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Sulfur and oxygen isotope fractionation during bacterial sulfur disproportionation under anaerobic haloalkaline conditions

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Key words: soda lakes; sulfate formation; sulfide formation

ABSTRACT

Sulfur and oxygen isotope fractionation of elemental sulfur disproportionation at anaerobic haloalkaline conditions were evaluated for the first time. Isotope enrichment factors of the strains *Desulfurivibrio alkaliphilus* and *Dethiobacter alkaliphilus* growing at pH 9 to 10 were significantly smaller compared to previously published values of sulfur disproportionators at neutral pH. We propose that this discrepancy iscaused by masking effects due to preferential formation of polysulfides at high pH leading to accelerated internal sulfur turnover rates, but cannot rule out distinct isotope effects due to specific enzymatic disproportionation reactions under haloalkaline conditions. The results imply that the microbial sulfur cycle in haloalkaline environments is characterized by specific stable sulfur and oxygen isotope patterns.

INTRODUCTION

In addition to sulfate and sulfur reduction as well as sulfide oxidation, the disproportionation of intermediately oxidized sulfur compounds including thiosulfate, sulfite and elemental sulfur, plays a major role in the global sulfur cycle (Canfield and Thamdrup 1996; Habicht et al. 1998; Philippot et al. 2007; Finster 2008). In disproportionation reactions the partially oxidized sulfur compounds are concomitantly oxidized to sulfate and reduced to sulfide similar to the classical fermentation process of organic compounds. Sulfur disproportionators shuttle electrons between different atoms of the same molecule, and it has been proposed that ATP is formed by both substrate-level and proton motive force-dependent phosphorylation (Krämer and Cypionka 1989; Finster et al. 2013). The disproportionation of elemental sulfur (eq. 1) is of special interest as this compound is a common and quantitatively important intermediate of sulfide oxidation processes at the oxic/anoxic interphase especially of marine sediments (Canfield and Thamdrup 1996).

$$4\ S^0 + 4\ H_2O \rightarrow SO_4^{2-} + 3\ HS^- + 5\ H^+$$

$$\Delta G^0 = +10.2\ \text{kJ mol}^{-1}\ (\text{per } S^0) \qquad (1)$$

Despite the fact that sulfur-disproportionating microbes have been enriched from marine and freshwater sediments (Canfield et al. 1998), only a few have been obtained in pure cultures (Finster 2008). For thermodynamic reasons the process is restricted to low sulfide concentrations and consequently the removal of sulfide, for instance by oxidation and/or precipitation with iron-bearing solids such as FeOOH, is essential, as it shifts the thermodynamics of this reaction in exergonic direction (Thamdrup et al. 1993; Frederiksen and Finster 2004). The addition of iron minerals such as FeOOH changes the stoichiometry of the reaction (eq. 2 and 3), as part of the produced sulfide is reoxidized chemically (Peiffer et al. 1992).

61

62 $$3\ HS^- + 2\ Fe^{III}OOH + 3H^+ \rightarrow S^0 + 2\ Fe^{II}S + 4\ H_2O$$

63 $$\Delta G^0 = -143.9\ kJ\ mol^{-1}\ (per\ S^0) \quad (2)$$

64

65 $$3\ S^0 + 2\ Fe^{III}OOH \rightarrow SO_4^{2-} + 2\ Fe^{II}S + 2\ H^+$$

66 $$\Delta G^0 = -30\ kJ\ mol^{-1}\ (per\ S^0) \quad (3)$$

67

68 Notably, under alkaline conditions, reaction (1) becomes also more exergonic due to the
69 production of protons.
70 Currently, the pathway of elemental sulfur disproportionation is poorly understood. Studies based
71 on enzyme assays by Frederiksen and Finster (2003) proposed a reaction scheme involving the
72 formation of sulfite as key intermediate, which could be subsequently oxidized to sulfate by the
73 reversed first steps of the dissimilatory sulfate reduction pathway. Notably, the genome of the
74 recently sequenced marine sulfur-disproportionating deltaproteobacterium *Desulfocapsa*
75 *sulfexigens* strain SB164P1 contains a complete set of genes necessary for sulfate reduction
76 (Finster et al. 2013). However, with the exception of *Desulfocapsa thiozymogenes*, no studied
77 sulfur-disproportionating strains were able to use sulfate as an electron acceptor. Therefore, these
78 genes may encode the reversed sulfate reduction pathway proteins involved in the
79 disproportionation of elemental sulfur (Frederiksen and Finster 2003).
80 One approach to investigate metabolic pathways is the analysis of stable isotopes.
81 Disproportionation reactions were reported to be accompanied by a considerable fractionation of
82 ^{34}S /^{32}S sulfur (from elemental sulfur to sulfide and sulfate) and ^{18}O /^{16}O oxygen (incorporation
83 of oxygen from water into sulfate) isotopes. In several studies it was observed that sulfide
84 produced during the disproportionation reaction was depleted in ^{34}S by -3.7 to -15.5 ‰ relative to

