Microbial diversity and sulfur cycling in an early earth analogue: From ancient novelty to modern commonality

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Abstract

Life emerged and diversified in the absence of molecular oxygen. The prevailing anoxia and unique sulfur chemistry in the Paleo-, Meso- and Neoarchean, and early Proterozoic eons may have supported microbial communities that are drastically different than those currently thriving on the earth's surface. Zodletone spring in southwestern Oklahoma represents a unique habitat where spatial sampling could substitute for geological eons: from the anoxic, surficial light exposed sediments simulating a preoxygenated earth, to overlaid water column where air exposure simulates the relentless oxygen intrusion during the Neo Proterozoic. We document a remarkably diverse microbial community in the anoxic spring sediments, with 340/516 (65.89%) of genomes recovered in a metagenomic survey belonging to 200 bacterial and archaeal families that were either previously undescribed or that exhibit an extremely rare distribution on the current earth. Such diversity is underpinned by the widespread occurrence of sulfite-, thiosulfate, tetrathionate-, and sulfur-reduction, and paucity of sulfate-reduction machineries in these taxa; hence greatly expanding lineages mediating reductive sulfur cycling processes in the tree of life. Analysis of the overlaying water community demonstrated that oxygen intrusion lead to the development of a significantly less diverse community dominated by well-characterized lineages and a prevalence of oxidative sulfur cycling processes. Such transition from ancient novelty to modern commonality underscores the profound impact of the great oxygenation event on the earth's surficial anoxic community. It also suggests that novel and rare lineages encountered in current anaerobic habitats could represent taxa once thriving in an anoxic earth, but have failed to adapt to earth's progressive oxygenation.

43 **Introduction**

44 Sulfur is one of the most abundant elements on earth, exhibiting a wide range of oxidation states
45 (-2 to +6). Microorganisms have evolved a plethora of genes and pathways for exploiting sulfur-
46 redox reactions for energy generation. Reductive processes employ sulfur oxyanions or
47 elemental sulfur as terminal electron acceptors in anaerobic respiratory schemes linked to
48 heterotrophic or autotrophic growth. Oxidative processes, on the other hand, employ sulfides or
49 elemental sulfur as electron donors, powering chemolithotrophic and photosynthetic growth.

50 Thermodynamic considerations limit reductive sulfur processes to habitats where oxygen
51 is limited. This is reflected in the global distribution of sulfate (SO_4^{2-}), sulfite (SO_3^{2-}), thiosulfate
52 ($S_2O_3^{2-}$), tetrathionate ($S_4O_6^{2-}$), and elemental sulfur (S^0) reducing microorganisms (henceforth
53 collectively referred to as SRM) in permanently and seasonally anoxic and hypoxic habitats in
54 marine [1, 2, 3], freshwater [4], terrestrial [5], and subsurface [6] ecosystems. Sulfate is highly abundant on
55 the current earth, and hence sulfate-reduction dominates reductive processes in the global sulfur
56 cycle; although reduction and disproportionation of the intermediate sulfur species, e.g. sulfur [7,
57 8], sulfite [8], thiosulfate and tetrathionate [9, 10] could be significant in localized settings.

58 The history of earth's sulfur cycle is a prime example of a geological-biological feedback
59 loop, where the evolution of biological processes is driven by, and dramatically impacts, the
60 earth's biogeochemistry. The earth's surface was completely anoxic during the first two billion
61 years of its history, and the availability and speciation of various sulfur species greatly differed
62 from its current values. Sulfate levels were significantly lower when compared to current values
63 in oceanic water (28 mM), with estimates of <200 μM-1mM from the Archean up to the
64 Paleoproterozoic (2.3 Gya) [11, 12, 13, 14]. On the other hand, intermediate sulfur species appear to
65 have played an important role in shaping the ancient sulfur cycle [15]. Modeling suggests that mM

levels of SO_3^{2-} were attained in the Archaean anoxic shallow surficial aquifers as a result of dissolution of the volcanic SO_2 prevailing in aquatic habitats [12]. Isotopic studies have demonstrated the importance of elemental sulfur, sulfite, and thiosulfate reduction in the Archean [15, 16].

The evolution of life (3.8 to 4.0 Gy) in the early Archean era and the subsequent evolution of major bacterial and archaeal clades in the Archean and early Proterozoic eons [17] occurred within this background of anoxia and characteristic sulfur-chemistry. As such, it has been speculated that organisms using intermediate forms of sulfur were likely more common than sulfate-reducing organisms [15]. However, while isotopic fractionation, modeling, and microscopic studies could provide clues on prevailing sulfur speciation patterns and prevalent biological processes, the identity of microorganisms mediating such processes is unknown. This is mostly due to constrains on preservation of nucleic acids and other biological macromolecules, with the oldest successful DNA sequenced sample being only 1.2 M years old [18]. Investigation of the microbial community in modern ecosystems with conditions resembling those prevailing in the ancient earth could provide important clues to the nature, identity, and evolutionary trajectory of microorganisms that thrived under conditions prevailing prior to earth's oxygenation and the associated changes in the sulfur cycle. In Zodletone spring, a surficial anoxic spring in southwestern Oklahoma, the prevailing conditions are analogous to those predominant on the earth's surface in the late Archean/early Proterozoic eons At the source of the spring, anoxic, surficial, light-exposed conditions are maintained in the sediments by constant emergence of sulfide-saturated water at the spring source from anoxic underground water formations in the Anadarko basin, along with gaseous hydrocarbons, which occurs in seeps in the general vicinity. These surficial anoxic conditions also support a sulfur chemistry

89 characterized by high levels of sulfide, sulfite, sulfur (soluble polysulfide), thiosulfate, and a low
90 level of sulfate. Further, the sediments at the source of the spring are overlaid by an air-exposed
91 water column, where oxygen intrusion leads to a vertical oxygen gradient (from oxic in the top 1
92 μm, to hypoxic in the middle, to anoxic in deeper layers overlaying the sediments) that simulates
93 oxygen intrusion into such habitats during the great oxygenation event (Figure S1). As such the
94 spring represents a readily accessible habitat where spatial gradients remarkably correspond to
95 temporal geological transitions. Here, we combined metagenomic, metatranscriptomic, and
96 amplicon-based approaches to characterize the microbial community in Zodletone spring. Our
97 results provide a glimpse of the community mediating the ancient sulfur cycle, significantly
98 expand the overall microbial diversity by the description of a wide range of novel lineages, and
99 greatly increase the number of lineages documented to mediate reductive sulfur processes in the
100 microbial tree of life.
101

102 **Results**

103 **Novel phylogenetic diversity in Zodletone sediments.** Metagenomic sequencing of the spring
104 sediments yielded 281 Gbp, 79.54% of which assembled into 12 Gbp contigs, with 6.8 Gbp
105 contigs longer than 1Kbp. 1,848 genomes were binned, 683 of which passing quality control
106 criteria, and 516 remaining after dereplication (Table S1). These MAGs represented 64 phyla or
107 candidate phyla (53 bacterial and 11 archaeal), 127 classes, 198 orders, and 300 families (Figure
108 1a-b). Diversity assessment utilizing small subunit ribosomal protein S3 from assembled contigs
109 (n=2079), as well as a complementary 16S rRNA illumina sequencing effort (n=309,074
110 amplicons), identified a higher number of taxa (82 phyla and 1679 species in the ribosomal
111 protein S3 dataset, and 69 phyla and 1050 species in 16S rRNA dataset) (Figure S2).
112 Nevertheless, the overall community composition profiles generated from all three approaches
113 were broadly similar (Figure S2), suggesting that the MAG list broadly reflects the sediment
114 microbial community.

115 Assessment of novelty and degree of uniqueness of sediment MAGs identified a
116 remarkably high number of previously undescribed lineages (1 phylum, 14 classes, 43 orders,
117 and 97 families), as well as Lineages exhibiting Rare global Distribution patterns compared to
118 other present time earth environments (defined as lineages represented by 5 genomes or less in
119 GTDB r95, henceforth LRD) (11 phyla, 24 classes, 45 orders, and 113 families) in the spring.
120 (Figure 1, 2a, b). At the family level, 132 (25.58%), and 208 (40.03%) genomes clustered into 97
121 novel and 113 LRD families respectively, bringing the proportion of genomes belonging to novel
122 or LRD families in Zodletone sediments to 65.89%. The high level of novelty in the sediment
123 MAGs is reflected in an average RED value of 0.76, a value that is slightly lower than the
124 median RED value for designation of a novel family (0.77) [19].

125 The Chloroflexota (n=69), Planctomycetota (n=47), Bacteroidota (n= 43),
126 Desulfobacterota (n= 43), Spirochaetota (n= 28 genomes), Patescibacteria (n=20 genomes), and
127 the archaeal phylum Nanoarchaeota (n=21) were the most abundant phyla in Zodletone spring
128 sediments, albeit representing only 52.52% of the total number of recovered genomes (S. Text,
129 Figure 1, 2C). An extreme paucity of genomes belonging to the Proteobacteria (6 genomes) and
130 Firmicutes (12 genomes), widely distributed and abundant taxa in current biomes[20], [20], and the
131 absence of oxygen-generating Cyanobacteria (0 genomes) were observed (Figure 1, 2C). Within
132 the Chloroflexota, 38/69 genomes belonged to 3 previously undescribed orders, 5 previously
133 undescribed families, and multiple LRD orders and families (Figure 3a). Within the
134 Planctomycetota, 17/47 genomes belonged to 2 previously undescribed orders, 8 previously
135 undescribed families, and multiple LRD orders and families (Figure 3b). Within the
136 Bacteroidota, 27/43 genomes belonged to 1 previously undescribed family, and multiple LRD
137 families (Figure 3c). Within the Spirochaetota, 19/28 genomes belonged to one previously
138 undescribed class, 2 previously undescribed orders, and 9 previously undescribed families, as
139 well as multiple LRD families (Figure 3d). Within the Desulfobacterota (Figure 3e), 35/43
140 genomes belonged to 3 previously undescribed classes, 10 previously undescribed orders, and 7
141 previously undescribed families, as well as multiple LRD families. Finally, an extremely diverse
142 community of Patescibacteria (13 different orders, 3 of which belonging to LRD orders, and 14
143 different families, including 6 previously undescribed and 2 LRD families), and Nanoarchaeota
144 (2 orders and 15 families, including 5 previously undescribed and 10 LRD families) were
145 identified in the spring sediments. A similar pattern of high proportion of previously undescribed
146 and LRD families was identified throughout all other lineages (S. Text, Figure 1a). Therefore, in
147 addition to expanding the number of novel lineages (classes, orders, and families), and greatly

148 enriching available genomes in rare, poorly represented taxa, our results highlight the uniqueness
149 and distinction of the microbial community thriving in Zodletone spring sediments, compared to
150 present earth environments studied so far.

