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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 is caused 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).

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).

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

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

Notably, under alkaline conditions, reaction (1) becomes also more exergonic due to the production of protons.

Currently, the pathway of elemental sulfur disproportionation is poorly understood. Studies based on enzyme assays by Frederiksen and Finster (2003) proposed a reaction scheme involving the formation of sulfite as key intermediate, which could be subsequently oxidized to sulfate by the reversed first steps of the dissimilatory sulfate reduction pathway. Notably, the genome of the recently sequenced marine sulfur-disproportionating deltaproteobacterium *Desulfocapsa sulfexigens* strain SB164P1 contains a complete set of genes necessary for sulfate reduction (Finster et al. 2013). However, with the exception of *Desulfocapsa thiozymogenes*, no studied sulfur-disproportionating strains were able to use sulfate as an electron acceptor. Therefore, these genes may encode the reversed sulfate reduction pathway proteins involved in the disproportionation of elemental sulfur (Frederiksen and Finster 2003).

One approach to investigate metabolic pathways is the analysis of stable isotopes.

Disproportionation reactions were reported to be accompanied by a considerable fractionation of $^{34}\text{S} / ^{32}\text{S}$ sulfur (from elemental sulfur to sulfide and sulfate) and $^{18}\text{O} / ^{16}\text{O}$ oxygen (incorporation of oxygen from water into sulfate) isotopes. In several studies it was observed that sulfide produced during the disproportionation reaction was depleted in ^{34}S by -3.7 to -15.5 ‰ relative to

the source (elemental sulfur) whereas sulfate was enriched by +11.0 to +35.3 ‰ (Canfield and Thamdrup 1994; Canfield et al. 1998; Böttcher et al. 2001; Böttcher and Thamdrup 2001; Böttcher et al. 2005). Thus, ^{32}S was preferentially used for the electron accepting part of the reaction (formation of sulfide) while ^{34}S was preferentially utilized in the electron-donating step (sulfate production); the remaining elemental sulfur pool maintained the initial isotope value (Canfield et al. 1998). In contrast, the spontaneous purely chemical disproportionation of elemental sulfur is associated with a relatively small sulfur isotope fractionation between the formed sulfate and sulfide ($\Delta^{34}\text{S}(\text{SO}_4\text{-H}_2\text{S})$); differences were shown to range between -0.4 to -3.0‰ (Smith 2000). The sulfur isotope fractionation of this reaction has been shown to depend on the reaction temperature, the duration of the reaction and the extent of the conversion (Smith 2000).

Since microbial sulfur disproportionation is a strictly anaerobic process, the oxygen atoms in the newly formed sulfate are fully derived from water, similar to the oxidation of sulfide to sulfate (Toran and Harris 1989). In addition to the canonical fractionation, an exchange of oxygen isotopes between cell-internal sulfur compounds (in particular sulfite) and ambient water could strongly influence the measured isotopic fractionation (Fritz et al. 1989; Brunner et al. 2005; Knöller et al. 2006; Turchyn et al. 2010; Müller et al. 2013a, b) depending on the exchange rates. Once the sulfate is formed, the oxygen isotope composition is conserved as the exchange of oxygen isotope between sulfate and water is extremely slow at neutral and alkaline conditions (Lloyd 1968; Mizutani and Rafter 1969a, b; Chiba and Sakai 1985). During the incorporation of oxygen into sulfate, an isotope fractionation in favor of ^{18}O between +8.2 to +21.6 ‰ was reported (Böttcher and Thamdrup 2001; Böttcher et al. 2001; Böttcher et al. 2005).

Taking all isotopic data into account, a conserved and uniform biogeochemistry of elemental 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 disproportionation 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 – N₂:H₂ (95:5); Coy

Laboratory Products Inc., USA) and sealed with Teflon-coated butyl rubber stoppers and aluminium crimps. Subsequently, the headspace of the serum bottles was flushed with nitrogen for 15 min to remove traces of hydrogen. Flowers of elemental sulfur were sterilized as described elsewhere (Thamdrup et al. 1993). Freshly prepared ferric oxyhydroxide (goethite / α -FeOOH) (Lovely and Phillips, 1986) was added in excess (0.2 M) to capture the produced sulfide for isotope measurements (Thamdrup et al. 1993; Böttcher et al. 2001; Böttcher and Thamdrup 2001). Each isotope fractionation experiment was carried out using nine active parallel cultures and two negative controls (anoxic, without biomass). For each strain, three independent experiments were performed. Culture medium was inoculated with 5% (vol/vol) of a preparatory culture pre-grown at sulfur-disproportionating conditions. Before inoculation, the preparatory culture was centrifuged and washed twice with DSMZ medium 1104 to remove remaining sulfate and iron sulfide. After approximately 30%, 60% and 100% of the elemental sulfur was consumed three out of the nine cultures were harvested for isotope analyses at each time point, respectively, to calculate sulfur and oxygen isotope discrimination. Before harvesting, the cultures were vigorously shaken to disperse the precipitated iron sulfide.

