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# ***In Situ* Bioremediation of Perchlorate**

## **SERDP Project CU-1163 Final Report**

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## 1.0 PROJECT BACKGROUND

Ammonium perchlorate ( $\text{NH}_4\text{ClO}_4$ ) has been used for several decades in the United States as an oxidant in solid propellants and explosives. It is the primary oxidant used in many rocket motors and boosters, such as those powering the space shuttle and intercontinental ballistic missiles (ICBMs). For example, a single rocket booster for the space shuttle contains approximately 350,000 kg of ammonium perchlorate. Various perchlorate salts (e.g., ammonium, potassium, and magnesium perchlorate) are also used in flares, fireworks, matches, and air bags as well as in leather tanning, electroplating, and for ionic strength adjustment in analytical chemistry (Gulick et al., 2001; USEPA, 2001a). Perchlorate is also present in a naturally-occurring nitrate formation that is mined in Chile (Chilean caliche) for use in some agricultural fertilizers (USEPA, 2001b). However, a majority of fertilizers used in the United States are not produced with this material and do not appear to contain environmentally significant levels of perchlorate (Renner, 2001). Rather, the primary sources of soil and groundwater contamination with perchlorate are related to the production of the compound for aerospace and military applications, the testing of rockets and munitions, and the periodic removal and replacement of solid fuels in rockets. The latter procedure, which is referred to as hog out, is required because solid perchlorate fuels have a limited shelf life and must be periodically removed and replaced. During the hog out procedure, solid propellant is initially washed from the missile or rocket casing using high-pressure water, then the solid fuel is replaced or the casing is discarded. The wastewater resulting from this operation contains high concentrations of perchlorate and other salts. The improper disposal of this wastewater as well as the disposal techniques traditionally used during manufacturing and testing has resulted in substantial perchlorate contamination in several states including Texas, California, Utah, New Mexico, and Nevada.

A sensitive detection method for perchlorate was developed by the California Department of Health Services (CDHS) in 1997 (CDHS, 1997). Because this technique has only been available for a few years, the total scope of perchlorate contamination in the United States is not yet known. However, perchlorate has now been detected in 14 states, and current estimates suggest that the drinking water of as many as 15 million people may be impacted by this compound (USEPA, 1999; Logan, 2001). For example, as of April 2002, CDHS had sampled 629 public water systems in California and found 69 (11 %) with detectable perchlorate ( $> 4 \mu\text{g/L}$ ) (CDHS, 2002a). Of the 3,864 non-public drinking water sources tested by the agency, 246 (6.4%) tested positive for the oxidant. Perchlorate has been manufactured or used in at least 44 states nationwide, so groundwater pollution may extend beyond recent reports (USEPA, 2001a; USEPA, 2002b). There is currently no federal action level for perchlorate in groundwater. However, several states, including Arizona, California, Nevada, and Texas have set provisional action levels ranging from 4 to 31  $\mu\text{g/L}$  (ppb), and site-specific clean-up levels of 1.5  $\mu\text{g/L}$  and below have been set by regulators. Based on results from a draft toxicological document prepared by

the USEPA, CDHS recently lowered the action level for perchlorate in groundwater from 18 µg/L to 4 µg/L (CDHS, 2002b; USEPA, 2002a). Perchlorate has also been placed on the USEPA Unregulated Contaminant Monitoring Regulation list (UCMR) and Contaminant Candidate List (CCL) for regulatory consideration (USEPA, 2000).

The potential human health risks of ammonium perchlorate are based largely on the ability of the perchlorate anion to inhibit the transport of iodide into the thyroid gland (Wolff, 1998). Because iodide regulates the synthesis of thyroid hormone (T<sub>2</sub>), exposure to perchlorate can disrupt T<sub>2</sub> regulation, and subsequently influence levels of thyroxine (T<sub>4</sub>) and thyroid-stimulating hormone (TSH) (OEHHA, 2002). The levels these two hormones are regulated in a feedback loop with T<sub>2</sub>. Because perchlorate salts disrupt iodide uptake, they have been used therapeutically in large doses to treat hyperthyroid conditions, such as that resulting from Graves' disease. Although a variety of different studies have been conducted during the past several years to evaluate the influence of perchlorate on human health (e.g., Lawrence et al., 2000; 2001, Lamm and Doemland, 1999), many questions remain concerning the risks of low levels of perchlorate exposure to humans through drinking water. The EPA has recently reevaluated the human health risks associated with perchlorate contamination (USEPA, 2002a). At the writing of this report, the EPA risk assessment document is still undergoing review. However, a draft reference dose (RfD) of 0.03 µg/kg/d was proposed in this document to ensure public protection from adverse effects of perchlorate in water over a lifetime. This value, which corresponds to a drinking water concentration of only 1 µg/L, is approximately 30-fold lower than the RfD proposed by EPA in a previous toxicological document in 1998 (0.9 µg/kg/d) (USEPA, 1998; CDHS, 2002b). The revised reference dose reflects new data concerning perchlorate toxicity, the need to protect the most sensitive populations, including pregnant women and their unborn children, and a level of uncertainty spanning about one order of magnitude based on current data gaps (USEPA, 2002b).

In addition to human health issues, perchlorate is anticipated to have toxicological effects on various terrestrial and aquatic species, including rodents, fish, and amphibians (York et al., 2001; Smith et al., 2001; Manzon and Youson, 1997). Perchlorate is known to influence metamorphosis, so amphibians, may be particularly sensitive to this compound. For example, Goleman et al. (2002) recently reported that perchlorate concentrations in the part-per-billion range caused significant impacts on forelimb emergence, tail resorption, and hindlimb growth in frogs (*Xenopus laevis*) undergoing metamorphosis. The compound, however, exhibited low toxicity to eggs and larvae of this species (LC<sub>50</sub> = 223 to 510 mg/kg). Although research is ongoing, the current database concerning the ecological impacts of environmentally-relevant concentrations of perchlorate is sparse. Thus, the environmental and human health effects resulting from long-term exposure to low levels of perchlorate remain somewhat unclear at the current time.

Perchlorate salts are highly soluble in water (e.g., ammonium perchlorate is soluble to ~ 200 g/L) and dissociate completely. The resulting perchlorate anion is nonvolatile, highly mobile, and chemically stable in aqueous systems under normal conditions present in ground and surface water. As a result, in areas where substantial quantities of perchlorate salts have been discarded, expansive groundwater plumes of perchlorate are often observed. Because of its physical characteristics (i.e., low reactivity, low volatility, high solubility), water treatment technologies including ultrafiltration, air-stripping, carbon adsorption, and advanced oxidation are not effective options for perchlorate removal from groundwater (Damian and Pontius, 1999; Logan, 1998; USEPA, 2001a). Ion exchange using one or more selective resins is a viable approach for removing low concentrations of perchlorate from water (e.g., Gu et al., 2000; 2002). However, the perchlorate anion is not destroyed during the ion exchange process, but rather is reversibly bound to the resin. The exchange resins eventually become saturated with the perchlorate (and other anions which also bind to the resin) and must then be replaced or regenerated using a high strength salt solution (Urbansky, 1998; Logan, 2001). If the latter procedure is used, the waste brine from the regeneration procedure contains concentrated perchlorate, which then must undergo additional treatment or disposal.

Unlike abiotic approaches, biological treatment represents a promising technology for the effective and economical removal of perchlorate from water (Logan, 2001; Urbansky, 1998). A number of bacteria have been isolated which are able to degrade perchlorate to the harmless products chloride and water (Rikken et al., 1996; Wallace et al., 1996; Coates et al., 1999; Achenbach et al., 2001). These bacteria grow through anaerobic respiration. During this process, the bacteria require an organic or inorganic electron donor (e.g., ethanol, acetate, hydrogen gas) for growth and utilize the perchlorate molecule as a terminal electron acceptor. A perchlorate reductase enzyme appears to catalyze an initial two-step reduction of perchlorate ( $\text{ClO}_4^-$ ) to chlorate ( $\text{ClO}_3^-$ ) and then chlorite ( $\text{ClO}_2^-$ ) (Kengen et al., 1999). The chlorite is then further reduced by the enzyme chlorite dismutase to chloride ( $\text{Cl}^-$ ) and oxygen ( $\text{O}_2$ ) (van Ginkel et al., 1996). Thus, microbial degradation of perchlorate yields two innocuous products, chloride and oxygen. Perchlorate respiration is similar to denitrification, where bacteria utilize a substrate and reduce nitrate as the terminal electron acceptor to nitrogen gas.

*Ex situ* biological treatment systems have been successfully developed at full-scale to treat perchlorate-contaminated water. Electron donors, such as ethanol and acetate, are supplied to perchlorate reducing bacteria in these reactors to promote biological reduction of the propellant. An initial bioreactor design was developed and tested in the early 1990s by researchers at Tyndall Air Force Base to treat heavily contaminated wastewater from hog out and other operations. This stirred-tank reactor utilizes the bacterium *Wolinella succinogenes* HAP-1 for perchlorate reduction (Attaway and Smith, 1994; Hurley et al., 1996). This design works well for low-flow, high-concentration perchlorate wastes, and has been

applied at full-scale for this application. However, the reactor is not well-suited for high-flow groundwater applications, where perchlorate concentrations are likely to be in the  $\mu\text{g/L}$  (ppb) to low  $\text{mg/L}$  (ppm) range, and flow rates of thousands of gallons per minute may be required. Other bioreactor designs, including packed bed reactors (Miller and Logan, 2000; Wallace et al., 1998; Logan, 2001) and fluidized bed reactors (Green and Pitre, 1999; Hatzinger et al., 2000; 2002) have subsequently been developed specifically for treatment of low levels of perchlorate in high-flow groundwater applications. Three commercial-scale fluidized bed reactors are currently treating perchlorate in groundwater at flow rates ranging from 50 to 4,000 gallons per minute (Hatzinger et al., 2002).

The success of *ex situ* biological treatment of perchlorate suggests that *in situ* treatment through electron donor addition may also be possible. For this technology to be successful, however, perchlorate reducing bacteria must be present in contaminated aquifers, and these bacteria must be stimulated to degrade perchlorate from existing levels to below state regulatory levels (e.g.,  $< 4 \mu\text{g/L}$  in California). A few recent papers suggest that perchlorate reducing bacteria are naturally-occurring in various environments, including soils, sludges, raw wastewater, and farm animal waste (Coates et al., 1999; Wu et al., 2001). However, few data exist concerning the presence and distribution of perchlorate reducing bacteria in groundwater aquifers. In addition, the most effective substrates to stimulate perchlorate reduction by these organisms have not been determined nor have geochemical factors that may influence this process. The key to utilizing perchlorate reducing bacteria for *in situ* remediation is understanding the conditions that limit their activity in subsurface environments and then devising effective technologies to overcome these limitations and subsequently stimulate perchlorate degradation. To date, little research has been conducted to develop an *in situ* technology for perchlorate bioremediation. The assessment and development of such a technology is the goal of this SERDP project.

This project was a collaborative effort between scientists at Envirogen Inc. (Envirogen) in Lawrenceville, NJ and the Indian Head Division, Naval Surface Warfare Center, Naval Sea Systems Command in Indian Head, Maryland. Envirogen is a leader in developing *in situ* and *ex situ* treatment technologies for hazardous wastes, and has constructed three full-scale *ex situ* reactor systems for perchlorate treatment. Scientists at Envirogen conducted microcosm, column, and pure culture studies to provide a better understanding of perchlorate biodegradation in subsurface aquifers and developed a mathematical model to describe the kinetics of perchlorate biodegradation in the presence of competing electron acceptors. The scientists and engineers at IHDIIV have a comprehensive understanding of the chemistry, analysis, and military applications of ammonium perchlorate, as this compound has been used at IHDIIV for more than 50 years to prepare solid rocket propellants. The researchers at IHDIIV developed an improved method for perchlorate analysis in saline environments, provided field samples for use in laboratory studies, and are currently funding a field demonstration of *in situ* perchlorate treatment as part

of the technology transfer scope of this SERDP project. The collaboration between researchers at Envirogen and IHDIIV has rapidly lead to an improved understanding of perchlorate biodegradation in subsurface environments. This research is now being used to develop and test effective bioremediation strategies for perchlorate-contaminated groundwater.

## 2.0 PROJECT OBJECTIVES

**The objective of this project is to develop a biological treatment technology for *in situ* remediation of perchlorate in subsurface environments.** The development of an effective technology for *in situ* perchlorate remediation requires a fundamental understanding of the conditions that limit biological perchlorate reduction in groundwater and the most effective means to overcome such limitations. This research effort is designed to provide this fundamental understanding. We hypothesize that four key factors may be contributing to the persistence of perchlorate at various subsurface sites. These key factors and our approach to their evaluation in this research effort are as follows:

- (1) **Absence of an appropriate substrate (electron donor) for growth of indigenous perchlorate degrading bacteria.** Based on preliminary studies, we believe that the absence of an oxidizable substrate is the key factor limiting biological perchlorate degradation at many subsurface sites. Therefore, experiments were conducted using aquifer samples from contaminated field sites to evaluate the potential of numerous organic and inorganic electron donors to stimulate perchlorate reduction *in situ*. The most promising electron donors were tested in a flow-through aquifer system to provide relevant kinetic data for modeling and field trials.
  
- (2) **Presence of alternative electron acceptors for bacterial respiration, including O<sub>2</sub>, NO<sub>3</sub><sup>-</sup>, and NO<sub>2</sub><sup>-</sup> in groundwater.** Perchlorate serves as an electron acceptor for bacteria during anaerobic respiration. The microbial reduction of one electron acceptor is frequently influenced by the presence of others (e.g., oxygen inhibits dissimilatory nitrate reduction). The general relationship between perchlorate and other common electron acceptors is unclear. However, nitrate, nitrite, and oxygen have been observed to inhibit perchlorate reduction by a few bacterial cultures (Attaway and Smith, 1993; Logan, 1998). Because each of these molecules as well as other electron acceptors such as sulfate and iron are frequently present in groundwater, understanding their influence on microbial perchlorate reduction is critical to successful remediation efforts. Experiments conducted during this project were

designed to assess the influence of common electron acceptors, such as oxygen and nitrate, on perchlorate degradation by naturally occurring bacteria in field samples and by microbial isolates.

(3) **Lack of an indigenous population of bacteria capable of perchlorate reduction.** In some environments, bacteria with the metabolic enzymes to reduce perchlorate to chloride may be absent. In such cases, augmentation with exogenous microorganisms will be required for *in situ* remediation. As part of this research effort, bacterial strains and consortia were isolated from Envirogen's FBR systems that are currently treating perchlorate and from aquifer samples collected from perchlorate-contaminated sites. The potential for these strains to degrade perchlorate *in situ* under relevant environmental conditions was then evaluated in microcosm studies. These cultures were also used to provide necessary parameters for a biodegradation model developed during this research project.

(4) **Unfavorable environmental conditions for activity of indigenous perchlorate degraders.** The role of environmental variables on *in situ* perchlorate degradation has not been extensively studied. In addition to evaluating the effect of electron acceptors such as nitrate on perchlorate reduction, experiments were undertaken to look at the effect of salinity (ionic strength), pH, and co-contaminants on microbial perchlorate degradation. These factors may be extremely important at specific sites (e.g., salinity in groundwater at coastal sites) but, as yet, they have not been investigated.

The research performed during this project was designed to provide extensive information on (1) the potential for successful perchlorate remediation at subsurface sites by addition of electron donors (i.e., biostimulation); (2) the most effective electron donors to use in biostimulation efforts, and the expected concentrations and remediation kinetics achievable with these donors; (3) the possibility for successful bioaugmentation (i.e., injection of bacterial isolates) for subsurface perchlorate remediation; and (4) the probable influence of alternate electron acceptors and environmental variables on perchlorate reduction during biostimulation and/or bioaugmentation efforts. These data will provide the fundamental knowledge required for the design and implementation of pilot-scale and full-scale remediation efforts at perchlorate contaminated sites.

### 3.0 TECHNICAL APPROACH

The research tasks conducted during this project are summarized in the following section.

**Task 1. Collect Aquifer Solids and Groundwater from Field Sites.** Aquifer samples from perchlorate-contaminated sites with widely differing geochemical characteristics and contaminant concentrations were obtained for use in enrichment, microcosm, and column studies. These samples were collected from five perchlorate-contaminated locations in California (2 sites), Texas, Maryland, and Utah, respectively. Samples of a perchlorate-contaminated surface soil were also obtained from one location for studies. A total of eight different environmental samples were obtained and tested during the project. These samples were used in microcosm studies to represent a range of different environments that have experienced perchlorate contamination. Column studies were also conducted with one set of these samples to evaluate perchlorate degradation kinetics in a flow-through system.

**Task 2. Obtain Microbial Consortia and Individual Bacterial Isolates Capable of Perchlorate Degradation.** Envirogen has constructed three full-scale fluidized bed reactor (FBR) biotreatment systems for degrading perchlorate in groundwater. The first of these facilities is located at the Aerojet facility in Rancho Cordova, CA. This reactor system, which uses granular activated carbon as a matrix and ethanol as an electron donor, has been reducing perchlorate levels in feed water from approximately 4 mg/L to non-detectable levels ( $< 4 \mu\text{g/L}$ ) at flow rates of greater than 4,000 gallons per minute for more than 2 years. Food processing waste was used as the original inoculum for the FBR system. The objective of this task was to isolate individual perchlorate degrading bacteria or a mixed bacterial culture from the FBR system as well as from some of the field sites. One perchlorate degrading culture was isolated and identified from the FBR during this project. This culture, designated *Dechlorospirillum* species FBR2, was subsequently used in several different microcosm studies during the course of this project. In addition, bacterial isolates were obtained from groundwater at Jet Propulsion Labs and from the Rocky Mountain site. The kinetics of perchlorate reduction and the influence of other terminal electron acceptors on this process were extensively studied using one of the isolates from JPL, designated *Dechlorosoma suillum* JPLRND. These data were then used as parameters in a kinetic model of perchlorate reduction (see Task 5).

**Task 3. Identify Conditions Required for In situ Biostimulation of Perchlorate Degradation.** The objective of this task was to develop an understanding of the factors promoting perchlorate degradation in subsurface environments as well as the conditions that inhibit the process. Small-scale laboratory microcosms were used to evaluate both biostimulation of indigenous perchlorate degrading bacteria and the addition of exogenous perchlorate degraders (strain FBR2 isolated during *Task 2*) for aquifer remediation. The factors that were evaluated in these studies include: (A) choice of electron donor (substrate) for growth of perchlorate degrading bacteria, (B) the influence of dissolved oxygen, nitrite, and nitrate on perchlorate removal, and (C) the role of environmental factors including salinity (ionic strength), groundwater pH, and presence of organic co-contaminants on perchlorate degradation. Several of these factors were further examined during column studies. Results from these studies revealed that a variety of different organic substrates, as well as hydrogen gas, can be used to stimulate perchlorate reduction at many sites. The most effective electron donor appeared to vary by site, although acetate, lactate, and molasses were generally effective. High salinity and low pH both appear to inhibit perchlorate reduction. Perchlorate reduction could not be stimulated in low pH aquifer materials and soils (three separate sample locations) by any organic or inorganic substrate. However, when the aquifer or soil samples were amended with carbonate to increase alkalinity and pH, perchlorate biodegradation occurred in all samples by naturally-occurring microorganisms.

**Task 4. Evaluate Perchlorate Transport and Biodegradation in Pilot-Scale Model Aquifers.** The most effective treatments for perchlorate degradation in the microcosm studies were further tested using pilot-scale flow through model aquifers. A flow-through model system better approximates *in situ* aquifer conditions than either an aqueous system or a static microcosm, and being continuous flow, inputs of perchlorate, substrates, and various groundwater constituents, including terminal electron acceptors such as oxygen and nitrate, can be controlled and varied. The model aquifers, which were designed at Envirogen to simulate subsurface conditions, were constructed from steel tubing. Columns of 50-cm and 30-cm total length were used in various studies. The columns were built with sampling ports at various distances from the bottom (upward flow) where aqueous subsamples could be withdrawn by syringe. The columns were packed with subsurface sediments from the Longhorn Army Ammunition Plant (LHAAP), and an artificial groundwater was prepared based on the geochemical characteristics of the LHAAP groundwater. A peristaltic pump supplied a continuous flow of groundwater from a reservoir to a port at the bottom of the columns. Separate syringe pumps were used to supply electron donor. The entire system was airtight so that anoxic conditions could be generated within the column.

The initial 50-cm column was run for more than 200 days. The flow characteristics in the column (including mixing at the influent port and groundwater transport) were initially quantified using bromide

as a conservative tracer. The column was then fed acetate as an electron donor, and the degradation of perchlorate, acetate, oxygen, and nitrate was quantified with time and with distance in the column. The concentrations of acetate, perchlorate and nitrate were varied during the column study, and the influence of these changes on the kinetics of perchlorate biodegradation was determined. The impacts of pH and chlorate addition were also examined. The data from this column were subsequently used to test a coupled biodegradation-transport model for perchlorate in the subsurface. An additional 30-cm column was constructed and used to determine the potential use of lactate as an electron donor, to evaluate the degradation of perchlorate in the absence of nitrate, to assess the potential for sustained biodegradation of very low perchlorate concentrations (50 – 250 µg/L), and to determine if perchlorate and a second explosive compound, hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), can be biodegraded simultaneously with lactate as an electron donor.

**Task 5. Modeling.** Biodegradation and reactive transport modeling were performed as part of this SERDP project. A biodegradation model was initially developed (Task 5a), parameters required for the model were determined experimentally in the laboratory using the bacterium *Dechlorosoma suillum* JPLRND (Task 5b), and a fully-coupled biodegradation and transport model for perchlorate was then developed using the software HydroBioGeoChem123D (HBGC123). This model was tested using data from the model aquifers described in Task 4.

Task 5a. Development of a Biodegradation Model for Perchlorate. A mathematical model was developed to describe the kinetics of perchlorate biodegradation. This concept of this model is based on the program RT3D developed by Battelle Pacific Northwest National Laboratory. The biodegradation of an electron donor and consumption of multiple electron acceptors are described using modified Monod equations. The rate of perchlorate degradation is described as a function of the electron donor utilization rate, the presence of alternate electron acceptors, and rates of biomass growth and decay. Inhibition factors are included in the model to describe the effect of alternate electron acceptors (nitrate and oxygen) on perchlorate degradation. The model links the dynamics of the microbial population to the consumption of electron donor and acceptors and describes bacterial growth and decay.

Task 5b. Quantification of Model Parameters. Microcosm experiments were conducted to determine the input parameters for the biodegradation model. The studies utilized a perchlorate degrading strain (*Dechlorosoma suillum* JPLRND) isolated from groundwater underlying the Jet Propulsion Laboratory in Pasadena, CA during previous work for this project. A series of batch experiments

were conducted with the strain using a range of starting donor (acetate) concentrations where oxygen, nitrate, or perchlorate were present in excess as electron acceptors. The maximum specific growth rate and half saturation constant for growth of the organism on acetate with each electron acceptor were determined. Similar experiments were performed to determine the growth rate parameters for each acceptor when starting donor concentrations were constant and not limiting (i.e., detectable donor remained at the end of the experiment), while acceptor concentrations were varied. Due to the low solubility of oxygen, these experiments were performed only for perchlorate and nitrate. Experiments were also conducted to evaluate potential inhibition effects of nitrate and oxygen on perchlorate biodegradation. Varying concentrations of each electron acceptor were added to flasks containing a culture that was actively-degrading perchlorate, and the subsequent rate of perchlorate degradation was quantified. Inhibition factors for each terminal electron acceptor were determined for the model using the results from these studies.

*Task 5c. Development of a Reactive Transport Model.* Groundwater flow and reactive transport modeling was conducted to verify degradation rates derived from laboratory studies and to aid design of field-scale applications. Groundwater flow modeling was initially performed to simulate perchlorate transport in aquifer columns. The software HydroBioGeoChem123D (HBGC123D) was used to describe the one-dimensional transport of bromide and perchlorate in the laboratory columns. This software was chosen because of its capability to describe the transport and consumption of multiple electron acceptors. Once non-reactive perchlorate transport was adequately simulated, the biodegradation model developed in Task 5a was incorporated into the program. The fully-coupled model was then used to simulate perchlorate biodegradation under flowing conditions. Data from the column studies were used for simulations. The model did not adequately describe biodegradation data from the column studies. Model simulations predicted no significant losses of acetate (electron donor), perchlorate, nitrate, or oxygen within the column. Inspection of the model data revealed that the simulated biomass within the column decayed much faster than it grew, resulting in the lack of electron donor or acceptor biodegradation.

This difference between the laboratory data (which showed degradation of acetate and all three electron acceptors along the column profile) and the model prediction suggests that one or more of the assumptions of the biodegradation model were critically violated. Factors that may contribute to the discrepancy between the model and experimental data include: 1) a lower biomass decay rate in the column than the value determined from the batch experiments; 2) enzyme induction rather than biomass growth during the lag period preceding biodegradation; 3) higher biomass concentrations in the column at the onset of biodegradation as compared to the value measured at the beginning of the

laboratory experiments; and 4) the decay of microbial populations to a minimum value (capable of sustaining acetate degradation and perchlorate utilization) rather than zero, as assumed by the model. Based upon results from this task, further research and investigation are needed to improve the coupling process between the perchlorate biodegradation model developed from microcosm experiments and the transport and utilization of perchlorate in column and groundwater flow experiments.

Detailed methods and results for each research task are provided in the following section.

## **4.0 PROJECT ACCOMPLISHMENTS**

### **4.1 SAMPLE COLLECTION**

Aquifer samples were collected from five perchlorate-contaminated locations: (1) the Jet Propulsion Laboratory (JPL) in Pasadena, CA; (2) the Indian Head Division Naval Surface Warfare Center (IHDIW) in Indian Head, MD (2 field sites), (3) the Longhorn Army Ammunition Plant (LHAAP) in Karnack, TX (2 field sites, one surface soil); (4) the Boeing Company, Sacramento, CA (2 field sites), and (5) a commercial facility in the Rocky Mountains, UT. Aquifer solids and groundwater were obtained from the first three locations, and groundwater only was obtained from the Rocky Mountain and the Boeing sites. Samples of a perchlorate-contaminated surface soil were also obtained from LHAAP. Samples were collected from multiple locations at many of the sites based on geochemistry and perchlorate concentrations; a total of nine different environmental samples were collected for this project. These samples were used in microcosm studies to represent a range of different environments that have experienced perchlorate contamination. In addition, one set of samples from LHAAP was used to prepare a series of flow-through aquifer columns. The details of sample collection as well as the geochemical characteristics of each sample are provided below.

### **4.2 ISOLATION AND IDENTIFICATION OF PERCHLORATE DEGRADING BACTERIA FROM FBRs AND FIELD SITES**

#### **Methods**

One objective of this task was to enrich and isolate consortia and pure cultures of perchlorate degrading bacteria for use in microcosm studies (i.e., evaluation of bioaugmentation for perchlorate degradation) as well as to better understand variables influencing perchlorate degradation at the cellular level. The cultures were also used to develop appropriate parameters for a model of perchlorate biodegradation (see section 4.5). Enrichment cultures were prepared from Envirogen bioreactors and from subsurface

samples collected at JPL, IHDIV, and the RM site. Samples were added to a phosphate-buffered enrichment medium containing ammonium chloride, numerous trace elements (Co, Mn, Cu, Al, etc), casamino acids (0.5 g/L) and yeast extract (0.5 g/L) as sources of vitamins and other growth factors potentially required by the organisms. This medium is a modified from that described by Hareland et al. (1975). The isolation medium was amended with ammonium perchlorate to 1000 mg/L ( $\text{ClO}_4^-$ ) and ethanol or acetate (JPL enrichment) to 500 mg/L. The samples were incubated on a rotary shaker operating at 100 rpm and 30°C in the dark.

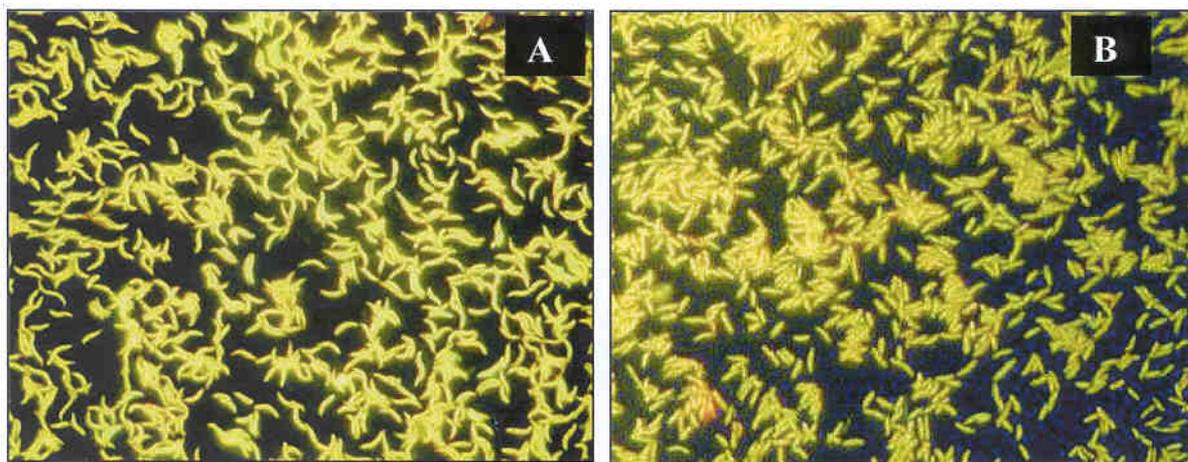
The bottles were periodically checked for signs of microbial growth (turbidity). Any samples showing turbidity were transferred to fresh, sterile media under anoxic conditions. To conduct a transfer, serum bottles were opened using aseptic conditions in the anaerobic chamber, and a small volume of the media (0.025 – 0.050 mL) was pipetted to a serum bottle with fresh media. After several transfers, perchlorate levels were checked in bottles showing microbial growth, and subsamples from each bottle showing perchlorate degradation were plated on two types of agar media. Liquid samples were plated on R2A agar, (a simple medium designed for culturing groundwater bacteria), and incubated aerobically, as most perchlorate degrading cultures are facultative anaerobes. Samples were also plated on a solid agar medium containing the same constituents as the enrichment media plus 15 g of agar per liter. Individual colonies were selected from solid agar plates and streaked on fresh plates several times in succession until each appeared to be a pure culture. The cultures were then inoculated from plates into liquid media with perchlorate, and perchlorate degradation was tested. Cultures that reduced perchlorate were rechecked for purity, then identified using 16S rRNA analysis (Acculab Inc., Newark, DE).

## Results

Some of the samples collected from IHDIV showed microbial growth after several days of incubation and were transferred. A few of these samples again became turbid after transfer, and were passed one or two additional times. However, when levels of perchlorate were tested in the enrichments, none showed appreciable perchlorate degradation. Thus, although some microbial growth was observed in these samples, the bacteria did not appear to be perchlorate degrading strains.

One pure culture was isolated from bioreactor samples initially collected from a fluidized bed bioreactor treating perchlorate in California (Figure 1A). The culture, which was identified by 16S rRNA analysis as a *Dechlorospirillum* sp., was used in several microcosm studies. The *Dechlorospirillum* sp. (FBR2) is very similar to a bacterium (strain WD) isolated from swine waste by Dr. John Coates at Southern Illinois University (SIU). The two strains have a 0.4 % nucleotide difference. This appears to be the only other organism in the available 16S rDNA databases that has reasonable similarity to strain FBR2. In addition to strain FBR2, two pure cultures were isolated from aquifer samples collected from

JPL. These cultures were each identified at the species level as *Dechlorosoma suillum*. This perchlorate degrading genus, which was recently named and described by Achenbach et al., (2001), appears to be widely dispersed in the environment. A photomicrograph of *D. suillum* JPLRND is given in Figure 1B. This bacterium was subsequently used in a series of studies to develop kinetic data for the biodegradation model. These studies evaluated the growth rate of JPLRND on acetate, with perchlorate, nitrate, and oxygen as terminal electron acceptors. Studies were also conducted with this strain to determine possible inhibition of perchlorate reduction by both nitrate and oxygen. A positive enrichment culture was also obtained from the RM groundwater sample using lactate as a carbon source, and two perchlorate degrading strains were purified from the enrichment culture. However, because *Dechlorospirillum* sp. FBR2 and *D. suillum* JPLRND were used extensively for laboratory studies and were adequate to fulfill the objectives of this project, the two bacteria isolated from the RM water were not identified or studied further. However, all of the strains isolated during this project were sent to Dr. Coates at SIU for further study and inclusion in his collection of perchlorate degrading bacteria. In addition, the two pure cultures isolated from JPL were supplied to Dr. Mark Losi from Foster Wheeler Corporation as a seed material to inoculate fixed film bioreactors for testing performed at the JPL facility.



**Figure 1. Photomicrograph of *Dechlorospirillum* sp. FBR2 (A) and *Dechlorosoma suillum* JPLRND (B). Cells are Stained with Acridine Orange.**

## Conclusions

The preliminary results of this project suggest that perchlorate degrading bacteria are widely-occurring in the environment. Pure cultures were isolated from groundwater at the Jet Propulsion Laboratory, from Envirogen reactors (initially seeded with food processing waste), and from the Rocky Mountain site. Although pure cultures were not isolated from the IHDIV samples, laboratory results showed that perchlorate degrading bacteria are present at this site. The enrichment media used for culture isolation

may not have been appropriate based on the physiology of the strains in this environment. In addition, although enrichment studies were not performed with samples from the other sites, perchlorate biodegradation was stimulated in all 9 environmental samples (8 aquifer samples and 1 soil sample) when appropriate electron donors were added, although pH adjustment was also required in acidic samples (see next section). Thus, naturally-occurring perchlorate reducing strains were present in all locations. Few studies exist regarding the occurrence and phylogeny of perchlorate degrading bacteria in natural environments. However, the strains identified during this project (*Dechlorospirillum* sp., *Dechlorosoma* sp.) are similar to bacteria recently discovered by John Coates and colleagues (Coates et al., 1999; Achenbach et al., 2001) in various environmental samples. Additional studies are necessary to better understand the natural distribution and role of this newly identified group of bacteria in the environment, and to determine why the perchlorate reductase and chlorite dismutase enzymes that are characteristic of these strains are so widely conserved.

#### **4.3 LABORATORY MICROCOSM STUDIES**

The objective of this task was to develop an understanding of the factors promoting perchlorate degradation in subsurface environments as well as the conditions that inhibit the process. Small-scale laboratory microcosms were used to evaluate both biostimulation of indigenous perchlorate degrading microbes and the addition of exogenous perchlorate degraders for aquifer remediation. The factors that were evaluated in these studies include: (A) choice of electron donor (substrate) for growth of perchlorate degrading bacteria, (B) the influence of dissolved oxygen, nitrite, and nitrate on perchlorate removal, and (C) the role of environmental factors including salinity (ionic strength), groundwater pH, and presence of organic co-contaminants on perchlorate degradation. The results from microcosm studies are reported in this section on a site-specific basis.

##### **4.3.1. JET PROPULSION LABORATORY (JPL)**

Groundwater samples and well-bottom sediments were collected from the Jet Propulsion Laboratory (JPL) on April 27, 2000. These samples were used in a series of microcosm studies to evaluate (1) the most effective electron donors for the stimulation of perchlorate reducing bacteria at the site (adding substrate but not bacteria); (2) the possibility for successful bioaugmentation (i.e., injection of bacterial isolates) for subsurface perchlorate remediation; (3) the influence of alternate electron acceptors (nitrate, nitrite, and oxygen) on perchlorate degradation; and (4) the roles of two environmental variables, pH and salinity, on perchlorate degradation.

#### 4.3.1.1 Sample Collection

*Groundwater:* Groundwater was collected from monitoring well 7 (MW-7) at the JPL site. Aseptic sampling techniques and sterile sample containers were used to prevent contamination of groundwater with non-native bacteria.

*Aquifer Solids:* Aquifer core samples were not collected for these studies. The extreme depth to contaminated groundwater at JPL (> 200 ft) makes collection of subsurface solids problematic and expensive. However, a bailing device was used to collect sediments from the bottom of MW-7. The well sediments provided sediment material (and associated microflora) for microcosms. Microcosms were set up using groundwater only and groundwater mixed with solids from the bottom of the well.

#### 4.3.1.2 Electron Donor Addition and Bioaugmentation

##### Methods

Small-scale laboratory microcosms were used to evaluate both biostimulation of indigenous perchlorate degrading microorganisms and the addition of exogenous perchlorate degraders for aquifer remediation at JPL (Figure 2). Microorganisms capable of degrading perchlorate utilize the molecule as an electron acceptor during growth on either an organic or inorganic substrate. The absence of an appropriate electron donor in subsurface aquifers contaminated with perchlorate is probably one of the key factors leading to its persistence *in situ*. The factors influencing the choice of substrate to promote perchlorate biodegradation are likely to include the physiology of the perchlorate degrading strains, the character of the natural microflora competing with those strains for growth, and the geochemistry at the site. The objective of this phase of work was to test a variety of substrates in groundwater samples collected from JPL and determine which substrates, if any, are most efficient at stimulating perchlorate reduction.

Microcosms to evaluate perchlorate degradation were prepared in sterile, 160-mL serum bottles. All experimental work was performed in a Coy Environmental Chamber with a nitrogen headspace. In one study, groundwater and well solids (silty material) were mixed together in a ratio of approximately 6:1 in a large sterile bottle. The slurry material was amended with a sterile stock of diammonium phosphate to provide nitrogen (5 mg/L as NH<sub>4</sub>) and phosphorus (4.5 mg/L as P) as nutrients for bacterial growth, then 120-mL volumes were added to serum bottles. Triplicate serum bottles were amended with one of the following substrates to 200 mg/L: methanol, ethanol, acetate, benzoate, lactate, sucrose, molasses or a mixture of ethanol/yeast extract (100 mg/L each). Triplicate bottles also received hydrogen gas or propane in the headspace as gaseous substrates. Several microcosms were inoculated with *Dechlorospirillum* sp. FBR2. Acetate and ethanol/yeast extract were tested as electron donors in these

samples. Triplicate samples were prepared without any substrate, and triplicate bottles received formaldehyde (1 %) to inhibit all biological activity. All bottles were crimp-sealed with sterilized Teflon-lined septa and incubated at 15°C to approximate *in situ* temperatures. After 10 and 21 days of incubation, a 20-mL subsample was removed from each bottle. The samples were analyzed for perchlorate by ion chromatography (IC) using EPA Method 314.0.

A second microcosm study was conducted using only groundwater collected from MW-7 (i.e., no sediment). Sterile serum bottles received 120-mL of groundwater and acetate, yeast extract, methanol, or molasses at a concentration of 200 mg/L. Microcosms without added substrate were also prepared as were killed controls (1 % formaldehyde). Sampling and analysis were conducted as described for the previous study.



**Figure 2. Photograph of Aquifer Microcosms.**

## Results

The water collected from well MW-7 contained perchlorate at 307  $\mu\text{g/L}$  (ppb). The water also contained nitrate at a starting concentration of 18.6 mg/L (as  $\text{NO}_3$ ), sulfate at 44 mg/L, 140 mg/L of alkalinity (as  $\text{CaCO}_3$ ) and dissolved oxygen at 2.6 mg/L.

*Sediment/Groundwater Microcosms:* The starting perchlorate concentration in microcosms prepared with groundwater and sediments was 310  $\mu\text{g/L}$ . The initial pH was 7.6. The microcosms also contained high levels of ferric iron ( $> 600$  mg/L), which was present in the sediment sample. The iron was probably well casing that had oxidized and settled to the well bottom. After 10 days of incubation at 15°C, perchlorate levels were below detection (PQL; 5  $\mu\text{g/L}$ ) in microcosms amended with acetate, ethanol, ethanol/yeast

extract, lactate and molasses (Table 1). The perchlorate concentration in all samples augmented with exogenous perchlorate degrading bacteria (*D. suillum* FBR2) was also below detection after 10 days. After 21 days of incubation, perchlorate was below detection in all live samples except those amended with benzoate as an electron donor. Interestingly, perchlorate was also degraded in samples without added electron donor. An organic or inorganic electron donor associated with the well sediments (e.g., reduced iron, natural organic matter) probably supported biological perchlorate reduction in these samples. This hypothesis is supported by the observation that perchlorate was not degraded in groundwater samples without electron donor added (see next section). No perchlorate loss was evident in samples that were treated with formaldehyde to inhibit biological activity. Nitrate was also degraded to below detection in the live aquifer microcosms, but not in killed controls (data not shown).

**Table 1. Perchlorate Degradation in JPL Sediment/Groundwater Microcosms Amended with Various Electron Donors or Perchlorate Degrading Bacteria.**

Treatment	Perchlorate Concentration ( $\mu\text{g/L}$ ) <sup>1</sup>		
	Day 0	Day 10	Day 21
<i>Electron Donors</i>			
Killed Control	310 ± 0	293 ± 6	320 ± 0
Benzoate	310 ± 0	297 ± 6	150 ± 135
Methanol	310 ± 0	77 ± 57	< 5
Hydrogen	310 ± 0	177 ± 61	< 5
Propane	310 ± 0	283 ± 6	< 5
No Addition	310 ± 0	14 ± 19	< 5
Sucrose	310 ± 0	92 ± 67	< 5
Ethanol	310 ± 0	< 5	NS <sup>2</sup>
Lactate	310 ± 0	< 5	NS
Molasses	310 ± 0	< 5	NS
Yeast Extract/Ethanol	310 ± 0	< 5	NS
Acetate	310 ± 0	< 5	NS
<i>Bioaugmentation</i>			
Killed + <i>Dechlorospirillum</i> FBR2 <sup>3</sup>	310 ± 0	385 ± 7	415 ± 7
<i>Dechlorospirillum</i> FBR2+ YE/Etoh	310 ± 0	< 5	NS
<i>Dechlorospirillum</i> FBR2+ Acetate	310 ± 0	< 5	NS

<sup>1</sup> Values are the mean ± standard deviation from triplicate microcosms.

<sup>2</sup> NS: Not sampled because previous sample point was below detection.

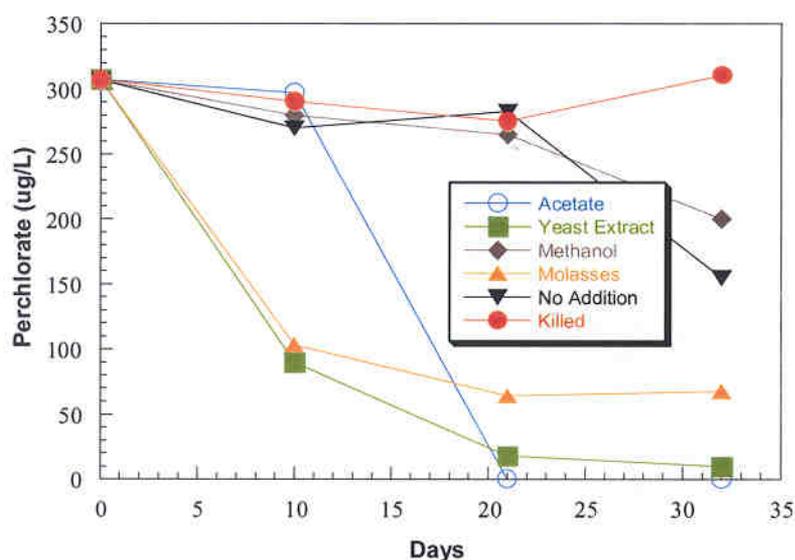
<sup>3</sup> *Dechlorospirillum* sp. FBR2 is a perchlorate degrading culture isolated from a fluidized bed bioreactor.

*Groundwater Microcosms:* Perchlorate degradation was somewhat slower in microcosms containing groundwater compared to those with sediments (Figure 3). However, after 21 days of incubation, perchlorate was below detection (PQL; 5  $\mu\text{g/L}$ ) in triplicate samples amended with acetate. Appreciable degradation of perchlorate was also observed in samples amended with yeast extract or molasses.

Perchlorate was not degraded in samples treated with methanol as an electron donor or in those without added electron donor. The killed samples (1 % formaldehyde) also showed no loss of perchlorate.

## Conclusions

The results from the microcosm study using aquifer samples from JPL suggest the following: (1) indigenous bacteria capable of degrading perchlorate are present in the aquifer underlying JPL; (2) these bacteria can be stimulated to degrade perchlorate by the addition of electron donors; and (3) perchlorate levels can be reduced to below 5  $\mu\text{g/L}$  through biostimulation. The fact that perchlorate degradation was observed in groundwater microcosms, without sediment, is very promising, since microbial biomass in aquifers is usually associated primarily with solids.



**Figure 3. Influence of Different Electron Donors on Perchlorate Biodegradation in Groundwater Microcosms from JPL.**

### 4.3.1.3. Influence of Alternate Electron Acceptors on Perchlorate Biodegradation

#### Methods

The objective of this study was to determine the influence of oxygen, nitrate, and nitrite on perchlorate degradation by natural microflora in aquifer samples from JPL. Aquifer microcosms were used to assess the role of these molecules on perchlorate reduction by natural microflora in the subsurface samples. Based on results from the previous study of electron donors, ethanol was chosen as the electron donor for these experiments.

The microcosms were set up as described in the previous section (160-mL serum bottles, 120-mL aquifer slurry of sediments and groundwater). Eight microcosms were initially amended with perchlorate

to provide a starting concentration of 100 mg/L. Duplicate microcosms at an initial concentration of 100 mg/L perchlorate received the following treatments: (1) ethanol only (100 mg/L); (2) ethanol (100 mg/L) and  $\text{NO}_3^-$  (100 mg/L); (3) nitrate only (100 mg/L) (i.e., no ethanol); or (4) ethanol (100 mg/L),  $\text{NO}_3^-$  (100 mg/L), and formaldehyde (*killed control*). To evaluate the role of nitrite ( $\text{NO}_2^-$ ) on perchlorate degradation, duplicate bottles were amended with the following (1) ethanol (100 mg/L) and 1 mg/L nitrite; (2) ethanol (100 mg/L) and 10 mg/L nitrite; or (3) ethanol only. A killed control was also prepared for this study by adding 1 % formaldehyde to one set of duplicate samples. In a third experiment, the effect of oxygen on perchlorate degradation was determined by oxygenating the headspace of two bottles containing 300  $\mu\text{g/L}$  perchlorate and ethanol (100 mg/L). The samples were incubated at 15°C. Aqueous subsamples were periodically removed from each microcosm for perchlorate analysis by EPA Method 314.0 and analysis of nitrate and nitrite by EPA 300.0 series methods.

## Results

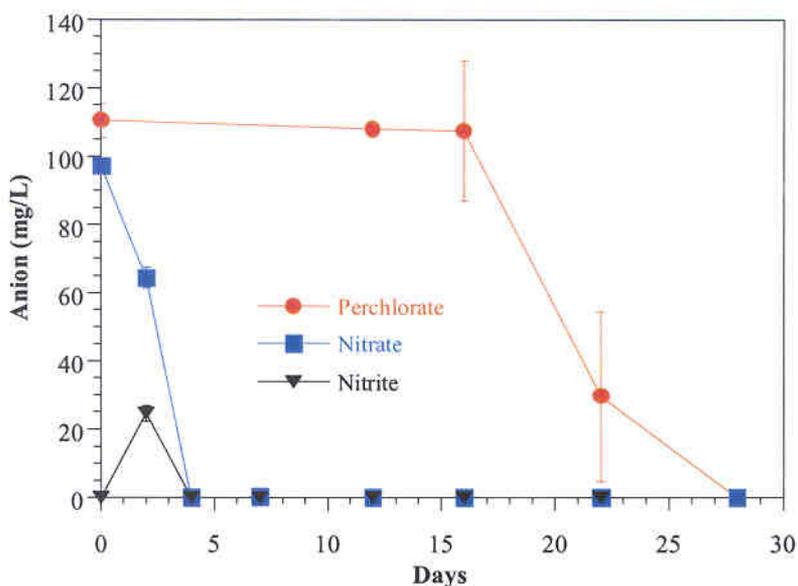
There was no loss of perchlorate, nitrate, or nitrite in any of the samples that were treated with formaldehyde to inhibit microbial activity. Thus, all reductions in the concentrations of these anions in aquifer samples are assumed to be biological. Nitrate was degraded before perchlorate in samples that received both anions at initial concentrations of 100 mg/L (Figure 4). Nitrate was reduced to below detection after only 4 days of incubation, with no apparent lag period. Nitrite, which is the initial product in biological denitrification and nitrate reduction, was detected in samples at day 4, but this anion was also degraded to below detection by day 7. A lag period of approximately 16 days occurred before perchlorate degradation commenced in these microcosms. However, perchlorate was reduced from 100 mg/L to below detection ( $< 5 \mu\text{g/L}$ ) between day 16 and day 28. Interestingly, the degradation of perchlorate was slightly more rapid in samples that were initially amended with nitrate to 100 mg/L compared to those that did not receive the anion (Figure 5). This may reflect the growth of a population of denitrifying bacteria (stimulated by nitrate addition) that subsequently degraded perchlorate.

Samples that were not amended with ethanol as an electron donor showed no perchlorate degradation during a 22-day incubation period (Figure 6). In these same samples, however, nitrate levels declined from 100 to approximately 40 mg/L during the initial 7 days of incubation. During this same time, levels of nitrite in the samples increased from below detection to nearly 40 mg/L. On a molar basis, this represents a nearly stoichiometric reduction of nitrate to nitrite. Thus, the data show that nitrate was biologically reduced to nitrite, but not further (i.e., to nitrogen gas or ammonia) in the absence of an added electron donor. The substrate supporting this reaction is unclear, but may be organic matter associated with the well-bottom sediments.

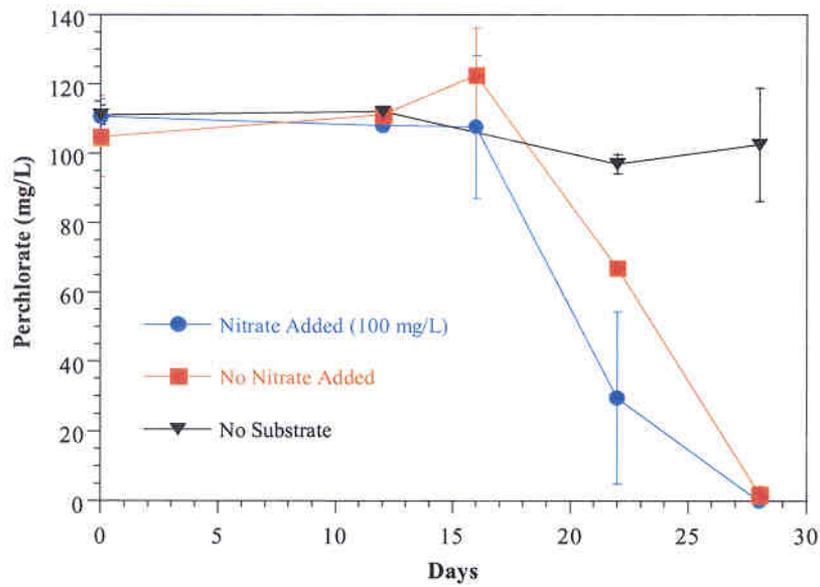
Like nitrate, nitrite added to aquifer microcosms at either 1 or 10 mg/L was degraded before perchlorate (data not shown). The addition of nitrite at these levels did not appear to influence the rate of perchlorate degradation (i.e. after the initial lag period, the rate of perchlorate reduction was the same in samples with and without added nitrite). The degradation of perchlorate was completely inhibited by the presence of oxygen in aquifer samples (Figure 7). This result confirms previous findings that perchlorate degradation occurs only under anoxic conditions.

## Conclusions

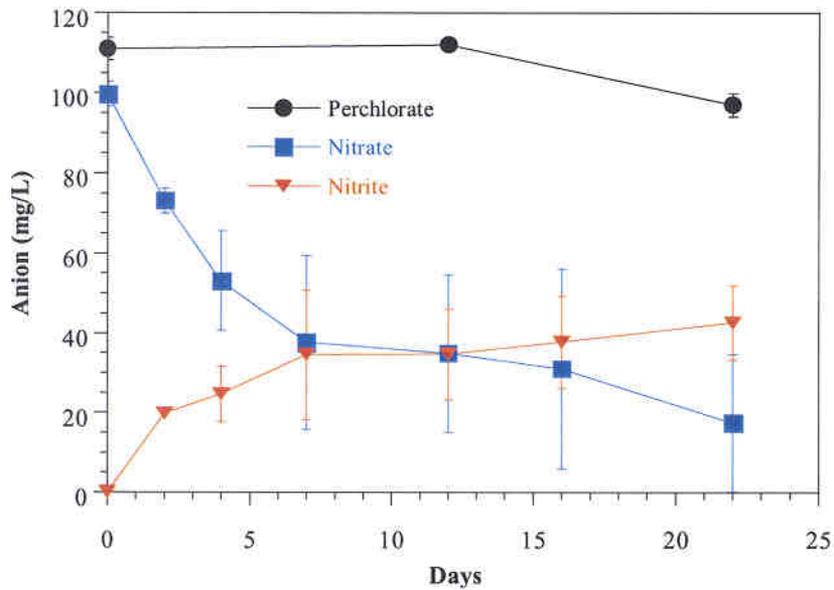
The data from this set of experiments suggest that nitrate and nitrite are degraded preferentially to perchlorate in this subsurface environment. It is unclear from these results whether the presence of nitrate or nitrite actually inhibits biological perchlorate degradation, however, in no instance was perchlorate degradation observed until both of these competing electron acceptors were degraded in the samples. In subsequent studies with a pure culture isolated from this site (*D. suillum* JPLRND), nitrate was observed to inhibit active perchlorate degradation, suggesting that it may be a biochemical inhibitor of biological perchlorate reduction (see section 4.5.6.4.1. *Influence of Nitrate on Perchlorate Reduction*). An understanding of the relationship between perchlorate and competing electron acceptors (e.g., oxygen, nitrate, nitrite, ferric iron) is important because these molecules frequently occur with perchlorate in groundwater. For example, the groundwater collected from JPL contained 18.6 mg/L of nitrate but only 300 µg/L of perchlorate. Therefore, an understanding of whether nitrate impedes perchlorate degradation (i.e., due to enzyme inhibition or other factors) may be important in evaluating treatment options at contaminated sites.



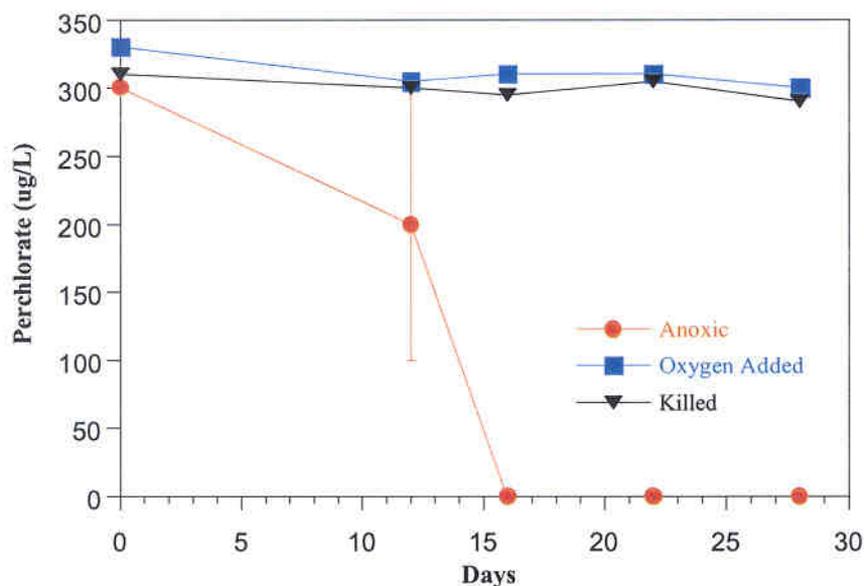
**Figure 4. Sequential Biodegradation of Nitrate and Perchlorate in Aquifer Microcosms from JPL.**



**Figure 5. Influence of Nitrate on Perchlorate Biodegradation in Aquifer Microcosms from JPL.**



**Figure 6. Biodegradation of Perchlorate (100 mg/L) and Nitrate (100 mg/L) in Aquifer Microcosms with No Substrate Added.**



**Figure 7. Influence of Oxygen on Perchlorate Biodegradation in Aquifer Microcosms from JPL.**

#### 4.3.1.4 Influence of pH and Salinity on Perchlorate Degradation

##### Methods

Little information exists on the influence of environmental variables such as temperature, pH, salinity, redox potential, alkalinity, and the presence of additional contaminants on biological degradation of perchlorate in groundwater. The influence of two environmental variables, pH and salinity, on perchlorate degradation was tested using aquifer samples from JPL. To assess the influence of salinity on perchlorate removal in field samples, a synthetic seawater medium (Atlas, 1995) was prepared at 0.5X, 1X, and 2X concentrations. The stocks were then mixed 1:1 with groundwater from the field site yielding salinities ranging from 0.25 X to 1 X that of seawater. The samples were then amended with perchlorate back to the initial concentration (~ 300  $\mu\text{g/L}$ ). Ethanol was used as the electron donor in these studies. Killed controls were prepared at each level of salinity by adding formaldehyde to samples to a final concentration of 1%. All microcosms were prepared and incubated under anoxic conditions. Aqueous subsamples were removed periodically and analyzed for perchlorate as described previously.

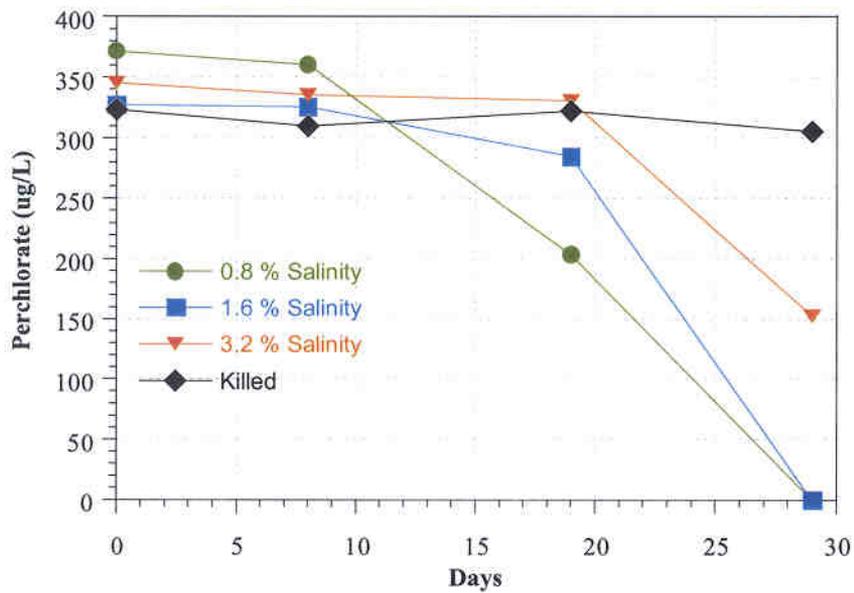
The role of pH on perchlorate biodegradation was evaluated essentially as described for salinity. In this case, however, the pH rather than the ionic strength of the aquifer material was manipulated. Because the buffering capacity of groundwater is limited, MES (2-[N-morpholino]ethanesulfonic acid;  $\text{pK}_a = 6.1$ ) buffer was added to samples at a concentration of 2 mM to maintain pH at desired levels. The

slurry material was then divided into 5 sterile beakers in the anaerobic chamber and the pH of each sample was adjusted using sterilized HCl or NaOH. The final pH levels of the slurries were 4.0, 5.0, 6.0, 7.0, or 8.0. The pH-adjusted slurry material was then added to sterile 160-mL serum bottles in triplicate. One bottle at each pH was amended with formaldehyde to a final concentration of 1% to inhibit microbial activity. Aqueous subsamples were removed from each sample at various times during incubation at 15°C and analyzed for perchlorate by EPA Method 314.0.

