

PHYTOREMEDIATION AND ENHANCED NATURAL ATTENUATION OF
PERCHLORATE AND N-NITROSODIMETHYLAMINE AS A SINGLE AND CO-
CONTAMINANTS

by

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(Under the Direction of Valentine Nzungu)

ABSTRACT

The fate of two emerging contaminants, perchlorate (ClO_4^-) and N-Nitrosodimethylamine (NDMA) ($\text{C}_2\text{H}_6\text{N}_2\text{O}$), in planted systems, was investigated using phreatophytes grown in hydroponic and soil bioreactors under greenhouse conditions. The recent detection of perchlorate in the food chain and growing concern over the recycling of perchlorate in the environment by decaying senescent leaves have been attributed to plant perchlorate uptake and phytoaccumulation. This study found higher perchlorate concentrations in mature and dry vegetation than in the young leaves collected from Longhorn Army Ammunition Plant (TX) and the Las Vegas Wash (NV), implying phytoaccumulation and possible recycling of perchlorate into the ecosystem. The study also investigated the feasibility of enhancement of rhizodegradation of perchlorate by willow trees (*Salix nigra*) using three electron donor sources of dissolved organic carbon (DOC). In planted hydroponic and soil bioreactors dosed with DOC, 40 mg L⁻¹ perchlorate was completely degraded to below the IC method detection limit of 2 µg

L⁻¹ in under 10 days. For planted control bioreactors, more than 70 days were required to remove the same amount of perchlorate. Addition of DOC to the root zone biostimulated rapid degradation of perchlorate and reduced the amount of perchlorate taken up and phytoaccumulated by an order of magnitude. Biostimulation and sustained rapid rhizodegradation will minimize the undesirable uptake and accumulation of perchlorate in agricultural products and possible recycling of perchlorate into the ecosystem.

Hybrid poplar (*Populus deltoides* × *nigra*, ND34) and black willow trees were used to remove 1 mg L⁻¹ NDMA to below the GC/MS method detection limit of 10 µg L⁻¹ in less than 60 days in hydroponic system. A linear correlation between the volume of water transpired by the plants and the mass of NDMA removed from the root zone indicated that the phytoremediation mechanism of NDMA is primarily by uptake and phytovolatilization. The calculated transpiration stream concentration factor of 0.28 ± 0.06 suggests passive uptake of NDMA. In experiments conducted with radiolabeled (¹⁴C-NDMA), $46.4 \pm 1.1\%$ of the total ¹⁴C-activity was recovered in the plant tissue while 47.5% was phytovolatilized. This study found no evidence of competitive uptake of perchlorate and NDMA, however rhizodegradation of perchlorate decreases in the presence of NDMA.

INDEX WORDS: Acetate, Bioremediation, Chicken litter extract, Dissolved Organic Carbon (DOC), Las Vegas Wash, Longhorn Army Ammunition Plant, Mushroom compost, N-Nitrosodimethylamine (NDMA), Perchlorate, Phytoaccumulation, Phytodegradation, Phytoremediation, Phytovolatilization, Plant-uptake, Rhizodegradation.

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DEDICATION

To Gashe, Eteye, Kidistye and Neb for their love and support.

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CHAPTER 1

INTRODUCTION

Environmental contamination by two emerging contaminants perchlorate (ClO_4^-) and N-Nitrosodimethylamine (NDMA) pose significant health risks to water resources in several areas of the United States. A growing number of ongoing research efforts are focused on the source, transport, health effects, and treatment options for these emerging contaminants. However, there is an immediate need for low-cost and sustainable treatment technologies that minimize the ecotoxicological risks of exposure to perchlorate and NDMA. In response to the latter need, the research presented in this dissertation focused on:

1. The evaluation of the potential of phytoremediation of NDMA,
2. Biostimulation and enhancement of rapid biodegradation of perchlorate in the root zone of plants (rhizodegradation) using dissolved organic carbon as additional source of carbon and electron donors for perchlorate-respiring bacteria,
3. The feasibility of minimizing the undesired uptake and phytoaccumulation of perchlorate by the enhancement of rhizodegradation of perchlorate,
4. Determination of the long-term fate of perchlorate in leaf tissues of vegetation growing over perchlorate-contaminated soils and water at the Longhorn Army Ammunition Plant (Karnack, TX) and the Las Vegas Wash (Las Vegas, NV).

Previous greenhouse hydroponics and soil studies provided evidence on the potential effectiveness of phytoremediation of perchlorate. Two main phytoremediation mechanisms identified by these studies include slow uptake and phytodegradation in the leaf tissue and rapid

degradation in the root zone (rhizodegradation). These mechanisms were shown to apply to both aquatic and terrestrial plants systems. The perchlorate taken up into the plants is mainly accumulated in the leaf tissue as it is slowly phytodegraded by perchlorate reductase enzyme to chloride *via* intermediate products chlorate (ClO_3^-) and chlorite (ClO_2^-). The slow phytodegradation results in accumulation of perchlorate in plant tissues, which may be re-released into the environment *via* leaching from decaying senescence leaves. Phytoaccumulation of a fraction of the perchlorate taken up into plants has been confirmed by the detection of perchlorate in the food crops, dairy milk and human breast milk. This research provides a feasible solution to the nationally recognized problem of perchlorate accumulation in plants. A method for the stimulation of perchlorate-degrading microorganisms in the rhizosphere using natural and artificial electron donors is described for hydroponics system and soil bioreactors. The findings of this study have so far been presented at seven national and international conferences and published in two conference proceedings (National Ground Water Association, 2005; and Battelle, 2005). Three manuscripts have been prepared and submitted for review and publication in the peer reviewed literature. The first two manuscripts that have been peer reviewed and revised for resubmission focused on the biostimulation of perchlorate degradation in the rhizosphere in hydroponics system (Chapters 3) and soil system (Chapter 4). The Battelle proceeding paper is presented as appendix A.

Previous experiments on the uptake of perchlorate by plants were performed in well-controlled laboratory conditions for relatively short durations. Therefore, little or no data was available for long-term experiments that simulate the length of the growing season experienced in the field and where environmental conditions may affect plant perchlorate uptake. Environmental factors that may influence plant perchlorate uptake include distance from source

of contamination, exposure duration, availability of organic carbon in the subsurface, and the recycling potential of perchlorate from deciduous leaves. Former rocket fuel manufacturing facilities at the Las Vegas Wash (LVW, Las Vegas, NV) and Longhorn Army Ammunition Plant (LHAAP, Karnack, TX) are among over 150 facilities contaminated by perchlorate nationwide. This study collected and analyzed multiple plant samples surrounding the LHAAP and the LVW to investigate the long-term fate of perchlorate accumulated in plant leaves, spatial and seasonal variability of perchlorate concentration in plants, and the difference in perchlorate concentrations among plant species. The findings of this study together with recommendations of approaches that should be included in the design of phytoremediation of perchlorate contaminated sites are reported in Chapter 5.

N-Nitrosodimethylamine is a very potent emerging carcinogen that has been detected in the drinking water wells near rocket engine testing facilities and in areas where chlorinated wastewater was used for aquifer recharge. The commonly used treatment technology for NDMA is UV radiation, which is expensive and cost-prohibitive in some applications. The United States Department of Defense has funded a number of initiatives to develop bioremediation methods for NDMA. Until this study, the efficacy of phytoremediation of NDMA was not known. In addition to the other phytoremediation of perchlorate studies described above, a set of experiments was conducted to test the hypothesis that because of its water miscibility, NDMA should be readily taken up by phreatophytes. A greenhouse experiment was conducted using black willow (*Salix nigra*) and hybrid poplar (*Populus deltoides* \times *nigra*) trees to remove NDMA from hydroponic bioreactors. Radiolabeled ^{14}C -NDMA was used as a tracer to investigate the fate of NDMA taken up by these trees and perform a mass balance analysis. Chapter 6 presents the findings of the first study on phytoremediation of NDMA and the fate of NDMA in plants.

NDMA and perchlorate frequently occur as co-contaminants at a growing number of sites because both were used in the production of rocket fuel. However, the fate of perchlorate and NDMA in the environment when present as co-contaminants is lacking in the literature. This study also investigated whether phreatophytes selectively take up perchlorate or NDMA and the effect of NDMA on the microbial degradation of perchlorate in the rhizosphere. The results of this experiment are also presented in chapter 6. The research presented in chapter 6 was submitted to the journal of Environmental Science and Technology in February 2006 for peer review and publication on a special issue that focuses on emerging contaminants.

CHAPTER 2

LITERATURE REVIEW

The USEPA has identified both perchlorate (ClO_4^-) and N-Nitrosodimethylamine ($((\text{CH}_3)_2\text{N}_2\text{O})$) as emergent contaminants with potential health risks. Perchlorate has impacted soils and groundwater in 44 states, and more than 15 million people have perchlorate in their drinking water at concentrations of $4 \mu\text{g L}^{-1}$ or higher (1). Perchlorate has also been shown to accumulate in the food chain, for example perchlorate has been detected in lettuce, cow milk and human breast milk. N-Nitrosodimethylamine (NDMA) has similarly contaminated drinking water supplies in many states (2,3). Both NDMA and perchlorate frequently occur together in the same location because these chemicals are used in the production of rocket fuel. Although a growing number of studies have focused on the fate and transport and cost-effective treatment technologies for perchlorate-contaminated soils and water, very few studies have successfully led to the development of treatment technologies for NDMA. Specifically, no study has focused on the effectiveness of phytoremediation of NDMA and phytoremediation of NDMA and perchlorate when they occur as co-contaminants.

1. Perchlorate

Perchlorate is an anthropogenic as well as naturally occurring emerging contaminant. The majority (~ 90%) of all perchlorate salts is manufactured as ammonium perchlorate (NH_4ClO_4) (4), an oxygen-adding component in propellants for rockets, missiles and fireworks. Perchlorate

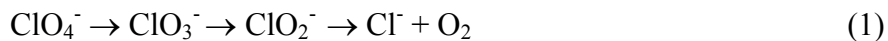
is also produced naturally from atmospheric sources and is present in evaporite deposits of arid and semi-arid climates (5,6) such as in Chilean caliche; a sodium nitrate fertilizer with perchlorate concentrations of about 0.5 - 2 mg g⁻¹ (7). Past disposal practices of perchlorate in the military and aerospace facilities, which include burning of perchlorate-containing materials and discharging wastewater produced during the replacement of expired propellant in missiles and rockets, has led to perchlorate contamination of soils and groundwater in many states. In addition, perchlorate has been detected in several plant species (8,9) and the food chain (10). These findings suggest that human exposure to perchlorate may come from sources beyond drinking water. A study conducted by the Environmental Working Group showed that 18 % of the lettuce samples purchased from supermarkets contained detectable levels of perchlorate (11). Perchlorate was also found in dairy milk samples purchased randomly in Lubbock, TX (12), in 36 samples of breast milk collected from women in 18 states and in human urine in Atlanta (13).

Environmental contamination by perchlorate has been linked to abnormal thyroid function and other human health problems (14). Perchlorate has similar size and charge as iodide (1), an essential component for normal thyroid hormone production. Therefore, the presence of perchlorate in the bloodstream interferes with human iodide uptake and production of thyroid hormone, which is vital for growth and metabolism. In January 2006, the U.S. Environmental Protection Agency issued a preliminary clean-up goal for perchlorate of 24.5 µg L⁻¹ in water (15).

The perchlorate anion is nonvolatile and its common salts are highly soluble in water (*e.g.*, aqueous solubility of ammonium perchlorate is 200 g L⁻¹). Ammonium perchlorate dissociates completely into ammonium (NH₄⁺) and perchlorate (ClO₄⁻) ions in water (14). Perchlorate is chemically stable in aqueous solution, and hardly reacts at all in the absence of

suitable electron sources (16,17). Common reducing agents do not reduce perchlorate, and common cations do not precipitate it (14). As a result, conventional water treatment technologies such as air stripping, activated carbon adsorption are not effective for removing perchlorate from water (14,18).

Research conducted over the past several years revealed that there are several species of bacteria capable of degrading perchlorate under anaerobic environmental conditions (19,20). Therefore bioremediation has become an approved and commonly used treatment technology for perchlorate contaminated soils and groundwater. Perchlorate is a highly oxidized compound with a +7 oxidation state and biological reduction to chloride is carried out by microorganisms which use perchlorate as a terminal electron acceptor (19,20). For effective bioremediation of contaminated sites, the presence of perchlorate degrading microbes, anaerobic conditions, adequate electron donors and sufficient nutrients are important. Perchlorate-respiring microorganisms are ubiquitous in the environment (20,21). Anaerobic environmental conditions are important because perchlorate is most rapidly biodegraded under anaerobic conditions to oxygen and chloride (19,22). Perchlorate (ClO_4^-) is biodegraded step wise to chlorate (ClO_3^-) and chlorite (ClO_2^-). These reactions are catalyzed by perchlorate reductase enzyme (23). The chlorite is further reduced by chlorite dismutase to chloride (Cl^-) and oxygen (O_2) (19,20).



During the reduction process, microorganisms need organic carbon (OC) and electron donors for growth. One possible electron donor for the reduction process are various organic compounds that occur naturally in soil organic matter and exudates of plant roots such as ethanol, sugars, and acetate (24). However, the amount of natural OC in soils and groundwater at some sites is limited and can not sustain the complete degradation of perchlorate at many field

sites (25). At such sites, the sustained degradation of perchlorate can only be maintained by providing suitable electron and nutrient sources to contaminated media. Biostimulation of perchlorate degradation can be achieved by adding ethanol, acetate, yeast extract, methanol or molasses in soils as sources of carbon and electrons (26,27).

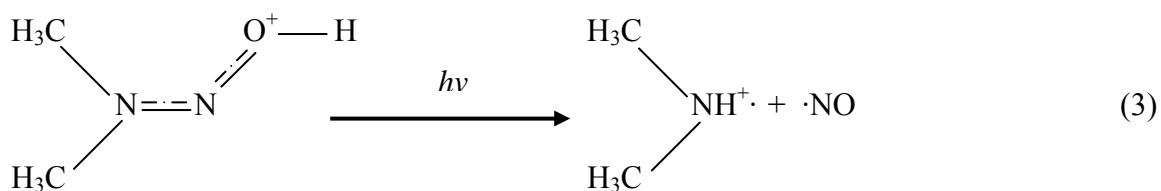
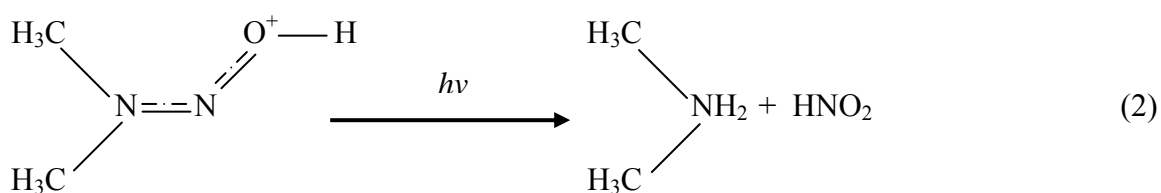
2. N-Nitrosodimethylamine (NDMA)

Similar to perchlorate, N-Nitrosodimethylamine (NDMA) is a naturally occurring as well as anthropogenic emergent contaminant. N-Nitrosodimethylamine can be produced by the oxidation of 1,1-dimethylhydrazine (UDMH), which is mainly used in rocket fuel production (2,28) or during disinfection of water and waste water by chlorine or monochloroamine (3,29). It is also produced in natural ecosystems by the reactions of amines such as dimethylamine and nitrite (30,31). Being highly soluble in water, NDMA is readily transported into various environmental compartments including groundwater. Contamination of soils and groundwater by NDMA is commonly recorded at facilities manufacturing or testing rocket fuels such as Sacramento County, CA (3) and Rocky Mountain Arsenal, CO (2,28). The full extent of NDMA contamination of ground water and soils in the United States is currently unknown as many sites are still undergoing testing.

N-Nitrosodimethylamine is of grave concern to municipal water suppliers because there exist sufficient and growing evidence of its carcinogenicity in experimental animals (32,33). It has been shown to cause lung, liver and kidney tumors in mice, rats, rabbits, guinea pigs, and ducks (34). Significant increase of liver tumors occurred in rats receiving NDMA in drinking water (35). But, only limited data is available to evaluate the carcinogenicity of NDMA in

humans. Chronic (long-term) exposure of humans to NDMA may cause liver damage and low platelet counts (34). The USEPA listed NDMA as a priority pollutant and the drinking water standard for NDMA is set at 0.7 parts per trillion (28).

Similar to perchlorate, NDMA is highly soluble in water with a Henry's law constant of $1.45 \times 10^{-3} \text{ atm M}^{-1}$ at 20 °C (36) and $\log K_{ow}$ value of -0.57 (37). Because it is hydrophilic, NDMA sorbs poorly to soils (2,38), activated carbon and other sorbents (2,28). Due to the high solubility and chemical stability of NDMA, removal by typical physical-chemical water treatment technologies is expensive. Currently, the most commonly applied aqueous treatment method for NDMA is photolysis by ultraviolet radiation (3). N-Nitrosodimethylamine absorbs light strongly at between 225 and 250 nm (3) and the absorption of UV results in the excitation of NDMA molecule followed by photocleavage of the N-N bond (39). Hydrolysis of the excited state produces the protonated form of dimethylamine (DMA) and HNO_2 (reaction 2) or rearrangement followed by dissociation of the excited state of NDMA to dimethylaminium radical and NO radical (reaction 3) (39).



N-Nitrosodimethylamine and perchlorate frequently occur together at the same Department of Defense sites because both compounds are used in the production of rocket fuel. For example, perchlorate and NDMA contamination were observed at Aerojet (Sacramento, CA) and in the Santa Clara valley (CA). So far, no research has been conducted on the remediation of NDMA and perchlorate when these compounds occur as co-contaminants.

3. Phytoremediation

Phytoremediation refers to a number of biotechnologies that involve the use of plants and microorganisms associated to the root zone of plants to either sequester or degrade contaminants or a combination of the two processes. Plants clean up contaminated soil and groundwater through several processes (40). These include:

- *Phytoaccumulation* - the uptake, translocation and concentration of contaminants in roots and aboveground shoots.
- *Phytovolatilization* - contaminant uptake and volatilization to the atmosphere.
- *Phytodegradation or phytotransformation* - the uptake and mineralization of contaminants by plants or incorporation of contaminants into plant tissues.
- *Rhizodegradation* - the break down of contaminants in the root zone through microbial activity. Plant exudation and other processes provide organic carbon and other nutrients to stimulate and promote microbial growth.
- *Phytostabilization or immobilization* - the holding of contaminated soils and sediments in place by vegetation and immobilization of contaminants in the rooted soils.

Phytoremediation has several advantages over most common remediation technologies. It is cost-effective, aesthetically pleasing to the public, environmentally compatible, and minimizes wind and water erosion. Phytoremediation has been used on a number of sites to clean up both organic and inorganic contaminants. This cleanup method can also be combined with source removal technologies to attenuate widespread contaminant concentrations to non-toxic levels.

Nzengung *et al.*, (16) showed that the removal of 22 to 100 mg L⁻¹ of perchlorate from planted hydroponic bioreactors to below the method detection limit of 2 µg L⁻¹ was attributed to plant uptake and phytodegradation which preceded the more rapid rhizodegradation phase. Aken and Schnoor (41) used radio-labeled ³⁶ClO₄⁻ to show the reduction of perchlorate in plant leaves *via* the same pathway observed for microbial mediated degradation (Equation 1). Plant uptake of perchlorate is not a desirable process because slow phytodegradation of perchlorate leads to phytoaccumulation. Some of the phytoaccumulated perchlorate either ends up in the food chain (10) or is re-released into the environment by leaching from the leaves (42). On the other hand, rhizodegradation is a desirable process that completely transforms perchlorate to chloride. Therefore, in order to avoid perchlorate recycling by deciduous plants, the uptake of perchlorate into plants should be minimized by biostimulation and maintaining high rates of perchlorate biodegradation by ubiquitous perchlorate degrading bacteria in the environment. The hypothesis of this research is that plant perchlorate uptake could be minimized by using dissolved organic carbon as electron source to biostimulate and enhance the rapid rhizodegradation of perchlorate.

Previous studies on the uptake of perchlorate by plants were performed in well-controlled laboratory and greenhouse conditions for relatively short durations. Therefore, little or no information is available from these studies to predict the fate of perchlorate in plants growing at perchlorate-contaminated field sites where environmental conditions may affect the uptake of

perchlorate by plants. Among the environmental factors that may influence plant uptake of perchlorate are the type of plant species, the duration of exposure to perchlorate, and the availability and amount of organic carbon in the rhizosphere.

Except for Dean-Raymond and Alexander (43) who showed that NDMA can be assimilated by the roots of lettuce and spinach with some fraction translocated to the stem and leaves, detailed studies on the mechanism(s) of phytoremediation of NDMA are lacking in the published literature.

The findings of this research, presented in the following four chapters address four objectives:

- 1) Evaluates the relative effectiveness of using natural and artificial electron donor such as organic compost, chicken litter extracts and acetate to biostimulate rapid rhizodegradation of perchlorate in hydroponics and soil bioreactors and minimize plant perchlorate uptake,
- 2) Determines the long-term fate of perchlorate taken up by plants in the field and the environmental factors affecting plant perchlorate uptake,
- 3) Investigates the potential of phytoremediation of NDMA, including the rate, fate, pathways, and phytoremediation mechanisms, and
- 4) Investigates whether plants selectively take up or rhizodegrade perchlorate or NDMA when these compounds occur together as co-contaminants.

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CHAPTER 3

USE OF DISSOLVED ORGANIC CARBON TO BIOSTIMULATE RAPID RHIZODEGRADATION OF PERCHLORATE: HYDROPONICS STUDIES ¹

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Abstract

Previous hydroponics and field studies show that phytodegradation and rhizodegradation are the two main mechanisms by which plants metabolize perchlorate. Plant uptake and phytodegradation of perchlorate is a slower and undesired process that poses ecological risks resulting from phytoaccumulation of some fraction of the perchlorate. By contrast, rhizodegradation is a more rapid and favored process involving perchlorate degraders utilizing dissolved organic carbon (DOC) as carbon and energy (electron) source to rapidly degrade perchlorate to innocuous chloride. In this study, rhizodegradation of perchlorate by willow trees (*Salix nigra*) was biostimulated and enhanced using electron sources obtained from organic mushroom compost, chicken litter extract and acetate. In bioreactors provided with electron sources as 500 mg L⁻¹ DOC, 25 to 40 mg L⁻¹ perchlorate was removed to below the ion chromatography (IC) method detection limit of 2 µg L⁻¹ in approximately 9 days. For planted controls provided with no electron donors, the time required for the complete removal of the same doses of perchlorate approximately 70 days. Enhancement of rhizodegradation reduced the phytoaccumulated fraction of perchlorate by an order of magnitude from about 430 mg kg⁻¹ to 20 mg kg⁻¹. High fraction uptake and phytoaccumulation of perchlorate in agricultural products and recycling of perchlorate into the ecosystem can be significantly curtailed by supplying electron donors derived from organic carbon sources to the root zone of plants.

Key words: perchlorate, phytodegradation, rhizodegradation, phytoaccumulation, dissolved organic carbon

1. Introduction

Perchlorate (ClO_4^-) is an anthropogenic as well as naturally occurring chemical in the environment. Over 90 % of anthropogenic perchlorate is manufactured as ammonium perchlorate (NH_4ClO_4) (1), an oxygen-adding component in propellants for rockets, missiles and fireworks. The natural sources of perchlorate include Chilean caliche, a sodium nitrate fertilizer with perchlorate concentrations of about 0.5 to 2 mg g⁻¹ (2). Recently, perchlorate was detected in many rain and snow samples indicating atmospheric origin (3). Anthropogenic and natural sources of perchlorate could be distinguished by isotopic analysis of oxygen and chlorine atoms (4). Perchlorate ions are quite stable under typical surface and groundwater conditions, especially under aerobic and high nitrate conditions.

Perchlorate contamination has been observed in surface water and groundwater at several sites where perchlorate salts have been manufactured, processed or used. In addition to drinking water, perchlorate has been detected in lettuce (5), dairy milk (6), and human breast milk (7). Perchlorate poses environmental concerns because it competitively blocks human thyroid iodide uptake. In January 2006, the U.S. Environmental Protection Agency (USEPA) issued a preliminary clean-up goal for perchlorate of 24.5 µg L⁻¹ in water (<http://epa.gov/newsroom/perchlorate.pdf>).

Anoxic bioremediation approaches including phytoremediation and microbial degradation show the greatest promise as potential low-cost sustainable technologies for cleanup of perchlorate-contaminated soils and water (8-10). For bioremediation approaches to be effective and sustainable, anaerobic conditions, perchlorate degraders and an adequate supply of carbon and electron sources should be present (11,12). Perchlorate-reducing microbes are ubiquitous (13). In the natural environment, the source of organic carbon utilized as electron

donors could be soil organic matter and plant root exudates such as acetate, organic acids, sugar, and dead root biomass. Under suitable conditions, biodegradation of perchlorate proceeds sequentially *via* chlorate (ClO_3^-) and chlorite (ClO_2^-) to chloride (Cl^-) (11,14). The dismutation of ClO_2^- into Cl^- and O_2 , an important step in the reductive pathways of perchlorate, is mediated by the enzyme chlorite dismutase (15). Degenerate primers were recently developed for the amplification of the chlorite dismutase gene *cld* (16).

Many species of terrestrial and aquatic plants can remove perchlorate from contaminated water and soils (17). Specifically, terrestrial [black willow (*Salix nigra*), eastern cottonwood (*Populus deltoides*)] and aquatic plants (water weed (*Elodea canadensis*), parrot-feather (*Myriophyllum aquaticum*), cattails (*Typha spp.*)] grown in hydroponic bioreactors dosed with low and high perchlorate concentrations effectively removed perchlorate to below the IC detection limit of $2 \mu\text{g L}^{-1}$ (18). Poplar trees (*Populus deltoides* \times *nigra*) reduced 25 mg L^{-1} perchlorate by 50 % during 30 days of incubation (19). The two predominant processes by which plants decontaminate perchlorate are uptake and phytodegradation, and rhizodegradation (20). Uptake and phytodegradation is a slower process that poses ecological risk due to uptake and temporal accumulation of perchlorate in plant leaves. In fact, uptake and slow phytodegradation of perchlorate is responsible for the accumulation of significant concentrations of perchlorate in grass eaten by herbivores and lettuce grown with perchlorate-contaminated irrigation water (21). Rhizodegradation is a more rapid process that involves perchlorate-respiring bacteria utilizing root exudates and soil organic carbon as electron source to rapidly degrade ClO_4^- to Cl^- .

The primary objective of this study was to develop a process that minimizes the fraction uptake of perchlorate by plants through the enhancement of rhizodegradation. Specifically, this research determined the efficacy of different electron sources provided as dissolved organic

carbon (DOC) derived from acetate, organic (mushroom) compost and poultry litter extract to: (I) reduce the lag-time (uptake and phytodegradation phase) that precedes rhizodegradation of perchlorate; (II) enhance biodegradation of perchlorate in the rhizosphere of trees (rhizodegradation); and (III) minimize the undesired uptake and temporal phytoaccumulation of perchlorate in plant tissues grown hydroponically.

2. Materials and Methods

2.1. Chemicals

Sodium perchlorate monohydrate ($\text{NaClO}_4 \cdot \text{H}_2\text{O}$) was purchased from Aldrich[®] (Milwaukee, WI). Sodium acetate trihydrate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) and sodium hydroxide (NaOH , 50% w/w) solution were obtained from J.T.Baker[®] (Phillipsburg, NJ). Sodium acetate trihydrate and sodium hydroxide were used to prepare dissolved organic carbon and ion chromatography eluent, respectively. Perchlorate standard solutions were purchased from AccuStandard[®] (New Haven, CT) and SPEX CertiPrep[®] (Metuchen, NJ). Peters Professional[®] (St. Louis, MO) (4.6% ammonical Nitrogen, 19.4% urea Nitrogen, 12% phosphate and 12% potash) was diluted to make the desired strength of nutrient solution. Organic (mushroom) compost was obtained from Advantage Organic Products (Duncanville, TX) in one gallon containers sold under the trade name 100 % Organic Compost Tea. Chicken litter was collected from the University of Georgia poultry farm. Almatris Ac. Inc. provided DD-6 alumina sorbent with 48×100 U.S. screen mesh and having $360 \text{ m}^2 \text{ g}^{-1}$ surface area.

