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# The impact of Fe(III)-reducing bacteria on uranium mobility

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**Abstract.** The ability of specialist prokaryotes to couple the oxidation of organic compounds to the reduction of Fe(III) is widespread in the subsurface. Here microbial Fe(III) reduction can have a great impact on sediment geochemistry, affecting the minerals in the subsurface, the cycling of organic compounds and the mobility of a wide range of toxic metals and radionuclides. The contamination of the environment with radioactive waste is a major concern worldwide, and this review focuses on the mechanisms by which Fe(III)-reducing bacteria can affect the solubility and mobility of one of the most common radionuclide contaminants in the subsurface, uranium. In addition to discussing how these processes underpin natural biogeochemical cycles, we also discuss how these microbial activities can be harnessed for the bioremediation of uranium-contaminated environments.

## Introduction

The potential for Fe(III)-reducing bacteria to limit uranium mobility in anaerobic subsurface environments is currently of great interest. Uranium contamination can be a major environmental problem due to the high degree of solubility and mobility of this key radionuclide in the oxidised form. Although chemical pump-and-treat processes have been used previously to try and limit the spread of contamination at various sites around the world, these techniques are often prohibitively expensive and give unsatisfactory results (Mackay and Cherry 1989; Macdonald and Kavanaugh 1994). By contrast, microbially mediated bioremediation strategies have the potential to be relatively simple, low cost and effective and there is now considerable interest in this area (for example in the Natural and Accelerated Bioremediation Research [NABIR] program, Office of Biological and Environmental Research, U.S. Department of Energy [http://www.lbl.gov/NABIR]). Some of the dissimilatory Fe(III)-reducing bacteria (DIRB) that are commonly found in radionuclide-contaminated environments can couple the oxidation of organic compounds to the reduction of U(VI), resulting in the precipitation of U(VI) from solution as

U(IV). At many contaminated sites it has been suggested that the addition of simple organic compounds (e.g. short chain fatty acids) to act as electron donors will stimulate the natural communities of Fe(III)-reducing bacteria and result in U(IV) precipitation as the very insoluble mineral uraninite. Other possible strategies include stimulating Fe(III) reduction so that the resulting biogenic Fe(II) minerals can abiotically reduce key radionuclide contaminants. Given the considerable potential of these bioremediation strategies, this review will focus on the direct enzymatic effects of Fe(III)-reducing bacteria and also the products of Fe(III) reduction on uranium mobility in subsurface environments.

## Fe(III) reduction

In aerobic environments, bacteria can couple the oxidation of organic matter to the reduction of the terminal electron acceptor, dissolved oxygen. However, the diffusion of oxygen into sediments is slow, resulting in this respiratory process being restricted to fringe areas around the sediment/water or oxic/anoxic interface. The utilisation of all available oxygen results in the onset of anaerobic conditions (Chapelle 1993). Under such conditions, $NO_3^-$, Mn(IV), Fe(III), $SO_4^{2-}$ and $CO_2$ can all be utilised successively as terminal electron acceptors (TEAs) in the oxidation of organic matter (Reeburgh 1983). Although classical diagrams show the formation of distinct regions of different TEA processes, it is becoming clear that these systems are far more complex than previously thought. For example, when predicting which substrates will be available for microbial reduction, one must consider the kinetic and thermodynamic factors of both solid and soluble electron acceptors (Roden 2003; Roden, 2004b).

The importance of Fe(III)-reducing microorganisms and Fe(III) reduction in a range of environmental settings and biotechnological applications cannot be underestimated (Methe and Fraser 2004). Iron is the fourth most abundant element in the Earth's crust, where it constitutes approximately 5% of the mass (Straub et al. 2001). Fe(III) can exist in the environment in a variety of states as oxides, hydroxides and oxyhydroxides, but for ease of expression, all these various forms will simply be referred to as 'Fe(III) oxides' for the remainder of this manuscript. Recent work has suggested that Fe(III) reduction may have been one of the first microbial respiratory processes on Earth (Vargas et al. 1998). Many new biotechnology applications also harness the unique enzymatic capabilities of DIRB for a range of *in situ* and *ex situ* processes (Lloyd et al. 2004) including the bioremediation of metal contaminated land and water (Lloyd and Lovley 2001), the oxidation of xenobiotics under anaerobic conditions (Lovley and Anderson 2000) and even the generation of electricity from sediments (Bond et al. 2002). In many environments, Fe(III) is the dominant terminal electron acceptor, resulting in both the processes and products of Fe(III) reduction having important effects on the biogeochemistry of these

areas. Indeed, some research has suggested that Fe(III) reduction accounts for the oxidation of up to 65% of organic matter in some anaerobic sediments (Canfield 1989). As well as playing a major role in organic matter cycling, the reduction of Fe(III) to Fe(II) can lead to other significant chemical changes in anaerobic soils, sediments and groundwaters, such as the release of soluble Fe(II) into water systems, the release of contaminants such as trace metals and radionuclides into water supplies and the inhibition of methane production (Lovley and Phillips 1986b, 1988).

### *Fe(III) reducing bacteria*

The first research identifying micro-organisms capable of conserving energy to support growth from Fe(III) reduction was published in 1979, then in English translation in 1980 (Balashova and Zavarzin 1980). Bacteria from both the delta and gamma subdivision of the *Proteobacteria* were subsequently shown to be able to reduce Fe(III) in pure culture. *Geobacter metallireducens* (formerly strain GS-15) is from the delta subdivision and was the first organism found to couple complete oxidation of acetate to $CO_2$ via the citric acid cycle with the reduction of Fe(III). In addition to acetate, *G. metallireducens* can oxidize ethanol, propionate, butyrate, valerate, pyruvate, propanol and toluene amongst others to carbon dioxide with Fe(III) as the electron acceptor (Lovley et al. 1993a), thus:

$$\text{pyruvate}^- + 2\text{Fe(III)} + 2\text{H}_2\text{O} \rightarrow \text{acetate}^- + \text{HCO}_3^- + 2\text{Fe(II)} + 3\text{H}^+$$

and

$$\text{acetate}^- + 8\text{Fe(III)} + 4\text{H}_2\text{O} \rightarrow 2\text{HCO}_3^- + 8\text{Fe(II)} + 9\text{H}^+$$

