PHYTOREMEDIATION OF PERCHLORATE CONTAMINATED SOILS AND WATER

COMPREHENSIVE PROJECT REPORT FOR COOPERATIVE AGREEMENT BETWEEN

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EXECUTIVE SUMMARY

For five years, a multitude of phytoremediation of perchlorate studies were conducted at the University of Georgia in Athens, Georgia, with funding provided by the Wright Patterson AFB in Dayton, Ohio. The data collected so far indicates that, selected woody, edible, and aquatic plants and microbial mats can be used to detoxify environments contaminated with perchlorate. In hydroponics systems, the initial slow uptake and phytodegradation of perchlorate by plants exposed to perchlorate changed to very rapid removal by rhizodegradation after several days depending on the plant physiology and root zone environmental conditions. The prolonged exposure of rooted green plants to perchlorate-dosed media stimulated the growth of perchlorate-degrading microorganisms in the rhizosphere. Exudates secreted by the plant roots supplied nutrients that sustained the growth of rich and diverse consortia of bacteria among which are perchlorate-degrading bacteria. In the presence of isolated and identified root-colonizing bacteria, perchlorate is rapidly degraded to chloride in the rhizosphere, minimizing uptake into the tree leaves and branches.

The rhizodegradation of perchlorate is influenced by both the concentration of nitrate in the growth media and the N-source. The fastest perchlorate degradation kinetics were observed in planted bioreactors with lower NO_3^- concentrations of <200 mg L^{-1} and slowest kinetics at higher NO_3^- concentration of 300 - 600 mg L^{-1}. The influence of NO_3^- on perchlorate reduction was attributed to competing reactions in which rhizosphere bacteria preferentially utilized NO_3^-, as terminal electron acceptor (TEA). This shortcoming was overcome by replacing the NO_3^- N-source (Hoagland's solution) with an ammonium and urea N-source (i.e., Miracle-Gro). As a result, perchlorate was rapidly reduced in the rhizosphere of the willow trees grown in miracle-Gro. Uptake of perchlorate by woody plants was minimized in medium with ammonium and urea serving as the N-sources.

The results of experiments conducted with ^{36}Cl perchlorate provided more direct evidence that the rapid removal of perchlorate ions from the rhizosphere of plants under anaerobic conditions is due to rhizodegradation. In experiments where diluted Miracle-Gro® (urea/ammonium N-source) was used as the growth media, the mass balance based on the ^{36}Cl activity in the root zone solution was closed at better than 95%. However, a lower recovery (80%) of ^{36}Cl activity in solution was observed if a nitrate nitrogen source (Hoagland solution) rather than a urea/ammonium nitrogen source (Miracle-Gro®) was used as the growth solution. The poor rhizosphere mass balance obtained in experiments with Hoagland solution was attributed to the greater uptake of perchlorate ions by plants in the presence of nitrate N-source. Also, the rate of rhizodegradation of perchlorate ions was faster for trees grown in Miracle-Gro® than those grown in Hoagland solution. Using ^{36}Cl labeled perchlorate it was confirmed that any perchlorate taken up into the green plants is not simply accumulated, but slowly transformed.

This study has obtained some of the first evidence on the efficacy of phytoremediation as a potentially effective technology for remediation of mixed contaminant plume containing perchlorate and trichloroethylene (TCE) and perchloroethene (PCE). It was observed that perchlorate was rapidly rhizodegraded to chloride while TCE and PCE were mostly taken up and phytovolatilized by the trees. Rhizodegradation of perchlorate was confirmed by the higher rate of perchlorate removal from solution and the formation of chlorate and chloride as an

intermediate and final product, respectively. The very small concentration of reductive dechlorination products (dichloroethenes (1,1-DCE and cis-DCE) and vinyl chloride (VC)) and chloroacetic acids detected in the root zone solution suggested that rhizodegradation did not account for the removal of a significant fraction of the parent compounds, TCE and PCE, under the greenhouse experimental conditions.

For phytoremediation studies conducted in soil and sand bioreactors maintained in the greenhouse and under natural conditions, the performance of the "controls" in both the bucket and drum studies proved that plants need not be present for perchlorate degradation to take place in a carbon- and nutrient-rich anaerobic environments. The results of the controls also attested to the importance and efficiency of microbial activity in the degradation of perchlorate.

A pseudo-first-order model described the kinetic data gathered from studies performed using planted and unplanted five gallon buckets in the green house. However, some of the later dosings exhibited zero-order kinetics, which led to the suggestion that with prolonged exposure to perchlorate, the number of perchlorate-reducing microorganisms reach a critical mass (provided favorable environs) that enables them to degrade perchlorate more proficiently.

The plant tissue analyses from the bucket and drum studies (simulating natural conditions) established that plants remove perchlorate from contaminated water through rhizodegradation, phytoaccumulation and phytodegradation. Although the phytoaccumulation and phytodegradation take place simultaneously, the plants examined in this study, accumulated perchlorate much more rapidly than they were able to degrade it within their tissue.

The drum study confirmed that the results of the bucket study could be achieved in a less controlled, outdoor environment, although the drum systems did tend to exhibit slower kinetics than the buckets. The "simulated wetland" drum showed the fastest perchlorate removal from the contaminated water, probably due to a combination of anaerobic microbial activity and hydrophyte phytoaccumulation. The five anion and oxidation-reduction potential measurements confirmed that chlorate did not accumulate within the drums during anaerobic perchlorate reduction.

The microbiological study showed that individual strains of bacteria, which have been isolated from the root zones of willow trees used in this study, are capable of perchlorate degradation. Specifically, one of four bacteria isolated from the rhizosphere of willow trees degraded perchlorate and most likely mediated the observed rhizodegradation reactions. Recent developments in microbial ecology; such as DGGE, (Terminal Restriction Fragment Length Polymorphism) (TRFLP) and total community 16S rDNA hybridization) were applied to provide a rapid means of evaluating the entire microbial community structure. T-RFLP allowed snapshots (fingerprint) of entire bacterial communities in the rhizosphere media to be compared after polymerase chain reaction (PCR) amplification of the extracted DNA using primers specific for the 16S rRNA in all eubacteria. The results obtained in this study suggest that as rhizodegradation of perchlorate progresses there is a shift in the microbial community in favor of perchlorate degrading bacteria populations.

1. Hydroponic Experiments

1.1. Proof of Concept Hydroponic Tests

The discovery that perchlorate can be remediated by plants was initiated during an ongoing collaborative study between the USEPA National Exposure Research laboratory and the University of Georgia, funded by Wright Patterson AFB in Dayton, Ohio. The initial objective of the research project was to provide a better understanding of phytoremediation of perchloroethylene (PCE) and trichloroethylene (TCE) in support of a demonstration/validation field study at Carswell AFB in Fort Worth, Texas. Motivated by the growing concerns on the recent detection of perchlorate in public drinking water in mostly western states, the US Air Force Wright Patterson AFB in Dayton, Ohio, requested that Dr. Nzengung of the University of Georgia evaluate the possible use of plants in remediation of perchlorate-contaminated water. In response to this request, Dr. Nzengung screened a number of plants for their suitability in phytoremediation of perchlorate, as describe below.

Plants and Other Photoautotrophs used in study
* Eastern Cottonwood …… Carswell AFB, Texas
* Willow (*Salix ssp*.) …… Carswell AFB, Texas, Athens, Georgia
* Willow (*Carolianana*) …… Tampa, FL
* *Eucalyptus cineria* …… Local Nursery, Athens, GA
*** Myriophyllum aquaticum ….. *Local Wetland, Athens, GA***
* *Artemisia dracunculus* …… Local Nursery, Athens, GA
(French tarragon)
* Spinach
* Green Algae
* Constructed Mixed-species microbial mats

1.2. Methodology of Screen Tests.

Perchlorate ion uptake by woody plants was investigated in 2-liter glass reactors with side sampling ports. One cutting was grown in each sand bioreactor. Pristine sand was placed at the bottom of each reactor to support the tree roots so that the branches and leaves were outside of the reactor. The sand occupied about half of the reactor volume. The growth solution was 50% of full strength Hoagland's solution and was spiked with a stock perchlorate solution to make an initial perchlorate concentration of 20 and 100 ppm, respectively, as ClO4-. This concentration is about two orders of magnitude higher than that reported for most perchlorate contaminated sites. Total volume of liquid media in each reactor was 600 - 900 mL. The reactor was wrapped in a sheet of PARAFILM® and the solution portion of the reactor was shielded from the light by aluminum foil. For a few initial experiments conducted in early Spring 1998, 65 watts plant growth lights illuminated the trees for 12/12 hours day and night cycle in the laboratory. Experiments conducted in summer months were located outside where the plants were exposed to natural environmental conditions. The water in the reactor was kept at a constant level by replenishing the medium lost by evapotranspiration with a known volume of half-strength Hoagland's solution. This necessitated adding diluted Hoagland's solution twice or thrice a day to each reactor. A daily record of the water uptake by each plant was maintained over the course of each study. A 1-mL aliquot was taken every day from the sampling port and was replaced

with an equivalent volume of half strength medium. The sample was diluted with deionized water to the IC measurement range of the perchlorate ion.

The distribution of perchlorate in different plant fractions was determined by sacrificing some of the study plants for extraction and analysis. At the end of each study the plant was removed from the medium, rinsed with deionized water and sectioned into roots, lower stem, upper stem, branches and leaves. Each fraction was extracted several times by blending for 30 - 60 min with a solution of 1 mM NaOH (pH = 11). The extract was separated from the aqueous-plant phase by centrifugation. The number of extractions needed to ensure complete removal of the extractable perchlorate and its analogs was dependent on the plant fraction and type of plant. On average three extractions were needed to completely extract the extractable perchlorate from the respective ground plant organs. The extract was analyzed for perchlorate, chlorate, chlorite and chloride ions. All experiments with each plant species were replicated.

Perchlorate ion uptake by herbs and microbial mats was investigated in 60-mL serum bottles. French tarragon and spinach were each minced before used in the perchlorate studies. One gram of the minced plant was added to 20-mL vials. The remaining space in the sampling vials were filled with deionized water and dosed with perchlorate to obtain an initial solution concentration of about 10 mg/L. The vial were mixed on a rotary shaker and sacrificed for analysis at predetermined intervals. Pieces of the wet mats were weighed into the vials and the headspace filled with deionized water, dosed with perchlorate to obtain an initial solution concentration of 10 mg/L and mixed on a rotary shaker. The pellets were separated from solution by centrifugation, and the liquid-phase was analyzed by IC. A total of 9 sample vials were used in each of the replicate experiments. Controls contained no plant matter and were dosed with the same concentration of perchlorate as the samples.

1.3. Results of Screen Tests.

Rooted cuttings of three woody plants (willow [*Salix spp*.], Eastern Cottonwood [*poplar*] *Eucalyptus cineria*) were observed to take up perchlorate ions from aqueous solution (Figure 1). When the plants were initially exposed to the perchlorate medium, the rate of perchlorate uptake was proportional to the water uptake rate of each plant. Figure 7 and Tables 1 – 3 show that the one-year-old Eucalyptus plant had the largest fraction root and leaf of 6.6 and 46%, respectively, and took up perchlorate from the nutrient medium at the fastest rate. The evapotranspiration rate of the Eucalyptus was also higher than that of the cottonwood and willow plants used in the latter study. The perchlorate concentration in the unplanted reactor did not change over the course of this study.

The results presented in Figure 1 also show that when each of the three plants was first exposed to perchlorate in the planted bioreactors, three distinct reaction phases could be distinguished. Phase 1 was described by a rapid decrease in perchlorate concentration in the medium, which was proportional to the volume of water transpired by each plant. In Phase 2, the progressive increase in water uptake was not accompanied by any significant loss of perchlorate from solution. Phase 2 persisted in studies with cottonwoods up till the conclusion of the experiment when the plants were sacrificed for extraction and analysis. Meanwhile, in Phase 3, a very rapid

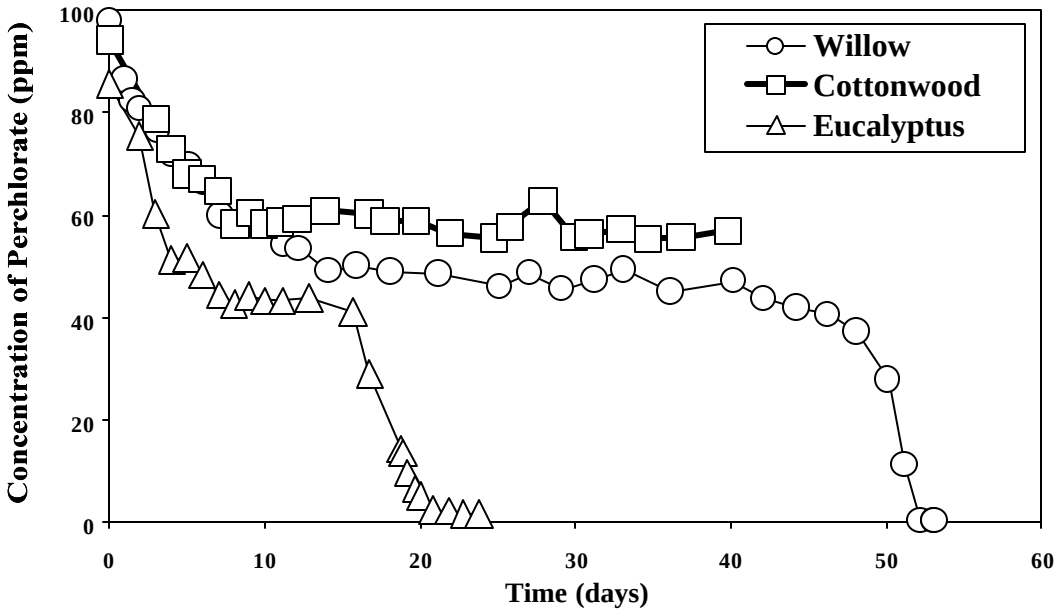


Figure 1. Removal of perchlorate from aqueous solution by cottonwood, willows and Eucalyptus trees.

decrease in perchlorate concentration in the medium was observed, but not proportional to water uptake by the plants. The disappearance of perchlorate from solution was initially described by first-order kinetics (i.e. data represented by Phase 1 and 2), which changed to zero order (Phase 3) towards the end of the run. The plateau in the data (Phase 2) was apparently attributed to higher ionic strength of nitrate in the medium. Since the half-strength Hoagland's solution was continuously added to the reactor to make up for the transpired water the rate of nutrient addition probably exceeded its utilization rate and nitrate out-competed perchlorate as the dominant terminal electron acceptor.

When the rhizosphere nitrate concentration in the willow bioreactor was diluted from 262 ppm to below 100 ppm a rapid decrease in perchlorate concentration in the medium was observed. This initial observation led us to suggest that the remediation of perchlorate by plants could be affected by the ionic strength of nitrate in the nutrient. As a result a number of subsequent studies were designed to investigate the effect on nitrate concentration and nitrogen source on phytoremediation of perchlorate. It is also possible that as the concentration of perchlorate increased in leafs to toxic levels the plants developed mechanisms to resist further uptake of perchlorate until the accumulated fraction had been detoxified. It is not clear from this study which of the latter processes caused the plateau observed in studies with all three plants. Because the control and sample plants continued to grow at the same rate, there is no clear evidence that the perchlorate concentration used in this study was toxic to the cottonwood, Eucalyptus or willow.

The distribution of perchlorate in the pore water in the sand layer, the liquid medium, and in various organs of the cottonwood, Eucalyptus and willow sacrificed for analysis at the

termination of the kinetic studies shown in Fig 1 is presented in Tables 1. The aqueous perchlorate concentration is higher because of the higher concentration recovered from the sand layer which was not in direct contact with the plant roots. Sorption studies confirmed that perchlorate was not sorbed by the sand. This means that the higher perchlorate concentration measured in the sand layer was due to dissolved perchlorate trapped in the pore water. If uptake was the predominant mechanism responsible for perchlorate loss from the bioreactor at that point, then the slow diffusion of perchlorate ions to the root zone should be the limiting process.

Table 1: Distribution of perchlorate in screen test woody plants grown in sand bioreactors for 26 days.

