016). In the light of this issue it seems plausible that distinct sets of chemolithotrophs more adept to growth in ASWTr medium are present across the sediment-samples within the 2-90 cmbsf, 120-175 cmbsf or 220-275 cmbsf zones, and it was only their characteristic rates of tetrathionate oxidation which were manifested as the *in vitro* tetrathionate oxidation rates of the individual communities.

Whatever may be the actual tetrathionate formation/oxidation rates of the sedimentary communities, *in vitro* or *in situ*, growths in the slurry culture experiments illustrated that tetrathionate-forming and oxidizing bacteria detected along SSK42/5 and SSK42/6 are alive *in situ*. In order to further verify whether these metabolic-types were metabolically active in their native habitat, whole metatranscriptome of the 275 cmbsf sediment-sample of SSK42/6 was sequenced, and the paired end reads assembled into contigs. The gene-catalog obtained via annotation of the assembled contigs was found to encompass homologs of thiosulfate dehydrogenase (*tsdA*), which is involved in the conversion of thiosulfate to tetrathionate, and sulfate thiol esterase (*soxB*) and sulfur dehydrogenase (*soxC*), involved in tetrathionate oxidation (Table S20). Furthermore, from 275 cmbsf of SSK42/6, 15 such bacterial strains were isolated (Table 1) that could form tetrathionate from thiosulfate and/or oxidize tetrathionate to sulfate (Fig. 2). 16S rRNA gene sequence-based taxonomic identification of the isolates clustered them under six species-level entities belonging to six distinct genera. The isolates belonging to the genera *Halomonas*, *Methylophaga*, *Pseudomonas* and *Stenotrophomonas* oxidized thiosulfate only up to tetrathionate; those belonging to *Pusillimonas* not only formed tetrathionate from thiosulfate but also oxidized tetrathionate to sulfate; the *Halothiobacillus* isolates did not form tetrathionate from thiosulfate but oxidized both thiosulfate and tetrathionate directly to sulfate (Table 1). Tetrathionate-forming and/or oxidizing phenotypes of one representative strain each from the six species-level clusters are shown in Fig. 2.

3.4 Active tetrathionate-reducing microorganisms in ASOMZ sediment

During anaerobic slurry incubation in RVTr medium, microbial communities of all the sediment-samples explored in SSK42/5 and SSK42/6 reduced tetrathionate to thiosulfate and/or sulfide at a rate of 0.5-1.5 μmol S day^{-1} g $sediment^{-1}$ (Figs. 3E and 3F, Tables S21 and S22). Notably, no tetrathionate reductase (*ttrABC*) or thiosulfate reductase (*phsAB* or *psrA*) was detected in the gene-catalog obtained via assembly and annotation of the metatranscriptomic data from 275 cmbsf of SSK42/6; but, the same catalog did contain many genes having highest sequence identities with functionally diverse genes belonging to the typical tetrathionate-reducer

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

585 *Salmonella*. Furthermore, anaerobic enrichment of the 275 cmbsf sediment-samples of SSK42/6, followed by isolation of pure cultures, in RVTr medium yielded four tetrathionate-reducing strains that reduced 30-32 mM S tetrathionate into equivalent amount of thiosulfate over 72 h anaerobic incubation in RVTr medium (Fig. 2H shows the tetrathionate-reduction kinetics of the representative strain RVSM5a). 16S rRNA gene sequence-based taxonomic identification of the 590 four isolates clustered them under a single species-level entity belonging to the genus *Enterobacter* (Table 1).

3.5 The tetrathionate-metabolizing bacteria isolated from 275 cmbsf of SSK42/6 are widespread across SSK42/5 and SSK42/6

595 Whole genome sequencing and annotation was carried out for the three tetrathionate-forming isolates *Halomonas* sp. MCC 3301, *Methylophaga* sp. MTCC 12599 and *Pseudomonas bauzanensis* MTCC 12600; the two tetrathionate-oxidizing isolates *Halothiobacillus* sp. SB14A, and *Pusillimonas ginsengisoli* MTCC12558; and the tetrathionate-reducing isolate *Enterobacter* sp. RVSM5a (see Table 1 and Table S23 for GenBank accession numbers and general features 600 of the genomes respectively). When metagenomic sequence data from the 25 distinct sediment-samples of SSK42/5 and SSK42/6 were mapped separately onto each of these six genomes, significant percentages of the metagenomic read-sets were found to match sequences from the individual genomes (Figs. 4 and 5, Table S23). In SSK42/5 and SSK42/6, 0.01-0.3% and 0.02-19.05% metagenomic reads from the individual sediment-samples mapped onto the different 605 genomes respectively. Expectedly, prevalence of reads matching sequences from the new isolates was relatively higher for the metagenomes of SSK42/6 (Fig. 5); and within this core it was highest for 275 cmbsf, i.e. the sample-site from where all the strains were isolated (Table S23). These data corroborated the significant prevalence of the tetrathionate-metabolizing bacterial strains across the sediment-horizons of SSK42/5 and SSK42/6.

610

3.6 Thiosulfate as the key source of tetrathionate in ASOMZ sediments

Findings of the present culture-independent and culture-dependent investigations showed that microbe-mediated oxidation of thiosulfate to tetrathionate (by members of the bacterial genera included under group A in Fig. 6) could be a major metabolic process in the sulfur cycle of 615 ASOMZ sediments. While genes for the metabolic conversion of thiosulfate to tetrathionate were present across the two cores, so were live microorganisms which could accomplish this process. Furthermore, metatranscriptomic data highlighted the potentially functional (metabolically active) state of thiosulfate to tetrathionate converting bacteria *in situ*. Tetrathionate formed in this way

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

can have a number of fates: it can either be converted to sulfate (via chemolithotrophic oxidation by members of the genera included under Group B in Fig. 6) or reduced to thiosulfate and/or sulfide (by members of the genera included in Group C of Fig. 6). Whilst culture-independent as well as culture-dependent data supported the feasibility of these metabolic processes *in situ*, copious hydrogen sulfide present in the pore-waters (Fernandes et al., 2018; also see Figs. 3G and 3H, Table S24) can potentially reduce tetrathionate to thiosulfate and elemental sulfur abiotically (Rowe et al., 2015). Corroborative to these possibilities, ion chromatographic analyses revealed up to 11.1 µM thiosulfate in the pore-waters of all the sulfide-containing sample-sites of SSK42/5 and SSK42/6 (Figs. 3G and 3H, Table S24; tandem absence of sulfide and thiosulfate in the upper 15 cmbsf of SSK42/6 could be due to potentially high rates of chemolithotrophic conversion of sulfide/thiosulfate to sulfate *in situ*). Tetrathionate, remarkably, was not found to exist freely in the pore-waters of SSK42/5 and SSK42/6; this is apparently attributable to the fact that its build-up to measurable quantities is generally debarred in natural environments due to high reactivity under the mediation of microbes (Kanao et al., 2007; Ghosh and Dam, 2009; Boden et al., 2010; Pyne et al., 2017, 2018) as well as naturally-occurring chemical substances such as sulfide (Schippers et al., 1999; Schippers and Jørgensen, 2001). From that perspective, the geomicrobiological information unearthed in this study illustrates the power of meta-omics in discovering such cryptic interfaces between the chemosphere and the biosphere that are almost impossible to decipher from geochemical records.

