Journal of Hazardous Materials Advances 9 (2023) 100246

Contents lists available at ScienceDirect

Journal of Hazardous Materials Advances

journal homepage: www.elsevier.com/locate/hazadv

Effective removal of trace 1,4-dioxane by biological treatments augmented with propanotrophic single culture versus synthetic consortium

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ARTICLE INFO

Keywords:
1,4-dioxane
Bioaugmentation
Propanotroph
Azoarcus
Consortium
Biomarker

ABSTRACT

1,4-Dioxane (dioxane) is historically used as a stabilizer for chlorinated solvents and has emerged with increasing attention due to its frequent detection at numerous sites in the US. However, trace concentrations of dioxane and intricate environmental conditions hinder its effective treatment in the field. A number of propanotrophic microorganisms are reputed for their ability to degrade dioxane via metabolism (e.g., *Pseudonocardia dioxanivorans* CB1190 and *Mycobacterium dioxanotrophicus* PH-06) and cometabolism (e.g., *Azoarcus* sp. DD4). In this study, we assessed the feasibility of the novel dioxane cometabolizer, *Azoarcus* sp. DD4, and the mixture of CB1190, PH-06, and DD4 at the ratio of 1:1:2 to remove dioxane in various water samples from a landfill site in northern New Jersey. After being fed with propane for 6~9 days, DD4 or the synthetic consortium can effectively degrade dioxane to below our method detection limit (i.e., 0.38 μg/L) in both influent and effluent samples from the existing pump-and-treat facility, as well as the contaminated groundwater from the monitoring well near the source zone of the plume. Relative abundances of *Azoarcus* and DD4's toluene monooxygenase gene *tmoA* were both positively correlated with dioxane degradation rates observed in microcosms, suggesting DD4's pivotal contribution. In light of both qPCR and 16S rRNA amplicon sequencing analyses, DD4 remained dominant (29~52%) in these three types of groundwater, indicating DD4's compatibility for both in situ and *ex situ* treatments. Interestingly, inoculation with the synthetic consortium did not elicit significant improvement in dioxane removal as compared with the treatments seeded with DD4 alone. Unlike DD4, CB1190 and PH-06 were absent after the complete course of the microcosm study, indicating the decay of both two exogenous species. This was probably due to outcompetion by DD4 and/or indigenous microbiomes and hindrance by field factors (e.g., inhibitors and nutrients). Native *Rhodococcus* species emerged and exhibited significant correlation with the *prmA* gene encoding the alpha subunit of propane monooxygenase of PH-06, suggesting them as possible recipients of this dioxane degradation gene via horizontal transfer. Collectively, this study demonstrated the DD4's high viability and excellent compatibility with diverse field samples suited as an effective inoculum to mitigate trace levels of dioxane in the subsurface or in bioreactors. Amendment of Gram-positive propanotrophic metabolizers has the potential to spread dioxane degradation genes though their viability can be low at certain sites.

1. Introduction

1,4-Dioxane (dioxane) is of paramount concern as an emerging contaminant in groundwater given its carcinogenicity (IARC 1999; USEPA 2017) and widespread occurrence (2012, Adamson et al., 2014a). On the basis of the animal and human toxicology assays, dioxane is classified as a probable human carcinogen (B2) by US Environmental Protection Agency (USEPA) and International Agency for Research on Cancer (IARC) (IARC 1999; USEPA 2017). USEPA also reported a guideline of 0.35 μg/L for dioxane in drinking water considering a cancer risk level of 10^{-6} (USEPA 2013). Unfortunately, dioxane has been reported at a broad concentration range from sub- μg/L level to 10,000 μg/L in groundwater at sites in the US and many other countries (Mohr 2004). Such extensive contamination mainly stemmed from its historical storage, transportation, and use as the stabilizer for chlorinated solvents, typically 1,1,1-trichloroethane (1,1,1-TCA) (Mohr et al., 2016). Therefore, demands to develop effective treatment methods are escalating to manage and remediate sites impacted by dioxane.

Clean-up of dioxane-contaminated sites is a challenging task because of its extreme persistency and its physicochemical properties that preclude effective removal by conventional treatment, such as air stripping and GAC sequestration (Nyer 1992; USEPA 2017). AMBERSORB™ is a new proprietary synthetic adsorbent exhibiting effective removal of dioxane (Woodard et al., 2014). Like other adsorbents, AMBERSORB™

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https://doi.org/10.1016/j.hazadv.2023.100246
Received 6 December 2022; Received in revised form 13 January 2023; Accepted 20 January 2023

are exhausted periodically and thus require routine regeneration or even replacement to prevent undesirable dioxane breakthroughs. The washing solvent derived from regeneration needs further disposal or treatment as it contains concentrated dioxane. This sequestration technique is also subject to flow limitations in order to maintain a satisfactory treatment efficiency (Chiang et al., 2016). Advance oxidation processes (AOPs) represent another effective alternative that has been commercialized for dioxane treatment. Two common AOPs include hydrogen peroxide with ozone and hydrogen peroxide with ultraviolet (UV) radiation, which both generate active hydroxyl radicals that attack dioxane. However, the treatment efficiency of AOPs is frequently affected by free radical scavengers (e.g., carbonate and bicarbonate) in the groundwater. Further, when bromide is present in the groundwater, AOPs can oxidize it to form bromate, which is a probable human carcinogen with a maximum contaminant level (MCL) of 100 $\mu g/L$. To reduce the release of excessive oxidants used during the treatment, amendment of quenching chemicals (e.g., sodium bisulfate) is likely required to secure the discharge (EPA 2010). Therefore, though effective, both AMBERSORB^{TM} and AOPs are relatively expensive for dioxane remediation considering high capital and Operation and Maintenance (O&M) cost, and liability for waste and byproduct management may linger to reduce potential environmental risks (Chiang et al., 2016).

