Fall 2009

Plant-assisted bioremediation of perchlorate and the effect of plants on redox conditions and biodiversity in low and high organic carbon soil

Garrett Cletus Struckhoff
University of Iowa

PLANT-ASSISTED BIOREMEDIATION OF PERCHLORATE AND THE EFFECT OF PLANTS ON REDOX CONDITIONS AND BIODIVERSITY IN LOW AND HIGH ORGANIC CARBON SOIL

by

Garrett Cletus Struckhoff

An Abstract

Of a thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Civil and Environmental Engineering
in the Graduate College of
The University of Iowa

December 2009

Thesis Supervisor: Professor Gene F. Parkin

ABSTRACT

Perchlorate is a known inhibitor of the human thyroid gland. Perchlorate is destroyed by ubiquitous perchlorate-reducing bacteria. The bacteria often lack sufficient electron donor. Research was undertaken to evaluate the relationship between plants and perchlorate-reducing bacteria. To what degree can plant-produced electron donors stimulate perchlorate reduction in low organic carbon (LOC) and high organic carbon (HOC) soil? A complication is that plants have been shown to influence redox conditions which may inhibit perchlorate reduction. The removal of perchlorate in a flow-through reactor was monitored with variables of soil organic carbon, hybrid poplar trees, and bioaugmentation. The biodiversity was monitored using denaturing gradient gel electrophoresis.

Low oxidation-reduction potential (ORP) was shown to indicate the capacity for greater perchlorate removal in soil. However, in planted LOC soil systems, evidence suggests that perchlorate reduction may also be possible at higher bulk redox conditions than previously observed. Increased hydraulic retention time was shown to both lower bulk ORP and increase perchlorate removal.

Radiolabeled perchlorate was used to find that in planted systems as much as 11.7% of the influent perchlorate mass was taken up into the tree and 82% of the perchlorate taken up was accumulated in the leaves. The plant contribution to total perchlorate removal in nonbioaugmented LOC soil was 39%, with the balance of the removal being attributed to microbial reduction. In bioaugmented soil the microbial contribution to perchlorate removal was increased.

Just planting poplar trees decreased the diversity of perchlorate reducers in the soil. However, when LOC soil was both planted and bioaugmented, the diversity of perchlorate reducers was not decreased. In HOC soil, the presence of an indigenous population of microorganisms competed with perchlorate reducers. At the increased

ORP observed in planted HOC soil, the non-perchlorate-reducing bacteria appear to outcompete the perchlorate reducers and perchlorate removal is decreased.

Engineering implications of this research are that perchlorate remediation in HOC soil does not benefit from planting hybrid poplar trees but that remediation in LOC soil is stimulated by planting and bioaugmentation.

Abstract Approved: _____
Thesis Supervisor

_____
Title and Department

_____
Date

PLANT-ASSISTED BIOREMEDIATION OF PERCHLORATE AND THE EFFECT OF PLANTS ON REDOX CONDITIONS AND BIODIVERSITY IN LOW AND HIGH ORGANIC CARBON SOIL

by

Garrett Cletus Struckhoff

A thesis submitted in partial fulfillment
of the requirements for the Doctor of
Philosophy degree in Civil and Environmental Engineering
in the Graduate College of
The University of Iowa

December 2009

Thesis Supervisor: Professor Gene F. Parkin

Graduate College
The University of Iowa
Iowa City, Iowa

CERTIFICATE OF APPROVAL

PH.D. THESIS

This is to certify that the Ph.D. thesis of

Garrett Cletus Struckhoff

has been approved by the Examining Committee
for the thesis requirement for the Doctor of Philosophy
degree in Civil and Environmental Engineering at the December 2009
graduation.

Thesis Committee: _____
Gene F. Parkin, Thesis Supervisor

_____
Chi-Lien Cheng

_____
Timothy E. Mattes

_____
Michelle M. Scherer

_____
Jerald L. Schnoor

To Haley

"The human race is challenged more than ever before to demonstrate our mastery - not over nature but of ourselves"

Rachel Carson

ACKNOWLEDGMENTS

I would like to thank the University of Iowa Center for the Health Effects of Environmental Contamination for a seed grant that made this research possible. I would like to thank the University of Iowa Center for Biocatalysis and Bioprocessing, and the National Institutes of Health, Department of Health and Human Services, Public Health Services, Grant Number: 5-T32-GM08365-17 for a Predoctoral Training Fellowship and financial assistance.

Deepest gratitude is due Gene Parkin, my thesis advisor. He has provided a great combination of support and discipline over the course of my doctoral work. Looking back now, I am in wonder at the deft hand he has used to help shape me into the researcher I am now. The everpresent question, "What exactly does this have to do with your thesis?" is one that I will keep in my repertoire as a future professor. I will need to learn how to golf just for the excuse to continue my association with him.

Special thanks are due my dissertation committee. They are Chi-Lien Cheng, Tim Mattes, Michelle Scherer, and Jerry Schnoor. Tim has been willing to discuss troubleshooting of my molecular techniques at any time, not to mention more mundane topics such as postdoctoral interviews. His attitude is the epitome of the "open door policy." Jerry has graciously provided numerous recommendation letters, advice, and great parties. In addition, as my academic grandfather, he trained Joel Burken, the best Masters advisor I could have asked for and the primary reason I came to Iowa. Michelle's greatest contribution to my research has been to provide the incentive for me to constantly keep in mind the role of ORP as little more than an indicator of a very complex system of reactions, and not a driving force behind perchlorate reduction. She also directly and indirectly provided a great deal of assistance with my electrochemical synthesis both in person and through the support of Drew Latta and Chris Gorski.

I am grateful to Lou Licht for providing me an internship at Ecolotree. Under his tutelage, working side-by-side, I gained insight into the workings of a small engineering consulting company that I know will have everlasting benefit. His enthusiasm and passion for phytoremediation is most contagious. His generosity is matched only by his love of macaroons.

My family is the absolute best. Though I do not call as often as I would like, they are always only a couple button pushes away. Any number of times I have decided at the last minute to visit St. Louis and never have they said anything other than, "We'll be here. Come on down. It's about time."

I am grateful to Judy Holland for her insight and assistance. I think that we students easily forget that someone is watching over us in the CEE office. Angie, Ginny, and Collin are also deserving of gratitude and praise. They have been the grease that keep the wheels of the CEE department rolling. Many thanks to Jerry, Rich, and Craig for providing social events at their homes that allow us EESers to meet each other outside of the lab. Many thanks to the faculty and students that made Friday after seminar a great time to learn the inner workings of academia. It was this particular form of education that will serve me in the future as I move out into that world.

Thank you to EES students present and past. They have made this a wonderful place to live and work. In fact, the students here are so great that I made virtually no friends outside of the department in the years that I have been here. I hesitate to do so, knowing that I will forget some people, but I would like to extend special recognition to: Heather and Jon, Sachiyo and Neil, Drew, Laura and Steve, Britney, Rob and Shannon, JV and Kim, Adina and Mike, Josh, Chris, Anne, Phil, Justine, Cassie, Carmen, Kate, Marcio, Jeremy, Rachel, Roopa, Bryan, Lydia and Steve, Tim P. and Ashley, Tim W., Andrea R. and Andrea K.

I would like to thank Haley. Her smile is the sun breaking through the clouds after a long day of frustrating lab results. Her inevitable curiosity in how my day went

kept me working hard to have something interesting to discuss. Her confidence in me has been as constant and immutable as the tide. I can only hope that I am at least a fraction as wonderful to her as she is to me.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION AND OBJECTIVES

Background

Perchlorate (ClO_4^-) contamination is a problem in several areas across the United States including perhaps Hills, Iowa (1-4). Groundwater concentrations as high as 3,700 mg L^{-1} have been reported (5). As of 2008, the US-EPA has declined to regulate perchlorate, but has issued an interim health reference level of 15 μg L^{-1} in drinking water (6). The state of California, where widespread perchlorate contamination was first reported, has recently established a maximum contaminant level (MCL) of 6 μg L^{-1} for drinking water (7). The State of Massachusetts Department of Environmental Protection promulgated an MCL of 2 μg L^{-1} in 2006 (8).

Perchlorate was first detected in wells at Superfund sites in California in 1985 (9). At that time, the detection limit was around 100 mg L^{-1}. In 1997, the California Department of Health Services developed a method to detect perchlorate at concentrations as low as 4 μg L^{-1} (10,11). With this development came the realization that perchlorate contamination is much more widespread than previously suspected.

Methods for effective remediation of perchlorate are being sought and the most promising possibilities appear to involve the use of bacteria that can reduce perchlorate through chlorate and chlorite to chloride (12-14). Treating perchlorate at the source of contamination more quickly eliminates potential health problems by destroying the contaminant at its point of highest concentration. This is much more cost effective than treating low-level contamination that has dispersed through the groundwater. One promising remedy is to treat perchlorate at sites of contamination by engineering an *in situ* bioremediation zone where perchlorate-reducing bacteria are fostered to degrade perchlorate (15).

Perchlorate Toxicology

Perchlorate has been shown to inhibit the thyroidal sodium-iodide symporter (NIS) (16,17). NIS inhibition leads to a decrease in iodide uptake by thyroid cells. As iodine uptake decreases, the levels of thyroid-synthesized hormones released into the bloodstream changes. In workers exposed long-term to perchlorate, thyroid stimulating hormone and thyroglobulin appear to be unaffected by acute doses of perchlorate, but serum T_4, free T_4 index, and total T_3 were shown to increase (18). T_4 and T_3 both contain iodine. Only 20% of the body's T_3 is produced in the thyroid. The balance of T_3, the more active of the two hormones, is made in other bodily tissues through the deiodination of T_4 (9).

Perchlorate is not the only NIS inhibitor. The common anions nitrate (NO_3^-) and thiocyanate (SCN^-) have both been shown to decrease thyroidal iodine uptake, although not as strongly as perchlorate (18-21). Nitrate is detectable in virtually all foods. Thiocyanate is naturally found in many foods including cabbage and mustard seed. In areas of the world where cassava is a staple food, it is the major dietary source of thiocyanate. In the rest of the world, smoking is the major thiocyanate source (22,23).

In the body, the three topical NIS inhibitors exhibit different renal elimination rates. The half-lives in the blood serum of healthy volunteers for perchlorate, nitrate, and thiocyanate have been shown to be approximately eight hours, seven hours, and three days; respectively (22,24,25). Smokers, with serum thiocyanate concentrations typically 2.5 times higher than that of non-smokers, exhibit serum thiocyanate half-lives around 6 days. The relative potency of perchlorate with regards to radioactive iodine uptake compared to thiocyanate and nitrate on a weight basis is 15 and 240, respectively. However, after taking into account the increased half-life of thiocyanate compared to perchlorate, thiocyanate is actually twice as potent at inhibiting the NIS as perchlorate (22).

Perchlorate Remediation

Contaminant remediation can be separated into several main categories. One major separation is that between biological and physicochemical processes to reduce concentrations. Within those two categories is also the separation between *in situ* and *ex situ* applications. For perchlorate, biologically catalyzed reactions appear to be the most suitable and economically feasible for treating contamination *it situ*. However, for well-head treatment before discharging into a drinking water distribution system, physical processes such as ion exchange have typically been utilized. The public has traditionally been opposed to the idea of biological treatment of drinking water. Considering the chance, however small, of pathogen contamination; those oppositions may not be entirely unfounded.

Ex Situ Physicochemical Perchlorate Treatment

Ex situ physical/abiotic treatment of perchlorate is typically used to treat drinking water before it enters a distribution system. In some regards, biological treatment is superior to abiotic, but biological treatment is popularly regarded as being unsafe for drinking water treatment. The primary abiotic treatment technologies for perchlorate are ion exchange, electrodialysis, and reverse osmosis. The possibility for precipitation of perchlorate with uncommon cations such as nitron ($C_{20}H_{16}N_4$) or tetraphenylarsonium chloride does exist. However, these compounds are toxic and not economically feasible (3).

Ion exchange has been identified as an optimal technology for treating low levels of perchlorate at a high flow rate (26). Ion exchange is the use of resins to entrap ionic contaminants by ion substitution. One drawback of ion exchange is that other anions will compete with perchlorate for binding sites. Examples include sulfate and nitrate. The particular binding strength of each anion is dependent upon the resin being used, so knowing the water chemistry at a site is critical to resin selection. As a common household example, a water softener uses ion exchange resins to replace calcium and

magnesium in water with sodium. Many anion exchange resins can be regenerated by backflushing the container with a strong base or concentrated salt solution (hence the salt in water softeners). The high ionic strength of the regenerant causes the contaminants to be driven off of the resin and washed out with the brine for disposal (27).

Electrodialysis and reverse osmosis are less commonly used for perchlorate removal because of their high cost. Electrodialysis and reverse osmosis are both semi-permeable membrane technologies, but their method of removing contaminants varies. Electrodialysis is driven by an electric potential to remove charged species from an aqueous solution. Essentially, anions (e.g. perchlorate) are removed from a solution by being drawn across an anion-exchange membrane toward an anode. At the same time, cations in the solution are drawn across a cation-exchange membrane toward a cathode. The result is that the process solution is deionized and the ions are removed to the waste stream (28). Reverse osmosis is similar to electrodialysis, except that rather than driving ions across membranes with electric potential, reverse osmosis uses high pressure potential to drive water across a membrane while leaving the ions behind. After a time, the solution (or brine) concentrated with ions and is discarded as a waste stream (28).

One problem common to the three physical perchlorate removal technologies discussed here is that they do not actually destroy perchlorate. Rather, ion exchange, electrodialysis, and reverse osmosis merely set contaminants aside during treatment and then expel them later as waste products. Fortunately, research has shown some promise regarding the use of biodegradation to treat these highly saline waste streams (29-32).

Biodegradation of Perchlorate

Biodegradation/bioremediation is the use of bacteria to degrade contaminants. Three main characteristics are required for the reduction of perchlorate: the presence of perchlorate-reducing bacteria, available electron donor, and low oxidation-reduction potential (ORP). Perchlorate-reducing bacteria have been shown to be naturally ubiquitous in soils (33). Low ORP naturally results from the presence of available

electron donors as other electron acceptors are used up. Therefore, it is not surprising that at most sites where perchlorate contamination remains, the electron donor concentration is too low to promote degradation. The primary challenge of perchlorate bioremediation, therefore, is to provide electron donor to bacteria without increasing the ORP more than necessary.

Ex Situ Bioremediation

Ex situ bioremediation is a developing technology used in "pump and treat" remediation strategies where hydraulic capture of a plume is necessary. Groundwater is pumped up from a contaminant plume, treated in a bioreactor, and either reinjected into the groundwater or discharged to surface waters. There are many design possibilities for *ex situ* bioremediation. The choices are limited by economics; regulatory standards; and the specifications that: electron donor must be added and the bioreactor must exhibit reducing conditions.

One reactor design that is currently being used to remediate perchlorate in the Las Vegas wash is known as a fluidized bed reactor. In this type of reactor, water flows upward through sand or another media at a velocity just greater than the settling velocity of the media particles. This keeps the media bed "fluidized." A fixed biofilm forms on the media and harbors the contaminant-degrading bacteria (34). At the referenced site, ethanol is provided as the electron donor, the media is sand in the first reactors; then changes to activated carbon in the last reactors. This system treats 1000 gallons per minute and reduces concentrations from 400 mg L^{-1} at the influent to 3.5 μg L^{-1} at the effluent (35). That is equivalent to 200 pounds of perchlorate destroyed per hour.

Fluidized bed reactors are normally used in situations with dilute contamination. For more concentrated streams, such as ion exchange or reverse osmosis waste, the use of suspended-growth continuous-flow stirred tank reactors (CSTR) is often preferred. In this system the contaminated stream flows into a large basin where it is thoroughly

mixed. The large basin has a suspended culture of contaminant-degrading bacteria. As solution flows into the basin, a corresponding volume flows out of the effluent and into a clarifier. In the clarifier suspended solids settle out and the resulting supernatant is discharged. The suspended-growth CSTR system at the Thiokol rocket production facility in Brigham City, Utah degrades perchlorate from 4500 mg L^{-1} to effluent concentrations below the maximum reporting level (~400 μg L^{-1} in that matrix) (11).

Another promising technology for *ex situ* treatment of perchlorate is the use of hollow-fiber membrane-biofilm reactors to deliver gaseous electron donors to perchlorate-reducing bacteria. Essentially, this technology grows bacteria on hydrogen-permeable membranes arranged into long hollow fibers. These bacteria use hydrogen as it diffuses through the membrane to degrade perchlorate from the solution circulating past the biofilm. Using hydrogen gas as an electron donor is appealing because it is sparsely soluble and nontoxic. The researchers have been able to reach drinking water standards while adding nothing to the water but hydrogen (36,37). Depending upon regulator and consumer support, this technology may even be suitable for the treatment of drinking water.

In Situ Bioremediation

In situ bioremediation of perchlorate is typically a rapid means of removing a significant mass of contaminant at low to moderate expense. Since the limitation to natural perchlorate reduction is usually a lack of electron donor, providing a ready supply of electron donor to the subsurface can stimulate bacteria to rapidly degrade the contaminant. One straightforward approach to this problem is to simply mix a source of electron donor into the surface soil and monitor to see that it reaches the perchlorate contamination. In the case of Nzengung and O'Neill (38), the electron donor sources were ethanol, chicken litter, and horse manure. In a large-scale "proof of concept" experiment, they observed 70% reduction of perchlorate mass on the site in nine months.

Another approach to providing electron donor to starved perchlorate-reducing bacteria is the patent-pending gaseous electron donor injection technology whereby mixtures of electron donors are injected directly into the contaminated zone (39). A more involved method of providing electron donor is to place a trench across a plume's path filled with organic matter such as vegetable oil, compost, or animal waste (40). This is known as a permeable reactive barrier. An agreeable characteristic of these technologies is that they are passive. Once the initial installment is finished, all that may be required is monitoring.

Phytoremediation of Perchlorate

Phytoremediation is the use of plants to perform or assist in remediation. Phytoremediation of perchlorate includes rhizofiltration, phytotransformation, and rhizodegradation. Rhizofiltration is the removal of a contaminant from soil by roots. Nzengung (41) showed that plants can uptake perchlorate through their roots. Phytotransformation is degradation of a contaminant within plant tissues. Van Aken and Schnoor (42) showed evidence of perchlorate reduction all the way to chloride in hybrid poplar tree tissues. Rhizodegradation/rhizoremediation is the plant-assisted bacteria-mediated destruction of contaminants. Rhizodegradation is a relationship between the plant and bacteria. The plant provides electron donors and the bacteria can use those to grow and degrade contaminants. In the case of perchlorate, rhizodegradation will almost certainly outweigh other degradation pathways due to the rapid kinetics of bacterial catalysis compared to phytotransformation. To engineers, rhizodegradation provides a cost-effective means of providing electron donors to the subsurface. All that is required is a brief time spent planting trees, water to keep them alive, and sunlight to let them grow and produce organic carbon (43-45).

Perchlorate-Reducing Bacteria

In order to utilize an electron acceptor, such as oxygen or perchlorate, an electron donor must be present and available (46,47). Many studies have examined laboratory-scale biodegradation of perchlorate, but few have addressed the ability of these organisms to respire perchlorate under conditions found at field-scale. At perchlorate-contaminated sites, the electron donors needed to allow reduction of perchlorate are generally found only at low levels. This is rational because the presence of electron donors, combined with the ubiquity of perchlorate-reducing bacteria, would preclude perchlorate remaining at significant levels, barring the occurrence of unusually high ORP. As interest in perchlorate has increased, many perchlorate-reducing bacteria have been isolated (48-51).

When sufficient electron donor is supplied, perchlorate reduction to chloride (Cl^-) can be quite rapid. Laboratory studies with both pure-strain and mixed-culture perchlorate-reducing bacteria have shown perchlorate degradation half-lives on the order of hours (14,49-52). However, exogenous electron donor must often be added in significant quantities (at significant cost). Therefore a challenge of perchlorate remediation is to provide sufficient cost-effective electron donor to promote complete perchlorate degradation.

Studies have shown that perchlorate-reducing bacteria can use a number of different electron donors. These include hydrogen gas, volatile fatty acids, humic materials, and sugars (52-55). Many isolates will not grow on hydrogen gas, but all of Shrout's isolates could use lactate for growth. In addition, work in the field of phytoremediation has demonstrated that plants provide electron donors and carbon sources to the subsurface. Reports are beginning to point out that these plant products can be used to stimulate perchlorate degradation (43,44).

Plant Contributions

Recent studies have shown that plants can uptake and transform perchlorate all the way to chloride (41,42). Studies with radiolabeled $^{36}ClO_4^-$ indicated that 27.4% of the ^{36}Cl was translocated to the leaves while the remainder was found in solution (outside of the plant). Sixty-eight percent of the ^{36}Cl remaining in solution was untransformed ClO_4^-. Results showed that reduction of ClO_4^- to Cl^- occurred, similar to bacterial reduction, and much of the Cl^- was found in solution. Reduction products chlorate (ClO_3^-), chlorite (ClO_2^-), and Cl^- were recovered in the leaves. Nzengung and McCutcheon (56) suggest that reduction by plants is slower than reduction by perchlorate-reducing bacteria. While perchlorate does not appear to be inherently toxic to poplar trees (Figure 1-1), it seems clear that complete perchlorate removal will require microbial perchlorate reduction. Fortunately, reported research has suggested the utility of root products as bacterial substrates for targeted remediation (45,57-61).

Root products result from two primary sources. First, as roots push their way through soil, they secrete mucigel from their tips as a lubricant. Second, as roots die off and decay, they release simple and complex organic molecules into the soil matrix around them. Several studies have reported the use of root products as electron donors and carbon sources for bacterial degradation of pollutants such as PCBs and atrazine (45,57). These results are significant to perchlorate remediation because they suggest a potential synergy of rhizodegradation, utilizing root-colonizing bacteria to reduce perchlorate contamination while using the transpiration of plants to hydraulically control the spread of perchlorate. The potential benefits of implementing a rhizodegradation scheme to intercept and treat perchlorate-contaminated waters could be significant.

Samples collected from a perchlorate-contaminated site (Longhorn Army Ammunition Plant (LHAAP) in Karnack, Texas) indicate that perchlorate-reducing bacteria are present but electron-donor limited. Research demonstrated that root exudates

and root homogenate (roots harvested, ground up into a slurry, and filter sterilized) can support perchlorate reduction (Figure 1-2)(44,61). These root homogenates mimic the electron donor contribution expected from root die-off. Several perchlorate-reducing bacteria were isolated from the LHAAP samples. These bacteria could use a variety of electron donors for growth while respiring perchlorate. To verify that the degradation of perchlorate is truly a function of bacterial metabolism, two sets of microcosms were set up; one with root homogenate and perchlorate alone and the other with root homogenate, perchlorate, and a perchlorate-reducing isolate (JDS4). These microcosms showed that perchlorate is stable in the presence of root homogenates and that bacteria are required for perchlorate reduction using root homogenate as an electron donor (Figure 1-3).

Another contribution from plants is their ability to uptake water through transpiration, thereby slowing infiltration and possibly halting the downward movement of soluble contaminants. This allows perchlorate-degrading bacteria more time to access perchlorate contamination in the unsaturated zone. This contribution could be called hydraulic control of infiltration, and is an important factor to keep in mind when considering the control of source zones. By preventing infiltration from carrying soluble contaminants such as perchlorate into the groundwater, the risk that those contaminants will reach drinking water wells is diminished.

Oxidation-Reduction Potential

Experiments with a microbial consortium enriched on lactate and perchlorate showed that perchlorate reduction decreases dramatically when measured ORP increases (Figure 1-4). In these experiments, electron donor (lactate) was provided in excess of the stoichiometric amount needed to completely reduce added perchlorate. Additional experiments with perchlorate concentrations of approximately 150 mg/L indicated that the presence of dissolved oxygen at concentrations as high as 3 mg/L did not significantly affect perchlorate reduction as long as sufficient electron donor was available. It should

be noted that oxygen and nitrate are competitive electron acceptors and at lower perchlorate concentrations, may slow the reduction of perchlorate (61). These results indicate that the redox environment significantly affects the rate of perchlorate reduction. Plants have been shown to possess the ability to affect ORP. For example, Nzengung showed diurnal fluctuations between -400 mV and + 300 mV for microbial mats and between -100 mV and +300 mV in willow-planted sealed bioreactors (43,62). This could possibly have negative implications for rhizoremediation of perchlorate, considering the importance of low ORP to biodegradation.

Objectives and Hypotheses

Rhizoremediation depends on (a) the ability of plants (hybrid poplar trees in this case) to uptake perchlorate, (b) the ability of perchlorate-reducing bacteria to use plant exudates and root products as electron donors and carbon sources for growth, and (c) the ability of the plant to support a redox environment conducive to microbial perchlorate reduction.

The primary objective of this research was to delineate the relationship between poplar trees and bacteria in phytoremediation systems treating high levels ($\geq$ 25 mg/L) of perchlorate contamination *in situ*. It is clear that if effective, sustainable rhizodegradation systems are to be designed, there is a need to better understand the role of the plants in providing a redox environment suitable for effective perchlorate remediation and the relative contributions of bacteria and plant to overall remediation. In pursuing this research, the following hypotheses will be tested:

1. While poplar trees will increase overall measured system ORP, they will also provide sufficient electron donors and carbon sources to ensure sustainable perchlorate reduction in soil with a low fraction of organic carbon.

2. In low organic carbon soil, poplar trees will play a greater role in perchlorate degradation. As soil organic carbon is increased, bacteria will begin to control the degradation of perchlorate.
3. The presence of plants will decrease the diversity of perchlorate-reducing bacteria, as measured using the *cld* gene, at population equilibrium; but will not affect the diversity of universal bacterial 16S rRNA.

In testing these hypotheses, the following specific objectives are proposed:

1. Assess the role of redox conditions on perchlorate degradation in rhizosphere and non-rhizosphere soils and the effect that trees have on rhizosphere ORP.
2. Assess the effect of soil organic carbon on perchlorate phytoremediation.
3. Determine, as soil organic carbon and conditions of planting or bioaugmentation are changed, what fraction of the perchlorate reduction is due to bacteria and what fraction is due to plants (i.e., construct a mass balance).
4. Determine what fraction of the influent mass of perchlorate is taken up into the tree under variable conditions of bioaugmentation, planting, and soil organic carbon.
5. Using molecular methods, examine the effect of rhizosphere conditions on the diversity of perchlorate-reducing and general bacterial populations.
6. Examine the effect of bioaugmentation on the diversity of perchlorate-reducing and general bacterial populations.
7. Examine the effect of soil organic carbon on the diversity of perchlorate-reducing and general bacterial populations.

Thesis Organization

This thesis is organized as three central chapters representing three separate manuscripts that have all been prepared in accordance with the author guidelines of *Environmental Science and Technology* for consistency. Chapter II describes the

determination of rhizosphere effects on measured ORP and perchlorate removal in low and high organic carbon soil. Chapter III outlines further research to delineate the fate and transport of perchlorate in planted and bioaugmented systems. Chapter IV explains observations about the diversity of both a gene essential to perchlorate reduction and 16S rRNA from general bacteria. Chapter V provides conclusions related to the three central chapters, engineering significance of the results, and potential future research to answer questions raised by this research. Appendices A through G provide supplemental data to the central manuscripts. Preliminary results of this research have been published (44).

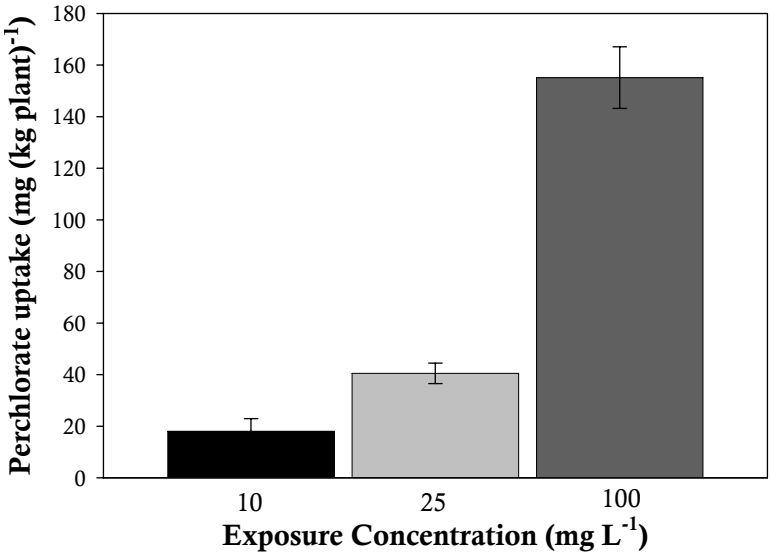


Figure 1-1. Cumulative mass uptake of perchlorate by poplar trees at different exposure concentration.

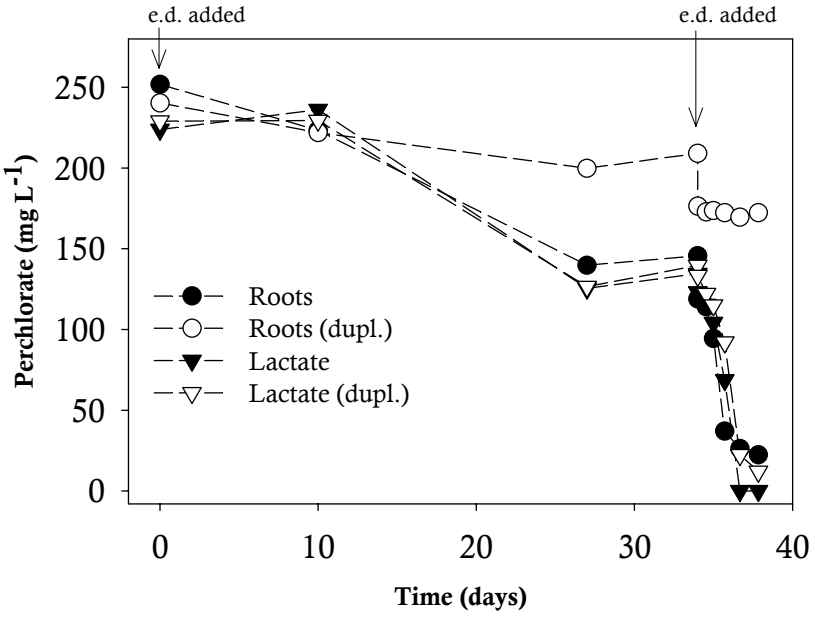


Figure 1-2. Use of root homogenate or lactate for perchlorate degradation in microcosms developed from samples taken from the LHAAP. Root homogenate or lactate (electron donor = e.d.) was added on Day 0 and Day 34.

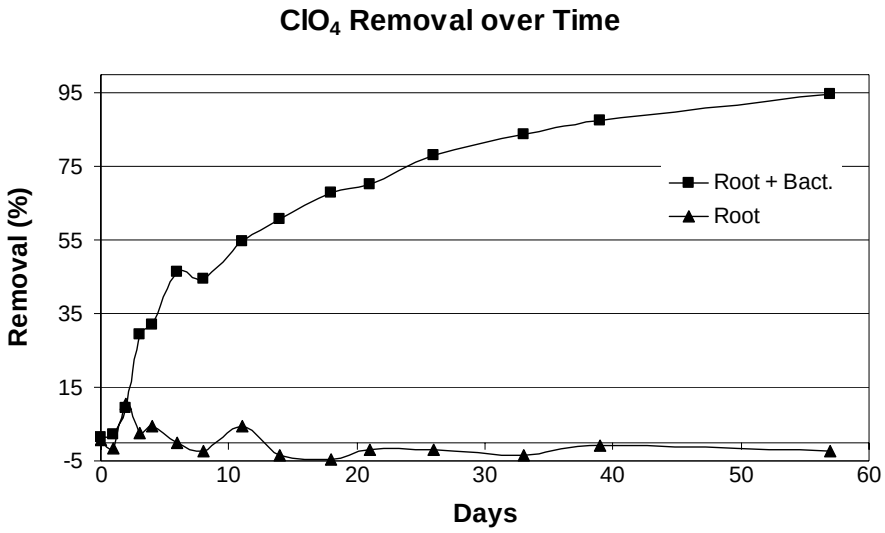


Figure 1-3. Poplar root homogenate alone does not catalyze the reduction of perchlorate in microcosms inoculated with a pure culture perchlorate reducer.

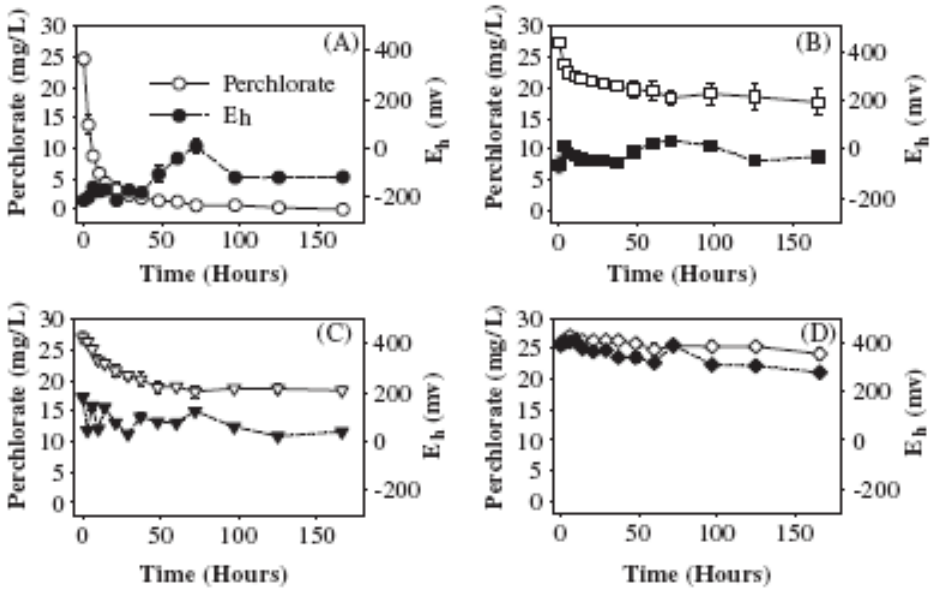


Figure 1-4. Perchlorate (closed symbols) and ORP (open symbols) over time for poised initial ORPs of (A) -220 mV, (b) -50 mV, (C) +180 mV, and (D) +390 mV. Initial lactate ≈ 18 mg/L; initial perchlorate ≈ 25 mg/L. Error bars represent ± one standard deviation for three replicates.

CHAPTER II

THE EFFECT OF PLANT-INFLUENCED VARIATION OF OXIDATION-REDUCTION POTENTIAL ON PERCHLORATE BIOREMEDIATION IN LOW AND HIGH ORGANIC CARBON SOIL

Abstract

Current techniques to treat perchlorate source zones focus on the use of pumping to remove the contaminant from the ground followed by treatment in aboveground bioreactors. This is done because indigenous perchlorate degraders often lack the electron donors necessary to reduce perchlorate to innocuous chloride. However, planting trees above a perchlorate source zone could provide electron donors directly to the indigenous bacteria at a lower cost and with an aesthetically pleasing effect. Plant root extracts have been shown to support the microbial-catalyzed reduction of perchlorate. However, plants have also been shown to raise the measured oxidation - reduction potential (ORP) in hydroponic systems, a mechanism that could have ill effects on the redox-sensitive reduction of perchlorate. The primary purpose of this study was to analyze the relationship between plants and bacteria in a soil-based perchlorate bioremediation system to determine if the overall effects of plants are beneficial or detrimental to the reduction of perchlorate.

We have demonstrated, in laboratory scale reactors, that planting hybrid poplars in a high organic carbon soil does not affect the degradation of perchlorate. Instead, the degradation of perchlorate is significantly related to the mean hydraulic retention time and the measured ORP. However, in low organic carbon (LOC) soil, evidence suggests that hybrid poplar trees can raise the ORP but also take up a significant quantity of perchlorate. The ORP in LOC soil was significantly different between planted and unplanted condition but was not significantly related to perchlorate removal. The mean hydraulic retention time was significantly related to perchlorate removal in LOC soil.

Chemical oxygen demand in the effluent of LOC soil reactors was found to be significantly greater from planted vs. unplanted reactors. In these soil-containing reactors, perchlorate removal efficiencies of 3% – 100% and ORPs between -410 and +268mV were observed.

Introduction

Recent innovations in analytical chemistry have allowed the detection of the monovalent oxyanion perchlorate (ClO_4^-) to levels previously unattainable (Clewell *et al.* 2000; Tsui *et al.* 2000). With this development and subsequent environmental surveys came the realization that perchlorate contamination is detectable at a multitude of sites; including many where there is no known source (Kalkhoff 2005). Some of these contaminations are reported to be the result of natural sources such as perchlorate-contaminated nitrate deposits or atmospheric deposition (Jackson *et al.* 2005; Urbansky *et al.* 2001). Conversely, the perchlorate contamination of highest concentration and most concern is found at anthropogenic sources such as manufacturing facilities, military sites, and other places where human activities (such as blasting and fireworks displays) have resulted in perchlorate releases to the environment (DENIX 2000; Fitzgerald *et al.* 2005). The focus of this study is on these source zone areas of high perchlorate concentration (around 25 mg/L).

Perchlorate has been shown to inhibit the thyroidal sodium-iodide symporter and subsequently uptake of iodide into the thyroid gland (Lawrence *et al.* 2000; Merrill *et al.* 2005). This results in the decreased production of thyroid hormones at very low perchlorate concentrations (Brechner *et al.* 2000; Carr *et al.* 2003; Greer *et al.* 1966; McNabb *et al.* 2004; Siglin *et al.* 2000). These thyroid hormones regulate metabolism in adults. In children, however, the appropriate balance of thyroid hormones and iodide is also essential for proper growth and mental development (Laurberg *et al.* 2002; NAS 2005). Compounding this problem is the fact that dietary iodide levels in the USA have

been steadily declining, one cause of which is ostensibly that processed-food manufacturers no longer use iodized salt (Hollowell *et al.* 1998; Matovinovic *et al.* 1973). The level of perchlorate consumption shown to have no measurable effect on thyroid function with a safety factor included for sensitive populations was reported by the National Research Council to be 24.5 μg/L (NAS 2005). However, this is only true if drinking water is the only route of perchlorate consumption. Unfortunately, drinking water is not the only source of perchlorate consumption. Recent studies that have generated a great potential for concern report on the presence of perchlorate in dairy milk, breast milk, and baby formula (Kirk *et al.* 2005). Thyroid hormones, produced by the mother during pregnancy and by the child in the first few years of life, are essential for neuronal differentiation and myelination, both of which are aspects of brain tissue development (Alvarez-Dolado *et al.* 2000; Dowling *et al.* 2000; Haddow *et al.* 1999). The Kirk *et al.* study is of such concern because it demonstrates that human breast milk, the first sustenance that many infants receive, can contain perchlorate. Even more alarming, every one of 36 samples of breast milk, from randomly selected mothers in 18 scattered states, contained perchlorate at detectable levels.