4 Discussions

4.1 Structure-function of the sedimentary microbiome in relation to tetrathionate metabolism

Metagenome analysis along SSK42/5 and SSK42/6 revealed tetrathionate-metabolizing potentials in all the bacterial communities present at various depths of the sediment-horizon. At the same time, metatranscriptome analysis for the deepest sediment-sample of SSK42/6 indicated that the potentially tetrathionate-metabolizing communities are active *in situ.* For instance, the thiosulfate to tetrathionate conversion-related gene *tsdA* was found to be present in the metagenomes analyzed from all but two of the 25 sample-sites explored along the two cores (Table S3). At the same time, the metatranscriptomic sequence dataset encompassed reads matching the *tsdA* genes of the thiosulfate-to-tetrathionate-converting isolates *Halomonas* sp. MCC 3301 and *Pseudomonas bauzanensis* MTCC 12600 (Table S25). Furthermore, *tsdA* homologs belonging to species of *Gramella* and *Marinobacter* were detected in the assembled metatranscriptome (Table S20). The tetrathionate-oxidation-related genes *soxB* and *soxC* were

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

found to be present in the metagenomes of all the sample-sites explored along the two cores (Table S4), while the metatranscriptomic dataset encompassed reads matching the *soxB* and *soxC* genes of the tetrathionate-oxidizing isolates *Halothiobacillus* sp. SB14A and *Pusillimonas ginsengisoli* MTCC 12558, according to mapping experiments (Table S25). *soxB* and *soxC* homologs belonging to species of *Gramella*, *Halothiobacillus* and *Marinobacter* were also detected in the assembled metatranscriptome (Table S20). While the tetrathionate-reduction-related genes *ttrABC*, *phsABC* and/or *psrA* were found to be present in the metagenomes of 24 out of 25 sample-sites (Table S5), no known tetrathionate-reduction-related gene was detected in the assembled and annotated metatranscriptome of the 275 cmbsf sediment-sample of SSK42/6. In this context it is further noteworthy that the genome of the tetrathionate-reducing isolate *Enterobacter* sp. RVSM5a was found to be lacking the *ttrABC* and *phsABC* genes, thereby indicating the presence of potentially novel genetic diversities for tetrathionate reduction. This said, genes other than *ttrABC* and *phsABC* ascribed to the typically tetrathionate-reducing genus *Salmonella* were detected in the assembled and annotated metatranscriptome (data not shown).

So far as the feasibility of the three tetrathionate-metabolizing processes in the highly-sulfidic sediments of SSK42/5 and SSK42/6 (Fernandes et al., 2018) is concerned, it is noteworthy that tetrathionate reduction is a mode of anaerobic respiration (Barrett and Clark, 1987; Hensel et al., 1999; Price-Carter et al., 2001), while thiosulfate to tetrathionate conversion by the action of *tsdA*-encoded thiosulfate dehydrogenase occurs under both aerobic and anaerobic conditions (Sorokin et al., 1999). However, most of the sulfur chemolithotrophic bacteria known thus far, including some of those which form tetrathionate from thiosulfate and/or oxidize tetrathionate to sulfate, use O_2 as the terminal electron acceptor (Ghosh and Dam, 2009; Wasmund et al., 2017; Patwardhan et al., 2018). Albeit it is peculiar that such microorganisms could be alive and active in these apparently zero-O_2 sediment-horizons, aerobic-respiration-related genes such as *aa*$_3$-/*cbb*$_3$-type cytochrome-*c* oxidases (*coxABCD* / *ccoNOPQ*) and cytochrome-*bd* ubiquinol oxidase (*cydABX* / *appX*) are abundant in the metagenomes of all the sample-sites explored in SSK42/5 and SSK42/6 (Bhattacharya et al., 2019). Furthermore, when the metatranscriptomic sequence dataset obtained for the 275 cmbsf sediment-sample of SSK42/6 was assembled and annotated, the resultant contigs were found to encompass several homologs corresponding to genes for aerobic respiration by *aa*$_3$-type and *cbb*$_3$-type cytochrome-*c* oxidases and cytochrome-*bd* ubiquinol oxidase (Table S26), together with those for other O_2-requiring (oxidase enzyme catalyzed) biochemical reactions (Table S27). All these data collectively indicated that potential cryptic O_2 source(s) supportive of aerobic metabolic processes are present in this sedimentary ecosystem.

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

4.2 Trends of geomicrobial parameters down the sediment-depths corroborated sulfur cycle functions centered on tetrathionate

Along both SSK42/5 and SSK42/6, prevalence of tetrathionate-forming, oxidizing as well as reducing bacteria increased with sediment-depth (Figs. 1A and 1B). This could be attributable to the corresponding overall down-depth increase in the concentration of thiosulfate (Figs. 3G and 3H), which is potentially the key source of biogenic tetrathionate in the sediment. Consistent with the trend of down-depth increase in thiosulfate concentration, along both the cores, increase was recorded in the concentrations of sulfide (Figs. 3G and 3H), which is not only a potential source of thiosulfate in marine sediments (Jørgensen, 1990) but also a product of tetrathionate reduction (Barrett and Clark, 1987; Price-Carter et al., 2001). On the other hand, down-depth decreases in sulfate concentration in tandem with increases in the relative abundance of tetrathionate-oxidizing bacteria (Figs. 1A, 1B, 3G and 3H) indicated that the quantities and rates of *in situ* sulfate production from potential tetrathionate oxidation were far less than the overall pore-water sulfate concentrations and rates of sulfate reduction respectively. Furthermore, in this context, it is noteworthy that in neither of the two cores, rates of *in vitro* tetrathionate formation or tetrathionate oxidation (in slurry culture experiments) exhibited any parity with the trends of fluctuation observed for the relative abundance of tetrathionate-forming or oxidizing bacteria (Figs. 1A, 1B, 3A-3D). This could be reflective of the fact that the substrate-utilization rates manifested in the slurry incubation experiments actually resulted from the activities of the few chemolithotrophic species that were potentially enriched in the specific media used in these experiments and not that of the whole community present *in situ*. In contrast, however, the rate of *in vitro* tetrathionate reduction exhibited overall parity with the trends of fluctuation observed for the relative abundance of tetrathionate-reducing bacteria (both parameters showing overall increases in the sediment-surface to core-bottom trajectories; Figs. 1A, 1B, 3E and 3F). This could be reflective of the fact that the tetrathionate reduction rates manifested in the slurry incubation experiments resulted from the activities of most of the tetrathionate-reducing species present *in situ*, which in turn indicates the general ability of all tetrathionate-reducers to grow in RVTr medium.

4.3 Tetrathionate, and sulfur cycling in global marine sediments

Sulfur cycling is a crucial component of sediment biogeochemistry within the marine realm. Apart from controlling *in situ* sulfide-sulfate balance, microbe-mediated processes of the sulfur cycle work in conjunction with those of the carbon cycle to remineralize organic matters sequestered in the sea-bed, and also influence metal deposition/mobilization. Tetrathionate is rarely investigated

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

as a central intermediate of sulfur cycling in marine sediments, even though thiosulfate is long known to be a central biogeochemical junction of sedimentary sulfur cycling across the global ocean (Jørgensen, 1990; Jørgensen and Bak 1991; Thamdrup et al., 1994). Thus far, only one study based on the Baltic Sea sediments has reported microbial production of tetrathionate and highlighted the role of tetrathionate in the sulfur cycle (Podgorsek and Imhoff, 1999). The present geomicrobiological exploration of the sediments underlying the approximate-center (530-580 mbsl) of the ~200-1200 mbsl vertical-expanse of the Arabian Sea OMZ revealed tetrathionate as a potent intermediate of the *in situ* sulfur cycle, and identified the microbial mechanisms that are plausibly involved in the formation and transformation of this polythionate. Albeit the cryptic biogeochemical roles of tetrathionate in the sulfur cycle were revealed here in the context of an oxygen minimum zone, there were no observable reasons to assume that such processes do not have their equivalents in other geomicrobiologically-distinct sediment-horizons of the marine realm.

Supplementary material

Supplemental material for this article may be found with the digital version of this manuscript.

Data availability

All nucleotide sequence data have been deposited in NCBI Sequence Read Archive (SRA) or GenBank under the BioProject accession number PRJNA309469: (i) the whole metagenome shotgun sequence datasets have the Run accession numbers SRR3646127 through SRR3646132, SRR3646144, SRR3646145, SRR3646147, SRR3646148, SRR3646150 through SRR3646153, SRR3646155 through SRR3646158, SRR3646160 through SRR3646165, and (ii) the metatranscriptome sequence dataset has the Run accession number SRR7991972. (iii) the whole genome sequences have the GenBank accession numbers SWAW01000000, SSXS01000000, RAPG00000000.1., SSXT01000000, SWAV01000000 and VTPT00000000.

Code availability. All data analysis codes used in this study are in the published domain, and have been appropriately cited in the text.

