A Field Trial of TCE Phytoremediation by Genetically Modified Poplars Expressing Cytochrome P450 2E1

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ABSTRACT: A controlled field study was performed to evaluate the effectiveness of transgenic poplars for phytoremediation. Three hydraulically contained test beds were planted with 12 transgenic poplars, 12 wild type (WT) poplars, or left unplanted, and dosed with equivalent concentrations of trichloroethylene (TCE). Removal of TCE was enhanced in the transgenic tree bed, but not to the extent of the enhanced removal observed in laboratory studies. Total chlorinated ethene removal was 87% in the CYP2E1 bed, 85% in the WT bed, and 34% in the unplanted bed in 2012. Evapotranspiration of TCE from transgenic leaves was reduced by 80% and diffusion of TCE from transgenic stems was reduced by 90% compared to WT. Cis-dichloroethene and vinyl chloride levels were reduced in the transgenic tree bed. Chloride ion accumulated in the planted beds corresponding to the TCE loss, suggesting that contaminant dehalogenation was the primary loss fate.

INTRODUCTION

Trichloroethylene (TCE) is one of the most common groundwater contaminants in the United States. Several studies have found that plants can take up and metabolize TCE, and that the presence of trees may lead to enhanced groundwater remediation. Trichloroethylene can enter trees via uptake with water and by diffusion into roots. Poplars metabolize TCE by an oxidative pathway, producing the metabolites trichloroethanol (TCOH), dichloroacetic acid (DCAA), trichloroacetic acid (TCAA), and a bound TCOH-glucoside. In addition to metabolism within tree tissues, TCE is subject to diffusion through trunk, branches, and leaves to the atmosphere and sorption to tissues, and possible transformation by endophytes.

Many studies of phytoremediation of TCE and other chlorinated volatile organic compounds (VOCs) have not attempted to obtain a mass balance that accounts for all possible fates or to determine levels of the products of VOC metabolism, such as chloride ion. Some studies have focused exclusively on volatile loss pathways. Studies of trees growing over contaminated groundwater plumes are inherently handicapped in their ability to draw conclusions about actual effects on groundwater TCE since TCE uptake has to be estimated. Some laboratory measurements have used batch exposures of trees in small open vessels, precluding the achievement of a steady exposure to TCE that is required for mass balances.

Another approach is to determine mass balances of TCE and its metabolic products by exposing trees in field settings to TCE in contained test beds. This technique permits direct measurement of TCE exposure and allows for the measurements of all fate pathways. Newman et al. applied water containing TCE to the subsurface of test beds planted with hybrid poplar (Populus trichocarpa x P. deltoides). The planted test bed removed approximately 99% of the TCE mass applied, whereas only 33% was removed in the unplanted control. A chlorine mass balance indicated that the TCE loss in the planted bed could be accounted for by increased soil chloride. Volatilization of the pollutants from leaves and stems was insignificant in this study, and subsequent test bed mass balance studies. This study and others suggest that trees may effectively treat VOCs in proportion to the groundwater they take up and transpire, and that the contaminant concentration in the root zone water is unchanged.

The advantages of phytoremediation include low cost and low energy input, but uses of phytoremediation have been limited, largely due to its uncertain effectiveness. Genetic transformations have been undertaken to increase the activities of pollutant transformations in plants. A series of
laboratory experiments investigated the effects of the expression of mammalian cytochrome P450 2E1 (CYP2E1) in hybrid poplars (*Populus tremula* x *P. alba*) and tobacco (*Nicotiana tabacum cv. Xanthii*) against VOCs\textsuperscript{31–34} that are substrates for CYP2E1 in the mammal. The transgenic plants degraded a wide variety of compounds such as TCE, vinyl chloride, carbon tetrachloride, chloroform, benzene, toluene, and bromodichloroethane at significantly higher rates compared to wild type or vector control plants. Others have engineered petunia\textsuperscript{35} and alfalfa\textsuperscript{36,37} with CYP2E1 for phytoremediation of TCE.

In order to evaluate the effectiveness of the transgenic poplars in a field setting, water containing an equivalent amount of TCE was added to three hydraulically contained test beds for six growing seasons. One test bed was planted with genetically modified hybrid poplars expressing CYP2E1, one test bed was planted with unmodified hybrid poplars, and one bed was not planted. A chlorine mass balance was performed to identify the fates of chlorine. Parameters such as the VOC and chloride concentration in the influent and effluent water, VOC and total organic halide (TOX) concentration in plant tissues, soil chloride concentration, and VOC volatilization from the soil, leaves, and stem were measured, allowing the computation of mass balances and direct comparison of the three treatments. We performed soil microcosm studies to estimate microbial removal of TCE in test bed soil and to differentiate between tree and microbial contributions to TCE removal. To our knowledge this is the first field-scale mass balance evaluation of the effectiveness of phytoremediation of an environmentally important VOC by transgenic trees.

