Imaging redox activity and Fe(II) at the microbe-mineral interface during Fe(III) reduction

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Abstract

Dissimilatory iron-reducing bacteria (DIRB) play an important role in controlling the redox chemistry of Fe and other transition metals and radionuclides in the environment. During bacterial iron reduction, electrons are transferred from the outer membrane to poorly soluble Fe(III) minerals, although the precise physiological mechanisms and local impact on minerals of these redox processes remain unclear. The aim of this work was to use a range of microscopic techniques to examine the local environment of Geobacter sulfurreducens grown on thin films of Fe(III)-bearing minerals, to provide insight into spatial patterns of Fe(III) reduction and electron transfer. Confocal fluorescence microscopy showed that sparse biofilms formed on the mineral coatings, while the selective Fe(II) probe RhoNox-1 revealed Fe(II) patches on the minerals sometimes co-located with cells. Atomic force microscopy highlighted thin filamentous structures extending radially from the cell surface. Further analysis using fluorescent redox dyes showed redox-active, linear nanowires that formed cell to cell connections, although they were not implicated in playing a dominant role in direct electron transfer to the Fe(III) minerals. Overall this paper provides new methods and insights on studying Fe(III) reduction and other redox transformations in situ.

Keywords

Electron transfer, nanowires, biofilm, ferric oxides, Fe(III) reduction, confocal microscopy
Introduction

Biogeochemical interactions between microorganisms and minerals at the nanoscale have a major impact on the geochemical cycling of bulk (e.g. Fe and Mn) and trace elements (e.g. As and Cr) in the subsurface. Fe(III) minerals are often the dominant terminal electron acceptor for microbial metabolism in anoxic subsurface sediments, and play a defining role in controlling the mobility of trace elements through the Fe(III)/Fe(II) redox couple [1]. Dissimilatory Fe(III) reduction is a feature of several known genera of bacteria [2] and a commonly studied organism of this group is the anaerobic, Gram-negative species, *Geobacter sulfurreducens* [3]. *Geobacter* species can become dominant in anaerobic Fe(III)-rich subsurface environments, where they readily reduce metals, such as Fe(III). This microbial Fe(III) reduction is coupled with the oxidation of organic inputs and pollutants to CO$_2$ [4]. The dominance of *Geobacter* spp. in these environments may be attributed to their very low maintenance energy demand compared with other heterotrophic bacteria [5].

To facilitate reduction of such extracellular Fe(III) minerals, *Geobacter* spp., and other Gram-negative Fe(III)-reducing bacteria, such as *Shewanella* spp. [6] have evolved electron transfer pathways that traverse the periplasm and outer membrane, negating the requirement for solubilisation and uptake of the electron acceptor into the cell [7]. To date, there has been much focus on understanding the physiology of *Geobacter* spp., including the application of omics techniques and systems biology to uncover the electron carrier proteins involved in extracellular metal reduction [8–10]. However, these proteins are poorly conserved among *Geobacter* spp., sometimes leading to conflicting hypotheses on the exact electron transfer mechanisms involved [7], perhaps partly because of the potential diversity of extracellular electron transfer systems involved [9,11]. C-type cytochromes are known to be associated with the periplasm and outer cell membrane where they can act as the final step in an electron relay system across the cell membranes to minerals in direct contact with these proteins [12]. It has been hypothesised that they could transport electrons to electrically conductive type IV pili, which in turn can create an electrically
conductive network suitable for long-range extracellular electron transport [13,14]. This mechanism seems to be highly conserved among diverse genera of bacteria [15].

*G. sulfurreducens* can grow in planktonic form and in biofilms [16,17]. Biofilm formation has been observed in electrochemical experimental systems where the cells are provided with anodes as electron sinks [16]. A genetically modified strain of *G. sulfurreducens* which over-produces pili and extracellular polymeric substances (EPS) has been found to form biofilms much more readily than wild type strains [18], potentially due to both increased pili [17,19] and EPS production [20]. In such systems, long-range electron transport through biofilms has been theorised to progress via pili, where aromatic amino acids are key to the transport process [9], rather than via direct transfer from outer membrane c-type cytochromes [21,22]. In environmental systems, the reduction of extracellular metals provides a much greater energetic challenge than growth on electrodes [7] and requires a combination of genetic studies and direct observation to understand the physiological adaptations to this challenge.