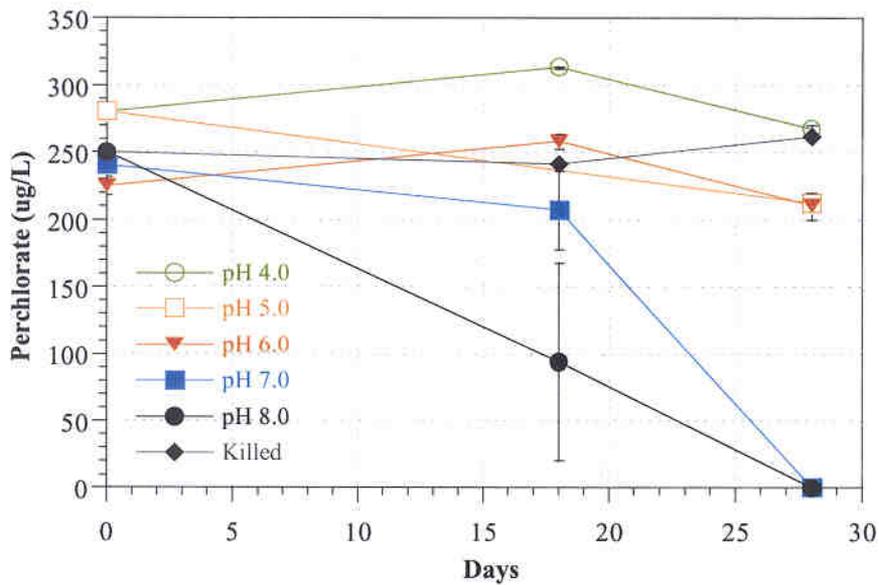
## **Results and Conclusions**

*Salinity:* The rate of perchlorate degradation in microcosms prepared from the JPL aquifer samples declined moderately with increasing salinity (Figure 8). During a 29-day incubation period, the perchlorate concentration in samples containing salinity at 25 or 50% that of seawater (0.8% and 1.6%, respectively) declined from a starting concentration of approximately 350 µg/L to below detection (PQL; 4 µg/L). The perchlorate concentration in samples brought to the salinity of seawater (~3.2% total salinity) also declined during 29 days, but approximately 150 µg/L remained at the end of the incubation period. There was no degradation in the killed controls. The results from this experiment show that perchlorate degradation is possible at salt levels at least as high as in sea water, although rates may be appreciably reduced compared to less saline environments. This observation confirms recent work by Logan et al. (2001) in which the authors successfully developed three perchlorate degrading enrichment cultures at a salinities ranging from 3 - 7%. Pure cultures were not obtained during this study. In addition, the rates of cell growth appeared to be extremely slow (with maximum cell doubling times of greater than 10 days) compared to non-salt-tolerant cultures. The 5 isolates obtained during this SERDP project were subsequently tested for salt tolerance by adding 1 – 5% NaCl to BSM medium. None of the cultures degraded perchlorate at salt levels of 1% or higher. Thus, while salt tolerant perchlorate degrading strains appear to exist based on our data from JPL and data from other laboratories, this trait appears not to be common among this group of organisms.

*pH:* The biodegradation of perchlorate was most rapid in JPL aquifer samples brought to a pH of 8.0 (Figure 9). Levels of perchlorate declined from approximately 250 µg/L to less than 4 µg/L in 28 days. Perchlorate was also completely degraded in samples at a pH of 7.0 during the 28-day incubation. However, at pH values of 4.0, 5.0, and 6.0, little or no perchlorate losses were observed in the aquifer microcosms. These results are supported by data from two additional sites (IHDIIV and LHAAP) that suggest that low pH is inhibitory to perchlorate reduction in environmental samples (see next two sections).



**Figure 8. Influence of Salinity on Perchlorate Biodegradation in Groundwater Microcosms from JPL.**



**Figure 9. Influence of pH on Perchlorate Biodegradation in Aquifer Microcosms from JPL.**

### **4.3.2 INDIAN HEAD DIVISION, NAVAL SURFACE WARFARE CENTER (IHDIV), INDIAN HEAD, MARYLAND**

#### **4.3.2.1 Sample Collection**

Aquifer solids and groundwater were collected from two separate locations at IHDIV on August 1, 2000 using a Geoprobe (Figure 10). The extent of perchlorate contamination in the shallow aquifer at this site was unknown, so sampling locations were chosen based on historical use and disposal of perchlorate at the site. The initial sample site was the drainage area behind a propellant mixing facility at IHDIV (Building 1190 site). The level of the water table at this site is approximately 4 ft below grade. Sediments were collected from 4 to 12 ft and groundwater from 6 to 12 ft below grade. The second sample location was an open meadow behind building 1419, the rocket “Hog Out” facility at IHDIV (Hog Out site). Solid fuel is removed from rockets and missiles in this building using a high-pressure washout procedure (i.e., Hog Out procedure). Before 1996, the washout water was discharged through the region where the field samples were collected. Sediment samples from 2 to 13 ft below grade were collected and homogenized. Groundwater was taken from 6 to 12 feet below grade at the site.

#### **4.3.2.2 IHDIV Building 1190 Site – Evaluation of Electron Donors and Electron Donor Concentration on Perchlorate Biodegradation**

##### **Methods**

*General Preparation and Sampling:* All experimental work was performed in a Coy Environmental Chamber with a nitrogen headspace. Sampling for analysis of perchlorate and other parameters was performed outside of the chamber. Prior to sample collection, a volume of nitrogen gas was added via syringe to the headspace of each microcosm bottle. The addition of nitrogen created backpressure in the bottle to facilitate sample withdrawal. More importantly, this method ensured that no oxygen was introduced into the bottles during sampling. All samples were analyzed for perchlorate by ion chromatography (IC) using EPA Method 314.0.

*Evaluation of Electron Donors:* Microcosms to evaluate the influence of different electron donors on perchlorate degradation were prepared in sterile, 160-mL serum bottles. Groundwater from the Building 1190 site was amended with a sterile stock of diammonium phosphate to provide nitrogen (5 mg/L as  $\text{NH}_4$ ) and phosphorus (4.5 mg/L as P) as nutrients for bacterial growth. Groundwater and sediment from the site were added to each 160-mL bottle at a ratio of approximately 3:1 (100-mL groundwater and 30-g sediment). Each bottle was spiked with a filter-sterilized sodium perchlorate stock solution to a final

perchlorate concentration of 125 mg/L. Triplicate serum bottles were amended with acetate, ethanol, or molasses to 200 mg/L. Triplicate bottles also received hydrogen in the headspace as a gaseous substrate. Triplicate bottles were inoculated with a perchlorate degrading culture (FBR2) isolated at Envirogen; ethanol was tested as electron donor in these bottles. Killed controls were prepared with acetate as a substrate and 1 % formaldehyde to inhibit all biological activity. The bottles were crimp-sealed with sterilized Teflon-lined septa and incubated at 15°C to approximate *in situ* temperatures. At 11, 19, and 34 days of incubation, a 15-mL subsample was removed from each bottle. Preservation of the samples was accomplished by passing the water through sterile nylon filters and storing at 4°C until analysis.



**Figure 10. Collection of Field Samples from the IHDIV Hog Out Site.**

*Electron Donor Concentration:* The objective of this study was to determine the amount of electron donor needed to support perchlorate reduction, and to compare the actual electron donor requirement to the theoretical requirement. Acetate was used as an electron donor (based on results from the previous study), and the quantity required to degrade a given quantity of perchlorate in aquifer microcosms was determined. Microcosms were prepared in sterile, 60-mL serum bottles. Nutrient-amended groundwater and sediment were combined in each bottle at a ratio of about 4.5:1 (45-mL groundwater and 10-g sediment). Each bottle was spiked with a filter-sterilized sodium perchlorate stock solution to a final perchlorate concentration of 100 mg/L (109 mg/L actual measured). Sodium acetate was added to triplicate bottles at concentrations of 0, 10, 25, 50, 75, and 100 mg/L as acetate. One killed control was prepared by adding 1% formaldehyde to a microcosm containing 100 mg/L acetate. All bottles were

crimp-sealed with sterilized Teflon-lined septa and incubated at 15°C to approximate *in situ* temperatures. At 4, 6, 8, 10, and 13 days of incubation, a 7-mL subsample was removed from each bottle. The samples were filtered and exposed to air, then frozen to inhibit any additional perchlorate or acetate degradation. Perchlorate concentrations were measured in each sample using EPA Method 314.0.

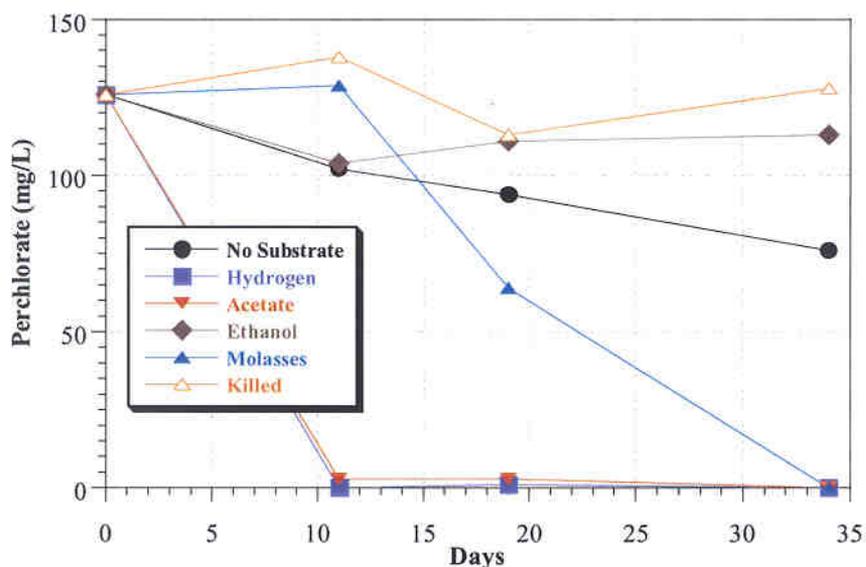
## Results

*Groundwater Analysis:* The groundwater collected from the Building 1190 site did not contain perchlorate (<4 µg/L), nitrate (<0.2 mg/L) or nitrite (<0.2 mg/L) above detection limits. Sulfate was present at 12 mg/L, chloride at 43 mg/L, and alkalinity was 40 mg/L (as CaCO<sub>3</sub>). The pH of the water was 5.9. A slurry containing 30 g of sediment and 100 mL of water had a pH of 6.1.

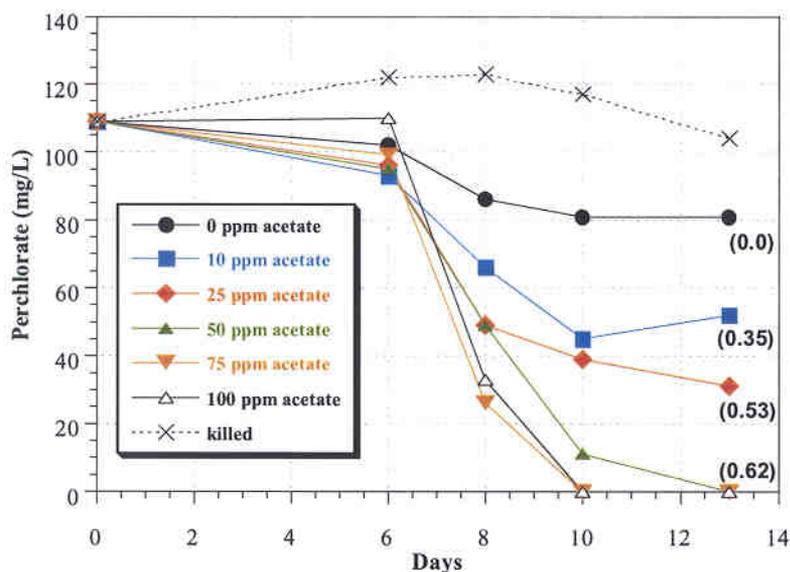
*Evaluation of Electron Donors:* Perchlorate was not detected in samples collected from the Building 1190 site, so the aquifer microcosms were amended with the anion to a starting concentration of ~125 mg/L. After 11 days of incubation at 15°C, perchlorate levels were below detection in microcosms amended with hydrogen gas (Figure 11). Samples that received acetate declined to 3 mg/L total perchlorate during this time. After 34 days of incubation, perchlorate was below detection in samples treated with molasses or acetate, as well as those receiving hydrogen as an electron donor. Samples receiving ethanol as an electron donor showed no appreciable decline in perchlorate levels. Likewise, no perchlorate loss was evident in acetate-amended microcosms that received formaldehyde to inhibit biological activity. The perchlorate concentration in live samples that did not receive any exogenous substrate declined from 126 to 76 mg/L during 34 days of incubation. A similar decline was previously observed with JPL microcosms containing groundwater and sediments (but not groundwater only). This decline suggests that an electron donor present at the site, such as natural organic matter or an organic co-contaminant, may support degradation of perchlorate at this location. The absence of detectable perchlorate in this region, which served as a deposition area for wash-down water from the Building 1190 facility, further supports this hypothesis.

*Electron Donor Concentration:* Based on stoichiometric calculations, the quantity of acetate required for a bacterium to degrade perchlorate is 0.61 mg per mg perchlorate. This ratio was tested in samples from the Building 1190 location by varying the acetate dose added to microcosms and evaluating perchlorate degradation. Microcosm samples initially received 100 mg/L of perchlorate and either 0, 10, 25, 50, 75, or 100 mg/L of acetate. After 10 days of incubation, samples amended with 100 or 75 mg/L of acetate no longer had perchlorate at detectable levels (Figure 12). After 13 days, concentrations of perchlorate in samples amended with 50 mg/L of acetate were also below detection and samples treated with 0, 10, and

25 mg/L acetate had mean perchlorate levels of 81, 52, and 41 mg/L, respectively. The quantity of acetate required for complete removal of perchlorate from the microcosm samples was less than determined from reaction stoichiometry. However, as observed in previous samples from this site, perchlorate degradation occurred in unamended samples, presumably supported by natural organic materials at the site. When this loss is taken into account, the perchlorate degradation observed with different levels of acetate become much closer to that expected based on theoretical calculations. These ratios are presented for 10, 25, and 50 mg/L acetate in Figure 12. Additional studies concerning the ratio of electron donor required for perchlorate degradation in natural samples will be conducted in flow-through column studies in Year 2.



**Figure 11. Influence of Different Electron Donors on Perchlorate Biodegradation in Aquifer Microcosms from the IHDIIV Building 1190 Site.**



**Figure 12. Biodegradation of Perchlorate in Aquifer Microcosms Amended with Different Concentrations of Acetate as Electron Donor. Values in Parentheses represent the ratio of Acetate/Perchlorate (mg/L).**

## Conclusions

The results from the microcosm study using aquifer samples from the Building 1190 site suggest the following: (1) indigenous bacteria capable of degrading perchlorate are present in the shallow aquifer in the vicinity of Building 1190, and (2) these bacteria can be rapidly stimulated to degrade perchlorate to below 4  $\mu\text{g/L}$  by the addition of several electron donors. The data also suggest that natural attenuation of perchlorate is possible at this location. This area was used for the disposal of perchlorate-containing wastewater from the mixing facility until 1998, yet the anion was not detected in subsurface samples, which suggests attenuation by either transport or biodegradation. In addition, samples amended with perchlorate but no electron donor showed significant losses of the anion in microcosm studies. A natural electron donor (e.g., humic material) or an organic co-contaminant most likely served as an electron donor for biological perchlorate reduction in these samples.

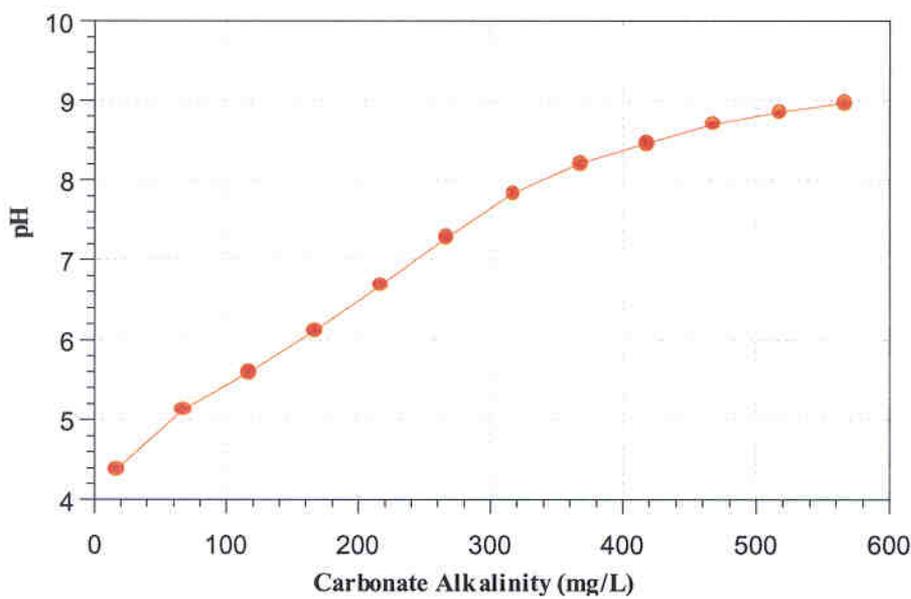
### 4.3.2.3 IHDIV Hog Out Facility – Evaluation of Electron Donors and pH on Perchlorate Reduction

#### Methods

*Electron Donor Addition:* A second microcosm study was conducted using groundwater and sediment collected from the Hog Out site at IHDIV (Building 1419). The experiment was prepared in the same manner as described for the previous study, except that no perchlorate addition was required. The starting perchlorate concentration in the mixed groundwater and sediment was approximately 45 mg/L. Triplicate serum bottles were amended with nutrients (N and P from diammonium phosphate) and one of the

following substrates at 200 mg/L: methanol, ethanol, acetate, benzoate, lactate, sucrose, molasses, or a mixture of ethanol and yeast extract (100 mg/L each). Triplicate bottles also received hydrogen gas or propane in the headspace as gaseous substrates. Triplicate bottles were inoculated with the perchlorate degrading enrichment FBR2; ethanol was used as an electron donor in these bottles. In addition, triplicate microcosms were prepared with nutrients (N and P) but no substrate, substrate (acetate) but no nutrients, and without addition of substrate or nutrients. Killed control samples were prepared with acetate and received formaldehyde (1 %) to inhibit all biological activity. All bottles were crimp-sealed with sterilized Teflon-lined septa and incubated at 15°C to approximate *in situ* temperatures. At 11, 20, 36, and 71 days of incubation, a 15-mL subsample was removed from each bottle. The samples were preserved and analyzed as described for the previous experiment.

*Influence of pH on Perchlorate Biodegradation:* An experiment was conducted to determine whether the low pH (4.3) of the Hog Out site samples was inhibiting perchlorate degradation at the site. Prior to adjusting the pH, the influence of increasing carbonate concentration on slurry pH was tested. The resulting titration curve showed that approximately 240 mg/L of additional carbonate was required to increase the pH of the slurry to 7.0 (Figure 13). Microcosms were prepared in sterile, 160-mL serum bottles. The groundwater was amended with a sterile stock of diammonium phosphate to provide nitrogen (1 mg/L NH<sub>4</sub> as N) and phosphorus (1 mg/L PO<sub>4</sub> as P) as nutrients for bacterial growth. Groundwater and sediment were added to each 160-mL bottle at a ratio of approximately 3:1 (100-mL groundwater and 30-g sediment). Acetate was added as the electron donor at 75 mg/L. Perchlorate was not added, as the perchlorate concentration in the mixed groundwater and sediment was approximately 45 mg/L. In eight of the fourteen bottles prepared, the pH was increased from 4.3 to approximately 7.0 by adding sodium carbonate. The pH of the remaining six microcosms was not adjusted (i.e., pH 4.3). Three of the bottles at pH 4.3 and three at pH 7.0 were inoculated with the perchlorate degrading culture FBR2, and three bottles at each pH remained uninoculated. Two of the bottles at pH 7.0 received formaldehyde (1 %) to inhibit all biological activity. The bottles were incubated on a rotary shaker at 15°C. After 7, 16, and 28 days of incubation, a 7-mL subsample was removed from each bottle. Each sample was initially centrifuged for approximately 30 minutes at 3,500 rpm to remove sediment fines. The supernatant was then passed through a nylon filter and placed at 4°C until analysis. A freshly grown inoculum of the FBR2 culture was re-added to three bottles at each pH on Day 10. This procedure was conducted to ensure that all bottles amended with the bacterium received active perchlorate degrading bacteria.



**Figure 13. Carbonate Titration Curve for Sediment Slurries from the IHDIV Hog Out Site.**

## Results

*Groundwater Analysis:* The groundwater collected from the Hog Out site contained perchlorate at 25 mg/L. In a slurry containing 30-g sediment and 100-mL groundwater, perchlorate was detected at 45 mg/L suggesting that the anion was present at a higher concentration in the sediments collected from the site than in the groundwater. This difference may represent perchlorate present in the unsaturated zone of the shallow aquifer. Nitrate and nitrite were not detected in samples. Sulfate was present at 88 mg/L, chloride at 26 mg/L, and alkalinity was 19 mg/L (as  $\text{CO}_3$ ). The pH of the water was 4.8, and a slurry of water (100 mL) and sediment (30 g) had a pH of 4.3.

*Electron Donor Addition:* There was no appreciable loss of perchlorate during the 71-day incubation period in any of the microcosms prepared from the Hog Out site samples (Table 2). Ten different electron donors did not stimulate perchlorate biodegradation in the samples. Bioaugmentation with an exogenous perchlorate degrading culture (FBR2) also did not reduce perchlorate levels. These results differ from those with the Building 1190 samples, where several electron donors quickly stimulated perchlorate degradation. Rapid reduction in perchlorate levels was also observed in aquifer microcosms from the Jet Propulsion Lab and a commercial site in the Rocky Mountains (see following section). The most apparent difference between the Hog Out samples and those from other sites is the comparatively low pH of the microcosms compared to other samples. The pH of the Hog Out site microcosms was

measured at 4.3. Other samples tested prior to this had pH values no lower than 6.1. An experiment was subsequently conducted to assess the influence of pH on perchlorate degradation in the Hog Out samples.

**Table 2. Perchlorate Degradation in Aquifer Microcosms from the IHDIV Hog Out Site.**

Treatment	Perchlorate Concentration (mg/L) <sup>1</sup>				
	Day 0	Day 11	Day 20	Day 36	Day 71
<b>Electron Donors</b>					
Killed Control	42 ± 4	41 ± 1	44 ± 2	36 ± 4	37 ± 2
No Substrate	42 ± 4	37 ± 1	36 ± 4	38 ± 1	39 ± 5
Nutrients Only	42 ± 4	38 ± 2	41 ± 4	42 ± 1	34 ± 1
Hydrogen	42 ± 4	38 ± 2	40 ± 4	32 ± 5	35 ± 2
Propane	42 ± 4	38 ± 1	39 ± 2	34 ± 2	37 ± 2
Ethanol	42 ± 4	39 ± 2	41 ± 2	36 ± 4	36 ± 3
Methanol	42 ± 4	41 ± 2	41 ± 1	32 ± 2	34 ± 2
Acetate	42 ± 4	39 ± 1	42 ± 2	33 ± 1	37 ± 1
Benzoate	42 ± 4	40 ± 1	43 ± 0	32 ± 1	38 ± 1
Lactate	42 ± 4	38 ± 3	43 ± 3	33 ± 2	37 ± 2
Molasses	42 ± 4	43 ± 2	43 ± 2	28 ± 1	36 ± 2
Sucrose	42 ± 4	44 ± 1	45 ± 0	31 ± 0	35 ± 0
Yeast Extract/Ethanol	42 ± 4	43 ± 2	44 ± 2	35 ± 3	37 ± 2
<b>Bioaugmentation</b>					
Inoculum FBR2+ Ethanol	42 ± 4	41 ± 1	44 ± 3	36 ± 2	36 ± 2

<sup>1</sup> Values are the mean ± standard deviation from triplicate microcosms.

**Influence of pH on Perchlorate Degradation:** The perchlorate levels in the samples at pH 4.3 did not decline appreciably during the study, regardless of whether the samples were bioaugmented (Figure 14). Conversely, the samples in which the pH was increased to 7.0 all showed perchlorate biodegradation. Perchlorate levels in samples receiving *Dechlorospirillum* sp. FBR2 declined from 43 to 9 mg/L from day 7 to day 16, and then to 0.16 mg/L by day 28. The perchlorate concentration in samples that were brought to pH 7.0 but not augmented with the culture declined more slowly, but perchlorate was below detection by day 28 of the experiment. Thus, the data suggest that low pH is inhibiting perchlorate degradation in the Hog Out site samples. It is interesting that indigenous perchlorate degrading microorganisms could be stimulated to degrade the anion at a pH of 7.0 but not at a pH of 4.3. These bacteria are obviously able to survive at the low pH, which occurs naturally at this site, yet appear not to degrade perchlorate at this pH. The results suggest that there may be a pH below which perchlorate biodegradation is physiologically inhibited.

## Conclusions

Data from experiments conducted with samples from the Hog Out site at IHDIV suggest that low pH is inhibitory to biological perchlorate reduction. Neither biostimulation nor bioaugmentation promoted perchlorate degradation at the site pH of 4.3. However, when the pH of the samples was increased to neutrality, perchlorate biodegradation was observed in samples receiving only acetate as well as those augmented with *Dechlorospirillum* sp. FBR2. The inhibition of perchlorate degradation at low pH in these field samples is consistent with previous observations at Envirogen during experiments with *ex situ* reactor systems. During a laboratory pilot study, perchlorate treatment in a fluidized bed reactor was observed to decline appreciably when the pH of the system declined below approximately 5.5. The performance was regained when the pH was increased to neutrality. Perchlorate inhibition at low pH was also observed in soil and groundwater samples from the Longhorn Army Ammunition Plant (LHAAP). These data are reported in section 4.3.4.

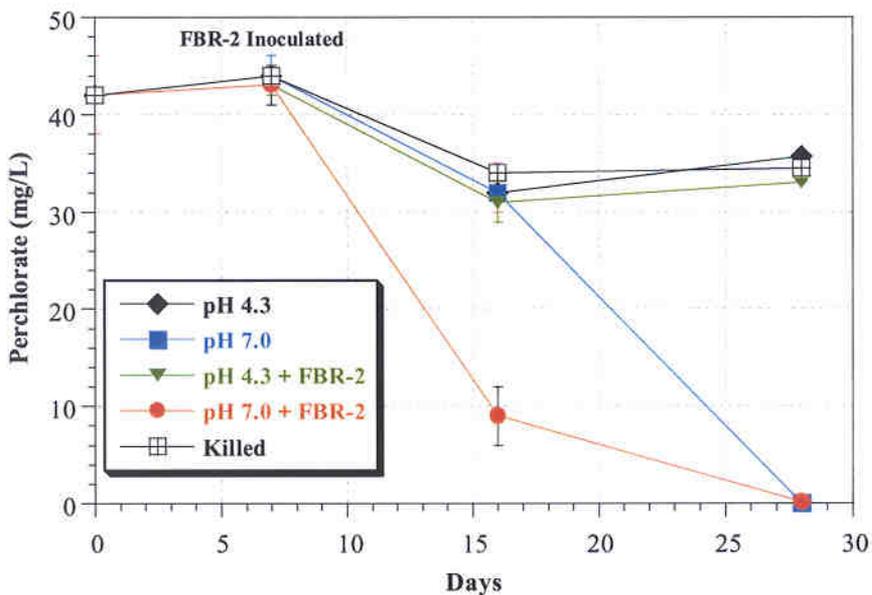


Figure 14. Influence of pH on Perchlorate Degradation in Aquifer Microcosms from the IHDIV Hog Out Site.

### **4.3.3 ROCKY MOUNTAIN COMMERCIAL FACILITY (RM), UTAH**

#### **4.3.3.1. Evaluation of Electron Donors and Influence of Chlorinated Solvents and BTEX on Perchlorate Reduction**

##### **Methods**

*Sample Collection:* Groundwater samples were collected by site personnel at an industrial manufacturing facility in the Rocky Mountains. Sediment samples were not available.

*Groundwater Microcosms:* Microcosms were used to evaluate the potential for perchlorate biodegradation in a subsurface aquifer in the Rocky Mountains. Subsurface sediments were not available for this study, so groundwater only was used in the experiments. The Rocky Mountain groundwater was amended with a sterile stock of diammonium phosphate to provide nitrogen (5 mg/L as NH<sub>4</sub>) and phosphorus (4.5 mg/L as P) as nutrients for bacterial growth. Each 160-mL bottle received 100 mL of site groundwater. The perchlorate concentration in the groundwater was approximately 57 mg/L. Duplicate serum bottles were amended with one of the following substrates to 100 mg/L: acetate, ethanol, methanol, benzoate, lactate, sucrose, molasses or a mixture of ethanol and yeast extract (100 mg/L each). Duplicate bottles also received hydrogen or propane in the headspace as gaseous substrates. Duplicate bottles were inoculated with *Dechlorospirillum* sp. FBR2 and ethanol as electron donor. Duplicate samples were also prepared with nutrients (N and P) but no substrate, no nutrients or substrate, or substrate (acetate) without nutrients. Killed controls received acetate as substrate and formaldehyde (1%) to inhibit all biological activity. All bottles were crimp-sealed with sterilized Teflon-lined septa and incubated at 15°C to approximate *in situ* temperatures. At 6, 14, 22, and 35 days of incubation, a 15-mL subsample was removed from each bottle. Preservation of the samples was accomplished by filtration and refrigeration, as described previously.

*Influence of Co-Contaminants:* Because chlorinated solvents such as trichloroethylene (TCE) and perchloroethylene (PCE) often occur in conjunction with perchlorate in contaminated groundwater, two experiments were conducted to determine the influence of these compounds on perchlorate degradation. The influence of high concentrations of PCE and TCE was initially examined, then, in a second study, the influence of several lower concentrations of these co-contaminants was evaluated. In the initial study, the role of BTEX contamination on perchlorate reduction was also examined.

In the first study, serum bottles were amended with lactate (100 mg/L), nutrients, and one of the following co-contaminants at a starting concentration of 100 mg/L: PCE, TCE, or the mixed gasoline

constituents benzene, toluene, ethylbenzene, and xylenes (BTEX). The bottles were sealed and placed on a rotary shaker operating at 15°C. Subsamples were periodically collected and analyzed for perchlorate. In the second study, the groundwater samples were amended with lactate (100 mg/L), nutrients, and either PCE or TCE at starting concentrations of 0, 5, 10, or 25 mg/L. The initial stocks of the chlorinated solvents were prepared in site groundwater. The bottles were sealed with Teflon septa and placed on a rotary shaker operating at 15°C. Subsamples were periodically collected and analyzed for perchlorate. Initial samples were also analyzed for TCE and PCE using a gas chromatograph equipped with a flame ionization detector (GC/FID).

## Results

*Groundwater Analysis:* Groundwater from the Rocky Mountain site was collected from an existing monitoring well screened to a depth of 89 – 99 ft below grade. The water contained perchlorate at 57 mg/L, which is consistent with historical levels in the well. Other anion levels included nitrate at 5.2 mg/L (as N), sulfate at 364 mg/L, chloride at 2,500 mg/L, and 285 mg/L of alkalinity (as CaCO<sub>3</sub>). The total dissolved solids (TDS) in the groundwater was 5,000 mg/L, and the pH was 7.7. Historical data provided to Envirogen by the commercial facility showed trichloroethene in the well water between 1 and 2 mg/L, and lesser chlorinated ethenes and ethanes at trace (ppb) levels. However, no volatile organic compounds were detected in the groundwater upon analysis by Envirogen's Analytical Lab. These compounds most likely volatilized during collection and shipment of the groundwater samples. The sampling techniques were designed to ensure aseptic collection of groundwater but not quantitative preservation of *in situ* VOC levels (since perchlorate is non-volatile).

*Groundwater Microcosms:* The starting perchlorate concentration in microcosms prepared with groundwater was 57 mg/L. After 6 days of incubation at 15°C, the perchlorate concentrations in samples augmented with exogenous perchlorate degrading bacteria (*Dechlorospirillum* sp. FBR2) had decreased to 15 mg/L. Perchlorate levels did not decline in any of the other treatments. After 14 days of incubation, perchlorate levels were below detection (PQL; 0.5 mg/L) in the FBR2-inoculated microcosms and in microcosms amended with sucrose, lactate, and molasses (Table 3). In microcosms amended with both ethanol and yeast extract, perchlorate levels had decreased to 1 mg/L after 14 days, and were non-detect (PQL; 0.5 mg/L) after 22 days. In microcosms receiving acetate, perchlorate levels declined to 31 mg/L after 14 days. After 35 days, perchlorate levels in the acetate bottles were less than 0.5 mg/L. However, no perchlorate loss was observed in microcosms prepared with acetate as electron donor but without nutrients (supplemental nitrogen and phosphorus), indicating that phosphorus may be a limiting nutrient for microbial growth in the groundwater. Nitrogen is probably not limiting because high levels of nitrate

are present in the water. No perchlorate loss was observed in those microcosms amended with hydrogen, benzoate, ethanol, methanol, or propane as electron donors. No perchlorate loss was evident in samples that were treated with formaldehyde to inhibit biological activity, nor in samples that were prepared with nutrients only (no substrate) or those receiving no nutrient or substrate addition.

*Influence of Co-Contaminants.* In the first study, perchlorate levels in samples that did not receive co-contaminants declined from 52.7 mg/L to 1.7 mg/L during the initial 15 days of incubation and were below detection by day 29 (Figure 15). In samples that received TCE at 100 mg/L, perchlorate degradation was slightly retarded compared to the samples without the co-contaminant, but perchlorate was also below detection by day 29. Conversely, during the 29-day study, samples receiving either BTEX or PCE showed no degradation of perchlorate.

In the second study, perchlorate levels in samples that did not receive co-contaminants declined from 52 mg/L to 19 mg/L during the initial 15 days of incubation and then to 0.9 mg/L by day 22. Interestingly, perchlorate levels in samples receiving TCE at 10 or 25 mg/L were all below detection (< 0.025 mg/L) after 15 days of incubation (Figure 16). Samples treated with TCE at 5 mg/L showed perchlorate levels of 10 mg/L after 15 days and were below detection for perchlorate after 22 days. Thus, in this study, TCE appeared to stimulate the rate of perchlorate degradation in groundwater samples. Analysis of TCE after 15 days of incubation showed no significant losses of the solvent in sample bottles. The mean levels of TCE in bottles initially receiving 5, 10, and 25 mg/L were 6, 12, and 26 mg/L after 15 days. Conversely, perchlorate degradation was somewhat inhibited in groundwater samples with PCE (Figure 17). After 15 days of incubation, perchlorate was detected at 36 mg/L in samples which were initially treated with 10 mg/L PCE and at 46 mg/L in samples receiving 25 mg/L PCE, compared to 10 mg/L in samples with no PCE. By 22 days, samples with 25 mg/L PCE still had more than 30 mg/L perchlorate, while all others were less than 1 mg/L. PCE levels in the sample bottles after 15 days were appreciably reduced compared to initial levels. Bottles initially receiving 5, 10, and 25 mg/L PCE showed 3, 3, and 9 mg/L, respectively, after 15 days. The method of analysis (GC/FID) did not allow accurate detection of PCE biodegradation daughter products (e.g., *cis*-DCE) and killed controls at each PCE level were not prepared, so it is unclear whether PCE was biologically degraded or lost due to volatilization or by another abiotic process from the samples.

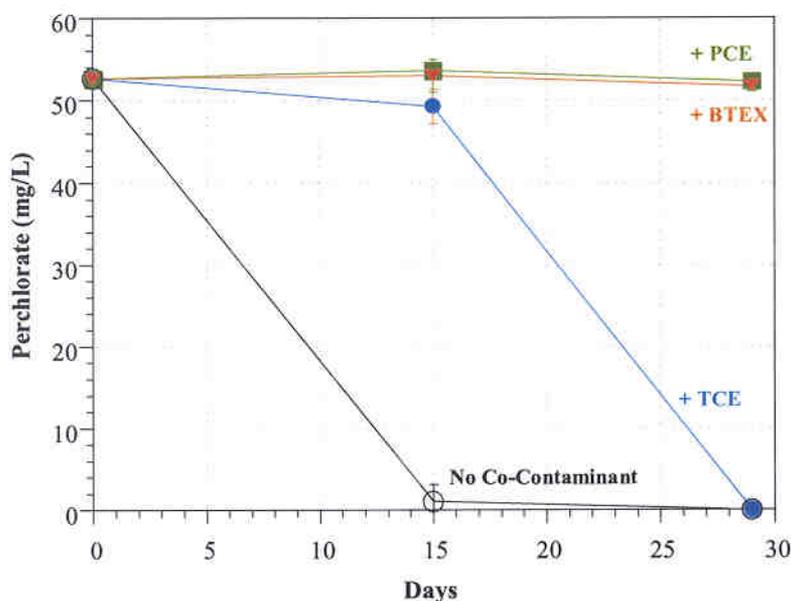
**Table 3. Perchlorate Degradation in Groundwater Microcosms from the Rocky Mountain Site.**

Treatment	Perchlorate Concentration (mg/L) <sup>1</sup>				
	Day 0	Day 6	Day 14	Day 22	Day 35
<i>Electron Donors</i>					
Killed	57 ± 2	60 ± 2	53 ± 2	60 <sup>3</sup>	55 <sup>3</sup>
No Addition	57 ± 2	60 ± 1	53 ± 1	53 ± 2	54 ± 1
Nitrogen/Phosphorus only	57 ± 2	62 ± 5	55 ± 1	59 ± 1	55 ± 2
Hydrogen	57 ± 2	61 ± 1	63 ± 10	52 ± 1	54 ± 1
Propane	57 ± 2	62 ± 0	66 ± 1	49 ± 0	53 ± 0
Benzoate	57 ± 2	62 ± 2	62 ± 1	49 ± 3	48 ± 2
Ethanol	57 ± 2	59 ± 3	63 ± 2	51 ± 0	43 ± 1
Methanol	57 ± 2	62 ± 2	63 ± 1	46 ± 4	47 ± 6
Acetate (no N or P)	57 ± 2	60 ± 5	54 ± 0	59 ± 1	49 ± 1
Acetate	57 ± 2	62 ± 1	31 ± 6	2 ± 2	< 0.5
Yeast Extract/Ethanol	57 ± 2	60 ± 0	1 ± 1	< 0.5	NS <sup>2</sup>
Lactate	57 ± 2	60 ± 1	< 0.5	< 0.5	NS
Molasses	57 ± 2	59 ± 1	< 0.5	< 0.5	NS
Sucrose	57 ± 2	61 ± 1	< 0.5	< 0.5	NS
<i>Bioaugmentation</i>					
Culture FBR2 + Ethanol	57 ± 2	15 ± 1	< 0.5	< 0.5	NS

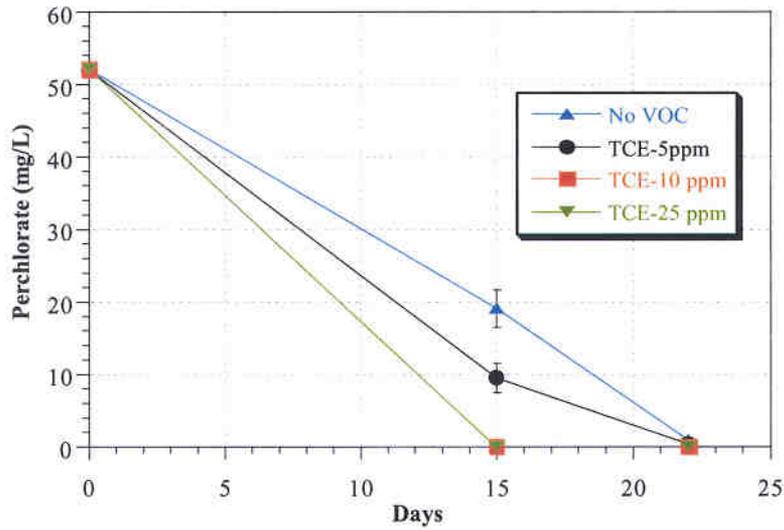
<sup>1</sup> Values are the means and standard deviations from duplicate microcosms.

<sup>2</sup> NS: Not sampled because perchlorate was previously below detection.

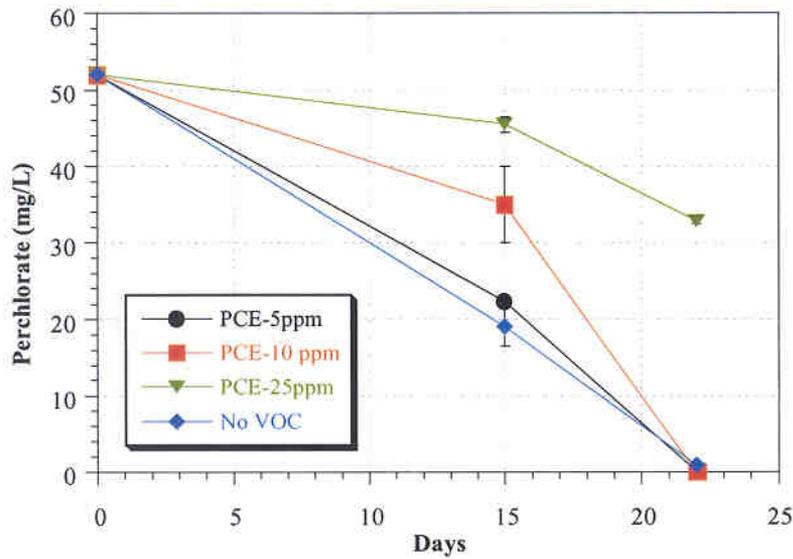
<sup>3</sup> Single analysis due to broken sample bottle.



**Figure 15. Influence of Co-Contaminants on the Biodegradation of Perchlorate in Groundwater from the RM Site.**



**Figure 16. Influence of Varying Concentrations of Trichloroethene (TCE) on Perchlorate Biodegradation in Groundwater Microcosms from the RM Site.**



**Figure 17. Influence of Varying Concentrations of Perchloroethene (PCE) on Perchlorate Biodegradation in Groundwater Microcosms from the RM Site.**

## **Conclusions**

Bioaugmentation with *Dechlorospirillum* sp. FBR2 caused the most rapid reduction in perchlorate levels in the Rocky Mountain samples. However, addition of some substrates (lactate, molasses, sucrose, yeast extract/ethanol) also promoted perchlorate biodegradation by indigenous bacteria. Other substrates that yielded rapid perchlorate biodegradation at the JPL site or the IHDIV Building 1190 Site, such as ethanol (JPL) and hydrogen gas (IHDIV), did not stimulate biodegradation of the anion at this site. Differences in the indigenous populations of perchlorate degrading bacteria at each site may account for the observed differences among sites in substrate effectiveness for perchlorate bioremediation.

The observed inhibition of perchlorate degradation by high concentrations of PCE and BTEX most likely reflects toxicity of these compounds on the perchlorate degrading strains in the RM groundwater. It is however, interesting that TCE appeared to be less toxic at 100 mg/L than PCE, since the former is more soluble and solvent toxicity often increases with solubility. An initial screening suggested that neither TCE or PCE were appreciably degraded during the course of the experiment. In the second study with lower solvent concentrations, PCE again inhibited perchlorate reduction, with the extent of inhibition varying directly with concentration. This likely reflects toxicity of the chlorinated solvent to perchlorate degrading bacteria.. It is also possible, although unlikely, that some perchlorate degrading strains also use PCE as an electron acceptor and that the observed inhibition reflects competition between the two electron acceptors. More detailed experiments are required to evaluate this possibility. Unlike PCE, TCE appeared to stimulate perchlorate degradation in the second study in a dose-dependent manner, even though the solvent was not apparently degraded. The reason for this effect is unclear, and requires additional experimentation. These studies are important because chlorinated solvents, including PCE and TCE, are often found with perchlorate at contaminated field sites.

### **4.3.4 LONGHORN ARMY AMMUNITION PLANT, KARNACK, TEXAS**

#### **4.3.4.1 Sample Collection**

Aquifer sediments and groundwater containing perchlorate were collected from two locations (Site 16, Site 25G) at the Longhorn Army Ammunition Plant (LHAAP) in Karnack, Texas. A Geoprobe rig was used for collection of sediments, and groundwater was taken from existing monitoring wells at the site. The samples were used for the final set of microcosm studies to be conducted during this project using natural sediments. Sediments from one of these locations are also being used for initial flow-through column studies. In addition, samples of a contaminated surface soil (Site 25C) at LHAAP were taken for testing. A total of seven different aquifer samples were collected from five sites across the country during the microcosm testing for this project.

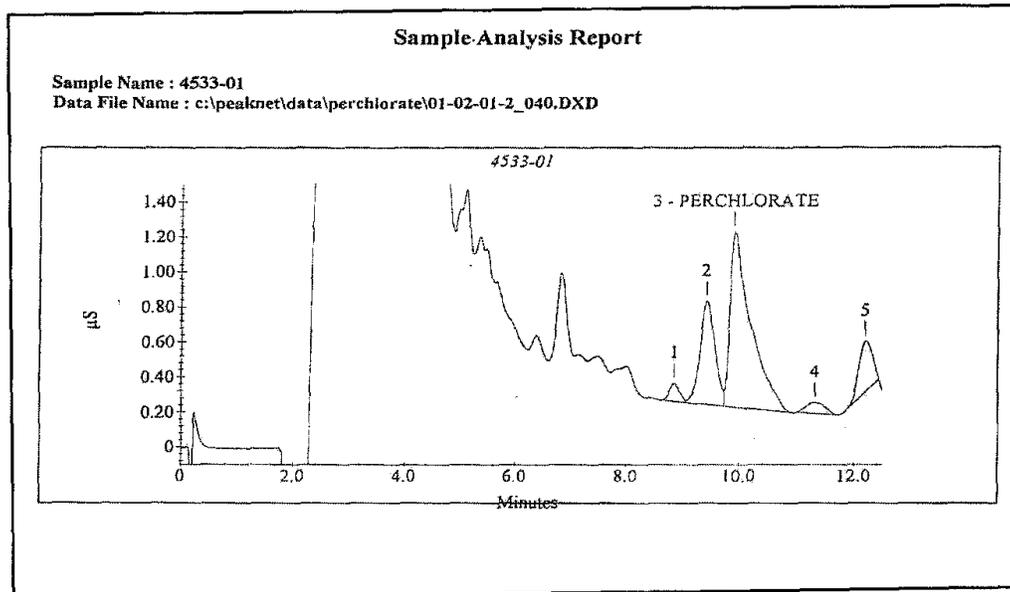
#### 4.3.4.2 LHAAP Site 16 –Landfill Leachate

##### Methods

Groundwater and sediment samples were collected from Site 16, which is a contaminated groundwater plume that is downgradient from a capped landfill at LHAAP. Sediments were taken from 22 – 26' below land surface using a Geoprobe rig and groundwater was collected from an existing monitoring well (EW-1). The groundwater at the site is bright yellow due to the presence of a tricarbonyl iron compound [(tetrahydrocyclopentadienone)tricarbonyliron(oxide)] leaching from the landfill. The initial concentration of perchlorate in the site water was approximately 0.7 mg/L and nitrate was present at 1.0 mg/L. Sulfate was present at 1600 mg/L, chloride at 810 mg/L, the alkalinity was 350 mg/L, and the pH was 6.7. To evaluate the influence of different electron donors on perchlorate degradation, microcosms were prepared in an anaerobic chamber using 30 g of sediment and 100 mL of site groundwater in 160-mL serum bottles. Duplicate bottles received acetate, ethanol, benzoate, molasses, lactate or soybean oil at 20 mg/L. The bottles also received diammonium phosphate to provide approximately 4 mg/L NH<sub>3</sub> as N and 5 mg/L PO<sub>4</sub> as P. Duplicate bottles were amended with acetate and 1% formaldehyde to inhibit all microbial activity, and duplicate bottles received no substrate or nutrients. The bottles were incubated at 15°C on a rotary shaker, and subsamples were periodically collected for perchlorate analysis by ion chromatography.

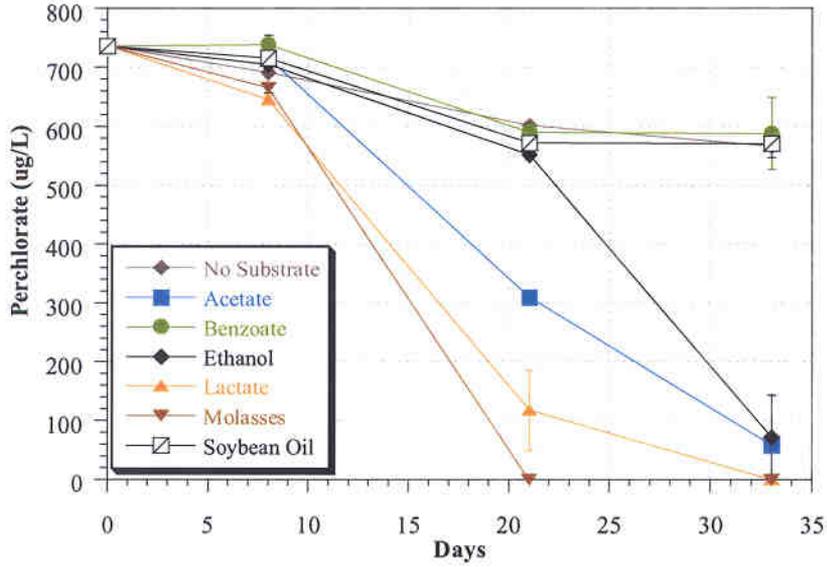
##### Results

Perchlorate analysis in the Site 16 samples proved to be difficult, particularly in samples showing appreciable biodegradation, due to the presence of one or two interfering ions (Figure 18). One of these compounds may be the tricarbonyl iron molecule causing the yellow color of the water. In some instances, the perchlorate ion and a second ion did not separate sufficiently during ion chromatography to allow accurate detection of perchlorate. The water was passed through several filters, including barium, silver, and humic acid filters, but these did not remove the competing ions or improve detection (the humic acid filter actually removed perchlorate at low concentrations). During some sample runs, depending on column pressure and other factors, the perchlorate could be sufficiently separated from the second peak to allow accurate detection. In instances where perchlorate could not be confirmed, samples were reanalyzed until accurate analytical results were obtained

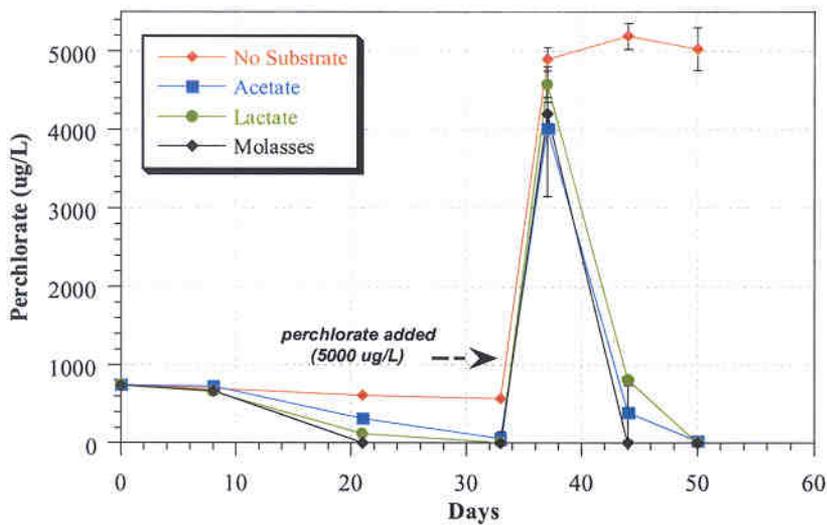


**Figure 18. Ion Chromatograph of Perchlorate and Interfering Anions in LHAAP Site 16 Groundwater.**

The average concentration of perchlorate in the samples at time 0 was 0.74 mg/L and nitrate was 0.96 mg/L. Nitrate was degraded in samples receiving all substrates except benzoate to near or below detection (0.1 mg/L) during the initial 8 days of the study. No loss of perchlorate was observed during this time. After 21 days of incubation, however, samples receiving molasses, lactate, and acetate showed appreciable perchlorate degradation (Figure 19). Microcosms amended with ethanol also showed perchlorate losses by day 33. Soybean oil and benzoate did not enhance perchlorate biodegradation at this site. On day 36, sample bottles with acetate, molasses, lactate, and no substrate were spiked with additional perchlorate to a concentration of approximately 5 mg/L to confirm that perchlorate degradation was occurring. Perchlorate in the bottles without substrate remained near 5 mg/L. However, those with the three substrates added declined to below detection by day 50 (14 days after perchlorate addition) (Figure 20). Thus, perchlorate biodegradation by indigenous bacteria was stimulated in the Site 16 samples using several substrates.



**Figure 19. Influence of Different Electron Donors on Perchlorate Levels in LHAAP Aquifer Microcosms.**



**Figure 20. Biodegradation of Perchlorate in LHAAP Microcosms after Respiking with Perchlorate.**

#### 4.3.4.3 LHAAP Site 25 – Propellant Mixing Facility

##### Methods

Site 25G at LHAAP is near a former perchlorate mixing facility. Soils and groundwater in the vicinity are heavily contaminated with the propellant. Sediment samples were collected by Geoprobe from a depth of 12 – 16 feet bls. Groundwater was from a nearby monitoring well (LHS-MW6C). The initial concentration of perchlorate in a slurry of the sediment and groundwater was 77 mg/L, nitrate was 1.0 mg/L, sulfate was 280 mg/L, chloride was 140 mg/L, the alkalinity was 51 mg/L, and the pH was 5.73. Microcosms were set up and incubated as described previously for Site 16 using ethanol, acetate, lactate, and molasses as substrates. The starting concentration of each was 200 mg/L. No interfering anions were observed in the Site 25G samples. Unlike Site 16, perchlorate was not degraded in any of the Site 25G microcosm bottles during the 33-day incubation period of the study (Table 4). Nitrate, however was degraded from 1 mg/L to less than 0.2 mg/L in all microcosms receiving ethanol, lactate, and molasses (but not acetate) after only 8 days of incubation. There are numerous differences in geochemistry between the two locations at LHAAP that could cause the difference in perchlorate degradation between the sites. However, one factor previously observed to be inhibitory to perchlorate degradation in samples from the Indian Head Division Naval Surface Warfare Center (IHDIV) was low pH. Although the pH in the Site 25G samples is not particularly low, and is appreciably higher than at IHDIV (5.7 vs 4.3, respectively), an experiment was conducted to determine whether pH adjustment could be used to enhance perchlorate degradation in the samples.

##### Results

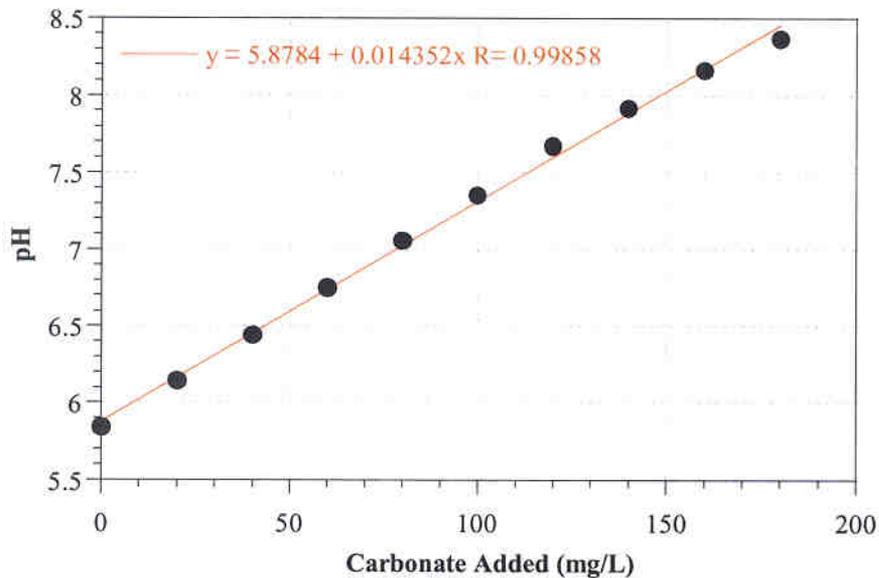
Initially, a titration with carbonate was performed using slurries of groundwater and sediment from site 25G to determine the amount of alkalinity required to increase the pH of the samples to 7. These data are shown in Figure 21. On day 36 of the microcosm study, individual microcosm bottles with ethanol and lactate were amended with carbonate to provide a final pH of 7.2. The duplicate sample microcosm remained at pH 5.7. After 24 days of additional incubation at 15°C (day 60 of the study), the perchlorate in the bottle with ethanol at pH 7.2 declined to 1 mg/L and then to 0.024 mg/L by day 33 (day 69 of the study) (Figure 22). The microcosm with lactate that was brought to a pH of 7.2 also showed appreciably decline in perchlorate by day 33 (< 5 mg/L). Unlike the pH-adjusted samples, no significant losses of perchlorate were observed in the microcosms with ethanol or lactate that remained at a pH of 5.7. Thus, the data clearly show that even a slightly acidic pH (i.e. 5.7), can completely inhibit perchlorate biodegradation in subsurface environments.

An additional study was conducted with Site 25G samples in which the pH was adjusted to 7.2 or left at 5.7, and the samples received one of two pure cultures of perchlorate degrading bacteria isolated during this project (*Dechlorosoma suillum* JPLRND or *Dechlorospirillum* sp. FBR2). Duplicate sample bottles received 15 g of sediment, 30 mL of site water, and one of the perchlorate degrading strains. Carbonate (4 mg) was added to adjust the pH to 7.2 in some samples, and all bottles were incubated at 15°C. In bottles brought to pH 7.2, perchlorate was completely degraded by the augmented strains after 5 days for JPLRND and 15 days for FBR2 (Figure 23). Conversely, no perchlorate degradation was observed at pH 5.7 in any of the samples.