### MATRERIALS AND METHODS

**Test Facility.** The field site was located at the University of Washington Phytoremediation Field Facility. Three adjacent, hydraulically isolated test beds were used, as described previously.\textsuperscript{6} The beds each measured 9 × 5.7 m, 1.4 m deep. The interior soil was removed from the beds in September 2006 and replaced with clean fill (sandy loam, 67.2% sand, 26.6% silt, 6.2% clay; total organic matter, 6.6%).

The transgenic hybrid poplar (*Populus tremula* x *P. alba*) expressed rabbit CYP2E1 under the cauliflower mosaic virus (CaMV) 35S promoter.\textsuperscript{31} Thirteen transgenic poplars (designated as clone 78 in the laboratory, herein referred to as r2E1) and 13 wild type (WT) INRA 717-1B4 clones (height approximately 1 m) were propagated in the laboratory, acclimated to exterior conditions, and planted at the field facility on April 11, 2007. One bed was planted with 12 r2E1 poplars, one with 12 WT poplars, and one left unplanted. One r2E1 and one WT poplar were planted in a separate test bed and not exposed to TCE. The presence of the transgene was verified by PCR on DNA extracted from poplar tissues following planting using the methods described previously.\textsuperscript{31}

**Water Supply and Chemical Dosing.** The three test beds received approximately equal concentrations of TCE during each growing season from 2007 to 2012; the 2012 growing season occurred from June 19, 2012 to October 28, 2012. The target influent TCE concentration was 15 mg L\textsuperscript{−1} from 2007 to 2011, and changed to 30 mg L\textsuperscript{−1} in 2012 to increase the sensitivity of metabolite detection. The dosing procedure was as described previously.\textsuperscript{6} Target water level depth as measured in the effluent wells was between 20 and 30 cm, although the unplanted bed water level was generally higher. A minimum of 16 L d\textsuperscript{−1} was pumped out of the test beds. Additional water was pumped out of the unplanted bed to match the volume transpired in the planted beds. Municipal water was used for irrigation and was added to the planted beds through the influent well or by surface watering as needed to maintain tree health. All test beds were open to rainfall. Rainfall levels were recorded on site.

**Sampling and Analysis.** Sampling and analysis of the influent and effluent water, soil chloride, volatilization of VOC from the soil and tree leaves and stem, and plant tissue analysis were performed as described previously.\textsuperscript{7,38} Influent water samples were collected daily and effluent samples at least weekly using methods described previously.\textsuperscript{6} A PerkinElmer Autosystem XL gas chromatograph (GC) 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. Concentrations of the chlorinated ethenes and ions were calculated based on external standards. Analysis for vinyl chloride (VC) was performed by collecting headspace samples from the effluent sample vials after they had been analyzed with the GC-ECD. All sampling was performed in triplicate. Analytical detection limit for VC was approximately 20 μg L\textsuperscript{−1}.

Chloride analysis was performed with a Dionex AS40 Automated Sampler connected to a Dionex DX-120 ion chromatograph (IC) with a Dionex IonPac AS14 4, 250 mm anion exchange column; eluent was 3.5 mM Na2CO3/1 mM NaHCO3 in degassed, deionized water. Analytical detection limit was approximately 0.1 mg L\textsuperscript{−1}.

The dissolved oxygen (DO) concentration and temperature were measured in the groundwater of each test bed approximately every 14 days. Prior to sampling, a minimum of 19 L of water was removed from each effluent well and wells were allowed to recharge for 1 h. DO analysis was performed with a YSI model 158 or 5000 DO meter.

Soil samples were collected three times each growing season. In 2012, samples were collected on May 22, 2012, August 21, 2012, and October 16, 2012. For each bed, a total of 24 soil core samples were collected, at six locations on a 2 × 3 grid, and at four depths for each location (10, 30, 60, and 100 cm below the soil surface). Analysis was as described previously.\textsuperscript{38} Briefly, soil samples were frozen prior to analysis, dried at 100 °C, extracted in water, shaken, centrifuged and analyzed using a Dionex DX-120 ion chromatograph. Analysis of TCE in the soil matrix was performed by collecting 10–15 g soil, preserved in sealed volatile organic analysis (VOA) vials with 10 mL methanol, and analyzing per EPA 8260B, and results reported as mg per kg dry weight.

Two sets of soil volatilization measurements were taken for the r2E1 and the WT beds in 2012. Soil volatilization from the unplanted bed was measured in 2008. Analysis was as described previously,\textsuperscript{39} using a soil vapor collection chamber.

