



## WATER RESOURCES RESEARCH GRANT PROPOSAL

**Title:** *Genetic Techniques for the Verification and Monitoring of Dihaloethane Biodegradation in New Mexico Aquifers*

**Priority Problem Area:** Water Quality

**Focus Category:** Groundwater, Methods, Toxic Substances

**Keywords:** EDB, EDC, PCR, xenobiotic, biodegradation, natural attenuation, carcinogen, horizontal gene transfer

**Duration:** 12 months (March 2000 – February 2001)

<b>2000 WRI Award:</b>	<u>\$36,950</u>	<u>\$25,000</u>	<u>\$11,950</u>
	Total	Direct	Indirect

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**Congressional District Number:** 2

**Critical Water Resource Problem**

Fresh water supplies throughout the world are threatened by the release of the dihaloethanes 1,2-dibromoethane (EDB) and 1,2-dichloroethane (EDC). These compounds are used for industrial, petrochemical, food-industry and agricultural applications. Dihaloethanes are carcinogens known to form adducts with glutathione, which can bind to DNA. Mutations introduced during the replication of adduct-bound DNA can cause cancer. In addition, dihaloethanes are cytotoxic and have been linked with a variety of acute health effects, including damage to the liver, stomach, and adrenal cortex along with significant reproductive system toxicity, particularly to the testes. According to the EPA's toxic release inventory database, approximately 2,670 pounds of EDB and 433,000 pounds of EDC were released onto land and into water between 1987 and 1993 in America. In New Mexico, approximately 175 locations have or have had EDB or EDC contaminated soil and groundwater, our primary drinking water source. The primary source of EDB and EDC contamination in New Mexico is associated with petroleum refining industries and fuel dispensing systems.

The minimum contaminant levels for EDB and EDC are 0.05 micrograms/L and 5 micrograms/L, respectively (EPA - National Primary Drinking Water Regulations). Laboratory studies indicate that half-lives for these compounds range from 1 day to 60 weeks in surface soils. Evaporation and photochemical reaction are the processes responsible for removal of the majority of EDB and EDC from surface soils. However, *in-situ* testing reveals that EDB can be detected in shallow soil strata 19 years after its last known application. The long persistence was contributed to entrapment in intraparticle micropores of the soil and low-rate biodegradation. Additionally, low octanol-water partitioning coefficient values and detection in many aquifers indicate that EDB and EDC can leach in soil. Microorganisms are responsible for the degradation of EDB and EDC in groundwater. In some natural systems, a small fraction of EDB/EDC may be hydrolyzed by geochemical and biological-byproduct reactions or volatilization. Recently, researchers from Dow Chemical performed experiments using microcosms prepared with EDC contaminated aquifer material and groundwater. EDC is biodegraded under aerobic, sulfate reducing and methanogenic conditions, indicating the involvement of microbes. The ability of microorganisms to degrade EDC reduces the risk factors associated with human exposure, and has potential application in the remediation of ground water. Results from an investigation performed at a contaminated Gulf Coast site indicates that EDC biodegraded through a series of steps that include the byproduct 2-chloroethanol, which indicates EDC is entering bacterial metabolism. These data verify the importance of natural attenuation and indicate the need for rapid methods of verification and monitoring of biodegradation.

## **Results and Benefits Expected**

A rapid and reliable scan for the presence of a gene necessary to degrade EDB and EDC will provide a means of verifying the capacity of natural systems to degrade these contaminants. The proposed research will provide an example of how biotechnology can aid the development of bioremediation methods. The biodegradation of EDB and EDC occurs through series of enzymatic reactions initiated by the hydrolytic enzyme, haloalkane dehalogenase (*dhla*). Our preliminary results demonstrate that microorganisms with the *dhla* gene are present in several EDB and EDC contaminated aquifers in New Mexico. We employed the polymerase chain reaction (PCR) method to detect the *dhla* gene in crude DNA extracted from ground water samples. Verification, optimization and refinement of this technique will provide a reliable method for detecting and tracking the *dhla* gene in aquifer systems. To increase the sensitivity of the PCR assay, fluorescent dye-labeled primers will be designed and used in conjunction with a Prism 310 Automated Genetic Analyzer. Once developed, ground water and soil samples can be quickly tested for the presence of the gene. This will facilitate the determination of the route of the gene within the aquifers, to track the progress of groundwater remediation plans such as natural attenuation or active bioremediation. Additionally, fluorescence-based sequencing of the 16SrRNA region for the identification of microbes will be applied to the same samples to determine microbial community structure. The methods developed will facilitate monitoring the *dhla* gene level and the community structure of contaminated regions. Horizontal gene transfer is an adaptive response to xenobiotic contamination that will be detected by comparing the level of the *dhla* gene

