1 Cr Isotope Fractionation Factors for Cr(VI) Reduction by a
2 Metabolically Diverse Group of Bacteria

4
5 Anirban Basu¹

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7 Thomas M. Johnson²

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9 Robert A. Sanford²

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11 Running Head: *Chromium Isotope fractionation*

12
13 ¹Department of Earth and Planetary Science, University of California, Berkeley,
14 Berkeley, CA, USA 94720

15 ²Department of Geology, University of Illinois at Urbana-Champaign, Urbana, IL, USA
16 61801

17
18
19 Corresponding Author:

20 Anirban Basu (anirbanbasu@berkeley.edu)

21 Department of Earth and Planetary Science

22 483 McCone Hall,

23 University of California, Berkeley

24 Berkeley, CA 94720

25 (510) 642-3993; FAX (510) 643-9980

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ABSTRACT

Reduction of Cr(VI) is an important process that determines the geochemical behavior, mobility and bioavailability of Cr in both terrestrial and marine environments. Many metabolically diverse microorganisms possess Cr(VI) reduction capacity. Cr(VI) reduction fractionates Cr isotopes and thus $^{53}\text{Cr}/^{52}\text{Cr}$ ratios can be used to monitor Cr(VI) reduction and redox conditions. The magnitude of isotopic fractionation (ϵ) for a variety of microbial reduction mechanisms must be known for accurate interpretation of observed shifts in $^{53}\text{Cr}/^{52}\text{Cr}$ ratios. We determined isotopic fractionation factors for Cr(VI) reduction by metal reducers *Geobacter sulfurreducens* and *Shewanella* sp. strain NR, a denitrifying soil bacterium *Pseudomonas stutzeri* DCP-Ps1, and a sulfate reducer *Desulfovibrio vulgaris*. All bacteria investigated in this study produced significant Cr isotope fractionation. The fractionation (ϵ) for *G. sulfurreducens*, *Shewanella* sp. (NR), *P. stutzeri* DCP-Ps1, and *D. vulgaris* were $-3.03\text{‰} \pm 0.12\text{‰}$, $-2.17\text{‰} \pm 0.22\text{‰}$, $-3.14\text{‰} \pm 0.13\text{‰}$, and $-3.01\text{‰} \pm 0.11\text{‰}$, respectively. Despite differences in microbial strains in this study, the ϵ did not vary significantly except for *Shewanella* sp. (NR). Our results suggest that strong isotopic fractionation is induced during Cr(VI) reduction under electron donor poor ($\sim 300\text{ }\mu\text{M}$) conditions.

1. INTRODUCTION

Redox cycling of chromium (Cr) is a critical process that controls its geochemical behavior in various settings including both ancient and modern terrestrial and marine systems (e.g., Berna et al., 2010; Frei et al., 2009). The oxidized species, Cr(VI) is a thermodynamically stable, soluble species at low temperature, circum-neutral pH conditions whereas the reduced trivalent species, Cr(III), is sparingly soluble and less toxic. The oxidation state of Cr determines the solubility, and therefore its mobility and toxicity in aqueous systems, and its geochemical cycling at various times in earth history.

Extensive industrial use of hexavalent chromium (Cr(VI)), a known carcinogen and mutagen (Losi et al, 1994; De Flora, 1999), has led to soil and groundwater Cr contamination in many parts of the world (Testa, 2004). Reduction of Cr(VI) to Cr(III) immobilizes Cr and bioremediation of contaminated sites offers promise in this regard. Enzymatic Cr(VI) reduction by numerous microbial species including facultative anaerobes (e.g., *E. coli*, *S. oneidensis* MR-1) (Myers et al., 2000; Bae et al., 2005), obligate anaerobes (e.g., *Desulfotomaculum reducens*) (Tebo and Obraztsova, 1998) and also aerobically grown bacteria (e.g., *Pseudomonas ambigua* G-1, *Pseudomonas putida*) (Ishibashi et al., 1990; Suzuki et al., 1992) was demonstrated in laboratory studies and successful bioremediation of Cr(VI) has been achieved in field-scale studies (Faybishenko et al., 2008; Hubbard et al., 2008). Although the electron transfer pathways for microbial Cr(VI) reduction are not fully understood, enzymatic Cr(VI) reduction may occur in the cytoplasm, periplasmic space, on the cell surface and also outside the cell, depending on the microbial species. Generally, the energy obtained from Cr(VI) reduction does not support microbial growth, with the exception of sulfate reducing

bacterium *Desulfotomaculum reducens* sp. nov. strain MI-1 (Tebo and Obraztsova, 1998).

The breakage of Cr–O bonds and production of unstable intermediate products Cr(V) and Cr(IV) during microbial Cr(VI) reduction (Suzuki et al., 1992; Myers et al., 2000; Kalabegishvili et al., 2003) lead to kinetic isotopic fractionation (Sikora et al., 2008). The lighter Cr isotopes (i.e., ^{52}Cr) are reduced at a faster rate leaving the remaining unreacted Cr(VI) enriched in the heavier isotopes (i.e., ^{53}Cr). Unlike Cr(VI) concentrations, $^{53}\text{Cr}/^{52}\text{Cr}$ ratios are only slightly affected by the processes like dilution, dispersion, and adsorption. Therefore, $^{53}\text{Cr}/^{52}\text{Cr}$ ratios are successfully employed to monitor Cr(VI) reduction in contaminated sites (Berna et al, 2009; Raddatz et al, 2010, Wanner et al., 2012 a, b). The magnitude of the isotopic fractionation is expressed by the observed overall isotopic fractionation factor, α :

$$\alpha = \frac{R_{\text{product}}}{R_{\text{reactant}}} \quad (1)$$

where R_{product} and R_{reactant} are the $^{53}\text{Cr}/^{52}\text{Cr}$ in the instantaneously formed reaction product and in the remaining reactant pool, respectively. The fractionation factor α is often represented by ε ($\varepsilon = (\alpha - 1)$), which is a more convenient way to express the size of the isotopic fractionation (see methods section). This parameter describes the relationship between the changes in $^{53}\text{Cr}/^{52}\text{Cr}$ ratios and the extent of reduction. Therefore, a precise determination of ε for microbial Cr(VI) reduction reactions is necessary for any quantitative interpretation of Cr isotope data from any setting where microbial action may reduce Cr(VI).

Although several genera of bacteria can reduce Cr(VI) to Cr(III) under aerobic and anaerobic conditions, isotopic fractionation of Cr associated with this microbial

process is not yet well understood. We are aware of only two published studies that have investigated Cr isotope fractionation during microbial Cr(VI) reduction. Sikora et al. (2008) reported strong fractionation ($\epsilon = -4.0$ to -4.5%) by anaerobically grown *Shewanella oneidensis* MR-1 in washed cell suspensions with 3–100 μM lactate added as an electron donor. The fractionation was variable, being significantly weaker ($\epsilon = -1.8\%$) in one experiment with 10.2 mM lactate. In a recent study on Cr(VI) reduction by aerobically grown *Pseudomonas stutzeri* strain RCH2, Han et al., (2012) have reported a weaker fractionation ($\epsilon \sim -2\%$) during reduction, and very little fractionation ($\epsilon \sim -0.4\%$) for reduction under denitrifying growth conditions. These studies suggest significant variations may exist between different Cr reducing bacteria and also between different reaction conditions for the same bacterium.

