FINAL REPORT

Utilizing the Plant Microbiome and Bioaugmentation to Degrade 1,4-Dioxane and Co-Contaminants

SERDP Project ER-2719

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14. ABSTRACT 1,4-Dioxane (dioxane) is a probable carcinogen and persistent groundwater pollutant often found comingled with chlorinated solvents (e.g., trichloroethylene, dichloroethylene, and trichloroethane). Because of dioxane's high mobility in groundwater, dioxane plumes tend to be large and dilute. State-issued clean-up guidelines for dioxane are on the order of 1 µg/L or less. Reaching these low clean-up guidelines through remediation has proven to be particularly difficult and costly. Utilizing aggressive pump-and-treat and ex-situ technologies such as advanced oxidation (AO) on dilute dioxane plumes is often prohibitively expensive. During this project, we evaluated bioaugmented phytoremediation, a promising, cost-effective clean-up strategy for dioxane-contaminated groundwater. The objective of this research project was to discover microbial strains that can degrade 1,4-dioxane to health advisory levels. In addition, we evaluated the performance of candidate organisms when bioaugmented into the poplar rhizosphere.

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LIST OF ACRONYMS

1,1-DCE – 1,1-dichloroethylene

AMS – ammonium mineral salts microbiological medium

AMSV – ammonium mineral salts microbiological medium with ATCC MD-VS vitamin mixture

ATCC – American Type Culture Collection

CB1190 – *Pseudonocardia dioxanivorans* strain CB1190

cis-DCE – cis-1,2-dichloroethylene

Bgs – Below ground surface

Dioxane – 1,4-dioxane

1,4-D – 1,4-dioxane

DSM – German Collection of Microorganisms and Cell Cultures

iChip – isolation chip (diffusion chip)

LLC – limited liability corporation

Ks – Kinetic half-saturation constant

PH-06 – *Mycobacterium dioxanotrophicus* PH-06

SERDP – Strategic Environmental Research and Development Program

TCE – trichloroethylene

TCA – 1,1,1-trichloroethane

THF – tetrahydrofuran

q_{max} – Maximum substrate degradation rate

KEYWORDS

1,4-dioxane, groundwater contamination, microbiome, bioaugmentation, phytoremediation, bacterial biodegradation, plant science, chlorinated solvents, trichloroethylene (TCE)

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1. ABSTRACT

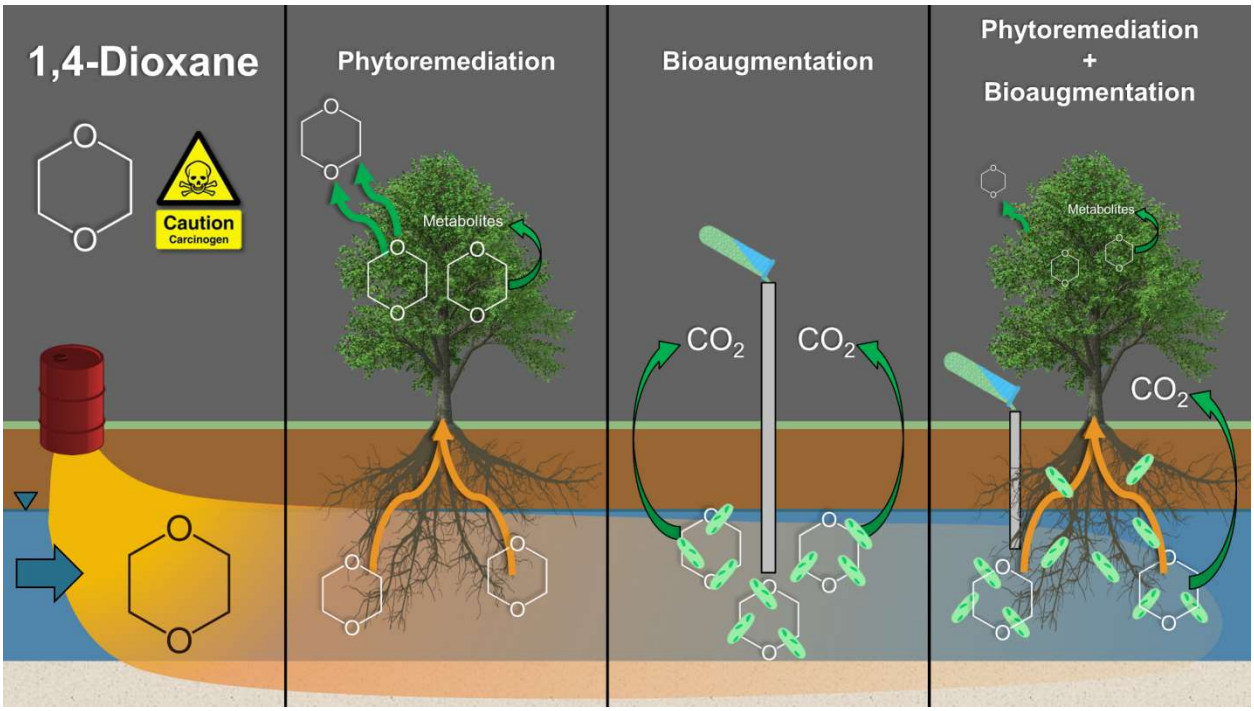
Introduction and Objectives. 1,4-Dioxane (dioxane) is a probable carcinogen and persistent groundwater pollutant often found comingled with chlorinated solvents (e.g., trichloroethylene, dichloroethylene, and trichloroethane). Because of dioxane's high mobility in groundwater, dioxane plumes tend to be large and dilute. State-issued clean-up guidelines for dioxane are on the order of 1 µg/L or less. Reaching these low clean-up guidelines through remediation has proven to be particularly difficult and costly. Utilizing aggressive pump-and-treat and ex-situ technologies such as advanced oxidation (AO) on dilute dioxane plumes is often prohibitively expensive. During this project, we evaluated bioaugmented phytoremediation, a promising, cost-effective clean-up strategy for dioxane-contaminated groundwater. The objective of this research project was to discover microbial strains that can degrade 1,4-dioxane to health advisory levels. In addition, we evaluated the performance of candidate organisms when bioaugmented into the poplar rhizosphere.

Technical Approach. During this work, we conducted bench-scale experiments to compare dioxane degradation rates of poplar bioaugmented with *Pseudonocardia dioxanivorans* CB1190 to that of metabolic dioxane-degrader *Mycobacterium sp.* PH-06. We completed experiments testing whether these bacterial strains can utilize root exudates as an auxiliary substrate. In addition, we evaluated the use of inexpensive B-vitamin supplements to accelerate dioxane metabolism by *Rhodococcus ruber* 219. We also tested R. ruber 219's ability to withstand chlorinated solvents. Finally, we conducted simulated aquifer experiments to evaluate whether phytoremediation and bioaugmentation can treat dilute plumes contaminated with dioxane to below health advisory levels over long periods.

Results. In our findings, we report the phytoremediation of dioxane by hybrid poplar to health advisory levels (~1 µg/L) in bench-scale experiments. Bioaugmentation with dioxane-degrading bacteria significantly increased the rate of removal by hybrid poplar. In addition, PH-06-bioaugmented poplar significantly outperformed all other treatments. However, growth curve experiments found that PH-06 could not utilize root extract as an auxiliary carbon source for growth. Despite this limitation, our findings suggest that PH-06 is a strong bioaugmentation candidate to enhance the treatment of dioxane by phytoremediation.

We also identified R. ruber 219 as a very strong candidate for field bioaugmentation. With the addition of B-vitamins, the strain is able to sustain growth in dilute dioxane concentrations (<100 µg/L) and degrade dioxane to below health advisory levels (<0.35 µg/L). We did observe 1,1-dichloroethene as inhibitory for dioxane degradation by *R. ruber* 219. Ongoing work aims to explore if bioaugmentation with *R. ruber* 219 in tandem with phytoremediation can overcome inhibition by 1,1,-DCE. Furthermore, poplar trees may release sufficient B-vitamins in root exudates, reducing the need for vitamin amendments.

Benefits. This project demonstrated that combining phytoremediation with bioaugmentation is a promising treatment alternative for dioxane-contaminated groundwater to achieve low concentrations (<0.35 µg/L) as recommended by health advisories. Dioxane-metabolizing microbes have been utilized, eliminating the need for auxiliary substrates required by cometabolic microorganisms. While challenges remain, the successful implementation of this strategy offers a green and cost-effective solution to a widespread problem of national and international importance.


1,4-Dioxane
Caution
Carcinogen
Phytoremediation
Metabolites
Bioaugmentation
CO_2
CO_2
Phytoremediation
+
Bioaugmentation
Metabolites
CO_2

2. EXECUTIVE SUMMARY

2.1. Introduction

1,4-Dioxane (dioxane) is a probable carcinogen and persistent groundwater pollutant often found comingled with chlorinated solvents (e.g., trichloroethylene, dichloroethylene, and trichloroethane). Because of dioxane's high mobility in groundwater, dioxane plumes tend to be large and dilute. Proposed EPA risk guidelines for dioxane in drinking water are as low as 0.35 µg/L. Reaching this low clean-up guideline through remediation has proven to be particularly difficult and costly. Utilizing aggressive pump-and-treat and *ex-situ* technologies such as advanced oxidation (AO) on dilute dioxane plumes is often prohibitively expensive. Monitored natural attenuation (MNA) is a low-cost alternative, but it can have difficulty reaching stringent clean-up concentrations. Dioxane plumes are found at many DoD sites, and they present a formidable cost for remediation.

Bioaugmentation with aerobic dioxane-degrading bacteria is an attractive option for the treatment of dioxane-contaminated groundwater as it is relatively inexpensive and well suited for dilute plumes. Metabolic bacteria, which utilize dioxane as a sole carbon and energy source, have many advantages over cometabolic strains, including higher transformation rates, lower oxygen demand, and no added costs due to additions of primary growth substrates required to induce dioxane degradation. However, metabolic dioxane degraders face challenges that may impede bioremediation. For example, the well-known metabolic dioxane-degrading bacterium *Pseudonocardia dioxanivorans* CB1190 can stall when exposed to low initial dioxane concentrations (<500 µg/L) commonly found at dioxane contaminated sites (Adamson et al., 2014; Li et al., 2010). This may be attributed to minimum substrate concentrations required by metabolic bacteria for sustained growth (Barajas-Rodriguez and Freedman, 2018; da Silva et al., 2018). Furthermore, the presence of chlorinated solvent co-contaminants can irreversibly inhibit dioxane degradation by CB1190 (Zhang et al., 2016). Finally, CB1190 tends to form clumps, which may prevent it from being transported throughout the subsurface plume during bioaugmentation (Grostern et al., 2012; He et al., 2018; Lippincott et al., 2015). Bioaugmented strains may also face stressors such as low temperatures, oligotrophic conditions, extreme pH, limited oxygen availability, washout, and competition and predation from indigenous microorganisms (Chan and Kjellerup, 2019; Stroo et al., 2012).

Phytoremediation is another green, cost-effective clean-up strategy that has been proposed for the treatment of dioxane-contaminated groundwater. This remediation technology offers many benefits, including aesthetics, low energy demand, and costs of 50 to 90% less than traditional remediation techniques (Aitchison et al., 2000; Dietz and Schnoor, 2001; Doty, 2008). Phytoremediation is well suited for sites with low-level contamination over a large area where other technologies might be prohibitively expensive. Mature poplar trees can "pump" up to 260 liters per day via transpiration (Barac et al., 2009). While poplars do possess P450 cytochrome monooxygenases capable of metabolizing dioxane, Aitchison et al. found that most (76.5 ± 3.9%) of the dioxane removed by poplar was not transformed but was transpired directly to the atmosphere (Aitchison et al., 2000; Dietz and Schnoor, 2001). Once volatilized, dioxane undergoes photodegradation via hydroxyl radicals in the atmosphere, with an estimated half-life of 6.7 to 9.6 hours (Ferro et al., 2013; Stepien et al., 2014). In addition, traditional phytoremediation is usually limited in treatment depth to shallow groundwater plumes (5-15 ft bgs).

Bioaugmenting the poplar rhizosphere alleviates many of the deficiencies that bioaugmentation and phytoremediation have separately. The poplar rhizosphere supplies a continual source of carbon-rich exudates, which stimulates the increased growth of bacteria compared to the adjacent bulk soil (Schnoor et al., 1995). Previous work has shown that *P. dioxanivorans* CB1190 can utilize these exudates as an auxiliary carbon source, thus reducing challenges associated with low dioxane concentrations (Kelley et al., 2001). Poplar roots also provide the microbial community with oxygen, allowing for the aerobic transformation of pollutants near roots (Kacprzyk et al., 2011; Schnoor et al., 1995). The poplar rhizosphere also provides habitat for bioaugmented bacteria, allowing for biofilm formation on the root surface, preventing washout, and reducing predation (Chan and Kjellerup, 2019). Previous work has shown that if augmented bacteria can colonize the roots, the growing roots can spread the bacteria throughout the subsurface (Kuiper et al., 2001). This may help distribute augmented strains that tend to clump during growth, such as CB1190. Phytoremediation has also been shown as an effective treatment for chlorinated solvents, allowing for the treatment of comingled plumes (Schnoor, 2002). Finally, increased dioxane metabolism in the rhizosphere by microbes minimizes the amount of dioxane transpired to the atmosphere by plants used in phytoremediation (Kelley et al., 2001).

2.2. Objectives

The objective of this research project was to discover microbial strains that can degrade 1,4-dioxane to health advisory levels (<0.35 μg/L). In addition, we evaluated the performance of candidate organisms when bioaugmented into the poplar rhizosphere.

2.3. Technical Approach

During the first two years of ER-2719, we compared the performance of two archetype degraders, *Pseudonocardia dioxanivorans* CB1190 and *Mycobacterium dioxanotrophicus* PH-06. Using bench-scale experiments, we examined the stimulatory effect that poplar root extract has on growth and dioxane degradation by CB1190 and PH-06. Also, we tested the ability of these two strains to enhance dioxane treatment through the bioaugmentation of hybrid poplar. We hypothesized that PH-06 would utilize root extract as an auxiliary carbon source, as previously observed with CB1190. We also hypothesized that PH-06 would outperform CB1190 in accelerating dioxane removal by poplar to low concentrations.

In the final year of ER-2719, our experiments centered around *Rhodococcus ruber* 219. Previous research on *R. ruber* 219 only described slow metabolism (Bernhardt and Diekmann, 1991) or cometabolism (Bock et al., 1996) of dioxane. However, novel work during ER-2719 at the University of Iowa demonstrated that *R. ruber* 219 rapidly degrades dioxane in the presence of b-vitamins. Experiments aimed to determine the growth and dioxane metabolism kinetics of *R. ruber* 219. Also, we examined the effects that common chlorinated solvent co-contaminants have on *R. ruber* 219. We conducted planted microcosm experiments to examine the potential of *R. ruber* 219 for bioaugmentation of the poplar rhizosphere (**Figure 1**).

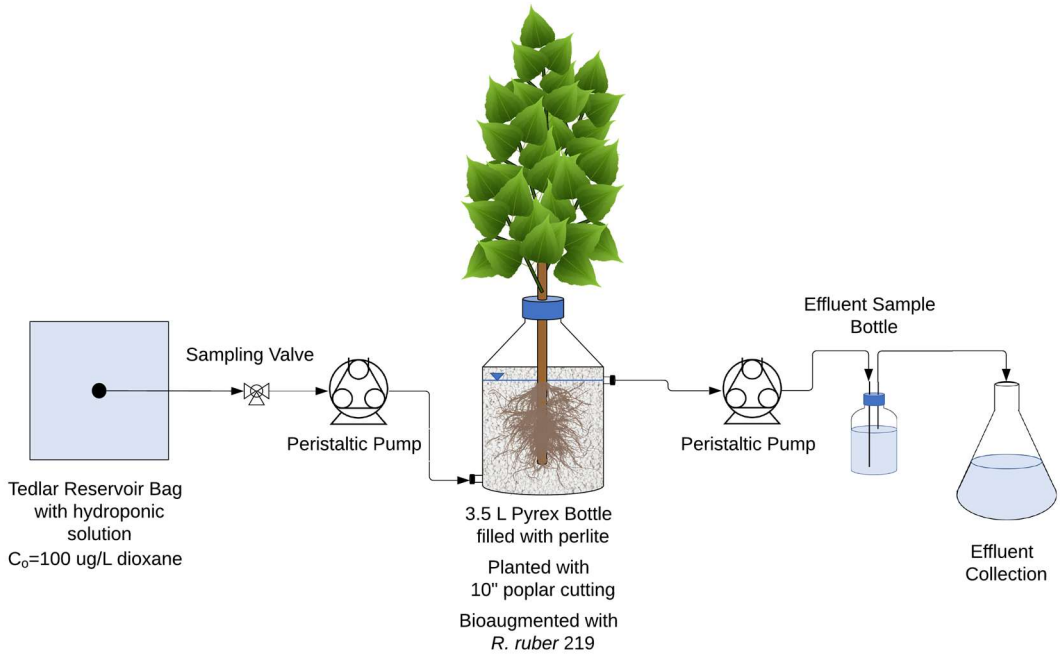


Figure 1. Flow-through experimental design used to evaluate bioaugmented phytoremediation as a strategy to treat dioxane-contaminated groundwater.

2.4. Results and Discussion

2.4.1. Bioaugmentation Experiments with Archetype Degraders

To compare the performance of CB1190 and PH-06, and to evaluate if root extract can serve as an auxiliary substrate for these strains, we conducted growth curve experiments in 500 mL Erlenmeyer flasks. As seen in **Figure 2**, root extract significantly increased the total growth (measured as protein) ($p = 0.017$) and dioxane degradation ($p = 0.0047$) of CB1190. Adding root extract also significantly increased the cell yield coefficients from 0.16 ± 0.04 mg-protein per mg-dioxane to 0.21 ± 0.03 mg-protein per mg-dioxane ($p = 0.006$). Interestingly, the addition of root extract decreased the specific degradation rate from 4.39 ± 1.20 g-dioxane per g-protein per day to 3.20 ± 0.62 g-dioxane per g-protein per day ($p = 0.045$), presumably due to the simultaneous utilization of root extract supplementing CB1190 growth. However, despite decreased specific degradation rates, overall degradation rates by CB1190 increased due to greater total biomass. These results align with Kelley et al. (2001), who concluded that root extract acts as an auxiliary substrate for the growth of CB1190 but does not induce dioxane monooxygenases. Previous work found that non-inducing, easily metabolized substrates can slow dioxane degradation by CB1190 due to the use of a preferred carbon source and repressed induction of dioxane monooxygenases (catabolite repression) (Li et al., 2017).

The addition of root extract did not significantly affect the total growth (measured as protein) ($p = 0.066$) or consumption of dioxane ($p = 0.14$) by PH-06 (**Figure 2**). These results suggest that PH-06 does not readily utilize root extract as an auxiliary carbon source or growth supplement. Root extract neither inhibits the specific dioxane degradation rate by PH-06, nor does it accelerate growth. Furthermore, the PH-06 dioxane degradation rate constants were significantly higher than with CB1190 for treatments without root extract ($p < 0.0001$) as well as treatments

with root extract added ($p < 0.0001$). PH-06 degrades dioxane faster than CB1190 under these experimental conditions. This aligns with previous research, which also found PH-06 degrades dioxane significantly faster than CB1190 (He et al., 2018).