151 **Oxygen intrusion reduces the proportion of novel and rare lineages in Zodletone spring.**
152 Metagenomic sequencing of the oxygen-exposed overlaying water column community yielded
153 323 Gbp, 80.07% of which assembled into 3.6 Gbp contigs with 3.1 Gbp contigs >1K. 883
154 genomes were binned, with only 114 remaining after dereplication. Of these, 62 belonged to
155 shared families with the sediment community, and 52 were water specific. Genomes recovered
156 from the water column belonged to a significantly lower number of phyla (n= 27), classes
157 (n=37), orders (n=52), and families (n=79) when compared to the euxinic sediments (Table S1).
158 The community exhibited a much lower level of novelty and rarity at the phylum, class, order,
159 and family levels when compared to the sediment community respectively (Figure 2 a, b). Water-
160 specific genomes (n=52) mostly belonged to well-characterized microbial lineages e.g. families
161 Rhodobacteraceae, and Rhodospirillaceae in the Alphaproteobacteria, families
162 Thiomicrospiraceae, Halothiobacillaceae, Acidithiobacillaceae, Burkholderiaceae,
163 Chromatiaceae, Methylothermaceae in the Gammaproteobacteria, families Sulfurimonadaceae,
164 Sulfurovaceae in phylum Camplylobacterota, and well described families in the phyla
165 Bacteroidota, Desulfobacterota (S. Text, Figure 1, Table S1). Collectively, this demonstrates a
166 pattern where the intrusion of oxygen is associated with a negative impact on previously
167 undescribed and LRD lineages that are prevalent in the sediment, and triggered the propagation
168 of proliferation of cosmopolitan communities within the bacterial tree of life.

169 ***Reductive sulfur processes dominate Zodletone spring sediment communities.*** A total of 149
170 genomes (28.9 % of all genomes), belonging to 32 phyla, 51 classes, 69 orders, and 97 families

171 were involved in at least one reductive sulfur processes (Figure 4, Table S2). By comparison,
172 only 21 sediment genomes (4.06% of all genomes) encoded at least one sulfur oxidation pathway
173 (Figure 4, Table S2). The reductive sulfur-community in the spring exhibited two unique traits:
174 First, a majority of genomes encoding such capacities belonged to novel (47 genomes) or LRD
175 (66 genomes) lineages (Figure 4), and second: sulfite-, polysulfide-, thiosulfate-, and
176 tetrathionate reduction appears to be more prevalent than sulfate-reduction capacities in the
177 sediment genomes.

178 Sulfate-reduction capacity was observed in only 18 sediment genomes (Figure 4, 5a), but
179 exhibited a unique community composition, when compared to well-studied marine and
180 terrestrial habitats [1, 2, 4, 21]. Sulfate-reduction capacities were observed in mostly previously
181 undescribed or LRD lineages within the Zixibacteria, Acidobacteriota (members of family
182 UBA6911, equivalent to Acidobacteria group 18), Myxococcota, Bacteroidota, Planctomycetota,
183 candidate phylum OLB16 (1 genome), as well as rare and novel lineages within the
184 Desulfobacterota (Figure 4, S3a). Organization of the sulfate reduction genes differed between
185 different phyla, with Myxococcota, Zixibacteria, OLB16, and Acidobacteriota genomes
186 encoding all genes for sulfate activation and reduction, dissimilatory sulfite reduction, as well as
187 energy conservation on one locus, while Desulfobacterota genomes encoded genes for sulfate
188 activation and reduction as well as energy conservation on one locus with genes for dissimilatory
189 sulfite reduction on another, as previously observed in cultured Desulfobacterota [22, 23, 24].

190 Sulfite (but not sulfate) reduction via the DsrAB+DsrC+DsrKMJOP system was
191 identified in only 8 genomes belonging to 7 families within the phyla Planctomycetes,
192 Chloroflexota, Spirochaetota, and Desulfobacterota (Figure 4, S3b). Gene organization of the *dsr*
193 locus in the above 9 genomes differed between different phyla, with Chloroflexota, and

194 Spirochaetota genomes encoding all genes for dissimilatory sulfite reduction (dsrABC plus
195 dsrKMJOP) on one locus, while Planctomycetota and Desulfobacterota genomes showed a split
196 *dsr* locus with dsrABC on one locus and dsrMKJOP on another.
197 On the other hand, sulfite-reduction capacity within Zodletone spring sediment solely via
198 the Asr/Hdr system was rampant, being encountered in 104 genomes belonging to 28 phyla, 43
199 (8 novel and 9 LRD) classes, 56 (18 novel, and 12 LRD) orders, and 72 (31 novel and 25 LRD)
200 families (Figure 4, S3b), with a gene organization of the *asr* locus adjacent to the *hdr* locus in the
201 majority of genomes. Asr-encoding genomes in the sediments included members of
202 predominantly previously undescribed and LRD lineages within the Chloroflexota,
203 Desulfobacterota. Planctomycetota, and Bacteroidota. The capacity was also rampant in the yet-
204 uncultured bacterial phyla, many of which have fairly limited distribution on the current earth,
205 e.g. the candidate phyla CSSED10-310, FCPU426, RBG-13-66-14, SM23-31, SZUA-182,
206 UBP14, Aureabacteria, Sumerlaeota. Interestingly, all genomes belonging to the novel phylum
207 Krumholzibacteriota, recently described from the spring sediment [25], encoded complete
208 anaerobic sulfite reductase systems. Zodletone dissimilatory sulfite reductase (Figure S3a) and
209 the anaerobic sulfite reductase (Figure S3b) sequences clustered with reference sequences from
210 the same phylum, generally showing no evidence of LGT (S. text).
211 Sulfur (polysulfide) reduction capacities were observed in twenty Zodletone sediment genomes
212 that encoded *psrABC* genes (Figure 4, S3c). These genomes belonged mostly to previously
213 undescribed and LRD families within the phyla Bacteroidota, Desulfobacterota, Myxococota,
214 Acidobacterota, Chloroflexota, and Campylobacterota (Figure 4), In addition, representatives of
215 the cytolpasmic sulfurhydrogenase I (HydABCD system), and/or II (ShyABCD system) were

216 identified in 119 Zodletone sediment genomes (Figure 4). However, as described above, direct
217 involvement of these enzymes in an ETS-associated respiration is not yet clear.
218 *Thiosulfate disproportionation and reduction.* The quinone-dependent membrane-bound
219 molybdopterin-containing thiosulfate reductase PhsABC was encoded in 11 genomes belonging
220 to 6 phyla, with Bacteroidota representing the major phsABC-encoding phylum (4 genomes).
221 Within these genomes, only two (a Chloroflexota family UBA6092 genome, and a
222 Desulfatiglandales family HGW15 genome) also encoded a dissimilatory sulfite reductase (the
223 Asr system) akin to the Gammaproteobacteria thiosulfate disproportionating pure culture
224 members, where the final products of thiosulfate disproportionation are expected to be only
225 hydrogen sulfide (Figure 4). On the other hand, 5 of the 11 phsABC-encoding Zodletone
226 genomes also encoded the sulfite dehydrogenase SoeABC system, akin to Desulfobacterota and
227 Firmicutes pure culture members, where the final products of thiosulfate disproportionation are
228 expected to be both hydrogen sulfide and sulfate. (Figure 4).
229 In addition to the phsABC system, 14 Zodletone genomes belonging to 6 phyla
230 (Desulfobacterota,Acidobacteriota, Chloroflexota, Bacteroidota, Spirochaetota, and
231 Myxococcota) encoded a rhodanase-like enzyme [EC: 2.8.1.1 or EC: 2.8.1.3] for thiosulfate
232 disproportionation, as well as enzymes for both sulfite oxidation (by means of reversal of sulfate
233 reduction via Sat+AprAB, or the sulfite dehydrogenase SoeABC), and sulfite reduction (via the
234 dissimilatory sulfite reductases Dsr or Asr), where the final products of thiosulfate
235 disproportionation are expected to be both hydrogen sulfide and sulfate (Figure 4).
236 *Tetrathionate reduction.* Seventy-three Zodletone sediment genomes encoded the octaheme
237 tetrathionate reductase (OTR) enzyme. These genomes belonged to 14 phyla with major
238 contribution from Bacteroidota (30 genomes), Chloroflexota (10 genomes), and Desulfobacterota

239 (10 genomes). In addition to Otr, 68 Zodletone genomes encoded the Ttr enzyme system. These
240 genomes belonged to 14 phyla with major contribution from Chloroflexota (22 genomes), and
241 Desulfobacterota (20 genomes). As shown previously in *Salmonella typhimurium* [26], in presence
242 of means for thiosulfate disproportionation/reduction and sulfite reduction, the thiosulfate
243 produced as a result of tetrathionate reduction could be further reduced to sulfide. Out of the 105
244 sediment genomes encoding the Otr, and/or Ttr enzymes, only 12 genomes also encoded
245 thiosulfate and sulfite reduction enzymes. These genomes belonged to the phyla Acidobacteriota,
246 Chloroflexota, Desulfobacterota, Myxococcota, and Spirochaetota.
247 *Substrates supporting sulfidogenic capacities at Zodletone spring.* Within lineages mediating
248 reductive sulfur processes in Zodletone sediments (n=98), a wide range of substrates supporting
249 sulfidogenesis were identified (Table S3, Figure 4). These included hexoses (26-87% of
250 sulfidogenic lineages), pentoses (30-41% of sulfidogenic lineages), amino acids and peptides
251 (39% of lineages), short chain fatty acids, e.g. lactate, propionate, butyrate, and acetate (22-73%
252 of lineages), long chain fatty acids (29% of lineages), aromatic hydrocarbons (3% of lineages),
253 and short chain alkanes (6% of lineages). Autotrophic capacities with hydrogen as the electron
254 donor were identified in 28% of sulfidogenic lineages.
255 **Transcriptomic analysis.** Transcriptional expression of genes involved in S-species
256 reduction/disproportionation was analyzed in the spring sediments (Figure 5). All S-species
257 reduction/ disproportionation genes discussed above were identified and mapped to 51 distinct
258 phyla. Total transcription levels of the Asr system were 4-times higher than the Dsr system,
259 consistent with the higher number of Zodletone sediment genomes encoding the Asr system
260 compared to the Dsr system. Transcription of Asr system genes were mapped to the phyla
261 Chloroflexota, Planctomycetota, Desulfobacterota, Bacteroidota, Fermentibacterota,

262 Acidobacteriota, CSSED10-310, Actinobacteriota, Spirochaetota, WOR-3, and Fibrobacterota;
263 while DSR genes to Desulfobacterota, Acidobacteriota, Zixibacteria, and Myxococcota (Dsr)
264 (Figure 5). Sulfate reduction genes (Sat, AprAB, and QmoABC) were also transcribed with
265 major contribution from Desulfobacterota, Myxococcota, Zixibacteria, and Acidobacteriota.
266 Transcription of the thiosulfate disproportionating rhodanese-like enzyme [EC: 2.8.1.1 or EC:
267 2.8.1.3], thiosulfate reductase *phsABC*, tetrathionate reduction genes *ttrABC*, octaheme
268 tetrathionate reductase *otr* , and *psrABC* for polysulfide reduction, and cytoplasmic
269 sulfurhydrogenases I and II (*hyd/shy* systems) was also identified (Figure 5, S. text for detailed
270 contributions of taxa).