TREE TYPE	**LOCATION**	**MASS** (mg)	**PERCENT**
Cottonwood	Roots & Stems	0.1	0.2
	Leaves & branches	4.2	4.9
	Bioreactor	78	92
	Phytoremediated	**2.6**	**3.1**
Eucalyptus	Roots & Stems	0.007	0.009
	Leaves & branches	22.8	29.6
	Bioreactor	23.3	30.2
	Phytoremediated	**31.1**	**40.2**
Willows	Roots & Stems	0.6	0.7
	Leaves & branches	0.5	0.6
	Bioreactor	76.2	87.7
	Phytoremediated	**9.5**	**11**

Additional studies were performed in bioreactors with perchlorate-dosed medium and no sand (Figure 2). Two phases of perchlorate removal from solution were observed in the latter study: a slower initial step attributed mostly to uptake followed by a rapid decrease attributed to both uptake and rhizodegradation.

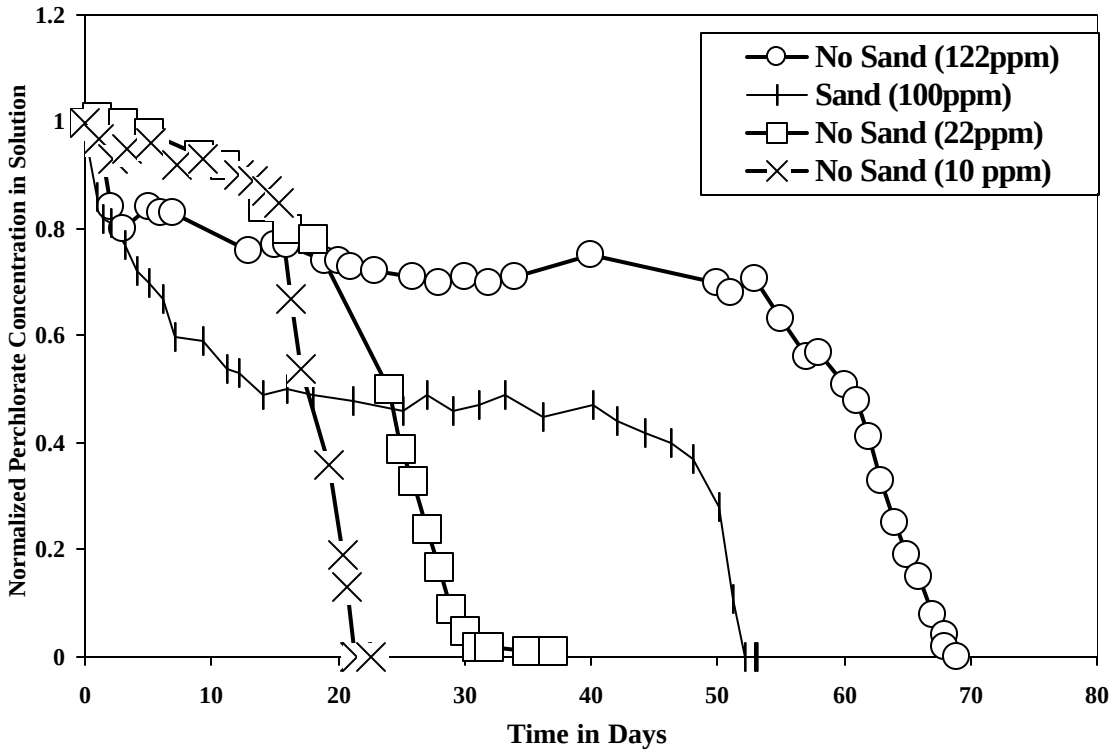


Figure 2. Removal of perchlorate from sand and hydroponics bioreactors by willow trees (pH 5.6 and nitrate <200 ppm).

Comparing the perchlorate concentration (mass/mass) extracted from the different plant organs, it is evident that the perchlorate taken up by all three woody plants was mostly accumulated in leafs and branches. The relatively small amount of perchlorate measured in the roots and lower stem (Tables 1) suggests that the perchlorate was not accumulated in these parts of the woody plants. The mass balance data in Table 2 shows that the Eucalyptus tree was relatively more effective in the uptake, accumulation and transformation of perchlorate than the cottonwood and willow. The 42% of unrecovered perchlorate in studies with Eucalyptus plants was assumed degraded to chloride or irreversibly bound to the plant tissue. The concentration of perchlorate detected in extracted fresh leafs of a willow tree dosed five times with perchlorate was 261 μg/g and 755 μg/g in leafs showing senile properties whereas the chloride concentration was 226 and 803 μg/g, respectively. We speculate that once in the tree leafs, the perchlorate ions were very slowly transformed by deoxygenase or reducing plant enzymes.

Direct evidence of phytodegradation was obtained from experiments in which the crude extracts and minced French tarragon and spinach were used to degrade perchlorate. Figure 3 shows representative results of perchlorate degradation by minced French tarragon. The degradation of perchlorate by the crude extract and minced herbs provided additional evidence the reactions were enzymatically catalyzed.

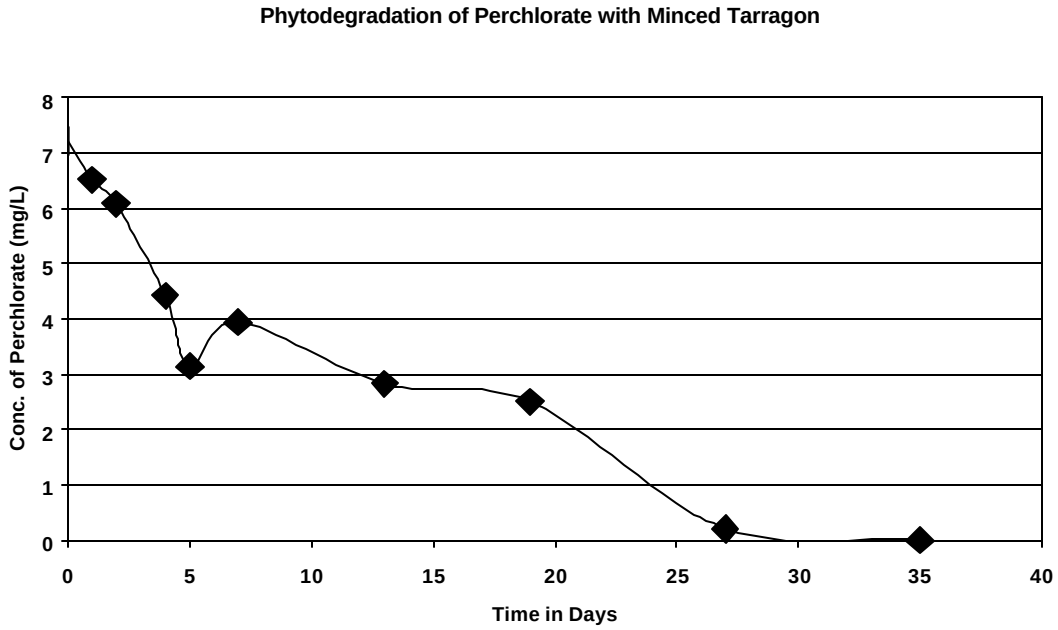


Figure 3. Evidence of phytodegradation of perchlorate by plant component(s).

In a further study, a cottonwood tree exposed to perchlorate for about one month was removed from the perchlorate-dosed medium, rinsed with deionized water and transferred to a perchlorate-free half-strength Hoagland's solution. The purpose of this investigation was to verify if the perchlorate accumulated in the tree leafs and branches can be released back into the remediated water by osmosis. After 7 days no detectable perchlorate was measured in the clean medium and analysis of the extracted plants confirmed that the perchlorate remained unchanged (641 ug/L) in the cottonwood leafs and branches. This means that if the perchlorate taken up by the woody plants is not transformed but simply accumulated, it is not likely to be released back into the remediated water by osmosis.

Figure 4 shows that following an initial exposure of the woody plants to perchlorate in a hydroponics reactor the predominant phytoremediation mechanism changed from uptake to degradation and the rate of perchlorate reduction in the medium increased by several orders of magnitude. For example, the rate of degradation of the second dose of perchlorate added to the willow bioreactor was much faster than that of the initial spike. In less than 72 hours 109 ppm of perchlorate was degraded to below the method detection limit of 2 ppb. When this reactor was dosed for a third time (initial concentration 86 ppm), the perchlorate was degraded to below the method detection limit in 43 hours. The rate of decrease of perchlorate in the bioreactor was described by zero order kinetics. After dosing the same reactor for a total of five times, the rate of perchlorate reduction by the willow showed no decrease (Figure 4). The estimated zero-order rate constants for the 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} spikes were 15.3, 20.5, 32.6, and 25.9 µM/h (Table 2). The average water uptake rate by the willow during the latter experiment was 64.5, 129, 93, 95, 80 mL/day for the 1^{st}, 2^{nd}, 3^{rd}, 4^{th}, and 5^{th} spikes, respectively. The very fast kinetics observed after the initial spike shown in Figure 4 suggested that the predominant reaction mechanism was degradation in the root zone (rhizotransformation) and not uptake and phytodegradation. The

latter was confirmed in subsequent studies by monitoring the chloride concentration in the rhizosphere. Based on these observations, radiolabeled studies were designed to confirm rhizodegradation of perchlorate, as described below.

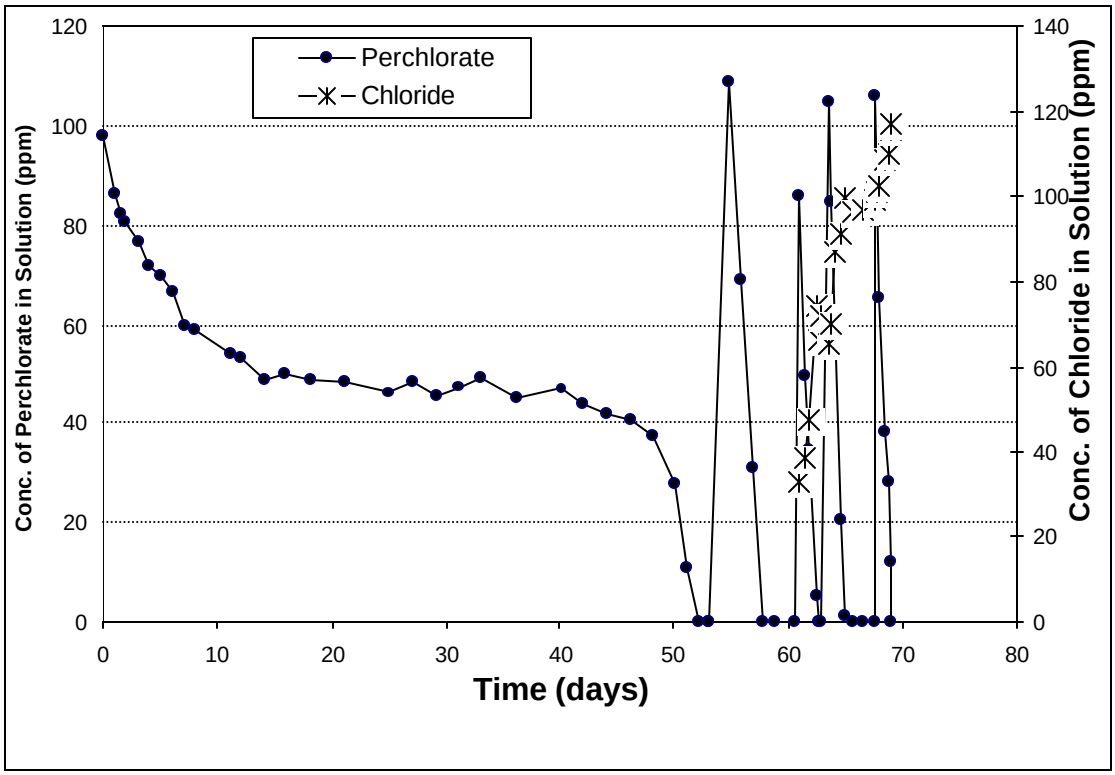


Figure 4. Removal of perchlorate from bioreactors dosed multiple times and progressive increase in chloride concentration in the rhizosphere.

Table 2. Kinetics and Chloride Mass Balance Results for One of Three Willow Trees Dosed Multiple Times with 100 mg/L of Perchlorate

Spike #	Water uptake rate (mL/day)	Initial perchlorate concentration (mg/L)	Zero-order rate constant (mg/L//h)	Cl^- mass balance[a] (%)
1	64.5	98	NA	ND
2	129	109	1.52 ± 0.42	84
3	93	86	2.04 ± 0.07	110.8
4	95	105	3.24 ± 0.22	92.2
5	80	106	2.58 ± 0.18	81.3
Average	92.3 ± 10.7	100.8 ± 4.1	2.35 ± 0.37	92.1 ± 6.7

NA, not applicable to the kinetic data. ND, not determined due of significant uptake into plant.
[a]Background chloride concentrations were subtracted before calculating individual and average percentages.

Possible transformation products of perchlorate such as chlorate, chlorite, hypochlorite and dichlorooxide were analyzed for but none was identified in the medium or plant extract. Complete transformation of perchlorate by the willow was confirmed by monitoring the change in chloride concentration over the course the study. The chloride concentration in the full strength Hoagland's solution was below the IC detection limit in the diluted sample. The chloride concentration in the medium was measured and observed to increase as each dose of perchlorate was completely degraded to below the IC detection limit. A chloride mass balance of 86% was obtained for the study whose results are presented in Figure 5 (6^{th} spike of the willow bioreactor). The stoichiometric reduction of perchlorate to chloride cannot be inferred from this data since; some chloride was taken up by the willow tree (Figure 5). Studies with the radiolabeled chemical should reveal whether the unrecovered perchlorate is completely transformed to chloride or simply bound to the plant tissue. It was observed that both nitrate and perchlorate utilization rates in the medium were similar and described by zero-order kinetics. Acetate was the only plant exudate identified in the medium at pH of 6.5. We inferred from the latter results that bacteria with perchlorate and nitrate acting as terminal electron acceptors oxidized acetate.

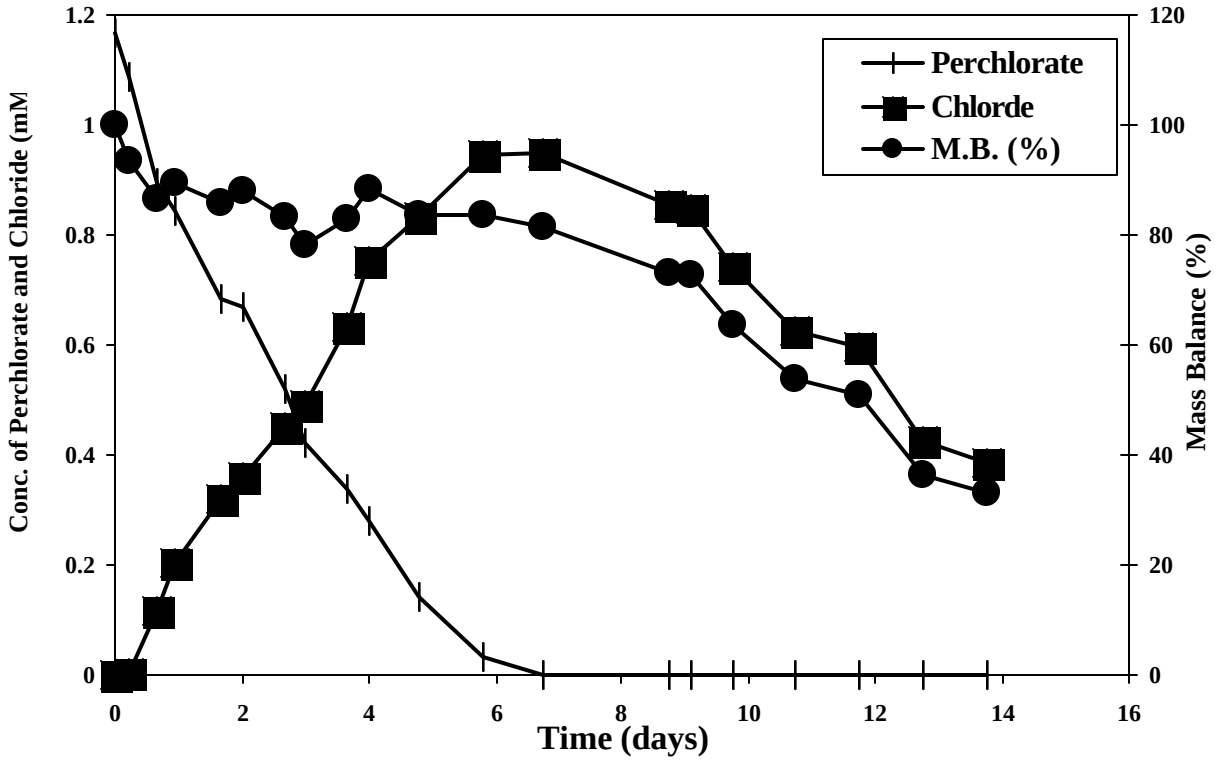


Figure 5. Chloride mass balance as an indicator of rhizodegradation of perchlorate by willow tree dosed multiple times, after biodegradation had been biostimulated. Background chloride considered in closing the mass balance at 92%.

