

Using stable isotope fractionation factors to identify Cr(VI) reduction pathway: metal-mineral-microbe interactions

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Abstract

Microbes interact with metals and minerals in the environment altering their physical and chemical states, whilst in turn metals and minerals impact on microbial growth, activity and survival. The interactions between bacteria and dissolved chromium in the presence of iron minerals, and how these impact on Cr isotope variations, were investigated. Cr(VI) reduction experiments were conducted with two bacteria, *Pseudomonas fluorescens* LB 300 and *Shewanella oneidensis* MR-1, in the presence of two iron oxide minerals, goethite and hematite. Both minerals were found to inhibit the rates of Cr(VI) reduction by *Pseudomonas*, but accelerated those by *Shewanella*. The Cr isotopic fractionation factors generated by *Shewanella* were independent of the presence of the minerals ($\epsilon = -2.3$ ‰). For *Pseudomonas*, the ϵ value was the same in both the presence and absence of goethite (-3.3 ‰); although, it was much higher ($\epsilon = -4.4$ ‰) in the presence of hematite. The presence of aqueous Fe(III) in solution had no detectable impact on either bacterial Cr reduction rates nor isotopic fractionation factors. While the presence of aqueous Fe(II) induced rapid abiotic reduction of Cr, it had little impact on the bacterial Cr reduction rates and the corresponding isotope fractionation factors. The different effects that the presence of Fe minerals had on the Cr fractionation factors for reduction by the different bacterial species may be attributed to the way each bacteria attached to the minerals and their different reduction pathways. SEM images confirmed that *Pseudomonas* cells were much more tightly packed on the mineral surfaces than were *Shewanella*. The images also confirmed that *Shewanella oneidensis* MR-1 produced nanowires. The results suggest that the dominant Cr(VI) reduction pathway for *Pseudomonas fluorescens* LB 300 may have been through membrane-bound enzymes, whilst for *Shewanella oneidensis* MR-1 it was probably via extracellular electron transfer. Since different minerals impact differentially on bacterial Cr(VI) reduction and isotope fractionation, variations of mineralogies and the associated changes of bacterial communities should be taken into consideration if using Cr isotopes as proxies in the environment.

Keywords:

Chromium, isotope fractionation, bacterial reduction, biogeochemistry, mineral, metal-mineral-microbe interaction

1. Introduction

Chromium occurs naturally in aqueous environments by weathering and leaching of rocks and can also originate from industrial anthropogenic sources. Cr(VI) and Cr(III) are the most common and stable species of chromium in the natural environment. When Cr(VI) in solution is reduced to Cr(III), which is less soluble and more particle-reactive, it is readily removed from solution. In such redox reactions, the lighter isotopes are more reactive and so when Cr is partitioned between two species, the products will have a lower $^{53}\text{Cr}/^{52}\text{Cr}$ ratios, and the remaining reactants will have higher ratios. The extent of isotope fractionation depends upon the specific reduction mechanisms involved.

Early studies found that Cr isotope fractionation factors were negligible for equilibrium adsorption processes, relative small for abiotic Cr(VI) reduction processes, and large for biotic Cr(VI) reduction processes [Ellis *et al.*, 2002, 2004]. Therefore, depending on the mechanism, Cr isotope signatures can be used to indicate the extent of Cr(VI) reduction [Ellis *et al.*, 2002] in the modern environment and even prevailing and historic redox conditions [Berna *et al.*, 2010; Ellis *et al.*, 2002]. They may be of particular useful in monitoring the reduction processes of anthropogenic contaminant plumes [Berna *et al.*, 2010]. Also, Cr isotopes in sedimentary deposits can be interpreted as reflecting atmospheric oxygen levels in the past, and have been used as an indicator of paleo-environmental conditions [Frei and Polat, 2013; Frei *et al.*, 2013]. However, with more data published over the past decade, it is now clear that Cr isotope fractionations processes are rather complicated and are controlled by many factors in the environment.

Abiotic reduction, which occurs both in the aqueous phase and on mineral surfaces, is important in controlling the behaviour of Cr and can be monitored using Cr isotopes. For example, Zink *et al.* [2010] reported Cr reduction by hydrogen peroxide in aqueous media under highly acidic and circum-neutral conditions, with fractionation factors lying between -3.5 ‰ and -5.0 ‰. Basu and Johnson [2012] conducted Cr reduction experiments with a range of different minerals and have reported fractionation factors from -2.11 to -3.91 ‰.

Microbial activity can also induce large difference in Cr isotope variations [Basu *et al.*, 2014; Han *et al.*, 2012; Sikora *et al.*, 2008]. Sikora *et al.* [2008] were the first to investigate the impact of microbial reduction on Cr isotope fractionation by *Shewanella oneidensis* MR-1 and found that the isotope fractionation factor ranged from -1.8 to -4.5 ‰. Han *et al.* [2012] investigated Cr fractionation factors (ϵ) during reduction by *Pseudomonas stutzeri* RCH2 under both aerobic and denitrifying conditions, and reported values of -2 ‰ aerobically and -0.4 ‰ respectively. Basu *et al.* [2014] examined Cr(VI) reduction by a metabolically diverse group of bacteria, and suggested that stronger isotopic fractionation was induced during Cr(VI) reduction under electron-donor-poor conditions. In our previous work, we found that the types of electron donors utilised and the bacterial respiration pathways

involved can result in significant differences in microbial Cr isotope fractionation factors [Zhang *et al.*, 2018a].

Considering the complexity of the environment, in order to improve the exploitation of Cr isotope signatures as a diagnostic tool of reduction processes, the relationship between environmental conditions and Cr isotope fractionation factors must be resolved. However, such relationships have not been well characterised to date. Therefore, further information is still required regarding the variation and mechanisms by which Cr isotope fractionation signatures are generated, in order to enhance its potential as an environmental diagnostic tool.

Previous studies of Cr(VI) reduction have mostly focused on either purely abiotic reactions or purely biotic reactions using suspended bacteria. Cr(VI) reduction involving metal-mineral-microbe interactions as seen in the natural environment have seldom been considered [Chorover *et al.*, 2007; Sparks, 2005]. Microbial processes can be significantly influenced by the presence of minerals, including nutrient acquisition, cell adhesion, biofilm formation, and energy generation [Brown *et al.*, 2008; Hochella, 2002]. Metals may also be adsorbed onto mineral surfaces, and can then be transformed by microbial activity [Chorover *et al.*, 2007; Gadd, 2010; Kraemer *et al.*, 1999]. Mineral surfaces have different physico-chemical properties that affect the biology of microbes, including micro-topography, composition, charge, and hydrophobicity. These properties play an important role in microbial attachment, which can critically affect colonization, biofilm formation, and other microbial metabolic processes [Brown *et al.*, 2008; Gadd, 2010; Vaughan *et al.*, 2002].

Fe minerals are ubiquitous in the natural environment as primary and secondary minerals, and are particularly interesting here because many Cr(VI)-reducing bacteria (*e.g.* *Shewanella oneidensis* MR-1) can also reduce Fe(III) to Fe(II), which can then readily reduce Cr(VI). In this case, reduction of Cr(VI) can be either the result of direct enzymatic reactions or of extracellular reactions with Fe(II) produced by bacteria. For instance, Stewart *et al.* [2007] studied microbially mediated chromate reduction in alkaline soil water systems and discovered that the percentage of Fe(II) to total Fe increased when nearly all the chromate had been reduced. Although it was suggested that Cr(VI) reduction was mediated directly through enzymatic processes, it is also possible that the reduction of Cr(VI) was caused by Fe(II) produced from microbially mediated iron reduction. These two pathways can be distinguished if these processes are found to have different isotope fractionation factors [Zhang *et al.*, 2018a]. Unfortunately, data are still limited on what impacts the presence of minerals, particularly Fe minerals, have on bacterial Cr(VI) reduction activity. Therefore, the objective of this study is to characterise the impacts of different forms of iron and minerals have on bacterial Cr(VI) reduction and isotope fractionation factors to further develop Cr isotopes as an environmental diagnostic tool. Specifically, anaerobic experiments were conducted to determine the differences in the rates of

microbial reduction of Cr(VI) to Cr(III) and the associated isotope fractionations in the presence of aqueous iron and iron minerals. The present study is the first to investigate Cr isotope fractionation factors associated with Cr-mineral-bacteria interactions under anaerobic conditions, and then to use Cr isotope signatures to indicate possible bio-reduction mechanisms and pathways.

2. Materials and Methods

2.1. Bacteria

The facultative anaerobes *Shewanella oneidensis* MR-1 (MR-1) and *Pseudomonas fluorescens* LB 300 (LB 300), were selected for this study. MR-1 was purchased from NCIMB Ltd (NCIMB 14063) and LB 300 was kindly provided by Prof. Yi-Tin Wang at the Department of Civil Engineering, University of Kentucky [Wang and Shen, 1995].

2.2. Cr reduction experiments

A series of experiments have been conducted anaerobically. In each experiment, dissolved Cr(VI) was combined with a microbial culture and either an aqueous or solid phase of iron (Table 1). Aliquots of the medium were periodically removed and analysed as Cr(VI) was progressively reduced.

The anaerobic growth medium and cell suspension medium were the same as used by Zhang *et al.* [2018a] The detailed compositions are listed in the supplementary materials section. Cells were cultured in large serum bottles containing degassed anaerobic growth medium and sealed with thick butyl rubber stoppers and aluminium crimp seals. The bottles were inoculated by transferring a single aerobically-grown colony. The cultures were then placed in a shaking incubator at 30 °C and 120 rpm until the desired cell density (5×10^{10} cells/mL) was reached. Ultra-high-purity N₂ gas (0.22 µm filtered) was used to fill headspaces, flush syringes prior to use, and inject into culture bottles to compensate for subsequent sample aliquot withdrawals.