271 **Oxidative sulfur processes dominate Zodletone water community.**

272 Reductive sulfur-processes were extremely sparse in the water community (S. text). In contrast
273 oxidative sulfur processes dominated the water community, with pathways encoding sulfide,
274 sulfur, thiosulfate, tetrathionate, and/or sulfite oxidation to sulfate present in 59/114 (51.8%) of
275 water genomes, belonging to 13 phyla, 16 classes, 25 orders, and 43 families, respectively. The
276 oxidative sulfur community in the water belonged to mostly well-characterized lineages (Table
277 S3, Figure 4), with only 8 and 10 genomes involved in oxidative sulfur processes belonged to
278 previously undescribed, and LDR families, respectively. A complete SOX system, putatively
279 mediating oxidation of a wide range of reduced sulfur-species to sulfate was encoded in genomes
280 belonging to well-characterized families within the Proteobacteria (11 genomes in
281 Acidithiobacillaceae, Burkholderiaceae, Halothiobacillaceae, Rhodobacteraceae, and
282 Thiomicrospiraceae) and Campylobacterota (3 genomes in the family Sulfurimonadaceae)
283 (Figure 4). The capacity for sulfide oxidation to sulfur (sulfide dehydrogenase and/or the
284 sulfide:quinone oxidoreductase Sqr) was encoded in thirty nine water genomes within the phyla

285 Bacteroidota, Proteobacteria, Campylobacterota, Chloroflexi, Desulfobacterota, Marinisomatota,
286 and Nitrospirota (S. Text, Figure 4). Only two of the above thirty-nine genomes (a Proteobacteria
287 genome and a Nitrospirota genome) encoded the capacity to further oxidize the
288 sulfur/polysulfide to sulfite via the reversal of the Dsr system, the sulfite dehydrogenase
289 (quinone) SoeABC, or the sulfite dehydrogenase (cytochrome) SorAB system was encountered
290 in 1, 22, and 3 genomes respectively, belonging to well characterized families within
291 Bacteroidetes, Proeobacteria, and Campilobacterota (S. text, Figure S4). Finally, for thiosulfate
292 oxidation, Eight water genomes (Proteobacteria and Flavobactereaceae) encoded thiosulfate to
293 tetrathionate oxidation capacities via either the thiosulfate dehydrogenase *tsdA* [EC: 1.8.2.2] or
294 *doxAD* [EC: 1.8.5.2]. Two of these 8 genomes (1 Rhodobacteraceae, and 1 Halothiobacillaceae
295 genomes) also encoded tetrathionate hydrolase (*tetH*) [27] known to cleave tetrathionate to
296 thiosulfate, sulfur, and sulfate. Simultaneous identification of the SOX system and both forms of
297 sulfide dehydrogenase (fccAB and Sqr) imply that these two genomes encode the capacity for
298 complete thiosulfate oxidation to sulfate.
299
300

301 **Discussion**

302 The microbial community in Zodletone spring sediments exhibited a high level of phylogenetic
303 diversity, novelty, and rarity (Figure 1-3). Conversely, representatives of lineages that
304 predominate in most present earth environments, e.g. Proteobacteria, Firmicutes, and
305 Cyanobacteria were absent or extremely sparse within the sediments. The community in the
306 spring sediments was also characterized by a high proportion of SRM, and the prevalence of
307 lineages mediating sulfur cycle intermediates (sulfite, thiosulfate, tetrathionate, and elemental
308 sulfur). Many of the organisms mediating reductive sulfur-cycling processes belonged to novel
309 and LRD lineages, hence greatly expanding the range of SRM within the tree of life (Figure 4).

310 What drives the assembly, propagation, and maintenance of such a diverse, novel, and
311 distinct community in the spring sediments? The high level of diversity, novelty, and rarity
312 within Zodletone spring sediment SRM community could be attributed to two main factors. First,
313 the availability of a wide range of sulfur cycle intermediates in concentrations much higher than
314 sulfate, in contrast to sulfate-predominance in current ecosystems [2]. Such pattern selects for a
315 more diverse community of SRM in the spring, when compared to predominantly sulfate-driven
316 marine and freshwater ecosystems (Figure 4). Second, additional factors usually constraining
317 SRM growth in several habitats, e.g. diel or seasonal intrusion of oxygen, Fe and NO_3 [1, 28, 29, 30],
318 recalcitrance of available substrates [6, 31, 32], temperature [33, 34], pH [21, 35, 36], salinity [37], and pressure
319 extremes [31, 38], or combinations thereof, are absent in the spring. Therefore, while the reductive
320 global sulfur cycle appears to be dominated by a few sulfate-reducing lineages within the
321 Desulfobacterota, and to a lesser extent the Firmicutes, as well as Thermodesulfobacteria and
322 *Archaeoglobus* in high temperature habitat, the SRM community in the spring is extremely
323 diverse, encompassing a wide range of previously undescribed and LRD lineages (Figure 4, 5,

324 S4-S6).

325 Sulfate-reducing organisms are the most prevalent component of the reductive sulfur
326 cycle in most marine and aquatic ecosystems. Aspects of the ecology [2], physiology [39], and
327 biochemistry [40, 41, 42] of dissimilatory sulfate reduction have been extensively investigated [43].
328 While not the most prevalent process, the sulfate-reducing community in Zodletone spring
329 sediment exhibited a unique composition, with members of the Zixibacteria, Acidobacteriota,
330 Myxococcota, Bacteroidota, Planctomycetota, and candidate phylum OLB16 constituting the
331 major players, as well as rare and novel lineages within the Desulfobacterota (Desulfatiglandales,
332 and Order C00003060), with scarce representation of canonical Desulfobacterota sulfate
333 reducers (1 genome). While the identification of the dissimilatory sulfate reducing machinery in
334 some of these lineages (e.g. Zixibacteria, Acidobacteriota, and Planctomycetota) has been shown
335 before [44, 45, 46], these members rarely appear to be the dominant players in a single ecosystem.

336 Compared to sulfate-reduction, the ecology and diversity of microbial dissimilatory
337 sulfite reduction has not been extensively studied. The biochemistry of the process has been
338 examined in sulfate-reducers, when grown on sulfite [43], as well as in a few other dedicated sulfite
339 reducers, e.g. *Desulfitobacterium* [47], *Salmonella* [48], *Shewanella* [49], and *Wolinella* [50]. A recent
340 study suggested the importance and ancient nature of sulfite reduction in an extreme
341 thermophilic environment in a limited diversity biofilm [51]. We document a plethora of
342 microorganisms within the phyla Planctomycetes, Chloroflexota, Spirochaetota, and
343 Desulfobacterota encoding the dissimilatory sulfite reductase DSR, as well as 72 additional
344 families (31 novel and 25 LRD) encoding the anaerobic sulfite reductase. These organisms
345 greatly expand the known sulfite reduction capacity within the domain Bacteria. Further, the

346 novelty or rarity of some of these families is a reflection of the dearth of present-time habitats
347 that could support this mode of metabolism, once predominant on ancient earth.
348 The bulk of knowledge on thiosulfate reduction and or disproportionation comes from
349 studies in pure cultures, e.g. members of the Desulfobulbaceae (e.g. *Desulfocapsa*) [52, 53] and the
350 genera *Desulfovibrio* and *Desulfomonile* [54, 55, 56] within Desulfobacterota, the
351 gammaproteobacterium *Pantoea agglomerans* [57], members of Thermodesulfobacteria [58] and
352 Firmicutes [59] . Radioisotope tracing of different sulfur atoms showed a significant contribution of
353 thiosulfate disproportionation to the sulfur cycle in marine [60], as well as freshwater sediments [61].
354 However, the lack of a marker gene for the process hinders ecological culture-independent
355 studies. Similar to sulfite, the high levels, and constant generation of thiosulfate in Zodletone
356 sediments sustains a highly diverse thiosulfate reducing (thiosulfate reductase plus a sulfite
357 reduction complex), or disproportionating (thiosulfate reductase plus both sulfite reduction and
358 sulfite oxidation systems) community with major contribution from novel or rare families in the
359 Acidobacteriota, Chloroflexota, Desulfobacterota, KSB1, Myxococcota, and Spirochaetota.
360 Finally, the extremely high levels of zero valent sulfur, available as soluble polysulfide, result in
361 enriching the community with a plethora of polysulfide-reducing organisms.
362 As described above, the prevailing conditions in Zodletone spring (anaerobic, surficial,
363 light-exposed, sulfidic, with abundance of S cycle intermediates) remarkably mimic the
364 conditions that were prevalent in the late Archean and early Proterozoic eons, when the evolution
365 of major bacterial and archaeal clades (based on recently refined timing estimates from [62]), with
366 the notable exception of Proteobacteria and Cyanobacteria) occurring prior to oxygen evolution.
367 This study infers that the microbial communities presumably thriving in surficial earth were
368 extremely diverse, with an abundance of SRM lineages. The evolution of oxygenic

369 photosynthesis has led to the steady and inexorable accumulation of O_2 in Earth's atmosphere
370 (the great oxidation event, GOE), with the rise of atmospheric O_2 to 1-5% of current levels
371 between 2.4 and 2.1 billion years (Gyr) ago, and its accumulation to values comparable to
372 modern values 500-600 Mya [63]. Due to the sensitivity and expected lack of adaptive mechanisms
373 to cope with atmospheric oxygen in multiple strict anaerobes, as well as the chemical instability
374 of multiple S species in an oxygenated atmosphere, the GOE exerted a profound negative impact
375 on anaerobic surficial life forms (the oxygen catastrophe) leading to the first and arguably most
376 profound extinction event in earth's history. In addition to suppressing anaerobiosis in
377 atmospherically-exposed habitats, the GOE also led to a significant change in the S cycle, from
378 one based on atmospheric inputs to one dependent on oxidative weathering leading to the release
379 of huge amount of sulfate derived from the oxidation of pyrite and the dissolution of sulfate
380 minerals [64], hitherto a minor byproduct of Archean abiotic and biotic reactions [15, 65]. Therefore, it
381 appears that loss of niches associated with geological transformations could be one of the
382 possible explanations for high extinction rates for microorganisms on earth, as well as the
383 constant identification of rare, novel taxa within anaerobic settings. It is notable that
384 phylogenetically novel branches with extremely rare distribution in earth (defined as phyla with
385 5 genomes or less in GTDB) have been consistently identified in anaerobic habitats.
386 The hypothesis that the GOE played an important role in the extinction of many novel
387 anaerobic lineages, specifically those mediating SCI reductive processes is bolstered by our
388 analysis of the overlaying microbial community in the spring. Here, spatial oxygen intrusion
389 provides a mimicking effect to the relentless temporal progression of oxygenation through the
390 Proterozoic (2.4-2.1 Gya) and Paleo-Phanerozoic (500-600 Mya). The analysis demonstrates a
391 community transitioning from ancient novelty to modern commonality, with the proportion of