Under suitable conditions, perchlorate is rapidly degraded in the environment. Suitable conditions include: low ORP, sufficient available electron donors, and the presence of perchlorate-reducing bacteria. Perchlorate-reducing bacteria have been shown to be nearly ubiquitous in soils (Waller *et al.* 2004). Low ORP often results from the presence of sufficient electron donors as microbial respiration uses up the more favorable electron acceptors (Coates and Achenbach 2004; Rikken *et al.* 1996). However, locations of perchlorate release have logically been somewhat devoid of electron donors. Many military and industrial sites were founded in the southwestern United States where the soil is very low in organic carbon. We believe that it is this low availability of organic electron donors at perchlorate sources that has led to the recalcitrance of perchlorate.

Recent studies have shown that plants can uptake and transform perchlorate (Nzengung *et al.* 1999; van Aken and Schnoor 2002). In addition, other reports point to the potential use of root products as microbial growth substrates (Burken and Schnoor 1996; Jordahl *et al.* 1997; Yoshitomi and Shann 2001). Several studies have reported the use of root products as electron donors and carbon sources for bacterial degradation of pollutants such as polychlorinated biphenyls, perchlorate, and atrazine (Burken and Schnoor 1996; Leigh *et al.* 2002; Shrout *et al.* 2006). These results are significant because they suggest a potential synergy of rhizodegradation, utilizing perchlorate-degrading bacteria to colonize the root zone of plants while using the plants to provide hydraulic control through evapotranspiration. Ultimately, plants would intercept perchlorate at its source by taking it up through the roots while providing electron donors for degradation by soil bacteria, thereby shielding the human populace from contaminated groundwater and the risk of thyroid dysfunction.

Perchlorate is an ideal compound for studying the interactions between plants and bacteria because of its physicochemical properties. Perchlorate does not appreciably absorb or adsorb to soil, it is essentially nonvolatile, and it is biologically recalcitrant under oxic conditions (Rao *et al.* 2007). In many ways it is a nonvolatile surrogate for the study of tetrachloroethene, trichloroethene, or other highly oxidized contaminants under varying redox conditions. These ideal properties of perchlorate allowed the testing of the effect of tree presence and bioaugmentation on perchlorate bioremediation in soil containing high levels of organic carbon without undue concern regarding volatilization or unknown perchlorate sinks in the system.

As previously stated, evidence suggests that plants could not only benefit perchlorate bioremediation by providing electron donors to starved bacteria, but also that plants could be a detriment to bioremediation by supplying alternative electron acceptors such as molecular oxygen and raising the measured subsurface ORP. The hypotheses tested were as follows: ORP will indicate the potential for perchlorate removal in soil,

with greater removal at lower ORP. Planting poplar trees in HOC soil will increase overall system ORP, but will not affect perchlorate removal. Greater hydraulic retention time will lead to more reduced redox conditions and increased perchlorate removal.

Materials and Methods

Reactor Construction

Flow-through reactors were used to assess the role of ORP on perchlorate degradation in soil. Both high organic carbon (HOC) soil and low organic carbon (LOC) soil were used as matrices for plant and bacterial growth. The HOC soil reactors, consisting of one-liter plastic jars, contained four horizontal layers alternating between chert gravel and high organic-carbon soil (f_{oc}=2.4%) with gravel on the bottom and soil on the very top (Figure 2-1). The purpose of the chert gravel was to prevent soil erosion and to be an inert layer of high hydraulic conductivity to ensure that the influent solution was evenly distributed across the cross section of the reactor. Nutrient solution was pumped into the upper layer of gravel and then flowed downward through the lower soil zone. The solution was then extracted from the bottom layer of gravel, passed through an ORP probe cell, volumetrically quantified, and disposed. After construction, the reactors were covered with aluminum foil to block light and prevent the growth of phototrophic oxygenic organisms. The reactors were designed to allow bioaugmentation with a previously characterized perchlorate-reducing enrichment culture.

Four experimental setups were examined with three reactors for each setup. In two setups, a hybrid poplar cutting (*Populus deltoides x nigra* DN34, Imperial Carolina) was planted into the reactor soil, where its roots provided rhizosphere conditions. One set of tree-planted reactors was inoculated with a perchlorate-reducing lactate-fed enrichment culture (LEC) developed from anaerobic digester sludge of the Iowa City, IA North Wastewater Treatment Plant (Shrout and Parkin 2006). This bioaugmentation greatly increased the likelihood of perchlorate-reducing bacteria in the rhizosphere. The

other set was not inoculated with a microbial culture and simulated real-world planting of a poplar cutting directly into contaminated soil. Solution entering these two systems was not deoxygenated, nor was oxygen added.

To simulate non-rhizosphere conditions, another group of reactors was initially set up the same way as previously described, but no poplar cutting was planted. To determine the activity of perchlorate-reducing bacteria without plants, this trio of reactors was also bioaugmented with LEC. In field conditions the solution infiltrating downwards would have been in contact with heterotrophic bacteria in the upper reaches of the soil and would likely become low in dissolved oxygen before reaching the deeper, non-rhizosphere soils where low-ORP perchlorate degradation could occur. To ensure low dissolved oxygen in these reactors, the nutrient solution was deoxygenated with compressed nitrogen for 2.5 minutes per liter before the solution was pumped into the system. Dissolved oxygen in the solution was verified to be less than 0.5 mg/L after deoxygenating through use of a dissolved oxygen probe.

A group of reactors was constructed to act as references. Before being filled with soil, the reactor vessels were sterilized with a dilute solution of sodium hypochlorite. The soil and gravel were autoclaved in 30-minute increments three times over the course of two days to allow resistant spores the opportunity to germinate and be neutralized. These reactors were fed perchlorate, but no bacteria were present, at least initially, and no tree cuttings were planted. The function of these reactors was to provide reference soil that was neither bioaugmented nor planted. Though the soil was autoclaved, evidence suggests that microbial activity likely returned to pre-autoclave levels in three weeks or less (Tuominen *et al.* 1994).

To determine the effect of hybrid poplar cuttings on perchlorate reduction in LOC soil, reactors were constructed as above but with the following changes. A sandy soil (f_{oc}=0.1%) was used in place of the HOC soil. The nutrient solution was deoxygenated with compressed nitrogen for all reactors in order to decrease variability among

experimental setups. An inline filter was installed on the reactor influent at the beginning of the experiment and replaced when algal growth was observed on the low-pressure side of the filter or when backpressure became too high for adequate flow.

All of the reactors were fed a nitrate-free nutrient solution modified from Hoagland's Solution (1938). Nitrate has been shown to be a competitive inhibitor of microbial perchlorate reduction for many perchlorate-reducing isolates, so care was taken to exclude it from the reactors (Herman and Frankenberger 1999; Shrout and Parkin 2006). Instead of nitrate, ammonium was used as a nitrogen source for plant growth. The nutrient solution consisted of 0.75mM $MgSO_4$, 1.00mM $CaCl_2$, 0.25mM NH_4ClO_4, 2.25mM NH_4Cl, 0.50mM $(NH_4)_2HPO_4$, 0.50mM K_2HPO_4, and 0.05mM Fe-EDTA. The average ORP of the influent solution was -6.84 ± 2.47 mV.

One possible concern with the reactor design was root flooding. When roots are flooded they can suffer from hypoxia potentially leading to death. Even before death, the metabolism of the root changes as its respiration shifts to fermentation. However, it has been shown that root hypoxia in *rubus ideaus* does not affect ion uptake (Redfield *et al.* 2004). This boded well for these experiments, where root-mediated anion uptake was one of the primary concerns. Additionally, hybrid poplars have been shown to perform well in low oxygen sediments. The formation of aerenchyma (root air channels) and other adaptations allow plant roots to survive flooding (Liu and Dickmann 1993; Siebel and Blom 1998). These aerenchyma probably also contribute to the plant's ability to influence subsurface measured ORP (Sorrell 1999).

Reactors were operated for an extended period to allow sufficient growth of the poplar cuttings and to achieve an equilibrium population of microorganisms prior to relevant sampling. The HRT, ORP, and perchlorate removal data presented herein were collected over an intensive sampling period of 123 and 102 hours for LOC and HOC soil, respectively. However, though the bulk of the data was collected in two intensive

periods, the reactors had been operated for a total of 240 and 300 days for LOC and HOC soil, respectively.

Determination of Hydraulic Retention Time

An iodide tracer study was undertaken to determine the mean hydraulic retention time (HRT) and overall flow regime in each reactor. Iodide was chosen as the conservative tracer ion because bromide, a more popular choice, co-eluted with chlorate on the ion chromatograph. The tracer study was performed by introducing a slug of iodide-containing solution into the reactor influent and monitoring the effluent for iodide and perchlorate and the suite of perchlorate daughter products. Initially, the effluent was sampled every two hours until the peak concentration had been reached and passed. Later, the effluent was sampled less frequently as the iodide concentration tailed off. HRT was calculated as the time-weighted mean of the tracer concentration curve.

A microcosm perchlorate reduction study was performed with iodide concentration as the master variable before undertaking the iodide tracer study. Perchlorate is known to interfere with iodide uptake into the thyroid and we needed to be certain that iodide would not inversely act to inhibit microbial perchlorate reduction. A comparison of bioaugmented, biostimulated serum bottles with control reactors showed that iodide had no inhibitory effect on microbial perchlorate reduction at a range of 0 – 900 mg L^{-1} I^- (Appendix A).

Ion Chromatography

Aliquots of the reactor waste flow were analyzed by ion chromatography for the presence of perchlorate and its metabolites. A Dionex (Sunnyvale, CA) AS50 auto sampler injected 50 µl sample aliquots into a Dionex ICS-2000. Separation was achieved with a Dionex AS16 4 mm column and a potassium hydroxide eluent at a 1.0 ml/min flow rate and a column temperature of 40 °C. An eluent gradient curve of 5 to 50 mM KOH with a curve number of eight (concave) was generated from 1 to 10 minutes of the sample run time. A Dionex DS6 conductivity detector was used to quantify perchlorate

and its daughter products and peak areas were integrated by Dionex Chromeleon software. Intermediates of perchlorate mineralization such as chlorate and chlorite were not observed in reactor effluents. This was not a concern because the rate-limiting step of perchlorate mineralization was expected to be the reduction of perchlorate to chlorate.

Oxidation-Reduction Potential

ORP was measured using flow-through redox cells, each assembled from one-half of a plumbing compression connector, two barbed fittings, a 1.5 cm square sheet of acrylic, and a Sensorex (Garden Grove, CA) ORP electrode (Figure 2-2). The redox cells were installed inline in either the influent or effluent of the reactors. A dedicated cell was provided for each reactor. The ORP electrode was connected to a millivolt meter which was in communication with a PC for ORP data collection at a 15-minute resolution. The electrode was calibrated based on the hydrogen electrode using 98 mV and 260 mV calibration solutions from Sensorex. ORP electrodes were cleaned of biofouling and recalibrated every two months.

Chemical Oxygen Demand

Chemical oxygen demand (COD) was measured using a reflux kit according to manufacturer protocol (Hach). Two mL of filtered effluent or influent was used as the sample and a potassium hydrogen phthalate serial dilution was used to construct a standard curve.

Calculation of Perchlorate Mass Removal Efficiency and Statistical Analysis

In planted reactors, the effluent perchlorate concentration at times exceeded the influent concentration. This was due to the preferential uptake of water into the poplar roots with the subsequent concentration of solutes. The effluent of the reactors was collected and measured to determine the true effluent flow rate. To calculate mass removal efficiency, the influent and effluent concentrations were multiplied by the respective influent and effluent flow rates. This converted the concentration data to

influent and effluent mass flow rates. Calculating the perchlorate mass removal efficiency was then a simple matter of dividing the difference between the influent and effluent mass flow rate by the influent mass rate.

Analysis of variance (ANOVA) was performed on sample data to determine relationships between factors such as planted, bioaugmented, relative ORP, relative HRT, and perchlorate removal. As ORP and HRT were not directly controlled, only measured, relative values such as high, mid, and low were used as factors in ANOVA.

Results and Discussion

The flow-through reactors allowed for the reproducible collection of perchlorate degradation data over time. The poplar cuttings thrived under conditions of 14 continuous hours of light per day. The cuttings did not suffer from saline toxicity, and all survived more than eight months with routine trimming to prevent over-transpiration, i.e. transpiration in excess of the amount of solution being pumped into the reactor.

Effluent Electron Donors

We hypothesized that in LOC soil, trees would provide electron donors to stimulate perchlorate degradation. COD was used as a measureable surrogate for electron donor concentration. The COD in the effluent of planted LOC soil reactors was observed to be greater than that from unplanted reactors. The COD averaged 24.7 ± 7.9 mg/L from planted reactors and 11.5 ± 3.3 mg/L from unplanted reactors. Shrout and Parkin (2006) reported that bacterial degradation of perchlorate using lactate occurred at a utilization rate of 0.83 mg ClO_4^- per mg COD over the course of 40 days. Our corrected effluent perchlorate concentration averaged 23.2 mg/L. The removal appears to be limited more by hydraulic retention time than by insufficient electron donor. Given a longer contact time, the available COD from planted reactors should allow for further degradation of perchlorate.

Plant Effect on Oxidation-Reduction Potential

In order to test the hypothesis that plants can raise measured ORP in the soil subsurface, ORP data were collected and plotted with time (Figure 2-3, Appendix B). In both HOC and LOC soil, the average ORP was greater in planted reactors than in unplanted reactors (112 and 158 mV greater, respectively). In addition, a clear relationship is observed in subsurface measured ORP compared to light and dark cycles in the planted reactors for both HOC and LOC soil. When the growth lights are turned on, a rapid increase in measured ORP is observed. Conversely, when the lights are turned off, a decrease in measured ORP is observed. These observations point to a potential tie between plant photosynthesis, which is known to generate oxygen, and an increase in subsurface ORP, which can be linked to oxygen concentration. Alder and olive trees have been shown to transport photosynthesis-derived molecular oxygen from the stem to the roots (Armstrong and Armstrong 2005; Mancuso and Marras 2003). Poplar trees are reasonably expected to do the same. Once in the roots, the oxygen partial pressure gradient from inside the root to the soil immediately exterior drives diffusion of oxygen into the subsurface with a potential subsequent effect on the soil redox environment. We suspect that root intrusion into subsurface strata could also open "paths of least resistance" for the introduction of atmospheric oxygen. However, this passive pathway for plant-assisted effects on subsurface redox conditions would not likely be stimulated by light.

Trees have also been shown to cause barometric pumping of gases through the subsurface through diurnal phreatic surface depression and recharge (Andraski *et al.* 2005). As the phreatic surface is lowered during the day the vadose zone becomes partially evacuated and can draw air downward. Groundwater recharge from unplanted areas overnight can then drive part of the vadose zone gasses into the atmosphere, much like an immense diaphragm pump. The reactors used in our experiments could potentially have had a similar barometric pumping effect. As transpiration increased

following morning light activation, the water level in the reactor likely dropped, followed by a small amount of air being pulled into the unsaturated zone.

Effect of HRT and ORP on Perchlorate Removal Efficiency

The data regarding perchlorate removal versus measured subsurface ORP are plotted in Figure 2-4. There appears to be no relationship between the presence or absence of bacteria or poplar trees and the removal of perchlorate in HOC soil. However, these results do show that perchlorate removal is more complete at lower measured ORP compared to the removal observed at higher measured ORP, thus corroborating previously reported work and supporting our first hypothesis (Nzengung *et al.* 2004; Shrout and Parkin 2006). Of note, however, is the outlier in the high ORP, moderate perchlorate removal sector of LOC soil (Figure 2-4 B). This planted reactor consistently demonstrated greater perchlorate removal compared to other LOC soil planted and unplanted reactors and had the highest transpiration rate.

The hypothesis that HRT would be a determining factor in perchlorate removal was supported. HRT was expected to slightly vary among reactors due to the imperfect balance that existed between the flow rates of the inlet and outlet peristaltic pumps. However, the variability among reactors within each experimental setup was greater than expected. A trend is observed when perchlorate removal is plotted as a function of HRT (Figure 2-5). As HRT increased, the perchlorate removal also increased. Similarly, a trend is observed when measured ORP is plotted as a function of the reactor HRT (Figure 2-6). As HRT increased, the measured ORP decreased. It is known that efficient perchlorate removal requires low ORP, and low ORP did indeed correlate to high removal in our experiments (Figure 2-4). However, it is noteworthy that greater HRT leads to lower ORP and is therefore likely to have even more effect on perchlorate removal. ANOVA showed that relative HRT had a significant effect on perchlorate removal efficiency in both HOC ($P = 0.007$) and LOC ($P = 0.003$) soil. In contrast, the reactor setup (the variable combination of plants and bioaugmentation in the reactors) did

not have a significant effect on HRT, measured ORP, or perchlorate removal in either LOC soil or HOC soil ($P > 0.05$). Two-factor ANOVA showed that relative HRT was more significant to removal than relative ORP in both LOC ($P = 0.008$) and HOC ($P = 0.009$) soil.

Each data point of ORP vs. perchlorate removal (Figure 2-4) is representative of the average value of at least 19 individual samples and concurrent ORP readings. The error bars demonstrate the 99% confidence interval of the data. These samples were taken over an intensive sampling period 8 and 10 months after the beginning of reactor operation, for HOC and LOC soil, respectively. Thus, though the individual points of each reactor setup do not always fall in the same part of the plot, each point has a significant amount of data supporting it. Some variability among biological reactors is to be expected and a fortunate aspect of the reactor design presented here was the ability to noninvasively sample relevant characteristics multiple times with strong reproducibility within each reactor.

Significance to Remediation

We have shown that plants increase measured ORP in real soil, and that measured ORP can vary over the course of a day from a high point when light impinges upon the leaves to a low point just before sunrise. We have also shown that perchlorate removal in real soil is generally lower at higher measured ORP, which corroborates previous results from culture and hydroponic studies. In addition, LOC soil reactors showed higher ORP when compared to HOC soil, with an expected decrease in perchlorate removal.

Hydraulic retention time was shown to be a controlling factor of perchlorate removal. Greater HRT led to increased removal, as would be expected, and also led to decreased ORP. Perchlorate removal is known to proceed more readily under conditions of low ORP. Therefore, the effect of greater HRT on perchlorate removal was multiplicative, with the decrease in ORP amplifying the increased removal potential resulting from longer contact time. In other words, combining longer contact time with

increased reaction rate yields a synergistic relationship between HRT and perchlorate removal that is greater than the sum of its parts.

It appears that the effect of plants on perchlorate degradation is minimal where bioaugmented perchlorate-reducing bacteria are present. However, in a setting where only limited numbers of perchlorate-reducing bacteria are present, the planted systems still achieve perchlorate removal. LOC soil planted reactors showed an average removal of 10%. The value of 10% removal is deceptively low and may lead to underestimation of the significance of these results. The average contact time was only 1.4 days. Assuming first order reaction kinetics in these plug flow reactors, 99% removal could occur in a larger reactor in as little as two months. Actual contact times in a phytoremediation site would only need to be designed to exceed two months to ensure substantial perchlorate remediation.

Subsurface drip irrigation is a potentially useful, relatively inexpensive technique to extend the potential for phytoremediation to treat deeper groundwater than that which poplar roots can reach unassisted. Pumps can be used to raise the groundwater to the surface before being distributed to the tree plot. The irrigation rate can be used to control the hydraulic retention time within the root zone. An HRT of four months would provide a twofold safety factor for complete removal of perchlorate under the conditions presented here. The reactor setup used in this research simulates the continuous irrigation of poplar trees planted in HOC and LOC soil. Planting trees and installation of subsurface drip irrigation will allow treatment of deep perchlorate-contaminated groundwater.

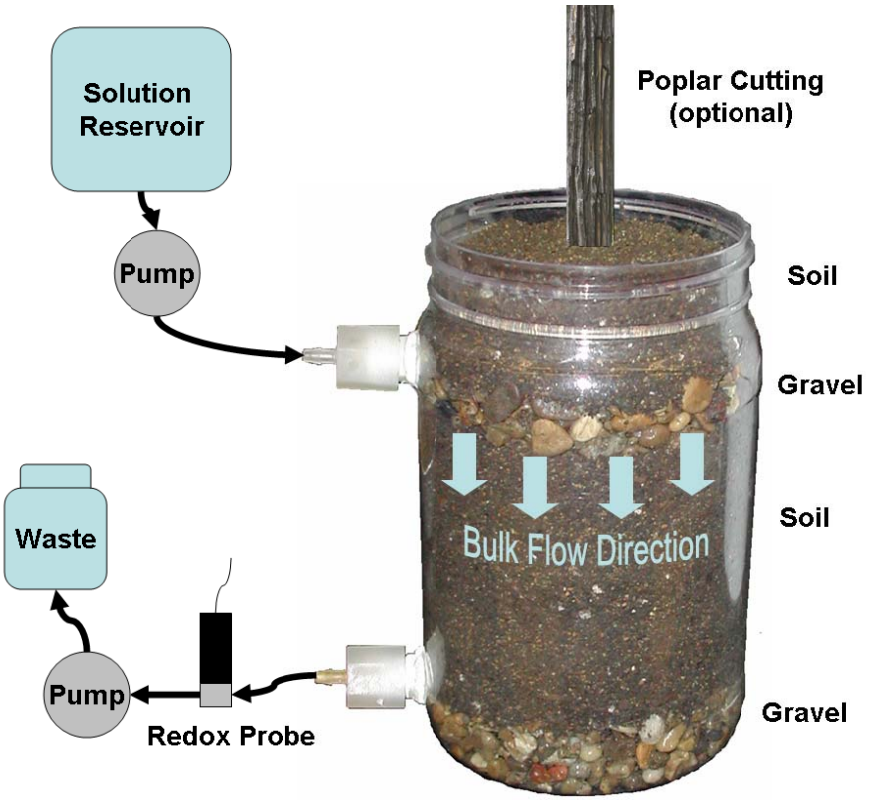


Figure 2-1. Schematic of reactor design and solution flow path. The downward flow of this reactor simulates infiltration of perchlorate from contaminated surface soils. Solution was pumped into the upper zone of the reactor through a ring-shaped influent. A separate pump removed solution through a similar effluent ring. Solution contact time averaged 1.2 days. Some reactors were either planted with a hybrid poplar cutting, bioaugmented with a known culture of perchlorate-reducing bacteria, both, or neither.

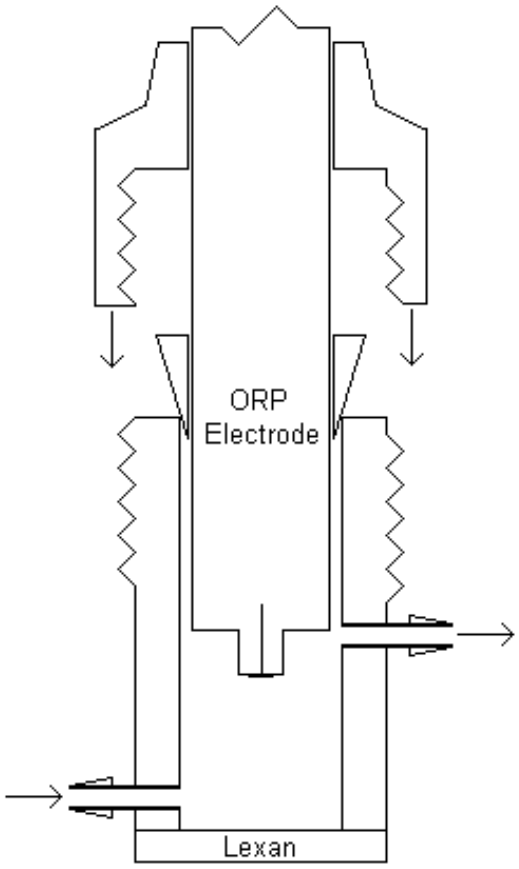


Figure 2-2. Flow-through redox cell (vertical cross section)

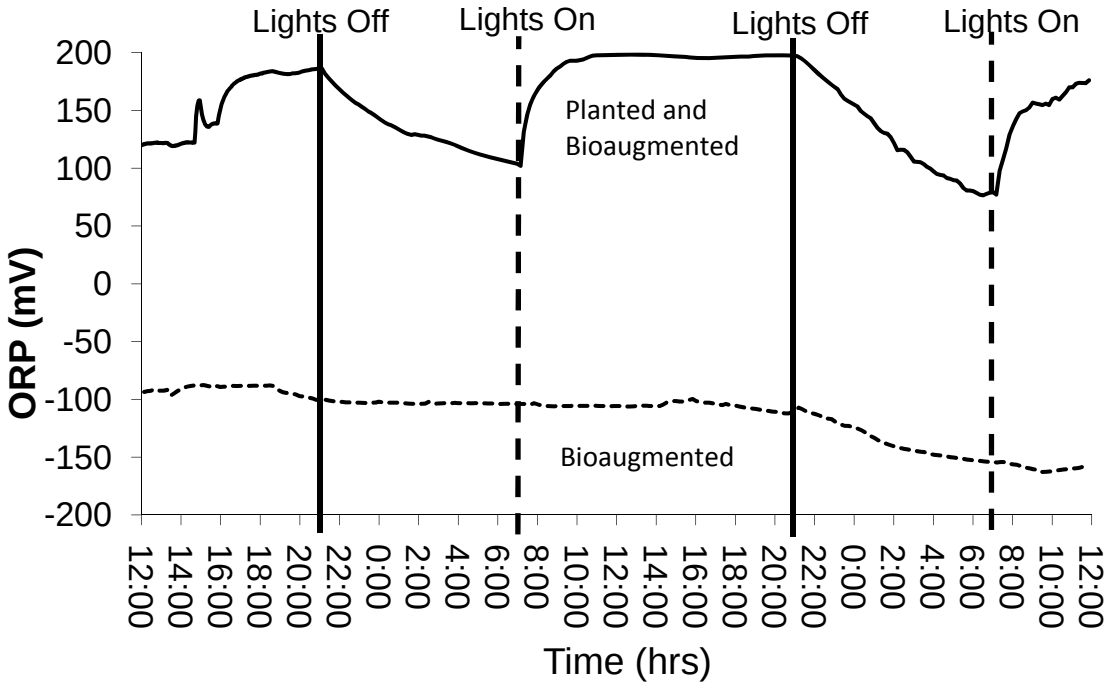


Figure 2-3. Representative plot showing diurnal variation of effluent ORP from individual reactors. The lower set shows data from a reactor that had been inoculated with LEC and contained no cutting. The upper set of data points represents ORP data collected from a reactor containing a hybrid poplar cutting and bioaugmentation. These data were obtained from HOC soil reactors and represent observations from both HOC and LOC soils. The light cycle was from 07:00 to 21:00.

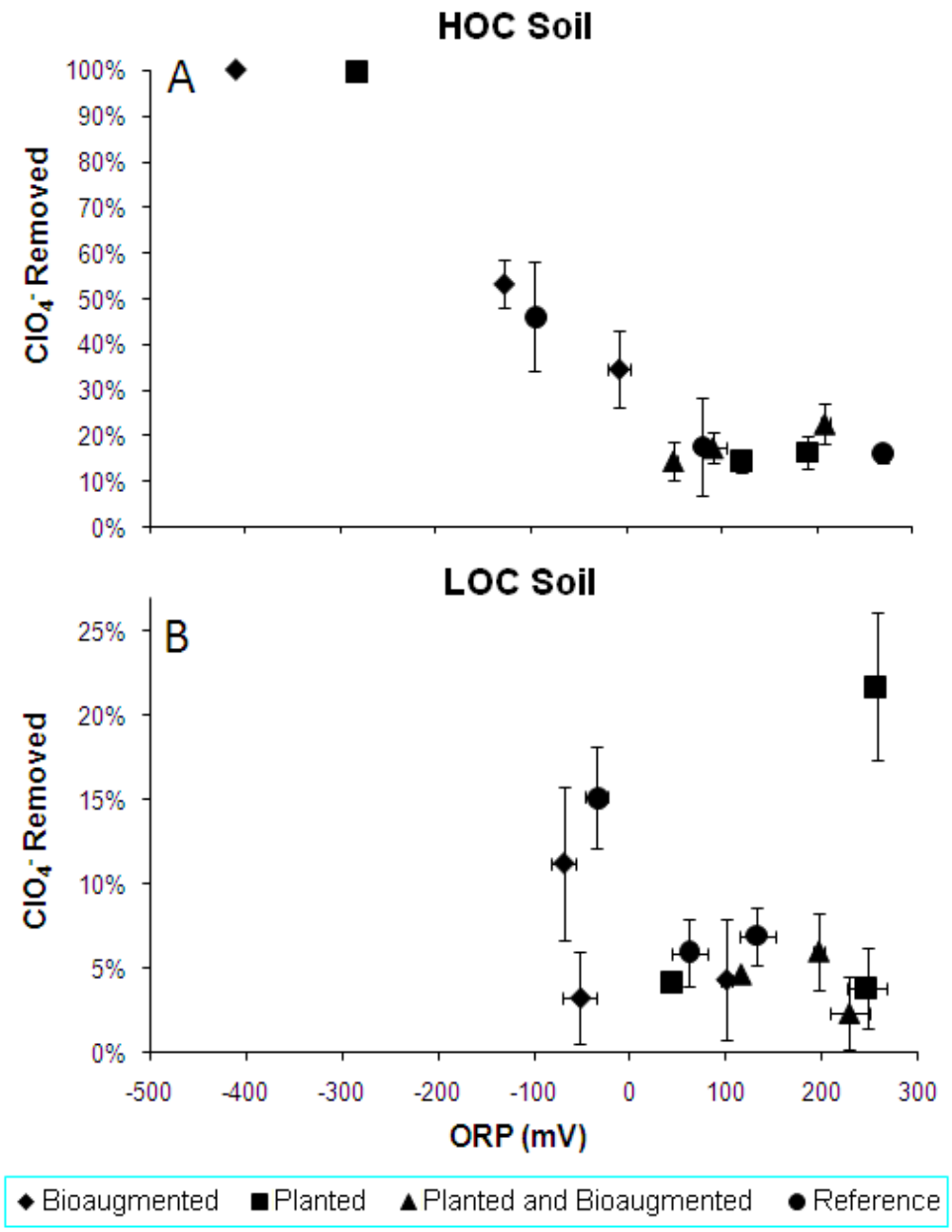


Figure 2-4. Perchlorate removal plotted as a function of ORP. Data plotted in (A) were calculated from observations in HOC soil reactors while (B) represents LOC soil. Please note difference in y-axis scale between (A) and (B). Error bars are the 99% confidence interval. Points in (A) represent 19 samples taken during 102 hours of sampling. Points in (B) represent 24 samples taken during 123 hours of sampling. Sampling took place after 240 and 300 days of operation for (A) and (B), respectively.

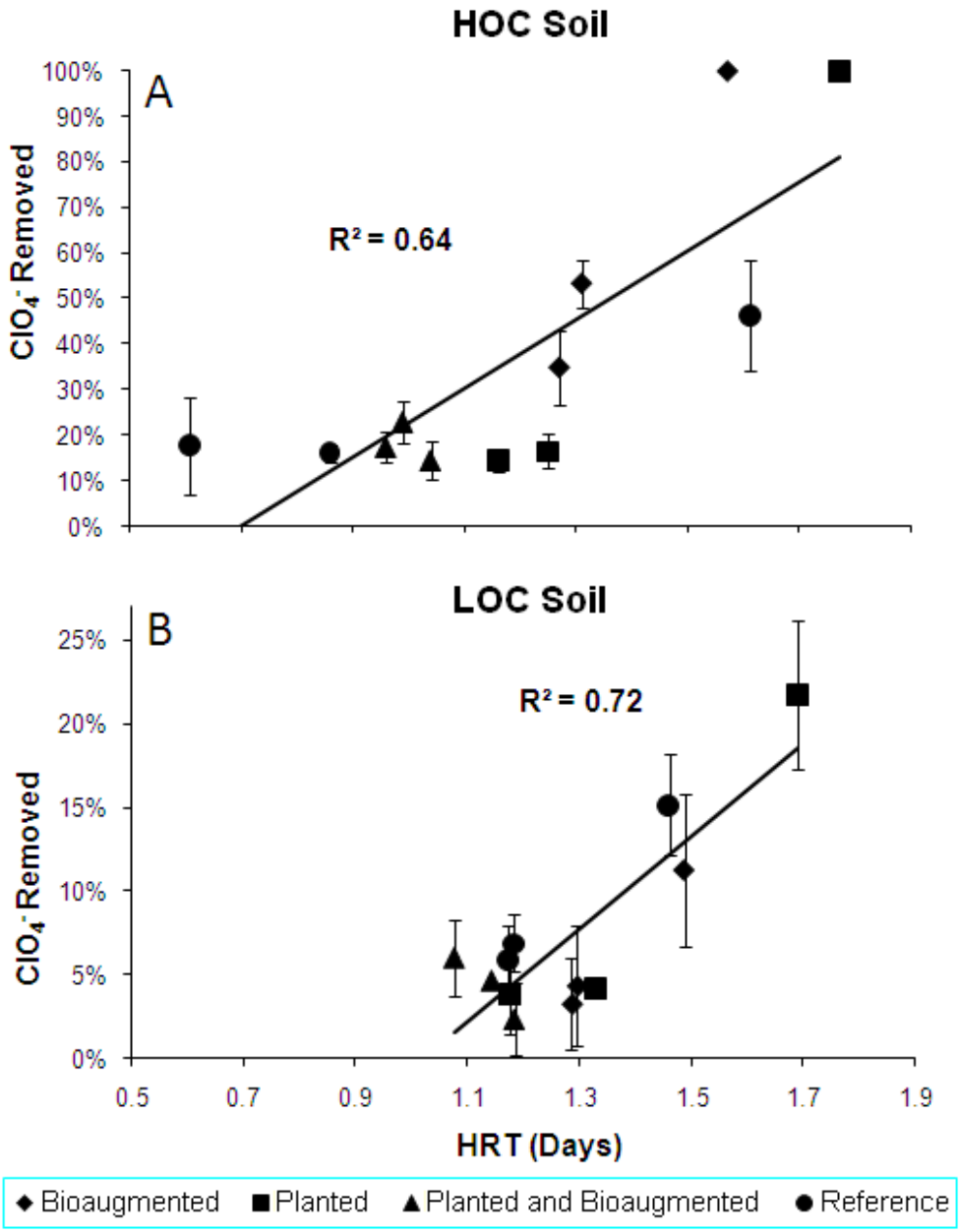


Figure 2-5. Perchlorate removal plotted as a function of the hydraulic retention time. Data plotted in (A) were calculated from observations in HOC soil reactors while (B) represents LOC soil. Please note difference in y-axis scale between (A) and (B). Error bars are the 99% confidence interval. Sample quantity and duration are the same as in Figure 2-4.

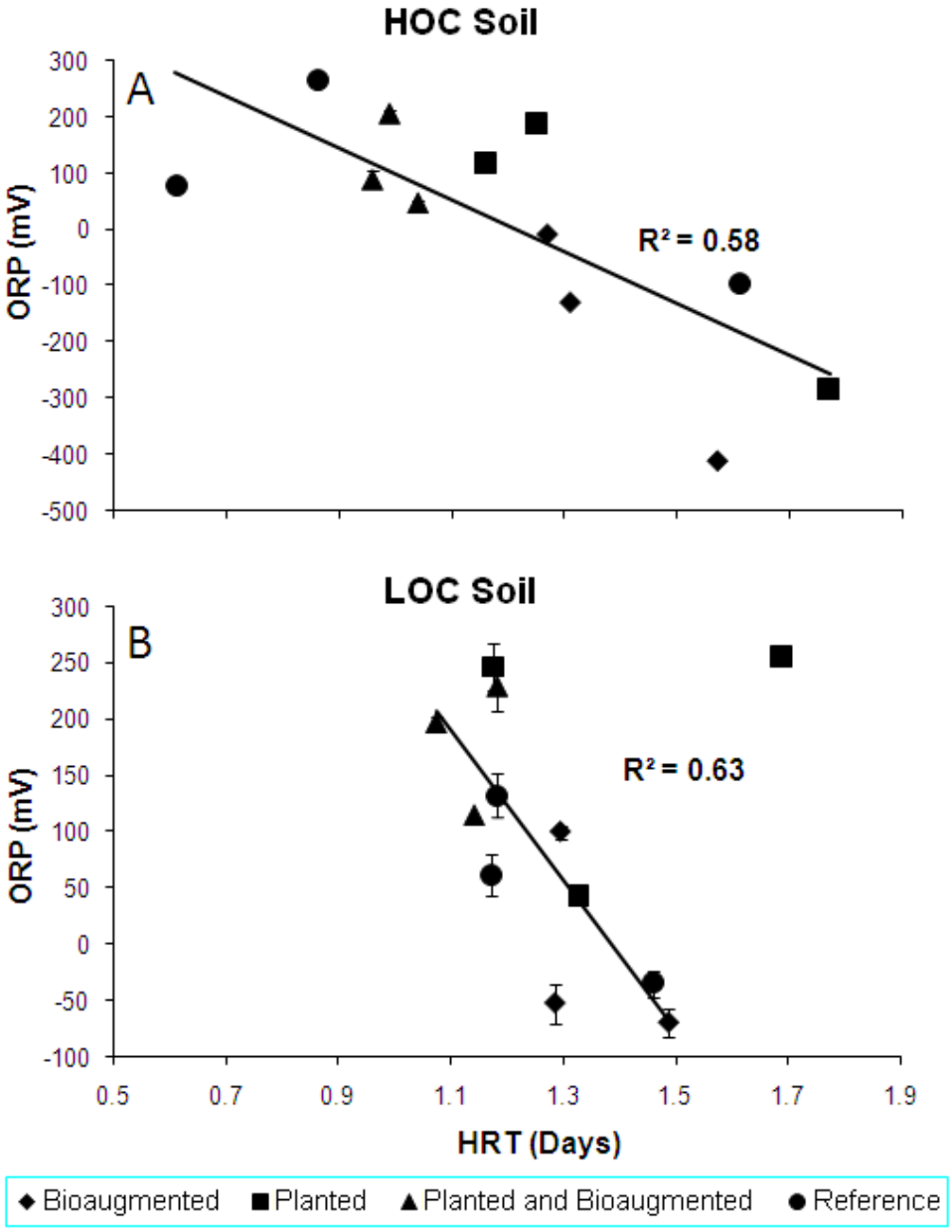


Figure 2-6. Reactor ORP plotted as a function of the hydraulic retention time. Data plotted in (A) were calculated from observations in HOC soil reactors while (B) represents LOC soil. The linear fit line of (B) does not include the outlier. Error bars are the 99% confidence interval. Sample quantity and duration are the same as in Figure 2-4.

CHAPTER III

FATE AND TRANSPORT OF PERCHLORATE DURING PHYTOREMEDIATION OF LOW AND HIGH ORGANIC CARBON SOIL

Abstract

Perchlorate is a known human toxin. Perchlorate-reducing bacteria have been shown to be ubiquitous in soils, but often lacking in electron donor. Bioremediation of perchlorate-contaminated soils and groundwater can be stimulated by the release of electron donors from plant roots. The overall objective of this research was to determine the relative roles of microorganisms compared to trees in the phytoremediation of perchlorate. A concern has existed that oxygen released into the subsurface through plant roots could raise measured oxidation-reduction potential (ORP) to a point where perchlorate reduction would be inhibited. We have found, through the use of radiolabeled perchlorate, that a system composed of a hybrid poplar tree planted into bioaugmented sandy soil can remediate perchlorate at much higher removal efficiency than expected based on predictions from the ORP. In planted, but not bioaugmented, low organic carbon (LOC) soil reactors the average perchlorate removal efficiency was 20.6% of which 12.6% was found to be attributable to microbial activity and 8.0% was accumulated in the tree. In bioaugmented, planted, LOC soil reactors, the removal averaged 30.3%. 25.6% was attributed to microbial activity and 4.7% was plant uptake. The mean hydraulic retention time in these plug-flow reactors was only 1.4 days, so perchlorate removal of 20% to 30% is quite reasonable. Perchlorate removal in planted reactors containing high organic carbon soil was found to be similar to that previously reported based on measured ORP. The molar ratio of chloride to perchlorate uptake was approximately equal to the ratio of chloride to perchlorate in the bulk solution. However, in bioaugmented sandy soil reactors, the transpiration stream concentration of perchlorate

was calculated to be half of that in nonbioaugmented systems. Rerelease of perchlorate that has accumulated into tree leaves can be blocked by two engineered processes. Bioaugmentation of the soil reduces the amount of perchlorate uptaken from the subsurface and burning collected perchlorate-contaminated leaves results in >99.8% destruction of accumulated perchlorate.

