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Geochimica et Cosmochimica Acta 276 (2020) 274–288

Geochimica et Cosmochimica Acta

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Mass-dependent selenium isotopic fractionation during microbial reduction of seleno-oxyanions by phylogenetically diverse bacteria

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Received 12 July 2019; accepted in revised form 26 February 2020; available online 4 March 2020

Abstract

Selenium (Se) isotope fractionation has been widely used for constraining redox conditions and microbial processes in both modern and ancient environments, but our knowledge of the controls on fractionation during microbial reduction of Se-oxyanions is based on a limited number of studies. Here we complement and expand the currently available pure culture data for Se isotope fractionation by investigating for the first time six phylogenetically diverse, mesophilic, and non-respiring bacterial strains that reduce Se-oxyanions to elemental Se [Se(0)]. Experiments were performed with either selenate [Se(VI)] or selenite [Se(IV)] at lower, more environmentally-relevant Se (9–47 μM) and carbon (500 μM) concentrations than previously investigated. *Enterobacter cloacae* SLD1a-1, *Desulfitobacterium chlororespirans* Co23 and *Desulfitobacterium sp.* Viet-1 were incubated with Se(VI) and Se(IV). *Geobacter sulfurreducens* PCA, *Anaeromyxobacter dehalogenans* FRC-W and *Shewanella sp.* (NR) were examined for their ability reducing Se(IV) to Se(0). Our data confirm that microbial reduction of both Se-oxyanions is accompanied by large kinetic isotopic fractionation (reported as $^{82/76}\varepsilon = 1000\times(^{82/76}\alpha\text{-}1)$ in ‰). Under our experimental conditions, microbial reduction of Se(VI) shows consistently greater isotope fractionation ($\varepsilon = -9.2$‰ to -11.8‰) than reduction of Se(IV) ($\varepsilon = -6.2$ to -7.8‰) confirming the difference in metabolic pathways for the reduction of the two Se-oxyanions. For Se(VI), an inverse relationship between normalized cell specific reduction rate (cSRR) and Se isotope fractionation suggests that the kinetic isotope effect for Se(VI) reduction is governed by an enzymatically-specific pathway related to the bacterial strain-specific physiology. In contrast, the lack of correlation between normalized cSRR and isotope fractionation for Se(IV) reduction indicates a non-enzyme specific pathway which is dominantly extracellular. Our study highlights the importance to understand microbially-mediated Se isotope fractionation depending on Se species, and cell-specific reduction rates before Se isotope ratios can become a fully applicable tool to interpret Se isotopic changes in modern and ancient environments.

Keywords: Selenium; Isotope fractionation; Microbial reduction; Cell-specific reduction rate

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https://doi.org/10.1016/j.gca.2020.02.036
0016-7037/

1. INTRODUCTION

Selenium (Se) isotope fractionation has been widely described in modern environments and in ancient settings preserved in sedimentary rocks (Mitchell et al., 2012; Wen and Carignan, 2011; Wen et al., 2014; Schilling et al., 2015; Stüeken et al., 2015a,b; Basu et al., 2016; Kipp et al., 2017). Selenium stable isotopes are particularly sensitive to redox reactions, which determine chemical Se speciation in the environment. The most mobile and bioavailable forms of Se are the water-soluble oxyanions selenate [Se(VI)] and (hydro)selenite [Se(IV)]. Selenate is the dominant redox state in modern surface waters (Martin et al., 2011) while Se(IV) is present in ocean surface water, but adsorbs strongly onto iron, manganese and aluminum oxides (Parida et al., 1997; Peak and Sparks, 2002; Peak, 2006) as well as clays. Sparingly soluble elemental Se [Se(0)] and selenide [Se(-II)] are the dominant redox states in anoxic environments. Typically, environmental Se concentrations are at sub-micromolar levels (Conde and San Alaejos, 1997; Fordyce, 2013) but can locally be elevated in sulfide ores and roll-front type uranium ores (Howard, 1977; Basu et al., 2016), shales (Pogge von Strandmann et al., 2015; Stüeken et al., 2015a) or by anthropogenic pollution (*e.g.,* Presser and Ohlendorf, 1987; Dreher and Finkelman, 1992; Lemly, 2004; Muscatello et al., 2008). Because the mobility and environmental impact of Se are determined by its chemical speciation, it is important to understand the environmental processes that control Se speciation and transitions between oxidation states.

Microbial reduction of Se-oxyanions is the primary set of reactions generating solid Se(0) in natural settings. Both Se-oxyanions are energetically favourable electron acceptors because the reduction of Se(IV) or Se(VI) can provide 90 to 150 times more free energy 'G (−8.9 to −15.5 kcal mol^{-1} e^{-1}) for bacteria than the reduction of sulfate to sulfide (Stolz and Oremland, 1999). Bacteria able to reduce Se-oxyanions are phylogenetically diverse, have different metabolic strategies, and have been isolated from oxic and anoxic environments (*e.g.,* Macy et al., 1993; Herbel et al., 2000; Stolz et al., 2006; Yee and Kobayashi, 2008; Pearce et al., 2009). All microorganisms are facultative and it has been shown that non-specific metabolic Se reduction can involve different enzyme systems, known for the for reduction of nitrite, nitrate, arsenate, sulfate and glutathione (*e.g.,* Switzer-Blum et al., 1998; Sabaty et al., 2001; Kessi and Hanselmann, 2004; Basaglia et al., 2007). Few Se(VI)-reducing bacteria have been identified to catalyze Se(VI) reduction by the Se-specific enzyme selenate reductase (Schröder et al. 1997; Bébien et al., 2002; Ridley et al., 2006; Theissen and Yee, 2014) and only two bacterial strains (*Tetrathiobacter kashmirensis* and *Pseudomonas sp.*) have a Se(IV)-specific enzyme (Hunter and Manter, 2008, 2009).

Limited published data on Se isotope fractionation during microbial Se reduction have revealed large variations (Herbel et al., 2000; Ellis et al., 2003; Clark and Johnson, 2008). A previous study with pure cultures (*Bacillus selenitireducens, Bacillus arsenicoselenatis* and *Sulfurospirillum barnesii*) was restricted to Se-respiring bacteria grown with very high millimolar levels of Se (10 – 20 mM) and with only lactate as electron donor at high concentrations (10 – 40 mM). For these conditions, the reported Se isotope fractionation varied greatly between −1.7 and −13.7‰ for the reduction of Se(IV) to Se(0) and −1.7 and −7.5 ‰ for reduction of Se(VI) to Se (IV) (Herbel et al., 2000). At contaminated sites, however, Se concentrations are two to three orders of magnitude lower with Se concentration up to 150 μM (= 12,000 μg/L) in agricultural drainage and irrigation water (Deverel and Fujii, 1988; Meseck and Cutter, 2012; Schilling et al., 2015) and up to 12 μM (= 955 μg/L) in waste water from Se-bearing phosphorite mining (Mars and Crowley 2003; Stillings and Amacher, 2010). Even lower Se concentrations occur in the modern ocean and aquifers with average values of 0.002 μM and 0.5 μM Se, respectively (*e.g.,* Conde and San Alaejos, 1997; Pearce et al., 2009; Basu et al., 2016).

Another study using sediments slurries reported Se isotopic fractionation by the resident Se-reducing microbial communities between −8.3 and −8.6‰ for the reduction of Se(IV) to Se(0) and −3.9 and −4.7‰ for the reduction of Se(VI) to Se(IV) (Ellis et al., 2003). Resident Se-reducing microbial communities comprise diverse groups of bacteria with different metabolic strategies. Although the magnitude of Se isotope fractionation by microbial reduction of Se-oxyanions has been previously studied, the cause of such large variation in ε values for pure cultures and their relevance for environmental settings remains unclear.

