Cryptic roles of tetrathionate in the sulfur cycle of marine sediments: Microbial drivers and indicators

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Abstract

To explore the potential role of tetrathionate in the sedimentary sulfur cycle, 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 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-culture 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, whereas tetrathionate remained undetected in assays based on its reaction with cyanide. While thiosulfate oxidation by chemolithotrophic bacteria prevalent *in situ* is the apparent source of tetrathionate in this ecosystem, high microbiological and geochemical reactivity of this polythionate could be instrumental in its cryptic status in the sulfur cycle. Potential abiotic origin of tetrathionate in the sediment-horizon explored could neither be ruled out nor confirmed from the geochemical information available for this territory. On the other hand, tetrathionate potentially present in the system can be either oxidized to sulfate () or reduced back to thiosulfate/sulfide via chemolithotrophic oxidation and respiration by native bacterial populations respectively. Up to 2.01 mM sulfide present in the sediment-cores may also reduce tetrathionate abiotically to thiosulfate and elemental sulfur. However, in the absence of measured data for O_2 or other oxyanions having possibilities of serving as electron acceptors, the biogeochemical modalities of the oxidative half of the *in situ* tetrathionate cycle remain unresolved.

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 the focus of such investigations has typically been on geomicrobial transformations of the two redox end-members sulfate and sulfide (Holmkvist et al., 2011; Jørgensen et al., 2019). Elemental sulfur and thiosulfate have also been envisaged as constituting key junctions in the network of sulfur species transformations 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 chemolithotrophs, 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 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 metabolism 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-culture of sediment-samples along two ~3-m-long cores collected from 530 and 580 meters below the sea level (mbsl), at sites having the GPS coordinates 16°50.03' N, 71°59.50' E and 16°49.88' N, 71°58.55' E respectively; sediment-samples were also analyzed for the presence of tetrathionate and thiosulfate in their pore-waters. *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).

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 investigated, two such slices or sample-replicates were collected for duplicate metagenome analyses (these were designated as sample-replicates 1 and 2, see Tables S1 and S2), a third one was taken for metatranscriptome analysis, while two more replicates were taken for culture-dependent investigations. All five replicates were collected in individual screw-capped bottles and those meant for metatranscriptomics were treated immediately with RNAlater (Ambion Inc., USA). The head-space of each sample-containing bottle was flushed with high-pure N_2, subsequent to which the bottles were sealed with Parafilm (Bemis Company Inc., Neenah, USA) and stored frozen or cool. 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 Bose Institute, and over subsequent preservation of samples, these temperatures were maintained.

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 crimp sealed 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 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 bicarbonate 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 from the absorbance of ferric thiocyanate ($\varepsilon = 5030\ M^{-1}\ cm^{-1}$, $\lambda = 460$ nm), which is formed by reacting the thiocyanate ion with ferric nitrate. Differences in the reactivity of thionates with cyanide enable their discrimination and quantitative characterization within mixtures of such compounds. For instance, trithionate is stable at high pH and reacts with cyanide only at elevated temperatures; thiosulfate reacts with cyanide at room temperature, albeit only in the presence of copper(II) catalyst; in contrast, the higher polythionates ($S_nO_6^{2-}$, where n = 4 or more) react rapidly with cyanide at room temperature to form SCN^-, $S_2O_3^{2-}$, SO_4^{2-} and HCN. Furthermore, in aqueous systems where intermediate sulfur compounds are cycling and dissolved sulfide is present, cyanide may also react with zero-valent sulfur components of the colloidal fraction of particulate elemental sulfur, polysulfides (S_n^{2-}), and their protonated forms, albeit when administered under hot and slightly acidic condition (Kamyshny, 2009), which is not the case in the cyanolytic procedure currently followed for tetrathionate estimation (Kelly and Wood, 1994). Here, 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 pre-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 on a spectrophotometer. The standard curve used for tetrathionate estimation was prepared using different concentrations (0.1, 0.5 1.0, 5.0 and 10.0

μM) of analytical grade tetrathionate (Sigma Aldrich, St. Louis, USA). Based on triplicate analyses of these standards, deviations from actual concentrations were less than 5%.

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

Total community DNA (metagenome) 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 (metatranscriptome) was extracted from an RNAlater-treated sample-replicate by 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 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 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) 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 reads from the duplicate metagenomic sequence datasets of each sediment-community were directly annotated for their taxonomic affiliation by searching the datasets individually against the NCBI non-redundant (*nr*) protein sequence database, using 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; percentage allocation of reads over various genera was taken as a direct measure of the relative abundance (prevalence) of those genera within the community (Tringe et al., 2005; Ghosh et al., 2015; Roy et al., 2020). Within MG-RAST, sequences were trimmed to contain no more than 5 successive bases with Phred quality score < 15. To classify reads using Organism Abundance tool, Best Hit Classification algorithm was followed, reporting only those BlastX search results which had minimum 45 nucleotides (15 amino acids) alignment, ≥ 60% identity, and *e*-value ≤ $1e^{-5}$; these cut-offs are stringent enough for genus-level classification of homologs of metabolically diverse genes, irrespective of their intrinsic levels of conservation.

2.8 Slurry-culture of sediment-samples

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 incubation of the samples in culture media supplemented with known concentrations of thiosulfate or tetrathionate. Within the slurry-culture set-ups, *in vitro* rates of redox transformations of sulfur involving tetrathionate were determined and expressed as μM S day^{-1} g $sediment^{-1}$. Since the culture set-ups involved different transformations between different redox species of sulfur (that have different valence states of S), every individual sulfur compound tracked within a culture was quantified in equivalence of sulfur atom concentrations (this facilitated an even comparison between the concentrations of all the substrates and product present in the system). Furthermore, in this context, it is noteworthy that the present experimental design is distinct from the typical slurry incubation experiments where no culture medium is used, instead rehydrated sediment slurries are spiked with known weights (mol) of the substrates, and transformation rates expressed in terms of mol day^{-1} g $sediment^{-1}$.

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 $K_2S_4O_6$, and 0.5 g sodium thioglycolate (used as an O_2 scavenger) and 0.1 mg resazurin (added to indicate the presence of any dissolved O_2).