Biological treatments relying on dioxane-degrading microorganisms are among the most cost-effective and eco-friendly approaches to manage dioxane contamination (Favara et al., 2016). In recent years, increasing number of microorganisms have been isolated given their ability to degrade dioxane via metabolism or cometabolism. Many of these dioxane degraders are propanotrophs that can grow with propane. Particularly, *Pseudonocardia dioxanivorans* CB1190 and *Mycobacterium dioxanotrophicus* PH-06 (Kim et al., 2009; Parales et al., 1994) are two archetypical metabolizers that can exploit dioxane as the sole carbon and energy source. The genome of CB1190 contains a *prm* gene encoding a group-5 propane monooxygenase, though it is the *thm*-coding tetrahydrofuran monooxygenase (THM) in charge of dioxane hydroxylation (Sales et al., 2011b; Sales et al., 2013). In PH-06, it can express a group-6 propane monooxygenase (PRM) to oxidize both dioxane and propane (Deng et al., 2018a; Deng et al., 2019). These metabolizers have many advantages at dioxane-impacted sites, as they can grow without the addition of substrates. They may also require lower oxygen demand and less chance of overgrowth (Polasko et al., 2019). Nevertheless, some challenges persist for field applications. For instance, low dioxane concentration in diluted plumes can be inadequate to support the growth of metabolizers; dioxane degradation may be kinetically restricted in dioxane metabolizers (Barajas-Rodriguez and Freedman 2018; Barajas-Rodriguez et al., 2019; Li et al., 2017). Thus, propanotrophic co-metabolizers, such as *Mycobacterium* sp. ENV421, *Pseudonocardia* sp. ENV478, *Azoarcus* sp. DD4 (Deng et al., 2018b; Masuda 2009; Steffan et al., 1997), have attracted increasing attention since they can grow rapidly with propane as the primary substrate and fortuitously degrade dioxane (Dalton and Stirling 1982). In this case, dioxane can be removed to very low levels, meeting the stringent cleanup requirements (e.g., 0.4 μg/L in New Jersey and 1 μg/L in New York).

Among the reported propanotrophic cometabolizers, DD4 is a gram-negative Proteobacterium with high growth rate and dioxane degradation ability when fueled with propane (Deng et al., 2018b) and soluble substrates, such as 1-propanol (Deng et al., 2022). It can oxidize dioxane by inserting a hydroxyl group to the α-carbon adjacent to oxygen by an unique group-2 toluene monooxygenase (TMO) (Deng et al., 2018b; Deng et al., 2020). In addition to its high cell yield and non-clumping behavior that favors subsurface distribution, DD4 can also degrade 1,1-dichloroethene [1,1-DCE] (Deng et al., 2018c), *cis*-1,2-dichloroethene [cDCE], and vinyl chloride [VC] (Li et al., 2021) that commonly co-occur with dioxane in groundwater (Adamson et al., 2014b).

In this present study, microcosm assays were set up to evaluate and compare the dioxane biodegradation effectiveness of the propanotrophic isolate DD4 versus a synthetic consortium constituted of CB1190, PH-06, and DD4 at environment-relevant conditions. As propanotrophic dioxane metabolizers, CB1190 and PH-06 can assist the dioxane mineralization and may accelerate the overall removal. Four groundwater samples were collected from monitoring wells and along the existing treatment train at a landfill site in New Jersey for the assessment of the feasibility of in situ and *ex situ* biological treatments, respectively. The abundance of these three exogenous propanotrophs were monitored using qPCR analysis with catabolic biomarkers that target different monooxygenase genes responsible for the initial hydroxylation of dioxane. Overall microbial community was profiled using 16S rRNA amplicon sequencing to investigate the shifting of the exogenous propanotrophs, as well as putative players in native microbiomes. This study advanced our knowledge on the environmental viability and compatibility of dioxane-degrading propanotrophs, unveiling their feasibility for in situ and *ex situ* treatments in the field.

2. Materials and methods

2.1. Sample collection

A landfill site located in northern NJ was selected for sampling (Figure S1). This site was identified about forty years ago due to the contamination of hazardous compounds such as benzene, methylene chloride, and chloroform. This site remains active for volatilized organic compound (VOC) and dioxane cleanup. The site is being treated by pump-and-treat at a groundwater extraction and treatment (GWET) facility that combines equalization, sedimentation, filtration, and carbon adsorption for VOC removal (Figure S2). This treatment train can efficiently remove VOCs and metals to below the NJDEP groundwater quality standards (GWQS) as shown in Table S1. When the existing groundwater treatment system was designed in the early 1990s, dioxane's presence was unknown. The current pump-and-treat system has minimal effect on reducing influent dioxane concentrations. Therefore, influent and effluent samples (INF and EFF) of the GWET facility were collected as the groundwater composite that was commingled with contaminated groundwater and that passed the treatment train in the effluent monitoring tank, respectively. In addition, two groundwater samples were collected directly from monitoring wells (MW1 and MW2 as shown in Figure S1), exhibiting typical high- and medium-level dioxane contaminations in the plume at the site. Samples were collected by HDR at this site in May 2019 and sent to NJIT on the same day of the sampling while being kept in a cooler with ice. All samples were stored at 4 °C in amber glass bottles without headspace prior to the microcosm setup.

2.2. Microcosm assays

Microcosm assays were conducted in triplicate to discern the rate and extent of dioxane biodegradation occurring in four groundwater samples of interest, enabling the assessment of the effectiveness of different biological treatment strategies (e.g., *ex situ* vs in situ). For each groundwater sample, an abiotic control and three biologically active treatments that mimic (1) biostimulation with propane, (2) bioaugmentation with the dioxane cometabolizer, DD4, and (3) bioaugmentation with a synthetic consortium consisting of two dioxane metabolizers (CB1190 and PH-06) and one cometabolizer (DD4) were prepared following the recipes outlined in Table 1.

All microcosms were prepared in 160-mL serum bottles sealed with rubber caps. DD4 was cultured in nitrate mineral salt (NMS) media with propane as the growth substrate. CB1190 and PH-06 grown on 100 mg/L of dioxane in ammonium mineral salts (AMS) media. All cultures were harvested at the exponential growth phase, washed for three times, and resuspended to OD_{600nm} of 2.0 with fresh AMS medium. For the DD4 bioaugmentation microcosms, 0.5 mL of the harvested cells were inoculated to achieve an initial total protein of 0.17 mg per vial. For the synthetic consortium inoculum, the seeding mixture was composed of DD4, CB1190, PH-06 at the biomass ratio of 2:1:1. Propane (0.15% v/v)

Table 1
Microcosm setup.

Treatment	Control	Propane	DD4	Syn
Propane (0.15% v/v in headspace)		√	√	√
Groundwater (60 mL in 160 mL serum bottle)	√	√	√	√
DD4 (0.17 mg as total protein)			√	
Synthetic Consortium of DD4, PH-06, and CB1190 (2:1:1) (0.17 mg as total protein)				√

was amended to all active treatments to supplement carbon and energy. Microcosms were incubated at room temperature (i.e., 24±3 °C) while being shaken at 150 rpm. At selected intervals, liquid and headspace samples were collected for the analysis of dioxane and propane, respectively. Propane was re-amended once when over 95% of the initial propane was consumed.

