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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

26 **ABSTRACT**

27 Sulfur and oxygen isotope fractionation of elemental sulfur disproportionation at anaerobic

28 haloalkaline conditions were evaluated for the first time. Isotope enrichment factors of the strains

29 *Desulfurivibrio alkaliphilus* and *Dethiobacter alkaliphilus* growing at pH 9 to 10 were

30 significantly smaller compared to previously published values of sulfur disproportionators at

31 neutral pH. We propose that this discrepancy iscaused by masking effects due to preferential

32 formation of polysulfides at high pH leading to accelerated internal sulfur turnover rates, but

33 cannot rule out distinct isotope effects due to specific enzymatic disproportionation reactions

34 under haloalkaline conditions. The results imply that the microbial sulfur cycle in haloalkaline

35 environments is characterized by specific stable sulfur and oxygen isotope patterns.

36

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).

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

$$\Delta G^0 = +10.2\ kJ\ mol^{-1}\ (per\ S^0) \tag{1}$$
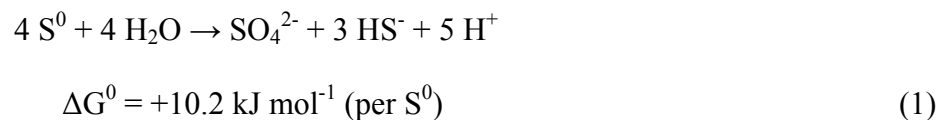
Despite the fact that sulfur-disproportionating microbes have been enriched from marine and

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

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

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

64

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

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

67
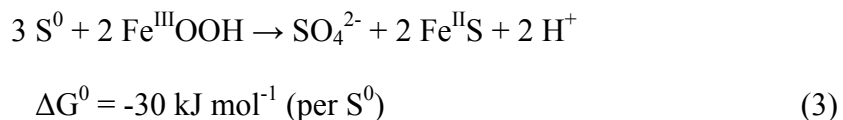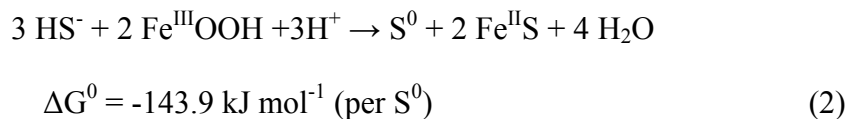
68 Notably, under alkaline conditions, reaction (1) becomes also more exergonic due to the

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 (Δ^{34}S(SO$_4$-H$_2$S)); 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

109 disproportionators of marine and fresh water origin. To test the validity of this hypothesis for

110 alkaline conditions, we performed isotope fractionation experiments with the haloalkaliphilic

111 deltaproteobacterium *Desulfurivibrio alkaliphilus*, and a representative of the order *Clostridiales*,

112 *Dethiobacter alkaliphilus*, isolated from soda lakes (Sorokin et al. 2008; 2010; 2011). In a

113 previous study, we could demonstrate that both species can grow by disproportionation of

114 elemental sulfur to sulfide and sulfate at pH 9-10, and that polysulfides were the actual substrate

115 of disproportionation under such conditions (Poser et al. 2013).

116 In the present study we measured the sulfur and oxygen isotope fractionation during alkaline

117 sulfur disproportionation to determine whether it is similar or different compared to the classical

118 fractionation described for neutral conditions. Such information may help to elucidate whether

119 sulfur disproportionation under neutral and alkaline conditions has a unique or different

120 biochemistry. Combined sulfur and oxygen isotopic fingerprints have furthermore a potential for

121 identifying sulfur disporportionation in natural alkaline habitats.