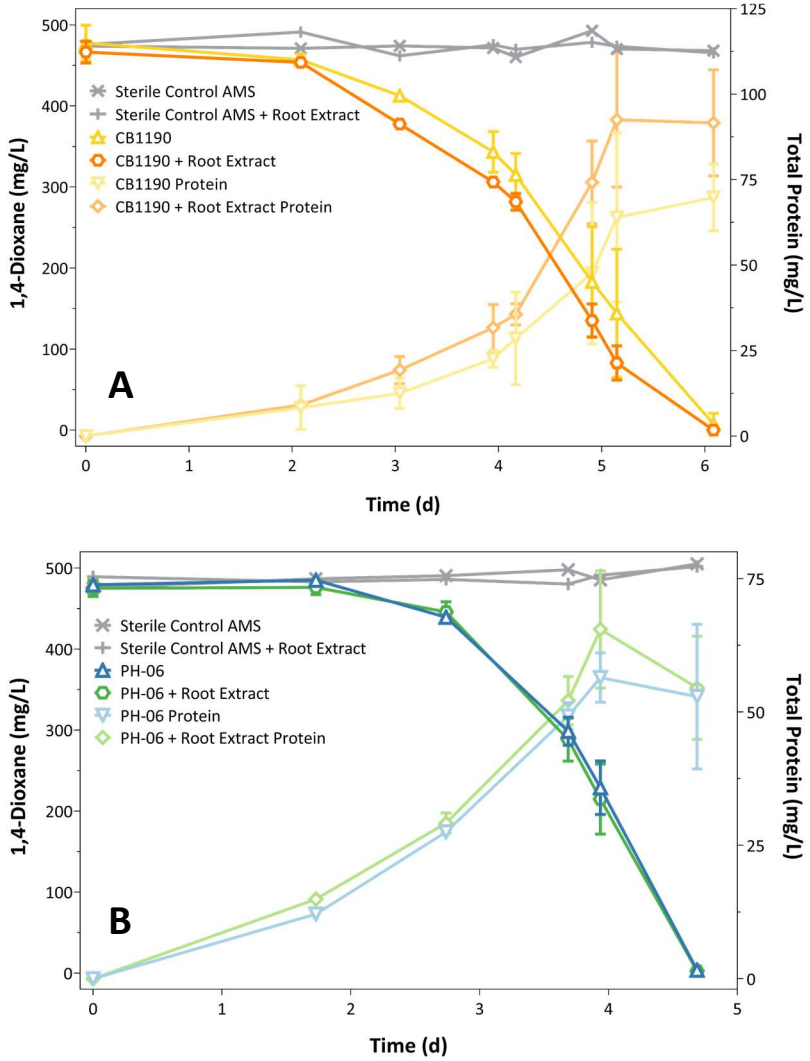


Figure 2. Bacterial growth and dioxane degradation experiments with (A) *Pseudonocardia dioxanivorans* CB1190 and (B) *Mycobacterium dioxanotrophicus* PH-06. Root extract significantly increased the total growth (measured as protein) ($p = 0.017$) and dioxane degradation ($p = 0.0047$) of CB1190. However, root extract did not significantly impact total growth ($p = 0.067$) or dioxane degradation ($p = 0.14$) of PH-06. Error bars represent the standard deviation from triplicate reactors.

To evaluate using archetype dioxane degraders CB1190 and PH-06 to speed phytoremediation, we conducted a batch hydroponic experiment in 500-mL Erlenmeyer bioreactors. As seen in Figure 2, all treatments tested removed 10 mg/L initial dioxane to below the LOQ of 4 µg/L. In planted experiments, non-bioaugmented poplar trees removed 10 mg/L initial dioxane to below 4 µg/L in 29 days. Dioxane removal followed first-order kinetics due to a directly proportional relationship between the transpiration rate and the rate of dioxane removal. This agrees with previous work that found that the majority (76.5 ± 3.9%) of dioxane removed by poplar trees was transpired through the leaves (Aitchison et al., 2000). Also, the transpiration

stream concentration factor (TSCF) for dioxane was approximately 1.0, suggesting dioxane moved freely across the root membrane and did not become concentrated in the bulk fluid. This TSCF value agrees with previous estimates, ranging from 0.72 to 0.98 (Aitchison et al., 2000; Dettenmaier et al., 2008; Ferro et al., 2013).

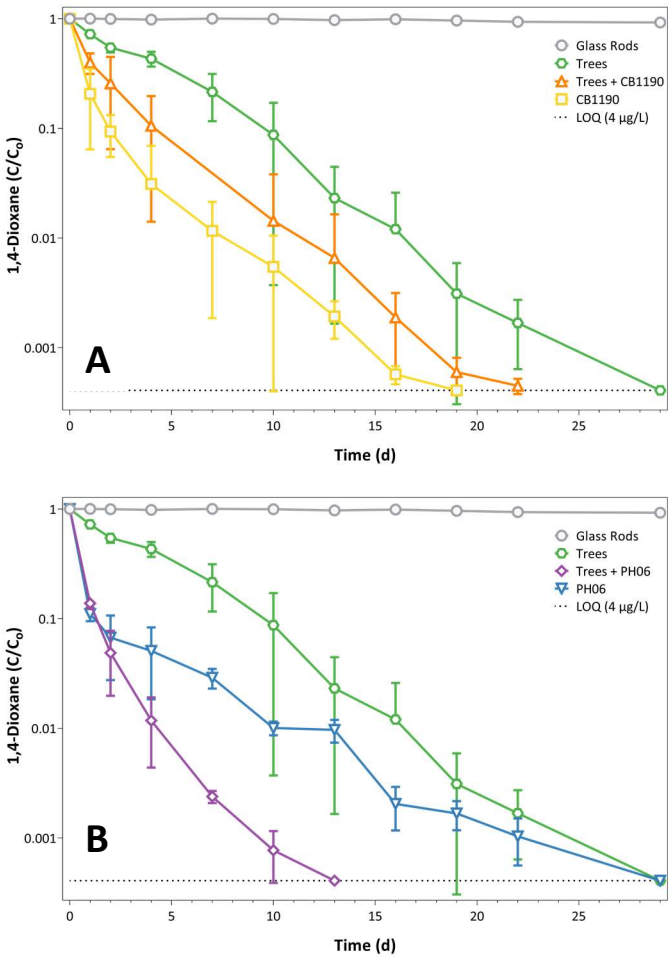


Figure 3. Planted bioaugmentation experiments conducted in modified Erlenmeyer bioreactors inoculated with either *Pseudonocardia dioxanivorans* CB1190 (A) or *Mycobacterium dioxanotrophicus* PH-06 (B). While all treatments reached the limit of quantification (4 µg/L), trees bioaugmented with PH-06 significantly outpaced all other treatments tested ($p < 0.05$). However, CB1190 in unplanted experiments removed dioxane significantly faster than planted treatments ($p = 0.014$). Error bars represent the standard deviation from triplicate reactors. C_o = 10 mg/L dioxane.

In bioaugmented planted experiments, CB1190 significantly enhanced bioremediation of dioxane by hybrid poplar (22 days vs. 29 days, p-value = 0.0017) (**Figure 3**). However, CB1190 in unplanted experiments removed dioxane significantly faster than planted treatments (19 days vs. 22 days, p-value = 0.014). One explanation for this unexpected result is that dioxane degradation by CB1190 was slowed by the consumption of poplar root exudates. In contrast, PH-06-bioaugmented poplars significantly outpaced all other treatments tested ($p < 0.05$), remediating dioxane to <4 µg/L in only 13 days (**Figure 3**). As PH-06 was not affected by root extract in growth curve experiments, we postulate that this increased rate is due to additive mechanisms between

degradation by PH-06 and uptake by the plant. Unexpectedly, PH-06 in unplanted reactors was significantly slower than all other bioaugmented treatments (p-value = 0.035), reaching non-detect levels in 29 days. Headspace oxygen remained above 19% across all treatments and was not limiting. Also, transpiration rates were not significantly different (p-value > 0.05).

2.4.2. Rhodococcus ruber 219 Experiments

Previous research on *R. ruber* 219 only described slow metabolism (Bernhardt and Diekmann, 1991) or cometabolism (Bock et al., 1996) of dioxane. However, novel work during ER-2719 at the University of Iowa demonstrated that *R. ruber* 219 rapidly degrades dioxane in the presence of b-vitamins. As seen in Figure 3, *R. ruber* 219 rapidly grew and consumed dioxane in cultures containing ATCC's MD-VS vitamin mixture, reducing dioxane concentrations by more than 99% in six days. In addition, while significantly slower (p-value = 0.0353), cultures containing only thiamine closely mirrored both the full MD-VS vitamin mixture's growth and depletion rates. Furthermore, no growth was observed in treatments containing Wolfe's mixture without thiamine or in cultures with no vitamins. Neither growth nor dioxane removal was observed in these cultures after 34 days (data not shown). This experiment demonstrates that *R. ruber* 219 rapidly degrades dioxane when b-vitamins are added to the media. Also, this experiment confirms that thiamine is the primary limiting co-factor for dioxane degradation by *R. ruber* 219.

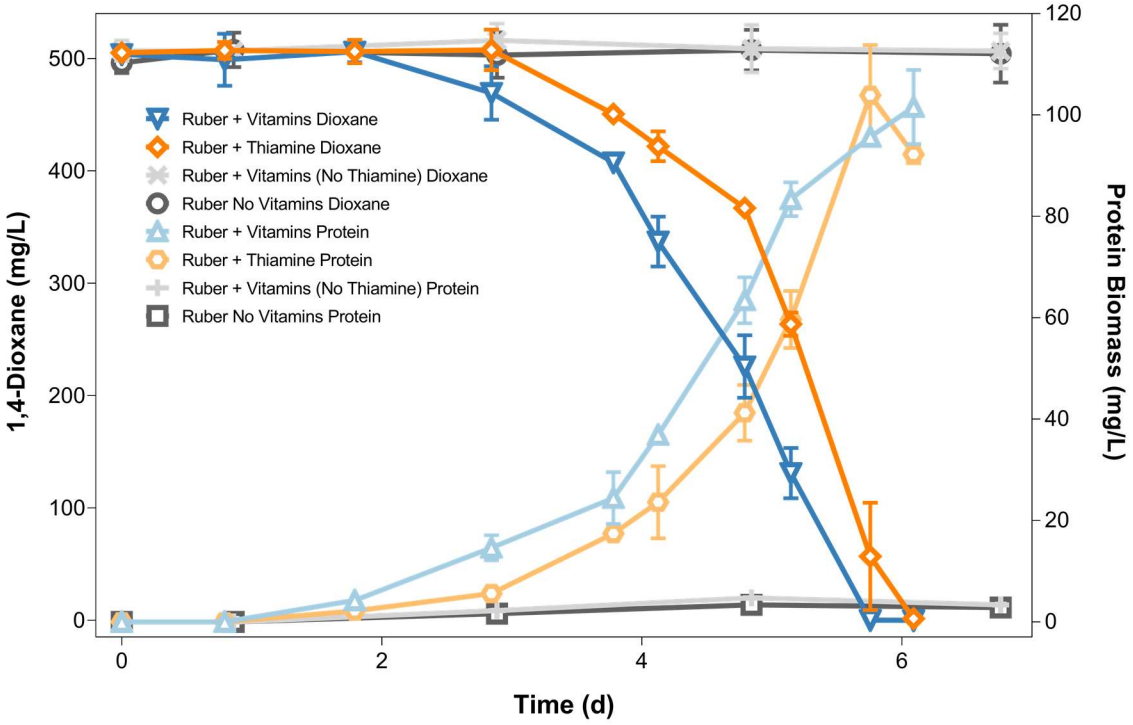


Figure 4. *R. ruber* DSM-44190 growth and depletion curve with and without the addition of B-vitamin mixtures. Experiment conducted in triplicate with ± 1 standard deviation error bars.

To obtain kinetic rate benchmarks for dioxane degradation by *R. ruber* 219, resting cell depletion curve experiments were carried out in 2-liter Erlenmeyer flasks. As seen in Figure 5, *R. ruber* 219 pre-grown with b-vitamins rapidly degraded dioxane in batch reactors to below 0.19 µg/L in less than 6 hours. The resulting Monod kinetic parameters can be seen in Table 1. The maximum degradation rate (q_{max}) for *R. ruber* 219 was similar to archetype degrader CB1190. However, in *R. ruber* 219 experiments, the half-saturation constant (Ks) was calculated to be 0.015 mg/L with substrate 1,4-dioxane, significantly lower than Ks values for both CB1190 and PH-06 (**Table 1**). This low Ks suggests that *R. ruber* DSM 219 can continually grow and degrade dioxane despite low dioxane concentrations (100 ppb or less), unlike CB1190 and PH-06, which can stall when exposed to such low concentrations (**Table 1**).

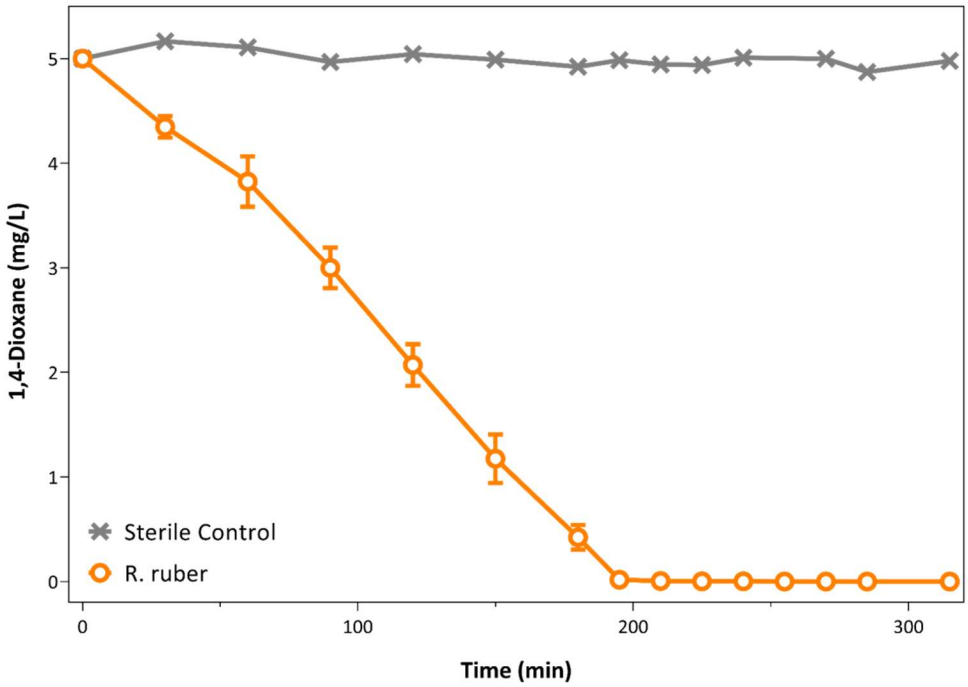


Figure 5. *R. ruber* DSM 219 depletion curve conducted to obtain kinetic degradation parameters. Experiment conducted in triplicate with ± 1 standard deviation error bars.

Table 1. Kinetic parameters of select metabolic dioxane-degrading bacteria. Values calculated using Aquasim 2.0, courtesy of Patrick Richards (University of Iowa).

Strain	**q_{max} (mg 1,4-dioxane/mg protein/day)**	**Ks (mg 1,4-dioxane /L)**	**Reference**
Pseudonocardia dioxanivorans CB1190	4.1 ± 0.14	6.3 ± 0.22	(Barajas-Rodriguez and Freedman, 2018)
Pseudonocardia dioxanivorans CB1190	26 ± 0.19	160 ± 44	(Mahendra and Alvarez-Cohen, 2006)
Mycobacterium dioxanotrophicus PH-06	Not reported	78 ± 10	(He et al., 2017a)
***Rhodococcus ruber* 219**	**4.8 ± 0.31**	**0.015 ± 0.065**	**This Work**

To observe the effect that exposure to chlorinated solvent co-contaminants has on *R. ruber* 219, inhibition tests were carried out in 500 mL Erlenmeyer flasks. As seen in Figure 6, *R. ruber* 219 rapidly degraded dioxane to <1 µg/L in 46 hours in all treatments except in flasks with 1,1-dichloroethylene (1,1-DCE). This result suggests that 1,1-DCE inhibits dioxane degradation by *R. ruber* 219. This compound is known to also inhibit dioxane degradation by CB1190 (Zhang et al., 2016). It was determined in the former study that 1,1-DCE reduced ATP production and dioxane-degrading enzyme expression in CB1190. However, further work is needed to determine the root cause of inhibition of *R. ruber* 219 by 1,1-DCE. It is unclear how greater solvent concentrations might impact *R. ruber* 219, but the concentrations of chlorinated solvents utilized in Figure 6 are representative of most field-contaminated sites. In addition, phytoremediation has also been shown as an effective treatment for chlorinated solvents, allowing for the treatment of comingled plumes (Schnoor, 2002). All lines of evidence indicate that bioaugmentation with *R. ruber* 219 in tandem with phytoremediation will allow for simultaneous treatment of 1,4-dioxane and chlorinated solvent co-contaminants.

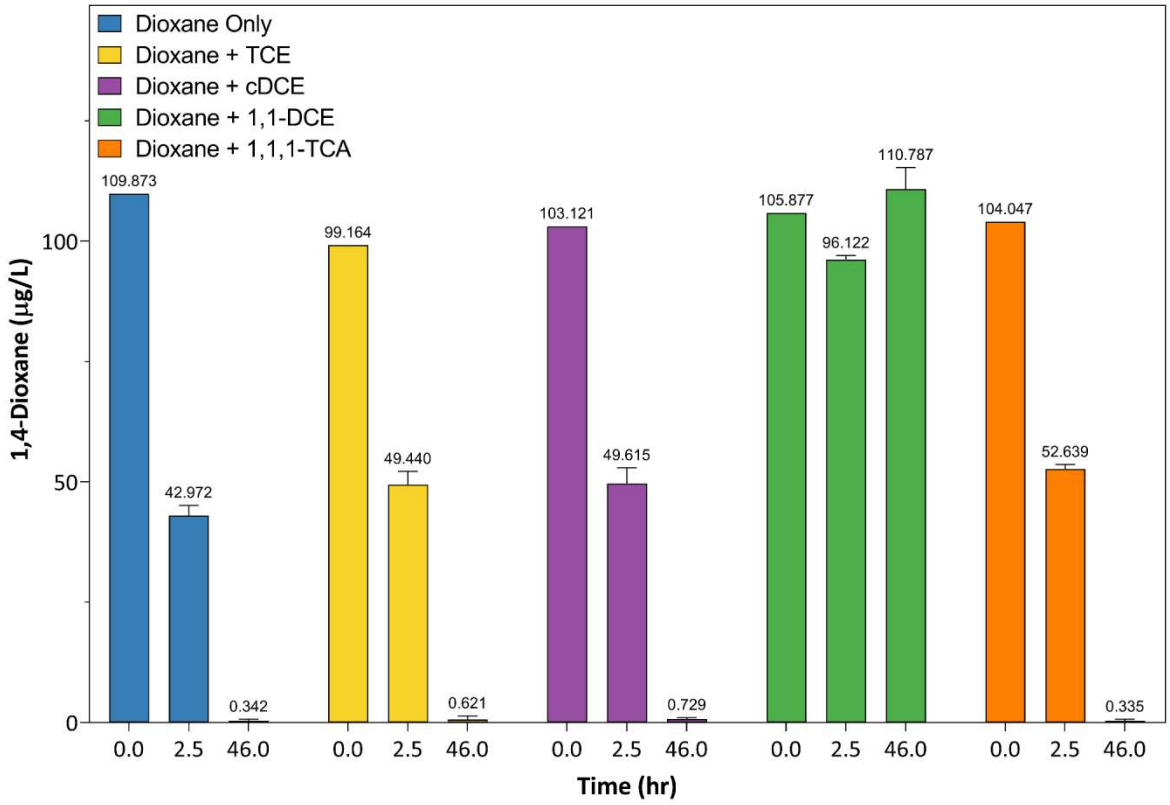


Figure 6. Dioxane degradation by *R. ruber* 219 measured over time in chlorinated solvent inhibition tests. Numbers above bars indicate the average dioxane concentration in µg/L. Experiment conducted in triplicate with ± 1 standard deviation error bars.

