Combined ^{34}S, ^{33}S and ^{18}O Isotope Fractionations Record Different Intracellular Steps of Microbial Sulfate Reduction

Gilad Antler[1,2], Alexandra V. Turchyn[1], Shuhei Ono[3] , Orit Sivan[4] and Tanja Bosak[3]

[1] Department of Earth Sciences,
University of Cambridge,
Cambridge CB2 3EQ, UK.

[2] Center for Geomicrobiology, Department of Biological Sciences,
Aarhus University,
Ny Munkegade, Bld. 1535, 8000 Aarhus C, Denmark

[3]Department of Earth, Atmospheric and Planetary Sciences,
Massachusetts Institute of Technology,
Cambridge MA 02139,USA,

[4]Department of Geological and Environmental Sciences,
Ben-Gurion University of the Negev,
P. O. Box 653, Beer-Sheva 84105, Israel

Phone	+44(0)1223333479
Fax	+44(0)1223333450
E-mail	giladantler@gmail.com

1. Abstract

Several enzymatic steps in microbial sulfate reduction (MSR) fractionate the isotope ratios of $^{33}S/^{32}S$, $^{34}S/^{32}S$ and $^{18}O/^{16}O$ in extracellular sulfate, but the effects of different intracellular processes on the isotopic composition of residual sulfate are still not well quantified. We measured combined multiple sulfur ($^{33}S/^{32}S$, $^{34}S/^{32}S$) and oxygen ($^{18}O/^{16}O$) isotope ratios of sulfate in pure cultures of a marine sulfate reducing bacterium *Desulfovibrio* sp. DMSS-1 grown on different organic substrates. These measurements are consistent with the previously reported correlations of oxygen and sulfur isotope fractionations with the cell-specific rate of MSR: faster reduction rates produced smaller isotopic fractionations for all isotopes. Combined isotope fractionation of oxygen and multiple sulfur isotopes also revealed the presence of rate-limiting enzymatic steps and a possible influence of the availability of the C subunit of the dissimilatory sulfite reductase. These experiments help reconstruct and interpret processes that operate in natural pore waters characterized by high $^{18}O/^{16}O$ and moderate $^{34}S/^{32}S$ ratios and suggest that some multiple isotope signals in the environment cannot be explained by microbial sulfate reduction alone. Instead, these signals support the presence of active, but slow sulfate reduction as well as the reoxidation of sulfide.

2. Introduction

Microbial sulfate reduction (MSR) is key process in the global carbon cycle, responsible for the oxidation of a substantial fraction of organic matter that reaches marine sediments (Kasten and Jørgensen, 2000; Bowles et al., 2014). The biochemical steps during MSR have been studied for more than 60 years (e.g. Hilz & Lipmann, 1955; Peck, 1962; Michaels et al., 1970; Pierik et al., 1992; Oliveira et al.,

2008; Santos et al., 2015). The reduction of sulfate is thought to occur in five major metabolic steps (Fig. 1). During the first step, extracellular sulfate is transported into the cytoplasm by sulfate permeases (Piłsyk & Paszewski, 2009). In the second step (step 2), intracellular sulfate is activated with adenosine triphosphate to form adenosine 5' phosphosulfate (APS) (Hilz & Lipmann, 1955) by ATP sulfurylase (Michaels et al., 1970). In the third step (step 3), the APS is reduced to sulfite (SO_3^{2-}). In the fourth step (step 4), sulfite reduced to sulfide by Dissimilatory sulfide reductase (Dsr) with two key subunits (DsrAB and DsrC) but also can be reduced to a variety of sulfur intermediates such as elemental sulfur (S^o), trithionate ($S_4O_6^{2-}$) and thiosulfate ($S_2O_3^{-2}$) (e.g. Kobayashi et al. 1969; Fitz and Cypionka, 1990; Akagi, 1995; Bradley et al. 2011). It was recently shown that these intermediates can form *in vitro* in the presence of excess sulfite and in the absence of the DsrC subunit. Under these conditions, sulfite is reduced to an S^{2+} intermediate, which is then converted to a DsrC trisulfide (Santos et al., 2015). The DsrC trisulfide is then reduced to sulfide by the membrane complex DsrMKJOP (Santos et al., 2015). Because most (if not all) intracellular steps during MSR are reversible, understanding the dynamics among different steps, the controls on their relative reversibilities, and the relationship of these steps with the measurable cell-specific sulfate reduction rate (often abbreviated as csSRR) is critical for understanding the activity and rates of microbial sulfate reduction in nature (Rees, 1973; Farquahr et al., 2003; Brunner and Bernasconi, 2005; Canfield et al., 2006).

The use of isotope geochemistry, specifically the stable sulfur ($^{33}S/^{32}S$ and $^{34}S/^{32}S$) and oxygen ($^{18}O/^{16}O$) isotope ratios (See appendix 1 for isotope notation), has given insights into the intracellular steps during MSR. Given that every step during MSR partitions each sulfur isotopologue to a different degree and in a different manner, the

isotope partitioning of the major or minor sulfur or oxygen isotopes in the extracellular sulfate pool should reflect the relationship between different steps and their reversibilities (assuming the first step is reversible). These are often called 'branching points' which refers to a point in the reaction pathway where isotopic fractionation occurs (e.g., Farquhar et al., 2003). Most studies have employed the ratio of ^{32}S and ^{34}S measured in sulfate, sulfide, or sulfur intermediates to investigate MSR and its branching points (e.g. Canfield et al., 2001b; 2006; 2010; Kamyshny et al., 2011; Knossow et al., 2015; Zerkle et al., 2010). Studies focusing on the fractionation of ^{34}S from ^{32}S during MSR have found that most of the enzymatic steps during MSR prefer the ^{32}S isotope, transferring ^{32}S into the produced sulfide pool and leaving ^{34}S behind in a Rayleigh-type isotope distillation. The magnitude of sulfur isotope fractionation during MSR can be as high as ~70 ‰ for $\delta^{34}S_{SO4}$ (Wortmman et al., 2007; Canfield et al., 2010; Sim et al., 2011a), and approaches the value expected for sulfur isotopic equilibrium between sulfate and sulfide (71 ‰ at 25ºC-- Tudge and Thode, 1950; Wing and Halevy, 2014).

More recently, studies have employed coupled ^{32}S, ^{33}S, and ^{34}S isotopes (e.g. Farquhar et al., 2003; 2008; Johnston et al., 2005; 2007; Sim et al., 2011a; 2011b; 2013; Leavitt et al., 2013; 2015; 2014; Ono et al., 2006; Pellerin et al., 2015a;2015b) or $^{34}S/^{32}S$ and $^{18}O/^{16}O$ isotope ratios (Brunner et al., 2005; 2012; Wortmann et al., 2007; Farquhar et al., 2008; Turchyn et al., 2006; 2010; Antler et al., 2013; Knöller et al., 2006; Mangalo et al., 2007;2008). The coupled isotope approaches are thought to provide information about up to two branching points in the MSR pathway (Fig. 1). During strictly mass-dependent fractionation, the magnitude of $\delta^{33}S_{SO4}$ is about half (0.5147 at 25ºC-- Farquhar et al., 2003) that of $\delta^{34}S_{SO4}$. Over the last 60 years, studies have shown that the magnitude of the sulfur isotope fractionation for either $^{34}S/^{32}S$ or

76 $^{33}S/^{32}S$ is a function of microbial metabolism and intracellular pathways which deliver
77 electrons to the sulfate reduction pathway (e.g. Sim, et al., 2011b; 2012; 2013;
78 Brüchert, 2004), the amount of sulfate available (e.g. Canfield, 2004; Habicht et al.,
79 2002; Farquhar et al., 2003; Bradley et al., 2015), the temperature (e.g. Hoek et al.,
80 2006; Bruchert et al., 2001; Canfield, et al., 2006) and the rate of sulfate reduction
81 (e.g., Chambers and Trudinger, 1975; Canfield, et al., 2001; Leavitt et al., 2013; 2015;
82 Sim et al., 2011a;2011b).