Several forms of iron were used, including aqueous Fe(III), aqueous Fe(II), an iron(III) oxyhydroxide (FeO(OH)) goethite, and an iron(III) oxide (Fe₂O₃) hematite. Initial experimental concentrations of Fe(III) were 1 mM/L. This is substantially below the amount of FeCl₃ that can be dissolved in the minimal salt medium, which was experimentally determined to be > 10mM/L.

Prior to the experiments, both goethite and hematite were cleaned in an ultrasonic bath (with 18.2 MΩ cm water) for 30 mins and dried at 40 °C until a constant weight was achieved. Both minerals have been characterised by using X-ray Diffraction (XRD) and Raman microspectroscopy (Supplementary materials). The Cr(VI) reduction experiments were all conducted in triplicate following standard

procedures to account for the variability in microbial systems. The cell cultures were washed with degassed sterile NaCl solution (0.9%) and transferred to 100 mL serum bottles containing degassed cell suspension medium, which were then closed with thick blue butyl rubber stoppers and aluminum crimp seals. The cell densities were adjusted to 10^9 - 10^{10} /mL. Then Cr(VI), aqueous FeCl₃ solution, FeCl₂ solution, goethite, and hematite were added into the media depending upon the experiment (Table 1). Aliquots of 1 mL of sample were periodically taken and sterilized by filtration (0.22 µm), transferred to a clean Eppendorf tube, and stored at -80 °C before further treatment. The Cr(VI) concentrations were measured by HPLC (Thermo Scientific™ Dionex™ ICS-3000) using the method described in [Zhang *et al.*, 2018a].

2.3. Cr isotope analysis

The preparation method of samples for isotope composition determinations largely followed that of Zhang *et al.* (2018). A mixture of ⁵⁰Cr and ⁵⁴Cr were employed as a double-spike to correct for isotopic fractionation occurring during sample preparation and mass spectrometry, and added to the sample to provide a ⁵⁰Cr/⁵⁴Cr ratio of approximately 1:1. Cr isotope measurements were performed on a Thermo Finnigan Triton TIMS instrument. The dried sample was dissolved in 1 µL 0.1 M HNO₃, and loaded onto a Re filament. One µL of silica gel activator was then added on top of the Cr sample. The data acquisition method used was described by [Zhang *et al.*, 2018a]. Briefly, filaments were slowly heated to a final temperature of between 1170 °C and 1230 °C. The ⁵⁰Cr/⁵²Cr, ⁵³Cr/⁵²Cr, and ⁵⁴Cr/⁵²Cr ratios were determined by simultaneous measurement of the ion beams on a multiple collector array. V and Fe interferences were monitored on mass 51 (99.76 % of V) and 56 (91.66 % of Fe) [May and Wiedmeyer, 1998; Zhang *et al.*, 2018a; Zhang *et al.*, 2018b], and both were found to be negligible. Chromium isotope data are reported as relative deviations from a standard reference material (NIST SRM 979) in parts per thousand (per mil or ‰):

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

The long-term instrumental reproducibility was determined by repeatedly measuring a spiked NIST SRM 979 Cr standard, obtaining a $\delta^{53}\text{Cr}$ value of 0 ± 0.049 ‰ (normalised to the daily average value). In this study, the Cr isotope standard was measured alongside each batch of samples.

2.4. Characterization of bacteria-mineral interaction by SEM

To gain a better understanding of the metal-microbe-mineral interactions in this study, the bacterial cells were imaged using a JEOL JSM-6390 Scanning Electron Microscope (SEM), after 24 hours of incubation with Fe minerals and 20 mg/L Cr(VI). The samples from each experimental group were

primarily fixed by using 2.5 % glutaraldehyde in a 0.1 M phosphate buffer at pH 7.2 to 7.4 and incubated at room temperature for 1 hour. They were then washed several times with phosphate buffer and fixed with 1 % OsO₄ buffer at 4 °C. After being washed several times with Milli-Q water, the samples were dehydrated with different concentrations of EtOH under ambient conditions and then dried using HMDS (Hexamethyldisilazane). Finally, they were mounted on carbon adhesive tape on SEM stubs and coated with gold before being examined by SEM at 10 kV.

3. Results

3.1. Fe minerals

The rates of microbial Cr(VI) reduction in the presence of goethite and hematite are plotted in Figure 1. All concentration data reported here are the average values for the group of triplicates of each experiment, with uncertainties given as the standard deviation on the three values. During each experiment, the dissolved Cr(VI) concentration progressively decreased. Compared to the control group, which contained only the minimal salts medium with bacteria and Cr(VI) solution, the presence of either goethite or hematite inhibited the reduction of Cr(VI) by *Pseudomonas*, and the reduction rates in the experiments with minerals were much lower than those without minerals. In the control group, 80% of the Cr(VI) was reduced in the first 4 hours, whereas in the groups with minerals only 50% was reduced. The specific reduction rates, in mg Cr(VI) reduced per hour per cell, are shown in Table 1. In this study, cell counts were kept the same in each bottle, and the optical densities were measured at the beginning of the experiments. The average time for 50% Cr(VI) reduction in each group is also shown in Table 1.

In contrast to the experiments with *Pseudomonas*, the presence of minerals greatly promoted bacterial Cr(VI) reduction activity by *Shewanella*. The calculated cellular reduction rates were one order of magnitude greater with minerals present in the medium, and so the time for 50% Cr(VI) reduction were much shorter (Table 1). Of the two minerals that were used in this study, goethite promoted a greater reduction rate of Cr(VI).

Throughout the Cr(VI) reduction experiments, no Fe(II) was detected by Ferrozine Assay [Carter, 1971] for experiments that had both bacteria, minerals, and Cr(VI) in the solution (Fig.S3). In the control experiments where only bacteria and either mineral were present (no Cr(VI)), Fe(II) was found to be generated by both bacteria (Fig. S3), although Fe(III) reduction rates by *Pseudomonas* were found to be much slower than those by *Shewanella* (Fig. S3). The isotope results are plotted in Figure 2. Significant isotopic fractionation associated with microbial Cr(VI) removal was observed in every experiment, in the presence and absence of Fe-bearing minerals in the medium. The $\delta^{53}\text{Cr}$ values of the remaining Cr(VI) increased progressively from the initial value of $\delta^{53}\text{Cr}$ ($0.00 \pm 0.11\text{‰}$) as the reaction

advanced (Fig. 2). In this study, Cr(VI) reduction experiments were performed in a well-mixed, closed environment. With no further source of Cr(VI) and no removal mechanisms for Cr(VI) other than by microbial reduction, if the isotopic fractionation factor α is constant over time under these conditions, the $\delta^{53}\text{Cr}$ value of the remaining Cr(VI) in the system evolves according to a Rayleigh distillation relationship, which can be expressed as:

$$R_{\text{Cr(VI)}} = R_{\text{Cr(VI)}}^0 f^{\alpha-1} \quad (2)$$

where $R_{\text{Cr(VI)}}$ is the $\frac{^{53}\text{Cr}}{^{52}\text{Cr}}$ ratio in the remaining Cr(VI), $R_{\text{Cr(VI)}}^0$ is that in the original Cr(VI) pool. α is the isotope fractionation factor, and f is the fraction of the Cr(VI) remaining after reduction. The best-fit values for α can be found by plotting a linearised version equation of Eq.2 by linear regression (Fig.S5-Fig.S7). Uncertainties were calculated as 2×standard errors on the best-fit slopes. Our results are expressed as ε

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

For *Pseudomonas*, there was no significant difference in ε between the values obtained for the control experiment and those for the experiment with goethite. The fractionation factors were -2.56 ± 0.51 and -2.96 ± 0.19 ‰ for the two experiments, respectively, indicating that the presence of goethite did not have a detectable impact on Cr isotope fractionation. For the experiment with hematite a greater fractionation factor (-4.26 ± 0.71 ‰) was found. It is not clear if the difference is due to microbial fractionation or mineral adsorption fractionation, since fractionation factors for adsorption on goethite and hematite were not investigated in this study.

For *Shewanella*, the isotopic fractionation factor in the absence of iron was -2.47 ± 0.07 ‰, and for those with goethite and hematite it was -2.13 ± 0.41 and -2.27 ± 0.29 ‰, respectively. This shows that the presence of these two minerals did not have a resolvable effect on the microbial Cr isotope fractionation factor and so likely had little influence on microbial Cr reduction pathways.

3.2. Aqueous Fe(III)

In the reduction experiment with *Pseudomonas*, the presence of aqueous Fe(III) in the medium had no detectable impact on the reduction process (Fig. 1). The reduction rate was similar in the experiment without Fe(III); both were very fast (Table 1), with all the Cr reduced within the first 8 hours. In the study with *Shewanella*, there was also no significant difference ($p > 0.05$) between the control (no iron) and the experiment with aqueous Fe(III). The Cr isotope data for *Pseudomonas* and *Shewanella* are both shown in Fig. 2. The best-fit fractionation factor for *Pseudomonas* is -3.0 ± 0.82 ‰, and -2.34 ± 0.07 ‰ for *Shewanella* with aqueous Fe(III) in the medium; both of these are essentially indistinguishable from

that in the correspondingly controls. Overall, this indicates that the presence of soluble Fe(III) had little impact on chromium isotope fractionation by bacteria.

