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Contrasted redox-dependent structural control on Fe isotope fractionation during its adsorption onto and assimilation by heterotrophic soil bacteria†

Aridane G. González,¹ Franck Poitrasson,² Felix Jiménez-Villacorta,³ Liudmila S. Shirokova⁴ and Oleg S. Pokrovsky⁴

Despite the importance of structural control on metal stable isotope fractionation in inorganic and abiotic systems, the link between metal structural changes and related isotopic fractionation during reactions with organic surfaces and live cells remains poorly established. We conducted reversible adsorption of Fe(II) and Fe(III) on the surface of exopolysaccharide (EPS)-rich and EPS-poor *Pseudomonas aureofaciens*, and we allowed Fe intracellular uptake by growing cells. We analyzed the Fe isotopic composition of the remaining fluid and cell biomass, and compared the isotopic fractionation during adsorption and assimilation reaction with relative changes in Fe structural status between aqueous solution and bacterial cells, based on available and newly collected X-ray absorption spectroscopy (XAS) observations. Iron(III) adsorption onto *P. aureofaciens* at $2.8 \leq \text{pH} \leq 6.0$ produced an enrichment of the cell surface in heavier isotopes with $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ ranging from +0.7 to +2.1‰, without a link to pH in EPS-rich cultures. In contrast, the magnitude of isotopic fractionation increased with pH in EPS-poor cultures. Iron(II) adsorption produced an even larger enrichment of the cell surface in heavier isotopes, by up to 3.2‰, tentatively linked to Fe(III) hydroxide precipitation. Intracellular assimilation of Fe(II) favored heavier isotopes and led to $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ of +0.8‰. In addition, Fe(III) cellular uptake produced an enrichment of the bacterial biomass in lighter isotopes with $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ of -1‰. The XAS analyses demonstrated the dominance of Fe(III)-phosphate complexes both at the cell surface and in the cell interior. We suggest that heavier isotope enrichment of the cell surface relative to the aqueous solution is due to strong Fe(III)-phosphoryl surface complexes and Fe complexation to ligands responsible for metal transfer from the surface to the inner cell. In case of Fe(II) adsorption or assimilation, its partial oxidation within the cell compartments may lead to cell enrichment in heavier isotopes. In contrast, loss of symmetry of assimilated Fe(III) relative to the aqueous Fe³⁺ ion and longer bonds of intracellular ions relative to aqueous Fe(III)-citrate or hydroxo-complexes could produce an enrichment of cells in lighter isotopes. The versatile nature of Fe(II) and Fe(III) fractionation without a distinct effect of pH and surface exopolysaccharide coverage suggests that, in natural soil and sedimentary environments, Fe isotope fractionation during interaction with heterotrophic bacteria will be primarily governed by Fe complexation with DOM and Fe redox status in the soil pore water.

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Environmental significance

This investigation provides valuable insights into the mechanisms governing Fe isotope fractionation in soil-fluid bacterial systems. Isotopic partitioning during the adsorption of Fe(II) and Fe(III) by a common soil bacterium is dominated by Fe complexation with DOM. The adsorption of Fe(III) results in an enrichment of heavier isotopes on the cell surface that is attributed to the stronger and more symmetrical complexation of Fe(III) with surface phosphoryl moieties compared to hydroxyl complexes in the aqueous solution. In natural environments, the $\delta^{57}\text{Fe}$ values in solution or microbial biomass may vary from approximately -1 to +3‰, depending on the Fe redox status in soil porewater, and this can occur rapidly over short time scales, independent of pH and the presence of microbial EPS.

¹Instituto de Oceanografía y Cambio Global, IOCG, Universidad de Las Palmas de Gran Canaria, ULPGC, Spain. E-mail: aridane.gonzalez@ulpgc.es; Tel: +34 928 45 44 51

²Géosciences Environnement Toulouse (GET), CNRS UMR 5563, UPS-IRD-CNES 14-16, Avenue Edouard Belin, 31400, Toulouse, France

³ESS Bilbao, Parque Tecnológico Bizkaia, Nave 201, 48170 Zamudio, Spain

⁴N.P. Laverov Federal Center for Integrated Arctic Research (FCI Arctic), Russian Academy of Sciences, Arkhangelsk, Russia

⁵BIO-GEO-CLIM Laboratory, National Research Tomsk State University, Tomsk, Russia