Samples for the volatilization of VOCs from leaves were collected from both planted test beds simultaneously using leaf bag sampling as described previously,\textsuperscript{6} except that several leaves were enclosed in Teflon bags loosely tied around the stem and air was drawn through the bag and into activated carbon tubes with TCE trapped for later analysis by GC-ECD. Leaf areas were measured. Six leaf bag measurements were performed in the r2E1 bed and seven in the WT bed. Volatilization from the tree stem was measured at 0.25, 0.4, and 0.57 m heights as described previously.\textsuperscript{38} Loss of volatiles from the stems (i.e., trunks) was measured using 26 cm\textsuperscript{2} diffusion traps strapped to the trunks with air flowing through carbon tubes to trap
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Table 1. Chlorine Balance for Test Beds Planted with Either Transgenic Hybrid Poplars Expressing Mammalian Cytochrome P450 2E1 (r2E1), or Wild Type (WT) Hybrid Poplars, or from an Unplanted Control, for the 2012 Mass Balance Period (June 19, 2012 to October 28, 2012)\textsuperscript{44}

<table>
<thead>
<tr>
<th>inputs (mol as Cl)</th>
<th>output/accumulation (mol as Cl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>transgenic r2E1 bed</td>
<td>WT bed</td>
</tr>
<tr>
<td>VOC accumulated in bed water</td>
<td>0.19 ± 0.00</td>
</tr>
<tr>
<td>bed water chloride</td>
<td>0.89 ± 0.02</td>
</tr>
<tr>
<td>water TCE</td>
<td>3.18 ± 0.08</td>
</tr>
<tr>
<td>water dDCE</td>
<td>ND</td>
</tr>
<tr>
<td>water DC</td>
<td>ND</td>
</tr>
<tr>
<td>water chloride</td>
<td>1.87 ± 0.03</td>
</tr>
<tr>
<td>soil chloride</td>
<td>4.29 ± 4.07</td>
</tr>
<tr>
<td>rain water chloride</td>
<td>0.008 ± 0.00</td>
</tr>
<tr>
<td>leaf volatilization</td>
<td>0.88 ± 0.03</td>
</tr>
<tr>
<td>trunk volatilization</td>
<td>1.87 ± 0.03</td>
</tr>
<tr>
<td>tissue metabolites</td>
<td>10.4</td>
</tr>
<tr>
<td>recovery</td>
<td>109%</td>
</tr>
</tbody>
</table>

\textsuperscript{44}Soil chloride is the calculated molar mass of chlorine that accumulated in each of the test beds over the growing season. Tissue metabolite estimations assumed a uniform concentration of metabolites throughout the tree tissue. ND = analyte was not present above the detection limit. NA = not applicable. TCE = trichloroethylene. dDCE = cis-dichloroethylene. VC = vinyl chloride. VOCs = \( \sum \) (TCE, dDCE, VC). \textsuperscript{*}Superscripts indicate the groupings of the data between beds using the Kruskal-Wallis test and the Steel-Dwass-Critchlow-Fligner procedure/Two-tailed test: (p < 0.05).

emitted TCE for later analysis by GC-ECD. Four measurements were taken at each height in both beds in 2012 with the exception of three measurements at 0.57 m in the WT bed. The detection limits for volatilization fluxes were about 0.8 nmol of TCE m\textsuperscript{-3} air.

Plant tissue samples were collected from the field and the laboratory plant growth room. Sterilization, sampling, and analysis were as described previously.\textsuperscript{31,32} Root material was not surface sterilized to avoid damaging the root tissue. Plant tissue analysis included samples of root, stem, branch, and leaf tissue collected from at least three different trees in each test bed. For stem tissue, two of the three samples were combined in one metabolite extraction.\textsuperscript{6,12} Leaf and core samples were collected and immediately frozen on-site in liquid nitrogen, then homogenized and stored on dry ice for transport. Analysis for TCE and free metabolites was performed with published allometric linear regression equations for poplar root,\textsuperscript{40} stem,\textsuperscript{31} branch,\textsuperscript{42} and leaves.\textsuperscript{43} A minimum of three biomass estimations were performed for each compartment with equations from different sources using the median value. Total leaf area was estimated with a published allometric linear regression equation for leaf area of Populus tremuloides.\textsuperscript{44}

Statistical analysis was performed using XLStat software (Addinsoft SARL, Paris France). Since the data were not always normally distributed, nonparametric procedures were used to compare distributions of VOCs and chloride (Table 1).

