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Comparison of 1-Propanol and Propane as Auxiliary Substrates on 1, 4-Dioxane Biodegradation via Bioaugmentation with *Azoarcus* sp. DD4 at a Landfill Site

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Abstract

1, 4-Dioxane (dioxane) is a persistent contaminant of concern due to its high mobility and stability in aquatic environments, posing significant environmental and public health risks. *Azoarcus* sp. DD4, a novel propanotrophic bacterium, has demonstrated an enhanced ability to cometabolize and degrade dioxane using propane or 1-propanol as the carbon source. In this study, we evaluated DD4's efficiency in dioxane degradation in groundwater samples from a landfill site at site-specific concentrations (41.7 ± 0.5 µg/L in MW-A and 12.3 ± 0.2 µg/L in MW-B). Bioaugmentation with DD4 reduced dioxane concentrations below the detection limit (0.13 µg/L) in both propane and 1-propanol treatments. Dioxane degradation rates were 2.23 and 0.72 µg/L/day in propane-amended treatments for MW-A and MW-B, respectively, and 1.94 and 0.57 µg/L/day in 1-propanol-amended treatments. While propane showed slightly higher degradation rates, the differences were not statistically significant. Parallel treatments mimicking natural attenuation and biostimulation with propane or 1-propanol showed minimal dioxane removal compared to abiotic losses. Although total biomass remained constant before and after DD4 bioaugmentation, DD4 proliferation was evident during incubation with both propane and 1-propanol. Microbial community analysis revealed that *Azoarcus* was abundant in bioaugmented samples, accounting for 5.6% to 45.4% of the overall biomass, highlighting substantial shifts in response to the auxiliary substrate used. These findings were supported by *tmoA* biomarker analysis specific to DD4 via qPCR. Overall, our results demonstrated the feasibility of 1-propanol as an auxiliary substrate alternative of propane for supporting dioxane cometabolic biodegradation by DD4 in complex landfill environments with trace levels of dioxane.

Water Impact

1,4-Dioxane has been widely detected in landfill leachates, where it coexists with numerous other contaminants. Remediating 1,4-dioxane, particularly at concentrations below 100 μg/L, poses significant challenges. This study presents cost-efficient remediation strategies designed to treat environmentally relevant concentrations of 1,4-dioxane in landfill leachate-impacted groundwater that lack native 1,4-dioxane degraders. The demonstrated equivalent effectiveness of 1-propanol and propane in facilitating 1,4-dioxane degradation underscores the flexibility and practicality of either auxiliary substrate for addressing this high-priority contaminant.

Key Words

1, 4-dioxane, landfill, microcosms, bioaugmentation, propane, 1-propanol

Introduction

1,4-Dioxane (dioxane) is a synthetic industrial chemical belonging to the class of cyclic ethers, characterized by its four alkyl carbon atoms linked in a cyclic structure through two ether bonds (1). It is commonly used as a stabilizer for chlorinated solvents, particularly 1,1,1-trichloroethane (1,1,1-TCA), as well as a solvent in paints, oils, pharmaceuticals and textiles (2-5). It has been detected as a byproduct during the manufacture of polyesters and various polyethoxylated compounds (6). Surface and groundwater contamination by dioxane has been a major concern due to its high solubility in water and resistance to biodegradation in environment, which allows it to persist in aquatic environments and poses risks of prolonged exposure leading to adverse health effects, such as liver and kidney damage (7, 8). Dioxane is classified as a probable human carcinogen by the U.S. Environmental Protection Agency (EPA) in 2010 (9, 10). Accordingly, EPA has established a health advisory level for dioxane in drinking water at 0.35 μg/L based on its 10^{-6} life-time cancer risk level (1). Similarly, the New Jersey Department of Environmental Protection (NJDEP) has set a stringent groundwater quality standard of 0.4 μg/L for dioxane (11).

Several abiotic techniques, such as advanced oxidation processes (AOP), granular activated carbon (GAC), air stripping, and pump-and-treat systems, have been used to treat dioxane (12-15). However, its strong hydrophilicity, low Henry's constant, and chemical stability often make these methods less effective and costly, while bioremediation has emerged as a more effective and cost-efficient alternative. An increasing number of bacteria capable of aerobically degrading dioxane via metabolism and cometabolism have been discovered in the past decade, highlighting the potential of bioremediation as a highly effective approach for dioxane removal (16-19). Cometabolic biodegradation is advantageous for treating dilute plumes of dioxane since low concentrations of dioxane may not be adequate to support the growth for dioxane metabolizers (5, 20). Particularly, *Azoarcus* sp. DD4 is a novel propanotrophic bacterium capable of efficiently degrading dioxane, as well as co-occurring chlorinated aliphatic hydrocarbons (CAHs), such as 1,1-dichloroethene (1,1-DCE), which can inhibit dioxane biodegradation (21). Cometabolism by DD4 can be fueled by auxiliary substrates such as propane and 1-propanol, which induce the expression of toluene monooxygenase (TMO), the key enzyme responsible for initiating the degradation of dioxane and CAHs (22, 23). Previous microcosm studies have demonstrated that both propane and 1-propanol effectively facilitate dioxane degradation. Propane, a short-chain alkane gas, exerts selective pressure on the microbial community in high-biomass environments (e.g., wastewater sludge and sediment), promoting DD4 dominance and inducing TMO activity. In contrast, 1-propanol may offer advantages in low-biomass environments, as it supports DD4 growth without directly competing for the active sites of TMO (23). Although both substrates induce *tmoA* expression in DD4, propane elicits a slightly stronger induction (2.93±0.47 fold) compared to 1-propanol (2.16±0.30 fold), suggesting that propane could be a more potent inducer. Correspondingly, resting DD4 cells pre-fed with propane or 1-propanol exhibited comparable dioxane biotransformation capacities (i.e., 26.2 ±3.9 and 22.8 ± 3.7 μg dioxane h^{-1} mg $protein^{-1}$, respectively). However, since 1-propanol is a downstream oxidation product of propane and not a direct substrate of TMO, it avoids direct enzymatic competition for TMO activity, which may enhance its compatibility with TMO-driven cometabolism (22). Future research is warranted to assess whether 1-propanol may cause any unintended interference with TMO function or overall metabolic efficiency.

