

# Variations of stable isotope fractionation during bacterial chromium reduction processes and their implications

Qiong Zhang<sup>a</sup>, Ken Amor<sup>a</sup>, Stephen J.G. Galer<sup>c</sup>, Ian Thompson<sup>b</sup>, Don Porcelli<sup>a</sup>

<sup>a</sup>*Department of Earth Sciences, University of Oxford, Oxford, United Kingdom*

<sup>b</sup>*Department of Engineering Science, Univeristy of Oxford, Oxford, United Kingdom*

<sup>c</sup>*Max Planck Institute for Chemistry, Climate Geochemistry Department, Mainz, Germany*

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## Abstract

Many chemical processes generate subtle but readily measured changes in isotope compositions of elements across the periodic table. The elements involved therefore carry diagnostic information about their chemical histories in complex geochemical or biochemical environments. Distinctive Cr isotope signatures can be used to identify immobilization processes of Cr in the environment, such as microbial Cr(VI) reduction, abiotic Cr(VI) reduction, and adsorption. Here we demonstrate that under well-controlled conditions, Cr isotopes can also be used to distinguish between different biological Cr(VI) reduction pathways. The reduction of Cr(VI) by two facultative anaerobic bacteria, *Pseudomonas fluorescens* LB 300 and *Shewanella oneidensis* MR 1, was investigated to determine the conditions under which Cr(VI) is reduced and to quantify the corresponding isotope signatures. The present study considers the effects of a broad range of parameters on Cr isotope fractionation, including bacterial species, electron donors, pH, and respiration pathways (aerobic vs. anaerobic) that must be considered for understanding Cr isotope variations under different experimental and environmental conditions.

In the bacterial Cr(VI) reduction experiments, the  $^{53}\text{Cr}/^{52}\text{Cr}$  isotope ratio of the remaining Cr(VI) increased by up to + 8‰, indicating that lighter isotopes of Cr were preferentially reduced. In aerobic experiments, although Cr reduction rates increased as pH increased from 4 to 8, the fractionation factor did not vary significantly ( $\varepsilon = -3.21 \pm 0.18\text{‰}$ ). Experiments using different electron donors demonstrated that citrate promoted the greatest Cr reduction rate compared with glucose, acetate, and propionate. Under aerobic conditions, although the Cr(VI) reduction rates varied substantially between different experimental settings, the isotope fractionation factors were indistinguishable between all the environmental conditions examined ( $\varepsilon = -3.1\text{‰}$ ), with the

exception of when citrate was the electron donor ( $\varepsilon = -4.3 \text{ ‰}$ ).

Cr reduction rates were generally much faster under anaerobic conditions for both bacteria investigated. The utilisation of different electron donors resulted in the same Cr reduction rates by the bacteria, but fractionated Cr with a broad range of isotope fractionation factors, from  $-1.58 \pm 0.16 \text{ ‰}$  to  $-4.93 \pm 0.36 \text{ ‰}$ . Although it has been proposed in many previous studies that there is an inverse relationship between reduction rates and the fractionation factors, no clear relationship between the reduction rates and fractionation factors was observed in this study.

The Cr isotope fractionation factors  $\varepsilon$  were insensitive to pH and electron donor concentration, but dependent on the type of electron donors and redox conditions in the cultures. This indicates that isotope variations may be used to identify when different biological pathways are involved, and so to investigate metabolic processes. The  $\varepsilon$  value from all experimental conditions examined ranged between  $-1.58$  to  $-4.93 \text{ ‰}$ , with a mean value at  $-3.3 \text{ ‰}$ . While Cr isotopes might be used to separate the effects of abiotic and microbially mediated reduction in environmental sites, the fractionation factors from reduction by individual bacterial species overlap with those from several individual abiotic reduction processes, suggesting that site-specific data (*e.g.*, fractionation factors associated with indigenous bacterial populations and local groundwater chemistry) are required in order to use Cr isotopes to distinguish between different reduction mechanisms.

*Keywords:*

Cr, bacterial reduction, biogeochemistry, stable isotope fractionation

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## 1. Introduction

Isotope compositions of many elements change during chemical reactions and therefore these compositions carry diagnostic information about the chemical histories of the elements in complex geochemical or biochemical environments. Techniques for chemical separation and high precision mass spectrometry can now achieve the necessary resolution for resolving the range of naturally occurring isotope ratios, typically in a few micrograms or less of the element of interest. However, the isotope fractionation factors for the different relevant processes must be documented. Within the last decade, variations in chromium isotope ratios have been documented and can be used to

understand the complex processes affecting the behaviour of Cr in the environment. This provides a powerful diagnostic tool for studies of environmental contamination, of the chemical cycles of redox-sensitive elements, and of paleoclimate. In particular, microbial processes reduce Cr(VI) using a range of mechanisms that are associated with different fractionation factors, indicating that Cr isotopes can be used not only to identify different microbial processes in the environment, but also to explore how microbes reduce Cr. These mechanisms are dependent on the environmental conditions under which the reduction processes occur. Therefore, information about the impacts of different environmental factors on microbial activity and corresponding isotope fractionation process would be enormously valuable in establishing isotopic fractionation as an effective indicator of prevailing conditions such as redox, pH, and concentration.

Chromium is relatively abundant among trace metals in the earth's crust with an average of 1.80 mmol/kg (*Rudnick and Gao, 2003*) and is naturally released to the environment by weathering and leaching from rocks. Cr exists in many different oxidation states (0 to VI), but only two of them, trivalent and hexavalent Cr, are stable in the environment(*Kotaś and Stasicka, 2000*).

The predominant oxidation state in igneous and metamorphic rocks is Cr(III), which is generally insoluble and surface-reactive, and therefore relatively immobile in the environment. When Cr is oxidized to Cr(VI), its solubility increases significantly, and, as a consequence, so does its mobility. Cr naturally occurs in rivers and lakes at concentrations of 0.5 to 100 nM and about 0.1 to 16 nM in seawaters (*Bradl, 2005*). In addition to inputs from natural sources, the presence of high levels of Cr can also be a consequence of its widespread use in industrial processes such as tanning leather, making stainless steel, and preventing engine corrosion.

Because Cr is spread widely in the environment, has high toxicity, and exhibits distinctive physico-chemical characteristics under different redox conditions, it is important to effectively monitor Cr distributions and identify the abiotic and biotic conditions that determine its chemical states. Cr stable isotopes can be of aid, since Cr has 4 of them, and redox reactions result in products that are significantly enriched in the heavier isotopes (*Ellis et al., 2002*). Thus, changes in isotopic composition may be used as indicators of redox conditions in the environment. Likewise, isotopic compositions enable differentiation between natural and anthropogenic local sources of

84 Cr (*Ellis et al.*, 2002, *Izbicki et al.*, 2012, *Johnson and Bullen*, 2004), and assessment of the  
85 effectiveness of remedial interventions.

86 Cr isotope data are usually reported as relative deviations from the standard (NIST SRM 979)  
87 in parts per thousand (per mil or ‰):

$$\delta^{53}\text{Cr} = \left[ \frac{(^{53}\text{Cr}/^{52}\text{Cr})_{\text{sample}}}{(^{53}\text{Cr}/^{52}\text{Cr})_{\text{standard}}} - 1 \right] \times 1000\text{‰} \quad (1)$$

88 The isotope fractionation factor is:

$$\alpha = \frac{(^{53}\text{Cr}/^{52}\text{Cr})_{\text{product}}}{(^{53}\text{Cr}/^{52}\text{Cr})_{\text{reactant}}} \quad (2)$$

89 which can also be expressed using  $\varepsilon$  notation:

$$\varepsilon \approx (\alpha - 1) \times 1000\text{‰} \quad (3)$$

90 An understanding of the mechanisms by which the isotopic ratios of Cr are modified may  
91 enable the monitoring and predictions of the fate of Cr(VI) in contaminated sites (*Berna et al.*,  
92 2010) or in the natural environment (*Ellis et al.*, 2002, *Gao and Schulze*, 2010, *Raddatz et al.*,  
93 2010, *Wanner et al.*, 2012). For instance, Cr(VI) reduction reactions, such as those performed  
94 by microbial communities, result in reaction of lighter isotopes at a faster rate than heavier ones,  
95 leaving diminished concentrations of Cr(VI) that is isotopically heavy. In contrast, if Cr(VI) in a  
96 contaminant plume were only diluted in the environment, the  $\delta^{53}\text{Cr}$  value would not change.

97 Cr isotopes are not only useful as indicators of redox conditions prevailing in modern environ-  
98 ments, but may also be used as a proxy of prevailing oxygenation conditions over geological time.  
99 The isotopic composition of Cr in sedimentary deposits can reflect oxygen levels in the hydrosphere  
100 and atmosphere in the past (*Frei and Polat*, 2013, *Frei et al.*, 2013, *Rotaru et al.*, 1992).