1.4. Effect of Nitrate Concentration and Source

The degradation of perchlorate by willow trees was affected by both the concentration of nitrate in the growth media and the N-source (Figure 6 and Table 3). The fastest perchlorate degradation kinetics were observed in planted bioreactors with lower NO_3^- concentrations of <200 mg L^{-1} and slowest kinetics at higher NO_3^- concentration of 300 - 600 mg L^{-1}. No perchlorate was removed from solution within the first 5 and 10 days by willow trees grown in nutrient solution containing the lower and higher nitrate concentrations, respectively. The influence of NO_3^- on perchlorate reduction was attributed to competing reactions in which rhizosphere bacteria preferentially utilized NO_3^-, as TEA. This shortcoming was overcome by replacing the NO_3^- N-source (Hoagland's solution) with an ammonium and urea N-source (i.e., Miracle-Gro). As a result, perchlorate was rapidly reduced in the rhizosphere of the willow trees grown in miracle-Gro. Uptake of perchlorate by woody plants was minimized in medium with ammonium and urea as the N-sources. In previous work, Nzengung and coworkers showed that increasing the concentration of the carbon (electron) source reversed the inhibition of perchlorate degradation under high nitrate concentrations.

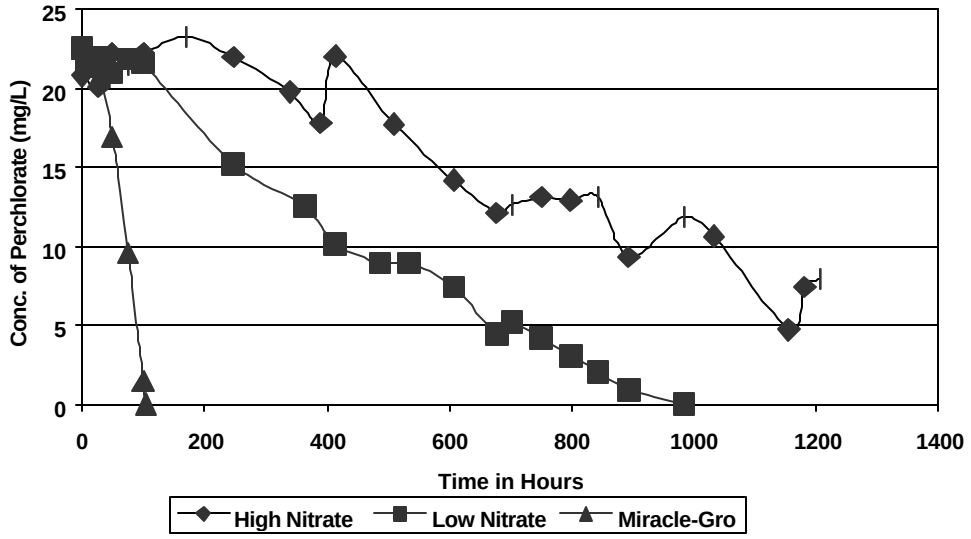


Figure 6. Rate of removal of perchlorate from willow planted bioreactors fed containing urea, low (<200 ppm NO_3^-) and high (300 – 600 ppm NO_3^-) nitrate in growth media.

Table 3. Summary Results of Perchlorate Treated and Control Willow Trees under Different Experimental Conditions

Bioreactor	Type of nutrient medium[a]	Ammonium or nitrate concentration in solution (mg/L)	Initial perchlorate concentration (mg/L)	Maximum perchlorate detected in leaf[c] (mg/kg)	Estimated zero-order rate constant (mg/L/h)
Hydroponic	Miracle-Gro 2 g/L	≤136[b]	22 - 23	15.0 ± 0.4	0.389 ± 0.005
Hydroponic	Miracle-Gro 0.5 g/L	≤34[b]	22 - 23	8.5 ± 0.9	0.284 ± 0.014
Control	Miracle-Gro 2 g/L	≤136[b]	0	0	NA
Hydroponic	Water only	<10	13	144.5 ± 5.4	0.007 ± 0.001
Hydroponic	Hoagland's	<200	10	100.0 ± 3.6	0.003 ± 0.000 [d] 0.053 ± 0.004 [d]
Hydroponic	Hoagland's	<200	23	132.0 ± 2.9	0.025 ± 0.001
Hydroponic	Hoagland's	<100	41	110.5 ± 2.8	0.311 ± 0.011
Hydoponic	Hoagland's	≥400	102	2219.5 ± 7.0	0.019 ± 0.001
Sand	Hoagland's	≤100	101	626.7 ± 7.4	2.35 ± 0.37[e]
Sand	Hoagland's	≤200	116	ND	0.802 ± 0.039[e]
Sand	Hoagland's	≤100 - 300	117	ND	0.232 ± 0.015[e]
Control	Hoagland's	≤200	0	0	NA
Control	Hoagland's	≥400	0	0	NA

These results are averages for multiple doses of perchlorate applied to each bioreactor when rhizodegradation was the dominant phytoprocess.
ND, not determined. NA, analytical method does not apply to the data.
[a]Stern's Miracle-Gro consists of 15% total nitrogen with 6.8% as ammonical nitrogen and 8.2% urea nitrogen. Hoagland's solution is a medium with nitrate as the nitrogen source. [b]Refers to NH_4^+ concentration. [c]Perchlorate concentrations in the leaves increased to a maximum during the experiment before decreasing to undetectable levels after perchlorate was completely removed from solution. [d]Removal of perchlorate from solution was described by two zero-order equations (slow followed by fast kinetics). [e]Applies only to the diluted Hoagland's (nutrient) solution phase above the sand layer in which the tree roots were submerged because perchlorate remained in the pore water of the sand layer after perchlorate was completely removed from the solution phase above.

1.5. Long-term Hydroponic Studies.

In additional greenhouse experiments, cuttings of *Salix nigra* (black willow) were planted in 2-liter (L) Erlenmeyer flasks outfitted with side sampling and feeding ports. Control flasks were set up in a similar manner, but without trees.

After the saplings were well established in their new environment, the trees and the control flasks were dosed with known amounts of perchlorate to achieve a 200 parts-per-million (ppm) concentration. The bioreactors were then sampled and analyzed on a regular basis until the perchlorate was below detectable levels.

The concentrations of perchlorate in planted hydroponics reactors decreased rapidly from the time that they were dosed. The two types of control bioreactors, both dosed and undosed, were monitored for perchlorate in solution for 3.5 months. The concentration of perchlorate in the dosed unplanted bioreactor stayed statistically constant, with a little variation, probably due to mixing, sampling, and instrument error. The solution of the unplanted control bioreactor that was not dosed with perchlorate never registered detectable levels of perchlorate. During the three and a half months that this experiment was monitored, no significant change in concentration occurred in either control bioreactor. Figures 6 and 7 show plots of perchlorate concentration (in ppm) versus time in all four hydroponics reactors.

The results of the hydroponics study suggest that the willow saplings and their associated microorganisms facilitated the removal of perchlorate from the planted bioreactors. These results are in agreement with those of previous studies on the phytoremediation of perchlorate.

The observed removal of perchlorate was attributed to a combination of processes: phytodegradation, phytoaccumulation and rhizodegradation. In the case of rhizodegradation, the willow saplings provided organic matter (sugars and other exudates from the roots, and dead root material itself) that acted as carbon or electron sources for perchlorate-degrading microorganisms. The microbes in the root zone of the trees used perchlorate as a terminal electron acceptor for the excess electrons, which resulted from the oxidation of organic matter for energy. Phytodegradation takes place within the plant tissue through the catalytic activity of oxidizing and reducing plant enzymes and other components in the leaves. Details of the above experiment are presented in Dondero 2001.

1.6 Phytoremediation of Perchlorate Under Different Redox Conditions

In order to examine the role of willow trees in perchlorate removal under different redox conditions, several bench-scale studies were performed. One set of experiments used an ebb-and-flow bioreactor that was aerated to sustain aerobic conditions while another set employed sealed bioreactors to simulate an anoxic vegetated environment.

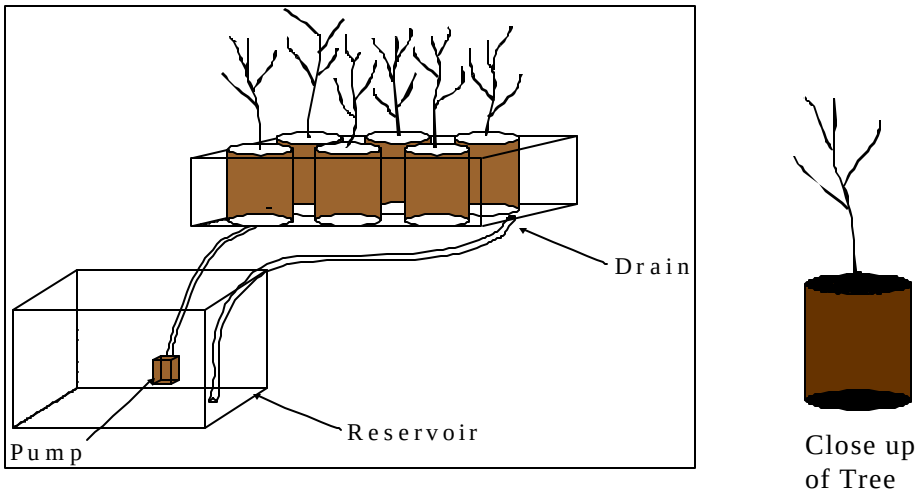


Figure 7. Ebb-and-flow bioreactor planted with six willow trees and rhizosphere aerated continuously throughout the experiment.

The ebb-and-flow hydroponics system (Figure 7) contained six mature trees and continuously circulated water from a reservoir tank through the rhizosphere of the trees. The water evapotranspired by the trees was replaced daily by adding an equivalent volume of deionized water to the reservoir. Samples were taken from the root zone in the upper tray and from the reservoir and analyzed for perchlorate routinely. To minimize the effect of dilution, samples for perchlorate analysis were taken prior to replenishing the evapotranspired water from the previous day. The reservoir was purged with air regularly to maintain aerobic conditions throughout the duration of the study. Leaves were harvested from the trees and analyzed for perchlorate during the experiment.

For the sealed bioreactor experiments, willow cuttings were used. For these sealed bioreactor experiments, one of the trees died as a result of a spider mite infestation. Measurements were continued, however, and this tree will be referred to as dead willow (DWT), with its live counterpart denoted as live willow (LWT). In both reactors redox and pH conditions were recorded every 15 minutes and samples for perchlorate analysis were taken at least every three days. Chlorate and chloride analyses were done at least every five days until perchlorate was completely removed from the rhizosphere.

The rate of perchlorate removal was slower in the ebb-and-flow bioreactor. This rate was modeled using a first-order expression, and the half-life of the aqueous phase perchlorate was

46.5 days ($r^2 = 0.87$). The relatively slow rate of removal of perchlorate in the ebb-and-flow bioreactor was attributed to the aerobic state of the circulated water, which is not favorable for perchlorate biodegradation. As a result, the decrease in perchlorate in the holding tray and reservoir is attributed mainly to uptake and accumulation in the plant leaves, and phytodegradation. Unlike rhizodegradation, uptake and phytodegradation are slower processes, as the perchlorate is mainly removed in the water taken up into the trees.

The complete removal of perchlorate from the LWT bioreactor took 31 days while approximately the same concentration of perchlorate was completely degraded in 17 days in DWT reactor. The removal of perchlorate from solution in both bioreactors was modeled using a zero-order expression. The half-lives were 14.2 days ($r^2 = 0.96$) for LWT and 8.2 days ($r^2 = 0.95$) for DWT, respectively. These results suggest that the biodegradation of perchlorate in the root zone of the dead willow tree was faster than for a living tree. This is expected because the anaerobic conditions present in the rhizosphere of decaying roots of dead trees are very rich in dissolved organic carbon (DOC) (sources of electrons) and microbial activity.

Compared to the ebb-and-flow system, the sealed bioreactor is a more efficient hydroponics system for the decontamination of perchlorate-contaminated water. The anaerobic conditions created in sealed bioreactors must be present for the specific enzymes to be stimulated in the ubiquitous perchlorate-reducing bacteria. The predominantly anaerobic conditions in the DWT-reactor throughout the experiment sustain a higher perchlorate biodegradation rate. The aerobic conditions that prevailed in the root zone of photosynthesizing willow trees result in a slower rhizodegradation rate. These results show that in case of sufficient supply of reductants (DOC), microbial degradation is controlled by the redox conditions. The role of plant enzymes in the rhizosphere reactions was not evaluated.

Overall, the results of these experiments suggest that under predominantly aerobic rhizosphere conditions, rhizodegradation may not be an important fate process for decontaminating perchlorate-contaminated soils and water. Under anaerobic conditions the dominant process is rhizodegradation by microorganisms, whereas phytoaccumulation and phytodegradation are the predominant phytoremediation mechanisms for trees grown in aerobic environments. Since rhizodegradation is a more efficient process for the decontamination of perchlorate-contaminated water, the design of phytoremediation systems should include the enhancement of rhizoprocesses. Additional details of this study are presented in Nzengung et al. 2003A (Submitted for peer review and publication).

1.7. Mass Balance and Pathway Confirmation Experiments

Direct evidence of perchlorate ion degradation to chloride by willow trees was obtained from experiments in which radiolabeled (^{36}Cl) ammonium perchlorate was used as a tracer. A1 milliliter (mL) dose of the 3.99 millicurie per millileter (μCi/mL) stock solution was placed in each 2 L planted bioreactor. The initial perchlorate concentration in each of the reactors was between 25 and 30 milligrams (mg) of perchlorate per liter (ClO_4^-/L). The concentration of perchlorate ions, chloride and ^{36}Cl activity in solution was monitored daily for the duration of the study. Perchlorate ions and chloride were measured by ion chromatography. At the termination of the study, the plants were sacrificed for a mass balance determination. The whole plant was

weighed and sectioned into roots, upper and lower stems, branches, and leaves. Each fraction was analyzed for perchlorate ion concentration and ^{36}Cl activity.

The results of experiments conducted with ^{36}Cl perchlorate provided more direct evidence that the rapid removal of perchlorate ions from the rhizosphere of plants under anaerobic conditions is due to rhizodegradation. Figures 8 and 9 show that the concentration of perchlorate ions decreased to non-detectable levels in the rhizosphere while the ^{36}Cl activity remained approximately the same. If the removal of perchlorate ions from solution was due to uptake and degradation in the plant (phytodegradation), the ^{36}Cl activity and concentration of perchlorate ions measured in solution should decrease proportionately. In experiments where diluted Miracle-Gro® (urea/ammonium N-source) was used as the growth media, the mass balance based on the ^{36}Cl activity in solution was better than 95%. However, a lower recovery (80%) of ^{36}Cl activity in solution was observed if a nitrate nitrogen source (Hoagland solution) rather than a urea/ammonium nitrogen source (Miracle-Gro®) was used as the growth solution. The poor rhizosphere mass balance obtained in experiments with Hoagland solution was attributed to the greater uptake of perchlorate ions by plants in the presence of nitrate N-source. Also, the rate of rhizodegradation of perchlorate ions was faster for trees grown in Miracle-Gro® than those grown in Hoagland solution (Figures 8 - 12). For a detailed look at this experiment see Nzengung et al. 2003B (Submitted for peer review and publication).