392 novel and rare taxa as well as lineages associated with reductive sulfur processes decreasing at
393 the expense of propagation of organisms mediating oxidative sulfur processes.
394 In summary, by examining microbial diversity in Zodletone spring, we greatly expand the
395 overall diversity within the tree of life via the discovery and characterization of a wide range of
396 novel lineages, and significantly enrich representation of a wide range of LRD lineages. We also
397 describe a unique sulfur-cycling community in the spring that is largely dependent on sulfite,
398 thiosulfate, sulfur, and tetrathionate, rather than sulfate, as an electron acceptor. Given the
399 remarkable similarity to conditions prevailing prior to the GOE, we consider the spring an
400 invaluable portal to investigate the community thriving on the earth's surface during these eons,
401 and posit that GOE-precipitated the near extinction of a wide range of phylogenetically distinct
402 oxygen-sensitive lineages and drastically altered the reductive sulfur-cycling community from
403 sulfite, sulfur, and thiosulfate reducers to predominantly sulfate reduction in the current earth.
404

405 **Materials and Methods**

406 **Site description and geochemistry.** Zodletone spring is located in the Anadarko Basin of
407 western Oklahoma (N34.99562° W98.68895°). The spring arises from underground, where water
408 is pumped out slowly along with sediments. Sediments settled at the source of the spring, a
409 boxed square $1m^2$ (Figure S1) are overlaid with water that collects and settles in a concrete pool
410 erected in the early 1900s. The settled water is 50-cm deep above the sediments and is exposed
411 to atmospheric air. Water and sediments originating from the spring source are highly reduced
412 due to the high dissolved sulfide levels (8-10 mM) in the spring sediments. Microsensor
413 measurements show a completely anoxic (oxygen levels < 0.1 μM) and highly reduced source
414 sediments. Oxygen levels slowly increase in the overlaid water column from 2–4 μM at the 2
415 mm above the source to complete oxygen exposure on the top of the water column [66]. The spring
416 geochemistry has regularly been monitored during the last two decades [66, 67, 68] and is remarkably
417 stable. The spring is characterized by low levels of sulfate (50-94 μM), with higher levels of
418 sulfite (0.21 mM), elemental sulfur (0.1 mM), and thiosulfate (0.52) [68, 69].

419 **Sampling.** Samples were collected from the source sediments and standing overlaid water in
420 sterile containers and kept on ice until brought back to the lab (~2h drive), where they were
421 immediately processed. For metatranscriptomics, samples were collected at three different time
422 points: morning (9:15 am), afternoon (2:30 pm), and evening (5:30 pm) in June 2019; stored on
423 dry ice until transferred to the lab where they were stored at -80ºC until processed for RNA
424 extraction within a week.

425 **Nucleic acid extraction.** DNA was directly extracted from 0.5 grams of source sediments. For
426 water samples, water was filtered on 0.2 μm sterile filters. DNA was directly extracted from
427 filters (20 filters, 10 L of water samples). Extraction was conducted using the DNeasy PowerSoil

428 kit (Qiagen, Valencia, CA, USA). RNA was extracted from 0.5 g sediment samples using
429 RNeasy PowerSoil Total RNA Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's
430 instructions.

431 **16S rRNA gene amplification, sequencing, and analysis.** Triplicate DNA extractions were
432 performed for both sediment and water samples from the Zoddletone spring. To characterize the
433 microbial diversity based on 16S rRNA gene sequences we used the Quick-16S™ NGS Library
434 Prep Kit (Zymo Research, Irvine CA), following the manufacturer's protocol. For amplification
435 of the V4 hypervariable region we used a mix of modified versions of primers 515F-806R [70],
436 tailored to provide better coverage for several under-represented microbial lineages. They
437 included 515FY (5'GTGYCAGCMGCCGCGGTAA) [71], 515F-Cren (5'
438 GTGKCAGCMGCCGCGGTAA, for Crenarchaeota) [72], 515F-Nano
439 (5'GTGGCAGYCGCCRCGGKAA, for Nanoarchaeota) [72], 515F-TM7 (5'
440 GTGCCAGCMGCCGCGGTCA for TM7/Saccharibacteria) [73] as forward mix and 805RB (5'
441 GGACTACNVGGGTWTCTAAT) [74] and 805R-Nano (5'GGAMTACHGGGGTCTCTAAT, for
442 Nanoarchaeota) [72] as reverse mix. Purified barcoded amplicon libraries were sequenced on an
443 Illumina MiSeq instrument (Illumina Inc., San Diego, CA) using a v2 500 cycle kit, according to
444 manufacturer's protocol. Demultiplexed forward and reverse reads were imported as paired fastq
445 files into QIIME2 v. 2020.8 [75] for analysis. The DADA2 plugin was used to trim, denoise, pair,
446 purge chimeras and select amplicon sequence variants (ASVs), using the command "qiime dada2
447 denoise-paired". Between 44k and 194k non-chimeric sequences were obtained for the individual
448 samples. The ASVs were taxonomically classified in QIIME2 using a trained classifier built
449 based on Silva-138-99 rRNA sequence database. The ASVs were assigned to 1643 taxonomic
450 categories corresponding to taxonomic level 7 (species and above) and to 932 genera (level 6).

451 Alpha rarefaction curves indicated saturation of observed sequence features (ASVs) at a
452 sequencing depth of 70-80k sequences.
453 **Metagenome sequencing, assembly, and binning.** Metagenomic sequencing was conducted
454 using the services of a commercial provider (Novogene, Beijing, China) using two lanes of the
455 Illumina HiSeq 2500 system for each of the water and sediment samples. Transcriptomic
456 sequencing using Illumina HiSeq 2500 2 × 150bp paired-end technology was conducted using
457 the services of a commercial provider (Novogene Corporation, Beijing, China). Metagenomic
458 reads were assessed for quality using FastQC followed by quality filtering and trimming using
459 Trimmomatic v0.38 [76]. High quality reads were assembled into contigs using MegaHit (v.1.1.3)
460 with minimum Kmer of 27, maximum kmer of 127, Kmer step of 10, and minimum contig
461 length of 1000 bp. Bowtie2 was used to calculate sequencing coverage of each contig by
462 mapping the raw reads back to the contigs. Assembled contigs were searched for ribosomal
463 protein S3 (rpS3) sequences using a custom hidden Markov model (HMM) built from Uniprot
464 reference sequences assigned to the Kegg Orthologies K02982, and K02984 (corresponding to
465 the bacterial, and archaeal RPS3, respectively) using hmmbuild (HMMER 3.1b2). rpS3
466 Sequences were clustered at 99% ID using CD-HIT as previously suggested for a putative
467 species cutoff for rpS3 data [77]. Taxonomic affiliations of (rpS3) groups were identified using
468 Diamond Blast against the GTDB r95 database [19].
469 Contigs from the sediment and water assemblies were binned into draft genomes using
470 both Metabat [78] and MaxBin2 [79]. DasTool was used to select the highest quality bins from each
471 metagenome assembly [80]. CheckM was used for estimation of genome completeness, strain
472 heterogeneity, and contamination [81]. Genomic bins showing contamination levels higher than
473 10%, were further refined based on the taxonomic affiliations of the binned contigs, as well as

474 the GC content, tetranucleotide frequency, and coverage levels using RefineM [82]. Low quality
475 bins (>10% contamination) were cleaned by removal of the identified outlier contigs, and the
476 percentage completeness and contamination were again re-checked using CheckM.
477 **Genomes classification, annotation, and metabolic analysis.** Taxonomic classifications
478 followed the Genome Taxonomy Database (GTDB) release r95 [19], and were carried out using the
479 classify_workflow in GTDB-Tk (v1.1.0) [83]. Phylogenomic analysis utilized the concatenated
480 alignment of a set of 120 single-copy bacterial genes, and 122 single-copy archaeal genes [19]
481 generated by the GTDB-Tk. Maximum-likelihood phylogenomic tree was constructed in
482 FastTree using the default parameters [84].
483 **Annotation and metabolic analysis.** Protein-coding genes were predicted using Prodigal [85].
484 GhostKOALA [86] was used for the functional annotation of every predicted open reading frame in
485 every genomic bin and to assign protein-coding genes to KEGG orthologies (KOs).
486 **Analysis of sulfur cycling genes.** To identify taxa mediating key sulfur-transformation
487 processes in the spring sediments, we mapped the distribution of key sulfur-cycling genes in all
488 genomes and deduced capacities in individual genomes by documenting the occurrence of entire
489 pathways (as explained below in details). This was subsequently confirmed by phylogenetic
490 analysis and examining contiguous genes organization in processes requiring multi-subunit
491 and/or multi-gene. Further, expression data was used from three time points to identify the
492 fraction of the community that is metabolically actively involved in the process. Analysis of S
493 cycling capabilities was conducted on individual genomic bins by building and scanning hidden
494 markov model (HMM) profiles as explained below. To build the sulfur-genes HMM profiles,
495 Uniprot reference sequences for all genes with an assigned KO number were downloaded,
496 aligned using Clustal-omega [87], and the alignment was used to build an HMM profile using

497 hmmbuild (HMMER 3.1b2) [88]. For genes not assigned a KO number (e.g. *otr*, *tsdA*, *tetH*), a
498 representative protein was compared against the KEGG Genes database using Blastp and
499 significant hits (those with e-values < e-80) were downloaded and used to build HMM profiles as
500 explained above. The custom-built HMM profiles were then used to scan the analyzed genomes
501 for significant hits using hmmscan (HMMER 3.1b2) [88] with the option -T 100 to limit the results
502 to only those profiles with an alignment score of at least 100. Further confirmation was achieved
503 through phylogenetic assessment and tree building procedures, in which potential candidates
504 identified by hmmscan were aligned to the reference sequences used to build the custom HMM
505 profiles using Clustal-omega [87], followed by maximum likelihood phylogenetic tree construction
506 using FastTree [84]. Only candidates clustering with reference sequences were deemed true hits
507 and were assigned to the corresponding KO. Details on the genes examined for evidence of
508 sulfate, sulfite, polysulfide, tetrathionate, and thiosulfate reduction, thiosulfate
509 disproportionation, and various sulfur oxidation processes are provided in the supplementary
510 document (S. text).
511 *Phylogenetic analysis and operon organization of S cycling genes.* The phylogenetic affiliation
512 of the S cycling proteins AsrB, Otr, PhsC, PsrC, and DsrAB was examined by aligning
513 Zodletone genome predicted protein sequences to Uniprot reference sequences using Mafft [89].
514 The DsrA and DsrB alignments were concatenated in MEGA X [90]. All alignments were used to
515 construct maximum likelihood phylogenetic trees in RAxML [91]. The R package genoPlotR [92]
516 was used to produce gene maps for the DSR and ASR loci in Zodletone genomes using the
517 Prodigal predicted gene starts, ends, and strand.
518 *Transcription of sulfur cycling genes.* A total of 21.4 M, 27.9 M, and 22.5 M 150-bp paired-end
519 reads were obtained from the morning, afternoon, and evening RNA-seq libraries. Reads were

