Chapter 2
Controls on sulfate reduction and sulfur isotope fractionation by natural microbial communities in sediments from the Schelde Estuary, The Netherlands
Chapter 2: The Schelde Estuary, The Netherlands

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Abstract
Stable sulfur isotopes are potential tracers of microbial activity with numerous geological and environmental applications. Here, I report concurrent measurements of potential sulfate reduction rates (SRRs) and $^{34}$S/$^{32}$S isotope fractionation effects ($\epsilon$) obtained with flow-through reactors containing intact, 2 cm thick, sediment slices sampled from an unvegetated, intertidal site adjoining a salt marsh along the Schelde Estuary, The Netherlands. A total of 30 reactors were run with sediments sampled in February, May and October 2006. The effects of incubation temperature (10, 20, 30 and 50°C), sediment depth (0-2, 4-6 and 8-10 cm), distance from the vegetated marsh and sampling time were systematically investigated. Sulfate was supplied in non-limiting concentrations via the reactor inflow solutions. No external electron donor was supplied. Data analysis was restricted to SSR and isotope fractionation effects ($\epsilon$) obtained under steady state conditions. Values of $\epsilon$ were derived from the measured differences in sulfate $\delta^{34}$S between in- and outflow of the reactors. Potential SRRs varied over one order of magnitude (5 to 49 nmol cm$^{-3}$ h$^{-1}$) and were highest in the 30°C incubations. SRRs systematically decreased with depth, and were highest in the sediments collected closest to the vegetated marsh. Steady state isotope fractionation effects ($\epsilon$) ranged from 9 to 34 ‰ and exhibited an inverse relationship with SRR, as predicted by the standard fractionation model for enzymatic sulfate reduction of Rees (1973). The $\epsilon$ versus SRR relationship, however, varied between sampling times, with higher $\epsilon$ values measured in February, at comparable SRRs, than in May and October. The observed $\epsilon$ versus SRR relationships also deviated from the previously reported inverse trend for sediments collected in a marine lagoon in Denmark (Canfield, 2001b). Thus, isotope fractionation during sulfate reduction is not uniquely determined by SRR, but is site and season specific. Possible factors affecting the $\epsilon$ versus SRR relationship include the community structure and abundance of sulfate reducers, and the nature and accessibility of organic substrates. The data imply that small ranges in sulfur isotope fractionation ($\epsilon \leq 15$ ‰) observed in the environment may be indicative of biogenic processes, reflecting high sulfate reducing activity.

2.1 Introduction
Sulfur isotopes have been used as tracers of sources, mixing processes and transformations of sulfur compounds in a variety of modern environments, including soils, sediments, ground waters, rivers, estuaries, oceans and acid mine drainage areas (e.g. Brüchert and Pratt, 1999; Mandernack et al., 2003; Böttcher et al., 2004; Knöller et al., 2004; Vokal-Nemec et al., 2006; Gu et al., 2008). Stable sulfur isotopes in sedimentary rocks from the early Archean onwards have also yielded essential constraints on palaeo-environmental conditions in ancient
oceans (Habicht et al., 2002; Canfield, 2004; Johnston et al., 2006; Johnston et al., 2008b), the emergence and development of sulfur-based metabolisms (Shen et al., 2001; Farquhar and Wing, 2003b; Strauss, 2003; Shen and Buick, 2004; Philippot et al., 2007; Johnston et al., 2008a) and the evolution of atmospheric oxygen (Kasting, 2001; Pavlov and Kasting, 2002; Farquhar and Wing, 2003a; Kaufman et al., 2007).

Sulfur isotope fractionation can result from either biotic or abiotic processes, but microbial activity has been shown to be the dominant mechanism for fractionation in low temperature sedimentary and diagenetic environments (< 200°C) (Ohmoto and Goldhaber, 1997; Canfield and Raiswell, 1999; Newton and Bottrell, 2007). Sulfate reducing prokaryotes (SRP) gain energy for growth and maintenance under anaerobic conditions by reducing sulfate to sulfide during dissimilatory sulfate reduction (Widdel, 1988). Because of their ability to use both organic (e.g. acetate, lactate, ethanol) and inorganic (e.g. H₂ or CO) electron donors, and their adaptation to a wide range of environmental conditions, sulfate reducing microorganisms are found in many environmental settings, including littoral sediments (Isaksen et al., 1994; Pallud and Van Cappellen, 2006), saline lakes (Brandt et al., 2001; Scholten et al., 2005), subareal and submarine hydrothermal vents (Tor et al., 2003; Roychoudhury, 2004; Amend and Teske, 2005), freshwater sediments (Bak and Pfennig, 1991) and anthropogenically polluted environments (Kleikemper et al., 2002; Roychoudhury and McCormick, 2006).

As sulfide produced by dissimilatory sulfate reduction is preferentially enriched in lighter $^{32}$S, sulfur isotope fractionation in ancient sedimentary rocks should in principle provide information about past sulfate reducing activity (Shen et al., 2001; Canfield, 2001a). The interpretation of sulfur isotope signals, however, is complicated by environment-specific effects on fractionation due, for instance, to variations in temperature, pore water chemistry, substrate and nutrient availability, degree of anoxia, and the composition of the active sulfate reducing microbial community (Brüchert, 2004).