**Table 4. Perchlorate Degradation in Sediment/Groundwater Microcosms from LHAAP Site 25G.**

<i>Electron Donors</i>	Perchlorate Concentration (mg/L) <sup>1</sup>			
	<i>Day 0</i>	<i>Day 8</i>	<i>Day 21</i>	<i>Day 33</i>
Killed Control (Acetate)	77 ± 0	72 ± 0	80 ± 0	79 ± 1
No Addition	77 ± 0	74 ± 2	81 ± 1	82 ± 4
Nutrients Only	77 ± 0	75 ± 1	80 ± 1	80 ± 3
Acetate	77 ± 0	73 ± 1	81 ± 1	78
Ethanol	77 ± 0	74 ± 0	82 ± 0	85 ± 8
Lactate	77 ± 0	74 ± 1	80 ± 1	85 ± 9
Molasses	77 ± 0	72 ± 0	80 ± 1	77 ± 3

<sup>1</sup> Values are the mean ± standard deviation from duplicate microcosms.



**Figure 21. Carbonate Titration Curve for Sediment Slurries from LHAAP Site 25G.**

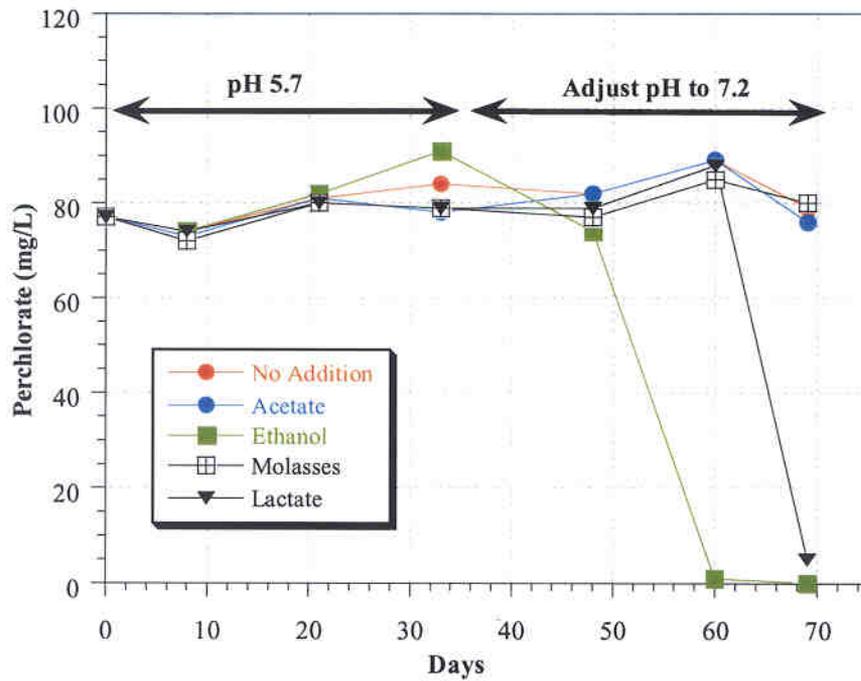


Figure 22. Influence of pH Adjustment on Perchlorate Biodegradation in LHAAP Site 25G Aquifer Microcosms.

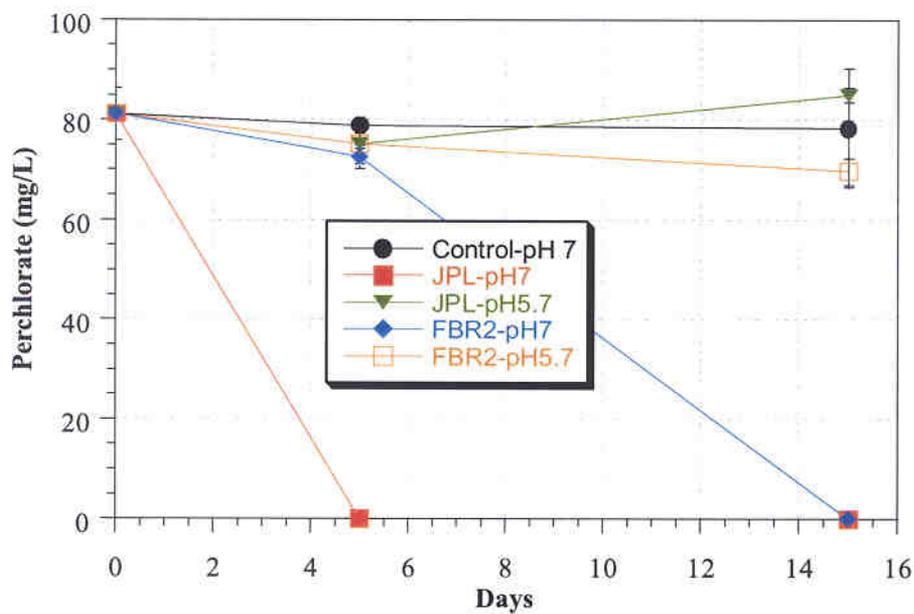


Figure 23. Influence of pH and Bioaugmentation (*Dechlorospirillum* sp. FBR2 or *D. suillum* JPLRND) on Perchlorate Biodegradation in LHAAP Site 25G Microcosms.

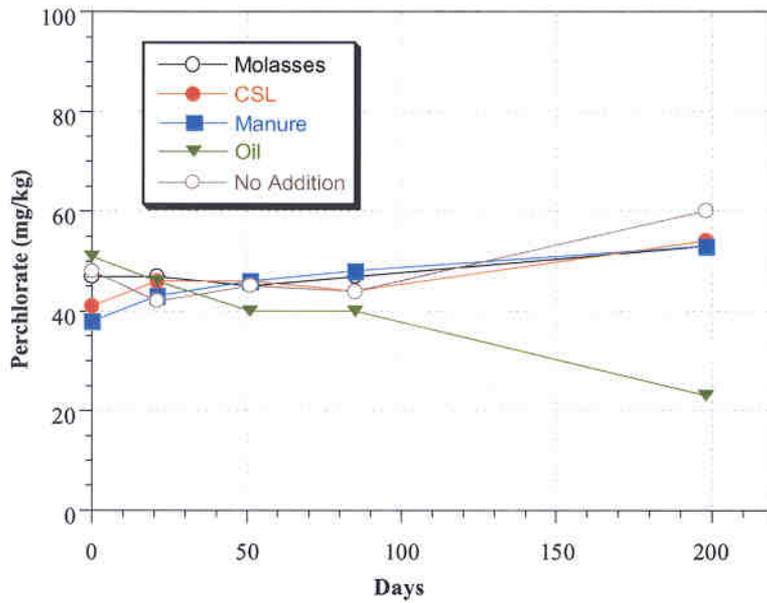
#### 4.3.4.4. LHAAP Site 25C – Biodegradation of Perchlorate in Surface Soils

##### Methods

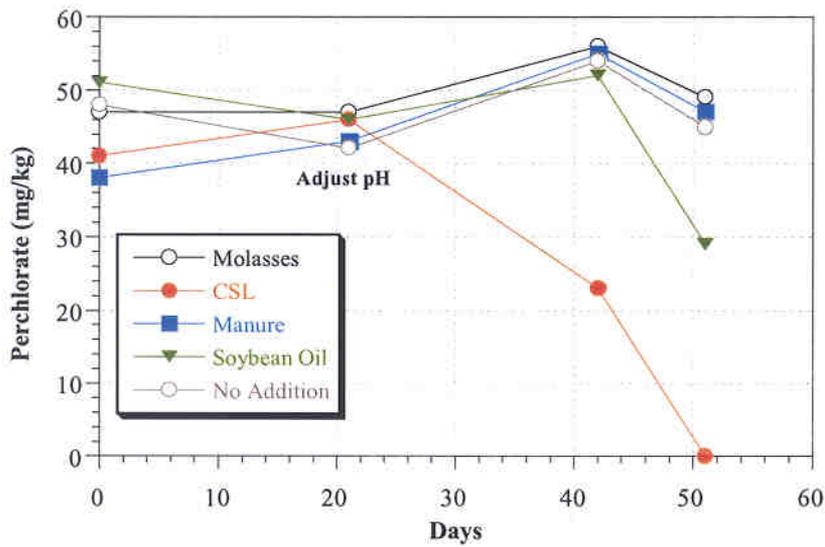
A surface soil was collected from Site 25C at LHAAP from an area with known perchlorate contamination in the high mg/kg range. The surface soils taken only had approximately 1 mg/kg perchlorate upon laboratory testing, so they were spiked with additional perchlorate to an initial level of about 50 mg/kg. The soil was homogenized and 500-g portions were placed in mason jars (1 qt) and amended with bulk substrates including corn-steep liquor, molasses, horse manure, and soybean oil at 1000 mg/kg. A control was prepared by adding mercuric chloride and sodium azide to inhibit microbial activity. The bottles were sealed and incubated at 15°C without shaking. Five-gram subsamples were removed from the bottles periodically in an anaerobic chamber. The samples were extracted by shaking for 1 hour with water, centrifuged to remove solids, and analyzed for perchlorate by IC.

##### Results

Perchlorate degradation was not observed in any of the surface soil samples from Site 25C during more than 80 days of incubation (Figure 24). An additional set of samples were collected after 198 days, and some perchlorate biodegradation was evident (levels reduced to 23 mg/kg) in samples amended with vegetable oil, but not in those with the other electron donors or samples with no electron donor added. The pH of the surface soil was measured at 4.8. Thus, as was observed previously with two sets of aquifer samples, it appears that acidic pH is inhibitory to perchlorate reduction in this surface soil. To test this hypothesis, 200-g subsamples of soil were removed from each sample jar after 21 days of incubation, and the pH in these samples was adjusted to neutrality using lime (calcium carbonate). As with site 25G, a titration was conducted to determine the lime required for appropriate pH adjustment. The increase in pH stimulated perchlorate biodegradation in the samples receiving corn-steep liquor after 20 days and perchlorate was below detection in the soil by 31 days (Figure 25). Soybean oil also stimulated perchlorate degradation in the pH-adjusted soil much more rapidly than in the acidic soil.



**Figure 24. Perchlorate Levels in Surface Soil from LHAAP Site 25C After Amendment with Different Electron Donors.**



**Figure 25. Influence of pH Adjustment on Perchlorate Biodegradation in LHAAP Site 25C Soil Samples Amended with Different Electron Donors.**

## **Conclusions**

The most important finding of the LHAAP microcosm studies is that even a slightly acidic pH (i.e. 5.7) can significantly or completely inhibit perchlorate degradation in site samples, regardless of electron donor addition or bioaugmentation. These results confirm initial findings at the IHDIV site in Maryland and clearly show the importance of evaluating the potential for perchlorate biodegradation using a variety of samples with differing geochemistries. If all samples tested during this project were from the western States where groundwater is usually neutral, this pH effect would not have been observed. The reason that an acidic pH inhibits perchlorate reduction is unclear. It is obvious from all data gathered at this time that perchlorate reducing strains are indigenous in acidic aquifers and soils. It is possible that the perchlorate reductase enzyme or another enzyme in the reduction pathway is inhibited at acidic pH values. Such inhibition could lead to a complete absence of perchlorate degradation (if perchlorate reductase is inhibited) or to the release of a toxic partial degradation product (e.g., chlorite) if a later enzyme is inhibited. This finding may also reflect a less obvious effect of low pH, such as increased solubility and toxicity of metals. Additional pure culture studies are required to better understand the influence of pH on perchlorate degradation.

### ***4.3.5 BOEING CORPORATION, SACRAMENTO, CA***

In the first year of this project, microcosm studies were conducted with samples from several different sites, including the Indian Head Naval Surface Warfare Center (MD), the Longhorn Army Ammunition Plant (TX), Rocky Mountain site (UT), and Jet Propulsion Laboratories (CA) (see previous sections). In the second year, studies focused primarily on flow-through model aquifers, pure cultures, and modeling. However, one additional set of site samples were collected for evaluation of perchlorate biodegradation. These samples were from the Boeing site near Sacramento, CA. The objective of these studies was to determine if perchlorate biodegradation could be stimulated in samples containing extremely low concentrations of perchlorate and without appreciable nitrate. In some instances, bacteria are known to have thresholds below which they will not biodegrade organic substrates (Alexander, 1994). It is unclear whether this threshold may also occur for electron acceptors such as perchlorate. Because there are many perchlorate plumes with concentration levels less than 100 µg/L that may have to be reduced further to less than 4 µg/L, a study was undertaken to evaluate the feasibility of biostimulation for perchlorate treatment at low initial concentrations.

#### **4.3.5.1 Sample Collection**

Groundwater samples were collected from the Boeing site into sterile 1L jars from two different contaminated wells and sent to Envirogen on ice. The starting concentration of perchlorate in these samples was 49.8 µg/L (Well 58) and 102 µg/L (Well 44A). Nitrate levels were less than 1 mg/L for each well. During sample collection, the pH of groundwater from well 44A was 7.0, and the redox was -125 mV. The pH of groundwater from well 58 was 6.7, and the redox was -150 mV. Dissolved oxygen was below detection in each well.

#### **4.3.5.2 Microcosm Studies to Evaluate Biodegradation of Low Perchlorate Concentrations and the Influence of Different Substrates on Sulfate Reduction**

##### **Methods**

Groundwater from each well was added in 120-mL volumes to sterile 160-mL serum bottles in a Coy Environmental Chamber (nitrogen headspace). Duplicate samples were then amended with acetate, molasses, or lactate at 20 mg/L each or hydrogen gas (5 mL in headspace). Microcosms were also prepared without added substrate or with 1% formaldehyde (killed controls). Bottles were incubated at 15°C with shaking, and 20-mL subsamples were collected after 9 and 21 days. Samples were filtered, then analyzed for perchlorate (EPA 314.0) and sulfate (EPA 300.0).

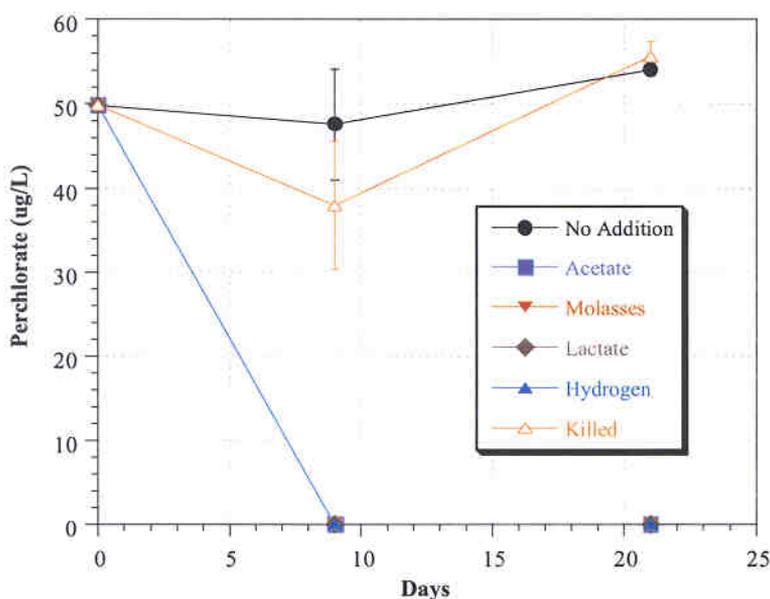
##### **Results**

The perchlorate levels in groundwater samples from Well 44A were below 8 µg/L in all bottles that received a substrate (acetate, hydrogen, lactate or molasses) after 9 days of incubation. (Figure 26). The concentration in samples without added substrate was 48 µg/L, and that in the killed controls was 38 µg/L after this time. The level of sulfate in all bottles was near the starting concentration of 9 mg/L. By 21 days, all samples with substrate added had perchlorate below 4 µg/L. The sulfate levels were below detection (< 0.4 mg/L) in bottles with molasses and lactate, and had dropped to 5.7 mg/L in samples with hydrogen. The formation of iron sulfide (black precipitate) was also apparent in these samples. Thus, perchlorate biodegradation preceded sulfate reduction in all samples. Interestingly, however, samples amended with acetate showed no sulfate reduction; levels remained near 9 mg/L, and were similar to levels in microcosms without added substrate and in killed controls.

Perchlorate reduction in samples from Well 58 was somewhat variable among replicate microcosms at day 9. For example, in duplicate microcosms with molasses, perchlorate in one bottle was below detection, while that in the second was nearly 100 µg/L. However, by day 21, perchlorate was below 4 µg/L in all sample bottles with organic substrate added. Perchlorate levels in the bottles with

hydrogen averaged 6.2  $\mu\text{g/L}$ . The starting sulfate concentration in samples from this well was approximately 2.5 mg/L. After 21 days of incubation, sulfate in all samples with molasses, lactate, and hydrogen was below detection ( $< 0.4$  mg/L), while that in samples with acetate remained at 2.5 mg/L. There was also no sulfate reduction in samples without added substrate or in killed controls.

After incubation for 75 days, aqueous samples were taken from each bottle and sulfate levels were determined. In well 44A, average sulfate levels were 8.7 mg/L and 9.0 mg/L in samples without electron donor and in killed controls, respectively. These levels were near the initial level of 8.5 mg/L. In samples amended with molasses, lactate, and hydrogen, sulfate levels were less than 0.4 mg/L and samples were black, showing that appreciable sulfate reduction had occurred. Conversely, sulfate levels in bottles amended with acetate averaged 7.4 mg/L. Thus, some sulfate was probably reduced in these samples (about 1 mg/L) based on control values, but the extent of this process was much less than observed with other substrates. In samples from well 58, the starting sulfate concentration was 2.7 mg/L. There was no appreciable loss of sulfate in killed controls (2.7 mg/L sulfate) or those without electron donor added (3.0 mg/L sulfate) after 75 days. The average sulfate was below detection ( $< 0.2$  mg/L) in samples receiving hydrogen, lactate, and molasses. The final sulfate concentration in acetate-amended samples was 0.65 mg/L. Thus, about 2 mg/L sulfate was reduced in these samples. Although there was some sulfate reduction in acetate-amended samples over 75 days of incubation, the data suggest that acetate is a much poorer substrate for sulfate-reducing bacteria than the others tested. Sulfate reduction is not a desired side effect in drinking water aquifers. These data suggest that acetate may be one substrate that effectively stimulates perchlorate reduction but does not yield reduction of sulfate.



**Figure 26. Biodegradation of Low Concentrations of Perchlorate in Groundwater Microcosms from Boeing Corp.**

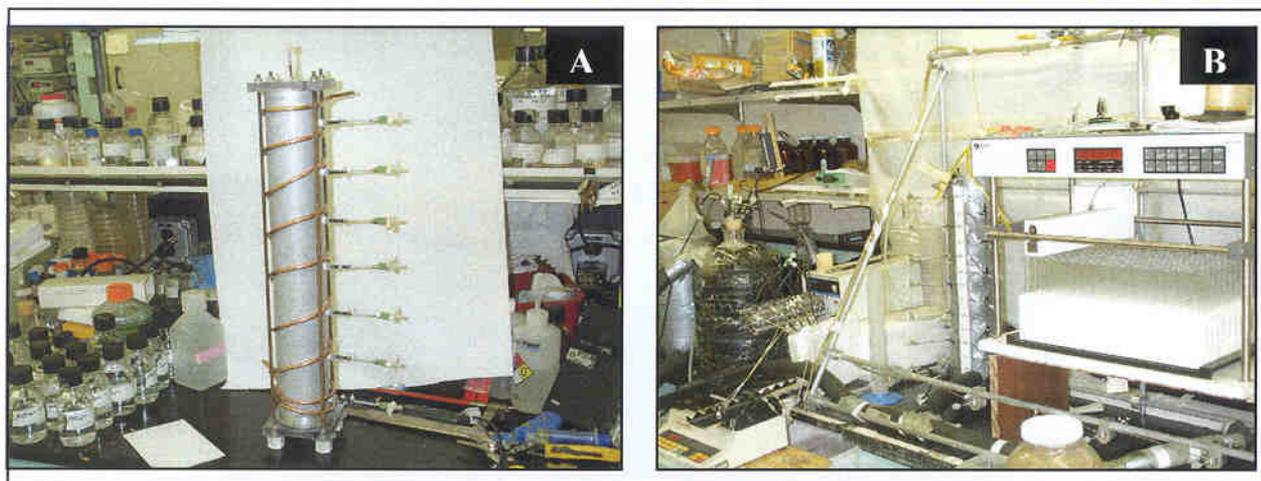
## Conclusions

Although there is now appreciable data from this and the two companion SERDP projects showing that perchlorate reduction can be stimulated in the subsurface by addition of various electron donors, the results of this study using groundwater samples from Boeing Corp. are significant for two reasons. First, these are the first data showing that perchlorate treatment to less than 4 µg/L is possible from starting concentrations as low as 50 µg/L using biostimulation. Second, the data reveal that lactate, molasses, and hydrogen support rapid and appreciable sulfate reduction after perchlorate is biodegraded, but that sulfate reduction is much less prevalent with acetate as a substrate. The formation of hydrogen sulfide from sulfate reduction may not be an acceptable endpoint from *in situ* perchlorate treatment, particularly in drinking water aquifers. These data suggest that acetate may be a better choice than several other substrates for perchlorate bioremediation.

## 4.4 EVALUATE PERCHLORATE BIODEGRADATION AND TRANSPORT IN PILOT-SCALE MODEL AQUIFERS

### 4.4.1 MODEL AQUIFER CONSTRUCTION

A flow-through model system better approximates *in situ* aquifer conditions than either an aqueous system or a static microcosm, and being continuous flow, inputs of perchlorate, substrates, and other groundwater constituents can be controlled and varied. A model aquifer was constructed and flow characteristics with sand and a natural subsurface sediment were tested. Two photos of the aquifer column are provided in Figure 27. The aquifer column was constructed from a stainless steel tube 50-cm long by 7.6-cm diameter (*see* Figure 28 for details). The bottom and top of the column are set in acrylic plates with ports for influent and effluent water flow. The two acrylic plates are held together with threaded rods on each corner. An aluminum diffuser plate is placed just above the influent flow port to provide mixing of water at the bottom of the column. Several experiments were conducted to determine the optimal design for the diffuser plate. The columns have sampling ports every 3.5 cm from the bottom (upward flow) which consist of an 18-gauge steel needle inserted to the center of the column through a barbed plastic fitting. Each needle is sealed with Norprene tubing to prevent leaks. Every other sample port is offset by 90 degrees. The entire column is wrapped in copper tubing over which is a layer of foam insulation. Water at 15°C is run through the copper tubing to maintain the entire column at groundwater temperature. A peristaltic pump supplies a continuous flow of groundwater from a reservoir (also temperature controlled) to the influent port at the bottom of the column. An additional syringe pump is attached in series to allow a slug of aqueous solution (containing substrate, electron acceptors, bacteria, etc.) to be independently applied to the column. The entire system is airtight so that anaerobic conditions can be generated within the column.



**Figure 27. Photographs of the 50-cm Model Aquifer Column During Construction (A) and Receiving Groundwater Flow During Tracer Studies (B).**

#### **4.4.2 EVALUATION OF INFLUENT WATER MIXING**

The column was initially packed with 3000 g of a silica quartz sand (99.4 % SiO<sub>2</sub>) of 0.45 – 0.50 mm diameter. The total pore volume of the sand column was measured at 946 mL, giving a porosity of 315 mL/kg sand. Initial bromide tracer tests were conducted to evaluate mixing of water at the bottom of the column. To conduct these studies influent water was amended with a pulse of bromide (~ 40 mg/L for 60 min) and passed through the column at a flow rate of approximately 50 mL/hr. Aqueous samples were removed with time from the sampling port 7 cm from the bottom of the column. The water was sampled from the center of the column, ½ the radius of the column, and at column outer edge, to determine the distribution of bromide (and thus water mixing) through the bottom section of the sand column. In the initial studies, the aluminum diffuser plate had 16 holes drilled at ½ the radius of the column. Thus, water entering the column was forced to distribute through these holes rather than moving in a slug up the center of the column from a single port. The initial mixing study showed that the water was traveling preferentially up the side of the column (Figure 29). The data suggested that, although the diffuser plate was fit tightly into the column, water was moving around as well as through the plate. The diffuser was sealed into the column with silicone caulk to prevent this flow path. The experiment was repeated and the results showed a much more equal distribution of bromide across the column, suggesting reasonable mixing (Figure 30). There was some lag in water flow at the edge of the column with the diffuser design, so a second diffuser with two rows of holes at 1/3 and 2/3 of the radius of the plate was tested to determine if mixing at the bottom of the column could be improved further. The results of the second diffuser were not appreciably different that the first. The mixing at the bottom of the column was deemed to be adequate for modeling work with either diffuser design.

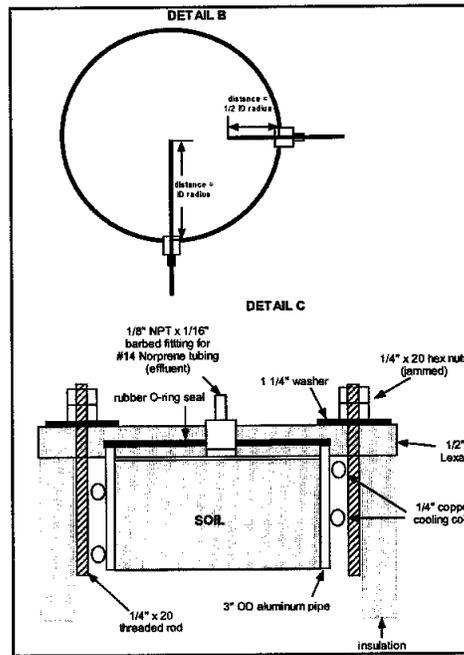
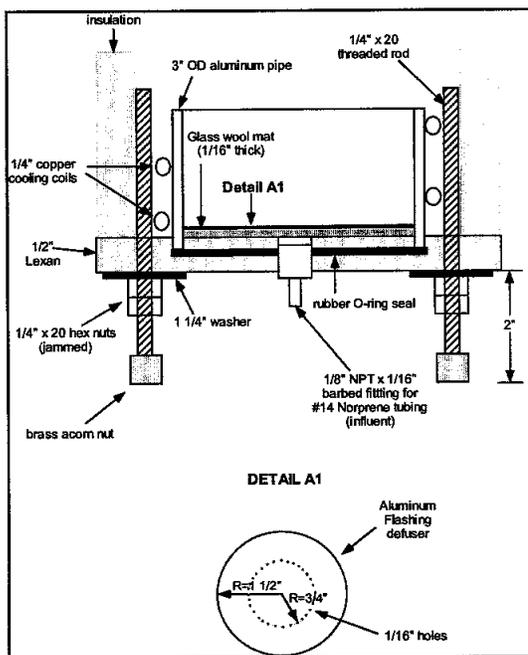
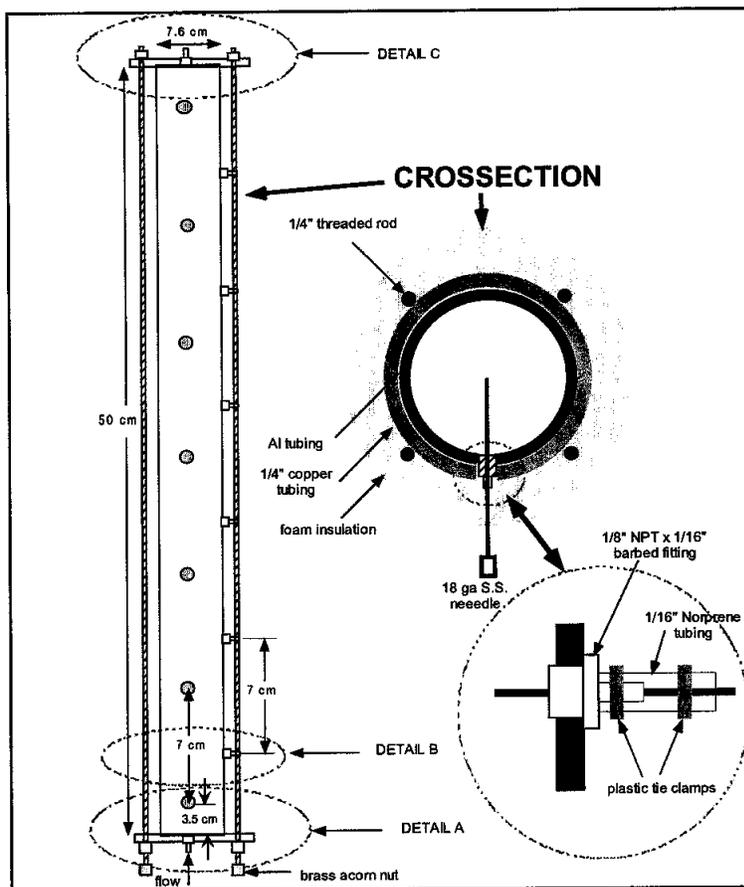
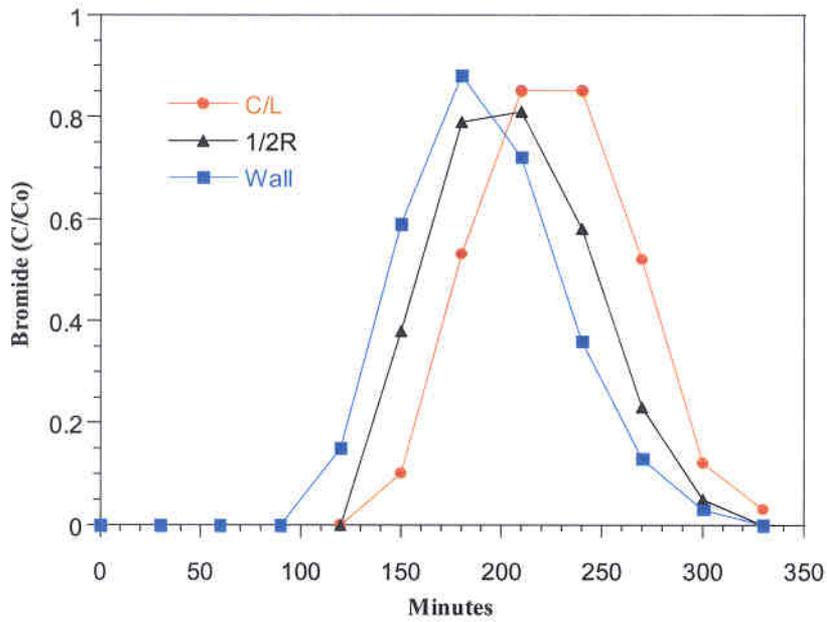
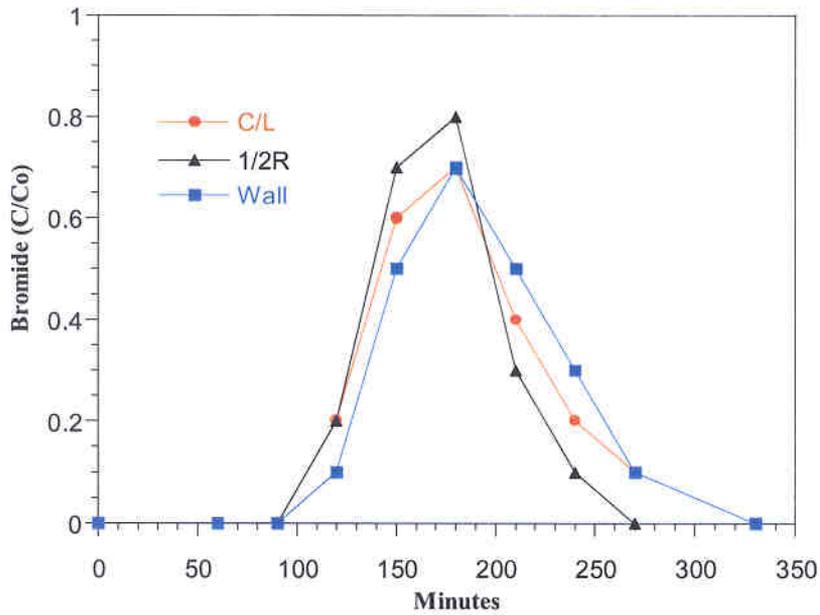


Figure 28. Design Specifications of Model Aquifer Column.



**Figure 29. Flow of Bromide Through the Model Aquifer with Unsealed Diffuser Plate. Notations are as Follows: C/L = Column Center; 1/2R = ½ Column Radius; Wall = Column Wall.**

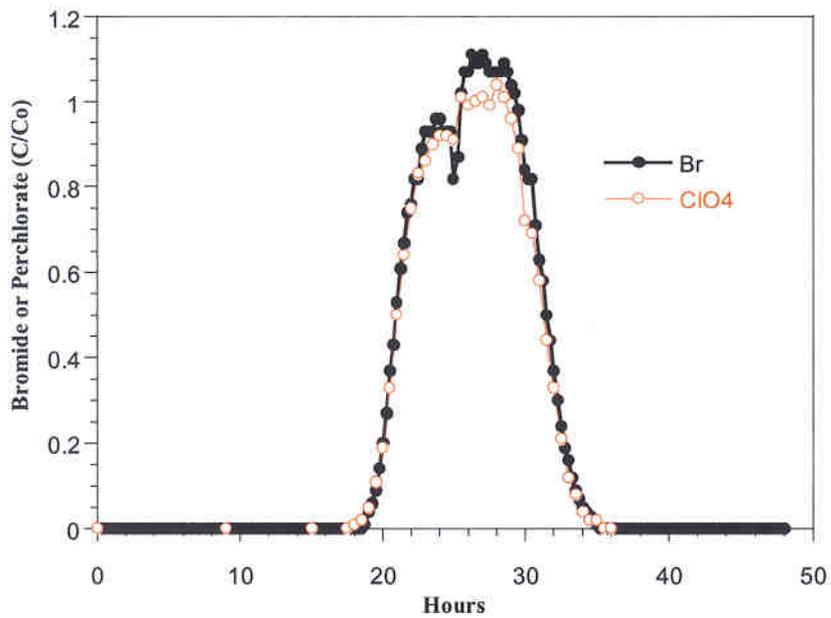


**Figure 30. Flow of Bromide Through the Model Aquifer with Sealed Diffuser Plate. Notations are as Follows: C/L = Column Center; 1/2R = ½ Column Radius; Wall = Column Wall.**

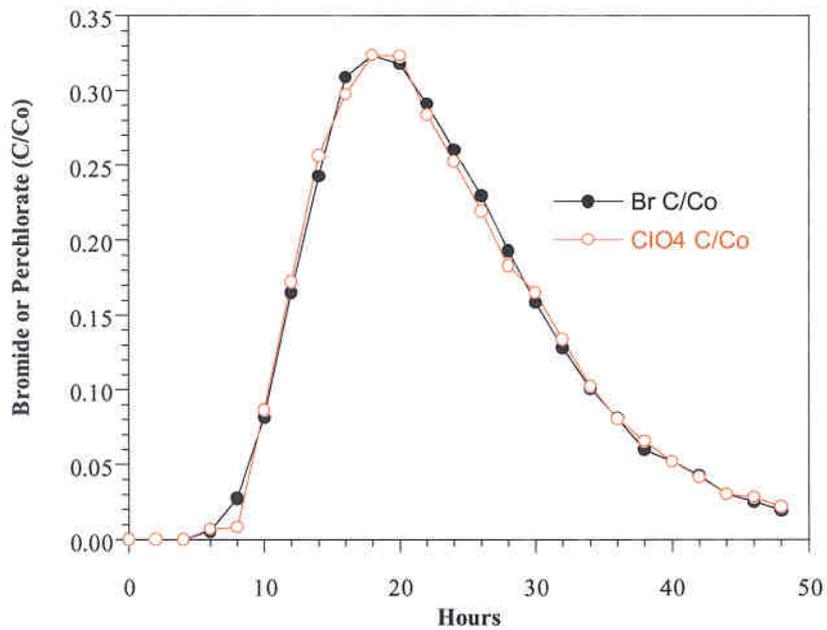
#### ***4.4.3. TRANSPORT OF PERCHLORATE THROUGH SILICA SAND AND AQUIFER SEDIMENT***

The transport of perchlorate through the sand column was tested using bromide as a conservative tracer. To conduct this study, approximately ½ pore volume (480 mL measured) of tracer containing bromide and perchlorate at 50 mg/L each was applied to the column in an artificial groundwater (AGW). The AGW recipe was designed to mimic site groundwater from a location in Virginia. The water was oxygenated to ensure that perchlorate was not biodegraded during the study. The initial pulse of perchlorate and bromide was followed by AGW only. Fractions were collected at the effluent port of the column at 15-min intervals and analyzed for both bromide and perchlorate using ion selective probes. The effluent data revealed that perchlorate and bromide moved very similarly through the sand column (Figure 31). Perchlorate breakthrough was slightly more rapid than for bromide, which may reflect exclusion of perchlorate in some small pores, as bromide is a smaller molecule. There was also a slightly higher fraction ( $C/C_0$ ) of bromide compared to perchlorate at the peak of the breakthrough curve. However, studies conducted after this tracer test revealed that the bromide probe was inaccurate (reading high) at higher bromide concentrations, even after appropriate standardization. Additional tracer experiments were analyzed by ion chromatography to verify probe results.

After the studies with silica sand were finished, the aquifer column was cleaned and packed with sediment from LHAAP Site 16. The sediments, which were collected from 16 – 26 ft bls, were removed from core liners, passed through a 4-mm sieve to remove rocks and debris, and thoroughly homogenized. The column was packed with 3258 g of field moist sediment (2891 g dry wt). The total pore volume in the column was 988 mL and the porosity was calculated as 303 mL/kg soil. An artificial groundwater was prepared based on the characteristics of water collected from EW-1 at Site 16 for use in column studies. A bromide tracer test was initially conducted to evaluate the flow characteristics of water through the LHAAP sediment core (Figure 32). This test was followed by two tracer studies to characterize the conservative transport of perchlorate through the column. In the initial test, the transport of perchlorate was measured at a flow rate of approximately 42 mL/hr. The second test was conducted at approximately half of this flow rate. As with the sand core, the tracer studies revealed that perchlorate and bromide moved in a similar fashion through the site sediments.



**Figure 31. Transport of Bromide and Perchlorate Through the Model Aquifer Column Packed with Silica Sand.**



**Figure 32. Transport of Bromide and Perchlorate Through a Model Aquifer Column Packed with LHAAP Site 16 Sediment.**

#### **4.4.4 EVALUATION OF PERCHLORATE AND NITRATE BIODEGRADATION**

After initial experiments to evaluate transport through the column, nitrate and perchlorate were added to influent groundwater and flow was initiated. For initial conditions in the column, perchlorate was added at 25 mg/L, nitrate was added at 16 mg/L, and oxygen was present at approximately 8 mg/L. These additions represent approximately equimolar quantities (0.25 mM) of each of these three electron acceptors. These values are not the same as those found naturally at LHAAP Site 16, but were used for laboratory experimentation. The other ions in the groundwater include sulfate at 1700 mg/L, chloride at 933 mg/L, calcium at 241 mg/L, magnesium at 176 mg/L, sodium at 989 mg/L. The alkalinity was 350 mg/L (as calcium carbonate), and the pH was 6.9.

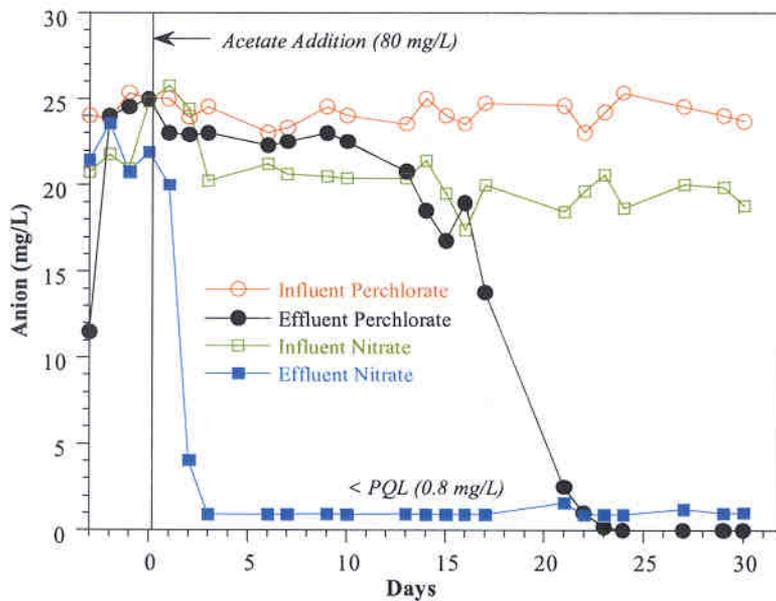
The initial groundwater flow rate to the column was set at 40 mL/hr which equals a residence time of approximately 24 hrs and a flow of 50 cm/day. Groundwater was passed through the column for 3 days without addition of acetate. The perchlorate and nitrate concentrations in the influent were the same as in the effluent water when the acetate flow was started (i.e., neither electron acceptor was degrading in the column). A separate syringe pump was used to add acetate to the influent flow line to the column. The pump was set initially to supply the electron donor at a concentration of 80 mg/L. This quantity of acetate is two times that stoichiometrically required to reduce oxygen, nitrate, and perchlorate in the feed water. The influent feed was also amended with diammonium phosphate to supply approximately 4 mg/L ammonia and 8 mg/L phosphate as inorganic nutrients.

The influent and effluent lines were sampled for perchlorate, nitrate, dissolved oxygen, and acetate 4 to 5 times per week. At least once per week, a profile was collected along the length of the column to determine the concentrations of each of the electron acceptors and acetate with distance up the column. The profile was taken by sampling points at 0, 3.5, 7, 14, 21, 28, 35, 42, and 50 cm from the bottom of the column. Because of the limited sample volume available, dissolved oxygen was analyzed using a colorimetric test kit (Chemets; Chemetrics, Inc., Calverton, VA). Nitrate was analyzed using ion chromatography (EPA 300.0) and perchlorate was analyzed using an ion-specific electrode and by ion chromatography (EPA 314.0) depending on concentration. Acetate was analyzed using gas chromatography with flame ionization detection (GC-FID). The lower detection limit for acetate was approximately 5 mg/L using this method. All water samples were passed through a 0.22-micron filter after collection. A subsample for acetate was collected from the original sample and further preserved using mercuric chloride.

The column was operated under the conditions described for a period of 48 days after acetate addition began. Levels of perchlorate and nitrate in the influent and effluent water are provided in Figure 33. The concentration of nitrate in the effluent declined from approximately 20 mg/L when the acetate

addition began to less than 1 mg/L (PQL) by day 3 after electron donor addition commenced. The nitrate remained below detection in the effluent throughout the duration of the 48-day period. The level of DO in the column influent varied between 7 and 9 mg/L. The effluent concentration prior to acetate addition was approximately 4 – 4.5 mg/L. This level declined to 2 mg/L by day 3, then to <1 mg/L by day 13. The oxygen remained at or below 1 mg/L according to the colorimetric assay. Perchlorate biodegradation began after approximately 2 weeks of operation. The level of perchlorate declined to 14 mg/L by day 17 (from an influent concentration of 25 mg/L), and was below detection by day 24 (PQL: 40 µg/L). Perchlorate remained below detection in the column effluent for the remainder of the 48-day duration of this experimental phase.

The target level of acetate in the influent water of the column was 80 mg/L. This level was expected to provide approximately 40 mg/L excess acetate based on stoichiometric calculations. Acetate measurements during the initial few weeks of column operation were not reliable because biodegradation was occurring in some of the samples during storage. Although the samples were filtered and stored at 4°C awaiting analysis, some were apparently contaminated during subsampling for perchlorate and nitrate. This problem was resolved by taking subsamples for acetate analysis at the time of collection and preserving them with mercuric chloride. When reliable data were obtained, the influent acetate levels varied from approximately 66 to 99 mg/L. When the column was in an apparent steady state on days 42 – 44, influent acetate values were 97, 99, and 95 mg/L and effluent concentrations were 23, 28, and 21 mg/L, on days 42, 43, and 44, respectively. Thus, the average acetate consumption during this period was 73 mg/L. This is approximately 30 mg/L greater than predicted from reaction stoichiometry, even taking biomass growth into account. Although there is a high concentration of sulfate in the groundwater (1700 mg/L), sulfate reduction was not occurring in the column based on periodic sulfate measurements and based on odor (no hydrogen sulfide was detected during sampling). Greater than expected acetate consumption has also been noted in previous serum bottle studies as well as in studies with fluidized bed reactors when nitrate and perchlorate were present. The reason for the extra consumption of acetate is not yet clear.



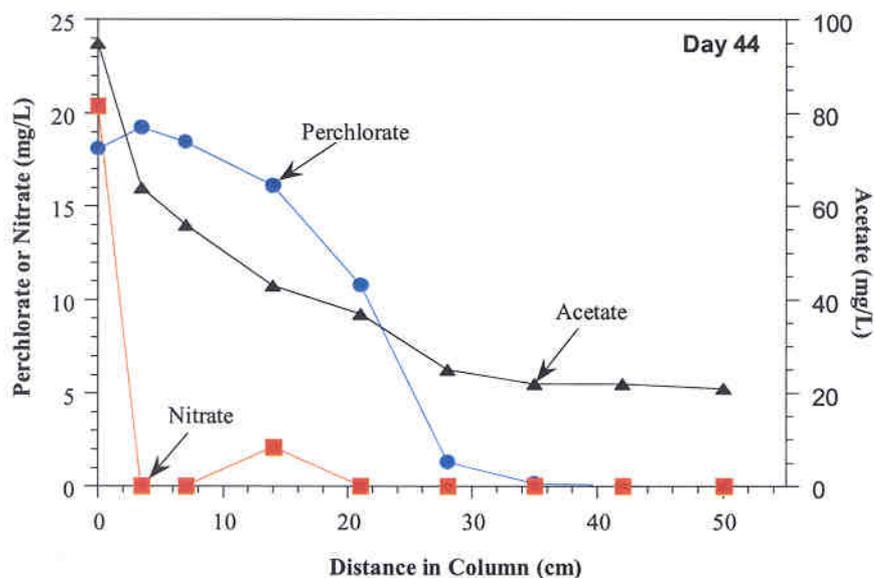
**Figure 33. Initial Biodegradation of Perchlorate and Nitrate in Model Aquifer with LHAAP Site 16 Sediment.**

The column profiles were taken beginning on day 14 after acetate addition. Most of the added nitrate was found to be degrading within the first 3.5 cm of the column in profiles taken from day 14 to day 48. Occasionally a small amount of nitrate was detected further in the column, but generally all was degraded by the first sample point. Conversely, perchlorate degradation was observed to begin well after the nitrate was biodegraded. A representative column profile from day 44 is provided in Figure 34. The profile of perchlorate degradation slowly moved down the column with time (i.e., the anion was degraded over a shorter column distance with time in the study). However, perchlorate degradation always occurred after nitrate, and thus appears to be appreciably slower than nitrate reduction. A series of perchlorate profiles in the model aquifer column with time (day 16 – day 44) are given in Figure 35.

After 48 days of groundwater flow and acetate addition, the acetate and nutrient feed pump was turned off. Without addition of electron donor, perchlorate in the effluent from the column increased from below detection at day 49 to 7 mg/L at day 50 and then to greater than 17 mg/L by day 52. Influent and effluent concentrations of the anion were the same from day 52 to day 59 (when the acetate feed was started again). Nitrate was also observed in the effluent water in the absence of added electron donor. Effluent concentrations of 4 and 13.5 mg/L were recorded on day 49 and day 52, respectively. The effluent concentration did not increase all the way back to the influent concentration by day 59, but remained in the vicinity of 14 mg/L, while the feed concentration was about 20 mg/L. Thus, some residual denitrification, but not perchlorate reduction, occurred during the period when electron donor

was not supplied. A graph showing perchlorate concentrations in feed and effluent water through greater than 100 days of operation is given in Figure 36.

The acetate feed pump was restarted at the previous rate on day 59 and the flow and anion concentrations were the same as before the electron donor was shut off. Perchlorate and nitrate levels in the column effluent again decreased to below detection within a few days. Between days 76 and 80, the nitrate concentration was increased from approximately 20 mg/L to 80 mg/L and then greater than 100 mg/L. When nitrate levels reached 100 mg/L, perchlorate biodegradation was no longer occurring in the column (i.e., influent and effluent concentrations were the same). Nitrate levels in the effluent water were approximately 25 mg/L during this time. No perchlorate degradation was observed during this time, rather all of the acetate supplied to the column (~ 80 mg/L) was consumed during reduction of oxygen and nitrate. In addition, the amounts of oxygen and nitrate consumed during acetate consumption was very close to that expected based on stoichiometry. Thus, the initial extra use of electron donor during the first phase of testing appears to reflect increased acetate consumption for perchlorate reduction. A column profile showing concentrations of acetate, nitrate, and perchlorate after nitrate levels were increased is provided in Figure 37 (day 84).



**Figure 34. Representative Profile of Perchlorate, Nitrate, and Acetate Biodegradation in Model Aquifer Column.**

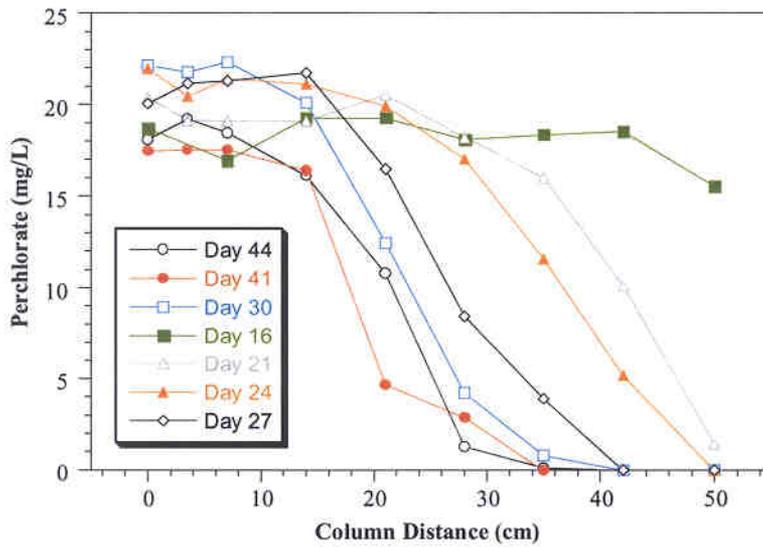


Figure 35. Perchlorate Profiles in Model Aquifer Column as a Function of Time.

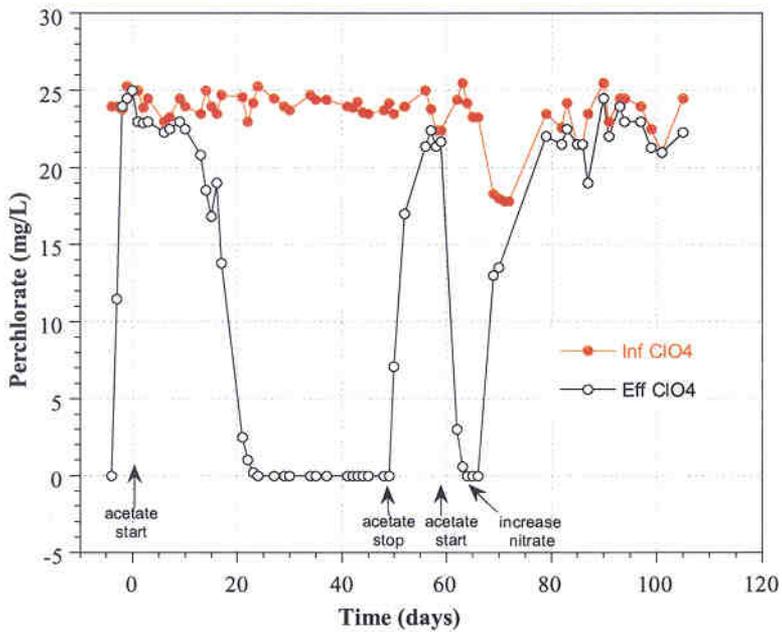
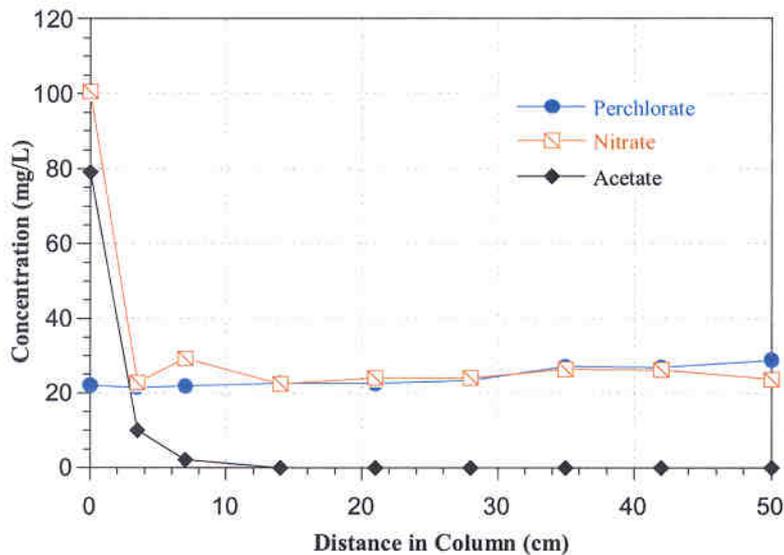


Figure 36. Influent and Effluent Concentrations of Perchlorate in Model Aquifer Column Packed with LHAAP Site 16 Sediment.



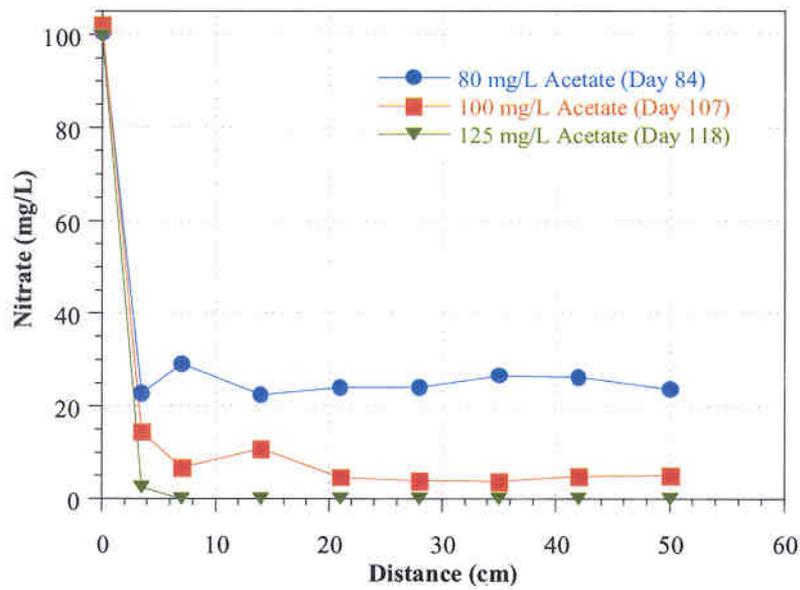
**Figure 37. Profile of Perchlorate, Nitrate, and Acetate Biodegradation in Model Aquifer Column After Nitrate Levels were Increased.**

The feed of electron donor (acetate) was increased from approximately 80 to 100 and then to 125 mg/L to determine if perchlorate reduction could be stimulated when nitrate was depleted, or if some perchlorate would degrade in the presence of low levels of nitrate. The increase in acetate supply was achieved by increasing the flow of the syringe pump supplying the electron donor, rather than changing concentration. The increase in electron donor from 80 to 100 mg/L caused residual nitrate levels to decline from 25 to approximately 5 mg/L (Figure 38). No reduction of perchlorate was observed after this increase in acetate (Figure 39), and all of the acetate was consumed within the first 7 cm of the column (Figure 40). Increasing the acetate feed to 125 mg/L resulted in perchlorate biodegradation. After this increase, all of the nitrate entering the column was degraded within the first 3.5 cm of the column and perchlorate biodegradation was then observed from approximately 14 to 28 cm in the 50-cm column. Thus, at 125 mg/L addition, the quantity of acetate entering the aquifer column was sufficient to support degradation of all nitrate (100 mg/L) and oxygen (8 mg/L), then approximately 80% of the perchlorate. Perchlorate reduction was not observed until each of these competing electron donors was consumed, and enough residual acetate was present to support perchlorate biodegradation.

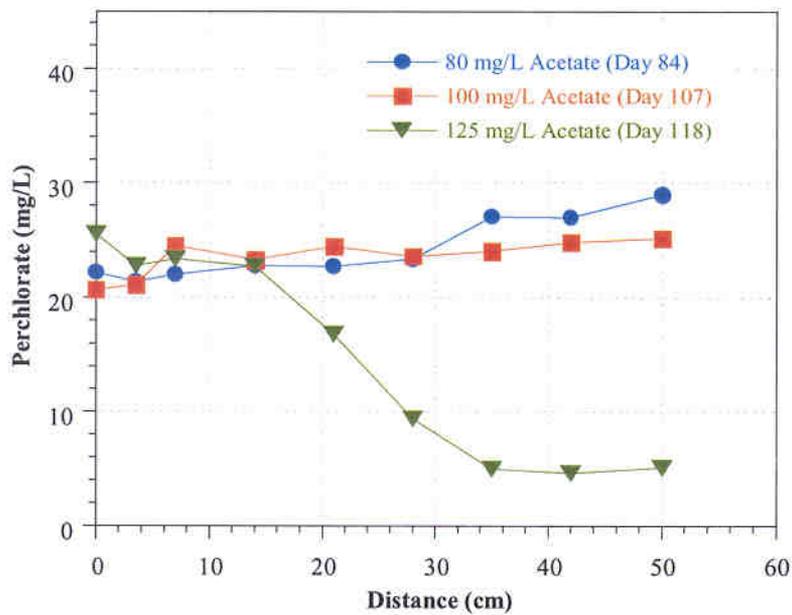
After this phase of testing, nitrate was removed from the artificial groundwater (prepared in the laboratory to simulate groundwater at LHAAP), the perchlorate concentration was increased to 50 mg/L, and the acetate feed was again reduced to 80 mg/L. Perchlorate was present in the effluent water at a concentration of 11.5 mg/L after 3 days without nitrate (day 125). The concentration then declined to 3.4 mg/L on day 4, and to below detection by day 5. After 12 days, nitrate was again added to the

groundwater at a concentration of approximately 100 mg/L. A column profile taken 18.5 hrs (1 day) after addition of nitrate revealed that both electron acceptors (nitrate and perchlorate) were degrading simultaneously within the column (Figure 41). However, denitrification rapidly replaced perchlorate reduction as the dominant microbial process within the 50-cm column. Perchlorate, which was below detection in the column effluent for several days prior to nitrate addition, was present in the effluent water at 3.7 mg/L 18.5 hrs after addition of nitrate (~ 0.8 pore volumes). The perchlorate concentration in the effluent increased to 26.4 mg/L two days after addition of nitrate, then to greater than 40 mg/L by day 5 after nitrate addition. The decline in perchlorate reduction after nitrate addition is apparent in column profiles taken during this period (Figure 42). One of the interesting findings of this phase of column testing is that biological nitrate reduction occurs preferentially to perchlorate reduction, even though the thermodynamics of the two processes are similar. The kinetics of nitrate reduction with acetate as electron donor appear to be favorable to the kinetics of perchlorate reduction. Thus, in general, when electron donor is limiting, all electron donor is consumed during denitrification, and perchlorate reduction does not occur. Another possible explanation for this finding is that nitrate actually inhibits perchlorate reduction through a biochemical mechanism (i.e., competitive or noncompetitive enzyme inhibition).

