Occurrence of *Rhodococcus* sp. RR1 *prmA* and *Rhodococcus jostii* RHA1 *prmA* across Microbial Communities and their Enumeration During 1,4-Dioxane Biodegradation

Zohre Eshghdoostkhatami and Alison M. Cupples*

Department of Civil and Environmental Engineering, Michigan State University, East Lansing, Michigan, USA
ORCID record: https://orcid.org/0009-0006-5508-1102

*Corresponding Author:
Alison M. Cupples
A135, 1449 Engineering Research Court, Michigan State University, East Lansing, MI 48824, cupplesa@msu.edu

Abstract

1,4-Dioxane, a likely human carcinogen, is a co-contaminant at many chlorinated solvent contaminated sites. Conventional treatment technologies, such as carbon sorption or air stripping, are largely ineffective, and so many researchers have explored bioremediation for site clean-up. An important step towards this involves examining the occurrence of the functional genes associated with 1,4-dioxane biodegradation. The current research explored potential biomarkers for 1,4-dioxane in three mixed microbial communities (wetland sediment, agricultural soil, impacted site sediment) using monooxygenase targeted amplicon sequencing, followed by quantitative PCR (qPCR). A BLAST analysis of the sequencing data detected only two of the genes previously associated with 1,4-dioxane metabolism or co-metabolism, namely propane monooxygenase (*prmA*) from *Rhodococcus jostii* RHA1 and *Rhodococcus sp.* RR1. To investigate this further, qPCR primers and probes were designed, and the assays were used to enumerate *prmA* gene copies in the three communities. Gene copies of *Rhodococcus* RR1 *prmA* were detected in all three, while gene copies of *Rhodococcus jostii* RHA1 *prmA* were detected in two of the three sample types (except impacted site sediment). Further, there was a statistically significant increase in RR1 *prmA* gene copies in the microcosms inoculated with impacted site sediment following 1,4-dioxane biodegradation compared to the control microcosms (no 1,4-dioxane) or to the initial copy numbers before incubation. Overall, the results indicate the importance of *Rhodococcus* associated *prmA*, compared to other 1,4-dioxane degrading associated biomarkers, in three different microbial communities. Also, the newly designed qPCR assays provide a platform for others to investigate 1,4-dioxane biodegradation potential in mixed communities and should be of particular interest to those considering bioremediation as a potential 1,4-dioxane remediation approach.

Keywords

1,4-dioxane, propane monooxygenases, qPCR, *Rhodococcus jostii* RHA1 *prmA, Rhodococcus sp.* RR1 *prmA*

1. Introduction

1,4-Dioxane, a likely human carcinogen, has been detected at many chlorinated solvents contaminated sites (particularly 1,1,1-trichloroethane) because of its widespread use as a stabilizer (Adamson et al., 2015, Adamson et al., 2014, EPA, 2013, EPA, 2014, Mohr et al., 2010, Mohr, 2001). There is no federal 1,4-dioxane drinking water Maximum Contaminant Level, however, federal screening levels, state health-based drinking water guidance values and federal occupational exposure limits have been established (EPA, 2014). A major challenge for 1,4-dioxane remediation concerns chemical characteristics that result in migration and persistence (Adamson et al., 2015, Mohr et al., 2010). Due to its physical and chemical characteristics, 1,4-dioxane is not effectively removed by conventional groundwater treatment technologies such as air stripping, carbon adsorption, air sparging or in-well stripping (Chiang et al., 2016, Mohr, 2010, Zenker et al., 2003). *Ex-situ* oxidation methods including ozone and hydrogen peroxide (Adams et al., 1994) or hydrogen peroxide and ultraviolet light (Stefan et al., 1998) have been commercially applied, but can be costly at high concentrations (Steffan et al., 2007). Full-scale treatment relying on aerobic 1,4-dioxane biodegradation has been effective (Bell et al., 2022). In contrast, there is limited evidence for anaerobic 1,4-dioxane biodegradation (Ramalingam et al., 2020).