Flow-through experiments were conducted to evaluate the long-term treatment of low initial dioxane concentrations (100 µg/L) by poplar trees bioaugmented with *R. ruber* 219 (**Figure 7**). As seen in Figure 7, bioaugmented flow-through microcosms with *R. ruber* 219 significantly treated the influent concentration of 100 µg/L dioxane. During the experiment, several adjustments were made to improve treatment performance. On day 10, the thiamine concentration was

increased from 5 µg/L to 50 µg/L in the influent hydroponic solution. This increase in thiamine improved dioxane treatment in unplanted reactors, but no change was observed in planted treatments.

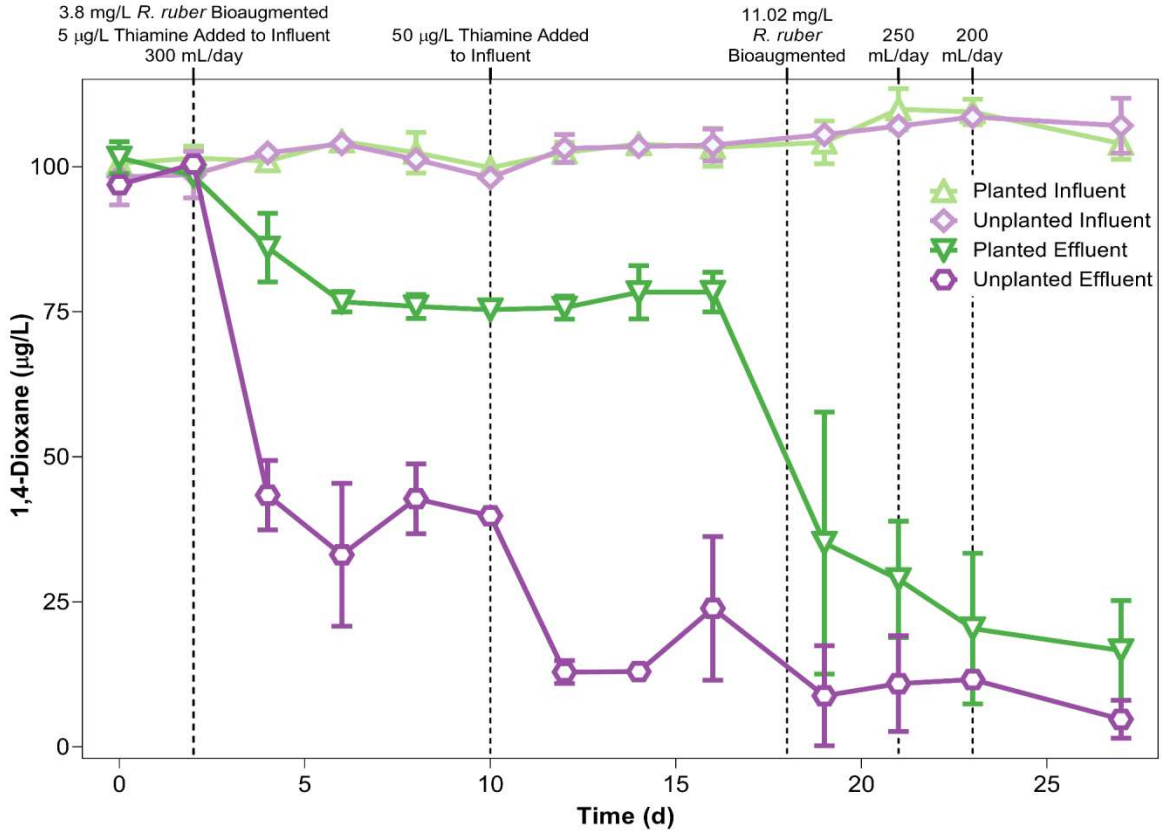


Figure 7. Planted flow-through experiments conducted in modified 3.5-liter glass bottles and bioaugmented with *Rhodococcus ruber* 219. Vertical dashed lines and top labels describe parameter changes made during the experiment to improve performance. Error bars indicate ± 1 standard deviation of replicates.

On day 18, additional *R. ruber* 219 (50 mL of 550 mg/L protein, approximately 11.02 mg/L final protein concentration in each reactor) was bioaugmented into all reactors. This, coupled with slowed flow rates (250 mL/d on 21 and 200 mL/d on day 23), caused a significant decrease in effluent dioxane concentrations in both planted and unplanted microcosms. Surprisingly, unplanted bioaugmented reactors outperformed planted bioaugmented treatments, although both achieved low effluent concentrations of dioxane (16.6 µg/L in planted reactors, 4.8 µg/L in unplanted reactors at day 27). One hypothesis for this phenomenon is dual substrate utilization (dioxane and root exudates) by *R. ruber* 219, as seen with CB1190 above, limiting dioxane consumption. An alternative hypothesis is that native rhizosphere microbes were competing with *R. ruber* for various macronutrients. Also, pressures from protozoans and other predators may have influenced dioxane degradation by *R. ruber* in planted reactors. Such pressures were not a factor in unplanted treatments, as these reactors were autoclaved before experimentation.

The findings of this study are especially field-relevant. In this experiment, *R. ruber* was able to sustain treatment of low initial dioxane concentrations (100 µg/L) commonly encountered in the field to near health advisory levels. Ongoing work aims to optimize this system to reach <1 µg/L dioxane.

2.5. Implications for Future Research and Benefits

Progress made during ER-2719 has been conclusive. Our work with archetype degraders, *Mycobacterium dioxanotrophicus* PH-06 or *Pseudonocardia dioxanivorans* CB1190, explained the energetics of dual substrate utilization, 1,4-dioxane plus root extract, which is critical when bioaugmentation is used in tandem with phytoremediation. Our team was the first to report that PH-06 cannot utilize root extract as primary substrates and confirmed that CB1190 can. We were also the first to demonstrate that PH-06-bioaugmented-poplar significantly outperformed poplar bioaugmented with CB1190. PH-06 was uninhibited by root extract, making the strain a strong candidate to speed phytoremediation of dioxane. However, it is possible that CB1190 would perform better in the field due to its capacity to utilize root extract and outcompete indigenous microorganisms. Finally, we have confirmed in 30-L fermentation runs that both CB1190 and PH-06 can be grown to the large quantities needed for field implementation.

We have also identified *R. ruber* 219 as a very strong candidate for field implementation. In our experiments, we demonstrated that this strain, with the addition of thiamine, can grow on low dioxane concentrations (<100 µg/L) without auxiliary substrates. Furthermore, we have demonstrated that despite extremely low initial biomass concentrations, *R. ruber* 219 can degrade 100 µg/L dioxane to below 0.35 µg/L health advisory level. This is the first metabolic dioxane-degrading bacteria reported in the literature to be able to sustain degradation under such dilute dioxane conditions. However, we have observed that dioxane degradation by *R. ruber* 219 slows somewhat in the presence of plant roots. We hypothesize this is due to dual substrate utilization, 1,4-dioxane and root exudates, as seen with CB1190. We have also identified 1,1-DCE as inhibitory for dioxane degradation by *R. ruber* 219. Ongoing work aims to explore if bioaugmentation with *R. ruber* 219 in tandem with phytoremediation can overcome inhibition by 1,1,-DCE. Furthermore, poplar trees may release sufficient thiamine in root exudates, reducing the need for vitamin amendments. Finally, as with CB1190 and PH-06, we have confirmed that *R. ruber* 219 can be grown to sufficient quantities for field bioaugmentation.

This project demonstrated that combining phytoremediation with bioaugmentation is a promising treatment alternative for dioxane-contaminated groundwater to achieve low concentrations (<0.35 µg/L) as recommended by health advisories. Dioxane-metabolizing microbes have been utilized, eliminating the need for auxiliary substrates required by cometabolic microorganisms. In the coming years, we will conduct a pilot-scale demonstration at the Twin Cities Army Ammunition Plant (TCAAP) in Minneapolis, MN, under ESTCP Project ER21-5096, titled "Bioaugmented Phytoremediation to Treat 1,4-Dioxane Contaminated Groundwater." We believe this study will validate that bioaugmented phytoremediation is an effective treatment strategy for dilute dioxane plumes. While challenges remain, the successful implementation of this strategy offers a green and cost-effective solution to a widespread problem of national and international importance.

3. OBJECTIVES

The overall objective of this research project is to discover and employ microbial strains that can degrade 1,4-dioxane and co-contaminants to health advisory levels in conjunction with hybrid poplar phytoremediation plantations at sites with contaminated groundwater. This objective speaks to the FY 2017 Statement of Need for ERSON-17-01 to 1) *develop a greater understanding of potential treatment synergies that could lead to cost savings and improved remedial strategies*; 2) *develop procedures to validate efficacy and implementability of potential treatment trains addressing mixed contamination in groundwater*; and 3) *develop procedures to maximize benefit from treatment interactions and to provide a systematic approach.*

Monooxygenase enzymes are known to be especially prevalent in the rhizobiome and are frequently required for the first step of aerobic biodegradation of cyclic ethers (e.g., 1,4-dioxane). Certain monooxygenase enzymes (dioxane monooxygenase, dioxane etherase, hydroxyethoxyacetate monooxygenase, soluble di-iron monooxygenases, and propane monooxygenases) are known to catalyze the oxidation of cyclic ether linkages like 1,4-dioxane and to also aerobically degrade trichloroethylene, a common co-contaminant (Chiang et al., 2012; Hand et al., 2015; He et al., 2017b; Stevenson and Turnbull, 2013). We are taking special advantage of the vast diversity of soluble di-iron monooxygenase genes (SDIMOs) among the strains utilized in this research. Most promising is the gene expression and metabolite pathways of *Rhodococcus ruber* 219, whose rapid biodegradation of 1,4-dioxane has not been previously reported. We have demonstrated in soil microcosms that *R. ruber* 219 can be bioaugmented into the root zone of hybrid poplar to enhance the degradation of low level 1,4-dioxane concentrations to health advisory levels (~1 µg/L).

4. BACKGROUND

1,4-Dioxane (dioxane) is a synthetic cyclic ether commonly used as a stabilizer for chlorinated solvents such as 1,1,1-trichloroethane (TCA) and trichloroethylene (TCE) (Anderson et al., 2012; Mohr et al., 2010). It is also used as an additive for paints and lacquers, as well as being a common unintended byproduct in the manufacturing of pesticides, herbicides, plastics, textiles, detergents, and cosmetics (Mohr et al., 2010; USEPA, 2017b). Dioxane is a contaminant of increasing concern due to its classification as a probable human carcinogen by the United States Environmental Protection Agency (US EPA) (USEPA, 2017b). While no enforceable federal guidelines for dioxane have currently been established, regulations have been proposed based on the IRIS risk assessment that 0.35 µg/L dioxane in drinking water represents a 1 x 10^{-6} lifetime cancer risk (USEPA, 2013). In addition, many states have passed drinking water and groundwater guidelines ranging from 0.25 µg/L in New Hampshire to 77 µg/L in Alaska (USEPA, 2017b).

Dioxane's prevalence as a contaminant (**Figure 1**) is exacerbated by its high mobility in water (log K_{ow} = -0.27), low tendency to sorb to aquifer materials (log K_{oc} = 0.4), and relatively low volatility (K_H = 2.0 x 10^{-4} mg/L air per mg/L water), which can result in large and/or dilute groundwater plumes (Adamson et al., 2014; Godri Pollitt et al., 2019; Zenker et al., 2003). These dilute plumes often make energy-intensive *ex-situ* strategies, such as advanced oxidation, economically impractical (Simon, 2015). Recent estimated capital costs for advanced oxidation treatment of dioxane range from $300,000 to near $2 million (Barndõk et al., 2018). As a result, there has been a push in recent years to develop cost-effective *in-situ* remediation techniques (Adamson et al., 2017; Chiang et al., 2016; USEPA, 2006).

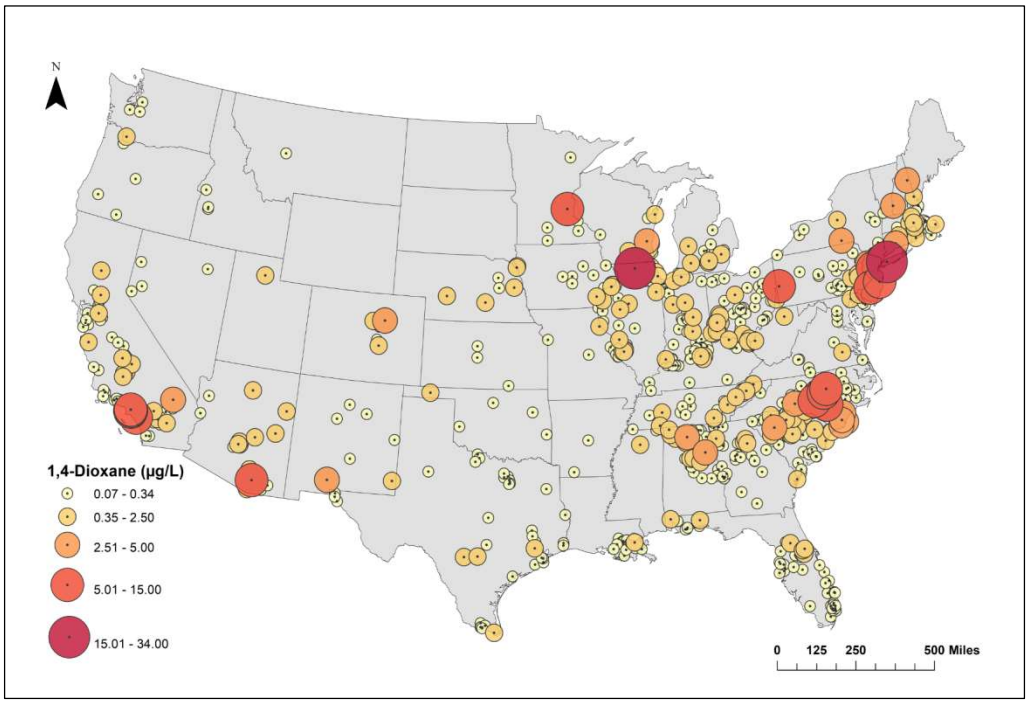


Figure 1. Locations with 1,4-dioxane concentrations in finished drinking water above Mandatory Reporting Limit (MRL) of 0.07 µg/L at Public Water Systems, 2013-2015. Data obtained from US EPA Unregulated Contaminant Monitoring Rule 3 (UCMR 3) (USEPA, 2017a). Map made using ArcGIS 10.4.1 (ESRI, Redlands, CA).

Phytoremediation is a cost-effective clean-up strategy of dioxane contaminated groundwater. This remediation technology offers many benefits, including appealing aesthetics, low energy demand, and costs of 50 to 90% less than traditional remediation techniques (Aitchison et al., 2000; Dietz and Schnoor, 2001; Doty, 2008). Phytoremediation is also well suited for sites with low-level contamination over a large area where other technologies might be prohibitively expensive (Gatliff et al., 2016). Phreatophytes such as poplar and willow are a common choice for phytoremediation applications due to their high growth rate, high transpiration rate, deep root systems, and resilience to contaminants (e.g., chlorinated solvents, BTEX, heavy metals, pesticides, and explosives) (Dietz and Schnoor, 2001; Ferro et al., 2013).

Previous work by Aitchison et al. demonstrated hybrid poplar tree cuttings readily removed dioxane in bench-scale experiments (Aitchison et al., 2000). While poplars do possess P450 cytochrome monooxygenases capable of degrading dioxane, Aitchison et al. found that most (76.5 ± 3.9%) of the dioxane removed by poplar was not transformed but was transpired directly to the atmosphere (Dietz and Schnoor, 2001). Once volatilized, dioxane may undergo photodegradation via hydroxyl radicals in the atmosphere (estimated half-life of 6.7 to 9.6 hours) (Ferro et al., 2013; Stepien et al., 2014). Several recent field studies have confirmed that phytoremediation can treat dioxane-contaminated groundwater to below 5 µg/L (Ferro et al., 2013; Gatliff et al., 2016).

Despite these promising results, questions remain if phytoremediation alone can be used to treat dioxane-contaminated groundwater to the low levels required by health advisories. Phytoremediation performance has been shown to vary significantly based on the tree hybrid or species used and co-contaminants present in the groundwater (Edwards et al., 2011; Silva, 2010). For example, ethylene glycol, a common co-contaminant of dioxane, has been shown to reduce the uptake of dioxane by poplar through osmotic inhibition (Edwards et al., 2011). Furthermore, phytoremediation may not be appropriate for all dioxane sites due to the large land area needed for tree plantations (Sorensen, 2013). Also, traditional phytoremediation is usually limited in treatment depth to shallow groundwater plumes (5-15 ft below ground surface). Finally, phytoremediation may be considered too passive due to lengthy treatment times and may need to be combined with other, more aggressive technologies to reach full site closures (Favara et al., 2016).

One possible technique to speed the treatment of dioxane by phytoremediation to low levels is to pump contaminated water onto plantations of trees (sub-surface irrigation) and to bioaugment the rhizosphere with dioxane degrading bacteria. Bioaugmentation itself is a promising *in-situ* technology to treat dioxane plumes. A number of dioxane-degrading bacteria have been identified, with some possessing the ability to utilize dioxane as a sole carbon and energy source (metabolic bacteria) (Bernhardt and Diekmann, 1991; Chen et al., 2016; Goodfellow et al., 2004; Huang et al., 2014; Kampfer and Kroppenstedt, 2004; Kim et al., 2009; Matsui et al., 2016; Nakamiya et al., 2005; Parales et al., 1994; Sei et al., 2013a). Metabolic bacteria have many advantages over cometabolic strains, including higher transformation rates, lower oxygen demand, and no added costs due to additions of primary growth substrates required to induce dioxane degradation co-metabolically (e.g., tetrahydrofuran (THF), propane, methane, toluene, 1-butanol, or isobutane) (Barajas-Rodriguez and Freedman, 2018; Hand et al., 2015; Hatzinger et al., 2017; Johnson et al., 2020; Kohlweyer et al., 2000; Lippincott et al., 2015; Mahendra and Alvarez-Cohen, 2006; Rolston et al., 2019; Sei et al., 2013b; Sun et al., 2011; Vainberg et al., 2006; Zenker et al., 2000).

In general, metabolic dioxane-degrading strains identified to date are strict aerobes that utilize soluble di-iron monooxygenases (SDIMOs) to oxidize and cleave the dioxane ring

(Grostern et al., 2012; Zhang et al., 2017). However, metabolic dioxane degraders face challenges that may impede bioremediation. For example, the well-known metabolic dioxane-degrading bacterium *Pseudonocardia dioxanivorans* CB1190 can stall when exposed to low initial dioxane concentrations (<500 µg/L) commonly found at dioxane contaminated sites (Adamson et al., 2014; Li et al., 2010). This may be attributed to minimum substrate concentrations required by metabolic bacteria for sustained growth (Barajas-Rodriguez and Freedman, 2018; da Silva et al., 2018). Also, CB1190 tends to form clumps, which may prevent it from being transported throughout subsurface plumes during bioaugmentation (da Silva et al., 2020). Finally, bioaugmented strains may also face stressors such as low temperatures, oligotrophic conditions, extreme pH, limited oxygen availability, washout, and competition and predation from indigenous microorganisms (Chan and Kjellerup, 2019; Stroo et al., 2012).

