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Sulfur isotope fractionation during growth of sulfate-reducing bacteria on various carbon sources

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Abstract—Stable sulfur isotope fractionation during microbial sulfate reduction is a potential tool to estimate sulfate reduction rates at field sites. However, little is known about the influence of the utilized carbon source on the magnitude of sulfur isotope fractionation. To investigate this effect, both a pure culture (strain PRTOL1) and enrichment cultures from a petroleum hydrocarbon (PHC)–contaminated aquifer were used and grown in batch cultures on various carbon sources with an initial sulfate concentration of 1 mmol/L. As sole carbon sources the PHC components naphthalene, 1,3,5-trimethylbenzene, and heating oil (enrichment culture) and the organic acids acetate, pyruvate, benzoate, and 3-phenylpropionate (enrichment culture and PRTOL1) were used. Sulfate reduction rates of all cultures ranged from 6 ± 1 nmol cm^{-3} d^{-1} (enrichment culture grown on 1,3,5-trimethylbenzene) to 280 ± 6 nmol cm^{-3} d^{-1} (enrichment culture grown on pyruvate). Cell-specific sulfate reduction rates ranged from 1.1×10^{-14} mol cell^{-1} d^{-1} (PRTOL1 grown on pyruvate) to 1.5×10^{-13} mol cell^{-1} d^{-1} (PRTOL1 grown on acetate). Sulfur isotope enrichment factors (ε) for the enrichment culture ranged from 16.1‰ (3-phenylpropionate) to 34.5‰ (1,3,5-trimethylbenzene) and for PRTOL1 from 30.0‰ (benzoate) to 36.0‰ (pyruvate). Cultures of PRTOL1 always showed higher ε values than the enrichment culture when grown on the same carbon source due to culture-specific properties. Higher ε values were obtained when the enrichment culture was grown on PHC components than on organic acids. No relationship between ε values and cell-specific sulfate reduction rate existed when all data were combined. When comparing the magnitude of ε values determined in this laboratory study with ε values measured at contaminated and uncontaminated field sites, it becomes evident that a multitude of factors influences ε values at field sites and complicates their interpretation. The results of this study help us assess some of the general parameters that govern the magnitude of ε in sulfate-reducing environments. *Copyright © 2004 Elsevier Ltd*

1. INTRODUCTION

Microbial SO_4^{2-} reduction is an important process in many petroleum hydrocarbon (PHC)-contaminated aquifers (Wiedemeier et al., 1999). To accurately estimate its contribution to contaminant degradation, in situ quantification of this process is essential (Madsen, 1991). Unfortunately, quantification of microbial SO_4^{2-} reduction in contaminated aquifers based on measurements of SO_4^{2-} consumption or S(-II) (here defined as the sum of S^{2-}, HS$^-$, and H$_2$S) production is often obscured by concurrent abiotic transformations, e.g., by dissolution of gypsum (CaSO$_4$) from the aquifer matrix (Stumm and Morgan, 1981) or by precipitation of S(-II) in form of iron sulfides (Anderson and Lovley, 2000). Hence, alternative tools are needed to investigate rates of SO_4^{2-} reduction.

Methods based on stable carbon isotopes were successfully used to characterize and quantify biologic processes in the subsurface (Hunkeler et al., 1999; Meckenstock et al., 2002). So far, sulfur isotope fractionation has been used qualitatively to indicate microbial SO_4^{2-} reduction in contaminated aquifers (Arneth and Hoefs, 1989; Bottrell et al., 1995; Alewell and Giesemann, 1996; Asmussen and Strauch, 1998; Schroth et al., 2001). However, to quantitatively use sulfur isotope fractionation at a field site, the enrichment factor (ε) has to be known a priori with reasonable accuracy (Aggarwal et al., 1997).

Nevertheless, the wide range of factors that influences sulfur isotope fractionation at field sites, e.g., heterogeneity, carbon source variations and limitations, microbial diversity, geochemical reactions, and reoxidation processes may complicate or rule out a quantitative application of sulfur isotope fractionation. Yet, in cases where e.g., reoxidation is not important, sulfur isotope fractionation data may provide quantitative estimates of sulfate removal (Spence et al., 2001).

Previously, a number of authors suggested that ε depends on carbon source, temperature, strain of sulfate-reducing bacteria (SRB), cell-specific sulfate reduction rate (sSRR), SO_4^{2-} and carbon source concentration, and complete or incomplete carbon source oxidation (Harrison and Thode, 1958; Kaplan and Rittenberg, 1964; Kemp and Thode, 1968; Chambers et al., 1975; Brüchert et al., 2001; Detmers et al., 2001; Habicht et al., 2002). The general range of ε values reported in the literature for pure and mixed SO_4^{2-}-reducing cultures was −3 to 46.9‰ (Harrison and Thode, 1958; Bolliger et al., 2001; Canfield, 2001; Detmers et al., 2001; Habicht et al., 2002). The influence of different carbon sources on isotope fractionation by the same pure culture was investigated only by few early researchers with contradictory results (Kaplan and Rittenberg, 1964; Kemp and Thode, 1968). Detmers et al. (2001) hypothesized that if one species was tested for several carbon sources, then a relationship between ε and sSRR might be shown. Such a relationship was demonstrated when pure cultures of *Desulfovibrio desulfuricans* were grown on lactate with varying temperature, lactate, and SO_4^{2-} concentrations (Harrison and

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Thode, 1958; Kaplan and Rittenberg, 1964; Chambers et al., 1975). However, when various strains were compared to each other a relation between ε and sSRR was not found (Brüchert et al., 2001; Detmers et al., 2001). Instead, strain-specific properties, complete or incomplete carbon source degradation (Brüchert et al., 2001; Detmers et al., 2001), and SO_4^{2-} concentration (Habicht et al., 2002) were found to be the most important factors influencing the extent of fractionation. Most of the studies published so far were conducted under optimal growth conditions. However, in PHC-contaminated freshwater environments, low SO_4^{2-} concentrations, low carbon source concentrations due to low solubility of most heating oil components in water (Wick et al., 2001), and complex carbon sources are frequently encountered.

In PHC-contaminated aquifers, ε values of 20 to 23‰ were obtained during single-well push-pull tests (Schroth et al., 2001). However, in such aquifers, SRB may not only directly degrade PHC components but also a range of organic acids which are metabolic products of fermenting bacteria (Cozzarelli et al., 1994). Injection of organic acids along with SO_4^{2-} in push-pull tests in a PHC-contaminated aquifer resulted in ε values of 16 to 26‰ (Kleikemper et al., 2002b), similar to those obtained by Schroth et al. (2001). Interestingly, these field data were in good agreement with data from Bolliger et al. (2001), who investigated sulfur isotope fractionation (average ε = 23.5‰) by a toluene-degrading enrichment culture. The study of Bolliger et al. (2001) focused on the effect of different SO_4^{2-} concentrations on sulfur isotope fractionation by pure and enrichment cultures and a single carbon source, toluene.

In contrast, our objective here was to quantify and compare sulfur isotope fractionation in batch cultures amended with various carbon sources. Conditions resembled freshwater field conditions in a PHC-contaminated aquifer (initial SO_4^{2-} concentration = 1 mmol/L). In particular, enrichment cultures from sediment of a PHC-contaminated aquifer were grown on naphthalene, 1,3,5-trimethylbenzene, heating oil, acetate, pyruvate, benzoate, and 3-phenylpropionate. A pure culture, strain PRTOL1, that had been isolated with toluene as sole carbon source from another PHC-contaminated freshwater aquifer by Beller et al. (1996), was used as model strain to explore the influence of carbon source on sulfur isotope fractionation by a single organism. PRTOL1 was incubated in parallel with the enrichment cultures on the same carbon sources (except for naphthalene, 1,3,5-trimethylbenzene, and heating oil, which PRTOL1 cannot use as carbon sources).

2. MATERIALS AND METHODS

2.1. Organisms and Cultivation

Active cultures of the SO_4^{2-}-reducing bacterium PRTOL1 (Beller et al., 1996) were purchased from the Oregon Collection of Methanogens (Portland, OR) and pregrown on pyruvate, benzoate or 3-phenylpropionate as sole carbon sources. The inocula for the enrichment cultures were obtained from a PHC-contaminated aquifer in Studen, Switzerland (Bolliger et al., 1999) and maintained under SO_4^{2-}-reducing conditions with either acetate, pyruvate, benzoate, 3-phenylpropionate, naphthalene, 1,3,5-trimethylbenzene, or weathered heating oil (recovered from the same site) as sole carbon sources. Each culture was transferred several times so that eventually sediment-free enrichment cultures were obtained.

The enrichment cultures and PRTOL1 were grown in basal media as described by Beller et al. (1996). For cultivation, SO_4^{2-} was added as

FeSO$_4$ (5 mmol/L) (MicroSelect, Fluka, Switzerland). For subsequent sulfur isotope fractionation experiments, SO_4^{2-} was added as NaSO$_4$ (MicroSelect, Fluka, Switzerland) except for experiments with naphthalene, 1,3,5-trimethylbenzene and heating oil, where FeSO$_4$ had to be employed due to severe growth inhibition by S(-II) when NaSO$_4$ was used. The media were supplemented with non-chelated trace element mixture, selenite-tungstate solution, bicarbonate solution, vitamin mixture, vitamin B$_{12}$ solution, and S(-II) solution as described previously (Widdel and Bak, 1992).