Since the isolation of *G. metallireducens*, a number of organisms able to conserve energy via the complete oxidation of acetate and reduction of ferric iron have either been isolated from environments or identified by screening culture collections. These bacteria include other *Geobacter* species such as *Geobacter sulfurreducens* (Caccavo Jr et al. 1994) and closely related *Desulfuromonas* sp. such as *D. palmitatis* (Coates et al. 1995). *D. acetoxidans* had previously been isolated because of its ability to couple the oxidation of acetate to the reduction of $S^0$ (Pfennig and Biebl 1976), but was found to be closely related to *G. metallireducens* (the genera *Geobacter* and *Desulfuromona* are both affiliates of the family *Geobacteraceae* (Lonergan et al. 1996)), and further work demonstrated that this organism could also grow on acetate with Fe(III) as the sole electron acceptor (Roden and Lovley 1993). Some *Pelobacter* species such as *P. carbinolicus* are able to grow using $H_2$ or ethanol as the electron donor and Fe(III) as the sole electron acceptor (Lovley et al. 1995). The same study also demonstrated that these bacteria could use $S^0$ as the terminal electron acceptor.

In the gamma subdivision of the *Proteobacteria*, organisms such as *Shewanella oneidensis* (formerly *S. putrefaciens*) MR-1 (Lovley et al. 1989), *Shewanella alga* (formerly strain BrY) (Caccavo et al. 1992; Rossello-Mora et al., 1994) and *Pseudomonas* sp. strain Z-731 (Balashova 1985; Balashova and Zavarzin 1980) can conserve energy from Fe(III) reduction but they have a limited ability to use organic electron donors. Although *Shewanella* species are unable to utilise acetate as an electron donor, they can couple the oxidation of lactate and hydrogen to the reduction of Fe(III) (Lovley et al., 1989).

Although the most intensively studied Fe(III)-reducers are found in the delta and gamma subdivisions of the *Proteobacteria*, the discovery of new species with Fe(III) reducing ability indicates that this capability is not just confined to these groups. *Geothrix fermentans* was initially isolated from aquifer sediments where Fe(III) reduction had been stimulated and is closely related to the acetogen *Holophaga foetida* (Coates et al. 1999) whilst another novel Fe(III)-reducing bacteria, *Geovibrio ferrireducens*, is not related to any metal-reducing bacteria in the Proteobacteria and forms a separate line of descent within the Bacterial Kingdom (Caccavo et al. 1996). In more extreme environments other Fe(III) reducing bacteria have been isolated including *Anaeromyxobacter*, *Paenibacillus*, *Brevibacillus* and *Acidiphilium* species from acidic sediments (Kusel et al. 1999; Petrie et al. 2003) and *Geothermobacterium ferrireducens* from a hydrothermal environment (Kashefi et al. 2002). Given the wide range of Fe(III)-reducing bacteria, it is therefore not surprising that they exhibit a high degree of metabolic diversity, with various species able to reduce Cr(VI) (Myers et al. 2000), Mn(IV) (Lovley and Phillips 1988; Myers and Nealson 1990), U(VI) (Lovley et al. 1991), Tc(VII) (Lloyd and Macaskie 1996), Co(III)EDTA complexes (Gorby et al. 1998), Au(III) (Kashefi et al. 2001) and V(V) (Ortiz-Bernad et al. 2004b).

Despite the apparent diversity of Fe(III)-reducing bacteria, microbial community analysis of subsurface environments where Fe(III) reduction occurs has suggested that in many cases, *Geobacter* species are the dominant Fe(III)-reducing microorganisms (Lovley et al. 2004). Also, in both laboratory (Rooney-Varga et al. 1999) and field-based experiments (Snoeyenbos-West et al. 2000; Holmes et al. 2002; Islam et al., 2004; North et al. 2004; Peacock et al. 2004) the stimulation of Fe(III) reduction by addition of electron donors such as acetate, a central intermediate in the breakdown of organic matter, promotes the enrichment of *Geobacter* strains.

### *Mechanism of reduction*

Dissimilatory Fe(III) reduction is the process by which the ferric iron reductase, probably located on the outer membrane in Gram-negative bacteria (Myers and Myers 1992; Myers and Myers 1997; Lloyd 2003) acts as the terminal reductase of an electron transport chain which is linked to the cytoplasmic membrane. Electrons are transferred down the transport chain to the

ferric iron reductase which then transfers them onto insoluble extracellular Fe(III) oxides. This movement of electrons is used to conserve energy for growth via the generation of ATP. This mechanism can be contrasted with assimilatory iron reduction, a process used for the uptake of iron in nearly all living organisms. Here, chelated Fe(III) is reduced by the ferric reductase either before or after uptake into the cell, thus forming a weak Fe(II)-chelate complex from which the iron can be easily dissociated for use by the cell.

Because the ferric iron reductase activity was thought to be localised on the surface of the cell in DIRB, it was previously hypothesised that cell contact with Fe(III) minerals was necessary for reduction to occur (Lovley and Phillips 1988) but this has since been shown not to be true in all situations. Recent work has in fact identified four possible mechanisms by which Fe(III) oxides can be reduced by Fe(III)-reducing bacteria and these are covered in great detail in the excellent review by Nevin and Lovley (2002b). Briefly however, these are the different mechanisms.

*Direct contact*

The most obvious mechanism is for the microorganism to contact the insoluble iron oxide and transfer an electron from the cell onto the Fe(III) oxide surface, with outer membrane bound *c*-type cytochromes playing some role in this process. This mechanism is thought to be employed by DIRB including *Geobacter* species. However, this mechanism is only possible if the bacteria are able to access the iron oxides directly. The production of flagellae which enable the organism to move towards Fe(III) minerals has been reported in *G. metallireducens* (Childers et al. 2002). Recent attention has focused on the role of pili in electron transfer onto an Fe(III) oxide surface (Reguera et al. 2005). Pili-deficient *G. sulfurreducens* mutants could attach to Fe(III) oxides but were unable to reduce them. Thus, some role in electron transfer from the cell to the mineral surface has been proposed for these highly conductive 'nanowires' in this microorganism. Despite this range of mechanisms for contacting mineral surfaces, in some environments the microbial population may not be able to access Fe(III) oxides due to a variety of reasons. If Fe(III) oxides are located in small pore spaces, out of reach of redox active cell surface assemblages, they may be inaccessible whilst the Fe mineral surface may become occluded due to the adsorption of (in)organic constituents (Roden and Urrutia 1999). In these instances, the microbes present must employ other mechanisms to facilitate Fe(III)-reduction.