520 pseudo-aligned to all Prodigal-predicted genes from all genomes using Kallisto with default
521 settings [93]. The calculated transcripts per million (TPM) were used to obtain total transcription
522 levels for genes identified from genomic analysis as involved in S cycling in the spring.
523 **Additional metabolic analysis**. For all other non-sulfur related functional predictions, combined
524 GhostKOALA outputs of all genomes belonging to a certain order (for orders with 5 genomes or
525 less; n=206), or family (for orders with more than 5 genomes; n=85) were checked for the
526 presence of groups of KOs constituting metabolic pathways (additional file 1). The list of these
527 291 lineages is shown in Table S2. The presence of at least 80% of KOs assigned to a certain
528 pathway in at least one genome belonging to a certain order/family was used as an indication of
529 the presence of that pathway in that order/family. Such criteria were used for the prediction of
530 autotrophic capabilities, as well as catabolic heterotrophic degradation capabilities of sugars,
531 amino acids, long-chain fatty acids, short chain fatty acids, anaerobic benzoate degradation,
532 anaerobic short chain alkane degradation, aerobic respiration, nitrate reduction, nitrification, and
533 chlorophyll biosynthesis. Glycolytic, and fermentation capabilities were predicted by feeding the
534 GhostKOALA output to KeggDecoder [94]. Proteases, peptidases, and protease inhibitors were
535 identified using Blastp against the Merops database [95], while CAZymes (glycoside hydrolases
536 [GHs], polysaccharide lyases [PLs], and carbohydrate esterases [CEs]) were identified by
537 searching all ORFs from all genomes against the dbCAN hidden Markov models V9 [96]
538 (downloaded from the dbCAN web server in September 2020) using hmmscan. FeGenie [97] was
539 used to predict the presence of iron reduction and iron oxidation genes in individual bins.
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542 **Figure legends:**

543 **Figure 1.** Phylogenomics of the 516 bacterial (A), and 114 archaeal (B) genomes analyzed in
544 this study. The maximum likelihood trees were constructed in FastTree [84] based on the
545 concatenated alignments of 120 (bacterial), and 122 (archaeal) housekeeping genes obtained
546 from Gtdb-TK [83]. The branches represent order-level taxonomy and are color coded by phylum.
547 For phyla with 4 orders or less branches are labeled as Phylum_Class_Order. For phyla with
548 more than 4 orders, the phylum is shown at the base of the colored wedge and the branches are
549 labeled as Class_Order. Lineages staring with ZN depict novel lineages (ZNC = novel class;
550 ZNO = novel order). Bootstrap support values are shown as bubbles for nodes with >70%
551 support. Tracks around the tree represent (from innermost to outermost): cultured status at the
552 order level (cultured versus uncultured), abundance in Gtdb based on the number of available
553 genomes (abundant with more than 5 genomes, rare with 5 genomes or less, and novel with no
554 genomes in Gtdb), percentage database enrichment (calculated as number of genomes belonging
555 to a certain order binned in the current study as a percentage of the number of genomes
556 belonging to the same order in Gtdb), energy conservation capabilities depicted by colored
557 circles (salmon, aerobic respiration; orange, Fe^{3+} respiration; yellow, nitrate/nitrite reduction;
558 dark green, reductive sulfur processes; lime green, nitrogen oxidation; cyan, oxidative sulfur-
559 processes; pink, respiratory hydrogen oxidation; and purple, photosynthesis), and the number of
560 MAGs belonging to each order binned from the sediment (blue bars) and the water (orange bars).
561 For orders with 20 or more genomes, the family-level delineation is shown in Figure 3. These
562 orders are: Anaerolineales (Fig. 3A), Bacteroidales (Fig. 3B), Sedimentisphaerales (Fig. 3C),
563 Spirochaetales (Fig. 3D), Syntrophales (Fig. 3E), and Woesearchaeales (Fig. 3F).

564

565 **Figure 2.** Novelty, rarity, and phylum-level makeup in Zodletone sediment and water
566 communities. Genomes belonging to novel (orange), and LRD (blue) lineages are shown as a
567 percentage of total binned genomes in the sediment (A) and the water (B) communities. The sum
568 of novel and LRD genomes is shown in grey. (C) Phylum-level affiliation for sediment versus
569 water genomes. Number of genomes belonging to each phylum is shown for the sediment (blue
570 bars on the left) and the water (orange bars on the right).
571
572 **Figure 3.** Family-level delineation for orders with 20 or more genomes. The maximum
573 likelihood trees were constructed in FastTree [84] based on the concatenated alignments of 120-,
574 and 122- single copy genes, respectively, obtained from Gtdb-TK [83]. Bootstrap support values
575 are shown as bubbles for nodes with >70% support. Families are color-coded. To the right of the
576 trees, tracks are shown for cultured status at the family level (cultured versus uncultured), and
577 abundance in Gtdb based on the number of available genomes (abundant with more than 5
578 genomes, rare with 5 genomes or less, and novel with no genomes in Gtdb).
579
580 **Figure 4.** Sulfur cycle in Zodletone spring. (A) Diagram of sulfur transformations predicted to
581 take place in the spring. Different sulfur species are shown in black boxes. Reduction reactions
582 are depicted by purple arrows, oxidation reactions are depicted by red arrows, while
583 disproportionation reactions are depicted by golden brown arrows. The gene(s) names are shown
584 on the arrows. (B) Phylum-level distribution of the S-cycling genes shown at the top of the figure
585 in sediment and water genomes. Processes involving more than one gene are highlighted by
586 horizontal bars and are color coded by reduction (purple), oxidation (red), or disproportionation
587 (golden brown), with the name of the process shown on top of the horizontal bar. (C) Family-

588 level distribution of the genomes involved in S cycling in the spring. The maximum likelihood
589 tree was constructed in FastTree [84] based on the concatenated alignments of 120 single-copy
590 genes obtained from Gtdb-TK [83]. The branches represent family-level taxonomy and are color
591 coded by phylum. For phyla with 2 families or less involved in S cycling, branches are labeled as
592 Phylum_Class_Order_Family. For phyla where 3 or more families are involved in S cycling, the
593 phylum is shown at the base of the colored wedge and the branches are labeled as
594 Class_Order_Family. Lineages staring with ZN depict novel lineages as follows: ZNC, novel
595 class; ZNO, novel order; and ZNF, novel family. Bootstrap support values are shown as bubbles
596 for nodes with >70% support. Tracks around the tree represent (from innermost to outermost):
597 heatmap for the number of genomes in each family, abundance in Gtdb based on the number of
598 available genomes (abundant with more than 5 genomes, rare with 5 genomes or less, and novel
599 with no genomes in Gtdb), pie charts of the breakdown of the number of genomes in the
600 sediment (cyan) versus water (magenta), sulfur reduction pathways (5 tracks in purple),
601 thiosulfate disproportionation pathways (1 track in golden brown), sulfur oxidation pathways (7
602 tracks in red), and substrates predicted to support growth depicted by colored stars (cyan, sugars;
603 lime green, complex carbohydrates; magenta, amino acids; orange, proteins; purple, CO2
604 fixation; red, short-chain fatty acids (SCFA); blue, beta oxidation of long-chain fatty acids;
605 brown, anaerobic benzoate/aromatic hydrocarbon degradation; black, anaerobic alkane
606 degradation; and grey, Hydrogen oxidation).
607 **Figure 5.** Phylum-level distribution for transcribed sulfur cycling genes in Zodletone spring
608 sediment. RNA-seq reads were pseudo-aligned to the S-cycling genes predicted in Zodletone
609 genomes to detect exact matches using Kallisto [93]. The transcripts per million are shown on the
610 Y-axis for the gene/ group of genes depicted at the top of the figure.

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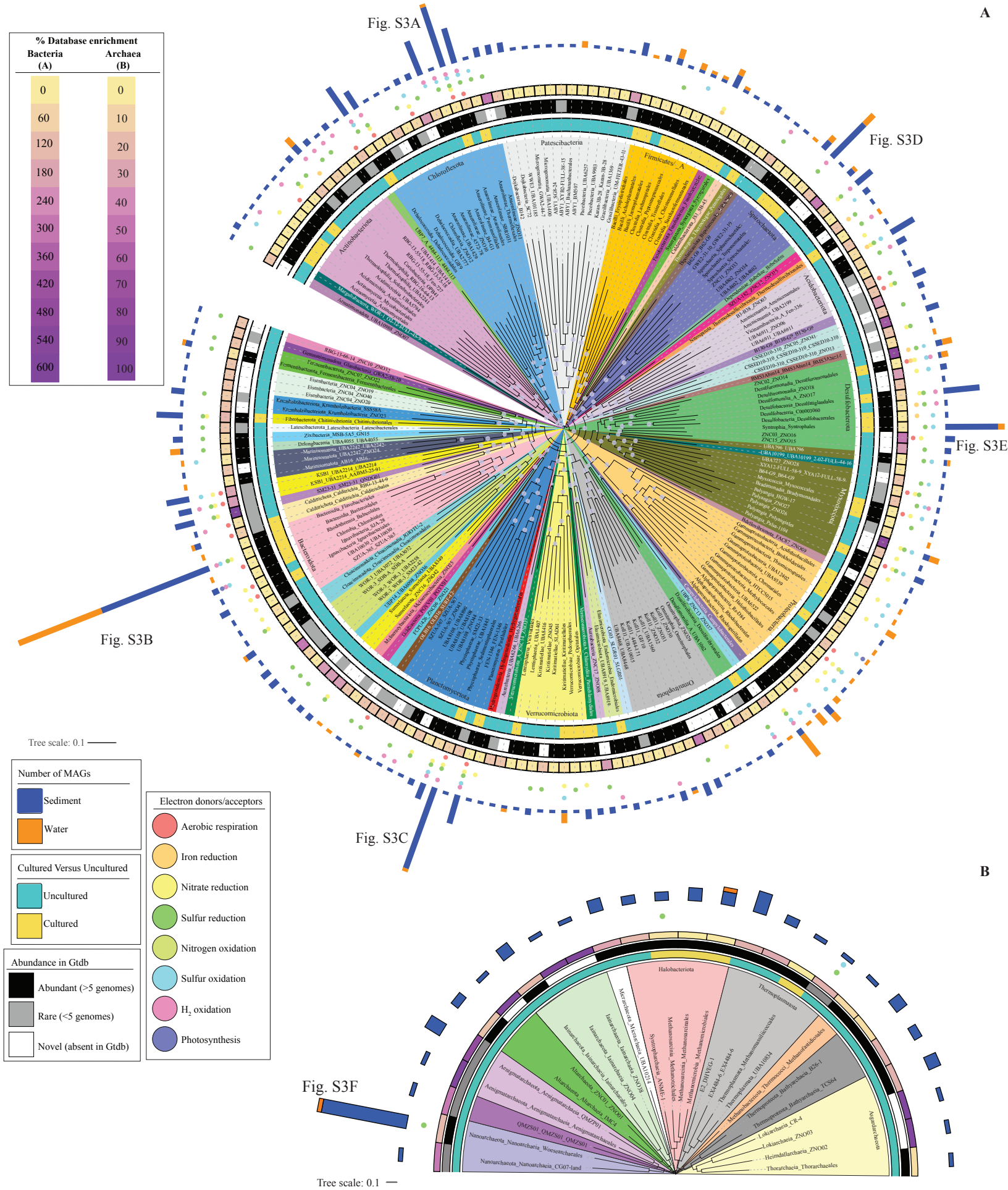