2.3. Quantitative polymerase chain reaction (qPCR)

Total DNA was extracted from samples collected after the completion of microcosm treatments. PowerSoil® DNA Isolation Kit (Qiagen, Hilden, Germany) was used following the manufacturer's instruction. The abundance of the toluene monooxygenase gene (*tmoA*) in DD4 (Deng et al., 2022), the propane monooxygenase gene (*prmA*) in PH-06 (He et al., 2017), and the tetrahydrofuran monooxygenase gene (*thmA*) in CB1190 (Li et al., 2014) was quantified by qPCR using designed probe/primers specifically targeting their alpha subunit components (Table S2). A set of 16S rRNA primers (341F and 534R) was used to enumerate the total biomass (Park et al., 2011). Each qPCR reaction (20 μL) consisted of 5 ng DNA extract, 300 nM of paired primers, 150 nM of probe (when applicable), and 10 μL of Power SYBR® Green or TaqMan® Mastermix (Thermo, Carlsbad, CA). qPCR was run with Quant Studio3 (Thermo, Carlsbad, CA) following the temperature program: initially held at 10 min for 95 °C, followed by 40 cycles of 15 s at 95 °C and 1 min at 60 °C. The relative abundance of each inoculated strain was calculated as the percent ratio of the specific dioxane degrading gene number to the total bacterial cell number, which is equivalent to the 16S rRNA gene number divided by 4.2 (i.e., average 16S rRNA gene copies per bacterial cell) (Větrovský and Baldrian 2013).

2.4. Microbial community analysis

16S rRNA amplicon sequencing was employed to profile the microbial communities in original and biotreated water samples. Bacterial V3-V4 hypervariable regions of 16S rRNA were amplified by PCR with primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) (Yu et al., 2005) following the standard MetaVx^{TM} library preparation process. PCR products were examined by gel electrophoresis on 2% agarose gel and recovered using the GeneJET^{TM} Gel Extraction Kit (Thermo, Carlsbad, CA). A second round PCR was run for limited cycles for the addition of sample-specific barcodes for multiplexing. Final libraries were pooled together with concentrations quantified by the Qubit® 2.0 Fluorometer (Thermo, Carlsbad, CA). Paired-end sequencing (2 × 250 bp) was performed using Illumina MiSeq (Illumina, San Diego, CA) at GENEWIZ (South Plainfield, NJ, USA). The raw sequencing data were filtered and analyzed using the Cutadapt (v1.9.1) (Martin 2011), Vsearch (1.9.6) (Rognes et al., 2016), Qiime (1.9.1) (Caporaso et al., 2010). After removing the chimera sequences, sequences of >200 bp in length were clustered when sequences' similarity was higher than 97%. Further, the taxonomy of representative OTUs was assigned using the Ribosomal Database Project (RDP) Classifier (Wang et al., 2007). Hierarchical clustering was carried out with *hclust* of stats package in R (Müllner 2013).

2.5. Analytical approaches

Dioxane in the aqueous phase was extracted using a frozen microextraction (FME) method (Li et al., 2011) with 1,4-dioxane-d_8 and tetrahydrofuran-d_8 as the surrogate and internal standard, respectively. Briefly, 3 mL of the filtered water sample was transferred to a 5-mL tube, which was further spiked with 6 μL of 25 mg/L 1,4-dioxane-d_8 to achieve a concentration of 50 μg/L. Then, 1 mL of GC-grade dichloromethane (DCM) was added to the tube and vigorously shaken for 10 min. The tube was then kept statically for 1 min to make sure all bubbles dissolved. Tubes were placed horizontally in −80 °C freezer for 20 min. After quickly removing the ice layer, 500 µL of the organic phase was transferred to a new GC analysis vial. Amendment of 5 µL of 5 μg/L tetrahydrofuran-d_8 was conducted prior to the gas chromatography/mass spectrometry (GC/MS) analysis. Propane in the headspace was monitored by GC-FID as previously described (Li et al., 2020).

Concentrations of co-occurring VOCs in original water samples were measured at an external commercial lab using the EPA Method 8260C (2006). This is a standard method for quantifying a wide span of VOCs in aqueous samples using purge-and-trap GC/MS.

3. Results

3.1. Dioxane biodegradation and propane utilization in microcosms

Dioxane was detected in the INF sample at an initial concentration of 34.3 ± 1.1 µg/L. Due to the high hydrophilicity and recalcitrance of dioxane, it was marginally removed through the existing treatment train at the GWET facility that includes an aerated bioreactor and two carbon-based adsorption units (Figure S2). Dioxane, thus, remained 27.4 ± 0.4 µg/L in the EFF sample, resulting in approximately 20% removal as compared to the INF concentration. Groundwater samples from two monitoring wells showed higher dioxane concentrations (130.0 ± 3.3 µg/L for MW1 and 83.2 ± 2.3 µg/L for MW2), in agreement with their locations in the center and middle of the dioxane plume, respectively.

Bioaugmentation with DD4 can effectively reduce trace dioxane contamination in both INF and EFF samples collected at the GWET facility. In microcosms prepared with the INF sample (Fig. 1A), dioxane was degraded to 2.5 ± 0.5 μg/L in 3 days, and subsequently to below the method detection limit (MDL, i.e., 0.38 μg/L) within 6 days of incubation. Fast dioxane degradation by DD4 was also observed in the microcosms prepared with the EFF sample (Fig. 1B). Dioxane was degraded from 27.4 ± 0.5 μg/L to 0.5 ± 0.1 μg/L within 3 days of incubation. Eventually, dioxane concentration was then below our MDL in day 6. At the same time, in both INF-DD4 and EFF-DD4 microcosms, over 300 μL of pure propane was consumed within 6 days (Figure S3A and S3B).

In microcosms prepared with the MW1 field water, dioxane was effectively removed from 130.0 ± 3.3 µg/L to below the MDL within 6 days (Fig. 1C). However, slow dioxane removal was observed in the DD4-bioaugmented microcosms prepared with the groundwater from MW2 (Fig. 1D). Dioxane was degraded by <30% of the initial dioxane concentration (83.2 ± 2.3 μg/L) after over 30 days of incubation, leaving a residual of 59.7 ± 2.4 μg/L. The quick propane depletion was observed in microcosms prepared with the MW1 sample as those with INF and EFF samples (Figure S3). Propane consumption in MW2 microcosms was greatly decelerated in contrast with the other three groundwater sam-

