TECHNICAL REPORT
TR-NAVFAC EXWC-EV-1806

ADVANCES IN THE STATE OF THE PRACTICE FOR ENHANCED IN SITU BIOREMEDIATION

Prepared for NAVFAC EXWC under Contract No. N62583-11-D-0515/0108

February 2018

REPORT DOCUMENTATION PAGE

FORM APPROVED
OMB NO. 0704-0188

1. REPORT DATE *(DD-MM-YYYY)*	2. REPORT TYPE	3. DATES COVERED *(From – To)*
28-02-2018	Technical Report	Feb 2018

4. TITLE AND SUBTITLE
Advances in the State of the Practice for Enhanced In Situ Bioremediation

5a. CONTRACT NUMBER
N62583-11-D-0515/0108

5b. GRANT NUMBER
N/A

5c. PROGRAM ELEMENT NUMBER
N/A

6. AUTHOR(S)
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5d. PROJECT NUMBER
N/A

5e. TASK NUMBER
Click here to enter text.

5f. WORK UNIT NUMBER
Click here to enter text.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)
Click here to enter text.

8. PERFORMING ORGANIZATION REPORT NUMBER
Click here to enter text.

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)
NAVFAC EXWC
1000 23rd Ave.
Port Hueneme, CA 93043

10. SPONSOR / MONITOR'S ACRONYM(S)
NAVFAC EXWC

11. SPONSOR / MONITOR'S REPORT NUMBER(S)
TR-NAVFAC EXWC-EV-1806

12. DISTRIBUTION / AVAILABILITY STATEMENT
Distribution Statement A: Approved for public release; distribution is unlimited

13. SUPPLEMENTARY NOTES
Click here to enter text.

14. ABSTRACT
Enhanced in situ bioremediation (EISB) is an engineered technology that introduces physical, chemical, and biological changes to the aquifer to create the conditions necessary for microorganisms to transform contaminants of concern (COCs) to innocuous byproducts. This document provides current industry-accepted best practices to design and apply EISB, with a primary focus on chlorinated ethene remediation, and introduces Remedial Project Managers (RPMs) to recent innovations and trends to facilitate successful application.

15. SUBJECT TERMS
Click here to enter text.

16. SECURITY CLASSIFICATION OF:			17. LIMITATION OF ABSTRACT	18. NUMBER OF	19a. NAME OF RESPONSIBLE PERSON
a. REPORT	b. ABSTRACT	c. THIS PAGE			Tony Danko
Click here	Click here	Click here	U	26	19b. TELEPHONE NUMBER *(include area code)* Click here to enter text.

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ACRONYMS AND ABBREVIATIONS

BIOPic	Bioremediation Pathway Identification Criteria
BTEX	Benzene, toluene, ethylbenzene, and xylene
COC	Contaminant of concern
CSIA	Compound-Specific Isotope Analysis
CSM	Conceptual site model
DCA	Dichloroethane
DCE	Dichloroethene
Dhc	*Dehalococcoides*
DNAPL	Dense non-aqueous phase liquid
DO	Dissolved oxygen
EISB	Enhanced in situ bioremediation
EK-BIO	Electrokinetic bioremediation
ELS™	Emulsified Lecithin Substrate
ERT	Electrical resistance tomography
ESTCP	Environmental Security Technology Certification Program
FISH	Fluorescent In Situ Hybridization
GPR	ground penetrating radar
HPFM	Heat pulse flow meter
HRSC	High-resolution site characterization
ITRC	Interstate Technology & Regulatory Council
LIF	Laser-induced fluorescence
MBT	Molecular biological tool
MIP	Membrane interface probe
MNA	Monitored natural attenuation
MTBE	Methyl tert butyl ether
NAPL	Non-aqueous phase liquid
OoM	Order of magnitude
PAH	Polycyclic aromatic hydrocarbon
PCE	Tetrachloroethylene
qPCR	Quantitative Polymerase Chain Reaction
RAO	Remedial action objective

RDX	Cyclotrimethylenetrinitramine
RG	Remedial goal
RPM	Remedial Project Manager
SERDP	Strategic Environmental Research and Development Program
SIP	Stable-Isotope Probing
SWQI	Secondary water quality impact
TCA	Trichloroethane
TCE	Trichloroethylene
TNT	Trinitrotoluene
U.S. EPA	United States Environmental Protection Agency
VC	Vinyl chloride

1.0 INTRODUCTION

Enhanced in situ bioremediation (EISB) is an engineered technology that introduces physical, chemical, and biological changes to the aquifer to create the conditions necessary for microorganisms to transform contaminants of concern (COCs) to innocuous byproducts. EISB of petroleum hydrocarbons and chlorinated solvents has been demonstrated and applied at sites for decades and, more recently, is being applied to treat emerging contaminants such as 1,4-dioxane and other COCs. Although remedial action objectives (RAOs) and remedial goals (RGs) are achieved at many sites, there have been sites where concentrations of COCs were not reduced significantly, elevated concentrations of harmful byproducts (e.g., vinyl chloride) were formed, or rebound of COCs prevented RAOs/RGs from being achieved. During the last several years, new tools and technologies have been developed and applied at sites and an improved understanding of technology- and site-specific challenges has been realized to facilitate successful application of this technology. This white paper provides current industry-accepted best practices to design and apply EISB, with a primary focus on chlorinated ethene remediation, and introduces Remedial Project Managers (RPMs) to recent innovations and trends to facilitate successful application.

