A Mass Balance Field Study of the Phytoremediation of Trichloroethylene with Transgenic Poplars Genetically Modified with Cytochrome P450 2E1

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A thesis
submitted in partial fulfillment of the
requirements for the degree of

Master of Science in Civil Engineering

University of Washington
2013

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Program Authorized to Offer Degree:
Civil and Environmental Engineering

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1 Abstract

In a 6-year field study, transgenic poplar (*Populas tremula x Populus alba*) genetically modified with mammalian cytochrome P450 2E1 (CYP2E1) was evaluated for its ability to enhance degradation of trichloroethylene (TCE) in the subsurface. In previous laboratory studies, the transgenic poplar demonstrated greatly increased metabolism towards a variety of organic contaminants.[1] Degradation of TCE in the field was studied in three test beds: a test bed containing twelve CYP2E1 hybrid poplar trees, a second test bed containing twelve wild-type hybrid poplar trees, and a third unplanted control bed. A mass-balance was performed to determine the fate of TCE and quantify primary loss pathways. Quantitative, real-time PCR (qPCR) assays targeting microbial genes involved in TCE degradation and field soil microcosm studies characterized microbial activity in bed soil. The transgenic CYP2E1 poplars demonstrated enhanced degradation of TCE in the field though not in proportion to enhanced removal observed in laboratory studies. Total chlorinated ethene removal was 87% in the CYP2E1 test bed, 85% in the wild-type, and 34% in the unplanted control bed in the 2012 growing season. Evapotranspiration of TCE from transgenic leaves was reduced by 80% and diffusion of TCE from transgenic stem was reduced by 90%. Chloride ion accumulated in the vadose zone soil of the planted test beds that approximately corresponded to the TCE loss, suggesting that dehalogenation was the primary loss fate. The application of a steady-state plant model indicated that the enhanced rate of metabolism in r2E1 roots was insufficient to substantially increase uptake of TCE in a field setting. This study demonstrates the importance of field tests of transgenic plants for phytoremediation applications. Inherent differences in mass transfer processes between laboratory and field experiments can limit the effectiveness of enhanced *in planta* metabolism.

2 Introduction

Due to its widespread use and unmitigated disposal, trichloroethylene (TCE) is among the most common soil and groundwater contaminants in the United States.[2] TCE is currently present in over 54% of US EPA Superfund sites.[3] Prior to awareness of its hazardous impacts, TCE was used extensively as a metal degreaser and dry cleaning solvent. Though it can be degraded biologically by aerobic and anaerobic microbial activity,[4] it is environmentally persistent. TCE is known to cause liver damage and central nervous system disruption and is a suspected human carcinogen.[5] As a result, efficient and cost-effective removal of TCE has become a priority for protection of human and environmental health nationwide.

Phytoremediation is a technology that utilizes plants for the treatment of pollutants in soil and groundwater. Plants can facilitate contaminant removal by chemical extraction, volatilization, stabilization, degradation *in planta* or by endophytes, and rhizodegradation.[6] Phytoremediation offers several advantages over conventional remediation techniques including low cost, minimal environmental impact, and low external energy requirements. Although phytoremediation has been implemented successfully, important drawbacks to its widespread use can include the substantial time necessary to achieve remediation goals or uncertainty regarding contaminant fate. [7] A potential strategy to alleviate both lengthy time requirements and chemical fate uncertainty is the use of transgenic plants with enhanced removal capabilities for specific pollutants. The use of transgenic plants in phytoremediation has been reviewed previously.[7-11]

A series of laboratory and field experiments investigated poplar removal of TCE. Plant-related processes for removal of TCE include rhizodegradation and, following plant uptake,

volatilization, sequestration, and metabolism. Hybrid poplars were shown to be directly capable of TCE biotransformation and mineralization in experiments with poplar axenic cell cultures.[12] Both laboratory and field studies have successfully demonstrated that poplar trees are capable of uptake and metabolism of TCE. [13-16] TCE can enter trees via uptake with transpiration water,[15, 17, 18] diffusion into roots from soil matrix air and water,[19] and diffusion into aerial plant tissue from the atmosphere. Poplars metabolize TCE in plant tissues by an oxidative pathway similar to that found in mammals, producing the metabolites trichloroethanol, dichloroacetic acid, trichloroacetic acid, and trichloroethanol-glucoside.[20] In addition to metabolism in tree tissues, TCE is subject to diffusion through trunk, branches, and leaves to the atmosphere and sorption to tree tissues. [21-26]

Degradation processes in the rhizosphere of trees may be important for TCE loss in a phytoremediation setting. The rhizosphere is known to support elevated microbial and fungal growth due to organic materials excreted by plant roots such as organic acids, amino acids, phenolic compounds, and carbohydrates.[27, 28] Phenolic compounds secreted by plant roots have been shown to support the growth of PCB-degrading bacteria.[29, 30] Several studies have reported enhanced degradation of TCE in vegetated soils as compared to unvegetated soils by both microbial cometabolism and anaerobic reductive dechlorination. [31-35] Anderson and Walton reported enhanced mineralization of [C^{14}]TCE in vegetated soils compared to unvegetated soils; the difference was attributed to the presence of root exudates.[32] Phytoremediation field studies have shown microbial reductive chlorination to be enhanced in planted areas as compared unvegetated areas.[33, 34] Eberts et al. reported that eastern cottonwood (*Populus deltoides*) input of organic carbon to the rhizosphere stimulated aerobic microbial activity, subsequently lowering

dissolved oxygen concentrations and generating methanogenic conditions suitable for reductive dechlorination of TCE.[34] Godsy et al. reported the development of an anaerobic ecosystem near eastern cottonwood trees planted over a TCE plume.[33] Root exudates appeared to provide organic carbon in low dissolved oxygen pockets to stimulate reductive dechlorination activities.

Transgenic poplar trees have been developed with enhanced capabilities for TCE degradation. A series of laboratory experiments tested the performance of hybrid poplar *(Populus tremula* x *Populus alba*) and tobacco (*Nicotiana tabacum cv. Xanthii)* expressing mammalian cytochrome 450 2E1 (CYP2E1). [1, 36, 37] The CYP2E1 modified plants were shown to degrade a wide range of volatile organic compounds (VOCs) at rates up to 640 times those of vector control cuttings. In the present study, we tested the performance of hybrid poplars expressing CYP2E1 under field conditions. TCE degradation was measured and compared in three test beds: one containing twelve CYP2E1 transgenic trees, the second containing twelve hybrid poplars (*Populus tremula* x *Populus alba*), and the third containing no trees.

In order to determine the fate of TCE and quantify important loss pathways, a chlorine mass balance was performed on each test bed. The following parameters were measured: (1) influent and effluent mass of chlorinated ethene and chloride for each test bed, (2) volatilization of TCE from the tree trunks and leaves, (3) accumulation of metabolites in plant tissues, (4) volatilization of TCE from the soil, and (5) accumulation of chloride ion in the soil. In addition, we performed quantitative qPCR assays and soil microcosm studies to enumerate microbial involvement in TCE degradation in the field soil and differentiate between tree and microbial contribution to TCE removal. A steady-state phytoremediation model was applied to gain insight into internal

tree processes. The study was conducted from 2007 – 2012. The 2012 mass balance is discussed in detail and is representative of growing season results each year following establishment of the trees.

To our knowledge, this study is among the first to test transgenic plant performance in phytoremediation of an important environmental organic contaminant under field conditions.

3 Materials and Methods

3.1 Field Site Description

Three separate test beds were located at the phytoremediation field site. Bed 6 contained twelve transgenic hybrid poplar trees, Bed 8 contained twelve hybrid poplar trees, and Bed 3 was an unplanted control that contained no trees. The transgenic hybrid poplar (*Populus tremula* x *Populus alba*) clones INRA 717-1B4 were transformed with rabbit *CYP2E1* under the cauliflower mosaic virus (CaMV) 35S promoter, as previously described.[1] Line 78 was determined to have the highest activity and was selected for field tests. Thirteen transgenic poplars (r2E1) and thirteen untransformed INRA 717-1B4 clones were propagated, hardened off, and planted at the field site on April 11, 2007. One r2E1 tree and one INRA-717 tree not dosed with TCE were planted in a separate location as controls. The presence of the transgene CYP2E1 was verified by PCR following planting.

The beds were adjacent and hydraulically independent from one another. The test beds were 1.5 m deep, 3.0 m wide, and 5.7 m long and filled with 0.3 m of coarse sand overlain by 1.2 m of soil. The soil was a sandy loam, consisting of 67.2% sand, 26.6% silt, and 6.2% clay; total organic matter content was 6.6%. Each test bed was lined with two 60 mil polyethylene liners and had influent and effluent wells. Both the influent and effluent wells consisted of perforated 2-inch PVC T-pipes located in the bottom sand layer of the bed. The influent end of each bed contained a 100-L polyethylene dosing tank connected by PVC to the T-pipe. The bottom of the bed was sloped at 1:40 to facilitate flow towards the effluent end.

3.2 Water Management and Chemical Dosing

The three test beds received equal amounts of TCE during the growing season, from May 10, 2011 – October 31, 2011 in 2011 and from June 19, 2012 - October 28, 2012 in 2012. The target influent TCE concentration was 15 mg L^{-1} in 2011 and 30 mg L^{-1} in 2012. A saturated aqueous TCE solution was diluted in the 100-L dosing tank to the target TCE concentration, well mixed, and immediately added directly to the bed through a ball valve system. The actual influent TCE concentration varied with ambient temperatures, but was equal for all beds. To simulate equivalent subsurface conditions, water levels were maintained between 25-30 cm in the planted beds. Water levels were measured daily by inserting a graduated rod into the well. Different volumes of water were pumped into and removed from the beds in order to maintain the target water level. The planted beds required more water due to the transpiration of the trees. Municipal water was used for irrigation and was added to the planted beds through the influent well or by surface watering.

3.3 Water Sampling and Analysis

Influent water samples of were collected from a sampling port located between the dosing tank and influent well. For irrigation water, influent samples were taken directly from the on-site municipal source. Effluent samples were collected each time water was pumped out from the bed, at least weekly. Prior to effluent sampling, a minimum of 19 liters of water was pumped out from the bed and the system was allowed to equilibrate for 45 minutes. Effluent samples were collected as previously described.[16]

Analysis of the volatile organic compounds (VOCs) TCE, cDCE, and VC in the influent and effluent water samples was according to EPA Method 8260A as previously described,[38] with modifications. The Perkin Elmer Autosystem XL Gas Chromatograph was connected to a Teledyne Tekmar AQUATek 70 Vial autosampler and Tekmar 3000 Purge & Trap Concentrator. Liquid samples were purged with helium for 11 min at 30°C onto the concentrator and desorbed at 225°C for 4 min for analysis on the GC.

Analysis of chloride ion in the influent and effluent water was performed with a Dionex AS40 Automated Sampler connected to a Dionex DX-120 ion chromatograph (IC) as previously described.[38] Flowrate was 1.20 ml/min.

Concentrations of chlorinated ethene and chloride were calculated based on seven-point calibration curves with external standards. Secondary verifications were also external.

Dissolved oxygen (DO) and temperature readings were measured approximately every two weeks in 2012 using a YSI 5000 Dissolved Oxygen Meter. Prior to measurement, a minimum of 19 L of water was pumped from each bed. The beds were allowed to equilibrate for 30 minutes prior to measurement. Water was pumped at from the base of the effluent well into a two-barb Erlenmeyer flask using a peristaltic pump and Teflon tubing. The flask was stirred using a magnetic stirrer with stirbar. During the measurement, water was pumped continuously. The YSI DO probe was placed in the Erlenmeyer flask and the dissolved oxygen measurement was recorded once readings became stable.

3.4 Soil Sampling and Analysis

Soil samples were taken at four depths with a T-handle soil auger: 10 cm, 30 cm, 60 cm, and 100 cm below the soil surface. Samples were taken at 6 locations in each bed and were equally spaced along the length and width of the bed in a 2 x 3 grid. Soil samples were analyzed for chloride ion and TCE. Samples were collected at the beginning and end of each growing season in order to measure soil chloride loss/accumulation. Samples were taken April 14, 2011 and October 17, 2011 for the 2011 growing season and on May 22, 2012, August 21, 2012, and October 16, 2012 for the 2012 growing season. Chloride was extracted from the soil samples as previously described.[39] To measure TCE concentration in the soil matrix, samples were placed into pre-weighed 40 mL volatile organic analysis (VOA) vials containing 10 mL purge and trap grade methanol. Samples were transported to the lab on ice and held at 4°C until analysis. Prior to analysis, samples were lightly vortexed and centrifuged at 200 g for five minutes. For analysis, an aliquot (250 µL) of methanol was transferred using gastight syringes to a 40 mL VOA vial containing de-ionized water without headspace by injection through the septa, and this sample was analyzed according to EPA Method 8260A as previously described.[38]

Chloride and TCE accumulation in the soil was determined by summing the mean concentration of the respective analyte in each soil sample multiplied by the volume of the bed represented by the sample in the unsaturated zone of the bed. The mass of soil per unit volume was determined by measuring soil porosity in each bed with a bulk density soil corer.

3.5 Soil and Stem Volatilization

Volatilization of TCE from the soil was measured in the 2012 growing season with a soil flux chamber, as previously described.[40] Two sets of volatilization measurements were taken for Bed 6 (r2E1) and Bed 8 (WT). Volatilization was not measured for Bed 3 (unplanted) but was approximated by the flux measured for Bed 8 (WT).The analysis of the mass of VOCs collected on activated carbon tubes was as previously described.[38] Tree stem (trunk) volatilization of TCE was measured with a glass diffusion trap at three stem heights as previously described.[39] Four measurements were taken at each height in both beds with the exception of three measurements at the 0.57 m stem height in Bed 8 (WT). Volatilization was not measured during the 2011 growing season.

3.6 Evapotranspiration

Leaf transpiration of TCE was measured in the 2012 growing season with Teflon leaf bags as previously described.[16] Leaf bags were set up simultaneously in the two planted beds to obtain measurements at similar conditions. Six sets of leaf bag measurements were performed in Bed 6 (r2E1) and seven samples in Bed 8 (WT). The transpiration leaf area was determined from the area of the leaves contained in the sampling bag. Total leaf area per bed was estimated with a published allometric linear regression equation for leaf area of *Populus Tremuloides.*[41]

3.7 TCE and Metabolites in Tree Tissue

Tree tissue samples were collected and analyzed in the 2012 growing season for TCE and metabolites trichloroethanol (TCOH), trichloroacetic acid (TCAA), dichloroacetic acid (DCAA), and trichloroethanol-glucoside (TCOH-glucoside) as previously described.[38] Root, branch, leaf,

and stem tissue samples were taken from three different trees in each planted bed for the analysis. Stem tissue was taken at breast height. For stem tissue, two of the three samples were combined in one metabolite extraction. Only leaf tissue was analyzed for TCOH-glucoside.

Contributions of tissue metabolites to the overall chlorine mass balance were estimated by multiplying the tissue metabolite concentration by the respective mass of the tree compartment. Biomass estimations were performed for root, stem, branch, and leaves with published allometric linear regression equations for poplar. Several biomass estimations (a minimum of three estimations) were performed for each compartment with equations from different sources; the median value was selected for final use in biomass estimation.

3.8 Root Chloride Efflux: Measurements and Analysis

In the 2012 growing season, tree roots were isolated to measure the efflux of chloride by roots in the vadose zone of the planted beds. Portions of roots at the surface in each bed were unburied, brushed to remove excess soil, sterilized with a 12 ppm Iodophor iodine liquid solution (the first experiment only, see below), enclosed in 8 x 14 inch FoodSaver© plastic bags with sterile sand, and sealed with a bag sealer. Aquarium sealant or Teflon tape was used to seal the bag around the root extruding from the two open ends of the bag. Prior to use, the sand was autoclaved, washed three times in the lab with de-ionized water, dried at 100°C for 24 hours, and tested for chloride ion in triplicate on the Dionex DX-120 IC as previously described.[39] The concentration of chloride in the sand was measured at the end of the experimental period as previously described.[39] Each sand sample was extracted in either duplicate or triplicate. Two experiments were performed: the first from August 28 - September 17 and the second from September 17 -

October 4. In the second experiment, roots were not sterilized with the Iodophor iodine solution. Four root sections were enclosed in each bed for the first experiment and three root sections were enclosed in each bed for the second experiment. Three roots were enclosed from the undosed control bed containing one r2E1 and one wild-type poplar for the second experimental period.

3.9 Bioavailable Copper in Soil

Bioavailable copper concentrations were measured in soil pore water using the method of diffusive gradients in thin films (DGT) as previously described.[42] Deployment time for copper flux into the DGT device was 24 hours. DGT measurements were performed on duplicate soil samples taken from each planted bed at the 30 cm depth.

3.10 Quantitative Real-Time PCR and PCR Assays

Quantitative real-time PCR (qPCR) and PCR was performed on DNA extracted from soil samples in each bed. Soil samples for qPCR/PCR analysis were taken from four depths in the bed as described above. Soil samples were placed in sterile 50 mL plastic centrifuge tubes, transported on ice, and stored at -80°C in the lab until analysis. Genes analyzed by qPCR were *Dehalococcoides* 16S rRNA, *pmoA* encoding for the α subunit of particulate methane monooxyganse (pMMO), toluene monooxygenase, toluene dioxygenase, phenol monooxygenase, and eubacterial 16S rRNA. A summary of primers and probes utilized for qPCR and PCR analyses are shown in

Table 1. All qPCR analyses were performed with the Applied Biosystems StepOnePlus Real-Time PCR System (Applied Biosystems). All qPCR cycle conditions were as follows: 2 minutes at 50°C, 10 minutes at 95°C, and 40 cycles of 15 seconds at 95°C followed by 1 minute at the specific annealing temperature. SYBR Green qPCR assays included a melting curve analysis.

DNA from soil samples was extracted and purified using a phenol/chloroform extraction method as previously described, [43] with modifications. The final extraction step was omitted since RNA analysis was not required. Soil was weighed into a lysis tube (Lysing Matrix E; MP Biomedicals) for extraction. Sample disruption was performed in a FastPrep-24 Cell Disrupter (MP Biomedicals). Following extraction, DNA soil extracts were cleaned with Zymo DNA Clean & Concentrator-5 Kit (Zymo Research). DNA extracts were routinely tested for quantity and quality on the Nanodrop ND - 1000 (ThermoFisher Scientific) by observance of the A260/230 and A260/280 ratios, visual spectra, and concentration. All DNA and primer/probe solutions were stored at -20°C until analysis. Working primer/probe solutions, DNA solutions, and qPCR/PCR reactions utilized molecular grade water.

DNA extraction efficiency was measured for samples analyzed for *pmoA* in order to quantify gene counts per gram of soil. Weighed soil samples were individually spiked with a known amount of an exogenous reference gene which was quantified following the extraction by qPCR. The utilized reference gene was *xplA*, a gene involved in microbial degradation of RDX.[44] Percent recovery of *xplA* was used to infer extraction efficiency and normalize targeted gene counts per gram of soil. Soil samples received 2 µL of *xplA* contained in either plasmid pJN105

or pCR-Blunt II-TOPO (Invitrogen) at a known concentration. The Taqman qPCR method for *xplA* detection was as previously described.[44]

The qPCR method for *Dehalococcoides* 16S rRNA followed a previously developed Taqman approach. [45] DNA for qPCR standards was obtained from *Escherichia coli* carrying plasmid TOPO-pCR2.1 (Invitrogen) with the cloned 16S rRNA gene of *Dehalococcoides* sp. strain BAV1. Clones were grown overnight and the plasmids were extracted with QIAprep Spin MiniPrep kit (Qiagen) according to the kit protocol. The plasmid concentration was measured with the Nanodrop ND – 1000. The DNA was used for preparation of standard curves for qPCR (1.69×10^8 copies/ng DNA) with a 10-fold dilution series. Cycle conditions, probe and primer concentrations, and materials were as previously described[45] with modifications. Total reaction volume was 20 µL with 2 µL DNA in each reaction tube. Two negative controls were included in each run and consisted of the reaction mix, probe, primers, and molecular grade water.

The cloned *Dehalococcoides* 16S rRNA was also used as a copy number standard for 16S rRNA gene amplification with universal eubacterial primers 27F[46] and 338R[47]. For 16S rRNA gene amplification, reaction mixtures (20 µL) contained: 2 uL of DNA, 7.2 µL of molecular grade water, 0.4 µL of each primer, and 10 µL of *Power* SYBR Green PCR Master Mix (Applied Biosystems).

DNA for qPCR standards for pMMO was obtained from the type II methanotroph *Methylosinus trichosporium* OB3b. The *pmoA* gene was amplified by PCR with a gene-specific primer pair[48] from total *M. trichosporium* OB3b total genomic DNA. After amplification, the *pmoA* gene

product was re-amplified by PCR to obtain high copy numbers for qPCR standard curves (1.85 x 10^9 copies/ng DNA). PCR was conducted on a BioRad iCycler. PCR reaction mixtures contained: 1 µL DNA, 0.1 µL Phusion Taq polymerase (Finnzymes), 4 µL 5X Phusion buffer solution (Finnzymes), 2 µL $MgCl_2$, 0.4 µL dNTP, 0.5 µL of each primer, and 11.5 µL of molecular-grade water. PCR cycle conditions were as follows: initial denaturation for 30 seconds at 98°C, 33-35 cycles of 6 seconds at 98°C, 22 seconds at the annealing temperature 63°C, 10 seconds at 72°C, and finally 7 minutes at 72°C. Gene concentration was measured with the Nanodrop ND – 1000. qPCR analysis for *pmoA* followed a previously developed Taqman approach.[49] Reaction mixtures (20uL) contained: 2 µL of DNA, 3.9 µL of molecular grade water, 1.8 µL of each primer, 0.5 µL of the Taqman probe, and 10 µL of Taqman Universal PCR Master Mix (Applied Biosystems).

The qPCR method for select aromatic oxygenases followed a previously developed SYBR Green approach, with modifications.[50] Reactions mixtures contained primers at final concentrations of 200 µM each. Targeted aromatic oxygenases were toluene monooxygenase, toluene dioxygenase, and phenol monooxygenase. DNA for toluene monooxgenase was obtained from *Escherichia coli* carrying the plasmid pSE380 with *Burkholderia* sp. strain JS150 toluene-4-monoxygenase (1.01 x 10^8 copies/ng DNA).[51] DNA for toluene diooxygenase was obtained from *Escherichia coli* carrying the plasmid pDTG601 with toluene dioxygenase genes *todC1C2BADE* from *Psuedomonas putida* F1 (1.06 x 10^8 copies/ng DNA).[52] DNA for phenol monooxygenase was obtained from *Escherichia coli* carrying plasmid pVI261 with phenol hydroxylase genes (*dmpKLMNOP*) of *Psuedomonas* CF600 (3.28 x 10^7 copies/ng DNA).[53] Clones were grown overnight and the plasmids were extracted with QIAprep Spin MiniPrep kit (Qiagen) according

to the kit protocol. Reaction mixtures (20 µL) contained: 2 uL of DNA, 7.2 µL of molecular grade water, 0.4 µL of each primer, and 10 µL of *Power* SYBR Green PCR Master Mix (Applied Biosystems).

Selected phenol monooxygenase qPCR products were run on 0.8% agarose gels containing 1% ethidium bromide to verify specificity of qPCR amplification. Additionally, PCR was performed on selected DNA samples as an additional verification of qPCR results. PCR reaction mixtures were as previously described. PCR cycle conditions were as follows: initial denaturation for 30 seconds at 98°C, 35 cycles of 6 seconds at 98°C, 22 seconds at 60.4°C, 5 seconds at 72°C, and finally 7 minutes at 72°C.