Bioaugmenting the poplar rhizosphere alleviates many of the deficiencies bioaugmentation and phytoremediation have separately. The poplar rhizosphere is a richer nutrient environment with higher dissolved oxygen suitable for obligate aerobes. Root exudates stimulate increased growth of bacteria compared to the adjacent bulk soil, allowing for metabolic activity and degradation of pollutants (Bais et al., 2006; Burken and Schnoor, 1996; Jones, 1998; Kuiper et al., 2004; Schnoor et al., 1995). Poplar roots also provide the microbial community with aerenchyma-transported oxygen, allowing for the aerobic transformation of pollutants in the rhizosphere (Kacprzyk et al., 2011a; Schnoor et al., 1995). The poplar rhizosphere provides habitat for bioaugmented bacteria, allowing for biofilm formation on the root surface, preventing washout, and reducing predation (Chan and Kjellerup, 2019). Finally, phytoremediation has also been shown as an effective treatment for chlorinated solvents, allowing for the treatment of comingled plumes (Schnoor, 2002).

A previous lab-scale study by Kelley et al. utilized CB1190 to bioaugment the rhizosphere of hybrid poplar (Kelley et al., 2001). The addition of this bacterium enhanced the degradation of dioxane by hybrid poplar, increasing removal by up to 35%. Bioaugmenting with CB1190 also increased the removal of dioxane in the rhizosphere, reducing the amount transpired by the plant. This phenomenon was seemingly due to parallel pathways for the uptake of dioxane by microbes and plants. The researchers also postulated that CB1190 utilized poplar root exudates as a non-inducing substrate, increasing their populations and thus accelerating dioxane degradation. Kelley et al. also demonstrated that CB1190 can be grown to large quantities in 10 L fermenters. This is significant as producing large cell quantities is a major challenge facing field-scale bioaugmentation (Stroo et al., 2012). While promising, this study was limited by an analytical limit of detection of 1 mg/L, which prevented observation of how the combined technologies performed in low dioxane conditions.

During the first two years of ER-2719, we compared the performance of two archetype degraders, *Pseudonocardia dioxanivorans* CB1190 and *Mycobacterium dioxanotrophicus* PH-06. Using bench-scale experiments, we examined the stimulatory effect poplar root extract has on growth and dioxane degradation by CB1190 and PH-06. Also, we tested the ability of these two strains to enhance dioxane treatment through the bioaugmentation of hybrid poplar. We hypothesized that PH-06 would utilize root extract as an auxiliary carbon source, as previously observed with CB1190. We also hypothesized that PH-06 would outperform CB1190 in accelerating dioxane removal by poplar to low concentrations.

In the final year of ER-2719, our experiments centered around *Rhodococcus ruber* 219. Previous research on *R. ruber* 219 only described slow metabolism (Bernhardt and Diekmann,

1991) or cometabolism (Bock et al., 1996) of dioxane. However, novel work during ER-2719 at the University of Iowa demonstrated that *R. ruber* 219 rapidly degrades dioxane in the presence of B-vitamins. Experiments aimed to determine the growth and dioxane metabolism kinetics of *R. ruber* 219. Also, we examined the effect common chlorinated solvent co-contaminants have of *R. ruber* 219. We also conducted planted microcosm experiments to explore the potential *R. ruber* 219 has for bioaugmentation of the poplar rhizosphere. Finally, work at the Center for Biocatalysis and Bioprocessing at the University of Iowa examined if CB1190, PH-06, and *R. ruber* 219 can be grown to sufficient quantities for field bioaugmentation. We believe that ER-2719 has helped validate bioaugmented phytoremediation as an accepted treatment technology for dioxane-contaminated groundwater.

5. MATERIALS AND METHODS

5.1. Chemicals

ACS grade 1,4-dioxane (anhydrous, >99.9%), 1,4-dichlorobenzene-d4 (2000 µg/mL in methylene chloride), and trichloroethylene (≥99.5%) were purchased from MilliporeSigma, Burlington, MA. Vitamin solution components, including biotin (≥99%), calcium pantothenate (≥98%), folic acid (≥97%), nicotinic acid (≥98%), p-aminobenzoic acid (≥99%), pydridoxine hydrochloride (≥98%), riboflavin (≥98%), thiamine hydrochloride (≥99%), thioctic acid (≥99%), and vitamin B12 (≥98%) were also purchased from MilliporeSigma. Cis-1,2-dichloroethylene (98.7%), 1,1-dichloroethylene (99.4%), and 1,1,1-trichloroethane (99.7%) were purchased from ChemService, West Chester, PA. 1,4-Dioxane (2,000 µg/mL in methylene chloride or P&T methanol), 1,4-dioxane-d8 (2000 µg/mL in P&T methanol), trichloroethylene (2000 µg/mL in P&T methanol), cis-1,2-dichloroethylene (2000 µg/mL in P&T methanol), 1,1-dichloroethylene (2000 µg/mL in P&T methanol), 1,1,1-trichloroethane (2000 µg/mL in P&T methanol), and tetrahydrofuran-d8 (2000 µg/mL in P&T methanol) were purchased from Restek Corporation, Bellefonte, PA. Methanol (≥99.9%, GC Resolv) and methylene chloride (≥99.9%, GC Resolv) were purchased from Fisher Scientific, Hampton, NH.

5.2. Bioaugmentation Experiments with Archetype Degraders

5.2.1. Growth of Hybrid Poplar in the Laboratory

Unrooted hybrid poplar cuttings (*Populus deltoides x nigra*, DN34) were purchased from Hramor Nursery (Manistee, MI). Before growth, each cutting (1/4 in x 10 in) was fitted with a pre-drilled screw cap with a PTFE liner and sealed with 100% silicone sealant (DAP Products Inc., Baltimore, MD). PTFE tape was used to wrap each cutting to ensure a snug fit between the cap and the trunk as well as prevent sealant from contacting the tree (**Figures 2, 3**). All buds were removed below the cap to prevent shoot growth within the reactor. Cuttings were grown in opaque plastic bins (25" x 18" x 7") containing 20 L of half-strength Hoagland's hydroponic solution (Burken and Schnoor, 1996). Bins were placed beneath grow-lights (Hydrofarm, Inc., Petaluma, CA) set to a 16-hour day length. Aquarium air stones were used to maintain aerobic conditions within the hydroponic solution. Once buds began to open (3-5 days), cuttings were pruned so that only the topmost bud could grow. Cuttings were pregrown for two weeks and selected for experimentation based on comparable size, leaf growth, and root density.

Figure 2. Hybrid poplar cuttings (10 in) grown hydroponically for use in phytoremediation experiments.

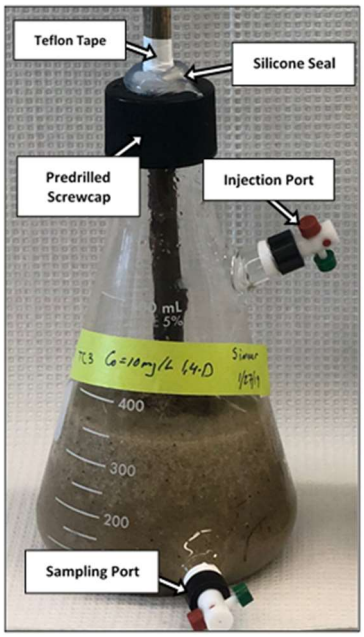


Figure 3. Hybrid poplar in a modified Erlenmeyer bioreactor.

5.2.2. Strain Cultivation

Pseudonocardia dioxanivorans CB1190 and *Mycobacterium dioxanotrophicus* PH-06 were precultivated in liquid Ammonium Mineral Salts (AMS) media with 500 mg/L 1,4-dioxane (Parales et al., 1994). All cultures were incubated aerobically at 30°C on an orbital shaker (150-200 rpm). Strain purity was routinely confirmed by Sanger sequencing. DNA was extracted using a DNeasy UltraClean Microbial Kit (Qiagen, Valencia, CA). The 16S gene was amplified by PCR using 27F and 1492R primers (Integrated DNA Technologies, Inc., Coralville, IA). Sequence data were processed using Sequence Scanner v2.0 (ThermoFisher Scientific, Waltham, MA) and matched by BLASTn using the NCBI database (www.ncbi.nlm.nih.gov).

5.2.3. Growth Curve Experiments and Poplar Root Extract as an Auxiliary Substrate

To compare the performance of CB1190 and PH-06, and to evaluate if root extract can serve as an auxiliary substrate for these strains, we conducted growth curve experiments in 500 mL Erlenmeyer flasks sealed with a screw cap. Due to challenges in producing root exudates in sufficient quantities and concentrations, root extract was used as a proxy (Kelley et al., 2001). Root extract was prepared by harvesting 5 g of wet roots from hydroponically grown poplar cuttings. Roots were thoroughly rinsed with deionized (DI) water, suspended in 1 L of DI water, and blended using a laboratory blender (Waring, Lancaster, PA). The resulting solution was vacuum-filtered through filters with a progressively finer pore size (Whatman 4 filter paper, Whatman GF/C glass fiber filter, and Whatman GF/F glass fiber filter) (Kelley et al., 2001). The solution was then filter-sterilized with a 0.2 µm bottle-top filter (Fisher Scientific, Hampton, NH) for use in microbiological media. The chemical oxygen demand (COD) of the final solution was measured using a Hach COD kit (Hach Co., Loveland, CO).

Experiments were initiated by adding 1 mL of active culture (late exponential phase) to 99 mL of fresh AMS media with a starting concentration of 500 mg/L dioxane (910 mg/L as COD). Culture volume was limited to 20% of the total flask volume (80% headspace) to ensure that oxygen was not limiting. Root extract was added to appropriate treatments at 9.1 mg/L as COD, a 1:100 COD ratio to that of 1,4-dioxane, ensuring that dioxane was utilized as the predominant substrate. Uninoculated sterile controls were included to account for unintended physical/chemical dioxane losses. Flasks were incubated at 30°C on an orbital shaker (200 rpm) for the duration of the experiment. Before sampling, cultures were sonicated for 10 minutes in a bath sonicator (Fisher Scientific, 40 kHz) to break up culture clumps. Samples (3 mL) were taken daily (twice daily during exponential growth) via sterile wide-orifice serological pipets within a laminar flow hood. Subsamples (1 mL) were sterile filtered and analyzed by GC-MS/MS, as described below. A portion of the remaining sample volume was extracted and analyzed for total protein. Cells were lysed following a modified cell lysis method from Coleman et al. (2002). Briefly, 450 µl of culture liquid was mixed with 150 µL of 10 M NaOH in a 1.5 mL centrifuge tube and heated (20 min at 90°C). The mixture was then cooled and neutralized by adding 110 µL of 10 M HCl and 290 µL 1 M phosphate buffer (pH 7). Finally, tubes were centrifuged (16,000 x g) for 5 minutes to remove cell debris. The resulting cell lysate was analyzed for total protein using a Pierce BCA Protein Assay Kit (ThermoFisher Scientific, Waltham, MA) with Bovine serum albumin (BSA) as a standard (six-point calibration, 0-250 mg/L, R^2>0.99).

5.2.4. Bioaugmentation of Hybrid Poplar to Remediate Dioxane

To evaluate using CB1190 and PH-06 to speed phytoremediation, we conducted a bench-scale hydroponic experiment in 500-mL Erlenmeyer bioreactors. Reactors were modified with a top injection port and bottom sampling port, sealed by Mininert valves (Valco Instruments Co. Inc., Houston, TX) (**Figure 3**). Each reactor was filled with 600 g of sterilized Ottawa silica sand (0.6 to 0.85 mm diameter, a proxy for a porous groundwater media) and 150 mL of sterile-filtered Hoagland's solution with a starting concentration of 10 mg/L of dioxane. While 10 mg/L is relatively high for groundwater, it was chosen because it provided more opportunity and time to observe differences between the various treatments. These treatments included: (1) planted reactors without bioaugmentation, (2) planted reactors bioaugmented with either CB1190 or PH-06, and (3) unplanted reactors bioaugmented with either CB1190 or PH-06. Glass rods (1/4 in x 10 in) were used in place of trees in unplanted reactors. Unplanted sterile controls were included to account for any unintended physical/chemical losses of dioxane. Cultures were harvested in mid- to late-exponential phase, centrifuged (5,000 x g) for 20 minutes, and triple washed with sterile 20 mM phosphate buffer. Washed cells were resuspended in Hoagland's solution, sonicated for 10 min, and homogenized using a magnetic stir bar. Reactors were bioaugmented by aliquoting resuspended cells by serological pipet. Initial optical densities (600 nm) were measured using a spectrophotometer (Hach Co., Loveland, CO) and averaged 0.077 ± 0.006 for CB1190 and 0.069 ± 0.008 for PH-06 (n=6). Using an optical density versus biomass (measured as protein) curve developed for both CB1190 and PH-06 (**Appendix A1**), the initial starting biomass was approximately 42.95 ± 1.18 mg/L for CB1190 and 59.84 ± 4.76 mg/L for PH-06. All reactors were wrapped in foil to prevent algal growth, cell death, and photolysis of dioxane.

For the duration of the experiment, reactors were placed within a reflective lined grow tent (Vivosun) under an LED grow light (ViparSpectra, Inc.) set to a 16-hour day length. Radiation intensity was measured with a quantum meter (Apogee Instruments Inc., Logan, UT) and averaged 270 µmol/M^2/day. The temperature within the grow tent averaged 23°C. Reactors were sampled

daily until day three, then every three days after that. Before sampling, reactors were weighed, and the transpired volume was replaced with sterile Hoagland's solution by syringe through the top injection port. Reactors were vigorously stirred for one minute to homogenize the solution. Samples (1 mL) were taken by syringe through the bottom sampling port, sterile filtered (0.2µm), and analyzed for dioxane by GC/MS, as described below. Headspace oxygen concentrations were monitored daily using a needle probe (OceanOptics, Inc., Largo, FL) through the top Mininert valve.

5.2.5. Statistical Analyses

Growth and degradation rate constants for growth curve experiments were estimated using logistic growth/decay model fitting. Degradation rate constants for bioaugmentation/phytoremediation experiments were calculated by fitting linear lines of best fit to log-linearized data. Statistical significance between treatments was evaluated by paired or unpaired Student's t-tests (two-tailed, 95% confidence interval) or by an extra sum-of-squares F-test (95% confidence interval). All statistical analyses were done using GraphPad Prism 8.3.0 (GraphPad Software, San Diego, California).

5.3. *R. ruber* 219 Experiments

5.3.1. Growth/Depletion Curve Experiments in Vitamin Mixtures

Known dioxane degrader, *Rhodococcus ruber* 219 (DSM #44190), was purchased from DSMZ (Braunschweig, Germany). Initially, no growth of *R. ruber* 219 was observed on dioxane in liquid cultures, as reported by (Bernhardt and Diekmann, 1991). However, rapid growth and degradation of dioxane by *R. ruber* 219 was observed in cultures supplemented with a vitamin solution (**Appendix A2**) prepared per the ATCC MD-VS™ vitamin supplement formulation (ATCC, Manassas, VA). Through testing each vitamin component of the mixture individually in AMS with 500 mg/L dioxane, thiamine (vitamin B1) was identified as the primary limiting nutrient for *R. ruber* 219. To confirm this finding, a growth/depletion curve experiment was conducted in liquid culture batch reactors (100 mL in 500 mL Erlenmeyer flask) using AMS media. Treatments included AMS with the MD-VS vitamin mixture, AMS with only thiamine (50 µg/L, per MD-VS formulation), AMS with the MD-VS vitamin mixture without thiamine, and AMS with no vitamins. All treatments had a starting dioxane concentration of 500 mg/L. All treatments were conducted in triplicate.

R. ruber 219 was pregrown in AMS with the MD-VS vitamin mixture (henceforth referred to as AMSV) and 500 mg/L dioxane. Cells were harvested in exponential phase and washed three times with 20 mM phosphate buffer. Reactors were inoculated with 1 mL (1% total culture volume) of washed cells. Flasks were incubated at 30ºC on an orbital shaker set to 150 rpm. Samples (3 mL) were taken regularly via a serological pipet and sterile filtered (0.2 µm). Subsamples were serially diluted to within the calibration range (<500 µg/L) and analyzed for dioxane by heated purge and trap GC-MS/MS. Deuterated tetrahydrofuran (THF-d8) was used as a surrogate standard to track errors caused by dilutions. In addition, each sample was analyzed for protein biomass by the method described above.

5.3.2. Kinetic Depletion Curve Experiments

To obtain kinetic rate benchmarks for dioxane degradation by *R. ruber* 219, resting cell depletion curve experiments were carried out in 2-liter Erlenmeyer flasks sealed with a 45 mm

screw cap. Cultures were pregrown on AMSV and 500 mg/L initial dioxane. Cells were harvested in exponential phase and washed three times with 20 mM phosphate buffer. Washed cells were diluted to an initial optical density (600 nm) of 1.0 (8.38 ± 0.57 mg/L protein biomass). The experiment was initiated by adding 100 mL of washed cells to 900 mL of 20 mM phosphate buffer with an initial dioxane concentration of 5 mg/L. Phosphate buffer was used instead of microbial media to ensure no growth occurred during the experiment and biomass remained constant. Flasks were incubated at 30°C on an orbital shaker set to 150 rpm. Samples (30 mL) were taken at regular intervals via serological pipet, sterile filtered (0.2 µm), and analyzed for dioxane by heated purge and trap GC-MS/MS. Monod kinetic model fitting of the resulting data was completed using Aquasim 2.0.

5.3.3. R. ruber 219 Chlorinated Solvent Inhibition Tests in Batch Reactors

To observe the effect of exposure to chlorinated solvent co-contaminants has on *R. ruber* 219, inhibition tests were carried out in 500 mL Erlenmeyer flasks sealed with a Duran 45 mm bromobutyl rubber stopper (DWK Life Sciences, Millville, NJ). *R. ruber* 219 was pregrown on AMSV and 500 mg/L initial dioxane. Cells were harvested in exponential phase and washed three times with 20 mM phosphate buffer. Washed cells were diluted to an initial optical density (600 nm) of 0.025. Washed cells (10 mL) were added to 80 mL of AMSV to a final optical density (600 nm) of 0.0025. The experiment was initiated by adding 10 mL of 20 mM phosphate buffer containing dioxane (100 µg/L) and a single chlorinated solvent co-contaminant from prepared aqueous stocks. Treatments included dioxane only and dioxane with either trichloroethylene, cis-1,2-dichloroethylene, 1,1-dichloroethylene, or 1,1,1-trichloroethane. Treatments were conducted in triplicate. Sterile controls were included to account for any unintended losses of dioxane or chlorinated solvents. Samples (40 mL) were taken by syringe, sterile filtered, and analyzed for dioxane chlorinated solvent co-contaminants by heated purge and trap GC-MS/MS.

5.3.4. Bioaugmented Phytoremediation Microcosm Batch Experiments

To evaluate *R. ruber* 219 as a bioaugmentation candidate for the poplar rhizosphere, microcosm experiments were conducted in 1-L Pyrex bottles (with and without poplar plants). Poplar cuttings (10") were affixed to 45 mm pre-drilled screw caps lined a Teflon-faced silicone septum using silicone sealant, as described above. Cuttings were pregrown for one month plastic totes filled with perlite and 20 L of half-strength Hoagland's hydroponic solution. Totes were placed under laboratory grow-lights set to a 16-hour day length.

Each microcosm contained approximately 400 mL (30 g) of perlite and 360 mL of half-strength Hoagland's solution with 100 µg/L initial dioxane. Before adding to each reactor, perlite was sifted with a #10 mesh sieve, triple washed with deionized water, and autoclaved (121°C, 15 min). Treatments included planted only, planted bioaugmented, bioaugmented with 50 µg/L thiamine, and planted bioaugmented with 50 µg/L thiamine. Sterile controls were also included to account for unintended losses of dioxane.

R. ruber 219 was pregrown on AMSV media and 500 mg/L initial dioxane. Cells were harvested in exponential phase and washed three times with 20 mM phosphate buffer. Treatment microcosms were bioaugmented 40 mL of washed cells (ten-fold dilution). The initial optical density (600 nm) in each bioaugmented reactor was 0.0015. Samples (25 mL) were taken by serological pipet, sterile filtered (0.2 µm), and analyzed for dioxane by heated purge and trap GC-MS/MS. Microcosm mass was also taken to measure transpiration. After each sample, planted reactors were backfilled to their initial mass with sterile deionized water.

