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Enhanced Degradation of TCE on a Superfund Site Using Endophyte-Assisted Poplar Tree Phytoremediation

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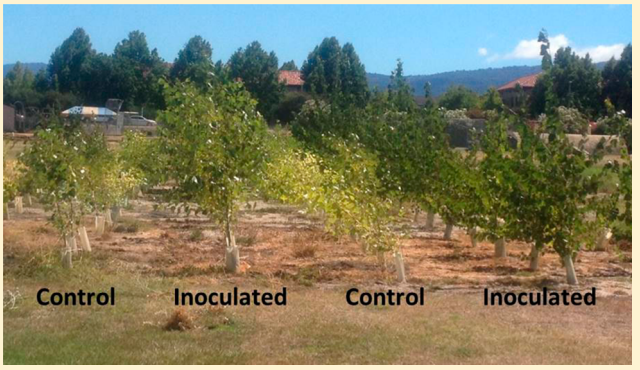
Supporting Information

ABSTRACT: Trichloroethylene (TCE) is a widespread environmental pollutant common in groundwater plumes associated with industrial manufacturing areas. We had previously isolated and characterized a natural bacterial endophyte, *Enterobacter* sp. strain PDN3, of poplar trees, that rapidly metabolizes TCE, releasing chloride ion. We now report findings from a successful three-year field trial of endophyte-assisted phytoremediation on the Middlefield-Ellis-Whisman Superfund Study Area TCE plume in the Silicon Valley of California. The inoculated poplar trees exhibited increased growth and reduced TCE phytotoxic effects with a 32% increase in trunk diameter compared to mock-inoculated control poplar trees. The inoculated trees excreted 50% more chloride ion into the rhizosphere, indicative of increased TCE metabolism *in planta*. Data from tree core analysis of the tree tissues provided further supporting evidence of the enhanced rate of degradation of the chlorinated solvents in the inoculated trees. Test well groundwater analyses demonstrated a marked decrease in concentration of TCE and its derivatives from the tree-associated groundwater plume. The concentration of TCE decreased from 300 μg/L upstream of the planted area to less than 5 μg/L downstream of the planted area. TCE derivatives were similarly removed with cis-1,2-dichloroethene decreasing from 160 μg/L to less than 5 μg/L and trans-1,2-dichloroethene decreasing from 3.1 μg/L to less than 0.5 μg/L downstream of the planted trees. 1,1-dichloroethene and vinyl chloride both decreased from 6.8 and 0.77 μg/L, respectively, to below the reporting limit of 0.5 μg/L providing strong evidence of the ability of the endophytic inoculated trees to effectively remove TCE from affected groundwater. The combination of native pollutant-degrading endophytic bacteria and fast-growing poplar tree systems offers a readily deployable, cost-effective approach for the degradation of TCE, and may help mitigate potential transfer up the food chain, volatilization to the atmosphere, as well as direct phytotoxic impacts to plants used in this type of phytoremediation.



INTRODUCTION

The United States Environmental Protection Agency (EPA) prioritizes sites for federal remediation assistance through the Superfund program based on criteria related to environmental risk and need for remediation.[1] One of the most common organic pollutants at Superfund sites is trichloroethylene (TCE),[2] a known human carcinogen.[3] Used extensively as a solvent and degreaser,[2] its unconfined use, spills, landfill leachate, and improper disposal resulted in contamination of the soil and groundwater. The U.S. EPA has estimated that TCE is a soil or water contaminant on more than 1000

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National Priorities List sites.[1] The EPA has set the drinking water maximum contaminant level at 5 μg/L.[4] The prevalence of TCE contamination coupled with the low action levels creates a substantial demand for effective remediation approaches.

Current remediation methods of contaminated groundwater rely on enhancing volatilization of TCE through air stripping or sparging, sorption using activated carbon or zerovalent iron barriers, or reductive dechlorination and degradation through bioremediation.[5] While remediation costs vary widely depending on site conditions and method, at an estimated capital cost of installing conventional systems (i.e., pump and treat) of between USD $700,000 and USD $3,000,000 per site plus annual operating costs averaging approximately USD $200,000,[6] the addressable market for TCE remediation on the currently identified NPL alone exceeds USD $2.5 billion. Phytoremediation is an alternative strategy that utilizes a plant's natural ability to take up chemicals from water and soil with its expansive root system, to degrade organic pollutants or sometimes to sequester inorganic pollutants.[7] Most phytoremediation projects anticipate a cost savings of approximately 50−75% compared to traditional engineering methods.[8] Poplar (*Populus* sp.) is an attractive plant for phytoremediation of TCE and other organic contaminants due to its compatibility with bioaugmentation systems, high growth rate, extensive root system, and high rates of water uptake from the soil.[9] Poplar's high water use can be used to contain contaminated groundwater plumes and also to take up and degrade TCE to less toxic forms.[10] However, in some cases, the pollutant concentration is phytotoxic, limiting the ability of the plant to effectively remove the chemical.