In this study, we extend the currently available experimental data by determining the isotopic fractionation during microbial reduction of Se-oxyanions [Se(VI), Se(IV)] that includes previously unexplored groups of mesophilic bacteria (Fig. 1), with the first results for bacteria that perform non-catabolic reduction of Se-oxyanions. To investigate whether different Se metabolic strategies affect the magnitude of isotopic fractionation, we selected bacterial strains based on their ubiquitous distribution and their well-studied metabolisms. For Se(VI) reduction, we conducted experiments with the Gram-negative bacterium *Enterobacter cloacae* SLD1a-1, and the Gram-positive bacteria *Desulfitobacterium sp.* Viet-1, and *Desulfitobacterium chlororespirans* Co23. For the reduction of Se(IV), we used pure cultures of three Gram-negative bacteria namely *Geobacter sulfurreducens* PCA, *Anaeromyxobacter dehalogenans* FRC-W and *Shewanella sp.* (NR) in addition to the three Se(VI)-reducing bacterial strains. Studying pure bacterial cultures has the advantage to eliminate any complexity from natural microbial communities such as competing strains with possibly different reduction rates and to determine isotopic fractionation linked to a single reduction mechanism. Further, we used lower electron acceptor and electron donor concentrations compared to previous studies to achieve slow Se reduction rates as it is well established that rapid reductions are transport limited and suppress the overall isotopic fractionation (Clark and Johnson, 2010). Our experimental conditions are very close to Se concentrations reported for Se contaminated ground-

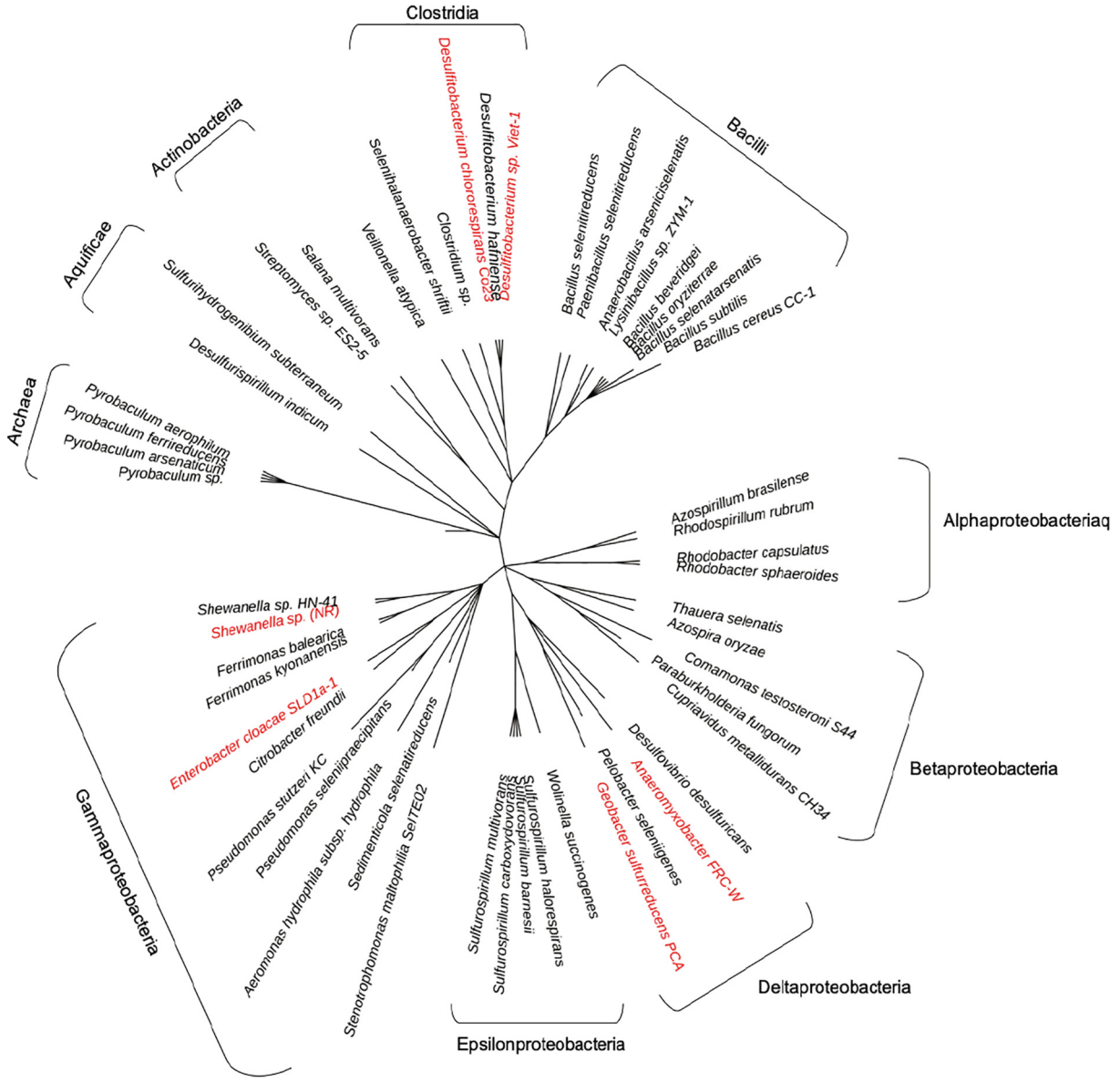

Fig. 1. Phylogenetic tree showing currently described Se(VI) and Se(IV)-reducing bacteria. Species names are shown in italics. Red marked species represent the bacterial strains studied in this work. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

water, soils, porewater (*e.g.*, Stillings and Amacher, 2010; Meseck and Cutter, 2012; Schilling et al., 2015; Basu et al., 2016).

2. MATERIALS AND METHODS

2.1. Microbial culture

The strains, *Desulfitobacterium sp.* Viet-1, *Desulfitobacterium chlororespirans* Co23, *Geobacter sulfurreducens* PCA, *Anaeromyxobacter dehalogenans* FRC-W and *Shewanella sp.* (NR) (Table 1) were supplied by Sanford, and *Enterobacter cloacae* SLD1a-1 (Table 1) was supplied by Pallud. For initial growth of the bacterial cultures and for the Se reduction experiments, we used a mineral-salt medium described by He and Sanford (2002). One liter of test medium was prepared with 10 mL buffer (12.5 g KH_2PO_4, 20.0 g K_2HPO_4 per liter), 10 mL trace salt (1.17 g $CaCl_2$, 2.00 g $MgCl_2 \times 6H_2O$, 0.70 g $FeSO_4 \times 7H_2O$, 0.50 g Na_2SO_4 per liter), 1 mL trace metals (0.05 g $ZnCl_2$, 0.5 g $MnCl_2 \times 4H_2O$, 0.03 g $CuCl_2 \times 2H_2O$, 0.05 g $CoCl_2 \times 6H_2O$, 0.05 g H_3BO_3, 0.05 g $NiSO_4 \times 6H_2O$, 0.01 g $Na_2MoO_4 \times 2H_2O$, 0.004 g Na_2WO_4), 1 mL ammonium chloride, 1 mL selenium-tungsten and 0.84 g $NaHCO_3$. The growth medium was supplemented with 0.03 g L-cysteine while the reductant L-cysteine was omitted for

Table 1
List of bacterial strains investigated in this study.

Bacterial strain	Gram strain	Electron acceptor	Electron donor
Enterobacter cloacae SLD1a-1	−	Se(VI)/Se(IV)	Acetate
Desulfitobacterium chlororespirans Co23	+	Se(VI)/Se(IV)	Lactate
Desulfitobacterium sp. Viet-1	+	Se(VI)/Se(IV)	Lactate
Geobacter sulfurreducens PCA	−	Se(IV)	Acetate
Anaeromyxobacter dehalogenans FRC-W	−	Se(IV)	Acetate
Shewanella sp. (NR)	−	Se(IV)	Lactate

the test medium used for Se reduction experiments. Anaerobic condition in both growth and test media was generated by boiling and degassing with N_2/CO_2 mix, the subsequent transfer into 120 mL glass serum bottles sealed with butyl rubber stoppers, and by autoclaving at 121 °C for 30 min.

In the growth medium, the bacterial strains *Desulfitobacterium chlororespirans* Co23 and *Desulfitobacterium sp.* Viet-1 were initially grown under fermentative conditions using 10 mM pyruvate and *Enterobacter cloacae* SLD1a-1 was grown using 2 mM glucose. *Shewanella sp*. (NR) was incubated with 1 mM nitrate and 2.5 mM lactate. *Anaeromyxobacter dehalogenans* FRC-W was incubated with 1.25 mM nitrate and 2mM acetate. *Geobacter sulfurreducens* PCA was grown with 10 mM fumarate and 3mM acetate. All anaerobic cultures were incubated at 30 °C for 3–5 days to achieve high cell densities ($\sim 10^{10}$ cells mL^{-1}) and complete consumption of the growth substrates.

2.2. Seleno-oxyanions reduction experiments

To test the reduction of Se-oxyanions, 10 mL of inoculum from the growth cultures, corresponding to bacterial densities of 10^5–10^7 cells mL^{-1} (Table 2) were transferred to the test medium. The microbial cultures were amended with 10–47 μM Se(VI) or Se(IV) as the sole terminal electron acceptor. Depending on the bacterial strain, 500 μM (2000 and 10,000 μM for *Geobacter sulfurreducens PCA*) acetate or lactate were added as electron donor to each reactor (Table 1 and 2). The experiments containing Se-oxyanions were conducted without any chemical reducing agent to minimize cell reproducibility and to avoid any abiotically mediated Se reduction. The "no-cell" control experiments were carried out using identical concentrations of electron donor and Se(VI) or Se(IV), but without any cell suspension. To identify if viable cells were responsible for Se reduction, a "heat-killed cell" control of *Desulfitobacterium chlororespirans* Co23 (autoclaved for 30 minutes) was inoculated with 18 μM Se(VI) and 500 μM lactate as electron donor. All cultures were incubated anaerobically at 30 °C under continuous shaking, and sampled at regular time intervals for periods ranging from 60 to 800 hours, depending on the bacterial strains. Subsamples were filtered through 0.2 μm nylon filters.