5.3.5. Flow-Through Experiments to Emulate Field-Scale Conditions

Flow-through experiments were conducted to evaluate the long-term treatment of low initial dioxane (100 µg/L) by poplar trees bioaugmented with *R. ruber* 219. These experiments were conducted in five modified 3.5-L Pyrex bottles filled with 400 g coarse perlite (**Figure 4**). Perlite was selected for its high porosity, low mass, and inertness. Before adding to each reactor, perlite was sifted with a #10 mesh sieve, triple washed with deionized water, and autoclaved (121ºC, 15 min). Experimental treatments included three planted reactors and two unplanted sterile reactors. Saturated reactors averaged approximately 2500 mL of Hoagland's hydroponic solution. Reactors were placed within a reflective lined grow tent (Vivosun) under an LED grow light (ViparSpectra, Inc.) set to a 12-hour day length. The grow tent temperature averaged 25ºC, and the relative humidity averaged 51.5%. Reactors were wrapped in aluminum foil to prevent algal growth.

Poplar cuttings (10") were affixed to 45 mm pre-drilled screw caps lined a Teflon-faced silicone septum with silicone sealant, as described above. Cuttings were pregrown for two months in the perlite-filled reactors to allow for tree growth and root development. Throughout this period, reactors were continually fed with half-strength Hoagland's solution through the bottom port using a peristaltic pump (**Figure 4**). Tedlar gas sampling bags were used as an air-tight reservoir for the hydroponic solution. To ensure consistent outflow, effluent lines from each reactor were fed back through the multi-channel peristaltic pump (**Figure 4**). The effluent was then pumped through in-line sample collection vials (100 mL serum bottles sealed with chlorobutyl rubber stoppers and pierced with two hypodermic needles). Effluent from collection vials was captured in a final waste reservoir (**Figure 4**).

R. ruber 219 was pregrown for bioaugmentation in AMSV with 500 mg/L initial dioxane within 2-L Erlenmeyer flasks. Cultures were regularly fed dioxane to maximize biomass growth. Cells were harvested in exponential phase and washed three times with 20 mM phosphate buffer, and protein biomass was measured by the method described above (**Figure 5**). Prior to bioaugmentation, reactor influent was switched to sterile (0.2 µm filtered) quarter-strength Hoagland's solution containing 100 µg/L dioxane and 5 µg/L thiamine. The initial influent flow rate was set to 300 mL per day. Baseline dioxane samples (30 mL) were collected from the influent (Tedlar reservoir bags) and effluent (in-line sample vials) by sterile syringe. Samples were sterile filtered (0.2 µm) and analyzed for dioxane by heated purge and trap GC-MS/MS.

The experiment was initiated by bioaugmenting each reactor with 50 mL washed *R. ruber* 219 by serological pipet through the reactors' top opening. Following the addition of the culture, reactors were gently stirred to distribute bioaugmented cells. Samples were collected regularly by syringe and analyzed for dioxane. The difference in influent (Tedlar reservoir bags) and effluent (in-line serum bottles and waste reservoir) hydroponic solution mass was used to estimate transpiration in planted reactors. Based on effluent concentrations, several parameters were adjusted over the course of the experiment to improve system performance. Firstly, thiamine (B1-vitamin) concentrations were raised to 50 µg/L to improve the growth of *R. ruber* 219. Also, additional *R. ruber* 219 was bioaugmented to increase dioxane degradation. Finally, the influent flow rate was slowed to increase reactor hydraulic residence time.

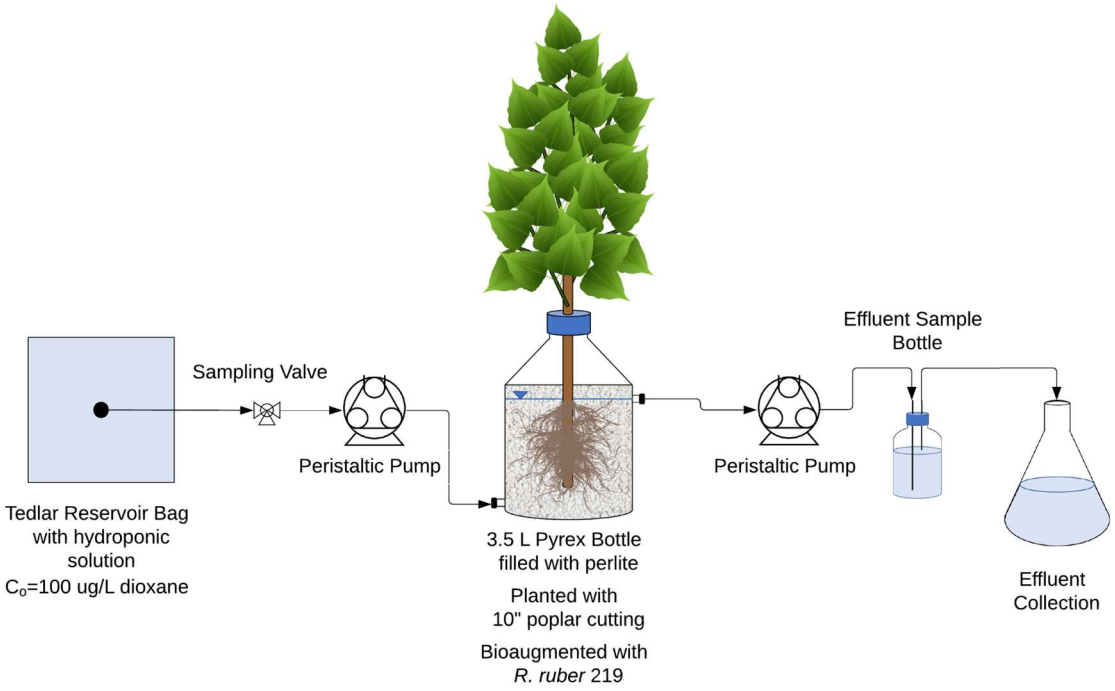


Figure 4. Flow-through experimental design conducted in modified 3.5-L Pyrex bottles filled with perlite.

Figure 5. Washed *Rhodococcus ruber* 219 used to bioaugment flow-through microcosms.

5.4. Large Scale Fermentation of Candidate Organisms

Scale-up feasibility experiments for CB1190, PH-06, and *R. ruber* 219 were conducted using a 30 L BIOSTAT® Cplus Fermenter (Sartorius, Goettingen, Germany). Before each fermentation run, strains were pregrown in AMS or AMSV (*R. ruber* 219) media with 500 mg/L initial dioxane. Fermentation runs were initiated by adding 400 mL of inoculum to 25 L of sterile AMS or AMSV (*R. ruber* 219) with a starting concentration of approximately 500 mg/L dioxane. The fermenter was set to 30ºC with 300 rpm of agitation. Antifoam 204 (MilliporeSigma, Burlington, MA) was added to control foaming. The aeration was set to 25 L per minute to maintain 20% dissolved oxygen. The pH was maintained at 6.8 using automated additions of 5N NH_4OH and 2N HCl. Culture samples were frequently monitored for changes in optical density (600 nm) and dioxane concentration, and dioxane was replenished as needed. Before sampling, agitation was increased to 700 rpm to homogenize the culture and break up clumps. Each culture was harvested by centrifugation (10,000 x g, 15 min) when the optical density (600 nm) reached 4.0, preserved by resuspending in 2 L of AMS media with 20% glycerol, and stored at -80º C.

5.5. Analytical Methods

5.5.1. Frozen Microextraction

Dioxane samples were extracted using a modified frozen microextraction (FME) method initially developed by Li et al. (2011). Filtered samples (400 µL) were mixed with 400 µl of dichloromethane (DCM) in a 2 mL screw-cap chromatography vial. 1,4-Dicholorobenzene-d4 (40 µL, 5 mg/L) was then added by a 100 µL gas-tight syringe as the surrogate standard. Samples were vortexed for 30 sec, inverted, and placed in a -40ºC freezer for 45 min. The liquid DCM was then removed by a 1 mL gas-tight syringe and transferred to a fresh 2 mL screw-cap vial with a 500 µL vial insert. Immediately preceding analysis, 40 µL of 5 mg/L 1,4-dioxane-d8 was added by a 100 µL gas-tight syringe as the internal standard. To prevent instrument contamination, dioxane samples expected to exceed 10 mg/L were serially diluted by micropipette before extraction.

5.5.2. GC-MS Analyses

Dioxane samples extracted by FME were analyzed by a GC/MS (HP 6890 GC with an HP 5973 MS) equipped with a DB-5ms column (30 m x 0.25 i.d. x 0.25 µm film thickness). Samples (2 µL) were injected into the inlet set to Pulsed Splitless mode with an inlet temperature of 200ºC and a pressure of 7.99 psi. The pulse pressure was set to 25 psi for 30 sec, followed by a purge flow of 150 mL/min at 1 min. The column flow was set to 1.1 mL/min. The oven was held initially at 38ºC for 3.5 min followed by a 75ºC/min ramp to 225ºC. The MS was operated in Selected Ion Monitoring (SIM) mode with a solvent delay of 3.5 min with an EM offset of 300. The limit of detection (LOD) for dioxane was 0.82 µg/L for the GC/MS. Due to an elevated baseline and instrument noise, a limit of quantification (LOQ) of 4 µg/L (five times higher than the LOD) was conservatively set for this instrument (**Appendix A3**).

5.5.3. GC-MS/MS Analyses

Dioxane samples extracted by FME were also analyzed by GC-MS/MS (Agilent Intuvo 9000 GC with an Agilent 7000C MS Triple Quad) equipped with an HP-5ms Ultra Inert column (30 m x 0.25 i.d. x 0.25 µm film thickness). Samples (2 µL) were injected into the inlet set to Pulsed Splitless mode with an inlet temperature of 220ºC and a pressure of 11.361 psi. The pulse

pressure was set to 25 psi for 30 sec, followed by a purge flow of 100 mL/min at 1 min. The column flow was set to 1.3 mL/min. The oven was initially held at 26ºC for 3.5 min, followed by a 100ºC/min ramp to 225ºC. The Intuvo Guard Chip was set to track the oven temperature. After each run, the oven was ramped to 280ºC and held for 2 min. The MS/MS was operated in Multiple Reaction Mode (MRM) with an EM offset of 300. The limit of detection (LOD) for dioxane was 0.11 µg/L for the GC-MS/MS (**Appendix A3**). Because of the high sensitivity with this instrument, a limit of quantification was not set as with the GC-MS.

5.5.4. Heated Purge and Trap GC-MS/MS Analyses

To streamline dioxane extractions and analyses, a heated purge and trap GC-MS/MS method for dioxane was also developed. Purge and trap settings were adapted from a method initially developed at North Carolina State University (Knappe, 2018; Sun et al., 2016). Samples were extracted from Teflon-lined 40 mL VOA vials by a Teledyne Tekmar AQUATek 100 autosampler and Lumin purge and trap concentrator equipped with a #9 trap (Teledyne Tekmar, Mason, OH). 1,4-Dioxane-d8 (2 µL) was automatically added to each 5 mL sample as an internal standard from a methanol stock by the autosampler. Complete purge and trap settings are listed in the **Appendix A4**. Extracted samples were transferred to the Agilent GC-MS/MS listed above equipped with a DB-624 Ultra Inert Inuvo column (30 m x 0.25 i.d. x 1.4 µm film thickness). The inlet was set to Split mode with an inlet temperature of 200ºC, a pressure of 14.078 psi, and a split ratio of 30:1. The column flow was set to 1.5 mL/min. The oven was held initially at 35ºC for 4 min followed by a 70ºC/min ramp to 175ºC. The Intuvo Guard Chip was set to a constant temperature of 200 ºC. The MS/MS was operated in Multiple Reaction Mode (MRM). Full MRM settings are listed in the **Appendix A5**. The limit of detection (LOD) for dioxane by this method was 0.19 µg/L (**Appendix A3**).

A second GC-MS/MS method was also developed for simultaneous analysis of dioxane trichloroethylene, cis-1,2-dichloroethylene, 1,1-dichloroethylene, and 1,1,1-trichloroethane. Purge and trap settings were the same as above. As with the dioxane-only method, extracted samples were transferred to the Agilent GC-MS/MS listed above equipped with a DB-624 Ultra Inert Inuvo column (30 m x 0.25 i.d. x 1.4 µm film thickness). The inlet was set to Split mode with an inlet temperature of 200ºC, a pressure of 14.078 psi, and a split ratio of 30:1. The column flow was set to 1.5 mL/min. The oven was held initially at 35ºC for 4 min followed by a 70ºC/min ramp to 210ºC. The Intuvo Guard Chip was set to a constant temperature of 200 ºC. The MS/MS was operated in Multiple Reaction Mode (MRM). MRM transitions for chlorinated solvents were adapted from Schulte et al. (2014) and are listed in the appendix. The limit of detection (LOD) for dioxane by this method was 0.19 µg/L (**Appendix A3**).

6. RESULTS AND DISCUSSION

6.1. Bioaugmentation Experiments with Archetype Degraders

6.1.1. Poplar Root Extract as an Auxiliary Substrate

In growth curve experiments, root extract significantly increased the total growth (measured as protein) ($p = 0.017$) and dioxane degradation ($p = 0.0047$) of CB1190 (**Figure 4**). Adding root extract also significantly increased the cell yield coefficients from 0.16 ± 0.04 mg-protein per mg-dioxane to 0.21 ± 0.03 mg-protein per mg-dioxane ($p = 0.006$) (**Table 1**). Interestingly, the addition of root extract decreased the specific degradation rate from 4.39 ± 1.20

g-dioxane per g-protein per day to 3.20 ± 0.62 g-dioxane per g-protein per day (p = 0.045), presumably due to the simultaneous utilization of root extract supplementing CB1190 growth. However, despite decreased specific degradation rates, overall degradation rates by CB1190 increased due to greater total biomass. These results align with Kelley et al. (2001), who concluded that root extract acts as an auxiliary substrate for the growth of CB1190 but does not induce dioxane monooxygenases. Previous work found that non-inducing, easily metabolized substrates can slow dioxane degradation by CB1190 due to the use of a preferred carbon source and repressed

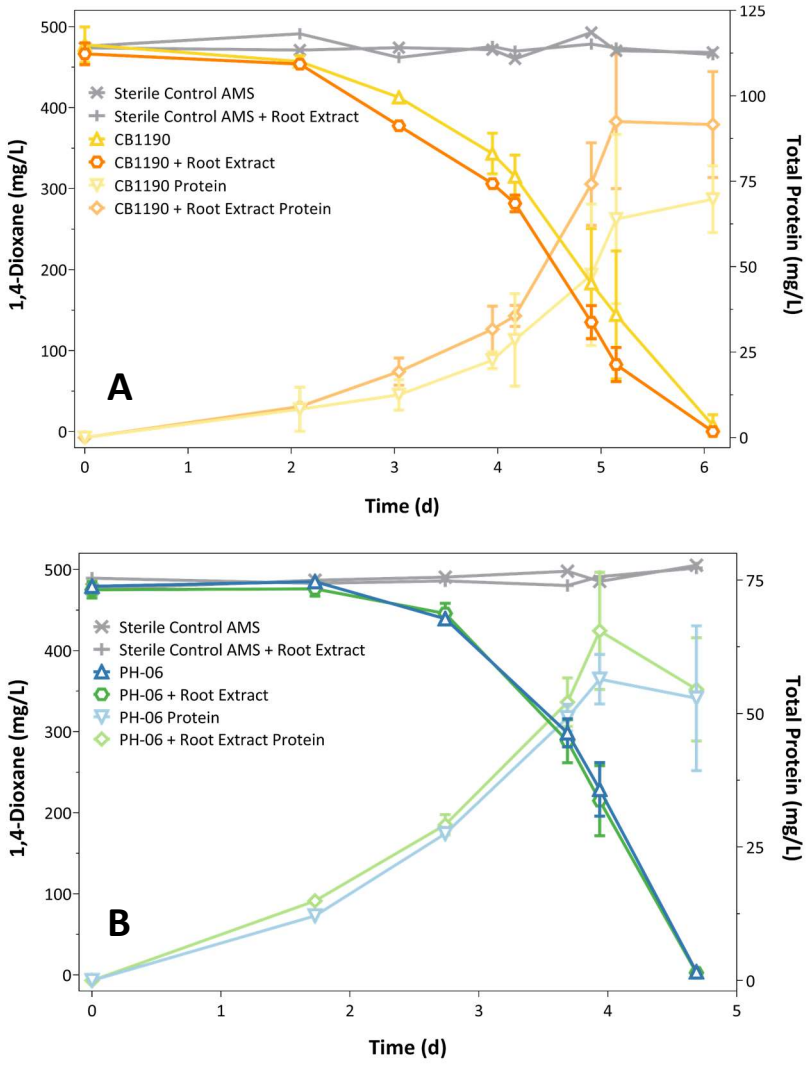


Figure 6. Bacterial growth and dioxane degradation experiments with (A) *Pseudonocardia dioxanivorans* CB1190 and (B) *Mycobacterium dioxanotrophicus* PH-06. Root extract significantly increased the total growth (measured as protein) (p = 0.017) and dioxane degradation (p = 0.0047) of CB1190. However, root extract did not significantly impact total growth (p = 0.067) or dioxane degradation (p = 0.14) of PH-06. Error bars represent the standard deviation from triplicate reactors.

induction of dioxane monooxygenases (catabolite repression) (Li et al., 2017).

The addition of root extract did not significantly affect the total growth (measured as protein) (p = 0.066) or consumption of dioxane (p = 0.14) by PH-06 (**Figure 6**). These results suggest that PH-06 does not readily utilize root extract as an auxiliary carbon source or growth

supplement. Root extract neither inhibits the specific dioxane degradation rate by PH-06, nor does it accelerate growth. Furthermore, the PH-06 dioxane degradation rate constants were significantly higher than with CB1190 for treatments without root extract ($p < 0.0001$) as well as treatments with root extract added ($p < 0.0001$) (**Table 1**). PH-06 degrades dioxane faster than CB1190 under these experimental conditions. This aligns with previous research, which also found PH-06 degrades dioxane significantly faster than CB1190 (He et al., 2018).

Table 1. Kinetic parameters from growth curve experiments with and without the addition of root extract. Plus and minus values equal the standard deviation from triplicate reactors. [a]Root extract added to medium; [b]no root extract added.

Strain	Growth Rate Constant (day^{-1})		Degradation Rate Constant (day^{-1})		Specific Degradation Rate (mg-dioxane mg-protein^{-1} day^{-1})		Cell Yield Coefficient (mg-protein mg-dioxane^{-1})	
	+ Root Extract[a]	- Root Extract[b]	+ Root Extract	- Root Extract	+ Root Extract	- Root Extract	+ Root Extract	- Root Extract
CB1190	1.78 ± 0.42	1.34 ± 0.47	1.72 ± 0.13	1.64 ± 0.21	3.20 ± 0.62	4.39 ± 1.20	0.21 ± 0.03	0.16 ± 0.04
PH-06	1.70 ± 0.43	1.68 ± 0.51	3.37 ± 0.44	3.41 ± 0.49	4.52 ± 0.55	4.54 ± 1.03	0.21 ± 0.07	0.22 ± 0.07

6.1.2. Bioaugmented Poplar Experiments

All treatments tested removed 10 mg/L initial dioxane to below the LOQ of 4 µg/L (**Figure 7**). In planted experiments, non-bioaugmented poplar trees removed 10 mg/L initial dioxane to below 4 µg/L in 29 days (**Figure 7**, **Table 2**). Dioxane removal followed first-order kinetics due to a directly proportional relationship between the transpiration rate and the rate of dioxane removal (**Appendix A6**). This agrees with previous work that found that the majority (76.5 ± 3.9%) of dioxane removed by poplar trees was transpired through the leaves (Aitchison et al., 2000). Also, the transpiration stream concentration factor (TSCF) for dioxane was approximately 1.0, suggesting dioxane moved freely across the root membrane and did not become concentrated in the bulk fluid (**Appendix A7**). This TSCF value agrees with previous estimates, which range from 0.72 to 0.98 (Aitchison et al., 2000; Dettenmaier et al., 2008; Ferro et al., 2013).