In light of the potential to use bioremediation, many researchers have identified the microorganisms responsible for 1,4-dioxane metabolism and co-metabolism. A number of microorganisms can use 1,4-dioxane as a sole source for carbon and energy, including *Pseudonocardia dioxanivorans* CB1190 (Mahendra et al., 2005, Parales et al., 1994), *Pseudonocardia benzenivorans* B5 (Mahendra et al., 2006), *Rhodococcus ruber* 219 (Bernhardt et al., 1991), *Pseudonocardia dioxanivorans* D17, *Afipia broomeae* D1 (Isaka et al., 2016, Sei et al., 2013), *Xanthobacter flavus* DT8 (Chen et al., 2016) and *Mycobacterium* sp. PH-06 (Kim et al., 2009). *Rhodanobacter* AYS5 can use 1,4-dioxane as a sole carbon source (Pugazhendi et al., 2015). Other strains, such as *Pseudonocardia* sp. strain ENV478, *Mycobacterium vaccae* JOB5 and *Rhodococcus jostii* RHA1 can degrade 1,4-dioxane following growth on other carbon sources (Burback et al., 1993, Hand et al., 2015, Vainberg et al., 2006). Co-metabolic 1,4-dioxane degradation, with growth supporting substrates such as tetrahydrofuran, propane,

toluene or ethanol, has previously been observed (Burback et al., 1993, Hand et al., 2015, Kohlweyer et al., 2000, Mahendra et al., 2006, Vainberg et al., 2006).

There has also been significant progress towards identifying the functional genes associated with 1,4-dioxane biodegradation (Deng et al., 2018, Gedalanga et al., 2014, Hatzinger et al., 2017, He et al., 2017, Li et al., 2017, Ramalingam et al., 2020, Rolston et al., 2019). Many researchers have noted the importance of soluble di-iron monooxygenases (SDIMOs) for the transformation of 1,4-dioxane (Deng et al., 2018, Deng et al., 2020, Gedalanga et al., 2014, Li et al., 2014, Yamamoto et al., 2018). In general, SDIMOs have been divided into six groups (Coleman et al., 2006, He et al., 2017). Given the importance of biomarkers for understanding 1,4-dioxane removal, a number of quantitative PCR (qPCR) or reverse transcriptase qPCR (RT-qPCR) methods have been developed to enumerate their occurrence in environmental samples. For example, a qPCR assay was designed to target the large hydroxylase subunit of tetrahydrofuran/dioxane monooxygenases (*thmA/dxmA*) using gene alignments from *Pseudonocardia dioxanivorans* CB1190, *Pseudonocardia tetrahydrofuranoxydans* K1, *Pseudonocardia* sp. ENV478 and *Rhodococcus* sp. YYL (Li et al., 2014) (group 5 SDIMOs). Assays have also been developed for *thmB/dxmB* from *Pseudonocardia dioxanivorans* CB1190 (Gedalanga et al., 2014), *thmC* from *Pseudonocardia* sp. strain D17 (Yamamoto et al., 2018), *prmA* (propane monooxygenase, *prmABCD* gene cluster, group 6 SDIMO) from *Mycobacterium dioxanotrophicus* PH-06 (Deng et al., 2018) and *tmoA* (toluene monooxygenase, *tmoABCDEF* gene cluster, group 2 SDIMO) from *Azoarcus* sp. strain DD4 (Deng et al., 2020). Assays have also been designed towards the genes encoding propane monooxygenase from *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA* (group 5 SDIMOs) to examine nitrosodimethylamine (NDMA) biodegradation (Sharp et al., 2010, Sharp et al., 2007).

Here, the overall objectives were 1) to determine which 1,4-dioxane degrading functional genes were present in three mixed microbial communities and 2) to develop qPCR assays to determine their abundance. Three different mixed microbial communities were investigated to provide a potentially diverse set of biomarkers. The newly designed qPCR assays were used to quantify the identified functional genes during 1,4-dioxane biodegradation in microcosms inoculated with sediment from an impacted site. The newly developed assays will be beneficial to those investigating the potential for 1,4-dioxane bioremediation at contaminated sites.