In this work, we experimentally determined isotopic fractionation by several well studied Cr(VI) reducers with a variety of reaction mechanisms. The ϵ values were determined for metal reducers *G. sulfurreducens*, *Shewanella* sp. (NR) and a denitrifying soil bacteria *Pseudomonas stutzeri* strain DCP-Ps1 in anaerobic batch incubations with up to 6 μM Cr(VI) as the sole electron acceptor and 300 μM electron donor. This electron donor concentration is lower compared to most of the previous experimental studies (e.g., Han et al., 2012) and is a better representation of environmentally relevant conditions. A second motivation for using this low electron donor concentration was to avoid muted isotopic fractionation as observed during microbial sulfate, nitrate, selenate, and chromate reduction under nutrient-rich conditions (e.g., Kaplan and Rittenberg, 1964; Chien et al., 1977; Herbel et al., 2000; Sikora et al., 2008). We determined the ϵ for a sulfate reducer *Desulfovibrio vulgaris* strain Hildenborough for Cr(VI) reduction in

continuously fed batch reactors with pyruvate supplied as an electron donor. Compared to the e^- donor concentrations, Cr(VI) concentrations were the limiting component of the redox couple in these experiments. Our main objectives were to determine i) the variability in ϵ during microbial Cr(VI) reduction with Cr(VI) as the sole electron acceptor ii) whether ϵ varies with the metabolic pathways (i.e. bacterial species) iii) whether reduction under electron donor-poor conditions ($\sim 300 \mu\text{M } e^-$ donor) generally yields large isotopic fractionation.

2. MATERIALS AND METHODS

2.1 Microbial cultures and medium

We obtained *G. sulfurreducens* strain PCA, *D. vulgaris* strain Hildenborough, and *P. stutzeri* strain DCP-Ps1 from one of the authors of this study (Sanford). *Shewanella* sp. strain NR, isolated from Neckar River sediment collected in Untertürkheim, Germany, was provided by Dr. Frank Löffler, University of Tennessee, Knoxville, TN. All cells were grown at 30°C in 160 mL glass serum bottles with a 100 mL working volume. The cultures of *G. sulfurreducens*, *Shewanella* sp. (NR) and *P. stutzeri* strain DCP-Ps1 were grown using an anaerobic mineral-salt medium described in He and Sanford (2002). The growth medium contained (per liter): 0.015 g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$, 0.02 g of $\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$, 0.007 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.005 g of Na_2SO_4 , 1 mL of trace metal solution, and 1 mL of selenium-tungsten solution. The trace metal solution contained (per liter): 5 g of $\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$, 0.5 g of H_3BO_3 , 0.5 g of ZnCl_2 , 0.5 g of $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$, 0.5 g of $\text{NiSO}_4 \cdot 6\text{H}_2\text{O}$, 0.3 g of $\text{CuCl}_2 \cdot 2\text{H}_2\text{O}$ and 0.1 g $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$. The selenium-tungsten solution contained (per liter): 0.003 g of NaSeO_4 and 0.008 g of Na_2WO_4 . The growth medium also contained 10 mM HCO_3^- , 2 mM NH_4Cl and the pH of the growth medium

was 7.2. In order to avoid abiotic Cr(VI) reduction by small amounts of growth medium carried over into experiments, we carefully reduced the amount of Na₂S to a final concentration of 50 µM in our medium for growing cultures. Any other reductant, including the resazurin solution was omitted from the medium. The medium was anaerobic, sterile and bicarbonate buffered (10 mM) with a N₂-CO₂ (80:20) headspace. The composition of the medium for Cr(VI) incubation experiments with *G. sulfurreducens*, *Shewanella* sp. (NR) and *P. stutzeri* strain DCP-Ps1 was identical with our growth medium with the exception of Na₂S concentration. Na₂S and all other reductants were completely excluded from the medium used for Cr(VI) incubations.

G. sulfurreducens growth cultures received 3 mM acetate as electron donor and 10 mM fumarate as electron acceptor. *Shewanella* sp. (NR) was grown on 2.5 mM lactate as electron donor and 1 mM NO₃⁻ as electron acceptor. *P. stutzeri* strain DCP-Ps1 cultures received 4 mM acetate as electron donor and 2 mM NO₃⁻ as electron acceptor. The stock cultures were grown to a cell density of about 10⁸ cells/mL which was diluted ten-fold into fresh media for Cr(VI) incubation experiments to obtain the final cell density (~10⁷ cells/mL) that was lower than the previous experimental studies (e.g., Han et al., 2012).

D. vulgaris were grown in batch cultures with a N₂-CO₂ (80:20) headspace using a sulfate-free mineral salt medium modified from He and Sanford (2002). The trace salt solution in the mineral salt medium described in He and Sanford (2002) was replaced with a sulfate-free trace salt solution containing 0.6 g/L CaCl₂, 1g/L MgCl₂. 6H₂O, and 0.5 g/L FeCl₂. 4H₂O. The sulfate-containing solution was omitted in order to avoid production of biogenic sulfide, which would readily reduce Cr(VI). All reductants,

including the resazurin solution were also excluded from the medium. The pH of the medium was 7.2. The same medium was used in Cr(VI) incubations with *D. vulgaris*. The cultures of *D. vulgaris* were grown on 5 mM pyruvate as a fermentable substrate and the stock culture was later diluted ten-fold for Cr(VI) incubation reactions.

2.2 Batch incubations with Cr(VI)

For anaerobic batch experiments with *G. sulfurreducens*, *Shewanella* sp. (NR), and *P. stutzeri* DCP-Ps1, we used glass serum bottle reactors with working volumes of 100 mL using the reductant-free mineral-salt medium described above. For each bacterium, the Cr(VI) reduction experiments included duplicate reactors with active cells and a no-cell abiotic control. The serum bottles were filled with 90 mL of reductant-free medium and inoculated with 10 mL of stock cultures (10% v/v). An aliquot of a degassed and sterile Na₂CrO₄ stock solution (0.96 mM) was injected in to the reactors to achieve a targeted initial Cr(VI) concentration (~5 µM). All reactors received ~300 µM of an appropriate electron donor (acetate or lactate). The control experiments for all reactors received the equal amount of Cr(VI) and the electron donor as the experiments with active cells. Throughout the course of the experiments, all reactors were shaken at 125 rpm, and incubated at 30°C in the dark to avoid potential photochemical reactions. All reactors were sampled at regular intervals using N₂-flushed syringes and the sampled volume was replaced by N₂. The samples were filtered with the 0.3 µm filter and stored at 4°C.