In bioaugmented planted experiments, CB1190 significantly enhanced bioremediation of dioxane by hybrid poplar (22 days vs. 29 days, p-value = 0.0017) (**Figure 7, Table 2**). However, CB1190 in unplanted experiments removed dioxane significantly faster than planted treatments (19 days vs. 22 days, p-value = 0.014). One explanation for this unexpected result is that dioxane degradation by CB1190 was slowed by the consumption of poplar root exudates, as observed in root extract-amended growth curve experiments (**Figure 6**). In contrast, PH-06-bioaugmented poplars significantly outpaced all other treatments tested ($p < 0.05$), remediating dioxane to <4 µg/L in only 13 days (**Figure 7, Table 2**). As PH-06 was not affected by the presence of root extract in growth curve experiments (**Figure 5**), we postulate that this increased rate is due to additive mechanisms between degradation by PH-06 and uptake by the plant. Unexpectedly, PH-06 in unplanted reactors was significantly slower than all other bioaugmented treatments (p-value = 0.035), reaching non-detect levels in 29 days. Headspace oxygen remained above 19% across all treatments and was not limiting. Also, transpiration rates were not significantly different (p-value > 0.05) (**Table 2**).

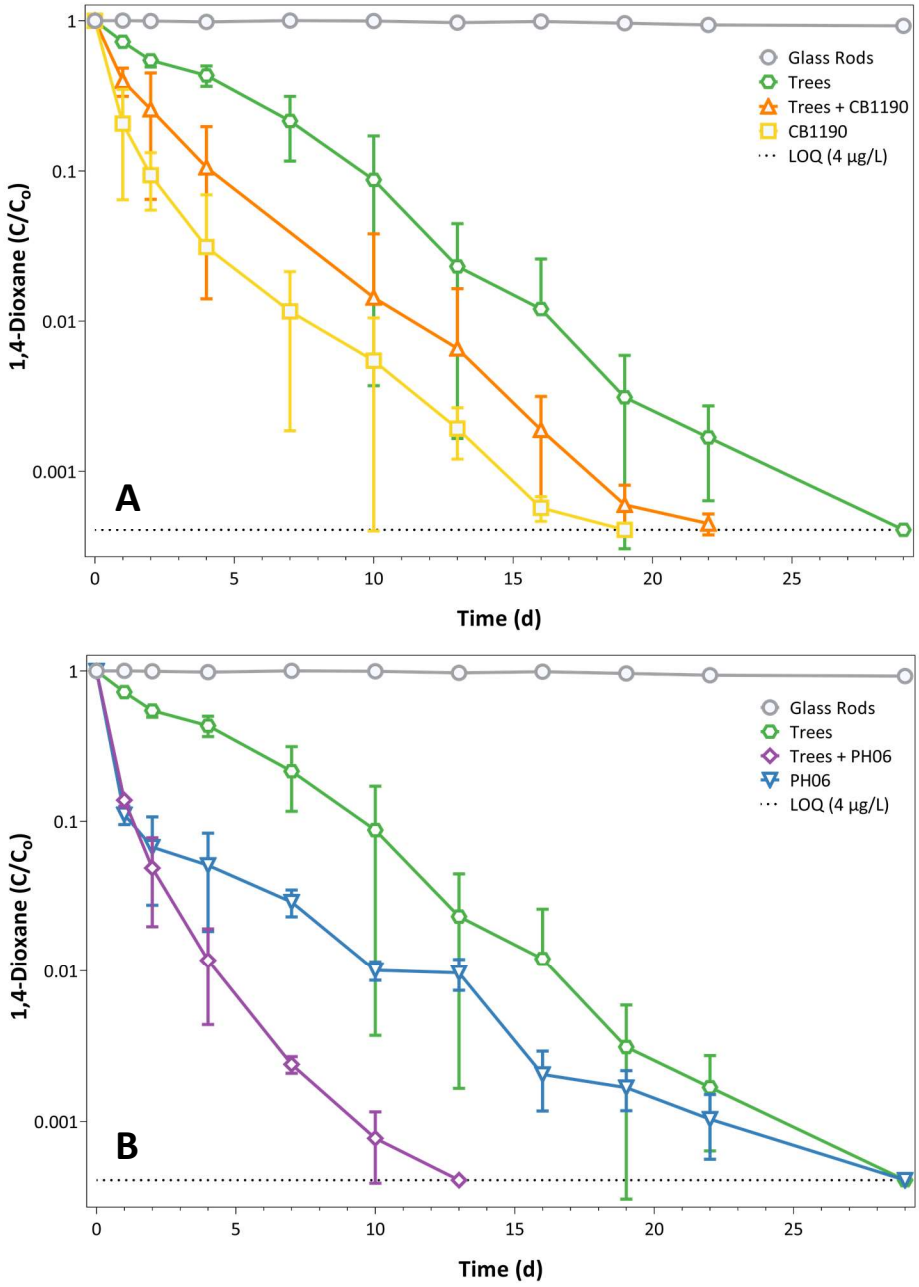


Figure 7. Planted bioaugmentation experiments conducted in modified Erlenmeyer bioreactors inoculated with either *Pseudonocardia dioxanivorans* CB1190 (A) or *Mycobacterium dioxanotrophicus* PH-06 (B). While all treatments reached the limit of quantification (4 µg/L), trees bioaugmented with PH-06 significantly outpaced all other treatments tested ($p < 0.05$). However, CB1190 in unplanted experiments removed dioxane significantly faster than planted treatments ($p = 0.014$). Error bars represent the standard deviation from triplicate reactors. C_o = 10 mg/L dioxane.

Table 2. Planted bioaugmentation experiments conducted in modified Erlenmeyer bioreactors inoculated with either *Pseudonocardia dioxanivorans* CB1190 or *Mycobacterium dioxanotrophicus* PH-06. Trees bioaugmented with PH-06 significantly outpaced all other reactors ($p < 0.05$). The transpiration rate did not significantly differ between treatments ($p > 0.05$). Error-values represent the standard deviation of triplicate reactors.

Treatment	Degradation Rate Constant (day^{-1})	Transpiration Rate ($mL\ day^{-1}$)
Trees Only	0.29 ± 0.013	32.81 ± 2.53
CB1190	0.37 ± 0.034	N/A
Trees + CB1190	0.34 ± 0.031	25.72 ± 9.64
PH-06	0.23 ± 0.015	N/A
Trees + PH-06	0.56 ± 0.046	27.87 ± 3.50

For bioaugmented poplar experiments, calculations were done to estimate the fraction of total removal performed by each mechanism (degradation by bacteria or plant uptake). The TSCF equation was used to calculate the amount of dioxane removed due to transpiration. The assumption was made that any remaining removal was due to degradation by bioaugmented strains in the rhizosphere (calculated by difference). These fractions were used to calculate cumulative dioxane removal by each process (**Figure 8**). It was estimated that CB1190 removed 79.3% ± 5.9%, while trees removed 20.6% ± 5.9%. Similarly, PH-06 removed an estimated 81.8% ± 4.3% of total dioxane compared to 18.2% ± 4.3% removed by trees. Detailed calculations can be found in SI. As seen in Figure 6, bioaugmented strains initially dominated removal for both CB1190 and PH-06. This was likely caused by low transpiration during the first 48 hours of the experiment, while the trees were adjusting to being planted in bioreactors (**Appendix A8**). While transpiration did increase and stabilize after the first 48 hours, the cumulative dioxane removed by trees did not exceed ~20% because the majority of dioxane had already been degraded by bioaugmented strains.

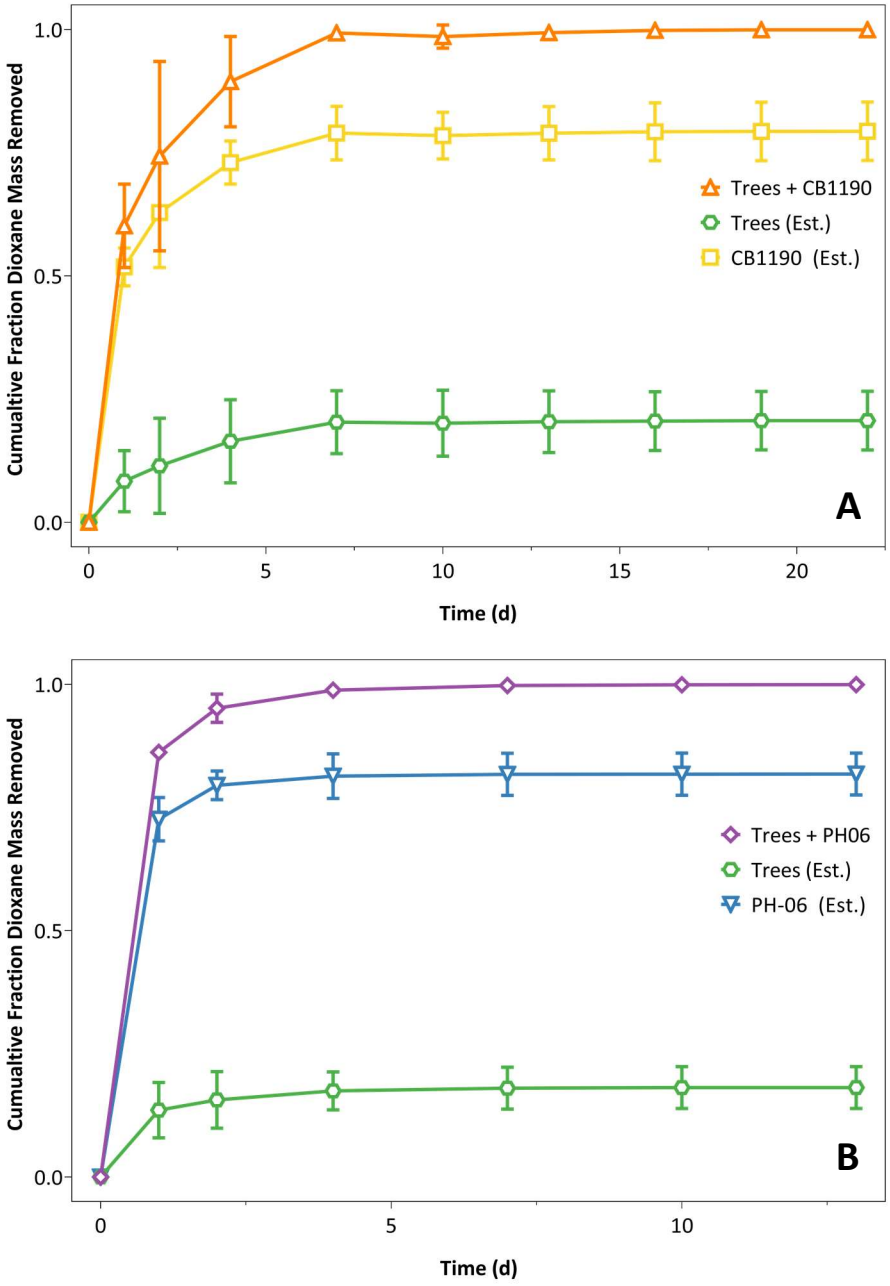


Figure 8. Estimated cumulative removal of dioxane by either bioaugmented strains or by plant uptake in planted bioaugmentation experiments. Reactors were bioaugmented with either *Pseudonocardia dioxanivorans* CB1190 (A) or *Mycobacterium dioxanotrophicus* PH-06 (B). CB1190 removed 79.3% ± 5.9% while trees removed 20.6% ± 5.9%. PH-06 removed 81.8% ± 4.3% of total dioxane compared to 18.2% ± 4.3% removed by trees. Error bars represent the standard deviation from triplicate reactors. C_o = 10 mg/L dioxane.

6.2. *R. ruber* 219 Experiments

6.2.1. Growth/Depletion Curve Experiments in Vitamin Mixtures

As seen in **Figure 9**, *R. ruber* 219 rapidly grew and consumed dioxane in cultures containing the MD-VS vitamin mixture, reducing dioxane concentrations by more than 99% in six days. In addition, while significantly slower (p-value = 0.0353), cultures containing only thiamine closely mirrored both the full MD-VS vitamin mixture's growth and depletion rates. Furthermore, no growth was observed in treatments containing the MD-VS mixture without thiamine or in cultures with no vitamins. Neither growth nor dioxane removal was observed in these cultures after 34 days (data not shown). This experiment demonstrates that *R. ruber* 219 rapidly degrades dioxane when B-vitamins are added to the media. Also, this experiment confirms that thiamine is the primary limiting co-factor for dioxane degradation by *R. ruber* 219.

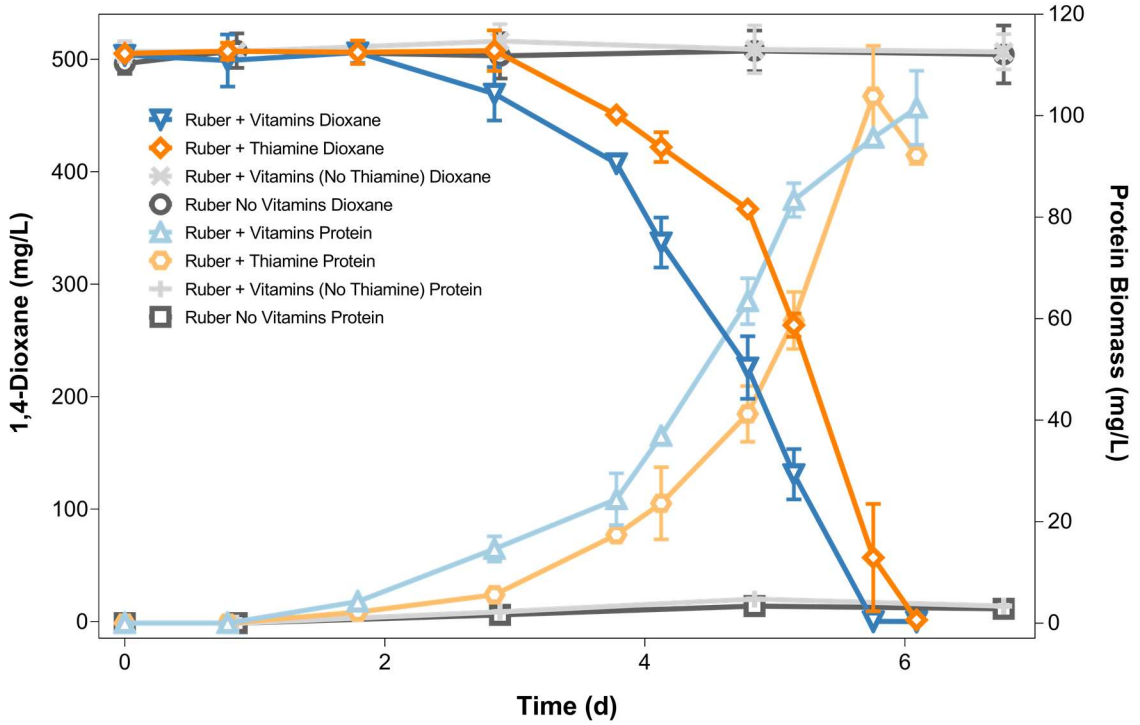


Figure 9. *R. ruber* DSM-44190 growth and depletion curve with and without the addition of B-vitamin mixtures. Experiment conducted in triplicate with ± 1 standard deviation error bars.

6.2.2. Kinetic Depletion Curve Experiments

As seen in Figure 10, *R. ruber* 219 pre-grown with B-vitamins rapidly degraded dioxane in batch reactors to below 0.19 µg/L in less than 6 hours. The resulting Monod kinetic parameters can be seen in **Table 3**. The maximum degradation rate (q_{max}) for *R. ruber* 219 was similar to archetype degrader CB1190. However, in *R. ruber* 219 experiments, the half-saturation constant (Ks) was calculated to be 0.015 mg/L 1,4-dioxane, significantly lower than both CB1190 and PH-06 (**Table 3**). This low Ks suggests that *R. ruber* DSM 219 can continually grow and degrade dioxane despite low dioxane concentrations (100 ppb or less), unlike CB1190 and PH-06, which can stall when exposed to such concentrations (Table 3).

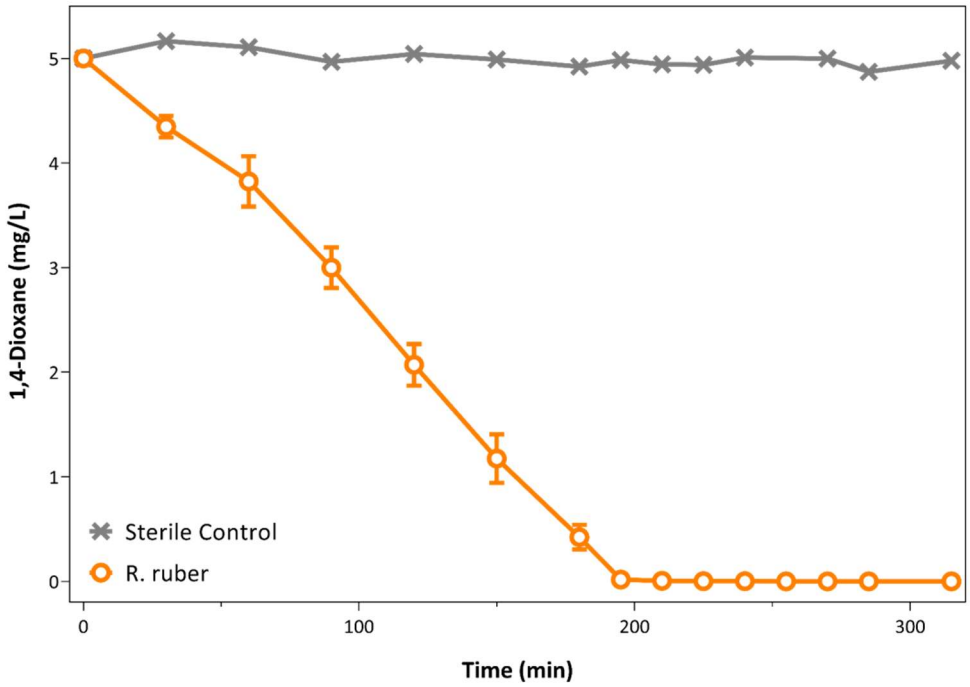


Figure 10. *R. ruber* DSM 219 depletion curve conducted to obtain kinetic degradation parameters. Experiment conducted in triplicate with ± 1 standard deviation error bars.

Table 3. Kinetic parameters of select metabolic dioxane-degrading bacteria. Values calculated using Aquasim 2.0, courtesy of Patrick Richards (University of Iowa).

Strain	q_{max} (mg 1,4-dioxane/mg protein/day)	Ks (mg 1,4-dioxane /L)	Reference
Pseudonocardia dioxanivorans CB1190	4.1 ± 0.14	6.3 ± 0.22	(Barajas-Rodriguez and Freedman, 2018)
Pseudonocardia dioxanivorans CB1190	26 ± 0.19	160 ± 44	(Mahendra and Alvarez-Cohen, 2006)
Mycobacterium dioxanotrophicus PH-06	Not reported	78 ± 10	(He et al., 2017a)
***Rhodococcus ruber* 219**	**4.8 ± 0.31**	**0.015 ± 0.065**	**This Work**

6.2.3. R. ruber 219 Chlorinated Solvent Inhibition Tests in Batch Reactors

Due to difficulty with the miscibility of chlorinated solvents in aqueous stocks, initial solvent concentrations differed from the target of 500 µg/L. These initial concentrations can be seen in Table 4. Despite this variability, all solvent concentrations are consistent with what is commonly found at dilute dioxane plumes at contaminated groundwater sites.

