Technical, Economical and Social Aspects of Moving Treatability Studies for In Situ Bioremediation of Contaminated Aquifers from the Laboratory to the Field

by

Tomasz Kalinowski

A Dissertation Presented in Partial Fulfillment
of the Requirements for the Degree
Doctor of Philosophy

Approved April 2013 by the
Graduate Supervisory Committee:

Rolf Halden, Chair
Rosa Krajmalnik-Brown
Paul Johnson
Ira Bennett

ARIZONA STATE UNIVERSITY

May 2013

ABSTRACT

This dissertation explores the use of bench-scale batch microcosms in remedial design of contaminated aquifers, presents an alternative methodology for conducting such treatability studies, and – from technical, economical, and social perspectives – examines real-world application of this new technology.

In situ bioremediation (ISB) is an effective remedial approach for many contaminated groundwater sites. However, site-specific variability necessitates the performance of small-scale treatability studies prior to full-scale implementation. The most common methodology is the batch microcosm, whose potential limitations and suitable technical alternatives are explored in this thesis. In a critical literature review, I discuss how continuous-flow conditions stimulate microbial attachment and biofilm formation, and identify unique microbiological phenomena largely absent in batch bottles, yet potentially relevant to contaminant fate. Following up on this theoretical evaluation, I experimentally produce pyrosequencing data and perform beta diversity analysis to demonstrate that batch and continuous-flow (column) microcosms foster distinctly different microbial communities.

Next, I introduce the *In Situ* Microcosm Array (ISMA), which took approximately two years to design, develop, build and iteratively improve. The ISMA can be deployed down-hole in groundwater monitoring wells of contaminated aquifers for the purpose of autonomously conducting multiple parallel continuous-flow treatability experiments. The ISMA stores all sample generated in the course of each

experiment, thereby preventing the release of chemicals into the environment. Detailed results are presented from an ISMA demonstration evaluating ISB for the treatment of hexavalent chromium and trichloroethene. In a technical and economical comparison to batch microcosms, I demonstrate the ISMA is both effective in informing remedial design decisions and cost-competitive.

Finally, I report on a participatory technology assessment (pTA) workshop attended by diverse stakeholders of the Phoenix 52nd Street Superfund Site evaluating the ISMA's ability for addressing a real-world problem. In addition to receiving valuable feedback on perceived ISMA limitations, I conclude from the workshop that pTA can facilitate mutual learning even among entrenched stakeholders.

In summary, my doctoral research (i) pinpointed limitations of current remedial design approaches, (ii) produced a novel alternative approach, and (iii) demonstrated the technical, economical and social value of this novel remedial design tool, i.e., the *In Situ* Microcosm Array technology.

ACKNOWLEDGMENTS

I would first and foremost like to thank my advisor, Rolf U. Halden, for his continual support throughout my doctoral studies. By both setting an example and providing candid feedback he has helped me become a better student, scholar, researcher, scientist, engineer, writer, and communicator. He gave me the autonomy, respect and trust to solve problems independently, but was always available to provide guidance when I needed it. I also must acknowledge his ability to create a pleasant, supportive and productive work environment, through recruiting people who are both capable and friendly into the group. I feel very fortunate to have had the opportunity to work under his guidance and leadership.

I also want to thank my committee members Paul C. Johnson, Rosa Krajmalnik-Brown (Dr. Rosy), and Ira Bennett for their invaluable suggestions during our semesterly meetings and elsewhere. I would like to thank Dr. Rosy for technical assistance with DehaloR^2 and all molecular techniques. I would like to thank Dr. Bennett for providing timely and appropriate advice in matters both professional and personal. I would in particular like to thank him for creating the wonderful experience I had during a two week workshop on science policy in Washington DC, as well as convincing me to take the Science, Technology and Public Affairs class co-taught by Dr.'s Sarewitz and Crow: both experiences have expanded my horizons and changed my outlook on the role of science in society.

I would like to thank my fellow ISMA creator, graduate student, co-worker and friend, Kristin McClellan for helping make the ISMA a reality, and making it fun in

the process. She has been a role model with her work ethic and consistent performance, which have been both inspiring and motivational. She has been an absolute pleasure to work along side.

I want to thank my fellow lab mates for their friendship and camaraderie, Isaac B. Roll, Benny Pycke, Michal Ziv-El, Tom A. Bruton, Hansa Done, Erica Hartmann, and Sam Supowit. I want to thank Isaac for generously helping with the ISMA deployments, including being willing to drive with us to San Diego (twice!). I am grateful for all the help Michal provided in training me on the chromatography instruments and in anaerobic techniques. I would like to thank Hansa for many thoughtful conversations (and the occasional baked good).

Next I would like to thank Maria Hanlin and Joann Williams for making the Biological Design PhD program what it is today. They were always available to give advice and provide feedback on various matters, and for that I am grateful.

I would like to thank Benjamin Duong, an undergraduate who I had the opportunity to mentor. His assistance in the lab was instrumental to the success of chapters 2 and 4 of this dissertation. I would also like to thank Diane Hagner for keeping the lab organized and well equipped, as well as helping me navigate the bureaucracy at ASU.

Finally, I would like to thank my wife, Sarah Fallon, who has been my greatest, most patient and most loving supporter throughout my graduate studies.

TABLE OF CONTENTS

CHAPTER Page

LIST OF TABLES

LIST OF FIGURES

Figure Page

Chapter 1

CRITICAL REVIEW ON THE NEED FOR REPLICATING NATURAL SUBSURFACE BIOFILMS IN GROUNDWATER MICROCOSM TREATABILITY STUDIES

Abstract

Bench scale microcosm studies are routinely performed to assess the potential for *in situ* bioremediation in saturated subsurface environments containing toxic chemical contaminants. These site-specific feasibility studies are performed because the functionality of microbial communities varies between different subsurface environments and is therefore difficult to predict *a priori*. Current methodological approaches for treatability studies include batch bottle microcosms and more sophisticated flow-through column studies. This review summarizes the state of knowledge about the salient differences between these two approaches from the perspective of microbial lifestyle. Unlike batch microcosms, continuous-flow columns allow for the development of 'steady-state' surface-associated biofilms, which are known to exhibit unique phenotypes and functionalities. Some biofilm features of potential importance to contaminant fate in the subsurface include: extracellular polymeric substances (EPS); metabolic, chemical, and physical heterogeneity within complex biofilm structures; and quorum sensing and other cell-to-cell interactions, all of which result in unique selective pressures that impact microbial community structure in unpredictable ways. Whereas the batch bottle approach is inexpensive and convenient, it is poorly suited to replicate the sessile lifestyle associated with a microbial biofilm, which is the prevalent phenotype of microbial communities in

saturated subsurface environments. We conclude that in order to increase the informational and predictive value of *in situ* bioremediation feasibility studies, it is essential to use an experimental approach reflecting the realities prevailing in natural subsurface environments, i.e., saturated subsurface material subjected to continuous flow conditions, a setting favoring the sessile microbial lifestyle in biofilms.

Introduction

In 2004, the U.S. EPA estimated there were 294,000 hazardous waste sites in the United States, with a total estimated cleanup cost of $209 billion (EPA 2004b). The same report identified a trend of increasing, but still limited, usage of *in situ* bioremediation to treat contaminated sediment and groundwater. The EPA survey found that bioremediation comprised 6% of implemented source control technologies at Superfund sites and 20% at Department of Defense National Priority List sites. *In situ* bioremediation (ISB) technology can be sustainable (Pandey, Chauhan, and Jain 2009), cost-effective (Kato and Davis 1996), and efficacious (Major et al. 2002), but also inconsistent in its ease of implementation, performance between and across sites, and secondary water quality impacts (Stroo, Major, and Gossett 2010; NSF 2005).

Need for Treatability Studies

Bench scale treatability studies are conducted to assess the potential of an *in situ* remediation technology at a given site (Morse et al. 1998). In some specific scenarios (e.g., Superfund sites), regulations mandate treatability studies prior to

implementation of ISB (EPA 2002), but even in cases where there is no regulatory requirement, they are still often performed for the valuable information they provide (ITRC 1998). Treatability studies are meant to mitigate some of the risks associated with ISB, including (i) outright failure of the remediation technology, sometimes attributed *ex post facto* to uncharacterized "inhibitors," (ii) suboptimal performance of the chosen bioremediation technology, as can happen with improper dosing or field application (iii) unnecessarily extended timeframes resulting from the missed opportunity of choosing the best available remediation strategy and (iv) negative water quality impacts resulting from unanticipated outcomes, like the mobilization of metals or the production of toxic metabolic byproducts. A treatability study yields data that can potentially enable good decision-making. However, the quality and utility of the data yielded by a treatability study is greatly dependent on the design and implementation of the study.

Treatability Study Methods

There are two common but distinct approaches of conducting microcosm treatability studies: batch bottles and sediment columns (ITRC 1998). In either case, fresh site groundwater and sediment is collected and transported to the laboratory. Care is taken to prevent exposure to oxygen in an effort to preserve the native microbial community (including any obligate anaerobes that may be present).

Batch Bottle Studies

Batch bottle microcosms are an industry-wide standard and the most common method for conducting bench-scale treatability studies to evaluate ISB (ITRC 1998).

The Environmental Protection Agency (EPA), the Environmental Security Technology Certification Program (ESTCP) and the Air Force Center for Environmental (AFCEE) recommend constructing batch microcosms in serum bottles loaded with freshly collected site materials, amended with proposed *in situ* bioremediation agents (unless the microcosm is for assessing natural attenuation), and capped with Teflon-lined butyl rubber stoppers (Wiedemeier et al. 1998; Morse et al. 1998; Air Force Research Laboratory 1998). Bottles are to be incubated for a period of time ranging from one week to over 6 months (in some cases, much more). During that time the bottles are periodically sampled and analyzed, sometimes sacrificially, for concentrations of contaminants and other parameters of interest (Findlay and Fogel 2000; Morse et al. 1998; Wiedemeier et al. 1998). Batch bottle studies have been instrumental to the prominent rise of anaerobic bioremediation as a viable technology for treatment of highly oxidized recalcitrant compounds, most notably trichloroethylene (TCE) (Maymo-Gatell 1997), polychlorinated biphenyls (PCBs) (Quensen, Tiedje, and Boyd 1988), and oxidized metals like Uranium (Lovley et al. 1991) and Chromium (Wang et al. 1989). The successes of the past three decades have established batch bottles as effective tools suitable for tasks ranging from routine, site-specific treatability studies to laboratory studies seeking to generate more fundamental, generalizable knowledge related to environmental microbiology. Nonetheless, institutions charged with site management and cleanup recommend that microcosm studies be used primarily to qualitatively assess bioremediation potential, implicitly (Morse et al. 1998) or explicitly (Wiedemeier et al. 1998) acknowledging that these microcosms studies are generally poor at replicating some field conditions relevant to microbial communities.

Limitations of Batch Bottle Studies

Batch reactors are closed systems with, for the most part, no exchange of materials allowing for the removal of waste products or introduction of fresh nutrients. This greatly simplifies the construction and operation of microcosms, but precludes the ability to create within them a steady-state as might be found in the subsurface where groundwater flows continuously past a fixed segment of sediment. Some researchers recognize this limitation and attempt to compensate for it by creating a 'pseduo steady-state' either by periodically replacing a fraction (e.g. 1/5) of the liquid (Ziv-El et al. 2012), or including a continuous-delivery source within the microcosm (Brennan and Sanford 2002). Batch culture however, pseudo steady-state or not, results in a profoundly different life-style for the bacteria within. Inside both columns and batch microcosms, a portion of the community will associate with surfaces and possibly form biofilms. Competing rates of bacterial surface attachment and detachment rates create a subpopulation of active bacteria that are attached at any one time (Characklis 1981). Within a continuous-flow system however, the unattached population is flushed out with the effluent, resulting in a selective pressure that selects for only surface-attached microbes. This impacts the composition of the community (due to differing attachment rates between species) (Komlos et al. 2005), which could consequently impact functionality.

Furthermore, continuous-flow conditions have been shown to actually stimulate biofilm growth by decreasing mass transfer limitations (Characklis 1981; Paul et al. 2012; Möhle et al. 2007; Korber et al. 1989; Herbert-Guillou 2001; Duddridge, Kent,

and Laws 1982; Melo and Vieira 1999; Vieira and Melo 1999). From a microbe's perspective, comparatively nutrient rich water continuously flowing over a surface creates an incentive for the microbe to attach to that surface, rather than travel with a flowing parcel of water in which nutrients would become depleted and wastes would accumulate to the detriment of the microbe trapped in these conditions. Batch bottles, with or without sediment, create the opposite incentive for microbes. A microbe attached to a surface within a batch bottle will, over the course of the experiment, experience lower nutrient concentrations than its planktonic counterparts due to diffusion being limited by the biofilm and the attachment surface (Trulear and Characklis 1982). This means that within a batch bottle, microbes will favor a planktonic existence (or, perhaps more accurately, planktonic microbes will experience more favorable conditions and outcompete their sessile counterparts), while in a subsurface environment with continuous groundwater flow, they will favor an attached existence. This tendency is supported by direct observation, where *P. aeruginosa* have been observed to form biofilms on the walls of a chemostat, but not in batch culture flasks (Davies and Marques 2009).

The claim that batch bottles do not foster surface associated biofilms is further supported by anecdotal observations made in our lab while studying a mutualistic consortia, DehaloR^2 (Ziv-El et al. 2011). DehaloR^2 is a dechlorinationg consortium in which *Dehalococcoides spp.* benefits from close proximity to homoacetogens that produces a substrate and essential metabolic cofactors (e.g., one bug's waste is another's food). When cultivated in batch microcosms the cells are sometimes observed to aggregate in free-floating flocs, but not on the interior surface

of the bottle. Since the consortia is comprised of over 100 environmental species, including *Pseudomonads,* it is unlikely the biofilm formers are not present. If biofilm growth was not imposing a fitness cost due to limiting diffusion, then one would expect that cells would equally distribute themselves between biofilms on the batch bottle surfaces and suspended flocks.

Sediment Column Studies

ITRC and others acknowledge that column studies more accurately simulate the subsurface environment than batch bottles, but that column studies are less frequently performed because of the additional time, money, and effort they require (ITRC 1998; McClellan 2012c). AFCEE further identified some other limitations of column studies (Air Force Research Laboratory 1998). Column studies tend to overestimate the oxygen demand *in situ* due to the perturbation of aquifer materials during column construction, and column studies can also have a high solids to water ratio, potentially requiring a long time before sorption of contaminants to sediment reaches equilibrium.

Due to their increased complexity, duration and labor needs, column treat ability studies are infrequently used. Consequently, at present, a standard configuration for column treatability studies comparable to that for 160 mL serum batch bottle microcosms does not exist. The consequent variability in column studies, both between experiments and labs, not only hinders comparisons and reproducibility, but also increases the labor needs of researchers, who must decide on additional experimental configuration details. Prior to beginning a column study, one must

determine: i) whether groundwater is to be supplied at constant head or a constant flow rate, and at what rate, ii) the dimensions of the column (often requiring custom manufacturing), iii) the design of, and arrangement of sampling ports, iv) how sediment is to be collected and processed and prior to column construction, v) how effluent and influent groundwater is to be stored and supplied to the columns, vi) selection of materials for column and tubing, vii) how amendments are to be supplied to the column, and viii) possibly develop new analytical chemistry methods to enable the sampling of the columns as effectively as batch bottles allow (Ziv-El et al. 2013).

Biofilms: State of the Field

One of the primary differences between batch and column studies is the presence of continuous-flow conditions, and consequently, the preponderance of biofilm growth in columns but not bottles. As such, it is appropriate to identify some of the differences between biofilm and planktonic cultures that may impact contaminant fate. There are a number of challenges with this task however.

While there is a robust body of research on biofilm functionality in applications of environmental biotechnology, there has been relatively little research focused on the specific differences between environmental samples cultivated in biofilm and planktonic cultures. Instead, much of the knowledge about these differences originates from studies of basic and medical microbiology, many of which stress the clinical relevance of the findings. Koenigsberg *et al.* recently identified a "trickle-down" effect of knowledge percolating from medical research to the study of

environmental communities (Koenigsberg, Hazen, and Peacock 2005). This is most apparent with "-omics" and molecular-based methodologies from human medical research being adopted for the study of environmental microbial communities. While methodologies seem to readily cross this disciplinary boundary, basic knowledge of the unique features of biofilm physiology has been slower to do so, as evidenced by the environmental restorations field's continued use of batch bottles to simulate subsurface environments with continuous flow. This is perhaps because, at first glance, much of the knowledge appears to have no immediate relevance to environmental biotechnology.

From a microbe's perspective, a diverse set of environmental changes accompanies surface attachment (Davey and O'Toole G 2000). High population densities and extremely close proximity to neighbors dramatically intensifies both competition and cooperation, while the ability to modify the local environment through the production and excretion of organic compounds augments the arsenal of mechanisms for adapting to the environment. Combined, these changes radically impact the dynamics of life for a microbe in a biofilm. In light of the fact that microbes have lived in biofilms for at least 3.4 billion years (Allwood et al. 2006), and that the majority of microbial life observed in the environment resides in biofilms (Costerton, Geesey, and Cheng 1978), it is not surprising that microbes have evolved a complex and diverse set of phenotypes and behaviors that enhance their competiveness in a biofilm community.

Most of the studies shedding light on complex biofilm phenomena originate from single species biofilms cultivated under controlled conditions in the laboratory. However, most environmental biofilms are composed of rich communities, with single species biofilms rarely observed in natural environments. Futhermore, the model organisms often used in many of these studies are primarily of clinical, but not necessarily environmental, importance. These aspects limit, but do not eliminate, the relevance of these studies to groundwater remediation. This literature review examines some of the salient known differences between sessile (biofilm-enclosed) and planktonic bacterial growth, and highlights their relevance to the practice of using batch bottle microcosms in treatability studies.

Differing gene and protein expression profiles

Important evidence indicating the physiological differences between biofilm and planktonic bacteria has come from analysis of transcriptome and proteome studies of biofilms. It is first worth noting that many of these studies (Schembri, Kjaergaard, and Klemm 2003; Whiteley et al. 2001; O'Toole and Kolter 1998; Sauer et al. 2002; Svensäter et al. 2001) come with a caveat: cells from biofilms are often collected, pelleted, and analyzed *en masse*, and as a result the significant heterogeneity that is observed within biofilms is averaged out. Nevertheless, the magnitude and variable nature of the differences observed is striking.

In study of *E. coli*, 581 of 4290 genes were observed to have ≥2.5 fold change in expression levels when cultivated in biofilm vs. stationary phase planktonic cultures (Schembri, Kjaergaard, and Klemm 2003). These differences were observed in

almost every functional category of genes, including those related to energy metabolism, transport proteins, protein biosynthesis including post-translational modifications, and a large category of genes whose function is still unknown. However, these drastic changes are not observed in all prokaryotes. *Pseudomonas aeruginosa*, a well-studied opportunistic pathogen, was found to only differentially express approximately 0.5% of expressed genes in biofilms, though the differences were important (Whiteley et al. 2001). Genes related to motility and attachment were downregulated in the biofilm cells (flagella and pili, while important for initial attachment (O'Toole and Kolter 1998) might not be necessary to maintain a mature biofilm), whereas genes related to metabolism, translation, membrane proteins, and secretion were largely upregulated. However, the relatively modest differences observed in gene expression contrasted with results from another group's analysis of the entire proteome which suggested as much as 50% of the proteome was differentially expressed during various stages of biofilm development (Sauer et al. 2002). Similarly large changes occur in translation and post-translational modifications between sessile and planktonic bacteria. In *Streptococcus mutans*, a major causative agent for dental carries, 135 of 694 analyzed proteins were observed to have a 1.3-fold or greater change in expression, with 22 of them not expressed in either sessile or planktonic communities (Svensäter et al. 2001). Many of the proteins with enhanced expression in biofilms were related to biosynthesis. However, it is worth noting that the function of many of the proteins identified is still unknown.

Though studies commonly examine genetic diversity in environmental biofilm samples, comprehensive transcriptomic and proteomic assays of environmental biofilms are not feasible at present because environmental biofilms are multi-species and largely lacking complimentary databases of complete genome sequence. However, differences of similar or larger magnitude are likely to be found in environmental biofilms.

Heterogeneity within single-species biofilms

Conditions within batch bottles are relatively homogenous, especially when bottles are agitated. In contrast, biofilms consist of a rich organic layer that limits diffusion of compounds into and out of them. Consequently, large concentration gradients of metabolic substrates and products can be observed across the depth of a biofilm. Oxygen is the most familiar example: its concentration profiles, often decreasing with depth in a biofilm, are routinely measured with microelectrodes or other methods (Revsbech 1989; von Ohle et al. 2010; Satoh et al. 2003). Chemical gradients will develop with any compound that is actively being consumed or produced by the biofilm population, or a subset of the biofilm population. Such gradients have been demonstrated with virtually every compound of significance to environmental biotechnology, including sulfate, nitrate, nitrite, hydrogen sulfide, methane, pH and various carbon sources (Damgaard, Nielsen, and Revsbech 2001; Ito et al. 2002; Kühl and Jørgensen 1992; Michael et al. 1998; Ramsing, Kühl, and Jørgensen 1993). With the exception of autotrophic activity, these concentration gradients generally accompany a redox gradient where progressively reducing conditions are observed with increasing biofilm depth.

Redox gradients are commonly present at a much larger scale in the subsurface during ISB of chlorinated compounds (Lovley 2003). In an aerobic contaminated environment, induction of anaerobic conditions is necessary before reductive dechlorination can proceed. However, it is important to realize that sufficiently reducing conditions can exist within a biofilm even when the bulk liquid cannot sustain reductive dechlorination. Environmental engineers already take advantage of this phenomenon by utilizing biofilms for simultaneous nitrification/denitrification (Helmer and Kunst 1998). In this manner, biofilm cultures can exhibit accelerated contaminant transformation. However, in comparison to a batch culture, a biofilm can also exhibit lower overall reaction kinetics due to limiting diffusion of substrate (contaminant or nutrient) into the biofilm. The overall impact of the biofilm matrix, whether it accelerates contaminant attenuation through the creation of additional reducing zones, or retards attenuation by limiting microbial access to substrate, is highly dependent on the biofilm composition and difficult to predict. This is one of the key reasons biofilms should be included in any laboratory treatability studies evaluating bioremediation.

Differentiation within biofilm communities

Partially as a result of the redox and chemical gradients within biofilms, subsets of single-species biofilms can undergo a process that is analogous to differentiation in multi-cellular organisms (Davey and O'Toole G 2000; Haagensen et al. 2007; O'Toole, Kaplan, and Kolter). Some of the functions acquired through this process of specialization are unique to biofilms, and not observed in the planktonic

counterparts (Hall-Stoodley, Costerton, and Stoodley 2004). Generally, these functions impart an enhanced ability to cope with environmental toxins and stress, though exceptions exist. For example, planktonic cells of *Staphylococcus aureus* can successfully detoxify and reduce oxyanions TeO_4^{2-}, TeO_3^{2-}, and SeO_3^{2-} to elemental Te^0 and Se^0, while some of their sessile counterparts are unable to carry out the reduction and as a result are more susceptible to these toxic metal species (Harrison et al. 2004).

Cell fate is not solely determined by substrate concentrations or redox conditions, and specialized subpopulations do not necessarily cluster together. Examples of a 'division of labor' between adjacent cells experiencing very similar conditions have been found in both pure cultures of *Bacillus subtilis* and *Pseudoalteromonas* spp. biofilms. Only a subset of the *B. subtilis* population participated in biofilm matrix production (Chai et al. 2008), while only a subset of the *Pseudoalteromonas* population expressed a chitinase gene when grown on a chitin-containing surface (Baty et al. 2000; Ace M Baty 2000). In both instances the non-participating cells were adjacent to the producing cells. It is hypothesized that stochastic determination of a bistable gene 'switch' may be responsible for differentiation (Chai et al. 2008; Elowitz et al. 2002; Maamar, Raj, and Dubnau 2007; Veening, Igoshin, et al. 2008).

Specialization, in some instances, is not readily reversible. Variant subpopulations detected through changes in colony morphology show that after only a few days, as much as 10% or more of the colonies from mature biofilm isolates differ from wildtype, with variants often producing wrinkly, small, or rough colonies (Boles and

Singh 2008; Kirisits et al.). These results have been reproduced in *P. aureginosa* (Kirisits et al. 2005b), *Streptococcus pneumonia* (Allegrucci and Sauer 2007), *Vibrio cholera* (Kirisits et al. 2005b), and many more (Proctor et al. 2006). In some instances (like *S. pneumonia*) the cause for the variant was mapped to a specific genetic change. Other studies have identified epigenetic regulation and stochastic processes as the basis for population diversity (Veening, Smits, and Kuipers 2008; Veening, Stewart, et al. 2008). It is unlikely that random genetic mutations alone sufficiently explain the high proportion of variants detected in biofilms (Stewart and Franklin 2008). Selective pressure within biofilms colonies promoting colony variants, and the nature of the advantages conferred by differentiation is still being researched.

Differentiation within single-species biofilms can often results in seemingly coordinated complex behaviors. Stalked fruiting bodies or similar structures have been observed in both gram-negative (Nierman et al. 2001; Reisner et al. 2003) and gram positive (Steven S Branda 2001; Goldman, Bhat, and Shimkets 2007) bacteria. A base layer of slower-growing, more toxin-resistant cells forms the 'stalk' in these bodies, atop which resides a mass of rapidly growing cells or sporulated cells forming the 'fruit'. Fruiting body development can culminate in a mass dispersion event where the 'fruit' detaches into the bulk liquid, leaving the 'stalk' remaining behind to potentially form another fruiting body. Concentration gradients, cell specialization, and fruiting body formation all contribute to physical heterogeneity in biofilms. Rather than growing as homogenous flat mats, biofilms tend to form lumpy uneven biofilms with a heterogeneous topology. The numerous pores and channels in

biofilms are hypothesized to lead to better overall mass transport within the biofilm (Flemming and Wingender 2010).

The complex structures surveyed here are meant to illustrate the many intricate processes ongoing within biofilms. In applications where biofilms are cultivated with high substrate concentrations (e.g., wastewater treatment), models that ignore these features are quite adept at predicting overall reaction kinetics. However, at low concentrations, cell differentiation and the resulting complex structures begin to play an increasingly larger role on biofilm structure, and models in these cases require further refinement and adaptation (Rittmann 2010).

In subsurface environments, the distribution of substrate can be very heterogeneous, and resulting biofilms will likewise vary in structure and functionality. Complex biofilm structures impact the biomass transport, groundwater flowpaths, and any associated permeability loss. Furthermore, at the micro-scale, biofilm detachment may not be a linear process, but rather characterized by dispersion events that are triggered when a 'threshold' condition is satisfied (discussed in more detail later, in *cell-to-cell signaling*). The exact impact of these complex structures and events will vary between different microbial communities and environmental conditions. In other words, at present, assessing the impact of cell differentiation and complex biofilms structure on contaminant fate in a specific subsurface environment is only possible with empirical data.

Extracellular polymeric substances (EPS)

Attached cells create a biofilm matrix by producing and excreting a variety of extracellular polymeric substances. The biofilm matrix participates in a wide variety of functions, including adhesion to surfaces and other cells, maintaining cohesion of the biofilm, retention of water, acting as a protective barrier against toxins and environmental stress, retention of organic and inorganic compounds through sorption, acting as an extracellular 'digestive system' though enzymatic activity, serving as a nutrient source, or as a sink for excess energy, enabling the exchange of genetic information between cells, and acting as an electron donor or acceptor (Flemming and Wingender 2010). EPS can make up as much as 90% dry weight of a mature biofilm. It can include fatty acids, lipids and glycolipids (also known as lipopolysacharides), fragments of DNA (extracellular DNA, or eDNA), peptides, proteins (enzymatic or otherwise), polysaccharides and other sugars, humic substances, toxins, cell-to-cell communication molecules, and other uncharacterized or poorly understood compounds (Flemming and Wingender 2010; Hall-Stoodley, Costerton, and Stoodley 2004).

Exopolysaccharides are a major fraction of most biofilms, and one of the most important components for producing and maintaining the biofilm's physical structure. They consist of long branched or linear sugar chains with numerous charged sites. The charged and partially charged sites allow the chains to complex with each other through a variety of weak physiochemical interactions, including van ders Walls interactions and hydrogen bonds, while repulsive forces prevent the chains from collapsing into tight balls (Wingender et al. 2001). The resulting

complex of long, interconnected chains full of charged sites not only is a major source of the cohesive strength of biofilms, but also acts as a scaffold that retains other biofilm components. The exact structure and production pattern of polysaccharides is highly variable between different conditions, species, strains of the same species, and even subpopulations of mono-culture biofilms (Vaningelgem et al. 2004). Accordingly, the shapes of the resulting scaffolds also vary from dense, random filamentous networks, to ordered honeycomb like structures (Schaudinn et al. 2007; Bontognali et al. 2008). Both polyanionic and polycationic (Götz 2002) exopolysaccharides have been observed in natural biofilms.

The charged nature of the EPS matrix has implications for the mobility of contaminants in the subsurface. Titration analysis of an EPS matrix found electrostatic charged sites ranging in pK_A values from 3 to 10, with 20 to 30-fold greater binding sites in the EPS component of the biofilm than present on cell membrane surfaces alone (Liu and Fang 2002). Of relevance to treatability studies, the composition and amount of EPS produced is strongly influenced by cell-to-cell interactions, often in unpredictable ways (Skillman, Sutherland, and Jones 2010). In a subsurface environment where the bulk of fauna has not been characterized (Rappé and Giovannoni 2003), site specific testing is necessary to accurately assess the role biosorbtion may play in biofilms formed by the native microbial community.

Another integral component of many biofilms is extracellular DNA. The amount of eDNA produced by different species varies greatly, in some instances making up a major structural component essential for biofilm formation and stability (as

measured by the ability of DNase to disrupt the biofilms) (Izano et al. 2008; Whitchurch 2002; Molin and Tolker-Nielsen 2003). While originally considered to originate solely from cell lysis due to a variety of reasons (Mann et al. 2009; Webb et al.), (including autolysis by cells hypothesized to be behaving altruistically (D'Argenio et al. 2002)), today we understand that it is in some instances it may also be actively produced and exported (Bockelmann et al. 2006). Recent advances in DNA origami illustrate the wide variety of structures DNA is capable of forming (Han et al. 2011; Ke et al. 2009; Rinker et al. 2008), and evidence suggest that microbial populations have exploited some of this potential. In some biofilms eDNA functions as an intracellular connector that aids in adhesion, forming random filamentous networks (Yang et al. 2007; Vilain et al.). It has also been observed to form highly ordered, grid-like structures (Bockelmann et al. 2006), or bundle into thicker 'ropes' spanning water channels (Jurcisek and Bakaletz 2007). A dense layer of eDNA can also act as a separator between the stalk and cap of a fruiting body (Allesen-Holm et al. 2006), with cells producing DNase to initiate dispersion. In addition to contribution to structural strength of biofilms, eDNA has other functions. It has exhibited the ability to aid in initial adhesion and surface aggregation (Das et al.), act as an antimicrobial, confer antibiotic resistance, and chelate Mg^{2+}, Ca^{2+}, and Mn^{2+} (Mulcahy, Charron-Mazenod, and Lewenza 2008). It can also serve as a mechanism for the exchange of genetic information (Molin and Tolker-Nielsen 2003; Dobrindt et al. 2004; Thomas and Nielsen 2005), including in the subsurface, where horizontal gene transfer has been found to promote resistance to radionucleides and metals (Martinez et al. 2006), as well as genes for dechlorination (Krajmalnik-Brown et al. 2007).

Surfactants and lipids comprise another major component of biofilms (Conrad et al. 2003). In some instances they enable attachment and subsequent biofilm formation on hydrophobic surfaces (Al-Tahhan et al. 2000; Neu et al. 1992). They also can have antibacterial and antifungal properties which are exploited by cells as a way to protect the biofilm community from invaders (Neu 1996; Ron and Rosenberg 2001). Similarly, fatty acids have been found to play an integral part in biofilm dispersion (Davies and Marques 2009) and the production of water channels and mushroom-like structures throughout a biofilm (Boles, Thoendel, and Singh 2005).

EPS composition is difficult to predict, but its properties can have a pronounced influence on contaminant fate and transport *in situ*. For instance, EPS can influence groundwater flow paths, and the sieve-like charged scaffold formed by exopolysaccharides and eDNA, biofilm dispersion events, and production of surfactants can either enhance or retard mobility of both hydrophobic and charged contaminants. Due to our still incomplete understanding of biofilms, it is difficult to accurately predict *a priori* the impact of a biofilm matrix on contaminant fate in any given environment without empirical data.

Cell-to-cell signaling

The major implication of cell-to-cell signaling for treatability studies is the fact is that it enables microbes within communities to coordinate activities, which in practice means functionality exhibited by the microbial population may not directly correlate to the population size, but rather may exhibit a step-like response with

functionality only exhibited once a minimum threshold of population density is reached. Because local population densities may be much higher within a biofilm than in a batch microcosm, there is a potential that microbial communities cultivated in batch microcosms may fail to perform some functions they otherwise might fulfill in a biofilm in the subsurface environment.

Many of the complex behaviors performed by sessile populations are hypothesized or known to be coordinated through extracellular signaling events. Signaling systems are defined as systems that have no known function or purpose other than to communicate information about the local environment to other organisms. They are a subset of the broader category of environmental cues, like pH, O_2 concentrations or metabolic byproducts, which are also used to regulate gene expression, but that might be incidental to other metabolic processes. The bulk of known signaling mechanisms fall into a category of regulation known as *quorum sensing* (QS), where cells constitutively express a low molecular weight diffusible compound, and then infer local population densities from the concentration of the that compound. QS has been observed to control a wide variety of activities, including virulence factor production (Latifi et al. 1995; Fouhy et al. 2007), biofilm development (Patriquin et al. 2008; Rice et al. 2005; Shrout et al. 2006), swarming motility (Patriquin et al. 2008; Shrout et al. 2006), biofilm dispersion (Davies et al. 1998; Davies and Marques 2009), expression of genes important for antibiotic or toxin resistance (Williams and Cámara 2009), and even fluorescence (Engebrecht, Nealson, and Silverman 1983). QS systems, in general, regulate genes and behaviors that are beneficial for bacteria

to express and perform in concert as a large group, rather than as autonomous individuals.

A diverse range of QS molecules are known (Bassler and Losick 2006). While some are species specific, many are universally conserved regulatory systems found in distant domains and lineages (Wang et al. 2003; Jintae Lee 2007; Ryan and Dow 2008; Shank et al. 2011), suggesting the long evolutionary history of these communication pathways. To illustrate the available complexity, we will profile one of the known major classes of QS molecules: *N*-acylated homoserine lactones (AHLs). All AHLs are composed of a conserved homoserine lactone, but can incorporate a variable length fatty acid side chain ranging from 4 to 18 carbon atoms as well as various side chain substitutions, resulting in several hundred different variants of AHLs. The variable side chain substitutions and variable fatty acid chain length confers specificity to the information communicated, and are utilized differently by different organisms. AHLs can have wildly different physical properties: molecular weight varies from 171.9 to 638.8 g/mol, K_{OC}:1.0 to 66,600, solubility: 3. 84 to 2.4 x 10^{-6} mol/L. AHLs also have wildly variable attenuation rates within biofilms, based on different rates of abiotic hydrolysis, enzymatic degradation, and oxidation. Consequently, based on the ratio of diffusion rates to hydrolysis half-life, AHLs have different distances over which they can be effectively used to communicate, ranging from less than 10 um to over 100 um (Decho, Frey, and Ferry 2011; Galloway et al. 2011). It is important to note that AHL variability is not only observed across species; for example, *Yersinia pseudotuberculosis* has been found to express up 24 different AHLs based on environmental conditions, though rarely more than 2 or 3

under any single set of environmental conditions (Ortori et al. 2006). Adding to this complexity is the fact that some organisms are known to produce compounds that can inhibit QS systems. For example, some species can produce brominated structural analogues that inhibit QS regulation (Manefield et al.). Alternatively, AHLs can also be degraded to tetramic acids, which can chelate and make bioavailable Fe, or exhibit antibacterial activity against other bacteria (Kaufmann 2005).

AHLs are only one class of QS molecules. Other major conserved QS systems with similar versatility and complexity include, furanosyl diesters (also known as autoinducer-2) (Galloway et al. 2011), multiple classes of autoinducer oligopeptides (Sturme et al. 2002; Schauder 2001), fatty acid messengers (Davies and Marques 2009), quinolone signals (Dubern and Diggle 2008; Diggle et al. 2007), and others (Llamas et al. 2008). These likely are only a small subset of the communication molecules used by bacteria in the environment.

QS systems make communities exhibit non-linear dynamics that are difficult to predict or model. Our understanding of communication pathways is far from complete and, at present, the only way to determine the full influence of cell-to-cell signaling on contaminant fate is to accurately reproduce biofilm conditions in the lab.

Multi-species biofilms

Community structure is intimately linked to the functions performed by that community. Community function, in turn, is influenced by environmental conditions. Environmental stresses experienced by bacteria in batch microcosms will be different from those experienced in continuous-flow sediment columns. Consequently, it is expected that different communities will develop within the two different microcosms types, even when controlling for different parameters. The difference in community structure, even when not directly impacting contaminant transformation rates, may still impact functions of importance to engineers, including robustness to stress or high toxin concentrations, functional stability during perturbations, and the development of new genetic capabilities.

One of the primary differences between biofilm and planktonic cultures is cell-to-cell physical proximity. Planktonic cells are in constant relative motion to each other due to Brownian motion or active motility. Biofilm cells, on the other hand, are much more limited in their mobility (Stoodley et al. 2002). The persistent proximity between cells in biofilms enhances a variety of cell-to-cell interactions, including exchange of genetic information, toxin warfare, and the development of more efficient cooperative consortia (Hansen et al. 2007). The latter is especially important for enhancing mutualistic relationships between cells. Most microorganisms have evolved to be part of microbial consortia. This is not only evidenced by the complex and various genes networks whose primary or only known function is to interact with other cells, but also that some organisms are completely dependent on others to provide essential metabolic cofactors. *Dehalococcoides*

mccartyi, one of the most important and best studied organisms for remediation, provides a striking example. Based on our current understanding, it is unable to use any carbon source other than acetate, unable to use any electron donor other than H_2, and unable to synthesize at least one essential metabolic co-factor, vitamin B_{12} (Löffler et al.). In other words, in the natural environment, it can only coexist with homoacetogens or possibly methanogens that provide these services for it (Ziv-El et al. 2012; Richardson et al. 2002; Rowe et al. 2008). It is still unclear how prevalent such obligate mutualists are, but the fact that researchers are only able to cultivate a small fraction of the total microbial fauna detected in the environment (Rappé and Giovannoni 2003), suggests they are very prevalent.

In addition to (and partially the result of) close cell-to-cell proximity, biofilm cultures undergo different stages of bacterial community succession and development from batch bottles. One of the key determinants of succession in biofilm maturation is related to the ability of a microorganism to attach. Early "pioneer colonizers" are able to initially attach to a surface and produce EPS, which allows for 2nd stage colonizers to attach, who then provide attachment sites for a 3rd group of microorganisms to attach, with each stage clearly distinct (Martiny et al. 2003). What follows has been described as a "ordered and sequential" colonization by different species as the biofilm matures (Martiny et al. 2003). This attachment-mediated dynamic is absent in planktonic cultures. In contrast, the primary determinant of microbial succession in batch cultures is related to water chemistry parameters: redox, pH, available e- acceptors and donors, etc. (Finstein et al. 1980). These mechanisms still apply to biofilms, but the stages are not delineated solely

temporally. Redox and chemical gradients form in biofilms, allowing different "succession stages" to coexist at different biofilm depths. This likely has an impact on overall efficiency, due to the greater amount of time that any given subset of the community has to adapt to a given set of conditions in a biofilm.

Concluding remarks

Laboratory microcosms exist on a continuum of 'realism,' with the most realistic most accurately recreating the most field phenomena. For *in situ* bioremediation, on the least realistic end of the continuum we might find a small batch bottle with a single carbon and electron source, a single electron acceptor, inoculated with a single species. On the opposite end of the continuum we might find a column constructed from a solid intact sediment core and fed real groundwater under constant head. It is important to recognize that microcosm studies seeking to generate fundamental, generalizable knowledge, and those seeking to predict contaminant fate at a specific site, must necessarily exist on opposite ends of this continuum. With one you are trying to isolate a single variable whereas in the other you are trying to capture all of the complexity. Batch bottle studies have been and will continue to be instrumental for fundamental research; they enable researchers, with relatively less effort, to create a controlled environment in which to isolate and characterize the specific microbe or process of interest. However, there is a wide chasm between microcosms constructed to study isolated phenomena, and the subsurface where those phenomena take place.

Humans have been managing microbial communities to perform beneficial functions since before recorded history. But, despite the advanced age of what we now call environmental biotechnology, the field and practice remain largely empirical, rather than theoretical. Foundational empirical equations like the Monod equation, while accurately predicting the aggregate behavior of microorganisms, often fail to account for frequently observed systems with oscillating (Schmid 2002), bi-stable, or otherwise non-linear dynamics (May 1975). Despite the increasingly more complex equations being continuously constructed to model microbial systems (de Silva and Rittmann 2000), the practice of environmental engineering continues to contain a "craft" component as a result of the as-yet not understood complexity (Curtis, Head, and Graham 2003). These statements are especially true for *in situ* bioremediation, which is a much younger field relative to environmental engineering.

Necessarily, treatability studies are subject to the law of diminishing marginal utility. On the continuum of complexity and cost there exists a point where a more thorough laboratory experiment will cost more than a less informed remedial decision. However, relatively little work has been done to identify this point, or to generally assess the overall predictive ability of these laboratory tests. The 'craft' component is clearly visible in the acceptance of batch microcosms as "good enough" despite the well-documented inaccuracy of their resulting predictions of subsurface phenomena. Instead of identifying and discarding the dated paradigm that planktonic and biofilm communities are equivalent (van Loosdrecht et al. 1990), practitioners have apparently resigned themselves to the fact that all lab microcosms are inherently poor at replicating subsurface microbial communities,

and henceward employed them primarily as qualitative screening tools, rather than for quantitative insights into contaminant transformation.

While the practice of bioremediation continues to retain a strong empirical component, the road to better performance will be through better data. As this review has hopefully conveyed clearly, our understanding of true subsurface complexity, particularly biofilm complexity, amounts to just the tip of the iceberg. Due to the complexity of microbial systems, and our consequent inability to accurately predict their activity, microcosm treatability studies will continue to be a necessary component of remedial design for the foreseeable future. However, to help ensure that microcosm studies produce reliable data, it is prudent for environmental engineers to embrace the true subsurface complexity when conducting site-specific treatability studies. For subsurface environments with continuous groundwater flow, the only microcosms that reproduce biofilms and their associated complexity are continuous flow sediment columns, and not batch bottles.