Although propane has demonstrated greater effectiveness in enhancing dioxane degradation, its high flammability and the requirement for specialized handling and equipment pose logistical challenges in field applications. On the other hand, 1-propanol, being water-miscible, simplifies injection and monitoring processes, making it a practical alternative for some field scenarios. Together, these substrates provide flexibility in addressing diverse environmental conditions, thereby broadening the applicability of DD4-based bioremediation strategies for dioxane removal. In this study, we performed a treatability study to assess the potential of DD4 bioaugmentation for degrading dioxane in microcosms prepared with groundwater samples collected from a landfill site located in Pennsylvania, USA. We compared the efficacy of two auxiliary substrates in enhancing dioxane degradation ranging between 12 to 42 μg/L. To simulate *in situ* treatment conditions, we conducted microcosm assays on a bench scale. These assays evaluated three treatment strategies: monitored natural attenuation (MNA)**,** biostimulation**,** and bioaugmentation with DD4. The study monitored both the removal of dioxane and the consumption of the auxiliary substrates over time. In the bioaugmentation treatments, the viability and dominance of DD4 were evaluated using quantitative PCR (qPCR**)** and microbial community analysis, allowing for a detailed understanding of microbial dynamics involved in dioxane degradation. This comprehensive approach enabled us to determine the effectiveness of bioaugmentation with DD4 and to understand how the auxiliary substrates, propane and 1-propanol, influenced the degradation process in a controlled bench-scale setting under environment-relevant conditions.

Materials and Method

Sample Collection and Microcosm Setup

Groundwater samples were collected from two wells, MW-A and MW-B, located near the source zone of a Superfund site in Pennsylvania, on October 10, 2023. Each sample, with a volume of 4 liters, was shipped on ice in plastic amber bottles to NJIT and stored at 4°C until the microcosm experiments were set up. To determine the baseline data, 150 mL from each original sample was stored at -20°C for subsequent analysis of the indigenous microbial community and initial dioxane concentration (time 0 measurement). Microcosm assays were initiated within 24 hours of sample collection. Prior to setup, each bottle was thoroughly shaken to ensure even distribution of the sample. Five treatments were prepared for each groundwater sample, as outlined in Table 1, to simulate monitored natural attenuation (MNA), biostimulation with propane or 1-propanol, and bioaugmentation with DD4 supplemented with either propane or 1-propanol. The MNA treatments contained only the collected groundwater to assess the native microbial activity for 1,4-dioxane degradation. For the bioaugmentation treatments, the DD4 culture was inoculated into the groundwater samples and amended with equimolar amounts of propane or 1-propanol. The microcosms were assembled in 200-mL amber glass bottles, sealed with two types of caps depending on the auxiliary substrates used for DD4 treatment (e.g., crimped rubber caps for propane-amended microcosms and Mininert® valve caps [Sigma-Aldrich, St. Louis, MI] for 1-propanol-amended microcosms). In parallel, abiotic controls were established by autoclaving groundwater samples and DD4 at 121°C for 1 hour to distinguish any abiotic losses of dioxane or the auxiliary substrates. All microcosm assays were conducted in triplicate to ensure reproducibility.

DD4 was cultured in Lysogeny broth (LB) and cells were harvested in the exponential growth phase, washed for three times, and resuspended to OD_{600} of 1.5 with fresh phosphate-buffered saline (PBS). Biomass concentration was estimated as the total protein quantified by Bradford assay (24). For the DD4 bioaugmentation microcosms, ~0.30 mL of the harvested cells was inoculated to achieve an initial total protein of 0.17 mg, making an initial viable DD4 concentration at as low as 4.6 × 10^3 cells/mL (23) (see detailed calculations in the SI).

Propane was amended to the concentration of 12.91 mg/L in headspace (equivalent to a percent volume of 0.7%) (21). Propane concentration was carefully selected to (1) ensure aerobic conditions in the microcosms (i.e., oxygen is six times greater than the oxidation demand for propane) and (2) minimize explosion risk (i.e., propane is capped at one third of its lower explosive limit) (see detailed explanations in the SI). To amend an equal molar amount of both substrates, 1-propanol was spiked at an initial concentration of 41 mg/L (see detailed calculations in the SI). Both substrates were respiked when 90% of initial concentration was consumed in microcosms. Prior to each substrate respiking, the headspace was refreshed to replenish oxygen to the microcosms.

Dioxane and Auxiliary Substrate Analysis

Approximately 3 mL aqueous sample was collected from the microcosms for dioxane extraction at time 0 and selected sampling points. Water samples were filtered using 0.2 µm, 13 mm Nylon membrane filters to remove biomass and suspended particles. Dioxane was extracted by using a frozen micro-extraction method developed in our laboratory (25). Isotopic labelled surrogate 1,4-dioxane-d8 was spiked to monitor the efficiency of the extraction procedure and tetrahydrofuran-d8 (THF-d8) was used as the internal standard prior to the gas chromatography mass spectrometry (GC/MS) analysis. Calibration curve was established using serially diluted standards in the concentration range between 0.39 and 100 µg/L. The method detection limit (MDL) was estimated as 0.13 µg/L.

Propane and 1-propanol concentrations were measured by gas chromatography with a flame ionization detection (GC-FID) in controls and treatments. For propane, 100 μL of the headspace was injected directly to the GC/FID inlet. For 1-propanol, 500 μL of filtered aqueous samples was analyzed by GC/FID. Propane and 1-propanol concentrations were measured every other day to monitor the substrate consumption by indigenous microbes in biostimulation treatments and mixed microbial communities amended with DD4 in bioaugmentation treatments.