2.3 Continuously fed batch incubations with Cr(VI)

The Cr(VI) incubations with *D. vulgaris* were done using an experimental set up where each reactor was provided with a continuous supply of the electron donor as a

substrate. The purpose of this continuous pyruvate injection was to minimize the toxicity of Cr(VI) as pyruvate induces increased Cr tolerance in *D. vulgaris* (Klonowska et al., 2008). The design and the working principles of this experimental set up are described by He and Sanford (2004). We set up the continuously fed batch system as an array of five 160 mL glass serum bottle reactors, each attached to a supply assembly containing a 2.5 mL glass syringe, connected to a cannula through PEEK tubing. The cannula from each supply assembly was inserted into a reactor. The syringes were filled with the stock sodium pyruvate solution, which was delivered in to the reactors using a syringe pump. The reactor array contained two active cell experiments, one experiment with air-treated cells (see below), one experiment with heat-killed cells and one abiotic control experiment with no bacteria. We included a control experiment in which it was intended to deactivate the *D. vulgaris* cells by exposure to air. The aeration of cells was done by injecting 20 mL of air in to the 60 mL headspace of a bottle containing 100 mL of stock culture of *D. vulgaris*.

We filled the serum bottles with 80 mL of sulfate- and reductant-free medium. The reactors were then inoculated with 16 mL of stock cultures (20% v/v). Two control experiments received an identical volume of inoculum as the reactors with active cells. The cells in the heat-killed control were autoclaved for 30 min. Before the Cr(VI) injection, each reactor was sampled for cell density measurements using LSR II (BD Biosciences) flow cytometry analyzer. We injected an appropriate amount of the Cr(VI) stock solution into the reactors for the targeted initial Cr(VI) concentration (~10 μ M). All reactors, including the controls, received a 5 mM pyruvate solution as an electron donor at a volumetric rate of 0.078 mL/hr (approximately 100 μ M/d). The reactor array was

incubated in the dark at 30°C and shaken constantly at all times. Sampling protocol and the storage of the samples were similar to the batch incubations described above. The pyruvate concentrations in samples were measured using a Shimadzu Prominence high-pressure liquid chromatograph with a BioRad Aminex HPx-87H column.

2.4 Analytical methods

2.4.1 Biomass concentrations, Cr concentration and isotope measurements

Prior to Cr(VI) injections, we collected 1 mL samples of culture medium from each serum bottle for cell density measurements. A 0.33 mL aliquot of 8% formaldehyde solution was added to each sample to preserve the cells. 1 mL of each preserved sample was treated with 1 µL of SYTO BC Bacteria Stain and 10 µL of the microsphere standard suspension (Bacteria Counting Kit, Molecular Probes, Eugene, OR) prior to concentration measurement using a LSR II (BD Biosciences) flow cytometry analyzer. The final cell concentration was calculated from the number of counting events for a known number of the microspheres in each sample.

We determined preliminary Cr(VI) concentrations by a colorimetric method using a Thermo Genesys 20 visible light spectrophotometer using diphenyl carbazide assay according to U.S. Environmental Protection Agency method 7196A. We obtained more precise Cr(VI) concentrations from isotope dilution calculations from Cr isotope measurements. The isotopic measurements were carried out using a Nu Instruments HR multicollector inductively coupled plasma mass spectrometer (MC-ICP-MS) following a method described by Schoenberg et al. (2008). We used a double isotope spike technique to correct for isotopic fractionation during sample purification and mass spectrometry (Ellis et al., 2002; Johnson and Bullen, 2004; Schoenberg et al., 2008). We added an

aliquot of a solution containing two spike isotopes, ^{54}Cr and ^{50}Cr to each sample prior to sample purification. Dissolved Cr (VI) in samples was purified for mass spectrometry by passing the samples through an anion exchange resin (AG1-X8 resin) where Cr (VI) was retained while matrix components were rinsed out, then reduced to Cr (III) and eluted. The isobaric interferences from Fe, V and Ti were measured and corrected for. The external precision (2σ) of the isotopic measurements was $\pm 0.10\text{‰}$ calculated from root mean square of the differences from 20 pairs of duplicate sample preparations.

2.4.2 Interpretation of Cr isotope data

The $^{53}\text{Cr}/^{52}\text{Cr}$ ratio is expressed using δ notation, which is a per mil deviation from the standard reference material NIST SRM-979. δ is defined as:

$$\delta^{53}\text{Cr} = \left(\frac{(^{53}\text{Cr}/^{52}\text{Cr})_{\text{Sample}}}{(^{53}\text{Cr}/^{52}\text{Cr})_{\text{SRM-979}}} - 1 \right) \times 1000\text{‰} \quad (2)$$

In the Cr(VI) incubation experiments, the concentration of the electron donor far exceeded the concentration of Cr(VI). The Cr(VI) reduction in well-mixed batch reactors with no back reaction follows a Rayleigh process and the enrichment of ^{53}Cr in the remaining Cr(VI) pool can be described according to the following relationship

$$\delta(t) = (\delta_0 + 1000\text{‰}) \left(\frac{c(t)}{c_0} \right)^{\alpha-1} - 1000\text{‰} \quad (3)$$

where δ_0 and c_0 are the initial isotopic composition and Cr(VI) concentration, $\delta(t)$ and $c(t)$ correspond to the isotopic composition and Cr(VI) concentration at time t , and α is the isotopic fractionation factor. For the Cr(VI) batch incubations with *G. sulfurreducens*, *Shewanella* sp. (NR) and *P. stutzeri* strain DCP-Ps1, α was determined from the slope of the best fit line in the plots of $\ln(\delta(t) + 1000\text{‰})$ vs. $\ln(c(t))$, linearized from equation (3). For the continuously fed batch reactors, the α values were determined in a slightly

different way. The continuous supply of pyruvate into these reactors resulted in the dilution of the Cr(VI) concentration with time. For this reason, we calculated the α values from the slopes of linearized plots equivalent to $\ln \frac{c(t) + 1000\text{‰}}{c_0 + 1000\text{‰}}$ vs. $\ln \frac{c(t)}{c_0}$ plots described in Scott et al., (2004). However, in this case, $c(t)$ represents the measured Cr(VI) in the sample, and c_0 represents the expected Cr(VI) concentration arising from dilution only. This is analogous to the approach we used to analyze the isotope data from Cr(VI) batch incubations. We calculated the uncertainties (2 x standard error) in α from the uncertainties of the slopes calculated from the scatter of points relative to the best-fit line, via standard linear estimation methods. Expressing the isotopic fractionation in terms of ϵ is particularly useful since this quantity approximates the difference in the $\delta^{53}\text{Cr}$ values between the remaining Cr(VI) and the reaction product flux.

$$\epsilon \gg (d_{\text{product}} - d_{\text{reactant}}) \quad (4)$$

3. RESULTS

3.1 Cr(VI) removal in batch Incubations with 3 microbial strains

Cr(VI) removal occurred in the batch incubations with *G. sulfurreducens*, *Shewanella* sp. (NR), and *P. stutzeri* strain DCP-Ps1 (Fig. 1a, 1c, 1e, Table 1). We observed Cr(VI) concentration decrease with time in all reactors except in the abiotic control experiments. In the experiments with *G. sulfurreducens*, the Cr(VI) concentration decreased by about 50% in the first ~5 hr. The Cr(VI) removal during this first 5 hr follows first order kinetics in *G. sulfurreducens* Exp 1 whereas the later data points deviate from first order kinetics. In *G. sulfurreducens* Exp 2, the Cr(VI) concentration decrease with time fits a first order model ($t_{1/2} = 5.83 \text{ hr}^{-1}$). In both experiments with

Shewanella sp. (NR), progressive Cr(VI) removal follow a first order kinetics ($t_{1/2} = 1.16$ hr⁻¹ and 1.87 hr⁻¹). We observed a ~43% decrease in Cr(VI) with respect to the initial Cr(VI) concentration in 19 hr in *P. stutzeri* DCP-Ps1 Exp 1. The Cr(VI) removal data from this experiment do not fit a first order model with an invariant rate constant; the rate constant for the later data points is larger compared to that for the earlier data. Similar to *G. sulfurreducens*, Cr(VI) removal in *P. stutzeri* DCP-Ps1 Exp 2 fits a first order model ($t_{1/2} = 11.11$ hr⁻¹).