During RVTr preparation, inside a Whitley H35 Hypoxystation preset to 0% partial pressure of O_2, pre-weighed amount of potassium tetrathionate salt was first dissolved in a premeasured volume of anoxic, deionized water (degassed for several hours inside the H35 Hypoxystation till the resazurine indicator added in the water became colorless). This anoxic tetrathionate solution was then added via filter-sterilization to a separate pre-autoclaved solution that contained the rest of the RVTr components in an appropriate volume and had cooled down to room temperature within the Hypoxystation. Thioglycolate that was there in the second solution had already reacted irreversibly, during autoclaving, with the dissolved O_2 present in the mixed-salts solution to form dithiodiglycolate. Post-autoclave cooling of this second solution within the Hypoxystation, therefore, did not breakdown the S-S bonds of dithiodiglycolate to regenerate the SH^--containing thioglycolate, so there was no possibility of thioglycolate attacking the incoming tetrathionate solution. Moreover, at neutral pH, thiol-group-containing reducing agents do not attack tetrathionate under non-enzymatic (abiotic) conditions (Pyne et al., 2018); and **z**ero hour reading for all the slurry-culture sets in RVTr medium showed the intact presence of the 10 mM tetrathionate originally supplied in the medium, while abiotic control incubations involving autoclaved sediment-samples showed that the 10 mM tetrathionate supplied to the RVTr medium was intact after prolonged incubation.

Concentrations of the various redox species of sulfur were estimated in the slurry-culture 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 by dividing the concentration of the substrate (in mM S) transformed eventually (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 $K_2S_4O_6$) supplemented variant of ASWM respectively. Concentrations of dissolved thiosulfate, tetrathionate and sulfate in the spent culture 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 mixture 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 of the Hypoxystation; biomasses were serially dilution streaked on to fresh plates and incubated aerobically until all colonies in the individual plates looked similar. Representative colonies from such pure-culture plates were taken 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. Concentrations of dissolved thiosulfate, tetrathionate and sulfide in the RVTr cultures were measured by the methods mentioned 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 (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*, *Guyparkeria*, *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. 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 these sediment-horizons (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*, *Guyparkeria*, *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 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-culture 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 these sediment-horizons 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 μM 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 μM 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 μM S day^{-1} g $sediment^{-1}$ (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 μM 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 μM 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 μM 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. This could be due to low number of metabolically active chemolithotrophic cells present in these samples. Notably, control incubation sets involving autoclaved sediment-samples in ASWT showed no change in the thiosulfate concentration of the medium.

Slurry-culture 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 μM S day^{-1} g $sediment^{-1}$ (Fig. 3C, Table S18). Slurry-culture of samples from the remaining five sediment-depths of SSK42/5 did not result in any oxidation of tetrathionate, plausibly due to low number of metabolically active chemolithotrophic cells present in these samples. 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 μM 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 μM 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 μM S day^{-1} g $sediment^{-1}$ (Fig. 3D, Table S19). Control incubations involving autoclaved sediment-samples in ASWTr showed no oxidation of tetrathionate.

The *in vitro* rates of tetrathionate formation, oxidation and reduction obtained by incubating the sediment-samples in specific media and culture conditions are expected to have little or no correspondence with the potential *in situ* rates of such processes. Rather, objective of these experiments was to check whether tetrathionate-metabolizing bacteria identified in this sediment system via culture-independent microbiological techniques were alive *in situ* (potential active state of the tetrathionate-metabolizers was subsequently corroborated by pure-culture isolations and metatranscriptome analysis). In the slurry-culture experiments it was peculiar to observe that the individual communities present within the sediment-depths 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 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 their natural habitat, growths and activities in the slurry-culture experiments illustrated that the 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 (taxonomically, these homologs were ascribed to species of *Gramella*, *Halothiobacillus* and *Marinobacter*; see 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 *Guyparkeria* 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-culture 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 μM S day^{-1} g $sediment^{-1}$ (Figs. 3E and 3F, Tables S21 and S22); control sets involving autoclaved sediment-samples exhibited no depletion of tetrathionate from the RVTr media. 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 *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 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

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 *Guyparkeria* 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 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 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.

3.6 Correspondence between genomic sequences of the tetrathionate-metabolizing isolates and metatranscriptomic sequences from their habitat

In order to check whether the newly isolated bacterial strains were metabolically active and growing in their natural habitat, the rRNA-sequence-free metatranscriptomic sequence dataset (i.e., all paired end mRNA reads) of the 275 cmbsf sediment-sample of SSK42/6 was mapped onto a number of tetrathionate-metabolizing and house-keeping genes of the individual strains. In this way, the metatranscriptomic sequence dataset was found to encompass reads matching the *tsdA* genes of the thiosulfate-to-tetrathionate-converting isolates *Halomonas* sp. MCC 3301 and *Pseudomonas bauzanensis* MTCC 12600, and the *soxB* and *soxC* genes of the tetrathionate-oxidizing isolates *Guyparkeria* sp. SB14A and *Pusillimonas ginsengisoli* MTCC 12558 (Table S24). In this context it is noteworthy that the annotated draft genome of the tetrathionate-forming isolate *Methylophaga* sp. MTCC 12599 did not contain any *tsdA* gene while that of the tetrathionate-reducing isolate *Enterobacter* sp. RVSM5a contained no *ttrABC* and *phsABC* genes; these could be attributable to either the incompleteness of the two genomes or the presence of novel thiosulfate-to-tetrathionate-converting and tetrathionate-reducing genes in the two bacteria respectively. On the other hand, mapping of the metatranscriptomic sequence dataset onto the individual isolates' gene-catalogs concerning core metabolisms such as (a) genetic information processing (transcription, translation, DNA replication and repair); (b) environmental information processing (ABC transporters, phosphotransferase system and bacterial secretion system) and (iii) cell growth and division (cell cycle), resulted in the concordant matching of up to 1.1 % read-pairs with the different catalogs. Table S24 shows the numbers of metatranscriptomic read-pairs that matched concordantly with some representatives of these house-keeping genes of the six isolates.

3.7 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

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 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 S25) 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 S25). These thiosulfate concentration values are consistent with those reported from a number of physicochemically similar sediment-horizons across the global ocean (Troelsen and Jorgensen, 1982; Zopfi et al., 2004). 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 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 [1 µM for the methods used in this as well as previous studies (Podgorsek and Imhoff, 1999)] could be 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 (Roy and Trudinger, 1970; Podgorsek and Imhoff, 1999; Schippers et al., 1999; Schippers and Jørgensen, 2001; Zopfi et al., 2004). From that perspective, the geomicrobiological information unearthed in this study illustrates the power of metaomics in discovering such cryptic interfaces between the chemosphere and the biosphere that are almost impossible to decipher from geochemical records alone.