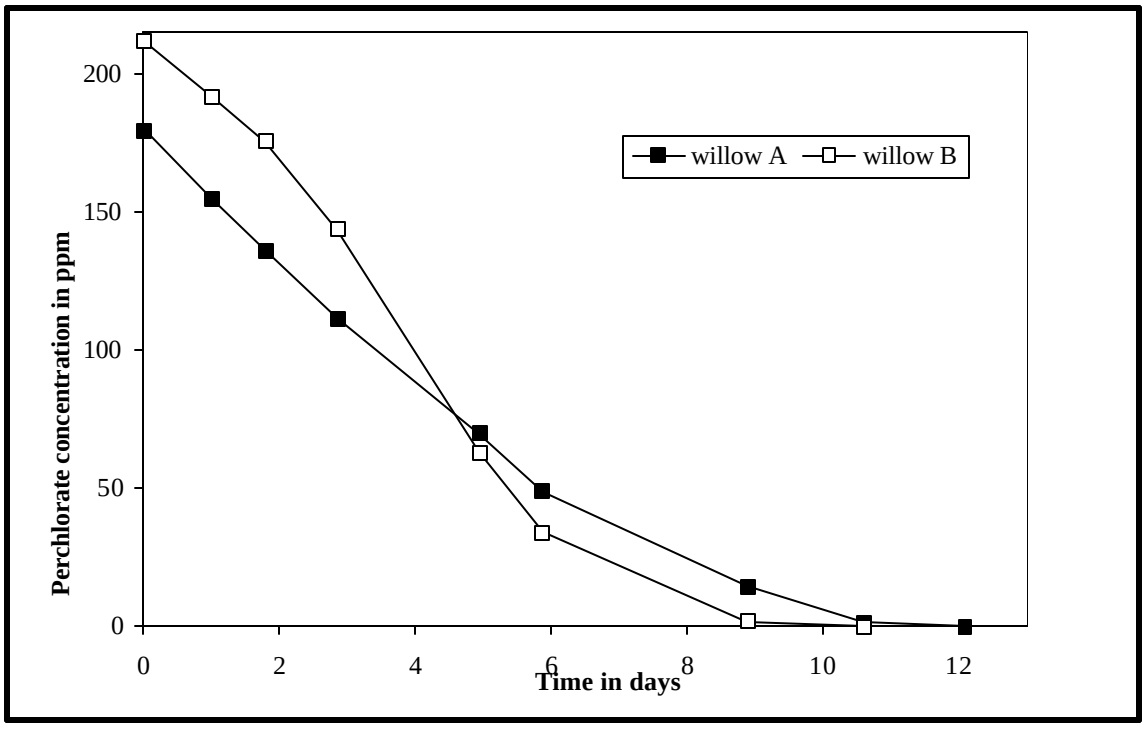


Figure 8. Results of perchlorate removal from hydroponics bioreactors.

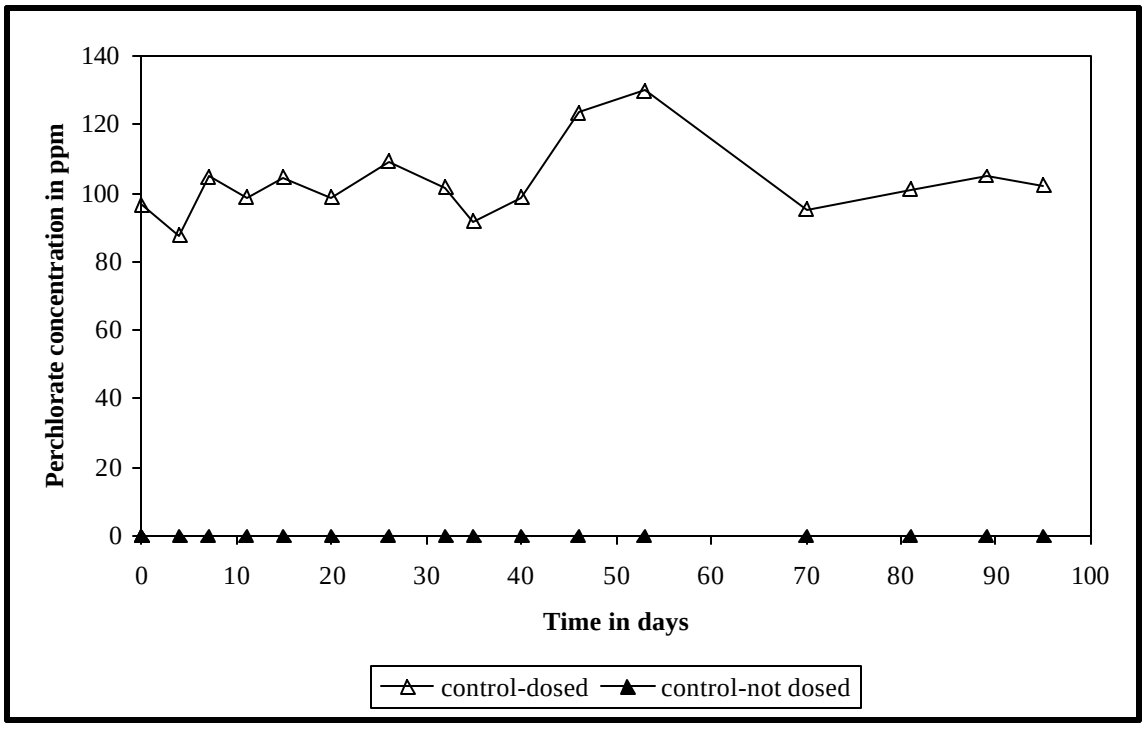


Figure 9. Results of perchlorate removal from unplanted hydroponics bioreactors.

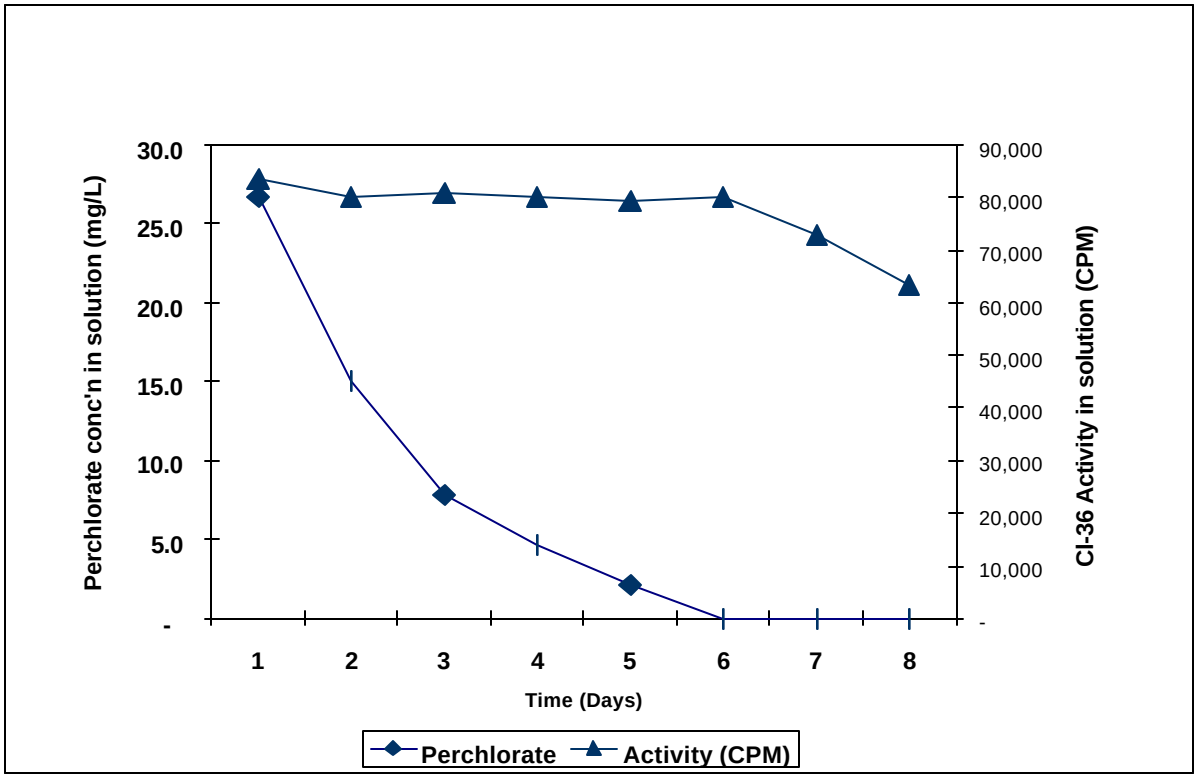


Figure 10. Evidence of rhizodegradation of perchlorate by willow tree.

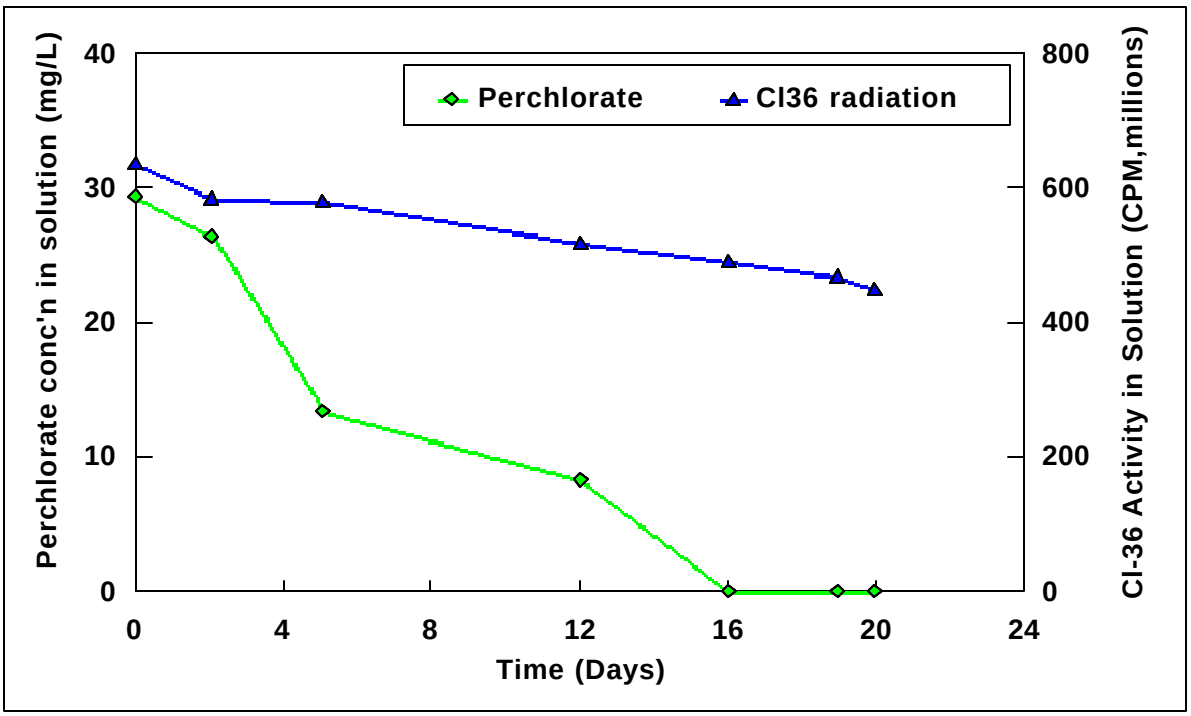
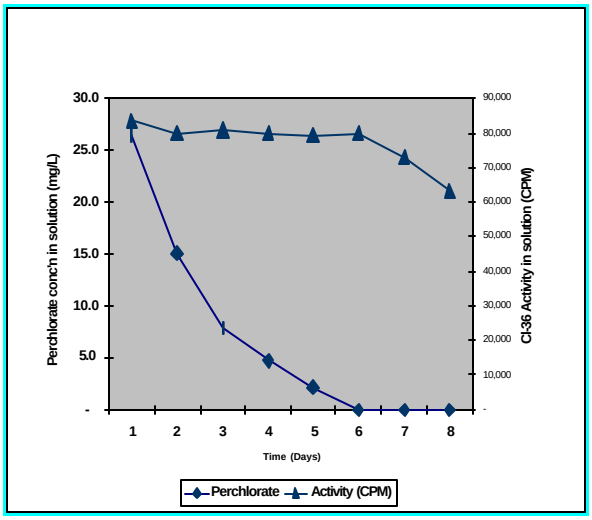


Figure 11. Evidence of rhizodegradation of perchlorate.



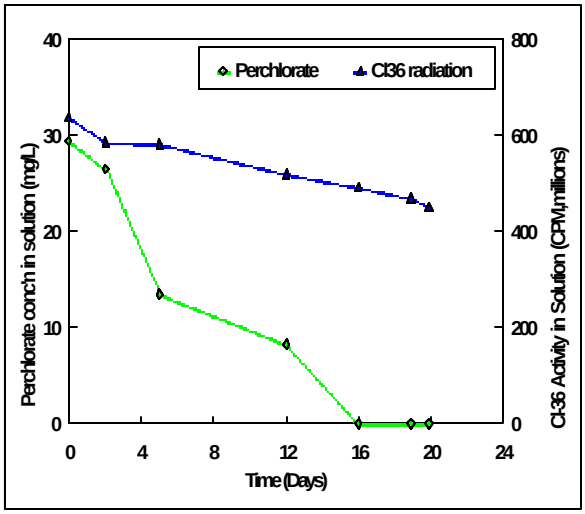


Figure 12. Relative rate of perchlorate degradation in MiracleGro (left) and Hoagland's Solution (right).

2. Sand & Soil Bioreactor Studies under Greenhouse and Natural Conditions

2.1. Bucket and Drum Studies

Black willow and cottonwood cuttings were rooted in potting soil. Six plastic 5-gallon buckets were outfitted with water gauges and sampling ports. The buckets were then filled with sand to within 3-5 centimeters (cm) of the rim. Two of the buckets were planted with one black willow cutting each, and two with one cottonwood cutting each. The last two buckets were labeled "control A" and "control B" and were not planted with any cuttings. Each of the buckets was filled with 10 L of deionized water. The resulting water level, which was right at or just below the soil surface, was marked on the manometer.

Once the saplings were established, the buckets were dosed with varying known concentrations of sodium perchlorate ($NaClO_4$) dissolved in deionized water. The pore-water (water within the saturated soil) in the buckets was then sampled and analyzed on a daily basis, with occasional exceptions. These samples were analyzed for perchlorate by ion chromatography. When the concentrations of perchlorate in the buckets reached non-detectable levels, the buckets were re-spiked with $NaClO_4$ dissolved in deionized water, and were sampled over subsequent days in the same manner described above.

The data in Table 2.A show that in the presence of abundant TOC and nutrients, perchlorate was rapidly degraded in the presence and absence of trees. Thus, the unplanted control bucket environment was just as conducive (if not more so) to the degradation of perchlorate as was the planted bucket environment.

Table 4. Half-lives of perchlorate removal in buckets.

Type of bucket setting	Average half-life*
Willow A	3.4 ± 2.3
Willow B	1.8 ± 1.0
Cottonwood A	1.4 ± 0.5
Cottonwood B	1.9 ± 1.0
Control A	3.0 ± 1.5
Control B	2.0 ± 1.3

*half-lives given in days ± standard deviation

For perchlorate, which does not volatilize, total removal from the pore water is equal to degradation and accumulation of perchlorate by any plants present, plus degradation by the microorganisms present:

$R_{total} = D_{plant} + A_{plant} + D_{microbe}$

where R is removal, D is degradation and A stands for accumulation. Thus, in the unplanted control buckets, D_{plant} and A_{plant} were negligible. Sorption data confirmed that the perchlorate did not simply bind to the sand and potting soil. Therefore, the total removal of perchlorate was attributed to microbial degradation.