Numerous laboratory studies have been carried out to investigate sulfur isotope fractionation effects ($\varepsilon$) during microbial sulfate reduction, with most reported $\varepsilon$ values, calculated from $\delta^{34}$S differences between sulfate and sulfide, falling in the range -3 to 47 ‰ (Harrison and Thode, 1958; Kaplan and Rittenberg, 1964; Kemp and Thode, 1968; Chambers et al., 1975; Habicht and Canfield, 1997; Detmers et al., 2001; Canfield, 2001b; Brüchert et al., 2001; Brüchert, 2004). Early results with pure cultures of sulfate reducing prokaryotes showed an inverse correlation between the extent of isotope fractionation and the rate of sulfate reduction (Kaplan and Rittenberg, 1964; Kemp and Thode, 1968), opening up the possibility that variations in $\varepsilon$ could be used as a tracer of sulfate reducing activity. The observed range of experimental $\varepsilon$ values, and the inverse relationship with the sulfate reduction rate, also led to the development of a conceptual model for isotope flow during dissimilatory sulfate reduction (Rees, 1973).

Several aspects of the original, or standard, Rees model for isotope fractionation during enzymatic sulfate reduction have been brought into question. First, more extreme variations in isotope fractionation, up to and exceeding 100 ‰, have been measured in sediment pore fluids (Rudnicki et al., 2001). Second, the inverse relationship between sulfur isotope fractionation and the rate of sulfate reduction in pure cultures was found to vary or be
completely absent when comparing one strain of sulfate reducer to another (Detmers et al., 2001; Canfield et al., 2006; Hoek et al., 2006; Mangalo et al., 2007). More recent studies considering the variations in all four stable sulfur isotopes by sulfate reducing and sulfur disproportionating microorganisms have provided new insights into the various enzymatic pathways and branching points involved in the flow of sulfur through the cells (Farquhar et al., 2003; Johnston et al., 2005; Farquhar et al., 2007; Johnston et al., 2007). These studies have resulted in modifications of the standard model, thereby allowing for values of $\varepsilon$ in excess of 47 ‰ (Brunner and Bernasconi, 2005).

While many pure culture studies have been carried out, far fewer coupled measurements of sulfate reduction rates and sulfur isotope fractionation are available for natural sulfate reducing communities. In this chapter, I present such coupled measurements using flow-through reactors containing intact slices of intertidal estuarine sediments collected at a variety of depth intervals, distances from a vegetated salt marsh, and at various times throughout the year from the same brackish water location within the Schelde Estuary in The Netherlands. This flow-through reactor approach has been used previously to investigate microbial reaction kinetics in sediments (Roychoudhury et al., 1998; Brüchert and Arnosti, 2003; Roychoudhury et al., 2003; Weston and Joye, 2005; Laverman et al., 2006; Pallud and Van Cappellen, 2006; Abell et al., 2009), but also to establish the link between sulfate reducing activity and sulfur isotope fractionation effects (Canfield et al., 2000; Canfield, 2001b; Habicht et al., 2002; Farquhar et al., 2008). However, little is known about the spatio-temporal variations in sulfate reduction and coupled isotope fractionation for a single sampling site. The flow-through reactor approach avoids the build-up of dissolved reaction products in the reactor, as well as artifacts resulting from the disruption of the sediment structure in slurry incubations (Pallud and Van Cappellen, 2006). The resulting reaction parameters, including isotope fractionation, should therefore closely approach the corresponding in situ values (Pallud et al., 2007).

### 2.2 Sampling and experimental methodology

#### 2.2.1 Sample selection and collection

Sediments were sampled in February, May and October 2006 from a brackish site located in the Schelde Estuary (51°24’04”N 04°07’04”E), close to the village of Waarde in The Netherlands (see Hyacinthe and Van Cappellen (2004) and Pallud and Van Cappellen (2006) for more specific details on the sampling site, pore water and sediment characteristics). Different depth intervals (0-2, 4-6 and 8-10 cm) were sampled at four locations along a 30 m transect from a mud-flat adjacent to the salt marsh, into the non-vegetated tidal flat of the estuary (Table 2.1). Most samples were collected in duplicate or triplicate (Table 2.1).

Intact sediment slices (2 cm thickness, 4.2 cm diameter) corresponding to different depth intervals were sampled directly into the Perspex reactor cells using a steel shuttle corer. Reactors were sealed with 0.2 μm pore size nitrocellulose filters and glass fiber filters at each end, to avoid sediment and bacterial outflow and to support radial flow in the reactor cell, respectively. Reactors were closed using O-rings and plastic caps which contained the
inflow and outflow channels. The sealed reactors were transported in anaerobic bags to the laboratory and were stored at 4°C for up to several days before starting the flow-through reactor experiments.