4 Discussions

4.1 Biogeochemical perspectives of tetrathionate metabolism in ASOMZ sediments

Concentrations and isotopic ratios of the various chemical constituents of sedimentary solid-phases and pore-fluids had long remained central to the deciphering of *in situ* biogeochemical pathways. Significant information on the carbon-sulfur cycles of modern as well as ancient, marine and lacustrine sediments have been generated in this way. Currently, however, there is an increasing consensus that several questions in biogeochemistry - such as those concerning sulfur compounds oxidation/disproportionation, relative importance of simple fatty acids

catabolism and anaerobic methane oxidation in sedimentary sulfate reduction, and biogeochemical processes within sulfate-methane transition zones - cannot be answered from preserved geochemical records alone. In recent times a lot of advancement has taken place in our overall understanding of carbon-sulfur cycling in marine systems by virtue of data obtained from metagenomic, metatranscriptomic, and *in situ* as well as *in vitro* geomicrobiological experiments. Forensic-level detection power of these approaches in unearthing such cryptic biogeochemical processes that do not get manifested, or leave their imprints, as detectable geological records, have been demonstrated in a number of recent papers (e.g., Canfield et al., 2010; Garcia-Robledo et al., 2017; Bhattacharya et al., 2019), which revealed such microbial community functions using metaomics approaches that were almost impossible to detect via geochemical analyses. So far as the present study is concerned, metagenome analysis along SSK42/5 and SSK42/6 revealed tetrathionate-metabolizing potentials in bacterial communities present at different depths of the sediment-horizons where tetrathionate is not detectable easily. At the same time, metatranscriptome analysis for the deepest sediment-sample of SSK42/6 indicated that these tetrathionate-metabolizing communities are potentially active *in situ.*

Regarding the biogeochemical feasibility of the three tetrathionate-metabolizing processes in the sulfidic sediments of SSK42/5 and SSK42/6 (Fernandes et al., 2018) 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 are alive and active in these apparently O_2-depleted 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). These data, together with the isolation of obligately aerobic strains from this sedimentary ecosystem (Bhattacharya et al., 2019), indicated that O_2 source(s) supportive of aerobic metabolic processes are likely to be present *in situ*.

The nature and origin of the oxidants necessary to comprehensively realize the thiosulfate to tetrathionate, and tetrathionate to sulfate, oxidation potentials of the ASOMZ sediment microflora remain unresolved. Whilst there aren't many biochemical options for *in situ* O_2 production, nor there are high chances of O_2 influx to the system (Breuer et al. 2009; Cavan et al. 2017; Jessen et al. 2017), a plausible scenario in the form of cryptic aerobiosis by perchlorate-respiring microorganisms have been envisaged by Bhattacharya et al. (2019). In that study it has been postulated based on metagenomic, metatranscriptomic and co-culture-based data that perchlorate-respirers could be sustaining aerobic life in these sulfidic sediment-systems via cryptic O_2 supply-consumption partnership similar to the one reported for picocyanobacteria and nitrite-oxidizers in the acutely hypoxic waters of eastern tropical North and South Pacific (Garcia-Robledo et al. 2017). This said, in the absence of measured data for any potential oxidant, including O_2 or other oxyanions having possibilities of serving as electron acceptors, only future investigations of biogeochemistry focused on possible pathways of cryptic aerobiosis can resolve the modalities of sulfur compounds oxidation in these acutely O_2-scarce environments.

4.2 Trends of thiosulfate/sulfide concentration and prevalence of tetrathionate-metabolizing bacteria, along the sediment-cores, implicate tetrathionate as a key intermediate of the sulfur cycle

Along both the sediment-cores, SSK42/5 and SSK42/6, relative abundance of tetrathionate-forming, oxidizing, as well as reducing bacteria increased with depth (Figs. 1A and 1B). This could be attributable to the corresponding overall increase in the concentration of thiosulfate (Figs. 3G and 3H), which plausibly is the key source of biogenic tetrathionate in the sediment. Sulfide concentration also increased with depth, consistent with the increase of thiosulfate concentration (Figs. 3G and 3H). Sulfide is not only a potential direct source of thiosulfate in marine sediments (Jørgensen, 1990) but can also be a product of microbial tetrathionate reduction (Barrett and Clark, 1987; Price-Carter et al., 2001). Furthermore, sulfide, when present in sediment-cores, can chemically (abiotically) reduce tetrathionate to thiosulfate and elemental sulfur (Roy and Trudinger, 1970; Rowe et al., 2015). In view of these dependencies, depth-trends of sulfide concentration are expected to show a certain degree of correlation with trends of thiosulfate concentration.

In both the sediment-cores, relative abundance of tetrathionate-oxidizing bacteria increased with depth even as sulfate concentration decreased along the same trajectory (Figs. 1A, 1B, 3G and 3H). This indicated that the amounts of sulfate produced from potential tetrathionate oxidation at individual sediment-depths were far less than the amounts of sulfate that were reduced to sulfide *in situ*. 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-culture experiments actually resulted from the activities of the few chemolithotrophic species/strains 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, rate of *in vitro* tetrathionate reduction along both the sediment-cores exhibited overall parity with the trends of fluctuation observed for the relative abundance of tetrathionate-reducing bacteria (both parameters showing overall increase with sediment-depth; Figs. 1A, 1B, 3E and 3F). This suggests that tetrathionate reduction rates observed in the slurry-cultures of the sediment-samples were attributable to all the tetrathionate-reducing species/strains present *in situ*, which in turn indicates a general ability of tetrathionate-reducers to grow in RVTr medium.

4.3 Potential abiotic drivers of tetrathionate formation in ASOMZ sediments

A number of other studies, based on geochemical experiments and preserved records, have also revealed the occurrence, and complex transformations, of tetrathionate and other intermediate sulfur species in ecologically diverse (but mostly non-sulfidic) environments, including marine sediments. Bak et al. (1993) measured tetrathionate, trithionate and thiosulfate in diverse natural samples, while Zopfi et al. (2004) used different techniques of analytical geochemistry to track the abiotic as well as biotic (microbe-mediated) transformations of sulfur cycle intermediates, including tetrathionate, in the sediments of Black Sea and North Sea. Findlay and Kamyshny (2017) envisaged the potential fates and transformation rates of intermediate sulfur species in lacustrine water-columns and sediments by introducing and tracking ^{35}S-labeled sulfur compounds in the samples. Furthermore, both Zopfi et al. (2004) and Findlay and Kamyshny (2017), in concurrence with previous reports (Schippers and Jørgensen, 2001), envisaged the *in situ* oxidation of pyrite (FeS_2) by MnO_2 as an abiotic source of tetrathionate in the sediments. Such microbes-independent processes are also not improbable in the marine sediments explored in this study as (i) Fe (9232-17234 ppm), Mn (71-172 ppm) and pyrite (0.05-1.09 wt %) were all detected in the solid phase of SSK42/5 and SSK42/6 samples; (ii) genes for Mn(II) to Mn(IV) oxidation were identified in the assembled metagenomes of all the sediment-samples explored; (iii) sequences corresponding to manganese oxidase (*cotA*) and other accessory proteins involved in Mn(II) to Mn(IV) oxidation were there in the assembled metatranscriptome analyzed; and (iv) high percentage of reads ascribed to MnO-depositing bacteria such as *Aeromonas*, *Citrobacter*, *Enterobacter Gallionella*, *Hyphomicrobium Leptothrix* and *Proteus* (that reduce Mn^{+4} to Mn^{+2} for anaerobic respiration; Ghiorse, 1984), and MnO-to-MnO_2-converting bacteria such as *Arthrobacter*, *Oceanospirillum* and *Vibrio* (Tebo et al., 2005; Sujith and Bharathi, 2011), were