Chapter 2

IMPACT OF BATCH AND CONTINUOUS-FLOW CONDITIONS ON THE STRUCTURE OF ANAEROBIC MICROBIAL COMMUNITIES BIOTRANSFORMING CHLORINATED SOLVENTS

Abstract

Two commonly accepted methods for conducting treatability studies evaluating *in situ* anaerobic bioremediation are sediment-slurry batch bottle microcosms and continuous-flow sediment columns, with the former being simpler to conduct and the latter acknowledged as being a more realistic simulation of the subsurface environment. Lacking any prior rigorous scientific comparison of these two important bioremediation assessment tools, we examined the structure of an anaerobic bacterial community capable of chloroethene biotransformation within replicate batch and column microcosms using pyrosequencing and dimensionless, weighted UniFrac distance beta diversity analysis. Key observations concerning microbial community structure included: (*i*) in batch and column microcosms, good agreement among biological replicates (average distance ± standard deviation of 0.11±0.03 and 0.11± 0.05, respectively); (*ii*) in batch microcosms, a relatively homogenous distribution (0.13±0.04) between sediment, slurry, and liquid fractions; (*iii*) in column microcosms, large differences between liquid and solid fractions (0.54± ≤0.01); and (*iv*) large differences between batch and column microcosms (0.44±0.05). This first systematic, qualitative and quantitative analysis of bioremediation assessment tools supports the notion of batch and column microcosms both representing useful tools to study microbial ecology phenomena in a reproducible

fashion. Yet, this work also reveals a lack in predictive power of batch microcosms to inform microbial community structure in flow-through environments, specifically sediment column microcosms and, by extension, the saturated subsurface environments extant in nature.

Introduction

In situ bioremediation can be a sustainable (Pandey, Chauhan, and Jain 2009), cost-effective (Kato and Davis 1996), and efficacious (Major et al. 2002) technology for the treatment of legacy hazardous waste sites. However, its implementation is known to produce results that are inconsistent and highly variable between different sites, and sometimes include unforeseen or undesirable outcomes (Stroo 2010). Consequently, bench-scale treatability studies evaluating the suitability of *in situ* bioremediation approaches for a specific site of interest are considered necessary due diligence prior to field implementation of this cleanup strategy (ITRC 1998).

Batch bottle microcosms are an industry-wide standard for conducting bench-scale treatability studies (ITRC 1998). To evaluate *in situ* remediation technologies, serum bottles, most commonly ranging in size from 60-250 mL, are loaded with groundwater, sediment and amendments proposed for field application, are capped with rubber stoppers or Teflon-sealed valves and, following incubation for some time at an appropriate temperature, are then sampled and analyzed for physical, chemical and biological parameters of interest (Findlay and Fogel 2000; Morse et al. 1998; Wiedemeier et al. 1998). Results of these studies serve to identify and rank multiple potentially effective *in situ* technologies under consideration, and to predict

if their implementation at small or full scale may produce unintended secondary water quality impacts.

Whereas this approach is widely practiced and considered to be fairly robust (Morse et al. 1998; Wiedemeier et al. 1998; Findlay and Fogel 2000) among cleanup practitioners, researchers routinely acknowledge that batch bottle microcosms are an imperfect tool to understand, diagnose and forecast subsurface phenomena, particularly when involving microbiology (Madsen 1991). As closed systems, batch reactors promote the accumulation of (metabolic) waste products and preclude the continuous stream of nutrients toward immobilized biomass. This precludes the ability to create within them the localized, steady-state biogechemical environments characteristic of saturated subsurface environments. In contrast, continuous flow sediment column microcosms are a more sophisticated experimental approach enabling the development and maintenance of steady-state conditions in simulated subsurface environments. In these artificial systems, groundwater (synthetic or natural) is continuously fed through a fixed bed of sediment to foster the formation of biofilms on sediment grains and to reach localized, steady-state conditions balanced by continuous inputs, metabolism and advective removal of groundwater constituents and metabolic waste products. However, primarily due to the increased effort, time and money required for these experimental systems, column studies are infrequently performed in basic research when compared to batch studies, and are quite rare in remediation practice (ITRC 1998; ESTCP 2005).

A large body of literature provides testimony to the unique physiology and morphology of sessile (biofilm-enclosed) bacteria relative to their free-living

(suspended) counterparts, implying potentially important consequences for the design and validity of remediation treatability studies. Diverse species of aerobic and anaerobic bacteria have been found to differentially express as much as 38% of their genome (Prigent-Combaret et al. 1999) and over 50% of their proteome (Sauer et al. 2002) when extant in biofilm vs. planktonic culture. Bacteria in biofilms are known to modify their microenvironment and create microniches via production and excretion of extracellular polymeric substances (EPS), a complex matrix which can include fatty acids, fragments of DNA, peptides, sugars, humic substances, and other uncharacterized or poorly understood compounds (Flemming and Wingender 2010; Hall-Stoodley, Costerton, and Stoodley 2004). Some of the compounds excreted by sessile bacteria are known to be conserved across multiple diverse species (Hardie and Heurlier 2008) (Nadell, Xavier, and Foster 2009). These substances are thought or hypothesized to control and coordinate complex behaviors as varied as enhancing resistance to toxic metals (Harrison, Ceri, and Turner 2007), entering sporulation (Steven S Branda 2001), fruiting body formation (Goldman, Bhat, and Shimkets 2007), development of other non-reversible physiological changes (Hinsa et al. 2003), production of toxins (Gonzalez-Pastor, Hobbs, and Losick 2003), and modulation of EPS production to coordinate either biofilm development (Davies et al. 1998) or its dispersion (Stoodley et al. 2002). Quorum sensing, the ability for microbial populations to infer local population densities from the concentration of a constitutively excreted, low-molecular weight compound (autoinducers), is just one of many nuanced and complex ways that microbes communicate with each other (Bassler and Losick 2006) and react to their environment. Other mechanisms by which sessile bacteria can seemingly coordinate mutualistic behavior include

exchanging genetic information (Chen, Provvedi, and Dubnau 2006), modifying and enforcing community structure through toxin-antitoxin genetic pathways (Claverys, Martin, and Havarstein 2007), and producing and exchanging essential metabolic cofactors (Yan et al.; He et al. 2007; Rowe et al. 2008).

The documented existence of fundamental phenotypic and metabolic differences between suspended and immobilized biomass and between the chemistry of batch versus continuous-flow environments, challenges us to examine the appropriateness of contemporary remedial design tools for contaminated aquifer restoration, namely the use of batch bottle and sediment column microcosms in laboratory treatability studies. In this work, we sought to elucidate the reproducibility of each of these two methodologies with a focus on microbial community structure. Additional objectives included an assessment of qualitative and quantitative differences in microbial community structure that may exist between biomass associated with the liquid phase (groundwater) and the solid phase (sediment) in both experimental approaches. As a model microbial community of relevance to the *in situ* remediation of subsurface sites impacted by chlorinated solvent spills, we chose the recently described anaerobic consortium DehaloR^2 (Ziv-El et al. 2011) that is capable of fully dechlorinating various chlorinated ethenes during dehalorespiration under strict anaerobic conditions.

Methods

Column Microcosm Construction, Operation and Performance Monitoring

Composite sediment from the drilling of multiple wells was collected from Naval Air Station – North Island and transported back to Arizona State University (ASU). In the ASU lab, the sediment was transferred into a shallow tray and allowed to air dry in the fume hood over a period of approximately three days. Particles 250-1000 µm in diameter were sifted from dried sediment and packed into three custom glass columns (250 mm length, 14 mm ID) with Teflon® screw caps and Viton® O-rings that provided a waterproof seal. Influent media was stored in a 10-L glass bottle connected to a 10-L Tedlar bag inflated with air. The bottle and bag were spiked with trichloroethene (TCE) to a final concentration of 15 mg/L in the aqueous column influent. Media was prepared as detailed in Löffler et al. (Löffler, Sanford, and Ritalahti 2005) with the following modifications: i) media was prepared under aerobic conditions of the ambient laboratory atmosphere; ii) the redox-state indicator compound rezazurin was omitted; iii) all reducing agents were omitted, namely *L*-cystine and sodium disulfide; iv) AATC vitamin supplements were reduced to 10% of the recommended dosing; v) no carbonate was added in addition to the buffering capacity provided by a 10-mM phosphate buffer at an initial pH of 7.3.

Column influent was supplied with a multi-channel peristaltic pump (Ismatec Reglo Digital, Ismatec Inc., Glattbrugg, Switzerland) in a pulsed influent feed cycle, with the pumps on for 90 seconds at a flow rate of 56 µL/min, followed by a 240-second pause, resulting in an effective flow rate of 0.91 mL/hour, which translates into a

residence time within the columns of 10.45 hours and a linear velocity of 0.54 m/day calculated using a porosity of 0.4.

Column effluent samples were analyzed for chlorinated ethenes and ethene using an automated headspace solid phase microextraction followed by gas chromatography and flame ionization detection method (HS SPME GC-FID) as detailed in (Ziv-El et al. 2013)). After five days of operation, TCE concentrations in column effluent had stabilized and matched the influent TCE concentration. The columns were then inoculated with DehaloR^2, a dechlorinating consortium containing *Dehalococcoides* that had been obtained by enrichment of sediment from a tributary to the Chesapeake Bay (Ziv-El et al. 2011). The inoculum was generated in a sediment-free 160mL batch bottle allowed to dechlorinate two separate spikes of TCE to ethene, as detailed in (Ziv-El et al. 2011). Inoculation was carried out with a gas-tight syringe by attaching it to the column inlet's Luer fitting and injecting approximately 3 mL of the microbial culture. Immediately after inoculation, the amendment of influent with sodium lactate commenced, producing an effective concentration of 50 µM lactate in each column's influent. After 7 days, complete conversion of influent TCE to *cis*-2-dichloroethene (cDCE) was observed in the columns. The columns were re-inoculated with DehaloR^2 to ensure presence of viable populations of obligate anaerobes. After approximately 120 days, column effluent consisted entirely of ethene, indicating complete degradation of TCE. One hundred forty days after initial inoculation, sediment and liquid effluent samples were collected from the columns. Liquid effluent was collected into a sterile glass container and stored on ice over a period of 10 days. Column sediment was collected by uncapping column ends and

collecting approximately 0.5 g from each column end with a sterile spatula in an anaerobic glove box (Coy laboratory products Inc. Grass Lake, MI).

Batch Bottle Microcosms

Sediment, media, amendments, and inoculum types were identical to column microcosms. Batch bottle microcosms were constructed in 250-mL glass serum bottles capped with butyl-rubber septa. Each batch microcosm was constructed by adding to each bottle 42 g of sediment, 60 mL of media, 5 mL DehaloR^2 inoculum. Further, lactate and TCE were added to match conditions in column microcosm influent. Bottles were stored inverted on an orbital shaker and sampled periodically by GC-FID as detailed elsewhere (Ziv-El et al. 2011). After 3 days, the bottles were similarly reinoculated after anaerobic conditions were evident from observed conversion of TCE to cDCE, at which time the headspace was flushed with nitrogen gas and the bottles were respiked with TCE. The bottles were incubated for an additional 22 days, during which time active dechlorination of TCE to ethene was observed. After 25 days the bottles were sacrificially harvested for DNA extraction. Bottles were taken off the orbital shaker and allowed to settle for 1 hour. The cap was removed and care taken to not resuspend any sediment. Liquid was removed from the bottle with a pasteur pipette and processed for DNA extraction. 'Slurry' sediment samples were collected from the surface of the settled sediment with a sterile spatula, while 'sediment' samples were collected from the bottom of the settled solids within the bottles also with a sterile spatula.

DNA Extraction

Genomic DNA was extracted from sediment and slurry sample with the PowerSoil DNA extraction kit (MoBio Laboratories, Inc., Carlsbad, CA). Genomic DNA was extracted from all liquid samples by filtering liquid through a 0.2-µm filter and extracting the filter cake according to the manufacturer's protocol using the UltraClean Water DNA kit (MoBio Laboratories, Inc., Carlsbad, CA).

Pyrosequencing and Data Analysis

To explore microbial communities in the column and batch microcosms, extracted genomic DNA samples were sent to Molecular Research DNA Laboratories (Shallowater, TX, USA), where bacterial tag-encoded FLX amplicon pyrosequencing (bTEFAP) was performed by the 454 Genome Sequencer FLX- Titanium System (Sun, Wolcott, and Dowd 2011). The V2 and V3 regions of the 16S rRNA gene were targeted with primers 104F (5′-GGCGVACGGGTGAGTAA-3′) and 530R (5′-CCGCNGCNGCTGGCAC-3′), and the amplicon was sequenced by the procedure described by Wolcott et al. (2009). Raw sequencing data were processed using Quantitative Insights Into Microbial Ecology (QIIME) 1.6.0 suite (Caporaso, Kuczynski, et al. 2010). Sequencing data were qualified by removing sequences with lengths shorter than 200 bps, primer and barcode mismatches, homopolymers of more than 8 bps, or an average quality score lower than 25. Operational taxonomic units (OTUs) were defined by clustering qualified sequencing readouts at 97% similarity with UCLUST algorithm (Edgar 2010), and the representative sequence of each OTU was aligned to the Greengenes Database using PyNAST (Caporaso, Bittinger, et al. 2010; DeSantis et al. 2006). Chimeric sequences were detected and

removed using ChimeraSlayer (Haas et al. 2011), and then OTUs that contain less than two sequences (singletons) were eliminated. By using the ribosomal database project (RDP) classifier and the Greengenes 12_10 release dataset, taxonomy was assigned to the representative sequences with a 50% confidence threshold (Cole et al. 2009).

To assess the microbial diversity, sequence numbers were matched across samples to eliminate heterogeneity associated with different sequencing reads among samples. The OTU table was sub-sampled by randomly sampling ten different times 1,430 sequences from each sample, which was the least number of sequences obtained in one sample. The microbial diversity within a sample (alpha diversity) was assessed by measuring ecological indices of observed species, Chao1 estimator, phylogenetic distance (PD), and equitability. Chao1 and PD indices describe the microbial richness and diversity, respectively. To evaluate the microbial diversity between samples (beta diversity), the fraction of unique branch lengths from the total branch length of the phylogenetic tree was quantified using the weighted UniFrac distance matrices (Lozupone and Knight 2005). Principal coordinate analysis (PCoA) plots were generated using jackknifed beta diversity that subsampled each sample at a depth of 1430 sequences.

Results

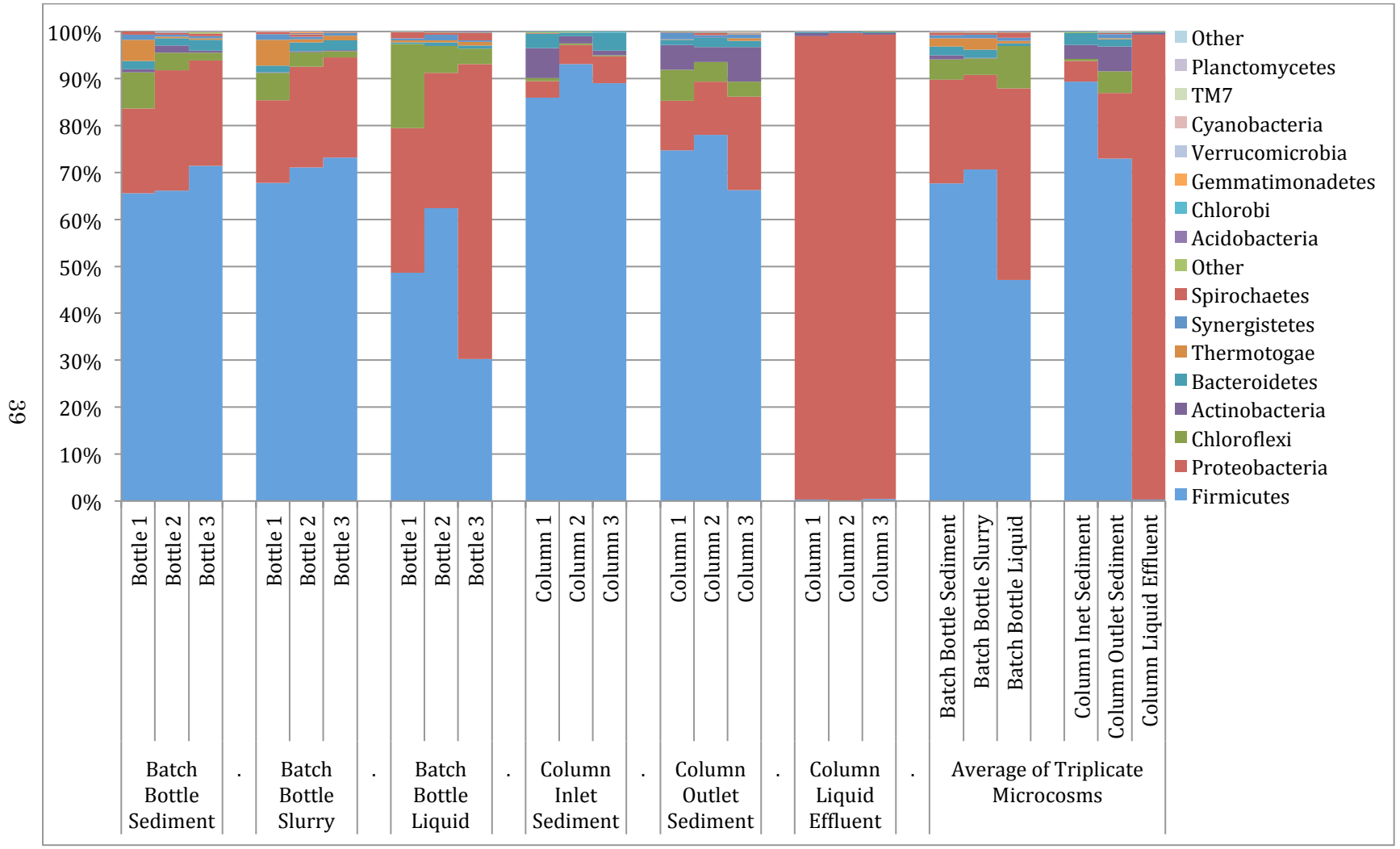

Figure 1. Relative abundance of phyla in column and batch microcosms.

Phylum Level Overview

The relative abundance of phyla present in the microcosms is presented in Figure 1. *Firmicutes* were found to dominate in both column and batch microcosms, but were preferentially attached to sediment. Lower relative abundance of *Firmicutes* was observed in the liquid fraction of both batch and column microcosms, but this difference was most pronounced in column liquid effluent, where *Firmicutes* comprised less than 1% of the community. *Firmicutes* were most dominant in column microcosms in sediment near the influent port, making up 89±3% (average ± standard deviation) of the community, with 98.7% of the *Firmicutes* belonging to the family *Veillonellaceae*. Near the column outlet, the *Firmicutes* were still dominant in numbers, but less so, constituting 73±5% of the community. However, the composition of the *Firmicutes* shifted dramatically, with *Veillonellaceae* now comprising only 13±5% of the total community (*Veillonellaceae* similarly comprised less than 1% in all other samples). Near the column outlet, the *Firmicutes* were dominated by *Acetobacterium*, making up 54.5±4% of the total community. Despite the dominance of *Firmicutes* in column sediment, *Firmicutes* were largely absent from column liquid effluent, representing only 0.2±0.1% of the respective community.

In batch microcosms, *Firmicutes* was also the most single most abundant phylum, comprising 43±12% in the liquid fraction, 61±4% in the sediment fraction, and 64±3% in the slurry fraction. Similar to column microcosm, we observed *Firmicutes* to be preferentially associated with solids, but the difference was less pronounced. Overall, the distribution of the community was much more homogenous within the

batch microcosms when compared to the columns. Among the *Firmicutes* present in the batch bottle microcosm, *Sedimentibacter* was most dominant, comprising 49±3% in the sediment, and 53±4% in the slurry fraction, and 36±13% in the liquid fraction.

Proteobacteria was the second most abundant phylum present in both batch and column microcosms, and was preferentially found in the liquid fractions therein. In the column microcosm effluent, the community was comprised primarily of *alphaproteobacteria* (53±12%), *betaproteobacteria* (21±5%) and *gammaproteobacteria* (24±9%). Within the batch microcosms the composition of *Proteobacteria* was shifted, with *deltaproteobacteria* (25±11%, 10±1%, and 7±1% of total community in liquid, sediment, slurry fractions, respectively) and *gammaproteobacteria* (7±2% and 10±5% in liquid and sediment, respectively) dominating.

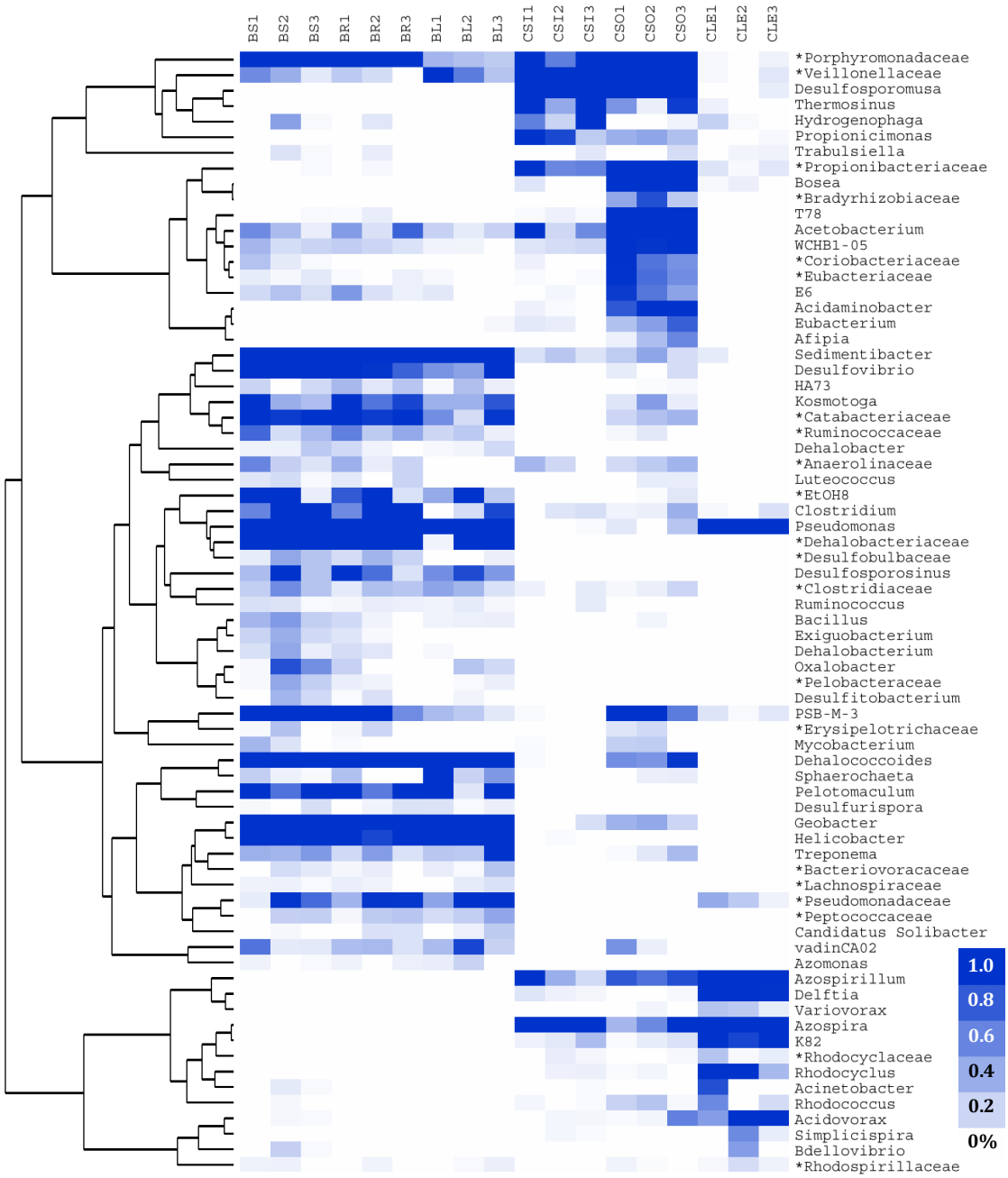

Figure 2. Distribution of microbial community at the genus level.

Genus Level Overview

The distribution of the microbial community at the genus level is presented in Figure 2. Within the column microcosms, we see that sediment near the inlet

contained only a subset of the microorganisms present near the outlet. Most notably, *Dehalococcoides* and *Geobacter*, the genera containing microorganisms responsible for dechlorination of TCE, were only detected in column microcosms in sediment located near the outlet, but not in the liquid fraction nor in sediment near the inlet. Even within the column sediment near the outlet, *Dehalococcoides* and *Geobacter* only made up a minor fraction of the community (0.7±0.2% and 0.3±0.1%, respectively). This is in stark contrast to the liquid fraction of batch microcosms, where *Dehalococcoides* and *Geobacter* constituted 9±6% and 24±10%, of the community, respectively. Contrasting with the column microcosms, the distribution of the community was relatively homogenous within the various fractions of the batch microcosms.

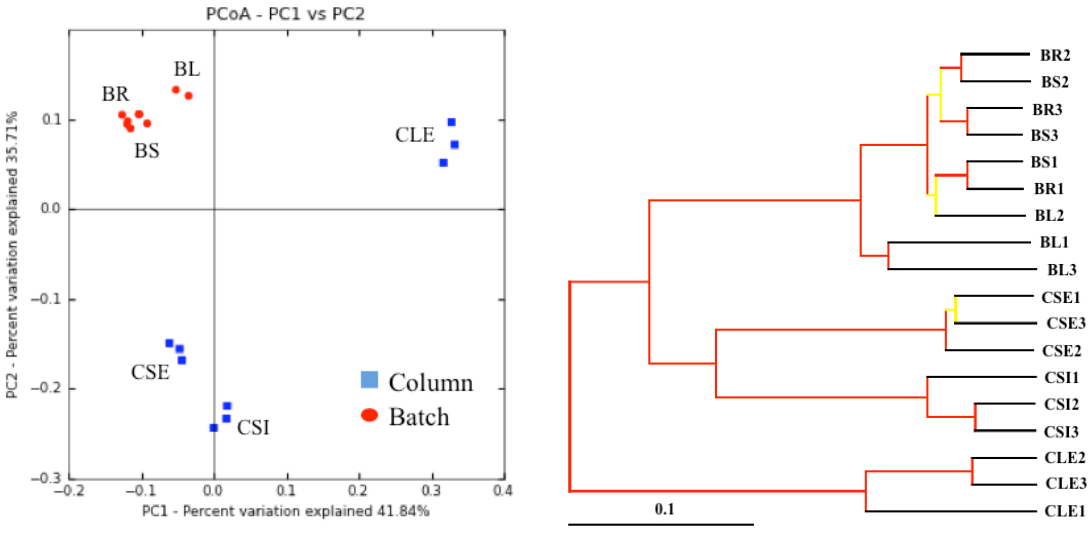

Figure 3. Results from weighted PCoA (left) and jackknifed weighted UniFrac analysis (right). Red nodes indicate 75-100% support, yellow 50-75%.

These general trends observed in Figure 2 were further supported by beta diversity analysis. Weighted principal coordinate analysis (PCoA) and UniFrac plots generated through jackknifed beta diversity analysis are presented in Figure 3. In the PCoA plot, column microcosms formed three distinct clusters belonging to replicates from column sediment near the outlet, sediment near the inlet, and effluent liquid. This is in contrast to the batch microcosms, where replicates from the three bottle fractions (sediment, slurry, and liquid) were observed to cluster together. Similar clustering was observed in the bootstrapped tree generated through jackknifed analysis.

Beta diversity was further analyzed by determining the average and standard deviation of UniFrac distances between various sample group subsets (presented in Figure 4). First we compared the average distance between replicates to assess the reproducibility of microcosms. An average distance of 0.11±0.04 was recorded between all replicates. Similar replicate distances were observed within columns and batch bottles (0.11±0.05 and 0.11±0.03, respectively), indicating that both microcosm types showed equally good reproducibility with respect to community structure.

The average distance observed between bottle and column microcosm samples was 0.44±0.05, indicating that batch bottles and column microcosms do not produce equivalent communities. To determine if the difference observed between batch and column microcosms was due to attachment to sediment, we compared only the sediment portions of both microcosms. There, a similarly large distance of 0.40±0.03,

was observed, indicating attachment alone was not responsible for the difference in microbial community structure.

We further investigated the role of cell attachment by comparing attached and unattached populations within the microcosms. A relatively homogenous distribution of the population was observed within the batch bottles, with an average distance of 0.17±0.05. Within the sediment columns, however, we observed a stark contrast between attached and unattached communities, with an average distance of 0.54±0.02.

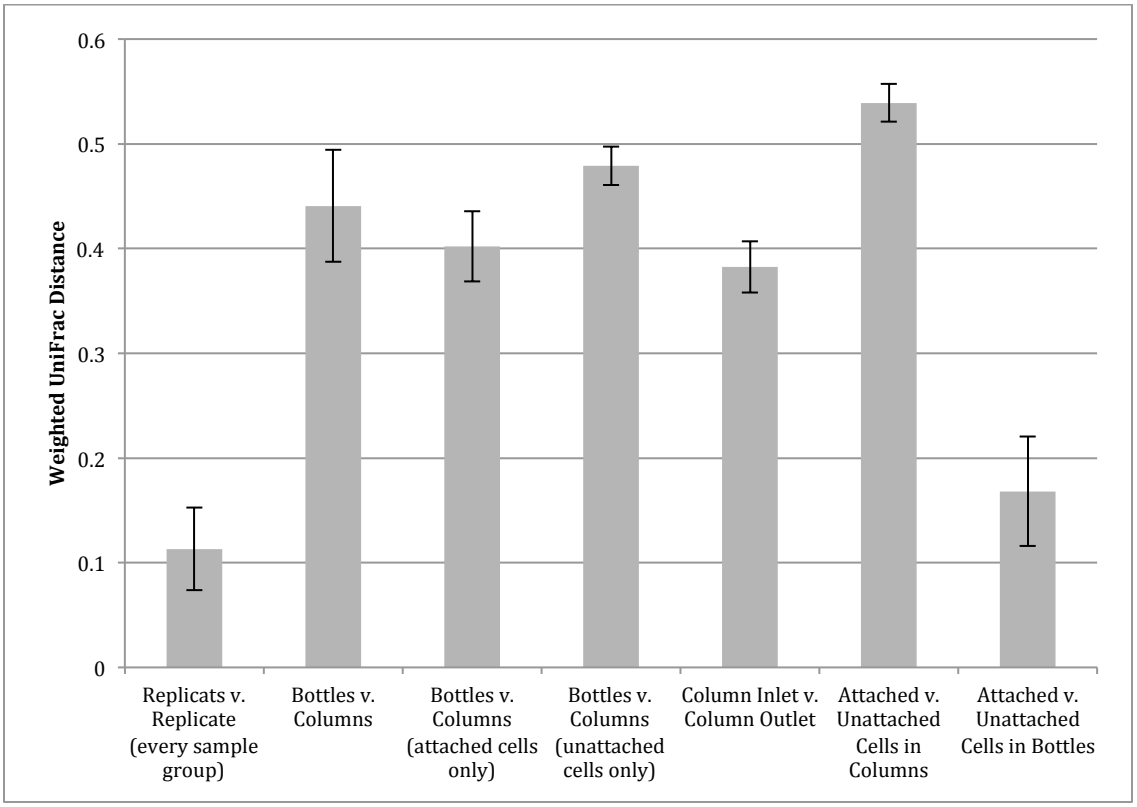

Figure 4. Average and standard deviation of weighted UniFrac distance (beta diversity) between bacterial communities profiled in select comparison groups. Higher numbers indicate greater difference.

Table 1. Microbial diversity indices obtained for batch and column microcosms. The p-values were determined by nonparametric Monte Carlo permutations.

	Batch (Ave)			Column (Ave)			(Ave±SD)		
	BS	BR	BL	CSI	CSO	CLE	Batch	Column	*p*-value
Observed species	191	170	153	108	169	83	171±23	120±41	0.012
Chao1 estimator	311	284	250	178	291	128	282±46	199±75	0.013
PD	15.0	13.4	11.4	6.6	12.4	5.9	13.3±1.8	8.3±3.2	0.004
Equitability	0.58	0.55	0.54	0.52	0.60	0.41	0.56±0.04	0.51±0.1	0.245

Results obtained from computing microbial diversity indices are summarized in Table 1. More unique OTUs were observed in the batch than in the column microcosms, which indicates higher richness of the bacterial community in the batch microcosms (171 vs. 120). As an alternative method to assess the richness, the Chao1 estimator was used to estimate the total number of OTUs with infinite sequence reads (Chao and Lee 1992). Similar to the observed species, nonparametric *t*-test revealed that Chao1 estimator was significantly higher in the batch samples than the column ones (p=0.013). Additionally, the Phylogenetic Diversity (PD) index was employed to estimate microbial diversity (Faith 1992); higher diversity was observed in the batch microcosms than the column microcosms (p=0.004). However, the two microcosms had comparable values of Equitability index, demonstrating the similar microbial evenness.

Discussion

Profiles of microbial communities derived from batch bottle microcosms are often used to inform our understanding of what might be found in the subsurface where continuous flow conditions exist and where microbes predominantly reside in biofilms (Y Shi 1999) (Ellis et al. 2000) (Mundle et al. 2012) (Futamata et al. 2007).

This study sought to inform the validity of using batch microcosms for understanding subsurface phenomena by examining the microbial communities of consortia maintained in batch and flow-through mode.

Reproducibility of Microcosms.

Microbial communities in biological reactors are known to be dynamic, varying in richness, structure, species diversity, and evenness over time. Dynamics is defined as the number of species that on average come to significant dominance at a given habitat during a defined time interval. Examples of high community dynamics during periods of relatively stable performance are present in the literature (Van Nostrand et al.). The non-stable nature of complex communities would suggest that identical biological replicate microcosms will, given enough time, produce divergent communities (K and C 2002). However, studies have demonstrated that, though unpredictable, community assembly is directional, non-random and reproducible (Ayarza, Guerrero, and Erijman 2010). For microcosms to be reproducible, however, it is necessary for conditions to be as controlled as possible. It has been found that even conducting an 'identical' experiment in different labs introduces enough variability to create different bacterial communities (Roeselers et al.). To overcome the latter limitation and qualitatively and quantitatively assess differences between batch and column microcosms, we performed both types of studies side-by-side in the same lab using identical microcosm materials and inocula.

This methodological approach did indeed yield good reproducibility between biological replicates of both experimental setups. We observed the development of

very similar communities among replicate microcosms (average UniFrac distance of 0.11±0.04).

Two conclusions can be drawn from the high degree of similarity between replicates: (i) the large observed difference between sample groups (i.e., batch vs. column microcosms) are unlikely to be caused by random processes of community dynamics; and (ii) generating multiple community profiles from biological replicate microcosms, while providing confidence in the reproducibility of the results generated, does not necessarily provide additional insights into the community present in the system under study. In situations where cost must be minimized (e.g., site-specific treatability studies), pyrosequencing analysis of replicate samples thus may not be necessary.

Homogeneity within batch microcosms

Surface attachment of cells can be regarded as a process in which cells seek to attain more favorable of 'better' environmental conditions. While the exact benefits cells derive from attachment vary (Hall-Stoodley, Costerton, and Stoodley 2004), they are in all cases fundamentally dependent on the presence of a microenvironment near a surface that differs from the bulk liquid (Stewart and Franklin 2008). Within a batch slurry microcosm as conducted here, especially one incubated on an orbital shaker, the potential for any significant chemical gradient is mostly diminished. Furthermore, a deep biofilm is unlikely to develop on a surface within a batch microcosm, because the microenvironment near the surface would have reduced substrate concentrations (i.e., unfavorable environmental conditions). A special circumstance where cells may be observed to aggregate in batch microcosms is when

mutualistic or commensal relationships are present. In these cases, bacteria benefit from close proximity to other bacteria that provide substrate or essential metabolic cofactors. *Dehalococcoides,* an obligate mutualist that depends primarily on *Firmicutes* to provide acetate, hydrogen, and vitamin B_{12}, is one such example (Ziv-El et al. 2012).

Paradoxically, we observed relatively higher amounts of *Firmicutes* attached to sediment, but relatively lower amounts of *Chloroflexi*, the phylum to which *Dehalococcoides* belongs (Löffler et al.). In batch bottle sediment, slurry and liquid, *Firmicutes* comprised 0.677±0.026%, 0.707±0.022%, and 0.471± 0.131%, respectively, of the community in the fractions, while *Chloroflexi* comprised 0.043±0.025%, 0.034±0.018%, and 0.090±0.064%. Parametric Student *t*-test statistical analysis reveled that the difference in abundance between attached fractions (sediment + slurry) and unattached (liquid) was significant for *Firmicutes* (p=0.01) but not for *Chloroflexi* (p= 0.17). It is worth noting that, regardless of statistical significance, the difference is minor. Two hypotheses may explain this observation: (i) cells are still aggregated while suspended in the liquid in free-floating flocs; and (ii) the chemicals derived from the mutualistic relationship (e.g., acetate, H_2, B_{12}) were not limiting, but rather was a substrate present in the bulk liquid (i.e., the metabolic incentive for mutualists to aggregate was absent).

Cell population size distributions were not quantified within the microcosms, so the relative sizes of attached v. unattached communities is unknown. Alpha diversity analysis, however, revealed a greater overall diversity in the attached community

than in the unattached liquid fraction. A consistent trend was observed between sediment, slurry, and liquid fractions: 191, 170 and 153 unique species were detected in the fractions, respectively. Similar trends were observed with other diversity indices (Table 1). This result indicates that a large subset of the community almost exclusively preferred to be attached to sediment particles. Because the inoculum (Dehalo^2) was derived from a sediment-free culture, the consortium potentially may have a preference for planktonic lifestyle. The preferential partitioning by a subset of the population to sediment particles supports the claim that surface associated growth will support more diverse communities.

Heterogeneity within column microcosms

The largest difference between communities observed in the study was between attached (sediment inlet and outlet) and unattached (column liquid effluent) cells, with a UniFrac distance of 0.54±0.02. The community within the liquid effluent also showed much lower alpha diversity, with only 83 species detected. The liquid effluent community was dominated by *Azospirillum* (68±12%) and *Azospira* (18±9%). *Azospirillum* is a *proteobacteria* that is primarily known as plant growth promoting rhizobacteria that is capable of fixing nitrogen under aerobic and micoaerobic conditions(Steenhoudt and Vanderleyden 2000). *Azospira* is similarly a nitrogen-fixing *proteobacteria(Bae et al.).* It is unclear why these species dominated in the effluent, as the media was not nitrogen limiting (5.6 mM ammonium supplied in the influent). *Azospirillum* and *Azospira* were much less dominant in the inlet and outlet communities, making up 0.7±0.3% and 1.7±1.1% of the community, respectively.

In contrast to biomass suspended in the liquid, the attached community, particularly near the outlet, was not dominated by a small number of species (equitability of 0.60). Similar to the batch bottles, the columns exhibited greater species richness and evenness in the attached community, supporting the claim that surface associated communities are more diverse, and that a large number of cells prefer a surface association. Similarly large differences have been observed between communities present in adjacent liquid and sediment samples collected fresh from the field (Vrionis et al.), suggesting that the presence of continuous flow impacts community structure.

Comparing communities from column inlet and column outlet, we see a relatively modest difference, with an average and standard deviation UniFrac distance of 0.38±0.02. Alpha diversity analysis revealed greater diversity in the outlet (169 species) than in the inlet (108 species) (Table 1). The greater diversity present towards the outlet, in part, may be due to the additional presence of obligate anaerobes that are unable to grow near the inlet where aerobic conditions prevailed. While columns were supplied aerobic media amended with lactate and TCE at the inlet, a redox gradient developed along the column length with anaerobic conditions present in the outlet evidenced by the presence of dechlorination products (cDCE, vinyl chloride and ethene) in the liquid effluent.

Differences between batch and column microcosms.

Large differences were observed between batch microcosm and column microcosm communities (average Unifrac distance of all column v. batch comparison pairs:

0.44±0.05). A similarly large difference was observed when comparing only the attached or unattached fractions of the microcosms (presented in Figure 4). A few factors may be causing this observed difference.

A column contains predominantly sessile bacteria (attached, in a biofilm), while a batch bottle can contain a mixture of bacteria attached to sediment, aggregated into suspended flocs, or dispersed into single planktonic cells. Previous studies have shown that, when comparing communities from sediment and liquid samples, a bias can be introduced either by differing extraction efficiencies (Martin-Laurent et al. 2001), or by variable attachment rates across the microbial community (Vrionis et al.). However, the relative homogeneity we observed within the batch microcosm suggests that neither of these biases was present in our experimental setup. The similarity between batch sediment and batch liquid samples (UniFrac distance of 0.17±0.05) indicates that attachment alone does not account for difference observed between batch and column microcosms.

Another source of the beta diversity differences observed between batch and column microcosms may be a high dynamic (Dy) (Marzorati et al. 2008) value for the community. Previous studies have found that biological replicate microcosm communities can diverge due to random or seemingly random dynamics. During the extended microcosm incubation time the communities may have developed differently, explaining the difference. However, if this caused the difference between column and batch microcosms, we would expect to see equally high differences

between biological replicates within the same experimental group, particularly in the column community, which was maintained for 140 days.

Additionally, the alpha diversity was greater in the batch bottles than the columns. The batch communities showed both greater species richness and evenness, on average (presented in Table 1). However, the greatest species evenness was observed in column sediment near the outlet. A possible explanation for of this (and a limitation of this study) is that column sediment was only analyzed from two fractions, the inlet and outlet. It is likely that a different community was present at different points along the column, corresponding to the redox and substrate concentrations at that point. Another possible cause for the decreased alpha diversity within columns is due to the fact that the inoculum, DehaloR^2, had already been adapted to growth in batch mode in the lab, and the introduction of the community to continuous-flow conditions resulted in a subset of the community being unable to compete. With no additional bank of diversity to replace the lost species, the columns thus would have been expected to exhibit a lower alpha diversity.

Study limitations

This study was conducted with a single consortium, DehaloR^2, and under a single set of environmental conditions. For this reason, any extrapolations to different communities or different conditions should be done with caution. Furthermore, only two sediment fractions from the column were sampled. This is likely inadequate to capture the full community (as suggested by the relatively low alpha diversity

values observed therein). Additionally, neither batch bottles nor columns were sampled over time to quantify community dynamics or observe species succession. It is possible that the observed differences between columns and bottles merely correspond to different successions stages.

Conclusions

Our results indicate that whether a microcosm is operated in batch or continuous-flow conditions has a sizable impact on microbial community structure, and that this difference cannot be solely attributed to attachment. Furthermore, large differences were observed in community profiles generated from sampling different fractions of microcosms, especially column microcosms, where the largest difference was observed between community profiles generated from sediment and liquid samples. Finally, this is the first study to report that DehaloR^2 maintains dechlorination activity upon reintroduction to sediment.

It appears that, for reasons of convenience, inertia and economy, the environmental field has in its industry's standard practices grandfathered in an outdated paradigm(van Loosdrecht et al. 1990): that there are no substantive differences between biofilm and planktonic microbial communities, and that conclusions derived from observations (in batch systems) of bacteria in one mode can be liberally applied to environments where the other mode of existence predominates (in flow-through columns and natural subsurface environments). This assumption is built into the practices of i) using batch bottle microcosm studies to simulate the subsurface, and ii) sampling groundwater only to profile the entirety of the community present in the

subsurface. Our results along with those of others (Lehman and O'Connell 2002; Middelboe et al. 1995; Unanue et al. 1992; Simon 1985) suggest that caution should be exercised when using batch bottle studies or only groundwater samples to inform our understanding of microbial communities in aquifers where continuous-flow conditions predominate.