Author contributions

W.G. conceived the study, designed the experiments, interpreted the results and wrote the paper in conjunction with S.M. S.M. anchored the whole microbiological work, performed the

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

755 experiments, analyzed the data and substantially contributed to the writing of the paper. A.M led the entire SSK42 mission and all geochemical investigations therein. S.B., T.M., M.J.R. and C.R. performed microbiological experiments and data analysis. S.F. and A.P. performed geochemical experiments. All authors read and vetted the manuscript.

760 **Acknowledgements**

Financial support for conducting the microbiological studies was provided by given by Bose Institute via internal faculty grants and Earth System Science Organization, Ministry of Earth Sciences (MoES), Government of India (GoI) via grant number MoES/36/00IS/Extra/19/2013. We thank the Director CSIR-National Institute of Oceanography for facilitating the geochemical
765 studies and the research cruise SSK42 for acquisition of sediment cores. MoES (GAP2303) also funded the research cruise. All the support received from the CSIR-NIO Ship Cell members and the crew members of SSK42 is gratefully acknowledged. S.B. received fellowship from Bose Institute. SM got fellowship from Department of Science and Technology, GoI. MJR and C.R. got fellowship from University Grants Commission, GoI. S.F. received fellowships from Council of
770 Scientific and Industrial Research, GoI.

References

Alam, M., Pyne, P., Mazumdar, A., Peketi A., and Ghosh, W.: Kinetic enrichment of ^{34}S during proteobacterial thiosulfate oxidation and the conserved role of SoxB in SS bond breaking,
775 Appl. Environ. Microbiol., 79, 4455-4464, https://doi.org/10.1128/AEM.00956-13, 2013.

Barrett, E. L., and Clark, M. A.: Tetrathionate reduction and production of hydrogen sulfide from thiosulfate, Microbiol. Rev., 51,192-205, 1987.

Baumgartner, L. K., Reid R. P., Dupraz, C., Decho, A. W., Buckley, D. H., Spear, J. R., Przekop, K. M., and Visscher, P. T.: Sulfate reducing bacteria in microbial mats: changing paradigms,
780 new discoveries, Sediment. Geol., 185, 131-145, https://doi.org/10.1016/j.sedgeo.2005.12.008, 2006.

Bhattacharya, S., Roy, C., Mandal, S., Rameez, M.J., Sarkar, J., Fernandes, S., Mapder, T., Alam, M., Roy, R., Mondal, N. and Pyne, P.: Metabolically-active obligate aerobes in anoxic (sulfidic) marine sediments, bioRxiv., https://doi.org/10.1101/728287, 728287, 2019.

785 Boden, R., Kelly, D. P., Murrell, J. C., and Schäfer, H.: Oxidation of dimethylsulfide to tetrathionate by *Methylophaga thiooxidans* sp. nov.: a new link in the sulfur cycle, Environ. Microbiol., 12, 2688-2699, https://doi.org/10.1111/j.1462-2920.2010.02238.x, 2010.

Boden, R., Scott, K. M., Williams, J., Russel, S., Antonen, K, Rae, A. W., and Hutt, L. P.: An evaluation of *Thiomicrospira*, *Hydrogenovibrio* and *Thioalkalimicrobium*: reclassification of
790 four species of *Thiomicrospira* to each *Thiomicrorhabdus* gen. nov. and *Hydrogenovibrio*, and reclassification of all four species of *Thioalkalimicrobium* to *Thiomicrospira*, Int. J. Syst. Evol. Microbiol., 67, 1140-1151, https://doi: 10.1099/ijsem.0.001855, 2017.

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Brito JA, Denkmann K, Pereira IA, Archer M, Dahl C.: Thiosulfate Dehydrogenase (TsdA) from *Allochromatium vinosum* structural and functional insights into thiosulfate oxidation, J Biol. Chem., 9222-9238, https://doi: 10.1074/jbc.M114.623397, 2015.

Cline, J. D.: Spectrophotometric determination of hydrogen sulfide in natural waters, Limnol. Oceanogr., 14, 454-458, 1969.

De Jong, G.A.H., Hazeu, W., Bos, P., Kuenen, G.: Polythionate degradation by tetrathionate hydrolase of *Thiobacillus ferrooxidans*, Microbiology., 143, 499-504. https://doi.org/10.1099/00221287-143-2-499, 1997.

Denkmann, K., Grein, F., Zigann, R., Siemen, A., Bergmann, J., van Helmont, S., Nicolai, A., Pereira, I. A., and Dahl, C.: Thiosulfate dehydrogenase: a widespread unusual acidophilic c-type cytochrome, Environ. Microbiol., 14, 2673-2688, https://doi.org/10.1111/j.1462-2920.2012.02820.x, 2012.

Euzéby, J. P.: List of Bacterial Names with Standing in Nomenclature: a folder available on the Internet, Int. J. Syst. Evol. Microbiol., 47, 590-592, https://doi:10.1099/00207713-47-2-590, 1997.

Fernandes, S., Mazumdar, A., Bhattacharya, S., Peketi, A., Mapder, T., Roy, R., Carvalho, M. A., Roy, C., Mahalakshmi, P., Silva, R., Rao, P. S., Banik, S., and Ghosh, W.: Enhanced carbon-sulfur cycling in the sediments of Arabian Sea oxygen minimum zone center, Sci. Rep., 8, 8665, https://doi.org/10.1038/s41598-018-27002-2, 2018.

Ghosh, W., Bagchi, A., Mandal, S., Dam, B., and Roy, P.: *Tetrathiobacter kashmirensis* gen. nov., sp. nov., a novel mesophilic, neutrophilic, tetrathionate-oxidizing, facultatively chemolithotrophic betaproteobacterium isolated from soil from a temperate orchard in Jammu and Kashmir, India, Int. J. Syst. Evol. Microbiol., 55, 1779-1787, https://doi:10.1099/ijs.0.63595-0, 2005.

Ghosh, W., and Roy, P.: *Mesorhizobium thiogangeticum* sp. nov., a novel sulfur-oxidizing chemolithoautotroph from rhizosphere soil of an Indian tropical leguminous plant, Int. J. Syst. Evol. Microbiol., 56, 91-97, https://doi:10.1099/ijs.0.63967-0, 2006.

Ghosh, W., and Roy, P.: Chemolithoautotrophic oxidation of thiosulfate, tetrathionate and thiocyanate by a novel rhizobacterium belonging to the genus *Paracoccus*. FEMS. Microbiol. Lett., 270, 124-131, https://doi.org/10.1111/j.1574-6968.2007.00670.x, 2007.

Ghosh, W., and Dam, B.: Biochemistry and molecular biology of lithotrophic sulfur oxidation by taxonomically and ecologically diverse bacteria and archaea, FEMS Microbiol. Rev., 33, 999-1043, https://doi.org/10.1111/j.1574-6976.2009.00187.x, 2009, 2009.

Ghosh, W., Roy, C., Roy, R., Nilawe, P., Mukherjee, A., Haldar, P. K., Chauhan, N. K., Bhattacharya, S., Agarwal, A., George, A., and Pyne, P.: Resilience and receptivity worked in tandem to sustain a geothermal mat community amidst erratic environmental conditions, Sci. Rep., 5, 12179, https://doi:10.1038/srep12179, 2015.

Gerhardt, P.: Methods for general and molecular bacteriology, American Society for Microbiology, 791. ASM Press, Washington DC, 1994.

Hedrich, S., and Johnson, D. B.: *Acidithiobacillus ferridurans* sp. nov., an acidophilic iron-, sulfur- and hydrogen-metabolizing chemolithotrophic gammaproteobacterium, Int. J. Syst. Evol. Microbiol., 63, 4018-4025, https://doi:10.1099/ijs.0.049759-0, 2013.

Hensel, M., Hinsley, A.P., Nikolaus, T., Sawers, G. and Berks, B.C.:The genetic basis of tetrathionate respiration in *Salmonella typhimurium*, Mol. Microbiol., 32, 275-287, https://doi.org/10.1046/j.1365-2958.1999.01345.x, 1999.

© Author(s) 2019. CC BY 4.0 License.

Hensen D, Sperling D, Trüper HG, Brune DC, Dahl C.: Thiosulphate oxidation in the phototrophic sulphur bacterium *Allochromatium vinosum*, Mol. Microbiol., 62, 794-810, https://doi.org/10.1111/j.1365-2958.2006.05408.x, 2006.