detected in all the metagenomes sequenced. Notably, however, manganese concentrations detected in the sediment-samples of SSK42/5 and SSK42/6 are orders of magnitude lower than the threshold [> 0.2% (w/w)] reported previously for FeS_2 dissolution (Schippers and Jørgensen, 2001), so to confirm MnO_2-FeS_2 interaction as a potent source of tetrathionate in these sediment-horizons, future studies of geochemistry yielding fine-resolution depth-trends for pyrite and MnO_2 contents of the sediments are necessary alongside comprehensive data for pore-water metal ions concentrations and pyrite-specific (tracer) slurry incubations.

4.4 Biogeochemical underpinnings of the cryptic status of tetrathionate in the sulfur cycle

Previous investigations based on the top few centimeters of Baltic Sea sediments had reported microbial production of tetrathionate and highlighted its role in the sulfur cycle (Podgorsek and Imhoff, 1999). Similar to the present scenario, in that study of sediments collected from coastal locations off Hiddensee (Germany), thiosulfate and tetrathionate were undetectable in the 7-8 mM sulfate- and 4-20 µM sulfide-containing native pore-water samples tested along a 6 cmbsf sediment-profile [detection limits for thiosulfate and tetrathionate, in the methods employed by Podgorsek and Imhoff (1999), were 0.5 and 1.0 µM, respectively]. But when an approximately 15 m^2 area of the same Baltic Sea location was covered with a plastic sheet for 5 months to artificially construct a stable anoxic condition, up to 5 µM tetrathionate was found to accumulate within the 6 cmbsf sediment-profile explored, concomitant with the build-up of 80-280 µM thiosulfate and 320-1200 µM sulfide, and depletion of sulfate to a concentration of 0.4 mM within the first 4 cmbsf. In the above findings of Podgorsek and Imhoff (1999) it is peculiar that tetrathionate was absent in the native Baltic Sea (off Hiddensee coast) sediment-samples containing 4-20 µM sulfide, but accumulated to a concentration of 5 µM under induced anoxia involving 320-1200 µM sulfide. Concurrently, in the other Baltic Sea study-site (Gotland Basin) explored by Podgorsek and Imhoff (1999), tetrathionate accumulation of up to 5 nmol cm^{-3} sediment was recorded, during a period of annual anoxia, concomitant with increased concentrations of sulfide, elemental sulfur and thiosulfate. To explain these data Podgorsek and Imhoff (1999) invoked the cyclic sequence of reactions that was proposed and demonstrated previously (Roy and Trudinger, 1970; Hansen, 1974; Sorokin et al., 1996) and which involved bacterial oxidation of thiosulfate to tetrathionate on one hand and chemical reaction between sulfide and tetrathionate, forming elemental sulfur and thiosulfate, on the other. While it was added that accumulation of tetrathionate to high concentrations is debarred in euxinic marine environments owing to its high chemical reactivity with sulfide, and that even low concentrations of thiosulfate and tetrathionate, acting as a catalytic couple, were sufficient to promote large-scale net oxidation of sulfide to elemental sulfur (Podgorsek and Imhoff, 1999), questions remained as to how tetrathionate accumulation in the Baltic Sea sediments occurred only amidst

heightened sulfide build-up. In the context of this apparent biogeochemical paradox, findings of the current study illuminate the centrality of microbe-mediated oxidation/reduction mechanisms (over and above the abiotic role of sulfide) in keeping the tetrathionate pool cryptic (below easily-detectable quantities) within the sediment systems.

4.5 Conclusion

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 and nitrogen cycles to remineralize organic matter sequestered in the sea-bed, and also influence metal deposition/mobilization. Of the various redox species of sulfur, tetrathionate is seldom considered to be an important intermediate of sulfur cycling in marine sediments. The present study of the eastern Arabian Sea sediment system used microbiological data to reveal tetrathionate as a key intermediate of the sulfur cycle and identify the potential biochemical pathways involved its formation and transformation *in situ*. Albeit the microbiological drivers and indicators underlying the potential status of tetrathionate as a key junction of sedimentary sulfur cycle were revealed here in the context of an oxygen minimum zone, there was no apparent reason to presume that such processes do not have their equivalents in other geomicrobiologically-distinct sediment-horizons of the marine realm. The absence of measured data for key *in situ* geochemical parameters disallowed any direct inference regarding the abiotic mechanisms potentially involved in the formation and transformation of tetrathionate in this sediment-system. However, a number of such microbiological markers were identified that could be indicative of pyrite (via abiotic reaction with MnO_2) being a source of tetrathionate in the system. Further appreciation of the scope and significance of this molecular and classical microbiology-based forecast remains subject to further biogeochemical substantiation, which among other things should explore the real time presence of tetrathionate in the system. Consistent with previous reports form Baltic Sea sediments, tetrathionate was also not detectable in the sulfidic sediment-system of the Arabian Sea explored in the current study. In the context of this cryptic nature of tetrathionate in the sulfur cycle, our data revealed the key role of microbial redox metabolisms in preempting the accumulation of this highly-reactive polythionate in sediment pore-fluids, over and above the known abiotic mechanisms of tetrathionate scavenging by *in situ* sulfide. However, in the absence of any measured data for O_2 or other oxyanions having possibilities of serving as electron acceptors, the biogeochemical modalities of the oxidative half of the *in situ* tetrathionate cycle remain subject to future investigations of biogeochemistry focused on possible pathways of cryptic aerobiosis in these acutely O_2-scarce environments.

Supplementary material

Supplemental materials for this article may be found with the digital version of this manuscript. These include an MS Word file named “1_Supplementary Information” and an MS Excel file named “2_Supplementary Dataset”.

