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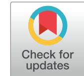
Bioaugmenting the poplar rhizosphere to enhance treatment of 1,4-dioxane



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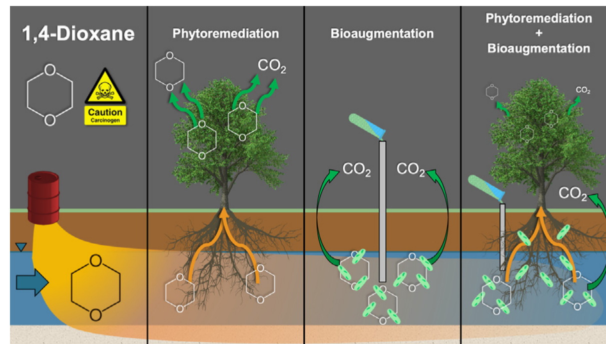
HIGHLIGHTS

- 1,4-Dioxane is a mobile groundwater pollutant that threatens human health.
- Phytoremediation with poplar removed 1,4-dioxane to low concentrations (~1 μg/L).
- Adding dioxane-degrading microorganisms accelerated dioxane removal by poplar.
- Select dioxane-degraders can utilize poplar root extract as an auxiliary substrate.
- Dioxane-degraders were grown in fermenters for field-scale implementation.

GRAPHICAL ABSTRACT



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ABSTRACT

1,4-Dioxane is a highly mobile and persistent groundwater pollutant that often forms large dilute plumes. Because of this, utilizing aggressive pump-and-treat and ex-situ technologies such as advanced oxidation can be prohibitively expensive. In this study, we bioaugmented the poplar rhizosphere with dioxane-degrading bacteria *Mycobacterium dioxanotrophicus* PH-06 or *Pseudonocardia dioxanivorans* CB1190 to enhance treatment of 1,4-dioxane in bench-scale experiments. All treatments tested removed 10 mg/L dioxane to near health advisory levels (<4 μg/L). However, PH-06-bioaugmented poplar significantly outperformed all other treatments, reaching <4 μg/L in only 13 days. Growth curve experiments confirmed 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. In addition, we confirmed that CB1190 could utilize both 1,4-dioxane and root extract as substrates. Finally, we demonstrated the large-scale production of these two strains for use in the field. Overall, this study shows that combining phytoremediation and bioaugmentation is an attractive strategy to treat dioxane-contaminated groundwater to low risk-based concentrations (~1 μg/L).

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1. Introduction

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

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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, 2017). 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, 2017). While no enforceable federal guidelines for dioxane have currently been established, regulations have been proposed based on the assessment that 0.35 μg/L dioxane in drinking water represents a 1×10^{-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, 2017).

Dioxane's prevalence as a contaminant (Fig. 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 ($K_H = 2.0 \times 10^{-4}$ 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).

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 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 (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 h) (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,