101 The dominant environmental processes that control Cr cycling include both biotic and abiotic  
102 reactions. Once data are available for isotope signatures generated from biotic and abiotic reac-  
103 tions, these signatures can be used to identify which reactions are the dominant controls on Cr  
104 reduction in the environment. Although isotopic data on abiotic Cr(VI) reduction are still limited,



105 it is clear that the values of the fractionation factors depend on the reduction mechanism. For  
 106 example, *Zink et al.* (2010) reported the isotope fractionation factors to be -3.5 ‰ and -5.0 ‰ for  
 107 Cr(VI) reduction by H<sub>2</sub>O<sub>2</sub> in aqueous media under highly acidic and circum-neutral conditions,  
 108 respectively. *Kitchen et al.* (2012) conducted Cr(VI) reduction experiments with aqueous Fe(II)  
 109 and reported fractionation factors to be -4.2 ‰ at pH values ranging between 4 and 5.3. *Dossing*  
 110 *et al.* (2011) reported values of -3.0 ‰ to -4.4 ‰ for reduction by aqueous Fe(II) at circum-neutral  
 111 pH, and of -1.5 ‰ when Cr was reduced by dissolved Fe(II) and Fe(II)+Fe(III) green rust together.  
 112 *Basu and Johnson* (2012) found  $\varepsilon$  values from -2.1 ‰ to -3.9 ‰ in Cr(VI) reduction experiments  
 113 with different forms of iron. Oxidation of Cr(III) by Mn oxides is also a dominant inorganic con-  
 114 trol on Cr behaviour in oxic environments (*Feng et al.*, 2006, *Landrot et al.*, 2012). In addition to  
 115 redox reactions, it is possible that adsorption may induce Cr isotope fractionation. However, *Ellis*  
 116 *et al.* (2004) investigated the effects of sorption of Cr(VI) onto goethite and  $\gamma$ -Al<sub>2</sub>O<sub>3</sub>, and found  
 117 that equilibrium fractionation of Cr stable isotopes during adsorption on these phases is negligible.  
 118 Cr(VI) isotope fractionation during adsorption onto other phases has not been determined.

119 Shifts in isotope fractionation due to microbial activity also provide valuable insight into the  
 120 mechanisms of metal cycling and can be used to quantify biogeochemically mediated redox pro-  
 121 cesses in the environment (*Basu et al.*, 2014, *Han et al.*, 2012, *Sikora et al.*, 2008). *Sikora et al.*  
 122 (2008) were the first to investigate the impact of microbial factors on Cr isotope fractionation.  
 123 They conducted anaerobic reduction experiments with *Shewanella oneidensis* MR 1 and deter-  
 124 mined that fractionation factors ranged from -4.0 to -4.5 ‰ when reduction rates were low, but  
 125 was -1.8 ‰ at greater reduction rates. *Han et al.* (2012) reported Cr isotope data during reduction  
 126 by *Pseudomonas stutzeri* RCH2 under both aerobic ( $\varepsilon$  = -2 ‰) and denitrifying conditions ( $\varepsilon$  =  
 127 -0.4 ‰). *Basu et al.* (2014) reported Cr(VI) reduction by a metabolically diverse group of bacteria,  
 128 with Cr isotope fractionation factors ranged from -2.17 to -3.14 ‰, and suggested that stronger  
 129 isotopic fractionation was induced during Cr(VI) reduction under electron-donor-poor conditions.

130 There are many factors that can impact the microbial processing of Cr, such as the presence of  
 131 specific electron donors (*Brodie et al.*, 2011) and Cr concentration. Such factors have been shown  
 132 to have an impact with S, where the extent of S microbial isotope fractionation is dependent on

133 S concentration and which electron donors are present (*Habicht and Canfield, 1997, Kaplan and*  
 134 *Rittenberg, 1964*). Similar effects may be reasonably expected for Cr isotope fractionation. How-  
 135 ever, such knowledge is still limited. Therefore, the aim of this study was to fill this knowledge gap  
 136 by determining Cr isotope fractionation factors associated with microbial activity under differing  
 137 culture conditions to identify the range of isotope signatures generated by bacteria and the factors  
 138 that have the greatest impact on fractionation factors. Specifically, the aim was to determine the  
 139 influence of bacterial species, type of electron donor, mode of bacterial metabolism (aerobic and  
 140 anaerobic), and pH, on both the rates of reduction and isotopic signatures generated. This data  
 141 are fundamental for interpreting Cr isotope data in the environment, including identifying the pro-  
 142 cesses responsible for reducing and immobilizing both natural and contaminant Cr(VI). Once this  
 143 data are available, measured isotope composition of Cr(VI) can be used to constrain the extent of  
 144 Cr reduction in the environment and under what conditions this occurred.

## 145 **2. Materials and Methods**

### 146 *2.1. Bacteria*

147 The bacterial strains selected in this study were *Shewanella oneidensis* MR 1 (MR 1) and  
 148 *Pseudomonas fluorescens* LB 300 (LB 300), both of which are common in the environment and  
 149 are reported to reduce Cr under specific conditions (*Sikora et al., 2008, Wang and Xiao, 1995*).  
 150 The two bacteria are both facultative anaerobes, so both have the potential to reduce Cr(VI) to  
 151 Cr(III) both aerobically and anaerobically. *Shewanella oneidensis* MR 1 (MR 1) was purchased  
 152 from NCIMB Ltd (NCIMB 14063), and *Pseudomonas fluorescens* LB 300 (LB 300) was kindly  
 153 provided by Prof. Yi-Tin Wang from Department of Civil Engineering, University of Kentucky  
 154 (*Wang and Shen, 1995*).

### 155 *2.2. Cr reduction experiments*

156 A series of experiments was performed in triplicate to determine the rates of reduction of Cr(VI)  
 157 to Cr(III) and the associated isotope fractionations by microbial action. In each experiment,  
 158 dissolved Cr(VI) was combined with the microbial culture under different conditions. At different  
 159 time points in the experiments, an aliquot of the experimental solution was collected, and the

160 remaining Cr(VI) in solution was separated using anion exchange chromatography. The isotope  
161 compositions were measured by thermal ionisation mass spectrometry (TIMS).

### 162 2.2.1. Aerobic reduction

163 The experiments, with different pH, electron donors, electron donor concentrations, and dif-  
164 ferent species, were conducted aerobically in 150 mL Erlenmeyer flasks. Two growth media were  
165 employed in the aerobic reduction study: LB (Luria Bertani) Broth (Sigma Aldrich) and a Minimal  
166 salts medium (0.03g NH<sub>4</sub>Cl, 0.03g K<sub>2</sub>HPO<sub>4</sub>, 0.05g KH<sub>2</sub>PO<sub>4</sub>, 0.01g NaCl and 0.01 g MgSO<sub>4</sub> in 1 L  
167 of Milli-Q water ). A Cr(VI) solution was prepared by dissolving potassium di-chromate in steril-  
168 ized Milli-Q water and filtering through a 0.22  $\mu$ m membrane before the experiments. When the  
169 cells reached the late exponential state, they were harvested by centrifugation at 4100 rpm and 4  
170 °C for 20 minutes. The cells were then washed 3-4 times using a sterile 0.9 % NaCl solution before  
171 adding to 50 mL of freshly made autoclaved minimal salts medium. The cell density was adjusted  
172 to approximately 10<sup>10</sup> cells/mL ( $1.0 \pm 0.2 \times 10^{10}$  cells/mL). Cell density was measured by using  
173 both optical density (OD 600) and plating. It should be noted that all the aerobic experiments  
174 with *Pseudomonas* have been conducted at the same time and from the same enriched culture of  
175 bacteria, so that the cell density for each group was approximately the same. For *Pseudomonas*  
176 *fluorescens* LB 300, glucose was the major electron donor employed in this study, except in the  
177 experiments with different electron donors, in which the effect of three other electron donors have  
178 also been investigated: citrate, acetate and propionate. The concentrations for all the electron  
179 donors were 5g/L. For *Shewanella oneidensis* MR 1, sodium succinate was the principle electron  
180 donor, with a concentration of 2.43 g/L. The electron donors used have been widely used in many  
181 previous studies and shown to efficiently mediate bacterial reduction (*Tekerlekopoulou et al.*, 2010,  
182 *Xu et al.*, 2011). All the electron donor solutions were sterilised by being passed through a 0.22  
183  $\mu$ m filter membrane. The pH of the media was adjusted using 1 M HCl or NaOH. Except for  
184 experiments on pH dependence, the pH for the experiments was  $7 \pm 0.2$ . The media were then  
185 incubated at 30 °C.