2.0 BACKGROUND

EISB is widely used to treat a variety of chemical classes including petroleum hydrocarbons such as benzene, toluene, ethylbenzene, and xylenes (BTEX) and polycyclic aromatic hydrocarbons (PAHs); chlorinated ethenes, such as tetrachloroethylene (PCE) and trichloroethylene (TCE); pesticides; and energetics, such as trinitrotoluene (TNT) or cyclotrimethylenetrinitramine (RDX). EISB is a process by which indigenous or inoculated microorganisms transform organic contaminants in groundwater with the goal to convert them into innocuous end products. This technology can be applied to treat and control the migration of dissolved phase plumes, as well as used to treat source areas. EISB can complement other technologies by using a treatment train approach where EISB is performed before or after another technology. For example, it can be applied for source area treatment before monitored natural attenuation (MNA), or it can be applied as a polishing step after in situ chemical oxidation or thermal treatment.

Several types of biodegradation processes (pathways) can be leveraged to degrade COCs. To a large extent, biodegradation pathways are dependent on the type of COCs (Table 1) and microorganisms present in the aquifer. For instance, petroleum hydrocarbon contaminants are readily degraded through an aerobic pathway. Aerobic biodegradation occurs in the presence of oxygen (air) and relies on the direct oxidation of the contaminant. Oxygen is used as an electron acceptor and the COCs serve as electron donors which are degraded for carbon and energy. Some constituents, such as benzene, can also be eliminated via direct microbial metabolic oxidation of the COC, which relies on other electron acceptors such as nitrate or sulfate. Amendments containing soluble sulfate (e.g., magnesium sulfate) can also be added to the affected area to stimulate sulfate-reducing conditions to help microbes metabolize the COCs.

Chlorinated solvents such as PCE and TCE are generally degraded through an anaerobic pathway, in which the COC is used as the electron accepter and the food source is another form of carbon such as emulsified vegetable oil, which typically is added to the aquifer. Anaerobic degradation

results in the reduction of chlorinated solvents and other COCs after the carbon amendment is fermented and hydrogen is generated. Hydrogen then serves as the electron donor for the reductive dechlorination process.

Lastly, chlorinated solvents (and other COCs such as 1,4-dioxane) may be degraded by another degradation process – cometabolic degradation. Cometabolic degradation occurs when microorganisms using one compound as an energy source fortuitously produce an enzyme that chemically transforms another compound (i.e., COC). As a result, organisms can degrade a contaminant without gaining any energy from the reaction. Cometabolic degradation may occur aerobically or anaerobically.

Table 1. Common Contaminants of Concern Degraded by each Biodegradation Process

Class	Common Contaminant	Aerobic Oxidation	Anaerobic		Cometabolic Processes
			Oxidation	Reduction	
Petroleum Hydrocarbons and Related COCs	Non-halogenated alkenes/alkanes	X			
	BTEX	X	X		X
	Simple PAHs (e.g., naphthalene)	X	X		X
	Cyclic PAHs		X		X
	Methyl tert butyl ether (MTBE)	X			X
Chlorinated Ethenes	PCE and TCE			X	X
	Dichloroethene (DCE) and vinyl chloride (VC)	X		X	X
Chlorinated Ethanes	1,1,1-trichloroethane (1,1,1-TCA), 1,2- dichloroethane (1,2-DCA), and 1,1-dichloroethane (1,1-DCA)			X	X
Chlorinated Methanes	Carbon tetrachloride				X
	Chloroform and methylene chloride			X	X
Pesticides	Select pesticides	X		X	X
Ethers	1,4-dioxane	X			X
Energetics	TNT	X			X

EISB often employs biostimulation and bioaugmentation to modify existing geochemical and biological conditions in an aquifer to facilitate biodegradation of COCs. Biostimulation refers to the introduction of an amendment into the aquifer for the purpose of stimulating microbial growth. In the case of aerobic biodegradation, the amendment may simply be air supplied to the subsurface. For anaerobic degradation, many types of amendments are available, including liquids (such as

emulsified vegetable oil, lactate, molasses, and other food-grade compounds) and solid materials (such as mulch or chitin).

Bioaugmentation refers to the introduction of microorganisms into the aquifer and can supply the site with the needed microbial community when it is necessary to lessen the time required to attain project goals or when (in the case of reductive dechlorination) sufficient microorganisms are not present at a site to overcome anticipated DCE and VC stall. Several commercially-available microbial consortia consist of one or more of *Dehalococcoides (Dhc)*, *Dehalobacter*, sulfate reducers, methanogens, and fermentative microbes, which can degrade chlorinated ethene, chlorinated ethane, and mixed plumes. These cultures should be added only after the necessary redox conditions have been achieved in the aquifer to ensure the consortia's survivability and proliferation.

In addition to electron donors and microorganisms, amendments such as nutrients, buffers, or other reagents may be used to enhance bioremediation and create/maintain optimum conditions for biodegradation to occur. Types of amendments, appropriate dosages, and application methods are site-specific. Various considerations and discussion of some of the recent advances pertaining to amendments and design of EISB remedies are presented in this fact sheet and additional information can be found in the references (AFCEC and NAVFAC, 2004; NAVFAC, 2015).

3.0 DESIGN CONSIDERATIONS

A detailed understanding of the conceptual site model (CSM) is paramount to ensure successful design and application of an EISB remedy. The CSM should include up-to-date knowledge of geochemical and lithologic characteristics of the site, flow, and mass transport, and information related to the transformation and retardation of COCs and proposed amendments. Failure to address these components in the design can have a negative impact on technology performance. However, it is important to acknowledge throughout the design process, as well as during the application process, that there are always unknowns and that understanding of the site may evolve over time. Therefore, it is important to acknowledge potential data gaps and to identify possible deviations that could occur during application to develop appropriate contingencies. More detailed information on CSM requirements for bioremediation sites can be found in NAVFAC's *Design Considerations for Enhanced Reductive Dechlorination* (NAVFAC, 2015).