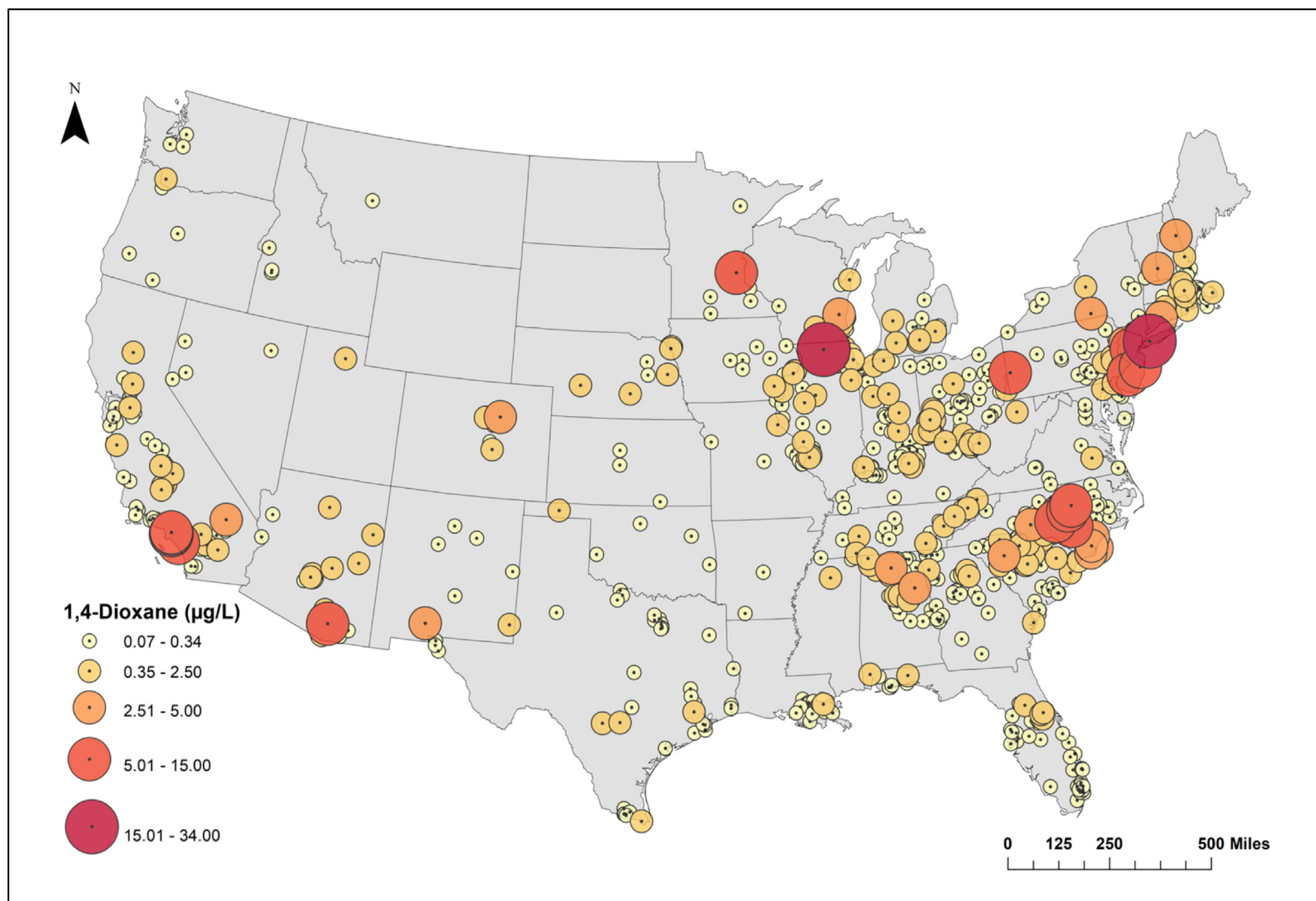

Fig. 1. Locations with 1,4-dioxane concentrations in finished drinking water above Mandatory Reporting Limit (MRL) of 0.07 μg/L at Public Water Systems, 2013–2015. Data obtained from US EPA Unregulated Contaminant Monitoring Rule 3 (UCMR 3) (USEPA, 2017). Map made using ArcGIS 10.4.1 (ESRI, Redlands,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

ethylene glycol, a common co-contaminant of dioxane, has been shown to reduce 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 (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., 2011; Schnoor et al., 1995). Finally, 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).

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.

In this study, we compare the performance of CB1190 to another promising bioaugmentation candidate, *Mycobacterium dioxanotrophicus* PH-06 (Kim et al., 2009). This metabolic dioxane-degrading bacterium has been shown to degrade dioxane faster than CB1190 in both high (500 mg/L) and low (300 μg/L) starting dioxane concentrations (He et al., 2018). Using bench-scale experiments, we examine the stimulatory effect poplar root extract has on growth and dioxane degradation by CB1190 and PH-06. Also, we test the ability of these two strains to enhance dioxane treatment through the bioaugmentation of hybrid poplar. We hypothesize that PH-06 will utilize root extract as an auxiliary carbon source, as previously observed with CB1190. We also hypothesize that PH-06 will outperform CB1190 in accelerating the removal of dioxane by poplar to low concentrations. Finally, we demonstrate that like CB1190, PH-06 can be grown to sufficient quantities for field bioaugmentation. We believe that this series of experiments will help solidify bioaugmented phytoremediation as an accepted treatment technology for dioxane-contaminated groundwater.

