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Phytoremediation and Bioremediation of Perchlorate at the Longhorn Army Ammunition Plant

Final Report

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Executive Summary

Phytoremediation is the use of vegetation for *in-situ* treatment of hazardous wastes. It is an emerging technology that has proven effective and relatively inexpensive at many pilot and full-scale sites. Plants have shown the ability to withstand high concentrations of some toxic chemicals and take-up contaminants quickly, converting them to innocuous end-products. In addition, plants can stimulate the degradation of chemicals in the rhizosphere by the release of root exudates and enzymes, and the build-up of organic carbon in the soil. Preliminary results have demonstrated hybrid poplars can remove ammonium perchlorate (NH_4ClO_4) from hydroponic solution or simulated groundwater. In addition, it has been demonstrated in the laboratory that facultative bacteria are capable of reducing perchlorate and chlorate under reducing conditions to chloride ions and dissolved oxygen, innocuous end-products.

Work done for this project has addressed objectives specific to the perchlorate concerns at the Longhorn Army Ammunition Plant (LHAAP) at Marshall, Texas. This work consisted of laboratory studies conducted to determine the uptake and transformation of perchlorate by plants and bacteria; also seven novel bacteria capable of perchlorate degradation were isolated from soil samples collected at the LHAAP. The potential for stimulation of bacteria utilizing poplar roots was also investigated.

This is the final report in a series describing our efforts to determine the fate of perchlorate in hybrid poplar trees and microorganisms. This fundamental research was conducted to investigate the possibility of treating the perchlorate issues present at the LHAAP by bioremediation, and to discover the primary design criteria. The work done on this project has shown the potential for success of phytoremediation/rhizoremediation system at the Longhorn Army Ammunition Plant.

Section

2

Introduction

General Background and Bioremediation Potential of Perchlorate

Perchlorate is an oxyanion having a net charge of negative one. Perchlorate salts are highly soluble in water (>200g/L), stable, and labile such that they do not readily react in solution (Urbansky, 1998). The primary military use of perchlorate is as an oxidant for solid rocket fuel. Crystalline particles of ammonium perchlorate salt are incorporated within a polymer binding matrix to comprise as much as 70% of solid rocket propellants (Herman and Frankenberger, 1998). The strong oxidative properties of perchlorate are also currently incorporated in explosives.

Perchlorate contamination at Longhorn Army Ammunition Plant (LHAAP) in Marshall, Texas stems from both the production and disassembly of rockets used for missile defense systems. The site was active with respect to rocket handling during Cold War buildup of missile defense systems as well as dismantling of these systems as part of disarmament stipulated in the INF Treaty of 1987. Production lines utilizing perchlorate at the Longhorn plant included Building 25-C. Destruction of rockets occurred near the INF Pond. This pond continues to be used for retention of site water that has been known to contain perchlorate. Site monitoring data collected from the LHAAP shows a number of locations with high concentrations of perchlorate during monitoring programs in April-May and September, 1999. The groundwater treatment plant at the site is not designed to remove or degrade perchlorate. Thus, there is a need for a positive remedy at the site that is cost-effective and efficient at cleaning groundwater and soils. The LHAAP treatment plant was designed to meet wastewater discharge requirements for recalcitrant organic compounds, primarily trichloroethene (TCE). The discovery of perchlorate subsurface contamination and the need for active treatment stems from investigation conducted after the design of the current LHAAP treatment plant. Concentrations of perchlorate were high in soils and groundwater sampled in the vicinity of Bldg. 25-C and other buildings. Concentrations in groundwater in the area of the burning ground were quite high (ranging up to 200,000 μg/liter), and the depth to groundwater is relatively shallow there (4-8 ft).

The known health risks associated with perchlorate include the ability to promote growth of thyroid tumor cells preventing the uptake of iodine, which impedes the synthesis of thyroid hormones (L-triiodothyronine (T_3) and L-tetraiodotyrosine (T4)) leading to cretinism, an iodine-deficient mental disease (Clark, 1999). Perchlorate is also known to target bone marrow and muscle and possibly cause aplastic anemia at high concentrations (Urbansky, 1998). Additional characterization of human and ecological toxicological and carcinogenic effects is a current focus of the USEPA (USEPA, 1999).

The provisional action level for perchlorate is 22 μg/L for drinking water and 270 μg/kg for soil in the state of Texas. The Texas Natural Resource Conservation Commission has

also adopted a groundwater action level of 66 μg/L for an industrial site (Cowan, 2000). The USEPA has proposed a drinking water maximum contaminant level (MCL) of 32 ppb for perchlorate. Perchlorate was placed on the contaminant candidate list (CCL) of the USEPA in 1998 to address the need to set a National Primary Drinking Water Regulation (NPDWR) for this compound (USEPA, 1998). With increasing concerns over perchlorate contamination, several typical physical and chemical water treatment technologies have been tested (e.g. ion exchange, air stripping, carbon adsorption, advanced oxidation, reverse osmosis), with no cost-effective satisfactory results (Logan, 1998).

More than thirty microorganisms have been isolated that can utilize perchlorate as an electron acceptor. These isolates have utilized various growth substrates and energy sources including: acetate, fumarate, ethanol, and hydrogen (Logan 1998; Herman and Frankenberger 1999; Coates et al., 1999). Initially, the best-characterized perchlorate-utilizing organism was a strain of *Wolinella succinogenes* that has been shown to reduce perchlorate in the presence of various substrates and hydrogen as an electron donor (Wallace, et al., 1996). *W. succinogenes* HAP-1 has been shown to be obligately microaerophilic and can grow in the presence of hydrogen with aspartate, fumarate, malate, pyruvate, or succinate. This organism appears not to grow on glucose, fructose, sucrose, ethanol, methanol, propionate, or formate (Wallace, et al., 1996). No perchlorate degrading enzymes from HAP-1 have been isolated to date. Other known organisms that have been shown to transform perchlorate have been identified—all appear to be facultative anaerobes that display conversion of perchlorate (or chlorate) to chloride with no observed intermediates. *Vibrio dechloraticus* Cuznesove B-1168 has been known, since the 1970s, to grow on acetate or ethanol while degrading perchlorate (Urbansky, 1998). While these isolated bacteria have been shown to grow on a host of electron donors, few have been shown to utilize hydrogen and none have grown autotrophically (using carbon dioxide as a carbon source) on hydrogen. *Dechlorimonas* sp. JM, for example, has been shown to utilize hydrogen as an electron donor but still requires acetate as a carbon source, indicating heterotrophic growth (Miller and Logan, 2000). Other identified perchlorate utilizing organisms capable of complete transformation perchlorate to chloride include strains of *Bacillus cereus* and *Staphylococcus epidermis* and more recently identified *Dechloromonas agitatus* Strain CKB, *Dechlorosporillum*, *Dechlorosoma*, and a proteobacteria, strain GR-1 (Urbansky, 1998; Herman and Frankenberger, 1998; Coates, 1999).

While perchlorate degradation is extremely energetically favorable, this is a reduction reaction requiring an anaerobic environment to proceed. However, few specific values of redox potential are published in the literature to suggest the specific redox requirments of perchlorate reduction. One study suggests that perchlorate reduction will not occur above a redox potential of –110 mV (Attaway and Smith, 1993). Redox chemistry is also of particular interest due to the transformation of perchlorate through its steps of reduction and dismutation, ultimately producing molecular oxygen. Currently, limited information is available to explain perchlorate degradation in the context of naturally occurring multi-component conditions. It is known that perchlorate-utilizing organisms do exist, the thermodynamics of perchlorate reduction are favorable, and the chemistry involved may be complex when anions other than chlorate species are present. However, the results that may be achieved under more varying natural or in-situ conditions are not well characterized.

Recent studies using flow-through biological reactors have shown that addition of hydrogen as an electron donor can sustain perchlorate reduction (Giblin, Herman et al.

2000; Miller and Logan 2000). Pure cultures of *Dechlorosoma* sp.KJ and *Wollinella succinogenes* have shown perchlorate degrading activity in the presence of added hydrogen (Wallace, Ward et al. 1996; Miller and Logan 2000). However, these and other perchlorate-degrading bacteria require organic carbon to support growth; none has shown an ability to utilize carbon dioxide for autotrophic growth (Urbansky 1998; Coates, Michaelidou et al. 1999; Giblin, Herman et al. 2000).

Organisms isolated based on their ability to use perchlorate or chlorate have shown remarkable diversity. Perchlorate reducing isolates have shown the ability to oxidize ferrous iron or sulfide, grow facultatively on oxygen, and in the case of *Dechloromonas* sp. JJ, degrade benzene in the presence of nitrate as an electron acceptor (Bruce, Achenbach et al. 1999; Coates, Michaelidou et al. 1999; Coates, Chakraborty et al. 2001). Many perchlorate-degrading isolates are clustered in the genera *Dechloromonas* and *Dechlorosoma*, yet, perchlorate degraders have been identified throughout the alpa, beta, gamma, and epsilon classes of Proteobacteria (Achenbach, Michaelidou et al. 2001; Logan, Zhang et al. 2001). This diversity of genotype and catabolic capability has justifiably piqued the interest of many environmental researchers. The diversity among perchlorate-reducing organisms is at best marginally identified, characterized, and understood.

Phytoremediation of Contaminated Soils and Waters

Biodegradation, pump and treat, and phytoremediation technologies have been researched and implemented as approaches for remediation of water and soil contaminated with perchlorate. Phytoremediation is the use of vegetation for *in-situ* treatment of hazardous wastes. It is a form of ecological engineering that has proven effective and relatively inexpensive at several pilot and full-scale sites. Phytoremediation is a promising option for contaminated sites with large areas of low level soil or surface water contamination because the operation and maintenance costs for plant based clean-up techniques are lower than the cost of most other treatment technologies.

Phytoremediation has applications at sites with shallow contamination of metals, organics or nutrients. Sites contaminated with metals or organics can be remediated with plants via phytoextraction, rhizofiltration, phytostabilization, phytodegradation, rhizodegradation, or phytovolatilization (EPA 1998) mechanisms:

- Phytoextraction involves the uptake and translocation of metals into the upper portions of the plant system (shoots and leaves). After a substantial amount of the metal has been “extracted” by the plant, the plants are harvested and typically incinerated and the ash is disposed of as hazardous waste. The volume of hazardous waste requiring special containment is greatly reduced using this procedure.

- Rhizofiltration is the adsorption of contaminants onto the surface of the roots or incorporation into the roots without subsequent translocation within the plant. After an acclimation period, the plants are placed in the contaminated area (usually a pond or wetland) and the roots remove the contaminant. When the roots become saturated with pollutants, the plants are harvested and taken to an appropriate facility for disposal.

- Phytostabilization seeks to immobilize contaminants in the groundwater or soil by sorption to roots or precipitation within the root zone. The technique reduces the mobility of the contaminants, thus preventing movement to the groundwater or air. The contaminants are hopefully stabilized after treatment, but it is possible for metals to chelate with soluble root exudates which could then migrate unexpectedly.

- Phytodegradation or phytotransformation involves the metabolic breakdown of contaminants after being uptaken into the plant tissues. It also involves contaminant breakdown outside the plant as a result of plant enzymes that have been exuded from the plant roots. Plant enzymes have been identified that degrade ammunitions, chlorinated solvents and herbicides.

- Rhizodegradation utilizes the synergism between the root zone and soil microbes to enhance the degradation of pollutants by the microbes. The sugars, alcohols, and acids released by the roots provide an easily accessible carbon source and other nutrients necessary to increase microbial activity.

- Phytovolatilization involves uptake and transport of a contaminant through the plant with subsequent release of the compound, or a modified form of the compound, to the atmosphere. A compound may be modified prior to volatilization either by plant metabolic processes or by photodegradation in translucent leaves.

Since plants induce changes in soil conditions (e.g. redox potential, pH, organic matter, microbial communities, hydrodynamics), phytoremediation encompasses a range of processes beyond direct plant metabolism of pollutants and may sometimes be best described as a “plant-assisted” remediation. The idea of using plants for cleaning-up polluted soils and water started with heavy metals. Under certain circumstances, plants exhibit a remarkable ability to concentrate elements from the environment in their tissues (Salt et al., 1998). In addition to uptake and storage of contaminants inside their tissues, plants are known to be able to metabolize some organic xenobiotic compounds. While most studies have addressed the transformation of pesticides an increasing number of reports state the capacities of plants to efficiently detoxify other environmental xenobiotic pollutants including nitroaromatic explosives (TNT, RDX, HMX), organochlorides, polychlorinated biphenyls (PCB), and polyaromatic hydrocarbons (PAH). The “green liver” model of Sandermann (1994) perhaps best describes the fate of organic contaminants within plant tissues. Unlike most microbes, plants, as autotrophic organisms, do not have an extensive array of catabolic pathways for using organic compounds for carbon, energy, and nitrogen. Thus, the metabolism of foreign compounds in plants may best be considered a detoxification mechanism. Initial transformation of xenobiotic compounds in plants (i.e. phase I) involves enzyme-catalyzed oxidation (hydroxylation), reduction, and hydrolysis reactions. The modified products may undergo tranferase-catalyzed conjugation (i.e. phase II) with a molecule of plant origin (e.g. glutathione, glucide, or peptide) forming an compound less toxic than the parent xenobiotic. The resulting conjugate can be sequestrated (e.g. stored in plant organelles, such as vacuoles, or incorporated into biopolymers, such as lignin) or excreted in the case of wetland plants (i.e. phase III) (Coleman et al., 1997).

Except for two recent reports showing a partial transformation of perchlorate inside plant tissues (Susarla et al., 2000, Nzengung et al., 1999), only bacteria have been reported to reduce perchlorate to chloride (Logan,1998, Coates et al., 1999). Nzengung et al. (1999) showed that rooted cuttings of woody plants (*Salix* spp., *Poplar* ssp., and *Eucalyptus cineria*) were able to remove perchlorate from solution. The identified phytoprocesses involved a primary slow uptake followed by a limited phytotransformation of perchlorate in branches and leaves. In a second phase, when the microbial population in the root zone had grown to a significant level, a rapid microbe-mediated degradation was observed, leading to a complete removal of perchlorate. The authors also reported a perchlorate accumulation in branches and leaves. The rate of perchlorate uptake was correlated to the transpiration rate of the trees.