*Fe(III) chelators*

Fe(III) oxides can be solubilised using Fe(III) chelators. The soluble chelated Fe(III) is then more accessible to Fe(III) reductases than insoluble Fe(III) oxides and can then be reduced more readily (Lovley et al. 1996). Lovley and Woodward (1996) demonstrated that the synthetic Fe(III) chelator, nitrilotriacetic acid (NTA), was able to increase the rate at which both poorly crystalline, and crystalline Fe(III) forms were reduced. However, it has also been

suggested that chelators may stimulate Fe(III) oxide reduction by removing inhibitory Fe(II) on the surface of either the Fe(III) oxide or the cell, thereby increasing the rate of Fe(III) reduction (Roden and Urrutia 1999; Urrutia et al. 1999). The production of a chelating agent by a DIRB, *Geothrix fermentans*, has also been reported (Nevin and Lovley 2002a).

*Electron-shuttling compounds*

Electron-shuttling compounds found in the environment provide another mechanism by which bacteria can reduce Fe(III) oxides without the need for direct contact with the mineral phase. An electron shuttle is able to accept an electron from an Fe(III)-reducing microorganism and transfer it to the Fe(III) oxide surface, regenerating itself in the oxidized form. As with chelated Fe(III), soluble electron shuttling compounds should be more accessible to terminal reductases than insoluble Fe(III) oxides. Humic substances and related compounds were the first electron-shuttling compounds reported to stimulate Fe(III)-oxide reduction (Lovley et al. 1996). The quinone moieties in the humic compounds accept electrons from Fe(III)-reducing bacteria (Scott et al. 1998), producing semiquinones which abiotically transfer the electrons to insoluble Fe(III) oxides. Following the transfer of the electron, the humic substance is reoxidized and can act as an electron acceptor again. Experiments using *G. metallireducens* and *S. alga* have demonstrated that respiration on humics as the sole electron acceptor can yield energy to support cell growth (Lovley et al. 1998, 1999). Humics have been shown to reduce a variety of Fe(III) oxide phases including poorly crystalline Fe(III) oxide as well as other Fe(III) forms, such as goethite and hematite that in some instances are resistant to reduction (Lovley and Phillips 1987). The reduction of structural Fe(III) present in clays such as smectite was also stimulated by the addition of humic acids and the humic analogue 2,6-anthraquinone disulfonate (AQDS) (Lovley et al. 1998).

*Production of electron-shuttling compounds*

Whereas some bacteria use natural electron shuttles such as humic compounds to transport electrons to Fe(III) oxides, other organisms may be able to produce electron-shuttling compounds themselves. As well as producing Fe(III) chelating molecules, work with *G. fermentans* demonstrated that this organism could release electron-shuttling compounds. The identity of the electron-shuttling compound was not discovered but heat and protease treatments suggested that it was not a protein. The compound also appears to be very specific for the reduction of Fe(III) oxides by *G. fermentans*. Whereas AQDS and humic compounds can stimulate Fe(III) oxide reduction by a number of DIRB, the *G. fermentans*-produced electron-shuttling compound could not be utilised by *G. metallireducens* in Fe(III) oxide reduction. Moreover, the addition of AQDS for additional electron-shuttling capacity had no effect on the Fe(III) oxide reduction rate (Nevin and Lovley 2002a).

Evidence for the production of an electron shuttling compound by *G. fermentans* has also been gathered in work looking at the reduction of

graphite electrodes by this microorganism (Bond and Lovley 2005). The potential for redox-active antibiotics to play a role in Fe(III) oxide reduction has also been recently reported by Hernandez et al. who noted that some antibiotics produced by common soil bacteria have similar aromatic ring structures and redox-active functional groups to other electron-shuttling compounds such as humics and AQDS (Hernandez et al. 2004).

*Fe minerals*

A poorly crystalline Fe(III) compound later known as ferrihydrite was first described by Towe and Bradley (1967). Whereas other common Fe(III) or Fe(III)-containing oxides such as goethite, hematite and magnetite occur in sufficient abundance to be considered rock-forming minerals, ferrihydrite, although common, occurs less frequently and in less abundance than these more crystalline oxides. Despite this, bulk experiments have indicated that in subsurface environments, ferrihydrite is the most bioavailable form of ferric iron available for microbial reduction (Lovley and Phillips 1986a; Glasauer et al. 2003) and often makes up around 20% of the total iron phase in a sediment (Thamdrup 2000). Ferrihydrite is extremely fine-grained and so exhibits a high surface area (several hundred square metres per gram). It often occurs as coatings on rock fragments in subsurface environments, or as suspended material in groundwaters (Schwertmann et al. 1982).

As ferrihydrite is thermodynamically unstable, Ostwald ripening and/or structural aggregation (based on thermodynamics) leads to the precipitation of goethite and hematite, which are more crystalline. (Johnston and Lewis 1983; Cornell and Schwertmann 1996; Schwertmann et al. 1999; Banfield et al. 2000). Crystalline Fe(III) minerals are the dominant Fe(III) oxide phases in soils and sediments but have significantly smaller surface areas than more poorly crystalline Fe(III) phases (Schwertmann and Taylor 1989). The enzymatic reduction of these more crystalline phases has also been demonstrated in several lab-based studies (Roden and Zachara, 1996; Zachara et al. 1998; Roden and Urrutia 1999; Hansel et al. 2004), with the passivation of the oxide surface by Fe(II) identified as a major control on long-term crystalline Fe(III) oxide reduction (Roden 2004a). Continuous flow systems have been used to investigate the effects of the removal of this passivating Fe(II). Far greater reduction of crystalline Fe(III) was observed in a continuous flow system where Fe(II) was removed than in a batch system where Fe(II) accumulated (Roden et al. 2000). These studies have also indicated that natural crystalline Fe(III) oxides could be subject to much greater degrees of microbial reduction when compared to synthetic crystalline forms (Zachara et al. 1998), possibly due to differences in crystalline disorder and defects between the natural and synthetic oxides. This clearly requires verification in field experiments.

As electrons are transferred from the cell to the Fe(III) oxide surface, Fe(II) is released which can lead to the formation of a variety of secondary Fe

minerals such as siderite, vivianite, magnetite and lepidocrocite. A wide range of factors including pH, $pCO_2$, electron donor and acceptor concentrations and $E_h$ influence the formation of different Fe(II) mineral phases and it is beyond the scope of this review to cover this area comprehensively. However, some excellent papers have recently investigated and discussed secondary Fe(II) mineral formation and are therefore recommended (Fredrickson et al. 1998; Glasauer et al. 2003; Hansel et al. 2003).

