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Emerging investigator series: Environment-specific Auxiliary Substrates Tailored for Effective Cometabolic Bioremediation of 1,4-Dioxane

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1,4-Dioxane is one of the most prevalent water contaminants in the world. This study provides cost-efficient bioremediation strategies to accelerate the mitigation of 1,4-dioxane contamination in a range of aquatic matrices. The formula that couples 1,4-dioxane degrading bacteria and appropriate substrates can be designed and tailored to optimize the overall treatment effectiveness in the field.

Emerging investigator series: Environment-specific Auxiliary Substrates Tailored for Effective Cometabolic Bioremediation of 1,4-Dioxane

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Abstract

Cometabolic bioremediation is trending for the treatment of 1,4-dioxane (dioxane) and other emerging contaminants to meet stringent regulatory goals (e.g., <10 μg/L) since the biodegradation activities can be fueled by the supplementation of auxiliary substrates. In this study, we compared and investigated the effectiveness of two types of common auxiliary substrates, short-chain alkane gases (e.g., propane and butane) and primary alcohols (e.g., 1-propanol, 1-butanol, and ethanol), for dioxane removal in diverse environmental matrices with *Azoarcus* sp. DD4 as the inoculum. Physiochemical characterization at the pure culture level revealed that propane and 1-propanol are advantageous for stimulating cell growth and dioxane biodegradation by DD4. Parallel microcosm assays were conducted to assess the compatibility of DD4 bioaugmentation in diverse microbiomes recovered from five different environmental samples, including shallow and deep aquifer groundwater, contaminated river sediment, and municipal activated sludge. Propane was effective in sustaining efficient dioxane removal and the dominance of DD4 across all environmental matrices. Notably, amendment of 1-propanol promoted superior dioxane degradation in the deep aquifer groundwater, in which low pre-treatment biomass and post-treatment diversity were observed, suggesting its potential for intrinsic field applications. The combination of microbial community analysis and differential ranking identified *Ochrobactrum* and several other indigenous bacteria were boosted by the inoculation of DD4, implying their commensal or mutualistic relationship. Collectively, propane and 1-propanol can be effective auxiliary substrate alternatives tailored for *in situ* bioaugmentation and their effectiveness is affected by the density and structure of environmental microbiomes.

Key Words

1,4-dioxane, cometabolic biodegradation, groundwater, *Azoarcus*, *Ochrobactrum*

Introduction

1,4-Dioxane (dioxane) is one of the most prevalent and persistent emerging water contaminants, posing prominent threats to human health and environmental safety (1). Animal tests revealed chronic and acute exposure to dioxane can impair nervous systems and elicit carcinogenic and non-carcinogenic responses in liver and kidney (2). Accordingly, dioxane is classified as a probable human carcinogen (Group B2) (3) and is subject to a stringent health advisory level of 0.35 μg/L at the 10^{-6} lifetime cancer risk (4). Unfortunately, dioxane has been detected in groundwater at sites (e.g., Superfund and Brownfield) impacted by chlorinated solvents, because dioxane was primarily used as a solvent stabilizer particularly for 1,1,1-trichloroethane (TCA) (5). Recent surveys also indicated that dioxane co-occurs with trichloroethene (TCE) at an exceedingly high frequency (> 90%) (6, 7). To date, over 1,000 out of the current or former National Priority List (NPL) sites (approximately 1,700 in total) have been reported with the contamination of TCA and/or TCE. Based on a conceivable extrapolation, dioxane contamination represents an imminent concern at a significant number of NPL sites, particularly for those that are subject to the renaissance of field monitoring and re-visiting (8).

Driven by the recent research advance on dioxane biodegradation, *in situ* cometabolic bioremediation is trending as a cost-efficient and environment-friendly treatment alternative to mitigate dioxane contamination (9-13). Through cometabolic biodegradation, the growth and activity of dioxane degrading microorganisms can be sustained by the continuous supplementation of auxiliary substrates. Thus, the removal of dioxane is independent of its contamination level and

can be achieved to extremely low levels (e.g., parts-per-billion) or even non-detect (14). For cometabolic bioremediation, there are three properties for desirable auxiliary substrates: (1) they are readily degradable and support fast growth of the exogenous inocula (for bioaugmentation) or indigenous degraders (for biostimulation); (2) they induce the expression of degradation enzymes; and (3) they are non-hazardous and cost-efficient. Previous studies on dioxane cometabolic degradation were centered on utilizing propane, iso-butane, or other short-chain alkane gases as the auxiliary substrates (13, 15-17). Application of gaseous substrates has been recently demonstrated with effective dioxane removal in the field (16, 18, 19). Groundwater recirculation is an effective way to deliver low levels of gaseous alkanes at precise concentrations with the assistance of gas-flow controllers (18).

Compared to gaseous alkanes, liquid alcohols (e.g., 1-propanol and 1-butanol) can be advantageous considering their ease of dosing at the injection wells and monitoring through routine water sampling. Liquid alcohols are highly soluble or even miscible, allowing direct injection of high concentrations without the need of specialized distribution systems to optimize the radius of influence. Liquid alcohols are also safer for storage and use, while gaseous alkanes like propane are a concern due to their flammable and explosive nature, requiring appropriate onsite management. However, liquid alcohols are not specific to dioxane degraders. Many native bacteria can grow with liquid alcohols, which thus may pose the potential to outcompete the dioxane degraders. Therefore, it is of great application value to discern the effectiveness of liquid alcohols for bioremediation in a range of environmental matrices consisting of different microbiomes.

