



Cite this: DOI: 10.1039/d2ew00524g

Emerging investigator series: environment-specific auxiliary substrates tailored for effective cometabolic bioremediation of 1,4-dioxane†

Daiyong Deng, Dung Ngoc Pham and Mengyan Li *

Cometabolic bioremediation is trending for the treatment of 1,4-dioxane (dioxane) and other emerging contaminants to meet stringent regulatory goals (e.g., $<10 \mu\text{g L}^{-1}$) since 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. Physicochemical 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 with 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 that *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.

Received 8th July 2022,
Accepted 13th September 2022

DOI: 10.1039/d2ew00524g

rsc.li/es-water

Water impact

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.

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 that chronic and acute exposure to dioxane can impair the nervous system and elicit carcinogenic and non-carcinogenic responses in the 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

$\mu\text{g L}^{-1}$ 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 have also indicated that dioxane co-occurs with trichloroethene (TCE) at an exceedingly high frequency ($>90\%$).^{6,7} To date, over 1000 out of the current or former National Priority List (NPL) sites (approximately 1700 in total) have been reported to be contaminated with 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 advances in dioxane biodegradation, *in situ* cometabolic bioremediation is

Department of Chemistry and Environmental Science, New Jersey Institute of Technology, Newark, NJ, 07102, USA. E-mail: mengyan.li@njit.edu;

Fax: +1 973 596 3586; Tel: +1 973 642 7095

† Electronic supplementary information (ESI) available. See DOI: <https://doi.org/10.1039/d2ew00524g>

trending as a cost-efficient and environmentally 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 extremely low levels (*e.g.*, parts-per-billion) or even non-detectable levels can be achieved.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, and³ 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 to achieve 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 for 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 *Rhodococcus 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⁻¹ 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 potential 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 bioremediation 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 has 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 characterization of DD4 in response to auxiliary substrates

The 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 an OD₆₀₀ value of 0.01. The individual substrate was amended at an initial concentration of 5.0 mM (or the equivalent in the aqueous phase for gaseous alkanes) in 20 mL nitrate mineral salt (NMS) medium in 160 mL serum bottles. All treatments were aerobically cultivated at 30 °C and sampled at select intervals to monitor the substrate and biomass concentrations. The cell yield was estimated from the plot of total protein produced against the substrate consumed. The specific growth rate (μ) was calculated by fitting an exponential growth model ($X = X_0 \cdot e^{\mu t}$) to the OD₆₀₀ data, and the doubling time was $\ln 2/\mu$.

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 the individual substrate. Glucose was used as the control substrate and the 16S rRNA gene was used as the housekeeping gene. The total RNA was extracted using a PureLink RNA Mini Kit (Thermo, Carlsbad, CA) according to the manufacturer's protocol, in combination with on-column PureLink DNase treatment (Thermo, Carlsbad, CA) to eliminate the interference from DNA. cDNA was synthesized using a High-Capacity cDNA Reverse Transcription Kit (Thermo, Carlsbad, CA) and then purified using a DNA Clean & Concentrator™-5 Kit (Zymo, Irvine, CA). RT-qPCR mixtures contained 1 μ L of diluted cDNA (5 ng μ L⁻¹), 10 μ L of 2 $\times$ Power SYBR Green PCR Master Mix (Thermo, Carlsbad, CA), 0.3 μ M of forward and reverse primers, and DNA-free water to yield a total volume of 20 μ L. RT-qPCR was performed with a QuantStudio™ 3 Real-Time PCR System (Thermo, Carlsbad, CA) with the following temperature setup: 95 °C for 10 min and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The primers and other materials used in the RT-qPCR procedures are described in our previous publication.³⁰ Differential gene expression was quantified using the $2^{-\Delta\Delta C_q}$ method³² with *tmoA* as the target gene and 16S rRNA as the housekeeping gene. The expression fold change was calculated with the following formula:

$$\Delta\Delta C_{q, \text{Targetgene}} = (C_{q, \text{Targetgene}} - C_{q, \text{Housekeepinggene}})_{\text{Treatment}} - (C_{q, \text{Targetgene}} - C_{q, \text{Housekeepinggene}})_{\text{Control}}$$