Holmkvist, L., Ferdelman, T. G., and Jørgensen, B. B.: A cryptic sulfur cycle driven by iron in the methane zone of marine sediment (Aarhus Bay, Denmark), Geochim. Cosmochim. Acta., 75, 3581-3599, https://doi.org/10.1016/j.gca.2011.03.033, 2011.

Huerta-Cepas, J., Szklarczyk, D., Forslund, K., Cook, H., Heller, D., Walter, M. C., Rattei, T., Mende, D. R., Sunagawa, S., Kuhn, M., and Jensen, L. J.: eggNOG 4.5: a hierarchical orthology framework with improved functional annotations for eukaryotic, prokaryotic and viral sequences, Nucleic Acids Res., 44, D286-D293, 2016.

Hyatt, D., Chen, G. L., LoCascio, P. F., Land, M. L., Larimer, F. W., and Hauser, L. J.: Prodigal: prokaryotic gene recognition and translation initiation site identification. BMC. Bioinformatics., 11, 119, https://doi.org/10.1186/1471-2105-11-119, 2010.

Jørgensen, B. B.: A thiosulfate shunt in the sulfur cycle of marine sediments, Science., 249, 152-154, https://doi:10.1126/science.249.4965.152, 1990.

Jørgensen, B. B., and Bak, F.: Pathways and microbiology of thiosulfate transformations and sulfate reduction in a marine sediment (Kattegat, Denmark), Appl. Environ. Microbiol., 57, 847-856, 1991.

Jørgensen, B. B., Findlay, A. J., and Pellerin, A.: The Biogeochemical Sulfur Cycle of Marine Sediments, Front. Microbiol., 10. https://doi.org/10.3389/fmicb.2019.00849, 2019.

Kaprálek, F.: The physiological role of tetrathionate respiration in growing *Citrobacter*, Microbiology, 71, 133-139, https://doi: 10.1099/00221287-71-1-133. 1972.

Kanao, T., Kamimura, K., and Sugio, T.: Identification of a gene encoding a tetrathionate hydrolase in *Acidithiobacillus ferrooxidans*, J. Biotechnol.,132, 16-22, https://doi.org/10.1016/j.jbiotec.2007.08.030, 2007.

Kanao, T., Kosaka, M., Yoshida, K., Nakayama, H., Tamada,T., Kuroki, R., Yamada, H., Takada, J., Kamimura, K.: Crystallization and preliminary X-ray diffraction analysis of tetrathionate hydrolase from *Acidithiobacillus ferrooxidans*, Acta. Cryst. F., 69, 692-694. https://doi.org/10.1107/S1744309113013419, 2013.

Kanehisa, M., Sato, Y., Kawashima, M., Furumichi, M., and Tanabe, M.: KEGG as a reference resource for gene and protein annotation, Nucleic Acids Res., 44**,** 457-462, 2016.

Kelly, D.P. and Wood, A.P.: Synthesis and determination of thiosulfate and polythionates, Methods. Enzymol., 243, 475-501, https://doi.org/10.1016/0076-6879(94)43037-3, 1994.

Kikumoto, M., Nogami, S., Kanao, T., Takada, J., Kamimura, K.: Tetrathionate-forming thiosulfate dehydrogenase from the acidophilic, chemolithoautotrophic bacterium *Acidithiobacillus ferrooxidans*, Appl. Environ. Microbiol., 79, 113-120. https://doi.org/10.1128/AEM.02251-12, 2013.

Lahiri, C., Mandal, S., Ghosh, W., Dam, B., and Roy, P.: A novel gene cluster *soxSRT* is essential for the chemolithotrophic oxidation of thiosulfate and tetrathionate by *Pseudaminobacter salicylatoxidans* KCT001, Curr. Microbiol., 52, 267-273, https://doi.org/10.1007/s00284-005-0176-x, 2006.

Langmead, B., and Salzberg, S.L.: Fast gapped-read alignment with Bowtie 2, Nat. Methods., 9, 357–359, https://doi.org/10.1038/nmeth.1923, 2012.

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Li, D., Liu, C. M., Luo, R., Sadakane, K., and Lam, T. W.: MEGAHIT: an ultra-fast single-node solution for large and complex metagenomics assembly via succinct de Bruijn graph, Bioinformatics., 31, 1674-1676, https://doi.org/10.1093/bioinformatics/btv033, 2015.

Mason, J. and Kelly, D.P.: Thiosulfate oxidation by obligately heterotrophic bacteria, Microb. Ecol., 15,123-134, https://doi.org/10.1007/BF02011707, 1988.

Meyer, F., Paarmann, D., D'Souza, M., Olson, R., Glass, E. M., Kubal, M., Paczian, T., Rodriguez, A., Stevens, R., Wilke, A., and Wilkening, J.: The metagenomics RAST server–a public resource for the automatic phylogenetic and functional analysis of metagenomes, BMC. Bioinformatics., 9, 386, https://doi.org/10.1186/1471-2105-9-386, 2008.

Müller, F.H., Bandeiras, T.M., Urich, T., Teixeira, M., Gomes, C.M. and Kletzin, A.: Coupling of the pathway of sulphur oxidation to dioxygen reduction: characterization of a novel membrane-bound thiosulphate: quinone Oxidoreductase, Mol. Microbiol.,53, 1147-1160, https://doi.org/10.1111/j.1365-2958.2004.04193.x, 2004.

Nurk, S., Bankevich, A., Antipov, D., Gurevich, A. A., Korobeynikov, A., Lapidus, A., Prjibelski, A. D., Pyshkin, A., Sirotkin, A., Sirotkin, Y., and Stepanauskas, R.: Assembling single-cell genomes and mini-metagenomes from chimeric MDA products, J. Comput. Biol., 20, 714-737, https://doi.org/10.1089/cmb.2013.0084, 2013.

Oltmann, L. F., and Stouthamer, A. H.: Reduction of tetrathionate, trithionate and thiosulphate, and oxidation of sulphide in *Proteus mirabilis*, Arch. Microbiol., 105, 135-142, https://doi.org/10.1007/BF00447128, 1975.

Parte, A. C.: LPSN—list of prokaryotic names with standing in nomenclature, Nucleic Acids Res., 42, 613-616, https://doi.org/10.1093/nar/gkt1111, 2013.

Patwardhan, S., Foustoukos, D.I., Giovannelli, D., Yucel, M. and Vetriani, C.: Ecological succession of sulfur-oxidizing Epsilon-and Gammaproteobacteria during colonization of a shallow-water gas vent, Front. Microbiol., 9, 2970, https://doi.org/10.3389/fmicb.2018.02970, 2018.

Podgorsek, L., and Imhoff, J. F.: Tetrathionate production by sulfur oxidizing bacteria and the role of tetrathionate in the sulfur cycle of Baltic Sea sediments, Aquat. Microb. Ecol., 17, 255-265, https://doi:10.3354/ame017255, 1999.

Price-Carter, M., Tingey, J., Bobik, T. A., and Roth, J. R.: The Alternative Electron Acceptor Tetrathionate Supports B12 Dependent Anaerobic Growth of *Salmonella* enteric Serovar *Typhimurium* on Ethanolamine or 1,2-Propanediol, J. Bacteriol., 183, 2463–2475, https://doi: 10.1128/JB.183.8.2463-2475.2001, 2001.

Pyne, P., Alam, M., and Ghosh, W.: A novel *soxO* gene, encoding a glutathione disulfide reductase, is essential for tetrathionate oxidation in *Advenella kashmirensis*, Microbiol. Res., 205, 1-7, 2017.

Pyne, P., Alam, M., Rameez, M. J., Mandal, S., Sar, A., Mondal, N., Debnath, U., Mathew, B., Misra, A. K., Mandal, A. K., and Ghosh, W.: Homologs from sulfur oxidation (Sox) and methanol dehydrogenation (Xox) enzyme systems collaborate to give rise to a novel pathway of chemolithotrophic tetrathionate oxidation, Mol. Microbiol., 109, 169-191, https://doi.org/10.1111/mmi.13972, 2018.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., Peplies, J., and Glöckner, F. O.: The SILVA ribosomal RNA gene database project: improved data processing and web-based tools, Nucleic Acids Res., 41, 590-596, https://doi.org/10.1093/nar/gks1219, 2012.