122

123 **2. METHODS**

124

125 **2.1. Cultivation and experimental setup**

126 *Desulfurivibrio alkaliphilus* and *Dethiobacter alkaliphilus* were taken from the strain collection

127 of D.Y. Sorokin. Detailed information about genetic, phylogenetic and morphologic aspects of

128 these strains can be found in Sorokin et al. (2008). Both strains were incubated under anaerobic

129 conditions at 37°C and pH 10 in modified DSMZ medium 1104 with sulfur (30 mM) and acetate

130 (4.9 mM) as energy and carbon sources as described by Poser et al. (2013). All experiments were

131 carried out in 120 ml glass serum bottles containing 100 ml medium and 20 ml head space. The

132 culture bottles were prepared inside an anaerobic glove box (gas atmosphere – N_2:H_2 (95:5); Coy

6

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 (δ^{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 δ^{34}S data was

180   carried out using the IAEA (International Atomic Energy Agency) materials IAEA-S1 (Ag_2S)

181 and NBS 127 ($BaSO_4$) as reference materials. The assigned values were -0.3‰ for IAEA-S1 and

182 +20.3‰ for NBS 127. Sulfur isotope compositions are reported in delta notation relative to

183 VCDT (Vienna Cañon Diablo Troilite) (eq. 4). Oxygen isotope analyses of barium sulfate and

184 ferric oxyhydroxide were performed by high temperature pyrolysis at 1450°C in a TC/EA (High

185 Temperature Conversion Elemental Analyzer) coupled to a delta plus XL mass spectrometer

186 (both ThermoFinnigan, Bremen, Germany). Precision of the oxygen isotope measurements was

187 ±0.6‰ (2σ). The normalization of the ^{18}O-SO_4^{2-} values was performed using the IAEA reference

188 material NBS 127 with an assigned $\delta^{18}O$ value of +8.7 ‰. The ^{18}O / ^{16}O ratio of the bulk water

189 was determined by laser cavity ring-down spectroscopy (Picarro L2120-i, Santa Clara, USA)

190 (Godoy et al. 2012) with an analytical error of ±0.2 ‰ (2σ). Oxygen isotope compositions are

191 reported relative to Vienna-Standard Mean Ocean Water (VSMOW) (eq. 4).

192

193 $$\delta^{18}O = (^{18}O/^{16}O_{sample} / \, ^{18}O/^{16}O_{standard}) -1 \qquad (4)$$

194

195 Isotope enrichment factors for sulfur and oxygen isotopes were calculated by subtracting the

196 initial isotope compositions of elemental sulfur ($\delta^{34}S$) and oxygen from water ($\delta^{18}O$) from the

197 final isotope compositions of sulfide and sulfate ($\delta^{34}S$) and oxygen from sulfate ($\delta^{18}O$). Since no

198 sulfide and sulfate was transformed from the initiating cultures no correction of the enrichment

199 factors was necessary.

200

201 **3. RESULTS AND DISCUSSION**

202

203 **3.1. Polysulfides as substrates for disproportionation**

204   We recently reported that polysulfides were formed during elemental sulfur disproportionation

205   under alkaline conditions (Poser et al. 2013), even in the presence of FeOOH, which reacts

206   immediately with sulfide and precipitates as FeS. The formation of polysulfides is favored at pH

207   > 9 by a chemical equilibrium reaction between sulfide and elemental sulfur (Schauder and

208   Müller 1993; eq. 5).

209

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

211

212   The concentrations of the (total) polysulfide species in our experiments were up to 0.9 mM (sum

213   of all polysulfides) in the presence of FeOOH (Poser et al. 2013). We also observed that the

214   polysulfide concentrations at the beginning of the incubation were close to zero, indicating that

215   sulfide first had to be formed by elemental sulfur disproportionation to form polysulfides.