Table 1. Primers and Probes for qPCR and PCR.

Primer Name	Target	Sequence	Use	Reference
Dhc1200F	*Dehalococcoides* 16S rRNA	5'-CTGGAGCTAATCCCCAAAGCT-3'	taqman	[45]
Dhc1271R	*Dehalococcoides* 16S rRNA	5'-CAACTTCATGCAGGCGGG-3'	taqman	[45]
Dhc1240-Probe	*Dehalococcoides* 16S rRNA	5'-FAM-TCCTCAGTTCGGAT TGCAGGCTGAA-TAMRA-3'	taqman	[45]
xplA-taqF	*xplA*	5'-GGAGGACATGAGATGACCGCT-3'	taqman	[44]
xplA-taqR	*xplA*	5'-CCTGTTGCAGTCGCCTATACC-3'	taqman	[44]
xplAtaq-Probe	*xplA*	5'-FAM-TCCCGAATTCAGGA ACAACCCCTATCC-BHQ1-3'	taqman	[44]
pmoA 189F	*pmoA*	5'-GGNGACTGGGACTTCTGG-3'	PCR	[48]
pmoA 682R	*pmoA*	5'-GAASGCNGAGAAGAASGC-3'	PCR	[48]
pmoA-taqF	*pmoA*	5'-TTCTGGGGCTGGACCTAYTTC-3'	taqman	[49]
pmoA-taqR	*pmoA*	5'-CCGACAGCAGCAGGATGATG-3'	taqman	[49]
pmoA-Probe	*pmoA*	5'-FAM-CAGCCTTGTGTTC CCGTCCGCBCT-TAMRA-3'	taqman	[49]
RDEG-F	Toluene monooxygenase	5'-T(C/T)TC(A/C/G)AGCAT(A/C/T)C A(A/G)AC(A/C/G)GA(C/T)GA-3'	SYBR Green	[50]
RDEG-R	Toluene monooxygenase	5'-TT(A/G/T)TCG(A/G)T(A/G) AT(C/G/T)AC(A/G)TCCCA-3'	SYBR Green	[50]
PHE-F	Phenol monooxygenase	5'-GTGCTGAC(C/G)AA (C/T)CTG(C/T)TGTTC-3'	SYBR Green, PCR	[50]
PHE-R	Phenol monooxygenase	5'-CGCCAGAACCA(C/T)TT(A/G)TC-3'	SYBR Green, PCR	[50]
TOD-F	Toluene dioxygenase	5'-ACCGATGA(A/G) GA(C/T)CTGTACC-3'	SYBR Green	[50]
TOD-R	Toluene dioxygenase	5'-CTTCGGTC(A/C)AGTAGCTGGTG-3'	SYBR Green	[50]
27F	Bacterial 16S rRNA	5'-AGAGTTTGATCMTGGCTCAG-3'	SYBR Green	[46]
338R	Bacterial 16S rRNA	5'-GCTGCCTCCCGTAGGAGT-3'	SYBR Green	[47]

3.11 Soil Microcosm Incubations

Microcosm experiments were conducted in the 2012 growing season to measure TCE biodegradation in bulk bed soil (including the rhizosphere) under aerobic conditions. Approximately 2 liters of soil was collected from each test bed at a depth of 30 cm. Prior to experimentation, the soil was left in an open container in a dark fume hood for three days to remove labile TCE in the soil matrix. Roots were removed manually from soil by visual inspection. Forty-mL VOA vials were filled with approximately 10 grams of soil per vial and sealed with mininert caps. Vials were dosed with 50 µL of a 1000 TCE mg L^{-1} aqueous solution by injection through the septa to obtain a target initial equilibrium TCE aqueous concentration of approximately 1.7 mg L^{-1}. Degradation activity was observed under seven different experimental conditions with each condition performed in triplicate for each planted bed: (1) TCE only, (2) TCE and methane, (3) TCE, methane, and 500 µL of *Methylomonas* sp. strain LW13 liquid culture, (4) TCE, methane, and acetylene, (5) TCE, methane, acetylene, and 500 µL of *Methylomonas* sp. strain LW13 liquid culture, (6) TCE only in sterile soil, and (7) TCE only in sodium azide treated soil. Triplicate vials with 10 mL water and TCE only (no soil) served as experimental controls for leakage. The methane concentration was 10% by volume and the acetylene concentration was 1% by volume. Sterile soil vials were autoclaved 3 times over a 3 day period. Sodium azide treated vials received 2 mL of a 200 mg L^{-1} sodium azide aqueous solution as a biocide. *Methylomonas* sp. strain LW13 was grown for 4 days at 30°C in NMS1 medium with phosphate buffer and methane prior to inoculation in experimental vials. The headspace concentration of TCE was measured every for 6 days by analysis on a Perkin-Elmer Autosystem GC-ECD, as previously described.[37] Vials were allowed to equilibrate for two hours

following dosing and prior to the time-zero measurement. Assumed first-order degradation rate constants were determined by linear regression.

Soil collected for the microcosm study (but not placed in vials for experimentation) was tested by qPCR for gene counts of pMMO, phenol monooxygenase, and *Dehalococcoides* 16S rRNA.

3.12 Phytoremediation Modeling

A published steady-state plant model was applied to r2E1 and WT trees to gain insight into internal processes contributing to TCE removal in the r2E1 and WT tree systems. The Fruit Tree Model utilized in modeling is described in detail in Trapp, S.[54] The model considers sorption to soil; uptake with transpiration water; diffusive flux in and out of root, stem, and leaf; metabolism in root, stem, and leaf; plant growth; and sorption to root and trunk wood. The model does not consider metabolism or flux in/out of branches, sorption to bark, or a difference in diffusivity between bark and wood. Flow rate of water through the tree was given by the 2012 water balance measurements of water transpiration. Metabolism rates for r2E1 root and leaf tissue and for dormant WT root tissue were measured previously.[55] Metabolism rate in the stem was approximated by that in the root for both r2E1 and WT trees. The WT metabolism rate for leaf was approximated by the difference in oxidative metabolite levels between the r2E1 and WT leaf tissue, resulting in 40-fold reduced rate for the WT leaf compartment. The empirically determined wood-water partitioning coefficient for TCE in poplar [22] was input directly into the model. Modeling was performed for the average tree size in each bed and also for the high and low root biomass estimations. Tree compartment masses were estimated from published allometric linear-regression equations as described. Root compartment volumes were calculated

utilizing the specific gravities of poplar leaf[56] and wood[57] for use in separate residence time calculations. The soil TCE concentration was the average observed TCE concentration over the growing season in the bottom, saturated layer. The water, lipid, and gas pore content in roots and the fraction of water and gas in the stem were as designated in the model. The water flow velocity in the stem was also as designated in the model.

4 Results

4.1 2011 Field Data

4.1.1 Water Use/Balance

Total inputs and removals of water are shown in Table 2. Transpiration was 143 L/day and 128 L/day for Bed 6 (r2E1) and Bed 8 (WT), respectively, a difference in water uptake of 10%. Bed 6 (r2E1) trees transpired more water due to their larger overall size. Average water levels in the beds during the growing season were 34.6 ± 17.9 cm, and 43.4 ± 20.3 cm, and 48.6 ± 23.1 cm for Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. Water levels are shown in Figure **1** - Figure 3.

Table 2. Water balance for the 2011 growing season showing all inputs and outputs of water into the field test beds from May 10, 2011 - November 3, 2011.

	Bed 6 (r2E1) (L)	Bed 8 (WT) (L)	Bed 3 (unplanted) (L)
Rainfall	3,688	3,688	3,688
Influent	21,880	19,741	5,016
Effluent	1,136	1,052	6,045
Bed Water Accumulation	-955	-358	-2,676
Transpiration/Volatilization	25,387	22,735	5,334
Transpiration/Volatilization per day	143	128	NA

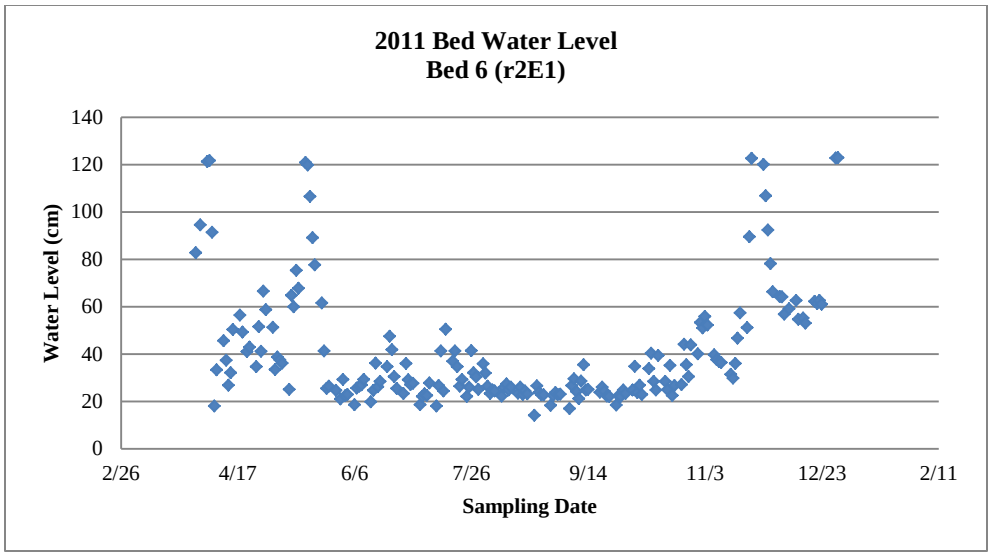

Figure 1. Water level in Bed 6 (r2E1) in 2011. Water levels are the height of water measured from the bottom of the field bed.

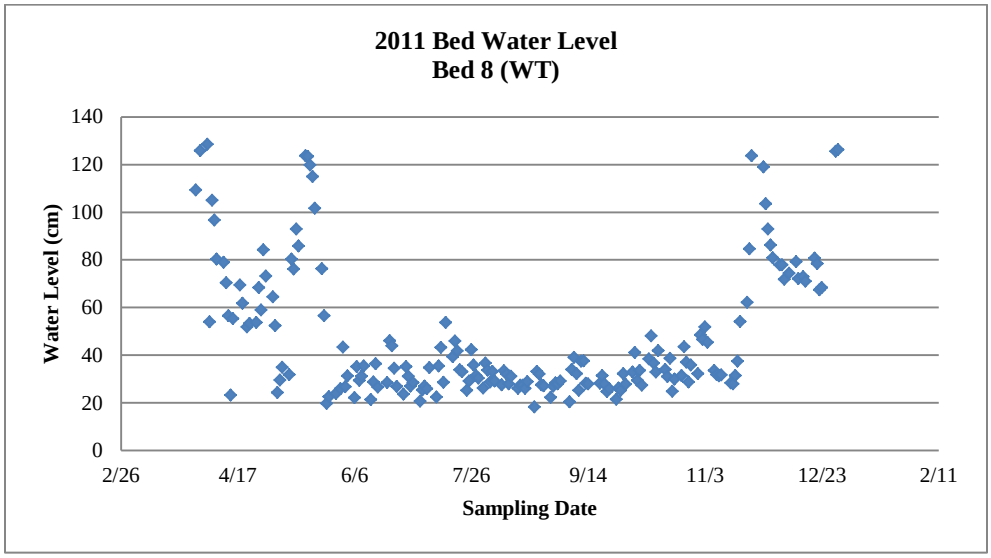

Figure 2. Water level in Bed 8 (WT) in 2011. Water levels are the height of water measured from the bottom of the field bed.

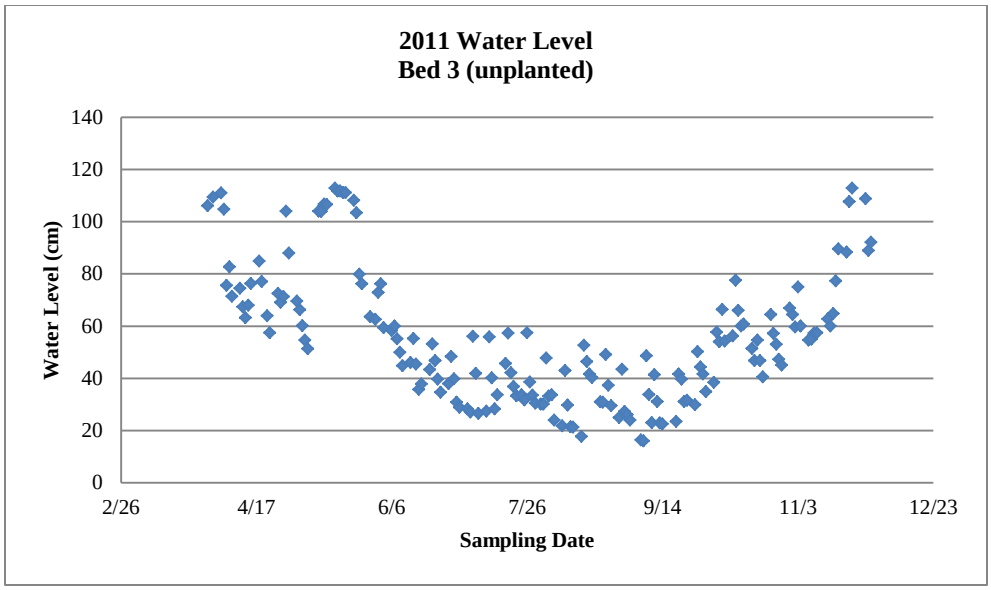

Figure 3. Water level in Bed 3 (unplanted) in 2011. Water levels are the height of water measured from the bottom of the field bed.

4.1.2 Influent and Effluent VOC and Chloride Concentrations

Influent TCE concentrations for all beds are shown in Figure 4. Effluent VOC concentration profiles of TCE, cDCE, and VC for the test beds are shown in Figure 5 - Figure 7. The average input TCE concentration was reduced by 86%, 83%, and 90% in the effluent of Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. The TCE concentration was lowest in Bed 3 (unplanted) due to dilution caused by a higher water level. Concentrations of cDCE accounted for 9%, 10%, and 19% of the total VOCs measured in effluent water and concentrations of VC accounted for 2%, 6%, and 16% of the total VOCs measured in effluent water of Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively.

In general, concentration trends in the two planted beds were similar to each other and different from the unplanted bed. Concentrations of TCE initially rose in all three beds but began to decrease at the beginning of August only in the planted beds. The downward trend in TCE concentration in the planted beds may have due to tree uptake as this trend followed the most active month of tree growth (July). In contrast, the TCE concentration increased for the entire mass balance period in the unplanted control bed. Concentrations of cDCE and VC, metabolites of anaerobic reductive dechlorination, were consistently higher in the unplanted control (Bed 3). Concentrations of cDCE began to increase in the planted beds in late October, coinciding with the beginning of leaf drop on November 3, 2011. A decrease in cDCE concentrations in Bed 3 (unplanted) in late September coincided with increasing VC concentrations. These results indicate that TCE removal is linked to tree metabolic state in the planted beds.

The effluent aqueous chloride ion concentration was generally higher in Bed 3 (unplanted) than in the planted beds and was higher in Bed 8 (WT) then in Bed 6 (r2E1) (Figure 8). These results are expected considering the respective effluent levels of cDCE and VC in each bed. Chlorine atoms are released in microbial reductive dechlorination, subsequently increasing the chlorine concentration in neighboring water. Higher levels of cDCE and VC corresponded to higher levels of chloride in the test bed effluent water.

Effluent concentrations of VOCs and chloride ion continued to be measured following the completion of the mass balance period (Figure 9 - Figure 12). Increased reductive dechlorination activity was observed in all test beds in the dormant season. Notably, output masses of cDCE and VC during the dormant season were comparable amongst the three beds. These results are consistent with a previous TCE phytoremediation field study which also observed increased reductive dechlorination during the dormant season.[34] The effluent chloride concentration increased substantially in the planted beds during the winter season. This was likely a result of heavy winter rains mobilizing chloride ion that had accumulated in the upper, unsaturated vadose zone of the planted beds during the growing season.

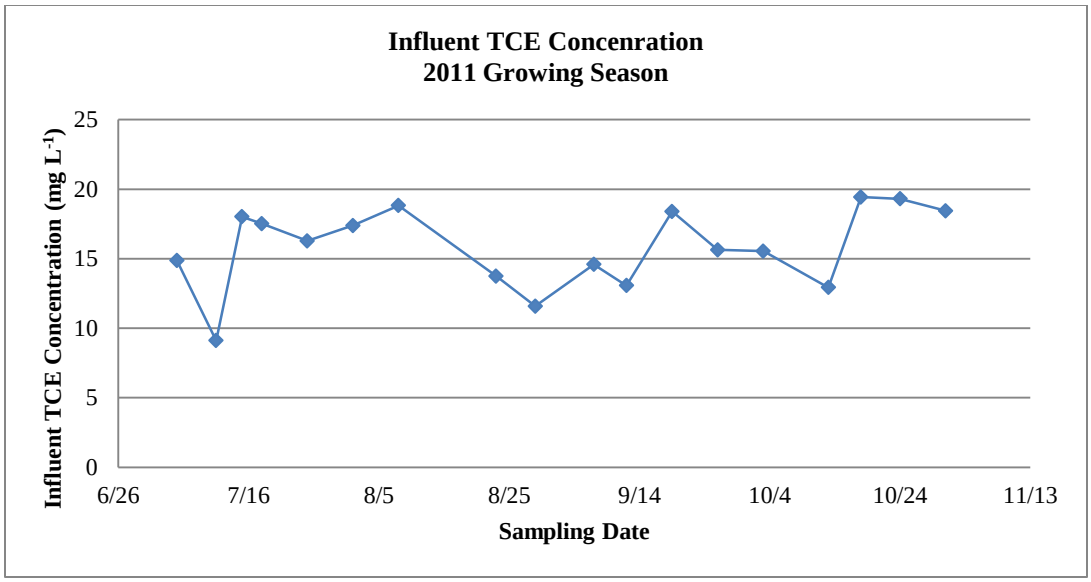

Figure 4. Influent TCE Concentration for the 2011 growing season.

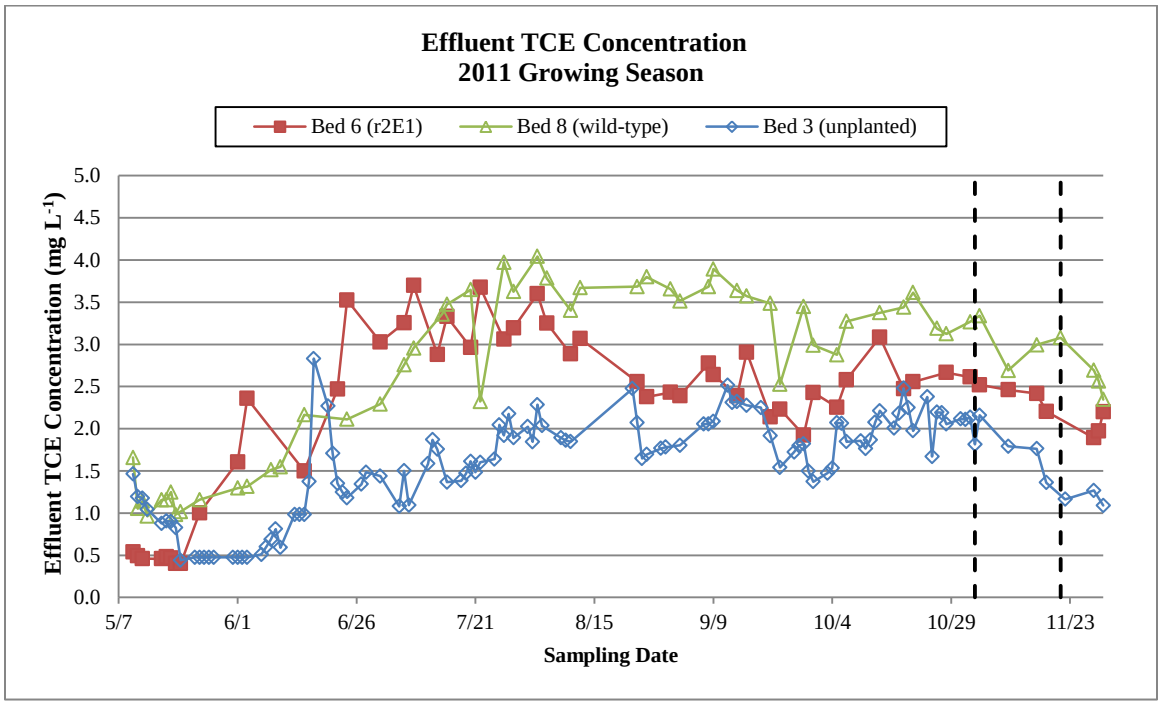

Figure 5. TCE concentration in the effluent water over the 2011 growing season in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted). Dashed black lines enclose the period of leaf fall.

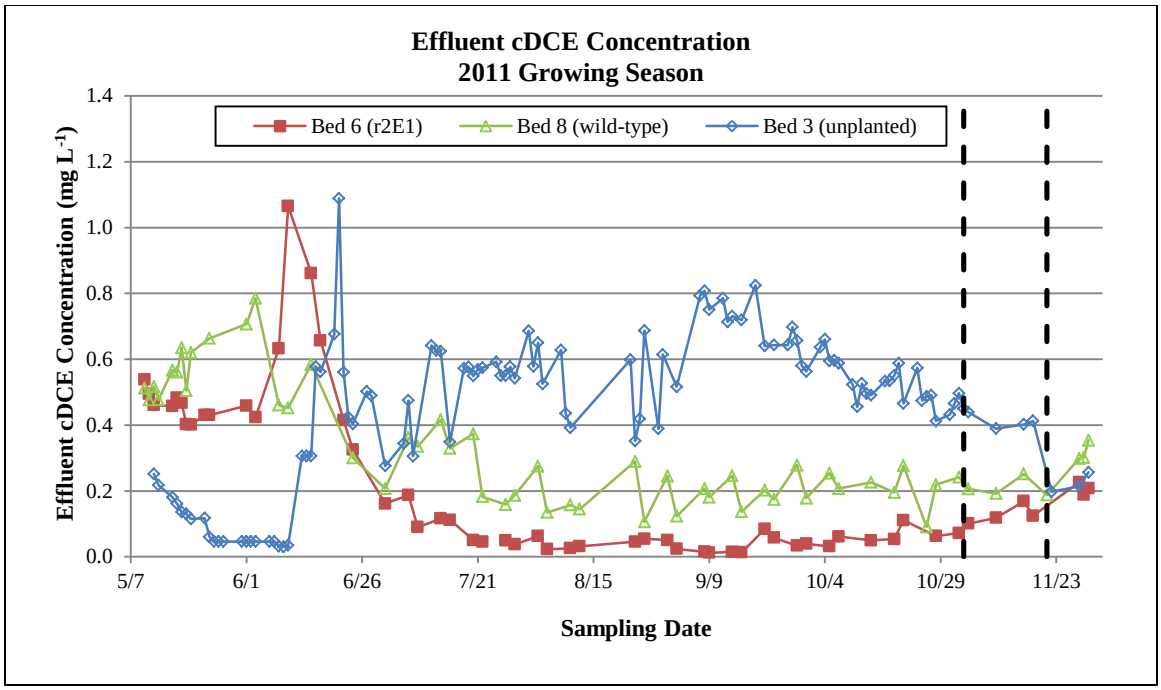

Figure 6. cDCE concentration in the effluent water over the 2011 growing season in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted). Dashed black lines enclose the period of leaf fall.