### 2.2.2 Flow-through reactor experiments

A detailed description of the flow-through reactor technique is given in Roychoudhury et al. (1998), Laverman et al. (2006), Pallud and Van Cappellen (2006) and Pallud et al. (2007). Inflow solutions consisted of deionized water containing 2 mM Na₂SO₄ and 180 mM NaCl, yielding sulfate and salt concentrations comparable to those at the sampling site (Pallud and Van Cappellen, 2006). The chosen sulfate concentration was in excess of the apparent sulfate half-saturation concentration, $K_m$, previously estimated to be in the range 0.37 to 0.87 mM (Pallud and Van Cappellen, 2006), in order to avoid anomalous isotopic effects associated with sulfate limitation (Kampara et al., 2008; Thullner et al., 2008). Bromide (2 mM NaBr) was used as a tracer to monitor fluid flow-through each reactor. Inflow solutions and tubing were purged with Argon before and during the experiments to maintain anaerobic conditions. The inflow solution was supplied using a peristaltic pump with a continuous flow rate of $1.0 \pm 0.1$ ml/h. Reactors and inflow solutions were kept in the dark during experimentation.

A range of incubation temperatures between 10 and 50°C was achieved using thermostatic water baths. In the February 2006 experiment, temperature was varied between reactors so that replicates were maintained at different but constant temperatures (10, 20, 30 and 50°C). In the May 2006 experiment, each reactor was successively exposed to different temperatures (10 → 20 → 30 → 50°C). Temperature was gradually increased, 5°C per day, only after outflow sulfate concentration had remained at steady state for at least 3 days.

Before starting the experiments, reactors were flushed for 24 h with 180 mM NaCl solution (corresponding to approximately 1.5 reactor pore space volumes) to remove the sampling site pore water remaining in the sediment. Outflow samples were collected every 2 h for the first 24 h of the experiment to obtain detailed breakthrough Br data. For the remainder of the experiment, outflow samples were collected every 12 h. One sample per 24 h sampling period was used for chemical and isotopic analysis and the other was stored at -18°C. All collection tubes were pre-filled with 2 ml zinc acetate (10 %) to trap sulfide as ZnS.

Effects of seasonal variations at the sampling site on sulfate reduction rates and isotope fractionation were assessed using a series of duplicate reactors containing sediment collected in February, May and October 2006 from the 0-2 cm depth interval at a distance of 30 m from the salt marsh (Table 2.1). These reactors were incubated at 20°C with remaining experimental conditions identical to the other experiments. Effects of sampling location were investigated for the 4-6 cm depth interval in February 2006 using reactors sampled 1, 10 and 20 m from the salt marsh (Table 2.1). These reactors were incubated at 20°C with remaining experimental conditions identical to the other reactors. Blank experiments were run using duplicate reactors sterilized with gamma radiation (25 kGy). Following irradiation, the reactors were stored at 4°C for 2 months to ensure the absence of enzymatic activity before starting the experiment. Irradiated reactors were run under similar experimental conditions to the other 20°C reactors.
2.2.3 Chemical analysis

SO\textsubscript{4}\textsuperscript{2-} and Br\textsuperscript{-} concentrations were determined in the inflow and outflow solutions by ion chromatography (Dionex DX120 equipped with an AS14 column). The detection limit was < 5 µM with a mean precision of approximately 4%. Sulfur isotope fractionation was measured in the same samples. Sulfate in the collection tubes was precipitated as BaSO\textsubscript{4} with BaCl\textsubscript{2} solution (10%), rinsed with deionized water and dried for several days at 50°C. \(\delta^{34}S\) was measured using an elemental analyzer Na 1500NCS coupled to a Finnigan MAT (Delta +) gas source mass spectrometer. BaSO\textsubscript{4} was converted to SO\textsubscript{2} by flash combustion in a tungstic oxide, ultra pure copper quartz tube at 1050°C (mean precision of approximately 0.5‰).

Sedimentary sulfide \(\delta^{34}S\) was measured on sediments collected in May 2006 at 1 m and 30 m from the salt marsh for all depth intervals (0-2, 4-6 and 8-10 cm). Samples were taken next to the sediment cores which were used for the flow-through reactor experiments (Table 2.1). Approximately 2 g freeze dried sediment was distilled using a chromium reduction method to isolate the reduced sulfur compounds (Canfield et al., 1986; Fossing and Jørgensen, 1989). Sulfide produced during distillation was trapped as Ag\textsubscript{2}S and analyzed using the techniques described above.

2.2.4 Sulfate reduction rates and isotope fractionation

Steady state potential sulfate reduction rates (SRRs) were calculated using equation 1:

\[
SRR = \frac{\Delta C \times Q}{V}
\]

(1)

where \(Q\) represents the flow rate of the solution through the reactor in ml h\textsuperscript{-1}, \(\Delta C\) is the difference between inflow (\(C_0\)) and outflow (\(C\)) sulfate concentration in mM and \(V\) is the volume of the sediment in cm\textsuperscript{3}, which for our reactor was 27.7 cm\textsuperscript{3}. A three-way analysis of variance (ANOVA) was performed for all steady state sulfate reduction rates to statistically investigate the effect of incubation temperature, sampling depth, and proximity to the salt marsh at the sampling site on SRR using the Sigmastat software package.