With liquid alcohols as the auxiliary substrates, cometabolic degradation of dioxane and co-existing chlorinated compounds has been recently demonstrated with a few bacterial cultures. *Mycobacterium vaccae* JOB5 and *Rhodoccoccus jostii* RHA1 were reported to cometabolize

dioxane and TCE when fed with 1-butanol (20). *Rhodococcus rhodochrous* ATCC 21198 exhibited continuous biotransformation of dioxane and TCA using 1-butanol, 2-butanol, and 2-propanol leaching from the hydrolysis of orthosilicates (21). Similar to other well-characterized dioxane degrading species (22-26), these strains belong to *Rhodococcus* and *Mycobacterium*, which are gram-positive Actinomycetes that tend to aggregate and the formed clumps can hinder subsurface distribution for bioaugmentation.

Azoarcus sp. DD4 is a gram-negative propanotroph that can effectively degrade dioxane in microcosm studies to below 0.4 μg/L without noticeable clumping behaviors (27). When grown with propane, DD4 can also decompose several chlorinated aliphatic hydrocarbons (e.g., 1,1-dichloroethene [1,1-DCE] (27), *cis*-1,2-dichloroethene [cDCE], and vinyl chloride [VC] (28)) that commonly co-occur with dioxane in groundwater as biotic and abiotic products from TCA and TCE attenuation (5). Further molecular studies revealed a diversity of soluble di-iron monooxygenase (SDIMO) genes in the genome of DD4 (29). Using knockout mutations and heterologous expression, a unique toluene monooxygenase (TMO) was identified to be responsible for the initial oxidation of dioxane and 1,1-DCE. Expression of this TMO appeared inducible by both propane and its primary oxidation product, 1-propanol, implying their potentials as auxiliary substrates for cometabolic bioremediation (30). Furthermore, unlike the majority of dioxane degrading Actinomycetes (31), DD4 has the *tmo* gene on its chromosome (29). This is important as it precludes the loss of the essential dioxane degradation genes when the culture is fed with liquid alcohols and other non-selective substrates that are readily biodegradable (32). Though effective dioxane removal by DD4 has been demonstrated mostly in its pure culture, knowledge remains scarce regarding the feasibility of using different auxiliary substrates (e.g., gaseous alkanes and liquid alcohols) for *in situ* bioaugmentation in a diversity of environments where

dioxane contamination has been frequently reported. Our central hypothesis is that the abundance and composition of native microbiomes in the environment can affect their competition with DD4 for substrates, particularly those like liquid alcohols that are readily biodegradable.

Though dioxane contamination is widespread, recent microbial ecology analyses revealed that dioxane degradation genes are not ubiquitous across environments (33, 34). When dioxane degradation genes are absent, *in situ* bioaugmentation can be a bioremedial option for site cleanup. In this study, we chose DD4 as the inoculum and investigated the effectiveness of short-chain alkanes and primary alcohols as auxiliary substrates to promote dioxane cometabolic degradation in three field groundwater samples and other matrices (e.g., wastewater activated sludge and river sediments) where dioxane have also been frequently detected (35-37) but extensive engineering can be challenging. A novel biomarker specific to TMO was developed and validated to facilitate the monitoring of DD4 in complex environmental samples. Furthermore, microbial community analysis enabled the evaluation of the shifting of environmental microbiomes in response to the amendment of different inoculum-substrate formulae. The differential ranking technique was used to uncover the native bacteria that are potential satellites of DD4, which may assist in dioxane or co-contaminant removal or some other associated processes. This study identified inoculum-substrate formulae that can be tailored for effective *in situ* cometabolic bioremediation, to control and mitigate dioxane contamination in a range of aquatic environments.

Materials and Methods

Physiological and molecular characterizations of DD4 in response to auxiliary substrates

Growth yield and doubling time of DD4 were assessed by growing cells on gaseous alkanes (propane and butane), alcohols (1-propanol, 1-butanol, and ethanol), and pyruvate. The initial inoculation biomass was adjusted to OD_{600} of 0.01. Individual substrate was amended at the initial

concentration of 5.0 mM (or equivalent in the aqueous phase for gaseous alkanes) in 20 mL nitrate mineral salt (NMS) media in 160-mL serum bottles. All treatments were aerobically cultivated at 30 °C and sampled at select intervals to monitor substrate and biomass concentrations. Cell yield was estimated from the plot of total protein produced against the substrate consumed. Specific growth rate (μ) was calculated by fitting an exponential growth model ($X=X_0 \cdot e^{\mu t}$) to the OD_{600} data, and the doubling time was ln2/μ.

Transcription of the *tmoA* gene was evaluated by reverse transcription-quantitative PCR (RT-qPCR) using RNA extracted from the DD4 cells harvested after the exponential growth with individual substrate. Glucose was used as the control substrate and 16S rRNA gene was used as the housekeeping gene. The total RNA was extracted using the PureLink RNA Mini Kit (Thermo, Carlsbad, CA) according to the manufacturer's protocol, in combination with an on-column PureLink DNase Treatment (Thermo, Carlsbad, CA) to eliminate the interference from DNA. cDNA was synthesized using the High-Capacity cDNA Reverse Transcription Kit (Thermo, Carlsbad, CA) and then purified using the DNA Clean & Concentrator™-5 Kit (Zymo, Irvine, CA). RT-qPCR mixtures contained 1 μL of diluted cDNA (5 ng/μL), 10 μL of 2 × Power SYBR Green PCR Master Mix (Thermo, Carlsbad, CA), 0.3 μM of forward and reverse primers, and DNA-free water to a total volume of 20 μL. RT-qPCR was performed with a QuantStudio™ 3 Real-Time PCR System (Thermo, Carlsbad, CA) with the following temperature setup: 95 ºC for 10 min, and 40 cycles of 95 ºC for 15 s and 60 ºC for 1 min. Primers and other materials used in the RT-qPCR procedures are described in our previous publication (30). Differential gene expression was quantified using the $2^{-\Delta\Delta Cq}$ method (32) with *tmoA* as the target gene and 16S rRNA as the housekeeping gene, respectively. The expression fold change was calculated with the following formula.

$$\Delta\Delta C_{q,\text{ Target gene}} = (C_{q,\text{ Target gene}} - C_{q,\text{ Housekeeping gene}})_{\text{ Treatment}} - (C_{q,\text{ Target gene}} - C_{q,\text{ Housekeeping gene}})_{\text{ Control}}$$

Resting cell assays were conducted to estimate the apparent specific biodegradation rates by DD4. DD4 cells were pre-cultured and harvested at the exponential growth phase in NMS media that contained individual substrate as the sole carbon and energy source. After being washed three times with NMS media, cells were concentrated to OD_{600} of 1 in 6 mL NMS in 35-mL serum bottles. Then, dioxane or 1,1-DCE was spiked at the initial concentration of 100 mg/L. The apparent specific biodegradation rates were estimated by the disappearance of dioxane or 1,1-DCE between time 0 and 2 h and normalized to the initial biomass of resting cells.