**Quantitative RT-PCR.** Leaf and root samples were collected on site, immediately frozen in liquid nitrogen, transported to the laboratory in dry ice, and stored at \(-80^\circ\text{C}\) until analysis. Samples were ground under liquid nitrogen. Approximately 100 mg of tissue was used with the RNeasy Plant Mini Kit (Qiagen) to extract total RNA. RNA was used to synthesize cDNA with the Bio-Rad iScript kit with SYBR Green labeling agent. Quantitative RT-PCR was performed with SYBR Green-labeled probes of CYP2E1 and 18S as the housekeeping gene and included negative controls. The primers for the rabbit cytochrome P450 gene (r2E1) were (forward) AATGTG-CACCGTGGACTT and (reverse) TATGATAGCAG-GACCGGTTG, and for the poplar 18S RNA gene (forward) AATGTTGTCTTTCAACGAGGA and (reverse) AAAGGCGAGGAGCAGTGC. A specific amplification was observed.

**Soil Microcosms.** Microcosm experiments were conducted to measure aerobic TCE removal in soil. Soil was collected from each test bed at a depth of 30 cm and left in an open container for 3 days to allow TCE volatilization. Roots were removed from soil by visual inspection. Forty-mL VOA vials were filled with approximately 10 g of soil and sealed with septum valve caps (Mininert) and dosed with 0.05 mg TCE. The soil moisture content was maintained at approximately 50%.
field conditions. Vials had sufficient headspace to remain aerobic during the experiment.

Biotransformation activity was evaluated under four different conditions, each in triplicate with soil from each planted bed: (1) TCE only, (2) TCE and 10% v/v methane, (3) TCE, 10% v/v methane, and 1% v/v ethyne, and (4) TCE and 2 mL of 200 mg L⁻¹ sodium azide. Triplicate vials with 10 mL water and TCE only (no soil) served as controls for leakage. Headspace of TCE was sampled every 24 h for 6 days and analyzed on a PerkinElmer Autosystem GC-ECD, as described previously. Vials were allowed to equilibrate for 2 h following dosing prior to the time-zero measurement.

Figure 1. Effluent water trichloroethylene (TCE) concentration from test beds planted with either transgenic hybrid poplars (r2E1), or wild type (WT) hybrid poplars, or from an unplanted control bed, for the 2012 growing season. Influent TCE concentration was equal for all test beds, approximately 31 mg L⁻¹. Dashed black lines indicate the period of leaf fall.

Figure 2. Reductive dechlorination products of TCE in the effluent water over the 2012 growing season in Bed 6 (r2E1), Bed 8 (Wild Type), and Bed 3 (unplanted). Dashed black lines indicate the period of leaf fall. (A) cis-Dichloroethylene (cDCE) concentrations and (B) vinyl chloride (VC) concentrations.
Root Enclosure Experiment. Tree roots were isolated to estimate the efflux of chloride by roots. Portions of roots at the surface in each bed were exposed while still attached to the trees, excess soil removed by gentle brushing, enclosed in 8 × 14 in. FoodSaver© plastic bags with sterile sand, and sealed with Aquarium sealant or Teflon tape. Three root sections were enclosed in each planted bed and also in the undosed control bed containing one r2E1 and one WT poplar. The roots were not surface sterilized to avoid damaging the root tissue. Prior to use, the sand was autoclaved, washed three times with deionized water, and dried at 100 °C for 24 h. Chloride concentration was measured at the beginning and end of the experimental period as described above and each sand sample was extracted in duplicate.

RESULTS

Water Use/Water Balance. WT and r2E1 trees grew equally rapidly during the experimental period. The r2E1 trees were about 6% larger than the WT trees at planting and remained slightly larger throughout the experimental period (Supporting Information (SI) Table S1). These differences were not significant.

SI Table S2 summarizes the water use for each test bed from June 19, 2012 through October 28, 2012. The accumulation of water in each test bed was calculated based on the change in water levels during this period. The r2E1 and WT poplars transpired approximately 175 and 170 L d⁻¹, respectively, during the 2012 growing season, while loss of water from the unplanted bed was much less. The average water levels over the growing season in the r2E1, WT, and unplanted beds were 28.4 ± 10.9 cm, 30.2 ± 8.5 cm, and 53 ± 13.9 cm, respectively.

The average DO concentrations in the groundwater were 4.1 mg L⁻¹, 1.0 mg L⁻¹, and 0.68 mg L⁻¹ for the r2E1 bed, WT bed, and unplanted bed, respectively. Groundwater temperature is shown in SI Figure S1.

