Harnessing microbial activities for environmental cleanup
Frank E Löffler¹ and Elizabeth A Edwards²

Human activities have released large amounts of toxic organic and inorganic chemicals into the environment. Toxic waste streams threaten dwindling drinking water supplies and impact terrestrial, estuarine and marine ecosystems. Cleanup is technically challenging and the costs based on traditional technologies are exceeding the economic capabilities of even the richest countries. Recent advances in our understanding of the microbiology contributing to contaminant transformation and detoxification has led to successful field demonstrations. Hence, harnessing the activity of naturally occurring bacteria, particularly the power of anaerobic reductive processes, is a promising approach to restore contaminated subsurface environments, protect drinking water reservoirs and to safeguard ecosystem health.

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Introduction
Industrial and military operations, inadvertent and accidental spills, and deliberate discharge in agriculture and private households, have resulted in the release of large quantities of toxic chemicals into the environment. Anthropogenic pollutants impact all ecosystems, terrestrial and aquatic, and compromise drinking water, food and air quality. Oil and pipeline spills in the 1970s and 80s triggered extensive research on the microbial degradation of petroleum hydrocarbons, and demonstrated for the first time that bioremediation can restore surface soil impacted with crude oil components [1]. More challenging is the remedy of contaminated subsurface environments. The extent and localization of subsurface contamination typically requires the application of in situ approaches which, in contrast to ex situ approaches, focus on contaminant detoxification in the natural setting [2]. The past decade has seen the successful implementation of in situ bioremediation applied to common pervasive subsurface and groundwater contaminants including chlorinated solvents, toxic metals and perchlorate. Crucial to these successes were basic scientific discoveries that enhanced understanding of the ecology, physiology and phylogeny of the key microbes involved in contaminant transformation and detoxification. This scientific progress cleared the way towards the design of innovative engineering solutions that exploit the activities of naturally occurring bacteria in bioremediation. This review focuses on the bioremediation of chloroethenes, uranium, and perchlorate because of their widespread occurrence. We use these examples to illustrate the current state-of-the-art and to highlight technology shortcomings and challenges.

Contaminant sources and properties
Tetrachloroethene (perchloroethylene, PCE) and trichloroethene (TCE) have been the solvents of choice for dry cleaning operations and degreasing applications since the 1930s, because they are non-flammable and chemically stable. Their frequent use and the lack of proper disposal practices has resulted in widespread subsurface contamination, and thousands of polluted sites exist (see the Comprehensive Environmental Response, Compensation and Liability Information System [CERCLIS] database of site information; http://cfpub.epa.gov/superpad/cursites/srchsites.cfm). Owing to their low water solubility and hydrophobicity, PCE and TCE can exist as dense, non-aqueous phase liquids (DNAPLs) in the subsurface complicating remediation efforts [3,4] (Table 1). PCE and TCE are toxic and suspected to cause cancer, but even more troubling is the accumulation of cancer-causing vinyl chloride (VC) (National primary drinking water regulations, list of contaminants and their maximum contaminant levels [MCLs]; www.epa.gov/safewater/mcl.html#mcls). VC is a transformation product of PCE and TCE formed through abiotic and biotic mechanisms in the subsurface (Figure 1a) [5].

Uranium contamination in the US can be traced back to uranium mill tailings and Department of Energy sites that were involved in the production of weapons grade uranium [6]. Uranium in the oxidation state +VI can be soluble and mobile, transported with the groundwater flow, and cause widespread subsurface contamination. The implementation of stringent regulations and appropriate disposal practices and, in the case of uranium, the end of the nuclear arms race, have significantly reduced or eliminated new contamination and most of the damage
was done decades ago. However, anthropogenic chemicals continue to be released into the environment, and new problems and challenges arise persistently. An example of an emerging contaminant is perchlorate.