### 186 2.2.2. Anaerobic reduction

187 All sets of anaerobic experiments were also conducted together, so cell density was also approx-  
188 imately the same for each group in the experiment. The impact of different electron donors was  
189 also investigated in the anaerobic experiments, in which the cells were first cultured in an anaer-  
190 obic growth medium and then transferred to the cell suspension medium. The anaerobic growth  
191 medium and cell suspension medium for *Shewanella oneidensis* MR 1, described by *Myers and*  
192 *Nealson* (1988), was modified by adding different electron donors and accepters. The composition  
193 of the growth medium was as follows: 9.0 mM  $(\text{NH}_4)_2\text{SO}_4$ , 5.7 mM  $\text{K}_2\text{HPO}_4$ , 3.3 mM  $\text{KH}_2\text{PO}_4$ ,  
194 2.0 mM  $\text{NaHCO}_3$ , 1.01 mM  $\text{MgSO}_4$ ,  $\text{CaCl}_2$ , 67.2  $\mu\text{M}$   $\text{Na}_2\text{EDTA}$ , 56.6  $\mu\text{M}$   $\text{H}_3\text{BO}_3$ , 10.0  $\mu\text{M}$   $\text{NaCl}$ ,  
195 5.4  $\mu\text{M}$   $\text{FeSO}_4$ , 5.0  $\mu\text{M}$   $\text{Ni}(\text{NH}_4)_2(\text{SO}_4)_2$ , 5.0  $\mu\text{M}$   $\text{CoSO}_4$ , 3.87  $\mu\text{M}$   $\text{Na}_2\text{MoO}_4$ , 1.5  $\mu\text{M}$   $\text{Na}_2\text{SeO}_4$ ,  
196 1.26  $\mu\text{M}$   $\text{MnSO}_4$ , 1.04  $\mu\text{M}$   $\text{ZnSO}_4$ , 0.2  $\mu\text{M}$   $\text{CuSO}_4$ , 20 $\mu\text{g}$  L-arginine HCl, 20  $\mu\text{g}$  L-glutamine, 40 $\mu\text{g}$   
197 DL-serine in milli-Q water. Throughout the study, 0.22- $\mu\text{m}$ -filtered ultra-high-purity  $\text{N}_2$  gas was  
198 used to fill headspaces, flush the syringes prior to use, and compensate for subsequent sample  
199 withdrawal.

### 200 2.2.3. Sample collection and Cr concentration analysis

201 At each sampling time, 1 mL of sample was taken and centrifuged at 9000 rpm at 4°C for  
202 10 min. The supernatant was filtered (to 0.22  $\mu\text{m}$ ) to remove all cells, transferred to a clean  
203 Eppendorf tube and stored at -80°C before further treatment. The Cr(VI) concentrations were  
204 measured by HPLC (Thermo Scientific<sup>TM</sup> Dionex<sup>TM</sup> ICS-3000), using a Dionex IonPac CG5A (4  
205  $\times$  50mm) guard column, a Dionex IonPac CS5A (4  $\times$  250 mm) column and a 375  $\mu\text{L}$  reaction coil.  
206 A calibration curve was done at the beginning of each measurement. The detection limit is 0.1  
207 mg/L. Cr(III) concentrations were determined by mass balance. A preliminary study was done to  
208 confirm the adsorption of Cr(III) onto cell and bottle walls are negligible.

### 209 2.3. Cr Isotope Analysis

210 The preparation method of samples for isotope composition determination largely followed that  
211 of *Ellis et al.* (2002). A double-spike method was employed for correction of isotopic fractionation  
212 occurring during sample preparation and mass spectrometry. The double spike used is a mixture

213 of  $^{50}\text{Cr}$  and  $^{54}\text{Cr}$  and was added to the sample to provide a  $^{50}\text{Cr}/^{52}\text{Cr}$  ratio of approximately 1:1.  
 214 Cr isotope measurements were performed on a Thermo Finnigan Triton TIMS instrument. The  
 215 dried sample was dissolved in 1  $\mu\text{L}$  0.1 M  $\text{HNO}_3$  and carefully loaded onto a Re filament. The Cr  
 216 sample was dried by passing a low current through the filament. One  $\mu\text{L}$  of activator, containing  
 217 silica gel,  $\text{H}_3\text{BO}_3$ , and Al, was then added on top of the Cr sample under the same conditions.  
 218 After this was fully dried, the mixture was heated to 1.8 A for 30 seconds to form a glass, which  
 219 helps to enhance  $\text{Cr}^+$  emission and stabilize small ion-beams. The data acquisition method used  
 220 was modified from *Trinquier et al.* (2008) and described briefly here. In the mass spectrometer,  
 221 filaments were slowly heated to a final temperature of between 1170  $^\circ\text{C}$  and 1230  $^\circ\text{C}$ .  $^{50}\text{Cr}/^{52}\text{Cr}$ ,  
 222  $^{53}\text{Cr}/^{52}\text{Cr}$  and  $^{54}\text{Cr}/^{52}\text{Cr}$  ratios were determined by simultaneous measurement of the ion beams  
 223 on a multiple collector array. V and Fe interferences were monitored on mass 51 (99.76 % of  
 224 V) and 56 (91.66 % of Fe) (*May and Wiedmeyer*, 1998), and neither was detectable. The long-  
 225 term instrumental reproducibility was determined by repeatedly measuring a spiked NIST SRM  
 226 979 Cr standard and the  $\delta^{53}\text{Cr}$  value is  $0 \pm 0.049$  ‰ (normalised to the daily average  $\delta^{53}$  value  
 227 of the standard). In this study, the Cr isotope standard was measured alongside each batch of  
 228 samples. During the reduction experiments and the sample treatment period, it was assumed that  
 229 no significant isotope exchange between Cr species occurred, as the equilibrium exchange reaction  
 230 rates between Cr species were found to be very low under these conditions ( $\times 10^{-8} \text{M day}^{-1}$ ) (*Wang*  
 231 *et al.*, 2015).

232 In closed systems, such as experiments in this study, if the isotopic fractionation factor,  $\alpha$ , is  
 233 constant with time, and there is no subsequent isotope exchange between Cr(VI) and Cr(III), then  
 234 the  $\delta^{53}\text{Cr}$  value of the remaining Cr(VI) in the system evolves according to Rayleigh fractionation  
 235 equation.

$$R_{\text{Cr(VI)}} = R_{\text{Cr(VI)}}^0 f^{\alpha-1} \quad (4)$$

236 Where  $R_{\text{Cr(VI)}}$  is the  $\frac{^{53}\text{Cr}}{^{52}\text{Cr}}$  in the remaining Cr(VI);  $R_{\text{Cr(VI)}}^0$  is the  $\frac{^{53}\text{Cr}}{^{52}\text{Cr}}$  in the original Cr(VI)  
 237 pool;  $\alpha$  is the isotope fractionation factor; and  $f$  is the fraction of the Cr(VI) remaining after  
 238 the reduction process. The conditions of a constant fractionation value and no isotope exchange  
 239 between Cr species in different Cr oxidation states (*Wang et al.*, 2015) are met with reasonable

certainty during the experiments, and therefore, as with the process of mixing, changes in Cr(VI) isotope composition can be confidently related to removal of Cr(VI) through a single simple process according to equation (4) (*Rayleigh*, 1896).

By plotting values using a linearised equation, the best-fit  $\alpha$  values can be found by using linear regression (*Kitchen et al.*, 2012). Uncertainties can be calculated as  $2 \times$  standard errors of the best-fit slopes. Our results are expressed in terms of  $\varepsilon$  (Eq. 3).

### 3. Results

#### 3.1. Biosorption and bioreduction

In order to determine the extent to which Cr(VI) was adsorbed onto cell surfaces, heat-killed cells were used as controls for each experiment. Less than 3% of the Cr(VI) was removed by the dead cells, indicating that the much higher observed reductions in Cr(VI) to Cr(III) were due to cellular activity and no corrections for this effect were required (Fig. 1a).

#### 3.2. The Effects of Electron Donors and Respiration Pathways

The experiments with different electron donors were done with *Pseudomonas fluorescens* LB 300 under both aerobic (Fig. 1a) and anaerobic conditions (Fig. 1b). All groups of experiments were aliquoted from the same culture in this experiment. Thus, they contained approximately the same amount of bacteria ( $\approx 10^{10}$  cells/mL), along with the same initial Cr(VI) concentrations. The reduction rates of each group were calculated as the Cr(VI) reduced over time and normalised to cell numbers.

For the first 24 hours, in the aerobic experiments, citrate promoted a significantly greater average Cr(VI) reduction rate ( $1.5 \times 10^{-9}$  mg·L<sup>-1</sup>·hour<sup>-1</sup>·cell<sup>-1</sup>,  $p < 0.01$ ) compared to those of glucose ( $6.6 \times 10^{-10}$  mg·L<sup>-1</sup>·hour<sup>-1</sup>·cell<sup>-1</sup>), acetate ( $4.9 \times 10^{-10}$  mg·L<sup>-1</sup>·hour<sup>-1</sup>·cell<sup>-1</sup>), and propionate ( $4.8 \times 10^{-10}$  mg·L<sup>-1</sup>·hour<sup>-1</sup>·cell<sup>-1</sup>). However, under anaerobic conditions, reduction rates on experiments with similar number of viable cells were not affected by the nature of the electron donors (Fig 1b). Generally, Cr(VI) reduction rates under anaerobic conditions were generally much greater, with over 70 % of Cr(VI) reduced within 24 hours (Fig. 1b), than those detected under

266 aerobic conditions, where cultures took 72 hours to reduce approximately 50% of the Cr(VI) in  
267 the medium (Fig 1a). However, reduction in the aerobic experiments with citrate was much faster.

268 In all experiments,  $\delta^{53}\text{Cr}$  of the remaining Cr(VI) increased as the concentration decreased;  
269 Cr(VI) became enriched in the heavier isotopes as microbial reduction proceeded (Fig. 2).