2. Methods

2.1. Chemicals

ACS grade 1,4-dioxane (anhydrous, >99.9%) and 1,4-dichlorobenzene-d_4 (2000 μg/mL in methylene chloride) were purchased from MilliporeSigma, Burlington, MA. 1,4-Dioxane (2000 μg/mL in methylene chloride) and 1,4-dioxane-d_8 (2000 μg/mL in P&T methanol) were purchased from Restek Corporation, Bellefonte, PA. Methylene chloride (≥99.9%, GC Resolv) was purchased from Fisher Scientific, Hampton, NH.

2.2. 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 × 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 (Figs. 2, 3). All buds were removed below the cap to prevent shoot growth within the reactor. Cuttings were grown in opaque plastic bins (25″ × 18″ × 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-h 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.

2.3. 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

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

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).

2.4. 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 min 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 ×g) for 5 min 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, $R^2 > 0.99$).

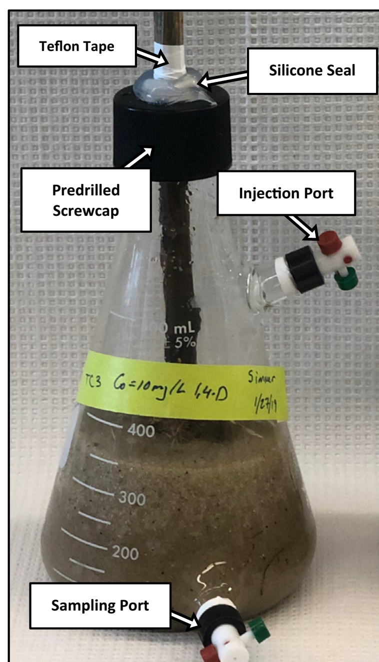

Fig. 3. Hybrid poplar in a modified Erlenmeyer bioreactor.

2.5. 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) (Fig. 2). 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 × 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 (5000 ×g) for 20 min, 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 (Figure SI1), 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-h 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 1 min to homogenize the solution. Samples (1 mL) were taken by syringe through the bottom sampling port, sterile filtered (0.2 μm), 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.

2.6. Strain scale-up production

Scale-up feasibility experiments for CB1190 and PH-06 were conducted using a 30 L BIOSTAT® Cplus Fermenter (Sartorius, Goettingen, Germany). Before each fermentation run, strains were pregrown in AMS media with 500 mg/L initial dioxane. Fermentation runs were initiated by adding 400 mL of inoculum to 25 L of sterile AMS with a starting concentration of approximately 500 mg/L dioxane. The fermenter was set to 30 °C 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 5 N NH_4OH and 2 N 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 ×*g*, 15 min) when the optical density reached 4.0, preserved by resuspending in 2 L of AMS media with 20% glycerol, and stored at −80 °C.

2.7. Analytical methods

Dioxane samples were extracted using a modified frozen microextraction 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 s, inverted, and placed in a −40 °C 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-d_8 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.

Dioxane samples were analyzed by either a GC/MS (HP 6890 GC with an HP 5973 MS) or GC/MS/MS (Agilent Intuvo 9000 GC with an Agilent 7000C MS Triple Quad). The GC/MS was equipped with a DB-5 ms column (30 m × 0.25 i.d. × 0.25 μm film thickness). Samples (2 μL) were injected into the inlet set to Pulsed Splitless mode with an inlet temperature of 200 °C and a pressure of 7.99 psi. The pulse pressure was set to 25 psi for 30 s, 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 38 °C for 3.5 min followed by a 75 °C/min ramp to 225 °C. 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 GC/MS/MS was equipped with an HP-5 ms Ultra Inert column (30 m × 0.25 i.d. × 0.25 μm film thickness). Samples (2 μL) were injected into the inlet set to Pulsed Splitless mode with an inlet temperature of 220 °C and a pressure of 11.361 psi. The pulse pressure was set to 25 psi for 30 s, 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 held initially at 26 °C for 3.5 min followed by a 100 °C/min ramp to 225 °C. The Intuvo Guard Chip was set to track the oven temperature. After each run, the oven was ramped to 280 °C and held for 2 min. The MS/MS was operated in Multiple Reaction Mode (MRM) with an EM offset of 300.

Limits of quantification (LOQ) for dioxane were determined to be 4 μg/L for the GC/MS and 0.55 μg/L for the GC/MS/MS. Calibration curves and reference standards (400 μL clean DCM with surrogate and internal standards) were used to calculate sample recovery. For added quality assurance, each sample was split, extracted, and analyzed in parallel. Final results were averaged between parallel samples. Instrument results were analyzed using Masshunter Qualitative Software B.08.00 (Agilent Technologies, Inc., Santa Clara, CA). Ion acquisition information, as well as LOQ and sample recovery calculations, and can be found in SI.