2.3. Determination of cell-specific reduction rate (cSRR)

One milliliter aliquot of each bacterial culture was collected for cell counting at the beginning of the experiment (t = 0). Bacterial cells were prefixed in 8% formaldehyde and stored at 4 °C until analyzed. The cells were stained with 1 μL of the STYO bacterial stain and 10 μL of microsphere standard (bacteria counting kit, Invitrogen). The cell counting was performed by flow cytometry analysis using a LSR II analyzer (BD Biosciences). The cell density was determined from the cell-counts for a known number of microspheres in each sample. Culture cell density values were used to calculate the initial cell density in the batch experiments and to calculate the cell specific Se reduction rate (cSRR) using the following expression:

$$cSRR = \Delta c / t_{1/2} \times d_0 \quad (1)$$

where Δc is the decrease in Se(VI) or Se(IV) concentration at the half-life $t_{1/2}$, and the initial cell density d_0. All cSRR values were normalized relative to the initial Se concentrations.

2.4. Transmission electron microscopy (TEM)

Microbial cultures of *Enterobacter cloacae* SLD1a-1 were incubated with Se(VI) or Se(IV) for 24 h. Afterwards the bacterial cells were pre-concentrated by centrifugation and fixed with 2% glutaraldehyde in a 0.04 M phosphate buffer. After the second cell fixation using 1% osmium tetraoxide, the samples were dehydrated with different concentrated ethanol solutions and embedded in resin. After sectioning of the bacterial cells, micrographs were taken with a Technai 12 transmission electron microscope (University of California, Berkeley).

2.5. Se isotope and concentration analysis

Initially, Se(VI) and Se(IV) concentrations were measured using hydride generation-inductively coupled plasma-mass spectrometry (ICP-MS). Prior to the analysis, Se(VI) was converted to Se(IV) by heating at 105 °C degrees for 60 minutes in a 5 M HCl matrix. The reported Se concentrations were calculated using an isotope dilution double-spike method by adding $^{74}Se + {}^{77}Se$ double spike of known isotope ratio and concentration to the sample with unknown Se concentration (*e.g.,* Heumann, 1992). We also used double spike isotope technique with an approximate sample spike proportion of 2 to correct for isotopic fractionation during sample purification and instrumental mass bias during the isotope measurement.

Selenium species separation from matrix solutes was performed using 1 mL AG1-X8 anion-exchange resin

Table 2
Reduction rate [$t_{50\%}$ in days] and normalized cell-specific reduction rate (cSRR) of investigated bacterial strains.

Bacterial strain	Electron acceptor	Initial Se (μM)	Time for 50% reduction ($t_{50\%}$) (d)	Normalized cSRR (10^{-17} mol cell^{-1} d^{-1})
Enterobacter cloacae SLD1a-1	Se(VI)	30	1.13	0.24
	Se(IV)	9	1.03	5.68
Desulfitobacterium chlororespirans Co23	Se(VI)	42	41.25	0.11
		9	2.31	0.29
			1.27	0.45
	Se(IV)	9	0.42	0.51
			0.71	0.31
Desulfitobacterium sp. Viet-1	Se(VI)	47	1.10	0.34
			0.79	1.22
		13	3.80	0.98
			3.85	1.30
	Se(IV)	9	3.10	0.65
			2.49	0.85
Geobacter sulfurreducens PCA	Se(IV)	13	3.10	0.28
		8	0.49	0.92
			0.66	1.14
		15	0.63	0.81
			0.55	1.32
Anaeromyxobacter dehalogenans FRC-W	Se(IV)	13	0.51	0.78
Shewanella sp. (NR)	Se(IV)	19	2.37	0.53
		13	1.60	2.17

(Eichrom, 200-400 mesh). Prior to anion-exchange separation step, all subsamples of Se(IV) were oxidized to Se (VI) with a 20 mM potassium persulfate solution (Schilling et al., 2014, 2015) by heating at 90 °C for 1 h. The sample purification procedure (including an oxidation step for Se(IV) and chromatographic separation for all samples) resulted in recoveries of >90%.

Selenium isotope ratios were measured using a Nu Plasma high resolution multiple collector-ICP-MS, connected to a custom-built hydride generation system described in previous studies (*e.g.,* Clark and Johnson, 2008; Mitchell et al. 2012; Zhu et al., 2014; Schilling et al., 2015; Mitchell et al., 2016). All $^{82}Se/^{76}Se$ ratios are reported as δ notation relative to the inter-laboratory standard NIST SRM 3149:

$$\delta^{82}Se(‰) = \left(\frac{(\,^{82}Se/\,^{76}Se)_{sample}}{(\,^{82}Se/\,^{76}Se)_{standard}} \right) \times 1000 \quad (2)$$

Blank solutions processed through the same sample purification procedure contained an average of 4.4 ± 2.9 ng Se ($n = 12$), less than 0.7% of the total Se in the sample. The uncertainty on $\delta^{82/76}Se$ was estimated by calculating the root mean square difference for samples prepared and analyzed in duplicate ($n = 25$). The in-house standard MH-495 was with −3.35 ± 0.1‰ (2σ, $n = 12$) relative to SRM-3149 in excellent agreement to previously reported values (Carignan and Wen, 2007; Zhu et al., 2008). Average external $\delta^{82/76}Se$ precision was ±0.16‰ based on repeated analysis of SRM-3149 standards ($n = 145$) over two years. The external reproducibility for $\delta^{82/76}Se$ of the samples, determined as twice root mean square, was ±0.17‰ ($n = 25$) across a range of $\delta^{82/76}Se$ values between −0.3‰ and +29.3‰.

2.6. Determination of the magnitude of isotopic fractionation (ε)

As all experiments were conducted in sealed batch reactors, the experiments were assumed to follow closed system behavior. Positive $\delta^{82/76}Se$ values of the remaining, unreacted Se thus indicate enrichment in heavy isotopes relative to the standard, whilst negative values represent depletion of heavy isotopes. Changes in $\delta^{82/76}Se$ can be directly related to the extent of Se(VI) or Se(IV) reduction. The magnitude of Se isotope fractionation was determined for each experiment by fitting the measured $\delta^{82/76}Se$ values to Rayleigh distillation model following the method described by Scott et al. (2004):

$$\delta^{82}Se_{(t)} = \left(\delta^{82}Se_0 + 1000\right) \times \left(\frac{c_{(t)}}{c_0}\right)^{\alpha-1} - 1000 \quad (3)$$

where $c(t)$ and $\delta(t)$ are the concentration and the isotopic composition of the remaining reactant (Se(VI) or Se(IV)) in solution as a function of reaction time. The fractionation factor (α) is defined as $\alpha = R_{product}/R_{reactant}$, where R is the measured $^{82}Se/^{76}Se$, and often expressed in terms of ε (a per mil quantity) as

$$\varepsilon = 1000 \times (\alpha - 1) \quad (4)$$

The magnitude of isotopic fractionation, ε, was calculated from the corresponding slope from the linear regression of $\ln(\delta^{82}Se + 1000)$ *versus* $\ln(c(t)/c_0)$.

2.7. Statistical analysis

One-way analysis of variance (ANOVA) with Tukey-HSD test ($\alpha = 0.05$) was used to evaluate the potential difference in the magnitude of Se isotope fractionation (ε) between Se(IV) or Se(VI) reduction by the different bacterial strains. The statistical analyses were performed using JMP software 13.1.0. with a statistical probability of $p < 0.05$.

3. RESULTS

3.1. Microbial reduction of Se(VI)

Fig. 2 shows Se(VI) removal over time during anaerobic microbial Se(VI)-reduction by three bacterial strains (*Enterobacter cloacae* SLD1a-1, *Desulfitobacterium chlororespirans* Co23, *Desulfitobacterium sp.* Viet-1) and different intital Se(VI) concentrations. In the presence of Se(VI)-reducing bacteria, the decrease in Se(VI) concentration ranged between 51% and 99% relative to the initial Se(VI) concentrations in the batch reactors. In all experiments, the decrease in Se(VI) concentration with time follows a first-order kinetic except for the latest sampling points. The "heat-killed" control with bacterial cells from *Desulfitobacterium chlororespirans* Co23 did not show any measurable Se(VI) removal after ca. 4 days of incubation (Fig. 2A). Further, we observed in the batch reactors an initial increase in Se(IV) resulting from the reduction of Se(VI), followed by Se(IV) reduction to Se(0) (Table A1).