DNA Extraction and Purification

Genomic DNA was extracted from two original groundwater samples (100 mL) and bioaugmentation treatments (50 mL). Extractions were performed using Qiagen DNeasy® UltraClean® Microbial kit, following the manufacturer's protocol. DNA concentration and quality were measured with a SpectraMax Plus 384 Microplate Reader using a Nanodrop Micro-Volume Microplate. DNA extracted from original samples had an A260/A280 ratio of >1.1 and A260/A230 ratio of ~0.8, probably due to minor protein and organic residuals from environmental matrices. DNA in bioaugmentation microcosms displayed improved DNA quality, with an A260/280 ratio of >1.7 and A260/A230 ratio of >1.6. Extracted DNA was stored at -20^{o} C for further analysis.

Quantitative Polymerase Chain Reaction (qPCR)

Quantitative polymerase chain reaction (qPCR) analysis was conducted to measure the quantity of *tmoA* and 16S rRNA genes, which represent DD4 and total bacteria, respectively, in original groundwater samples and samples after bioaugmentation treatments. This involved the use of our newly designed probe/primers that specifically target the alpha subunit of the toluene monooxygenase gene *tmoA* in DD4 (26). Additionally, a set of 16S rRNA primers (341F and 534R) was employed to quantify the overall biomass (27). Primer and probe sequences are indicated in Table S1. The PCR mixture comprised 1 μL of 10 ng/μL DNA, 0.1 μL each of forward and reverse primers (and probe for *tmoA*), and 10 µL of either TaqMan® Universal Master Mix (for *tmoA*) or Power SYBR® Green Master Mix (for 16S rRNA), along with DNA-free water, resulting in a total volume of 20 µL. The qPCR was carried out with the following temperature program initially held for 10 minutes at 95°C, followed by 40 cycles of 15 seconds at 95°C and 1 minute at 60°C. The quantification of target genes (i.e., *tmoA* for DD4 and 16S rRNA for the total biomass) were measured by employing calibration curves, which were generated by serially diluting genomic DNA from DD4 ranging from 0.0001 to 10 ng/µL in an increment factor of 10. As shown in Figure S1 and S2, R^2 value of both curves were above 0.98. The total bacteria was estimated as the 16S rRNA gene number divided by 4.2 (i.e., the average 16S rRNA gene copies per bacterial cell) (28).

Microbial Community Analysis

The microbial community compositions of the original groundwater samples and after the bioaugmentation treatments were analyzed using 16S rRNA gene sequencing. Specifically, the V3-V4 region of the 16S rRNA genes was amplified using PCR with 338F and 806R primers (29, 30). Illumina MiSeq paired-end sequencing, with an approximate read length of 500 bp, was conducted by Azenta (South Plainfield, NJ). Subsequently, the sequence data underwent processing using Vsearch, a well-established clustering algorithm for metagenomics that uses the Greengenes 2 database. Sequencing reads obtained from Illumina MiSeq sequencing were processed using Vsearch (v2.15.2) and Usearch (v10.0.240) with conda (23.3.1) deployed in the linux_x86_64 (Ubuntu 18.04.3 LTS). First, the paired-end reads were joined, followed by primer cutting and quality control, which ensured that only high-quality sequences (score ≥ 20 or fastq_maxee_rate = 0.01) were retained. Next, identical sequences were collapsed during the dereplication step to eliminate redundancy. Chimeras, which can form during PCR amplification, were removed using Usearch. Instead of generating Operational Taxonomic Units (OTUs) through clustering, Amplicon Sequence Variants (ASVs) were identified for greater precision in sequence resolution. A count table was created by mapping the merged reads back to the ASVs. Taxonomic assignments were performed by annotating the ASVs against the Greengenes2 (gg2, v2022.10) and NCBI databases, ensuring accurate identification of the microbial taxa present in the samples. The final ASV table was then formatted for downstream analyses. This comprehensive processing workflow encompassed merging, quality control, dereplication, chimera removal, ASV generation, count table creation, and taxonomy assignment (31-33).

Results

Dioxane Biodegradation by Bioaugmentation with *Azoarcus* sp. DD4

Among the five treatments applied to both samples, significant dioxane degradation occurred in two bioaugmentation treatments where DD4 was initially inoculated. The initial dioxane concentrations in MW-A and MW-B were 41.7±0.5 and 12.3±0.2 µg/L, respectively. As shown in Figure 1, it took 12 and 18 days for DD4 to remove dioxane concentration in MW-A below the detection limit (i.e., 0.13 µg/L) when fed with propane and 1-propanol, respectively. Similarly, complete dioxane removal was achieved within 15 and 18 days in MW-B when propane and 1-propanol was used as the auxiliary substrates for DD4, respectively (Figure 2).

Fit with the zero-order decay kinetics (Table S2), dioxane degradation rates were estimated as 2.23 and 1.94 µg/L/day for DD4 in MW-A and 0.72 and 0.57 µg/L/day in MW-B with propane and 1-propanol, respectively. After normalizing the initial protein concentrations, the averaged dioxane degradation rates were 0.03 and 0.024 µg dioxane/h/mg protein by DD4 with propane and 1-propanol, respectively (Table 2). In contrast, first-order decay kinetics didn't show a good fit

($R^2 < 0.8$) to the observed dioxane degradation data (Table S3). The comparable dioxane degradation rates corroborated that both propane and 1-propanol were effective in supporting DD4 to remove dioxane in these two groundwater samples collected at the landfill site. Although propane may result in a strong substrate competition effect on TMO biodegradation of dioxane, propane is slightly more effective compared to 1-propanol, probably due to higher induction to *tmoA* gene and less substrate divergence to native microorganisms (22). Propane's higher effectiveness compared to 1-propanol in our study aligns with previous findings (detailed in Table 2), where gaseous substrates like isobutane, also led to higher dioxane degradation than liquid alcohols (1-butanol, 2-butanol, and 2-propanol) by *Rhodococcus rhodochrous* ATCC 21198 (34, 35). This similarity suggests that gaseous substrates could be more selective in promoting dioxane cometabolism, though the prominence of this advantage varies across environmental matrices. Furthermore, though no lag phases were experienced in either groundwater, DD4 performed better in MW-A compared to MW-B. This faster kinetics in MW-A was probably due to its higher initial dioxane concentration.