The reduction of Fe(III) in minerals has profound consequences on the mineralogy and aqueous geochemistry of subsurface environments. Some common ferric minerals in the environment are susceptible to Fe(III) reduction, examples include ferrihydrite, goethite, lepidocrocite and akaganeite [23]. One outcome of bioreduction of these minerals is the reductive dissolution of Fe(III), which forms soluble Fe(II) and is thought to be capable of secondary Fe(III) mineral transformation by sorbing to mineral surfaces [23,24]. The soluble Fe(II) can also recrystallize to form new Fe(II)-bearing minerals such as magnetite, siderite or vivianite depending on the pH, temperature and the other elements present during the reaction [25]. Magnetite nanocrystals may have a secondary function in facilitating electron transfer along pili during extracellular electron transfer [26]. Microbial Fe(III) reduction can also control the fate of metals, radionuclides and organics via a range of coupled processes described in [4] although the precise mechanisms employed remain poorly understood.
Microscopy has provided important insights in the field of microbe-metal interactions, particularly for estimating cell abundance [27], and understanding the structural components of individual cells [28], or the spatial distribution of metals, minerals and biofilm components around the cells [29]. Overall, these studies have greatly improved our understanding of the function of metal reducing bacteria. Many different imaging techniques have been applied to these systems and confer different advantages and challenges. Atomic force microscopy (AFM) measures 3D height profiles at very high resolution and has been used to study Geobacter cells deposited on smooth surfaces [30]. Previous studies have used AFM to examine Geobacter cell capsules or extracellular features such as pili [13,31]. Force microscopy involves direct contact between the sample and a probe and can therefore be used to measure interaction forces between a probe and the sample. For example, by mounting bacterial cells on cantilevers and tapping them on surfaces, it has been possible to measure adhesion forces between bacteria and minerals [32]. Bacteria can be imaged in solution with AFM, but this normally requires the addition of an adhesive layer to the surface to ensure immobilization of the cells [33].

Numerous optical microscopy methods have been employed for studying microbes on surfaces [34]; these are often relatively non-invasive techniques, and are therefore suitable for imaging biofilms in their native state. Confocal laser scanning microscopy offers the potential to probe the chemistry of the bacterial microenvironment with the addition of fluorescent probes to understand the intracellular and extracellular environments in hydrated systems. Significant changes to bacterial structures can occur during sample preparation and drying, including changes to the extracellular proteins and EPS [35]. Common systems for studying electrically-active biofilms include microbial fuel cells and bio-electrochemical systems [36], which has allowed pH imaging of Geobacter sulfurreducens biofilms at low resolution with the probe C-SNARF-4 [37]. Similarly, mapping extracellular redox processes would be beneficial and could enable further understanding of the extent of electron transfer from bacteria and biofilms. Previous studies have shown that
preparing a thin film of a suitable Fe(III) mineral affords a method of studying the microbe mineral-interface while the bacteria are still visible on the surface [38,39].

Studying Fe(III)-reducing bacteria systems with extracellular Fe(III) minerals as the terminal electron acceptor is challenging due to the opacity of the minerals. When grown in batch cultures with amorphous mineral particles such as ferrihydrite, the bacteria can become coated in dense minerals making them difficult to observe spectroscopically, unless advanced and time-consuming techniques such as synchrotron source X-ray microscopy [40] are employed. Ferrihydrite is thought to be present in redox-active sediments as coatings on larger particles of, for example, quartz grains [41]. Therefore, preparing a thin film of a suitable Fe(III) mineral affords a method of studying the microbe mineral-interface while the bacteria are still visible on the surface [38,39].