2.8. Statistical analyses

All treatments were conducted in triplicate. 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).

3. Results and discussion

3.1. 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 (Fig. 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 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 (Fig. 4). 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

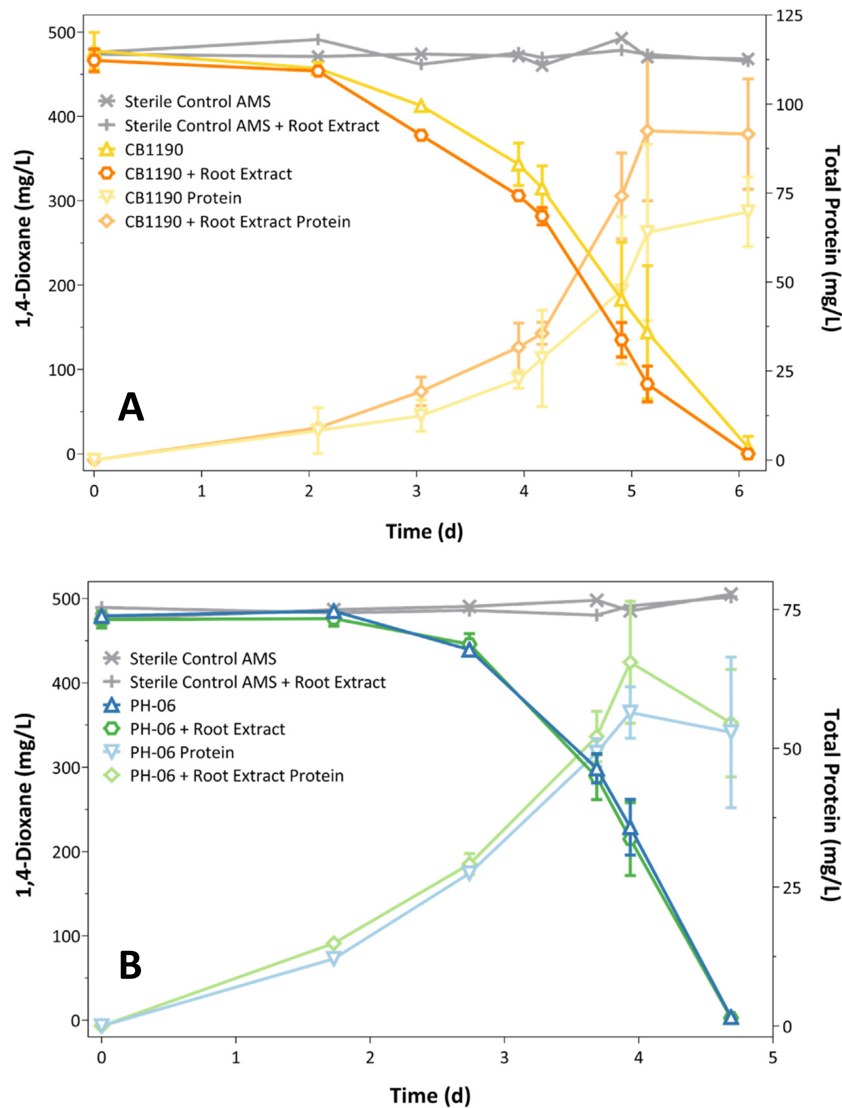

Fig. 4. Bacterial growth and dioxane degradation experiments with (A) *Pseudonocardia dioxanivorans* CB1190 and (B) *Mycobacterium dioxanotrophicus* PH-06. Root extract significantly increased the total growth (measured as protein) ($p = 0.017$) and dioxane degradation ($p = 0.0047$) of CB1190. However, 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.

conditions. This aligns with previous research, which also found PH-06 degrades dioxane significantly faster than CB1190 (He et al., 2018).