2.2. Electron Sources

Aqueous solutions of acetate and ethanol were used as commonly available synthetic carbon and electron sources. Meanwhile, 100 % organic compost and chicken litter extracts were used as model carbon and electron sources derived from agricultural waste products. The organic compost was filtered through a 0.45 μm filter (Pall Corp., Ann Arbor, MI). Chicken litter extract solution was prepared by mixing 100 g of dry chicken litter with 1 L of deionized water. After shaking the mixture overnight and centrifugation at 1000 $\times g$ for 30 minutes, the supernatant was sampled and filtered through 0.45 μm filter papers. To prepare 500 mg L^{-1} aqueous solutions of DOC, different serial dilutions of the filtered extracts were measured. This required 5 and 20 fold dilutions of the concentrated raw mushroom compost and chicken litter extracts, respectively. A 500 mg L^{-1} solution of DOC was prepared by dissolving 2.84 g of $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ in 1 L of deionized water. For some experiments, the mushroom compost and chicken litter extracts were sterilized by autoclaving to eliminate the microbial contribution derived from the extracts prior to use in the phytoremediation experiments. The carbon content in each media was measured with a Shimadzu 5050 Total Organic Carbon analyzer. For sterilized experiments, DOC measurements were made after autoclaving.

2.3. Hydroponic Bioreactors

The selection of willow trees for this study was based on their high rate of survival under our greenhouse conditions. Several willow (*Salix nigra*) tree cuttings were harvested from a local garden in Athens, Georgia, and pre-rooted in hydroponic growth medium under natural light in a

greenhouse at ambient temperature. For each experiment, pre-rooted cuttings with similar stem diameter, root system and total biomass were selected. The pre-rooted trees were transferred to 2 L Erlenmeyer[®] flasks (bioreactors) wrapped in aluminum foil and containing 0.39% (w/w) diluted Peters Professional[®] plant growth solution dissolved in perchlorate-containing DI water or perchlorate-contaminated groundwater. The septum and tree cutting interface was sealed with Parafilm[®] and DAP[®] aquarium sealant (100 % silicon) in order to prevent water loss by volatilization. The volume of water in the bioreactors was maintained at the 2 L mark by adding nutrient solution dissolved in deionized water or groundwater. The volume of water transpired by the willow plants from each bioreactor and the weight of each tree was recorded throughout the course of the experiments.

Initially, the experiments were conducted with deionized water containing approximately 25 mg L⁻¹ perchlorate. The experiments were repeated with approximately 40 mg L⁻¹ perchlorate-contaminated groundwater collected in the summer 2004 from the Longhorn Army Ammunition Plant, Karnack, TX. Each planted bioreactor was dosed with acetate, ethanol, diluted organic compost or chicken litter extract to obtain 500 mg L⁻¹ DOC as the electron source. Triplicate planted bioreactors and corresponding controls with no DOC amendment were prepared and handled in parallel. A 1.5 ml sample of rhizosphere solution was taken for analysis from each bioreactor until the concentration of perchlorate decreased below the ion chromatography (IC) method detection limit (MDL) of 2 µg L⁻¹.

At the termination of each experiment, the plants were harvested, sectioned into leaves, stem and roots, weighed, and washed with deionized water before drying overnight at 105 °C. The dried samples were weighed and pulverized with a mortar and pestle. The pulverized samples were sieved with a 500 µm mesh screen prior to extraction. The plant tissues extraction

and clean-up procedure described in detail by Ellington and Evans (22) was followed. To confirm the identity of perchlorate peaks, some of the duplicate samples prepared for IC analysis were spiked with $100\ \mu\text{g L}^{-1}$ perchlorate prior to analysis.

2.4. Analytical Methods

Water samples taken from the rhizosphere of dosed willow trees were diluted as needed to the working perchlorate concentration range of 0.002 to $1.5\ \text{mg L}^{-1}$. Each prepared sample was placed in two $5\ \text{ml}$ Dionex[®] autosampling vials. All samples were stored at $4\ ^\circ\text{C}$ between preparation steps and until the samples were analyzed. Analysis was performed on a Dionex[®] DX-500 IC outfitted with an IONPAC[®] AG16 guard column ($4 \times 50\ \text{mm}$) and an IONPAC[®] AS16 analytical column ($4 \times 250\ \text{mm}$). The IC was equipped with a Dionex[®] AI-450 Chromatography Automation System and the Advanced Computer Interface Module (ACI). The system was run using an ASRS-ULTRA II Self-Regenerating Suppressor ($4\ \text{mm}$) at a $300\ \text{mA}$ setting. A $100\ \text{mM}$ and $50\ \text{mM}$ sodium hydroxide (NaOH) eluent at a flow rate of $1\ \text{ml min}^{-1}$ and a $500\ \mu\text{l}$ sample loop were used to measure high (mg L^{-1}) and low ($\mu\text{g L}^{-1}$) perchlorate concentrations, respectively. The eluent was made using J. T. Baker[®] $50\ \%$ (w/w) NaOH solution and deionized, degassed (in the VWR Scientific Aquasonic, Model 150D) water. Calibration and check standards were made by diluting $1000\ \mu\text{g mL}^{-1}$ perchlorate anion standard (SPEX CertiPrep, Inc.[®]) and $0.5\ \mu\text{g mL}^{-1}$ and $1\ \mu\text{g mL}^{-1}$ standards (AccuStandard, Inc.[®]). A new calibration curve was created each time the IC was turned on, or after the eluent had been changed (every 2 to 3 days). For quality control, all samples were run in duplicate, and an external standard and a blank were run after every two samples. The check standards were used

to ensure that the percent error remained below 5 %, and to monitor any instrumental drift, while the blank checked for any carry-over from the previous sample. Student's t-test was used to determine if differences between treatments were significant.

The presence of heavy metals in organic compost, chicken litter extract and acetate dosed bioreactors was determined using a VG Elemental Plasma Quad III ICPMS. Concentrations of NO_3^- , NO_2^- , NH_4^+ , total P and SO_4^{2-} was quantified using a Bran-Luebbe Auto Analyzer II continuous flow system.

2.5. Bacterial Cell Counts and Polymerase Chain Reaction (PCR) Amplification

Standard cell counts were performed on plate count agar (Difco). Plates were incubated at room temperatures under aerobic conditions for 2 days before the colony counts. DNA was extracted from the samples using the Ultra Clean Soil DNA Kit (MO BIO, Laboratories, Inc., Ca) and amplified with the primers 238F (5'-T(C/T)GA(A/C/G)AA(A/G)CA(C/T)AAGGA(A/T/C)AA(A/C/G)GT AND UCD646R (5'-GAGTGGTA(A/C/G)A(A/G)(C/T)TT(A/C/G)CG(C/T)TT) (16). Polymerase chain reaction (PCR) was performed from 100 ng of purified sample DNA or from 1 μl of cell suspension of the bacterial isolates transferred directly to a 30 μl PCR mixture containing 50 mM KCl, 10 mM Tris-HCl, 2 mM MgCl_2 , 0.1 μM each primer, 200 μM each deoxynucleoside triphosphate (Promega), and 0.5 U of Taq polymerase (Promega). Samples were denatured for 4 minutes at 94 °C, and amplified for 35 cycles at 94 °C for 30 seconds, 53 °C for 1 minute, 72 °C for 30 seconds and finally held at 4 °C. PCR products were analyzed by agarose gel electrophoresis (15).

3. Results and Discussion

The efficacy of four electron sources to biostimulate and enhance rapid rhizodegradation of perchlorate was evaluated in hydroponic bioreactors dosed with the same perchlorate concentration in deionized and ground water. The electron sources were provided in equal concentrations (500 mg L^{-1}) of DOC to the growth solution of willow trees as acetate, ethanol, organic compost, or chicken litter extract. The performance of the DOC sources were evaluated based on the capability to: (I) reduce the long lag-time that normally precedes the rhizodegradation phase of perchlorate, (II) the overall time required for complete removal of the initial perchlorate concentration in bulk solution to below the method detection limit (MDL), and (III) the amount of perchlorate phytoaccumulated in plant leaves at the end of the experiment. Of the four types of carbon and electron sources tested, only ethanol was phytotoxic to the young willow trees under our greenhouse conditions. The 500 mg L^{-1} aqueous DOC solutions of ethanol caused plant mortality within 7 days of dosing the hydroponically grown willow trees.

No significant difference in plant biomass was observed between trees grown with and without the electron sources. Specifically, the average increase of fresh biomass for trees exposed to 500 mg L^{-1} DOC was 1.17 g day^{-1} and the controls grown without DOC was 1.15 g day^{-1} . This suggested that none of the three DOC sources (*i.e.*, acetate, organic compost nor chicken litter extract) utilized as electron sources was phytotoxic to the young willow trees at the concentration applied. Volumes of water transpired by the trees during the experiment were similar for planted bioreactors dosed with DOC ($87 \pm 5 \text{ ml day}^{-1}$) and without DOC ($83 \pm 5 \text{ ml day}^{-1}$). The concentration of a likely competing terminal electron acceptor, nitrate, was below the

method detection limit (MDL) of 0.2 mg L^{-1} throughout these experiments (Table 3.1).

Elemental analysis of samples from the chicken litter extract and organic compost bioreactors showed that both are rich in plant nutrients such as N, P, Na, K, Ca and Fe. Except for arsenic, the amount of heavy metals in diluted chicken litter extract and organic compost bioreactors was below the USEPA maximum contaminant level (MCL). The source of heavy metals in the acetate bioreactors is from the diluted Peters Professional[®] nutrient solution.

3.1. Electron Sources from Agricultural Waste Products

Evidence that perchlorate degraders mediated the rhizodegradation of perchlorate was obtained from the chlorite dismutase gene (*clt*) detected in the DNA extracts from bacteria isolated in the unsterilized DOC sources are presented in Figure 3.1. Unlike synthetic acetate, the agricultural wastes apparently provided additional bacteria to the root zone solution. For each media, the following bacterial counts as colony forming units (CFU) on nutrient agar plate were estimated: $1.6 \times 10^8 \text{ ml}^{-1}$ in diluted chicken litter extract, $6.1 \times 10^5 \text{ ml}^{-1}$ in dilute mushroom compost, $1.2 \times 10^8 \text{ g}^{-1}$ in soil, and no bacteria isolates detected in the freshly prepared aqueous acetate solution.

The removal of perchlorate from solution in willow planted bioreactors amended with 500 mg L^{-1} DOC from organic compost and chicken litter extract was observed to be rapid (Figures 3.2 and 3.3). The rapid removal of perchlorate, which did not correlate with water uptake by the willow trees, was attributed to degradation by rhizosphere bacteria or rhizodegradation. As shown in Figure 3.2, 25 mg L^{-1} perchlorate was completely removed from

Table 3.1. Concentration of heavy metals and plant nutrients in the various dilutions of DOC sources: chicken litter extract (CL) (100 % and 5 %), organic (mushroom) compost (MC) (100 % and 20 %), and acetate (A) (1000 mg L⁻¹ DOC). The Maximum Contaminant Levels (MCL) (Hazardous Site Inventory, Environmental Protection Division, Georgia Department of Natural Resources July 1, 1995) for the different elements are also presented. With the exception of arsenic, heavy metal concentrations are below the MCL.

Sample	Total N	Total P	NO ₃	TOC	TIC	Cr	Cu	Zn	As	Se	Sr	Cd	Pb
	(mg L ⁻¹)	(mg L ⁻¹)	(mg L ⁻¹)	(mg L ⁻¹)	(mg L ⁻¹)	(µg L ⁻¹)	(µg L ⁻¹)	(µg L ⁻¹)	(µg L ⁻¹)	(µg L ⁻¹)	(µg L ⁻¹)	(µg L ⁻¹)	(µg L ⁻¹)
CL (100 %)	1727.0	704.8	28.8	6708.4	251.2	131.2	24950	15280	2521	65.2	886.1	9.5	16.8
CL (5 %)	339.2	127.2	< 0.2	543.5	246.3	14.5	2835	1107	87.2	5.1	30.2	0.7	0
MC (100 %)	346.9	3.9	< 0.2	2408.2	735.5	67.5	145.3	192.6	484.7	33.9	482.9	0.4	0
MC (20 %)	260.6	127	< 0.2	572.9	56.0	5.2	850.4	1884	49.8	7.6	147.6	0.4	0
Acetate	260.2	92.3	< 0.2	796.5	139.5	8.6	485.1	1324	1.2	2.9	30.6	0.4	0
MCL			10 ^a			100	1300	2000	50	50		5	15

^awww.epa.gov/safewater/consumer/mcl/pdf

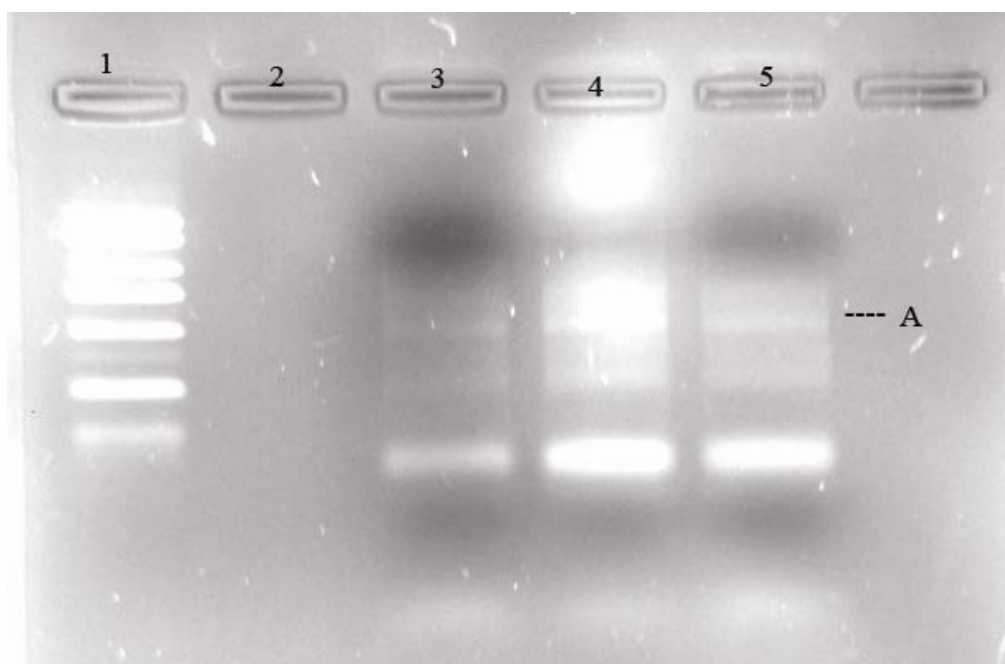
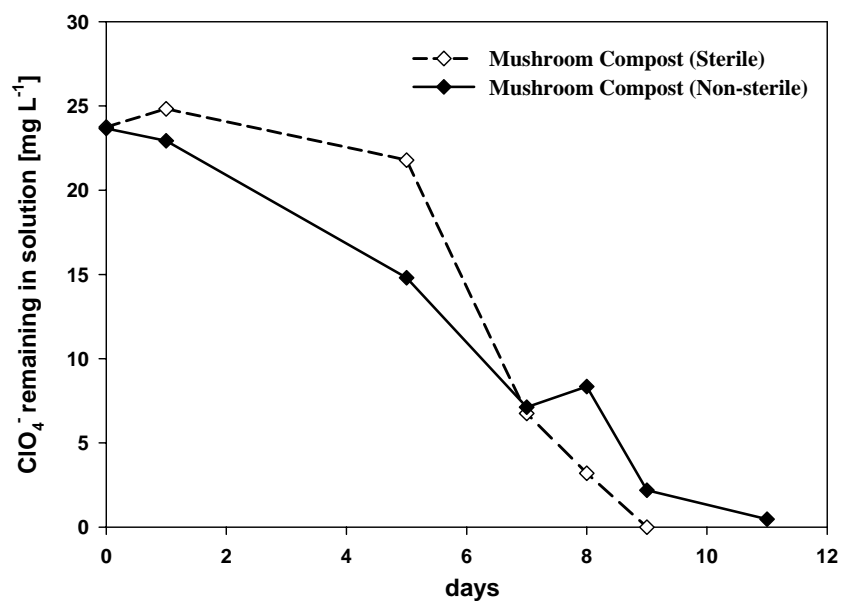
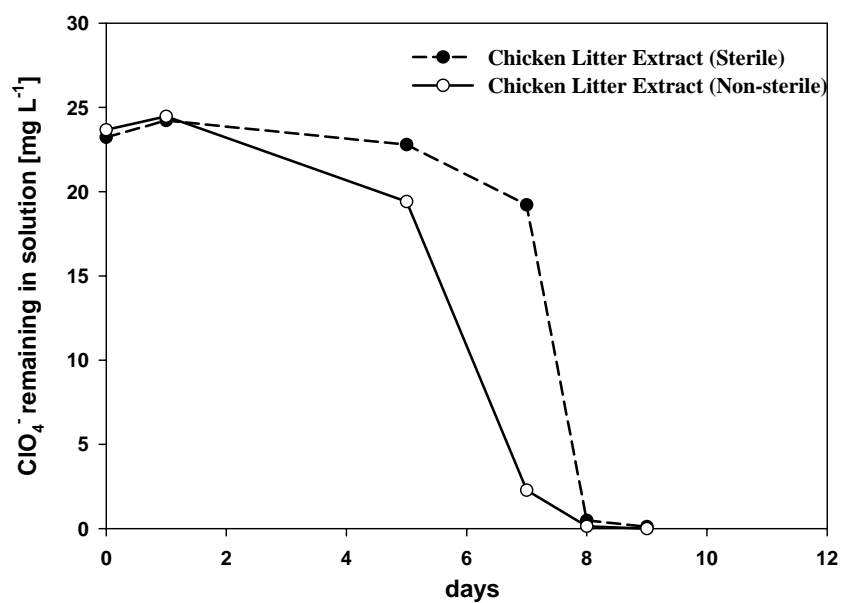


Figure 3.1. PCR detection of a 408-base pairs segment of the *cld* gene using the UCD-238F/ucd-646R primer set. Lane 1, molecular weight ladder (2000 base pairs to 200 base pairs); lanes 3 and 4 are diluted mushroom compost and chicken litter extract, respectively, added to the hydroponic reactors, and lane 5 is soil from Athens, GA.



a



b

Figure 3.2. The relative rate of biostimulation of rhizodegradation of perchlorate in the root zone solution of willow (*Salix nigra*) planted bioreactors amended with sterilized and unsterilized electron sources provided as 500 mg L⁻¹ DOC from chicken litter extract and organic (mushroom) compost tea.

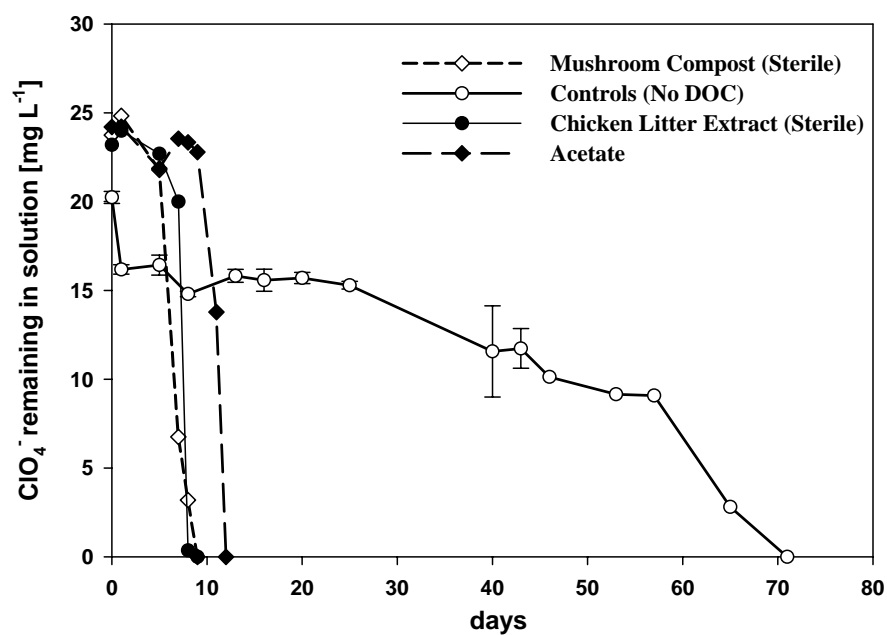


Figure 3.3. The relative rates of biostimulation of rhizodegradation of perchlorate in the root zone solution of control willow (*Salix nigra*) planted hydroponic bioreactors and enhanced treatments provided with different electron sources as 500 mg L⁻¹ DOC from mushroom compost, chicken litter extract and acetate. The controls received dilute Peters Professional[®] plant growth solution and no external electron sources.

planted bioreactors within 9 to 11 days. A noticeable difference between experiments conducted with the sterilized and unsterilized electron sources was the short lag-time (the time before the beginning of the faster rhizodegradation phase) of only 1 day in bioreactors supplied with unsterilized DOC.

Contrarily, in bioreactors dosed with the sterilized carbon and electron sources, a lag-phase of about 5 days preceded rhizodegradation with the complete removal of perchlorate to the MDL within 3 to 5 days, thereafter. The rhizodegradation phase started earlier in bioreactors with unsterilized DOC sources because the microbial population rapidly used all the terminal electron acceptors such as dissolved oxygen (DO) and nitrate in the rhizosphere. As soon as these terminal electron acceptors were completely used, microorganisms shifted to use perchlorate as a terminal electron acceptor. In bioreactors supplied with sterilized DOC, longer time was required for the microbial community to increase in number and use terminal electron acceptors such as DO and nitrate before beginning to use perchlorate as a terminal electron acceptor. The short lag-time observed in the unsterilized experiments indicated that unsterilized electron donors derived from agricultural waste products supply additional perchlorate-degrading microbes to the rhizosphere, resulting in faster biostimulation of rhizodegradation of perchlorate.

Rhizodegradation of perchlorate followed zero-order kinetics. An average zero-order rhizodegradation rate of $7.4 \pm 0.2 \text{ mg L}^{-1} \text{ day}^{-1}$ was estimated for experiments with 500 mg L^{-1} DOC from organic compost utilized as the electron source and $7.6 \pm 0.1 \text{ mg L}^{-1} \text{ day}^{-1}$ for electron donors provided as sterilized or unsterilized chicken litter extract.

The detection of perchlorate and the metabolites chlorate, chlorite and chloride in the harvested leaves reported elsewhere has provided direct evidence of phytodegradation of some fraction of perchlorate taken up into the leaves (18, 19). Because the degradation process is slow,

Table 3.2. The amount of perchlorate removed from each bioreactor and measured perchlorate concentration in the plant tissues.

Treatment	ClO ₄ ⁻ removed (mean ± 1SD) (mg L ⁻¹)	Recovered ClO ₄ ⁻ in plant tissues (mg Kg ⁻¹ fw) (mean ± 1SD)			ClO ₄ ⁻ mass balance (mg) (mean ± 1SD)			
		Leaves	Stem	Roots	Initial	Phytoaccumulated	Rhizodegraded	% Rhizodegraded
Sterilized chicken litter extract	23.2 ± 0.1	35.4 ± 5.4						
Unsterilized chicken litter extract	23.7 ± 0.1	30.3 ± 0.1						
Sterilized chicken litter extract *	39.3 ± 0.2	65.6 ± 0.3	2.3 ± 0.1	0.73 ± 0.1	78.6 ± 0.5	0.35 ± 0.1	78.2 ± 0.7	99.55
Sterilized Mushroom compost	23.7 ± 0.2	32.5 ± 0.2						
Unsterilized mushroom compost	23.7 ± 0.0	32.6 ± 0.1						
Acetate (500 mg L ⁻¹)	24.0 ± 0.3	18.0 ± 4.8						
Acetate (500 mg L ⁻¹) *	41.2 ± 0.2	92.4 ± 0.4	4.4 ± 0.1	0.37 ± 0.1	82.4 ± 0.2	0.50 ± 0.1	81.9 ± 0.3	99.40
Acetate (250 mg L ⁻¹)	24.0 ± 0.1	16.5 ± 0.1						
Acetate (1000 mg L ⁻¹)	23.3 ± 0.2	26.2 ± 0.8						
Control 1	04.2 ± 0.0	117.1 ± 0.4						
Control 2	23.4 ± 0.2	424.1 ± 1.4						
Control 3 ^{b,*}	41.1 ± 0.3	428.3 ± 0.5	3.3 ± 0.1	2.8 ± 0.1	82.2 ± 0.6	11.9 ± 0.1	70.3 ± 0.9	85.52

^b Control plant #3 was sacrificed after 25 days of the experiment during which the concentration of perchlorate in solution was below the method detection limit of 2 µg L⁻¹.

* Experiment conducted with groundwater collected from Longhorn Army Ammunition Plant, Karnack, Texas.

plants accumulate perchlorate mainly in the leaves where maximum transpiration takes place.

The concentration of perchlorate measured in plant leaves harvested at the end of the experiment from bioreactors dosed with mushroom compost and chicken litter extracts were $32.6 \pm 0.1 \text{ mg kg}^{-1} \text{ fw}$ (fresh weight) and $33.7 \pm 5.4 \text{ mg kg}^{-1} \text{ fw}$, respectively (Table 3.2). Using the Tukey standardized range (HSD) test, these values were not significantly different at $\alpha = 0.01$.

Perchlorate recoveries in spiked plant samples were 95+ %.

For each planted control experiment, a prolonged slow initial perchlorate removal phase attributed mainly to plant uptake in transpired water preceded the rapid rhizodegradation phase, similar to previous observations (17,20). Thus, the removal of the 23 mg L^{-1} perchlorate solution to the MDL took 71 days (Figures 3.3), an order of magnitude longer than for the DOC-amended willow-planted bioreactors. The observed slow removal of perchlorate was attributed mainly to uptake into the willow leaves where the perchlorate is phytoaccumulated and slowly phytodegraded to chloride (18). As a result, a decrease in initial perchlorate concentration of $23 \pm 0.2 \text{ mg L}^{-1}$ by only 4.2 mg L^{-1} during 8 days of phytoremediation was accompanied by uptake and phytoaccumulation of about $117 \text{ mg kg}^{-1} \text{ fw}$ in the willow leaves. This increased to $424 \pm 1.4 \text{ mg kg}^{-1} \text{ fw}$ after the initial perchlorate was removed to below the MDL. The faster rhizodegradation began after 57 days with an average perchlorate degradation rate of $0.8 \text{ mg L}^{-1} \text{ day}^{-1}$, an order of magnitude less than estimated for experiments in which external sources of electron donors were provided.

3.2. *Electron Source Provided as Acetate*

The complete removal of 25 mg L⁻¹ perchlorate from the aqueous growth solution in hydroponic bioreactors amended with 250 to 1000 mg L⁻¹ DOC as a dilute solution of acetate took 8 to 12 days (Figure 3.4). The faster perchlorate removal from bioreactors amended with 250 and 1000 mg L⁻¹ DOC is possibly due to higher population of microorganisms in these bioreactors. The lag times for experiments with acetate as the DOC source were similar to sterilized organic compost tea and chicken litter extract-amended bioreactors. However, once biostimulation in the rhizosphere was achieved, perchlorate removal was relatively faster with a zero-order rate constant of 11.6 ± 0.3 mg L⁻¹ day⁻¹. The faster rhizodegradation rate in acetate-amended bioreactors compared to organic compost and chicken litter extract may be attributed to the bioavailability and relatively higher rate of metabolism of acetate than the DOC derived from agricultural waste. Acetate is among the suite of natural dissolved organic compounds exuded by plants (23). The perchlorate concentration in plant leaves harvested from 500 mg L⁻¹ acetate-amended bioreactors was 18.05 ± 4.82 mg kg⁻¹ fw; a factor of two less than the perchlorate concentration measured in willow leaves from organic compost- and chicken litter-amended bioreactors (Table 3.2).