We observed a minor loss of Cr(VI) within the first minute of the experiments with *G. sulfurreducens* and *P. stutzeri* DCP-Ps1. The initial concentrations of Cr(VI) were not well constrained in these experiments due to uncertainties in the delivered amount of the Cr(VI). In the reactors with *Shewanella* sp. (NR), we observed a loss of ~35% of the initial Cr(VI) in the first minute of the experiments. After the first minute, the Cr(VI) reduction by *Shewanella* sp. (NR) was slow and took ~5 hours to reach near completion.

We calculated the cell-specific reduction rate for first ~ 3 hours of the experiment when the Cr(VI) removal rate was maximum in each batch incubation experiment. This approach is similar to that of Sikora et al. (2008) and Han et al. (2012). The cell-specific reduction rate, determined for each reactor, was defined as

$$\text{Cell-specific Reduction Rate} = \frac{\Delta c}{\Delta t \times d} \quad (5)$$

Where Δc is the Cr(VI) concentration decrease in the time interval Δt (i.e., 3.1 hr) and d is the density of the bacterial population in the reactor. The cell-specific reduction rates in Cr(VI) batch incubation experiments ranged from 179×10^{-18} mol cell⁻¹ d⁻¹ to 893×10^{-18}

mol cell⁻¹ d⁻¹. We observed significant variation in the cell-specific reaction rates between different bacteria and also between the duplicate reactors with the same bacteria.

3.2 Cr(VI) removal in continuously fed batch reactors with *D. vulgaris*

In duplicate reactors with active cells and in the reactor containing the air-treated cells, Cr(VI) concentrations decreased significantly with time (Fig. 1g, Table 2). During the course of the experiment, the continuous supply of pyruvate into each reactor caused substantial dilution. We accounted for the dilution by carefully measuring the volume of sample withdrawn and the volume of pyruvate injected, for each reactor. The measured decrease in the Cr(VI) concentration in the samples from the reactors in the continuously fed batch system is due to a combination of dilution and reduction. We represent our Cr(VI) concentration data as a ratio of measured Cr(VI) to the expected Cr(VI) due to dilution only (Cr(VI)_{measured}/Cr(VI)_{dilution}). This approach is similar to normalizing Cr(VI) concentrations by the initial Cr(VI), which represents the fraction of Cr(VI) reduction. In the absence of any Cr(VI) reduction, Cr(VI)_{measured}/Cr(VI)_{dilution} should always be equal to 1. The reduction of Cr(VI) is the only process that drives this ratio to a value less than 1. We observed a steady decrease in the Cr(VI)_{measured}/Cr(VI)_{dilution} ratio with time in the reactors with active and air-treated cells. A small decrease in Cr(VI) concentrations was observed in both control experiments. The cell-specific reduction rates were calculated for the first 2.4 days using equation 5. This time interval was chosen as the change in cell density due to dilution was negligible after 2.4 d. The cell-specific reduction rates low and varied from 80×10^{-18} mol cell⁻¹ d⁻¹ to 172×10^{-18} mol cell⁻¹ d⁻¹ in the reactors with active and air-treated cells (Table 3).

We observed an accumulation of pyruvate with time in the reactors. After an incubation time of 11 d, the pyruvate concentration was 1.81-2.39 mM in the reactors with active cells and in the reactor with the air-treated cells (Table 2). We did not observe any increase in the Cr(VI) reduction rate in these reactors even growth leading to increased active biomass was possible.

3.3 Cr isotope results

For every bacterium tested in this study, we observed significant isotopic fractionation associated with microbial Cr(VI) removal. The $\delta^{53}\text{Cr}$ values of the remaining Cr(VI) in the reactors progressively increased from the initial $\delta^{53}\text{Cr}$ (-0.02‰) as the reaction advanced (Fig 1b, 1d, 1f, 1h). The ϵ values for all experiments, determined by fitting to Rayleigh distillation models (equation 3) are given in Table 3. The uncertainties (2*s.e.) in ϵ ranged from $\pm 0.11\text{‰}$ to $\pm 0.22\text{‰}$. There were no significant differences between ϵ values obtained from duplicate reactors.

The magnitudes of isotopic fractionations for Cr(VI) removal by *G. sulfurreducens*, *P. stutzeri* DCP-Ps1, and *D. vulgaris* were fairly strong ($\epsilon \approx -3\text{‰}$). The ϵ values did not vary significantly between the bacteria except that *Shewanella* sp. (NR) was substantially lower ($\epsilon = -2.17\text{‰} \pm 0.22\text{‰}$) (Fig. 1d, Table 3). For experiments with *D. vulgaris*, a single ϵ fits the data from the reactors with the anaerobically-maintained and air-treated cells (Fig. 1h). In the continuously fed batch system, the ϵ values obtained for the Cr(VI) removal in the no-cell control and the heat-killed control experiments are $-2.2\text{‰} \pm 0.74\text{‰}$ and $-2.01\text{‰} \pm 0.75\text{‰}$. The ϵ did not change during the course of any experiment within our ability to resolve such changes, as the data fit single values of ϵ within analytical uncertainties. We did not observe any systematic relationship between

the cell-specific reduction rate and the magnitude of the isotopic fractionation in our microbial Cr(VI) incubation experiments. However, the weakest fractionation was observed in the experiments with the highest cell-specific reduction rate.

In the two *Shewanella* sp. experiments, the $\delta^{53}\text{Cr}$ values of the samples taken one minute after the Cr(VI) addition were significantly elevated ($\delta^{53}\text{Cr}_{\text{Exp1}} = 1.30\text{‰}$, $\delta^{53}\text{Cr}_{\text{Exp2}} = 1.11\text{‰}$) relative to the starting Cr(VI) solution ($\delta^{53}\text{Cr} = -0.02\text{‰}$). We do not have any data between the initial $\delta^{53}\text{Cr}$ value and these elevated $\delta^{53}\text{Cr}$ values. However, after the first minute, the $\delta^{53}\text{Cr}$ values increased smoothly in both reactors. A single ϵ fits the data from both the early rapid Cr(VI) removal within the first minute and the later data from *Shewanella* sp. experiments.

