Division of Science, Research and Environmental Health

Research Project Summary

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Applying Innovative Diagnostic Tools at New Jersey Publicly Funded Sites

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Abstract

This project demonstrated the use of Environmental Molecular Diagnostic Tools (EMDs) for detecting microbial biodegradation of contaminants and identifying bacteria responsible for contaminant biodegradation or biotransformation at three contaminated sites in New Jersey. These sites were unique based on the contamination present, and EMDs were selected to address a particular issue at each site.

EMDs is a collective term that describes a group of advanced and emerging techniques used to analyze biological and chemical characteristics of soils, sediments, groundwater, and surface water. Many of these tools were originally developed for applications in medicine, defense, and industry. Over the last decade, great advances have been made in adapting and applying EMDs for site characterization, remediation, monitoring, and closure. EMDs are important and valuable because they can provide key information not available using traditional analytical methods (e.g., groundwater analysis for volatile organic compounds). While they are intended to complement these traditional methods, EMDs can bring a new perspective to all stages in the environmental management decision-making process.

As a result of this work, a bio-augmentation/bio-stimulation design was developed for an organic solvent plume at one site. At a second site, Stable Isotope Probing (SIP) was used to confirm the presence of dehalogenating organisms and bio-stimulation demonstrated rapid reductive dechlorination. Finally, aniline degrading organisms were studied using SIP. Aniline biodegradation was demonstrated and the specific bacteria responsible for biodegradation were identified.

Background

Management of contaminated sites encompasses everything from site characterization to ultimate selection, implementation, and completion of a site remedial action plan and then finally, site closure. The investigation begins with a characterization that includes specifying the contaminants, concentrations and media (e.g., groundwater aquifer, sediments or soils) of interest, assessing exposure pathways, and determining remediation goals. The estimated volumes or areas of the site to be remediated must be defined. Finally, detail concerning the equipment, methods, and locations to be evaluated for each possible site remediation approach alternative (e.g., potential natural recovery processes, potential remediation plan, need for monitoring and/or institutional controls, etc.) must be determined. To the extent possible the time frame(s) in which alternatives are expected to achieve cleanup levels are also estimated.

Remediation of contaminated sites may be performed using physical-chemical processes such as ground water pump and treat with sorption or volatilization, soil excavation or sediment dredging and landfilling, or chemical oxidation. More sustainable remediation options include bioremediation approaches to degrade or transform pollutants to benign end products.

To assess the potential for application of bioremediation to a site or to select the type of bioprocess to be used in bioremediation, a variety of assessment protocols have been developed that provide guidance for monitored natural attenuation (MNA) or enhanced bioremediation based on geochemical indicators such as pH, prevailing redox conditions as indicated by the presence of various electron acceptors, and presence of transformation products of the original contaminant. Powerful new environmental molecular diagnostics (EMDs) have been developed that can detect and quantify the microorganisms, genes, and enzymes

responsible for specific processes, and are recommended for use in site monitoring and management. The use of EMDs thus allow a direct assessment of the potential natural or enhanced biological processes for contaminant removal.

Objectives

The overarching goal of this project was to demonstrate applications for innovative EMDs for improving bioremediation at contaminated sites in New Jersey. The specific objectives were:

1. Use emerging EMDs (at Rutgers and commercially available) to facilitate and guide site assessment to direct a bioremediation approach.
2. Assess a site that is or has undergone unsuccessful or stalled bioremediation and use diagnostic tools for re-assessment to re-screen and re-initiate remedial actions.
3. Provide data through EMD analyses to support site closure at one or more sites that have been successfully remediated or which are undergoing monitored natural attenuation.
4. Perform research in support of development and application of EMDs for novel bioremediation approaches for emerging contaminants or for those with no commercially available EMDs.