The similarity in the rate of rhizodegradation in bioreactors dosed with 250, 500, and 1000 mg L⁻¹ DOC as acetate (Figure 3.4) suggests that the optimum concentration of DOC needed to biostimulate and enhance rhizodegradation could be less than 250 mg L⁻¹. The total organic carbon (TOC) measurement taken at the beginning and end of the experiment indicated a decrease in TOC as perchlorate was biodegraded. For example, in acetate-amended planted bioreactors, the TOC concentration measured in solution decreased from 583 mg L⁻¹ at the

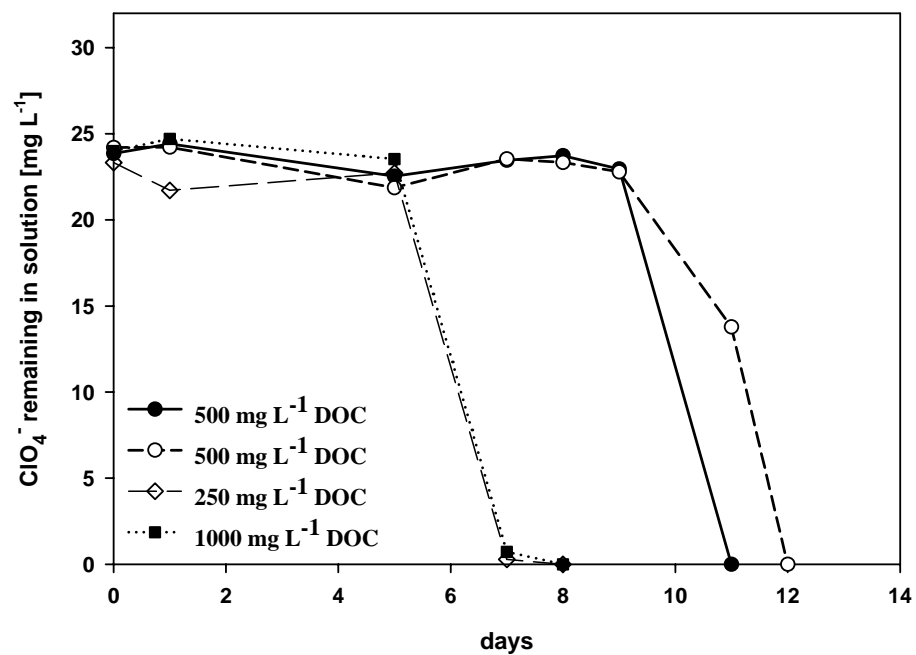


Figure 3.4. Relative rates of perchlorate removal by rhizodegradation in willow (*Salix nigra*) planted bioreactors amended with three concentrations of electron sources: 250, 500, and 1000 mg L⁻¹ DOC as acetate.

beginning of the experiment to 393 mg L^{-1} when 40 mg perchlorate was completely degraded to chloride.

3.3. Groundwater Experiments

For experiments conducted with perchlorate-contaminated groundwater, the complete removal of an initial 40 mg L^{-1} perchlorate to below the MDL was achieved in 7 days with a lag-time of 2 to 3 days preceding rhizodegradation, as the primary phytoremediation mechanism (Figure 3.5). Except for the planted controls, each willow bioreactor was provided with an electron source as either 500 mg L^{-1} DOC from sterilized chicken litter extract or acetate. Based on the lag-times for the three sets of experiments conducted with groundwater (Figure 3.5), biostimulation of rhizodegradation was most rapidly achieved in bioreactors treated with sterilized chicken litter extract (2 to 3 days) and acetate (7 days) than in the planted controls (22 days).

The complete removal of 23 mg L^{-1} perchlorate from growth solution prepared with deionized water required 71 days (Figure 3.3) compared to 25 days for 40 mg L^{-1} perchlorate-contaminated groundwater (Figure 3.5). The shorter treatment time for perchlorate-contaminated groundwater is attributed to the documented presence of ubiquitous perchlorate degrading bacteria in the groundwater that may not be present in the deionized water.

Willow leaves harvested after the complete treatment of 40 mg L^{-1} perchlorate-contaminated groundwater dosed with sterilized chicken litter extract phytoaccumulated $65.65 \pm 0.26 \text{ mg kg}^{-1}$ fw perchlorate, which is twice the perchlorate concentration measured in leaves harvested from trees used to treat 23 mg L^{-1} . In the control bioreactors dosed with 40 mg L^{-1}

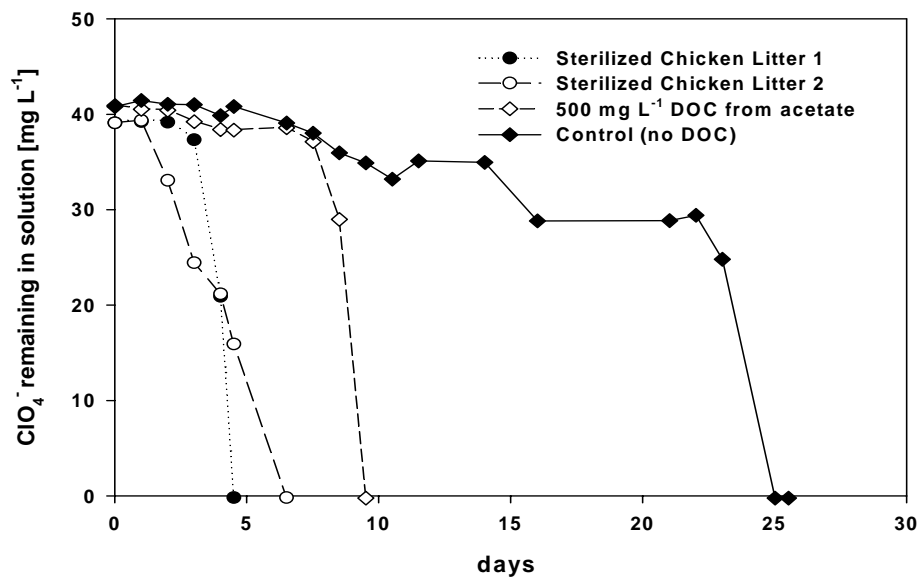


Figure 3.5. The relative rates of biostimulation and rhizodegradation of perchlorate in willow (*Salix nigra*) planted control bioreactors and enhanced treatments provided with 500 mg L⁻¹ DOC from chicken litter extract or acetate as the electron sources. The perchlorate-contaminated groundwater from LHAAP in Karnack, Texas, used in this experiment also contained 0.39 % (w/w) Peters Professional[®] plant growth media as plant nutrient.

perchlorate, the concentration of perchlorate phytoaccumulated in the willow leaves was $428.35 \pm 0.53 \text{ mg kg}^{-1} \text{ fw}$, which is 4- to 6-fold higher than in the leaf tissues of plants provided with the different electrons sources. This suggested that for the same plant species, the bioconcentration factor is determined by the initial concentration of perchlorate in the rhizosphere and duration of the slow uptake phase during phytoremediation. From these experiments, the three distinct differences that emerge between the control and the enhanced rhizodegradation experiments include: (I) the longer lag-time that precedes rhizodegradation in the control bioreactors; (II) the low perchlorate mass removal rate from the control bioreactors; and (III) the high fraction perchlorate phytoaccumulated in the leaf tissue of control plants, if the concentration of electron donors is low and not augmented.

3.4. Estimation of Phytoaccumulated Fraction

The likely presence of initially higher concentrations of oxygen in the plant root zone at the start of phytoremediation experiments suggests that perchlorate removal from the plant growth solution prior to biostimulation of rhizodegradation should be attributed mainly to uptake. If the latter assumption is correct, the uptake rate of perchlorate and other water miscible chemicals from the plant nutrient solution can be estimated as follows:

$$\text{Uptake rate of perchlorate ions} = \frac{(C_i - C_{\text{lag}}) * V}{T_{\text{lag}}} \quad (1)$$

where C_i is the initial concentration of perchlorate in the 2 L bioreactor, C_{lag} is the perchlorate concentration in the bioreactors at the end of the estimated lag-time, V is volume of growth solution in the bioreactor and T_{lag} is the number of days before the faster rhizodegradation begins. Table 3.3 presents the estimated lag time from time course data for experiments

Table 3.3. Calculated perchlorate uptake rate and transpiration stream concentration factor (TSCF) for the different treatments.

Treatment	T _{lag} (days)	Initial ClO ₄ ⁻ concentration (mg L ⁻¹)	Mass uptake rate (mg day ⁻¹)	Plant uptake mass (mg)	Transpiration (L day ⁻¹)	TSCF
Chicken litter	4.0	39.3	0.89	3.5	0.087	0.259
Acetate	7.5	41.2	1.01	7.6	0.087	0.282
Control	25.0	41.09	0.91	22.8	0.083	0.268
Control	57.0	20.5	0.40	22.8	0.08	0.244
Mushroom compost	5.0	23.8	0.45	2.3	0.08	0.240
Average ± 1 SD						0.26±0.02

conducted during this study, the total uptake mass of perchlorate, and the uptake rate of perchlorate for representative experiments. There is a notable agreement in the uptake rate of perchlorate for the different sets of experiments with similar initial concentration.

Using the average uptake rate estimated from (Table 3.3), the contaminant specific fractional efficiency of uptake (transpiration stream concentration factor, TSCF) was calculated as follows (24):

$$TSCF = \frac{U}{C * T} \quad (2)$$

where TSCF is the ratio of the contaminant concentration in the transpiration stream to the concentration in the external solution (dimensionless), U is uptake rate of contaminant (mg day^{-1}), C is the aqueous concentration of the contaminant (mg L^{-1}), and T is the transpiration rate of vegetation (L day^{-1}). The calculated TSCF of perchlorate for the uptake dominated phase (*i.e.*, prior to biostimulation of rhizodegradation) in willow bioreactors provided with an electron source was 0.26 ± 0.02 , which is similar to the TSCF calculated for the controls 0.25 ± 0.02 (Table 3.3). Hydrophilic or polar compounds like perchlorate tend to have low TSCF values because these compounds do not sorb to plant roots and are not actively transported through the lipid membranes of the roots (25,26). In fact, a TSCF of about 0.2 is expected for very polar compounds like perchlorate (25). The similar TSCF values obtained for both DOC amended and control willow trees means that for the same concentration of perchlorate in the rhizosphere, the duration of lag-phase will determine the mass of perchlorate taken up by each tree.

3.5. Implication of Enhancing Rhizodegradation of Perchlorate

Plant perchlorate uptake in transpired water coupled with the slow rate of phytodegradation leads to the accumulation of perchlorate in the food chain. Cattle raised on perchlorate-contaminated fields will ingest large quantities of perchlorate-contaminated vegetation. As a result, dairy and beef products raised on such vegetation could become contaminated by perchlorate. Kirk *et al.* (23) showed the presence of perchlorate in all seven milk samples purchased randomly in Lubbock, TX. Hogue (21) found perchlorate in all 18 samples of California lettuce, presumably from Colorado River irrigation water, which carries perchlorate from a former industrial plant near Las Vegas. The high bioconcentration of perchlorate by some field plants could potentially result in perchlorate recycling when senescent deciduous leaves drop during the fall season (27). To minimize ecotoxicological concerns posed by uptake and phytoaccumulation of perchlorate due to the slow rate of phytodegradation, enhancement of rhizodegradation should be incorporated when planting vegetation on perchlorate contaminated sites and when designing phytoremediation of perchlorate.

The results of this study highlight the benefit of using commonly available cheap sources of DOC as electron donors needed to reduce the lag-time that precedes rhizodegradation and enhance the rhizodegradation of perchlorate. Providing electron sources to the rhizosphere may increase the rate of rhizodegradation by as much two orders of magnitude which should significantly reduce the time to achieve cleanup goals in the field. The electron sources obtained from agricultural waste such as chicken litter and organic compost extracts have an added advantage because these supply perchlorate degraders to the rhizosphere of trees where the

concentration of these desired bacteria may be low. The successful biostimulation and enhancement of rhizodegradation caused the uptake and phytoaccumulated fraction of perchlorate to reduce by an order of magnitude from 430 mg kg^{-1} to 20 mg kg^{-1} fw. Thus, manipulation of the root zone environment to create favorable conditions for rhizodegradation minimizes plant uptake of perchlorate. An important benefit of this study is that the potential ecotoxicological concerns posed by plant uptake and phytoaccumulation of perchlorate in food crops and other vegetation grown on perchlorate-contaminated soils and water could be mitigated by increasing the concentration of electron sources or DOC content in the rhizosphere.

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CHAPTER 4

USE OF DISSOLVED ORGANIC CARBON TO BIOSTIMULATE RAPID RHIZODEGRADATION OF PERCHLORATE: SOIL EXPERIMENTS¹

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Abstract

The main limitation to biodegradation of perchlorate in most soils and groundwater tends to be the availability of an adequate supply of organic carbon or electron donors. This study investigated the use of electron sources provided as dissolved organic carbon (DOC) from chicken litter extract and acetate to enhance and sustain rhizodegradation of perchlorate in contaminated soils. This approach should reduce the residence time of perchlorate in soils and minimize the well-documented plant uptake of perchlorate at contaminated field sites. Willow trees (*Salix nigra*) planted in 20 L soil bioreactors were dosed with perchlorate-contaminated water multiple times. The rate of biodegradation of perchlorate in willow-planted soil bioreactors provided with electron sources as 300 mg L⁻¹ DOC was very rapid and described by zero-order kinetics with a maximum rate constant of 24 mg L⁻¹ day⁻¹. For planted control experiments in which DOC was limiting, the removal of perchlorate primarily by biodegradation was described by pseudo-first-order kinetics with a maximum rate constant of 0.35 day⁻¹. The fraction of perchlorate phytoaccumulated in the control plants was an order of magnitude higher than in plants grown in the DOC-dosed bioreactors. The results of this study indicate that the slow build up of DOC in the rhizosphere by root exudation and organic matter decomposition is insufficient to sustain a high rate of rhizodegradation of perchlorate and perhaps other degradable contaminants in vegetated contaminated soils. Therefore, an optimum design of phytoremediation of perchlorate should include enhancement of rhizodegradation by providing an optimum and sustained supply of electron donors.

1. Introduction

Contamination of freshwater resources, fresh produce and some processed foods by perchlorate (ClO_4^-) is a growing problem in the United States. The anion perchlorate is a naturally occurring as well as an anthropogenic chemical which occurs as salts of ammonium, sodium and potassium. The natural sources of perchlorate include Chilean caliche which is mined for use as a natural source of NaNO_3 in fertilizer and evaporite deposits in arid climates. Fertilizer manufactured from Chilean caliche may contain approximately 0.5 to 2 mg g^{-1} perchlorate (1). The main anthropogenic source of perchlorate is ammonium perchlorate (NH_4ClO_4), which is used as an oxygen-adding component in propellants for rockets, missiles and fireworks. Ammonium perchlorate is highly soluble in water and dissociates to ammonium (NH_4^+) and perchlorate (ClO_4^-) ions. Perchlorate is very stable under typical surface and ground water conditions.

Perchlorate is not only found in the drinking water supplies of over 15 million people in the USA (2), but is also found in foods such as lettuce (3), dairy milk (4) and breast milk (5). The health concerns over perchlorate result from the interference with the function of the human thyroid gland. Perchlorate competitively inhibits thyroid iodide uptake, which results in disruption of the normal thyroid hormone production. In January 2006, the U.S. Environmental Protection Agency (USEPA) set a preliminary cleanup goal for perchlorate of $24.5 \text{ } \mu\text{g L}^{-1}$ in drinking water (6).

Anoxic bioremediation and phytoremediation processes have shown great promise as cost-efficient and sustainable approaches for the removal of perchlorate from contaminated soils and water (7). Under favorable root zone conditions, an adequate supply of carbon and electron

sources is required to mineralize perchlorate. In the natural environment, the sources of electrons and organic carbon may include soil organic matter, exudates of plant roots such as ethanol, acetate, organic acids, sugars, and dead root biomass. Perchlorate reducing microbes are ubiquitous in soils, sediment and water (8). Under reducing conditions, microbes use perchlorate as a terminal electron acceptor in the absence of nitrate, thereby degrading perchlorate to innocuous chloride ion. When suitable environmental conditions exist, perchlorate degradation follows the pathway: $\text{ClO}_4^- \rightarrow \text{ClO}_3^- \rightarrow \text{ClO}_2^- \rightarrow \text{Cl}^-$ (9,10). The dismutation of ClO_2^- into Cl^- and O_2 , an important step in the reductive pathways of perchlorate, is mediated by an enzyme chlorite dismutase (11). Degenerate primers were recently developed by Bender *et al.*, (12) for the amplification of the chlorite dismutase gene *cld*.

At the bench scale, phytoremediation has been shown to be a promising technology for the clean up of perchlorate-contaminated surface water and groundwater (13,14). Previous research has identified two predominant mechanisms of phytoremediation of perchlorate as uptake and phytodegradation, and rhizodegradation (13,15). Uptake and phytodegradation poses ecological risk due to uptake and slow phytodegradation resulting in phytoaccumulation of a fraction of the perchlorate taken-up into plants. A growing number of studies have documented the uptake and accumulation of perchlorate in plant leaf tissues, including tobacco (16), lettuce (3,17), grass (18), terrestrial and aquatic plants (19). The detection of perchlorate in dairy and breast milk (4,5) provides direct evidence of perchlorate accumulation in the food chain. Unlike uptake and phytodegradation, rhizodegradation is a desirable process because perchlorate-respiring microbes in the root zone rapidly degrade ClO_4^- to Cl^- . The most important benefits of rhizodegradation are the rapid removal of perchlorate from the root zone and minimization of perchlorate accumulation in plants.

Although previous research has shown that plant exudates enhance rhizodegradation of perchlorate, the natural supply of plant exudates alone may not be sufficient to sustain rapid rhizodegradation of perchlorate at some field sites. To overcome this potential limitation, this study evaluated the use of external sources of DOC derived from agricultural waste and synthetic sources to biostimulate rapid biodegradation and rhizodegradation of perchlorate in planted soil systems. Specifically, two sources of organic carbon were applied to perchlorate-contaminated soils in planted bioreactors. The lag-time, rates of perchlorate removal and the fractions of perchlorate measured in leaf tissue of willows (*Salix nigra*) grown in acetate (synthetic DOC source), and chicken litter extract (natural DOC source) amended soil bioreactors were compared to results of the no DOC amended controls. The lag-time was considered as the duration of slow phase of perchlorate removal *via* plant uptake of the soil pore water, which usually preceded the rapid rhizodegradation phase. The presence of the chlorite dismutase gene (*cld*) in the DNA extracts of bacteria isolated from soil pore water was analyzed to confirm that perchlorate degraders mediated the degradation reactions.

2. Materials and Methods

2.1. Chemicals

Sodium acetate trihydrate ($\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$) and sodium hydroxide (NaOH , 50% w/w) solution were obtained from J.T.Baker[®] (Phillipsburg, NJ) and used as a synthetic organic carbon source and to prepare the ion chromatography eluent, respectively. Multiple concentrations of perchlorate standard solutions were purchased from AccuStandard[™] (New

Haven, CT) and SPEX CertiPrep (Metuchen, NJ). Peters Professional[®] (St. Louis, MO) (4.6% ammonical nitrogen, 19.4% urea nitrogen, 12% phosphate and 12% potash) was diluted to make the desired strength of nutrient solution. Almatris Ac. Inc. provided DD-6 alumina sorbent with 48×100 U.S. screen mesh and having a surface area of $360 \text{ m}^2 \text{ g}^{-1}$.

2.2. Preparation of Dissolved Organic Carbon Sources

Solutions of acetate and chicken litter extracts were used as carbon and electron sources. Chicken litter extract solution was prepared by mixing 100 g of solid chicken litter obtained from a University of Georgia poultry farm, with 1 L of deionized water. After shaking the mixture overnight and centrifugation for 30 minutes at $1000 \times g$, the supernatant was filtered sequentially through a Whatman[®] 42 and then a $0.45 \mu\text{m}$ filter paper (Pall Corp., Ann Arbor, MI). The raw extract was diluted as needed to provide the desired 300 mg L^{-1} of DOC to the root zone solution. The 300 mg L^{-1} DOC source from acetate (CH_3COO^-) was prepared by dissolving 1.7 g of $\text{CH}_3\text{COONa} \cdot 3\text{H}_2\text{O}$ in 1L of perchlorate contaminated groundwater added to the soil bioreactors. To ensure uniform distribution of the DOC, all pore water was drained from each bioreactors and the stock solution of DOC mixed in before reapplying at the soil surface. The concentrations of DOC in solution were measured on a Shimadzu 5050 Total Organic Carbon analyzer.

2.3. Trees

Willow tree (*Salix nigra*) cuttings purchased from Ernst Conservation Seed (PA) were pre-rooted in 0.39 % aqueous solutions of Peter's Professional[®] plant growth media under natural light in a greenhouse maintained at a temperature of approximately 28 °C. For each experiment, pre-rooted cuttings having similar stem diameter, root system and total biomass were used. Willow trees were selected for this study because of a high water uptake and high rate of survival under the humid greenhouse conditions.

2.4. Soil Bioreactors

The pre-rooted trees were transferred to 20 L buckets which were filled with layers of sand and soil (Figure 4.1). The bottom 7 cm of the bucket was filled with clean sand topped by 25 cm of sandy loam collected from Athens, Georgia. The soil has a pH of 5.79 and contains 4.96 % organic carbon. The sandy loam contains 16.7 % clay, 21.3 % silt and 62 % sand. The soil was obtained from depths of 50 to 100 cm from a site that has not been used for agriculture nor received any fertilizer or pesticide treatment in recent years. The 25 cm layer of soil in each bucket was topped with a 3 cm layer of clean sand. The plant growth nutrients were provided as 0.39 % diluted Peters Professional[®] solution. Triplicate planted soil bioreactors were prepared for acetate, chicken litter and control experiments. A water gauge (manometer) prepared from vinyl tubing was used to monitor the water level inside each soil bioreactor. A sampling port of perforated PVC was installed in each bioreactor on the opposite side of the water gauge.

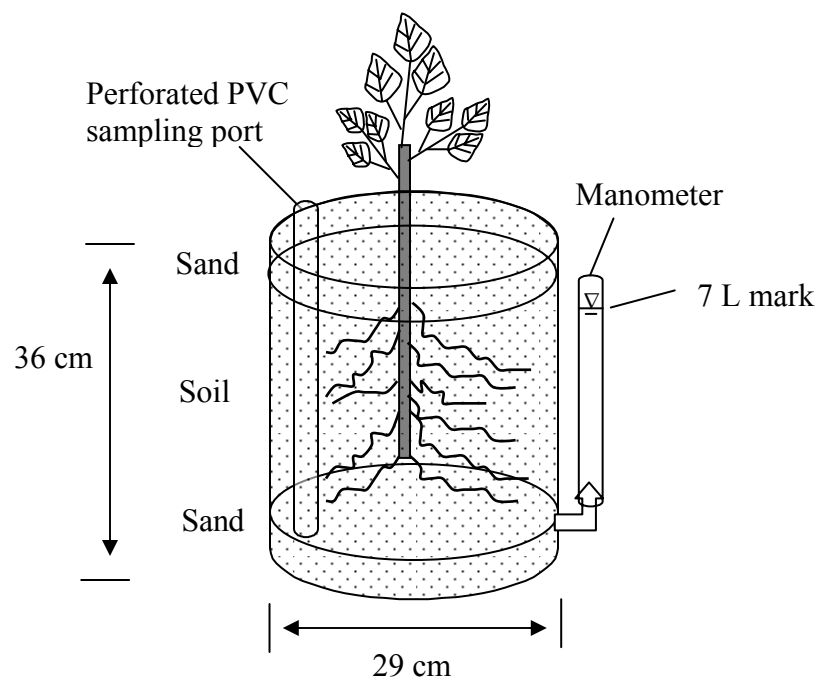


Figure 4.1. Schematic view of the 20 L soil bioreactors.

Once the trees were acclimated for about two weeks, each planted bioreactor was dosed with either chicken litter extract or acetate and perchlorate-contaminated groundwater from the Longhorn Army Ammunition Plant, Karnack, TX. The concentration of perchlorate in the groundwater was 32 mg L^{-1} and the DOC source was provided as either approximately 300 mg L^{-1} acetate or chicken litter extract. In order to get similar concentration of perchlorate in all bioreactors, a NaClO_4 solution prepared in the lab was added to some of the bioreactors. The constant volume of water in the bioreactors was maintained by replacing the evapotranspired water with nutrient solution dissolved in deionized water. The perchlorate-contaminated water and the DOC were homogenized in each bioreactor by pumping from the bottom of the sampling port and expelling the water at the soil surface. The pore-water (water within the saturated soil) in the bioreactors was then sampled and analyzed on a daily basis, with occasional exceptions.

Before taking samples from each soil bioreactor, deionized water was added on soil surface to replace the evapotranspired water and to bring the water level up to approximately 7 L water mark. A 10 ml pipette (Costar Corp., Cambridge, MA) was used to purge 40 to 50 ml of water from multiple depths within the sampling port, which was disposed on the soil surface. This procedure was performed prior to taking a sample in order to thoroughly mix the water in the bioreactor and take a representative sample. The 1.5 ml sample was diluted with deionized water to the working calibration concentration range of the ion chromatograph. These samples were analyzed for perchlorate on a Dionex[®] DX 500 ion chromatography (IC). Each planted soil bioreactor was sampled on a daily basis until the concentration of perchlorate decreased to below IC method detection limit of $2 \text{ } \mu\text{g L}^{-1}$.

Once the concentrations of perchlorate in the willow-planted soil bioreactors had reached non-detectable levels, a fraction of the willow tree leaves were harvested before the buckets were

re-spiked with perchlorate. This process was repeated four times. For the second and subsequent dosing events, the water within the bioreactors was pumped out and dosed with perchlorate before slowly being reapplied onto the soil surface. The bioreactors were not dosed with DOC during the second and third dosing events. During the fourth dosing, DOC was added to selected bioreactors to achieve an initial concentration of approximately 300 mg L⁻¹.

2.5. Bacterial Cell Counts and Polymerase Chain Reaction (PCR) Amplification

Water samples were collected for DNA extraction from each set of experiments. Standard cell counts were performed on plate count agar (Difco). Plates were incubated at room temperatures under aerobic conditions for 2 days before colony counting. DNA was extracted from the samples using the Ultra Clean Soil DNA Kit (MO BIO, Laboratories, Inc., CA) and amplified with the primers 238F (5'-T(C/T)GA(A/C/G)AA(A/G)CA(C/T)AAGGA(A/T/C)AA(A/C/G)GT AND UCD646R (5'-GAGTGGTA(A/C/G)A(A/G)(C/T)TT(A/C/G)CG(C/T)TT) (12). Polymerase chain reaction (PCR) was performed on 100 ng of purified sample DNA or on 1 µl of cell suspension of the bacterial isolates transferred directly to 30 µl PCR mixture containing 50-mM KCl, 10-mM Tris-HCl, 2-mM MgCl₂, 0.1 µM each primer, 200 µM each deoxynucleoside triphosphate (Promega), and 0.5 U of Taq polymerase (Promega). Samples were denatured for 4 minutes at 94 °C, and amplified for 35 cycles at 94 °C for 30 seconds, 53 °C for 1 minute, 72 °C for 30 seconds and finally hold at 4 °C. The PCR products were analyzed by agarose gel electrophoresis (11).

2.6. Plant Tissue Extraction

Plant leaves harvested for perchlorate analysis during and immediately after the complete removal of each dose of perchlorate added to the bioreactors were weighed and washed with deionized water before drying overnight at 75 °C. The dried samples were weighed and pulverized with a mortar and pestle. The extraction of leaves and clean-up procedure of Ellington and Evans (20) was followed. The extracted perchlorate concentrations were measured in mg L^{-1} and converted to the mass of perchlorate recovered per mass of dried or fresh weight (dw or fw) of the leaf material (mg kg^{-1}).

2.7. Analysis

Water samples taken from the rhizosphere were diluted as needed to the working perchlorate concentration range of 0.002 to 1.5 mg L^{-1} . Each prepared sample was placed in two 5 ml Dionex[®] autosampling vials and was analyzed immediately after sampling. Perchlorate analysis was performed using a Dionex[®] DX-500 IC outfitted with an IONPAC[®] AG16 guard column (4 × 50 mm) and an IONPAC[®] AS16 analytical column (4 × 250 mm). The IC was equipped with a Dionex[®] AI-450 Chromatography Automation System and the Advanced Computer Interface Module (ACI). An auto sampler with a holding capacity of sixty 5 ml vials was used. The system was run using an ASRS-ULTRA II Self-Regenerating Suppressor (4 mm) at a 300 mA setting. A 100 mM and 50 mM sodium hydroxide (NaOH) eluent at a flow rate of 1 ml min^{-1} were used to measure perchlorate in mg L^{-1} and $\mu\text{g L}^{-1}$ levels respectively. The eluent was made using J. T. Baker[®] 50 % (w/w) solution and deionized, degassed water (in the VWR

Scientific Aquasonic, Model 150D). Calibration and check standards were made by diluting 1000 $\mu\text{g mL}^{-1}$ perchlorate anion standard (SPEX CertiPrep, Inc.[®]) and 0.5 $\mu\text{g mL}^{-1}$ and 1 $\mu\text{g mL}^{-1}$ standards (AccuStandard, Inc.[®]). A new calibration curve was created each time the ion chromatograph was turned on, or after the eluent had been changed (every 1 to 2 days). For quality control, all samples were run in duplicate, and an external standard and a blank were run after every two samples. The standard was used to ensure that the percent error remained below 5 %, and to monitor any instrumental drift, while the blank checked for any carry-over from the previous sample. To confirm the perchlorate peak in plant tissue extracts, some duplicate samples were spiked with a solution containing 100 $\mu\text{g L}^{-1}$ perchlorate.