270 Under aerobic conditions, relatively small variations between fractionation factors were detected  
271 using different electron donors (Fig. 2a). The experiments with glucose, acetate, and propionate  
272 yielded isotopic fractionation factors ( $\varepsilon$ ) of  $-3.12 \pm 0.10$  ‰,  $-3.21 \pm 0.20$  ‰, and  $-3.90 \pm 0.95$  ‰, re-  
273 spectively, which were not distinguishable from one another. The experiment with citrate yielded  
274 an isotope fractionation factor of  $-4.32$  ‰, but with only two points determined in this experiment  
275 (the rapid Cr(VI) reduction rate made collecting samples at intermediate Cr concentrations im-  
276 possible), an error cannot be assigned to this. It is therefore not possible to categorically establish  
277 this fractionation factor as greater than those of the other experiments until further experiments  
278 confirm that the error is no greater than for the other values.

279 Under anaerobic conditions, the variation in isotopic fractionation signatures as a result of using  
280 different electron donors was much more significant (Fig. 2b), despite the reduction rates being  
281 similar (Fig. 1b). The best-fit  $\varepsilon$  value was  $-4.93 \pm 0.36$  ‰ for glucose,  $-4.63 \pm 0.37$  ‰ for citrate,  
282  $-3.37 \pm 0.18$  ‰ for acetate, and  $-1.58 \pm 0.16$  ‰ for propionate.

283 The extent of Cr reduction and the corresponding isotope fractionations under aerobic and  
284 anaerobic conditions are directly compared for each electron donor in Fig. 3. The best-fit  $\varepsilon$   
285 value for the combined aerobic and anaerobic rate is  $-4.71 \pm 0.38$  ‰ for cultures with citrate ( $r^2$   
286  $= 0.9875$ ) and  $-3.26 \pm 0.13$  ‰ for those with acetate ( $r^2 = 0.9981$ ). However, greater differences  
287 were observed when glucose and propionate were the electron donors. With glucose, the isotopic  
288 fractionation factor increased from  $-3.12 \pm 0.10$  ‰ under aerobic conditions to  $-4.93 \pm 0.36$  ‰ ( $r^2$   
289  $= 0.9910$ ) under anaerobic conditions; in contrast, when using propionate, it was much smaller  
290 under anaerobic ( $-1.58 \pm 0.16$  ‰) than aerobic conditions ( $-3.90 \pm 0.95$  ‰).

291 Cr reduction with *Shewanella oneidensis* MR 1 was also examined. The anaerobic reduction  
292 rates of Cr(VI) were much faster for this species than under aerobic conditions, but much lower  
293 than that recorded for *Pseudomonas fluorescens* LB 300 (Fig. 4a). The isotope data are shown in

Fig 4b. Best-fit Rayleigh distillation curves correspond to  $\varepsilon$  of  $-3.43 \pm 0.05$  ‰ ( $r^2 = 0.9989$ ) under aerobic and  $-2.47 \pm 0.07$  ‰ ( $r^2 = 0.9996$ ) under anaerobic conditions. It should be noted that due to the limited extent of reduction, the isotope fractionation data for only a limited range of Cr(VI) depletion (f) during aerobic reduction were obtained, and therefore a more precisely constrained Rayleigh fractionation curve could not be obtained. The best-fit value for both experiments together is  $-2.6$  ‰ ( $r^2 = 0.98$ ).

### 3.3. *The Effects of Electron Donor Concentration*

During the 48 hours of incubation in these experiments, both the rates of Cr(VI) reduction and the fractions reduced were higher in samples with greater glucose concentrations (Fig. 5a). There was little reduction in the experiments in the absence of glucose in the medium, indicating that there were also no endogenous electron donors in the system.

As bacterial Cr(VI) reduction proceeded in the presence of glucose, the  $\delta^{53}\text{Cr}$  value increased (Fig. 5b). The best-fit Rayleigh distillation curve using data for all experiments in this study regardless of donor concentration in the medium corresponds to  $\varepsilon = -3.19 \pm 0.34$  ‰.

### 3.4. *The Effects of pH*

At pH 4 and 6, the bacterial Cr(VI) reduction rates were similar, whilst at pH 8, the reduction rate was much faster within the first 24 hours, and the fraction of reduced Cr(VI) was also much greater than in the other two groups.

Isotope data from the pH experiments are presented in Fig 6b. Under all conditions, with the reduction of Cr(VI), the  $\delta^{53}\text{Cr}$  value increased. There were no significant differences with respect to pH, and all data points from the three experiments fit on one Rayleigh distillation curve ( $\varepsilon = -3.21 \pm 0.18$  ‰).

## 4. Discussion

### 4.1. *The Effects of Electron Donors and Respiration Pathways*

In this study, the nature of electron donors was found to be of great importance in affecting Cr(VI) reduction rates and isotope fractionation under both aerobic and anaerobic conditions.



Cr(VI) reduction rates similar to those in this study have also been reported by Wang and Shen (1995), employing the same bacteria but only glucose as the electron donor. Of the 4 electron donors tested in this study, citrate promoted the greatest Cr(VI) reduction rate for *Pseudomonas fluorescens* LB 300. However, it must be noted that the effect of electron donors on Cr(VI) reduction is also dependent on the bacterial species; the electron donor that is effective for one bacteria in catalysing Cr reduction may not be as useful for other bacterial species. For instance, Xu et al. (2011) investigated Cr(VI) reduction by another bacteria (*Pannonibacter phragmitetus* Lsse-09) and found that acetate resulted in the greatest reduction rate compared with other electron donors employed, including lactate, formate, pyruvate, citrate, and glucose.

Microbial reduction and isotope fractionation of other elements has also been found to be dependent on which electron donors are available. For example, in a previous study, S isotope fractionation during sulphate reduction was found to be directly proportional to reduction rate with molecular hydrogen as the electron donor, and inversely proportional to that with lactate and ethanol as electron donors (Kaplan and Rittenberg, 1964). However, far less is known of the extent to which different electron donors impact Cr isotope fractionation. So far, the only data available, reported by Sikora et al. (2008), suggest that anaerobic Cr isotope fractionation by *Shewanella oneidensis* MR 1 was similar for two different electron donors, formate and lactate. However, in that experiment, very little external electron donor was added (less than 100  $\mu$ M of formate and lactate). Under such conditions, the bacteria may also have utilised their endogenous reserves as electron donors (Cheung and Gu, 2007, Sikora et al., 2008). It is not clear how much the endogenous electron donors have facilitated the bacterial Cr(VI) reduction processes and affected the fractionation factors obtained. Unfortunately, no isotope data from the control group (without any external electron donor) were reported. The present study demonstrates for the first time that, in substantial concentrations, different electron donors result in different Cr isotope fractionations, especially under anaerobic conditions. The differences that electron donors induce in bacterial isotope fractionation reflect different reduction pathways. However, the underlying mechanisms remain uncertain. As multiple enzymes have been found to efficiently catalyse Cr(VI) reduction in microbial systems (Michel et al., 2001, 2003), such as various types of C-cytochromes, [Fe]-

hydrogenase and other metalloenzymes containing hemes or (Fe/S) centers, it is highly likely that multiple reduction pathways co-exist in the same bacteria, each possibly with a different fractionation factor, and the efficiencies of each of these pathways may be different for different electron donors. Although different pathways (enzymes) may be employed by the bacteria when using different electron donors, the kinetic Cr(VI) reduction rates by these pathways may be similar. Therefore, bacterial Cr(VI) reduction rates may be the same with different electron donors, as were observed in this study. Further work is needed to measure the expression and activity of different enzymes in bacteria when using different electron donors, and the isotope signatures generated when Cr(VI) is reduced by these enzymes.

In this study, the substantially different isotopic fractionation factors were generated by *Pseudomonas* in the presence and absence of oxygen, but with the same electron donor. This indicates that the respiration pathways can have a significant impact on microbial isotopic fractionations, and that there are very different reduction mechanisms involving electron transfer processes under aerobic and anaerobic conditions.

For aerobes, different enzymes may be responsible for Cr(VI) reduction. The reductase for Cr(VI) may be associated with the cell membrane and can mediate the transfer of electrons from NADH to chromate (*Bopp and Ehrlich, 1988*), while the reductases in many other bacteria capable of reducing Cr(VI) are soluble in the cytosol (*Cheung and Gu, 2007*). For example, ChrR, one of the best studied reductases of *Pseudomonas putida*, transiently reduces Cr(VI) with a one-electron shuttle to form Cr(V), followed by a two electron transfer to generate Cr(III) (*Cheung and Gu, 2007*). YieF Cr(VI) reductase, another well characterised protein extracted from *E.coli*, is similar to ChrR in terms of kinetic and physicochemical properties, but is unique in that it catalyzes the reduction of Cr(VI) to Cr(III) via a four-electron transfer, in which three electrons are consumed in reducing Cr(VI) to Cr(III) and the fourth one is transferred to molecular oxygen, generating Reactive Oxygen Species (ROS) (*Cheung and Gu, 2007*). It is possible that the isotope fractionation factors associated with the activity of these very different enzymes may be different. The production of these enzymes is an adaptive mechanism promoted by recent chromate exposure, and their reduction of Cr(VI) is through co-metabolism, which requires electron donors in addition

376 to Cr(VI). The types of electron donors may impact the production rate of the different Cr(VI)-  
377 reducing enzymes that can be produced in the cell, which then impacts the reduction pathway and  
378 isotope fractionation for Cr(VI).