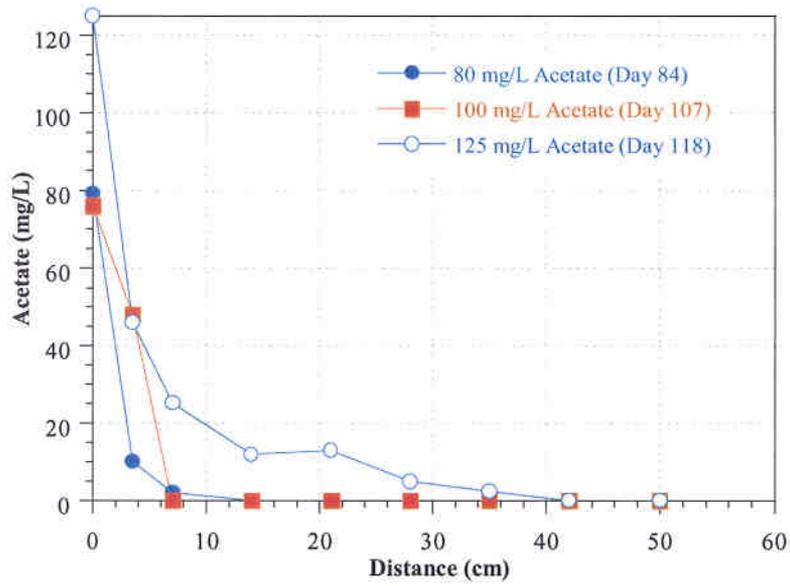
As described in section 4.5.6, a pure culture study was conducted to evaluate whether the prevalence of denitrification over perchlorate reduction reflects the relative kinetics of the two processes or a biochemical inhibition. In this experiment, *Dechlorosoma suillum* JPLRND (pure culture isolated from groundwater at the Jet Propulsion Laboratory) was incubated with perchlorate (and acetate as electron donor) until active degradation of perchlorate was observed. At this time, the culture was quickly split into several flasks, and these flasks were amended with different concentrations of nitrate. The data from this study showed that the rate of perchlorate reduction was reduced dramatically by the addition of nitrate, and that this reduction was directly proportional to nitrate concentration. The results suggest that nitrate is a biochemical inhibitor of perchlorate reduction, (probably an inhibitor of the perchlorate reductase enzyme). The data also provide one explanation for the preferential degradation of nitrate compared to perchlorate in the flow-through columns.



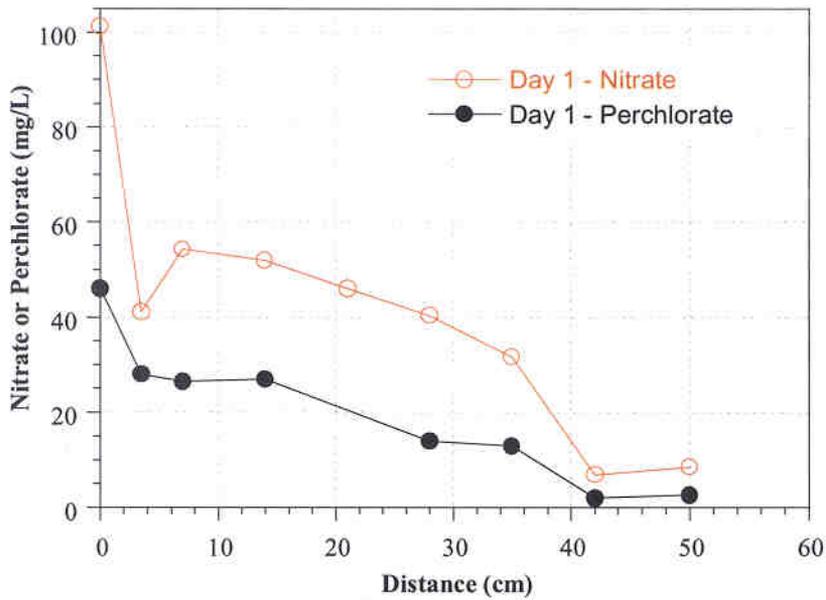
**Figure 38. Profiles of Nitrate in Aquifer Column with Increasing Levels of Electron Donor (Acetate).**



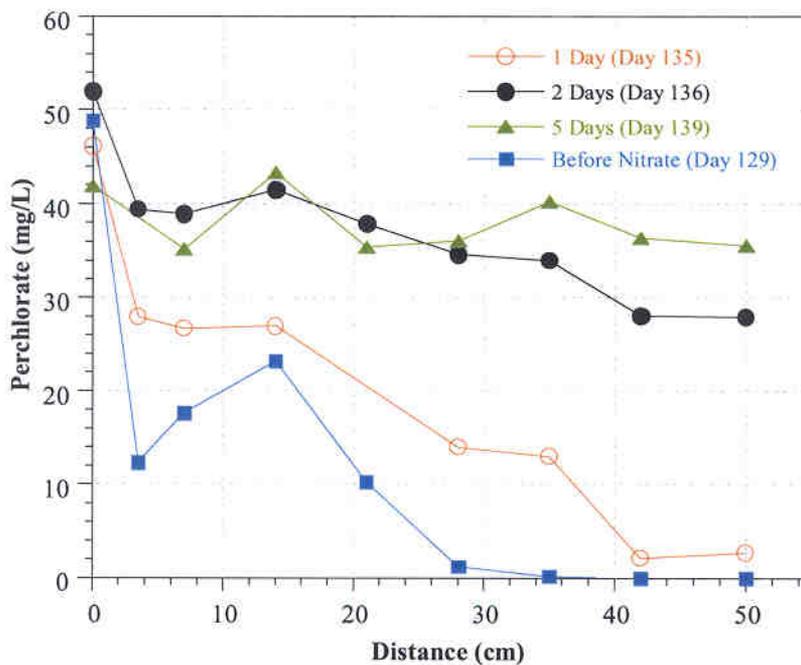
**Figure 39. Profiles of Perchlorate in Aquifer Column with Increasing Levels of Electron Donor (Acetate).**



**Figure 40. Profile of Acetate in Aquifer Column with Increasing Levels of Electron Donor (Acetate).**



**Figure 41. Profile of Nitrate and Perchlorate in the Aquifer Column 1 Day (18.5 hrs) after Nitrate was Added to Groundwater.**



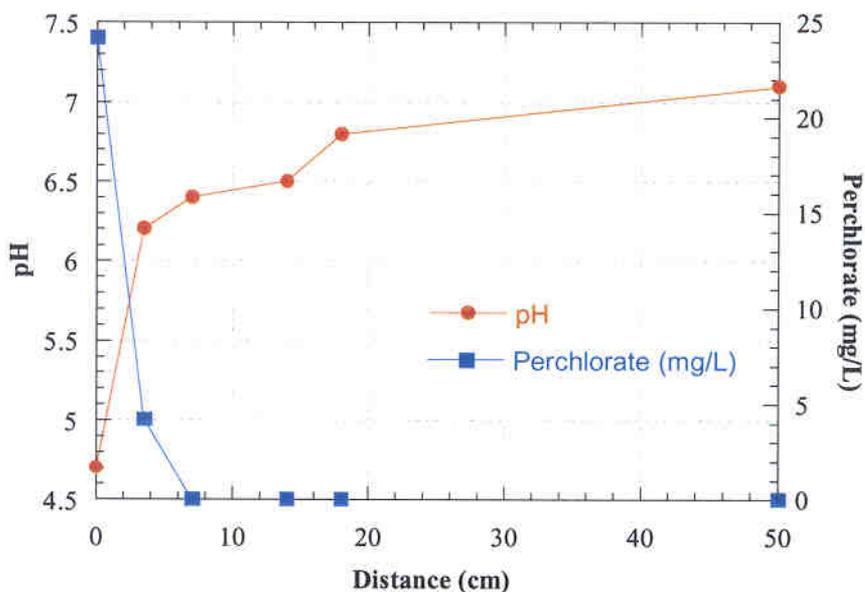
**Figure 42. Profiles of Perchlorate in the Aquifer Column with Time after Nitrate (100 mg/L) was Added to Groundwater.**

#### 4.4.5. INFLUENCE OF pH ON PERCHLORATE REDUCTION

At the conclusion of studies with nitrate, the influence of groundwater pH on perchlorate reduction was evaluated in the 50-cm aquifer column. Previous microcosm studies with site samples from LHAAP and IHDIV revealed that perchlorate degradation is inhibited below a pH of approximately 5.7, but that degradation can be quickly stimulated with buffering to pH 7. An attempt was made to explore this effect further using the model aquifer. To do this, sodium bicarbonate was initially removed from the artificial groundwater used in the column studies. This reduced the pH from 7 to approximately 5. Prior to this modification, the starting perchlorate level was adjusted down to approximately 25 mg/L and the nitrate was adjusted to 16 mg/L (approximately 0.25 mM each). The initial acetate concentration was 80 mg/L. Prior to pH adjustment, both perchlorate and nitrate were degrading from initial levels to below detection within the first 7 cm of the 50-cm column. The adjustment in pH did not influence the kinetics of perchlorate reduction. However, the buffering capacity of the soil in the column was quickly bringing the pH of the groundwater back up to 7.0. In an attempt to overcome this effect, the buffer MES (2-[N-morpholino]ethanesulfonic acid; pKa = 6.1) was added to the water at a concentration of 2 mM, and the

groundwater pH was adjusted to between 4.5 and 5.0. The buffered groundwater was run through the column for approximately 33 days.

The acidification of the groundwater did not influence denitrification. Nitrate was degraded from a starting concentration of 16 mg/L to below detection within the first 3.5 cm of the column throughout the study period. For the first week that the buffered groundwater was added to the column, perchlorate was also degraded to below detection within the first few centimeters of the aquifer column. However, profiles collected after 8 and 14 days showed a moderate reduction in the rate of perchlorate degradation. The column profiles also showed that the groundwater pH was still quickly buffered in the column, increasing from approximately 4.5 in the influent to 6.2 within 3.5 cm (Figure 43). Because we were unable to dramatically alter the pH across the profile of the column with buffered water, and did not want to attempt more rigorous methods (e.g., acid addition), the study was discontinued after 33 days. The data show some reduction in the rate of perchlorate degradation (but not denitrification) upon moderate acidification in the first few cm of the column, but because of the high buffering capacity of the sediments, the results of this phase of the study were inconclusive.



**Figure 43. Groundwater pH and Perchlorate Levels in Column Profile.**

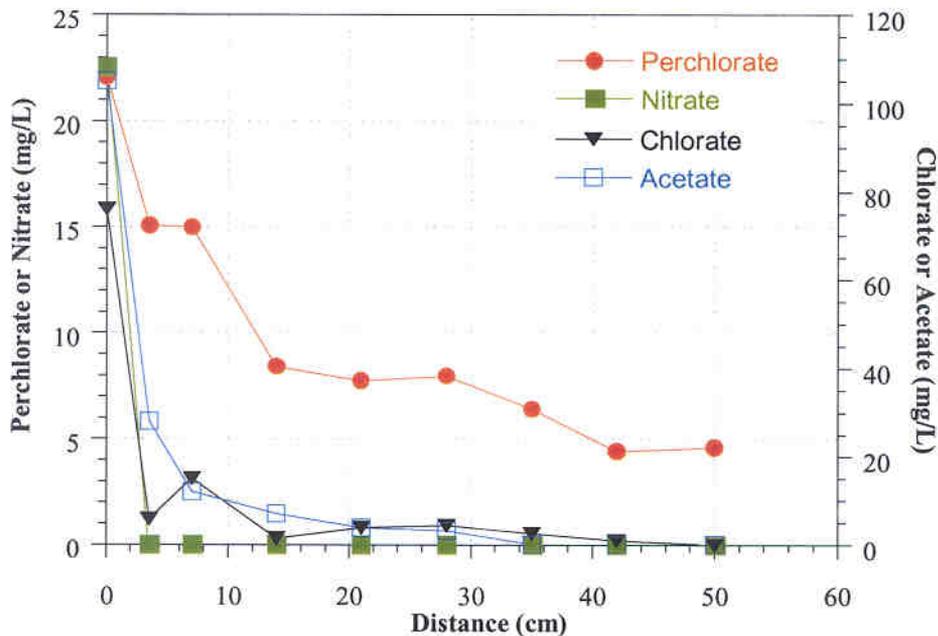
#### **4.4.6 INFLUENCE OF CHLORATE ON PERCHLORATE REDUCTION**

An additional experiment was conducted with the aquifer column to evaluate the influence of chlorate addition on perchlorate reduction. Chlorate and perchlorate appear to be degraded by the same enzyme pathway in perchlorate reducing bacteria, therefore the degradation of both of these anions together in a natural system is interesting. In addition, chlorate has been found experimentally to inhibit nitrate reduction in some instances. Thus, the presence of chlorate in groundwater could reduce nitrate reduction, and based on our previous findings, the residual nitrate could then impact perchlorate degradation. To evaluate the influence of chlorate, the anion was added to the influent groundwater in the column at a concentration of approximately 80 mg/L (1 mM). The influent perchlorate concentration was approximately 25 mg/L (0.25 mM), the nitrate concentration was approximately 16 mg/L (0.25 mM), and the acetate feed was supplying approximately 100 mg/L to the column influent.

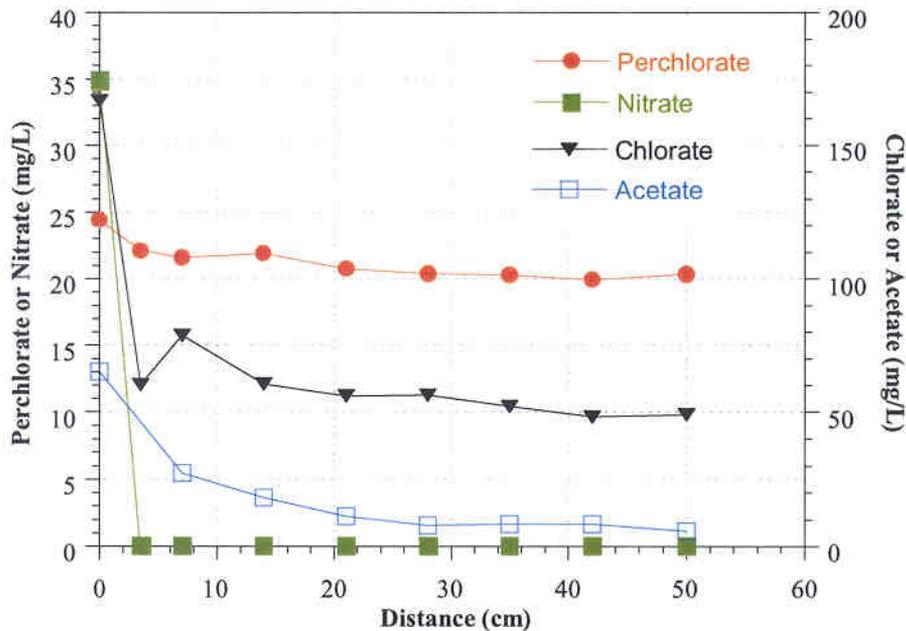
Prior to chlorate addition, both perchlorate and nitrate were degrading to below detection within the first 3.5 cm of the aquifer column. There was approximately 40 mg/L of residual acetate. Within one day after chlorate addition, perchlorate was detected in the column effluent (50 cm sample). Nitrate, however, was still biodegrading within the first 3.5 cm of the column. Seven days after chlorate was added to the influent groundwater, nitrate was still degraded in the first 3.5 cm of the column (Figure 44). A majority of the chlorate was also biodegraded within this initial zone of the column. The concentration dropped from 76 mg/L in the influent to 5.8 mg/L after 3.5 cm of transport in the column, a loss of 94 %. Perchlorate biodegradation occurred throughout the length of the column. The perchlorate concentration declined from 22 to 15 mg/L in the first 3.5 cm, then to 7.7 mg/L by the middle of the column (21 cm), and 4.6 mg/L remained in the effluent. Acetate was below detection after 35 cm in the column. The profile for the three electron acceptors remained approximately the same in additional profiles taken during the following several days.

The chlorate concentration was increased to 160 mg/L (2 mM) approximately 3 weeks after chlorate was initially added. This increase did not appear to influence nitrate degradation. Nitrate was still completely degraded in the initial 3.5 cm of the column sediment (Figure 45). The rate and extent of perchlorate biodegradation, however, were each further reduced by the increased chlorate concentration. After 5 days, perchlorate levels were only declining marginally through the column. Most of the chlorate degradation continued within the initial few cm of the column, but a residual level of approximately 50 mg/L remained after this initial degradation. Although the acetate profile showed low residual concentrations of the electron donor in the latter half of the column, it is likely that it was actually all consumed within the first half of the column. The detection limit for acetate by the GC method employed is approximately 5 mg/L, but the method has proven not to be reliable below 10 mg/L.

The data from this study showed that chlorate did not influence the biodegradation of nitrate in the aquifer column. Nitrate was degraded within the first few cm of the column in the absence of chlorate as well as in the presence of 160 mg/L (2 mM) of the anion. The rate and extent of perchlorate reduction, however, were dramatically affected by chlorate addition. Perchlorate reduction was not completely inhibited by chlorate, as was observed for nitrate, but it was decreased substantially. Before chlorate addition, all of the added perchlorate (~ 25 mg/L) was degraded in the first 3.5 cm of the column. After addition of 80 mg/L chlorate, perchlorate was observed to be present in the effluent of the 50-cm column at approximately 5 mg/L. This residual increased to about 20 mg/L after the addition of 160 mg/L chlorate. Pure culture studies with JPLRND to evaluate the influence of chlorate on perchlorate reduction were not conducted. However, based on previous findings with other strains, it is likely that both of these anions are substrates for the (per)chlorate reductase enzyme. Thus, the influence of chlorate on perchlorate reduction probably results from competitive effects of the two anions at the active site of the enzyme.



**Figure 44. Biodegradation of Perchlorate, Chlorate, and Nitrate in Model Aquifer with Acetate as Electron Donor.**



**Figure 45. Biodegradation of Perchlorate, Chlorate, and Nitrate in Model Aquifer After Chlorate Levels were Increased to 160 mg/L.**

#### **4.4.7 SUSTAINED BIODEGRADATION OF PERCHLORATE AT LOW CONCENTRATIONS**

An additional flow-through model aquifer was constructed to evaluate perchlorate biodegradation at low concentrations (in the absence of nitrate), and to test the influence of co-contaminants on perchlorate biodegradation. The second column was constructed as described originally except that the length of the column was reduced from 50 cm to 30 cm, and the sampling points were placed at 2.5, 5.0, 10.0, 15.0, 20.0, 25.0, and 30-cm from the bottom of the column. After construction, the column was packed with sediments from the Longhorn Army Ammunition Plant (LHAAP) and a bromide tracer experiment was conducted to quantify flow characteristics. Artificial groundwater was prepared with similar chemistry as described for the initial column experiments. The sustained biodegradation of low concentrations of perchlorate in the absence of nitrate was tested. Initially, perchlorate was added to the influent groundwater at a concentration of 250  $\mu\text{g/L}$ , and oxygen was present at approximately 8 mg/L. These conditions are similar to those at the location from which the sediments were originally collected (LHAAP Site 16 Landfill). The geochemistry of the artificial groundwater is also based on this location. Unlike the previous column, in which acetate was used as an electron donor, this column received lactic acid as an electron donor. The target concentration in the influent groundwater was 20 mg/L.

The column was initially packed with 2280 g of sieved, moist sediments from LHAAP Site 16 (1750 g dry wt). These sediments contained much more clay than the sample used to pack the 50-cm column. The pore volume of the column was estimated at 540 mL. During the initial tracer test, bromide and perchlorate were added to the column at 50 mg/L each in a 180 mL (~1/3 pore volume) pulse. The flow rate was approximately 45 mL/hr. The breakthrough curves for bromide and perchlorate are provided in Figure 46. The two anions moved through the column together, as observed previously for the 50-cm column. The breakthrough curves displayed a more significant tail than was observed for the 50-cm column, suggesting that there may be some preferential flow in the column.

There was no appreciable reduction in perchlorate through the column for the first 14 days after the lactate addition was initiated. By day 22, however, perchlorate was below detection in the column effluent (Figure 47). The minimum detection limit for perchlorate for this phase of testing was 8 µg/L. The column effluent remained below detection throughout the remainder of the study period. Column profiles taken on days 36 and 37 showed that most of the perchlorate was degrading within the first 10 cm of the column (Figure 48). At day 56, the influent perchlorate concentration was reduced to 50 µg/L. The effluent perchlorate concentration remained below detection after the influent perchlorate was reduced to this level. The minimum detection limit was 4 µg/L during this phase of testing. The data show that lactic acid will support biological perchlorate reduction at this site. The data also show that perchlorate biodegradation can be stimulated and sustained (to < 4 µg/L) even when initial concentrations of the anion in groundwater are very low (i.e., 50 – 250 µg/L) and nitrate, an alternate electron acceptor for most perchlorate-respiring bacteria, is not initially present. There are a number of large perchlorate plumes in which the initial levels of the anion in groundwater are several to several hundred µg/L, and in which nitrate is not present. The data from this experiment suggest that sustained *in situ* perchlorate bioremediation may be possible for these sites. If a lower threshold for perchlorate biodegradation exists, as has been found for many organic contaminants acting as electron donors, it appears that this level is below the low µg/L levels which are relevant for current regulatory requirements.

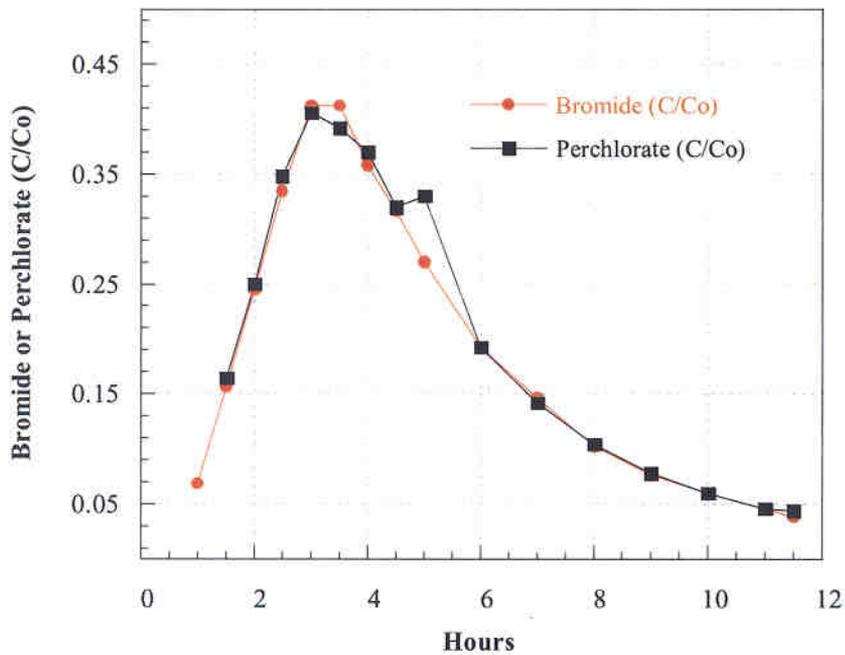


Figure 46. Transport of Bromide and Perchlorate Through the 30-cm Model Aquifer Column Packed with LHAAP Site 16 Sediment.

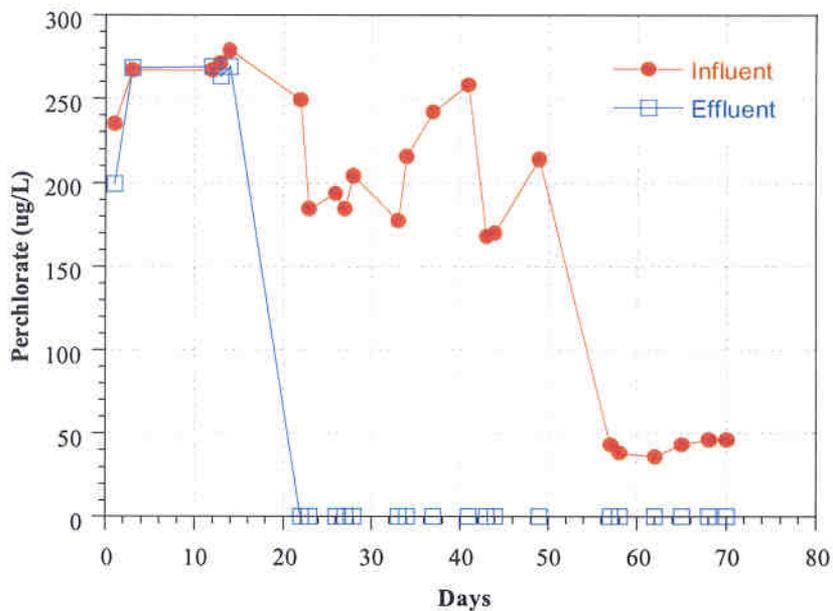
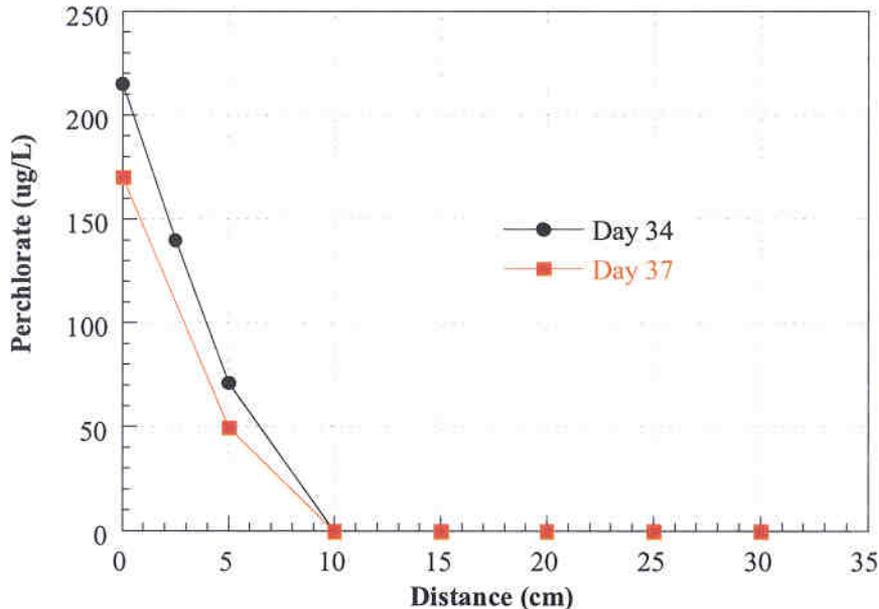


Figure 47. Sustained Biodegradation of Perchlorate at Low Influent Concentrations with Lactate as Electron Donor.



**Figure 48. Profiles of Perchlorate Biodegradation in the 30-cm Aquifer Column with Lactate as Electron Donor.**

#### ***4.4.8 INFLUENCE OF RDX ON PERCHLORATE BIODEGRADATION AND POTENTIAL FOR COMBINED TREATMENT***

Previous microcosm experiments performed during this project evaluated the influence of chlorinated solvents (PCE and TCE) on perchlorate degradation. In addition, in one of the companion projects, Geosyntec researchers have been examining the joint bioremediation of chlorinated solvents and perchlorate in laboratory and field studies. Therefore, rather than looking at chlorinated solvents in a column experiment, we decided to examine the influence of a second co-contaminant, the nitramine explosive hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX), on perchlorate degradation. RDX, and a similar explosive, octahydro-1,3,5,7-tetranitro-1,3,5,7-tetraazocine (HMX) are widespread environmental contaminants at many current and former military facilities. For example, a recent report suggests that at least 28 U.S. Army installations and more than 200 facilities in Germany are contaminated with these compounds (Stahl et al., 2001). The majority of this contamination is the result of historical manufacturing, handling, and disposal procedures for munitions. However, past and current activities on military training ranges may also be resulting in releases of these energetics into the environment. For example, groundwater underlying impact ranges at the Massachusetts Military Reservation (MMR) on Cape Cod has recently been found to be contaminated with various explosives, including both RDX and

HMX (Burt, 1999). This groundwater is also contaminated with low levels of perchlorate from training activities or from another source (e.g., burn pits). It is likely that other sites also have co-contamination with perchlorate and RDX.

Based on this information as well as the current interest in RDX treatment methods, we conducted a column study to examine (1) whether RDX impacts perchlorate treatment, and (2) whether both RDX and perchlorate can be biodegraded using electron donor addition. There is a growing body of literature on the biodegradation of nitramine explosives, and although the mechanism by which bacteria utilize these compounds (i.e., as growth substrates, cometabolites, or terminal electron acceptors) remains unclear, the preponderance of scientific evidence suggests that the most rapid degradation of RDX and HMX occurs under anoxic or anaerobic conditions. In addition, the application of a suitable organic growth substrate is often required to achieve rapid and complete degradation of the nitramine explosives (Boopathy and Manning, 1998; Boopathy et al., 1993). Therefore, treatment methods to stimulate perchlorate biodegradation in groundwater may also stimulate RDX biodegradation.

To examine this possibility, RDX was added at ~ 5 mg/L in the influent water of the 30-cm aquifer column. The explosive was added in crystalline form from a military stock containing 93% RDX and 7 % HMX. The column used was the same column described previously to evaluate degradation of low concentrations of perchlorate (previous section). The influent feed of perchlorate remained at approximately 50 µg/L and lactate addition was continued at 20 mg/L. Subsamples were taken periodically from the influent and effluent of the column, and from the sampling points along the profile. The samples were analyzed for perchlorate by IC (EPA 314.0) and RDX by HPLC (EPA 8330).

There was no apparent decline in the rate or extent of perchlorate biodegradation upon adding RDX to the influent water to the column (Day 70 after column flow was started). The anion continued to degrade from 50 µg/L to near or below detection within the first 10 cm of the 30-cm column. Interestingly, RDX levels also declined appreciably during transport through the aquifer column. The influent concentrations varied from about 2.5 to 5 mg/L, but losses of approximately 70% to 90% were observed across the column. A profile of perchlorate and RDX in the aquifer column 22 days after RDX was initially added is given in Figure 49. Perchlorate was reduced from 45 to less than 4 µg/L in the first 2.5 cm of the column, and RDX levels declined from 5.17 mg/L to 1.27 mg/L across the 30-cm column length.

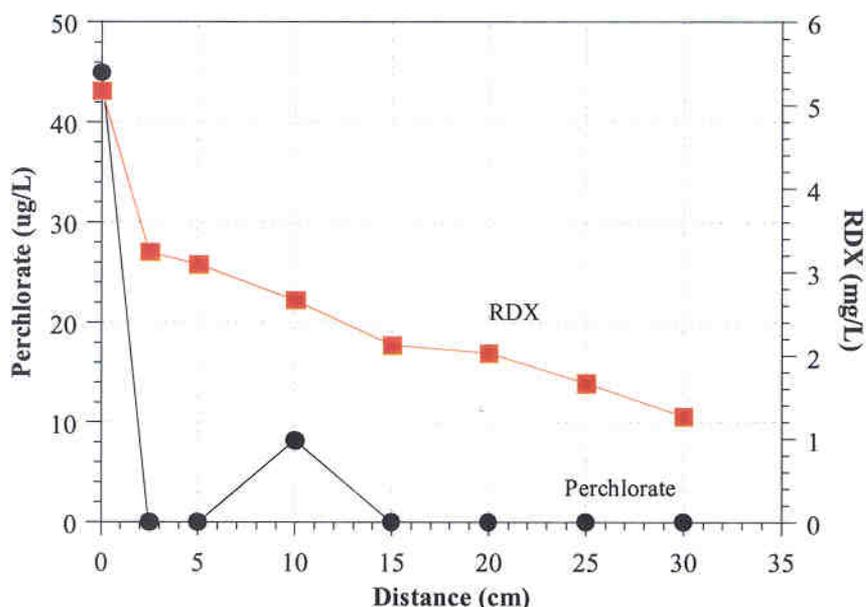
Because electron donor was being supplied to the column at the time that RDX was added, it is unclear whether the initial losses of the explosive in the column represented biodegradation or merely adsorption to aquifer sediments. RDX has a low octanol-water partition coefficient ( $\text{Log } K_{ow} = 0.86$ ) and does not sorb appreciably to most constituents of soils or sediments (Schumacher et al., 1992; Sheremata et al., 2001). However, sorption of nitramines to some clays has been observed. Therefore, to examine

whether the decline in RDX levels in the column was due to biodegradation (supported by lactate addition) or to sorption only, the electron donor feed to the column was discontinued on day 27 after RDX addition. Three days after the lactate feed was shut off (approximately 6 pore volumes), perchlorate was observed in the column effluent. The RDX levels across the column also increased, although far more gradually than for perchlorate. A profile for both RDX and perchlorate before and after the lactate feed was shut off (Day 27 and Day 30, respectively) is given in Figure 50. The lactate feed remained off for 33 days. After several days, influent and effluent perchlorate levels were similar (i.e., biodegradation no longer occurred). However, levels of RDX in the effluent continued to decline by 60 – 70% across the column during the period that the lactate feed was off. A column profile for day 44 (17 days after lactate feed was shut off) is provided in Figure 51. The data suggest that RDX was biodegraded in the absence of added electron donor, although the extent of degradation was less than in the presence of lactate.

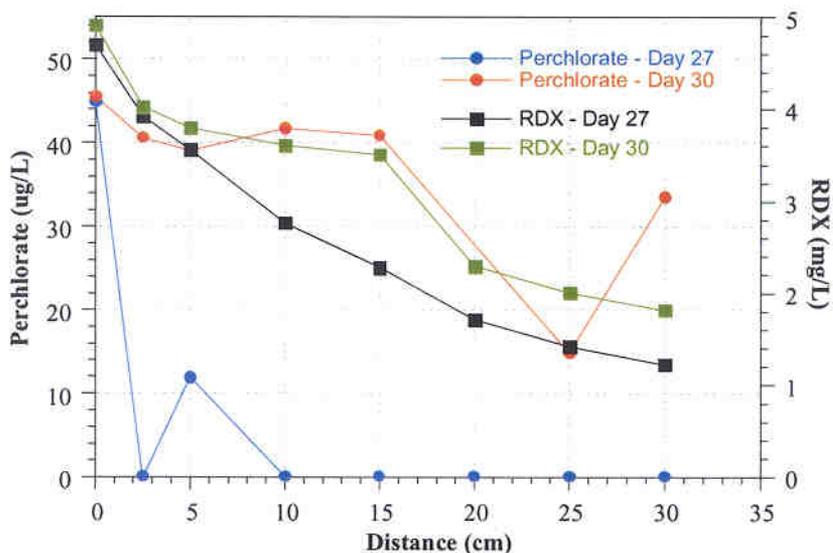
To evaluate whether the extent of biodegradation of RDX could be enhanced by electron donor addition, the lactate feed was restarted on day 50 at a concentration of 40 mg/L (compared to 20 mg/L in the previous phase). The addition of lactate did not appear to appreciably influence the loss of RDX across the column. After 13 days, the flow rate to the column was cut approximately in half from 45 mL/hr to 24 mL/hr in order to increase the hydraulic retention time (HRT) of groundwater within the column from approximately 12 to 23 hrs. When the retention time was increased, effluent levels of RDX declined appreciably (Figure 52). The effluent levels of RDX from the column declined to approximately 200 -300 µg/L after the HRT was increased. The degradation across the column was greater than 92% of the influent RDX after the HRT was increased to 23 hrs, compared to approximately 70% when the HRT was 12 hrs.

The biodegradation pathway for RDX under anoxic conditions is postulated to proceed by sequential reduction of the nitro (NO<sub>2</sub>) groups to nitroso (-NO) groups, resulting in the formation of hexahydro-1,3,5-trinitroso-1,3,5-triazine (TNX) (e.g., Hawari et al., 2000). In addition to monitoring RDX, the initial nitroso- breakdown products of RDX [hexahydro-1-nitroso-3,5-dinitro-1,3,5-triazine (MNX), hexahydro-1,3-dinitroso-5-nitro-1,3,5-triazine (DNX), and TNX] were quantified by HPLC for some of the column profiles (beginning on Day 58 after initial RDX addition) using appropriate standards for each metabolite. TNX and MNX were routinely detected throughout the column during analyses. Conversely, DNX was either below detection or was observed only in a few samples in the column profiles that were collected. A column profile showing RDX degradation and levels of each of the three metabolites at an influent flow rate of 45 mL/hr (12 hr HRT) is provided in Figure 53 and a similar profile at a flow rate of 24 mL/hr (23 hr HRT) is provided in Figure 54. The column was being supplied with lactate at 40 mg/L when each profile was taken. At the more rapid flow rate, the maximum levels of TNX and MNX in the column were 0.3 mg/L. DNX was not detected. The flow to the column was reduced on

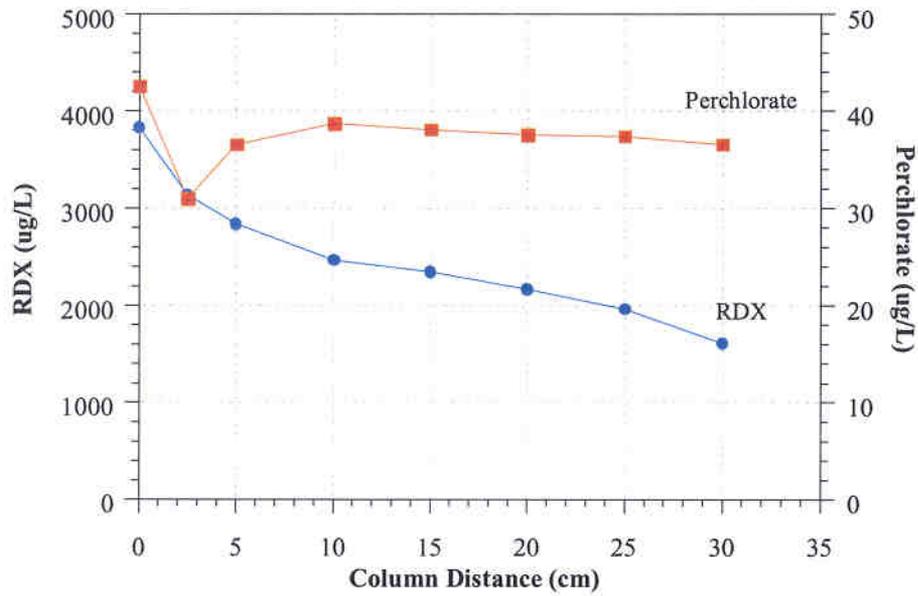
Day 63. On day 84 when the second profile was collected, levels of MNX exceeded 1.5 mg/L through the first 10 cm of the column. Levels of TNX increased gradually through the first several centimeters of the column, reaching a maximum concentration at the 10 cm point, and decreasing thereafter. A small amount of DNX was detected at the 10-cm sample point, but nowhere else in the column. The metabolite data clearly show that RDX biodegradation is occurring within the column in conjunction with perchlorate degradation.



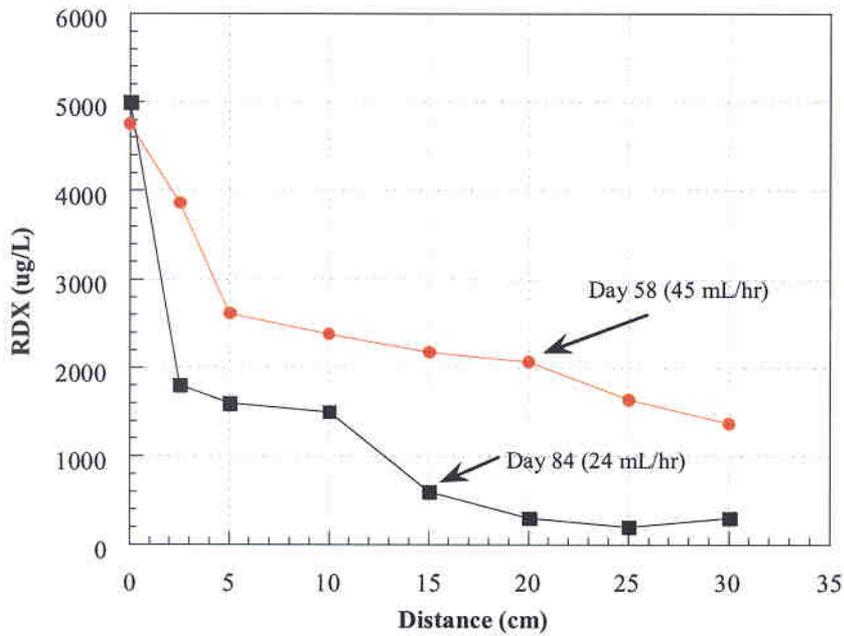
**Figure 49. Profiles of Perchlorate and RDX in Aquifer Column 22 Days after Initial Addition of RDX.**



**Figure 50. Profile of Perchlorate and RDX in Aquifer Column on Day 27 (+ Lactate Feed) and Day 30 (- Lactate Feed) after Initial Addition of RDX to Groundwater.**



**Figure 51. Profile of Perchlorate and RDX in Aquifer Column with Electron Donor Feed Off (Day 44).**



**Figure 52. Biodegradation of RDX in Aquifer Column at Flow Rates of 45 mL/hr (12 hr HRT) and 24 mL/hr (23 hr HRT).**

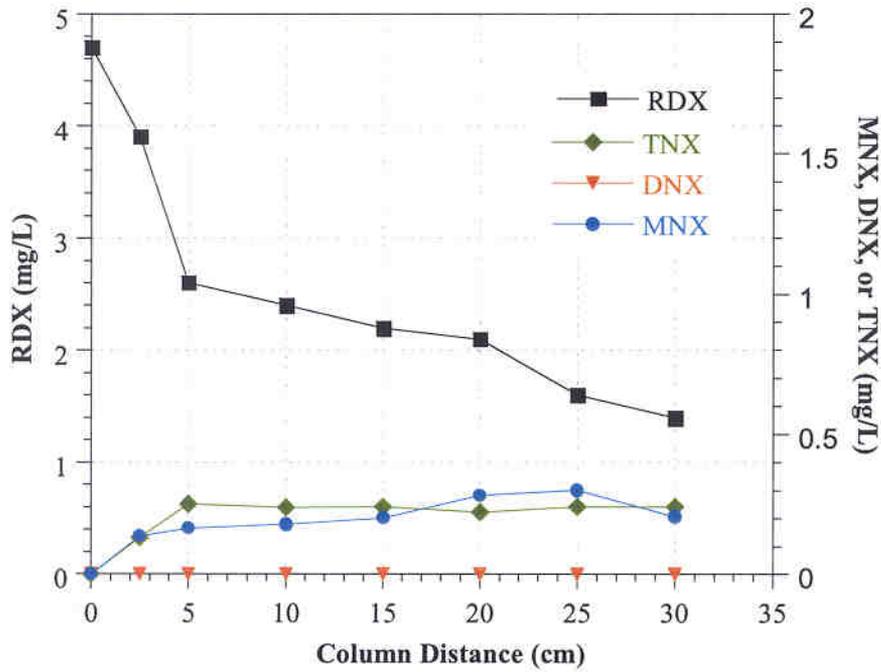


Figure 53. Biodegradation of RDX and Formation of RDX Metabolites in Aquifer Column at Flow Rate of 45 mL/hr (12 hr HRT; Day 58).

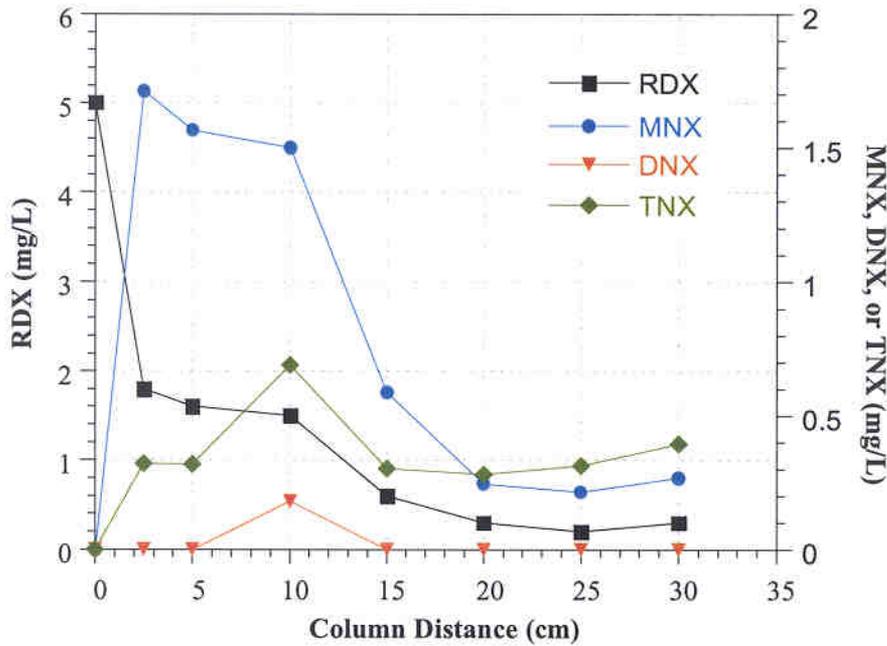


Figure 54. Biodegradation of RDX and Formation of RDX Metabolites in Aquifer Column at Flow Rate of 24 mL/hr (23 hr HRT; Day 84).

## **4.5 PERCHLORATE BIODEGRADATION MODEL**

### ***4.5.1 MODEL DESCRIPTION***

A mathematical model has been developed to describe the biodegradation kinetics of perchlorate (an electron acceptor) in the presence of an electron donor and other alternate electron acceptors. The model is based on the modeling program RT3D, developed by Battelle Pacific Northwest National Laboratory. The rate of perchlorate degradation is described as a function of the electron donor (acetate) utilization rate, presence and utilization of alternate electron acceptors (oxygen and nitrate), and rates of biomass growth and decay. The kinetics of biomass growth are described using a modified Monod model. To describe the effect of alternate electron acceptors on perchlorate degradation an inhibition factor is included as a modification to the Monod model. Specific details of the model are given in the sections below.

### ***4.5.2 ELECTRON DONOR CONSUMPTION***

The model describes sequential degradation of acetate (the electron donor) in the presence of oxygen, nitrate, and perchlorate (the electron acceptors). Also, the influence of biomass populations on the utilization of acetate is included in the model (see Section 4.5.3 below). In the model, it is assumed that the total amount of acetate consumed is equal to the sum of three terms: 1) the amount of acetate consumed using oxygen as an electron acceptor; 2) the amount of acetate consumed using nitrate as an electron acceptor; and 3) the amount of acetate consumed using perchlorate as an electron acceptor. The rate of acetate consumption using each electron acceptor is written as a function of acetate concentration, electron acceptor concentration, bacterial population, the maximum specific rate of degradation, and the half-saturation constant for that reaction. For the reaction where nitrate is utilized as an electron acceptor, oxygen is modeled as a competitive inhibitor to the reaction. Similarly, for the reaction where perchlorate is utilized as the electron acceptor, nitrate and oxygen are modeled as competitive inhibitors of the reaction. The complete model equations are provided in Appendix B (section 7.2).

### ***4.5.3 MICROBIAL POPULATIONS***

In the model, two primary processes are used to describe changes in the microbial populations: 1) growth due to electron donor consumption; and 2) indigenous cell decay. The rate of change in the biomass due to growth is dependent upon the starting biomass concentration, the biomass yield, and the rate of electron donor utilization. Biomass concentrations are included in the mathematical expression for calculating the rate of utilization of the electron donor and each electron acceptor.

#### **4.5.4 ELECTRON ACCEPTORS**

In the model, changes in the electron acceptor concentrations are directly linked to the consumption of the electron donor. The change in concentration of each acceptor is calculated as a function of the specific electron donor utilization rate for that acceptor, the mass of acceptor consumed per mass of substrate consumed (the donor/acceptor yield coefficient), the biomass concentration, and the concentration of the other electron acceptors. The donor/acceptor yield coefficients were determined theoretically based upon the stoichiometric half-reactions for each electron acceptor and compared to observations in the laboratory. Inhibition among the different electron acceptors was modeled using the assumption that the electron acceptors are used sequentially as follows: oxygen, nitrate, and perchlorate. Previous experimental work has demonstrated this sequential electron acceptor use is relatively accurate. Microcosm experiments were performed during this study to further evaluate this relationship.

#### **4.5.5 MODEL ASSUMPTIONS**

Some of the basic assumptions made to develop the model include the following:

1. Maximum specific growth rate and the half saturation constant do not significantly change with the different electron acceptors.
2. Cell yield does not change with different electron acceptors.
3. Competition among the different electron acceptors is a continuous function, i.e., not based on "threshold concentrations".
4. The electron donor and electron acceptors described do not volatilize into air or sorb onto soil.
5. Any lag periods observed during the microcosm studies are due to microbial growth only.
6. Biomass may decay to zero or grow indefinitely.

#### **4.5.6 EXPERIMENTAL DETERMINATION OF MODEL PARAMETERS**

##### **Electron Donor Parameters**

Experiments were performed to estimate the different input parameters for the model. These studies utilized a perchlorate degrading strain, *Dechlorosoma suillum* JPLRND, hereafter referred to as JPLRND, which was isolated from groundwater underlying the Jet Propulsion Laboratory in Pasadena, CA, during previous work in this project. A series of batch experiments were conducted with this strain using a range of starting electron donor (acetate) concentrations where individual electron acceptors (oxygen, nitrate, or perchlorate) were constant and not limiting (i.e., supplied in excess). The electron donor levels and the microbial population density were measured over time in each microcosm. Using the biomass versus time graph, the biomass growth rate for each microcosm was determined from the exponential growth

stage of each graph. Consequently, a “Monod growth” curve was constructed by plotting the exponential growth rate of the bacterium versus the starting electron donor concentration for that microcosm. The maximum specific growth rate ( $k_{\max}$ ) and half-saturation constant ( $K_s$ ) for JPLRND using acetate as each electron donor were determined from each Monod curve. These values represent the maximum specific growth rate ( $k_{\max}$ ) of the culture on the acetate (donor), whereas  $K_s$  represents the concentration where the specific growth rate is half the  $k_{\max}$  value. These two parameters were determined for each electron acceptor. As stated in the Model Assumptions (Section 4.5.5), it was assumed that the maximum specific growth rate ( $k_{\max}$ ) and the half saturation constant ( $K_s$ ) do not vary significantly among the different electron acceptors. This assumption was tested by fitting  $k_{\max}$  and  $K_s$  values for acetate and each electron acceptor and comparing the variability in these parameters. The notation for  $k_{\max}$  values for the three electron acceptors is as follows:  $k_{\max}^{\text{don/oxy}}$ ,  $k_{\max}^{\text{don/nit}}$ , and  $k_{\max}^{\text{don/per}}$ . As discussed below, the average value of these parameters (designated as  $k_{\max}$ ) was used for subsequent modeling of perchlorate biodegradation.

Similarly, three  $K_s$  values for acetate, corresponding to the three electron acceptors, were fit to the data and examined for variability. The notation for these three parameters is as follows:  $K_s^{\text{don/oxy}}$ ,  $K_s^{\text{don/nit}}$ , and  $K_s^{\text{don/per}}$ . As discussed below, the average of these three parameters, designated as  $K_s$ , was used for subsequent modeling of perchlorate biodegradation.

### Electron Acceptor Parameters

Experiments were performed to determine the growth rate parameters for the bacterium when the electron acceptor concentrations (nitrate or perchlorate only) were limiting to the biodegradation process while the electron donor (acetate) was present in excess (i.e., did not influence the biodegradation kinetics). The maximum growth rate and half saturation constant for growth under these conditions were determined. These parameters were determined for the electron acceptors nitrate and perchlorate only. Experiments where oxygen was limiting were not performed because of the difficulty of measuring oxygen concentrations in microcosm experiments. The notations for the maximum growth rate and half saturation constant for these fits are as follows:  $k_{\max}^{\text{nit/don}}$ ,  $k_{\max}^{\text{per/don}}$ ,  $K_s^{\text{nit/don}}$ , and  $K_s^{\text{per/don}}$ . It is expected that  $k_{\max}^{\text{nit/don}}$  and  $k_{\max}^{\text{per/don}}$  should equal the values  $k_{\max}^{\text{don/nit}}$  and  $k_{\max}^{\text{don/per}}$ . On the other hand, given that  $K_s^{\text{nit/don}}$  represents a nitrate concentration,  $K_s^{\text{per/don}}$  represents a perchlorate concentration,  $K_s^{\text{don/nit}}$ ,  $K_s^{\text{don/per}}$  represent acetate concentrations, it is expected that these half-saturation constants will be different in value.

#### 4.5.6.1 Experimental Quantification of Model Parameters

##### Electron Donor Parameters

###### Methods

Three groups of experiments were conducted to determine the different  $K_S$  and  $k_{max}$  values for JPLRND growing on acetate. In each group, one electron acceptor (oxygen, nitrate, or perchlorate) was tested using seven flasks initially prepared with basal salts medium (BSM) and supplied with varying acetate concentrations, ranging from 0 to 600 mg/L. The starting concentration of nitrate or perchlorate in each of the flasks was 1000 mg/L, while oxygen was maintained at saturation (approximately 8 mg/L). These concentrations were used to ensure that the electron acceptors did not become limiting during these experiments. For the nitrate and perchlorate groups, an additional control flask was prepared, to which no acceptor (nitrate or perchlorate) was added. This control flask was setup to confirm that the culture was not using oxygen as an electron acceptor in those experiments. All flasks were prepared in a Coy Environmental Chamber with nitrogen headspace. After preparation of the flasks for each group, two subsamples (8 mL) were extracted from each flask and added to 10-mL screw-cap spectrophotometer tubes. These tubes were then inoculated with JPLRND to an initial optical density (OD) of ~0.03. The tubes were sealed (to prevent oxygen intrusion) and incubated at 22°C. In the experiment in which growth on oxygen was tested, the culture was inoculated into 250-mL Erlenmeyer flasks rather than the 10-ml spectrophotometer tubes to minimize the potential for oxygen depletion. In addition, the flasks were placed on a rotary shaker operating at 200 rpm to ensure that oxygen transfer did not become limiting during the experiment. The OD was used as a measure of the microbial population in each experiment. For the nitrate and perchlorate experiments, the OD was measured by simply placing the 10-ml tube in the spectrophotometer. For the oxygen experiment, the OD was measured by collecting a 5-mL subsample from the experimental flasks and placing it in an OD-tube for OD measurement.

###### Results

The specific growth rate of JPLRND at each acetate (the electron donor) concentration was determined by plotting the natural log of the optical density (x 1000) versus time. The rate of change of optical density (i.e., the slope of the line in the plot) represents the growth rate of JPLRND at that acetate concentration. The growth of JPLRND on acetate at 250 mg/L with two different electron acceptors, perchlorate and oxygen, is presented in Figure 55. The slope of each curve shown in the figure was taken from the steepest portion of the OD versus time curve. Again, the slope value represents the growth rate of the culture at the donor concentration at the beginning of the experiment. A plot of the growth rate versus starting donor concentration can be constructed for each electron acceptor (Figures 56, 57, 58). This plot

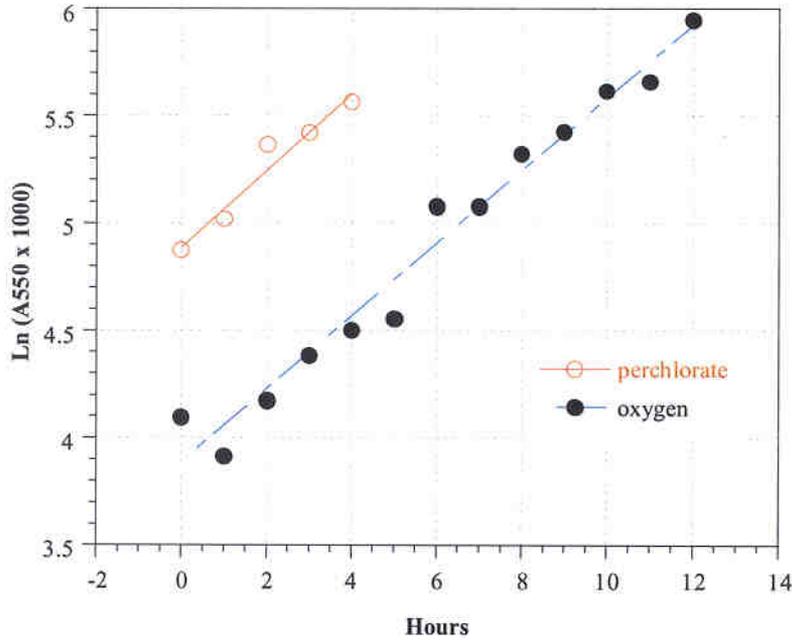
represents the Monod growth curve for JPLRND using acetate. Non-linear regression analysis was used to fit the Monod curves in Figures 56, 57, and 58 using the model presented in Appendix B.

The model was fit to the Monod curve by numerically solving the equations presented in Appendix B and varying values of the maximum specific growth rate ( $k_{max}$ ) and the half-saturation constant ( $K_S$ ) to minimize the difference between data and model. The model fits are shown in Figures 56, 57, and 58. The fitted  $k_{max}$  and  $K_S$  values are presented in Table 5. The maximum growth rates of the culture on acetate when it was grown in the presence of perchlorate and nitrate were  $0.14 \text{ h}^{-1}$  and  $0.15 \text{ h}^{-1}$ , respectively. The growth rate of the culture in the presence of oxygen was slightly higher ( $0.21 \text{ h}^{-1}$ ). The half-saturation constants for acetate with perchlorate, nitrate, and oxygen as electron acceptors were 120 mg/L, 70 mg/L, and 90 mg/L, respectively. The relatively low variability of these parameters among the different electron acceptors suggests that our assumption that the different electron acceptors do not significantly influence the biodegradation kinetics is valid.