Microcosm assays using different environmental matrices

To further assess the effectiveness of different auxiliary substrates for *in situ* bioaugmentation of DD4 and the compatibility with native microbiomes, five environmental samples were collected, including two groundwater samples (SGW1 and SGW2) from two shallow aquifers (less than 10 ft below water table [BWT]) in Florida and South Carolina, respectively, one groundwater sample (DGW) from a deep bedrock aquifer (48~75 ft BWT for the screen interval) in New Jersey, one activated sludge sample (SLU) from the aeration tank of a local wastewater treatment plant (WWTP) in New Jersey, and one sediment sample (SDT) in the Hackensack River, New Jersey, which is contaminated with organic compounds, including polycyclic aromatic compounds (PAHs) and polychlorinated biphenyl compounds (PCBs) above New Jersey's state sediment screening benchmarks. To collect biomass for even distribution in the aerobic microcosms, all samples were centrifuged at 10,000 ×g for 10 min at 4 °C. Then, 0.3 g of the pellets (wet weight) from each of the four samples (SGW1, SGW2, SLU, and SDT) was measured, washed twice using PBS to remove the excess organics that may greatly hinder dioxane degradation, and suspended in 230 mL of their original waters. An aliquot of 25 mL mixed slurry,

consisting of 32.6 mg solid pellet/bottle, was transferred into a 160-mL serum bottle. Biomass in the DGW samples was extremely low and thus dosed at 2 mg pellet/bottle. Due to the trace dioxane contamination detected in all environmental samples (< 10 μg/L) and limited volume of samples we received, dioxane concentration was dosed to 10 mg/L in all treatments so that time series sampling can be conducted over a long period of incubation time. DD4 inoculum (0.010±0.001 mg of protein/bottle, equivalent to 6.6×10^2 CFU/mL) and 100 mg/L of each test substrate (total concentration equivalent as in the aqueous phase) were then amended. Substrates were amended again when >90% was consumed. Aliquots and headspace samples were periodically collected to monitor the depletion of dioxane and consumption of auxiliary substrates (propane, 1-propanol, and ethanol) by GC-FID. At the beginning and end of the aerobic microcosm assays, pellets were collected after centrifugation at 10,000 ×g for 10 min at 4 °C. Genomic DNA was extracted using the PowerSoil® DNA Isolation Kit (Qiagen, Hilden, Germany) for qPCR analysis and 16S rRNA amplicon-based sequencing as detailed below. Positive treatment with DD4 in NMS media was prepared to discern the impacts of environmental matrices and indigenous microbiomes. Negative controls were conducted using DD4 cells killed by autoclave to distinguish the abiotic loss of dioxane and substrate compounds. All treatments were performed in triplicate.

Monitoring of inoculated DD4 using quantitative PCR (qPCR)

The copy numbers of *tmoA* and 16S rRNA genes were used for the enumeration of DD4 cells and total biomass in microcosms, respectively. TaqMan quantitative PCR assay was employed to quantify the *tmoA* gene in DD4. The PCR mixture contained 10 ng DNA, 300 nM forward and reverse primers, 150 nM probe, 10 μL of TaqMan universal master mix II (Applied Biosystems, Foster City, CA), and DNA-free water, yielding a total volume of 20 μL. DD4 specific primers and probe used was specifically designed with the sequences: 5'-GGC GGA TGG CTG

TAC TCA ACA GAA TG-3' for DD4tmo_F, 5'-AAA TCG CCG GAA AGC TTG GGC-3' for DD4tmo_R, and 5'-/6-FAM/CGA CCT GGC /ZEN/ CAG GAG TAC GAA C/IABkFQ/-3' for DD4tmo_P. To determine the total bacteria, SYBR Green qPCR was conducted using 16S rRNA as a target gene representing the total bacteria accordingly. The sequences for the universal 16S rRNA primers are 5'-CCT ACG GGA GGC AGC AG-3' for 341F and 5'-ATT ACC GCG GCT GCT GG-3' for 534R. qPCR was performed with a Quant Studio 3 Real-Time PCR system (Thermo, Waltham, MA) using the following temperature program: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions (10^{-5} to 1 ng/μL) of the extracted genomic DNA of DD4 were used to prepare the calibration curves for both *tmoA* (one copy per genome) and 16S rRNA (four copies per genome) genes.

Microbial community analysis

After amplification and library construction, 16S rRNA sequence reads were obtained by the Illumina HiSeq2500 platform and then processed using the QIIME2 pipeline (v2020.2.0) (38) with the Divisive Amplicon Denoising Algorithm 2 (DADA2) (39) for sequence pairing, denoising, and chimera elimination. For the taxonomy assignment, operational taxonomic units (OTUs) were generated at 97% of nucleotide sequence similarity and searched against the GenBank database using the NCBI BLASTN for top hits with the lowest e values (40).

