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# Monitoring of microbial metal transformations in the environment

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The biotransformation of metals is an exciting, developing strategy to treat metal contamination, especially in environments that are not accessible to other remediation technologies. However, our ability to benefit from these strategies hinges on our ability to monitor these transformations in the environment. This involves monitoring metals in both solid and aqueous samples, distinguishing between different chemical states, and obtaining information on the activities of specific microbial taxa in communities that inhabit the treated site. Accomplishing these goals requires cooperation among scientists from various disciplines and would benefit from both new, innovative approaches and the tailoring of established methods to control metal mobility in the environment.

### Addresses

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### Introduction

Metals and radionuclides contaminate vast tracts of land in the USA as a result of industrial activities and nuclear weapons manufacturing. Although regulations have reduced the input of new metals to the environment, the old sites continue to leach toxic metals. A driving force behind cleaning up these sites is the high degree of public awareness regarding metal contamination and the potential consequences toward human health. Not only did the 2000 movie 'Erin Brockovich' garner an Oscar for Julia Roberts, it also educated the public about the dangers of chromate [Cr(VI)] in the water supply. (Throughout this manuscript the chemical symbol is used when speciation is known; the generic names for metallic elements, such as chromium, is used when speciation is unknown or when relating to the element in general.) This trend is not likely to reverse, as there is an increasing availability of products that bring the diagnosis and treatment of heavy metal poisoning out of the realm of

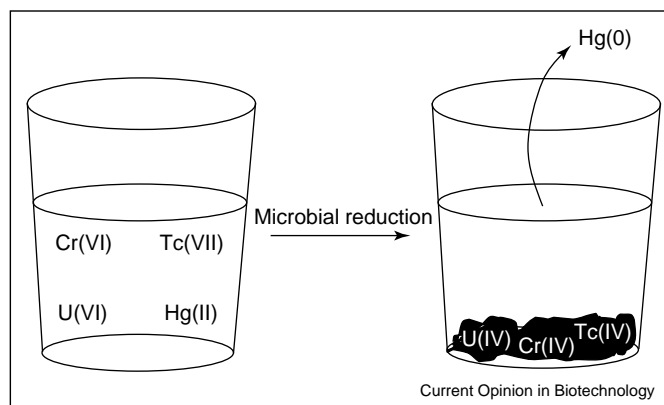
physicians and environmental health specialists directly to the consumer. Websites such as <http://www.extreme-healthusa.com> and <http://www.Awakennutrition.com> offer inexpensive hair analysis kits, and subsequently sell the client home chelation therapy. Although there is no doubt that true metal poisoning is dangerous to human health — mercury causes renal damage and severe developmental delays in children and Cr(VI) is a potent carcinogen — chelation therapy is offered to a naive public as a cure for many poorly defined maladies, such as attention deficit disorder, autism, hypoglycemia, and chronic fatigue syndrome. Far from remaining in the realm of the desperate and the wacky, self-diagnosis of metal poisoning became fashionable this past fall when Greenpeace teamed up with Aveda hair salons to offer mercury hair tests to people who had come in for a cut and color [1,2]. As more voters become convinced that many ailments they suffer from are a direct result of metal poisoning, there will be less tolerance for trace amounts of metals in drinking water, and increased pressure to tackle even the most intractable problems.

Metals provide several unique challenges for remediation. Unlike organic contaminants, they are elements and cannot be degraded into innocuous products. Additionally, metals in subsurface environments, which can leach into ground water aquifers and contaminate drinking water, are unreachable by the many methods available to decontaminate surface soils. One option currently being explored is to immobilize metals by microbial transformations to insoluble states [3]. Specifically, oxidized forms of chromium, technetium and uranium are water soluble, but upon reduction form solid precipitates with lower mobility. By contrast, oxidized inorganic mercury Hg(II), although water soluble, sorbs onto sediment particles and forms ligands with organic matter. The reduced form, Hg(0), is a volatile gas with poor water solubility that can be transported to the atmosphere (Figure 1). To harness microbial transformations to immobilize metals in the environment, information on metal concentrations and speciation and the status of the microbial community in the treated environment are needed. This review focuses on the methods and the challenges involved in monitoring microbial metal transformations in environmental samples, specifically for chromium, technetium, uranium and mercury, for which bioremediation strategies are in development.