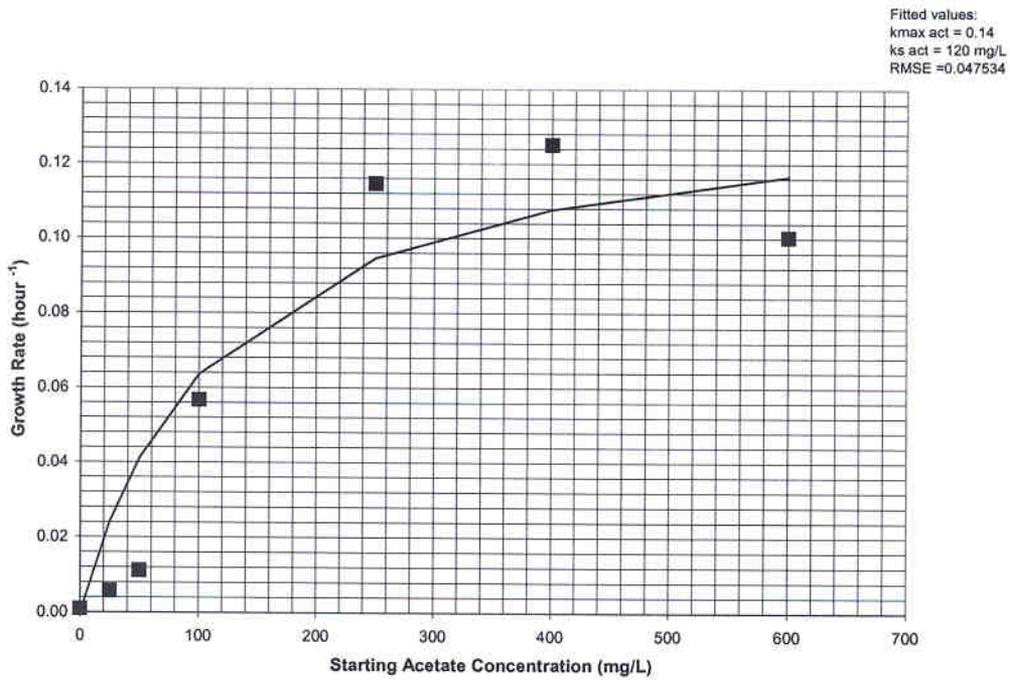
## **Electron Acceptor Parameters**

### **Methods and Results**

Similar experiments were performed to determine the kinetic parameters for each electron acceptor when the starting acetate concentration was constant and not limiting (i.e., excess acetate remained at the end of the experiment), while the electron acceptor was limiting. The parameters were determined from the experimental data similarly to the method used for obtaining the electron donor parameters. The growth rate of JPLRND at each electron acceptor concentration was taken as the steepest part in the optical density ( $\times 1000$ ) versus time graph. A Monod curve for each electron acceptor was made by plotting the growth rate (i.e., the slopes of the steepest part of the curve) versus the starting electron acceptor concentration. Non-linear regression was then used to fit of the model presented in Appendix B to the Monod curve. As described above, the equations in Appendix B were solved numerically and fit to the data by varying the maximum specific degradation rate and the half saturation constant for the electron acceptors. The “variable electron acceptor” experiments were performed only for perchlorate and nitrate, and not for oxygen. The Monod curves for these two experiments are presented in Figures 59 and 60 with the corresponding fitted model parameters presented in Table 6. The maximum specific growth rates for nitrate and perchlorate were  $0.21 \text{ h}^{-1}$  and  $0.071 \text{ h}^{-1}$ , respectively. These values are within a factor of two of the maximum specific growth rates determined during the electron donor studies above. As discussed above, it was assumed that the maximum specific growth rate of the culture does not significantly vary with the electron acceptor being used by the culture. Based upon the fact that the maximum specific growth rates vary only over a factor of two for all the different experiments conducted (see Tables 5 and 6), this assumptions seems to be valid. The half-saturation constants for nitrate and perchlorate during these experiments were 180 mg/L and 150 mg/L, respectively.



**Figure 55. Growth of JPLRND on Acetate with Either Oxygen or Perchlorate as Electron Acceptor.**



**Figure 56. Growth Rate of JPLRND on Acetate with Perchlorate as Electron Acceptor: Acetate Varied; Determination of  $K_s$  and  $k_{max}$ .**

Fitted values:  
k<sub>max act</sub> = 0.21  
k<sub>s act</sub> = 90 mg/L  
RMSE: 0.028561

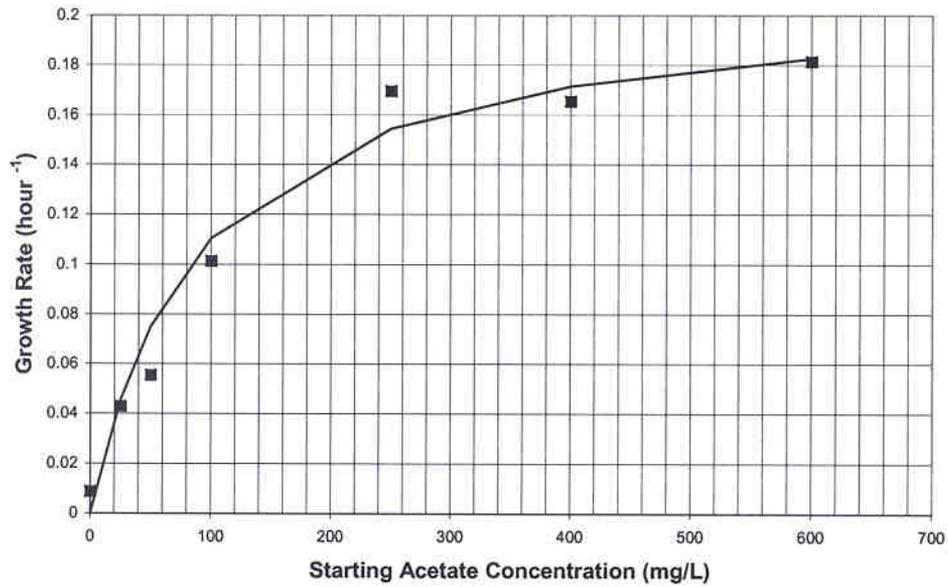


Figure 57. Growth Rate of JPLRND on Acetate with Oxygen as Electron Acceptor: Acetate Varied; Determination of  $K_s$  and  $k_{max}$ .

Fitted values:  
k<sub>max act</sub> = 0.145  
k<sub>s act</sub> = 70  
RMSE= 0.03066

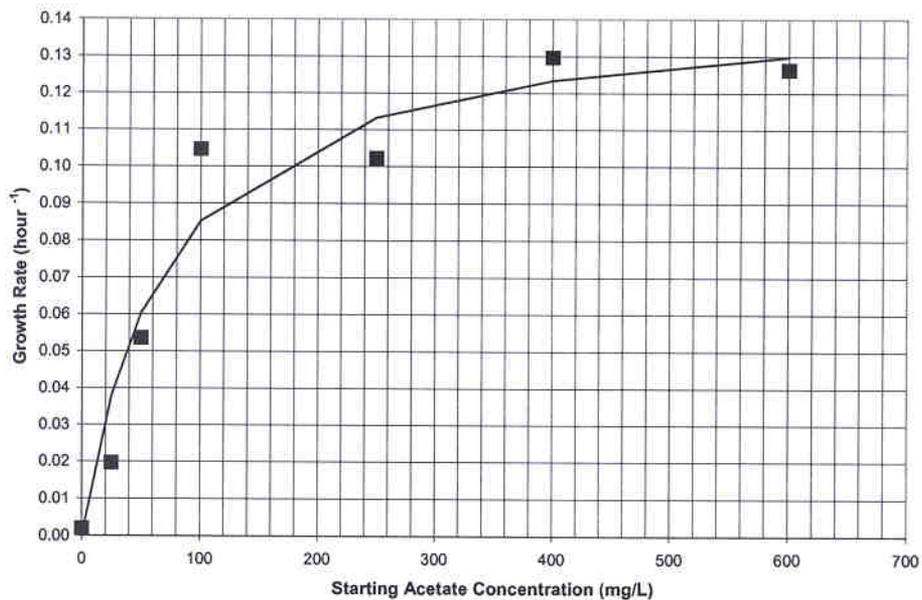


Figure 58. Growth Rate of JPLRND on Acetate with Nitrate as Electron Acceptor: Acetate Varied; Determination of  $K_s$  and  $k_{max}$ .

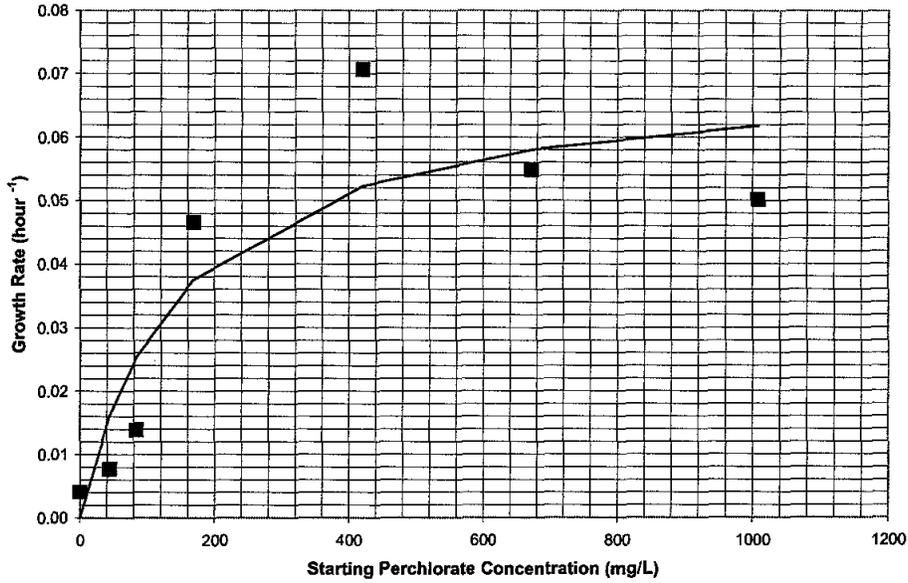
**Table 5. Growth Rate Parameters with the Electron Donor (Acetate) Varied.**

Parameter (units)	Value	Method of Determination
$k_{\max}^{\text{don/per}} \text{ (h}^{-1}\text{)}$	0.140	Determined by measuring OD550 values of the culture with acetate (substrate) varied and acceptor in excess, constructing the Monod curve for acetate, and fitting the model to this curve (Figure 56). This parameter represents the “asymptotic” rate value observed in Figure 56.
$k_{\max}^{\text{don/nit}} \text{ (h}^{-1}\text{)}$	0.145	Determined by measuring OD550 values of the culture with acetate (substrate) varied and acceptor in excess, constructing the Monod curve for acetate, and fitting the model to this curve (Figure 58). This parameter represents the “asymptotic” rate value observed in Figure 58.
$k_{\max}^{\text{don/oxy}} \text{ (h}^{-1}\text{)}$	0.21	Determined by measuring OD550 values of the culture with acetate (substrate) varied and acceptor in excess, constructing the Monod curve for acetate, and fitting the model to this curve (Figure 57). This parameter represents the “asymptotic” rate value observed in Figure 57.
$K_S^{\text{don/per}} \text{ (mg/L)}$	120	Determined as the acetate concentration where the growth rate in the Monod curve for acetate and perchlorate (Figure 56) is $\frac{1}{2} k_{\max}$ .
$K_S^{\text{don/nit}} \text{ (mg/L)}$	70	Determined as the acetate concentration where the growth rate in the Monod curve for acetate and nitrate (Figure 58) is $\frac{1}{2} k_{\max}$ .
$K_S^{\text{don/oxy}} \text{ (mg/L)}$	90	Determined as the acetate concentration where the growth rate in the Monod curve for acetate and oxygen (Figure 57) is $\frac{1}{2} k_{\max}$ .

**Table 6. Growth Rate Parameters with the Electron Acceptors (Nitrate and Perchlorate) Varied.**

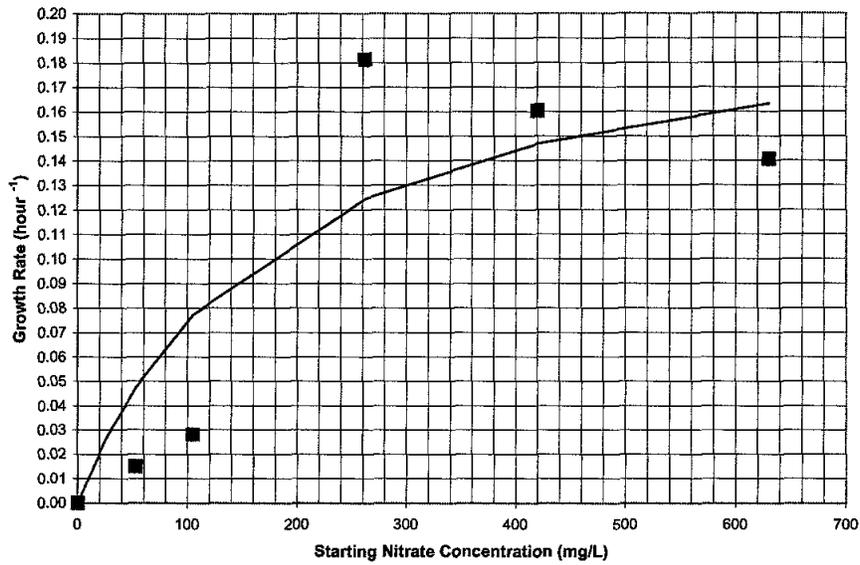
Parameter (units)	Value	Method of determination
$k_{\max}^{\text{per/don}} \text{ (h}^{-1}\text{)}$	0.071	Determined by measuring OD550 values of the culture with acetate (substrate) in excess and perchlorate (electron acceptor) varied, constructing the Monod curve for perchlorate, and fitting the model to this curve (Figure 59). This parameter represents the “asymptotic” rate value observed in Figure 59.
$k_{\max}^{\text{nit/don}} \text{ (h}^{-1}\text{)}$	0.21	Determined by measuring OD550 values of the culture with acetate (substrate) in excess and nitrate (electron acceptor) varied, constructing the Monod curve for nitrate, and fitting the model to this curve (Figure 60). This parameter represents the “asymptotic” rate value observed in Figure 60.
$K_S^{\text{per/don}} \text{ (mg/L)}$	150	Determined as the perchlorate concentration where the growth rate in the Monod curve for perchlorate and acetate (Figure 59) is $\frac{1}{2} k_{\max}$ .
$K_S^{\text{nit/don}} \text{ (mg/L)}$	180	Determined as the nitrate concentration where the growth rate in the Monod curve for nitrate and acetate (Figure 60) is $\frac{1}{2} k_{\max}$ .

Fitted values:  
 $k_{max}$  PCL = 0.071  
 $K_s$  PCL = 150  
 RMSE = 0.02805



**Figure 59. Growth Rate of JPLRND on Acetate with Perchlorate as Electron Acceptor: Perchlorate Varied; Determination of  $K_s$  and  $k_{max}$ .**

Fitted values:  
 $k_{max}$  NIT = 0.21  
 $K_s$  NIT = 180 mg/L  
 RMSE: 0.09212



**Figure 60. Growth Rate of JPLRND on Acetate with Nitrate as Electron Acceptor: Nitrate Varied; Determination of  $K_s$  and  $k_{max}$ .**

#### 4.5.6.2. Experimental Quantification of Cell Yield and Decay

##### Cell Yield

To determine the biomass yield on perchlorate, nitrate, and oxygen, flasks were prepared with acetate as the electron donor and one of these three compounds as electron acceptor. The electron acceptor was added in excess based on stoichiometric requirements for acetate metabolism. In the case of oxygen, the culture was incubated aerobically with vigorous shaking to ensure adequate oxygen supply. After measuring the starting acetate concentration in each flask, the flasks were inoculated with JPLRND, and growth was measured by periodically taking optical density measurements on subsamples collected from each flask. When the culture reached the late phase of its exponential growth, the concentrations of electron donor and acceptor (nitrate or perchlorate) in each flask were quantified. At that point, a 500 – 750 mL volume from each flask was centrifuged to concentrate the bacterial cells. This concentrate was resuspended in 5 – 10 mL of water and filtered under vacuum through an oven-dry cellulose filter (47 mm diameter, 0.45 micron pore-size; Gelman Sciences). The filter was oven-dried (at 105°C for 24 hours) to determine the weight of biomass in each flask. Consequently, the mass of dried biomass produced per mass of electron donor (i.e., the yield coefficient) was determined.

*Dechlorosoma suillum* JPLRND was grown on 1250 mg/L acetate and 1750 mg/L perchlorate to determine the yield of the culture while using perchlorate as an electron acceptor. During this experiment, JPLRND consumed 1246 mg of acetate and 961 mg of perchlorate, giving a ratio of 1.30 mg acetate/mg perchlorate utilized. The consumption of acetate by JPLRND was nearly twice that expected based on stoichiometric calculations (0.69 mg acetate per mg perchlorate). The cell yield on acetate was 0.173 mg dry biomass per mg acetate utilized. This corresponds to 0.225 mg dry biomass per mg perchlorate consumed.

The yield of JPLRND during growth on acetate while utilizing nitrate was slightly lower than for perchlorate. The strain metabolized 560 mg of acetate and 682 mg of nitrate to produce 73.3 mg of biomass. Based on these numbers, the yield of the strain was 0.131 mg biomass/mg acetate and 0.107 mg biomass/mg nitrate. The ratio of electron donor to electron acceptor was 0.82 mg acetate/mg nitrate consumed. This value is close to the calculated stoichiometric ratio of 0.76 mg acetate/mg nitrate consumed.

Two yield studies were performed with oxygen as the electron acceptor. In the first study, JPLRND utilized 1030 mg acetate to produce 326 mg of biomass, giving a yield of 0.317 mg dry biomass per mg acetate consumed. In the second study, the cells consumed 966 mg acetate to produce 313 mg biomass for a yield of 0.324 mg dry biomass per mg acetate consumed. Oxygen consumption was not

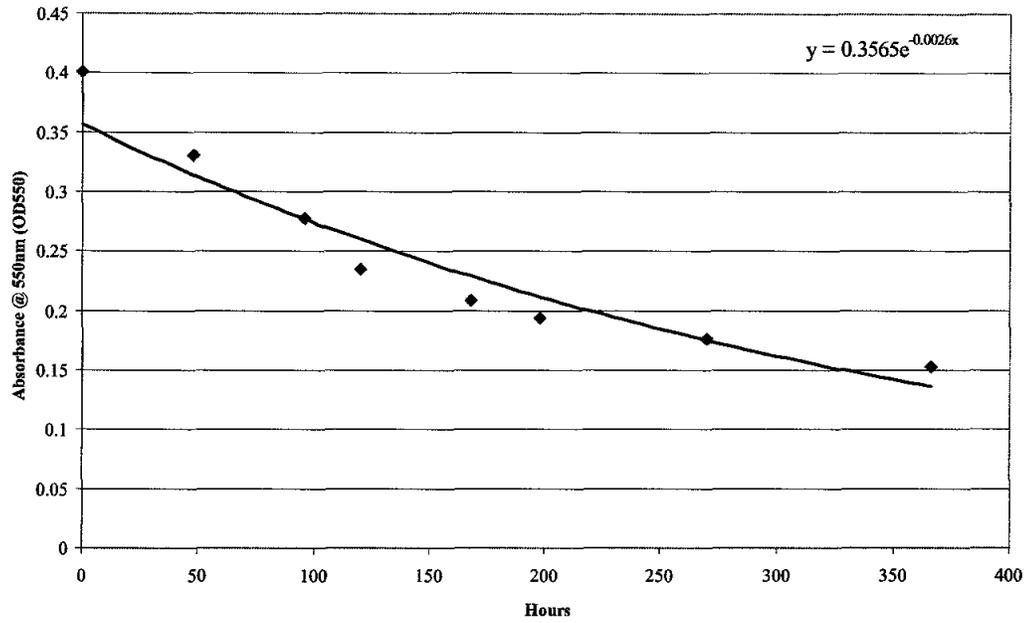
measured in these studies. Overall, the cell yield varied between 0.131 to 0.324 mg biomass/mg acetate, with an average value of 0.236 mg biomass/mg acetate.

### **Cell Decay**

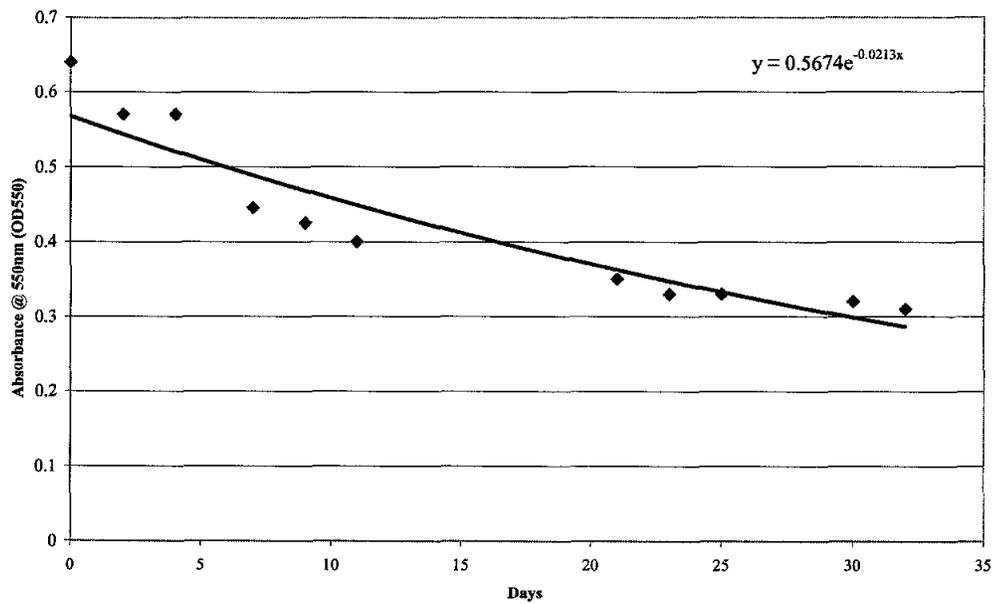
Two experiments were conducted to quantify the cell decay rate of *D. suillum* JPLRND after growth on acetate with oxygen as the electron acceptor. In the first experiment, the bacterium was inoculated into an Erlenmeyer flask containing BSM and 500 mg/L acetate. In the second experiment, the bacterium was inoculated into an Erlenmeyer flask containing BSM and 1,500 mg/L acetate. The flasks were shaken at 22°C and 200 rpm. When the culture reached the stationary phase of growth (acetate was depleted), cell numbers were measured by optical density (absorbance at 550 nm). Dilution plating was also done at that point to compare absorbance measurements with actual microbial populations. The decay curves after growth on acetate and oxygen are shown in Figures 61 and 62. The decay rate obtained from these graphs were 0.0026 h<sup>-1</sup> and 0.0213 h<sup>-1</sup>, respectively.

Two experiments were conducted to quantify the cell decay rate of *D. suillum* JPLRND after growth on acetate with nitrate as the electron acceptor. In the first experiment, the culture was grown on 750 mg/L nitrate and 500 ppm acetate. In the second experiment, the culture was grown on 750 mg/L nitrate and 1000 ppm acetate. When the culture reached stationary phase, and the acetate was depleted, cell numbers were measured by optical density (absorbance at 550 nm). Dilution plating was also done to verify absorbance measurements. The decay curves after growth on acetate and nitrate are shown in Figure 63 and 64. The decay rates obtained from these graphs were 0.0066 h<sup>-1</sup> and 0.0026 h<sup>-1</sup>, respectively.

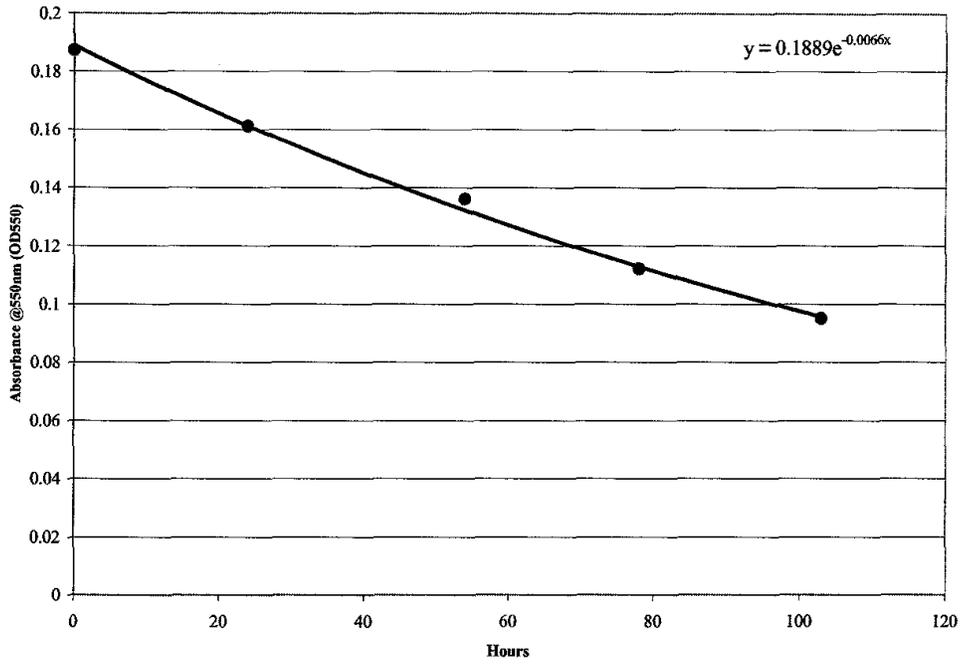
One experiment was conducted to quantify the cell decay rate of *D. suillum* JPLRND after growth on acetate with perchlorate as the electron acceptor. The culture was grown on 1000 mg/L perchlorate and 600 mg/L acetate. Measurements were taken as described for the other decay experiments. The decay curve after growth on acetate and perchlorate is shown in Figure 65. The decay rate obtained from this graph was 0.0388 h<sup>-1</sup>. The decay rates for the *D. suillum* JPLRND after growth on the three of electron acceptors ranged from 0.0026 to 0.0388 h<sup>-1</sup>, with an average of 0.014 h<sup>-1</sup>.



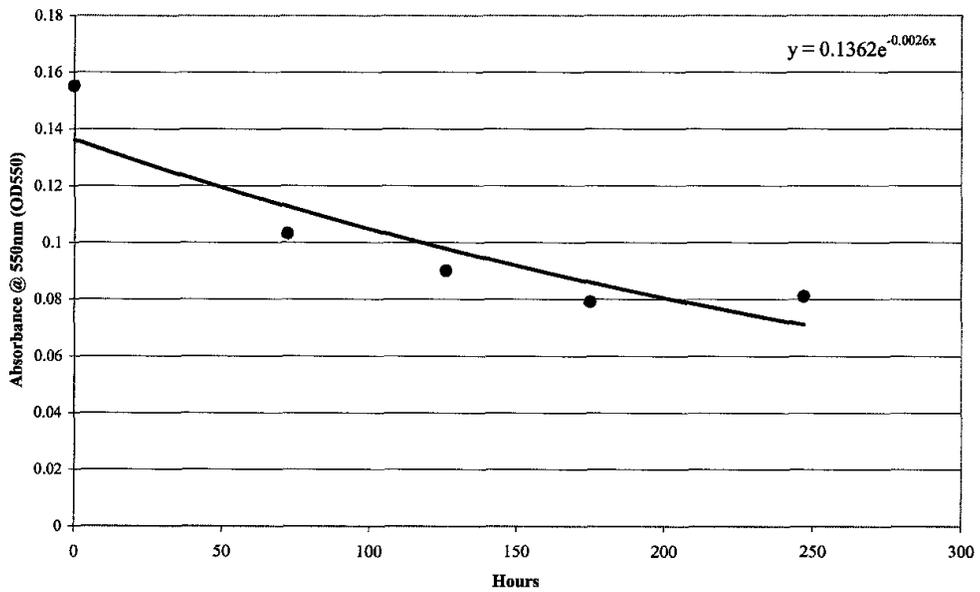
**Figure 61. Microbial Decay Curve for JPLRND after Growth on Oxygen and 500 mg/L Acetate.**



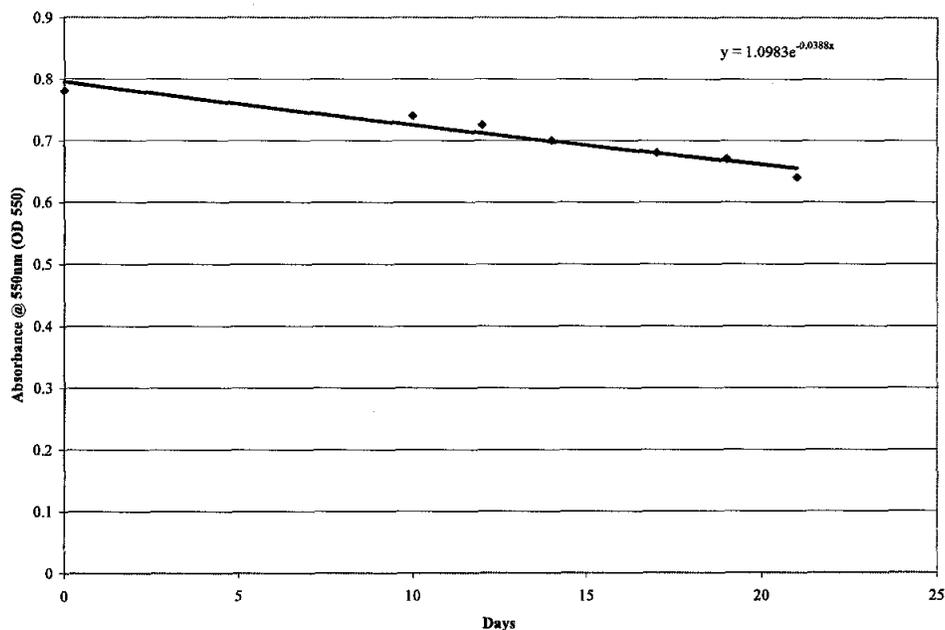
**Figure 62. Microbial Decay Curve for JPLRND after Growth on Oxygen and 1500 mg/L Acetate.**



**Figure 63. Microbial Decay Curve for JPLRND after Growth on Nitrate (750 mg/L) and Acetate (500 mg/L).**



**Figure 64. Microbial Decay Curve for JPLRND after Growth on Nitrate (750 mg/L) and Acetate (1000 mg/L).**



**Figure 65. Microbial Decay Curve for JPLRND after Growth on Perchlorate (1000 mg/L) and Acetate (600 mg/L).**

#### 4.5.6.3 Utilization of Competing Electron Acceptors

Laboratory studies were conducted to better understand the relationship among competing electron acceptors, particularly nitrate, perchlorate, and oxygen. These studies are important as a conceptual basis for the perchlorate biodegradation model and for a more thorough understanding of factors influencing perchlorate biodegradation in subsurface environments.

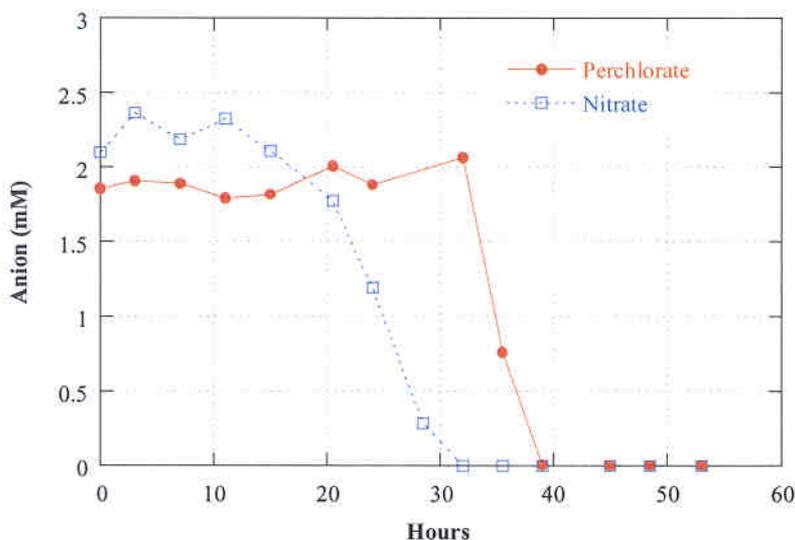
#### Methods

In one experiment, nitrate and perchlorate were added together in flasks with acetate as the electron donor. The degradation of the two electron acceptors by three JPLRND cultures grown on nitrate, perchlorate, and oxygen, respectively, was evaluated. These experiments were conducted to determine whether the initial growth conditions of the culture (i.e., which electron acceptor was used) influence the rate or order of degradation of nitrate and perchlorate by the strain. In this study, *D. suillum* JPLRND was grown to early stationary phase in media containing acetate as an electron donor and perchlorate, nitrate, or oxygen as the electron acceptor. Each culture was then centrifuged, resuspended in BSM media to the same density, then inoculated into sterile 1000-mL Erlenmeyer flasks containing 400 mL of BSM with acetate (8 mM) and a mixture of nitrate and perchlorate at 2 mM each. The flasks were incubated with shaking in a Coy Environmental Chamber with a nitrogen headspace. Subsamples were

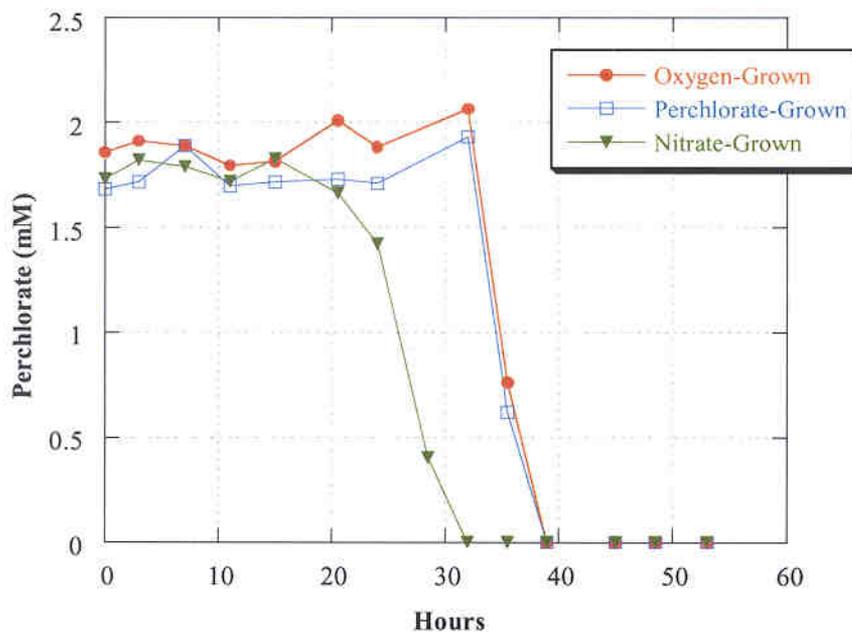
periodically removed from the flasks, filtered through a 0.22-micron syringe filter, and analyzed for nitrate, perchlorate, and acetate.

## Results

There was a lag period of 20 hrs or more before degradation of perchlorate or nitrate was observed. Then, in each treatment (oxygen-, nitrate-, or perchlorate-grown cells), the bacterium degraded nitrate prior to reducing perchlorate. A graph of perchlorate and nitrate degradation by the oxygen-grown strain is provided in Figure 66. Data for the other two electron acceptors were similar, although the lag periods for both nitrate and perchlorate reduction were appreciably shorter for the nitrate-grown strain (perchlorate data are presented in Figure 67). These data show that, regardless of the electron donor upon which JPLRND initially grows, it will degrade nitrate first, followed by perchlorate. These results are similar to those observed for a different strain isolated by John Coates at Southern Illinois University (SERDP Project CU-1162).



**Figure 66. Degradation of Nitrate and Perchlorate by an Oxygen-Grown Culture of *D. suillum* JPLRND.**



**Figure 67. Biodegradation of Perchlorate by *D. suillum* JPLRND after Growth on Nitrate, Perchlorate, or Oxygen (Electron Acceptors) with Acetate as Electron Donor.**

#### 4.5.6.4. Influence of Nitrate and Oxygen on Perchlorate Biodegradation

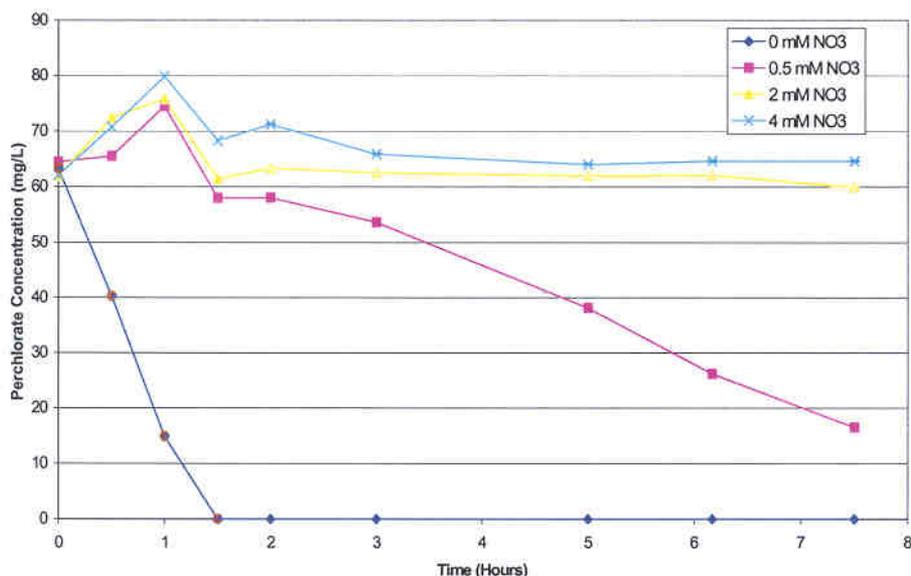
Experiments were conducted to evaluate the influence of nitrate and oxygen on perchlorate degradation by *D. suillum* JPLRND. Unlike the previous experiments, in which both nitrate and perchlorate were added to flasks simultaneously, in these studies, the culture was allowed to begin degrading perchlorate, then the second electron acceptor (oxygen or nitrate) was added.

##### 4.5.6.4.1 Influence of Nitrate on Perchlorate Reduction

###### Methods

An experiment was conducted to evaluate the influence of nitrate on perchlorate biodegradation. *Dechlorosoma suillum* JPLRND was grown in a 1-L flask using 750 mg/L acetate as electron donor and 500 mg/L perchlorate as electron acceptor. Subsamples of the culture media were collected and tested periodically using an ion specific probe to confirm that the culture was actively degrading perchlorate. When the perchlorate concentration dropped below 25 mg/L, additional perchlorate (200 mg/L; 2 mM) and acetate (460 mg/L; 8 mM) were added to the culture. After one hour, the perchlorate concentration had decreased to approximately 100 mg/L (~ 1 mM). At this point, 100 mL of the active culture was added to 4 sterile 125-mL flasks, and nitrate was added at approximately 0, 0.5, 2.0, or 4.0 mM. An

initial sample was taken from each flask, and subsequent 20-mL subsamples were taken every 0.5 hours for 2 hours, then at increasing time intervals for 22.5 hours. These subsamples were collected and filter-sterilized in the anaerobic chamber and analyzed for perchlorate and nitrate by ion chromatography. Select samples were also analyzed for acetate by gas chromatography. Data from this experiment are presented in Figure 68.



**Figure 68. Influence of Nitrate on Perchlorate Degradation by *D. suillum* JPLRND.**

## Results

After the addition of nitrate, perchlorate degradation quickly stopped in the flasks amended with 2 or 4 mM nitrate, and slowed dramatically in flasks receiving 0.5 mM nitrate. No change occurred in perchlorate concentrations in any of the flasks after 7.5 hrs, therefore, only data prior to that time are presented. Perchlorate degradation continued in the flask to which no nitrate was added, and the anion was below detection (< 5 mg/L by probe) by 1.5 hrs. Degradation of perchlorate did not resume in the flasks spiked with 2 mM or 4 mM nitrate, and nitrate was not degraded in these flasks during the experiment. In the flask to which 0.5 mM nitrate was added, perchlorate degradation ceased temporarily, but then continued at a slower rate. Perchlorate concentrations in this flask decreased from approximately 60 mg/L at 2 hrs to 16 mg/L after 7.5 hrs. Interestingly, nitrate was present in this flask at the end of the experiment at approximately 20 mg/L (the initial nitrate concentration in this flask was approximately 30

mg/L). About 300 mg/L (5 mM) acetate remained in each of the flasks at the end of the experiment (acetate data not shown). This indicates that sufficient acetate remained to support nitrate and/or perchlorate degradation.

The data indicate that, at least for this bacterial strain, nitrate is an inhibitor of perchlorate reduction at nitrate concentrations as low as 0.5 mM. The mechanism of this inhibition requires further study, which is beyond the scope of this research project. However, based upon the data collected, it is likely that nitrate is a competitive inhibitor of perchlorate reduction. Because of the short time required for nitrate to completely inhibit perchlorate degradation, it is unlikely that nitrate was inhibiting enzyme synthesis (i.e., of (per)chlorate reductase). It is more likely that nitrate was directly inhibiting enzyme activity.

#### 4.5.6.4.2 Estimation of a Nitrate Inhibition Factor for Perchlorate Reduction.

Nitrate inhibition of perchlorate utilization was described in the biodegradation model by including a nitrate inhibition factor ( $K_i^{nit}$ ) in the model equations (Appendix B, Equation 1d). This factor was estimated from experimental data from a flask study in which both nitrate and perchlorate were present and using all known or experimentally-estimated parameters in the biodegradation model. The known values included the starting nitrate and perchlorate concentrations (oxygen concentration was < 1 mg/L). Experimentally-estimated parameters included: the ratio of substrate consumed per mass of acceptor utilized,  $k_{max}$ , the half-saturation constants for each compound, the starting biomass concentration, the biomass yield coefficient and the biomass decay rate. Given all these parameters, the  $K_i^{nit}$  value was varied to obtain the best visual model fit to the data.

Initially, variation of  $K_i^{nit}$  alone did not result in good fits to the data. This indicated that one or more of the other estimated parameters did not represent conditions in the flasks. Upon investigation of experimental conditions, it was determined that the estimate for the initial biomass (30 mg/L) was the least accurate value in the model. This initial biomass value was measured at the start of the experiment, prior to enzyme induction and biomass growth (i.e., prior to the lag period). In the model, it is assumed that the biomass immediately undergoes exponential growth and decay. Thus, the initial biomass value input into the model must represent the biomass concentrations immediately before exponential growth starts. Consequently, it was determined that the measured initial biomass value (i.e., 30 mg/L) likely underestimated the actual biomass in the flasks when biodegradation commenced. Thus, the starting biomass value was increased in the model. This increase greatly improved model fits to the data. The biomass concentration was increased by an order of magnitude to 500 mg/L. This increase is believed to be reasonable because, in several experiments, including the one used to evaluate  $K_i^{nit}$ , the biomass increased by 1 to 2 orders of magnitude during the lag phase of the experiments. Given the higher initial

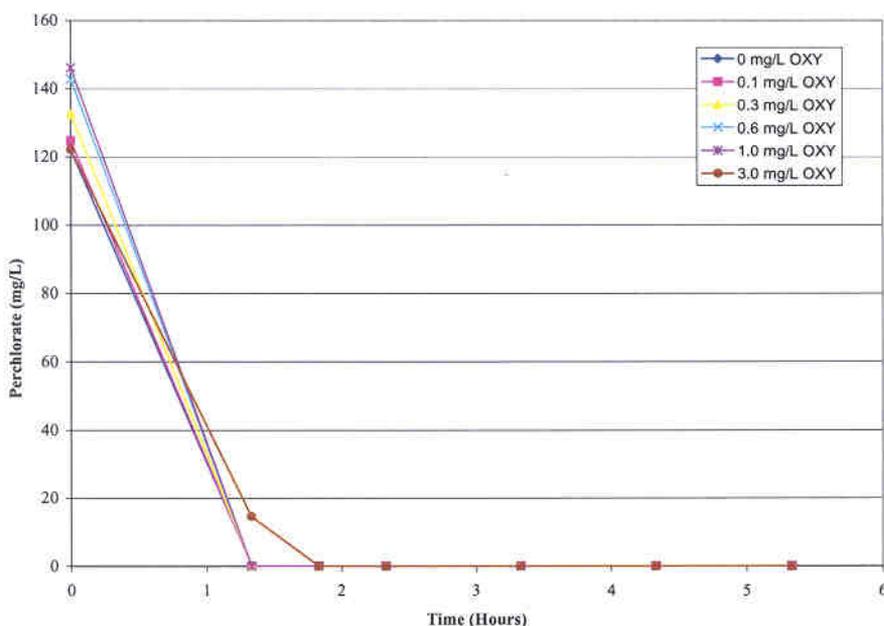
biomass value,  $K_i^{nit}$  was varied to obtain the optimal fit. The best-fit  $K_i^{nit}$  value was determined to be 7 mg/L. This is consistent with experimental data that indicate that nitrate concentrations as low as 0.5 mM (30 mg/L) inhibit perchlorate degradation. However, the effects of nitrate concentrations below 30 mg/L on perchlorate degradation were not directly examined.

#### 4.5.6.4.3 Influence of Oxygen on Perchlorate Reduction

An experiment was conducted to evaluate the effect of oxygen on perchlorate utilization. Although perchlorate degradation is known to be an anoxic process, data related to the minimum oxygen concentration at which perchlorate reduction occurs are not currently available in the literature. These values, however, have been reported for denitrification (i.e., inhibition of nitrate reductase enzyme(s) by oxygen). The experiments for evaluating the effect of oxygen on perchlorate utilization were performed in a similar fashion to the nitrate experiments described above, except that different amounts of oxygen (rather than nitrate) were added to a culture that was actively degrading perchlorate.

A culture of *Dechlorosoma suillum* JPLRND was originally grown on 1000 mg/L each of perchlorate and acetate. When the perchlorate was degraded to less than 10 mg/L, the culture was spiked with an additional 500 mg/L of perchlorate. The perchlorate was then degraded to approximately 100 mg/L, at which point an additional 2 mM perchlorate (approximately 200 mg/L) and 8 mM acetate (approximately 460 mg/L) were added. When the perchlorate concentration reached approximately 200 mg/L, 100 mL of the active culture was added to each of six sterile 160-mL serum bottles. The bottles were sealed with butyl rubber stoppers and crimp caps. A time-zero sample was taken from each bottle for perchlorate analysis, and the bottles were then removed from the anaerobic chamber. Differing volumes of air were added to each bottle based on the assumption that if the headspace of each bottle were completely filled with air and at equilibrium, the dissolved oxygen concentration in the liquid would be approximately 8.2 mg/L. Different fractions of the nitrogen headspace in each bottle were removed and replaced with an equivalent volume of air to establish the following dissolved oxygen concentrations: 0 mg/L, 0.1 mg/L, 0.3 mg/L, 0.6 mg/L, 1.0 mg/L, and 3.0 mg/L. The bottles were shaken vigorously by hand for approximately 5 minutes to speed dissolution of the headspace air into the liquid phase. The bottles were then placed on a rotary shaker in the anaerobic chamber for 1.3 h before the next sample was taken. A colorimetric test method (Chemets; Chemetrics, Calverton, VA) was used to measure the initial dissolved oxygen concentration in each bottle. However, the results from this test were determined to be inaccurate based on results from control samples. It is likely that the mineral salts solution used for the study interfered with measurement of oxygen by this technique. Therefore, the initial oxygen concentrations in solution could not be experimentally determined.

Perchlorate was degraded to below detection (< 5 mg/L by probe) by 1.3 hrs in all bottles except in the bottle to which the greatest volume of air was added. In this bottle, the rate of perchlorate biodegradation slightly decreased (Figure 69). This result suggests that oxygen inhibits perchlorate degradation at 3 mg/L, not at lower concentrations. However, because accurate measurements of dissolved oxygen concentrations could not be obtained, the results of this study may have several explanations. It is possible that the target levels of dissolved oxygen were not achieved in the 5 minutes of vigorous shaking, that the range of oxygen concentrations used was too low, or that the culture actually degraded the oxygen while continuing to degrade perchlorate.



**Figure 69. Influence of Oxygen on Perchlorate Degradation by *D. suillum* JPLRND.**

#### 4.5.6.5 Model Simulation of Perchlorate and Nitrate Biodegradation by *D. suillum* JPLRND

Experimental data from a flask study in which both nitrate and perchlorate were initially present were used for comparison with model simulations. The data were presented previously in Figure 66. Several simulations of the model were run with varying parameter values to determine the sensitivity of the biodegradation model to changes in individual parameters. All known or experimentally-estimated parameters were initially used in the model. After the optimal fit was obtained (by varying the initial

biomass and  $K_i^{nit}$ ), the sensitivity analysis was performed. The sensitivity analysis was performed on the following parameters: nitrate inhibition factor ( $K_i^{nit}$ ), biomass decay rate ( $b$ ), and the starting biomass concentration ( $B_0$ ). The sensitivity analysis included the latter two parameters because each varied by more than an order of magnitude during the fitting process. All other parameters were held constant during this analysis. The results of eight simulations are presented in Figures 70 through 77. In each of these simulations, one of the three parameters is varied, while the other two are kept at their “best-fit” value. The best-fit values for the three parameters are:  $K_i^{nit} = 7$  mg/L,  $b = 0.014$  hr<sup>-1</sup>, and  $B_0 = 500$  mg/L.

In Figures 70 through 73, the starting biomass concentration ( $B_0$ ) is varied, while the nitrate inhibition factor ( $K_i^{nit}$ ) remains constant at 7 mg/L and the decay rate ( $b$ ) is set at 0.014 hr<sup>-1</sup>. At  $B_0 = 30$  mg/L (Figure 70), the biomass curve is linear, and very little nitrate or perchlorate degradation is observed. Degradation of both compounds improves when  $B_0$  is increased to 100 mg/L (Figure 71), and the biomass curve remains relatively linear. At  $B_0 = 300$  mg/L (Figure 72), decay begins to dominate when nitrate and perchlorate are depleted. At  $B_0 = 500$  mg/L (Figure 73), the model fit with the data is optimal. The model is sensitive to starting biomass concentration, particularly when  $B_0$  is less than 300 mg/L. The starting biomass concentration used in the optimal model simulation (Figure 73) was 500 mg/L.

In Figures 74 and 75, the nitrate inhibition factor ( $K_i^{nit}$ ) is varied, while  $B_0$  is set at 500 mg/L and  $b$  remains constant at 0.014 hr<sup>-1</sup>. In each of these simulations, perchlorate degradation is affected by a change in  $K_i^{nit}$ , while nitrate degradation does not change. In addition, changes in  $K_i^{nit}$  resulted in only slight changes to the biomass curve. At  $K_i^{nit} = 0.7$  mg/L, the modeled perchlorate degradation was slower than the experimental data show (Figure 74). At  $K_i^{nit} = 70$  mg/L, the modeled perchlorate degradation is more rapid than the experimental data suggest (Figure 75). At  $K_i^{nit} = 7$  mg/L (Figure 73) the modeled perchlorate degradation curve gives a close fit to the experimental data. Therefore, the nitrate inhibition factor used in the optimal model simulation was  $K_i^{nit} = 7$  mg/L.

In Figures 76 and 77, the cell decay rate ( $b$ ) was varied, while  $B_0 = 500$  mg/L and  $K_i^{nit} = 7$  mg/L. At  $b = 0.0014$  hr<sup>-1</sup> (Figure 76), perchlorate and nitrate degradation simulated by the model are a close fit to the data with biomass growth and decay dominating the biomass curve. At  $b = 0.14$  hr<sup>-1</sup> neither perchlorate nor nitrate degradation are simulated well by the model, and biomass decay dominates the biomass curve (Figure 77). The decay value used in the optimal model simulation was 0.014 hr<sup>-1</sup>.

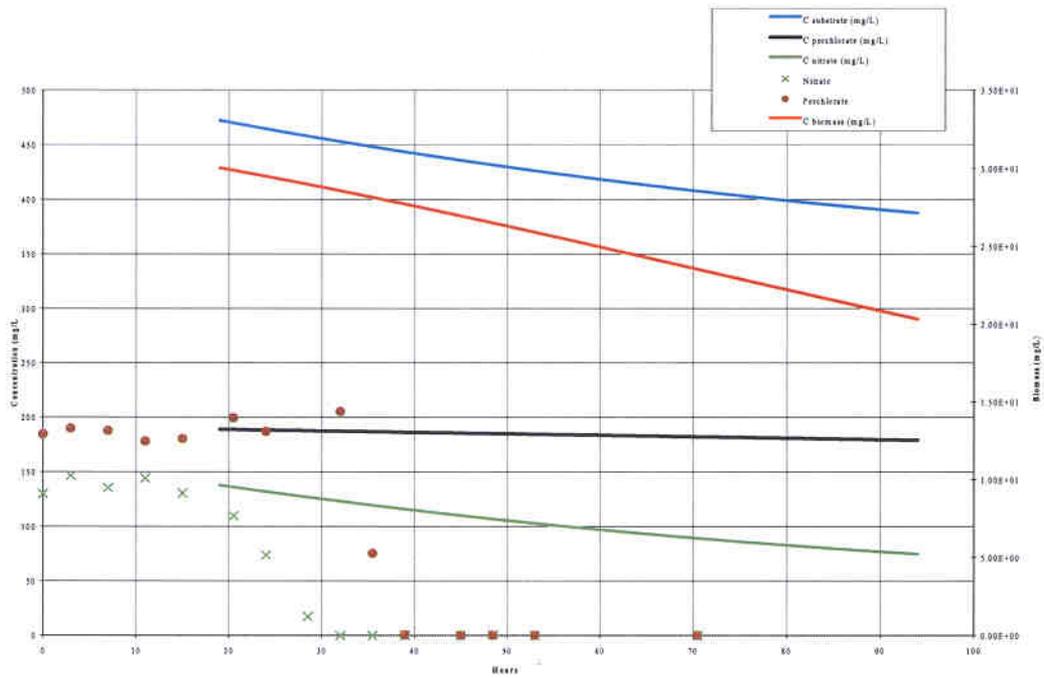


Figure 70. Model Simulation of Perchlorate and Nitrate Biodegradation by *D. suillum* JPLRND in Batch Culture:  $K_i^{nit} = 7$  mg/L,  $B_0 = 30$  mg/L, and  $b = 0.014$  hr<sup>-1</sup>.

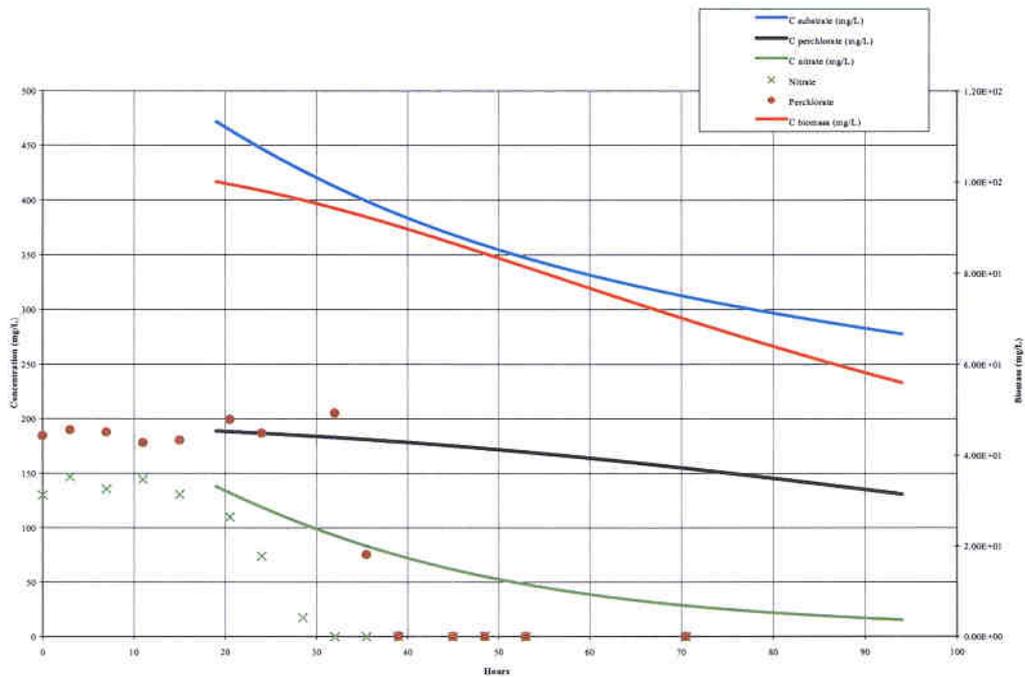


Figure 71. Model Simulation of Perchlorate and Nitrate Biodegradation by *D. suillum* JPLRND in Batch Culture:  $K_i^{nit} = 7$  mg/L,  $B_0 = 100$  mg/L, and  $b = 0.014$  hr<sup>-1</sup>.

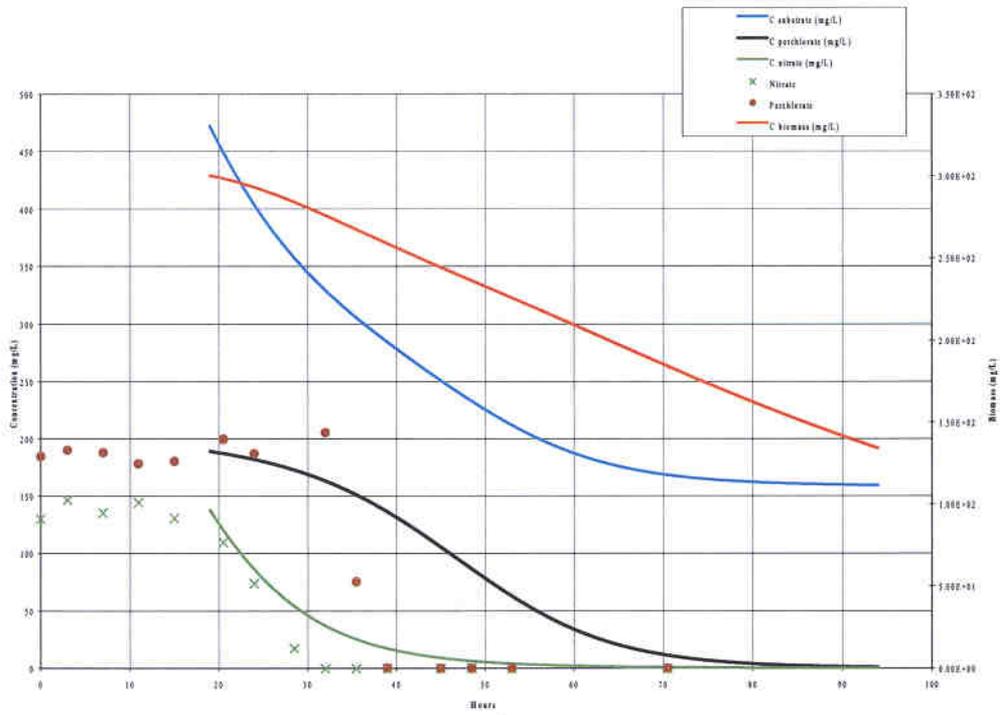


Figure 72. Model Simulation of Perchlorate and Nitrate Biodegradation by *D. suillum* JPLRND in Batch Culture:  $K_i^{nit} = 7$  mg/L,  $B_0 = 300$  mg/L, and  $b = 0.014$  hr<sup>-1</sup>.