Differential ranking analysis was performed to identify taxa favored by different auxiliary substrates (i.e., propane or 1-propanol) using Songbird (v1.0.3) (41). First, the term "differential" in this algorithm refers to the logarithm of the fold change in abundance of a taxa between two conditions (i.e., propane or 1-propanol versus initial). Then, coefficients computed by multinomial regression analysis are used to rank the relative differentials of all taxa and determine those with greatest changes. Feature ranking and log-fold change were subsequently visualized using Qurro

(v0.7.3) (42). High-rank taxa (i.e. positive log-fold change) were recognized as potential "propane-associated" or "1-propanol-associated" since they are important contributors with significant increases in propane or 1-propanol treatment relative to the initial communities. Ranking taxa on the basis of their log-fold changes mitigates compositional artifacts caused by the variance in total microbial loads among samples as the bias is uniformly distributed across the differential (41). Details about the microbial community analysis are in the supporting information (SI).

Results and Discussion

Propane and 1-propanol as superior auxiliary substrates

Physiological characterization of DD4 (Table 1) suggested that propane and 1-propanol were better auxiliary substrates than butane, 1-butanol, and ethanol, considering the cell yield, growth rate, and induction of the key enzyme (i.e., TMO) responsible for dioxane biodegradation. First, cell yield was highest for the growth with propane (0.25±0.00 mg protein/mg substrate), indicating propane is most energy-proficient for DD4. 1-Propanol, butane, and 1-butanol generated relatively lower yields between 0.19 and 0.22 mg protein/mg substrate, followed by ethanol and pyruvate. Second, doubling time was shortest when DD4 was fed with 1-propanol (4.81±0.51 h), followed by 1-butanol (6.33±0.30 h). This suggested the assimilation of primary alcohols is more efficient than their corresponding alkanes, probably because terminal oxidation of alkanes to form primary alcohols consumes energy and requires robust enzymes (e.g., SDIMOs) for catalysis. In addition, all four C3 and C4 auxiliary substrates (i.e., propane, 1-propanol, butane, and 1-butanol) can effectively upregulate the transcription of the *tmoA* gene in similar fashions (~3 folds compared to the housekeeping gene). In contrast, lower and marginal inductions were observed for ethanol (1.38±0.22 folds) and pyruvate, respectively.

Based on biotransformation assays using resting cells, for both dioxane and 1,1-DCE, propane and 1-propanol-grown DD4 cultures exhibited higher apparent degradation rates than those fed with butane, 1-butanol, or ethanol. Neither dioxane nor 1,1-DCE was degraded by pyruvate-fed DD4, since pyruvate could not activate the transcription of *tmoA*. As shown in Figure S1, 1,1-DCE cometabolic degradation was demonstrated by growing DD4 cells in NMS media. Interestingly, 1-propanol is most efficient in stimulating 1,1-DCE biotransformation. Within 4 days of incubation, propane-fed DD4 showed ~50% less 1,1-DCE removal than those grown with 1-propanol. Since biomass-normalized 1,1-DCE transformation rates were similar between DD4 resting cells fed with propane and 1-propanol (69.4±1.1 vs 66.4±7.4 μg 1,1-DCE h^{-1} mg $protein^{-1}$ in Table 1), the greater 1,1-DCE removal observed in growing cell assays in 1-propanol-fed microcosms was probably due to its rapid assimilation and growth (shortest doubling time at 4.81±0.51 h in Table 1). Ethanol was found ineffective for promoting 1,1-DCE cometabolism. Thus, converging lines of evidence corroborated that propane and 1-propanol are better suited for stimulating the growth of DD4 and its activity of degrading dioxane and 1,1-DCE.

Distinct dioxane removal in environmental matrices

To further assess the efficacy of DD4 bioaugmentation, propane, 1-propanol, and ethanol were selected and compared as the auxiliary substrates in five environmental matrices with microbiomes of different abundances and compositions. Propane was found the most effective in sustaining the dioxane removal (Figure 1) and DD4 population (Figure 2), followed by 1-propanol and then ethanol, in shallow groundwater (SGW1 and SGW2), activated sludge (SLU), and river

sediment (SDT). Notably, in deep groundwater (DGW), 1-propanol amendment stimulated fast dioxane removal in a similar fashion to propane amendment (Figure 1c). Complete dioxane removal was achieved within 3 days after a quick consumption of 1-propanol on the first day. Such efficient dioxane removal was repeated after the re-spiking of dioxane and 1-propanol. On Day 6, DD4 population reached $8.45\pm1.97\times10^5$ *tmoA* copies/mL, accounting for nearly half of the total bacteria (Figure 2). These results demonstrate that 1-propanol can be used as an effective auxiliary substrate for DGW. There are two possible explanations. First, DGW has a much lower concentration of indigenous biomass as compared to other environmental matrices. Secondly, native microbiomes in DGW can be predominantly oligotrophic and do not adapt well with 1-propanol as a substrate. This was evident by the discernable decrease in richness (Chao1, from 1045 to 68) and diversity (Shannon, from 6.46 to 2.90) after the amendment of 1-propanol as compared to the initial condition (Table S1). Such postulations can further validated in the future with tests with more samples from deep aquifers.

In the two shallow groundwater samples (SGW1 and SGW2), dioxane degradation was prolonged for all three substrates (Figure 1a and 1b) as compared to DGW and the positive control in NMS (Figure 1c and 1f). When propane was used as the auxiliary substrate, a minimum lag of 3 days for dioxane degradation was experienced, probably due to the delayed propane consumption. Complete removal of dioxane was observed after 7 days of incubation. However, dioxane degradation was greatly accelerated after the re-amendment of dioxane and propane. Both dioxane and propane were removed within 3 days between Day 7 and 10, leading to the increase of DD4 population to $5.58\pm2.83\times10^5$ (SGW1) and $9.61\pm1.55\times10^5$ (SGW2) *tmoA* copies/mL (Figure 2a). Thus, repetitive amendments of propane to shallow groundwater samples can acclimate DD4 and promote dioxane removal efficiencies. In the activated sludge and sediment samples (SLU and

SDT), dioxane degradation rates were further decreased probably due to the complexity of native microbiomes in both samples (Table S1, see discussion in SI). After two amendments of propane, dioxane was completely removed in SLU by Day 12. However, neither propane consumption nor dioxane degradation occurred in SDT until Day 7. At Day 17, only 63% of the initially dosed propane was consumed and dioxane was degraded from 10.6±0.2 to 4.0±0.5 mg/L. DD4 population was estimated as $1.72\pm0.66\times10^5$ *tmoA* copies/mL in SDT at Day 17, which was significantly lower than other environmental matrices that also received propane.