The objectives of this study were (1) to culture G. sulfurreducens on thin films of ferrihydrite for optical imaging of the cell/mineral interface at high resolution (sub-micron) to help quantify cell colonisation of the mineral surface; (2) to analyse the spatial chemical microenvironment surrounding G. sulfurreducens cells grown using such a method and (3) to quantify the spatial abundance of Fe(II) after Fe(III) bioreduction to gain insight into the dynamic processes of electron transfer to insoluble Fe(III)-bearing minerals in hydrated samples with minimal disturbance. We employed a range of fluorescent probes for the extracellular environment where, in the case of Geobacter spp. and other metal-reducing bacteria, important metabolic reactions take place. The Fe(II)-selective probe RhoNox-1, was applied to detect Fe(II) on mineral films. RhoNox-1 employs a switching mechanism based on N-oxide conversion to a tertiary amine through Fe(II)-mediated deoxygenation [42]. Collectively these experiments have shown that microbial Fe(III) reduction can be viewed directly, in situ, at the sub-micron scale alongside the extracellular structures that are thought to be involved in such processes. We also suggest that such techniques can be useful for dissecting a range of complex redox processes that are coupled to Fe(III) reduction in the subsurface.
Materials and methods

Bacterial strains and culture conditions

G. sulfurreducens strains PCA, KN400 [43] and ΔpilA [44] were grown anaerobically to late log phase in a modified minimal medium [3] with 40 mM fumarate as the electron acceptor and 25 mM acetate as the electron donor. The cells were washed once in 30 mM NaHCO$_3$ and added to experiment bottles to achieve an OD$_{600}$ of 0.3 at time point 0.

Mineral synthesis

Poorly-crystalline Fe(III)-oxyhydroxide was produced using the method of Lovley & Phillips [45], where a 0.4 M solution of FeCl$_3$ was neutralised by 10 N NaOH to pH 7 and washing the solid by centrifugation six times until no Cl$^-$ ions remained.

Preparation of thin film experiment bottles

A spin coating technique was used to produce surfaces with thin films of Fe(III) mineral. A suspension of 0.15 M moles per litre Fe(III)-oxyhydroxide and methanol at a v:v ratio of 3:1 was added to microscope slide cover glasses fixed to the base of a salad spinner. They were spun vigorously for 15 seconds and allowed to air dry at room temperature and were then were cut into 1 cm$^2$ pieces using a diamond-tipped scriber. The final mass of Fe(III) on the slides was 4.5 ± 0.4 µg (n=6) and the coatings were found to be saturating but variable in thickness. Mineral-coated cover slides were added to serum bottles and placed vertically in slide holders prior to inoculation. Anaerobic modified minimal medium [3] with 25 mM acetate as the electron donor and no electron acceptor was added to the bottles to cover the films.

Geochemical analysis

Fe(II) formation was monitored using the ferrozine assay [45,46] on both samples of the medium in the incubation bottles and from the mineral phase on the glass cover slides after extraction with 0.5 M HCl.
Synthesis and characterisation of RhoNox-1 probe dye

According to a modification of a literature procedure [47], 3-chloroperbenzoic acid (270 mg, 1.53 mmol, Sigma Aldrich) was slowly added to a solution of Rhodamine B base (660 mg, 1.49 mmol, Sigma Aldrich) with sodium bicarbonate (125 mg, 1.49 mmol, Sigma Aldrich) in ethyl acetate (66 mL) at 0°C under a dinitrogen atmosphere. The mixture was slowly warmed to room temperature and then stirred at room temperature for 5 hours. After this time, the insoluble materials were removed by filtration with a pad of celite and the solvent removed under reduced pressure. The residue was purified using silica gel chromatography (CHCl₃:MeOH = 10:1) to obtain RhoNox-1 as a purple solid in 33% yield (0.211 g). ¹H NMR and mass spectroscopy were used to characterise the compound as RhoNox-1 and were consistent with literature values. ¹H NMR (400 MHz) (CDCl₃) δH (ppm): 8.06 (d, 3JHH = 7.2 Hz, 1H, ArH), 7.99 (d, 4JHH = 2.4 Hz, 1H, ArH), 7.71 (t, 3JHH = 7.6 Hz, 1H, ArH), 7.66 (t, 3JHH = 7.6 Hz, 1H, ArH), 7.26 (br, 1H, ArH), 7.23 (d, 3JHH = 7.6 Hz, 1H, ArH), 6.87 (d, 3JHH = 8.8 Hz, 1H, ArH), 6.49 (d, 3JHH = 8.8 Hz, 1H, ArH), 6.41 (dd, 3JHH = 9.2 Hz, 4JHH = 2.4 Hz 1H, ArH), 3.77 (br, 4H, CH₂NO), 3.38 (q, 3JHH = 7.2 Hz, 4H, CH₂N), 1.20 (overlapping peaks, 12H, CH₃). ES⁻-MS, m/z calculated: 459.23. Found: 459.3 [M-H⁻](100%).