In all Se(VI) experiments, an enrichment of ^{82}Se occurred in the remaining unreacted Se(VI) with progressive Se(VI) reduction (Fig. 3). The largest $\delta^{82/76}$Se value of +38.6‰ was observed at 99% reduction of Se(VI) for the experiment with *Desulfitobacterium sp.* Viet-1 (Fig. 3C). The $\delta^{82/76}$Se values for the intermediate Se(IV) varied between −9.6‰ for 1.5% Se(IV) and 16.6‰ for 15% Se(IV) relative to the initial Se(VI) concentration (9 µM) (Table A1). The magnitudes of Se isotopic fractionation (ε) for microbial Se(VI) reduction, obtained by fitting $\delta^{82/76}$Se data to Eq. (3), are illustrated in Fig. 3 and reported in Table 3. Among the three Se(VI)-reducing bacterial cultures, the ε values varied between −9.2‰ and −11.8‰ with a mean value of -10.6 ± 1.3‰. The ε values did not deviate in duplicate reactors within a 2σ uncertainty limit. The initial cell densities varied by two orders of magnitude in the Se(VI) incubation with the highest cell density of 1.9×10^8 cell ml^{-1} for the batch of *Enterobacter cloacae* SLD1-1a. The normalized cSRR ranged from 0.11×10^{-17} to 1.30×10^{-17} mol Se(VI) cell^{-1} d^{-1} (Table 2). We observed a strong inverse relationship ($r^2 = 0.91$) between normalized cSRR and ε for Se(VI) reduction showing decreasing ε values with increasing values of cSRR (Fig. 4A).

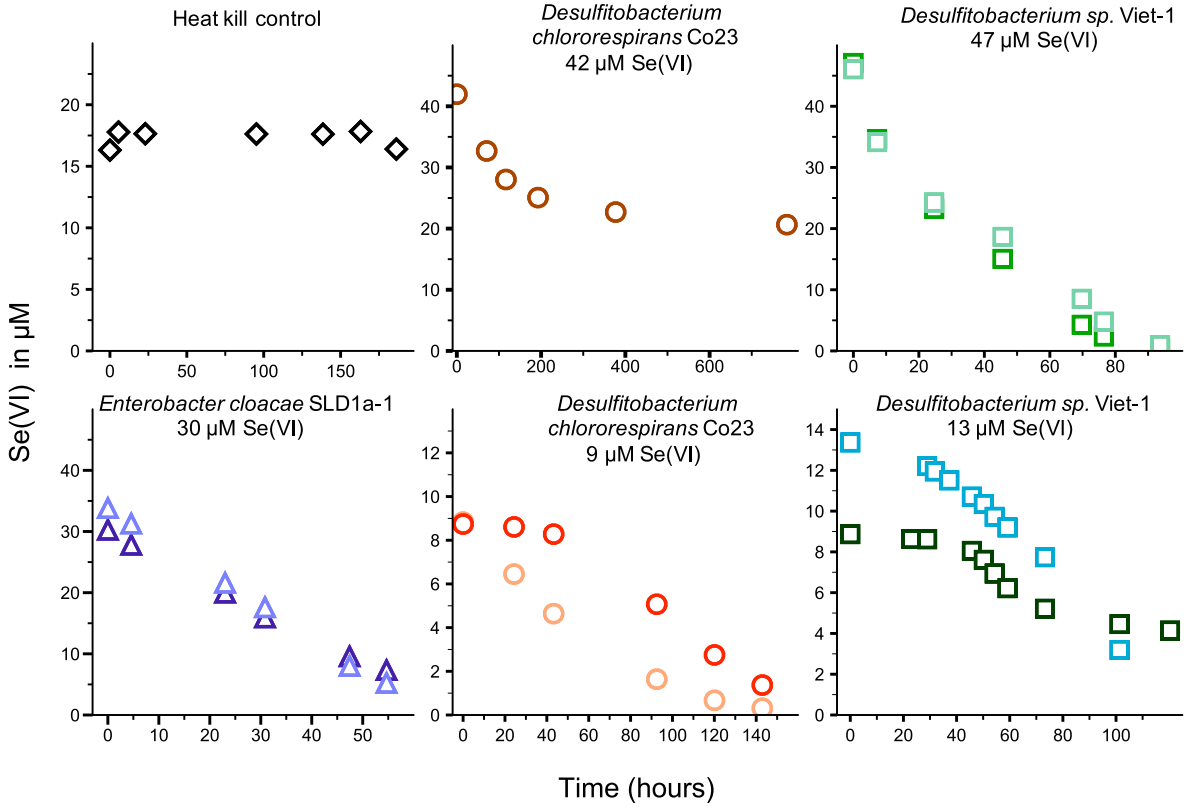

Fig. 2. Time series (batch experiments) of Se(VI) reduction at 30 °C with 500 µM of acetate (*Enterobacter cloacae* SLD1a-1) or lactate (*Desulfitobacterium chlororespirans* Co23, *Desulfitobacterium sp.* Viet-1) as electron donor. Heat kill control with cells from *Desulfitobacterium chlororespirans* Co23 incubated at 30 °C with 500 µM of acetate. The analytical uncertainty for the Se concentrations is less than 1% and close to the size of the symbols.

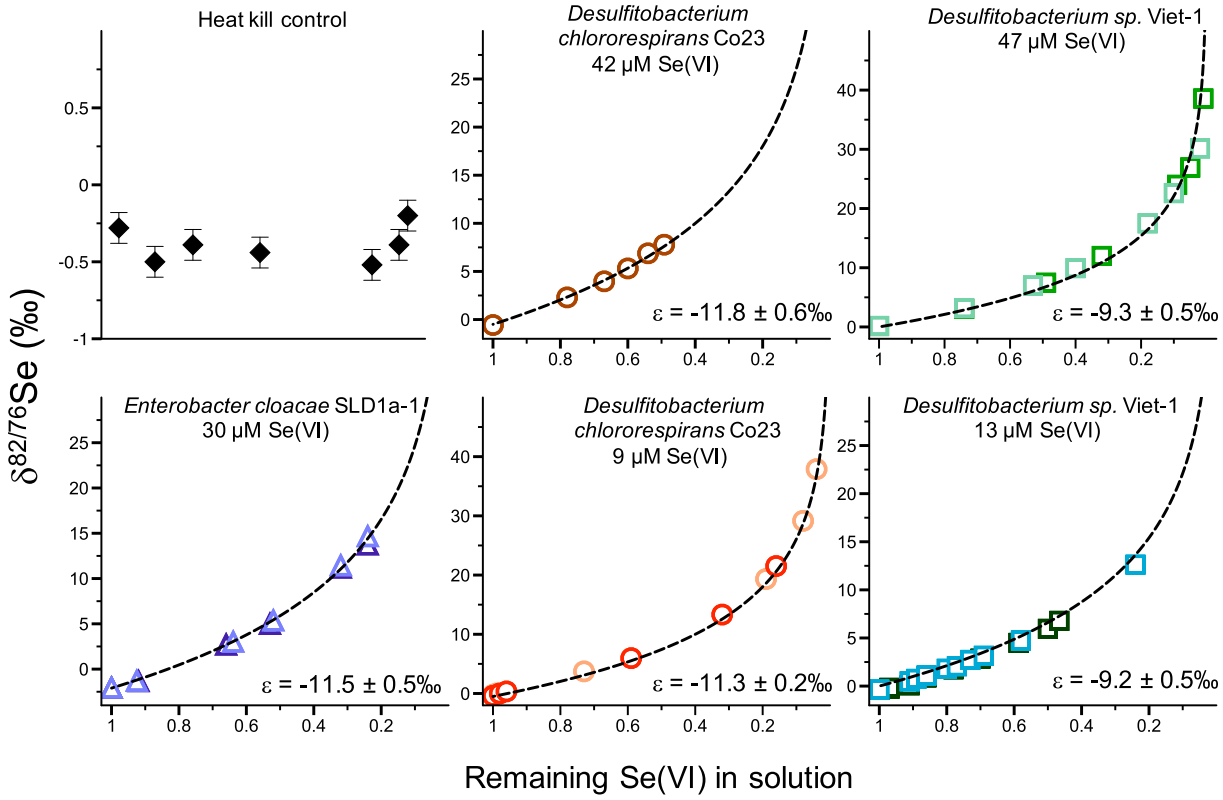

Fig. 3. Values of $\delta^{82/76}$Se of Se(VI) versus remaining Se(VI) during microbial reduction in closed system (batch experiments). Modelled lines (dashed) follow a predicted Rayleigh fractionation process. Uncertainties (±2 SD) are close to the size of the symbols. For the heat-kill control the error bars show the twice root mean square (RMS) of replicate measurements as described in Section 2.5.

Table 3
Magnitude of isotopic fractionation ε of Se(VI) and Se(IV) reduction by various bacterial strains.