Aromatic hydrocarbons (naphthalene, 1,3,5-trimethylbenzene; purum, Fluka) were added as dilute solutions in an inert lipophilic solvent used as carrier phase (Mineral oil, MicroSelect; Fluka) to maintain nearly constant hydrocarbon concentrations in the aqueous phase of 0.1 mmol/L during cultivation and batch culture experiments (Rabus et al., 1993). Three ml of carrier phase containing 0.69 mmol naphthalene or 0.79 mmol 1,3,5-trimethylbenzene were added per 100 mL of medium. One ml of heating oil was added to the respective batch cultures. Organic acids were added from anaerobic, autoclaved stock solutions to give final concentrations of 5 mmol/L (acetate, pyruvate) or 1.0 mmol/L (benzoate, 3-phenylpropionate). All carbon sources were non-limiting in our batch culture experiments. The enrichment cultures were incubated with all carbon sources and strain PRTOL1 with organic acids only. The final pH of the media was ~7.1. The media were inoculated with either 10% (v/v) of pregrown cultures (naphthalene, 1,3,5-trimethylbenzene, heating oil) or 5% (v/v) (all other carbon sources). Bacteria were cultured in 120-mL serum bottles with a headspace of ~17 mL (90% N$_2$, 10% CO$_2$) at 28°C inverted in the dark.

2.2. Batch Culture Experiments

Batch culture experiments were prepared in 120-mL serum bottles from basal media and carbon sources as described in the previous section. The initial SO_4^{2-} concentration was ~1 mmol/L in all batch cultures. Two independent control experiments were performed for each set of batch cultures. For the first control experiment we prepared batch cultures as described above except that the carbon source was omitted. In the second control experiment we prepared batch cultures as described above except that culture inoculation was omitted. All experiments were conducted at 28°C in the dark. Sulfate concentrations in test cultures were periodically monitored during the experiments, and at certain intervals sets of three serum bottles per culture and employed carbon source were analyzed for SO_4^{2-}, S(-II), and cell numbers and sacrificed for sulfur isotope analyses. Experiments were terminated when the initially supplied SO_4^{2-} was consumed or SO_4^{2-} reduction ceased.

2.3. Anion Concentrations

For the analysis of S(-II) concentrations, 0.2 mL of medium was removed from the serum bottles after vigorous shaking, and immediately dispensed in 4.8 mL of 20 mmol/L zinc acetate solution for S(-II) analysis. Cline reagent (0.5 mL) was immediately added and 20 min later absorbance was measured at 670 nm by spectrophotometry (Cline, 1969). An additional 2 mL of medium was withdrawn for SO_4^{2-} measurement. After centrifugation (10 min at 13,000 rpm), the supernatant was used for anion (SO_4^{2-}, organic acids) measurement by ion chromatography (IC-320, Dionex) according to Kleikemper et al. (2002b). The remaining pellet was further treated for counting of bacterial cells (see below). Analytical reproducibility was ~±5% for SO_4^{2-} and organic acids and ±2% for S(-II).

2.4. Bacterial Cell Numbers

Bacterial cell numbers were determined in batch cultures sacrificed at all sampling times of the respective experiments, and the arithmetic mean of these data were used to represent average cell numbers for each experiment. In experiments with naphthalene, 1,3,5-trimethylbenzene, and heating oil cell numbers were determined only at the first sampling point since FeS precipitates rendered cell counting under the microscope impossible at later times.

The pellet remaining from centrifugation (see previous section) was fixed over night in 4% paraformaldehyde in phosphate buffered saline solution (PBS; Sambrook et al., 1989) and then washed twice with

PBS. Cell suspensions were stored at −20°C in 50% (v/v) ethanol/PBS. Before application to slides, the paraformaldehyde-fixed cell suspensions were centrifuged, the supernatant removed, and the pellet was dispersed in sodium pyrophosphate (0.1%) by mild sonication for 3 min in a sonication bath. Ten microliters of suspension was subsequently spotted onto slides, dried at room temperature and finally dehydrated in 50, 80, and 100% ethanol for 3 min each. The cells were stained with DAPI (4′,6-diamidino-2-phenylindole, final concentration 500 ng ml⁻¹) at 42°C for 2 h. After staining and washing, slides were mounted with Citifluor solution (Citifluor, Caterbury, UK) and examined with a Zeiss Axiophot microscope fitted for epifluorescence with a high pressure mercury bulb (Zarda et al., 1997). Cells were counted in 40 images per sample.

2.5. Isotope Analyses of Sulfate and Sulfide

For isotope measurements S(-II) was precipitated as ZnS by addition of 5 mL 1 mol/L zinc acetate solution to the cultures. Serum bottles were vigorously shaken before 1 mL of 2 mol/L NaOH was added. The contents of the bottles were then filtered using a 0.45-μm HVLP membrane filter (Millipore). Sulfate was subsequently precipitated as $BaSO_4$ by first adding 2 mL 2 mol/L HCl and then 5 mL 1.2 mol/L $BaCl_2$ solutions to the filtrate and the precipitate was recovered on a separate 0.45-μm HVLP membrane filter. Both filters were dried at 60°C overnight. Excess mineral or heating oil was removed from filtrates using hexane.

For stable sulfur isotope ratio measurements ~400 to 600 μg of $BaSO_4$ or 150 or 200 μg of ZnS were weighed into tin cups. Vanadium pentoxide was added as catalyst in the amount of about twice the weight of the sample. Sulfur isotopes were subsequently measured on a FISONS OPTIMA mass spectrometer (Fisons, Middlewich, Chesire, UK) coupled in continuous-flow with a Carlo Erba elemental analyzer (CE Instruments, Milan, Italy). Data are reported in the conventional δ-notation relative to the Vienna-Canyon Diabolo Troilite (V-CDT) standard according to

$$\delta^{34}S(\text{‰}) = \left[\left(\frac{[^{34}S/^{32}S]_{sample}}{[^{34}S/^{32}S]_{V-CDT}} \right) - 1 \right] \times 1000 \qquad (1)$$

The system was calibrated using the international standards IAEA-S1 ($\delta^{34}S = -0.3$‰) and IAEA-S2 ($\delta^{34}S = 22.66$‰) (Ding et al., 2001). The mean $\delta^{34}S$ values obtained for the international standards NBS127 and NBS123 were 21.2 ± 0.2‰ ($n = 47$) and 17.7 ± 0.1‰ ($n = 13$), respectively.

2.6. Determination of Sulfate Reduction Rates

We computed SO_4^{2-} reduction rates (SRR, units of nmol cm⁻³ d⁻¹) for each culture and carbon source based on SO_4^{2-} consumption measured during the experiments assuming zero-order kinetics. Thus, values of SRR were obtained from the slope of a straight line fitted to data of remaining SO_4^{2-} concentration vs. time using linear regression analysis.

For the enrichment culture grown on pyruvate and PRTOL1 incubated with 3-phenylpropionate we also computed first-order SO_4^{2-} reduction rate coefficients (k, units of d⁻¹). For these computations we assumed a first-order-type reaction $dc/dt = -kc$, where c is reactant (here SO_4^{2-}) concentration and t is time. Hence, values of k were obtained from the slope of a straight line fitted to data of $\ln(c)$ vs. t using linear regression analysis. Employing a generic linear regression tool we computed standard deviations for both SRR and k, and coefficients of determination (R^2) were used as a measure of goodness of the respective fits. Finally, we computed values of sSRR (mol cell⁻¹ d⁻¹) according to Bolliger et al. (2001) by dividing SRR values by average cell numbers determined for the respective cultures. Values of sSRR may also be calculated according to Detmers et al. (2001) for the log phase growth. However, sSRR values obtained in this way were not substantially different from those calculated using average cell numbers (±23% difference on average) considering the sometimes large standard deviations found for sSRR values. Most importantly, calculating sSRR according to Detmers et al. (2001) did not change the way or significance of the correlation of sSRR with ε values (shown below).

2.7. Determination of Isotope Enrichment Factors

Stable isotope fractionation during a reaction is commonly quantified in terms of the fractionation factor α or the isotope enrichment factor ε (in per mil, e.g., Hoefs, 1997). The expression $1000 \times \ln\alpha$ gives a thermodynamically accurate measure of the extent of isotope fractionation (Clark and Fritz, 1997). A simplified form is $\varepsilon = (\alpha - 1) \times 1000$, which introduces a relative error ≤ 2% for ε values ≤ 40‰, which is typically smaller than the error of ε values determined in batch experiments (10%; Bolliger et al., 2001). Hence, we decided to use ε to quantify stable sulfur isotope fractionation throughout this study. In a closed system, values of ε can be determined using Rayleigh distillation equations (Mariotti et al., 1981). Using linear regression analysis, values of ε were obtained from the slope of a straight line simultaneously fitted to measured $\delta^{34}S$ values of remaining, unconsumed SO_4^{2-} ($\delta^{34}S[SO_4^{2-}]$) and accumulated S(-II) ($\delta^{34}S\{S[-II]\}$) according to Böttcher et al. (1999):

$$\delta^{34}S(SO_4) = \delta^{34}S(SO_4)_0 + \varepsilon \ln f \qquad (2)$$

$$\delta^{34}S[S(-II)] = \delta^{34}S(SO_4)_0 - \varepsilon(f \ln f)/(1-f) \qquad (3)$$

In Eqn. 2 and 3, f denotes the fraction of unconsumed SO_4^{2-}, and $\delta^{34}S(SO_4^{2-})_0$ represents the initial isotope composition of dissolved SO_4^{2-} (equal to 8.4 ± 1.6‰ in our experiments). Enrichment factors were calculated both by linear regression for each serum bottle separately and then averaged, and by linear regression of all data points together. Measured $\delta^{34}S(S[-II])$ data were corrected for the initial S(-II) concentration contained in the batch cultures and its isotope composition. For a detailed derivation of Eqn. 2 and 3 the reader is referred to Mariotti et al. (1981). All experimental data can be obtained in tabular form from the corresponding author upon request.