4. DISCUSSION

4.1 Reduction as Cr(VI) removal mechanism

We are confident that Cr removal in all our experiments is driven by Cr(VI) reduction by the bacteria. Although the reaction products were not characterized in this study, previous studies demonstrated Cr(VI) reduction to Cr(III) by the strains of *Geobacter* (Inoue et al., 2010; Chovanec et al., 2012), *Shewanella* (Middleton et al., 2013), *P. stutzeri* (Zawadzka et al., 2007; Han et al., 2010) and *D. vulgaris* (Goulhen et al., 2006). The presence of competing anions such as phosphate, carbonate, and sulfate (except for *D. vulgaris*) in the bacterial medium should inhibit Cr(VI) adsorption as a Cr removal mechanism (Stollenwerk and Grove, 1985; Zachara et al., 1987). Also, adsorption of Cr(VI) would reach equilibrium within minutes (Ellis et al., 2004) and does not explain slow decreases in Cr(VI) over several hours to days in our experiments. If indeed a small amount of non-reductive removal of Cr(VI) occurred, it would necessarily

have to be proportional to removal via reduction in order to produce a constant isotopic fractionation factor. For example if sorption occurred during the first part of an experiment, but reached equilibrium, then its effect on the fractionation factor would cease and thus an increase in fractionation would be observed. We did not observe any such increase in fractionation factors during the course of the experiments. Therefore, non-reductive Cr(VI) removal was negligible in our experiments.

4.2 Rapid early reduction and Cr(VI) removal in control experiments

A rapid Cr(VI) removal, accounting for ~35% of the initial Cr(VI), was observed in the duplicate reactors with *Shewanella* sp (NR) within the first minute since the start of the experiment. We attribute this Cr(VI) removal to rapid reduction within the first minute of the beginning of the experiment as suggested by significantly elevated $\delta^{53}\text{Cr}$ values after the first minute relative to the starting solution. Similarly, in one *G. sulfurreducens* experiment and in *P. stutzeri* DCP-Ps1 experiments, we observed elevated $\delta^{53}\text{Cr}$ for the time point after the first minute (Table 1). This rapid reduction may happen due to presence of large amount of activated enzymes in bacteria from the previous growth cycle. In our Rayleigh models, we considered the elevated $\delta^{53}\text{Cr}$ measured for the second sampling points to be the effective starting points for our experiments. Accordingly, we normalized the remaining Cr(VI) fractions to the Cr(VI) concentrations corresponding to the elevated $\delta^{53}\text{Cr}$ values. We are confident that from this time onward, our isotopic data represent the magnitude of isotopic fractionation by *Shewanella* sp. (NR) accurately. The early, rapid reduction is thus not considered further.

The experiment with the air-treated *D. vulgaris* cells was initially designed as a control because these bacteria would theoretically be deactivated by oxidative stress (e.g.,

Wildschut et al., 2006). Significant Cr(VI) reduction (59%) in this reactor suggests that the amount of oxygen injected into the headspace was not enough to deactivate the cells. Furthermore, *D. vulgaris* is capable of surviving oxidative stress due to short oxygen exposure using a mechanism involving periplasmic enzymes (Fournier et al., 2004; Lamrabet et al., 2011). Due to the observed significant Cr(VI) reduction activity and concomitant isotopic fractionation comparable to that seen with the active anaerobically-maintained cells, we treat the air-treated reactor as a third, effectively identical experiment with active cells of *D. vulgaris*.

We observed a small Cr(VI) concentration decrease with time in the control experiments for the continuously fed batch system. In the heat-killed and no-cell control reactors, the Cr(VI) loss, with dilution effects considered, was 25% and 22% of the initial Cr(VI) concentrations, respectively. This is a matter of concern since any Cr(VI) reduction in the no-cell control implies reduction by the medium itself. The elevated final $\delta^{53}\text{Cr}$ values in the heat-killed ($\delta^{53}\text{Cr} = 0.85\text{‰}$) and no-cell control ($\delta^{53}\text{Cr} = 0.64\text{‰}$) experiments confirmed a small amount of reduction in these reactors. This reduction possibly originated from the slow chemical breakdown of pyruvate. All reactors including the controls received a continuous supply of 5 mM sodium pyruvate solution as electron donor at all times during the 29 d incubation period. The pyruvate solution is known to be unstable at temperatures higher than -20°C at neutral pH and can slowly decarboxylate to form acetic acid (Margolis and Coxon, 1986). The slow degradation of sodium pyruvate solution at 30°C incubation temperature might have generated enough reduction capacity to cause a small decrease of the initial Cr(VI) in the no-cell control experiment. An additional minor concentration decrease in the heat-killed control is

expected to occur from the reductant released from the killed cells. This is similar to Cr(VI) reduction from endogenous decay of the *S. oneidensis* MR-1 cells reported by Sikora et al. (2008). However, Cr(VI) reduction in triplicate active reactors with live *D. vulgaris* cells is much larger (41%-77%) and the highest $\delta^{53}\text{Cr}$ value from these reactors is about 80% larger than $\delta^{53}\text{Cr}$ of the heat-killed and no-cell controls. This leads us to believe that live *D. vulgaris* cells, as opposed to the medium and the lysed cells, actively mediated most of the Cr(VI) reduction and associated isotopic fractionation in triplicate reactors.

Within the uncertainty of our calculations, the ϵ values for the abiotic Cr(VI) reduction in the control experiments in the continuously fed batch system are similar to that obtained from the experiments with live cells. This suggests that abiotic reduction causes little error in our determination of the ϵ for microbial Cr(VI) reduction by *D. vulgaris*.

4.3 Microbial Cr(VI) reduction mechanisms

Mechanisms of Cr(VI) reduction vary depending on the microbial species, and this may have an influence on the Cr isotope fractionation. Our results can be better understood in light of what is known about reduction mechanisms in different bacteria. Differing reduction mechanisms for Cr(VI) reduction have been identified for *G. sulfurreducens*, *S. oneidensis* MR-1, *P. stutzeri*, and *D. vulgaris*. *G. Sulfurreducens* couples the oxidation of acetate with Cr(VI) reduction (Lloyd, 2006), although this reduction does not support growth of *G. sulfurreducens*. Previous studies have demonstrated the active role of an outer membrane cytochrome OmcZ (Inoue et al., 2010) and a periplasmic cytochrome (Lloyd, 2003) in Cr(VI) reduction by *G.*

446 *sulfurreducens*. The Cr(VI) reduction activity in *Shewanella oneidensis* MR-1 is co-
447 metabolic and involves both intracellular and extracellular mechanisms (Meyer et al.,
448 2000; Viamajala et al., 2002; Middleton et al., 2003). However, Cr(VI) as the sole
449 terminal electron acceptor does not support growth of *Shewanella oneidensis* MR-1
450 (Middleton et al., 2003). The Cr(VI) reduction mechanism by *Shewanella* sp. (NR) is not
451 yet characterized, but we surmise that multiple extra- and intra-cellular enzymes likely
452 play an active role in Cr(VI) reduction by this strain.