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1. Introduction

Iron (Fe) is the fourth most abundant element in the Earth's crust and it is crucial for most living organisms because of its implication in a number of metabolic processes.^{1–5} Besides, Fe is a limiting factor of primary productivity in many aquatic systems^{6,7} because of very low free-ion concentration and low solubility of its hydroxides.⁸ Due to the high abundance of Fe in soil systems,⁹ versatile Fe redox status,^{10–12} strong complexation with natural organic matter,¹³ and the ability of Fe isotopes to track sources and processes, Fe isotope fractionation in natural Earth-surface systems has been at the forefront of isotopic studies for the past two decades.^{14,15}

Iron has four stable isotopes (⁵⁴Fe, ⁵⁶Fe, ⁵⁷Fe, and ⁵⁸Fe), known to fractionate mass-dependently during abiotic and bacterially mediated processes in low temperature environments.^{16–19} Iron isotope fractionation during Fe interaction with bacteria has received significant attention, mostly with regard to anaerobic phototrophic Fe-oxidizing,²⁰ aerobic neutrophilic Fe-oxidizing,²¹ and heterotrophic Fe-reducing bacteria.²² Most of these studies demonstrated significant isotopic fractionation occurring during Fe assimilation by the cells and the production of bacterially induced Fe biominerals. For example, Croal *et al.*²⁰ investigated the Fe(II) isotopic fractionation in anaerobic conditions caused by the photoautotrophic bacteria of genus *Thiodictyon*. The hydrous ferric oxide (HFO) metabolic products yielded $\delta^{57}\text{Fe}$ values with heavier isotopic composition than the initial Fe(II), with a fractionation factor ($\Delta^{57}\text{Fe}_{\text{Fe(III)-oxides-Fe(II)_{aq}}}$) of 2.2‰. Crosby *et al.*²³ reported that the Fe dissimilatory reduction promoted by *Geobacter sulfurreducens* and *Shewanella putrefaciens* strains caused Fe isotopic fractionation of approximately 2.2‰, with final Fe(II) species enriched in light isotopes. Beard *et al.*,²⁴ Wiesli *et al.*²⁵ and Johnson *et al.*^{26,27} showed that Fe(II)_{aq} produced by the dissimilatory reduction exhibited lower ⁵⁷Fe/⁵⁴Fe ratios when compared to the initial ferrihydrite substrates, with a fractionation factor ($\Delta^{57}\text{Fe}_{\text{Fe(III)-oxide-Fe(II)_{aq}}}$) ranging from 1.9 to 4‰.

Bacterial adsorption and assimilation of metals, in particular Fe, play an essential role in overall metal biogeochemical cycling in soils and waters.^{4,28–30} It is known that, during equilibrium isotope fractionation processes, heavier Fe isotopes are preferentially adsorbed onto solid surfaces.^{23,31–33} Generally, the binding affinity of metals on bacteria cell walls and their exopolymer substances (EPS) is controlled by proton-active surface functional groups such as carboxyl, hydroxyl, sulfhydryl, phosphoryl and amine moieties that undergo deprotonation and bind metal ions to form stable ligand–metal surface complexes.^{30,34–40} However, while the main molecular mechanisms controlling metal adsorption onto bacterial surfaces and metal assimilation into microbial cells are identified, this is not so for metal isotope–bacteria reaction. Rarely have researchers combined structural study of metal speciation in the cell or solution and its isotope fractionation measurements and this was mostly applied to Zn and Cu isotopes.^{38,41–44} To our knowledge, the combined XAS and Fe isotope approach on bacteria was used only twice so far. Mulholland *et al.*³² investigated the

Fe isotope fractionation during the interaction of aqueous Fe with three species of cyanobacteria (*Gloeocapsa* sp., *Synechococcus* sp., and *Planthothrix* sp.) conducted in batch experiments using two distinct Fe oxidation states (Fe(II) and Fe(III)). In these cultures, the structural status of adsorbed Fe was characterized in a concomitant *in situ* X-ray Absorption Spectroscopy (XAS) study.⁴⁰ It was demonstrated that in the presence of surface organic ligands, the oxidation of divalent iron occurs, but the polymerization of Fe(III) oxy(hydr)oxides is partially inhibited. As a result, the adsorbed iron stays in the form of both Fe–O–Fe polymers and individual Fe atoms which are attached to phosphoryl moieties. We also showed that the presence of EPS in solution reduces metal-cell surface binding capacity and enhances Fe polymerization in the form of Fe(III) oxy(hydr)oxides at the bacterial surface. The isotopic results showed a systematic enrichment in heavy Fe isotopes upon iron adsorption onto bacterial cell surfaces, and this was particularly strong when Fe oxidation was involved in the process.³² Subsequently, Swanner *et al.*²¹ expanded on this Fe isotopic study of Fe oxidation by *Synechococcus* sp. and focused the XAS investigation on the characterization of the Fe-oxide minerals produced.