Paired with dioxane cometabolism, propane and 1-propanol were consumed quickly in these bioaugmentation microcosms. Over 18 days of incubation, 3 to 4 doses of propane or 1-propanol were (almost) fully consumed. In general, 1-propanol was consumed faster than the equivalent molar dose of propane regardless of the groundwater sources. This could be due to the limited solubility of propane and the requirement of initial hydroxylation catalysis for the consumption. This could be due to the limited solubility of propane and the requirement of initial hydroxylation catalysis for the consumption. Furthermore, the continuous substrate consumption observed in all bioaugmentation microcosms suggests that oxygen availability was sufficient, supporting aerobic conditions for biodegradation in both landfill samples.

Little Dioxane Removal by Native Microbiomes

Contrary to the bioaugmentation treatments, no significant dioxane removal was noted in treatments that mimic MNA or biostimulation in both groundwater samples, as compared to the abiotic control (Figure 1 and 2). Throughout the incubation, approximately 30% and 20% abiotic loss of dioxane was observed in the autoclaved controls prepared for MW-A and MW-B, respectively. This was probably due to evaporation during autoclaving at elevated temperature and pressure (121 °C and 15 psi).

In biostimulation treatments, propane consumption occurred after day 9 in both samples. However, 1-propanol was completely consumed within 5 to 6 days in both biostimulation treatments. These results indicated the presence of indigenous propanotrophs in the groundwater, though their ability for dioxane cometabolism was not observed.

DD4 Remained Dominant Post Bioaugmentation

Based on qPCR analysis, the total bacterial biomass in the original groundwater samples were estimated as 1.1×10^7 cells/mL in MW-A and 7.3×10^6 cells/mL in MW-B, with no detectable DD4 cells (Figure 3 and Table S4 and S5). After 18 days of bioaugmentation treatments, the total biomass remained stable, ranging from 1.0×10^6 to 7.6×10^6 cells/mL, while DD4 cells were observed at concentrations between 1.7×10^3 and 1.6×10^5 cells/mL, indicating successful colonization and growth as compared to its initial inoculum (i.e., 4.6×10^3 cells/mL). Microbial community analysis revealed that an Amplicon Sequence Variant (ASV) within the *Azoarcus* genus, sharing nearly 100% identity with *Azoarcus* sp. DD4, became a prominent community member post-bioaugmentation. This ASV accounted for 45.4%, 5.6%, 39.7%, and 17.4% of the microbial communities in the bioaugmented microcosms MW-A DD4 + propane, MW-A DD4 + 1-propanol, MW-B DD4 + propane, and MW-B DD4 + 1-propanol, respectively.

Both qPCR and 16S rRNA-based amplicon sequencing supported the dominance of DD4 following bioaugmentation treatments. However, some discrepancies were observed between the two methods. Specifically, 16S rRNA-based community profiling generally indicated higher relative abundances of DD4 compared to qPCR. These differences may stem from methodological biases, including variations in primer coverage and amplification efficiency. Additionally, it is important to note that 16S rRNA gene sequencing cannot reliably differentiate DD4 from other closely related *Azoarcus* species that do not possess the *tmoA* gene, potentially contributing to the overestimation of DD4 abundance by this method.

Overall, the findings suggest that DD4 can survive and grow in both groundwater samples using either propane or 1-propanol as substrates. This growth enables cometabolic degradation of dioxane, achieving the cleanup goal within a reasonable timeframe. The decrease in the Shannon diversity index (from 6 to 4) after DD4 inoculation reflects its dominance and its impact on the microbial community structure (Table S6). This decline likely results from competition for resources or selective pressures arising from DD4's metabolic activities. The reduced diversity indicates that DD4 effectively established itself and shifted the microbial population structures by the end of incubation. DD4 inoculation and substrate addition also stimulated the growth of several bacteria, such as *Mycobacterium, Rhodococcus, Pseudoxanthomonas,* and *Azospirillum*. This conclusion is further supported by Principal Coordinate Analysis (PCoA), which revealed a distinct shift in microbial community structure after DD4 introduction (Figure S3). While both DD4 inoculation and substrate addition influenced community structure, propane had a more pronounced effect than 1-propanol, although dioxane removal was not significantly different between the two substrates.

Growth of Other Microorganisms Coupled with DD4 Bioaugmentation

Microbial community analysis revealed that all four bioaugmented samples exhibited a significant abundance of DD4, accompanied by a notable presence of *Mycobacterium* species, with relative abundances ranging from 4% to 13%. These species were undetectable in the original samples prior to treatment. The results suggest that the addition of gaseous alkanes and alcohols promoted the growth of *Mycobacterium*. Interestingly, this operational taxonomic unit (OTU) shared 90% sequence similarity with *Mycobacterium dioxanotrophicus* PH-06, a known dioxane degrader (36). However, its role in dioxane metabolism remains unclear, as no dioxane degradation was observed in microcosms simulating natural attenuation. This finding indicates a potential mutualistic relationship, where *Mycobacterium*, known to utilize propane and 1-propanol as carbon sources, may contribute to substrate utilization and community stability. The presence of *Mycobacterium*, likely originating from the natural environment, underscores the importance of synergistic interactions in enhancing overall substrate utilization (37-39). Furthermore, the low dioxane concentrations in the field (<50 μg/L) may be insufficient to support the proliferation of dioxane degraders like as PH-06 (40, 41).

Consistent with our previous studies, co-existence of two satellite microbes, *Rhodococcus* and *Pseudoxanthomonas*, were found in the MW-B DD4 + 1-propanol bioaugmentation treatment, accounting for 9.6% and 12.8% in the community, respectively (23, 26). In contrast, their abundance in the MW-B DD4 + propane sample was found to be below 1%. These species were not detectable in both original groundwater samples and MW-A bioaugmentation treatments, probably due to their minimal abundance.