High-resolution site characterization (HRSC) can be a particularly useful aid to develop the CSM and to design an appropriate bioremediation strategy for the site. Some of the more common HRSC methods include geophysical techniques such as ground penetrating radar (GPR), cross-borehole radar, electrical resistance tomography (ERT), seismic reflection, and electrical induction techniques. Cone penetrometers coupled with various detectors such as laser-induced fluorescence (LIF) or membrane interface probes (MIPs) are effective screening techniques to understand lithology and the extent of residual non-aqueous phase constituents. Other techniques include the use of various colorimetric indicators such as ribbon samplers or dyes to detect the presence of non-aqueous phase liquids. Geophysical tools, such as heat pulse flow meters (HPFMs), optical and acoustic televiewers, and gamma loggers, provide detailed information to characterize bedrock sites.

The design of an EISB remedy is developed using information provided in the CSM. It should include an amendment delivery plan detailing the method and procedures for introducing amendments, amendment dosing and longevity, number of injection events, injection/extraction point well layout, equipment specification, process and performance monitoring requirements, health and safety requirements, and any regulatory issues. The design should include RAOs, RGs, treatment milestones, treatment endpoints, and contingencies for potential deviations. All project stakeholders should agree to and approve the design prior to its implementation.

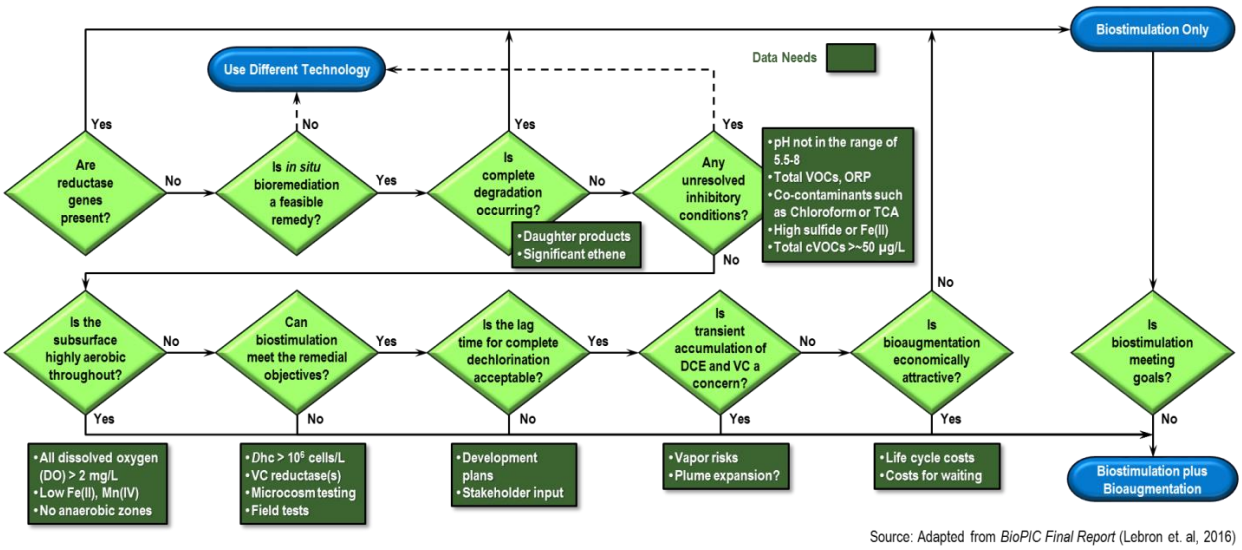
Several questions regarding site conditions need to be answered prior to EISB implementation for contaminants such as chlorinated ethenes:

3.1 Are Conditions Favorable for an EISB Remedy?

Aerobic biodegradation of petroleum hydrocarbon constituents is relatively straightforward. The required microorganisms are ubiquitous in the environment and, in general, the only amendment needed to facilitate degradation is oxygen. Anaerobic biodegradation of chlorinated solvents (reductive dechlorination) and other compounds can be more involved and sensitive to a wide range of aquifer conditions. In addition to achieving adequate contact of introduced amendments with chlorinated ethenes, aquifer properties including geochemistry and microbiology can strongly influence and impact the success of a remedy.

Figure 1 presents a flowchart to assess if site conditions are favorable for reductive dechlorination and if biostimulation and/or bioaugmentation will be required. This flowchart was adapted from the Environmental Security Technology Certification Program (ESTCP) BioPIC Tool decision-making process as outlined in the final report (Stroo et al., 2013; Lebrón et al., 2016). BioPIC stands for Bioremediation Pathway Identification Criteria. It is an Excel-based tool that can help practitioners choose and apply the most appropriate bioremediation approach at sites impacted with chlorinated solvent and is an update to the original MNA protocol adopted by the United States Environmental Protection Agency (U.S. EPA, 1998).

Based on this updated protocol, Figure 1 presents a series of questions (light green diamonds) to guide the practitioner to determine if bioremediation will be effective at a site and if bioaugmentation will be required. The dark green squares indicate the data that are needed to answer each of the questions, which can be obtained through analysis of groundwater samples for a variety of parameters and through microcosm and/or field testing. Although the BioPIC tool was initially developed to evaluate if MNA is applicable at a site, the tool is also a useful aid to evaluate the mechanism by which bioremediation is occurring. It considers both biotic and abiotic processes to assess the potential for bioremediation and/or biogeochemical transformation. This information can be useful to determine when EISB can be transitioned from active bioremediation to MNA.



Source: Adapted from *BioPIC Final Report* (Lebron et. al, 2016)

Figure 1. Stepwise Approach to Evaluate Suitability of Reductive Dechlorination and Need for Bioaugmentation (Adapted from Lebrón et al., 2016)

3.2 Should Bioaugmentation be Utilized in Addition to Biostimulation?

Microorganisms necessary to perform complete degradation of the contaminant can be native to a site. For example, the presence of *Gordonia sp*. strain KTR9 microorganisms has been linked to the degradation of RDX and the presence of *Dhc*-related microorganisms has been linked to complete dechlorination of PCE and TCE to ethene in field conditions. However, the preferred microorganisms may not always be present or abundant. Bioaugmentation may be considered at a site when an appropriate microbial population is not present or is not sufficiently active to stimulate complete degradation of the COC. In these cases, microbial cultures of non-native microorganisms known to degrade the contaminant of interest are introduced into the aquifer.