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. S.M. anchored the whole microbiological work, performed the experiments, analyzed the data and contributed substantially 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.

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Legend of figures

Figure 1. Relative abundance of the tetrathionate-metabolizing bacterial groups plotted pair-wise against each other or against sediment-depth, along (A) SSK42/5 and (B) SSK42/6. Relative abundances are expressed as the percentages of metagenomic reads matching protein-coding genomic sequences from the genera of tetrathionate-forming (*Pseudomonas* and *Halomonas*), tetrathionate-oxidizing (*Acidithiobacillus*, *Guyparkeria*, *Halothiobacillus* and *Thiomicrospira*) and tetrathionate-reducing (*Citrobacter*, *Proteus* and *Salmonella*) 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 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 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 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 *Guyparkeria* sp. SB14A. (**H**) reduction of tetrathionate to thiosulfate by *Enterobacter* sp. RVSM5a. **—●—, —▲—** and **—▼—** indicate the concentrations of sulfur (mM S) present in the medium at a given time-point of incubation in the form of thiosulfate, sulfate and tetrathionate respectively. **—■—** denotes the pH of a culture at a given time-point of incubation.

Figure 3. Rates of transformation of thiosulfate and/or tetrathionate in slurry-cultures of the sediment-samples, and concentrations of different sulfur species recorded along the two sediment-cores. (**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 respectively. (**E** and **F**) *in vitro* rates of tetrathionate reduction in RVTr medium, for the different sedimentary communities of SSK42/5 and SSK42/6 respectively. (**G** and **H**) concentrations of sulfur species measured along SSK42/5 and SSK42/6 respectively. 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 genomic sequences of the six tetrathionate-metabolizing bacterial isolates: (*Ent*) *Enterobacter* sp. RVSM5a, (*Gpk*) *Guyparkeria* sp. SB14A, (*Hlm*) *Halomonas* sp. MCC 3301, (*Mtp*) *Methylophaga* sp. MTCC 12599, (*Psb*) *Pseudomonas bauzanensis* MTCC 12600, and (*Psg*) *Pusillimonas ginsengisoli* MTCC12558. Scales for sediment-depth (plotted in y-axis) and percentage of metagenomic reads from a given depth matching genomic sequences of an isolate (represented by bubble size) are both linear; the individual isolates are placed along the x-axis.

Figure 5. Bubble plot showing the percentages of metagenomic reads from individual sediment-samples of SSK42/6 that matched genomic sequences of the six tetrathionate-metabolizing bacterial isolates: (*Ent*) *Enterobacter* sp. RVSM5a, (*Gpk*) *Guyparkeria* sp. SB14A, (*Hlm*) *Halomonas* sp. MCC 3301, (*Mtp*) *Methylophaga* sp. MTCC 12599, (*Psb*) *Pseudomonas bauzanensis* MTCC 12600, and (*Psg*) *Pusillimonas ginsengisoli* MTCC12558. Scales for sediment-depth (plotted in y-axis) and percentage of metagenomic reads from a given depth matching genomic sequences of an isolate (represented by bubble size) are both linear; the individual isolates are placed along the x-axis.

Figure 6. Schematic diagram showing the network of biotic and abiotic process that are apparently involved in the formation and transformation of tetrathionate in the Arabian Sea sediments explored in this study.

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	*Guyparkeria* 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

Figures

Figure 1.