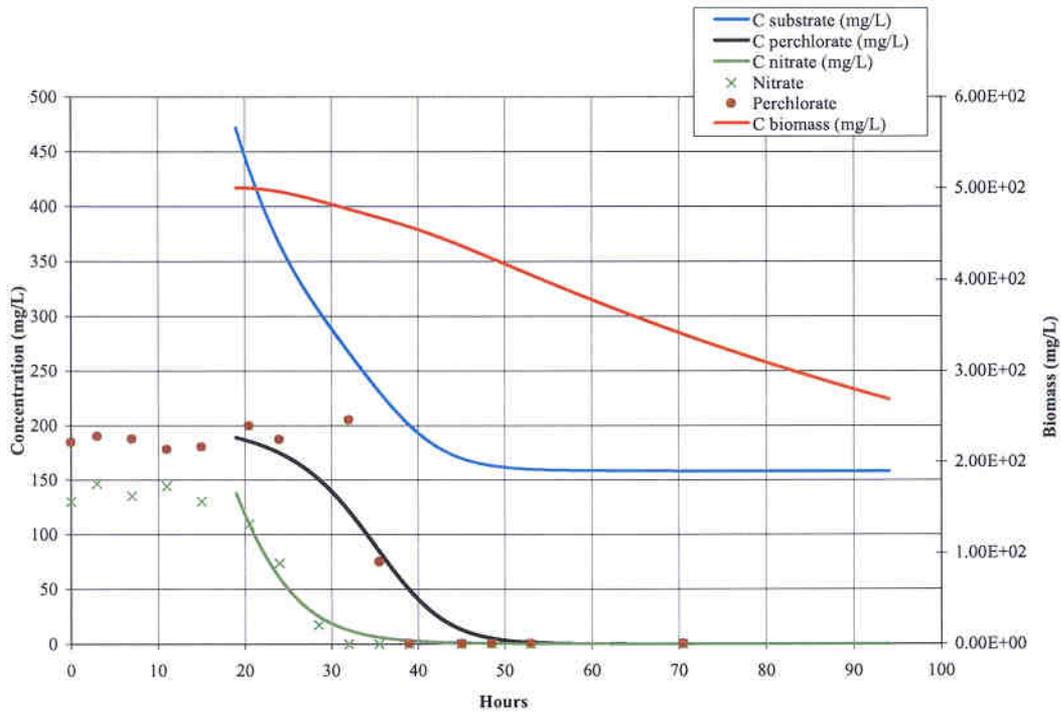


Figure 73. Model Simulation of Perchlorate and Nitrate Biodegradation by *D. suillum* JPLRND in Batch Culture:  $K_i^{nit} = 7$  mg/L,  $B_0 = 500$  mg/L, and  $b = 0.014$  hr<sup>-1</sup>.

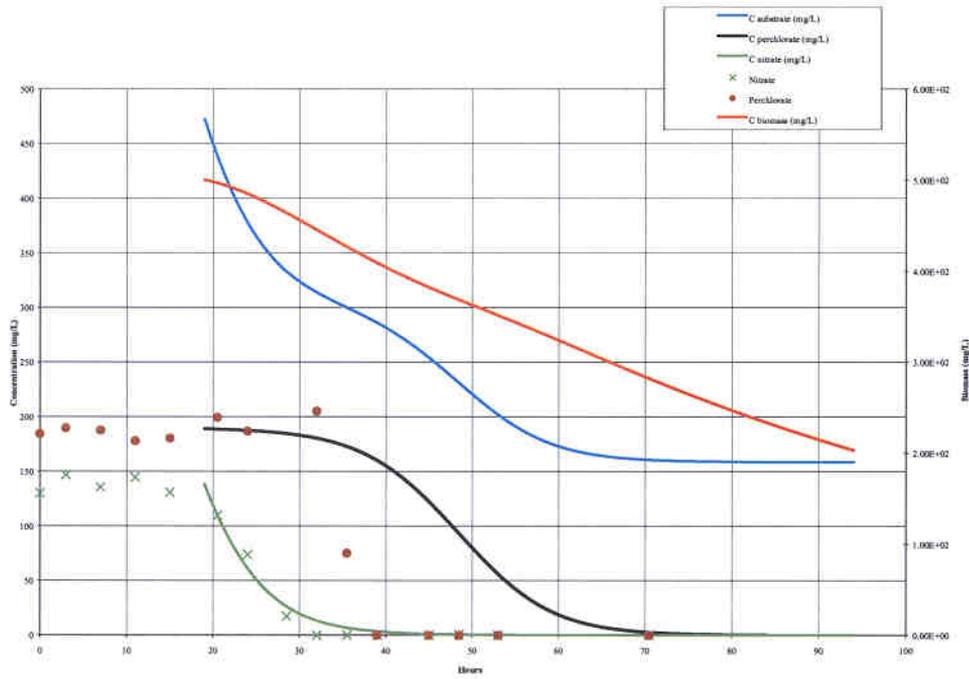


Figure 74. Model Simulation of Perchlorate and Nitrate Biodegradation by *D. suillum* JPLRND in Batch Culture:  $K_i^{nit} = 0.7$  mg/L,  $B_0 = 500$  mg/L, and  $b = 0.014$  hr<sup>-1</sup>.

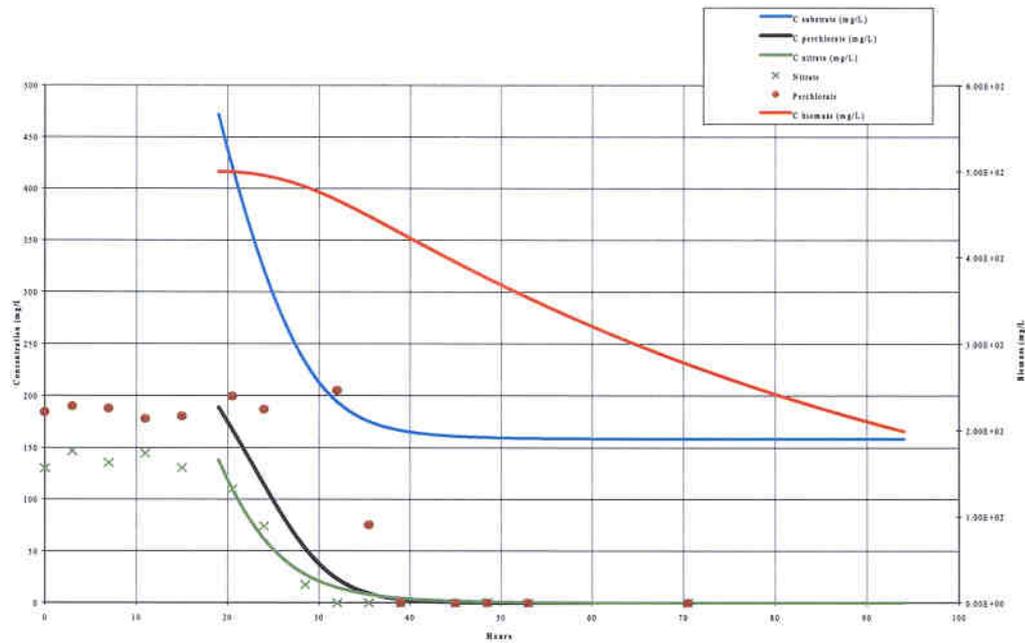


Figure 75. Model Simulation of Perchlorate and Nitrate Biodegradation by *D. suillum* JPLRND in Batch Culture:  $K_i^{nit} = 70$  mg/L,  $B_0 = 500$  mg/L, and  $b = 0.014$  hr<sup>-1</sup>.

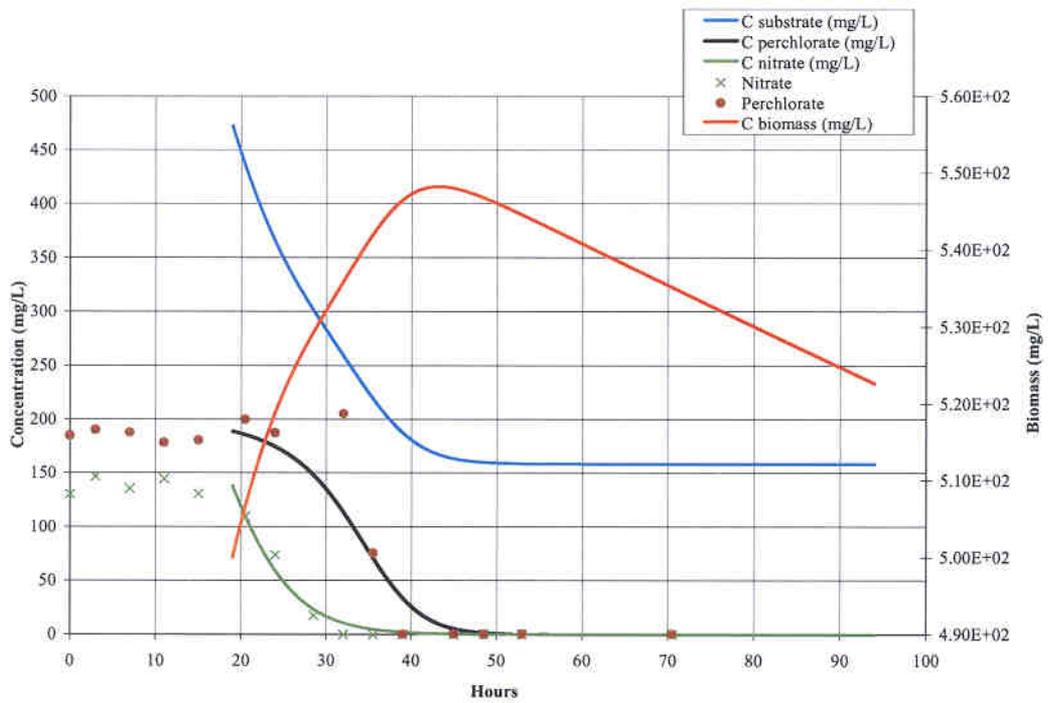


Figure 76. Model Simulation of Perchlorate and Nitrate Biodegradation by *D. suillum* JPLRND in Batch Culture:  $K_i^{nit} = 7 \text{ mg/L}$ ,  $B_0 = 500 \text{ mg/L}$ , and  $b = 0.0014 \text{ hr}^{-1}$ .

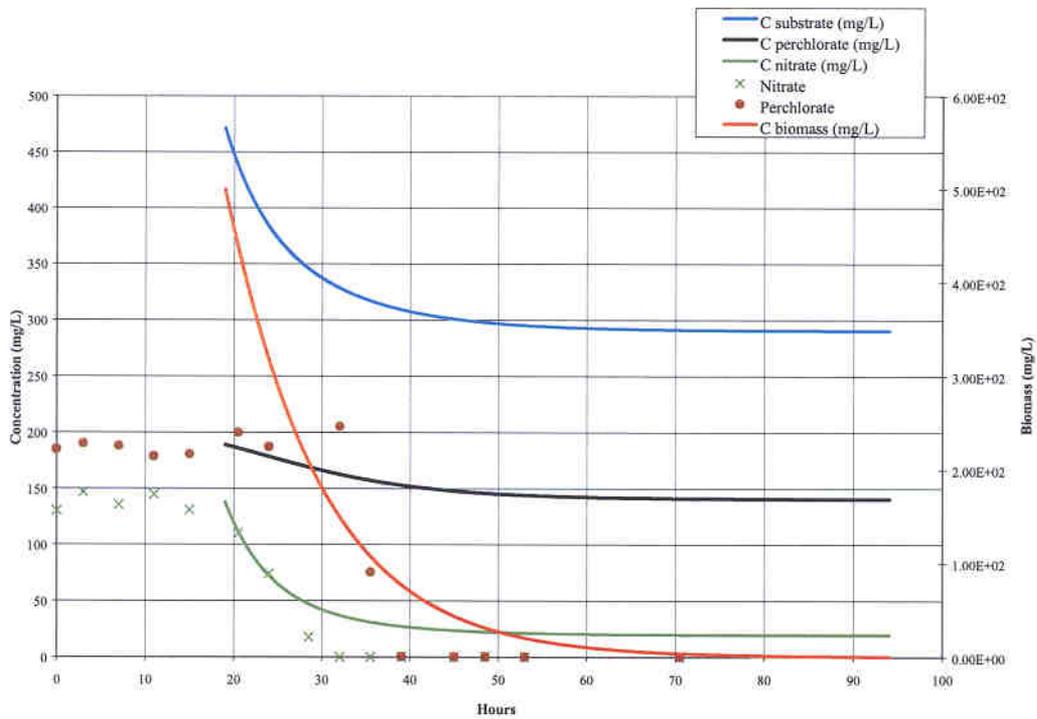


Figure 77. Model Simulation of Perchlorate and Nitrate Biodegradation by *D. suillum* JPLRND in Batch Culture:  $K_i^{nit} = 7 \text{ mg/L}$ ,  $B_0 = 500 \text{ mg/L}$ , and  $b = 0.14 \text{ hr}^{-1}$ .

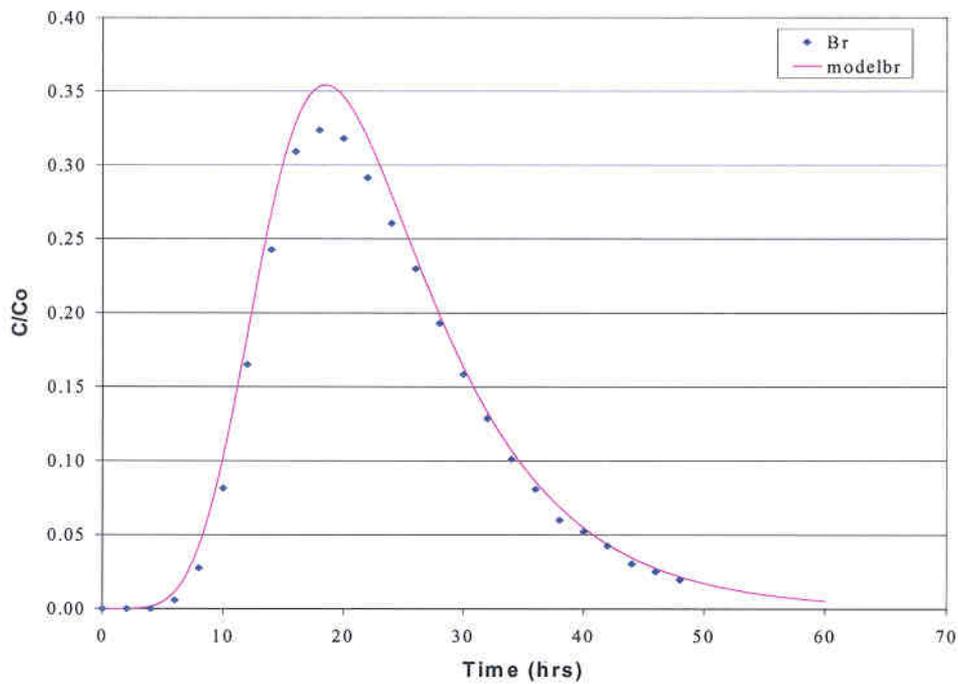
## 4.6 PERCHLORATE TRANSPORT MODEL

### 4.6.1 MODEL SIMULATION OF PERCHLORATE AND BROMIDE TRANSPORT IN THE MODEL AQUIFER

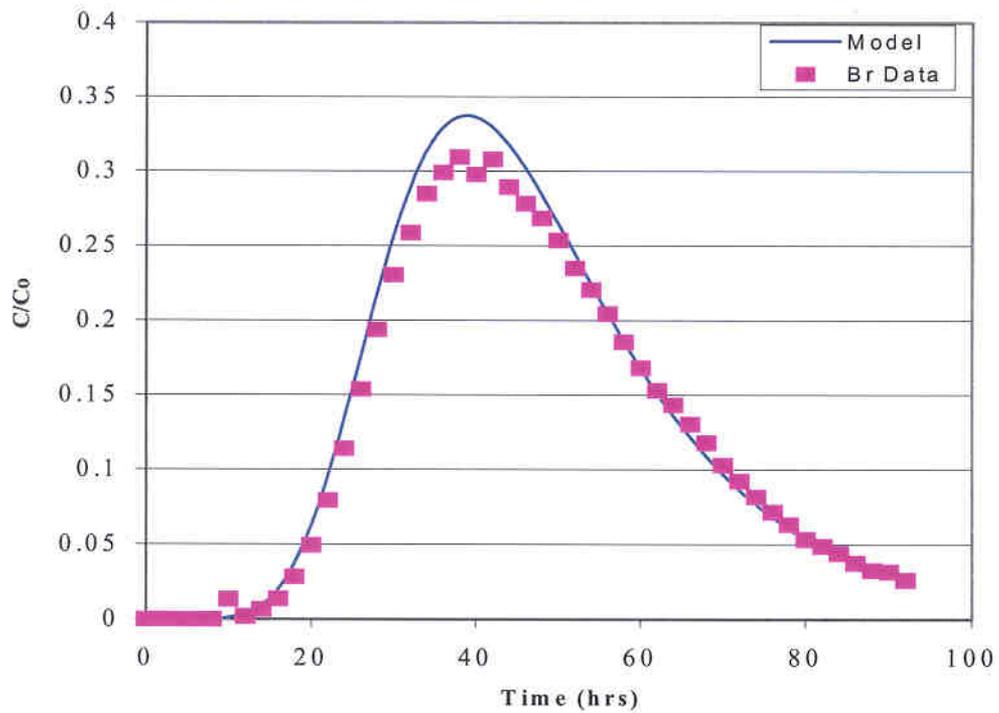
The software HydroBioGeoChem123D (HBGC123D) was used to describe the one-dimensional transport of bromide and perchlorate in a 50-cm laboratory column. This software was chosen because of its capability to describe the transport and consumption of multiple electron acceptors in groundwater. Several conservative transport experiments using sand and aquifer sediments were conducted using the 50-cm column prior to commencing biodegradation studies, as described in Section 4.4.3.

The transport of perchlorate in two column experiments was simulated using HBGC123D. For each of these experiments, the 50-cm column was packed with sediments from LHAAP. In the first experiment, the groundwater flow rate was 42 mL/hr and the pulse of perchlorate and bromide (added together at 50 mg/L each) was 7 hrs. In the second study, the flow was decreased by half to 21 mL/hr, and the addition of bromide and perchlorate lasted 13 hrs. The conservative transport of perchlorate and bromide was nearly identical in these studies. The following parameters were measured or calculated and used for modeling chemical breakthrough curves from each experiment: column length = 50 cm; column diameter = 7.3 cm; cross-sectional area = 42 cm<sup>2</sup>; bulk density = 1.41 g/cm<sup>3</sup>; and porosity = 0.47.

To describe the bromide and perchlorate breakthrough curves, the dispersion coefficient of these compounds through the columns is needed. This parameter is related to the average pore velocity according to the equation  $D = a \cdot v$ , where  $D$  is the dispersion coefficient (cm<sup>2</sup>/hr),  $a$  is the dispersivity (cm); and  $v$  is the average pore velocity (cm/hr). The average pore velocity is calculated by the model based upon the water flux, the column cross-sectional area, and the porosity. To obtain the best fits between the model and observed data, the dispersivity,  $a$ , was varied in HBGC123D. Model fits for the two experiments are provided in Figures 78 and 79, respectively. The bromide data are presented in the figures, but perchlorate data were identical and are simulated by the same model fits. The dispersivity obtained for each curve fit is also given in the figures. These results represent the initial curve fitting exercise with HBGC123D. As seen in the figures, model simulations compared very well with the data, although the maximum breakthrough concentration for each experiment was overestimated slightly by the model.



**Figure 78. Model Fit of Bromide Breakthrough Curves in Experiment 1 (42 ml/hr) with a Dispersivity of 7 cm.**



**Figure 79. Model Fit of Bromide Breakthrough Curves in Experiment 2 (21 ml/hr) with a Dispersivity of 5.5 cm.**

#### **4.7. COUPLED TRANSPORT AND BIODEGRADATION MODEL FOR PERCHLORATE**

Column transport experiments were conducted where perchlorate utilization as an electron acceptor was promoted inside the column. Perchlorate, nitrate, and oxygen were fed into a 50-cm soil column packed with sediments collected from LHAAP (see section 4.4). After measured inlet and outlet perchlorate concentrations were similar (i.e., steady-state concentration conditions), acetate was added to the influent. Perchlorate concentrations were measured at the sampling ports along the length of the column and at the outlet of the column. The software HBGC123D was used to model the transport and biodegradation of acetate, oxygen, nitrate, and perchlorate during these experiments. This software has the capability of modeling the transport and biodegradation of multiple electron donors and acceptors in ground water. In HBGC123D, chemical biodegradation can be described using Monod kinetics and biomass growth. Further, this software can describe inhibition of the consumption of one electron acceptor (e.g., perchlorate) due to the presence of other electron acceptors (e.g., oxygen or nitrate).

##### ***4.7.1. EXPERIMENTAL APPROACH***

The biodegradation function described above and presented in Appendix B was incorporated into HBGC123D to describe the transport and biodegradation of perchlorate in the 50-cm soil column experiment. As described previously (Section 4.4.3), the column was packed using soil materials collected from the Longhorn Army Ammunition Plant (LHAAP). During these experiments, perchlorate was added to influent groundwater at 25 mg/L, nitrate was added at 16 mg/L, and oxygen was present at approximately 8 mg/L. These additions represent approximately equimolar quantities (0.25 mM) of each of these three electron acceptors. Acetate (electron donor) was not initially added. After reaching steady-state conditions for nitrate and perchlorate, (i.e., equal influent and effluent concentrations), acetate was added to the inflow water. The concentration of acetate in the inflow water was set at 80 mg/L using a separate syringe pump. In the model, the time when acetate was first added is designated as  $t = 0$ . Data collected during this experiment include the concentrations of acetate, oxygen, nitrate and perchlorate in influent and effluent water, and at sampling ports along the column.

##### ***4.7.2. MODEL PARAMETERS***

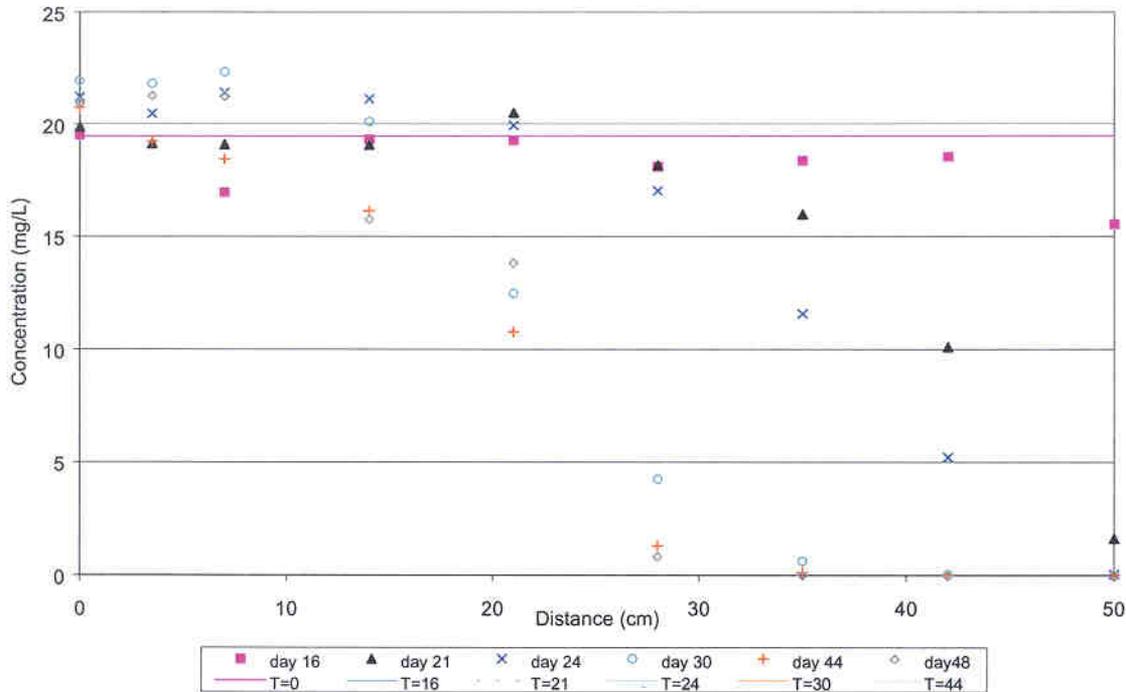
The input parameters used in HBGC123D to simulate the column data are summarized in Table 7. These parameters were measured either directly from the column or obtained from the batch experiments conducted during this project.

**Table 7. Input Parameters for Coupled Model**

	Parameter	Value	Units	Method of Determination
<b>Physical Parameters</b>	Darcy velocity	10	cm/hr	Calculated as the volume of water input to column divided by the column cross-sectional area.
	Dispersivity	6.25	cm	Average of values fit to the bromide breakthrough curve.
	Bulk density	1.41	g/cm <sup>3</sup>	Calculated using the mass of dry soil added to the column and the total volume of the column.
	Porosity	0.47	cm <sup>3</sup> water/ cm <sup>3</sup> total	Calculated using the bulk density and assuming the density of the soil particles as 2.65 g/cm <sup>3</sup> .
<b>Biological Parameters</b>	$k_{max}$	0.165	1/hr	Average of $k_{max}^{don/oxy}$ , $k_{max}^{don/nit}$ and $k_{max}^{don/per}$ values fit to batch data (see Table 5).
	$K_s^{don}$	93.3	mg/L	Average of $K_s^{don/oxy}$ , $K_s^{don/nit}$ and $K_s^{don/per}$ values fit to batch data (see Table 5).
	$K_s^{oxy/don}$	1	mg/L	Assumed.
	$K_s^{nit/don}$	180	mg/L	Value fit to batch data (see Table 6)
	$K_s^{per/don}$	150	mg/L	Value fit to batch data (see Table 6)
	$b$	0.014	1/hr	Average of values fit to batch data in Section 4.5.6.2
	$Y$	0.236	g biomass/ g acetate	Average of values fit to batch data in Section 4.5.6.2
Initial biomass, $B_0$	0.01	mg biomass/ g soil	Calculated based on an initial cell population of $1 \times 10^7$ cells/g soil and assuming a cell density of 1 g/cm <sup>3</sup> and a cell volume of 1 $\mu\text{m}^3$	

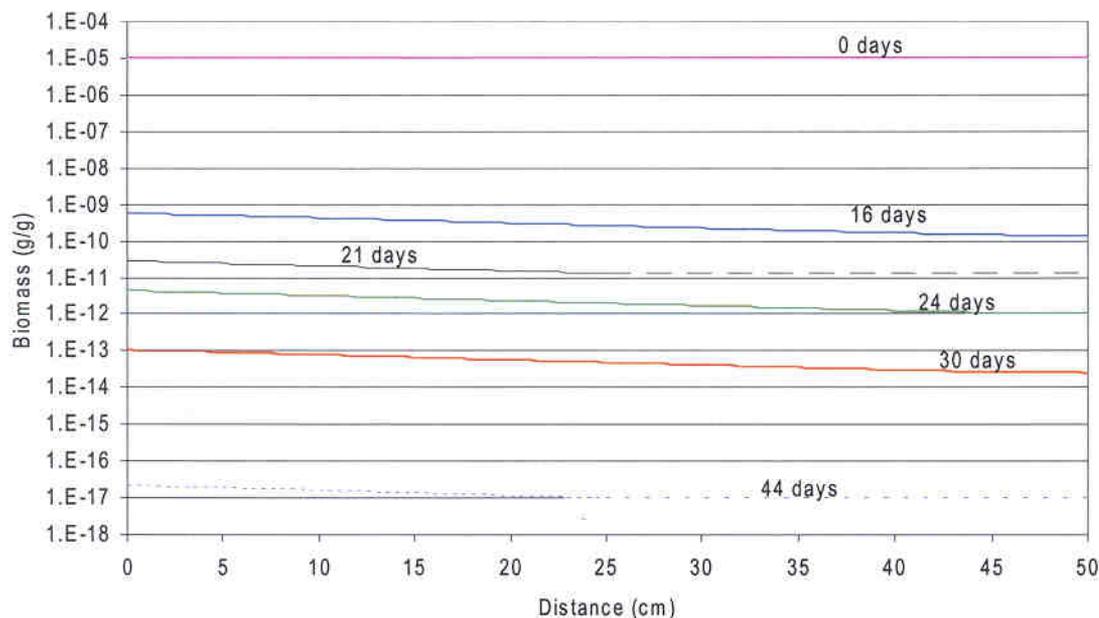
#### 4.7.3. MODEL RESULTS

Model simulations predict that no significant biodegradation occurs inside the column. Modeled acetate breakthrough curves show that the concentration front moves through the column with little or no detectable decrease. Also, no detectable decreases in oxygen, nitrate or perchlorate concentrations were calculated in the column simulations. A comparison between measured and modeled perchlorate concentrations is shown in Figure 80.



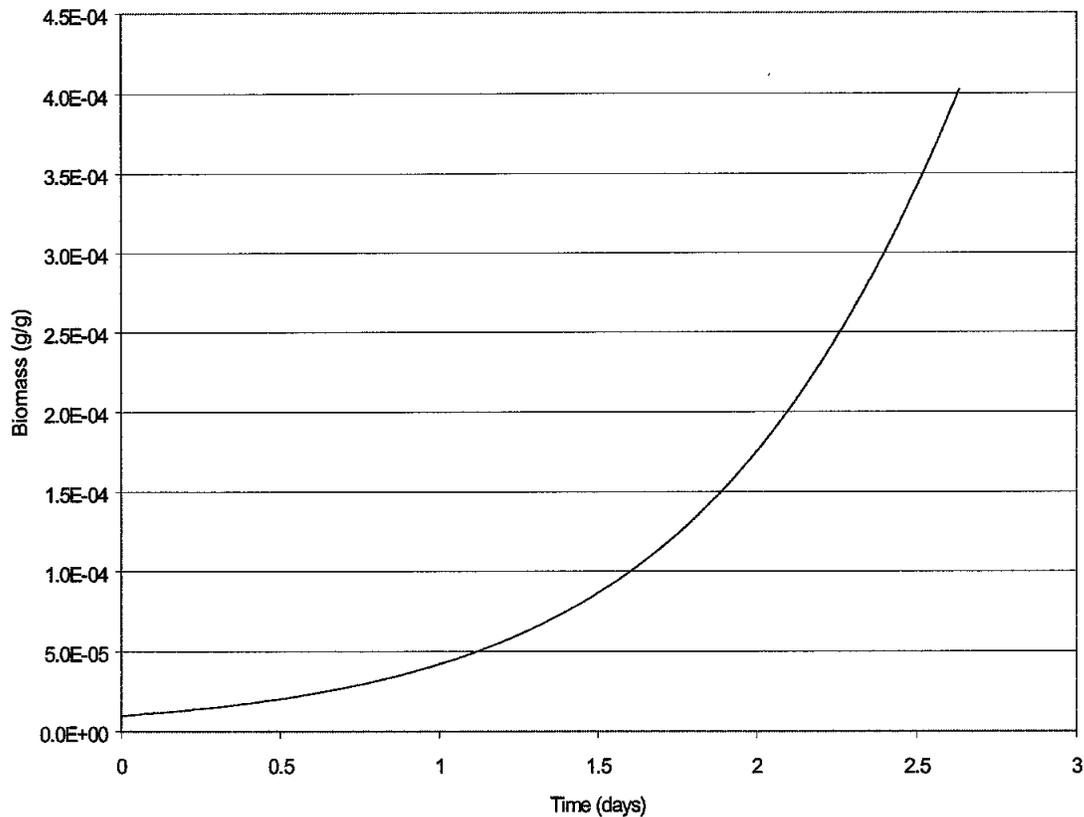
**Figure 80. Comparison between Model Simulation and Measured Perchlorate Profiles in the 50-cm Column Experiment at Different Times.**

Inspection of model results showed that the calculated biomass within the column decayed much faster than it grew. This phenomenon appears to account for the absence of biodegradation of acetate or any of the three electron acceptors within the column. The modeled biomass profiles in the column at different times are presented in Figure 81a. As can be seen in the figure, the modeled microbial population decreased by five orders of magnitude during the first 16 days. The initial biomass concentration in the column was estimated to be  $1 \times 10^7$  cells/g, which is equal to  $1 \times 10^{-5}$  g/g. Because the model calculated fast decay of the microbial population, the model predicted that insignificant consumption of acetate and perchlorate over time would occur. In the actual column experiment, biomass in the column was able to sustain activity and degrade acetate, oxygen, nitrate and perchlorate during the experiment. The difference between the data and model suggests that some of the biomass modeling assumptions were not valid or were critically violated (see below).



**Figure 81a. Modeled Biomass Levels Across the 50-cm Column.**

In an attempt to prevent the calculated microbial population from rapidly decreasing to zero, the biomass decay rate was decreased by a factor of 10 (i.e., the parameter  $b$  was decreased to  $0.0014 \text{ hr}^{-1}$ ) and additional simulations were performed. After simulating approximately 2.5 days, the model failed to converge and the simulation stopped. This occurred because, after 2.5 days, the net rate of biomass growth resulted in a rapid increase in microbial populations causing the numerical iterative scheme within the model to fail to reach convergence. Figure 81b shows the simulated biomass over time at a point 0.5 cm from the column inlet. The calculated biomass increased by 1.5 orders of magnitude during the first 2.5 days of simulation time. The sharp increase in biomass that was observed in the column simulations but not in microcosm simulations reflects the constant feed of acetate to the column. Thus, in the column simulations, the bacteria are allowed to grow indefinitely as long as acetate is being supplied. In reality, the growth of high bacterial populations will be limited eventually by the availability of substrates and nutrients on the microscale, and by physical space on the soil particles.



**Figure 81b. Modeled Biomass Levels at a Point 2-cm from the Column Inlet.**

A violation of one or more of the following assumptions most likely contributed to the inability of the fully coupled model to simulate column data:

1. *The decay rate,  $b$ , in the column is equal to the value determined from the batch experiments.*  
 A wide deviation from this assumption is the most likely reason for simulated biomass in the column to decay quickly to low numbers, and thus for electron donors and acceptors to show no biodegradation in simulations. It was not possible to determine biomass levels or biomass decay in the column study without destructively sampling the sediment matrix. The actual biomass decay rate in the column may be very different from that determined in batch experiments with JPLRND. These two environments are very different, and the decay of organisms in a well-mixed system (batch studies) is likely to differ widely from that of an attached biofilm (sediment column). To determine the “true” biomass decay rate in a soil environment, a new methodology that accounts for the impact of soil on biomass decay needs to be developed.

2. *There is no lag period for biomass activity.* In the model, biomass activity and decay were assumed to occur immediately. This differs from the column data, in which there was a significant lag period prior to perchlorate biodegradation, and a lesser, but measurable lag before nitrate biodegradation was observed. There was also a significant lag period in some of the batch studies performed before the onset of microbial activity and subsequent consumption of perchlorate. Because biomass growth and decay are exponential functions, a short lag period in the column experiment will cause the modeled biomass to quickly diverge from actual values, resulting in incorrect estimates of the magnitude of biodegradation and a large discrepancy between the data and simulations.
3. *The initial biomass in the column is  $1 \times 10^7$  cells/g soil.* The initial microbial population in the column was assumed to be  $1 \times 10^7$  cells/g soil. Though this number is believed to be representative of microbial population in the column, the actual biomass in the soil may be appreciably higher. If this assumption is significantly violated (i.e. actual biomass is 1 or 2 orders of magnitude different than the assumed value) it can result in the observed discrepancy between the model and data.
4. *There is no maximum allowable biomass population in the column.* In the model, biomass growth is limited only by the concentration of acetate, oxygen, nitrate and perchlorate. Given that these components are always abundant at the inlet side of the column, in the absence of a high biomass decay rate, microbial populations will increase indefinitely at column points close to the inlet of the column. In reality, once high microbial populations are present, additional microbial growth will likely be inhibited by the availability of micronutrients and, eventually, physical space on soil particles. To avoid this pitfall, the biodegradation model presented in Appendix B needs to be modified to include a maximum biomass level in soil.

### **Modeling Summary**

In summary, the biodegradation kinetic model for perchlorate was successfully applied to describe consumption of an electron donor (acetate), bacterial growth and decay, and the dynamics of multiple electron acceptors, including consumption, sequential utilization, and inhibition. In addition, the non-reactive transport of perchlorate through natural sediment was successfully simulated using the modeling software HBGC123D. However, when the biodegradation and transport models were coupled using HBGC123D, the simulated kinetics of perchlorate biodegradation under flow conditions failed to describe laboratory data collected from column studies. We have evaluated potential reasons for failure of the

coupled model (see above), and determined that the failure of the model reflects difficulties with coupling HBGC123D with the kinetic model rather than problems with either of these individual components. In addition, gaps in column data, particularly with respect to biomass levels, cell growth, and cell decay, also contributed to the failure of the coupled model. As part of an upcoming field demonstration of *in situ* perchlorate treatment (see section 5.2), we intend to refine the coupling process, collect additional data concerning biomass dynamics, and/or incorporate the biodegradation model into another fate and transport model. The biodegradation model has also been used by researchers at the Air Force Institute of Technology to describe perchlorate biodegradation in a complex flow regime (see section 4.8).

#### **4.8. ADDITIONAL MODEL DEVELOPMENT**

The biodegradation model constructed during this project and the growth parameters obtained from pure culture studies were provided to Lt. Jeffrey Parr at the Air Force Institute of Technology (AFIT). Lt. Parr is a graduate student under the supervision of Dr. Mark Goltz. As part of his Master's Thesis, Lt. Parr incorporated the biodegradation model with a groundwater transport model developed previously by Dr. Goltz. This effort resulted in a new fate and transport model for perchlorate biodegradation. Publications resulting from this effort will be provided to the SERDP Office.

#### **4.9 ANALYTICAL METHOD DEVELOPMENT**

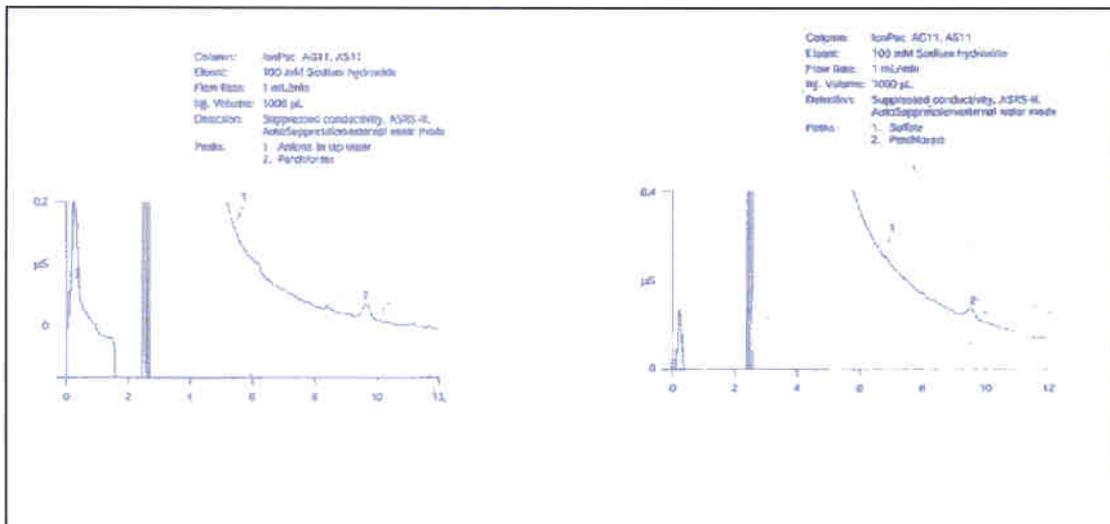
##### **4.9.1 BACKGROUND**

As a partner in this SERDP effort, scientists at the Indian Head Division, Naval Surface Warfare Center (IHDI) evaluated techniques to improve perchlorate detection at low concentrations and in matrices where strong interference is anticipated (e.g., high salinity environments). The method tested during this work, EPA Method SW9058, is an accepted method for analysis of perchlorate in water and wastewater, and is essentially the same ion chromatography procedure as described by EPA Method 314.0 for drinking water.

The recent focus on the presence of perchlorate as a contaminant in groundwater has led to the advent of several methodologies to measure the ion in a variety of matrices. The separation from other matrix bound components and sensitivity of detection afforded by approaches based on ion chromatography have made these analyses the most widely used for perchlorate determination at trace levels. Accordingly, the current EPA method for measurement of perchlorate in groundwater (EPA Method SW9058) uses ion chromatography with chemically suppressed/conductivity detection and an ion exchange separation mode.

The application of SW9058 and similar methods to matrices with high ionic content, apart from the target analyte, has proven difficult due to interference provided by these anionic moieties. While stated detection limits lie in the low parts per billion range (i.e.,  $\mu\text{g/L}$ ), the effective detection is at least ten times higher in more aggressive matrices. The latter resulting in the recent presentation of an amendment to the procedure requiring the dilution of specimens with a high conductivity, due to high ionic content, prior to analysis (Jackson, 2000).

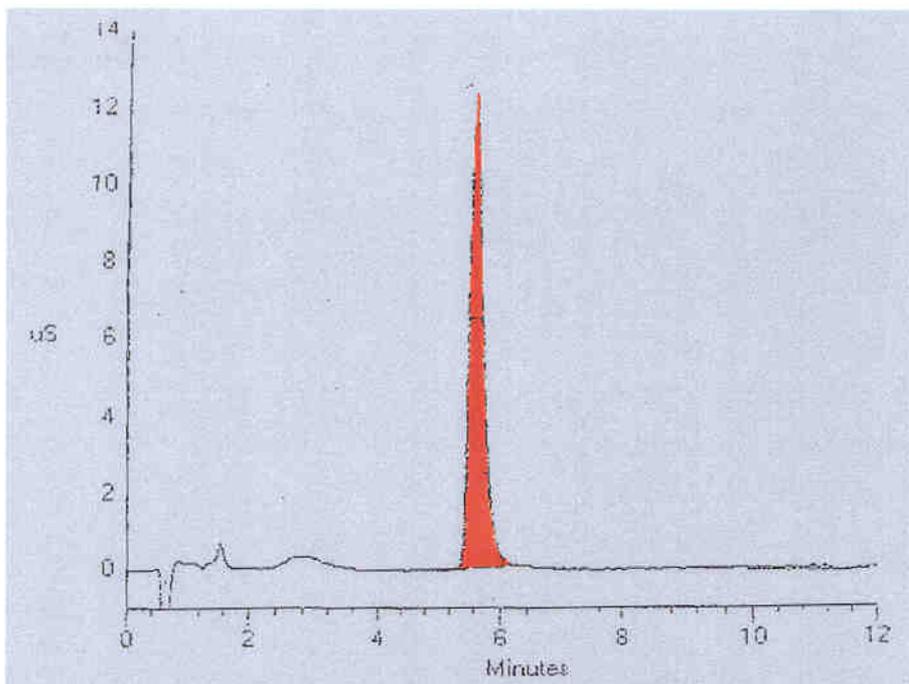
The analysis of specimens taken at different sites at the Naval Surface Warfare Center as part of SERDP Project CU-1163 included groundwater, extracts of soil, brackish water, and extracts of aquatic vegetative and animal matter. Earlier attempts at analysis of some of these matrices with SW9058, and similar methodologies, failed due to the levels of interferants found in the specimens. While the analyses appeared to be capable of detection in the low parts per billion range in the presence of a few hundred parts per million of interferants, some of the matrices contained other ionic moieties at greater than 30,000 parts per million. Interference was due to the overload of the separator used in the system or the earlier eluting species generating signals of such magnitude as to obscure perchlorate detection. Figure 82 shows detection of perchlorate in tap water and in the presence of 300 parts per million sulfate using the current EPA method.



**Figure 82. EPA Method SW9058: Chromatograms of Tap Water Sample and Sample with High Sulfate Content.**

The figure illustrates the limitation of SW9058 and similar methods (e.g., EPA 314.0). The perchlorate appears as a small signal on the tailing edge of a signal with greater amplitude by several orders of magnitude. The leading signal represents common anions such as fluoride, chloride, nitrate, or sulfate in the range of a few hundred parts per million. An increase in the level of these anions by a factor of two approximately doubled the height and breadth of the signal and effectively masked the signal generated for perchlorate. In the face of tens of thousands parts per million of other ionic species, the entire chromatographic field would be obscured. Dilution of the specimen to lower the intensity of the interferants would have proportionally lowered the perchlorate signal below detection limits.

Methods based on mobile phase ion chromatography (MPIC) have been successfully used in the detection of low levels of perchlorate in environmental matrices (Figure 83) (Basom, 1993). The low background of the eluants, low peak volume, and resistance to interference provide for sensitive detection in specimens with high ionic content. The method, however, possesses a detection limit of approximately 100 parts per billion in typical groundwater and less sensitivity in specimens such as seawater containing several thousand parts per million of ionic content.



**Figure 83. MPIC: Chromatogram of Groundwater Containing Two Parts Per Million Perchlorate.**

The limitations of the aforementioned procedures, in terms of sensitivity in aggressive matrices, prevented their direct use for determining perchlorate at requisite levels (low parts per billion) in the various specimens to be tested in the core project. The development of a procedure with the required capabilities and successful analysis of test specimens were the objectives of this work.

#### ***4.9.2. OBJECTIVES OF ANALYTICAL METHOD DEVELOPMENT***

The objective of this phase of research was to develop an analytical methodology capable of detecting perchlorate ion in the low parts per billion range in environmental specimens containing potential interference at concentrations up to  $\sim 1 \times 10^7$  times greater than that of the target analyte. The development of such a methodology is to be used to test the samples collected at the IHDIIV installation as part of the core project. The developed method had to meet the following criteria:

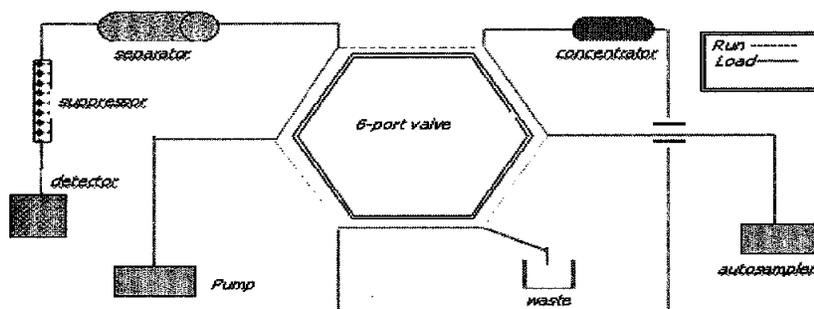
- Detection limits in low parts per billion range (1 - 10 parts per billion)
- Detection not compromised by relatively high levels of interferences including ionic or organic components in specimens
- Durability of approach: system integrity not compromised by repeated analysis of specimens with high levels of contaminants
- High analytical accuracy and precision: to ensure the validity of test results

#### ***4.9.3. TECHNICAL APPROACH***

- 1) **Literature review.** Technical journals and other materials were reviewed to provide candidate methods that could perform the required analysis. Literature reviews consisted of materials contained at the laboratory, literature from suppliers of scientific equipment, and database searches by the technical library at IHDIIV.
- 2) **Testing of candidate methods.** Candidate methods were initially used on test specimens to determine applicability. Methodologies based on approaches other than ion chromatography were eliminated due to inherent limitations. Eventually, conventional ion chromatographic methods employing ion exchange were eliminated due to limitations imposed by the conductivity of the eluants, the nature of different media in the separator, and other factors.
- 3) **Selection of a "core" analytical approach.** As the candidate methodology with the greatest potential for meeting the previous criteria, test matrices were analyzed using the previously

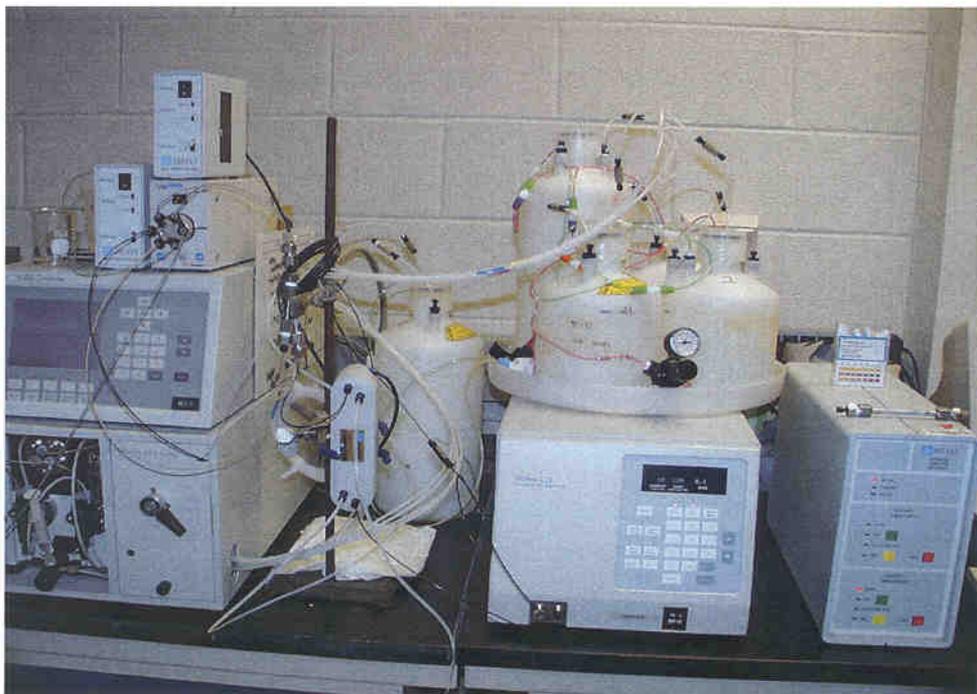
referenced MPIC methodology. Specimens were diluted to lower the levels of interfering anions and to prevent overloading and coelution phenomena. Required detection levels were not achieved; however, the method demonstrated potential as a basis for a hybrid approach coupling MPIC with ancillary technologies.

- 4) **Modification of analytical methodology.** To effectively concentrate the target analyte and lower the levels of interferants, a chromatographic system was configured employing a concentrator column, six-port valve, and pressure assisted autosampler. The system allowed for effective concentration of perchlorate, by a factor of ~500 times versus a fixed volume injection, while reducing the concentration of other ionic species prior to injection. The system was configured to allow for "back-flushing" of the perchlorate onto the separator to prevent band spreading and subsequent reduction in sensitivity. Figure 84 is a schematic of the chromatographic system.



**Figure 84. Diagram of Ion Chromatographic System.**

The solid line in the flow path depicts the valve ports that are interconnected during sample loading and flushing of the concentrator. The broken lines in the flow path depict the valve ports that are interconnected when the concentrator is brought into the liquid circuit and the sample is swept onto the separator column. Figure 85 is a photograph of the system in the depicted configuration.



**Figure 85. Photograph of Ion Chromatographic System.**

Initially, the media used in the concentrator was similar to the media contained in the separator. System instability following injection and other operational problems associated with the hydrophobic media led to the construction of a concentrator using a mixed-bed ion exchange media. The concentration of components in the eluant and other operational parameters were subsequently modified to allow for the use of the concentrator media in the system. Method validation consisting of measurement of accuracy, system recovery, and precision were performed on the system.

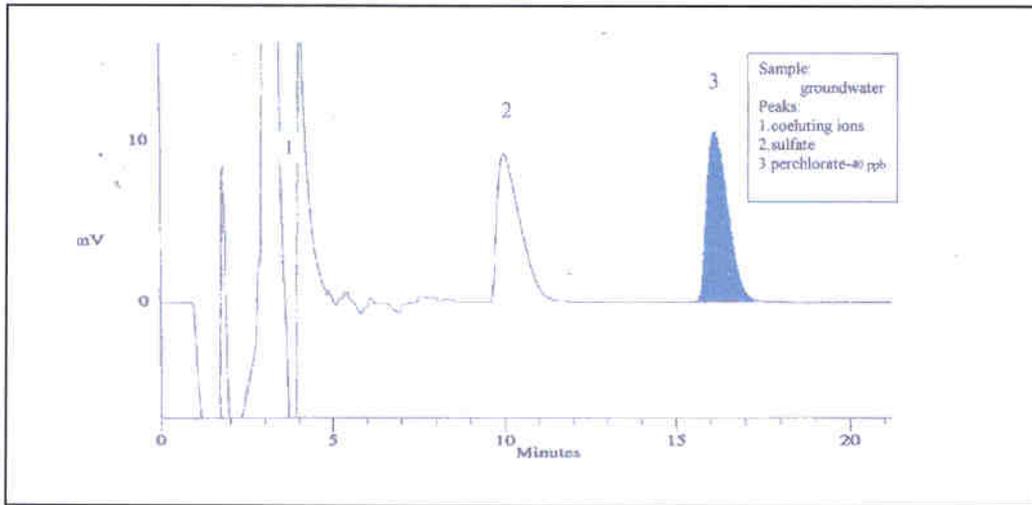
#### ***4.9.4 RESULTS OF ANALYTICAL METHOD***

##### **4.9.4.1. Construction of Analytical System**

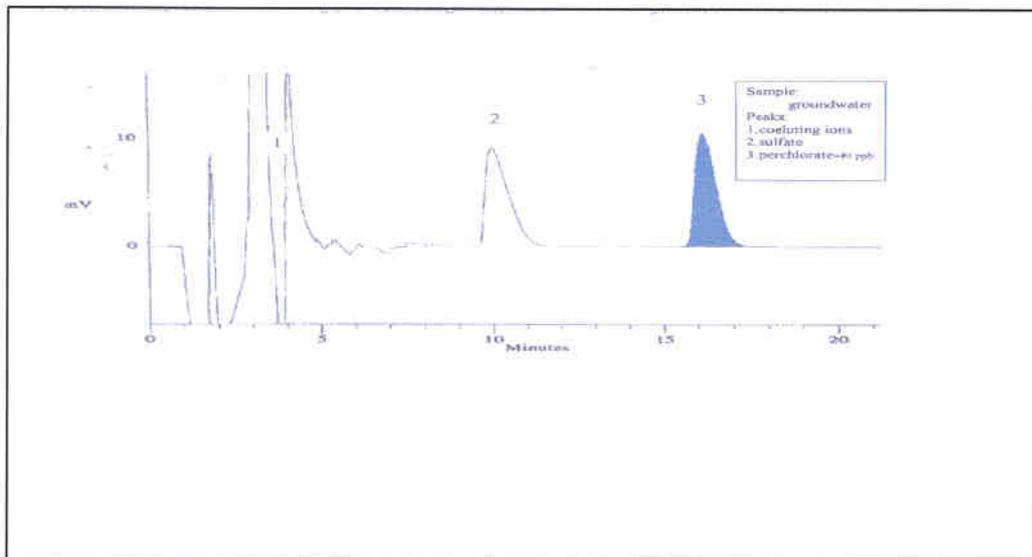
The described system provided for the accurate measurement of perchlorate in the low parts per billion range in samples with ionic content of several thousand parts per million. In-house validation using standards and sample spikes showed the procedure to be accurate (>99% accuracy) and precise (>99% relative precision) when measuring perchlorate at from 5 to ten parts per billion in matrices with up to thirty thousand parts per million of ionic interferants. The detection of the target analyte at these levels in aggressive test matrices rendered the system the most sensitive known for detection of perchlorate in environmental samples.

#### 4.9.4.2. Results and Conclusions from Sample Analysis

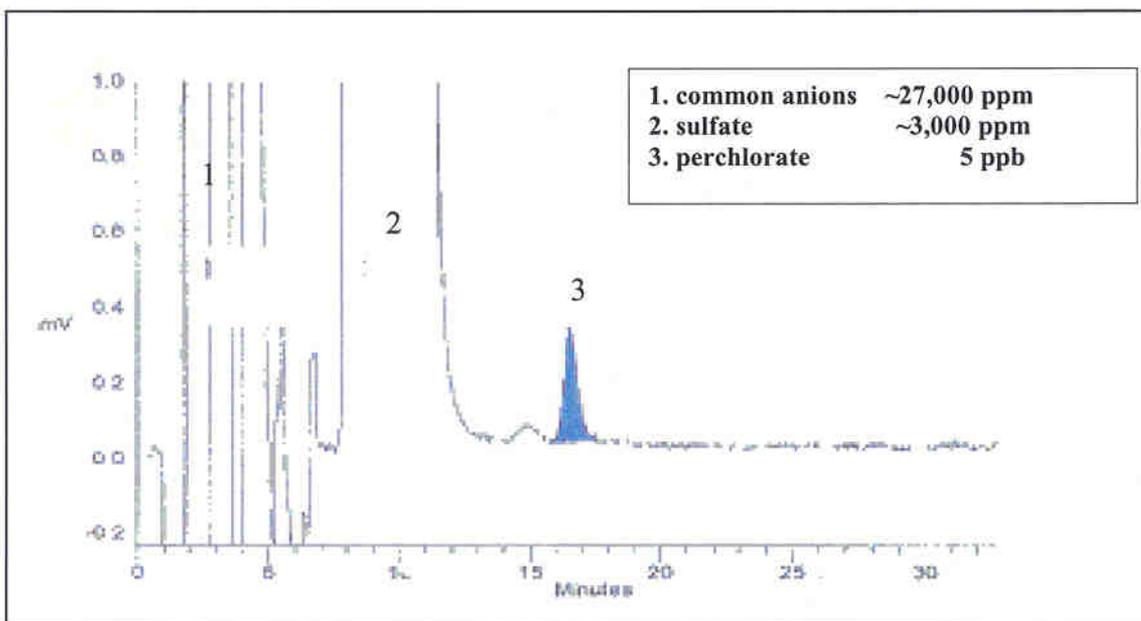
The chromatographic system was used to analyze all samples withdrawn at IHDIV as part of the core project including groundwater and extracts of soil, vegetative, and animal matter with results reported to the liaison on station. None of the interferences displayed by any of the specimens prevented the detection or measurement of perchlorate as determined by sample spikes as all specimens were analyzed for perchlorate at less than or equal to five parts per billion. Figures 86, 87, and 88 are chromatograms of tap, ground, and seawater generated by the system.



**Figure 86. Chromatogram of Perchlorate in Tap Water Using IC Equipped with Perchlorate Concentrator.**



**Figure 87. Chromatogram of Perchlorate in Groundwater Using IC Equipped with Perchlorate Concentrator.**



**Figure 88. Chromatogram of Perchlorate in Seawater Using IC Equipped with Perchlorate Concentrator.**

As observed in the chromatograms, the signal corresponding to perchlorate was well separated from other ionic components and detectable at the low parts per billion level even in matrices with thirty thousand parts per million of ionic content. Unlike chromatograms generated by current methods, the signal appeared as a discrete, symmetrical chromatographic signal which provided for accurate measurement of perchlorate content in all test specimens.

The developed methodology provided accurate measurement of perchlorate in all samples withdrawn from IHDIIV during the core project with the results being used for the research into the remediation of perchlorate in subsurface environments. The notable attributes of the method, including sensitivity, accuracy, and resistance to interference, should allow for the analysis to be used in environments in which interfering anions or other constituents in a sample prohibit sensitive perchlorate detection by EPA Methods SW9058 and 314.0.