### Controlled microbial metal transformations as a bioremediation strategy

The following is a brief description of microbial metal transformations and their potential in environmental

Figure 1



The likely fates of microbially reduced metals and radionuclides. Cr(VI), U(VI), Tc(VII) and Hg(II) are water soluble. Upon microbial reduction, Cr(IV), Tc(IV) and U(IV) form solid precipitates, whereas Hg(0) forms a volatile gas. Both mechanisms remove these metals from the aqueous phase.

remediation. The interested reader may find more comprehensive discussion of these issues in recent reviews cited herein.

Microbial transformations of mercury have long been of interest, because they control the production of the potent neurotoxin methylmercury ( $\text{CH}_3\text{Hg}$ ) in the environment [4,5]. Very low concentrations of  $\text{CH}_3\text{Hg}$  are biomagnified and bioaccumulated more than a million times in predatory fish that occupy the top levels of the aquatic food chain. Microorganisms transform mercury between the ionic Hg(II) and elemental Hg(0) forms, convert Hg(II) to  $\text{CH}_3\text{Hg}$ , and degrade  $\text{CH}_3\text{Hg}$ . Some of these activities have been integrated into remedial strategies for mercury [5,6]. Most importantly, microbial reduction of Hg(II) to Hg(0) has enabled the removal of mercury from waste streams [7\*\*] and contaminated soils [8].

As with mercury, the oxidation state of chromium has long been known to influence toxicity and mobility. For example, Cr(III), an important micronutrient, is poorly soluble in water, but the oxidized form Cr(VI) is highly water soluble and is a potent mutagen and carcinogen (reviewed in [9]). Biological reduction of Cr(VI) to Cr(III) has been demonstrated for diverse organisms such as fungi, bacteria and algae [10\*].

More recently, there has been interest in biological transformations of uranium and technetium. Several anaerobic microorganisms, many dissimilatory metal-reducing bacteria, utilize one or both of these metals as a terminal electron acceptor in pure culture [10\*,11,12]. A strategy for remediation of these metals in the subsurface is to harness this ability by providing electron donors to stimulate growth of indigenous microorganisms [11–13]; the microbes, in turn, reduce the oxidized metals to their

insoluble form. In addition to pure culture work, microcosm studies have demonstrated that biological reduction of U(VI) and Cr(VI) can occur in sediments under anoxic conditions [14–16].

Initial field studies on remediation of U(VI) and Tc(VII) have been promising. Small-scale studies in boreholes at the US Department of Energy (DOE) field research center in Oak Ridge, TN, have demonstrated microbial reduction of U(VI) and Tc(VII) [17\*\*,18]. In a field site at Old Rifle, CO, which had been contaminated by Uranium mill tailings, a team of researchers set up injection galleries in which they added acetate as an electron donor to stimulate growth of dissimilatory metal-reducing bacteria [19\*\*]. After nine days, a decrease in soluble U(VI) in the groundwater was noted and, in some wells, concentrations fell to below regulated levels within 50 days. Concurrent with U(VI) reduction, microorganisms related to those known to reduce U(VI) in pure culture were enriched in the treated aquifer.

### Monitoring metal transformations in natural waters

To a large extent, our ability to measure soluble, oxidized metals in natural waters is well developed, and numerous techniques are available. However, most analytical methods do not distinguish between metal oxidation states and manipulations that separate metals according to their chemical form are needed before the analysis of environmental samples. For example, highly sensitive quantitation of mercury is achieved using fluorescence spectrometry of Hg(0). To distinguish various chemical forms of mercury, water samples are briefly bubbled to drive out Hg(0), reduced with tin to obtain the 'reactive' Hg(II) form, and subjected to organic mercury extraction followed by ethylation and gas chromatography separation to quantitate  $\text{CH}_3\text{Hg(I)}$  and  $(\text{CH}_3)_2\text{Hg}$  [20].

However, as the ultimate goal of remedial treatments is to decrease metal mobility by affecting solubility, metal disappearance from the aqueous phase, concurrent with other analyses, may be sufficient to imply precipitation and immobilization.

One technique that is applicable to nearly all metals is inductively coupled plasma mass spectrometry (ICP-MS). ICP-MS has been used to detect uranium, mercury and chromium in water [21,22], and is better suited for the detection of uranium than radiochemical methods [23]. One major advantage of using ICP-MS is that it performs solidly on environmental samples contaminated with mixed wastes. Because identification of the elements relies on the mass-to-charge ratios of their nuclei, a single instrument analyzes many different metals. Although ICP-MS units are not portable, they are small enough that they can be housed in a laboratory or near a field site. An additional benefit of ICP-MS is that some instruments distinguish various metal isotopes [24\*].