3. RESULTS

3.1. Microbial Sulfate Reduction

Strain PRTOL1 and the enrichment culture consumed SO_4^{2-} in all batch cultures amended with different carbon sources and with SO_4^{2-} as sole electron acceptor (Fig. 1). Concomitantly, we observed production of S(-II) during these experiments. On the other hand, SO_4^{2-} concentrations remained unchanged in the controls during up to 132 d of incubation (not shown).

The enrichment cultures consumed most of the SO_4^{2-} within ~25 d of incubation in batches amended with acetate, benzoate, and 3-phenylpropionate (Figs. 1a, 1c, and 1d), and within ~6 d of incubation in batches amended with pyruvate (Fig. 1b). Conversely, SO_4^{2-} was consumed only within 100 to 130 d of incubation in enrichment cultures amended with naphthalene, 1,3,5-trimethylbenzene, and heating oil (Figs. 1i–1k). Using data presented in Figure 1 we performed a mass balance on the sum of SO_4^{2-} and S(-II) in each sacrificed batch culture (Table 1). In batch cultures amended with acetate, pyruvate, phenylpropionate, and benzoate close to 100% of the sum of initially added SO_4^{2-} and S(-II) was recovered, while in naphthalene, 1,3,5-trimethylbenzene, and heating oil batch cultures, only 68 to 76% were recovered. Calculations using Henry's Law constant at 28°C ($9.31 \cdot 10^{-2}$ mol/L atm⁻¹; Sander, 1999) for H_2S indicated that 2.8% of total S(-II) was present as H_2S in the headspace. A partial loss of gaseous H_2S, as may have occurred when the serum bottles were briefly opened to add ZnAcetate to precipitate ZnS, would result in a change in mass balances (Table 1) by ~1% and hence, was not taken into account.

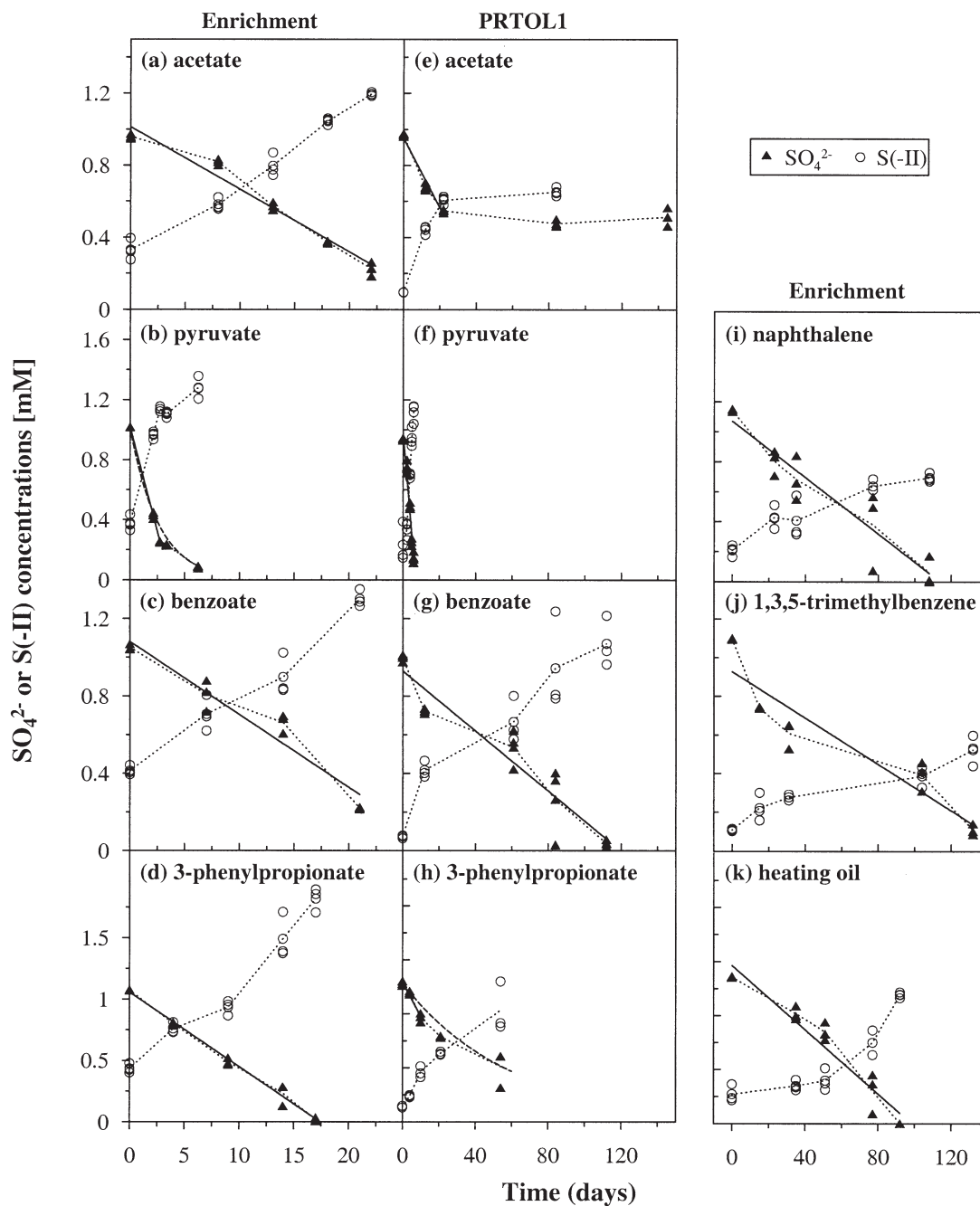Strain PRTOL1 consumed only ~50% of SO_4^{2-} within 85 d

Fig. 1. Concentrations of SO_4^{2-} (▲) and S(-II) (○) during batch culture experiments for (a–d, i–k) the enrichment cultures and (e–h) PRTOL1 grown on different carbon sources. Solid lines represent the fit of measured SO_4^{2-} concentrations vs. time used to compute zero-order SO_4^{2-} reduction rates (SRR). Data of enrichment batch cultures amended with pyruvate (b) and PRTOL1 batch cultures amended with 3-phenylpropionate (h) were additionally fitted assuming first-order kinetics to obtain rate coefficients (k) (dashed lines). Dotted lines connect averages of triplicate data points.

of incubation in batch cultures amended with acetate (Fig. 1e). Thereafter, SO_4^{2-} concentrations remained nearly constant for another 60 d. Conversely, nearly the entire SO_4^{2-} was consumed by PRTOL1 within 6 d in experiments with pyruvate (Fig. 1f), within 120 d with benzoate (Fig. 1g), and within 45 d with 3-phenylpropionate (Fig. 1h). In all PRTOL1 experiments, slightly > 100% of the sum of initially added SO_4^{2-} and S(-II) was recovered in the sacrificed batch cultures (Table 1).

3.2. Carbon Source Consumption

Concomitantly to SO_4^{2-} reduction and S(-II) production, organic acids were consumed in enrichment and PRTOL1 batch cultures grown on acetate, benzoate, and 3-phenylpropionate, and presumably, also on pyruvate (Fig. 2). Pyruvate concentrations were erratic for unknown reasons (not shown in Figs. 2b and 2f). Furthermore, we observed an evolution of

Table 1. Mass balance of initially added SO_4^{2-} and S(-II), observed stoichiometry of degraded carbon source:degraded SO_4^{2-}, and qualitative observed CH_4 production. Values are presented as average $\pm$ standard deviation.

Culture	Carbon source	% recovery of initially added SO_4^{2-} and S(-II)[a]	Observed stoichiometry carbon source:SO_4^{2-}[b]	CH_4 production observed
Enrichment cultures	Acetate	109 $\pm$ 3	2.32 $\pm$ 2.71	+
	Pyruvate	98 $\pm$ 3	3.90 $\pm$ 2.48[c]	+
	Benzoate	105 $\pm$ 5	0.78 $\pm$ 0.24	+
	3-phenylpropionate	109 $\pm$ 12	0.54 $\pm$ 0.10	+
	Naphthalene	76 $\pm$ 16	n.a.[d]	n.a.
	1,3,5-trimethylbenzene	68 $\pm$ 11	n.a.	n.a.
	Heating oil	75 $\pm$ 8	n.a.	n.a.
PRTOL1	Acetate	107 $\pm$ 2	1.43 $\pm$ 0.69	—
	Pyruvate	101 $\pm$ 6	n.a.	—
	Benzoate	109 $\pm$ 7	0.53 $\pm$ 0.07	—
	3-phenylpropionate	102 $\pm$ 5	0.19 $\pm$ 0.05	—

[a] Calculated by a mass balance of the sum of SO_4^{2-} and S(-II) as an average for all sampling points and all replicates (n = 12–15).

[b] Calculated based on data presented in Figures 1 and 2.