As seen in Figure 11, *R. ruber* 219 rapidly degraded dioxane to <1 µg/L in 46 hours in all treatments except in flasks with 1,1-dichloroethylene (1,1-DCE). This result suggests that 1,1-

DCE inhibits dioxane degradation by *R. ruber* 219. This compound is known to also inhibit dioxane degradation by CB1190 (Zhang et al., 2016). It was determined in the former study that 1,1-DCE reduced ATP production and dioxane-degrading enzyme expression in CB1190. However, further work is needed to determine the root cause of inhibition of *R. ruber* 219 by 1,1-DCE. Furthermore, it is unclear how increasing solvent concentration may also impact *R. ruber* 219. Nevertheless, phytoremediation has also been shown as an effective treatment for chlorinated solvents, allowing for the treatment of comingled plumes (Schnoor, 2002). All lines of evidence indicate that bioaugmentation with *R. ruber* 219 in tandem with phytoremediation will allow for simultaneous treatment of 1,4-dioxane and chlorinated solvent co-contaminants.

Table 4. Chlorinated solvent concentrations measured in inhibition experiments.

Compound	Average Concentration (µg/L)
TCE	37.38 ± 1.86
cDCE	329.11 ± 20.78
1,1-DCE	45.17 ± 1.79
1,1,1-TCA	15.65 ± 0.856

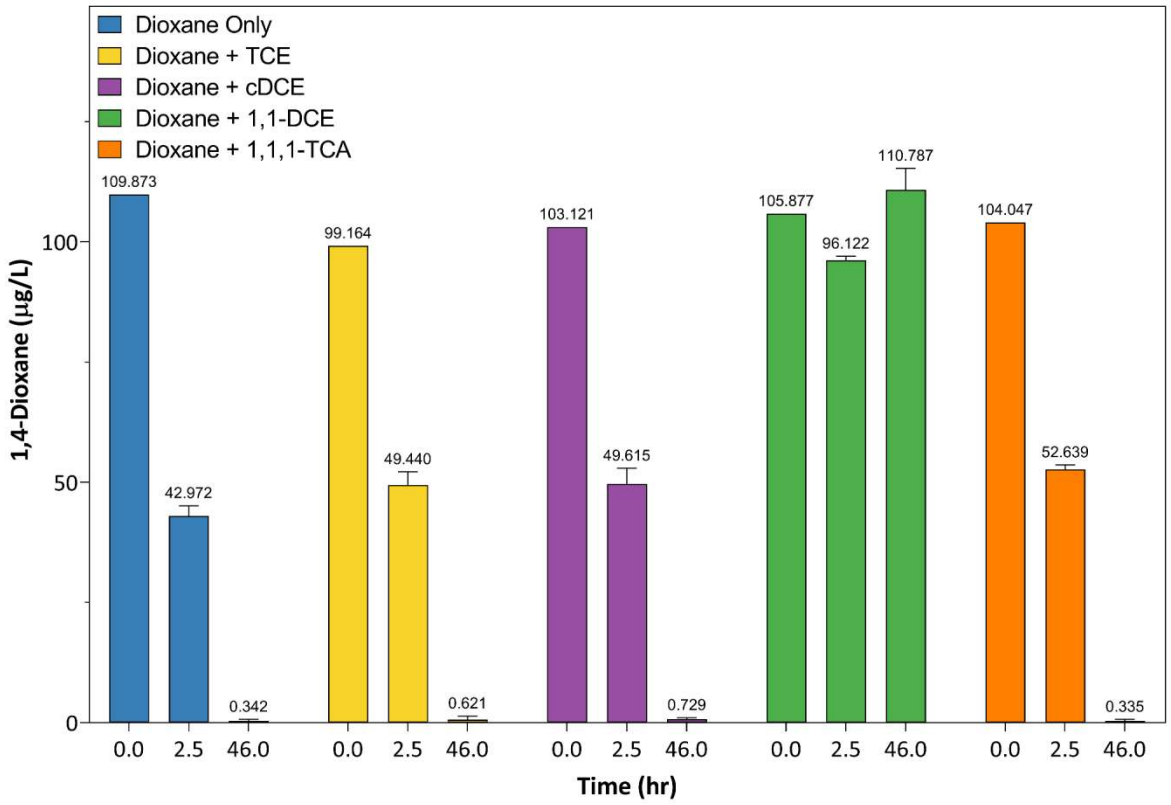


Figure 11. Dioxane degradation by *R. ruber* 219 measured over time in chlorinated solvent inhibition tests. Numbers above bars indicate the average dioxane concentration in µg/L. Experiment conducted in triplicate with ± 1 standard deviation error bars.

6.2.4. Bioaugmented Phytoremediation Microcosm Batch Experiments

As seen in Figure 12, all treatments bioaugmented with *R. ruber* 219 degraded 100 µg/L initial dioxane to below 1 µg/L in 18 days. This was despite an extremely low initial biomass (OD_{600} = 0.0015). Non-bioaugmented trees also removed approximately 30% of the initial

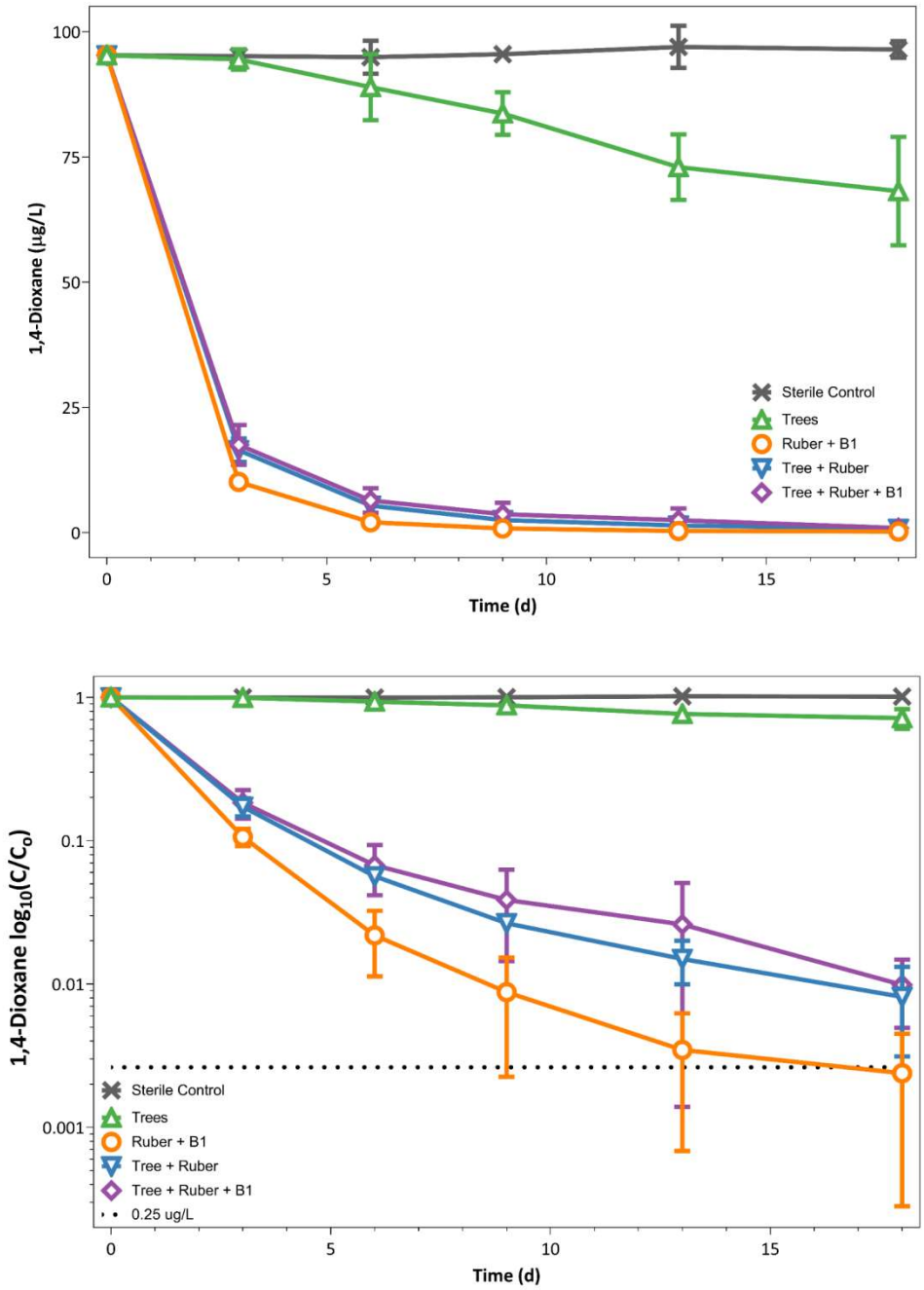


Figure 12. Planted microcosm experiments conducted in 1-liter glass bottles filled with perlite and bioaugmented with *Rhodococcus ruber* 219. Top: dioxane concentrations graphed over time; Bottom: log-transformed dioxane concentrations graphed over time. Experiment conducted in triplicate with ± 1 standard deviation error bars.

dioxane, presumably through transpiration. No significant difference was found between *R. ruber*-bioaugmented planted and unplanted treatments (p-value > 0.05). Curiously, the lack of thiamine did not limit the degradation of dioxane in this experiment. We hypothesize that *R. ruber* could have assimilated sufficient thiamine from the liquid culture media to consume the low concentration of dioxane during this short experiment completely. However, plants are known to release thiamine in root exudates (Curl and Truelove, 1986). Thus, another hypothesis is that poplars exude sufficient thiamine to support dioxane degradation by *R. ruber* 219. Ongoing research aims to elucidate this relationship.

6.2.5. Flow-Through Experiments to Emulate Field-Scale Conditions

As seen in Figure 13, bioaugmenting flow-through microcosms with *R. ruber* 219 significantly increased continual treatment of influent 100 µg/L dioxane. During the experiment, several adjustments were made to improve treatment performance. On day 10, the thiamine concentration was increased from 5 µg/L to 50 µg/L in the influent hydroponic solution. This increase in thiamine improved dioxane treatment in unplanted reactors, but no change was observed in planted treatments.

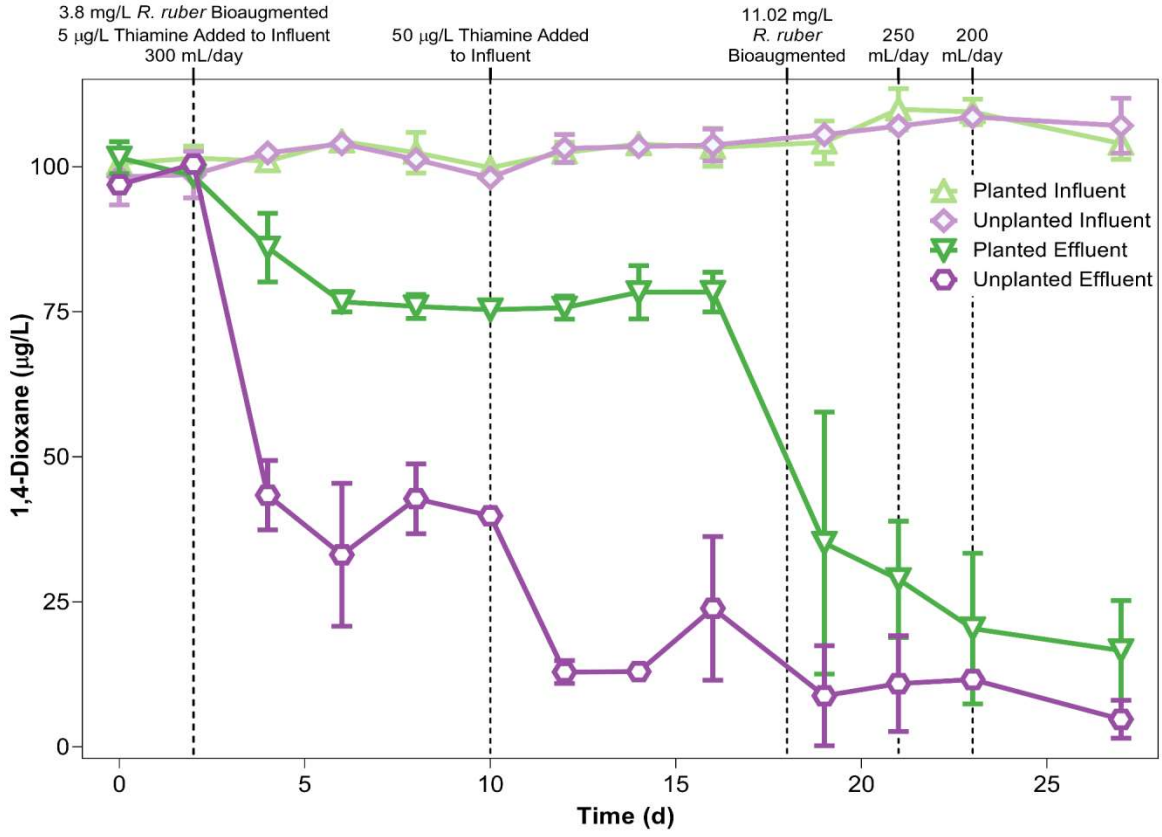


Figure 13. Planted flow-through experiments conducted in modified 3.5-liter glass bottles and bioaugmented with *Rhodococcus ruber* 219. Vertical dashed lines and top labels describe parameter changes made during the experiment to improve performance. Error bars indicate ± 1 standard deviation of replicates.

On day 18, additional *R. ruber* 219 (50 mL of 550 mg/L protein, approximately 11.02 mg/L final protein concentration in each reactor) was bioaugmented into all reactors. This, coupled with slowed flow rates (250 mL/d on 21 and 200 mL/d on day 23), caused a significant decrease in effluent dioxane concentrations in both planted and unplanted microcosms. Surprisingly, unplanted bioaugmented reactors outperformed planted bioaugmented treatments, although both achieved low effluent concentrations of dioxane (16.6 µg/L in planted reactors, 4.8 µg/L in unplanted reactors at day 27). One hypothesis for this phenomenon is dual substrate utilization (dioxane and root exudates) by *R. ruber* 219, as seen with CB1190 above, limiting dioxane consumption. An alternative hypothesis is that native rhizosphere microbes were competing with *R. ruber* for various macronutrients. Also, pressures from protozoans and other predators may have influenced dioxane degradation by *R. ruber* in planted reactors. Such pressures were not a factor in unplanted treatments, as these reactors were autoclaved before experimentation.

The findings of this study are especially field-relevant. In this experiment, *R. ruber* was able to sustain treatment of low initial dioxane concentrations (100 µg/L) commonly encountered in the field to near health advisory levels. Ongoing work aims to optimize this system to reach <1 µg/L dioxane.

6.3. Strain Scale-Up Production

As previously discussed, producing bioaugmentation strains in sufficiently high quantities is a major limiting factor for successful field implementation (Stroo et al., 2012). In production runs conducted in 30 L fermenters, we confirmed that both CB1190, PH-06, and *R. ruber* 219 could be grown in large quantities (**Figure 14**). CB1190 was harvested after 14 days, yielding 425 g of biomass. In contrast, PH-06 required more additions of dioxane, 18 days to reach a similar optical density, and only yielded 350 g of biomass. *R. ruber* 219 reached an OD of 4 in 15 days and had an OD of 5 at the time of harvest. However, the harvested biomass totaled only 90 g. Further work is needed to confirm what caused this discrepancy.

Previous work by Kelley et al. (2001) used tetrahydrofuran (THF) as a growth substrate during the fermentation of CB1190. THF is a structural analog of dioxane that CB1190 can use as a primary growth substrate while still inducing dioxane degrading monooxygenases. CB1190 grows much faster on THF than dioxane (11 hr vs. 30 hr doubling time) (Parales et al., 1994). Because of this increased growth rate, Kelley et al. were able to grow CB1190 to a higher optical density than observed in the current study (OD of 13.6 in only 13 days vs. OD of 4.0 in 14 days). However, due to THF's high volatility relative to dioxane (vapor pressure of 114 mm Hg for THF vs. 38.1 mm Hg for dioxane) and associated health risks, dioxane was chosen as the primary growth substrate for this study. Alternatively, future work could also grow strains on 1,4-butanediol, a non-toxic substrate that also induces dioxane-degrading enzymes (Inoue et al., 2018).

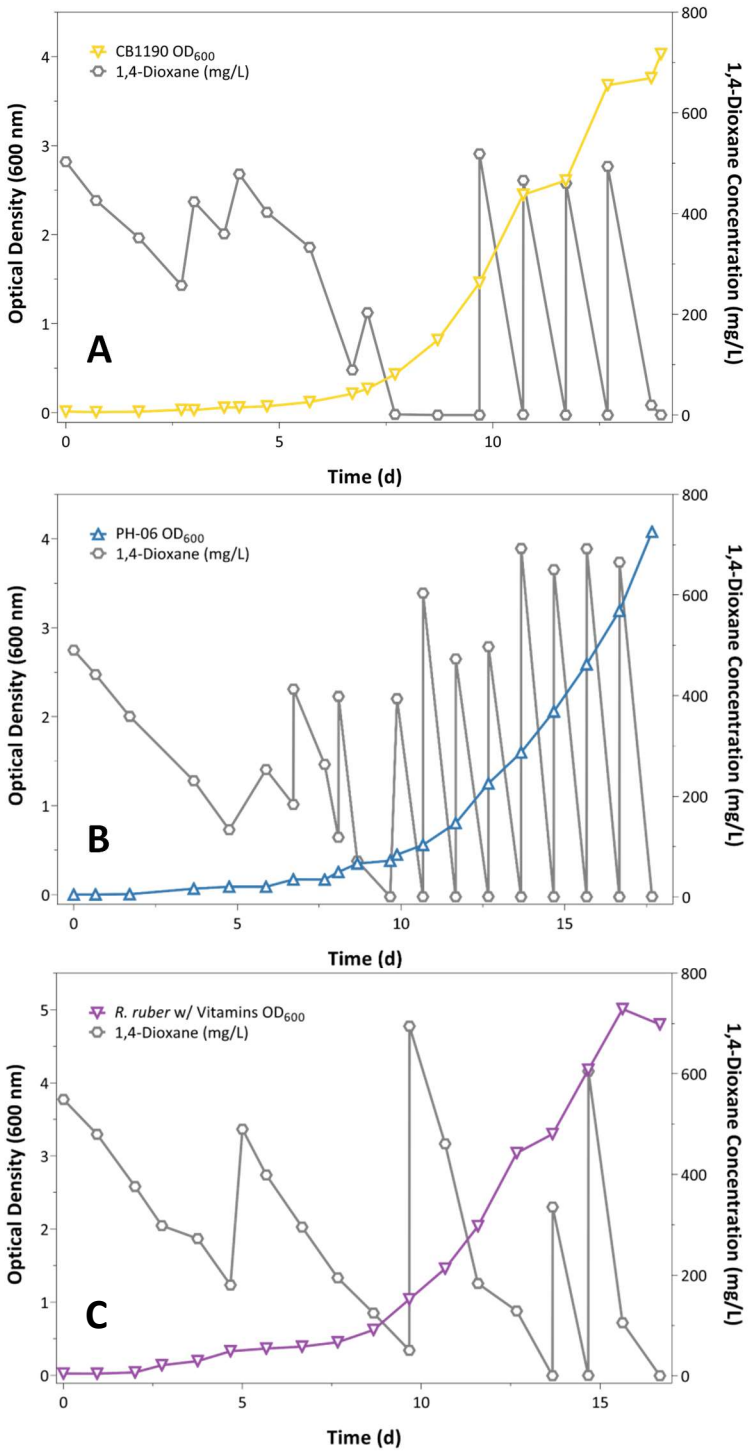


Figure 14. Fermentation runs using (A) *Pseudonocardia dioxanivorans* CB1190, (B) *Mycobacterium dioxanotrophicus* PH-06, and (C) *Rhodococcus ruber* 219 (with B-vitamins). Dioxane was replaced as needed. CB1190 reached an optical density of 4 in 14 days, while PH-06 needed 18 days to reach a similar optical density. *R. ruber* reached an optical density of 4 in 15 days.

7. CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH/IMPLEMENTATION

Progress made during ER-2719 has been conclusive. Our work with archetype degraders, *Mycobacterium dioxanotrophicus* PH-06 or *Pseudonocardia dioxanivorans* CB1190, explained the energetics of dual substrate utilization, 1,4-dioxane plus root extract, which is critical when bioaugmentation is used in tandem with phytoremediation. Our team was the first to report that PH-06 cannot utilize root extract as primary substrates and confirmed that CB1190 can. We were also the first to demonstrate that PH-06-bioaugmented-poplar significantly outperformed poplar bioaugmented with CB1190. PH-06 was uninhibited by root extract, making the strain a strong candidate to speed phytoremediation of dioxane. However, it is possible that CB1190 would perform better in the field due to its capacity to utilize root extract and outcompete indigenous microorganisms. Finally, we have confirmed in 30-L fermentation runs that both CB1190 and PH-06 can be grown to the large quantities needed for field implementation.

We have also identified *R. ruber* 219 as a very strong candidate for field implementation. In our experiments, we demonstrated that this strain, with the addition of thiamine, can grow on low dioxane concentrations (<100 µg/L) without auxiliary substrates. Furthermore, we have demonstrated that despite extremely low initial biomass concentrations, *R. ruber* 219 can degrade 100 µg/L to below 0.35 µg/L health advisory level. This is the first metabolic dioxane-degrading bacteria reported in the literature able to sustain degradation under such dilute dioxane conditions. However, we have observed that dioxane degradation by *R. ruber* 219 slows somewhat in the presence of plant roots. We hypothesize this is due to dual substrate utilization, 1,4-dioxane and root exudates, as seen with CB1190. We have also identified 1,1-DCE as inhibitory for dioxane degradation by *R. ruber* 219. Ongoing work aims to explore if bioaugmentation with *R. ruber* 219 in tandem with phytoremediation can overcome inhibition by 1,1,-DCE. Furthermore, poplar trees may release sufficient thiamine in root exudates, reducing the need for vitamin amendments. Finally, as with CB1190 and PH-06, we have confirmed that *R. ruber* 219 can be grown to sufficient quantities for field bioaugmentation.

This project demonstrated that combining phytoremediation with bioaugmentation is a promising treatment alternative for dioxane-contaminated groundwater to achieve low concentrations (<0.35 µg/L) as recommended by health advisories. Dioxane-metabolizing microbes have been utilized, eliminating the need for auxiliary substrates required by cometabolic microorganisms. In the coming years, we will conduct a pilot-scale demonstration at the Twin Cities Army Ammunition Plant (TCAAP) in Minneapolis, MN, under ESTCP Project ER21-5096, titled "Bioaugmented Phytoremediation to Treat 1,4-Dioxane Contaminated Groundwater." We believe this study will validate that bioaugmented phytoremediation is an effective treatment strategy for dilute dioxane plumes. While challenges remain, the successful implementation of this strategy offers a green and cost-effective solution to a widespread problem of national and international importance.

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Zhang, S., Gedalanga, P.B., Mahendra, S. Biodegradation Kinetics of 1,4-Dioxane in Chlorinated Solvent Mixtures. Environ Sci Technol 2016; 50: 9599-607 DOI: 10.1021/acs.est.6b02797.

Zhang, S., Gedalanga, P.B., Mahendra, S. Advances in bioremediation of 1,4-dioxane-contaminated waters. J Environ Manage 2017; 204: 765-774 DOI: 10.1016/j.jenvman.2017.05.033.

9. APPENDICES

Appendix A: Supporting Information

Appendix A1: Correlation between Optical Density and Protein for CB1190 and PH-06

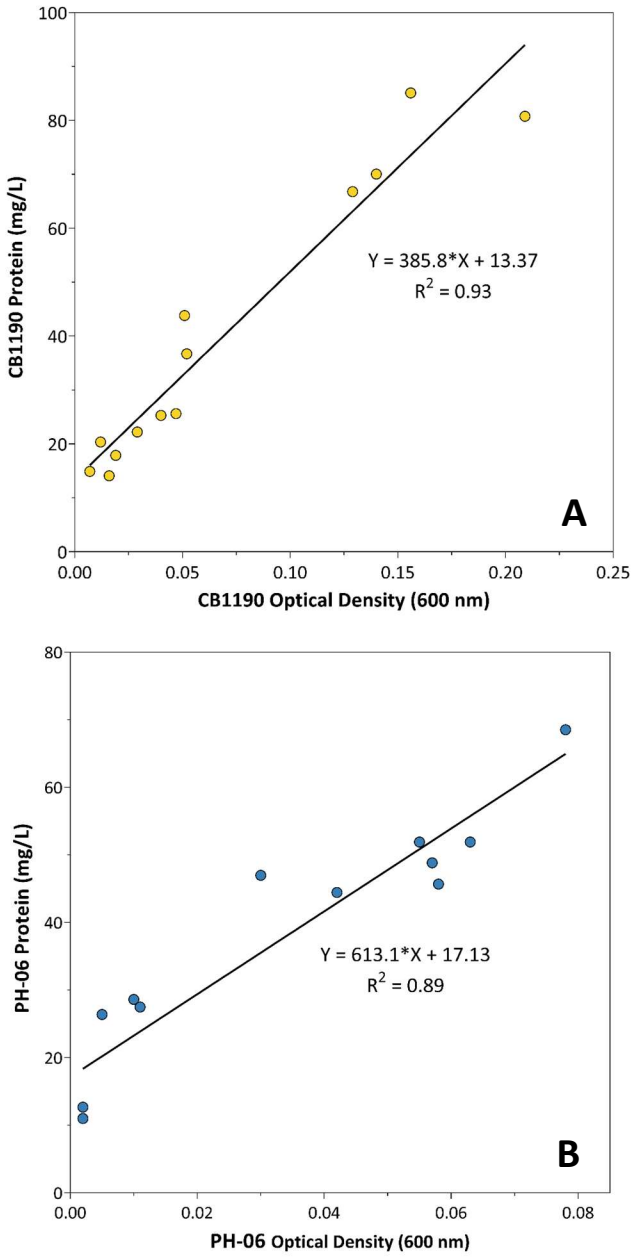

Figure A1. Correlation between optical density (600 nm) and biomass measured as protein for *Pseudonocardia dioxanivorans* CB1190 (A) and *Mycobacterium dioxanotrophicus* PH-06 (B). Strains grown in Ammonium Mineral Salts media with C_o =500 mg/L dioxane. Reactors were sonicated for 10 minutes prior to sampling.

Appendix A2: Vitamin Supplement Formula

Table A1. ATCC MD-VS™ vitamin supplement formulation based on Wolfe's vitamin solution (ATCC, Manassas, VA). Prepared as 100x stock in 20 mM phosphate buffer (ph = 6.8) for laboratory experiments.

Component	**Concentration**
Folic Acid	2.0 mg/L
Pyridoxine hydrochloride	10.0 mg/L
Riboflavin	5.0 mg/L
Biotin	2.0 mg/L
Thiamine	5.0 mg/L
Nicotinic acid	5.0 mg/L
Calcium Pantothenate	5.0 mg/L
Vitamin B12	0.1 mg/L
p-Aminobenzoic acid	5.0 mg/L
Thioctic acid	5.0 mg/L

Appendix A3: Limit of Detection Calculations

The limits of detection (LOD) were determined by measuring the standard deviation of seven replicate samples multiplied by 3.14 (Student's t value for 99% confidence interval, n-1 degrees of freedom) (USEPA, 2016).

Appendix A4: Purge and Trap Settings

Table A2. Teledyne Tekmar AquaTek 100 and Lumin Concentrator settings for analysis of 1,4-dioxane and chlorinated solvents. Adapted from Knappe (2018) and Sun et al. (2016).

Setting Name	**Setting**	**Setting Name**	**Setting**
Valve Oven Temp	180ºC	Desorb Time	1.00 min
Transfer Line Temp	180 ºC	Drain Flow	300 mL/min
Sample Mount Temp	90 ºC	Bake Time	6 min
Purge Ready Tempp	40 ºC	Bake Temp	280 ºC
MCS Purge Temp	20 ºC	MCS Bake Temp	200 ºC
Purge Temp	20 ºC	Bake Flow	300 mL/min
Purge Time	12 min	Sample Loop Time	0.35 min
Purge Flow	40 mL/min	Sample Transfer Time	0.35 min
Dry Purge Flow	100 mL/min	Rinse Loop Time	1.00 min
Sample Temp	60ºC	Sweep Needle Time	1.00 min
Pre-Purge Time	0.50 min	Presweep Time	0.25 min
Pre-Purge Flow	40 mL/min	Water Temp	90 ºC
Preheat Time	2.00 min	Bake Rinse Cycles	3
Desorb Preheat Temp	245 ºC	Bake Rinse Drain Time	0.35

Appendix A5: Ion Acquisition Information

Table A3. Ion acquisition information for GC/MS analyses of frozen micro-extracted samples

Compound	SIM Ions (m/z)
1,4-Dioxane	88, 58
1,4-Dioxane-d_8	96, 64
1,4-Dichlorobenzene-d_4	115

Table A4. Ion acquisition information for GC/MS/MS analyses of frozen micro-extracted samples

Compound	MRM Transitions (m/z)
1,4-Dioxane	88 → 58
1,4-Dioxane-d_8	96 → 64
1,4-Dichlorobenzene-d_4	115

Table A5. Ion acquisition information for GC/MS/MS analyses extracted by heated purge and trap. Adapted from (Schulte et al., 2014).

Compound	Transition 1 (m/z)	Transition 2 (m/z)
1,4-Dioxane	88 → 57	88→ 43
1,4-Dioxane-d_8	96 → 64	96→62
1,4-Dichlorobenzene-d_4	115→78	
1,1-Dichloroethene	96→61	98→63
cis-Dichloroethene	96→61	98→63
1,1,1-Trichloroethane	97→61	99→63
Trichloroethene	130→95	132→97

Appendix A6: Relationship Between Dioxane Removal and Transpiration in Non-Bioaugmented Planted Experiments

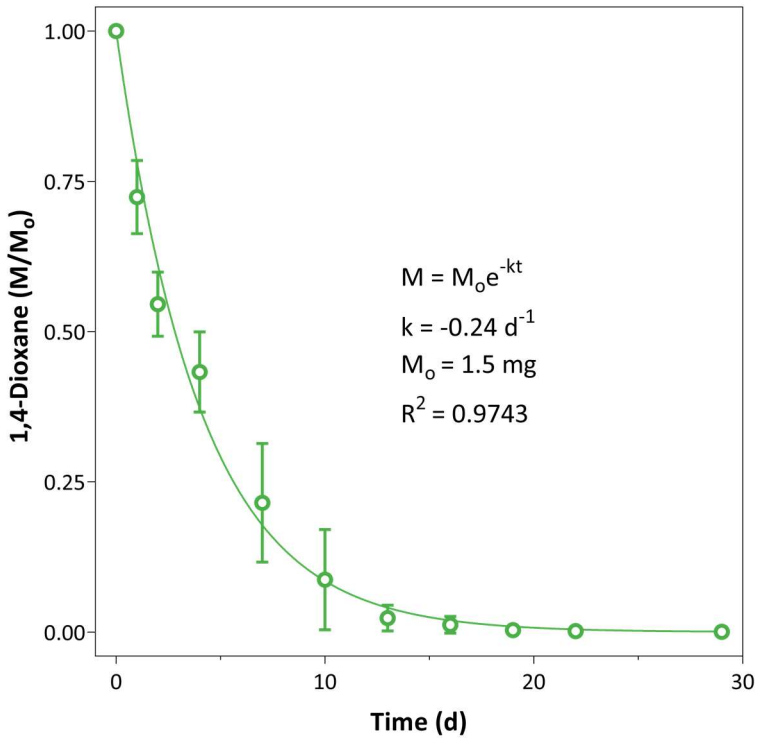

Figure A2. Dioxane mass removal vs. time by hybrid poplar in non-bioaugmented planted experiments. Dioxane removal followed first-order kinetics due to a directly proportional relationship between the transpiration rate and the change in concentration. Error bars represent the standard deviation from triplicate reactors.

Appendix A7: Transpiration Stream Concentration Factor in Non-bioaugmented Planted Experiments

Equation 6: $TSCF = \frac{k*V}{T}$

Where:

- TSCF = Transpiration Stream Concentration Factor (dimensionless)
- k = Rate constant for dioxane removal (d^{-1}) = 0.24 d^{-1} (from Figure S5)
- V = Volume of solution in flask (mL) = 150 mL (total volume in bioaugmentation experiment)
- T = Average transpiration rate (mL/d) = 32.815 mL/d (from change in mass during bioaugmentation experiment)

Plugging into Equation 6:

$$TSCF = \frac{0.24\ \mathrm{d^{-1}} * 150\ \mathrm{mL}}{32.815\ \mathrm{mL/d}} = 1.097 \approx 1.0$$

Appendix A8: Estimated Dioxane Fraction Removed by Each Mechanism in Bioaugmented Planted Reactors

From Above:

- $TSCF \approx 1$ (From above)
- $V = 150\ mL$ (total volume in bioaugmentation experiment)
- T = transpired volume over sampling interval (mL per day)

Plugging into Equation 6:

Equation 7: $k = \frac{1*T}{150\ mL} = \frac{T}{150\ mL}$

Dioxane fraction removed by each mechanism:

Equation 8: $\Delta M_{Total,t} = \Delta M_{trees,t} + \Delta M_{bacteria,t}$

Where:

- $\Delta M_{Total,t}$ = change in total dioxane mass over time, t
- $\Delta M_{trees,t}$ = change in mass due to plant uptake over time, t

- $\Delta M_{bacteria,t}$ = change in mass due to degradation by bacteria over time, t

Dioxane fraction removed by plant uptake:

Combining Equations 7 and 8:

$$\text{Equation 9:} \quad \Delta M_{trees,t} = \Delta M_{Total,t} * \frac{T}{150\ mL}$$

Fraction removed by bacterial degradation:

Combining Equations 8 and 9:

$$\text{Equation 10:} \quad \Delta M_{bacteria,t} = \Delta M_{Total,t} - (\Delta M_{Total,t} * \frac{T}{150\ mL})$$

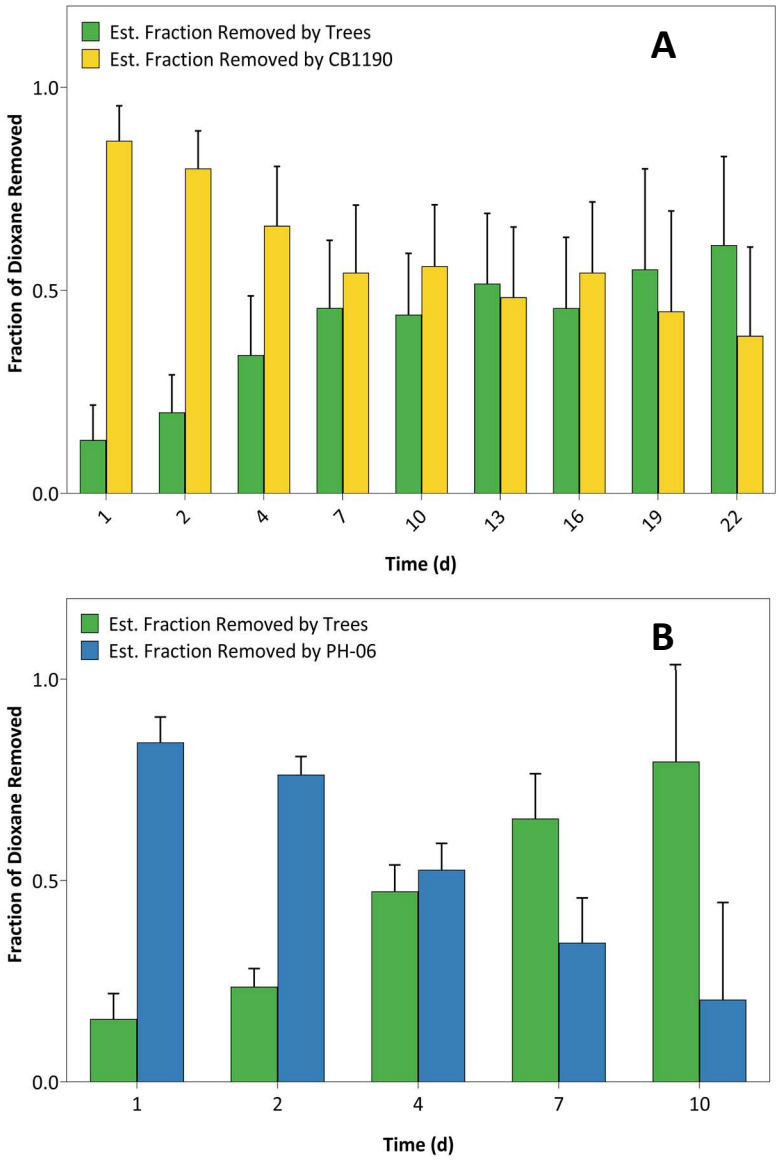

Figure A3. Estimated fraction of dioxane removed at each time period in planted bioaugmentation experiments. Reactors were bioaugmented with either *Pseudonocardia dioxanivorans* CB1190 (A) or *Mycobacterium dioxanotrophicus* PH-06 (B). Error bars represent the standard deviation from triplicate reactors.

Appendix A9: Strain Biosafety Classification

CB1190, PH-06, and *R. ruber* 219 were declared as biosafety level 1 (BSL-1) by University of Iowa Biosafety Officers for our use. This agrees with the American Type Culture Collection's (ATCC) biosafety designation for CB1190 and previous work by He et al. (2017a), who found no evidence of pathogenicity in the genome of PH-06.

Appendix B: Scientific/Technical Publications

Simmer, R., Mathieu, J., da Silva, M. L., Lashmit, P., Gopishetty, S., Alvarez, P. J., & Schnoor, J. L. (2020). Bioaugmenting the poplar rhizosphere to enhance treatment of 1, 4-dioxane. *Science of the Total Environment*, 744, 140823.

Simmer, R., Richards, da Silva, M. L., P., Mathieu, J., Alvarez, P. J., & Schnoor, J. L. (2021). Rapid metabolism of 1,4-dioxane to below health advisory levels by *Rhodococcus ruber* strain 219, a thiamine auxotroph. *Environmental Science & Technology Letters.* In Review.

Simmer, R., Richards, P., Ewald, J., Mathieu, J., Alvarez, P. J., & Schnoor, J. L. (2021). Complete genome sequence of *Rhodococcus ruber* 219, a rapid metabolizer of 1,4-dioxane. Manuscript in progress.

Simmer, R., Richards, P., Mathieu, J., Alvarez, P. J., & Schnoor, J. L. (2021). Bioaugmented phytoremediation with *Rhodococcus ruber* 219 to treat 1,4-dioxane contaminated groundwater. Manuscript in progress.

Appendix C: Patents

Simmer, R. (2021) Vitamin supplements speed the metabolism of 1,4-dioxane by *Rhodococcus ruber* 219. Patent application in progress.