Chapter 3

AUTONOMOUS SCREENING OF GROUNDWATER REMEDIATION TECHNOLOGIES IN THE SUBSURFACE USING THE IN SITU MICROCOSM ARRAY (ISMA)

Abstract

Science and engineering lack tools for accurately predicting the effect of anthropogenic perturbations on aquatic ecosystems. We introduce the *In Situ* Microcosm Array (ISMA) as a new method for conducting contained experiments in the subsurface, demonstrated here for the remedial design of two contaminated aquifers. An array of flow-through sediment column microcosms is deployed *in situ* in contaminated aerobic groundwater to evaluate the feasibility of in situ anaerobic bioremediation. Candidate technologies evaluated successfully in triplicate within the device included (i) monitored natural attenuation, (ii) biostimulation, and (iii) in situ bioaugmentation to remediate contamination with trichloroethene, hexavalent chromium, and perchlorate. Results demonstrate the ISMA's utility to perform cost-effective, risk-free, high-throughput screening of multiple intervention strategies *in situ*, without impacting in any way the subsurface environment examined. The ISMA opens the door to cost-effective, high-throughput, parallel screening of multiple technologies *in situ*, including risky approaches.

Introduction

Cleaning up millions of hazardous waste sites worldwide is a daunting task. In the U.S. alone, remediation of some 294,000 U.S. locations is projected to take over 30

years and consume US$200,000,000,000 (EPA 2004a). To address the enormous challenges posed by legacy hazardous waste sites worldwide, the U.S. government alone spends over $500M annually towards research and development of innovative remediation technologies (EPA 2012; Under Secretary of Defense for Acquisition 2011). These efforts have been only partially effective. Despite continuing efforts to reduce the use of energy intensive pump & treat (P&T) operations, this unsustainable treatment still represented the only remedial action implemented at 65% of U.S. priority contaminated aquifers 2002 (EPA 2004a).

In situ remediation technologies (ISRTs) are comparatively inexpensive alternatives intended to replace or augment conventional P&T operations. However, real-world performance of these is difficult to predict from laboratory feasibility studies using batch microcosms (Suarez and Rifai 1999) because microorganisms behave very differently under laboratory conditions compared to their natural environments (Madsen 1991). The performance of ISRTs like *in situ* chemical oxidation and *in situ* bioremediation is site-specific and difficult to predict, particularly for low-cost bioremediation approaches, which can consist of monitored natural attenuation (MNA), the injection of nutrients (biostimulation), or injection of both nutrients and microorganisms (bioaugmentation). In addition to the variable performance, contributing to the barrier facing ISRTs are risk-averse site stakeholders (potentially responsible parties, affected communities, site regulators), which are, understandably, wary of expending significant resources to field-test yet unproven ISRTs, especially when such field-tests carry the risk of making conditions worse through unforeseen secondary water quality impacts (Dyer, van Heiningen, and Gerritse 2000).

To address these obstacles to effective remediation, we have taken a proven laboratory approach (flow-through sediment column studies, Figure 5), reduced it in scale, and relocated it into a submersible down-hole field-deployable device called the *In Situ* Microcosm Array or ISMA (Figure 6).

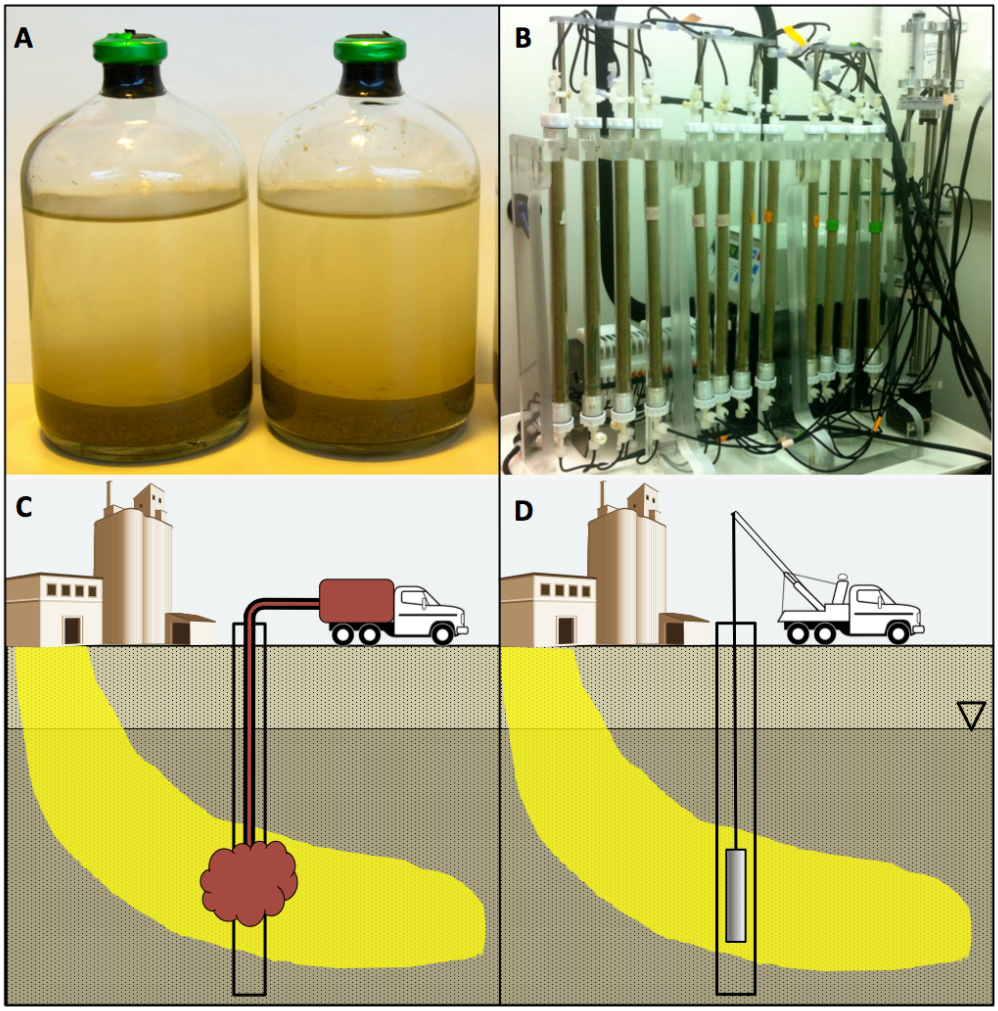

Figure 5. Conceptual representation of treatability study methods. (**a**), Sediment slurry batch bottles, the industry standard in treatability studies. (**b**), Sediment column study, a more realistic but infrequently used approach to treatability studies. (**c**), Small-scale field trial of an *in situ* remediation technology. (**d**), Conceptual representation of an ISMA deployment in which the self-contained device is deployed in a groundwater well, i.e., *in situ*. Presently, the common practice is to move directly from **a** to **c**. Approach **b** is scientifically more rigorous (gold standard approach) but expensive and reliant on groundwater stored for extended periods of time in the laboratory. Here, we leverage the advantages of **b** by packaging it into a self-contained, field-deployable device to arrive at a new feasibility study approach, **d**, that offers many of the advantages of multiple deployments of **c**, yet does so in a single deployment, economically, and without the risk of contaminating the aquifer studied.

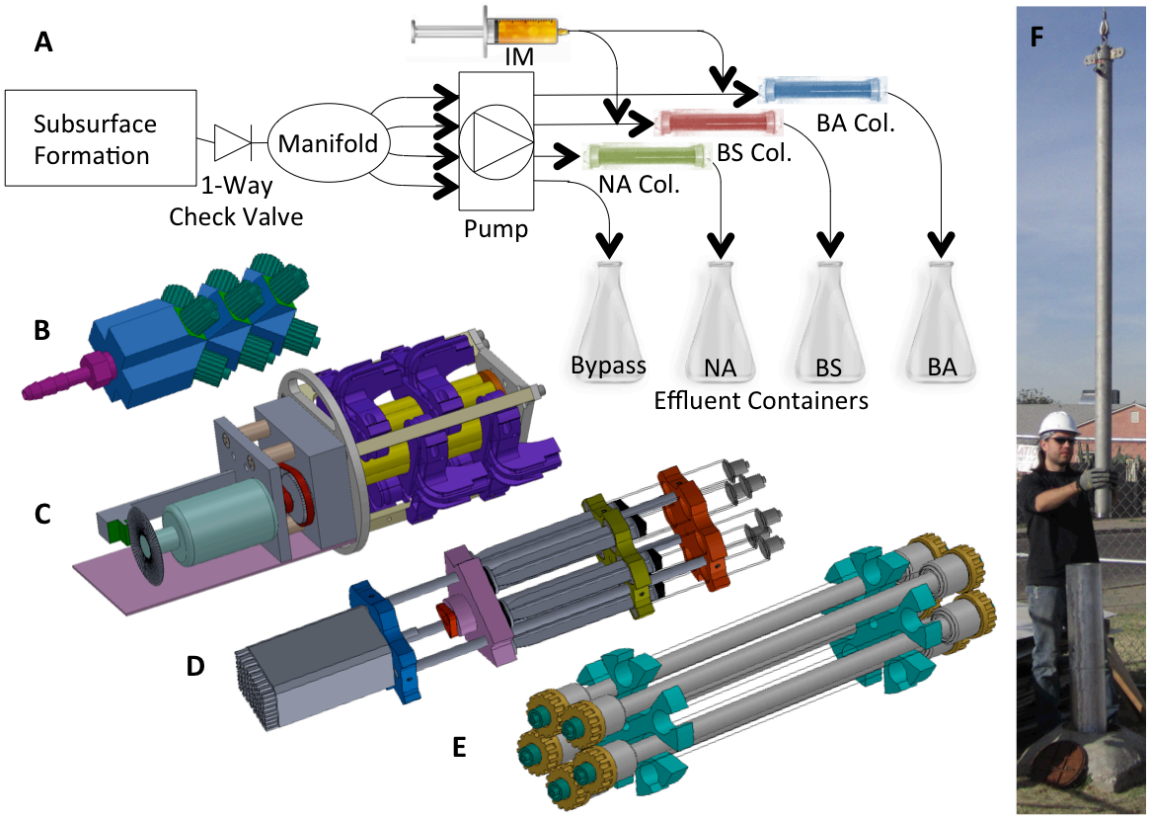

Figure 6. Schematics of ISMA components and groundwater flow paths. **a**), Groundwater flow paths and experimental setup: water is drawn directly from the aquifer. One-way check valve, along with secondary containment system (not shown, 8.9-cm OD cylindrical shell), ensure that nothing is released into the environment. A manifold (**b**) splits water into 12 separate lines (n=3 for each line drawn). Each line has a dedicated channel in a peristaltic pump (**c**). Biostimulation (BS) and Bioaugmentation (BA) lines are amended with sodium lactate throughout the deployment via an Injection Module (**d**). BA columns were inoculated with KB-1® prior to ISMA incubation *in situ*. All columns are housed in a column array (**e**). All groundwater is stored in individual effluent capture vessels, which are analyzed upon ISMA retrieval. Effluent vessels are preloaded with a quencher and biostatic agent to capture the chemical and biological signatures of reactions occurring in the sediment column only. (**f**), Researcher guides ISMA into a groundwater well as it is being field deployed. Schematic of complete ISMA assembly in Figure 7.

The ISMA generates data on the field performance of *in situ* remediation technologies without releasing anything into the environment. It does so by conducting trials of ISRTs autonomously in the subsurface in continuous-flow mode using sediment column microcosms with groundwater drawn directly from the aquifer. In the configuration shown (Figure 6), the unit simultaneously conducts 9 separate trials, allowing for the statistically significant comparison of up to three ISRTs performed in triplicate at the small-scale *in situ*.

The device is housed within an 8.9-cm outer-diameter (OD) stainless steel cylindrical shell and contains: an intake with a one-way check valve, a 1-to-12 splitting manifold, 2 multi-channel peristaltic pumps regulating flow rates in 12 liquid lines, a step-motor delivering treatment agents used in the various ISRTs to 6 of the lines, 9 continuous-flow column microcosms packed with site-sediment, 12 separate liquid effluent capture vessels, 12 sorbent-based in-line cartridges for volatiles capture, secondary liquid containment system, and assorted control electronics and line management systems. The device is deployable in standard 10.2-cm inner diameter wells commonly installed at hazardous waste sites for groundwater monitoring. The stringent size limitations of 8.9-cm OD, necessary for the ISMA to be practical for field use, required the custom design and machining of the tubular device with all the mechanical components shown in Figure 6 (expanded views in Figure 7).

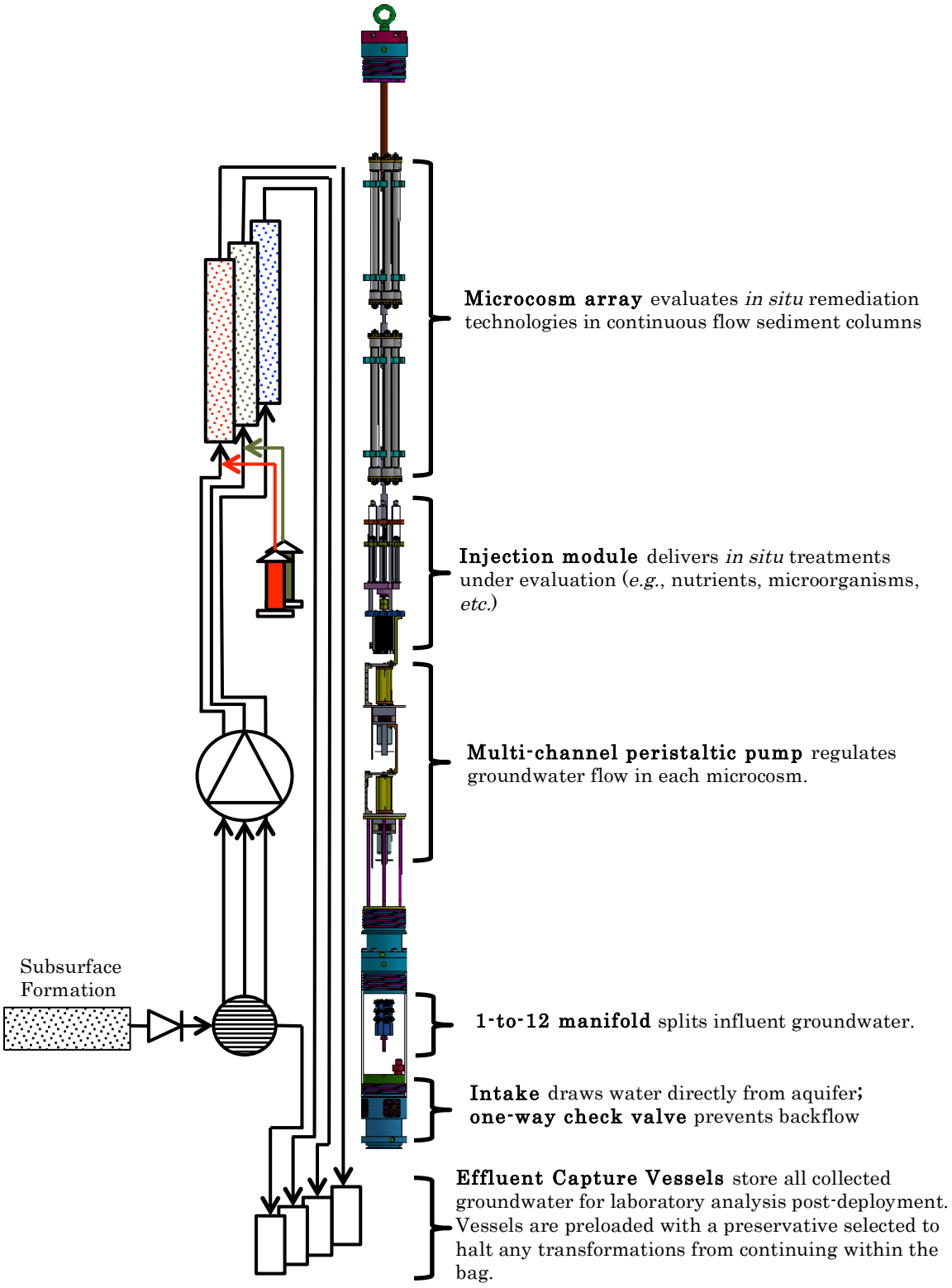

Figure 7. Expanded schematic of main ISMA section depicting groundwater flow paths within the device

In a standard deployment, three 183-cm long sections are preconfigured in the lab, and then assembled in the field, stacked sequentially as the device is lowered into the well. The three sections are linked by custom-designed quick-connects that are load-bearing, waterproof, and propagate all individual liquid and electric lines. Internally, all materials that contact field materials were chosen for their compatibility with aggressive solvents: glass, stainless steel, Teflon®, and Viton®. During deployment, the ISMA is tethered to the surface and powered from the grid or from batteries recharged by solar panels.

Nothing is released during an ISMA deployment *in situ*. This allows for zero-risk testing of new technologies, and for conducting multiple trials and deployments within the same well. It also takes advantage of existing infrastructure present at many legacy hazardous waste sites (10.2-cm OD wells) without impacting their long-term functionality as compliance monitoring locations.

Effluent produced by the column microcosms is stored in individual Teflon® vessels preloaded with a biostatic and/or quenching agent to prevent *in situ* transformation reactions from continuing once the groundwater enters the effluent bags. To accommodate for the potentially significant volumes of gas produced within microcosms (e.g., CO_2, N_2), vent lines were connected to each effluent bag; offgas from the bags during deployment is passed through sorbent cartridges to capture volatile analytes and improve mass balances.

The ISMA is a versatile new addition to existing *in situ* feasibility study approaches that include bio-trap samplers (Busch-Harris et al. 2008), *in situ* microcosms (Nielsen et al. 1996), and single-well push-pull tests (Istok et al. 1997). Distinguishing features of the ISMA technology include (a) the use of flow-through sediment microcosms in which the flow conditions can be tightly controlled, and (b) the isolation of the *in situ* experiments from the aquifer to improve mass balances and to prevent environmental release of either biological or chemical agents during feasibility testing of candidate technologies.

Field validation of the new technology took place at two hazardous waste sites, where we assessed the feasibility of implementing bioremediation to simultaneously treat TCE and hexavalent chromium [Cr(VI)] (Site 1), and perchlorate (Site 2).

TCE is one of the most common and recalcitrant contaminants worldwide. Under reducing conditions (E_h <100 mV), it can undergo biological reductive dechlorination by specialized microorganisms that use chlorinated ethenes as electron acceptors (Maymo-Gatell 1997) (Figure 8). Mixed microbial communities capable of dechlorination are commercially available (e.g., KB-1® (Duhamel et al. 2002)). Of particular concern during bioaugmentation with dechlorinating microorganisms is the potential accumulation of vinyl chloride (VC), which poses a greater threat than the original contaminant of concern, TCE. Thus, proof of successful dechlorination beyond VC is highly desirable to convince stakeholders of the suitability of *in situ* bioremediation for site remediation, particularly when co-contaminant mixtures are present, as at Site 1, where hexavalent chromium and TCE co-occur. Hexavalent

chromium can be detoxified under reducing conditions (Leita et al. 2011; Rai, Sass, and Moore 1987). However, due to its high toxicity, it is commonly believed that Cr(VI) concentrations of >5 mg/L necessitate injection of a chemical reductant to first reduce Cr(VI) before biological reductive dechlorination of TCE to ethene can commence (Sandrin and Hoffman 2007).

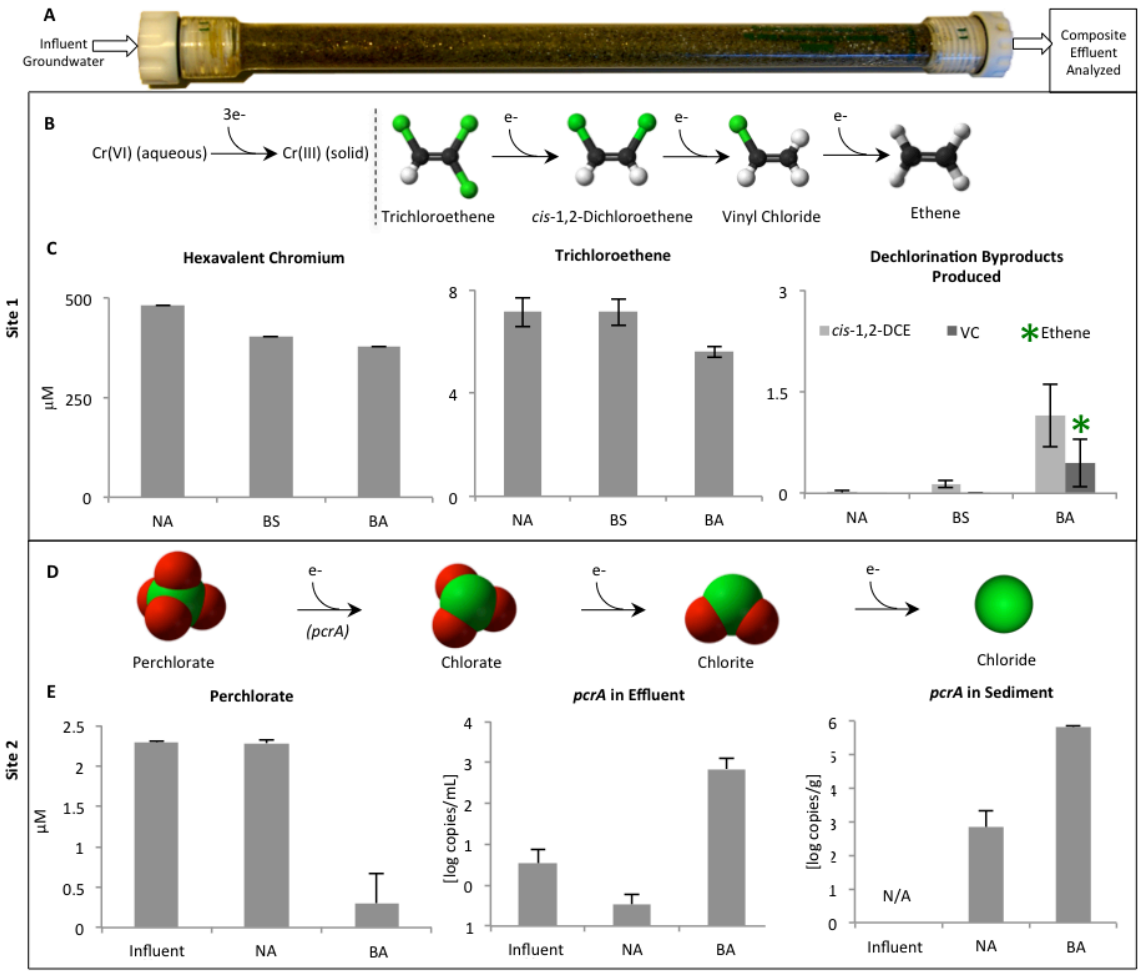

Figure 8. Subsurface chemistry captured in the ISMA.(**a**), Sediment column containing materials from the subsurface formation. Groundwater drawn directly from the aquifer was pumped through columns continuously for 35 days. (**b**, **d**), Contaminant transformations observed under reducing conditions. (**c**), Contaminant concentrations in composite effluent collected over the duration of ISMA deployment *in situ* at Site 1 comparing three remediation technologies: NA (natural attenuation), BS (biostimulation), and BA (bioaugmentation with KB-1®). Error bars represent standard error (n=3). Analysis of ethene was qualitative only due to lack of gas-trapping sorbent materials. (**e**), Concentrations of perchlorate and perchlorate reductase (*pcrA*) in composite effluent, and *pcrA* in column sediment.

The additional complexity introduced by a contaminant mixture like the one extant at Site 1 often increases the cost and time-to-completion of remedial actions, due to

the need for designing and testing customized solutions. Complex sites also increase the risk of undesirable outcomes, like the solubilization of toxic subsurface constituents or the production of harmful byproducts (here VC), resulting in a reluctance to implement innovative technologies such as enhanced *in situ* bioremediation.

To reduce the uncertainty associated with implementation of *in situ* bioremediation at Site 1, we evaluated the relative performance of three remediation strategies: natural attenuation (monitoring only, no technical intervention), biostimulation (amendment with sodium lactate), and bioaugmentation (amendment with sodium lactate and KB-1®, a dechlorinating culture). Sediment columns were operated and characterized in the laboratory for 75 days prior to placement in the ISMA device and incubation *in situ* at a depth of 8 meters below ground surface for 35 days.

After *in situ* incubation, the collected composite column effluent was analyzed for metals, chlorinated volatiles, and ethene (Figure 8). Relative to MNA, significantly reduced amounts of TCE (homoscedastic 2 tailed student *t*-test, $p<0.05$), and elevated levels of cDCE ($p=0.08$), VC ($p<0.05$), and ethene detected post-deployment in bioaugmented sediment microcosm effluent provided multiple lines of evidence for a successful conversion of aerobic site groundwater to anaerobic conditions that facilitated the reductive dehalogenation of TCE by the strict anaerobic bacteria added to the sediment. The reductive dechlorination of TCE in the presence of high concentrations of Cr(VI) (>5 mg/L) is a notable secondary outcome of this study. The observed biological removal of TCE in the presence of 24 mg/L of Cr(VI) in

groundwater entering the ISMA extends the reported spectrum of conditions conducive for reductive dechlorination of chloroethenes via bioaugmentation.

At Site 2 we demonstrated the ability to track microorganisms in the ISMA. Perchlorate is a salt that is stable under environmental conditions (Urbansky 2002), but can be biologically reduced by a range of perchlorate reducing bacteria (Coates and Achenbach 2004; Coates et al. 1999) (Figure 8, **d**). We quantified the initiatory enzyme catalyzing the reduction of perchlorate, *pcrA,* between 2 simultaneously evaluated remediation treatments: natural attenuation and bioaugmentation (amendment with perchlorate-reducing-bacteria and sodium acetate) (Figure 8, **d**, **e**). Field data collected with the ISMA indicate that enhanced *in situ* bioremediation is a potentially feasible remedial actions both at hazardous waste Sites 1 and 2.

Field demonstrations at Site 1 and 2 illustrate the ability of the ISMA technology to generate data of similar sophistication and reproducibility as a traditional laboratory study, but with the added benefit of being performed *in situ.* The ISMA reduces opportunities for laboratory artifacts and enables the determination of *in situ* processes under ambient subsurface conditions, as opposed to those extant in commonly employed laboratory batch bottles studies.

Conclusions

In summary, we report the successful development and field demonstration of the ISMA, an innovative remedial design tool suitable for parallel *in situ* screening of multiple, mutually exclusive treatment technologies at the same time in the same

place. Use of the device requires access to only a single groundwater well, which is left unimpaired by the ISMA deployment and thus can continue to serve as a compliance monitoring location in the future. The ISMA opens the door for accelerated translation of remediation technologies from the lab to the field by reducing the risk associated with generating *in situ* performance data. The device may also find additional applications in bioprospecting in extreme environments (e.g., hot springs), risk assessment of genetically modified microorganisms, and fate and transport studies of new materials prior to mass production and environmental release.

Methods Summary

Chlorinated ethenes, ethene, and hexavalent chromium were analyzed, respectively, by EPA methods 8260B, RSK 175, and 7196A at EMAX laboratories (Torrance, CA). Perchlorate was analyzed following EPA method 314.0. DNA was extracted from water according to the manufacturer's protocol using the UltraClean Water DNA kit from MoBio Laboratories, Inc., Carlsbad, CA. DNA from sediment was extracted using the PowerSoil DNA extraction kit from MoBio Laboratories, Inc., Carlsbad, CA in combination with the DNeasy Blood and Tissue kit from Qiagen Inc., Valencia, CA. Quantitative PCR was performed according to a previously published protocol (Zhao et al. 2011).

Chapter 4

DEMONSTRATION DEPLOYMENT OF THE IN SITU MICROCOSM ARRAY (ISMA) AT A TRICHLOROETHENE AND HEXAVALENT CHROMIUM CONTAMINATED SITE

Sections of this chapter appeared in ESTCP Project # 200914 Final Report titled "Parallel *In Situ* Screening of Remediation Strategies for Improved Decision Making, Remedial Design, and Cost Savings"

Abstract

This chapter summarizes results from a demonstration deployment of the ISMA at Naval Air Station North Island (NAS NI) performed for the Department of Defense (DoD) Environmental Security Technology Certification Program (ESTCP). The ISMA was deployed *in situ* to conduct a treatability study to simultaneously assess natural attenuation, *in situ* biostimulation, and *in situ* bioaugmentation for the treatment of groundwater containing two comingling contaminants, trichloroethene (TCE) and hexavalent chromium [Cr(VI)]. Dechlorination of TCE to ethene was observed in bioaugmented microcosms; Cr(VI) reduction was observed in both bioaugmented and biostimulated microcosms. Results generated by the ISMA were compared to and found consistent with complimentary datasets produced from batch bottle treatability studies, laboratory column studies, and field pilot trials. From these results it is concluded that the ISMA is a suitable novel technology for conducting treatability studies that can compliment or replace contemporary bench-scale treatability study methods.

Introduction

The *In Situ* Microcosm Array (ISMA) technology is an innovative remedial design whose conceptual, structural and functional details were introduced in the preceding chapters. This chapter focuses on a closer examination of the data obtained with this new device and its limitations when seeking to investigate the appropriateness of engineered *in situ* reductive dechlorination for the bioremediation of groundwater impacted by co-mingling contaminants, in this case chlorinated ethene species and hexavalent chromium.

Strictly anaerobic consortia performing reductive dechlorination can biologically transform chlorinated ethenes, including PCE, TCE, DCE and VC. In these reactions, chlorinated ethenes undergo a stepwise dechlorination process to ultimately yield the innocuous end product ethene. The bacterial consortia performing these reactions contain *Dehalococcoides* type organisms that gain energy in this detoxification reaction by using the chlorinated ethenes as electron acceptors. Since the metabolic capabilities of *Dehalococcoides* are very limited, they rely on an association with other bacteria in order to obtain suitable carbon sources, vitamins and hydrogen required for dehalorespiration (Ziv-El et al. 2012; Löffler et al.).

It has been observed that dechlorination reactions at hazardous waste sites frequently are limited by unfavorable redox conditions (Air Force Center for Engineering and the Environment 2007) and a lack of bacteria featuring the metabolic capability of transforming TCE to ethene (Major et al. 2002). Therefore, it

is common practice to add carbon and electron sources to the subsurface in a process termed biostimulation to foster anaerobic conditions conducive to reductive dechlorination. At many sites, the reductive dechlorination process will stall at cis-DCE (McGuire et al. 2004), thereby necessitating the addition of microbial consortia containing *Dehalococcoides* (Stroo 2010).

Favorable redox conditions and the presence of *Dehalococcoides* are no guarantee for effective in situ bioremediation of chloroethenes, however, as the presence of inhibitors can slow or completely stall reductive dechlorination. One such known inhibitor of anaerobic microbial activity is hexavalent chromium, a heavy metal (Freedman, Lehmicke, and Verce 2005). Reduction of hexavalent chromium renders it less toxic, less soluble and less inhibitive to microbial metabolism (Richard and Bourg 1991; Rai, Eary, and Zachara 1989; Losi, Amrhein, and Frankenberger 1994). The reduction of Cr(VI) can be initiated by the addition of reductants to the subsurface (Freedman, Lehmicke, and Verce 2005; Hawley et al. 2004) or by stimulation of organisms capable of reducing it (Owlad et al. 2009). A number of studies show that Cr(VI), when present at elevated concentrations in the ppm range, can inhibit reductive dechlorination of chloroethenes for extended periods (Freedman, Lehmicke, and Verce 2005; Viamajala et al. 2004). However, the threshold of Cr(VI) toxicity for inhibition of reductive dechlorination at present is ill-defined (Arias and Tebo 2003), varies by hazardous waste sites and their resident microbial communities (U S EPA 2000), and thus cannot be predicted *a priori*.

In this project we employed the ISMA to determine in a field feasibility study whether reductive dechlorination of TCE will occur at a contaminated site in San Diego, CA. The work entailed pre-conditioning of ISMA sediment columns in the laboratory followed by their transfer into the field and operation for several weeks in the subsurface contained in the ISMA device. The objectives of this demonstration deployment were to:

(i) Demonstrate ISMA capability of conducting mutually exclusive experiments in the same well

(ii) Demonstrate no residue was released into monitoring well during testing

(iii) Demonstrate ISMA ability to determine potential side effects of remediation strategies

(iv) Reproduce outcome of prior lab studies in the ISMA

(v) Reproduce outcome of prior field trials in the ISMA

Materials and Methods

Deployment Location, Site Description, and History of Prior Work Leading Up to the ISMA Deployment

NAS North Island (NAS NI) is located in San Diego County, California, southwest of the city of San Diego, on the tip of the Silver Strand peninsula adjacent to the city of Coronado. The remainder of NAS North Island is surrounded by water, with the Pacific Ocean to the south and San Diego Bay on the west and north (Figure 9). North Island was commissioned in 1917 and is currently an active military base.

Since 1935, NAS North Island has been occupied exclusively by the Navy. Operable Unit 20 (OU-20) is located on the northeast portion of the island.

Industrial processes performed in Buildings 1 and 2 at OU-20 are the likely source of hexavalent chromium in groundwater (Figure 10). Past operations at Building 1 were related to helicopter blade repair and maintenance, as well as the manufacture and repair of fiberglass components. Activities included parts grinding, cleaning, anodizing, paint stripping, and painting. Liquid wastes and rinse waters from these operations were piped to the Industrial Waste Treatment Plant via an industrial waste pipeline that was discovered to have breaks in it. Additional contributions to the subsurface contamination may have included overflow of subsurface pits used for temporary waste storage, and outdoor aircraft fuel tank washing.

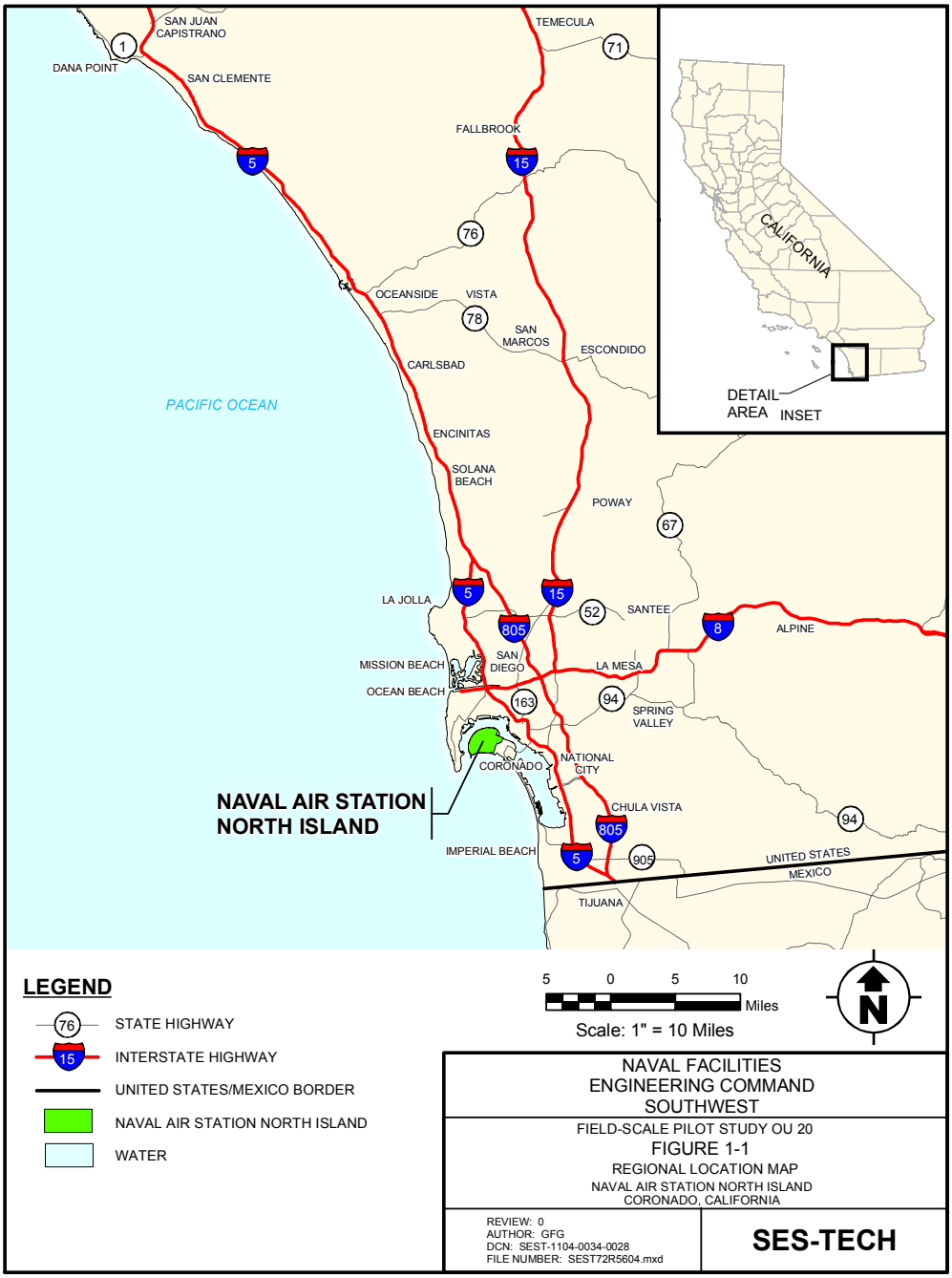

Figure 9. Regional Location Map of NAS NI in California.

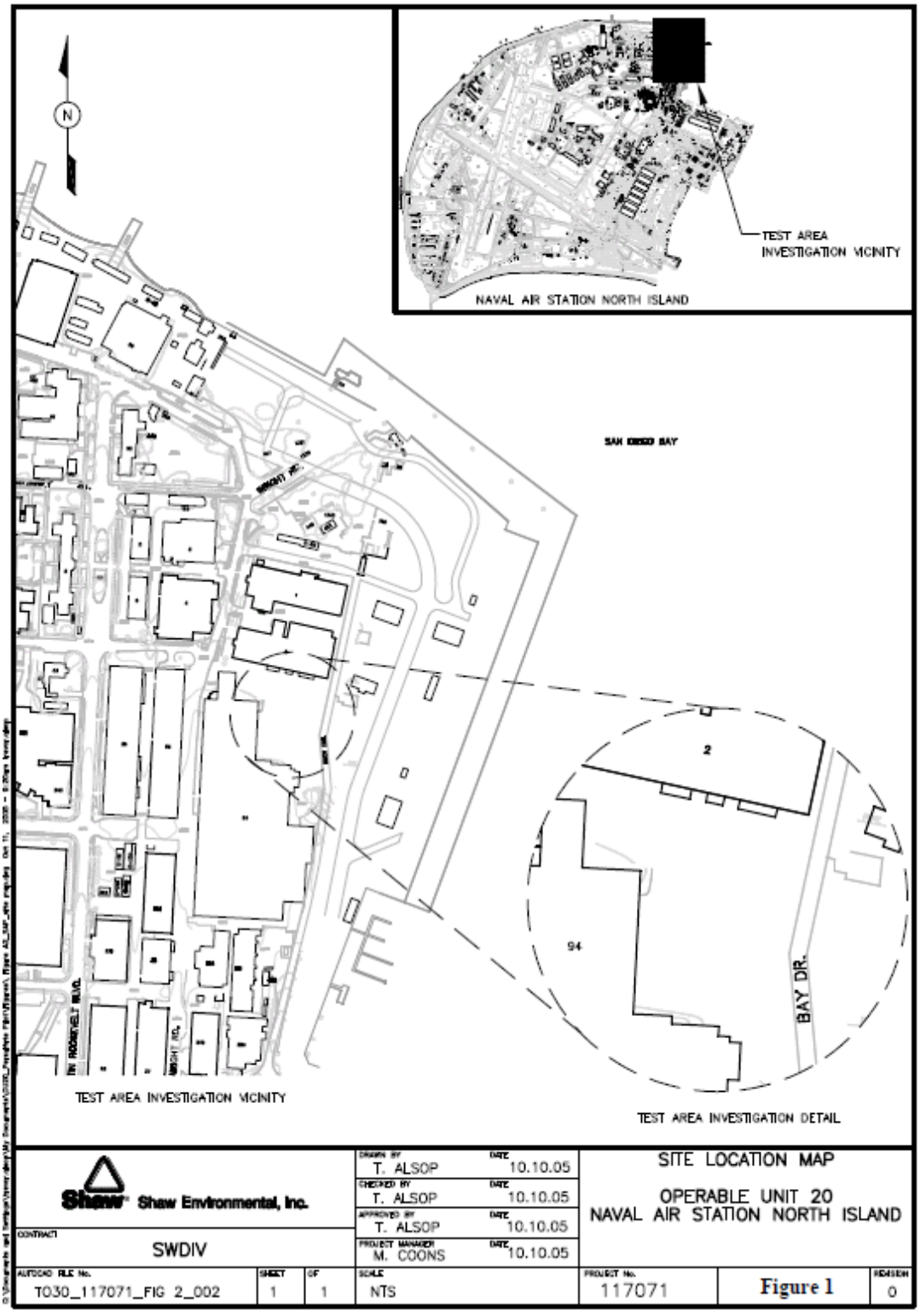

Figure 10. Site location map: OU 20

Site Geology and Hydrogeology

NAS NI is located on relatively flat land with an average elevation of approximately 20 feet above sea level. The island was enlarged beginning in the 1930s through placement of hydraulic fill dredged from San Diego Bay onto tidal flats and nearshore areas. All of NAS NI has been graded for development, and the area surrounding Buildings 1 and 2 is covered with asphalt, concrete, or maintained landscaping. The hydraulic fill used to construct much of NAS NI consists of medium-grained to coarse-grained, poorly graded sands and silty sands. In some areas, the fill is underlain by organic silts and clays.

Since most of NAS NI is paved, groundwater recharge is minimal and occurs primarily from irrigation. Shallow groundwater beneath NAS NI is unconfined, and groundwater occurs at depths from approximately 4 to 25 feet below ground surface (bgs). Groundwater in the investigation area flows northeast and discharges into San Diego, not accounting for temporary fluctuations due to tidal influence.
The groundwater level in OU-20 is approximately 5 feet above mean sea level (msl). The groundwater gradient across the study area is relatively flat and ranges from 0.001 to 0.002 foot per foot. Groundwater flow direction is to the north/northeast. Aquifer transmissivity values calculated from slug and pumping tests in the Building 379 area ranged from 0.5 to 1,116 square feet per minute (ft^2/min), with an approximate value of 418.5 ft^2/min calculated nearest to the ISMA deployment location (well S1-MW-9) (SES-TECH 2010a).

Contaminant Distribution

The OU-20 VOC and Cr(VI) plumes are located in the northeastern portion of NAS North Island. The VOC plume originates from the vicinity of Building 379 and extends downgradient to the northeast approximately one half-mile, with several sources contributing. The Cr(VI) plume originates in the vicinity of building 2, with the former anodizing shop in Building 2 as the most likely source of Cr(VI), and extends downgradient approximately 700 ft (Figure 11).

The ISMA deployment well OU20-PEW-01 is located on the southwest edge of the chromium plume, in the parking lot located between buildings 2 and 94, and marked in Figure 11 with a red circle. This well was chosen because it was (i) preexisting, (ii) sufficiently sized to accommodate the ISMA, (iii) outside and up-gradient of the field pilot-test areas, and (iv) minimally disruptive to traffic and logistically easy to access due to its location in a parking lot.