83 The $^{18}O/^{16}O$ ratio in dissolved sulfate ($\delta^{18}O_{SO4}$) also increases as MSR progresses,
84 but often reaches an apparent isotopic equilibrium with water and ceases to increase
85 (Fritz et al, 1989; Böttcher et al., 1998, 1999; Turchyn et al, 2006; 2010; Wortmann,
86 et al, 2007; Aller et al, 2010; Zeebe, 2010). Pure culture studies have shown that
87 oxygen atoms from water are incorporated into sulfate during MSR (Fritz et al, 1989;
88 Mizutani and Rafter 1973; Brunner et al., 2005; Mangalo et al, 2007; Mangalo et al,
89 2008) much more rapidly than would be expected from the purely abiotic oxygen
90 isotope exchange between water and sulfate under normal surface conditions (pH>1,
91 temperature <100ºC) (Chiba and Sakai 1985; Lloyd, 1968; Rennie and Turchyn,
92 2014). This rapid isotope exchange during MSR is attributed to the intracellular
93 exchange of oxygen atoms between water and sulfur redox intermediate species such
94 as sulfite (Mizutani and Rafter, 1973; Fritz, et al., 1989) and occurs within minutes
95 (Betts and Voss, 1970; Horner and Connick, 2003; Wankel et al., 2014; Muller et al.,
96 2013). If a portion of intracellular sulfite is reoxidized back to sulfate and added to
97 the extracellular sulfate pool, the exchange of oxygen isotopes between sulfite and
98 water will be observed. The observed (and modelled) oxygen isotope enrichment of
99 sulfate relative to the isotopic composition of the water ($\delta^{18}O_{SO4}$- $\delta^{18}O_{H2O}$) is between
100 20 to 28‰, reflecting this intracellular oxygen isotope exchange and subsequent

reoxidation (e.g. Böttcher et al., 1998, 1999; Turchyn et al, 2006;2010 Wortmann et al, 2007; Zeebe, 2010).

Some experiments with natural populations and observations in the environment show a linear trend between $\delta^{18}O_{SO4}$ and $\delta^{34}S_{SO4}$, demonstrating that both oxygen and sulfur isotopes undergo kinetic isotope fractionation and that the aforementioned isotope equilibrium between sulfate and water is not always observed (e.g. Antler et al., 2015; Sivan et al., 2014). The magnitude of this kinetic isotope fractionation for $\delta^{18}O_{SO4}$ has been suggested to be 25% of the magnitude of $\delta^{34}S_{SO4}$ (Rafter and Mizutani 1967; Mandernack et al., 2003). Therefore, the oxygen isotope fractionation observed during MSR is thought to result from a combination of the kinetic isotope effect associated with enzymatic steps during MSR (similar to sulfur isotopes), the equilibration of oxygen isotopes between sulfur species in intermediate valence state and water, and the contribution of these sulfur species to the extracellular sulfate pool.

This study aims to: 1. Test whether the major and minor isotopes of sulfur and the oxygen isotope system record complementary information about intracellular processes; and 2. Assess the magnitude of the equilibrium and kinetic oxygen isotope effects. To do this, we experimentally explore the respective evolution of three isotope ratios: $^{33}S/^{32}S$, $^{34}S/^{32}S$ and $^{18}O/^{16}O$ during MSR in pure culture batch experiments as a function of the csSRR and $\delta^{18}O_{H2O}$. These measurements are then used to constrain models of $\delta^{34}S_{SO4}$, $\delta^{33}S_{SO4}$ and $\delta^{18}O_{SO4}$.

3. The use of models of sulfur and oxygen isotope fractionation during microbial sulfate reduction

Numerical models of MSR use sulfur ($^{33}S/^{32}S$ and $^{34}S/^{32}S$) and oxygen ($^{18}O/^{16}O$) isotope ratios in dissolved sulfate as experimental constraints, add assumptions about

126 electron flow and maximum isotope fractionations imparted by individual enzymes
127 (Fig. 1), and then solve for the magnitudes of backward and forward fluxes associated
128 with the modelled reactions. These models have been used to reconstruct
129 environmental processes and physiological conditions from observed isotope
130 fractionations (e.g. Farquhar et al., 2003; 2008; Sim et al., 2011b; Brunner et al.,
131 2012). However, the problem is under-constrained, as there are more variables than
132 equations. Many studies work around this limitation by merging several steps
133 together. Most notably, this is done by considering the reduction of sulfite to sulfide
134 in a single step. Brunner et al. (2012) modelled the sulfur and oxygen isotope
135 evolution during MSR, and in doing so determined that a single-step sulfite reduction
136 to sulfide (step 4 and 5— Fig. 1) is not consistent with the isotope data. Combining
137 multiple sulfur and oxygen isotopes should help constrain the problem and provide an
138 equivalent number of variables (the flux ratios at each step—ϕ_i in figure 1) and mass
139 balance equation for each isotope ratio. However, only a few studies have combined
140 multiple sulfur and oxygen isotopes (^{32}S, ^{33}S, ^{34}S and ^{18}O and ^{16}O) (e.g. Farquhar et
141 al., 2008). The following section summarizes past modeling efforts for sulfur and
142 oxygen isotopes during MSR.

143 The overall sulfur isotope fractionation during MSR is modelled as a
144 superposition of the various forward and backward fluxes at each step with any
145 isotope partitioning occurring at each step (Rees, 1973; Brunner and Bernasconi,
146 2005; Farquhar et al., 2003; Sim et al., 2011b) and is given mathematically by (after
147 Brunner et al 2012):