When 1-propanol was used as the auxiliary substrate, approximately one-third of the initially dosed dioxane was removed in SGW1 (40.6%), SGW2 (33.7%), SLU (34.6%), and SDT (22.9%) by Day 17, while 1-propanol was consumed rapidly without lag. Ethanol was found least effective to support dioxane biodegradation by DD4. Multiple ethanol amendments resulted in significantly lower dioxane removal in SGW1 (25.7%), SLU (21.2%), and SDT (15.1%) by Day 17. No dioxane removal was observed in SGW2. Accordingly, qPCR results (Figure 2a and 2b) revealed decreasing trends for DD4 populations (both absolute and relative) when the auxiliary substrate was alternated from propane to 1-propanol to ethanol. Native microorganisms in environmental samples were likely competitive in consuming these alcoholic compounds that are readily biodegradable and/or generating factors that hinder the growth of DD4 or its activity. No significant removal of dioxane or substrates in abiotic controls (Figure S2). Collectively, both propane and 1-propanol can stimulate dioxane degradation by DD4 in all environmental matrices, though their effectiveness may vary greatly as affected by the native microbiome compositions and their total biomass.

Coupled growth of DD4 and Ochrobactrum

The dominance of DD4 at the beginning and the end of all microcosms was evident by the microbial community analysis (Figure 2 and S3). The OTU_1 sequence showed 100% identity to the 16S rRNA gene of DD4 (Table S2), counting for 0.07~0.11% in the initial samples, 10.35~60.58% after the propane amendments, and 0.19~37.20% after the 1-propanol amendments (Figure 2). Further, based on combined results from all environmental matrices, a positive correlation was established between relative abundances of DD4 estimated via the 16S rRNA amplicon-based sequencing (x-axis) and qPCR (y-axis) (Figure 3). The slope of the linear correlation was 0.93±0.13, approaching 1. This validated the specificity and effectiveness of using our designed *tmoA* biomarker for the monitoring and quantification of DD4 in diverse environments.

16S rRNA sequencing analysis revealed 43,225 to 148,588 bacterial taxa in 15 samples collected at the beginning and end of incubation in microcosms that mimic active treatments. As the 2D-PCoA plot shown in Figure S4, the microbial communities shifted greatly but differently (except DGW) in response to the propane and 1-propanol amendments. By using differential ranking, 4 bacterial taxa were identified with strong association with DD4 bioaugmentation across all 5 environmental matrices when propane or 1-propanol was fed as the auxiliary substrate (Figure 4). Notably, *Azoarcus* sp. DD4 (OTU_1) and *Ochrobactrum* sp. (OTU_2) were positively enriched after the bioaugmentation treatment (log ratios between "propane" and "initial" were 1.68 and 0.57, respectively, and log ratios between "1-propanol" and "initial" were 2.47 and 0.73, respectively).

In contrast, *Rhodococcus erythropolis* (OTU_5) was negatively influenced (log ratio between "propane" and "initial" was -4.70 and log ratio between "propanol" and "initial" was -1.91). Regardless of the auxiliary substrate, the enrichment of the indigenous *Ochrobactrum* sp. in all environmental matrices after the introduction of DD4 implied a communal or mutualistic relationship between these species. However, the other indigenous species, *Rhodococcus erythropolis*, is likely a propanotroph (43, 44) and was outcompeted by the exogenous inoculum DD4. These results indicated that DD4 was not only able to sustain its dominance across different environmental matrices with propane or 1-propanol as the auxiliary substrate, but can also alter and coordinate with the indigenous microbiomes to possibly promote its viability and biodegradation activity.

It is interesting to identify this *Ochrobactrum* sp. as a native satellite for DD4 when it was introduced to all environments. Members of *Ochrobactrum* are mostly aerobic. Some isolates are likely facultative anaerobes given the observation of their activity under anaerobic conditions. *Ochrobactrum cytisi* was reported for its ability for the aerobic degradation of methyl tert-butyl ether (MTBE) as the sole carbon source (45). Similarly, another *Ochrobactrum* isolate demonstrated effective removal of MTBE when introduced as a pure culture or a mixed consortium (46). A clone group phylogenetically related to *Ochrobactrum anthropi* was found as the candidate

for anaerobic tert-butyl alcohol (TBA) mineralization in fuel-contaminated aquifer materials under iron- and sulfate-reducing conditions (47). Furthermore, no studies have clearly demonstrated the growth of *Ochrobactrum* with propane or 1-propanol. Thus, its enrichment across all treatments is less likely due to the auxiliary substrate amendment. Based on these previous reports on recalcitrant ether and alcohol degradation, we postulate a potential role of *Ochrobactrum* sp. in degrading 2-hydroxyethoxyacetic acid (HEAA), a cometabolic metabolite of dioxane by DD4 that consists of ether and alcohol moieties (15). Though genomes of *Ochrobactrum* sp. include abundant oxygenase and other degradation genes (48, 49), further molecular and physiological characterization is needed to understand their role in HEAA transformation, and to reveal their relationship with DD4.