Therefore, the described phytoremediation of perchlorate involved two successive phases. The first one was mediated directly by the plant and included uptake and partial phytotransformation. After a continuous removal from the nutrient solution, the concentration of perchlorate stabilized to a level corresponding to 40-60 % of the initial (100 mg/L) within about 10 days, suggesting that plants possess mechanisms inhibiting the perchlorate uptake after a prolonged exposure (Nzengung et al., 1999). The second phase appears to be related primarily to the development of a perchlorate-degrading microbial population, which led to a complete reduction of the remaining perchlorate into chloride within a few days. This second step started after a lag period (15-40 days) and might be explained by several factors. Two potential factors for this observed lag time involve time for growth and/or the adaptation of perchlorate-degrading populations and, secondly, the development of anaerobic conditions in the root zone to provide for suitable microbial perchlorate-degrading conditions. It is speculated that efficient rhizodegradation outside the plant limited the uptake of perchlorate by plant and its potential subsequent accumulation in the plant tissues (Nzengung et al., 1999).

Direct evidence for limited phytotransformation of perchlorate by plant cells or enzymes has been obtained by incubating crude extracts of French tarragon and spinach in the presence of perchlorate (Nzengung and Wang, 2000). Since the transformation started immediately after the addition of perchlorate, the authors suggested that plant tissues possess mechanisms and constitutive biocatalysts able to efficiently reduce perchlorate. High nitrate concentrations (300-600 mg/L) in the nutrient solution were reported to considerably reduce the degardation process, likely because nitrate competed with perchlorate as terminal electron acceptor under anaerobic conditions (Nzengung et al., 1999).

Susarla et al. (2000) studied the phytoremediation capacities of six plant species, including two tree species (*Liquidambar Styraciflua*, *Salix nigra*), two herbaceous wetland species (*Allenrolfea occidentalis*, *Polygonum punctatum*), and two herbaceous aquatic species (*Nymphea odorata*, *Spirodela polyrhyza*). Working with perchlorate concentrations of 0.2, 2.0, and 20 mg/L, the authors observed uptake rates ranging from 0.0 to 100 % from the hydroponic solution depending on the plant species, the culture conditions (i.e. unwashed sand, washed sand, or no sand), the presence of nutrients, and the perchlorate concentration. The uptake rates were also shown to be higher when other anions were not present or significantly low (e.g. nitrate or chloride). The concentration of perchlorate in the solution remained unchanged when high concentrations of chloride were present. Because no microbial contamination was detected over the time of the experiments and reported lag times for microbial biodegradation of perchlorate are about 1-2 weeks (Wallace et al., 1998), the authors

concluded that the observed transformation of perchlorate resulted solely from the action of vascular plants. The analysis of perchlorate and related metabolites in the plant tissues revealed an accumulation of perchlorate as high as 120 mg/kg wet material for *S. nigra* in the presence of washed sand and nutrients and 981 mg/kg for *P. punctatum* in the presence of washed sand without nutrients. Reduction products of perchlorate have also been detected in the plant tissues upon initial uptake, suggesting an enzymatic stepwise reduction of perchlorate by plant cells (Susarla et al., 2000).

The benefits of reduced site management costs and revegetation provided by phytoremediation may not offset concerns related to the fate of contaminants in the plant tissues. Therefore, research related to this issue must be completed if phytoremediation is to gain full acceptance as a viable treatment technology.

Section

3

Methods

Hybrid Poplar Trees

Poplar tree (genus *Populus*, family Salicaceae) is one of the most studied woody plants, due to its potential for pulp and paper production and its particular usefulness in phytoremediation, e.g. fast growth, large transpiration flux, and re-growth from cut stems (Schnoor et al., 1995, Thompson et al., 1998; Burken and Schnoor, 1999). Small trees used for *in vivo* degradation experiments were produced as previously described (Burken and Schnoor, 1999). In short, 8-inch dormant cuttings of hybrid poplar trees (*Populus deltoides* x *nigra* DN34) were grown in hydroponic solution, under a 16-h/8-h (light/dark) photoperiod. The growth medium consisted of half-strength modified Hoagland nutrient solution at pH 7.0 (Hoagland and Arnon, 1950).

In Vivo Degradation Experiments

After about two weeks, the pre-rooted cuttings, possessing both shoots and roots, were transferred individually into bioreactors consisting in 250-ml conical flasks. Before the transfer, the lower parts of the plant (lower stem and roots in contact with the solution) were sterilized by immersion successively for 30 s in 70 % aqueous ethanol and for 30 s in 1.0 % commercial bleaching solution (5.25 % initial sodium hypochloride, NaClO) containing 0.1 % Tween 80, before being rinsed for 5 min in sterilized distilled water. Each flask contained 200 ml of the growth medium described above and was supplemented with 25 mg l^{-1} radio-labeled perchlorate ($^{36}ClO_4^-$, sodium salt) exhibiting a specific activity of 400,000 dpm mg^{-1}. The bioreactors were sterilized by autoclaving 20 min at 121 °C, 1 atm before introducing the plant. They were closed with a drilled teflon-lined septum and screw cap fitting with the stem passing through. The septum was sealed to the wood segment with teflon tape and silicon caulk. Conical flasks possessed lateral tubing in the upper part, which was stoppered with a cotton plug, allowing gas exchanges with external air. The conical flasks, housing the submerged parts of the plants, were wrapped with aluminum foil in order to prevent light infiltration. The flasks were re-filled every 1-3 days with sterilized distilled water, in order to compensate for transpiration by the leaves. The transpiration volumes were recorded. The concentrations of ClO_4^- and its reduction derivatives, i.e. chlorate (ClO_3^-), chlorite (ClO_2^-), and Cl^-, in the nutrient solution were monitored by removing periodically one-ml aliquots. Samples were frozen at -40 °C overnight and filtered on 0.22-μm Acrodisk® filters (Pall Gelman Laboratory, Ann Arbor, MI) before being analyzed by ion chromatography (IC). Manipulations involving opened flasks were performed under a sterile laminar flow hood. After four weeks, the cultures were sacrificed in order to determine the distribution of ClO_4^- and potential metabolites in the different plants fractions (Nzengung et al., 1999). Plant material was extracted as follows: The different plant fractions, i.e. roots, lower stems (i.e. submerged stems), upper stems (i.e. aerial stems), and leaves were washed

with distilled water, wiped, and weighed, before being dried overnight at 105 °C. Plant fractions were ground mechanically. The resulting raw powdered material was then mixed with a defined volume (25-75 ml) of sodium hydroxide (NaOH, 0.1 M) and homogenized for two min. The suspension was introduced in centrifugation tubes and sonicated overnight. The samples were centrifuged at 15,000 rpm for 30 min. Then, the supernatant was frozen overnight, filtered (0.22-μm), and stored at 4 °C for further analysis. Two further extractions on remaining pellets did not allow the recovery of more than 1.0 % of the ClO_4^- extracted in the first step, indicating that one extraction procedure was sufficient. Bioreactors supplemented with ClO_4^-, but containing a plant whose stem had been cut off at the surface of the screw cap, were used as controls. Another control consisted of bioreactors containing a whole tree incubated without ClO_4^-. Experiments were performed with three replicates. In order to ensure the absence of microbial contamination during the degradation experiments, 0.1-ml aliquots of the nutrient solutions were plated both on nutrient agar 2.3 % (Difco, Sparks, MD) and on yeast-extract agar 2.0 % (Difco). Inoculated Petri dishes were incubated for one week at 37 °C prior to observation.

Production of Nodules

Explants from a hybrid poplar tree (*Populus deltoides* x *nigra* DN34), consisting of 1-cm length pieces of young stems or 1-cm^2 pieces of young leaves, were sterilized by immersion while stirring successively in 70 % aqueous ethanol (30 s), in 5.0 % bleaching solution containing 0.1 % Tween 80 (15 min), in 0.05 % mercuric chloride ($HgCl_2$) (15 min), and rinsed several times in sterilized distilled water. Sterilized explants were grown on solid medium, Murashige and Skoog (1962) culture medium (MS) at pH 5.8 supplemented with 30 g l^{-1} sucrose, 0.9 g l^{-1} agar, and 5.0 mg l^{-1} 2,4-dichlorophenoxyacetic acid (2,4-D) and 1.0 mg l^{-1} 6-furfurylaminopurine (kinetin) as growth regulators. After about one month of growth under a 16-h/8-h photoperiod, cell calli developed actively. About 1-cm^3 callus material was introduced in 500-ml conical flasks containing 300 ml MS liquid medium (without agar) supplemented with 30 g l^{-1} sucrose and the same combination of growth regulators. The flasks were then incubated under agitation at 125 rpm on an orbital shaker and under a 16-h/8-h photoperiod. After about 1-month, the callus material developed in the form of a cell suspension (free cells and small aggregates) containing 20-50 spherical green plant cell aggregates of about 2-20 mm diameter, referred as *nodules* (McCown, 1986). Cell suspension and nodules were separated by filtration on cheesecloth. Nodules were shown to be photosynthetic. Microscopic observation revealed a radial cell organization and a chaotic vascularization. Manipulations were performed under strict sterile conditions.

In Vitro Degradation Experiments

About 10 cm^3 nodule material (3-4 pieces) was introduced into 250-ml conical flasks stoppered with a cotton plug and containing 100 ml of MS liquid medium supplemented with 30 mg l^{-1} sucrose, 5 mg l^{-1} 2,4-D, 1 mg l^{-1} kinetin, and 25 mg l^{-1} $^{36}ClO_4^-$ (sodium salt, 400,000 dpm mg^{-1}). Flasks were incubated under agitation at 125 rpm under a 16-h/8-h photoperiod. One-ml aliquots of medium were collected periodically and treated as described above. Nodules were separated form a light cell suspension by filtrating the solution on cheesecloth. Nodule material was extracted as described above for the plant

fractions. Control experiments were conducted under the same conditions, but contained either autoclaved nodules in the presence of ClO_4^- or living nodules without ClO_4^-. Experiments were performed in triplicate.

Effect of Nitrate on the Uptake of Perchlorate by Plants and Nodules

In order to investigate the influence of nitrate (NO_3^-) in the nutrient solution on the uptake of perchorate by both plants and nodules, modified Hoagland and MS solutions were prepared where nitrate (NO_3^-) was replaced by ammonia (NH_4^+), keeping the total inorganic nitrogen unchanged. Modified solutions were supplemented with increasing amounts of NO_3^- (sodium salt): 0.0, 50, 100, 200, and 400 mg l^{-1}. Degradations experiments using cuttings or nodules were performed as described above. The initial concentration of ClO_4^- (sodium salt) in the solution was 25 mg l^{-1}.

Toxicity Assessments

Two-day pre-grown fresh cuttings bearing two green developing buds but no roots were transferred in the previously-described 250-mL conical flasks, containing 240 mL of modified half-strength Hoagland solution at pH 7.0 supplemented with increasing concentrations of $NaClO_4$ ranging from 0.0 to 500 mg/L. In order to compensate the increase of ionic strength due to $NaClO_4$, NaCl was added to the solution in concentrations such that the sum of chloride and perchlorate equivalents was maintained constant throughout the different solutions. The lower parts of the plant in contact with the solution and the conical flasks were sterilized as previously described. Flasks were closed with a drilled teflon-lined septum and screw cap fitting the stem passing through and were wrapped with aluminum foil. Small trees were allowed to grow under a 16-h/8-h (light/dark) photoperiod. Every 4-6 days, the plants were weighted under sterile atmosphere and reintroduced in fresh sterile Hoagland solutions containing the same concentrations of $NaCl/NaClO_4$. The transpiration volumes over the previous four days were recorded. Experiments were performed in triplicate. After 24 days, plants were sacrificed, washed with distillate water, and the fresh weight of the different parts (roots, lower stems, upper stems, and leaves) was determined.

Analysis

Analysis of ClO_4^- and reduction metabolites, i.e. ClO_3^-, ClO_2^-, and Cl^-, were performed by ion chromatography (IC) using a Dionex DX-500 ion chromatograph (Dionex Co., Sunnyvale, CA) equipped with a Dionex ASRS suppressor operating in external water mode. Twenty to 200 μl-injections were made using a Dionex AS50 autosampler. Separation was achieved on a Dionex AS11 column maintained at 40°C using a NaOH eluent flowing at 1 ml min^{-1}. For the quantitative determination of ClO_4^- alone, the injection volume was 50 μl and the eluent was an isocratic solvent system consisting of 25 mM NaOH. For the simultaneous determination of ClO_4^- and ClO_4^- metabolites, the injection volume was 20 μl and the eluent was a gradient solvent system. The initial solvent composition was 100 % H_2O, a linear gradient up to 2 mM NaOH was run over 20 min, then a linear gradient up to 25 mM NaOH was run over the next 5 min and held for 5 min. Finally, a linear gradient came back to the initial solvent of 100 % H_2O and the column was equilibrated for 5 min. Intermediates and products were identified by comparison of

their retention times with authentic standards. The total ^{36}Cl radioactivity in the solutions and in the extracts was determined by mixing 100 μl sample and 900 μl H_2O with 9 ml scintillation cocktail (Ultima Gold XR, Packard Instrument Co., Meriden, CA) and by analyzing the radioactivity (dpm) using a Beckman liquid scintillation counter (LSC) LS 6000IC (Beckman Coulter Inc., Fullerton, CA). The total radioactivity, expressed in dpm, was processed using the Auto DPM method (Beckman). Distribution of the radioactivity in the samples was analyzed by IC using the gradient system described above. The injection volume was 200 μl sample and 1-ml fractions of outflow were collected every min on a fraction collector Dynamax FC-1 (Rainin Instrument Inc., Woburn, MA). One-ml fractions were mixed with 9 ml scintillation cocktail before being assayed by LSC as described above.