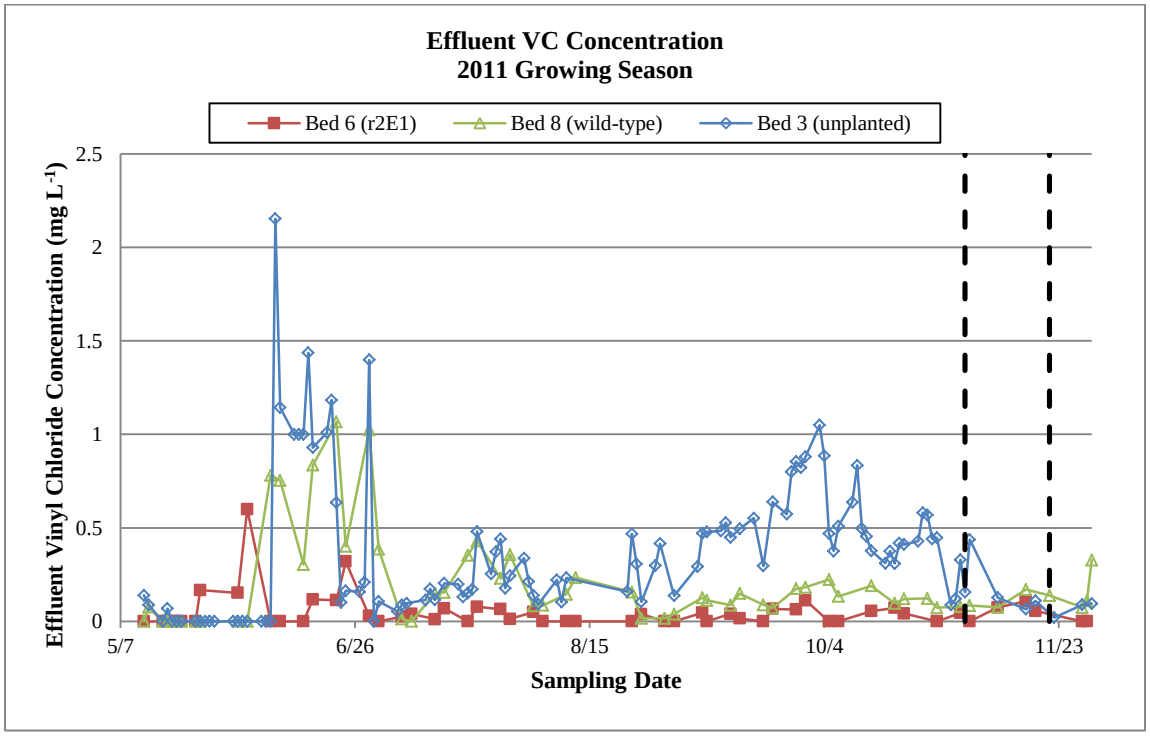

Figure 7. VC concentration in the effluent water over the 2011 growing season in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted). Dashed black lines enclose the period of leaf fall.

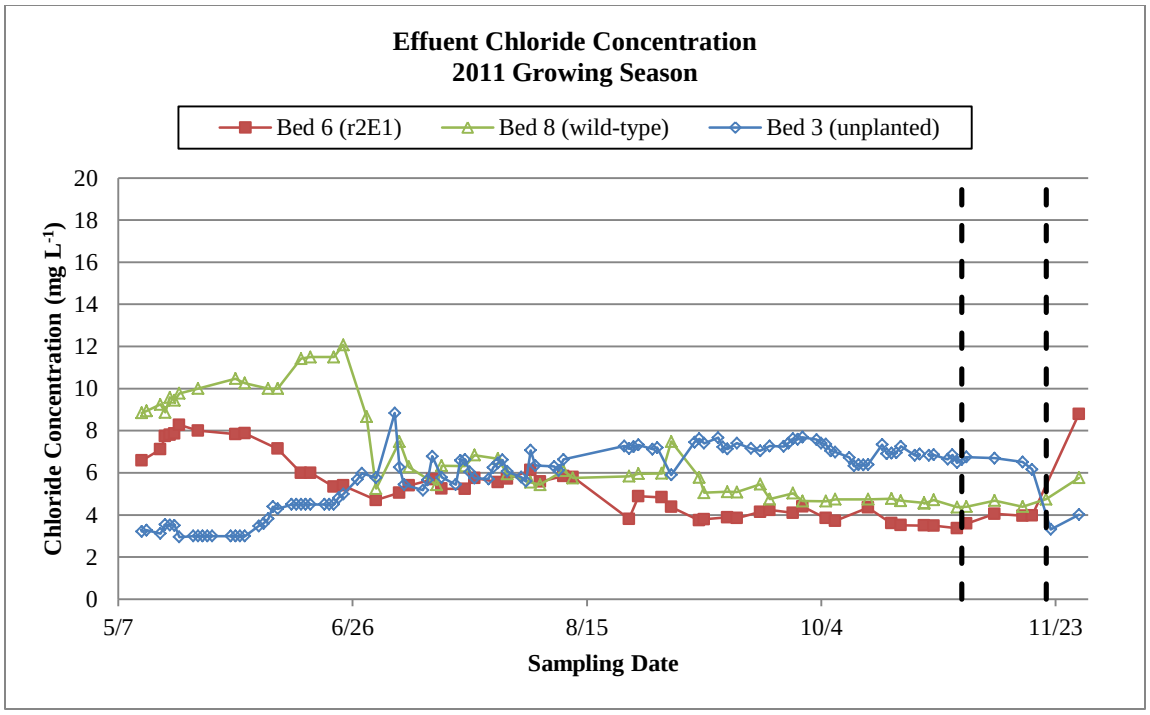

Figure 8. Chloride ion concentration in the effluent water over the growing season in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted). Dashed black lines enclose the period of leaf fall.

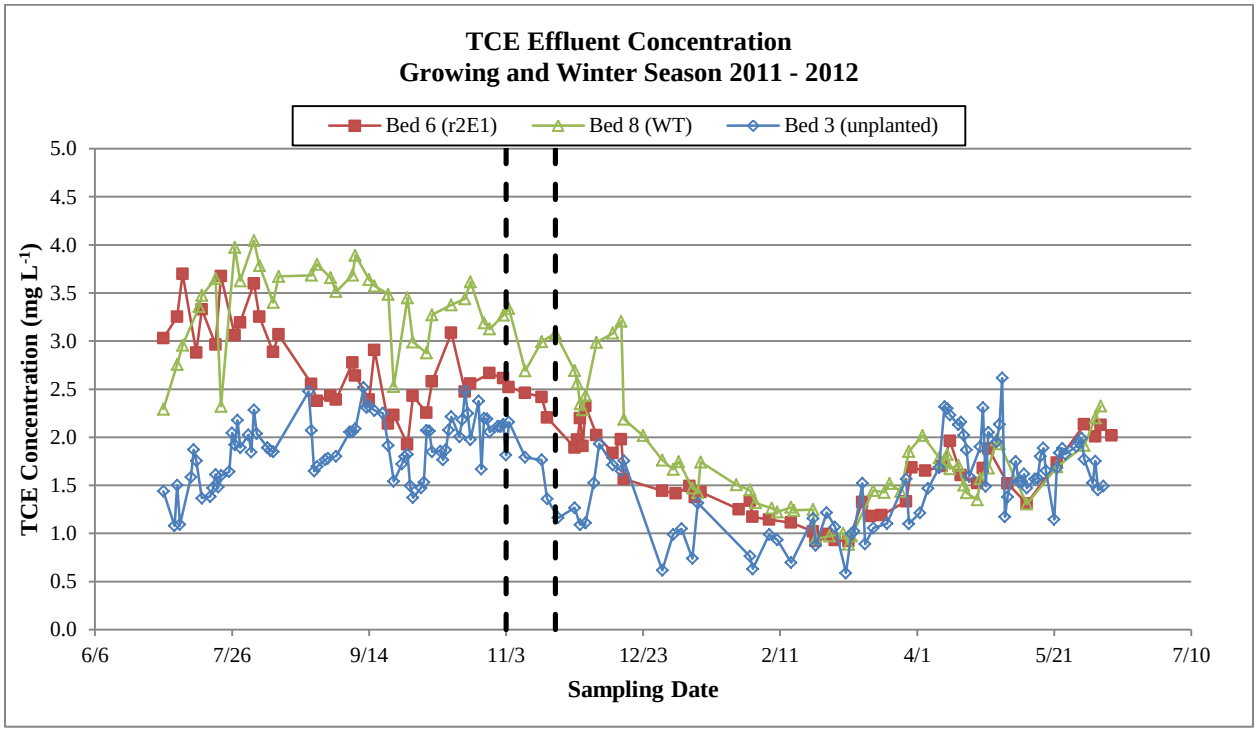

Figure 9. Effluent TCE concentration in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted) during the 2011 growing and winter season. Dashed black lines enclose the period of leaf drop.

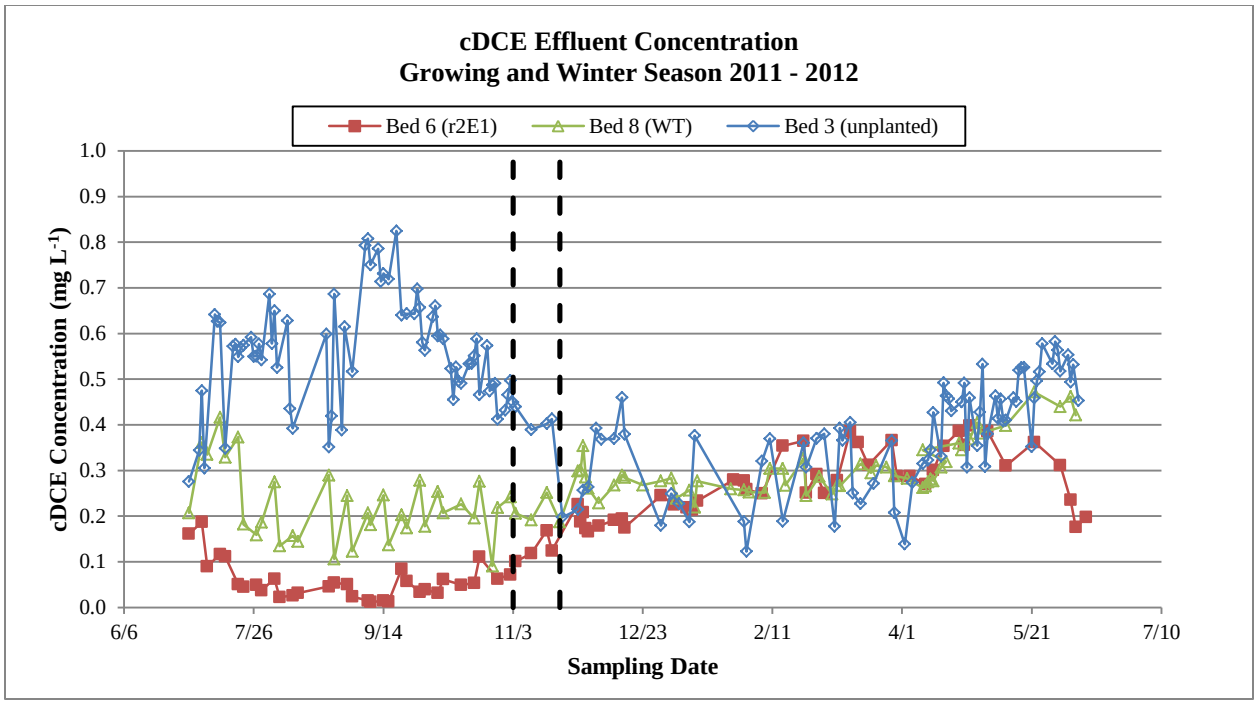

Figure 10. Effluent cDCE concentration in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted) during the 2011 growing and winter season. Dashed black lines enclose the period of leaf drop.

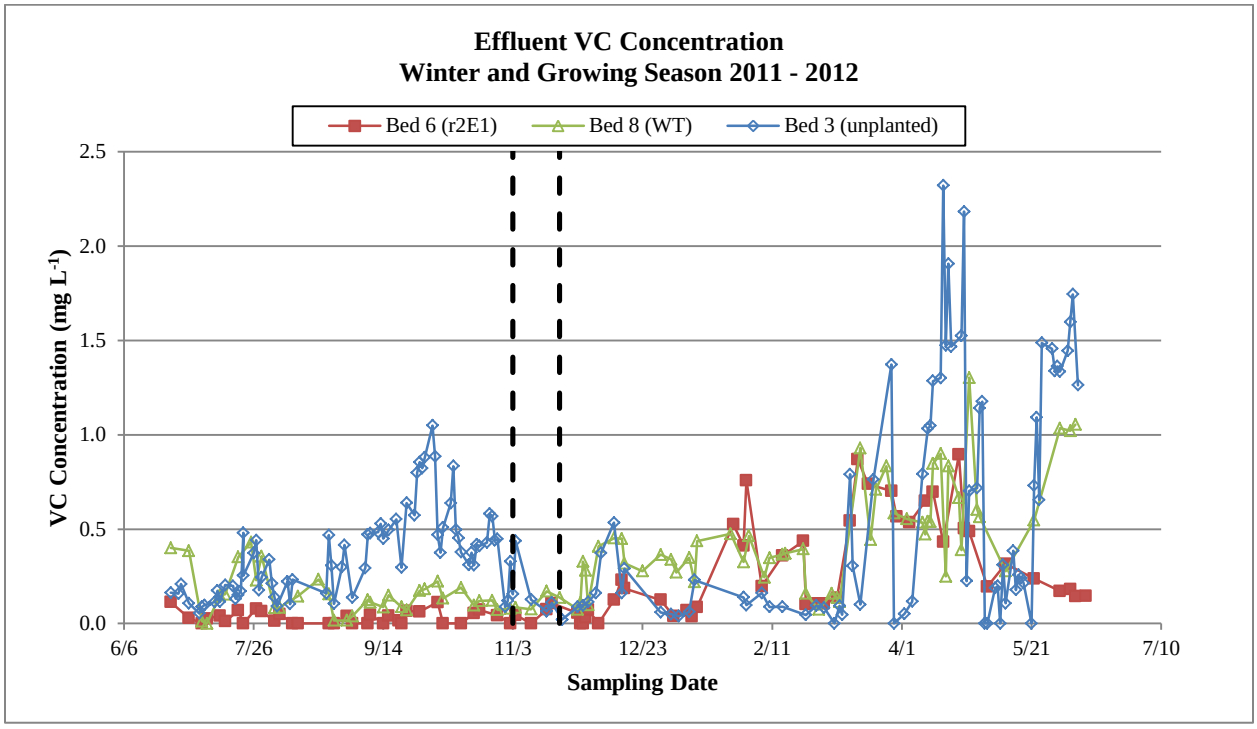

Figure 11. Effluent VC concentration in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted) during the 2011 growing and winter season. Dashed black lines enclose the period of leaf drop.

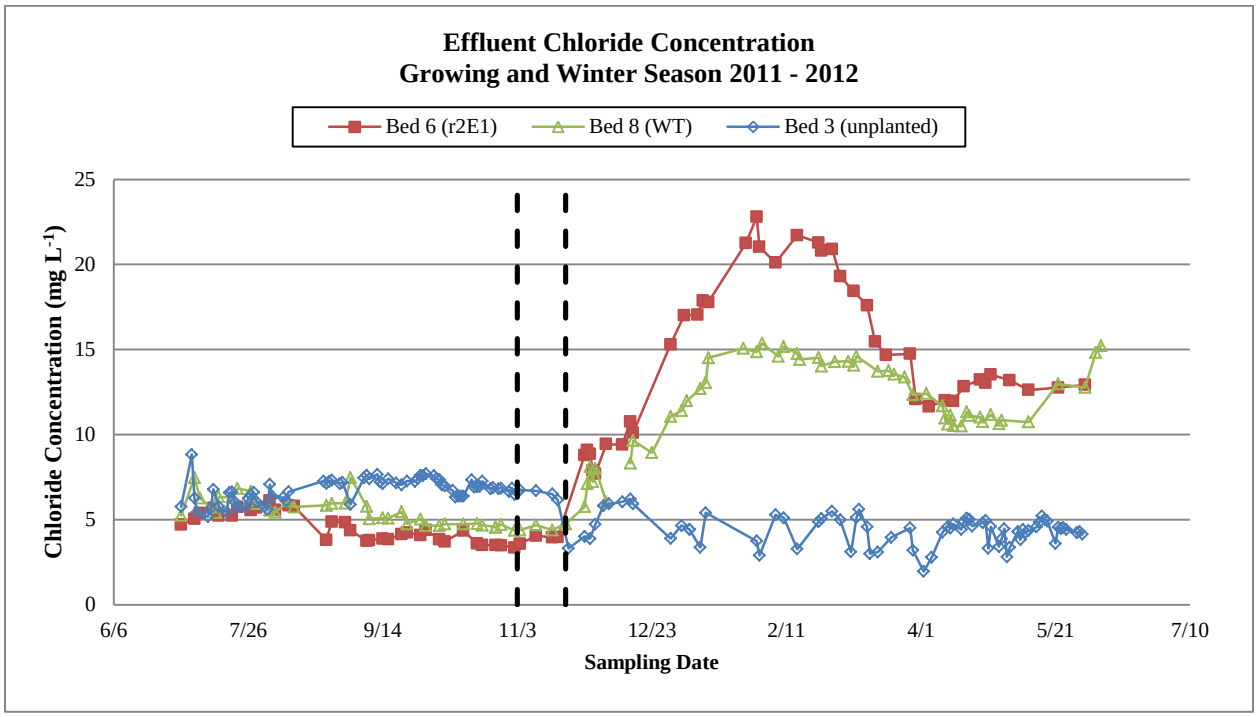

Figure 12. Effluent chloride concentration in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted) during the 2011 growing and winter season. Dashed black lines enclose the period of leaf drop.

4.1.3 Chlorinated Ethene Mass Balance

Total inputs and outputs of VOCs are displayed in Table 3. Percent recoveries of VOCs in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted) were 6%, 15%, and 28%, respectively. Bed 6 (r2E1) had the highest removal efficiency of TCE of 94%.

The effluent masses of cDCE and VC accounted for 2%, 4%, and 18% of the input TCE mass in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. Bed 3 (unplanted) had the highest recovery of cDCE and VC, products of microbial anaerobic reductive dechlorination, indicating that reductive dechlorination was a more active process in the unplanted bed.

Table 3. Total inputs and outputs of VOCs from the three field test beds during the mass balance period (May 5, 2011 - November 3, 2011). Values in parenthesis were estimated with measurements taken during the 2012 growing season.

	Inputs			**Output/Accumulation**		
	Bed 6 (r2E1)	Bed 8 (WT)	Bed 3 (unplanted)	Bed 6 (r2E1)	Bed 8 (WT)	Bed 3 (unplanted)
TCE in water (mol)	0.494	0.494	0.494	0.0197	0.0243	0.0667
cDCE in water (mol)	ND	ND	ND	0.0031	0.0052	0.0252
VC in water (mol)	ND	ND	ND	0.0007	0.0031	0.0333
VOCs Accumulated in Bed Water	NA	NA	NA	0.0037	0.0401	0.0120
Soil Volatilization (mol)	NA	NA	NA	(6.06E-05)	(2.48E-04)	(2.48E-04)
Trunk Volatilization (mol)	NA	NA	NA	(1.97E-06)	(1.38E-05)	NA
Leaf Volatilization (mol)	NA	NA	NA	(1.27E-05)	(5.26E-05)	NA
Total VOCs (mol)	0.494	0.494	0.494	0.0273	0.0731	0.1375
VOC Recovery	6%	15%	28%			

4.1.4 Soil Chloride Concentration

Average soil chloride concentrations are displayed at four depths for the beginning and end of the growing season (Figure 13 and Figure 14). The average bed soil chloride concentration increased from 2.10 ± 0.8 mg Cl^- kg^{-1}, 1.79 ± 0.9 mg Cl^- kg^{-1}, and 1.16 ± 0.6 mg Cl^- kg^{-1} soil on April 15 in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively, to 10.8 ± 7.2 mg Cl^- kg^{-1}, and 6.49 ± 7.7 mg Cl^- kg^{-1}, and 1.84 ± 0.4 mg Cl^- kg^{-1} soil in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. The high standard deviation was in part due localized biological processes specific to depth. The beds were maintained with an upper, unsaturated zone and an underlying saturated zone approximately 0.4 m in depth. There was a substantial increase in soil chloride concentration in the unsaturated vadose zone of the planted beds (Figure 14). However, no increase was observed in the vadose zone soil of the unplanted bed, indicating that the accumulation of chloride ion in the planted beds was related to the presence of the tree roots. The accumulation of chloride ion was a result of *in planta* degradation of TCE and subsequent efflux of excess chloride from roots or microbial mineralization of TCE in the bed soil. Mean chloride concentration in the bottom saturated sandy layer did not change substantially in any bed over the growing season because free chloride ions were dissolved and removed in the effluent flow of the test bed. These results are consistent with previous field studies which found an increase in soil chloride concentrations in the vadose zone over the growing season.[16, 38, 39]

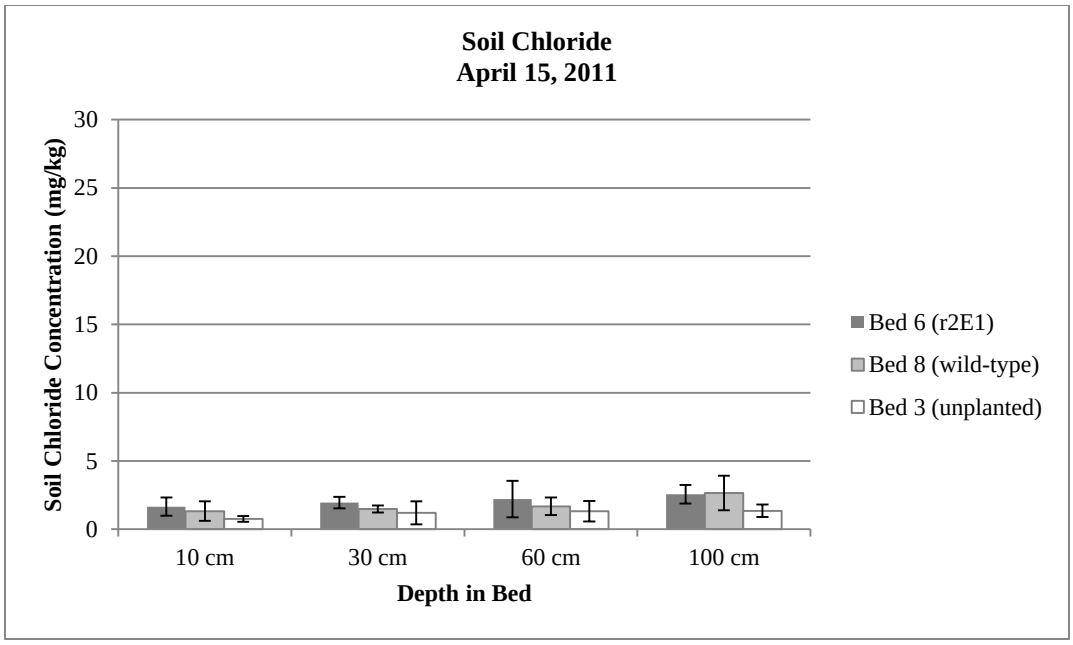

Figure 13. Average soil chloride concentration at depths of 10, 30, 60, and 100 cm for the 2011 growing season on April 15, 2011. Data bars show mean and standard error of six samples taken across the bed at the specified depth.