3. Results and Discussion

Over a period of 90 days, the planted bioreactors treated four doses of 17 to 53 mg L^{-1} perchlorate to below the method detection limit (MDL) of 2 $\mu\text{g L}^{-1}$. At the termination of each experiment, there was no significant difference in the biomass of willow trees grown with and without an external supply of electron source. Similarly, the volume of water evaporated from the soil surface and transpired by the willow trees in each experiment was similar at a rate of approximately 300 ml day^{-1} .

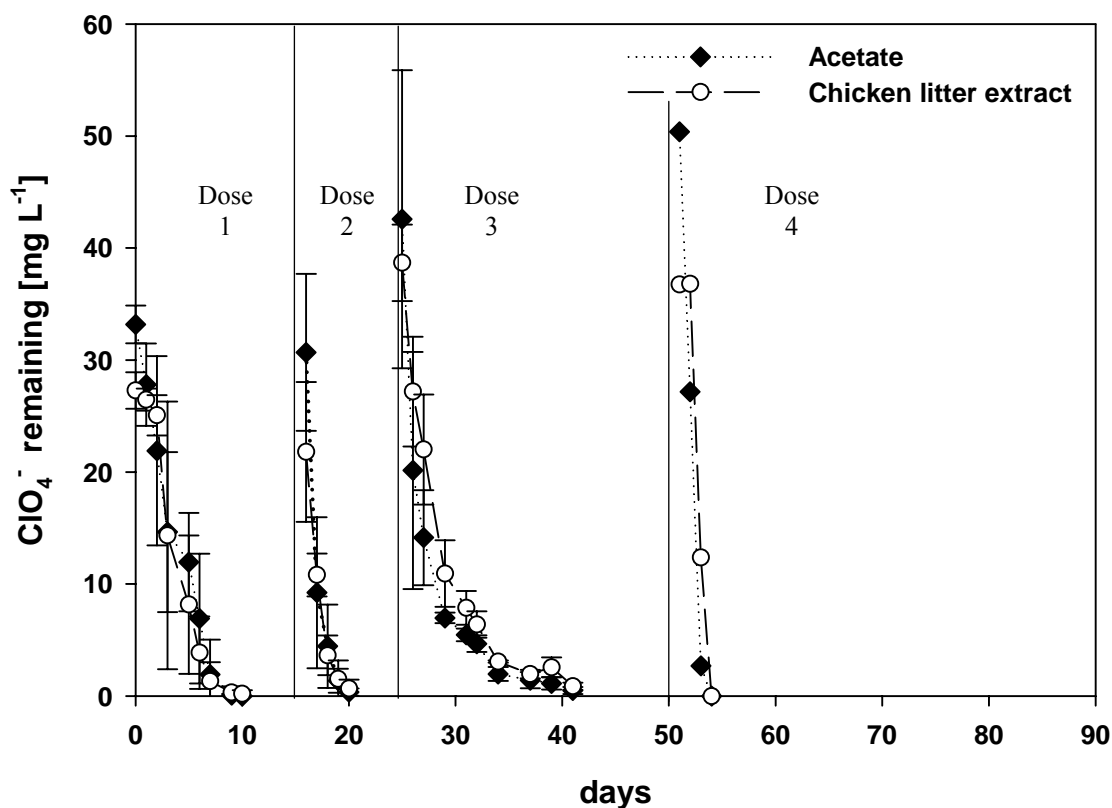


Figure 4.2. Removal of perchlorate from three 20 L soil bioreactors planted with one willow (*Salix nigra*) tree each and dosed with 300 mg L⁻¹ DOC derived from chicken litter extract or acetate. DOC was added to the bioreactors during the first and fourth perchlorate dosing events to biostimulate and sustain rhizodegradation. Peaks correspond to the initial concentrations measured in the rhizosphere immediately after the reactors were dosed with perchlorate. The slow rate of removal of the third dose of perchlorate is attributed to the low concentration of DOC remaining in the pore water.

3.1. Dose 1

Initial perchlorate concentrations of approximately 33 mg L^{-1} was reduced to the MDL within 9 days in planted bioreactors treated with DOC as chicken litter extract and acetate (Figure 4.2). There was no significant difference in the effectiveness of the two DOC sources to stimulate perchlorate reduction by bacteria. In bioreactors supplied with acetate, there was no lag-time preceding the faster rhizodegradation phase, while a lag-phase attributed to slow plant uptake of perchlorate lasted for 3 days for chicken litter extract amended bioreactors. The 3 days lag-phase was likely the time needed for perchlorate degraders to adapt to metabolizing the DOC provided as chicken litter extract. Following the biostimulation of rhizodegradation, perchlorate removal was very fast in both acetate and chicken litter bioreactors and was described by a zero-order kinetic equation with a degradation rate constant of $4.53 \pm 0.65 \text{ mg L}^{-1} \text{ day}^{-1}$. The concentration of perchlorate in willow leaves harvested immediately after the removal of the first dose of perchlorate was $11.7 \pm 2.7 \text{ mg kg}^{-1} \text{ fw}$.

The planted soil bioreactors provided with no external electron sources (controls) contained an initial DOC concentration of $54.6 \pm 5.3 \text{ mg L}^{-1}$ derived from residual soil organic carbon and plant exudates. Removal of the first dose of perchlorate required more than 13 days compared to 9 days for chicken litter or acetate amended bioreactors with a first-order rate constant of $0.36 \pm 0.02 \text{ day}^{-1}$ (Figure 4.3). As a result, the concentration of perchlorate in willow leaves harvested immediately after treatment of the first perchlorate dose was $103.7 \pm 16.8 \text{ mg kg}^{-1} \text{ fw}$, an order of magnitude higher than in the leaves of DOC amended trees. The slower rate of perchlorate removal amounted to longer residence times and higher perchlorate fraction uptake and phytoaccumulation by the willow trees.

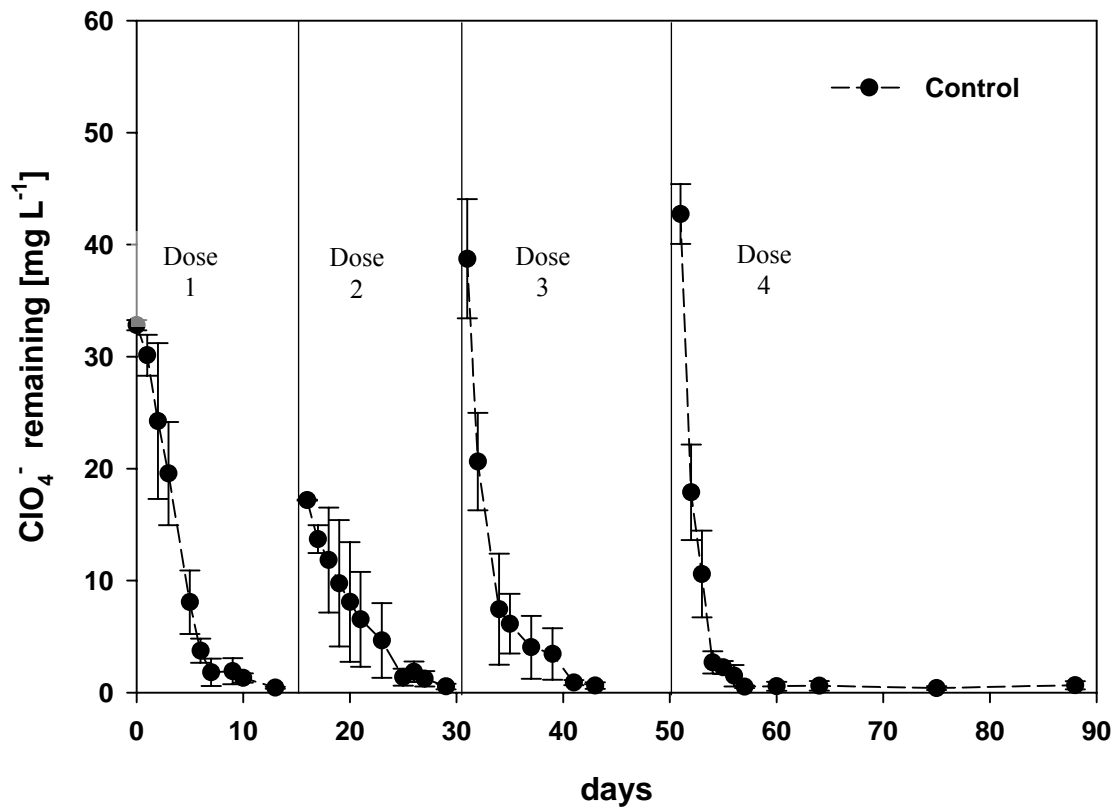


Figure 4.3. Removal of four doses of perchlorate from the willow (*Salix nigra*) planted control bioreactors. The DOC concentration in the control bioreactors remained low throughout the experiments as no external DOC was provided.

3.2. Dose 2

The second dose of 30 mg L^{-1} perchlorate applied to DOC-amended bioreactors was completely degraded within 5 days. The kinetic data was still described by the zero-order equation and the degradation rate constant for the second dose of perchlorate increased from $4.53 \pm 0.65 \text{ mg L}^{-1} \text{ day}^{-1}$ during the first dose to $8.66 \pm 3.23 \text{ mg L}^{-1} \text{ day}^{-1}$. The doubling of the perchlorate removal rate observed during the second dosing suggested that microbial activity was higher in the rhizosphere. As the second dose was being completely removed from solution, the DOC concentration reduced from an average of 309.1 ± 21.7 to $280.7 \pm 71.6 \text{ mg L}^{-1}$, while the perchlorate concentration in the willow leaves increased to $39.0 \pm 18.5 \text{ mg kg}^{-1} \text{ fw}$. Complete removal of the second dose of 17.2 mg L^{-1} perchlorate from the control bioreactors required 15 days, compared to only 5 days for DOC amended bioreactors. Because perchlorate was available for plant uptake for longer time, the amount of perchlorate in the control leaves increased to $185.6 \pm 88.5 \text{ mg kg}^{-1}$. At the end of the second dose, the concentration of DOC remaining in solution was $32.0 \pm 12.0 \text{ mg L}^{-1}$.

3.3. Dose 3.

In the DOC-amended bioreactors, the removal of the third dose of perchlorate, initial concentration of 42 mg L^{-1} , was observed to be slower. More than 18 days was required to reach the MDL and the kinetic data was poorly described by the zero-order kinetic model and better described by the first-order model (Table 4.1). As the microbial population increased and the available DOC was progressively consumed or oxidized by bacteria to yield electrons used in

Table 4.1. The average initial concentration of perchlorate and dissolved organic carbon in solution, and concentration of perchlorate in willow leaves. CLE: chicken litter extract. The leaves were collected immediately following the complete treatment of perchlorate dose.

Experiment	Average initial ClO ₄ ⁻ [mg L ⁻¹]	Average initial DOC [mg L ⁻¹]	ClO ₄ ⁻ in leaves [mg kg ⁻¹ fw]	Zero order K [mg L ⁻¹ day ⁻¹]	R ²	1 st order K [day ⁻¹]	R ²
Acetate 1 st dose	33.2 ± 1.7	293.2 ± 12	11.9 ± 1.8	4.21 ± 0.61	0.93		
Acetate 2 nd dose	30.7 ± 7.0	246.3 ± 82	33.8 ± 25.1	10.81 ± 1.9	0.85		
Acetate 3 rd dose	42.6 ± 13.3	29.1 ± 1.3	69.4 ± 58.3	1.80 ± 0.58	0.58	0.24 ± 0.04	0.95
Acetate 4 th dose	50.4	300.0	104.7	23.8	0.99		
CLE 1 st dose	27.3 ± 1.7	325 ± 16.6	11.7 ± 3.8	4.85 ± 0.62	0.98		
CLE 2 nd dose	21.8 ± 6.2	315.1 ± 50.3	44.1 ± 12	6.50 ± 2.94	0.91		
CLE 3 rd dose	38.7 ± 3.4	36.4 ± 9.3	120.8 ± 29.6	1.73 ± 0.24	0.71	0.23 ± 0.05	0.95
CLE 4 th dose	37.0	300.0	136.4	18.4	0.97		
Control 1 st dose	32.8 ± 0.5	54.6 ± 5.3	103.7 ± 16.8	3.23 ± 0.91	0.85	0.36 ± 0.02	0.94
Control 2 nd dose	17.2 ± 0.1	32 ± 12	185.6 ± 88.5	1.31 ± 0.10	0.85	0.35 ± 0.05	0.90
Control 3 rd dose	38.7 ± 5.4	18.3 ± 5.6	210.6 ± 97.8	2.68 ± 0.43	0.68	0.33 ± 0.03	0.93
Control 4 th dose	42.8 ± 2.7		386.6 ± 27	3.2 ± 0.72	0.50	(0.17 ± 0.1)[†]	0.88

Values presented in table are mean ± standard deviation based on three soil bioreactors. The degradation kinetics is better described by the values in bold.

[†] - second order degradation rate constant [L mg⁻¹ day⁻¹] for the 4th dose in the control bioreactors.

perchlorate mineralization, the rates of rhizodegradation of subsequent doses of perchlorate decreased and were progressively poorly described by the zero-order kinetic model. This led to the persistence of perchlorate in the root zone of the trees as confirmed by the higher perchlorate concentration measured in the leaves, which increased from $39.0 \pm 18.5 \text{ mg kg}^{-1} \text{ fw}$ to $95.1 \pm 50 \text{ mg kg}^{-1} \text{ fw}$. The slower rate of perchlorate degradation and increased perchlorate concentration in the willow leaves corresponded to the low DOC concentration remaining in solution, which had reduced from $280.7 \pm 71.6 \text{ mg L}^{-1}$ to $32.8 \pm 7.2 \text{ mg L}^{-1}$ when the third dose was completely removed to below the MDL.

Likewise, perchlorate removal from the control bioreactors was described by a first-order degradation rate constant of $0.33 \pm 0.03 \text{ day}^{-1}$ and perchlorate phytoaccumulated in the leaves of the control trees increased to $210.6 \pm 97.8 \text{ fw}$. The similarity of the biodegradation kinetics of the third dose in DOC-amended and control experiments further confirmed the limiting effect of electron sources at low concentrations of DOC during phytoremediation of perchlorate-contaminated soils.

3.4. Dose 4

At the fourth dosing, 300 mg L^{-1} DOC as chicken litter extract or acetate was added together with 45 mg L^{-1} perchlorate to the willow planted bioreactors. Unlike the response to the third dose, the rhizodegradation was described by the zero-order kinetic model and the rhizodegradation rate increased to the highest observed rate of $21.1 \pm 3.8 \text{ mg L}^{-1} \text{ day}^{-1}$. Immediately after the complete removal of the fourth dose of perchlorate from solution, the concentration of perchlorate phytoaccumulated increased to $120.5 \pm 22.4 \text{ mg kg}^{-1} \text{ fw}$. In the

control bioreactors no DOC was added with the fourth dose of perchlorate. As a result, the rate of perchlorate removal progressively became slower and was described by a second-order kinetic model with rate constant of $0.17 \pm 0.09 \text{ L mg}^{-1} \text{ day}^{-1}$. The willow leaves accumulated $387 \pm 27 \text{ mg kg}^{-1} \text{ fw}$, a factor of three higher than in plants grown in the DOC-amended bioreactors.

The results of the control experiments provide good evidence that electron sources normally available as DOC is limiting at most field sites where plants growing over perchlorate-contaminated soil and groundwater are found to phytoaccumulate significant amounts of perchlorate. Therefore an external source of DOC is required to minimize plant perchlorate uptake and stimulate microbial degradation of perchlorate. Thus, it is clear from measuring the rate of perchlorate removal and amount of perchlorate in the leaf tissue that a higher fraction uptake of perchlorate correlates directly with the residence time of perchlorate in the rooted media. The residence time of perchlorate in the rhizosphere is longest when the DOC concentration in soils is insufficient to sustain a high rate of rhizodegradation.

3.5. *Bacteria.*

The polymerase chain reaction (PCR) results confirmed the presence of the chlorite dismutase gene (*cld*) in pore water collected from planted bioreactors dosed with DOC and the control (Figure 4.4). The presence of chloride dismutase gene (*cld*) in the control soil bioreactors suggested that the soils already harbored a high population of the ubiquitous perchlorate-respiring microbes required for biodegradation of perchlorate. This means that perchlorate degradation was not limited by the availability of perchlorate degraders, but by the electron donors.

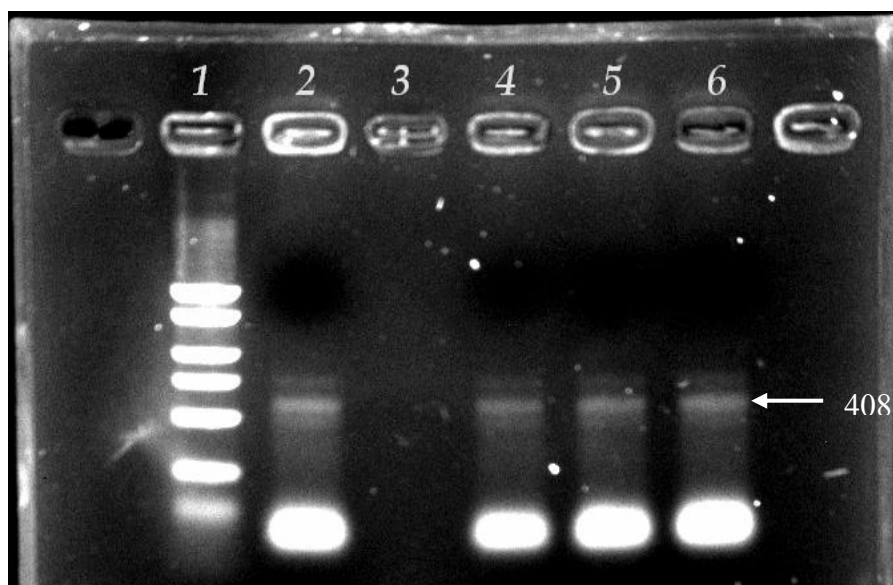


Figure 4.4. PCR detection of a 408-base pair segment of the *cld* gene using the UCD-238F/ucd-646R primer set. Lane 1, molecular weight ladder (2000 base pairs to 200 base pairs); lane 2 is dilute mushroom compost solution, lane 3 is dilute chicken litter extract, lane 4 is solution from the control bioreactors; lane 5 is solution from acetate-amended bioreactors and lane 6 is solution from chicken litter extract amended soil bioreactors. The bright bands at the bottom of the gel in lanes 2,4,5 and 6 are primer dimers. In a separate sample of chicken litter extract, the *cld* gene was detected by PCR.

3.6. Sorption of Perchlorate onto Soil.

At the end of the experiments, when the concentration of perchlorate in pore water had decreased to the MDL, multiple soil samples were collected from below the top 5 cm in each of the 20 L planted soil bioreactors and analyzed for perchlorate. Perchlorate was detected in all soil samples with the lowest concentration of $0.40 \pm 0.29 \text{ mg kg}^{-1}$ measured in bioreactors amended with acetate and the highest in the control bioreactors ($3.19 \pm 0.88 \text{ mg kg}^{-1}$). The soil in the chicken litter-amended bioreactors contained $1.11 \pm 0.35 \text{ mg kg}^{-1}$ perchlorate. In a previous study, Susarla et al., (1999) observed and reported the pH dependence of sorption of perchlorate to soil with maximum sorption occurring at pH 6 (22). The latter authors also showed that at lower and higher pH values, the sorption of perchlorate to soil decreases. The soil used in our study had a pH of 5.79, which is close to the optimum pH for sorption of perchlorate to soils.

3.7. Implications for Biostimulation of Perchlorate Degradation.

The detection of the chlorite dismutase gene (*cld*) in the soil solution and agricultural waste products used as electron source confirms the documented ubiquity of perchlorate-degrading microbes (8). This means that the limitation to rhizodegradation of perchlorate in most perchlorate-contaminated soils is the lack of an adequate supply of DOC consumed by perchlorate degraders and utilized as electron sources to mineralize perchlorate. The control experiments have further confirmed that the concentration of readily available DOC provided by most aquifer natural soils and exuded by plants may be insufficient to sustain rhizodegradation of perchlorate in soil and groundwater at vegetated perchlorate-contaminated sites (23). The slow

rate of rhizodegradation observed when electron sources are limiting increases the residence time of perchlorate in the rhizosphere and results in higher uptake, slower phytodegradation and phytoaccumulation of perchlorate in plant leaf tissue. Therefore, biostimulation of rhizodegradation is necessary by providing cheap and commonly available carbon and electron sources, such as acetate and organic rich waste products.

This study has provided evidence that the slow build up of DOC in the rhizosphere by root exudation and organic matter decomposition is insufficient to sustain a high rate of rhizodegradation of perchlorate and perhaps other degradable contaminants. An optimum design of phytoremediation of perchlorate should include enhancement of rhizoremediation by providing a sustained supply of electron donors to the root zone. The ecological benefits of enhancement of rhizodegradation include rapid attainment of site cleanup goals, minimization of the undesired uptake and phytoaccumulation of perchlorate, and avoidance of the potential recycling of perchlorate during phytoremediation.

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CHAPTER 5

FATE OF PERCHLORATE IN LOCAL PLANT SPECIES GROWING AT CONTAMINATED SITES IN HUMID AND SEMI-ARID CLIMATES¹

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Abstract

Previous greenhouse and field studies show that terrestrial and aquatic vegetation including trees, grasses and agricultural produce grown on perchlorate contaminated soil and irrigation water can readily accumulate perchlorate mostly in the leaves. The phytoaccumulated perchlorate poses ecological risk by either contaminating the food chain of humans and animals or recycling in the ecosystem during litter fall. In this study, several species of terrestrial and aquatic vegetation growing over contaminated sites at the Longhorn Army Ammunition Plant (LHAAP) in Karnack, Texas and the Las Vegas Wash (LVW), Nevada were harvested during multiple growing seasons and analyzed for perchlorate. All vegetation species collected from these sites contained quantifiable levels of perchlorate. The plant perchlorate concentrations varied with the type of plant species, initial perchlorate concentration in the subsurface, season, and stage of plant maturity. The highest perchlorate concentration was measured in willows (*Salix nigra*), Crabgrass (*Digitaria* spp.), and Bermuda grass (*Cynodon dactylon*) at the LHAAP, while salt cedar (*Tamarix ramosissima*) at the LVW phytoaccumulated the highest amount of perchlorate. For the same plant species, those growing nearest the source areas consistently phytoaccumulated the highest amounts of perchlorate than those farther away from the sources. The slow rate of phytodegradation of perchlorate taken up by plants during the growing season explained the detection of higher perchlorate concentrations in leaves collected later in the growing season (fall) and senesced leaves than in the young and live leaves. This provided evidence that the decaying of perchlorate-tainted vegetation potentially recycles perchlorate by releasing some fraction back into the soils on which plant litter collects. To minimize the potential recycling of perchlorate during phytoremediation, harvest and subsequent bioremediation of the plant leaves containing perchlorate and the enhancement of

rhizodegradation should be incorporated into the design, installation and management of the technology.

1. Introduction

Environmental contamination by perchlorate (ClO_4^-) has been identified at the Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas (1,2) and the Las Vegas Wash (LVW), Nevada (3,4). The sources of perchlorate contamination at both sites are from the manufacture and use of ammonium perchlorate (NH_4ClO_4) in solid rocket propellant, as well as loading, packing, and assembly of pyrotechnics and rocket motors (2,5). In addition to the LHAAP and the LVW, perchlorate contamination is a serious problem in more than 150 sites in the United States. As of September 2004, perchlorate contamination has been reported in 44 states with about 16 million people or more estimated to have perchlorate in their drinking water supplies (6). Many studies have reported cases of perchlorate contamination of vegetable crops grown with perchlorate-tainted soil or irrigation water or fertilized with Chilean nitrate (7,8). The detection of perchlorate in dairy milk is attributed to perchlorate contamination of forage crops (9). Meanwhile, perchlorate was recently detected in human breast milk (10); the likely source of which was from food crops or drinking perchlorate contaminated water. Perchlorate poses health concerns because by blocking iodide uptake by the thyroid (11). A chronically impaired thyroid function could potentially lead to abnormal development in children and disruption of adult metabolism. Growing concerns over perchlorate contamination led the U.S. Environmental Protection Agency (EPA) to issue a preliminary cleanup goal for perchlorate of $24.5 \mu\text{g L}^{-1}$ in drinking water (12).

In-situ and *ex-situ* bioremediation including phytoremediation are the most effective treatment technologies for perchlorate contaminated soils and groundwater. In the presence of suitable carbon and electron sources, perchlorate is rapidly degraded by bacteria under anaerobic

conditions. Bacteria have been shown to use perchlorate as a terminal electron acceptor while oxidizing organic substrates (13,14). The degradation pathway for perchlorate includes a three-step reduction of perchlorate to chlorate, chlorite and chloride ($\text{ClO}_4^- \rightarrow \text{ClO}_3^- \rightarrow \text{ClO}_2^- \rightarrow \text{Cl}^-$) and none of the intermediate products (ClO_3^- or ClO_2^-) accumulates in solution (13,15).

Phytoremediation of perchlorate has been shown to be potentially effective in many laboratory hydroponics and a few field studies (16-19). Many terrestrial and aquatic plant species have the ability to remove perchlorate from contaminated water and soils. Specifically, terrestrial plants [black willow (*Salix nigra*), eastern cottonwood (*Populus deltoides*), spinach (*Spinacia oleracea*) and French tarragon (*Artemisia dracunculus*)] and aquatic plants [water weed (*Elodea canadensis*), parrot-feather (*Myriophyllum aquaticum*), cattails (*Typha latifolia*) and duck weed (*Spirodela polyrhiza*)] were shown to be effective in decontaminating low and high perchlorate concentrations, ranging from 20 to 122 mg L⁻¹ to below the ion chromatography (IC) detection limit of 2 µg L⁻¹ (19). Plants take up and metabolize perchlorate by phytodegradation in plant tissues or microbial-mediated rhizodegradation occur in the root zone. Uptake and phytodegradation of perchlorate is a relatively slower process, which follows a stepwise reduction of perchlorate to chloride in plant tissues (16,17). Uptake and slow phytodegradation in plants results in phytoaccumulation of some fraction of the perchlorate taken up by plants, which could create additional ecotoxicological risks if the phytoaccumulated perchlorate is recycled by senesced leaves or introduced into the food chain. On the other hand, rhizodegradation is a faster process that rapidly degrades perchlorate in the rhizosphere, with root exudates providing the electrons used to reduce perchlorate to chloride.

Under most field environmental conditions, electron donors are in short supply at vegetated perchlorate-contaminated sites. Therefore, the uptake phase lasts longer leading to

higher phytoaccumulation of perchlorate. For example, the detection of perchlorate in lettuce, cucumber and soybean is due to plant uptake and phytoaccumulation. The other environmental impact associated with phytoaccumulation is when plants act as a reservoir of perchlorate and re-release perchlorate into the ecosystem during litter fall. Smith *et al.*, (2004) indicated that leaf litter collected beneath salt cedar trees from the LVW contained higher perchlorate concentrations than leaves collected from the live trees (4).

This study compliments previous work on the uptake and phytoaccumulation of perchlorate at field sites. The study focused on the fate of perchlorate taken up by different species of terrestrial and aquatic plants growing in perchlorate-impacted media at two sites located in a humid and a semi-arid climate, the LHAAP and the LVW, respectively. Specifically, we investigated: (1) the long term fate of perchlorate accumulated in different plant species, (2) the spatial and seasonal variability of perchlorate in plants, and (3) the differences in concentration of perchlorate among plant species.