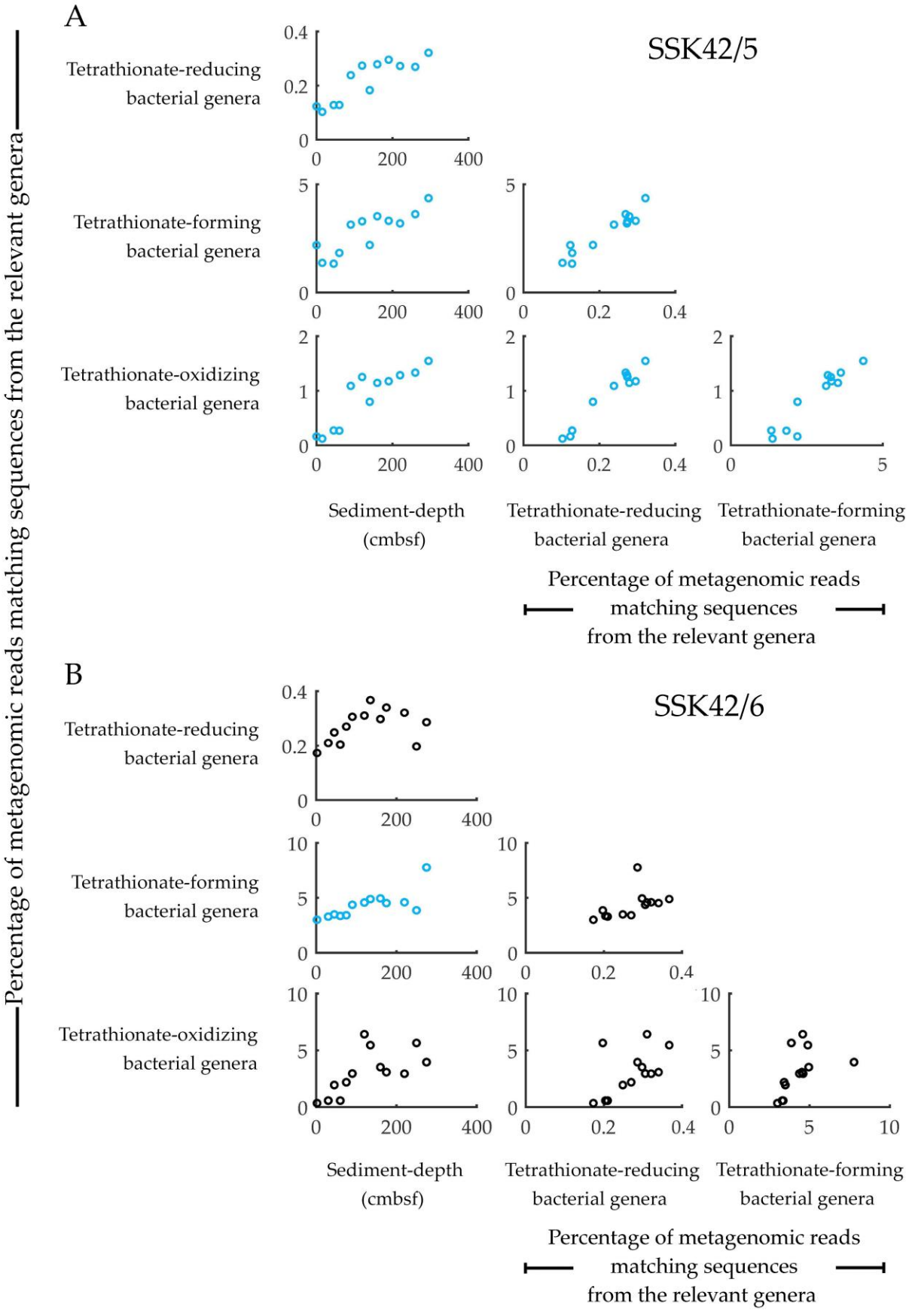
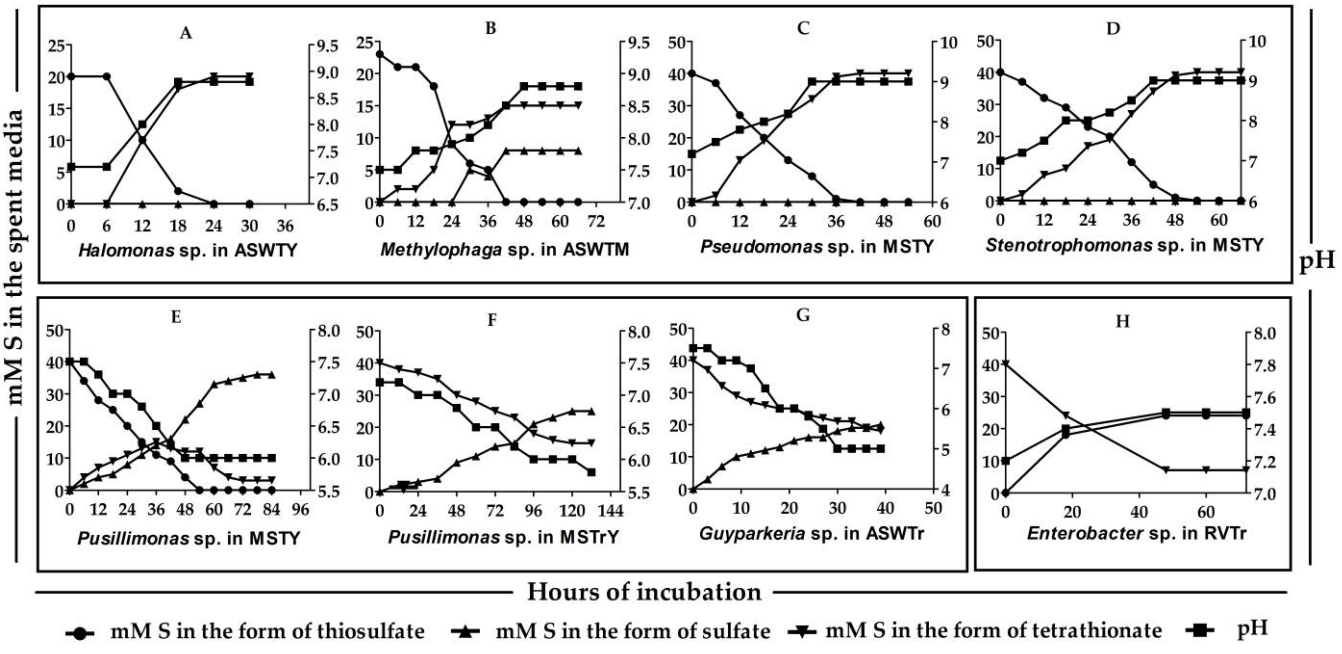


Figure 2.



1300

Figure 3.