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 medium that contained the individual substrate as the sole carbon and energy source. After being washed three times with NMS medium, cells were concentrated to an OD₆₀₀ value of 1 in 6 mL NMS in 35

mL serum bottles. Then, dioxane or 1,1-DCE was spiked at an initial concentration of 100 mg L⁻¹. 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 their 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 the 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 10000 $\times$ 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 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. The 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 the environmental samples (<10 μ g L⁻¹) and the limited volume of samples we received, the dioxane concentration was dosed to 10 mg L⁻¹ in all the treatments so that time series sampling can be conducted over a long period of incubation time. The DD4 inoculum (0.010 $\pm$ 0.001 mg of protein/bottle, equivalent to 6.6 $\times$ 10² CFU mL⁻¹) and 100 mg L⁻¹ of each test substrate (total concentration equivalent as in the aqueous phase) were then amended. The substrates were amended again when >90% was consumed. Aliquots and headspace samples were periodically collected to monitor the depletion of dioxane and the 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 10000 $\times$ g for 10 min at 4 °C. Genomic DNA was extracted using a 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 medium was conducted to discern the impacts of environmental matrices and indigenous microbiomes. Negative controls were utilized using DD4 cells killed with an autoclave to

distinguish the abiotic loss of dioxane from substrate compounds. All the 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 the 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 μ L of TaqMan Universal Master Mix II (Applied Biosystems, Foster City, CA), and DNA-free water, yielding a total volume of 20 μ L. The DD4 specific primers and probe used were specifically designed with the following 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 were 5'-CCT ACG GGA GGC AGC AG-3' for 341F and 5'-ATT ACC GCG GCT GG-3' for 534R. qPCR was performed with a QuantStudio 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 μ L $^{-1}$) 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 using an 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% nucleotide sequence similarity and searched against the GenBank database using the NCBI BLASTN program 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 the greatest changes. Feature ranking and log-fold changes 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 ESI.†

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, the cell yield was the highest for the growth with propane (0.25 ± 0.00 mg protein per mg substrate), indicating that propane is the most energy-efficient for DD4. 1-Propanol, butane, and 1-butanol generated relatively lower yields between 0.19 and 0.22 mg protein per mg substrate, followed by ethanol and pyruvate. Second, the doubling time was the shortest when DD4 was fed with 1-propanol (4.81 ± 0.51 h), followed by 1-butanol (6.33 ± 0.30 h). This suggested that the assimilation of primary alcohols is more efficient than that of 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 fold compared to the housekeeping gene). In contrast, lower and marginal inductions were observed for ethanol (1.38 ± 0.22 fold) 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 Fig. S1,† 1,1-DCE cometabolic degradation was demonstrated by growing DD4 cells in NMS medium. Interestingly, 1-propanol was the most efficient in stimulating 1,1-DCE biotransformation. Within 4 days of incubation, propane-fed DD4 showed $\sim 50\%$ 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 μ g 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 (the 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 for degrading dioxane and 1,1-DCE.

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. The 16S rRNA gene of DD4 was used as the housekeeping gene for error control. ^b The degradation rate was estimated at an initial concentration of 100 mg L⁻¹ (equivalent concentration for 1,1-DCE assuming all dissolved in the aqueous phase).

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 to be the most effective in sustaining the dioxane removal (Fig. 1) and DD4 population (Fig. 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 (Fig. 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, the DD4 population reached $8.45 \pm 1.97 \times 10^5$ tmoA copies per mL, accounting for nearly half of the total bacteria (Fig. 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 in the discernible decrease in richness (Chao1, from 1045 to 68) and diversity (Shannon, from 6.46 to 2.90) after the amendment with 1-propanol as compared to the initial condition (Table S1†). Such postulations can be further