Steady state emission spectra of RhoNox-1 were recorded at 295 K using an Edinburgh Instrument FP920 Phosphorescence Lifetime Spectrometer equipped with a 450 W xenon lamp (with single 300 mm focal length excitation and emission monochromators in Czerny Turner configuration) and a red sensitive photomultiplier in a Peltier housing (air cooled, Hamamatsu R928P). All data were corrected for the instrument response function.

Similarly, steady state excitation-emission maps over the range 350-540 nm of Fe(III)-oxyhydroxide were recorded in optically dilute solutions (with a maximum absorbance below 0.5 absorption units) and in the solid state as a thin film on a quartz slide to check if there could be any background fluorescence from the Fe(III)-oxyhydroxide slide coating. No emission corresponding to Fe(III) could be detected and only scatter and non-linear effects could be measured (Figure SI 2).
The ability of RhoNox-1 to optically detect labile Fe(II) under our instrumental set up was determined by fluorescence spectroscopy by incremental addition of FeSO$_4$ in 50 mM HEPES buffer at pH 7.4 to a 2 micromolar solution of Rhonox-1 in deionised H$_2$O at room temperature using an excitation wavelength of 540 nm. The pH remained stable throughout the experiment. Fig. SI 1 displays the titration data on the above instrumental set-up.

**Fluorescence microscopy**

Cover glasses were removed from serum bottles, under a stream of argon gas in a box, and placed mineral side up onto microscope slides inside a window of liquid blocker, which had been drawn on with “liquid blocker PAP pen” (Sigma-Aldrich). A solution of 150 µl of anaerobic 30 mM NaHCO$_3$ buffer and fluorescent probes was added to the mineral film, the excess removed, and a cover slide was placed on top and the edges sealed to the slide using nail varnish. The probes were DAPI, SYTO9 and Redox Sensor Green (all from Invitrogen) used at final concentrations of 25 µg l$^{-1}$, 20 µM and 1 µM in 30 mM NaHCO$_3$, respectively. To visualise Fe(II), we synthesised the highly selective turn-on fluorescent probe RhoNox-1 [53] and used it at a final concentration of 5 µM in 30 mM NaHCO$_3$. The slides were imaged using a Zeiss Axio Scope A1 microscope with CCD monochrome camera and EC PLAN 100 × 1.3 oil lens. Confocal laser scanning microscopy was performed using a Leica SP5 inverted tandem head microscope and HCX PL APO lambda blue 63× 1.40 oil lens. The excitation and emission wavelengths were as follows; DAPI: Ex. 350 nm, Em. 420 – 480 nm; SYTO9: Ex. 488 nm, Em. 495 – 550 nm; Redox Sensor Green: Ex. 488 nm, Em. 500 – 540 nm; RhoNox-1: Ex. 543 nm, Em. 560 – 600 nm. Multiple channels were recorded sequentially with 2 × line averaging.

**Atomic force microscopy**

After 12 days of incubation in the experiment bottles, slides were removed from the serum bottles and mounted directly onto the Veeco NanoScope V. Samples were scanned in tapping mode...
using a gold coated silicon nitride cantilever, with a scan resolution of 256 samples/line. Data were analysed using WSxM 5.0 software [48].

**Image analysis**

Image analysis was performed using the software imageJ [49]. Cells were counted by measuring the area of representative individual *G. sulfurreducens* cells (n=29) in segmented images. In other images the total area of bacteria fluorescence was divided by the mean area of 1 cell to estimate the total number of cells in an image [50] of a known area. The first step of comparing the location of *G. sulfurreducens* cells with the intensity RhoNox-1 fluorescence (and therefore Fe(II) concentration) and the location of thick regions of mineral coating was to segment the cells, patches of Fe(II) and thick regions of mineral from the background in respective channels of the same image. These were saved as regions of interest (ROIs) and the RhoNox-1 fluorescence intensity for each ROI was measured and categorised into areas which corresponded with a cell, a thick piece of mineral, with one of these or without either. Statistical analyses were performed using OriginPro 8.5. Extracellular appendages were measured from 10 confocal images using the straight line tool in image J; pili extending beyond the field of view were not included and a total of 80 features were measured.
Results