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Quatrini, R., Appia-Ayme, C., Denis, Y., Jedlicki, E., Holmes, D. S., and Bonnefoy, V.: Extending the models for iron and sulfur oxidation in the extreme acidophile *Acidithiobacillus ferrooxidans*, BMC. Genomics., 10, 394, https://doi.org/10.1186/1471-2164-10-394, 2009.

Rameez, M.J., Pyne, P., Mandal, S., Chatterjee, S., Alam, M., Bhattacharya, S., Mondal, N., Sarkar, J. and Ghosh, W.: Two pathways for thiosulfate oxidation in the alphaproteobacterial chemolithotroph *Paracoccus thiocyanatus* SST, Microbiol. Res., 230,126345, https://doi.org/10.1016/j.micres.2019.126345, 2019.

Rowe Annette, R., Chellamuthu, P., Lam, B., Okamoto, A., and Nealson, K. H.: Marine sediments microbes capable of electrode oxidation as a surrogate for lithotrophic insoluble substrate metabolism, Front. Microbiol., 5, 784, https://doi.org/10.3389/fmicb.2014.00784, 2015.

Roy, C., Alam, M., Mandal, S., Haldar, P.K., Bhattacharya, S., Mukherjee, T., Roy, R., Rameez, M.J., Misra, A.K., Chakraborty, R. and Nanda, A.K.: Global association between thermophilicity and vancomycin susceptibility in bacteria, Front. Microbiol., 7, 412, https://doi.org/10.3389/fmicb.2016.00412, 2016.

Rudnicki, M. D., Elderfield, H., and Spiro, B.: Fractionation of sulfur isotopes during bacterial sulfate reduction in deep ocean sediments at elevated temperatures, Geochim. Cosmochim. Acta., 65, 777-789, https://doi.org/10.1016/S0016-7037(00)00579-2, 2001.

Rzhepishevska, O.I., Valdés, J., Marcinkeviciene, L., Gallardo, C.A., Meskys, R., Bonnefoy, V., Holmes, D.S., Dopson, M.: Regulation of a novel *Acidithiobacillus caldus* gene cluster involved in metabolism of reduced inorganic sulfur compounds, Appl. Environ. Microbiol., 73, 7367-7372, https://doi.org/10.1128/AEM.01497-07, 2007.

Schippers, A., Rohwerder, T., and Sand, W.: Intermediary sulfur compounds in pyrite oxidation: implications for bioleaching and biodepyritization of coal, Appl. Microbiol. Biotechnol., 52, 104-110, https://doi.org/10.1007/s002530051495, 1999.

Schippers, A., and Jørgensen, B. B.: Oxidation of pyrite and iron sulfide by manganese dioxide in marine sediments, Geochim. Cosmochim. Acta., 65, 915-922, https://doi.org/10.1016/S0016-7037(00)00589-5, 2001.

Sorokin, D.Y., Teske, A., Robertson, L.A. and Kuenen, J.G.: Anaerobic oxidation of thiosulfate to tetrathionate by obligately heterotrophic bacteria, belonging to the *Pseudomonas stutzeri* group, FEMS. Microbiol. Ecol., 30, 113-123, https://doi.org/10.1111/j.1574-6941.1999.tb00640.x, 1999.

Sorokin, D.: Oxidation of inorganic sulfur compounds by obligatory organotrophic bacteria, Mikrobiologiia, 72, 725-739, https://doi.org/10.1023/B:MICI.0000008363.24128.e52003, 2003.

Stoffels, L., Krehenbrink, M., Berks, B. C., and Unden, G.: Thiosulfate Reduction in *Salmonella enterica* Is Driven by the Proton Motive Force, Appl. Environ. Microbiol., 194, 475–485. https://doi:10.1128/JB.06014-11, 2011.

Tuttle, J. H.: Thiosulfate oxidation and tetrathionate reduction by intact cells of marine pseudomonad strain 16B, Appl. Environ. Microbiol., 39, 1159-1166, 1980.

Thamdrup, B., Finster, K., Fossing, H., Hansen, J. W and Jørgensen, B. B.: Thiosulfate and sulfite distributions in porewater of marine sediments related to manganese, iron, and sulfur geochemistry, Geochim. Cosmochim. Acta., 58, 67-73, https://doi:1016/0016-7037(94)90446-4, 1994.

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

970 Tostevin, R., Turchyn, A. V., Farquhar, J., Johnston, D. T., Eldridge, D. L., Bishop, J. K., and McIlvin, M.: Multiple sulfur isotope constraints on the modern sulfur cycle, Earth. Planet. Sci. Lett., 396, 14-21, https://doi.org/10.1016/j.epsl.2014.03.057, 2014.

Vassiliadis, P.: The Rappaport-Vassiliadis (RV) enrichment medium for the isolation of salmonellas: An overview, J. Appl. Bacteriol., 54, 69-76, https://doi.org/10.1111/j.1365-
975 2672.1983.tb01302.x, 1983.

van Zyl, L.J., van Munster, J.M., Rawlings, D.E. Construction of *arsB* and *tetH* mutants of the sulfur-oxidizing bacterium *Acidithiobacillus caldus* by marker exchange, Appl. Environ. Microbiol., 74, 5686-5694. https://doi.org/10.1128/AEM.01235-08, 2008.

Vishniac, W., and Santer, M.: The *Thiobacilli*. Bacteriol. Rev., 21, 195-213, 1957.

980 Wasmund, K., Mußmann, M. and Loy, A.: The life sulfuric: microbial ecology of sulfur cycling in marine sediments, Environ. Microbiol. Rep., 9, 323-344, https://doi.org/10.1111/1758-2229.12538, 2017.

Watsuji, T. O., Had, E., Miyazaki, M., Ichimura, M., and Takai, K.: *Thiomicrospira hydrogeniphila* sp. nov., an aerobic, hydrogen-and sulfur-oxidizing chemolithoautotroph isolated from a
985 seawater tank containing a block of beef tallow, Int J Syst Evol Microbiol, 66, 3688-3693, https://doi: 10.1099/ijsem.0.001250, 2016.

Zhu, W., Lomsadze, A., and Borodovsky, M.: *Ab initio* gene identification in metagenomic sequences, Nucleic Acids Res., 38, e132, https://doi.org/10.1093/nar/gkq275, 2010.

990

Legend of figures

Figure 1. Scatter plots showing the down-depth variations in the prevalence of key metabolic-types, or the pair-wise associations between the prevalence of the metabolic-types, along (1A) SSK42/5 and (1B) SSK42/6. Parameters considered include sediment-depth
995 (in cmbsf), and percentages of metagenomic reads matching protein-coding genomic sequences from the genera of tetrathionate-reducing (*Citrobacter*, *Proteus* and *Salmonella*), tetrathionate-forming (*Pseudomonas* and *Halomonas*) and tetrathionate-oxidizing (*Acidithiobacillus*, *Halothiobacillus* and *Thiomicrospira*) bacteria. Presence of these genera in the various sediment-samples of SSK42/5 and SSK42/6 was corroborated via manual scrutiny of the amplified 16S
1000 rRNA gene sequence-based diversity data reported previously for these cores (Fernandes et al., 2018) as well as by searching the individual metagenomic sequence datasets against the 16S rRNA gene sequence database of the Ribosomal Database Project (using BlastN with minimum alignment length 50 bp, minimum identity cut-off 90% and maximum e-value cut-off $1e^{-5}$). Plots corroborated by Pearson correlation coefficient (CC) and/or Spearman rank correlation
1005 coefficient (RCC) values ≥ + 0.8 with $P < 0.05$ are shown in blue. Whereas none of the plots were corroborated by negative CC or RCC values numerically ≥ 0.8 with $P < 0.05$, those corroborated by positive/negative CC and/or RCC values numerically ≤ 0.8 are shown in black, irrespective of

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

whether P is < 0.05. All CC and RCC values pertaining to the above plots are given in Table S6 and Table S7 respectively.