Interestingly, a specific strain of *Azospirillum* showed unusual dominance in samples amended with 1-propanol constituting approximately 37.3% of the microbial community in sample MW-A, compared to 4.5% in MW-B. This genus was absent in the bioaugmentation treatments with propane. *Azospirillum* exhibited significant growth in the presence of 1-propanol, suggesting its ability to utilize this carbon source. Previous research (42, 43) has shown that *Azospirillum* spp. can tolerate heavy metal contamination and may participate in xenobiotic biodegradation. However, heavy metals in both groundwater samples are relatively low (Table S7). Their increased abundance with the amendment of 1-propanol makes *Azospirillum* possible indigenous competitors for 1-propanol. This could be attributed to the non-specific nature of 1-propanol as a substrate, which is readily assimilated by other microbes, reducing its availability for DD4 and potentially lowering its degradation efficiency.

Discussion

Our results demonstrate that dioxane was completely degraded to levels below the method detection limit (0.13 µg/L) in microcosms containing landfill leachate-impacted groundwater samples. Bioaugmentation with an active DD4 culture not only confirmed DD4's dominance in natural environments but also exhibited its ability to degrade dioxane in field samples, even when dioxane concentrations were as low as tens of ppb. The sustained dioxane cometabolism by DD4 was evident through multiple rounds of propane or 1-propanol amendments with the total consumption of propane (12.91 mg/L) or 1-propanol (41 mg/L). Moreover, MNA and biostimulation treatments failed to achieve significant dioxane removal during the study period, highlighting the inability of indigenous microbes in these environmental samples to degrade dioxane effectively.

DD4 exhibited compatibility with both substrates, aligning with previous findings in which gaseous alkanes (e.g., propane, isobutane) and their alcohols (e.g., 1-propanol and 1-butanol) were supplemented as auxiliary carbon sources for the cometabolic degradation of dioxane (Table 2) (26, 34, 35). Both types of substrates have distinct advantages and limitations. Gaseous substrates, such as propane or isobutane, are often more specific in enriching dioxane-degrading populations, as they are less likely to be utilized by non-target native bacteria. This specificity can lead to higher efficiency in promoting cometabolic biodegradation. However, their application in the field may require specialized delivery systems to ensure uniform distribution and prevent losses due to volatilization (19). Liquid alcohols, such as 1-propanol or 1-butanol, are easier to handle and apply but may be less specific, as they can also be consumed by non-dioxane-degrading bacteria. This non-target consumption can reduce their availability to the amended degraders. Nevertheless, with proper monitoring and management, the effectiveness of alcohols may be enhanced. For instance, microbial populations can be tracked to guide timely re-amendments, and the use of slow-release formulations, such as coatings or embedded matrices, can help ensure a sustained and controlled supply of alcohol to the degrader populations over time (34). These strategies may make liquid alcohols as effective as gaseous substrates in many scenarios, particularly when site-specific conditions are considered. Beyond substrate choice, several other factors influence the success of cometabolic biodegradation. The concentration of dioxane in the environment is low at ppb levels, hindering fortuitous reactions with enzymes expressed by cometabolic degraders. Inhibitory compounds, such as heavy metals and co-contaminants like CAHs, particularly 1,1-DCE, can interfere with cometabolic pathways (26, 44, 45). Additionally, the structure and functionality of the microbial community play a pivotal role; communities with a higher abundance of competitors for the auxiliary substrates can negatively affect the viability of the amended degraders (23, 26, 46). Taking together, while gaseous substrates often exhibit higher specificity, liquid alcohols may remain a viable alternative with proper management strategies. Careful consideration of site-specific conditions, including substrate delivery, microbial community dynamics, and environmental inhibitors, is essential for optimizing cometabolic bioremediation.

While DD4 biomass showed effective growth and achieved complete dioxane removal within 18 days, previous bioaugmentation studies reported challenges on persistence of inoculated strain for longer treatment duration in field scale application. For example, a study by Miao et al. (47) showed the bioaugmentation of *Rhodococcus ruber* ENV425 initially performed rapid dioxane degradation but the species abundance declined over time and disappeared after 60 days of incubation. Similar observation was reported by Hatzinger et al. (48) where ENV425 augmented to a field-scale fluidized bed bioreactor to treat N-nitrosodimethylamine (NDMA) and N-nitrodimethylamine (NTDMA) became undetectable after 112 days of operation, while native *Mycobacterium* species increased in abundance by Day 249. In contrast, a study done by Li et al. (26) demonstrated that among the inoculated strains (*Mycobacterium* sp. PH-06, *Pseudonocardia* sp. CB1190 and DD4), only DD4 persisted throughout the experimental period. DD4 not only outcompeted other inoculated strains and but also remained dominant in all treatment and co-existed with other indigenous propanotrophs when oxygen and substrate were fed continuously. Though DD4 showed stable growth and maintained dominance under bench scale conditions, field scale application is essential to warrant its persistence in natural environment. To prevent the potential loss of this strain, strategies like monitoring of target gene (e.g., *tmoA* for DD4), consistent supply of substrate and oxygen, and periodic reinoculation of DD4 should be consider ensuring effective *in situ* dioxane remediation by DD4.

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We declare no competing financial interest.