It is noted that some practitioners routinely bioaugment their sites as a precautionary measure based on the additional cost and time to procure and then remobilize cultures. The rationale is that the additional cost to bioaugment may be offset by faster remediation timeframes leading to lower project life-cycle costs and the reduced risk of accumulating undesirable intermediate byproducts (e.g., VC). However, site-specific conditions should be considered as noted in Figure 1 before undertaking this approach.

There are a wide range of bioaugmentation cultures on the market, most of which have been developed to treat chlorinated ethenes and ethanes. These cultures are usually a consortium of microbes consisting of *Dhc*, *Dehalobactor*, and other various types of microbes. Table 2 provides a list of some commercially-available cultures used for reductive dechlorination.

Recent research has focused on developing cultures, such as KB-1 Plus, which are tolerant to low pH environments. In general, *Dhc* does not survive and proliferate in groundwater with pH less than 6 and, therefore, the aquifer may need to be amended periodically with buffer. Research is also being performed to identify microorganisms other than *Dhc* that can completely degrade chlorinated ethenes. For instance, it has recently been reported that the *cerA* gene expressed by *Dehalogenimonas* can anaerobically degrade VC to ethene (Löffler, 2017). Recent developments

also include the development of cultures to treat other types of contaminants. For instance, UCLA developed the CB190 culture that can directly metabolize 1,4-dioxane under aerobic conditions (Mahendra and Alvarez-Cohen, 2006). Similarly, organizations are investigating and developing cultures for anaerobic degradation of benzene. The 1,4-dioxane culture is commercially available now and the benzene culture will be available soon.

Table 2. Commercially-Available Bioaugmentation Cultures for Reductive Dechlorination[1]

Vendor	Culture	Target Contaminants	Other
SiRem	KB-1	Chlorinated ethenes, ethanes, methanes, propanes, RDX, chloroflourocarbons	
	KB-1 Plus		Well-suited for low pH (5.8 to 6.3) aquifers
Regenesis	BDI Plus	Chlorinated ethenes/ethanes	
EOS Remediation	BAC-9	PCE, TCE, *cis*- & *trans*-DCE, VC, Freon 113, mixed plumes containing 1,1,1-TCA & 1,1,2-TCA, dichloroethane isomers, carbon tetrachloride, chloroform, and bromine compounds	
BCI Inc.	BCI-e	Chlorinated ethenes	Variations of cultures are available, which are not inhibited by chloroform, high PCE levels, TCA, and brackish water
	BCI-a	TCA, 1,1-DCA, and chlorinated ethenes	
	BCI-t	Trichlorobenzene and dichlorobenzenes	
Terra Systems	TSI DC	Chlorinated ethenes, ethanes, 1,1,1-trichloroethane, 1,1-dichloroethane, chloroethane, carbon tetrachloride and chloroform	
	TSI DC-TCA	Chlorinated ethenes, 1,1,1-TCA, 1,1,2-trichloroethane (1,1,2-TCA), 1,2-DCA, and 1,1-DCA	Equal parts *Dhc* and *Dehalobactor*
Redox Tech	RTB-1	Chlorinated ethenes	

3.3 What Types of Amendments Should I Consider?

3.3.1 Electron Donors

Many types of electron donor substrates are available and have been used to stimulate anaerobic biodegradation of COCs. The selection of an appropriate electron donor is based on site-specific factors, objectives, and the practitioner's experience applying EISB remedies. Substrates can be divided into two categories consisting of aqueous and slow-release compounds.

Aqueous compounds include amendments such as lactate, sodium benzoate, molasses, and whey. They are highly soluble and are easily distributed across large areas. However, they also are readily

[1] Other cultures may be available. Selection should be based on site-specific conditions and project objectives. Please see the disclaimer accompanying this document.

bioavailable, and, therefore, are consumed in a relatively short time. Slow-release compounds, including compounds such as emulsified vegetable oils (HRC® and EHC®), mulch, and compost, tend to have low solubility limits and greater viscosities than their aqueous counterparts, making them more difficult to emplace in the aquifer (AFCEC, 2007). However, because slow-release compounds are less soluble (and less bioavailable), they persist much longer in the aquifer.

Vendors have developed oil-water emulsion formulations that include both aqueous and slow-release compounds. In these formulations, the aqueous compounds are degraded rapidly, generating the conditions necessary for reductive dechlorination to occur, while the slow-release compounds provide a long-term source of electron donor for the dechlorinating microbial population, which increases the time required between applications of the substrate. All electron donors act by stimulating microbial processes that deplete dissolved oxygen (DO) and other terminal electron acceptors, thus lowering the oxidation-reduction potential of groundwater and producing the electron donor (hydrogen) necessary to support anaerobic biodegradation.