[c] Value calculated from measured acetate (Fig. 2) since pyruvate concentrations were erratic; a theoretical (degraded pyruvate):(produced acetate) ratio of 1:1 was assumed.

[d] n.a. = not available.

acetate in enrichment cultures grown on pyruvate, benzoate, 3-phenylpropionate and in PRTOL1 batch cultures grown on benzoate (Figs. 2b, 2c, 2d, and 2g). Increasing acetate concentrations to up to ~0.50 mmol/L were also observed in enrichment batch cultures grown on naphthalene and heating oil, but not on 1,3,5-trimethylbenzene (Fig. 2i–2k). The stoichiometries of degraded carbon source : degraded SO_4^{2-} for the same carbon source were always higher for the enrichment cultures than for PRTOL1 (Table 1). Sampling and analysis of headspace gas of enrichment and PRTOL1 cultures grown on organic acids revealed the presence of CH_4 in some of the incubations (Table 1).

3.3. Bacterial Cell Numbers

Average bacterial cell numbers for the experiments ranged from 1.1×10^5 to 1.8×10^7 cm^{-3} (Table 2), associated with fairly large standard deviations (σ_{cells}, often > 50% of the mean ($\bar{x}$)) due to changes of cell numbers during the experiments (Fig. 3). For enrichment cultures grown on naphthalene, 1,3,5-trimethylbenzene, and heating oil, cell numbers are only available for the first data point (Table 2) since FeS precipitates in these batch cultures at later time points made cell counts impossible.

Bacterial cell numbers increased during experiments with the enrichment culture (Fig. 3a). In particular, increases in cell numbers of almost one order of magnitude were observed in enrichment culture experiments with acetate and pyruvate. An increase of cell numbers in cultures of PRTOL1 was only observed for the pyruvate-amended culture (Fig. 3b). In the experiment with PRTOL1 grown on acetate, cell numbers decreased with time, and on day 84, cells were undetectable despite sample concentration. Conversely, in the PRTOL1 experiments with benzoate and phenylpropionate, cell numbers showed a transient decrease. This temporary decline was possibly due to the high sulfide sensitivity of this strain (Beller et al., 1996) since in contrast to the pregrown cultures, in the batch culture experiments emerging sulfide was not precipitated as FeS. Nevertheless, these cultures were active since

SO_4^{2-} was reduced at a constant rate (Fig. 1) and carbon sources were consumed (Fig. 2).

3.4. Sulfate Reduction Rates

Sulfate reduction rates, determined from linear regression analyses of SO_4^{2-} concentration vs. time (Fig. 1), varied by up to a factor of 47 between different cultures and carbon sources (Table 2). Highest SRR were obtained for cultures grown on pyruvate (280 nmol cm^{-3} d^{-1} for the enrichment culture and 143 nmol cm^{-3} d^{-1} for PRTOL1), lowest values were observed for PRTOL1 grown on benzoate (8 nmol cm^{-3} d^{-1}) or the enrichment culture grown on 1,3,5-trimethylbenzene (6 nmol cm^{-3} d^{-1}). Furthermore, values of SRR were characterized by small standard deviations (σ_{SRR}, $\leq$10% of SRR in most cases), and coefficients of determination (R^2) were > 0.9 in all but three cases (Table 2).

Sulfate reduction in the enrichment culture incubated with pyruvate and PRTOL1 incubated with 3-phenylpropionate appeared to follow first-order kinetics (Figs. 1b and 1h) with SO_4^{2-} concentrations declining exponentially. Using linear regression analyses (dashed lines in Figs. 1b and 1h), we obtained first-order rate coefficients $k = 0.398 \pm 0.019$ d^{-1} for the enrichment culture, and $k = 0.018 \pm 0.002$ d^{-1} for PRTOL1. Thus, values of k were characterized by standard deviations < 13% of k, and coefficients of determination were $R^2 = 0.98$ (number of data points, $n = 15$) for the enrichment culture on pyruvate, and $R^2 = 0.84$ ($n = 15$) for PRTOL1 on 3-phenyl-propionate.

Computed values of sSRR ranged from 1.1×10^{-14} to 1.5×10^{-13} mol cell^{-1} d^{-1} and thus varied by a factor of up to 13 between experiments (Table 2). Differences in sSRR values between the cultures were smaller than differences in SRR values. Values of sSRR were in the same range for the enrichment cultures and PRTOL1.

3.5. Sulfur Isotope Fractionation

Values of $\delta^{34}S(SO_4^{2-})$ increased from 8.4‰ ($\delta^{34}S[SO_4^{2-}]_0$) to values of up to 88.5‰ during the experiments (Fig. 4).
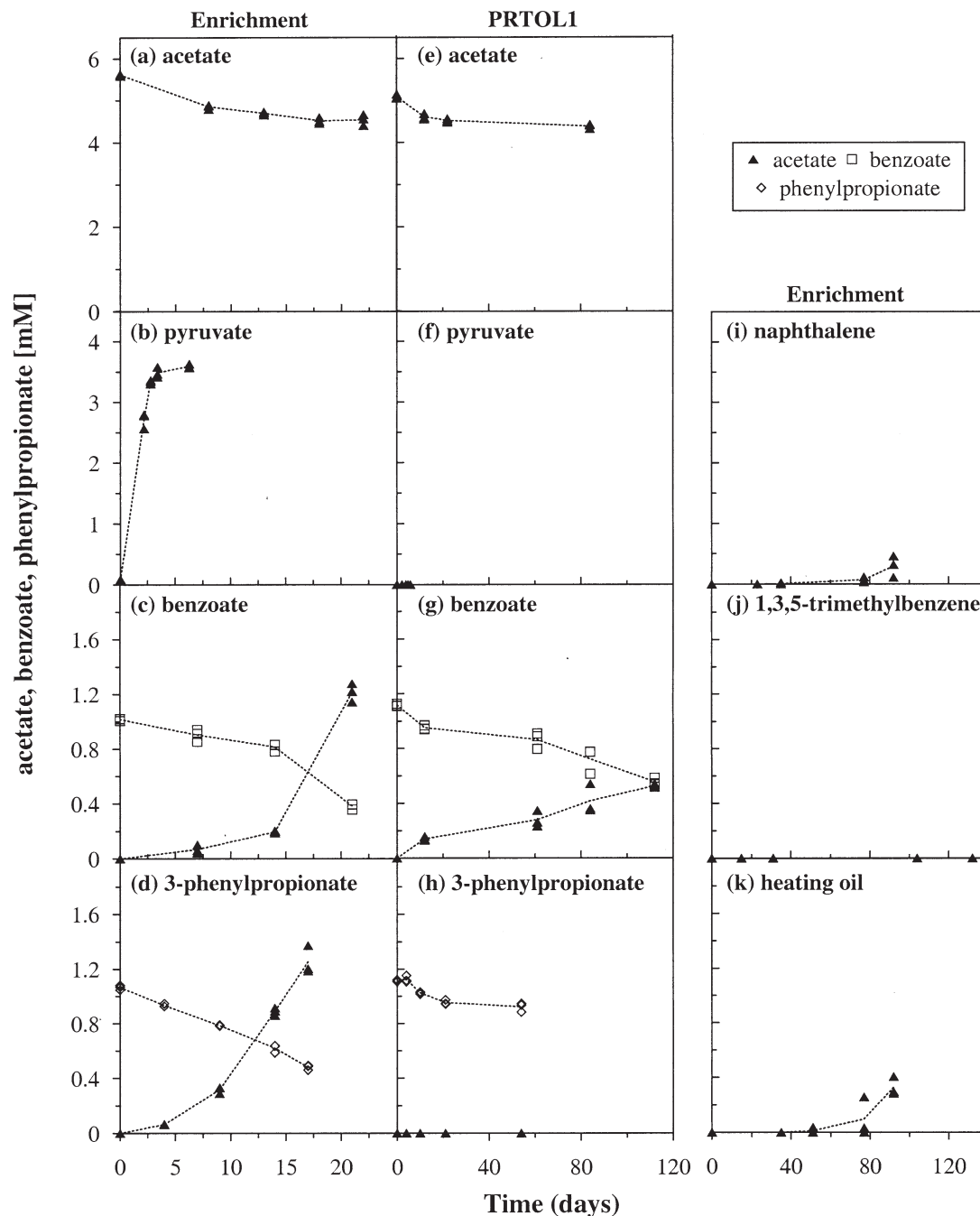
Fig. 2. Concentrations of acetate, benzoate, and 3-phenylpropionate during batch culture experiments for (a–d, i–k) the enrichment culture and (e–h) PRTOL1 grown on different carbon sources. Pyruvate concentrations were erratic for unknown reasons and were omitted from the figure. Dotted lines connect averages of triplicate data points.

Simultaneously to increases in $\delta^{34}S(SO_4^{2-})$, values of $\delta^{34}S$ (S[-II]) increased and, in general, approached the initial isotope composition of SO_4^{2-} over the course of the experiments. The latter was not the case in the experiment with PRTOL1 grown on acetate in which SO_4^{2-} reduction had ceased before consumption of most of the supplied SO_4^{2-} (Fig. 1e). In this experiment, $\delta^{34}S(S[-II])$ remained substantially more negative than $\delta^{34}S(SO_4^{2-})_0$ (Fig. 4e).