Introduction

Perchlorate is a major component of solid rocket fuel and similar combustible solid products such as flares, matches, and fireworks. Natural sources of perchlorate have been identified including atmospheric deposition and contaminated nitrate deposits of the Atacama (Jackson *et al.* 2005; Urbansky *et al.* 2001). However, poor disposal practices of the mid 20th century have also led to anthropogenic perchlorate contamination measurable at a multitude of current and former industrial and military sites, particularly in the southwest United States (Fitzgerald *et al.* 2005). Perchlorate is a known inhibitor of iodide uptake into the human thyroid and as such can upset the balance and release of thyroid hormones (Brechner *et al.* 2000; Greer *et al.* 2002). The balance of these hormones has been shown to be essential to proper growth and mental development (Laurberg *et al.* 2002; NAS 2005).

Perchlorate-reducing bacteria have been shown to be nearly ubiquitous in soils (Waller *et al.* 2004). The recalcitrance of perchlorate appears then to stem from an insufficient supply of electron donor at sites where contamination is found. Considering that much of the southwest is arid and overlain by low organic carbon soils, it is perhaps no surprise that perchlorate contamination persists. The ubiquity and metabolic diversity of perchlorate reducing bacteria has stimulated interest in natural attenuation and enhanced bioremediation of perchlorate source zones. However, if the goal of enhanced bioremediation is to provide electron donors to perchlorate-reducers *in situ*, then a reasonable choice to achieve maximal economy is to plant trees on the site. Trees can be

modeled as solar-powered pumps that provide hydraulic control of contamination in the subsurface while releasing electron donors from their roots. Previous research has shown conclusively that willow and hybrid poplar trees are able to take perchlorate up into the plant, that perchlorate reduction can occur within plant tissues, and that hybrid poplar roots produce materials that are able to stimulate perchlorate reduction by a pure culture of perchlorate reducing bacteria (Nzengung *et al.* 1999; Shrout *et al.* 2006; van Aken and Schnoor 2002).

A complication of plant-mediated bioremediation of perchlorate is that while plants are primary producers capable of converting light and carbon dioxide into organic carbon substrates, they also produce oxygen. Molecular oxygen has been shown to be released from plant roots. This release of oxygen is likely related to observations that plants are able to increase the subsurface measured oxidation-reduction potential (ORP) (Nzengung *et al.* 2004). Microbial perchlorate reduction has been shown to be inhibited at high relative ORP. Though perchlorate reduction itself is thermodynamically favorable, oxygen and nitrate are typically utilized as terminal electron acceptors before perchlorate removal is observed. The concern then is that plants will provide electron donors for perchlorate reduction but that oxygen leaked from the roots will allow aerobic organisms to proliferate and outcompete the perchlorate reducing bacteria for resources. Evidence of this competition between aerobes and perchlorate reducers was indeed observed in planted high organic carbon (HOC) soil, but not in planted low organic carbon (LOC) soil (Chapter IV).

Perchlorate is ideal for the study of fate and transport in planted bioremediation systems. Like chlorinated solvents, perchlorate is highly oxidized and more easily reduced at low measured ORP. However, perchlorate is nonvolatile and is not significantly adsorbed to or absorbed by soil. These physical properties allow the use of perchlorate in examining the interaction between plants and bacteria during its

bioremediation without concern regarding mass loss due to volatilization or other unknown sinks in the system.

Previous research in our laboratory demonstrated that planting a hybrid poplar tree in HOC soil does not increase perchlorate removal compared to reactors without trees and that low ORP, modulated somewhat by hydraulic retention time (HRT), is required for high perchlorate removal (Chapter II). Conversely, planting a tree in LOC soil may lead to a small increase in perchlorate removal compared to reactors without trees. There was one exception to these general observations: the reactor with the most active tree (highest transpiration rate) consistently had the highest perchlorate removal even though it also had the highest measured ORP. The general objective of the research described herein is to elucidate the relative contributions of plants and bacteria in perchlorate phytoremediation systems. We hypothesize that perchlorate remediation will be dominated by bacteria in HOC soil, but plant uptake will contribute more to overall removal in LOC soil. This will be shown using radiolabeled perchlorate. In addition, we provide general recommendations for *in situ* phytoremediation of perchlorate as a function of existing site conditions.

Materials and Methods

Reactor Construction

Reactors were constructed from one-liter plastic wide-mouthed jars. Pea-sized chert gravel and soil were layered sequentially as follows: 2 cm gravel, 6 cm soil, 2 cm gravel, and 3 cm soil. An experimental variable was soil organic carbon content and the soil used in reactor construction was either topsoil or quartz sand. The topsoil "fraction organic carbon" (f_{oc}) was 2.4% and the quartz sand had f_{oc} of 0.1%. The soil and gravel media were autoclaved twice with a two-day pause in between to allow spore-forming bacteria to germinate. Nitrate-free modified Hoagland's solution was introduced into the top gravel layer and removed from the bottom gravel layer, leading to a predominantly

downward flow. The solution was allowed to equilibrate with ambient oxygen concentration. Two other experimental variables were the presence of an inoculum of perchlorate-reducing bacteria and/or rhizosphere conditions. The inoculum was from an enrichment culture derived from municipal activated sludge provided lactate and perchlorate in a medium with no added chloride. Rhizosphere conditions were achieved by planting a hybrid poplar cutting (*Populus deltoides x nigra,* DN-34) directly into the reactor soil, with no hydroponic intermediate step. These reactors are referred to as planted. The reactors that were both planted and bioaugmented with an inoculum of perchlorate reducers hereafter referred to as bioaug./planted. Complete construction details are reported elsewhere (Chapter II).

Radiolabeled Perchlorate Synthesis

Chlorine-36-labeled NaCl (American Radiolabeled Chemicals, St. Louis, MO) was used to synthesize perchlorate through hypochlorite, chlorite, and chlorate by a combination of chemical and electrolytic oxidation. Final electrolysis conditions were as follows: 3 mL of 50 mM sodium chloride solution in a one-dram vessel were subjected to a constant current of 25 mA with a platinum anode and stainless steel cathode. The anode was made by winding 4 cm of 0.25 mm diameter thermocouple-grade platinum wire around a 21 gauge needle to form a spring-like shape that allowed maximum surface area in the allowable length. The solution was rapidly stirred with a magnetic stirrer. The background electrolyte concentration was 0.25 M Na_2SO_4 and sufficient 0.1 M NaOH was added to raise the pH to 10.0. At low pH, the reaction of chlorite and hypochlorite can lead to the generation of chlorine gas through the reaction:

$$Cl^- + OCl^- + 2H^+ = Cl_2 + H_2O$$

By raising the pH, the equation was shifted towards the reactants and the stoichiometric mass of chlorine remained constant during the electrolysis. The overall electrolyte concentration yielded a potential around 3 V at a current of 25 mA. After 6 hours, the system total chlorine was present as approximately 90% chlorate and 10% perchlorate.

To drive the synthesis to completion, ammonium persulfate was added to a final concentration of 0.1 M and the current was increased to 40 mA. 2.5 hours of electrical and chemical oxidation led to a final perchlorate molar yield of 90%.

Plant Tissue Perchlorate Extraction

Perchlorate was extracted from plant tissues in the following fashion. Plant fractions were excised, weighed and dried overnight at 102 °C. After drying, the fractions were reweighed and ground with a mortar and pestle to a fine powder. One gram or less of the material was added to a 15 mL centrifuge tube along with 10 mL of deionized water. The centrifuge tubes were then boiled for 60 minutes before freezing at -80 °C. Once frozen, the tubes were boiled again for 30 minutes and then refrigerated overnight. The tubes were frozen to break up cell walls and boiled to precipitate proteins. The tubes were centrifuged at 12,000 x g for 90 minutes following refrigeration to remove the solid fraction. The supernatant was then filtered through a 0.2 µm syringe filter. The described plant extract was then quantified through ion chromatography (IC) and liquid scintillation counting (LSC). To backcalculate the perchlorate concentration of the plant fractions on a dry tissue basis the final extract concentration (mg/L) was divided by the sample dry tissue mass, multiplied by the total dry fraction mass, and multiplied by 0.010 L (the water added to the vial) to end with mg/g perchlorate. Total radioactivity of the tissue was quantified in a similar fashion except that the final multiplication factor was 10 to correct for the fact that only one mL of the 10 mL added was actually counted.

Plant Tissue Combustion

A fraction of the finely-ground plant tissue was combusted at 500 °C to simulate collection and burning of fallen leaves. The combustion took place in aluminum weigh dishes for two hours. Following combustion, the ash was placed into 15 mL centrifuge tubes with 8 mL of deionized water, shaken for one hour and refrigerated overnight prior to filtration and analysis by LSC and IC.

Liquid Scintillation Counting

Sample radioactivity was measured using a Beckman 6000 IC scintillation counter. One mL of the liquid sample was added to a 20-mL glass scintillation vial followed by nine mL of Ultima Gold scintillation cocktail and shaking to mix. To determine the fraction of radiolabeled perchlorate reduced to chloride in the effluent, samples were slowly forced through Onguard Ag cartridges using manufacturer suggested protocol (Dionex, Sunnyvale, CA). The before and after samples were then monitored by LSC and IC. The IC was used to verify that chloride was removed from solution and that perchlorate was not.

Reactors were operated for 310 days prior to dosing to allow plant growth and equilibration of the microbial population. After dosing with the radiolabel, the effluent solutions were sampled 24 times over six days to obtain ORP, HRT, and perchlorate removal data. The plant material was sacrificed and sampled at the end of the six-day dosing period to determine fate and transport.

Ion Chromatography

Perchlorate, chlorate, chlorite, and chloride were measured by ion chromatography. The IC used was a Dionex (Sunnyvale, CA) ICS-2000 equipped with an AS-50 autosampler, AS-16 4mm analytical column held at 40 °C, ASRS Suppressor, and DS6 conductivity detector held at 31 °C. A potassium hydroxide eluent gradient was used to both separate the daughter products at the early end of the run and reduce the total run time for perchlorate elution. A flow of one mL per minute was used with a starting eluent concentration of 5 mM which was then increased to 50 mM from 1 to 10 minutes with a curve number of eight (concave). Total acquisition time was 16.5 minutes.

Quality Control

No persulfate was detectable in the perchlorate synthesis cell after electrolysis. An expected increase in the concentration of sulfate was measured. The method detection limit of perchlorate was measured to be 571 µg/L (5.74 µM). The detection

limit of LSC while maintaining an error of 1% or less was 43.8 cpm. A quench curve was generated by using increasing plant extract concentration and constant added radiolabel. It was found that so long as the H# was below 250 there was no decrease in counting efficiency. Perchlorate was shown to have undetectable sorption to plant tissues when following the tissue extraction protocol outlined above.

A complication of phytoremediation is that the contaminant concentration can actually increase in treated water compared to untreated. This is due to the preferential uptake of water by plants to support many of the necessary life systems. In order to correct the influence of transpiration on perchlorate effluent concentration in our systems, we used a modified ratio of the influent and effluent chloride concentration. A mass balance on chloride can be written as:

$$C_{Cl,in}V_{in} = C_{Cl,out}V_{out} + 24 \times V_{trans} - rxns$$

where $C_{Cl,in}$ and $C_{Cl,out}$ are the influent and effluent chloride concentrations (μM), V_{in}, V_{out}, and V_{trans} are the influent, effluent, and transpired volumes (L) and 24 is the transpiration stream concentration of chloride (μM) as reported later. The mass of chloride produced by the reduction of perchlorate was found to be experimentally insignificant because chloride had 40 times higher concentration than perchlorate. Rearranging the equation and removing the reaction term yields the transpiration concentration factor, which is equivalent to V_{out} over V_{in}:

$$TF = \frac{C_{Cl,in}}{C_{Cl,out} + \frac{24 \times V_{trans}}{V_{out}}} = \frac{V_{out}}{V_{in}}$$

While the more complicated transpiration factor (TF) equation is equivalent to the ratio of effluent and influent volumes, the effluent volume is difficult to precisely quantify due to evaporation and sampling losses. The true perchlorate removal efficiency, which accounts for both plant uptake and bacterial reduction, was calculated as:

$$Perchlorate\ Removal(\%) = (1 - \frac{C_{ClO4,in}}{TF \times C_{ClO4,out,measured}}) \times 100\%$$

The perchlorate concentration correction was calculated in a slightly different way during the fate and transport study than that described earlier (Chapter II). This was due to a somewhat different sampling protocol. Previously, perchlorate removal samples were taken directly from the effluent by diverting the flow into a syringe. However, in this study a much large volume of sample was required for IC and LSC of both raw and chloride-free samples. To gain sufficient sample volume in a reasonable time, samples were taken directly from the waste reservoir and cumulative effluent was monitored. For incremental sampling as used previously, the correction method described before was not sensitive to sample volume. However, the former method was very sensitive to the sample volume when measuring the cumulative effluent, and sample volume was not precisely recorded. Correction based on chloride concentrations in influent and effluent was found to be insensitive to sample volume and was thus used when cumulative effluent sampling was done.

Results and Discussion

Perchlorate Fate and Microbial Contribution to Removal

Radiolabeled perchlorate was used to construct a stoichiometric mass balance on the reactor system and to identify the sinks of perchlorate in a planted system with and without inoculated bacteria. Radiolabel recovery efficiency was 100.9 ± 1.5% in the four dosed reactors, with a range of 98.8 – 102.3%. The planted LOC soil reactor had overall perchlorate removal of 30.4% with 11.7% of the removal shown to be perchlorate accumulation into the poplar cutting and 18.6% of the removal due to reduction of the contaminant in the soil (Figure 3-1). The bioaug./planted LOC soil reactor had overall removal of 33.2% with 5.1% in the tree and 28% reduced in the soil. The planted HOC soil reactor had overall removal of 19.9% with 1.6% in the tree and 18.3% reduced in the soil. The bioaug./planted LOC soil reactor had overall removal of 21.1% with 0.77% in the tree and 20.3% reduced in the soil. Planted LOC soil reactors were shown to have

greater overall perchlorate removal than the planted HOC soil reactors (with or without bioaugmentation). We had observed this previously in one reactor (Chapter II). As expected, bioaug./planted reactors showed more in-soil reduction than planted reactors. Unexpectedly, bioaug./planted reactors also showed less plant uptake, even in plants with very similar transpiration rates, HRT, and ORP (Figure 3-1, Table 3-1).

As described above, the objective of these experiments was to determine the relative contributions of plants and microorganisms during phytoremediation of perchlorate. Though perchlorate removal efficiency was found to be greater in planted LOC soil than planted HOC soil, the microbial contribution was greater in HOC soil (Figure 3-2, Table 3-1). As expected, the microbial contribution was greater in bioaug./planted soil than in nonbioaugmented. In planted, nonbioaugmented LOC soil, the bacteria accounted for more than 60% of the total perchlorate removal. Both bioaugmentation and an increase in soil organic carbon content served to further increase the bacterial contribution to total perchlorate removal.

Results from these experiments with $^{36}ClO_4^-$ agree well with previous research in our laboratory when perchlorate removal is plotted as a function of measured ORP (Figure 2-4, Figure 3-3). In HOC soil, we showed previously that perchlorate removal efficiency was related to measured ORP and that planting a tree did not appear to affect removal compared to unplanted reactors. In contrast, perchlorate removal was much lower in LOC soil than in HOC soil overall, but there was evidence that planting a tree in LOC soil may positively impact remediation. One reactor, which contained the most transpirationally active tree, achieved 20% perchlorate removal at high measured ORP. Current results support this observation. Half of the LOC soil planted reactors were shown to have unexpectedly high perchlorate removal at high measured ORP (Figure 3-3). Again, these reactors also had the most actively transpiring trees. The HOC soil data point furthest to the right represents a reactor that had very similar transpiration rate and ORP, but much less perchlorate removal. These combined results provide sufficient

evidence to reveal the influence of plants on perchlorate remediation in LOC soil; an influence that is strengthened by bioaugmentation and is not present in HOC soil.

Implications Toward a Perchlorate Uptake Mechanism

Careful comparison of total average perchlorate and chloride concentration in the reactors, radiolabeled perchlorate and chloride concentration in the reactors, and plant radiolabeled perchlorate and chloride mass allowed us to calculate the overall uptake of chloride, perchlorate, and water during the course of the radiolabel dosing. The chloride uptake was shown to be 38.24 ± 6.09 times higher than the perchlorate uptake. In other words, approximately 38 chloride ions were taken up into the plant for every perchlorate ion. The mean ratio of chloride to perchlorate in the bulk solution was calculated to be 39.9 ± 3.4 which indicates that neither perchlorate nor chloride is preferentially taken up into the plant. The water to perchlorate uptake ratio was twice as high in bioaug./planted vs. planted, nonbioaugmented LOC soil reactors, meaning that though the trees had the same transpiration rate (Table 3-1), only half as much perchlorate was taken up into the plant in the bioaugmented system. The overall mean water to perchlorate ratio was 1.55 ± 0.61 x (10^6) and ranged from 1.20 to 2.47 x (10^6) water molecules taken up per perchlorate molecule. The observations that perchlorate uptake is relatively constant among reactors compared to chloride but not constant compared to water indicate that perchlorate uptake into root cells is probably mediated by a membrane ion transporter rather than leaky aquaporins or an otherwise hydrophilically-leaky membrane. Perhaps uptake of perchlorate takes place through a membrane transporter similar to that of nitrate or chloride (Tsay *et al.* 2007). Chloride is a known plant micronutrient (White and Broadley 2001). The potential also exists that perchlorate is able to be substituted into the plant's native symplastic chloride uptake pathway. The observation that perchlorate appears to be taken up through a membrane transporter means that it is possible that perchlorate-reducing bacteria associated with the root surface or interior can block or shut down plant uptake of perchlorate either physically or enzymatically. If the uptake of

perchlorate were the result of a leaky membrane or aquaporin, then it would be much less likely that bacteria could directly interfere, other than by reducing bulk concentration.

Implications for Remediation and Engineering Significance

Based on our research results, it appears that *in situ* perchlorate remediation is most efficient in a bioaugmented, moist, organic-rich soil where the activity of heterotrophic bacteria is expected to lead to low ORP and suitable conditions for microbial perchlorate reduction (Chapter II). However, perchlorate contamination is not likely to be observed in soil with high organic carbon and the cost of raising the organic carbon content in the existing soil or adding electron donor to a high enough level for efficient remediation could be prohibitive. The more economical choice appears now to be bioaugmentation and phytoremediation while leaving the soil organic carbon low.

The LOC soil reactors planted with highly active trees demonstrated perchlorate removal at ORP higher than that previously believed possible. Conversely, planted HOC soil did not show the same result, even under similar conditions of ORP, HRT, and transpiration (Figure 3-3, Table 3-1). The factor limiting microbial perchlorate reduction is often a lack of electron donor. The defining characteristic of the HOC soil used in these experiments is high organic carbon and corresponding high electron donor content. Why is it then that planted HOC soil did not exhibit high perchlorate removal except at low ORP? The low perchlorate removal in planted HOC soil can perhaps be explained through considerations of microbial ecology. A comparison of 16S rRNA sequences from these reactors showed that a richer and more diverse population of bacteria was found in HOC soil than in LOC soil. In contrast, the sequence diversity of the gene coding for an enzyme essential to perchlorate reduction was the same in LOC and HOC soil (Chapter IV). In HOC soil therefore, a population of microorganisms exists that is already adjusted to utilization of the available soil electron donors, even after autoclaving (Tuominen *et al.* 1994). This diverse community of soil organisms has access to oxygen released from the plant roots, so has little demand for perchlorate as an electron acceptor.

The perchlorate reducing bacteria are unable to compete against such a large population of microorganisms for resources, and the increased ORP observed in planted reactors is not overcome by the electron donors released from the roots. In LOC soil, however, a less diverse population of competitors to perchlorate reduction allows perchlorate-reducing bacteria to overcome the higher ORP of a planted system in order to utilize the plant-derived electron donors.

Evidence indicates that bioaugmenting the soil with an enrichment culture of known perchlorate degraders not only increases the efficiency of perchlorate reduction in the subsurface but also decreases the amount of perchlorate taken up into the tree. This evidence yields yet another tie between plant roots and microbes. The organisms appear to degrade perchlorate and therefore decrease its uptake into tree roots. It is known that competition exists for plant root products (Bowen and Rovira 1976). To effectively compete, the perchlorate-reducing bacteria being stimulated by plant products must be in close proximity of the root surface.

Bioaugmentation does not completely limit the uptake of perchlorate into plant tissues and the vast majority of accumulated perchlorate is found in the leaves when compared to stem and root tissues. One potential way to limit perchlorate release from fallen leaves back into the soil is to collect the leaves and burn or compost them. To verify the efficacy of perchlorate destruction during leaf burning, dried leaf samples were combusted at 500 °C for two hours and the ash was extracted into water as described above. For all samples, < 0.2% of the initial perchlorate mass was recovered from the ash. When the combustion experiment was performed with radiolabeled perchlorate 41.0 ± 29.3% of the radiolabel was not recovered and is assumed to have volatilized from the sample. Also of note is the observation that destruction of solid $NaClO_4$ was not observed under the same thermal conditions.

Based on this research, we recommend that for full scale phytoremediation of perchlorate at source zones the following precautions be taken. Bioaugment the soil with

an inoculum of known perchlorate-reducing bacteria to decrease the uptake of perchlorate into plant tissues and to increase the microbial contribution to remediation. Add minimal compost to encourage healthy plant growth, but do not add enough that the indigenous microorganisms will outcompete the bioaugmented perchlorate reducers. Calculate removal based on mass loss rather than concentration change or use a relatively conserved analyte such as chloride is available to calculate a transpiration correction factor. To prevent the release of perchlorate from plant tissues, collect leaves in the fall and burn, compost (in a leakproof container), or otherwise dispose of them. A fortuitous property of hybrid poplar DN-34 is its propensity to drop nearly all of its leaves in a very short time span which further simplifies the task of collecting leaves prior to significant degradation (Licht 2006).

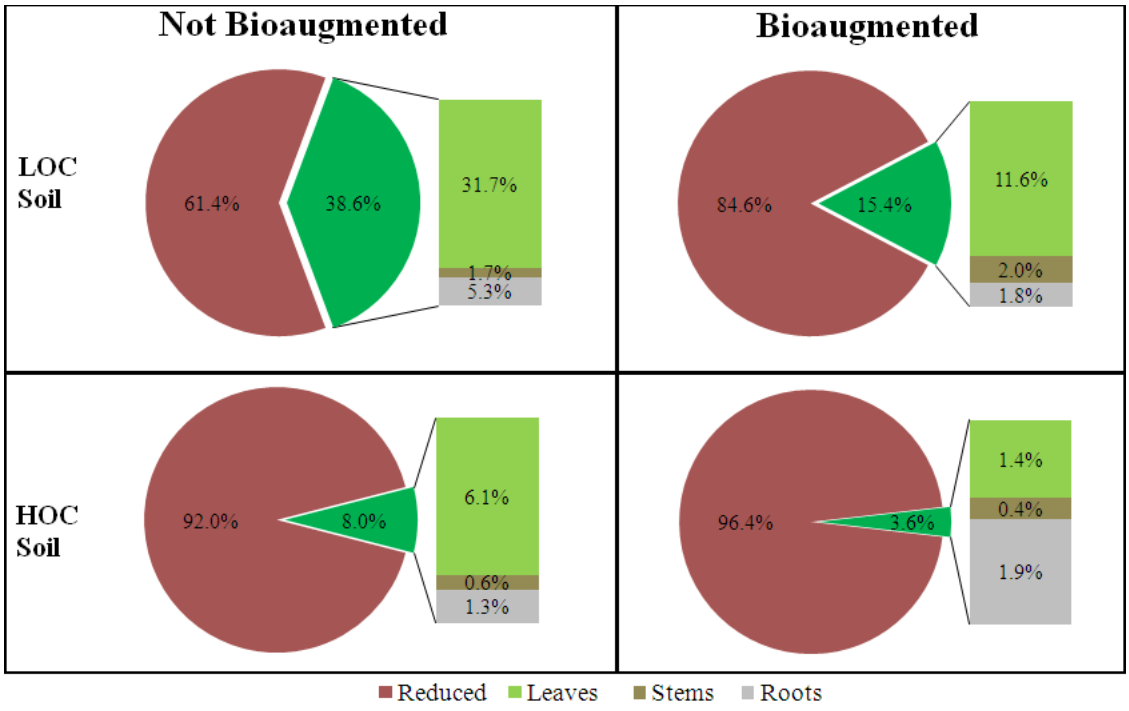


Figure 3-1. The fate of radiolabeled perchlorate removed from planted reactors (i.e. not in the effluent solution). The red and green sections of the pie chart indicate microbial reduction and plant accumulation, respectively. The green, tan, and gray sections of the bar chart indicate leaf, stem, and root accumulation, respectively.

Table 3-1. The contribution of plants and bacteria to overall perchlorate removal in planted reactors

		Not Bioaugmented	Bioaugmented
LOC Soil	ClO_4^- Removal (%)	22.0	32.2
	Plant Contribution (%)	38.6	15.4
	Bact. Contribution (%)	61.4	84.6
	Average ORP* (mV)	325	328
	Transpiration* (ml/hr)	4.14	3.91
	Hydr. Ret. Time (hr)	55.0	51.7
HOC Soil	ClO_4^- Removal (%)	14.9	15.9
	Plant Contribution (%)	8.0	3.6
	Bact. Contribution (%)	92.0	96.4
	Average ORP* (mV)	64.5	4.72
	Transpiration* (ml/hr)	1.03	0.49
	Hydr. Ret. Time (hr)	51.6	60.5

*Average ORP and transpiration rate are shown to provide comparison of physical parameters among reactors.

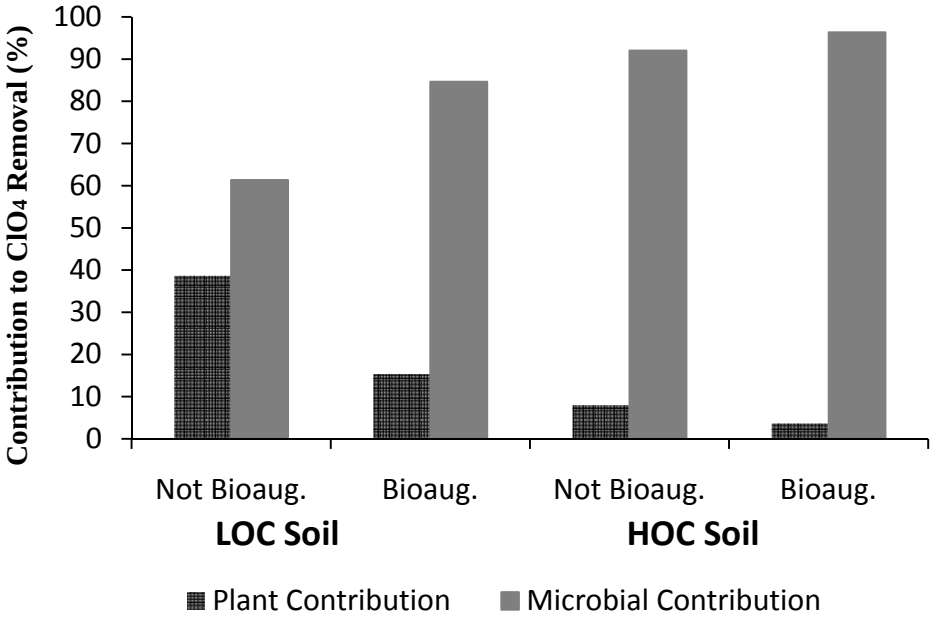


Figure 3-2. The relative contribution of plant and microbes to overall removal in planted reactors. Bioaugmented reactors showed a greater microbial contribution compared to non bioaugmented.

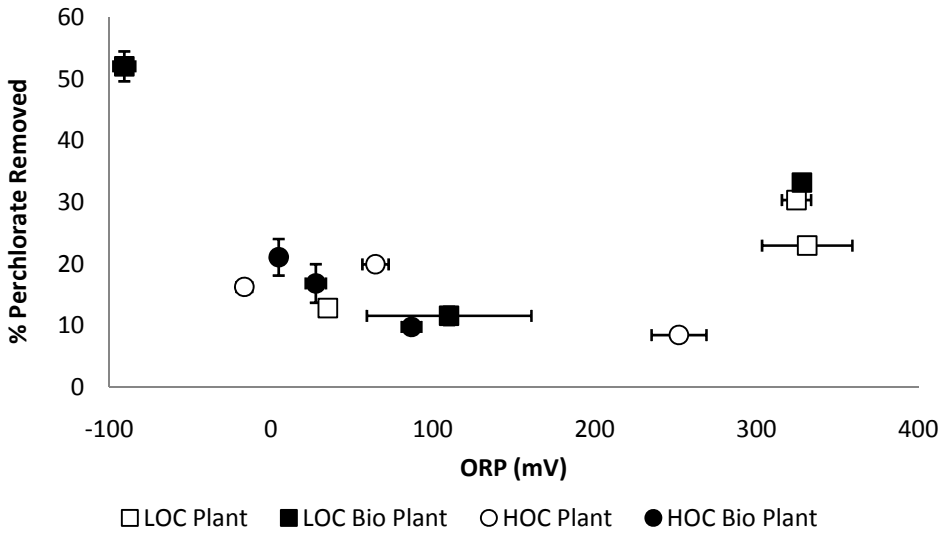


Figure 3-3. Perchlorate removal in planted reactors as a function of ORP. LOC (□) and HOC (○) refer to soil organic carbon content, while Bio refers to bioaugmentation with a known perchlorate degrading consortium (filled points). Error bars show the 95% confidence interval. Data points represent 24 samples taken over 147 hours. Sampling began after 310 days of operation. The data plotted here match well with previously reported results (Figure 2-4).

CHAPTER IV
DIVERSITY OF THE CHLORITE DISMUTASE GENE IN RHIZOSPHERE SOIL COLONIZED BY PERCHLORATE-REDUCING BACTERIA

Abstract

Chlorite dismutase (*cld*) is an essential enzyme in the microbial degradation pathway of perchlorate. Of interest to us is the change in sequence diversity of the *cld* gene in soil samples under varying conditions of high and low organic carbon, bioaugmentation, and plant influence. To gain an understanding of general bacterial diversity under the same conditions, universal eubacterial primers were used to quantify 16S rRNA. We found that *cld* gene diversity, quantified using the Shannon Index, was not different between high organic carbon (HOC) and low organic carbon (LOC) soil. Combining results from HOC and LOC soil, diversity of the *cld* gene was decreased in soil that had been bioaugmented but not planted. Diversity of the *cld* gene was also decreased in soil that had been planted but not bioaugmented. However, in soil that had been both bioaugmented and planted the *cld* diversity was not decreased. These observations were repeated when focusing on LOC soil reactors alone. However, in HOC soil the *cld* diversity was not decreased by bioaugmention, planting, or the combination of planting and bioaugmentation. General bacterial diversity as measured with 16S rRNA was significantly greater in HOC soil than in LOC soil, but no significant difference was observed between reference soil and planted or bioaugmented soil.

Introduction

Perchlorate (ClO_4^-) is a powerful oxidant. It is used in the aerospace industry as a solid rocket propellant and other uses include matches, flares, and fireworks. Unregulated disposal of the mid to late 20th century has led to relatively widespread occurrence of perchlorate contamination. Evidence suggests that though perchlorate is

naturally occurring (Jackson *et al.* 2005; Urbansky *et al.* 2001), the sites of highest concentration and therefore the most concern were contaminated by human activity, such as solid rocket motor decommission (Fitzgerald *et al.* 2005). Many of these sites in the United States occur in semi-arid to arid regions of the southwest.

Perchlorate is known to inhibit the uptake of iodide into the human thyroid. Specifically, perchlorate is a competitive inhibitor of the sodium-iodide symporter (NIS). Under equimolar conditions, perchlorate is 30 times more likely than iodide to be transported into the thyroid by the NIS (NAS 2005; Tonacchera *et al.* 2004). Increased perchlorate levels in blood have been associated with decreased thyroid function including decreased production and excretion of thyroid hormones (Braverman *et al.* 2005).

It has previously been established that perchlorate-reducing bacteria are nearly ubiquitous in soils (Waller *et al.* 2004). Why then is perchlorate contamination still measurable at a multitude of sites? The answer to that question lies in both the anabolic preferences of perchlorate reducers and the lack of electron donor in many of the arid sites where contamination has occurred. Perchlorate-reducing bacteria have been shown to prefer molecular oxygen and nitrate to perchlorate (Shrout 2002). A perchlorate-contaminated sandy soil in an arid climate has converging detriments to natural attenuation of the contaminant. Not only is there a lack of electron donor, an abundant supply of oxygen prohibits the reduction of perchlorate to chloride. It is these conditions that lead to the recalcitrance of a relatively labile chemical. Additionally, arid sandy soil is not an ideal medium for bacterial growth.

The ability to reduce perchlorate is found in a widely varied group of bacteria (Coates *et al.* 1999). Indeed, bacteria that would be considered closely related based on 16S rRNA phylogeny may or may not be capable of perchlorate reduction. This leads to problems with 16S-targetted approaches to determining the diversity of perchlorate-degraders. To alleviate these problems, we focused instead on diversity of the chlorite-

dismutase (*cld*) gene. Our interest is the function of perchlorate reduction, so it is logical to monitor the presence of a gene essential to the perchlorate-reduction pathway (Bender *et al.* 2002). The chlorite dismutase gene was chosen over other genes in the pathway because no other enzyme has been identified that is able to convert chlorite into chloride and oxygen, and our interest is in the complete degradation of perchlorate to chloride.

An intuitive approach to the assessment of microbial diversity and community structure is to isolate members of the community and study their metabolism and physiology. Current estimates indicate that we are able to culture only 0.3% of soil bacteria (Amann *et al.* 1995). This means that a strictly culture-dependent approach to the determination of microbial diversity will vastly underestimate the true diversity of an environmental sample. Denaturing gradient gel electrophoresis (DGGE) is a culture-independent tool to qualify sequence diversity among polymerase chain reaction (PCR) amplicons of similar length (Hurst and Crawford 2002; Muyzer *et al.* 1993). It is dependent only upon the ability to extract nucleic acids from a sample and amplify genes of interest with some degree of fidelity.

Plants are natural sources of electron donors and can stimulate microbial activity. However, they also produce molecular oxygen as a byproduct of photosynthesis. Some of that oxygen is transported to the root system and leaked into the subsurface. Our interest is whether plants produce sufficient electron donor to stimulate perchlorate degradation without raising redox conditions in the subsurface enough to inhibit degradation. We have shown in previous work that plants can affect the oxidation-reduction potential (ORP) in the subsurface of a laboratory-scale reactor with a maximum value observed as the growth lights turn off in the evening and the minimum observed just before the lights came back on in the morning (Chapter II). Will this diurnal variation of redox conditions select for oxygen-tolerant perchlorate degraders and ultimately lead to less microbial diversity in planted systems or will the plant electron donors allow a more diverse community due to a lack of competition? We hypothesize

that a planted system will lead to a less diverse population of chlorite dismutase sequences if not also bioaugmented. We further hypothesize that a HOC soil environment will yield a more diverse population of general bacteria when compared to LOC soil. We assume, based on previous literature, that chlorite dismutase genes of different sequence are present in different species of bacteria (Bender *et al.* 2002). With that in mind, a diversity of *cld* gene sequences is assumed to be a direct indicator of a diverse community of perchlorate degraders.

Materials and Methods

Overview of Reactor Setup

Soil-filled reactors were used to determine perchlorate removal under conditions of variable bioaugmentation (the inoculation of known perchlorate-degrading bacteria), planting, and soil organic carbon. All variables were binary, either present or not. Bioaugmentation was accomplished by adding a lactate-fed enrichment culture derived from municipal wastewater treatment plant activated sludge to the reactor soil (Shrout 2002). Trees planted were hybrid poplar clone *Populus deltoides x nigra* (DN-34). Herein reactors will be referred to as: bioaugmented, planted, bioaug./planted (both bioaugmented and planted), and reference. Soil in reference reactors was autoclaved, but was not chemically sterilized or dosed with biocide. Evidence suggests that the microbial activity could return to pre-autoclave levels within three weeks, thus these reactors acted as untreated soil (Tuominen *et al.* 1994). Soil organic carbon was quantified as fraction of organic carbon (f_{oc}) which was 2.4% in HOC soil reactors and 0.1% in LOC soil reactors. A perchlorate-containing nitrate-free growth medium based on Hoagland's solution was pumped into a gravel layer in the upper region of the reactor. The solution passed downwards through the bulk soil media (either HOC or LOC soil depending upon the experiment) and was pumped out of another gravel layer at the bottom of the reactor. A more complete description of reactor operation is given elsewhere (Chapter II).

DNA Extraction

DNA was extracted from soil microorganisms using the Ultraclean Soil DNA Extraction kit (MO BIO, Carlsbad, CA) according to manufacturer instructions. Approximately one gram of soil was used. DNA was extracted from soil approximately 240 and 300 days after the experiment startup for LOC and HOC soil, respectively. Because of this long exposure time, we made the assumption that the microbial population was at or near equilibrium. Cyclic population dynamics have been observed (Vandermeer 2006), but the use of six samples per reactor setup served to reduce the effects of variability. After extraction, amplification problems indicated that PCR inhibitors remained at high levels in the HOC soil DNA extracts. To further remove these inhibitors, the HOC soil extracts were passed through a resin column (Epicentre, Madison, WI) and diluted twofold. These procedures allowed amplification of the desired genes.

PCR Conditions

PCR conditions for the chlorite dismutase gene were according to those of Bender et. al. (2004). A nested PCR approach was followed because it is known to increase yield and specificity on environmental samples. The first primers used were the DCD set. An aliquot of this mixture containing 5-10 ng of DNA was then used as template DNA in another PCR mix containing the UCD primer set. The UCD-238F primer was modified to include a 40 base-pair GC-rich region on the 5' end (GC-clamp: [5'-CGCCCGCCCGGCCCCCGGCCCGGCCCGCCGCCCCCGCCCC-3']).

PCR conditions were modified slightly from those of Bender et al. The DCD primer set was used with an annealing temperature of 40°C and 60 cycles. This temperature was determined from a gradient PCR run with annealing temperature ranging from 39 – 49°C. The DCD reaction mixture was then used as template DNA with the UCD-GC primer set and an annealing temperature of 50°C with 60 cycles. In order to improve specificity, the UCD-GC reaction mixture was used as a template with the UCD-

GC primer set at 58.5°C and 35-40 cycles yielded a single band of approximately 440 base pairs on agarose gel.