216   Disproportionation of polysulfides is indicated by sulfide: sulfate ratios of ~ 4 (3.6 to 4.3)for S_4^{2-}

217   to S_8^{2-} (Milucka et al. 2012). However, the stoichiometry is altered by precipitation and re-

218   oxidation of the produced sulfide with Fe-oxyhydroxide, which might be the reason why the

219   expected ratios were not observed in our previous study (Poser et al. 2013). The observed sulfide:

220   sulfate ratio of ~ 2:1 during elemental sulfur disproportionation by the tested alkaline cultures fits

221   well with the theoretical value shown by equation (3) and should cause, depending on the

222   fractionation, an isotope mass balance at a ratio of -1:2. Notably, the calculated stoichiometry

223   based on enrichment factors is in the range of -1:4 for both alkaline strains (Table 1). However,

224   the discrepancy between mass and isotope balance is not an appropriate indicator for polysulfide

225   disproportionation as the isotope balance is likely influenced by sulfur isotope exchange and

226   equilibrium reactions between sulfur and sulfide (probably via polysulfides) resulting in a pool of

227   [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

11

251   conditions, or (iii) abiotic isotope effects due to alkaline polysulfide chemistry. We will discuss

252   these possible effects in the following.

253   *(i) Masking of isotope fractionation*

254   Masking of kinetic isotope fractionation can take place if mass transfer-dependent, non-

255   fractionating processes by which the substrate is transferred to the enzyme catalysing the reaction

256   become rate-limiting. Such masking can be due to high concentration of enzymes (Templeton et

257   al. 2006) and occurs also at low substrate concentrations due to limited substrate bioavailability

258   (Thullner et al. 2008; Kampara et al. 2008). However, sulfur disproportionation is thought to

259   consist of multiple different enzymatic steps (Finster 2008), and the isotope fractionation

260   measured in the final products of the pathway, sulfate and sulfide, is thus the sum of isotope

261   fractionation of each step of this metabolic network. In biochemical pathways, the flow of

262   substrates and hence, the magnitude of isotope fractionation is usually controlled by various

263   environmental and physiological factors; more complicating, many reactions are reversible and

264   characterized by considerable backward reactions. For example, the magnitude of sulfur isotope

265   fractionation associated with dissimilatory sulfate reduction in a single sulfate reducer depends

266   largely on the cell specific sulfate reduction rate and corresponding growth rate: the lower these

267   rates (due to limited available energy), the higher the sulfur isotope fractionation, and vice versa

268   (Bradley et al. 2011; Sim et al. 2011; Wing and Halvey 2014). Upon low energy conditions, the

269   enzymes of the sulfate reduction pathway operate maximally reversible leading to near

270   equilibrium conditions resulting in maximal sulfur isotope fractionation (Brunner and

271   Bernasconi, 2005). Analogously, sulfur isotope fractionation during sulfur disproportionation

272   might be controlled as well by the cell-specific disproportionation rates; notably, the reactions of

273   the oxidative branch of the pathway forming sulfate from sulfite may be similar to the

274   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 incorporation 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

413

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LEGENDS OF FIGURES AND TABLES

633

Figure 1: Progressing sulfur isotope fractionation during the disproportionation of elemental

sulfur to sulfide (AVS = Acid-Volatile Sulfide) and sulfate by *Desulfurivibrio alkaliphilus* (DSV)

and *Dethiobacter alkaliphilus* (DTB) at pH 10 and 37°C. The initial isotope value of elemental

sulfur was +5.3 ‰.

638

Table 1: Isotope enrichment factors (ε 34S) for sulfur disproportionation by pure cultures of

neutrophilic Deltaproteobacteria (*Desulfocapsa thiozymogenes, Desulfocapsa sulfexigens* and

Desulfobulbus propionicus) obtained by Canfield et al. (1998) compared to the factors obtained

for the haloalkaliphilic strains *Desulfurivibrio alkaliphilus* and *Dethiobacter alkaliphilus* in this

study. In addition, ratios of sulfide to sulfate sulfur enrichment factors are shown.