3.3. Fe(II)

To investigate further the influence of iron, experiments with added aqueous Fe(II) (0.5mM) were conducted (Fig. 3). The reduction rates were found to be extremely fast in all of the groups with added Fe(II); about 65 % of Cr(VI) was reduced by Fe(II) in the first minute. After this initial rapid loss, the rest of the Cr(VI) was gradually reduced by the bacteria (*Pseudomonas*) over the following 4 hours. Bacterial reduction rates in the experiments having only aqueous Fe(II) were slightly higher than those with both Fe(II) and Fe(III) minerals, but the differences were not significant ($p > 0.05$, Fig. 3, Table 1).

The Cr isotope data are also presented in Fig. 3. After the quick initial loss, subsequent reduction of Cr(VI) was due to microbial activity, with Cr isotope fractionation factors that were much higher. The best-fit ϵ value for microbial reduction was -3.3 ‰ in all experiments with Fe(II) (considering the point of initial loss as the start of microbial reduction, solid line in Fig. 3), regardless of whether there were any minerals in the system. The presence of iron minerals or aqueous iron species in the environment did not appear to greatly affect the magnitude of microbial Cr(VI) isotope fractionation.

3.4. SEM Analysis

In order to obtain further insights into microbial Cr reduction mechanisms and microbe-mineral interactions, scanning electron microscopy (SEM) studies were undertaken, and selected images are shown in Fig. 4. Suspended cells of *Pseudomonas* and *Shewanella* in the two control groups, without minerals and Cr(VI), are illustrated in Figs. 4A and 4B, respectively. *Pseudomonas* displayed rough surfaces and possessed multiple polar flagella that aid the motility of the cells. The flagella have also been found to be essential for cells to interact with abiotic surfaces [O'Toole George and Kolter, 1998a; b]. *Pseudomonas* is about 1 to 2 μm in size, and is smaller than *Shewanella*, which is about 2 to 3 μm . The cell surfaces of *Shewanella* were much smoother compared to *Pseudomonas*. Furthermore, nanowires produced by *Shewanella* were observed in this study (Fig.4B, Fig.S4 (white arrows)). These have been shown to be conductive and able to transfer electrons extracellularly [Pirbadian et al., 2014]. Figs. 4C and 4E show the attachment of *Pseudomonas* onto goethite and hematite surfaces. With both minerals, it was very clear that the bacteria produced many flagella and extracellular polymeric substances (EPS) that helped the cells adhere to the grain surfaces and to each other. The attached cells formed several layers on the mineral surfaces. The interaction of *Shewanella oneidensis* MR-1 with the two minerals is shown in Figs. 4D (with goethite) and 4F (with hematite). The species is known to form

biofilms under various conditions; however, compared with *Pseudomonas*, the attachment of *Shewanella* on the mineral surfaces were much looser and no EPS was observed.

4. Discussion

4.1. Does biogenic Fe(II) contribute to Cr(VI) reduction?

Very different effects of iron oxides on the microbial Cr(VI) reduction rates by the two different bacteria were observed. The presence of minerals largely increased the reduction rates by *Shewanella* but significantly slowed down the rates by *Pseudomonas*. To explain these differences, one obvious question was whether any Fe(II) had been produced by bacterial Fe(III) reduction and the extent it contributed to Cr(VI) reduction. Several studies have confirmed that the Fe present in the minerals have been shown to be bioavailable to bacteria such as *Shewanella oneidensis* [Lower *et al.*, 2001], *Geobacter sulfurreducens* [Wu *et al.*, 2009], *Shewanella putrefaciens* CN32 [Liu *et al.*, 2001], and *Shewanella alga* BrY [Roden and Zachara, 1996]. However, this has been challenged by other studies [Cutting *et al.*, 2009] which showed that in crystalline form the iron was not available and only amorphous coatings or nanoscale granular substrates were biologically reduced. Nonetheless it is feasible that the presence of those minerals in the environment, particularly where amorphous surfaces are likely to be present, may significantly impact Cr(VI) reduction on both biotical and abiotical processes. In bacterial reduction experiments when both an Fe(III)-bearing mineral and Cr(VI) were present in solution, no Fe(II) was detected by the Ferrozine assay (Fig. S3). This is consistent with the bacteria reducing Cr(VI) first, when dissolved Cr(VI) and Fe(III) minerals were both present in the system. However, it is also possible that the bacteria reduced Fe(III) first, generating Fe(II) which rapidly re-oxidised by Cr(VI) and serving as an electron shuttle in the system. In this case, the rates of Cr(VI) reduction depended on the rates of Fe(II) generated by the bacteria, and calculations must be made to determine if biogenic Fe(II) made a substantial contribution to microbial Cr(VI) reduction.

In the Fe(III)-mineral reduction experiment with *Pseudomonas fluorescens* (Fig. S3), extremely slow Fe(III) reduction rates were detected: $2 \pm 1 \times 10^{-13}$ mg/L Fe(II)/hour/cell for goethite and $1.5 \pm 0.2 \times 10^{-14}$ mg/L Fe(II)/hour/cell for hematite, respectively. Even if it is assumed that the rate of Fe(II) reduction Cr(VI) was extremely fast, which has indeed been suggested in previous studies [Buerge and Hug, 1997; Kitchen *et al.*, 2012], with such a low rate of Fe(II) generation, the associated Cr(VI) reduction rate would be orders of magnitude slower than the rate observed in this experiment. This suggests that biogenic Fe(II) was unlikely to have been the main reductant for Cr(VI).

For *Shewanella oneidensis* MR-1, a known iron-reducer, similar calculations can be made. The bacteria generated Fe(II) at a rate of $9 \pm 5 \times 10^{-12}$ mg/L/hour/cell for goethite, and $2.0 \pm 0.1 \times 10^{-12}$ mg/L/hour/cell for hematite respectively. At such rates, less than 5% of Cr(VI) was reduced by the biogenic Fe(II).

Therefore, biogenic Fe(II) from *Shewanella oneidensis* is unlikely to have been the main reductant for Cr(VI) either. Here Cr isotope data can also provide some insight into the process. If the bacteria reduced Fe(III) first, generating Fe(II), which then reduced Cr(VI), the isotope fractionation factor should be close to -1.5 ‰ (dotted line in Fig. 2, lower panel; (Dossing et al., 2011)), which was determined in abiotic Cr(VI) reduction experiments with Fe(II) at pH conditions close to those in this study. If Cr(VI) reduction was due to bacterial activity, then the isotope fractionation factors measured in the presence of minerals should be $-2.47 \pm 0.07\text{‰}$, as shown in Table 1. The results are consistent with a Cr isotope fractionation factor of $-2.13 \pm 0.41\text{‰}$ in the presence of goethite, and $-2.27 \pm 0.29\text{‰}$ in the presence of hematite. These values are indistinguishable from the value for bacterial reduction, and therefore is consistent with Cr(VI) reduction being completely attributed to bacterial directly reducing Cr(VI). In the presence of mineral phases, Cr(VI) reduction rates were much greater than that in the absence of minerals, and such an increase in reduction by an order of magnitude clearly cannot be explained by abiotic Cr(VI) reduction, given the isotope constraints. Therefore, it is likely that *Shewanella* substantial contribution to the observed accelerated Cr(VI) reduction rates.

4.2. Cr reduction pathways - insights from microbial-mineral interactions

Since biogenic Fe(II) from Fe(III) reduction did not make a substantial contribution to Cr(VI) reduction, the rate changes associated with the presence of minerals in the system were likely due to the impact of the minerals on the pathways the bacteria employed for Cr(VI) reduction. Most bacteria, including *Pseudomonas* and *Shewanella*, have negatively charged surfaces, and both goethite and hematite have positive surface charges under neutral pH conditions [Kumpulainen et al., 2008; Pan et al., 2014; Plaza et al., 2001; Zeltner and Anderson, 1988], so the bacteria can naturally adhere to the mineral surfaces by electrostatic attachment. This was confirmed by SEM imaging (Fig. 4). The electron-transfer pathways and the production of effective Cr reduction enzymes may have differed for the two strains under the growth conditions employed.

Bacterial Cr(VI) reduction mechanisms have been studied for over 20 years, resulting in three reduction pathways being identified (Fig. 5 and Table 2).

1) Both *Pseudomonas fluorescens* LB 300 and *Shewanella oneidensis* MR-1 have membrane-bound or intracellular enzymes that are responsible for Cr(VI) reduction [Bopp and Ehrlich, 1988; Michel et al., 2001; Myers et al., 2000]. As illustrated in Fig. 5A, when the enzymes are on the outer membrane, Cr(VI) can be reduced directly from outside the cell. In contrast, when the enzymes are localised on the inner membrane, Cr(VI) must be transported into the cell and then reduced [Myers and Myers, 1992; Myers et al., 2000]. If cells are attached and stacked on the mineral surface, the contactable surface area between bacteria and Cr(VI) decreases, and Cr(VI) reduction rates also decrease.

2) Bacteria can use self-made nanowires for electron transfer [El-Naggar *et al.*, 2010; Gorby *et al.*, 2006; Pirkadian *et al.*, 2014]. Even if the cells are immobilised, they can transfer electrons to Cr(VI) over long distances (see Fig. 5B). The electron transfer processes can be between bacteria and abiotic substrates, or even between two different bacteria. These electron transfer processes might be enhanced by the mineral, with positive charged surfaces enhancing the electron transfer gradient along the bacterial nanowires, so the rate increases.

3) Bacteria can produce extracellular electron shuttles or water-soluble enzymes for Cr(VI) reduction [Lies *et al.*, 2005; Michel *et al.*, 2001] (see Fig. 5C). This pathway cannot be significantly impacted by the presence of the minerals.

Overall, the effect of the presence of mineral on the rate of Cr reduction reflected the mechanism involved.