85 the source (elemental sulfur) whereas sulfate was enriched by +11.0 to +35.3 ‰ (Canfield and
86 Thamdrup 1994; Canfield et al. 1998; Böttcher et al. 2001; Böttcher and Thamdrup 2001;
87 Böttcher et al. 2005). Thus, ^{32}S was preferentially used for the electron accepting part of the
88 reaction (formation of sulfide) while ^{34}S was preferentially utilized in the electron-donating step
89 (sulfate production); the remaining elemental sulfur pool maintained the initial isotope value
90 (Canfield et al. 1998). In contrast, the spontaneous purely chemical disproportionation of
91 elemental sulfur is associated with a relatively small sulfur isotope fractionation between the
92 formed sulfate and sulfide ($\Delta^{34}S(SO_4\text{-}H_2S)$); differences were shown to range between -0.4 to -
93 3.0‰ (Smith 2000). The sulfur isotope fractionation of this reaction has been shown to depend on
94 the reaction temperature, the duration of the reaction and the extent of the conversion (Smith
95 2000).
96 Since microbial sulfur disproportionation is a strictly anaerobic process, the oxygen atoms in the
97 newly formed sulfate are fully derived from water, similar to the oxidation of sulfide to sulfate
98 (Toran and Harris 1989). In addition to the canonical fractionation, an exchange of oxygen
99 isotopes between cell-internal sulfur compounds (in particular sulfite) and ambient water could
100 strongly influence the measured isotopic fractionation (Fritz et al. 1989; Brunner et al. 2005;
101 Knöller et al. 2006; Turchyn et al. 2010; Müller et al. 2013a, b) depending on the exchange rates.
102 Once the sulfate is formed, the oxygen isotope composition is conserved as the exchange of
103 oxygen isotope between sulfate and water is extremely slow at neutral and alkaline conditions
104 (Lloyd 1968; Mizutani and Rafter 1969a, b; Chiba and Sakai 1985). During the incorporation of
105 oxygen into sulfate, an isotope fractionation in favor of ^{18}O between +8.2 to +21.6 δ‰ was
106 reported (Böttcher and Thamdrup 2001; Böttcher et al. 2001; Böttcher et al. 2005).
107 Taking all isotopic data into account, a conserved and uniform biogeochemistry of elemental
108 sulfur disproportionation has been suggested by Canfield et al. (1998) for neutrophilic sulfur

disproportionators of marine and fresh water origin. To test the validity of this hypothesis for alkaline conditions, we performed isotope fractionation experiments with the haloalkaliphilic deltaproteobacterium *Desulfurivibrio alkaliphilus*, and a representative of the order *Clostridiales*, *Dethiobacter alkaliphilus*, isolated from soda lakes (Sorokin et al. 2008; 2010; 2011). In a previous study, we could demonstrate that both species can grow by disproportionation of elemental sulfur to sulfide and sulfate at pH 9-10, and that polysulfides were the actual substrate of disproportionation under such conditions (Poser et al. 2013).

In the present study we measured the sulfur and oxygen isotope fractionation during alkaline sulfur disproportionation to determine whether it is similar or different compared to the classical fractionation described for neutral conditions. Such information may help to elucidate whether sulfur disproportionation under neutral and alkaline conditions has a unique or different biochemistry. Combined sulfur and oxygen isotopic fingerprints have furthermore a potential for identifying sulfur disporportionation in natural alkaline habitats.

2. METHODS

2.1. Cultivation and experimental setup

Desulfurivibrio alkaliphilus and *Dethiobacter alkaliphilus* were taken from the strain collection of D.Y. Sorokin. Detailed information about genetic, phylogenetic and morphologic aspects of these strains can be found in Sorokin et al. (2008). Both strains were incubated under anaerobic conditions at 37°C and pH 10 in modified DSMZ medium 1104 with sulfur (30 mM) and acetate (4.9 mM) as energy and carbon sources as described by Poser et al. (2013). All experiments were carried out in 120 ml glass serum bottles containing 100 ml medium and 20 ml head space. The culture bottles were prepared inside an anaerobic glove box (gas atmosphere – N_2:H_2 (95:5); Coy