453 Several strains of *Pseudomonas stutzeri* species are capable of Cr(VI) reduction,
454 but the reduction mechanisms vary significantly. For example, *P. stutzeri* strain KC can
455 reduce Cr(VI) secreting a soluble siderophore (Zawadzka et al., 2007) whereas Cr(VI)
456 reduction by *P. stutzeri* strain RCH2 is cometabolic both under aerobic and denitrifying
457 conditions (Han et al., 2010). The exact mechanism of Cr(VI) reduction by *P. stutzeri*
458 DCP-Ps1 is not known. However, extracellular Cr(III) precipitates, with some occasional
459 intracellular deposits, are observed during anaerobic Cr(VI) reduction by two strains of *P.*
460 *stutzeri* (Badar et al., 2000) suggesting multiple Cr(VI) reduction mechanisms may exist
461 in *P. stutzeri*. We are not aware of any study demonstrating growth of *P. stutzeri* on
462 Cr(VI) as the sole electron acceptor.

463 Cr(VI) reduction by *Desulfovibrio* genera also involves multiheme cytochromes
464 coupled to the use of several electron donors (e.g. lactate, formate, or pyruvate). In *D.*
465 *vulgaris*, Cr(VI) reductase activity is associated with membrane-bound cytochrome c3
466 and hydrogenase (Lovley and Phillips, 1994; Michel et al., 2001; Chardin et al., 2003).
467 Cr(III) products are observed in the periplasmic space and associated with the cell
468 membranes (Goulhen et al., 2006). Cr(VI) as the sole electron acceptor does not support

growth of *D. vulgaris* (Michel et al., 2001; Chardin et al., 2002; Goulhen et al., 2006). Pyruvate has also been suggested to possibly induce increased Cr tolerance and protect the cells from Cr toxicity (Klonowska et al., 2008).

As the isotopic fractionation during microbial Cr reduction is related to reconfiguration of Cr–O bonds, and the expression of this kinetic isotope effect depends on the interplay of Cr(VI) reduction and the transport of Cr(VI) to the sites of reduction (see below), variation in reduction mechanism and location of enzymatic activities might have the potential to influence the magnitude of Cr(VI) isotopic fractionation.

4.4 General systematics of isotopic fractionation for microbial processes

The reduction of Cr(VI) to Cr(III) requires transfer of three electrons, and the reaction progresses in a stepwise fashion via unstable intermediates, Cr(V) and Cr(IV). Analogous to microbial sulfate or nitrate reduction, both the kinetics of individual steps and the reaction mechanism control the magnitude of the isotopic fractionation. Based on previous studies on microbial S isotope fractionation (e.g. Rees, 1973; Canfield, 2001; Brunner and Bernasconi, 2005, Johnston et al., 2007) and C and H isotope fractionation during photosynthesis (e.g. Hayes 2001), a conceptual model has been developed to explain Cr and Se isotopic fractionation during microbial Cr(VI) and Se(VI) reduction (Sikora et al., 2008; Johnson and Bullen, 2004; Johnson, 2011). Here we briefly summarize the important points of this model: 1) The magnitude of isotopic fractionation for a reaction chain comprised of multiple steps is the sum of the fractionations for all intermediate steps up to and including the rate-limiting step. 2) The steps after the rate-limiting step do not contribute to the overall isotopic fractionation even if these

individual steps may generate some isotopic fractionation. This general approach is helpful in understanding the isotopic fractionation during microbial Cr(VI) reduction.

According to this model, reduction kinetics, location of the reduction reaction, and the manner of electron transfer from the enzymes may play important roles. For extracellular Cr(VI) reduction occurring on the outer-membrane of a cell, Cr(VI) receives one or multiple electrons at a time from the terminal part of the electron transport chain of the cell. This causes preferential reduction of $^{52}\text{Cr(VI)}$ leading to an enrichment of ^{53}Cr in the remaining Cr(VI) at the sites of reduction. In a well-mixed system, the resulting isotopically fractionated Cr(VI) freely mixes with the bulk Cr(VI) pool and thus the kinetic isotope effect should be fully expressed. It is not limited by diffusion/transport of the fractionated Cr(VI). The overall isotopic fractionation, therefore, is expected to be relatively large and independent of reduction rate when reduction is extracellular.

In contrast, isotopically fractionated Cr(VI) generated by intracellular Cr(VI) reduction must leak out and mix with the bulk Cr(VI) pool outside the cell for a kinetic isotope effect to be expressed in the bulk Cr(VI) pool. In this case, the degree of leakage from the cells limits the chemical communication between the intracellular fractionated Cr(VI) pool and the bulk Cr(VI) pool outside the cell. The rate of intracellular Cr(VI) reduction relative to the rate of diffusion or active transport of Cr(VI) becomes important in determining the magnitude of isotopic fractionation. A strong isotopic fractionation is produced during electron transfer steps that reconfigure Cr bonds. Therefore, if enzymatic Cr(VI) reduction steps are rate-limiting, relatively strong isotopic fractionation is expected. On the other hand, if diffusion or transport of Cr(VI) into the cells is the rate-

limiting step, an overall weaker isotopic fractionation results, as transport does not involve significant bonding change or isotopic fractionation.

4.5 Variability in ϵ among bacterial strains

Using the general framework described above, we discuss the results from our microbial Cr(VI) reduction experiments in comparison with previous microbial Cr isotope studies in order to understand the systematics of the isotopic fractionation during microbial Cr(VI) reduction.

Previous studies reported Cr isotope fractionation during microbial Cr(VI) reduction. The ϵ for Cr(VI) reduction by *S. oneidensis* MR1 was $-4.2 \pm 0.2\text{‰}$ under electron donor-poor ($< 100 \mu\text{M}$ lactate) conditions (Sikora et al., 2008). The cell specific reduction rate observed within the first 24 hr in these experiments ranged from $37 \times 10^{-18} \text{ mol cell}^{-1} \text{ d}^{-1}$ to $92 \times 10^{-18} \text{ mol cell}^{-1} \text{ d}^{-1}$. In one donor-rich (10.2 mM lactate) experiment with a much higher cell-specific reduction rate ($450 \times 10^{-18} \text{ mol cell}^{-1} \text{ d}^{-1}$ in the first 24 hr), the fractionation was much weaker ($\epsilon = 1.8\text{‰}$). These results imply a dependence of isotopic fractionation on the cell-specific Cr(VI) reduction rate. Sikora et al. (2008) suggested that, analogous to models of S isotope fractionation during sulfate reduction, the fractionation observed for MR-1 in the low-donor, low-rate experiments ($\epsilon = -4.2\text{‰}$) was probably the highest possible fractionation for Cr(VI) reduction by the MR-1 strain. Conversely, they suggested weaker fractionation should be expected for more favorable reducing conditions like those of the high-donor experiment.

Han et al. (2012) also observed variation in the magnitude of Cr isotope fractionation during Cr(VI) reduction. The ϵ for *P. stutzeri* RCH2 was $-2.0 \pm 0.4\text{‰}$ during aerobic Cr(VI) reduction and $-0.4 \pm 0.2\text{‰}$ during Cr(VI) reduction under

denitrifying conditions. The cell-specific Cr(VI) reduction rates under aerobic and denitrifying conditions were 3.5×10^{-19} mol cell⁻¹ d⁻¹ and 1.8×10^{-19} mol cell⁻¹ d⁻¹, respectively. Han et al. (2012) suggested that transport of Cr(VI) into the cell was the rate-limiting step for Cr(VI) reduction under denitrifying conditions leading to minimal isotopic fractionation.