Phytoremediation can be potentially improved by using genetic engineering and by bioaugmenting with pollutant-degrading bacteria.[11−18] Transgenic trees[19−23] and other suitable phytoremediation plant species[24,25] engineered to overexpress key enzymes can potentially have substantially improved pollutant detoxification and removal. Alternatively, partnering plants with effective pollutant-degrading microorganisms has also shown promise, and research in this area has increased dramatically in the past decade[26] demonstrating near-term application potential without the need for the high-cost and lengthy deployment process associated with transgenic plants.

In natural systems, plants can use symbiosis with internal microorganisms, termed endophytes,[27] to adapt to environmental challenges, including pollutants.[28−30] Plants in contaminated areas have a high number of endophytes which are capable of degrading the pollutant.[31−34] This effect can be plant genotype-specific, suggesting that some plant species or ecotypes are better able to recruit or stably interact with the beneficial microorganisms.[35−38] With the first demonstration that inoculation of plants with a specific pollutant-degrading endophyte can lead to improved removal and detoxification of the pollutant,[39] lab-scale tests have demonstrated that specific endophyte strains improve host plant removal and/or detoxification of PAHs,[40−43] toluene,[44−47] diesel and petroleum,[36,48] TCE,[49,50] explosives,[51,52] textile effluent,[53] and metals.[54−59] The use of endophytes potentially also increases phytoremediation success with indirect benefits[60,61] including increased nutrient acquisition, more extensive root systems, and increased environmental stress tolerance.[62−65]

Despite these numerous lab or greenhouse-based studies, there are few reports of field trials of endophyte-assisted phytoremediation.[26] The in situ addition of a poplar root endophyte strain engineered to degrade TCE resulted in reduced evapotranspiration of TCE.[66] However, no direct evidence was provided on increased removal of groundwater TCE or degradation of TCE in planta. Axenic plants inoculated with a TNT-degrading strain of *Pseudomonas putida* resulted in increased removal of TNT under laboratory conditions but had no impact under field conditions.[67] Bioaugmentation in general is thought to have less success at field scale due to the possibility of native soil microorganisms harming or outcompeting the artificially added strains.[5] The complexity of field trials where the pollutants are often heterogeneous can also make precise assessments of phytoremediation success challenging.

We previously reported on the isolation and characterization of a TCE-degrading endophyte strain.[68] This *Enterobacter* sp. strain PDN3, isolated from hybrid poplar collected from a TCE-contaminated site, grows well on high levels of TCE. Strain PDN3 aerobically dechlorinated TCE without the addition of cometabolite inducers. We report herein on the first successful phytoremediation field trial of bioaugmentation with this natural TCE-degrading endophyte strain, demonstrating a low-cost, simple approach to improving tree growth and pollutant degradation.

MATERIALS AND METHODS

Bacterial Strain. Endophyte strain *Enterobacter sp.* PDN3 was originally isolated from hybrid poplar (*Populus deltoides* x *nigra*) in a screen for endophytes tolerant to 5.5 mM TCE.[68] Cryogenically stored microbial stocks had been prepared previously by growing the strain in MG/L broth[69] containing approximately 300 μg/mL TCE, freezing in 33% sterile glycerol, and storing at −70 °C.

Verification of Colonization Ability. Prior to the field testing, the ability of strain PDN3 to colonize hybrid poplar clone OP367 (*P. deltoides* x *nigra*) was tested using fluorescent microscopy. The pBHR:*gfp* plasmid[70] was introduced into strain PDN3 using triparental mating[69] and transconjugants were selected on MG/L agar containing carbenicillin to select for PDN3 and kanamycin to select for the plasmid. *gfp*-PDN3 was grown in MG/L broth for 24 h and washed twice in deionized water by centrifugation. The pellet was resuspended in 0.5× Hoagland's solution,[71] adjusted to an optical density (OD) at 600 nm of 0.1 using Unico spectrophotometer model 1000 (Fisher), and exposed to poplar roots for 1 week at room temperature. Colonization was visualized after 48 h and 1 week using a Zeiss Imager M2 equipped with an AxioCam MRM, and recorded with Zeiss AxioVision software (Carl Zeiss, LLC). The microscopy experiments were done twice with two biological replicates.

Field Trial Experimental Design and Tree Plot Establishment. A field site at Moffett Field/NASA-Ames Research Park was selected for a demonstration site, and permitting was completed with assistance from NASA environmental division and facilities groups. The Middlefield-Ellis-Whisman (MEW) Superfund Study Area was contaminated due to industrial, manufacturing, and military operations, and has been the subject of remediation studies since the 1980s.[72] As shown in Figure 1 and Supporting Information (SI) Figure S1, planting sites were chosen in three areas near the center of the plume where the TCE concentration was in the 100−1000 μg/L range.