133 Laboratory Products Inc., USA) and sealed with Teflon-coated butyl rubber stoppers and
134 aluminium crimps. Subsequently, the headspace of the serum bottles was flushed with nitrogen
135 for 15 min to remove traces of hydrogen. Flowers of elemental sulfur were sterilized as described
136 elsewhere (Thamdrup et al. 1993). Freshly prepared ferric oxyhydroxide (goethite / α-FeOOH)
137 (Lovely and Phillips, 1986) was added in excess (0.2 M) to capture the produced sulfide for
138 isotope measurements (Thamdrup et al. 1993; Böttcher et al. 2001; Böttcher and Thamdrup
139 2001). Each isotope fractionation experiment was carried out using nine active parallel cultures
140 and two negative controls (anoxic, without biomass). For each strain, three independent
141 experiments were performed. Culture medium was inoculated with 5% (vol/vol) of a preparatory
142 culture pre-grown at sulfur-disproportionating conditions. Before inoculation, the preparatory
143 culture was centrifuged and washed twice with DSMZ medium 1104 to remove remaining sulfate
144 and iron sulfide. After approximately 30%, 60% and 100% of the elemental sulfur was consumed
145 three out of the nine cultures were harvested for isotope analyses at each time point, respectively,
146 to calculate sulfur and oxygen isotope discrimination. Before harvesting, the cultures were
147 vigorously shaken to disperse the precipitated iron sulfide.
148
149 **2.2. Microscopy**
150 Cells were counted by epifluorescence microscopy (Adrian et al. 2007). Eighteen µL of a well-
151 mixed cell suspension was mixed with 1 µL SYBR Green (Bio Rad) with a pipette and stored for
152 15 min in the dark. Afterwards, this mixture was immobilized on agarose-coated slides, sealed
153 with a cover slip and examined by epifluorescence microscopy (Nikon Eclipse TE300). To
154 guarantee accurate counting, each sample/slide was scanned in a z-pattern and 10 pictures were
155 taken with a Nikon DXM 1200F digital camera (fixed focus and aperture). Cell count data were
156 produced through analysis of the pictures with the ImageJ software. To preclude an interference

157 of the solid phases of FeOOH/FeS on the cell-counting, we adjusted the measurement by the
158 ImageJ software to the size of the cells.
159
160 **2.3. Chemical and isotopic analyses**
161 The concentration of sulfide plus sulfane-sulfur of polysulfides was determined
162 spectrophotometrically with the methylene blue method according to Cline (1969) using
163 modifications described by Herrmann et al. (2008). Samples were taken under anoxic conditions
164 and fixed immediately with zinc acetate. Sulfate in the supernatant was analyzed by ion
165 chromatography (DX 500 Dionex) using an IonPacAS18 / AG18 column and KOH (23 mM) as
166 eluent; samples were taken under anoxic conditions and subsequently filtered (0.20 µm pore
167 size).
168 For isotopic measurements, the formed FeS was separated from the remaining solution by
169 vacuum filtration (cellulose-acetate filters with 0.45 µm pore size). The FeS-containing filters
170 were stored inside an anaerobic jar until further analysis. To determine the sulfur isotope
171 composition ($\delta^{34}S$) of sulfide and sulfate, the acid-volatile fraction of sulfide (AVS) was distilled
172 with 6 N HCl and the liberated hydrogen sulfide was first precipitated as zinc sulfide (reaction
173 with zinc acetate) and subsequently converted to silver sulfide after reaction with silver nitrate.
174 Notably, AVS was the only major sulfide pool. The formed sulfate was precipitated as barium
175 sulfate after reacting with a barium chloride solution (Canfield et al. 1998; Böttcher and
176 Thamdrup 2001; Knöller et al. 2008). Both compounds (AVS and sulfate) were converted and
177 measured as SO_2 using an elemental analyzer coupled with an isotope ratio mass spectrometer
178 (DeltaS, ThermoFinnigan, Bremen, Germany). The analytical precision of the sulfur isotope
179 measurement was better than ±0.4‰ (2σ). Calibration and normalization of the $\delta^{34}S$ data was
180 carried out using the IAEA (International Atomic Energy Agency) materials IAEA-S1 (Ag_2S)

and NBS 127 ($BaSO_4$) as reference materials. The assigned values were -0.3‰ for IAEA-S1 and +20.3‰ for NBS 127. Sulfur isotope compositions are reported in delta notation relative to VCDT (Vienna Cañon Diablo Troilite) (eq. 4). Oxygen isotope analyses of barium sulfate and ferric oxyhydroxide were performed by high temperature pyrolysis at 1450°C in a TC/EA (High Temperature Conversion Elemental Analyzer) coupled to a delta plus XL mass spectrometer (both ThermoFinnigan, Bremen, Germany). Precision of the oxygen isotope measurements was ±0.6‰ (2σ). The normalization of the ^{18}O-SO_4^{2-} values was performed using the IAEA reference material NBS 127 with an assigned $\delta^{18}O$ value of +8.7 ‰. The ^{18}O / ^{16}O ratio of the bulk water was determined by laser cavity ring-down spectroscopy (Picarro L2120-i, Santa Clara, USA) (Godoy et al. 2012) with an analytical error of ±0.2 ‰ (2σ). Oxygen isotope compositions are reported relative to Vienna-Standard Mean Ocean Water (VSMOW) (eq. 4).

$$\delta^{18}O = (^{18}O/^{16}O_{sample} / {}^{18}O/^{16}O_{standard}) - 1 \quad (4)$$

Isotope enrichment factors for sulfur and oxygen isotopes were calculated by subtracting the initial isotope compositions of elemental sulfur ($\delta\ ^{34}S$) and oxygen from water ($\delta\ ^{18}O$) from the final isotope compositions of sulfide and sulfate ($\delta^{34}S$) and oxygen from sulfate ($\delta\ ^{18}O$). Since no sulfide and sulfate was transformed from the initiating cultures no correction of the enrichment factors was necessary.