Methods

With the assistance of the NJDEP Division of Site Remediation, three sites were selected for this study. First, sediment cores were collected from the contaminated aquifer at the former Pagan-Martinez site (Jersey City, NJ) to aid an on-going bioremediation effort. The former Pagan -Martinez site has perchloroethene and trichloroethene along with fuel oil contamination. Initally, the leaking fuel oil tank was removed along with fuel-oil contaminated soils. A work plan concentrating on dense non-aqueous phase liquids (DNAPLs) was developed for the site and microcosms were established using the aquifer core materials. After six months, no dechlorination of PCE or TCE was detected in the microcosms from the site. EMD tools were applied to determine the next course of action at the site.

In the second project, Stable Isotope Probing (SIP) was used to identify dehalorespiring bacteria in sediments from the organochloride-contaminated Hackensack River, NJ. SIP targets metabolically active bacteria using isotope incorporation (e.g., ^{13}C) into newly biosynthesized macromolecules. The ^{13}C DNA can be recovered separately from the unlabeled DNA from other organisms. Certain dehalorespiring bacteria do not assimilate carbon from the chlorinated compounds they use as electron acceptors. To label DNA from dechlorinators using SIP, site materials were exposed to ^{13}C-based carbon sources that are assimilated by the bacteria (i.e., ^{13}C-acetate and ^{13}C-labeled lactate which is fermented to ^{13}C-labeled acetate and hydrogen). The bacterial community in active Hackensack River microcosms included *Dehalococcoides*, *Dehalogenimonas, Desulfobacterium, Geobacteraceae,* and *Desulfuromonas* as identified in the clone library derived from ^{13}C-heavy fraction. This method could also be used to guide and monitor the bioremediation effort at the Pagan-Martinez site after bioaugmentation (since no dechlorination was observed in the native sediments).

Finally, in the third effort supported by this project, bacteria involved in the biodegradation of aniline at a contaminated industrial chemical site were identified. Results from microcosms operated over many months as part of an earlier project indicated loss of aniline in site sediments. However, the biodegradation of aniline was not conclusively demonstrated in this earlier project because intermediates were not identified and loss from sorption could not be ruled out. Operating microcosms in varying redox conditions were performed to determine if biodegradation was occurring.

Collection of Environmental Samples

Aquifer materials. Sediment cores and groundwater samples were collected from the former Pagan-Martinez site in Jersey City, NJ in March 2013 under the direction of Land Planning Associates, Inc. (see **Figure 1**). At this site a leaking fuel oil tank was excavated and the excavation was back-filled with soil containing a variety of chlorinated solvents. After the tank was removed, ground water analysis detected tetrachloroethene (PCE) and trichloroethene (TCE) and other chlorinated ethanes. The sediment cores collected for this study include those from the contaminated groundwater plume and from uncontaminated portions of the aquifer as controls. In all, four sediment cores from different locations and two groundwater samples from different monitoring wells were collected. Samples were transported immediately to the Department of Environmental Sciences at Rutgers University and stored at 4°C until use.

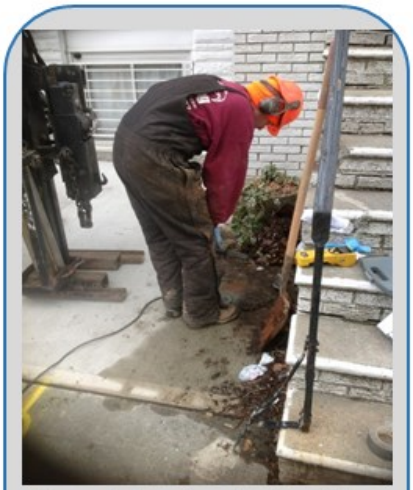

Figure 1. Sample collection at the Pagan-Martinez site in Jersey City, NJ.