2.2. Microscopy

Cells were counted by epifluorescence microscopy (Adrian et al. 2007). Eighteen μ L of a well-mixed cell suspension was mixed with 1 μ L SYBR Green (Bio Rad) with a pipette and stored for 15 min in the dark. Afterwards, this mixture was immobilized on agarose-coated slides, sealed with a cover slip and examined by epifluorescence microscopy (Nikon Eclipse TE300). To guarantee accurate counting, each sample/slide was scanned in a z-pattern and 10 pictures were taken with a Nikon DXM 1200F digital camera (fixed focus and aperture). Cell count data were produced through analysis of the pictures with the ImageJ software. To preclude an interference

of the solid phases of FeOOH/FeS on the cell-counting, we adjusted the measurement by the ImageJ software to the size of the cells.

2.3. Chemical and isotopic analyses

The concentration of sulfide plus sulfane-sulfur of polysulfides was determined spectrophotometrically with the methylene blue method according to Cline (1969) using modifications described by Herrmann et al. (2008). Samples were taken under anoxic conditions and fixed immediately with zinc acetate. Sulfate in the supernatant was analyzed by ion chromatography (DX 500 Dionex) using an IonPacAS18 / AG18 column and KOH (23 mM) as eluent; samples were taken under anoxic conditions and subsequently filtered (0.20 µm pore size).

For isotopic measurements, the formed FeS was separated from the remaining solution by vacuum filtration (cellulose-acetate filters with 0.45 µm pore size). The FeS-containing filters were stored inside an anaerobic jar until further analysis. To determine the sulfur isotope composition ($\delta^{34}\text{S}$) of sulfide and sulfate, the acid-volatile fraction of sulfide (AVS) was distilled with 6 N HCl and the liberated hydrogen sulfide was first precipitated as zinc sulfide (reaction with zinc acetate) and subsequently converted to silver sulfide after reaction with silver nitrate. Notably, AVS was the only major sulfide pool. The formed sulfate was precipitated as barium sulfate after reacting with a barium chloride solution (Canfield et al. 1998; Böttcher and Thamdrup 2001; Knöller et al. 2008). Both compounds (AVS and sulfate) were converted and measured as SO_2 using an elemental analyzer coupled with an isotope ratio mass spectrometer (DeltaS, ThermoFinnigan, Bremen, Germany). The analytical precision of the sulfur isotope measurement was better than $\pm 0.4\text{‰}$ (2σ). Calibration and normalization of the $\delta^{34}\text{S}$ data was carried out using the IAEA (International Atomic Energy Agency) materials IAEA-S1 (Ag_2S)

and NBS 127 (BaSO₄) 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 ¹⁸O-SO₄²⁻ values was performed using the IAEA reference material NBS 127 with an assigned δ¹⁸O value of +8.7 ‰. The ¹⁸O / ¹⁶O 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).

$$\delta^{18}\text{O} = \left(\frac{{}^{18}\text{O}/{}^{16}\text{O}_{\text{sample}}}{{}^{18}\text{O}/{}^{16}\text{O}_{\text{standard}}} \right) - 1 \quad (4)$$

Isotope enrichment factors for sulfur and oxygen isotopes were calculated by subtracting the initial isotope compositions of elemental sulfur (δ³⁴S) and oxygen from water (δ¹⁸O) from the final isotope compositions of sulfide and sulfate (δ³⁴S) and oxygen from sulfate (δ¹⁸O). 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).

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

react. Thus, the pool of elemental sulfur may change its isotope composition, and the apparent enrichment factor for sulfide formation may change as well, indicated by a relatively large error (Table 1). . As discussed below in section 3.2, indirect indicators of polysulfide disproportionation under alkaline conditions are the high substrate turnover rates of *Desulfurivibrio alkaliphilus* and *Dethiobacter alkaliphilus*, which might be caused by enhanced uptake and cell internal transport of polysulfides which are water soluble in contrast to elemental sulfur.