3. RESULTS AND DISCUSSION

3.1. Polysulfides as substrates for disproportionation

We recently reported that polysulfides were formed during elemental sulfur disproportionation under alkaline conditions (Poser et al. 2013), even in the presence of FeOOH, which reacts immediately with sulfide and precipitates as FeS. The formation of polysulfides is favored at pH > 9 by a chemical equilibrium reaction between sulfide and elemental sulfur (Schauder and Müller 1993; eq. 5).

$$HS^- + (n-1)\ S^0 \leftrightarrow S_n^{2-} + H^+ \qquad (5)$$

The concentrations of the (total) polysulfide species in our experiments were up to 0.9 mM (sum of all polysulfides) in the presence of FeOOH (Poser et al. 2013). We also observed that the polysulfide concentrations at the beginning of the incubation were close to zero, indicating that sulfide first had to be formed by elemental sulfur disproportionation to form polysulfides. Disproportionation of polysulfides is indicated by sulfide: sulfate ratios of ~ 4 (3.6 to 4.3)for S_4^{2-} to S_8^{2-} (Milucka et al. 2012). However, the stoichiometry is altered by precipitation and re-oxidation of the produced sulfide with Fe-oxyhydroxide, which might be the reason why the expected ratios were not observed in our previous study (Poser et al. 2013). The observed sulfide: sulfate ratio of ~ 2:1 during elemental sulfur disproportionation by the tested alkaline cultures fits well with the theoretical value shown by equation (3) and should cause, depending on the fractionation, an isotope mass balance at a ratio of -1:2. Notably, the calculated stoichiometry based on enrichment factors is in the range of -1:4 for both alkaline strains (Table 1). However, the discrepancy between mass and isotope balance is not an appropriate indicator for polysulfide disproportionation as the isotope balance is likely influenced by sulfur isotope exchange and equilibrium reactions between sulfur and sulfide (probably via polysulfides) resulting in a pool of ^{34}S-depleted sulfur, sulfide and polysulfides as produced ^{34}S-enriched sulfate does not further

228 react. Thus, the pool of elemental sulfur may change its isotope composition, and the apparent
229 enrichment factor for sulfide formation may change as well, indicated by a relatively large error
230 (Table 1). . As discussed below in section 3.2, indirect indicators of polysulfide
231 disproportionation under alkaline conditions are the high substrate turnover rates of
232 *Desulfurivibrio alkaliphilus* and *Dethiobacter alkaliphilus*, which might be caused by enhanced
233 uptake and cell internal transport of polysulfides which are water soluble in contrast to elemental
234 sulfur.
235
236 **3.2. Fractionation of stable sulfur isotopes during sulfur disproportionation**
237 Disproportionation of elemental sulfur by *Desulfurivibrio alkaliphilus* (DSV) and *Dethiobacter*
238 *alkaliphilus* (DTB) in the presence of FeOOH resulted in the production of sulfide (HS^-+ sulfane
239 atoms of polysulfides) and sulfate in a ratio of 1.8:1 and 2.1:1, respectively (Poser et al. 2013).
240 These values are close to the theoretical value of 2:1 (eq. 3) (Thamdrup et al. 1993). For both
241 strains, the produced sulfate was enriched in ^{34}S over time, whereas sulfide became ^{34}S depleted
242 as it was reported for sulfur disproportionation at neutral pH (Canfield and Thamdrup 1994;
243 Canfield et al. 1998, Böttcher et al. 2001; Böttcher and Thamdrup 2001). However, the
244 fractionation values of the sulfur isotopes were significantly lower than those reported in previous
245 studies for neutrophilic bacteria (Table 1). We observed a fractionation of -0.9 ± 0.3 ‰ (DSV)
246 and -1.0 ± 0.5 ‰ (DTB) for the formed sulfide and +4.7 ± 0.4 ‰ (DSV) and +3.6 ± 1.3 ‰ (DTB)
247 for sulfate, respectively (Table 1). The difference to the values observed for neutrophilic
248 disproportionators might have been caused by various factors: (i) sulfur disproportionation at
249 neutral and alkaline conditions is biochemically similar, but isotope fractionation is considerably
250 masked under alkaline conditions, (ii) a different biochemistry of the process under haloalkaline

conditions, or (iii) abiotic isotope effects due to alkaline polysulfide chemistry. We will discuss these possible effects in the following.