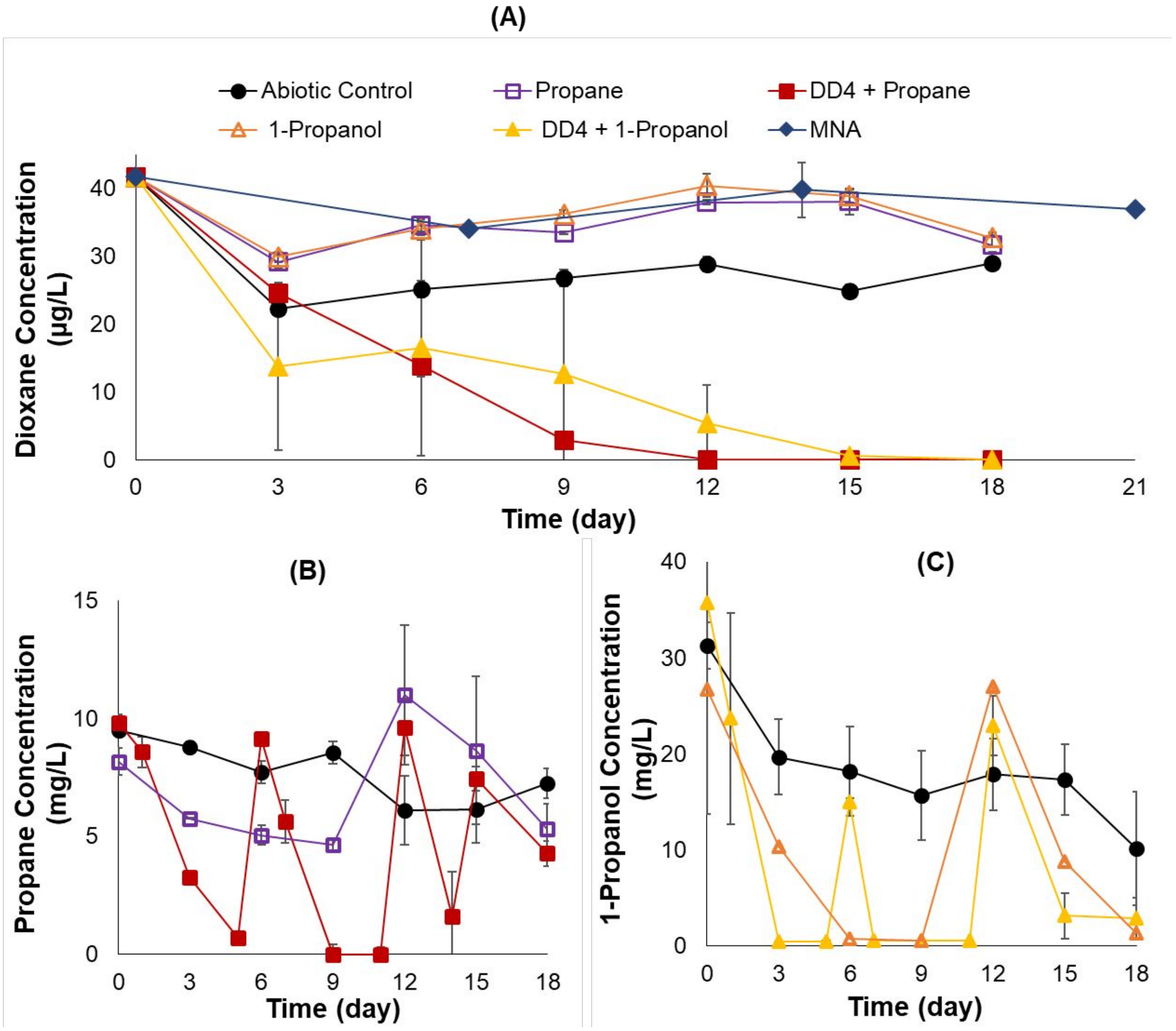

Figure 1. Biodegradation of dioxane (A) and substrate consumption (B and C) by DD4 in microcosms prepared with the MW-A sample.