2. Methods

2.1. Incubation Conditions and Analytical Methods

The biodegradation of 1,4-dioxane, with and without the addition of 1-propanol, was examined in sediment from an impacted site in California (West Coast Naval Station). 1-Propanol was selected as an amendment because previous research indicated this substrate enhanced 1,4-dioxane removal (Deng et al., 2022). Microcosms were established in triplicate, with the following treatments: 1) with 1,4-dioxane and 1-propanol, 2) with 1,4-dioxane and without 1-propanol, 3) without 1,4-dioxane and without 1-propanol, and 4) without 1,4-dioxane and with 1-propanol. Also, for the treatments amended with 1,4-dioxane, three abiotic controls were included (autoclaved consequently for three days). The microcosms were set up in 160 mL serum bottles with 10 grams of sediment and 30 mL of media. The media recipe was as previously reported (Parales et al., 1994), but without nitrilotriacetic acid, included 100 mL of buffer stock [K_2HPO_4 (32.4 g/L), KH_2PO_4 (10 g/L), NH_4Cl (20 g/L)], 100 mL of trace metal stock [$MgSO_4 \cdot 7H_2O$ (2 g/L), $FeSO_4 \cdot 7H_2O$ (0.12 g/L), $MnSO_4 \cdot H_2O$ (0.03 g/L), $ZnSO_4 \cdot 7H_2O$ (0.03 g/L), and $CoCl_2 \cdot 6H_2O$ (0.01 g/L)] and water to a final volume of 1L, then the pH was adjusted to 7.4. Both 1,4 dioxane (99.8%) and 1-propanol were purchased from Sigma-Aldrich (MO, USA). The final concentrations of 1-propanol and 1,4-dioxane were 120 mg/L and 3 mg/L, respectively. The microcosms were incubated at room temperature, sealed with rubber seal and aluminum crimp cap and set on a shaker (110 rpm) at 21°C. The microcosms were opened for one hour every five days to maintain aerobic conditions. Liquid samples (0.7 mL) were withdrawn (sterilized disposable needles and a 1 mL syringe), filtered (0.22 mm, 4 mm nylon syringe filter, Thomas Scientific, New Jersey) and then injected (1 μL) into a gas chromatograph with a flame ionization detector (Hewlett Packard 5890) equipped with a capillary column (Restek, Stabilwax-DB, 30 m, 0.53 mm ID, 1 m) (Myers et al., 2018). Nitrogen was the carrier gas (purity > 99%). The injector temperature was maintained at 220°C and the detector temperature was maintained at 250°C. Initially, the oven's temperature was programmed at 80°C for one minute, then it incrementally increased to 140°C with a ramp of 20°C/minute. The retention time was approximately 1.5 min.

153 **2.2. DNA Extraction and Amplicon Sequencing**

154 DNA was extracted (triplicate DNA extracts from each microcosm) from the impacted sediment
155 microcosms before and after 1,4-dioxane biodegradation in all treatments (except the abiotic
156 controls) (1 g dry or wet weight) using the DNA extraction kit (DNeasy PowerLyzer PowerSoil
157 Kit, Mo Bio, USA) according to the manual protocol. DNA was also extracted from two other
158 sample types, not previously exposed to 1,4-dioxane. One sample was agricultural soil was
159 collected from six replicate plots of the Main Cropping System Experiment at the Kellogg
160 Biological Station Long-Term Ecological Research (KBS LTER), in southwest Michigan. More
161 details can be found at https://lter.kbs.msu.edu/research/long-term-experiments/main-cropping-
162 system-experiment. The other sample, wetland sediments, were collected from the surface (top 5
163 inches) of Lake Lansing (Mi). The concentration of DNA in each extract was quantified using
164 the Quant-iT™ dsDNA High-Sensitivity Assay Kit. For the DNA extracts from the three sample
165 types (site sediment, agricultural soil, wetland sediment), one DNA extract for each was
166 submitted in triplicate for sequencing and the other extracts were used for qPCR (as described
167 below). The DNA extracts from the 1,4-dioxane biodegradation experiment were only subject to
168 qPCR.
169
170 For sequencing, a two-step library preparation first involved PCR with target-specific primers
171 with tags on the 5 prime ends (Fluidigm common oligos CS1/CS2) to facilitate the second PCR
172 for barcoding. The target-specific primers were two degenerate primers (NVC57 and NVC66,
173 target size 420 bp, Supplementary Table 1) previously designed to target conserved regions in
174 the SDIMO alpha subunit gene (Coleman et al., 2006). The second PCR step, as well as the
175 sequencing itself, was performed by the Genomics Core at the Research Technology Support
176 Facility at Michigan State University (MSU). For the second PCR, triplicate PCR reactions were
177 performed for each, to ensure enough total product was available for the next step. Amplicons
178 were batch-normalized using Invitrogen SequalPrep DNA Normalization plates and recovered
179 product was pooled. The pool was QC'd and quantified using a combination of Qubit dsDNA
180 HS, Agilent 4200 TapeStation HS DNA1000 and Invitrogen Collibri Library Quantification
181 qPCR assays. This pool was loaded onto one (1) Illumina MiSeq v2 Standard flow cell and
182 sequencing was carried out in a 2x250bp paired end format using a MiSeq v2 500 cycle reagent
183 cartridge. Custom sequencing and index primers complementary to the Fluidigm CS1 and CS2

oligomers were added to appropriate wells of the reagent cartridge. Base calling was done by Illumina Real Time Analysis (RTA) v1.18.54 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.20.0.