Several metals can be detected using atomic absorption, atomic fluorescence or atomic emission spectroscopy. Mercury is quantified by cold vapor atomic absorbance or fluorescence techniques. A highly specific kinetic phosphorescence assay that detects soluble U(VI) was used to monitor the disappearance of uranium from groundwater [19\*\*].

Because uranium and technetium are radionuclides, they may be detected by radiochemical methods. Several protocols are in use for determining  $^{99}\text{Tc}$  levels by counting disintegrations [25,26], and this is the standard method for determining the presence of technetium in natural waters [27,28]. Typically, this method only detects the oxidized soluble form, as interference from other  $\beta$  emitters is eliminated by filtration and ion chromatography before analysis.

Spectrophotometry has been used to measure U(VI) following its complexation with arsenazo III [29] in suspended solids in groundwaters [30]. This method was used to detect the microbial removal of U(VI) from soil microcosms [31]. The classic diphenylcarbohydrazine spectrophotometric assay for Cr(VI) and Cr(V) [32] has been incorporated into several field kits and hand-held analyzers; however, both of these assays are subject to numerous interferences from other elements that can be present in natural waters.

### Detection of insoluble metals in soils and sediments

Analysis of immobilized metals in their solid phase is essential for the basic understanding of the immobilization process and the potential for remobilization as conditions and microbial activities change. In addition, many microbes precipitate metals within their biomass and the

chemical form of the cell-associated metal and its ligands provide evidence for the precipitation processes. However, quantitation and speciation of metals in the solid phase presents many challenges to the investigator. There are two major approaches to analyzing metals in the solid phase: extraction into the aqueous phase followed by analyses as described above (sequential extractions); and direct analysis of the solid matrix itself.

### Sequential extractions

Pioneered by Tessier 1979 [33], the sequential extraction approach partitions metals in soils and sediments into fractions based on their solubility in solutions of varying pH, carbonate content, and redox active reagents. An attempt is then made to correlate the presence of the metal in a given extract to the chemical form of that metal *in situ*. Despite the fact that the weaknesses of this approach were recognized early on, variations of this method are still in broad use today [34,35]. For a comprehensive review regarding the vast array of sequential extraction protocols and reagents see Filgueiras *et al.* [36]. For an example of methods used with a single metal, Cr(VI), see Parks *et al.* [37\*].

One conceptual problem with the sequential extractions approach is that acid extractions transform insoluble reduced metals to soluble oxidized states, and one never directly analyzes the reduced metal. For example, oxidized metals may sorb to solid matrices and the acid-extractable fraction could then be a mixture of both oxidized and reduced metals, thus giving an overestimation of microbial reduction. This is known to be an issue for uranium, for which new more reproducible methods for the sequential extraction of biologically reduced uranium have recently been developed [38]. A more critical problem is the difficulty of assigning an oxidation state or a chemical form to sequentially extracted fractions. Despite caveats, sequential extraction remains in wide use because of its ease, low cost and reproducibility. These advantages may have a greater impact on the assessment of metal transformations in the environment if more work is done to correlate fractions with biologically meaningful chemical forms.

### Solid phase based methods for metal analysis

Laser ablation ICP-MS determines total metals in intact solid-phase matrices, whereby a solid sample is vaporized with a laser beam and sent into a detector. This approach has been successfully used to detect depleted uranium and plutonium in soils [39,40]. Unfortunately, this technique does not give any information on the oxidation state of the metal.

Another method that detects metal precipitates inside and surrounding bacterial cells, but which does not distinguish between metal oxidation states, is scanning electron microscopy or transmission electron microscopy,

followed by energy dispersive X-ray spectroscopy. This approach has been used to visualize cell-associated precipitates of uranium and technetium in pure culture [41] and cell-associated uranium in a microcosm study [42\*].

Energy dispersive X-ray fluorescence spectroscopy (XRF) analyzes, but does not speciate, a wide variety of metals in solid phase. Field kits employing portable equipment are available from several manufacturers, and methods have been developed to utilize these with soils [43]. Although the detection limits of these kits might be too high for some applications, bench top models have a 10- to 20-fold higher sensitivity, and can easily be deployed in a trailer or van [44]. As XRF is a non-destructive technique, samples can be screened on-site and then sent to the laboratory for more sophisticated testing.