with the microbial community structure at different sites. This estimation of the rate of horizontal gene transfer in natural communities is an important factor when considering genetically-based bioremediation methods, but this rate has not yet been measured.

The use of fluorescence-based techniques to detect genes involved in biodegradation can be expanded. For example, a PCR method that combines multiple pairs of compatible fluorescent dye-labeled primers can be developed to simultaneously detect other genes in this metabolic pathway, such as *dhaA*, another dehalogenase. Also, genes involved in the metabolism of the xenobiotic compounds can be detected with this method. The proposed gene detection system can also be used to monitor the success of new bioremediation approaches. For example, studies of horizontal transmission suggest that it may be possible for genetic information to be transmitted to native bacteria from cultured specimens added to wells rather than injected in aquifers. If the *dhlA* gene could be transmitted to aquifer bacteria by adding living *Xanthobacter autotrophicus* strain GJ10 to a contaminated monitoring well, the gene might be transmitted throughout the contaminated region by natural processes. It will be critical to have a technique that can determine if the gene transfer occurs irrespective of aquifer properties such as pore space and hydraulic conductivity. Such a remediation method overcomes traditional setbacks to approaches that attempt to inject or disperse engineered microbes into aquifer systems. In the past, these techniques have failed due to inadequate cell filtration through natural sediments resulting in insignificant influence around injection points, clogging, and biofouling. Finally, this project will increase our understanding of the role that horizontal genetic transmission plays in microbial adaptation.

### **Nature, Scope and Objectives**

Microorganisms readily degrade naturally occurring hydrocarbon compounds such as petroleum. The phenomenal growth of industry experienced during the late twentieth century has introduced many man-made compounds (xenobiotics) into the environment. Some microbes have developed pathways that use xenobiotic compounds, despite the fact that these compounds have been in the environment for a relatively short period of time. Once the genes necessary to utilize a compound evolve, they can be transferred between species by horizontal transmission. Although this process has not been well characterized, it is of interest because of its role in bioremediation and the possible transfer of genetic information from genetically engineered organisms. In this case, molecular genetic techniques can be applied to water resource issues and, simultaneously, to investigate the role of horizontal transmission of genes in microbial adaptation. A gene associated with degradation of the dihaloethanes; 1,2-dibromoethane (EDB) and 1,2-dichloroethane (EDC) was detected in microbes harvested from contaminated aquifers in New Mexico. This gene was not detectable in portions of the aquifer that were not contaminated with EDB and EDC. This may be an example of the ability of aquifer microbes to horizontally transfer DNA between dissimilar species. An aquifer is a symbiotic environment in which natural microbes share the necessary genetic material to

survive in the same way disease-causing microbes can adapt to antibiotics by developing and transmitting resistance genes in medical facilities.

Ethylene dibromide (1,2-dibromoethane or EDB) and ethylene dichloride (1,2-dichloroethane or EDC) are halogenated aliphatic hydrocarbons, a category of xenobiotic compounds. Halogenated hydrocarbons cover a broad range of compounds with one or more halogen (fluorine, chlorine, bromine, iodine, and/or astatine). These compounds are heavily used in manufacturing industries and agriculture and are common aquifer pollutants. Although some microbes have developed the ability to use halogenated hydrocarbons as a carbon source, these compounds are toxic to eukaryotic cells. Inside the nucleus, EDB is conjugated to glutathione by glutathione S-transferase. This complex can bind to DNA, forming DNA-adducts. During DNA replication, the strand containing the DNA-adduct may be misread, resulting in base substitutions (Cmarik et al. 1992; Kim and Guengerich 1997; Kim and Guengerich 1998). The resulting mutations can cause cancer. EDB can also be metabolized by cytochrome P450, but this pathway is not as well characterized (Wormhoudt et al. 1997). EDB also has been found to be carcinogenic to fish (Hawkins et al. 1998). In addition to being carcinogenic, EDB causes neural tube damage in rat embryo culture (Brown-Woodman et al. 1998), and has been implicated in liver and kidney damage, and reproductive lesions such as reduced sperm levels (Scharder et al. 1988).