148
$$\alpha^{3x}S_{total}=\frac{\begin{array}{c}\phi_1\cdot\phi_2\cdot\phi_3\cdot\phi_4\cdot\left(1-\alpha^{3x}S_{f\_5}\right)+\dots\\ \phi_1\cdot\phi_2\cdot\phi_3\cdot\alpha^{3x}S_{f\_5}\cdot\left(1-\alpha^{3x}S_{f\_4}\right)+\dots\\ \phi_1\cdot\phi_2\cdot\alpha^{3x}S_{f\_5}\cdot\alpha^{3x}S_{f\_4}\cdot\left(1-\alpha^{3x}S_{f\_3}\right)\end{array}}{\alpha^{3x}S_{f\_5}\cdot\alpha^{3x}S_{f\_4}\cdot\alpha^{3x}S_{f\_3}}+1 \quad (1)$$

where $\alpha^{3x}S_{total}$ is the total expressed sulfur isotope fractionation factor for isotope 3x (x=3,4), $\alpha\ ^{3x}S_{i\_j}$ is the sulfur isotope fractionation during the forward (i=f) and backward (i=b) reaction j (where j=1…5) and ϕ_k (where k=1…4) is the ratio between the fluxes of the four intracellular steps summarized in Figure 1:

$$\phi_k = \frac{b_k}{f_k} \quad (2)$$

Thus, models of MSR (Rees, 1973; Brunner and Bernasconi, 2005; Farquhar et al., 2003; Sim et al., 2011b; Brunner et al., 2012) assign values for sulfur isotope fractionation factors in the forward steps 1,3,4 and 5, respectively, and assume that all other steps (and backward reactions) do not fractionate sulfur isotopes – e.g., Rees, (1973). Since the isotope fractionation in step 1 is very small, and there is no fractionation in step 3, all solution are virtually symmetrical for both of these steps. Therefore, using isotopic techniques, it is impossible to distinguish one from another.

For minor isotopes (such as ^{33}S), the relationship between the isotope fractionation for ^{33}S /^{32}S (compared with $^{34}S/^{32}S$) is commonly given as (e.g. Harrison and Thode, 1965; Young, 2002; Farquhar et al., 2003)

$$\ln(\alpha^{33}S) = \vartheta^* \cdot \ln(\alpha^{34}S) \quad (3)$$

where ϑ^* is the calculated temperature-dependent equilibrium isotope fractionation between sulfate and sulfide (0.5147-- Farquhar et al., 2003). The deviation between the calculated value for $\alpha^{33}S$ and the expected mass-dependent relationship between $\alpha^{33}S$ and $\alpha^{34}S$ is defined as:

$$E^{33}S = 1000 \cdot \left(\alpha^{33}S - \alpha^{34}S^{0.515}\right) \quad (4)$$

The partitioning of ^{33}S in every step during MSR does not deviate from a mass-dependent fractionation with respect to ^{34}S (equation 2), but the overall expressed isotope fractionation can deviate from a purely mass-dependent relationship. The

magnitude of this offset is a function of the relative forward and backward fluxes of every step during MSR and stems from the fact that the mixing between two pools is linear, but the mass-dependent fractionation obeys a power law (Farquhar et al., 2003; Ono et al., 2006; Farquhar et al., 2007; Johnston et al, 2007). Mixing between two pools with variable branching points is common in metabolisms such as MSR, and it has been used in the past to calculate the dynamics of the forward and backward fluxes of each step during MSR (e.g., Farquhar et al., 2003; Sim et al., 2011b; Johnston et al., 2007). Figure 2a shows an example of these calculations.

Oxygen isotopes in dissolved sulfate ($\delta^{18}O_{SO4}$ values) are thought to record information complementary to that revealed by sulfur isotopes. The relative change between $\delta^{18}O_{SO4}$ and $\delta^{34}S_{SO4}$ has been used as a tracer of pyrite oxidation (e.g. Balci et al., 2007; Brunner, et al., 2008; Heidel and Tichomirowa, 2011; Kohl and Bao, 2011), sulfur disproportionation (e.g. Cypionka et al., 1998; Böttcher et al, 2001; Böttcher and Thamdrup, 2001; Böttcher et al, 2005; Poser et al., 2016), cryptic cycling of sulfur (e.g., Aller et al, 2010; Johnston et al., 2014; Riedinger et al., 2010; Mikucki et al., 2009) and sulfate-driven anaerobic oxidation of methane (e.g. Aharon an Fu, 2000; 2003; Antler et al., 2014;2015; Deusner et al., 2014; Sivan et al., 2014). Changes in the reversibility of all steps during MSR (Fig. 1) change the composition of oxygen and sulfur isotopes of extracellular sulfate as MSR progresses. This is expressed mathematically as (after Brunner et al., 2012; Antler et al., 2013):

$$\delta^{18}O_{SO4(t)}=\begin{cases} \frac{\varepsilon^{18}O_{total}}{\varepsilon^{34}S_{total}}\cdot\left(\delta^{34}S_{SO4(t)}-\delta^{34}S_{SO4(0)}\right)+\delta^{18}O_{SO4(0)} & \phi_1\cdot\phi_2\cdot\phi_3=0 \\ \delta^{18}O_{SO4(A.E)}-\exp\left(-\theta_O\cdot\frac{\delta^{34}S_{SO4(t)}-\delta^{34}S_{SO4(0)}}{\varepsilon^{34}S_{Stotal}}\right)\ldots & 0<\phi_1\cdot\phi_2\cdot\phi_3<1 \\ \qquad\cdot\left(\delta^{18}O_{SO4(A.E)}-\delta^{18}O_{SO4(0)}\right) & \end{cases} \quad (5)$$

where $\varepsilon^{34}S_{total}$ and $\varepsilon^{18}O_{total}$ are the measured sulfur and oxygen isotope fractionation, respectively, and $\delta^{34}S_{SO4(t)}$, $\delta^{34}S_{SO4(0)}$, $\delta^{18}O_{SO4(t)}$ and $\delta^{18}O_{SO4(0)}$ are the isotopic

196 compositions of sulfur and oxygen in the residual sulfate at time t and time 0,
197 respectively. $\delta^{18}O_{SO4(A.E)}$ is the isotopic composition of oxygen in the residual sulfate
198 at apparent equilibrium, and θ_O is a parameter initially formulated by Brunner et al.
199 (2005). This parameter measures the ratio between the apparent oxygen isotope
200 exchange and sulfate reduction rate (Brunner et al., 2012):

201 $$\theta_O = \frac{\phi_1 \cdot \phi_2 \cdot \phi_3}{1 - \phi_1 \cdot \phi_2 \cdot \phi_3} \quad (6)$$

202 Factorization of equation (5) suggests that there are two distinct stages on a cross-plot
203 of $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$ during bacterial sulfate reduction:
204
205 1. Apparent linear phase. The initial segment of the $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$ cross-plot
206 during MSR can be approximated by a linear line. The mathematical term for this line
207 can be described by the first term of the Taylor series of Equation 5 around $\delta^{34}S_{SO4(0)}$
208 and $\delta^{18}O_{SO4(0)}$ (Antler et al., 2013). The slope of this apparent linear (SALP) phase
209 can therefore be written as:

210 $$SALP = \theta_O \frac{\delta^{18}O_{SO4(A.E)} - \delta^{18}O_{SO4(0)}}{\varepsilon^{34}S_{total}} \quad (7)$$

211 The value of the SALP can vary between 0.25 to over 10 and depends on the sulfate
212 reduction rate (Böttcher et al., 1998; 1999; Brunner et al., 2006; Aharon and Fu, 2000;
213 Antler et al., 2013) and the type and supply rate of the electron donor (Antler et al.,
214 2014; Antler et al., 2015).
215 2. Apparent equilibrium phase. This is the last segment on the $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$
216 cross plot, where the $\delta^{18}O_{SO4}$ reaches a constant value while the $\delta^{34}S_{SO4}$ continues to
217 increase. In the natural environment, the $\delta^{18}O_{SO4}$ equilibrium value has been observed
218 to be between 22‰ to 28‰ larger than the $\delta^{18}O$ value of the water (e.g. Wortmann et
219 al., 2007; Turchyn et al., 2006; Knöller et al., 2006). This large range of oxygen