Cell proliferation on Fe(III) films over time

The Fe(III) mineral coated slides inoculated with cells of *G. sulfurreducens* were monitored over a period of 12 days by removing slides from incubation bottles after 1, 2, 3, 7 and 12 days and imaging them by epifluorescence microscopy, to allow analysis of cell density (shown in blue) on the surface (grayscale) (Fig. 1). One day after addition of the cells to the bottles, there was a mean cell density of $2.4 \times 10^4$ cells mm$^{-2}$ ($n = 3$) which decreased after 2 days, but after 3 days there was an increase and a further increase after 7 days to a maximum cell density of $1.4 \times 10^5$ cells mm$^{-2}$ ($n = 5$). This remained constant until the final sample point at 12 days ($n = 3$). Fluorescence microscopy revealed that the bacteria formed a “biofilm” which appeared to be a monolayer in most places. These results suggested that this was an appropriate method for quantifying the growth of *G. sulfurreducens* on supported Fe(III) oxyhydroxide films, and cellular interactions with the Fe(III) minerals.

Investigating physical changes to Fe-bearing minerals during Fe(III) reduction

In order to probe the physical properties of the cell-mineral interface at a sub-micron scale, we performed atomic force microscopy (AFM) on Fe(III)-coated slides supporting the growth of *G. sulfurreducens*, after 12 days of incubation. Tapping mode AFM allowed profiling of the height of surfaces with bacterial cells, but also provided more information on the cell/mineral interface from the interaction of the tip with the sample surface. We observed 32 bacterial cells and in 75% of cases, the cells appeared to be on top of the surface of the mineral film, however in 25% of cases we observed cells or groups of cells on the surface of the glass with a gap between the cell and the Fe-mineral (Fig. 2A). In the example shown, there is a gap of up to 200 nm between the edge of the cells and the start of the mineral film, which we suggest could be due to reductive dissolution of the Fe(III) in the mineral. The material in this region has a higher tip adhesion and surface deformation than the cell or mineral surfaces, and most likely has a different chemical composition, for example due to the presence of EPS. In cases where there was no space between the cell membrane and mineral film,
the tip adhesion and energy dissipation measurements showed around the cell had higher values than mineral film not adjacent to cells (Fig. 2B). In regions without cells or on control samples with no cells, the Fe(III) mineral surface had some thickened regions on the micron scale but minimal variation in tip adhesion and surface deformation. In some cases we observed cells on the mineral surface with appendages extending radially from the cell surface across the mineral (n = 11, e.g. Fig. 2C). We also imaged a number of regions with thin filaments but no cells, which were especially obvious in areas where there were breaks in the mineral coating (Fig. 2D). Again, these were not observed in control samples with no cells (data not shown).

**Imaging Fe(III) reduction**

Fe(III) reduction on the slides was monitored using the ferrozine assay [45,46]: 12 days after adding bacteria to the experiment bottles, this technique showed that 28 % ± 3 % (n=6) of the Fe on the slides had been reduced to Fe(II) (supplementary table 1). However this did not give any information on the site of Fe(III) reduction, in relation to the bacterial cells. To identify regions of Fe(II) on the mineral films, we synthesised a highly selective turn-on fluorescent probe for Fe(II), RhoNox-1 (RN-1), using the method described by Hirayama et al. [47]. The probe was applied to the slides prior to imaging using confocal laser scanning microscopy. Along with RN-1 (red), we also imaged the living cells using the probe Syto9 (green) and the mineral surface by detecting reflected light (grayscale). The results showed a sparse layer of living cells and high intensity areas of Fe(II) co-localised with the living cells. We also found areas of Fe(II), often the same size and shape as G. sulfurreducens cells that were separate from living cells, and these were on thin or thick regions of the mineral surface (Fig. 3). Using image analysis, we segmented the spots, or regions of interest (ROIs) (n = 394), of bright RN-1 fluorescence and measured the intensity of each spot. We used this to determine whether each ROI was co-located with a living cell (n = 116), on a thick region of mineral, both with a living cell and on a thick region of mineral or not with a cell and on a thin mineral coating. We found that ROIs with the brightest RN-1 fluorescence (corresponding to the highest concentration of Fe(II)) were associated with living cells and on thick regions of mineral. ROIs
on cells on thin mineral regions and ROIs not on cells but on thick mineral regions had the similar
Fe(II) concentrations, lower than ROIs on cells on thick pieces of mineral. ROIs not associated with
cells or with thick mineral regions had the lowest Fe(II) concentrations (Fig. SI 3). Analysis of variance
(ANOVA) confirmed that the mean fluorescence intensity from the 4 types of regions showed
significant differences at the 0.05 level (F value = 66.1) and a post hoc Tukey test showed that the
means were all significantly different from one another, with the exception of groups 2 and 3 where
the means were not significantly different (Fig. SI 3). When *G. sulfurreducens* cells were grown in
planktonic culture with no extracellular Fe(III) present (using fumarate as the electron acceptor) and
imaged with RN-1, they showed a very weak but detectable fluorescence.