3.4 What Are Some Recent Advances in Amendment Formulations?

Several innovative amendments are commercially available and represent a wide array of applications:

3.4.1 New Generation of Electron Donors

- **Emulsified Lecithin Substrate (ELS™)** – This amendment is a microemulsion of a food-grade carbon source. It is amphiphilic, meaning it has both hydrophobic and hydrophilic ends. This allows it to sequester hydrophobic compounds, while still having a hydrophilic end, making it soluble in water for distribution into the aquifer. It is composed of a fast- and slow-release electron donor to promote the development of reducing conditions, while also providing for a longer-term electron donor. ELS™ also provides nitrogen and phosphorus nutrients to the microorganisms.
- **Quick Release Electron Donors** – This amendment is rapidly fermented to decrease the oxidation-reduction potential in an aquifer, thus providing the necessary conditions for reductive dechlorination. These donors are depleted within weeks to a few months. Therefore, blending with other long-lasting substrates (which persist for several months to years) may be necessary to maintain conditions for an extended duration. Quick release donors are formulated with food-grade carbon sources, nutrients, cofactors, and vitamins. One type is Newman Zone QR, which contains lactate, complex carbohydrates, phospholipids, soluble proteins, micronutrients, and phosphate.
- **Colloidal Liquid Activated Carbon** – This amendment is a colloidal biomatrix of activated carbon particles that can be distributed under low pressures, applied with an electron donor, and is resistant to clumping. Colloidal activated carbon may address problems with matrix diffusion due to its longevity at the site. The activated carbon binds to the aquifer matrix, then captures and concentrates dissolved-phase contaminants. Simultaneously, the activated carbon becomes colonized by bacteria, which can degrade the contaminants. As COCs are degraded, sorption sites on the active carbon are available to more COCs.

3.4.2 Methane Inhibiting Amendments

Production of methane (CH_4) is a good indicator that biodegradation (e.g., reductive dechlorination) is occurring. However, excessive production of CH_4 can consume a significant portion of the electron donor. Other drawbacks of excessive CH_4 production include contributing CH_4 as a source to greenhouse gas emissions and vapor intrusion (possibly resulting in explosive concentrations in some instances).

Proprietary amendments have been developed to mitigate production of CH_4. For example, Provect-CH4™, which is a food-grade, natural source of Monacolin K, is used to prevent CH_4 production by inhibiting the growth and proliferation of methanogenic Archaea. It is supplied as a water-soluble powder that can be mixed on site and added in conjunction with the electron donor. Other amendments are available on the market as well that directly incorporate this proprietary methane inhibitor without the need for on-site mixing.

3.5 What Types of Substrate Delivery Methods Are Available?

A main design consideration is the type of delivery approach used to introduce the amendments into the aquifer. Delivery approaches are highly site-specific and to a large extent are based on RAOs and RGs. Principal delivery methods, which may be used independently or combined to achieve project goals, include the following:

- Direct injection – Involves introducing the reagents directly into the subsurface with a specified volume of water from an external source. This process displaces groundwater corresponding to the volume of reagent injected.
- Recirculation – Relies on a forced gradient to introduce the amendments over an extended time. Groundwater is extracted from one set of points or wells, amended with the reagents, and reinjected into another set of wells.
- Hydraulic or Pneumatic Fracturing – Applies hydraulic or pneumatic pressure to the formation to induce fractures in low permeability formations (e.g., clays and bedrock) through which amendments may be introduced using direct injection or recirculation approaches.

There are advantages and disadvantages of direct injection and recirculation approaches. Direct injection approaches tend to be less expensive than active approaches, can be implemented rapidly, and require less equipment. However, because groundwater is displaced, there sometimes is a concern that COCs will be displaced outside of the treatment area. Since water is not typically withdrawn from the treatment area, water is required from an external treatment source, which can make this approach less "green" than a recirculation approach. Recirculation methods tend to be costly and more equipment intensive compared to direct injection; however, much better hydraulic control can be obtained. In addition, with recirculation, it typically is possible to achieve a greater radius of influence than can be achieved using a direct injection approach. The risk of amendments traveling to the surface during application is reduced, but there may be a greater likelihood of creating preferential pathways in the aquifer material, which hinder contact between the reagents and COCs. Fouling and channeling may be more problematic with recirculation approaches than direct injection approaches. Best practices and general guidance to apply these techniques can be found in the document *Best Practices for Injection and Distribution of Amendments* (NAVFAC, 2013).

4.0 TECHNOLOGICAL ADVANCES FOR EISB AT COMPLEX SITES

Meeting restoration goals has been challenging at complex contaminated sites (i.e., fractured bedrock, large dilute plumes, non-aqueous phase liquid [NAPL] source zones, emerging contaminants, etc.). Additional refinements and enhancements to EISB continue to be developed that can help to address these challenging sites. Several emerging technologies to support EISB remedies at challenging site types are described in the subsections below.

4.1 Shear Thinning Fluids

It is challenging to adequately distribute amendments into low permeability (k) silts and clays. At many sites, COCs residing in low-k portions of an aquifer act as continuing sources of contamination, even after treatment has been performed to remove the COCs in the more permeable zones.

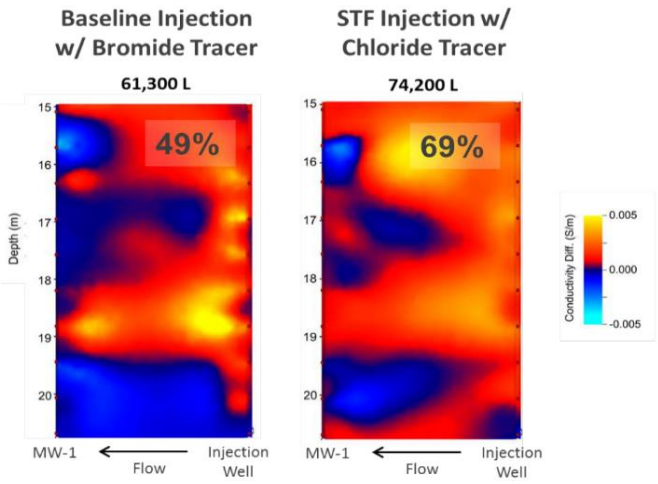


Figure 2. Enhanced Sweep Efficiency of Electron Donor Achieved using Shear Thinning Fluids at Joint Base Lewis-McChord (ESTCP ER-200913) (ESTCP 2015a)

Shear thinning fluids can help to improve the distribution of amendments into low-k zones to achieve better treatment. Shear thinning fluids include food-grade water-soluble polymers, such as xanthan gum, that exhibit non-Newtonian behavior, meaning that their viscosities exhibit a temporary drop when the applied shear rate is increased. This shear-thinning behavior causes a greater viscosity reduction of the fluid flowing through the low-k zones relative to the viscosity reduction of the fluid flowing through the high-k zones. Therefore, preferential flow through the more permeable zones is significantly reduced while the flow into the low-k zone is increased. Furthermore, a transverse pressure gradient is created that generates cross-flow of fluids from the high permeability into the low-k zones.