## The impact of biotic/abiotic reactions on U cycling

### *Introduction*

The issues surrounding radionuclide mobility in the environment are becoming increasingly important as the problems surrounding long-term storage of nuclear wastes are addressed and remediation strategies developed for contaminated sites. Radionuclide contamination has come from a variety of sources including the nuclear power industry, the production of nuclear weapons and the mining of uranium (Lloyd and Macaskie 2000; Lloyd and Renshaw, 2005). Wastes can range from low-level radioactive liquids and gases to high-level radioactive wastes produced in nuclear fuel reprocessing. $^{226}Ra$, $^{222}Rn$, $^{238}U$ and $^{230}Th$ are frequent contaminants at former uranium mills (Morrison and Cahn 1991) while uranium is the primary contaminant at a majority number of low- and medium-level waste storage sites. Great amounts of nuclear waste were produced during the Cold War and in many instances priority was given to weapons production over waste management. In the United States, two of the largest nuclear weapons complexes were the Hanford Engineering Works in Washington and the Oak Ridge site in Tennessee, both of which produced the enriched uranium and plutonium for the first atomic bomb. Other large complexes include Savannah River in South Carolina and the Idaho National Engineering and Environmental Laboratory (Crowley and Ahearne 2002). At many of these sites, problems associated with nuclear waste storage have led to concerns about environmental contamination. For example, at the Hanford site 177 large underground tanks store about 200,000 cubic metres of high-level waste whilst at Savannah River 48 tanks store around 130,000 cubic metres of high-level waste. At both sites, leaks from these tanks have occurred, with 1.5 million gallons of waste estimated to have leaked from 67 tanks into the subsurface at the Hanford site. Once released into the subsurface, uranium and other radionuclide contaminants can then move considerable distances, in some instances being transported into aquifers and rivers (McKinley et al. 2001; Moser et al. 2003; Flury et al. 2004).

Because of the large scale of contamination and the subsequent migration of radionuclides through the subsurface, interest is focusing on the potential effects of microbial processes on radionuclide solubility. Microorganisms are able to impact upon radionuclide mobility via a range of mechanisms but

discussing all these processes is beyond the scope of this review. The reader is referred to other reviews dealing with some of these aspects (Macaskie 1991; Banaszak et al. 1999; Lloyd and Macaskie 2000; Macaskie and Lloyd, 2002; Gadd 2005).

Many microorganisms that are able to couple the oxidation of organic compounds to the reduction of Fe(III) are also able to reduce various radionuclides enzymatically. In many cases this reduction results in a decrease in the radionuclide solubility and the precipitation of insoluble minerals (e.g. the reduction of U(VI) to U(IV) to give insoluble $UO_2 \cdot 2H_2O$). However, in other cases, the reduction of the radionuclide can sometimes result in a soluble species (e.g. the reduction of Np(V) to Np(IV)). Direct enzymatic reduction was first demonstrated with U(VI) (Lovley et al. 1991) but since this discovery the enzymatic reduction of Tc(VII) (Lloyd and Macaskie 1996), Np(V) (Lloyd et al. 2000b) and Pu(IV) (Rusin et al. 1994) have all been reported. The microbial reduction and biogeochemistry of a range of metals and radionuclides in addition to U have been recently reviewed by Lloyd (2003) and Lloyd and Renshaw (2005).

### *Enzymatic uranium reduction*

The mobility of U in anaerobic subsurface environments is dependent on its ability to form insoluble precipitates or strongly sorbing species. Although the oxidation state of uranium can vary from III to VI (Seaborg 1993), under environmental conditions only the IV and VI states are stable. Uranium is very redox sensitive and in oxidised environments, exists as the soluble uranyl [$U(VI)O_2^{2+}$] species. By contrast, under anoxic conditions it can form insoluble U(IV) precipitates such as $UO_2 \cdot 2H_2O$ (Lovley et al. 1991; Rai et al. 2003) The III oxidation state is very easily oxidised whilst the V oxidation state is prone to disproportionation.

The majority of Fe(III)-reducing microorganisms that can conserve energy by coupling the oxidation of hydrogen or organic compounds to the reduction of Fe(III) also have the ability to reduce soluble U(VI) to insoluble U(IV) (Lovley and Anderson 2000). These microorganisms include the well studied *G. metallireducens*, *S. oneidensis* and *S. alga* species (Lovley et al. 1991; Caccavo et al. 1992). In addition, some sulfate-reducing bacteria including *D. desulfuricans* and *D. vulgaris* are able to reduce Fe(III) and U(VI) enzymatically without conserving energy from these processes (Lovley and Phillips 1992b; Lovley et al. 1993c), while *Desulfotomaculum reducens* can grow with either Fe(III) or U(VI) as the sole electron acceptor (Tebo and Obraztsova 1998).

Microbial reduction of U(VI) by the Fe(III)-reducing microorganism *G. metallireducens* (formerly GS-15) was first reported by Lovley et al. (1991). Prior to this discovery, U(VI) reduction was simply considered an abiotic reaction, with sulfide, organic compounds or molecular hydrogen acting as the reductant. Microbial U(VI) reduction occurs much faster than abiotic reduc-

tion and helps to explain the presence of uranium deposits in areas of Fe(III) reduction (Bonatti et al. 1971; Langmuir 1978). The U(VI) reduction rate has been studied for a number of U(VI) reducing bacteria including *G. metallireducens*, *S. alga*, *S. oneidensis* and *D. desulfuricans* (Lovley and Phillips 1992a). Although work with *S. alga* under non-growing conditions demonstrated that the U(VI) reduction rate proceeds at only 30% of the Fe(III) reduction rate, *Shewanella* species have been shown to have slightly faster reduction rates than *G. metallireducens* and *D. desulfuricans* (Truex et al. 1997; Liu et al. 2002).

The pathway by which U(VI) is enzymatically reduced has been studied in *D. vulgaris* (Lovley and Phillips 1994), *G. sulfurreducens* (Lloyd et al. 2003) and *S. oneidensis* (Wade Jr. and DiChristina 2000) with *c*-type cytochromes identified as playing a role in two of these microorganisms.