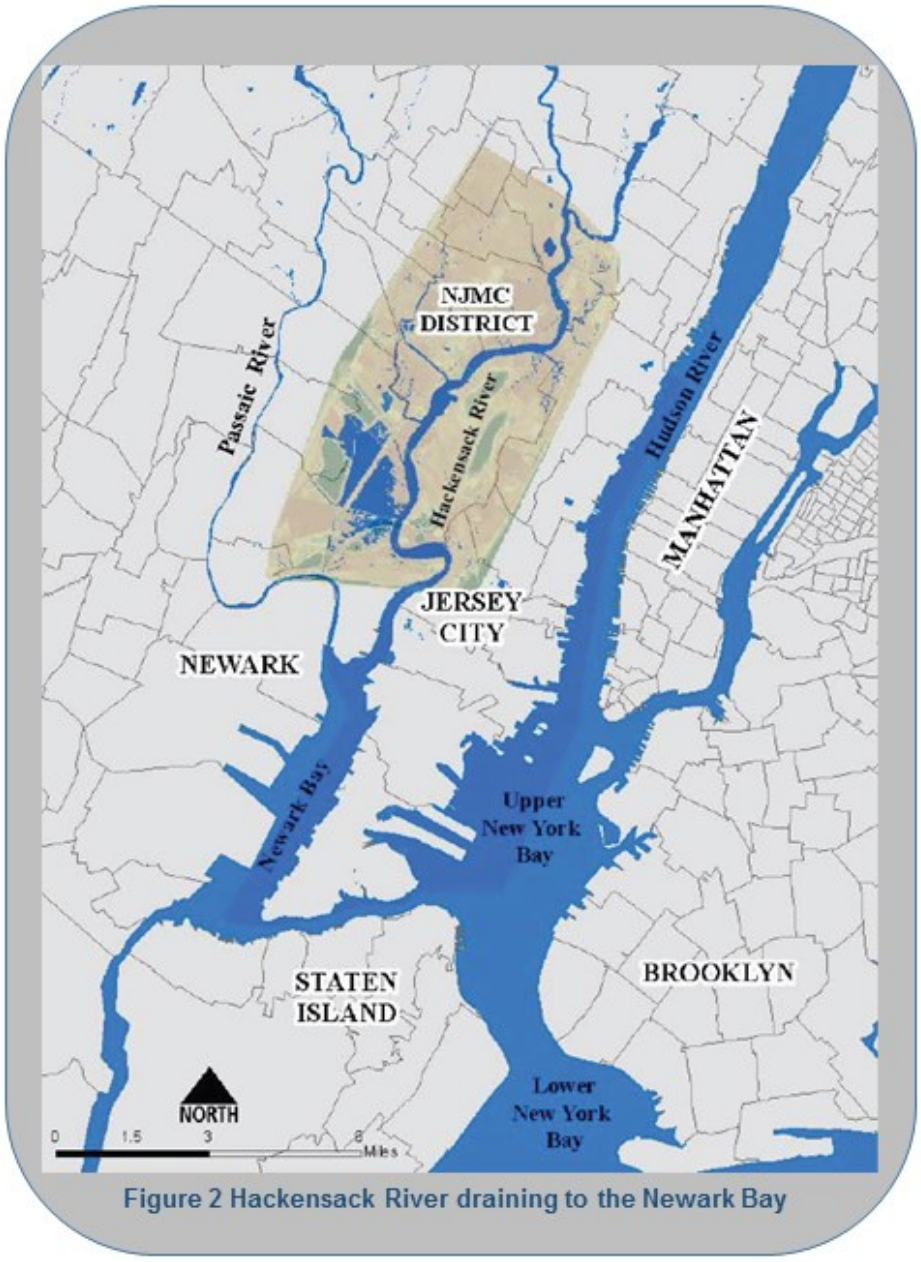

Figure 2 Hackensack River draining to the Newark Bay

Hackensack River sediments. Surficial grab samples were obtained from the Hackensack River (**Figure 2**). Samples were placed in sterile jars, transported immediately to the Department of Environmental Sciences and stored at 4°C until used within the established holding times.

Microcosm protocols

Dechlorinating microcosms. Anaerobic microcosms were used to identify the interacting and interdependent microorganisms involved in dechlorinating communities with tetrachloroethene (PCE) used as a model chlorinated compound. Organohalide contaminated river sediments (Hackensack River) and PCE contaminated aquifer sediments (Pagan-Martinez site, Jersey City, NJ) were studied.