The application of shear thinning fluids has been demonstrated at Area D of a TCE plume at the Joint Base Lewis McChord (ESTCP Project ER-200913) (ESTCP, 2015a). An electron donor, ethyl lactate, was amended with a chloride tracer and xanthan gum. Resulting data showed an improvement in the uniformity of the amendment distribution (Figure 2), along with removal of TCE to below action levels without rebound. The study concluded that the use of shear thinning fluids is applicable for aquifers that have less than two orders of magnitude difference between the low and high-k zones.

4.2 Electrokinetic Bioremediation

Electrokinetic bioremediation (EK-BIO) is another technology that can facilitate amendment distribution and improve treatment at sites with COCs present in low-k zones. EK-BIO leverages the electrical properties of soil, groundwater, and amendments to promote the distribution of

electron donors and cultures into low-k zones. An electric current is applied to the ground, which facilitates the transport of amendments from the electrode and/or supply wells into and throughout the formation regardless of the stratigraphy encountered. The process is highly efficient in clay-rich strata, resulting in the migration of ions and dissolved compounds at a rate of several meters per month in tight clays. For heterogeneous systems, where significant contaminant mass remains in low-k regions, the application of EK results in enhanced delivery of reagents into these low-k layers.

A successful demonstration of EK-BIO was performed at a PCE-contaminated site in Denmark, where lactate flow was generated through clay particles with a rate of 3 to 5 cm/day. At Naval Air Station Jacksonville, Florida (ESTCP ER-201325) (ESTCP, 2016b), the technology is currently being implemented to distribute potassium lactate and the KB-1 culture. Results to date have demonstrated that the concentration of total organic carbon has increased at all locations within the low-k zone and that reductive dechlorination is occurring. Concentrations of TCE have decreased and the formation of ethene has been observed across the treatment area. In addition, the concentrations of *Dhc* also were noted to increase by one or more orders of magnitude at monitoring wells.

4.3 Heat-Enhanced Bioremediation

Bioremediation can be coupled with thermal technologies to raise the temperature of the aquifer to stimulate biodegradation. As the aquifer temperature is increased, reaction kinetics (i.e., biodegradation rates) increase. Laboratory studies performed as part of ESTCP project ER-200719 (ESTCP, 2015c) showed that at about 40ºC, the concentration of *Dhc* and *vcrA* genes (responsible for anaerobic metabolic conversion of vinyl chloride to ethene) increased substantially compared to concentrations at ambient groundwater temperatures. In addition to facilitating biodegradation, heat also offers several other advantages. For instance, it can enhance dense non-aqueous phase liquid (DNAPL) dissolution, enhance desorption from soil to the aqueous phase, and increase the rate of volatilization, all of which serve as mechanisms to enhance removal of COCs. Also, unlike using high temperature thermal treatment technologies, aboveground treatment is not necessary since the majority of COCs are degraded in situ.

Heat is generated in a similar manner to other heating technologies such as thermal conductive heating or electrical resistance heating. The primary difference is that the target treatment temperature is much lower than that of other heating technologies. Results of a pilot test performed at the Joint Base Lewis-McChord Landfill (ESTCP, 2015c) demonstrated effective removal of TCE. Elevated concentrations of TCE were initially noted in the treatment area, presumably due to desorption from soil; however, concentrations rapidly declined by the end of the heating phase. The genes *tceA*, *bvcA*, and *vcrA* were also analyzed to track EISB progress. The genes *tceA* and *bvcA* are responsible for metabolic conversion of PCE to TCE, TCE to DCE, and DCE to VC under anaerobic conditions, while *vcrA* is responsible for metabolic conversion of VC to ethene under anaerobic conditions. Low detections of *Dhc* and these genes were observed during the baseline sampling event; however, concentrations were observed to increase by one to two orders of magnitude following the onset of heating.

4.4 Bioremediation in Fractured Bedrock

Bioremediation in fractured bedrock presents a number of unique challenges. The distribution of COCs and the behavior of remedial systems are less likely to be understood. These sites typically require a longer treatment time to address back diffusion from the rock matrix, and in general, it is difficult to achieve good hydraulic connectivity to deliver the necessary amendments. Naturally-occurring organic carbon likely is low, and therefore, larger dosages of electron donor may be required compared to other types of sites.

A number of best practices can be employed to improve the likelihood of a successful EISB application at a fractured bedrock site. Recent development of various geophysical and other HRSC techniques and tools (e.g., heat pulse flow meter, rock matrix characterization, optical and acoustic televiewers) can be applied to identify the horizontal and vertical extent of COCs and groundwater flow pathways and velocities, with the objective to develop a more accurate CSM and better design an appropriate remedy. Other techniques, such as applying amendments in discrete zones and using a dense injection grid, can be employed to target the intervals having high levels of contamination. Long-lasting amendments should be used as opposed to water-soluble amendments that can be easily transported away from the treatment area. Also, it is advantageous to inject the amendments over extended time intervals at low flowrates to achieve better distribution. Hydraulic or pneumatic fracturing may be necessary to create additional flow pathways and multiple injection events should be anticipated. More information can be found in the Interstate Technology Regulatory Council (ITRC) guidance on *Characterization and Remediation of Fractured Rock* (ITRC, 2017).