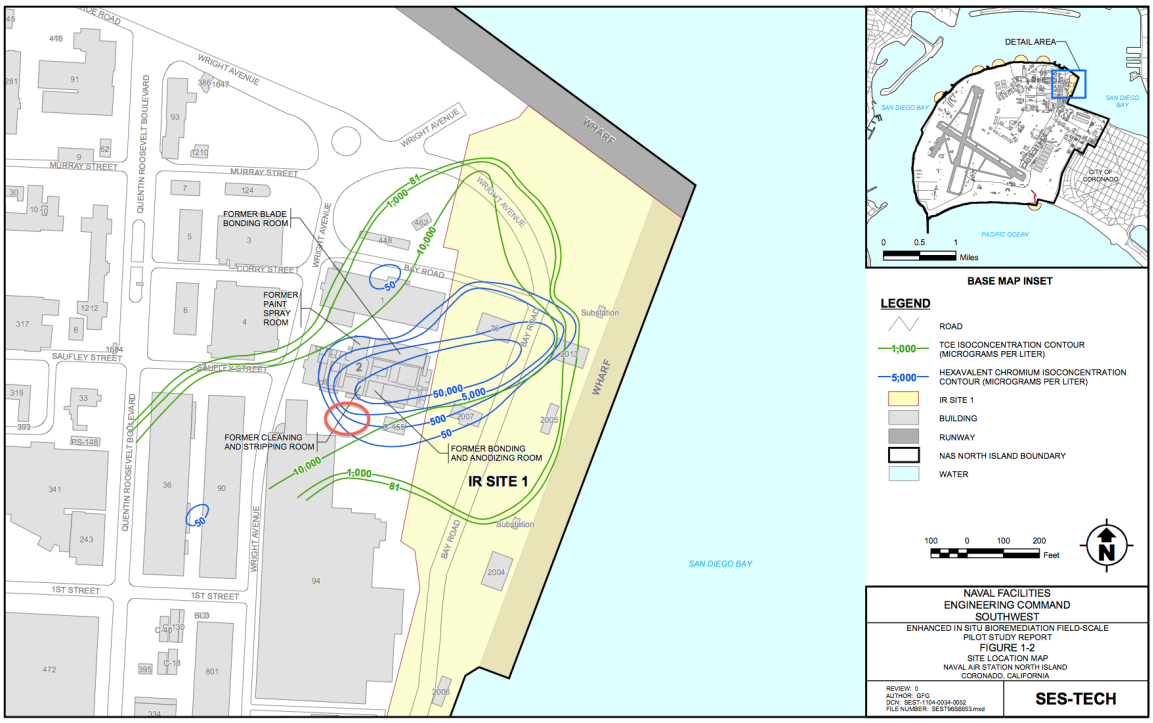

Figure 11. OU 20 TCE and Cr(VI) Plumes at OU-20.

Prior Laboratory Treatability Studies

The following subsection is a brief summary of the relevant laboratory treatability studies investigating *in situ* treatments for OU-20 prior to 2010 (SES-TECH 2010b).

SiRem Inc. was retained to evaluate 5 *in situ* treatments for the Cr(VI) and TCE present at OU-20 in bench-scale batch bottle tests. The slow-release substrate SRS–M (Terra Systems Inc., Wilmington, DE) in conjunction with bioaugmentation culture KB-1® (SiRem Inc., Guelph, Ontario Canada) was identified as the best performing and most cost-effective remediation strategy (associated batch microcosm result presented in Figure 12). Below are the manufacturers' descriptions of the chosen amendments:

SRS®-M contains a proprietary food grade reductant compound plus 60% soybean oil, food grade emulsifiers, sodium lactate, and organic and inorganic nutrients including nitrogen, phosphorus, and vitamin B12. Additionally a reductant reacts directly with hexavalent chromium to reduce it to the trivalent state. SRS®-M provides a readily degradable carbon (lactate) to rapidly generate reducing conditions and a long-lasting carbon source (soybean oil) to maintain the reducing conditions.

KB-1® is a bioaugmentation culture that contains *Dehalococcoides* (Dhc), the only group of microorganisms documented to promote the complete dechlorination of chlorinated ethenes to non-toxic ethene.

More detailed analysis of lab treatability study results is presented in results.

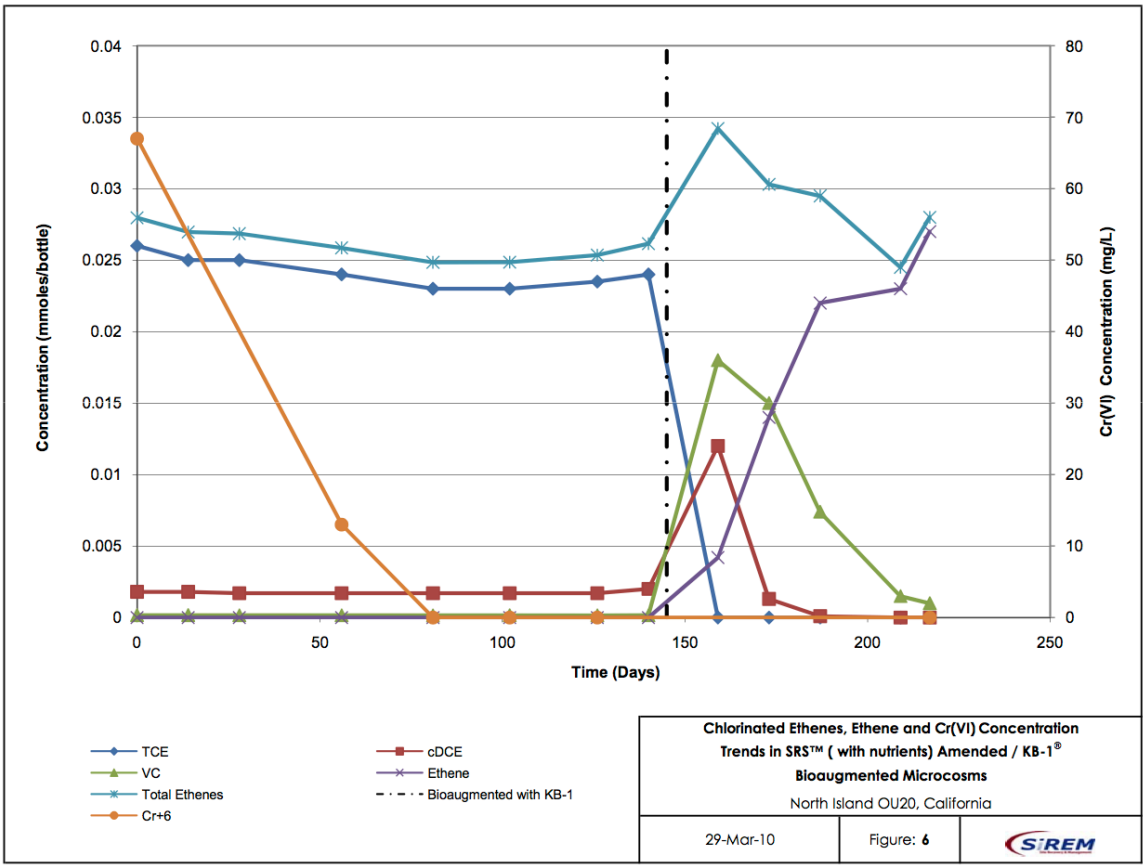

Figure 12. Sampling results from a batch bottle microcosm study performed by SiRem. Data demonstrates the effectiveness of bioaugmentation with KB-1® for treatment of groundwater from OU-20 at NAS NI.

Prior Field-Scale Pilot Study

A brief summary of the relevant feasibility study objectives is presented here (SES-TECH 2011):

Stated objectives of the field-scale pilot test were to:

(i) Evaluate the capacity of the formation to receive the injected amendments.

(ii) Evaluate the distribution and survivability of injected bioaugmentation cultures.

(iii) Evaluate radius of donor delivery (RODD).

(iv) Evaluate the effectiveness of the donor in reducing concentrations of Cr(VI) and TCE in groundwater.

(v) Evaluate the potential for contaminant presence in vadose zone soils and effectiveness of the amendment in reducing contaminant levels in soils.

The two injection methods tested - liquid atomized injection and direct-push injection - were both found to be effective at distributing the donor and culture in the aquifer; direct-push injection was chosen as the delivery method for full-scale implementation based on a cost analysis.

Reductions in Cr(VI) and chlorinated ethene concentration were observed within one to three months of injecting amendments *in situ*. SRS-M and KB-1® injections were recommended for full-scale implementation. See results section for a detailed comparison between field-scale, bench-top laboratory, and ISMA results.

Conceptual Experimental Design

The primary goal of this ISMA demonstration was to showcase the functionality of the ISMA by studying TCE dechlorination in the presence of hexavalent chromium. Secondary goals were to compare the data output of the ISMA to the extant data sets associated with the deployment site. Accordingly, the treatability experiment conducted in the ISMA was designed to be as comparable as possible to the extant lab and field treatability data sets associated with the deployment location.

The deployment of the ISMA technology encompassed: (i) the delivery of the self-contained ISMA device into the screened interval of a deployment well; (ii) incubation of the device for a period of several days to weeks; (iii) removal of the device from the deployment well; and (iv) analysis of the miniature sediment columns contained therein, and of each column's effluent that is also stored in the device and retrieved from the well together with the ISMA apparatus after testing (Miller 2005).

The hardware used in this demonstration had 12 liquid flow channels that were allocated between experimental groups to balance the desires for a large number of experimental groups (i.e., number of treatments tested) and a large number of replicates per experimental group (i.e., statistical significance of results). In an effort to meet both primary and secondary demonstration objectives, the allocation of liquid flow channels in the field demonstrations detailed here balanced both desires and thus featured 3 experimental groups conducted in triplicate (Table 2).

Table 2. Experimental plan for NAS North Island.

Experimental Group	Replicates	Column Medium	Inoculum	Amendment
Monitored Natural Attenuation (MNA)	3	Site Sediment	-	-
Biostimulation	3	Site Sediment	-	Sodium Lactate
Bioaugmentation	3	Site Sediment	KB-1®	Sodium Lactate
Influent Control	3	-	-	-

Column Construction and Preconditioning to Facilitate Conditions Suitable for Reductive Dechlorination

The following is a summary of sediment column construction and operation in the laboratory at ASU prior to column deployment *in situ* at NAS NI.

Column construction: On Aug. 22, 2011, composite sediment from the drilling of multiple wells the previous week at NAS NI was collected into a 5 gallon bucket and transported back to ASU. In the ASU lab, the sediment was transferred into a shallow tray and allowed to air dry in the fume hood over a period of approximately 3 days. Dried sediment was then sifted to collect particles ranging in size from 1000 to 250 μm in diameter, that were then packed into 9 glass ISMA columns.

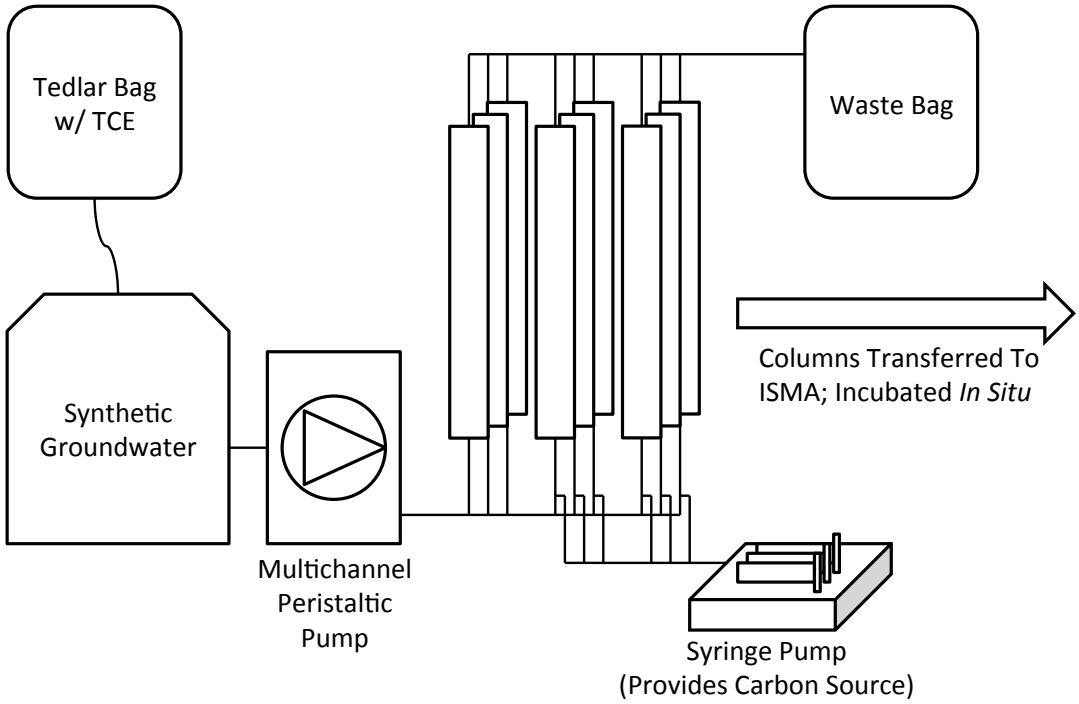

Figure 13. Schematic of laboratory column setup. To ensure a stable TCE concentration in the influent, a Tedlar bag, filled with air already at equilibrium with the headspace in the groundwater bottle was connected to the groundwater bottle so that it supplied the bottle with air as the groundwater was pumped out. Columns were fed with mineral media (as detailed in chapter 2) in a pulsed influent-feed cycle, with the pumps on for 90 seconds at a flow rate of 56 μL/min, followed by a 240 second pause, resulting in an effective flow-rate of 0.91 mL/hour, which translates into a residence time of 10.45 hours and a linear velocity of 0.54 m/day, assuming a porosity of 0.4. Annotated pictures of column array are presented in Figure 14 and Figure 15.

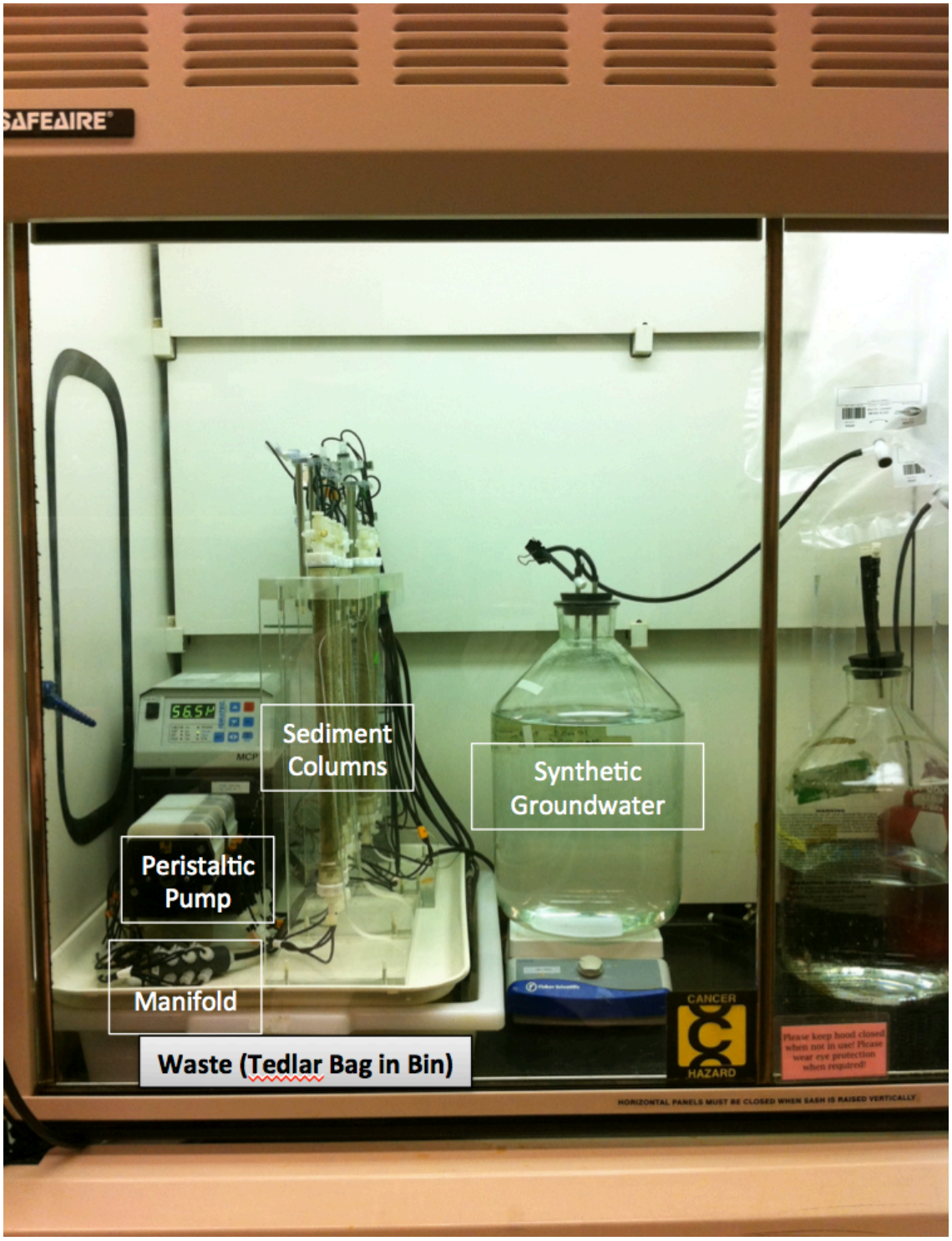

Figure 14. Picture of laboratory column setup.Columns filled with sediment from NAS NI being operated under continuous flow conditions in the laboratory. Entire assembly takes up approximately 5 sq. ft. in a fume hood.

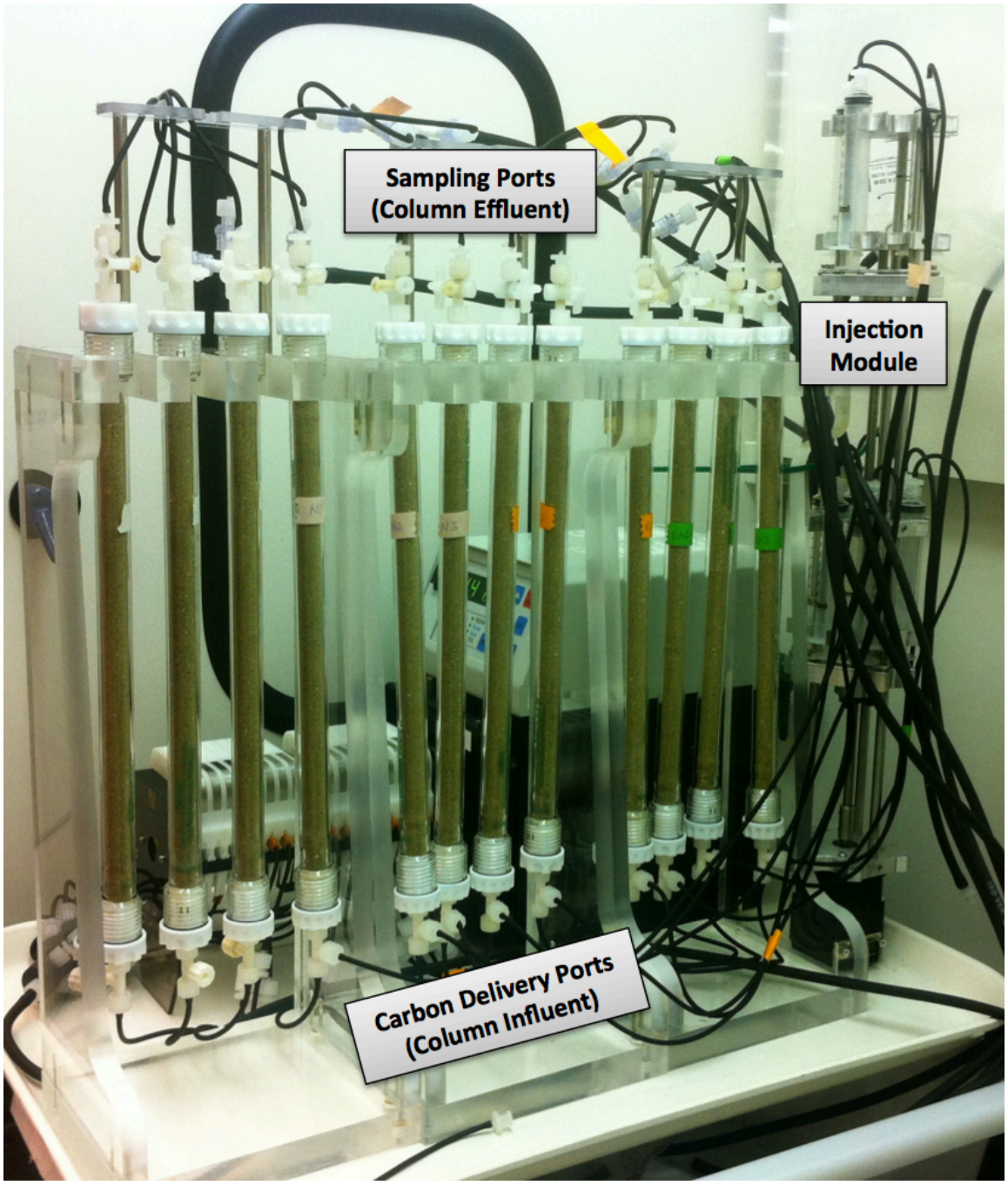

Figure 15. Closeup view of the sediment columns and the Injection Module shown in Figure 14.

Column effluent samples were analyzed for chlorinated ethenes and ethane using an automated headspace solid phase microextraction followed by gas chromatography

and flame ionization detection method (HS SPME GC-FID) developed in our laboratory that enabled accurate measurements with only 0.2 mL of liquid [28]. After 5 days, once TCE concentrations in column effluent had stabilized and matched the 15 µg TCE/L supplied in influent, the three columns comprising the bioaugmentation experimental group were inoculated w/ KB-1®. Inoculation was performed with a gas-tight syringe by injecting approximately 3 mL of the microbial culture as received from SiRem Inc. in a serum bottle into the influent (bottom) port of the column. Immediately after inoculation, the influent of the six columns comprising the bioaugmention and biostimulation experimental groups began to be amended with sodium lactate. The amendment, a 10% w/v sodium lactate solution, was continuously dispensed to each column influent at flowrate of 0.231 µL/min from an array of six 10 mL plastic syringe powered by the ISMA injection module, resulting in an effective concentration of 50 µM lactate in each column's influent. On Day 12, after complete conversion of influent TCE to cis-2-dichloroethene (cDCE) was observed in the bioaugmented columns, the columns were reinoculated with KB-1® to ensure presence of viable populations of obligate anaerobes.

Figure 16 shows the results for molar fractions of chlorinated ethenes and ethene detected in column effluent. Each graph represents the average of 3 columns. For each graph, mass is normalized to the total molar mass of TCE, cDCE, VC, and ethene collected at that sampling event. On day 75, 70 days after the initial inoculation event, all bioaugmented columns were successfully converting all influent TCE to ethene. In the same timeframe, biostimulated columns were only converting approximately half of influent TCE to ethene, and unamended columns

showed no evidence of reductive dechlorination. After 80 days of operation in the laboratory, columns were transferred in situ to NAS NI.

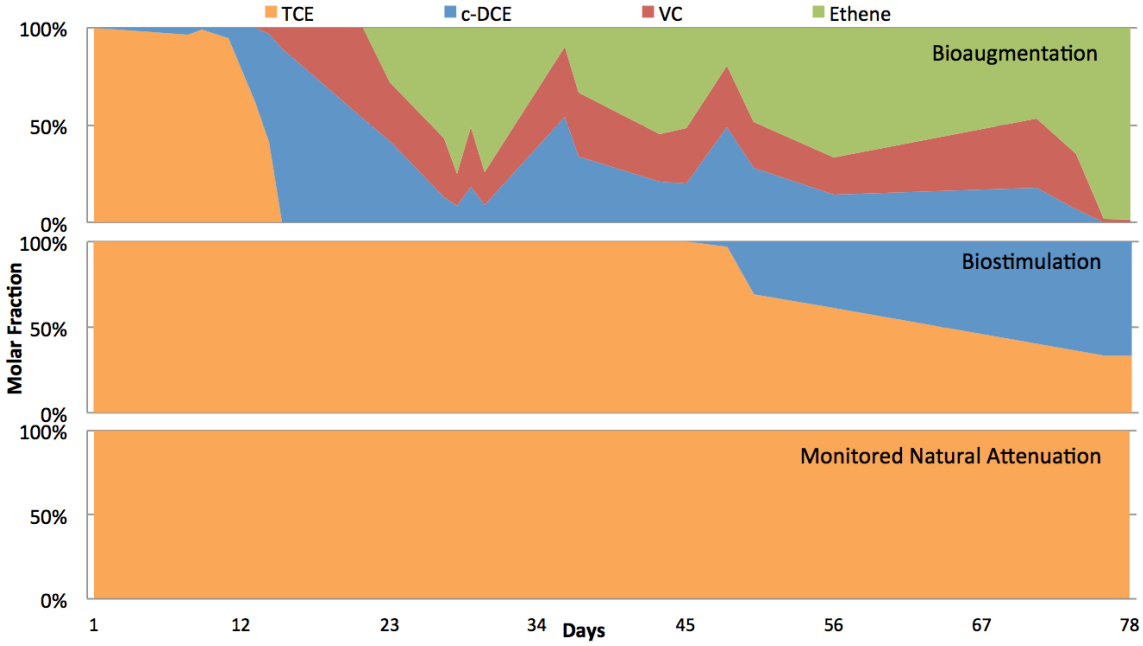

Figure 16. Results from laboratory column effluent samples.

Field Testing

The individual sections of the ISMA were pre-assembled in the lab prior to field-testing. Upon transferring the active conditioned columns to the field ISMA unit, the ISMA pumps were recalibrated while the ISMA was suspended vertically from the custom-build ISMA stand. All the flow channels were checked for any leaks or loose fittings prior to assembly. The ISMA was then transported by truck to NAS North Island for deployment in the subsurface. A Boom truck was used to lower the unit into the ground where it was incubated for 35 days in well OU-20-PEW-01.

Throughout the deployment period, grid power (110V) supplied by NAS NI was used to power the device. The schedule of deployment activities is presented in Table 3.

Table 3. Schedule of deployment activities.

Project start: 05/01/2009	2011				2012	
Tasks	**Q1**	**Q2**	**Q3**	**Q4**	**Q1**	**Q2**
Column Pre-conditioning for NAS NI		X	X	X		
Deployment of ISMA at NAS NI				X		
Incubation at NAS NI				X	X	
Retrieval of ISMA at NAS NI					X	

Water Sampling

Prior to deployment of the ISMA, a water sample was retrieved from the deployment well and analyzed for dissolved oxygen (DO), redox potential (ORP) and pH in the field using a pre-calibrated multi-parameter probe (YSI Inc., Yellow Springs, OH). A pre-deployment well grab sample was also shipped to a commercial laboratory (EMAX laboratories, Torrance, CA) that analyzed for concentrations of chlorinated ethenes (TCE, cDCE, VC) as well as concentrations of dissolved metals that are relevant for drinking water (arsenic, chromium, iron, manganese, selenium).

After incubation in the subsurface for 35 days, the ISMA device was retrieved from the well. Effluent samples were retrieved from the device and sent to the analytical lab for analysis, along with a post-deployment well-grab sample. All samples were handled using proper chain-of-custody procedures. The sampling plans and methods are presented in Table 4 and Table 5.

Table 4. Water Sampling performed for ISMA deployment at NAS NI.

Component	Sample Collected	# of Samples	Sample Volume	Analytes	Comments
Prior to deployment	Groundwater from deployment well	1	1L	TCE, c-DCE, VC, TDS, drinking water metals, inorganic anions	To assess groundwater quality before deployment
During deployment of ISMA	Untreated groundwater collected from bypass channels (ISMA captured effluent)	3	0.75L	TCE, c-DCE, VC, TDS, drinking water metals, inorganic anions	To assess groundwater quality during deployment
During deployment of ISMA	Groundwater flowing through sediment column without amendment (ISMA captured effluent)	3	0.75L	TCE, c-DCE, VC, TDS, drinking water metals, inorganic anions	To assess potential for Monitored Natural Attenuation
During deployment of ISMA	Groundwater flowing through sediment column with amendment 1 (ISMA captured effluent)	3	0.75L	TCE, c-DCE, VC, TDS, drinking water metals, inorganic anions	To assess treatment potential of amendment 1
During deployment of ISMA	Groundwater flowing through sediment column with amendment 2 (ISMA captured effluent)	3	0.75L	TCE, c-DCE, VC, TDS, drinking water metals, inorganic anions	To assess treatment potential of amendment 2
After deployment	Groundwater from deployment well	1	1L	TCE, c-DCE, VC, TDS, drinking water metals, inorganic anions	To assess groundwater quality after deployment

Table 5. Analytical Methods used for ISMA deployment at NAS NI.

Matrix	Analyte	Method	Container	Preser-vative	Analytical Laboratory	Holding time
Ground water	TCE, cDCE, VC	EPA Method 524.2 (Capillary Column GC/MS)	Gastight glass bottle	Maleic acid (0.625 g/L); Ascorbic acid (5g/L)	Columbia Analytical Services, Phoenix, AZ	14 days
Ground water	Total Dissolved Solids	Standard Methods, Section 2540C	HDPE bottle	As required	Columbia Analytical Services, Phoenix, AZ	7 days
Ground water	Drinking Water Metals (As, Mn, Cr, Fe, …)	EPA Method 200.7 (ICP/AES)	HDPE bottle	Nitric acid (pH 2)	Columbia Analytical Services, Phoenix, AZ	6 months
Ground water	Inorganic Anions (Cl^-, SO_4^{2-}, NO_3^{2-}, NO_2^{2-})	EPA Method 300.1 (IC)	HDPE bottle	As required	Columbia Analytical Services, Kelso, WA	48 hrs

Rate Calculations

An important performance objective of this deployment was to reproduce the outcome of prior laboratory studies in the ISMA. However, batch microcosms, ISMA microcosms, and field pilot trials produce results in forms that are not immediately comparable. In order to enable a direct comparison, it is first necessary to reduce the data sets to a common data-reduction endpoint. For this objective the first-order rate degradation constants were chosen as the equivalency basis on which to make a comparison between the data sets.

Batch Microcosms:

Laboratory Batch Bottle rate constants were calculated as follows:

$$k = \frac{\ln(C_{baseline}) - \ln(C_{teatment})}{t_{treatment} - t_{baseline}}$$

where $C_{baseline}$ and $t_{baseline}$ were the concentration and time point prior to amendment, respectively, and $C_{treatment}$ and $t_{treatment}$ were the contaminant concentration and time point when the contaminant was no longer detectable, or at the last sampling point, whichever was sooner.

The rate was calculated in this manner for each replicate bottle. The average and standard deviation of the rate constants is reported in Table 9 and Table 10. Note, for the SRS + nutrients + KB-1® experimental group, $C_{treatment}$ and $t_{treatment}$ for the TCE rate constant was taken from the last sampling point prior to KB-1® amendment; the associated graph is shown in Figure 12.

ISMA:

ISMA rate constant values generated from the deployment were calculated as follows:

$$k_{Composite} = \frac{\ln(C_{i_{\text{Influent}}}) - \ln(C_{i_{\text{Effluent}}})}{\Delta T_{\text{Column}}}$$

where, ΔT_{Column} is the residence time within the column, $C_{iInfluent}$ is the average concentration in the MNA experimental group effluent, and $C_{iEffluent}$ is the concentration of the contaminant in the column effluent collected throughout the in situ incubation period.

This is similar to the approach taken for batch bottle calculations, with the additional correction that concentrations detected in the influent and effluent are composite samples. The MNA experimental group is taken as the influent baseline due to the fact that an incomplete mass balance might be the result of volatilization losses through the column assembly, and attributing those losses to biodegradation would yield an overly optimistic rate constant.

Field:

Field pilot trial results performed at NAS NI previously by other parties were inconsistent. A few of the monitoring wells showed relatively rapid reduction, but some showed no appreciable differences, or rapid rebound after SRS-M injections. Consequently, only the maximum rate constant calculable from a single monitoring well is reported. Variable sourcing for rate calculates are summarized in Table 6.

Table 6. Variable Sourcing for Rate Calculations at NAS NI.

Variable	Lab Batch Bottle	Field Pilot Trial	ISMA
$C_{control}$	Baseline concentration at T_0	Baseline concentration at T_0	Concentration in bypass (influent concentration)
$C_{treatment}$	Concentration after treatment and after no further activity was observed	Lowest concentration detected in treatment well	Concentration in treated effluent
ΔT	Time between $C_{control}$ and $C_{treatment}$	Time between $C_{control}$ and $C_{treatment}$	Calculated column residence time

Results

The ISMA was incubated in well OU20-PEW-01 at NAS NI for 35 days. I first assessed whether the ISMA deployment had any impact on the well. I compared pre- and post-deployment well grab samples and observed that concentrations in the well fluctuated significantly between deployments. The deployment well is in a tidal zone and as such multiple parameters will fluctuate over time, as shown in Figure 17 and Table 7. Despite the fluctuations due to tidal influence, no leaks were evident in the ISMA or ISMA housing, and no materials were released from the ISMA into the well.

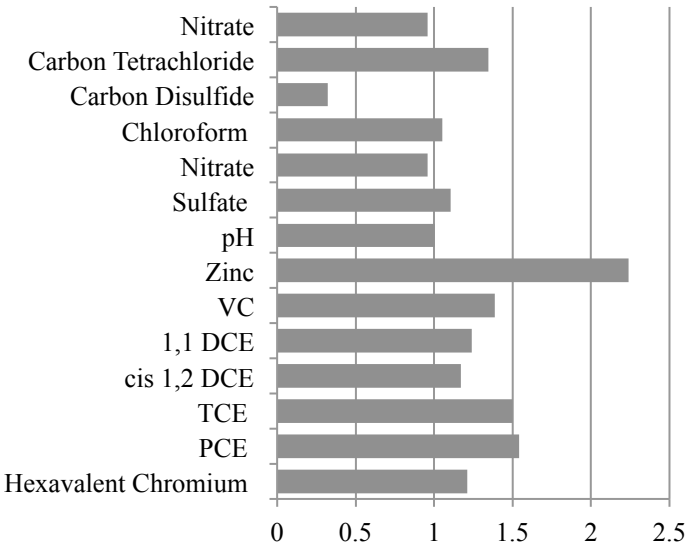

Figure 17. Groundwater chemistry in well grab samples before and after ISMA deployment at site 1. Change in concentration after ISMA deployment represented as C/C0. Fluctuations observed are due to tidal influence, additional parameters presented in Table 7.

Table 7. Characteristics of groundwater quality parameters determined pre- and post-deployment of the ISMA. Any difference observed were within the range expected in a well that is subject to tidal movement of groundwater.

	Pre-deployment	Post-deployment
Depth to water (ft)	3.3	3.6
Temperature (°C)	20.46	21.1
Dissolved Oxygen (mg/L)	9.92	5.96
ORP (mV)	83.1	240.7
pH	7.85	7.7
Conductivity (µS/cm)	1683	1148
Salinity (%)	0.94	0.62

During the incubation period the ISMA pumps operated in a pulsed mode analogous to pumps operated in the laboratory during column preconditioning: pumping for 90 seconds at a flow rate of 69.2 µL/min (as calibrated in the laboratory), then pausing for 284 seconds. Target net flow rate was 16.6 µL/min with a target collected effluent volume of 840 mL and a target column residence time of 9.54 hours. Actual volumes collected were 20% lower than targeted, with an average and standard deviation of 665.5 ± 57.4 mL (greater detail in Table 8). The discrepancy between targeted and collected volumes suggests that lab calibration procedure failed to account for the backpressure pumps experienced when the ISMA was fully assembled. However, simulating the full hydraulic head differential in the laboratory for pump calibration is not practical. Future deployments should benefit from the empirically derived 20% correction factor when calibrating pumps.

Table 8. Groundwater collected during ISMA incubation.Column parameters of residence time, groundwater linear velocities, and pore volumes exchanged assume a porosity value of 0.4.

	Bypass			MNA			Biostimulation			Bioaugmentation		
Channel #	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
Volume collected	644.0	745.4	621.6	559.0	681.8	593.0	681.1	713.3	751.2	680.0	701.3	614.0
Effective flowrate (ul/min)	12.78	14.79	12.33	11.09	13.53	11.77	13.51	14.15	14.90	13.49	13.91	12.18
Column residence time (hours)				14.28	11.71	13.46	11.72	11.19	10.63	11.74	11.38	13.00
Average Linear Velocity (ft/day)				1.38	1.68	1.46	1.68	1.76	1.85	1.68	1.73	1.51
Pore volumes exchanged				58.82	71.74	62.40	71.67	75.06	79.05	71.55	73.80	64.61

After ISMA retrieval, collected effluent was subsequently analyzed for hexavalent chromium (Figure 18) as well as chlorinated ethenes and ethene (Figure 19). Relative to the collected influent, no reduction of hexavalent chromium concentrations was observed in MNA effluent, while both biostimulation and bioaugmentation showed approximately 20% lower concentrations in effluent. These results indicate that stimulation with sodium lactate facilitates chromium reduction, but that additional bioaugmentation with KB-1® does not further enhance chromium reduction. These results are consistent with the available site-specific bench-top batch bottle treatability studies. A detailed comparison of attenuation rates between batch bottles and in situ column data is presented in the results section.

Chlorinated ethene results showed approximately 20% lower concentrations of TCE in MNA effluent relative to the influent. This difference can be the result of abiotic TCE attenuation processes (Lee and Batchelor 2002), or may be a result of additional mass loss due to volatilization in the additional length of tubing, fittings, and column apparatus that groundwater must traverse in the MNA experimental lines. Effluent from biostimulation columns showed no difference in detected TCE concentration. Slightly elevated *cis*-DCE concentrations were observed in bioaugmentation samples relative to MNA, however the difference was not statistically significant (homoscedastic 2 tailed student t-test, $p=0.1$), and the mass of TCE that may have been lost to biological reduction to cis-DCE was smaller than the overall variability in TCE concentrations detected. Effluent from bioaugmentation columns, however, contained significantly reduced concentrations of TCE ($p<0.05$), and elevated levels of cDCE ($p=0.08$), VC ($p<0.05$), relative to MNA.

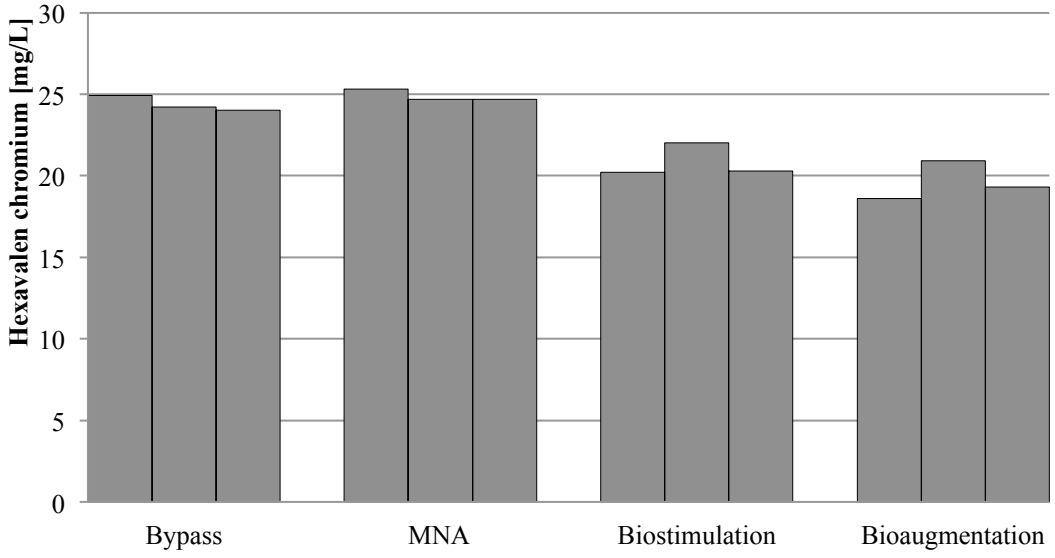

Figure 18. Hexavalent chromium detected in ISMA effluent post *in situ* incubation.

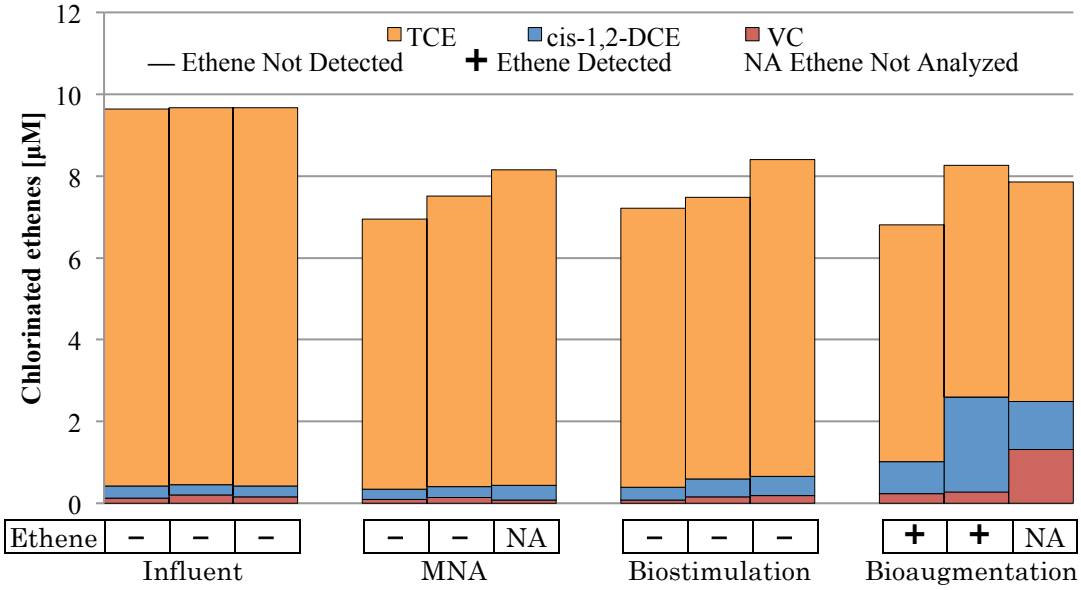

Figure 19. Chlorinated ethenes and ethene detected in ISMA effluent post *in situ* incubation.

Quantification of ethene is challenging due to ethene's extremely high volatility and the fact that it does not sorb well to activated carbon or other sorbents. As mentioned in the previous chapter, a sorbent cartridge installed in the ISMA assists with capture of volatile organics, but unfortunately, not with ethene. As a result, quantification of ethene is not possible due to the fact that the bulk of any ethene produced will volatilize and escape through the vent line installed in each effluent capture vessel. Nevertheless, liquid effluent was analyzed for any traces of ethene remaining. Ethene was detected in two bioaugmentation effluent samples at levels below the commercial lab's reporting limit of 1.2 µg/L (0.04 µM), but above the detection limit of 0.6 µg/L (0.02 µM). Unfortunately, ethene analysis was not possible for the effluent from the 3rd bioaugmentation column effluent, due to the fact that all sample was consumed for the analysis for chlorinated ethenes, which was given higher priority. However, analysis of column pore water withdrawn from

the column post deployment indicates that the third bioaugmentation column likely had the highest amounts of ethene produced (Figure 20). No ethene was detected in any other column pore water examined after ISMA retrieval.

The effluent data collected illustrate some of the challenges that come from using a miniaturized sediment column that is only 25cm in length. Many of the attenuation processes important for remedial design are only measurable over longer, sometimes much longer distances. A column 25cm in length can practically only simulate processes that take place over only 25cm, and can only provide limited, if any, insights into process that take place over kilometers. However, the converse of this implies that, if only a comparatively minor change is observed over a 25cm column, then the implications of that result are huge when the results are extrapolated to the subsurface environment, where a plume can span a much longer distance. In the context of this study, the short column length limits any conclusions that can be drawn about MNA, because MNA processes, if present, would be much slower than the ISMA is currently capable of measuring. In contrast, the successful bioaugmentation treatment observed, while seemingly modest at 20%, is actually quite significant in light of the fact that it was observed over only 25cm.