Influent and Effluent Water VOCs and Chloride. Influent water for all beds contained an average of 31.4 ± 4.4 mg L⁻¹ TCE during the 2012 growing season. The effluent TCE concentrations are shown in Figure 1. The average effluent concentration of TCE from June 19, 2012 through October 28, 2012 was 3.24 ± 1.3, 2.93 ± 0.96, 2.36 ± 0.97 mg L⁻¹ for the r2E1, WT, and unplanted beds, respectively. Interestingly, the temporal changes in concentration varied over the season. The system appeared to reach 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 for the r2E1, WT, and unplanted beds, respectively. The effluent water cDCE and VC concentrations are shown in Figure 2.

The average influent TCE fluxes into the beds were 3.6, 3.7, and 0.73 g d⁻¹ for the r2E1, WT, and unplanted beds, respectively. The average effluent TCE fluxes from the beds were 0.39 ± 0.16, 0.36 ± 0.12, and 0.06 ± 0.02 g d⁻¹ for the r2E1, WT, and unplanted beds, respectively. Thus, the loss rates of TCE from the beds were about 3.3, 3.3, and 0.67 g d⁻¹ for the r2E1, WT, and unplanted beds, respectively.

The average influent chloride concentration for 2012 was 2.66 ± 0.31 mg L⁻¹. Effluent water chloride concentration measurements for 2012 are shown in SI Figure S2. Effluent aqueous chloride concentrations increased over the growing season in the unplanted bed but not in the planted beds.

The effluent chloride concentrations from June 2011 through June 2012 are shown in SI Figure S3. Chloride concentration in the effluent from the r2E1 planted bed increased most during the winter season, compared to the WT and unplanted beds, suggesting a higher rate of accumulation of chloride in bed soil in the previous growing season. This is consistent with soil chloride measurements of the unsaturated layers of the three beds (SI Figure S5).

Soil TCE and Chloride. The season average concentrations of soil TCE were 0.77 ± 0.27 mg kg⁻¹, 0.68 ± 0.25 mg kg⁻¹, and 0.18 ± 0.03 mg kg⁻¹ for the r2E1 bed, WT bed, and unplanted bed, respectively (Figures S7–S8).

Soil chloride increased in the vadose zone of the planted beds (less than 100 cm deep) during the growing season (Figures S9–S11). A similar increase was not observed in the unplanted bed. On May 22, 2012, chloride concentration at 30 cm was
3.19 ± 1.17, 2.64 ± 0.50, and 1.64 ± 0.33 mg kg⁻¹ for the r2E1 bed, WT bed, and unplanted bed, respectively. By October 16, 2012, concentrations at the 30 cm depth had increased 6-fold from the beginning of the season in the r2E1 bed and 8-fold in the WT bed to 19.8 ± 17.2 and 21.3 ± 30.3 mg kg⁻¹, respectively. The increase in soil chloride in the vadose zone of the planted beds occurred in each growing season in this study and was also observed other phytoremediation field studies with poplar and chlorinated solvents. The accumulated chloride ion in the vadose zone was mobilized in the winter season due to heavy winter rain and possibly root decomposition (SI Figure S3).

**TCE and Metabolutes in Plant Tissue.** The leaves, stems, and branches of the r2E1 poplars had significantly higher concentrations of TCOH and TCOH-glucoside than the WT tissues and significantly lower concentrations of TCE for all tissues (P < 0.05, Two-Factor ANOVA; Figure 3). These results suggest that metabolism of TCE was enhanced in the r2E1 trees. TOX analysis in 2008 found no significant increase in the leaf tissues in the r2E1 or WT poplars compared to unexposed controls.

**Volatilization from Soil, Plant Stem, and Leaves.** The flux of TCE from soil was 19.9 ± 2.2 nmol m⁻² d⁻¹ and 81.6 ± 33.6 nmol m⁻² d⁻¹ for the r2E1 and WT beds, respectively (SI Figure S4). The soil volatilization flux was reduced 4-fold in the r2E1 bed compared to the WT bed. Flux of TCE from soil in the unplanted bed, measured in 2008, was 60 ± 41 nmol m⁻² d⁻¹.

Flux of TCE from stems (i.e., the trunks, collected at three heights), and from leaves is shown in SI Figure S4. The average flux of TCE from stem tree (trunk) was 7.2 ± 8.9 and 54 ± 20 nmol m⁻² d⁻¹ for the r2E1 bed and WT bed, respectively, an 87% reduction in r2E1 stem volatilization compared to WT. The difference in flux from the stem between r2E1 and WT trees was significant at all heights (P < 0.05). Stem flux did not change significantly with height for either the WT or r2E1 trees. Fluxes were higher from trees planted at the infuuent end of a test bed compared to trees planted at the effluent end (P < 0.05).