To determine the possible pathways, we identified multiple genes that are responsible for Cr(VI) reduction in the bacteria studied here. We conducted BLAST searches (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>) on published genome sequences of both bacteria [Heidelberg *et al.*, 2002; Silby *et al.*, 2009], to identify sequences of multiple (membrane-bound or intracellular) enzymes [Michel *et al.*, 2001] known to reduce Cr(VI) and the genes related to extracellular electron transfer [Gorby *et al.*, 2006] (see Table 2). A number of bacteria that were used in previous Cr isotope studies were also analysed here for comparison.

Clearly, *Pseudomonas fluorescens* does not have the genes to allow an extracellular electron transport pathway (Table 2), and there was no evidence to suggest that their flagella were conductive. Therefore, pathway 1 was the most likely route for Cr reduction by *Pseudomonas fluorescens* [Bopp and Ehrlich, 1988], and this may explain why a significant decrease of Cr(VI) reduction was observed in the experiment in the presence of minerals (Fig. 1, Fig 5A). The same effects are expected to be seen for other bacteria that require intracellular Cr(VI) reduction, and for these exogenous electron donors may be important to enable the expression of the different enzymes. Interestingly, when bacteria (using intracellular pathway) that were taxonomically distinct employed the same electron donor (acetate), this induced identical Cr isotope fractionation factor ($3.12 \pm 0.08 \text{ ‰}$) (Fig. 6A).

For *Shewanella oneidensis* MR-1, the three pathways have all been shown to be responsible for Cr(VI) reduction (Table 2, Fig. 4) [Gorby *et al.*, 2006; Lies *et al.*, 2005; Myers *et al.*, 2000], but the relative importance of the three pathways have not been investigated before. In this study, the presence of the minerals clearly increased Cr(VI) reduction rates, indicating that the electron transfer had been significantly enhanced. Of the three pathways, only the nanowire mechanism would be enhanced by the presence of minerals. This may explain the greater reduction rate observed for *Shewanella*

oneidensis MR-1 observed in this study. The unchanged ϵ value (-2.09 ± 0.33 ‰) under various conditions, in the presence and absence of minerals, or with different exogenous electron donors, suggests that the pathway the bacteria used for Cr(VI) reduction remained the same (Fig. 6B), indicating that extracellular electron transfer may be the dominant pathway for *Shewanella* under all anaerobic conditions. However, when exogenous electron donors are limited, the pathway may change, and so alter the Cr isotope fractionation factor [Sikora *et al.*, 2008; Zhang *et al.*, 2018a].

4.3. The impact of abiogenic iron

In our previous experiments [Zhang *et al.*, 2018a] utilizing the same bacteria but without Fe(II), fast initial loss of Cr(VI) was not detected. This suggests that this loss may be ascribed to the abiotic reaction with Fe(II). Chromium isotope fractionation during reduction of Cr(VI) by aqueous Fe(II) has been investigated previously by Dossing *et al.* [2011] and Kitchen *et al.* [2012]. The fractionation factors were found to be -1.5 ‰ when pH ranged between 6.8 and 8.1 [Dossing *et al.*, 2011], and -4.2 ‰ when pH ranged from 4.0 to 5.3 [Dossing *et al.*, 2011; Kitchen *et al.*, 2012]. In this study, the pH in the buffer solution was maintained at pH 7, which is closer to the conditions used by Dossing *et al.* [2011]. The best-fit Rayleigh distillation curve is shown in Figure 3, with $\epsilon = -1.9$ ‰, and consistent with the data from Dossing *et al.* [2011]. After the initial Cr(VI) loss, the subsequent reduction of chromium was attributed to microbial activity, and the best-fit ϵ value for the microbial reduction is -3.3 ‰ in all groups with Fe(II), regardless of whether there were any minerals in the system. As with the experiments without Fe(II), although the remaining Cr(VI) concentrations were quite low after reduction by Fe(II), the specific reduction rates by bacteria in groups with minerals were lower than those without minerals, demonstrating the presence of minerals slowed down the reduction of Cr(VI) by *Pseudomonas*. The isotope data, are unlike those in experiments without Fe(II), where hematite had a more significant impact on bacterial Cr(VI) isotope fractionation. In this experiment, the presence of both iron minerals did not affect microbial Cr(VI) isotope fractionation, suggesting that the reduction pathways were identical under these conditions. This might have been due to the reaction of Fe(II) and Cr(VI) at the beginning of the experiment. The reaction generated the same Fe(III) minerals on surfaces of the two different minerals present in the medium, and therefore had the same impact on bacterial Cr(VI) isotope fractionation.

4.4. Perspectives on the results

4.4.1. In the field of geochemistry

Our current study identified the complexity of bacteria-mineral interactions on Cr isotope fractionation, and suggested that many factors need to be taken into consideration when using Cr isotope as proxies in the environment where various bacteria and minerals may be present. The potential of using Cr

isotopes as a powerful redox proxy for environmental and paleo-environmental studies [Frei and Polat, 2013; Frei *et al.*, 2014; Frei *et al.*, 2013; Gueguen *et al.*, 2016] depends upon understanding the constraints on isotope fractionations associated with different processes, such as oxidative weathering and riverine transport of Cr and the reduction of Cr(VI) during burial [Gueguen *et al.*, 2016]. For example, recent studies by Wu *et al.* [2017] and D'Arcy *et al.* [2016] on Cr isotopes in rivers revealed that the variations of Cr isotopes were not all inherited from rocks in the catchment, suggesting that other factors, such as prevailing redox conditions and dissolved organic matter, may also be responsible for the observed variability. Here we demonstrate that minerals in the environment may have a significant impact on microbial Cr fractionation factors, and this may provide an additional explanation for the variability of Cr isotopes in rivers where microbial Cr reduction occurs.

4.4.2. In the field of bioremediation

The two bacteria studied here are representative for two major groups that employ different electron transport pathways (intracellular/extracellular) in the contaminated area. Depending on the pathways, different methods may be used to enhance the reduction activity by these different bacteria for bioremediation. The dominant species in the local area is an important factor in determining the remediation strategy. If the dominant bacteria use intracellular reduction pathways, the types of electron donors are important [Xu *et al.*, 2011; Zhang *et al.*, 2018a]. If the bacteria predominantly use extracellular electron transfer, an electric field may be used to enhance their activity [Yin *et al.*, 2015].

4.4.3. In the field of microbiology.

The observations and interpretations from this study can be expanded into studying specific molecular pathways in order to obtain a fundamental understanding of the electron transfer processes in cells. Further work can be done to study the ability to reduce Cr(VI) by genetic modified bacteria (*e.g.* *Shewanella*, [Gorby *et al.*, 2006]) which lack the function of making nanowires, or to specifically overexpress certain enzyme, to determine the detailed molecular pathways associated with Cr isotope fractionation, and further develop Cr isotope signatures to precisely indicate different functions in biological metabolisms.

5. Conclusions

Although metal-mineral-microbe interactions are important in the natural environment, there are limited data available on the specific processes involved. In this study, we used isotope fractionations to identify differences in Cr(VI) reduction processes, and obtained the first isotope data about the impact of different forms of Fe, including aqueous Fe(III), aqueous Fe(II), goethite and hematite, on microbial Cr reduction processes.

The presence of both goethite or hematite inhibited Cr(VI) reduction rates by *Pseudomonas fluorescens* LB 300, but accelerated those by *Shewanella oneidensis* MR-1. These differences were most likely related to the mode of bacterial attachment to the minerals, as well as differences in the underlying reduction mechanisms of each bacterial species. The Cr isotopic fractionation factors were independent of the presence of minerals for *Shewanella*. However, for *Pseudomonas*, the value of ϵ was higher in the presence of hematite. Under the same laboratory conditions, the isotope fractionation factors for different bacteria were may have significant impacts on microbial Cr fractionation factors. Therefore, to estimate the extent of Cr reduction in particular environments, data from natural samples are required. For example, where isotope shifts are used to determine the extent of Cr removal by microbial reduction in Cr contaminated areas [Ellis *et al.*, 2002], site-specific ϵ values must be obtained from experiments using natural samples and the local microbial populations. If using Cr isotopes as a paleo-redox proxy, variations in mineralogy and the associated changes of bacterial communities should be taken into account.

Aqueous Fe(III) in solution did not have a detectable effect on either bacterial Cr reduction rates nor the Cr isotopic fractionation factors, indicating that reduction of Cr(VI) occurs prior to that of Fe(III). By contrast, the presence of aqueous Fe(II) induced rapid reduction of Cr(VI), but has little impact on bacterial Cr(VI) reduction rates or isotopic fractionation.

SEM images confirmed that the attachment of *Pseudomonas fluorescens* LB 300 onto mineral surfaces was much closer than that of *Shewanella oneidensis* MR-1, and this was most likely due to the flagella on the cell surfaces and the EPS produced by *Pseudomonas*. These images also showed that *Shewanella oneidensis* MR-1 can produce bacterial nanowires, which may be involved in electron transfer processes for the bacteria.

Overall, bacterial Cr isotope fractionation processes are independent of the presence of aqueous iron, but may be impacted by the presence of minerals in the surrounding environment. In addition, the results in this study show that Cr isotopes are useful in distinguishing between biological Cr(VI) reduction and abiotic Cr(VI) reduction (by Fe(II)), and between different biological processes. The results indicate that the dominant Cr(VI) reduction pathway for *Pseudomonas fluorescens* LB 300 is possibly membrane-bound enzymes, while for *Shewanella oneidensis* MR-1 it may be via bacterial nanowires. The observations and interpretations from this study have great potential to be used in the field of geochemistry, bioremediation, and microbiology.