220 isotope equilibrium values may reflect isotope exchange at different temperatures
221 (Fritz et al. 1989; Zeebe, 2010) or reflect the combined effect of kinetic and
222 equilibrium oxygen isotope fractionations (Wortmann et al., 2007; Turchyn et al.,
223 2010). The latter is named apparent equilibrium ($\delta^{18}O_{SO4(A.E)}$). Several sulfur
224 intermediates have been suggested to exchange oxygen isotopes with water; most
225 notably APS (e.g. Mizutani and Rafter, 1973; Fritz, et al., 1989), sulfite (e.g. Wankel
226 et al., 2014; Muller et al., 2013) and when sulfite is bound to adenosine mono-
227 phosphate (e.g. Wortmann et al., 2007; Wankel et al., 2014). Recent studies have
228 ruled out the equilibrium between APS and water (Brunner et al., 2012; Kohl et al.,
229 2012). Under cytoplasmic pH (6-7), sulfite reaches isotopic equilibration in matters of
230 minutes (Betts and Voss, 1970; Wankel et al., 2014); the rapidity of the oxygen
231 isotope equilibrium implies that sulfite in the cell is fully equilibrated with water. The
232 value of apparent equilibrium, including the effect of the kinetic oxygen isotope
233 fractionation, is expressed mathematically as:
234
235 $$\delta^{18}O_{SO4(A.E)}=\delta^{18}O_{H2O}+\varepsilon^{18}O_{ex}+\frac{\varepsilon^{18}O_{f\_1}}{\phi_1\cdot\phi_2\cdot\phi_3}+\frac{\varepsilon^{18}O_{f\_3}}{\phi_3} \quad (8)$$
236
237 A useful way to study the mutual evolution of $\delta^{18}O_{SO4}$ and $\delta^{34}S_{SO4}$ with respect to
238 the progress of MSR (e.g. the decrease in sulfate concentration with time during
239 MSR) is to plot θ_O vs. $\varepsilon^{34}S_{total}$ (Fig. 2b). Previous studies have used this cross-plot to
240 investigate the mechanism of MSR (Brunner, et al., 2005; Knöller et al., 2006;
241 Turchyn, et al., 2010; 2016; Brunner et al., 2012; Antler et al., 2013) and sulfate-
242 driven anaerobic methane oxidation (Deusner et al., 2014). Because both $\varepsilon^{34}S_{total}$ and
243 θ_O are functions of the forward and backward fluxes during MSR (Equations 1 and 6,

244 respectively), this plot can be used to relate sulfur and oxygen isotope measurements
245 to the intracellular MSR fluxes. This is because for every set of given forward and
246 backward fluxes, there are specific values of θ_O and $\varepsilon^{34}S_{total}$. However, because there
247 are more branching points in the framework of MSR than the solution for θ_O and
248 $\varepsilon^{34}S_{total}$, the ratio of between the forward and backward fluxes of every branching
249 point cannot be solved uniquely. Figure 2b also demonstrates the relationship
250 between θ_O vs. $\varepsilon^{34}S_{total}$ and the ratio of intracellular fluxes.

251 The plot of θ_O vs. $\varepsilon^{34}S_{total}$ (Fig. 2b) has similarities to the plot of $E^{33}S$ vs. $\varepsilon^{34}S_{total}$
252 (Fig, 2a), but typically only one of these two is used. The central question asked by
253 this study is whether the use of combined θ_O vs. $\varepsilon^{34}S_{total}$ and $E^{33}S$ vs. $\varepsilon^{34}S_{total}$ diagrams
254 can enable the probing of different processes and reaction rates during MSR. We
255 hypothesize that this combined isotope approach can explore a wider range of steps in
256 the modeled MSR network, not all of which may be inferred by using θ_O vs. $\varepsilon^{34}S_{total}$
257 or $E^{33}S$ vs. $\varepsilon^{34}S_{total}$ plots alone.

258

259 **4. Methods**

260 Pure cultures of marine sulfate reducing bacterium *Desulfovibrio* sp. (strain DMSS-
261 1—Sim et al. 2011b) were incubated in batch at room temperature (22ºC) in the dark
262 (see Sim et al. 2011b for the medium recipe). DMSS-1 was grown on five different
263 organic substrates (lactate, malate, ethanol, fructose and glucose at limiting
264 concentrations) that served as both the electron donors and carbon sources. Each
265 incubation experiment with different organic substrates was repeated three times
266 using isotopically enriched water with different initial oxygen isotope compositions
267 (Table 1). The use of ^{18}O-enriched water serves a way to enhance the signal in the
268 measured $\delta^{18}O_{SO4}$. Furthermore, by comparing the change in $\delta^{18}O_{SO4}$ from

experiments using different $\delta^{18}O_{H2O}$ we can distinguish the contribution of oxygen exchange with water from other isotopic effects (such as kinetic isotope fractionation).

Bacteria were pre-cultured on the respective electron donors before inoculation: about 1 ml of preculture was spun down anaerobically, the pellet was washed with fresh medium three times to remove sulfide and then transferred to 100 ml of fresh medium. Then, this medium was transferred in 10-ml aliquots to seven or eight 15 ml vials. Each of the vials was sacrificed at different time points during the experiment by removing 1 ml for cell counts and sulfide measurements and injecting 2 ml of 20% Zn acetate to precipitate zinc sulfide. Most of this solution was used to measure multiple sulfur isotopes. Two milliliters of the Zn-treated culture were filtered. Half of the filtrate was used to measure the sulfate concentration and the other half was mixed with 1 ml of the saturated BaCl solution to precipitate barite ($BaSO_4$).

4.1 Analytical methods

Sulfate concentrations were measured by ion chromatography (IC, Dionex DX5000) with an error of 3% between duplicates. Sulfide concentrations were measured by spectrophotometer using a modified methylene blue assay (Cline, 1969). Cell density was measured by epifluorescence microscopic counts of cells stained by SYTOX® Green nucleic acid stain (Life Technologies, Grand Island, NY, USA).

For the analysis of $\delta^{18}O_{SO4}$, barite was pyrolyzed at 1450°C in a temperature conversion element analyzer (TC/EA), producing carbon monoxide. Carbon monoxide was measured by continuous helium flow in a GS-IRMS (Thermo Finnegan Delta V Plus, at the Godwin Laboratory, University of Cambridge). To

294 analyse the $\delta^{34}S_{SO4}$, barite was combusted at 1030°C in a flash element analyzer (EA),
295 and the resulting sulfur dioxide (SO_2) was measured by continuous helium flow on a
296 GS-IRMS (Thermo Finnegan Delta V Plus Godwin Laboratory, University of
297 Cambridge). Analyses of $\delta^{18}O_{SO4}$ were conducted in replicates (n=3-5) and the
298 standard deviation was determined using the standard NBS 127 (~0.3‰ 1σ). The
299 error for $\delta^{34}S_{SO4}$ was determined using the standard deviation of standards run at the
300 beginning and the end of each run (~0.3‰ 1σ). Measurements of $\delta^{18}O_{SO4}$ and $\delta^{34}S_{SO4}$
301 were corrected to NBS 127 and IAEA-SO-6 ($\delta^{18}O_{SO4}$ of 8.6‰, -11.35‰ and $\delta^{34}S_{SO4}$
302 of 20.3‰, -34.1‰, respectively). $\delta^{34}S_{SO4}$ is reported with respect to Vienna Canyon
303 Diablo Troilite (VCDT) and $\delta^{18}O_{SO4}$ is reported relative to the Vienna Standard Mean
304 Ocean Water (VSMOW). Appendix 3 discusses the validation of measurements of
305 exceptionally high $\delta^{18}O_{SO4}$.