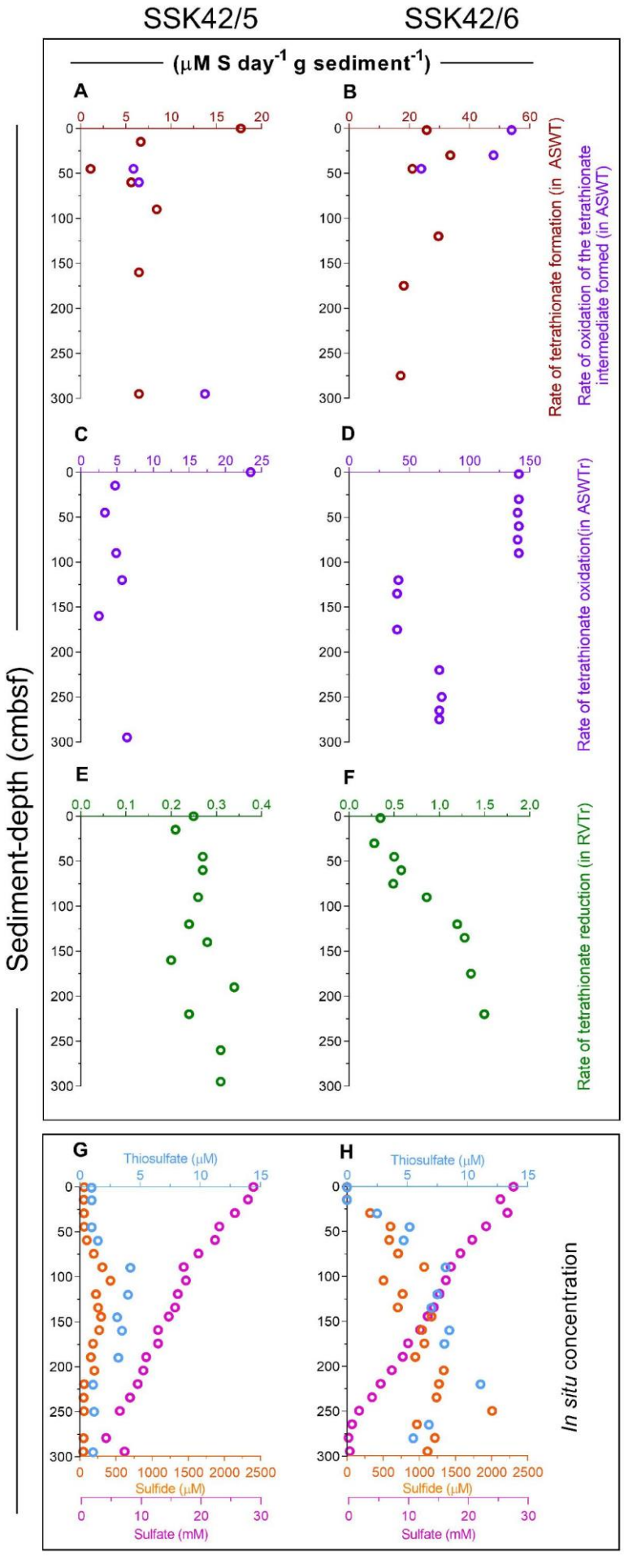
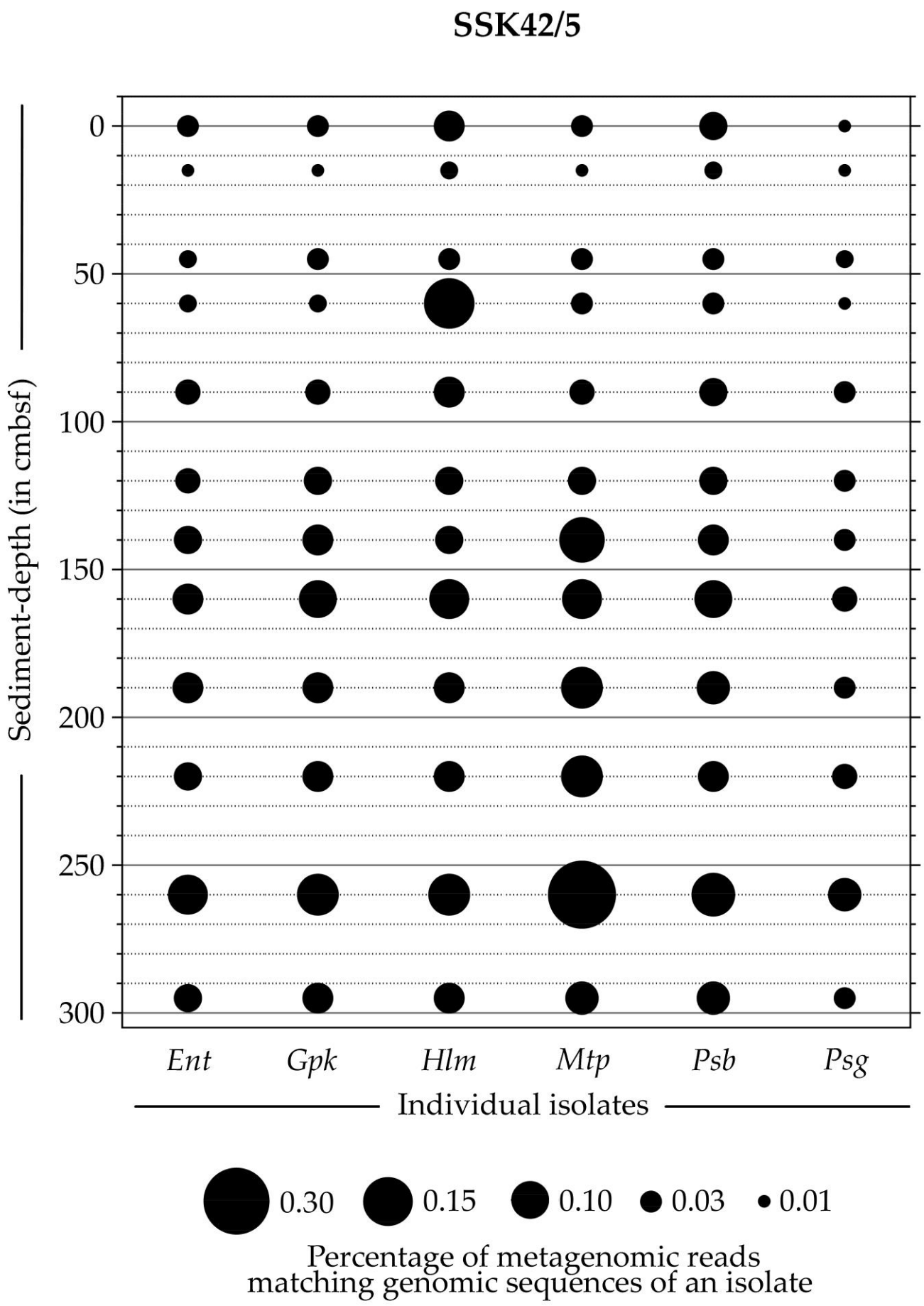

SSK42/5
SSK42/6
(μM S day-1 g sediment-1)
A
B
C
D
E
F
G
H
Sediment-depth (cmbsf)
Rate of tetrathionate formation (in ASWT)
Rate of oxidation of the tetrathionate intermediate formed (in ASWT)
Rate of tetrathionate oxidation(in ASWTr)
Rate of tetrathionate reduction (in RVTr)
Thiosulfate (μM)
Sulfide (μM)
Sulfate (mM)
In situ concentration

Figure 4.



1305

Figure 5.