Perchlorate was first detected in drinking water in 1997, when laboratory methods became available to detect low concentrations. Perchlorate salts are used in the manufacturing of propellants, explosives and

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**Table 1**

<table>
<thead>
<tr>
<th>Contaminant</th>
<th>Properties relevant to remediation</th>
<th>MCLs in drinking water and toxicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCE or TCE</td>
<td>Volatile, low aqueous solubility, hydrophobic; significant retardation relative to groundwater flow; often present as free organic phase (DNAPL)</td>
<td>5 µg/L Liver problems; increased risk of cancer</td>
</tr>
<tr>
<td>Perchlorate</td>
<td>Highly soluble salt (ClO₄⁻); little retardation results in large plumes of low concentration</td>
<td>6–14 µg/L Blocks iodide uptake by the thyroid</td>
</tr>
<tr>
<td>Uranium</td>
<td>Soluble in the oxidized +VI form (UO₂⁺²⁺); sparingly soluble in the reduced +IV form (UO₂)</td>
<td>30 µg/L Increased risk of cancer, kidney toxicity</td>
</tr>
</tbody>
</table>

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**Figure 1**

Reductive transformation pathways for chloroethenes, oxidized uranium and perchlorate. (a) Documented reductive dechlorination pathways leading to PCE detoxification catalyzed by reductively dechlorinating bacteria. The thick arrows indicate the predominant pathway. Apparently, dechlorination beyond DCE is restricted to some members of the Dehalococcoides group. (b) The reduction of uranium(VI) to uranium(IV). Although biological processes cannot destroy metals and radionuclides, many microbes can change their redox state, thus affecting metal speciation and solubility. This can lead to a form with reduced mobility and prevent plume migration. (c) Microbially catalyzed perchlorate reduction. Many bacteria can reduce perchlorate to non-toxic products (i.e. inorganic chloride and oxygen). The dismutation step is an internal redox reaction that does not require an electron donor. Oxygen is subsequently reduced to water; all perchlorate reducers grow with oxygen as electron acceptor.
pyrotechnic devices by the chemical, aerospace and defense industries [7**]. The main source of contamination in groundwater is the past unregulated discharge of ammonium perchlorate (NH₄ClO₄), an oxidizer used in solid rocket fuels [7**]. The high solubility of ammonium perchlorate coupled with its chemical stability in water has led to numerous, expansive plumes. Perchlorate has been detected in drinking water reservoirs across the US and, more recently, in crops such as lettuce and even in milk [7**].

Owing to their toxicity and increased risk of cancer, chloroethenes, radioactive uranium and perchlorate concentrations in drinking water are regulated (Table 1). To meet the remediation challenge, innovative, economically feasible technologies that harness the diverse metabolisms of naturally occurring microbes are needed.

**Contaminant detoxification: the microbiology**

Bioremediation based on metabolic processes, in which the organisms benefit and derive energy for growth from contaminant transformation, are generally preferable over fortuitous, co-metabolic processes [8,9]. Metabolic processes are easier to stimulate or implement, sustain, and control under field conditions. In metabolic processes, contaminants can be either electron donors or electron acceptors, depending on the oxidation state of the contaminant. The contaminants reviewed herein are all relatively oxidized, and serve as energetically favorable electron acceptors in microbial metabolism in anaerobic environments (Table 2).

**Chloroethenes**

Under anaerobic conditions, PCE can be reductively dechlorinated in a stepwise manner to less chlorinated ethenes following the pathway shown in Figure 1a. Members of phylogenetically diverse bacterial groups use PCE and TCE as metabolic electron acceptors and couple reductive dechlorination to cis-1,2-dichloroethene (cis-DCE) to energy capture and growth [10,11]. The current wisdom is that dechlorination beyond cis-DCE to environmentally benign ethene requires the presence of certain members of the *Dehalococcoides* group [5*,12–19]. The known *Dehalococcoides* organisms are strictly hydrogenotrophic (de)chlororespiers (i.e. require chloroorganic electron acceptors) and cannot grow with other redox couples. Genetic and genomic analyses revealed the presence of multiple reductive dehalogenase genes [20*,21,22] suggesting that the range of chloroorganic substrates supporting growth of these organisms is large and remains to be determined. *Dehalococcoides* are fastidious organisms and maintenance in pure culture is challenging [5*,23]; they are more easily maintained in consortia, as long as conditions remain anaerobic and a chloroorganic electron acceptor and a fermentable electron donor as a source of hydrogen are provided [16]. Unfortunately, not all *Dehalococcoides* organisms act on chloroethenes and their presence does not guarantee efficient ethene formation (Table 3). Growth-linked aerobic oxidation of the dechlorination products cis-CDE and VC is also feasible [24], but PCE and TCE are stable under aerobic conditions and do not support growth of aerobic microorganisms. Thus, the reductive transformation of PCE and TCE is critical to initiate detoxification, and a sequential anaerobic–aerobic process might also lead to detoxification.