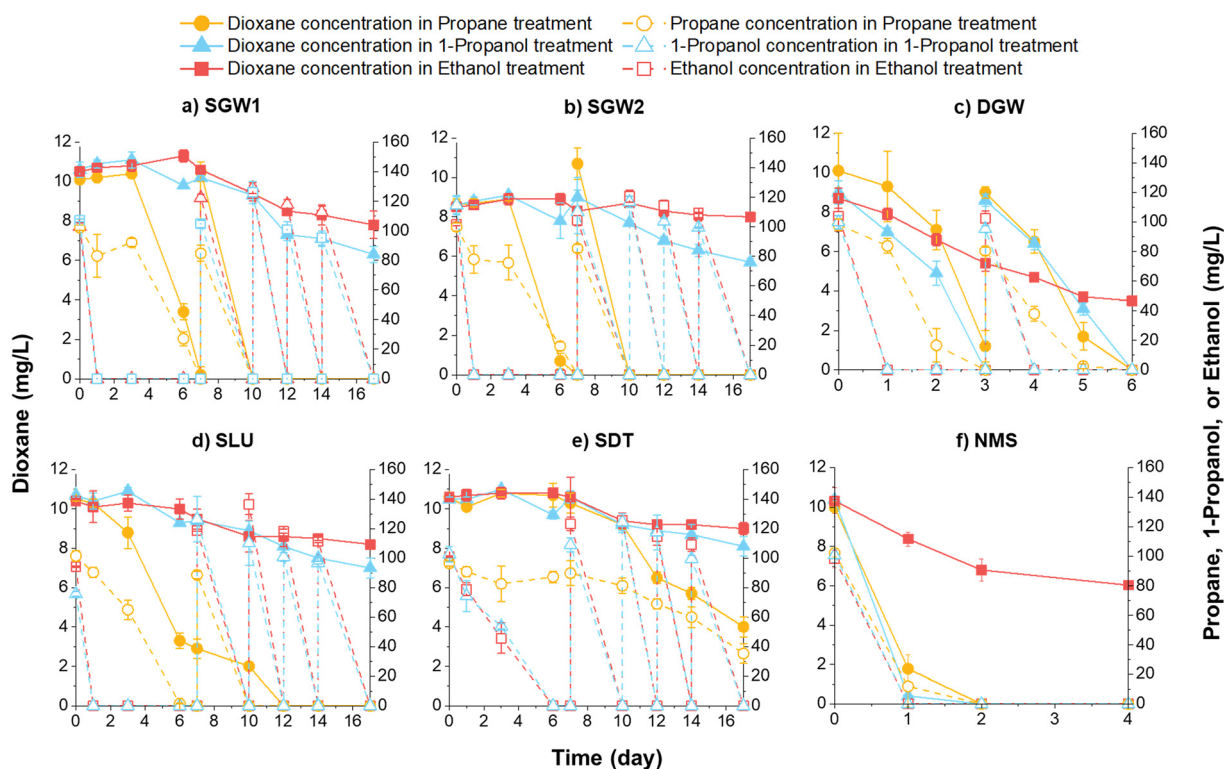


Fig. 1 Dioxane degradation and substrate (*i.e.*, propane, 1-propanol, or ethanol) consumption in DD4-bioaugmented microcosms prepared with two surface groundwater samples (SGW1 and SGW2), deep bedrock groundwater (DGW), activated sludge (SLU), and river sediment (SDT), versus nitrate mineral salt medium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

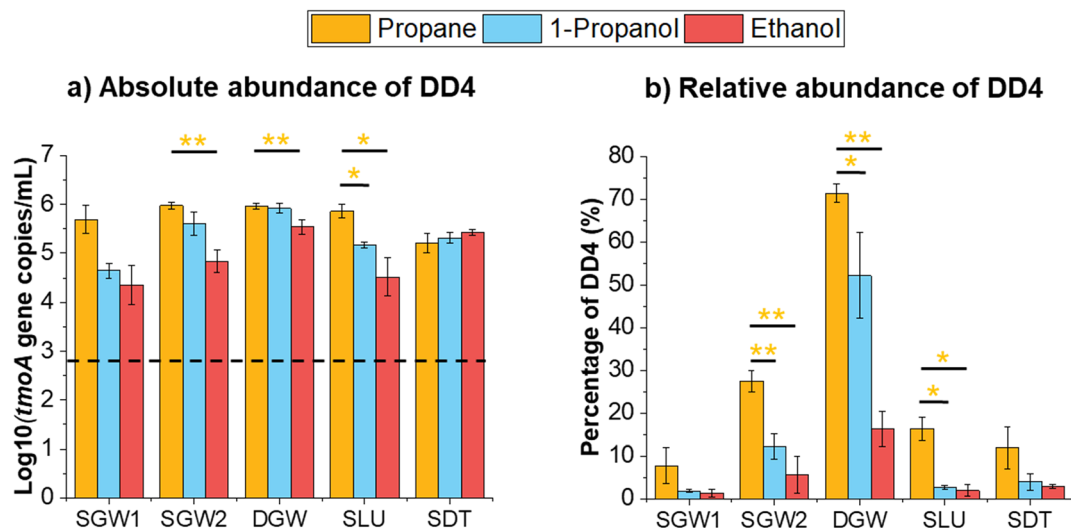


Fig. 2 Absolute (a) and relative (b) abundances of DD4 enumerated as the *tmoA* gene copies in groundwater and other environmental matrices that received the amendment of propane, 1-propanol, or ethanol on day 17. The black dash line depicts the initial dose of DD4. Significant differences between different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