4.5 Bioremediation of 1,4-Dioxane

1,4-Dioxane, an emerging contaminant, is a likely human carcinogen which has been found in groundwater at sites throughout the United States. It is highly mobile and is not readily biodegraded in the environment. However, 1,4-dioxane can be degraded either by direct or cometabolic oxidation. Microbial species such as *Pseudonocardia dioxanivorans sp*. Strain CB1190, *Rhodococcus sp*, *Amycolata sp*., and *Mycobacterium vaccae* have been demonstrated to carry out direct metabolic oxidation of 1,4-dioxane. In addition, species including *Pseudonocardia sp*. strain ENV487, *Mycobacterium sp*. ENV421, and *Nocardia sp*. ENV425 oxidize 1,4-dioxane via a cometabolic process using substrates such as ethane, propane, and toluene. New developments for bioremediation of this emerging contaminant have been reported. For example, Strategic Environmental Research and Development Program (SERDP) Project ER-2307 (SERDP, 2016c) showed a positive correlation between increasing oxygen concentrations in groundwater and 1,4-dioxane attenuation and a negative correlation between high levels of metals and chlorinated volatile organic compounds. Several other SERDP studies (ER-2303, ER-2306) are ongoing to address in situ biodegradation of 1,4-dioxane with branched hydrocarbons and cometabolic aerobic biodegradation of 1,4-dioxane by methanotrophs in commingled chlorinated solvent plumes (SERDP, 2016a; SERDP, 2016b). ESTCP Project ER-201733 is a follow-up to SERDP Project ER-2303 to perform a field demonstration using isobutane-oxidizing bacteria to treat high concentrations of 1,4-dioxane commingled with chlorinated ethenes.

5.0 MONITORING BEST PRACTICES AND INNOVATIONS

A comprehensive monitoring program helps to ensure successful application of EISB. As part of an EISB design, a monitoring plan must be developed that includes the types of process and performance monitoring that will be performed. Process monitoring includes those measurements necessary to evaluate amendment distribution and to confirm that the remedy is applied according to design. Performance monitoring is conducted after the amendments are added to gauge the progress of the remedy toward achieving RGs and to determine if additional application of amendments or a transition to an alternative technology would be beneficial. The process and performance monitoring measurements shown in Table 3 should be considered and incorporated as applicable into the monitoring plan.

Table 3. Recommended Process and Performance Monitoring for EISB Application

Measurement	Process	Performance	Common Evaluation Purposes
Pressures, Volumes, & Flowrates	X		Amendment dosage, formation of fractures, fouling
COC Concentrations		X	Treatment progress, rebound
Soil Gas Vapors	X	X	Biodegradation and vapor intrusion
Groundwater Levels	X	X	Distribution of amendments, preferential pathways, fouling, radius of influence
Groundwater Quality and Alkalinity		X	Amendment distribution, suitability of aquifer for survival and proliferation of microorganisms (e.g., suitable pH and buffering)
Total Organic Carbon	X	X	Electron donor distribution and supply
Visual Observations	X		Amendment distribution (e.g., presence in wells, gas bubbles)
Dissolved Hydrocarbon Gases		X	Degradation progress, high methane can be hazardous to bacteria and present health and safety issues.
Dissolved Metals		X	Evaluate redox conditions, metals mobilization
Bacteria & Gene Counts		X	Assess quantities of microorganisms and/or specific degradative genes

Common questions related to EISB monitoring practices are noted in the subsections below.

5.1 What Period of Long-Term Monitoring is Adequate?

ESTCP Project ER-201210 (ESTCP, 2017b) evaluated the performance of in situ remediation technologies including EISB. The authors evaluated data from 117 sites to ascertain an appropriate monitoring period after applying the remedy. The results of this investigation indicated that there is little change in concentrations of COCs beyond the first three years of long-term monitoring, and therefore three years of monitoring data likely are sufficient to demonstrate the efficacy of the

remedy. The data imply that it should be possible to make site decisions (e.g., closure, additional injection events, transition to an alternative technology) based on the first three years of monitoring data. However, it is important to note that the initial post-application sampling event should be performed within a year of completing amendment application for comparison to data collected in subsequent years.

5.2 What About Monitoring for Rebound?

The potential for rebound of COCs at sites where EISB has been performed is a common concern. Another objective of ESTCP ER-201120 (ESTCP, 2016a) was to evaluate the potential for rebound. Figure 3 shows results from 37 EISB sites for which 3 to 12 years of monitoring data were available. The first line of each pair of lines in Figure 3 represents the order of magnitude (OoM) reduction measured one year after biostimulation and/or bioaugmentation. The second line is the percent reduction based on the last monitoring event. The OoM reduction at 14 sites improved over the long-term monitoring period, while eight sites remained the same. Twelve sites had increasing concentrations (indicative of rebound) during the post-remediation period. At all but two sites, the final concentration was less than the pretreatment concentration even if rebound did occur. Results suggest that 65% of the sites may have exhibited sustained treatment, while the remaining 35% of the bioremediation sites exhibited rebound. At sites where rebound occurred, the median concentration was reduced from 90% to 67%.