**Uranium**

Actinides such as uranium are least soluble at the +IV oxidation state, and the reduction of soluble and mobile U(VI) to relatively immobile U(IV) uraninite (UO₂) precipitates is a promising remedial strategy to prevent uranium plume migration (Figure 1b) [25]. Important bacterial groups contributing to U(VI) to U(IV) reduction include dissimilatory metal-reducing and sulfate-reducing bacteria [25]. Extensive efforts are underway to

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**Table 2**

<table>
<thead>
<tr>
<th>Reaction</th>
<th>ΔG° (kJ/electron equivalent for half reaction)*</th>
<th>ΔG° (kJ/electron equivalent for overall reaction with H₂ as electron donor)*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aerobic respiration</td>
<td>1/2O₂ + H⁺ + e⁻ → 1/2H₂O</td>
<td>-78.72</td>
</tr>
<tr>
<td>Perchlorate reductionab</td>
<td>1/2ClO₄⁻ + H⁺ + e⁻ → 1/2Cl⁻ + 1/2H₂O</td>
<td>-75.12</td>
</tr>
<tr>
<td>Iron reduction</td>
<td>Fe³⁺ + e⁻ → Fe²⁺</td>
<td>-74.27</td>
</tr>
<tr>
<td>Denitrification</td>
<td>2NO₃⁻ + 3/2H⁺ + e⁻ → 3/2N₂ + 3/2H₂O</td>
<td>-72.20</td>
</tr>
<tr>
<td>Chloroethene respiration</td>
<td>2C₂H₅Cl₂ + H⁺ + e⁻ → 2C₂H₄ + 1/2H₂ + 1/2Cl⁻</td>
<td>-36.35</td>
</tr>
<tr>
<td>Uranium reduction</td>
<td>1/2UO₂⁶⁺ + e⁻ → 1/2UO₂(solub)</td>
<td>-25 to -35</td>
</tr>
<tr>
<td>Sulfate reduction</td>
<td>1/4SO₄⁴⁻ + 1/2H⁺ + e⁻ → 1/4HS⁻ + 1/2H₂O</td>
<td>+20.85</td>
</tr>
<tr>
<td>Methanogenesis</td>
<td>1/4CO₂ + H⁺ + e⁻ → 1/4CH₄ + 1/2H₂O</td>
<td>+25.53</td>
</tr>
</tbody>
</table>

* Source references [9,30].

ab The reduction of perchlorate to chloride involves the transfer of eight electrons (Figure 1c). The Gibbs free energy values listed for perchlorate accounts for the fact that no energy capture occurs from the chlorite to chloride plus oxygen dismutation step [31].

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characterize the underpinning mechanisms and pathways. The current focus is on *Geobacter* spp., *Shewanella* spp., *Anaeromyxobacter* spp. and sulfate-reducing *Desulfovibrio* spp., although the diversity of organisms contributing to uranium reduction reactions in contaminated subsurface environments is probably much larger. An important issue under investigation is the stability of the reduced radionuclides. The long half-life of radioactive uranium requires lasting immobilization, which could be challenging to achieve. Abiotic [26,27] and microbially catalyzed [28–30] reoxidation might occur under field conditions and interfere with remedial efforts to achieve plume control.