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- 463 Basu, A., and T. M. Johnson (2012), Determination of Hexavalent Chromium Reduction Using
464 Cr Stable Isotopes: Isotopic Fractionation Factors for Permeable Reactive Barrier Materials,
465 *Environ Sci Technol*, 46(10), 5353-5360.
- 466 Basu, A., T. M. Johnson, and R. A. Sanford (2014), Cr isotope fractionation factors for Cr(VI)
467 reduction by a metabolically diverse group of bacteria, *Geochim Cosmochim Ac*, 142(0), 349-
468 361.
- 469 Berna, E. C., T. M. Johnson, R. S. Makdisi, and A. Basui (2010), Cr Stable Isotopes As Indicators
470 of Cr(VI) Reduction in Groundwater: A Detailed Time-Series Study of a Point-Source Plume,
471 *Environ Sci Technol*, 44(3), 1043-1048.
- 472 Bopp, L., and H. Ehrlich (1988), Chromate resistance and reduction in *Pseudomonas*
473 *fluorescens* strain LB300, *Arch Microbiol*, 150(5), 426-431.
- 474 Brown, G. E., T. P. Trainor, and A. M. Chaka (2008), Geochemistry of Mineral Surfaces and
475 Factors Affecting Their Chemical Reactivity, *Chemical Bonding at Surfaces and Interfaces*, 457-
476 509.
- 477 Buerge, I. J., and S. J. Hug (1997), Kinetics and pH Dependence of Chromium(VI) Reduction by
478 Iron(II), *Environ Sci Technol*, 31(5), 1426-1432.
- 479 Carter, P. (1971), Spectrophotometric Determination of Serum Iron at Submicrogram Level
480 with a New Reagent (Ferrozine), *Anal Biochem*, 40(2), 450-&.
- 481 Chakraborty, R., et al. (2017), Complete genome sequence of *Pseudomonas stutzeri* strain
482 RCH2 isolated from a Hexavalent Chromium [Cr(VI)] contaminated site, *Stand Genomic Sci*,
483 12.
- 484 Chorover, J., R. Kretzschmar, F. Garcia-Pichel, and D. L. Sparks (2007), Soil biogeochemical
485 processes within the Critical Zone, *Elements*, 3(5), 321-326.
- 486 Cutting, R. S., V. S. Coker, J. W. Fellowes, J. R. Lloyd, and D. J. Vaughan (2009), Mineralogical
487 and morphological constraints on the reduction of Fe(III) minerals by *Geobacter*
488 *sulfurreducens*, *Geochim Cosmochim Ac*, 73(14), 4004-4022.
- 489 D'Arcy, J., M. G. Babechuk, L. N. Dossing, C. Gaucher, and R. Frei (2016), Processes controlling
490 the chromium isotopic composition of river water: Constraints from basaltic river catchments,
491 *Geochim Cosmochim Ac*, 186, 296-315.
- 492 Dossing, L. N., K. Dideriksen, S. L. S. Stipp, and R. Frei (2011), Reduction of hexavalent
493 chromium by ferrous iron: A process of chromium isotope fractionation and its relevance to
494 natural environments, *Chem Geol*, 285(1-4), 157-166.
- 495 El-Naggar, M. Y., G. Wanger, K. M. Leung, T. D. Yuzvinsky, G. Southam, J. Yang, W. M. Lau, K.
496 H. Nealson, and Y. A. Gorby (2010), Electrical transport along bacterial nanowires from
497 *Shewanella oneidensis* MR-1, *P Natl Acad Sci USA*, 107(42), 18127-18131.
- 498 Ellis, A. S., T. M. Johnson, and T. D. Bullen (2002), Chromium isotopes and the fate of
499 hexavalent chromium in the environment, *Science*, 295(5562), 2060-2062.
- 500 Ellis, A. S., T. M. Johnson, and T. D. Bullen (2004), Using chromium stable isotope ratios to
501 quantify Cr(VI) reduction: Lack of sorption effects, *Environ Sci Technol*, 38(13), 3604-3607.
- 502 Frei, R., and A. Polat (2013), Chromium isotope fractionation during oxidative weathering-
503 Implications from the study of a Paleoproterozoic (ca. 1.9 Ga) paleosol, Schreiber Beach,
504 Ontario, Canada, *Precambrian Res*, 224, 434-453.
- 505 Frei, R., D. Poiré, and K. M. Frei (2014), Weathering on land and transport of chromium to the
506 ocean in a subtropical region (Misiones, NW Argentina): A chromium stable isotope
507 perspective, *Chem Geol*, 381(0), 110-124.

508 Frei, R., C. Gaucher, D. Stolper, and D. E. Canfield (2013), Fluctuations in late Neoproterozoic
 509 atmospheric oxidation — Cr isotope chemostratigraphy and iron speciation of the late
 510 Ediacaran lower Arroyo del Soldado Group (Uruguay), *Gondwana Research*, 23(2), 797-811.
 511 Gadd, G. M. (2010), Metals, minerals and microbes: geomicrobiology and bioremediation,
 512 *Microbiol-Sgm*, 156, 609-643.
 513 Gorby, Y. A., et al. (2006), Electrically conductive bacterial nanowires produced by *Shewanella*
 514 *oneidensis* strain MR-1 and other microorganisms, *P Natl Acad Sci USA*, 103(30), 11358-11363.
 515 Gueguen, B., C. T. Reinhard, T. J. Algeo, L. C. Peterson, S. G. Nielsen, X. L. Wang, H. Rowe, and
 516 N. J. Planavsky (2016), The chromium isotope composition of reducing and oxic marine
 517 sediments, *Geochim Cosmochim Ac*, 184, 1-19.
 518 Han, R. Y., L. P. Qin, S. T. Brown, J. N. Christensen, and H. R. Beller (2012), Differential Isotopic
 519 Fractionation during Cr(VI) Reduction by an Aquifer-Derived Bacterium under Aerobic versus
 520 Denitrifying Conditions, *Appl Environ Microb*, 78(7), 2462-2464.
 521 Heidelberg, J. F., et al. (2004), The genome sequence of the anaerobic, sulfate-reducing
 522 bacterium *Desulfovibrio vulgaris* Hildenborough, *Nature Biotechnology*, 22(5), 554-559.
 523 Heidelberg, J. F., et al. (2002), Genome sequence of the dissimilatory metal ion-reducing
 524 bacterium *Shewanella oneidensis*, *Nature Biotechnology*, 20, 1118.
 525 Hochella, M. F. (2002), Sustaining Earth: Thoughts on the present and future roles of
 526 mineralogy in environmental science, *Mineral Mag*, 66(5), 627-652.
 527 Kim, D. H., S. Jiang, J. H. Lee, Y. J. Cho, J. Chun, S. H. Choi, H. S. Park, and H. G. Hur (2011),
 528 Draft Genome Sequence of *Shewanella* sp Strain HN-41, Which Produces Arsenic-Sulfide
 529 Nanotubes, *J Bacteriol*, 193(18), 5039-5040.
 530 Kitchen, J. W., T. M. Johnson, T. D. Bullen, J. M. Zhu, and A. Raddatz (2012), Chromium isotope
 531 fractionation factors for reduction of Cr(VI) by aqueous Fe(II) and organic molecules, *Geochim*
 532 *Cosmochim Ac*, 89, 190-201.
 533 Kraemer, S. M., S. F. Cheah, R. Zapf, J. D. Xu, K. N. Raymond, and G. Sposito (1999), Effect of
 534 hydroxamate siderophores on Fe release and Pb(II) adsorption by goethite, *Geochim*
 535 *Cosmochim Ac*, 63(19-20), 3003-3008.
 536 Kumpulainen, S., F. von der Kammer, and T. Hofmann (2008), Humic acid adsorption and
 537 surface charge effects on schwertmannite and goethite in acid, sulphate waters, *Water Res*,
 538 42(8-9), 2051-2060.
 539 Lies, D. P., M. E. Hernandez, A. Kappler, R. E. Mielke, J. A. Gralnick, and D. K. Newman (2005),
 540 *Shewanella oneidensis* MR-1 uses overlapping pathways for iron reduction at a distance and
 541 by direct contact under conditions relevant for biofilms, *Appl Environ Microb*, 71(8), 4414-
 542 4426.
 543 Liu, C. X., S. Kota, J. M. Zachara, J. K. Fredrickson, and C. K. Brinkman (2001), Kinetic analysis
 544 of the bacterial reduction of goethite, *Environ Sci Technol*, 35(12), 2482-2490.
 545 Lower, S. K., M. F. Hochella, and T. J. Beveridge (2001), Bacterial recognition of mineral
 546 surfaces: Nanoscale interactions between *Shewanella* and alpha-FeOOH, *Science*, 292(5520),
 547 1360-1363.
 548 May, T. W., and R. H. Wiedmeyer (1998), A table of polyatomic interferences in ICP-MS, *Atom*
 549 *Spectrosc*, 19(5), 150-155.
 550 Methe, B. A., et al. (2003), Genome of *Geobacter sulfurreducens*: Metal reduction in
 551 subsurface environments, *Science*, 302(5652), 1967-1969.
 552 Michel, C., M. Brugna, C. Aubert, A. Bernadac, and M. Bruschi (2001), Enzymatic reduction of
 553 chromate: comparative studies using sulfate reducing bacteria - Key role of polyheme
 554 cytochromes c and hydrogenases, *Applied Microbiology and Biotechnology*, 55(1), 95-100.

Myers, C. R., and J. M. Myers (1992), Localization of Cytochromes to the Outer-Membrane of Anaerobically Grown *Shewanella-Putrefaciens* Mr-1, *J Bacteriol*, 174(11), 3429-3438.

Myers, C. R., B. P. Carstens, W. E. Antholine, and J. M. Myers (2000), Chromium(VI) reductase activity is associated with the cytoplasmic membrane of anaerobically grown *Shewanella putrefaciens* MR-1, *J Appl Microbiol*, 88(1), 98-106.