A
Fig. S3A
Fig. S3B
Fig. S3C
Fig. S3D
Fig. S3E
% Database enrichment
Bacteria (A)
Archaea (B)
0
60
120
180
240
300
360
420
480
540
600
0
10
20
30
40
50
60
70
80
90
100
Patescibacteria
Chloroflexota
Firmicutes_A
Spirochaetota
Acidobacteriota
Desulfobacterota
Myxococcota
Bacteroidota
Planctomycetota
Verrucomicrobiota
Omnitrophota
Tree scale: 0.1
Number of MAGs
Sediment
Water
Cultured Versus Uncultured
Uncultured
Cultured
Abundance in Gtdb
Abundant (>5 genomes)
Rare (<5 genomes)
Novel (absent in Gtdb)
Electron donors/acceptors
Aerobic respiration
Iron reduction
Nitrate reduction
Sulfur reduction
Nitrogen oxidation
Sulfur oxidation
H2 oxidation
Photosynthesis
B
Fig. S3F
Halobacteriota
Methanosarcinales_Methanosaetaceae
Methanosarcinales_Methanosarcinaceae
Methanomicrobia_Methanomicrobiales
Methanomicrobia_Methanocellales
Methanomicrobia_Methanosalinales
Methanomicrobia_Methanosphaerales
Methanomicrobia_Methanomassiliicoccales
Methanomicrobia_Methanomicrobiales
Methanomicrobia_Methanomicrobiales
Methanomicrobia_Methanomicrobi

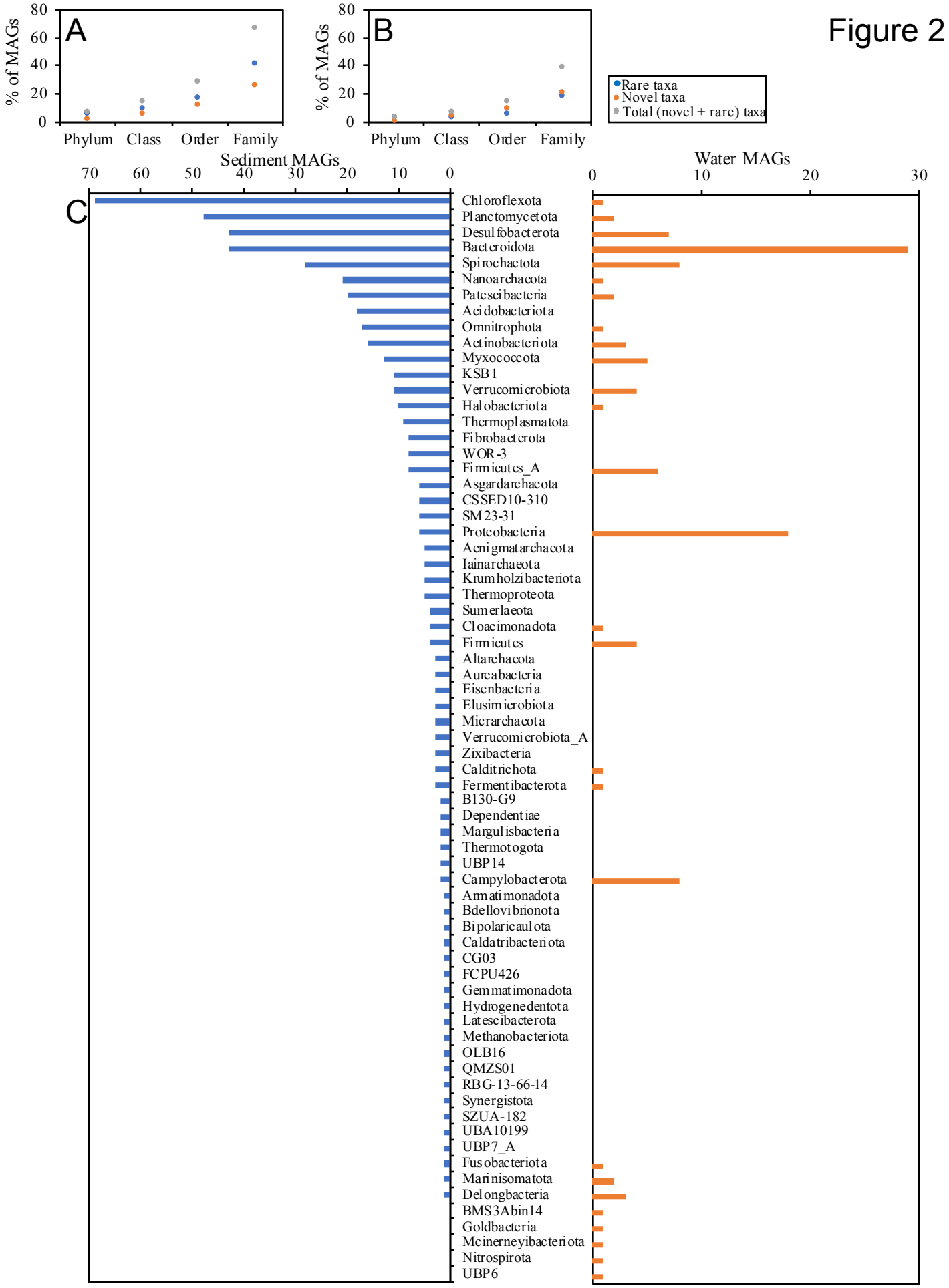
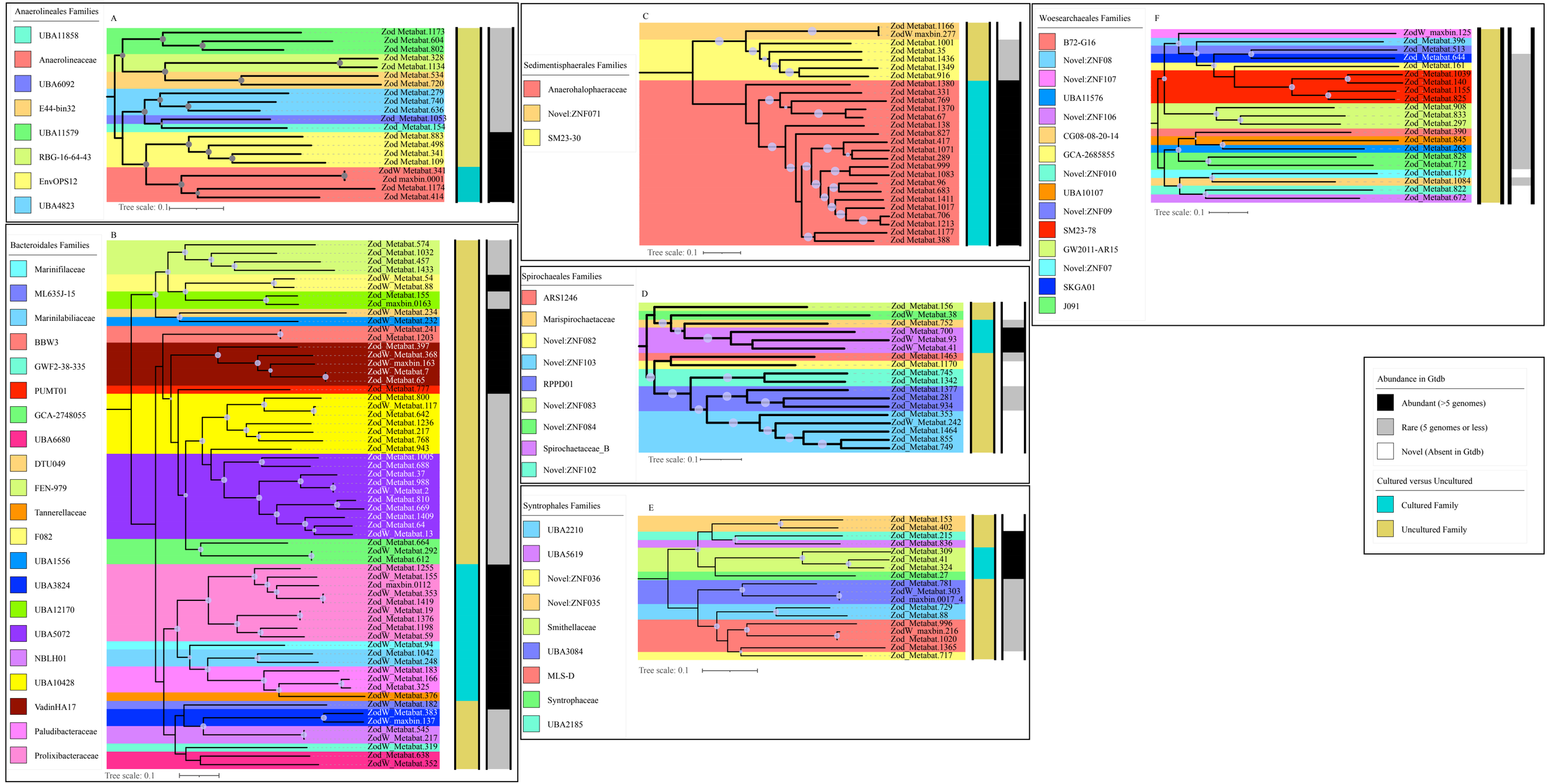
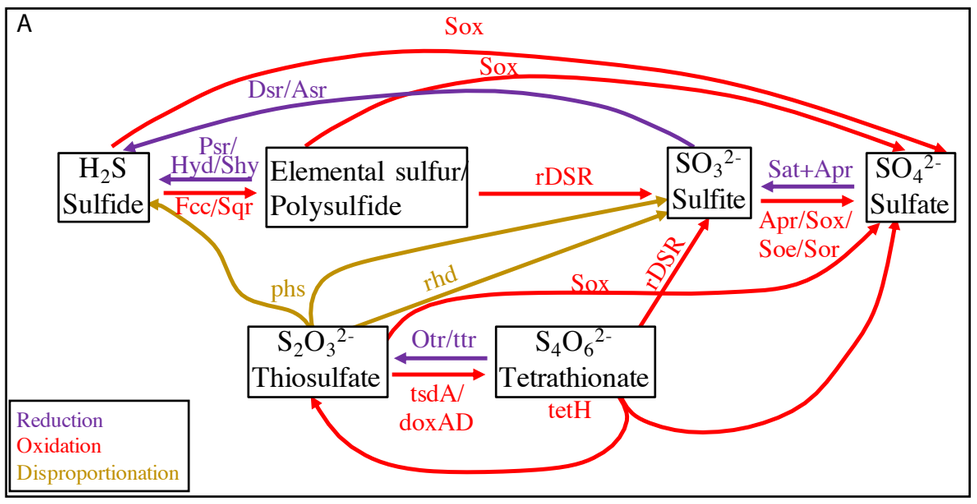
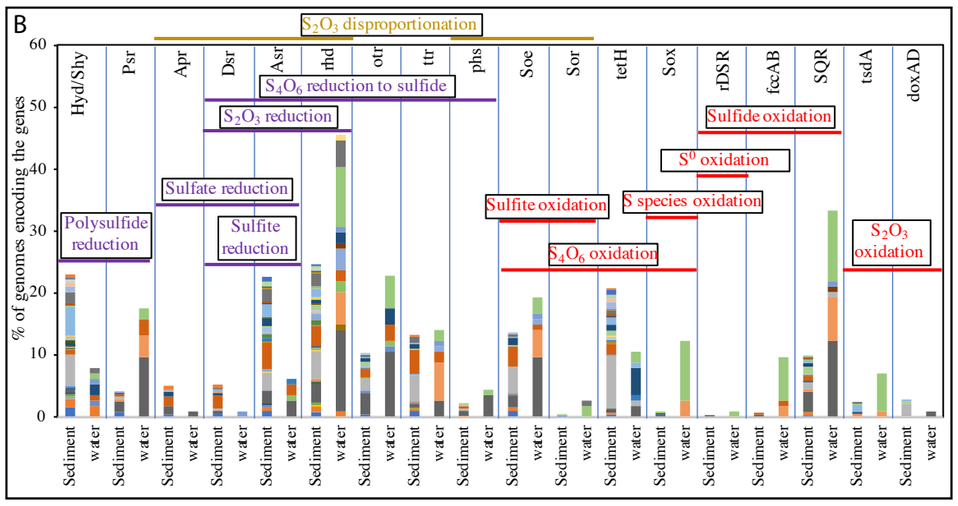