**Perchlorate**

The reduction of perchlorate to chloride is associated with a substantial change in free energy (Table 2). Dis-similatory perchlorate-reducing bacteria use perchlorate (ClO₄⁻) as an electron acceptor in their metabolism when provided with a suitable electron donor such as acetate or hydrogen [7**]. Perchlorate is reduced sequentially via chlorite (ClO₂⁻) and chloride (Cl⁻). Chlorite undergoes a dismutation reaction to chloride (Cl⁻) and oxygen (O₂) (Figure 1c) [31]. The enzymes and corresponding genes have been identified for each dechlorination step, including perchlorate reductase [32], chlorite reductase [33] and chloride dismutase [34]. Perchlorate-reducing mixed and pure cultures are easily obtained from pristine and contaminated environments [7**], suggesting that the ability to catabolize perchlorate is widely distributed or even ubiquitous. For example, isolates were obtained from all 13 locations investigated in a survey of perchlorate-contaminated sites [35]. Phylogenetic analyses based on the 16S rRNA gene sequences of known perchlorate-reducing bacteria indicate that they are distributed among the Proteobacteria. Interestingly, the known perchlorate reducers share similar characteristics and are Gram-negative, non-spore-forming, facultative anaerobes.

**From the laboratory to the field: process scale-up**

**Site assessment**

A prerequisite for the implementation and management of successful subsurface bioremediation is a thorough site characterization. This entails the collection of physical (e.g. hydrogeological), chemical and microbiological information obtained from representative monitoring well groundwater samples and, if possible, subsurface soil samples. The comprehensive chemical analysis of groundwater includes parameters such as concentrations of contaminants, possible degradation products, alternate electron acceptors (e.g. oxygen, nitrate and sulfate), dissolved hydrogen, dissolved minerals, trace metals and bioavailable organic carbon, as well as data on redox conditions, pH, temperature and salinity. Obviously relevant, is information on the presence or absence of microorganisms catalyzing the desired transformation reactions. Traditionally, time-consuming, laboratory-based microcosm treatability studies were performed to obtain microbiological information [36]. Research over the past decade has generated a wealth of information on bacteria involved in specific detoxification processes, and a variety of approaches, in particular nucleic acid based molecular biological tools, are now available for the specific detection and quantification of the key microbes involved in the process of interest [37**,38–41]. To complement molecular detection of microbial activity, isotopic approaches for field-validation of contaminant degradation are being explored. For example, microbial degradation of contaminants including chlorinated ethenes or perchlorate can result in measurable shifts in the stable isotope composition of the contaminant that is diagnostic of the microbial degradation process [42**].

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**Table 3**

<table>
<thead>
<tr>
<th>Dehalococcoides strain</th>
<th>Metabolic chloroethene electron acceptors</th>
<th>Major end product(s)</th>
<th>Substrate range includes</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Strain 195</td>
<td>PCE, TCE, cis-DCE, 1,1-DCE</td>
<td>trans-DCE VC</td>
<td>PCBs, PCDDs, chlorinated naphthalenes, chlorobenzenes</td>
<td>[23,84,85]</td>
</tr>
<tr>
<td>Strain BAV1</td>
<td>cis-DCE, trans-DCE, 1,1-DCE, VC</td>
<td>PCE, TCE</td>
<td>Ethene ND</td>
<td>[5*]</td>
</tr>
<tr>
<td>Strain FL2</td>
<td>TCE, cis-DCE, trans-DCE</td>
<td>PCE, VC</td>
<td>Ethene ND</td>
<td>[14]</td>
</tr>
<tr>
<td>Strain VS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TCE, cis-DCE, 1,1-DCE, VC</td>
<td>ND</td>
<td>Ethene ND</td>
<td>[19,75,86]</td>
</tr>
<tr>
<td>Strain CBDB1</td>
<td>PCE, TCE</td>
<td>ND</td>
<td>trans-DCE Chlorobenzenes, PCDDs</td>
<td>[87,88]</td>
</tr>
<tr>
<td>Strain KB-1/VC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>TCE, cis-DCE, VC</td>
<td>ND</td>
<td>Ethene ND</td>
<td>[16]</td>
</tr>
<tr>
<td>Strain GT</td>
<td>TCE, cis-DCE, VC</td>
<td>None</td>
<td>Ethene ND</td>
<td>[19]</td>
</tr>
<tr>
<td>Strain RC&lt;sup&gt;a&lt;/sup&gt;</td>
<td>None</td>
<td>None</td>
<td>Chloropropanes</td>
<td>[73]</td>
</tr>
<tr>
<td>Strain KS&lt;sup&gt;a&lt;/sup&gt;</td>
<td>None</td>
<td>None</td>
<td>Chloropropanes</td>
<td>[73]</td>
</tr>
</tbody>
</table>