Isotope fractionation, expressed in isotope fractionation effects (\(\varepsilon\)), were derived using the Rayleigh distillation model assuming that laminar flow in the reactor approximated closed system behavior (Canfield, 2001b; Fry, 2006). Isotope fractionation effects (\(\varepsilon\)), approximately equal to the difference in fractionation between outflow sulfate and sulfide, were calculated from the fractionation factor (\(\alpha\)) and measured \(\delta^{34}S\) values using equations 2 and 3:

\[
\alpha = 1 + \frac{(\ln \delta_{SO4_{in}} + 1000) - (\ln \delta_{SO4_{out}} + 1000)}{\ln(f_{SO4})}
\]

(2)

and

\[
\varepsilon = 1000(\alpha - 1)
\]

(3)
where $\delta_{SO_4\text{in}}$ represents the isotopic composition of the inflow solution, $\delta_{SO_4\text{out}}$ is the isotopic composition of the outflow solution, and $f_{SO_4}$ is the fraction of sulfate remaining in the outflow solution compared to the inflow solution,

$$f_{SO_4} = \frac{[SO_4\text{out}]}{[SO_4\text{in}]}$$

The accurate determination of $\varepsilon$ requires sufficient consumption of substrate in order to generate $\delta^{34}S$ values that are outside analytical error of $\delta^{34}S$ for the inflow sulfate. Excessive consumption of sulfate can also lead to inaccuracy since fractionation may be reduced at low substrate concentration. To take account of these problems $\varepsilon$ was determined when $f$ was between 10 and 90%.

Equations (1), (2) and (3) could only be applied when outflow sulfate concentration was constant, implying that the system was thriving under non changing conditions. Therefore all sulfate reduction rates and isotope fractionation data presented in the results and discussion were obtained from these periods at (or near) steady state. In our experiments steady state was defined as the first time interval, after applying a new experimental parameter, where at least 3 measurements (across three days) showed a constant outflow sulfate concentration within a maximum error of approximately 10%. Our measured sulfate reduction rates should be considered as potential rates since sulfate was the only electron acceptor supplied to the reactors, resulting in a probable, but small, overestimation compared with site representative values (Pallud and Van Cappellen, 2006).

2.3 Results

2.3.1 Reactor hydrodynamics and abiotic controls

Measured bromide breakthrough curves, i.e. outflow Br concentrations ($C$) normalized to the inflow Br concentration ($C_0$) plotted versus time (Figure 2.1), were in good agreement with theoretical curves predicted by a one-dimensional advective-dispersive model for a finite, radially homogeneous porous medium (Pallud and Van Cappellen, 2006; Pallud et al., 2007), with the exception of 5 out of the 30 reactor experiments. Results of these 5 flow-through reactors were not used in further data interpretation. Longitudinal dispersion coefficients ($D$) and pore water velocities ($v$) derived from the model fits fell in the ranges 0.017 to 0.14 cm$^2$ h$^{-1}$ and 0.039 to 0.17 cm h$^{-1}$, respectively.

Sulfate concentrations in the outflow of the blank, gamma-irradiated, reactor experiments were indistinguishable from the inflow concentrations, implying the absence of sulfate reducing activity. The measured $\delta^{34}S$ of sulfate in inflow and outflow solutions were also identical, indicating the absence of abiotic sulfur isotope exchange between the inflow solution and pre-existing phases in the sediments.
Figure 2.1: Comparison of simulated (line) and experimental (black circles) bromide breakthrough curves, with bromide concentrations (C) normalized to inflow concentrations (C₀). Insets indicate differences between simulated and experimental C/C₀. Panel 2.1a shows an example of data considered as acceptable, with a dispersion coefficient (D) of 0.019 cm² h⁻¹ and a pore water velocity (v) of 0.10 cm h⁻¹. Panel 2.1b shows an example of data considered not acceptable (D = 0.018 cm² h⁻¹, v = 0.12 cm h⁻¹).
2.3.2 Potential sulfate reduction rates

Steady state potential sulfate reduction rates (SRRs) ranged from 5 to 49 nmol cm\(^{-3}\) h\(^{-1}\) (Table 2.1). The rates listed in Table 2.1 are averages of rate determinations for duplicate or triplicate reactors, where available, that were run under identical experimental conditions. The corresponding relative standard deviations of the SRRs ranged from 1 to 30%. It took typically 3 to 9 days for the outflow sulfate concentration to stabilize after a change in conditions, e.g., a change in temperature.

SRRs measured in the 0-2 cm interval reactors of February and May exhibited the same systematic response to temperature (Figure 2.2), despite the fact that a slightly different approach was used for comparing this parameter in the February and May experiments (see experimental section). The temperature dependence of SRR was also similar for the May reactors collected at the locations furthest (30 m) and closest (1 m) to the edge of the salt marsh (Figures 2.2a and 2.2b). For any given depth interval, the lowest SRR was typically measured at 10°C and the highest at 30°C. Increasing the temperature to 50°C caused SRR to decrease to values similar to, or smaller than those measured at 20°C on the same sediment slice.