**Imaging redox of bacterial extracellular environment**

To investigate the extracellular redox conditions of the wider *G. sulfurreducens* cell
environment during the reduction of insoluble Fe(III), we applied the redox sensitive probe “Redox
Sensor Green” (Invitrogen) to Fe film samples before confocal imaging. The probe highlighted many
bacterial cells (also visualised using DAPI). It also showed straight, thin, linear redox-active features
connecting between cells (Fig. 4). There was large variation in the lengths of these features, with a
mean of 22 (SE ± 1.8) µm, minimum 6 µm and maximum 78 µm (69 features measured from 14
images).

To test the hypothesis that these features were type (IV) pili implicated in extracellular Fe(III)
reduction, we carried out the same experiment using genetically modified strains of *G.
sulfurreducens* by growing the different strains on ferrihydrite films as described. First, we used *G.
sulfurreducens* ΔpilA2 which lacks type(IV) pili [50] and secondly, *G. sulfurreducens* KN400 which
forms biofilms more readily and has been shown to produce more current in biofilms growing on the
anode of microbial fuel cells [51]. We found no evidence of these features in the ΔpilA2 strain, and
the appearance of the Redox Sensor Green signal on the cells was different from the wild type strain
images: There were bright spots of high redox activity within the cells, often at the poles of the cells,
compared to the wild type strain where the whole cells had a bright constant redox-active signal (Fig.
In *G. sulfurreducens* KN400 samples, we found more large aggregates of cells compared to the wild type and there were redox-active linear features (Fig. SI 4).

**Discussion**

This paper highlights the value of studying bacterial processes in situ, allowing direct imaging of reduced Fe(II) and redox-active pili. This has been facilitated by refining methods of culturing DIRB on Fe(III) oxyhydroxide films [39] into a novel setup featuring spin-coated slides and imaging of fully hydrated, living cells. AFM has been used previously to image EPS and bacteria [38], including *Geobacter* [30], but we have further exploited the capabilities of the technique using the tip contact “soft” measurements for assessing the physical properties of minerals combined with EPS. We have shown examples where the mineral has been transformed by bacteria through reductive dissolution or has become more adhesive near the cells. The capability for sub-nanometer scale resolution of the technique also makes it very suitable for studying fine features such as pili [52] and their properties such as hydrophobicity [53], and here we have further shown that pili can be studied during interactions with minerals. Bacterial EPS has been implicated in sorbing toxic metal ions [29] promoting Fe(III) reduction [40], increasing clay particle aggregation [54] and therefore using AFM to map the spatial distribution of EPS, metals and minerals in such systems could further help to study these interactions.