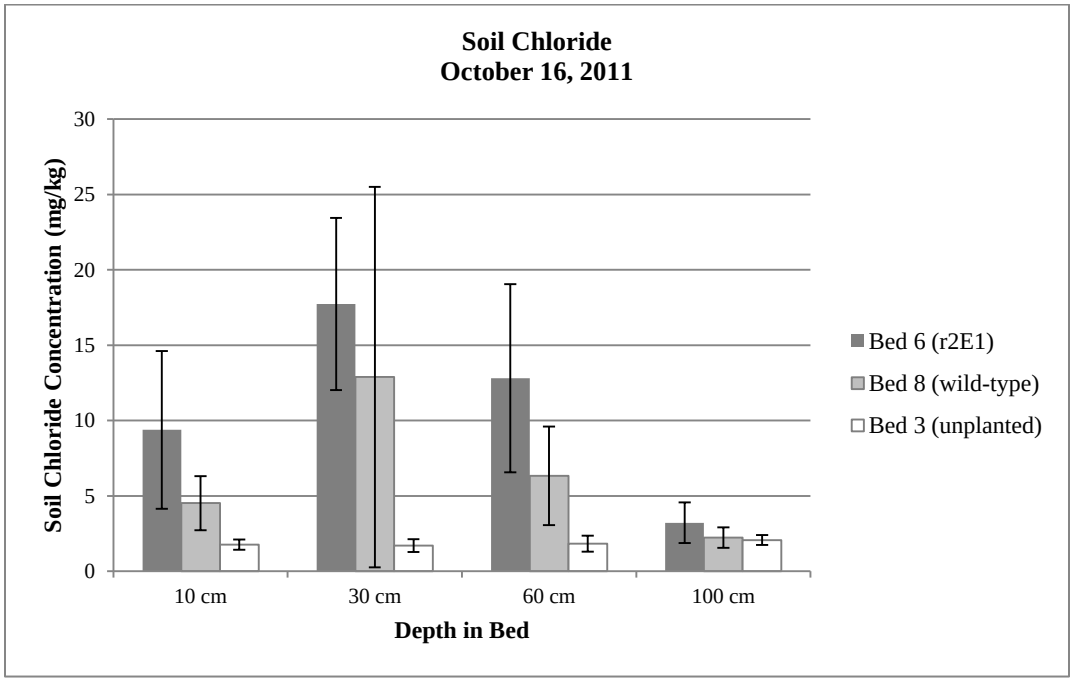

Figure 14. Average soil chloride concentration at depths of 10, 30, 60, and 100 cm for the 2011 growing season on October 16, 2011. Data bars show mean and standard error of six samples taken across the bed at the specified depth.

4.1.5 Soil TCE Concentration

An increase in soil TCE concentration occurred in the vadose zone of only the planted beds during the growing season (Figure 15).This increase may be due to a diffusional efflux of TCE from tree roots following uptake from the bottom saturated soil layer. Another possible explanation is that greater porosity generated by plant roots enhanced upward diffusive transfer of TCE in the subsurface.

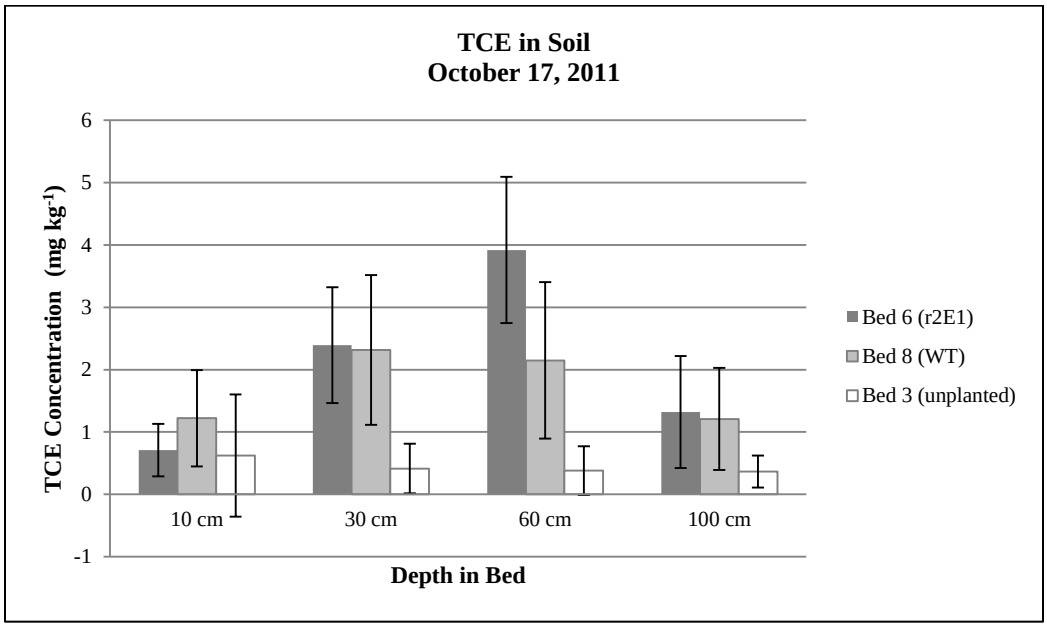

Figure 15. Average soil matrix TCE concentrations on October 17, 2011. Data bars show mean and standard error of six samples taken across the bed at the specified depth.

4.1.6 Chlorine Mass Balance

Total inputs and removals of chlorine during the mass balance period are shown in Table 4. Bed 6 (r2E1) had the highest input chlorine mass due to the larger volume of water required for irrigation. Chloride ion accumulation in soil accounted for 88%, 80%, and 30% of recovered chlorine in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. In Bed 3 (unplanted),

the largest source of recovery of chlorine was as chloride ion in the effluent and bed water (52% of input chlorine).

Chlorine recovery was 126%, 90%, and 100% in Beds 6 (r2E1), 8 (wild-type), and 3 (unplanted), respectively. The error in recoveries in Bed 6 (r2E1) and Bed 8 (WT) are likely due to the large error associated with soil chloride measurements.

Table 4. Mass balance of total input and output chlorine from the three test beds for the 2011 mass balance period (May 10, 2011 - November 3, 2011). Bed Water TCE, DCE, VC and Bed Water Chloride refer to the mass of VOCs and chloride contained in the water in each test bed at the beginning (input) and end (ouput) of the mass balance period. Values in parenthesis were estimated with measurements taken during the 2012 growing season.

	Inputs (mol as Cl)			**Output/Accumulation (mol as Cl)**		
	Bed 6 (r2E1)	Bed 8 (WT)	Bed 3 (unplanted)	Bed 6 (r2E1)	Bed 8 (WT)	Bed 3 (unplanted)
Bed Water TCE, DCE, VC	0.269	0.232	0.380	0.294	0.361	0.396
Bed Water Chloride	1.05	1.10	0.857	0.483	0.525	1.32
Water TCE	1.48	1.48	1.48	0.059	0.073	0.200
Water DCE	ND	ND	ND	0.006	0.010	0.050
Water VC	ND	ND	ND	0.001	0.003	0.033
Water chloride	2.87	2.59	0.657	0.170	0.218	0.890
Soil chloride	1.58	1.36	0.869	8.11	4.88	1.38
Rain water chloride	0.009	0.009	0.009			
Soil Volatilization				(1.82E-04)	(7.43E-04)	(7.43E-04)
Leaf Transpiration				(3.81E-05)	(1.58E-04)	NA
Trunk Volatilization				(5.90E-06)	(4.13E-05)	NA
Tissue Metabolites				NA	NA	NA
Total	7.25	6.77	4.25	9.12	6.07	4.27
Recovery	126%	90%	100%			

4.2 2012 Field Data

4.2.1 Water Use/Balance

Water use for each bed is summarized in Table 5. The r2E1 trees were larger and transpired an average of 175 L d^{-1} while the wild-type trees transpired 170 L d^{-1}, a difference in transpiration of 3%. The average water levels over the growing season in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted) were 28.4 ± 10.9 cm, 30.2 ±8.51 cm, and 53 ± 13.9 cm, respectively. Water levels are shown in Figure 16 - Figure 18.

Table 5. Water Balance for the 2012 Growing Season

	Bed 6 (r2E1) (L)	Bed 8 (WT) (L)	Bed 3 (unplanted) (L)
Rainfall	2,116	2,116	2,116
Influent	22,769	22,959	4,543
Surface Irrigation	2,226	2,092	469
Water Accumulation	3,283	4,025	1,232
Effluent	691	702	4,741
Transpiration/Volatilization	23,137	22,440	1,155
Transpiration/Volatilization per day	175	170	NA

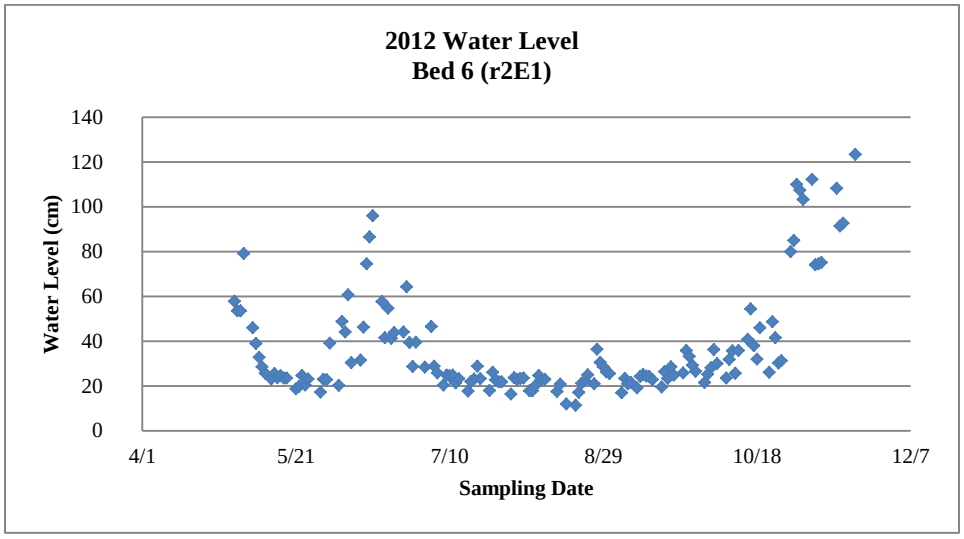

Figure 16. Water level in Bed 6 (r2E1) in 2012. Water levels are the height of water measured from the bottom of the field bed.

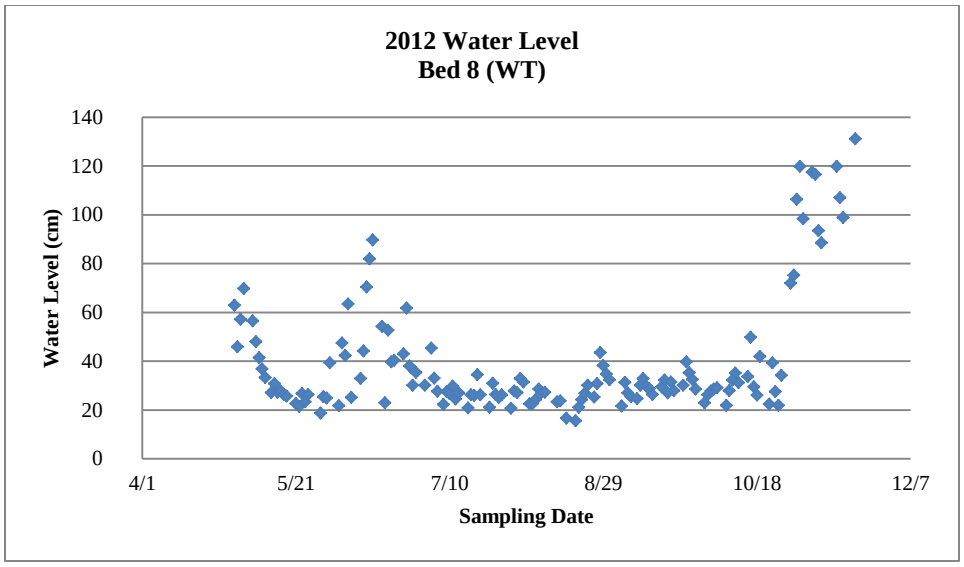

Figure 17. Water level in Bed 8 (WT) in 2012. Water levels are the height of water measured from the bottom of the field bed.

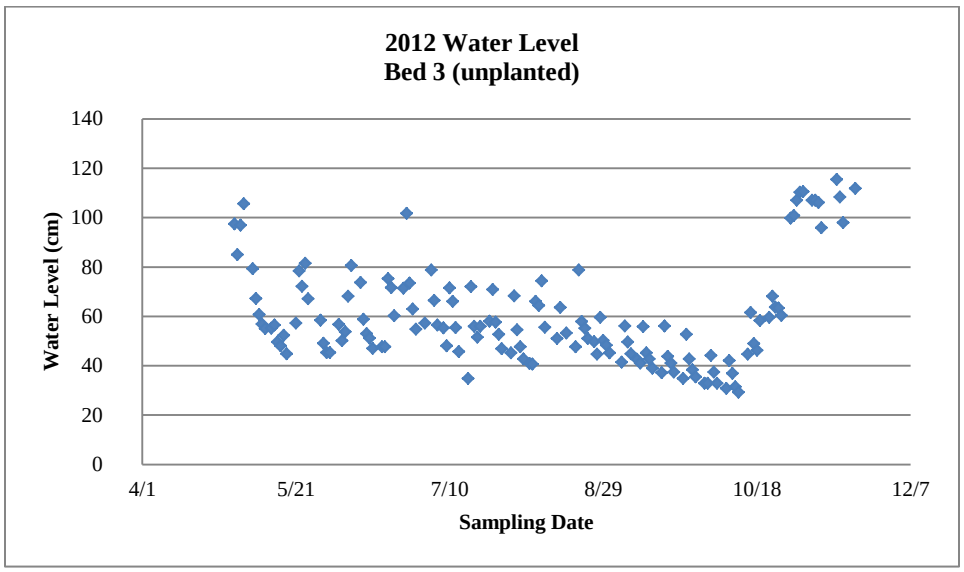

Figure 18. Water level in Bed 3 (unplanted) in 2012. Water levels are the height of water measured from the bottom of the field bed.

4.2.2 Tree Growth

Trees were healthy and showed no signs of distress due to TCE exposure or genetic modification. Tree circumferences measured in April 2012 are shown in Table 6.

Table 6. Tree circumference measured at breast height (1.37 m) in April 2012. Diameters are calculated from circumference.

	Bed 6 (r2E1)		Bed 8 (WT)	
	Circumference (cm)	Diameter (cm)	Circumference (cm)	Diameter (cm)
Tree 1	29	9.36	30	9.68
Tree 2	16	5.03	19	5.98
Tree 3	29	9.17	24	7.51
Tree 4	22	7.00	8	2.61
Tree 5	34	10.9	30	9.42
Tree 6	26	8.28	25	8.09
Tree 7	30	9.42	20	6.37
Tree 8	20	6.37	18	5.67
Tree 9	27	8.59	17	5.41
Tree 10	24	7.64	26	8.21
Tree 11	11	3.37	22	7.13
Tree 12	16	5.09	26	8.15

4.2.3 Dissolved Oxygen and Temperature

Dissolved oxygen concentrations were consistently highest in Bed 6 (r2E1) and lowest in Bed 3 (unplanted) (Figure 19).These results are consistent with measured levels of anaerobic microbial degradation products and methane concentrations in each bed. Temperature of bed water is shown in Figure 20.

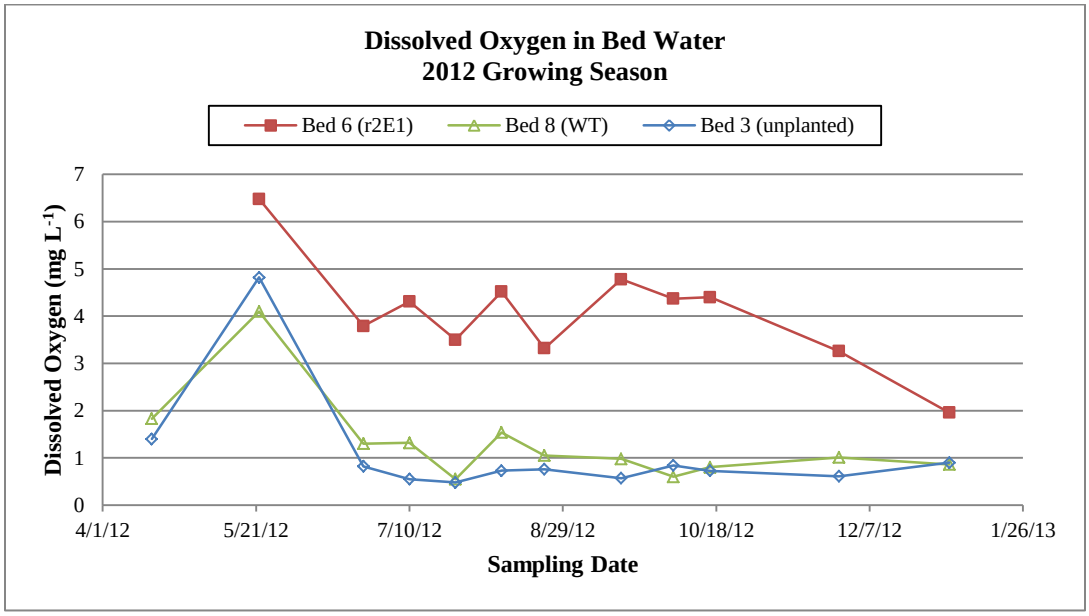

Figure 19. Dissolved oxygen concentrations measured in bed water of Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted) over the 2012 growing season.

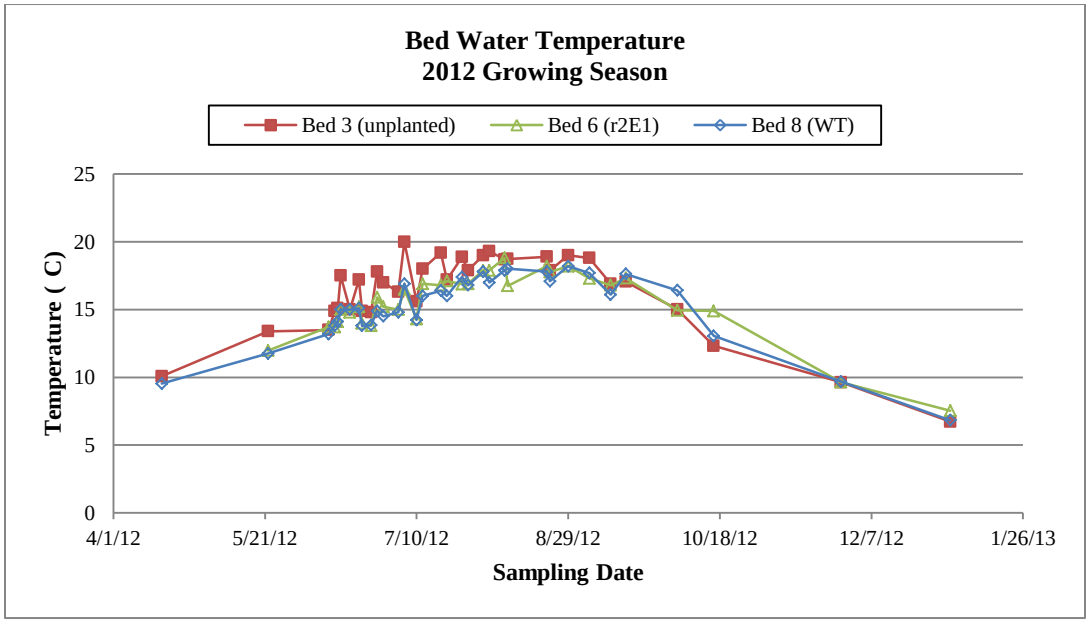

Figure 20. Temperature measured in bed water of Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted) over the 2012 growing season.

4.2.4 Influent and Effluent VOC and Chloride Concentrations

Influent TCE concentrations are shown in Figure 21. The average influent concentration for all beds was 31.4 ± 4.4 mg L^{-1}. The average effluent TCE concentration from June 19, 2012 through October 28, 2012 was 3.24 ± 1.3 mg L^{-1}, 2.93 ± 0.96 mg L^{-1}, 2.36 ± 0.97 mg L^{-1} in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. The TCE concentration increased substantially in all beds following the initiation of dosing. The system appeared to reach semi-steady-state conditions in the second half of the season; a moderate downward trend was observed in both planted beds and an upward trend in the unplanted bed from August 15, 2012 to October 28, 2012. Average effluent TCE concentrations during this period were 3.56 ± 0.45 mg L^{-1}, 3.55 ± 0.39 mg L^{-1}, and 3.07 ± 0.32 mg L^{-1} in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. A greater water volume in the unplanted bed resulted in lower TCE concentrations. A major rainfall event on October 29, 2012 coincided with a decrease in TCE concentrations in all beds.

The effluent cDCE and VC concentrations were substantially higher in Bed 3 (unplanted) compared to the unplanted beds (Figure 23 and Figure 24), indicating that microbial reductive dechlorination was considerably more active in the unplanted bed. In the planted beds, reductive dechlorination appeared to be more active in Bed 8 (WT) as cDCE and VC concentrations were consistently 1 or 2 orders of magnitude greater in Bed 8 (WT) than in Bed 6 (r2E1).

Effluent aqueous chloride concentrations increased over the growing season in Bed 3 (unplanted) but not in the planted beds (Figure 25). This was likely a result of chlorine release during anaerobic reductive dechlorination and subsequent mobilization in effluent water.

Methane was detected in all test beds (Figure 26) and suggested that sufficiently anaerobic conditions had developed for methanogenesis. Bed 3 (unplanted) maintained substantially higher methane concentrations than in the planted beds; these results are consistent with the higher effluent concentrations of microbial reductive metabolites cDCE and VC and the dissolved oxygen levels in Bed 3. Conditions appeared to be more strongly-reducing in the unplanted bed.

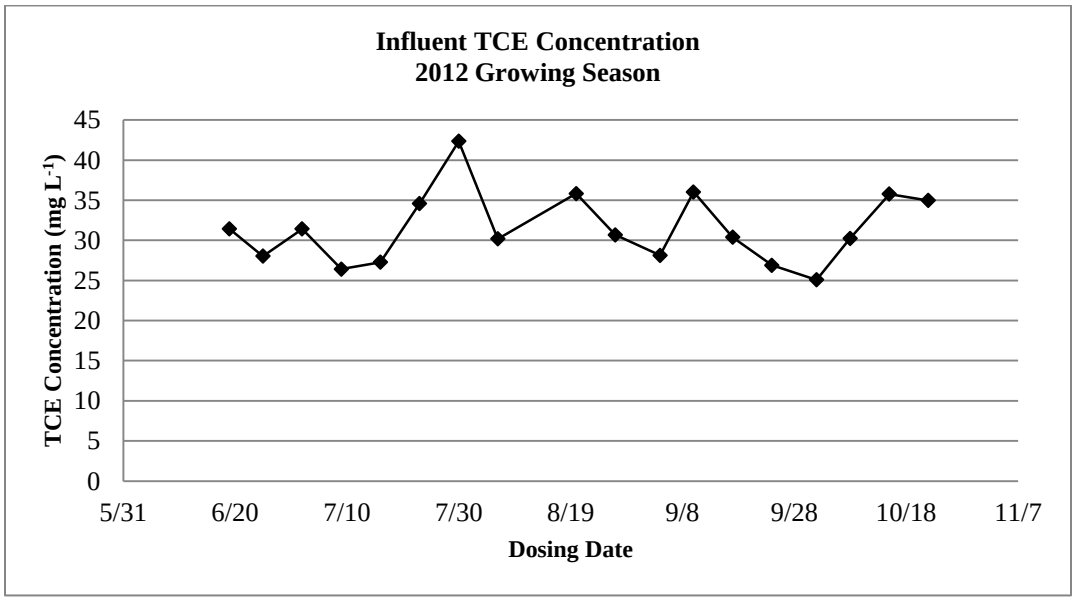

Figure 21. Influent TCE Concentration for the 2012 growing season.