Upon assimilation inside the cell organelles, metals, including Fe, can be subjected to redox reactions such as a reduction to form S-bearing proteins, complexation with strong ligands in the cytoplasm^{45–47} or scavenging in the form of insoluble carbonates, phosphates and oxalates.⁴⁸ Any single reaction involving metals inside the cell has its specific equilibrium isotopic fractionation, in addition to the kinetic isotope effect which can operate in each reaction of intracellular Fe transformation. This may set serious limitations on the capacity to interpret the isotopic fractionation measured between the aqueous solutions and intracellular assimilated metals, even though the average structural status of metal can be determined. In contrast, adsorption processes are typically fast, reversible and rarely include more than 2–3 major binding sites, thus allowing a more straightforward assessment of the structural control on metal isotope fractionation. Considering these intrinsic limitations and the information available at the present time for Fe and other metal interaction with microorganisms, the goal of the current investigation was to test the hypothesis that both Fe(II) and Fe(III) adsorption onto the cell surface brings about preferential removal of the heavier isotopes from solution, whereas cell growth in Fe(II) or Fe(III)-bearing media leads to cell enrichment in lighter Fe isotopes inside the cells. In other words, adsorption of Fe can favor heavier isotopes and incorporation might enrich the cells with lighter Fe isotopes. Relative to previous work where we combined structural (XAS) and isotopic measurements during Fe adsorption on phytoplankton cells,^{32,40} here we further intended to test the impact of bulk Fe structural status assessed by synchrotron-based spectroscopy (oxidation state, coordination number, nature of atomic neighbors) on Fe isotopic fractionation between the bacteria and aqueous solution, both at the cell surface and inside the cells. For this, we used this time a well-known soil heterotrophic bacterium, *Pseudomonas aurifaciens*. Examination of the link between the speciation of



$$\delta^{57}\text{Fe}_{\text{solution}} = (100 \times \delta^{57}\text{Fe}_{\text{initial}} - A \times \delta^{57}\text{Fe}_{\text{cell}})/(100 - A) \quad (4)$$

In this work, we define the isotopic fractionation factor between Fe in solution and Fe adsorbed onto or assimilated by bacteria as:

$$\Delta^{57}\text{Fe}_{\text{cell-solution}} = \delta^{57}\text{Fe}_{\text{cell}} - \delta^{57}\text{Fe}_{\text{solution}} \quad (5)$$

The values of isotopic signature ($\delta^{57}\text{Fe}$ and $\delta^{56}\text{Fe}$) and isotopic fractionation factor ($\Delta^{57}\text{Fe}_{\text{cell-solution}}$) and those of the starting products are listed in Table 1. In the adsorption experiments, 6 pairs of samples (cell biomass and corresponding aqueous solution) were processed for isotopic analyses. This allowed straightforward calculation of $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ using eqn (4) (Table 1). In these experiments, we also calculated $\delta^{57}\text{Fe}_{\text{cell}}$ using eqn (3); the values of $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ obtained from such indirect calculation agreed within propagated analytical uncertainties with directly measured $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ values for 4 out of the 6 experiments. For the remaining two experiments (EPS-poor-1 and 2), it is possible the proportion of adsorbed Fe on the cell surfaces is only known with an uncertainty larger than our 3% estimate. With this caveat in mind, we computed $\delta^{57}\text{Fe}_{\text{cell}}$ using eqn (3) when only $\delta^{57}\text{Fe}_{\text{aqueous}}$ was available (exp. EPS-rich-3 and EPS-poor-Fe2 in Table 1). Similarly, in the assimilation experiments, coupling of cell and solution measurements in EPS-rich-5 samples yielded direct $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ ($+0.767 \pm 0.32\%$ by eqn (4)), which is similar within uncertainty to the calculated value ($+0.739 \pm 0.25\%$ by eqn (3) and (4)). This allowed using other measured $\delta^{57}\text{Fe}_{\text{cell}}$ to calculate $\delta^{57}\text{Fe}_{\text{solution}}$ and $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ via eqn (4) and (5), respectively.