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

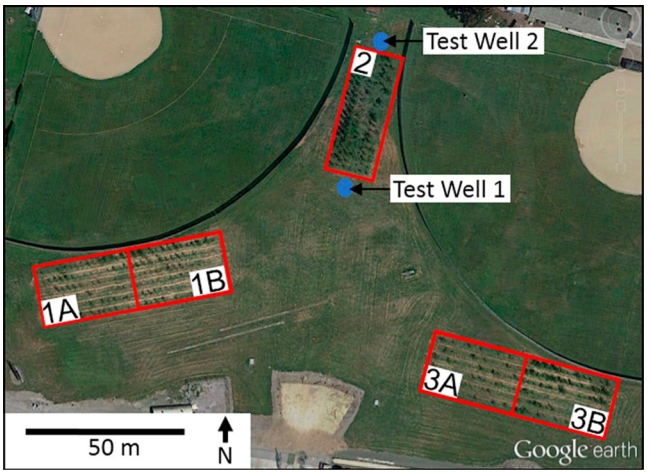

Figure 1. Location of field trial test area showing the layout of MEW plume poplar TCE phytoremediation field trial blocks.

In 2013, the planting areas were prepared by mowing and removal of existing vegetation. Three commercial varieties of poplar were selected for the study based on the geographical area and site conditions. Nine-inch dormant cuttings of OP-367, 15−029, and 311−93 were obtained for planting from Segal Nurseries (Grandview, WA). Half the number of cuttings of each variety were soaked in quarter-strength Hoagland's solution (Control Poplar, CP) and the other half were inoculated (Endophyte-inoculated Poplar, EP) with the endophyte strain by placing the cuttings upright in an 8 L container and soaking in a 0.1 OD_{600} suspension of the PDN3 culture in quarter-strength Hoagland's solution at a depth of approximately 10 cm for 24 h prior to planting. Details of the planting and maintenance are in the SI.

Tree Growth Assay. Tree growth was measured periodically throughout the study using tree base and breast height trunk diameter measurements.

Assessing Chloride Level in the Rhizosphere. As a means of assessing phytoremediation performance with respect to TCE degradation, chloride levels were measured as a breakdown product from the TCE in samples collected from the rhizosphere of Control Poplar (CP) and Endophyte-Inoculated Poplar (EP) trees 16 months from the beginning of the field trial. The samples were collected by moving aside the surface of the soil localized around the tree stems, collecting the rhizospheric soil from around the visible roots, and placing the samples in sterile 50-mL conical tubes. Approximately 2.0 g of soil were ground with a mortar and pestle to pass through a 10 mesh sieve (<2 mm) and mixed in order to homogenize the sample. Two-hundred mg of homogenized soil were weighed using an analytical balance (resolution ±0.0001 mg) and suspended in 25 mL of 4% acetic acid solution in 125 mL Erlenmeyer flasks. The flasks were sealed and incubated at room temperature under shaking conditions at 110 rpm for 30 min. Soil particles were then removed by filtering the suspension through a Whatman No. 1 filter paper (Sigma-Aldrich). Filtered samples were collected into glass tubes, sealed, and stored at 4 °C for further analysis. Chloride (Cl) levels were assessed using a chloride-selective electrode (Thermo-Fisher Scientific). A calibration curve for low-level measurements was prepared as described by the manufacturer using a NaCl solution (chloride standard) prepared in deionized water (DQ3, Millipore) supplemented with 1 mL of Ionic Strength Adjustor (ISA) solution (1.0 M $NaNO_3$). After each increment, millivolt potential was registered and plotted against Cl concentration. Finally, chloride concentration of each sample was calculated interpolating millivolt values to the corresponding calibration curve.

Tree Core Measurements of TCE and Breakdown Products. Tree cores were taken using standard coring methods from both EP and CP trees by using a 5 mm diameter increment borer inserted to a minimum depth of 1.5 inches (4 cm). Within seconds of collection, the cores were placed into 20 mL PTFE sealed headspace vials. Samples were shipped the next day to the Missouri University of Science and Technology Environmental Engineering Lab. Analysis of TCE and other chlorinated solvents in the transpiration stream of the trees was carried out using solid-phase micro extraction (HS-SPME) coupled with gas chromatography and electron capture detection (GC-μECD). Quantification was accomplished using a 5 point standard curve with external standards. An $R^2 > 0.98$ was deemed acceptable. This equilibrium passive sampling method was developed specifically for tree core cVOC sampling using a 100 μm polydimethylsiloxane (PDMS) fiber.[73] Method detection limits were below 8 ng/L for TCE and 0.5 ng/L for PCE in the aqueous solution in plant tissues.[74]