## **5.0 TRANSITION PLAN - FIELD DEMONSTRATIONS**

The laboratory results from this SERDP Project suggest that *in situ* biodegradation of ammonium perchlorate through electron donor addition is likely to be a viable remediation approach at numerous DoD sites. The next step in transitioning this technology is to perform one or more field demonstrations. The data from these demonstrations can then be used to provide cost information on *in situ* perchlorate remediation and to evaluate and resolve potential problems with the technology. Envirogen currently has one *in situ* field demonstration underway and a second demonstration is planned to begin in June, 2002. These two demonstrations will test different technologies for electron donor addition and mixing in subsurface aquifers, and will be performed at sites with very different hydrogeological and geochemical characteristics. A brief description of each field demonstration is provided below.

### **5.1 EVALUATION OF ELECTRON DONOR ADDITION FOR PERCHLORATE TREATMENT AT IHDIV**

Based on laboratory results from SERDP Project 1163, the Indian Head Division Naval Surface Warfare Center (IHDIV) provided additional funding for a field demonstration of electron donor injection for perchlorate remediation. This work was contracted to Envirogen through Booze, Allen & Hamilton Consultants, a primary contractor at IHDIV. The field location for this demonstration is located behind Building 1190 (Hog Out Facility) at IHDIV. The laboratory results from this site are provided in section 4.3.2.3. In summary, the data revealed the following: 1) naturally-occurring perchlorate degrading bacteria are present in the groundwater aquifer underlying this site, and 2) these organisms can be stimulated to degrade perchlorate from > 50 mg/L to below detection using acetate or lactate as electron donors but only if the pH of the aquifer is buffered from 4.3 to neutrality. Based on laboratory studies, if the pH of the groundwater is not adjusted, perchlorate biodegradation will not occur. Thus, one of the main technological challenges of this demonstration will be aquifer buffering.

A general description of the field demonstration is provided in Appendix A (Cramer et al., 2002). Characterization work at this site was completed in March, 2002. The field investigation revealed a shallow, narrow plume of perchlorate contamination behind Building 1419. Groundwater analysis from 17 Geoprobe points showed perchlorate levels ranging from below detection to approximately 430 mg/L (Table 8). With a few exceptions, the pH of the site was below 5, and the dissolved oxygen levels were < 2 mg/L. Based on the Geoprobe data, permanent monitoring wells were installed at the site, and pump tests were conducted to characterize groundwater flow and hydraulic conductivity at the site. These data were then used to develop a preliminary system design for the demonstration (see Appendix A; Cramer et al, 2002). Two recirculation cells will be installed at the site. These cells will consist of two extraction wells, three injection wells, and 4 – 6 nested monitoring wells. Groundwater will be removed from each

cell through the extraction wells and reapplied to the aquifer through the injection wells. In the test plot, groundwater will be amended with sodium carbonate as a buffer and lactate as an electron donor prior to reinjection. The control plot will receive neither amendment. Perchlorate, nitrate, pH, and dissolved oxygen will be measured in monitoring wells within each plot during the 6-month demonstration period.

The study is anticipated to reach completion in December, 2002. The data from this demonstration will be used to provide technical guidance and cost information for treating perchlorate in shallow aquifers using a recirculation cell design. The trial will also provide valuable information on the potential for perchlorate treatment in low pH environments using a combination of aquifer buffering and electron donor addition. It is anticipated that the results of this project will be presented to DoD site managers outside of IHDIV and to SERDP/ESTCP personnel.

**Table 8. Groundwater Chemistry at the IHDIV Demonstration Site**

<b>Geoprobe Boring</b>	<b>Perchlorate (mg/L)</b>	<b>Nitrate as N (mg/L)</b>	<b>Sulfate (mg/L)</b>	<b>pH</b>	<b>DO (mg/L)*</b>
GP-1	120	0.6	66	4.67	NA
GP-2	<2.5	3.0	220	8.08	NA
GP-3	8.2	1.9	280	5.23	NA
GP-4	57	0.3	110	4.54	NA
GP-5	65	0.1	130	4.21	1
GP-6	280	11	69	5.62	1
GP-7	35	1.5	66	4.21	0.1
GP-8	430	14	62	4.57	ND
GP-9	73	0.4	56	4.44	0.8
GP-10	300	12	70	4.31	1
GP-11	230	14	72	4.71	0.8
GP-12	55	2.0	110	6.46	ND
GP-13	230	3.8	64	4.61	1.5
GP-14	14	1.5	250	4.97	ND
GP-15	9.8	<0.2	160	5.34	0.2
GP-16	270	2.8	74	4.16	1
GP-17	<5	<0.2	140	4.83	0.2

**Notes:**

- DO: Dissolved oxygen
- ND: Not determined
- NA: Not analyzed
- \*: Colorimetric field method

## **5.2 EVALUATION OF ELECTRON DONOR ADDITION FOR PERCHLORATE USING HORIZONTAL FLOW TREATMENT WELLS**

A second field demonstration is planned to begin in June, 2002. This project, which will be funded by ESTCP, is a collaborative effort among Envirogen, Inc., the Air Force Institute of Technology (AFIT), and the University of New Mexico (UNM). The objective of this technology is to demonstrate that electron donor addition can be used to efficiently and cost-effectively treat perchlorate in subsurface groundwater to below the current minimum detection limit (MDL) of 4 µg/L. One of the most critical issues in adding an electron donor or other amendment to the subsurface is how to achieve mixing of that chemical with contaminated groundwater. If sufficient mixing is not achieved, the treatment technology will be ineffective. Therefore, a recirculating well system (sometimes referred to as a horizontal flow treatment well (HFTW) system) will be tested in this ESTCP Project as a delivery and mixing technology for electron donor addition. A similar system has previously been field tested for the addition of co-substrate (toluene) and oxygen for aerobic remediation of TCE. We believe that this technology will be highly effective for the *in situ* treatment of perchlorate in the subsurface, and will be widely applicable at many DoD sites.

### **5.2.1 TECHNICAL DESCRIPTION, SCHEDULE, AND TECHNOLOGY TRANSFER FOR ESTCP DEMONSTRATION**

The site for this ESTCP demonstration has not yet been selected. The project will demonstrate and validate the combined use of two innovative technologies: (1) bioremediation of perchlorate contaminated groundwater through electron donor addition, and (2) horizontal flow treatment wells to achieve *in situ* mixing of the electron donor with the perchlorate-contaminated water, and delivery of the mixture to indigenous perchlorate-degrading bacteria. An HFTW system combines the best features of pump-and-treat and funnel-and-gate technologies to contain and treat contaminated groundwater. As an *in situ* technology, contaminant destruction occurs below ground, and there is no need to pump contaminated water to the surface for treatment. On the other hand, since the HFTW system uses pumping wells, the contaminant plume is actively contained, and the limitations of funnel-and-gate systems (restricted to relatively shallow contamination depths, potential for plume to bypass the treatment system) are overcome. In the field evaluation proposed for this study, two treatment wells will be installed (Figure 89). Each treatment well will have two screens, one an injection screen, the other an extraction screen, with one well pumping in an upflow mode and the other in a downflow mode, so that the water will circulate between the wells. Note that in the HFTW system, due to hydraulic conductivity anisotropy such as is typically seen in aquifers, groundwater flow between the injection and extraction screens of a well pair is horizontal. This is in contrast to conventional groundwater circulation wells (GCWs) that

depend on vertical flow between the injection and extraction screens of a single well. With each pass of perchlorate-contaminated water through a treatment well, electron donor will be added through an in-well static mixer. The donor-amended water will be injected into the aquifer, where a bioactive zone is established and indigenous microorganisms will degrade the donor, using perchlorate as the electron acceptor. Disinfectant may also be added into the in-well mixer to control biogrowth at the injection screens. Due to the circulation between wells, the contaminated water is treated multiple times, so that perchlorate removal efficiencies (comparing concentrations upgradient and downgradient of the treatment wells) can be greatly increased over the removal achieved by a single-pass of perchlorate-contaminated water through the reactor.

The proposed test will demonstrate whether or not perchlorate can be successfully bioremediated in the field for a prolonged period using electron donor addition. To our knowledge, no field demonstrations of *in situ* perchlorate remediation for deep subsurface sites have been conducted over a sufficient length of time to demonstrate and validate the application of this technology. The performance issues that will be documented and validated with the HFTW system include the following: (1) *in situ* biological perchlorate treatment is feasible in the field using electron donor addition; (2) perchlorate can be treated for a sustained period to  $< 4 \mu\text{g/L}$ ; (3) the zone of influence and efficiency of the HFTW are sufficient to make the system a viable, cost-effective option at many sites; (4) biofouling can be effectively controlled by one or several measures that are easily implemented; and (5) co-contaminants, including nitrate and VOCs, can be treated using the same HFTW technology (optional based on site conditions). As with any full-scale technology demonstration, a main objective of this field project is to collect and document information that is relevant to site managers and regulators who are responsible for choosing and implementing technologies. The demonstration is designed to validate the use of HFTWs and electron donor addition for *in situ* perchlorate treatment and to determine the potential problems and costs associated with implementation. This information will be made available to interested DoD and regulatory personnel through technology transfer efforts.

The proposed project will be performed over a period of 24 months, including a 1-year field demonstration. During the initial 6 months of the project, a field site will be selected and characterized as required based on existing hydrogeological data. Samples from the site will be collected and assayed to confirm the existence of naturally-occurring perchlorate-degrading bacteria, to determine the most effective electron donors for perchlorate degradation, and to estimate electron donor consumption and perchlorate degradation kinetics. A conceptual and complete system design will be developed for the field site during this period. In addition, a fate and transport model designed to simulate perchlorate biodegradation at the field site will be developed during this period. The HFTW System will be installed and tested during the second half of Year 1. The field demonstration will begin in Month 11 of Year 1

and continue for 12 months. Data collection and analysis, and model testing and verification will occur during this period. During the demonstration, system modifications will be made as indicated by the field data.

After this demonstration, Envirogen with AFIT and UNM will prepare and submit a technology protocol document at the completion of the project. This document will provide information that can be used to assess the hydrogeological, technical, and design requirements for pilot- or full-scale *in situ* perchlorate treatment using HFTW technology. A cost-benefit comparison with competing technologies will also be provided. The objective of this document will be to provide all necessary information so that the technology can be applied by others throughout the DoD. The document may be supplemented by user-friendly software. Such software has been shown to help remedial project managers select innovative technologies that may be appropriate for their sites. An example of the above described documentation and software may be found at the following website: <http://en.afit.edu/env/geem/insitubio.htm>, where similar products were prepared for the *in situ* aerobic cometabolic bioremediation technology and made available both within and outside of DoD. The above technology transfer material will be in addition to “traditional” avenues of technology transfer, such as publication in peer-reviewed journals and presentation at conferences, which will also be pursued.

### **5.3 OTHER MEANS OF TECHNOLOGY TRANSFER**

In addition to the planned field demonstrations, the laboratory data resulting from this SERDP Project have been presented at seven different conferences (see Appendix A). A portion of the data have been published in a recent journal article, and at least two additional publications will be submitted to appropriate journals in 2002. The results of this project have also been presented to numerous aerospace and chemical companies and/or their primary environmental consultants (including Boeing, Aerojet, Thiokol, Kerr-McGhee, Lockheed-Martin, and Environmental Chemical Corporation) as well as personnel involved in the restoration of perchlorate-contaminated government/military sites, including the Longhorn Army Ammunition Plant, the Massachusetts Military Reservation, the Indian Head Division Naval Surface Warfare Center, and Jet Propulsion Laboratories. These presentations are anticipated to lead to one or more additional pilot demonstrations and/or full-scale *in situ* perchlorate remediation efforts.

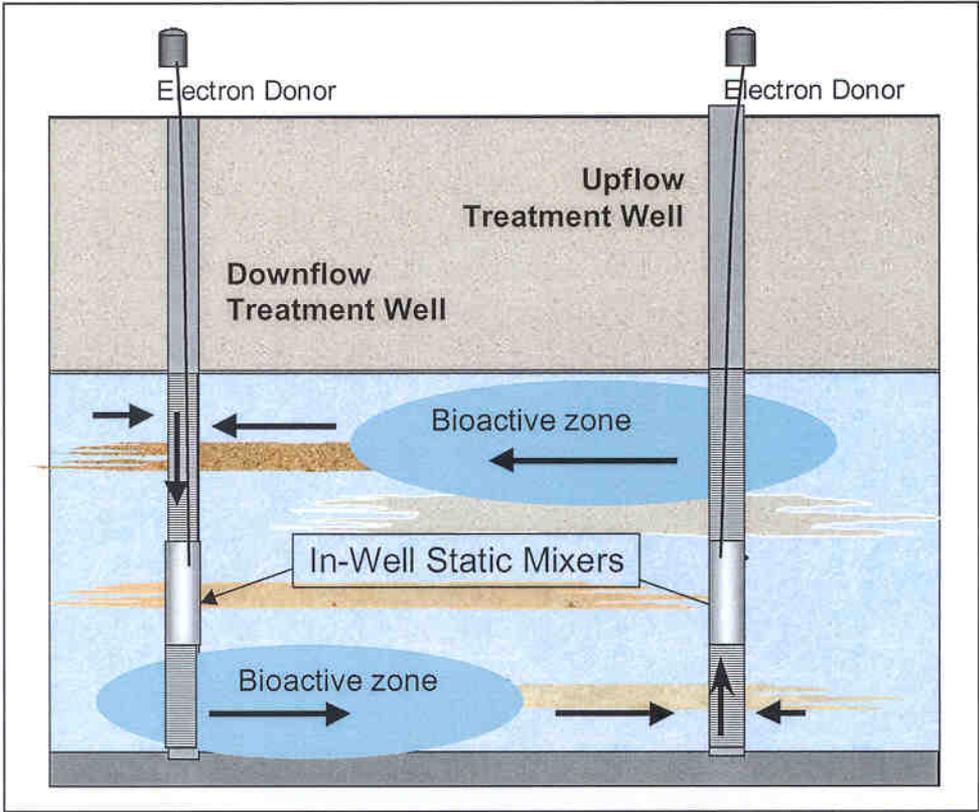


Figure 89. Diagram of Paired Horizontal Flow Treatment Wells.

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## 7.0 APPENDICES

## 7.1. APPENDIX A: TECHNICAL PRESENTATIONS, PUBLICATIONS, AND ABSTRACTS

### 7.1.1. CONFERENCES AND SYMPOSIA

The research resulting from this SERDP project has been presented at seven conferences in 2000 - 2002. The conference citations are listed below. Published abstracts and papers from these meetings are provided where applicable.

1. **Hatzinger, P. B.** 2000. Biotreatment of Perchlorate in Groundwater. Partners in Environmental Technology Symposium and Workshop. Arlington, VA. November 28 – 30, pp. 38.
2. **Hatzinger, P. B.** 2000. *In situ* Bioremediation of  $\text{ClO}_4^-$  in Groundwater. Fifth Annual Joint Services Pollution Prevention & Hazardous Waste Management Conference. San Antonio, TX. August 21-24, available on CD-ROM.
3. **Hatzinger, P. B., M. D. Arkins, Y. H. Farhan, and R. J. Steffan.** 2001. *In situ* Bioremediation of Perchlorate in Groundwater. Partners in Environmental Technology Symposium and Workshop. Washington, D. C. November 27 – 29, pp. 45.
4. **Hatzinger, P. B., M. D. Arkins, M. Tugusheva, and R. J. Steffan.** 2001. *In situ* Bioremediation of Perchlorate in Groundwater. *In situ* and On-Site Bioremediation. The Sixth International Symposium, San Diego, CA. June 4-7.
5. **Hatzinger, P., M. Arkins, W. Guarini, and J. Manning.** 2001. *In situ* Bioremediation of Perchlorate in Groundwater. Society of Environmental Toxicology and Chemistry (SETAC) 22<sup>nd</sup> Annual Meeting, Baltimore, MD. November 11 – 15, pp. 313.
6. **Togna, A. P. and P. B. Hatzinger.** 2002. Perchlorate: Bioremediation of Perchlorate in Groundwater and Soil. AFCEE Cleanup Technology Transfer Workshop, San Antonio, TX. March 4 – 7.
7. **Cramer, R. J., C. A. Yates, P. B. Hatzinger, J. Diebold, and M. Giovanelli.** 2002. Field Demonstration of *In situ* Perchlorate Bioremediation at NSWC Indian Head. Joint Army-Navy-NASA-Air Force (JAANF) Interagency Propulsion Committee, 30<sup>th</sup> Propellant Development and Characterization Subcommittee Meeting, Colorado Springs, CO, March 18 – 21. (proceedings paper *in press*).

### 7.1.2. JOURNAL ARTICLES

One journal paper has been published from this work. Two additional papers are currently being prepared for journal submission in 2002. The citations for these papers are provided below. The full text of the published manuscript is also included.

1. **Hatzinger, P. B., W. C. Whittier, M. Arkins, and C. W. Bryan.** 2002. In-situ and Ex-situ Bioremediation Options for Treating Perchlorate in Groundwater. *Remediation* 12:69-85.
2. **Hatzinger, P. B., J. Pollock, M. Arkins, and J. R. Coates.** 2002. Potential for *In situ* Bioremediation of Perchlorate-Contaminated Groundwater. *Environmental Science and Technology* (*in preparation*).

3. **Farhan, Y. H., M. Arkins, C. Condee, and P. B. Hatzinger.** 2002. Development of a Kinetic Model for Describing Perchlorate Degradation in the Presence of Competing Electron Acceptors. *Journal of Environmental Quality* (*in preparation*).

**7.1.3. REPRINTS OF PUBLISHED JOURNAL ARTICLES, CONFERENCE PROCEEDINGS, AND ABSTRACTS**

The following section contains reprints and/or preprints of journal articles, conference proceedings, and abstracts from this project.

# In-Situ and Ex-Situ Bioremediation Options for Treating Perchlorate in Groundwater

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Paul B. Hatzinger

M. Casey Whittier

Martha D. Arkins

Chris W. Bryan

William J. Guarini

*Perchlorate has been identified as a water contaminant in 14 states, including California, Nevada, New Mexico, Arizona, Utah, and Texas, and current estimates suggest that the compound may affect the drinking water of as many as 15 million people. Biological treatment represents the most-favorable technology for the effective and economical removal of perchlorate from water. Biological fluidized bed reactors (FBRs) have been tested successfully at the pilot scale for perchlorate treatment at several sites, and two full-scale FBR systems are currently treating perchlorate-contaminated groundwater in California and Texas. A third full-scale treatment system is scheduled for start-up in early 2002. The in-situ treatment of perchlorate through addition of specific electron donors to groundwater also appears to hold promise as a bioremediation technology. Recent studies suggest that perchlorate-reducing bacteria are widely occurring in nature, including in groundwater aquifers, and that these organisms can be stimulated to degrade perchlorate to below the current analytical reporting limit (< 4 µg/l) in many instances. In this article, in-situ and ex-situ options for biological treatment of perchlorate-contaminated groundwater are discussed and results from laboratory and field experiments are presented. © 2002 Wiley Periodicals, Inc.*

## INTRODUCTION

Ammonium perchlorate ( $\text{NH}_4\text{ClO}_4$ ) is the primary oxidant used in the propellant for many solid rocket motors and boosters, including those providing propulsion for space shuttles and intercontinental ballistic missiles. A single rocket booster for the space shuttle contains approximately 500,000 kg of propellant, of which 70 percent is ammonium perchlorate. Perchlorate salts are also used in munitions, explosives, fireworks, matches, and air bags, as well as in leather tanning, electroplating, and lubrication oils (Gulick et al., 2001; USEPA, 2001a). The only confirmed natural source of perchlorate is Chilean caliche, a nitrate salt that is mined in that country for use in agricultural fertilizers (USEPA, 2001b). Most fertilizers in the United States, however, are not produced with this material, and do not appear to contain significant levels of perchlorate. Rather, the primary sources of perchlorate groundwater contamination are related to the manufacture of the compound for military applications, testing and disposal of rocket motors and munitions, and the periodic removal and replacement of solid fuels in missiles and rockets. The latter procedure, which is referred to as "hog-out," is required because perchlorate fuels have a limited shelf life and must be periodically replaced. During fuel replacement, solid propellant is washed from the missile casing using water under high pressure. The wastewater resulting from this operation contains high concentrations of perchlorate. Improper disposal of this water, as well as disposal practices during per-

chlorate manufacturing and testing, has resulted in substantial perchlorate contamination in several states including Texas, California, Utah, New Mexico, and Nevada.

The California Department of Health Services (CDHS) developed a sensitive detection method for perchlorate in 1997 (CDHS, 1997). Because this technique has only been available for a few years, the total scope of perchlorate contamination in the United States is not yet clear. However, perchlorate has now been detected in 14 states, and current estimates suggest that the drinking water of as many as 15 million people may be affected by this compound (USEPA, 1999; Logan, 2001). For example, as of November 2001, CDHS had sampled 570 public water systems in California and found 65 (11 percent) with detectable perchlorate ( $> 4 \mu\text{g}/\text{l}$ ; CDHS, 2001). Of the 3,434 nonpublic drinking water sources tested by the agency, 226 (6.6 percent) tested positive for the oxidant. Perchlorate has been manufactured or used in 44 states nationwide, so groundwater pollution may extend beyond recent reports (USEPA, 2001a). There is currently no federal action level for perchlorate in groundwater. However, several states, including Arizona, California, Nevada, and Texas, have set provisional action levels ranging from 4 to 31  $\mu\text{g}/\text{l}$  (ppb), and site-specific cleanup levels of 1.5  $\mu\text{g}/\text{l}$  and below have been set by regulators. Perchlorate has also been placed on the contaminant candidate list (CCL) of the EPA (Urbansky & Schock, 1999).

The public health concern regarding ammonium perchlorate is based largely upon the effect of the perchlorate anion on thyroid function. Perchlorate blocks the production of thyroid hormone by inhibiting the transport of iodide into the thyroid gland (Wolff, 1998). Because of this inhibitory action, perchlorate salts have been used therapeutically in large doses to treat hyperthyroid conditions, such as that resulting from Graves' disease. The potential risks of low levels of perchlorate exposure to humans through drinking water are not fully understood. However, the EPA has recently evaluated the human health risks associated with perchlorate contamination, and is expected to issue a revised reference dose for the compound in the near future. A tentative reference dose of 0.9  $\mu\text{g}/\text{kg}/\text{d}$  was proposed by the EPA in a draft toxicological document in 1999, but a review panel determined that additional information was required before a final level could be established (Logan, 2001; USEPA 2001a). There have been a few recent papers concerning the potential for toxicological effects of perchlorate on terrestrial and aquatic species (York et al., 2001; Smith et al., 2001; Manzon & Youson, 1997). However, much more research is necessary to evaluate the potential ecological effects of this contaminant. Thus, the environmental and human health effects resulting from long-term exposure to low levels of perchlorate remain largely unknown.

Perchlorate salts are highly soluble in water (e.g., ammonium perchlorate is soluble to 200 g/l) and dissociate completely. The resulting perchlorate anion is nonvolatile, highly mobile, and chemically stable in aqueous systems under normal conditions present in groundwater and surface water. As a result, in areas where substantial quantities of perchlorate salts have been disposed, large groundwater plumes of perchlorate are often observed. Because of its physical characteristics (i.e., low reactivity, low volatility, high solubility), water treatment technologies including ultrafiltration, air stripping, carbon adsorption, and advanced oxidation are not effective options for perchlorate removal from groundwater (Damian & Pontius, 1999; Logan, 1998; USEPA, 2001a). Ion exchange using one or more selective resins is a viable approach for removing low concentrations of perchlorate from water (e.g., Gu et al., 2000). However, the perchlorate anion is not destroyed during the ion-exchange process, but rather is reversibly bound to the resin. The exchange resins eventually become saturated with the perchlorate

. . . perchlorate has now been detected in 14 states, and current estimates suggest that the drinking water of as many as 15 million people may be affected by this compound . . .

(and other anions which also bind to the resin) and must then be replaced or regenerated using a high-strength salt solution (Urbansky, 1998; Logan, 2001). If the latter procedure is used, the waste brine from the regeneration procedure contains concentrated perchlorate, which then must undergo additional treatment or disposal. Because of the necessity for regeneration or periodic replacement of ion-exchange resins, the operation and maintenance of ion-exchange treatment systems can be expensive compared to other options.

Unlike abiotic approaches, biological treatment represents a promising technology for the effective and economical removal of perchlorate from water (Logan, 2001; Urbansky, 1998). A number of bacteria have been isolated which are able to degrade perchlorate to the harmless products chloride and water (Rikken et al., 1996; Wallace et al., 1996; Coates et al., 1999; Achenbach et al., 2001). These bacteria grow through anaerobic respiration. During this process, the bacteria require an organic or inorganic electron donor (e.g., ethanol, acetate, hydrogen gas) for growth and utilize the perchlorate molecule as a terminal electron acceptor. A (per)chlorate reductase enzyme appears to catalyze an initial two-step reduction of perchlorate ( $\text{ClO}_4^-$ ) to chlorate ( $\text{ClO}_3^-$ ) and then chlorite ( $\text{ClO}_2^-$ ) (Kengen et al., 1999). The chlorite is then further reduced by the enzyme chlorite dismutase to chloride ( $\text{Cl}^-$ ) and oxygen ( $\text{O}_2$ ) (van Ginkel et al., 1996). Thus, microbial degradation of perchlorate yields two innocuous products, chloride and oxygen. Perchlorate respiration is similar to denitrification, in which bacteria utilize a substrate and reduce nitrate as the terminal electron acceptor to nitrogen gas.

Several different types of bioreactor systems have been evaluated for perchlorate treatment during the past several years. An initial bioreactor design was developed and tested in the early 1990s by researchers at Tyndall Air Force Base to treat heavily contaminated wastewater from hog-out and other operations. This stirred-tank reactor utilizes the bacterium *Wolinella succinogenes* HAP-1 for perchlorate reduction (Attaway & Smith, 1994; Hurley et al., 1996). This design works well for low-flow, high-concentration perchlorate wastes, and has been applied at full-scale for this application. However, the reactor design is not well suited for high-flow groundwater applications, in which perchlorate concentrations are likely to be in the  $\mu\text{g}/\text{l}$  (ppb) to low  $\text{mg}/\text{l}$  (ppm) range, and flow rates of thousands of gallons per minute may be required. Several researchers have tested laboratory-scale and pilot-scale packed-bed bioreactors for perchlorate treatment (e.g., Miller & Logan, 2000; Wallace et al., 1998). These systems appear to show promise at the laboratory scale. However, one traditional problem with packed-bed systems is the potential for clogging and channeling with long-term use. Because there is no efficient mechanism to remove biomass from the packed bed, if clogging occurs, the media in the reactor must be replaced. To date, no full-scale packed-bed systems for perchlorate treatment in groundwater have been constructed, so the reliability, treatment efficiency, and cost of these systems at the field scale are not known.

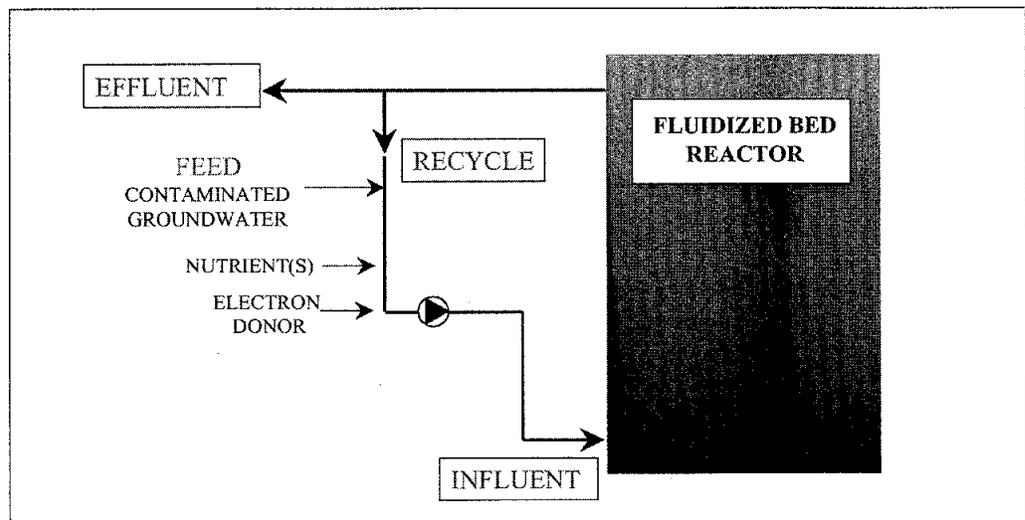
The third biological reactor design that has been tested for perchlorate is the fluidized bed reactor (FBR). Envirogen and USFilter Envirex Products have performed laboratory and pilot-scale tests at several sites across the country. The data from one of these tests is described herein. In addition, two full-scale FBR systems are currently treating perchlorate-contaminated groundwater in California and Texas, respectively, and a third system is under construction. To our knowledge, these are the only full-scale bioreactors currently being used to remediate perchlorate-contaminated groundwater in the United States. The design and treatment efficiency of these systems will be briefly discussed.

A number of bacteria have been isolated which are able to degrade perchlorate to the harmless products chloride and water.

In addition to ex-situ biological treatment using FBRs, in-situ bioremediation of perchlorate through electron donor addition also appears to hold promise for groundwater remediation. For in-situ treatment to be successful, perchlorate-reducing bacteria must be present in contaminated aquifers, and these bacteria must be stimulated to degrade perchlorate from existing levels to below regulatory requirements. A few recent studies show that chlorate- and perchlorate-reducing bacteria are present in soils, sediments, sludges, wastewater, animal waste, and other environments (Coates et al., 1999; Wu et al., 2001). Recent data from our laboratory suggest that these organisms are also native to many groundwater aquifers. In addition, it appears that specific electron donors can be used to promote perchlorate reduction by these bacteria, although the most-effective donors may vary by site. A case study of the effectiveness of electron donor addition for perchlorate and nitrate removal from groundwater is discussed and potential in-situ remediation alternatives are described.

## EX-SITU GROUNDWATER TREATMENT FOR PERCHLORATE USING FLUIDIZED BED REACTORS

Biological fluidized bed reactors (FBRs) have been used for the treatment of nitrate in wastewater beginning in the 1970s (Sutton & Mishra, 1994). Since this time, FBRs have been successfully applied for the treatment of a variety of organic chemicals as well, including petroleum hydrocarbons and pentachlorophenol. An FBR system is a highly efficient, fixed-film bioreactor (Exhibit 1). It consists of a reactor vessel containing media with a large surface area (usually sand or granular activated carbon (GAC)) that is colonized by a film of active bacterial biomass. This media is “fluidized” by the upward flow of wastewater or groundwater into the vessel, with the lowest density particles (those with highest attached biomass) moving to the top. Fluidization is achieved by passing influent water through a distribution system at the bottom of the bed. This system provides a uniform upflow velocity with a flow rate sufficient to achieve a 25–30 percent expansion of the sand or GAC media within the FBR. As biomass grows on the media, the particles become less dense and the bed expands further. A control system is used to remove excess biomass and, thus, maintain the height of the expanded bed. To



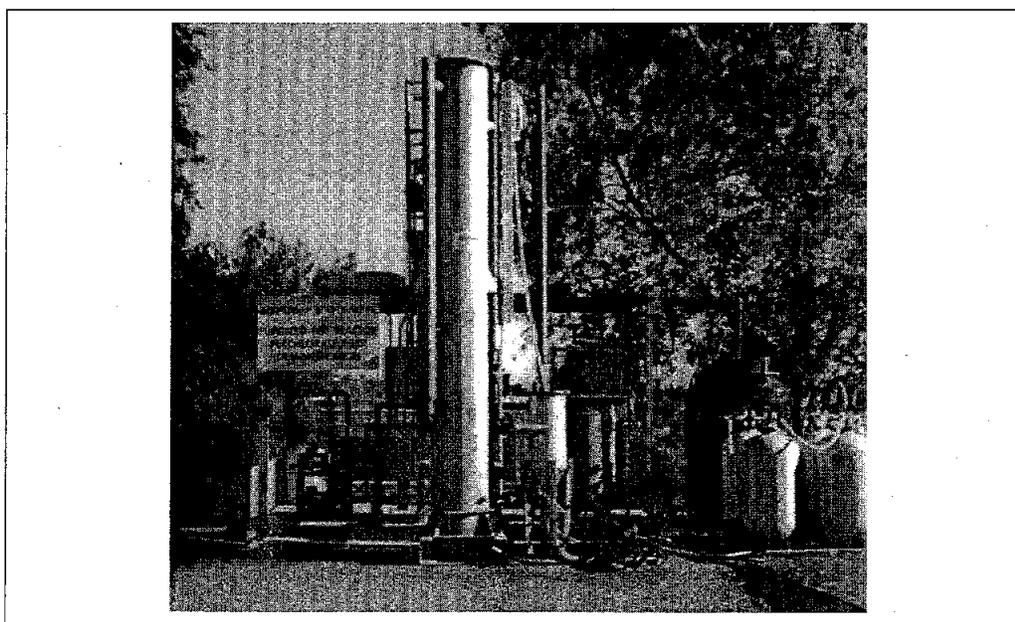
**Exhibit 1.** Process Schematic for a Fluidized Bed Reactor

achieve the required upflow velocity of water for proper fluidization of the media, influent groundwater can be combined with recycled (i.e., treated) water from the reactor. The high biomass maintained within the FBR bed makes it significantly more efficient for water treatment than many other types of biological systems, and allows reactors to be considerably smaller (USEPA, 1993). In addition, the fluidization of the media combined with the presence of effective systems of biomass control prevents clogging and/or channeling in the reactor, allowing efficient performance over long time periods.

## FBR DEMONSTRATION

### *Materials and Methods*

A field pilot test was conducted at a perchlorate-contaminated site in southern California. This study was designed to evaluate reactor performance under site conditions and to develop parameters necessary to size and cost a full-scale system at the site. The pilot FBR system used for the demonstration consisted of a single stainless steel FBR vessel that was 15 feet tall by 20 inches in diameter (Exhibit 2). GAC was used as the fluidization media, and the expanded bed volume was approximately 25 cubic feet. The unit included a complete fluidization system, a biomass control device, media separator, pumps and controls required for addition of the electron donor and nutrients, and online pH control. In addition to the FBR unit for perchlorate treatment, GAC vessels were used to remove low concentrations of volatile organic compounds (VOCs) from the groundwater, a filter was used to remove suspended solids, and a post-aeration tank was supplied to aerate effluent from the FBR. Ethanol (9 percent solution) was used as the electron donor for biological perchlorate reduction. In addition, nitrogen and phosphorus (soluble nutrients necessary for bacterial growth) were added such that a small residual amount of each was detected in the FBR effluent.



**Exhibit 2.** Pilot-Scale Fluidized Bed Reactor

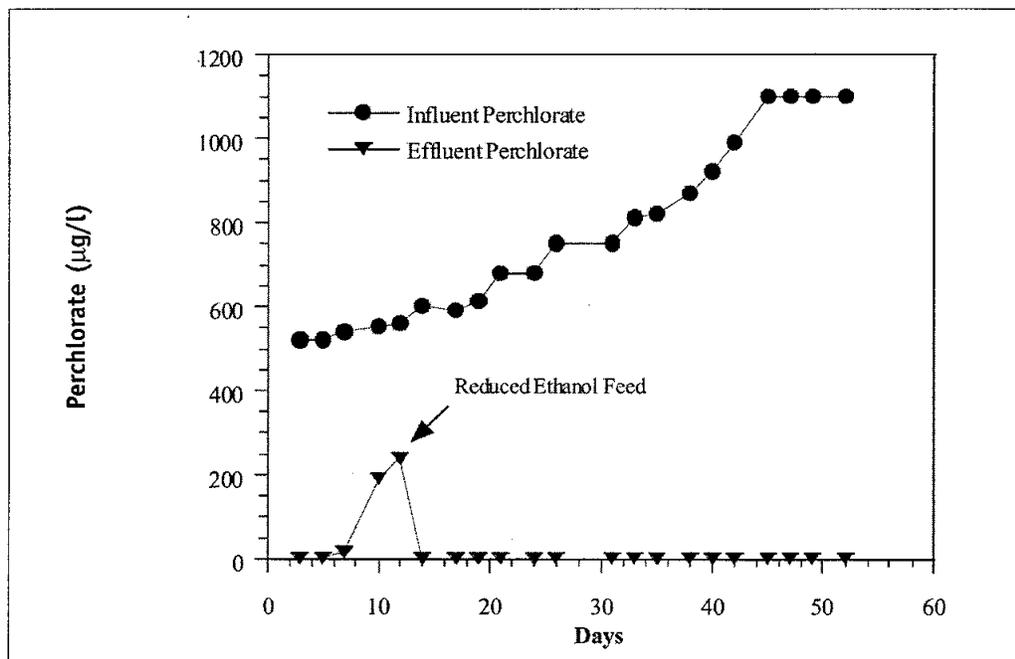
The initial start-up of the FBR system consisted of mechanical start-up, inoculation with a biological seed material, and water flow through the FBR system. The seed material consisted of 20 gallons of GAC media from a full-scale operating FBR that is currently treating perchlorate at the Aerojet Corporation in Rancho Cordova, California. Initial adsorption of perchlorate by the GAC system (used to remove low levels of VOCs in the influent water) and delays in state permitting postponed the steady introduction of perchlorate-contaminated groundwater to the FBR for approximately seven weeks. The FBR was fed groundwater from the site for the initial few weeks of this period, then the unit was operated in recycle mode and fed doses of ethanol (growth substrate) and nutrients daily to sustain biological growth. Air was periodically added to the recycled water by pumping it through the post-aeration tank and back to the FBR. This provided the microbial biomass with oxygen as an alternate electron acceptor. The FBR was spiked with perchlorate prior to beginning steady flow of groundwater to saturate the GAC media with the anion. An effluent sample collected from the FBR after the spike of perchlorate showed a level of 1,100  $\mu\text{g}/\text{l}$ , suggesting that the GAC was saturated with the anion.

FBR systems usually require two to three weeks after start-up before perchlorate levels in effluent reach target levels.

During the demonstration, aqueous samples were collected from the influent to the FBR and from the effluent line. The samples were analyzed for perchlorate using EPA Method 314.0 (ion chromatography) and for nitrate by EPA Method 300.0. In addition, selected samples were analyzed for chemical oxygen demand (COD) as a measure of ethanol concentration by EPA Method 410.4; for total phosphorus by Method E365.1; and for ammonia nitrogen ( $\text{NH}_3\text{-N}$ ) by EPA Method 350.1. Laboratory analyses were performed by Montgomery Watson Laboratories in Pasadena, California. The groundwater well pump feeding the FBR pilot operated at maximum capacity throughout the pilot study. The maximum flow rate achieved was 5.3 gallons per minute (gpm) and the minimum flow rate was 3.6 gpm. This water was combined with recycle flow from the FBR to maintain a fluidization flow of approximately 30 gpm. The ethanol feed rate was varied based on the influent flow rate and the influent nitrate and perchlorate concentrations. Once steady groundwater flow was initiated (day zero), the pilot test was conducted for 52 days.

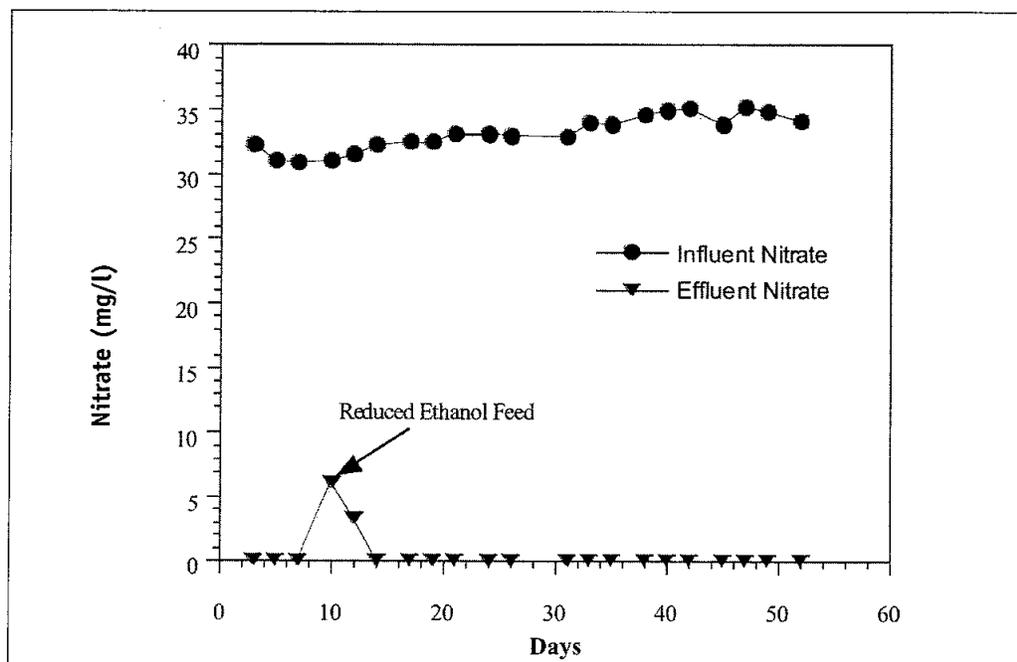
## **Results and Discussion**

There was no appreciable lag period in perchlorate treatment once groundwater flow was initiated (Exhibit 3). Based on previous pilot- and full-scale experience, FBR systems usually require two to three weeks after start-up before perchlorate levels in effluent reach target levels. Rapid colonization of media with microbial biomass occurs during the period. However, it is likely that such biomass growth occurred during the initial period that the reactor was operated and during the period that it was fed in recycle. The results of the FBR effluent analysis after three days of operation showed 520  $\mu\text{g}/\text{l}$  of perchlorate in the influent and less than 4  $\mu\text{g}/\text{l}$  in the effluent. The level of nitrate ( $\text{NO}_3^-$ ) in the influent was 32.3 mg/l at the same time, and the effluent concentration was below 0.4 mg/l (Exhibit 4). To demonstrate that biological treatment rather than adsorption by the GAC media was reducing levels of nitrate and perchlorate, the ethanol feed rate was reduced by 16 percent (from 2.5 to 2.1 ml/min) on day seven. The perchlorate in the FBR effluent increased from below detection ( $< 4 \mu\text{g}/\text{l}$ ) to 15  $\mu\text{g}/\text{l}$ , 190  $\mu\text{g}/\text{l}$ , and 240  $\mu\text{g}/\text{l}$ , during the next three sampling events (days seven, ten, and 12, respectively). The levels of nitrate in the effluent water increased from less than 0.4 mg/l to 6.11 and 3.32 mg/l on day ten and day 12, respectively. The break-



**Exhibit 3.** Influent and Effluent Concentrations of Perchlorate during the Pilot FBR Test

through of both nitrate and perchlorate when the ethanol feed was reduced verifies that biological reduction rather than GAC adsorption was responsible for the removal of perchlorate and nitrate within the FBR. The ethanol feed rate was increased on day 13 and the FBR effluent levels of perchlorate and nitrate returned to less than 4 µg/l and less than 0.4 mg/l, respectively, by day 14. This consistent level of treatment was maintained for the remainder of the study. The influent flow to the reactor was discontinued after 52 days of steady-state operation.

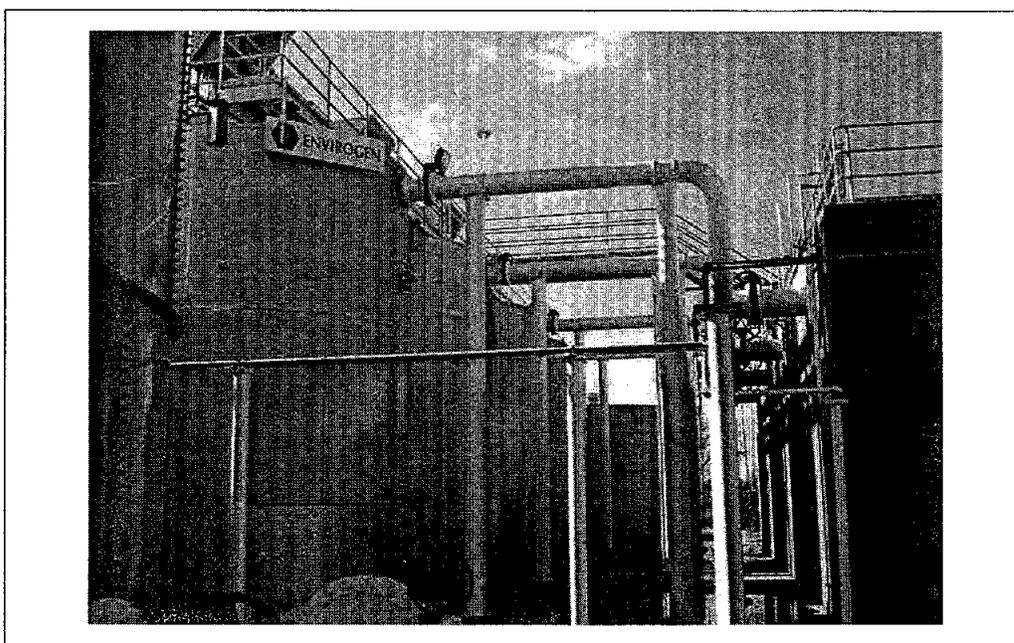


**Exhibit 4.** Influent and Effluent Concentrations of Nitrate during the Pilot FBR Test

As illustrated in Exhibit 3, the FBR received a steady increase in perchlorate concentration during the pilot demonstration. The perchlorate concentration in the influent groundwater increased from 360  $\mu\text{g}/\text{l}$  to 1.10  $\text{mg}/\text{l}$  and then leveled off. The concentrations of  $\text{NO}_3$  in the influent water also increased marginally during the test period. The increase in influent concentrations occurred naturally as the pumping progressed at the site. The FBR consistently reduced both perchlorate and nitrate to below detection during the entire test period (after the initial ethanol dosing test) irrespective of changes in the influent concentration of the two contaminants.

The average influent nitrate and perchlorate concentrations for the pilot test were 33.2  $\text{mg}/\text{l}$  and 770  $\mu\text{g}/\text{l}$ , respectively. The average influent flow rate and ethanol feed rate over the same period were 4.1  $\text{gpm}$  and 3.9  $\text{ml}/\text{min}$ , respectively. The calculated ethanol feed rate of the 9 percent solution is 1.2 pounds per day and the  $\text{NO}_3$  loading rate is 1.64 pounds per day. The amount of ethanol required is largely based on the nitrate loading because it is more than 30 times that of the perchlorate.

The data from the pilot demonstration suggest that an FBR is a viable technology for treating both nitrate and perchlorate in groundwater at the test site. The FBR system consistently reduced both contaminants to below detection throughout the 52-day treatment period (except when the ethanol dose was intentionally reduced for testing). These test data support findings at several other sites where pilot tests with FBRs have been conducted (Hatzinger et al., 2000; Greene & Pitre, 2000). In addition, there are currently two full-scale FBR systems treating perchlorate-contaminated groundwater in the United States. The first system, which is in operation at the Aerojet facility in Rancho Cordova, California, consists of four FBR units, each 14 feet in diameter by 22 feet high (Exhibit 5). This system has been treating perchlorate ( $\sim 3.5 \text{ mg}/\text{l}$ ) and nitrate ( $\sim 6.5 \text{ mg}/\text{l}$ ) in groundwater to below detection limits for more than two years. The design flow rate for this system is 4,000  $\text{gpm}$ . A second system has been effectively treating perchlorate (15–35  $\text{mg}/\text{l}$  influent) at the Longhorn Army Ammunition Plant, in Karnack, Texas, for more than nine months. The design flow of this system is 50  $\text{gpm}$ . A third FBR



**Exhibit 5.** Full-Scale Fluidized Bed Reactor System Treating Perchlorate in Groundwater

system is currently under construction for perchlorate treatment in groundwater at a U.S. naval facility in Texas. This system is expected to be in operation in early 2002.

## IN-SITU REMEDIATION OF PERCHLORATE IN GROUNDWATER

Three separate projects were funded by the Strategic Environmental Research and Development Program (SERDP) of the Department of Defense (DOD) beginning in 2000 to evaluate the potential for in-situ remediation of perchlorate in groundwater. These projects were designed to assess the occurrence and diversity of perchlorate-reducing bacteria in groundwater, to evaluate the most-effective substrates to stimulate biological perchlorate reduction and the geochemical parameters that may influence the process, and to design and test applicable field techniques for in-situ remediation of the propellant. As a recipient of one of these SERDP projects, we have examined the potential for in-situ remediation of perchlorate at several sites across the United States. These studies have included evaluations of the following: (1) the occurrence of perchlorate-reducing bacteria in groundwater; (2) the most-effective electron donors for the stimulation of these bacteria; and (3) the influence of alternate electron acceptors (e.g., nitrate, chlorate, and oxygen) and environmental conditions on perchlorate degradation. The laboratory results from these studies suggest that perchlorate-reducing bacteria are widespread in groundwater aquifers and that, with addition of specific electron donors, these bacteria can be stimulated to degrade perchlorate from existing levels to below current detection limits. However, environmental conditions including low pH, high salinity, and the presence of co-contaminants can influence the rates and extents of degradation. Laboratory data generated using aquifer samples collected from a site in southern California are presented in the following section. This site is at the same location where the previously described FBR pilot test was conducted. In-situ and ex-situ remediation options are being considered together for this location.

... these studies suggest that perchlorate-reducing bacteria are widespread in groundwater aquifers and that, with addition of specific electron donors, these bacteria can be stimulated to degrade perchlorate from existing levels to below current detection limits.

### *Materials and Methods*

Groundwater was collected from a monitoring well at the southern California site. Aseptic sampling techniques and sterile sample containers were used to prevent contamination of groundwater with non-native bacteria. Microcosms to evaluate the effectiveness of different substrates for promoting in-situ perchlorate degradation were prepared in sterile, 160-ml serum bottles. All experimental work was performed in a Coy Environmental Chamber with a nitrogen headspace. Groundwater was amended with a sterile stock of diammonium phosphate to provide nitrogen (5 mg/l as  $\text{NH}_4$ ) and phosphorus (4.5 mg/l as P) as nutrients for bacterial growth, then 120-ml volumes were added to the serum bottles. Triplicate bottles were amended with acetate, yeast extract, methanol, or molasses as electron donors (i.e., growth substrates) to 200 mg/l. Triplicate samples were prepared without substrate, and triplicate bottles received formaldehyde (1 percent) to inhibit all biological activity. All bottles were crimp-sealed with sterilized Teflon-lined septa and incubated at 15°C to approximate in-situ temperatures. After various incubation times, a 20-ml volume was removed from each bottle. The samples were analyzed for perchlorate and nitrate by ion chromatography (EPA Method 314.0 and 300.0, respectively).

Additional microcosm studies were conducted to assess the relative reduction kinetics of alternate electron acceptors including nitrate and nitrite, and to evaluate the

influence of these anions on perchlorate reduction. The microcosms were prepared as described in the previous section (160-ml serum bottles, 120-ml groundwater). Ethanol was added as the electron donor to 100 mg/l, and diammonium phosphate was provided as a source of nitrogen and phosphorus. A separate study (data not presented) showed that ethanol was an effective electron donor for microbial perchlorate reduction in site samples. In experiments conducted to evaluate the relative kinetics of nitrate and perchlorate degradation, the perchlorate concentration in the groundwater was brought to 115 µg/l in some samples and others were left at the site level of 310 µg/l. Duplicate microcosms at each perchlorate concentration then received either no additional nitrate or 100 mg/l of nitrate (NO<sub>3</sub>) in solution. In addition, one set of microcosm bottles containing perchlorate at 310 µg/l received 10 mg/l of nitrite (NO<sub>2</sub>) in solution, and two additional bottles received oxygen rather than nitrogen in the headspace to examine perchlorate reduction under oxic conditions. A killed control for each treatment was prepared by adding 1 percent formaldehyde to the groundwater samples. The samples were incubated at 15°C, and aqueous subsamples were removed periodically and tested for perchlorate (EPA 314.0), nitrate (EPA 300.0), and nitrite (EPA 300.0).

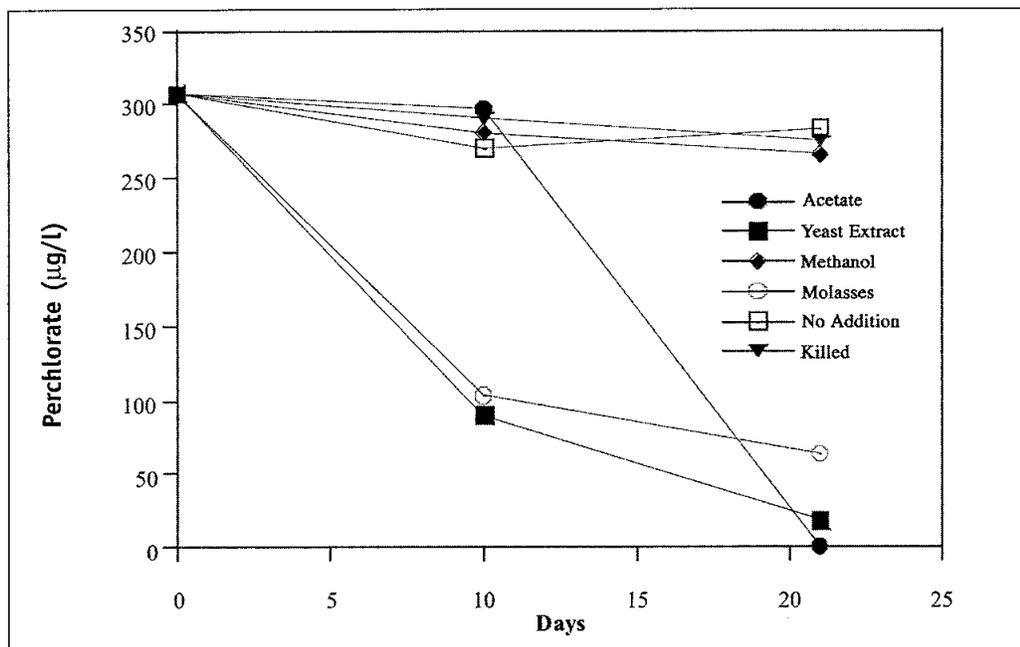
## Results and Discussion

... perchlorate levels can be reduced to below 5 µg/l through biostimulation with some substrates.

The groundwater initially collected from the site contained perchlorate at 310 µg/l, nitrate at 18.6 mg/l, sulfate at 44 mg/l, 140 mg/l of alkalinity (as CaCO<sub>3</sub>), and dissolved oxygen at 2.6 mg/l. The initial pH was 7.6. In microcosms prepared to evaluate the effectiveness of different electron donors, after 21 days of incubation perchlorate was below < 5 µg/l in triplicate samples amended with acetate (Exhibit 6). Appreciable degradation of perchlorate was also observed in samples amended with yeast extract and those receiving molasses. After 21 days, samples amended with yeast extract had an average of 18 µg/l of perchlorate remaining, while those receiving molasses had 64 µg/l. Perchlorate was not appreciably degraded in samples with methanol added as an electron donor or in those without an electron donor. The killed samples (1 percent formaldehyde) also showed no loss of perchlorate.

The results from this microcosm study reveal the following: (1) indigenous bacteria capable of degrading perchlorate are present in the aquifer underlying the site (see additional information below); (2) these bacteria can be stimulated to degrade perchlorate by the addition of specific electron donors; and (3) perchlorate levels can be reduced to below 5 µg/l through biostimulation with some substrates. A perchlorate-degrading bacterium was subsequently isolated from the groundwater at this location by traditional microbial enrichment and plating techniques. This pure culture was then identified as *Dechlorosoma suillum* strain JPLRND by 16S rRNA analysis (Acculabs, Newark, Delaware). Similar perchlorate-degrading strains of this species were recently identified by researchers at Southern Illinois University (Achenbach et al., 2001). Additional experiments are ongoing to determine kinetic parameters and to evaluate the influence of environmental variables and alternate electron acceptors on perchlorate degradation by this strain.

In experiments conducted to assess the relative reduction kinetics of nitrate and perchlorate in site samples, nitrate was consistently degraded before perchlorate in groundwater microcosms, irrespective of the initial concentrations of the two anions. For example, in the samples spiked with nitrate (100 mg/l) and perchlorate (115 mg/l),

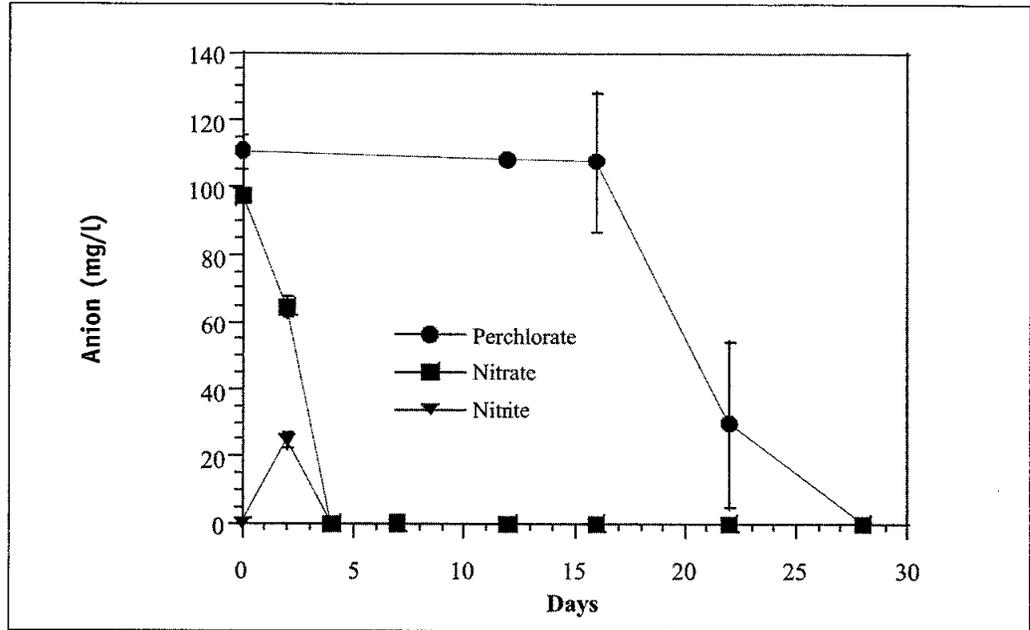


**Exhibit 6.** Biodegradation of Perchlorate in Groundwater Microcosms Amended with Different Electron Donors

nitrate was reduced to below detection after only four days of incubation, with no apparent lag period (Exhibit 7). Nitrite, which is the initial product in biological denitrification and nitrate reduction, was detected in samples at day four, but this anion was also degraded to below detection by day seven. A lag period of approximately 16 days occurred before perchlorate degradation commenced in these microcosms, then perchlorate was rapidly reduced to below detection. A similar pattern was observed in samples with nitrate at 100 mg/l and perchlorate at the initial concentration of 310 µg/l; nitrate was degraded within a few days, whereas perchlorate showed an initial lag period of approximately two weeks prior to degradation (data not shown).