<sup>a</sup> Characterized in mixed culture. ND, not determined; PCBs, polychlorinated biphenyls; PCDDs, polychlorinated dibenzo-p-dioxins.
Identifying bottlenecks

Prior to the implementation of a successful remedy, the limits (i.e. controls) of the detoxification process must be identified. Common restrictions on microbial detoxification activity in oligotrophic subsurface environments include unfavorable pH and redox conditions, nutrient limitations (e.g. nitrogen and phosphorus), high salinity and, for reductive processes such as those described herein, insufficient concentrations of suitable electron donors. To stimulate desired microbial activity, adjustments of pH and redox conditions are feasible through the addition of base (e.g. bicarbonate or NaOH) [43] or easily oxidizable organic carbon substrates, respectively. A variety of organic substrates, including alcohols, organic acids, emulsified vegetable oil, and complex organic materials (e.g. molasses, corn cobs, newsprint, wood chips, microbial biomass, chitin, etc.) have been supplied as sources of reducing equivalents [44]. An issue that has received less attention to date involves the effects of inhibitors on the desired activity. For instance, co-contaminants like carbon tetrachloride, chloroform and 1,1,1-trichloroethane inhibit chloroethene dechlorination catalyzed by *Dehalococcoides* organisms [45,46].

An obvious prerequisite is the presence of microbes capable of catalyzing the desired detoxification reaction(s). Perchlorate- and metal-reducing bacteria appear to be ubiquitously distributed in subsurface environments, but a different scenario applies to bacteria involved in chloroethene reductive dechlorination. Although *Dehalococcoides* are often found in anaerobic freshwater habitats, these organisms might not be present in all chloroethene-contaminated sites or are patchily distributed [18,47]. To overcome this bottleneck, consortia containing organisms capable of PCE-to-ethene dechlorination are used to augment sites where the microbiology is limiting the detoxification process. Bioaugmentation is a promising approach when the key players (e.g. *Dehalococcoides* spp.) involved in detoxification are absent or present in numbers too low to allow contaminant removal at appreciable rates. Bioaugmentation adds to the complexity of the remediation effort and must be clearly justified. With the availability of molecular site assessment and monitoring tools, together with a deeper understanding of the genetics and physiology of the key microbes catalyzing the detoxification reactions, it is likely that bioaugmentation strategies will be implemented at many more sites in the near future [15,37,48].

Delivery of electron donors

In addition to establishing and maintaining reducing conditions, the addition of organic substrate(s) increases the flux of acetate and hydrogen, which are the relevant direct electron donors for many reductive detoxification processes. A critical issue is ensuring that the electron donor(s) are available to the organisms where the contaminants are located; this is a major engineering challenge and requires innovative solutions. Small-scale field demonstrations often rely on closed-loop recirculation systems (Figure 2), which give the operator maximum control and management options (e.g. dosing sufficient substrate to promote detoxification while avoiding excessive microbial growth that could cause fouling and clogging). Recirculation systems can be expensive to operate...
and large-scale efforts rely on ‘slow release’ electron donors such as the poly lactate ester HRC\textsuperscript{1} \textsuperscript{[49]}, emulsified vegetable oil \textsuperscript{[44]}\textsuperscript{/C15}, chitin \textsuperscript{[50]}, biomass \textsuperscript{[51]} and others. These substrates can provide a lasting source of fermentable substrates to maintain increased levels of organic acids (i.e. acetate) and hydrogen (Figure 3).