Groundwater Test Wells. Well borings were drilled to a depth of 17-feet below ground surface (bgs) using 8-in. hollow stem augers. The well screens, casings and completion materials were installed as per the attached well completion logs. The water samples were collected via "Hydro-sleeves", which are plastic-like baggies that are collapsed when deployed to the bottom of the well, and then raised quickly for approximately 1-foot which causes the sleeve to open at the top allowing the well water to completely fill the sleeve within the screened aquifer zone (zone of interest). The wells were installed on 9/20/16. Test well 1 is approximately 20-feet south of the plot (the up-gradient/incoming well), while test well 2 is to the north of the plot (the downgradient/outgoing well). Groundwater samples were collected on 9/28/16 and again on 11/21/2016 immediately prior to leaves turning yellow and winter senescence. The analytical method for the groundwater samples was EPA Method 624 normally utilized for Volatile Organic Compounds (VOCs) analysis.

Verification of PDN3 Colonization of the Field Site Trees. To verify that the EP plants at the field test site had been colonized with PDN3, branch samples collected from the trees in spring 2016 were shipped from the field site to the University of Washington, surface sterilized with 0.6% sodium hypochlorite for 10 min and rinsed four times with sterile water. We chose to test branch samples because PDN3 can effectively colonize poplar vascular tissue, migrating from root to shoot or shoot to root depending on inoculation site (unpublished data). One node per tree sample was weighed and extracted with 0.5 mL 0.1× Hoagland's solution per gram of tissue in separate sterile mortars. Resulting extracts were stored at −70 °C. Samples of the extracts from 6 CP trees and 7 EP trees were randomly chosen, with strain PDN3 included as a reference, and inoculated into M9 medium containing 0.4% peptone[4] and 0.5% mannitol. After 3 days at 30 °C, 1 mL samples were pelleted in a microcentrifuge, the cells resuspended in 1 mL M9 with peptone, and the OD_{600} was measured. Cultures were adjusted to an OD_{600} of 0.1 in 3 mL M9 medium with 0.4% peptone and 730 μg/mL TCE in 40-ml amber VOA vials. After 15 days exposure to the high level of TCE, samples were plated on M9 with peptone, and restreaked

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

for colony purification. Colonies of the dominant morphology and similar to PDN3 were subjected to colony PCR using 16S rRNA gene primers as described previously.[75] PCR products were treated with ExoSAP-IT (Affymetrix) after verification by electrophoresis to be single 1.5 Kb products with no PCR product from a water blank control. Samples were sequenced by GeneWiz (Seattle) and identified using NCBI BLAST.[76] For samples identified as *Enterobacter* species, PCR was repeated and the products cloned into pGEM-T-Easy (Promega), and sequenced twice. The 16S rRNA sequences were compared to that of PDN3 (GenBank #JN634853) using the alignment program of NCBI BLAST.

Comparative Genomic Analysis of *Enterobacter* sp. Strain PDN3. The PDN3 genome was sequenced and assembled at the Department of Energy Joint Genome Institute (JGI) as part of the research topic "Defining the poplar root microbiome". Genome annotation, performed at the JGI, was carried out using the DOE-JGI Microbial Annotation Pipeline (DOE-JGI MAP).[77]

Enzyme Commission (EC) numbers were used as annotation terms to define a functional profile to investigate the genetic potential of PDN3 to metabolize or cometabolize TCE. The chloroalkane and chloroalkene degradation pathway and the metabolism of xenobiotics by cytochrome P450 from the KEGG database were used as reference pathways. Once the functional profile was defined, the "missing" enzymes were further analyzed using the 'Find Candidate Genes for Missing Function' tool from the IMG/ER platform. This step was performed using two different approaches; for search by homology, a percent identity cutoff of 30% and an E-value cutoff of $1e^{-2}$ were used, while a search by orthologs was performed with a percent identity cutoff of 10%. Both steps were executed by querying the IMG database. Similarly, the genetic potential of PDN3 to regulate heavy metal homeostasis was investigated.

Statistical Analysis. Data analysis was carried out in R v3.2.3, performing a Tukey's HSD (Honest Significant Difference) as posthoc test in conjunction with a One Way ANOVA. For TCE levels in core samples the nonparametric test Kruskal–Wallis rank sum test was used.

RESULTS AND DISCUSSION

Verification of the Colonization Ability of Endophyte Strain PDN3. To verify that PDN3 was capable of colonizing the hybrid poplar, the fluorescently tagged PDN3 (pBHR:gfp) strain was used. After 48h of cocultivation with poplar roots, the strain was observed to begin colonization through the formation of a biofilm at the junctions between the primary and secondary roots (Figure 2a). Crack entry at lateral root junctions is a common mode of colonization by endophytes.[60,78] After 1 week of coinoculation, gfp-PDN3 was verified to be within the host plant (Figure 2b). Since strain PDN3 was originally isolated from another hybrid *P. deltoides* x *nigra* poplar clone, it was expected that it would be able to effectively colonize the hybrid poplar used in the study.