(i) Masking of isotope fractionation

Masking of kinetic isotope fractionation can take place if mass transfer-dependent, non-fractionating processes by which the substrate is transferred to the enzyme catalysing the reaction become rate-limiting. Such masking can be due to high concentration of enzymes (Templeton et al. 2006) and occurs also at low substrate concentrations due to limited substrate bioavailability (Thullner et al. 2008; Kampara et al. 2008). However, sulfur disproportionation is thought to consist of multiple different enzymatic steps (Finster 2008), and the isotope fractionation measured in the final products of the pathway, sulfate and sulfide, is thus the sum of isotope fractionation of each step of this metabolic network. In biochemical pathways, the flow of substrates and hence, the magnitude of isotope fractionation is usually controlled by various environmental and physiological factors; more complicating, many reactions are reversible and characterized by considerable backward reactions. For example, the magnitude of sulfur isotope fractionation associated with dissimilatory sulfate reduction in a single sulfate reducer depends largely on the cell specific sulfate reduction rate and corresponding growth rate: the lower these rates (due to limited available energy), the higher the sulfur isotope fractionation, and vice versa (Bradley et al. 2011; Sim et al. 2011; Wing and Halvey 2014). Upon low energy conditions, the enzymes of the sulfate reduction pathway operate maximally reversible leading to near equilibrium conditions resulting in maximal sulfur isotope fractionation (Brunner and Bernasconi, 2005). Analogously, sulfur isotope fractionation during sulfur disproportionation might be controlled as well by the cell-specific disproportionation rates; notably, the reactions of the oxidative branch of the pathway forming sulfate from sulfite may be similar to the dissimiliatory sulfate reduction pathway (Frederiksen and Finster 2003). At haloalkaline

275 conditions, polysulfides - which are dispropotionated by the used model strains rather than
276 elemental sulfur (Poser et al. 2013; see also section 3.1) – are much better bioavailable compared
277 to neutral conditions. Polysulfides are ionic linear molecules and, therefore, much more reactive
278 and mobile than hardly water-soluble cyclic molecules of elemental sulfur. Therefore,
279 solubilzation of the crystalline ring sulfur with sulfide to form polysulfides stable at high pH
280 increases the whole sulfur-dependent conversion strongly as has been shown for a specialized
281 polysulfide-respiring haloalkaliphilic bacterium *Desulfurispira natronophila* isolated from soda
282 lakes (Sorokin and Muyzer 2010). Due to high concentrations and increased stability of
283 polysulfides, cultures of DSV and DTB showed doubling times of six to seven hours (Poser et al.
284 2013), which is significantly faster than the doubling times reported for neutrophilic elemental
285 sulfur disproportionating strains (24 to 48 h; Thamdrup et al. 1993; Finster et al. 1998; Janssen et
286 al. 1996; Canfield and Thamdrup 1996). Elemental sulfur disproportionation at neutral pH
287 conditions is likely driven by polysulfides, too. It is known that at pH values > 6, a small pool of
288 polysulfides develops in the presence of excess elemental sulfur and moderate concentrations of
289 sulfide (1 mM) (Schauder and Müller 1993); furthermore, sulfur transferase systems have been
290 described binding and transporting polysulfides effectively even at low concentrations (Klimmek
291 et al. 1999; Lin et al. 2004). If polysulfides are actually used as substrate by neutrophilic
292 disproportionators (rather than elemental sulfur), the uptake of polysulfides is expected to be a
293 rate-limiting step due to their limited bioavailability at neutral pH. Consequently, the cell-internal
294 concentration of polysulfides in neutrophilic disproportionators is expected to be low, resulting in
295 low disproportionating rates and reversible enzymatic reactions; under these conditions, sulfur
296 isotope effects may be considerably expressed due to equilibrium isotope fractionation processes
297 – similar as described for sulfur isotope fractionation upon dissimilatory sulfate reduction. Here,
298 high fractionation seems to be possible even at very low substrate (here: sulfate) concentrations

299 as long as the rate of dissimilatory sulfate reduction is low enough (Wing and Halvey 2014).
300 Notably, this scenario contradicts to the general rule of thumb for isotope fractionation that
301 isotope fractionation effects downstream of a rate-limiting step are not expressed. By contrast,
302 isotope fractionation might be masked under haloalkaline conditions due to the higher sulfur
303 disproportionation rates and reduced reversibility of polysulfide disproportionation steps.
304 *(ii) Different biochemical pathways*
305 A different isotope fractionation pattern would probably also evolve if the enzymatic pathway of
306 sulfur disproportionation under alkaline conditions is different compared to pH neutral
307 conditions. However, the exact mode of electron flow during elemental sulfur disproportionation
308 is currently not completely understood, especially the reductive branch of the pathway leading to
309 sulfide formation, and the oxidative part resulting in sulfite formation (Frederiksen and Finster
310 2003; Finster 2008; Finster et al. 2013). Thus, possible effects caused by different enzymatic
311 reactions are currently purely speculative. Moreover, sulfur isotope fractionation upon elemental
312 sulfur disproportionation is not expected to be controlled by the activity of a single enzyme, in
313 analogy to dissimilatory sulfate reduction (Wing and Halvey 2014).
314 The extent of isotope fractionation was nearly similar under different alkaline pH regimes and
315 temperatures. At pH 9 (drop from pH 10 in our experiments) and 37°C, the sulfur isotope
316 fractionation shifted slightly towards more ^{34}S depleted values for sulfide and sulfate (Table 2)
317 although the growth characteristics did not change considerably (data not shown). When
318 incubated at pH 10 and 22°C both cultures showed extended lag phases (data not shown) and
319 sulfide and sulfate isotope values again became slightly more ^{34}S depleted (Table 2) compared to
320 values detected at pH 10 and 37°C.
321 *(iii) Abiotic isotope effects*