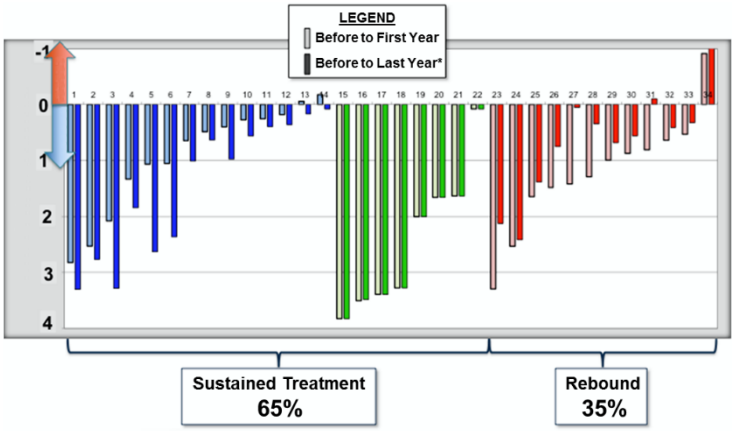


Figure 3. Change in COC Concentrations after Treatment (Courtesy of ESTCP, 2017b)

5.3 Are Secondary Groundwater Impacts a Concern?

SERDP Project ER-2341 (SERDP, 2016c) evaluated the potential for secondary water quality impacts (SWQIs) resulting from EISB, which can include changes to oxygen, nitrate, sulfate, sulfide, manganese, dissolved iron, arsenic, pH, methane, and total organic carbon. The objective was to develop an improved understanding of the near- and long-term impacts to groundwater quality after implementation of in situ anaerobic bioremediation processes. The study concluded that SWQIs attenuate rapidly immediately outside of the injection area and that they are not likely to have an adverse impact on drinking water wells. SWQIs that were considered great enough to be "significant" were primarily located within 10 m of the treatment area, the greatest impacts being within the treatment area itself. Immediately outside of the treatment area, the greatest impacts were due to manganese, although changes in sulfide, dissolved iron, arsenic, and total organic carbon were noted. Some lesser magnitude impacts were also noted greater than 10 m downgradient of the injection area. Furthermore, over 90% of impacts coincided with elevated levels of COCs so even if SWQIs were to impact a groundwater well, it is likely that there would also be additional impacts due to the COCs.

5.4 To What Extent Should Molecular Biological Tools and Other Advanced Techniques be Incorporated into the Monitoring Program?

Molecular biological tools (MBTs) and other advanced monitoring techniques are powerful tools to aid the practitioner to better understand the biodegradation processes occurring at a site. MBTs can measure how the EISB remedy impacts these biodegradation processes, as well as help to identify optimization opportunities to achieve RGs and lower life-cycle costs. Table 4 summarizes a variety of MBTs that are available and some of the questions that each tool can help answer. It is important to note that these tools are meant to complement, not replace, traditional data collection methods.

Table 4. Advanced Monitoring Tools to Assess EISB Performance

Tool	Overview	Example Questions Answered
Compound-Specific Isotope Analysis (CSIA)	Analyze relative abundance of isotopes (^{13}C & ^{12}C)	Is biodegradation occurring?
Quantitative Polymerase Chain Reaction (qPCR)	Quantification of target genes	Are the necessary organisms present in sufficient quantity? What impact does amendment addition have on the community?
Fluorescent In Situ Hybridization (FISH)	A fluorescent dye is appended to a gene of interest. Fluorescent light emitted is then used to determine the gene's abundance	What other microorganisms are present in the environment, and what impact do they have on the microorganisms or processes of interest? Does the microbial community change in response to an amendment?
Microarrays	Evaluate community composition based on the presence of 16S rRNA genes	How diverse is a community and what functional genes are present? What competing organisms are present?
Stable-Isotope Probing (SIP)	Placement of isotopically modified (^{13}C & ^{15}N) contaminants in aquifer, followed by subsequent analysis of byproducts	Is biodegradation occurring? Can biodegradation occur under modified conditions? Are organisms present capable of degrading the contaminant?
Enzyme Activity Probes	Uses surrogate compounds that are transformed by target enzymes into distinct and readily detectable products	Which known organisms are present and active? What is the rate of containment degradation?
Metagenomics	Provides information on the genomes present in a soil or groundwater sample	How diverse is a community and what microorganisms are present?
Proteomics	Analysis of the proteins (enzymes) produced by a microbial community	Is a specific organism actively degrading the COC?

Recent advances have extended the ability to rapidly perform large-scale genome sequencing referred to as metagenomics. Metagenomics provides information on the microbial community composition based on the gene sequences present in a given sample (Figure 4). Integration of the analyses of proteins via metaproteomics or whole community proteomics provides a snapshot of community metabolic activity at the time of sampling. While metagenomic sequencing can define the microbial and/or gene composition, it does not reveal details on actual microbial activity (i.e., active bioremediation processes). Metaproteomics provides the most direct measure of microbial activity. It allows detection of proteins of interest, providing direct evidence on active bioremediation. These two techniques represent the cutting-edge of experimental genome science and, with further developments, have the potential to determine in situ biodegradation rates and provide additional lines of evidence for natural attenuation, especially when geochemical data are mixed or varied. ESTCP research is currently being conducted to validate metagenomic and metaproteomic methods for enhanced performance monitoring of bioremediation and MNA sites (ER-201588 and ER-201726) (ESTCP, 2015b; ESTCP, 2017a).

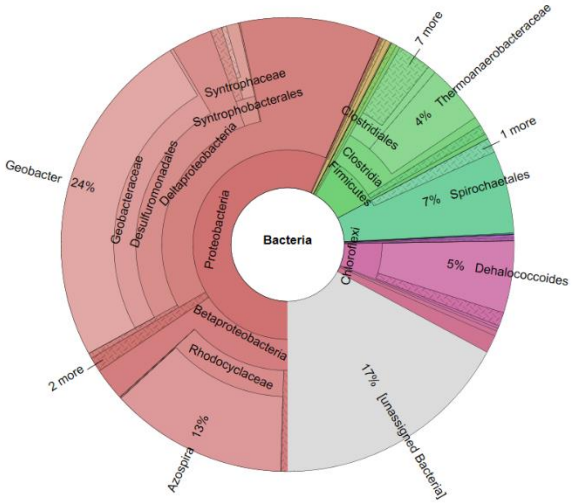


Figure 4. Metagenomic Characterization of Microbial Community Composition (Courtesy of Battelle)

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