O'Toole George, A., and R. Kolter (1998a), Flagellar and twitching motility are necessary for *Pseudomonas aeruginosa* biofilm development, *Molecular Microbiology*, 30(2), 295-304.

O'Toole George, A., and R. Kolter (1998b), Initiation of biofilm formation in *Pseudomonas fluorescens* WCS365 proceeds via multiple, convergent signalling pathways: a genetic analysis, *Molecular Microbiology*, 28(3), 449-461.

Pan, X., Z. Liu, Z. Chen, Y. Cheng, D. Pan, J. Shao, Z. Lin, and X. Guan (2014), Investigation of Cr(VI) reduction and Cr(III) immobilization mechanism by planktonic cells and biofilms of *Bacillus subtilis* ATCC-6633, *Water Res*, 55(0), 21-29.

Pirbadian, S., et al. (2014), *Shewanella oneidensis* MR-1 nanowires are outer membrane and periplasmic extensions of the extracellular electron transport components, *P Natl Acad Sci USA*, 111(35), 12883-12888.

Plaza, R. C., F. Gonzalez-Caballero, and A. V. Delgado (2001), Electrical surface charge and potential of hematite/yttrium oxide core-shell colloidal particles, *Colloid Polym Sci*, 279(12), 1206-1211.

Roden, E. E., and J. M. Zachara (1996), Microbial reduction of crystalline iron(III) oxides: Influence of oxide surface area and potential for cell growth, *Environ Sci Technol*, 30(5), 1618-1628.

Sikora, E. R., T. M. Johnson, and T. D. Bullen (2008), Microbial mass-dependent fractionation of chromium isotopes, *Geochim Cosmochim Acta*, 72(15), 3631-3641.

Silby, M. W., et al. (2009), Genomic and genetic analyses of diversity and plant interactions of *Pseudomonas fluorescens*, *Genome Biology*, 10(5), R51.

Sparks, D. L. (2005), Toxic metals in the environment: The role of surfaces, *Elements*, 1(4), 193-197.

Stewart, D. I., I. T. Burke, and R. J. G. Mortimer (2007), Stimulation of microbially mediated chromate reduction in alkaline soil-water systems, *Geomicrobiol J*, 24(7-8), 655-669.

Vaughan, D. J., R. A. D. Patrick, and R. A. Wogelius (2002), Minerals, metals and molecules: ore and environmental mineralogy in the new millennium, *Mineral Mag*, 66(5), 653-676.

Wang, Y. T., and H. Shen (1995), Bacterial Reduction of Hexavalent Chromium, *J Ind Microbiol*, 14(2), 159-163.

Wu, L. L., B. L. Beard, E. E. Roden, and C. M. Johnson (2009), Influence of pH and dissolved Si on Fe isotope fractionation during dissimilatory microbial reduction of hematite, *Geochim Cosmochim Acta*, 73(19), 5584-5599.

Wu, W. H., X. L. Wang, C. T. Reinhard, and N. J. Planavsky (2017), Chromium isotope systematics in the Connecticut River, *Chem Geol*, 456, 98-111.

Xu, L., M. F. Luo, W. L. Li, X. T. Wei, K. Xie, L. J. Liu, C. Y. Jiang, and H. Z. Liu (2011), Reduction of hexavalent chromium by *Pannonibacter phragmitetus* LSSE-09 stimulated with external electron donors under alkaline conditions, *J Hazard Mater*, 185(2-3), 1169-1176.

Yin, X., S. Qiao, and J. Zhou (2015), Using electric field to enhance the activity of anammox bacteria, *Applied Microbiology and Biotechnology*, 99(16), 6921-6930.

Zeltner, W. A., and M. A. Anderson (1988), Surface-Charge Development at the Goethite Aqueous-Solution Interface - Effects of Co₂ Adsorption, *Langmuir*, 4(2), 469-474.

601 Zhang, Q., K. Amor, S. J. G. Galer, I. Thompson, and D. Porcelli (2018a), Variations of stable
602 isotope fractionation during bacterial chromium reduction processes and their implications,
603 *Chemical Geology*, 481, 155-164.
604 Zhang, Q., J. T. Snow, P. Holdship, D. Price, P. Watson, and R. E. M. Rickaby (2018b), Direct
605 measurement of multi-elements in high matrix samples with a flow injection ICP-MS:
606 application to the extended *Emiliana huxleyi* Redfield ratio, *J Anal Atom Spectrom*, 33(7),
607 1196-1208.
608 Zink, S., R. Schoenberg, and M. Staubwasser (2010), Isotopic fractionation and reaction
609 kinetics between Cr(III) and Cr(VI) in aqueous media, *Geochim Cosmochim Ac*, 74(20), 5729-
610 5745.
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Table 1: Cell specific reduction rates and isotopic fractionation factors

Experiments	Cell specific reduction rate ($10^{-10} \text{ mg}\cdot\text{h}^{-1}\cdot\text{Cell}^{-1}$)	ϵ (‰)	Time for 50% Cr(VI) reduction (Hours)
<i>P. fluorescens</i>			
No Fe	4.62 ± 0.08	-2.56 ± 0.51	1.65
Aqueous Fe(III)	5.64 ± 0.09	-3.00 ± 0.82	1.57
Goethite	3.70 ± 0.59	-2.96 ± 0.19	3.43
Hematite	1.64 ± 0.07	-4.26 ± 0.71	4.66
Aqueous Fe(II)	2.94 ± 0.02	-3.39 ± 1.99	1.05
Goethite and Fe(II)	2.70 ± 0.00	-3.24 ± 0.39	0.68
Hematite and Fe(II)	1.71 ± 0.56	-3.29 ± 0.28	0.88
<i>S. oneidensis</i>			
No Fe	0.29 ± 0.07	-2.47 ± 0.07	30.34
Aqueous Fe(III)	0.30 ± 0.02	-2.34 ± 0.07	54.09
Goethite	2.59 ± 0.95	-2.13 ± 0.41	1.78
Hematite	1.65 ± 1.12	-2.27 ± 0.29	2.59

All experiments were done in triplicates.

Errors for cell-specific reduction rates are shown as standard deviations between the triplicates.

ϵ : Isotope fractionation factor. Errors are calculated based on least-squares fitting method.

All isotope analysis consists of measurement of 800 isotope ratios.

Table 2: BLAST search for chromium reductase in bacteria (<https://blast.ncbi.nlm.nih.gov/Blast.cgi>)

✓ means it has the sequence cover for the target protein, E. value < 10⁻⁵

Enzyme	Cr(VI) reduction rate (purified protein)	<i>S. oneidensis</i>	<i>P. fluorescens</i>	<i>G.sulfurreducens</i>	<i>Shewanella sp</i>	<i>P. stutzeri</i>	<i>D.vulgaris</i>
Cytochrome c3 (<i>D.Vulgaris</i>)	377 ± 32 ^{a*}	✗	✗	✗	✗	✗	✓
Cytochromes c3 (<i>D. norvegicum</i>)	739 ± 56 ^a	✗	✗	✓	✗	✗	✓
Cytochrome c7 (<i>D. acetoxidans</i>)	557 ± 25 ^a	✗	✗	✓	✗	✗	✓
(Fe) Hydrogenase (<i>D. vulgaris</i>)	7679 ± 4 ^a	✓	✓	✓	✓	✓	✓
(Ni-Fe-Se) Hydrogenase (<i>D. norvegicum</i>)	161 ± 3 ^a	✓	✗	✓	✓	✗	✓
Extracellular							
MtrA (<i>S. oneidensis</i>)	ND	✓	✗	✓	✓	✗	✗
MtrC (<i>S. oneidensis</i>)	ND	✓	✗	✗	✓	✗	✗
MtrF (<i>S. oneidensis</i>)	ND	✓	✗	✓	✓	✗	✗
OmcA (<i>S. oneidensis</i>)	ND	✓	✗	✗	✓	✗	✗

a: Data are from [Michel et al., 2001] (unit: μmol Cr(VI) min⁻¹ mg⁻¹ enzyme)

a*: Calculated as average Cr(VI) reduction rates by Cytochrome c3 extracted from three different strains of *Desulfovibrio vulgaris* (unit: μmol Cr(VI) min⁻¹ mg⁻¹ enzyme).

Reference: *S. oneidensis* [Heidelberg et al., 2002], *P. fluorescens* [Silby et al., 2009], *G.sulfurreducens* [Methe et al., 2003], *Shewanella sp.* [Kim et al., 2011], *P. stutzeri* [Chakraborty et al., 2017], *D. vulgaris* [Heidelberg et al., 2004].