Under optimum temperature conditions (20-30°C), SSRs were highest in the top layer of sediment (0-2 cm) and generally decreased with increasing depth (Table 2.1 and Figure 2.2). Sulfate reducing activity was most sensitive to temperature in the 0-2 cm depth interval sediment slices. The difference between maximum and minimum SRRs decreased systematically with depth (Figure 2.2). Hence, it was not possibly to uniquely define an activation energy or Q\(_{10}\) value of sulfate reduction for the entire set of variable temperature experiments carried out.

A three-way ANOVA analysis was performed for all steady state SRRs to statistically investigate the effect of incubation temperature, sampling depth, time of the year when sampling took place and location with respect to the edge of the salt marsh. The analysis confirmed that incubation temperature (P < 0.001), sampling depth (P < 0.001) and sampling season (P < 0.001) had significant effects on SRRs, whereas location had no significant effect (P = 1.00).

2.3.3 Sulfur isotope fractionation effects

Sulfur isotope fractionation effects (ε), calculated using equations 2 and 3, ranged from 9 to 34‰ (Table 2.1). Note that only sulfate δ\(^{34}\)S was used to calculate ε. Relative errors on individual ε values were calculated using standard error propagation methods, and were typically on the order of approximately 12% RSD.

Frequency distributions of ε values obtained at the four incubation temperatures are shown in Figure 2.3. Each panel combines data from February, May and October reactors and shows the May data separately. For 20 and 30°C (Figures 2.3a and 2.3b), the distributions exhibit a well-defined maximum, less so at 10 and 50°C (Figures 2.3c and 2.3d). This is also the case when considering only the May data, which represent the bulk of the data (13 out of 25 reactors). The mean ε values for the entire data set were 19, 18, 15 and 15‰ at 10, 20, 30 and 50°C, respectively.
### Table 2.1: Overview of samples collected and experiments performed in February, May and October 2006 showing average sulfate reduction rates (SRR) and corresponding isotope fractionation effects ($\epsilon$). All data were produced under steady state conditions. Errors are reported for agreement between multiple reactors run under identical conditions, where appropriate. Where no replicates were made, errors represent agreement between measurements made within the steady state area of one reactor, typically for 3-5 data points.

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<th>Location distance from the salt marsh</th>
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<th>sd (nmol cm$^{-3}$ h$^{-1}$)</th>
<th>$\epsilon$ (‰)</th>
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Isotope fractionation generally increased with depth in the sediment. This is illustrated in Figure 2.4a, where average \( \varepsilon \) values are plotted versus sampling depth for the May reactors collected 1 and 30 m away from the salt marsh edge. The large error bars are due to the fact that data from the four incubation temperatures (10, 20, 30 and 50°C) were averaged together. As Figures 2.4a and 2.4b show, the increasing trend of \( \varepsilon \) with depth tracked a decrease in SSR. No systematic effect of sampling location on \( \varepsilon \) values was observed.

Isotope fractionation correlated negatively with SSR (Figure 2.5). Data collected for all the May reactors at 20 and 30°C exhibited a single, near-linear \( \varepsilon \) versus SSR trend. The fractionation measured in the 20°C October reactor experiment fell on the same trend. In contrast, the February reactors run at 20 and 30°C, yielded \( \varepsilon \) values exceeding those measured in May at comparable SRRs. The 10 and 50°C reactors defined a somewhat steeper inverse relationship between \( \varepsilon \) and SSR, at the lower end of SRRs.

Sedimentary sulfide, extracted from sediments collected in May 2006, gave \( \delta^{34}S \) in the range of -15 to -20 ‰. There was no systematic variation in isotope fractionation observed in sediments collected at the different depth intervals. Average isotope fractionation of sediments sampled 1 m from the salt marsh showed \( \delta^{34}S \) values which were approximately 2 ‰ lighter compared to the 30 m location.

2.4 Discussion

2.4.1 Sulfur isotope fractionation effects
Sulfur isotope fractionation effects (\( \varepsilon \)) during microbial sulfate reduction in brackish estuarine sediments were studied using an experimental flow-through reactor approach designed to preserve the original physical, geochemical and microbial structure of the sediment (Pallud et al., 2007). The work builds on an earlier detailed study of the kinetics of sulfate reduction in sediments from the same site (Pallud and Van Cappellen, 2006). As no external electron acceptor other than sulfate was supplied to the reactors, sulfate reduction to sulfide, coupled to the oxidation of naturally-occurring electron donors, was the predominant respiratory process taking place in the reactors. The experimental approach minimizes isotope effects due to sulfide reoxidation and sulfur disproportionation reactions. Abiotic controls with sterilized sediment further confirm that the observed isotope fractionation was due to the metabolic activity of microorganisms inhabiting the sediment.