Figure 2
A
B
C
% of MAGs
80
60
40
20
0
Phylum
Class
Order
Family
Rare taxa
Novel taxa
Total (novel + rare) taxa
Sediment MAGs
70
60
50
40
30
20
10
0
Water MAGs
0
10
20
30
Chloroflexota
Planctomycetota
Desulfobacterota
Bacteroidota
Spirochaetota
Nanoarchaeota
Patescibacteria
Acidobacteriota
Omnitrophota
Actinobacteriota
Myxococcota
KSB1
Verrucomicrobiota
Halobacteriota
Thermoplasmatota
Fibrobacterota
WOR-3
Firmicutes_A
Asgardarchaeota
CSSED10-310
SM23-31
Proteobacteria
Aenigmatarchaeota
Iainarchaeota
Krumholzibacteriota
Thermoproteota
Sumerlaeota
Cloacimonadota
Firmicutes
Altarchaeota
Aureabacteria
Eisenbacteria
Elusimicrobiota
Micrarchaeota
Verrucomicrobiota_A
Zixibacteria
Calditrichota
Fermentibacterota
B130-G9
Dependentiae
Margulisbacteria
Thermotogota
UBP14
Campylobacterota
Armatimonadota
Bdellovibrionota
Bipolaricaulota
Caldatribacteriota
CG03
FCPU426
Gemmatimonadota
Hydrogenedentota
Latescibacterota
Methanobacteriota
OLB16
QMZS01
RBG-13-66-14
Synergistota
SZUA-182
UBA10199
UBP7_A
Fusobacteriota
Marinisomatota
Delongbacteria
BMS3Abin14
Goldbacteria
Mcinerneyibacteriota
Nitrospirota
UBP6


Anaerolineales Families
UBA11858
Anaerolineaceae
UBA6092
E44-bin32
UBA11579
RBG-16-64-43
EnvOPS12
UBA4823
A
Zod Metabat.1173
Zod Metabat.604
Zod Metabat.802
Zod Metabat.328
Zod Metabat.1134
Zod Metabat.534
Zod Metabat.720
Zod Metabat.279
Zod Metabat.740
Zod Metabat.636
Zod_Metabat.1053
Zod_Metabat.154
Zod Metabat.883
Zod Metabat.498
Zod Metabat.341
Zod Metabat.109
ZodW Metabat.341
Zod maxbin.0001
Zod Metabat.1174
Zod Metabat.414
Tree scale: 0.1
Bacteroidales Families
Marinifilaceae
ML635J-15
Marinilabiliaceae
BBW3
GWF2-38-335
PUMT01
GCA-2748055
UBA6680
DTU049
FEN-979
Tannerellaceae
F082
UBA1556
UBA3824
UBA12170
UBA5072
NBLH01
UBA10428
VadinHA17
Paludibacteraceae
Prolixibacteraceae
B
Zod_Metabat.574
Zod_Metabat.1032
Zod_Metabat.457
Zod_Metabat.1433
ZodW_Metabat.54
ZodW_Metabat.88
Zod_Metabat.155
Zod_maxbin.0163
ZodW_Metabat.234
ZodW_Metabat.232
ZodW_Metabat.241
Zod_Metabat.1203
Zod_Metabat.397
ZodW_Metabat.368
ZodW_maxbin.163
ZodW_Metabat.7
Zod_Metabat.65
Zod_Metabat.777
Zod_Metabat.800
ZodW_Metabat.117
Zod_Metabat.642
Zod_Metabat.1236
Zod_Metabat.217
Zod_Metabat.768
Zod_Metabat.943
Zod_Metabat.1005
Zod_Metabat.688
Zod_Metabat.37
Zod_Metabat.988
ZodW_Metabat.2
Zod_Metabat.810
Zod_Metabat.669
Zod_Metabat.1409
Zod_Metabat.64
ZodW_Metabat.13
Zod_Metabat.664
ZodW_Metabat.292
Zod_Metabat.612
Zod_Metabat.1255
ZodW_Metabat.155
Zod_maxbin.0112
ZodW_Metabat.353
Zod_Metabat.1419
ZodW_Metabat.19
Zod_Metabat.1376
Zod_Metabat.1198
ZodW_Metabat.59
ZodW_Metabat.94
Zod_Metabat.1042
ZodW_Metabat.248
ZodW_Metabat.183
ZodW_Metabat.166
Zod_Metabat.325
ZodW_Metabat.376
ZodW_Metabat.182
ZodW_Metabat.383
ZodW_maxbin.137
Zod_Metabat.545
ZodW_Metabat.217
ZodW_Metabat.319
Zod_Metabat.638
ZodW_Metabat.352
Tree scale: 0.1
Sedimentisphaerales Families
Anaerohalophaeraceae
Novel:ZNF071
SM23-30
C
Zod Metabat.1166
ZodW maxbin.277
Zod Metabat.1001
Zod Metabat.35
Zod Metabat.1436
Zod Metabat.1349
Zod Metabat.916
Zod Metabat.1380
Zod Metabat.331
Zod Metabat.769
Zod Metabat.1370
Zod Metabat.67
Zod Metabat.138
Zod Metabat.827
Zod Metabat.417
Zod Metabat.1071
Zod Metabat.289
Zod Metabat.999
Zod Metabat.1083
Zod Metabat.96
Zod Metabat.683
Zod Metabat.1411
Zod Metabat.1017
Zod Metabat.706
Zod Metabat.1213
Zod Metabat.1177
Zod Metabat.388
Tree scale: 0.1
Spirochaeales Families
ARS1246
Marispirochaetaceae
Novel:ZNF082
Novel:ZNF103
RPPD01
Novel:ZNF083
Novel:ZNF084
Spirochaetaceae_B
Novel:ZNF102
D
Zod_Metabat.156
ZodW_Metabat.38
Zod_Metabat.752
Zod_Metabat.700
ZodW_Metabat.93
ZodW_Metabat.41
Zod_Metabat.1463
Zod_Metabat.1170
Zod_Metabat.745
Zod_Metabat.1342
Zod_Metabat.1377
Zod_Metabat.281
Zod_Metabat.934
Zod_Metabat.353
ZodW_Metabat.242
Zod_Metabat.1464
Zod_Metabat.855
Zod_Metabat.749
Tree scale: 0.1
Syntrophales Families
UBA2210
UBA5619
Novel:ZNF036
Novel:ZNF035
Smithellaceae
UBA3084
MLS-D
Syntrophaceae
UBA2185
E
Zod_Metabat.153
Zod_Metabat.402
Zod_Metabat.215
Zod_Metabat.836
Zod_Metabat.309
Zod_Metabat.41
Zod_Metabat.324
Zod_Metabat.27
Zod_Metabat.781
ZodW_Metabat.303
Zod_maxbin.0017_4
Zod_Metabat.729
Zod_Metabat.88
Zod_Metabat.996
ZodW_maxbin.216
Zod_Metabat.1020
Zod_Metabat.1365
Zod_Metabat.717
Tree scale: 0.1
Woesearchaeales Families
B72-G16
Novel:ZNF08
Novel:ZNF107
UBA11576
Novel:ZNF106
CG08-08-20-14
GCA-2685855
Novel:ZNF010
UBA10107
Novel:ZNF09
SM23-78
GW2011-AR15
Novel:ZNF07
SKGA01
J091
F
ZodW_maxbin.125
Zod_Metabat.396
Zod_Metabat.513
Zod_Metabat.644
Zod_Metabat.161
Zod_Metabat.1039
Zod_Metabat.140
Zod_Metabat.1155
Zod_Metabat.825
Zod_Metabat.908
Zod_Metabat.833
Zod_Metabat.297
Zod_Metabat.390
Zod_Metabat.845
Zod_Metabat.265
Zod_Metabat.828
Zod_Metabat.712
Zod_Metabat.157
Zod_Metabat.1084
Zod_Metabat.822
Zod_Metabat.672
Tree scale: 0.1
Abundance in Gtdb
Abundant (>5 genomes)
Rare (5 genomes or less)
Novel (Absent in Gtdb)
Cultured versus Uncultured
Cultured Family
Uncultured Family


A
Sox
Sox
Dsr/Asr
H_2S Sulfide
Psr/ Hyd/Shy
Fcc/Sqr
Elemental sulfur/ Polysulfide
rDSR
SO_3^{2-} Sulfite
Sat+Apr
Apr/Sox/ Soe/Sor
SO_4^{2-} Sulfate
phs
rhd
Sox
rDSR
$S_2O_3^{2-}$ Thiosulfate
Otr/ttr
tsdA/ doxAD
$S_4O_6^{2-}$ Tetrathionate
tetH
Reduction
Oxidation
Disproportionation


B
% of genomes encoding the genes
Hyd/Shy
Psr
Apr
Dsr
Asr
rhd
otr
ttr
phs
Soe
Sor
tetH
Sox
rDSR
fccAB
SQR
tsdA
doxAD
S_2O_3 disproportionation
S_4O_6 reduction to sulfide
S_2O_3 reduction
Sulfide oxidation
S^0 oxidation
Sulfate reduction
S species oxidation
Sulfite oxidation
Polysulfide reduction
Sulfite reduction
S_4O_6 oxidation
S_2O_3 oxidation
Sediment
water
Acidobacteriota
Asgardarchaeota
Bdellovibrionota
Cloacimonadota
Eisenbacteria
Fusobacteriota
Latescibacterota
Nanoarchaeota
Planctomycetota
Sumerlaeota
UBP14
Zixibacteria
Actinobacteriota
Aureabacteria
Bipolaricaulota
CSSED10-310
FCPU426
Halobacteriota
Marinisomatota
Nitrospirota
Proteobacteria
Synergistota
UBP6
Aenigmatarchaeota
B130-G9
Calditrichota
Delongbacteria
Fermentibacterota
Armatimonadota
BMS3Abin14
Chloroflexota
Desulfobacterota
Firmicutes_A
KSB1
Myxococcota
Patescibacteria
Spirochaetota
Thermoproteota
WOR-3
Iainarchaeota
Margulisbacteria
OLB16
RBG-13-66-14
SZUA-182
UBP7_A
Altarchaeota
Bacteroidota
Campylobacterota
Dependentiae
Fibrobacterota
Krumholzibacteriota
Methanobacteriota
Omnitrophota
SM23-31
Thermoplasmatota
Verrucomicrobiota