In addition, *Ferruginibacter alkalilentus* (OTU_3) and *Pseudoxanthomonas indica* (OTU_4) exhibited high positive correlations when comparing the propane treatment with the initial sample (log ratios were 1.53 and 0.91, respectively), but were negatively or not affected by 1-propanol treatment (Figure 4). This finding suggested the potential contribution of these two bacterial taxa to the oxidation of propane, but not 1-propanol. Though there is no direct evidence to support these taxa in utilizing propane as a substrate, *Ferruginibacter* was enriched in propane-fed biofilms that

actively reduced selenite (50). A metagenomics study reported *Pseudoxanthomonas* co-existed with propanotrophic bacteria, such as *Nocardioides*, *Xhanthobacter*, and *Mycobacterium*, in the proximity of gas stations and may play a role in the degradation of MTBE in the contaminated groundwater (51). Further research is needed to assess their metabolic roles in propane assimilation.

Conclusions

The foregoing findings demonstrated the compatibility of DD4 and gaseous alkanes with indigenous microbiomes for effective biodegradation of dioxane under laboratory conditions. These positive findings showed promise for the potential success of field deployment of cometabolic bioaugmentation for *in situ* biodegradation of dioxane in groundwater. Short-chain alkane gases and several associated alcohols are two types of auxiliary substrates that are known to concomitantly support the growth of the inocula and stimulate the contaminant degradation activity. Propane and other short-chain alkane gases can exert selective pressure to maintain the dominance of the inocula in environments (e.g., shallow aquifers, activated sludge, and river sediment) that are rich in native microbiomes and with complex compositions. Previous microcosm assays prepared with groundwater samples from sites in California indicated DD4 can degrade dioxane to below the method detection limit (i.e., 0.38 μg/L) when fed with propane (15,

28). One additional concern is the inhibition between short-chain alkane substrates (e.g., propane) and the contaminants (e.g., dioxane) as they compete for the same enzyme (e.g., TMO in DD4), particularly in the field when large amount of inocula and substrates mingle in the proximity of the injection well(s). Accordingly, pulse injection is recommended as it creates periods with low or minimal alkane residuals in the aquifers allowing substantial cometabolic biotransformation of target contaminants to occur right after the inocula are fueled by substrates (18, 52).

Inhibition by alcohol substrates on cometabolism is precluded since the degrading enzymes are not engaged in alcohol assimilation. In DD4, though 1-propanol can induce the expression of TMO, it is the product of the oxidation of propane by TMO, which thus doesn't compete with dioxane for the active sites of TMO (30). Further, alcohol substrates are miscible with water and can be easily operated for field injection and monitoring. However, 1-propanol and other alcohol substrates are less selective to the inocula and thus more suited for low biomass environments dominated by oligotrophic microbiomes that respond poorly to the addition of auxiliary substrates, such as deep bedrock aquifers. The combination of DD4 and 1-propanol may potentially address the immense need for the cleanup of dioxane contamination in deep aquifers since most of the existing remediation approaches are either ineffective or costly to handle large and dilute plumes

with limited accessibility. Note that 1-propanol and other liquid alcohols may not be compatible for bioaugmentation cultures whose dioxane degradation genes are located on the plasmids. Previous studies revealed the curing of the *thm* gene and its carrying plasmid in CB1190 after long term growth with 1-butanol and other substrates that are readily biodegradable (32). To mitigate the impacts of native microbiomes and other factors in high biomass environments, cometabolizing microorganisms and alcohol substrates (or alcohol releasing particles) can be co-encapsulated to promote the performance of long-term bioaugmentation (53).

Cometabolic bioremediation is also advantageous in coping with commingled contamination. DD4, ATCC 21198, and many other cultures are known for their abilities to degrade dioxane and co-existing chlorinated chemicals. The co-existence of 1,1-DCE and other chlorinated chemicals can affect the choice of inoculum-substrate formula considering their influence on the inocula, as well as on the native microbiomes (54), at the molecular, single cellular, and community levels. This calls for site-specific inoculum-substrate formula tailored to optimize the overall treatment effectiveness in the field.

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Table 1. DD4 growth and co-oxidation parameters when fed with different auxiliary substrates.

Auxiliary Substrate	*Propane*	*1-Propanol*	*Butane*	*1-Butanol*	*Ethanol*	*Pyruvate*
Cell yield (mg protein mg substrate^{-1})	0.25±0.00	0.22±0.01	0.19±0.02	0.21±0.02	0.16±0.03	0.13±0.02
Doubling time (h)	8.61±0.66	4.81±0.51	10.35±0.19	6.33±0.30	6.00±0.13	6.40±0.54
TmoA gene expression fold change[a] (number of doublings)	3.34±0.84	3.01±0.91	3.58±0.74	2.95±1.01	1.38±0.22	ND
Apparent dioxane degradation rate[b] (μg dioxane h^{-1} mg protein^{-1})	26.2±3.9	22.8±3.7	20.0±2.2	18.5±3.4	18.9±1.5	ND
Apparent 1,1-DCE degradation rate[b] (μg 1,1-DCE h^{-1} mg protein^{-1})	69.4±1.1	66.4±7.4	49.2±6.3	52.2±8.4	50.7±4.2	ND

[a]Data were normalized to the treatment in which DD4 was fed with glucose. 16S rRNA gene of DD4 was used as the housekeeping gene for error control.
[b]Degradation rate was estimated at an initial concentration of 100 mg/L (equivalent concentration for 1,1-DCE assuming all dissolved in the aqueous phase).