379 The reduction activities of anaerobes have been reported to be associated with electron transfer  
380 systems that catalyse the electron shuttle along the respiratory chains (*Cheung and Gu, 2007*). In  
381 the absence of oxygen, Cr(VI) can serve as a terminal acceptor for many electron donors such as  
382 carbohydrates, proteins, fats, hydrogen, and even endogenous electron reserves. Under anaerobic  
383 conditions, it has been demonstrated that the cytochromes are responsible for the enzymatic  
384 reduction of different metals, such as Fe(III) and Cr(VI). Several types of  $c_3$  cytochromes were found  
385 responsible for Cr(VI) reduction in bacteria (*Lovley, 1995, Michel et al., 2001, 2003, Sundararajan*  
386 *et al., 2011*).

387 These enzyme studies provide evidence that bacteria can use several different pathways to  
388 reduce Cr(VI), depending upon the environmental conditions and the electron donors present.  
389 These biological properties modify the isotopic fractionation factors, and therefore Cr isotope  
390 signatures may be useful for investigating Cr(VI) reduction mechanisms and physiological states  
391 of microbial communities. Further, if the isotope fractionation factors related to reduction by  
392 individual enzymes are identified, then which enzymes are involved within pathways might be  
393 constrained.

#### 394 4.2. The Effects of Electron Donor Concentration

395 The relationship between Cr(VI) reduction rates and Cr isotope fractionation factors was ad-  
396 dressed with the experiment that only changed the reduction rates by changing the electron donor  
397 concentrations in the media under aerobic conditions, using glucose as an example (Fig 5). In this  
398 study, the concentration of glucose had a great impact on bacterial Cr reduction rates, but no  
399 significant change in the Cr isotope fractionation factor of  $-3.19 \pm 0.34$  ‰. A similar finding was  
400 reported by *Xu et al. (2015)*, who investigated the effect of glucose concentration on Cr reduction  
401 activity by *Bacillus sp.* (black circles in Fig 5b) and also found that its impact on the isotope frac-  
402 tionation factor was insignificant, with a best-fit  $\varepsilon$  value of  $-2.00 \pm 0.21$  ‰. The different  $\varepsilon$  values  
403 determined in these two studies may be due to the difference in bacterial species investigated.

404 The concentrations and distributions of electron donors vary considerably in the environment,  
405 and this can have a significant impact on microbial metabolic rates (*Xu et al.*, 2011). Some previous  
406 studies have suggested that higher bacterial metabolic rates resulted in lower isotope fractionation  
407 factors (*Sikora et al.*, 2008, *Wing and Halevy*, 2014), in contrast, in this study, while higher electron  
408 donor concentrations led to higher Cr(VI) reduction rates, higher Cr(VI) reduction rates did not  
409 result in lower isotope fractionation factors.

410 One possible factor that influences both reduction rates and isotope fractionation factors is  
411 the electron donor employed. *Sikora et al.* (2008) reported that the concentration of lactate  
412 affected the isotopic fractionation factor during Cr reduction by *Shewanella oneidensis* MR 1.  
413 The fractionation factor was -4.3 ‰ when the electron donor concentration was low and -1.8 ‰  
414 when it was high. It may be the case that the difference in isotope fractionation factors was due  
415 to the difference in reduction rates, but it is also possible that such a difference was caused by the  
416 different electron donor employed by the bacteria. Under anaerobic conditions, some bacteria can  
417 use internally stored reserves as electron donors, so even if there are no external electron donors  
418 present, it still can reduce Cr(VI) (*Xu et al.*, 2011). This has also been confirmed in the *Sikora*  
419 *et al.* (2008) study, in which high Cr(VI) reduction rates were also found in the control groups  
420 (no electron donors), indicating that *Shewanella oneidensis* MR 1 can get electrons from the  
421 endogenous reserves to catalyse the reduction of Cr(VI). Thus the different fractionation factors  
422 reported between low and high lactate concentrations may have reflected reduction pathways that  
423 utilized different electron donors (endogenous electron donors vs. lactate). To investigate such a  
424 possibility, further study can be done to determine Cr isotope fractionation factors by *Shewanella*  
425 *oneidensis* with no external electron donors added under anaerobic conditions.

#### 426 4.3. The Effects of pH

427 The chemical state of Cr depends on pH (*Campanella*, 1996, *Zink et al.*, 2010). It is also a  
428 critical factor in determining the optimum conditions for bacterial growth and metabolism. In  
429 this study, the optimal pH for *Pseudomonas fluorescens* was investigated by examining the growth  
430 and Cr(VI) reduction rates over a range of pH (4, 6 and 8). The pH range was determined by the  
431 viability of the bacteria; within this range, the redox state of Cr was unchanged in the medium,

432 as determined by measuring the Cr(VI) concentration in the control medium (without bacteria).  
433 The dominant Cr(VI) species at pH 4 is  $\text{HCrO}_4^-$  (Pourbaix, 1974), but at higher pH, the species  
434  $\text{CrO}_4^{2-}$  becomes more significant (Trama and Benoit, 1960, Zink et al., 2010).

435 It has been observed that both the reduction rate and the fraction of reduced Cr(VI) were much  
436 greater at pH 8 than at pH 4 or 6. Similar trends were also observed by Wang and Shen (1995)  
437 for both *Pseudomonas fluorescens* and *Bacillus sp.*. The different reduction rates determined at  
438 different pH may have been due to Cr toxicity. Cr(VI) is able to penetrate cellular membranes,  
439 inducing oxidative stress that damages cell membranes and DNA-protein complexes and altering  
440 the physiological functions of bacteria and other biota (Xu et al., 2004, 2005). Many previous  
441 studies regarding Cr(VI) have confirmed that a decrease in the pH resulted in increased toxicity  
442 of Cr(VI) to various species, such as earthworms (Sivakumar and Subbhuraam, 2005), common  
443 carp (Stouthart et al., 1995), and rainbow trout (Van Der Putte et al., 1981). To explain these  
444 observations, it has been suggested that the level of acute Cr(VI) toxicity is attributable to the  
445 speciation of Cr, where  $\text{HCrO}_4^-$  is much more toxic than  $\text{CrO}_4^{2-}$ , because a) monovalent ions tend  
446 to be more readily taken up by the cells than divalent ions, and b)  $\text{HCrO}_4^-$  is more oxidizing and  
447 reactive than  $\text{CrO}_4^{2-}$  (Trama and Benoit, 1960, Van Der Putte et al., 1981). Therefore, the changes  
448 in reduction rates with changes with pH may be because either the bacterial Cr(VI) reduction  
449 activity is impacted by the levels of toxicity of the different species, or the bioavailabilities of the  
450 different Cr(VI) species are different under those pH conditions.

451 Importantly, the consistent isotope fractionation factor found at all different pH values suggests  
452 that pH and the speciation of Cr(VI) had little impact on bacterial Cr isotope fractionation factors,  
453 which are consistent with the reduction pathways of Cr(VI) being identical under different pH  
454 conditions.

#### 455 4.4. Differences between bacterial species and comparison with abiotic reduction

456 The fractionation factor reflects the difference in the rates at which the different isotopes are  
457 being removed by reduction. As reduction progresses, the isotope ratio of the Cr(VI) will evolve  
458 following the Rayleigh fractionation relationship (see e.g., Figure 2). Therefore, if Cr(VI) has  
459 been reduced by a single process, the ratio measured in Cr(VI) will depend upon the fractionation

factor of the mechanism involved and the extent of reduction. Where the latter is known in a lab experiment, the former can be calculated. In the environment, in situations where the extent of reduction can be constrained (*e.g.*, through changes in concentration in groundwater along a flowline), the fractionation factor can be calculated, and the mechanisms that have been found to be associated with such a factor can be identified. Conversely, where the dominant mechanism, and hence fractionation factor, can be identified (*e.g.*, microbial reduction), the extent of reduction can be calculated. An example has been reported by *Berna et al.* (2010), where they used Cr isotope measurements as indicators of Cr(VI) reduction in groundwater with a point-source plume.

Although several genera of bacteria have been reported to have the ability to reduce Cr(VI), there have been only a few studies focused on comparing Cr isotopic fractionation associated with the reduction process by different species, as shown in Fig. 7 (For direct comparison, the  $\delta^{53}\text{Cr}$  values were normalised to zero at the starting point of each experiment). *Basu et al.* (2014) studied several Cr(VI) reducing bacteria and found that the Cr isotope fractionation factor  $\varepsilon$  was -3.03 for *G. sulfurreducens*, -2.17‰ for *Shewanella. sp.*, -3.14‰ for *P. stutzeri* and -3.01‰ for *D. vulgaris*. In our study, the fractionation factor for *Pseudomonas fluorescens* LB 300 fell in the range of -1.5 to -5 ‰ and was -2.5‰ for *Shewanella oneidensis* MR 1. *Sikora et al.* (2008) also studied *Shewanella oneidensis* MR 1, the same strain reported here, but found different fractionation factors. *Sikora et al.* (2008) used formate and lactate as the electron donors, while in this study, sodium succinate was used as the electron donor. The difference in the Cr isotopic fractionation factors may have been due to the different electron donors employed, which further proves that the importance of different electron donors in bacterial Cr(VI) isotope fractionations.