Synthesis of ^{36}Cl Radio-Labeled Perchlorate

^{36}Cl radio-labeled ClO_4^- was synthesized from ^{36}Cl radio-labeled sodium chloride ($Na^{36}Cl$) (DuPont NEN, Boston, MA) by electrochemical oxidation of chloride ($^{36}Cl^-$) according to a modified method described by Walton (1948). In short, $Na^{36}Cl$ (1.0 mCi ml^{-1}) was mixed with non-labeled NaCl in aqueous solution to a concentration of 165 mg l^{-1} NaCl (i.e. 100 mg l^{-1} Cl^-) and a radioactivity of 2.2 10^8 dpm ml^{-1}. The solution was slightly acidified with a few drops of HCl 0.1 N. Three ml of the reaction mixture underwent electrolysis in a stirred 10-ml sealed cell. Electrodes were platinum (Pt) wires and the system worked at a fixed current intensity of 0.025 A for 2 days, increased to 0.05 A on the third day. The potential difference was about 3 V. Samples were collected periodically to monitor the reaction advancement, i.e. the stepwise oxidation of Cl^- into hypochlorite (ClO^-), ClO_2^-, ClO_3^-, and ClO_4^-. After two weeks, the reaction yield was 100 % formation of ClO_4^-. The reaction mixture was further purified by recrystallization and treatment on OnGuard-Ag cartridge (Dionex). Labeled and non-labeled ClO_4^- were mixed to obtain a stock solution with a final concentration of 5.0 mg ml^{-1} ClO_4^- and a specific radioactivity of 2.0 10^6 dpm ml^{-1} (i.e. 400,000 dpm mg^{-1} ClO_4^-).

Chemicals

All chemicals were of analytical grade and purchased from Fluka (Ronkonkoma, NY) or Sigma (St Louis, MO). Plant growth regulators were from Sigma. ^{36}Cl radio-labeled sodium chloride ($Na^{36}Cl$) was purchased from DuPont NEN.

Development of Soil Microcosms

Soil, sediment, and water samples were collected at the Longhorn Army Ammunition Plant in Karnack, Texas from areas believed or known to have perchlorate contamination (Smith, Theodorakis et al. 2001). Two soil samples were collected within the top 10 cm of soil near "Building 25-C" and two sediment samples were collected from the top 5 cm of sediment near the shore of the "INF Holding Pond". Surface water was also collected from the INF Holding Pond. One groundwater sample was collected from Extraction Well IBJ-004. All samples were collected in glass or plastic containers leaving no additional headspace and stored on ice.

Within 24 hours, batch soil microcosm slurries were started from 2 g of site soil or sediment with 50 mL site water or reduced nutrient media spiked with perchlorate in 60 mL serum bottles. Site water contained 30-250 mg L-1 perchlorate and required no perchlorate addition. All bottles were sealed with Teflon coated rubber stoppers and sparged with N2/CO2 gas (80/20, v/v). Lactate or hydrogen was added as an electron donor to some conditions. All bottles were incubated at 20ºC in the dark on a shaker table at 160 rpm.

Isolation of Perchlorate Reducing Bacteria

Bacteria were isolated from soil microcosms displaying repeated reduction of perchlorate spikes that received hydrogen or lactate. Standard anaerobic techniques were used for all isolations (Hungate 1969). "Freshwater" media was heated near boiling under N_2/CO_2 gas (80/20, v/v), dispensed into 25 mL anaerobic pressure tubes, capped with thick, butyl-rubber stoppers, and sterilized by autoclaving. Media contained 5mM sodium perchlorate as an electron acceptor and 10 mM lactate, 10 mM acetate, or 40% (headspace) H_2 as an electron donor. Soil microcosm slurry (0.1-0.5 mL) was inoculated into ten milliliters sterile media and incubated at 30ºC in the dark (Table).

Positive enrichments were identified by an increased optical density and were transferred (1 in 10) into fresh media. After two transfers, isolated colonies were obtained by growth of positive enrichments in 1.2% Noble agar shake tubes containing 5 mM perchlorate and 10 mM lactate, 10 mM acetate, or 40% (headspace) H_2 (Hungate 1969).

Media

A reduced anaerobic nutrient media used for some soil microcosms contained NH_4HCO_3 (1200 mg L^{-1}), $KHCO_3$ (540 mg L^{-1}), $NaS\bullet 9H_20$ (300 mg L^{-1}), $FeO_4P\bullet 4H_20$ (45 mg L^{-1}), $MgHPO_4\bullet 3H_20$ (575 mg L^{-1}), $CaCO_3$ (17 mg L^{-1}), $(NH_4)_2HPO_4$ (160 mg L^{-1}), $CoBr_2$ (2.2 mg L^{-1}), KI (2.5 mg L^{-1}), $(NaPO_3)6$ (10 mg L^{-1}), $MnBr_2$ (0.5 mg L^{-1}), NH_4VO_3 (0.5 mg L^{-1}), $ZnBr_2$ (0.5 mg L^{-1}), $NaMoO_4\bullet 2H_20$ (0.5 mg L^{-1}), H_3BO_3 (0.5 mg L^{-1}), $NiBr_2$ (0.5 mg L^{-1}), and cysteine (10 mg L^{-1}).

The "Freshwater" media used for isolation of perchlorate degrading bacteria is described by Bruce et al. (Bruce, Achenbach et al. 1999).

Growth of Isolates

Multiple 160 mL serum bottles containing 100 mL Freshwater media with perchlorate and a headspace of N_2, CO_2, and H_2 gas were used to monitor increases in cell mass during growth on hydrogen. Due to concerns of maintaining the integrity of headspace (and available hydrogen), each serum bottle in a series was treated as one sample and was sacrificed at a given time to determine the hydrogen, perchlorate, and dry cell weight for each recorded data point. A 600 mL Pyrex bottle, fitted with a special cap utilizing a butyl-rubber stoppered port to allow syringe sampling, containing 500 mL Freshwater media with perchlorate and lactate was used to monitor increases in cell mass during growth on lactate. Aqueous samples were collected over time to measure perchlorate concentrations, dry cell weight, and optical density. All bottles were autoclaved prior to inoculation.

The ability of all isolates to utilize lactate, acetate, hydrogen, succinate, propionate, butyrate, formate, casamino acids, glucose, ethanol, benzoate, benzene, or ferrous iron as electron donors for growth on 5 mM perchlorate was investigated. Additionally, the ability of these strains to grow using lactate, acetate, hydrogen, glucose, ethanol, benzoate, or benzene with 5 mM nitrate was investigated. Standard anaerobic techniques were used as cultures were inoculated into 10 mL

sterile "Freshwater" media under N_2/CO_2 gas (80/20, v/v) in 25 mL anaerobic pressure tubes (Hungate 1969). Media contained 5mM sodium perchlorate as an electron acceptor and electron donor in the form of dissolved sodium salt or acid, or 40% (headspace) in the case of hydrogen. Similarly, all isolates were investigated for the potential to grow using the electron acceptors chlorate, nitrate, oxygen, fumarate, sulfate, sulfite, selenate, ferrous iron, tetrachloroethene (PCE), and trichloroethene (TCE) with 10 mM acetate. The ability of isolates to grow on some electron acceptors with 10mM lactate or 40% hydrogen headspace was also investigated. All tubes containing PCE, TCE, or benzene were stoppered with Teflon coated rubber septa to limit sorption.

^{14}C experiments

One μCi $H^{14}CO_3$ (Sigma Chemical) was added to 750 mL of Freshwater media containing 10 mM perchlorate and 40% H_2 (headspace) in a one-liter Pyrex bottle fitted with a special cap utilizing a butyl-rubber stoppered port to allow syringe sampling and was autoclaved prior to inoculation. Aqueous samples were collected and 100 μL were dissolved in 10 mL Scintiverse (Fischer Scientific) scintillation cocktail for quantification of bulk radioactivity by a Beckman (Fullerton, CA) model LS 6000IC liquid scintillation system. Additional samples were analyzed for perchlorate. Gaseous samples of 200 μL were slowly passed through 10 mL RJ Harvey (Hillsdale, NJ) scintillation cocktail for counting. At the end of the experiment, contents of the Pyrex bottles were sacrificed and filtered through Whatman (Clifton, NJ) GF/C 1.2 μm pore glass filters. The filters were combusted in a RJ Harvey model OX-600 biological oxidizer at a temperature of 900°C with nitrogen and oxygen flows of 350 mL min-1 each. RJ Harvey cocktail was used as the ^{14}CO2 trapping solution and liquid scintillation fluid.

Chlorite Utilization Assays

Wet, unwashed, cell suspensions were examined for activity to degrade chlorite. Chlorite utilizing activity was determined by assaying the oxidation of O-dianisidine by horseradish peroxidase at 450 nm of samples collected over time from a master reaction containing washed cells, 10 mM chlorite, and 1 M phosphate buffer (pH 7.3) as outlined by Coates, et al. (Coates, Michaelidou et al. 1999).

Growth of Bacteria on Root Products

Root exudate was prepared by collecting the hydroponic solution remaining for 12 poplar cuttings grown in a single batch reactor for 30 days. Cuttings were grown and maintained as described previously, as Hoagland's solution was added to the growth vessel as necessary to account for transpiration of the plants. After 30 days, the remaining liquid in the growth vessel was recovered and filter sterilized to form the resultant root exudate.

Root homogenate was prepared by homogenizing poplar roots from healthy growing poplar cuttings with a hand blender into a slurry. This slurry was further filter sterilized to form the root homogenate used for degradation studies.

Reduction of perchlorate using Strains JDS 1—JDS7 and perchlorate-acclimated culture LEC-PH was investigated using sealed, 25 mL test tubes were purged with N_2/CO_2 gas (80/20, v/v) and sterilized by autoclave. Eight milliliters of root exudate or four milliliters of root homogenate was added to concentrated Hoagland's solution to reach a final volume of 10 mL with approximately ½

Hoagland's solution strength containing 5mM perchlorate. Sterile techniques were used to inoculate each of the tubes which were incubated in the dark at 30°C.

Analyses

Analysis for perchlorate, chlorate, chlorite, and chloride was performed using a Dionex (Sunnyvale, CA) DX-500 ion chromatograph equipped with a Dionex ASRS suppressor operating in external water mode with a regenerant of 10 mM sulfuric acid. Separation was achieved with a Dionex AS11 column, maintained at 40°C, by an eluent gradient of 2 mM sodium hydroxide to 50 mM sodium hydroxide flowing at 1 mL min-1 over twenty minutes controlled by a Dionex GP50 gradient pump. A Dionex CD20 conductivity detector performed detection and peak areas were integrated by Dionex PeakNet software. Injection volumes depended upon the initial concentration of perchlorate. The detection limit for perchlorate was approximately 0.1 mg/L. The detection levels for chlorite and chlorate were approximately 1 mg/L. Analysis for chloride required a separate injection of samples diluted 1 in 10. The detection limit for chloride was approximately 0.01 mg/L.

Hydrogen analysis was performed using a Hewlett Packard 5890 series II gas chromatograph equipped with a thermal conductivity detector. Manual injections of 40 μL gas using a Pressure-lock gas syringe were separated using an Alltech HayesepQ 8-foot stainless steel packed column and nitrogen carrier gas of 60 mL min^{-1}. The detection limit of hydrogen was approximately 0.001 mmol/L.

Analysis for PCE, TCE, DCE, and vinyl chloride was conducted using an Agilent 6890 gas chromatograph equipped with an O-I Analytical 5320 electrolytic conductivity detector. Manual injections of 100 μL were separated using a JW Scientific 60m×0.35mm DB-VB phase capillary column and a helium carrier gas velocity of 42 cm sec^{-1}. The detection limit of these halogenated ethenes was approximately 0.01 μmol/L.

Analysis for ethene and ethane was conducted using a Hewlett Packard 5890 series II gas chromatograph equipped with a flame ionization detector. Manual injections of 100 μL were separated using a JW Scientific 30m×0.53mm GCQ phase capillary column and a nitrogen carrier gas velocity of 8.6 cm sec^{-1}. The detection limit of ethene and ethane was approximately 0.01 μmol/L.

Analysis for benzene was conducted using a Hewlett Packard 5890 series II gas chromatograph equipped with a flame ionization detector. Manual injections of 100 μL were separated using a JW Scientific 30m×0.32mm DB1 phase capillary column and a nitrogen carrier gas of 2 mL min^{-1}. The detection limit for benzene was approximately 0.01 mmol/L.

Dry cell mass was determined by measuring the volatile suspended solids (VSS) or total suspended solids (TSS) by standard wet chemistry techniques (APHA, AWWA et al. 1989). TSS was utilized only when volatile cell fractions were required for additional analyses (i.e. recovery of ^{14}C). Dry cell mass was used as the primary measure of cell quantification due to the heterogeneous growth of JDS5 and JDS6 as discussed under Results.

Chemical Oxygen Demand (COD) was determined by standard wet chemistry techniques by the closed reflux method (APHA, AWWA et al. 1989).

Optical density at a wavelength of 600 nm and other absorbance measurements were determined using a Spectronic (Rochester, NY) Genesys 5 spectrophotometer.

Microscopic Investigation

Wet cell mounts and Gram stains of all strains were investigated optically using a Zeiss (Göttingen, GDR) AxioTech microscope and Zeiss Axiovision software installed on a PC.