Combined evaluation of measured sulfur isotope data re-

vealed approximately linear relationships for all experiments when data were plotted as $\delta^{34}S(SO_4^{2-})$ vs. $(-\ln f)$ (Eqn. 2) and $\delta^{34}S(S[-II])$ vs. $(f \ln f)/(1- f)$ (Eqn. 3, Fig. 4). Using linear regression analysis, we determined ε values that ranged from 16.1 to 36.0‰ (Table 3). A partial loss of gaseous H_2S (see section 3.1), taking into account isotope fractionation between liquid and gas phases (Fry et al., 1986), would have changed ε values only in the second decimal and hence, was not included in calculations of ε. Enrichment factors were computed from 21

Table 2. Calculated average bacterial cell numbers, SO_4^{2-} reduction rates (SRR), and specific SO_4^{2-} reduction rates (sSRR) for batch culture experiments.

Culture	Carbon source	Average bacterial cell numbers $\bar{x} \pm \sigma_{cells}$[a] ($10^6$ cells cm^{-3})	n[b]	SRR $\pm \sigma_{SRR}$[a] (nmol cm^{-3} d^{-1})	R^2[c]	sSRR $\pm \sigma_{sSRR}$[a] (10^{-14} mol $cell^{-1}$ d^{-1})
Enrichment cultures	Acetate	0.62 ± 0.46	15	35 ± 2	0.971	5.6 ± 4.1
	Pyruvate	18 ± 14	9	280 ± 6	0.999	1.5 ± 1.2
	Benzoate	2.3 ± 1.5	12	38 ± 3	0.936	1.6 ± 1.1
	3-phenylpropionate	1.9 ± 1.3	15	61 ± 2	0.989	3.1 ± 2.1
	Naphthalene	0.13 ± 0.0067[d]	15	9 ± 1	0.887	7.1 ± 0.84
	1,3,5-trimethylbenzene	0.11 ± 0.0091[d]	15	6 ± 1	0.871	5.6 ± 0.80
	Heating oil	0.17 ± 0.03[d]	15	12 ± 1	0.936	7.0 ± 1.3
PRTOL1	Acetate	0.13 ± 0.10	9	19 ± 2	0.970	15 ± 11
	Pyruvate	13 ± 11	15	143 ± 8	0.965	1.1 ± 0.95
	Benzoate	0.13 ± 0.12	15	8 ± 1	0.900	6.1 ± 5.8
	3-phenylpropionate	0.20 ± 0.13	9	26 ± 3	0.953	13 ± 8.3

[a] Standard deviation.
[b] Number of data points included in SRR computation.
[c] Coefficient of determination for linear regression analyses performed to obtain SRR.
[d] Determined for the first sampling point only.

to 27 independent data points each and were generally characterized by small standard deviations ($\leq 8\%$ of ε in all cases), with R^2 values > 0.95 in all cases (Table 3). The averages of ε values calculated for each serum bottle separately were similar to ε values calculated by overall linear regression, although usually slightly lower (by 0.5‰), and the standard deviations were somewhat larger (on average 13.8% of ε, with a minimum of 5.5% and a maximum of 30.9%; data not shown).

4. DISCUSSION AND CONCLUSIONS

4.1. Microbial Sulfate Reduction

In our study SO_4^{2-} was consumed during all but the two control experiments. However, even though acetate and SO_4^{2-} were initially degraded and S(-II) was produced when PRTOL1 was incubated with acetate (Fig. 1e), SO_4^{2-} reduction apparently ceased, with about half of the initially added SO_4^{2-} remaining in aqueous solution. Since acetate was still present (Fig. 3), substrate limitation can be excluded as a reason for cessation of SO_4^{2-} reduction. Hence, the physiology of PRTOL1 was likely responsible for this behavior (see section 4.2).

Mass balances on SO_4^{2-} and S(-II) (Table 1) indicated that consumed SO_4^{2-} was entirely converted to S(-II) during many of our experiments (Fig. 1). However, in enrichment cultures amended with naphthalene, 1,3,5-trimethylbenzene, and heating oil, mass balances indicated that significantly $< 100\%$ of consumed SO_4^{2-} was converted to S(-II) (Table 1). Differences between the sum of initially supplied and recovered SO_4^{2-} and S(-II) were too large to be explained by analytical uncertainty alone. Possibly, SO_4^{2-} was reduced to sulfur species other than S(-II) such as e.g., sulfite or thiosulfate (Chambers and Trudinger, 1979; Sass et al., 1992). Apart from the quantification of produced S(-II), however, no effort was made here to further elucidate the fate of initially supplied SO_4^{2-}.

4.2. Carbon Source Consumption

Acetate production in some of the enrichment culture experiments (Fig. 2) and the high carbon source:SO_4^{2-} stoichiometries (Table 1) indicated the presence of SRB that can only incompletely degrade the carbon sources to acetate. Strain PRTOL1 is known to degrade carbon sources completely to CO_2 (Beller et al., 1996). Complete or incomplete degradation of carbon sources by SRB may have an influence on the extent of sulfur isotope fractionation (Detmers et al., 2001; see section 4.4.4).

Upon incubation with acetate, cell numbers of PRTOL1 decreased (Fig. 3b). Beller et al. (1996) previously observed that PRTOL1 neither showed cell growth on acetate nor degraded acetate, even though SO_4^{2-} was reduced. In contrast to their results, we observed a decrease in acetate concentration during our experiment (Fig. 2e). A similar behavior has been observed for other completely oxidizing SRB such as *Desulfosarcina variabilis*, *Desulfococcus* and *Desulfobacterium* species or the closest relative of strain PRTOL1, *Desulforhabdus amnigenus*, whereas *Desulfobacter* species grow well on acetate (Widdel, 1988; Oude-Elferink et al., 1995). The latter use the citric acid-cycle which does not require ATP to oxidize acetate. The former organisms, however, possess an incomplete citric acid-cycle and use the ATP-requiring carbon monoxide dehydrogenase pathway for acetate oxidation. Hence, such a "slow-acetate" physiology may as well be applicable to the complete carbon source degrader PRTOL1. This may have implications for isotope fractionation by this strain (see section 4.4.5).

The higher acetate:SO_4^{2-} stoichiometry for the enrichment culture than for PRTOL1 (Table 1) indicated that some of the added acetate in the enrichment cultures may have been degraded by methanogenic microorganisms (Zinder, 1993). Acetate produced during incomplete degradation of the carbon sources in the other enrichment cultures may have met the same fate. Methanogenesis was corroborated by detection of CH_4 in some enrichment batch cultures (Table 1).
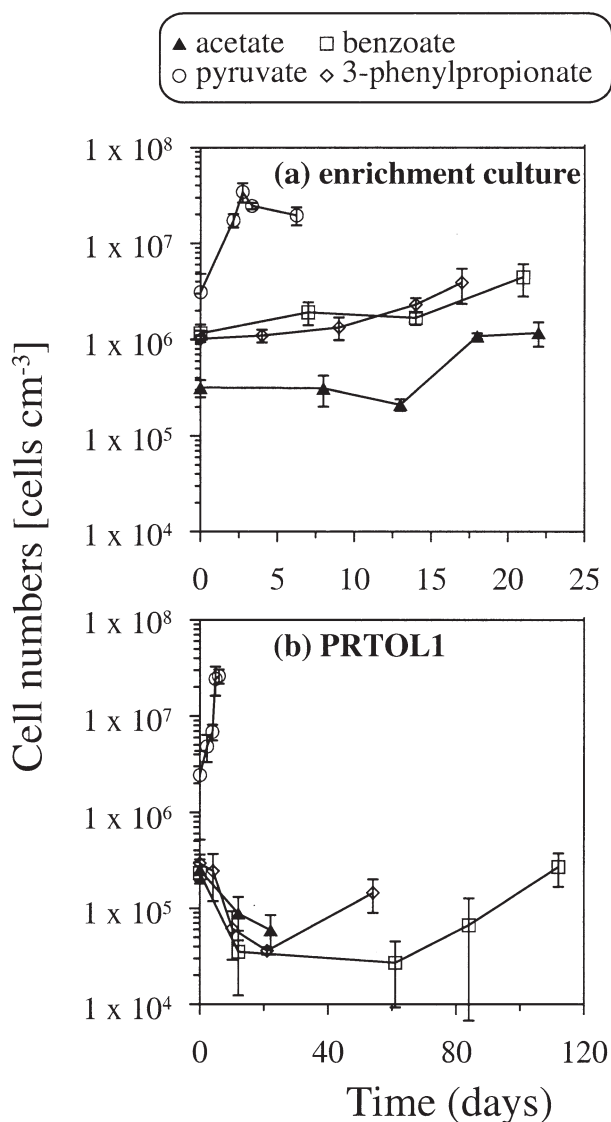
Fig. 3. Bacterial cell numbers determined by DAPI-staining/microscopy from sacrificed batch cultures during experiments for (a) the enrichment cultures and (b) PRTOL1. Each data point represents the average of three replicate batch cultures. Error bars represent standard deviations.

4.3. Sulfate Reduction Rates

A wide range of SRR was obtained for the different experiments (Table 2). However, SRR depend to a large extent on the cell density in the respective batch cultures. In our experiments, inoculants had different cell densities and initial (Fig. 3) and average bacterial cell numbers (Table 2) varied between different experiments, which may explain some of the observed variability in SRR. Interestingly, the first-order rate coefficients for SO_4^{2-} reduction determined in this study ($k = 0.398 \pm 0.019$ d^{-1} for the enrichment culture grown on pyruvate, and $k = 0.018 \pm 0.002$ d^{-1} for PRTOL1 grown on 3-phenylpropionate) mark the upper and lower end of first-order rate coefficients that have been determined for SO_4^{2-} reduction in

PHC-contaminated aquifers (0.02–0.32 d^{-1}; Chapelle et al., 1996; Schroth et al., 2001; Kleikemper et al., 2002b).