Universal eubacterial 16S rRNA was targeted using the 27F/519R primer pair. The forward primer was modified with a 40 base pair GC clamp (Reeson *et al.* 2003). PCR conditions were taken from literature except that an optimal annealing temperature of 54.5 °C was chosen based on gradient PCR using the actual DNA extracts to be amplified.

Denaturing Gradient Gel Electrophoresis (DGGE)

DGGE gels were cast using an 8% solution of 37.5:1 acrylamide:bis-acrylamide. A urea-formamide gradient of 20-50% was used for both *cld* and 16S amplicons. The 1x TAE run buffer was held at 60 °C during electrophoresis. Gels were run at 200V for 900 volt-hours (V-hrs). Gels were stained for 30-40 minutes in a 1X solution of SYBR Gold (Invitrogen, Carlsbad, CA) in 1X TAE buffer. Following staining, the gels were photographed on a UV transilluminator (Figure 4-1, Appendix C).

Some literature suggests that short runtimes provide optimal results, while other literature takes a contrary view and suggests long runtimes at lower potential (Gejman *et al.* 1998; Sigler *et al.* 2004). A comparison of gels run at variable potentials (60, 100, 200V) for 900 V-hrs showed that 200V gave the best separation and sharpest bands. This was in agreement with Sigler *et al.* (Sigler *et al.* 2004), who suggest that the decrease in clarity at lower potential could result from a diffusive loss of denaturant gradient resolution. The DCODE system (Bio-Rad, Hercules, CA) was used for DGGE analysis. The buffer tank was placed into a fiberglass-insulated cardboard box to improve performance in the rear gel. Insulation led to no observable difference between front and rear gels. For the front gel, size standards were placed into the 1st well and one well in from the end. For the rear gel, size standards were placed into the 1st well and two wells in from the end. This allowed a very rapid determination of each gel and its correct orientation after staining and imaging was complete.

Verification of Amplicons

The TOPO/TA kit with pCR®4 vector (Invitrogen, Carlsbad, CA) was used to clone PCR products into chemically competent TOP10 cells following manufacturer instructions. Transformation efficiency was excellent with approximately 150 white colonies and 10 blue colonies for each cloning reaction. Six clones each were sequenced from HOC soil reactors (three *cld* and three 16S) and LOC soil reactors (three *cld* and three 16S) for a total of six *cld* clones and six 16S clones (Appendix D).

All six of the randomly selected putative *cld* clone sequences showed >98% homology to known chlorite dismutase sequences using BLAST searches. The six *cld* clone amplicons represented five unique positions on DGGE gels. Sequences from 16S clones were identified using the Ribosomal Database Project as: two unclassified bacteria, delta proteobacteria, Porphyrobacter, Alicycliphilus, and Sphingobacteriales (Cole *et al.* 2009).

Maximizing SYBR Gold Economy

Literature suggests that SYBR Gold staining solution can be used for multiple gels (Molecular Probes 2006). However, staining efficiency appeared to decrease after staining as few as two gels. An aliquot of the fresh SYBR Gold solution was scanned on a UV/Vis spectrophotometer blanked with 1X TAE and a maximum absorbance of 0.058 was observed at a wavelength of 485nm in plastic 1-cm cuvettes. After staining one gel the solution was found to have an absorbance of only 0.030. We found that SYBE Gold has a linear relationship between concentration and absorbance. 1X TAE showed no absorbance at 485nm when deionized water was used as a blank, so either could be used for this analysis. We found that maximal economy can be achieved by simply measuring the absorbance of a used solution before staining and adding just enough dye to reach some predetermined concentration.

The Use of Size Standards as Denaturant Gradient Standards

We discovered during DGGE optimization that commercially available DNA size standards can be used as denaturant gradient standards. A 100 base pair size standard (New England Biolabs) was run alongside PCR amplicons in a preliminary experiment to determine the optimal V-hrs required for separation of the bands. The DGGE gel was run for a total of 1000 V-hrs, but samples and size standards were added incrementally, 50 V-hrs apart, rather than all at the beginning (Muyzer *et al.* 1993). The PCR products showed optimal separation at 900 V-hrs, but a surprising discovery was that several of the size standard bands halted migration at discrete denaturant concentrations. This observation was used later during band analysis to compare bands in different gels by using the gel imaging software to assign migrations to the sample bands based on measured migrations of the relevant size standard bands

Software

Kodak Molecular Imaging (Carestream Health, Rochester, NY) was used to analyze DGGE image data. Bands were identified using the default sensitivity followed by minimal band realignment and minimal changes to background reference points. The default settings were used to avoid introducing human error that could affect the ability to compare between gels. Band information was then exported, formatted in Microsoft Excel, and used in both DGGE STAT and SPSS 17 to develop cluster analyses and non-metric multidimensional scaling based on Euclidean distance (Appendix C). Euclidean distance uses the distance formula in *s*-dimensional space, where *s* is the total number of bands per lane, to calculate how far apart two samples are (Fushuku and Fukuda 2008; van Hannen *et al.* 1999). The Euclidean distance between samples can then be visually compared by plotting points in one, two, or three dimensional space at positions that satisfy most of the distance constraints (Figure 4-2, Appendix C). To compare between gels The Gimp 2.0 (www.gimp.org) was used to scale images so that ladders were

aligned. This scaling did not affect calculation of the band diversity and was only used for the calculation of Euclidean distance. No other aspect of images was altered during band analysis.

Results and Discussion

Diversity of *cld* and 16S genes

Two parameters were used to quantify diversity and population characteristics of chlorite dismutase and 16S rRNA genes: DGGE band diversity as measured by Shannon Index and band richness. The Shannon Index, which is analogous to entropy, essentially measures the chance that an organism randomly selected from a population will be different from an organism selected previously. The Shannon Index is calculated as:

$$Shannon\ Index = H = -\sum_{i=1}^{s} p_i \log_2 p_i$$

where p_i is the relative intensity of each band in a sample and s is the total number of bands (Janczyk *et al.* 2007; Spellerberg and Fedor 2003). The richness is the total number of bands measured present in a sample. The diversity and richness of the bands from each sample were compared using Student's T tests at a 95% confidence level.

Chlorite Dismutase

Chlorite dismutase band diversity and richness in LOC soil, HOC soil, and inoculum were not shown to be significantly different (Figure 4-3). However, the inoculum *cld* richness was found to be significantly lower than those of the LOC soil or HOC soil.

To examine the effect of reactor setup without the complication of soil organic carbon, results from LOC soil and HOC soil were combined (Figure 4-4, Appendix E). The diversity of *cld* DGGE bands was found to have two underlying statistical populations when comparing reactor conditions such as bioaugmentation and planting. Planted soil and bioaugmented soil *cld* diversities were not different from each other.

Likewise, the bioaug./planted reactor, reference reactor, and inoculum *cld* diversities were not different from each other. However, the planted soil and bioaugmented soil were significantly different from the bioaug./planted soil, reference soil, and the inoculum. These observations support the hypothesis that a planted system will lead to a less diverse population of *cld* sequences. In planted soil, the *cld* diversity was significantly less than that of the reference soil. Only when the planted soil was also bioaugmented was the *cld* diversity not decreased. In contrast to the observations of *cld* diversity, *cld* richness was not significantly different between reactor setups; all of which showed *cld* richness greater than that of the inoculum.

When focusing on LOC soil alone or HOC soil alone, three differences were revealed compared to the observations discussed above (Figures 4-5 and 4-6, Appendix E). The differences are: *cld* diversity in planted HOC soil is not significantly different from reference HOC soil or inoculum, *cld* richness in bioaugmented LOC soil is significantly lower than the reference soil, and *cld* richness of the inoculum is not different from that of planted LOC soil and bioaugmented LOC soil.

Interestingly, when *cld* diversity and richness are compared within the same reactor setup between LOC soil and HOC soil, the same trend as was seen in Figure 4-3 can be seen again. The *cld* richness and diversity are not significantly different between planted LOC soil and planted HOC soil (Figures 4-5 and 4-6, Appendix E). The same is true between the other reactor setups. Soil organic carbon does not affect *cld* diversity or richness.

16S rRNA

A hypothesis was that HOC soil would yield a more diverse population of bacteria, as determined by 16S rRNA sequences. This hypothesis was supported by the data. Diversity and richness of 16S DGGE bands were significantly greater from HOC soil than from LOC soil (Figure 4-3). Diversity and richness of the LOC and HOC soil

16S bands were also greater than those of the enrichment culture (inoculum) used to bioaugment half of the reactors.

The variable of soil organic carbon content was removed by combining results from LOC soil and HOC soil as above. 16S diversity was not shown to be significantly different between the reactor setups, though all setups had greater 16S diversity than the inoculum (Figure 4-4).

Only one difference was observed when focusing on LOC soil and HOC soil individually. Specifically, the 16S diversity and richness of the bioaugmented LOC soil and planted LOC soil was greater than that of the references, though the difference was not significant in all cases (Figures 4-5 and 4-6, Appendix E). In contrast, the 16S diversity and richness from HOC soil were not significantly different between any reactor setup, as was seen when LOC and HOC soil results were combined.

Ecological and Engineering Significance

The chlorite dismutase gene was identified and amplified from soil DNA extracts and differences in the population of *cld* sequences were quantified among reactor conditions. The above ecological parameters (diversity and richness) for both *cld* and 16S were compared to physical and chemical parameters and observations of the reactors such as oxidation-reduction potential (ORP), hydraulic retention time, tree transpiration rate, and perchlorate removal efficiency. Somewhat surprisingly, none of the parameter combinations showed a significant linear relationship ($R^2 > 0.6$) (Appendix F).

However, comparison of diversity to ORP showed a trend that could be fit to a parabolic curve, which may be indicative of an edge effect (Figure 4-7). An edge effect can be visualized as the diversity observed where a forest abuts a field. At the boundary, species that can thrive in both habitats will be present while further from the boundary only those species that can survive in one habitat will be found. Similarly, ORP can indicate the potential for a complex, diverse population of microorganisms. At high ORP, aerobic organisms will thrive and outcompete facultative organisms. At moderate

ORP, aerobic, facultative, and anaerobic organisms will likely exist in suitable niches, and at low ORP only anaerobic organisms will thrive.

The potential edge effect is apparent with maximal 16S diversity in HOC soil around 0 mV and maximal *cld* diversity in LOC soil around 100 mV (Figure 4-7). A parabolic regression of edge effect has been observed previously (Galli *et al.* 1976; Gorman *et al.* 2009). Diversity of *cld* in HOC soil appears to follow the competitive exclusion principle. The points on the negative side of the x-axis represent reactors that were initially bioaugmented and also show the lowest *cld* diversity. It is likely that bacteria from the inoculum were able to gain a foothold in the reactor before extraneous bacteria had a chance to either migrate in or regain activity after autoclaving. Those established bacteria were of high enough population and influence to drive competing bacteria to extinction. The observation of very similar *cld* richness between LOC soil bioaugmented reactors and the inoculum supports this reasoning (Figure 4-6).

A founding hypothesis of this work was that the diversity of a functional gene would be useful as a master variable indicating microbial stress in a similar fashion to the use of rRNA gene and catabolic diversity reported in literature (Degens *et al.* 2001; Torsvik and Øvreås 2002). As expected, 16S ribosomal RNA gene diversity did vary among experimental conditions. However, though the diversity of *cld* genes exhibited some variability, the changes in *cld* diversity were not correlated to changes in 16S diversity. In addition, no significant relationship could be established between reactor performance and *cld* or 16S band diversity or richness. There is some evidence that *cld* and 16S diversity may decrease in more reducing conditions (Figure 4-7). Considering that perchlorate removal is negatively correlated to measured ORP, efficient perchlorate removal could be reasonably predicted to be favorable under conditions of low diversity. Literature suggests that high diversity is correlated to a healthy and stable reactor or treatment system, but perhaps the leap to correlate diversity and performance should be

made with caution (Degens *et al.* 2001; McMahon *et al.* 2007; Nannipieri *et al.* 2003). This is especially true when measuring diversity of a functional gene.

The major goal of this research was to answer the questions: Do rhizosphere conditions affect the diversity of bacteria and chlorite dismutase in an *in situ* perchlorate bioremediation system? Is that effect confounded or assisted by soil organic carbon? We found that *cld* diversity decreased in soil that had been bioaugmented or planted, compared to the inoculum and the references (Figure 4-4). However, bioaugmentation and planting (i.e. bioaug./plant) led to significantly greater *cld* diversity than just bioaugmenting or just planting. This held true when looking at LOC and HOC soil separately, though it was less significant in HOC soil (Figure 4-5). These results pertaining to chlorite dismutase infer that combining bioaugmentation with tree planting will result in greater diversity of perchlorate-reducing bacteria compared to only planting or only bioaugmenting.

The increase in *cld* diversity in bioaug./planted soil compared to just bioaugmented or just planted soil is an important finding. Diversity is a measure of population complexity. Plant roots provide more than electron donors to the subsurface. They also provide attachment surface and promote water movement. Plant roots can be expected to introduce more microbial niches and more niches leads to more habitat complexity. Bioaugmentation adds a large amount of bacteria to the soil. These bacteria were, in this case, already acclimated to the efficient transformation of perchlorate. The combination of a large number of bacteria, plus a large number of niches for those bacteria to occupy, leads to a more complex and diverse population. In soil that has been planted but not bioaugmented, the diversity may be low due to the diurnal variation in ORP. Unlike in the case of bioaug./planted, this potential stress is not countered by the sheer number of bacteria introduced during bioaugmentation. This indicates that plants may be both an energy source for, and a repressor of, microbial diversity during perchlorate bioremediation.

Bioaugmentation and/or rhizosphere conditions had no significant effect on bacterial (16S rRNA) diversity (Figures 4-4 and 4-5). However, HOC soil contained both a more diverse and richer 16S rRNA population compared to LOC soil (Figure 4-3). In contrast, even though soil organic carbon was a factor in general bacterial diversity, it did not affect the diversity or richness of the chlorite dismutase gene (Figures 4-3). Previous work in our lab has shown that bioaug./planted LOC soil can exhibit quite high perchlorate removal efficiency (20-50% in 2.5 days), at higher ORP than previously thought suitable (Chapters II and III). We have now observed a diverse population of *cld* in bioaug./planted reactors and this diversity did not depend upon soil organic carbon (Figure 4-5). Perhaps more interestingly, and contrary to some literature (Grayston *et al.* 1998; Mavingui *et al.* 1992), we observed that universal bacterial diversity was not significantly affected by rhizosphere conditions or bioaugmentation in HOC soil. A recommendation for *in situ* treatment of perchlorate in soil with low organic carbon is to both plant hybrid poplars and bioaugment the soil. The trees will stimulate perchlorate reduction and the bioaugmentation will ensure a diverse population of perchlorate degraders able to outcompete non-perchlorate-reducing microorganisms for available resources.

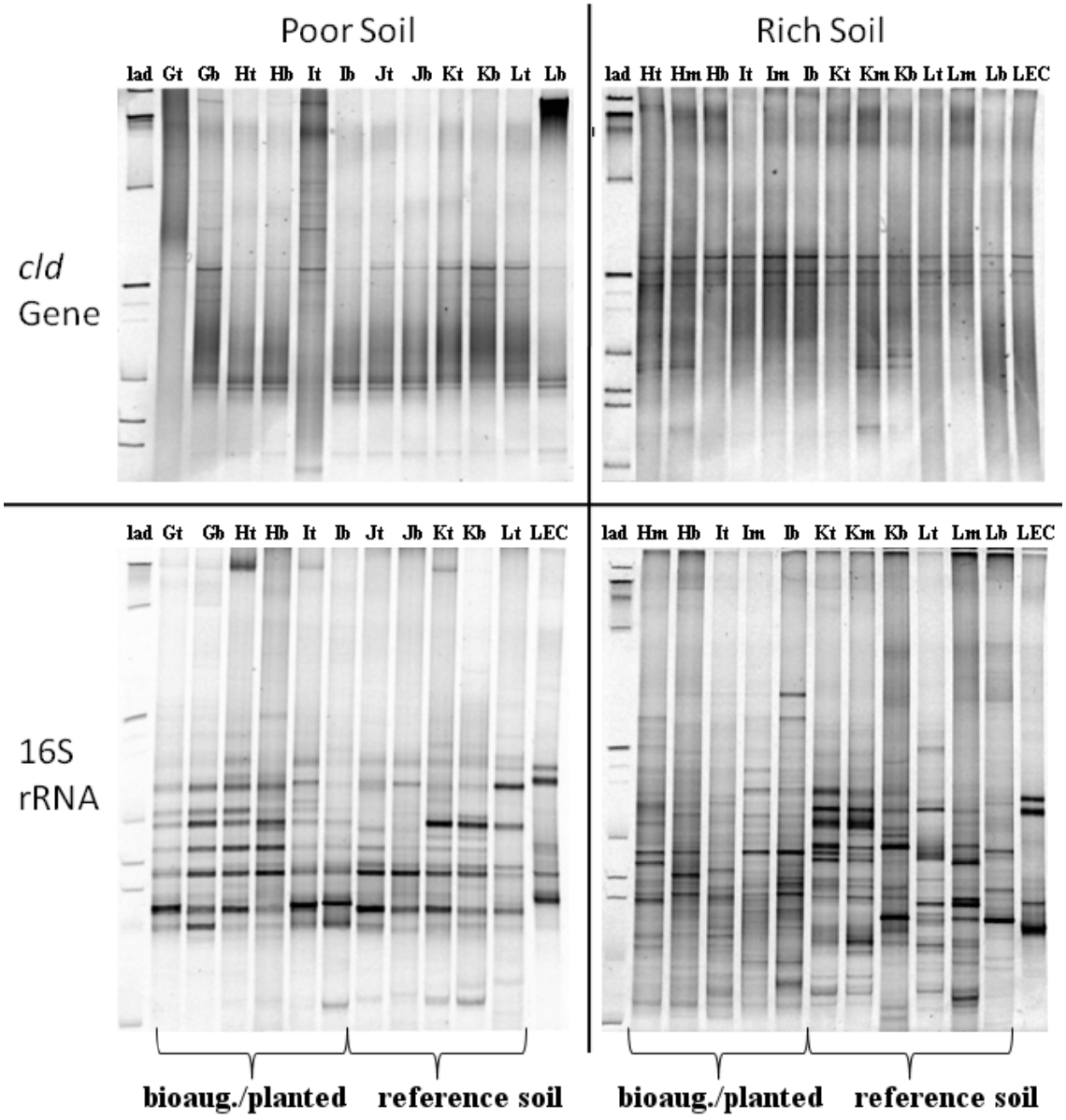


Figure 4-1. Representative DGGE gel images from bioaug./planted reactors (G-I), reference reactors (J-L), and the enrichment culture used to inoculate the bioaugmented reactors (LEC). Lane markers are as follows: lad is the size standard, uppercase letters designate the reactor ID while lowercase letters represent the location from with each sample was taken (t = top, m = middle, b = bottom). These images have been modified to remove extra size standard lanes for reporting purposes only.

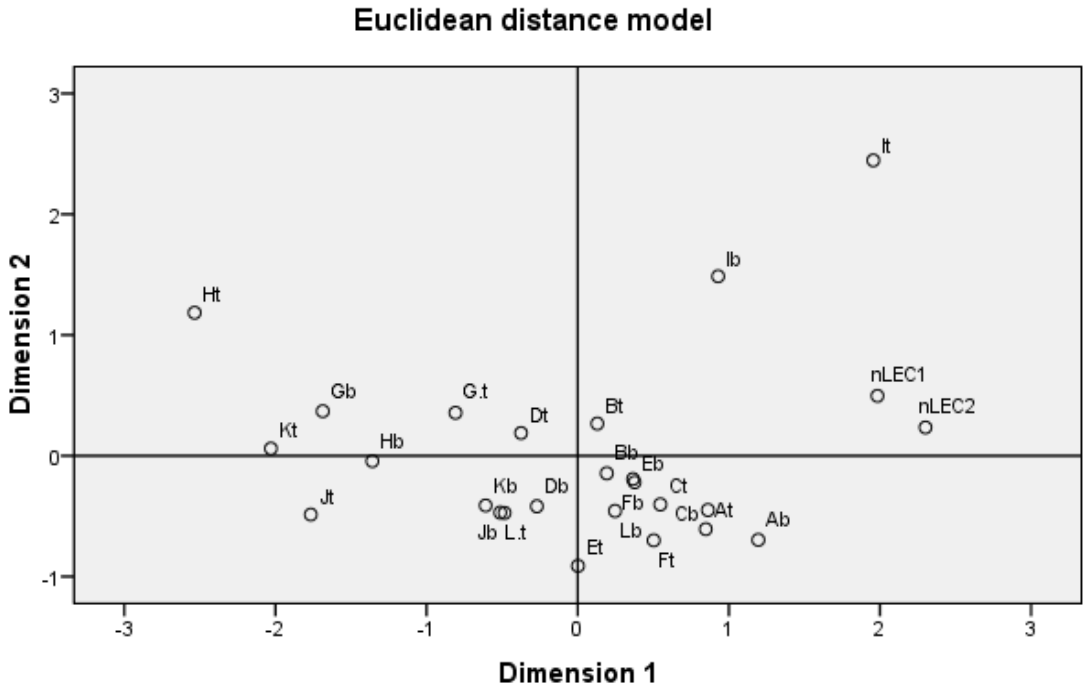


Figure 4-2. Representative non-dimensional scaling of Euclidian distances between LOC soil 16S samples based on DGGE band mobility. Symbol labels are the same as those of Figure 4-1 with the addition of bioaugmented (A-C) and planted (D-F). Note that while many of the reactor samples cluster together, the enrichment culture used to inoculate half of the reactors (nLEC) lies outside the group. This figure shows that 16S populations among reactors were not identical and not randomly distributed. A random distribution would show a clear elliptical pattern.

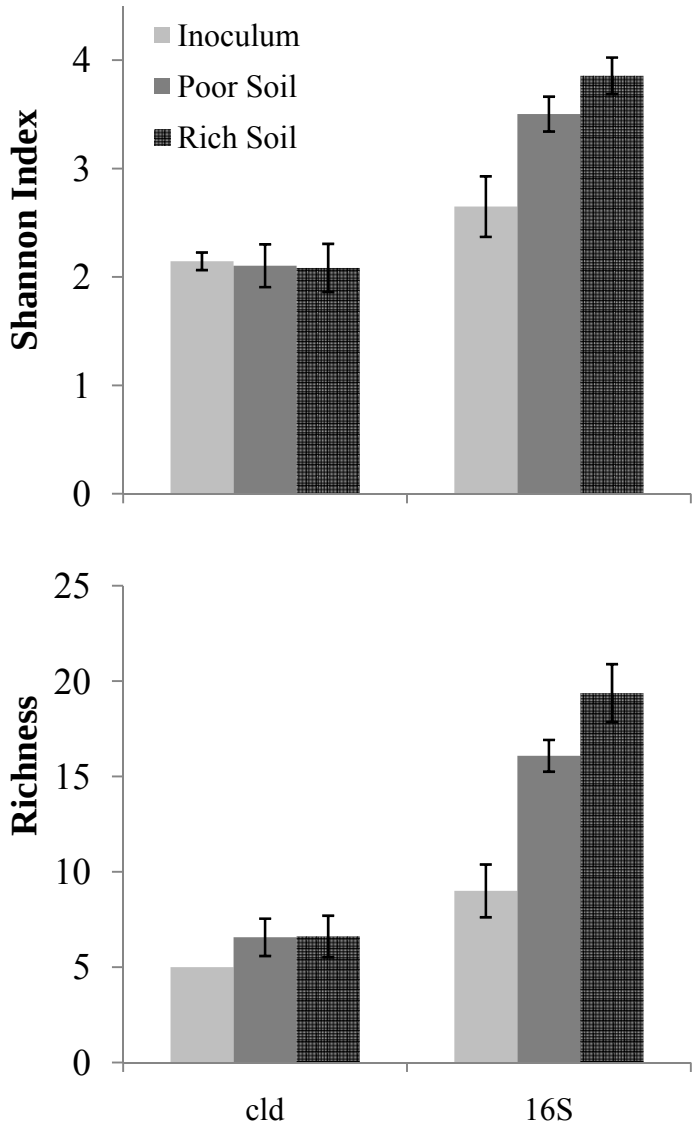


Figure 4-3. Diversity and richness of *cld* and 16S bands from samples of: inoculum, LOC soil, and HOC soil. Error bars represent the 95% confidence interval.

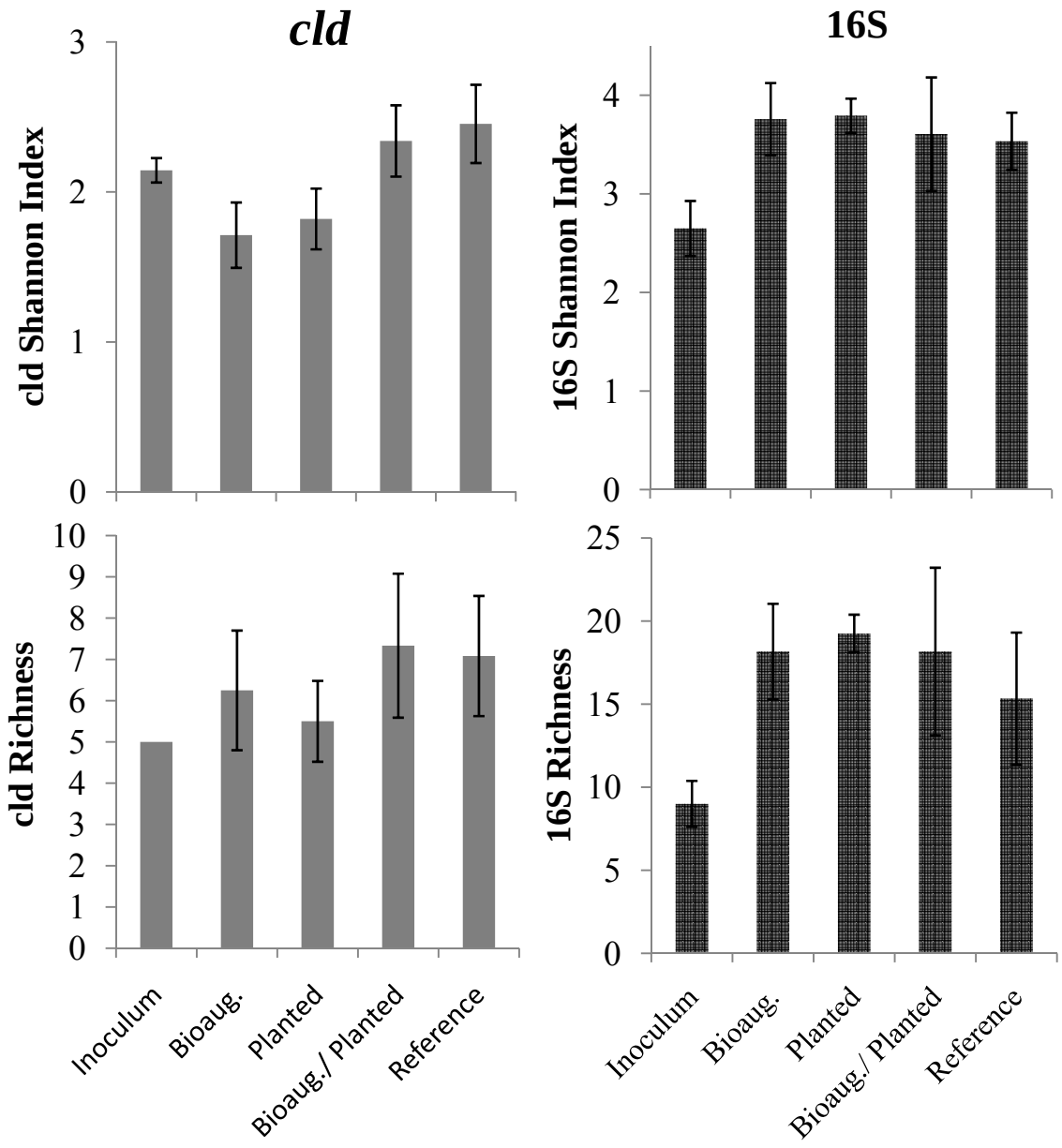


Figure 4-4. *cld* and 16S band diversity and richness separated by reactor conditions and the inoculum used in bioaugmented reactors. Diversity and richness from both LOC soil and HOC soil are combined. Error bars represent the 95% confidence level.

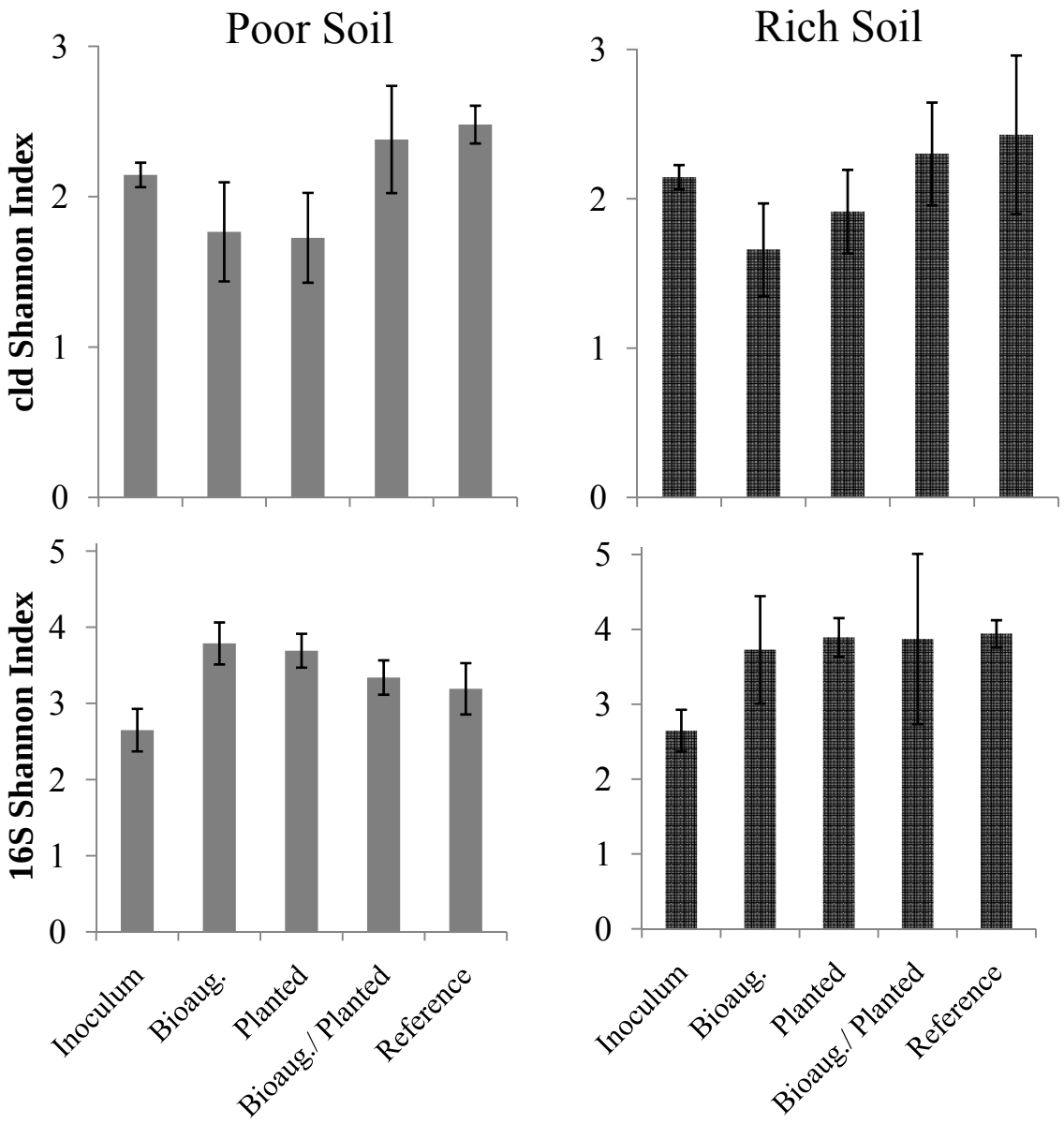


Figure 4-5. *cld* and 16S diversity in LOC and HOC soil separated by reactor setup. Error bars represent the 95% confidence interval.

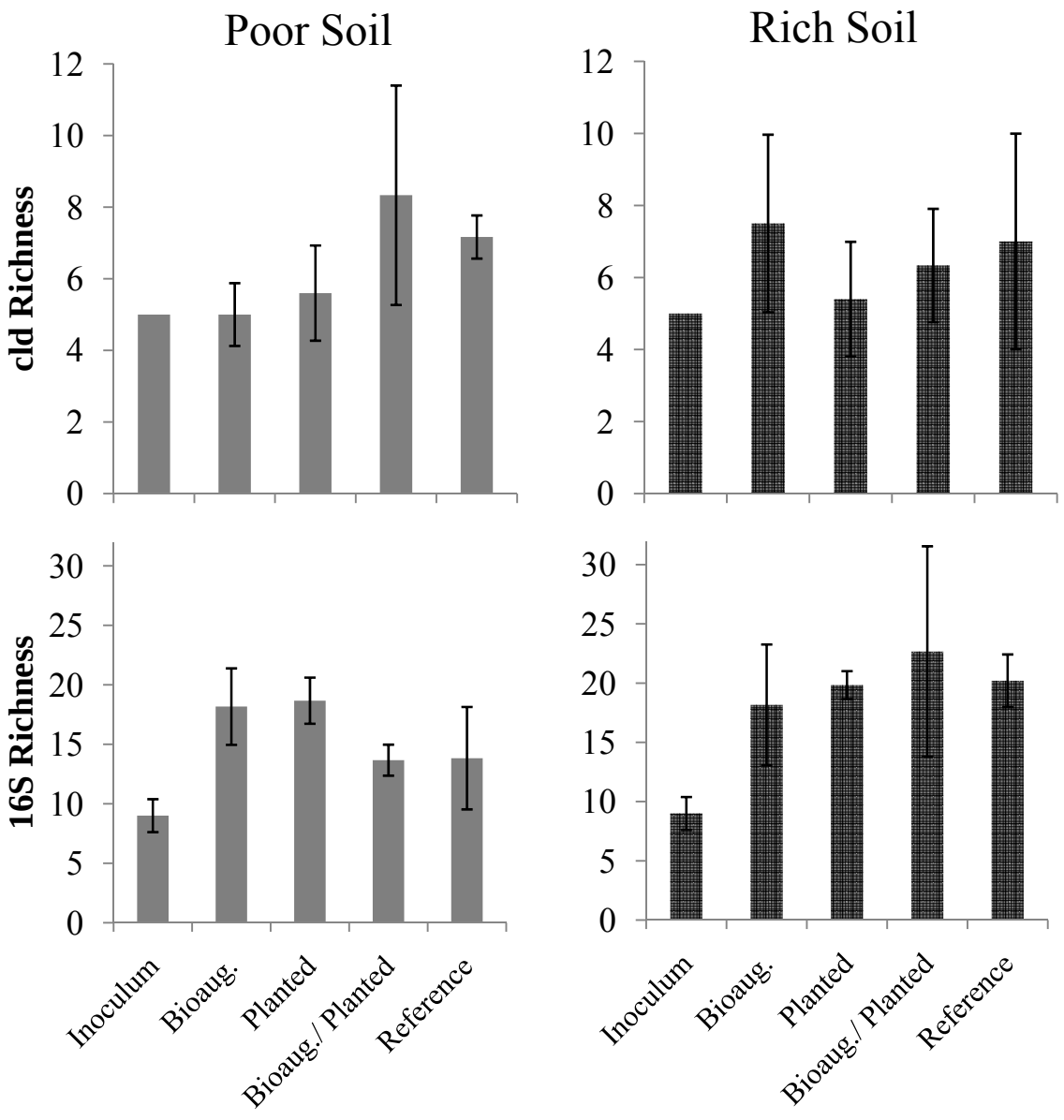


Figure 4-6. *cld* and 16S richness in HOC and LOC soil separated by reactor setup. Error bars represent the 95% confidence interval.

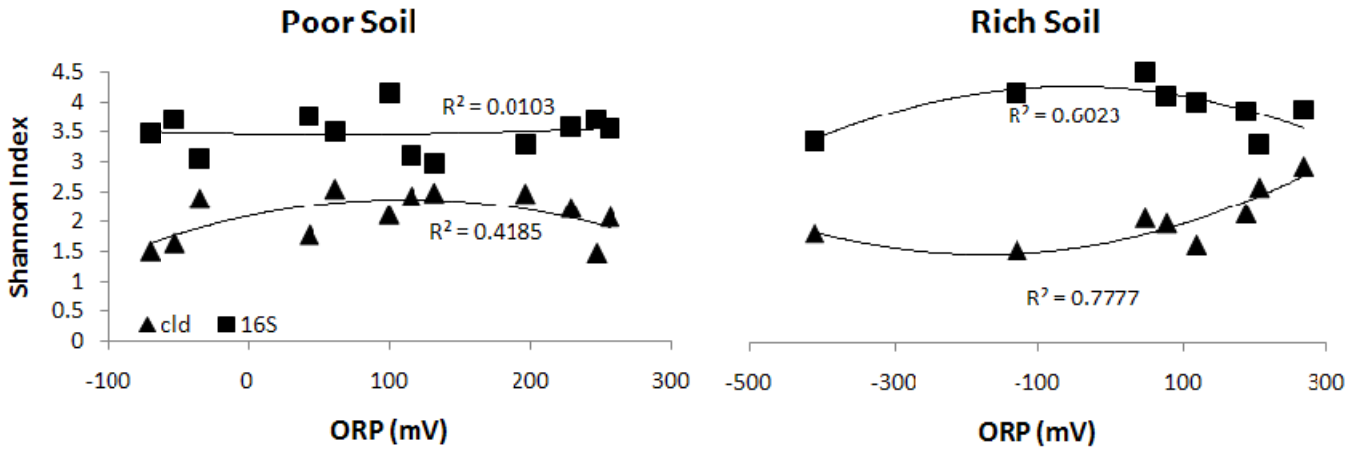


Figure 4-7. Plots comparing ORP and diversity of individual reactors separated into poor and rich soil. Poor soil diversity data points represent the average of two samples per reactor. Rich soil diversity data represents three samples per reactor. *cld* and 16S diversity appear to follow trends that can be described using ecological theory. An edge effect is observed for 16S diversity in rich soil and *cld* diversity in poor soil. The principal of competitive exclusion could explain the lower *cld* diversity observed at low ORP in rich soil.

CHAPTER V

CONCLUSION AND ENGINEERING SIGNIFICANCE

Results are summarized and conclusions are drawn below and separated into relevant hypotheses and objectives.

Hypothesis 1

While poplar trees will increase overall measured system ORP, they will also provide sufficient electron donors and carbon sources to ensure sustainable perchlorate reduction in soil with a low fraction of organic carbon.

Objectives:

1. Assess the role of redox conditions on perchlorate degradation in rhizosphere and non-rhizosphere soils and the effect that trees have on rhizosphere ORP.
2. Assess the effect of soil organic carbon on perchlorate phytoremediation.