Observable Growth Differences in EP and CP Trees. The preparation and plantings on the MEW plume resulted in successful establishment of the three poplar test plots directly over the MEW plume in an area of TCE concentrations ranging from 100 to 1000 μg TCE/L (Figure 1). In 2014 after one year of growth, it was apparent that the PDN3 inoculated poplar (EP) trees were more robust than the uninoculated control (CP) trees (Figure 3). This may have been due to several factors. The endophyte may have reduced the TCE load within the trees by rapidly metabolizing the pollutant taken up by the plant, thereby directly reducing phytotoxic effects. Although the TCE concentration in the plume itself may not have been high enough to be phytotoxic, the condition of the CP trees indicated that TCE did impact plant growth. TCE may have

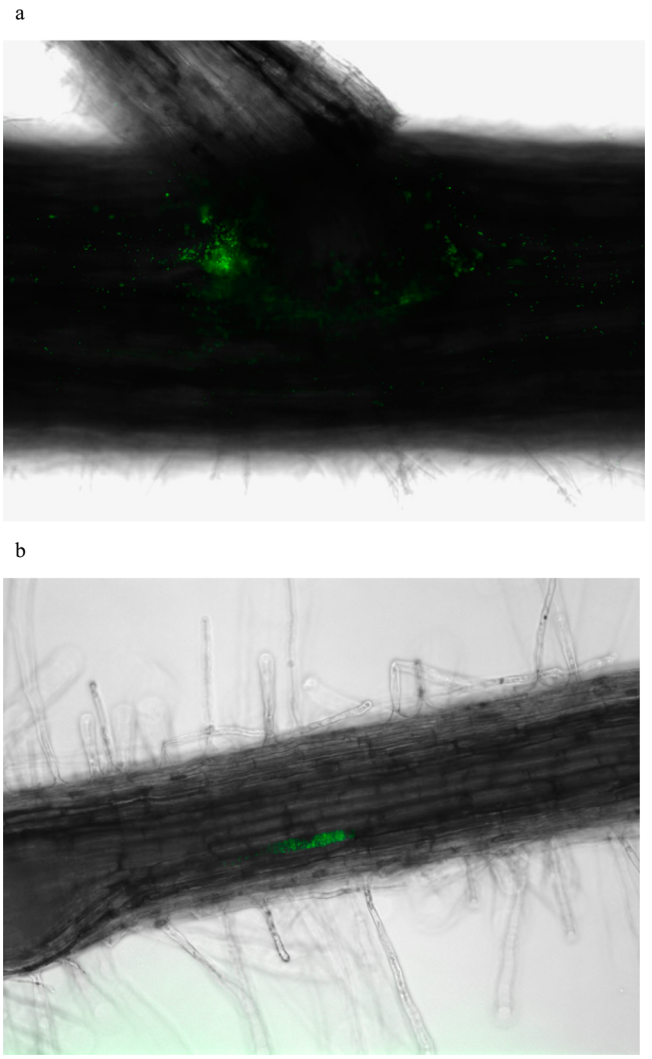

Figure 2. Colonization of hybrid poplar OP367 by *gfp*-PDN3. (a) After 48h of cocultivation, *gfp*-PDN3 can be viewed colonizing the lateral root junctions. (b) After 1 week of coinoculation, *gfp*-PDN3 is seen within the host plant roots.

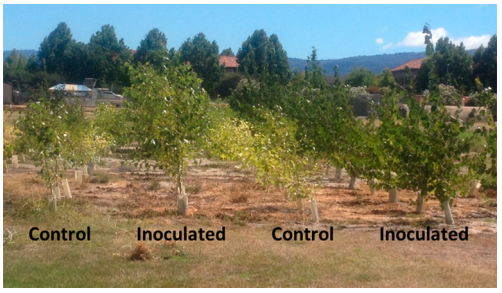

Figure 3. Successful establishment of the EP and CP trees. Visible growth and health differences in inoculated and uninoculated poplar trees (Plot 2) after one year's growth. Photo courtesy of Edenspace Systems Corporation (M. Blaylock).

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX

been present in the upper rhizosphere possibly due to the nature of the volatile organic contaminant, a persistent dry soil condition and an unconfirmed volatilization up through cracked dry soil. In addition to benefiting the plant through detoxification of TCE, the endophyte may have enhanced root growth and overall health due to its ability to produce phytohormones. PDN3 has the biosynthetic genes for butane 2,3-diol and acetoin,[79] volatile organic compounds shown to be important for increasing plant stress tolerance.[80−82] The strain also produces indole acetic acid (unpublished data), an auxin that induces root formation and plant growth.[64] The combination of both the endophyte strain and the right tree variety was necessary for success. The OP367 variety was the hybrid of choice for this site due to its larger, more robust growth when compared to the other two varieties.