Our results show that *G. sulfurreducens* and *P. stutzeri* strain DCP-Ps1, induced identical isotopic fractionation ($\epsilon \approx -3.0$ ‰) during anaerobic Cr(VI) reduction under similar electron donor concentrations (~300 μM acetate). Despite slightly different reaction conditions in the continuously fed batch system, the fractionation produced by *D. vulgaris* ($\epsilon = -3.01$ ‰) also matches this value. This similarity is remarkable despite major differences in microbial metabolisms. The $\epsilon \approx -3.0$ ‰ is considerably weaker than the fractionation ($\epsilon = -4.2$ ‰) observed so far for *S. oneidensis* MR-1. The cell-specific reduction rates for *G. sulfurreducens* and *P. stutzeri* DCP-Ps1 experiments were much higher than that for MR-1 with ~100 μM lactate. However, the difference in the cell-specific reduction rate alone cannot explain our data. We observed a difference of a factor of 1.5 in cell-specific reduction rate in the duplicate reactors with *G. sulfurreducens*, but a single ϵ fit all data from duplicate reactors. Similarly, there was no significant variation in fractionations between *G. sulfurreducens* and *P. stutzeri* DCP-Ps1, although the cell-specific reduction rate in these reactors varied by about a factor of 2. Furthermore, two experiments with *D. vulgaris* yielding almost identical fractionation ($\epsilon = -3.01$ ‰) occurred at cell-specific reduction rates ($\sim 80 \times 10^{-18}$ mol cell⁻¹ d⁻¹) similar to those in the donor-poor experiments with MR-1. The reason for the similar magnitude of isotopic fractionation for metabolically diverse organisms is not quite clear. We suggest

that fairly large fractionations observed for *G. sulfurreducens*, *P. stutzeri* DCP-Ps1, and *D. vulgaris* may have resulted from predominantly extracellular Cr(VI) reduction and are probably characteristic of the microbial species studied here under donor-poor conditions.

The fractionation for *Shewanella* sp. (NR) is significantly weaker ($\epsilon = -2.2\text{‰}$) compared to other bacteria studied here and that reported for *S. oneidensis* MR-1 ($\epsilon = -4.2\text{‰}$) under donor-poor conditions. Interestingly, ϵ for *Shewanella* sp. (NR) is similar to the fractionation ($\epsilon = -1.8\text{‰}$) observed for MR-1 in the rapid, donor-rich experiment. This similarity in fractionations may arise from comparable large cell-specific reduction rates in these experiments. The average cell-specific reduction rate for *Shewanella* sp. (NR) experiments was $\sim 783 \times 10^{-18} \text{ mol cell}^{-1} \text{ d}^{-1}$ whereas the same reported for the donor-rich experiment with MR-1 was $450 \times 10^{-18} \text{ mol cell}^{-1} \text{ d}^{-1}$ in the first 24 h of the incubation time. Alternatively, the difference between the high fractionation ($\epsilon = -4.2\text{‰}$) expressed by MR-1 and our observed weaker ϵ for *Shewanella* sp. (NR) may be due to differences in the reduction mechanism (e.g., manner of electron transfer).

Our results show that during Cr(VI) reduction under anaerobic conditions with Cr(VI) as the sole electron acceptor, *P. stutzeri* DCP-Ps1 produced a high isotopic fractionation ($\epsilon = -3.14\text{‰}$). This is in contrast to *P. stutzeri* RCH2, which yielded a much weaker isotopic fractionation ($\epsilon = -2\text{‰}$) for aerobic Cr(VI) reduction and a minimal isotopic fractionation ($\epsilon = -0.4\text{‰}$) during cometabolic Cr(VI) reduction under denitrifying conditions. The cell-specific reduction rate reported for the experiments with *P. stutzeri* RCH2 were much lower compared to our strain DCP-Ps-1 experiments. Further research is necessary to determine whether this difference in isotope fractionation arises due to differences in bacterial physiology or reduction mechanism.

The concentration of electron donor did not have a significant effect on Cr(VI) reduction by *D. vulgaris*. We observed a steady accumulation of pyruvate reaching a concentration of about 2 mM after 11 d of incubation in *D. vulgaris* reactors. We did not measure the final pyruvate concentration or the increase in cell density in these reactors. However, given the accumulation rate of pyruvate observed in the first 11 days, the projected pyruvate concentration at the end of the 29 d incubation should be about 7.5 mM. The high concentration of electron donor (> 2 mM) had little effect on the isotopic fractionation by *D. vulgaris*. A possible explanation for the observed relation between electron donor and Cr isotopic fractionation is this: Since *D. vulgaris* cultures were grown using pyruvate as a fermenting substrate, it is likely that in our experiments, cells maintained physiological activity and probably a small amount of growth by fermenting pyruvate at high pyruvate concentrations and this pyruvate consumption was not directly coupled with Cr(VI) reduction. Therefore slow pyruvate fermentation provided reducing power required for Cr(VI) reduction which was only a minor fraction of total reducing equivalent of the accumulated pyruvate. The resulting Cr(VI) reduction rate as well as the Cr isotope fractionation for *D. vulgaris* was thus virtually independent of the pyruvate concentration. We cannot evaluate the effect of electron donor concentrations for *G. sulfurreducens*, *P. stutzeri*, and *Shewanella* sp. (NR) since the electron donor concentrations in these experiments were similar (~ 300 μ M).

5. CONCLUSIONS

The results from our experiments demonstrate significant isotopic fractionation during Cr(VI) reduction by all bacteria investigated in this study. The magnitude of isotopic fractionation for Cr(VI) reduction by the metal reducer *G. sulfurreducens*,

denitrifier *P. stutzeri* DCP-Ps1 and sulfate reducer *D. vulgaris* was $\sim -3.0\text{‰}$. These organisms produced similar fractionation at varying cell-specific reduction rate. The reason for this similarity in ϵ is not clear and may have resulted from a predominantly extracellular Cr(VI) reduction characteristic to the microbial species.

The ϵ yielded by *Shewanella* sp. (NR) was significantly weaker (-2.17‰) but comparable to $\epsilon = -1.8\text{‰}$ reported for *S. oneidensis* MR-1 under donor-rich conditions that induced rapid reduction. We attribute this weaker fractionation to a high cell-specific reduction rate for *Shewanella* sp. (NR). In contrast to the results reported by Han et al. (2012), *P. stutzeri* DCP-Ps1 produced a strong isotopic fractionation of -3.14‰ during incubations with Cr(VI) as the sole electron acceptor. This suggests that under radically different reaction conditions (e.g. presence of other electron acceptors), similar strains probably employ different reduction and/or transport mechanisms.

The accumulation of the supplied electron donor (i.e., pyruvate) in our continuously fed batch experiments did not impact the Cr(VI) reduction rate or the magnitude of Cr isotope fractionation. We suggest a decoupling of pyruvate oxidation and Cr(VI) reduction in our experiments. However, for other experiments in this study, electron-donor poor conditions generally yielded strong isotopic fractionations.

These results are instrumental for interpretation of Cr isotope data from Cr(VI) bioremediation sites for detection and possible quantification of bioremediation. In addition, the results will also be applicable to any study on modern or palaeo-environments (e.g., ancient oceans, lakes) involving microbial Cr(VI) reduction.