Bacterial strain	Electron acceptor	Initial Se concentration (µM)	Electron donor concentration (µM)	ε (‰) $^{82/76}$Se ± 2 s.e.	Number of experiments
Enterobacter cloacae SLD1a-1	Se(VI)	30	500	−11.5 ± 0.5	2
	Se(IV)	9	500	−7.6 ± 0.3	1
Desulfitobacterium chlororespirans Co23	Se(VI)	42	500	−11.8 ± 0.6	1
		9	500	−11.3 ± 0.2	2
	Se(IV)	9	500	−7.8 ± 0.8	2
Desulfitobacterium sp. Viet-1	Se(VI)	47	500	−9.3 ± 0.5	2
		13	500	−9.2 ± 0.2	2
	Se(IV)	9	500	−7.3 ± 0.2	2
Geobacter sulfurreducens PCA	Se(IV)	13	500	−6.3 ± 0.6	2
		8	2000	−7.0 ± 0.4	3
		15	10,000	−7.3 ± 0.5	3
Anaeromyxobacter dehalogenans FRC-W	Se(IV)	13	500	−6.3 ± 0.5	1
Shewanella sp. (NR)	Se(IV)	19	500	−6.9 ± 0.2	1
		13	500	−6.2 ± 0.4	1

TEM images of a washed cell suspension of *Enterobacter cloacae* SLD1a-1 after the reduction of Se(VI) showed intracellular Se(0) precipitates (Fig. 5A). The particle sizes were spherical, <0.2 µm in diameter and located in the periplasmic space.

3.2. Microbial reduction of Se(IV)

Anoxic batch incubations of six Se-reducing bacterial strains with Se(IV) showed a decrease in Se(IV) concentration as a function of time (Fig. 6 and Table A2). The Se(IV)

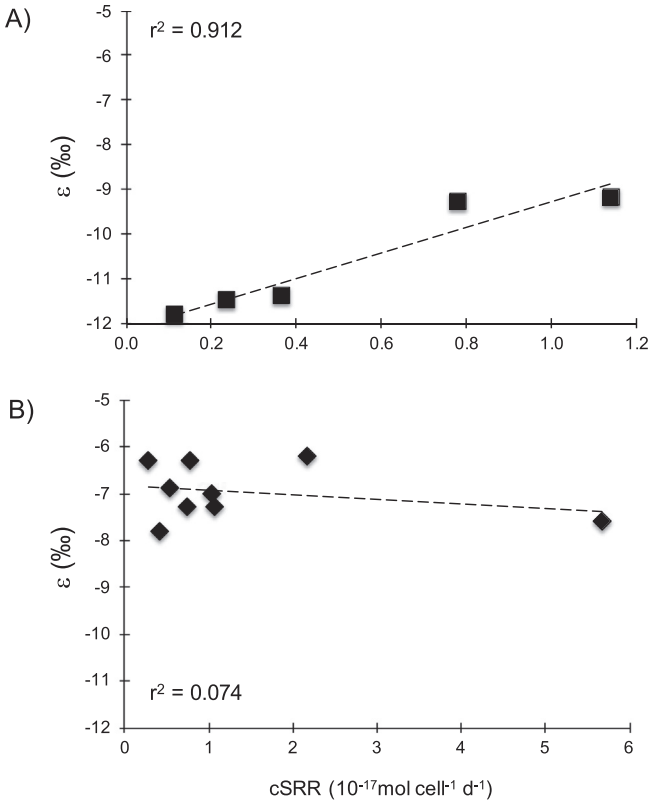

Fig. 4. Normalized cell-specific reduction rate (cSRR) and Se isotopic fractionation (ε) of phylogenetically diverse bacteria for the **(A)** reduction of Se(VI) and **(B)** reduction of Se(IV).

removal ranged between 56% and 92% over a period of 0.4–6.1 days. The no-cell control experiments did not show any change in Se(IV) concentrations with time. In each experiment, a first order kinetic model with a single rate constant fits all Se(IV) concentration data. The time interval for 50% removal of the initial Se(IV) concentration varied from 0.42 to 3.1 days. *Geobacter sulfurreducens* PCA assayed with varying electron donor concentrations (2000–10,000 μM) exhibited an approximately 50% Se(IV) removal after similar time periods ($t_{1/2}$ 0.49–0.66 days).

The initial cell densities varied by approximately two-orders of magnitude depending on the volume of inoculum. The highest cell density was found for the Se(IV) batch of *Anaeromyxobacter dehalogenans* FRC-W (1.3×10^8 cell ml^{-1}) and the lowest for *Enterobacter cloacae* SLD1a-1 (8.8×10^6 cell ml^{-1}). The calculated cell-specific reduction rate ranged from 0.28 to 5.7×10^{-17} mol $cell^{-1}$ d^{-1} with no correlation between normalized cSRR and ε values ($r^2 = 0.13$; Fig. 3B).

While the Se(IV) concentration in the batch reactors decreased, $\delta^{82/76}$Se of Se(IV) progressively increased with time (Fig. 7) relative to the starting Se(IV) isotope composition. The duplicate batch reactors showed similar ε values within the 2σ uncertainty level of ±0.12‰ to ±0.43‰. (Table 3). The largest $\delta^{82/76}$Se value of +15.7‰ was observed for *Shewanella sp.* (NR) after 91% Se(IV) removal. Overall, the ε values for Se(IV) reduction span a narrow range of −6.2‰ to −7.8‰ with a mean value of −7.0 ± 0.6‰ for all tested bacterial strains. We observed no significant difference in ε values among the six different bacterial strains ($p = 0.376$; Fig. 7).

TEM images of *Enterobacter cloacae* SLD1a-1 incubated with Se(IV) showed exogenous precipitates, presumably Se(0) (Fig. 5A) which are smaller than the intracellular Se(0) particles observed as a product of Se (VI) reduction (Fig. 5B).

4. DISCUSSION

Our results demonstrate that the magnitude of Se isotope fractionation by microbial reduction of Se-oxyanions depends mainly on two factors (1) growth conditions and (2) the Se-reduction by cometabolic or Se-respiring pathway. Below we discuss variation in Se isotope fractionation based on these two factors, specifically in the context of previous studies. Differences in the magnitude of Se isotope fractionation in our study compared to the previous studies (Herbel et al., 2000; Ellis et al., 2003; Clark and Johnson, 2008) is attributed to differences in the experimental approach and selection of bacterial strains.

4.1. Effect of experimental conditions on ε

4.1.1. Comparison to pure culture studies

Environmentally-relevant conditions, more specifically much lower Se substrate concentrations, result in a

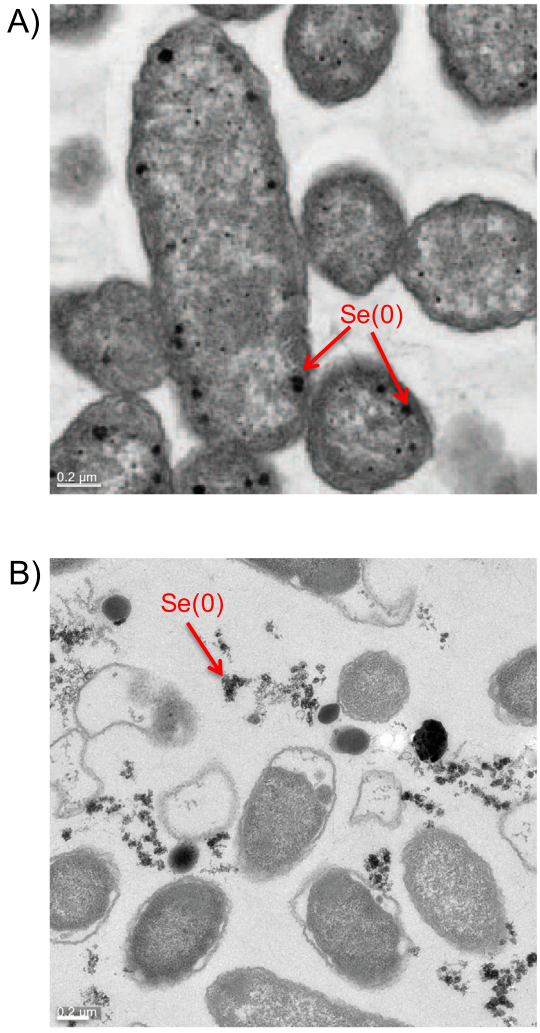

Fig. 5. TEM images of *Enterobacter cloacae* SLD1a-1 grown at 30 °C under anoxic conditions in presence of acetate as electron donor and **(A)** Se(VI))or **(B)** Se(IV). Red arrows indicate the presence of intracellular (**A**) or extracellular (**B**) Se(0) as reduction product. Scale bars represent 0.2 µm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