Hybrid poplar trees were shown to induce an increase in measured ORP in soil and a diurnal variation with the lowest ORP measured just before the growth lights were turned on in the morning and a highest measured just prior to the lights turning off at night. As had been previously shown, measured ORP was an indicating factor to the bioremediation of perchlorate with removal occurring to a greater extent under reducing conditions. However, previous results were derived from hydroponic and culture studies, not from trees planted into actual soil. Hydraulic retention time was also shown to be a contributing factor to perchlorate remediation, in part due to a negative relationship between HRT and ORP. As HRT increased, ORP decreased and perchlorate removal increased.

Planting a hybrid poplar tree did not affect perchlorate removal in HOC soil. Instead, redox conditions and HRT were shown to have much more effect on overall perchlorate removal in HOC soil. However, in LOC soil the highest perchlorate removal

was observed in a planted reactor. Based on transpiration data, that tree was the most active of those planted into LOC soil. Interestingly, that reactor had the highest measured ORP as well, an observation that did not follow the general rule of low removal at higher ORP. The observation of high perchlorate removal in planted LOC soil was repeated in later work. A conclusion drawn from these experiments is that poplar trees influence the subsurface microbial conditions enough to overcome the inhibition of perchlorate reduction at the resulting high ORP in LOC soil. Furthermore, plant-induced perchlorate reduction may not have been observed in HOC soil as a result of competition from a dominant population of non-perchlorate-reducing bacteria. The general bacterial diversity was greater in HOC soil than LOC soil but *cld* diversity was not different. The competing population of non-perchlorate-reducers likely used more kinetically favorable electron acceptors than perchlorate to utilize electron donors and nutrients and thus inhibited the activity of perchlorate-reducing bacteria.

Hypothesis 2

In low organic carbon soil, poplar trees will play a greater role in perchlorate degradation. As soil organic carbon is increased, bacteria will begin to control the degradation of perchlorate.

Objectives:

1. Determine, as soil organic carbon and conditions of planting or bioaugmentation are changed, what fraction of the perchlorate reduction is due to bacteria and what fraction is due to plants (i.e., construct a mass balance).
2. Determine what fraction of the influent mass of perchlorate is taken up into the tree under variable conditions of bioaugmentation, planting, and soil organic carbon.

Radiolabeled perchlorate was synthesized from chloride using a combination of electrolytic and chemical oxidation with a molar yield of 90%. Using the radiolabeled perchlorate, a mass balance was constructed to delineate the relative contribution of bacteria and plants to perchlorate phytoremediation. Even in planted reactors, the microbial contribution to total perchlorate removal outweighed that of the plants. The lowest microbial contribution (60%) was observed in LOC soil that had been planted but not bioaugmented. Higher microbial contribution was observed both in HOC soil and in reactors that were bioaugmented. A bioaugmented tree in LOC soil was shown to accumulate half as much perchlorate as a nonbioaugmented tree. LOC soil reactors that had been both bioaugmented and planted also showed significantly higher perchlorate removal than reactors that had been only planted. Bioaugmentation serves to both increase the microbial contribution to perchlorate removal and reduce plant uptake of perchlorate. Therefore, the conclusion is that bioaugmentation greatly increases the benefits of planting trees in perchlorate-contaminated LOC soil.

Hypothesis 3

The presence of plants will decrease the diversity of perchlorate-reducing bacteria, as measured using the *cld* gene, at population equilibrium; but will not affect the diversity of universal bacterial 16S rRNA.

Objectives:

1. Using molecular methods, examine the effect of rhizosphere conditions on the diversity of perchlorate-reducing and general bacterial populations.
2. Examine the effect of bioaugmentation on the diversity of perchlorate-reducing and general bacterial populations.
3. Examine the effect of soil organic carbon on the diversity of perchlorate-reducing and general bacterial populations.

Reactors that had been only planted and reactors that had been only bioaugmented were shown to have significantly lower chlorite dismutase diversity than reference soil. However, both planting and bioaugmenting the same reactor led to no difference in *cld* diversity compared to reference soil. Soil organic carbon content did not affect the *cld* diversity, but higher diversity and richness of general bacteria were observed in HOC soil than in LOC. In contrast, rhizosphere conditions and bioaugmentation did not affect the diversity or richness of general bacteria. LOC soil yields a less complex population of general bacteria, but does not affect the population of perchlorate reducers compared to HOC soil. Only planting or only bioaugmenting both lead to a less diverse and less complex population of perchlorate reducers, but do not affect the general bacterial population. Not surprisingly, the inoculum used to bioaugment some of the reactors showed *cld* diversity and richness that were similar to that of the reactors. In contrast, though also not surprisingly, the inoculum 16S rRNA diversity and richness were significantly lower than that of the reactors.

Engineering Significance

The purpose of this research was to determine whether planting trees at a perchlorate-contaminated site will benefit or inhibit remediation. Previously, it was shown that trees could increase the ORP in hydroponic solution and that root products could stimulate microbial perchlorate reduction. Literature had also suggested that microbial reduction of perchlorate is inhibited under oxidizing conditions. What was not known was how this combination of factors would affect remediation in actual soil. The answer to that question is reasonably straightforward. In soil with a high fraction of organic carbon, planting a tree does not improve perchlorate removal compared to unplanted. Instead, the hydraulic retention time appears to be much more important and the potential for remediation can be inferred from the ORP. Planting a tree may actually decrease the potential for remediation in HOC soil due to the increased ORP observed in

planted systems. However, a mitigating factor is that trees provide hydraulic control of contaminants, so though the reaction rate may be decreased by higher ORP in HOC soil, the contact time is increased by transpiration uptake. Mature poplar plots can cause a cone of depression in groundwater that will limit the potential for perchlorate to leave the source zone.

Planting a tree in LOC soil yielded substantial perchlorate removal at higher ORP than previously thought possible. This removal was assisted by bioaugmentation. A study of the diversity of chlorite dismutase and 16S rRNA genes in the LOC soil reactors also indicated that *cld* diversity was increased when soil was both planted and bioaugmented. In addition, 16S diversity was decreased in LOC soil but *cld* diversity was unaffected, when compared to HOC soil. The salient point is that an equivalent complexity of perchlorate reducers existed in LOC soil and HOC soil, but that in LOC soil there was a less complex population of competitors. From the point of view that a remediation system works best when there is a diverse population of contaminant-degrading microorganisms with a low population of competing organisms, the best choice is to bioaugment and plant LOC soil without increasing the organic carbon fraction. Increasing the organic carbon is often accomplished with compost, and compost harbors a population of organisms that could compete with perchlorate reducers for resources.

Considering that perchlorate contamination is assumed to be predominantly found in LOC soil, these results are quite promising. It appears that the original goal of this research, to show that hybrid poplars could act as a source for electron donors during perchlorate bioremediation without inhibiting perchlorate reducers through increased ORP, has been fulfilled. In addition, this research provides further evidence to the usefulness of bioaugmenting phytoremediation sites with perchlorate-reducing bacteria. On the other hand, it does not appear useful to add an excess of compost or other

microorganism-rich organic carbon source to a phytoremediation site due to the potential for aerobic bacteria to outcompete perchlorate reducers.

Potential Future Research

Perchlorate is a model compound for phytoremediation of highly oxidized contaminants that are microbially reduced and often electron-donor limited. The chlorinated solvents perchloroethene (PCE) and trichloroethene (TCE) are examples of chemicals that meet those criteria and are of environmental relevance. A potential question could be, "Are observations of increased microbial contaminant removal at high ORP under the influence of hybrid poplar trees during phytoremediation of perchlorate in LOC soil also valid for reductive dechlorination of PCE and TCE?" Planted and unplanted glass reactors filled with sandy soil and continuously fed a moderate level of PCE could be used to answer this question. Reactor lids would need to be Teflon-lined and the lid opening caulked around the plant stem. A long vertical glass tube could be used to allow pressure equilibration in the reactor without the diffusive losses of a bare opening. All of the reactors would be bioaugmented with a culture of halorespirers following autoclaving of the sand. ORP would be monitored at the reactor effluent. A longer HRT may be required to observe significant PCE degradation. Sampling and data analysis would be similar to that described in the text other than the necessary caution to prevent loss of volatile constituents (glass syringes, Teflon tubing, etc).

Bioaugmented planted LOC soil reactors demonstrated higher microbial perchlorate reduction than nonbioaugmented reactors. How are the perchlorate-reducing bacteria associated with the roots? Are the microbes in the bulk soil, attached to the root surface, or inside of root cells? Future research could examine the location of perchlorate reducers in relation to the roots. Bioaugmented and nonbioaugmented poplar trees could be grown in an agar medium using the modified Hoagland's solution described previously. After growing, thin sections of the agar could be cut and microscopically

visualized to observe bacterial colonization in either the agar or on the root surface using fluorescence *in situ* hybridization or DAPI staining.

An observation was made of significant perchlorate removal in planted LOC soil. A potential future question could be, "Can the perchlorate – chloride and/or perchlorate - chlorate redox couples control the measured ORP in LOC soil during bioremediation?" LOC soil may lack redox buffers, thus potentially allowing efficient removal at high measured ORP because the high ORP is not actually indicative of the dominance of an oxygen redox couple but of perchlorate instead. This question could be answered by growing trees in LOC soil dosed with perchlorate as described earlier, identifying significant perchlorate removal at high measured ORP and then rigorously analyzing the effluent for the common redox couples at the measured ORP (e.g. nitrate/nitrite, perchlorate/chlorate, perchlorate/chloride, O_2/H_2O).

Trees with greater transpiration rates tended to show higher removal rates. An unfortunate side effect of that was the observation of replicate reactors that did not have similar perchlorate removal or ORP. A goal of future research could be to select for trees with similar transpiration rates prior to the beginning of an experiment. The focus could be on determining whether cutting characteristics can be used to determine the performance of the growing tree. For example, characteristics such as density (and water content by correlation), diameter, color, parent tree age, or time from harvest could be compared to the transpiration rates and new biomass of growing trees over time. Hybrid poplar trees are propagated though cuttings and are assumed to have nearly identical genetics, yet some trees grow more rapidly than others. Selecting cuttings that will grow at similar rates will allow more reproducibility in the science of phytoremediation.

On average, planted LOC soil showed perchlorate removal at higher ORP than expected. However, some individual reactors showed removal as high as 35% at high ORP while others did not exceed 5%. What caused this discrepancy? In chapter II, hydraulic retention time is shown to have a significant effect on perchlorate removal. It

is possible, however, that different microbial communities in the soil also contributed to the disparity. Indeed, it could be very interesting to see if a change in hydraulic retention time could lead to different microbial communities. In the reported work, diversity of 16S and *cld* was quantified at equilibrium. Monitoring the actual microbial community over time may allow an understanding of why planted reactors with very similar transpiration rate and ORP can show different perchlorate removal. One means of monitoring the actual microbial community is the use of emerging next-generation sequencing technologies such as 454 pyrosequencing. Pyrosequencing is currently $8,500+ per run, but each run yields approximately one million 400 base pair reads. Using primer bar-coding and 27 different bar codes, over 18,500 sequences could be identified from each sample of six reactors, sampled at three levels, at three time points. The DNA would be extracted from simple reactor soil cores in advance and only the samples from reactors that showed desirable ORP and perchlorate removal attributes would be pyrosequenced. Pyrosequencing allows diversity analysis but also provides sequence information so that the microorganisms that constitute the observed diversity profile can be identified based on their chlorite dismutase gene sequence or 16S rRNA sequence.

APPENDIX A

IODIDE DOES NOT INHIBIT MICROBIAL PERCHLORATE REDUCTION

Iodide was used as the tracer analyte because bromide coeluted with chlorate on the ion chromatograph. However, perchlorate inhibits iodide uptake into the thyroid, so a potential concern was that iodide may inhibit perchlorate reduction by bacteria. Fortunately, high concentrations of iodide were not shown to inhibit perchlorate reduction (Figure C-1). The study was setup using a 50% inoculum of LEC-PH in anaerobic culture bottles along with 0.2% (vol/vol) lactate, 70 mg/L perchlorate, and varying iodide (0 – 900 mg/L). The media was filter-sterilized prior to inoculation. Controls were also set up without inoculum. Bottles were vigorously stirred on a shaker table. Samples were taken at 5.5 hours and 20 hours. Perchlorate was completely degraded in nearly all of the cultures after 20 hours, so only the 5.5-hour time point is reported.

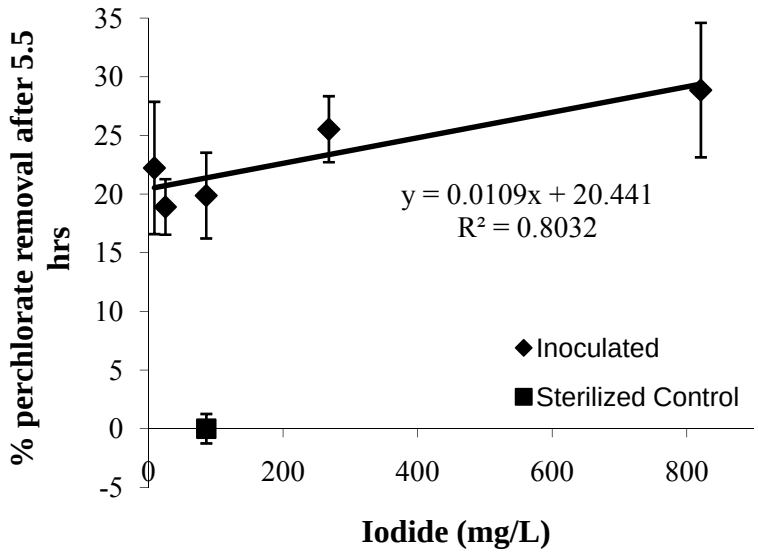


Figure A-1. Inhibition of perchlorate reduction by iodide was not observed. Error bars represent one standard deviation. Filter sterilized controls demonstrate the observed removal to be of microbial origin.

APPENDIX B

OXIDATION-REDUCTION POTENTIAL IN REACTOR EFFLUENT

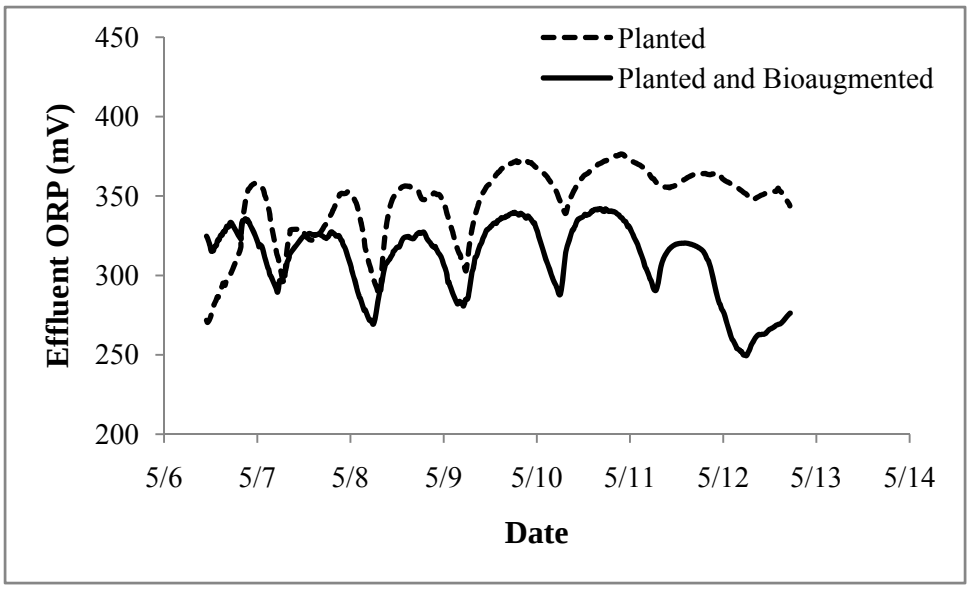


Figure B-1. Effluent ORP in LOC soil reactors over the course of six days during the radiolabel dosing described in Chapter III.

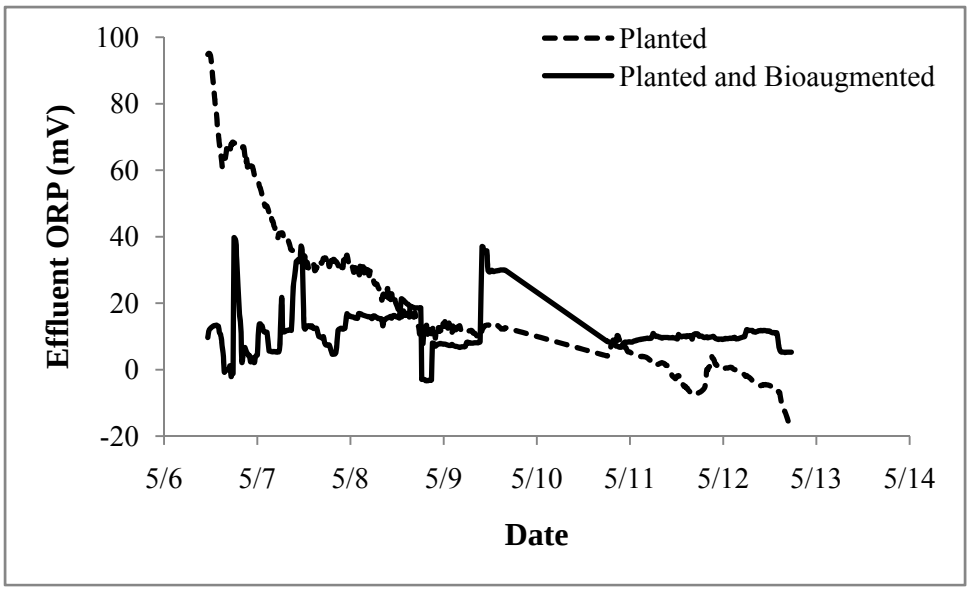


Figure B-2. Effluent ORP in HOC soil reactors over the course of six days during the radiolabel dosing described in Chapter III.

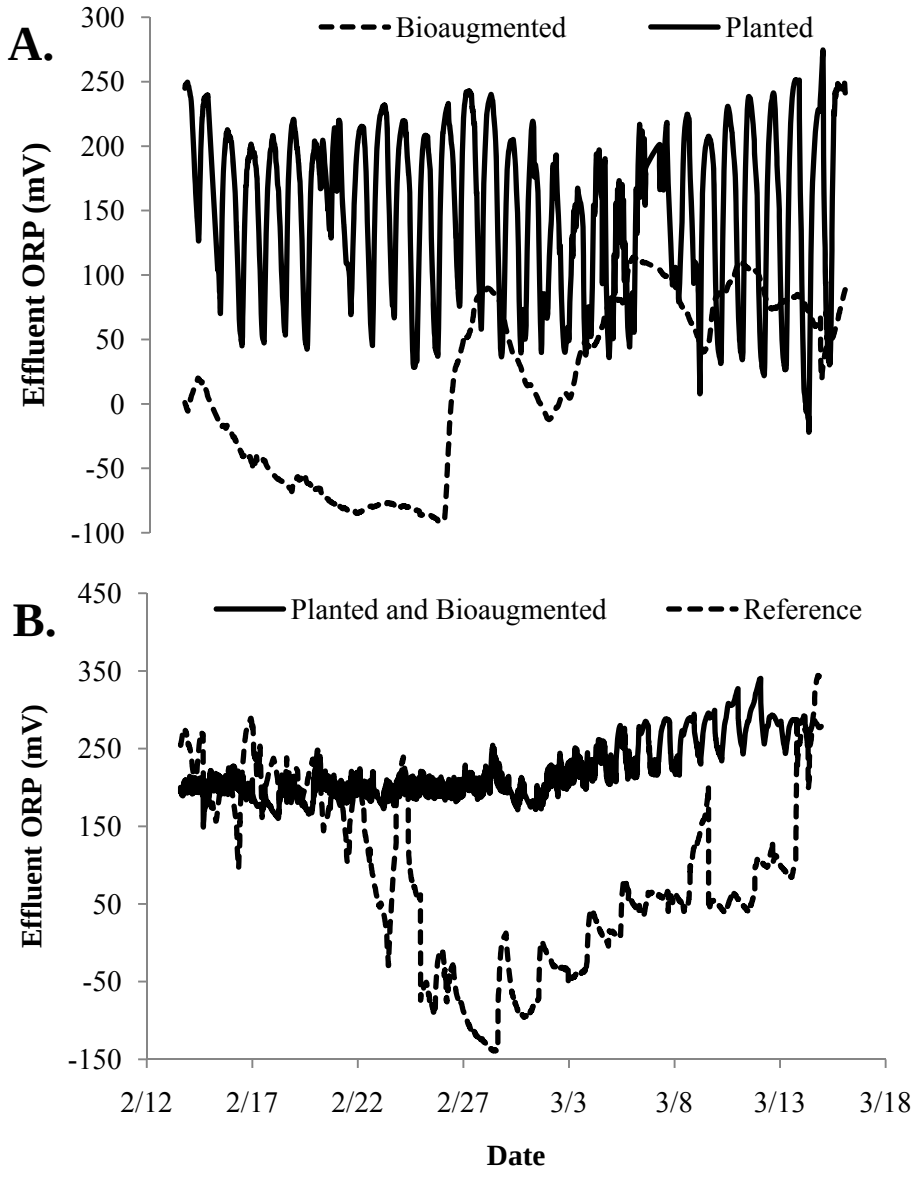


Figure B-3. Effluent ORP in LOC soil reactors over the course of a month in 2008. The diurnal variation in ORP can be observed in planted reactors.

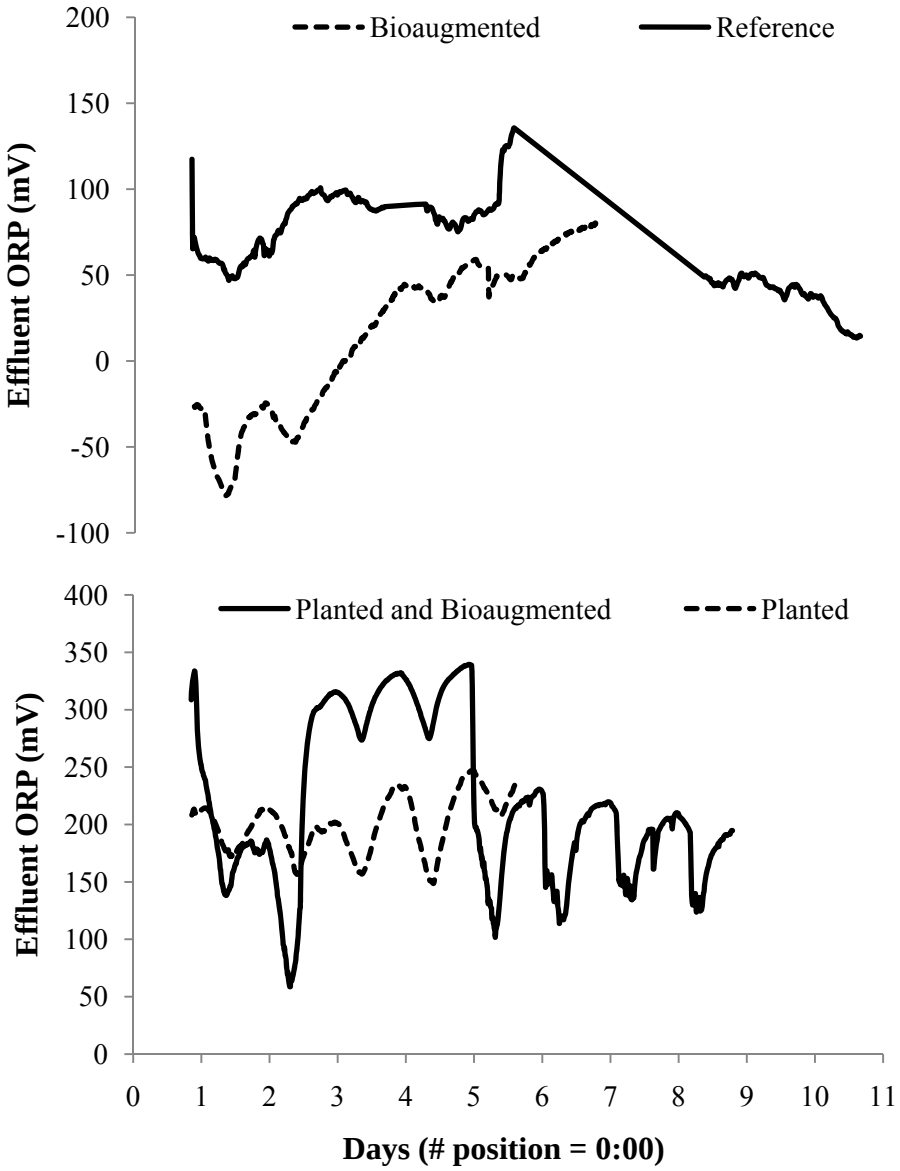


Figure B-4. Effluent ORP in HOC soil reactors. ORP data was not taken simultaneously, hence the normalization to "days" instead of date. Note that peaks and valleys of ORP in planted reactors match quite closely even though the Planted and Bioaugmented data were taken in December 2005 and the Planted data were taken in February 2006.

APPENDIX C

SUPPLEMENTAL DATA TO DIVERSITY STUDY

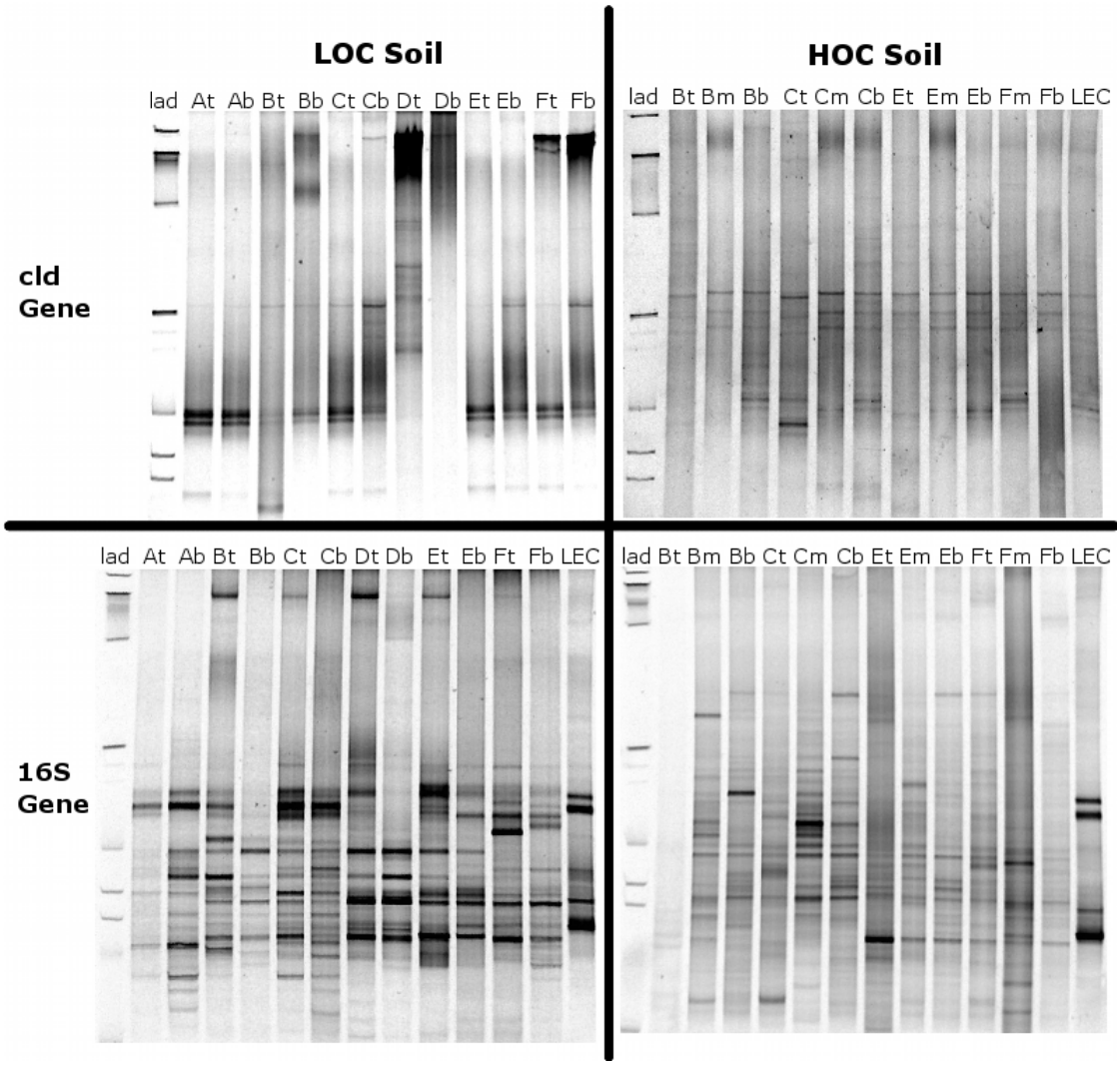


Figure C-1. Representative DGGE gel images from bioaugmented reactors (A-C), planted reactors (D-F), and the enrichment culture used to inoculate the bioaugmented reactors (LEC). Lane markers are as follows: lad is the size standard, uppercase letters designate the reactor ID while lowercase letters represent the location from with each sample was taken (t = top, m = middle, b = bottom). These images have been modified to remove extra size standard lanes for reporting purposes only.

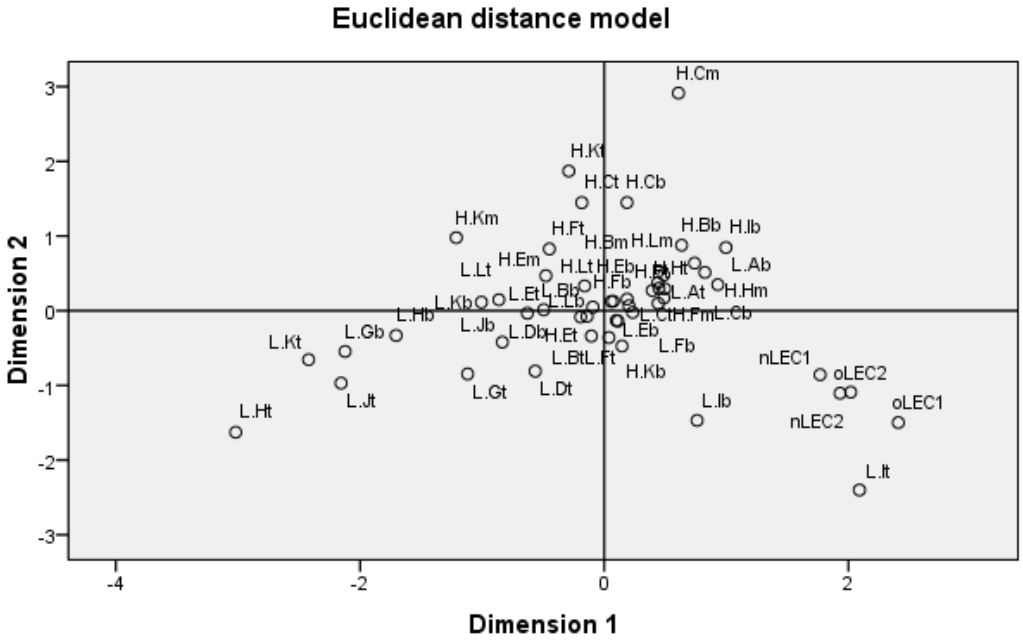


Figure C-2. Non-dimensional scaling of Euclidean distances from DGGE band analysis of bacterial 16S rRNA. HOC soil is delineated by a H.Xx (H=high organic carbon) and LOC soil by L.Xx (L=low organic carbon). The Xx designation is the same label as in Figures 4-1 and 4-2.

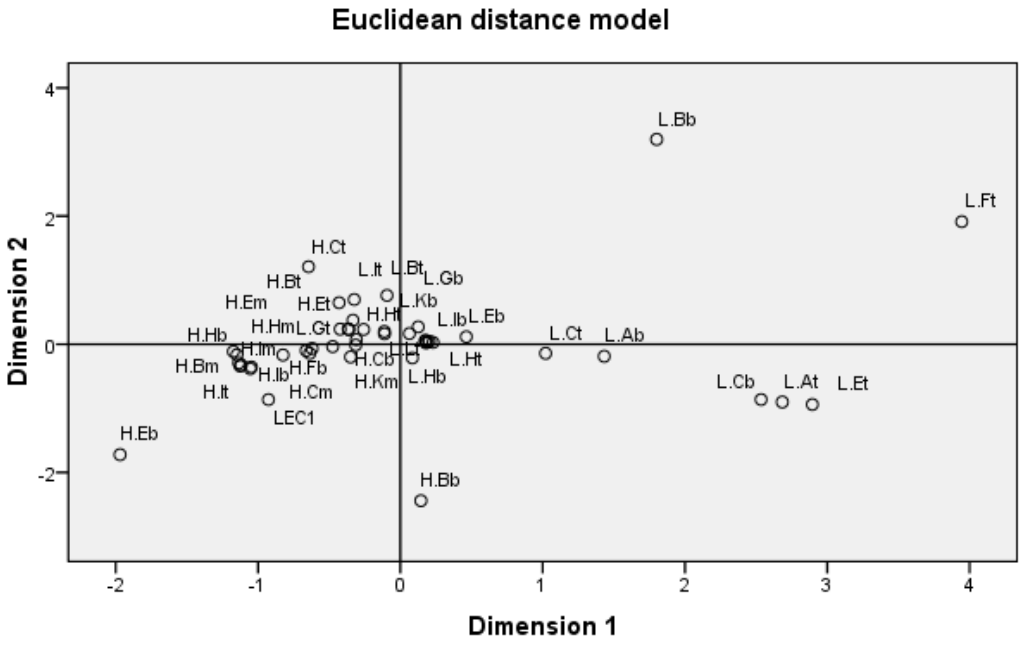


Figure C-3. Non-dimensional scaling of Euclidean distances from DGGE band analysis of chlorite dismutase. HOC soil is delineated by a H.Xx (H=high organic carbon) and LOC soil by L.Xx (L=low organic carbon). The Xx designation is the same label as in Figures 4-1 and 4-2.

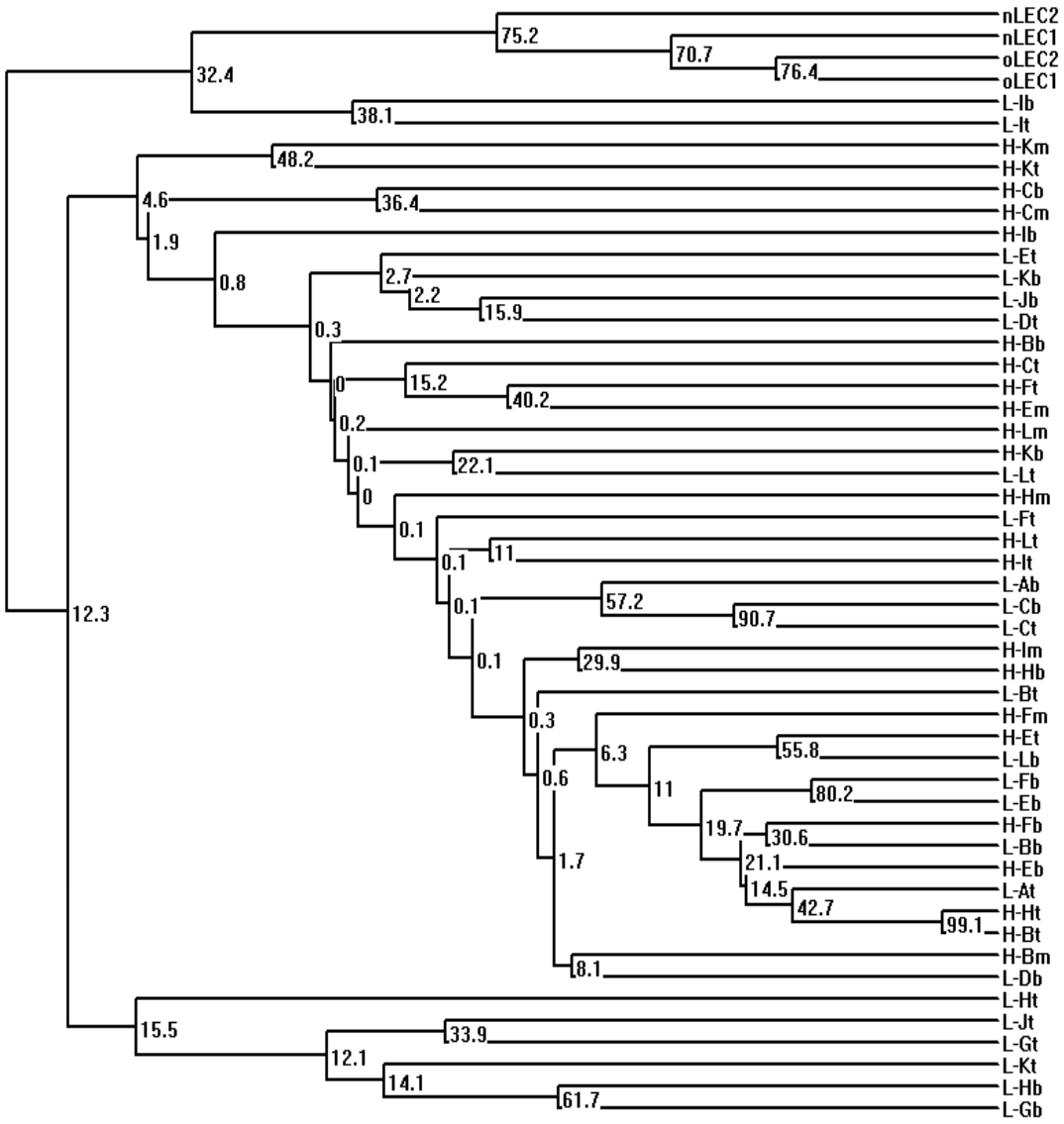


Figure C-4. Cluster analysis of 16S rRNA bands based on Euclidean distance. The symbols are the same as described in Figures 4-1 and 4-2.