16S rDNA gene sequencing and analysis

Cells from 10-mL cultures of perchlorate reducing bacteria were harvested by centrifugation, resuspended in 1 mL sterile water, and lysed by adding 20 μL of chloroform and incubating the preparations for 10 min at 90ºC. Primers specific to bacterial 16S rDNA were used (primer 8F [5'-AGAGTTTGATCCTGGCTCAG-3'] and primer 1525R [5'-AGGAGGTGATCCAGCC-3']) (Coates, Michaelidou et al. 1999). 5 μL lysed cells were added in 50 μL of 10% DMSO, Eppendorf (Hamburg, GDR) Taq Buffer, 2.5mM dNTPs, 1mM 8F and 1525R primers, and 0.1 U mL^{-1} Taq polymerase. The samples were incubated in an Eppendorf Mastercycler with an initial period of 7 minutes at 94°C (during which the Taq polymerase was added); 30 cycles of 1 minute at 94°C, 1 minute at 55°C, and 2 minutes at 72°C; and an end cycle of 10 minutes at 72°C. Products (20 μL) from these incubations was mixed with 4 μL of "loading dye" containing 15% ficoll, 0.25% bromo blue, and 0.25% xylene cyanol FF; loaded onto a 0.8% agarose gel containing ethidium bromide; and separated by electrophoresis using a fixed voltage of 55V for 1 hour 15 minutes. All bands were approximately 1.5 kilobases in length. DNA was extracted using a Qiagen (Valencia, CA) QIAquick Extraction Kit and submitted for sequencing by Sanger-based fluorescent identification of bases performed using an ABI 3700 electrophoresis detector by the DNA Facility at the University of Iowa.

A phylogenetic tree was prepared for comparison recovered 16S rDNA sequences and compared against 16S rDNA gene sequences. Phred (Ewing, Hillier et al. 1998) was used to extract sequence from raw sequencing chromatographs for the seven isolates (Isolate JDS1 - Isolate JDS7) obtained from ABI 3700 sequences. A multiple sequence global alignment was then performed on these sequences using clustalw (Jeanmougin, Thompson et al. 1998). The aligned sequences were then edited using BioEdit (http://jwbrown.mbio.ncsu.edu/BioEdit/bioedit.html) to retain only the commonly shared region across all 56 species. This edited alignment was then utilized by MEGA2 (http://www.megasoftware.net/) to perform a maximum parsimony analysis. Bootstrapping with 100 replicates was performed, with boot-strap values indicated at the branch nodes.

GenBank sequences from the following organisms were used to construct the phylogentic tree (Achenbach, Michaelidou et al. 2001; Logan, Zhang et al. 2001): *Wollinella succinogenes* ATCC 29543 (M26636), *Treponema pallidum* (M88726), *Magnetospirillum gryphiswaldense* (Y10109), *Dechlorosporillum* sp.WD (AF170352), *Azospirillum brasilense* (Z29617), *Azospirillum* sp. TTI (AF170353), *Comamomas testosteroni* (M11224), *Ideonella dechloratans* (X72724), *Dechloromonas* sp. FL2 (AF288771), *Dechloromonas* sp. FL8 (AF288772), *Dechloromonas* sp. FL9 (AF288773), *Dechloromonas agitus* strain CKB (AF047462), *Dechloromonas* sp. CL (AF170354), *Dechloromonas* sp. NM (AF170355), *Dechloromonas* sp. CL24 (AF288775), *Dechloromonas* sp. CL24+ (AF288774), *Dechloromonas* sp. JJ (AY032611), *Dechloromonas* sp. JM (AF323489), *Dechloromonas* sp. MissR (AF170357),

Dechloromonas sp. CCO (AF288776), *Dechloromonas* sp. SIUL (AF170356), *Ferribacterium limneticum* strain CDA (Y17060), *Rhodocyclus tenuis* strain DSM109 (D16208), *Rhodocyclus tenuis* strain DSM110 (D16209), *Rhodocyclus purpureus* strain DSM168 (M34132), *Azoarcus evansii* (X77679), *Azoarcus denitrificans* strain Td-17(L33689), *Thauera selenatis* (X68491), *Azoarcus indigens* strain VB32 (L15531), *Duganella zoogloeoides* (previously *Zoogloea ramigera*; X74913), *Dechlorosoma suilla* strain PS (AF170348), *Dechlorosoma* sp. SDGM (AF170349), *Dechlorosoma* sp. Iso1 (AF170350), *Dechlorosoma* sp. Iso2 (AF170351), *Dechlorosoma* sp. PDX (AF323490), *Dechlorosoma* sp. KJ (AF323491), Gill symbiont of *Thyasira flexuosa* (L01575), *Dechloromarinus chlorophilus* strain NSS (AF170359), *Pseudomonas stutzeri* (U26415), *Pseudomonas* sp. PK (AF170358), *Pseudomonas* sp. CFPBD (AF288777), *Helicobacter pylori* (M88157), *Propionivibrio limicola* strain GolChi1 T.(AJ307983), bacterium DCE10 (AJ249226), *Sulfurospirillum deleyianum* (Y13671), *Campylobacter* sp. (L14632), *Dehalospirillum multivorans* (AF218076), *Geospirillum barnesii* (U41564), and *Propionibacter pelophilus* (AF016690).

The sequences determined for strains JDS1-JDS7 have been deposited in the GenBank database with accession numbers AY084082 to AY084088.

Section

4

Results

Degradation Experiments with Trees and Nodules

Both trees and nodules (Pictures 1 and 2) grew and appeared healthy over the time of the experiments (30 d). The average growth of the trees contacted by perchlorate, assessed by the increase of fresh biomass, was 0.24 ± 0.07 g /day (or 0.61 ± 0.22 % of the initial weight /day) and does not shown significant differences with the controls growing without perchlorate (0.24 ± 0.09 g/day or 0.74 ± 0.34 % per day). Similarly, the transpiration volumes over the time of the experiment were not significantly different for the plants growing either in the presence or in the absence of perchlorate (Figure 1).

Picture 1: Poplar "nodule" cultures.

Picture 2: Poplar tree "cutting" cultures.

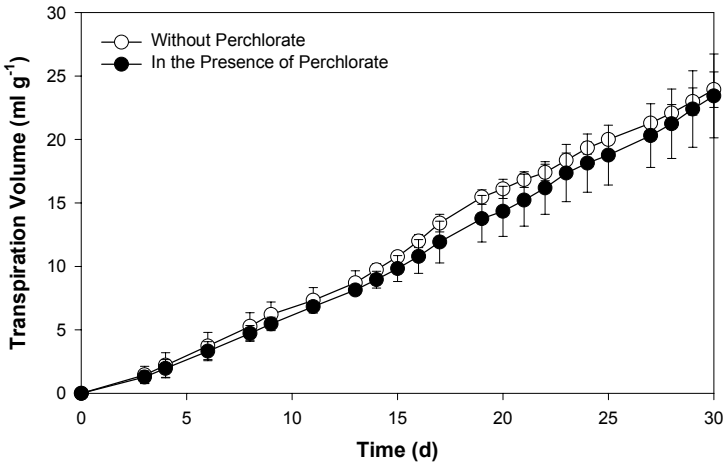

Figure 1. Transpiration of plants growing in the presence and in the absence of ClO_4^- (initial concentration 25 mg/L).

The nodule biomass grew in a comparable manner in the presence of perchlorate (0.23 ± 0.03 g /day or 3.03 ± 1.31 % of the initial weight /day) and in the controls without perchlorate (0.25 ± 0.08 g /day or 3.27 ± 1.45 % /day).

The increase of nodule biomass in the presence of ClO_4^- was 0.23 ± 0.03 g d^{-1} (or 3.03 ± 1.31 % of the initial weight d^{-1}), which was comparable to the increase of the biomass observed in the controls without ClO_4^- (0.25 ± 0.08 g d^{-1} or 3.27 ± 1.45 % d^{-1}).

Over the 30-d experiment, trees were shown to reduce continuously the ClO_4^- concentration in the hydroponic solution to 51.6 ± 1.2 % of the initial 25 mg l^{-1}, which corresponds to a specific reduction rate of 1.9 ± 0.2 ng d^{-1} g^{-1} of fresh plant biomass in average over the period. At the same time, the radioactivity in solution decreased to 67.2 ± 2.3 % of the initial concentration after 30 d (Figure 2). Neither the ClO_4^- concentration nor the radioactivity decreased significantly in the control solutions with excised trees, i.e. consisting only of lower stem and roots.

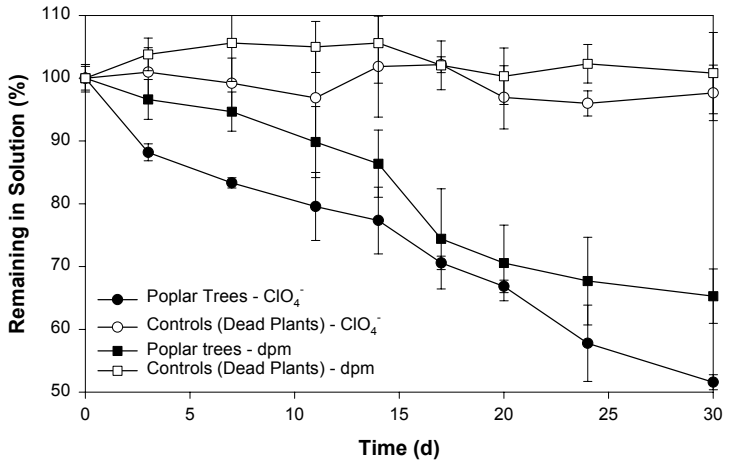

Figure 2. Uptake of ClO_4^- (initial concentration 25 mg l^{-1}) by poplar trees as measured by the ClO_4^- concentration (ion chromatography) and by the radioactivity (LSC) remaining in solution.

The submerged plant nodules were shown to reduce the initial ClO_4^- concentration from the liquid medium to 50.4 ± 5.8 % of the initial 25 mg l^{-1} after 30 d. Radioactivity in solution decreased to 53.1 ± 7.0 % of the initial concentration over the same period of time (Figure 3). The average specific rate of ClO_4^- reduction was 2.6 ± 0.3 ng d^{-1} g^{-1} of fresh biomass. No significant change of ClO_4^- concentration or radioactivity was recorded in the control solutions containing autoclaved nodules.

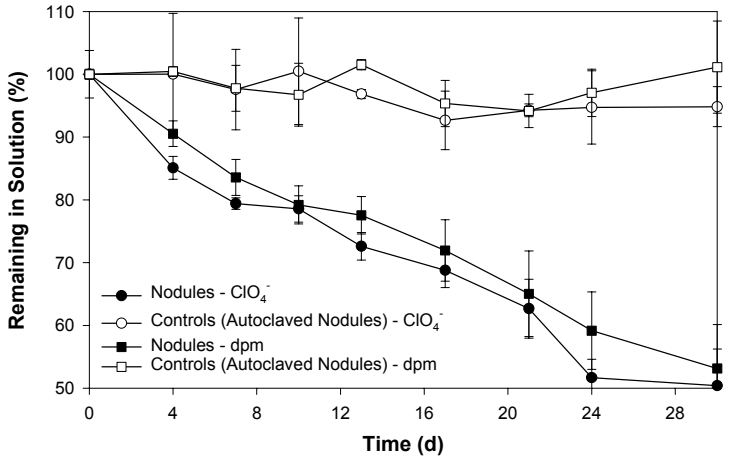

Figure 3. Uptake of ClO_4^- (initial concentration 25 mg l^{-1}) by plant nodules as measured by the ClO_4^- concentration (ion chromatography) and by the radioactivity (LSC) remaining in solution.

Analysis of the distribution of the radioactivity in the different parts of the plants showed that 27.4 ± 4.0 % of the initial radioactivity was translocated to the leaves, which represents 91.5 ± 13.4 % of the total radioactivity taken up by the plants. Very little radioactivity (2.6 ± 0.8 % of the initial) was detected in the other parts of the plants (i.e. roots, lower stems, and upper stems) (Table 1).

Table 1. Distribution of ClO_4^- (Initial Concentration 25 mg l^{-1}) in Different Plant Fractions after 30 d of Exposure

Fraction	Leaves	Roots	Upper Stems	Lower Stems	Hydroponic Solution	Balance
Plants Exposed to $^{36}ClO_4^-$						
Radioactivity ^{36}Cl (% of the Initial)	27.35 ± 4.01	1.27 ± 0.32	0.84 ± 0.33	0.44 ± 0.19	66.93 ± 2.03	96.83 ± 2.48
Controls Exposed to $^{36}ClO_4^-$						
Radioactivity ^{36}Cl (% of the Initial)				2.53 ± 1.10	100.81 ± 6.49	103.34 ± 7.37

Table 2. Distribution of ClO_4^- (Initial Concentration 25 mg l^{-1}) in Different Fractions of Nodule Cultures after 30 d of Exposure

Fraction	Nodules	Free Cells	Nutrient Solution	Balance
Nodules Exposed to $^{36}ClO_4^-$				
Radioactivity ^{36}Cl (% of the Initial)	43.00 ± 2.04	0.00 ± 0.00	53.13 ± 7.00	96.13 ± 8.19
Controls Exposed to $^{36}ClO_4^-$				
Radioactivity ^{36}Cl (% of the Initial)	1.37 ± 0.23	0.00 ± 0.00	101.14 ± 7.33	102.51 ± 7.41

Looking at the chemical speciation of radioactive ^{36}Cl revealed that 32.0 % of the radioactivity recovered in the solution after 30 d (i.e. 19.7 % of the total) consisted of $^{36}Cl^-$ and 68.0 % (i.e. 41.8 % of the total) of non-transformed $^{36}ClO_4^-$. On the other hand, the radioactivity recovered in the leaf extracts was distributed into $^{36}Cl^-$ (1.6 % of the total), $^{36}ClO_2^-$ (2.4 % of the total), $^{36}ClO_3^-$ (4.8 % of the total), $^{36}ClO_4^-$ (21.6 % of the total), and an unidentified, likely organic compound (1.4 % of the total) (Table 3 and Figure 4).