2. Materials and Methods

2.1. Site Description – Longhorn Army Ammunition Plant (LHAAP), Karnack, Texas

The LHAAP is located in east central Texas in the northeast corner of Harrison County (Figure 5.1), approximately 65 km west of Shreveport, Louisiana. Founded in 1942, the LHAAP occupies an area of approximately 3500 ha (8500 acres) with all surface and storm water draining into Caddo Lake, located at the northeast border of the plant. Caddo Lake is a drinking water source for seven public drinking water systems downstream from the LHAAP in the state

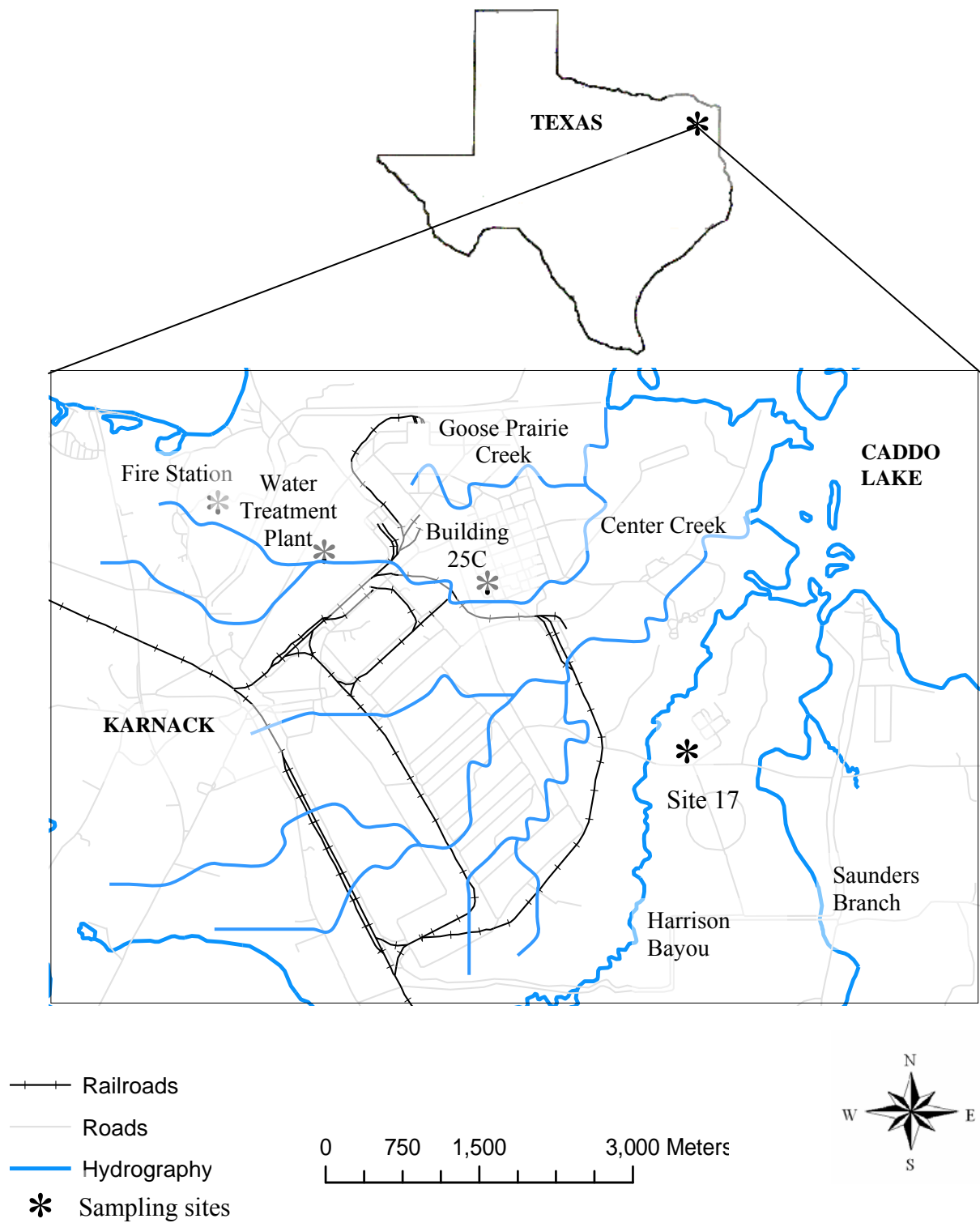


Figure 5.1. Location of study site at the Longhorn Army Ammunition Plant, Karnack, Texas.

of Louisiana (20). In addition, groundwater near the LHAAP is also used as source of drinking water. The LHAAP operated between 1942 to 1997, loading, assembling and packaging pyrotechnic and illuminating signal munitions and manufacturing and demilitarizing of 2,4,6-trinitrotoluene munitions and solid propellant rocket motors (1). In addition to trinitrotoluene and perchlorate, other contaminants at the LHAAP include trichloroethylene, methylene chloride and trinitrobenzene (20).

Previous reports showed that water, soil, sediment, vegetation and animal tissue samples collected from several locations in the LHAAP are contaminated with perchlorate (2). As a result, the site is on the USEPA national priority list (20) and both *in-situ* (bioremediation with dissolved organic substrate amendment) and *ex-situ* (fluidized bed reactor) treatment technologies are being used to treat perchlorate. Several locations have been identified within the LHAAP where intense perchlorate contamination was observed. These include Building 25C (former perchlorate grinding facility), Site 17 (burning ground), the old fire station and the waste water treatment plant (Figure 5.1). Previous studies showed that soil and vegetation samples collected from near Building 25C contained up to 0.3 mg kg^{-1} and 5557 mg kg^{-1} dry weight (dw), respectively (2). In January 2001, soil and groundwater samples collected by Solutions To Environmental Problems, Inc. from near Site 17 contained as high as 0.3 mg kg^{-1} and 320 mg L^{-1} perchlorate, respectively.

2.2. Site Description – The Las Vegas Wash (LVW), Nevada

The LVW is located in southeast Nevada, approximately 15 km southeast of the city of Las Vegas (Figure 5.2). In 1997 elevated levels of perchlorate were discovered in the waters of

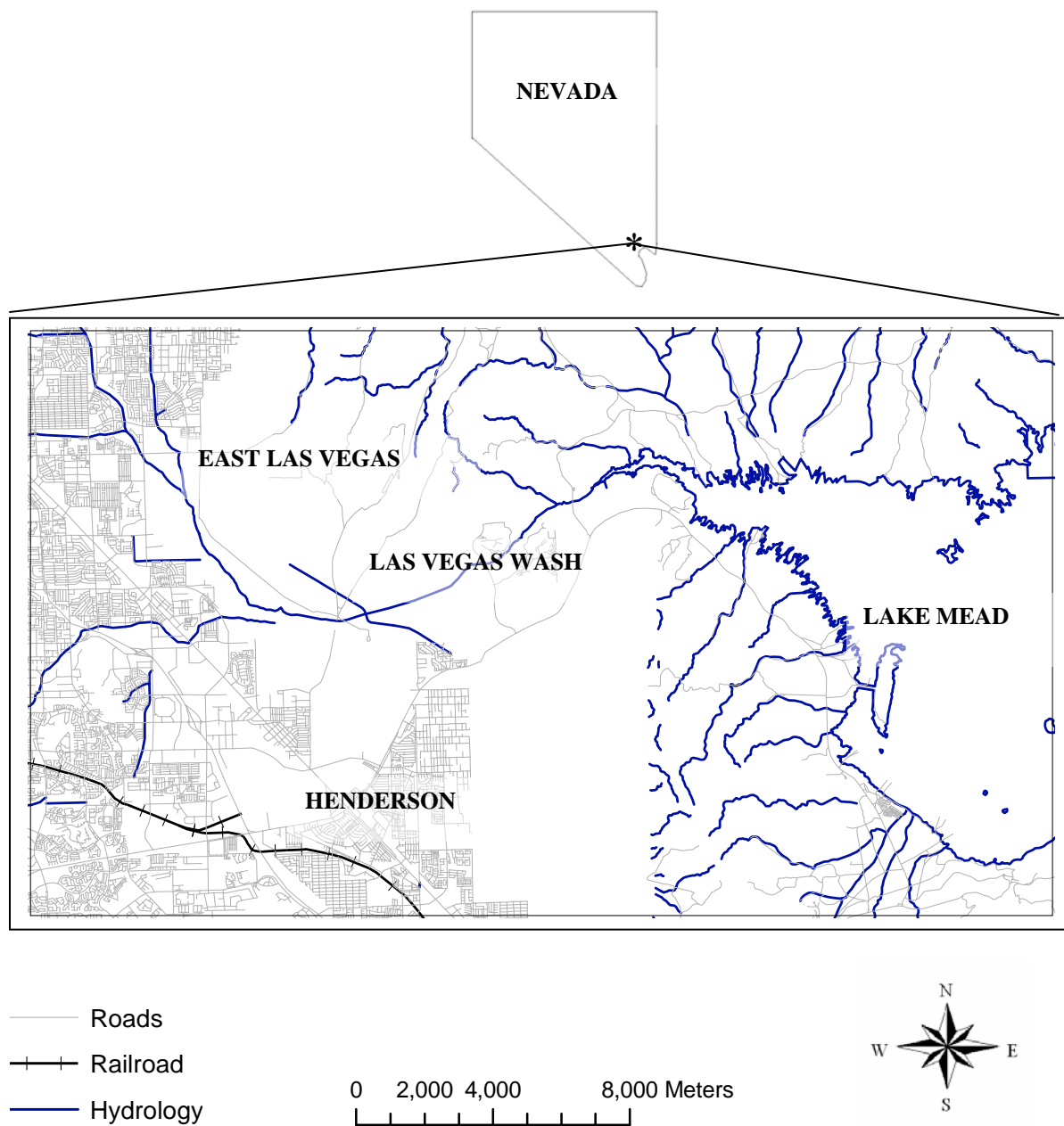


Figure 5.2. Study site location at the Las Vegas Wash, Nevada.

the Wash and in Lake Mead, the source of drinking water for the Las Vegas Valley, and one of the largest reservoirs of the Colorado River. In addition to the surface water, perchlorate contamination has been detected in ground water, soil, vegetation and rodents in the LVW (3,4). The source of contamination is a plume of contaminated shallow groundwater from Kerr McGee Chemical Corporation, an ammonium perchlorate producing plant located in Henderson, NV, approximately 6 miles west of Lake Mead (5). Prior to installation of a treatment system, groundwater at the site contained up to 3700 mg L^{-1} perchlorate (1). A fluidized bed reactor installed in 2004 is treating 350 mg L^{-1} perchlorate before discharging the treated water into the Wash. Despite a pump and treat remediation effort at the site, about 230 kg of perchlorate enters the Wash each day (5). Perchlorate contamination of soil and vegetation at the LVW were reported to be 0.5 mg kg^{-1} and 4460 mg kg^{-1} , respectively (4).

Surface and ground water from the LVW enters Lake Mead, which is formed by Hoover Dam on the Colorado River. The Colorado River is the source of water supply for communities in parts of California, Arizona and Nevada (3). Perchlorate-contaminated Colorado River water is also used to irrigate more than 1.8 million acres of land producing some 15 % of the nation's crop and about 13 % of its livestock (21). Irrigation by perchlorate-contaminated water has led to the uptake and accumulation of perchlorate in the food chain. For example, lettuce grown with perchlorate contaminated Colorado River water contained detectable levels of perchlorate (8).

2.3. Vegetation Sampling

Vegetation samples were collected from three locations at the LHAAP site. Building 25C, near the old fire station and the waste water treatment plant, and Site 17. Plant samples

collected from Building 25C represented plants growing over a site with contaminated soils and groundwater that was not remediated during the duration of the study. The plant samples harvested adjacent to the old fire station and near the former wastewater treatment plant grew over *in-situ* bioremediated plots and control plots that had not been bioremediated. Similarly, plants harvested from Site 17 included samples harvested from the treated and untreated plots. The soils from these sites were actively bioremediated from September 2000 to October 2001. Sampling from the LHAAP was conducted in April 2004, September 2004, January 2005 and May 2005. Both senesced leaf litter and live vegetation samples were collected from trees and grasses. The samples were then placed in polyethylene bags, labeled and transported to the laboratory in coolers. The vegetation samples from LHAAP included willow (*Salix nigra*), pine (*Pinus* spp.), sweet gum (*Liquidambar styraciflua*), oak (*Quercus* spp.), goldenrod (*Solidago* spp.), crabgrass (*Digitaria* spp.) bermuda grass (*Cynodon dactylon*), bullrush (*Juncus effusus*) and an assortment of grasses. These vegetation were not planted for phytoremediation purposes.

Samples from the LVW were collected in May 2004 and October 2004 from sites near the Kerr-McGee seepage point (where treated water is discharged into the Wash) and the Wash. The types of vegetation collected from LVW include salt cedar (*Tamarix ramosissima*), cattail (*Typha latifolia*), algae (spp. unidentified), black willow (*Salix nigra*) and sedge (*Carex* spp.). Similar to the LHAAP trees, the vegetation in the LVW was not planted for phytoremediation purposes. Groundwater and soil concentration data was available from prior site assessment events and other published work. Thus limited effort focused on these tasks.

2.4. Chemicals

Sodium hydroxide (NaOH) 50 % (w/w) solution was obtained from J.T.Baker™ (Phillipsburg, NJ) to prepare the eluent for the ion chromatography (IC). Certified perchlorate standards of 1 mg L⁻¹ and 0.5 mg L⁻¹ were purchased from AccuStandard™ (New Haven, CT) to prepare calibration standards. Almatris Ac. Inc. provided DD-6 alumina sorbent with 48 × 100 U.S. screen mesh and a surface area of 360 m² g⁻¹. The alumina was used to cleanup the plant extracts prior to filtration with 0.22 µm syringe filters.

2.5. Perchlorate Extraction from Plant Leaves

The harvested plant leaves were weighed and washed with deionized water before drying overnight at 75 °C. The dried samples were weighed and then pulverized with a mortar and pestle. The mortar and pestle was carefully washed with deionized water and dried between samples to avoid cross contamination. The samples were sieved using a 35 mesh (500 µm) screen. Sample preparation procedure and analysis of perchlorate in plants follow the procedure of Ellington and Evans (22). About 600 mg of dried and ground plant samples were extracted with 30 ml of deionized water and heated for 30 minutes to precipitate proteins. The samples were then placed in a refrigerator overnight with occasional shaking. After shaking overnight at 4 rpm, the samples were centrifuged at 1000 ×g for 30 minutes and the supernatant was filtered through Kim wipes and 0.22-µm filters. A 2-ml sample extract was mixed with 1 g of DD-6 alumina and kept overnight in a refrigerator. The supernatant was removed, diluted and filtered through a 0.22 µm filter and On Guard® RP cartridge (Dionex®, Sunnyvale, CA) to produce a

clear water extract for IC analysis. The first 1-ml of the extract was discarded before dispensing the latter extract directly into 5-ml autosampling vials.

2.6. Analytical Methods

Perchlorate analysis was performed by ion chromatography using a Dionex[®] DX-500 ion chromatography (Dionex[®] Co., Sunnyvale, CA). The IC was equipped with an IonPac AS 16 analytical column (4 x 250 mm), an IonPac AG 16 guard column (4 x 50 mm), a Dionex[®] ASRS suppressor, an advanced computer interface module (ACI) and a Dionex AS-40 auto sampler. The analytical conditions developed by Dionex[®] Corporation for analysis of low concentrations of perchlorate in drinking and groundwater by ion chromatography was followed in the experiment. A 100 mM NaOH eluent solution flowing at 1 ml min⁻¹ was used for perchlorate analysis in µg L⁻¹ level. The eluent was prepared using 50 % (w/w) NaOH solution and deionized and degassed water. The run time for perchlorate analysis was 15 minutes. For quality control, all samples were run in duplicate. To validate calibration of the instrument through out the analysis, standards were analyzed every 4 samples. This also ensures that the percentage of analytical error is less than 5 %. Blank deionized water was used to clean up the system between 2 sample measurements. Calibration curves were determined each day to ensure accurate quantification of perchlorate. To confirm the perchlorate peak in plant tissue extracts, some duplicate samples were spiked with a solution containing 100 µg L⁻¹ perchlorate. Comparisons of mean perchlorate concentrations between different plant species and between different sampling sites at LHAAP were conducted using Student's t-test.

3. Results and Discussion

The concentrations of perchlorate measured in plant leaves growing over contaminated soils at multiple LHAAP locations did not reveal the strong seasonal variability observed for the LVW samples. After only two seasonal sampling events, the plants monitored at the LVW accumulated and recycle perchlorate to the top-soil. Therefore, only two sampling events were needed at the LVW. On the other hand, the plants at the LHAAP were monitored for a longer period to better evaluate the fate of perchlorate in plant species growing at the site. A total of four sampling events were conducted during two growing seasons at the LHAAP.

3.1. Longhorn Army Ammunition Plant

The type of plant species, sampling time and the mean and standard deviation of perchlorate concentrations in the vegetation collected from LHAAP are presented in Table 5.1. All plant species sampled contained detectable concentrations of perchlorate. The highest perchlorate concentration of 866.3 ± 2.1 mg kg⁻¹ dry weight (DW) was measured in grass samples growing on untreated plots near the old fire station. Perchlorate concentrations in willow and sweet gum leaves collected next to Building 25C were as high as 168.1 and 51.8 mg kg⁻¹ DW, respectively. Grass samples growing over bioremediated soils at Site 17 contained 10.5 ± 4.8 - 45.8 ± 3.0 mg kg⁻¹ DW perchlorate, 43 months after the source area treatment study was terminated.

Table 5.1. Perchlorate concentrations in terrestrial plants (dry weight basis) collected from multiple sites at the Long Horn Army Ammunition Plant, Karnack, Texas.

Site	Species	Month	Concentration [mg kg ⁻¹]
Building 25C	Willow in building	April 04	79.1 ± 3.3 ^a
	Willow 25m away from building	April 04	5.3 ± 0.2 ^a
	Sweet gum next to building	April 04	18.6 ± 0.1 ^a
	Sweet gum away from building	April 04	6.5 ± 0 ^a
	Willow south of the building	September 04	168.1 ^b
	Willow next to building	September 04	134.5 ^b
	Pine south east of Building	September 04	3.0 ^b
	Pine 30m down gradient	September 04	2.6 ^b
	Sweet gum south east of building	September 04	51.8 ^b
	Sweet gum 30m down gradient	September 04	2.2 ^b
	Dry willow leaves from concrete	January 05	8.2 ^b
	Pine	January 05	7.4 ^b
	Dry sweet gum from ground	January 05	7.0 ^b
	Dry oak down gradient	January 05	< DL ^b
	Goldenrod	January 05	45.4 ^b
	Willow next to building	May 05	54.2 ± 1.8 ^a
	Willow next to building	May 05	12.4 ± 5.6 ^a
	Pine next to building	May 05	15.4 ± 2.2 ^a
	Sweet gum 30m down gradient	May 05	< DL ^b
Site 17	Willow near well 130	April 04	6.70 ± 0.2 ^a
	Pine	April 04	6.8 ± 0.2 ^a
	Pine well 130	April 04	5.4 ± 1.3 ^a
	Bermuda grass near well 01	April 04	5.2 ± 0.3 ^a
	Willow North of well 130	September 04	2.2 ^b
	Willow 100 ft from well 130	September 04	< DL ^b
	Pine 30 ft North well 130	September 04	1.5 ^b
	Sweet gum north of well 130	September 04	2.2 ^b
	Bull rush inside dry wetland	September 04	2.4 ^b
	Pine	January 05	< DL ^b
	Live grass near Site 17 east	January 05	29.4 ^b
	Goldenrod from North boundary	January 05	61.2 ^b
	Goldenrod from South boundary	January 05	34.7 ^b
	Goldenrod from well 130	January 05	39.0 ^b
	Dry Oak near well 06	January 05	7.2 ^b
	Willow at North boundary	May 05	31.3 ± 4.0 ^a
	Willow at wetland creek	May 05	17.2 ± 3.4 ^a
	Pine near wetland	May 05	< DL ^a
	Pine near wetland	May 05	< DL ^a
	Sweet gum near wells 05 & 06	May 05	< DL ^a
	Composite grass North of site 1	May 05	45.8 ± 3.0 ^a
	Composite grass, South of site	May 05	10.5 ± 4.8 ^a
Old WWTP	Grass (treated plot)	April 04	84.2 ± 2.0 ^a
	Grass (treated plot)	April 04	15.5 ± 4.4 ^a
	Grass (control plot)	April 04	248.3 ± 3.5 ^a
	Grass (treated plot)	May 05	58.3 ± 1.9 ^a
	Grass (control plot)	May 05	28.3 ± 4.3 ^a
	Grass (control plot)	May 05	866.3 ± 2.1 ^a

^a Sample size (n = 2)

^b Sample size (n = 1)

The concentration of perchlorate in the field vegetation varied with proximity to and concentration in the source areas soils. For example, willow trees growing nearest to and inside Building 25C contained higher perchlorate concentrations than willow trees located 25 m down gradient of the building. Similarly, sweet gum trees nearest to Building 25C contained higher perchlorate concentrations than sweet gum trees located 50 m down gradient of the building. At Site 17, the leaves collected from plants growing on the southern plot where the in-situ bioremediation had less than 10 % of the initial perchlorate in soil generally contained lower concentrations of perchlorate than the same plant species growing on the northern plot containing higher residual concentrations of perchlorate. The groundwater concentration of perchlorate at Site 17 revealed higher concentrations on the southern plot (200 mg L^{-1}) than the northern plot (150 mg L^{-1}). These results suggested that the concentration of perchlorate in source areas in soil influenced the concentrations in the plant tissues to a greater extent than the groundwater concentrations.

The phytoaccumulation of perchlorate also varied with the plant species (Table 5.1). Thus, a statistically significant difference was observed between perchlorate concentrations in willow, pine and sweet gum trees collected from Building 25C ($\alpha = 0.05$) and between willow and pine trees at Site 17 ($\alpha = 0.1$). Generally, willow trees and grasses took up and phytoaccumulated higher concentrations of perchlorate than the rest of the plant species monitored.

The spatial distribution of perchlorate in soils at the LHAAP was quite heterogeneous. Soils collected from 0 to 6-cm depth at Building 25C during a remedial investigation/feasibility study conducted in January, 2001 by Solutions To Environmental Problems Inc. (STEP) contained up to 1.45 mg kg^{-1} perchlorate whereas soils at Site 17 contained 0.34 mg kg^{-1}

perchlorate. Similarly, vegetation collected from Building 25C contained higher perchlorate concentrations than vegetation from Site 17 (Table 5.1). Specifically, the perchlorate concentrations measured in willow trees at Building 25C were significantly higher than in willow trees at Site 17 ($\alpha = 0.05$) whose soils were bioremediated. This suggests that higher concentrations of perchlorate in the rhizosphere soils results in higher phytoaccumulation. The data obtained from greenhouse hydroponics experiments also confirmed a higher uptake and phytoaccumulation of perchlorate in willow leaves grown on high concentrations in the hydroponics growth solution (Table 5.2).

In-situ soil bioremediation of perchlorate was conducted at the old waste water treatment plant to reduce the perchlorate concentration in the top 1 m. The perchlorate-contaminated soils in three pairs of plots were amended with chicken litter and horse manure compost and diluted solutions of ethanol. A control plot was not treated with any nutrient amendment, except for irrigated water added at the same volume as applied to the treated plots. The perchlorate concentration in the control plot soils was an order of magnitude higher than in the treated plots. The active treatment of the site soils was completed in 2001. Three years after the treatment ended, the concentration of perchlorate measured in grass samples harvested from the bioremediated plots ranged from 15.5 ± 4.4 to 84.2 ± 2.0 mg kg⁻¹ DW compared to the much higher concentrations (248.3 ± 3.5 mg kg⁻¹ DW) detected in soils at the control plot. A follow up analysis of perchlorate in grasses from treated and untreated plots the following spring (2005) yielded 58.3 ± 1.9 mg kg⁻¹ DW and 866.3 ± 2.1 mg kg⁻¹ DW in the treated and untreated soils, respectively. This confirmed that lowering the perchlorate concentration in the vegetated soils using in situ bioremediation significantly reduces the perchlorate concentration taken up and phytoaccumulated in vegetation. Yifru and Nzengung (23) showed that treating willow planted

Table 5.2. Concentration of perchlorate in willow leaves (wet weight basis) as a function of initial concentration of perchlorate in the contaminated media.

Initial ClO_4^- [mg L^{-1}]	Leaf ClO_4^- [mg kg^{-1}]
32.8	103.66 ± 16.80
41.1	428.35 ± 0.53
100	813.1 ± 11.2 ‡
167.8	975.3 ± 89.9

‡ From Nzengung et al., (1999)

perchlorate-contaminated soils with cheap agricultural waste such as chicken litter and organic mushroom compost extracts reduces the phytoaccumulated perchlorate in willow leaves by an order of magnitude.

Perchlorate was detected in the leaves of the live plants as well as their senesced and dry leaves. The concentration of perchlorate detected in dry willow, sweet gum and oak leaves collected from Building 25C and Site 17 was an average of $5.1 \pm 4.3 \text{ mg kg}^{-1} \text{ DW}$. The presence of detectable concentration of perchlorate in the senesced dry leaves is attributed to the reported slow rate of phytodegradation of perchlorate in plant tissues (16). Slow phytodegradation generally results in phytoaccumulation of some fraction of perchlorate taken up into the plant leaves.

Greenhouse experiments conducted using willow (*Salix nigra*) and poplar (*Populus deltoides* \times *nigra*) trees planted in 20 L soil bioreactors showed that for the first 24 days, the concentration of perchlorate in leaves increased (Figure 5.3). Thereafter, the perchlorate concentration in the willows and poplar tree leaves approached a maximum before decreasing progressively. The decrease was attributed to phytodegradation of perchlorate in the leaves and the biostimulation of rapid rhizodegradation which minimizes plant perchlorate uptake (23). The mass of perchlorate accumulated in willow leaves increased with cumulative mass of perchlorate added to the soil bioreactors (Figure 5.4).

Vegetation collected from Building 25C showed temporal variation in the concentration of perchlorate. The highest leaf concentration of perchlorate occurred in September, late in growing season when the plants have taken up large volumes of the perchlorate contaminated soil pore water, meanwhile the lowest concentrations was observed in January. A similar

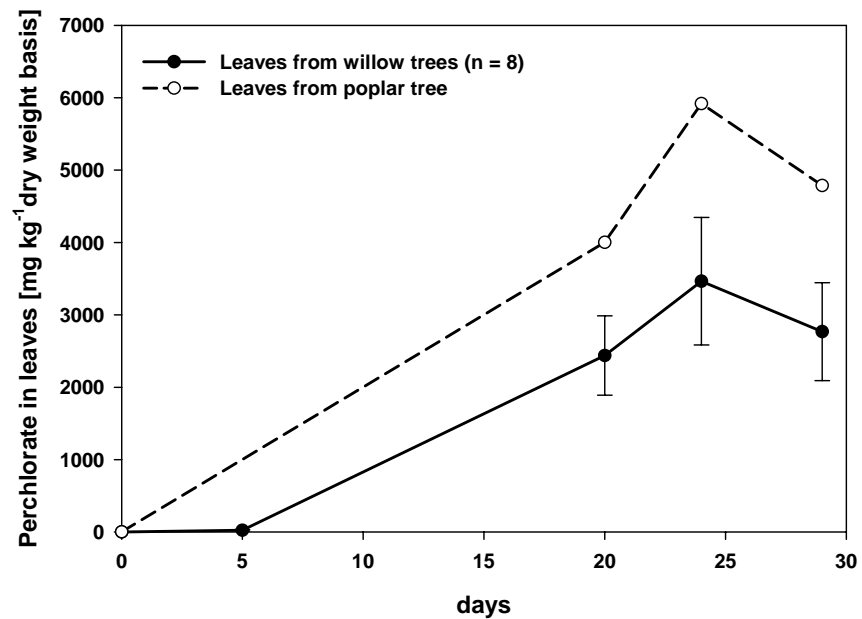


Figure 5.3. Perchlorate concentrations measured in leaves harvested from willow (*Salix nigra*) and poplar (*Populus deltoides* × *nigra* DN-34) trees planted in 20L soil bioreactors dosed with 10 mg kg⁻¹ perchlorate.