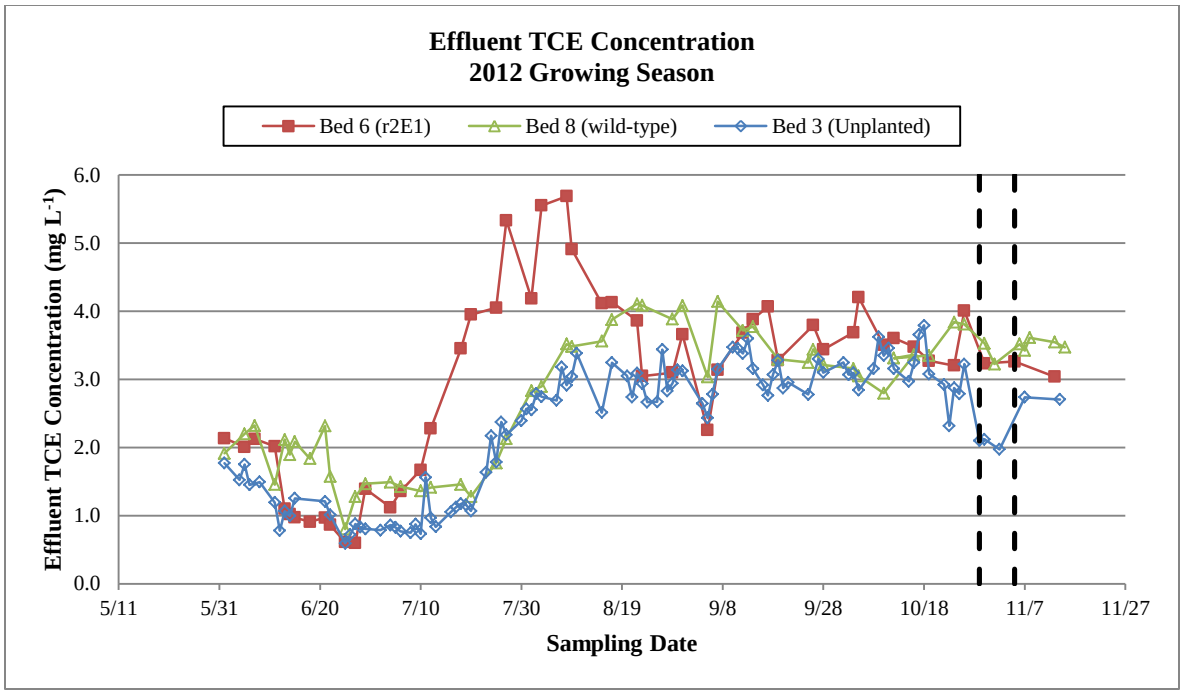

Figure 22. TCE concentrations in the effluent water over the 2012 growing season in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted). Dashed black lines enclose the period of leaf fall.

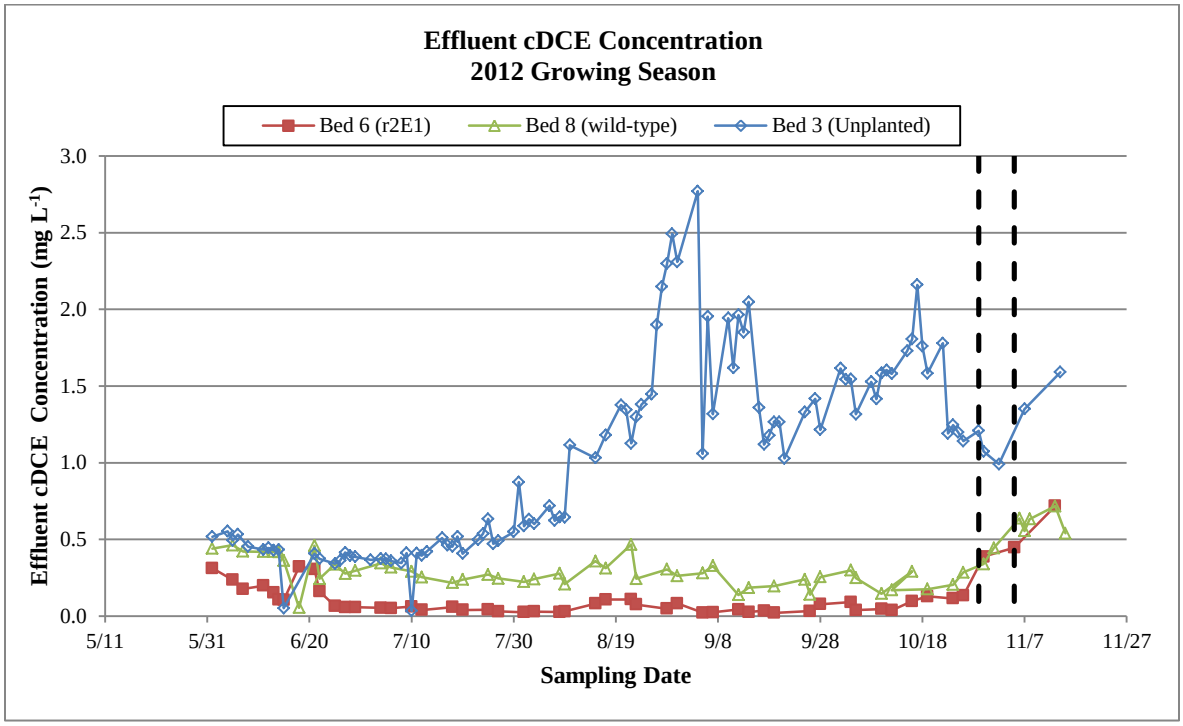

Figure 23. cDCE concentrations in the effluent water over the 2012 growing season in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted). Dashed black lines enclose the period of leaf fall.

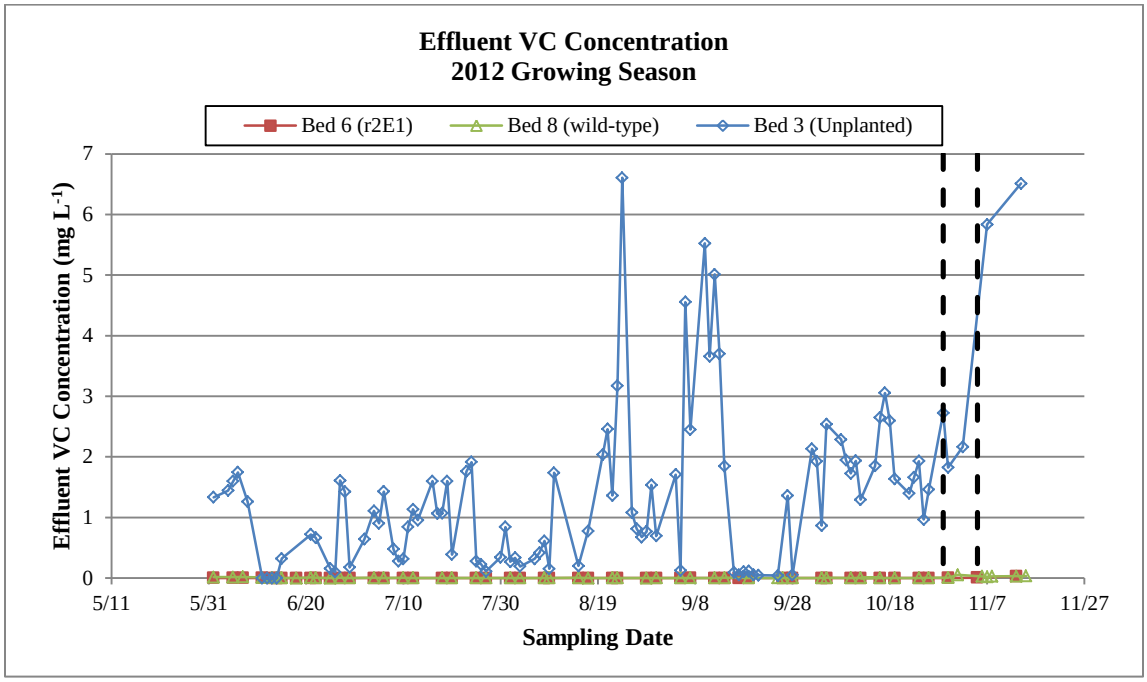

Figure 24. VC concentrations in the effluent water over the 2012 growing season in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted). Dashed black lines enclose the period of leaf fall.

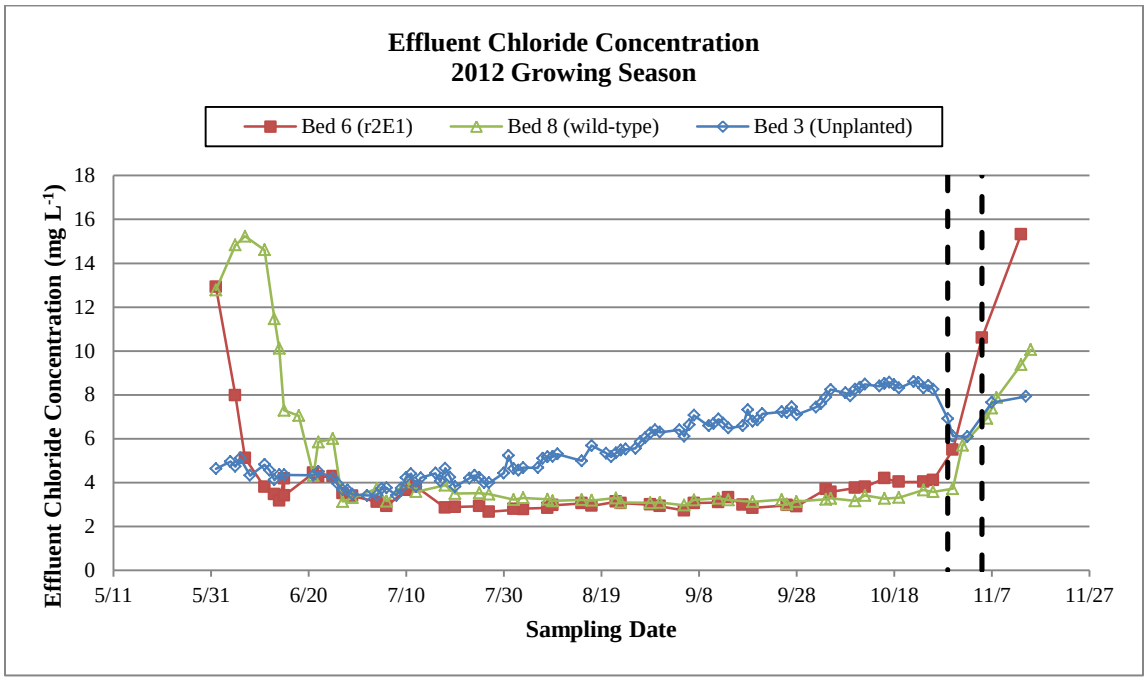

Figure 25. Chloride concentrations in the effluent water over the 2012 growing season in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted). Dashed black lines enclose the period of leaf fall.

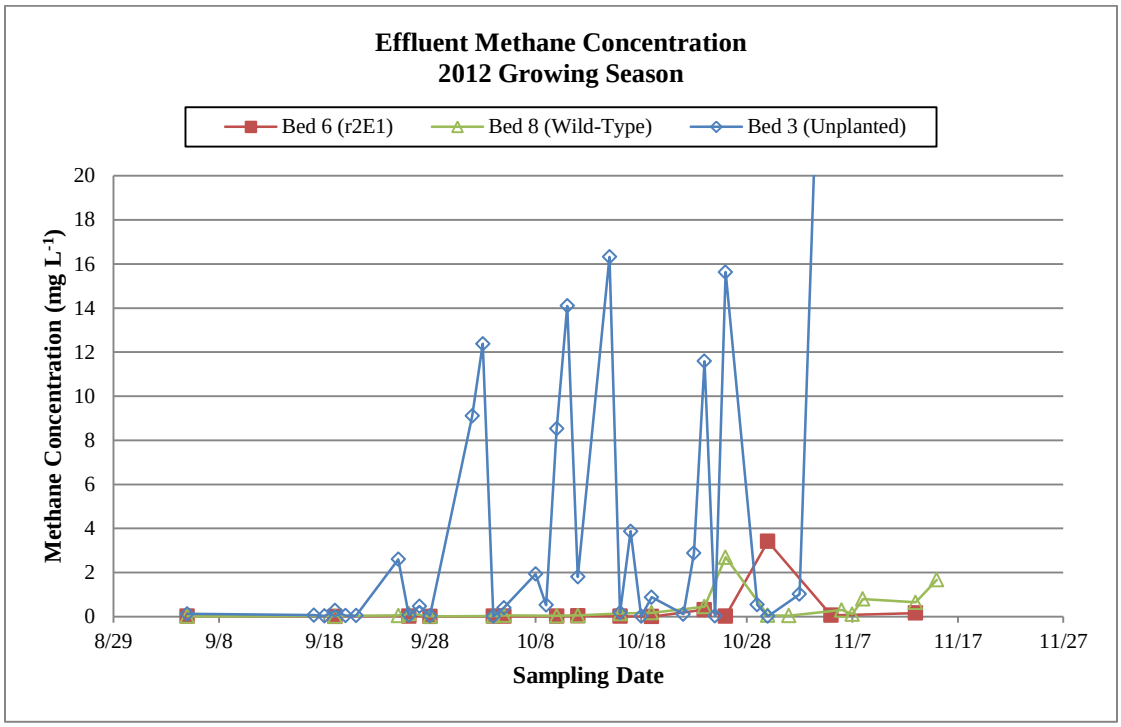

Figure 26. Methane concentrations in the effluent water in from September to November in the 2012 growing season in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted).

4.2.5 Soil Chloride Concentration

Soil chloride concentration increased in the vadose zone of the planted beds during the growing season period (Figure 27 - Figure 29). A similar increase was not observed in the unplanted bed. The increase was the greatest at the 30 cm depth. On May 22, 2012, chloride concentration at 30 cm was 3.19 ± 1.17 mg kg^{-1}, 2.64 ± 0.50 mg kg^{-1}, and 1.64 ± 0.33 mg kg^{-1} in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. Soil concentration at the 30 cm depth more than doubled in the planted beds by August 21, 2012. By October 16, 2012, concentrations at the 30 cm depth had increased 7-fold from the beginning of the season in Bed 6 (r2E1) and 8-fold in Bed 8 (WT) to 19.78 ± 17.18 mg kg^{-1} and 21.31 ± 30.26 mg kg^{-1}, respectively. An increase in soil chloride in the vadose zone of the planted beds occurred in each growing season in the

present study and has also been observed other mass-balance phytoremediation field studies with hybrid poplars and chlorinated solvents TCE, CT, and PCE. [10, 16, 39] The accumulated chloride ion in the vadose zone was dissolved and mobilized in effluent water in the winter season due to heavy winter rains; this occurrence can be seen in the 2011-2012 winter season effluent chloride concentration profile (Figure 12).

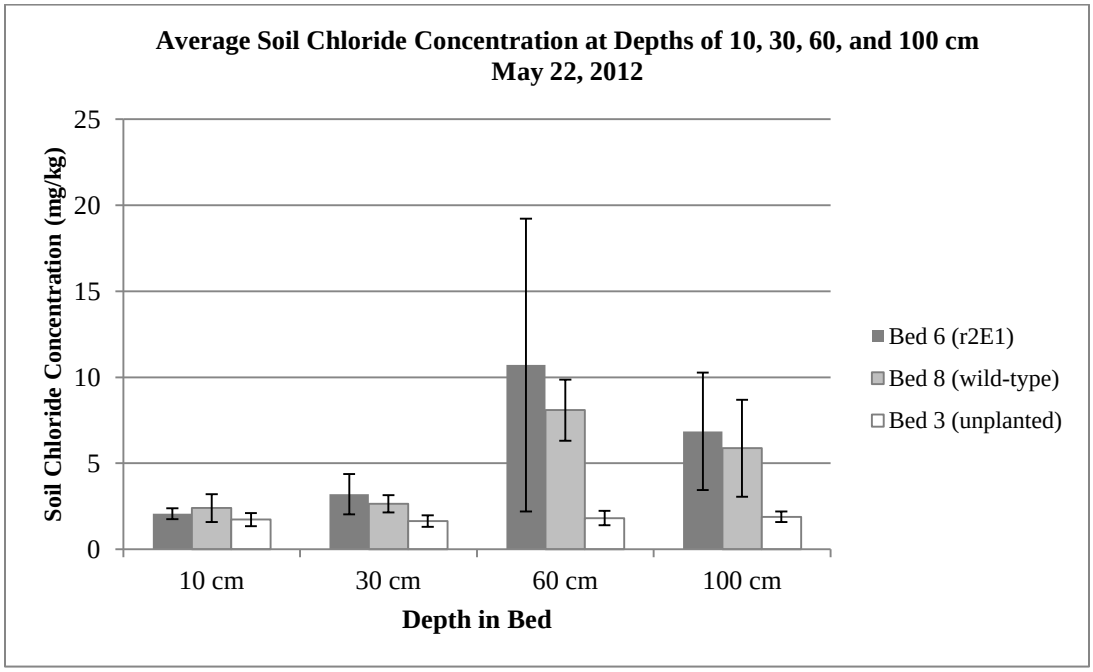

Figure 27. Average soil chloride concentration at depths of 10, 30, 60, and 100 cm on May 22, 2012. Data bars show mean and standard error of six samples taken across the bed at the specified depth.

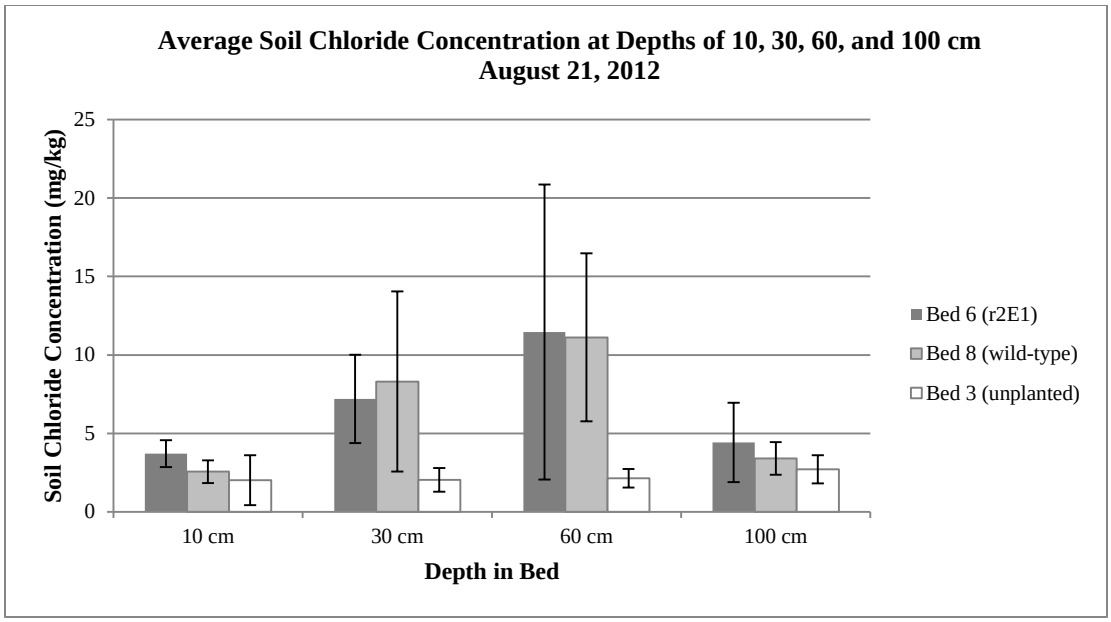

Figure 28. Average soil chloride concentration at depths of 10, 30, 60, and 100 cm on August 21, 2012. Data bars show mean and standard error of six samples taken across the bed at the specified depth.

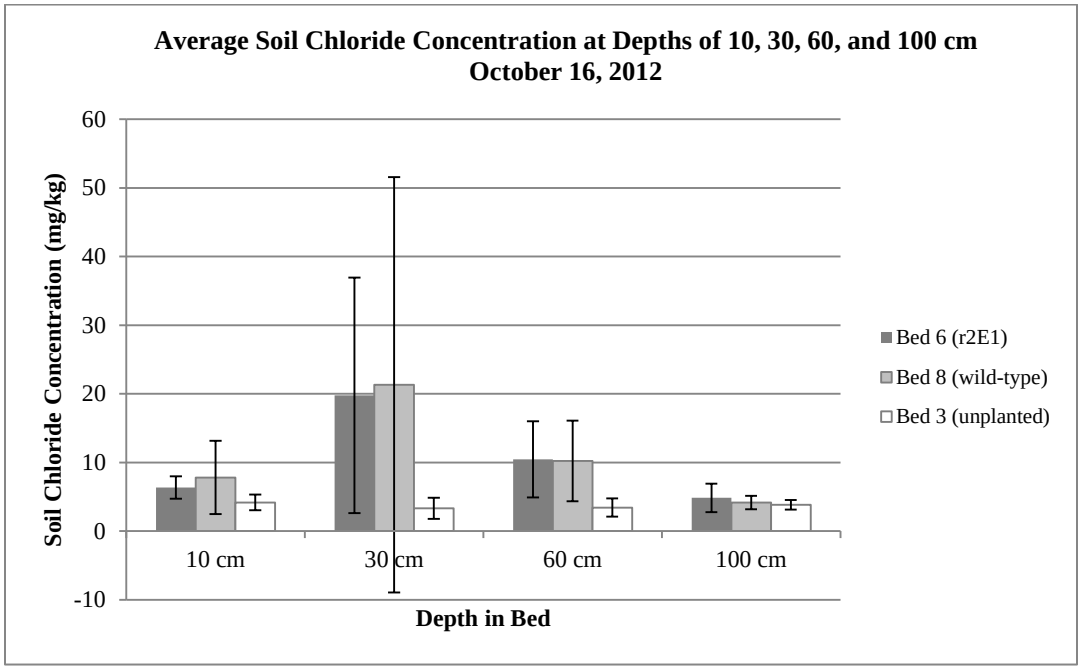

Figure 29. Average soil chloride concentration at depths of 10, 30, 60, and 100 cm for the 2012 growing season on October 16, 2012. Data bars show mean and standard error of six samples taken across the bed at the specified depth.