**State of practice: chloroethene bioremediation**

Given that *Dehalococcoides* were only discovered in 1997 \textsuperscript{[23]}, it is remarkable how quickly this science has moved from the laboratory to the field. A recent review of the current state of practice affirmed that enhanced anaerobic bioremediation is ‘a promising technology for the *in situ* remediation of chlorinated aliphatic compounds in groundwater, which has been and is being applied at many sites’ \textsuperscript{[44]}\textsuperscript{/C15}. Although scientific questions remain to be explored and technological challenges to be addressed, the anaerobic biological treatment of chloroethene plumes is an excellent example of how scientific discovery coupled with aggressive technology transfer and implementation leads to innovative solutions in environmental biotechnology. For example, at the Bachman Road site in Oscoda, Michigan, the *Dehalococcoides* required for efficient reductive dechlorination to ethene were already present, and bioaugmentation with lactate was sufficient to promote detoxification. A parallel study performed at the same site demonstrated the benefits of bioaugmentation: the addition of a dechlorinating consortium containing ethene-producing *Dehalococcoides* organisms promoted faster contaminant removal \textsuperscript{[47]}\textsuperscript{/C15}. Moreover, at sites where key *Dehalococcoides* organisms were not detected, bioaugmentation was essential to achieve detoxification \textsuperscript{[15,18,48,52]}. Robust PCE-to-ethene dechlorination is achieved in consortia that have been maintained for years with chloroethenes as electron acceptors. For instance, the commercial Bio-Dechlor INOCULUM\textsuperscript{TM} (Regenesis; http://www.regenesis.com/) and KB-1\textsuperscript{TM} (SiREM; http://www.siremlab.com/) consortia contain multiple *Dehalococcoides* strains together with other bacterial groups (e.g. other PCE-to-cis-DCE dechlorinators, acetogens, fermenters) and are stably maintained \textsuperscript{[13,16]}. These bioaugmentation consortia are produced in stainless steel or plastic batch reactors, shipped to the site in sealed containers, and injected into delivery wells or delivered via drive point injection (Geoprobe, Salinas, KS) \textsuperscript{[15]}. *Dehalococcoides* organisms are sensitive to oxygen, and exposure to air must be avoided when handling the inoculum and during injection into the subsurface. Hence, electron donor delivery to establish reducing conditions typically precedes bioaugmentation with *Dehalococcoides*-containing consortia. Laboratory-based column experiments and field pilot studies suggest that *Dehalococcoides* strains distribute readily in porous medium aquifers, but also colonize the solids \textsuperscript{[18,47]}\textsuperscript{/C15},\textsuperscript{53,54]. These are desirable properties and have been exploited to treat
dissolved-phase chloroethene plumes [18,52] and to establish biobarriers that intersect the plume [47,55]. Particularly challenging are scenarios with high contaminant loading, for instance DNAPL source zones [56]. Recent findings indicate that dechlorinators can be active near source zones and that biological enhancement of PCE dissolution is possible [49,53], but the high electron acceptor concentrations demand effective electron donor delivery strategies to dechlorinating bacteria in the vicinity of the DNAPLs [57].

**State of practice: uranium bioremediation**

Acetate additions to stimulate dissimilatory metal-reducing bacteria promoted the reduction of soluble and mobile U(VI) to sparingly soluble U(IV) uranium precipitates in situ [58]. Metal-reducing bacteria are ubiquitously distributed in anoxic subsurface environments [59], and bio-stimulation was sufficient to reduce U(VI) concentrations and meet regulatory limits at a U(VI)-contaminated aquifer located in Rifle, Colorado. Acetate additions boosted the *Geobacteraceae* population size, which comprise members implicated in U(VI) precipitation [60,61]. A pilot study at the Field Research Center (FRC) in Oak Ridge, Tennessee, also demonstrated the in situ immobilization of U(VI) following aquifer conditioning and bio-stimulation with ethanol [62]. Besides *Geobacter* spp., *Anaeromyxobacter* spp. are implicated in uranium reduction at the FRC [63,64]. Uranium immobilization prevents U(VI) plumes from spreading; however, the contaminant remains in the subsurface and the long-term stability of immobilized uranium is of concern. The maintenance of reducing conditions is critical to prevent re-oxidation of U(IV) to mobile U(VI) by oxidants such as oxygen and N-oxyniogens [65]. Hence, the removal of precipitated uranium is desirable and could be achieved at some sites by microbial [66] and chemical [67] processes or excavation. An innovative approach used electrodes poised at −60 mV to deliver electrons to *Geobacter sulfurreducens* capable of U(VI) to U(IV) reduction. The U(IV) precipitated onto the electrode and recovery was feasible upon removal of the electrode from laboratory-based flow-through columns [68]. Demonstration studies are needed to evaluate if microbially catalyzed electrode precipitation and U(IV) recovery will be productive in the field.