2.3. Sequencing Data Processing and Analysis

The sequencing files were processed using usearchv11 (Edgar, 2010) on the High Performance Computing Cluster (HPCC) at MSU. This involved an inspection of the quality data and using the commands -fastx_info and fastq_eestats2. Sequencing triplicates were pooled using -fastq_mergepairs. Quality filtering, with a maximum expected error threshold set to 1.0, was achieved using -fastq_filter. The sequences were then dereplicated using the -fastx_uniques command. The cluster_otus command was used to perform 97% operational taxonomic units (OTU) clustering using the UPARSE-OTU (Edgar, 2013) algorithm and to filter chimeras. OTU tables with OTU abundance values were created using the -otutab command. The sequences of twelve genes previously associated with 1,4-dioxane metabolism and co-metabolism, as previously summarized (He et al., 2017) were obtained from NCBI. Each sequence was then uploaded for a nucleotide-nucleotide blastn search to find highly similar sequences to create a blast database for each (Altschul et al., 1990). The resulting databases were filtered using a percent identity and query length threshold of greater than or equal to 95% to ensure only highly similar sequences were selected. The occurrence of each gene in the files generated by usearch were determined using blastn (BLAST/2.10.0-Linux_x86_64 on HPCC).

The results from the blastn search for the twelve target genes were filtered to include matches of > 90% sequence identity (the sequence identity was reduced to capture a wide diversity of gene matches) and alignment length of more than 400 bps. The number of OTUs aligning to each gene for each sample was determined and the data were used to construct the phylogenetic trees discussed below. As only two of the twelve genes (*Rhodococcus jostii* RH1 *prmA* and *Rhodococcus* sp. RR1 *prmA*) were detected in the three sample types, only two trees were produced. A blastn search was also conducted against the nt database from NCBI. For this, the formatted database was downloaded to the HPCC and the resulting files were analyzed with R

(Version 4.2.1) (R Core Team, 2018) in RStudio (Version 2022.12.0) (RStudio_Team, 2020). The data were filtered using a >95% sequence identity and an alignment length of more than 400 bps. The data were analyzed (in RStudio) to determine which gene matches aligned to the greatest number of OTUs. The data were ranked and the sequences of the twenty most abundant were collected for inclusion in the phylogenetic trees. The protein ID numbers for each were obtained from NCBI. The sequences of the most abundant OTUs were also obtained from the blastn output file for inclusion into the phylogenetic trees.

The output files from the blastn analysis of the twelve genes and the nt database were downloaded from the HPCC directory for data manipulation with R (Version 4.2.1) (R Core Team, 2018) in RStudio (Version 2022.12.0) (RStudio_Team, 2020). For this, the following R packages were utilized: tidyverse (Version 1.3.1) (Wickham et al., 2019), ampir (Version 1.1.0) (Fingerhut L. et al., 2021), writexl (Version 1.4.2) (Ooms, 2023), readxl (Version 1.4.2) (Wickham et al., 2023), writexl (Ooms, 2023), ggplot2 (Wickham, 2016) and phylotools (Version 0.2.2) (Zhang, 2017).

2.4. Phylogenetic Trees

Sequences were submitted for MAFFT (multiple alignment using fast Fourier transform) alignment using an online server (https://mafft.cbrc.jp/alignment/server/) (Katoh et al., 2019) (Version 7). The alignments (obtained using the Neighbor-Joining method and Jukes-Cantor model) were exported in Newick format. The downloaded tree files were uploaded to the Interactive Tree of Life (https://itol.embl.de) (Letunic et al., 2021) (Version 6.7.2). OTU abundance values were added to the trees using the datasets function called multi value bar chart. Presence and absence values were added using the datasets function called binary.

2.5. Quantitative PCR

Gene copies of *Rhodococcus* sp. RR1 *prmA, Rhodococcus jostii prmA*, *Mycobacterium dioxanotrophicus* PH06 *prmA* and the Bacterial 16S rRNA gene were determined using qPCR. Although *Mycobacterium dioxanotrophicus* PH06 *prmA* was not detected in the amplicon sequencing data, it was investigated with qPCR as it represents another propane monooxygenase associated with 1,4-dioxane biodegradation (He et al., 2017). Gene copies were investigated in

the three sample types (impacted site sediment, agricultural soil and wetland sediment) as well as before and after 1,4-dioxane biodegradation in the impacted site inoculated microcosms, in all treatments. Both previously designed primers/probes and newly designed primers/probe were used (Supplementary Table 1). Primers and probes for the two *Rhodococcus prmA* gene targets were designed using Primer-BLAST (Ye et al., 2012) and the PrimerQuest™ Tool from Integrated DNA Technologies. The specificities of the primers and probe were investigated using blastn (Altschul et al., 1990). For this, sequences with more than 95% sequence identity to the standard plasmid insert for RR1 *prmA* were downloaded from blastn and were then aligned in MEGA X (version 10.1.8) (Kumar et al., 2018) to determine the number of mismatches to the primers and probe. In addition, seven sequences previously classified in the same group as *Rhodococcus* sp. RR1 *prmA* (group 5 large hydroxylase of SDIMO) (He et al., 2017, Li et al., 2014) were also aligned to the target sequences.