306 To conduct multiple sulfur isotope measurements ($\delta^{33}S$ and $\delta^{34}S$), Ag_2S samples
307 were reacted with an excess of fluorine gas at 300°C, following the procedure
308 described in Ono et al. (2006). This produced SF_6 gas, which was purified by gas
309 chromatography and transferred into Thermo Finnegan MAT 253 for multiple sulfur
310 isotope measurements. Sulfide generated during growth was extracted by acidifying
311 the zinc sulfide precipitate with 6N HCl at 80°C under nitrogen gas for two hours.
312 H_2S(g) produced during this distillation was re-precipitated as zinc sulfide in a zinc–
313 acetate solution (0.18 M). After the extraction of sulfide, the samples were purged by
314 nitrogen gas for an additional hour to ensure the complete removal of sulfide. Sulfate
315 in the remaining medium was reduced to sulfide by reacting with 30 ml of the
316 reducing agent (mixture of HI, H_3PO_2 and HCl, Thode et al., 1961). The samples were
317 boiled and purged by N_2 gas. The volatile products were passed through a condenser
318 and a trap containing distilled water and sulfide gas generated by sulfate reduction

was collected in the zinc–acetate trap. The analytical reproducibility of measurements using the fluorination method, as determined by repeated analyses of international reference material, is ±0.1‰ and ±0.2‰ for $\delta^{33}S$ and $\delta^{34}S$, respectively.

$\delta^{18}O_{H2O}$ was measured by a Continuous Flow Gas Source Isotopic Ratio Mass Spectrometer (CF-GS-IRMS Thermo, at the Godwin Laboratory, University of Cambridge) coupled to a Gas Bench II (GBII) interface. Vials containing 0.5 ml of the sample were flushed with helium and 0.4% CO_2 gas-mixture and the samples were measured after equilibrating with the gas mixture for 24 hours. Samples were corrected to three standards (-7.3, 0.2 and 11.2‰). The error of the measurement was ± 0.1‰. $\delta^{18}O_{H2O}$ reported versus Vienna Standard mean Ocean water (VSMOW).

5. Results

All results are tabulated in the supporting online material. DMSS-1 grew and reduced sulfate under all tested conditions. Figure 3 is a composite figure showing sulfate concentrations and $\delta^{34}S_{SO4}$ and $\delta^{18}O_{SO4}$ measured in experiments with all different electron donors in experiments with the $\delta^{18}O_{H2O}$ of 72 ± 1‰. The results of the cultures grown in water with different $\delta^{18}O_{H2O}$ show very similar trends and are presented in the supplementary material. Sulfate concentrations decreased with time, as expected during MSR. Cell densities, and $\delta^{34}S_{SO4}$ and $\delta^{18}O_{SO4}$ increased with time in all experiments. The largest and the smallest decrease in sulfate concentration were observed in the experiments with lactate and glucose, respectively. The $\delta^{34}S_{SO4}$ and $\delta^{18}O_{SO4}$ showed the opposite trend, where sulfur and oxygen isotope ratios changed faster when DMSS-1 grew on glucose than when the bacterium grew on lactate (Fig. 3).

344 To compare sulfur isotope enrichments among the experiments, we plot the
345 change in $\delta^{34}S_{SO4}$ from its initial value ($\delta^{34}S_{SO4}(t)$- $\delta^{34}S_{SO4}(0)$) against the natural
346 logarithm of the ratio of the remaining sulfate (Fig. 4a); The more rapidly the $\delta^{34}S_{SO4}$
347 changes with the decreasing sulfate concentration, the bigger the sulfur isotope
348 enrichment or fractionation. The growth on glucose resulted in the highest sulfur
349 isotope fractionation. Decreasing sulfur isotope fractionation occurred in cultures
350 grown on fructose, malate, ethanol and lactate. The cross-plot of $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$
351 (Fig. 4b) demonstrates the relative changes of the $\delta^{18}O_{SO4}$ and $\delta^{34}S_{SO4}$ in different
352 experiments. The $\delta^{18}O_{SO4}$ exhibited the largest and smallest changes, respectively,
353 relative to the changes in $\delta^{34}S_{SO4}$ during growth on glucose and lactate, respectively.
354
355

6. Discussion

6.1 Placing a limit on kinetic oxygen isotope fractionation

356 Studies on MSR use $\delta^{18}O_{SO4}$ mainly to target the reoxidation of intracellular reduced
357 sulfur species, although the potential importance of kinetic oxygen isotope
358 fractionation is becoming increasingly recognized (Brunner et al., 2005; 2012;
359 Farquhar et al., 2008; Wortmann et al., 2007, Turchyn et al., 2006; Aller et al., 2010;
360 Antler et al., 2013; Wankel et al., 2014), because it can influence the $\delta^{18}O_{SO4}$ at
361 'apparent equilibrium' (Wortmann et al., 2007; Turchyn et al., 2010; Antler et al.,
362 2013—see also equation 8). Some studies assume that kinetic oxygen isotopic
363 fractionation does not occur (Brunner et al., 2006; 2012), and some estimate an
364 overall kinetic oxygen isotope fractionation as high as 10‰ during MSR (Wankel et
365 al., 2014). Turchyn et al. (2010) suggested that the kinetic oxygen isotope
366 fractionation could not exceed 4‰. All these assumptions lead to differing

368 conclusions about cellular fluxes of sulfur and electrons during MSR and complicate
369 interpretations of environmental data.

370 Kinetic fractionation of oxygen isotopes during MSR can only be studied
371 when the effect of water-isotope equilibrium on the $\delta^{18}O_{SO4}$ value is minimal. Even
372 then, we must assume that the timescale for oxygen isotope exchange between water
373 and sulfite (minutes in intracellular pH of 6-7—Wankel et al., 2014) is longer than the
374 residence time of sulfite in the cell. We explore this by plotting the slope of the
375 apparent linear phase ('SALP', section 2, equation 7) against the oxygen isotope
376 composition of water for all our studied cultures (Fig. 5a). Our experiments
377 demonstrate that the oxygen isotopic composition of water affects the calculated
378 SALP in all experiments, including conditions that previously would have been
379 interpreted as primarily kinetically driven. Therefore, an equilibrium component
380 contributes to the total oxygen isotope fractionation between sulfate and water under
381 all tested conditions.