The application of the Fe(II) probe RhoNox-1 (RN-1) to bio-reduced Fe mineral films has highlighted the spatial orientation of Fe(II) in relation to living cells. The pattern of Fe(II) distribution on the mineral surface showed patches of bright fluorescence corresponding to the size and shape of *G. sulfurreducens* cells where the brightest patches were co-located with living cells and less bright patches were not co-located with cells (Fig. 3). We found that *G. sulfurreducens* cells themselves were weakly fluorescent, possibly explained by the Fe in heme groups of outer membrane cytochrome proteins responsible for electron transfer through the cell membrane to extracellular electron acceptors. C-type cytochromes, OmcB, OmcE and OmcS are all present on the outer
membrane and/or pili of *G. sulfurreducens* cells and therefore could be amenable to reduction by the RN-1 probe. There are also a host of periplasmic c-type cytochromes, such as ppcA, which could be accessible to the RN-1 probe. From similar imaging studies, we have seen evidence of non-living membrane shells of *G. sulfurreducens* or “ghost bacteria” [55] which remain attached to mineral surfaces (data not shown). These would not take up the styo9 probe, but may still contain cytochromes that could reduce RN-1.

Given the weak fluorescence of RN-1 with *G. sulfurreducens* cells, the majority of RN-1 fluorescence in samples where the bacteria were grown on ferrihydrite films came from extracellular Fe(II). Fe(II) bound to Fe-oxide surfaces has been found to have a high reactivity to reducible compounds [56] such as organic pollutants [57], and Cr(VI) [58]. Minerals with Fe(II) in their lattice structure may be intrinsically reactive, have reactive layers or, similar to Fe(III) minerals, have reactive surface bound Fe(II) [23,56], any of which could be reduced by RN-1, resulting in fluorescence. Our data suggest that Fe(II) was mostly produced in direct association with cells (which could be alive, dead, or no longer on the mineral surface at the time of imaging) and subsequently sorbed onto the Fe(III)-oxyhydroxide surface or transformed into Fe(II) bearing minerals. We cannot rule out the possibility of long-range electron transfer through nanowires or conductive minerals contributing to the presence of Fe(II) patches not co-located with living cells.

The development of metal- and oxidation-specific fluorescent probes is a rapidly expanding area of research. In the case of RN-1 [47], it is described as a probe for labile, intracellular Fe(II), but it is clear that it can also detect extracellular Fe(II) (Fig. 3). There are many fluorescent probes for elements of interest in subsurface, reducing environments available commercially or described in the literature. For example, Hao *et al.* [29] reviewed rhodamine-based probes for heavy metal species such as Cd$^{2+}$, Cu$^{2+}$, Hg$^{2+}$, Ni$^{2+}$ and Pd$^{2+}$. We have presented methods for directly imaging Fe biogeochemistry, but future studies combining these techniques with other metal probes could allow
significant advances in the understanding of the mechanisms controlling toxic metal mobility in the environment, especially when coupled to Fe redox transformations.

There have been numerous studies showing bacteria and mammalian tissue systems with redox-sensitive fluorescent proteins for intracellular imaging [59]. This paper aims to progress exploration of the extracellular redox environment, which is as yet much less well understood.

Reporter fluorescent protein (e.g. GFP or mCherry) expression is currently applicable for intracellular imaging and would not give information on extracellular biogeochemistry, hence the use of externally applied probes. The fluorescent probe “redox sensor green” has shown, for the first time using such techniques, extracellular appendages consistent with redox-active nanowires that may transfer electrons cell to cell, between cells and extracellular minerals or anodes, and also syntrophic partners [16]. As there was no clear correlation between RN-1 fluorescence and the location of redox-active appendages (Fig. 4), the appendages observed here were unlikely to be the dominant mechanism of electron transfer to the minerals in our experiments. Their main role in this system could be related to biofilm maintenance [60] and the presence of nanowire-like structures in a fully hydrated G. sulfurreducens biofilm was shown by fluorescence microscopy for the first time here. It has been suggested that the physiology of Geobacter spp. changes in the presence of (semi) conductive iron oxides from EPS rich biofilm forming mode to a low EPS network of mineral particles and microbial cells [61], which could be consistent with our results.