*D. Vulgaris*. Work with *D. vulgaris* identified the tetraheme cytochromes $c_3$ as a U(VI) reductase *in vitro* when supplied with hydrogen gas as the electron donor (Lovley and Phillips 1994). A cytochrome $c_3$ mutant strain of a close relative to *D. vulgaris*, *D. desulfuricans*, was then used in *in vivo* experiments to further confirm the role of this cytochrome in U(VI) reduction (Payne et al. 2002). However, the authors noted that their results indicated that there are additional pathways for U(VI) reduction in this strain with organic electron donors. More recent work looking at the effects of uranium on the transcription and translation of cytochrome $c_3$ has produced inconclusive results (Payne et al. 2004).

*G. sulfurreducens*. In the well characterised Fe(III)-reducing microorganism *G. sulfurreducens*, the triheme periplasmic cytochrome $c_7$ has been identified as playing a role in the transfer of electrons from acetate to U(VI) (Lloyd et al. 2002, 2003), although more recent studies also suggest a role for other periplasmic and outer membrane cytochromes (Shelobolina and Lovley, pers comm.). It was previously assumed that *G. sulfurreducens* reduced U(VI) to U(IV) via a two-electron transfer from the U(VI) terminal reductase. However, recent work carried out by Renshaw et al. (2005) has suggested that this microorganism transfers one electron to U(VI), reducing it to U(V). As mentioned earlier, U(V) is unstable and prone to disproportionation, forming U(VI) and U(IV). When *G. sulfurreducens* and uranyl (VI) acetate were incubated together, U(V) accounted for 60% of the total U after 4 h incubation whereas after 8 h, about 80% of the U was in the U(IV) state as $UO_2$. *G. sulfurreducens* was unable to enzymatically reduce the pentavalent actinide Np(V), suggesting that the electron transfer chain of the organism is specific for hexavalent actinides, and that the reduction of U(V) to U(IV) is abioitc (disproportionation) (Figure 1).

*Shewanella* species. Preliminary work on another well characterised Fe(III)-reducing bacterium, *Shewanella putrefaciens* (strain 200) indicated that, in this microorganism at least, part of the nitrite-reduction pathway may be involved in U(VI) reduction (Wade Jr. and DiChristina 2000). Mutants that were unable to respire on U(VI) were found to be unable to respire on $NO_2^-$.

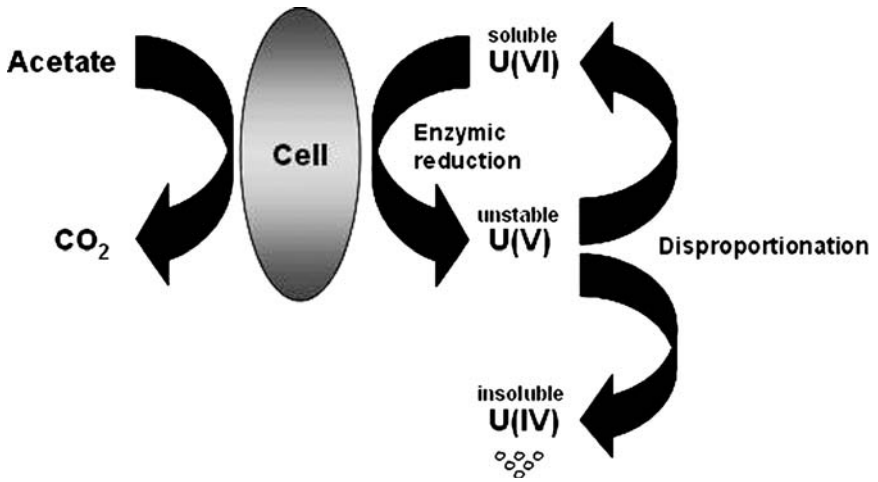


*Figure 1.* Potential route via which U(VI) is reduced and U(IV) formed by *G. sulfurreducens*.

Following the discovery of an enzymatic pathway in DIRB for U(VI) reduction, Gorby and Lovley (1992) demonstrated that it was possible for Fe(III)-reducing bacteria to precipitate U(IV) from water via U(VI) reduction. These experiments suggested that there is potential for the *in-situ* remediation of U(VI)-contaminated subsurface sites simply by stimulating the indigenous Fe(III)-reducing microbial community. When Fe(III)-reducing bacteria reduce U(VI) to U(IV), insoluble uraninite ($UO_2$) is precipitated (Lovley et al. 1991). Recent work has shown that the initial particles of uraninite are very small, ca 1.5–3 nm in diameter, and at first this discovery led to concerns that the U(IV) would be mobile in porous sediments and therefore not immobilised in the environment (Suzuki et al. 2002). However, it now seems likely that these small uraninite particles quickly aggregate after formation into much larger U(IV) particles which are very insoluble and far less mobile than was initially predicted (Lovley and Phillips 1992a; Suzuki et al. 2002). Indeed, the analysis of sediments at an inactive uranium mine revealed near-surface areas that were highly enriched in stable microbially reduced U(IV) (Suzuki et al. 2005).

The potential for stimulating microbial U(VI) reduction in contaminated aquifer sediments at circumneutral pH via the addition of electron donors has been assessed (Finneran et al. 2002a). Although the addition of the electron donors formate, lactate or benzoate to the sediments had little effect on U(VI) concentrations, the addition of acetate or glucose led to soluble U(VI) concentrations decreasing from 10 μM to below detection limits in 15 days. While U(VI) reduction occurred concurrently with Fe(III) reduction in the aquifer material, no sulfate reduction was observed until U(VI)- and Fe(III)-reduction had ceased. These data, coupled with molecular analysis of the microbial community which showed a large enrichment of microorganisms in the family *Geobacteraceae* (Holmes et al. 2002), suggested that Fe(III)-reducing bacteria

rather than sulfate-reducing microorganisms were responsible for the U(VI) reduction. The addition of the electron shuttling compound AQDS led to an increase in Fe(III) reduction but had no effect on U(VI) concentrations when the majority of U(VI) was in solution. This contrasts with another study which concluded that AQDS increased both the rate and extent of U(VI) reduction in sediment microcosm studies where the majority of U(VI) was sorbed to mineral surfaces (Jeon et al. 2004). In this work, AQDS also had negligible influence on the reduction of the small amount of aqueous U(VI) present in the experiments. It has therefore been suggested that whilst $AH_2DS$ reacts more rapidly with Fe(III) than aqueous U(VI), it is able to access sorbed U(VI) on sediments in areas that are inaccessible to terminal reductases in microorganisms (Jeon et al. 2004). In the absence of Fe(III), some recent work has indicated that natural humic acids in laboratory microcosms can also increase the rate of U(VI) reduction ten-fold (Gu et al. 2005b).