Total organic carbon (TOC) was measured in all of the buckets about one third of the way through the study. Each bucket had a very high concentration of TOC, which attests to the fact

that organic carbon (used by microorganisms as an electron source) was not a limiting factor to microbial activity in the buckets (as is often the case in groundwater). The periodic doses of perchlorate added to the buckets provided an abundant supply of terminal electron acceptors. For these reasons, it is concluded that the limiting factor in perchlorate degradation in the buckets was the amount of perchlorate degrading microorganisms present. Therefore, as a critical mass of microorganisms accumulated, the perchlorate removal kinetics shifted from first- to zeroth-order, and in some cases faster perchlorate removal was observed. (For a complete description of the rate laws used, please see the *Sorption Data Analysis* description in Section 3.1, page 12.)

A simultaneous study was conducted to go beyond the bucket study in approaching field conditions using 55-gallon (approximately 193 L) drums in an open-sided outdoor structure with a slatted roof that offered 50% shade. A thick piece of transparent painter's plastic slipcover was stapled to the slatted roof to prevent rain from entering the study area.

Each drum was outfitted with a manometer and 6 sampling ports. The sampling ports were of such lengths that water could be taken from shallow, medium or deep levels either in the middle or at the edge of the drum. Play sand was added to each drum to a depth of 30 cm. The sand was smoothed to create a flat surface, and the drum was filled to within 4-5 cm from the rim with potting mix.

Two of the drums were left unplanted, one was planted with an assortment of wetland species: *Typha latifolia* L. (cattail), *Spirodela polyrhiza* (L.) Shield (duck weed), and *Myriophyllum aquaticum* (parrot feather), and the other seven were planted with one sapling each. The remaining seven drums were planted with the following saplings: three with black willow, two with cottonwood, and two with *Pinus taeda* (loblolly pine). Half way through the study, the original loblolly pine planted in drum "pine A" was removed after showing signs of dying for over a month. When it was removed, it was discovered that pine A's roots had never spread from its original root ball, indicating that it had never fully established in the drum. Another loblolly pine was planted in the drum shortly after the first was taken out. Although the initial pine A died and was replaced with another loblolly pine, the results do not reflect these transitions; rather, they match the other planted drums' results.

Each drum was dosed with 150 mL of 50,000-ppm perchlorate stock solution, made up of sodium perchlorate ($NaClO_4$) dissolved in de-ionized water. The resulting perchlorate concentration was calculated to be approximately 250 ppm, if evenly distributed in the drum.

The drums were sampled twice per week. These pore water samples were then analyzed for perchlorate twice per week and for chlorate, chloride, nitrate, and sulfate once per week.

The simulated wetland drum showed the most rapid removal of perchlorate from the pore water. Not only was it a strongly anaerobic environment, which would require bacteria and other microorganisms to utilize alternate electron acceptors such as perchlorate, it contained a number of individual hydrophytes that phytoaccumulated large amounts of perchlorate, while all other drums only had one plant each. Generally, the highly reducing conditions and multiple plant

species combined to achieve the rapid removal of perchlorate from the soil, surface and groundwater in the simulated wetland drum.

The performance of the "controls" in both the bucket and drum studies prove that plants need not be present for perchlorate degradation to take place in a carbon- and nutrient-rich anaerobic environment. The results of the controls also attested to the importance and efficiency of microbial activity in the degradation of perchlorate.

The kinetic data gathered during the bucket study suggested that the degradation of perchlorate in the bucket study (for both planted and unplanted buckets) could be described by a pseudo-first-order reaction. However, some of the later dosings exhibited zero-order kinetics, which led to the suggestion that with prolonged exposure to perchlorate, the number of perchlorate-reducing microorganisms reach a critical mass (provided favorable environs) that enables them to degrade perchlorate more proficiently.

The plant tissue analyses from the bucket and drum studies established that plants remove perchlorate from contaminated water through rhizodegradation, phytoaccumulation and phytodegradation. Although the phytoaccumulation and phytodegradation take place simultaneously, the plants examined in this study accumulated perchlorate much more rapidly than they were able to degrade it within their tissue.

The drum study confirmed that the results of the bucket study could be achieved in a less controlled, outdoor environment, although the drum systems did tend to exhibit slower kinetics than the buckets. The "simulated wetland" drum showed the fastest perchlorate removal from the contaminated water, probably due to a combination of anaerobic microbial activity and hydrophyte phytoaccumulation. The chlorate, chloride and oxidation-reduction potential measurements confirmed that chlorate did not accumulate within the drums during anaerobic perchlorate reduction. For more details on the bucket and drum experiments, see Dondero 2001.

2.2. Bermuda Grass

A greenhouse study was conducted to investigate whether Bermuda grass might be a suitable phytoremediation agent for perchlorate at shallow depths. Six five-gallon buckets were equipped with manometers and sampling ports. Three of the buckets were filled with clean play sand and the three remaining buckets were filled with Georgia red clay collected next to the UGA Crop and Soil Sciences greenhouse facilities. Bermuda grass seeds were sown in two of the sand buckets and two of the clay buckets, leaving one bucket of both sand and clay as controls. Crab grass grew as a weed, ostensibly from dormant seeds in the collected soil.

After the grass had established itself, each of the buckets was dosed to obtain an initial perchlorate concentration of 70 ppm. The bottom third of the clay buckets contained sand that was fully saturated with water for the duration of the experiment. Deionized water was added on a daily basis to maintain the water level in each bucket. Aqueous samples from each bucket were sampled on a regular basis and analyzed for perchlorate content. When perchlorate concentrations fell to below the detectable limits of the instrument, the bucket was re-dosed at ~70ppm perchlorate.

The degradation of perchlorate by Bermuda grass planted in soil was much faster (by a factor of 2) than in the sand-filled buckets planted with the same grass (Figures 13 and 14). This was attributed to the healthy growth of Bermuda grass in clay as opposed to sand media. In the clay buckets the healthy growth of grass corresponded with a high root mass leading to a higher rate of rhizodegradation. Thus, although the total organic carbon (TOC), total phosphorus (TP), and total nitrogen (TN) concentrations were higher in the sand than in the clayey soils, the higher root mass was a more important influence on the degradation of perchlorate.

As the buckets were dosed multiple times, the rate of perchlorate degradation in each bucket increased with each subsequent dosing. The removal of perchlorate from the control buckets was attributed to the ubiquity of perchlorate-degrading bacteria. As expected, the degradation of perchlorate in the clay control bucket was much faster than in the sand control bucket; the clay should be richer in bacteria than the sand. These results suggest that perchlorate is rapidly attenuated in wet, vegetated soils.

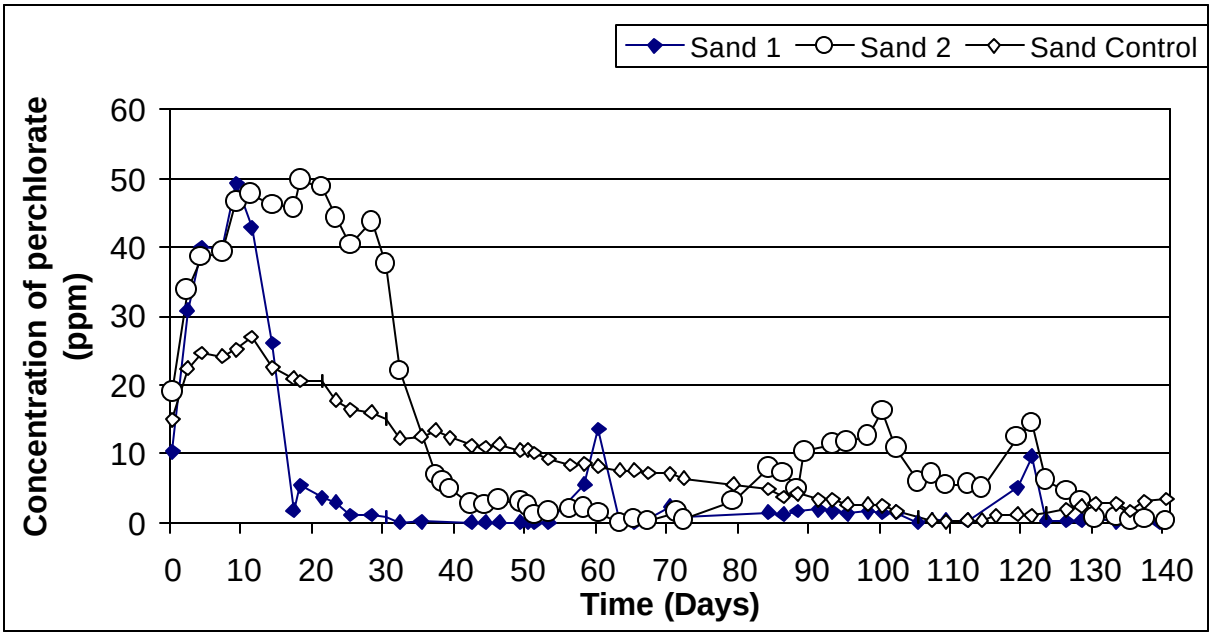


Figure 13. Removal of perchlorate in five gallon planted and unplanted sand bioreactors. Bermuda grass was used in this experiment and the vegetated bioreactors were dosed multiple times.

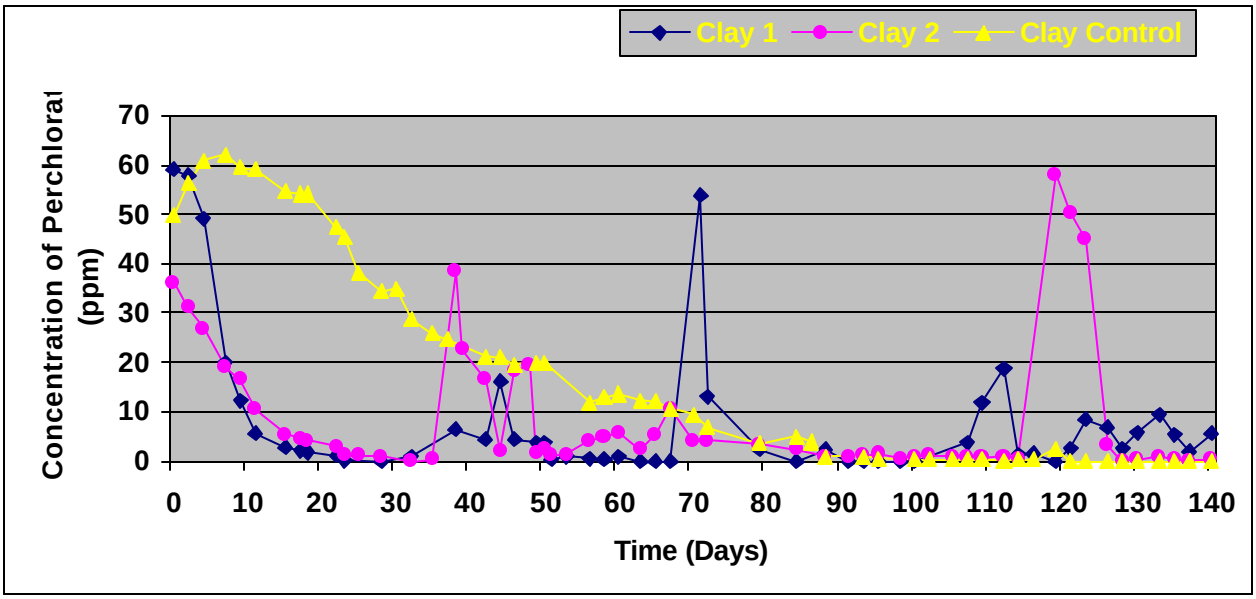


Figure 14. Removal of perchlorate in five gallon planted and unplanted clayey-soil bioreactors. Bermuda grass was used in this experiment and the vegetated bioreactors were dosed multiple times (denoted by spikes in concentration).

3. Batch Sorption Studies

3.1. Sorption Data Analysis

Kinetic sorption studies were performed to determine the time needed to attain sorption equilibrium. Sorption isotherm experiments were performed to determine the extent to which perchlorate sorbed onto sand, soil and peat moss. Column experiments were run to evaluate the effectiveness of pine mulch as a sorbent for perchlorate. In the column experiments, it was observed that perchlorate does not sorb to pine mulch under our experimental conditions, so these experiments were not pursued further.

First-order sorption rates for the batch microcosm experiments were determined by the method of least squares optimization. For each kinetic experiment, the parameter measured was the amount of perchlorate left in solution at a given time t (C_t) relative to the concentration present at time t = 0 (C_0).

Zero-order biodegradation rates were modeled by the following equation:

$$\frac{d[A]}{dt} = -k[A]^0 = -k$$

Integration of the zero order equation yields:

$$[A] = [A]_0 - k \cdot t$$

Plotting the C_t vs time yields the zero-order rate constant $k\left(\frac{mg}{L \cdot d}\right)$.

The half-life of disappearance for the zero-order reactions was calculated from the equation:

$$t_{\frac{1}{2}} = \frac{0.5[A]_0}{Co}$$

The first-order reaction rates were modeled by the equation:

$$\frac{d[A]}{dt} = -k[A]$$

Rearranging and integrating the first order equation yields:

$$[A] = [A]_0 \cdot e^{-kt}$$

Plotting $\ln(C_t/C_0)$ verse time yields the first order rate constant k (d^{-1}).
The half-life of disappearance for the first order reactions was calculated from the equation:

$$t_{\frac{1}{2}} = \frac{0.693}{k}$$

The difference method was used to calculate the concentrations of perchlorate sorbed to the sand, soil and peat moss when sorption equilibrium was attained. Se (mg/Kg) is the sorbed concentration calculated using the difference method. The difference method data were calculated by subtracting the final liquid phase concentrations (Ce in mg/l) from the initial concentrations (C_0 in mg/l) and attributing this difference to sorption. Regression analyses of the final liquid phase concentrations (Ce) versus the sorbed concentrations (Se) were used to determine the partitioning coefficient (Kd in ml/g) between the solids and aqueous media.

3.2. Peat Moss

A kinetic sorption study was performed to determine the sorption equilibrium time for perchlorate to peat moss. Twelve 100-ml vials were filled with 20g of peat moss and 80ml of 10ppm perchlorate solution. Another twelve vials were filled with the same amount of peat moss and 80ml of 100ppm perchlorate solution. One vial from each of the two batches was sampled at pre-determined time intervals and analyzed for perchlorate content. Based on the results of this experiment, 12 hours was determined to be the optimal equilibrium time to use in the isotherm study.

To determine the sorption coefficient of peat moss, seven 100-ml vials containing 25g of peat moss received 90ml of perchlorate solution with concentrations ranging from 0 (control) to 100ppm. The vials were put on the shaker, and then sampled after 12 hours. From the resulting data, we found the sorption coefficient to be 3.62L/kg (Figure 15), which means that one kg of peat moss, is needed to treat 3.6L of the perchlorate-contaminated water. The mechanism by which perchlorate is sorbed to peat moss is partitioning. This means that perchlorate binds reversibly to peat moss. Kinetically, perchlorate sorbs to peat moss very rapidly, with equilibrium achieved in less than four hours.

Changes in pH from ~5.8 (beginning of experiment) to ~3.8 (end of experiment) were detected during sorption. This is attributed to the release of organic acids into solution by the peat moss. Further studies might verify that we can increase the sorption by optimizing pH and ionic strength.

3.3. Sand and Soil

Six 50-ml vials were filled with 10 grams (g) of soil, 30 ml of deionized water and 10 ml of varying perchlorate solutions, giving final solutions with concentrations ranging from 0 ppm (Control) to 20 ppm. The pH of the stock perchlorate solutions ranged from 6.6 to 7.3. Six vials of sand were prepared in the same fashion. The samples were allowed to equilibrate for 72 hours, and were then analyzed for perchlorate content.

The results for soil were inconclusive (Figure 16) and showed little correlation between the initial perchlorate concentration and the amount sorbed. Most likely there was bacterial degradation of perchlorate in the soil, which we have documented before. Future studies should include gamma irradiation of the soil to kill off bacteria before attempting to determine the sorption coefficient of soil.

The results of the sand sorption tests (Figure 16) showed a fairly strong correlation between perchlorate solution concentration and sorption amount (r^2=0.91) with a sorption coefficient of 0.4 L/kg. This indicates that sand is not necessarily a suitable sorbent for perchlorate, since it takes one kg of sand to treat only 0.4 L of perchlorate-contaminated water.