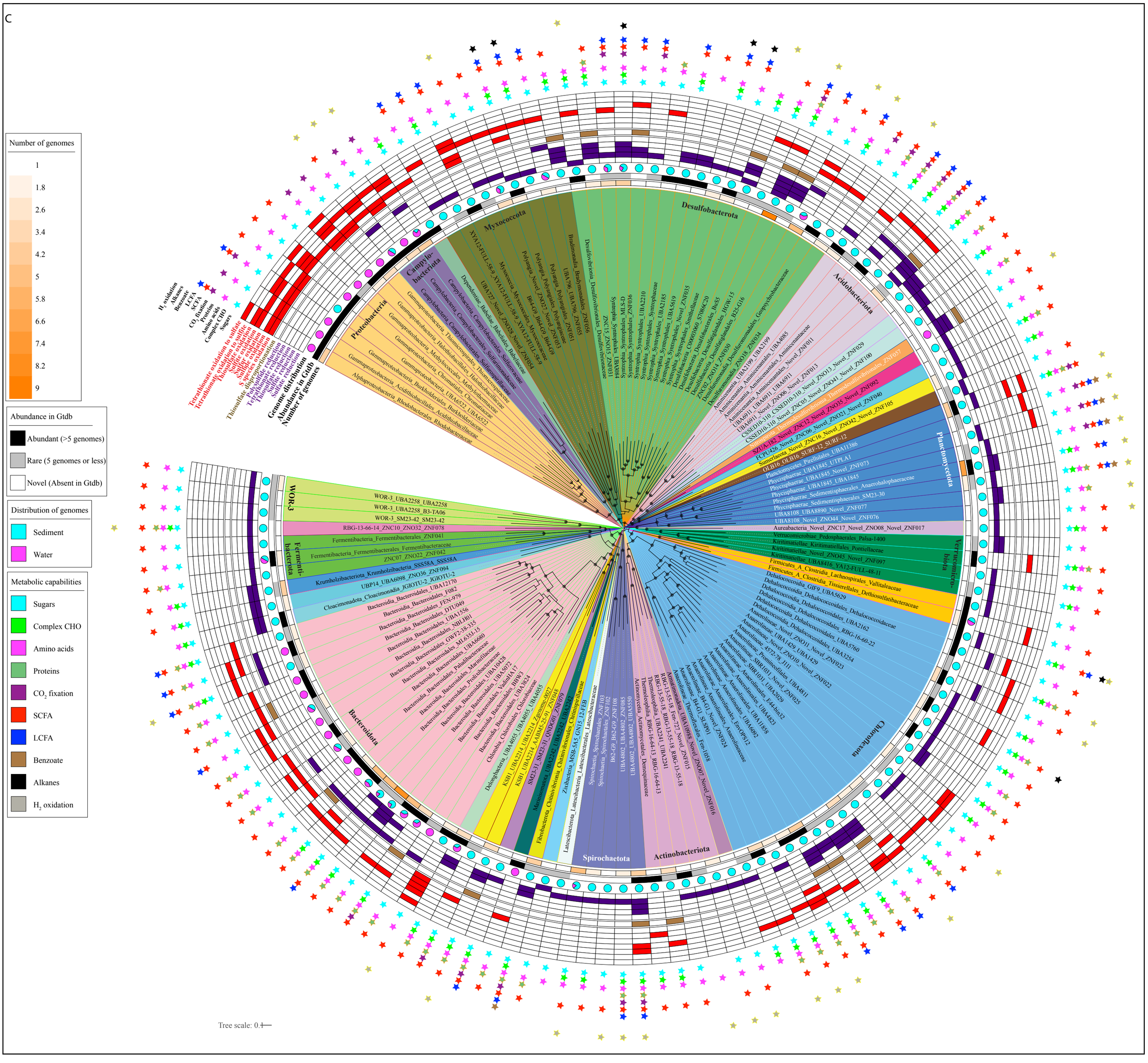
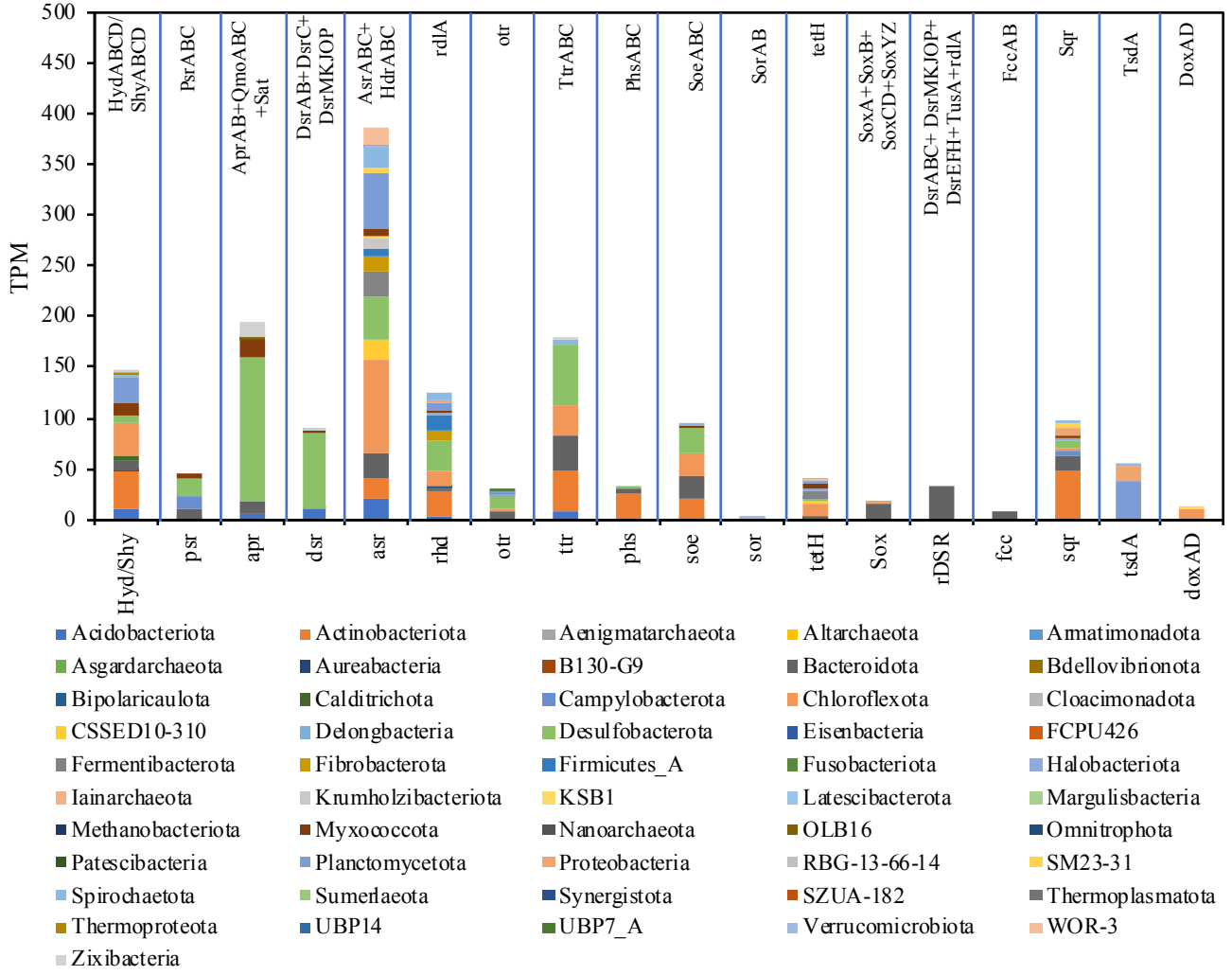

C
Number of genomes
1
1.8
2.6
3.4
4.2
5
5.8
6.6
7.4
8.2
9
Abundance in Gtdb
Abundant (>5 genomes)
Rare (5 genomes or less)
Novel (Absent in Gtdb)
Distribution of genomes
Sediment
Water
Metabolic capabilities
Sugars
Complex CHO
Amino acids
Proteins
CO_2 fixation
SCFA
LCFA
Benzoate
Alkanes
H_2 oxidation
H_2 oxidation
Alkanes
Benzoate
LCFA
SCFA
CO_2 fixation
Proteins
Amino acids
Complex CHO
Sugars
Tetrathionate oxidation to sulfate
Tetrathionate oxidation to sulfite
Thiosulfate oxidation
Sulfur oxidation
Sulfide oxidation
S species oxidation
Thiosulfate disproportionation
Polysulfide reduction
Tetrathionate reduction
Sulfate reduction
Thiosulfate reduction
Sulfite reduction
Genome distribution
Abundance in Gtdb
Number of genomes
Desulfobacterota
Acidobacteriota
Myxococcota
Campylo-bacteriota
Proteobacteria
Planctomycetota
Verrucomicro-biota
Chloroflexota
Actinobacteriota
Spirochaetota
Latescibacterota
Zixibacteria
Fibrobacterota
Marinisomatota
Bacteroidota
Fermenti-bacteriota
WOR-3
Tree scale: 0.1

Figure 6


TPM
500
450
400
350
300
250
200
150
100
50
0
HydABCD/ ShyABCD
PsrABC
AprAB+QmoABC +Sat
DsrAB+DsrC+ DsrMKJOP
AsrABC+ HdrABC
rdlA
otr
TtrABC
PhsABC
SoeABC
SorAB
tetH
SoxA+SoxB+ SoxCD+SoxYZ
DsrABC+ DsrMKJOP+ DsrEFH+TusA+rdlA
FccAB
Sqr
TsdA
DoxAD
Hyd/Shy
psr
apr
dsr
asr
rhd
otr
ttr
phs
soe
sor
tetH
Sox
rDSR
fcc
sqr
tsdA
doxAD
Acidobacteriota
Actinobacteriota
Aenigmatarchaeota
Altarchaeota
Armatimonadota
Asgardarchaeota
Aureabacteria
B130-G9
Bacteroidota
Bdellovibrionota
Bipolaricaulota
Calditrichota
Campylobacterota
Chloroflexota
Cloacimonadota
CSSED10-310
Delongbacteria
Desulfobacterota
Eisenbacteria
FCPU426
Fermentibacterota
Fibrobacterota
Firmicutes_A
Fusobacteriota
Halobacteriota
Iainarchaeota
Krumholzibacteriota
KSB1
Latescibacterota
Margulisbacteria
Methanobacteriota
Myxococcota
Nanoarchaeota
OLB16
Omnitrophota
Patescibacteria
Planctomycetota
Proteobacteria
RBG-13-66-14
SM23-31
Spirochaetota
Sumerlaeota
Synergistota
SZUA-182
Thermoplasmatota
Thermoproteota
UBP14
UBP7_A
Verrucomicrobiota
WOR-3
Zixibacteria