322 Amrani et al. (2006) investigated the distribution of the sulfur isotopes of polysulfide ions with
323 an artificial polysulfide solution [equilibrium reaction between S^0 and $(NH_4)_2S$] at pH 9 and
324 reported that polysulfides were enriched in ^{34}S and that this enrichment increased with increasing
325 polysulfide chain length. Furthermore, the authors showed that polysulfides are a highly dynamic
326 and complex sulfur pool due to sulfur isotope exchange between elemental sulfur and sulfide
327 within the polysulfide chain and sulfur isotope exchange between the polysulfides and the
328 remaining sulfur species in the system. Interestingly, an enrichment of ^{34}S in the zero valent
329 sulfur moiety compared to the sulfane moiety of the chain was observed. Given that polysulfides
330 have a $S\text{-}S^0_n\text{-}S^-$ structure, it can be speculated that the heavier S^0 atoms are located medial or
331 subterminal between the two terminal (sulfane) sulfur atoms of the chain, which would increase
332 the chains stability by stronger S-S bonds. Thus, the heavier sulfur isotopes in the middle part of
333 the chain might not be as reactive as the lighter sulfur isotopes at the terminal sites. Therefore, the
334 value of the elemental sulfur isotope composition (in our case: +5.3‰) depends on the dynamics
335 of the above-described processes of polysulfides formation and depletion and might be variable.
336 Such dynamic processes of polysulfide chain formation could also explain the observed
337 increasing (for sulfate) and decreasing (for sulfide) sulfur isotope fractionation values observed in
338 the present study.
339 Notably, we observed an increasing sulfur isotope fractionation of sulfide and sulfate for both
340 strains over time (Figure 1). A similar trend has been demonstrated in studies by Canfield et al.
341 (1998) and Böttcher et al. (2001). A trend to lighter sulfur isotopes for sulfide is explainable by a
342 partial chemical reoxidation of the produced ^{34}S-depleted sulfide to elemental sulfur by ferric iron
343 (eq. 2); the hereby formed ^{34}S-depleted elemental sulfur could have been disproportionated again
344 to sulfide and sulfate.. However, this scenario is inconsistent to the observed heavier sulfur
345 isotope values of sulfate (which should become also lighter with time) and therefore implausible.

346 The trend of increasing fractionation of sulfide and sulfate with time is explainable by cell
347 growth: due to increases in cell numbers and enzymes, polysulfides become more limited, leading
348 to a higher reversibility of the process, resulting in a higher fractionation.
349
350 **3.3. Stable oxygen isotope effects during the incorporation of water into sulfate**
351 Disproportionation of elemental sulfur is usually accompanied by an oxygen isotope
352 discrimination favoring the enrichment of ^{18}O in the formed sulfate by about +17 ‰ (for
353 *Desulfocapsa thiozymogenes*) and up to +22 ‰ (for *Desulfobulbus propionicus*) (Böttcher et al.
354 2001; Böttcher et al. 2005). Similar to the results for sulfur isotope fractionation, the enrichment
355 of ^{18}O in the formed sulfate was significantly lower under alkaline conditions than under neutral
356 conditions. We measured a fractionation of +7.8 ± 3.9 ‰ for culture DSV and +4.3 ± 2.8 ‰ for
357 culture DTB, respectively; no evidence was found that the adjusted pH or temperatures changed
358 the extent of oxygen isotope fractionation (Table 2). Similar values of oxygen enrichment factors
359 - ranging between 0 and +4 ‰ - were reported for biological and abiotic sulfide oxidation to
360 sulfate under anoxic conditions (Lloyd 1968; Toran and Harris 1969; Taylor et al. 1984a, b; van
361 Everdingen and Krouse 1985; van Stempvoort and Krouse 1994; Balci et al. 2007). In contrast, a
362 slight depletion in ^{18}O of formed sulfate relative to the isotope composition of water was recently
363 reported for *Thiobacillus denitrificans* and *Sulfurimonas denitrificans* upon sulfide oxidation
364 under nitrate-reducing conditions, which was presumably linked to exchange reactions of nitrite
365 (formed during nitrate reduction) and water (Poser et al. 2014). Sulfate might be formed by
366 similar biochemical reaction during sulfide oxidation and elemental sulfur disprorportionation,
367 involving sulfite and adenosine 5′-phosphosulfate (APS) as intermediates (Friedrich et al. 2001;
368 Finster 2008; Finster et al. 2013; Poser et al. 2014); thus, oxygen isotope fractionation of anoxic
369 sulfide oxidation and disproportionation might be in a comparable range. However, neutrophilic