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 the three substrates (Fig. 1a and b) as compared to DGW and the positive control in NMS (Fig. 1c and f). 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 with dioxane and propane. Both dioxane and propane were removed within 3 days between day 7 and day 10, leading to the increase of DD4 population to $5.58 \pm 2.83 \times 10^5$ (SGW1) and $9.61 \pm 1.55 \times 10^5$ (SGW2) *tmoA* copies per mL (Fig. 2a). Thus, repetitive amendments with 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 the ESI†). After two amendments with propane, dioxane was completely removed in SLU by day 12. However, neither propane consumption nor dioxane degradation occurred in SDT until day 7. On 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⁻¹. The DD4 population was estimated to be $1.72 \pm 0.66 \times 10^5$ *tmoA* copies per mL in SDT on day 17, which was significantly lower than in 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 to be least effective in supporting 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, the qPCR results (Fig. 2a and b) 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 was observed (Fig. 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 from the microbial community analysis (Fig. 2 and S3†). The OTU_1 sequence showed 100% identity to the 16S rRNA gene of DD4 (Table S2†), accounting 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 (Fig. 2). Further, based on combined results from all environmental matrices, a positive correlation was established between the relative abundances of DD4 estimated *via* 16S rRNA amplicon-based sequencing (*x*-axis) and qPCR (*y*-axis) (Fig. 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

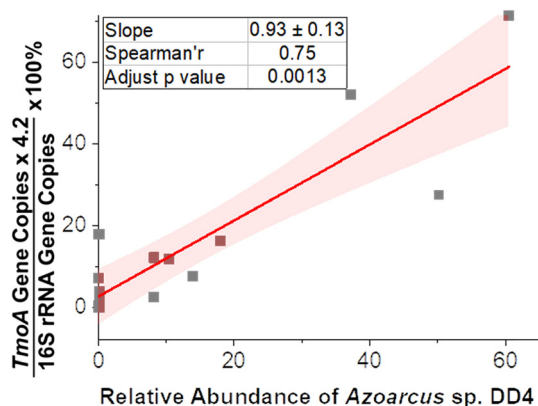


Fig. 3 Positive Spearman correlation of the relative abundances of *Azoarcus* sp. DD4 as quantified by plotting the *tmoA* gene biomarker (y-axis) versus the 16S rRNA amplicon-based sequencing (x-axis). Linear regression was observed at the slope of 0.93 with the red shadow depicting the 95% confidence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

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 shown in the 2D-PCoA plot in Fig. 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 to show strong association with DD4 bioaugmentation across all 5 environmental matrices when propane or 1-propanol was fed as the auxiliary substrate (Fig. 4). Notably, *Azoarcus* sp. DD4 (OTU_1) and *Ochrobactrum* sp. (OTU_2) were positively enriched after the bioaugmentation treatment (the log ratios between “propane” and “initial” were 1.68 and 0.57, respectively, and the log ratios between “1-propanol” and “initial” were 2.47 and 0.73, respectively). In contrast, *Rhodococcus erythropolis* (OTU_5) was negatively influenced (the log ratio between “propane” and “initial” was −4.70, and the 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 to be a 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 alkalilientus* (OTU_3) and *Pseudoxanthomonas indica* (OTU_4) exhibited high positive