For the Pagan-Martinez site, 24 microcosms seeded from sediment cores were set up to test *in situ* dechlorination capability. A top surface sediment and bottom sediment from each core were used as inoculum to set up triplicate microcosms. For the Hackensack sediments triplicate microcosms were established from several locations. Microcosms were amended with PCE as the electron acceptor and either acetate or lactate was added as electron donors/carbon sources or electron-hydrogen donors/carbon sources, respectively, for the dechlorinators. ^{13}C-acetate and ^{13}C-lactate were provided to parallel dechlorinating microcosms in order to label the population of interest for further analysis. All original sediments and digester sludge were sub-sampled for later molecular analysis and stored at -80ºC.

Aniline-degrading microcosms. Aerobic and anaerobic microcosms prepared from environmental media collected for a previous study from a contaminated site in New Jersey were used for development of protocols for this project and for scientific study. These sediments and microcosms were part of an existing project at Rutgers. Sediment slurries of 100 mL containing 20 mL of sediment and 80 mL of site water or minimal media were constructed under sterile conditions. Microcosms seeded with contaminated sediments either from (freshwater canal

or groundwater aquifer) were prepared under aerobic, denitrifying or methanogenic conditions. The Stable Isotope Probing (SIP) experiment incorporated nine microcosms for each treatment. Among them, triplicate microcosms were amended with labeled aniline (1µl, ring-$^{13}C_6$ aniline, Cambridge Isotope Laboratories, Inc. Andover, U.S.) or unlabeled aniline (1µl, Sigma Aldrich, St. Louis, U.S.). Triplicate abiotic autoclaved controls amended with unlabeled aniline were also included. The abiotic controls were sterilized in an autoclave for 45 min for three consecutive days. The microcosms were incubated at room temperature (20°C) with reciprocal shaking.

Chemical analytical methods

PCE and its daughter products vinyl chloride (VC) and ethene, and methane, were determined using an Agilent Technology 6890N (Agilent Technologies, Inc. Santa Clara, CA, USA) gas chromatograph (GC) with a flame ionization detector (FID). 250 µL headspace samples were injected into the GC-FID equipped with a GS-GasPro capillary column (30 m × 0.32 mm I.D.; Agilent Technologies, Inc. Santa Clara, CA, USA) with helium as the carrier gas.

Microcosms were sampled for aniline, initially weekly and thereafter, periodically. Analysis was by an Agilent 1100 high performance liquid chromatography (HPLC) system equipped with a diode array detector operating at 244 nm for detection of aniline and PCA. Isocratic separations were made on a Luna 5m C18 (2) 120 column (250*2 mm) (Phenomenex, Torrance, CA). The column was held at 40°C. A water: acetonitrile (ACN) mixture (45:55 volume:volume) was supplied at a flow rate of 0.33 mL min^{-1} as the mobile phase. Aniline eluted at 2.8 min and was identified by comparison of the retention time to a known standard. Aniline concentrations in samples were quantified using a five point calibration. The microcosms were amended with approximately 1500 µM aniline and the concentration of the standards ranged from 100 µM to 2000 µM.

Molecular methods

Methods to target metabolically active bacteria have been developed using isotope incorporation into newly biosynthesized macromolecules. This is termed stable isotope probing (SIP). For example, active bacteria can be detected in environmental samples by following ^{13}C substrates into ^{13}C DNA, RNA or proteins and is termed DNA-SIP, RNA-SIP and protein-SIP, respectively. Stable isotope probing (SIP) of DNA and sequencing of this labeled DNA were used as a major means to identify bacteria responsible for biodegradation at contaminated sites in NJ. The overall work flow for SIP is shown in **Figure 3**.

Results and Discussions

Microbial Investigation of the Former Pagan-Martinez Site

A work plan was developed in conjunction with Engineering and Land Planning Associates, Inc. (E&LP) for remediation of the Pagan-Martinez site. For the Pagan-Martinez site both microcosms for PCE dechlorination and community analysis of the aquifer were evaluated.