Table 3. Distribution (Percent of the Total) of the Radioactivity Originating from the Initial $^{36}ClO_4^-$ (25 mg l^{-1}) among Reduced Metabolites in Different Extracts of the Plants after 30 d of Exposure

Fraction	ClO_4^-	ClO_3^-	ClO_2^-	Cl^-	Unidentified	Total
Solution t = 0	100.00	0.00	0.00	0.00	0.00	100.00
Solution t = 30 d	41.75	0.00	0.00	19.67	0.00	61.42
Leaf Extract	21.59	4.81	2.42	1.64	1.37	31.83
Control Solution t = 30 d	102.30	0.00	0.00	0.0	0.00	102.30

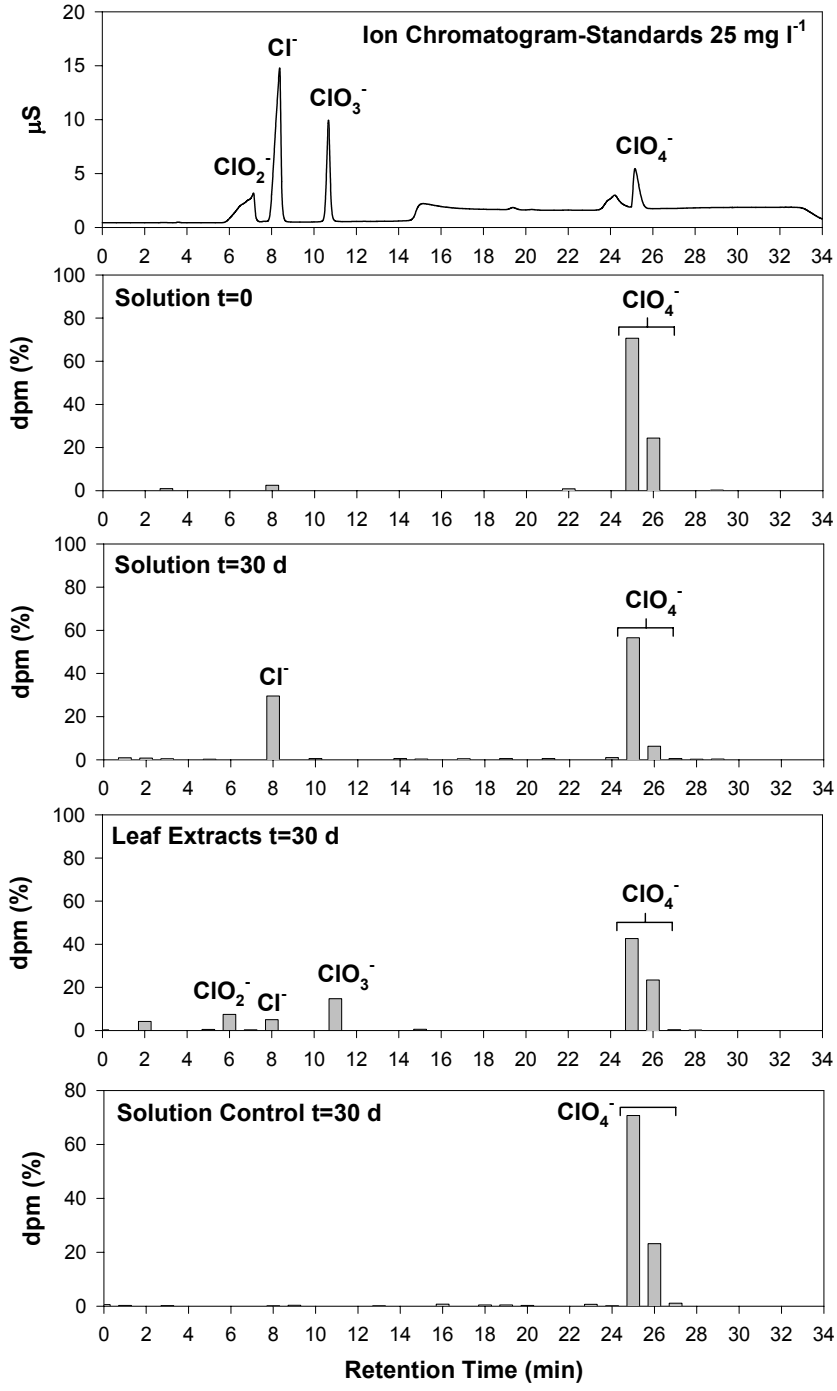

Figure 4. Distribution of the radioactivity (percent) originating from the initial $^{36}ClO_4^-$ (25 mg l^{-1}) among reduced metabolites in different extracts of plant cultures after 30 d of exposure.

The radioactivity recovered in the solution containing submerged nodules consisted of $^{36}Cl^-$ (6.4 % of the total), $^{36}ClO_3^-$ (1.3 % of the total), and non-transformed $^{36}ClO_4^-$ (51.5 % of the total). The radioactivity detected in the nodule extracts was distributed into $^{36}Cl^-$ (2.0 % of the total), $^{36}ClO_2^-$ (5.2 % of the total), $^{36}ClO_3^-$ (6.4 % of the total), $^{36}ClO_4^-$ (22.7 % of the total), and an unidentified organic compound (0.5 % of the total) (Table 4 and Figure 5).

Table 4. Distribution (Percent of the Total) of the Radioactivity Originating from the Initial $^{36}ClO_4^-$ (25 mg l^{-1}) among Reduced Metabolites in Different Extracts of Nodule Cultures after 30 d of Exposure

Fraction	ClO_4^-	ClO_3^-	ClO_2^-	Cl^-	Unidentified	Total
Solution t = 0	100.00	0.00	0.00	0.00	0.00	100.00
Solution t = 30 d	51.51	1.31	0.00	6.64	0.00	57.52
Nodule Extract	22.67	6.41	5.15	1.97	0.52	36.71
Control Solution t = 30 d	93.13	0.00	0.00	0.00	0.00	93.13

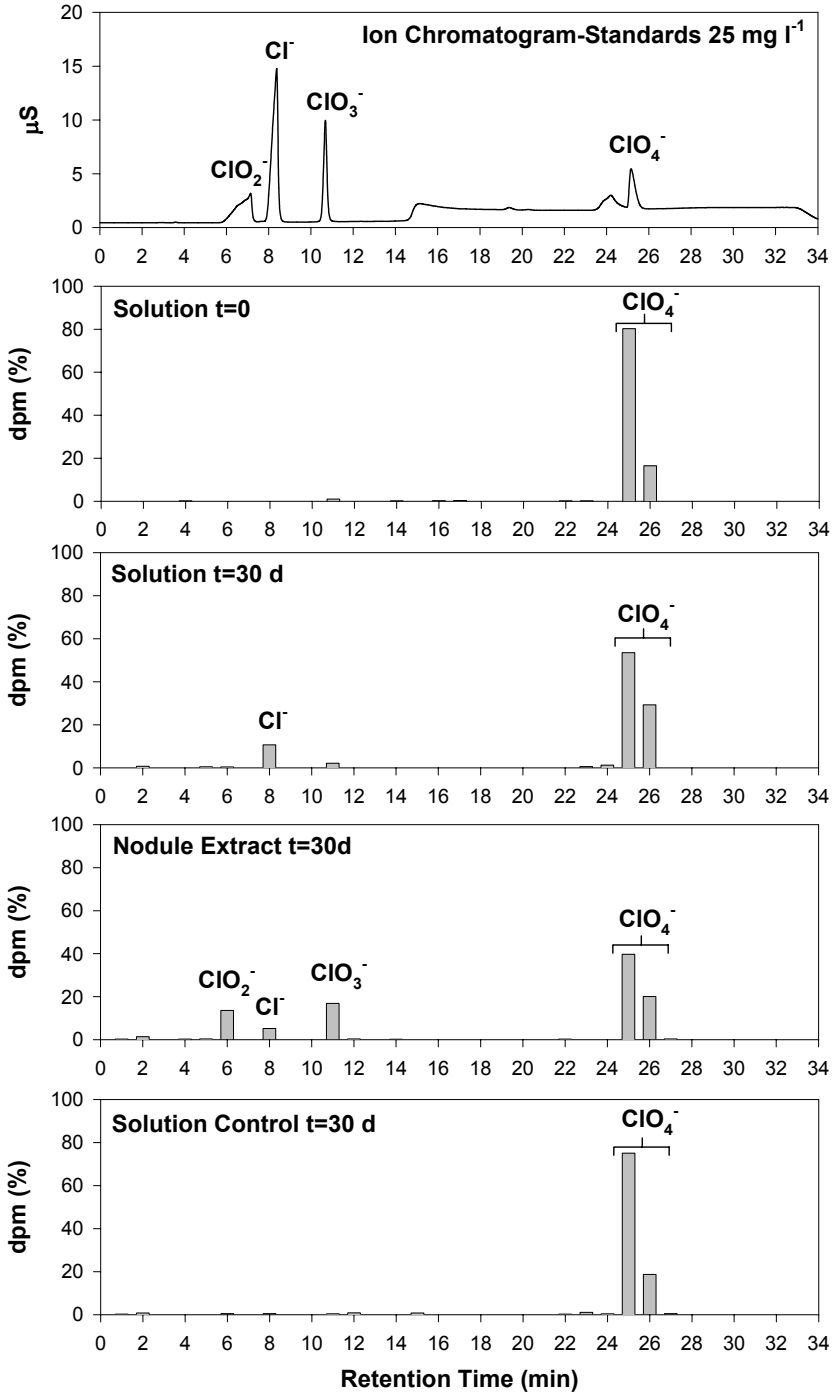

Figure 5. Distribution of the radioactivity (percent) originating from the initial $^{36}ClO_4^-$ (25 mg l^{-1}) among reduced metabolites in different extracts of nodule cultures after 30 d of exposure.

Effect of Nitrate on Perchlorate Uptake by Small Plants and Nodule Cultures

Degradation experiments conducted with pre-grown cuttings in the presence of increasing nitrite concentrations showed a significant reduction of the disappearance rate of perchlorate in the solution when nitrate levels above 200 mg l^{-1} (Figure 6).

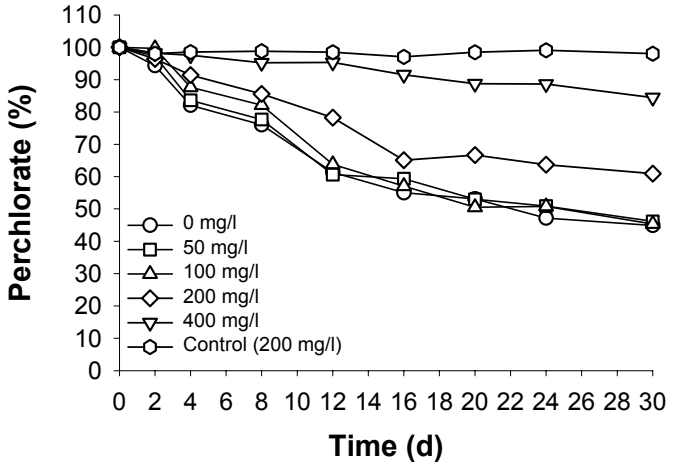

Figure 6. Effect of NO_3^- concentration in the nutrient solution on perchlorate uptake by small poplar trees.

On the other hand, degradation experiments uisng nodule cultures showed only a sligth decrease of the perchlorate disappearance when nitrate concentration increased. The differences do not seem statistically significant (Figure 7).

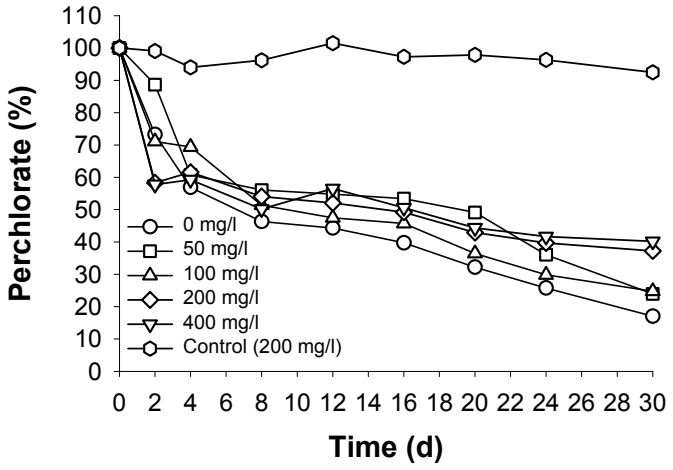

Figure 7. Effect of NO_3^- concentration in the nutrient solution on perchlorate uptake by nodule culture.

Toxicity Assessement

Toxicity of perchlorate on young developing cuttings, as assessed by the transpiration volumes, did not show any significant toxic effect when growing in solutions containing up to 250 mg/L perchlorate. However, the volumes transpirated over the time of the experiment droped from about 4 mL/g to 1.7 mL/g when the perchlorate concentration reached 500 mg/L (Figure 8).

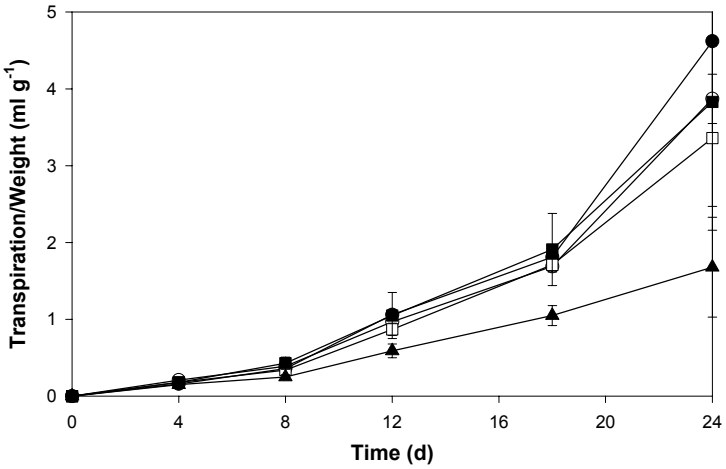

Figure 8. Effect of ClO_4^- concentration in the nutrient solution on the transpiration of poplar trees.

Growth rates (measured by the increase of fresh weight) did not show any significative differences when the trees grew in perchlorate concentrations up to 100 mg/L and corresponded to about 6.5 g after 24 d (or to about 0.27 g/d). However, in the presence of 250 and 500 mg/L perchlorate, the growth rates dropped to 3.3 and 0.7 g over the time of the experiment (or to 0.14 and 0.03 g/d) respectiveley (Figure 9). Chlorose marks were visible on the leaves of plants exposed to perchlorate at concentrations equal or higher than 250 mg/L.