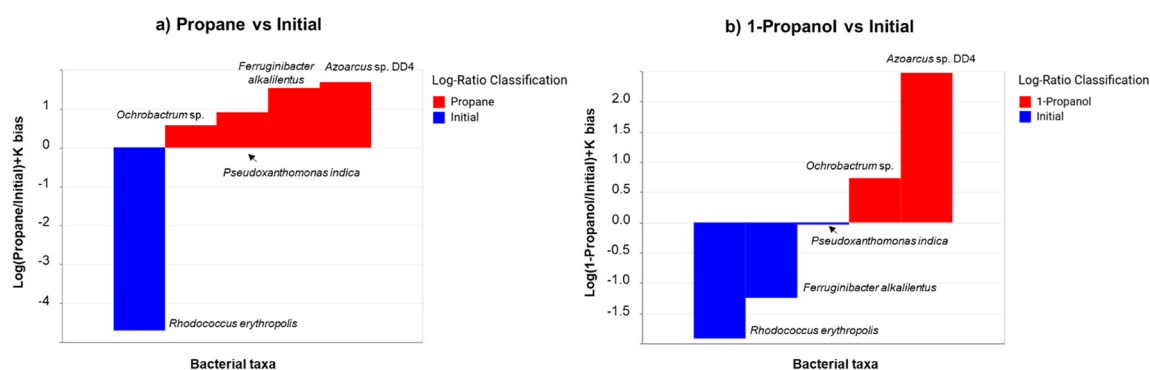


Fig. 4 Differential ranking revealed five taxa associated with DD4 bioaugmentation with a) propane and b) 1-propanol across all five environmental matrices. The y-axis represents the log-fold change that is known up to some bias constant *K*, and the x-axis numerically orders the ranks of each taxon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

correlation when the propane treatment was compared with the initial sample (the log ratios were 1.53 and 0.91, respectively) but were negatively or not affected by 1-propanol treatment (Fig. 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 that these taxa utilize propane as a substrate, *Ferruginibacter* was enriched in propane-fed biofilms that actively reduced selenite.⁵⁰ A metagenomics study reported that *Pseudoxanthomonas* co-existed with propanotrophic bacteria, such as *Nocardioides*, *Xanthobacter*, and *Mycobacterium*, in the proximity of gas stations and may play a role in the degradation of MTBE in 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 show 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 that DD4 can degrade dioxane to below the method detection limit (*i.e.*, $0.38 \mu\text{g L}^{-1}$) 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 aquifers allowing substantial cometabolic biotransformation of target contaminants to occur right after the inocula are fueled by substrates.^{18,52}

Inhibition of cometabolism by alcohol substrates 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.

Conflicts of interest

We declare no competing financial interest.

Acknowledgements

This work was supported by the National Science Foundation (NSF, CAREER CBET-1846945, CHE-2203616), the United States Geological Survey (USGS) State Water Resources Research Act Program (2018NJ400B), the 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.

References

- 1 T. Mohr, J. Stickney and W. DiGuseppi, *Environmental Investigation and Remediation: 1,4-Dioxane and Other Solvent Stabilizers*, CRC Press, 2010, p. 552.
- 2 ATSDR, in *Toxicological Profile for 1,4-Dioxane*, ed. Services UDoHaH, 2006.