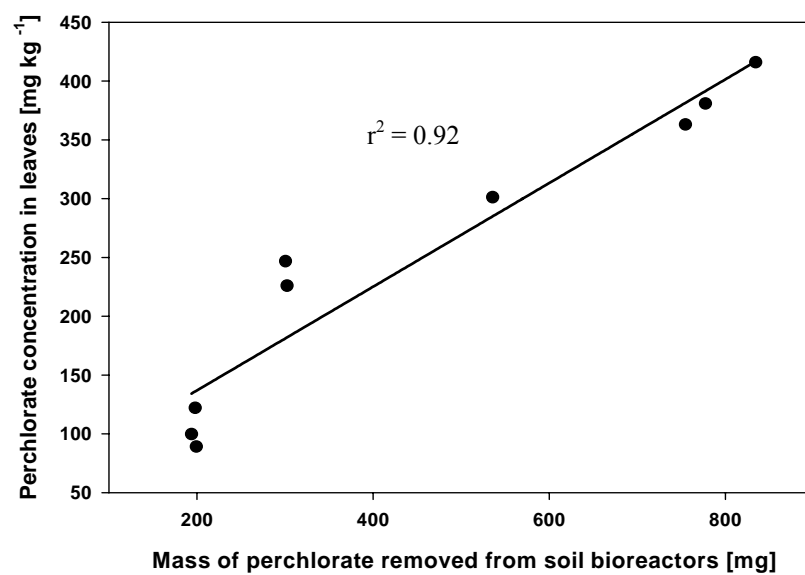


Figure 5.4. Increase in uptake and phytoaccumulated perchlorate in willow (*Salix nigra*) leaves as a function of the initial perchlorate concentration in the soil bioreactors increase.

observation was made by Tan et al., (24) who observed the highest perchlorate concentration in terrestrial vegetation late in the growing cycle.

3.2. The Las Vegas Wash

The leaves of terrestrial and wetland plants at the LVW contained detectable concentrations of perchlorate (Table 5.3). A generally higher concentration of phytoaccumulated perchlorate was observed in leaf tissue of plants growing at the LVW than at the LHAAP. The perchlorate concentration measured in salt cedar tree leaves and cattails were $463.4 \pm 2.6 \text{ mg kg}^{-1}$ DW and $105.5 \pm 0.5 \text{ mg kg}^{-1}$ DW, respectively. Salt cedar contained the highest perchlorate concentrations than any other plant species analyzed at the LVW.

The groundwater at the site contained perchlorate concentrations in the range of 350 mg L^{-1} and the treated water discharged to the Wash contain less than $20 \text{ } \mu\text{g L}^{-1}$ perchlorate. As a result, the highest concentrations of phytoaccumulated perchlorate detected in terrestrial plant species were attributed to their use of groundwater to satisfy their evapotranspired water needs. The aquatic plants whose water needs are satisfied by surface water containing lower concentrations of perchlorate phytoaccumulated lower concentrations of perchlorate. Plants growing down gradient of the Kerr McGee seepage point where treated water is discharged contained less perchlorate than the plants north of the seepage point. For the same general location, plants harvested in October contained higher concentrations of perchlorate than those harvested in spring. The same increase in concentration of phytoaccumulated perchlorate was observed at the LHAAP which is located in a humid climate.

Table 5.3. Perchlorate concentrations (dry weight basis) in terrestrial plants collected from Las Vegas Wash. Live and dry leaves collected from same vegetation are shown in bold.

Species	Month	Concentration [mg/kg]
Salt cedar at Kerr McGee seepage	May 04	118.0 ± 13.3 ^a
Salt cedar at Kerr McGee seepage (Upstream)	May 04	233.5 ± 10.1 ^a
Salt cedar at Kerr McGee well (Downstream)	May 04	166.2 ± 6 ^a
Salt cedar flowers and seeds, North of seepage	May 04	98.3 ± 1.5 ^a
Salt cedar opposite side of wash (control)	May 04	26.4 ^b
Cat tail opposite side of wash (control)	May 04	9.8 ^b
Cat tail	May 04	7.3 ^b
Cat tail	May 04	29.2 ^b
Cat tail	May 04	105.5 ± 0.5 ^a
Algae growing at Kerr McGee seep (Upstream)	May 04	2.59 ^b
Algae growing at Kerr McGee seep	May 04	2.5 ^b
Salt cedar 10ft North of Kerr McGee seepage	October 04	295.9 ± 1.9 ^a
Salt cedar 5 ft from Kerr McGee seepage of treated water	October 04	12.4 ± 0.1 ^a
Salt cedar down gradient Kerr McGee seepage point	October 04	134.2 ± 3.6 ^a
Salt cedar from a dry wetland 250 ft N of seepage point	October 04 }	44.6 ± 1.9 ^a
Senesced salt cedar 250 ft N of seepage point	October 04 }	463.4 ± 2.6 ^a
Salt cedar east of wetland 250 ft N of seepage point	October 04	189.8 ± 5.9 ^a
Salt Cedar South of seepage points near edge of wash	October 04 }	44.8 ± 1.7 ^a
Senesced salt cedar south of seepage points	October 04 }	233.2 ± 0.4 ^a
Senesced salt cedar	October 04	305.5 ^b
Green salt cedar	October 04 }	138.9 ± 2.8 ^a
Senesced salt cedar	October 04 }	326.6 ± 4.6 ^a
Cat tail down gradient of Kerr McGee seepage	October 04	8.2 ± 0.2 ^a
Willow leaves south of seepage of treated water	October 04 }	1.5 ^b
Senesced willow leaves south of seepage of treated water	October 04 }	5.0 ± 1.8 ^a
Sedge from 250 ft N of seepage point	October 04 }	73.1 ± 0.7 ^a
Senesced sedge 250 ft N of seepage point	October 04 }	133.8 ± 1.3 ^a
Sedge down gradient of Wash	October 04	0.03 ^b
Senesced algae Kerr McGee seep	October 04	19.0 ± 0.5 ^a

^a Sample size (n = 2)

^b Sample size (n = 1)

Senescent vegetation of all species collected from the LVW contained higher amounts of perchlorate than the live growing vegetation (Table 5.3). Similarly, Smith et al., (4) also observed higher perchlorate concentrations in leaf litter of salt cedar trees than in the leaves from live trees. These observations provide evidence that the terrestrial plants growing at LVW are recycling perchlorate through the leaf litter that is deposited onto the top soil. Confirmation was obtained from the analysis of composite soil samples collected from under salt cedar trees. The topsoil sample (0-5 cm) contained 203.8 mg kg^{-1} perchlorate compared to 9.8 mg kg^{-1} at depths of 6-10 cm below ground surface. The higher perchlorate concentration in the top soil were in the range of the plant litter accumulated on the topsoil. Also, senesced sedge (aquatic plant) sampled from 100-m north of the seepage point contained up to $134 \pm 2.8 \text{ mg kg}^{-1} \text{ DW}$ perchlorate as did the senesced willow leaves planted in the restored wetland area down gradient of Kerr McGee's seepage point in the Wash (Table 5.3).

4. Summary and Conclusions

All of the terrestrial vegetation species sampled at the LHAAP and LVW sites were found to contain detectable concentrations of perchlorate, implying uptake of perchlorate by the different plant species growing at these sites. The slow rate of phytodegradation of perchlorate taken up by plants therefore results in phytoaccumulation of some fraction in mostly the plant leaves. The observed high concentrations of phytoaccumulated perchlorate and published data of laboratory studies suggest that the sustainment of rhizodegradation is limited by the insufficient supply of carbon and electron donors from root zone exudation. The phytoaccumulated perchlorate poses ecological risks because deciduous vegetation at perchlorate contaminated sites recycle the phytoaccumulated perchlorate. This study has documented generally higher

concentrations of perchlorate in leaf tissue of plants growing over perchlorate-contaminated soils in arid climates. The monitored plants growing in arid climate at the LVW contained significantly higher concentrations of perchlorate than plants growing in sub-humid to humid climate of the LHAAP. The analysis of soil and plant litter at the LVW confirmed that salt cedar has contributed significantly to the recycling of perchlorate at this site. The amount of perchlorate taken up and accumulated by terrestrial and aquatic plants is influenced by several factors, including: 1. the perchlorate concentration in the source area, 2. the distance of the plant to the source of contamination, 3. the type of plant species, 4. the season, and 5. the sampling time during the growing season. Plants closer to the source of contamination where the perchlorate concentrations are highest took up and phytoaccumulated higher amounts of perchlorate. Grasses and salt cedar trees accumulated significantly higher concentrations of perchlorate than woody plants. Plant leaves harvested in late summer contained higher amounts of perchlorate than leaves harvested in spring and early summer. Senescent leaves and litter fall contained phytoaccumulated perchlorate to a greater extent than the live leaves, an indication that plants could potentially recycle perchlorate in an ecosystem. To minimize the potential recycling of perchlorate during phytoremediation, it is recommended that plant leaves containing perchlorate be collected and composted and rhizodegradation enhancement be incorporated into the design, installation and management of the technology.

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CHAPTER 6

UPTAKE OF N-NITROSODIMETHYLAMINE (NDMA) FROM WATER BY PHREATOPHYTES IN THE ABSENCE AND PRESENCE OF PERCHLORATE AS S CO- CONTAMINANT¹

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Abstract

The uptake and fate of N-Nitrosodimethylamine (NDMA) in phreatophytes was studied in hydroponics system under greenhouse conditions. The rate of removal of NDMA from solution by rooted cuttings of black willow (*Salix nigra*) and hybrid poplar (*Populus deltoides* × *nigra*, DN34) trees varied seasonally, with faster removal in summer months when transpiration rates were highest. A linear correlation between the volume of water transpired and mass of NDMA removed from the root zone was observed, especially at higher NDMA concentrations. In bioreactors dosed with both NDMA ($0.7 - 1.0 \text{ mg L}^{-1}$) and perchlorate (27 mg L^{-1}), no competitive removal of NDMA and perchlorate was observed. While NDMA was primarily removed from solution by plant uptake, perchlorate was predominantly removed by rhizodegradation. In the presence of NDMA, a slower rate of rhizodegradation of perchlorate was observed, but still significantly faster than the rate of NDMA uptake. For experiments conducted with radiolabeled NDMA, $46.4 \pm 1.1 \%$ of the total ^{14}C -activity was recovered in the plant tissues and 47.5% was phytovolatilized. The $46.4 \pm 1.1 \%$ in the plants was distributed as follows: $18.8 \pm 1.4 \%$ (leaves), $15.9 \pm 5.9 \%$ (stems), $7.6 \pm 3.2 \%$ (branches), and $3.5 \pm 3.3 \%$ (roots). The poor extractability of NDMA with methanol-water (1:1 v/v) from stem and leaf tissues suggested that NDMA is assimilated by plants. The calculated transpiration stream concentration factor (TSCF) of 0.28 ± 0.06 suggests that NDMA is passively taken up by phreatophytes, and mainly phytovolatilized.

1. Introduction

N-Nitrosodimethylamine (NDMA) is an emergent contaminant and a probable human carcinogen of natural and anthropogenic origin. NDMA can be produced by the oxidation of 1,1-dimethylhydrazine (UDMH), which is mainly used in rocket fuel production (1,2) or during disinfection of water and waste water by chlorine or monochloroamine (3,4). NDMA is also produced in natural ecosystems by the reactions of secondary amines such as dimethylamine and nitrite (5,6). NDMA has contaminated groundwater near aerospace facilities and where treated wastewater is used for irrigation and groundwater recharge. Recently, several drinking water wells have been closed in California as a result of NDMA contamination (4). NDMA and perchlorate frequently occur as co-contaminants at a growing number of sites because both are used in the production of rocket fuel.

NDMA is of concern to municipal water suppliers because there is sufficient and growing evidence of its carcinogenicity in experimental animals (7,8). Chronic ingestion and inhalation of NDMA may cause an increase in liver, kidney and other types of tumors (9). NDMA is highly soluble in water (Table 5.1) with Henry's law constant of $1.45 \times 10^{-3} \text{ atm m}^3 \text{ M}^{-1}$ at 20 °C (10) and log K_{ow} value of -0.57 (11). Due to its hydrophilicity, NDMA sorbs poorly to soils (2,12), activated carbon and other sorbents (1,2). So far, there is no state or federal drinking water maximum contaminant level (MCL) for NDMA. However, the U.S. Environmental Protection Agency (USEPA) Integrated Risk Information Services has indicated that higher than 0.7 ng L^{-1} result in an increased cancer risk (13).

Similarly, perchlorate is an anthropogenic as well as naturally occurring emergent contaminant. Ammonium perchlorate is an oxygen-adding component in propellants for rockets,

Table 6.1. Physical and Chemical Properties of NDMA and Ammonium perchlorate.

Property	NDMA	Ammonium perchlorate
Synonyms	Nitrosodimethylamine, Dimethylamine N-nitroso Dimethylnitrosamine	Ammonium perchlorate
Molecular formula	$C_2H_6N_2O^a$	NH_4ClO_4
Molecular weight	74.08 ^a	117.5
Density at 20°C	1.0048 ^a	1.95 ^g
Boiling point	151 – 153°C ^a	
Water solubility	Very soluble ^a	200 g L ^{-1g}
Vapor pressure	2.7 mm Hg at 20°C ^b	Not volatile
Henry's Law constant	2.6×10^{-4} atm M ⁻¹ at 20°C ^c 1.45×10^{-3} atm M ⁻¹ at 20°C ^d	Not applicable
log K _{ow}	-0.57 ^e	-5.84 for ClO ₄ ^{-h}
UV absorption maxima (water)	230 nm, 332 nm ^f	

^a Ref 25. ^b Ref 26. ^c Ref 27. ^d Ref 9. ^e Ref 10. ^f Ref 28. ^g Ref 29. ^h Ref 30.

missiles and fireworks which is highly soluble in water and dissociates into ammonium and perchlorate ions. Perchlorate is produced naturally from atmospheric sources and occurs in evaporite deposits of arid and semi-arid climates (14,15) such as in Chilean caliche; a sodium nitrate fertilizer with perchlorate concentrations of about 0.5 to 2 mg g⁻¹ (16). Perchlorate contamination has been observed in surface water and groundwater at several sites where perchlorate salts have been manufactured, processed or used. In addition to drinking water, perchlorate has been detected in lettuce (17), dairy milk (18), and breast milk (19). Perchlorate poses environmental concerns because it competitively blocks iodide uptake by the thyroid. In January 2006, the USEPA set a preliminary cleanup goal for perchlorate of 24.5 µg L⁻¹ in water (20).

Although Tate and Alexander (6) showed that microorganisms could not metabolize NDMA due to the resistance of nitrogen-nitrogen bonding in NDMA, a recent study by Sharp et al., (21) indicated that certain monooxygenase expressing bacterial strains have the capability to degrade NDMA. Due to their high aqueous solubility, both NDMA and perchlorate are well-suited for phytoremediation using phreatophytes. It is known from multiple previous laboratory studies that phytodegradation and rhizodegradation are the two main mechanisms by which plants remove perchlorate from water (22,23). Except for Dean-Raymond and Alexander (24) who showed that NDMA can be assimilated by the roots of lettuce and spinach with some fraction translocated to the stem and leaves, detailed studies on the mechanism(s) of phytoremediation of NDMA are lacking in the published literature. This study represents the first detailed study that focuses on the potential of green plants to remove NDMA from contaminated water. Specifically, this study investigated the mechanisms of decontamination of NDMA-contaminated water by poplars and willows in hydroponics system, as well as the competitive

removal of NDMA and perchlorate as co-contaminants. In a subset of the experiments, radiolabeled [^{14}C] - NDMA was used as a tracer to verify the accuracy of GC/MS analysis results, close mass balance, and determine the fate of NDMA in the plant tissues.

2. Materials and Methods

2.1. Chemicals

Reagent grade N-Nitrosodimethylamine (99+ %) was purchased from Acros Organics (Pittsburg, PA). Radiolabeled (^{14}C) NDMA (0.1mCi, purity > 98 %) was purchased from Moravsek Biochemicals Inc., (Brea, CA) and used as a tracer in a subset of the experiments. Hoagland[®] plant nutrient solution was obtained from Carolina Biological Supply Co. (Burlington, NC). Miracle-Gro[®] nutrient was purchased from a local grocery. HPLC grade methanol was purchased from J.T. Baker[®] (Phillipsburg, NJ). Methylene chloride (HPLC grade) and Sintisafe[™] 30% LSC cocktail were purchased from Fisher Scientific (Fairlawn, NJ). An OX161 ^{14}C cocktail purchased from RJ Harvey Instrument Corporation (Hillsdale, NJ) was used to trap $^{14}\text{CO}_2$ in the biological oxidizer. Ambersorb[®]-572 was purchased from Sigma Aldrich (St. Louis, MO), while activated carbon was obtained from Fisher Scientific (Fairlawn, NJ). Almatris Ac. Inc. provided DD-6 alumina sorbent (48×100 U.S. screen mesh and surface area of $360 \text{ m}^2 \text{ g}^{-1}$) that was used to cleanup the plant extracts. Pelletized NaOH, liquid sodium hydroxide (NaOH, 50 % w/w), and 1-Octanol (99% HPLC grade) were obtained from J.T. Baker[®] (Phillipsburg, NJ). Sodium perchlorate monohydrate ($\text{NaClO}_4 \cdot \text{H}_2\text{O}$) was purchased from

Aldrich[®] (Milwaukee, WI). The perchlorate calibration and check standard solutions were purchased from AccuStandard[®] (New Haven, CT) and SPEX CertiPrep[®] (Metuchen, NJ).

2.2. Hydroponics Experiments

Pre-rooted black willow (*Salix nigra*) and hybrid poplar (*Populus deltoides* × *nigra*, DN34) cuttings were selected for this experiment due to their very high water uptake rate. Black willow cuttings purchased from Ernst Conservation Seed (Meadville, PA) and hybrid poplar obtained from Segal Ranch hybrid poplars (Grandview, WA), were rooted in a greenhouse in aerated quarter-strength Hoagland[®] nutrient solution. After three weeks, the pre-rooted trees were transferred to 2 L Erlenmeyer[®] flasks (bioreactors) containing quarter-strength Hoagland[®] solution. To document the effect of nitrate-N in Hoagland[®] solution on the removal of perchlorate, some planted bioreactors were provided with an ammonical-N growth solution as 0.39% (w/w) Miracle-Gro[®]. The sampling port on each Erlenmeyer[®] flask was closed using Mininert sampling valves (Supelco, Bellefonte, PA) and each bioreactor was wrapped with aluminum foil to prevent algae growth and NDMA photolysis. A predrilled screw cap with Teflon-lined septum was placed around each cutting. The septum and tree cutting interface was sealed with Parafilm[®] tape (Chicago, IL) and covered with aluminum foil. DAP[®] aquarium sealant (100 % silicone, Baltimore, MD) was used to seal openings between the septum and aluminum foil. The aquarium sealant was also placed around the cuttings (up to 2 cm from the septum) to prevent any loss of water by volatilization from the bioreactors.

The uptake of NDMA from the hydroponic bioreactors was initially conducted in winter months and then repeated in summer months. The planted bioreactors and corresponding

unplanted control bioreactors were dosed with 0.7 to 1 mg L⁻¹ NDMA and the rate of decrease of NDMA concentration was measured periodically. Prior to each sampling event, the volume of water transpired by the plants was replenished by adding quarter-strength Hoagland nutrient solution up to the 2 L mark on the bioreactors. The volume of water added to the bioreactors was recorded for the entire duration of the experiment. Exactly 2.5 ml of the dosed hydroponic growth solution in each bioreactor was sampled using a 3 ml syringe every 2 to 3 days for GC/MS analysis.

To simulate the uptake of NDMA from water containing perchlorate as a co-contaminant, a subset of the planted bioreactors were dosed with 0.65 mg L⁻¹ NDMA and 27 mg L⁻¹ perchlorate. Quarter-strength Hoagland® solution was used as the plant growth medium. The concentration of NDMA and perchlorate was monitored every 2 to 3 days. After the concentrations of both NDMA and perchlorate decreased below the method detection limit (MDL) of 10 µg L⁻¹ and 2 µg L⁻¹, respectively, the plants were harvested for analysis.

Plant-mediated removal of 27 mg L⁻¹ perchlorate alone was investigated in growth solution containing nitrate-N (Hoagland) and ammonical-N (Miracle-Gro®). A 1.5 ml sample of rhizosphere solution was taken from each bioreactor for analysis until the concentration of perchlorate decreased below the MDL. At the termination of each experiment, the plants were harvested, sectioned into leaves, branches, stem, and roots prior to extraction. The plant tissues extraction and clean-up procedure described in detail by Ellington and Evans (31) was followed. The concentration of perchlorate was measured with a Dionex® DX-500 ion chromatography (IC) analytical method described elsewhere (22,32). To confirm the identity of perchlorate peaks, some duplicate samples were spiked with 100 µg L⁻¹ perchlorate prior to analysis.

2.3. Radiolabeled Experiments

Poplar planted bioreactors were dosed to obtain an initial concentration of 1 mg L^{-1} NDMA using a predetermined volume of [^{12}C]-NDMA stock solution containing $10 \text{ } \mu\text{Ci}$ [^{14}C]-NDMA as a tracer. The canopy of each tree was enclosed in an inverted 4 L Erlenmeyer flask placed over the root zone bioreactor to fit snugly around the screw cap, which created a closed aerial compartment (Figure 6.1). Layers of parafilm were wrapped around each cutting to obtain a snugly fit inside the Teflon-lined septum in the screw cap. A layer of aluminum foil was wrapped around the parafilm to minimize losses of NDMA due to sorption. A vacuum pump was connected to the 4-L Erlenmeyer flask to withdraw headspace air at a rate of approximately 1 to 2 L min^{-1} . The growth chamber outlet was fitted through a ball and socket joint to a series of four screw-top traps. Purged air from the growth chamber was bubbled through 25 ml of 1N NaOH solution to capture any [^{14}C]- CO_2 produced from [^{14}C]-NDMA mineralization. The air then flowed through one Ambersorb[®]-572 and two granular activated carbon traps (GAC) to capture some of the phytovolatilized [^{14}C]-NDMA and degradation products. The traps were changed every 7 days. Silicone seal was applied to all joints to prevent leakage. The hydroponics growth solution was slowly and continuously mixed throughout the experiment with a magnetic bar and stir plate (Figure 6.1).

To determine the ^{14}C -activity in the rhizosphere solution, 1 ml sample was placed in a scintillation vial and filled with 15 ml Sintisafe[™] 30 % scintillation cocktail. The ^{14}C -activity in the vials was counted on a Wallac Winspectral α/β 1414 Liquid Scintillation Counter (LSC) with 3 minutes counting time. At the termination of the experiments, five sub-samples

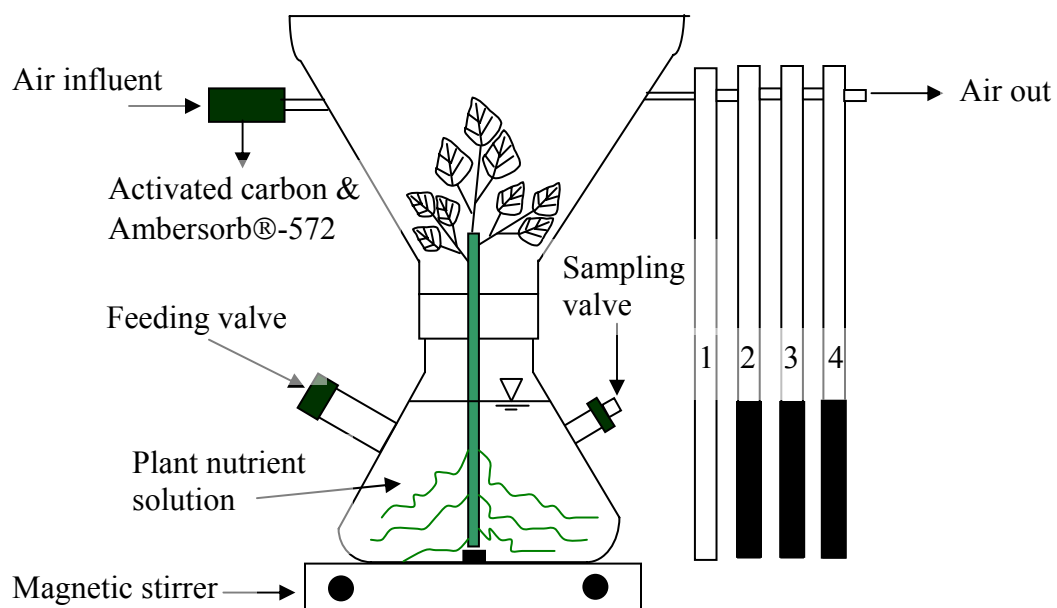


Figure 6.1. Schematic diagram of the hydroponic bioreactor used in the experiment. Trap 1 was filled with 25-mL 1N NaOH solution, Trap 2 was filled with 15-g Ambersorb® 572, Traps 3 and 4 were filled with 15-g activated carbon.

(approximately 0.6 g) of each homogenized plant tissue were combusted for 3 min in a biological oxidizer (RJ Harvey Instrument Corporation OX-500, Hillsdale, NJ) at 900 °C to convert all ^{14}C in the tissue to $^{14}\text{CO}_2$, which was collected in 15 ml OX161 $^{14}\text{CO}_2$ cocktail for assay.

2.4. Plant Tissue Extraction

At the termination of each experiment, the plants were harvested, sectioned into leaves, stem, branches, and roots, dried with paper towel, weighed, and flash frozen with liquid nitrogen. For radiolabeled experiments, the plants were sacrificed for analysis when $94.9 \pm 4.8\%$ of the ^{14}C -activity in the bioreactors was removed. The leaves, roots and branches were pulverizing with a mortar and pestle. The stems were first minced in a blender before pulverized with a mortar and pestle. A 2 g sample of each homogenized plant tissue was extracted with 15 ml of methanol-water (1:1 v/v) by sonication for one hour and then mixed continuously overnight. The glass vials were covered with aluminum foil to prevent photodegradation of NDMA. The supernatant was separated from the plant residue by centrifugation for 30 minutes at $1000 \times g$. A 10 ml extract was concentrated to 2 ml in a hot water bath and extracted into methylene chloride for GC/MS analysis (21). The extraction efficiency of methanol-water (1:1 v/v) and methylene chloride was verified by comparing the ^{14}C activity in plant extracts from ^{14}C -NDMA experiments with the activity measured in sub-samples combusted in the biological oxidizer.

2.5. Gas Chromatography Analysis

A Shimadzu gas chromatograph/mass spectrometer (GC/MS) was used for quantitative analysis of NDMA extracted from aqueous solution and plant extracts using the method of Choi and Valentine (33). All analyses were by split-splitless injection of 1 μl of the methylene chloride fraction using an autosampling injector. Helium was used as the carrier gas, at a flow rate of 23.4 ml min^{-1} . The oven temperature was programmed at 35°C for 1 minute, followed by a temperature increase of $15^{\circ}\text{C min}^{-1}$ to 150°C and then ramped at a rate of $30^{\circ}\text{C min}^{-1}$ to 250°C and held for 3 minutes for a total run time of 18 min. The injector and transfer line temperatures were 200°C and 250°C , respectively. The separation column was an HP-5MS 30-m \times 0.25 mm \times 0.25 μm capillary column. NDMA was quantified by mass detection of NDMA's molecular ion ($m/z = 74.1$) while products were identified in the scan mode. The efficiency of the liquid-liquid extraction was 45 % with a variance of less than 5 % between duplicate samples. The retention time for NDMA was 3.4 minutes.

2.6. Thin Layer Chromatography (TLC)

Dichloromethane extracts of ^{14}C -activity in stem and leaves samples were further analyzed by TLC to separate NDMA and its transformation products based on hydrophobicity. 100 μl of the plant extracts were spotted onto $5 \times 20 \text{ cm}$ TLC plates coated with 250- μm silica gel (Whatman[®], Florham, NJ). The dosed TLC was placed in ethanol:benzene:water (4:1:1 v/v) to separate out the different components. The silica gel was scraped off the plate at 1 cm

intervals directly into scintillation vials, mixed with 10 ml of Sintisafe™ 30 % scintillation cocktail and assayed for total ^{14}C -activity.