Our sSRR values (1.1×10^{-14} to 1.5×10^{-13} mol cell^{-1} d^{-1}, Table 2) are in the upper range of sSRR values obtained in previous studies (e.g., Kaplan and Rittenberg, 1964; Chambers et al., 1975; Detmers et al., 2001) and in the same range of sSRR values determined by Bolliger et al. (2001). However, caution is required when comparing sSRR values between various studies as discussed in Bolliger et al. (2001). The accuracy of sSRR values depends on the variability of the underlying parameters, i.e., SRR values and bacterial cell numbers. Cell numbers varied during most of our experiments (Fig. 3). Consequently, computed average bacterial cell numbers, and hence, sSRR values, were associated with fairly large uncertainties (Table 2).

4.4. Sulfur Isotope Fractionation

Stable sulfur isotope ratios of unconsumed SO_4^{2-} and produced S(-II) changed during our experiments as expected for a reaction in a closed system (Fig. 4) (Chambers and Trudinger, 1979; Thode, 1991). In general, the range of ε values obtained in this study was comparable to those reported by other authors for various strains under different growth conditions (Kaplan and Rittenberg, 1964; Chambers et al., 1975; Böttcher et al., 1999; Bolliger et al., 2001; Detmers et al., 2001). The influence of potential intermediate (e.g., sulfite) formation during sulfate reduction (see section 4.1) on ε values may be calculated exemplarily for the experiment with naphthalene. If ε calculated from $\delta^{34}S(SO_4^{2-})$ isotope data (ε_1) was 28‰ and if 24% of the SO_4^{2-} was reduced to an intermediate such as sulfite, with an assumed ε of 25‰ (ε_2) (Rees, 1973), ε_3 calculated from S(-II) isotope data would deviate from ε_1 by only 0.9‰. The deviation would be even lower if the difference between ε_1 and ε_2 was smaller. Hence, a small difference between ε_1 and ε_2 as is the case for our range of ε values (Table 3) and an ε_2 value of 25‰ (Rees, 1973) will mask potential isotopic effects of intermediate formation. In addition, an isotope effect of intermediate formation would be visible as an inconsistency between SO_4^{2-} and S(-II) isotope data. This however, does not seem to be the case for our data (Fig. 4).

In this study a range of ε values were determined and in the following sections this variation is related to the type of organism studied and the influence of carbon source, which in turn is controlled by the variability of cell-specific SO_4^{2-} reduction rates, the energy yield of the reaction (ΔG values), and complete or incomplete carbon source degradation.

4.4.1. Influence of Culture

Cultures of PRTOL1 always showed higher ε values than the enrichment cultures when both were grown on the same carbon source (Table 3). This is in agreement with Bolliger et al. (2001) who cultivated PRTOL1 and an enrichment culture derived from the same field site with toluene as sole carbon source. Two reasons may explain this result. Firstly, an enrichment culture containing a range of differentially fractionating SRB will show an average enrichment factor which may be expected to be lower than that of a pure culture that tends to show high enrichment factors (Detmers et al., 2001). Secondly,
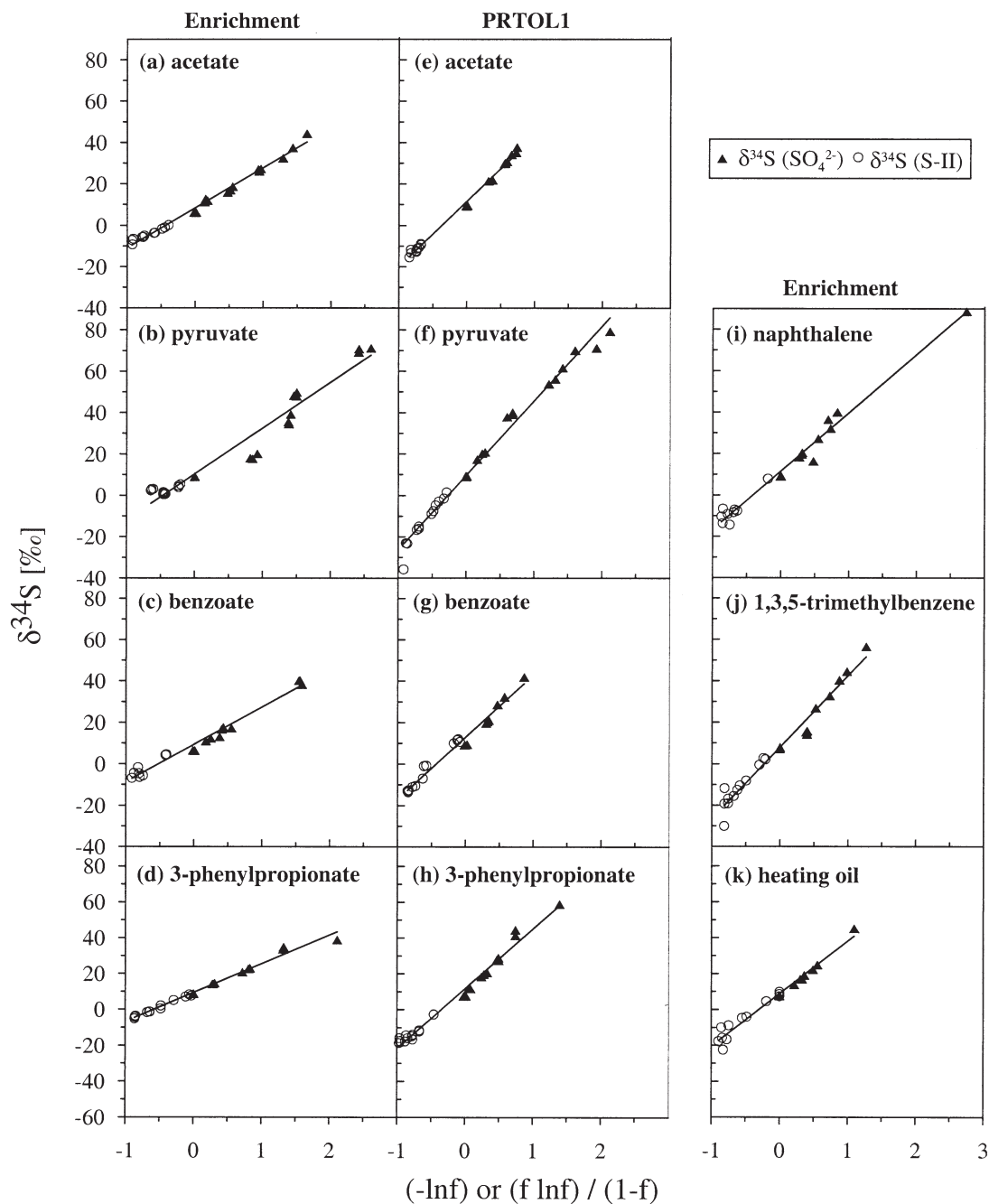
Fig. 4. Sulfur isotope ratios in SO_4^{2-} and S(-II) during batch culture experiments for (a–d, i–k) the enrichment cultures, (e–h) PRTOL1 grown on different carbon sources. Values of $\delta^{34}S(SO_4^{2-})$ (▲) are plotted vs. $-\ln f$ (Eqn. 2), whereas values of $\delta^{34}S(S[-II])$ (○) are plotted vs. $(f \ln f)/(1 - f)$ (Eqn. 3). Solid lines represent the linear fit used to compute isotope enrichment factors (ε).

complete or incomplete carbon source degradation may have influenced ε values as discussed below.

4.4.2. Influence of Cell-Specific Sulfate Reduction Rate

Several authors have suggested that sulfur isotope fractionation is controlled by sSRR rather than SRR since for fractionation, processes occurring on the cellular level are important (Chambers and Trudinger, 1979; Habicht and

Canfield, 1997). The SO_4^{2-} reduction pathway consists of several different steps, each of which is associated with a different isotope fractionation (Rees, 1973; Habicht and Canfield, 1997). The overall enrichment factor depends on which step is rate-limiting and represents the sum of the enrichment factors from each step until the rate-limiting reaction is reached. For example, at high sSRR with excess SO_4^{2-}, the reduction of adenosine-5′-phosphosulfate to sul-

Table 3. Calculated sulfur isotope enrichment factors (ε) obtained during microbial SO_4^{2-} reduction in batch culture experiments with various carbon sources.

Culture	Carbon source	n^a	$\varepsilon \pm \sigma_\varepsilon^b$ (‰)	R^{2c}
Enrichment cultures	Acetate	27	19.3 ± 0.4	0.990
	Pyruvate	27	22.2 ± 1.0	0.950
	Benzoate	21	18.0 ± 0.7	0.976
	3-phenylpropionate	24	16.1 ± 1.3	0.986
	Naphthalene	24	28.1 ± 0.9	0.981
	1,3,5-trimethylbenzene	24	34.5 ± 1.3	0.970
	Heating oil	22	29.3 ± 1.2	0.972
PRTOL1	Acetate	21	31.5 ± 0.6	0.995
	Pyruvate	27	36.0 ± 0.9	0.986
	Benzoate	21	30.0 ± 1.1	0.975
	3-phenylpropionate	27	33.3 ± 0.9	0.983

[a] Number of data points included in computation of ε values.
[b] Standard deviation.
[c] Coefficient of determination for linear regression analyses performed to obtain ε values.

fite (SO_3^{2-}) might become rate-limiting, and a fractionation of 22‰ will be observed (Rees, 1973; Habicht and Canfield, 1997). In contrast, at low specific rates, the reduction of

SO_3^{2-} to S(-II), the last reaction step, is rate-limiting, yielding maximum fractionation.