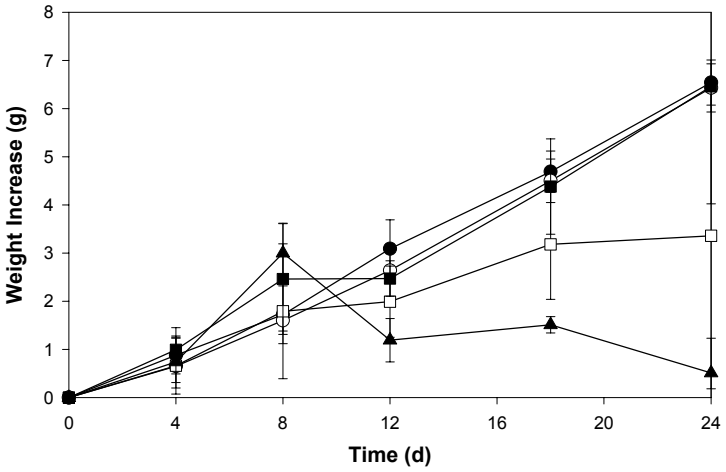

Figure 9. Effect of ClO_4^- concentration in the nutrient solution on the growth of poplar trees.

Enrichment and Purification of Perchlorate-Reducing Bacteria

Seven bacteria were enriched and isolated from three soil microcosms shown to consistently degrade perchlorate when hydrogen or lactate was added (Table 5). These microcosms were maintained for 400 days prior to attempting isolation of perchlorate degrading organisms.

Table 5. Inoculum and enrichment conditions used for isolation of seven perchlorate degrading isolates

Isolate	Source Microcosm	Enrichment Conditions
JDS1	INF sediment+anaerobic media+lactate	5mM perchlorate +10mM lactate in Freshwater media
JDS2	INF sediment+anaerobic media+lactate	5mM perchlorate +10mM lactate in Freshwater media
JDS3	INF sediment+anaerobic media+lactate	5mM perchlorate +10mM lactate in Freshwater media
JDS4	Building 25C soil+IBJ-004 water+lactate	5mM perchlorate +10mM acetate in Freshwater media
JDS5	Building 25C soil+IBJ-004 water+hydrogen	5mM perchlorate +40% H_2 headspace in Freshwater media
JDS6	Building 25C soil+IBJ-004 water+hydrogen	5mM perchlorate +40% H_2 headspace in Freshwater media
JDS7	INF sediment+anaerobic media+lactate	5mM perchlorate +10mM lactate in Freshwater media

All isolates displayed consistent growth on perchlorate and lactate and a decrease in perchlorate could be coupled to an increase in cell mass (Table 6, Figure10). For growth on 5mM perchlorate and lactate, doubling times for the isolates were between 4.5 hours (JDS5) and 6.9 hours (JDS1). Comparatively, *Dechloromonas agitus* strain CKB and *Dechlorosoma suilla* strain PS, two previously well described perchlorate degrading bacteria, isolated and characterized by Achenbach, et al. (Achenbach, Michaelidou et al. 2001), were grown along side the seven isolates and the doubling times were 4.1 hours and 6.9 hours, respectively. Analysis for chloride was not conducted for all collected samples, however a corresponding increase in chloride was observed for all perchlorate degraded in samples analyzed, accounting for 92±21% of initial perchlorate added on a molecular mass basis (data not shown). No chlorate or chlorite was detected. All seven isolates were Gram-negative rods of approximately 1 μm in length. All isolates displayed motility, however, isolate JDS4 was visually observed as having significantly faster motility than the other six isolates. JDS5 and JDS6 did not grow uniformly in suspension. Under all growth conditions, particularly when grown on hydrogen, isolates JDS5 and JDS6 tended to grow in flocs or clumps and also tended to form films along the side and bottom of the growth tube or vessel. For this reason, the measured dry cell weight may be less than the actual cell weight present for these isolates as removed aliquots of cell suspension were assumed to be homogeneous; however, adherence of bacteria to the sides of growth vessels persisted even after vigorous shaking.

Table 6. Growth of perchlorate degrading isolates on 5mM perchlorate and 10mM lactate at 30°C after 66 hours

Isolate	Final Perchlorate Level (mM)	End dry cell mass (mg/L)
JDS1	BDL	40 ±6
JDS2	BDL	40 ±5
JDS3	BDL	45 ±3
JDS4	BDL	90 ±11
JDS5	BDL	140 ±15
JDS6	BDL	33 ±3
JDS7	0.59 ±0.14	46 ±3
CKB (Positive Control)	BDL	220 ±6
PS (Positive Control)	BDL	26

BDL= Below Detection Limit (0.001 mM ClO_4^-). All conditions performed in triplicate, except for Strain PS.

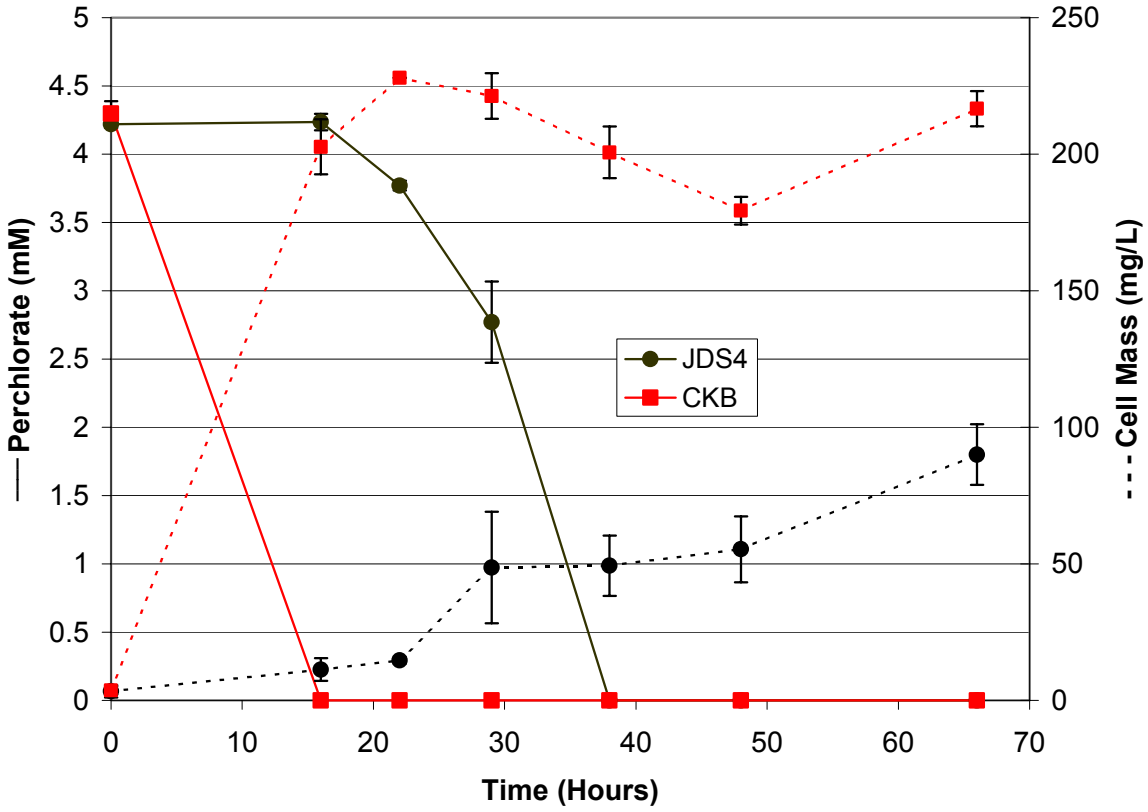

Figure 10. Reduction of perchlorate and increase in biomass for Strain JDS4 and Dechloromonas agitus strain CKB grown in triplicate on 5mM perchlorate and 10mM lactate at 30°C.

All isolates were found to utilize a lactate, acetate, and propionate as electron donors when using perchlorate as an electron acceptor (Table 7). Growth was characterized by an observable increase in optical density at 600 nm within a two-week period or less.

Strains JDS 4, JDS 5, and JDS6 showed the same electron donor utilization with 5 mM nitrate as with 5 mM perchlorate, with the exception of growth being observed by JDS4 with 5mM ethanol and 5mM nitrate but not 5 mM ethanol and 5mM perchlorate. No isolates were able to grow on or degrade 0.1 mM benzene with 5 mM perchlorate or 5 mM nitrate. No isolates were able to ferment lactate or acetate.

Table 7. Electron donors tested for growth with 5 mM perchlorate at 30°C

Electron Donor	JDS1	JDS2	JDS3	JDS4	JDS5	JDS6	JDS7
Lactate (10mM)	+	+	+	+	+	+	+
Acetate (10mM)	+	+	+	+	+	+	+
Hydrogen (40% headspace)	-	-	-	-	+	+	-
Succinate (5mM)	+	+	+	+	-	-	+
Propionate (5mM)	+	+	+	+	+	+	+
Butyrate (5mM)	-	-	-	+	+	+	-
Formate (5mM)	-	-	-	-	*	*	-
Casamino Acids (1 g L^{-1})	*	*	*	*	*	*	*
Glucose (5mM)	-	-	-	+	-	-	+
Ethanol (5mM)	-	-	-	-	-	-	-
Benzoate (5mM)	-	-	-	-	-	-	-
Benzene (0.1mM)	-	-	-	-	-	-	-
Fe (II) (5mM)	-	-	-	-	-	-	-

- No Growth observed, + Growth observed, *Only poor growth observed

All isolates could use 5 mM perchlorate or 5 mM chlorate for growth with 10 mM acetate (

Table 8). Strains JDS1, JDS2, JDS3, and JDS7 were unable to grow on nitrate with any electron donor with the exception of growth of JDS7 on 5 mM glucose and 5 mM nitrate. Isolates JDS5 and JDS6 were unable to grow aerobically (or in a closed headspace with 20% oxygen) on 10 mM acetate, 10 mM lactate or 40 % hydrogen. Isolates JDS4 and JDS7 were able to grow aerobically using lactate or acetate; while aerobic growth of JDS1, JDS2, and JDS3 was observed initially but could not be repeated from anaerobically maintained inocula at a later time. Strains JDS1, JDS2, and JDS3 showed an increase in optical density on TCE or PCE with 10mM lactate or 10mM acetate as the electron donor. Ethene was detected in some replicates, however, this could not account for the disappearance of PCE or TCE observed. No DCE compounds or vinyl chloride were detected.

Table 8. Electron acceptors tested for growth with 10mM acetate at 30°C

Electron Acceptor	JDS1	JDS2	JDS3	JDS4	JDS5	JDS6	JDS7
Perchlorate (5mM)	+	+	+	+	+	+	+
Chlorate (5mM)	+	+	+	+	+	+	+
Nitrate (5mM)	-	-	-	+	+	+	-
Oxygen (20%--atm)	-	-	-	+	-	-	+
Sulfate (5mM)	-	-	-	-	-	-	-
Fumarate (5mM)	-	-	-	+	+	+	-
Sulfite (5mM)	-	-	-	-	-	-	-
Selenate (5mM)	-	-	-	-	-	-	-
Fe (III) (5mM)	-	-	-	-	-	-	-
TCE (0.1mM)	*	*	*	*	*	*	*
PCE (0.1mM)	*	*	*	*	*	*	*

- No Growth observed, + Growth observed, *Only poor growth observed

Isolates JDS5 and JDS6 showed an ability to grow using hydrogen as the sole electron donor, perchlorate as the electron acceptor, and carbon dioxide as the carbon source. No organic carbon source was added or required for growth under these conditions. Confirmation of autotrophic activity was established by observing that hydrogen and perchlorate utilization over time was coupled to an increase in cell mass of these cultures (Figure 11). As discussed previously, the measured dry cell weight may be less than the actual cell weight that was present for these isolates as bacterial films were observed to adhere to the sides of serum bottles even after vigorous shaking. In additional experiments, uptake of $^{14}CO_2$ into cell mass was also observed to confirm use of inorganic carbon for growth. While the decrease of radioactivity in the aqueous phase could not be statistically verified, recovery of 2-3% initial radioactivity from filtered solids (i.e. cell mass) was obtained as compared to non-radioactive inoculated controls and a radioactive non-inoculated control after 140 hours(Table 9). Assuming that molecular hydrogen donates 2 electrons per mole and complete reduction of perchlorate consumes 8 electrons per mole, 85-104% of hydrogen can be accounted by perchlorate reduction by growth of JDS5 and JDS6 (Table10). Utilization of hydrogen and perchlorate by these strains was patterned the same as that observed in previous growth experiments, with or without added $H^{14}CO_3$ (data not shown), however observed growth was faster, presumably due to better hydrogen transfer from headspace in a larger reaction vessel. Strains JDS5 and JDS6 were unable to grow on hydrogen and perchlorate in Freshwater media modified to contain no bicarbonate or carbon dioxide buffered to pH 7.2 with 0.1 M phosphate buffer to provide further support of the growth observed under normal media conditions as utilizing inorganic carbon.