382 The experiment exhibiting the smallest influence of the oxygen isotope
383 equilibrium can place an upper limit on the potential magnitude of the total kinetic
384 oxygen isotope fractionation. Figure 5a shows that the kinetic isotope effect has the
385 largest contribution to the measured $\delta^{18}O_{SO4}$ in cultures grown on lactate. This
386 experiment, grown in water with a $\delta^{18}O_{H2O}$ of -5.3‰, yielded the smallest calculated
387 slope of the apparent linear phase (SALP, Fig. 3). This slope, 0.009 ± 0.03, suggests
388 that the kinetic isotope fractionation of oxygen isotopes relative to the fractionation of
389 sulfur isotopes is negligible at high cell specific sulfate reduction rates. However, the
390 variation in $\delta^{18}O_{SO4}$ in the lactate experiment is very small (~1‰) and the error of the
391 analytical measurement of $\delta^{18}O_{SO4}$ values is high (about 0.4‰), requiring a more
392 conservative calculation. Therefore, we consider the experiment with malate, which

393 exhibited the second smallest experimental slope (Malate- Fig. 5a). In this case, the
394 slope was still smaller than 0.25, which suggests that the magnitude of the kinetic
395 oxygen isotope fractionation cannot be larger than 25% of the magnitude of the
396 kinetic isotope fractionation for sulfur isotopes. Overall, we suggest that the kinetic
397 oxygen isotope fractionation ($\varepsilon^{18}O_{total}$) is between 0 and 5‰ (between 0-25% of the
398 kinetic sulfur isotope fractionation $\varepsilon^{34}S_{total}$-- 20‰ for the experiment with malate).
399 This value is in agreement with Brunner et al. (2005; 2012) and estimates derived by
400 Turchyn et al. (2010) in pure culture studies, but different from some environmental
401 studies of sites where methane is highly abundant and sulfate reduction rate are high,
402 and linear correlations on the $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$ cross-plot have slopes between 0.34-
403 0.5 (Antler et al., 2015; Sivan et al., 2014). These observations indicate that: 1. MSR
404 in nature can be associated with kinetic oxygen isotope fractionations larger than 5‰
405 at high rates of sulfate reduction or 2. processes other than MSR control the observed
406 oxygen isotope fractionation.

407 The calculated $\delta^{18}O_{SO4(A.E)}$ (Fig. A.4, Appendix 2) for each of our experiments
408 are presented in Figure 5b as a function of the csSRR. There is an inverse correlation
409 between the $\delta^{18}O_{SO4(A.E)}$ and the csSRR. Because our experiments were conducted at
410 the same temperature (~22ºC) and at the same time, we can rule out temperature
411 effects on these different isotope equilibria. Instead, factors related to cell physiology
412 and growth conditions are likely to modify the value of $\delta^{18}O_{SO4(A.E)}$. This correlation
413 validates the idea that MSR can generate a range of apparent equilibrium values,
414 rather than a fixed value (Wortmann et al., 2007; Turchyn et al., 2010; Wankel et al.,
415 2014; Antler et al., 2013). This correlation also demonstrates that the kinetic oxygen
416 isotope fractionation impacts the $\delta^{18}O_{SO4}$ much more than the equilibrium
417 fractionation under growth conditions that favour high csSRRs. Equation 8 predicts

418 this, because at high csSRRs, the uptake of sulfate and its reduction to sulfite are not
419 reversible and the $\delta^{18}O_{SO4}$ at equilibrium approaches infinity.
420
421 **6.2 Tracing of intracellular sulfur metabolism during microbial sulfate reduction**
422 The sulfur isotope fractionation factor in our experiments was calculated using
423 Rayleigh-type distillation by plotting the change in the isotopic composition of sulfur
424 against the natural log of the fraction of the remaining sulfate (Fig. 4a). Typically,
425 studies report an inverse correlation between $\varepsilon^{34}S_{total}$ and the sulfate reduction rate
426 (e.g. Chambers and Trudinger, 1975; Canfield, et al., 2001; Leavitt et al., 2013; 2015;
427 Sim et al., 2011a;2011b; Ono et al., 2015). Our experiments used different organic
428 donors to change the csSRR and reproduced the same inverse relationship between
429 the csSRR and the magnitude of sulfur isotope fractionation (Fig. 6a). This is
430 consistent with previous culture studies of DMSS-1 (Sim et al., 2011a; 2011b).
431 Models attribute small overall sulfur isotope fractionations to the lower fluxes of
432 intracellular sulfur intermediates that are being oxidized back to the sulfate pool (e.g.
433 Rees 1973; Brunner and Bernasconi, 2005; Farquhar et al., 2003; Canfield 2006;
434 Wing and Halevy, 2014).
435 Minor sulfur isotopes ($^{34}S/^{32}S$ and $^{33}S/^{32}S$) provide information about the
436 mixing among different pools of metabolites during MSR (see Fig. 2a). Figure 6b
437 plots the $E^{33}S$ and $\varepsilon^{34}S_{total}$ values calculated from our experimental results. Our results
438 fall within the grey mesh of model space for ϕ_4=1 (the flux ratio of step 4 - the
439 reduction of sulfite to polysulfide — Fig. 1). However, the plot of our data together
440 with previously published data using the same strain and electron donors (DMSS-1 --
441 Sim et al., 2011a; 2011b) shows that the $E^{33}S$ vs. $\varepsilon^{34}S_{total}$ data cannot be explain solely
442 by assuming ϕ_4=1 or ϕ_4=0 (since not all data fall within the envelopes for the two end

443 member solutions: ϕ_4=0 and ϕ_4=1) and that additional information is needed to solve
444 the intracellular fluxes uniquely.

445 Changes in the oxygen isotope composition of sulfate can provide some
446 additional information about MSR. Figure 6c shows θ_O calculated from the data
447 obtained in the five different electron donor experiments (see equation A.5, appendix
448 2) plotted against $\varepsilon^{34}S_{total}$ (similar to Fig. 2b). The larger θ_O are associated with the
449 larger $\varepsilon^{34}S_{total}$. Notably, not all the data fall within the envelopes for the two end
450 member solutions (ϕ_4=0 and ϕ_4=1, black lines and grey lines, respectively, Figure 6c).
451 The presence of some data points outside the modelled space in $E^{33}S$ vs. $\varepsilon^{34}S$ and θ_O
452 vs. $\varepsilon^{34}S_{total}$ plots (Fig. 6a, b, c) is consistent with observations made by Brunner et al.
453 (2012) and Sim et al. (2011b). In practice, this indicates that previous models that
454 account for two branching point during MSR, as constrained by stable isotopes of
455 sulfur and csSRRs, do not adequately explain all observations that have been made in
456 pure culture and in the natural environment. Therefore, our model considered three
457 branching points instead of two, which is in line with recent revision of the sulfate
458 reduction pathway (Santos et al., 2015). The sulfur isotope fractionation was taken as
459 $\alpha^{34}S$= 0.975 at each of the commonly-used branching points (e.g. Brunner et al.,
460 2012).