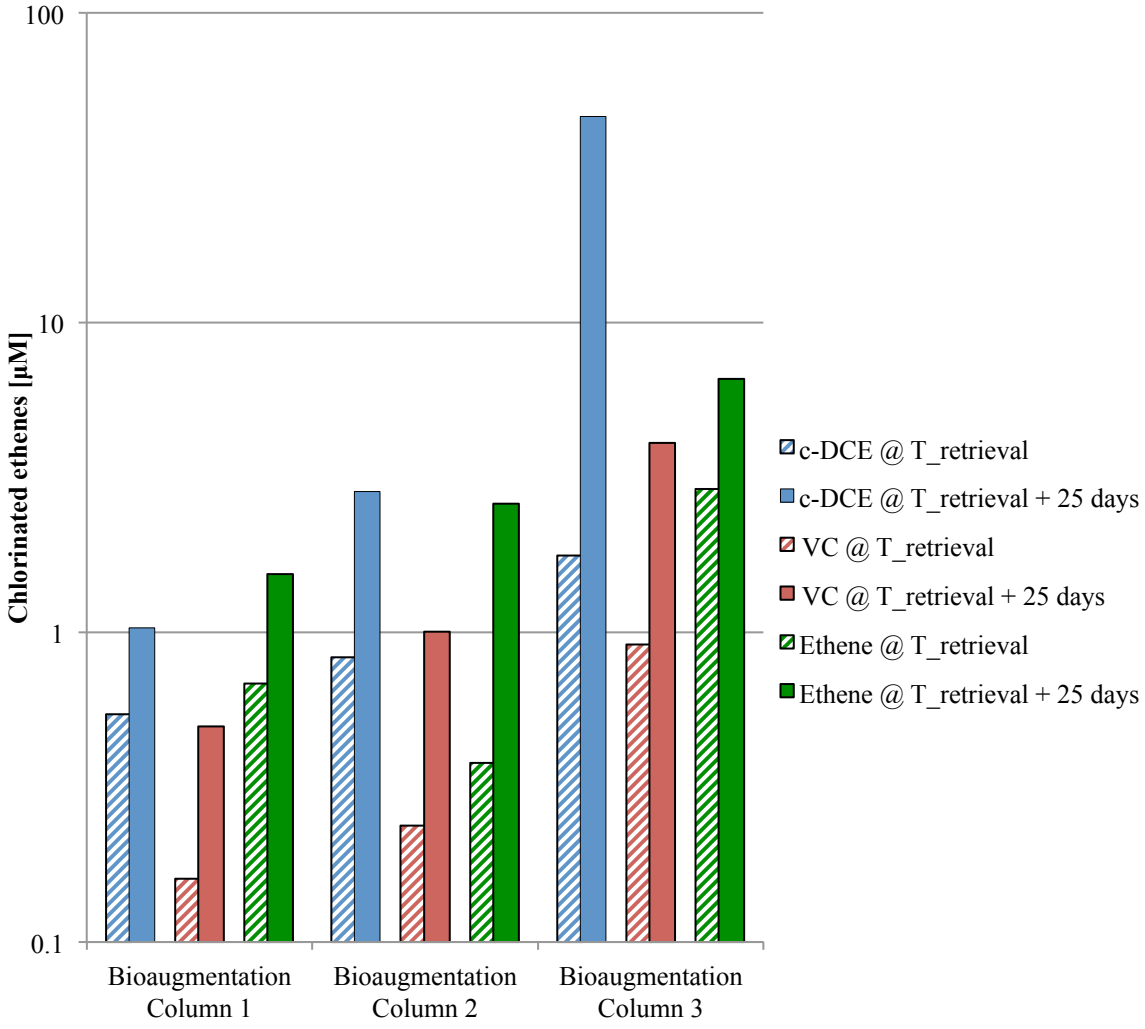

Figure 20. Column pore water analysis for chlorinated ethenes and ethene after retrieval and again after 25 days without flow. Increased concentrations of TCE dechlorination products in all the columns indicate that columns were still biologically active and dechlorinating after *in situ* incubation and exposure to Cr(VI).

Additional work was carried out to establish unequivocally that detected ethene was indeed the product of ongoing biotransformation by the bioaugmented, strictly anaerobic microbial community. After ISMA retrieval and transport back to the lab, the sealed sediment columns were incubated in the laboratory without flow at 20°C,

which is equivalent to the temperature of the groundwater in situ at the deployment site. The column pore water was then sampled 5 times over a period of 25 days and analyzed for the presence of chlorinated ethenes. Over the sampling period, the bioaugmentation columns showed trends of decreasing TCE concentrations and increasing VC, *cis*-DCE, and ethene concentrations. The first and last sampling points are presented in Figure 20. Results show production of dechlorination products during the post-deployment incubation, indicating that all biological activity in the columns was ongoing after *in situ* incubation.

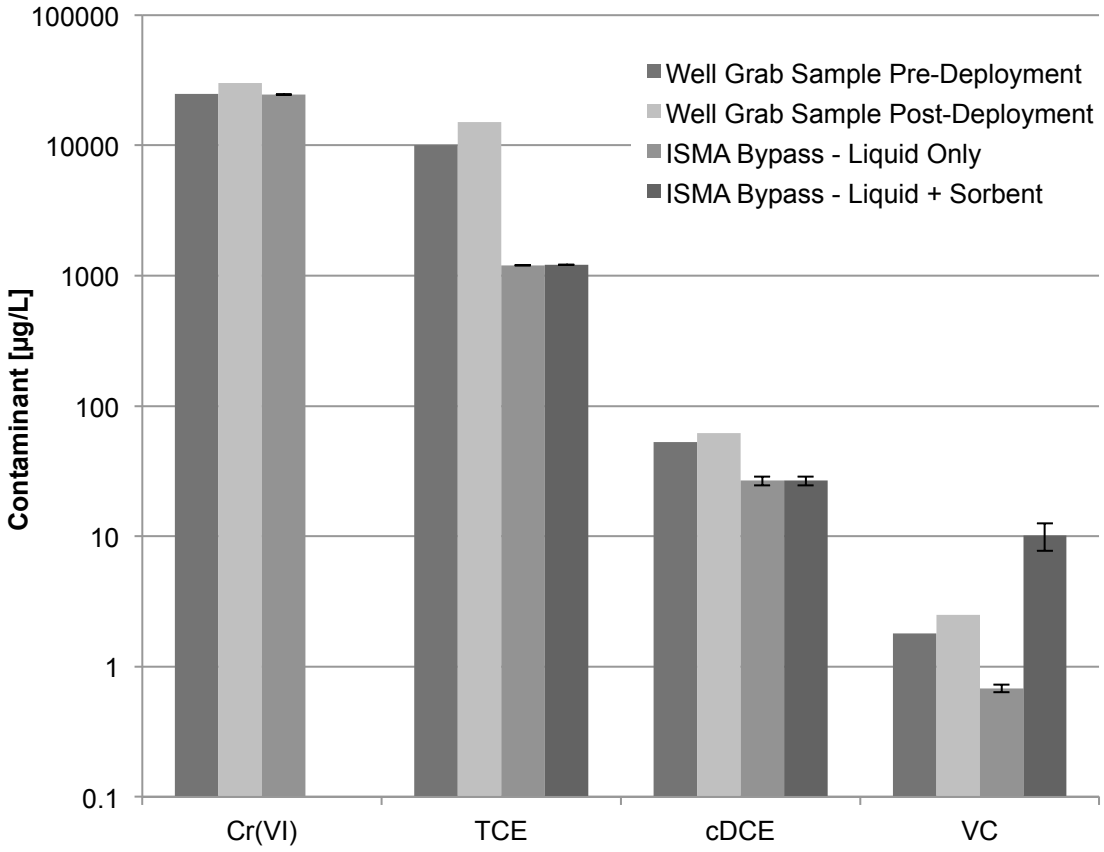

Figure 21. Concentrations of primary contaminants detected in the ISMA deployment well compared to those found in ISMA bypass channels.

Further work was done to assess the ISMA's performance at mass capture. Comparisons between ISMA influent and well grab samples demonstrate that the ISMA has excellent capture ability of non-volatile and stable compounds like hexavalent chromium. These results are consistent with other parameters analyzed from the NAS NI deployment (not shown). Results shown in Figure 21 also demonstrate that recovery and *in situ* preservation of volatile compounds like TCE is challenging. Concentrations of volatile compounds detected in the influent stored in the ISMA were up to an order of magnitude lower than those detected in well grab samples. These known losses have to be attributed to the extended holding period of groundwater in the effluent capture vessels. This result is supported by the fact that concentrations of chlorinated ethenes detected in groundwater from column pore water were in the same order of magnitude as those found in the groundwater sampled at the site and shipped to the commercial laboratory for analysis (Figure 22).

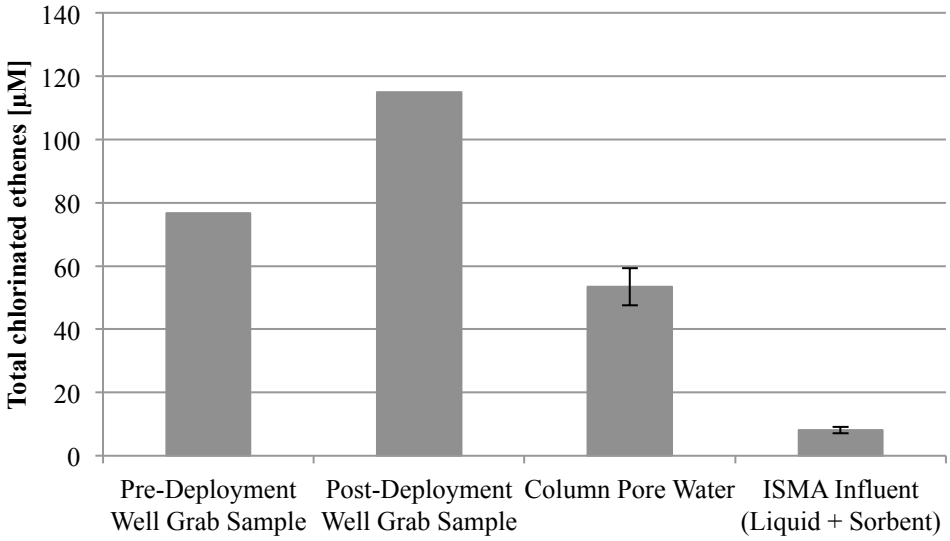

Figure 22. Sum of chlorinated ethenes (TCE, cDCE, and VC) detected in different sample types.Results indicate that columns were exposed to those concentrations of volatile organics found in grab samples of the groundwater and that the lower concentrations observed in captured effluent are a result of losses due to extended effluent storage in the ISMA.

In addition, we observed a potentially unforeseen and side effect of ISB. Elevated concentrations of acetone (Figure 23) and 2-butanone (Figure 24) (also commonly referred to as methyl ethyl ketone) were detected in effluent from bioaugmentation and biostimulation experimental lines. These are fermentation products that have in the past been detected transiently immediately after biostimulation was implemented (Fowler, Thompson, and Mueller 2011). They may also be laboratory artifacts arising from analyzing samples with high VFA content (Adventus Group 2009).

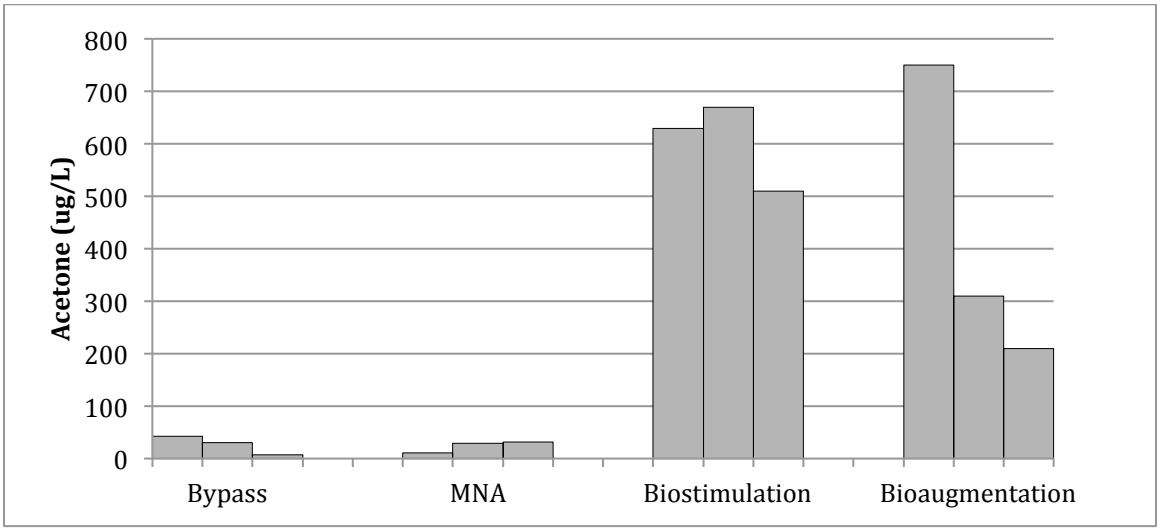

Figure 23. Acetone concentrations detected in ISMA effluent after incubation *in situ* at NAS NI

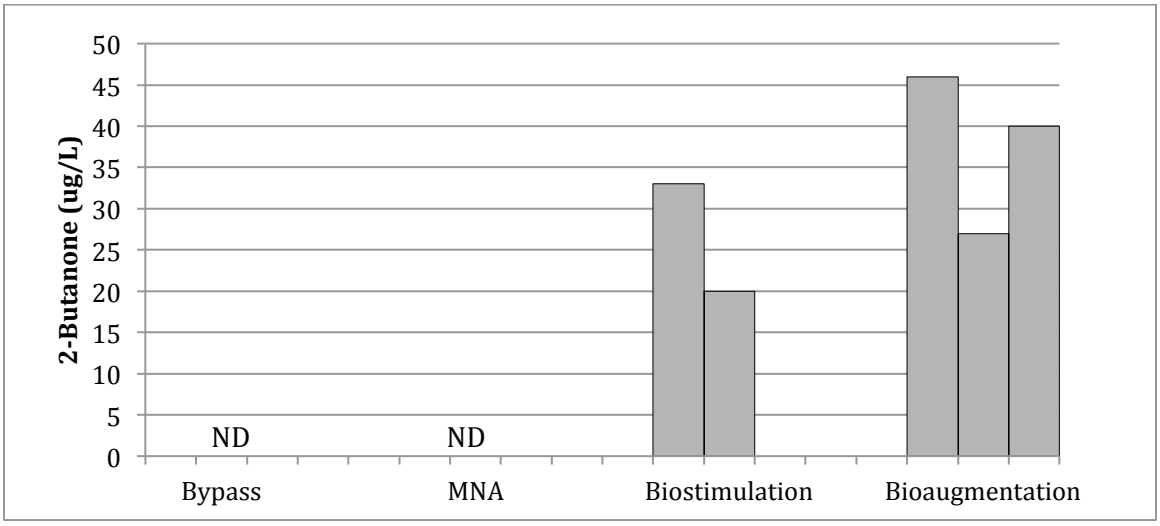

Figure 24. 2-Butanone concentrations detected in ISMA effluent after incubation *in situ* at NAS NI.

Comparison to preexisting site data

No rates were calculated in either the bench-scale treatability study or the field scale pilot study conducted previously by third parties at NAS NI, so we calculated rates where applicable to generate a basis on which to compare treatability study

methods. Table 9 and Table 10 report the various first-order rate constants calculated.

Table 9. TCE: calculated first-order degradation constants (k day^{-1}).

Amendments	Lab Batch Bottle	Field Pilot Trial	ISMA
Lactate	0.051 ± 0.043	-	-0.001 ± 0.157
Lactate + KB-1®	-	-	0.481 ± 0.048
SRS + nutrients + KB-1®	0.524 ± 0.002	-	-
SRS-M + nutrients + KB-1®	3.358 ± 0.169 (in mineral medium)	0.240 (maximum rate detected)	-

Table 10. Cr(VI): calculated first-order degradation constants (k day^{-1}).

Amendments	Lab Batch Bottle	Field Pilot Trial	ISMA
Lactate	0.086 ± 0	-	0.385 ± 0.104
Lactate + KB-1®	-	-	0.479 ± 0.113
SRS + nutrients	0.117 ± 0	-	-
SRS-M + nutrients + KB-1®	7.948 ± 1.218 (in mineral medium)	0.247 (maximum rate detected)	-

Discussion and Conclusions

These results provide multiple lines of evidence for a successful conversion of aerobic site groundwater to anaerobic conditions that facilitated the reductive dehalogenation of TCE by the strict anaerobic bacteria (*Dehalococcoides*) added to the sediment. The reductive dechlorination of TCE in the presence of high concentrations of Cr(VI) (>5 mg/L) is a notable secondary outcome of this study. The observed biological removal of TCE in the presence of 24 mg/L of Cr(VI) in groundwater entering the ISMA extends the reported spectrum of conditions conducive to reductive dechlorination of chloroethenes via bioaugmentation.

While we can confidently conclude that we observed reductive dehalogenation in our biostimulation experiments, unfortunately, no such claims can be made about any attenuation processes that may have transpired in the MNA experiments. A 20% reduction of TCE mass was observed in MNA effluent, relative to the influent. However, the overall poor mass capture of volatiles in collected samples prevents one from drawing any definitive conclusions from this finding. Until better mass balance of volatiles in the effluent storage vessels can be achieved, the possibility cannot be ruled out that the observed 20% reduction in TCE mass simply was lost in the device via volatilization through the additional length of tubing and the column apparatus that the liquid had to traverse prior to collection in the effluent storage container. It should be noted that we tried to minimize this loss by choosing compatible materials (Teflon and glass). Furthermore, any MNA processes that may have occurred in the sediment columns likely would have been relatively slow in comparison to losses observed in the bioaugmentation and biostimulation microcosms. To accurately quantify these processes one would need (i) a complete mass balance of TCE, or an alternative tracer compound to track attenuation, and (ii) a longer column residence time, and therefore a longer deployment time.

Results gathered in the course of the project indicate that the ISMA is a suitable alternative to contemporary treatability or feasibility study methods. Qualitatively, results from ISMA and batch-bottle studies led to similar conclusions: both indicated that bioaugmentation was effective at treating comingled Cr(VI) and TCE, while that biostimulation was effective at treating Cr(VI). Furthermore, with the ISMA

deployment, we were able to absolutely prove that bioaugmentation stimulated dechlorination of TCE all the way to ethene *in situ*.

This conclusion is consistent with the results from all relevant site-specific data sets, including (i) data gathered in our laboratory at Arizona State University from both complimentary batch-bottle studies and flow-through column studies; (ii) results generated from a batch-bottle study conducted by an outside consulting firm, (iii) and results generated from a field pilot trial. A quantitative comparison of first-order degradation rate constants found that batch bottles overestimated field rates by over an order of magnitude (>10), while the degradation rates observed in the ISMA differed from those observed in the field only by a factor of two. In addition, the ISMA was able to identify a potential unintended side effect of ISB (acetone and MEK formation). This result indicates that the ISMA more accurately reproduces field phenomena, and may potentially be used to quantitatively and accurately assess the field performance of *in situ* remediation technologies.

Chapter 5

COMPARATIVE COST ANALYSIS OF THE IN SITU MICROCOSM ARRAY TECHNOLOGY AS A TOOL FOR DETERMINING THE BIOTREATABILITY OF CONTAMINATED GROUNDWATER

Abstract

The *In Situ* Microcosm Array (ISMA) technology is an innovative remedial design tool suitable for evaluating the feasibility of *in situ* bioremediation to effect environmental cleanup of aquifers impacted by natural or anthropogenic contamination. To characterize the economic value proposition of this technology, we performed a cost analysis using conventional feasibility study approaches as a measure of comparison, specifically laboratory batch bottles, laboratory flow-through column microcosms, and small-scale pilot tests conducted in the field. The methodology employed in this work involved the compilation of material costs for capital instrumentation, consumables and labor for ISMA assembly, deployment, post-deployment disassembly, data analysis and reporting. A groundwater monitoring well at the Naval Air Station – North Island (NAS NI) in San Diego, CA served as the field deployment location. For comparative purposes, cost information on alternative feasibility study approaches were extracted from the literature and identified via communication with cleanup industry representatives and service providers. This comparative cost analysis study found that the use of the ISMA technology as a feasibility study tool for design of *in situ* bioremediation ($67-100K) is less expensive than both a field pilot trial ($559K) and a conventional column study ($198K), but slightly more expensive than a batch microcosm study ($54K). In

consideration of the superior data generated by the ISMA when compared to a batch microcosm, this study identifies the ISMA as a cost-effective technology for conducting treatability studies.

Introduction

Treatability studies are designed to prevent costly mistakes in remedial design, and as such, are fundamentally a cost-saving measure (ESTCP 2005; EPA 2002). In light of this, the cost of a treatability study is equally as important as the data it produces and the decision making it enables (Halden 2013). In order to be successful, novel treatability study methods need to provide some combination of superior data output and cost savings over existing methods. The previous chapters have introduced the *in situ* microcosm array (ISMA) technology as an alternative treatability study method, presented illustrative data output, and compared it to contemporary treatability methods, using a demonstration deployment at the Naval Air Station – North Island (NAS NI) as a case study (Kalinowski 2010, 2012a, 2012b; McClellan 2009, 2011a, 2011b, 2012a, 2012c).

The present chapter concentrates on the economic viability of the ISMA technology by drawing on available data from competing, contemporary remedial design approaches.

In 1992, the US EPA issued a guidance document detailing how to conduct treatability studies under CERCLA regulation (EPA 2002). They suggested a three-tiered approach to treatability testing, with distinct motivations guiding the design

treatability studies for each stage of remedial design: remedy screening (early remedial investigation / feasibility study [RI/FS]), remedy selection (late RI/FS), and remedial design / remedial action (post record of decision). It is recommended that as remedial actions proceed, treatability studies correspondingly progress with increasing complexity, cost, and duration.

During remedy screening, it is recommended that batch microcosms be conducted with only 1-2 replicates. Duration of testing is recommended to only take a few days, and it is suggested the data is only to be interpreted qualitatively, not quantitatively. These limited-in-scope studies are suggested to cost $10,000-$50,000, which converted to 2013 dollars is approximately $16K – $82K (inflations adjustments calculated using data from the Consumer Price Index provided by the US Bureau of Labor Statistics).

During Remedy Selection, it is recommended that more complex continuous flow treatability studies be conducted. It is suggested these treatability studies be conducted in duplicate or triplicate, and over a period of weeks. These more complex studies are estimated to cost $50K-$100K, which adjusted for inflation is $82K-$165K. Full scale (onsite) treatability studs during RD/RA continue this trend, and are reported to cost $250K-$1M ($413K-$1.6M in 2013 dollars).

In 2004, AFCEE, NAVFAC and ESTCP(2004) issued a report similarly recommending that properly conducted batch microcosms (for ISB of TCE) cost $50K-$100K ($61K-$122K in 2013 dollars) and last 8-12 months. However, a follow-

up AFCEE report in 2007 (Air Force Center for Engineering and the Environment 2007) suggested microcosm studies for ISB of TCE cost $10K-$40K ($11K-$44K in 2013 dollars).

Part of the overall trend towards reduced or limited scope of treatability studies is due to the fact that alternative sources of qualitative data are now available. Initially, microcosm studies were the only available way to assess the metabolic potential of the indigenous population at a site (ESTCP 2005). Modern molecular tools can determine the presence of *Dehalococcoides* or other important species *in situ* to provide evidence the indigenous population can carry out the requisite biotransformation. In some cases, these methods can replace treatability studies (ITRC 2011). However, it is important to note that molecular tools do not provide reliable data on *in situ* rates of contaminant transformation, and without additional data cannot be used to actually prove biotransformations are occurring *in situ.*

Based on the available information, it is clear that treatability studies vary widely in scope, duration, and cost. It is important to note, that despite the potentially large dollar values associated with these treatability studies, they in many cases are only a small percentage (>10%) of the overall cost of remediation (EPA and US Army Corps of Engineers 2000). Reaching the right remedial design decision has a monetary value that is larger than the cost of the treatability study. Nevertheless, cost is a factor that will influence acceptance of the ISMA. For this reason, it is important to place the ISMA on the continuum of cost by comparing it to other treatability studies.

Methods

Materials Cost

Commercial costs for batch microcosm and column microcosm studies were gathered from informal conversations and other communications with practitioners knowledgeable with the matter, and confirmed by a review of available case reports, technical and regulatory guidance documents, and peer-reviewed literature. All ISMA-associated costs related to the NAS NI demonstration deployment were directly recorded as they were incurred and provided the basis for direct costs in the cost analysis.

Labor Cost Model

A labor costs model was constructed in consultation with the Biodesign Impact Accelerator and In Situ Well, LLC, a startup company formed in partnership with the Biodesign Institute at Arizona State University to bring the ISMA technology to the marketplace. Labor costs were calculated by estimating the effort required from personnel to conduct an ISMA deployment in the future, based on experience with the NAS NI demonstration deployment and other previous ISMA deployments.

Results

Where applicable, costs are provided for the ISMA demonstration deployment at NAS NI, but the focus in the cost assessment is to determine projected costs of future ISMA deployments, and to compare them to alternative methods of conducting treatability studies. Direct costs for materials are compiled in Table 11. The primary cost drivers for material costs consist of consumable ISMA components, primarily liquid channel tubing and effluent storage containers.

Table 11. Direct material costs incurred during NAS NI deployment.

Cost Element	Unit Cost	NAS NI QTY	Total cost NAS NI deployment
ISMA consumables			
Viton tubing 0.89 mm ID	$105 / 50 ft	100 ft	$210
Viton tubing 3.17 mm ID	$44 / 25 ft	12 ft	$22
Effluent containers	$40 / piece	12	$480
GAC cartridges	$68 / 50 tubes	12	$17
Subtotal			$729
Field equipment			
Cable Ramps	$68/3ft / month	60ft for 1 month	$1,360
YSI meter	450 / week	2 weeks	$900
Boom truck + operator	$100/hr + travel	6	$760
Subtotal			$3,020
Total			$3,749

ISMA samples generated during the course of the demonstration deployment at NAS NI were analyzed by a commercial laboratory (EMAX laboratories, Torrance, CA). Costs for sample analysis are summarized in Table 12.

Sample Analysis - Method	$ / Sample	NAS NI QTY	Total cost NAS NI deployment

VOC - 8260B	100	14	$1,400.00
CAM (17) Metals - 6010/7000	150	14	$2,100.00
Anions (3 anions) - 300.0	45	14	$630.00
pH - 9040	15	14	$210.00
TDS - 160.1	15	14	$210.00
VFA - 300 Modified	100	14	$1,400.00
Hexavalent Chromium - 7196	60	14	$840.00
Subtotal			$6,790.00

.

Table 12. Direct costs for sample analyses by commercial laboratory incurred during NAS NI deployment.

Sample Analysis - Method	$ / Sample	NAS NI QTY	Total cost NAS NI deployment
VOC - 8260B	100	14	$1,400.00
CAM (17) Metals - 6010/7000	150	14	$2,100.00
Anions (3 anions) - 300.0	45	14	$630.00
pH - 9040	15	14	$210.00
TDS - 160.1	15	14	$210.00
VFA - 300 Modified	100	14	$1,400.00
Hexavalent Chromium - 7196	60	14	$840.00
Subtotal			$6,790.00

Additionally, a labor needs model was constructed to estimate the total labor costs involved with an ISMA deployment (Table 13). Based on previous experience, we estimated the required efforts by personnel, differentiating personnel between four distinct skill sets and levels of expertise.

Table 13. Projected labor needs for future deployments.

Deployment Activities	Project Manager	Senior Technical Advisor	Environmental Scientist / Engineer	ISMA Technician
Prepare ISMA Configuration for Deployment (includes	4 wk @ 10%	4 wk @ 10%	4 wk @ 20%	4 wk @ 100% FTE

mechanical build, systems check, column construction)				
Pack/Ship ISMA to Customer Site	1 wk @ 10%			1 wk @ 80% FTE
Receive/Secure ISMA at Customer Site	1 wk @ 10%			
Deploy ISMA Down-Hole and Initiate Process Run (includes travel time)	1 wk @ 100%			1 wk @ 100%
Stop Process Run; Retrieve ISMA Samples and deliver to commercial laboratory for analysis	1 wk @ 100%			1 wk @ 100%
Data reduction and analysis; Reporting	4 wk @ 25%	4 wk @ 50%	4 wk @ 50%	4 wk @ 55%
Subtotal (Person-Months)	0.975	0.6	0.7	2.3

Based on the calculated costs for materials, sample analysis and labor, the total cost for an ISMA deployment were calculated, and presented in Table 14.

Table 14. Projected ISMA costs.

Cost element	Present	Future
Labor costs	$41,515	$20,757
Consumable and Equipment Costs (not including ISMA leasing)	$7,989	$1000
Laboratory analysis	$14,000	$12,000
Travel	$4,000	$3,000
Facility and Administrative costs	$43,210	$29,924
Subtotal	$110,713	$66,681

The calculated ISMA cost was compared with the actual costs incurred by NAS NI at OU20. NAS NI project costs are presented in

Table 15, and the comparison of the ISMA to NASNI costs and other costs is

presented in Figure 25.

Table 15. Feasibility study project costs: OU-20, NAS NI.

Cost Element	$
Project Management	$71,435
Plans	$88,633
Installation of Wells and Associated Sampling	$80,527

Bench-scale Treatability Evaluation	$53,424
Field-scale (Pilot) Treatability Evaluation	$223,731
Reporting	$94,883
Total	$612,633

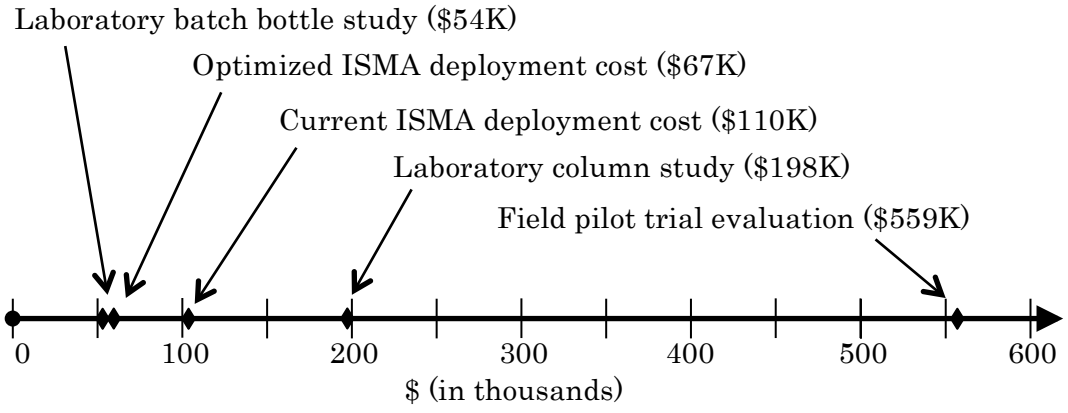

Figure 25. Cost comparison of treatability study methods. Values for the batch bottle and field pilot study are actually costs incurred during NAS NI remediation.

Discussion

Cost Drivers

There are three primary drivers determining ISMA costs: materials, sample analysis, and labor.

Material.

Materials are defined as consumables (i.e., non-reusable) ISMA components. The bulk of the ISMA device - including the columns, pumps, motors, internal skeleton, outer shell, and electrical connectors - is reusable. However, to minimize the risk of cross-contamination between deployments, internal components that come into

contact with field materials are replaced. These materials are: flexible tubing (used to route groundwater throughout the device), peristaltic pump tubing, tubing connectors and fittings, check valves, syringes for amendment injection, effluent storage containers, and activated carbon sorbent cartridges for capture of volatile organics (if applicable).

Materials costs also include the cost for field equipment associated ISMA deployment / retrieval as well as with storage of ISMA equipment on site. At present, rental of a boom-truck and operator is necessary to deploy and retrieve the ISMA, and this cost does not vary much between sites. However, the costs associated with storing the ISMA on site are, necessarily, site-specific. For a deployment location that is secure and sparsely used, no such costs exist. At NAS NI the deployment location was in an active parking lot and cable ramps along with a custom well box cover were necessary to avoid impacting traffic. Examples of costs that might fall into this category at other locations may include installing a temporary shed or a fence to protect ISMA equipment. Direct material costs incurred during the ISMA demonstration deployment at NAS NI are presented in Table 11.

Sample analysis.

In future deployments, the analytics can be performed by a certified commercial laboratory, by the site owner, or at the research laboratory at Arizona State University. Sample analysis costs will likely differ between deployments based on data needs and relationships with commercial labs.

Labor.

Direct labor costs incurred during the demonstration deployments are not reported or computed, partly due to the difficulty of quantifying the exact effort expended on any single deployment, and of differentiating from efforts towards ISMA development and iterative design, and from concurrent associated laboratory studies, but also because such a computation would not be instructive of future costs. With two ISMAs built, and over 10 individual deployments that were used to iteratively improve on the ISMA and identify and correct failure modes, the one-time capital and labor costs have been incurred, and future deployments will be significantly less expensive. The activities necessary for an ISMA deployment and the associated projected personnel time are listed in Table 13. Activity categories are described and discussed below.

Laboratory Labor: Column microcosm assembly and preparation consists of sediment processing (drying, homogenizing, crushing and sifting as necessary) and then manually packing the columns with processed sediment. ISMA assembly consists of replacing and installing all the consumable materials, testing all channels for consistent flow rates and leaks, and loading the materials and reagents necessary for the test (*in situ* treatment technology, preservative, and sediment columns, etc.).

Column operation in the laboratory (including, potentially, column preconditioning) is not included in the labor model of an ISMA deployment due to the fact that it can and should be considered as a stand-alone laboratory column treatability study. It is complementary, but not strictly necessary, to an ISMA deployment.

Field Labor: A boom truck and operator are necessary for approximately 2-3 hours during both ISMA deployment and retrieval. Additional support is required from one ISMA technician. Field tasks performed by the ISMA technician include (i) taking a well grab sample before deployment and after retrieval, and determining field parameters with a pre-calibrated multi-parameter probe, (ii) installing the ISMA in the well and retrieving it after field incubation (with assistance from the boom truck operator), (iii) ensuring all electronic components (solar panels, battery array, controls for ISMA pumps and motor) function properly, (iv) securing ISMA equipment on site in such a way that it minimizes impact on site activities and minimizes risk of vandalism or theft, (v) external decontamination of the ISMA upon retrieval from the well, (vi) sample extraction from the ISMA, including transfer of samples to the appropriate containers and adding any necessary sample preservatives, and (vii) transporting samples to a laboratory for analysis, or coordinating transport with another courier.

Additional Cost Drivers

There are relatively few site-specific cost drivers that may drive up the cost of an ISMA deployment. Beyond column preparation, and the chosen amendment and quencher, ISMA assembly and preparation is not specifically sensitive to cost

variation based on deployment site. The largest site-specific cost driver is the type and number of sample analyses required. This is dependent on the data needs of the customer, and may also include the need for additional ISMA deployments or laboratory studies. At present, a full ISMA deployment produces fourteen liquid samples (twelve column effluent composites, and pre- and post-deployment well grab samples). Additionally, based on data needs, sediment from up to ten separate columns can be analyzed (with the potential to fraction each of the columns into 4 or more distinct samples).

An additional cost driver not incurred during the demonstration deployments but recommended for future deployments is the cost of collection of fresh sediment for microcosm construction. This cost of drilling a well and collecting the sediment is highly site-specific, and therefore not enumerated in this cost analysis.

One of the largest overall cost drivers for a treatability study that incorporates the ISMA will hinge on the decision of whether to conduct a complementary laboratory study. A laboratory column study prior to field deployment will yield empirically generated column operation parameters. Data generated from such a laboratory study can maximize the utility of the field deployment by informing the experimental design of the field experiment on dosing requirements, column residence times, and other design parameters. A complementary laboratory column study may also be particularly beneficial if the *in situ* treatment technology being evaluated is dependent on a slow-growing microbial culture that may require an extended acclimation period in the column before demonstrating significant activity.

Projected ISMA Costs

Table 14 lists the calculated projected costs for ISMA deployments in the immediate future, along with potential deployment costs once certain process optimizations and economies of scale are realized. As mentioned in the previous subsection, there are relatively few site-specific cost drivers, thus the costs listed are representative of those that might be incurred during a typical deployment. Assumptions underlying this claim are that the ISMA study site is similar to the demonstration locations, namely that

- A single deployment may satisfy the initial data needs
- There is a pre-existing 4"-ID monitoring well that can accommodate the ISMA
- ISMA surface components can be accommodated safely for the deployment period

The projected future cost reductions identified in Table 14 can be attributed to:

1. Labor reduction: economies of scale and efficiency will result from having multiple ISMA deployments ongoing concurrently (i.e., it does not take twice as much effort to assemble two ISMAs as opposed to one). The reduced labor costs presented are estimates based on a labor model which assumes three ongoing ISMA deployments at any one time. Other contract laboratories to

which the ISMA technology is compared here already realize similar economies of scale.

Labor needs will also be reduced with further modifications to the ISMA. Three people were present during the demonstration deployment at NAS NI, in addition to the boom truck operator. However, the ISMA is compact enough that in the future, deployment may be feasible with only a tri-pod or custom hoist, thereby eliminating the additional expenditure linked to boom truck operation and making a manual ISMA deployment requiring only two people possible.

2. Consumables and Equipment Cost: additional engineering effort can lead to refinements and reduced material needs per ISMA deployment. These modifications can be based on a redesigned, and reusable, effluent storage array, as well as hard-wired (reusable) and easily serviceable liquid flow channels in the ISMA.

3. Laboratory analysis: the modest savings listed are primarily due to a customer-loyalty program and reduced unit cost when ordering a large number of analyses. This number will fluctuate based on customer needs, and is only included as an estimate assuming a standard suite of analyses chemical analyses for 14 samples (12 ISMA effluent channels, and deployment well samples before and after deployment).

4. Facility and Administrative (F&A): These are a fixed percentage cost based on modified total direct costs. These are based on the costs at Biodesign-ASU, but are comparable to the overhead charges incurred in other academic or commercial settings.

Comparison to Alternatives

Comparing costs incurred by NAS NI to projected costs for a comparable ISMA deployment ($67K) we see that an ISMA deployment is more expensive than a laboratory batch bottle treatability study ($54K) but significantly less so than a field pilot trial ($559K). This is acceptable due to the fact that the ISMA produces results that are more representative of the field than a laboratory study, but generates them with significantly less impact than a field pilot trial.

It is also instructive to compare ISMA costs to those of a traditional column study; one commercial laboratory quoted a column study examining bioaugmentation at $22,000 / column. At this rate, a lab study comparable to the ISMA (that is, with 9 columns) is estimated to cost $198,000. On a true comparison of flow-through to flow-through treatability study, the use of the ISMA can realize significant cost savings. Furthermore, due to the standardized ISMA components, the marginal cost of additional columns in study will be significantly less than the fixed cost of $22K / column, and this cost-savings realized by the ISMA will significantly increase with increasing complexity of the proposed study.

Limitations

This cost analysis does not take into account data quality produced by the competing treatability study methods. The relationship between data quality and realized cost savings for the remedial project is fundamental to the proposition of the ISMA, but is an unknown that can only be quantified in cases where a costly mistake has been made – and even then impossible to generalize for other sites. Due to the *in situ* nature of the ISMA, it is expected to produce superior data than batch microcosms, and thus potentially realize cost savings. It may in other situations also be more cost-effective than field pilot trials at identifying unintended or unforeseen consequences of *in situ* bioremediation, due to its contained, and therefore risk-free, experimental design.

Another limitation of this cost analysis is that it does not take into account any profit margins included in the reported costs for commercial treatability studies.

Conclusions

The ISMA is cost-efficient in comparison to alternative treatability study methods. Treatability studies, which can be thought of as a form of insurance against poor decisions, are part of a larger effort to minimize the total cost and effort involved with site remediation. Due to its *in situ* nature, ISMA data output should more accurately predict performance of *in situ* remediation technologies than conventional batch microcosms, and therefore could help reduce overall costs of remediation. An additional benefit of the ISMA technology is that it can also be used to conduct a column study in the laboratory for lower cost than conventional methods.

Chapter 6

FACILITATING MUTUAL PROBLEM UNDERSTANDING: PARTICIPATORY TECHNOLOGY ASSESSMENT OF AN EMERGING TECHNOLOGY

Abstract

The Motorola/52nd Street (M52) Superfund Site, an approximately 7-mile long chlorinated solvent plume in downtown Phoenix, has been included in the National Priorities List (NPL) since 1989, and is projected to require continued remedial action for centuries. The current mode of communication between M52 stakeholders—responsible parties, regulatory agencies, and community members—often leaves all parties feeling dissatisfied, misunderstood, and demotivated about working toward a site-wide remedy. The goal of this project was to increase transparency, facilitate civil dialogue, build mutual problem understanding and arrive at a common vision of the future among M52 stakeholders. This goal informed our research question of whether a participatory technology assessment (pTA) can facilitate shared learning and problem understanding among a diverse set of stakeholders. The study employed a participatory workshop focused on exploring the potential usefulness and applicability of a new technology, the *In Situ* Microcosm Array (ISMA), at the M52 Superfund Site. Participants included stakeholders representing engineering firms working on behalf of potentially responsible parties, regulatory agencies, environmental justice advocates, and citizens. The workshop established a solutions-focused dialogue with an emerging technology as the focal point, while creating a non-confrontational space. Participants worked together to assess the applicability of the technology. In so doing, they initiated a dialogue that

explored the problem understanding from their differing perspectives and enabled a discussion on a final remedy, or future vision, for the M52 Superfund Site. One participant described the technology assessment workshop process as a way to "demystifying the technology," while others agreed that social processes, such as trust and political will, presented challenges to the technology. This research informs two divergent knowledge sets. First, it models a way to develop shared problem understanding at contaminated sites that are constrained by social factors, such as lack of trust and power asymmetries. Second, the work informs technology assessment methodologies by taking an emerging technology out of the lab and into a place where a community of stakeholders may assess and critically evaluate it on both technical and social merits.

Introduction

The ISMA is designed to provide data that can be used to make decisions about optimal remediation strategies. However, decision-making for remedial design often involves a diverse set of stakeholders experiencing different constraints, operating under different motivations, and desiring different outcomes. It is to be expected then, that different stakeholders will have different perceptions of the ISMA. Whereas the previous chapters have explored the technical and economical merits of the ISMA, this chapter is focused on exploring the perceptions of the ISMA held by different stakeholders of a local hazardous waste site, the Motorola 52nd street Superfund site (M52). This was an effort by the technology creator (myself) to explore the societal barriers the ISMA may face, in addition to the technical and economical barriers discussed in the previous chapters. This was accomplished by

partnering with students and faculty from the Consortium for Science Policy and Outcomes at ASU (CSPO), and the Global Institute of Sustainability at ASU.