**State of practice: perchlorate bioremediation**

Perchlorate reduction has proven to be relatively easy to stimulate under field conditions through the injection of electron donors, including sugars, alcohols, vegetable oils, mulch and organic acids. Several dozen large pilot tests and field-scale demonstrations completed in the US have clearly shown that in situ bioremediation is technically feasible and has the capability to remove perchlorate to levels below prevailing action levels in most jurisdictions (e.g., < 6 μg/L in California). Given that perchlorate-reducing microorganisms have been detected at almost all sites investigated (e.g. [35]), bioaugmentation is not likely to be required or have added value. Because perchlorate frequently contaminates aerobic, oligotrophic (i.e. electron-donor-limited) drinking water aquifers, perchlorate bioremediation requires the addition of sufficient electron donor to consume the dissolved oxygen, nitrate, chloride (if also present) and perchlorate in the groundwater. However, excess electron donor can promote iron, manganese and sulfate reduction and methanogenesis that deteriorate groundwater quality [69]. In industrial settings, these secondary water quality impacts may be of less concern and, therefore, passive bioremediation approaches that inject large volumes of slow-release electron donors might be acceptable. Passive in situ bioremediation systems involve the injection of slow-release electron donors into the affected aquifer, in many cases to create in situ biobarriers that curtail perchlorate migration (Figure 3). Successful examples of both active and passive full-scale bioremediation systems have been documented in recent years [69,70]. One of the largest systems, treating 500 gallons of perchlorate-contaminated groundwater per minute, has recently been constructed in Nevada [71].

**Bioremediation monitoring**

Process monitoring is crucial for science-based site management decisions and for successful bioremediation. A variety of analytical and molecular biology tools that directly or indirectly monitor the detoxification process have become available (for a summary see [37]). A common target for qualitative and quantitative assessment of a target bacterial population is the 16S rRNA gene. Monitoring of changes in population size following treatment using this phylogenetic marker gene has been very successful, particularly for *Dehalococcoides* [18,47,72]. However, the incongruence between phylogeny (e.g. the 16S rRNA gene sequence) and phenotype (i.e. detoxifying activity) often blurs conclusions on the organism’s metabolic capabilities [73,74]. To overcome these limitations, process-specific indicator genes are being sought. For example, assessing reductive dehalogenase genes involved in chloroethene detoxification more accurately characterizes the dechlorinating *Dehalococcoides* community [19,38,39,41,75,76]. For a more direct measure of activity, gene transcripts (mRNA) [77,78] or proteins [79] have been targeted; however, the applicability of these approaches for field monitoring has yet to be established. The current site assessment and bioremediation monitoring approaches only provide basic information, and knowledge of additional process-specific biomarkers is critical to describe the biodegrading community and its activity with much greater resolution. The genomes of key dechlorinators (e.g. *Dehalococcoides* spp.) and metal reducers (e.g. *Anaeromyxobacter* spp., *Geobacter* spp., *Shewanella* spp. and *Desulfovibrio* spp.) have been sequenced, and current efforts are using high-throughput nucleic acid (i.e. microarrays) and protein screening (i.e. mass-spectrometry-based) technologies to identify process-specific biomarkers. Hence, improved prognostic site assessment and
diagnostic site monitoring tools for subsurface bioremediation are likely to emerge in the short-term. Successful subsurface bioremediation is a young technology, particularly for the contaminants reviewed herein, and has yet to build its reputation as a viable and often superior alternative to traditional physical-chemical (e.g. pump-and-treat) approaches. Hence, establishing cause-and-effect relationships that unequivocally establish a link between enhanced anaerobic bioremediation and contaminant removal and detoxification is crucial. To this end, advanced molecular tools that monitor the key players catalyzing the detoxification reactions are indispensable both to establish confidence in the technology and to demonstrate to practitioners, stakeholders and regulators that enhanced anaerobic bioremediation is a powerful technology for environmental cleanup.