*Han et al.* (2012) also investigated Cr isotope fractionation under denitrifying conditions by an aquifer-derived bacterium *Pseudomonas stutzeri* RCH2 and found that the Cr(VI) was reduced at a very high rate (about 75% within 2 hours). The data presented in their study revealed a small  $\delta^{53}\text{Cr}$  value range and considerable variance, suggesting that additional processes were affecting Cr reduction and isotope fractionation in that study.

Overall, after analysing all the data available, the Cr isotope fractionation factors resulting from bacterial reduction are found to lie in a narrow range. A best-fit for all the data yields a value of

about -3.3‰ (Fig. 7; excluding the *Han et al.* (2012) data). Some abiotic Cr(VI) adsorption and reduction data are also plotted in Fig. 7. The fractionation factors due to adsorption processes are quite distinct from those due to reduction. The fractionation factors due to abiotic reduction processes are quite variable depending on the reductants and experimental conditions and overlap with the  $\epsilon$  range from bacterial reduction processes.

Early work (*Ellis et al.*, 2002, *Sikora et al.*, 2008) suggested that there may be differences in the isotope fractionation factors for abiotic and biotic reduction. If the variations are distinct, then the isotope signatures could be used to identify what was controlling Cr(VI) reduction and thus mobility. However, with additional data it is clear that isotopic variation cannot be simplistically interpreted. In the natural environment, depletion of Cr(VI) is likely the result of a combination of different processes (*Paulukat et al.*, 2016, *Wu et al.*, 2017). Under most circumstances the Cr isotope composition can be used to identify where depletion of Cr has occurred due to reduction (Fig. 7). Even so, in order to distinguish between different reduction processes, more site-specific data are required. This requires determining the fractionation factors associated with indigenous bacterial populations and local groundwater chemistry, including the composition of available electron donors and organic ligands. Experiments on the fractionation onto surfaces of local materials by inorganic processes are also necessary.

## 5. Conclusions

Cr isotope variations can be used as a valuable tool for understanding microbially mediated Cr reduction and transport processes. However, a firm understanding of the effects of various processes on Cr isotope fractionation is needed. Based on the present and previous studies, the isotope fractionation factors  $\epsilon$  of Cr by microbial reduction range from -1.5 to -5 ‰, with a mean value of -3.3 ‰. Changes in pH and electron donor concentration had little significant impact on the fractionation factors under the experimental conditions employed. However, different electron donors and bacterial respiration pathways had significant effects on microbial metabolism, thus affecting Cr isotope fractionation.

It has been generally assumed that lower cell specific reduction rates would result in higher fractionation factors (*Basu et al.*, 2014, *Habicht and Canfield*, 1997, *Sikora et al.*, 2008); however, in

the conditions tested in this study, this was not always the case. Overall, it may be that different reduction pathways, rather than different rates of reduction, are responsible for the observed differences in the fractionation factors. The clearly resolved isotopic variations determined by high-precision mass spectrometry can be utilised to probe biological processes by clearly indicating when different biological pathways occur. This is a significant finding; now that we have established that there are isotopic variations reflecting variations in Cr(VI) reduction mechanisms, the isotope signatures associated with different mechanisms can be explored and quantified further. Eventually Cr isotope signatures may be an effective diagnostic tool for investigating microbial activities over a range of environmental conditions.

An important implication for this work is that while there is the prospect of using isotopic variations for identifying the relative importance of adsorption and microbial reduction for limiting the migration of Cr at contaminated sites (*Ellis et al.*, 2002, 2004), the complexities of microbial Cr isotope fractionation mean that the isotope fractionation factors must be determined for each site using cultures of the microbial communities present and available electron donors. Additional site-specific factors (*e.g.*, local groundwater chemistry) must also be determined to separated the effects of those processes and quantify the extent of Cr reduction.

Our study provides fundamental information for interpreting Cr isotope fractionations in the environment, thus further developing the potential for using Cr isotopic fractionation as an environmental diagnostic tool for assessing the overall extent of Cr(VI) reduction and immobilisation by biotic and abiotic processes.

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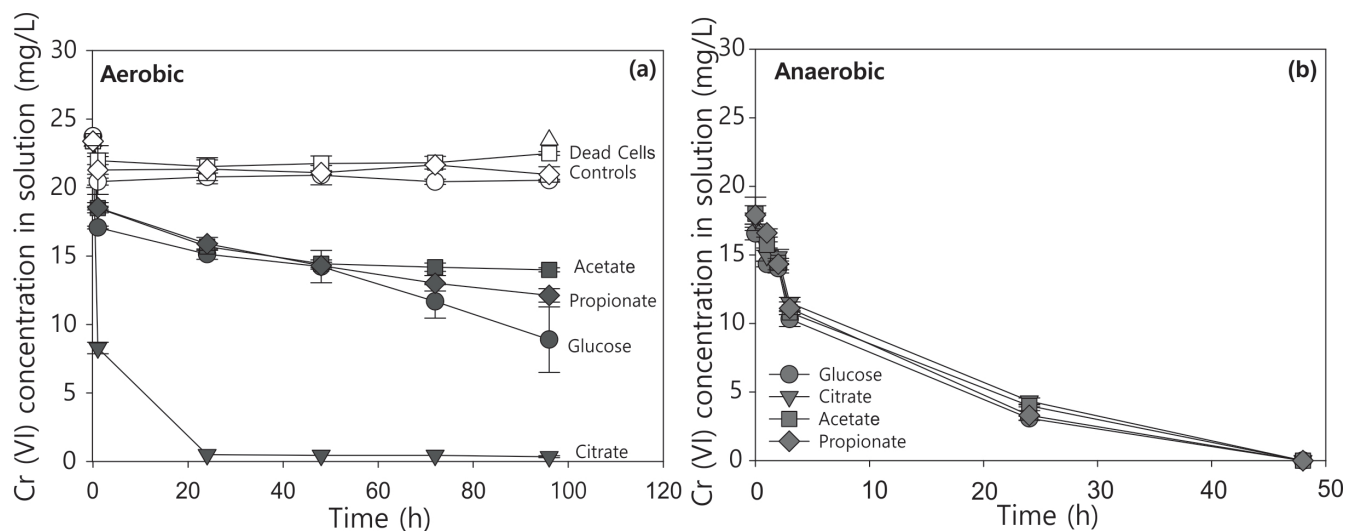


Figure 1: Cr(VI) reduction experiments by *Pseudomonas fluorescens* LB 300 under aerobic conditions (a) and anaerobic conditions (b), at neutral pH and using different compounds as electron donors. Data plotted are the average of triplicate experiments. During aerobic reduction, the reduction rate was fastest with citrate as the electron donor, and there were no significant differences between the other three donors ( $p > 0.5$ ). The reduction rates under anaerobic conditions were generally greater than those under aerobic conditions. There were no differences between different electron donor groups when the reduction occurred anaerobically ( $p > 0.5$ ). Uncertainties in each experiment were expressed as the standard deviation between the triplicates in each experimental group. Controls were prepared with the same concentration of cells as in experimental groups, though the cells were thermally killed. The dead cell controls in the anaerobic experiments were the same as those in the aerobic experiments.

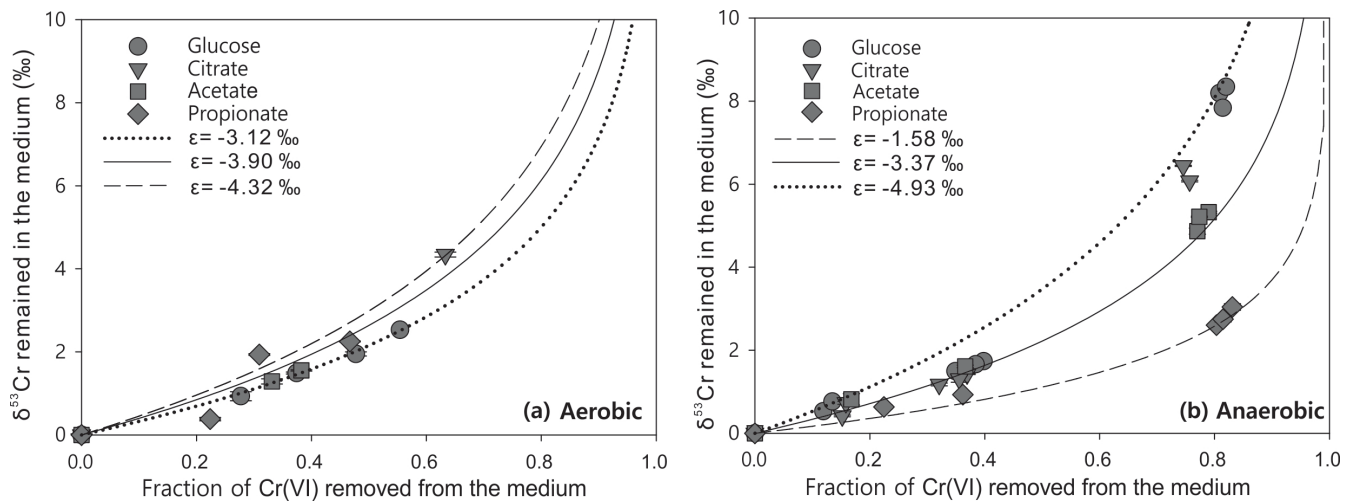


Figure 2: The impact of different electron donors on Cr isotope fractionation by *Pseudomonas fluorescens* LB 300. Under aerobic conditions and neutral pH,  $\epsilon$  values for Rayleigh fractionation range from -3.12 ‰ to -4.32 ‰ for calculated best-fit values for each electron donor, with the best-fit value of 3.8 ‰ for all electron donors. Under anaerobic conditions and neutral pH,  $\epsilon$  was -4.93, -4.63, -3.37 and -1.58 ‰ for experiments with glucose, citrate, acetate and propionate, respectively. Error bars are  $2 \times$  standard error for the measurement, which are generally smaller than the sizes of the symbols.