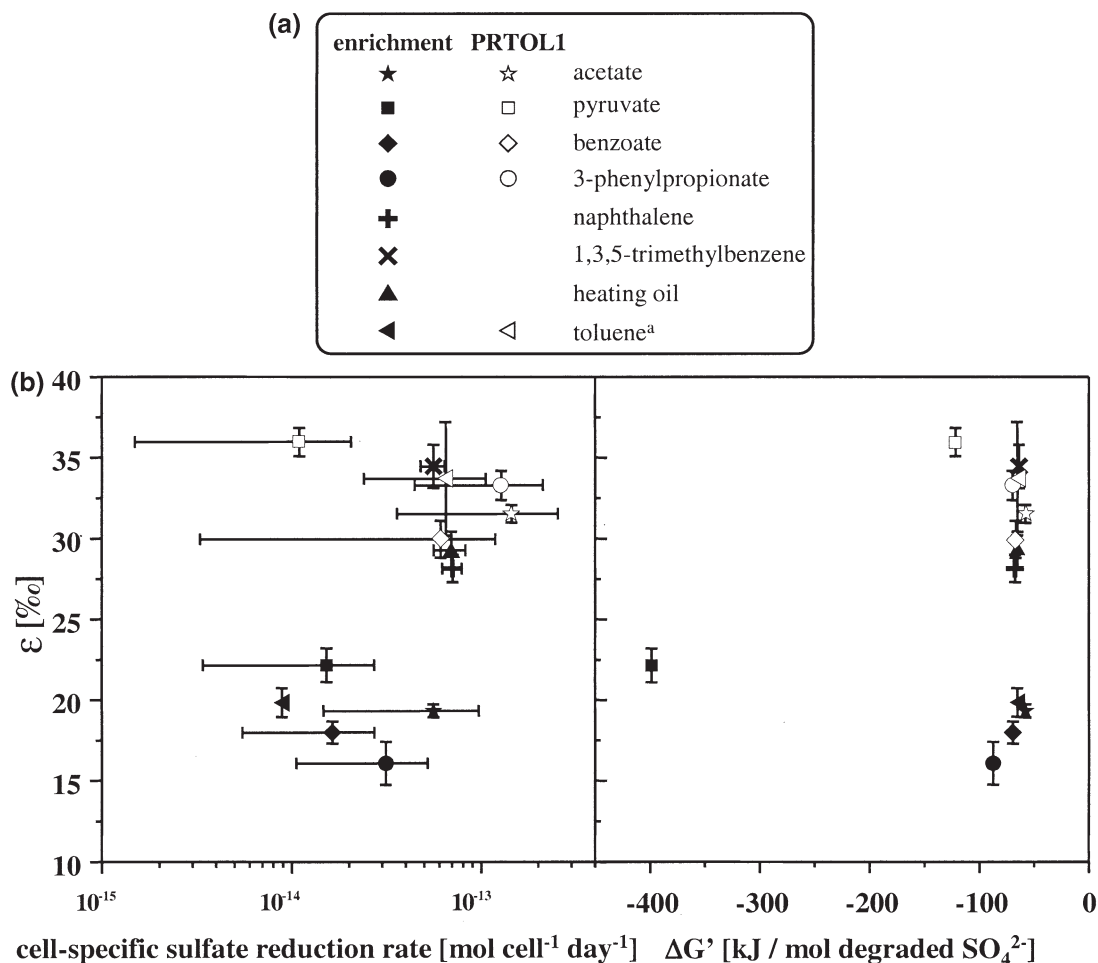Sulfur isotope fractionation and sSRR for PRTOL1 tended

Fig. 5. Calculated enrichment factors (ε) for all batch culture experiments as a function of (a) cell-specific SO_4^{2-} reduction rate (sSRR) and (b) actual free energy yield ($\Delta G'$). Error bars represent standard deviations in ε and sSRR values. [a]Data from Bolliger et al. (2001) were included for comparison.

to be inversely related ($R^2 = 0.23$, Fig. 5a), however, the relationship was not significant ($p = 0.05$). Such an inverse relationship was also found by Bolliger et al. (2001) for strain PRTOL1. However, more data are needed to confirm the prediction of Detmers et al. (2001) that a correlation between sSRR and fractionation may be found if various substrates were tested for one organism. For the enrichment culture data or when data from all batch culture experiments were combined, there was no obvious correlation between ε values and sSRR (Fig. 5a). This agrees with findings of Detmers et al. (2001), who concluded that there is little or no correlation between ε and sSRR values for a broad range of SRB.

4.4.3. Influence of Energy Yield

The energy yields ($\Delta G_0'$) of the respective reactions may influence the extent of isotope fractionation as has been suggested previously by Detmers et al. (2001). Higher energy yields are generally associated with lower ε values. One possible explanation for this correlation is that for a reaction yielding more free energy (more negative ΔG) the redox potential difference ($\Delta E_0'$) is also higher and the reaction equilibria will be shifted towards the product side, i. e. S(-II) (Brüchert et al., 2001; Detmers et al., 2001). Thus, with the reaction being faster, enzymes involved in the sulfate reduction pathway will discriminate sulfur isotopes to a lesser extent and isotope fractionation decreases.

However, we suggest that instead of using standard $\Delta G_0'$ values (Detmers et al., 2001), it may be more appropriate to consider actual $\Delta G'$ values under the conditions the reaction is occurring (Table 4, Fig. 5b). The stoichiometric reactions shown in Table 4 were chosen based on observed stoichiometries derived from measured SO_4^{2-} and organic acid concentrations in batch culture experiments (Figs. 1, 2; Table 1). For example, for the enrichment culture experiment with benzoate, we observed that 0.78 mmol benzoate was degraded per mmol of degraded SO_4^{2-} (Table 1). This number best agrees with a theoretical stoichiometry of 0.57, implying that two acetate molecules were formed per degraded benzoate molecule (Table 4). For the adoption of theoretical stoichiometries shown in Table 4, biomass and CH_4 formation were not taken into account.

Nevertheless, using actual $\Delta G'$ instead of $\Delta G_0'$ values did not significantly improve the relationship between energy yield and ε values for our data (Fig. 5b). Hence, we tested this hypothesis for a larger dataset by recalculating data reported by Kaplan and Rittenberg (1964), Bolliger et al. (2001), and Detmers et al. (2001) (not shown). Results indicated that for completely degrading strains energy yields tended to be lower (less negative $\Delta G'$ values) and ε values higher than for incompletely degrading strains, as had been observed by Detmers et al. (2001) using $\Delta G_0'$ values. However, the relationship between energy yield and ε values did not improve. Furthermore, the data from Kaplan and Rittenberg (1964) show that incompletely degrading strains can also produce large fractionations up to 46‰. Thus, it seems that in addition to energy yield, the physiology of a certain strain under certain conditions plays a major role for the magnitude of ε.

4.4.4. Influence of Complete or Incomplete Carbon Source Degradation

Several authors observed that completely degrading pure strains tended to show higher ε values than incompletely degrading strains, due to generally lower energy yields (less negative $\Delta G_0'$ values per mole of degraded SO_4^{2-}) of complete substrate oxidations (Brüchert et al., 2001; Detmers et al., 2001). While PRTOL1 is a completely degrading strain (Beller et al., 1996), the enrichment cultures grown on organic acids (except for acetate) likely also contained incompletely degrading SRB since acetate was produced in many of our experiments (Table 1). The lower energy yields ($\Delta G'$) for the complete oxidations of strain PRTOL1 as compared to incomplete carbon source degradation by the enrichment cultures may be the second reason to explain the higher ε values for strain PRTOL1 (Table 4, see section 4.4.1).

In our enrichment cultures all carbon sources except acetate and 1,3,5-trimethylbenzene were—at least to some degree—incompletely degraded (production of acetate; Fig. 2). Hence, ε values for these two substrates may be expected to be higher. Indeed, growth of cultures on 1,3,5-trimethylbenzene was associated with high ε values (34.5‰), explained by the comparatively low energy yield of the reaction (Table 4). However, while the energy yield for acetate was the lowest of all substrates (Table 4), ε was quite low (19.3‰, Table 3). Hence, the different SRB communities that developed on different carbon sources likely influenced ε values in addition to complete or incomplete carbon source degradation.

4.4.5. Influence of Carbon Source

This study is the first investigation of sulfur isotope fractionation when the PHC naphthalene, 1,3,5-trimethylbenzene, and heating oil served as sole carbon sources. Interestingly, ε values obtained for these PHC compounds (average = 30.6‰) were significantly larger ($p = 0.05$) than for enrichment cultures grown on organic acids (average = 18.9‰). Different SRB communities were possibly enriched on the different carbon sources (Parkes et al., 1993; Kleikemper et al., 2002a) which likely affected fractionation.