We also obtained $\delta^{56}\text{Fe}$ values, though slightly less precisely than $\delta^{57}\text{Fe}$ values on a per amu basis. Given that the relationships between $\delta^{56}\text{Fe}$ and $\delta^{57}\text{Fe}$ of both adsorbed and assimilated samples plot on a single mass fractionation line (Fig. ESI-1†), only $\delta^{57}\text{Fe}$ values are discussed in this paper. The resulting linear regression for $\delta^{57}\text{Fe}$ vs. $\delta^{56}\text{Fe}$ (Fig. ESI-1†) was:

$$\delta^{57}\text{Fe} = (0.016 \pm 0.009) + (1.456 \pm 0.022) \times \delta^{56}\text{Fe} \quad (R^2 = 0.996) \quad (6)$$

This is consistent with $\delta^{57}\text{Fe}$ being equal to $\sim 3/2$ of $\delta^{56}\text{Fe}$. Given the findings reported by Amor *et al.*⁶⁴ on mass-independent Fe isotope fractionation during magnetotactic bacteria metabolism, we searched for such mass independent Fe isotope fractionation on our experimental products to see whether such an effect could also occur with other type of bacteria. However, we could not detect any significant mass independent effect outside 0.1‰ uncertainty.

3. Results

3.1. Isotope fractionation during Fe adsorption onto and assimilation by the bacteria

In case of Fe^{3+} adsorption onto EPS-rich and EPS-poor cells (see Table ESI-1†), the $\delta^{57}\text{Fe}_{\text{cell}}$ was always higher than $\delta^{57}\text{Fe}_{\text{solution}}$ (Table 1). However, for the two sets of experiments having the same pH range (close to 4 and 4.6–4.9, respectively), the

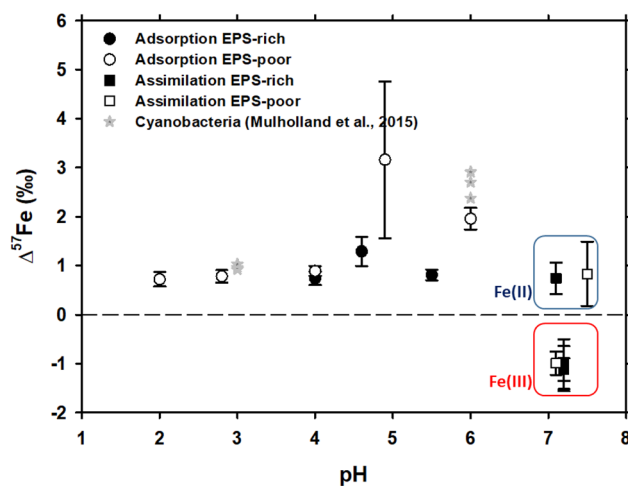


Fig. 1 $\delta^{57}\text{Fe}$ values of the initial solution calculated by the mass balance between cell and solution Fe concentrations and isotopic compositions. Asterisks represent the data on Fe isotope fractionation during adsorption onto cyanobacteria, as reported by Mulholland *et al.*, 2015.³² Note that the isotopic offset during assimilation is strongly dependent on the initial Fe redox status – negative in case of Fe(III) and positive in case of Fe(II) as distinguished by red and blue rectangles, respectively.

$\Delta^{57}\text{Fe}_{\text{cell-solution}}$ is slightly higher for EPS-poor cultures compared to the EPS-rich ones, although the difference is comparable to uncertainties. Iron(III) adsorption onto *P. aur-eofaciens* at $2.8 \leq \text{pH} \leq 6.0$ produced an enrichment of the cell surface in the heavier isotope with $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ ranging from +0.72 to +2.0‰ (Table 1 and Fig. 1). The EPS-poor culture exhibited a possible impact of pH on the isotopic offset during adsorption, which increased from +0.72‰ at pH of 2 to +1.96‰ at pH of 6 (Table 1 and Fig. 1).