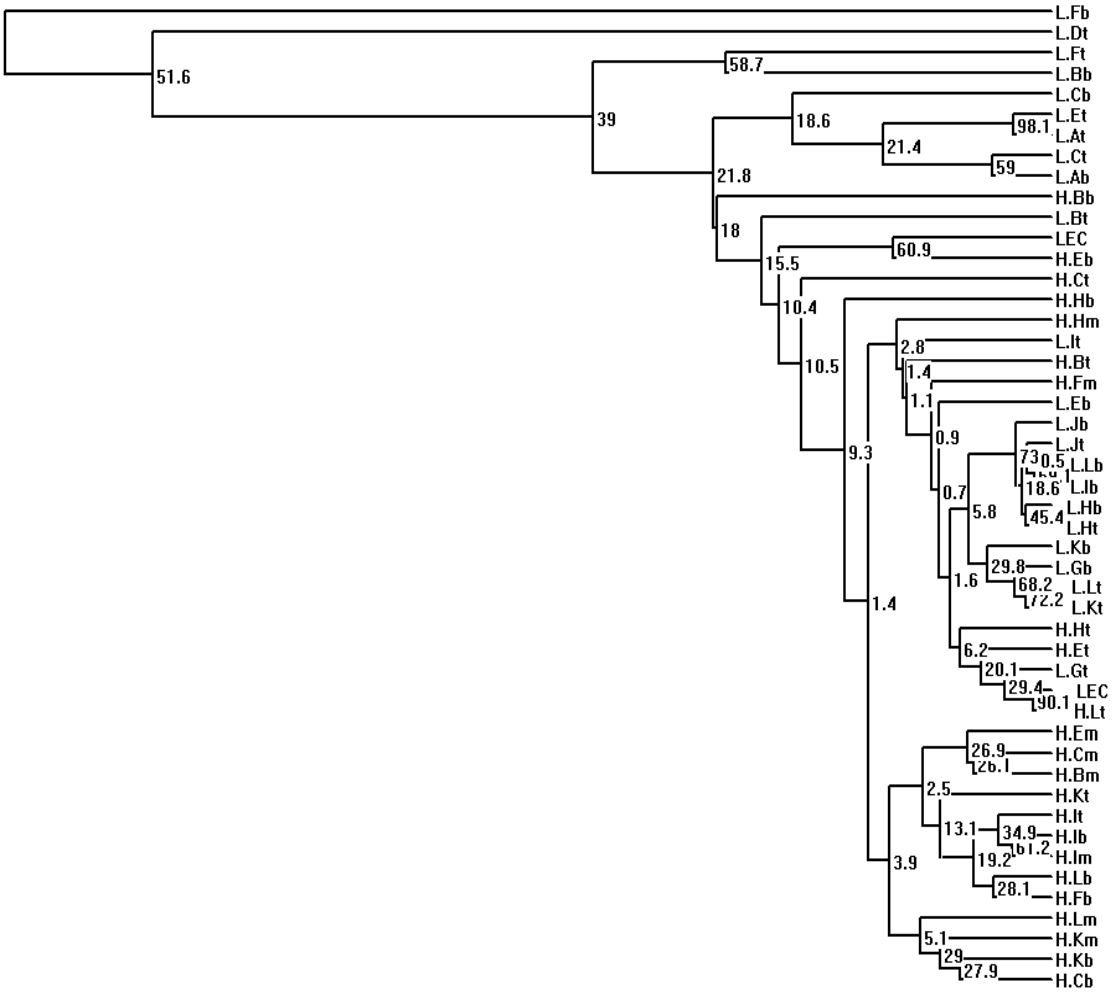


Figure C-5. Cluster analysis of *cld* bands based on Euclidean distance. The symbols are the same as described in Figures 4-1 and 4-2.

APPENDIX D

CHLORITE DISMUTASE AND 16S R-RNA SEQUENCES TAKEN FROM CLONES

The first six sequences were from clones of 16S rRNA amplicons. The results from a search on the Ribosomal Database Project are on the line above the sequence. The primer sequences are not shown here. All sequences are in order from base pair number 27 to 519 (as found in E. coli).

Alphaproteobacteria Sphingomonadaceae Porphyrobacter
5'-AACGAACGCTGGCGGCATGCCTAACACATGCAAGTCGAACGAGACCTTCG
GGTCTAGTGGCGCACGGGTGCGTAACGCGTGGGAACCTGCCTTTAGGTTCGG
AATAACTCCCCGAAAGGGGTGCTAATGCCGAATAATGTCTTCGGACCAAAGA
TTTATCGCCTTTAGATGGGCCCGCGTTGGATTAGCTAGTTGGTGGGGTAAAGG
CCTACCAAGGCGACGATCCATAGCTGGTCTTAGAGGATGATCAGCCACACTG
GGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTG
GACAATGGGCGAAAGCCTGATCCAGCAATGCCGCGTGAGTGATGAAGGCCTT
AGGGTTGTAAAGCTCTTTTACCAGGGATGATAATGACAGTACCTGGAGAATA
AGCTCCGGCTAACTCCGTGC-3'

Betaproteobacteria, Burkholderiales, Comamonadaceae, Alicycliphilus
5'-ATTGAACGCTGGCGGCATGCTTTACACATGCAAGTCGAACGGCAGCGGGG
GCTTCGGCCTGCCGGCGAGTGGCGAACGGGTGAGTAATACATCGGAACGTGC
CCGATCGTGGGGGATAACGGAGCGAAAGCTTTGCTAATACCGCATACGATCT
ACGGATGAAAGCGGGGGATCTTCGGACCTCGCGCGGACGGAGCGGCCGATG
GCAGATTAGGTAGTTGGTGGGATAAAAGCTTACCAAGCCGACGATCTGTAGC
TGGTCTGAGAGGATGATCAGCCACACTGGGACTGAGACACGGCCCAGACTCC
TACGGGAGGCAGCAGTGGGGAATTTTGGACAATGGGCGCAAGCCTGATCCAG
CCATGCCGCGTGCAGGATGAAGGCCTTCGGGTTGTAAACTGCTTTTGTACGG
AACGAAAAGGTGCCTTCTAATAAAGGGCGCTCATGACGGTACCGTAAGAATA
AGCACCGGCTAACTACGTGC-3'

Bacteroidetes, Sphingobacteria, Sphingobacteriales
5'-GATGAACGCTAGCGGCAGGCTTAATACATGCAAGTCGAGGGGTAACAGGT
CCTTCGGGATGCTGACGACCGGCAGACGGGTGAGTAACACGTATGCAATCTA
CCTCTAACCGGGGCACAACTTCGGGAAACTGGAGCTAATTCCCCTTAACATT
ATGGATCCGCATGGATCTATAATTAAAATTCCGATGGTTAGAGATGAGCGTG
CGTCCGATTAGATAGTTGGCGGGGTAACGGCCCACCAAGTCGACGATCGGTA
GCTGATGTGAGAGCATGATCAGCCACACGGGCACTGAGACACGGGCCCGACT
CCTACGGGAGGCAGCAGTAGGGAATATTGGGCAATGGAGGAAACTCTGACC
CAGCCATGCCGCGTGAAGGATGAAGGTCCTACGGATTGTAAACTTCTTTTATT

AGGGAAGAAACCCTCGCATTCATGTGAGGCTGACGGTACCTAAGGAATAAGC
ACCGGCTAACTCCGTGC-3’

unclassified deltaproteobacteria
5’-AACGAACGCTGGCGGCGTGCCTAACACATGCAAGTCGAACGAGAAAGGG
GCTTCGGCTCCGAGTAAAGTGGCGCACGGGTGAGTAACACGTGGGTAACCTG
CCTCTAAGATTGGGACAACTCGTCGAAAGATGAGCTAATACCGAATAAGACC
ACAGTTCCTGCGGGAACAGCGGTCAAAGGTAGCCTCTGCTTGCAAGCTATCA
CTTAGAGATGGGCCTGCGCACCATTAGCTAGTTGGTAGGGTAACGGCCTACC
AAGGCAAAGATGGTTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTG
AGACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCGCAATG
GGCGAAAGCCTGACGCAGCGACGCCGCGTGGAGGATGAAGGTCTTAGGATT
GTAAACTCCTGTCAGATGGAAAGAAAAGTTTGTGTTTAATAGACACAAAATA
TGACGGTACCATCAGAGGAAGCACCGGCTAACTTCGTGC-3’

unclassified bacteria
5’-GACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCTACGAGAACGAGGT
AGCAATATCTTTAGTAAAGTGGCGTACGGGTGCGTAACACGTAAGTAATCTA
CCTTTGGGTTCGGAATAACTTCGGGAAATCGGAGCTAATACCGGATAATGCA
GCGGCACCGCATGGTGATGTTGTTAAAGTTTGTGTAATGCCTAAAGATGAAC
TTGCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCTGCGATCA
GTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGAGGAATATTGGGCAATGCCCGAAAGGGTG
ACCCAGCAACGCCGCGTGGAGGATGAAGGTCGTAAGATTGTAAACTCCTGTT
AGAGGGGAAGAATAACTAAGTTTGGAGCTTAGTATGACTGTACCCTCAGAGA
AAGCCCCGGCTAACTACGTGC-3’

unclassified bacteria
5’-GACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCTACGAGAACGAGGT
AGCAATATCTTTAGTAAAGTGGCGTACGGGTGCGTAACACGTAAGTAATCTA
CCTTTGGGTTCGGAATAACTTCGGGAAATCGGAGCTAATACCGGATAATGCA
GCGGCACCGCATGGTGATGTTGTTAAAGTTTGTGTAATGCCTAAAGATGAAC
TTGCGTCTGATTAGCTAGTTGGTGAGGTAATGGCTCACCAAGGCTGCGATCA
GTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGGTCCAG
ACTCCTACGGGAGGCAGCAGTGAGGAATATTGGGCAATGCCCGAAAGGGTG
ACCCAGCAACGCCGCGTGGAGGATGAAGGTCGTAAGATTGTAAACTCCTGTT
AGAGGGGAAGAATAACTAAGTTTGGAGCTTAGTATGACTGTACCCTCAGAGA
AAGCCCCGGCTAACTACGTGC-3’

The following six sequences were obtained from *cld* clones. The homology to known *cld* sequences is specified on the line preceding each sequence. As before, the primer sequence is not shown but the sequence is reported as the first base after the forward primer to the last base before the reverse primer.

>*cld*-1
5'-TCTGGTTGATCTCTACCTAACACGTGGCCTAGAAACCAATTCCGACTTTTT
CTTCCGCATCAACGCCTACGACTTGGCAAAAGCCCAGACCTTCATGCGCGAA
TTCCGCTCGACAACGGTCGGGAAGAATGCCGATGTTTTTGAGACCCTTGTCGG
TGTAACAAAGCCCTTAAACTACATCAGCAAAGACAAGTCTCCAGGGCTGAAT
GCGGGACTTAGTTCGGCTACCTATAGCGGGCCGGCACCAAGATATGTGATCG
TAATCCCCGTCAAGAAAAACGCGGAATGGTGGAACATGTCGCCGGAAGAGC
GTTTGAAGGAGATGGAAGTCCACACGACACCTACCTTAGCCTATCTCGTGAA
CGTC-3'

>*cld*-2
5'-CAAGGCTGAAGCCTACCTGACACGGGGATTCGAGGCACAGAGCGACTTTT
TCCTGCGCATCCACTCTTACGACATGGCTGCCACCCAGGCATTTTTGGTTGAC
TTCCGTGCCTCACGCTTTGGTATGAATGCCGAGGTGACCGAGAATCTGGTCGG
CATGACGAAGGCATTGAACTACATCACAAAAGATAAATCGCCAAATCTCAAC
GCCGGTCTGACTGGCGCCACCTATAGCGACGCAACACCCCGTTACGCCTTCG
TGATACCCGTGAAGAAAAATGCGGACTGGTGGAATCTGACCGACGAGCAAC
GTCTTAAGGAAATGGAGACCCATACCCTGCCGACGCTGGCCAATCTGGTCAA
TGTT-3'

>*cld*-3
5'-TCTGGTTGATCTCTACCTGACACGTGGCCTGGAAACCAATTCTGACTTTTTC
TTCCGCATCAACGCCTACGACTTGGCAAAAGCCCAGACCTTCATGCGCGAAT
TCCGCTCGACTACAATCGGCAAGAATGCCGATGTTTTCGAGACCCTTGTCGGT
GTTACAAAGCCCTTGAACTACATCAGCAAAGACAAGTCTCCTGGGCTGAATG
CGGGGCTTAGTTCGGCTACCTATAGTGGGCCGGCTCCACGATATGTGATCGTG
ATTCCCGTCAAGAAAAACGCGGAATGGTGGAACATGTCGCCGGAAGAGCGTT
TGAAGGAGATGGAAGTTCACACGACACCTACCTTAGCCTATCTCGTGAACGT
C-3'

>*cld*-4
5'-CAAGGCTGAAGCCTACCTGACACGGGGATTCGAGGCACAGAGCGACTTTT
TCCTGCGAATCCACTCTTACGACATGGCTGCCACCCAGGCATTTTTGGTTGAC
TTCCGTGCCACACGCTTTGGTATGAGTGCCGAGGTGACCGAGAACCTGGTCG
GCATGACGAAGGCCTTGAACTACATCACAAAAGATAAATCGCCAAATCTCAA
CGCCGGTCTGACTGGCGCCACCTATAGCGACGCAACACCCCGTTGCGCCTTC
GTGATACCCGTGAAGAAAAATGCGGACTGGTGGAATCTGACCGACGAGCAA

CGTCTCAAGGAAATGGAGACCCATACCCTGCCGACGCTGGCCAATCTGGTCA
ATGTA-3’

>*cld*-5
5’-TCTGGTTGATCTCTACCTGACACGTGGCCTGGAAACCAATTCTGACTTTTTC
TTCCGCATCAACGCCTACGACTTGGCAAAAGCCCAGACCTTCATGCGCGAAT
TCCGCTCGACTACTATCGGCAAGAATGCCGATGTTTTCGAGACCCTTGTCGGT
GTTACAAAGCCCTTGAACTACATCAGCAAAGACAAGTCTCCTGGGCTGAATG
CGGGGCTTAGTTCGGCTACCTATAGTGGGCCGGCTCCACGATATGTGATCGTA
ATTCCCGTCAAGAAAAACGCGGAATGGTGGAACATGTCGCCGGAAGAGCGTT
TGAAGGAGATGGAAGTTCACACGACACCTACCTTAGCCTATCTCGTGAACGT
C-3’

>*cld*-6
5’-TAAGGCTGAAGTTTACCTGATACGGGGATTCGAGGCACAGAGCGACTTTT
TCCTGCGAATCCATTCTTACGACATGGCTGCCACCCAAGCATTTTTGATTAAC
TTCCATGCCACACGCTTTGGTATGAATGCCGAAGTGACCGAGAACCTGGTCA
GCATAACAAAAGCCTTGAACTACATCACAAAAGATAAATCGCCAAATCTCAA
CGCCGGTCTGACTGGCGCCACCTATAGCGACGCAACACCCCATTACGCCTTC
GTGATACCCGTGAAGAAAAATGCGGACTGGTGAAATCTGACCGACGAGCAA
CGTCTCAAGGAAATAAAAACCCATACCCTGCCGACGCTGGCCAATCTGGTCA
ATGTA-3’

APPENDIX E

SIGNIFICANCE TABLES FOR 16S R-RNA AND CHLORITE DISMUTASE DIVERSITY

Table E-1. Two-tailed Student's T tests of *cld* and 16S diversity

		***cld* Diversity**				**16S Diversity**				
		Bioaug.	Planted	Bioaug. /Plant	Reference	Bioaug.	Planted	Bioaug. /Plant	Reference	
LOC & HOC	Bioaugmented	1.0				1.0				Bioaugmented
	Planted	0.48	1.0			0.87	1.0			Planted
	Bioaug./Plant	0.0009	0.0039	1.0		0.67	0.55	1.0		Bioaug./Plant
	Reference	0.0003	0.0013	0.53	1.0	0.37	0.16	0.83	1.0	Reference
HOC Soil	Bioaugmented	1.0				1.0				Bioaugmented
	Planted	0.26	1.0			0.68	1.0			Planted
	Bioaug./Plant	0.022	0.12	1.0		0.84	0.97	1.0		Bioaug./Plant
	Reference	0.040	0.13	0.70	1.0	0.59	0.78	0.91	1.0	Reference
LOC Soil	Bioaugmented	1.0				1.0				Bioaugmented
	Planted	0.87	1.0			0.61	1.0			Planted
	Bioaug./Plant	0.033	0.022	1.0		0.034	0.054	1.0		Bioaug./Plant
	Reference	0.0064	0.0050	0.63	1.0	0.024	0.039	0.49	1.0	Reference

Indicates a significant difference at the 95% confidence level.
Identifies a relationship that would be significant in a one-tail test and therefore indicate greater than or less than significance.

Table E-2. Two-tailed Student's T tests of *cld* and 16S richness

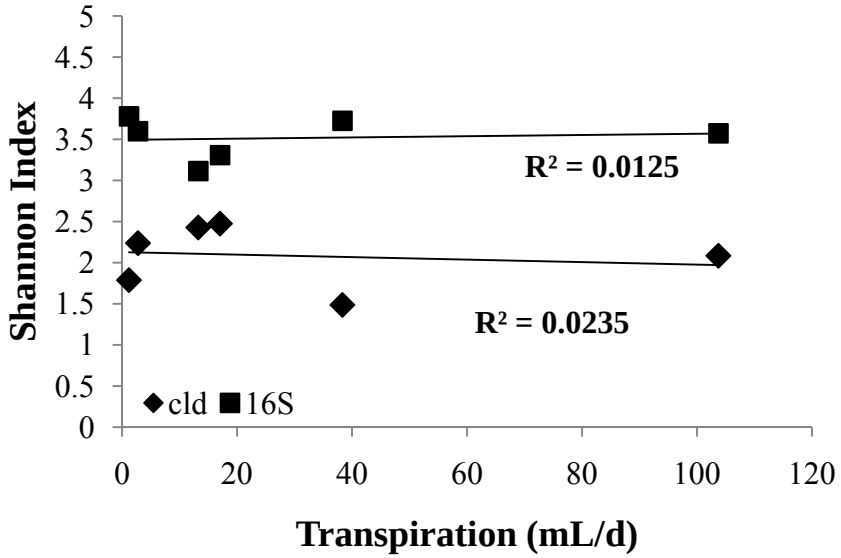
		***cld* Richness**				**16S Richness**				
		Bioaug.	Planted	Bioaug. /Plant	Reference	Bioaug.	Planted	Bioaug. /Plant	Reference	
LOC & HOC	Bioaugmented	1.0				1.0				Bioaugmented
	Planted	0.41	1.0			0.41	1.0			Planted
	Bioaug./Plant	0.36	0.090	1.0		0.82	0.80	1.0		Bioaug./Plant
	Reference	0.44	0.094	0.83	1.0	0.41	0.073	0.42	1.0	Reference
HOC Soil	Bioaugmented	1.0				1.0				Bioaugmented
	Planted	0.20	1.0			0.37	1.0			Planted
	Bioaug./Plant	0.46	0.44	1.0		0.52	0.83	1.0		Bioaug./Plant
	Reference	0.81	0.38	0.71	1.0	0.39	1.0	0.84	1.0	Reference
LOC Soil	Bioaugmented	1.0				1.0				Bioaugmented
	Planted	0.48	1.0			0.94	1.0			Planted
	Bioaug./Plant	0.088	0.15	1.0		0.51	0.43	1.0		Bioaug./Plant
	Reference	0.0032	0.083	0.49	1.0	0.012	0.0010	0.15	1.0	Reference

■ Indicates a significant difference at the 95% confidence level.
□ Identifies a relationship that would be significant in a one-tail test and therefore indicates greater than or less than significance.

APPENDIX F

COMPARISON OF BIODIVERSITY TO REACTOR PERFORMANCE

AND PHYSICAL PARAMETERS



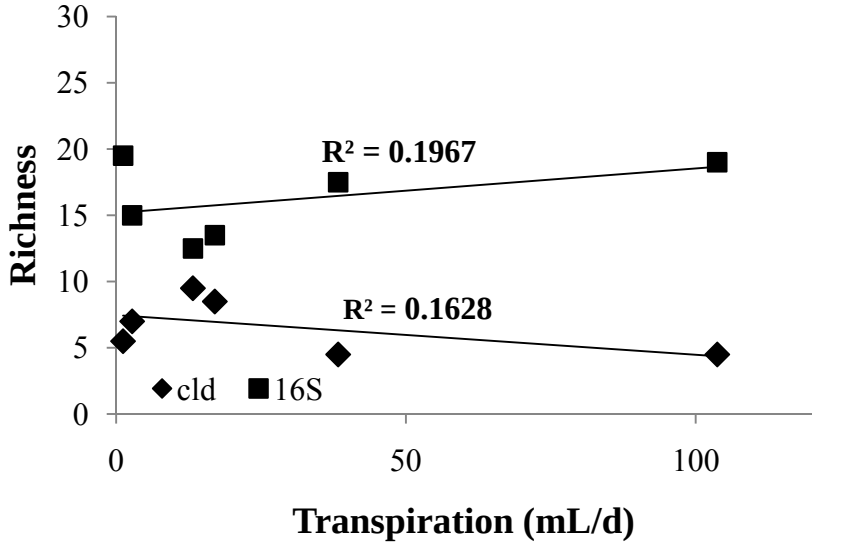


Figure F-1. Diversity (Shannon Index) and richness of *cld* and 16S as a function of transpiration in LOC soil. Transpiration does not appear to influence LOC soil biodiversity.

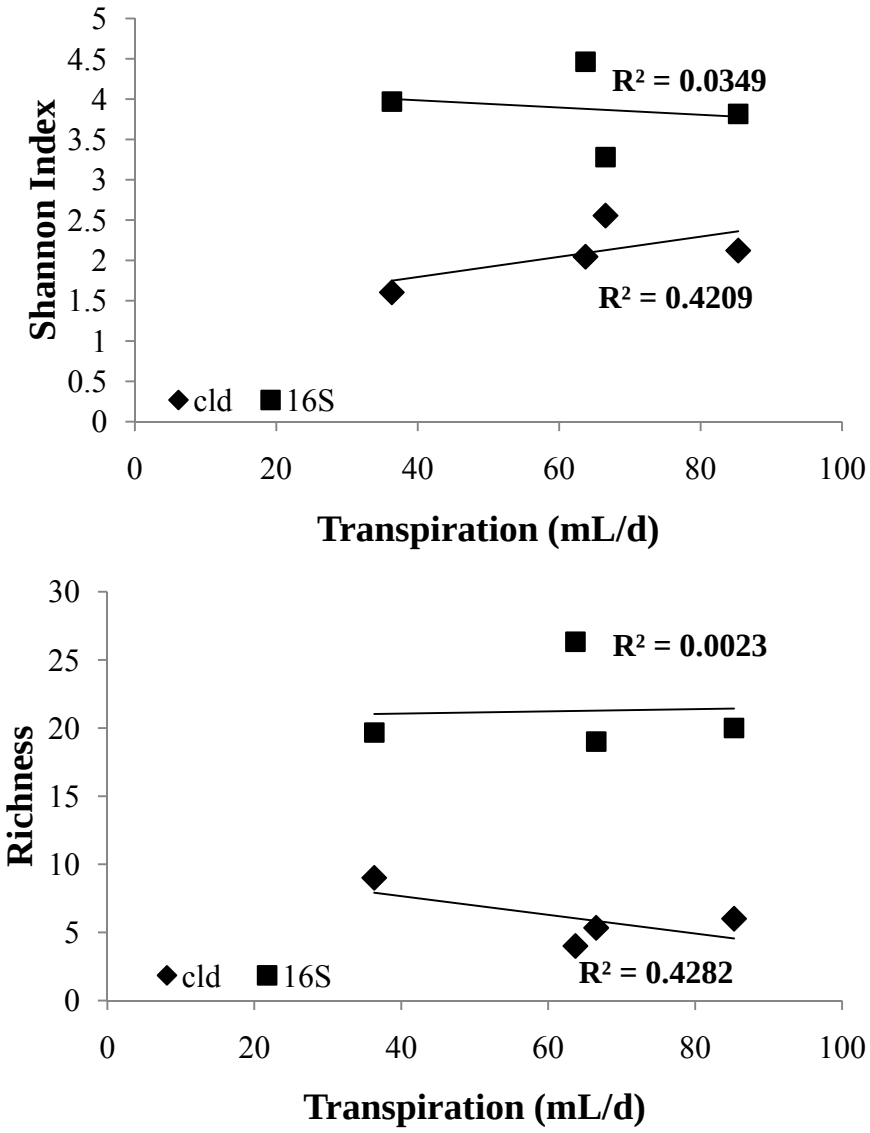


Figure F-2. Diversity (Shannon Index) and richness of *cld* and 16S as a function of transpiration in HOC soil. Transpiration does not appear to influence HOC soil biodiversity.

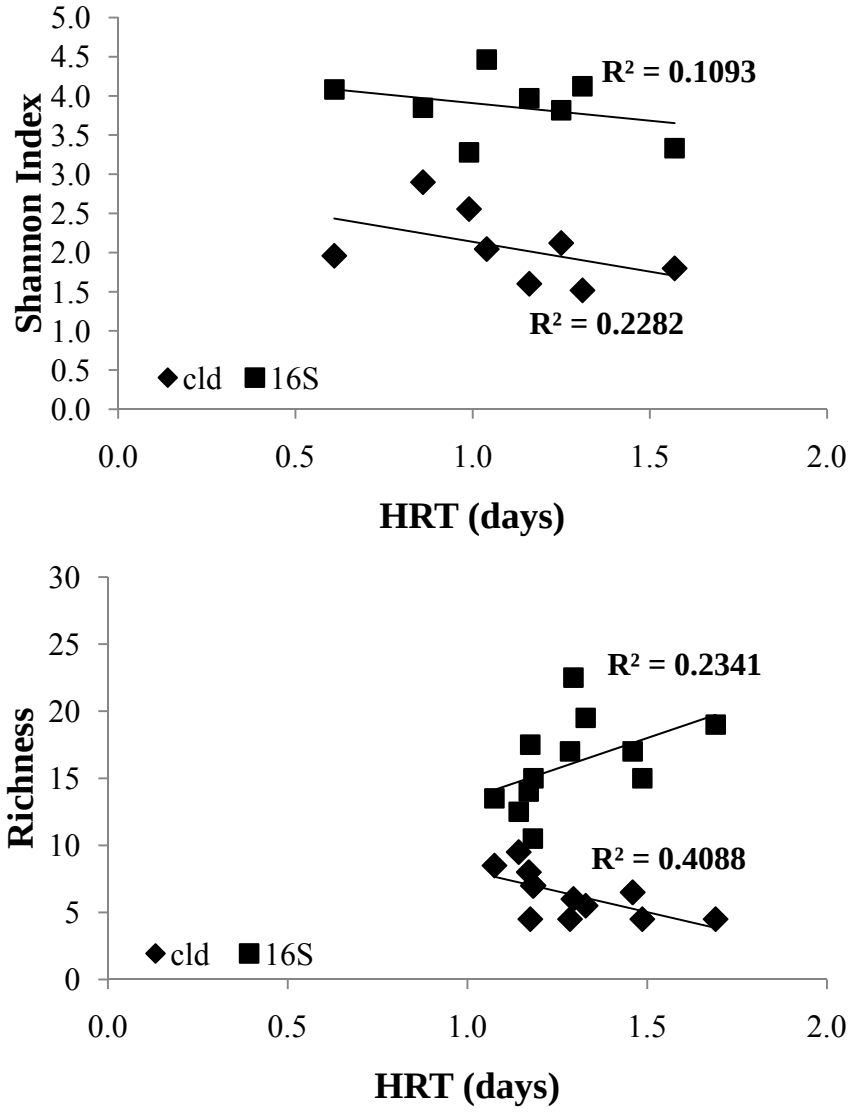


Figure F-3. Diversity (Shannon Index) and richness of *cld* and 16S as a function of hydraulic retention time in LOC soil. HRT does not appear to influence LOC soil biodiversity.

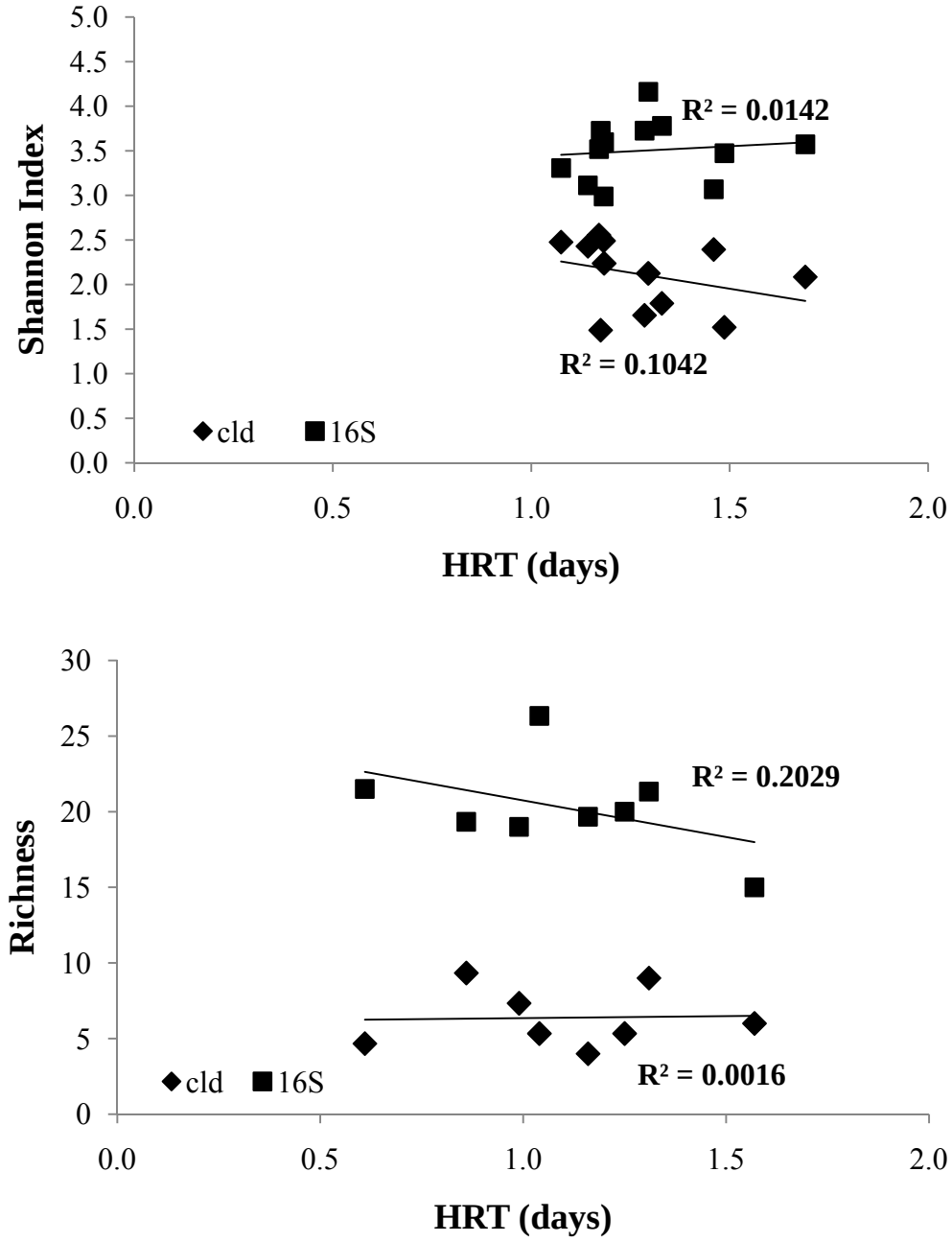


Figure F-4. Diversity (Shannon Index) and richness of *cld* and 16S as a function of hydraulic retention time in HOC soil. HRT does not appear to influence HOC soil biodiversity.

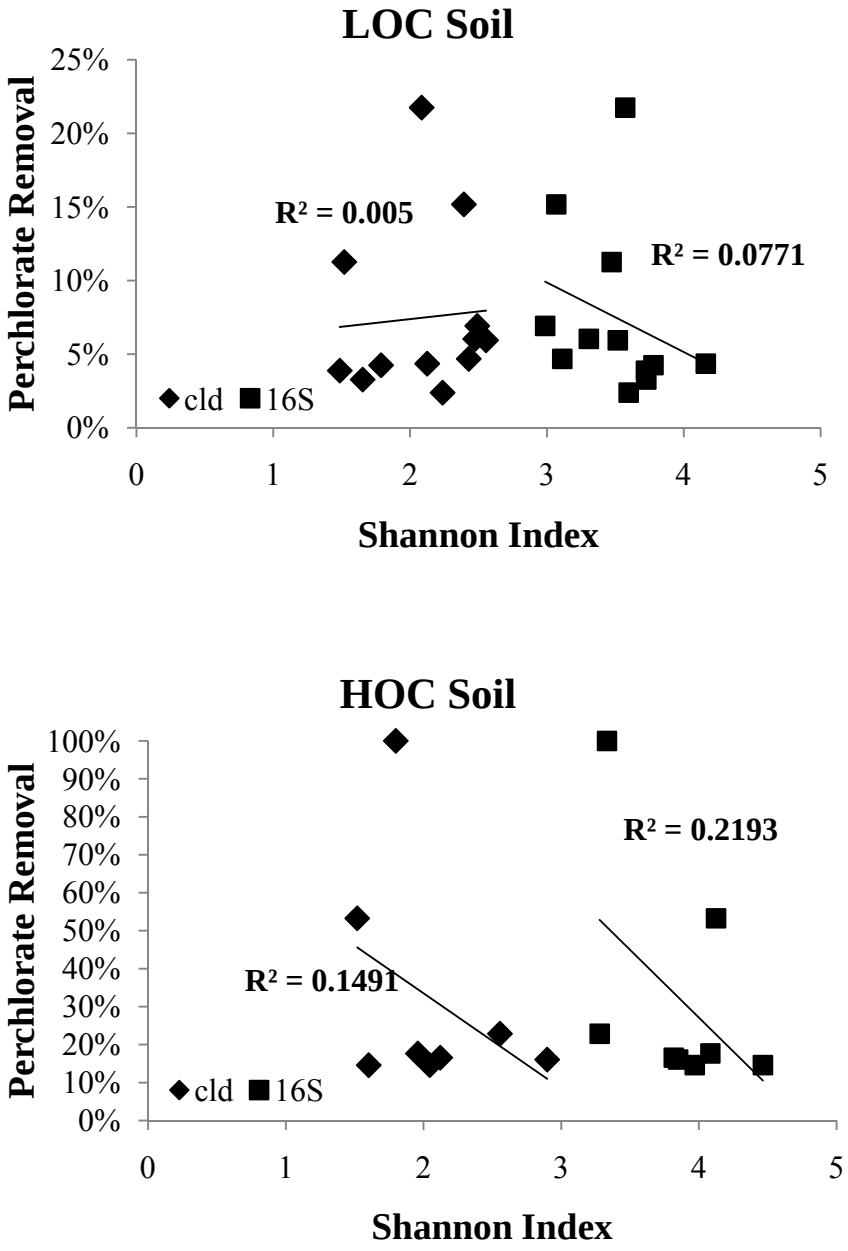
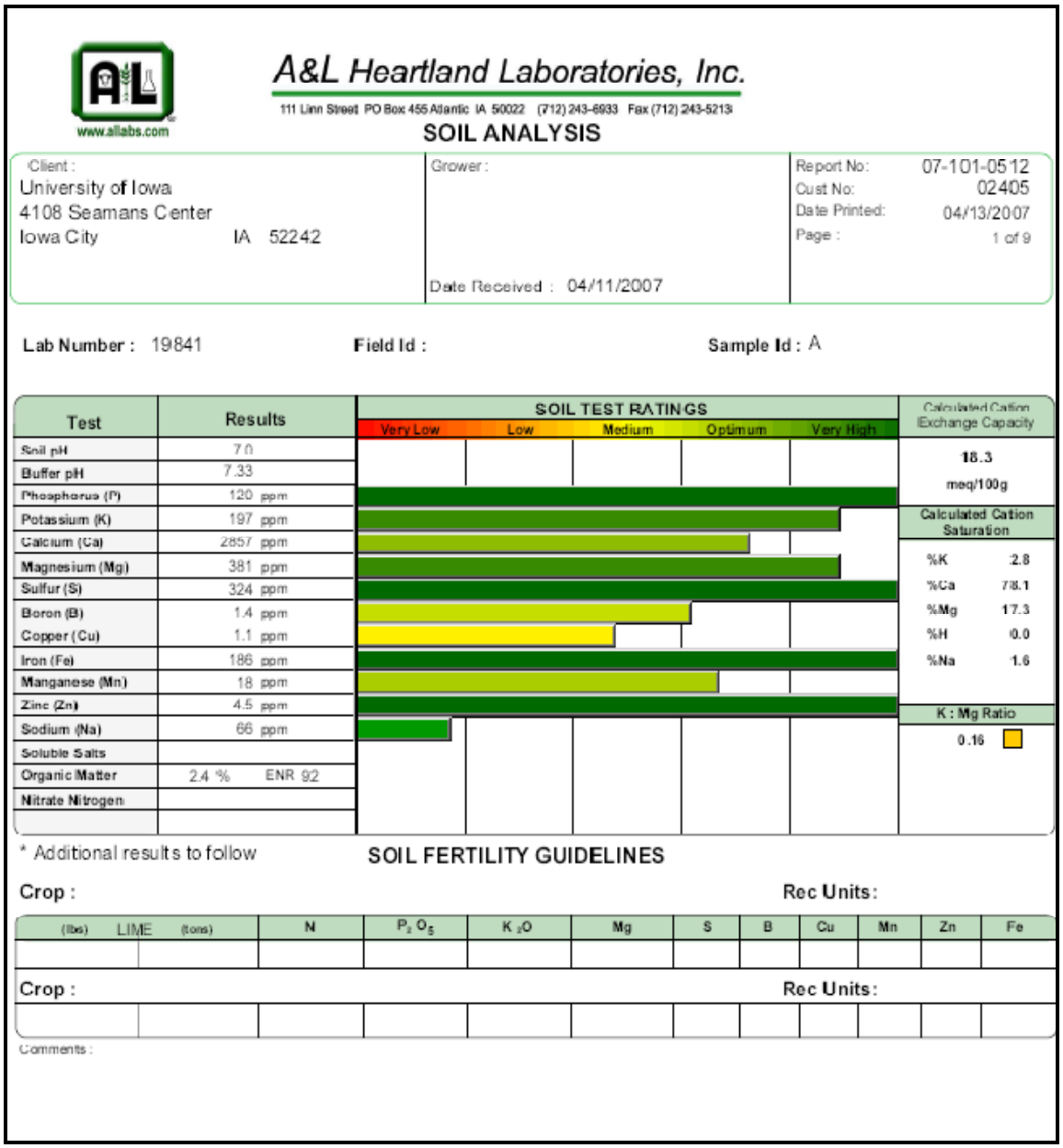


Figure F-5. Perchlorate removal as a function of diversity (Shannon Index) for both cld and 16S in LOC soil and HOC soil. Diversity does not appear to affect perchlorate removal.

APPENDIX G

SOIL ANALYSIS REPORTS

Table G-1. Two parts topsoil to one part sand that was added to HOC reactors prior to experiments



A&L Heartland Laboratories, Inc.