Since dihaloethanes are toxic, they have been studied intensively and a large body of literature is available. The persistence of these chemicals in the environment has been studied (Steinberg et al. 1987) and abiotic mechanisms of attenuation have been described (Barbash and Reinhard 1989). By far, the greatest bulk of literature is on the biotic degradation by microbes (Belay and Daniels 1987; Bouwer and McCarty 1985; Dos Santos et al. 1996; Egli et al. 1987; Herbst and Weismann 1966; Janssen et al. 1994a; Janssen et al. 1994b; Klecka et al. 1999; Pignatello 1987; Poelarends et al. 1999).

Microbial utilization of halogenated hydrocarbons as a substrate requires that the halogens are first removed, leaving behind an easily degradable carbon skeleton. Carbon-halogen bonds can be cleaved through enzymatic processes. The enzymes referred to as dehalogenases are responsible for breaking carbon-halogen bonds and are specific to the type of compound they degrade. Haloalkane dehalogenase catalyzes the removal of a halogen group from halogenated aliphatic hydrocarbons. This initial reaction is the rate-limiting step in the biodegradation of EDB and EDC (Schanstra et al. 1996). Both compounds can enter the metabolic pathway of microbes that contain the gene for haloalkane dehalogenase (*dhla*). The *dhla* gene is on a 200 kilobase plasmid, pXAU1, isolated from *Xanothbacter autrophicus* strain GJ10 (Tardif et al. 1991). The *dhla* gene has been cloned and sequenced from *X autotrophicus* (Janssen et al. 1989), the kinetics of the enzyme have been studied (Schanstra et al. 1996), and the structure of the protein has been established by x-ray crystallography (Verschueren et al. 1993). Site-directed mutagenesis has been used to determine the critical amino acids (Kennes et al. 1995; Krooshof et al. 1997; Pries et al. 1994). Thus, the structure and catalytic activity of the *dhla* gene product is well characterized.

Recently, another haloalkane dehalogenase capable of degrading EDB but not EDC, was discovered in *Rhodococcus rhodochrous* (Kulakova et al. 1997). The enzyme is coded for by the plasmid gene *dhaA*, which was cloned and sequenced (Kulakova et al. 1997; Poelarends et al. 1998; Poelarends et al. 1999). The *dhlA* and *dhaA* gene products exhibit some structural similarities, but contain limited homology at the nucleic acid level. The *dhaA* gene has been isolated and sequenced from both gram-positive and gram-negative bacteria, an indication that it can be passed between species, a process known as horizontal transfer.

Horizontal (or lateral) gene transfer is the exchange of genetic material across species boundaries and has been detected in prokaryotes and eukaryotes. The processes of transformation, transduction and conjugation are well-established microbial characteristics that contribute to horizontal gene transfer. Transformation involves the uptake of free DNA from the environment (Lorenz and Wackernagel 1994, Chamier, 1993 #56), transduction is the viral-mediated transfer of DNA (Jiang and Paul 1998), and conjugation requires cell-to-cell contact (Syvanen 1994). Recent interest in gene transfer has been stimulated, in part, by the release of genetically modified organisms into the environment and is the subject of several recent reviews (Syvanen 1994; Syvanen and Kado 1998). As microbial genomes are sequenced, DNA sequence comparisons detect the genetic footprint of horizontal gene transfer (Brown and Doolittle 1999; Brown et al. 1994; Garcia-Vallve et al. 1999; Teichmann and Mitchison 1999). Horizontal transfer has been detected in natural environments, including marine (Hermansson and Linberg 1994), soil (Paget and Simonet 1994), and aquifers (Zhou and Tiedje 1995). In the environment, free DNA can remain intact when bound to particles (Romanowski et al. 1993) and may be available to microbes by transformation. An assay to detect the movement of a gene between bacterial species has been developed for laboratory studies (Jaenecke et al. 1996). Horizontal transmission is a fundamental characteristic of microbial genetics that makes the definition of a species difficult. A modification of the definition of species suggests that bacterial taxonomy be based only on those genes that are not exchangeable over evolutionary time and ignore that portion of the genome that can be shared (Vellai et al. 1999). The proposed study will facilitate the detection of horizontal transmission of a gene in a natural environment.