3.2. Bioaugmented poplar experiments

All treatments tested removed 10 mg/L initial dioxane to below the LOQ of 4 μg/L (Fig. 5). In planted experiments, non-bioaugmented poplar trees removed 10 mg/L initial dioxane to below 4 μg/L in 29 days (Fig. 5, Table 2). Dioxane removal followed first-order kinetics due to a directly proportional relationship between the transpiration rate and the rate of dioxane removal (Fig. S5). 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 (SI). 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.

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[a]	− Root[b]	+ Root	− Root	+ Root	− Root	+ Root	− Root
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	3.365 ± 0.44	3.412 ± 0.49	4.52 ± 0.55	4.54 ± 1.03	0.21 ± 0.07	0.22 ± 0.07

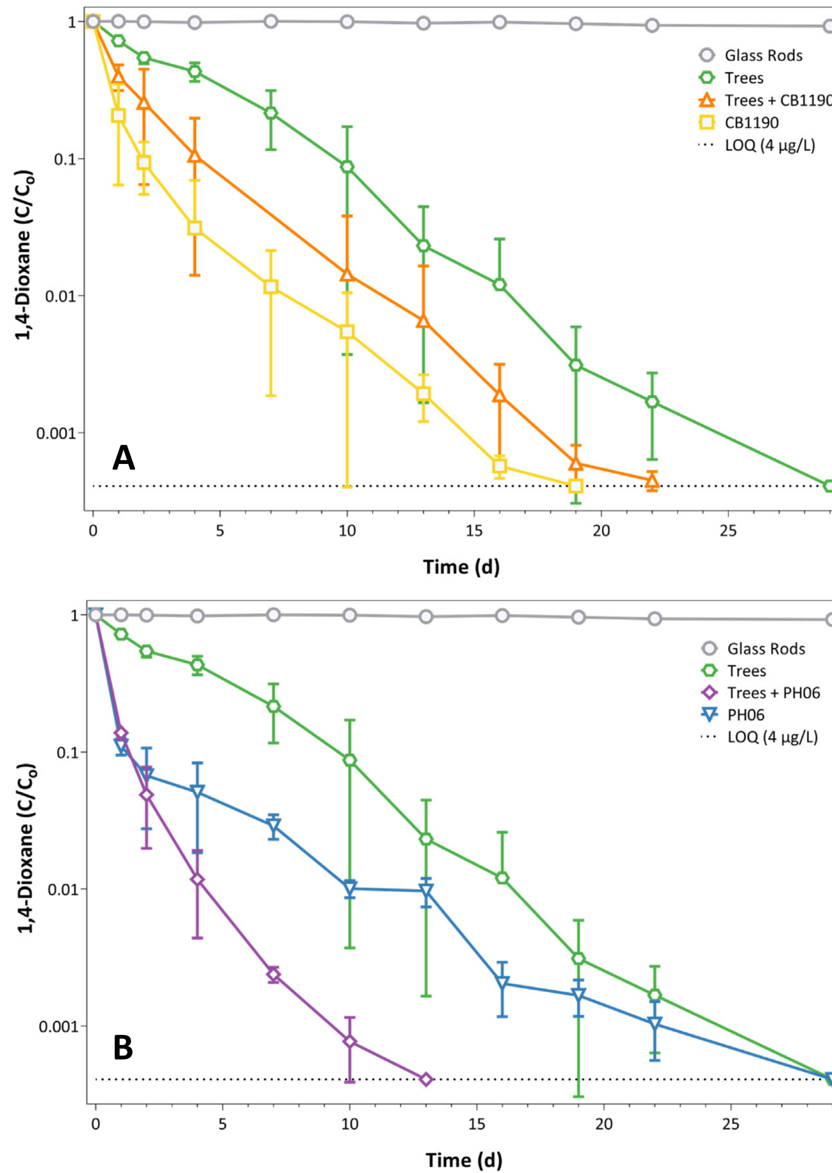

Fig. 5. Planted bioaugmentation experiments conducted in modified Erlenmeyer bioreactors inoculated with either *Pseudonocardia dioxanivorans* CB1190 (A) or *Mycobacterium dioxanotrophicus* PH-06 (B). While all treatments reached the limit of quantification (4 μg/L), trees bioaugmented with PH-06 significantly 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.

29 days, p-value $= 0.0017$) (Fig. 5, 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 (Fig. 4). 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 (Fig. 5, Table 2). As PH-06 was not affected by the presence of root extract in growth curve experiments (Fig. 4), 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 between planted treatments ($p > 0.05$) (Table 2).