111 Linn Street PO Box 455 Atlantic IA 50022 (712) 243-6933 Fax (712) 243-5213

www.allabs.com

SOIL ANALYSIS

Client :
University of Iowa
4108 Seamans Center
Iowa City IA 52242

Grower :

Date Received : 04/11/2007

Report No: 07-101-0512
Cust No: 02405
Date Printed: 04/13/2007
Page : 1 of 9

Lab Number : 19841 Field Id : Sample Id : A

Test	Results	SOIL TEST RATINGS (Very Low / Low / Medium / Optimum / Very High)
Soil pH	7.0	
Buffer pH	7.33	
Phosphorus (P)	120 ppm	
Potassium (K)	197 ppm	
Calcium (Ca)	2857 ppm	
Magnesium (Mg)	381 ppm	
Sulfur (S)	324 ppm	
Boron (B)	1.4 ppm	
Copper (Cu)	1.1 ppm	
Iron (Fe)	186 ppm	
Manganese (Mn)	18 ppm	
Zinc (Zn)	4.5 ppm	
Sodium (Na)	66 ppm	
Soluble Salts		
Organic Matter	2.4 % ENR 92	
Nitrate Nitrogen		

Calculated Cation Exchange Capacity: 18.3 meq/100g

Calculated Cation Saturation

%K	2.8
%Ca	78.1
%Mg	17.3
%H	0.0
%Na	1.6

K : Mg Ratio: 0.16

* Additional results to follow

SOIL FERTILITY GUIDELINES

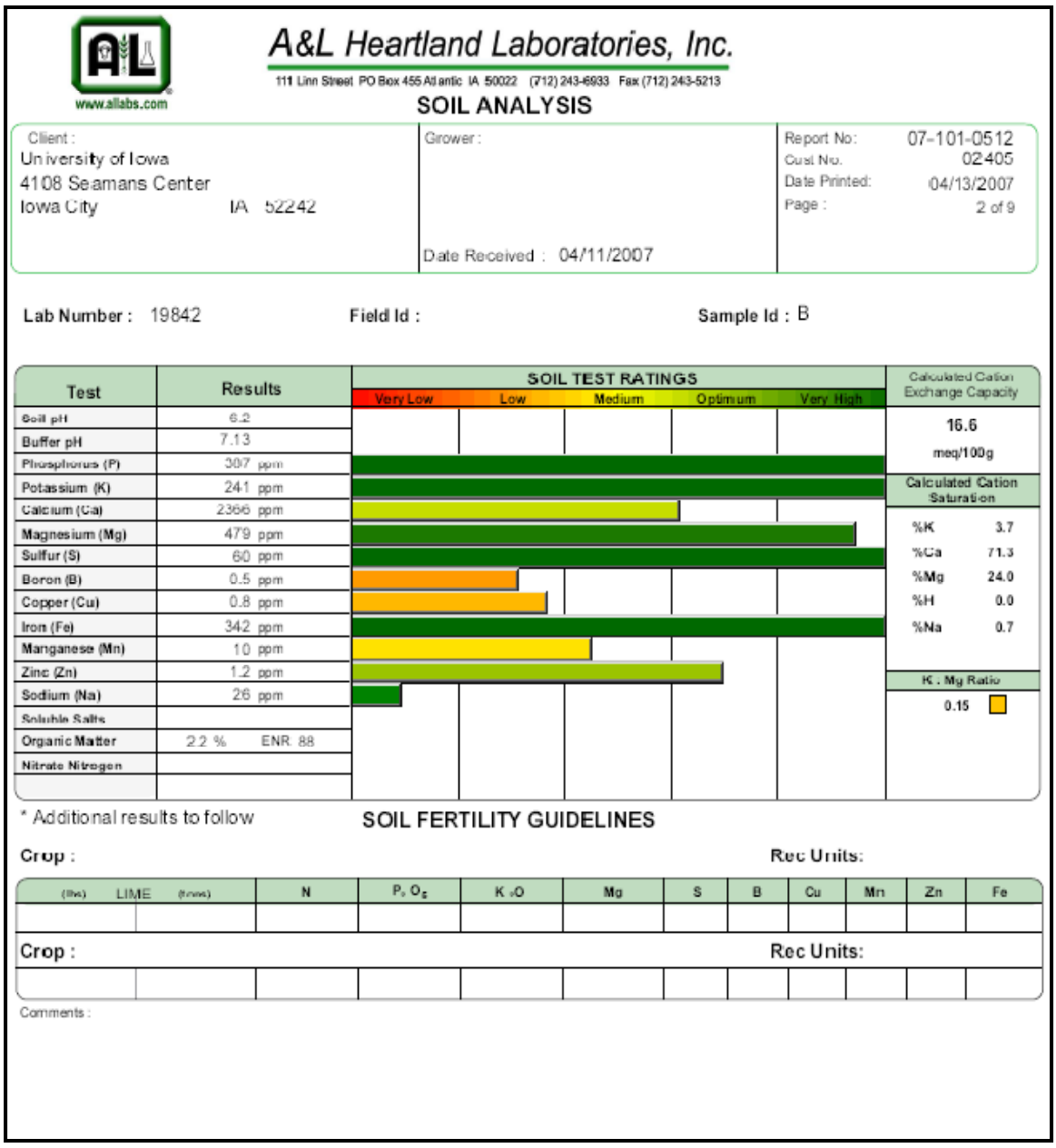
Crop : Rec Units:

(lbs) LIME (tons)	N	P_2O_5	K_2O	Mg	S	B	Cu	Mn	Zn	Fe

Crop : Rec Units:

Comments :

Table G-2. HOC soil from bioaugmented reactor B after 240 days of operation



A&L Heartland Laboratories, Inc.

111 Linn Street PO Box 455 Atlantic IA 50022 (712) 243-6933 Fax (712) 243-5213

www.allabs.com

SOIL ANALYSIS

Client :
University of Iowa
4108 Seamans Center
Iowa City IA 52242

Grower :

Date Received : 04/11/2007

Report No: 07-101-0512
Cust No. 02405
Date Printed: 04/13/2007
Page : 2 of 9

Lab Number : 19842 Field Id : Sample Id : B

Test	Results	SOIL TEST RATINGS (Very Low / Low / Medium / Optimum / Very High)
Soil pH	6.2	
Buffer pH	7.13	
Phosphorus (P)	307 ppm	
Potassium (K)	241 ppm	
Calcium (Ca)	2366 ppm	
Magnesium (Mg)	479 ppm	
Sulfur (S)	60 ppm	
Boron (B)	0.5 ppm	
Copper (Cu)	0.8 ppm	
Iron (Fe)	342 ppm	
Manganese (Mn)	10 ppm	
Zinc (Zn)	1.2 ppm	
Sodium (Na)	26 ppm	
Soluble Salts		
Organic Matter	2.2 % ENR 88	
Nitrate Nitrogen		

Calculated Cation Exchange Capacity: 16.6 meq/100g

Calculated Cation Saturation:

%K	3.7
%Ca	71.3
%Mg	24.0
%H	0.0
%Na	0.7

K : Mg Ratio: 0.15

* Additional results to follow

SOIL FERTILITY GUIDELINES

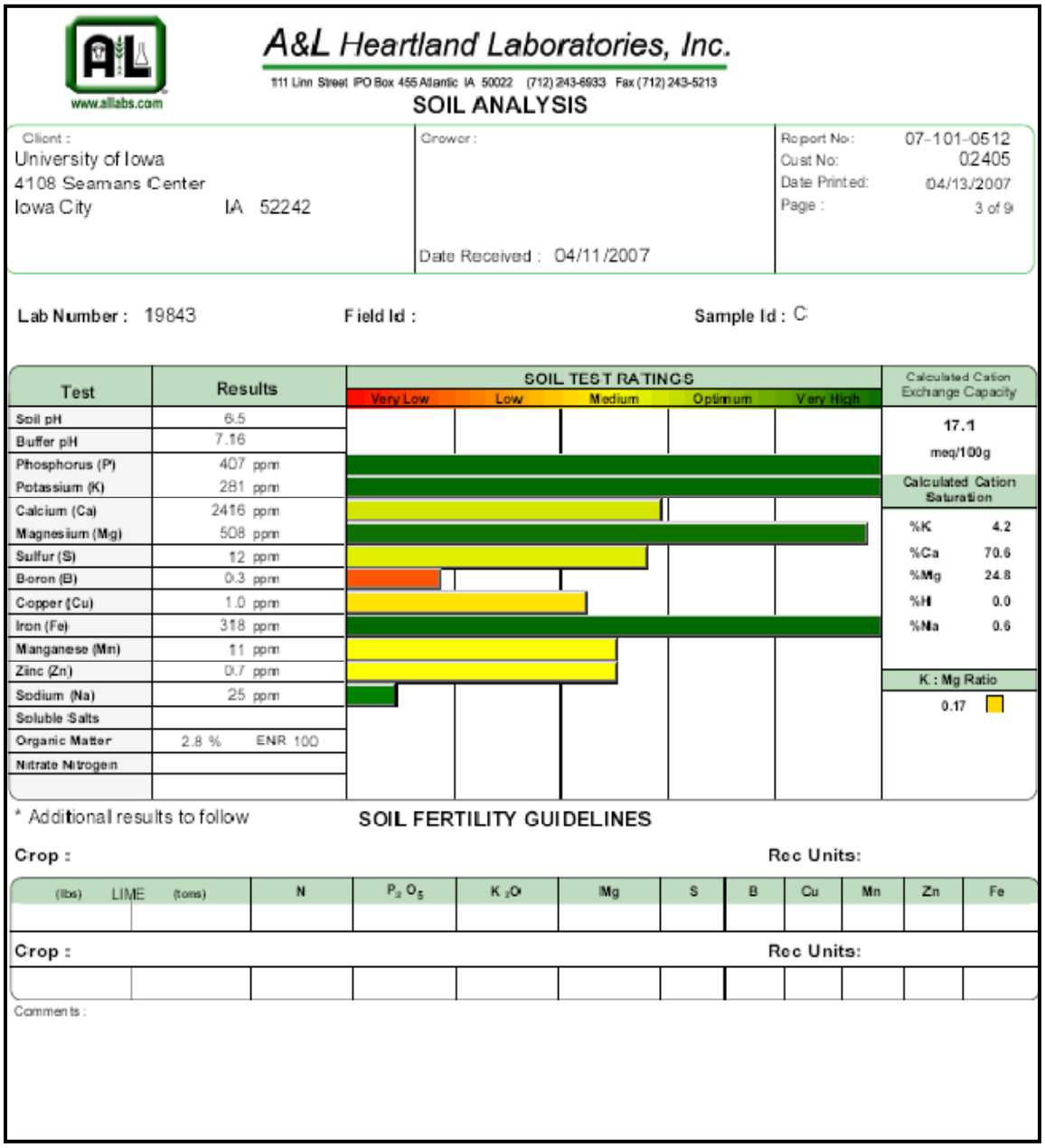
Crop : Rec Units:

(lbs) LIME (tons)	N	P_2O_5	K_2O	Mg	S	B	Cu	Mn	Zn	Fe

Crop : Rec Units:

Comments :

Table G-3. HOC soil from bioaugmented reactor C after 240 days of operation



A&L Heartland Laboratories, Inc.

111 Linn Street PO Box 455 Atlantic IA 50022 (712) 243-6933 Fax (712) 243-5213

www.allabs.com

SOIL ANALYSIS

Client :
University of Iowa
4108 Seamans Center
Iowa City IA 52242

Grower :

Date Received : 04/11/2007

Report No: 07-101-0512
Cust No: 02405
Date Printed: 04/13/2007
Page : 3 of 9

Lab Number : 19843 Field Id : Sample Id : C

Test	Results	SOIL TEST RATINGS (Very Low / Low / Medium / Optimum / Very High)
Soil pH	6.5	
Buffer pH	7.16	
Phosphorus (P)	407 ppm	
Potassium (K)	281 ppm	
Calcium (Ca)	2416 ppm	
Magnesium (Mg)	508 ppm	
Sulfur (S)	12 ppm	
Boron (B)	0.3 ppm	
Copper (Cu)	1.0 ppm	
Iron (Fe)	318 ppm	
Manganese (Mn)	11 ppm	
Zinc (Zn)	0.7 ppm	
Sodium (Na)	25 ppm	
Soluble Salts		
Organic Matter	2.8 % ENR 100	
Nitrate Nitrogen		

Calculated Cation Exchange Capacity: 17.1 meq/100g

Calculated Cation Saturation

%K	4.2
%Ca	70.6
%Mg	24.8
%H	0.0
%Na	0.6

K : Mg Ratio: 0.17

* Additional results to follow

SOIL FERTILITY GUIDELINES

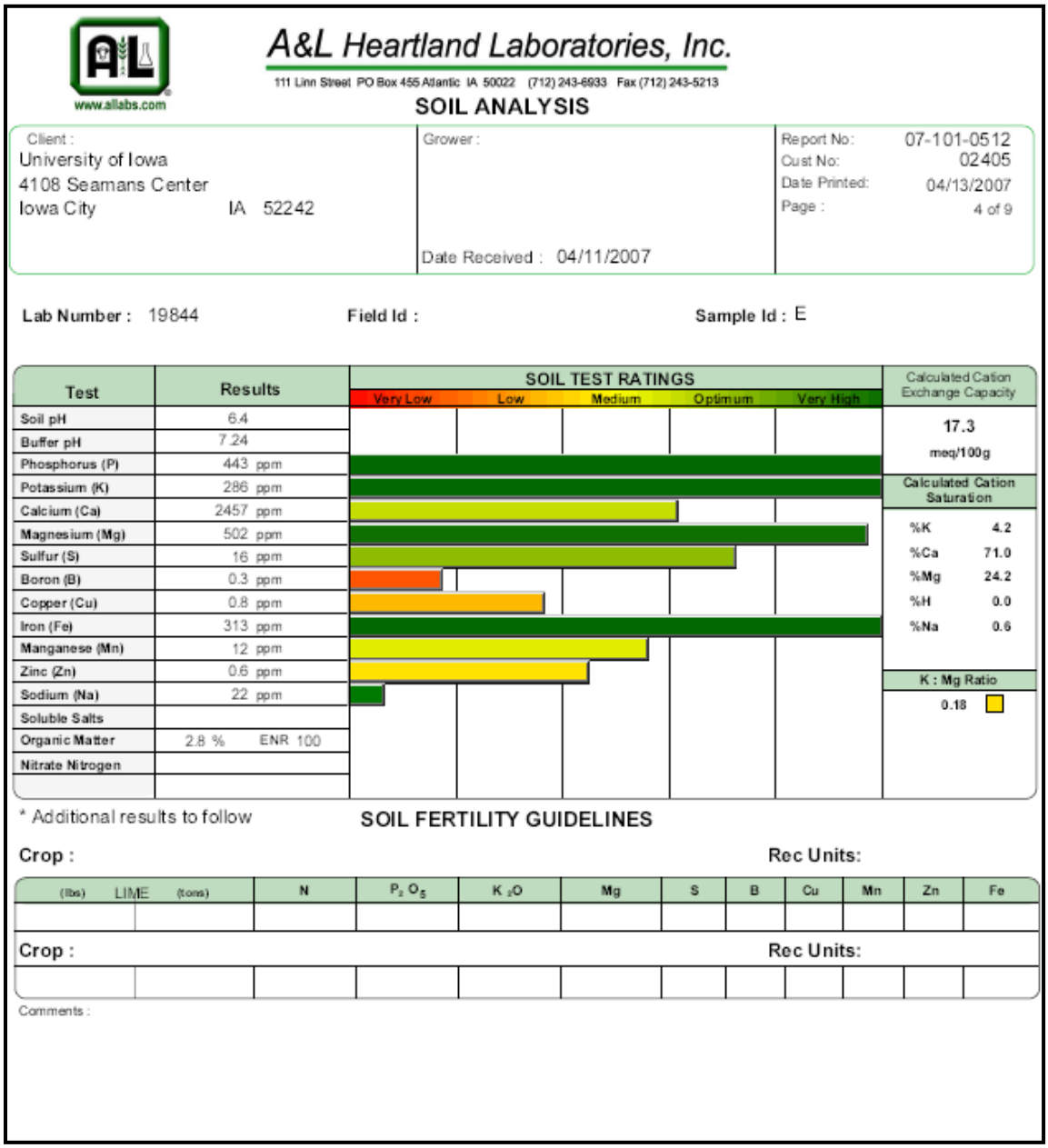
Crop : Rec Units:

(lbs) LIME (tons)	N	P_2O_5	K_2O	Mg	S	B	Cu	Mn	Zn	Fe

Crop : Rec Units:

Comments :

Table G-4. HOC soil from planted reactor E after 240 days of operation



A&L Heartland Laboratories, Inc.

111 Linn Street PO Box 455 Atlantic IA 50022 (712) 243-6933 Fax (712) 243-5213

www.allabs.com

SOIL ANALYSIS

Client :
University of Iowa
4108 Seamans Center
Iowa City IA 52242

Grower :

Date Received : 04/11/2007

Report No: 07-101-0512
Cust No: 02405
Date Printed: 04/13/2007
Page : 4 of 9

Lab Number : 19844 Field Id : Sample Id : E

Test	Results
Soil pH	6.4
Buffer pH	7.24
Phosphorus (P)	443 ppm
Potassium (K)	286 ppm
Calcium (Ca)	2457 ppm
Magnesium (Mg)	502 ppm
Sulfur (S)	16 ppm
Boron (B)	0.3 ppm
Copper (Cu)	0.8 ppm
Iron (Fe)	313 ppm
Manganese (Mn)	12 ppm
Zinc (Zn)	0.6 ppm
Sodium (Na)	22 ppm
Soluble Salts	
Organic Matter	2.8 % ENR 100
Nitrate Nitrogen	

SOIL TEST RATINGS: Very Low | Low | Medium | Optimum | Very High

Calculated Cation Exchange Capacity: 17.3 meq/100g

Calculated Cation Saturation

%K	4.2
%Ca	71.0
%Mg	24.2
%H	0.0
%Na	0.6

K : Mg Ratio: 0.18

* Additional results to follow

SOIL FERTILITY GUIDELINES

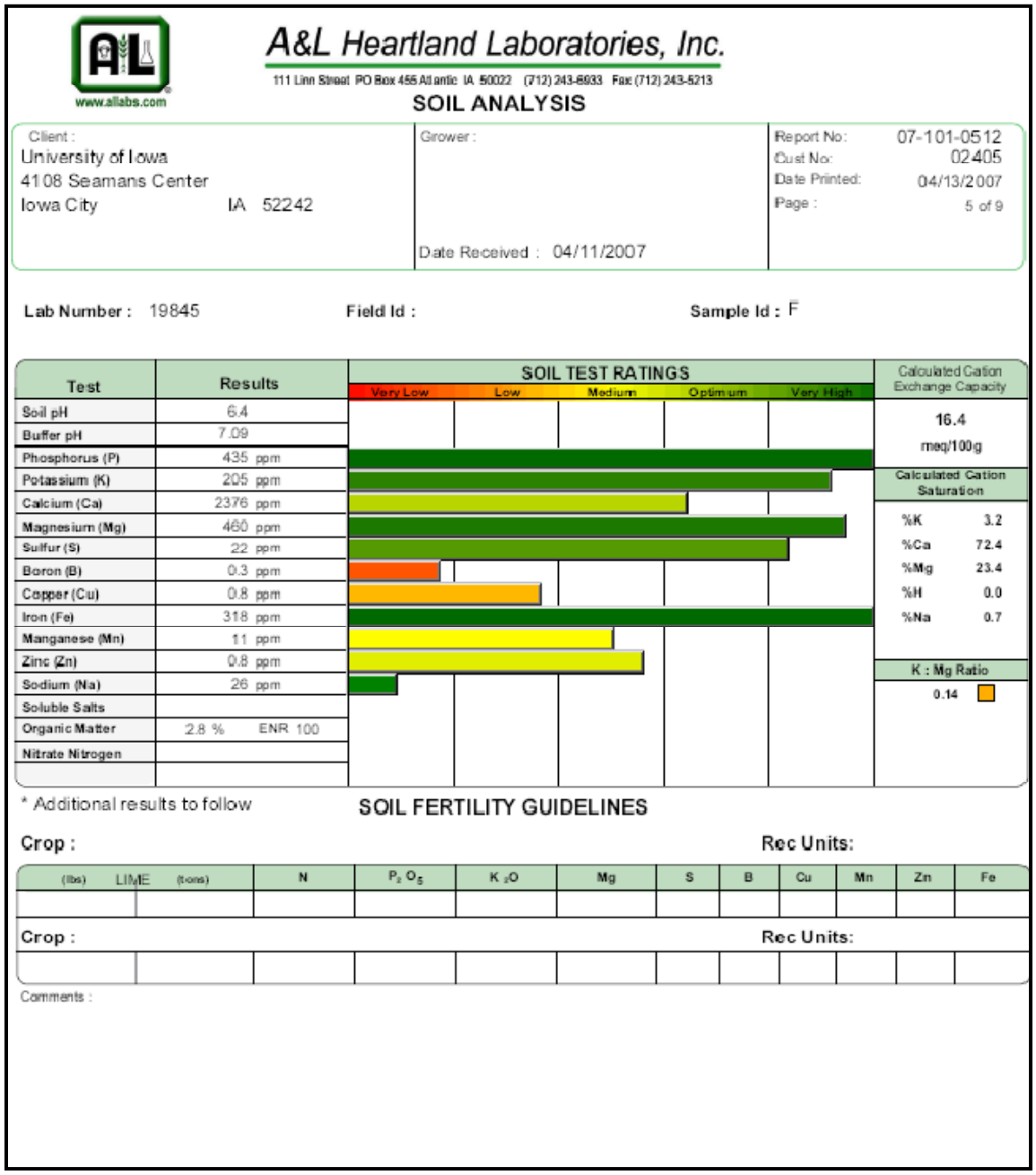
Crop : Rec Units:

(lbs) LIME (tons)	N	P_2O_5	K_2O	Mg	S	B	Cu	Mn	Zn	Fe

Crop : Rec Units:

Comments :

Table G-5. HOC soil from planted reactor F after 240 days of operation



A&L Heartland Laboratories, Inc.

111 Linn Street PO Box 455 Atlantic IA 50022 (712) 243-6933 Fax (712) 243-5213

www.allabs.com

SOIL ANALYSIS

Client :
University of Iowa
4108 Seamans Center
Iowa City IA 52242

Grower :

Date Received : 04/11/2007

Report No: 07-101-0512
Cust No: 02405
Date Printed: 04/13/2007
Page : 5 of 9

Lab Number : 19845 Field Id : Sample Id : F

Test	Results
Soil pH	6.4
Buffer pH	7.09
Phosphorus (P)	435 ppm
Potassium (K)	205 ppm
Calcium (Ca)	2376 ppm
Magnesium (Mg)	460 ppm
Sulfur (S)	22 ppm
Boron (B)	0.3 ppm
Copper (Cu)	0.8 ppm
Iron (Fe)	318 ppm
Manganese (Mn)	11 ppm
Zinc (Zn)	0.8 ppm
Sodium (Na)	26 ppm
Soluble Salts	
Organic Matter	2.8 % ENR 100
Nitrate Nitrogen	

SOIL TEST RATINGS

Very Low | Low | Medium | Optimum | Very High

Calculated Cation Exchange Capacity
16.4 meq/100g

Calculated Cation Saturation

%K	3.2
%Ca	72.4
%Mg	23.4
%H	0.0
%Na	0.7

K : Mg Ratio
0.14

* Additional results to follow

SOIL FERTILITY GUIDELINES

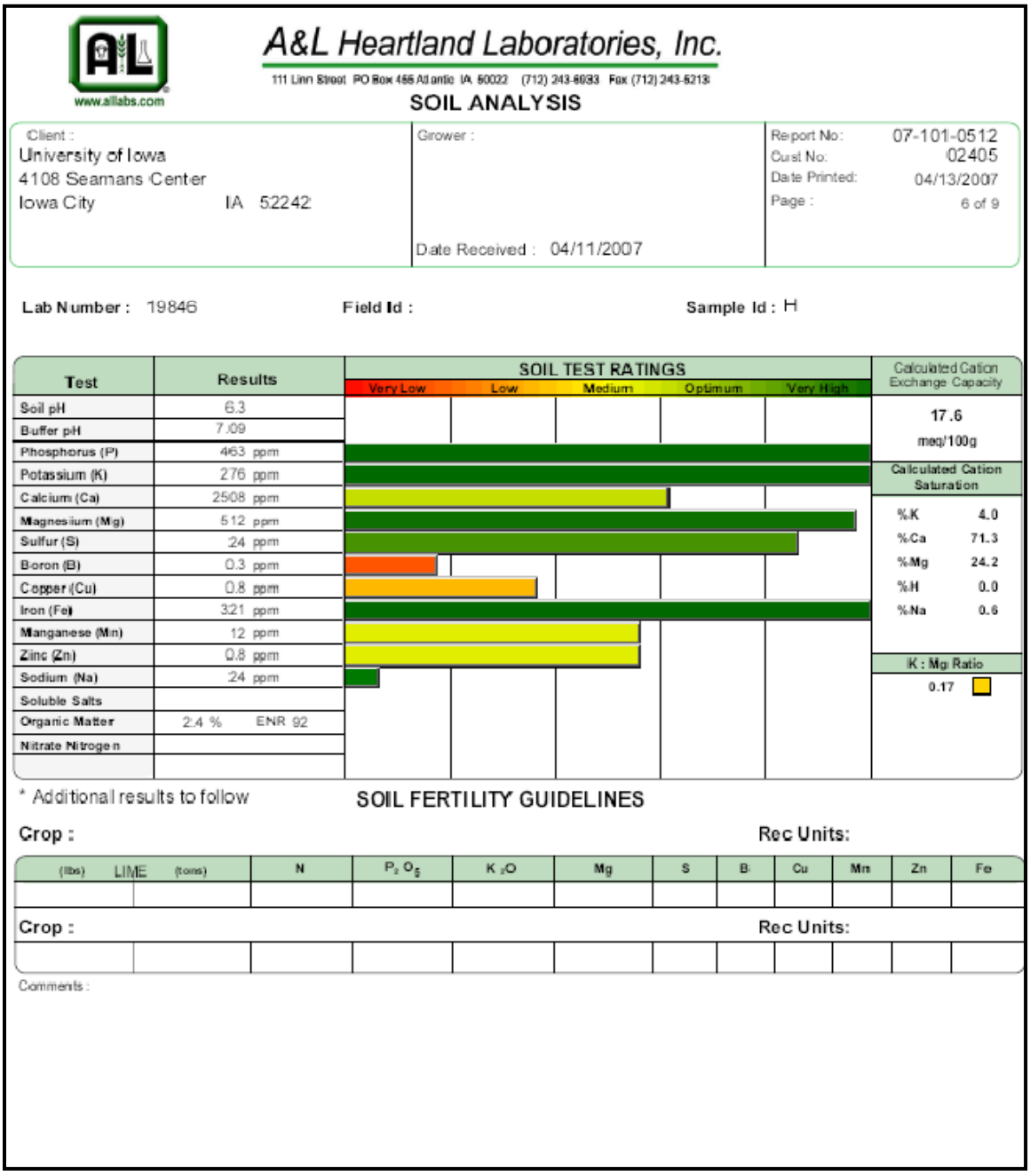
Crop : Rec Units:

(lbs) LIME (tons)	N	P_2O_5	K_2O	Mg	S	B	Cu	Mn	Zn	Fe

Crop : Rec Units:

Comments :

Table G-6. HOC soil from bioaug./planted reactor H after 240 days of operation



A&L Heartland Laboratories, Inc.

111 Linn Street PO Box 458 Atlantic IA 50022 (712) 243-6983 Fax (712) 243-5213

www.allabs.com

SOIL ANALYSIS

Client :
University of Iowa
4108 Seamans Center
Iowa City IA 52242

Grower :

Date Received : 04/11/2007

Report No: 07-101-0512
Cust No: 02405
Date Printed: 04/13/2007
Page : 6 of 9

Lab Number : 19846 Field Id : Sample Id : H

Test	Results
Soil pH	6.3
Buffer pH	7.09
Phosphorus (P)	463 ppm
Potassium (K)	276 ppm
Calcium (Ca)	2508 ppm
Magnesium (Mg)	512 ppm
Sulfur (S)	24 ppm
Boron (B)	0.3 ppm
Copper (Cu)	0.8 ppm
Iron (Fe)	321 ppm
Manganese (Mn)	12 ppm
Zinc (Zn)	0.8 ppm
Sodium (Na)	24 ppm
Soluble Salts	
Organic Matter	2.4 % ENR 92
Nitrate Nitrogen	

SOIL TEST RATINGS

Very Low | Low | Medium | Optimum | Very High

Calculated Cation Exchange Capacity
17.6
meq/100g

Calculated Cation Saturation

%K	4.0
%Ca	71.3
%Mg	24.2
%H	0.0
%Na	0.6

K : Mg Ratio
0.17

* Additional results to follow

SOIL FERTILITY GUIDELINES

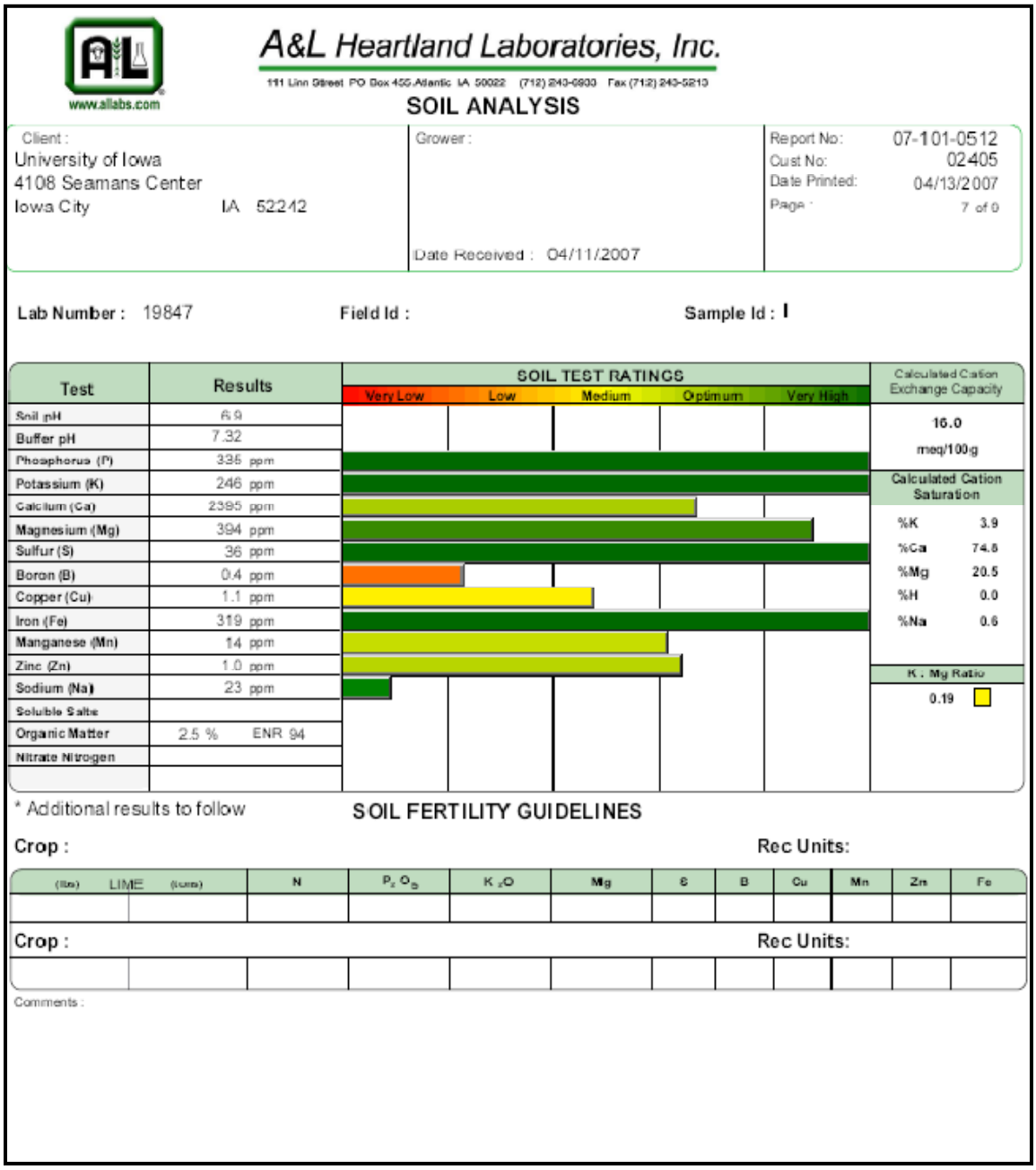
Crop : Rec Units:

(lbs) LIME (tons)	N	P_2O_5	K_2O	Mg	S	B	Cu	Mn	Zn	Fe

Crop : Rec Units:

Comments :

Table G-7. HOC soil from bioaug./planted reactor I after 240 days of operation



A&L Heartland Laboratories, Inc.

111 Linn Street PO Box 458 Atlantic IA 50022 (712) 243-6933 Fax (712) 243-5213

www.allabs.com

SOIL ANALYSIS

Client :
University of Iowa
4108 Seamans Center
Iowa City IA 52242

Grower :

Date Received : 04/11/2007

Report No: 07-101-0512
Cust No: 02405
Date Printed: 04/13/2007
Page : 7 of 9

Lab Number : 19847 Field Id : Sample Id : I

Test	Results	SOIL TEST RATINGS (Very Low / Low / Medium / Optimum / Very High)
Soil pH	6.9	
Buffer pH	7.32	
Phosphorus (P)	335 ppm	
Potassium (K)	246 ppm	
Calcium (Ca)	2395 ppm	
Magnesium (Mg)	394 ppm	
Sulfur (S)	36 ppm	
Boron (B)	0.4 ppm	
Copper (Cu)	1.1 ppm	
Iron (Fe)	319 ppm	
Manganese (Mn)	14 ppm	
Zinc (Zn)	1.0 ppm	
Sodium (Na)	23 ppm	
Soluble Salts		
Organic Matter	2.5 % ENR 94	
Nitrate Nitrogen		

Calculated Cation Exchange Capacity: 16.0 meq/100g

Calculated Cation Saturation

%K	3.9
%Ca	74.8
%Mg	20.5
%H	0.0
%Na	0.6

K : Mg Ratio: 0.19

* Additional results to follow

SOIL FERTILITY GUIDELINES

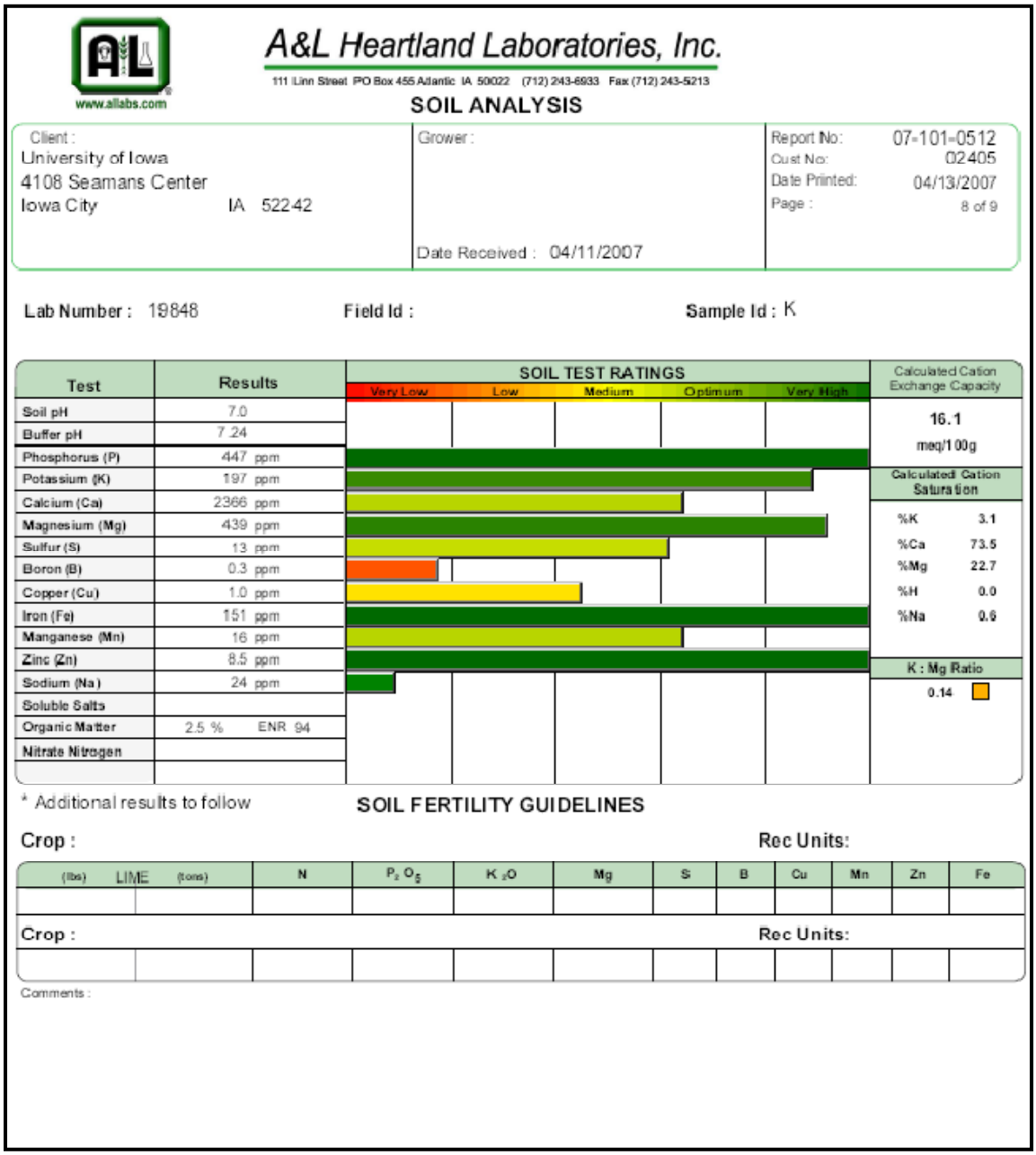
Crop : Rec Units:

(lbs) LIME (tons)	N	P_2O_5	K_2O	Mg	S	B	Cu	Mn	Zn	Fe

Crop : Rec Units:

Comments :

Table G-8. HOC soil from reference reactor K after 240 days of operation



A&L Heartland Laboratories, Inc.