Dechlorination results. No PCE dechlorination was detected in the aquifer microcosms after six months of monitoring. E&LP sent additional samples to a

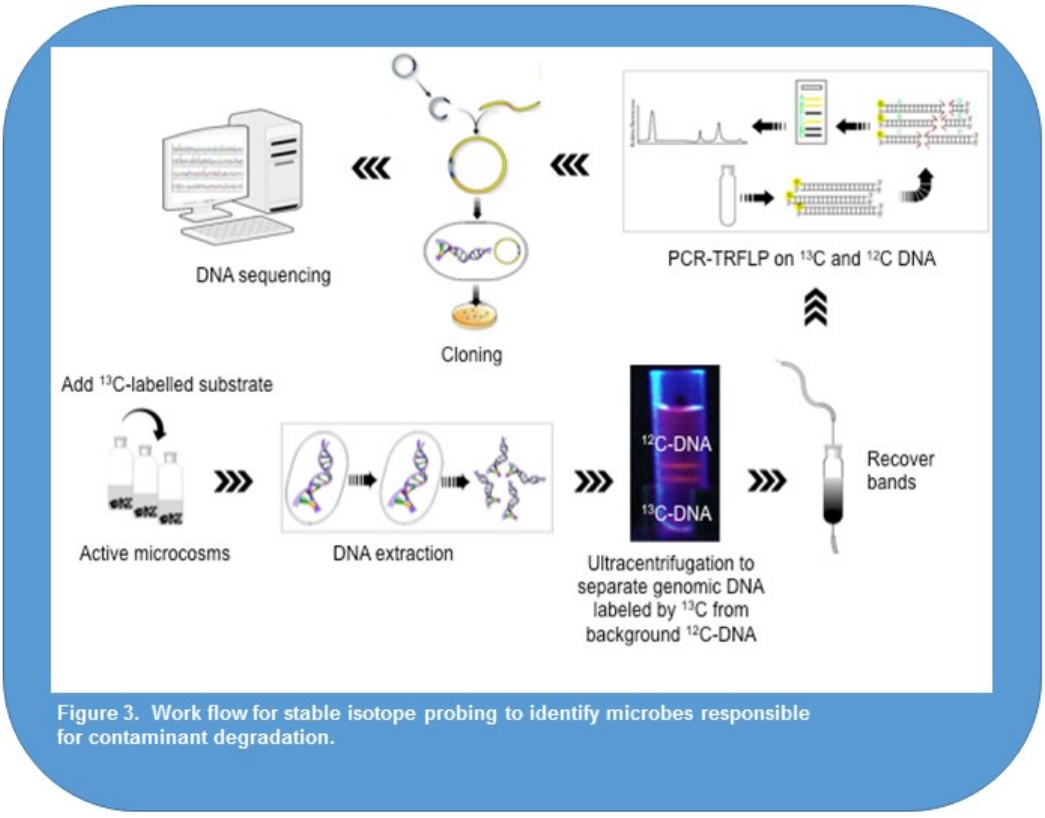

Figure 3. Work flow for stable isotope probing to identify microbes responsible for contaminant degradation.

commercial laboratory (Microbial Insights) for a variety of molecular analyses and the results confirmed low numbers of known dechlorinating bacteria at the site. Additional work at the site was performed and a bioaugmentation pilot test was initiated. This project ended before any results of the bioaugmentation were available. This study provided the baseline information on the microbial community, thus better targeting the bioaugmentation.

Microbial community analysis. To determine whether *in situ* dechlorinating bacteria exist and to understand how microorganisms in the aquifer respond to contamination, a depth-resolved microbial community analysis for the different portions of the site was commenced. Genomic DNA was extracted for six different points with various depths within the heavily contaminated plume and for five different points with different depths at a pristine sampling point. Ground water samples from two different monitoring wells were also collected. Phylogenetic information from the background microbial community analysis was performed. The results of this showed an absence of *Dehalococcoides* bacteria since this was a recent release of contaminants. As a result of this work, they decided to incorporate an in-situ bioreactor (ISBR) (bioaugmentation) in concert with bio-stimulation with an electron donor. The ISBR served to incubate and sustain the target degraders and deliver them into the formation. Without the work done by this project, a straight injection of electron donor may have failed to produce the desired results since there were no in-situ bacteria capable of degradation.