Research Design and Methods

Workshop Design And Implementation

A workshop modeled after known pTA practices was implemented. Email invitations to the workshop were sent out to members of the Community Information Group (CIG) distribution list via the regulatory agency, while clearly stating that this particular event was not sponsored by regulatory agencies. The mailing list included interested local citizens, consultants, responsible parties, and regulatory personnel. In this way, established stakeholders previously connected to the site were solicited for participation. Although the M52 Site is a large urban Superfund site, the site residents are, for the most part, unaware of its existence. Even though invitations were sent out over the CIG distribution list, the list is a pre-selected small percentage (<1%) of site residents and stakeholders, and therefore the invitations had limiting access to the 'general public'. The stated purpose in the invitation was to introduce a technology, "explain its functionality, and explore community perceptions of the technology and potential applications" as well as "to gather feedback from the general public, and other interested parties to better understand how the ISMA may (or may not) play a role in the future remediation of contaminated groundwater sites." The invitation included a link to the EPA website where interested participants could access a recorded webinar on the technology prior to the workshop. The invitation also made clear that none of the presenters

stood to profit from the manufacturing, distribution or sale of the ISMA. To ensure easy access, the workshop was hosted at 6 PM, after standard working hours, and at a local community college connected to both light rail and bus services.

Participants, upon entering the workshop venue, signed the requisite forms and were seated in a horseshoe configuration with a facilitator standing at the front. The workshop began with a brief introduction as to the purpose and scope of the event (the introduction, largely, repeated the initial invitation). The next fifteen-minutes were devoted to a presentation by myself (Tomasz Kalinowski), a member of the development and evaluation team for the ISMA technology. The ISMA was described in non-technical language that had been reviewed beforehand with the Technical Advisor to the CIG for language and content. In that way, the presentation was vetted and deemed appropriate by the person hired by the community to translate technical reports and information. The remainder of the workshop time consisted of a structured, facilitator-led discussion. The facilitator began by distributing a small sheet of paper with an open-ended, opinion-soliciting question. Time and silence was given for participants to respond in written form and then the opportunity was provided for participants to express their answers verbally. This first captured individuals' contributions and secondarily forced all the participants to listen to others' perspectives on the question. Once the discussion question was exhausted, the facilitator moved on to the next question by distributing the next sheet of paper, until a total of ten questions had been asked and responses had been elicited. Participants were encouraged to go back to previous questions, in the event that the discussion (or later questions) prompted new thoughts and ideas. The questions

progressed from identifying the positive and negative aspects of the ISMA, generally, to more specific questions focused on social barriers, and then back into open-ended, general questions. The questions and responses are presented below in Table 16.

Case Study Profile: Motorola 52nd Street Superfund Site

The Motorola 52nd Street (M52) Superfund Site is a contaminated groundwater plume underlying a portion of downtown and east-central Phoenix. In 1983, workers at the former Motorola facility at 52th Street and McDowell in east-central Phoenix discovered contamination of local soil and groundwater with 1,1,1-trichloroethane. Further investigations at the former Motorola facility discovered numerous contaminants had entered the groundwater table and were migrating towards downtown Phoenix (Burnell et al. 2011; URS Corporation 2011). Historical documents record an estimated 93,000 gallons or about 500,000 kg of trichloroethylene (TCE)—a known carcinogen—had been disposed of in unlined dry wells (EPA 2011). Investigations down gradient from the release points revealed additional sources of groundwater contamination not attributable to the Motorola facility (Burnell et al. 2011), necessitating a larger groundwater contamination study and eventually expanding the site (URS Corporation 2011). The M52 Superfund Site is approximately 7 miles long, extending from 52nd Street to 7th Avenue in Phoenix, and 2 miles wide, extending from Palm Lane to the north and Buckeye Road to the south. The entire site is approximately 11.4 square miles. The regulatory agencies divided the M52 Site into three operable units (OUs), in part due to the large geographic size - see map (Figure 26).

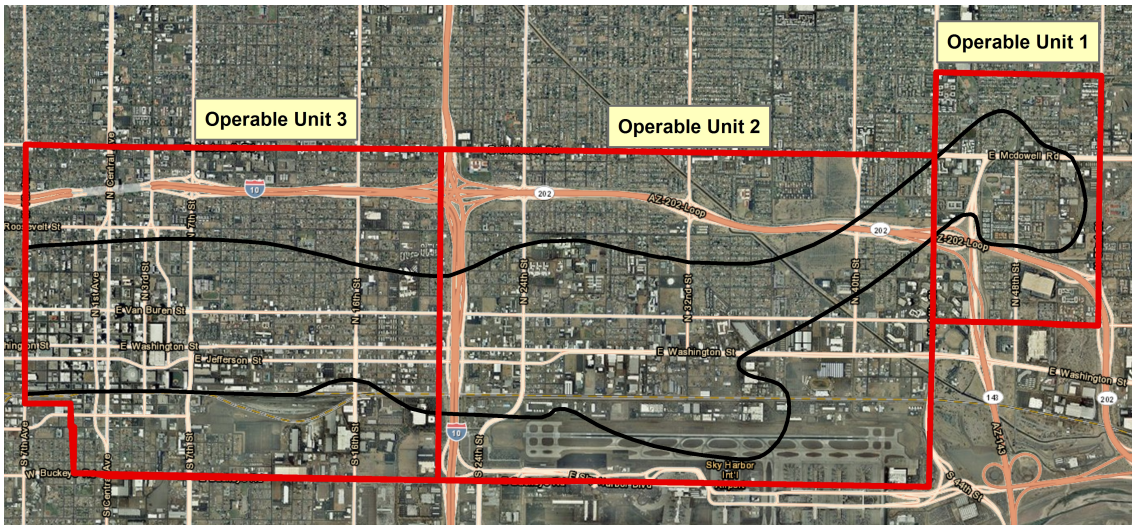

Figure 26. Site map of M52 Superfund Site. Black lines indicate areas of known contamination and red lines indicate the boundaries of operable units within the M52 Superfund Site. Note: Sky Harbor International Airport in Phoenix, Arizona in the bottom right of the map is visible.

Since 1992 when the M52 Superfund Site in Phoenix, Arizona was added to the National Priorities List, citizens demanded scientific proof that toxic vapors were not intruding through the foundations of their homes. In 2010, the U.S. Environmental Protection Agency (EPA) assumed control of community involvement, due to a lack of responsiveness by state agencies. To address citizens' concerns, EPA initiated soil vapor monitoring. Validated soil vapor tests, reported in June 2011, revealed that numerous sampling locations exceeded risk-based screening levels (RBSL) for trichloroethylene (TCE). This initiated indoor air quality sampling within community residences. In November 2011, EPA published validated data revealing that of the 40 sampled homes, 6 contained levels of gaseous TCE, a known human carcinogen, necessitating the installation of sub-slab (sub-foundation) depressurization systems to remediate indoor air. The existence of groundwater

contamination and associated toxic soil vapor urgently demand remedial action via innovative technological solutions.

However, the soil gas vapor intrusion is just one of numerous instances where technological solutions to site problems have not been explored with community members, evoking distrust in responsible parties and government agencies. Recently, site-wide controversy erupted over a proposed effluent discharge pipeline to be built by the responsible parties through a nearby elementary school property. While the proposal ultimately failed by vote of the local school board, there was little to no participatory engagement by the governing agencies or responsible parties regarding the technological alternatives to the proposed effluent discharge plan during the several years long planning period. Despite requests by the M52 Superfund Site's Community Involvement Group (CIG) to evaluate the proposal with responsible parties, a pTA of the effluent discharge plan and its technology was eschewed in favor of direct approval by a local school board. Interestingly, the Baltz School Board ultimately sided with the community over safety concerns, despite public requests to evaluate the proposal with responsible parties.

Technology Profile: *In Situ* Microcosm Array

The *In Situ* Microcosm Array (ISMA), is a field-deployable device which fits into a standard 4" monitoring well. The device consists of a stainless steel pipe that houses an array of sediment columns (microcosms), along with an assortment of pumps and electronics to feed the columns with groundwater drawn directly from the aquifer. In a standard configuration, the ISMA simultaneously feeds nine continuous-flow

microcosms, allowing for the comparison of up to three different approaches to *in situ* remediation in triplicate, at the same time in the same well. During the course of the deployment, effluent from each column is captured in separate vessels. After *in situ* incubation, all of the effluent is transported back to the lab for chemical analyses.

The ISMA addresses a critical gap in the capabilities of contemporary methods for assessing the effectiveness of a particular *in situ* treatment technology for a particular site. Treatability studies conducted in the laboratory are often not representative of field conditions, whereas more informative field pilot tests are expensive and thus are conducted only on a select few candidate technologies (typically no more than one or two). Furthermore, the inherent heterogeneity of the subsurface prevents a fair comparison between two technologies tested in field pilot trials, as observed differences may be attributed to either treatment performance or the distinct monitoring wells in which tests were conducted. Additionally, because an ISMA deployment releases no chemicals into the environment, it lowers the barrier of entry for experimental and unproven *in situ* treatment technologies.

By generating *in situ* performance snapshots the ISMA equips remediation engineers with higher quality data enabling them to more accurately perform comparative evaluations of remedial technologies, thus paving the way for more sustainable and effective environmental restoration actions.

Results

This study demonstrates that strong, formal, institutional support mechanisms are required to enable effective communication among stakeholders at this legacy hazardous waste site. Secondarily, the pTA workshop, itself, opened the opportunity to robust dialogue and discussion about the technical and societal aspects of the ISMA within the place-based context of the M52 Superfund Site. Additionally, the ISMA was held as a 'boundary object' in the workshop and allowed for communication between stakeholders, who were previously at odds (i.e. have been engaged in decades-long legal battles). And finally, the workshop created a cohort of people that were learning together, with many of the larger power asymmetries set aside for the duration of the workshop. This was due in large part to the setting, the newness of the ISMA and to active, strong facilitation. It should be noted here, that immediate, local policy implication arose, as the technology creator (Tomasz Kalinowski) was invited back to present to the larger M52 Superfund Site's Community Involvement Group (CIG). This early outcome demonstrated the immediate benefits recognized that federal officials sought to build from as they continue to struggle to manage citizen-responsible party interactions.

pTA Workshop Results

The pTA workshop created an informal and unscripted space to allow for a mutual exchange of perspectives. We have identified some of the workshop features that we believe are responsible for enabling a candid, civil, and productive dialogue focused on solutions for the long-term future.

The pTA workshop was attended by nine people, representing the following groups:

- 4 citizens and citizen-advocates
- 2 regulatory agency officials
- 3 engineering consultants working with responsible parties.

Table 16 presents the participant responses during the course of the workshop, distinguishing between the three stakeholder groups identified. For brevity, responses were paraphrased and aggregated where applicable. Questions are presented in order they were asked, and the table thus presents a narrative of the workshop.

Question	Regulatory	Consultant/PRP	Community
What do you think is good about the ISMA?	It reduces the risk of experimenting.	It can conduct better, more realistic experiments.	[Initially tentative to participate]
How could the ISMA be improved? What features should be added?	Consensus: Ability to perform better experiments Detailed Comments / questions related to ISMA functionality (e.g., can it transmit data electronically? how long does it take to generate data?) Consultant suggestions, comparatively, exhibited the greatest specificity.		
What are the barriers to implementing the ISMA at the M52 site?	Access, funds, interest from leadership	Additional costs worth the perceived improvement in data quality? Well size requirement. "Sociopolitical implications of conducting 'experiments in populated areas" (1 consultant)	Political will, "is it dangerous?"
What might support the implementation of the ISMA at the M52 site?	Established tracks new technologies enter to become more mainstream – pilot testing at DoD or EPA sites, ITRC, to: "demonstrate low risk and intrusiveness" "build public interest in supporting experiments" "get an approval – EPA or ASTM test method designation"	"Regulations [that] allow for new tech", funds, available trial sites. Very bottom-line objective focused responses – "potential for moving [the] project forward and achieving site cleanup faster than current technology at lower overall remedial cost"	Communicating the benefits of technology to community members
Do you think that the ISMA can help identify potential final remedies at the m52 site?	Yes	Qualified maybes – concern that it may take more effort explaining the ISMA than the *in situ* technology being tested, concerns related to in *situ* remediation technologies in general, concerns about ability to scale up.	Yes

Questions (continued)	Regulatory	Consultant/PRP	Community
Do you think that the ISMA remedies all the problems at the M52 site?	"This is only a technology solution – does not address political + [bureaucratic] issues that prevent cleanup, address community concerns affected by groundwater contamination or financial constraints" Another official, alternatively said "no, does not address these issues: [list of technical limitations]"	No. Listed all the technical problems with using in situ. One mentioned "untested tech does have community / technical hurdles"	"Potentially. It just shows us what technology should be used. Someone / agency has to implement the actual remedy. It should be presented as a way to save money _ time to lower relative [impacts]."
What did you not like about the workshop?	Small number of participants, difficulty finding location Nothing, maybe need a little more description	Presentation brief, more background and technical detail desired. Parking issues. Limited discussion relevant to m52	
What did you enjoy about the workshop?	"Relaxed way it was administered, open discussion, facilitator did a great job of drawing people out, easy access to public transit" "Small group size, no judgment, respectful, informative"	Good questions – good meeting time. Food! Enjoyed discussion "good mix of experience / ?'s" "good idea to have the workshop"	Very interactive / brainstorming. Honestly seeking info + opinions

Table 16. Workshop participant feedback to facilitated discussion questions.

Discussion & Conclusion

Stakeholder Perceptions of the ISMA

Participant responses illustrated the differing constraints that stakeholders operate under. PRP/Engineers focused on the technical and fiscal features and limitations of the technology, and regulatory personnel included institutional and bureaucratic constraints in their evaluation of the technology, while community participants focused more on the perceived risks of the technology, and on the need for effective communication to complement any potential use of the technology.

Overall, many of the limitations of the ISMA identified by workshop participants, particularly engineering consultants, were similar to those communicated to the ISMA team by practitioners at professional conferences and during formal peer-review of official reports and journal publications. Furthermore, the ISMA creators, myself included, were already aware of most of these limitations. In that regard, not much additional insight was gained by the technology creator, about required future development needs of the ISMA.

An additional observation, not altogether surprising, but noteworthy nonetheless, is that the ISMA did not seem entirely relevant to many of the community members. The ISMA is very removed from the day-to-day concerns of the community members, who are more concerned about health and safety issues within their homes and community. However, it appeared that exposing community members to the ISMA, and particularly hearing engineering and regulatory professional discuss merits of

the technology, helped convey the complexity, enormity, and intractability of the contamination problem in a way that was perhaps not apparent before. In this manner, the ISMA served as a launchpad for what turned out to be an educational experience for community members about the M52 Superfund Site.

The format of the pTA workshop allowed for the mutual sharing of constraints experienced by participants in a productive manner that was not possible via existing communication methods. Prior and existing communications are in the form of official mailings or lecture style presentations at community informational meetings. These are primarily unidirectional exchanges of information, and only of information that has already been filtered, condensed and approved. This mode of communication conflates, and therefore hinders recipients' ability to accurately distinguish between technical, financial, bureaucratic, cultural, and social constraints. This promotes misunderstanding by clouding the constraints of all stakeholders from each other, which in turn marginalizes the affected community and consequently reinforces a pattern of contentious relationships.

The pTA workshop created an informal and unscripted space to allow for a mutual exchange of perspectives. Three features of the pTA we feel are responsible for the candid, civil, and productive nature of the dialogue are that it focused the conversation on (i) solutions (ii) for the long-term future (iii) around a semi-hypothetical boundary object. The focus on the future minimized the history of contentious relationships, while the focus on solutions established from the outset that all participants were united by a shared goal: site-cleanup.

pTA and the Maturity of the Technology

One of the central questions of citizen engagement (CE) events identified by Delgado et al. (2011) is to determine at what stage in a technology's development CE should occur, with options existing on a continuum of what is commonly referred to as 'upstream' or 'downstream.' Upstream participation is frequently considered the superior approach because it takes place prior to significant investments in technological development, and before the formation of technology stakeholders. Downstream citizen participation, while still practiced, is considered to be less effective because it is "too late" to have much impact.

However, the ideal of upstream public participation is difficult to implement in reality. Upstream citizen deliberations are vulnerable to manipulation by the organizing institution, who can selectively provide the context and narrow the topics of discussion to restrict the dialogue in such a way that it can only enhance the legitimacy of existing technological aims and current progress. Downstream participation, on the other hand, is not as vulnerable to framing by institutional actors as upstream participation events, yet technology stakeholders are already entrenched, with certain interests and relationships informing their perceptions of the issues, limiting the potential impact of such events.

Sites with preexisting stakeholders and a long history of contentious relations disrupt this dynamic of tradeoffs between upstream and downstream public participation. Citizen engagement events are less vulnerable to framing by institutional actors for legitimizing ongoing activities because, regardless of whether

the CE activity takes place upstream or downstream, stakeholders with a preexisting understanding of the problem provide their own context, preempting event organizers from doing so. In this manner, problems with an already defined set of diverse stakeholders help ensure that citizen participation is an honest exercise.

Even in cases of context-specific problems however, upstream events can suffer from a lack of specificity. Technology creators prefer to present emerging technologies in terms of the potential benefits they can provide, but the further 'upstream' the discussion, the more vulnerable it is to 'over-selling' by the technology creator. Facilitating an amorphous discussion about the hypothetical benefits of an emerging technology is not the best approach to highlight the very context-specific problems of a legacy problem, because the narrative of the technology is still too malleable, leaving the technology creator (and presenter) free to paint an overly rosy vision of the technology's capabilities. Such discussions of a 'silver bullet' can actually hinder progress because they discourage stakeholders from doing the hard work of resolving social problems if they perceive a technological solution is just around the corner.

Downstream events, however, come with their own tradeoffs. Participants identify the contours of the problem when they articulate desired technological solutions. The binary nature of "does the technology solve the problem today?" discourages participants from articulating suggestions for technology development, and in so doing stifles the discussion from fully exploring the nuances of the problem. Furthermore, by centering the discussion in the present, downstream events limit

participates from fully articulating visions of the future. Based on these series of tradeoffs in the context of a specific problem, we identified *midstream* intervention as the ideal time for a citizen engagement activity. A conceptual understanding of the balance of these trade offs is presented in Figure 27.

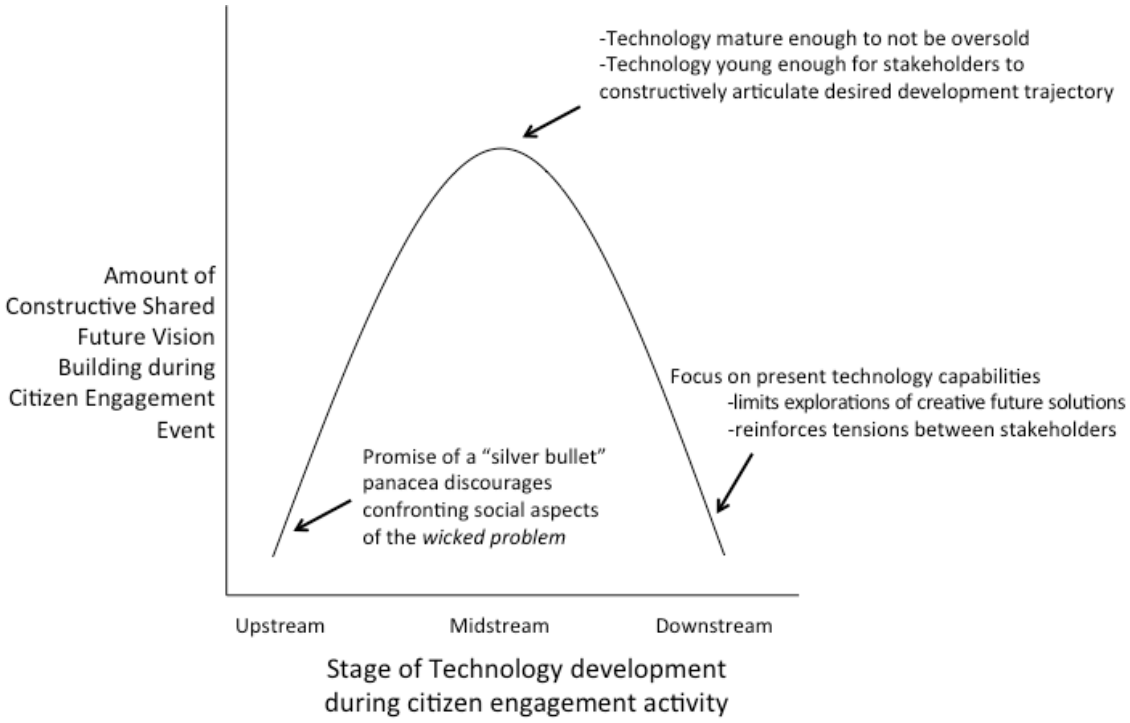

Figure 27. Conceptual representation of the relationship between the maturity of a technology and the amount of future vision building between diverse stakeholders during CE event.

ISMA as a Quasi-hypothetical Remedial Design Solution

The midstream status of the ISMA allowed it to act as a *quasi-hypothetical.* It was far enough along in development that an actual physical artifact was available for participants to look and touch while they discussed it. However, the initial fifteen-minute presentation showcased the ISMA as a technology under active development and framed its use at the site only as a hypothetical possibility in the not too distant

future. It was not stated or implied that the technology was yet ready for actual use at the site, even if a consensus emerged that it should. This shielded the participants from feeling like they were being sold something, and in so doing kept the event honest.

This framing of the technology as a semi-hypothetical facilitated a civil yet candid discussion. The 'work-in-progress' state of the technology allowed participants to freely criticize the technology without fear of offending people already vested in it. It also enabled people to express support for aspects of the technology without true commitment, because actual advocacy for use of the technology was not a true option (at least, as it was framed in the workshop). Nevertheless, because the technology already existed and was under active development, it prevented participants from feeling like the entire discussion was just an exercise in hypotheticals, but rather, that it was directed with a potential solution on the horizon. Despite the presentation of potential solutions to workshop participants, it should be noted that the participants were stakeholders in an on-going cleanup site with legally mandated remediation technologies in place therefore potential solutions to on-site problems occupy a very small solution space, chiefly how to evaluate future expansion of current remediation activities.

ISMA as a Boundary Object

Star and Griesemer (1989) define *boundary objects* as objects that are "both adaptable to different view points and robust enough to maintain identity across them." These are objects that sit between different social worlds, and individuals

within each can use the object for specific purposes without losing their own identity. The ISMA is unique in that it was explicitly designed to aid in decision making for the cleanup of contaminated groundwater wasted sites, and as such it, it is explicitly designed to act as a boundary object between all groups involved in the decision making process.

While M52 stakeholders frequently interact around boundary objects, the objects are primarily official mailings and reports, or lecturer style presentations. These are primarily unidirectional exchanges of information, and only of information that has already been filtered, condensed and approved. These modes of communication conflate, and therefore hinder recipients' ability to accurately distinguish between technical, financial, bureaucratic, cultural, and social constraints. By obfuscating the constraints of all stakeholders from each other, these promote misunderstanding, which in turn marginalizes the affected community and consequently reinforce a pattern of contentious relationships.

The pTA workshop allowed participants to break out of this pattern, which had become a hindrance to meaningful community engagement at the M52 Site. Participants then were able to fully articulate the different constraints they operate under in terms of the ISMA, which served as a *quasi-hypothetical boundary object.* This was apparent in the approach that different stakeholders assessed the ISMA. Potentially responsible parties (PRPs) and engineers focused on the technical and fiscal features and limitations of the technology, regulatory personnel included institutional and bureaucratic constraints in their evaluation of the technology,

while community participants focused more on the perceived risks of the technology, and on the need for effective communication to complement any potential use of the technology. Participants from public agencies focused on the bureaucratic challenges to implementing new technologies within their institutions.

pTA Workshop and Cohort Building

The pTA format is designed to help build a cohort, defined here as a group of individuals that share a learning experience together. Lawerence (2002) discusses some of the benefits of cohort building in the context of adult learning. In cohorts, members participate in a process of co-creating knowledge (understanding) that is greater than the sum of the individuals in the group. Particularly relevant to pTA exercises with entrenched stakeholders, cohorts help create and sustain communities by establishing peer networks that persist after the shared learning experience ends.

This persistence was observed first-hand immediately after the event. Although we finished promptly on time, all except one participant lingered after the workshop for over half an hour to continue informal conversations. Participants broke off into 2-4 person groups to continue the discussion with the technology creator, the workshop facilitator, or each other. There was even an eager student who wanted to stay for the after-workshop discussion so long that his mother eventually had to insist on leaving. We believe the rapport participants established during the pTA workshop should persist for some time. It may facilitate better relations in the future,

particularly at official CIG meetings that have otherwise limited capabilities for similar rapport building and constructive informal interactions.

Additional Outcomes of the pTA Workshop

Following the workshop, the technology creator (myself) received an invitation to present the ISMA technology at a formal CIG meeting. The nature of the interactions at the CIG meeting changed during and after the presentation. Whereas community questions of regulators and PRP consultants usually focus on why they made particular decisions or why they chose to represent data in a certain way, the nature of the questioning of regulators and PRP consultants as a result of the ISMA presentation changed to questions of how change can be implemented on the site to foster more efficient cleanup, and how new remedial solutions can be implemented on-site to complement existing remedial solutions.

Limitations of the Study

This small workshop was faced with a number of limitations that hampered the research, but in no way invalidate the results. The mechanism to invite participants, email, is timely and efficient, but excludes potential participants that lack routine access to email, do not have email and those who chose not to partake in that form of communication. This invitation, sent out by the federal agency, stated that the event was, in no way, sanctioned by their agencies. This may have sent a 'mixed message' to potential participants. This also limited the invitation to those subscribed to the listserv that serves as the formal communication mechanism at the M52 Superfund Site to the CIG. This limitation is not inherent in this study, but electronic mail is

the standard communication form for the federal agency overseeing the site, unless citizens specify an alternative communication form (e.g., postal mail). Another barrier was the lack of a timely response by key stakeholders who demanded to review the invitation and then never responded, which caused a delay of the invitations and lower than expected attendance.

Additional limitations include the fact that this was an entirely graduate student led endeavor that garnered no formal regulatory support. And while the project had a budget to perform the workshop, the resources limited this to a single event held on one evening, rather than a series of events scheduled for different dates and times. That leads to many of the known and often discussed limitations, inherent in community engagement (pTA or otherwise): childcare, work schedules, apathy and lack of salience (Cobb 2011). Couple this with a general lack of awareness of the M52 Superfund Site by the community at large (Foley 2012) and the result was a small group of participants. Yet, the previous CIG meetings had seen attendance by two, four and three persons, respectfully. Therefore, this event garnered three to four times the usual number of participants. Additionally, despite the fact that this effort was not supported by the federal agency overseeing the CIG meetings, they immediately saw the value afterwards, and attempted to replicate the event at their next CIG meeting by inviting graduate student Kalinowski to present for a second time.

Concluding Remarks

With this study we have demonstrated that pTA activities can serve as a tool for creating a forum wherein diverse stakeholders can mutually exchange problem understandings, and constructively build a shared vision that identifies critical social and technological changes needed to address the problem holistically.

Chapter 7

CONCLUSIONS AND FUTURE WORK

In this dissertation, four distinct tasks have been accomplished:

(i) Creation of generalizable knowledge by demonstrating that batch microcosms and flow-through microcosms create different environments for microbes and lead to the development of notably different microbial communities;

(ii) Design and development of the *In Situ Microcosm Array* (ISMA), a down-hole deployable device for, among other things, conducting feasibility studies of *in situ* remediation. The ISMA is a superior alternative to batch microcosms for simulating subsurface conditions in a controlled manner, due to the ISMA's inclusion of continuous-flow conditions, as well as its use of fresh groundwater drawn directly from the aquifer during the course of the experiment.

(iii) Validation of the performance of the ISMA with a demonstration deployment, during which the ISMA was used to successfully cultivate and assess the metabolic potential of strict anaerobes in an aerobic aquifer.

(iv) Assessment of the suitability of the ISMA to address a real-world, local groundwater problem by inviting local stakeholders to participate in an ISMA assessment workshop

Together, these accomplishments each exist at different stages of technology development, from far upstream 'basic' research, to applied engineering, to assessment of societal barriers of the technology. Additional work is necessary at each of these stages in order to advance the practice of groundwater cleanup. The following is a short, but far from exhaustive, list of future work that can pick up where this dissertation leaves off. For the sake of continuity, the list is split into different stages of technology development.

Upstream, 'basic' knowledge about treatability methods

While this dissertation has pointed out the differences between batch and flow-through microcosms and the microbial ecology implications, additional comparative work is necessary to affirm the preliminary conclusions reached.

(i) The results of Chapter 2 need to be reproduced with different microbial cultures, different metabolic processes, and different experimental conditions.

(ii) There needs to be a detailed profile of metabolic processes and microbial communities that develops along the length of the sediment column microcosm, and this differentiation over *distance* in the column should be compared to succession stages that develop over *time* in batch bottles.

(iii) A comparison is necessary between identical lab and field flow-through experiments, in order to assess the importance of using fresh groundwater drawn directly from the aquifer during *in situ* incubation of the ISMA.

Design and development of the ISMA

The ISMA is fully functional at the writing of the dissertation, but further design efforts will make the ISMA both more user friendly, and more robust. Immediate design needs include:

(i) A redesign of the effluent storage vessels that both minimizes losses of volatile compounds, as well makes the vessels more robust and less prone to leakage.

(ii) Replacement of consumable tubing in the main section of the ISMA with hard-shelled reusable channels. This will reduce the cost of consumable materials between deployments, as well as significantly reduce the required effort to prepare the ISMA for field deployment.

Validate the performance of the ISMA

In this dissertation, one demonstration deployment of the ISMA is reported on. While this initial demonstration is promising and indicates the ISMA functions suitably, this conclusion needs to be supported by reproducing these results at different sites with different conditions.

(i) An exhaustive comparison between full-scale implementations of *in situ* remediation, field-pilot trials, ISMA studies, lab flow-through studies, and batch microcosm studies are necessary to determine whether the ISMA consistently delivers superior site-specific assessments of *in situ* remediation.

Employ in real-world remediation

As of this writing, the ISMA is in the early stages of becoming a commercial offering for the engineering market. Much more work will be necessary to ensure the ISMA is adopted and used for remediation.

(i) Continued collaboration with industry partners and customers. Solicit and incorporate customer feedback to inform future ISMA design and development needs.

(ii) Ongoing economic analysis of whether early investments in superior feasibility studies will lead to substantial cost savings and risk reduction during full-scale remediation.

REFERENCES

Ace M Baty, III, Callie C Eastburn Somkiet Techkarnjanaruk Amanda E Goodman Gill G Geesey. 2000. "Spatial and Temporal Variations in Chitinolytic Gene Expression and Bacterial Biomass Production during Chitin Degradation." *Applied And Environmental Microbiology* no. 66 (8):3574.

Adventus Group. 2009. Considerations for Planning an In Situ Injection Project - An open, honest review of lessons learned from remediation challenges edited by Adventus Group.

Air Force Center for Engineering and the Environment. 2007. Protocol for In Situ Bioremediation of Chlorinated Solvents Using Edible Oil.

Air Force Research Laboratory. 1998. Installation Restoration Program Aerobic Cometabolic In Situ Bioremediation Technology Guidance Manual and Screening Software User's Guide. edited by Air Force Research Laboratory.

Al-Tahhan, R. A., T. R. Sandrin, A. A. Bodour, and R. M. Maier. 2000. "Rhamnolipid-induced removal of lipopolysaccharide from Pseudomonas aeruginosa: effect on cell surface properties and interaction with hydrophobic substrates." *Appl Environ Microbiol* no. 66 (8):3262-8.

Allegrucci, M., and K. Sauer. 2007. "Characterization of colony morphology variants isolated from Streptococcus pneumoniae biofilms." *J Bacteriol* no. 189 (5):2030-8. doi: 10.1128/JB.01369-06.

Allesen-Holm, M., K. B. Barken, L. Yang, M. Klausen, J. S. Webb, S. Kjelleberg, S. Molin, M. Givskov, and T. Tolker-Nielsen. 2006. "A characterization of DNA release in Pseudomonas aeruginosa cultures and biofilms." *Mol Microbiol* no. 59 (4):1114-28. doi: 10.1111/j.1365-2958.2005.05008.x.

Allwood, A. C., M. R. Walter, B. S. Kamber, C. P. Marshall, and I. W. Burch. 2006. "Stromatolite reef from the Early Archaean era of Australia." *Nature* no. 441 (7094):714-8. doi: 10.1038/nature04764.

Arias, Y. Meriah, and Bradley M. Tebo. 2003. "Cr(VI) Reduction by Sulfidogenic and Nonsulfidogenic Microbial Consortia." *Applied And Environmental Microbiology* no. 69 (3):1847-1853. doi: 10.1128/aem.69.3.1847-1853.2003.

Ayarza, J. M., L. D. Guerrero, and L. Erijman. 2010. "Nonrandom assembly of bacterial populations in activated sludge flocs." *Microb Ecol* no. 59 (3):436-44. doi: 10.1007/s00248-009-9581-1.

Bae, H. S., B. A. Rash, F. A. Rainey, M. F. Nobre, I. Tiago, M. S. da Costa, and W. M. Moe. 2007. "Description of Azospira restricta sp. nov., a nitrogen-fixing bacterium isolated from groundwater." *Int J Syst Evol Microbiol* no. 57 (Pt 7):1521-6. doi: 10.1099/ijs.0.64965-0.

Bassler, B. L., and R. Losick. 2006. "Bacterially speaking." *Cell* no. 125 (2):237-46. doi: 10.1016/j.cell.2006.04.001.

Baty, A. M., 3rd, C. C. Eastburn, Z. Diwu, S. Techkarnjanaruk, A. E. Goodman, and G. G. Geesey. 2000. "Differentiation of chitinase-active and non-chitinase-active subpopulations of a marine bacterium during chitin degradation." *Appl Environ Microbiol* no. 66 (8):3566-73.

Bockelmann, U., A. Janke, R. Kuhn, T. R. Neu, J. Wecke, J. R. Lawrence, and U. Szewzyk. 2006. "Bacterial extracellular DNA forming a defined network-like structure." *FEMS Microbiol Lett* no. 262 (1):31-8. doi: 10.1111/j.1574-6968.2006.00361.x.

Boles, B. R., and P. K. Singh. 2008. "Endogenous oxidative stress produces diversity and adaptability in biofilm communities." *Proc Natl Acad Sci U S A* no. 105 (34):12503-8. doi: 10.1073/pnas.0801499105.

Boles, B. R., M. Thoendel, and P. K. Singh. 2005. "Rhamnolipids mediate detachment of Pseudomonas aeruginosa from biofilms." *Mol Microbiol* no. 57 (5):1210-23. doi: 10.1111/j.1365-2958.2005.04743.x.

Bontognali, Tomaso R. R., Crisógono Vasconcelos, Rolf J. Warthmann, Christophe Dupraz, Stefano M. Bernasconi, and Judith A. McKenzie. 2008. "Microbes produce nanobacteria-like structures, avoiding cell entombment." *Geology* no. 36 (8):663. doi: 10.1130/g24755a.1.

Brennan, R. A., and R. A. Sanford. 2002. "Continuous steady-state method using tenax for delivering tetrachloroethene to chloro-respiring bacteria." *Appl Environ Microbiol* no. 68 (3):1464-7.

Burnell, Daniel K, JW Mercer, M Van Oort, Thomas R Suriano, and R Douglas Bartlett. 2011. "Stochastic Model to Estimate Travel Times From the 52 nd Street Facility in Phoenix, AZ." *Journal of Earth Science and Engineering* no. 1 (2):2-10.

Busch-Harris, Jennifer, Kerry Sublette, Kenneth P Roberts, Carla Landrum, Aaron D Peacock, Greg Davis, Dora Ogles, William E Holmes, David Harris, Christopher Ota, Xiaomin Yang, and Arati Kolhatkar. 2008. "Bio-Traps Coupled with Molecular Biological Methods and Stable Isotope Probing Demonstrate the In Situ Biodegradation Potential of MTBE and TBA in Gasoline-Contaminated Aquifers." *Ground Water Monitoring And Remediation* no. 28 (4):47-62. doi: 10.1111/j.1745-6592.2008.00216.x.

Caporaso, J. G., K. Bittinger, F. D. Bushman, T. Z. DeSantis, G. L. Andersen, and R. Knight. 2010. "PyNAST: a flexible tool for aligning sequences to a template alignment." *Bioinformatics* no. 26 (2):266-7. doi: 10.1093/bioinformatics/btp636.

Caporaso, J. G., J. Kuczynski, J. Stombaugh, K. Bittinger, F. D. Bushman, E. K. Costello, N. Fierer, A. G. Pena, J. K. Goodrich, J. I. Gordon, G. A. Huttley, S. T. Kelley, D. Knights, J. E. Koenig, R. E. Ley, C. A. Lozupone, D. McDonald, B. D. Muegge, M. Pirrung, J. Reeder, J. R. Sevinsky, P. J. Turnbaugh, W. A. Walters, J. Widmann, T. Yatsunenko, J. Zaneveld, and R. Knight. 2010. "QIIME allows analysis of high-throughput community sequencing data." *Nat Methods* no. 7 (5):335-6. doi: 10.1038/nmeth.f.303.

Chai, Y., F. Chu, R. Kolter, and R. Losick. 2008. "Bistability and biofilm formation in Bacillus subtilis." *Mol Microbiol* no. 67 (2):254-63. doi: 10.1111/j.1365-2958.2007.06040.x.

Chao, Anne, and Shen-Ming Lee. 1992. "Estimating the Number of Classes via Sample Coverage." *Journal of the American Statistical Association* no. 87 (417):210. doi: 10.2307/2290471.

Characklis, W. G. 1981. "Bioengineering report: Fouling biofilm development: A process analysis." *Biotechnology and Bioengineering* no. 23 (9):1923-1960. doi: 10.1002/bit.260230902.

Chen, I., R. Provvedi, and D. Dubnau. 2006. "A macromolecular complex formed by a pilin-like protein in competent Bacillus subtilis." *J Biol Chem* no. 281 (31):21720-7. doi: 10.1074/jbc.M604071200.

Claverys, J. P., B. Martin, and L. S. Havarstein. 2007. "Competence-induced fratricide in streptococci." *Mol Microbiol* no. 64 (6):1423-33. doi: 10.1111/j.1365-2958.2007.05757.x.

Coates, John D, and Laurie A Achenbach. 2004. "Microbial perchlorate reduction: rocket-fueled metabolism." *Nature Reviews Microbiology* no. 2 (7):569-580. doi: 10.1038/nrmicro926.

Coates, John D., Urania Michaelidou, Royce A. Bruce, Susan M. O'Connor, Jill N. Crespi, and Laurie A. Achenbach. 1999. "Ubiquity and Diversity of Dissimilatory (Per)chlorate-Reducing Bacteria." *Applied And Environmental Microbiology* no. 65 (12):5234-5241.

Cobb, Michael D. 2011. "Creating informed public opinion: citizen deliberation about nanotechnologies for human enhancements." *Journal of Nanoparticle Research* no. 13 (4):1533-1548.

Cole, J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, G. M. Garrity, and J. M. Tiedje. 2009. "The Ribosomal Database Project: improved alignments and new tools for rRNA analysis." *Nucleic Acids Res* no. 37 (Database issue):D141-5. doi: 10.1093/nar/gkn879.

Conrad, A., M. K. Suutari, M. M. Keinanen, A. Cadoret, P. Faure, L. Mansuy-Huault, and J. C. Block. 2003. "Fatty acids of lipid fractions in extracellular polymeric substances of activated sludge flocs." *Lipids* no. 38 (10):1093-105. doi: 10.1007/s11745-006-1165-y.

Costerton, J. W., G. G. Geesey, and K. J. Cheng. 1978. "How bacteria stick." *Sci Am* no. 238 (1):86-95.

Curtis, T. P., I. M. Head, and D. W. Graham. 2003. "Theoretical ecology for engineering biology." *Environ Sci Technol* no. 37 (3):64A-70A. doi: 10.1021/es0323493.

D'Argenio, D. A., M. W. Calfee, P. B. Rainey, and E. C. Pesci. 2002. "Autolysis and Autoaggregation in Pseudomonas aeruginosa Colony Morphology Mutants." *Journal of Bacteriology* no. 184 (23):6481-6489. doi: 10.1128/jb.184.23.6481-6489.2002.

Damgaard, L. R., L. P. Nielsen, and N. P. Revsbech. 2001. "Methane microprofiles in a sewage biofilm determined with a microscale biosensor." *Water Res* no. 35 (6):1379-86.

Das, T., P. K. Sharma, H. J. Busscher, H. C. van der Mei, and B. P. Krom. 2010. "Role of extracellular DNA in initial bacterial adhesion and surface aggregation." *Appl Environ Microbiol* no. 76 (10):3405-8. doi: 10.1128/AEM.03119-09.

Davey, M. E., and A. O'Toole G. 2000. "Microbial biofilms: from ecology to molecular genetics." *Microbiol Mol Biol Rev* no. 64 (4):847-67. doi: 10.1128/MMBR.64.4.847-867.2000.

Davies, D. G., and C. N. Marques. 2009. "A fatty acid messenger is responsible for inducing dispersion in microbial biofilms." *J Bacteriol* no. 191 (5):1393-403. doi: 10.1128/JB.01214-08.

Davies, D. G., M. R. Parsek, J. P. Pearson, B. H. Iglewski, J. W. Costerton, and E. P. Greenberg. 1998. "The involvement of cell-to-cell signals in the development of a bacterial biofilm." *Science* no. 280 (5361):295-8. doi: 10.1126/science.280.5361.295.

de Silva, D G Viraj, and Bruce E Rittmann. 2000. "Nonsteady-State Modeling of Multispecies Activated-Sludge Processes." *Water Environment Research* no. 72 (5):554-565.

Decho, A. W., R. L. Frey, and J. L. Ferry. 2011. "Chemical challenges to bacterial AHL signaling in the environment." *Chem Rev* no. 111 (1):86-99. doi: 10.1021/cr100311q.

Delgado, Ana, Kamilla Lein Kjølberg, and Fern Wickson. 2011. "Public engagement coming of age: From theory to practice in STS encounters with nanotechnology." *Public Understanding of Science* no. 20 (6):826-845.

DeSantis, T. Z., P. Hugenholtz, N. Larsen, M. Rojas, E. L. Brodie, K. Keller, T. Huber, D. Dalevi, P. Hu, and G. L. Andersen. 2006. "Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB." *Appl Environ Microbiol* no. 72 (7):5069-72. doi: 10.1128/AEM.03006-05.

Diggle, S. P., S. Matthijs, V. J. Wright, M. P. Fletcher, S. R. Chhabra, I. L. Lamont, X. Kong, R. C. Hider, P. Cornelis, M. Camara, and P. Williams. 2007. "The Pseudomonas aeruginosa 4-quinolone signal molecules HHQ and PQS play multifunctional roles in quorum sensing and iron entrapment." *Chem Biol* no. 14 (1):87-96. doi: 10.1016/j.chembiol.2006.11.014.

Dobrindt, U., B. Hochhut, U. Hentschel, and J. Hacker. 2004. "Genomic islands in pathogenic and environmental microorganisms." *Nat Rev Microbiol* no. 2 (5):414-24. doi: 10.1038/nrmicro884.

Dubern, J. F., and S. P. Diggle. 2008. "Quorum sensing by 2-alkyl-4-quinolones in Pseudomonas aeruginosa and other bacterial species." *Mol Biosyst* no. 4 (9):882-8. doi: 10.1039/b803796p.

Duddridge, J. E., C. A. Kent, and J. F. Laws. 1982. "Effect of surface shear stress on the attachment of Pseudomonas fluorescens to stainless steel under defined flow conditions." *Biotechnol Bioeng* no. 24 (1):153-64. doi: 10.1002/bit.260240113.

Duhamel, Melanie, Stephan D Wehr, Lawrence Yu, Homa Rizvi, David Seepersad, Sandra Dworatzek, Evan e Cox, and Elizabeth A Edwards. 2002. "Comparison of anaerobic dechlorinating enrichment cultures maintained on tetrachloroethene, trichloroethene, cis-dichloroethene and vinyl chloride." *Water Research* no. 36 (17):4193-4202.