Spectroscopy that determines X-ray absorption fine structure (XAFS), if used with skill and appropriate references, reveals the oxidation state of most metals, as well as their complexation with other ions. XAFS measurements can be performed on solid or liquid samples, and can be used on nearly any matrix with very little sample preparation. Because X-ray spectroscopic techniques do not destroy the sample, spectra can be obtained for several different elements in the same sample and the sample can be subsequently used for further experiments. X-ray spectroscopic techniques have been used to determine uranium speciation in soils from the deep subsurface in the northwestern USA [45] and from Kuwait [46], to analyze mercury speciation in soils [47\*], and to determine the chemistry of technetium in wastes [48]. Oliver *et al.* [49,50] used X-ray spectroscopy to monitor Cr(VI) reduction in soil microcosms and this method was also used to monitor the microbial reduction of technetium [51] and uranium [52] by pure cultures, as well as to study uranium in microcosm studies [42\*]. The biggest limitation of X-ray spectroscopy is its accessibility. It requires a synchrotron, highly skilled operators, and intensive data analysis. These requirements prohibit the use of X-ray absorption spectroscopy for high-throughput analysis [53] and thus for regular monitoring of extensive bioremediation efforts.

Transmission electron microscopy (TEM) followed by electron energy loss spectroscopy (EELS) elucidates the oxidation state of chromium, but is not a suitable technique for all metals. Although the equipment is expensive, no special facilities such as a synchrotron are required. The oxidation states of precipitates in and around cells of *Shewanella oneidensis* MR-1 during the dissimilatory reduction of Cr(VI) was revealed by TEM-EELS, with the added benefit of the production of micrographs that show cellular association of the reduced metal [54–56].

### Microbial community analysis as evidence of biotic metal transformations

Relating changes that occur in microbial community structure during biostimulation to the dynamics of metal transformation facilitates optimization and the safe application of remedial approaches [19\*\*]. Thus, monitoring microbial communities is an essential part of metal bioremediation and the application of methods that do not depend on culturing have been of high priority. These methods are based mostly on the analysis of macromolecules such as DNA, RNA or phospholipid fatty acids (PLFA) that are extracted from the microbial communities of the treated environment.

Changes in community structure can be followed through the construction of clone libraries of 16S ribosomal DNA genes and the analysis of PLFA fingerprints that are then related to the function of the microbes whose signatures have been identified in the environment. These approaches were taken at the US Department of Energy sites in Old Rifle (CO) [19\*\*] and in Oak Ridge (TN) [57,58] to show an increase in sequences of dissimilatory metal-reducing bacteria, among them *Geobacter* spp. and *Anaeromyxobacter* spp., concurrent with U(VI) reduction. One problem with these approaches is that, although enrichment of known dissimilatory metal reducers indicates a potential for metal immobilization, their absence may not be conclusive. Because dissimilatory metal reduction is found broadly distributed among prokaryotes, it is entirely possible that as yet uncharacterized organisms could possess this capability.

A more direct way to analyze microbial community activities during metal transformations is by targeting the presence and expression of functional genes. This approach requires familiarity with the genes that specify metal transformations and is currently only a reality for mercury, as genes responsible for some mercury transformations are well characterized [5]. This method was demonstrated in a bioreactor designed for the removal of Hg(II) as Hg(0) from industrial wastewaters [59] and in mercury-contaminated surface waters [60]. Unfortunately, the genetics of dissimilatory metal reduction remains poorly understood even in model organisms, and it appears as though different microorganisms may use different enzymes. Nevertheless, progress towards understanding of this topic has been brisk and, hopefully, in the near future molecular tools that enable interrogation of microbial communities during the reduction of U(VI), Tc(VII) and Cr(VI) will become available.

Interrogating the respiratory pathway that is employed by the microbial community while metal reduction is stimulated identifies the specific metabolic group that is involved in the remediation process. For example, the presence of two genes employed during denitrification, *nirK* and *nirS* [61], as well as genes involved in sulfate

reduction [62,63<sup>•</sup>], were followed during the stimulation of uranium reduction *in situ*. The treated aquifer was contaminated with nitrate as well as radionuclides and metals. Results showed that U(VI) reduction did not occur when nitrate reduction was the major respiratory pathway employed by the community [17<sup>••</sup>]. Rather, it depended on the creation of iron-reducing conditions and the appearance of *Geobacter* species [19<sup>••</sup>,57,58,64]. It is possible that a predominance of a nitrate-reducing community in the absence of known metal reducers could indicate that uranium remediation is failing, whereas the continued presence of metal reducers would indicate progression. The combined probing for structural and functional molecular signatures as well as the integration of biogeochemical parameters is likely to result in a better understanding of community dynamics during metal transformations.