#### *Preliminary Studies at NM Tech*

EDB and EDC are subject to aerobic biodegradation, however, degradation of these compounds by anaerobic cultures can occur (Tandol et al. 1994). Batch reactor studies were undertaken to determine the rate of degradation of EDB and EDC under anaerobic conditions, which are commonly encountered in the contaminated subsurface. Silt and groundwater were collected from the bottom of two monitor wells located in Albuquerque, New Mexico. Although each well was contaminated with gasoline, EDB and EDC was not detectable in either sample. However, one site was formerly contaminated with EDB and EDC (site 1), but aeration-based soil and groundwater remediation was recently completed. Dihaloethane contamination was never detected at the other site (site 2). The ground water and sediments were used as media in batch reactors. EDB and EDC were added to each sample and the level of the dihaloethanes

was monitored by electron-capture gas chromatography. Half-life values for EDB were 39 days and 87 days for sites 1 and 2, respectively. EDC half-lives were longer at 96 days and >110 days for the two sites. These results correspond with published data and indicate that these compounds were being biodegraded (Pignatello 1987).

Based on these batch-reactor results, the presence of a microbe capable of utilizing EDB and EDC was suspected. Rather than attempt to culture the microbes responsible for degradation, we chose to try to detect the presence of the haloalkane dehalogenase gene. The sequence of the *dhlA* gene was obtained from GenBank (accession #M26950), and primers (*dhlA*F and *dhlA*B) were designed to amplify a 903 basepair fragment from the gene. The primers were tested on DNA isolated from *Xanthobacter autotrophicus* strain GJ10, and the expected band was observed. Ground water samples were obtained from five wells contaminated with EDB and EDC that exhibit biodegradation, and five areas upstream of the contaminated wells. Total DNA was extracted from these samples and used as a template for PCR with the *dhlA*F and *dhlA*B primers. The gene was detected in all five contaminated samples, but it was not detected in any of the non-contaminated samples.

Currently, an undergraduate is being supported by funding from the Hispanic Collaborative for Research and Education in Science and Technology (HiCREST) to confirm the association of the *dhlA* gene with EDB and EDC contaminated wells. In addition, the PCR product from New Mexico aquifers will be sequenced to determine the relationship to the original *dhlA* gene, which was isolated from activated sludge in the Netherlands. HiCREST funding only supports undergraduates, and it ends in May, 1999. Funding from WRRI is being requested to continue and expand this study, with the following objectives:

1. Determine the route of the *dhlA* gene in aquifers, and
2. determine which microbes may harbor and transmit the *dhlA* gene.

#### Objective 1: Determine the Route of the Gene (*dhlA*) in Aquifers

Our current hypothesis is that the gene is present at very low levels until selection pressure is increased. Dihaloethanes can provide a rich carbon source for those microbes that harbor the genes to metabolize these compounds, so there is a strong selective advantage for those microbes that contain the *dhlA* gene in contaminated environments. Therefore, an increase in the genes will be observed in areas contaminated with EDB and EDC. To determine if the gene is present at low levels in non-contaminated sites, a method more sensitive than gel electrophoresis is necessary. Increased sensitivity of the PCR assay will be accomplished using a Perkin-Elmer Prism 310 automated DNA analyzer. Fluorescent dye-labeled primers will be designed for use with the automated Prism 310 Genetic analyzer. Once this assay is developed, ground water samples can be quickly analyzed for the presence and relative concentration of the gene. Comparison of

the DNA data with spatial and temporal data from the study sites will facilitate the determination of the route of the gene within the aquifers.