- 3 IARC, *Monograph on 1,4-Dioxane*, International Agency for Research on Cancer, Lyon, France, 1999.
- 4 EPA, <https://www.epa.gov/ncea/iris/subst/0326.htm#woe>, 2010.
- 5 D. T. Adamson, S. Mahendra, K. L. Walker, S. R. Rauch, S. Sengupta and C. J. Newell, A multisite survey to identify the scale of the 1,4-dioxane problem at contaminated groundwater sites, *Environ. Sci. Technol. Lett.*, 2014, **1**(5), 254–258.
- 6 R. H. Anderson, J. K. Anderson and P. A. Bower, Co-occurrence of 1,4-dioxane with trichloroethylene in chlorinated solvent groundwater plumes at US Air Force installations: Fact or fiction, *Integr. Environ. Assess. Manage.*, 2012, **8**(4), 731–737.
- 7 U. Karges, J. Becker and W. Püttmann, 1,4-Dioxane pollution at contaminated groundwater sites in western Germany and its distribution within a TCE plume, *Sci. Total Environ.*, 2018, **619–620**, 712–720.
- 8 K. J. Godri Pollitt, J.-H. Kim, J. Peccia, M. Elimelech, Y. Zhang and G. Charkoftaki, *et al.*, 1,4-Dioxane as an emerging water contaminant: State of the science and evaluation of research needs, *Sci. Total Environ.*, 2019, **690**, 853–866.
- 9 M. Li, S. Fiorenza, J. R. Chatham, S. Mahendra and P. J. Alvarez, 1,4-Dioxane biodegradation at low temperatures in Arctic groundwater samples, *Water Res.*, 2010, **44**(9), 2894–2900.
- 10 M. Li, J. Mathieu, Y. Yang, S. Fiorenza, Y. Deng and Z. He, *et al.*, Widespread distribution of soluble di-iron monooxygenase (SDIMO) genes in Arctic groundwater impacted by 1,4-dioxane, *Environ. Sci. Technol.*, 2013, **47**(17), 9950–9958.
- 11 M. Li, E. T. Van Orden, D. J. DeVries, Z. Xiong, R. Hinchee and P. J. Alvarez, Bench-scale biodegradation tests to assess natural attenuation potential of 1,4-dioxane at three sites in California, *Biodegradation*, 2015, **26**(1), 39–50.
- 12 S. Y. Chiang, R. Mora, W. H. Diguseppi, G. Davis, K. Sublette and P. Gedalanga, *et al.*, Characterizing the intrinsic bioremediation potential of 1,4-dioxane and trichloroethene using innovative environmental diagnostic tools, *J. Environ. Monit.*, 2012, **14**(9), 2317–2326.
- 13 P. B. Hatzinger, R. Banerjee, R. Rezes, S. H. Streger, K. McClay and C. E. Schaefer, Potential for cometabolic biodegradation of 1,4-dioxane in aquifers with methane or ethane as primary substrates, *Biodegradation*, 2017, **28**(5–6), 453–468.
- 14 T. C. Hazen, *Cometabolic bioremediation*, 2010.
- 15 D. Deng, F. Li, C. Wu and M. Li, Synchronic biotransformation of 1,4-dioxane and 1,1-dichloroethylene by a gram-negative propanotroph Azoarcus sp. DD4, *Environ. Sci. Technol. Lett.*, 2018, **5**(8), 526–532.
- 16 D. Lippincott, S. H. Streger, C. E. Schaefer, J. Hinkle, J. Stormo and R. J. Steffan, Bioaugmentation and propane biosparging for in situ biodegradation of 1,4-dioxane, *Groundwater Monit. Rem.*, 2015, **35**(2), 81–92.
- 17 H. M. Rolston, M. R. Hyman and L. Semprini, Aerobic cometabolism of 1,4-dioxane by isobutane-utilizing microorganisms including *Rhodococcus rhodochrous* strain 21198 in aquifer microcosms: Experimental and modeling study, *Sci. Total Environ.*, 2019, **694**, 133688.