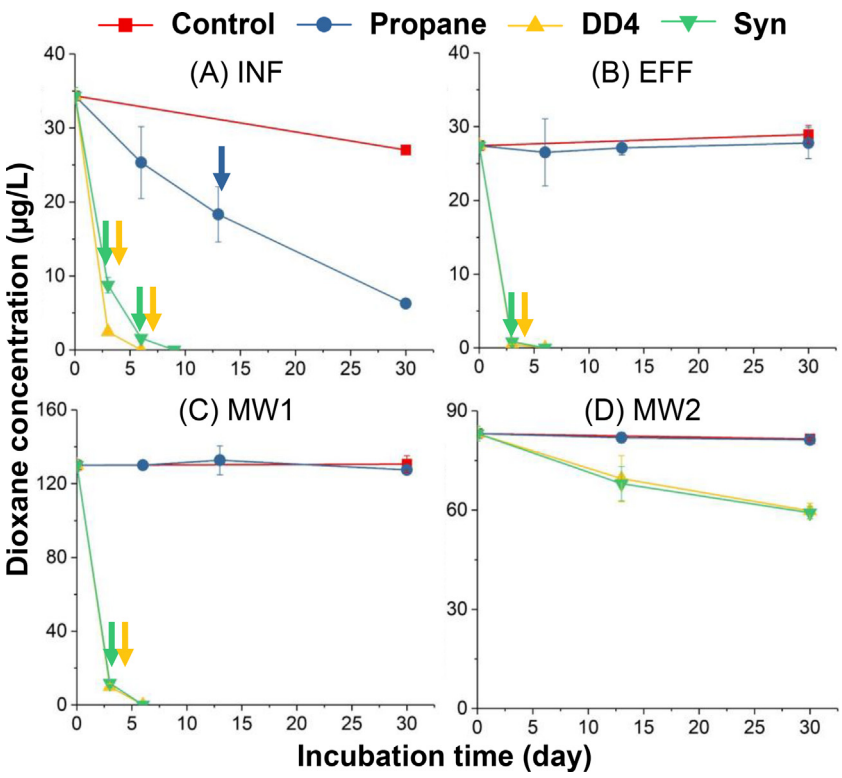

Fig. 1. Dioxane degradation in microcosms amended with propane, DD4, and synthetic consortium in comparison with the abiotic control. Microcosms were prepared with varying groundwater samples, including (A) the influent (INF) and (B) effluent (EFF) of the GWET facility and two monitoring wells (C) MW1 and (D) MW2. Arrows indicate repeated propane amendments when over 90% of propane was consumed. The colors of the arrows correspond to different treatment as depicted in the legend.

ples, and the initially amended propane was not fully consumed until Day 30 (Figure S3D).

In microcosm assays, the synthetic consortium inoculum did not elicit significant differences in either dioxane degradation or propane consumption as compared to the corresponding microcosms bioaugmented with mere DD4 in all four types of field groundwater samples that were tested in this study (Fig. 1). Dioxane in INF-Syn was degraded from 34.3 ± 1.1 μg/L to 1.6 ± 0.4 μg/L in 6 days and was fully depleted in 9 days; in EFF-Syn, dioxane was degraded from 27.4 ± 0.5 μg/L to 0.9 ± 0.1 μg/L in 3 days and was completely removed in 6 days; in MW1-Syn, dioxane was degraded from 130.0 ± 3.3 μg/L to 11.8 ± 0.9 μg/L in 3 days and fully disappeared in 6 days; in MW2-Syn, dioxane remained 59.2 ± 1.9 μg/L after the 30-day incubation. Accordingly, propane consumption in the synthetic consortium-inoculated treatments showed similar trends to the DD4 treatments in all four groundwater samples (Figure S3).

Interestingly, in the propane biostimulation treatment prepared with the INF water sample but no amendment of exogenous dioxane degraders, significant dioxane removal was observed in comparison with the abiotic control (Fig. 1A). On day 30, dioxane concentration dropped from 34.3 ± 1.1 μg/L to 6.3 ± 0.6 μg/L in the INF-Propane treatment, while it remained as high as 27.0 ± 0.6 μg/L in INF-Control microcosms. At the same time, propane removal was observed after 9 days in all biostimulation microcosms with four different groundwater samples, particularly in INF-Propane that showed two rounds of complete consumption of propane (>300 μL) (Figure S3A). Within 30 days, propane was degraded from 3.5 ± 0.0 mg/L to 2.4 ± 0.6 mg/L, 1.7 ± 0.9 mg/L, 2.71±0.7 mg/L in EFF-Propane, MW1-Propane, and MW2-Propane, respectively. However, no significant dioxane removal was observed in these three sets of microcosms.

3.2. Biomarker abundances in microcosms by the qPCR analysis

The qPCR analysis revealed the absence of *tmoA* in either original water samples (i.e., INF, EFF, MW1, and MW2) or propane amended treatments (i.e., INF-Propane, EFF-Propane, MW1-Propane, and MW2-Propane) (data not shown). As a catabolic biomarker for DD4 (Deng et al., 2022), *tmoA* was abundant in the DD4-bioaugmented microcosms prepared with INF, EFF, and MW1, which occupied 29.9%, 28.6%, and 35.4% of the total bacteria. In contrast, *tmoA* only accounted for 2.2% in MW2-DD4, which was in line with the observation of low dioxane degradation and propane utilization rates. The abundances of *tmoA* in synthetic consortium-bioaugmented treatments prepared by INF, EFF, MW1, and MW2 were comparable with those that received DD4 as the single inoculum, which were 30.7%, 37.9%, 37.1%, and 4.7%, respectively.

In the synthetic consortium amended treatments, the abundances of PH-06 and CB1190 were tracked by the enumeration of *prmA* and *thmA* genes by specific biomarkers (He et al., 2017; Li et al., 2014). However, the abundance of *prmA* and *thmA* became surprisingly low after 30-day incubation as compared with the initial inoculation ratio to DD4 (i.e., 2:1 for DD4:PH-06) (Fig. 2). *PrmA* was undetectable in INF-Syn, 0.1% in EFF-Syn, 1.1% in MW1-Syn, and 0.8% in MW2-Syn. Similarly, the relative abundances of *thmA* were undetectable in INF-Syn, 0.1% in EFF-Syn, 0.1% in MW1-Syn, and 1.3% in MW2-Syn. The low detection of *prmA* and *thmA* indicated the significant decay of PH-06 and CB1190, which may be resulted from the competition of DD4 and/or native microbiomes.