The isotope fractionation induced by Fe(II) and Fe(III) assimilation during bacterial growth demonstrated the following features. The EPS-poor-8 cells exhibited higher $\delta^{57}\text{Fe}_{\text{cell}}$ values of assimilated Fe compared to those of the EPS-rich-6 ones for the same pH range (Table 1), even though the starting Fe(III)-citrate of the former was isotopically lighter than the starting FeCl_3 of the latter (Table 1). This feature of Fe(III) assimilation is not confirmed for the case of Fe(II) assimilation since the $\delta^{57}\text{Fe}_{\text{cell}}$ values in EPS-poor-6 and EPS-rich-5 experiments were undistinguishable (Table 1). Intracellular assimilation of Fe(II) led to preferential uptake of heavier isotopes producing $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ of +0.77 and +0.83‰ for EPS-rich-5 and EPS-poor-6 experiments. In contrast, intracellular uptake of Fe(III) yielded an enrichment of the cell in lighter isotopes with $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ close to -1% as calculated from EPS-poor-8 and EPS-rich-4,6,7 experiments (Table 1). It is noteworthy that the Fe(III) uptake from both initial FeCl_3 and Fe-citrate salts yielded similar isotopic offsets (Tables ESI-1† and 1). Considering the propagated uncertainties (see Table 1), the magnitude of isotopic offset ($\Delta^{57}\text{Fe}_{\text{cell-solution}}$) in the EPS-rich cells (from -1.01 to -1.12%) was not distinguishable from that in the EPS-poor cells ($\Delta^{57}\text{Fe}_{\text{cell-solution}} = -0.99\%$) for the experiments using Fe(III) salts (Tables ESI-1† and 1).



3.2. XAS characterization of Fe bound to soil bacteria

The structural characterization of Fe adsorbed onto *P. aurifaciens* was based on XAS (EXAFS and XANES) analysis of

samples as was also described in previous work.⁴⁰ These results are directly used for testing structural control on adsorbed Fe(III) and Fe(II) from aqueous solution as described in Section 4.1 below. The intracellularly assimilated Fe was not