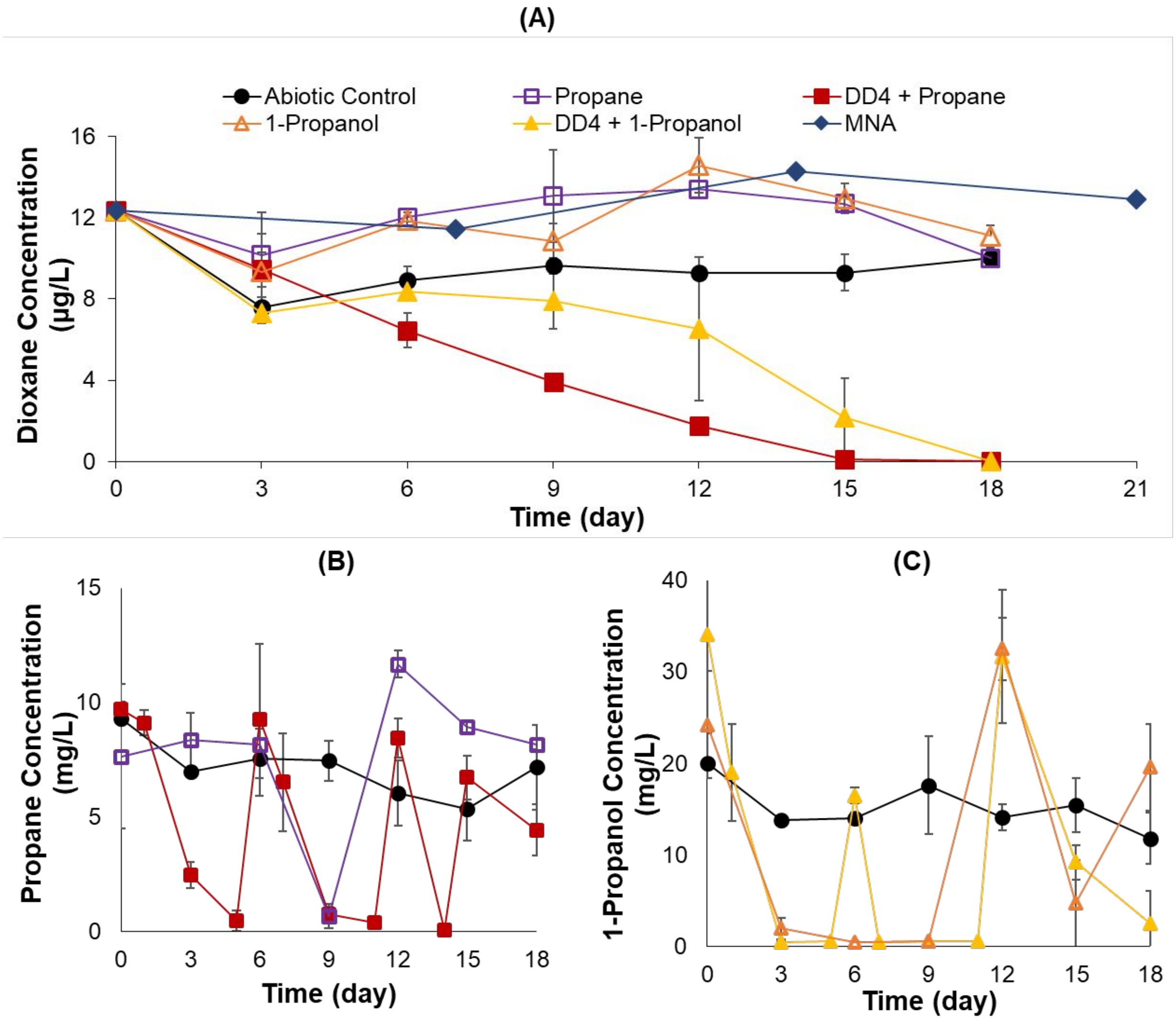

Figure 2. Biodegradation of dioxane (A) and substrate consumption (B and C) by DD4 in microcosms prepared with the MW-B sample.

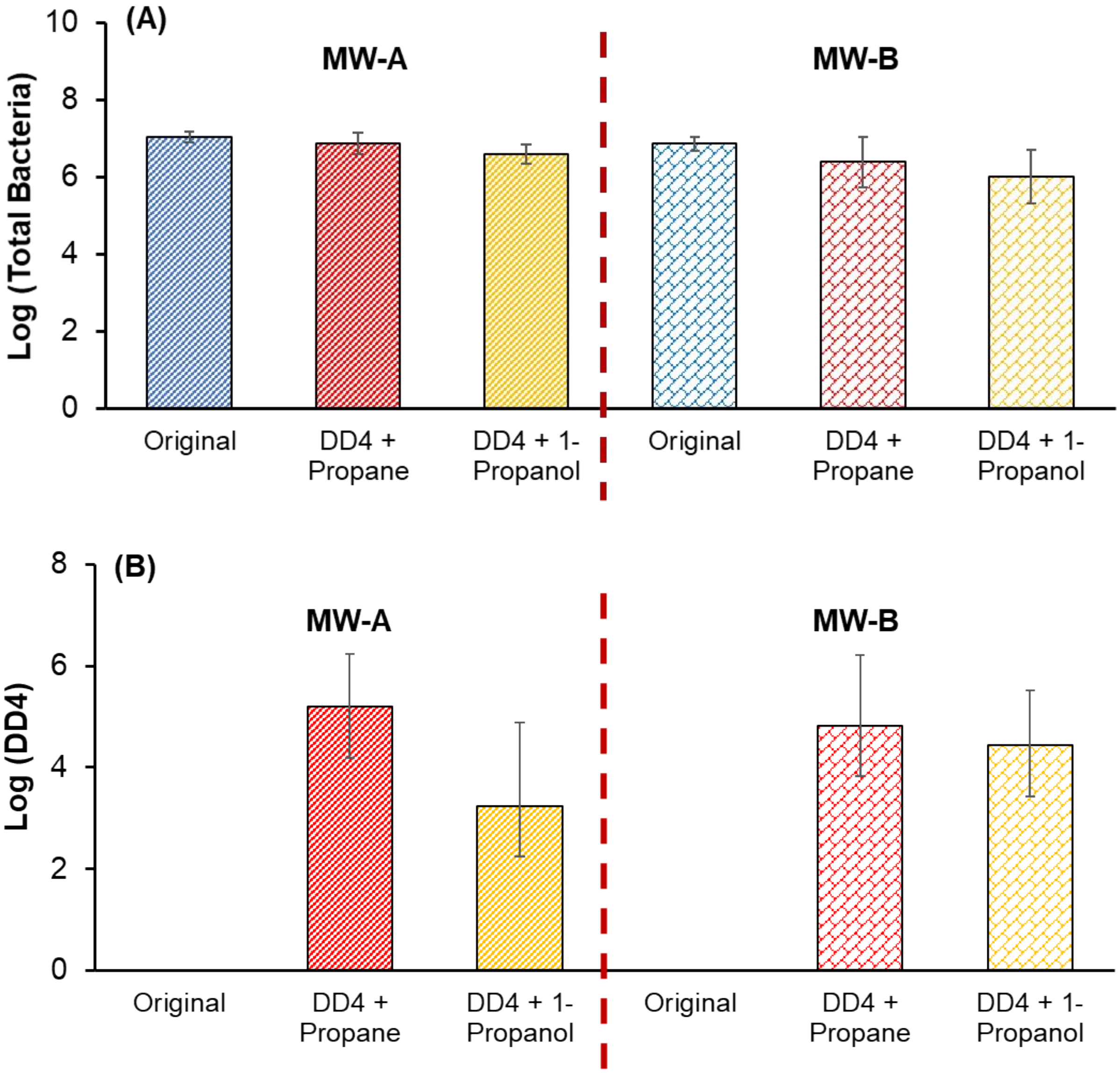

Figure 3. Total bacteria (A) and the abundance of DD4 (B) in original groundwater samples and microcosms after DD4 bioaugmentation treatments by qPCR analysis.