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 (Fig. 6). 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 Fig. 6, bioaugmented strains initially dominated removal for both CB1190 and PH-06. This was likely caused by low transpiration during the first 48 h of the experiment, while the trees were adjusting to being planted in bioreactors (Figure SI6). While transpiration did increase and stabilize after the first 48 h, the cumulative dioxane removed by trees did not exceed ~20% because the majority of dioxane had already been degraded by bioaugmented strains.

3.3. 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 and PH-06 can be grown to large quantities (Fig. 7). 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. Furthermore, CB1190 had a much higher cell yield coefficient than PH-06 (4.12 g-biomass per g-dioxane consumed vs. 2.34 g-biomass per g-dioxane consumed). This disagrees with the cell yield coefficients observed during growth curve experiments, where PH-06 exceeded CB1190 (Table 1). CB1190's higher cell yield during fermentation may have been due to the strain's tendency to clump during growth. As a result, considerable cell aggregate accumulated on the walls of the fermenter, reducing the amount of cell density in the bulk fluid. Thus, the optical density measured for this culture may have been artificially low and not a representative measure of the total biomass at the time of harvest.

Previous work by Kelley et al. (2001) used tetrahydrofuran (THF) as a growth substrate during the fermentation of CB1190. THF is a

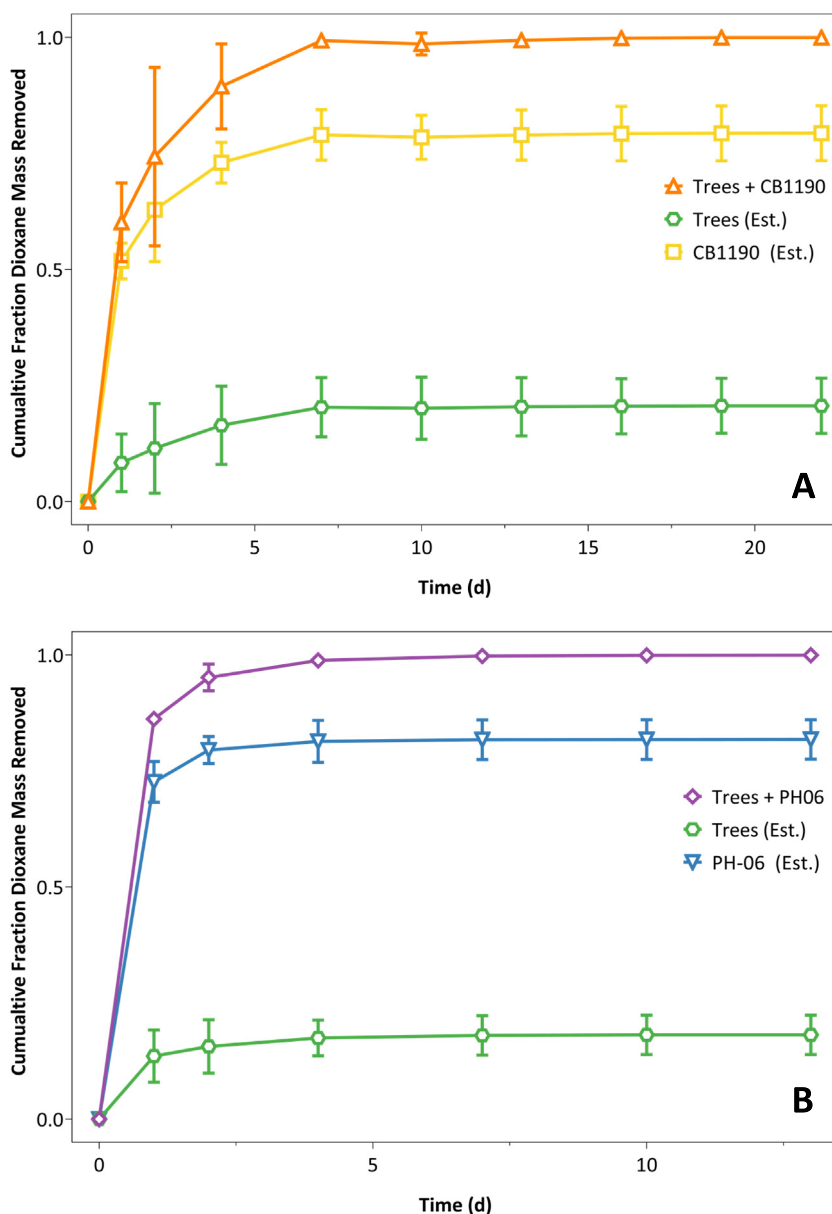

Fig. 6. Estimated cumulative removal of dioxane by either bioaugmented strains or by plant uptake in planted bioaugmentation experiments. Reactors were bioaugmented with either *Pseudonocardia dioxanivorans* CB1190 (A) or *Mycobacterium dioxanotrophicus* PH-06 (B). CB1190 removed 79.3% ± 5.9% while trees 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.

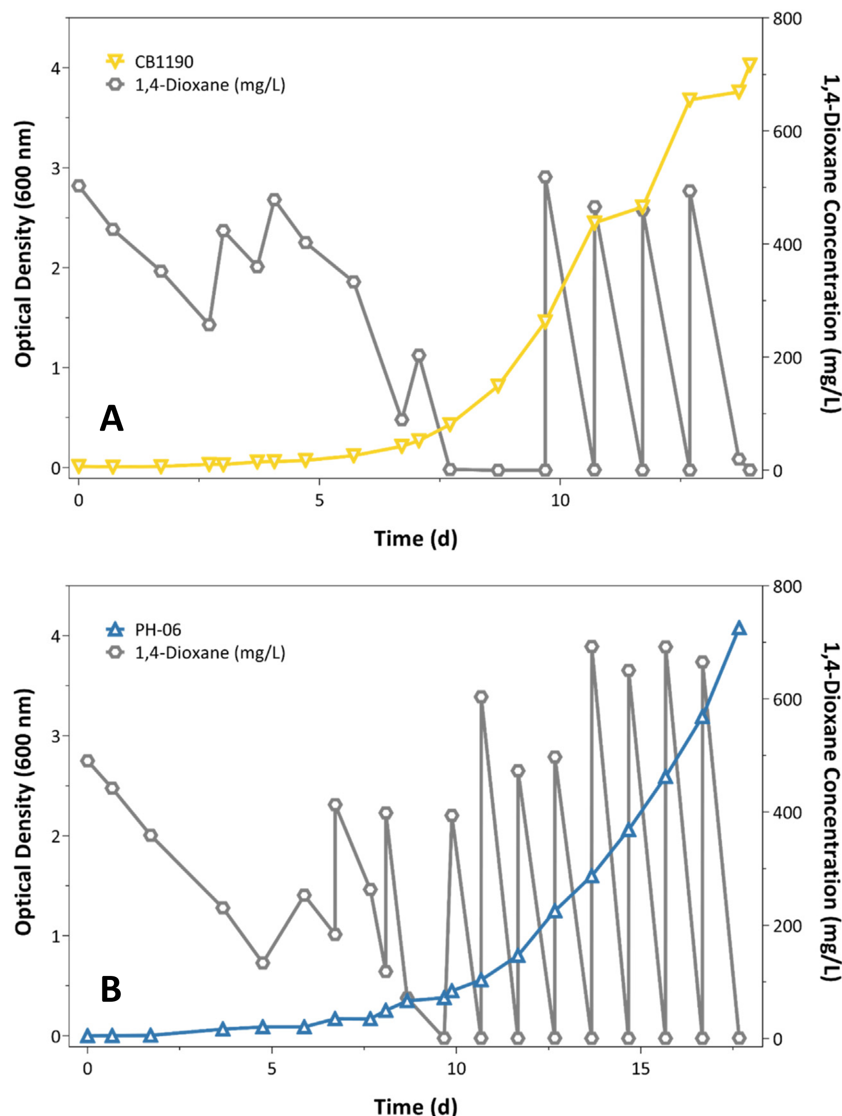

Fig. 7. Fermentation runs using (A) *Pseudonocardia dioxanivorans* CB1190 and (B) *Mycobacterium dioxanotrophicus* PH-06. Dioxane was replaced as needed. CB1190 reached an optical density of 4 in 14 days, while PH-06 needed 18 days to reach a similar optical density.

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 h vs. 30 h 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 mmHg for THF vs. 38.1 mmHg 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).