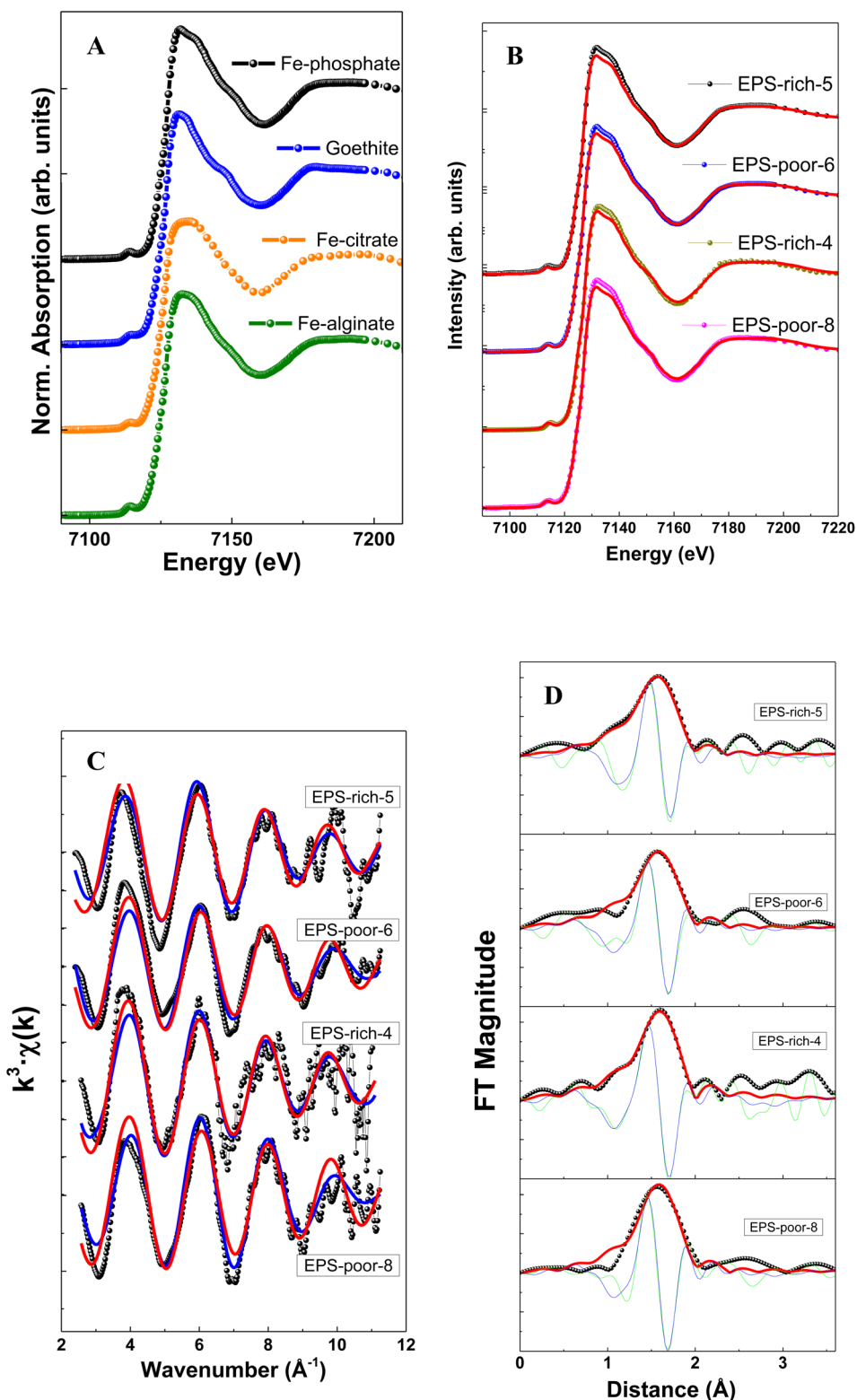


Fig. 2 Fe K-edge XANES spectra at the Fe K-edge energies (A) and (B), presenting Fe K-edge oscillations (C) and Fourier transform magnitudes (D) of the Fe adsorbed on cells.



Table 2 Summary of Fe structural parameters of Fe(II) and Fe(III) assimilation experiments. Experimental details are listed in Table ESI-1

Sample	pH	Fe(III) phosphate (%)	Goethite (%)	R (Å) (± 0.05 Å)	N (atom) (± 0.5)	σ^2 (Å ²) (± 0.001 Å ²)	R -factor
EPS-rich-5	7.1	95	5	2.07	5.9	0.007	0.08
EPS-poor-6	7.8	100		2.07	5.1	0.006	0.13
EPS-rich-4	7.2	100		2.07	6.0	0.007	0.05
EPS-poor-8	7.1	100		2.06	5.6	0.005	0.15

characterized previously and newly obtained results are presented below.

The XANES spectra of assimilated Fe–bacteria samples are illustrated in Fig. 2 and Table 2. When comparing the samples with reference spectra, the analysis clearly shows that the unique organic component in most of the samples (and main component in all cases) is Fe(III)–phosphate, corresponding to Fe binding to the phosphoryl group. The relative proportion of Fe(III)–phosphate and goethite was estimated from linear combination fits (LCF analysis) of XANES spectra and using Fe(III) reference compounds. The choice of the best corresponding standard for each spectrum was made using automated combinational fitting (Athena software).

The EXAFS results allowed extracting the first shell of Fe (Table 2) which exhibited similar features among samples (Fig. 2). Note that the modeling of EXAFS spectra in biological samples is very difficult due to distorted geometries and the presence of different neighbors, like O, N and S, light elements with similar weak cross section values, in the nearest metal coordination shell. In this sense, XANES results may complement the EXAFS *via* identifying the ligands using the edge features.

The filtered Fourier transform curves (EXAFS signals filtered for the first coordination sphere) were fitted using Fe–O bonds. The inelastic loss factor was estimated/set, based on references and the EPS-rich-4 sample, as $S_0^2 = 0.87$. Results show a coordination number of around 6 in all cases. This is in good agreement with an octahedral geometry of 6 oxygen atoms surrounding Fe cations, with a Fe–O distance of ~ 2.07 Å. We could not quantify the relative contribution of Fe(III)–phosphoryl or carboxyl complexes that are typical in samples of microorganisms,^{38,65,66} although the samples showed an important contribution of Fe(III)–phosphate like groups in the XANES analysis (peak at ~ 1.9 – 2.0 Å⁻¹). Overall, the EXAFS spectra confirmed the XANES results.