461 Our experiments with DMSS-1 grown on different electron donors probe
462 csSRRs that vary over two orders of magnitude. The increase in csSRR and the
463 accompanying variations in the stable sulfur ($^{33}S/^{32}S$ and $^{34}S/^{32}S$) and oxygen
464 ($^{18}O/^{16}O$) isotope ratios can be used to further explore the dynamics of MSR and solve
465 uniquely the fluxes at three branching points within the cells. This flux ratio solution
466 (ϕ_1, ϕ_3 and ϕ_4) is shown in Figure 6d as a function of the csSRR; the ratios of fluxes
467 at all assumed branching points are inversely correlated with the csSRR. Our

468 experiments can be divided into three broad categories based on the key branching
469 reaction: 1) Lactate experiment, with high csSRR and minimal reoxidation of sulfur
470 intermediates; 2) Malate, ethanol and fructose, with moderate csSRR, where the
471 reduction of APS to sulfite is likely to be the key branch point; 3) Glucose, with slow
472 csSRR where the last step (the reduction to sulfide) is the key branching point.
473 Oliveira et al. (2008) suggested that a subunit of dissimilatory sulfite
474 reductase, DsrC, plays a key role in the reduction of S^0 produced by DsrAB. More
475 recently it was confirmed that the csSRR is indeed determined at the cellular level by
476 the DsrC subunit (Santos et al., 2015). Our calculations (Fig. 6d) show that: 1. fluxes
477 at all three modelled branching points exhibit an inverse correlation with the overall
478 csSRR with similar slopes between the ratios of fluxes and csSRR and 2. the first four
479 branching points or steps do not appear to limit the overall MSR process. Given our
480 model assumption that the MSR machinery consists of three branching points and that
481 sulfur isotope fractionations at steps 3,4 and 5 are $\alpha^{34}S= 0.975$, we therefore suggest
482 that the csSRR directly influences the reversibility at all branching points under our
483 experimental conditions. This implies that the last step (step 5, the reduction of S^{2+} to
484 sulfide—Fig. 1) is the rate-limiting step. Our results therefore, are consistent with the
485 observation made by Santos et al. (2015). We suggest the availability of the organic
486 matter, and therefore electrons, controls the availability of the DsrC subunit which is
487 directly linked to the expression of sulfur and oxygen isotope fractionation during
488 MSR.
489
490 **6.3 Environmental implications**
491 To what extent do results from pure culture experiments such as ours explain the
492 geochemical variability in the natural environment? This section addresses this

493 question by applying our insights from pure culture experiments to $\delta^{18}O_{SO4}$ and
494 $\delta^{34}S_{SO4}$ measured in sedimentary pore fluids. In this discussion, the depth below the
495 seafloor of total consumption of sulfate is used to calculate the net sulfate reduction
496 rate with the observation that sulfate is often consumed within three meters below the
497 seafloor in environments with high sulfate reduction rates (on the order of 10^{-4} to 10^{-5}
498 mol cm^{-3} $year^{-1}$) such as estuaries and methane seeps (Aharon and Fu, 2000 & 2003).
499 On the other hand, sulfate is consumed at depths between three and ten meters in
500 environments with moderate sulfate reduction rates (on the order of 10^{-6} mol cm^{-3}
501 $year^{-1}$), including the continental shelf and river deltas (Aller et al., 2010). When
502 sulfate is consumed deeper than ten meters below the seafloor, the environment is
503 considered to have low sulfate reduction rates (lower than 10^{-7} mol cm^{-3} $year^{-1}$). The
504 latter environments include organic-poor deep-sea sediments (Turchyn et al., 2006;
505 Wortmann et al., 2007).

506 Figure 7 plots $\delta^{18}O_{SO4}$ and $\delta^{34}S_{SO4}$ of pore fluids for these three types of sites
507 (color coded), and overlays the curves for the relative evolution of $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$
508 based on our experiments. Figure 7 shows a number of notable features. First, the
509 overall variation in the data from pore fluids follows our findings, where at lower
510 rates of microbial sulfate reduction, the $\delta^{18}O_{SO4}$ increases rapidly relative to the
511 $\delta^{34}S_{SO4}$. Second, most of the pore fluid data fall above our laboratory-derived
512 estimate for kinetic oxygen isotope fractionation. Lastly, data points from at least
513 eleven field sites with moderate to low sulfate reduction rates fall above the curve of
514 our experiment with the slowest csSRR (glucose experiment) although this
515 experiment is record the highest oxygen isotope fractionation (θ_o) in sulfate measured
516 in pure culture to date.

517 In an open system such as marine sediments, transport (e.g. diffusion and
518 advection through the pore fluids) should modify the measured $\delta^{18}O_{SO4}$ and $\delta^{34}S_{SO4}$
519 (Jørgensen, 1979; Wortmann et al., 2007; Chernyavsky and Wortmann et a., 2007).
520 Therefore, if we want to compare results from batch cultures (a closed system) to pore
521 fluid data, it is important to consider the variations of $\delta^{18}O_{SO4}$ and $\delta^{34}S_{SO4}$ in open
522 systems that are modified by transport. Given that the $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$ relationship
523 is always concave-down (except the special case when it is linear), by definition, any
524 mixing between two points on the plot will results in a value lower than the original
525 value. This means that the effect of transport can only result in a more moderate
526 SALP. This effect was modelled by Antler et al. (2013). Therefore, transport
527 (diffusion and advection) by itself cannot explain the discrepancy between our
528 experiments and the pore water results and most likely will exacerbate this
529 discrepancy.

530 Overall, pore fluids and pure culture experiments of microbial sulfate reducers
531 exhibit similar trends. Some gaps between them can be explained by the much lower
532 csSRR relative to the batch experiments (e.g. Holmkvist et al., 2011). However, some
533 features, such as the high oxygen isotope equilibrium (which can be more than 5‰
534 higher than expected from our experiment—Fig. 7), with high apparent SALP in pore
535 fluids from sites with slow sulfate reduction rates may not be explained only by MSR
536 even if we consider any potential temperature effect on the oxygen isotope
537 equilibrium between sulfur intermediates and water. Therefore, we suggest that
538 different processes control this relationship in those cases. For instance, in gas seeps,
539 where sulfate-driven anaerobic oxidation of methane is present, the plot of $\delta^{18}O_{SO4}$
540 and $\delta^{34}S_{SO4}$ shows a linear correlation with moderate slopes (Aharon an Fu,
541 2000;2003; Rubin-Blum et al., 2014; Wehrmann et al., 2011) due to little reoxidation

of reduced sulfur species during net sulfate reduction (Antler et al., 2015). In contrast, in organic-poor sediments where the sulfate reduction rate is low and complex extracellular cycling between sulfur and iron, sulfur and manganese cycling or disproportionation is possible, the slopes of $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$ are much steeper as $\delta^{18}O_{SO4}$ increases with minor changes in $\delta^{34}S_{SO4}$ (e.g. Aller et al., 2011, Blake et al., 2006 Böttcher and Thamdrup, 2001; Böttcher et al., 2001; Böttcher et al., 2005; Mills et al., 2016). From this perspective, oxygen isotopes in sulfate are a more reliable indicator of extracellular reoxidation than sulfur isotopes. An alternative speculation is that with extreme low availability of organic carbon, DsrC is also much less available, which might result in the formation of other intermediates including trithionate ($S_4O_6^{2-}$) and thiosulfate ($S_2O_3^{-2}$) (Santos et al., 2015; Bradley et al., 2011) which will ultimately will alter the oxygen and sulfur isotope fractionation.