4.2.6 Soil TCE Concentration

In general, concentrations of TCE measured in the soil matrix greater in the planted beds (Figure 30 – Figure 32). On May 22, 2012, average soil TCE concentrations were 0.53 ± 0.4 mg kg^{-1}, 0.43 ± 0.2 mg kg^{-1} and 0.16 ± 0.3 mg kg^{-1}, for Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. On August 21, 2012, average soil TCE concentrations were 1.1 ± 1.0 mg kg^{-1}, 0.58 ± 0.4 mg kg^{-1} and 0.21 ± 0.3 mg kg^{-1}, for Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. On October 17, 2012, average soil TCE concentrations were 0.64 ± 0.4 mg kg^{-1}, 1.02 ± 0.7 mg kg^{-1} and 0.15 ± 0.1 mg kg^{-1}, for Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. Concentrations appeared to increase in the vadose zone of the planted beds over the course of the growing season. This increase was likely due either to diffusional efflux of TCE from tree roots or increased upward diffusion of TCE in the planted beds due to a greater soil porosity generated by tree roots.

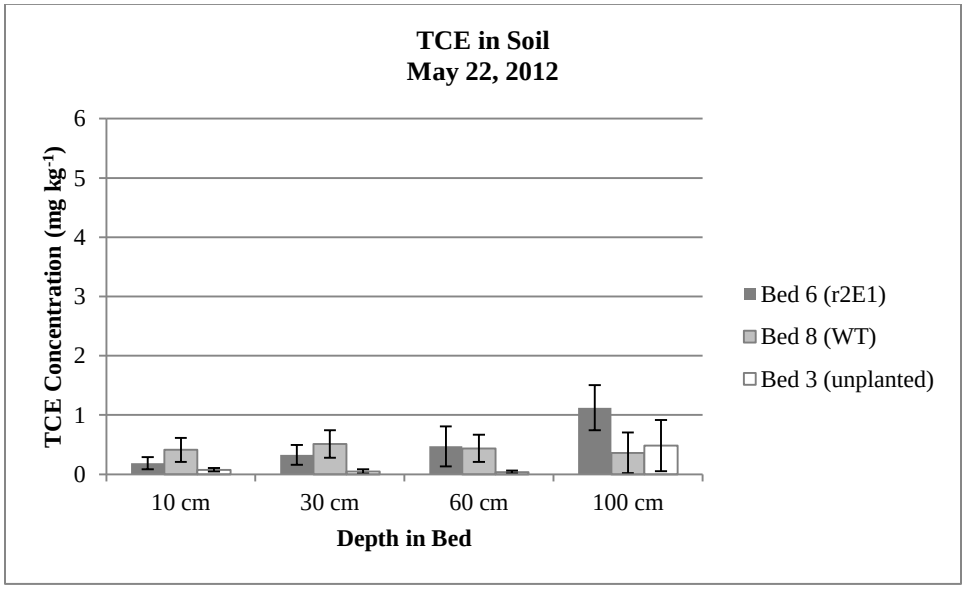

Figure 30. Average soil matrix TCE concentrations on May 22, 2012. Data bars show mean and standard error of six samples taken across the bed at the specified depth.

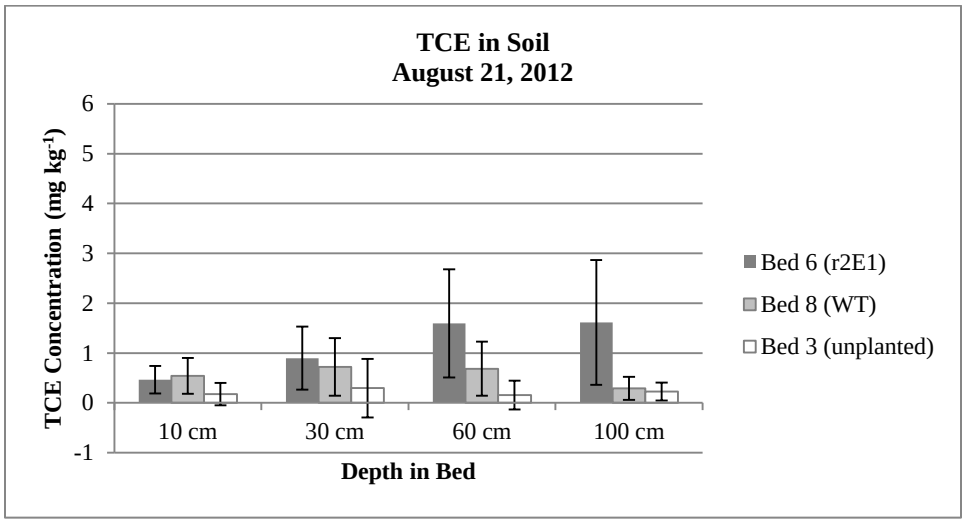

Figure 31. Average soil matrix TCE concentrations on August 21, 2012. Data bars show mean and standard error of six samples taken across the bed at the specified depth.

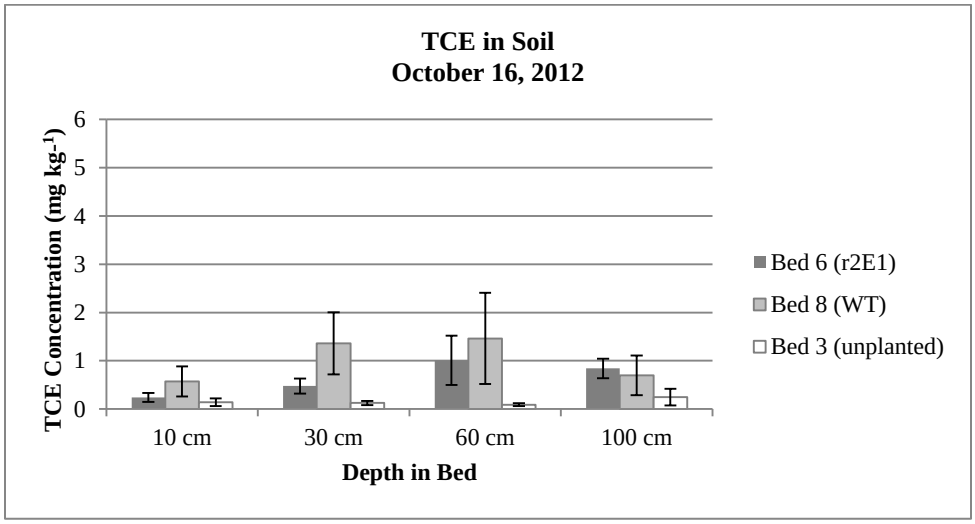

Figure 32. Average soil matrix TCE concentrations on October 16, 2012. Data bars show mean and standard error of six samples taken across the bed at the specified depth.

4.2.7 Volatilization from Soil

The flux of TCE from soil in Bed 6 (r2E1) and Bed 8 (WT) was $8.3\times10^{-14} \pm 9.3\times10^{-15}$ mol h^{-1} cm^{-2} and $3.4\times10^{-13} \pm 1.4\times10^{-13}$ mol h^{-1} cm^{-2}, respectively (Figure 33, Table 7). The flux was reduced 4-fold in Bed 6 (r2E1). The measurements were one order of magnitude smaller than measurements taken four years prior (one-year following tree planting).[55] These results are as expected given that the trees were older and larger and therefore were able to take up greater amounts of TCE from the subsurface, reducing TCE diffusion to the atmosphere.

4.2.8 Volatilization from Stem and Leaves

Flux of TCE from tree stem at heights 0.25, 0.4, and 0.57 meters from the ground was $1.87\times10^{-14} \pm 1.1\times10^{-14}$, $1.77\times10^{-14} \pm 1.4\times10^{-14}$, and $5.36\times10^{-14} \pm 1.1\times10^{-15}$ mol h^{-1} cm^{-2} in Bed 6 (r2E1), respectively, and $2.18\times10^{-13} \pm 7.0\times10^{-14}$, $2.42\times10^{-13} \pm 1.0\times10^{-13}$, and $2.11\times10^{-13} \pm 6.6\times10^{-14}$ mol h^{-1} cm^{-2} in Bed 8 (WT), respectively (Figure 33, Table 7). The difference in flux from tree stem between r2E1 and WT trees was significant at all heights ($P<0.05$). Flux was reduced from r2E1 trees by 12, 14, and 4-fold at the 0.25, 0.40, and 0.57 m heights, respectively. On average, this corresponded to an 87% reduction in r2E1 stem volatilization of TCE. Results from previous research have shown a decrease in volatilization of TCE with increasing stem height.[21] Our results follow this trend in some instances, though no significant differences were found between fluxes at different stem heights in either bed. Differences were significant between the influent and effluent end measurements for the stem measurements ($P<0.05$); this may explain the higher flux measurement at the 0.57 m height in Bed 6 (r2E1) as all measurements for this height were taken from trees at the influent end.

Flux of TCE from tree leaves in Bed 6 (r2E1) and Bed 8 (WT) was $2.8\times10^{-15} \pm 2.7\times10^{-15}$ mol h^{-1} cm^{-2} and $1.4\times10^{-14} \pm 5.0\times10^{-15}$ mol h^{-1} cm^{-2}, respectively. Evapotranspiration of TCE from r2E1 tree leaves was reduced 5-fold compared to WT leaves. The reduction was significant ($P<0.05$). Reduced volatilization of TCE from both the r2E1 stem and leaves is evidence that metabolism of TCE was enhanced in the r2E1 trees. Evapotranspiration measurements were two orders of magnitude lower for r2E1 and one order of magnitude lower for WT trees than measured four growing seasons earlier in the present study.[55]

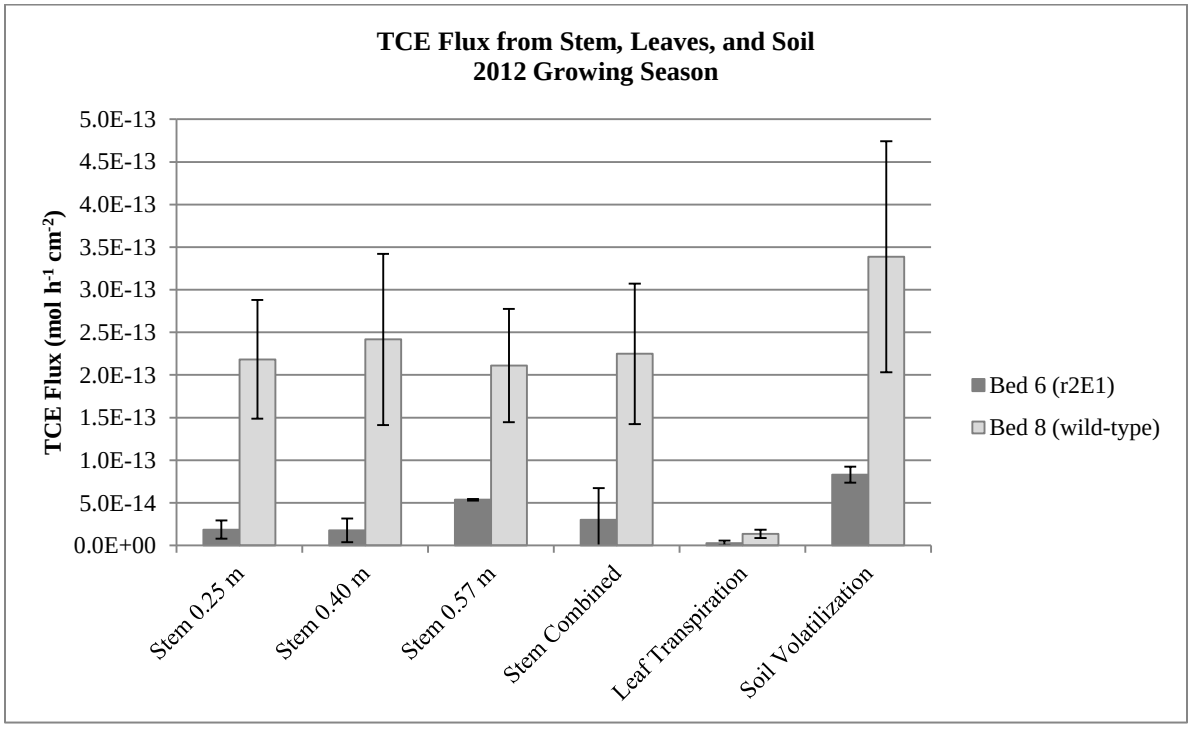

Figure 33. Flux of TCE measured from the soil, trunk, and leaves. Values reported are in mol h^{-1} cm^{-2} and bars are the mean and standard error for all measurements taken during the 2012 mass balance period. Measurements were taken at both influent and effluent ends of test beds. Differences between Bed 6 (r2E1) and Bed 8 (WT) were statistically significant ($P<0.05$) for all measurements with the exception of soil volatilization.

Table 7. Flux of TCE measured from the soil, trunk, and leaves. Values reported are in mol h^{-1} cm^{-2} and are the mean and standard error for all measurements taken during the 2012 mass balance period. Measurements were taken at both influent and effluent ends of test beds. Differences between Bed 6 (r2E1) and Bed 8 (WT) were statistically significant ($P<0.05$) for all measurements with the exception of soil volatilization.

	Bed 6 (r2E1) Flux			Bed 8 (WT) Flux			Number of Observations	
							Bed 6	Bed 8
Trunk 0.25 m	1.87E-14	±	1.09E-14	2.18E-13	±	6.97E-14	4	4
Trunk 0.40 m	1.77E-14	±	1.40E-14	2.42E-13	±	1.00E-13	4	4
Trunk 0.57 m	5.36E-14	±	1.05E-15	2.11E-13	±	6.64E-14	4	3
Trunk Combined	3.00E-14	±	3.72E-14	2.25E-13	±	8.25E-14	12	11
Leaf transpiration	2.83E-15	±	2.71E-15	1.35E-14	±	4.95E-15	6	7
Soil Volatilization	8.30E-14	±	9.26E-15	3.39E-13	±	1.36E-13	2	2

4.2.9 TCE and Metabolites in Tissues

Levels of TCOH were significantly greater in leaf, root, branch, and stem of r2E1 poplars ($P<0.05$, Two-Factor ANOVA) (Figure 34). TCE concentration in leaf, root, branch, and stem tissue was significantly lower in r2E1 trees ($P<0.05$, Two-Factor ANOVA). TCOH-glucoside levels in Bed 6 (r2E1) leaf samples were significantly greater than in Bed 8 (WT) ($P<0.05$). Dichloroacetic acid was below detection limits in all samples. Trichloroacetic acid was detected only in leaf tissue. These results suggest that metabolism of TCE in was enhanced in the r2E1 trees. Additionally, the results suggest enhanced metabolism of TCE in the trunk of the r2E1 poplars. TCOH levels were the highest in the r2E1 trunk tissue samples, and there was a notable decrease in TCE concentration from the r2E1 trunk to the branch.

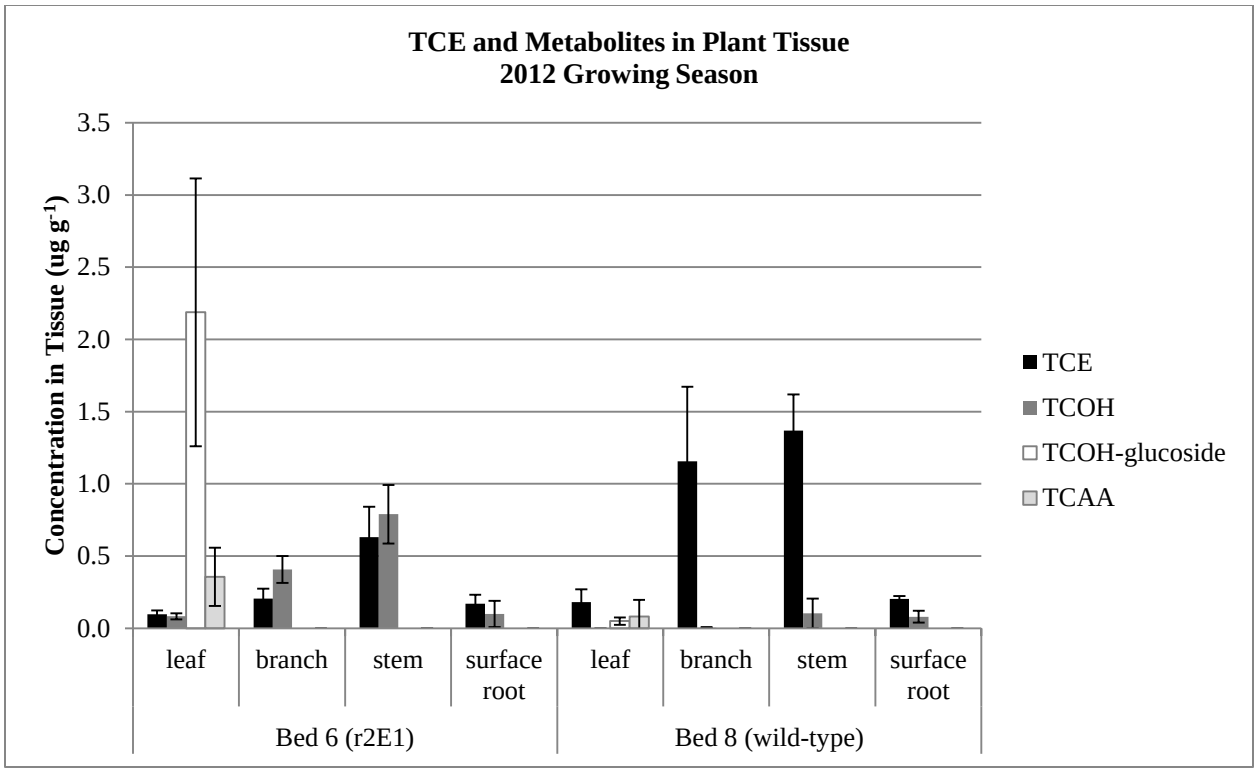

Figure 34. Levels of TCE metabolites measured in leaf, branch, stem, and surface root of r2E1 and WT trees in the 2012 growing season. Data bars show mean and standard error of three tissue samples taken from different trees with the exception of stem. Stem data bars show mean and standard error of two tissue samples; one sample was combined from two different trees.

4.2.10 Chlorinated Ethene Mass Balance

Total inputs and outputs of VOCs are displayed in Table 8. Percent recoveries of VOCs over the growing season were 13%, 15%, and 66% in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. Bed 6 (r2E1) had the highest removal efficiency of input TCE. Accumulation in bed water was the largest source of VOC recovery in all beds. In Bed 3 (unplanted), 60% of total recovered chlorinated ethene was as VC, indicating a high degree of reductive transformation of both TCE and cDCE.

Table 8. Total inputs and outputs of VOCs from the three field test beds during the mass balance period (June 19, 2012 – October 29, 2012)

	Inputs			**Output/Accumulation**		
	Bed 6 (r2E1)	Bed 8 (WT)	Bed 3 (unplanted)	Bed 6 (r2E1)	Bed 8 (WT)	Bed 3 (unplanted)
TCE in water (mol)	1.060	1.060	1.060	0.0171	0.0151	0.0840
cDCE in water (mol)	ND	ND	ND	0.0005	0.0019	0.0516
VC in water (mol)	ND	ND	ND	0.0003	0.0018	0.1022
VOCs Accumulated in Bed Water	NA	NA	NA	0.1229	0.1387	0.4609
Soil Volatilization (mol)	NA	NA	NA	6.06E-05	2.48E-04	2.48E-04
Trunk Volatilization (mol)	NA	NA	NA	1.46E-06	1.02E-05	NA
Leaf Volatilization (mol)	NA	NA	NA	9.41E-06	3.90E-05	NA
Total VOCs (mol)	1.060	1.060	1.060	0.1407	0.1578	0.699
VOC Recovery	13%	15%	66%			

4.2.11 Chlorine Mass Balance

Total input, output, and accumulation of chlorine for the 2012 growing season is shown in Table 9. Biomass estimations used for calculation of accumulated metabolite mass are shown in Table 10. All biomass estimations performed are shown for Bed 6 (r2E1) in Appendix B. Estimates of accumulation of metabolites in plant tissue assumed uniform concentration of metabolite throughout tree tissue. Concentrations of TCOH-glucoside measured in branch and root in 2008 were used for estimations of TCOH-glucoside accumulation in branch and root of the planted beds.

Chlorine recoveries were 109%, 102%, and 106% for Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. Major sources of input chlorine were as free chloride ion present in soil in planted beds and TCE and chloride ion in influent water. The largest source of recovery of chlorine was as chloride ion in the vadose zone soil of all three beds, accounting for 70%, 85%, and 42% of total recovered chlorine in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. Oxidative metabolite accumulation in the tissue of the r2E1 trees was a major source of chlorine recovery in Bed 6 (r2E1). However, metabolite accumulation estimations were made assuming uniform concentration throughout plant tissue, which may not be valid. Metabolite accumulation in tissue accounted for 57% and 6% of input TCE in Bed 6 (r2E1) and Bed 8 (WT), respectively. Evapotranspiration of TCE was a minor loss pathway. Volatilization of TCE from trunk, leaf, and soil together represented less than 0.01% of input TCE for Bed 6 (r2E1) and less than 0.1% for Bed 8 (WT).

Table 9. Mass balance of total input and output chlorine from the three test beds for the 2012 mass balance period (June 19, 2012 - October 28, 2012).

	Inputs (mol as Cl)			**Output/Accumulation (mol as Cl)**		
	Bed 6 (r2E1)	Bed 8 (WT)	Bed 3 (unplanted)	Bed 6 (r2E1)	Bed 8 (WT)	Bed 3 (unplanted)
Bed Water TCE, DCE, VC	0.194	0.202	0.387	0.558	0.557	1.073
Bed Water Chloride	0.89	0.63	0.994	1.055	0.618	1.59
Water TCE	3.18	3.18	3.18	0.051	0.045	0.252
Water DCE	ND	ND	ND	0.001	0.004	0.103
Water VC	ND	ND	ND	0.000	0.002	0.102
Water chloride	1.87	1.88	0.38	0.065	0.068	0.753
Soil chloride	4.29	3.57	1.33	7.79	8.17	2.77
Rain water chloride	0.008	0.008	0.008			
Soil Volatilization				1.82E-04	7.43E-04	7.43E-04
Leaf Transpiration				2.82E-05	1.17E-04	NA
Trunk Volatilization				4.38E-06	3.06E-05	NA
Tissue Metabolites				1.808	0.196	NA
Total	10.43	9.46	6.27	11.33	9.66	6.64
Recovery	109%	102%	106%			

Table 10. Leaf area and median estimations of dry mass of root, leaf, trunk, and branch for r2E1 and WT trees used in modeling and mass balance calculations. Estimations were calculated from allometric linear-regression equations based on diameter at breast height (DBH). DBH of individual trees was measured in April 2012.

	Bed 6 (r2E1)	**Bed 8 (WT)**	Unit	Reference
Root	29	24	kg	[58]
Leaf	5.7	3.4	kg	[59]
Trunk	84	70	kg	[60]
Branch	39	9.6	kg	[61]
Leaf Area	210	183	m^2	[41]

4.2.12 Root Chloride Efflux

Results from root enclosure experiments are shown in Table 11. There was a significant increase in chloride concentration in one sample (root sample D) in Bed 6 (r2E1) for the first experiment ($P<0.01$). There was a significant increase in chloride concentration in the r2E1 and WT sand for all samples in the second experiment ($P<0.05$). There was no change in chloride concentration in control root sand ($P<0.05$). The roots were not rinsed with water following application of Iodophor iodine solution in the first experiment. The iodine solution may have caused the death of root cells and therefore a decrease in root activity. This may explain the why chloride accumulated in only one sample in the first experiment, but there was a significant increase in all root samples in the second experiment.