4. Discussion

4.1. Heavier isotope enrichment of cells during Fe(II) and Fe(III) adsorption

The adsorption of Fe onto *P. aureofaciens* allowed quantifying the adsorption constants and surface binding site concentrations. The total amount of proton-binding sites was two times higher in the EPS-rich cells compared to the EPS-poor ones (5.5 and 2.6 mmol g_{wet}⁻¹), whereas the concentration of phosphoryl-like groups was higher in the EPS-poor cells and the concentration of carboxyl-like groups was higher in the EPS-rich cells. The adsorption of Fe(III) started at acidic pH (~ 1.5) and increased with the pH, following the “universal pH-edge” for

metals.^{67,68} González *et al.*⁴⁰ provided chemical characterization of Fe³⁺ adsorbed onto soil bacteria by X-ray absorption spectroscopy (XAS), and demonstrated that Fe(III)–phosphate was the predominant Fe-binding compound on the cell surfaces of the EPS-poor cultures, at pH from 2.0 to 6.0. In addition, the goethite-like component was present in the EPS-rich cells (pH from 3.0 to 4.6). Therefore, EXAFS spectra supported the XANES results.

The present study demonstrated that, during Fe(III) adsorption, the effect of pH was strongly pronounced for the EPS-poor culture ($\Delta^{57}\text{Fe}_{\text{cell-solution}}$ increased from +0.72‰ at pH = 2.0 to +1.96‰ at pH = 6.0; Table 1 and Fig. 1). The pH effect could not be tested for the EPS-rich culture, for which our two experiments yielded significantly different $\Delta^{57}\text{Fe}_{\text{cell-solution}}$ values (+0.74 \pm 0.14‰ and +1.29 \pm 0.30‰; Table 1 and Fig. 1) at similar $4 \leq \text{pH} \leq 4.6$. The exact reasons for these differences between these EPS-rich and EPS-poor cultures in the circum-neutral pH range (Fig. 1) are not clear. It can be hypothesized that, in the case of EPS-poor strain, the phosphoryl moieties of the cell wall are not protected from solution and adsorption occurs essentially on these high affinity sites. This could produce stronger fractionation at higher pH, where phosphorylated groups are mostly deprotonated. However, this seems to be inconsistent with XAS-based bulk speciation of Fe at the cell surface, because the surface-bound Fe(III) was dominated by Fe–phosphoryl ligands, regardless of pH and the presence or absence of the EPS. At the same time, one should keep in mind that XAS treatment provides dominant (*ca.* 90%) speciation of an element. The remaining 10% are not detectable by spectroscopy but it is not excluded that they can be responsible for observed isotope fractionation, provided that the isotopic offsets of reactions with these minor compounds strongly exceed those of the major binding constituents of Fe. Recently, Oleinikova *et al.*⁶⁹ studied the adsorption of organo-ferric colloids onto *P. aureofaciens* and demonstrated an enrichment in $\delta^{57}\text{Fe}$ (+0.4‰) of cell surfaces compared to the remaining solution. Consistent with the results of Gonzalez *et al.*⁴⁰ and of the present study, this could be explained by the dominance of Fe(III)–complexes with phosphoryl groups on cell surfaces. Therefore, the only possible explanation for the observed difference in isotopic offset between the aqueous solution and cell biomass of EPS-rich an EPS-poor cultures is the shielding effect of carboxylate-rich EPS on aqueous Fe ions. These rather inert EPS layers can prevent direct interaction and binding of Fe²⁺(aq.) and Fe³⁺(aq.) with the strong phosphoryl moieties of the external cell membrane.

Globally, the XAS analyses of adsorbed Fe demonstrated a dominance of Fe(III)–phosphate complexes at the cell surface



stronger bonds with phosphoryl moieties in cell organelles compared to nutrient media.

Taken together, the results obtained indicate that the mechanisms controlling Fe isotope fractionation in the soil fluid–bacterial system are better constrained. In natural settings, the $\delta^{57}\text{Fe}$ in solution or microbial biomass may vary from *ca.* -1 to $+3\text{‰}$ depending primarily on the Fe redox status in soil and sediment porewater and these changes may occur over a short time scale (hours to days) regardless of pH and the presence or not of microbial extracellular substances.

Conflicts of interest

There are no conflicts to declare.

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