Flux of TCE from tree leaves and petioles in both the r2E1 and WT beds was significantly lower than from the stems; 0.672 ± 0.648 and 3.36 ± 1.2 nmol m⁻² d⁻¹, respectively. Flux of TCE from r2E1 tree leaves was reduced by 79% compared to WT (P < 0.05). Reduced volatilization of TCE from the r2E1 stem and leaves is evidence of enhanced metabolism in the r2E1 trees.

**Chlorinated Ethene Mass Balance.** Total inputs and outputs of chlorinated VOCs are shown in SI Table S3. Percent recoveries of chlorinated VOCs over the growing season were 13%, 15%, and 66% in the r2E1 bed, WT bed, and unplanted bed, respectively. A higher proportion of chlorinated ethenes was recovered in the unplanted bed compared to the planted beds.

**Chlorine Balance.** Table 1 lists the chlorine inputs, losses and accumulations for each test bed for the mass balance period. The total recovery was 109% from the r2E1 bed, 102% from the WT bed, and 106% from the unplanted bed. The largest source of recovery was as chloride ion in the vadose zone soil of all three beds, accounting for 31%, 47%, and 22% of total recovered chlorine in the r2E1, WT, and unplanted beds, respectively. The mass of metabolite accumulation was calculated assuming a uniform concentration throughout the tree tissue. Oxidative metabolite accumulation in tree tissue was a major source of chlorine recovery in the r2E1 bed, accounting for 16% of total recovered chlorine. Measured volatilization of TCE from stem, leaf, and soil was a minor loss pathway in all three beds, and represented less than 0.002% and 0.009% of total recovered chlorine for the r2E1 and WT beds, respectively.

**Plant Tissue RNA Analysis.** In order to confirm transcription activity of the r2E1 gene in the field trees, leaf and root samples were collected from transgenic trees in the field and from a specimen of clone 78 growing in the laboratory plant room that had not been exposed to TCE. Samples were analyzed for CYP2E1 mRNA and 18S rRNA expression by qRT-PCR in triplicate. The average 18S rRNA concentration in the leaf samples (0.74 ± 0.30 ng μL⁻¹) was significantly greater than in root samples (0.18 ± 0.10 ng μL⁻¹; P < 0.05). The relative levels of CYP2E1 transcript in the field samples and in the unexposed transformed plant from the laboratory were not significantly different (SI Figure S12).

**Plant Tissue Microcosms.** Root tissue samples were collected during tree dormancy in March 2008 from r2E1 and WT trees, and from r2E1 poplars actively growing in a plant growth room, and exposed to TCE in sealed VOA vials. The half-life of TCE lost from WT roots collected from dormant trees was not significantly different than from sterile controls. The TCOH and trichloroethanal (chloral) concentration in hydroponic media was monitored in a separate set of root samples. TCOH and chloral were detected in all r2E1 root samples at an average concentration of 0.35 ± 0.08 μg mL⁻¹ and 0.02 ± 0.002 μg mL⁻¹, respectively. TCOH was detected in only one WT root sample at 0.09 μg mL⁻¹; chloral was not detected above the analytical detection limit.

The calculated transformation rates from leaf, root, and stem samples collected from r2E1 trees (either from the test bed or the laboratory), and the wild type test bed, are shown in SI Table S4.

**Soil Microcosms.** Microbial transformation of TCE was observed in soil microcosms. TCE concentration profiles for selected microcosms are shown in SI Figure S13. Log-transformed data indicated that removal followed first-order kinetics (r² = 0.94 ± 0.05, r2E1 TCE-only vials; r² = 0.89 ± 0.1, WT TCE-only vials). Calculated first-order rate-constants per gram of soil are shown in SI Table S5; there was no significant difference between r2E1 and WT microcosms (P < 0.05, Tukey test). Removal was inhibited by sodium azide, suggesting that the majority of TCE removal was due to biological transformation rather than sorption. TCE appeared to be oxidized since neither DCE nor VC were detected.

**Estimation of Microbial Transformation in Soil.** The first-order rate constants calculated from the TCE-only microcosms were used to estimate the contribution of microbial removal in vadose zone bed soil to TCE removal in the planted beds. Sorption losses were approximated by removal observed in the sodium azide vials, and the TCE-only microcosm rate constants were corrected to account for sorptive loss rates. Microbial transformation was estimated assuming a batch, first-order system. The TCE concentration in the soil water was determined from the average of the measured field soil TCE concentrations and by assuming equilibrium partitioning in the soil matrix between soil, air, and water. TCE soil concentration was assumed to be at steady-state during the growing season. The average, steady-state TCE concentration in the soil water was determined to be 0.19 mg L⁻¹ and 0.21 mg L⁻¹ for the r2E1 bed and WT bed, respectively.