narrower-range Se isotope fractionation factors for the reduction of both Se(VI) and Se(IV) compared to previously published values obtained for *Bacillus selenitireducens*, *Bacillus arsenicoselenatis* and *Sulfurospirillum barnesii* (Herbel et al. 2000). Indeed, experiments by Herbel et al. (2000) were conducted under highly optimum growth conditions for anaerobic Se respiration *i.e.*, using high initial Se-oxyanion concentrations (10–20 mM) and high carbon concentrations (10–40 mM). This led to 100-fold increase in cell density and thus fast reduction rates during the experiment, which might explain the large span in isotopic fractionation they observed for Se(VI) reduction ($\varepsilon = -1.7$ to -7.5‰; Herbel et al., 2000). Se reduction rates in the previous study were one-order of magnitude (10^{-16} mol cell^{-1} d^{-1}) higher than the cSRRs observed in our experiments (10^{-17} mol cell^{-1} d^{-1}). In contrast to the large increase in cell density in the experiment by Herbel et al (2000), natural microbial consortia generally maintain a rather steady state population where cell decay balances cell growth (Brock, 1971). The more uniform ε values in our study correspond to minor cell growth or decay evident from single first-order rate constants fitting the time series from each experiment. Moreover, three-orders of magnitude lower initial Se-oxyanions concentrations, together with 20 times lower carbon concentrations as electron donor (10 mM *vs.* 0.5 mM), led to significantly larger ε values ($\varepsilon = -9.2$‰ to -11.8‰). Likewise, our data on microbial Se(IV) reduction result in an overall narrow distribution of ε values ($\varepsilon_{mean} = -7 \pm 0.6$‰) compared to the previous study ($\varepsilon = -1.7$ to -13.7‰; Herbel et al., 2000). This narrow range of ε values is likely when the tested strains are not actively respiring Se. Instead, the reduction of Se-oxyanions is a response to cope with the element's toxicity. Lower initial Se(IV) concentration also affect the results in two different ways by producing lower reduction rates and lower Se concentrations are less toxic for the cells and thus cell viability can be maintained.

Whether bacteria reduce Se by respiration (Herbel et al., 2000) or a cometabolic pathway determines the magnitude of isotope fractionation. All bacterial strains (*Bacillus selenitireducens, Bacillus arsenicoselenatis* and *Sulfurospirillum barnesii)* used by Herbel et al. (2000) are capable of actively metabolizing Se-oxyanions. This means that the bacteria are able to harness the energy derived from coupling reduction of Se(VI)/Se(IV) and oxidation of lactate to synthesize biomass. This leads to bacterial growth (increase of cell density by about two-orders of magnitude) during the experiments which changes the cSRR and thus affects the ε values. For example, isotope fractionation by *Bacillus selenitireducens* varied between -2.6 and -13.7‰ (Herbel et al., 2000). This also explains why the cSRR and ε relationship breaks down for Se-respiring bacteria. Further, both Se(VI)-respiring bacterial strains, *Bacillus arsenicoselenatis* and *Sulfurospirillum barnesii*, only reduce a very small amount of Se(IV) to Se(0) (Herbel et al., 2000), while our tested bacterial strains reduce Se(VI) all to Se(0) (Table A1).

Although the tested Se-reducers are not confined to any particular group of bacteria (Fig. 1), we demonstrate that the metabolic mechanisms control Se isotope fractionation rather than the their phylogenetic differences. There is no systematic difference in ε between the tested non-respiring Gram-positive (*Desulfitobacteria*) and Gram-negative bacteria (*Enterobacter cloacae* SLD1a-1). Gram-negative bacteria possess two membranes separated by the periplasmic space. The selenate reductase of Gram-negative bacterium *Enterobacter cloacae* SLD1a-1 is a membrane-bound enzyme situated in the cytoplasmic (inner) membrane (Schröder et al., 1997; Bébien et al., 2002; Ma et al., 2009; Watts et al., 2003). The location for the Se(VI)- reducing enzymes of *Desulfitobacterium chlororespirans* Co23 and *Desulfitobacterium sp.* Viet-1 are not known but are probably also membrane-bound as described for other Se(VI)-reducing Gram-positive bacteria (Kuroda et al., 2011). Hence, the diffusive transport across the outer membrane

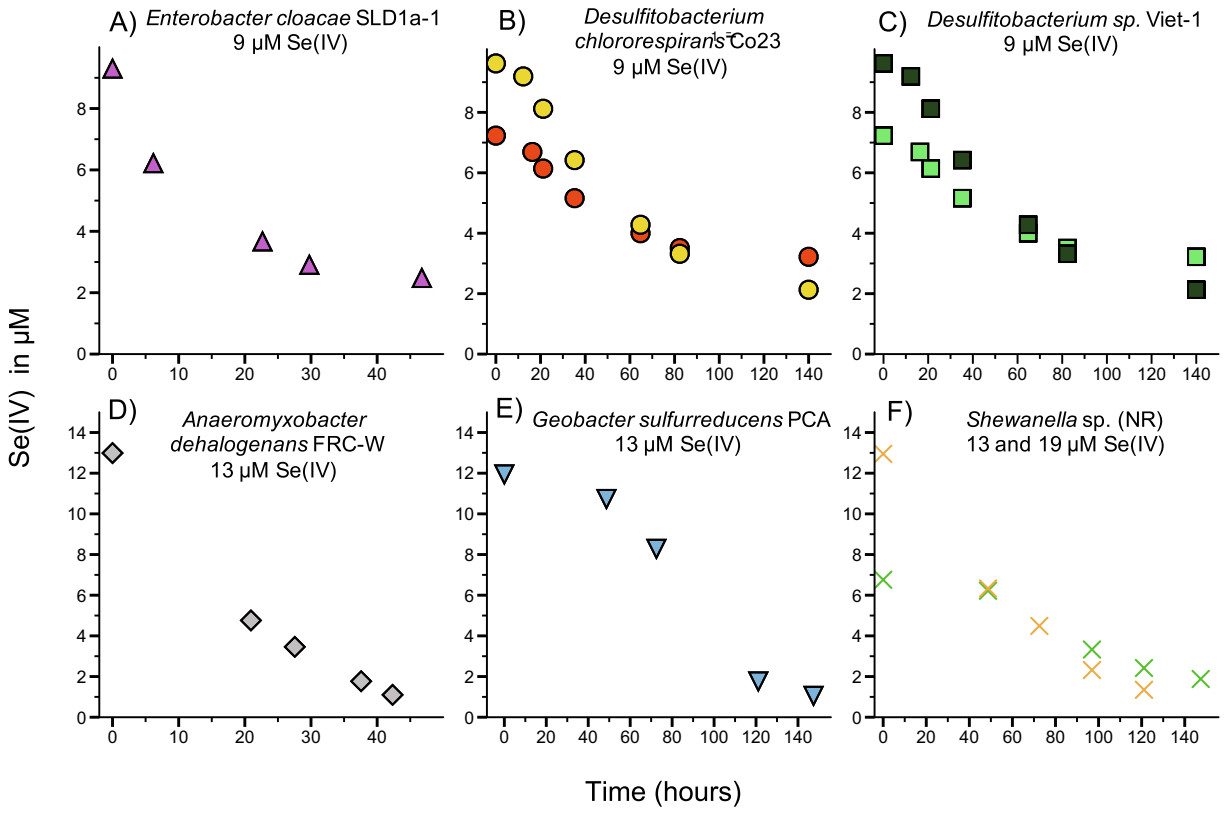

Fig. 6. Time series (batch experiment) of Se(VI) reduction at 30 °C with 500 µM of lactate (*Desulfitobacterium chlororespirans* Co23, *Desulfitobacterium sp.* Viet-1, Shewanella sp. (NR)) or acetate (*Enterobacter cloacae* SLD1a-1, *Anaeromyxobacter* FRC-W, *Geobacter sulfurreducens* PCA) as electron donor. The analytical uncertainty for the Se concentrations is less than 1% and close to the size of the symbols.

for Gram-negative bacteria seems not to affect the reduction rate and Se isotope fractionation for Se(VI) reduction.

For bacterial strains with no Se-specific enzymatic pathway, the reduction is carried out by various enzyme systems, e.g. nitrite reductase, nitrate reductase, arsenate and sulfate reductases, or the reduction of Se(IV) by glutathione (*e.g.,* Switzer-Blum et al., 1998; Sabaty et al., 2001; Kessi and Hanselmann, 2004; Basaglia et al., 2007; Nancharaiah and Lens, 2015). These enzyme systems have mainly a detoxifying function of Se and the energy released by the redox reaction is not generally utilized to synthesize biomass. Therefore, we assume that the obtained isotope fractionation factors can be extrapolated to a much wider group of microorganisms because the studied pure cultures include bacteria with different cell membranes (Gram positive and Gram negative), different enzymes in the electron transfer chain (*e.g.,* selenate reductase for *Enterobacter cloacae*) and carbon substrates (acetate and lactate) but show nearly identical Se isotope fractionation for the reduction of the respective Se-oxyanion.