In order to assay the difference between CP and EP trees after two and three years of tree growth, the tree trunk diameter at breast height was recorded for all blocks on site for the OP-367 variety. This data showed a statistically significant 32% increase in trunk diameter growth of the PDN3 inoculated trees after two and three years of growth when compared directly to the control uninoculated trees (Figure 4. $p < 0.01$)

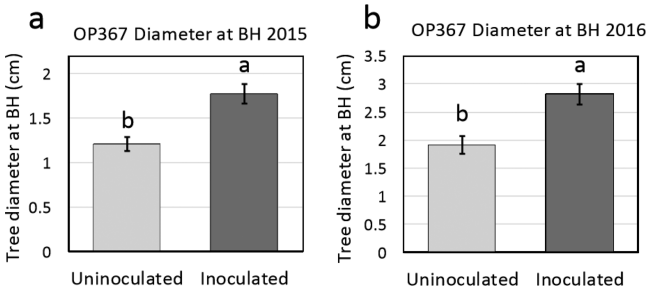

Figure 4. Tree trunk diameters at breast height (BH) in (cm) for inoculated and uninoculated hybrid poplar OP367 measured in 2015 (a) and 2016 (b). Means ± SE shown (n = 84 and 86 for uninoculated, and n = 100 and 104 for inoculated, for 2015 and 2016, respectively). Letters represent groups with statistically significant differences (alpha = 0.001) between means. Statistically different groups were determined using a Tukey's HSD posthoc test.

Soil Chloride in the Rhizosphere of CP and EP. Chloride is an essential co-ion that exerts important physiological functions in higher plants. However, the efflux of Cl from roots as an effect of regulatory systems which regulate Cl concentration inside and outside plant tissues has been observed.[83−85] Assessing the accumulation of chloride anions in the rhizosphere as a consequence of TCE degradation in the endosphere can be used to monitor the biodegradation of chlorinated solvents.[86] For comparison, basal levels of chloride ion in soil were assessed by analyzing 10 soil samples collected from a non-contaminated area located nearby the contaminated site. After 16 months, chloride levels in the rhizosphere soil collected from CP and EP, and chloride level in control soils were statistically significantly different (Figure 5). High levels of chloride were detected in the rhizosphere soil collected from poplar plants inoculated with PDN3 (EP), where chloride concentration was 0.5-fold higher compared to poplars uninoculated with PDN3 (CP). Such experimental observation strongly suggests that the TCE dechlorination was prevalent in poplars colonized by PDN3. Similarly, there was increased chloride in the rhizosphere of poplar trees genetically

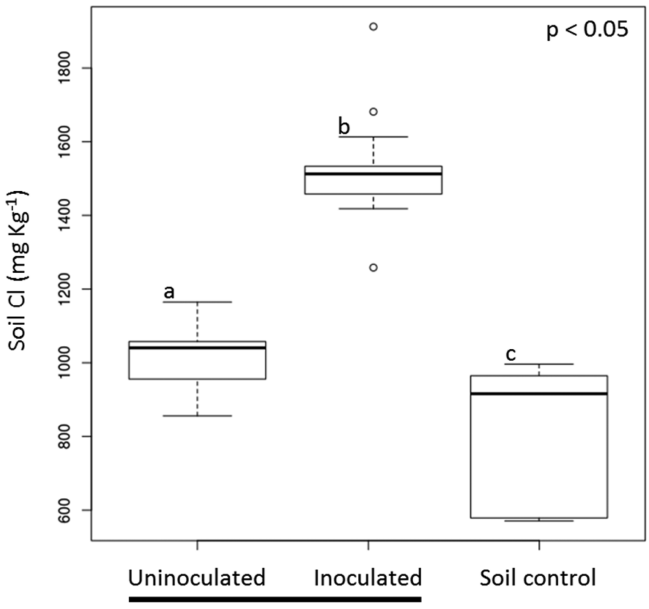

Figure 5. Box plot representing median (solid lines), interquartile ranges (boxes) and ranges (whiskers) of chloride levels in rhizosphere soil collected from inoculated (n = 13) and uninoculated hybrid poplars (n = 10), and chloride concentration in a soil control (n = 10). Letters represent groups with statistically significant differences between means (α = 0.05). Statistically different groups were determined using a Tukey's HSD posthoc test. Open circles, outliers. ANOVA output and R code used to run this analysis are reported in SI Tables 2 and 3 respectively.

enhanced to degrade TCE,[23] supporting the concept that excess chloride within the plant is secreted into the rhizosphere.