Quantitative PCR was conducted with the CFX96™ Real-Time PCR System (Bio-Rad, Hercules, CA) with 20 µL total volume containing 10 µL PrimeTime™ Gene Expression Master Mix, 0.3 µM of each primer (IDT Integrated DNA Technologies, Coralville, IA), 0.2 µg/mL bovine serum albumin (Thermo Fisher Scientific), 0.15 µM of the probe (IDT Integrated Technologies), 6.4 µL of PCR grade water (IDT Integrated DNA Technologies), and 2 µL DNA extract or PCR grade water (for the negative controls). BSA was added as it has been shown to mitigate inhibition in environmental samples (Gedalanga et al., 2014, Kreader, 1996, Wang et al., 2007). The thermal cycler program was an initial activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, then annealing at 60°C for 1 minute. Each target gene was incorporated into a plasmid for use as qPCR standards (GenScript Biotech Corporation). Each qPCR assay was performed in triplicate with DNA templates, no template controls (NTCs), and 5-fold serial dilutions of the standards to create calibration curves. DNA extract concentrations (Supplementary Table 2), as well as data concerning the qPCR assays (e.g. dilution range, qPCR efficiency, as suggested by MIQE guidelines) (Bustin et al., 2009) (Supplementary Table 3) has been summarized.

3. Results

3.1. SDIMO Amplicon Sequencing

The BLAST analysis of the amplicon sequencing data detected only two of the twelve genes previously associated (He et al., 2017) with 1,4-dioxane metabolism or co-metabolism (Figure 1). Both genes, detected in all three sample types, were associated with *Rhodococcus* species, namely *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA*. The alignments of the two *Rhodococcus prmA* genes were further investigated (Figure 2). The largest number of OTUs for the *Rhodococcus jostii* RHA1 *prmA* database aligned with *Rhodococcus jostii* RHA1 *prmA* and functional genes associated with *Rhodococcus imtechensis* strain RKJ300 and *Rhodococcus* sp. SMV152 (Figure 2A). The OTU alignments to the *Rhodococcus* sp. RR1 *prmA* database were primarily to *Rhodococcus* sp. RR1 *prmA* and functional genes associated with *Rhodococcus* sp. 11-3, *Rhodococcus* sp. DMU1, *Rhodococcus* sp. M8, *Rhodococcus* sp. PSBB049, *Rhodococcus aetherivorans* isolate PSBB011, *Rhodococcus* sp. PSBB066, *Rhodococcus aetherivorans* strain N1 and *Rhodococcus* sp. WB1 (Figure 2B). For both alignments, the OTUs from the wetland soils illustrated the largest number of alignments and the impacted site samples illustrated the lowest number.

3.2. Quantitative PCR Assay Design

Based on the amplicon sequencing results, two qPCR assays were designed towards *Rhodococcus* sp. RR1 *prmA* (hereafter called RR1 *prmA*) and *Rhodococcus jostii* RHA1 *prmA* (hereafter called RHA1 *prmA*) (Figure 3). The RR1 *prmA* primers and probe illustrated five or more mismatches to group five large hydroxylases of SDIMOs (Figure 3B). One sequence matched the forward primer completely, however, had five mismatches to the probe and reverse primer. The RHA1 *prmA* primers and probe illustrated two or more mismatches to this group of sequences (Figure 3C). The closest match was to *Gordonia* sp. TY-5 *prmA* with two mismatches in the forward primer. The newly designed primers and probe exhibited between 29 and 30 mismatches (RR1 *prmA* assay) and between 26 and 28 mismatches (RHA1 *prmA* assay) to *Pseudonocardia dioxanivorans* CB1190 *thmA*, *Pseudonocardia tetrahydrofuranoxydans* K1 *thmA* and *Pseudonocardia* sp. ENV478 *thmA* and *Rhodococcus* sp. YYL *thmA*.