3. Result and Discussion

3.1. Plant Uptake of NDMA

The uptake of NDMA by willow and poplar trees was studied in hydroponic experiments lasting up to 100 days. For the experiments conducted during summer months, $98.3 \pm 1.7 \%$ of the initial 1 mg L^{-1} NDMA was taken up by the trees in 80 days while $81.4 \pm 10.3 \%$ of the initial 1 mg L^{-1} NDMA was taken up by the trees in 102 days during winter months (Figure 6.2). Excised willow trees and unplanted controls were used to verify NDMA losses from the bioreactors due to sorption to plant roots and reactor seals, microbial- and photo-transformation, and evaporation. For the excised controls, 10.5 % of the initial concentration ($0.6 \mu\text{g L}^{-1}$) was not recovered after 50 days, while only $3.5 \pm 0.9 \%$ of the initial 1.1 mg L^{-1} NDMA was removed after 86 days from the unplanted control experiments. Comparison of the recoveries from excised and unplanted controls showed that sorption to the reactor compartments and transformation of NDMA in the bioreactors were minimal during the experiment. The good recovery from the controls suggest that the removal of NDMA from the planted bioreactors was dominated by the plant uptake. The analysis of ^{14}C -NDMA by liquid scintillation assay provided an independently confirmation of the accuracy of results for ^{12}C -NDMA analysis by liquid-liquid extraction followed by GC/MS analysis.

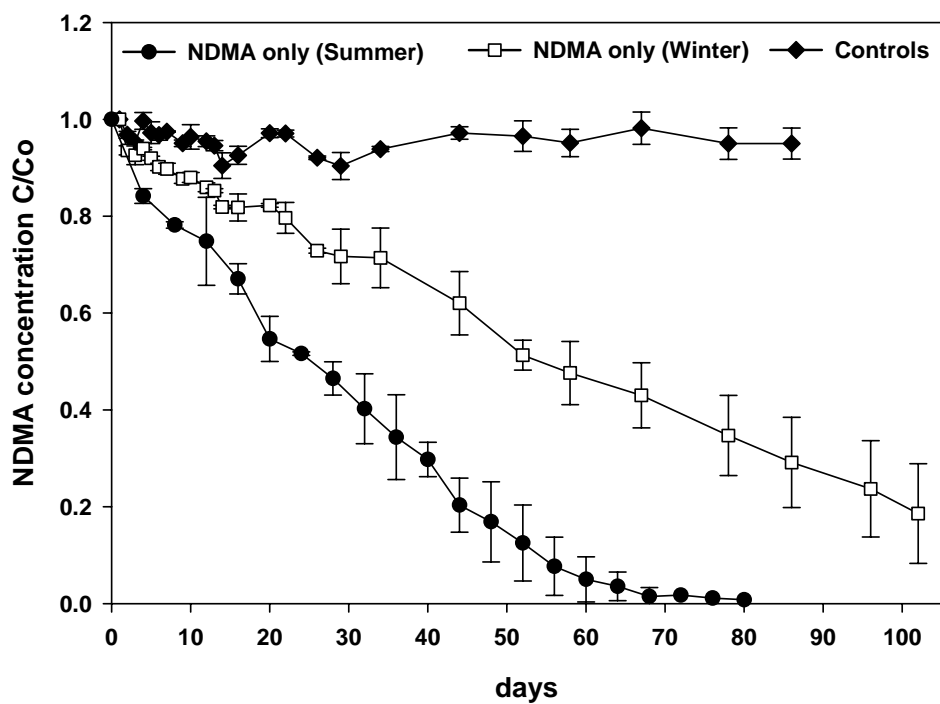


Figure 6.2. Representative plots for the rate of uptake of NDMA from hydroponic bioreactors planted with hybrid poplar and willow trees. The data is plotted as averages of three bioreactors ± 1 SD.

The mass removal of NDMA from solution by black willow and hybrid poplar trees increased linearly with the volume of water transpired, until about 90 % of the initial concentration of NDMA in the bioreactors was removed (Figures 6.3). After approximately 90 % of the initial NDMA mass removal, the rate of uptake decreased significantly and deviated from the initial linear correlation. Because there was no continuous supply of NDMA to the bioreactors in these experiments, the concentration of NDMA in the bioreactors decreased over time due to plant uptake. As a result, the mass of NDMA removed per volume of transpired water decreased as the total mass of NDMA taken up asymptotically reached the initial mass of NDMA added to the bioreactors. The strong linear relationship between the mass of NDMA removed and total volume of water transpired was more evident during the winter months, as shown by the pooled data for the removal of 89.3 ± 3.8 % of the initial mass of NDMA from multiple experiments (Figure 6.3). The slower uptake rates of NDMA observed in experiments conducted during winter months are attributed to the low transpiration rates during cooler months (Figure 6.2, Table 6.2). The implication of these observations is that the mass removal of NDMA via uptake from groundwater by phreatophytes at some sites can be predicted using the transpiration rate.

The measured daily and cumulative volume of water transpired was used to calculate the transpiration rates for the experiments listed in Table 6.2. Using this data, the transpiration stream concentration factor (TSCF) for NDMA, which is the ratio of a chemical concentration in the transpiration stream of plants to its concentration in the root zone (34,35), was estimated by applying the following model (35):

$$TSCF = \frac{U}{C * T}$$

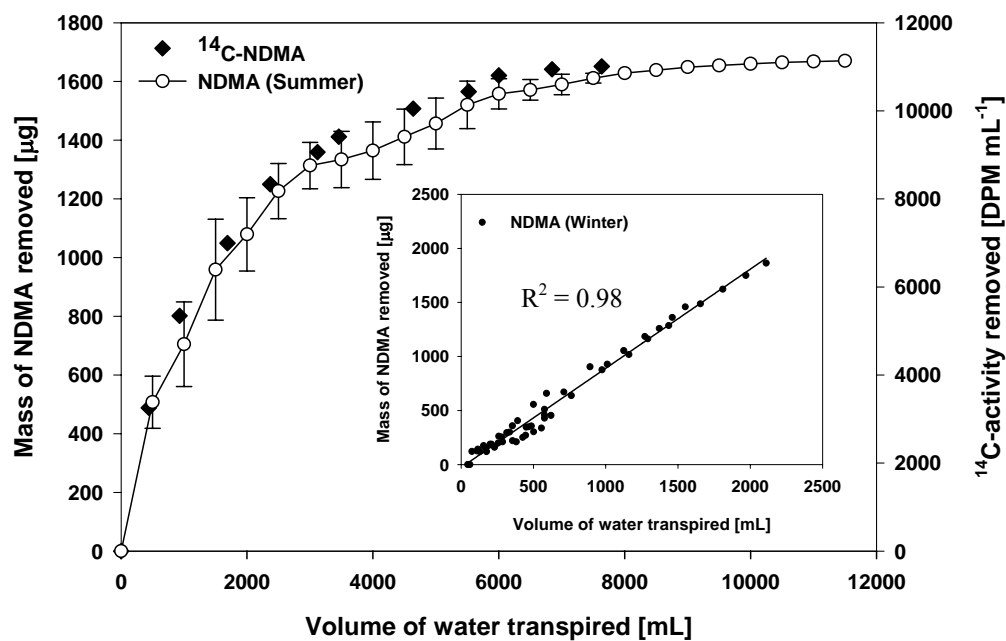


Figure 6.3. Relationship between the total volume of water transpired and mass of NDMA removed from hydroponic growth solution. Inset: strong linear relationship between mass removed and total transpiration from multiple winter experiments.

Table 65.2. Calculated transpiration stream concentration factor (TSCF) and linear regression coefficients.

Bioreactor	Treatment	Slope	Intercept	R ²	Cumulative transpiration (ml)	Transpiration rate (ml day ⁻¹)	Mass NDMA removed (µg)	TSCF
N-1 (Poplar)	NDMA	-10.0	694.65	0.95	11505	155	1683.6	0.24
N-2 (Poplar)	NDMA	-12.3	726.6	0.96	7235	128	1623.4	0.32
N-3 (Poplar)	NDMA	-15.6	597.07	0.95	5105	186	1408.5	0.32
N-4 (Willow)	NDMA	-23.6	771.2	0.89	3145	113.8	1467.8	0.26
N-5 (Willow)	NDMA	-23.3	763.9	0.90	3312	113	1488.2	0.29
N-6 (Willow)*	NDMA	-9.2	1034.4	0.99	2110	22.6	1908.2	0.38
N-7 (Willow)*	NDMA	-7.0	960.3	0.99	1551	22.0	1503.4	0.33
NP-1 (Poplar)	NDMA and perchlorate	-18.0	680.94	0.96	8290	260.5	1298.4	0.18
NP-2 (Willow)	NDMA and perchlorate	-13.1	502.38	0.93	11015	253	1044.2	0.22
NP-3 (Poplar)	NDMA and perchlorate	-16.3	483.09	0.91	5952	227	1027.8	0.27
NP-4 (Poplar)	NDMA and perchlorate	-11.0	559.27	0.96	6883	123.3	1042.1	0.27
Average								0.28
Standard deviation								0.06

*- experiments conducted during winter months when transpiration was low.

where C is NDMA concentration in aqueous solution ($\mu\text{g L}^{-1}$), T is the rate of transpiration (L day^{-1}), and U is uptake ($\mu\text{g day}^{-1}$). The average transpiration rates (T) and NDMA uptake (U) for individual trees (Table 6.2) was used to calculate the average TSCF for eleven trees. The estimated TSCF for NDMA was 0.28 ± 0.06 . Our experimentally determined $\log K_{ow}$ for NDMA was -0.60, which is not significantly different from the $\log K_{ow}$ value of -0.57 reported by Hansch et al. (10). The $\log K_{ow}$ value of a compound, which is related to its hydrophobicity, is often used to predict concentrations of a compound in the transpiration stream of plants. Hydrophilic compounds with small $\log K_{ow}$ values, do not pass through the lipid membranes of the roots and translocation to shoots is low (34,35). Very lipophilic compounds with $\log K_{ow} > 4.5$, rapidly adsorb to the extracellular wall of the roots and thus are not readily transported in plants (34,35). Maximum translocation to shoots has been observed for moderately lipophilic compounds with $\log K_{ow}$ values in the range of 1.0 to 3.5 (34,35). Using our experimentally determined $\log K_{ow}$ for NDMA of -0.6, the TSCF values calculated using the models proposed by Briggs et al., (34) and Burken and Schnoor, (35) were 0.08 and 0.02, respectively. Our experimentally determined values are one order of magnitude higher than the TSCF- $\log K_{ow}$ model predicted values. It appears that the model under predicts the TSCF values of highly water-soluble compounds. Similarly, Doucette et al., (36) observed that their experimental TSCF values were higher than predicted using the TSCF- $\log K_{ow}$ relationship models.

The similarity of the TSCF from multiple experiments, 90% recoveries in excised planted control experiments, and the good agreement in the results of parallel radiolabeled and non-radiolabeled experiments suggested that rhizodegradation did not contribute significantly to the removal of NDMA in these experiments. The higher rate of NDMA removal during the first 48

hours of each experiment was attributed to rapid adsorption of NDMA to the organic cuticles of the tree roots.

3.2. Fate of ^{14}C -NDMA in Plant Tissues

The mean recovery of ^{14}C -activity in the plant tissues and the four traps in Figure 6.1 was $52.6 \pm 1.6\%$. The total fraction of ^{14}C -NDMA recovered by biological oxidation of the different plant tissues was $46.4 \pm 1.1\%$, of which $18.8 \pm 1.4\%$ was taken up and translocated into the leaves, $15.9 \pm 5.9\%$ was in the stem, $7.6 \pm 3.2\%$ in the branches, and $3.5 \pm 3.3\%$ in the roots, while $3.7 \pm 0.6\%$ and $1.3 \pm 1\%$ was recovered in Ambersorb[®]-572 and the two activated carbon traps, respectively. Although NDMA should sorb poorly to Ambersorb[®]-572 and GAC due to its high solubility and very low $\log K_{ow}$, the three sorption traps were used mainly to confirm phytovolatilization. The poor sorption of NDMA to GAC has been reported by Gunnison et al., (2) while Choi and Valentine (33) recovered only 25 % of NDMA in aqueous solution in solid phase extraction with Ambersorb[®]-572 (R. Valentine-personal communication). The $42.5 \pm 1.6\%$ of the ^{14}C -activity not recovered in the plant and traps was likely translocated into leaves and phytovolatilized to the atmosphere. In previous studies by Dean-Raymond and Alexander (24), 0.02 to 5.06 % of the ^{14}C -activity applied to lettuce and spinach seedlings was recovered after 2 to 15 days of exposure. Meanwhile, Arienzo et al., (9) recovered 0.07 to 2.85 % of the applied ^{14}C -activity in turf grass grown in sandy loam and loamy sand lysimeters after 14 days of exposure. The higher recoveries of ^{14}C -NDMA in this study are attributed to: 1) the high transpiration rates of the willow and hybrid poplar trees used in this study, 2) the limited effect of

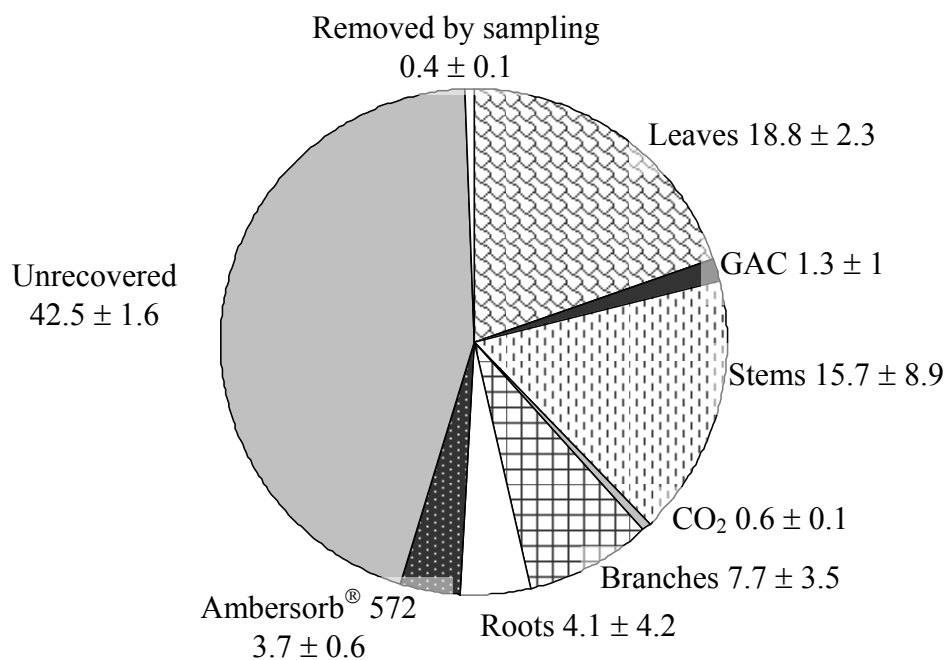


Figure 6.4. Distribution of 1.0 mg L⁻¹ NDMA (containing 10-μCi ¹⁴C-NDMA) taken up into plant tissues and in various traps. The ¹⁴C-activity in plants, Amborsorb®-572 and GAC was quantified by combustion in a biological oxidizer. Values indicate average for three bioreactors ± 1 SD.

mass transfer on plant uptake in the hydroponics system, and 3) limited or no influence of volatilization losses.

The ^{14}C -NDMA fraction extracted from the plants using either methanol-water mixture or methylene chloride was compared to the total activity measured in the plant by combustion of the different plant tissues in a biological oxidizer (95 % recovery efficiency). The extraction efficiency of methanol-water was 32.3 ± 4.5 % compared to only 5.8 ± 3.3 % for methylene chloride.

The results of thin layer chromatography analysis of the leaf and stem extracts did not provide evidence of NDMA transformation in the plant tissues to hydrophobic products. The recovery of approximately 85 % of initial ^{14}C -activity in the extracts within the top 1 cm of the chromatogram was similar to controls. The enzyme cytochrome P450, which is found in many plant species (37) catalyzes the oxidation of NDMA to formaldehyde and methyldiazonium ion (38). Since both NDMA and formaldehyde are readily soluble in water they are not separable using TLC. The recovery of 0.6 ± 0.1 % of the total activity in the 1N NaOH trap suggests that small amounts of NDMA could have been mineralized to $^{14}\text{CO}_2$ in the plant tissues. At the low concentrations used in these experiments, the concentration of metabolites formed by phytodegradation of NDMA should be too small to confirm by GC/MS analysis.

3.3. Phytoremediation of NDMA and Perchlorate as Co-Contaminants

The uptake of NDMA from aqueous solution in the presence of perchlorate was studied in hydroponic bioreactors dosed with 0.65 mg L^{-1} NDMA and 27 mg L^{-1} perchlorate. The uptake of an initial 0.65 mg L^{-1} NDMA to below method detection limit (MDL) of $10 \text{ } \mu\text{g L}^{-1}$ was

achieved in about 45 days, while removal of 27 mg L^{-1} perchlorate by uptake and rhizodegradation required 70 days in the nitrate-N rich Hoagland solution (Figures 6.5a,b). No difference was observed in the time to achieve complete removal of NDMA to below the GC/MS MDL of $10 \text{ } \mu\text{g L}^{-1}$ in the presence or absence of perchlorate (Figure 6.5a). While passive uptake and rhizodegradation characterized the removal of perchlorate from solution, NDMA was primarily removed by passive plant uptake. As a result, the removal of NDMA as a single and co-contaminant showed the same good linear correlation between the mass of NDMA removed and total volume of water transpired as in Figure 6.3.

The removal of 27 mg L^{-1} perchlorate to below the MDL of $2 \text{ } \mu\text{g L}^{-1}$ required about 70 days in the presence of NDMA, and about 50 days when the bioreactors were dosed with perchlorate alone (Figure 6.5b). The slower rate of removal of perchlorate in the presence of NDMA can be attributed to the latter's effect on rhizodegradation of perchlorate. Although it has been reported that NDMA is toxic to natural population of microorganisms at concentrations of up to 10 g L^{-1} (39), it is possible that in the presence of NDMA optimum conditions for the growth of perchlorate degraders were not created.

Figure 6.5b illustrates the characteristic slow initial perchlorate removal phase attributed mainly to plant uptake in transpired water and phytodegradation which precedes the rapid microbial degradation or rhizodegradation phase (22,32). The duration of the uptake dominated phase was 25 - 36 days when nitrate-rich dilute Hoagland[®] solution was used as the plant growth media compared to 8 days for non-nitrate growth media (Miracle-Gro[®]) (result not presented). Thus, the same initial concentration of perchlorate was removed in a much shorter time (16 to 29 days) if a Miracle-Gro[®] was used to prepare the growth solution. The trees grown on nitrate-N source (Hoagland[®] nutrient solution) were observed to phytoaccumulate higher concentrations

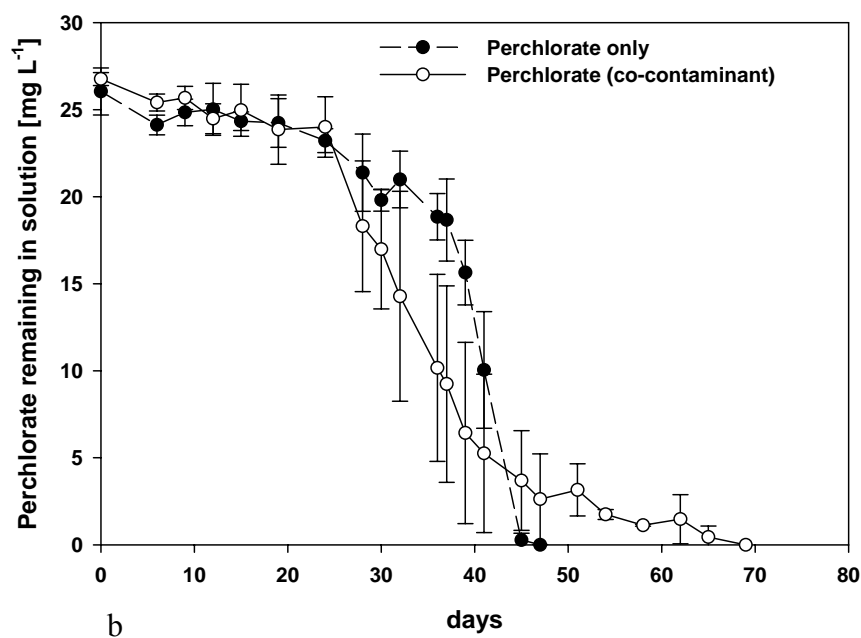
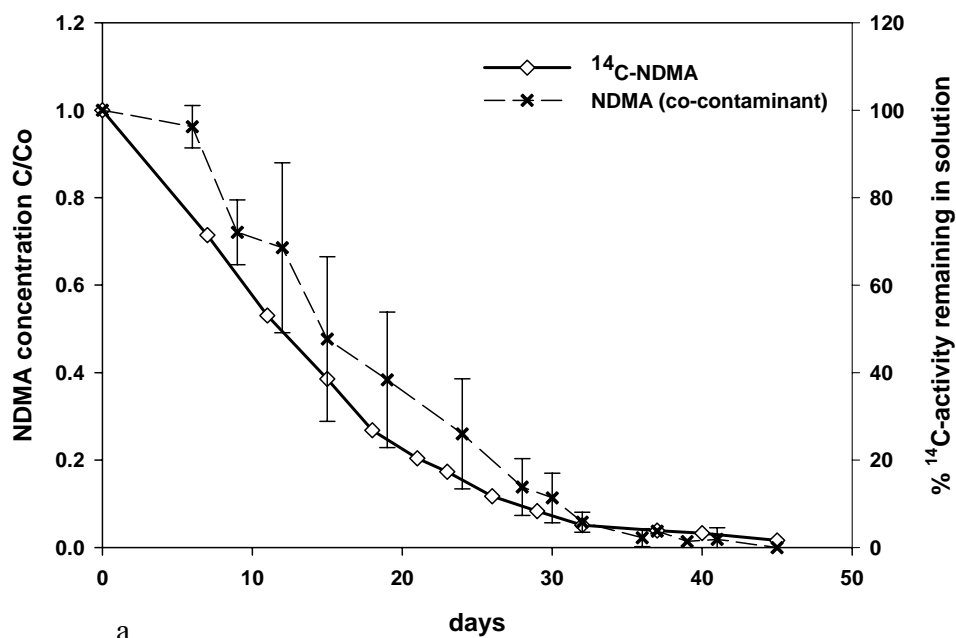


Figure 6.5. Uptake of NDMA by phreatophytes from hydroponic bioreactors containing only NDMA (¹⁴C-NDMA) and ¹²C-NDMA and perchlorate as co-contaminants (Figure 6.5a). The results in Figure 6.5b show the uptake and rhizodegradation of 27 mgL⁻¹ perchlorate from similar hydroponic bioreactors containing only perchlorate and NDMA as a co-contaminant. The data points represent average concentrations from 3 bioreactors \pm SD.

of perchlorate than trees grown on Miracle-Gro[®] solution (Table 6.3). This is because perchlorate persisted in the root zone solution in the presence of nitrate which is a competing terminal electron acceptor. Although the detection of chloride in plant leaves does not offer direct evidence of phytodegradation of perchlorate to chloride, the source of the chloride may be inferred for experiments conducted with Hoagland and Miracle-Gro[®] growth solutions. Despite the higher uptake fraction of perchlorate measured in experiments conducted with plants grown on Hoagland solution, there was an order of magnitude less chloride recovered in the plant leaves. The higher concentration of chloride detected in plants leaves grown with Miracle-Gro[®] nutrient solution is partly attributed to the uptake of potassium chloride contained in the Miracle-Gro[®].

The amounts of perchlorate phytoaccumulated in trees grown with perchlorate and NDMA were relatively lower than in trees grown with perchlorate only (Table 6.3). Unlike NDMA which was more evenly distributed in the leaves, stems and branches, 98.1 ± 0.3 % of the perchlorate fraction taken up into the plants was recovered in the leaves with only 0.2 ± 0.1 %, 1.3 ± 0.4 and 0.5 ± 0.4 % measured in the roots, stem and branches, respectively. Because perchlorate is more hydrophilic ($\log K_{ow} = -5.84$) than NDMA ($\log K_{ow} = -0.57$), it is easily translocated to the leaves where there is maximum transpiration. NDMA on the other hand is less hydrophilic than perchlorate and may be potentially conjugated to the various organic components of the plant. The TSCF of NDMA calculated for experiments with perchlorate as a co-contaminant was similar to values for experiments conducted with only NDMA. We concluded from the above observations that the removal of NDMA from groundwater primarily by uptake should not be affected by the presence of a co-contaminant. The results of this study provide evidence that phreatophytes, such as hybrid poplar and willow trees, can be used to

Table 6.3. Perchlorate concentrations in plant tissue of trees spiked with perchlorate and NDMA. Plant perchlorate concentrations are in mg kg⁻¹ dry weight.

Bioreactor	Treatment	Initial ClO ₄ ⁻ [mg L ⁻¹]	ClO ₄ ⁻ [mg kg ⁻¹]				Cl ⁻ [mg kg ⁻¹]
			Leaves	Stem	Branches	Roots	Leaves
P5 (Poplar) ^a	Perchlorate	25.1	1145.9	5.5	3.9	15.7	349.1
P6 (Poplar) ^a	Perchlorate	29.3	823.8	4.1	4.4	5.5	457.6
P7 (Willow) ^a	Perchlorate	27.0	1941.1	6.6	35.5	10.9	438.9
P8 (Poplar) ^b	Perchlorate	29.5	458.7	3.4	3.9	6.9	2963.6
P9 (Poplar) ^b	Perchlorate	25.3	673.0	3.1	3.7	3.7	4152.8
NP1 (Poplar) ^a	NDMA and Perchlorate	27.0	313.0	3.9	6.9	14.1	360.9
NP2 (Willow) ^a	NDMA and Perchlorate	26.7	899.5	2.7	6.1	6.0	1054.1
NP3 (Poplar) ^a	NDMA and Perchlorate	27.0	496.6	3.7	3.3	10.4	322.4
NP4 (Poplar) ^a	NDMA and Perchlorate	26.3	615.2	5.7	6.5	7.6	399.0

^a bioreactors with Hoagland® nutrient solution. ^b: bioreactors with Miracle-Gro® nutrient solution.

manage NDMA groundwater plumes at sites where phytoremediation is applicable. NDMA is removed from water primarily by passive plant uptake and phytovolatilization. High concentrations of nitrate and NDMA in the rhizosphere tend to inhibit the rapid rhizodegradation of perchlorate. There is no evidence that perchlorate-degrading bacteria used NDMA as carbon/electron source to rapidly degrade perchlorate.

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CHAPTER 7

CONCLUSION

This study has provided some of the first evidence that the rhizosphere can be manipulated to enhance rhizodegradation and minimize the uptake of perchlorate by plants. Under natural rhizosphere conditions, the slow buildup of organic carbon by root exudation and organic matter decay leads to a longer lag-phase during which perchlorate uptake and phytoaccumulation dominates. By providing external dissolved organic carbon sources to the rhizosphere, the lag-phase that precedes the faster rhizodegradation of perchlorate could be shortened and the amount of perchlorate accumulated in plant leaves could be reduced by an order of magnitude. One of the main findings of this study is development of a process to enhance rhizodegradation of perchlorate and minimize the undesirable uptake and accumulation of perchlorate in the food chain, which also reduces the potential for recycling of perchlorate in the ecosystem.

This research investigated the long term fate of perchlorate in vegetation collected from two sites where perchlorate was manufactured or used: LHAAP (Karnack, TX) and the LVW (Las Vegas, NV). All plant species from these sites contained detectable concentrations of perchlorate. The plant perchlorate uptake was influenced by many factors including: (1) the plant proximity to perchlorate source areas, (2) type of plant species, and (3) the maturity of the vegetation. Plants growing closer to the source of contamination contained higher perchlorate concentrations. Significant perchlorate uptake was observed in grass and salt cedar samples collected from LHAAP and the LVW, respectively. Senescent leaves and litter fall contained

higher perchlorate concentrations than the young leaves, implying slow degradation of perchlorate in plant tissue and possible recycling of perchlorate in the ecosystem. In order to curtail these environmental problems, biostimulation of rhizodegradation using DOC and harvest and subsequent bioremediation of the plant material should be included in the design of phytoremediation of perchlorate.