By sampling various intertidal locations near the salt marsh, at different depths and different times of the year, and imposing a range of incubation temperatures, sulfur isotope fractionation was measured over a relatively large span of potential sulfate reduction rates (SRRs). Only SRRs and \( \varepsilon \) values corresponding to steady state conditions are included in the analysis. In the following sections, the results are compared to predictions of existing metabolic fractionation models, and to isotope fractionation obtained using the same experimental approach on sediments from a marine lagoon site in Denmark (Canfield, 2001b).
The SRRs follow a temperature trend that would be expected for mesophilic microorganisms, with the highest rates measured at 30°C and the lowest at 10 and 50°C. This trend is explained by a combination of 1) the physiological response of individual strains of sulfate reducing prokaryotes (SRP) to temperature, which affects metabolic rates such as

![Figure 2.2: Effect of temperature on steady state potential sulfate reduction rates (SRR) measured with flow-through reactor experiments for different sediment depth intervals (squares: 0-2 cm, triangles: 4-6 cm and circles: 8-10 cm) sampled 30 m from the salt marsh in May (black symbols) and February (white symbols) 2006 (Panel 2.2a) and 1 m from the salt marsh in May 2006 (Panel 2.2b). Error bars represent the standard deviation calculated from replicate reactors, where available (Table 2.1), with a minimum of 3 measurements per reactor or error within a single reactor when no replicates were measured. When not visible, the size of the y-error bars fall within the size of the symbols.](image)

The SRRs follow a temperature trend that would be expected for mesophilic microorganisms, with the highest rates measured at 30°C and the lowest at 10 and 50°C. This trend is explained by a combination of 1) the physiological response of individual strains of sulfate reducing prokaryotes (SRP) to temperature, which affects metabolic rates such as
reduction of cellular sulfite to sulfide, or the transport of substrates and nutrients through cell membranes (Brüchert et al., 2001; Canfield, 2001b; Rabus et al., 2002), 2) a change in (labile) electron donor supply, either released from the sediment or due to a temperature-dependent shift in the activity of fermenting microorganisms (Macdonald et al., 1995; Zogg et al., 1997; Andrews et al., 2000), and 3) the variable composition of the active part of the sulfate reducing microbial community as the growth versus temperature behavior is strain

Figure 2.3: Distribution plots of isotope fractionation effects ($\varepsilon$) versus the number of samples analyzed for incubations at 20°C (Panel 2.3a), 30°C (Panel 2.3b), 10°C (Panel 2.3c) and 50°C (Panel 2.3d). Plots contain data from February, May and October 2006 (grey and black) and only May data (grey). These plots were made using the individual data points produced under steady state conditions.
The transient times observed after switching to a new temperature are similar to those observed in previous studies with intertidal sediments (Nedwell and Floodgate, 1971; Canfield, 2001b). The results further indicate that an active community of sulfate reducers was present in all sediment samples and was able to respond immediately to changes in temperature.

Whereas the entire data set confirms the existence of a general inverse relationship between $\varepsilon$ and SRR, a detailed comparison of the results from individual reactors also illustrates the natural variability of the relationship, even within a single environmental setting (Figure 2.5). Particularly at lower SRRs ($\leq 15$ nmol cm$^{-3}$ h$^{-1}$), $\varepsilon$ values tend to exhibit significant scatter (compare error bars on Figure 2.5). At 10°C, differences in $\varepsilon$ of up to 30% were observed between parallel reactors run under identical conditions. The spread in $\varepsilon$ values among these reactors is real and not caused by analytical error. The correlation between $\varepsilon$ and SRR is also weaker for the deeper sediment intervals sampled, where sulfate reduction rates were lower, most likely because of a drop in availability of organic substrates with depth in the sediment.

**Figure 2.4:** Isotope fractionation effects ($\varepsilon$) (Panel 2.4a) and steady state potential sulfate reduction rates (SRR) (Panel 2.4b) averaged for all temperatures selected by depth intervals (0-2, 4-6 and 8-10 cm) obtained for tidal flat sediments sampled in May 2006.
Chapter 2: The Schelde Estuary, The Netherlands

(Pallud and Van Cappellen, 2006). A similar increasing spread of \( \varepsilon \) with decreasing SRR has been reported in other laboratory studies (see Habicht and Canfield (1997) and references therein).

The \( \varepsilon \) versus SRR relationship also appears to depend on the time of sampling during the year (Figure 2.5). Although the size of the February data set is limited, there is an offset to higher \( \varepsilon \) values, compared to the May and October data. Possibly, this offset was due to variations in the nature and supply of organic substrates (Brüchert et al., 2001; Canfield, 2001b), or in the composition and size of the active fraction of the sulfate reducing microbial community (Detmers et al., 2001). However, experimental artifacts may have also played a role, as it took longer to achieve steady state in the February than in the May experiments. In fact, for several February reactors steady state was not reached at all, and the corresponding data were rejected.

Although the isotope fractionation effects, derived from the \( \delta^{34}S \) values of aqueous sulfate, show a relatively broad range in the experiments (9 to 34 ‰), most in situ sulfate reduction activity is likely restricted to the period from late spring to early fall, when temperatures in the field (12 to 23°C) are closest to the optimum temperature (Figure 2.2). Thus, from among the entire data set, the \( \varepsilon \) values obtained for the May and October sediments at 20°C are expected be the most representative of the in situ isotope fractionation due to sulfate reduction to sulfide at the site. The corresponding average \( \varepsilon \) value is 17 ± 3 ‰. A limited effective range of \( \varepsilon \) is consistent with the narrow range in \( \delta^{34}S \) values determined on the extracted whole sediment pyrite fraction (-15 to -20 ‰). However, as is generally observed, the measured \( \varepsilon \) values in the flow-through reactor experiments and the \( \delta^{34}S \) values of the sedimentary sulfides also imply that redox processes in addition to the microbial reduction of sulfate to sulfide are needed to explain the isotopic composition of early diagenetic pyrite (Goldhaber, 2003). The location water sulfate has a \( \delta^{34}S \) value of approximately 20 ‰ suggesting \( \varepsilon \) values of approximately 40 ‰ which are a factor of two larger than the fractionation effects obtained in our experiments.