3.3. Microbial community analysis by 16S rRNA sequencing

Microbial community analysis revealed that *Azoarcus* was the most abundant genus in INF, EFF, and MW1 microcosms that were bioaugmented with DD4 or the synthetic consortium (Fig. 3). Particularly, OTU_1 was assigned as *Azoarcus* sp. DD4 given their high sequence similarity (>99%) (Fig. 4). OTU_1 accounted for 39.6%, 51.4%, and 32.9% in INF-DD4, EFF-DD4, and MW1-DD4 microcosms, respectively (Table S3 and Fig. 4), supporting the dominant role of DD4 in dioxane degradation in these microcosms that were initially inoculated with DD4. Interestingly, though the initial DD4 inoculation dosage was half in the synthetic consortium as compared to the pure DD4 treatment, the relative abundances of OTU_1 in INF-Syn, EFF-Syn, and MW1-Syn microcosms were

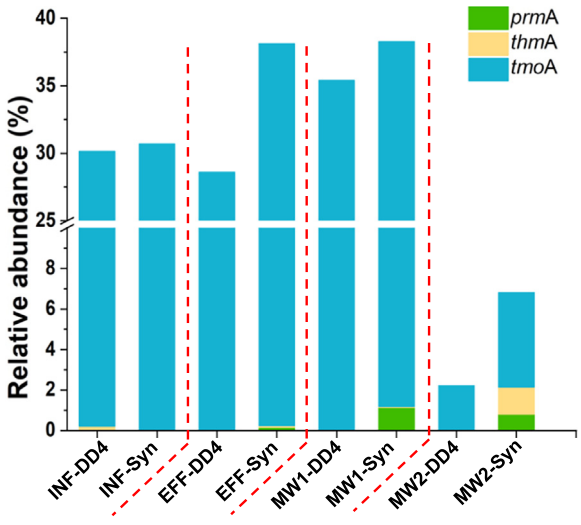

Fig. 2. The relative abundance of *tmoA, prmA*, and *thmA* by qPCR to target the toluene monooxygenase in DD4, propane monooxygenase in PH-06, and tetrahydrofuran monooxygenase in CB1190. All types of gene clusters are normalized by the total bacteria (equivalent to the total 16S rRNA gene copies divided by 4.2).

38.3%, 52.4%, and 34.9%, respectively (Table S3), which were quite similar to those observed in microcosms that were only inoculated with DD4. These results were in good agreement with the qPCR data by targeting the *tmoA* biomarker gene, indicating the rapid growth of DD4 in these microcosms. However, OTU_1 was relatively low in MW2 microcosms bioaugmented with DD4 (3.7%) and the synthetic consortium (3.1%), in which dioxane biodegradation or propane consumption was also slow.

Notably, no sequences were recovered in the genus of *Pseudonocardia* in any microcosms including those that were inoculated with the synthetic consortium consisting of 25% of CB1190 (Fig. 3). As the genus for the other inoculum, *Mycobacterium* accounted for up to 7.3% of total biomass across all samples (Fig. 3). However, no OTU was phylogenetically assigned as PH-06 at the sequence identity threshold of 99% (Fig. 4). The microbial community analysis revealed the absence of CB1190 and PH-06 after the bioaugmentation treatment. This echoes the low or no detection of *prmA* and *thmA* genes by qPCR. The low abundance of these catabolic genes may be the residual DNA from the decayed cells and/or those transmitted to native microbiomes by horizontal gene transfer (see more discussion below).

Among all detected *Mycobacterium* taxa, OTU_52 shares the highest sequence identity of 97.3% with PH-06. This OTU has the identity of 100% to the 16S rRNA genes of *Mycobacterium* sp. NBB3. NBB3 can express a group-3 soluble di-iron monooxygenase (encoded by *smoXYB1C1Z*) that can initiate the oxidation of propane and other short-chain alkanes and alkenes, as well as VC and 1,2-DCA (Coleman et al., 2012; Martin et al., 2014). As shown in Fig. 4, OTU_52 was found at low abundance of 0.5% in MW1 and INF. After propane biostimulation, OTU_52 enriched to 7.3% in MW1-Propane, where dioxane degradation was not observed. It was also detected in other samples (e.g., INF-Propane and INF-DD4) in which propane was amended. These results

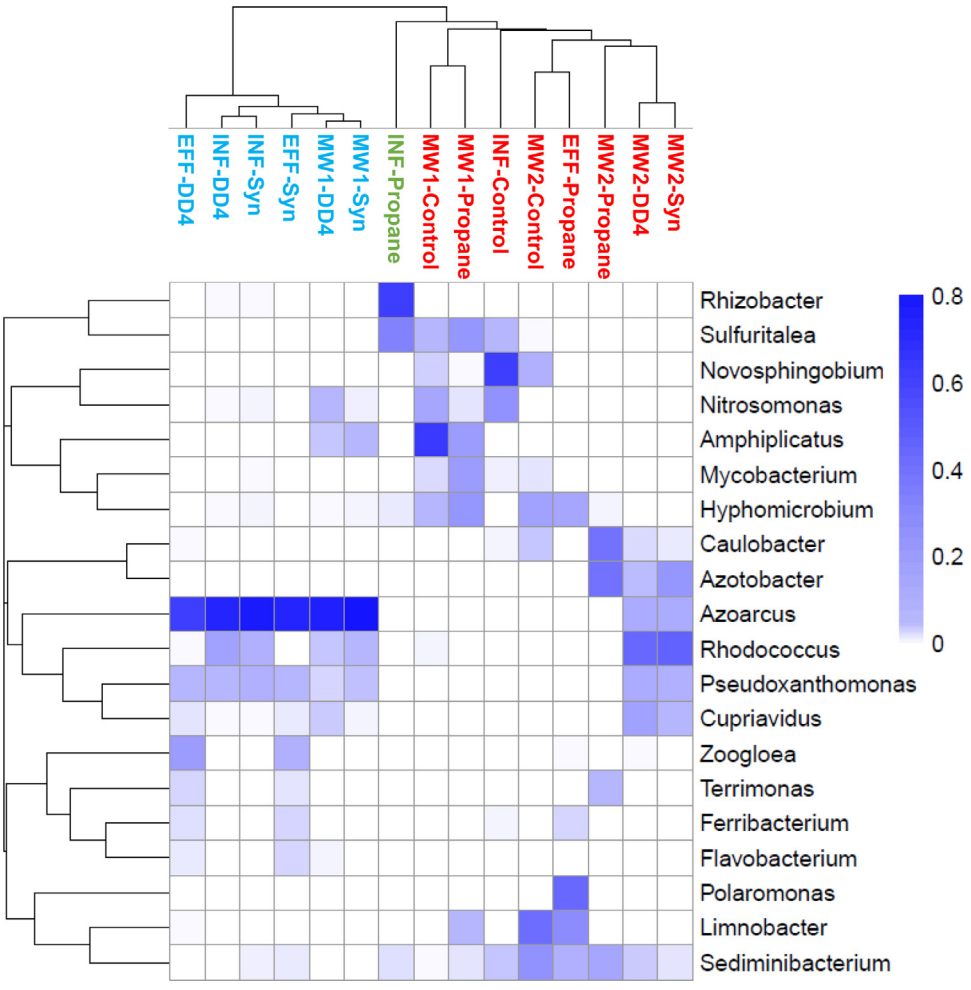

Fig. 3. Heat map showing the dominant genera detected in different treatments. Color in the heat map is scaled in accordance with the relative sequence abundance of a specific genus. The dendrogram on the left reveals the similarity in the distribution for individual genera. The dendrogram on the top represents the clustering of microcosm treatments based on their microbial community structure and compositions. Treatments that showed complete dioxane removal are highlighted in blue. Treatments with no observable dioxane degradation are highlighted in red. Dioxane in INF-propane (marked in green) was partially degraded by indigenous microorganisms.