Dyer, Mark, Erwin van Heiningen, and Jan Gerritse. 2000. "In situ bioremediation of 1,2-dichloroethane under anaerobic conditions." *Geotechnical & Geological Engineering* no. 18 (4):313-334. doi: 10.1023/A:1016699701105.

Edgar, R. C. 2010. "Search and clustering orders of magnitude faster than BLAST." *Bioinformatics* no. 26 (19):2460-1. doi: 10.1093/bioinformatics/btq461.

Ellis, David E., Edward J. Lutz, J. Martin Odom, Ronald J. Buchanan, Craig L. Bartlett, Michael D. Lee, Mark R. Harkness, and Kim A. DeWeerd. 2000. "Bioaugmentation for Accelerated In Situ Anaerobic Bioremediation." *Environ Sci Technol* no. 34 (11):2254-2260. doi: 10.1021/es990638e.

Elowitz, M. B., A. J. Levine, E. D. Siggia, and P. S. Swain. 2002. "Stochastic gene expression in a single cell." *Science* no. 297 (5584):1183-6. doi: 10.1126/science.1070919.

Engebrecht, J., K. Nealson, and M. Silverman. 1983. "Bacterial bioluminescence: isolation and genetic analysis of functions from Vibrio fischeri." *Cell* no. 32 (3):773-81.

Environmental Security Technology Certification Program (ESTCP). 2004. Principles and Practices of Enhanced Anaerobic Bioremediation of Chlorinated Solvents.

EPA, U.S. Environmental Protection Agency. 2002. Guide for Conducting Treatability Studies Under CERCLA, Final. edited by Office of Research and Development US EPA, Office of Solid Waste and Emergency Response.

———. 2004a. Cleaning Up the Nations Waste Sites, Markets and Technology Trends. Natl Technical Information.

———. 2004b. Cleaning Up the Nations Waste Sites, Markets and Technology Trends. edited by EPA: Natl Technical Information.

———. 2011. Final Health Assessment for TCE. edited by EPA. Washington, D.C.: EPA.

———. 2012. FY 2012 Budget in Brief. US Environmental Protection Agency Washington, DC.

EPA, U.S. Environmental Protection Agency, and US Army Corps of Engineers. 2000. A Guide to Developing and Documenting Cost estimates during the feasibility study. edited by EPA.

ESTCP, Environmental Security Technology Certification Program. 2005. Bioaugmentation For Remediation Of Chlorinated Solvents:
Technology Development, Status, And Research Needs. edited by Department of Defense: GeoSyntec Consultants.

Faith, Daniel P. 1992. "Conservation evaluation and phylogenetic diversity." *Biological Conservation* no. 61 (1):1-10. doi: 10.1016/0006-3207(92)91201-3.

Findlay, Margaret, and Samuel Fogel. 2000. Microcosm Test for Natural Attenuation Of Chlorinated Solvents. Watertown, MA: Bioremediation Consulting, Inc.

Finstein, M S, J Cirello, D J Suler, M L Morris, and P F Strom. 1980. "Microbial Ecosystems Responsible for Anaerobic Digesting and Composting." *Journal of Water Pollution Control Federation* no. 52 (11):2675-2685.

Flemming, H. C., and J. Wingender. 2010. "The biofilm matrix." *Nat Rev Microbiol* no. 8 (9):623-33. doi: 10.1038/nrmicro2415.

Foley, R. W., Kay, B., Rushforth, R. & Wiek, A. . 2012. Can Nanotechnology Decontaminate Water in a Morally Contested Context? . Paper read at The 2012 International Symposium on Sustainable Systems and Technology, May 16-18, at Boston, MA.

Fouhy, Y., K. Scanlon, K. Schouest, C. Spillane, L. Crossman, M. B. Avison, R. P. Ryan, and J. M. Dow. 2007. "Diffusible signal factor-dependent cell-cell signaling and virulence in the nosocomial pathogen Stenotrophomonas maltophilia." *J Bacteriol* no. 189 (13):4964-8. doi: 10.1128/JB.00310-07.

Fowler, Troy, Bruce Thompson, and Jim Mueller. 2011. "Acetone and 2-butanone creation associated with biological and chemical remediation of environmental contamination." *Remediation Journal* no. 22 (1):9-28. doi: 10.1002/rem.21296.

Freedman, David L., Leo Lehmicke, and Matthew F. Verce. 2005. "Reductive Dechlorination of Tetrachloroethene Following Abiotic Versus Biotic Reduction of Hexavalent Chromium." *Bioremediation Journal* no. 9 (2):87-97. doi: 10.1080/10889860500276474.

Futamata, H., N. Yoshida, T. Kurogi, S. Kaiya, and A. Hiraishi. 2007. "Reductive dechlorination of chloroethenes by Dehalococcoides-containing cultures enriched from a polychlorinated-dioxin-contaminated microcosm." *ISME J* no. 1 (6):471-9. doi: 10.1038/ismej.2007.42.

Galloway, W. R., J. T. Hodgkinson, S. D. Bowden, M. Welch, and D. R. Spring. 2011. "Quorum sensing in Gram-negative bacteria: small-molecule modulation of AHL and AI-2 quorum sensing pathways." *Chem Rev* no. 111 (1):28-67. doi: 10.1021/cr100109t.

Goldman, B., S. Bhat, and L. J. Shimkets. 2007. "Genome evolution and the emergence of fruiting body development in Myxococcus xanthus." *PLoS ONE* no. 2 (12):e1329. doi: 10.1371/journal.pone.0001329.

Gonzalez-Pastor, J. E., E. C. Hobbs, and R. Losick. 2003. "Cannibalism by sporulating bacteria." *Science* no. 301 (5632):510-3. doi: 10.1126/science.1086462.

Götz, Friedrich. 2002. "Staphylococcus and biofilms." *Molecular Microbiology* no. 43 (6):1367-1378.

Haagensen, Janus A J, Mikkel Klausen, Robert K Ernst, Samuel I Miller, Anders Folkesson, Tim Tolker-Nielsen, and Søren Molin. 2007. "Differentiation and distribution of colistin- and sodium dodecyl sulfate-tolerant cells in Pseudomonas aeruginosa biofilms." *Journal of Bacteriology* no. 189 (1):28-37. doi: 10.1128/JB.00720-06.

Haas, B. J., D. Gevers, A. M. Earl, M. Feldgarden, D. V. Ward, G. Giannoukos, D. Ciulla, D. Tabbaa, S. K. Highlander, E. Sodergren, B. Methe, T. Z. DeSantis, J. F. Petrosino, R. Knight, B. W. Birren, and Consortium Human Microbiome. 2011. "Chimeric 16S rRNA sequence formation and detection in Sanger and 454-pyrosequenced PCR amplicons." *Genome Research* no. 21 (3):494-504. doi: 10.1101/gr.112730.110.

Halden, R U; Mcclellan, K; Kalinowski, T. 2013. "Parallel In Situ Screening of Remediation Strategies for Improved Decision Making, Remedial Design, and Cost Savings: ESTCP Project # 200914."

Hall-Stoodley, Luanne, J William Costerton, and Paul Stoodley. 2004. "Bacterial biofilms: from the Natural environment to infectious diseases." *Nature Reviews Microbiology* no. 2 (2):95-108. doi: 10.1038/nrmicro821.

Han, D, S Pal, J Nangreave, Z Deng, Y Liu, and H Yan. 2011. "DNA Origami with Complex Curvatures in Three-Dimensional Space." *Science (New York, NY)* no. 332 (6027):342-346. doi: 10.1126/science.1202998.

Hansen, Susse Kirkelund, Paul B Rainey, Janus A J Haagensen, and Søren Molin. 2007. "Evolution of species interactions in a biofilm community." *Nature* no. 445 (7127):533-536. doi: 10.1038/nature05514.

Hardie, Kim Rachael, and Karin Heurlier. 2008. "Establishing bacterial communities by 'word of mouth': LuxS and autoinducer 2 in biofilm development." *Nature Reviews Microbiology* no. 6 (8):635-643. doi: 10.1038/nrmicro1916.

Harrison, Joe J, Howard Ceri, Carol Stremick, and Raymond J Turner. 2004. "Differences in biofilm and planktonic cell mediated reduction of metalloid oxyanions." *FEMS microbiology letters* no. 235 (2):357-362. doi: 10.1016/j.femsle.2004.05.005.

Harrison, Joe J, Howard Ceri, and Raymond J Turner. 2007. "Multimetal resistance and tolerance in microbial biofilms." *Nature Reviews Microbiology* no. 5 (12):928-938. doi: 10.1038/nrmicro1774.

Hawley, Elisabeth L, Rula A Deeb, Michael C Kavanaugh, and James A Jacobs. 2004. "Treatment technologies for chromium (VI)." *Chromium (VI) Handbook*:275.

He, Jianzhong, Victor F Holmes, Patrick K H Lee, and Lisa Alvarez-Cohen. 2007. "Influence of vitamin B12 and cocultures on the growth of Dehalococcoides isolates in defined medium." *Applied And Environmental Microbiology* no. 73 (9):2847-2853. doi: 10.1128/AEM.02574-06.

Helmer, C., and S. Kunst. 1998. "Simultaneous nitrification/denitrification in an aerobic biofilm system." *Water Science and Technology* no. 37 (4-5):183-187. doi: 10.1016/s0273-1223(98)00102-4.

Herbert-Guillou, D. 2001. "Influence of the hydrodynamics on the biofilm formation by mass transport analysis." *Bioelectrochemistry* no. 53 (1):119-125. doi: 10.1016/S0302-4598(00)00121-5.

Hinsa, Shannon M, Manuel Espinosa-Urgel, Juan L Ramos, and George A O'Toole. 2003. "Transition from reversible to irreversible attachment during biofilm formation by Pseudomonas fluorescens WCS365 requires an ABC transporter and a large secreted protein." *Molecular Microbiology* no. 49 (4):905-918. doi: 10.1046/j.1365-2958.2003.03615.x.

Istok, J D, M D Humphrey, M H Schroth, M R Hyman, and K T O'Reilly. 1997. "Single-Well, "Push-Pull" Test for In Situ Determination of Microbial

Activities." *Ground Water* no. 35 (4):619-631. doi: 10.1111/j.1745-6584.1997.tb00127.x.

Ito, T, J L Nielsen, S Okabe, Y Watanabe, and P H Nielsen. 2002. "Phylogenetic Identification and Substrate Uptake Patterns of Sulfate-Reducing Bacteria Inhabiting an Oxic-Anoxic Sewer Biofilm Determined by Combining Microautoradiography and Fluorescent In Situ Hybridization." *Applied And Environmental Microbiology* no. 68 (1):356-364. doi: 10.1128/AEM.68.1.356-364.2002.

ITRC. 1998. Technical and Regulatory Requirements for Enhanced In Situ Bioremediation of Chlorinated Solvents in Groundwater. Interstate Technology and Regulatory Cooperation Workgroup: In Situ Bioremediation Subgroup.

———. 2011. Environmental Molecular Diagnostics Fact Sheets. The Interstate Technology & Regulatory Council: Environmental Molecular Diagnostics Team.

Izano, Era A, Matthew A Amarante, William B Kher, and Jeffrey B Kaplan. 2008. "Differential roles of poly-N-acetylglucosamine surface polysaccharide and extracellular DNA in Staphylococcus aureus and Staphylococcus epidermidis biofilms." *Applied And Environmental Microbiology* no. 74 (2):470-476. doi: 10.1128/AEM.02073-07.

Jintae Lee, Arul Jayaraman Thomas K Wood. 2007. "Indole is an inter-species biofilm signal mediated by SdiA." *BMC Microbiology* no. 7:42. doi: 10.1186/1471-2180-7-42.

Jurcisek, Joseph A, and Lauren O Bakaletz. 2007. "Biofilms formed by nontypeable Haemophilus influenzae in vivo contain both double-stranded DNA and type IV pilin protein." *Journal of Bacteriology* no. 189 (10):3868-3875. doi: 10.1128/JB.01935-06.

K, Kaewpipat, and Grady Jr C. 2002. "Microbial population dynamics in laboratory-scale activated sludge reactors." *iwaponline.com*.

Kalinowski, T., McClellan, K., Bruton, T.A., Halden R. U. 2010. In Situ Microcosm Array: A Novel Tool For Conducting Treatability Studies In Situ. Paper read

at Partners in Environmental Technology – Technical Symposium & Workshop, , at Washington, DC.

Kalinowski, T., McClellan, K., Bruton, T.A., Roll, I.B., Halden R. U. . 2012a. In Situ Microcosm Array: A Novel Tool For Conducting Treatability Studies In Situ. Paper read at AEHS Foundation Annual Meeting, at San Diego, CA.

Kalinowski, T., McClellan, K., Bruton, T.A., Roll, I.B., Krajmalnik-Brown, R., Halden R. U. . 2012b. Bioremediation of TCE and Hexavalent Chromium Contamination: Comparing Bench-Scale Treatability Studies to the In Situ Microcosm Array. Paper read at Eighth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, at Monterey, CA.

Kato, Kinya, and Kimberly L Davis. 1996. "Current use of bioremediation for TCE cleanup: Results of a survey." *Remediation Journal* no. 6 (4):1-14. doi: 10.1002/rem.3440060402.

Kaufmann, G F. 2005. "Revisiting quorum sensing: Discovery of additional chemical and biological functions for 3-oxo-N-acylhomoserine lactones." *Proceedings of the National Academy of Sciences* no. 102 (2):309-314. doi: 10.1073/pnas.0408639102.

Ke, Yonggang, Jaswinder Sharma, Minghui Liu, Kasper Jahn, Yan Liu, and Hao Yan. 2009. "Scaffolded DNA Origami of a DNA Tetrahedron Molecular Container." *Nano Letters* no. 9 (6):2445-2447. doi: 10.1021/nl901165f.

Kirisits, M. J., L. Prost, M. Starkey, and M. R. Parsek. 2005a. "Characterization of colony morphology variants isolated from Pseudomonas aeruginosa biofilms." *Appl Environ Microbiol* no. 71 (8):4809-21. doi: 10.1128/AEM.71.8.4809-4821.2005.

Kirisits, Mary Jo, Lynne Prost, Melissa Starkey, and Matthew R Parsek. 2005b. "Characterization of colony morphology variants isolated from Pseudomonas aeruginosa biofilms." *Applied And Environmental Microbiology* no. 71 (8):4809-4821. doi: 10.1128/AEM.71.8.4809-4821.2005.

Koenigsberg, Stephen S, Terry C Hazen, and Aaron D Peacock. 2005. "Environmental biotechnology: A bioremediation perspective." *Remediation Journal* no. 15 (4):5-25. doi: 10.1002/rem.20057.

Komlos, J, A B Cunningham, A K Camper, and R R Sharp. 2005. "Interaction of Klebsiella oxytoca and Burkholderia cepacia in Dual-Species Batch Cultures and Biofilms as a Function of Growth Rate and Substrate Concentration." *Microbial Ecology* no. 49 (1):114-125. doi: 10.1007/s00248-003-1066-z.

Korber, Darren R, John R Lawrence, Ben Sutton, and Douglas E Caldwell. 1989. "Effect of laminar flow velocity on the kinetics of surface recolonization by Mot+ and Mot− Pseudomonas fluorescens." *Microbial Ecology* no. 18 (1):1-19. doi: 10.1007/BF02011692.

Krajmalnik-Brown, Rosa, Youlboong Sung, Kirsti M Ritalahti, F Michael Saunders, and Frank E Löffler. 2007. "Environmental distribution of the trichloroethene reductive dehalogenase gene (tceA) suggests lateral gene transfer among Dehalococcoides." *FEMS Microbiology Ecology* no. 59 (1):206-214. doi: 10.1111/j.1574-6941.2006.00243.x.

Kühl, M, and B B Jørgensen. 1992. "Microsensor measurements of sulfate reduction and sulfide oxidation in compact microbial communities of aerobic biofilms." *Applied And Environmental Microbiology* no. 58 (4):1164-1174.

Latifi, A, M K Winson, M Foglino, B W Bycroft, G S Stewart, A Lazdunski, and P Williams. 1995. "Multiple homologues of LuxR and LuxI control expression of virulence determinants and secondary metabolites through quorum sensing in Pseudomonas aeruginosa PAO1." *Molecular Microbiology* no. 17 (2):333-343.

Lee, Woojin, and Bill Batchelor. 2002. "Abiotic Reductive Dechlorination of Chlorinated Ethylenes by Iron-Bearing Soil Minerals. 2. Green Rust." *Environmental Science & Technology* no. 36 (24):5348-5354. doi: 10.1021/es0258374.

Lehman, R. M., and S. P. O'Connell. 2002. "Comparison of extracellular enzyme activities and community composition of attached and free-living bacteria in porous medium columns." *Appl Environ Microbiol* no. 68 (4):1569-75.

Leita, Liviana, Alja Margon, Tania Sinicco, and Claudio Mondini. 2011. "Glucose promotes the reduction of hexavalent chromium in soil." *Geoderma* no. 164 (3–4):122-127. doi: 10.1016/j.geoderma.2011.05.013.

Lipson Lawrence, Randee. 2002. "A small circle of friends: Cohort groups as learning communities." *New Directions for Adult and Continuing Education* no. 2002 (95):83-92. doi: 10.1002/ace.71.

Liu, Hong, and Herbert H P Fang. 2002. "Characterization of electrostatic binding sites of extracellular polymers by linear programming analysis of titration data." *Biotechnology and Bioengineering* no. 80 (7):806-811. doi: 10.1002/bit.10432.

Llamas, María A, Marlies J Mooij, Marion Sparrius, Christina M J E Vandenbroucke-Grauls, Colin Ratledge, and Wilbert Bitter. 2008. "Characterization of five novel Pseudomonas aeruginosa cell-surface signalling systems." *Molecular Microbiology* no. 67 (2):458-472. doi: 10.1111/j.1365-2958.2007.06061.x.

Löffler, FE, RA Sanford, and KM Ritalahti. 2005. "Enrichment, cultivation, and detection of reductively dechlorinating bacteria." *Methods in enzymology* no. 397:77-111.

Löffler, Frank E, Jun Yan, Kirsti M Ritalahti, Lorenz Adrian, Elizabeth A Edwards, Konstantinos T Konstantinidis, Jochen A Müller, Heather Fullerton, Stephen H Zinder, and Alfred M Spormann. 2013. "Dehalococcoides mccartyi gen. nov., sp. nov., obligately organohalide-respiring anaerobic bacteria relevant to halogen cycling and bioremediation, belong to a novel bacterial class, Dehalococcoidia classis nov., order Dehalococcoidales ord. nov. and family Dehalococcoidaceae fam. nov., within the phylum Chloroflexi." *International Journal of Systematic and Evolutionary Microbiology* (63):625–635. doi: 10.1099/ijs.0.034926-0.

Losi, M. E., C. Amrhein, and W. T. Frankenberger, Jr. 1994. "Environmental biochemistry of chromium." *Rev Environ Contam Toxicol* no. 136:91-121.

Lovley, Derek R. 2003. "Cleaning up with genomics: applying molecular biology to bioremediation." *Nature Reviews Microbiology* no. 1 (1):35-44. doi: 10.1038/nrmicro731.

Lovley, Derek R, Elizabeth JP Phillips, Yuri A Gorby, and Edward R Landa. 1991. "Microbial reduction of uranium." *Nature* no. 350 (6317):413-416.

Lozupone, C., and R. Knight. 2005. "UniFrac: a new phylogenetic method for comparing microbial communities." *Applied and Environmental Microbiology* no. 71 (12):8228-8235. doi: 10.1128/aem.71.12.8228-8235.2005.

Maamar, Hédia, Arjun Raj, and David Dubnau. 2007. "Noise in Gene Expression Determines Cell Fate in Bacillus subtilis." *Science (New York, NY)* no. 317 (5837):526-464. doi: 10.1126/science.1140818.

Madsen, E L. 1991. "Determining in situ biodegradation." *Environmental Science and Technology* no. 25 (10).

Major, David W, Michaye L McMaster, Evan e Cox, Elizabeth A Edwards, Sandra M Dworatzek, Edwin R Hendrickson, Mark G Starr, Jo Ann Payne, and Lois W Buonamici. 2002. "Field Demonstration of Successful Bioaugmentation To Achieve Dechlorination of Tetrachloroethene To Ethene." *Environmental Science & Technology* no. 36 (23):5106-5116. doi: 10.1021/es0255711.

Manefield, Michael, Thomas Bovbjerg Rasmussen, Morten Henzter, Jens Bo Andersen, Peter Steinberg, Staffan Kjelleberg, and Michael Givskov. 2002. "Halogenated furanones inhibit quorum sensing through accelerated LuxR turnover." *Microbiology* (148):1119–1127.

Mann, Ethan E, Kelly C Rice, Blaise R Boles, Jennifer L Endres, Dev Ranjit, Lakshmi Chandramohan, Laura H Tsang, Mark S Smeltzer, Alexander R Horswill, and Kenneth W Bayles. 2009. "Modulation of eDNA release and degradation affects Staphylococcus aureus biofilm maturation." *PLoS ONE* no. 4 (6):e5822. doi: 10.1371/journal.pone.0005822.

Martin-Laurent, F, L Philippot, S Hallet, R Chaussod, J C Germon, G Soulas, and G Catroux. 2001. "DNA Extraction from Soils: Old Bias for New Microbial Diversity Analysis Methods." *Applied And Environmental Microbiology* no. 67 (5):2354-2359. doi: 10.1128/AEM.67.5.2354-2359.2001.

Martinez, R J, Y Wang, M A Raimondo, J M Coombs, T Barkay, and P A Sobecky. 2006. "Horizontal Gene Transfer of PIB-Type ATPases among Bacteria Isolated from Radionuclide- and Metal-Contaminated Subsurface Soils." *Applied And Environmental Microbiology* no. 72 (5):3111-3118. doi: 10.1128/AEM.72.5.3111-3118.2006.

Martiny, Adam C, Thomas M Jørgensen, Hans-Jørgen Albrechtsen, Erik Arvin, and Søren Molin. 2003. "Long-term succession of structure and diversity of a biofilm formed in a model drinking water distribution system." *Applied And Environmental Microbiology* no. 69 (11):6899-6907. doi: 10.1128/AEM.69.11.6899–6907.2003.

Marzorati, Massimo, Lieven Wittebolle, Nico Boon, Daniele Daffonchio, and Willy Verstraete. 2008. "How to get more out of molecular fingerprints: practical tools for microbial ecology." *Environmental Microbiology* no. 10 (6):1571-1581. doi: 10.1111/j.1462-2920.2008.01572.x.

May, Robert M. 1975. "Biological populations obeying difference equations: Stable points, stable cycles, and chaos." *Journal of Theoretical Biology* no. 51 (2):511-524. doi: 10.1016/0022-5193(75)90078-8.

Maymo-Gatell, X. 1997. "Isolation of a Bacterium That Reductively Dechlorinates Tetrachloroethene to Ethene." *Science (New York, NY)* no. 276 (5318):1568-1571. doi: 10.1126/science.276.5318.1568.

McClellan, K., Kalinowski, T., Bruton, T. A., Roll, I.B., Halden, R. U. 2011a. Bringing the Lab to the Field - the In Situ Microcosm Array. Paper read at Annual Conference of the NIEHS Superfund Program, at Lexington, KY.

———. 2011b. In Situ Microcosm Array (ISMA) vs. Standard Laboratory Assessment of Candidate Remediation Technologies – A Perchlorate Case Study. . Paper read at Geological Society of America Annual Meeting & Exhibition, at Minneapolis, MN.

McClellan, K., Kalinowski, T., Bruton, T. A., Roll, I.B., Halden, R. U. . 2012a. In Situ Microcosm Array vs. Laboratory Based Feasibility Studies for Remediation Technology Selection. . Paper read at Eighth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, at Monterey, CA.

———. 2012b. Lowering the Barrier for Novel In Situ Remediation Approaches - The In Situ Microcosm Array. . Paper read at AEHS Foundation Annual Meeting, at San Diego, CA.

McClellan, K., Kalinowski, T., Halden, R. U. 2009. ESTCP Project 200914: A New Technology for Remedial Design. . Paper read at Partners in Environmental Technology – Technical Symposium & Workshop, at Washington, DC.

McClellan, K., Kalinowski, T., Madrid, V., Verce, A., Sublette, K., Rays, E., Halden, R. U. . 2012c. Characterizing and Understanding Remediation Processes In Situ: The In Situ Technological Toolbox. . Paper read at Eighth International Conference on Remediation of Chlorinated and Recalcitrant Compounds, at Monterey, CA.

McGuire, Travis M., Charles J. Newell, Brian B. Looney, Karen M. Vangelas, and Claire H. Sink. 2004. "Historical analysis of monitored natural attenuation: A survey of 191 chlorinated solvent sites and 45 solvent plumes." *Remediation Journal* no. 15 (1):99-112. doi: 10.1002/rem.20036.

Melo, L. F., and M. J. Vieira. 1999. "Physical stability and biological activity of biofilms under turbulent flow and low substrate concentration." *Bioprocess Engineering* no. 20:363-368.

Michael, K, C Steuckart, E Gabriele, and P Jeroschewski. 1998. "A H2S microsensor for profiling biofilms and sediments: application in an acidic lake sediment." *Aquat. Microb. Ecol.* no. 15:201-209.

Middelboe, M., M. Søndergaard, Y. Letarte, and N. H. Borch. 1995. "Attached and free-living bacteria: Production and polymer hydrolysis during a diatom bloom." *Microbial Ecology* no. 29 (3):231-248. doi: 10.1007/bf00164887.

Miller, T. R. . 2005. Enrichment of Xenobiotic-degrading Bacteria Using a New In Situ Device. . Paper read at SETAC, at Baltimore, MD.

Möhle, Roland B, Timo Langemann, Marian Haesner, Wolfgang Augustin, Stephan Scholl, Thomas R Neu, Dietmar C Hempel, and Harald Horn. 2007. "Structure and shear strength of microbial biofilms as determined with confocal laser scanning microscopy and fluid dynamic gauging using a novel rotating disc biofilm reactor." *Biotechnology and Bioengineering* no. 98 (4):747-755. doi: 10.1002/bit.21448.

Molin, Søren, and Tim Tolker-Nielsen. 2003. "Gene transfer occurs with enhanced efficiency in biofilms and induces enhanced stabilisation of the biofilm structure." *Current Opinion in Biotechnology* no. 14 (3):255-261. doi: 10.1016/S0958-1669(03)00036-3.

Morse, Jeff J, Bruce C Alleman, James M Gossett, Stephen H Zinder, and Donna E Fennell. 1998. "Draft Technical Protocol: A Treatability Test for Evaluating

the Potential Applicability of the Reductive Anaerobic Biological in Situ Treatment Technology (Rabitt) to Remediate Chloroethenes." *oai.dtic.mil.ezproxy1.lib.asu.edu*.

Mulcahy, Heidi, Laetitia Charron-Mazenod, and Shawn Lewenza. 2008. "Extracellular DNA chelates cations and induces antibiotic resistance in Pseudomonas aeruginosa biofilms." *PLoS pathogens* no. 4 (11):e1000213.

Mundle, Scott O C, Tiffany Johnson, Georges Lacrampe-Couloume, Alfredo Pérez-de-Mora, Melanie Duhamel, Elizabeth A Edwards, Michaye L McMaster, Evan Cox, Kinga Révész, and Barbara Sherwood Lollar. 2012. "Monitoring Biodegradation of Ethene and Bioremediation of Chlorinated Ethenes at a Contaminated Site Using Compound-Specific Isotope Analysis (CSIA)." *Environmental Science & Technology* no. 46 (3):1731-1738. doi: 10.1021/es202792x.

Nadell, Carey D, Joao B Xavier, and Kevin R Foster. 2009. "The sociobiology of biofilms." *FEMS Microbiology Reviews* no. 33 (1):206-224. doi: 10.1111/j.1574-6976.2008.00150.x.

Neu, T R. 1996. "Significance of bacterial surface-active compounds in interaction of bacteria with interfaces." *Microbiological reviews* no. 60 (1):151.

Neu, T R, T Dengler, B Jann, and K Poralla. 1992. "Structural studies of an emulsion-stabilizing exopolysaccharide produced by an adhesive, hydrophobic Rhodococcus strain." *Journal of general microbiology* no. 138 (12):2531-2537.

Nielsen, P H, T.H. Christensen, H.J. Albrechtsen, and R.W. Gillham. 1996. "Performance of the in situ microcosm technique for measuring the degradation of organic chemicals in aquifers." *Ground Water Monitoring And Remediation* no. 16 (1):130-140.

Nierman, W C, T V Feldblyum, M T Laub, I T Paulsen, K E Nelson, J Eisen, J F Heidelberg, M R K Alley, N Ohta, J R Maddock, I Potocka, W C Nelson, A Newton, C Stephens, N D Phadke, B Ely, R T DeBoy, R J Dodson, A S Durkin, M L Gwinn, D H Haft, J F Kolonay, J Smit, M B Craven, H Khouri, J Shetty, K Berry, T Utterback, K Tran, A Wolf, J Vamathevan, M Ermolaeva, O White, S L Salzberg, J C Venter, L Shapiro, and C M Fraser. 2001. "Complete genome sequence of Caulobacter crescentus." *Proceedings of the National Academy of Sciences* no. 98 (7):4136-4141. doi: 10.1073/pnas.061029298.

NSF, National Science Foundation: Committee on Source Removal of Contaminants in the Subsurface. 2005. *Contaminants in the Subsurface: Source Zone Assessment and Remediation*: National Academy Press.

O'Toole, George A, and Roberto Kolter. 1998. "Flagellar and twitching motility are necessary for Pseudomonas aeruginosabiofilm development." *Proceedings of the National Academy of Sciences* no. 30 (2):295-304. doi: 10.1046/j.1365-2958.1998.01062.x.

O'Toole, George, Heidi B Kaplan, and Roberto Kolter. 2000. "Biofilm formation as microbial development." *Annu. Rev. Microbiol.* no. 54:49–79.

Ortori, Catharine A, Steve Atkinson, Siri Ram Chhabra, Miguel Cámara, Paul Williams, and David A Barrett. 2006. "Comprehensive profiling of N-acylhomoserine lactones produced by Yersinia pseudotuberculosis using liquid chromatography coupled to hybrid quadrupole–linear ion trap mass spectrometry." *Analytical and Bioanalytical Chemistry* no. 387 (2):497-511. doi: 10.1007/s00216-006-0710-0.

Owlad, Mojdeh, MohamedKheireddine Aroua, WanAshriWan Daud, and Saeid Baroutian. 2009. "Removal of Hexavalent Chromium-Contaminated Water and Wastewater: A Review." *Water, Air, and Soil Pollution* no. 200 (1-4):59-77. doi: 10.1007/s11270-008-9893-7.

Pandey, Janmejay, Archana Chauhan, and Rakesh K Jain. 2009. "Integrative approaches for assessing the ecological sustainability of in situbioremediation." *FEMS Microbiology Reviews* no. 33 (2):324-375. doi: 10.1111/j.1574-6976.2008.00133.x.

Patriquin, Glenn M, Ehud Banin, Christie Gilmour, Rivka Tuchman, E Peter Greenberg, and Keith Poole. 2008. "Influence of quorum sensing and iron on twitching motility and biofilm formation in Pseudomonas aeruginosa." *Journal of Bacteriology* no. 190 (2):662-671. doi: 10.1128/JB.01473-07.

Paul, Etienne, Juan Carlos Ochoa, Yoan Pechaud, Yu Liu, and Alain Liné. 2012. "Effect of shear stress and growth conditions on detachment and physical properties of biofilms." *Water Research* no. 46 (17):5499-5508. doi: 10.1016/j.watres.2012.07.029.

Prigent-Combaret, C, O Vidal, C Dorel, and P Lejeune. 1999. "Abiotic surface sensing and biofilm-dependent regulation of gene expression in Escherichia coli." *Journal of Bacteriology* no. 181 (19):5993-6002.

Proctor, Richard A, Christof von Eiff, Barbara C Kahl, Karsten Becker, Peter McNamara, Mathias Herrmann, and Georg Peters. 2006. "Small colony variants: a pathogenic form of bacteria that facilitates persistent and recurrent infections." *Nature Reviews Microbiology* no. 4 (4):295-305. doi: 10.1038/nrmicro1384.

Quensen, J F, J M Tiedje, and S A Boyd. 1988. "Reductive dechlorination of polychlorinated biphenyls by anaerobic microorganisms from sediments." *Science (New York, NY)* no. 242 (4879):752-754. doi: 10.1126/science.242.4879.752.

Rai, D., L. E. Eary, and J. M. Zachara. 1989. "Environmental chemistry of chromium." *Science of The Total Environment* no. 86 (1–2):15-23. doi: http://dx.doi.org/10.1016/0048-9697(89)90189-7.

Rai, D., B.M. Sass, and D.A. Moore. 1987. "Chromium (III) hydrolysis constants and solubility of chromium (III) hydroxide." *Inorganic Chemistry* no. 26 (3):345-349.

Ramsing, N B, M Kühl, and B B Jørgensen. 1993. "Distribution of sulfate-reducing bacteria, O2, and H2S in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes." *Applied And Environmental Microbiology* no. 59 (11):3840-3849.

Rappé, Michael S, and Stephen J Giovannoni. 2003. "The uncultured microbial majority." *Annual Review Of Microbiology* no. 57:369-394. doi: 10.1146/annurev.micro.57.030502.090759.

Reisner, Andreas, Janus A J Haagensen, Mark A Schembri, Ellen L Zechner, and Søren Molin. 2003. "Development and maturation of Escherichia coli K-12 biofilms." *Molecular Microbiology* no. 48 (4):933-946. doi: 10.1046/j.1365-2958.2003.03490.x.

Revsbech, Niels Peter. 1989. "Diffusion characteristics of microbial communities determined by use of oxygen microsensors." *Journal Of Microbiological Methods* no. 9 (2):111-122. doi: 10.1016/0167-7012(89)90061-4.

Rice, S A, K S Koh, S Y Queck, M Labbate, K W Lam, and S Kjelleberg. 2005. "Biofilm Formation and Sloughing in Serratia marcescens Are Controlled by Quorum Sensing and Nutrient Cues." *Journal of Bacteriology* no. 187 (10):3477-3485. doi: 10.1128/JB.187.10.3477-3485.2005.

Richard, Françoise C., and Alain C. M. Bourg. 1991. "Aqueous geochemistry of chromium: A review." *Water Research* no. 25 (7):807-816. doi: http://dx.doi.org/10.1016/0043-1354(91)90160-R.

Richardson, Ruth E, Vishvesh K Bhupathiraju, Donald L Song, Tanuja A Goulet, and Lisa Alvarez-Cohen. 2002. "Phylogenetic Characterization of Microbial Communities That Reductively Dechlorinate TCE Based upon a Combination of Molecular Techniques." *Environmental Science & Technology* no. 36 (12):2652-2662. doi: 10.1021/es0157797.

Rinker, Sherri, Yonggang Ke, Yan Liu, Rahul Chhabra, and Hao Yan. 2008. "Self-assembled DNA nanostructures for distance-dependent multivalent ligand–protein binding." *Nature Nanotechnology* no. 3 (7):418-422. doi: 10.1038/nnano.2008.164.

Rittmann, Bruce E. 2010. "The significance of biofilms in porous media." *Water Resources Research* no. 29 (7):2195-2202. doi: 10.1029/93WR00611.

Roeselers, G., B. Zippel, M. Staal, M. van Loosdrecht, and G. Muyzer. 2006. "On the reproducibility of microcosm experiments - different community composition in parallel phototrophic biofilm microcosms." *FEMS Microbiol Ecol* no. 58 (2):169-78. doi: 10.1111/j.1574-6941.2006.00172.x.

Ron, Eliora Z, and Eugene Rosenberg. 2001. "Natural roles of biosurfactants. Minireview." *Environmental Microbiology* no. 3 (4):229-236. doi: 10.1046/j.1462-2920.2001.00190.x.

Rowe, A R, B J Lazar, R M Morris, and R E Richardson. 2008. "Characterization of the Community Structure of a Dechlorinating Mixed Culture and Comparisons of Gene Expression in Planktonic and Biofloc-Associated "Dehalococcoides" and Methanospirillum Species." *Applied And Environmental Microbiology* no. 74 (21):6709-6719. doi: 10.1128/AEM.00445-08.

Ryan, R P, and J M Dow. 2008. "Diffusible signals and interspecies communication in bacteria." *Microbiology* no. 154 (7):1845-1858. doi: 10.1099/mic.0.2008/017871-0.

Sandrin, ToddR, and DouglasR Hoffman. 2007. "Bioremediation of Organic and Metal Co-contaminated Environments: Effects of Metal Toxicity, Speciation, and Bioavailability on Biodegradation." In *Environmental Bioremediation Technologies*, edited by ShreeN Singh and RudraD Tripathi, 1-34. Springer Berlin Heidelberg.

Satoh, Hisashi, Yoshiyuki Nakamura, Hideki Ono, and Satoshi Okabe. 2003. "Effect of oxygen concentration on nitrification and denitrification in single activated sludge flocs." *Biotechnology and Bioengineering* no. 83 (5):604-607. doi: 10.1002/bit.10717.

Sauer, K, A K Camper, G D Ehrlich, J W Costerton, and D G Davies. 2002. "Pseudomonas aeruginosa Displays Multiple Phenotypes during Development as a Biofilm." *Journal of Bacteriology* no. 184 (4):1140-1154. doi: 10.1128/jb.184.4.1140-1154.2002.

Schauder, S. 2001. "The languages of bacteria." *Genes & Development* no. 15 (12):1468-1480. doi: 10.1101/gad.899601.

Schaudinn, Christoph, Paul Stoodley, Aleksandra Kainović, Teresa O Keeffe, Bill Costerton, Douglas Robinson, Marc Baum, Garth Ehrlich, and Paul Webster. 2007. "Bacterial biofilms, other structures seen as mainstream concepts." *Microbe* no. 2 (5):231.

Schembri, Mark A, Kristian Kjaergaard, and Per Klemm. 2003. "Global gene expression in Escherichia coli biofilms." *Molecular Microbiology* no. 48 (1):253-267. doi: 10.1046/j.1365-2958.2003.03432.x.

Schmid, Andreas. 2002. "Increasing the microbial activity in activated sludge by the phenomenon of 'biological resonance." *Environmental science and pollution research international* no. 9 (4):227-229.

SES-TECH. 2010a. Enhanced in situ Bioremediation Field-scale Pilot Study Work Plan. San Diego, CA.

———. 2010b. Report of Laboratory Biotreatability Study to Assess Degradation of Hexavalent Chromium and Chlorinated Ethenes in Groundwater. San Diego, CA.

———. 2011. Enhanced in situ Bioremediation Field-scale Pilot Study Report.

Shank, Elizabeth A, Vanja Klepac-Ceraj, Leonardo Collado-Torres, Gordon E Powers, Richard Losick, and Roberto Kolter. 2011. "Interspecies interactions that result in Bacillus subtilis forming biofilms are mediated mainly by members of its own genus." *Proceedings Of The National Academy Of Sciences Of The United States Of America* no. 108 (48):E1236-43. doi: 10.1073/pnas.1103630108.

Shrout, Joshua D, David L Chopp, Collin L Just, Morten Hentzer, Michael Givskov, and Matthew R Parsek. 2006. "The impact of quorum sensing and swarming motility on Pseudomonas aeruginosa biofilm formation is nutritionally conditional." *Molecular Microbiology* no. 62 (5):1264-1277. doi: 10.1111/j.1365-2958.2006.05421.x.

Simon, MEINHARD. 1985. "Specific uptake rates of amino acids by attached and free-living bacteria in a mesotrophic lake." *Applied And Environmental Microbiology* no. 49 (5):1254-1259.

Skillman, L C, I W Sutherland, and M V Jones. 2010. "The role of exopolysaccharides in dual species biofilm development." *Journal of Applied Microbiology* no. 85 (S1):13S-18S. doi: 10.1111/j.1365-2672.1998.tb05278.x.

Star, Susan Leigh, and James R Griesemer. 1989. "Institutional ecology,translations' and boundary objects: Amateurs and professionals in Berkeley's Museum of Vertebrate Zoology, 1907-39." *Social studies of science* no. 19 (3):387-420.

Steenhoudt, O, and J Vanderleyden. 2000. "Azospirillum, a free-living nitrogen-fixing bacterium closely associated with grasses: genetic, biochemical and ecological aspects." *FEMS Microbiology Reviews* no. 24 (4):487-506.

Steven S Branda, José Eduardo González-Pastor Sigal Ben-Yehuda Richard Losick Roberto Kolter. 2001. "Fruiting body formation by Bacillus subtilis." *Proceedings Of The National Academy Of Sciences Of The United States Of America* no. 98 (20):11621. doi: 10.1073/pnas.191384198.

Stewart, Philip S, and Michael J Franklin. 2008. "Physiological heterogeneity in biofilms." *Nature Reviews Microbiology* no. 6 (3):199-210. doi: 10.1038/nrmicro1838.

Stoodley, P, K Sauer, D G Davies, and J W Costerton. 2002. "Biofilms as complex differentiated communities." *Annual Review Of Microbiology* no. 56:187-209. doi: 10.1146/annurev.micro.56.012302.160705.

Stroo, Hans F. 2010. "Bioremediation of Chlorinated Solvent Plumes." In *In Situ Remediation of Chlorinated Solvent Plumes*, edited by H. F. Stroo and C. H. Ward, 309-324. Springer New York.

Stroo, Hans F, David W Major, and James M Gossett. 2010. "Bioaugmentation for Anaerobic Bioremediation of Chlorinated Solvents." In *In Situ Remediation of Chlorinated Solvent Plumes*, edited by H. F. Stroo and C. H. Ward, 425-454. Springer New York.

Sturme, Mark H J, Michiel Kleerebezem, Jiro Nakayama, Antoon D L Akkermans, Elaine E Vaughan, and Willem M de Vos. 2002. "Cell to cell communication by autoinducing peptides in gram-positive bacteria." *Antonie van Leeuwenhoek* no. 81 (1/4):233-243. doi: 10.1023/A:1020522919555.

Suarez, M.P., and H.S. Rifai. 1999. "Biodegradation rates for fuel hydrocarbons and chlorinated solvents in groundwater." *Bioremediation Journal* no. 3 (4):337-362.

Sun, Yan, Randall D. Wolcott, and Scot E. Dowd. 2011. "Tag-Encoded FLX Amplicon Pyrosequencing for the Elucidation of Microbial and Functional Gene Diversity in Any Environment." In *High-Throughput Next Generation Sequencing: Methods and Application*, edited by Y. M. Kwon and S. C. Ricke, 129-141. Humana Press Inc, 999 Riverview Dr, Ste 208, Totowa, Nj 07512-1165 USA.

Svensäter, G, J Welin, J C Wilkins, D Beighton, and I R Hamilton. 2001. "Protein expression by planktonic and biofilm cells of Streptococcus mutans." *FEMS microbiology letters* no. 205 (1):139-146. doi: 10.1111/j.1574-6968.2001.tb10937.x.

Thomas, Christopher M, and Kaare M Nielsen. 2005. "Mechanisms of, and Barriers to, Horizontal Gene Transfer between Bacteria." *Nature Reviews Microbiology* no. 3 (9):711-721. doi: 10.1038/nrmicro1234.

Trulear, Michael G, and William G Characklis. 1982. "Dynamics of biofilm processes." *Journal (Water Pollution Control Federation)*:1288-1301.

U S EPA, Office of Research and Development. 2000. In Situ Treatment of Soil and Groundwater Contaminated with Chromium. Technical Resource Guide. edited by Office of Research and Development U S EPA.