Figure 2. Metabolic transformations of thiosulfate and/or tetrathionate by representative strains of the various species-level entities isolated from 275 cmbsf of SSK42/6. (**A** through **D**) thiosulfate to tetrathionate conversion by *Halomonas* sp. 15WGF, *Methylophaga* sp. SBPC3, *Pseudomonas* sp. SBBB and *Stenotrophomonas* sp. SBPC3 respectively. (**E** and **F**) oxidation of thiosulfate to sulfate via tetrathionate, and oxidation of tetrathionate to sulfate, by *Pusillimonas* sp. SBSA respectively. (**G**) oxidation of tetrathionate to sulfate by *Halothiobacillus* sp. SB14A. (**H**) reduction of tetrathionate to thiosulfate by *Enterobacter* sp. RVSM5a.
—●—, —▲— and **—▼—** denote the concentrations of sulfur (mM S) present in the media, at a given time-point of incubation in the form of thiosulfate, sulfate and tetrathionate respectively.
—■— denotes pH of the media at a given time-point of incubation.

Figure 3. Transformation of thiosulfate and/or tetrathionate in slurry incubation experiments, and down-depth concentrations of different sulfur species. (**A** and **B**) *in vitro* rates of tetrathionate formation and its downstream oxidation (if any) in ASWT medium, for the different sedimentary communities of SSK42/5 and SSK42/6 respectively. (**C** and **D**) *in vitro* rates of tetrathionate oxidation in ASWTr medium, for the different sedimentary communities of SSK42/5 and SSK42/6. (**E** and **F**) *in vitro* rates of tetrathionate reduction in RVTr medium, for the different sedimentary communities of SSK42/5 and SSK42/6. (G and H) concentrations of sulfur species measured along SSK42/5 and SSK42/6. In **A** through **F**, brown, violet and green circles represent tetrathionate formation, oxidation and reduction rates respectively. In **G** and **H**, orange, light blue and purple circles represent the concentrations of sulfide, thiosulfate and sulfate respectively (sulfide and sulfate data taken from Fernandes et al., 2018).

Figure 4. Bubble plot showing the percentages of metagenomic reads from individual sediment-samples of SSK42/5 that matched with genomic sequences of the six tetrathionate-metabolizing bacterial isolates: (Ent) *Enterobacter* sp. RVSM5a, (*Hlm*) *Halomonas* sp. MCC 3301, (*Htb*) *Halothiobacillus* sp. SB14A, (*Mtp*) *Methylophaga* sp. MTCC 12599, (*Psb*) *Pseudomonas bauzanensis* MTCC 12600, and (*Psg*) *Pusillimonas ginsengisoli* MTCC12558. Scales for the two independent variables in x-axis (individual isolates) and y-axis (sediment-depth), and the dependent variable (percentage of metagenomic reads matching genomic sequences of an isolate) represented by the size of the bubbles, are all linear.

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Figure 5. Bubble plot showing the percentages of metagenomic reads from individual sediment-samples of SSK42/6 that matched with genomic sequences of the six tetrathionate-metabolizing bacterial isolates: (Ent) *Enterobacter* sp. RVSM5a, (*Hlm*) *Halomonas* sp. MCC 3301, (*Htb*) *Halothiobacillus* sp. SB14A, (*Mtp*) *Methylophaga* sp. MTCC 12599, (*Psb*) *Pseudomonas bauzanensis* MTCC 12600, and (*Psg*) *Pusillimonas ginsengisoli* MTCC12558. Scales for the two independent variables in x-axis (individual isolates) and y-axis (sediment-depth), and the dependent variable (percentage of metagenomic reads matching genomic sequences of an isolate) represented by the size of the bubbles, are all linear.

Figure 6. Schematic diagram showing the network of biotic and abiotic process that are potentially involved in the formation and transformation of tetrathionate in the Arabian Sea OMZ sediments.

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Table 1. Bacteria isolated from 275 cmbsf of SSK42/6, and their tetrathionate-metabolizing properties.

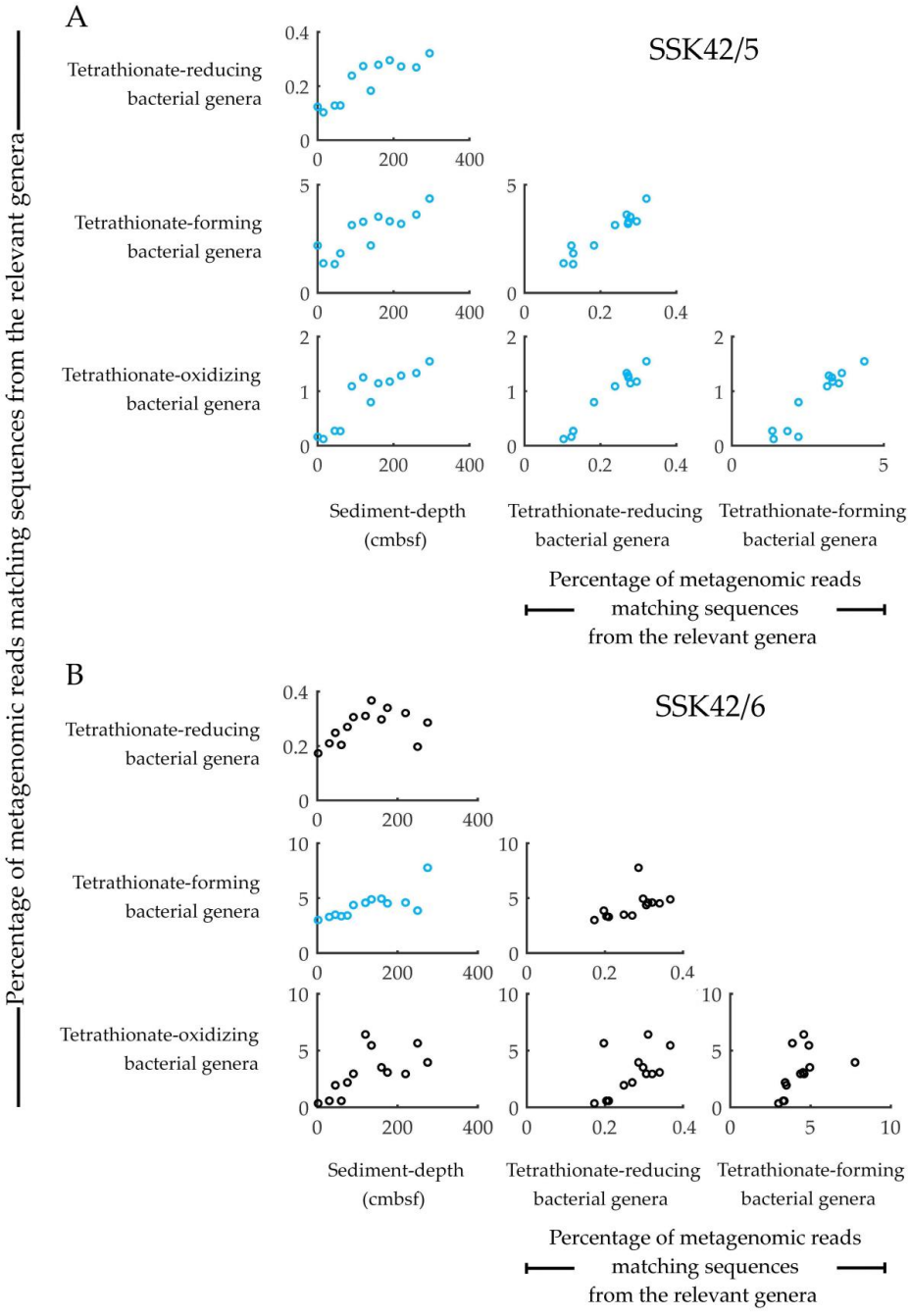
	Bacteria isolated in ASWT		**Bacteria isolated in ASWTY**	**Bacteria isolated in MSTY**			**Bacteria isolated in RVTr**
Identification up to lowest taxonomic level possible	*Halothiobacillus* sp.	*Methylophaga* sp.	*Halomonas* sp.	*Pseudomonas* sp.	*Stenotrophomonas* sp.	*Pusillimonas ginsengisoli*	*Enterobacter* sp.
Total number of strains isolated for the species-level cluster	6	2	2	2	1	2	4
Name of the representative strain	SB14A	SB9B = MTCC12599	15WGF = MCC3301	SBBB = MTCC12600	SBPC3	SBSA = MTCC12558	RVSM5a
16S rRNA gene sequence accession number of the representative strain	LN999387	LN999390	LT607031	LN999396	LN999400	LN999398	MH593841
Tetrathionate-metabolizing phenotype (medium in which phenotype was tested)	(i) Tetrathionate to sulfate (ASWTr)	(i) Thiosulfate to tetrathionate (ASWTM)	(i) Thiosulfate to tetrathionate (ASWTY)	(i) Thiosulfate to tetrathionate (MSTY)	(i) Thiosulfate to tetrathionate (MSTY)	(i) Thiosulfate to tetrathionate (MSTY) (ii) Tetrathionate to sulfate (MSTrY)	(i) Tetrathionate to thiosulfate (RVTr)
NCBI accession number for whole genome sequence	SWAW01000000	SSXS01000000	SSXT01000000	SWAV01000000	-	RAPG00000000	VTPT00000000