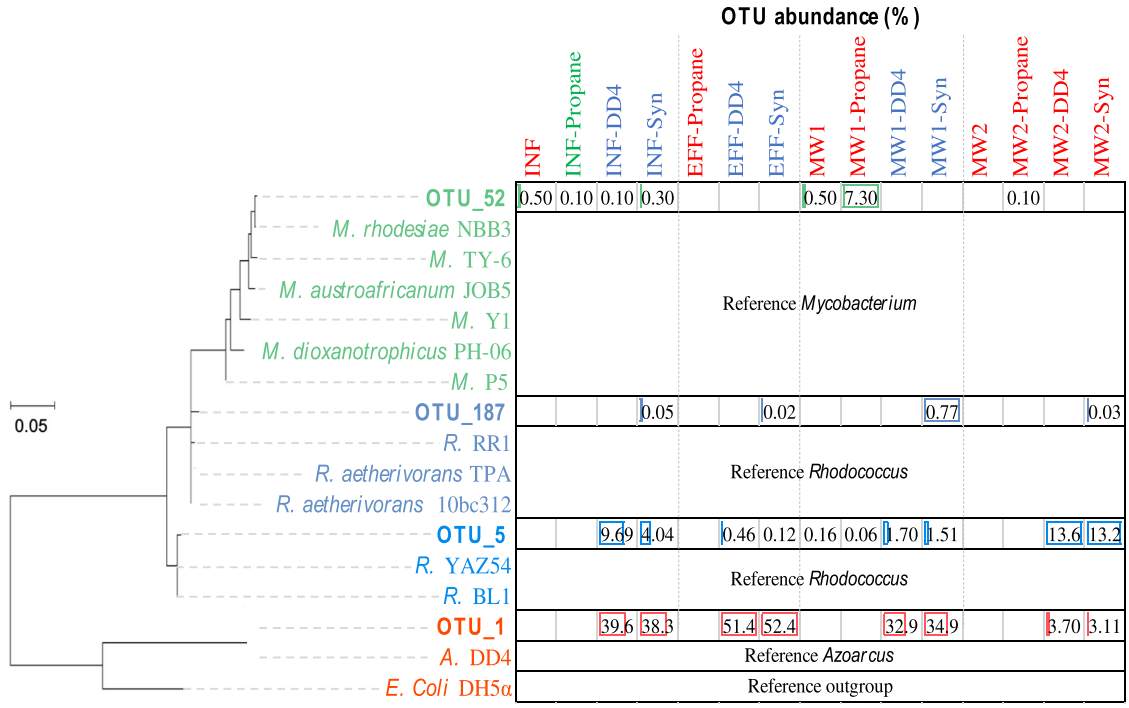

Fig. 4. Neighbor-joining phylogenetic tree with representative OTUs and relevant propanotrophs and dioxane degraders that belong to *Azoarcus* (shaded by red), *Mycobacterium* (shaded by blue), and *Rhodococcus* (shaded by green). Phylogeny is based on the V3-V4 region of 16S rRNA sequences with *E. coli* DH5a as the outgroup reference. Numbers in the table on the right are the percentage abundances of four OTUs in treatments based on the 16S rRNA amplicon sequencing results. Blanks indicate non-detection. The treatments on top are labeled in colors that are consistent with Fig. 3 (i.e., blue for treatments with complete dioxane degradation, green for treatments with partial dioxane degradation, and red for treatments with no dioxane degradation).

suggest that OTU_52 as a propanotrophic *Mycobacterium* naturally occurring at the site, though its contribution to dioxane degradation could be minimal.

As another genus potentially involved in propane and/or dioxane degradation, *Rhodococcus* was abundant as one of the top 10 genera in bioaugmented treatments prepared with INF, MW1, and MW2 groundwater (Table S3). However, *Rhodococcus* was barely detected in the original water samples from INF and MW1, as well as the treatments that received propane amendment. Two OTUs (i.e., OTU_5 and 187) as *Rhodococcus* were classified into two sub-branches (Fig. 4). OTU_187 was exclusively detected in all four groundwater samples that were bioaugmented with the synthetic consortium, though its relative abundances were quite low at 0.05%, 0.02%, 0.77%, and 0.03% in INF-Syn, EFF-Syn, MW1-Syn, and MW2-Syn, respectively. OTU_187 is phylogenetically close (>99%) to a dioxane degrader *Rhodococcus aetherivorans* 10bc312 (=JCM 14,343=DSMZ 44,752) (Inoue et al., 2018) and a propane-utilizing bacteria *Rhodococcus aetherivorans* TPA (Cappelletti et al., 2018). Thus, OTU_187 may serve as a dioxane degrader and/or propane consumer in microcosms bioaugmented with the synthetic consortium. It is likely that OTU_187 is a recipient of *thm* or *prm* genes actively or passively transmitted from CB1190 and PH-06, though such hypothesis requires further validation using advanced microbial ecology tools, such as EpicPCR and stable isotope probing (SIP).

In contrast, OTU_5 was more widespread and abundant across all samples and treatments. OTU_5 was particularly high when DD4 or the synthetic consortium was inoculated, including INF-DD4 (9.7%), INF-Syn (4.0%), EFF-DD4 (0.5%), EFF-Syn (0.1%), MW1-DD4 (1.7%), MW1-Syn (1.5%), MW2-DD4 (13.6%), and MW2-Syn (13.2%). It was also detected in MW1-Initial (0.2%) and MW1-Propane (0.1%). OTU_5 has >99% identity in comparison with a polychlorinated biphenyl (PCB) degrading species *Rhodococcus* sp. YAZ54 (Hara et al., 2016) and a hexahydro-1,3,5-trinitro-1,3,5-triazine (RDX) degrading species *Rhodococcus* sp. BL1 (Chong et al., 2014). As shown in the phylogenetic tree (Fig. 4), OTU_5, YAZ54, and BL1 form a branch that is distinctly divergent with other known propanotrophic *Rhodococcus* (e.g., TAP and RR1). Thus, OTU_5 may be less related to dioxane degradation or propane consumption, but more associated with the downstream assimilation of dioxane or propane metabolites (i.e., *β*-hydroxyethoxyacetic acid [HEAA] and 1-propanol) through the cross-feeding by DD4 and other propanotrophic degraders.