## In situ *projects*

Following successful experiments demonstrating U(VI) reduction in the laboratory, a number of studies have now attempted to stimulate U(VI) reduction *in situ*. These test sites have been at a US Department of Energy (DOE) uranium mill tailings remedial action (UMTRA) site in Rifle, CO (USA) and the US DOE NABIR Field Research Centre site at Oak Ridge, TN (USA).

### *UMTRA Rifle site*

At the UMTRA site in Rifle, an injection gallery was constructed to allow the injection of acetate into the subsurface (Anderson et al. 2003). Following the addition of this electron donor, Fe(III) reduction was stimulated and a drop in U(VI) concentrations was recorded after only 9 days. U(VI) concentrations fell by about 70% over the first 50 days of the experiment. These geochemical changes were associated with a significant enrichment of *Geobacter* species in the treatment zone. After about 40 days however, more changes were observed in the subsurface microbial community. Whereas the community had been '*Geobacteraceae*-dominated' following acetate addition, as time went on, a shift towards microorganisms that could couple the oxidation of acetate to sulfate reduction was seen. By Day 80 of the study, members of the *Desulfobacteraceae* comprised about 45% of the microbial community and products of sulfate reduction such as sulfide were increasing in concentration. This switch from Fe(III) reduction to sulfate reduction was associated with an increase in U(VI) concentrations down-gradient of the acetate-injection zone, suggesting that the acetate-oxidising sulfate-reducing bacteria present were not able to maintain the removal of U(VI). Although some sulfate-reducing bacteria have been shown to reduce U(VI) (Lovley and Phillips 1992a; Lovley et al. 1993b), this has been with hydrogen or lactate rather than acetate serving as the electron donor.

*Oak Ridge FRC site*

At the Oak Ridge site, attempts have also been made to stimulate the activity of Fe(III)-reducing bacteria *in situ* (Istok et al. 2004; North et al. 2004). In one set of experiments, glucose and ethanol were added to the subsurface and the resulting geochemical and microbial changes were monitored (North et al. 2004). As with the work at Rifle, microorganisms from the *δ-proteobacteria* were enriched following the addition of the carbon sources until they accounted for *ca.* 40% of the microbial community. More specifically, *Geobacter*-type microorganisms were shown by quantitative PCR to have increased in numbers by between one and two orders of magnitude in the amended sediments. Another dissimilatory Fe(III)-reducing member of the *δ-proteobacteria*, *Anaeromyxobacter dehalogenans*, was also significantly enriched in the biostimulated sediment. Associated with this microbial enrichment in the electron donor-amended sediments was a decrease in U(VI) groundwater concentrations from *ca.* 5 μM to less than 1 μM within 200 h. Other work at the Oak Ridge site investigated microbial changes following electron donor addition to the subsurface by developing a downwell microbial sampling system (Peacock et al. 2004). Synthetic Bio Sep beads and glass wool were used as a solid support matrix for microbial growth and were suspended down wells at the site before being removed for analysis. Again, the addition of ethanol, glucose or acetate resulted in the enrichment of members of the *Geobacteraceae* and also of nitrate-reducing microorganisms, due to the high nitrate concentrations at the site. The removal of U(VI) from groundwater at the Oak Ridge site was observed following the stimulation of these Fe(III)-reducing microorganisms by the addition of acetate, ethanol and glucose to the subsurface (Istok et al. 2004).

*Sorbed U(VI) reactivity*

Whilst some of the studies described above demonstrated that soluble U(VI) can be removed from groundwater by microbial enzymatic reduction, the reducibility of sorbed U(VI) is unknown. In many subsurface environments, Fe(III) oxides will be the mineral phases responsible for the greatest removal of U(VI) from solution via adsorption. Ferrihydrite is very effective at removing U(VI) from solution (Hsi and Langmuir 1985; Langmuir 1978) due to its large surface area whilst the more crystalline Fe oxides such as goethite and hematite are still able to sorb U(VI) but have lower adsorption capacities than ferrihydrite. Fredrickson et al. (2000) initially reported that the reducibility of the U(VI) mineral metaschoepite decreased significantly when it was associated with goethite. Other research has since suggested that sorbed U(VI) is only bioavailable once soluble U(VI) is almost exhausted (Elias et al. 2003b). Jeon et al. (2004) studied synthetic Fe(III) oxides and natural Fe(III) oxide-containing solids in the presence of *G. sulfurreducens*, where more than 95% of U(VI) was sorbed, and discovered that the extent of U(VI) reduction varied

significantly between the different materials. Using X-ray absorption near-edge structure (XANES) data, it was reported that U(VI) sorbed onto the surface of synthetic hematite, goethite and ferrihydrite is reduced at a similar rate to aqueous U(VI), whereas both the rate and extent of U(VI) reduction is lower for U(VI) sorbed onto natural solids. As briefly mentioned earlier, the addition of AQDS to the natural solids results in the complete reduction of sorbed U(VI), leading to the suggestion that some U(VI) is sorbed in enzymatically inaccessible locations on the natural mineral surfaces. In the absence of an electron shuttle, terminal reductases cannot access the U(VI), leading to a decrease in U(VI) reduction. Calculations of the total microporosities for the different materials indicated that the values for the natural materials were 5- to 10-fold greater than those for the synthetic hematite. Recent work carried out by Ortiz-Bernad et al. (2004a) on sediments taken from the *in situ* bioremediation project at Rifle suggests that much of the U(VI) reduction seen at this site may have resulted from the reduction of soluble U(VI). Although Jeon et al. (2004) previously reported that U(VI) sorbed onto natural solids undergoes limited microbial reduction, it now appears that in these Rifle sediments, the portion of U(VI) sorbed to subsurface material is not available for microbial reduction (Ortiz-Bernad et al. 2004a). Due to the recent nature of the work indicating limited microbial reduction of sorbed U(VI), the consequences for microbial bioremediation programmes have not yet been fully evaluated. However, as discussed previously, the adsorption of U(VI) on sediments is a major sink for soluble U(VI) in subsurface environments, with one calculation estimating that there is eight times more U(VI) associated with sediment than in the groundwater at the Rifle site (Ortiz-Bernad et al. 2004a). Although the U(VI) sorbed onto mineral surfaces is immobile, desorption processes could later play a role in the remobilization of U(VI) into the aqueous phase.