#### Objective 2: Determine which Microbes may Harbor the *dhlA* Gene

Since the preliminary experiments involved isolating total DNA from water and sediment samples as a template for PCR, we do not know which microbes harbor this gene, or whether it is chromosomal or extrachromosomal. We hypothesize that the gene will be found in a variety of naturally-occurring microbes due to their ability to transfer genetic information laterally. The vast diversity of microbes is just beginning to be realized, so this objective will be challenging. This is important information for the continued development of remediation methods. The community profile of contaminated and non-contaminated areas will be determined by amplification and sequencing of the 16S rRNA. Microbes absent from contaminated regions can be ruled out, and those at high frequency in contaminated regions will be studied further. Enrichment cultures will be established, which can be tested for the presence of the *dhlA* gene.

To achieve these goals in the one-year time period, we will concentrate on a few local sites. These areas are around the Socorro area, making funding for travel to distant sites unnecessary. Travel funds will be used for travel to one conference to present results. Salaries will include one month summer salary for R.A. Reiss, a nine month graduate student stipend is included for P. Guerra, to finish a Master's of Science, and funding to support an undergraduate Environmental Science major for one year. Supplies for this project include polymer, capillaries, primers and fluorescent dyes for the automated DNA sequencer and reagents for the extraction of DNA from environmental samples.

We believe that meeting these objectives will elucidate methods applicable to monitoring and remediating EDB and EDC contaminated aquifers. Understanding the natural response of indigenous microbes to contamination is essential for designing remediation procedures and to monitor the progress of biological attenuation.

#### **Methods, Procedures and Facilities**

##### ***Ground-water Sample Collection and Preservation:***

Ground-water sampling is accomplished using a sterile, Teflon-lined bailer. The bailer is lowered into a well using a stainless-steel cable. In general, the bailer is submerged into the top third of the water column in the well to obtain a relatively sediment free sample. Upon retrieval of the bailer to the surface, the ground-water sample is transferred to 40-ml vials with Teflon-lined septa, which are sealed with zero-headspace. Samples are immediately packed on ice and shipped to the laboratory. As with the bailer equipment, the vials are sterilized prior to use.

A cluster of EDB and EDC contaminated sites associated with retail fuel businesses exists along California Street in Socorro, New Mexico. Presently, several of these sites

are under the direct supervision of the New Mexico Environment Department Underground Storage Tank Bureau (USTB) and are State Lead Sites funded by the Corrective Action Fund (CAF). In cooperation with the USTB, groundwater samples (contaminated and uncontaminated) will be collected from two or more of these sites. Groundwater from the one of these Socorro sites by P. Guerra as well as sites in Albuquerque, Cliff, and Santa Clara, New Mexico has already been collected for the preliminary experiments.

### *Genetic analysis*

In order to meet the objectives, two genetic assays will be performed on each sample:

- the detection and quantification of the *dhlA* gene by PCR and
- the determination of the microbial community structure by DNA sequencing

Microbes are separated from the sample by centrifugation or filtration. DNA is extracted by chemical lysis of the cells, organic extraction of the DNA and concentration by ethanol precipitation. DNA is quantified by UV spectrophotometry. Polymerase chain reaction (PCR) is performed in a thermal cycler using the *dhlA* gene primers, *dhlAF* and *dhlAB*. These primers produce a 903 basepair (bp) fragment, which can be visualized on agarose gels. In order to increase sensitivity, primers labeled with fluorescent dyes will be used and the product detected by capillary electrophoresis on a Perkin-Elmer Prism 310 Genetic Analyzer. To further increase sensitivity, a set of internal primers will be designed and a nested PCR strategy applied. Templates with very low concentrations may not be amplified efficiently in the first round. However, by amplifying the first round product using internal (nested) primers, enough product can be produced for analysis. The use of nested primers insures that product produced by false-priming in the first round are not amplified in the second round. This assay will be quantified using purified pAXU1 plasmid from *Xanthobacter autotrophicus* as a standard. Since the plasmid is 200,000 bp long, the number of target molecules is determined from the concentration of the plasmid. A standard curve will be produced by measuring the fluorescence signal for pre-determined amounts of plasmid. With this information, it will be possible to calculate the concentration of the gene per volume of sample.

Sequence analysis of the 16SrRNA gene will be performed to determine the taxonomy of the microbes present in the sample. The Microseq® kit from Perkin-Elmer will be used since they it is designed for automated fluorescent DNA sequencing. Primers specific for prokaryotic 16SrRNA are used to amplify a product, which is cloned and sequenced individually. The sequence is then compared with the rRNA database to identify microbial species.