Table 11. Accumulation of chloride ion in sealed bags containing sterilized tree root and sand. Roots were sealed for 19 days in experiment 1 (August 29, 2012 to September 17). A second set of different roots were sealed in the second experiment for 16 days (September 17, 2012 to October 4, 2012). The controls were sealed roots from the undosed control bed containing one wild-type poplar and one 2E1 poplar. Values reported are the mean and standard error of either duplicate or triplicate chloride extractions.

Experiment 1 (sterilized)

	Root Sample	Initial Chloride Concentration (mg kg^{-1})	Final Chloride Concentration (mg kg^{-1})
Bed 6 (r2E1)	A	0.545 ± 0.097	1.112 ± 0.536
	B	0.545 ± 0.097	0.616 ± 0.094
	C	0.545 ± 0.097	0.673 ± 0.137
	D	0.545 ± 0.097	0.980 ± 0.046
Bed 8 (WT)	A	0.545 ± 0.097	0.554 ± 0.246
	B	0.545 ± 0.097	0.569 ± 0.273
	C	0.545 ± 0.097	0.369 ± 0.045
	D	0.545 ± 0.097	0.793 ± 0.256

Experiment 2 (unsterilized)

	Sample	Initial Chloride Concentration (mg kg^{-1})	Final Chloride Concentration (mg kg^{-1})
Bed 6 (r2E1)	A	0.545 ± 0.097	2.102 ± 0.346
	B	0.545 ± 0.097	1.580 ± 0.468
	C	0.545 ± 0.097	1.416 ± 0.041
Bed 8 (WT)	A	0.545 ± 0.097	1.137 ± 0.212
	B	0.545 ± 0.097	2.124 ± 0.061
	C	0.545 ± 0.097	4.267 ± 1.920
Control	A	0.545 ± 0.097	0.485 ± 0.123
	B	0.545 ± 0.097	0.493 ± 0.010
	C	0.545 ± 0.097	0.502 ± 0.017

4.2.13 Bioavailable Copper

Bioavailable copper concentrations were very low, ranging from 0.16-0.27 µM (Table 12). Expression of the soluble form of MMO is has been shown to be inhibited at copper concentrations greater than 0.25 µM.[62] The rate of TCE oxidation of sMMO decreases at copper

concentrations approaching 0.25 µM.[62] Therefore, if sMMO was expressed at these copper concentrations, its rate of TCE oxidation was likely low. TCE oxidation rates for pMMO are also influenced by copper concentrations.[63] Oxidation of TCE by pMMO was not measurable at copper concentrations below 2.5 µM.[63] These results indicate that the rate of TCE oxidation by either pMMO or sMMO (if expressed) was likely very low.

Table 12. Bioavailable copper concentrations from duplicate soil samples taken at the 30 cm depth in Bed 6 (r2E1) and Bed 8 (WT).

Sample	Bed 6 (r2E1) (µM Cu)	Bed 8 (WT) (µM Cu)
1	0.263	0.232
2	0.265	0.161

4.2.14 qPCR and PCR Assays

Gene counts for *Dehalococcoides* 16S rRNA were normalized by eubacterial 16S rRNA for each soil extraction. *Dehalococcoides* 16S counts were generally found to be higher in Bed 3 (unplanted) (Figure 35). *Dehalococcoides* 16S counts were the greatest at the 100 cm soil depth in all three beds. Additionally, Bed 6 (r2E1) had the lowest *Dehalococcoides* 16S numbers of the three beds. These results are consistent with the greater recovered mass of reductive dechlorination metabolites cDCE and VC in Bed 3 (unplanted) and the lowest mass recovered from Bed 6 (r2E1).

The presence of *pmoA* was verified at all depths in all three beds (Figure 36 and Table 13). Gene counts of *pmoA* were normalized by extraction efficiencies as inferred by spiked *xplA* recovery. Extraction efficiencies ranged from 0.01% to 90%; the average extraction efficiency was 16%

(41 samples). Gene counts of *pmoA* were generally found to be higher in Bed 3 (unplanted), with the exception of the 100 cm depth where gene counts were the lowest. This result is expected given that the unplanted bed had the lowest dissolved oxygen concentrations. There were no significant differences between *pmoA* counts in the planted beds.

Toluene monooxygenase and dioxygenase were below detection limits in all samples. Phenol monooxygenase was detected in both the planted beds and in Bed 3 (unplanted) (Figure 37 - Figure 38). Quantification of gene counts was not possible for phenol monooxygenase due to poor performance of qPCR standard curves. In general, the fluorescence level of the amplicon band was very weak, indicating that the phenol monoxygenase levels were likely low in bed soil.

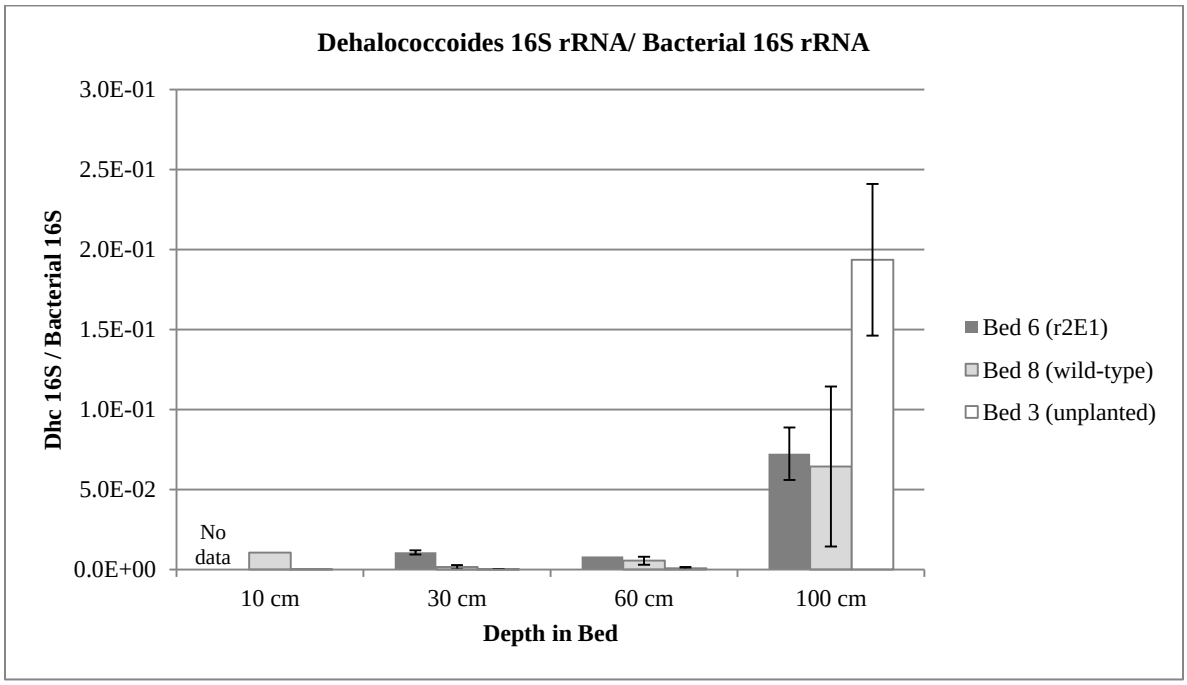

Figure 35. *Dehalococcoides* 16S rRNA copy number per eubacterial 16S rRNA copy number contained in the soil sample extraction. Mean and standard error are calculated from either 2, 3, or 4 soil sample replicates.

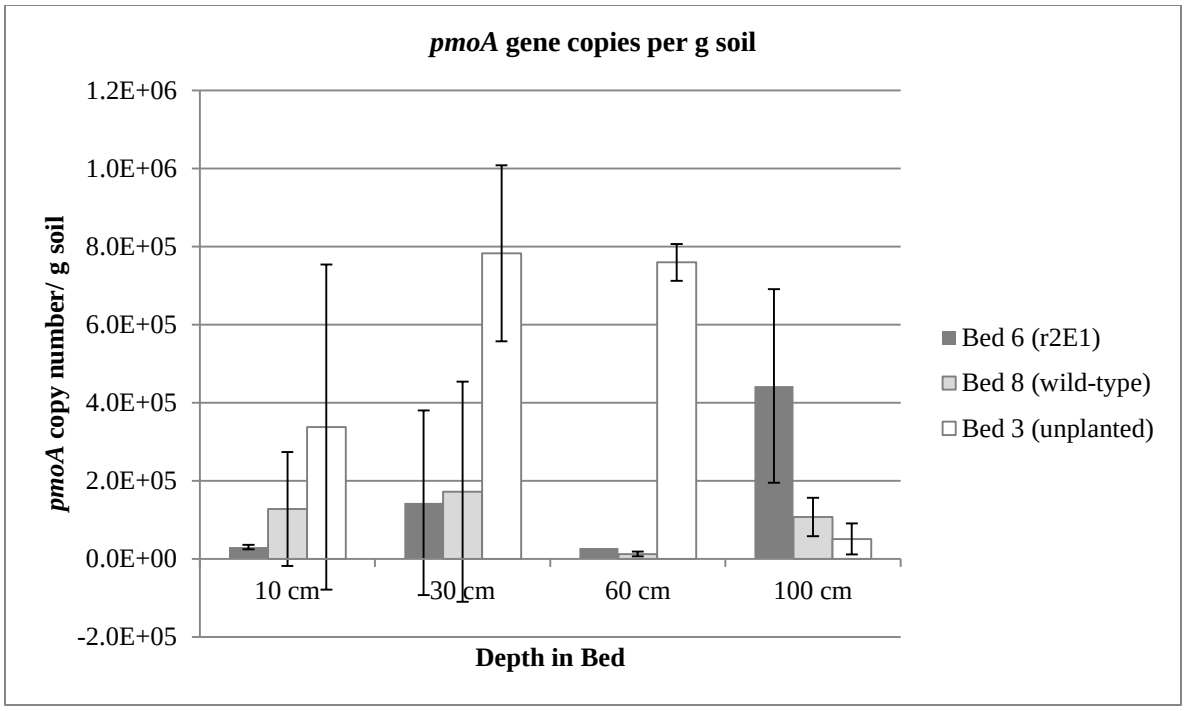

Figure 36. *pmoA* counts per gram of soil at depths 10 cm, 30 cm, 60 cm, and 100 cm in all test beds. *pmoA* counts were normalized by the DNA extraction efficiency for each sample as determined by recovery of spiked exogenous reference gene, *xplA.*

Table 13. *pmoA* counts per gram of soil at depths 10 cm, 30 cm, 60 cm, and 100 cm in all test beds. *pmoA* counts were normalized by the DNA extraction efficiency for each sample as determined by recovery of spiked exogenous reference gene, *xplA*. "n" is number of replicates. Average and standard error given for all measurements.

	Bed 6 (r2E1)			n	Bed 8 (WT)			n	Bed 3 (unplanted)			n
10 cm	30,411	±	5,455	3	127,753	±	146,106	3	337,464	±	416,497	4
30 cm	143,703	±	236,716	5	171,962	±	282,169	4	782,900	±	225,263	3
60 cm	28,140	±		1	12,643	±	5,913	2	759,707	±	46,979	2
100 cm	442,853	±	247,922	3	107,310	±	49,234	2	51,229	±	39,913	2

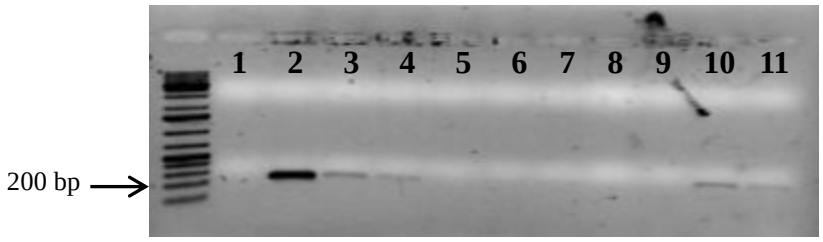

Figure 37. PCR results confirming the presence of phenol monooxygenase. The expected size was 206 bp. Lane 2 contains the phenol hydroxylase genes of *Psuedomonas* CF600. Lanes 3 and 4 are for Bed 6 (r2E1). Lanes 10 and 11 are for Bed 8 (WT).

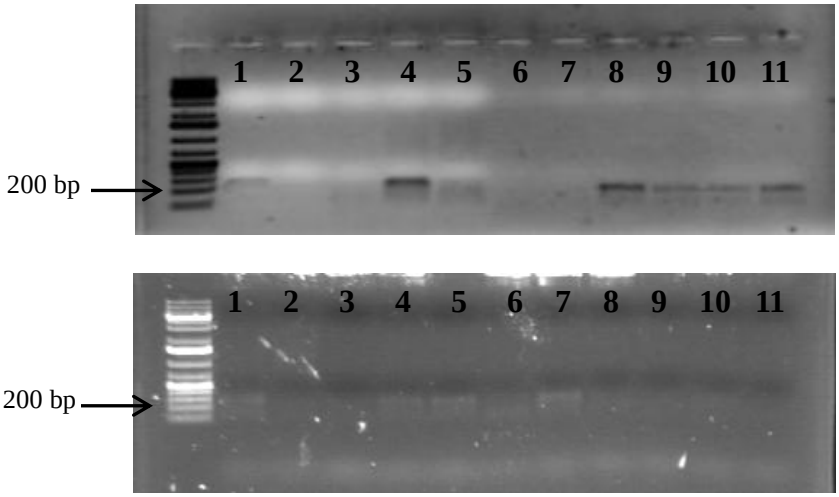

Figure 38. Results of SYBR Green qPCR assays confirming the presence of phenol monooxygenase. The expected size is 206 bp. qPCR results are shown in lanes 4-11 of the top gel and in lanes 1-11 of the bottom gel. In the top gel, lanes 4 and 5 are for Bed 8 (WT). Lanes 8-11 are for Bed 6 (r2E1). Lanes 6 -7 are for Bed 3 (unplanted). In the bottom gel, all positives (lanes 1, 4-7) are for Bed 8 (WT).

4.2.15 Soil Microcosm Incubations

Microbial degradation of TCE was observed in soil microcosm incubations in Bed 6 (r2E1) and Bed 8 (WT). Select microcosm TCE concentrations profiles are shown in Figure 39 - Figure 40. Log-transformed data indicated that removal followed first-order kinetics ($r^2 = 0.94 \pm 0.05$, r2E1 TCE-only vials; $r^2 = 0.89 \pm 0.1$, WT TCE-only vials). Calculated first-order rate-constants per gram of soil are shown in Figure 41. Degradation was inhibited in sodium azide vials,

demonstrating that the majority of TCE removal in other microcosms was due to biological degradation and not sorption. TCE appeared to be oxidized since additional chloroethene peaks were not observed in the GC-ECD analysis. The addition of methane did not enhance the degradation rate of TCE. It is unclear as to why the addition of acetylene did not inhibit removal in vials that received TCE, CH_4, and C_2H_2 while removal was inhibited in vials that received *Methylomonas* sp. strain LW13, TCE, CH_4, and C_2H_2. There was no significant difference between r2E1 and WT microcosm first-order rate-constants ($P<0.05$, tukey test).

The microcosm degradation rates likely overestimate actual activity in the field due to the effects of soil disturbance. Previous research has found that disturbance of field soil increases microbial oxidation rates due to the release of organic matter in soil aggregates.[64] Short term increases in CO_2 production and a decline in soil carbon were observed following tillage disturbance.[65, 66] Additionally, the microcosm degradation rates may overestimate the actual microbial contribution to TCE degradation in the field because of the exclusion of plant roots; plant uptake of TCE would reduce TCE available for microbial degradation. However, the exclusion of plant roots may also underestimate field degradation due to the exclusion of continual root exudate input into the rhizosphere, though this effect would likely be small over the microcosm experimental period.

Results from qPCR on soil collected for the microcosms confirmed the presence of *pmoA*, *Dehalococcoides* 16S, and phenol monoxygenase. *Dehalococcoides* 16S was detected only in Bed 8 (WT) soil. Gene counts for *pmoA* per gram of soil are shown in Table 14.

4.2.15.1 *Modeling*

The microcosm first-order rate-constants were utilized in modeling to determine the potential contribution of microbial degradation in bed soil to overall removal of TCE in the planted beds. Rate-constants determined from the TCE-only microcosms were utilized in modeling calculations. Removal due to sorption was approximated by removal observed in the sodium azide vials and rate-constants were corrected accordingly. Modeling was performed with a batch, first-order model (Appendix A, equation 1). The initial concentration of TCE in the soil water for use in modeling was calculated by performing a mass balance on TCE concentration in the soil matrix, as measured over the 2012 growing season (Figure 30 - 32). The soil matrix mass balance considered sorption into soil and partitioning into both soil air and water (Appendix A, equation 2). TCE soil concentration was assumed to be at steady-state over the growing season. The steady-state TCE concentration in the soil water was determined to be 0.185 mg L^{-1}, 0.210 mg L^{-1}, and 0.046 mg L^{-1} in Bed 6 (r2E1), Bed 8 (WT), and Bed 3 (unplanted), respectively. TCE degradation over a 1-day period was calculated. The removal per day was then multiplied by the length of the growing season to obtain total potential removal of TCE over the growing season. Calculations were performed for the volume of soil represented by the entire vadose zone. Modeling was performed with average rate-constants and also with the high and low rate-constants as given by standard deviation. Results are shown in Table 15.

For the volume of soil represented by the entire vadose zone, degradation of 14% and 15% of input TCE was predicted on average for Bed 6 (r2E1) and Bed 8 (WT), respectively. These results indicate that microbial degradation in the vadose zone of the planted beds (including the

rhizosphere) may have contributed to degradation of TCE in the 2012 growing season and that the contribution accounted for 15% to 20% or less of input TCE for both planted beds.

Validation of Modeling

The selected modeling time-period of one day assumes that the change in TCE in soil water can be replenished within a day. Calculations of diffusive flux of TCE verify this assumption given the relative diffusivity of TCE in soil with the specific test bed porosity, water content, and depth to the saturated zone containing TCE. Calculations of the diffusive flux of TCE through the unsaturated zone in the planted beds given the average aqueous TCE concentration in the bottom saturated zone (assuming no TCE present in the air or water of soil pores in the unsaturated zone) indicated that between 620 and 920 mg of TCE can diffuse upwards into the unsaturated layer daily (for a porosity ranging from 0.45 to 0.5, respectively) (Appendix A, equation 3). The calculated steady-state soil water concentration of TCE multiplied by the total average water volume in the vadose zone indicated that at steady-state, 340 mg and 390 mg of TCE were present in the vadose zone water of Bed 6 (r2E1) and Bed 8 (WT), respectively. The daily potential upward diffusive flux of TCE could therefore approximately replenish depleted soil water TCE due to modeled microbial degradation.

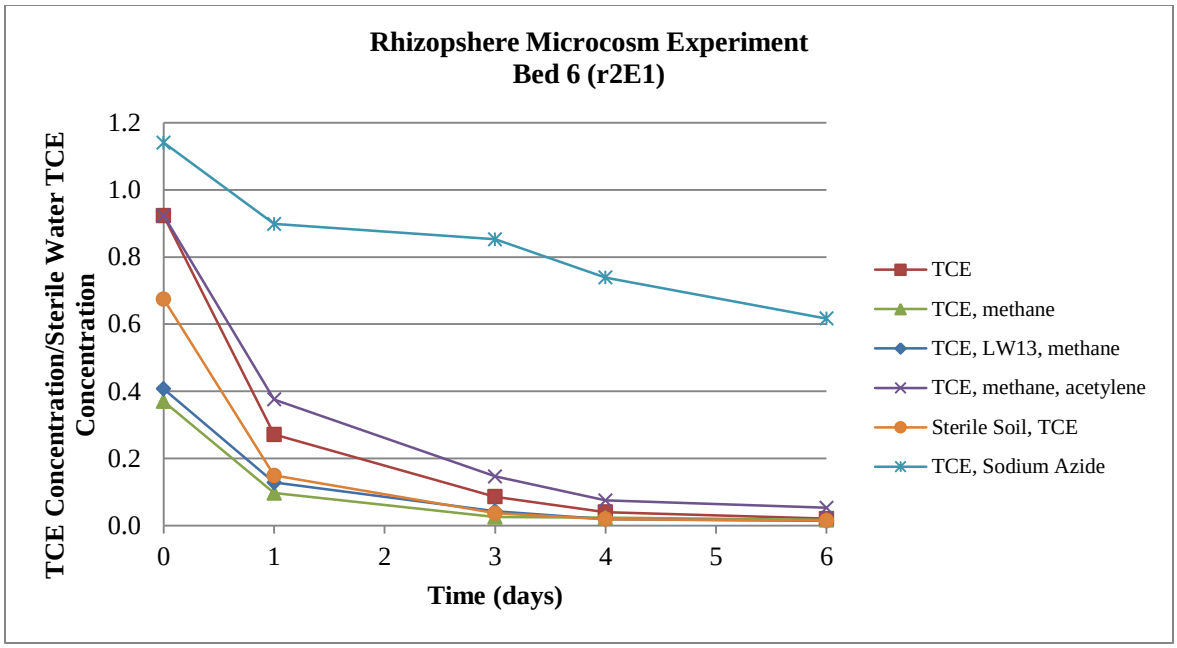

Figure 39. TCE degradation in soil microcosm incubations for field test bed soil collected at the 30 cm depth in Bed 6 (r2E1). Chemical dosing occurred two hours prior to time zero. Six of the seven vial experiment types are shown below. Points are measurements from one of the three vials for each experiment type.

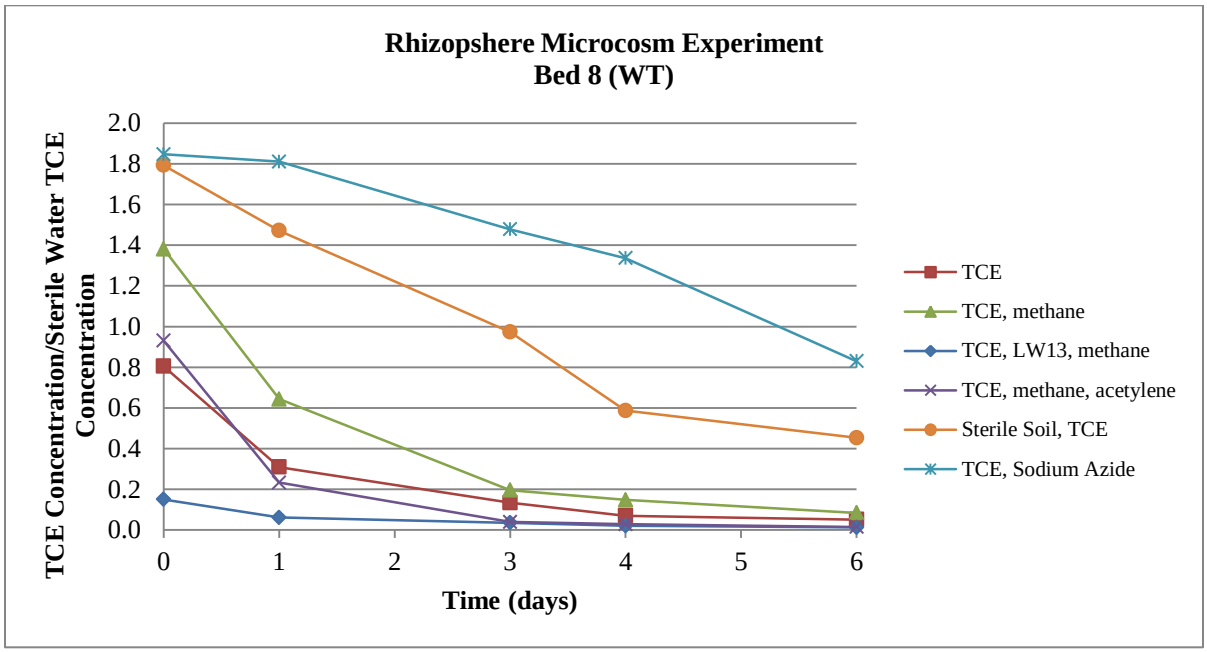

Figure 40. TCE degradation in soil microcosm incubations for field test bed soil collected at the 30 cm depth in Bed 8 (WT). Chemical dosing occurred two hours prior to time zero. Six of the seven vial experiment types are shown below. Points are measurements from one of the three vials for each experiment type.