4.1.2. Comparison to natural microbial consortia in sediment slurries and cores

Our pure culture microbial Se(VI) reduction experiments induced significantly larger Se isotope fractionation than experiments with sediment slurries and sediment cores involving complex microbial communities (Ellis et al., 2003; Clark and Johnson, 2008). Here reported ε values relate to suspensions of free-living cells with maximized mass transfer and accessibility of Se-oxyanions for each bacterial cell. Mass transfer limitation in sediment slurries and cores is expected to decrease the exchange between Se(VI) in solution and the particle-bound bacteria. This in turn should reduce selectivity for an isotopologue (heavy vs. light). Generally, mass transfer is faster for the isotopically light ^{76}Se-oxyanions than for the isotopically heavy ^{82}Se-oxyanions. If the probability for Se(VI) selectivity of an isotopologue is limited for the particle-bound bacteria the mass transfer affects the Se isotope fractionation. This presumably explains the relatively small Se isotope fractionation observed in sediment slurries (Ellis et al., 2003) where the contact between bacteria and Se-oxyanions in solution is limited but still higher than for sediment cores (Clark and Johnson, 2008). Selenium isotope signals in sediments controlled by the diffusion of Se from the overlying water have the highest mass transfer limitation determined by incomplete solution exchange at the water-sediment interface and the lowest Se isotope fractionation of 0.4‰ for microbial Se reduction. In contrast, pure cultures in our study are more selective to a particular Se isotopologue (heavy vs. light) as they are not particle-bound. However even if in porewaters, diffusion limitation yields a smaller Se isotope

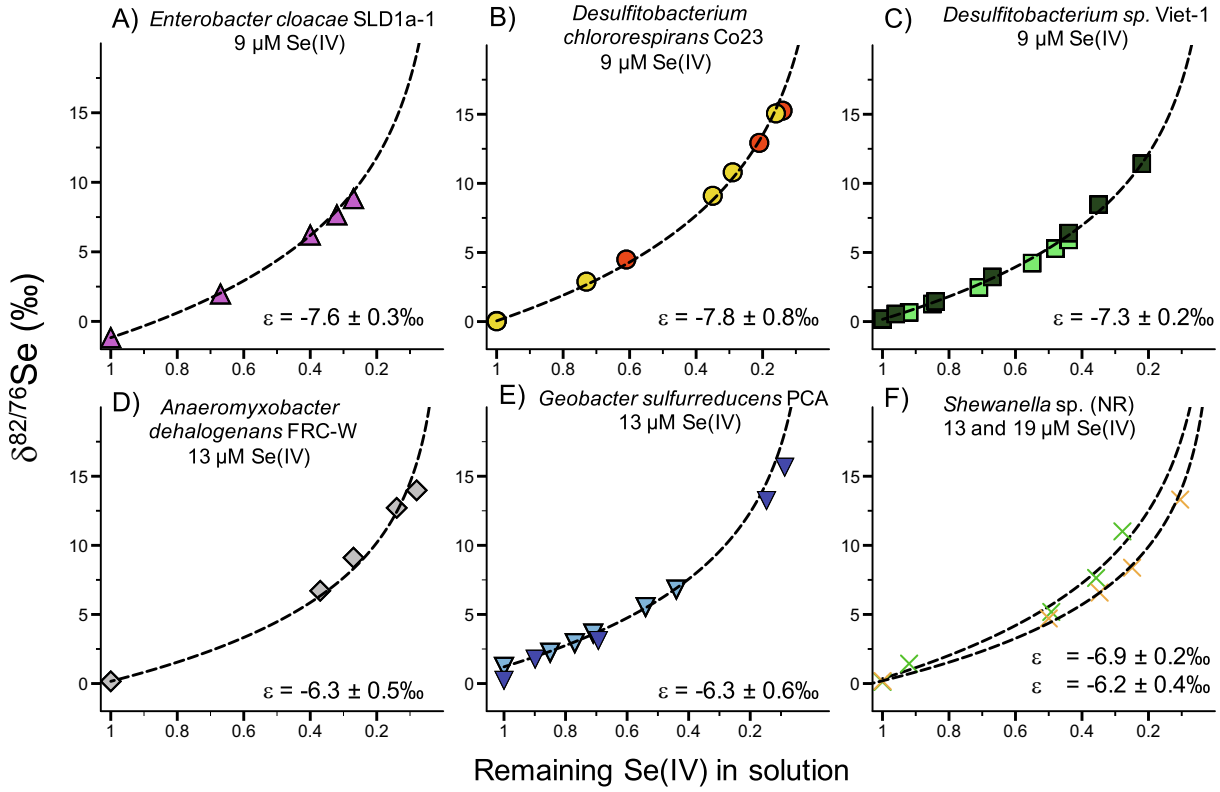

Fig. 7. Values of $\delta^{82/76}$Se of Se(IV) versus remaining Se(IV) during microbial reduction in closed system (batch experiments). Modelled lines (dashed) show predicted Rayleigh fractionation. Uncertainties (±2 SD) are close to the size of the symbols.

fractionation the reduction of Se(VI) and Se(IV) are still detectable because the shift in δ values from the initial value is significant.

4.2. Effect of metabolic pathway on Se isotope fractionation

Reduction of Se-oxyanions occurs intracellularly in the periplasmic space for Se(VI) (*i.e.,* Ridley et al., 2006; Nancharaiah and Lens, 2015) or extracellularly for Se(IV) (*e.g.,* Pearce et al., 2009; Nancharaiah and Lens, 2015). Our data and the pure culture study by Herbel et al., (2000) confirm that the pathways for reduction of Se(VI) or Se(IV) determine the Se isotope fractionation. Microbial Se(VI) reduction induces significantly larger fractionation than Se(IV) reduction for the six tested bacterial strains ($p > 0.01$).

4.2.1. Intracellular Se(VI) reduction

The reduction Se(VI) to Se(0) is a sequential two-step reaction which leads to significantly larger fractionation than Se(IV) reduction for the tested bacterial strains ($p > 0.01$). The reduction of Se(VI) to Se(IV) *via* two electron transfer is followed by a four electron transfer to form Se(0). The "heat-killed" control experiment with *Desulfitobacterium chlororespirans* Co23, which did not show Se(VI) reduction and any concomitant Se isotopic fractionation, confirms that Se(VI) reduction is enzymatically-mediated by viable cells. Correlation between normalized cSRR and ε for Se(VI) reduction indicates that the rate of electron transfer depends on the abundance of bacteria and their enzymes (*e.g.,* selenate reductase) (Yee and Kobayashi, 2008). Mechanistically, diffusion transport brings Se(VI) to the reduction site of the bacterial cell where Se(VI) is then reduced intracellularly. As diffusive transport of Se (VI) does not involve changes in coordination of oxygen around Se, any discrimination between the isotopologes in the Se-oxyanions will be minor compared to the enzymatic reduction. If the reduction rate of Se(VI) at these reduction sites is very slow either due low abundances of bacteria and their enzymes it is expected that ε reaches a maximum value. Future studies can help determining the maximum value as well as the more in-depth understanding of the relevant enzymes involved in the cometabolic Se(VI) reduction and the related Se isotope fractionation.

4.2.2. Extracellular Se(IV) reduction

The four electron transfer by only one reduction step for Se(IV) explains the smaller Se isotope fractionation compared to the reduction of Se(VI). Electron transfer for the reduction of Se(IV) is driven by either an exogenous electron shuttle, extracellular proteins or pili structures (Pearce et al., 2009). This also explains why the magnitude of Se isotope fractionation does not correlate with normalized cSRRs (Fig. 4C) because an exogenous electron transfer does not require a direct contact between the bacterial cell and the substrate *via* a specific enzyme. Such extracellular reaction is also most likely decoupled from electron donor oxidation, so that different donor types or concentra-

tions do not affect the isotope fractionation factor. This is clearly shown for the experiments with *Geobacter sulfurreducens* PCA, known for reducing Se(IV) extracellularly by outer membrane cytochromes (Pearce et al., 2009). Varying concentrations of electron donor (500–10,000 μM) have no effect on ε values (Table 3) for Se(IV) reduction by *Geobacter sulfurreducens* PCA. This is consistent with the observation for microbial Cr(VI) reduction where an extracellular Cr reduction pathway results in uniform Cr isotope fractionation at different electron donor concentrations (*i.e.,* Sikora et al., 2008; Basu et al., 2014; Zhang et al., 2019). Further, the extracellular reduction pathway for Se(IV) is not impacted by mass transfer limitation of Se(IV) to the bacterial cell and this explains the relatively good agreement for isotope fractionation between sediment slurry experiments for particle-bound natural microbial consortia ($\varepsilon = -8.4‰$; Ellis et al., 2003) and our pure culture study with free-living cells ($\varepsilon = -6.2$ to $-7.8‰$).