2.4.2 Isotope fractionation models

Microbial sulfate reduction can be divided into four steps: 1) the uptake of sulfate into the cell (Cypionka, 1995), 2) the reaction of sulfate with adenosine-5'-triphosphate (ATP) to form adenosine-3'-phosphate-5'-phosphosulfate (APS), 3) the reduction of APS to sulfite, and 4) the reduction of sulfite to sulfide with subsequent export from the cell (Harrison and Thode, 1958; Kaplan and Rittenberg, 1964; Rees, 1973). Steps 1, 2 and 3 are reversible, while step 4 is believed to be irreversible, although reversibility has also been suggested (Brunner and Bernasconi, 2005). Large variations in \( \varepsilon \) can be produced within steps 3 (up to 25 ‰) and 4 (up to 25 ‰) whereas little or no fractionation is associated with steps 1 (up to -3 ‰) and 2 (0 ‰) (Rees, 1973). According to this standard model outlined by Rees (1973) based on experimental work by Harrison and Thode, 1958, Kaplan and Rittenberg, 1964 and Kemp and Thode, 1968, at low rates of sulfate reduction all backward and forward reactions are close to equilibrium, resulting in large isotope fractionation. As rate increases, cell sulfate demand increases, intermediate reactions become increasingly irreversible, exchange between internal sulfur pools is minimized, and transport of sulfate across the cell membrane ultimately
Figure 2.5: Relationship between isotope fractionation effects (ε) and steady state potential sulfate reduction rates (SRR). In Panel 2.5a, ε and SRR were measured at 20°C (triangles) and 30°C (diamonds) in February (white symbols), May (black symbols) and October (gray symbols) 2006 and in Panel 2.5b, ε and SRR were measured at 10°C (squares) and 50°C (circles) in February and May 2006. Error bars represent 1 standard deviation calculated from replicate reactors or from steady state areas within reactors where no replicates were made (Table 2.1).
becomes the rate limiting step, resulting in a decrease in fractionation. In general, the further along the reduction process the rate determining step is, the larger the expected fractionation (Rees, 1973; Brunner and Bernasconi, 2005). The standard model thus implies that the physiology of the cell, which is controlled by environmental parameters such as temperature and organic substrate availability, regulates the rate of sulfate reduction and the associated isotope fractionation in a predictable way. When the system is in complete equilibrium, the maximum fractionation should be about 47 ‰.

Most studies have yielded ε values less than or equal to 47 ‰, although more recent field observations show that sulfide formed as a result of microbial sulfate reduction has corresponding ε values exceeding 47 ‰ (Rudnicki et al., 2001; Wortmann et al., 2001). Brunner and Bernasconi (2005) therefore proposed a revised model in which the reduction of sulfite to sulfide proceeds not in a single step but in a series of reversible steps called the trithionate pathway. This more complex reaction network can potentially result in fractionation of up to 70 ‰.

Pure culture (Harrison and Thode, 1958; Kaplan and Rittenberg, 1964; Kemp and Thode, 1968; Chambers et al., 1975) and natural sediment studies (Habicht and Canfield, 1997; Canfield, 2001b; this study) have shown systematic inverse correlations between SRR and ε, in accordance with the standard model. However, other studies found that this relationship was either absent (Detmers et al., 2001; Brüchert et al., 2001; Mangalo et al., 2007) or more complex than expected from the standard fractionation model (Canfield, 2001b; Canfield et al., 2006; Hoek et al., 2006). For example, Canfield, 2001b measured small isotope fractionation at low temperatures and correspondingly low SRR. The low observed fractionation was explained by a reduction in the fluidity of the cell membrane, thereby rendering transport across the cell membrane rate limiting. Furthermore, Canfield et al. (2006) and Hoek et al. (2006) reported a positive relationship between rate and fractionation at low and high temperatures. These authors proposed a new model, built on the model introduced by Farquhar et al. (2003) and Johnston et al. (2005), in which they consider variations in mass flow and associated fractionation at two branching points: 1) transport of sulfate in and out the cell, and 2) sulfur exchange between the different internal sulfur pools. The magnitude and balance between these branching points at different temperatures could vary among different microorganisms leading to variable responses for different pure cultures or natural communities of sulfate reducing prokaryotes (Johnston et al., 2007).