3.2. Fractionation of stable sulfur isotopes during sulfur disproportionation

Disproportionation of elemental sulfur by *Desulfurivibrio alkaliphilus* (DSV) and *Dethiobacter alkaliphilus* (DTB) in the presence of FeOOH resulted in the production of sulfide (HS^- + sulfane atoms of polysulfides) and sulfate in a ratio of 1.8:1 and 2.1:1, respectively (Poser et al. 2013). These values are close to the theoretical value of 2:1 (eq. 3) (Thamdrup et al. 1993). For both strains, the produced sulfate was enriched in ^{34}S over time, whereas sulfide became ^{34}S depleted as it was reported for sulfur disproportionation at neutral pH (Canfield and Thamdrup 1994; Canfield et al. 1998, Böttcher et al. 2001; Böttcher and Thamdrup 2001). However, the fractionation values of the sulfur isotopes were significantly lower than those reported in previous studies for neutrophilic bacteria (Table 1). We observed a fractionation of $-0.9 \pm 0.3 \text{ ‰}$ (DSV) and $-1.0 \pm 0.5 \text{ ‰}$ (DTB) for the formed sulfide and $+4.7 \pm 0.4 \text{ ‰}$ (DSV) and $+3.6 \pm 1.3 \text{ ‰}$ (DTB) for sulfate, respectively (Table 1). The difference to the values observed for neutrophilic disproportionators might have been caused by various factors: (i) sulfur disproportionation at neutral and alkaline conditions is biochemically similar, but isotope fractionation is considerably 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 dissimilatory sulfate reduction pathway (Frederiksen and Finster 2003). At haloalkaline

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

as long as the rate of dissimilatory sulfate reduction is low enough (Wing and Halvey 2014).

Notably, this scenario contradicts to the general rule of thumb for isotope fractionation that isotope fractionation effects downstream of a rate-limiting step are not expressed. By contrast, isotope fractionation might be masked under haloalkaline conditions due to the higher sulfur disproportionation rates and reduced reversibility of polysulfide disproportionation steps.

(ii) Different biochemical pathways

A different isotope fractionation pattern would probably also evolve if the enzymatic pathway of sulfur disproportionation under alkaline conditions is different compared to pH neutral conditions. However, the exact mode of electron flow during elemental sulfur disproportionation is currently not completely understood, especially the reductive branch of the pathway leading to sulfide formation, and the oxidative part resulting in sulfite formation (Frederiksen and Finster 2003; Finster 2008; Finster et al. 2013). Thus, possible effects caused by different enzymatic reactions are currently purely speculative. Moreover, sulfur isotope fractionation upon elemental sulfur disproportionation is not expected to be controlled by the activity of a single enzyme, in analogy to dissimilatory sulfate reduction (Wing and Halvey 2014).

The extent of isotope fractionation was nearly similar under different alkaline pH regimes and temperatures. At pH 9 (drop from pH 10 in our experiments) and 37°C, the sulfur isotope fractionation shifted slightly towards more ³⁴S depleted values for sulfide and sulfate (Table 2) although the growth characteristics did not change considerably (data not shown). When incubated at pH 10 and 22°C both cultures showed extended lag phases (data not shown) and sulfide and sulfate isotope values again became slightly more ³⁴S depleted (Table 2) compared to values detected at pH 10 and 37°C.

(iii) Abiotic isotope effects

Amrani et al. (2006) investigated the distribution of the sulfur isotopes of polysulfide ions with an artificial polysulfide solution [equilibrium reaction between S^0 and $(NH_4)_2S$] at pH 9 and reported that polysulfides were enriched in ^{34}S and that this enrichment increased with increasing polysulfide chain length. Furthermore, the authors showed that polysulfides are a highly dynamic and complex sulfur pool due to sulfur isotope exchange between elemental sulfur and sulfide within the polysulfide chain and sulfur isotope exchange between the polysulfides and the remaining sulfur species in the system. Interestingly, an enrichment of ^{34}S in the zero valent sulfur moiety compared to the sulfane moiety of the chain was observed. Given that polysulfides have a $S-S^0_n-S^-$ structure, it can be speculated that the heavier S^0 atoms are located medial or subterminal between the two terminal (sulfane) sulfur atoms of the chain, which would increase the chains stability by stronger S-S bonds. Thus, the heavier sulfur isotopes in the middle part of the chain might not be as reactive as the lighter sulfur isotopes at the terminal sites. Therefore, the value of the elemental sulfur isotope composition (in our case: +5.3‰) depends on the dynamics of the above-described processes of polysulfides formation and depletion and might be variable. Such dynamic processes of polysulfide chain formation could also explain the observed increasing (for sulfate) and decreasing (for sulfide) sulfur isotope fractionation values observed in the present study.