The addition of nitrate (100 mg/l) or nitrite (10 mg/l) to groundwater did not appreciably influence the rate of perchlorate degradation in site samples. The data for samples containing 115 mg/l of perchlorate, with or without amendment with additional nitrate (100 mg/l), are presented in Exhibit 8. In fact, perchlorate reduction may have been stimulated slightly by the addition of nitrate. Conversely, the degradation of perchlorate was completely inhibited by the presence of oxygen in aquifer samples (Exhibit 9). This result confirms previous findings that perchlorate degradation occurs only under anoxic conditions (e.g., Attaway & Smith, 1993; Rikken et al., 1996). However, if enough of the electron donor was added for the indigenous bacteria to consume all of the oxygen in the samples via aerobic respiration, it is likely that perchlorate reduction would soon follow.

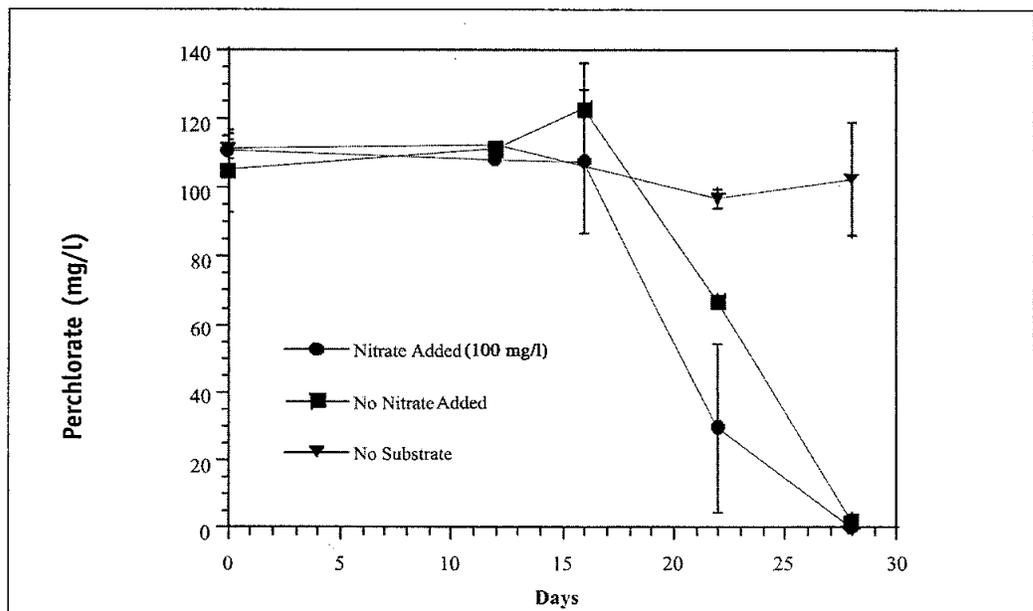
The data from these experiments suggest that nitrate and nitrite are degraded preferentially to perchlorate in this subsurface environment. It is possible that this pattern of degradation reflects a larger population of denitrifying bacteria in the groundwater samples compared to perchlorate-reducers. Most known perchlorate-reducing strains can also grow using nitrate as an electron acceptor, but the reverse is not true. It is also possible that nitrate and/or nitrite physiologically inhibit biological perchlorate degradation in perchlorate-reducing strains, and that perchlorate reduction cannot commence until



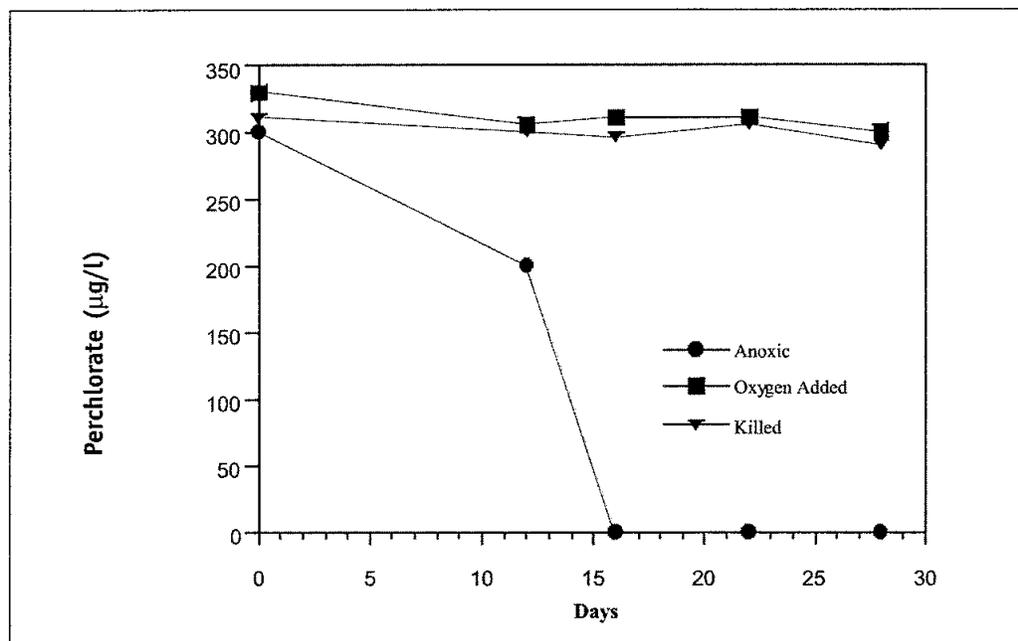
**Exhibit 7.** Biodegradation of Perchlorate and Nitrate in Groundwater Microcosms

these molecules have been reduced below a certain concentration. Nitrate has been shown to inhibit chlorate degradation in enrichment cultures (van Ginkel et al., 1995). Recent studies in our laboratory with the *D. suillum* strain isolated from this site have shown that the rate of perchlorate reduction can be reduced by the addition of nitrate when the culture is actively degrading perchlorate, and that the amount of reduction is dependent on nitrate concentration (unpublished data). This finding suggests that nitrate may be a competitive inhibitor of perchlorate reduction in this bacterium.

An understanding of the relationship between perchlorate and other electron acceptors (e.g., oxygen, nitrate, ferric iron, sulfate) is important because these molecules



**Exhibit 8.** Influence of Nitrate on Perchlorate Biodegradation in Groundwater Microcosms



**Exhibit 9.** Influence of Oxygen on Perchlorate Biodegradation in Groundwater Microcosms

frequently occur with perchlorate in groundwater. For example, the groundwater collected from the southern California site contained 18.6 mg/l of nitrate but only 310 µg/l of perchlorate. This situation is not uncommon due to nitrate contamination of groundwater from agricultural and other sources. Therefore, an understanding of whether nitrate influences perchlorate degradation (e.g., due to enzyme inhibition or other factors) may be important in evaluating, designing, and applying treatment options at some contaminated sites.

The data from microcosm studies suggest that in-situ bioremediation of perchlorate through electron donor addition may be an option for perchlorate treatment at this location. Data collected from other sites during this project have generally supported these data. Overall, the results have shown that perchlorate-degrading bacteria are widely distributed in groundwater environments, and that these organisms can be stimulated to degrade perchlorate by several different electron donors, although the most-effective donors may vary by location.

There are currently few published data concerning the effectiveness of in-situ perchlorate treatment in groundwater at the pilot- or field-scale. One of the significant challenges for in-situ perchlorate treatment to be an effective and cost-competitive technology at many locations is the development of methods to effectively deliver and mix the electron donor with groundwater. Much of the perchlorate-contaminated groundwater in California, Utah, New Mexico, and other western states is present in deep aquifers (100 to 700 feet below surface); therefore, many current in-situ technologies that work well in shallow groundwater, such as treatment trenches and barrier walls, are unlikely to be applicable or cost-effective. The addition of slow-release substrates (e.g., vegetable oil, polylactate) is also unlikely to be economical or effective at many of these sites. Drilling depths greater than 100 feet can be prohibitively expensive if many wells are required for injection, as would likely be the case with poorly dispersed oils or other slow-release substrates. In addition, poor mixing of these electron donors with perchlo-

rate-contaminated groundwater is likely to lead to ineffective treatment of large plumes.

Systems that effectively meter and mix the electron donor with a large zone of influence are anticipated to be the most-effective in-situ remediation options for perchlorate. One design, which has been tested at the pilot-scale with promising results at the Aerojet site in Rancho Cordova, California, is a groundwater injection-extraction system (Cox et al., 2001). In a field demonstration, contaminated groundwater was pumped up from the aquifer and mixed with the electron donor. The water with the electron donor was then reinjected through a second well into the aquifer, creating a treatment zone between the injection and extraction wells. The injection and extraction wells were placed perpendicular to groundwater flow. The study results showed rapid biodegradation of perchlorate in situ, with concentrations of perchlorate declining from 15 mg/l to less than 4 µg/l within 15 feet of the electron-donor delivery well. In a second phase of testing, a system consisting of two extraction wells and one injection well is being tested as a means to capture the core of a perchlorate plume (a width of about 600 feet). In this case, groundwater is pumped through the two extraction wells, amended with the electron donor (ethanol), and then recharged to the aquifer via the single recharge well. In initial data from this test, perchlorate concentrations have been reduced from approximately 8 mg/l to less than 4 µg/l within 35 feet of the electron-donor delivery wells.

A second design which holds promise for the treatment of perchlorate in deep sites is a horizontal flow treatment well (HFTW) system, such as that applied by McCarty et al. (1998) for remediation of TCE at Edwards Air Force Base. The HFTW system employs dual-screened treatment wells placed in pairs to create a recirculation cell within a contaminated aquifer (i.e., water is not brought to the surface). The electron donor can then be mixed with groundwater passing through each well to promote perchlorate treatment within the recirculation zone. A field demonstration of this technology for perchlorate treatment will be conducted by Envirogen in collaboration with the University of New Mexico and the Air Force Institute of Technology beginning in 2002.

In-situ and ex-situ biological treatment technologies represent the most-promising alternatives for cost-effective treatment of perchlorate in groundwater.

## SUMMARY AND CONCLUSIONS

Perchlorate has been detected in water supplies in 14 states, including California, Arizona, Nevada, Utah, and Texas. In-situ and ex-situ biological treatment technologies represent the most-promising alternatives for cost-effective treatment of perchlorate in groundwater. Fluidized bed reactors (FBRs) have proven to be an effective reactor design for ex-situ treatment of perchlorate in groundwater. In the study reported herein, an ethanol-fed FBR consistently reduced perchlorate concentrations from influent levels averaging 770 µg/l to below the analytical reporting limit (< 4 µg/l). The FBR also consistently treated nitrate from 33.2 mg/l to nondetectable levels (< 0.4 mg/l). These performance data are consistent with those from two full-scale FBR systems treating perchlorate in groundwater and from several other pilot tests. The high biomass maintained within an FBR system, its ability to efficiently handle high groundwater flow rates and changing concentrations of perchlorate and nitrate, and effective mechanical devices to prevent biological fouling and channeling make the FBR an optimal reactor design for perchlorate treatment in groundwater.

In addition to bioreactor technologies such as the FBR, in-situ treatment of perchlorate-contaminated groundwater through electron donor addition is likely to be a viable remediation option at many sites. Laboratory data suggest that perchlorate-reducing bacteria are widely occurring in natural environments, including groundwater aquifers, and

that these organisms can be stimulated to degrade perchlorate to below detection through addition of specific electron donors. Pilot-scale or full-scale field data are presently inadequate to evaluate the cost and effectiveness of different in-situ treatment options for perchlorate in groundwater. However, initial pilot tests of in-situ perchlorate bioremediation conducted at the Aerojet site in California are showing promising results, and several additional demonstrations of technologies for perchlorate treatment are planned beginning in 2002 (funded through the Environmental Security Technology Certification Program (ESTCP)). These field tests should provide the necessary cost and performance data to evaluate the applicability and economics of in-situ treatment technologies for perchlorate at full scale.

Based on current information, it is likely that both in-situ and ex-situ bioremediation will be employed at many sites as part of an overall strategy for treatment of perchlorate in groundwater. The facilities where perchlorate-containing fuels have been manufactured, tested, or replaced frequently have several different sources of groundwater contamination, including testing grounds, burn areas, and hog-out facilities. The factors that influence perchlorate remediation options at these sites are varied, and include the characteristics of the perchlorate plume (i.e., concentration, depth, extent), the presence of co-contaminants (such as chlorinated solvents and heavy metals, and existing systems to remove these contaminants), the necessity to protect public or private drinking water wells from plume migration, regulatory requirements, and cost. In cases where well protection is required, where existing pump-and-treat systems are already in place, or where plumes are very deep and/or expansive, ex-situ treatment is likely to be the bioremediation option of choice. Conversely, in well-defined source areas, shallow or narrow zones of contamination, and/or regions where drinking water is unlikely to be affected, in-situ perchlorate treatment may prove to be the best treatment option.

## ACKNOWLEDGMENTS

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## Field Demonstration of In Situ Perchlorate Bioremediation at NSWC Indian Head

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### ABSTRACT

Biological treatment of ammonium perchlorate is a promising technology for remediation of ground and surface water. A wide variety of microbial strains have been isolated with the ability to degrade perchlorate to chloride and water. These organisms require an electron donor (substrate) for growth and utilize the perchlorate molecule as a terminal electron acceptor. The key to utilizing perchlorate-reducing bacteria for *in situ* bioremediation is the understanding and control of the conditions that promote the activity in subsurface environments. As part of a SERDP-funded research project, laboratory microcosm and column studies were conducted using aquifer samples collected from several different perchlorate-contaminated sites across the United States. These studies were designed to evaluate the most effective substrates for stimulating biological perchlorate reduction and to assess the influence of environmental variables on the process. Perchlorate-degrading bacteria were found to be widespread in subsurface aquifers, and pure cultures of the genera *Dechlorosoma* and *Dechlorospirillum* were isolated. Several substrates, including acetate, lactate, molasses, ethanol, and hydrogen effectively stimulated perchlorate reduction in aquifer microcosms. Starting perchlorate levels ranging from 0.31 mg/L to greater than 100 mg/L were reduced to below detection (< 0.004 mg/L) in aquifer samples amended with appropriate electron donors. The laboratory studies revealed that *in situ* bioremediation is a promising technology for perchlorate treatment at many DoD sites. In order to build upon the successful laboratory studies, a field demonstration of *in situ* bioremediation of perchlorate is planned for 2002 at Naval Surface Warfare Center Indian Head Division's Building 1419, otherwise known as the Hogout Facility. Laboratory studies as well as the geological and hydrogeological conditions observed at this site indicate that it is likely to be suitable for *in situ* bioremediation. Laboratory results from this site and an overview of the planned field demonstration are presented.

### INTRODUCTION

Ammonium perchlorate is extensively used as an oxidizer in solid rocket motor and booster propellant formulations and has been found as a contaminant in the environment.<sup>1</sup> The perchlorate salts are quite soluble in water and exceedingly mobile in aqueous systems. The  $\text{ClO}_4^-$  anion is stable and can persist for many decades due to its low reactivity with other constituents. The current database on the health effects and toxicity of  $\text{ClO}_4^-$  anion is very limited.<sup>2</sup> However, current data confirm the potential of perchlorate for disrupting thyroid hormones. The National Center for Environmental Assessment (NCEA) has recently issued a draft toxicological risk assessment based on a reevaluation of the toxicological data for this compound.

Since perchlorate is very unreactive with most reducing agents, the development of suitable remediation and treatment technologies is difficult. However, specific types of bacteria have been found to metabolize perchlorate as a terminal electron acceptor during growth on either organic or inorganic substrates (e.g., lactate, acetate, hydrogen gas), subsequently reducing perchlorate completely to chloride ion and oxygen.<sup>3</sup> Biological treatment technologies for remediation of groundwater with perchlorate are based on the use of these naturally-occurring microbes.<sup>4</sup>

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Both *ex situ* and *in situ* groundwater and soil remediation techniques have been evaluated to some extent.<sup>5,6</sup> Full-scale fluidized bed bioreactors are currently being used successfully to remediate perchlorate-contaminated groundwater at several sites in the United States.<sup>6</sup> In addition, application of appropriate substrates (electron donors) to subsurface aquifers to stimulate indigenous perchlorate-degrading bacteria holds promise as an *in situ* technology for perchlorate remediation. As part of a SERDP-funded research effort, the potential for *in situ* perchlorate remediation at several different sites across the United States was evaluated. The occurrence of perchlorate-reducing bacteria at these sites was tested, the effectiveness of various donor substrates for stimulating perchlorate reduction by these bacteria was quantified, and the influence of environmental variables, including groundwater pH and the presence of co-contaminants, on the rate of microbial perchlorate degradation was determined. Data from this research effort were then used to design a pilot-scale system for a field demonstration of *in situ* bioremediation of perchlorate in groundwater at NSWC Indian Head.

## METHODS, RESULTS AND DISCUSSION

### Site Selection

Biological treatment of ammonium perchlorate is a promising technology for remediation of groundwater. Several microbial strains with the ability to degrade perchlorate by using the molecule as a terminal electron acceptor have been isolated. The enzymatic pathways involved in perchlorate reduction have yet to be fully studied. However, it appears that a perchlorate reductase enzyme catalyzes an initial two-step reduction of perchlorate ( $\text{ClO}_4^-$ ) to chlorate ( $\text{ClO}_3^-$ ) and then chlorite ( $\text{ClO}_2^-$ ).<sup>7</sup> The chlorite is further reduced by chlorite dismutase to chloride ( $\text{Cl}^-$ ) and oxygen ( $\text{O}_2$ ). Thus, microbial degradation of perchlorate yields two innocuous products, chloride, and oxygen. In addition, the reduction of perchlorate to chloride is a very favorable process from a thermodynamic perspective. Thus, bacteria capable of using perchlorate are likely to have a distinct ecological advantage in contaminated environments.

The key in utilizing perchlorate-reducing bacteria for *in situ* bioremediation is to understand the conditions that limit their activity in subsurface environments and then effectively devise technologies that overcome these limitations, subsequently stimulating perchlorate degradation activity. The presence and occurrence of perchlorate-reducing microbes, the ability to stimulate perchlorate degradation with the appropriate electron donors, and the potential factors which may inhibit metabolic degradation of perchlorate have been evaluated during this study. Results from studies at NSWC Indian Head are reported herein.

Two sites where perchlorate use and disposal activities prominently existed were selected for this study. Core and aquifer samples were obtained near the location of a large propellant (300-gallon) mixer facility, and near a facility where water washout of perchlorate propellant rocket motors was performed. The results of the chemical analysis of site samples are shown in Table 1. Slurry samples are a mixture of groundwater and site sediments. Other data are from groundwater only.

Table 1. Chemical Characteristics of Site Samples

	Hogout Facility Bldg. 1419	300-Gallon Mixer Bldg. 1190
Depth	6 – 13 ft (BLS)	4 – 12 (BLS)
Perchlorate	45 mg/L (slurry)	<0.004 mg/L (slurry)
pH	4.3 (slurry)	6.1 (slurry)
Alkalinity	19 mg/L	40 mg/L
Sulfate	88 mg/L	12 mg/L
Nitrate	<0.4 mg/L	<0.2 mg/L
Nitrite	<0.4 mg/L	<0.4 mg/L
Chloride	26 mg/L	43 mg/L
Co-Contamination	NA (binder/metals)	Fuel?

The groundwater was shallow at each of these sites. Although a large quantity of perchlorate has been used and disposed of at the mixer site (Bldg. 1190), perchlorate was not detected in groundwater near this facility. Samples from this location were amended with perchlorate to perform microcosm studies, but the site was dropped from consideration for a field demonstration. Perchlorate was detected in groundwater and sediment samples near the Hogout facility at approximately 45 mg/L. Additional site characterization work has shown perchlorate levels in shallow groundwater exceeding 300 mg/L in specific locations near this facility. Therefore, this area has been identified for use as the primary field demonstration site.

### Microcosm Studies

Aquifer microcosm studies were performed with samples from the above sites to determine the effectiveness of various electron donors for stimulation of perchlorate degradation. To prepare microcosms, groundwater and homogenized sediment from the sites was added to 160-mL serum bottles (100-mL groundwater and 30-g sediment) in a Coy Environmental Chamber with a nitrogen headspace. The groundwater was initially amended with a nutrient solution to provide nitrogen and phosphorus, then the serum bottles received one of several different substrates, including acetate, ethanol, molasses, and lactate. Some bottles were amended with hydrogen as an inorganic substrate for perchlorate reduction. Killed controls received acetate as a substrate and 1 % formaldehyde to inhibit microbiological activity. The bottles were sealed with sterile septa and incubated at 15°C with gentle shaking. At various times, aqueous subsamples were collected and analyzed for perchlorate by ion chromatography using EPA Method 314.0.

The results from the Building 1190 site are shown in Figure 1. As previously noted, perchlorate was not detected in samples from this site. Therefore, perchlorate was added to microcosms at a starting concentration of approximately 125 mg/L. After 11 days, perchlorate levels were below detection in microcosms amended with hydrogen gas, and samples that received acetate declined to 3 mg/L perchlorate during this time. After 34 days, perchlorate was below detection in samples treated with molasses or acetate, as well as those receiving hydrogen. No perchlorate loss was evident in samples receiving ethanol or in acetate-amended microcosms that were treated with formaldehyde to inhibit biological activity. The latter result shows that the decline in perchlorate levels was due to biodegradation rather than any abiotic process.

In a separate study using samples from the Hogout facility, there was no significant loss of perchlorate during more than 2 months of incubation in any of the aquifer microcosms, irrespective of substrate added (ten different substrates were tested) (*data not shown*). The most obvious difference between the two sets of samples was the much lower pH of the Hogout site compared to Building 1190 (pH 4.3 vs. 6.1 in slurries, respectively). Therefore, a second study was conducted in which some of the Hogout samples were amended with acetate, then brought to a pH of 7.0 using sodium carbonate. When the pH was increased, perchlorate degradation was observed in microcosms from this site (Figure 2). Perchlorate was below detection by day 28 in samples at pH 7.0, whereas little decline was observed in acetate-amended samples that remained at pH 4.3 or in formaldehyde-killed controls. Thus, the data suggest that perchlorate-degrading bacteria are present at the site, but that low pH is inhibiting perchlorate degradation by these strains.

Figure 1. Influence of Electron Donors on Perchlorate Levels in Aquifer Microcosms from Building 1190

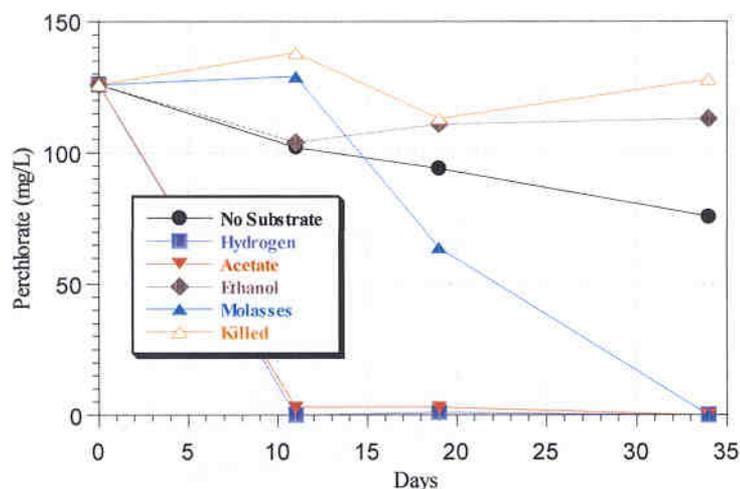
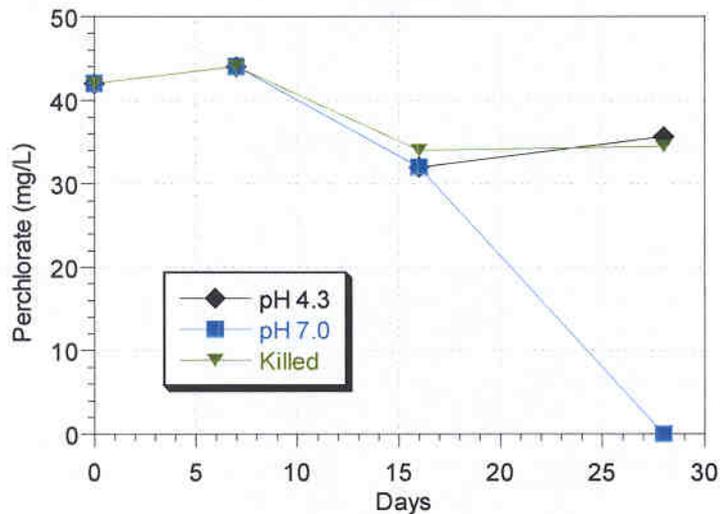


Figure 2. Influence of pH on Perchlorate Degradation in Aquifer Microcosms from the Hogout Facility



The results of microcosm studies performed at Indian Head suggest the following: (1) perchlorate-reducing bacteria are naturally present in the shallow aquifer underlying the facility; (2) these bacteria can be stimulated to biodegrade perchlorate from high mg/L levels to at or below current reporting limits for the compound (~ 0.004 mg/L) using specific substrates; and (3) low pH is inhibitory to perchlorate biodegradation at the Hogout facility. The information gained during the laboratory phase of this research is currently being applied in the field. An *in situ* treatment system has been designed and is currently being installed at the Hogout facility. As described in the next section, this system will provide both buffer and substrate to the shallow groundwater to stimulate perchlorate treatment at this location. This is one of only a few field demonstrations of *in situ* perchlorate treatment that have been attempted, and the only one at a low pH site.

#### In Situ Treatment System

The objective of this project is to build upon the microcosm results by conducting a field demonstration of *in situ* bioremediation of perchlorate at NSWC Indian Head Division's Hogout facility. The observed geological and hydrogeological conditions at this site indicate that it is a suitable candidate for *in situ* bioremediation. Based on the results of laboratory microcosm studies, this site will require pH adjustment and substrate amendment to promote *in situ* bioremediation of perchlorate. The site will need to be buffered to increase pH levels into a range suitable for microbial perchlorate degradation (> 6.0). Preliminary studies indicated that the low pH of the Hogout site (4.3) is inhibiting the biological breakdown of perchlorate. The ability to maintain a buffered subsurface reaction zone is critical in maintaining the biological destruction of perchlorate. Injection wells will be used to supply both buffer and substrate. Lactate or acetate will be added as substrates. Preliminary results indicate these compounds are suitable electron donors for supporting the biological breakdown of perchlorate at the Hogout site. A recirculation cell design will be applied at the site to add and mix substrate and buffer into groundwater (Figure 3). Two plots will be installed, a test plot in which substrate and buffer will be applied and a control plot in which neither amendment is added. If the initial demonstration is successful, and perchlorate levels in the test plot are reduced below detection and sustained at this level, additional studies will be performed using the control plot. In particular, a novel hydrogen-generating system (low-voltage proton reduction) will be tested at this site to determine if it can be used to provide hydrogen gas as a substrate for perchlorate reduction. If this demonstration is successful, this type of system could be applied at this or other sites as a flow-through bio barrier with alternating injection and extraction wells, as shown in Figure 4.

Figure 3. Plan View of Field Demonstration Recirculation Cells

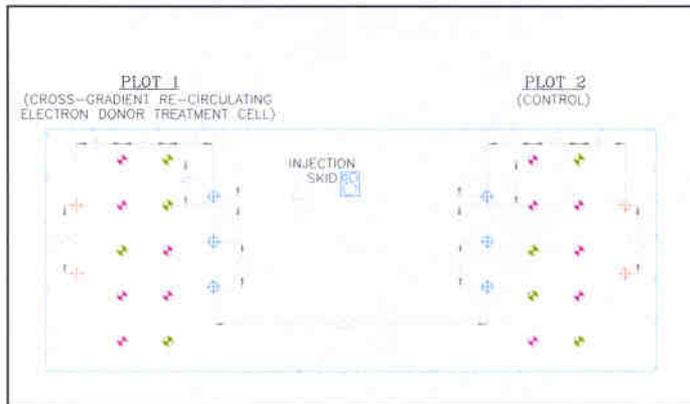
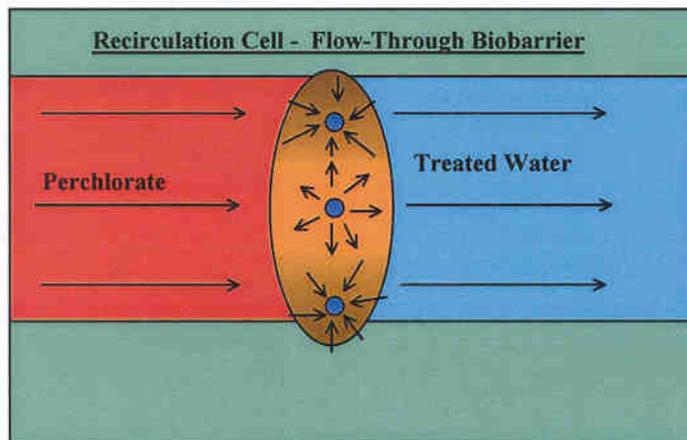


Figure 4. Flow-through Biobarrier for Full-Scale Perchlorate Treatment



This field demonstration conducted at NSWC Indian Head will test the effectiveness of a simple injection/extraction system to supply and mix an electron donor in groundwater, to adjust subsurface pH to optimal levels, and to subsequently promote and maintain microbial breakdown of perchlorate. The effectiveness of the recirculating cell system to supply a buffering agent combined with a low voltage proton reduction system to produce hydrogen gas as a substrate for perchlorate reduction will also be evaluated. This data will provide the field scale scientific as well as the practical knowledge required for the design and implementation of full-scale remediation efforts at perchlorate-contaminated sites. The demonstration system, if successful, is designated to become part of a full-scale system for *in situ* perchlorate remediation at the Hogout site

#### SUMMARY AND CONCLUSIONS

A low cost and effective method for remediation of groundwater containing the propellant formulation ingredient perchlorate is needed. Laboratory studies have shown that naturally-occurring microorganisms can be stimulated to reduce perchlorate to chloride and oxygen. This microbial activity requires the addition of an appropriate electron donor, and can be influenced by a variety of environmental factors. A field

demonstration of *in situ* perchlorate bioremediation is underway at the NSWC Indian Head, at a site in which perchlorate ion has been detected in shallow groundwater. This system uses a recirculating cell design equipped with injection wells for introducing electron donor and buffer. The data from this demonstration will provide the knowledge required for the design and implementation of full-scale *in situ* remediation efforts at perchlorate-contaminated sites.

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**ABSTRACT BOOK**



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in various size and density fractions of the Sunflower River dredged materials. Overall, the results were used to synthesize and correlate data to assess the availability and treatability of DDT in dredged sediments.

**N265 *In situ* Bioremediation of Perchlorate in Groundwater.** Hatzinger, P.<sup>1</sup>, Arkins, M.<sup>1</sup>, Guarini, W.<sup>1</sup> and Manning, J.<sup>1</sup> <sup>1</sup>Envirogen, Inc.. Ammonium perchlorate ( $\text{NH}_4\text{ClO}_4$ ) has been used since the 1940s as an oxidizer in solid propellants and explosives. In recent years, perchlorate contamination of groundwater has been reported in fourteen states, including California, Utah, Nevada, and Texas. Traditional groundwater remediation methods, such as carbon adsorption, air-stripping, and chemical oxidation have proven to be ineffective for perchlorate remediation. However, several microbial strains have been isolated that can utilize and subsequently degrade the molecule to chloride and water. The objective of this research is to explore the potential for *in situ* bioremediation of perchlorate and to determine factors influencing biodegradation of the compound in subsurface environments. Laboratory microcosm studies were conducted using aquifer samples collected from several locations throughout the United States. Rapid perchlorate degradation was observed by indigenous bacteria in substrate-amended aquifer samples from five of the seven sites tested, but the most effective substrates varied by site. For example, in aquifer samples from a site in Utah, amendment with lactate, sucrose, or molasses caused perchlorate concentrations to decline from 57 mg/L to less than 0.1 mg/L in 14 days. However, several other electron donors, including hydrogen gas and benzoate, did not stimulate perchlorate degradation. Experimental data showed that perchlorate biodegradation is inhibited at pH values below 6 and declines rapidly with increasing salinity. Oxygen inhibits perchlorate reduction, and nitrate appears to be preferentially degraded to perchlorate, but it is currently unclear whether nitrate actually inhibits microbial perchlorate reduction. The results suggest that *in situ* biostimulation is a promising technology for perchlorate remediation at many sites.

**W266 Biodegradation of Jet Fuel in a Karst Aquifer.** Byl, T.D.<sup>1,3</sup>, Allison, A.<sup>2,3</sup>, Minor, K.<sup>3</sup>, Roy, S.<sup>3</sup>, Haynes, S.<sup>3</sup>, Darlington, R.<sup>3</sup>, Morris, N.<sup>3</sup>, Elliot, L.<sup>3</sup> and Geymang, D.<sup>3</sup> <sup>1</sup>U.S. Geological Survey, Nashville, TN. <sup>2</sup>U.S. Army Corps of Engineers, Nashville, TN. <sup>3</sup>Tennessee State University, Nashville, TN. Complex hydrogeologic conditions coupled with poorly understood biodegradation processes in karst aquifers have led many to believe that the potential for natural attenuation of petroleum fuel hydrocarbons is limited. This research addressed the capacity for biodegradation processes in a karst aquifer. Ground-water samples were collected for bacteria and geochemical analysis from two monitoring wells (MCI-1 and MCI-4) in a karst bedrock aquifer. Water from the MCI-1 well has consistently tested positive for fuel contamination during the past 3 years of semi-annual monitoring. Water from MCI-4 has been relatively uncontaminated during the same time period. Bacteria concentrations were five-times greater in ground-water samples from the fuel-contaminated well. The bacteria community in the clean well was dominated by gram-positive cocci bacteria, whereas the bacteria community in the contaminated well was dominated by gram-negative, flagellated rods. Additional tests indicate the rod-shaped bacteria are Pseudomonads. Additionally, bacteria isolated from the fuel-contaminated ground-water samples readily grew in Petri dishes with dissolved toluene and benzene as the only source of food. Water from the less contaminated MCI-4 well had a greater dissolved oxygen concentration (6.4 milligrams per liter) than the fuel-contaminated water (dissolved oxygen less than 0.1 milligrams per liter). Also, where the oxygen concentrations were diminished, geochemical evidence indicated that anaerobic processes were active. This evidence includes elevated levels of ammonia, sulfide, and ferrous iron in the fuel-contaminated ground-water samples. Microcosms set up using water from the contaminated well established a half-life of 7 days for toluene and benzene. Based on these results, biodegradation of fuel constituents in the karst aquifer is indicated, and therefore, natural attenuation should not be disregarded because of preconceptions about low microbial activity in karst aquifers.

**PW267 Biodegradation of Chlorinated Contaminants Using Gel Encapsulated Bacterial Cultures.** Govind, R.<sup>1</sup> and Tian, F.<sup>1</sup> <sup>1</sup>Chemical Engineering, University of Cincinnati. Chlorinated chemicals, such as chlorinated aliphatic hydrocarbons (CAHs) and chlorinated aromatics, have been used widely for a variety of industrial applications, such as degreasing of aircraft engines, automobile parts, electronic components, and clothing and in transformer heat transfer fluids. Due to water solubilities exceeding drinking water standards and densities higher than water, these compounds migrate downward through soils and water bodies contaminating ground water, sediments, and penetrate deeply into aquifers forming dense non aqueous phase liquids (DNAPLs) on aquifer bottoms. Ground water toxicity problems associated with CAHs occur at over 358 major hazardous waste sites and many minor sites across the nation. Chlorinated contaminants, such as PCBs, are also found in sediments. Most of the CAHs are aerobically degradable. Some CAHs, such as TCE require cometabolites or specialized organisms for aerobic degradation. Full-scale field applications of cometabolic destruction of CAHs are greatly limited by the availability, cost, and potential adverse environmental impacts of the secondary substrates needed for induction of cometabolic activity. In this paper, the use of specially formulated silica gel beads with active biomass encapsulated within the bead, has been shown to biodegrade TCE without any organic substrates. Bench-scale experimental studies have shown high rates of TCE degradation without any release of intermediates, such as vinyl chloride. Kinetic models have been developed to obtain the kinetic coefficients from experimental data.

**PW268 Biomonitoring of an Oil Terminal Effluent; Baseline Studies.** Roddie, B.D.<sup>1</sup> <sup>1</sup>ERT Caspian. The recent re-development of the oil and gas industry in Azerbaijan has led to an increasing amount of onshore activity to provide oil reception and transportation facilities. The most extensive development to date is the Sangachal oil terminal, operated by BP. This terminal currently receives oil and gas from the Chirag platform, and further developments at this location are planned to accommodate output from the Shah Deniz field. Terminal expansion inevitably implies increased waste generation and discharges, and it was considered important to establish baseline conditions in the receiving waters in advance of any further developments. Baseline coastal marine and fisheries surveys have been conducted to establish the current status of the receiving environment. However, BP wished to implement a more proactive approach to effluent control, and commissioned the Caspian Environmental Laboratory to implement a programme of work aimed at a) establishing a procedure for determining 'end of pipe' ecotoxicity for the current effluent discharges from the terminal b) conducting baseline end-of-pipe toxicity tests c) developing and implementing an *in situ* biomonitoring approach based on the use of sublethal effects assessment in field-deployed bivalves. Both programmes of work were completed in 2000, and provide a baseline for effluent toxicity and receiving water quality against which future impacts can be evaluated. Both methodologies are practical, robust and require no expensive or sophisticated technology. They will, in future, provide a cost-effective method of monitoring effluent impact in real time, and will offer the possibility of detection and intervention before any harm is done to the environment.

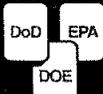
**PW269 Bioavailability of Particle and Sediment-sorbed PAH in Unionid Mussels.** Thorsen, W.A.<sup>1</sup>, Shea, D.<sup>1</sup> and Cope, G.<sup>1</sup> <sup>1</sup>North Carolina State University. Unionid Mussels (*Elliptio* spp.) were deployed in two different freshwater sites in Gaston County, North Carolina. One site was a fast-flowing system with low polycyclic aromatic hydrocarbon (PAH) concentrations and low sediment organic carbon ( $f_{oc}$  0.64%). The second site was a more stagnant wetland site with higher PAH concentrations and higher sediment organic carbon ( $f_{oc}$  3.23%). Mussels were collected at various time intervals along with samples of water, sediment,

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**Program Guide**

## IN SITU BIOREMEDIATION OF PERCHLORATE IN GROUNDWATER

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Ammonium perchlorate ( $\text{NH}_4\text{ClO}_4$ ) has been used since the 1940s in the United States as an oxidizer in solid propellants and explosives. The discharge of contaminated effluents from the manufacture of this compound and from the replacement of outdated fuels in military missiles and rockets has resulted in perchlorate contamination in groundwater in at least 14 states, including California, Utah, Texas, New Mexico, and Nevada. Biological treatment represents the most promising and cost-effective technology for remediation of perchlorate in groundwater. A variety of microbial strains have been isolated with the ability to degrade perchlorate to the innocuous products chloride and water. These organisms require an organic or inorganic electron donor (substrate) for growth and utilize the perchlorate molecule as a terminal electron acceptor. Two full-scale fluidized bed bioreactor systems have been successfully implemented for *ex situ* treatment of perchlorate-contaminated groundwater in California and Texas. The objective of this SERDP-funded research is to explore factors influencing perchlorate bioremediation in the subsurface, and to develop an effective technology for *in situ* treatment of this energetic compound.

Laboratory microcosm and column studies were conducted using aquifer samples collected from several different perchlorate-contaminated sites across the United States. These studies were designed to evaluate: (1) the occurrence of indigenous perchlorate-degrading bacteria in subsurface environments; (2) the most effective electron donors for stimulating perchlorate reduction by these bacteria; and (3) the influence of alternate electron acceptors and environmental variables on biological perchlorate reduction. Perchlorate-degrading bacteria were found to be widespread in subsurface aquifers, and pure cultures of the genera *Dechlorisoma* and *Dechlorospirillum* were isolated from samples during the studies. Several electron donors, including acetate, lactate, molasses, ethanol, and hydrogen effectively stimulated perchlorate reduction in aquifer microcosms, although the most effective amendments varied by site. Starting perchlorate levels ranging from 0.31 mg/L to greater than 100 mg/L were reduced to below detection ( $< 0.004$  mg/L) in aquifer samples amended with appropriate electron donors. Oxygen, nitrate, and nitrite were degraded prior to perchlorate in microcosm and column studies. Other electron acceptors, including ferric iron and sulfate, were reduced after perchlorate and did not influence its rate or extent of degradation. Perchlorate biodegradation was not observed in acidic aquifer samples (pH values below 5.7), but the process could be rapidly stimulated through pH adjustment. The laboratory studies conducted during this SERDP project reveal that *in situ* bioremediation is a promising technology for perchlorate treatment at many DoD sites. A field demonstration of *in situ* perchlorate bioremediation is planned for Spring 2002.

**POSTER ABSTRACTS**

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## IN SITU REMOVAL OF PERCHLORATE FROM GROUNDWATER

W. J. Hunter (USDA-ARS, Fort Collins, Colorado, USA)

Perchlorate is a groundwater contaminant that affects over 20 million people in the southwestern US. Laboratory column studies suggest that *in situ* barriers might be used to remove perchlorate from groundwater. Previously, we demonstrated that when a vegetable oil emulsion is injected onto a sand column the oil became trapped in the matrix and formed a stationary organic zone or barrier. Native denitrifying bacteria removed nitrate from the contaminated groundwater when the water was pumped through sand columns injected with vegetable oil. The present laboratory scale study shows that the same process works with perchlorate. For this study a moderately-hard water containing 20 ppm (0.2 mM) perchlorate was pumped, at a rate of ~25 ml/day, through 1.5 by 30 cm columns containing sand as a matrix. The water supplied to the columns was fully oxygenated. No attempt was made to remove oxygen from the supply water. At the start of the study all columns were inoculated with bacteria from a soil extract. After 14 days operation 0.47 mg of soybean oil was injected onto one group of columns, the treatment group, while a second group of columns, the control group, received no oil. Water containing perchlorate was pumped through both groups of columns and samples of the effluent water were collected at regular intervals and analyzed for chloride and perchlorate. In the control columns, perchlorate was present in the column effluents throughout the study. In the treatment columns, perchlorate in the effluent decreased by ~99% over a 17 week period following the addition of the vegetable oil. Also, the concentration of chloride in the treatment column effluents increased by ~0.2 mM after the addition of oil indicating the stoichiometric conversion of perchlorate to chloride. In the control columns perchlorate levels remained high and chloride levels low throughout the study. These results suggest that permeable barriers containing innocuous vegetable oils, other carbon substrates, or other electron donors might be used *in situ* to remove perchlorate from contaminated groundwater.

## IN SITU BIOREMEDIATION OF PERCHLORATE IN GROUNDWATER

Paul B. Hatzinger, Martha D. Arkins, Marina Tugusheva, and Robert J. Steffan  
(Envirogen, Inc., Lawrenceville, NJ, USA)

Biological treatment represents a promising technology for perchlorate remediation in subsurface environments. Several microbial strains have been isolated with the ability to degrade perchlorate to the innocuous products chloride and water. These organisms require an organic or inorganic electron donor (e.g., ethanol, acetate, hydrogen gas) for growth and utilize the perchlorate molecule as a terminal electron acceptor. Full-scale bioreactor systems have been successfully developed and implemented for *ex situ* perchlorate treatment. The objective of this research is to explore factors influencing perchlorate bioremediation in the subsurface, and to develop effective methods for *in situ* treatment of this contaminant.

Laboratory microcosm studies were performed using subsurface samples collected from the Jet Propulsion Laboratory (JPL) in Pasadena, CA, the Indian Head Division Naval Surface Warfare Center (IHDIIV) in Indian Head, MD, and a commercial facility in the Rocky Mountains. These studies were designed to evaluate: (1) the most effective electron donors (substrates) for growth of indigenous perchlorate-degrading bacteria, (2) the requirement for addition of exogenous perchlorate-degrading bacteria to aquifer samples, and (3) the influence of alternate electron acceptors (e.g., oxygen, nitrate, nitrite, sulfate) and environmental conditions on biological perchlorate reduction. Several electron donors rapidly stimulated perchlorate degradation by indigenous bacteria in JPL site samples. Perchlorate levels were reduced from 0.31 mg/L to less than the MDL (0.005 mg/L) in 10 days. Oxygen completely inhibited perchlorate degradation in these samples. Nitrate and nitrite were degraded prior to perchlorate and did not appreciably influence its rate of degradation. Perchlorate biodegradation was completely inhibited at pH values below 6.0, and declined appreciably with increasing salinity. Perchlorate was readily degraded in one set of subsurface samples collected from IHDIIV when hydrogen gas or acetate (but not ethanol) were added as electron donors. Conversely, perchlorate degradation was not observed in a second set of subsurface samples from IHDIIV after amendment with ten different electron donors or a culture of perchlorate-degrading bacteria. Experiments are underway to evaluate the factors inhibiting perchlorate biodegradation at this location. In samples from the Rocky Mountain site, bioaugmentation was found to provide the most rapid removal of perchlorate from groundwater. Lactate and sucrose, but not several other electron donors, also stimulated perchlorate degradation in these samples. Thus, current data from microcosm studies show that *in situ* bioremediation is a promising technology for perchlorate remediation, but that the remediation approach, including the choice of electron donors, may vary by site.

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## BIOTREATMENT OF PERCHLORATE IN GROUNDWATER

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**B**iological treatment represents the most promising approach for perchlorate remediation in groundwater. Several microbial strains have been isolated with the ability to degrade perchlorate to the innocuous products chloride and water. These organisms require an organic or inorganic electron donor (e.g., ethanol, acetate, hydrogen gas) for growth and utilize the perchlorate molecule as a terminal electron acceptor. Full-scale bioreactor systems have been successfully developed and implemented for ex situ perchlorate treatment. The design and operational data from one such treatment system, a fluidized bed reactor treating groundwater in California, will be presented.

With support from SERDP, research is being conducted to develop effective methods for in situ treatment of perchlorate in subsurface environments. Laboratory microcosm studies were performed using subsurface samples collected from the Jet Propulsion Laboratory (JPL) in Pasadena, CA, the Indian Head Division Naval Surface Warfare Center (IHDIV) in Indian Head, MD, a commercial facility in the Rocky Mountains, and a pristine site in Virginia. These studies were designed to evaluate: (1) the most effective electron donors (substrates) for growth of indigenous perchlorate-degrading bacteria, (2) the requirement for addition of exogenous perchlorate-degrading bacteria to aquifer samples, and (3) the influence of environmental conditions on biological perchlorate reduction. Rapid perchlorate biodegradation was observed in substrate-amended aquifer samples from three of the sites. The most effective substrates for stimulating perchlorate reduction varied by site. For example, in samples from the Rocky Mountain site, amendment with lactate, sucrose, or molasses caused perchlorate concentrations to decline from 57 mg/L to less than 0.1 mg/L in 14 days. However, several other electron donors, including hydrogen gas and benzoate, did not stimulate perchlorate degradation in these microcosms, even after several weeks of incubation. Perchlorate biodegradation was not observed in subsurface samples from a one location at IHDIV after amendment with ten different electron donors or a culture of perchlorate-degrading bacteria. Experiments are underway to evaluate the factors inhibiting perchlorate biodegradation at this location. The current data show that in situ biotreatment is a promising technology for perchlorate remediation, but that the remediation approach, including the choice of electron donors, may vary by site. An assessment of appropriate applications for in situ and ex situ biotreatment technologies will be given.

## **7.2 APPENDIX B**

### **Kinetic Model to Describe Perchlorate Biodegradation**

#### ***7.2.1. MODEL DESCRIPTION***

This appendix presents the mathematical model developed by Envirogen to describe the kinetics of perchlorate biodegradation. Based on laboratory data, perchlorate is used as an electron acceptor by microorganisms during consumption of available carbon and electron sources in soil. The model presented below describes the biodegradation kinetics of perchlorate in the presence of other, more favorable, electron acceptors.

The model links the dynamics of the microbial population to the consumption of electron donor and acceptors. The model for the microbial population describes bacterial growth and decay. Biodegradation of the electron donor (which is also the growth compound), and consumption of multiple electron acceptors are described using a modified Monod model. Within the hierarchy of electron acceptor use, perchlorate is typically utilized after oxygen and nitrate and before sulfate. Thus, in environments where oxygen or nitrate is present, perchlorate utilization may be reduced and/or inhibited until these electron acceptors are consumed.

#### ***7.2.2 MODEL DEVELOPMENT***

##### **7.2.2.1 Electron Donor**

The model describes sequential degradation of electron donor in the presence of oxygen, nitrate, and perchlorate. The rate of utilization of the electron donor per unit biomass is described by equations (1a, b, c, and d). Essentially, the rate of utilization of electron donor is equal to the sum of the donor degradation rates due to each electron acceptor (Eq. 1a). For simplification purposes, it is assumed that mass is expressed in units of milligrams (mg), volume in units of liters (L), and time in units of hours. Also, it is assumed that the compounds described by the equations below do not volatilize into air or sorb onto soil.

The specific rate of electron donor consumption ( $r_{\text{donor}}$ ) is:

$$r_{\text{donor}} = \frac{1}{B} \times \frac{dC^{\text{don}}}{dt} = r_{\text{don,oxy}} + r_{\text{don,nit}} + r_{\text{don,per}} \quad (1a)$$

where:

$$r_{\text{don,oxy}} = k_{\text{max}} \left[ \frac{C^{\text{don}}}{K_S^{\text{don}} + C^{\text{don}}} \right] \times \left[ \frac{C^{\text{oxy}}}{K_S^{\text{oxy}} + C^{\text{oxy}}} \right] \quad (1b)$$

$$r_{\text{don,nit}} = k_{\text{max}} \left[ \frac{C^{\text{don}}}{K_S^{\text{don}} + C^{\text{don}}} \right] \times \left[ \frac{C^{\text{nit}}}{K_S^{\text{nit}} + C^{\text{nit}}} \right] \times \left[ \frac{K_i^{\text{oxy}}}{K_i^{\text{oxy}} + C^{\text{oxy}}} \right] \quad (1c)$$

$$r_{\text{don,per}} = k_{\text{max}} \left[ \frac{C^{\text{don}}}{K_S^{\text{don}} + C^{\text{don}}} \right] \times \left[ \frac{C^{\text{per}}}{K_S^{\text{per}} + C^{\text{per}}} \right] \times \left[ \frac{K_i^{\text{oxy}}}{K_i^{\text{oxy}} + C^{\text{oxy}}} \right] \times \left[ \frac{K_i^{\text{nit}}}{K_i^{\text{nit}} + C^{\text{nit}}} \right] \quad (1d)$$

and

$r_{\text{don,oxy}}$  is the specific rate of electron donor consumption using oxygen as an electron acceptor (mg donor/mg biomass/hr);

$r_{\text{don,nit}}$  is the specific rate of electron donor consumption using nitrate as an electron acceptor (mg donor/mg biomass/hr);

$r_{\text{don,per}}$  is the specific rate of electron donor consumption using perchlorate as an electron acceptor (mg donor/mg biomass/hr);

$k_{\text{max}}$  is the maximum specific growth rate (mg donor/mg biomass/hr);

$C^{\text{don}}$  is the concentration of the electron donor (acetate) (mg/L);

$C^{\text{oxy}}$  is the concentration of oxygen (mg/L);

$C^{\text{nit}}$  is the concentration of nitrate (mg/L);

$C^{\text{per}}$  is the concentration of perchlorate (mg/L);

$K_S^{\text{don}}$  is the half saturation constant for the electron donor (acetate) (mg/L);

$K_S^{\text{oxy}}$  is the half saturation constant for oxygen (mg/L);

$K_S^{\text{nit}}$  is the half saturation constant for nitrate (mg/L);

$K_S^{\text{per}}$  is the half saturation constant for perchlorate (mg/L);

- $K_i^{oxy}$  is the oxygen inhibition coefficient;
- $K_i^{nit}$  is the nitrate inhibition coefficient;
- $B$  is the concentration of biomass (mg/L); and
- $t$  is time (hours).

### 7.2.2.2 Microbial Populations

Two primary processes were expected to influence changes in the microbial populations during our experiments: population growth due to electron donor consumption and indigenous cell decay. These two processes are mathematically represented by the two terms on the right hand side of Equation (2), respectively.

$$\frac{1}{B} \times \frac{dB}{dt} = Y_{biomass} r_{don} - b \quad (2)$$

where:

- $Y_{biomass}$  is the biomass produced per mass of donor consumed (mg biomass/mg electron donor)
- $b$  is the biomass decay rate (1/hour)

### 7.2.2.3 Electron Acceptors

Changes in the electron acceptor concentrations are directly linked to the consumption of the electron donor. The relationships linking consumption of electron donor and oxygen, nitrate, and perchlorate are given in Equations 3.

Oxygen:

$$r_{oxy} = \frac{1}{B} \times \frac{dC^{oxy}}{dt} = Y_{oxy} r_{don,oxy} \quad (3a)$$

Nitrate:

$$r_{nit} = \frac{1}{B} \times \frac{dC^{nit}}{dt} = Y_{nit} r_{don,nit} \quad (3b)$$

Perchlorate:

$$r_{per} = \frac{1}{B} \times \frac{dC^{per}}{dt} = Y_{per} r_{don,per} \quad (3c)$$

where

- $r_{oxy}$  is the specific rate of oxygen consumption (mg oxygen/mg biomass/hr);
- $r_{nit}$  is the specific rate of nitrate consumption (mg nitrate/mg biomass/hr);
- $r_{per}$  is the specific rate of perchlorate consumption (mg perchlorate/mg biomass/hr);
- $Y_{oxy}$  is the yield coefficient for the donor-oxygen reaction (mg oxygen/mg donor);
- $Y_{nit}$  is the yield coefficient for the donor-nitrate reaction (mg nitrate/mg donor); and
- $Y_{per}$  is the yield coefficient for the donor-perchlorate reaction (mg perchlorate/mg donor).

These coefficients were determined theoretically and compared to observations in the laboratory.

### **7.3 MODEL ASSUMPTIONS**

Some of the basic assumptions made to develop the model include:

1. Maximum specific growth rate and the half saturation constant do not significantly change with the different electron acceptors.
2. Cell yield does not change with different electron acceptors.
3. Competition among the different electron acceptors is a continuous function, i.e., not based on “threshold concentrations”.
4. The electron donor and electron acceptors described do not volatilize into air or sorb onto soil.
5. Any lag periods observed during the microcosm studies are due to microbial growth only.
6. Biomass may decay to zero or grow indefinitely.

### **7.4 PARAMETER DETERMINATION**

Batch experiments were conducted to determine the parameters needed for the equations above.

### 7.4.1 Absence of Inhibition

#### Acetate (Electron Donor)

To determine  $k_{max}$  and  $K_S^{don}$ , a series of batch experiments were conducted using a range of starting donor concentrations where oxygen is not limiting. Bacteria in these experiments were pre-grown on the electron donor. By determining the maximum rate of bacterial growth and measuring the electron donor concentrations in each experiment, a curve of specific growth rate versus donor concentration was constructed. The maximum specific growth rate on this curve is  $k_{max}$ .  $K_S^{don}$  is equal to the donor concentration value where the specific growth rate is half the  $k_{max}$  value.

To estimate  $Y_{biomass}$  the change in biomass in the batch experiments due to a given change in donor concentration was measured. In turn,  $Y_{biomass}$  was estimated by taking the ratio of the change in biomass to the change in donor concentration. The decay coefficient,  $b$ , was estimated by monitoring the decay of a given microbial population over time. This was also done in a batch experiment.

#### Oxygen

To determine  $K_S^{oxy}$ , batch experiments can be conducted using a range of starting oxygen concentrations where donor was not limiting. By measuring the change in oxygen concentrations and microbial populations over time a curve of specific growth rate versus oxygen concentration may be constructed. The maximum specific growth rate value on this curve should equal  $k_{max}$ , thus providing a check for the value determined during the donor experiments, and  $K_S^{oxy}$  is equal to the oxygen concentration value where the specific growth rate is equal half the  $k_{max}$  value. However, because it was experimentally difficult to accurately measure oxygen levels in the microcosm experiments,  $K_S^{oxy}$  was assumed to be 1 mg/L during this work.

### Nitrate

For  $K_S^{nit}$ , batch experiments were conducted using a range of starting nitrate concentrations where donor was not limiting and oxygen was not present. By measuring the change in nitrate concentrations and microbial populations over time a curve of specific growth rate versus nitrate concentration was constructed. The maximum specific growth rate on this curve is  $k_{max}$ , which is assumed/expected not to be significantly different from the  $k_{max}$  value determined from the donor experiments.  $K_S^{nit}$  is equal to the nitrate concentration value where the specific growth rate is half the  $k_{max}$ .

### Perchlorate

To determine  $K_S^{per}$ , batch experiments were conducted using a range of perchlorate concentrations where donor was not limiting and both oxygen and nitrate were absent. By measuring the change in perchlorate concentrations and microbial populations over time a curve of specific growth rate versus perchlorate concentration was constructed. The maximum specific growth rate on that curve is  $k_{max}$  (which is also a check of the second assumption made) and  $K_S^{per}$  is equal to the perchlorate concentration where the specific growth rate is half the  $k_{max}$  value.

## **7.4.2 Modeling Electron Acceptor Competitive Inhibition**

### Oxygen

The set of experiments described above for perchlorate were repeated with the addition of constant concentrations of oxygen to assess the role of oxygen inhibition on perchlorate utilization. The approach for these experiments was to be as follows:

- a) Four or five oxygen concentrations that would be suspected to cause varying degrees of perchlorate inhibition were chosen.
- b) Each oxygen concentration was to be inputted along with a range of perchlorate concentrations in batch experiments to construct a curve of specific growth rate versus perchlorate concentration in the presence of the starting oxygen concentration. Four or five such curves were to be obtained.

- c) These curves were to be mathematically described by maintaining  $k_{max}$  constant and allowing  $K_S^{nit}$  to vary. According to the formulation given in Eq. (1b), oxygen will inhibit perchlorate utilization by reducing the effective rate of perchlorate consumption.

The above experiments assessing the role of oxygen inhibition on perchlorate utilization were not completed, as described in the Final Report text.

### Nitrate

The set of experiments described above for perchlorate was repeated with the addition of constant concentrations of nitrate to assess the role of nitrate inhibition on perchlorate utilization.

The approach for these experiments was as follows:

- a) Four or five nitrate concentrations that would be suspected to cause varying degrees of perchlorate inhibition were chosen.
- b) Each nitrate concentration was inputted with a range of perchlorate concentrations to batch experiments to construct a curve of specific growth rate versus perchlorate concentration at each chosen nitrate concentration.
- c) These curves were mathematically described by maintaining  $k_{max}$  constant and allowing  $K_S^{per}$  to change. According to the formulation given in Eq. (1c), nitrate will inhibit perchlorate utilization by decreasing the effective rate of perchlorate utilization.