4. Discussion

4.1. Azoarcus sp. DD4 can retain high degradation activity and dominance during the bioaugmentation

Microcosm results showed that dioxane was completely degraded within 6 days in the microcosms prepared with INF, EFF, and MW1 when amended with DD4 and propane. It revealed that DD4 was able to exert its degradation capability in *ex situ* field samples (i.e., INF and EFF) as well as some specific in situ field samples (i.e., MW1), as the result of its adaptability to groundwater constituents and indigenous microbiomes (Wenderoth et al., 2003). Note that the growth of DD4 was greatly hindered in MW2, as evident by the slow propane consumption and partial dioxane degradation (Fig. 1). Field characterization detected VOCs and metals in the groundwater samples from this investigated site (Table S1). Some VOCs (e.g., trichlorethlyene [TCE] and VC) were only at single-digit μg/L levels, though exceeded their groundwater standard. Although TCE and other VOCs were reported as important inhibitors for dioxane degradation (Li et al., 2020; Mahendra et al., 2013; Zhang et al., 2017), the low concentrations of VOCs in MW2 may modestly contribute to incomplete degradation by either DD4 or the synthetic consortium. However, iron and aluminum were particularly high in MW2 at 40 and 0.33 mg/L, respectively (Table S1). Such high metal concentrations may pose negative effects on DD4, underscoring further investigation. Though uncharacterized in the collected samples, other

abiotic factors, particularly hydrogeochemical parameters (e.g., nitrate and sulfate), can also affect microbial behaviors and thus the biodegradation of dioxane in water, warranting assessments in future studies. Furthermore, the community diversity analysis revealed that MW2 initially has the highest Shannon Index (6.00) among all the samples (Table S5. The Shannon Index slightly dropped to 5.55 and 5.49 after DD4 and the consortium was introduced, though remain much higher than other bioaugmented samples (Table S5). Such high diversity in MW2 may reflect its microbial community stability and functional resilience with external perturbation (Girvan et al., 2005). Thus, the exogenous inoculum DD4 or the synthetic consortium might have been outcompeted by the complex native microbiomes in MW2.

16S rRNA sequencing and qPCR analysis independently verified that DD4 became dominant after the bioaugmentation treatments. These results well support the dioxane degradation observed in the microcosms, confirming the significant role of DD4 in dioxane biodegradation. It also suggests the adaptability and compatibility of DD4 in these dioxane-impacted water samples. Therefore, single DD4 culture would be effective in treating dioxane in either influent or effluent samples as an addendum to the GWET system being operated at the site. This upgrade of the existing pump-and-treat system can be applicable at many sites impacted by both VOCs and dioxane. In addition, effectiveness of in situ bioaugmentation with DD4 and propane can be site or location-specific to mitigate dioxane contamination. However, due to the specificity and complexity of field conditions, feasibility tests are recommended to discern the effectiveness at different sites or even different locations at a single site.

4.2. Exogenous metabolizers may decay and transmit dioxane-degrading genes to the native microbiomes

The addition of two dioxane metabolizers (i.e., PH-06 and CB1190) didn't greatly improve dioxane degradation. On the contrary, neither PH-06 nor CB1190 remained detectable by the 16S rRNA amplicon sequencing after the treatment. Thus, direct contribution of these two exogenous metabolizers to the observed dioxane removal might be minimal. The noticeable decay of CB1190 and PH-06 was likely due to the outcompetition by DD4 and/or native microbiomes. First, DD4 is quite efficient in propane assimilation, which supports a high growth rate (1.95 ± 0.01 day^{-1}) (Deng et al., 2018b). In contrast, CB1190 and PH-06 are Actinomycetes, reputed for slow growth. As the hierarchical clustering dendrogram shown in Fig. 3, microbial communities after the bioaugmentation with DD4 and the synthetic consortium showed high similarity, indicating the minimal ecological impacts resulted from the addition of CB1190 and PH-06. Previous study on bioaugmentation with CB1190 or *Rhodococcus ruber* ENV425 with the initial dioxane concentration of ~250 μg/L also indicated that exogenous dioxane degraders decreased at the end of the treatment especially for metabolizer CB1190 comparing with co-metabolzer ENV425. CB1190 rapidly decreased from 26.96% to 1.12% only in 3 days. ENV425, however, remained dominant at day 30 with the abundance of 23.38%. The persistence of ENV425 in varying environmental conditions was possibly attributed to its diverse substrate range and ability of producing bioactive steroids and other chemicals that promote their viability (Miao et al., 2020).

Though PH-06 and CB1190 diminished at the end of incubation, it is interesting that *prm* and *thm* remained detectable in the synthetic consortium-inoculated treatments (EFF-Syn, MW1-Syn, and particularly MW2-Syn). This suggested these dioxane degradation genes were retained in the communities, even though at low levels. Considering the *prm* and *thm* genes are both located on plasmids (Garbisu et al., 2017; Sales et al., 2011a), these plasmids or gene fragments were likely to be horizontally transferred to the phylogenetically close relatives (e.g., *Mycobacterium, Pseudonocardia, Rhodococcus*) that are native in the field (Leahy et al., 2003; Popa et al., 2011).

One OTU belonging to *Rhodococcus* (i.e., OTU_187) is postulated as the potential recipient of these dioxane degradation genes or their carrying plasmids. OTU_187 is unique to synthetic consortium-bioaugmented treatments indicating its relevance with the inoculation of PH-06 and CB1190. OTU_187 exhibits high similarity with *Rhodococcus aetherivorans* 10bc312 (= JCM 14,343), *Rhodococcus aetherivorans* TPA, and *Rhodococcus* sp. RR1. 10bc312 is an isolate from a methyl tert-butyl ether enrichment (Goodfellow et al., 2004) and contains the gene cluster for a group-5 propane monooxygenase (Inoue et al., 2016). Previous report has confirmed that this strain can use dioxane as sole carbon source, although it appeared not to be an effective dioxane degrader considering the low degradation rate (0.0073 mg-dioxane/mg-protein/h) and affinity (59.2 mg/L) (Inoue et al., 2018). Besides, *R. aetherivorans* TPA is a propane-utilizing bacterium (Cappelletti et al., 2018) and *R.* RR1 is a potential propanotroph because it harbors a complete gene cluster encoding a group-5 propane monooxygenase (He et al., 2015). Moreover, OTU_187 is found correlated with *prmA* (evaluated by the Spearman's coefficient 0.5 and p value 0.056) (Fig. 4 and Table S4), but not *thmA*. Thus, it is likely that OTU_187 received the *prm*–carrying plasmid or fragment from PH-06 and emerged with the dioxane degradation ability in groundwater samples tested in this study.