111 Linn Street PO Box 455 Atlantic IA 50022 (712) 243-6933 Fax (712) 243-5213

www.allabs.com

SOIL ANALYSIS

Client :
University of Iowa
4108 Seamans Center
Iowa City IA 52242

Grower :

Date Received : 04/11/2007

Report No: 07-101-0512
Cust No: 02405
Date Printed: 04/13/2007
Page : 8 of 9

Lab Number : 19848 Field Id : Sample Id : K

Test	Results
Soil pH	7.0
Buffer pH	7.24
Phosphorus (P)	447 ppm
Potassium (K)	197 ppm
Calcium (Ca)	2366 ppm
Magnesium (Mg)	439 ppm
Sulfur (S)	13 ppm
Boron (B)	0.3 ppm
Copper (Cu)	1.0 ppm
Iron (Fe)	151 ppm
Manganese (Mn)	16 ppm
Zinc (Zn)	8.5 ppm
Sodium (Na)	24 ppm
Soluble Salts	
Organic Matter	2.5 % ENR 94
Nitrate Nitrogen	

SOIL TEST RATINGS: Very Low | Low | Medium | Optimum | Very High

Calculated Cation Exchange Capacity: 16.1 meq/100g

Calculated Cation Saturation

%K	3.1
%Ca	73.5
%Mg	22.7
%H	0.0
%Na	0.6

K : Mg Ratio: 0.14

* Additional results to follow

SOIL FERTILITY GUIDELINES

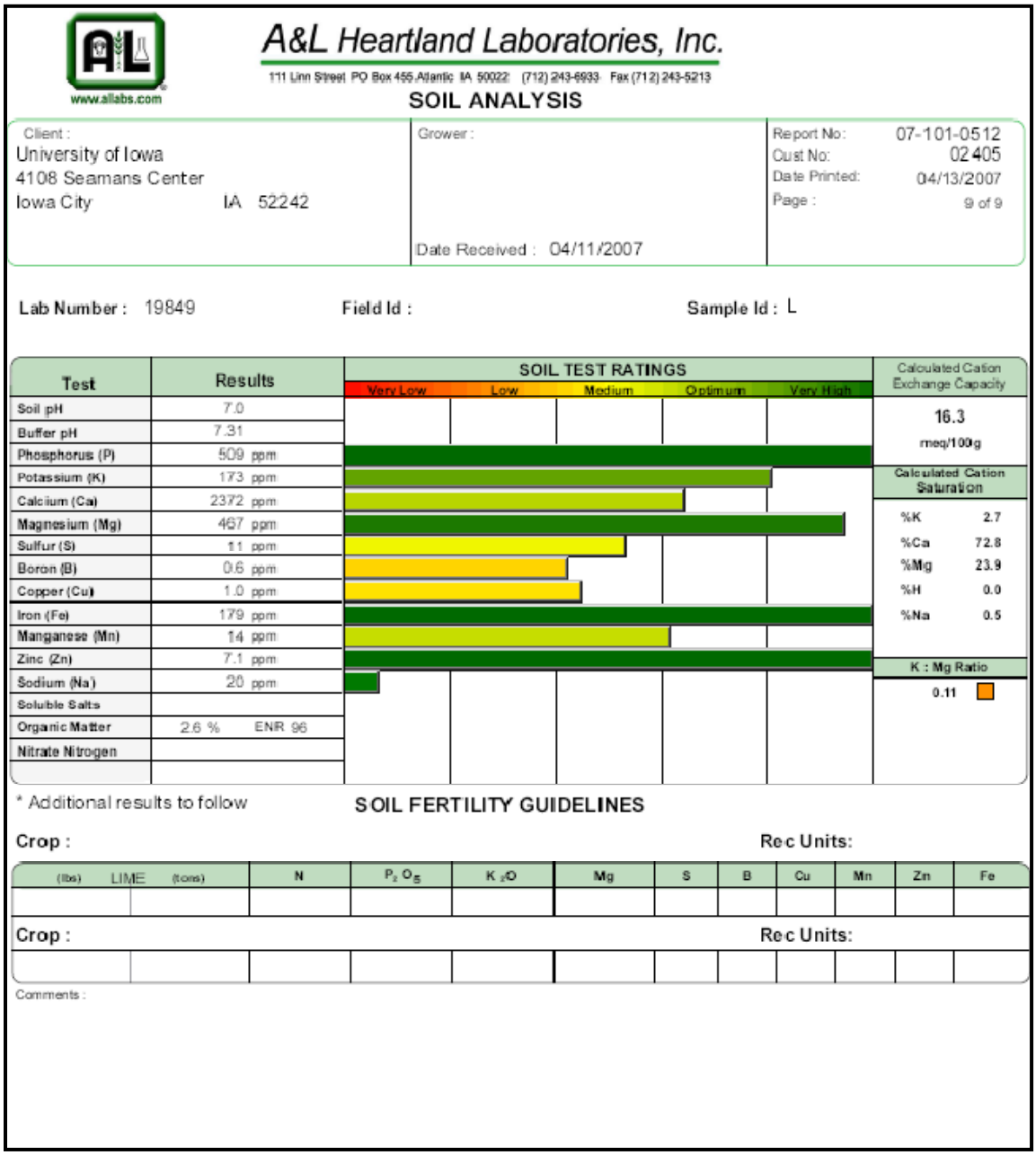
Crop : Rec Units:

(lbs) LIME (tons)	N	P_2O_5	K_2O	Mg	S	B	Cu	Mn	Zn	Fe

Crop : Rec Units:

Comments :

Table G-9. HOC soil from reference reactor L after 240 days of operation



A&L Heartland Laboratories, Inc.

111 Linn Street PO Box 455 Atlantic IA 50022 (712) 243-6933 Fax (712) 243-5213

www.allabs.com

SOIL ANALYSIS

Client :
University of Iowa
4108 Seamans Center
Iowa City IA 52242

Grower :

Date Received : 04/11/2007

Report No: 07-101-0512
Cust No: 02405
Date Printed: 04/13/2007
Page : 9 of 9

Lab Number : 19849 Field Id : Sample Id : L

Test	Results
Soil pH	7.0
Buffer pH	7.31
Phosphorus (P)	509 ppm
Potassium (K)	173 ppm
Calcium (Ca)	2372 ppm
Magnesium (Mg)	467 ppm
Sulfur (S)	11 ppm
Boron (B)	0.6 ppm
Copper (Cu)	1.0 ppm
Iron (Fe)	179 ppm
Manganese (Mn)	14 ppm
Zinc (Zn)	7.1 ppm
Sodium (Na)	20 ppm
Soluble Salts	
Organic Matter	2.6 % ENR 96
Nitrate Nitrogen	

SOIL TEST RATINGS: Very Low | Low | Medium | Optimum | Very High

Calculated Cation Exchange Capacity: 16.3 meq/100g

Calculated Cation Saturation

%K	2.7
%Ca	72.8
%Mg	23.9
%H	0.0
%Na	0.5

K : Mg Ratio: 0.11

* Additional results to follow

SOIL FERTILITY GUIDELINES

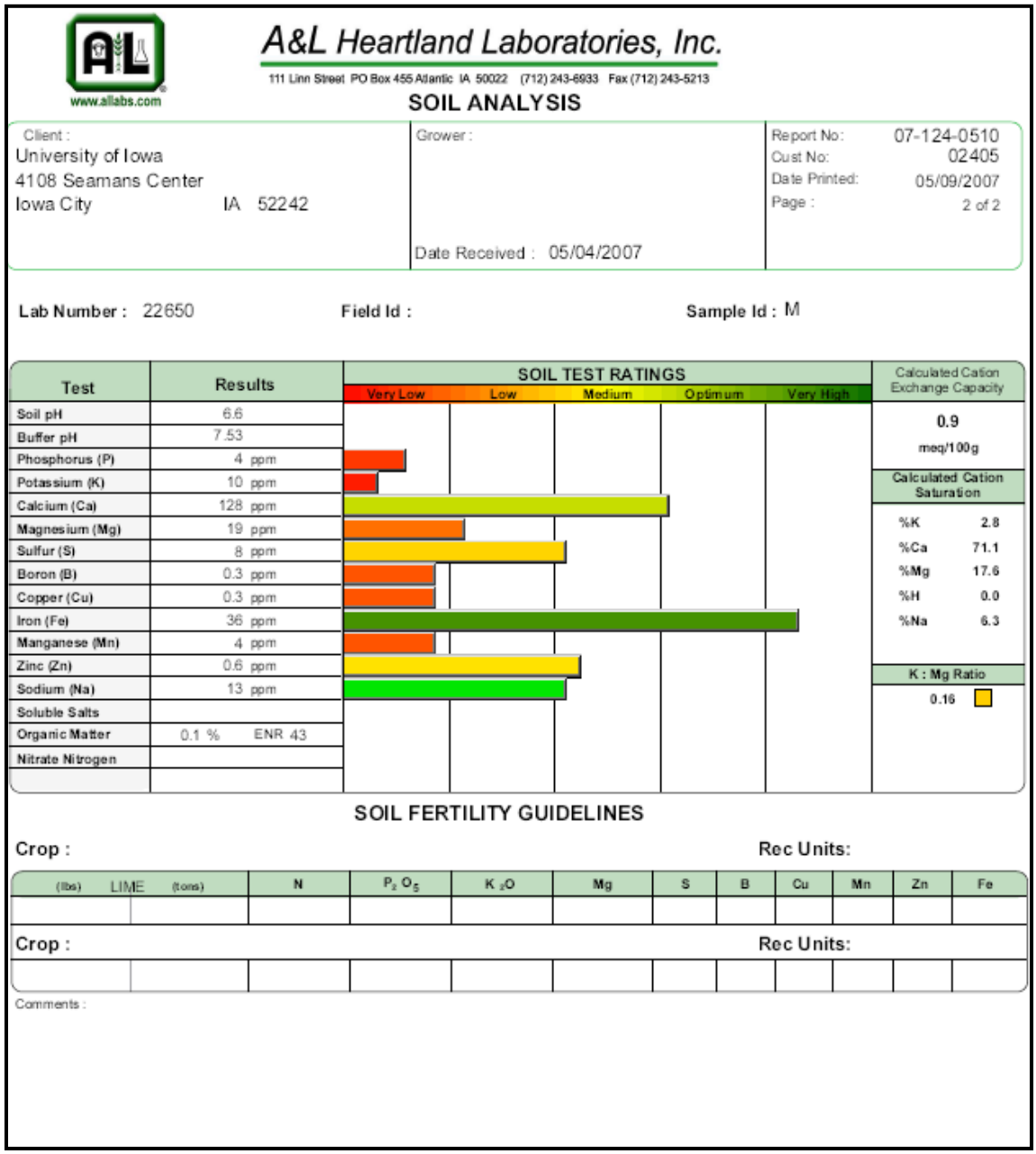
Crop : Rec Units:

(lbs) LIME (tons)	N	P_2O_5	K_2O	Mg	S	B	Cu	Mn	Zn	Fe

Crop : Rec Units:

Comments :

Table G-10. Multipurpose sand used to fill LOC reactors prior to operation



A&L Heartland Laboratories, Inc.

111 Linn Street PO Box 455 Atlantic IA 50022 (712) 243-6933 Fax (712) 243-5213

www.allabs.com

SOIL ANALYSIS

Client :
University of Iowa
4108 Seamans Center
Iowa City IA 52242

Grower :

Date Received : 05/04/2007

Report No: 07-124-0510
Cust No: 02405
Date Printed: 05/09/2007
Page : 2 of 2

Lab Number : 22650 Field Id : Sample Id : M

Test	Results	SOIL TEST RATINGS (Very Low / Low / Medium / Optimum / Very High)
Soil pH	6.6	
Buffer pH	7.53	
Phosphorus (P)	4 ppm	
Potassium (K)	10 ppm	
Calcium (Ca)	128 ppm	
Magnesium (Mg)	19 ppm	
Sulfur (S)	8 ppm	
Boron (B)	0.3 ppm	
Copper (Cu)	0.3 ppm	
Iron (Fe)	36 ppm	
Manganese (Mn)	4 ppm	
Zinc (Zn)	0.6 ppm	
Sodium (Na)	13 ppm	
Soluble Salts		
Organic Matter	0.1 % ENR 43	
Nitrate Nitrogen		

Calculated Cation Exchange Capacity: 0.9 meq/100g

Calculated Cation Saturation

%K	2.8
%Ca	71.1
%Mg	17.6
%H	0.0
%Na	6.3

K : Mg Ratio: 0.16

SOIL FERTILITY GUIDELINES

Crop : Rec Units:

(lbs) LIME (tons)	N	P_2O_5	K_2O	Mg	S	B	Cu	Mn	Zn	Fe

Crop : Rec Units:

Comments :

BIBLIOGRAPHY

Alvarez-Dolado, M., A. Cuadrado, C. Navarro-Yubero, P. Sonderegger, A. J. Furley, J. Bernal and A. Munoz (2000). "Regulation of the L1 Cell Adhesion Molecule by Thyroid Hormone in the Developing Brain." Molecular & Cellular Neuroscience **16**(4): 499-514.

Amann, R. I., W. Ludwig and K. H. Schleifer (1995). "Phylogenetic Identification and in-Situ Detection of Individual Microbial-Cells without Cultivation." Microbiological Reviews **59**(1): 143-169.

Andraski, B. J., D. A. Stonestrom, R. L. Michel, K. J. Halford and J. C. Radyk (2005). "Plant-Based Plume-Scale Mapping of Tritium Contamination in Desert Soils." Vadose Zone J **4**(3): 819-827.

Armstrong, W. and J. Armstrong (2005). "Stem Photosynthesis Not Pressurized Ventilation Is Responsible for Light-Enhanced Oxygen Supply to Submerged Roots of Alder (Alnus Glutinosa)." Annals of Botany **96**(4): 591-612.

AWWA (1999). Water Quality and Treatment: A Handbook of Community Water Supplies. New York, McGraw-Hill.

Banerjee, K. K., P. Marimuthu, P. Bhattacharyya and M. Chatterjee (1997). "Effect of Thiocyanate Ingestion through Milk on Thyroid Hormone Homeostasis in Women." British Journal of Nutrition **78**(5): 679-81.

Bender, K. S., S. M. O'Connor, R. Chakraborty, J. D. Coates and L. A. Achenbach (2002). "Sequencing and Transcriptional Analysis of the Chlorite Dismutase Gene of Dechloromonas Agitata and Its Use as a Metabolic Probe." Applied and Environmental Microbiology **68**(10): 4820-4826.

Bender, K. S., M. R. Rice, W. H. Fugate, J. D. Coates and L. A. Achenbach (2004). "Metabolic Primers for Detection of (Per) Chlorate-Reducing Bacteria in the Environment and Phylogenetic Analysis of Cld Gene Sequences." Applied & Environmental Microbiology **70**(9): 5651-5658.

Bowen, G. D. and A. D. Rovira (1976). "Microbial Colonization of Plant Roots." Annual Review of Phytopathology **14**(1): 121-144.

Braverman, L. E., X. M. He, S. Pino, M. Cross, B. Magnani, S. H. Lamm, M. B. Kruse, A. Engel, K. S. Crump and J. P. Gibbs (2005). "The Effect of Perchlorate, Thiocyanate, and Nitrate on Thyroid Function in Workers Exposed to Perchlorate Long-Term." Journal of Clinical Endocrinology & Metabolism **90**(2): 700-706.

Brechner, R. J., G. D. Parkhurst, W. O. Humble, M. B. Brown and W. H. Herman (2000). "Ammonium Perchlorate Contamination of Colorado River Drinking Water Is Associated with Abnormal Thyroid Function in Newborns in Arizona." Journal of Occupational and Environmental Medicine **42**(8): 777-782.

Burken, J. G. and J. L. Schnoor (1996). "Phytoremediation: Plant Uptake of Atrazine and the Role of Root Exudates." ASCE Journal of Environmental Engineering **122**(11): 958-963.

CA-DPH (2007). R-16-04 - Perchlorate in Drinking Water. California Dept. of Public Health. http://www.cdph.ca.gov/services/DPOPP/regs/Pages/R-16-04-PerchlorateinDrinkingWater.aspx

Carr, J. A., L. J. Urquidi, W. L. Goleman, F. Hu, P. N. Smith and C. W. Theodorakis (2003). "Ammonium Perchlorate Disruption of Thyroid Function in Natural Amphibian Populations: Assessment and Potential Impact." ASTM Special Technical Publication **STP 1443** (Multiple Stressor Effects in Relation to Declining Amphibian Populations): 130-142.

Clewell, R. A., S. Chaudhuri, S. Dickson, R. S. Cassady, W. M. Wallner, J. E. Eldridge and D. T. Tsui (2000). "Analysis of Trace Level Perchlorate in Drinking Water and Ground Water by Electrospray Mass Spectrometry." Environmental Science Research **57**(Perchlorate in the Environment): 45-58.

Coates, J. D. and L. A. Achenbach (2004). "Microbial Perchlorate Reduction: Rocket-Fueled Metabolism." Nature Reviews Microbiology **2**(7): 569-580.

Coates, J. D., K. A. Cole, R. Chakraborty, S. M. O'Connor and L. A. Achenbach (2002). "Diversity and Ubiquity of Bacteria Capable of Utilizing Humic Substances as Electron Donors for Anaerobic Respiration." Applied & Environmental Microbiology **68**(5): 2445-2452.

Coates, J. D., U. Michaelidou, R. A. Bruce, S. M. O'Connor, J. N. Crespi and L. A. Achenbach (1999). "Ubiquity and Diversity of Dissimilatory (Per)Chlorate-Reducing Bacteria." Applied & Environmental Microbiology **65**(12): 5234-5241.

Cole, J. R., Q. Wang, E. Cardenas, J. Fish, B. Chai, R. J. Farris, A. S. Kulam-Syed-Mohideen, D. M. McGarrell, T. Marsh, G. M. Garrity and J. M. Tiedje (2009). "The Ribosomal Database Project: Improved Alignments and New Tools for rRNA Analysis." Nucleic Acids Research **37**: D141-D145.

Cox, E. E., E. Edwards and S. Neville (2000). "In Situ Bioremediation of Perchlorate in Groundwater." Environmental Science Research **57** (Perchlorate in the Environment): 231-240.

Degens, B. P., L. A. Schipper, G. P. Sparling and L. C. Duncan (2001). "Is the Microbial Community in a Soil with Reduced Catabolic Diversity Less Resistant to Stress or Disturbance?" Soil Biology and Biochemistry **33**(9): 1143.

DENIX. (2000). "U.S. Perchlorate Releases." Retrieved 6/13/2005, 2005, from https://www.denix.osd.mil/denix/Public/Library/Water/Perchlorate/releases.html.

Dowling, A. L. S., G. U. Martz, J. L. Leonard and R. T. Zoeller (2000). "Acute Changes in Maternal Thyroid Hormone Induce Rapid and Transient Changes in Gene Expression in Fetal Rat Brain." Journal of Neuroscience **20**(6): 2255-2265.

Evans, P. J. and W. J. Weaver (2005). In Situ and Ex Situ Treatment of Perchlorate in Source Areas. NGWA Conference on MTBE and Perchlorate, San Francisco, CA, National Ground Water Association.

Fitzgerald, J., E. Kunce and C. R. West (2005). The Occurrence and Regulation of Perchlorate in Massachusetts. NGWA Conference on MTBE and Perchlorate, San Francisco, CA, National Groundwater Association.

Foss, O. P. and P. G. Lund-Larsen (1986). "Serum Thiocyanate and Smoking: Interpretation of Serum Thiocyanate Levels Observed in a Large Health Study." Scandinavian Journal of Clinical & Laboratory Investigation **46**(3): 245-51.

Fushuku, S. and K. Fukuda (2008). "Inhomogeneity of Fecal Flora in Separately Reared Laboratory Mice, as Detected by Denaturing Gradient Gel Electrophoresis (Dgge)." Experimental Animals **57**(2): 95-99.

Galli, A. E., C. F. Leck and R. T. T. Forman (1976). "Avian Distribution Patterns in Forest Islands of Different Sizes in Central New Jersey." The Auk **93**(2): 356.

Gejman, P. V., Q. H. Cao, F. Guedj and S. Sommer (1998). "The Sensitivity of Denaturing Gradient Gel Electrophoresis: A Blinded Analysis." Mutation Research-Genomics **382**(3-4): 109-114.

Giblin, T. L., D. C. Herman and W. T. Frankenberger, Jr. (2000). "Removal of Perchlorate from Ground Water by Hydrogen-Utilizing Bacteria." Journal of Environmental Quality **29**(4): 1057-1062.

Gingras, T. M. and J. R. Batista (2002). "Biological Reduction of Perchlorate in Ion Exchange Regenerant Solutions Containing High Salinity and Ammonium Levels." Journal of Environmental Monitoring **4**(1): 96-101.

Gorman, A. M., R. S. Gregory and D. C. Schneider (2009). "Eelgrass Patch Size and Proximity to the Patch Edge Affect Predation Risk of Recently Settled Age 0 Cod (Gadus)." Journal of Experimental Marine Biology and Ecology **371**(1): 1.

Grayston, S. J., S. Wang, C. D. Campbell and A. C. Edwards (1998). "Selective Influence of Plant Species on Microbial Diversity in the Rhizosphere." Soil Biology and Biochemistry **30**(3): 369.

Greer, M. A., G. Goodman, R. C. Pleus and S. E. Greer (2002). "Health Effects Assessment for Environmental Perchlorate Contamination: The Dose Response for Inhibition of Thyroidal Radioiodine Uptake in Humans." Environmental Health Perspectives **110**(9): 927-937.

Greer, M. A., A. K. Stott and K. A. Milne (1966). "Effects of Thiocyanate, Perchlorate and Other Anions on Thyroidal Iodine Metabolism." Endocrinology **79**(2): 237-47.

Gu, B., Y.-K. Ku and G. M. Brown (2005). "Sorption and Desorption of Perchlorate and U(Vi) by Strong-Base Anion-Exchange Resins." Environmental Science and Technology **39**(3): 901-907.

Guarini, W. J. (2005). Biologicial System for Treating Perchlorate-Contaminated Ground Water in Henderson, Nevada. NGWA Conference on MTBE and Perchlorate, San Francisco, CA, National Ground Water Association.

Haddow, J. E., G. E. Palomaki, W. C. Allan, J. R. Williams, G. J. Knight, J. Gagnon, C. E. O'Heir, M. L. Mitchell, R. J. Hermos, S. E. Waisbren, J. D. Faix and R. Z. Klein (1999). "Maternal Thyroid Deficiency During Pregnancy and Subsequent Neuropsychological Development of the Child." New England Journal of Medicine **341**(8): 549-555.

Hatzinger, P. B. (2005). "Perchlorate Biodegradation for Water Treatment." Environmental Science & Technology **39**(12): 239A-247A.

Hegde, R. S. and J. S. Fletcher (1996). "Influence of Plant Growth Stage and Season on the Release of Root Phenolics by Mulberry as Related to Development of Phytoremediation Technology." Chemosphere **32**(12): 2471-2479.

Herman, D. C. and W. T. Frankenberger (1999). "Bacterial Reduction of Perchlorate and Nitrate in Water." Journal Of Environmental Quality **28**(3): 1018-1024.

Hollowell, J. G., N. W. Staehling, W. H. Hannon, D. W. Flanders, E. W. Gunter, G. F. Maberly, L. E. Braverman, S. Pino, D. T. Miller, P. L. Garbe, D. M. DeLozier and R. J. Jackson (1998). "Iodine Nutrition in the United States. Trends and Public Health Implications: Iodine Excretion Data from National Health and Nutrition Examination Surveys I and III (1971-1974 and 1988-1994)." Journal of Clinical Endocrinology and Metabolism **83**(10): 3401-3408.

Hunter, W. J. (2002). "Bioremediation of Chlorate or Perchlorate Contaminated Water Using Permeable Barriers Containing Vegetable Oil." Current Microbiology **45**(4): 287-292.

Hurst, C. J. and R. L. Crawford (2002). Manual of Environmental Microbiology. Washington, D.C., ASM Press.

Jackson, W. A., S. K. Anandam, T. Anderson, T. Lehman, K. Rainwater, S. Rajagopalan, M. Ridley and R. Tock (2005). "Perchlorate Occurrence in the Texas Southern High Plains Aquifer System." Ground Water Monitoring & Remediation **25**(1): 137-149.

Janczyk, P., R. Pieper, H. Smidt and W. B. Souffrant (2007). "Changes in the Diversity of Pig Ileal Lactobacilli around Weaning Determined by Means of 16S rRNA Gene Amplification and Denaturing Gradient Gel Electrophoresis." Fems Microbiology Ecology **61**(1): 132-140.

Jin, G. and A. J. Englande (1997). "Effects of Electron Donor, Dissolved Oxygen, and Oxidation-Reduction Potential Biodegradation of Carbon Tetrachloride by Escherichia Coli K-12." Water Environment Research **69**(6): 1100-1105.

Jordahl, J. L., L. Foster, J. L. Schnoor and P. J. J. Alvarez (1997). "Effect of Hybrid Poplar Trees on Microbial Populations Important to Hazardous Waste Bioremediation." Environmental Toxicology and Chemistry **16**(6): 1318-1321.

Kalkhoff, S. J. (2005). Perchlorate Occurrence in Ground Water in the North Central United States – Do Agricultural Practices Contribute Perchlorate to Shallow Aquifers. NGWA Conference on MTBE and Perchlorate, San Francisco, CA, National Groundwater Association.

Kirk, A. B., P. K. Martinelango, K. Tian, A. Dutta, E. E. Smith and P. K. Dasgupta (2005). "Perchlorate and Iodide in Dairy and Breast Milk." Environmental Science and Technology **39**(7): 2011-2017.

Lamm, S. H., L. E. Braverman, F. X. Li, K. Richman, S. Pino and G. Howearth (1999). "Thyroid Health Status of Ammonium Perchlorate Workers: A Cross-Sectional Occupational Health Study." Journal of Occupational & Environmental Medicine **41**(4): 248-60.

Laurberg, P., S. Andersen, N. Knudsen, L. Ovesen, S. B. Nohr and I. Bulow Pedersen (2002). "Thiocyanate in Food and Iodine in Milk: From Domestic Animal Feeding to Improved Understanding of Cretinism." Thyroid **12**(10): 897-902.

Lawrence, J. E., S. H. Lamm, S. Pino, K. Richman and L. E. Braverman (2000). "The Effect of Short-Term Low-Dose Perchlorate on Various Aspects of Thyroid Function." Thyroid **10**(8): 659-663.

Leigh, M. B., J. S. Fletcher, X. O. Fu and F. J. Schmitz (2002). "Root Turnover: An Important Source of Microbial Substrates in Rhizosphere Remediation of Recalcitrant Contaminants." Environmental Science & Technology **36**(7): 1579-1583.

Licht, L. A. (2006). Difference in Leaf Drop Characteristics between Hybrid Poplar Cultivars DN-34 and DN-21. G. C. Struckhoff. North Liberty, IA.

Liu, Z. J. and D. I. Dickmann (1993). "Responses of 2 Hybrid Populus Clones to Flooding, Drought, and Nitrogen Availability.2. Gas Exchange and Water Relations." Canadian Journal of Botany-Revue Canadienne de Botanique **71**(7): 927-938.

Logan, B. E. (1998). "A Review of Chlorate- and Perchlorate-Respiring Microorganisms." Bioremediation Journal **2**(2): 69-79.

Logan, B. E. (2001). "Assessing the Outlook for Perchlorate Remediation." Environmental Science & Technology **35**(23): 483A-487A.

Logan, B. E., K. Kim, J. Miller, P. Mulvaney, J. Wu, H. Zhang and R. Unz (1999). "Factors Affecting Biodegradation of Perchlorate Contaminated Waters." Preprints of Extended Abstracts presented at the ACS National Meeting, American Chemical Society, Division of Environmental Chemistry **39**(2): 112-114.

Logan, B. E., J. Wu and R. F. Unz (2001a). "Biological Perchlorate Reduction in High-Salinity Solutions." Water Research **35**(12): 3034-3038.

Logan, B. E., H. S. Zhang, P. Mulvaney, M. G. Milner, I. M. Head and R. F. Unz (2001b). "Kinetics of Perchlorate- and Chlorate-Respiring Bacteria." Applied and Environmental Microbiology **67**(6): 2499-2506.

Lovley, D. R., J. L. Fraga, J. D. Coates and E. L. Blunt-Harris (1999). "Humics as an Electron Donor for Anaerobic Respiration." Environmental Microbiology **1**(1): 89-98.

MaDEP (2006). 310 Cmr. Mass. Department of Environmental Protection. http://www.mass.gov/dep/water/laws/perchlorate-310CMR22-07282006.pdf

Mancuso, S. and A. M. Marras (2003). "Different Pathways of the Oxygen Supply in the Sapwood of Young Olea Europaea Trees." Planta **216**(6): 1028-1033.

Matovinovic, I., F. L. Trowbridge, M. Z. Nichaman and M. A. Child (1973). "Iodine Nutriture and Prevalence of Endemic Goiter in USA." Acta Endocrinologica **179**: 17-8.

Mavingui, P., G. Laguerre, O. Berge and T. Heulin (1992). "Genetic and Phenotypic Diversity of Bacillus Polymyxa in Soil and in the Wheat Rhizosphere." Applied and Environmental Microbiology **58**(6): 1894-1903.

McCarty, P. L. and T. E. Meyer (2005). "Numerical Model for Biological Fluidized-Bed Reactor Treatment of Perchlorate-Contaminated Groundwater." Environmental Science & Technology **39**(3): 850-858.

McMahon, K. D., H. G. Martin and P. Hugenholtz (2007). "Integrating Ecology into Biotechnology." Current Opinion in Biotechnology **18**(3): 287.

McNabb, F. M. A., C. T. Larsen and P. S. Pooler (2004). "Ammonium Perchlorate Effects on Thyroid Function and Growth in Bobwhite Quail Chicks." Environmental Toxicology and Chemistry **23**(4): 997-1003.

Merrill, E. A., R. A. Clewell, P. J. Robinson, A. M. Jarabek, J. M. Gearhart, T. R. Sterner and J. W. Fisher (2005). "PBPK Model for Radioactive Iodide and Perchlorate Kinetics and Perchlorate-Induced Inhibition of Iodide Uptake in Humans." Toxicological Sciences **83**(1): 25-43.

Molecular Probes, I. (2006, April 24, 2006). "SYBR Gold Nucleic Acid Gel Stain." Retrieved June 17th, 2009, from http://probes.invitrogen.com/media/pis/mp11494.pdf.

Motzer, W. E. (2001). "Perchlorate: Problems, Detection, and Solutions." Environmental Forensics **2**(4): 301-311.

Muyzer, G., E. C. de Waal and A. G. Uitterlinden (1993). "Profiling of Complex Microbial Populations by Denaturing Gradient Gel Electrophoresis Analysis of Polymerase Chain Reaction-Amplified Genes Coding for 16S rRNA." Applied and Environmental Microbiology **59**(3): 695-700.

Nannipieri, P., J. Ascher, M. T. Ceccherini, L. Landi, G. Pietramellara and G. Renella (2003). "Microbial Diversity and Soil Functions." European Journal of Soil Science **54**(4): 655-670.

NAS (2005). Health Implications of Perchlorate Ingestion. Washington D.C., The National Academies Press.

Nerenberg, R. and B. E. Rittmann (2004). "Hydrogen-Based, Hollow-Fiber Membrane Biofilm Reactor for Reduction of Perchlorate and Other Oxidized Contaminants." Water Science and Technology **49**(11-12, Biofilm Systems V): 223-230.

Nerenberg, R., B. E. Rittmann and I. Najm (2002). "Perchlorate Reduction in a Hydrogen-Based Membrane-Biofilm Reactor." Journal American Water Works Association **94**(11): 103-114.

Nzengung, V. A. and S. C. McCutcheon (2003). "Phytoremediation of Perchlorate." Phytoremediation: 863-885.

Nzengung, V. A. and W. O'Niell (2005). In Situ Bioremediation of Explosives and Perchlorate in Vadose Zone Source Areas. NGWA Conference on MTBE and Perchlorate, San Francisco, CA, National Ground Water Association.

Nzengung, V. A., W. L. O'Niell, S. C. McCutcheon and N. L. Wolfe (2003). "Sequestration and Transformation of Water Soluble Halogenated Organic Compounds Using Aquatic Plants, Algae, and Microbial Mats." Phytoremediation: 499-528.

Nzengung, V. A., H. Penning and W. O'Niell (2004). "Mechanistic Changes During Phytoremediation of Perchlorate under Different Root-Zone Conditions." International Journal of Phytoremediation **6**(1): 63-83.

Nzengung, V. A., C. Wang and G. Harvey (1999). "Plant-Mediated Transformation of Perchlorate into Chloride." Environmental Science and Technology **33**(9): 1470-1478.

Okeke, B. C., T. Giblin and W. T. Frankenberger (2002). "Reduction of Perchlorate and Nitrate by Salt Tolerant Bacteria." Environmental Pollution (Oxford, United Kingdom) **118**(3): 357-363.

Park, C. and E. A. Marchand (2002). Biological Perchlorate Reduction of High-Salinity Wastewaters. Third International Conference on Remediation of Chlorinated and Recalcitrant Compounds, Monterey, CA, USA.

Rao, B., T. A. Anderson, G. J. Orris, K. A. Rainwater, S. Rajagopalan, R. M. Sandvig, B. R. Scanlon, D. A. Stonestrom, M. A. Walvoord and W. A. Jackson (2007). "Widespread Natural Perchlorate in Unsaturated Zones of the Southwest United States." Environmental Science & Technology **41**(13): 4522-4528.

Redfield, E. B., S. M. Durnie and J. J. Zwiazek (2004). "Effects of Hypoxia on Ion Accumulation in Wild Raspberry (Rubus Idaeus) Exposed to Water Released from Saline Oil Sands Mine Tailings." Environmental and Experimental Botany **52**(1): 1-9.

Reeson, A. F., T. Jankovic, M. L. Kasper, S. Rogers and A. D. Austin (2003). "Application of 16s rDNA-DGGE to Examine the Microbial Ecology Associated with a Social Wasp Vespula Germanica." Insect Molecular Biology **12**(1): 85-91.

Renner, R. (1999). "EPA Draft Almost Doubles Safe Dose of Perchlorate in Water." Environmental Science & Technology **33**(5): 110A-111A.

Reynolds, T. D. and P. A. Richards (1996). Unit Operations and Processes in Environmental Engineering. Boston, PWS Pub. Company.

Rikken, G. B., A. G. M. Kroon and C. G. van Ginkel (1996). "Transformation of (Per)Chlorate into Chloride by a Newly Isolated Bacterium: Reduction and Dismutation." Applied Microbiology and Biotechnology **45**(3): 420-426.

Schulz, V., R. Bonn and J. Kindler (1979). "Kinetics of Elimination of Thiocyanate in 7 Healthy Subjects and in 8 Subjects with Renal Failure." Klinische Wochenschrift **57**(5): 243-7.

Shrout, J. D. (2002). Characteristics and Electron Donor Requirements of Perchlorate Degradation by Mixed and Pure-Culture Bacteria. Civil and Environmental Engineering, University of Iowa**:** 163 pp.

Shrout, J. D. and G. F. Parkin (2006). "Influence of Electron Donor, Oxygen, and Redox Potential on Bacterial Perchlorate Degradation." Water Research **40**(6): 1191-1199.

Shrout, J. D., T. E. Scheetz, T. L. Casavant and G. F. Parkin (2005). "Isolation and Characterization of Autotrophic, Hydrogen-Utilizing, Perchlorate-Reducing Bacteria." Applied Microbiology and Biotechnology **67**(2): 261-8.

Shrout, J. D., G. C. Struckhoff, G. F. Parkin and J. L. Schnoor (2006). "Stimulation and Molecular Characterization of Bacterial Perchlorate Degradation by Plant-Produced Electron Donors." Environmental Science & Technology **40**(1): 310-317.

Siebel, H. N. and C. Blom (1998). "Effects of Irregular Flooding on the Establishment of Tree Species." Acta Botanica Neerlandica **47**(2): 231-240.

Sigler, W. V., C. Miniaci and J. Zeyer (2004). "Electrophoresis Time Impacts the Denaturing Gradient Gel Electrophoresis-Based Assessment of Bacterial Community Structure." Journal of Microbiological Methods **57**(1): 17-22.

Siglin, J. C., D. R. Mattie, D. E. Dodd, P. K. Hildebrandt and W. H. Baker (2000). "A 90-Day Drinking Water Toxicity Study in Rats of the Environmental Contaminant Ammonium Perchlorate." Toxicological Sciences **57**(1): 61-74.

Sorrell, B. K. (1999). "Effect of External Oxygen Demand on Radial Oxygen Loss by Juncus Roots in Titanium Citrate Solutions." Plant, Cell & Environment **22**(12): 1587-1593.

Spellerberg, I. F. and P. J. Fedor (2003). "A Tribute to Claude Shannon (1916 - 2001) and a Plea for More Rigorous Use of Species Richness, Species Diversity and the 'Shannon - Wiener' Index." Global Ecology & Biogeography **12**(3): 177-179.

Tonacchera, M., A. Pinchera, A. Dimida, E. Ferrarini, P. Agretti, P. Vitti, F. Santini, K. Crump and J. Gibbs (2004). "Relative Potencies and Additivity of Perchlorate, Thiocyanate, Nitrate, and Iodide on the Inhibition of Radioactive Iodide Uptake by the Human Sodium Iodide Symporter." Thyroid **14**(12): 1012-1019.

Torsvik, V. and L. Øvreås (2002). "Microbial Diversity and Function in Soil: From Genes to Ecosystems." Current Opinion in Microbiology **5**(3): 240.

Tsay, Y. F., C. C. Chiu, C. B. Tsai, C. H. Ho and P. K. Hsu (2007). "Nitrate Transporters and Peptide Transporters." FEBS Letters **581**(12): 2290-2300.

Tsui, D. T., R. A. Clewell, J. E. Eldridge and D. R. Mattie (2000). "Perchlorate Analysis with the AS-16 Separation Column." Environmental Science Research **57**(Perchlorate in the Environment): 59-80.

Tuominen, L., T. Kairesalo and H. Hartikainen (1994). "Comparison of Methods for Inhibiting Bacterial-Activity in Sediment." Applied and Environmental Microbiology **60**(9): 3454-3457.

Urbansky, E. T. (1998). "Perchlorate Chemistry: Implications for Analysis and Remediation." Bioremediation Journal **2**(2): 81-95.

Urbansky, E. T., S. K. Brown, M. L. Magnuson and C. A. Kelty (2001). "Perchlorate Levels in Samples of Sodium Nitrate Fertilizer Derived from Chilean Caliche." Environmental Pollution **112**(3): 299-302.

USEPA (2008). Interim Drinking Water Health Advisory for Perchlorate. Health and Ecological Criteria Division. Office of Science and Technology. Office of Water. http://www.epa.gov/ogwdw000/contaminants/unregulated/pdfs/healthadvisory_perchlorate_interim.pdf

van Aken, B. and J. L. Schnoor (2002). "Evidence of Perchlorate (ClO4-) Reduction in Plant Tissues (Poplar Tree) Using Radio-Labeled (ClO4-)-Cl-36." Environmental Science & Technology **36**(12): 2783-2788.

van Hannen, E. J., G. Zwart, M. P. van Agterveld, H. J. Gons, J. Ebert and H. J. Laanbroek (1999). "Changes in Bacterial and Eukaryotic Community Structure after Mass Lysis of Filamentous Cyanobacteria Associated with Viruses." Applied and Environmental Microbiology **65**(2): 795-801.

Vandermeer, J. (2006). "Oscillating Populations and Biodiversity Maintenance." BioScience **56**(12): 967-975.

Vanderpas, J., P. Bourdoux, R. Lagasse, M. Rivera, M. Dramaix, D. Lody, G. Nelson, F. Delange, A. M. Ermans and C. H. Thilly (1984). "Endemic Infantile Hypothyroidism in a Severe Endemic Goitre Area of Central Africa." Clinical Endocrinology **20**(3): 327-40.

Wallace, W., S. Beshear, D. Williams, S. Hospadar and M. Owens (1998). "Perchlorate Reduction by a Mixed Culture in an up-Flow Anaerobic Fixed Bed Reactor." Journal of Industrial Microbiology & Biotechnology **20**(2): 126-131.

Wallace, W., T. Ward, A. Breen and H. Attaway (1996). "Identification of an Anaerobic Bacterium Which Reduces Perchlorate and Chlorate as Wolinella Succinogenes." Journal of Industrial Microbiology **16**(1): 68-72.

Waller, A. S., E. E. Cox and E. A. Edwards (2004). "Perchlorate-Reducing Microorganisms Isolated from Contaminated Sites." Environmental Microbiology **6**(5): 517-527.

White, P. J. and M. R. Broadley (2001). "Chloride in Soils and Its Uptake and Movement within the Plant: A Review." Annals of Botany **88**(6): 967-988.

Yoshitomi, K. J. and J. R. Shann (2001). "Corn (Zea Mays L.) Root Exudates and Their Impact on C-14- Pyrene Mineralization." Soil Biology & Biochemistry **33**(12-13): 1769-1776.