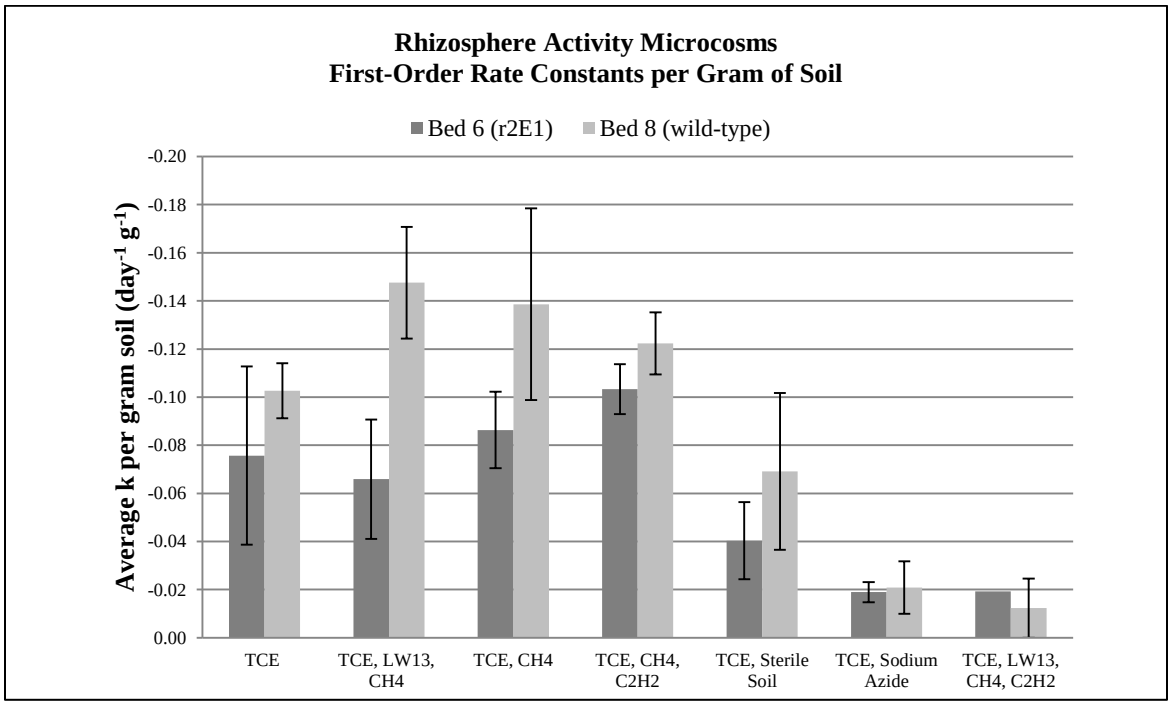

Figure 41. First-order rate constants (per gram of soil) determined by linear-regression from soil microcosm experiments. Bars represent the average and standard error of triplicate vials.

Table 14. Measured *pmoA* copies per gram of soil in soil used in soil microcosm experiments for Bed 6 (r2E1) and Bed 8 (WT). Mean and standard error are for duplicate measurements.

	pmoA copies per g soil		
Bed 6 (r2E1)	368,589		
Bed 8 (WT)	205,866	±	54,321

Table 15. Estimated mass of TCE degraded in the bed vadose zone soil by first-order, batch modeling for the 2012 growing season. Modeling utilized experimentally determined first-order rate constants (k) from the TCE-only soil microcosm incubations (corrected for sorption). The average, high, and low estimations result from modeling incorporating the standard error of the first-order rate constants.

	Average k		**High k**		**Low k**	
	TCE Degradation (mol)	Percent of Input TCE	TCE Degradation (mol)	Percent of Input TCE	TCE Degradation (mol)	Percent of Input TCE
Bed 6 (r2E1)	0.145	14%	0.200	19%	0.070	7%
Bed 8 (r2E1)	0.157	15%	0.212	20%	0.148	14%

4.2.16 Phytoremediation Modeling

Tree modeling results are shown in Table 16 for the combined removal predicted for all 12 trees in each bed for the median root biomass estimation. Modeling results are also shown for the median, high, and low root biomass estimations in Table 17. Modeling predicted the steady-state concentration entering and leaving each compartment (root, stem, and leaf). To obtain TCE removed over the entire growing season, the difference between the concentration in and out of each compartment was multiplied by the average transpiration flow per day and the length of the growing season (Appendix A, equation 4). The modeling predicted that 0.52 and 0.50 mol of TCE were taken up and metabolized by the r2E1 and WT trees, respectively. These values are 49% and 47% of total input TCE over the 2012 growing season in Bed 6 (r2E1) and Bed 8 (WT), respectively.

Interestingly, the model shows that the majority of tree removal of TCE occurs in the stem for both r2E1 and WT trees: 89% and 62% of total removal occurred in the stem for Bed 6 (r2E1) and Bed 8 (WT), respectively. Despite the reduction in WT metabolism rate, the WT tree system is capable of removing the majority of the TCE that enters the stem compartment. The model also predicts a greater amount of TCE removal in the leaves of the WT tree than in the r2E1 trees. This result may explain why total percent removals of input chlorinated ethene were similar for r2E1 and WT beds in 2012.

The modeling results shown ignore loss due to volatilization from both stem and leaves. When modeling was performed with diffusional loss from stem included, a loss of approximately 0.2 mol of TCE (20% of all 2012 growing season input TCE) was predicted for the planted beds

over the course of the growing season. Our field measurements contradict this prediction; loss of TCE from tree stem accounted for only 1.46×10^{-6} mol in Bed 6 (r2E1), less than 0.001% if total input TCE. A possible explanation for this discrepancy may be that the model does not account for decreased permeability and increased sorption capacity of bark for TCE. Gopalakrishan et al. found that bark diffusion coefficients for TCE were 2-10 times lower than in wood for five different tree species.[26] Additionally, sorption capacity for TCE was found to be higher in bark than in wood due to the greater lipid content of bark.[26] *Populus Tremula* bark thickness increases with age.[67] Therefore, the contribution of bark to inhibition of TCE loss from stem was likely substantial, given that the trees were six years old and that minimal amounts of TCE were found to volatilize from the stem.

Table 16. Fruit Tree Model results. C_{in} and C_{out} are the steady-state TCE concentrations entering and leaving each tree compartment, as predicted by the model. Total TCE removed is the total removed by all 12 trees in each test bed over the entire growing season. Modeling was performed for the median estimation of root biomass.

Tree Compartment	C_{in} (mg/L)	C_{out} (mg/L)	Total TCE Removed over Growing Season (mol)
Roots			
Bed 6 (r2E1)	2.94	2.60	0.059
Bed 8 (WT)	2.94	2.81	0.021
Stem			
Bed 6 (r2E1)	2.60	0.00048	0.46
Bed 8 (WT)	2.81	1.0	0.31
Leaves			
Bed 6 (r2E1)	0.00048	NA	0.000085
Bed 8 (WT)	1.0	NA	0.17
Total TCE Removed (mol)			
Bed 6 (r2E1)			0.52
Bed 8 (WT)			0.50

Table 17. Fruit Tree Model results for TCE removal predicted over the 2012 growing season for low, median, and high root mass estimations. Total TCE removed is the total removed by all 12 trees in each test bed over the entire growing season

Tree Compartment	Total TCE Removed over Growing Season (mol)	Total TCE Removed over Growing Season (mol)	Total TCE Removed over Growing Season (mol)
Roots	**Median Root Mass**	**High Root Mass**	**Low Root Mass**
Bed 6 (r2E1)	0.059	0.18	0.01
Bed 8 (WT)	0.021	0.08	0.00
Stem			
Bed 6 (r2E1)	0.46	0.34	0.51
Bed 8 (WT)	0.31	0.27	0.32
Leaves			
Bed 6 (r2E1)	0.000085	0.000063	0.000094
Bed 8 (WT)	0.17	0.15	0.18
		Total TCE Removed (mol)	
		Bed 6 (r2E1)	0.52
		Bed 8 (WT)	0.50

5 Discussion

The objective of this research was to evaluate the effectiveness of poplars genetically modified with CYP2E1 to enhance degradation of TCE under field conditions. To evaluate primary processes responsible for TCE removal, fate and transformation of input TCE was monitored with a chlorine mass balance. Percent recovery of input TCE described respective test bed removal efficiency. Metabolite concentration in plant tissue and volatilization of TCE from stem and leaf were important indicators of plant degradation activity. Soil microcosm experiments and qPCR results allowed for estimation of microbial contribution to TCE removal in the mass balances. A steady-state phytoremediation plant model provided insight into internal tree processes and the role of each tree compartment in TCE removal.

The trees were healthy and were actively transpiring. The r2E1 trees transpired greater amounts of water, presumably due to their larger size. Previous work confirmed the expression of the CYP2E1 transgene in field-planted r2E1 trees.[55] Expression levels were comparable to those observed in the laboratory. Plant tissue microcosms for leaf and root indicated that activity was higher in r2E1 leaf tissue, suggesting that CYP2E1 expression may be higher in leaf than in root.[55]

In order to evaluate the effectiveness of the phytoremediation of TCE in each planted test bed, several data sets were considered: TCE concentration in effluent water and soil, chloride concentration in effluent water and soil, TCE volatilization and evapotranspiration from trees, and oxidative metabolite concentration in tree tissue. In 2012, no difference was observed between effluent TCE concentrations from the r2E1 and WT beds. Average TCE concentrations

were higher in the r2E1 bed water. Average 2012 TCE soil concentrations were also higher in Bed 6 (r2E1) than in Bed 8 (WT). A reduction in TCE water concentration would be a key indicator of enhanced TCE degradation in the r2E1 roots. Increased diffusive uptake into roots from soil water and air would subsequently reduce the aqueous concentration in the bottom saturated layer. However, no difference was observed between r2E1 and WT bed TCE concentrations. Average water levels of r2E1 and WT beds differed by no more than 6%. Mass of recovered reductive metabolites cDCE and VC was greater in effluent water from the WT bed and may explain the greater rate of decrease in TCE concentration during the second half of the 2012 growing season. The TCE concentration levels in the planted beds do not support substantially increased uptake of TCE into r2E1 roots.

In both 2011 and 2012, dehalogenation was the primary fate of input TCE in both planted beds. In 2011, the accumulation of chloride ion in the soil accounted for 99% and 98% of the dehalogenation of TCE in Bed 6 (r2E1) and Bed 8 (WT), respectively. Other loss pathways including volatilization and recovery in effluent water accounted for less than 5% of input TCE in the planted beds. In 2012, the accumulation of chloride ion in the soil accounted for 95% and 99% of the dehalogenation of TCE in Bed 6 (r2E1) and Bed 8 (WT), respectively. Metabolite accumulation in r2E1 tissue was a major source of recovery of input TCE in Bed 6 (r2E1). The accumulation of chloride in the vadose zone soil was either a result of *in planta* degradation of TCE and subsequent export of excess chloride from roots or of microbial mineralization of TCE in soil. Chloride influx and efflux is regulated across plasma membranes of plants[68-70] and efflux of chloride ion from roots has been observed.[71] The field root enclosure experiments suggest that chloride ion was excreted by r2E1 and WT tree roots. However, though roots were brushed

thoroughly to remove soil and were sterilized in the first experiment, it is possible that bacteria colonizing the surface or interior of roots may have degraded TCE as it diffused out of the root. Methanotrophic bacteria have been shown to colonize root surface and interior of wetland plant species[35] as well as *Lespedeza cuneata* and *Pinus taeda.*[72]

Soil microcosm results indicated that the combined microbial contribution to degradation of TCE likely accounted for no more than 20% of total input TCE in the planted beds over the 2012 growing season. Differences in removal between Bed 6 (r2E1) and Bed 8 (WT) soil were not significant. For modeling with the soil microcosm high-scenario first-order rate constants, microbial activity accounted for 33% and 23% of accumulated chloride ion in vadose zone soil in 2012 in Bed 6 (r2E1) and Bed 8 (WT), respectively. Therefore, it appears that the majority of chloride ion accumulation in soil and dehalogenation of TCE in field test beds was due to *in planta* metabolism of TCE and subsequent efflux of excess chloride from roots. Importantly, this result also shows that microbial activity was not a more dominant process in the WT bed and that TCE removal efficiency was similar for WT and r2E1 poplar trees. Endophytic degradation of TCE in plant tissues may have contributed to *in planta* mineralization of TCE; however, this potential loss pathway was not investigated in this study.

TCE volatilization measurements and metabolite levels were clear evidence of increased *in planta* metabolic activity in the r2E1 poplars. Greater r2E1 oxidative metabolite levels, lower TCE levels in r2E1 plant tissue, and lower TCE volatilization from trees support that transformation of TCE was greater in the r2E1 trees. The flux of TCE from r2E1 trees was

significantly reduced 5-fold for evapotranspiration measurements (an 80% reduction) and up to 14-fold for stem measurements (an average reduction of 90%).

Plant modeling simulations were used to assess the relative contribution of each tree compartment to TCE removal in the r2E1 and WT trees. The phytoremediation modeling results are most appropriately utilized to provide general insight into internal tree removal processes rather than for inferring precise masses of TCE removed, given the steady-state assumption and the approximation of both stem and WT leaf and root (active) rate-constants. Several important observations can be made regarding the effect of enhanced transgenic metabolism on TCE removal in the field from model results. First, the r2E1 trees enhanced removal of TCE in the field, though only modestly: a 1% increase in removal of total input TCE in the 2012 growing season was predicted. This difference was due primarily to enhanced metabolism in the r2E1 root compartment (for median root mass estimations), given that all translocated TCE was degraded in either the stem or leaf compartment of both r2E1 and WT trees. Second, the WT trees can also remove a substantial portion of input TCE due to metabolism in the stem and leaf compartments. Third, a minimal amount of translocated TCE enters the leaf compartment in the r2E1 trees where the highest metabolism rate of TCE was measured in plant tissue microcosms.[55] This demonstrates that in order for the transgenic trees to have a more pronounced impact on TCE removal in the field, the rate of metabolism in the root would need to be greater. Because uptake of TCE into the root occurs by diffusion from soil as well as advection, the root compartment is not subject to the same mass transfer limitations as the aerial tree compartments and therefore has the greatest potential to impact TCE removal.

Field measurements appear to support model predictions. At the lowest stem height, volatilization of TCE was significantly reduced in r2E1 trees. This may be due to greater degradation of TCE in roots and therefore a reduced xylem TCE concentration. TCE concentrations were lower in both stem and branch of r2E1 tissue than in WT tissue, perhaps due to greater r2E1 metabolism rates in both root and stem compartments. Previous work with tobacco found that glycosylation of TCOH occurs primarily in roots and is transported to leaves;[20] our r2E1 leaf TCOH-glucoside levels were 40-fold higher than in WT leaves, indicating that greater amounts of TCOH may have been produced and glycosylated in roots prior to transport to shoots. TCE concentrations in r2E1 and WT leaf were not significantly different, although TCE concentrations were higher in WT stem and branch tissue, and may therefore indicate that greater amounts of TCE were degraded in the WT leaf (as predicted by the model), given that volatilization of TCE from WT leaves accounted for less than 0.01% of input TCE.

The assumption that metabolism occurs in the tree stem is important to drawing conclusions from model results. Results from hydroponic studies from Sharon Doty's lab indicate that stem tissue is metabolically active and may be more active than either root or leaf tissue (personal communication). In hydroponic studies with TCE and transgenic cottonwood, TCOH levels per gram of tissue were often highest in stem with levels up to 5-fold higher than in root or leaf tissue (data not shown). In this study, TCOH was found in both r2E1 and WT stem tissue. Additionally, because volatilization of TCE from leaves in the field was an essentially negligible sink in the overall TCE mass balance, metabolism of TCE likely occurred in the tree stem, given that volatilization from tree stem was also negligible and that TCE was being translocated. If

metabolism in stem had not occurred, we would expect greater amounts of TCE to have volatilized from leaves.

Implicit in TCE removal estimated from plant modeling is the role of plant tissue residence time. A comparison of the residence time and reaction half-life of TCE in tree root tissue indicated that residence time dampened the effect of enhanced r2E1 root metabolism. For the median estimated root volumes and measured transpiration rates, the residence time of water in roots was determined to be 0.45 and 0.40 days for r2E1 and WT trees, respectively. The half-life of TCE in r2E1 root was previously determined to be 3.1 days.[55] Therefore, the residence time in roots appears to limit the impact of enhanced r2E1 root metabolism in the field.

Importantly, this study shows that phytoremediation of TCE was enhanced by the r2E1 trees. Evapotranspiration and diffusion of TCE from tree stem were significantly reduced in r2E1 trees. However, the increase in metabolism rate in the r2E1 root appeared to be insufficient to substantially enhance removal in the field. It was hypothesized that increased *in planta* metabolism in roots would result in a larger concentration gradient across plant tissues, increasing diffusive flux of TCE into plant roots. Plant uptake of non-ionic compounds is a passive process, occurring by mass flow with transpiration water and by simple molecular diffusion across plant membranes. An increase in the spatial gradient of TCE due to more rapid metabolism would increase the mass flux per unit area per unit time into plant roots.
Given the residence time in root tissue, the enhanced CYP2E1 metabolic rate appeared to be insufficient to increase diffusive flux into the root tissue. The highest rates of metabolism were observed in r2E1 leaf tissue, which due to mass transfer limitations cannot significantly increase

diffusive uptake into the plant. Transport mechanisms therefore appear to limit the effect of enhanced *in planta* metabolism in the field. This study demonstrates the importance of field studies to corroborate laboratory results in phytoremediation. Field studies are complex with a variety of factors affecting plant performance that are not present in the laboratory.

Recommendations for future research include measurement of stem metabolism of TCE in both r2E1 and WT tissue and more thorough investigation of the rhizosphere microbial community to understand the specific involvement of different bacteria in TCE degradation, perhaps through metagenomic or proteomic approaches.

Acknowledgements

I would like to thank Dr. Marina Kalyuzhnaya, Dr. Rebecca Parales, Dr. Kirsti Ritalahti, Dr. Frank Loeffler, Dr. Vicky Shingler, and Dr. Jim Spain for generous provision of bacterial genes for use in PCR and qPCR. I would also like to thank Eric Nassau, Quyen Nguyen, Long Zhang, Peter Andeer, Keith Stewart and Manmeet Waria for assistance in laboratory technique and Carolina Parada for her assistance with microcosms. Thanks to Indulis Muizneks all his excellent help in the field, thanks to Andy James for helpful discussions, and thanks to Stefan Trapp for the use of his Fruit Tree Model. Finally, I would like to thank Dr. Stuart Strand for his guidance, patience, and encouragement.

Funding was provided by the University of Washington Superfund Basic Research Program, Grant: NIEHS P42ES04696

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Appendix A - Equations

Equation	Parameter Calculated	Formula	Variable Definition	Variable Formulae
1	Change in TCE concentration, batch first-order modeling	$C(t) = C_o e^{-kt}$	C(t) – concentration of TCE at time t C_o – initial aqueous TCE concentration k – first order rate constant, d^{-1} t - time	
2	TCE concentration in soil water	$C_{TCE,soil\ matrix} M_{soil\ matrix} = C_{w,TCE} V_w + C_{a,TCE} V_a + K_{D,TCE} M_{soil}$	$C_{TCE,soil\ matrix}$ – concentration of TCE in soil matrix $M_{soil\ matrix}$ – mass of soil matrix $C_{TCE,w}$ – aqueous concentration of TCE V_w – volume of water in soil matrix C_{aTCE} – concentration of TCE in air of soil matrix V_a – volume of air in soil matrix $K_{D,TCE}$ – solids partitioning coefficient M_{soil} – mass of soil in soil matrix	$K_{D,TCE} = f_{oc} K_{oc}$ f_{oc} – fraction of organic carbon in soil K_{oc} – organic carbon partitioning coefficient
3	Flux of TCE through the unsaturated soil zone, calculated at 15°C	$\theta_g D_{uz,15°C} \frac{C_{a,15°C}^{eq}}{\delta}$	D_{uz} - TCE diffusivity in unsaturated zone C_a^{eq} - [TCE] in air in equilibrium with saturated zone τ_g –tortuosity δ - depth of unsaturated zone	$D_{uz,15°C} = D_{a,15°C}/\tau_g$ $C_a^{eq} = [TCE]_{sat} \times K_{aw,15°C,TCE}$ $\tau_g = \frac{\varphi^{5/2}}{\theta_g^4}$ ϕ - porosity θ - volumetric gas content K_{aw} – TCE partitioning coefficient for air, water
4	TCE removed in tree compartment - phytoremediation modeling	$(C_{in} - C_{out}) \times Q \times t$	C_{in} – TCE concentration of flow into compartment C_{out} – TCE concentration of flow out of compartment Q – transpiration flow through tree per day	

Appendix B – Biomass estimations for Bed 6 (r2E1) tree root, leaf, stem, and branch

*Asterisks indicate median estimations selected for use in chlorine mass balance calculations and phytoremediation modeling.

Root	**Estimation 1**	**Estimation 2***	**Estimation 3**
Species	*Populus Tremuloides*	*Populus Deltoides*	*Populus Deltoides*
Location	Manitoba, Canada [59]	India [58]	India [73]
Mass of Compartment, dried (kg)	3.0	289	233
Volume of Compartment (L)	8.5	79	647

Leaf	**Estimation 1***	**Estimation 2**	**Estimation 3**	**Estimation 4**	**Estimation 5**
Species	*Populus Tremuloides*	*Populus Tremuloides*	*Populus Deltoides*	*Populus Tremuloides*	*Populus Tremuloides*
Location	Manitoba, Canada [59]	Wisconsin [74]	Maine[75]	Calgary, Alberta [76]	British Colombia [77]
Mass of Compartment, dried (kg)	5.7	560	9.5	3.5	4.0
Volume of Compartment (L)	1,417	140,108	2,372	866	992

Stem	**Estimation 1**	**Estimation 2**	**Estimation 3***	**Estimation 4**
Species	*Populus Tremuloides*	*Populus Tremuloides*	*Populus Tremuloides*	*Populus Tremuloides*
Location	Manitoba, Canada [59]	Wisconsin[74]	Nova Scotia[60]	Nova Scotia[78]
Mass of Compartment, dried (kg)	20	2785	84	96
Volume of Compartment (L)	54	7736	234	266

Branch	**Estimation 1**	**Estimation 2***	**Estimation 3**
Species	*Populus Tremuloides*	*Populus Tremuloides*	*Populus Tremuloides*
Location	Nova Scotia[60]	Northern Wisconsin[61]	Calgary, Alberta[76]
Mass of Compartment, dried (kg)	56	14	11
Volume of Compartment (L)	155.4	38.8	30.3