- 18 M. Y. J. Chu, P. J. Bennett, M. E. Dolan, M. R. Hyman, A. D. Peacock and A. Bodour, *et al.*, Concurrent Treatment of 1,4-Dioxane and Chlorinated Aliphatics in a Groundwater Recirculation System Via Aerobic Cometabolism, *Groundwater Monit. Rem.*, 2018, **38**(3), 53–64.
- 19 C. H. Bell, J. Wong, K. Parsons, W. Semel, J. McDonough and K. Gerber, First Full-Scale In Situ Propane Biosparging for Co-Metabolic Bioremediation of 1,4-Dioxane, *Groundwater Monit. Rem.*, 2022, DOI: [10.1111/gwmr.12511](https://doi.org/10.1111/gwmr.12511).
- 20 S. Hand, B. Wang and K. H. Chu, Biodegradation of 1,4-dioxane: effects of enzyme inducers and trichloroethylene, *Sci. Total Environ.*, 2015, **520**, 154–159.
- 21 R. A. Murnane, W. Chen, M. Hyman and L. Semprini, Long-term cometabolic transformation of 1,1,1-trichloroethane and 1,4-dioxane by *Rhodococcus rhodochrous* ATCC 21198 grown on alcohols slowly produced by orthosilicates, *J. Contam. Hydrol.*, 2021, **240**, 103796.
- 22 Y. M. Kim, J. R. Jeon, K. Murugesan, E. J. Kim and Y. S. Chang, Biodegradation of 1,4-dioxane and transformation of related cyclic compounds by a newly isolated *Mycobacterium* sp. PH-06, *Biodegradation*, 2009, **20**(4), 511–519.
- 23 R. E. Parales, J. E. Adamus, N. White and H. D. May, Degradation of 1,4-dioxane by an Actinomycete in pure culture, *Appl. Environ. Microbiol.*, 1994, **60**(12), 4527–4530.
- 24 S. Mahendra and L. Alvarez-Cohen, Kinetics of 1,4-dioxane biodegradation by monooxygenase-expressing bacteria, *Environ. Sci. Technol.*, 2006, **40**(17), 5435–5442.
- 25 S. Vainberg, K. McClay, H. Masuda, D. Root, C. Condee and G. J. Zylstra, *et al.*, Biodegradation of ether pollutants by *Pseudonocardia* sp. strain ENV478, *Appl. Environ. Microbiol.*, 2006, **72**(8), 5218–5224.
- 26 D. Deng, F. Li and M. Li, A novel propane monooxygenase initiating degradation of 1,4-dioxane by *Mycobacterium dioxanotrophicus* PH-06, *Environ. Sci. Technol. Lett.*, 2018, **5**(2), 86–91.
- 27 D. Deng, F. Li, C. Wu and M. Li, Synchronic Biotransformation of 1,4-Dioxane and 1,1-Dichloroethylene by a Gram-Negative Propanotroph Azoarcus sp. DD4, *Environ. Sci. Technol. Lett.*, 2018, 526–532.
- 28 F. Li, D. Deng, L. Zeng, S. Abrams and M. Li, Sequential anaerobic and aerobic bioaugmentation for commingled groundwater contamination of trichloroethene and 1,4-dioxane, *Sci. Total Environ.*, 2021, **774**, 145118.
- 29 D. Deng, F. Li, L. Ye and M. Li, Complete genome sequence of Azoarcus sp. strain DD4, a gram-negative propanotroph that degrades 1,4-dioxane and 1,1-dichloroethylene, *Microbiol. Resour. Announce.*, 2019, **8**(33), e00775-19.
- 30 D. Deng, D. N. Pham, F. Li and M. Li, Discovery of an Inducible Toluene Monooxygenase That Cooxidizes 1,4-Dioxane and 1, 1-Dichloroethylene in Propanotrophic Azoarcus sp. Strain DD4, *Appl. Environ. Microbiol.*, 2020, **86**(17), DOI: [10.1128/AEM.01163-20](https://doi.org/10.1128/AEM.01163-20).