5. IMPLICATIONS

Given the distinctive amounts of Se isotope fractionation for microbial reduction of the two Se-oxyanions, Se isotope ratios can, in principle, shed light on the processing of Se-oxyanions in both modern and ancient environments.

5.1. Modern environments

Selenium stable isotope ratios have been previously used as indicators for Se sources and cycling in aquifers, lakes, soils and sediments (Clark and Johnson 2010; Schilling et al., 2015; Basu et al., 2016). Microbial reduction of Se reduces the mobility of Se from soluble poorly adsorbed Se(VI), to soluble strongly adsorbed Se(IV), to solid Se(0).

Our ε values can be understood as reference values to estimate the extent of Se reduction for bacterial groups most commonly found in the environment. A clear indicator of the reduction of Se(VI) to Se(IV) is the enrichment of ^{82}Se in Se(VI) or Se(IV) in groundwater or porewater while the reduced Se species in sediments and soils is enriched in ^{76}Se. If microbial Se reduction is the dominant reaction mechanism in nature, this should be reflected by shifts in $\delta^{82/76}$Se ratios according to the aqueous speciation of Se and the ε for that particular reaction.

In groundwater systems, we can infer the ε for Se removal mechanism from $\delta^{82/76}$Se of Se(VI) and Se(IV) measured in the same sample (Schilling et al., 2015; Basu et al., 2016). Microbial reduction of Se-oxyanions in a groundwater plume moving through a redox gradient will fractionate $\delta^{82/76}$Se of both the reactant and the product. This fractionation combined with the reactive transport of Se should lead to a systematic pattern of $\delta^{82/76}$Se induced by a distillation effect in the groundwater. With progressive reduction along the flow path, Se-oxyanions will become isotopically heavy (^{82}Se). For instance, Basu et al. (2016) observed increasing $\delta^{82/76}$Se with decreasing Se(VI) concentrations in an aquifer along the redox gradient at an *in-situ* recovery mining site. However, along the flow path of groundwater the rate of microbial Se reduction may vary depending on the organic carbon content of the aquifer, and the bacterial population density. This variation in the Se reduction rates can systematically affect the ε, which is determined by the difference in $\delta^{82/76}$Se between Se(VI) and Se(IV) of the same sample.

The dependence of ε on the Se(VI) reduction rate has important implications for predicting Se removal/accumulation patterns in modern settings based on Se isotope ratios ($\delta^{82/76}$Se). Our experimental results suggest a higher ε at low Se(VI) reduction rate generally found in terrestrial sediments with low organic carbon. In contrast, lower ε is expected in geochemical settings with high Se(VI) reduction rate commonly found after organic carbon amendment (*i.e.*, acetate) at active bioremediation sites. Our results suggest that the ε inferred from water samples may be used to estimate the Se(VI) reduction rate, which is difficult to determine accurately in open systems. Similarly, if the Se(VI) reduction rate is known, an appropriate ε can be determined for calculating the extent of remediation for active remediation sites using rate-ε relationship. Therefore, any quantitative interpretation of the groundwater Se isotope ratios is predicated on the knowledge of the size of the intrinsic ε and the factors that control ε at a geochemical setting.

Selenium isotope ratios in soils and sediments may provide a complimentary view of the relationship between reduction rate of Se-oxyanions and ε. The $\delta^{82/76}$Se values of different Se soil pools in agricultural seleniferous soils vary up to 13‰ (Schilling et al., 2015). The isotopically heavy adsorbed Se(IV) in the agricultural seleniferous soils suggest Se isotope fractionation by microbial reduction of Se(VI) in irrigation water prior to scavenging of the reaction product Se(IV) by reactive minerals in the soil. Therefore, our laboratory-derived ε values for microbial reduction are essential to quantitative determination of the extent of microbial reduction in the field. Nevertheless, site-specific ε values might still be obtained from experiments using the resident Se-reducing microbial community.

5.2. Ancient environments

The Se isotope signature preserved in rocks and sediments can be used to constrain the evolution of the biosphere and redox conditions in near surface environments through geological time (Wen and Carignan, 2011; Mitchell et al., 2012, 2016; Wen et al., 2014; Stüeken et al., 2015a,b; Kipp et al., 2017). However, interpreting these Se isotope signatures is still difficult as (1) bulk rock Se isotope data mask the variability in Se isotope ratio of various Se phases (*i.e.,* organically-bound, pyritic, adsorbed) in the rocks and (2) the local versus global controls affect the Se cycling due to the short oceanic residence time (10^3 years) of Se species and their low concentrations (<1 nM). Therefore, the application of Se isotopes as a paleoredox proxy relies on experimentally determined ε values for microbial reduction of Se-oxyanions. High-resolution isotope analyses with an analytical precision of 0.2‰ allows to resolve different reaction pathways and fingerprint Se sources preserved within the rock record.

Selenium isotope signature in bulk shales range between −1.5 and +2.2‰ over geological time (Wen and Carignan, 2011; Mitchell et al., 2012; Pogge von Strandmann et al.,

2015; Stüeken et al., 2015a,b; Mitchell et al., 2016). The sequestration of isotopically light Se has been reported for shale deposits (Wen and Carignan, 2011; Mitchell et al., 2012; Pogge von Strandmann et al., 2015; Stüeken et al., 2015a,b) suggesting the partial reduction of Se-oxyanions in suboxic basins. Our results suggest a lower ε during very rapid removal of Se(VI), the dominant Se species in the ocean (Conde and San Alaejos, 1997), during anoxia which is consistent with small variations in the Se isotope signature for black shales. However, to disentangle microbial reduction of Se-oxyanions from other reaction pathways (*e.g.*, adsorption and assimilation) it is necessary to extract phase-specific Se (*i.e.*, adsorbed, organic, pyritic) from rocks and sediments and determine their Se isotope compositions. It should be noted that muting of ε values are expected in semi-closed or open flow through systems (Shrimpton et al., 2018) like microbial Se reduction in porewater in ancient oceans compared to ε values typically observed during Rayleigh distillation model for closed systems.

In our study, we demonstrate that decreasing microbial activity results in smaller cSRR for Se(VI) reduction, which causes larger Se isotope fractionation and this could ultimately be reflected in lighter Se isotopic signature in sedimentary Se reservoirs. Despite uncertainties about the Se concentrations and its speciation in ancient oceans, our results imply a systematic relationship between the rate of Se(VI) reduction and the observed Se isotope fractionation which must be considered when interpreting $\delta^{82/76}$Se signatures. This relationship can also be of great importance because Se reduction rates may be derived from Se isotope signature preserved in the rock record. Thus, the Se isotope signatures recorded in ancient sediments should be re-examined given the influence of Se(VI) reduction rate on the magnitude of Se isotope fractionation.

We also note that future studies and experiments should be designed to constrain Se isotope fractionation at microbial Se reduction rates relevant to ancient marine conditions and reconstruct $\delta^{82/76}$Se ratios imprinted in the rock record. Additionally, phase-specific Se isotope analysis for rocks and sediments, and quantitative modeling approaches can help to provide further insight into the microbial Se cycling in the ancient ocean.

6. SUMMARY AND CONCLUSIONS

This study provides the first insights on the variation of Se isotope fractionation for non-respiring Se reduction by six different bacterial strains (*Geobacter sulfurreducens* PCA, *Anaeromyxobacter dehalogenans* FRC-W, *Shewanella* sp. (NR), *Enterobacter cloacae* SLDa1-1, *Desulfitobacterium chlororespirans* Co23 and *Desulfitobacterium sp.* Viet-1). We demonstrate that under more environmentally-relevant experimental conditions (<42 μM Se and 500 μM electron donor), Se isotope fractionation factors reveal a relatively narrow range for both Se(VI) and Se(IV) reduction with consistently larger Se isotope fractionation for Se(VI) ($\varepsilon_{mean} = -10.6 \pm 1.3$‰) than for Se(IV) reduction ($\varepsilon_{mean} = -7 \pm 0.6$‰). Based on the present and previous studies on microbial reduction of Se-oxyanions, we conclude that Se isotopic fractionation during microbial reduction is controlled by the co-metabolic reaction pathway(s).

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

ACKNOWLEDGEMENT

The authors wish to thank Linden Schneider for helping with the experiment. The authors also thank E. Stueken and one anonymous reviewer for their constructive comments. This study was financially supported by the Netherlands Organisation for Scientific Research grant number 820.02.007.

APPENDIX A. SUPPLEMENTARY MATERIAL

Supplementary data to this article can be found online at https://doi.org/10.1016/j.gca.2020.02.036.

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Associate editor: Claudine Stirling