3.3. Quantitative PCR Results in Three Sample Types

Copy numbers for the three target genes, as well as the Bacterial 16S rRNA gene, were determined in DNA extracted from the three sample types. Only two of the three functional

genes were detected (Figure 4). Gene copies from the RR1 *prmA* assay were highest for the agricultural soil samples, followed by the wetland samples, then the impacted site samples. No amplification was observed from the RHA1 *prmA* assay in the impacted site samples. However, amplification occurred in the other two sample types, with the agricultural soil producing the highest levels. Notably, gene copies were higher for RR1 *prmA* assay compared to the RHA1 *prmA* assay. *Mycobacterium dioxanotrophicus* PH06 *prmA* was only detected in two DNA extracts (each in one of three replicates) with cycle threshold values below the lowest plasmid standard. Copy numbers of the Bacterial 16S rRNA gene were similar for the agricultural soil and wetland soil, but lower for the impacted site samples (Figure 4).

3.4. Quantitative PCR Following 1,4-Dioxane Biodegradation

The biodegradation of 1,4-dioxane appeared to occur in two phases in the impacted site microcosm study, with an initial lag phase followed by a rapid decline (Figure 5). This pattern is perhaps suggestive of growth-related biodegradation. From the data collected, the addition of 1-propanol did not appear to impact removal trends. Gene copies of *Rhodococcus* sp. RR1 *prmA, Rhodococcus jostii* RHA1 *prmA*, *Mycobacterium dioxanotrophicus* PH06 *prmA* and the Bacterial 16S rRNA gene were investigated following 1,4-dioxane biodegradation, for each treatment.

Only the RR1 *prmA* and Bacterial 16S rRNA assays produced cycle thresholds above the lowest plasmid standards. When the gene ratios (*prmA*/16S rRNA) were determined for each treatment, several interesting trends were observed ($p < 0.05$, multiple pairwise comparisons of means using least significant differences test) (Figure 6). There was a statistically significant increase in the gene ratio between the initial conditions and the microcosms amended with 1,4-dioxane (no 1-propanol), a trend suggestive of growth related 1,4-dioxane biodegradation. Other trends concerned the differences between the microcosms amended with and without 1,4-dioxane. Specifically, the microcosms amended with 1-propanol and 1,4-dioxane illustrated significantly higher gene ratios compared to those amended with 1-propanol, but without 1,4-dioxane. A similar trend (higher gene ratio) was noted for the microcosms amended with 1,4-dioxane, without 1-propanol and those amended without 1,4-dioxane or 1-propanol. Further, the gene ratios of both sets of microcosms without 1,4-dioxane were not statistically significantly different from the ratios at the initial conditions. The gene copy numbers for both RR1 *prmA* and

Bacterial 16S rRNA gene assays have also been summarized (Supplementary Figure 1). When detected (one or two extracts, in only one of three replicates), cycle threshold values for both *Rhodococcus jostii* RHA1 *prmA* and *Mycobacterium dioxanotrophicus* PH06 *prmA* after 1,4-dioxane biodegradation in the impacted site microcosm study were at or below the lowest plasmid standard.

3.4. Amplicon Sequencing Matches to nt Database

The closest matches from the nt database to the amplicon sequences from the impacted site sediment primarily included those classifying as propane monooxygenase hydroxylase large subunit from *Mycobacterium hodleri* strain B, aromatic/alkene/methane monooxygenase hydroxylase/oxygenase alpha subunits from *Bradyrhizobium japonicum, B. barranii* and *B. septentrionale* as well as those classifying as SDIMOs from enrichment culture clones (Figure 7). The closest matches to the amplicon sequences from the wetland sediment primarily included those classifying as methane monooxygenases from *Rhodococcus opacus* strains. Similar to the impacted site, some sequences classified similar to SDIMOs from enrichment culture clones (Figure 8). Functional genes associated with *Mycobacterium hodleri* strain B, *Mycobacterium* sp. JS623 and *Methylibium* sp. Pch-M were also among the top alignments for the wetland amplicons. The largest number of matches were found for propane monooxygenase hydroxylase large subunit from *Mycobacterium hodleri* strain B. The trend for the agricultural soil amplicons was similar to that from the impacted site, including similar *Bradyrhizobium* species and enrichment clones (Figure 9). The agricultural soil amplicons also illustrated matches to functional genes from *Mycobacterium hodleri* strain B (propane monooxygenase hydroxylase large subunit) and *Amycolatopsis* sp. DAM 110486 (aromatic/alkene/methane hydroxylase/oxygenase subunit alpha monooxygenase).