Tree Core TCE Analysis of CP and EP. TCE concentrations in trees that interact with groundwater plumes containing TCE have been observed, and accumulation of TCE in plant tissues has led to symptoms of toxicity. However, with the addition of TCE degrading endophyte PDN3, the concentration of TCE in OP367 tree core tissues was reduced (Figure 6) for all plots (Plot 1, p = 0.07, not shown,). Whether considering plots individually (Figure 6a,b) or combined (Figure 6c,d), hybrid poplar OP367 inoculated with PDN3 exhibited statistically significant reductions in TCE in tree core tissues after three years of growth. Hybrid poplar OP367 inoculated with PDN3 in Plot 2 did not have any measurable TCE in tree core tissue remaining. In contrast, the uninoculated CP group exhibited the highest TCE concentrations in tree core samples of any plot (Figure 6a). Combined with tree growth differences and Cl exudation into the soil, the data demonstrate that hybrid poplar trees inoculated with PDN3 have higher tolerance to elevated TCE concentrations and increased degradation of TCE, thus limiting volatilization of the VOCs to the surrounding atmosphere.[74]

Test Well Data of TCE in Groundwater Plume. A broad range of volatile organic compounds (VOCs) in well water samples from both the up-gradient (test well 1) and down-gradient (test well 2) wells flanking Plot 2 (Figure 1) were analyzed (Table 1). TCE and degradation products, along with PCE, chloroform and Freon 113, were all detected in test well 1 samples taken at two different dates after three years of site establishment. Interestingly, all detected VOCs from samples taken from test well 1 were not detected (ND) in test well 2,

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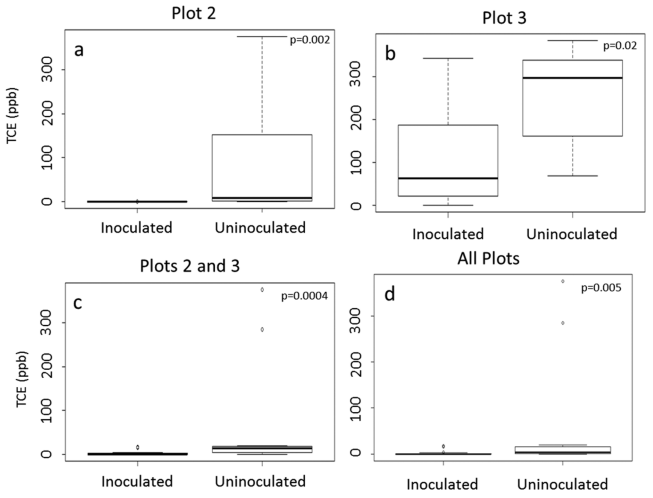

Figure 6. TCE levels in tree core samples (TCE ppb) grouped for inoculated and uninoculated hybrid poplar OP367 from Plot 2 (a), Plot 3 (b), a combination of Plots 2 and 3 (c), and All Plots 1, 2, and 3 combined (d).. Statistically significant differences determined using a Kruskal–Wallis rank sum test, with p values included in the upper right. Open circles, outliers. Kruskal–Wallis rank sum test outputs and R codes are reported in SI Tables 4 and 5 respectively.

Table 1. TCE Levels, Their Degradation Products, And Other Contaminants in Well Water Samples[a]

	August 28, 2016		November 21, 2016			
	test well		test well		reporting	
	1	2	1	2	MDL	limit
	(μg/L)					
trichloroethene	280	ND	300	ND	1.1	5
trans-1,2-dichloroethene	1.7	ND	3.1	ND	0.13	0.5
cis-1,2-Dichloroethene	140	ND	160	ND	1.5	5
1,1-dichloroethene	7.6	ND	6.8	ND	0.092	0.5
1,1-dichloroethane	7.9	0.64	9	0.58	0.12	0.5
vinyl chloride	0.54	ND	0.77	ND	0.17	0.5
tetrachloroethene	0.85	ND	0.88	ND	0.12	0.5
chloroform	0.38	ND	ND	ND	0.13	0.5
1,1,2-trichloro-1,2,2-trifluoroethane (Freon 113)	1.5	ND	1.5	ND	0.15	0.5

[a]Groundwater samples taken from test wells 1 (up-gradient of plume flow) and 2 (down-gradient of plume flow) of plot 2 at two different sampling dates. Individual sample (n = 1) analysis values shown in μg/L or not detected (ND). Minimum detection level (MDL) and reporting limit also shown.

except for 1,1 dichloroethane which was dramatically reduced and very close to the reporting limit.

Because of variations in underlying strata, groundwater flow and physical features, it would be difficult to have an equivalent unplanted control or to have separate CP and EP plots. However, the upgradient well (Well 1) immediately prior to the tree plot provides the best control prior to interception of groundwater by the trees. The concentrations and degradation products found in Well 1 are representative of what is observed in the other test wells on the site away from the planted area, whereas Well 2 immediately downgradient from the trees has much lower concentrations than found elsewhere throughout the site including immediately adjacent further downgradient wells. These data demonstrate that phytoremediation as a whole was effective at this Superfund site.