Growth of PRTOL1 on various carbon sources resulted in a narrow range of ε values (32.7 ± 2.6‰, average ± standard deviation, Table 3). More variable ε values were reported for PRTOL1 when grown on toluene and three different SO_4^{2-} concentrations (Bolliger et al., 2001) ($\varepsilon = 32.1$–46.9‰). Hence, for strain PRTOL1, SO_4^{2-} concentration seemed to have a greater influence on ε than carbon source type when the same range of sSRR values was considered. Furthermore, the ε value for PRTOL1 incubated with acetate, where no cell growth was observed, was within the range of ε values for the other carbon sources (Table 3). Therefore, non-optimal growth conditions as represented by the acetate culture as compared to more ideal conditions did not seem to influence ε values for PRTOL1.

Only few other authors investigated sulfur isotope fractionation by the same strain grown on different carbon sources. For example, Kaplan and Rittenberg (1964) found that *Desulfovibrio desulfuricans* showed a higher fractionation on ethanol than on lactate. They explained their results in terms of the substrate-induced variability in sSRR, which also may explain part

Table 4. Stoichiometric equations of the degradation of the carbon sources used in this study under SO_4^{2-}-reducing conditions and free-energy changes (kJ/mol degraded SO_4^{2-}) under standard conditions ($\Delta G_0'$; pH = 7, T = 298.15 K, aqueous concentrations except H^+ = 1 mol/L)[a] and actual conditions ($\Delta G'$)[b]. The reactions were chosen based on observed stoichiometries derived from measured SO_4^{2-} and organic acid concentrations in batch culture experiments (Table 1).

Carbon source	Complete/incomplete carbon source degradation[c]	Culture	Stoichiometric reaction	$\Delta G_0'$	$\Delta G'$[b]	ε (‰)
Acetate	Complete	PRTOL1	$CH_3COO^- + SO_4^{2-} \Rightarrow 2HCO_3^- + HS^-$	−48	−58	31.5
		Enrichment				19.3
Pyruvate	Complete	PRTOL1	$CH_3COCOO^- + H_2O + 1.25SO_4^{2-} \Rightarrow 3HCO_3^- + 0.75H^+ + 1.25HS^-$	−106	−121	36.0
	Incomplete (1)	Enrichment	$CH_3COCOO^- + H_2O + 0.25SO_4^{2-} \Rightarrow HCO_3^- + 0.75H^+ + 0.25HS^- + CH_3COO^-$	−341	−398	22.2
Benzoate	Incomplete (1)	PRTOL1	$C_7H_5O_2^- + 4H_2O + 2.75SO_4^{2-} \Rightarrow 5HCO_3^- + 2.25H^+ + 2.75HS^- + CH_3COO^-$	−43	−67	30.0
	Incomplete (2)	Enrichment	$C_7H_5O_2^- + 4H_2O + 1.75SO_4^{2-} \Rightarrow 3HCO_3^- + 2.25H^+ + 1.75HS^- + 2CH_3COO^-$	−40	−69	18.0
3-phenylpropionate[d]	Complete	PRTOL1	$C_9H_9O_2^- + 4H_2O + 5.25SO_4^{2-} \Rightarrow 9HCO_3^- + 2.75H^+ + 5.25HS^-$	−51[d]	−70[d]	33.3
	Incomplete (3)	Enrichment	$C_9H_9O_2^- + 4H_2O + 2.25SO_4^{2-} \Rightarrow 3HCO_3^- + 2.75H^+ + 2.25HS^- + 3CH_3COO^-$	−57[d]	−87[d]	16.1
Naphthalene[e]	Complete	Enrichment	$C_{10}H_8 + 6H_2O + 6SO_4^{2-} \Rightarrow 10HCO_3^- + 4H^+ + 6HS^-$	−52[g]	−67[g]	28.1
1,3,5-trimethylbenzene	Complete	Enrichment	$C_9H_{12} + 3H_2O + 6SO_4^- \Rightarrow 9HCO_3^- + 3H^+ + 6HS^-$	−48[g]	−64[g]	34.5
Heating oil[ef]	Complete	Enrichment	$C_{17}H_{36} + 13SO_4^{2-} \Rightarrow 17HCO_3^- + 4H^+ + 13HS^- + H_2O$	−52[g]	−65[g]	29.3

[a] Values of standard free energy are from Madigan et al. (2003) and Yaws (1999).

[b] Calculated using the Nernst equation: $\Delta G' = \Delta G_0' + RT \ln c(products)/c(reactants)$ (e.g., Jakobsen and Postma, 1999). Only conditions at the beginning of each experiment were considered for calculation of $\Delta G'$ values since concentration variations during the experiments changed $\Delta G'$ values by only 14% on average, much less than the difference between $\Delta G_0'$ and $\Delta G'$.

[c] Numbers in brackets refer to number of acetate molecules produced per carbon source molecule degraded.

[d] Standard free energy of formation for 3-phenylpropionate was not available; an approximate value of −200 kJ/mol was assumed based on values for related compounds (Yaws, 1999). Hence, these values may only be compared with each other and not with the other values.

[e] Even though some acetate was produced during naphthalene and heating oil degradation (Table 1), the greater proportion of these carbon sources was degraded by a complete mechanism as calculated using measured SO_4^{2-} and acetate concentrations.

[f] Heptadecane was chosen for calculation of ΔG values since it represents the average carbon number of heating oil (Lecomte and Mariotti, 1997).

[g] ΔG values for PHC compounds were calculated using Gibbs free energies of formation corrected for dissolution in water (Schwarzenbach et al., 1993).

of the variability in our data (Fig. 5). Nevertheless, experiments with similar sSRR, e.g., PRTOL1 grown on acetate and 3-phenylpropionate on the one hand or on toluene (Bolliger et al., 2001) and benzoate on the other hand (Fig. 5) showed different ε values, suggesting that factors other than sSRR also influence the extent of sulfur isotope fractionation. To explain the remaining variability of sulfur isotope fractionation data and to explore the mechanism of sulfur isotope fractionation in greater detail, an investigation of the processes at the molecular (enzymatic) level will be necessary.

4.5. Relevance to Field Studies at Contaminated Sites

To use sulfur isotope fractionation for a quantification of microbial sulfate reduction at contaminated sites, the enrichment factor for the site has to be known (Aggarwal et al., 1997). A general equation for calculating first-order rate coefficients (k) from stable isotope fractionation data were given by Aggarwal et al. (1997):

$$\delta_t - \delta_0 = -\varepsilon \, k(t - t_0) \quad (4)$$

where δ_t and δ_0 are the stable isotope ratios of an electron acceptor determined at times t and t_0, respectively. For example, this equation may be applied to the data of Strebel et al. (1990) for an anaerobic, SO_4^{2-}-reducing aquifer. Using the reported ε value of 9.7‰, half-lives for SO_4^{2-} reduction calculated in this way (67–88 yr) are similar to those determined by the authors using the first-order rate law based on SO_4^{2-} concentrations (75–100 yr; Strebel et al., 1990). Likewise, Spence et al. (2001) were able to quantify sulfate removal in a phenol-contaminated aquifer since they could demonstrate that S(-II) reoxidation did not occur.

Despite these two encouraging examples, the mechanisms governing sulfur isotope fractionation at field sites are not well understood. While detailed laboratory studies may help us define the most important parameters influencing ε, care has to be taken to interpret field data due to additional parameters, e.g., the general heterogeneity of aquifers, the wide range of indigenously used carbon sources and carbon source limitations, the diversity of subsurface microbial communities, the wealth of occurring geochemical reactions, temperatures deviating from those of laboratory experiments, and reoxidation processes where the Rayleigh model is no longer applicable. This becomes particularly evident when comparing laboratory with field studies and field studies with each other.

For example, our enrichment culture grown on heating oil showed a surprisingly high ε value (28.10‰) compared to values determined in field push-pull tests conducted in the sulfate-reducing zone of the PHC-contaminated aquifer from which the inocula for the enrichment cultures were obtained ($\varepsilon = 20.2$–22.8‰; Schroth et al., 2001). Here, differences between field and batch culture experiments were possibly due to different temperatures, which may have lead to selective enrichment of SRB in the heating oil cultures and may explain the different ε values (Brüchert et al., 2001). Furthermore, field SRB were possibly using other carbon sources (natural organic compounds) in addition to heating oil. In contrast, the ε value for the acetate-grown enrichment culture in this study (19.3‰) was similar to ε determined in a push-pull test with acetate as carbon source in the same

aquifer (20.8‰; Kleikemper et al., 2002b). In this case, several effects, e.g., temperature and growth conditions, may have influenced ε in opposite directions and cancelled out each other, leading to similar ε values.

In our experiments, enrichment cultures grown on PHC showed higher ε values than on organic acids. Interestingly, this tends to agree with the fact that ε values at some contaminated sites (9.5–21‰; Bottrell et al., 1995; Asmussen and Strauch, 1998; Spence et al., 2001) seemed to be higher than in uncontaminated freshwater aquifer environments, where organic acids may be dominant carbon sources (9.7–15.5‰; Strebel et al., 1990; Robertson and Schiff, 1994). However, addition of organic acids (acetate, lactate, propionate, and butyrate) lead to similarly high ε values for SO_4^{2-} reduction ($\varepsilon = 16.1$–25.7‰; Kleikemper et al., 2002b) as when heating oil was the main carbon source ($\varepsilon = 20.2$–22.8‰; Schroth et al., 2001) during field push-pull tests in the same aquifer.

Hence, to better understand field data on sulfur isotope fractionation, additional experimental approaches are needed, e.g., steady-state systems such as chemostats and laboratory columns that mimic field conditions more closely.

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