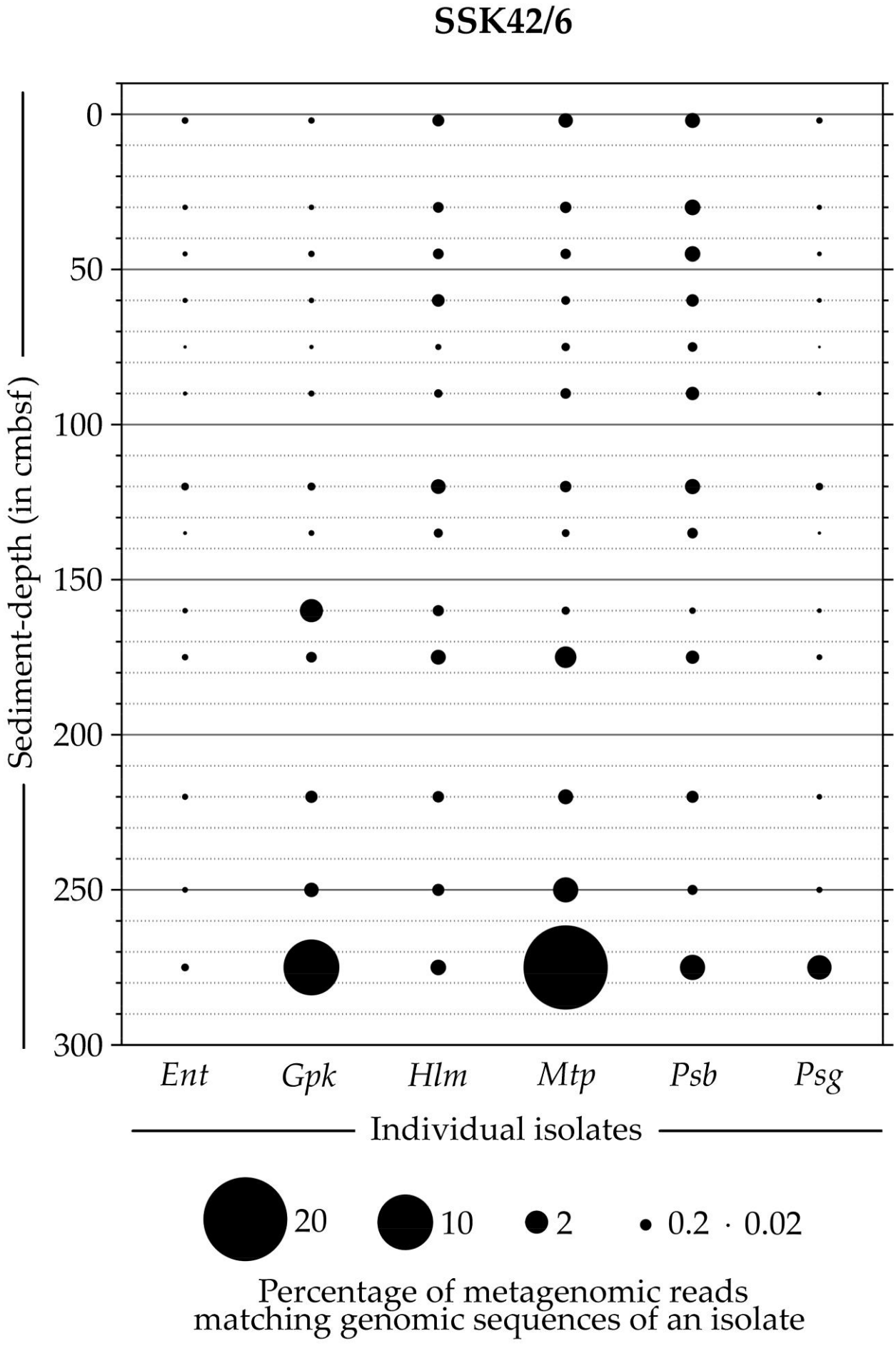
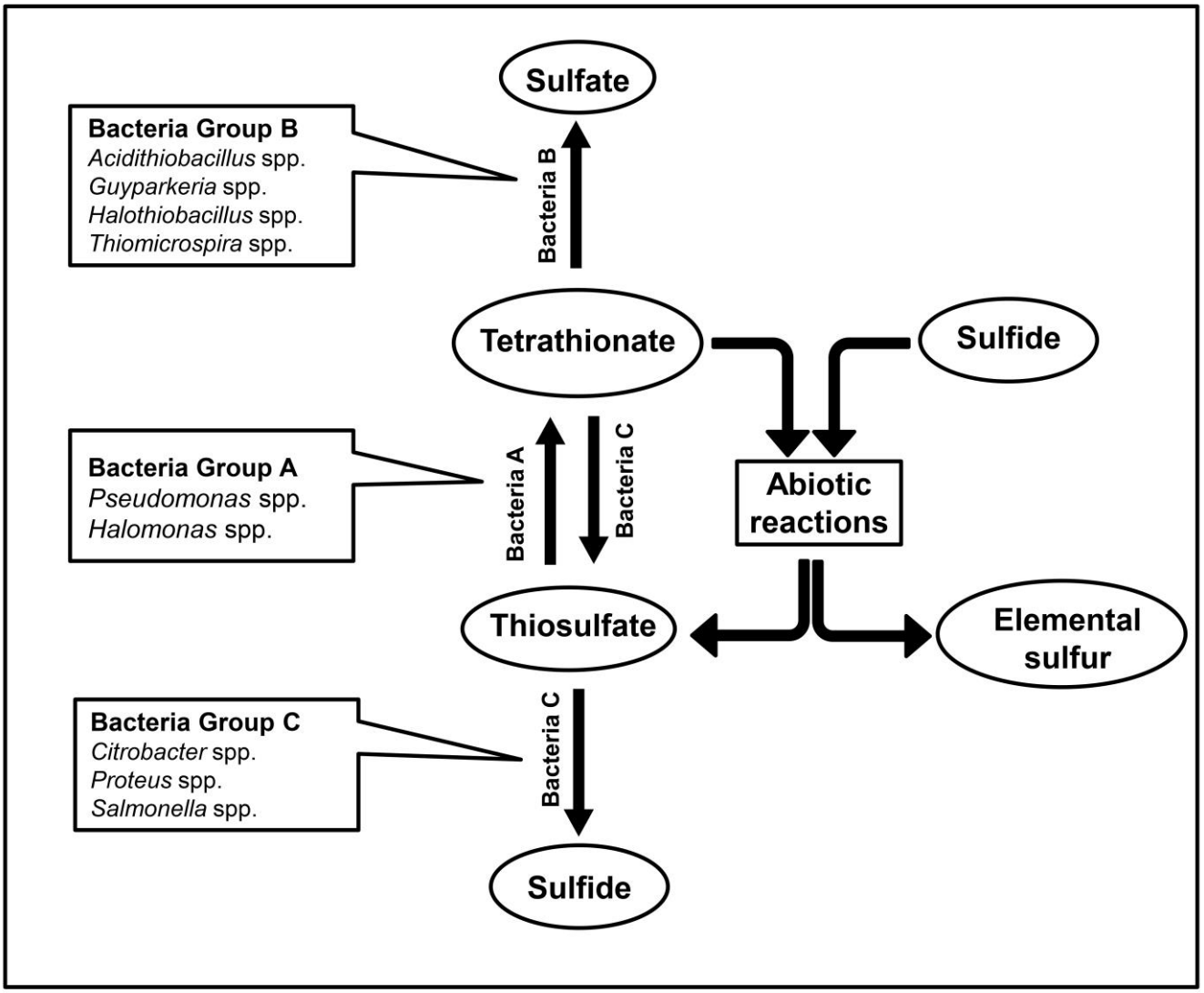

SSK42/6
Sediment-depth (in cmbsf)
0
50
100
150
200
250
300
Ent
Gpk
Hlm
Mtp
Psb
Psg
Individual isolates
20
10
2
0.2 · 0.02
Percentage of metagenomic reads
matching genomic sequences of an isolate

Figure 6.


Sulfate
Bacteria Group B
Acidithiobacillus spp.
Guyparkeria spp.
Halothiobacillus spp.
Thiomicrospira spp.
Bacteria B
Tetrathionate
Sulfide
Bacteria Group A
Pseudomonas spp.
Halomonas spp.
Bacteria A
Bacteria C
Abiotic reactions
Thiosulfate
Elemental sulfur
Bacteria Group C
Citrobacter spp.
Proteus spp.
Salmonella spp.
Bacteria C
Sulfide