644

Table 2: Sulfur and oxygen isotope fractionation during elemental sulfur disproportionation by

Desulfurivibrio alkaliphilus and *Dethiobacter alkaliphilus* under different experimental

conditions. Presented are the enrichment factors and the standard deviation in for sulfide, sulfate

and oxygen (95% confidence level / 2σ, n = 2 - 9). The oxygen atoms in sulfate are completely
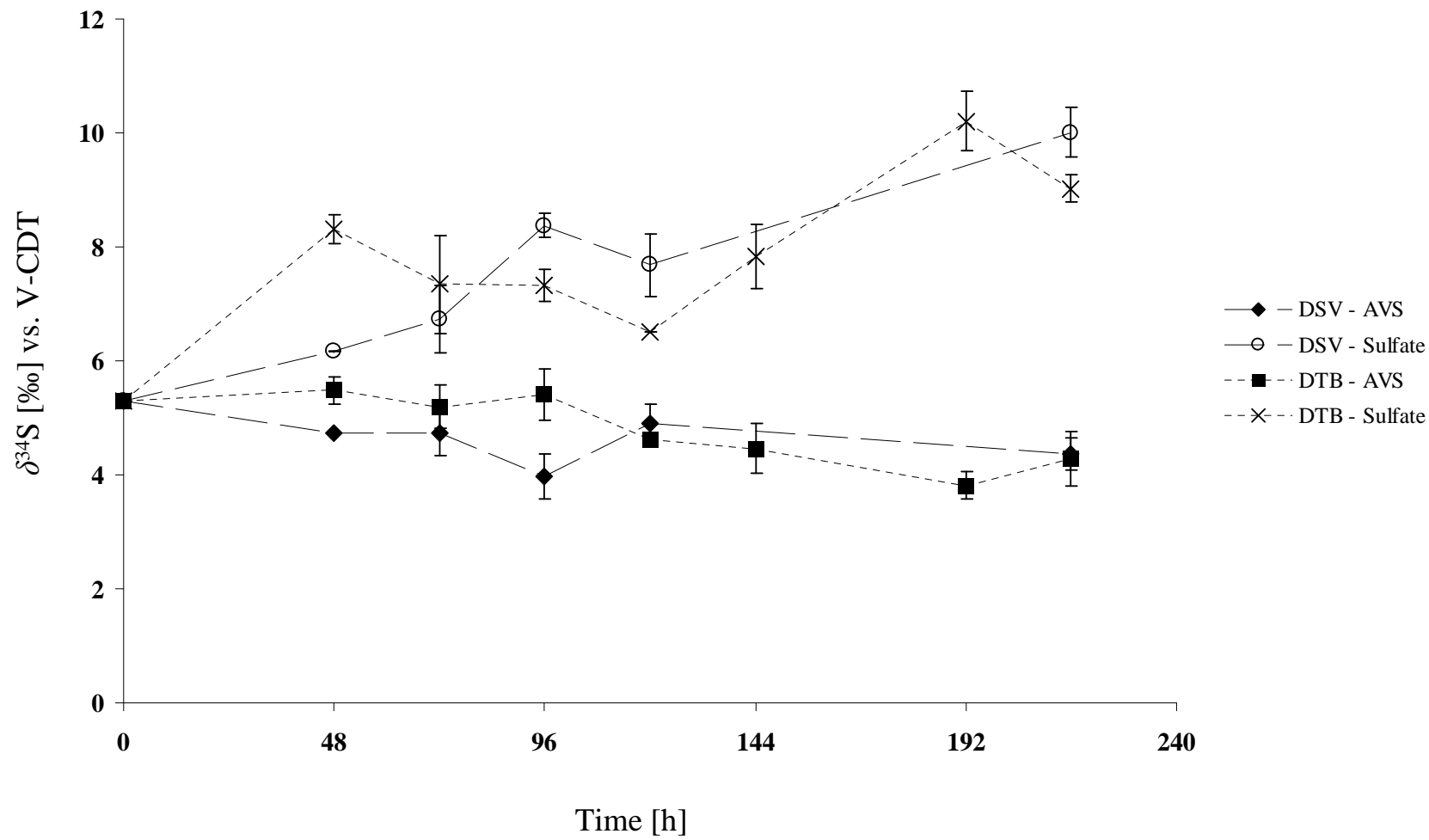
derived from water.

Figure 1

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