Unanue, Marian, Begoña Ayo, Iñigo Azúa, Isabel Barcina, and Juan Iriberri. 1992. "Temporal variability of attached and free-living bacteria in coastal waters." *Microbial Ecology* no. 23 (1):27-39.

Under Secretary of Defense for Acquisition, Technology and Logistics. 2011. Defense Environmental Programs Annual Report to Congress FY2010.

Urbansky, Edward Todd. 2002. "Perchlorate as an environmental contaminant." *Environmental science and pollution research international* no. 9 (3):187-192.

URS Corporation. 2011. 2011 Sitewide Five-Year Review Report: Motorola 52nd Street Superfund Site Phoenix, Arizona. Phoenix, AZ.: URS Corporation.

van Loosdrecht, M C, J Lyklema, W Norde, and A J Zehnder. 1990. "Influence of interfaces on microbial activity." *Microbiological reviews* no. 54 (1):75-87.

Van Nostrand, J. D., L. Wu, W. M. Wu, Z. Huang, T. J. Gentry, Y. Deng, J. Carley, S. Carroll, Z. He, B. Gu, J. Luo, C. S. Criddle, D. B. Watson, P. M. Jardine, T. L. Marsh, J. M. Tiedje, T. C. Hazen, and J. Zhou. 2011. "Dynamics of microbial community composition and function during in situ bioremediation of a uranium-contaminated aquifer." *Appl Environ Microbiol* no. 77 (11):3860-9. doi: 10.1128/AEM.01981-10.

Vaningelgem, Frederik, Medana Zamfir, Fernanda Mozzi, Tom Adriany, Marc Vancanneyt, Jean Swings, and Luc De Vuyst. 2004. "Biodiversity of exopolysaccharides produced by Streptococcus thermophilus strains is reflected in their production and their molecular and functional characteristics." *Applied And Environmental Microbiology* no. 70 (2):900-912.

Veening, J W, E J Stewart, T W Berngruber, F Taddei, O P Kuipers, and L W Hamoen. 2008. "Bet-hedging and epigenetic inheritance in bacterial cell development." *Proceedings of the National Academy of Sciences* no. 105 (11):4393-4398. doi: 10.1073/pnas.0700463105.

Veening, Jan-Willem, Oleg A Igoshin, Robyn T Eijlander, Reindert Nijland, Leendert W Hamoen, and Oscar P Kuipers. 2008. "Transient heterogeneity in extracellular protease production by Bacillus subtilis." *Molecular Systems Biology* no. 4. doi: 10.1038/msb.2008.18.

Veening, Jan-Willem, Wiep Klaas Smits, and Oscar P Kuipers. 2008. "Bistability, epigenetics, and bet-hedging in bacteria." *Annual Review Of Microbiology* no. 62:193-210. doi: 10.1146/annurev.micro.62.081307.163002.

Viamajala, Sridhar, Brent M. Peyton, Rajesh K. Sani, William A. Apel, and James N. Petersen. 2004. "Toxic Effects of Chromium(VI) on Anaerobic and Aerobic Growth of Shewanella oneidensis MR-1." *Biotechnology Progress* no. 20 (1):87-95. doi: 10.1021/bp034131q.

Vieira, M.J., and L.F. Melo. 1999. "Intrinsic kinetics of biofilms formed under turbulent flow and low substrate concentrations." *Bioprocess Engineering* no. 20:369-375.

Vilain, S., J. M. Pretorius, J. Theron, and V. S. Brozel. 2009. "DNA as an adhesin: Bacillus cereus requires extracellular DNA to form biofilms." *Appl Environ Microbiol* no. 75 (9):2861-8. doi: 10.1128/AEM.01317-08.

von Ohle, Christiane, Armin Gieseke, Laura Nistico, Eva Maria Decker, Dirk deBeer, and Paul Stoodley. 2010. "Real-time microsensor measurement of local metabolic activities in ex vivo dental biofilms exposed to sucrose and treated with chlorhexidine." *Applied And Environmental Microbiology* no. 76 (7):2326-2334. doi: 10.1128/AEM.02090-09.

Vrionis, H. A., R. T. Anderson, I. Ortiz-Bernad, K. R. O'Neill, C. T. Resch, A. D. Peacock, R. Dayvault, D. C. White, P. E. Long, and D. R. Lovley. 2005. "Microbiological and geochemical heterogeneity in an in situ uranium bioremediation field site." *Appl Environ Microbiol* no. 71 (10):6308-18. doi: 10.1128/AEM.71.10.6308-6318.2005.

Wang, Lian-Hui, Yawen He, Yunfeng Gao, Ji En Wu, Yi-Hu Dong, Chaozu He, Su Xing Wang, Li-Xing Weng, Jin-Ling Xu, Leng Tay, Rong Xiang Fang, and Lian-Hui Zhang. 2003. "A bacterial cell-cell communication signal with cross-kingdom structural analogues." *Proceedings of the National Academy of Sciences* no. 51 (3):903-912. doi: 10.1046/j.1365-2958.2003.03883.x.

Wang, P C, T Mori, K Komori, M Sasatsu, K Toda, and H Ohtake. 1989. "Isolation and Characterization of an Enterobacter cloacae Strain That Reduces Hexavalent Chromium under Anaerobic Conditions." *Applied And Environmental Microbiology* no. 55 (7):1665-1669.

Webb, J. S., L. S. Thompson, S. James, T. Charlton, T. Tolker-Nielsen, B. Koch, M. Givskov, and S. Kjelleberg. 2003. "Cell death in Pseudomonas aeruginosa biofilm development." *J Bacteriol* no. 185 (15):4585-92.

Whitchurch, C B. 2002. "Extracellular DNA Required for Bacterial Biofilm Formation." *Science (New York, NY)* no. 295 (5559):1487-1487. doi: 10.1126/science.295.5559.1487.

Whiteley, Marvin, M Gita Bangera, Roger E Bumgarner, Matthew R Parsek, Gail M Teitzel, Stephen Lory, and E P Greenberg. 2001. "Gene expression in Pseudomonas aeruginosa biofilms." *Nature* no. 413 (6858):860-864. doi: 10.1038/35101627.

Wiedemeier, Todd H, M A Swanson, D E Moutoux, E Kinzie Gordon, J T Wilson, B H Wilson, D H Kampbell, P E Haas, R N Miller, and J E Hansen. 1998. "Technical protocol for evaluating natural attenuation of chlorinated solvents in ground water."

Williams, Paul, and Miguel Cámara. 2009. "Quorum sensing and environmental adaptation in Pseudomonas aeruginosa: a tale of regulatory networks and multifunctional signal molecules." *Current Opinion in Microbiology* no. 12 (2):182-191. doi: 10.1016/j.mib.2009.01.005.

Wingender, Jost, Martin Strathmann, Alexander Rode, Andrew Leis, and Hans-Curt Flemming. 2001. "Isolation and biochemical characterization of extracellular polymeric substances from Pseudomonas aeruginosa." In *Extracellular Polymers*, 302-314. Elsevier.

Wolcott, R. D., V. Gontcharova, Y. Sun, and S. E. Dowd. 2009. "Evaluation of the bacterial diversity among and within individual venous leg ulcers using bacterial tag-encoded FLX and titanium amplicon pyrosequencing and metagenomic approaches." *BMC Microbiol* no. 9:226. doi: 10.1186/1471-2180-9-226.

Y Shi, M D Zwolinski M E Schreiber J M Bahr G W Sewell W J Hickey. 1999. "Molecular Analysis of Microbial Community Structures in Pristine and Contaminated Aquifers: Field and Laboratory Microcosm Experiments." *Applied And Environmental Microbiology* no. 65 (5):2143.

Yan, J., K. M. Ritalahti, D. D. Wagner, and F. E. Loffler. 2012. "Unexpected specificity of interspecies cobamide transfer from Geobacter spp. to organohalide-respiring Dehalococcoides mccartyi strains." *Appl Environ Microbiol* no. 78 (18):6630-6. doi: 10.1128/AEM.01535-12.

Yang, L, K B Barken, M E Skindersoe, A B Christensen, M Givskov, and T Tolker-Nielsen. 2007. "Effects of iron on DNA release and biofilm development by Pseudomonas aeruginosa." *Microbiology* no. 153 (5):1318-1328. doi: 10.1099/mic.0.2006/004911-0.

Zhao, He-Ping, Steve Van Ginkel, Youneng Tang, Dae-Wook Kang, Bruce Rittmann, and Rosa Krajmalnik-Brown. 2011. "Interactions between Perchlorate and Nitrate Reductions in the Biofilm of a Hydrogen-Based Membrane Biofilm Reactor." *Environmental Science & Technology* no. 45 (23):10155-10162. doi: 10.1021/es202569b.

Ziv-El, M, T Kalinowski, R Krajmalnik-Brown, and R U Halden. 2013. "Simultaneous Determination of Chlorinated Ethenes and Ethene in Groundwater Using Headspace Solid-Phase Microextraction with Gas Chromatography." *Journal of Chromatographic Science*. doi: 10.1093/chromsci/bms258.

Ziv-El, Michal, Anca G Delgado, Ying Yao, Dae-Wook Kang, Katherine G Nelson, Rolf U Halden, and Rosa Krajmalnik-Brown. 2011. "Development and characterization of DehaloR^2, a novel anaerobic microbial consortium performing rapid dechlorination of TCE to ethene." *Applied Microbiology And Biotechnology*. doi: 10.1007/s00253-011-3388-y.

Ziv-El, Michal, Sudeep C Popat, Prathap Parameswaran, Dae-Wook Kang, Alexandra Polasko, Rolf U Halden, Bruce E Rittmann, and Rosa Krajmalnik-

Brown. 2012. "Using electron balances and molecular techniques to assess trichoroethene - induced shifts to a dechlorinating microbial community." *Biotechnology and Bioengineering*.

APPENDIX A

CAN STRESS ENHANCE PHYTOREMEDIATION OF POLYCHLORINATED BIPHENYLS?

ENVIRONMENTAL ENGINEERING SCIENCE
Volume 00, Number 00, 2012

DOI: 10.1089/ees.2012.0089

PERSPECTIVE

Can Stress Enhance Phytoremediation of Polychlorinated Biphenyls?

Tomasz Kalinowski* and Rolf U. Halden

The Swette Center for Environmental Biotechnology and The Center for Environmental Security, The Biodesign Institute at Arizona State University, Tempe, Arizona.

Received: March 2, 2012 *Accepted in revised form: August 7, 2012*

Abstract

Phytoremediation—plant-facilitated remediation of polluted soil and groundwater—is a potentially effective treatment technology for the remediation of heavy metals and certain organic compounds. However, contaminant attenuation rates are often not rapid enough to make phytoremediation a viable option when compared with alternative treatment approaches. Different strategies are being employed to enhance the efficacy of phytoremediation, including modification to the plant genome, inoculation of the rhizosphere with specialized and/or engineered bacteria, and treatment of the soil with supplementary chemicals, such as surfactants, chelators, or fertilizers. Despite these efforts, greater breakthroughs are necessary to make phytoremediation a viable technology. Here, we introduce and discuss the concept of integrating controlled environmental stresses as a strategy for enhancing phytoremediation. Plants have a diverse suite of defense mechanisms that are only induced in response to stress. Here, we examine some stress-response mechanisms in plants, focusing on defenses involving physiological changes that alter the soil microenvironment (rhizosphere), and outline how these defense mechanisms can be co-opted to enhance the effectiveness of phytoremediation of polychlorinated biphenyls and other contaminants.

Key words: inducible defense mechanisms; PCB; phytoremediation; rhizodegradation; root exudates; stress response

Introduction

Phytoremediation, the remediation of contaminated soil and groundwater facilitated by vegetation, is a promising technology and has seen growing interest in the scientific community such that the last decade has seen an average 20% year-over-year increase in the number of publications in the field: in 2001, there were 150 phytoremediation articles indexed by ISI; in 2011, there were 588. Phytoremediation can enhance contaminant attenuation in a number of ways. Phytoextraction, the removal of contaminants via incorporation into plant root biomass that is subsequently harvested, has been a long-standing mode of phytoremediation, especially for metals (Sheoran *et al.*, 2009). Phytovolatilization, the transpiration-mediated volatilization of contaminants (typically volatile organic pollutants [VOC], with trichloroethene [TCE] being the prime example), has also been successful (Gordon *et al.*, 1998).

Although promising results have been observed in the lab (Liu and Schnoor, 2008) and field (Mackova *et al.*, 2009), greater breakthroughs are necessary for the successful phytoremediation of polychlorinated biphenyls (PCBs) (van Aken *et al.*, 2010). Attenuation rates are limited in part by the tendency of PCBs (and other compounds with a high octanol-water partitioning coefficients, K_{OW}) to sorb strongly to soils and sediments, thereby limiting their bioavailability. Remediation is also limited by slow transformation rates, due to the stability imparted on the structure by chlorine substituents and aromatic rings. These are some of the challenges and limitations that still should be overcome before full-scale implementations of phytoremediation can become practical more frequently.

Consequently, numerous efforts are being made to enhance the rate and effectiveness of phytoremediation. The initial and most sustained efforts have focused on genetic modifications of the plant (Dietz and Schnoor, 2001; Harms *et al.*, 2011). Typically, bacterial genes are expressed in plant cells, thereby conferring the ability to metabolize, accumulate, or simply tolerate the contaminant. For recalcitrant contaminants, such as PCBs, entire suites of genes must be transformed into the plant genome before contaminant attenuation is observed.

However, the creation of transgenic plants may be unnecessary. The rhizosphere, the soil area adjacent to plant roots, is one of the most diverse and rich microbial ecosystems known. Plants release an array of organic molecules from their root structure, thereby allowing the resident soil microbial community to flourish (Coleman *et al.*, 2004). Researchers have long been investigating how microbial communities residing on plant roots can be exploited for remediation purposes (Donnelly *et al.*, 1994). Much progress has been made, but it is

**Corresponding author:* Swette Center for Environmental Biotechnology, The Biodesign Institute at Arizona State University, 1001 S. McAllister Avenue, Tempe, AZ 85287. *Phone:* 480-727-0698; *Fax:* 480-727-0889; *E-mail:* tkalinow@asu.edu

clear that we are only beginning to understand the complex relationships between plants and the rhizosphere.

The relationship between roots and the resident microbial community of the rhizosphere can simultaneously be described as symbiotic, commensal, mutualistic, and parasitic. The most studied of the symbiotic relationship-forming microorganisms have been nodule-forming *Rhizobium* spp., capable of fixing nitrogen in legumes, and arbuscular mycorrhizae—strains which provide an extension of the root network and aid in the harvest of nutrients, primarily phosphorus (P). More recently, it has come to light that the rhizosphere imparts many additional benefits to the plant, including protection from bacterial parasites (Bais *et al.*, 2001; Salem, 2003; Badri *et al.*, 2009), from toxic organics in the soil, and from macro parasites (i.e., grazing caterpillars) (Bezemer and van Dam, 2005).

In exchange for these services that the rhizosphere provides, the plant exudes photosynthetically fixed carbon at the roots, primarily in the form of organic acids, as well as free amino acids. In addition, it is known that plants exude many other, more complex organic molecules (e.g., flavonoids, terpenoids) whose function is still debated. Microbial PCB degradation has been shown to be stimulated by many of these compounds, including citric acid (White *et al.*, 2006), linalool, terpenoids (Luo *et al.*, 2007), phenolics, and flavonoids (Narasimhan *et al.*, 2003; Leigh *et al.*, 2006).

Most relevant to remediation efforts is the fact that plants can modulate the composition and amount of exudates released in response to environmental stresses they experience. It has long been thought that plant exudates are intended to directly mitigate environmental stress, for example, excretion by plant roots of citric acid for chelation of free metal ions in the presence of excess aluminum ions (Al^{3+}) (Ma *et al.*, 1997a). However, recent evidence suggests that root exudates may also function by enhancing the rhizosphere's microbial community's ability to mitigate chemical and environmental stress.

The manipulation of innate plant stress responses may represent a novel and ecologically sustainable approach that stimulates the rhizosphere, thereby increasing contaminant attenuation rates. Here, we examine a number of innate plant stress responses, with a focus on how these might influence phytoremediation. To this end, we have included only stresses that could feasibly be integrated into existing or future phytoremediation applications.

In addition, we are focusing primarily on the remediation of PCBs as a proxy for hydrophobic organic pollutants in general. This selection was guided by both the challenges posed by this group of widespread pollutants and recent advances in the use of phytoremediation as a remediation tool for PCBs. Polychlorinated biphenyls have been identified as carcinogens and endocrine disruptors that reduce the primary productivity of aquatic and terrestrial ecosystems and bioaccumulate up the food chain (van den Berg *et al.*, 2006). They are U.S. Environmental Protection Agency (EPA) priority pollutants and are ranked number five on the list of priority pollutants in the 2007 Comprehensive Environmental Response, Compensation, and Liability Act (CERCLA). An estimated 1.5 million tons of PCBs have been produced worldwide (van Aken *et al.*, 2010). The majority of this chemical mass is believed to be still in use and, thus, poses a risk of further release into the environment (Diamond *et al.*, 2010), despite the 1970s ban of these compounds in the United States (Pieper, 2005). Nowadays, more than 400 soil and sludge sites in the United States are known to be contaminated with PCBs (Varanasi *et al.*, 2007).

Environmental Stresses

In this section, environmental stresses with the potential to positively or negatively affect phytoremediation are discussed. These environmental stresses and their potential impacts are summarized in Table 1.

Aluminum

The phytotoxicity of aluminum (Al) depends on its speciation. The ionic form, Al^{3+}, is much more toxic than the chelated form, presumably because the chelated form is bound and unable to participate in reactions with sensitive plant proteins. The primary physical manifestation of Al stress in plants is the suppression of root elongation. However, to counter the toxicity effect, some plant species exude Al-chelating substances, most often citric, malic, or other organic acids. The ability of an organic acid to alleviate these toxicity effects is dependent on its chelating ability; citric acid, when present in concentrations equimolar to Al, has been shown to completely negate the ability of Al to inhibit root elongation (Hue *et al.*, 1986; Ma *et al.*, 1997b).

It has already been demonstrated that the addition of citric acid to PCB-contaminated soil increases the bioavailability of PCBs (White *et al.*, 2006). The additional input of organic acids also drastically changes the microenvironment immediately adjacent to the root: pH decreases, total biological oxygen demand (BOD) increases, and many other ionic compounds in addition to Al, such as phosphorus, are chelated, and often made more bioavailable (Cesco *et al.*, 2010; Jones, 1998). Furthermore, some of these stresses may have synergistic effects; Ma *et al.* observed that Al presence coupled with P deficiency

Table 1. Potential Impacts of Environmental Stresses on Phytoremediation

Stress	*Plant responses that may enhance contaminant attenuation*	*Plant responses that may retard contaminant attenuation*
Ionic aluminum	Secretion of citric, malic, and other organic acids	Suppression of root elongation
Nutrient deficiency	Exudates of organic acids, additional symbiotic relationships with AM, release of flavonoids, architectural changes in root development (more density); increased fine root hair turnover	Decreased plant primary productivity
Grazing	Production of monoterpenes, alkaloids	Decreased photosynthesis
Anoxia	Oxygenation of the roots, oxidative burst (ROS)	Decreased primary productivity
Gamma-irradiation	Increased growth rate	Plant death

ROS, reactive oxygen species; AM, arbuscular mycohrhizae.

enhanced the excretion of citric acid more than P deficiency alone.

Additional research is necessary to determine what the relative magnitude of these phenomena is on these processes. It is difficult to directly compare studies measuring citric acid secretion with studies measuring the effects that citric acid have on PCB bioavailability and soil microflora, because studies examining the former are often conducted under hydroponic (soil-free) conditions, while the latter are necessarily conducted in soil. Nevertheless, it is possible to get a rough estimate; under 50 μM Al^{+} concentrations, *Cassia tora* L. roots secreted approximately 6–8 μM citric acid per hour per g of root dry weight (Ma *et al.*, 1997a). Meanwhile, plant-soil microcosms amended with a 1 mM citric acid solution showed approximately 600% increased leaf content of PCBs, and a 65% increase in total removal of PCBs, compared with unamended controls (White *et al.*, 2006). In the soil microenvironment immediately adjacent to the root, where mobility of exudates is relatively low, it is conceivable that concentrations of exudates can reach 1 mM or higher at secretion rates of 6 μM per hour per g of root dry weight. However, a study directly examining the effect of this phenomenon on PCB removal in soil is necessary before any conclusions can be drawn.

In light of these known effects, amending PCB contaminated soil with low concentrations of Al may be an alternative to amending with a low-molecular-weight organic acid. The direct application of such chelating agents to the soil introduces the risk of mobilizing contaminants; whereas inducing plants to secrete their own chelating agent directly at the roots minimizes these risks. Furthermore, a degradable amendment such as citric acid would need to be applied repeatedly, thus increasing cost; an elemental amendment such as Al, however, would only need to be applied once and would function on a continuing basis. Replacing a degradable amendment with an elemental amendment could significantly reduce the recurring cost of long-term phytoremediation projects.

Phosphorus/nitrogen deficiency

Currently, plants in phytoremediation projects are routinely fertilized with nitrogen (N) and phosphorus (P) (Sheoran *et al.*, 2009). However, there have been no studies conducted that determine whether this is a sound practice for the remediation of organic pollutants.

N and P are the two principal limiting nutrients in terrestrial ecosystems. This is not due to lack of presence, but lack of availability. Both N and P are often present in abundant amounts, though in a form that is inaccessible to plants. As a result, plants have evolved elaborate mechanisms for increasing the availability of these nutrients.

Phosphorus is often unavailable for root uptake. At low pH (<6), P forms insoluble compounds by reacting with Al, iron (Fe), and organic matter. Meanwhile, in alkaline environments, it binds with calcium (Ca) and magnesium (Mg) to form only slightly more soluble phosphates (Jones, 1998; López-Bucio *et al.*, 2002). Nitrogen, on the other hand, is often abundant but unavailable to plants in the form of di-nitrogen.

Plants have numerous adaptive traits for coping with limiting N and P conditions: alteration of pH through secretion exudates (Dakora and Phillips, 2002; Bertin *et al.*, 2003), cultivation of symbiotic relationships with arbuscular mycohrhizae (AM) or *Rhizobia* spp. (Parniske, 2008), release of flavonoids (Cesco *et al.*, 2010), and architectural changes in root development (Fig. 1). Under nutrient limitation, plants drastically increase their branching and lateral root density, thereby increasing the total root surface area as well as total volume of soil canvassed (López-Bucio *et al.*, 2003).

All these stress responses to nutrient limitation are known or hypothesized to enhance PCB attenuation. It has already been demonstrated that AM and *Rhizobia* spp. enhance phytoremediation of PCBs (Teng *et al.*, 2010). Flavonoids and other plant phenolic compounds, which can be structurally similar to PCB, are known to support the growth of PCB-degrading microorganisms (Donnelly *et al.*, 1994; Fletcher and Hegde, 1995). Greater canvasing of the soil by the root structure should also enhance PCB removal.

The turnover rate of fine root hairs also increases with nutrient deficiency. It is known that in perennial plants, as much as 70% of the fine root hairs (defined as less than 2 mm in diameter) produced in a single growing season die and constitute a significant source of energy for the rhizosphere. This input of dead root hairs is believed to be a source of biomass for the rhizosphere throughout the growing season, not just at the end. Further, the phenolic content of these fine root hairs doubles immediately before root death (Leigh *et al.*, 2002). We suggest that the large input of phenolic-laden biomass from fine root hair turnover could stimulate PCB degradation by creating additional selective pressure for microbes adapted to phenolic and aromatic compounds.

The increased input of organic matter into the rhizosphere, the increase in the total soil in contact with roots and consequently the size of the rhizosphere, and the increased phenolic content of roots due to N/P deficiency lead us to suggest that such environmental stress may positively impact net PCB transformation rates in the rhizosphere, or perhaps at a minimum, offset the loss in phytoremediation efficacy due to reduced overall plant growth resulting from not applying fertilizers.

Grazing

Throughout their evolutionary history, plants have developed a variety of adaptations for coping with grazing: some have increased production of lignin and other compounds that make leaf matter unpalatable; others have increased total biomass production to offset losses due to grazing (Oesterheld and McNaughton, 1991). Generally, defense mechanisms against herbivores can be separated into two categories: inducible and constitutive. Inducible defenses typically come at a great cost to the plant, often decreasing reproductive ability in exchange for robustness (Baldwin *et al.*, 2001). This presents an intriguing opportunity where by maintaining a constant moderate grazing stress, a plant can be coaxed toward diverting resources from growth and reproduction toward robustness and defense. In practice, this often results in the growth of elaborate root systems that produce a suite of complex organic defense molecules—compounds which may potentiate the rhizodegradation of halogenated aromatics (Kessler and Baldwin, 2002; Singer *et al.*, 2003; Luo *et al.*, 2007).

For example, *Nicotiana tabacum*, when experiencing grazing-induced stress, increases production of nicotine and monoterpenes, despite the increased metabolic cost. Nicotine alone can account for as much as 8% of the plant's nitrogen sink, and nicotine is only one alkaloid in a suite of inducible

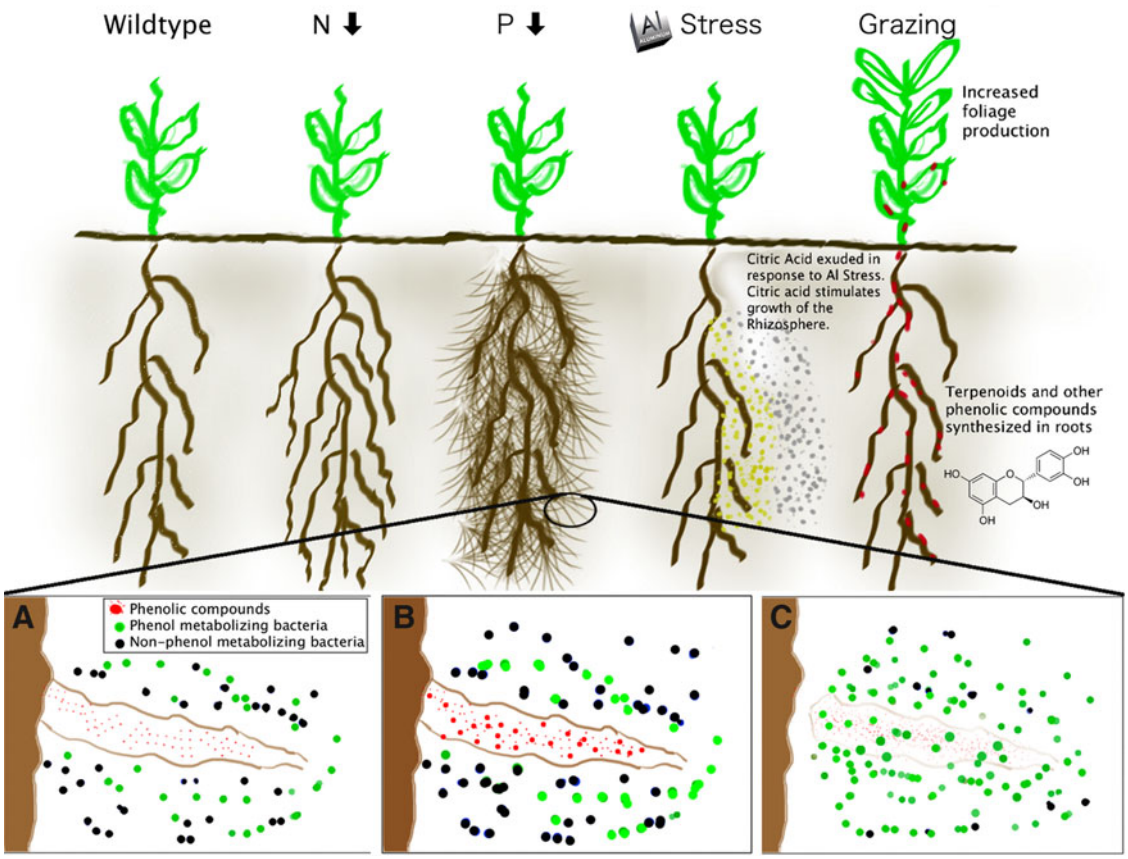

FIG. 1. Schematic drawing illustrating changes in plant physiology in response to environmental stress. Nitrogen deficiency (N ⬇): increased lateral root elongation. Phosphorous deficiency (P ⬇): increased fine root hair production, which may promote growth of polychlorinated biphenyls-degrading microorganisms. Aluminum stress: citric and other organic acids exuded, stimulating growth of the rhizoshphere. Grazing/parasite stress: phenolic compounds synthesized both in roots and leaves. **(A)** Fine root hair with background concentrations of phenolic compounds. **(B)** Phenolic content of root hairs increases before senescence. **(C)** After senescence of fine-root hair, phenol-metabolizing bacteria have increased presence in the rhizosphere due to the phenolic loading from fine root hair turnover. Adapted from Lopez-Bucio *et al.*, 2003.

defense mechanisms (Baldwin, 2001; Bezemer and van Dam, 2005). Interestingly, nicotine is primarily produced in the roots of plants, and then transported to leaves; it is unknown how much of the alkaloids produced at the roots are released, through fine root hair turnover or other means, into the rhizosphere.

Terpenoids represent another class of secondary plant metabolites that have been shown to facilitate PCB degradation in microcosm studies (Hernandez *et al.*, 1997; Kim *et al.*, 2003). Their production is only induced in response to stress: Terpenoids are metabolically expensive due to their need for extensive reduction, and they can build up in leaf matter and cause toxicity in the plant itself. Inducing the production of secondary plant metabolites through controlled, low-level grazing stress may be one way to potentiate the rhizosphere's remedial power by providing selective pressure for microorganisms with the ability to transform complex aromatic structures.

Anoxia

Anoxia, and its lesser cousin hypoxia, could be considered the most interesting stress treatment. An anaerobic environment is necessary for the metabolic reductive dehalogenation of higher chlorinated PCBs and other haloorganics by microbes, while an aerobic environment is needed for the complete mineralization of lower chlorinated PCBs. Consequently, sequential anaerobic–aerobic treatments have the most potential for removing PCBs (Vasilyeva and Strijakova, 2007).

However, anoxia poses a challenge for phytoremediation, because anoxia is detrimental to the health of almost all macroflora. Nevertheless, plants often have to deal with anoxic environments, and have developed elaborate coping mechanisms that could be co-opted for remediation purposes. These defense strategies are most developed in plants adapted to growing in marshes, bogs, and other environments that routinely are flooded and become anaerobic, but they are present in a limited form in other flora as well, including *N. tabacum* (Drew, 1997; Vartapetian and Jackson, 1997).

One strategy for coping with anoxia is oxygenation of the roots. For example, in the seagrass *Zostera marina* [another promising candidate for phytoremediation of PCBs (Huesemann *et al.*, 2009], oxygen is supplied to the roots during times of photosynthesis. However, roots still experience anoxia every night (Smith *et al.*, 1988). This daily supply

of oxygen to a normally anaerobic environment may create precisely the series of oxidative states necessary for the complete metabolic mineralization of PCBs.

γ-irradiation

More than 50 years ago, it was discovered that small doses of gamma (γ) irradiation can stimulate plant growth, and lead to more robust plants in general (Sax, 1955, 1963). Increased presence of oxidases within plant cells, faster growth rates, and additional branching in both shoots and roots are all gamma-ray induced changes in plant physiology (Wi *et al.*, 2007) that, technically, may positively impact PCB rhizodegradation. Further, γ-irradiation would be relatively inexpensive to implement in the field with a handheld device.

Conclusions and Future Work

Plants have stress-induced responses that are known to influence the microbial community of the rhizosphere. Some of these innate stress responses may enhance the attenuation rate for PCBs and other contaminants of concern through a variety of mechanisms, (i.e., stimulation of the rhizosphere through root exudates, change in root growth patterns, etc.) and, as such, provide a potential mechanism by which implementations of phytoremediation may be improved. We have reviewed here what is known about plant responses to certain stresses, and what is known about how these responses may impact the transformation of certain contaminants. However, the majority of our knowledge in these areas comes from research in which the two phenomena are studied separately. In addition, many publications of phytoremediation applications omit detailed information on the stresses that the plants experienced, making a literature meta-analysis of field results not possible. Further research directly aimed at studying the relationship between environmental stress and contaminant attenuation rates is necessary.

Intentionally exposing plants to any environmental stress in phytoremediation applications will involve balancing a series of trade offs; stress will impose a burden on the plant and can lead to less plant growth. However, it is clear that some of the stress responses may also enhance contaminant attenuation. Directed studies will be necessary to determine exactly to what extent these stress responses will off-set the overall reduced growth of the plant in phytoremediation applications. Some strategies for mitigating the negative impacts of stress may include only introducing them for brief periods, or in succession. Included in these investigations should also be the relative costs for implementing environmental stress mitigation measures (e.g., cost of applying fertilizer).

If future research demonstrates a positive association with stress-induced plant defense mechanisms and phytoremediation effectiveness, then the controlled use of environmental stresses could be used in concert with existing phytoremediation-enhancement strategies to address the challenges that should be overcome to establish phytoremediation as an effective environmental restoration technology.

Acknowledgments

This project was supported in part by Award Number R01ES015445 from the National Institute of Environmental Health Sciences (NIEHS). The content is solely the responsibility of the authors and does not necessarily represent the official views of the NIEHS or the National Institutes of Health (NIH).

Author Disclosure Statement

No competing financial interests exist.

References

Badri, D.V., Weir, T.L., van der Lelie, D., and Vivanco, J.M. (2009). Rhizosphere chemical dialogues: Plant-microbe interactions. *Curr. Opin. Biotechnol.* 20, 642.

Bais, H., Loyola-Vargas, V., Flores, H., and Vivanco, J. (2001). Root-specific metabolism: The biology and biochemistry of underground organs. *In Vitro Cell. Dev. Biol. Plant* 37, 730.

Baldwin, I.T. (2001). An ecologically motivated analysis of plant-herbivore interactions in native tobacco. *Plant Physiol.* 127, 1449.

Baldwin, I.T., Halitschke, R., Kessler, A., and Schittko, U. (2001). Merging molecular and ecological approaches in plant-insect interactions. *Curr. Opin. Plant Biol.* 4, 351.

Bertin, C., Yang, X., and Weston, L. (2003). The role of root exudates and allelochemicals in the rhizosphere. *Plant Soil* 256, 67.

Bezemer, T.M., and van Dam, N.M. (2005) Linking aboveground and below ground interactions via induced plant defenses. *Trends Ecol. Evol.* 20, 617.

Cesco, S., Neumann, G., Tomasi, N., Pinton, R., and Weisskopf, L. (2010). Release of plant-borne flavonoids into the rhizosphere and their role in plant nutrition. *Plant Soil* 329, 1.

Coleman, D.C., Crossley, D.A., and Hendrix P.F. (2004). *Fundamentals of Soil Ecology*, 2nd edn. San Diego, CA: Elsevier Academic Press.

Dakora, F., and Phillips, D. (2002). Root exudates as mediators of mineral acquisition in low-nutrient environments. *Plant Soil* 245, 35.

Diamond, M.L., Melymuk, L., Csiszar, S.A., and Robson, M. (2010). Estimation of PCB stocks, emissions, and urban fate: Will our policies reduce concentrations and exposure? *Environ. Sci. Technol.* 44, 2777.

Dietz, A.C., and Schnoor, J.L. (2001). Advances in phytoremediation. *Environ. Health Perspect* 109 (Suppl. 1), 163.

Donnelly, P., Hegde, R., and Fletcher, J. (1994). Growth of PCB-degrading bacteria on compounds from photosynthetic plants. *Chemosphere* 28, 981.

Drew, M.C. (1997). Oxygen deficiency and root metabolism: Injury and acclimation under hypoxia and anoxia. *Ann. Rev. Plant Physiol. Plant Mol. Biol.* 48, 223.

Fletcher, J.S., and Hegde, R.S. (1995). Release of phenols by perennial plant roots and their potential importance in bioremediation. *Chemosphere* 31, 3009.

Gordon, M., Choe, N., Duffy, J., Ekuan, G., Heilman, P., Muiznieks, I., Ruszaj, M., Shurtleff, B., Strand, S., Wilmoth, J., and Newman, L. (1998). Phytoremediation of trichloroethylene with hybrid poplars. *Environ. Health Perspect.* 106, 1001.

Harms, H., Schlosser, D., and Wick, L.Y. (2011). Untapped potential: Exploiting fungi in bioremediation of hazardous chemicals. *Nat. Rev. Microbiol.* 9, 177.

Hernandez, B., Koh, S., Chial, M., and Focht, D. (1997). Terpene-utilizing isolates and their relevance to enhanced biotransformation of polychlorinated biphenyls in soil. *Biodegradation* 8, 153.

Hue, N.V., Craddock, G.R., and Adams, F. (1986). Effect of organic acids on aluminum toxicity in subsoils. *Soil Sci. Soc. Amer. J.* 50, 28.

Huesemann, M.H., Hausmann, T.S., Fortman, T.J., Thom, R.M., and Cullinan, V. (2009). *In situ* phytoremediation of PAH- and PCB-contaminated marine sediments with eelgrass (*Zostera marina*). *Ecological Eng.* 35, 1395.

Jones, D.L. (1998). Organic acids in the rhizosphere: A critical review. *Plant Soil* 205, 25.

Kessler, A., and Baldwin, I.T. (2002). Plant responses to insect herbivory: The emerging molecular analysis. *Ann. Rev. Plant Biol.* 53, 299.

Kim, B., Oh, E., So, J., Ahn, Y., and Koh, S. (2003). Plant terpene-induced expression of multiple aromatic ring hydroxylation oxygenase genes in *Rhodococcus* sp. strain T104. *J. Microbiol.* 41, 349.

Leigh, M.B., Fletcher, J.S., Fu, X., and Schmitz, F.J. (2002). Root turnover: An important source of microbial substrates in rhizosphere remediation of recalcitrant contaminants. *Environ. Sci. Technol.* 36, 1579.

Leigh, M.B., Prouzová, P., Macková, M., Macek, T., Nagle, D.P., and Fletcher, J.S. (2006). Polychlorinated biphenyl (PCB)-degrading bacteria associated with trees in a PCB-contaminated site. *Appl. Environ. Microbiol.* 72, 2331.

Liu, J. and Schnoor, J.L. (2008). Uptake and translocation of lesser-chlorinated polychlorinated biphenyls (PCBs) in whole hybrid poplar plants after hydroponic exposure. *Chemosphere* 73, 1608.

López-Bucio, J., Hernández-Abreu, E., Sánchez-Calderón, L., Nieto-Jacobo, M.F., Simpson, J., and Herrera-Estrella, L. (2002). Phosphate availability alters architecture and causes changes in hormone sensitivity in the *Arabidopsis* root system. *Plant Physiol.* 129, 244.

López-Bucio, J., Cruz-Ramírez, A., and Herrera-Estrella, L. (2003). The role of nutrient availability in regulating root architecture. *Curr. Opin. Plant Biol.* 6, 280.

Luo, W., D'Angelo, E., and Coyne, M. (2007). Plant secondary metabolites, biphenyl, and hydroxypropyl-[beta]-cyclodextrin effects on aerobic polychlorinated biphenyl removal and microbial community structure in soils. *Soil Biol. Biochem.* 39, 735.

Ma, J., Zheng, S., and Matsumoto, H. (1997a). Specific secretion of citric acid induced by Al stress in Cassia tora L. *Plant Cell Physiol.* 38, 1019.

Ma, J., Hiradate, S., Nomoto, K., Iwashita, T., and Matsumoto, H. (1997b). Internal detoxification mechanism of Al in *Hydrangea macrophylla.* Identification of Al form in the leaves. *Plant Physiol.* 113, 1033.

Mackova, M., Prouzova, P., Stursa, P., Ryslava, E., Uhlik, O., Beranova, K., Rezek, J., Kurzawova, V., Demnerova, K., and Macek, T. (2009). Phyto/rhizoremediation studies using long-term PCB-contaminated soil. *Environ. Sci. Pollut. Res.* 16, 817.

Narasimhan, K., Basheer, C., Bajic, V.B., and Swarup, S. (2003). Enhancement of plant-microbe interactions using a rhizosphere metabolomics-driven approach and its application in the removal of polychlorinated biphenyls. *Plant Physiol.* 132, 146.

Oesterheld, M., and McNaughton, S. (1991). Effect of stress and time for recovery on the amount of compensatory growth after grazing. *Oecologia* 85, 305.

Parniske, M. (2008). Arbuscular mycorrhiza: the mother of plant root endosymbioses. *Nat. Rev. Microbiol.* 6, 763.

Pieper, D.H. (2005). Aerobic degradation of polychlorinated biphenyls. *Appl. Microbiol. Biotechnol.* 67, 170.

Salem, S., (2003). *Impact of mycorrhizal fungi and other symbiotic microbes as biocontrol agents on soil borne pathogens and some ecophysiological changes in tomato roots* [Ph.D. dissertation]. Gödöllő, Hungary: Szent István University.

Sax, K., (1955). The effect of ionizing radiation on plant growth. *Am. J. Botany* 42, 360.

Sax, K. (1963). The stimulation of plant growth by ionizing radiation. *Radiat. Botany* 3, 179.

Sheoran, V., Sheoran, A.S., and Poonia, P. (2009). Phytomining: A review. *Minerals Eng.* 22, 1007.

Singer, A.C., Crowley, D.E., and Thompson, I.P. (2003). Secondary plant metabolites in phytoremediation and biotransformation. *Trends Biotechnol.* 21, 123.

Smith, R., Pregnall, A., and Alberte, R. (1988). Effects of anaerobiosis on root metabolism of *Zostera marina* (eelgrass): Implications for survival in reducing sediments. *Mar. Biol.* 98, 131.

Teng, Y., Luo, Y., Sun, X., Tu, C., Xu, L., Liu, W., Li, Z., and Christie, P. (2010). Influence of arbuscular mycorrhiza and *Rhizobium* on phytoremediation by alfalfa of an agricultural soil contaminated with weathered PCBs: A field study. *Int. J. Phytoremediation* 12, 516.

van Aken, B., Correa, P.A., and Schnoor, J.L. (2010) Phytoremediation of polychlorinated biphenyls: New trends and promises. *Environ. Sci. Technol.* 44, 2767.

van den Berg, M., Birnbaum, L., Denison, M., de Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., and Haws, L. (2006). The 2005 World Health Organization reevaluation of human and mammalian toxic equivalency factors for dioxins and dioxin-like compounds. *Toxicol. Sci.* 93, 223.

Varanasi, P., Fullana, A., and Sidhu, S. (2007). Remediation of PCB contaminated soils using iron nano-particles. *Chemosphere* 66, 1031.

Vartapetian, B., and Jackson, M. (1997). Plant adaptations to anaerobic stress. *Ann. Botany* 79 suppl. A, 3.

Vasilyeva, G., and Strijakova, E. (2007). Bioremediation of soils and sediments contaminated by polychlorinated biphenyls. *Microbiology* 76, 639.

White, J.C., Parrish, Z.D., Isleyen, M., Gent, M.P.N., Iannucci-Berger, W., Eitzer, B.D., Kelsey, J.W., and Mattina, M.I. (2006). Influence of citric acid amendments on the availability of weathered PCBs to plant and earthworm species. *Int. J. Phytoremediation* 8, 63.

Wi, S.G., Chung, B.Y., Kim, J.-S., Kim, J.-H., Baek, M.-H., Lee, J.-W., and Kim, Y.S. (2007). Effects of gamma irradiation on morphological changes and biological responses in plants. *Micron* 38, 553.