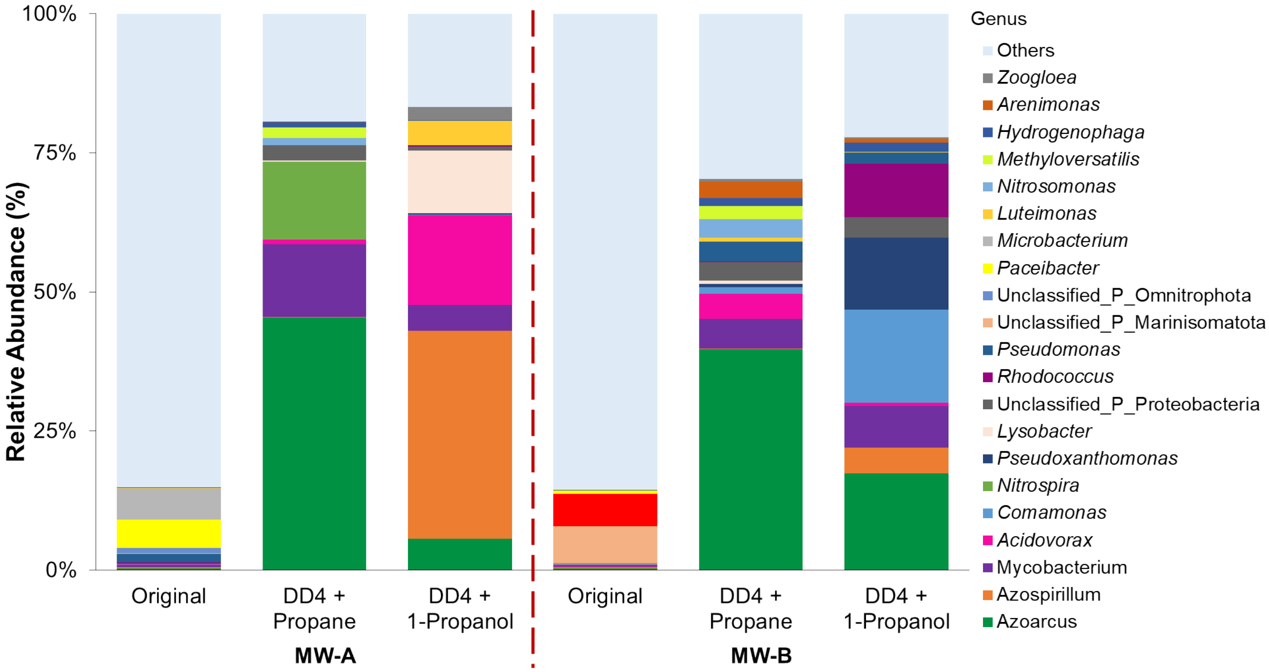

Figure 4. Microbial community distribution at the genus level in original groundwater samples and microcosms after DD4 bioaugmentation treatments.

Table 1. Microcosm setup and compositions.

Microcosm Composition	**Abiotic Control**	**MNA**	**Propane**	**1-Propanol**	**DD4 + Propane**	**DD4 + 1-Propanol**
Propane (0.7% in headspace*)	√		√		√	
1-Propanol (41 mg/L in liquid)	√			√		√
Groundwater (60 mL)	Autoclaved	√	√	√	√	√
DD4 (0.17 mg as total protein)	Autoclaved				√	√

*The headspace volume for all microcosms was 140 mL.

Table 2. Cometabolic degradation of dioxane via bioaugmentation with pure cultures fed with gaseous alkanes and alcohols.

Auxiliary Substrate		**Treatment Outcomes**	**Degradation Rates***	**Reference**
Alkane	Propane	Successive amendment of *Rhodococcus ruber* ENV425 reduced dioxane to below the detection limit (4 µg/L) over 165 days of incubation.	0.837 µg dioxane/h/mg protein[e]	(19)
		Mycobacterium vaccae JOB5 degrades dioxane without a lag phase when using propane as a carbon source.	31.8 µg dioxane/h/mg protein[e]	(49)
		DD4 effectively degraded dioxane in deep and shallow matrices with propane as the carbon source.	26±3.9 µg dioxane/h/mg protein	(23)
		DD4 degraded 99% of dioxane concentration when propane was used as carbon source.	0.03 µg dioxane/h/mg protein	this study
	Isobutane	*Rhodococcus rhodochrous* ATCC 21198 effectively degraded environmentally relevant concentrations of dioxane using isobutane as a carbon source in addition of inorganic nutrients like cations and anions (ca^{++}, NO_3^- etc.).	1.11 µg dioxane/h/mg protein[e]	(35)
Alcohol	1-propanol	Amending DD4 using 1-propanol as carbon source in deep aquifer that have low biomass can degrade 1.4-dioxane effectively.	22.8±3.9 µg dioxane/h/mg protein	(23)
		DD4 degraded 98% of 1,4-dioxane when 1-propanol was used as carbon source.	0.024 µg dioxane/h/mg protein.	this study
	2-propanol and 1-butanol	*Rhodococcus rhodochrous* ATCC 21198 exhibited rapid transformation of dioxane after its second addition, using 2-propanol released by SRC[a] with T2POS[b] as a substrate. Similarly, it transformed dioxane following its second addition by utilizing 1-butanol released by SRC with TBOS[c].	0.058 and 0.036 µg dioxane/h/mg TSS[e] respectively	(34)
	2-butanol	*Rhodococcus rhodochrous* ATCC 21198 rapidly transformed dioxane following its third addition by utilizing 2-butanol released from SRC with T2BOS[d] as a substrate.	0.018 µg dioxane/h/mg TSS[e]	

[a] Slow Releasing Compounds; [b]Tetra Isopropoxy Silane; [c]Tetra Butyl Orthosilicate; [d]Tetra-S-Butyl Orthosilicate; [e]Converted unit of degradation rates

* Dioxane degradation rates reported in different studies were unified in the unit of μg dioxane/h/mg protein or TSS for comparison. Dioxane degradation effectiveness may be considerably affected by factors, such as dioxane concentration, choice of primary substrate, and the nature of slow releasing compound (SRC).

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Data Availability

Data will be made available upon request.