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Table 1

Table 1. Results from Cr(VI) batch incubations with *G. sulfurreducens*, *Shewanella* sp. (NR), e) *P. stutzeri* DCP-Ps1

G. sulfurreducens Exp 1					
Time (hr)	Cr(VI) (μM)	Electron Donor	% Reduced	δ ⁵³ Cr(‰)	Half life of Cr(VI)
0	5.22	Acetate	0%	-0.02‰	
0.6	4.93	(300 μM)	6%	0.31‰	
1.6	4.11		21%	0.79‰	
3.1	3.27		37%	1.53‰	
4.7	2.73		48%	2.14‰	
8.1	2.17		58%	2.79‰	
18.9	1.06		80%	4.85‰	
8.1 Duplicate				2.84‰	
G. sulfurreducens Exp 2					
0	5.97	Acetate	0%	0.44‰	5.83 hr
0.6	5.56	(300 μM)	7%	0.63‰	
1.6	4.91		18%	1.03‰	
3.1	4.08		32%	1.54‰	
4.7	3.36		44%	2.14‰	
8.1	2.29		62%	3.35‰	
4.7 duplicate				2.12‰	
Shewanella sp. (NR) Exp 1					
0	3.28	Lactate	0%	1.30‰	1.16 hr
0.6	2.36	(300 μM)	28%	2.15‰	
1.6	1.33		60%	3.29‰	
3.1	0.51		84%	5.30‰	
Shewanella sp. (NR) Exp 2					
0	3.86	Lactate	0%	1.11‰	1.87 hr
1.6	1.77	(300 μM)	54%	3.09‰	
3.1	1.02		74%	4.30‰	
4.7	0.68		82%	4.94‰	
P. stutzeri DCP-Ps1 Exp 1					
0	6.04	Acetate	0%	0.12‰	
1.6	5.58	(300 μM)	8%	0.34‰	
3.1	5.21		14%	0.46‰	
4.7	4.78		21%	0.91‰	
8.1	4.23		30%	1.16‰	
18.9	3.45		43%	1.92‰	
3.1 duplicate				0.59‰	
P. stutzeri DCP-Ps1 Exp 2					
0	5.75	Acetate	0%	0.18‰	11.11 hr
0.6	5.62	(300 μM)	2%	0.19‰	

1.6	5.39	6%	0.33‰
3.1	5.01	13%	0.61‰
4.7	4.60	20%	0.86‰
8.1	3.92	32%	1.41‰
18.9	1.77	69%	3.86‰
8.1 duplicate			1.49‰

Table 2. Cr(VI) concentration, pyruvate concentration and Cr isotope results from continuously fed batch reactors with *D. vulgaris*.

Cr(VI) _{measured} (μM)					
t (d)	Exp 1	Exp 2	Exp 3 (air-treated)	Heat-killed Control	No Cell Control
0.0	9.69	9.49	9.95	9.65	9.83
1.2	7.89	7.50	8.51	9.29	9.73
2.4	7.66	7.11	8.06	9.09	9.66
4.0	7.00	6.22	7.11	8.14	8.78
7.2	6.17	5.10	6.19	7.35	7.84
10.9	5.74	4.08	5.50	6.83	7.81
18.3	4.85	2.79	4.25	6.25	6.42
22.2	4.22	2.03	3.37	4.91	5.12
29.0	3.64	1.41	2.60	4.23	4.59
Cr(VI) _{dilution} (μM)					
0.0	9.69	9.49	9.95	9.65	9.83
1.2	9.46	9.26	9.71	9.42	9.59
2.4	9.25	9.06	9.50	9.21	9.38
4.0	8.99	8.80	9.23	8.95	9.12
7.2	8.64	8.46	8.87	8.60	8.76
10.9	8.18	8.01	8.40	8.15	8.30
18.3	7.25	7.10	7.44	7.22	7.35
22.2	6.81	6.67	6.99	6.78	6.91
29.0	6.18	6.05	6.34	6.15	6.27
δ ⁵³ Cr(‰)					
0.0	-0.05‰	-0.04‰	-0.09‰	-0.15‰	0.03‰
1.2	0.61‰	0.67‰	0.43‰	0.09‰	-0.11‰
2.4		0.70‰	0.59‰	0.12‰	-0.11‰
4.0	0.81‰	1.14‰	0.77‰	0.17‰	0.09‰
7.2		1.64‰	1.11‰	0.51‰	-0.05‰
10.9	1.15‰	2.04‰	1.28‰	0.44‰	0.18‰
18.3	1.29‰	3.00‰	1.81‰	0.60‰	0.47‰
22.2	1.52‰	3.54‰	2.07‰	0.63‰	0.52‰
29.0	1.54‰	4.55‰	2.41‰	0.85‰	0.64‰
Electron Donor: Pyruvate (mM)					
0.0	0.15	0.10	0.14	0.13	0.00
4.0	0.38	0.38	0.39	0.39	0.51
7.2	1.17	1.27	1.21	1.26	1.18
10.9	1.81	2.39	2.37		

Table 3

Table 3. Isotopic Fractionations for *G. sulfurreducens*, *Shewanella* sp. (NR), *P. stutzeri* DCP-Ps1, and *D. vulgaris*.

Bacterial strain	Experiment 1				Experiment 2				Combined	
	ϵ (‰)	2 s.e. (‰)	Cell density $\times 10^7$ (mL ⁻¹)	Cell-specific reduction rate $\times 10^{-18}$ mol cell ⁻¹ d ⁻¹)	ϵ (‰)	2 s.e. (‰)	Cell density $\times 10^7$ (mL ⁻¹)	Cell-specific reduction rate $\times 10^{-18}$ mol cell ⁻¹ d ⁻¹)	ϵ (‰)	2 s.e. (‰)
<i>G. sulfurreducens</i>	-3.02	0.12	8.42	179	-3.03	0.07	5.37	274	-3.03	0.12
<i>Shewanella</i> sp. (NR)	-2.12	0.11	3.19	674	-2.22	0.27	2.76	893	-2.17	0.22
<i>P. stutzeri</i> DCP-Ps1	-3.23	0.34	2.03	317	-3.15	0.06	1.65	348	-3.14	0.13
<i>D. vulgaris</i>	-3.08	0.29	0.83	84	-3.08	0.13	0.49	172	-3.01*	0.11
<i>D. vulgaris: Exp 3 (aerated)</i>	-2.78	0.27	0.79	80						

* 3 experiments combined

FIGURE CAPTION

Fig. 1. The left panel shows the Cr(VI) reduction by a) *G. sulfurreducens* c) *Shewanella* sp. (NR), e) *P. stutzeri* DCP-Ps1, g) *D. vulgaris*. Note that the *D. vulgaris* x-axis is in days. The right panel shows concomitant increase in $\delta^{53}\text{Cr}$ plotted against fraction of remaining Cr(VI) during reduction for b) *G. sulfurreducens* d) *Shewanella* sp. (NR), f) *P. stutzeri* DCP-Ps1, h) *D. vulgaris*. The error bars for $\delta^{53}\text{Cr}$ are smaller than the symbols. The best fit Rayleigh models are shown by the dotted lines.

Figure 1