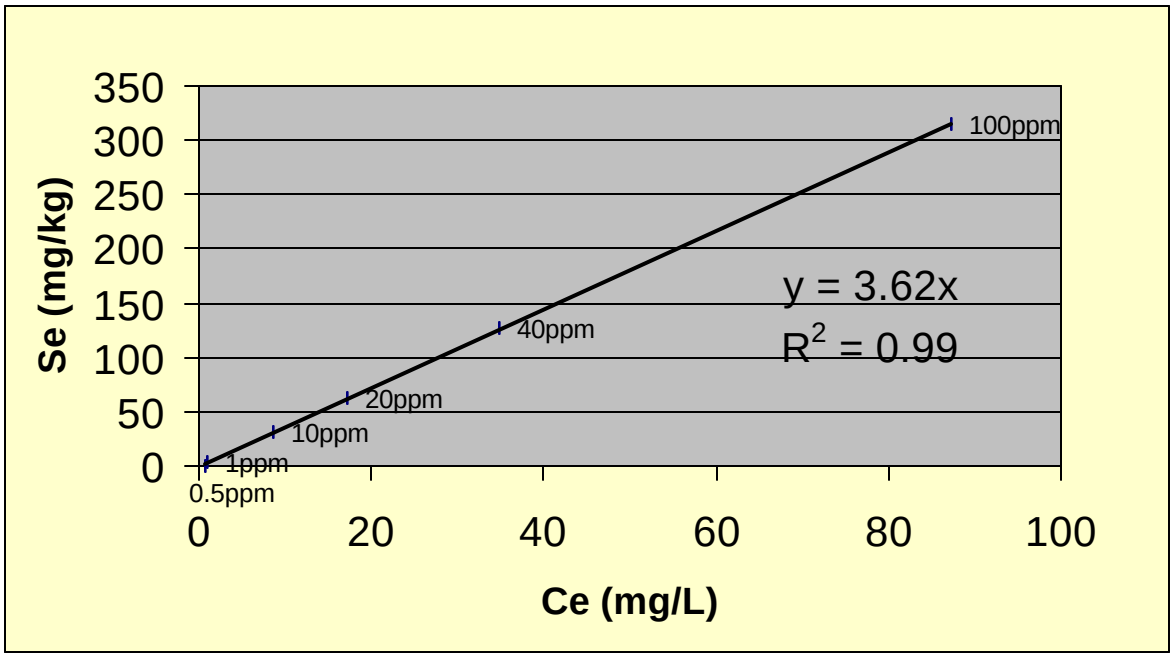


Figure 15. Sorption of Perchlorate to Peat Moss

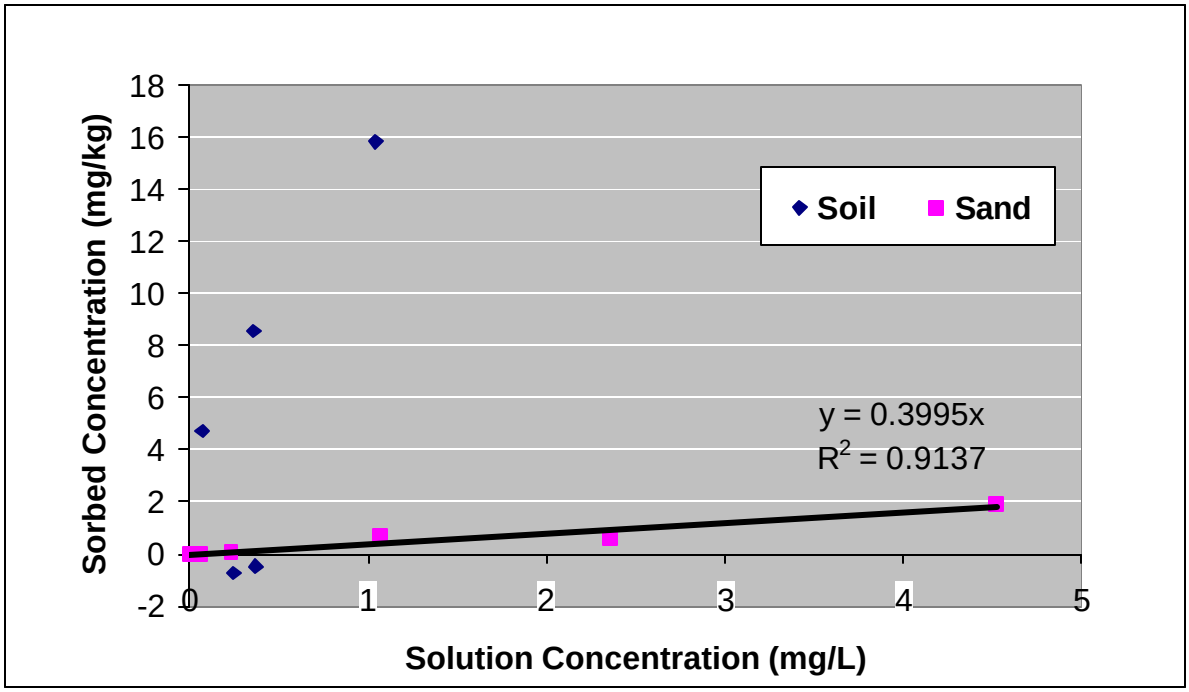


Figure 16. Sorption of Perchlorate to Soil and Sand.

4. Bacteria Isolation and Culture Studies

4.1. Evidence of Root Zone Microbial Activity

Growth media withdrawn from the rhizosphere of willow trees previously used in hydroponics experiments degraded perchlorate to chloride (Figure 17). Nutrient media supplemented with $NaNO_3$ to achieve 3.2 mM NO_3^- showed no activity. However, the unamended media or media amended with acetate or both acetate and NO_3^- degraded perchlorate to chloride. This suggests that a high concentration of the carbon (electron) source is not inhibitory and may reverse the inhibitory effects observed at high NO_3^- activity in the root zone. Figure 18 shows that the boiled media lost all of its perchlorate degradation activity. Additionally, one of four bacteria isolated from the rhizosphere of willow trees degraded perchlorate and most likely mediated the observed rhizodegradation reactions (Nzengung and Wang, 2000).

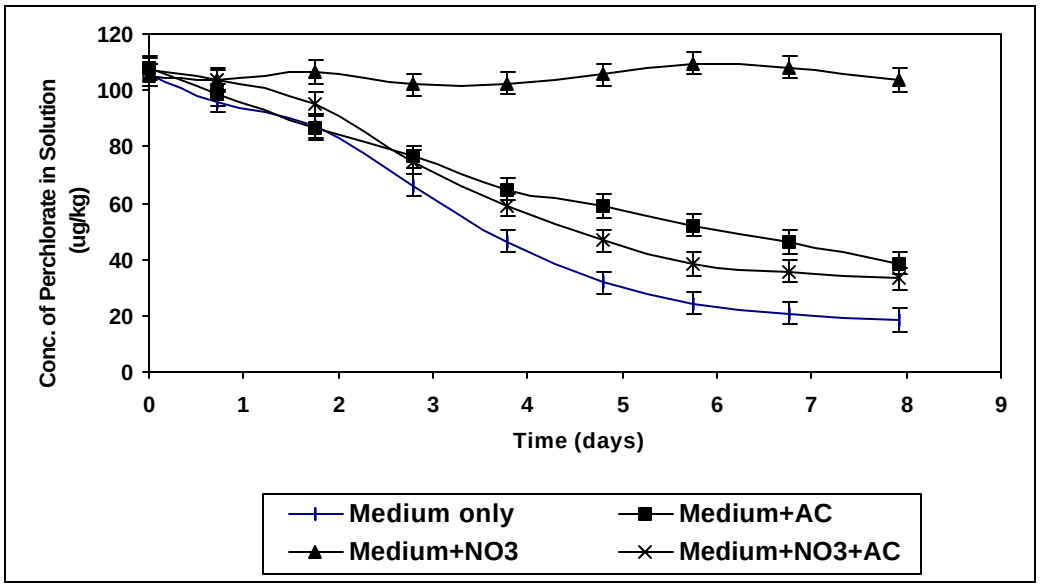


Figure 17. Biodegradation of perchlorate in root zone solution obtained from willow trees actively degrading perchlorate. The root zone solution was amended in some cases with nitrate, nitrate plus acetate, and acetate, respectively.

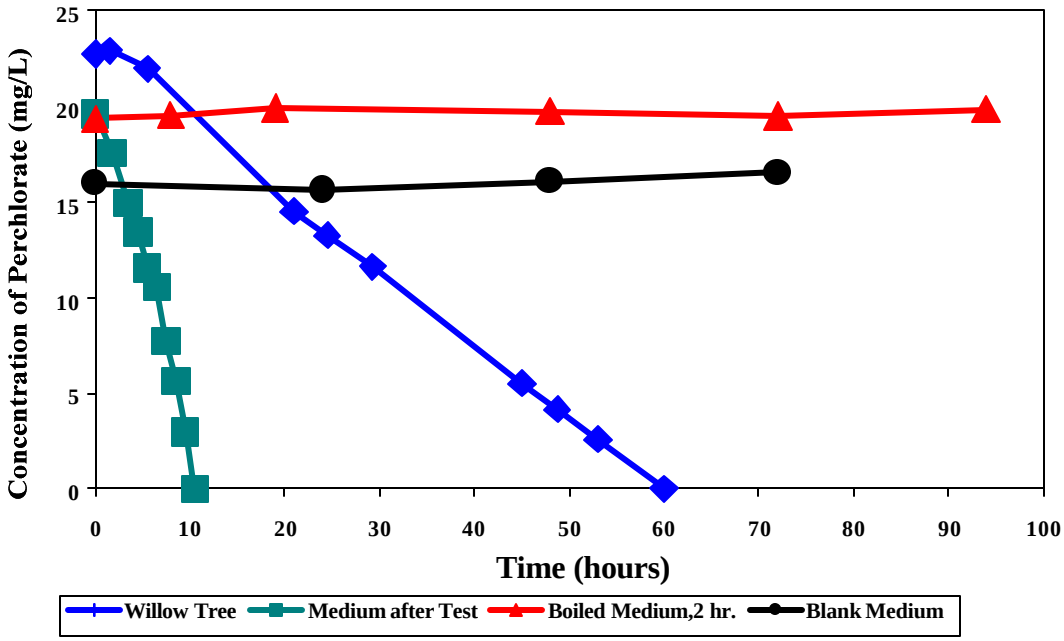


Figure 18. Additional evidence on microbial involvement in phytoremediation of perchlorate.

4.2. Isolation.

Based on the indirect evidence obtained to show microbial dominance during phytoremediation of perchlorate, further direct evidence was sought by focusing on the root zone consortia. Water and root samples were taken from hydroponics willows actively breaking down perchlorate. Pore water and root samples were also taken from perchlorate-degrading willow and cottonwood trees planted in soil. The aqueous samples were streaked onto agar plates (Table 5) and the root samples were cut into small pieces and placed directly onto the media plates to allow the root zone colonizing bacteria to grow out.

Resulting bacterial colonies were transferred from the agar plates to shake flasks (Table 5) in order to evaluate any perchlorate-degrading potential. The isolates grew in the shake flasks and after a predetermined time interval, the solution was tested for perchlorate degrading activity by any of the isolated (cultured) bacterial strains

A total of 12 bacterial strains (Table 6) were isolated, but none of the isolates or combination thereof has been confirmed to be a perchlorate degrader in shake flask tests. It appears that either none of these isolates is of the perchlorate-degrading variety, or more likely, the optimum experimental conditions to induce perchlorate degradation have not been identified. From our earlier studies we know that these are facultative bacteria that will only break down perchlorate in anaerobic conditions. Ongoing trials are focusing on optimizing these conditions.

Table 5. Contents of Plate and Shake Flask Media

Plate Media	Shake Flask Media
Hoagland's Solution Deionized water Sodium perchlorate Sodium acetate Potassium phosphate Glucose Bacto-Agar	Hoagland's Solution Deionized water Sodium perchlorate Sodium acetate Potassium phosphate Glucose

Table 6. Bacterial Strains Isolated From Rhizosphere

Sample Type	Sample Origin	Bacterial Strains (identified by number)
Aqueous	Hydroponic system	#1, #4, #5
Root	Hydroponic system	#2, #3
Root	Tree planted in soil	#6, #7, #8, #9, #10, #11
Aqueous	Pore water from tree planted in soil	#12

B. Bacterial Community Structure. It is well recognized that pollutants such as petroleum hydrocarbons (PHC), chlorinated solvents, perchlorate, etc exert selective pressures that shape diversity within microbial communities (richness and relative abundance of species). Thus, the microbial community response will provide a much more comprehensive indicator of environmental health. Microbial ecologist have tried to monitor the microbial community response to chemicals (e.g. PHC) by culture dependent techniques which included; determining the relative abundance of PHC-degraders (plate counts, Most probable number of oil degraders) to the total bacterial abundance (Heitzer and Sayler. 1993). Unfortunately, culturing techniques are only able to assess <10% of the microbial community in environmental samples (Colwell et al. 1985). Recent developments in microbial ecology; such as DGGE, (Terminal Restriction Fragment Length Polymorphism) (TRFLP) and total community 16S rDNA hybridization) now provide a rapid means of evaluating the entire microbial community structure (Bachoon et al. 2001).

T-RFLP allows snapshots (fingerprint) of entire bacterial communities in a sample to be compared after polymerase chain reaction (PCR) amplification of the extracted DNA using primers specific for the 16S rRNA in all eubacteria. This microbial community scan can be used in two ways (1) for direct statistical comparison of microbial community profiles from pristine (control) and polluted environments to assess significant differences between these community structure and (2) to indicate bacterial groups that are responsive (negative; death and positive; stimulated) to environmental stress caused by perchlorate. Once a method has been successfully

developed for a limited group of contaminants, the technique can be easily adapted for other groups of pollutants not included in the proposed study.

Recent research has demonstrated that many bacterial groups in the rhizosphere of plants in polluted soils that reduce perchlorate (e.g. *Proteobacteria*, *Pseudomonas*, *Bacillus, etc.*) are capable of degrading PHC and TCE. Therefore, we hypothesize that indicator bacterial groups will be revealed in the soils by TRFLP fingerprinting that are positively responsive to PHC, TCE and perchlorate contamination. Conversely, we expect to find bacterial groups, which are negatively affected by perchlorate. To complement these molecular (culture independent) techniques, enrichment cultures of the numerically important perchlorate degraders will be isolated. These bacterial isolates will be characterized by the sequencing of their 16S rRNA gene and the TRFLP pattern of all isolates, which grew in all three enrichments, will be determined. Herein, our primary objective is to use T-RFLP and DNA-hybridization to evaluate the microbial community response and contribution to biodegradation in the rhizosphere (Rhizodegradation) during phytoremediation of perchlorate. The T-RFLP fingerprints will be used to monitor and track the response of both culturable and non-culturable bacterial phylotypes to phytoremediation of perchlorate contaminated water.

Experimental Procedure. A pre-rooted willow tree grown hydroponically was dosed with perchlorate to stimulate the biodegradation of perchlorate by root zone bacteria. Water samples were taken from the root zone and analyzed for perchlorate three times a week. After the first dose of perchlorate was completely removed from the bioreactor, water sample "A" was taken from the rhizosphere of the dosed willow for the profiling of the rhizosphere microbial community. The complete removal of perchlorate from the willow bioreactor indicated that rhizodegradation of perchlorate had been initiated and the likelihood of detecting perchlorate-degrading bacteria in solution was high.

Another aqueous sample was taken for microbial community profiling after one more month of sustaining the degradation of perchlorate in the bioreactor. During the latter period the willow tree was dosed with perchlorate multiple times. The DNA profiles of Samples "A" and "B" were profiled and compared to determine if there is a change in the microbial community during rhizodegradation of perchlorate.

Briefly, community DNA was extracted form the bioreactors and 16S rDNA from bacteria present in the community were PCR amplified. This technique uses a PCR in which one of the primers is fluorescently labeled. Primers (27F and 785R) were designed to be nondiscriminating, amplifying nearly all 16S rDNAs (Figure 19). After amplification, the PCR products were digested with the restriction enzyme *Alu*, generating fragments with different lengths, depending on the 16S DNA sequence of the bacteria analyzed and on the specificity of the enzymes. The fluorescent end-labeled fragments were separated by capillary electrophoresis and detected with laser-induced fluorescence on automated DNA-sequencers (ABI 310). The resulting frequency distribution of terminal restriction fragment sizes from the DNA digestion represents a phylotypic profile (fingerprint) of the community that reflects the community diversity, composition and structure.