Verification of PDN3 Colonization of the Field Site Trees. Samples of the field site trees were tested for the presence of wild-type PDN3 in the leaf buds. Of the six CP plant extracts tested, only three yielded growth under the selective conditions of 730 μg/mL TCE. Colonies from two of the resulting cultures were identified as *Pseudomonas libanensis* (99%) and one as *Rhizobium* sp. (99%). Since the CP trees at the site suffered from TCE phytotoxic effects and contained significantly less TCE metabolites, these TCE-tolerant strains were apparently less effective at TCE degradation. Of the 7 EP plant extracts tested, all seven yielded growth in the TCE-containing medium. Colonies from two of the cultures were identified as *Pseudomonas* sp. whereas those of the other five were identified as *Enterobacter* sp. Two of these were a 100% match to the 16S rRNA of strain PDN3, confirming the ability of the strain to colonize the trees and remain stable within the host tree even after 3 years, and to retain its TCE-tolerant phenotype. Considering that only a single bud was assayed for the presence of PDN3 from each of the trees, being able to reisolate it in any of the samples 3 years after inoculation strongly suggests it was stable in the trees. The other 3 strains were identified as other *Enterobacter* sp. with 98% identity to PDN3. It is possible that horizontal gene transfer from PDN3 led to the ability of these other *Enterobacter* strains to gain the ability to dechlorinate TCE. This phenomenon has been reported previously from a toluene-degrading strain.[45] However, the mechanism by which strain PDN3 metabolizes TCE is currently unknown (see below); therefore, we were unable to test for the presence of the catabolic genes in the other strains. Until it is known which genes are required for TCE metabolism in this strain, it is not relevant to screen for genetic evidence of plasmid transfer into the host microbiome since the required genes may be chromosomal. Only after knowing the genes required from PDN3 for TCE metabolism, can we test if the relevant genes transferred into the resident microbiota.

Despite the close proximity of the CP and EP trees, the endophyte remained stable within the inoculated trees without apparent migration from one to the other. Since PDN3 was originally isolated as an endophyte of poplar, it is reasonable that it would remain within this natural host, colonizing throughout the host tree rather than outward, through the soil, to reach other hosts. This may be a characteristic of endophytes of trees compared to endophytes of annual plants. Since PDN3 is a natural strain and was originally isolated from hybrid poplar, it was able to persist within the inoculated poplar even three years into the field trial. Therefore, the strain was apparently able to compete with the indigenous microbial population at the site.

Genome Analysis for Genes Related to Bioremediation. In an effort to determine the candidate genes involved in TCE degradation by PDN3, an extensive genome analysis was performed. Interestingly, despite the presence of nine different genes encoding for monooxygenases, none of these encode for proteins known to be involved in TCE degradation, meaning that TCE degradation in PDN3 likely occurs through a novel noncanonical dechlorination pathway. The genomic analysis confirmed the earlier report that no TOM (toluene monooxygenase) genes could be amplified by PCR and none of the typical aerobic TCE degradation intermediates could be detected by GC-TOF-MS.[68] RB-TnSeq experiments[87] are underway to determine the genes required by PDN3 to grow

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Environ. Sci. Technol. XXXX, XXX, XXX–XXX

in TCE-containing medium. Heavy metals are common co-contaminants of polluted soils and are usually found as trace elements in non-contaminated soils.[88] Plants colonized by endophytes with heavy metal tolerance have decreased phytotoxic effects and increased growth.[54,59,89] Microorganisms have developed different strategies to avoid the cytotoxicity induced by the exposure to these elements. PDN3 carries genes which are part of copper, cadmium, arsenate, tellurite and hexavalent chromium detoxification systems (SI Table 1), suggesting that it may be an effective inoculant for phytoremediation of mixed polluted sites.

■ ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.est.7b01504.

Details of poplar planting and tree maintenance Supporting Information Table 1. Heavy metals resistance genes in *Enterobacter* strain PDN3 Supporting Information Table 2. Analysis of Variance (ANOVA) Table of Chloride levels data analysis in rhizosphere soil collected from inoculated and uninoculated hybrid poplars, and chloride concentration in a soil control Supporting Information Table 3. Code used in R v3.2.3, to perform a Tukey's HSD (Honest Significant Difference) as posthoc test in conjunction with a One Way ANOVA for chloride data analysis reported in Figure 5 Supporting Information Table 4. Kruskal–Wallis rank sum test outputs from data analysis of TCE levels in tree core samples. Supporting Information Table 5. Code used in R v3.2.3, to determine the level of normality (Q-Q Plots) and perform a Kruskal–Wallis rank sum test for TCE levels in tree core samples. Supplementary Figure S1. EPA reported TCE plume concentration maps (PDF)

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Notes

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Environ. Sci. Technol. XXXX, XXX, XXX−XXX