Biogeosciences Discussions
Open Access
EGU

https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Figures

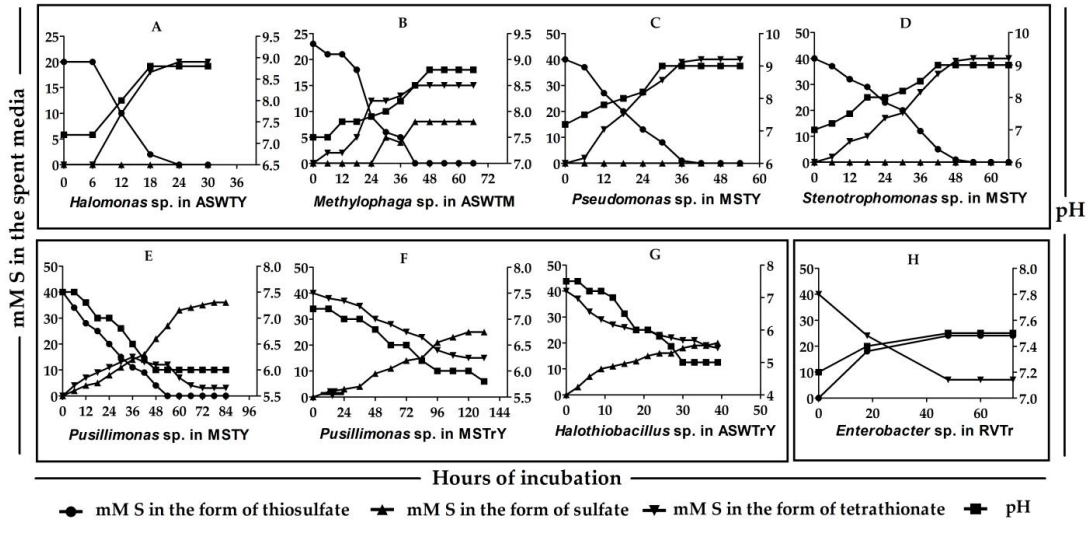
Figure 1.



https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Biogeosciences
Discussions
Open Access
EGU

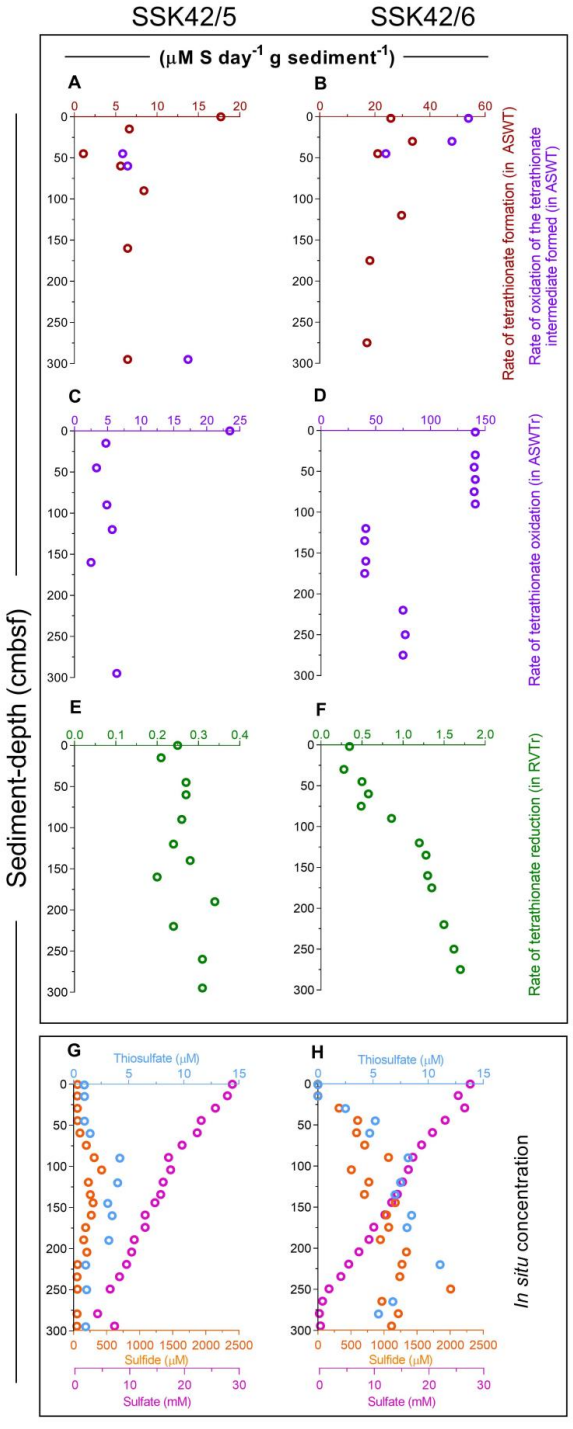
Figure 2.



https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

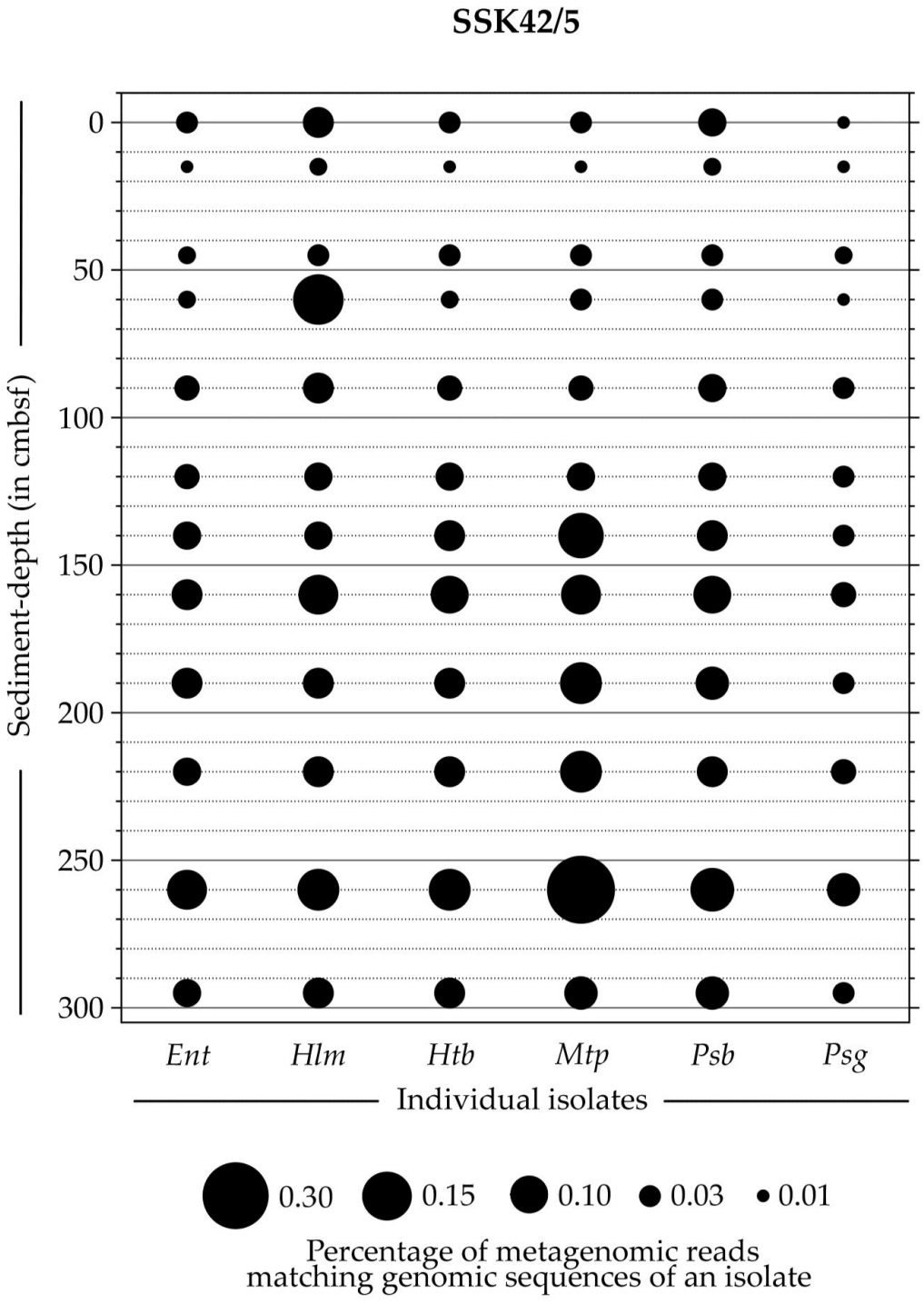
Biogeosciences
Discussions
Open Access
EGU