### *Factors limiting U(VI) reduction*

Whilst in many sediments the enzymatic reduction of U(VI) is an efficient process for removing highly mobile uranium from solution, in some subsurface environments the presence of inorganic ions may have a negative impact on microbial U(VI) reduction. An increase in bicarbonate ion concentrations from about 30 to 100 mM can lead to a significant decrease in enzymatic U(VI) reduction (Phillips et al. 1995) due to the lowering of the half-cell potential of the U(IV)–U(VI) couple, while in environments containing calcium at millimolar concentrations, the formation of ternary Ca–U(VI)–$CO_3$ complexes causes a drop in both the rate and extent of U(VI) reduction by *D. desulfuricans, S. oneidensis* and *G. sulfurreducens* (Brooks et al. 2003). This fall in U(VI) reduction is thought to arise because the complexation of U(VI) with calcium makes it a less energetically favourable electron acceptor than U(VI)-carbonato complexes, rather than because of any direct interaction of

Ca with the cells or electron donor. Ganesh et al. (1997) studied the effect of U(VI) complexation with the aliphatic ligands acetate, malonate, oxalate and citrate and the catechol analogue tiron (4,5-dihydroxy-1,3-benzene disulfonic acid) and discovered significant variation in the reduction of the U(VI)-complexes. Uranium complexed with malonate, oxalate, citrate and tiron formed multidentate aliphatic complexes whereas a monodentate aliphatic complex could be obtained with acetate and U(VI). Uranium present in a multidentate aliphatic complex was reduced more rapidly by *S. alga* than that present in monodentate aliphatic complexes, whilst the chelating effect of the multidentate complexes was highlighted by the decrease in the U(VI) reduction rate as the amount of multidentate complexes fell. Only a very small amount of U(VI) was reduced from the tiron complex by *S. alga*.

As Fe(III) oxides act as terminal electron accepting compounds, some research has suggested that their presence in the subsurface may delay the reduction and precipitation of U(VI) by DIRB (Wielinga et al. 2000). This work suggested that U(VI) reduction was hindered by the presence of ferrihydrite while a lesser effect was seen in the presence of goethite. Although free energy change values indicate that U(VI) should be preferentially reduced over Fe(III) (Francis et al. 1994), when this process is studied experimentally it becomes clear that other factors such as enzyme specificity, kinetics and the complexing of the uranyl ion with carbonate and hydroxo ligands also play a role in determining the order in which Fe(III) and U(VI) are reduced (Morris 2002). In natural environments, factors such as the relative abundance of Fe(III) and U(VI) will affect the sequence in which these metals are utilised, and this is reflected in results from natural sediments where Fe(III) and U(VI) are reduced concurrently (Finneran et al. 2002a).

The potential for microbial reduction of U(VI) in high nitrate environments has been assessed in a number of studies. Work on a number of sediment types has demonstrated that nitrate has the ability to inhibit microbial U(VI) reduction (Finneran et al. 2002b; Senko et al. 2002; Elias et al. 2003a). However, experiments using both sediment microcosms and pure cultures of *G. metallireducens* showed that once the nitrate has been reduced, Fe(III) and U(VI) are reduced concurrently (Finneran et al. 2002a). Subsequent *in situ* studies on a nitrate-contaminated aquifer similarly demonstrated that U(VI) reduction only occurred once Fe(III)-reducing conditions were obtained (Istok et al. 2004). Further problems of nitrate in these environments were highlighted by Shelobolina et al. (2003) who attempted to stimulate microbial U(VI) reduction in low pH (pH 4), high nitrate (55 mM) sediment samples by adding organic electron donors. In these experiments, nitrate reduction occurred slowly, with only about 20% of nitrate removed after 120 days and U(VI) precipitates forming. No microbial reduction of U(VI) to U(IV) was observed and metal-reducing bacteria, although detected in the sediments, were not stimulated by the addition of the electron donors. A recent paper described a potential method to treat U(VI)-containing sediments contaminated with high concentrations of nitrate (Gu et al. 2005a). Contaminated material in a

column was first flushed with an acidified salt solution (pH 4) to remove the nitrate, then neutralised with bicarbonate (60 mM) and finally biostimulated by adding glucose. However, despite the presence of Fe(III)-reducing bacteria in the sediment, no glucose utilisation and no enzymatic U(VI) reduction was observed until an additional bacterial culture was added to the column. This various research demonstrates the practical problems facing any attempt to promote both *ex situ* and *in situ* U(VI) reduction in a high nitrate environment.

### *Reoxidation of U(IV)*

Although it has been shown that the U(IV) mineral uraninite is stable under reducing conditions, the stability of U(IV) precipitates under oxidising conditions has recently been investigated more thoroughly. As previously discussed, U(VI) reduction will not occur until the nitrate in an environment has been utilised by nitrate-reducing microorganisms. However, both Senko et al. (2002) and Istok et al. (2004) demonstrated that should nitrate enter a reduced zone where U(IV) is present, the subsequent reduction of nitrate can lead to the remobilization of U(IV) as U(VI). Experiments on heat-killed sediments indicated that the intermediates of dissimilatory nitrate reduction (nitrite, nitrous oxide, and nitric oxide) were all able to oxidize and mobilise U(IV). Subsequent work by Senko et al. (2005) then suggested that freshly oxidized Fe(III) was able to oxidize U(IV) at a greater rate than nitrite (130 and 10 μM U(IV)/day, respectively). During nitrate reduction, oxidised Fe(III) can be produced either by the oxidation of Fe(II) by nitrite or by the enzymatic oxidation of Fe(II) coupled to nitrate reduction. The picture was further complicated by the observation that mineralogical differences in the oxidized Fe(III) affected the rate and extent of U(IV) oxidation (Senko et al. 2005).

Controversially, recent long-term (17 months) column studies on U(IV) stability in Oak Ridge sediments have indicated that even under reducing conditions in the presence of known U(VI)-reducing microorgansims, U(IV) may become reoxidised and solubilised (Wan et al. 2005). Here it was suggested that carbonate accumulation arising from microbial respiration promotes the formation of highly stable carbonato-U(VI) complexes, thereby increasing the thermodynamic favourability of U(IV) oxidation. Residual Fe(III) and possibly Mn(IV) in the columns were suggested to be the TEAs for the U(IV) reoxidation.