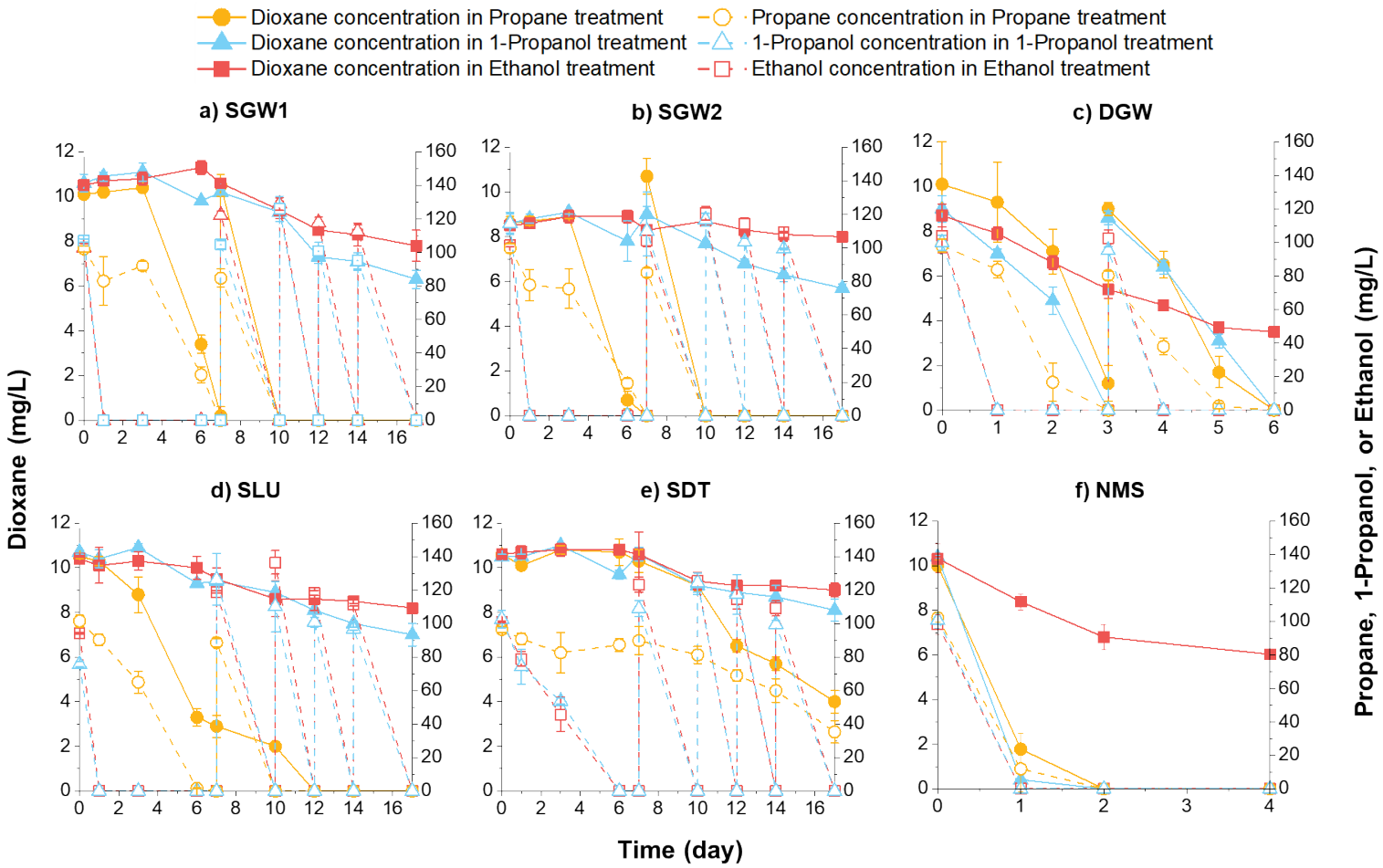

Figure 1. Dioxane degradation and substrate (i.e., propane, 1-propanol, or ethanol) consumption in DD4-bioaugmented microcosms prepared with two surface groundwater (SGW1 and SGW2), deep bedrock groundwater (DGW), activated sludge (SLU), river sediment (SDT), versus nitrate mineral salt media (NMS). The method detection limits for dioxane, propane, 1-propanol, and ethanol were 0.1, 0.01, 0.1, and 0.1 mg/L, respectively.