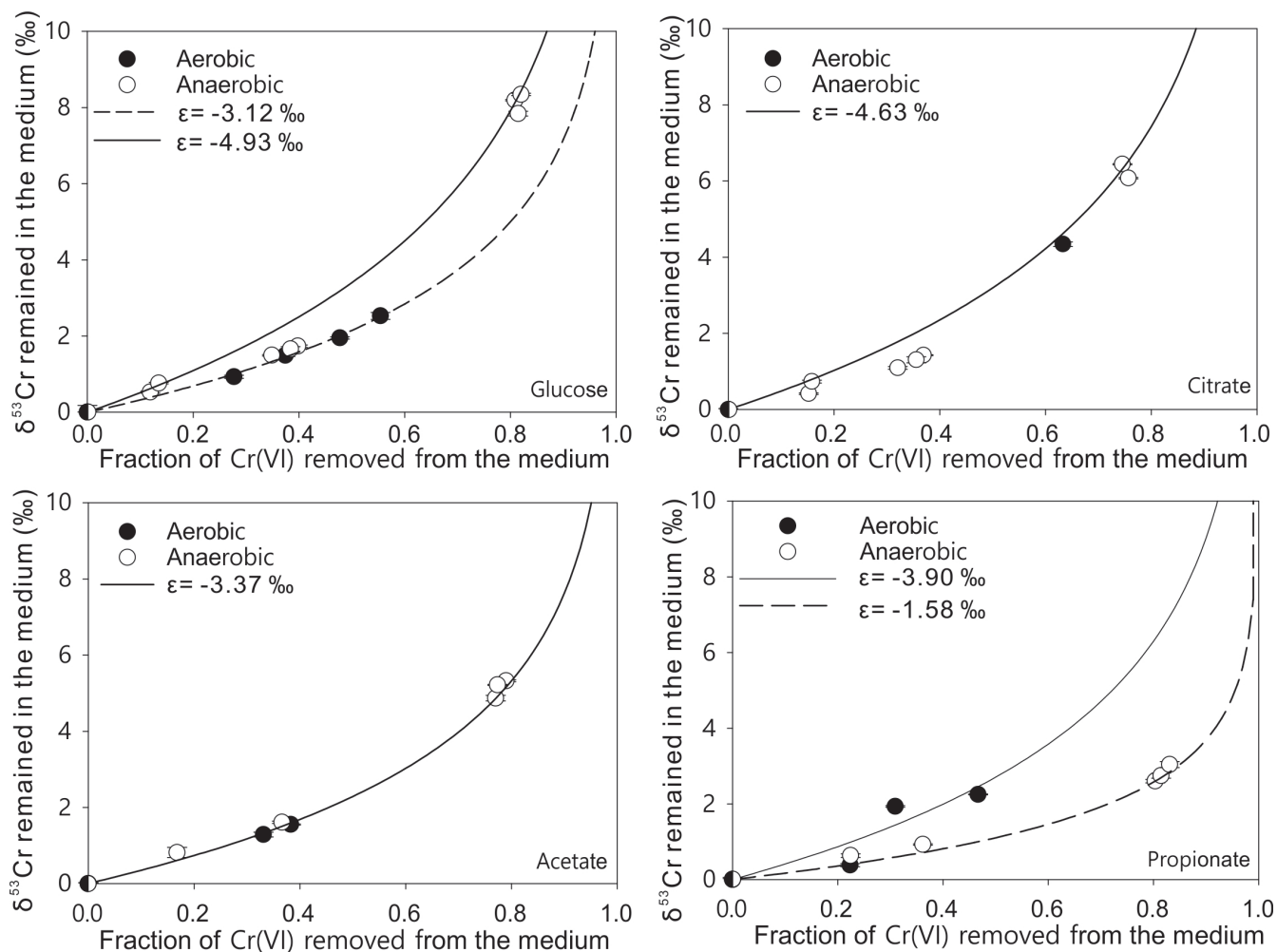


Figure 3: The impact of different respiration pathways on Cr isotope fractionation by *Pseudomonas fluorescens* LB 300. Data are the same as in Fig. 2. Rayleigh distillation curves corresponding to the fractionation factors calculated for individual experiments are plotted here. Significant differences in the fractionation factors for aerobic and anaerobic reduction are found when glucose or propionate are the electron donors, but not for acetate or citrate. Error bars are  $2 \times$  standard error for the measurements, which are generally smaller than the sizes of the symbols.

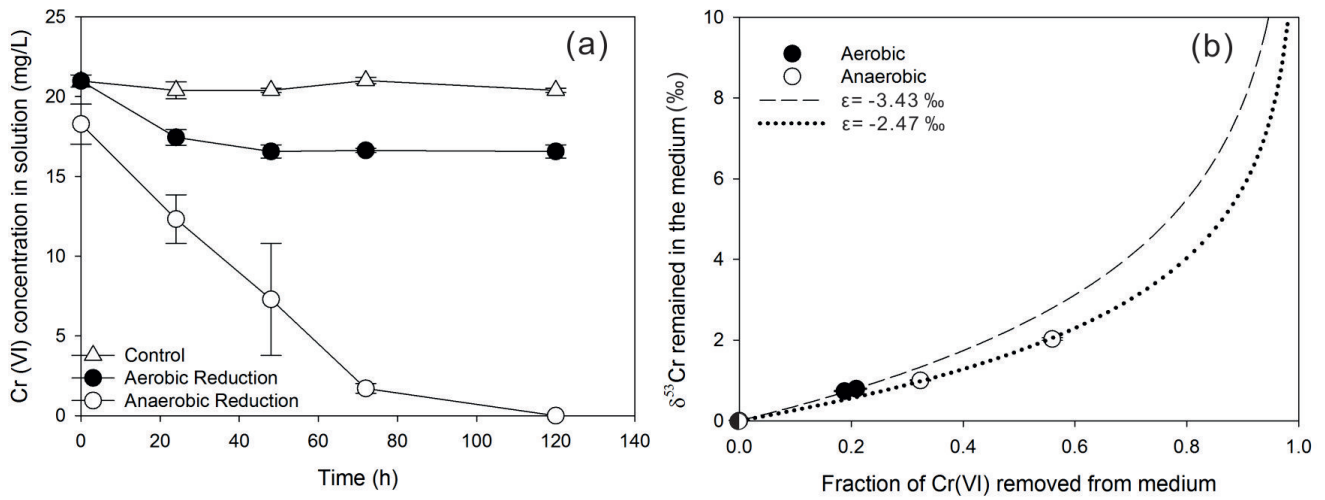


Figure 4: (a) Comparison of the aerobic and anaerobic reduction of Cr(VI) by *Shewanella oneidensis* MR 1, at neutral pH and with succinate as the electron donor. An autoclaved control was prepared under the same lab conditions and with the same concentration of cells as in experimental groups, but the cells in the control group were thermally killed. Error bars are shown as the standard deviations between the triplicates in each experimental group. (b): Cr isotope fractionation results from both aerobic and anaerobic reduction experiments with *Shewanella oneidensis* MR 1. Best-fit Rayleigh distillation curves are shown corresponding to the fractionation factor  $\varepsilon = -3.43$  for the aerobic reduction and  $-2.47\text{‰}$  for anaerobic reduction. Error bars are  $2 \times$  standard error for the measurement, which are generally smaller than the sizes of the symbols.