The next challenge will be to resolve the gap between the pure cultures experiments and *in situ* pore fluid $\delta^{34}S_{SO4}$ and $\delta^{18}O_{SO4}$. This might be enabled by the increasing the availability of coupled measurements of $\delta^{34}S_{SO4}$, $\delta^{33}S_{SO4}$ and $\delta^{18}O_{SO4}$ values together with csSRR. Studies of microbial cultures at even lower csSRR may also yield even higher $\delta^{34}S_{SO4}$, $\delta^{33}S_{SO4}$ and $\delta^{18}O_{SO4}$ signals and expand the known range of oxygen and sulfur isotope fractionations accessible to MSR.

7. Summary

This study presents measurements of $^{33}S/^{32}S$, $^{34}S/^{32}S$ and $^{18}O/^{16}O$ in pure cultures of *Desulfovibrio* sp. (strain DMSS-1). The cell-specific sulfate reduction rates vary over three orders of magnitude in DMSS-1 cultures growing on these different electron donors. The data show that isotopic fractionations of sulfur and oxygen isotopes in dissolved sulfate record different processes under controlled conditions and depend

on sulfate reduction rates. As previously shown, the $^{34}S/^{32}S$ isotope fractionation varies between 7 and 61‰ and correlates with the cell specific sulfate reduction rates. The combination of sulfur and oxygen isotope data also demonstrates for that the values of oxygen isotope fractionation at apparent equilibrium are a function of the cell specific sulfate reduction rates. These results are used to calculate the ratios of fluxes at each individual step during microbial sulfate reduction.

Compared to the previously reported environmental observations, the culture results cannot explain some combinations of sulfur and oxygen isotope fractionations in nature. We propose that the gap between lab experiments and the natural environment may arise from the much lower availability of organic matter in nature than culture. In addition, processes such as the oxidation of intermediate sulfur redox species to sulfate, sulfur disproportionation and sulfate-driven anaerobic methane oxidation likely impact the isotopic correlation in the environment, but not in pure cultures of sulfate reducing microbes. Experiments with natural populations and sulfate reducers limited by very low concentrations of organic matter can explore the combined effects of processes that impart isotopic signatures on sulfur and oxygen in dissolved sulfate.

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9. Figure captions

Figure 1: Schematic of the microbial sulfate reduction pathway. Steps of MSR and the presumed points of oxygen and sulfur isotope fractionation. i_{j_j}, $\alpha^{34}S_{i\_j}$ and $\alpha^{18}O_{i\_j}$ are the fluxes and the isotope fractionation factors for sulfur and oxygen, respectively, for the forward (i=f) and backward (i=b) reactions j (j=1...5). ϕ_k (k=1,2 and 4) is the ratio between the backward and forward fluxes. $\varepsilon^{18}O_{ex}$ is the fractionation of oxygen isotopes between water and sulfur intermediates.

Figure 2: Model predictions of flux ratios that produce different $E^{33}S$, $\varepsilon^{34}S_{total}$ and θ_O values. (a) $E^{33}S$ vs. $\varepsilon^{34}S_{total}$ diagram. (b) θ_O vs. $\varepsilon^{34}S_{total}$ diagram. The black and the gray meshes, respectively, in both panels are the solutions where ϕ_4 (the ratio of fluxes in step 4, the reduction of sulfite to S^{2+}—Fig. 1) is minimal (=0) and maximal (=1), respectively. The arrows show the direction by which each flux ratio (Figure 1) changes in the diagrams. According to numerical models, if two of the three values of $E^{33}S$, $\varepsilon^{34}S_{total}$, and θ_O are measured, the result will plot within the black and the grey meshes and enable solving for the relative fluxes at two steps during MSR. Here, we assume isotope fractionations at steps 3,4 and 5 to be $\alpha^{34}S$= 0.975 (Brunner et al., 2012) and $\vartheta^* = 0.5147$ (Farquhar et al., 2003).

929 Figure 3: Sulfate (SO_4^{2-}) and sulfide (H_2S) concentrations (a) and $\delta^{34}S_{SO4}$ and $\delta^{18}O_{SO4}$
930 values (b) in DMSS-1 cultures grown on lactate (#.1), malate (#.2), ethanol (#.3)
931 Fructose (#.4) and glucose (#.5) (where # indicates panels a and b). In all cultures,
932 $\delta^{18}O_{H2O}$ =~75 ‰. Results for other examined $\delta^{18}O_{H2O}$ can be found in table S1 in the
933 supplemental online material.
934
935 Figure 4: Enrichments of oxygen and sulfur isotopes in sulfate. Sulfur isotope ratios
936 vs. the natural logarithm of the residual sulfate fraction left in the experiment after
937 bacterial sulfate reduction (a), $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$ (b). The rightmost panel shows an
938 enlargement of the middle panel (c).
939
940 Figure 5: The slope of the apparent linear phase on the $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$ cross-plot
941 (SALP) plotted against the oxygen isotopic composition of water used in the
942 experiments (a). The apparent equilibrium value of $\delta^{18}O_{SO4}$ ($\delta^{18}O_{SO4(A.E)}$- $\delta^{18}O_{H2O}$) in
943 each experiment plotted against the sulfur isotopic fractionation $\varepsilon^{34}S_{total}$ in each
944 experiment (b) . The dashed lines represent the rang of $\delta^{18}O_{SO4(A.E)}$- $\delta^{18}O_{H2O}$ from pore
945 fluids sulfate.
946
947 Figure 6: Isotopic fractionation. (a) Fractionation of $^{34}S/^{32}S$ as a function of the cell
948 specific sulfate reduction rate in this study. Data from Sim et al. (2011a,b) are plotted
949 for comparison. $E^{33}S$ vs. $\varepsilon^{34}S_{total}$ (b) and the θ_O vs. $\varepsilon^{34}S_{total}$ (c) diagram; The black and
950 the gray meshes, respectively, in both panels are the solutions where ϕ_4 (the ratio of
951 fluxes in step 4, the reduction of sulfite to S^{2+}—Fig. 1) is minimal (=0) and maximal
952 (=1), respectively. The bottom right panel (d) is the calculated flux ratio of steps 1,3

953 and 4 (Fig. 2.1—methods section) as a function of the cell specific sulfate reduction
954 rate.
955
956 Figure 7: $\delta^{18}O_{SO4}$ vs. $\delta^{34}S_{SO4}$ data from pore fluids. (Data were taken from Aharon and
957 Fu, 2000; 2003; Aller et al., 2010 Antler et al., 2013; Böttcher et al., 1998; 1999;
958 2006; Blake et al., 2006; Wehrmann et al., 2011; Wortmann 2006; 2007). Broadly, the
959 blue symbols (sulfate depletion above 3m) are data from gas seeps sites and estuaries.
960 The green symbols (sulfate depletion between 3 to 10m) are data from continental
961 shelf. The red symbols (sulfate depletion below 10m) are data from deep-sea
962 sediments. The area for 'kinetic fractionation' was calculated based on the
963 fractionations of sulfur and oxygen in the lactate and malate experiment (section 6.1).
964