### *Automated DNA Analysis at NM Tech*

The instrumentation to perform the genetics experiments is housed in the lab of Dr. Reiss. This includes a Perkin-Elmer Prism 310 automated DNA analyzer, a thermal cycler, and all other equipment for molecular genetics. Fluorescent DNA sequencing and DNA molecular weight determination on the Prism are standard operating procedures in this lab.

Because PCR can be used to detect small amounts of DNA, special attention must be paid to potential sources of DNA contamination. The DNA analysis facility is designed to meet specifications for human forensic DNA work, following the guidelines developed by the FBI's Technical Working Group on DNA Analysis Methods (TWGDAM 1995). These extremely stringent standards were developed so DNA data can be admissible as evidence in court. Adherence to these standards and procedures will insure accurate results for any experiments performed in this facility.

### **Related Research**

Available research regarding the degradation of EDB and EDC in soil and ground water fall into two categories; (1) engineered systems such as biological trickle filters and (2) degradation-rate measurements performed on sediments collected at contaminated sites. A technical report published in 1987 discusses the natural biodegradation rate of EDB in sediments collected in Windsor Locks and Simsbury, Connecticut (Pignatello 1987). The objective of this study was to determine the importance of microbial degradation of EDB contaminated ground-water located beneath farmland. EDB was used as a soil fumigant in agriculture between 1950 and 1984. Degradation experiments were carried out at environmentally significant concentrations (<5 micrograms/L). Results were quite favorable; first-order half-lives of EDB degradation under aerobic and anoxic conditions ranged between 35 days and 350 days. At one of the sites, rates were faster in samples collected from within the EDB plume, suggesting that the microbial consortia had adapted to EDB as an efficient substrate. However, the report concluded with a reoccurring theme: The presence of EDB in the subsurface is not consistent with the rates determined in the lab. Nonetheless, this report does indicate that EDB is degradable by native microbes that may express the *dhla* gene.

EDC degrading microorganisms that were enriched and isolated under ideal conditions were used to inoculate a full-scale ground-water remediation system (Stucki and Thuer 1995). Treatment for incoming ground-water pumped from the subsurface consisted of a rotating biological contactor (RBC). RBC technology has been extensively applied in wastewater treatment. Further treatment and polishing was accomplished through a dual media (sand followed by activated carbon) system. Results from four years of operation indicate that more than 90% of the EDC is removed by biodegradation. The integration of the biological component to this adsorption system has lowered operating costs by more than 50%.

A recent accident on the Gulf Coast of the United States released 150,000 gallons of EDC into the environment (Lee et al. 1999; Sehayek et al. 1999). The focus of this study was to establish the decay order and rate of EDC degradation. Three methods including

laboratory batch reactors, long-term field investigation showing reduction in EDC levels and biodegradation byproducts, and general water-quality screening were used by the researchers. Results indicated that EDC was degrading at first-order half-lives ranging between 0.2 years to 4 years. In addition, 2-chloroethanol, the byproduct of haloalkane dehalogenase interaction with EDC, was detected in several of the wells tested.

Environmental scientists are beginning to consider genetic techniques for management of sites with contaminated subsurface. Traditional methods include physical and chemical analysis campaigns designed to characterize the hydrogeology, geochemistry, and contaminate phase and distribution. To date, these techniques have been refined and standardized to generate reliable and repeatable results. However, biological interaction with contamination has become increasingly important in assessing the fate and ultimate risks associated with contaminated soils and aquifers. Currently, the biological response in the subsurface can be estimated by measuring byproducts, electron acceptors, and other physical and chemical parameters associated with microbial mediated reactions. These assays may not provide a direct measurement of biological parameters. Due to the complex geochemical environment of aquifer systems, physical and chemical parameters can be effected by a myriad of reactions, which may or may not be biological. The methods that we propose provide a direct analysis of groundwater samples for the presence and concentration of a gene associated with the biodegradation of EDB and EDC, and a survey of the microbes that may be responsible. Results from this bioassay can be used to reduce the uncertainty in assessing the response of complex aquifer environments. In addition, new bioremediation strategies can be designed and tested with this bioassay. And finally, this study will aid in the understanding of the importance of horizontal transmission of genetic information for the adaptation of a natural community.

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