The extent to which NDMA is taken up and translocated into the above-ground portion of phreatophytes was also investigated. The NDMA is taken up by phreatophytes such as hybrid poplar and willow trees and transported into the foliar portion of the plants as a function of the volume of water transpired by these trees. The mass of NDMA removed from the bioreactors correlated linearly to the volume of water transpired, suggesting that the major decontamination mechanism for NDMA was passive plant uptake and phytovolatilization. Unlike perchlorate, rhizodegradation did not play a significant role in the removal of NDMA from the hydroponic bioreactors. The TSCF value for NDMA was 0.28 ± 0.06 , much higher than would be predicted from previously published models based on hydrophobicity as expressed by $\log K_{ow}$ values. The relationship between TSCF and $\log K_{ow}$ underestimates the TSCF values for highly water-soluble organic compounds and should be reevaluated. The results of this study provided the first evidence that phreatophytes such as hybrid poplar and willow trees can be used to manage NDMA groundwater plumes and NDMA contaminated soils at sites where phytoremediation is applicable.

The removal of NDMA is not influenced by the presence of perchlorate as a co-contaminant, because the main mechanism of NDMA removal is uptake and phytovolatilization. There is no evidence that perchlorate-degrading bacteria in the rhizosphere used NDMA as a

carbon or electron source to rapidly degrade perchlorate. Instead, the rate of rhizodegradation of perchlorate was slower when NDMA was present as a co-contaminant.

APPENDIX A

ENHANCEMENT OF MICROBIAL DEGRADATION OF PERCHLORATE (ClO_4^-) IN
THE RHIZOSPHERE¹

¹ Dawit D. Yifru and Valentine A. Nzengung, 2006. Reprinted with permission of the Battelle Press. Copyright 2005.

Abstract

Phytodegradation and rhizodegradation are the two predominant mechanisms by which plants metabolize perchlorate (ClO_4^-). Uptake and phytodegradation is the slower process and poses ecological risk because it involves uptake and temporal accumulation of perchlorate in plant leaves. Rhizodegradation uses perchlorate-respiring microbes and plant exudates/enzymes in the root zone to rapidly degrade ClO_4^- to innocuous Cl^- . In this study, rhizodegradation of perchlorate by willow trees (*Salix nigra*) was enhanced by biostimulation of the root zone perchlorate degrading microbes with dissolved organic carbon (DOC). Mushroom compost, chicken litter extract, and acetate were used as DOC sources. The concentration of DOC in solutions was about 500 mg L^{-1} . In reactors amended with DOC, perchlorate was completely degraded from an initial concentration of $23 - 30 \text{ mg L}^{-1}$ to below the IC method detection limit of $2 \text{ } \mu\text{g L}^{-1}$ in under 16 days. The average rate of perchlorate removal from the root zone was estimated as $2.41 \text{ mg L}^{-1} \text{ day}^{-1}$. In contrast, the rate of decontamination in the planted controls that received no DOC was as low as $0.13 \text{ mg L}^{-1} \text{ day}^{-1}$. Also, the concentration of perchlorate in plant tissues harvested from the control bioreactors is an order of magnitude higher than the DOC-amended bioreactors. This study has provided evidence that the addition of DOC to the root zone reduces the lag-time that precedes rhizodegradation of perchlorate by root zone microbes. The ecological significance of this research is that enhancing rhizodegradation minimizes the undesirable uptake and accumulation of perchlorate in plant leaves and possible recycling of perchlorate into the ecosystem. Rhizodegradation is a sustainable phytoremediation technologies that reduces clean-up time and costs.

1. Introduction

Environmental contamination by perchlorate (ClO_4^-) is a growing problem in the United States since the late 1990s. Recently, concern about the occurrence of perchlorate in drinking water, lettuce, dairy and breast milk is becoming a major public health concern. Perchlorate is produced when perchlorate salts such as ammonium, sodium or potassium perchlorate dissociate in aqueous solution. Over 90 % of perchlorate salts are manufactured as ammonium perchlorate (NH_4ClO_4) (Motzer, 2001), an oxygen-adding component in propellants for rockets, missiles and fireworks. Perchloric acid (HClO_4) and perchlorate salts are also used extensively in many commercial and industrial processes such as pyrotechnics, electronic tubes and car airbags, etc. Perchlorate also occurs naturally in Chilean caliche, a sodium nitrate fertilizer with perchlorate concentrations of about $0.5\text{--}2\text{ mg g}^{-1}$ (Urbansky et al., 2001). Contamination by perchlorate has been observed in surface water (such as the Colorado River and Lake Mead) and ground water at more than 44 states where perchlorate salts have been manufactured, processed or used. Perchlorate is also found in lettuce (Hogue, 2003) and dairy milk (Kirk et al., 2003). Recent study by Kirk et al., (2005) found perchlorate in breast milk at concentrations five times higher than in dairy milk.

The health concern of perchlorate is its effect on human thyroid iodide uptake. Perchlorate has similar ionic radius and charge to iodide and thus can competitively block iodide uptake by the thyroid gland. At relatively high doses, perchlorate is known to interfere with the thyroid's ability to produce hormones, required for fetal and neonatal neurodevelopment and may cause lower IQ and even mental retardation. In adults, deficiency of iodide may lead to hypothyroidism. Additionally, perchlorate contaminated surface and groundwater has ecotoxicological effects (Smith et al., 2004). Recently, the U.S. Environmental Protection

Agency (USEPA) set a reference dose for perchlorate of $0.0007 \text{ mg kg}^{-1} \text{ day}^{-1}$, which is equivalent to a drinking water level of $24.5 \text{ } \mu\text{g L}^{-1}$ (USEPA national news, 2005).

Perchlorate salts are readily soluble in water and produce ClO_4^- upon dissociation. The negative charge is dispersed evenly over the four oxygen atoms, which makes perchlorate stable (Espenson, 2000). As a consequence, water treatment technologies such as carbon adsorption and binding to positively charged ions and precipitation are not cost-effective for removal of perchlorate to current drinking water levels. Treatment technologies that have shown great promise as low-cost sustainable technologies for treatment of perchlorate-contaminated soil and water are anoxic bioremediation and phytoremediation.

Bioremediation has been identified as among the most effective methods for remediation of perchlorate-contaminated soils and water (Urbansky, 1998; Losi et al., 2002). For effective bioremediation, perchlorate degrading microbes, anaerobic environment and adequate supply of carbon and electron sources are necessary (Rikken et al., 1996) Coates et al., (1999) showed that perchlorate reducing microbes are ubiquitous. Microorganisms use dissolved organic carbon (electron donor) for growth and utilize perchlorate anions as terminal electron acceptors. In the natural environment, the source of organic carbon could be soil organic matter and exudates of plant roots such as ethanol, acetate, organic acids, sugars, and dead root biomass. Under suitable environmental conditions, biodegradation of perchlorate proceeds sequentially via chlorate (ClO_3^-), chlorite (ClO_2^-) to innocuous chloride (Cl^-) (Ginkel et al., 1996; Rikken et al., 1996).

Phytoremediation is a potentially promising bioremediation technology to remove perchlorate from soils, surface water and groundwater. Nzengung and McCutcheon (2003) have shown that many species of terrestrial and aquatic plants have the ability to remove perchlorate from contaminated water and soils. Aken et al., (2002) have also shown that poplar trees were

able to reduce perchlorate from 25 mg L⁻¹ initial concentration by 50 % after 30 days of incubation. The two major mechanisms by which plants decontaminate perchlorate are phytodegradation and rhizodegradation (Nzengung et al., 2004). Uptake and phytodegradation is a slower process and poses ecological risk because it involves temporal accumulation of perchlorate in plant tissue. This process is responsible for the presence of detectable concentrations of perchlorate in lettuce grown with perchlorate-contaminated irrigation water. Furthermore, uptake of perchlorate by vegetation grown over contaminated sites and consumed by cows and humans may be one route by which perchlorate got into dairy and breast milk (Kirk et al., 2003; 2005). Rhizodegradation on the other hand, is a desirable process because it uses anaerobic microbes and plant exudates/enzymes in the root zone to rapidly degrade ClO₄⁻ to Cl⁻ while minimizing uptake of perchlorate into the plant tissues, mainly leaves.

This study has been initiated to develop a process that minimizes the potential ecotoxocological risks posed by plant uptake and temporal phytoaccumulation of perchlorate during phytoremediation. Specifically, we investigated the efficacy of natural and artificial dissolved organic carbon (DOC) sources such as extracts of mushroom compost and chicken litter, ethanol and acetate, to (a) enhance biodegradation of perchlorate in the rhizosphere, and (b) reduce the undesired uptake and temporal phytoaccumulation of perchlorate in plant tissues.

2. Materials and Methods

2.1. Chemicals

Sodium perchlorate monohydrate (NaClO₄·H₂O) was purchased from Aldrich Chemical Company (Milwaukee, WI). Sodium Acetate Trihydrate (CH₃COONa·3H₂O) and sodium

hydroxide (NaOH, 50 % w/w) solution were obtained from J.T.Baker™ (Phillipsburg, NJ). Perchlorate standard solutions were purchased from AccuStandard™ (New Haven, CT) and SPEX CertiPrep (Metuchen, NJ). Peters Professional® was obtained from local plant nursery. Almatris Ac. Inc. provided DD-6 alumina sorbent with 48×100 U.S. screen mesh and surface area of $360 \text{ m}^2 \text{ g}^{-1}$.

2.2. Carbon and Electron Sources

The DOC source was ethanol, acetate, mushroom compost and chicken litter extracts. Mushroom compost leachate, marketed as 100 % organic compost tea, was obtained from Advantage Organic Products, Duncanville, TX. The organic compost tea was centrifuged at $1000 \times g$ for 30 minutes and filtered with a $0.45 \mu\text{m}$ filter (Pall Corp., Ann Arbor, MI). Chicken litter extract solution was prepared by mixing 100 g of dry chicken litter with 1 L of deionized water. After shaking the mixture overnight and centrifugation at $1000 \times g$ for 30 minutes, the supernatant was filtered through a $0.45 \mu\text{m}$ filter paper. To prepare 500 mg L^{-1} DOC, mushroom compost and chicken litter extracts were diluted 5 and 20 times, respectively, with deionized water. DOC in solution was measured with a Shimadzu 5050 Total Organic Carbon (TOC) analyzer.

2.3. Experimental Setup

The selection of willow trees for this study is due to their robustness and high rate of survival under greenhouse conditions. Several willow tree (*Salix nigra*) cuttings, harvested from

University of Georgia garden were pre-rooted in hydroponics growth media under natural light in a greenhouse at about 25 °C. The pre-rooted trees were transferred to a 2 L Erlenmeyer® flasks wrapped with aluminum foil and containing 0.39 percent diluted Peters Professional® plant growth solution. The volume of water in the reactors was maintained at 2 L by adding nutrient solution dissolved in deionized water. The volume of water transpired by plants from each reactor and the weight of plants were recorded through out the course of the experiment.

At the beginning of the experiment, willow trees were planted in water containing 25 mg L⁻¹ perchlorate. The experiments were repeated with perchlorate-contaminated groundwater collected from Karnack, Texas. The groundwater contained 40 mg L⁻¹ perchlorate. Each planted bioreactor was made to contain 500 mg L⁻¹ DOC solution of either of the amendments. Duplicate planted bioreactors were prepared for each DOC amendment and control. A 1.5 ml sample of rhizosphere solution was taken from each flask until the concentration of perchlorate decreased to below the ion chromatography (IC) detection limit of 2 µg L⁻¹. At the termination of the experiment, plants were harvested for perchlorate analysis. For perchlorate extraction from plant tissues, the procedure of Ellington and Evans (2000) was followed.

2.4. Sample Preparation and Ion Chromatography

The samples were analyzed on a Dionex® DX-500 Ion Chromatograph (IC) outfitted with an IONPAC® AG16 guard column (4 × 50 mm) and an IONPAC® AS16 analytical column (4 × 250 mm). An autosampler with a holding capacity of sixty 5 ml vials was used. The system was run using an ASRS-ULTRA II Self-Regenerating Suppressor (4 mm) at a 300 mA setting. A 100 mM and 50 mM sodium hydroxide (NaOH) eluent at a flow rate of 1 mL min⁻¹ and a 500 µl

sample loop were used to measure perchlorate in mg L^{-1} and $\mu\text{g L}^{-1}$ levels respectively. The eluent was made using J. T. Baker® 50 % (w/w) solution and deionized, degassed (in the VWR Scientific Aquasonic, model 150D) water. Calibration and check standards were made by diluting $1000 \mu\text{g mL}^{-1}$ perchlorate anion standard (SPEX CertiPrep, Inc.®) and $0.5 \mu\text{g mL}^{-1}$ and $1 \mu\text{g mL}^{-1}$ standards (AccuStandard, Inc.®). A new calibration curve was created each time the ion chromatograph was turned on, or after the eluent had been changed (every 1-2 days). For quality control, all samples were run in duplicate, and an external standard and a blank were run after every two samples. The standard was used to ensure that the percent error remained below 5 %, and to monitor any instrumental drift, while the blank checked for any carry-over from the previous sample. To confirm the perchlorate peak in plant tissue extracts, some duplicate samples were spiked with a solution containing $100 \mu\text{g L}^{-1}$ perchlorate.

3. Results and Discussion

Of the four types of DOC sources, only ethanol applied at concentration of 500 mg L^{-1} DOC from ethanol caused plant mortality within 7 days from the start of the experiments. The estimated daily average tree growth for the rest of the bioreactors showed that there was no significant difference in plant biomass between trees grown with and without DOC. This implies that at a dosing of 500 mg L^{-1} DOC, neither of the amendments was phytotoxic to the willow trees. The volume of water transpired by trees during the experiment was also similar for planted bioreactors amended with and without DOC. The total organic carbon (TOC) measurement taken at the beginning and end of the experiment indicated a decrease in TOC with time.

3.1. DOC Amended Bioreactors

In reactors dosed with 500 mg L⁻¹ DOC from acetate, perchlorate was completely removed from an initial concentration of 30 mg L⁻¹ to below detection limit within 16 days (Figure A-1). In acetate amended bioreactors, there was no lag time preceding the faster microbial rhizodegradation. The rate of perchlorate removal from solution amended with acetate was 2.25 mg L⁻¹ day⁻¹. The average concentration of perchlorate in plant leaves harvested from 500 mg L⁻¹ acetate amended reactors was 17.29 mg kg⁻¹ (fresh weight, FW) (Table A-1). For the mushroom compost and chicken litter experiments, perchlorate was completely removed from solution within 9 days (Figure A-2). The slow initial decrease in perchlorate concentration was followed by much faster kinetics. For mushroom compost and chicken litter-amended bioreactors, the initial rate of perchlorate removal was 0.39 and 0.57 mg L⁻¹ day⁻¹ respectively. After 5-7 days however, biostimulation of rhizodegradation was achieved with an average degradation rate constant of 5.5 mg L⁻¹ day⁻¹ and 9.6 mg L⁻¹ day⁻¹ for mushroom compost and chicken litter extracts, respectively. The average concentration of perchlorate measured in plant leaves harvested at the end of the experiment from mushroom compost and chicken litter-amended reactors was 32.58 mg kg⁻¹ and 32.88 mg kg⁻¹ FW respectively (Table A-1). Because of the lag time preceding rhizodegradation in mushroom compost and chicken litter amended bioreactors, the concentration of perchlorate in the leaves from these bioreactors is about two-fold that of acetate amended bioreactors (Table A-1). The concentration of perchlorate in various tissues of plants showed that the majority of perchlorate is accumulated in the leaves. In the DOC supplied reactors, more than 70% of the total perchlorate was recovered in the leaves while 29% was recovered in the stems and less than 1% was recovered from the roots.

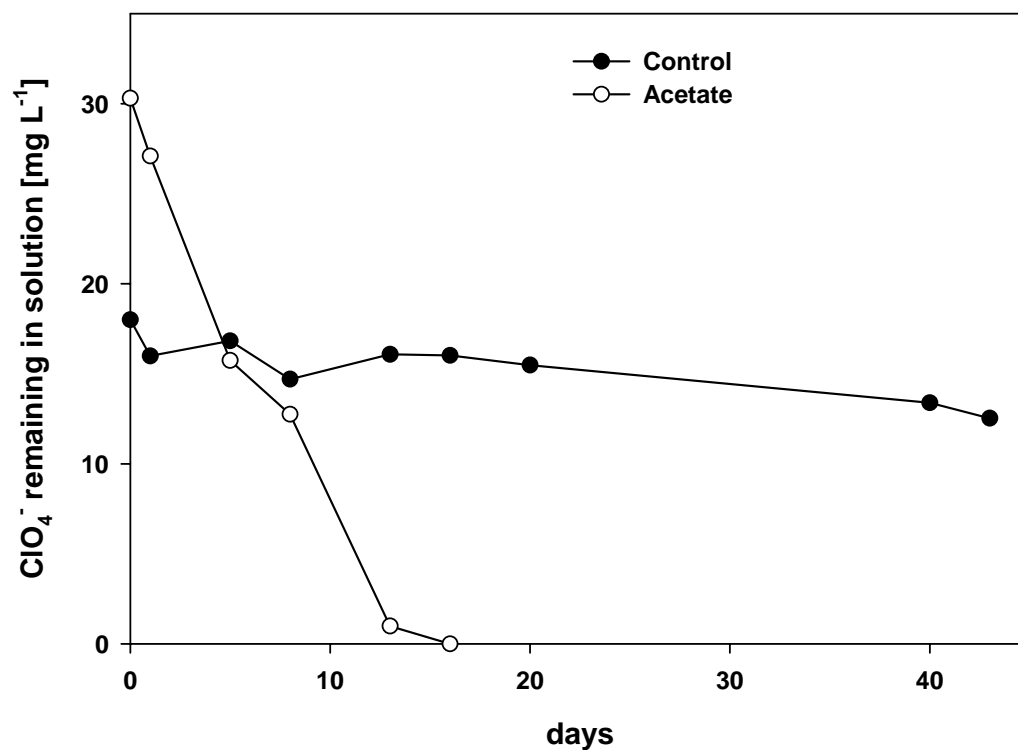


FIGURE A-1. Perchlorate concentration in solution as a function of time in bioreactors supplied with 500 mg L⁻¹ DOC from acetate. The control bioreactor contained a willow plant but no DOC.

TABLE A-1. Concentration of perchlorate removed from solution and phytoaccumulated in willow leaves. CL: chicken litter, MC: mushroom compost, A: acetate, C: control. All plant leaves except C were harvested after all the perchlorate in solution was completely degraded.

Treatment	Days plants exposed to ClO_4^- before leaf tissue harvested	Initial concentration of ClO_4^- in bioreactor [mg L^{-1}]	ClO_4^- concentration remaining in solution at harvesting [mg L^{-1}]	ClO_4^- concentration in leaves [mg kg^{-1} FW] (Fresh weight)
CL	9	23	Below detection	32.88
MC	9	24	Below detection	32.58
A	16	30	Below detection	17.29
C	8	18	14	116.35

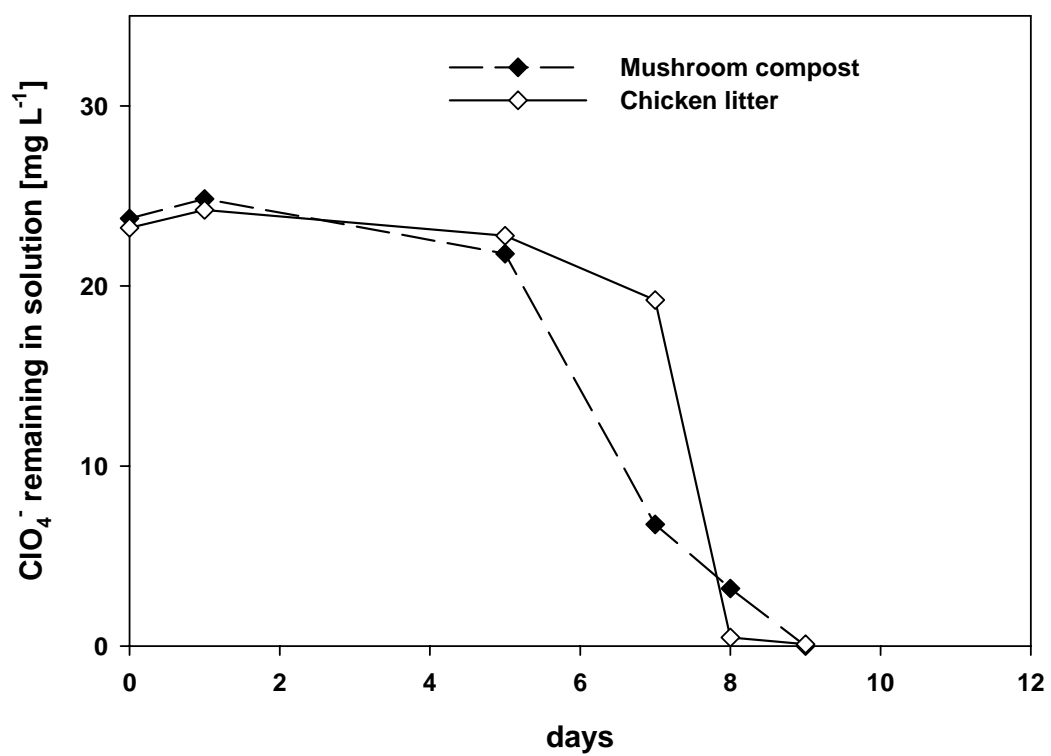


FIGURE A-2. Perchlorate degradation in planted bioreactors supplied with diluted mushroom compost and chicken litter extracts.

3.2. Controls

In the planted controls where no DOC was added, the rate of perchlorate removal was slow process (less than $0.13 \text{ mg L}^{-1} \text{ day}^{-1}$). The slow uptake and phytodegradation persisted longer than those supplied with DOC (Figure A-1). This resulted in higher concentration of perchlorate in plant tissue than DOC supplied reactors (Table A-1). For example, in a control bioreactor where the concentration of perchlorate reduced by only 4 mg L^{-1} after 8 days of the experiment, the concentration of perchlorate accumulated in the leaves was more than $116 \text{ mg kg}^{-1} \text{ FW}$. In the controls, 99 % of the total perchlorate was recovered in the leaves whereas only 1 % was recovered in the stem and roots.

3.3. Uptake and Phytodegradation

Generally, willow trees were observed to remove perchlorate from aqueous solution. In the planted controls where no DOC was added, the concentration of perchlorate in willow leaves increased with time (Figure A-3). The initial phase due to uptake via the transpiration stream of the trees and phytodegradation is characterized by slower kinetics. The presence of perchlorate and its metabolites (chlorate, chlorite and chloride) in the harvested leaves has provided evidence for uptake and phytodegradation of perchlorate (Nzengung et al., 2004). Transformation of ClO_4^- to Cl^- in plant leaves is a slow process relative to rhizodegradation. This results in temporary accumulation of perchlorate in plants which poses potential ecological risk. For example, perchlorate was found in California lettuce, grown with Colorado River irrigation water, which carries perchlorate from a former industrial plant near Las Vegas (Hogue, 2003). Cattle raised on

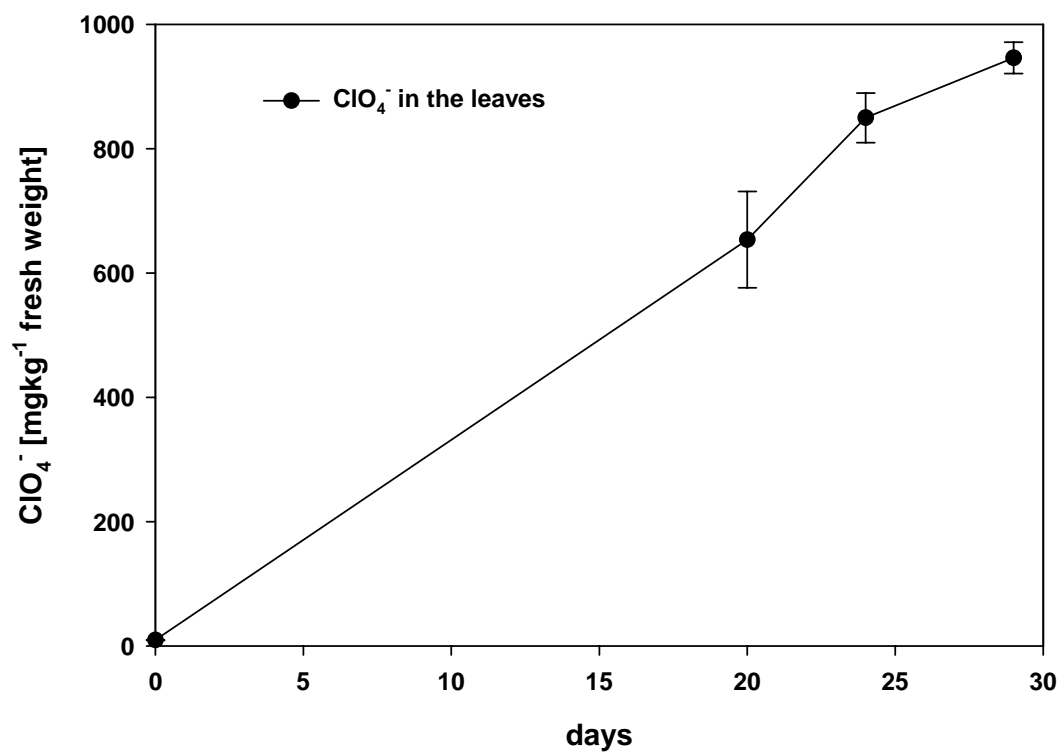


Figure A-3. The concentration of perchlorate in willow leaves increased with time. Therefore, enhancing rhizodegradation is the solution to minimize uptake and phytoaccumulation.

perchlorate-impacted areas potentially ingest large quantities of perchlorate containing leaves and grass. As a result, dairy and beef products from these areas are potential pathways to expose humans to perchlorate. Kirk et al., (2003) showed the presence of perchlorate in all seven milk samples purchased randomly in Lubbock, Texas. In addition, uptake and phytodegradation potentially recycles perchlorate in plant-soil system as the degradation of perchlorate in plant leaves is a slow process. Due to the latter potential ecotoxicological concerns, uptake and phytodegradation is not a desirable process.

3.4. Enhancement of Rhizodegradation

Rhizodegradation is a faster process and uses microbes to completely transform ClO_4^- to Cl^- without posing any ecological risk. Rhizodegradation is preceded by biostimulation when dissolved oxygen decreases to about 0.1 mg L^{-1} (Nzengung et al., 2004), nitrate if present has been mostly used up, anoxic or anaerobic zones are formed, and a critical mass of perchlorate degrading bacteria is present in the root zone. Unless rhizodegradation is biostimulated, uptake followed by slow phytodegradation would predominate and result in phytoaccumulation of higher concentrations in the plant leaves (Figure A-3). DOC in the rhizosphere is important because it is metabolized by bacteria rapidly while using up oxygen and creating favorable anoxic conditions for perchlorate reduction. Additionally, metabolization of DOC provides electron for perchlorate-respiring microbes to use perchlorate as a terminal electron acceptor. Under natural environmental conditions, the concentration of DOC from plant exudates is not sufficient for microbial growth. Addition of DOC-containing substances such as acetate, chicken litter and mushroom compost extracts are of major importance to microbial growth.

Among the factors that slow down rhizodegradation is the presence of nitrate and/or oxygen in the root zone. Nitrate has been to compete with perchlorate as a terminal electron acceptor (Nzengung et al., 1999). The concentration of nitrate in diluted chicken litter and mushroom compost solutions was observed to be below the method detection limit of 0.2 mg L^{-1} . Therefore, biostimulation using these DOC sources does not inhibit perchlorate degradation. The amount of heavy metals in diluted chicken litter extract and mushroom compost is lower than EPA's maximum contaminant level, except the $87.2 \text{ } \mu\text{g L}^{-1}$ arsenic found in diluted chicken litter solution. It is important to monitor the concentration of undesired and regulated chemicals in any DOC source prior to use to avoid future liabilities from contamination of soils and water resources.

4. Conclusions

This study has provided some of the first evidence that the rhizosphere environment can be manipulated to enhance rhizodegradation and minimize the uptake of perchlorate into plant tissues. Under natural rhizosphere conditions, the slow build up of DOC by root exudation allows for uptake and slow phytodegradation. The persistence of the latter process explains the observed phytoaccumulation of perchlorate at higher concentrations in the control plants. Meanwhile, providing an external DOC source to the root zone reduces the lag-time that precedes rhizodegradation of perchlorate by root zone microbes. As a result, the concentration of perchlorate accumulated in plant tissue reduces significantly. The ecological significance of this research is that enhancing rhizodegradation minimizes the undesirable uptake and accumulation of perchlorate in plant tissue and the potential for recycle of perchlorate back into the ecosystem.

Enhancement of rhizodegradation also shortens the clean-up time, is sustainable and cost-effective.

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