3.4. Technical implications

This study demonstrated that bioaugmenting the poplar rhizosphere is an effective method to speed the treatment of dioxane-contaminated groundwater. In bench-scale testing, bioaugmenting poplar with either CB1190 or PH-06 significantly accelerated the removal of dioxane to less than 4 μg/L compared to poplar alone (Fig. 5). We also estimated that in planted bioaugmented treatments, approximately 80% of the total dioxane removed was due to degradation by either CB1190 or PH-06, while the remaining 20% was removed by plant uptake (Fig. 6). However, this ratio was dependent on the experimental conditions, including the transpiration rate, the concentration of bioaugmented strains, and the starting dioxane concentration. Ongoing work aims to understand how changing these conditions may affect the ratio of removal between degradation and plant uptake.

This study also demonstrated that PH-06 does not use root extract as an auxiliary carbon source (Fig. 4). Despite this limitation, we observed that PH-06-bioaugmented poplar significantly outperformed all other treatments in removing dioxane, including poplar bioaugmented with CB1190 (Fig. 5). We postulate that PH-06 was uninhibited by root exudates, resulting in additive mechanisms between phytoremediation plus biodegradation. In contrast, bioaugmenting poplar with CB1190 did not significantly increase removal rates compared to unplanted treatments. This was likely the result of simultaneous utilization of root exudates and dioxane by CB1190, as observed in root extract-amended cultures (Fig. 4).

Despite our laboratory results, CB1190 has several qualities that make it a strong candidate for field bioaugmentation of the poplar rhizosphere.

CB1190's ability to utilize root extract as an auxiliary carbon source may allow CB1190 to overcome minimum substrate requirements for growth when exposed to low dioxane concentrations. Also, the ability to utilize root extract allows CB1190 to be better equipped to colonize the rhizosphere and outcompete indigenous microorganisms (Feng et al., 2017; Thijs et al., 2016; Yergeau et al., 2014). Furthermore, CB1190's tendency to clump may allow the strain to form biofilms on the poplar root surface and prevent washout (Chan and Kjellerup, 2019). On the other hand, CB1190's clumping poses a significant challenge for traditional bioaugmentation due to limited mobility in the subsurface (da Silva et al., 2020). But previous work has shown that if augmented bacteria can colonize the roots, rapidly growing roots can spread the bacteria throughout the subsurface (Kuiper et al., 2001). Finally, CB1190 can also degrade cis-1,2-dichloroethene, a common dioxane co-contaminant, as well as survive prolonged anaerobic conditions (Polasko et al., 2018).

4. Conclusions

This study explores the energetics of dual substrate utilization, 1,4-dioxane plus root extract, which is important when bioaugmentation is used in tandem with phytoremediation. We are the first to report that PH-06 cannot utilize root extract as primary substrates and confirmed CB1190 can. We are also the first to demonstrate that PH-06-bioaugmented poplar significantly outperforms poplar bioaugmented with CB1190. PH-06 is uninhibited by root extract, making the strain a strong candidate to speed phytoremediation of dioxane. However, it is possible that CB1190 would fare better in the field due to its capacity to utilize root extract and outcompete indigenous microorganisms. Whereas pilot studies may be needed to determine which of these two strains is a better bioaugmentation choice for a specific case, this study demonstrates that combining phytoremediation with bioaugmentation is a promising treatment alternative for dioxane-contaminated groundwater to achieve low concentrations (~1 μg/L) as recommended by health advisories. Ongoing work aims to optimize this technology for field-scale implementation. While challenges remain, the successful implementation of this strategy offers a potentially feasible and cost-effective solution to a widespread problem of national and international importance.

CRediT authorship contribution statement

Reid Simmer: Conceptualization, Methodology, Validation, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Jacques Mathieu:** Conceptualization, Methodology, Resources, Writing - review & editing, Funding acquisition. **Marcio L.B. da Silva:** Conceptualization, Methodology, Resources, Writing - review & editing. **Philip Lashmit:** Methodology, Investigation, Data curation. **Sridhar Gopishetty:** Methodology, Investigation, Data curation, Writing - review & editing, Supervision. **Pedro J.J. Alvarez:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition. **Jerald L. Schnoor:** Conceptualization, Methodology, Writing - review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in the online version, at https://doi.org/10.1016/j.scitotenv.2020.140823. These data include the Google map of Fig. 1.

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