Table 9. ^{14}C Radioactivity recovered from $H^{14}CO3$ or $^{14}CO2$ added during growth of JDS5 and JDS6 on 10 mM perchlorate and 40% hydrogen headspace at 30°C. Results in μCi

Aqueous Fraction	Start	End (140 hours)
No Inocula Control with $H^{14}CO_3$	1.57	1.28
JDS5 (cold control—no ^{14}C added)	0.44	0.33
JDS5 + $H^{14}CO_3$	1.20	1.15
JDS6 (cold control—no ^{14}C added)	0.33	0.37
JDS6 + $H^{14}CO_3$	1.04	1.22
Gaseous Fraction		
No Inocula Control with $H^{14}CO_3$	0.13	0.09
JDS5 (cold control—no ^{14}C added)	0.05	0.10
JDS5 + $H^{14}CO_3$	0.13	0.13
JDS6 (cold control—no ^{14}C added)	0.05	0.08
JDS6 + $H^{14}CO_3$	0.13	0.08
Cell Mass Fraction (filtered solids)		
No Inocula Control with $H^{14}CO_3$	NA	0.000 (0.0%)[C]
JDS5 (cold control—no ^{14}C added)	NA	0.000 (0.0%)[C]
JDS5 + $H^{14}CO_3$	NA	0.038 (3.0%)[C]
JDS6 (cold control—no ^{14}C added)	NA	0.000 (0.0%)[C]
JDS6 + $H^{14}CO_3$	NA	0.028 (2.1%)[C]
System Total (Sum of all Fractions)		
No Inocula Control with $H^{14}CO_3$	1.70	1.37 (81%)[R]
JDS5 (cold control—no ^{14}C added)	0.48	0.43 (90%)[R]
JDS5 + $H^{14}CO_3$	1.34	1.31 (98%)[R]
JDS6 (cold control—no ^{14}C added)	0.38	0.45 (118%)[R]
JDS6 + $H^{14}CO_3$	1.17	1.33 (114%)[R]

[C] %^{14}C Converted into cell mass from initial $H^{14}CO_3$ measured for system. [R] %Recovery from all fractions compared to initial measured radioactivity. NA—Not applicable, initial cell mass was not measured.

Table 10. Electron mass balance for strains JDS5 and JDS6 grown on 40% hydrogen atmosphere and 10mM perchlorate under autotrophic conditions at 30°C (milli electron equivalents $(L)^{-1}$)

	Hydrogen Consumed	Perchlorate Reduced	Cell Mass Produced	Mass Balance of Electrons
JDS5	38.2 ±2.3	29.6 ±0.3	6.34 ±2.89	94 ±12%
JDS6	40.2 ±1.2	30.0 ±1.1	3.83 ±0.26	87 ±4%

All values are the difference between measurements of 0 and 142 hours for three replicates. Based upon energetics (Sawyer, McCarty et al. 1994), 1 mole H_2 = 2 moles electrons; 1 mole cells (as $C_5H_7O_2N$) = 20 moles electrons = 113 g cells; assuming mineralization of perchlorate and products ($ClO_4^- + 8e^- + 8H^+ \rightarrow Cl^- + 4H_2O$), 1 mole ClO_4^- = 8 moles electrons. Cell mass produced was estimated as recovered dry cell weight from VSS measurements. The electron mass balance was determined by the sum of Perchlorate reduced and Cell Mass Produced divided by Hydrogen Consumed.

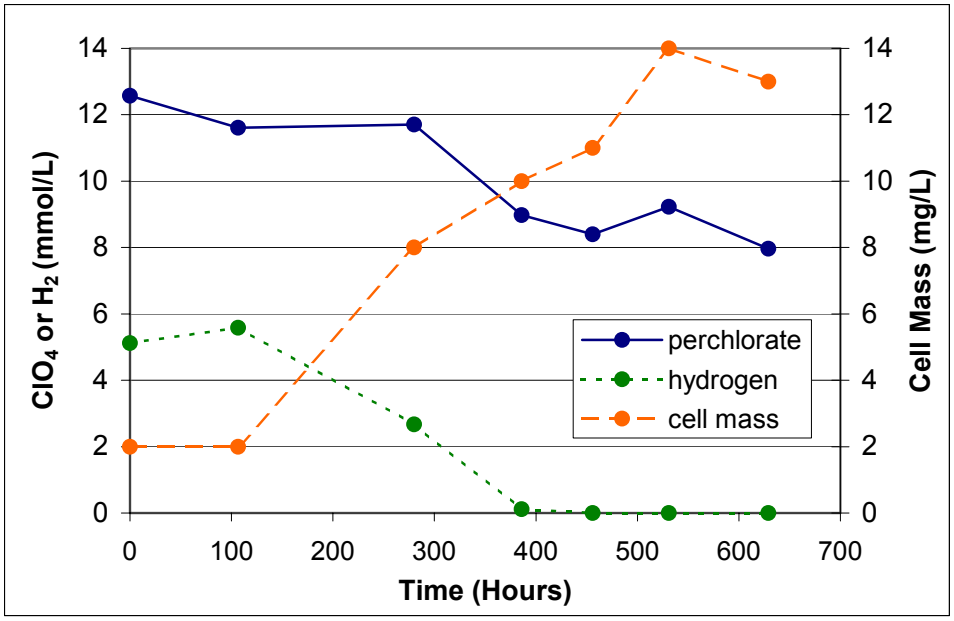

Figure 11a. Growth of JDS5 on 40% hydrogen atmosphere and 10mM perchlorate at 30°C.

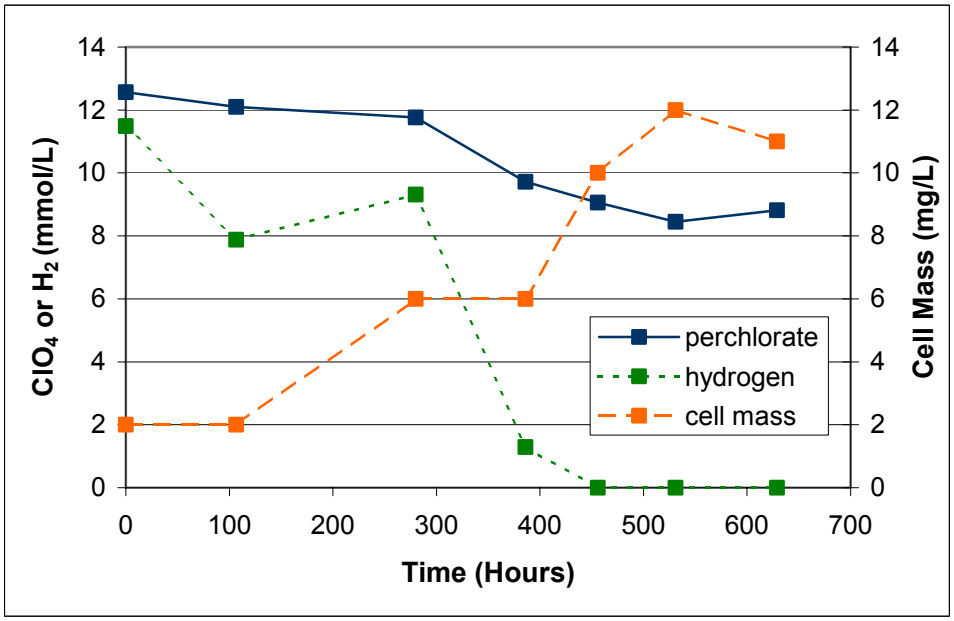

Figure 11b. Growth of JDS6 on 40% hydrogen atmosphere and 10mM perchlorate at 30°C.

Due to the unique ability of perchlorate and chlorate degrading organisms to dismutate chlorite to chloride and oxygen as described by Coates, et al. (1999) and Rikken, et al. (1996), isolates were examined for their ability to utilize chlorite. Wet cell suspensions of all isolates showed an ability to transform 10 mM chlorite. The maximum rate of cells grown on 10 mM lactate and 5 mM perchlorate to utilize 10 mM chlorite ranged from 0.5

mmol ClO_2^- (mg dry cell mass)$^{-1}$ (sec)$^{-1}$ (JDS6) to 11.6 mmol ClO_2^- (mg dry cell mass)$^{-1}$ (sec)$^{-1}$ (JDS7). Strains JDS5 and JDS 6 when grown on hydrogen and 5 mM perchlorate showed a maximum chlorite utilization of 5.1 mmol ClO_2^- (mg dry cell mass)$^{-1}$ (sec)$^{-1}$ and 1.7 mmol ClO_2^- (mg dry cell mass)$^{-1}$ (sec)$^{-1}$, respectively. Comparatively, maximum chlorite utilization of *Dechloromonas agitus* strain CKB and *Dechlorosoma suilla* strain PS grown alongside these strains on 10 mM lactate and 5 mM perchlorate were 4.7 mmol ClO_2^- (mg dry cell mass)$^{-1}$ (sec)$^{-1}$ and 7.9 mmol ClO_2^- (mg dry cell mass)$^{-1}$ (sec)$^{-1}$, respectively. Production of oxygen was not measured as part of this experiment; however, bubbles were observed to form during the assay signifying gas production that was likely due to dismutation activity producing molecular oxygen.

The 16S rDNA sequences recovered from these bacteria suggests they are members of the Proteobacteria class, and three different sub-groups of organisms have been isolated from the three microcosms used as inocula (Figure 12). From phylogenetic analysis of partial 16S rDNA sequence, isolates JDS1, JDS2, JDS3, and JDS7, which includes enrichments potentially showing dechlorinating activity, appear most closely related to other known dechlorinating organisms in the epsilon subclass of Proteobacteria. Within the beta subclass of Proteobacteria, JDS4 may be most highly related to *Dechlorosoma* or *Rhodocyclus* while isolates JDS5 and JDS6 may be most highly related to the genus *Dechloromonas*.

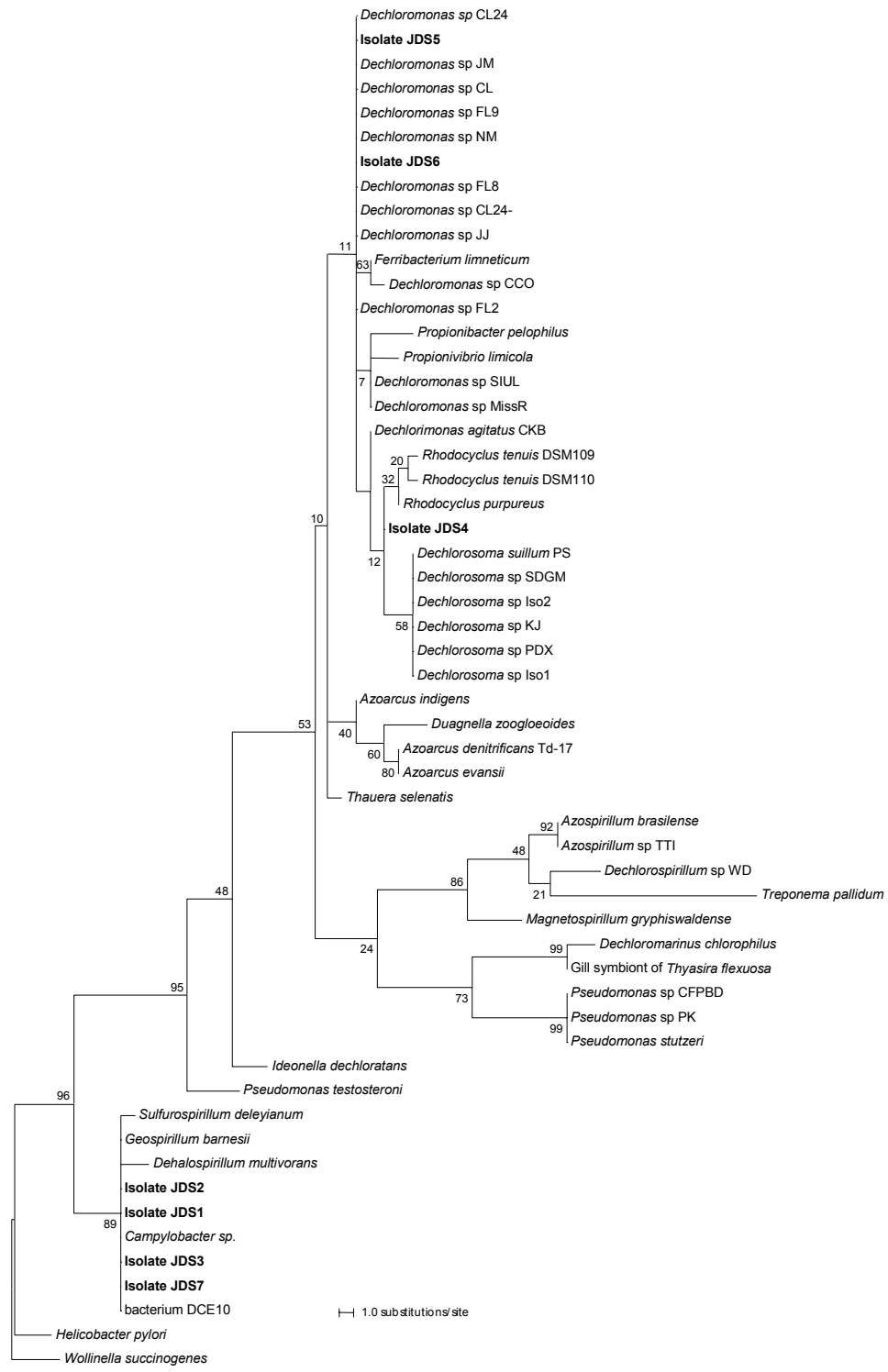

Figure 12. Tree of known species and newly obtained isolates based upon Bootstrap analysis of commonly shared regions of partial or whole 16S rDNA gene sequences.

Degradation of Perchlorate by Bacteria Using Root Products

As anticipated, the COD content of the root products were significantly different from each other. The COD of the recovered filtered root exudate was 25 mg/L and the COD of the filtered root homogenate was 670 mg/L. Subsequently, the concentrations of root exudate and root homogenate examined for growth were 20 mg/L COD and 270 mg/L COD, respectively, with 5mM perchlorate.

All seven isolates identified as part of this project (JDS1- JDS7) were investigated for their ability to use poplar roots as a carbon and energy source for perchlorate degradation. In addition, a perchlorate-acclimated culture, LEC-PH was also investigated for the ability to utilize root exudate for perchlorate degradation. After 18 days incubation, all inocula showed decreases in perchlorate concentration using the root products. Results using the root homogenate were pronounced as 10%-25% of initial perchlorate was degraded, and the growth of bacteria was observed visually in the tubes (Table 11). While incubations using root exudate did not show distinct visual results, the analytical results suggest some perchlorate degradation did occur. These results are extremely encouraging as this is the first known report of degradation of perchlorate using solely root products as a carbon and energy source by perchlorate-reducing bacteria.