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TCE removal over a 1 day period was calculated and then multiplied by the length of the growing season to estimate total potential removal of TCE. Calculations were performed for the volume of soil in the vadose zone. Calculations used average rate-constants and also the high and low rate-constants as given by the standard deviation from the microcosms (SI Table S6). Results suggest approximately 15% of input TCE was lost through microbial transformation in the planted beds.

The microcosm removal rates likely overestimated actual activity in the field soils due to the effects of soil disturbance. Previous research has found that disturbance of field soil increases microbial oxidation rates.

**Root Enclosure Experiment.** Results from root enclosure experiments are shown in SI Table S7. There was a significant increase in chloride concentration in the r2E1 and WT sand for all samples (P < 0.05). There was no change in chloride concentration in control root sand (P < 0.05).

## DISCUSSION

The purpose of this work was to determine whether transgenic poplars expressing mammalian CYP2E1 were more effective than WT poplars for the phytoremediation of TCE-containing water in a field setting. Growth was vigorous in both beds and there was no evidence of deleterious effects from TCE exposure or the genetic modification. The conclusions we could draw from this study were limited by the lack of replicate field beds, but data acquired from two seasons provided confidence when differences between beds were large.

Expression of the transgene was confirmed through RT-qPCR and activity in plant tissue microcosms. The CYP2E1 transgene was expressed in the r2E1 trees planted in the field at a level that was equivalent to the transformed trees in the laboratory. Transgene expression in plants is generally stable, though it may decrease with environmental stress. No decrease was observed in this study. Transgene activities in plant tissue microcosms were consistent with previous findings. TCE transformation rates appeared to be higher in leaves compared to roots, with more TCOH and TCAA detected in the leaves (SI Table S4), consistent with studies showing higher transgene expression under the CaMV 35S promoter in leaves compared to roots. However, translocation of metabolites from roots or stem tissue to the leaves cannot be ruled out.

To evaluate the effectiveness of phytoremediation of TCE-containing groundwater several end points were considered, including the effluent water TCE concentration, the water, soil, and leaf chloride concentrations, and TCE transpiration and volatilization values. In 2012, no difference was observed between effluent TCE concentrations from the r2E1 and WT beds. A reduction in TCE water concentration would be a key indicator of enhanced TCE removal in the r2E1 roots as increased diffusive uptake into roots would subsequently reduce the aqueous concentration. Concentrations of cDCE and VC concentrations were significantly less in effluent water from the r2E1 bed compared to the WT (p < 0.05, Figure 2), suggesting that reductive dechlorination may have been a more important process in the WT bed. The lower measured DO concentration in WT bed suggested conditions were more favorable for reductive dechlorination compared to the r2E1 bed, possibly due to the greater oxidative activity of the transgenic tree roots although this conclusion cannot be completed due to the lack of replicate text beds.

The extent of TCE transformation in the unplanted control was unexpected as it had not been observed in previous field studies. The presence of cDCE and VC in the test bed effluent water, and the low DO concentrations, suggest that reductive dechlorination was responsible for TCE loss in the unplanted test bed.

The chlorine mass balance indicated that the primary fate of input TCE in both planted beds was dehalogenation. Only 13% and 15% of input TCE in the r2E1 and WT bed, respectively, was recovered as VOCs. The largest mass of chloride was recovered as increased free chloride in the vadose zone soil, accounting for 31% and 47% of the recovered chloride in the r2E1 bed and WT bed, respectively (compared to 22% in the unplanted bed). Our measurements indicated that volatilization of chlorinated ethenes from leaf, stem, or soil was not a significant loss mechanism in any of the test beds, although the uncertainty of the mass balance (especially of the chloride measurements) allows for a possible greater role for volatile losses that our methods did not detect. The accumulation of metabolites in tree tissues accounted for a major fraction of the TCE chloride in the r2E1 bed mass balance.

The accumulation of chloride in the vadose zone soil was a result of in planta transformation of TCE and subsequent export of excess chloride from roots, release from decomposing roots or leaves after leaf drop, or of microbial dehalogenation of TCE in soil. The test beds had two distinct layers: a lower saturated zone and an upper, unsaturated vadose zone. Dechlorination of TCE in the rhizosphere would result in an increase in chloride in the unsaturated layer, as was observed. Dechlorination activity in planta may also result in soil accumulation due to internal chloride regulation, although internal accumulation has also been reported. With the exception of heavy rainfall events, which may create downward chloride migration, it is unlikely that free chloride that accumulated in the unsaturated zone would be remobilized into the saturated zone during most of the growing season when the bulk of the planted soils were desiccated causing chloride salts to be dry deposited on soil surfaces. The 2011 winter effluent aqueous chloride data (SI Figure S3) confirm that chloride ion accumulated in the vadose zone over the growing season as large amounts of chloride washed out of the unsaturated zone with heavy winter rains.