### Future perspectives

One major challenge with using established methods to monitor microbial transformations is that these methods were originally designed with other goals in mind. For example, determining the bioavailability of trace elements has offered a treasure trove of techniques that can be used to monitor metal transformations, provided the investigator has a thorough understanding of the strengths and weaknesses of each technique. It is therefore beneficial to examine existing methods and their possible potential to become integrated into strategies for monitoring metal transformations in the environment. Microarray technology for microbial community profiling including functional gene arrays is in development and could potentially provide rapid analysis of microbial communities in environmental samples [65,66]. Recent work correlating array data with geochemical analysis [63<sup>•</sup>] is a step toward realizing this potential. Blake and coworkers [67–69] have developed antibodies specific to metals and their chelates, which are implemented in hand held immunosensors to allow the detection of uranium, lead, mercury and cadmium in groundwater in real-time in the field.

One technology not currently in broad use for monitoring microbial transformations of metals, but which has great promise, is stable isotope analysis. The use of isotopes in environmental monitoring has increased dramatically in recent years, owing to technological advances that allow the detection of specific elemental isotopes by ICP-MS. Stable isotopes can be used as tracers to follow a specific element as it is transported and transformed during its passage in the environment of interest. This is similar in principle to the practice of using radioactive isotopes, but offers two advantages: avoiding exposure to radioactivity, which would prohibit controlled release to an ecosystem, and the expense associated with frequent purchasing of short-lived isotopes. For example, the fate of mercury that reaches forested ecosystems by atmospheric deposi-

tion was investigated using <sup>202</sup>Hg. The isotope was sprayed over the forest canopy and followed in runoff water, foliage, the atmosphere, and soil over a period of several months. The results implicated the deterioration of plant litter as the major route by which atmospherically deposited mercury is integrated into the impacted forested ecosystem [70].

The true potential of stable isotopes as a tool for monitoring transformations of specific elements in the environment is in the use of isotopic ratios to distinguish sources and pathways. Because both biological and chemical transformations show a preference for specific isotopes, sources may exhibit isotopic ratios that reflect the process by which they were formed rather than the natural abundance of the isotope. These ratios can therefore be used as signatures to implicate a specific source or transformation pathway. This approach was recently demonstrated by Christensen *et al.* [24<sup>•</sup>,71] who used the ratios of uranium isotopes measured at trace concentrations (femtogram to nanogram per mL [71]) to implicate leaking storage tanks at a nuclear weapons production facility as a source of uranium in ground water, but not as the source of contamination in the nearby unsaturated soil. This research highlights the potential of using ratios, of both stable and slowly decaying radioactive isotopes, as signatures for sources and transformations of metal and radionuclide contaminants in the environment. The idea that metal transformations can be followed by a shift in isotopic ratio between a substrate and its transformation product was demonstrated for the reduction of Cr(VI) by magnetite: the <sup>53</sup>Cr/<sup>52</sup>Cr ratio of the precipitated product was  $3.4 \pm 0.1$  per mL lighter than that of the substrate [72]. Testing isotopic fractionation during microbial reduction of Cr(VI) is an obvious next step in this research. Such fractionation, indicated by a decreased <sup>202</sup>Hg/<sup>200</sup>Hg ratio relative to that of the substrate during the microbial reduction of Hg(II) to Hg(0), was recently shown with a bacterium expressing mercuric reductase [73]. With further developments, this could become a tool for monitoring mercury reduction in environmental samples.

### Conclusions

Metal transformations are gaining importance as a tool for the environmental management of metal-contaminated environments and as part of remedial strategies that seek to control metal mobility. The need to monitor immobilization processes is driving the development of new methods for the detection of metals in their various oxidation states and their interactions with the transforming microbes. Future developments in the application of state-of-the-art methodologies, such as high-resolution metal analysis on a microscopic scale and the use of stable isotopes, will advance our understanding of the interactions between microbes, metals and their environment, and consequently promote our ability to manage metal contamination.

## Acknowledgements

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