**Technology risks and challenges**

Risks associated with enhanced anaerobic bioremediation include technology failure (i.e. the implementation of enhanced bioremediation does not decrease contaminant concentrations), incomplete contaminant removal without meeting regulatory standards, the stimulation of undesirable processes (e.g. sulfidogenesis or methanogenesis), excessive microbial growth causing well fouling or aquifer clogging, as well as permanent changes to the microbial community with possibly negative impacts on overall ecosystem function. Although these risks are real, the technology has already matured to a level where failures are avoided with proper site assessment and bioremediation management. To date, properly managed enhanced anaerobic bioremediation efforts have not caused any long-term adverse effect on the environment. Microbial community monitoring at the Bachman Road site demonstrated community shifts during the active phase of bioremediation, but the community rebounded to its original state following the completion of the pilot test [47].

Major challenges include the efficient delivery and distribution of organisms and optimum concentrations of electron donor(s) to the subsurface, especially in heterogeneous settings. Further, the complex interactions of multiple contaminants on the biodegrading microbiota need to be described in far greater detail so that bioremediation at mixed waste sites can be implemented with confidence. Another area that warrants further exploration is the combined application of physical-chemical and biological remedies. For instance, treatment strategies that couple surfactant flushing, thermal treatment or chemical oxidation with microbial reductive dechlorination show promise to enhance mass removal and reduce contaminant mass flux emanating from treated PCE-DNAPL source zones [4,54,80,81].

**Conclusions**

Nature provides a blueprint for innovative solutions, and recent efforts demonstrate that harnessing the metabolisms of naturally occurring bacteria provides effective, economically feasible solutions for environmental cleanup and restoration. Laboratory-based, fundamental scientific discovery will remain a crucial prerequisite for developing new technologies and well-instrumented field-scale demonstrations will guide their successful implementation. In concert with traditional microbiology, biochemistry, molecular biology and engineering approaches, the rapid progress in high-throughput screening technologies, computational and systems biology, biogeochemical modeling and database development will enhance current progress. Clear examples of how the integration of genomics [82**], proteomics [79*] and systems biology [83**] can significantly enhance our understanding of complex subsurface microbial processes are emerging. As an illustration, genome sequences are now being used to build sophisticated metabolic flux models for a variety of organisms, including the groundwater microbe *G. sulfurreducens* [83**]. The application of combined experimental and computational approaches will be invaluable for elucidating the roles of individual organisms in complex subsurface systems. These integrated approaches will significantly contribute to moving bioremediation from a relatively empirical practice to a predictable science with widespread application.

**Update**

A successful field demonstration of U(VI) bioremediation has been recently completed at the Field Research Center (FRC) in Oak Ridge, Tennessee. The low pH and the presence of co-contaminants required conditioning of the treatment zone, which was accomplished by flushing, above ground removal of inhibitors and clogging agents, and pH adjustment [89]. Subsequent biostimulation with ethanol demonstrated that the native microflora efficiently reduced soluble U(VI) to immobile U(IV) [90]. This excellent field study demonstrates that engineering design based on scientific principles and accomplished by interdisciplinary teams leads to innovative and successful solutions for environmental restoration and stewardship.

**Acknowledgements**

The authors acknowledge financial support from the US Department of Defense through the Strategic Environmental Research and Development and the Environmental Security Technology Certification Programs, the National Science Foundation, the Office of Science (BER) of the US Department of Energy, the National Science and Engineering Research Council of Canada, Regenesis, and GeoSyntec Consultants.

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