Experimental Results. The Terminal Restriction Length Polymorphism (TRFLP) profiles indicate that there is a difference in the microbial community structures between Figures 19A and B. Recall that sample A was taken at the on-set of rhizodegradation and sample B when rhizodegradation predominated in the experiments. The number of bacterial populations appears higher in B than A (see figures 20A, B). This suggests that, as rhizodegradation of perchlorate progresses there is a shift in the microbial community. Figures A and B indicate that the bacterial community of A is dominated by bacterial populations which in some case is not active in B. These results suggest that the bacterial community is responsive to perchlorate degradation in the root zone and the presence of perchlorate degrading bacteria populations.

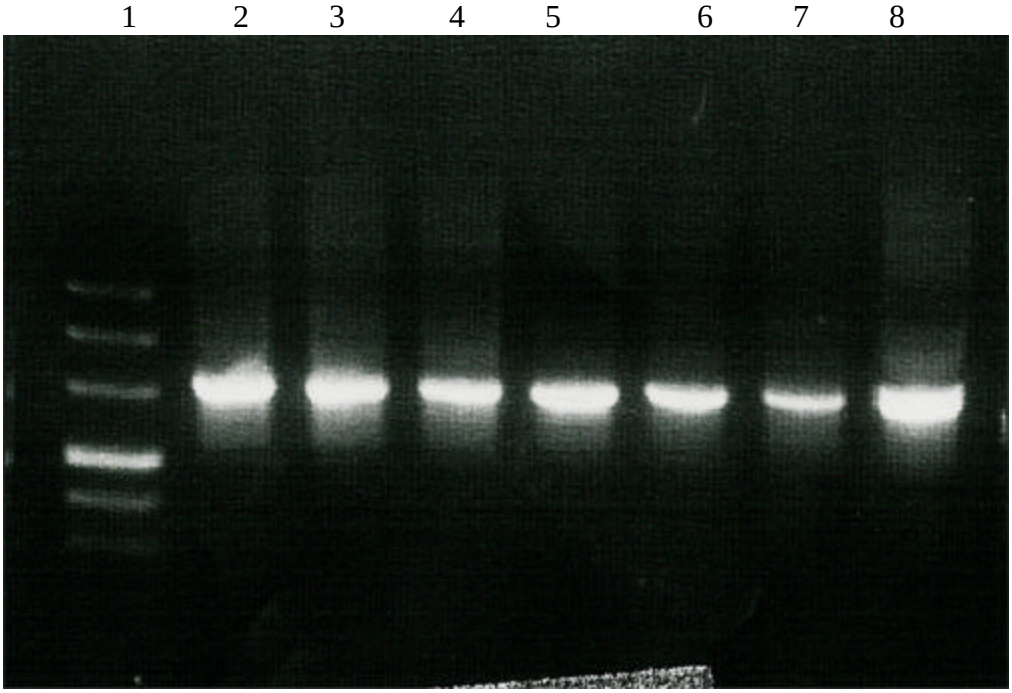


Figure 19. PCR amplification of extracted DNA with Fluorescent primers. Lane 1, molecular weight marker VIII, lanes 2-4, sample A; lanes 5-7 sample B, lane 8, positive control

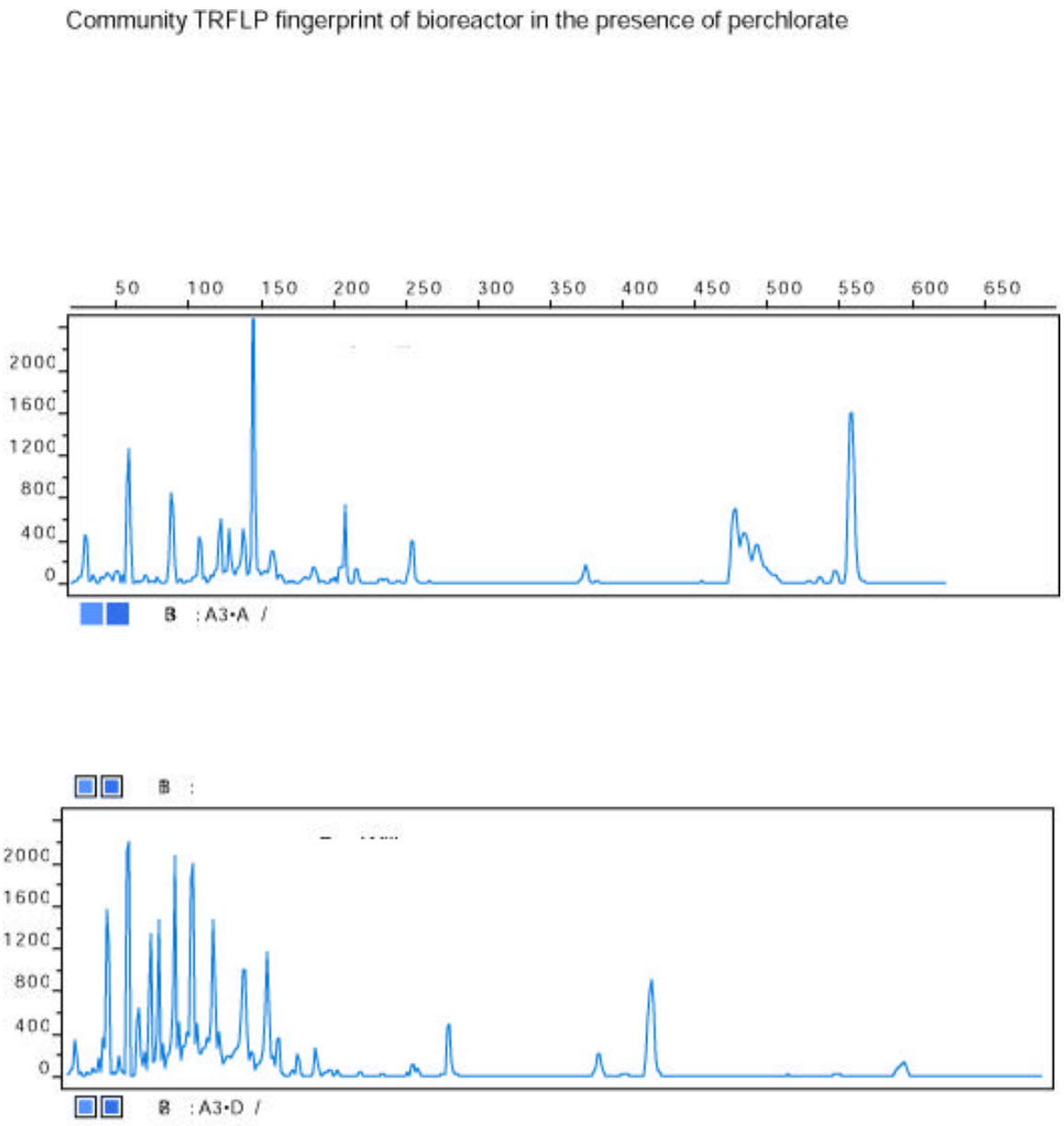


Figure 20. Bacteria community fingerprint of willow bioreactors dosed with perchlorate.

5. Phytoremediation of Mixed Contaminants (TCE and Perchlorate)

Two different willow species, *Salix caroliniana* (coastal plain willow) and *Salix nigra* (black willow) were planted in hydroponics, sealed bioreactors consisting of 2 L Erlenmeyer glass flasks outfitted with side sampling and feeding ports. Each reactor was dosed using aqueous stocks of perchlorate and trichlorothylene (TCE). To clarify many aspects of the removal of the individual contaminants from the mixed-contaminant system, four different sets of experiments were conducted (Table 7). The first set of experiments was conducted in the Spring of 2000, which initiated the design of the more detailed experiments conducted in 2001.

Table 7. Summary of experiments used in this study.

Study	salix species	number of reactors	perchlorate concentration [mg/L]	TCE concentration [mg/L]	duration of study
1	S. caroliniana	2	200	30	76 & 95
	S. caroliniana	2	200	40	95
	control	1	200	40	14
2	S. nigra	2	200	30	10 & 58
	S. nigra	2	200 & 275	40	38 & 45
	contol	1	200	35	75
3	S. nigra	2	0	40	15 & 46
	control	1	0	40	46
4*	S. nigra	2	10	10	32
	S. nigra	2	20	10	32
	S. nigra	2	100	10	32
	control	2	10	10	32

* preliminary experiments conducted in 2000

The removal of TCE from the rhizosphere of the reactors is shown in Figure 21. Kinetic data were analyzed using the zero-order and pseudo first-order models, respectively. The term "pseudo-first-order" is used in this study rather than a simple first-order kinetic model, since many variables are involved in contaminant removal including root mass, weather, plant health, level of microbial activity, and the contaminant concentration. Removal of TCE was described by a pseudo-first-order kinetic model ($r^2 > 0.88$) for all reactors in Study 1 (Table 8).

Table 8. Kinetics of contaminant removal from the rhizosphere in Studies 1, 2, and 3.

STUDY 1	Kinetics of TCE removal				
	reactor	30/200	30/200	40/200	40/200
	duration of exp [d]	95	76	95	95
	order of reaction	first	first	first	first
	correlation coef.	0.93	0.88	0.99	0.98
	rate constant k	0.015	0.016	0.022	0.041
	half-life [d]	46.8	42.8	31.1	17.1
	TSCF	0.80	1.13	0.49	0.29

STUDY 1	Kinetics of perchlorate removal				
	reactor	30/200	30/200	40/200	40/200
	duration of exp [d]	95	76	95	95
	order of reaction	zero	zero	zero	zero
	correlation coef.	0.92	0.92	0.95	0.77
	rate constant k	1.34	7.80	1.73	1.75
	half-life [d]	74.5	12.8	57.7	57.3
	mean (half-lives) = 50.6; SD 26.4				

STUDY 2	Kinetics of TCE removal					
	reactor	30/200	30/200	40/200	40/275	35/200 blank
	duration of exp [d]	58	10	45	38	75
	order of reaction	zero	zero	zero	zero	zero
	correlation coef.	0.97	0.92	0.97	0.95	0.18
	rate constant k	0.273	0.473	0.339	0.344	0.026
	half-life [d]	40.9	22.2	42.1	40.6	472.4
	TSCF	1.29	1.67	1.68	2.08	

STUDY 2	Kinetics of perchlorate removal				
	reactor	30/200	30/200	40/200	40/275
	duration of exp [d]	58	10	45	38
	order of reaction	zero	zero	zero	zero
	correlation coef.	0.92	0.98	0.91	0.86
	rate constant k	2.90	17.72	3.58	5.11
	half-life [d]	34.5	5.6	27.9	26.9
	mean (half-lives) = 23.7; SD 12.5				

STUDY 3	Kinetics of TCE removal		
	reactor	40/0	40/0
	duration of exp [d]	46	15
	order of reaction	zero	zero
	correlation coef.	0.97	0.83
	rate constant k	0.386	0.461
	half-life [d]	31.2	30.3

Reactors with higher TCE concentrations exhibited shorter half-lives, though the half-lives for duplicate experiments are very different. In Studies 2 and 3, TCE removal was described by the zero-order kinetics model for all reactors ($r^2 > 0.83$) suggesting that the reaction kinetics did not depend on the initial TCE concentration. Half-lives of the dosed planted bioreactors ranged from 22 to 42 days and the reactions were described by zero-order kinetics. The half-lives (30 days) obtained for experiments in Study 3 suggest that these plants exhibited similar kinetics, though the reactors were dosed with only one contaminant. This suggests that TCE removal from the rhizosphere is not influenced by the presence of perchlorate at the concentrations and conditions used in these experiments. The estimated loss of TCE in control bioreactors was about 20%, which was attributed to sorption to the submerged roots and stem.

The rate of removal of perchlorate from the rhizosphere of willow trees dosed with different concentrations of TCE is shown in Figure 22. No significant change in the concentration of perchlorate was observed in the blank control. The kinetic data in Table 8 indicate that faster rates of perchlorate removal from the planted bioreactors were observed with *S. nigra* (mean half-life = 23.7 $\pm$ 12.5 days) than with *S. caroliniana* (mean half-life = 50.6 $\pm$ 26.4 days). The average observed half-lives of perchlorate for *S. caroliniana* is greater than that for *S. nigra* by a factor of two. Despite the high values of standard deviation for *S. caroliniana* it appears that *S. nigra* were better suited to phytoremediation of perchlorate under the conditions of this study. Overall, the half-lives ranged from 5.6 to 74.5 days with a mean of 37.2 $\pm$ 23.9 days. The wide variability in the half-lives for experiments conducted under the same environmental conditions using the same plant species suggests that other factors influence the kinetics of perchlorate removal.

Based on visual inspection of the plants, willows with a higher root mass performed better than willows with a smaller root mass. Compared to similar experiments conducted only with perchlorate-dosed willows, the half-lives for bioreactors dosed with both perchlorate and TCE were longer than those observed in willow planted bioreactors dosed with only perchlorate (See Appendices A and D). The longer half-lives obtained in this study could be due to the competition for electrons by perchlorate and TCE, which are both terminal electron acceptors (TEAs) or to the inhibitory effects of TCE and its metabolites in the rhizosphere to the growth of perchlorate-degrading microorganisms. TCE may not be toxic to the young willow trees, but may slow the development of root-zone colonizing bacteria. As shown in Table 8, no correlation between the rate of removal of perchlorate and the initial TCE concentration in the bioreactor was observed.

It was observed in our most recent studies that the degradation of perchlorate in the rhizosphere produces oxygen and leads to an increase in Eh. We hypothesized that the change of redox potential caused by the degradation of perchlorate also influences the degradation of TCE. The aerobic conditions during perchlorate removal and in the period immediately following could favor the oxidative degradation pathway of TCE. Further studies are needed to test these hypotheses.

The main phytoremediation mechanisms for the mixed-contaminants systems included phyto-uptake (phytoaccumulation and phytodegradation) and rhizodegradation. Phyto-uptake appears to predominate at the beginning of the experiments until the root zone colonizing microorganisms have been biostimulated to degrade perchlorate (see Figure 23 as an example). If the removal were dominated by phyto-uptake, the pseudo-first-order equation would be expected to describe the kinetic data. Good mass balances based on root zone metabolites suggest that most of the perchlorate is degraded in the rhizosphere rather than phytoaccumulated or phytodegraded in the plant.

The good plant growth observed during these experiments indicated that the roots were sufficiently supplied with oxygen, despite the increasingly reducing atmosphere of the bulk solution. The detection of metabolites in the rhizosphere and in extracts of different organs of the willow trees provided evidence of oxidative transformation, while the presence of small concentrations of reductive dechlorination products indicated phytoreduction. The non-competitive removal of perchlorate and TCE from the same contaminated water by willow trees was observed. For the same initial concentration, perchlorate was removed from solution at a faster rate than TCE. The rate of TCE removal from solution increased with the evapotranspiration rate of the tree. Aerobic, anoxic and anaerobic conditions were created in the rhizosphere of trees during phytoremediation. Redox conditions in the rhizosphere were influenced by the natural light and dark cycles, weather, and the type and amount of contaminant present in the rhizosphere.

TCE was primarily removed from the bioreactors by uptake and biosorption, thus the presence of perchlorate did not impede the removal of TCT from solution by plants. However, the removal of perchlorate from solution was plant-microbe mediated (rhizodegradation) and the presence of TCE in the root zone solution appeared to have slowed, but not completely inhibited, the biostimulation of root zone colonizing perchlorate degraders. This led to slower perchlorate

removal rates than those observed in planted bioreactors dosed only with perchlorate. Given the above findings, we believe that phytoremediation has potential applications in the clean up of mixed-contaminant plumes of chlorinated organic solvents and perchlorate.