Identification of Dechlorinating Communities in Hackensack River, NJ

DNA-SIP was used to identify active bacteria during PCE dechlorination in contaminated sediments from the Hackensack River, NJ. The sediments of the Hackensack River are impacted by contamination of chlorinated benzenes, dioxins and PCBs, among other contaminants (ATSDR, 2005) from nearby sites. PCE was used as a model compound to stimulate the dehalogenating microbial community to obtain more rapid results for activity. The process of complete dechlorination of PCE to ethene is currently known only to be carried out by *Dehalococcoides* (Löffler et al., 2013). These organisms exclusively use hydrogen as an electron donor and chlorinated compounds as electron acceptors. Many other dehalorespiring bacterial genera are known which can dechlorinate the higher chlorinated ethenes to intermediate daughter products (Holliger et al., 2003).

This project showed Hackensack River microcosms included *Dehalococcoides*, *Dehalogenimonas, Desulfobacterium, Geobacteraceae,* and *Desulfuromonas* as identified in the clone library derived from ^{13}C-heavy fraction. *Dehalococcoides* was detected when ^{13}C-labeled lactate was added to microcosms, but not when ^{13}C-labeled acetate alone was added. This indicated that both hydrogen and acetate were needed to stimulate dechlorination by *Dehalococcoides*.

The growth of *Dehalococcoides* is dependent on interaction with other microbial groups such as fermenters, acetogens and methanogens. The *Dehalococcoides* only use hydrogen as an electron donor and short chain organic compounds such as acetate as a carbon sources and thus are dependent on these other community members to produce these substances through fermentation reactions of more complex organic matter (e.g., in this study, lactate) (**Figure 4**). Thus by adding labeled acetate, which may be taken up in an assimilatory (biosynthesis) process, dechlorinators may be identified by SIP. The dechlorinating groups in a real contaminated site may be more complicated than some established laboratory-scale dehalogenating enrichments, and SIP may be able to capture this complexity. It is believed that this is the first time this technique has been used to characterize NJ sites.

Microcosms seeded from the Hackensack River showed rapid dechlorination. PCE was degraded to *cis*-1,2-dichloroethene in less than one month and was subsequently dechlorinated to ethene during a five-month incubation (data not shown).

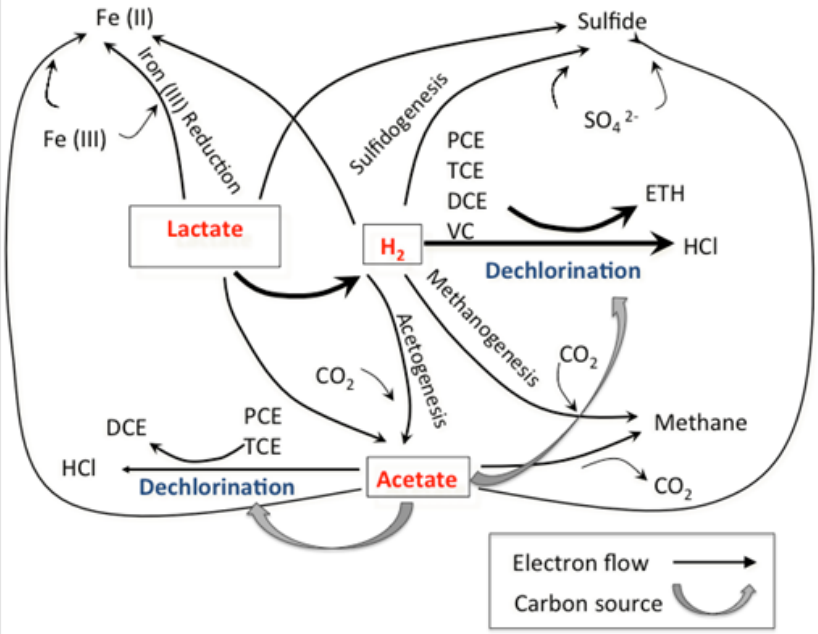

Figure 4 Interactions between chloroethene, dehalorespiring bacteria, and other microbes in the environment.