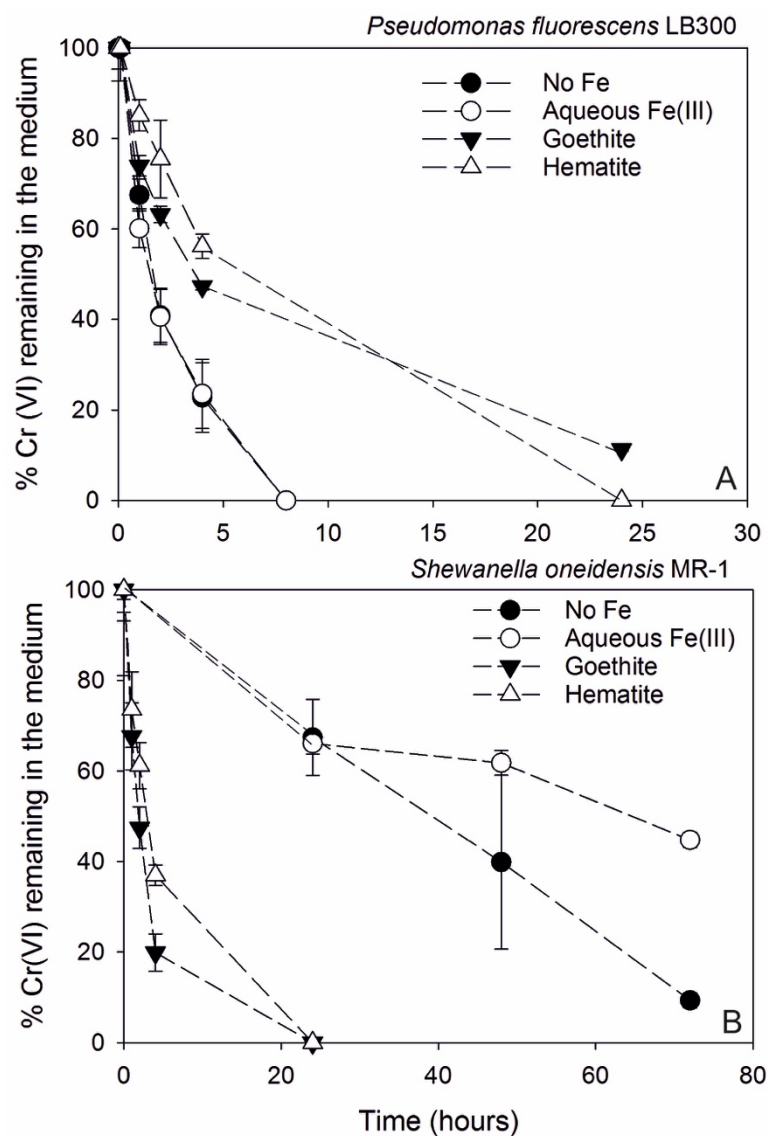


Figure 1: Microbial chromium reduction experiments with *Pseudomonas fluorescens* LB 300 (A) and *Shewanella oneidensis* MR-1 (B), in the presence of aqueous Fe(III) or iron minerals. Compared to the absence of Fe or the present of only aqueous Fe(III), in the presence of goethite and hematite decreases Cr(VI) reduction in *Pseudomonas* but increases it in *Shewanella*. There is no difference between the effects of goethite and hematite. Uncertainties are shown as standard deviations between the triplicates in each group. Note difference in x-axis scale between the two panels.

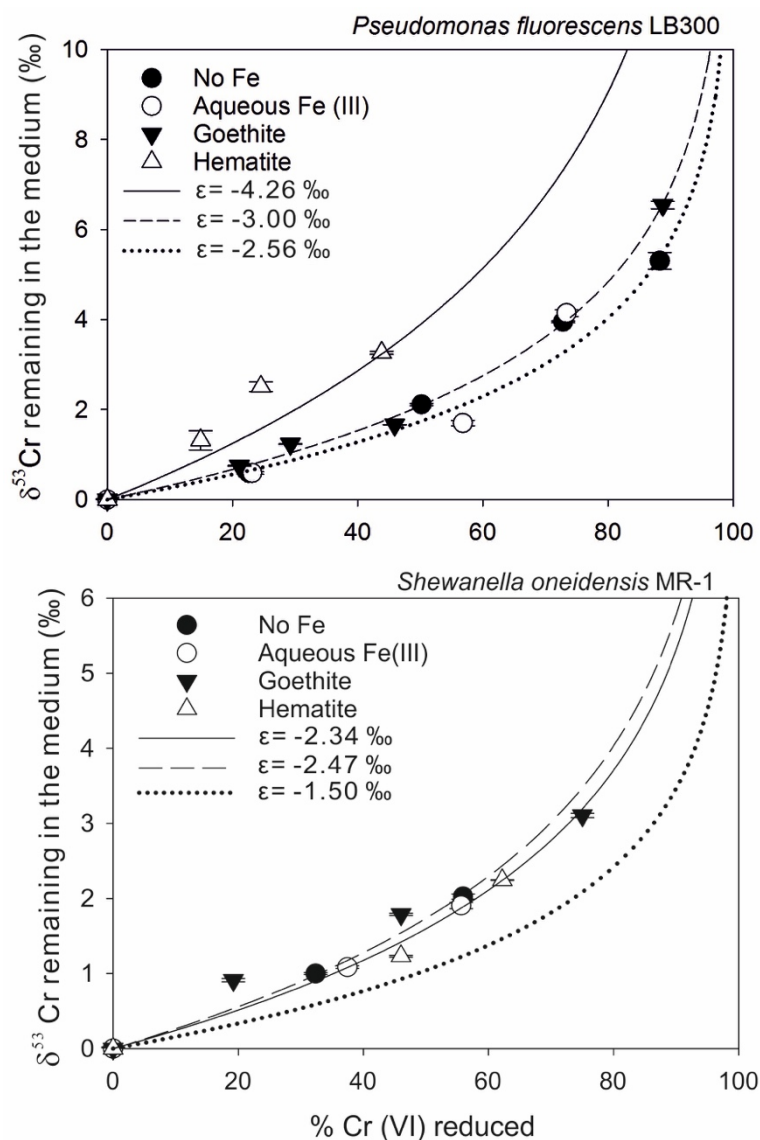
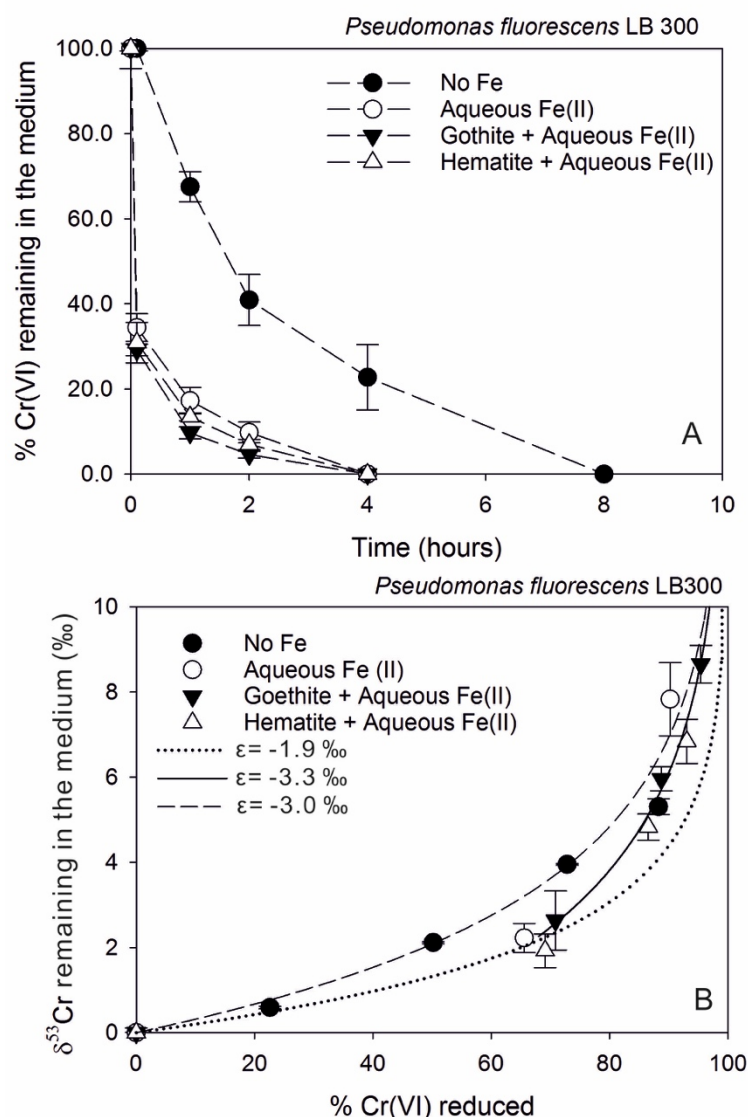


Figure 2: Observed isotopic fractionations during microbial Cr(VI) reduction experiments using *Pseudomonas fluorescens* LB 300 (A) and *Shewanella oneidensis* MR-1 (B). The presence of aqueous Fe(III) and both iron minerals does not have significant impact on Cr isotopic fractionation factors generated by *Shewanella*. For *Pseudomonas*, ϵ value is much higher in the presence of hematite. Uncertainties were shown at $2 \times$ of standard deviations of the isotope measurement.