370 disproportionating cultures showed significantly higher oxygen isotope fractionation (Böttcher et
371 al 2001, 2005).
372 Analogously to reactions of the dissimilatory sulfate reduction pathway, oxygen isotope effects in
373 the formed sulfate during disproportionation of sulfur are probably controlled by oxygen isotope
374 exchanges via water during polysulfide oxidation to sulfite (formed by sulfur oxidation), sulfite
375 oxidation to sulfate, and possible back-reactions if these reactions are reversible. For
376 dissimilatory sulfate reduction, rapid oxygen isotope exchange for cases where sulfur isotope
377 fractionation is large and slow exchange for cases where sulfur isotope fractionation is small was
378 recently predicted (Brunner et al. 2012). Such a model could also explain large oxygen isotope
379 fractionation in slow growing, large sulfur fractionating neutrophilic disproportionators, and
380 small oxygen isotope fractionation in fast growing, small sulfur fractionating alkalphilic
381 disproportionators. Notably, under alkaline conditions, the incorporated oxygen stems
382 preferentially from hydroxyl ions (OH^-) and not from water (H_2O), influencing oxygen isotope
383 fractionation under alkaline conditions considerably as the $\delta^{18}O$ of OH^- is 35–40 ‰ lower than
384 that of H_2O at 25°C. For example, alkaline phosphatase and acid phosphatase produce phosphate
385 with different oxygen isotope composition due to this reason (von Sperber 2014). Thus, oxygen
386 the observed small oxygen isotope effect during sulfur disproportionation under alkaline
387 conditions could be also due to preferential incorportation of isotopically light OH^- ions.
388
389 **4. CONCLUDING REMARKS**
390 In this study we report enrichment factors for sulfur and oxygen isotope fractionation during
391 bacterial sulfur disproportionation under haloalkaline conditions. The ^{34}S and ^{18}O isotope
392 fractionation was significantly lower compared to data reported for elemental sulfur
393 disproportionating bacteria at neutral pH. Under haloalkaline conditions, the concentration of

394 polysulfides, the proposed actual substrate of elemental sulfur disproportionation, is considerably
395 higher compared to neutral pH conditions due to the chemical stability of polysulfides at high pH.
396 We suggest that the better bioavailability of polysulfides leads to increased cell-specific growth
397 and sulfur disproportionation rates under haloalkaline conditions, resulting finally in a masking of
398 sulfur and oxygen isotope fractionation. However, as the biochemical pathways for sulfur
399 disproportionation in neutrophilic and alkaliphilic disproportionators are not fully elucidated yet,
400 the observed differences in sulfur and oxygen isotope fractionation might be also caused by
401 different set of enzymes. Oxygen isotope fractionation might be also influenced by the
402 preferential incorporation of OH^- ions under alkaline conditions. The measured sulfur and oxygen
403 isotope fractionation factors are furthermore valuable model culture data usable for estimating
404 sulfur disproportionation processes in haloalkaline environments by stable sulfur and oxygen
405 isotope analyses.
406

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631

632 **LEGENDS OF FIGURES AND TABLES**

633

634 Figure 1: Progressing sulfur isotope fractionation during the disproportionation of elemental
635 sulfur to sulfide (AVS = Acid-Volatile Sulfide) and sulfate by *Desulfurivibrio alkaliphilus* (DSV)
636 and *Dethiobacter alkaliphilus* (DTB) at pH 10 and 37°C. The initial isotope value of elemental
637 sulfur was +5.3 ‰.

638

639 Table 1: Isotope enrichment factors (ε 34S) for sulfur disproportionation by pure cultures of
640 neutrophilic Deltaproteobacteria (*Desulfocapsa thiozymogenes, Desulfocapsa sulfexigens* and
641 *Desulfobulbus propionicus*) obtained by Canfield et al. (1998) compared to the factors obtained
642 for the haloalkaliphilic strains *Desulfurivibrio alkaliphilus* and *Dethiobacter alkaliphilus* in this
643 study. In addition, ratios of sulfide to sulfate sulfur enrichment factors are shown.

644

645 Table 2: Sulfur and oxygen isotope fractionation during elemental sulfur disproportionation by
646 *Desulfurivibrio alkaliphilus* and *Dethiobacter alkaliphilus* under different experimental
647 conditions. Presented are the enrichment factors and the standard deviation in for sulfide, sulfate
648 and oxygen (95% confidence level / 2σ, n = 2 - 9). The oxygen atoms in sulfate are completely
649 derived from water.