Notably, we observed an increasing sulfur isotope fractionation of sulfide and sulfate for both strains over time (Figure 1). A similar trend has been demonstrated in studies by Canfield et al. (1998) and Böttcher et al. (2001). A trend to lighter sulfur isotopes for sulfide is explainable by a partial chemical reoxidation of the produced ^{34}S -depleted sulfide to elemental sulfur by ferric iron (eq. 2); the hereby formed ^{34}S -depleted elemental sulfur could have been disproportionated again to sulfide and sulfate.. However, this scenario is inconsistent to the observed heavier sulfur isotope values of sulfate (which should become also lighter with time) and therefore implausible.

The trend of increasing fractionation of sulfide and sulfate with time is explainable by cell growth: due to increases in cell numbers and enzymes, polysulfides become more limited, leading to a higher reversibility of the process, resulting in a higher fractionation.

3.3. Stable oxygen isotope effects during the incorporation of water into sulfate

Disproportionation of elemental sulfur is usually accompanied by an oxygen isotope discrimination favoring the enrichment of ^{18}O in the formed sulfate by about +17 ‰ (for *Desulfocapsa thiozymogenes*) and up to +22 ‰ (for *Desulfobulbus propionicus*) (Böttcher et al. 2001; Böttcher et al. 2005). Similar to the results for sulfur isotope fractionation, the enrichment of ^{18}O in the formed sulfate was significantly lower under alkaline conditions than under neutral conditions. We measured a fractionation of $+7.8 \pm 3.9$ ‰ for culture DSV and $+4.3 \pm 2.8$ ‰ for culture DTB, respectively; no evidence was found that the adjusted pH or temperatures changed the extent of oxygen isotope fractionation (Table 2). Similar values of oxygen enrichment factors - ranging between 0 and +4 ‰ - were reported for biological and abiotic sulfide oxidation to sulfate under anoxic conditions (Lloyd 1968; Toran and Harris 1969; Taylor et al. 1984a, b; van Everdingen and Krouse 1985; van Stempvoort and Krouse 1994; Balci et al. 2007). In contrast, a slight depletion in ^{18}O of formed sulfate relative to the isotope composition of water was recently reported for *Thiobacillus denitrificans* and *Sulfurimonas denitrificans* upon sulfide oxidation under nitrate-reducing conditions, which was presumably linked to exchange reactions of nitrite (formed during nitrate reduction) and water (Poser et al. 2014). Sulfate might be formed by similar biochemical reaction during sulfide oxidation and elemental sulfur disproportionation, involving sulfite and adenosine 5'-phosphosulfate (APS) as intermediates (Friedrich et al. 2001; Finster 2008; Finster et al. 2013; Poser et al. 2014); thus, oxygen isotope fractionation of anoxic sulfide oxidation and disproportionation might be in a comparable range. However, neutrophilic

disproportionating cultures showed significantly higher oxygen isotope fractionation (Böttcher et al 2001, 2005).

Analogously to reactions of the dissimilatory sulfate reduction pathway, oxygen isotope effects in the formed sulfate during disproportionation of sulfur are probably controlled by oxygen isotope exchanges via water during polysulfide oxidation to sulfite (formed by sulfur oxidation), sulfite oxidation to sulfate, and possible back-reactions if these reactions are reversible. For dissimilatory sulfate reduction, rapid oxygen isotope exchange for cases where sulfur isotope fractionation is large and slow exchange for cases where sulfur isotope fractionation is small was recently predicted (Brunner et al. 2012). Such a model could also explain large oxygen isotope fractionation in slow growing, large sulfur fractionating neutrophilic disproportionators, and small oxygen isotope fractionation in fast growing, small sulfur fractionating alkaliphilic disproportionators. Notably, under alkaline conditions, the incorporated oxygen stems preferentially from hydroxyl ions (OH^-) and not from water (H_2O), influencing oxygen isotope fractionation under alkaline conditions considerably as the $\delta^{18}\text{O}$ of OH^- is 35–40 ‰ lower than that of H_2O at 25°C. For example, alkaline phosphatase and acid phosphatase produce phosphate with different oxygen isotope composition due to this reason (von Sperber 2014). Thus, oxygen the observed small oxygen isotope effect during sulfur disproportionation under alkaline conditions could be also due to preferential incorporation of isotopically light OH^- ions.

4. CONCLUDING REMARKS

In this study we report enrichment factors for sulfur and oxygen isotope fractionation during bacterial sulfur disproportionation under haloalkaline conditions. The ^{34}S and ^{18}O isotope fractionation was significantly lower compared to data reported for elemental sulfur disproportionating bacteria at neutral pH. Under haloalkaline conditions, the concentration of

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

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LEGENDS OF FIGURES AND TABLES

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 ‰.

Table 1: Isotope enrichment factors ($\epsilon^{34}\text{S}$) 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.

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

Table 2

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