Figure 3.



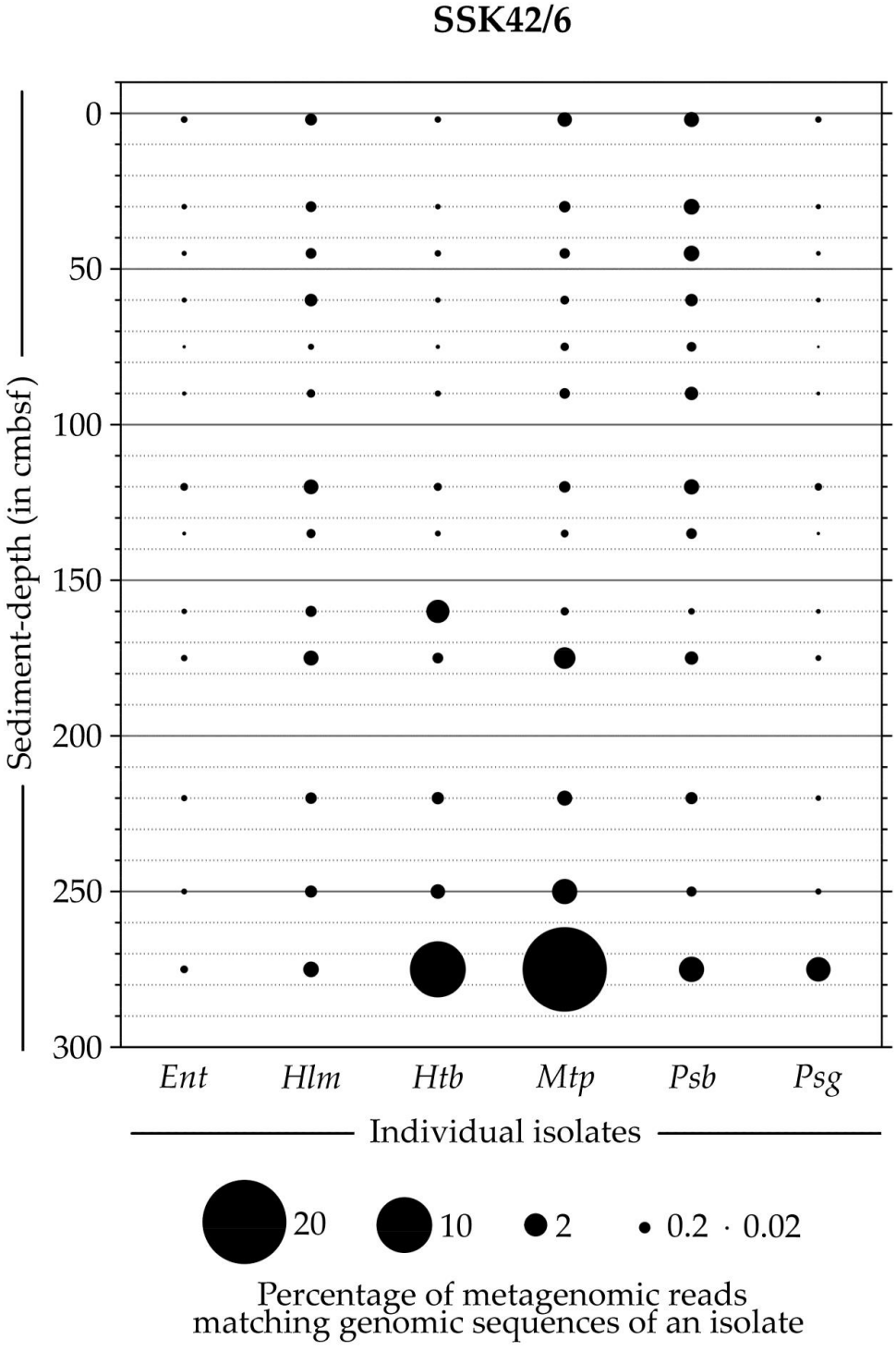
https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Figure 4.



https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

Figure 5.



https://doi.org/10.5194/bg-2019-471
Preprint. Discussion started: 17 December 2019
© Author(s) 2019. CC BY 4.0 License.

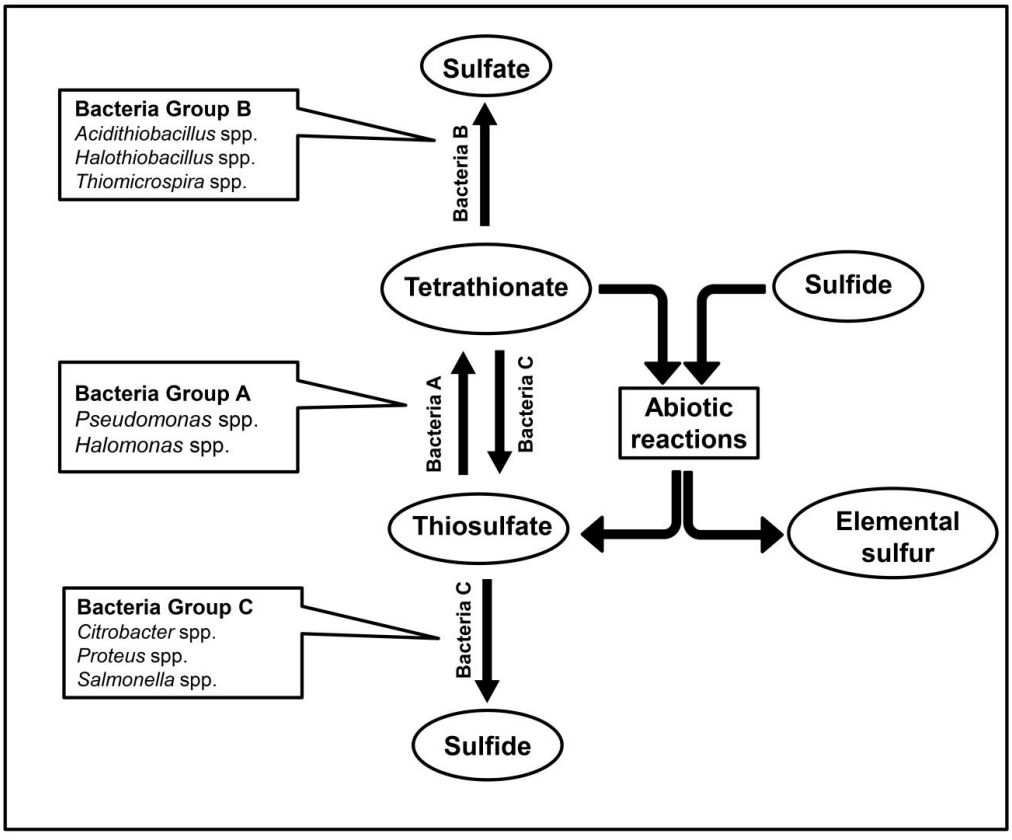



Figure 6.