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644 Figure 3: (A): The impact of aqueous Fe(II) on Cr(VI) reduction in experiments using *Pseudomonas*
 645 *fluorescens* LB 300, with different iron minerals in the system. (B): Corresponding $\delta^{53}\text{Cr}$ vs. Cr(VI)
 646 reduced during the experiments. Cr reduction and isotope fractionation in the experiments with Fe(II)
 647 involve a two-step process; the initial loss of Cr is from abiotic reduction by aqueous Fe(II), and the
 648 following reduction is caused by bacteria. The dotted-line shows the Rayleigh distillation curve for
 649 abiotic Cr isotope fractionation by aqueous Fe(II), and the solid line represents that for microbial Cr
 650 isotope fractionation (considering the point of initial loss as the start of microbial reduction). Note the
 651 difference in x-axis scale between the two panels

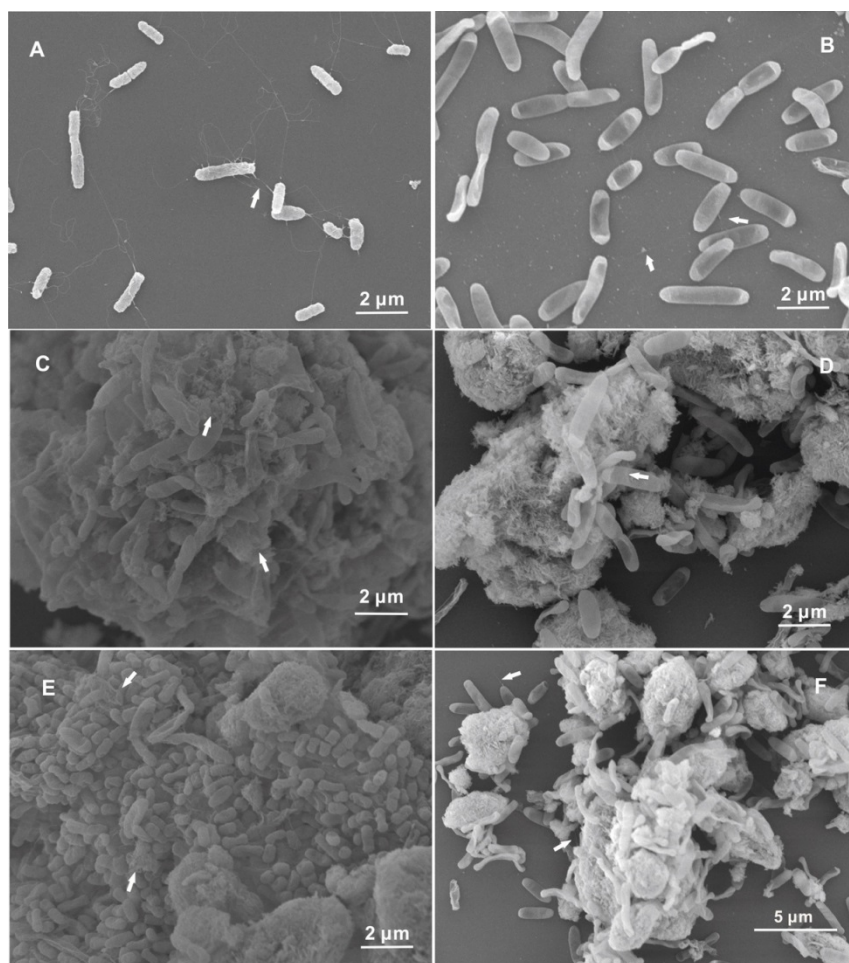


Figure 4: SEM images. A: Suspended cells of *Pseudomonas fluorescens* LB 300, with multiple flagella aiding their mobility (White arrows). B: Suspended cells of *Shewanella oneidensis* MR-1. The cells were flat and have thick polars. The cells produced nanowires to help electron transfer (white arrows). C: The attached cells of *Pseudomonas fluorescens* LB 300 on goethite surfaces. The flagella produced and EPS helped the attachment of the cells to the abiotic surfaces and also to each other (white arrows). D: The cells of *Shewanella oneidensis* MR-1 attached on goethite surfaces. No EPS was observed, but the cells produced some nanowires (white arrows). E: The cells of *Pseudomonas fluorescens* LB 300 attached on hematite surfaces. The flagella produced and EPS helped the attachment of the cells onto the abiotic surfaces and also to each other (white arrows). The cells attached closely and formed several layers of biofilm. F: The loosely attached cells of *Shewanella oneidensis* MR-1 on hematite surfaces. No EPS was observed, but the cells produced some nanowires (white arrows).

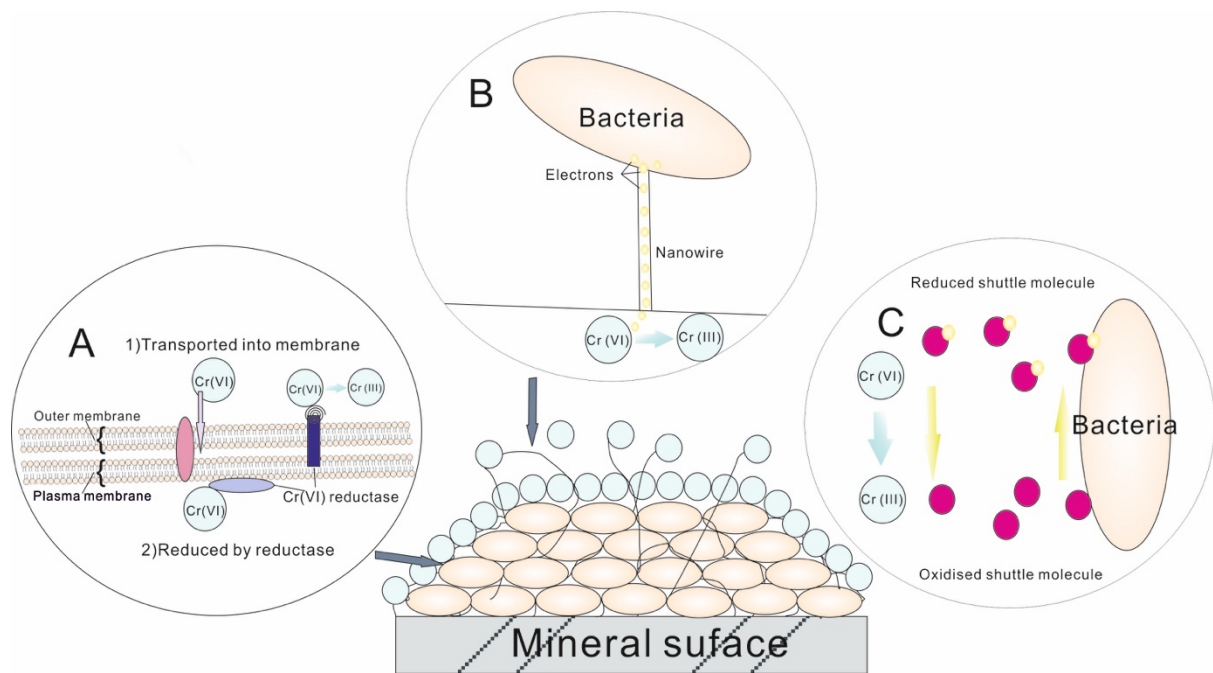


Figure 5: Three possible pathways for microbial Cr(VI) reduction. A: The enzymes responsible for Cr(VI) reduction are membrane-bound. If the enzymes are on the outer membrane, Cr(VI) can be reduced on the surface of the cell. However, if they are on the inner-membrane of the bacteria, Cr(VI) needs to be transported into the cell first, and then be reduced. B: *Shewanella oneidensis* MR-1 can produce nanowires to facilitate electron transfer processes. The nanowires are extensions of outer and periplasmic membrane, and they can aid electron transfer between bacteria and mineral substrate, bacteria and metals, and between two bacteria. C: *Shewanella oneidensis* MR-1 can also produce soluble "electron shuttles" to aid electron transfer at a distance.

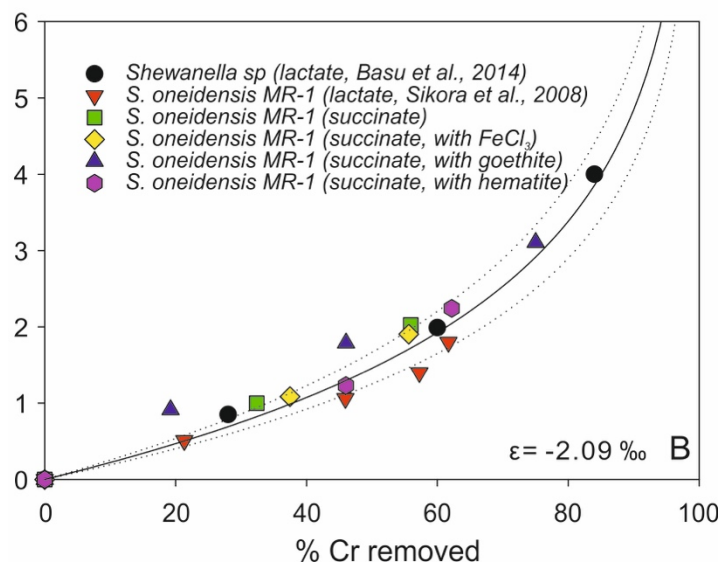
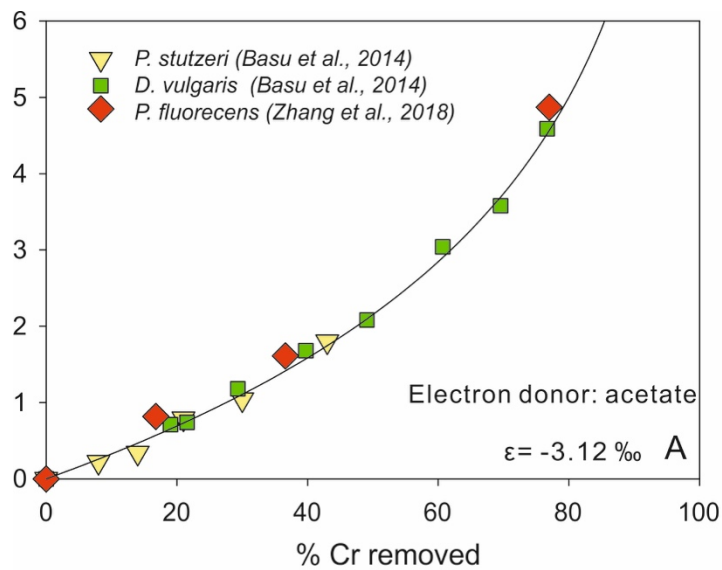


Figure 6: (A): Same Cr isotope fractionation factor was found for a diverse of bacteria (using intracellular reduction pathway, Table 2) when same electron donor (acetate) was employed. (B) Cr isotope fractionation factors are indistinguishable from one another for different *shewanella* strains under various conditions and different electron donors (when exogenous electron donors are abundant).