### *Abiotic U(VI) reduction*

A number of studies have suggested that Fe(II) can potentially reduce U(VI) to U(IV) and thus aid in the retention of contaminant U in subsurface environments (Wersin et al. 1994; Charlet et al. 1998b; Liger et al. 1999; Fredrickson et al. 2000; Missana et al. 2003). Indeed, it has been suggested that in some

sediments that are saturated with biogenic Fe(II) following long-term microbial Fe(III) reduction, abiotic reduction of U(VI) may be a more important process governing U mobility than direct microbial reduction (Fredrickson et al. 2000). Charlet et al. (1998a, b) found that although Fe(II) present in solution is unable to reduce U(VI), Fe(II) sorbed onto magnetite and hematite surfaces is able to reduce U(VI) to U(IV) which is then present as a $UO_{2(s)}$/$Fe(OH)_{3(S)}$ solid solution. Work on particulate matter sampled from the hypolimnion of a seasonally stratified lake suggested that Fe(II) sorbed onto ferrihydrite in these environmental samples caused the rapid reduction of U(VI) (Liger et al. 1999). Missana et al. (2003) demonstrated that U(VI) sorbing onto magnetite surfaces was reduced rapidly, with a large fraction of the total U precipitating as U(IV) within the first day of the experiment, while Behrends and Van Cappellen (2005) suggested that in some instances, abiotic reduction of U(VI) by biogenic Fe(II) could be the dominant reductive pathway.

However, other experiments carried out with natural sediments have been unable to identify any role for biogenic Fe(II) in U(VI) reduction. Work using heat-killed sediments containing 60% bioavailable iron as Fe(II) indicated that this abiotic mechanism did not significantly contribute to U(VI) reduction in the aquifer sediments tested (Finneran et al. 2002a). The analysis of some Oak Ridge sediments revealed that the reduction of U(VI) by Fe(II) was only a minor process, possibly due to the complexation of U(VI) with carbonate (Liu et al. 2005). Although Jeon et al. (2005) showed that both pure Fe(III) oxide phases and high Fe(III) oxide-enriched (18–35 wt.% Fe) sediments could rapidly and extensively reduce U(VI), abiotic reduction was far less efficient in natural sediments with lower Fe content (1–5 wt.% Fe). Thus, it appears that although the abiotic reduction of U(VI) by Fe(II) is possible, it is not always seen in environmental studies where the Fe content of natural sediments is relatively low.

## Other radionuclides

As with uranium, the enzymatic reduction of soluble Tc(VII) to insoluble Tc(IV) has been demonstrated in pure culture (Lloyd and Macaskie 1996; Lloyd et al. 2000a) and in both *in situ* (Istok et al. 2004) and *ex situ* (Wildung et al. 2004; Burke et al. 2005) in sediments. Unlike uranium however, Tc(VII) can clearly undergo efficient abiotic reduction to Tc(IV) by Fe(II) phases such as biogenic magnetite and green rust (Lloyd et al. 2000a; Pepper et al. 2003; Fredrickson et al. 2004; Wildung et al. 2004). Lloyd et al. (2002) also demonstrated that microbially formed U(IV) was able to act as an electron shuttle to reduce Tc(VII) abiotically in studies using *G. sulfurreducens*. Differences in the behaviour of U and Tc are also noted following the reoxidation of a reduced environment. Whereas U(IV) is reoxidised to U(VI) and therefore

remobilised, Tc appears to remain immobilised following the reoxidation of an environment with oxygen (Pepper et al. 2003).

Very little is known about the interactions of Fe(III)-reducing microorganisms and plutonium in the environment. However, a paper by Rusin et al. (1994) reported how a *Bacillus* species was able to reduce 91% of insoluble Pu(IV) to soluble Pu(III). The abiotic reduction of Pu(V) to Pu(IV) by magnetite has also been recently described. Powell et al. (2004) reported that Pu(IV) becomes more stable on the mineral surface over time, resulting in reduced mobility of Pu in the subsurface.

Whereas some radionuclides such as U and Tc are enzymatically reduced in a one-step process, others such as Np require a multi-step process. Lloyd et al. (2000b) demonstrated how following the initial reduction of Np(V) ($NpO_2^+$) by *S. oneidensis* to soluble Np(IV), a *Citrobacter* sp. was then able to remove Np(IV) from solution as a phosphate biomineral. As with Tc and Pu, the abiotic reduction of Np(V) by Fe(II) in magnetite has been observed (Nakata et al. 2002).

## Conclusions/future work

Although it is clear that the processes associated with Fe(III) reduction can have a great impact on reducing uranium contamination in the environment, many related factors and effects still need to be investigated. The stability and potential reoxidation of reduced U(IV) is of great importance and further research is needed on the impact of bacterial oxidation reactions in the subsurface in general. The role of Fe(III) as an oxidant for U(IV) has been proposed, and warrants further investigation while the potential for immobilising U through anaerobic bio-oxidation of Fe(II) with associated sequestration of contaminants in biogenic Fe(III) oxides has been proposed recently. Microbially produced antioxidants may also help in stabilizing reduced U(IV) and so also require further attention. Similarly, both the abiotic reduction of U(VI) by Fe(II) and the potential for the enzymatic reduction of sorbed U(VI) are areas that clearly require more attention so that possible bioremediation strategies can be optimised. It is still unclear why abiotic reduction of U(VI) by Fe(II) is seen in laboratory experiments using pure phases but not in natural sediment studies, while varying results have been obtained by researchers trying to assess the bioavailability of sorbed U(VI). Although the effects of carbonate-U(VI) complexation have been studied extensively with regard to contaminant migration, the effects of complexation on microbial U(VI) reduction rates in the subsurface also need to be investigated more closely. While the work of Brooks et al. (2003) has so far suggested that the formation of U-carbonate-calcium complexes will retard bioreduction, the effects *in situ* remain to be studied. So, despite many areas still needing to be investigated, it is clear that the predicted effects of Fe(III) reduction must be backed-up by experimental work using real sediments and environmentally relevant microorganisms. Only

when these systems are properly understood can this process be efficiently applied to the *in situ* remediation of U(VI) contaminated areas. It is also worth noting that significant advances in our understanding of the physiology of key subsurface microorganisms, including Fe(III)-reducing bacteria, are imminent based on the application of the latest genomics-enabled techniques. These results will also have a major impact in uncovering the microbiological basis of radionuclide-microbe interactions in the subsurface.

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