- 31 F. Li, D. Deng and M. Li, Distinct Catalytic Behaviors between Two 1,4-Dioxane-Degrading Monooxygenases: Kinetics, Inhibition, and Substrate Range, *Environ. Sci. Technol.*, 2020, **54**(3), 1898–1908.
- 32 M. Li, Y. Liu, Y. He, J. Mathieu, J. Hatton and W. DiGuseppi, *et al.*, Hindrance of 1,4-dioxane biodegradation in microcosms biostimulated with inducing or non-inducing auxiliary substrates, *Water Res.*, 2017, **112**, 217–225.
- 33 K. L. Goff and L. A. Hug, Environmental Potential for Microbial 1,4-Dioxane Degradation Is Sparse despite Mobile Elements Playing a Role in Trait Distribution, *Appl. Environ. Microbiol.*, 2022, e02091-21.
- 34 H. Dang, Y. H. Kanitkar, R. D. Stedtfeld, P. B. Hatzinger, S. A. Hashsham and A. M. Cupples, Abundance of chlorinated solvent and 1,4-dioxane degrading microorganisms at five chlorinated solvent contaminated sites determined via shotgun sequencing, *Environ. Sci. Technol.*, 2018, **52**(23), 13914–13924.
- 35 M. Sun, C. Lopez-Velandia and D. R. Knappe, Determination of 1,4-dioxane in the Cape Fear River watershed by heated purge-and-trap preconcentration and gas chromatography-mass spectrometry, *Environ. Sci. Technol.*, 2016, **50**(5), 2246–2254.
- 36 D. K. Stepien, P. Diehl, J. Helm, A. Thoms and W. Püttmann, Fate of 1,4-dioxane in the aquatic environment: From sewage to drinking water, *Water Res.*, 2014, **48**, 406–419.
- 37 M. J. Zenker, R. C. Borden and M. A. Barlaz, Occurrence and treatment of 1,4-dioxane in aqueous environments, *Environ. Eng. Sci.*, 2003, **20**(5), 423–432.
- 38 E. Bolyen, J. R. Rideout, M. R. Dillon, N. A. Bokulich, C. C. Abnet and G. A. Al-Ghalith, *et al.*, Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2, *Nat. Biotechnol.*, 2019, **37**(8), 852–857.
- 39 B. J. Callahan, P. J. McMurdie, M. J. Rosen, A. W. Han, A. J. A. Johnson and S. P. Holmes, DADA2: high-resolution sample inference from Illumina amplicon data, *Nat. Methods*, 2016, **13**(7), 581.
- 40 E. W. Sayers, J. Beck, J. R. Brister, E. E. Bolton, K. Canese and D. C. Comeau, *et al.*, Database resources of the national center for biotechnology information, *Nucleic Acids Res.*, 2020, **48**(D1), D9.
- 41 J. T. Morton, C. Marotz, A. Washburne, J. Silverman, L. S. Zaramela and A. Edlund, *et al.*, Establishing microbial composition measurement standards with reference frames, *Nat. Commun.*, 2019, **10**(1), 1–11.
- 42 M. W. Fedarko, C. Martino, J. T. Morton, A. González, G. Rahman and C. A. Marotz, *et al.*, Visualizing omic feature rankings and log-ratios using Qurro, *NAR: Genomics Bioinf.*, 2020, **2**(2), lqaa023.
- 43 A. Kulikova and A. Bezborodov, Assimilation of Propane and Characterization of Propane Monooxygenase from *Rhodococcus erythropolis* 3/89, *Appl. Biochem. Microbiol.*, 2001, **37**(2), 164–167.
- 44 B. Zou, Y. Huang, P.-P. Zhang, X.-M. Ding, H. J. Op den Camp and Z.-X. Quan, Horizontal gene transfer of copper-containing membrane-bound monooxygenase (CuMMO) and di-iron soluble monooxygenase (SDIMO) in ethane-and propane-oxidizing *Rhodococcus*, *Appl. Environ. Microbiol.*, 2021, 00227-21.
- 45 C.-W. Lin, Y.-W. Cheng and S.-L. Tsai, Influences of metals on kinetics of methyl tert-butyl ether biodegradation by *Ochrobactrum cytisi*, *Chemosphere*, 2007, **69**(9), 1485–1491.
- 46 F. Abbasi, A. Azhdarpoor and M. R. Samaei, Kinetic modeling of Methyl Tert-Butyl Ether (MTBE) removal by bacterial isolated from contaminated soils, *Int. J. Environ. Anal. Chem.*, 2020, 1–16.
- 47 N. Wei and K. T. Finneran, Microbial community composition during anaerobic mineralization of tert-butyl alcohol (TBA) in fuel-contaminated aquifer material, *Environ. Sci. Technol.*, 2011, **45**(7), 3012–3018.
- 48 P. Wanapaisan, N. Laothamteep, F. Vejarano, J. Chakraborty, M. Shintani and C. Muangchinda, *et al.*, Synergistic degradation of pyrene by five culturable bacteria in a mangrove sediment-derived bacterial consortium, *J. Hazard. Mater.*, 2018, **342**, 561–570.
- 49 X. Pan, T. Xu, H. Xu, H. Fang and Y. Yu, Characterization and genome functional analysis of the DDT-degrading bacterium *Ochrobactrum* sp. DDT-2, *Sci. Total Environ.*, 2017, **592**, 593–599.
- 50 C.-Y. Lai, Y. Song, M. Wu, X. Lu, Y. Wang and Z. Yuan, *et al.*, Microbial selenate reduction in membrane biofilm reactors using ethane and propane as electron donors, *Water Res.*, 2020, **183**, 116008.
- 51 K. H. Kucharzyk, H. V. Rectanus, C. M. Bartling, S. Rosansky, A. Minard-Smith and L. A. Mullins, *et al.*, Use of omic tools to assess methyl tert-butyl ether (MTBE) degradation in groundwater, *J. Hazard. Mater.*, 2019, **378**, 120618.
- 52 Y. Kim, D. J. Arp and L. Semprini, Kinetic and inhibition studies for the aerobic cometabolism of 1,1,1-trichloroethane, 1,1-dichloroethylene, and 1,1-dichloroethane by a butane-grown mixed culture, *Biotechnol. Bioeng.*, 2002, **80**(5), 498–508.
- 53 M. T. Rasmussen, A. M. Saito, M. R. Hyman and L. Semprini, Co-encapsulation of slow release compounds and *Rhodococcus rhodochrous* ATCC 21198 in gellan gum beads to promote the long-term aerobic cometabolic transformation of 1,1,1-trichloroethane, cis-1,2-dichloroethene and 1,4-dioxane, *Environ. Sci.: Processes Impacts*, 2020, **22**(3), 771–791.
- 54 S. Malik, M. Beer, M. Megharaj and R. Naidu, The use of molecular techniques to characterize the microbial communities in contaminated soil and water, *Environ. Int.*, 2008, **34**(2), 265–276.