Table 11. Degradation of perchlorate by Perchlorate-Degrading Bacteria using root products after 18 days of incubation.

Strain	Electron Donor	Day 0	Day 18	Δ	% reduction
JDS1	root homogenate	962	851	-111	11.5%
	root exudate	659	657	-1	0.2%
JDS2	root homogenate	1181	1051	-130	11.0%
	root exudate	667	665	-2	0.3%
JDS3	root homogenate	969	845	-124	12.8%
	root exudate	737	708	-28	3.9%
JDS4	root homogenate	992	891	-101	10.2%
	root exudate	687	665	-22	3.2%
JDS5	root homogenate	922	792	-131	14.2%
	root exudate	650	632	-18	2.8%
JDS6	root homogenate	972	848	-124	12.8%
	root exudate	695	684	-12	1.7%
JDS7	root homogenate	949	741	-208	22.0%
	root exudate	842	811	-31	3.7%
LEC-PH	root homogenate	1011	754	-258	25.5%
	root exudate	709	690	-19	2.7%

All Results in mg/L Perchlorate

- **Figure 2**

Section

5

Conclusions

Phytotransformation Experiments

Our results have shown that both trees in hydroponic solution and nodules in liquid suspension were able to remove perchlorate (ClO_4^-) from the nutrient solution. Since the uptake rates did not seem to slow significantly at the end of the experiment, it can be assumed that the process might continue over a longer period of time and that no toxic effect of perchlorate occured at the concentration applied (25 mg/L). The transpiration and growth rates observed in plants growing either in the presence or in the absence of perchlorate also did not show any toxic effect. The comparable growth rates observed between nodules incubated both in the presence and in the absence of perchlorate lead to a similar conclusion. This is consistent with previous observations reporting no phytotoxic effect of perchlorate in the solution below 2,500 mg/L (Nzengung et al., 1999, Susarla et al., 2000).

The initial concentration of perchlorate was reduced to half of the initial by both trees and nodules over the time of the experiment suggesting an active uptake by the roots, but also by the photosynthetic tissue in the periphery of nodules. This suggests that besides the specialized root tissue, photosynthetic tissue, mimicing leaf cells, possesses ion transporters able to take up perchlorate.

Using willow trees growing in diluted Hoagland solution, Nzengung et al. (1999) showed an almost complete disappearance of the initial 22 mg/L perchlorate after 30 days. However, the transfomation kinetics of that study showed two distinct phases: The first phase was slow (disappearance of about 20 % in 15 days) and the second one faster (disappearance of the remaining 80 % in 15 days). Since the experiments were not performed under sterile conditions, the first phase has been described as the result of a plant-mediated uptake, while the second as the result of a plant-assisted microbial transformation of perchlorate (i.e. rhizodegradation). The first two weeks could correspond to the lag phase required for the adaptation of perchlorate-reducing bacteria (Wallace et al., 1998)--recently reported to be ubiquitous in the environment (Coates et al., 1999)--and/or to the development of anaerobic conditions in the root zone (Nzengung et al., 1999). In a separate report, sweet gum trees growing in sand were shown to reduce the initial 20 mg/L perchlorate to undetectable levels, while willow trees, growing both in sand and in hydroponic solution, were shown to reduce the same concentration about 50 % over the same period of time (Susarla et al., 2000). These variable results may be explained by the use of different plant species and by the application of different experimental conditions. Even though poplar trees were reported to be poor at removing perchlorate by comparison to other species, our results suggest that they are at least in midway of the range of performances reported using other terrestrial trees. Poplar trees therefore continue to constitute a good model for phytoremediation studies, and because

of other valuable properties from a phytoremediation viewpoint, continue to be a leading candidate for cleaning up perchlorate-contaminated environments. More extensive screening of species and environmental conditions may be necessary to optimize a phytoremediation scheme for perchlorate contaimination.

Because our experiments were performed under sterile conditions and because no microbial contamination was detected, it can be considered that the reduction of perchlorate concentration observed in the solution was the result of a pure plant-mediated process (i.e. uptake and/or phytotransformation).

Most of the radioactivity recovered from the plants tissues at the end of the experiment was located in the leaves (more than 90 %), while insignificant amounts were recovered from the other fractions. Using willow trees growing in diluted Hoagland solution and in sand, Nzengung et al. (1999) found similarly that the major part of perchlorate (initial concentration 100 mg/L) taken up by the plants after 26 days exposure was found in the leaves (46 %), even though significant proportions were also recovered from the upper (33 %) and the lower stems (17 %). On the other hand, in similar phytoremediation experiments using sweet gum trees growing in sand (initial concentration of perchlorate 20 mg/L), perchlorate metabolites were observed in the leaf, the root, and the stem tissues at levels of 355, 191, and 83 mg/kg fresh material, respectively. However, using willow trees in the same experiments, only 51 mg/kg of perchlorate metabolites were extracted from the leaves, while 102 and 127 mg/kg were extracted from the roots and the stems, respectively (Susarla et al., 2000). The large variations reported in the repartition of perchlorate and perchlorate metabolites between the different plant organs may be explained by the use of different plant species and of different experimental conditions (e.g. the time of exposure), which seems to correlate the proportion of perchlorate and perchlorate metabolites accumulated in the leaves. Together these results suggest an active uptake of perchlorate by the roots followed by a translocation to the leaves where it can be either stored or transformed.

Both small trees growing in hydroponic solution and nodule cultures have been shown to reduce the concentration of $^{36}ClO_4^-$ in solution by 50 % in 30 days. However, the radio-activity recovered in the solutions corresponded to 67 and 53 % for the experiments using trees and nodules respectively. This suggests that a part of the ^{36}Cl in the solutions did not account for $^{36}ClO_4^-$. Analysis of the distribution of the radioactivity recovered in the solutions after one month showed that a significant portion (33 % and 10 % in experiments with trees and nodules, respectively) was accounted for by chloride. On the other hand, the radioactivity extracted from the leaves and from nodules showed distribution between non transformed perchlorate (66 %) and various proportions of reduced metabolites (i.e. ClO_3^-, ClO_2^-, and Cl^-). Because experiments were performed under sterile conditions, these results showed that plant tissues are able, qualitatively speaking, to entirely reduce perchlorate to chloride. The identified metabolites inside the plant tissues suggest a stepwise reduction of perchlorate to chloride, through a pathway similar to the reductive microbial metabolism and likely involving (per)chlorate reductase and chlorite dismutase. Differences between microbial enzymes involved in perchlorate reduction, which are oxygen-sensitive, and plant enzymes seem likely as these as yet unidentified plant enzymes appear to act mainly inside the leaves (or in photosynthetic cells of nodules), where oxygen is present due to production by photosynthesis. However, the relatively limited transformation of perchlorate observed over the time of the experiment suggests a slow activity by comparison to microbial reduction, which might be the price to pay for a reductive enzyme working under aerobic conditions. These results

are in agreement with other reports about phytoremediation of perchlorate. Based on the comparison of the perchlorate concentration extracted from the leaves of willow trees in short term (< 30 day) and long-term (> 60 day) experiments, Nzengung et al. (1999) concluded that accumulation of perchlorate in the leaf tissues occurs first, followed by a subsequent reduction into chloride. The other perchlorate reduction metabolites, in variable proportions, were also recovered from the different parts of sweet gum and willow trees (Susarla et al., 2000). In contrast to our results, large proportions of perchlorate reduction products were recovered not only from the leaves, but also from the other parts of the plant, suggesting either a translocation of the metabolites upon formation in the leaves or transformation by cells located in other parts of the plant.

Because no transformation could take place in the solution, the large proportion of the radioactivity recovered from the solution as chloride, as well as the low chloride titers extracted from the leaves, suggest a release of chloride in the solution upon its reduction in the leaf tissues. Nzengung et al. (1999) also reported an increase of the chloride concentration in the solution following the reduction of the perchlorate concentration, but suggested this originated from a microbial-mediated rhizodegradation of perchlorate inside the solution. The absence of chloride observed elsewhere in the leaves extracts from willow and sweet gum trees supports this hypothesis.

Nitrate NO_3^- has been shown to inhibit the reduction of perchlorate by bacterial strains. Apparently, NO_3^- ions compete with ClO_4^- for reductive enzymes (Logan 1998). In addition, it has been shown that NO_3^- inhibits the rate of disappearance of perchlorate by willow trees growing in Hoagland solution (Nzengung et al. 1999). However, whether this observation was the result of an inhibition of the uptake of perchlorate by trees or the result of an inhibition of the perchlorate-reducing activity of microbes remains unclear. Our degradation experiments using cuttings showed similarly a reduction of the perchlorate removal from the solution by small poplars. However, because our experiments were performed under sterile conditions, the observed effect was attributed to an inhibition of the active uptake mechanism of perchlorate by the roots. On the other hand, nodule uptake of perchlorate was only slightly affected by increasing nitrate concentration in the solutions, suggesting the existence of uptake mechanisms independent from the inhibition or the competition by nitrate.

Our results obtained using radio-labeled perchlorate and working under sterile conditions provide additional evidence that stepwise reduction, and subsequent detoxification, of perchlorate previously believed to be exclusively reduced only by facultative microorganisms, can also be achieved by higher plants. This is also the first time in the short history of phytoremediation that plants were reported, besides uptake, to actually transform an inorganic pollutant. Further experiments however are required in order to ensure that the remaining perchlorate detected in the plant tissues are susceptible to complete reduction to chloride.

Enrichment and Characerization of Perchlorate-Reducing Bacteria

Seven isolates capable of reducing perchlorate to chloride with added lactate or hydrogen were obtained from three soil microcosms. The results provide evidence that two of these isolates, JDS5 and JDS6, are capable of growth on hydrogen and perchlorate without an organic source of carbon. These are the first reported perchlorate-degrading autotrophs. The inability of these strains to grow in the presence of oxygen suggests they

may be microaerophilic or strictly anaerobic. In general, these two isolates appear very robust, as they were also able to grow heterotrophically using a variety of electron donors and acceptors. A phylogenetic analysis of partial 16S rDNA sequence from these organisms suggests they are most similar to other known perchlorate degraders in the beta subclass of Proteobacteria.

The ability of isolates JDS1, JDS2, and JDS3 to potentially degrade or grow using PCE or TCE is the first report of this activity among perchlorate-degrading organisms. However, these preliminary results require further investigation to determine the actual use and fate of chlorinated aliphatics by these bacteria. There is ecological and phylogenetic evidence to support the use of PCE or TCE by these organisms. The Longhorn Army Ammunition Plant, the original source of material for the perchlorate-degrading microcosms, is known to have TCE contaminated areas, which may have contributed to the natural selection of these perchlorate-utilizing bacteria. Comparison of 16S rDNA sequence recovered from these strains agrees with the observed phenotype as the closest phylogenetic 16S rDNA sequences were known dechlorinating organisms in the epsilon subclass of Proteobacteria. The bacteria *Sulfurospirillum deleyianum*, *Dehalospirillum multivorans,* and bacterium DCE10 were all isolated due to their ability to dechlorinate PCE, so it is logical that close phylogenetically related bacteria might share dechlorinating activity.

The existence of bacteria capable of autotrophic growth on hydrogen and perchlorate and bacteria capable of using either chlorinated aliphatics or perchlorate expands the previously known activity of perchlorate degraders. While perchlorate-respiring bacteria continue to be shown as abundant in nature, the ubiquity of these particular isolates (or similar phenotypes) has not been investigated. Nonetheless, the characteristics of these isolates may lend themselves to applications of both in-situ and ex-situ bioremediation strategies. Additionally, continued discovery of novel perchlorate-degrading bacteria may help to uncover unique physiological and/or genetic themes present in this increasingly significant group of organisms.

The ability of these bacteria to utilize root products for perchlorate degradation is very encouraging for the prospects of rhizodegradation at the LHAAP. All seven bacteria isolated were able to degrade perchlorate after 18 days of incubation, suggesting that products from plants can serve to promote a combined rhizodegradation zone for perchlorate degradation.

Project Summary

The work conducted for this project has culminated in discovery of new information fundamental to understanding biological perchlorate degradation by plant and bacterial systems and practical information directly applicable to the perchlorate issues of the Longhorn Army Ammunition Plant. The results suggest that biological degradation does occur under certain environmental conditions and the potential exists to create a passive phytoremediation/rhizodegradation system at the site.

Poplar trees were shown capable of both uptake and conversion of perchlorate. Trees growing in hydroponic solution were shown to reduce the concentration of $^{36}ClO_4^-$ in solution by 50 % in 30 days. Uptake of perchlorate was verified by recovery of radioactivity from the leaves. Additionally, conversion of perchlorate in these semi-sterile conditions was shown by the recovery of perchlorate metabolites containing ^{36}Cl. Of

radio-labeled ^{36}Cl recovered from the leaves, 68%, 15%, 8%, and 15% was recovered as ClO_4^-, ClO_3^-, ClO_2^-, and Cl, respectively. Additionally, 33 % of radio-labeled compound remaining in solution was recovered as chloride, which appeared not to be due to microbial conversion.

Additionally, seven new perchlorate-degrading bacteria were isolated from soil microcosm enrichments developed from soil and water samples collected at a perchlorate-contaminated site. Two of these isolates (JDS5 and JDS6) represent the first known hydrogen-utilizing, autotrophic, perchlorate degraders. Four other isolates obtained from this research (JDS1, JDS2, JDS3, and JDS7) display phylogeny most similar to dechlorinating bacteria, groups not previously described to show perchlorate-degrading activity.

The degradation of perchlorate by both the plant and bacterial systems utilized for this work lends great promise to the development of passive bioremediation systems to treat perchlorate contamination. Furthermore, the demonstration of utilization of root products by the newly identified bacteria adds merit to the potential of an engineered phytoremediation system to operate as a synergistic rhizodegradation zone.

Section

6

References

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