The field root enclosure experiments (SI Table S8) suggest that chloride ion was exported by r2E1 and WT tree roots in the vadose zone, though it is possible that bacteria colonizing the roots may have degraded TCE as it diffused out of the root. Soil microcosm results suggested that microbial transformation in the vadose zone accounted for no more than 20% of input TCE, indicating that tree-related activity was primarily responsible for the observed removal of input TCE. Differences in apparent microbial removal between the r2E1 and WT beds were not significant. The estimated microbial transformation accounted for 33% and 23% of accumulated chloride ion in vadose zone soil in the r2E1 bed and the WT bed, respectively. Microbial anaerobic reduction in the saturated zone contributed to some TCE dehalogenation in both planted beds, but was not likely a significant removal mechanism of TCE given that accumulation of DCE and VC in bed water over the growing season accounted for 0.7% and 4.1% of input TCE in the r2E1 bed and the WT bed, respectively (compared to 44% in the unplanted bed). Given the measurements showing limited microbial transformation of TCE in the unsaturated zone, it appears that the majority of chloride ion accumulation...
in soil and dehalogenation of TCE in field test beds is perhaps was due to plant-related removal of TCE by in planta metabolism and subsequent efflux of excess chloride from roots, or by removal in the rhizosphere.

Although similar phytoremediation studies with TCE, carbon tetrachloride, and PCE have shown that VOCs entrained in the groundwater taken up by trees are generally dehalogenated\textsuperscript{6,25,26} no mechanism was identified. Chloride accumulation in the unsaturated zone in these studies accounted for the majority of VOC dehalogenation, consistent with the results reported here.

There was evidence of increased in planta metabolic activity in the r2E1 poplars compared to the WT. Plant tissue analysis found a higher concentration of known oxidative metabolites of TCE in r2E1 leaf, stem, branch, and root, and lower TCE concentrations (Figure 3). The tissue analysis suggests that there were higher levels of free chloride in the transgenic leaves compared to WT leaves. Flux of TCE from r2E1 leaf and stem to the atmosphere was significantly reduced. These data indicate that the r2E1 poplars were transforming TCE at a greater rate than the WT poplars. However, it should be noted that the lack of replication of the plant beds weakens our ability to reach definite conclusions. This weakness is partially compensated by the consistency of the test bed performance over the multiple years of the study.

Field measurements of higher oxidative metabolite concentrations and lower TCE concentrations in r2E1 stem tissue compared to WT stem tissue suggests that transgenic stem r2E1 activity was increased in the stem tissue and that less translocated TCE would enter the leaf compartment in the r2E1 trees compared to the WT trees and so leaf associated transformation would be limited in the transgenic leaves.

The independent contributions of rhizosphere versus in planta activity to TCE dehalogenation in each planted bed could not be directly measured. However, greater oxidative metabolite concentrations in r2E1 tissues suggest that in planta metabolism contributed to the removal of a greater amount of TCE in the r2E1 test bed compared to the WT test bed. Rhizosphere removal of TCE may have dominated in the WT test bed. The field results indicated that increased in planta metabolic activity may not have been sufficient to markedly increase the uptake or transformation of TCE in the subsurface. In order for in planta metabolic activity to affect the subsurface TCE concentration, TCE uptake into root tissues would have to increase. Uptake of organic contaminants by trees is likely a passive process, resulting from molecular diffusion across at least one cellular membrane.\textsuperscript{10,52} Fick’s law indicates that an increase in the concentration gradient across the cell membrane would increase diffusive flux into the plant. In planta CYP2E1 activity could cause a decrease in cytoplasm TCE concentration, resulting in an increase in the concentration gradient. 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 would not have affected diffusive uptake into the plant. Transport mechanisms therefore appear to limit the effect of enhanced in planta metabolism in the field.

Importantly, the results demonstrate enhanced phytoremediation of TCE by hybrid poplars genetically modified to express mammalian CYP2E1. Diffusion of TCE from tree stem and leaf was significantly reduced in r2E1 trees, and VC and cDCE levels in the effluent of the r2E1 planted bed were significantly reduced. However, the increase in metabolism rate in the r2E1 root appeared to be insufficient to substantially enhance TCE removal in the field. This study demonstrates the importance of field studies to corroborate laboratory results in phytoremediation.

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.5b04758.

Additional tables and figures (PDF)

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**Notes**

The authors declare no competing financial interest.

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