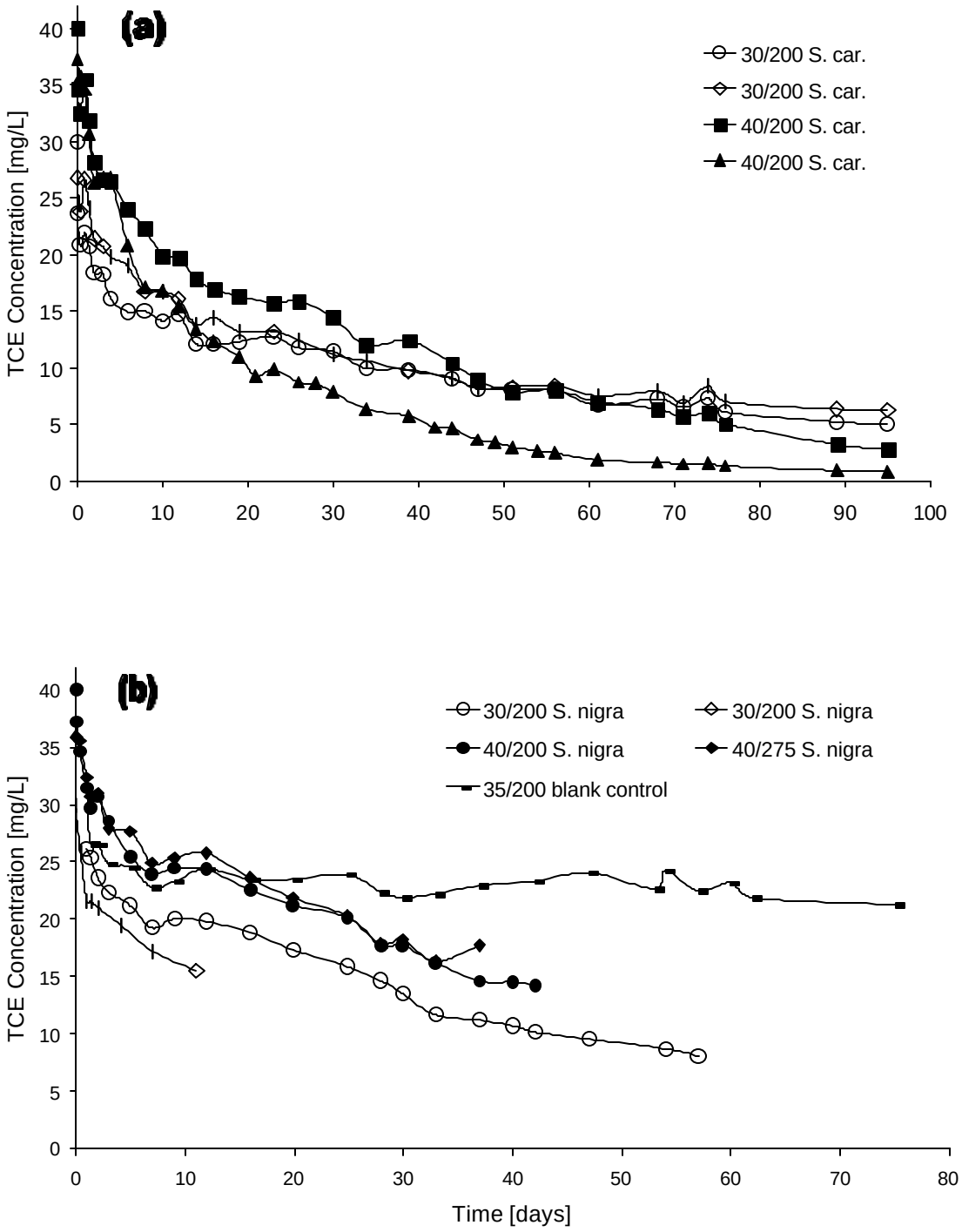


Figure 21. Removal of TCE from rhizosphere dosed with mixed contaminants (TCE, Perchlorate). (a) Study 1 with *S. caroliniana*. (b) Study 2 with *S. nigra*.

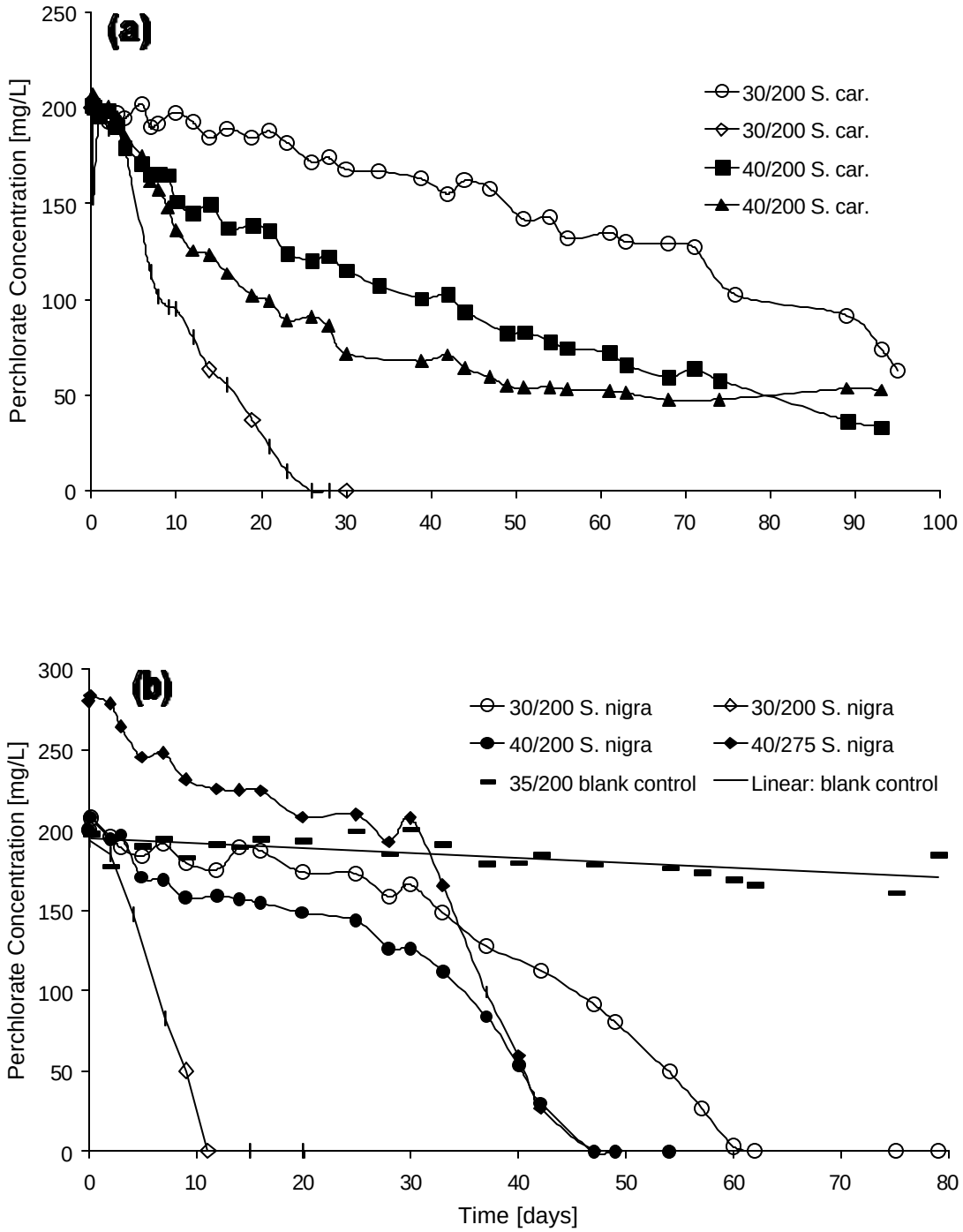


Figure 22. Removal of perchlorate from rhizosphere dosed with mixed contaminants (TCE, Perchlorate). (a) Study 1 with *S. caroliniana*. (b) Study 2 with *S. nigra*.

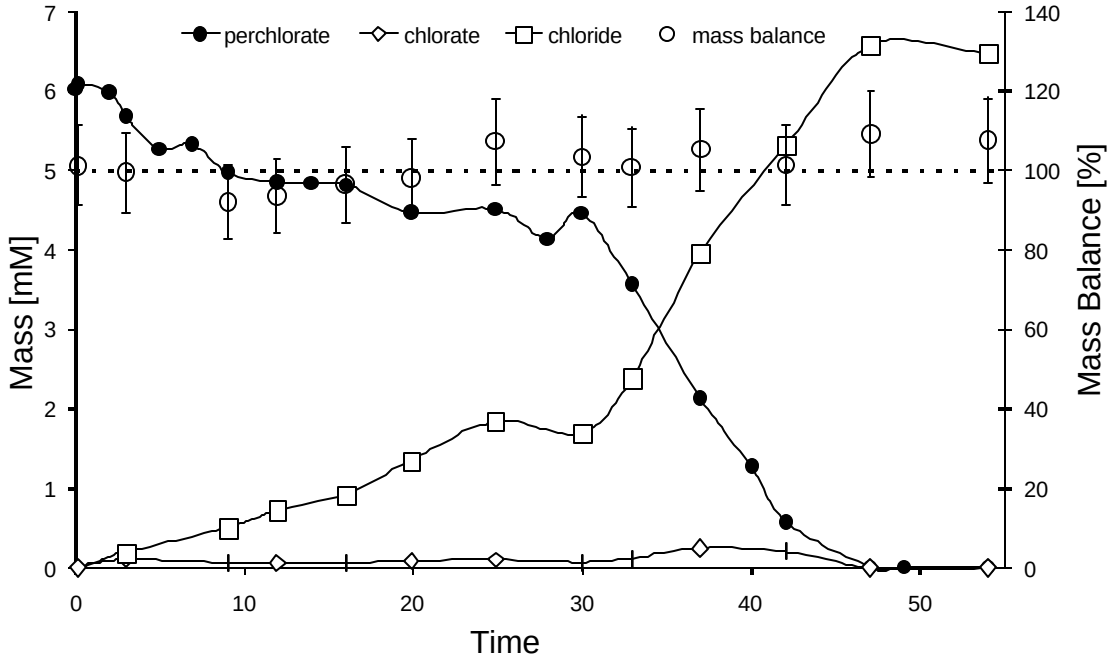


Figure 23. Mass Balance: Changes of molar mass of perchlorate and its degradation products chlorate, and chloride in the rhizosphere during the experiment. Mass balance is given in % (second y-axis). Data were corrected for background chloride. The greater than 100% mass recovery of chloride based solely on rhizodegradation of perchlorate is attributed to chloride contributed by rhizodegradation of a small fraction of TCE. 40/275 *S. nigra*.

6. Publications from Study

- Nzengung, V.A., Penning, H., and O'Niell, W. 2002. Phytoremediation of Perchlorate-Contaminated Water by Willow Trees Grown under Different Redox Conditions (In Review).
- Penning, H. and Nzengung, V.A. 2002. Decontamination of Perchlorate and Trichloroethylene Contaminated Water using Willow Trees (In review).
- Nzengung, V. A. and McCutcheon, S.C. Chapter 29: Degradation of Perchlorate by Plants. IN Phytoremediation: Scientific Advances to Manage Contamination by Organic Compounds. Editors: Steven C. McCutcheon and Jerald L. Schnoor. (IN PRESS)
- Dondero, Anna Christina. 2001. Phytoremediation of perchlorate under greenhouse and natural conditions. Masters Thesis University of Georgia Library 2001.
- Nzengung, V. A., Wang C. 2000. Influences on Phytoremediation of Perchlorate Contaminated Water. American Chemical Society (ACS) Special Symposium Series: Perchlorate in the Environment. Editor: Urbansky. Kluwer Academic/Plenum Publishers, New York. Chapter 21, pp 219 - 229.
- Nzengung, V. A., O'Niell, L.W., Adesida A. 2000. Treatment of Perchlorate Contaminated Water in Microbial Mat, Algae, and Ebb-and-Flow Hydroponic Bioreactors. Symposium Series: Case Studies in the Remediation of Chlorinated and Recalcitrant Compounds. Editors: Godage B. Wickramanayake, Arun R. Gavaskar, James T. Gibbs, and Jeffrey L. Means. Battelle Press, Columbus, Ohio. 2(7), Pp 101-106
- Nzengung, V. A., Wang, C., Harvey, G., McCutcheon, S.C., and Wolfe, N.L. 1999. Phytoremediation of Perchlorate Contaminated Water: Laboratory Studies. Symposium Series: Fifth International Symposium on *In Situ* and *On-Site* Bioremediation: Phytoremediation. Editors; Leeson Andrea and B. C. Alleman. Battelle Press, pp 239-244.

- Nzengung, V. A., Wang, C., Harvey, G., 1999. Plant-mediated transformation of perchlorate into chloride, *Environmental Science & Technology*, vol. 33, pp. 1470-1478.

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- Nzengung, V.A. 1999. Phytodegradation Kinetics and Pathways of Perchlorate. Presented to USEPA-NERL, Athens, GA.
- Nzengung, V.A. 1998. Laboratory Characterization of Phytotransformation Products of PCE, TCE and Perchlorate. For US Airforce, Wright Patterson AFB, Dayton, OH and USEPA-NERL, Athens, GA.

Papers presented at meetings, conferences, seminars, etc.

- Nzengung, V. A., Anna Dondero and O'Niell, W. 2001. Phytodegradation and Rhizodegradation of Trichloroethylene and Perchlorate. In-Situ and On-Site

Bioremediation. The Sixth International Symposium, San Diego, California. June 4 – 7, 2001.

- Nzengung, V. A., Anna Dondero. 2001. The role of green plants in the removal of perchlorate from hydroponics and organic-rich soils systems. SETAC 22nd Annual Meeting. 11-15 November, 2001. Baltimore, MD.

- Nzengung, V. A., O'Niell, L.W., Adesida A. 2000. Treatment of Perchlorate Contaminated Water in Microbial Mat, Algae, and Ebb-and-Flow Hydroponic Bioreactors. Symposium Series: Case Studies in the Remediation of Chlorinated and Recalcitrant Compounds. Editors: Godage B. Wickramanayake, Arun R. Gavaskar, James T. Gibbs, and Jeffrey L. Means. Battelle Press, Columbus, Ohio. 2(7), Pp 101-106.

- Nzengung, V. A., C. Wang, Harvey, G., McCutcheon, S.C., and Wolfe, N. L. 1998. Phytoremediation of perchlorate contaminated water. 4th Annual Strategic Environmental Research and Development Program (SERDP) Symposium, Washington, DC. December 1 - 3, 1998.

- Nzengung, V. A., Anna Dondero and O'Niell, W. 2001. Phytodegradation and Rhizodegradation of Trichloroethylene and Perchlorate. In-Situ and On-Site Bioremediation. The Sixth International Symposium, San Diego, California. June 4 – 7, 2001.

- Nzengung, V. A., O'Niell, W., Adesida, A. 2000. Treatment of perchlorate contaminated water in microbial mat, algae, and ebb-and-flow hydroponic bioreactors. Symposium Series. 2nd International Conference on Remediation of Chlorinated and Recalcitrant Compounds. Monterey, California, May 22-25, 2000.

- Nzengung, V.A., Das, K.C., Kastner, J., and Browder, G.A. 2001. Pilot scale in-situ bioremediation of perchlorate-contaminated soils at the Longhorn Army Ammunition Plant in Karnack, Texas. SETAC 22nd Annual Meeting. 11-15 November, 2001. Baltimore, MD.

- Nzengung, V. A., Anna Dondero. 2001. The role of green plants in the removal of perchlorate from hydroponics and organic-rich soils systems. SETAC 22nd Annual Meeting. 11-15 November, 2001. Baltimore, MD.

Briefings

- Nzengung, V. A. 1998. Phytoremediation of perchlorate contaminated water. Briefing to Perchlorate Working Group at Wright Patterson Air Force Base, Dayton, OH. August 19th, 1998.

- Nzengung, V. A. 1998. Phytoremediation of perchlorate contaminated water. Briefing to Perchlorate Working Group at Brooks Air Force Base, San Antonio, TX. December 16 - 17th, 1998.

Participants

- Anna Dondero: Graduated with a MSc. Degree in May 2001
- Andre Haynes: Undergraduate Research Assistant, Graduated with a BSc. Degree in Biological Sciences in December 2001
- Grace Browder: Research Technician
- Stacey Lewis: Student/Research Assistant