Taken together, the data of the Schelde estuarine sediments are consistent with the standard model of Rees (1973). The measured values of ε of 9 to 34 ‰, fall within the permissible range of the standard model, and they correlate inversely with SRR (Rees, 1973). Though no evidence was found for the larger isotope fractionation suggested by the modified model of Brunner and Bernasconi (2005), this model cannot be excluded. To test which model is most suitable, additional information on Δ33S and Δ36S values produced during the experiments would be needed (Farquhar et al., 2008). A systematic decrease in fractionation at the lowest temperature (10°C), which could indicate an effect of reduced fluidity of the cell membrane, was not clearly observed either.
2.4.3 Comparison with isotope fractionation in a Danish coastal sediment

Flow-through reactor experiments similar to the ones described here have been carried out with a small number of sediments sampled in a semi-enclosed marine lagoon at the northern tip of the Island of Fyn in Denmark (Canfield, 2001b; Farquhar et al., 2008). When no external electron donor was added, these experiments yielded comparable volume-based sulfate reduction rates as obtained in the present study of 2 to 38 nmol cm\(^{-3}\) h\(^{-1}\) (Canfield, 2001b) and 3 to 15 nmol cm\(^{-3}\) h\(^{-1}\) (Farquhar et al., 2008). Isotope fractionation effects, however, were substantially larger, ranging from 19 to 40 ‰ (Canfield, 2001b) and 37 to 45 ‰ (Farquhar et al., 2008).

The relationship between ε and SRR obtained by Canfield (2001b) with a single reactor, containing 0-2 cm depth interval sediment, is shown in Figure 2.6. The data points included are those in which the resident sulfate reducing prokaryotes utilize the naturally occurring electron donors present in the sediment (i.e., no external electron donor was supplied via the inflow solution). For comparison, the results from the multiple May reactors run at 20 and 30°C are also plotted. The figure implies that the ε versus SRR relationship is site-specific, and may reflect differences in the nature and availability of organic matter, or in the structure and abundance of the sulfate reducing community, between the two sites (Detmers et al., 2001). Similar considerations also apply to the differences in isotope fractionation observed among different sampling times at the Schelde Estuary site. In fact, the 20 and 30°C February data define a trend that is intermediate between the two relationships displayed on Figure 2.6.

As pointed out by Habicht and Canfield (1997), for comparative purposes it would make more sense to relate the isotope fractionation effects to cell-specific rates of sulfate reduction. This, however, requires accurate estimates of the in situ density of the active sulfate reducing community, which are not routinely accessible with currently available culturing and molecular techniques. Nonetheless, a quantitative characterization of natural sulfate reducing communities will be needed to fully interpret the observed variations in isotope fractionation in field settings, and to relate them to the large body of data available from laboratory experiments with pure cultures.

2.5 Conclusions

Potential sulfate reduction rates (SSRs) and corresponding \(^{34}\text{S}/^{32}\text{S}\) isotope fractionation effects (ε) produced by natural sulfate reducing communities were measured under steady state conditions using flow-through reactors containing undisturbed slices of intertidal estuarine sediments collected next to a salt marsh in the Schelde Estuary (Waarde, The Netherlands). Isotope fractionation effects (ε) and SRRs correlate inversely. Their variations are mainly related to the incubation temperature, sediment depth and sampling time, while sampling location with respect to the adjacent salt marsh has little effect. The potential SRRs range from 5 to 49 nmol cm\(^{-3}\) h\(^{-1}\), and exhibit an optimum temperature around 30°C. Isotope fractionation ranged from 9 to 34 ‰. The SRRs systematically decrease with depth in the sediments while ε values simultaneously increase.
The observed inverse relationship between $\varepsilon$ and SRR is consistent with the standard Rees model of isotope fractionation during microbial sulfate reduction. The correlation is strongest for the data measured at 20 and 30°C, but weaker at suboptimal temperatures (10 and 50°C). In addition, the $\varepsilon$ versus SRR relationship obtained for the sediments sampled in February shows a positive offset of several ‰, relative to the relationship obtained for the May and October sediments. The overall consequence is a range in $\varepsilon$ values of about 20 ‰ when SRR drops below 15 nmol cm$^{-3}$ h$^{-1}$. At higher SRRs, $\varepsilon$ exhibits a narrower range (~5 ‰) around an average value of 17 ‰. The value of 17 ‰ is probably representative of the bulk in situ isotope fractionation in Schelde sediments produced by a single step of sulfate reduction.

At comparable SRRs, $\varepsilon$ values in the present study are systematically lower than those measured previously by Canfield (2001b) in a near shore marine sediment in Denmark, using a similar flow-through reactor approach. Although in both cases $\varepsilon$ and SRR are inversely related, the relationships are site-specific, possibly reflecting differences in the size and structure of the microbial communities, or in the nature and availability of electron donor substrates. Quantitative information on the composition and activity of the sulfate reducing community will be needed in order to fully elucidate the mechanisms controlling variations in biogenic sulfur isotope fractionation.

Figure 2.6: Sulfate reduction rate (SRR) versus isotope fractionation effects ($\varepsilon$) measured in the present study (black diamonds and solid line) compared to data obtained for a Danish coastal sediment by Canfield (2001b) (white circles and dotted line), for incubation temperatures ranging from 15 to 35°C.
References


Chapter 2: The Schelde Estuary, The Netherlands


*Image of Mono Lake, California, USA*