4. Discussion

An understanding of the *in-situ* capabilities of bacteria to biodegrade 1,4-dioxane at contaminated sites is important for effective and timely remediation. Towards this goal, the current study utilized monooxygenase targeted amplicon based sequencing to investigate 1,4-dioxane associated functional genes in three mixed microbial communities. Following the detection of two genes (*Rhodococcus prmA*, group 5 SDIMOs) previously associated with 1,4-

dioxane biodegradation (Hand et al., 2015, Mahendra et al., 2006), qPCR assays were developed. These genes were selected not only because of their detection in the current work, but also because other recent sequencing studies also indicated their common occurrence in mixed communities during 1,4-dioxane biodegradation (Inoue et al., 2022, Inoue et al., 2020, Ramalingam et al., 2020). New primers and probes were designed towards these biomarkers as previous assays targeting these genes generated a short gene product (64 bp) or did not include a probe (Supplementary Table 4) (Sharp et al., 2010, Sharp et al., 2007). Also, previous assays were tested during NDMA biodegradation (Sharp et al., 2010, Sharp et al., 2007).

Other researchers have also investigated biomarkers for 1,4-dioxane biodegradation using the same monooxygenase targeted degenerate primers (NVC57, NVC66) (Coleman et al., 2006) used in the current study. For example, using all four SDIMO degenerate primers (NVC65, NVC58, NVC57, NVC66) (Coleman et al., 2006) on two 1,4-dioxane degrading consortia, the majority of SDIMO genes in one consortia corresponded to group 6 SDIMOs, while the majority in the other consortia aligned with both groups 5 and 6 (He et al., 2018). When the same primers (NVC57, NVC66) were used on 1,4-dioxane degrading enrichment cultures from landfill leachate, the resulting OTUs were classified into three group 5 SDIMOs subclusters (Inoue et al., 2020). These included subcluster A containing *prm/mmo* genes in Gram-positive bacteria (e.g. *Pseudonocardia* sp. CB1190, *Rhodococcus* sp. RR1), subcluster B containing *prm/mmo* genes in Gram-negative bacteria (e.g. *Methylibium* sp. PM1, *Rhodobacter* sp. MBTLJ-13), and subcluster C containing *thm/dxm* genes (e.g. *Rhodococcus* sp. T1, *Rhodococcus* sp. YYL) (Inoue et al., 2020). They reported the dominance of group 5 SDIMO subcluster C and smaller proportions of group 5 subclusters A and B in 1,4-dioxane and tetrahydrofuran enrichment cultures (Inoue et al., 2020). The same group classified OTUs, this time from a 1,4-dioxane enrichment culture inoculated with activated sludge, within group 5 subcluster A, aligning close to *Rhodococcus jostii* RHA1 *prmA, Rhodococcus* sp. RR1 *prmA* and *Gordonia* sp. TY-5 *prmA* (Inoue et al., 2022).

From the SDIMO degenerate primer amplicon based studies discussed above, it appears the genes encoding for group 5 SDIMOs are present and/or dominant in many 1,4-dioxane degrading communities. These results are consistent with the current study. Specifically, from the

401 comparison of OTUs from all three sample types to the twelve genes previously associated with
402 1,4-dioxane metabolism and co-metabolism (He, Mathieu, Yang, Yu, da Silva , et al., 2017),
403 only those aligning with *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA* were
404 detected. These trends are also consistent with previous research in our group (using shotgun
405 sequencing) that reported the importance of *Rhodococcus* sp. RR1 *prmA* and *Rhodococcus jostii*
406 RHA1 *prmA* over other known 1,4-dioxane degrading genes in 1,4-dioxane degrading mixed
407 communities inoculated with uncontaminated and contaminated soils (Ramalingam et al., 2020).
408
409 The qPCR assays developed in the current study add to the toolbox of qPCR or reverse
410 transcriptase (RT) qPCR assays already developed for other 1,4-dioxane degrading functional
411 genes. A summary of these qPCR assays has been provided (Supplementary Table 4). The
412 summary includes a qPCR assay targeting the large hydroxylase subunit of
413 tetrahydrofuran/dioxane monooxygenases, developed using gene alignments from
414 *Pseudonocardia dioxanivorans* CB1190, *Pseudonocardia tetrahydrofuranoxydans* K1,
415 *Pseudonocardia* sp. ENV478 and *Rhodococcus* sp. YYL (Li et al., 2014) (group 5 SDIMOs).
416 The researchers observed an increase in *thmA/dxmA* copy numbers during 1,4-dioxane
417 biodegradation in groundwater samples from five different dioxane-impacted sites, indicating
418 growth of 1,4-dioxane degraders. Another qPCR assay targets *dxmB/thmB* within the gene
419 cluster of the metabolic 1,4-dioxane degrader, *Pseudonocardia dioxanivorans* CB1190, referred
420 to as dioxane monooxygenase (DXMO) (Gedalanga et al., 2014). A third qPCR assay targets
421 *thmC* from *Pseudonocardia* sp. strain D17, a strain capable of constitutively degrading 1,4-
422 dioxane, using it as a carbon and energy source (Yamamoto et al., 2018). The authors *in silico*
423 analysis suggested the assay would also produce amplicons of the same size from strain
424 *Pseudonocardia dioxanivorans* CB1190, *Pseudonocardia* sp. ENV478, *Pseudonocardia* sp. K1,
425 and *Rhodococcus* sp. YYL (Yamamoto et al., 2018). The primers and probe developed in the
426 current study were designed to select against *Pseudonocardia dioxanivorans* CB1190 *thmA*,
427 *Pseudonocardia tetrahydrofuranoxydans* K1 *thmA*, *Pseudonocardia* sp. ENV478 *thmA* and
428 *Rhodococcus* sp. YYL *thmA*. Specifically, the newly designed primers and probe exhibited
429 between 29 and 30 mismatches (RR1 assay) and between 26-28 mismatches (RHA1 assay) to
430 these four group 5 SDIMOs.
431