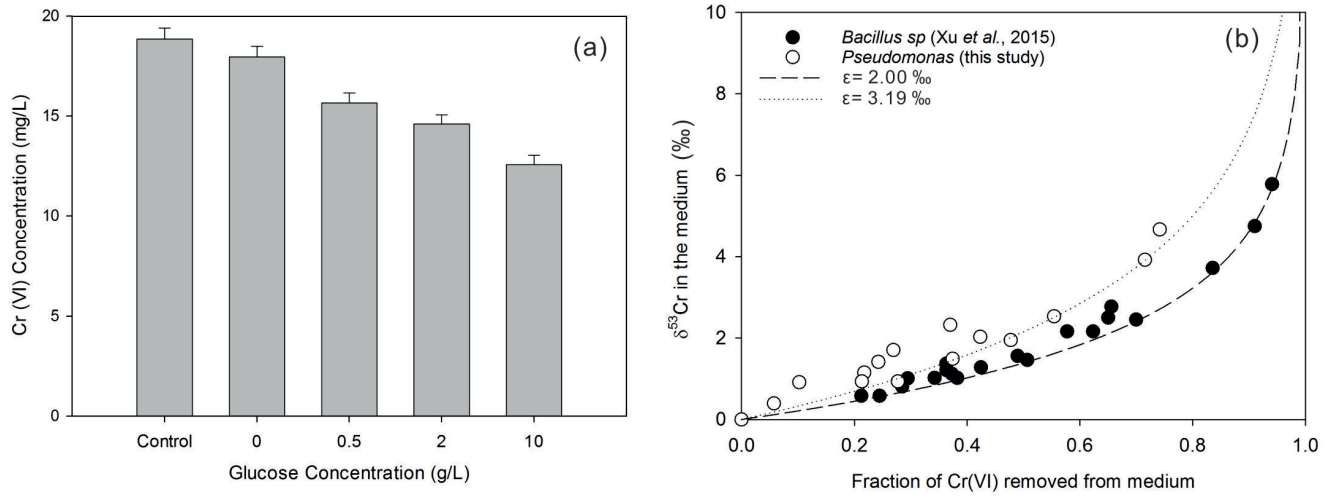


Figure 5: (a): 48 hour aerobic Cr(VI) reduction experiments with *Pseudomonas fluorescens* LB 300, at neutral pH and using different concentrations of the electron donor glucose. The fraction of Cr(VI) reduced was larger in cultures with higher concentrations of glucose. Uncertainties shown are the standard deviations between the triplicates in each experimental group. Controls were prepared with the same concentration of cells, though dead. (b): The fraction of Cr(VI) that has been reduced to Cr(III) versus the isotope composition of the remaining Cr(VI). The fractionation factor  $\epsilon = -3.19$  ‰ for all 5 experiments in this study, and is  $-2.0$  ‰ in the study with *Bacillus sp.* (Xu et al., 2015).

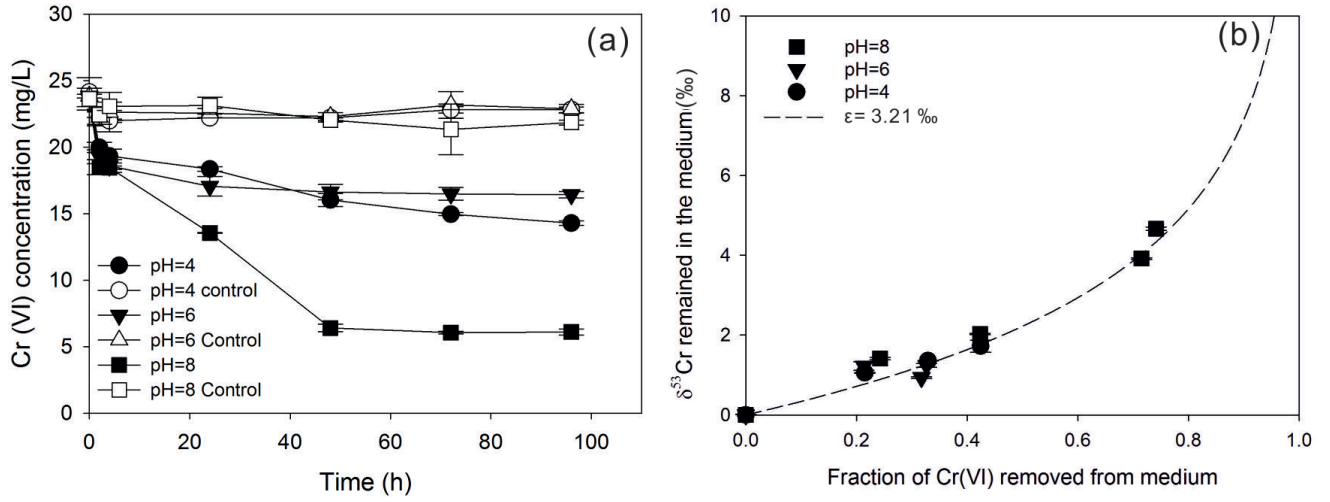


Figure 6: Cr(VI) reduction experiments for *Pseudomonas fluorescens* LB 300 at different pH, under aerobic condition and with glucose as the electron donor. Controls were prepared with the same concentration of cells though thermally killed. Uncertainties are the standard deviation between the triplicates in each group. (a): Cr(VI) was reduced at all pH conditions, though reduction was much faster, and occurred to a much greater extent, at pH = 8. (b): The fraction of Cr(VI) that has been reduced to Cr(III) versus the isotope composition of Cr of the remaining Cr(VI). A single Rayleigh distillation curve corresponding to the best-fit fractionation factor  $\epsilon = -3.21 \text{ ‰}$  (dashed line) is plotted. Error bars are shown as  $2 \times$  standard error for the measurement, which are smaller than the sizes of the symbols.

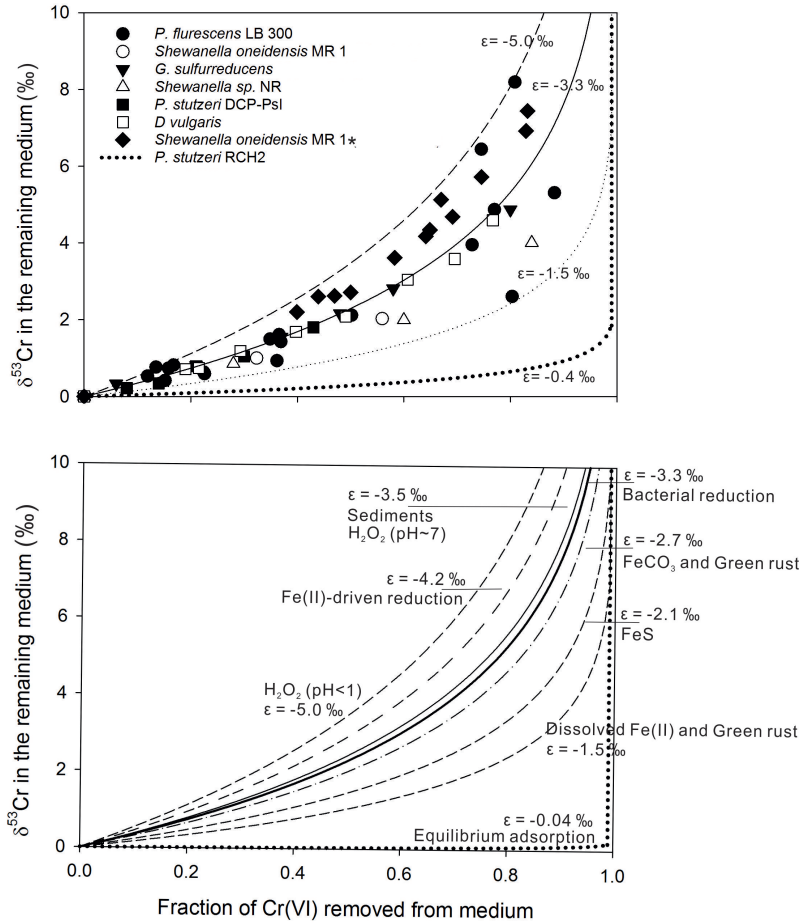


Figure 7: Upper panel: Isotope results for anaerobic reduction experiments with different species of bacteria. All values were normalised to the starting composition of each experiment. A Rayleigh distillation fractionation factor  $\varepsilon = -2.0$  ‰ fits the data for *Shewanella. sp* (white circles, (Basu *et al.*, 2014)), while  $\varepsilon = -4.3$  fits the Sikora *et al.* (2008) data for *Shewanella oneidensis* MR 1 (black diamonds). The isotope data from our study (*P. fluorescens*) and Basu *et al.* (2014) study (*G. sulfurreducens*, *P. stutzeri* and *D. vulgaris*) fall on a single Rayleigh distillation model with a best-fit value for  $\varepsilon = -2.8$  ‰ ( $r^2=0.98$ ). Black dots represent the data from Han *et al.* (2012) ( $\varepsilon = -0.4$  ‰). The best-fit value for  $\varepsilon$  for all data points in this figure was  $-3.3$  ‰ (solid line,  $r^2= 0.86$ ). Lower panel: Rayleigh distillation curves corresponding to fractionation factors from various abiotic Cr(VI) reduction experiments. All the data fall between  $\varepsilon = -1.5$  ‰ and  $-5$  ‰. Data on bacterial reduction are from this study, with FeS, FeCO<sub>3</sub>, and green rust are from Basu and Johnson (2012), with dissolved Fe(II) and green rust are from Dossing *et al.* (2011), with H<sub>2</sub>O<sub>2</sub> and Fe(II)-driven reduction are from Kitchen *et al.* (2012), with natural sediments are from Ellis *et al.* (2002) and Basu and Johnson (2012), and with equilibrium adsorption data from Ellis *et al.* (2004)