4.3. Native mycobacterium may participate in propane utilization

Propane consumption occurred after 9 days in all propane-stimulated treatments, indicating the extensive existence of propanotrophs in groundwater samples collected at this site. Widespread occurrence of native propanotrophs has been previously reported in aquifers or other environments (Kotani et al., 2006; Li et al., 2013; Tupa and Masuda 2018). However, no significant dioxane removal was observed in these propane-fed treatment except in INF-Propane. Correspondingly, 16S rRNA sequencing results in Fig. 3 showed that INF-Propane (printed in green) has a distinct microbial community structure that is divergent from the well-treated subgroup (printed in blue) and the poorly treated subgroup (printed in red). Thus, INF-Propane possesses a unique indigenous bacterial community associating with propane utilization and dioxane degradation. However, after re-examining the 10 most abundant genera in INF-Propane (e.g., *Rhizobacter* and *Sulfuritalea* in Table S3), to our best knowledge, no relevant research reported their degradation capacities related to dioxane or propane. We intend to continue the enrichment and make efforts to identify and isolate the indigenous dioxane degrading propanotroph(s), which may be well suited for both in situ and *ex situ* treatments at this site (Inoue et al., 2021).

To be noted, the abundance of OTU_52, a native *Mycobacterium*, was very high in MW1-Propane, accounting for 7.3% of the total community. Although dioxane degradation was not observed in MW1-Propane, 52% of propane has been removed from day 9 to day 30. It suggests that this indigenous species (i.e., OTU_52) might first utilize the relatively high organic carbon (12,000 μg/L) and then propane supported their growth after 9 days. The OTU_52 was also frequently detected in initial field samples and those that were biostimulated with propane (Fig. 4). Considering its close phylogeny with NBB3, this native *Mycobacterium* species is likely a propanotroph without the ability of degrading dioxane. The other reason to explain absence of dioxane degradation in some treatments is due to the greater affinity to propane than dioxane for group-6 propane monooxygenases expressed by propanotrophic dioxane-degraders (Barajas-Rodriguez and Freedman 2018; Li et al., 2017) In this case, propane is preferably used prior to dioxane. Thus, dioxane degradation may occur if the incubation time is extended after the majority of propane is consumed.

4.4. TmoA is a suitable biomarker for the quantitative assessment of dioxane removal rates by DD4 bioaugmentation

The correlation analysis in Fig. 5 demonstrated *tmoA* as an effective biomarker to assess dioxane degradation rates observed in different treatments that received the inoculation of DD4 or the synthetic consortium. The absolute copy numbers of *tmoA* are significantly correlated

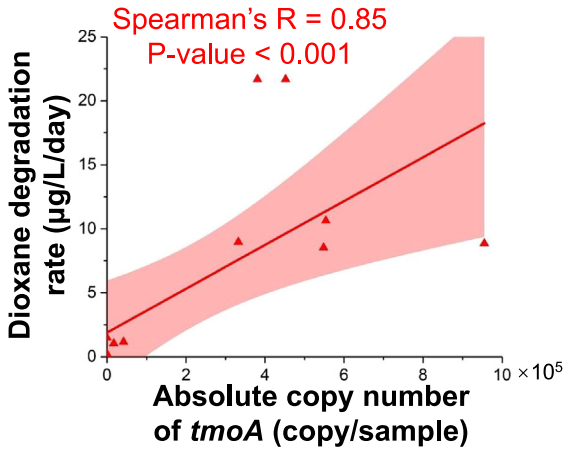

Fig. 5. Positive linear correlation between dioxane degradation rate (μg/L/day) and absolute copy number of *tmoA* ($\times 10^5$ copy/sample). The fitted line is forced through the origin.

with the dioxane degradation rates according to the Spearman's coefficient of 0.85. This indicated that the *tmoA* probe/primers specifically target to the *tmo* gene cluster in DD4 and can accurately reflect the abundance of DD4 and their activity in the environmental samples. The slope of the correlation is estimated as 2×10^{-5}, which may be useful for the prediction of dioxane biodegradation rate by DD4 bioaugmentation based on the *tmoA* copy number detected in the environmental samples.

5. Conclusions

Considering the low dioxane concentrations at the contaminated plume and treated waters (<100 μg/L), the cometabolizer, *Azoarcus* sp. DD4, is a potent candidate for dioxane cleanup at this site via in situ or *ex situ* approaches. DD4 exhibits its competitive capability and compatibility to different site samples with or without artificially induced dioxane metabolizers, PH-06 and CB1190. Interestingly, the dioxane degradation gene clusters from these two metabolizers have been partially retained in the communities possibly through horizontal gene transfer despite the complete disappearance of their host cells. According to the molecular results, *Rhodococcus* may involve in acquiring catabolic genes during the incubation. An indigenous propanotrophic *Mycobacterium* was found possibly to take part in propane consumption, but not dioxane degradation. The significant correlation between the dioxane degradation gene abundance in DD4 and dioxane degradation rate observed in microcosms indicates *tmoA* is a suitable biomarker to monitor and assess DD4 bioaugmentation in future applications.

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.

Data availability

Data will be made available on request.

Acknowledgments

This work was supported by US Environmental Protection Agency Region 2, National Science Foundation (NSF, CAREER CBET-1846945, CHE-2203616), United States Geological Survey (USGS) State Water Resources Research Act Program (2018NJ400B), Strategic Environmental Research and Development Program (ER23–3830), and the start-up fund from the Department of Chemistry and Environmental Science at NJIT. We sincerely appreciate Dr. Jim Claffey and Dr. Karnam Ramanand (Brown and Caldwell) for field sample collection and Sumbel Yaqoob (NJIT) for the graphic assistance. The funders had no role in study design, data collection and interpretation, or the decision to submit the work for publication.

Funding

This work was supported by US Environmental Protection Agency Region 2, National Science Foundation (NSF, CAREER CBET-1846945, CHE-2203616), United States Geological Survey (USGS) State Water Resources Research Act Program (2018NJ400B), Strategic Environmental Research and Development Program (ER23–3830), and the start-up fund from the Department of Chemistry and Environmental Science at NJIT.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.hazadv.2023.100246.

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