SIP Investigation of Aniline Degraders from a NJ Contaminated Site under Different Redox Conditions

Aniline is a widely detected groundwater pollutant at chemical industrial sites because of its use as a precursor compound in chemical manufacturing. Very little is known about the microorganisms responsible for aniline biodegradation under anoxic/anaerobic conditions. To date, only a single sulfate-reducing bacterium has been thoroughly characterized for its ability to degrade aniline (Schnell et al., 1989). This lack of information is perhaps a reflection of the delicate syntrophic chains involved in anaerobic aniline metabolism and the resulting long incubation times needed to establish suitable microbial populations with this functional ability. The *in situ* metabolism of aniline-degrading bacteria is likely to be controlled and shaped by the availability of electron acceptors. Aniline biodegradation was investigated in sediment microcosms from a chemical manufacturing site using aerobic and anaerobic aniline-degrading microcosms that were established from a contaminated aquifer and sediments from an adjacent contaminated canal in an earlier project. To investigate the diversity of bacteria involved, the microcosms were established under a variety of redox conditions.

Results using SIP with ^{13}C-labeled aniline conclusively demonstrated that aniline biodegradation occurred and the specific bacteria, particularly *Ignavibacterium*, for biodegradation under different redox conditions were identified. The use of this molecular method therefore provided evidence for bioremediation and specifically identified bacterial targets for monitoring further bioremediation efforts.

APPLICATIONS

The EMD tools used in this project provided additional knowledge about the microbial activity at the respective sites. These tools are meant to compliment current analytical methods by providing insight to the conceptual site model, the performance of remedial activities, as well as providing definitive information to help decide if monitored natural attenuation is an option for closure.

Conclusions

EMDs were successfully used at the three sites selected for this research and included:

- The assessment of the lack of biodegrading bacteria at the Pagan-Martinez site laid the groundwork for bio-augmentation and bio-stimulation of the aquifer, ultimately leading to a successful remediation.
- This research successfully characterized active dechlorinating bacteria from contaminated Hackensack River sediments.
- Finally, active aniline degraders from a NJ industrial site were identified and bioremediation confirmed by using SIP.

One of the major findings to emerge from this work is that EMDs such as SIP can not only identify microbes responsible for biodegradation and biotransformation, but it can also help elucidate important pathways. For example in the Hackensack River sediments, we observed that adding lactate—(presumably fermented to hydrogen (electron donor) and acetate (carbon source)) stimulated *Dehalococcoides*, but addition of acetate alone did not. While this is not entirely surprising, it does show that SIP could also be used as a rapid means to assess success of different amendment strategies, in addition to identifying active microbes to be tracked for remedial efforts. In the case of the aniline-contaminated site, SIP conclusively proved that aniline was biodegraded and incorporated into cellular biomass. Further, there appear to be many bacteria involved in anaerobic degradation at the site and candidate bacteria were identified. In particular, the identification of *Ignavibacterium* as a likely aniline degrader under anaerobic conditions was novel. These are critical pieces of information supporting the effectiveness of bioremediation that had been difficult to prove by tracking the metabolites or matching electron acceptor consumption. Compounds such as aniline may undergo sorption or polymerization, which are difficult to quantify. SIP could be used to confirm biodegradation is occurring rather than physical-chemical sequestration. At the Pagan-Martinez site EMDs accurately assessed the cause of a DCE stall in the bioremediation process and demonstrated the need for both bio-augmentation and bio-stimulation within the contaminated aquifer.

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