Figure 1

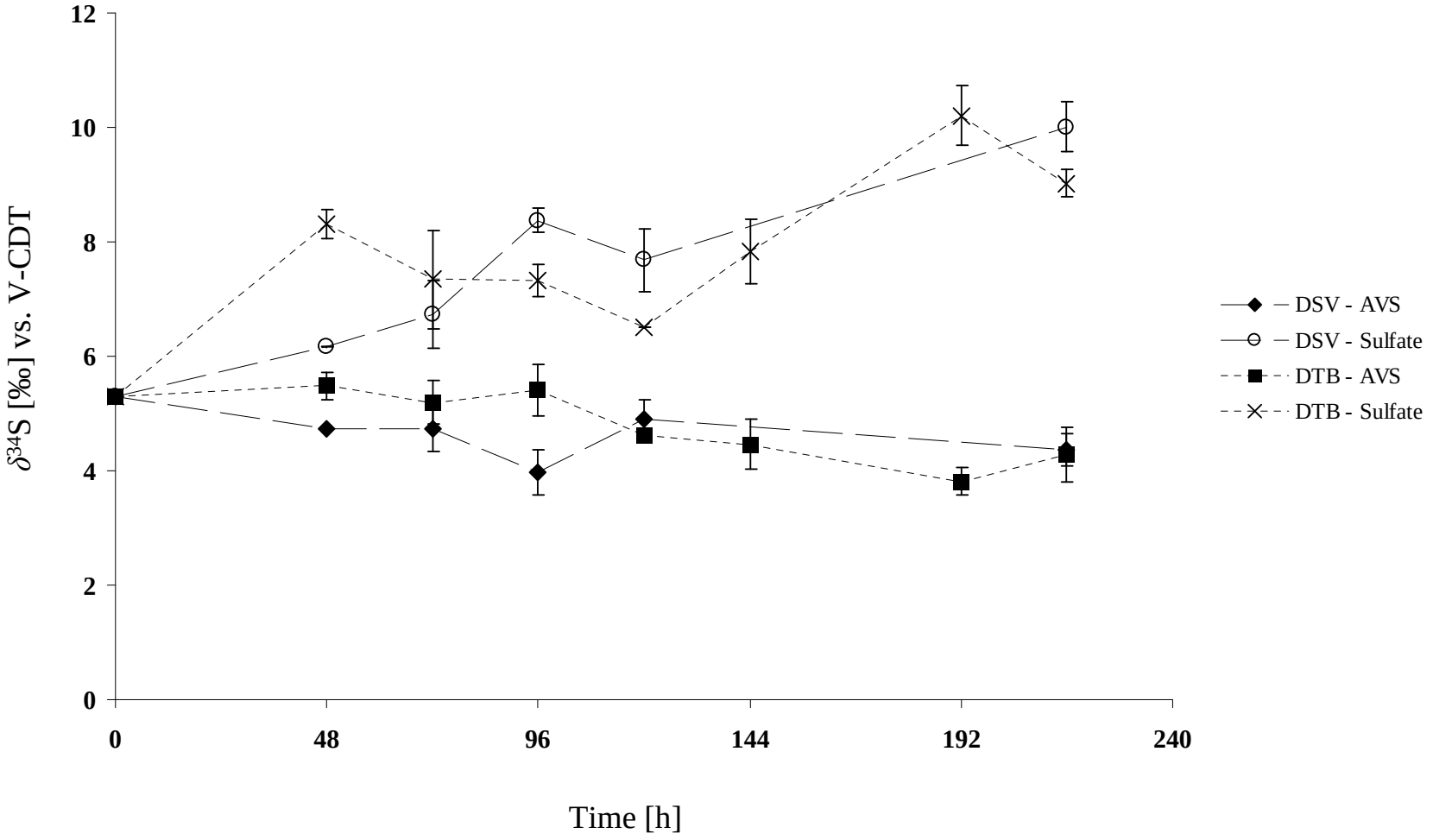
12
10
8
6
4
2
0
0
48
96
144
192
240
δ34S [‰] vs. V-CDT
Time [h]
DSV - AVS
DSV - Sulfate
DTB - AVS
DTB - Sulfate

Table 1

Strain	Sulfur compound	$^{34}\varepsilon$ [‰]	Ratio	Reference
Desulfocapsa thiozymogenes	Sulfide Sulfate	-5.9 17.3	-1 : 2.9	Canfield et al., 1998
Desulfocapsa sulfexigens	Sulfide Sulfate	-5.8 16.0	-1 : 2.8	Canfield et al., 1998
Desulfobulbus propionicus	Sulfide Sulfate	-15.5 30.9	-1 : 2.0	Canfield et al., 1998
Dethiobacter alkaliphilus	Sulfide Sulfate	-1.0 3.6	-1 : 3.6	This study
Desulfurivibrio alkaliphilus	Sulfide Sulfate	-0.9 4.7	-1 : 5.2	This study

Table 2

	pH 10, 37°C	pH 9, 37°C	pH 10, 22°C
Desulfurivibrio alkaliphilus			
$^{34}\varepsilon_{\text{elemental sulfur-sulfide}}$	-0.93 ± 0.28	-1.36 ± 0.29	-1.92 ± 1.27
$^{34}\varepsilon_{\text{elemental sulfur-sulfate}}$	+4.71 ± 0.42	+2.38 ± 0.42	+1.23 ± 0.28
$^{18}\varepsilon_{\text{water-sulfate}}$	+7.73 ± 3.86	+5.75 ± 4.97	+3.47 ± 3.18
Dethiobacter alkaliphilus			
$^{34}\varepsilon_{\text{elemental sulfur-sulfide}}$	-0.98 ± 0.53	-2.19 ± 0.45	-2.72 ± 0.49
$^{34}\varepsilon_{\text{elemental sulfur-sulfate}}$	+3.56 ± 1.27	+2.68 ± 0.32	+1.45 ± 0.47
$^{18}\varepsilon_{\text{water-sulfate}}$	+4.28 ± 2.76	+7.67 ± 4.60	+5.80 ± 1.56