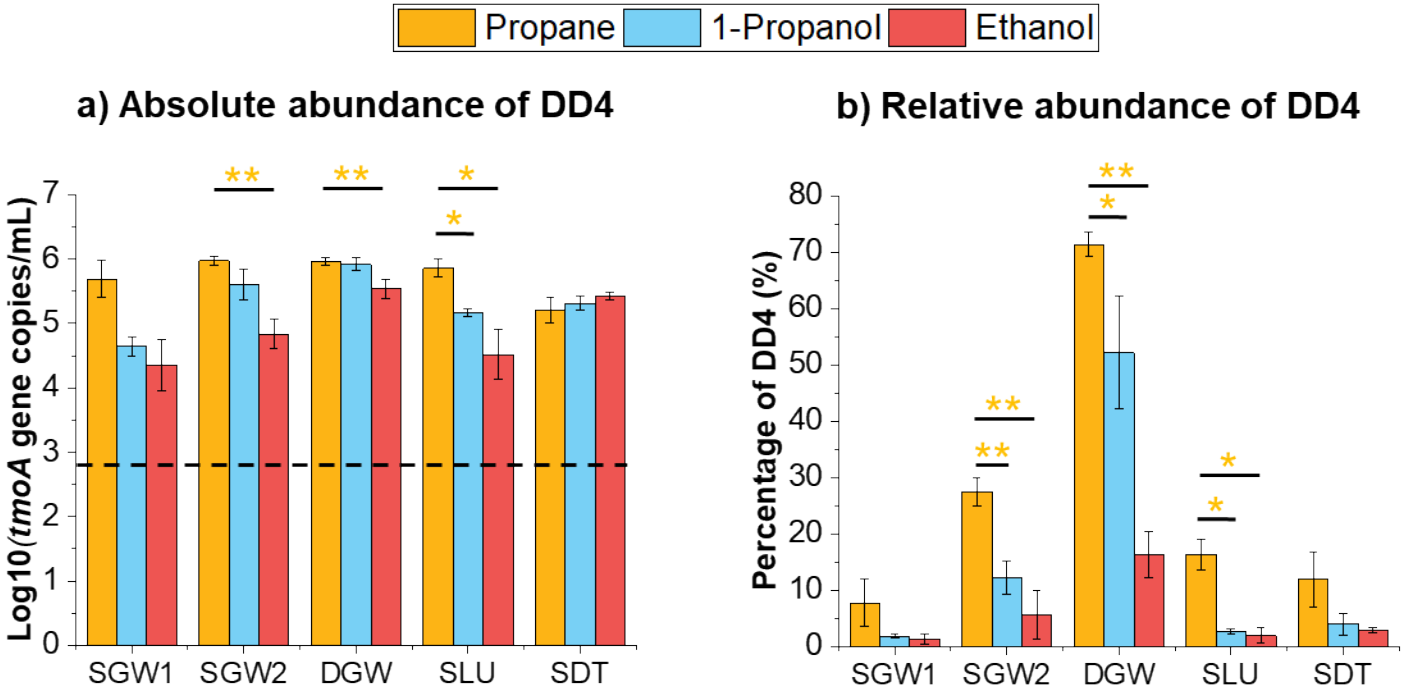

Figure 2. Absolute (a) and relative (b) abundance of DD4 as enumerated as the *tmoA* gene copies in groundwater and other environmental matrices that received the amendment of propane, 1-propanol, or ethanol on Day 17. The black dash line depicts the initial dose of DD4. Significant differences between different treatments were indicated with asterisks in yellow when compared to propane treatment based on the two-way Student's t-test (*, $p < 0.05$; **, $p < 0.01$).

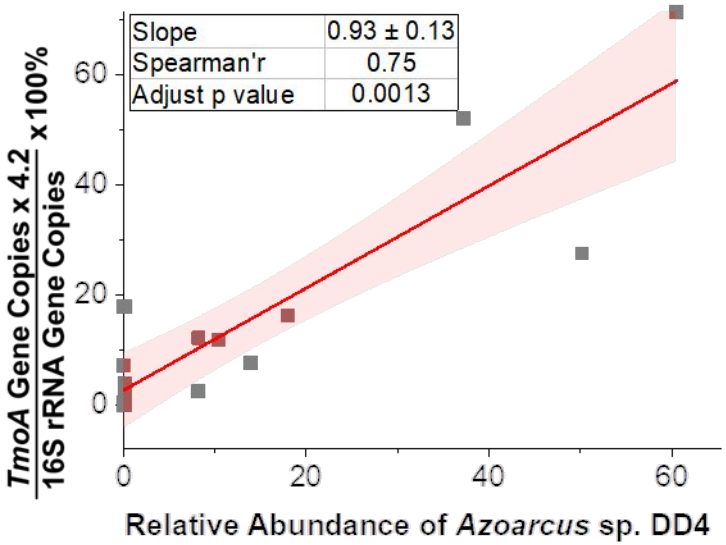

Figure 3. Positive Spearman correlation of relative abundances of *Azoarcus* sp. DD4 as quantified by the *tmoA* gene biomarker (y-axis) versus the 16S rRNA amplicon-based sequencing (x-axis). Linear regression was observed at the slope of 0.93 with the red shadow depicting the 95% confidence band.

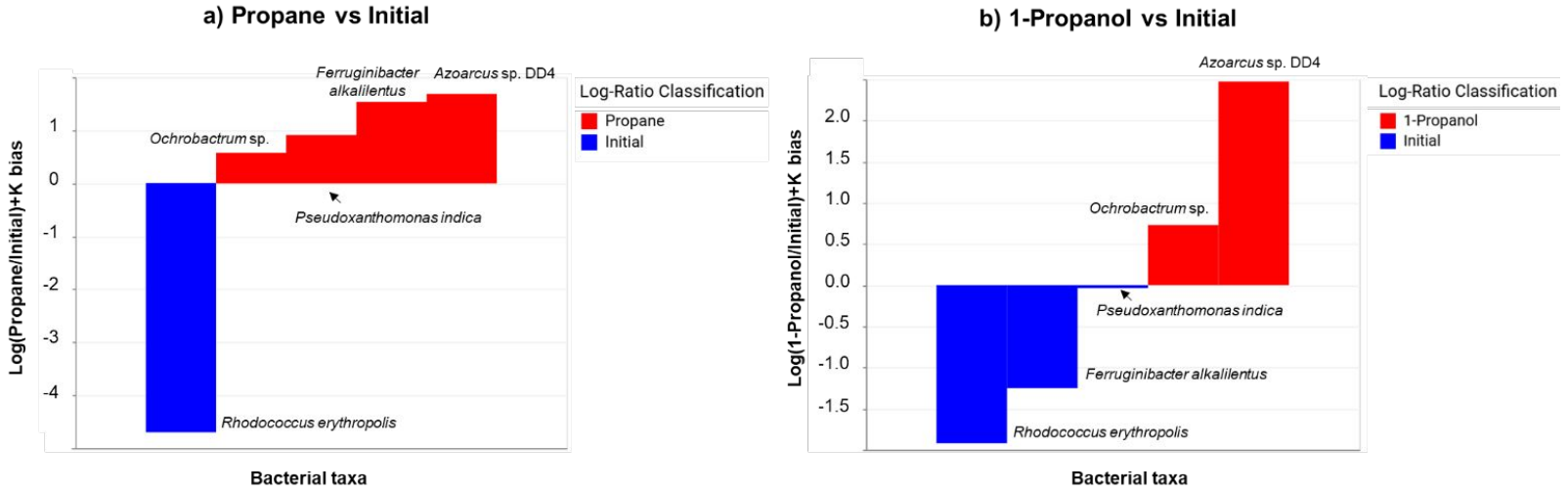

Figure 4. Differential ranking revealed five taxa associated with DD4 bioaugmentation with a) propane and b) 1-propanol across all five environmental matrices. The y-axis represents the log-fold change that is known up to some bias constant K, and the x-axis numerically orders the rank of each taxon in the analysis.

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