In conclusion, our results support the direct-contact model for electron transfer from G. sulfurreducens to Fe(III)-minerals because of the high Fe(II) concentration co-localised with bacterial cells and of cell-shaped and -sized Fe(II) patches on the mineral surface where cells have made contact with the surface, then potentially moved off or died. Changes to the mineral physical properties in the immediate vicinity of the cell surface are also consistent with this model and suggest some short-range electron transfer through the minerals themselves. Extracellular linear structures were identified and appeared to be connecting cells with other cells, often at relatively
long distances. These finding represent an important insight into electron transfer processes at the

cell-metal interface in hydrated conditions that mimic the subsurface environment. To further the
understanding of the processes of microbial Fe(III) reduction, complementary studies focussing on
imaging the dynamic development of *G. sulfurreducens* biofilms over time, their interaction with
other Fe(III)-bearing minerals and finally innovations towards imaging Fe(III) reduction in undisturbed
sediment samples (rather than laboratory analogues) would be beneficial. This would advance
further our understanding of the biogeochemical cycling of Fe, including the mechanisms involved
and the local and long range impacts on coupled processes.
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We have no conflict of interest to declare.
References


[40] Coker VS, Byrne JM, Telling ND, G VDL, Lloyd JR, Hitchcock AP, et al. Characterisation of the dissimilatory reduction of Fe(III)-oxyhydroxide at the microbe-


Figure 1

Proliferation of Geobacter sulfurreducens cells on thin amorphous iron oxide mineral film over 12 days. Cells were counted using an automated image analysis procedure. Scale bar = 10 µm. Error bars show standard error, n ≥ 3 at each time point.
AFM analysis of *Geobacter sulfurreducens* interaction with thin amorphous iron oxide films. (A) Cells “burrowing” into layer of ferrihydrite, (i) height profile, (ii) tip adhesion and (iii) energy dissipation measurements. Scale bar = 500 nm. (B) Cells on the surface of a film causing changes in physical properties of adjacent minerals (i) height profile, (ii) tip adhesion and (iii) energy dissipation measurements. Scale bar = 500 nm. (C) Mineral coated cells with appendages extending radially from the cells, highlighted with arrowheads in (i) height and (ii) energy dissipation measurements. Scale bar = 500 nm. (D) Filaments were also commonly found on the mineral surfaces where no cells were present. Height profile, scale bar = 2 µm.
Figure 3

Distribution of *Geobacter sulfurreducens* cells (green) and Fe(II) (red) on ferrihydrite film after 12 days, as highlighted by probes syto9 and RhoNox-1, respectively. (Ai-iii) Fe(II) and living cells are not always co-located. Scale bar = 5 µm. (Bi-ii) Fe(II) patches are similar size and shape to *G. sulfurreducens* cells. (Bii) marking cell positions (living cells shown in green in Bi) as outlines shows that the brightest patches of Fe(II) are co-located with cells.
Extracellular redox imaging shows arrangement of redox-active pili in *G. sulfurreducens* biofilm. (A-B) Clusters of cells (blue) and pili (green) forming connections between cells at long range. (C-i-iii) Redox probe highlighting cells and pili (green) and Fe(II) patches (red). Scale bar = 5 µm.
Figure S1. Fluorescence response of 2 µM RhoNox-1 in deionized H$_2$O (pH 7.4) with increasing addition of FeSO$_4$ in 50 mM HEPES buffer following excitation at 540 nm.
Figure S2. Steady state excitation-emission maps of 2 samples of Fe(III)-oxyhydroxide as a solution in H₂O at pH 7 with an absorbance of less than 0.5 absorption units (A) and as a thin film in the solid state (B) measured with a 450 W Xe lamp. The peaks visible are a result of scattering from the sample and from non-linear effects from the excitation source. No emission from the samples was detected with excitation between wavelengths 360 – 540 nm.
Figure S3. RhoNox-1 intensity, as a measure of concentration of Fe(II), in bright patches of RhoNox-1 signal (ROIs) that correspond with living cells and thick mineral (4) (n = 46), thick mineral but no cell (3) (n = 90), cell but no thick mineral (2) (n = 70) and no thick mineral or cell (1) (n = 188). Boxes show median, 25, 75 perc. Whiskers show 5 and 95 perc. Dotted line represents mean RhoNox-1 fluorescence of whole image.
Figure S4. (A) Redox imaging (green) of biofilms of *G. sulfurreducens* ΔpilA (blue) prepared by growing cells on thin amorphous iron oxide films showed no extracellular pili and a different pattern of redox activity: bright spots on the cell surfaces, often at the cell poles. (B) Redox imaging did reveal pili connections between cells in *G. sulfurreducens* KN400.