Other quantitative assays have targeted group 6 SDIMOs associated with 1,4-dioxane biodegradation (Supplementary Table 4). One RT qPCR assay targeted *prmA* (encoded by the propane monooxygenase *prmABCD* gene cluster) in *Mycobacterium dioxanotrophicus* PH-06 (Deng et al., 2018), an organism able to use 1,4-dioxane as a sole source of carbon and energy (Kim et al., 2009). This gene cluster exhibits a low level of amino acid sequence identity (< 40% for α subunits) with tetrahydrofuran/dioxane monooxygenases (Deng et al., 2018) discussed above and is not expected to be amplified with the newly developed RR1 and RHA1 assays. As discussed above, in the current study, using the previously designed qPCR assay (Deng et al., 2018), cycle threshold values for *Mycobacterium dioxanotrophicus* PH06 *prmA* were at or below the lowest plasmid standard. A RT-qPCR assay was also developed for a 1,4-dioxane degrading isolate containing a group 2 SDIMO (Deng et al., 2020) (Supplementary Table 4). The assay was designed towards a toluene monooxygenase (encoded by *tmoABCDEF* gene cluster) in *Azoarcus* sp. strain DD4, a Gram-negative propanotroph, capable of 1,4-dioxane co-metabolism (Deng et al., 2020).

Here, following the design of the two new qPCR assays, the assays were used to quantify genes in three mixed communities and in a microcosm study, inoculated with sediment from an impacted site, following 1,4-dioxane biodegradation. The highest RR1 *prmA* gene copy numbers were detected in the agricultural soil, followed by the wetland sediments and then the impacted site samples. Gene copies of RHA1 *prmA* were only detected in the agricultural soil and wetland samples and these values were lower than those of RR1 *prmA*. It is of interest to note that although amplicon sequencing detected RHA1 *prmA* in the impacted site sediment, qPCR did not. It is likely the amplicon sequences detected (detection threshold was > 90% sequence identity to each target gene) included mismatches to the *prmA* sequence targeted via qPCR.

An interesting trend was the statistically significant increase in RR1 *prmA* gene copies in the impacted site inoculated microcosms following 1,4-dioxane biodegradation compared to the control microcosms (no 1,4-dioxane) or to the initial copy numbers before incubation. Such a trend is perhaps indicative of growth of the microorganism harboring RR1 *prmA* on 1,4-dioxane. When the amplicons from the current study were compared to sequences similar to *Rhodococcus* sp. RR1 *prmA*, there were close matches to several *Rhodococcus aetherivorans* strains,

significant because *Rhodococcus aetherivorans* JCM 14343(T) is able to use 1,4-dioxane as a sole source of carbon and energy (Inoue et al., 2016). Also, 1,4-dioxane removal in the current study occurred in two phases, an initial lag phase followed by a rapid decline, a pattern typical of growth related metabolism. However, contrary to the results here, others have previously reported co-metabolic 1,4-dioxane biodegradation by *Rhodococcus* sp. RR1 (Mahendra et al., 2006). More research is needed to establish if growth related 1,4-dioxane metabolism by *Rhodococcus* sp. RR1 is indeed occurring.

When the SDIMO sequencing amplicons in the current study were blasted against the entire nt database from NCBI, matches to propane monooxygenase hydroxylase large subunit, aromatic/alkene/methane monooxygenase hydroxylase/oxygenase alpha subunits, methane monooxygenases or SDIMOs were observed. The top matching taxa for each sample type included *Mycobacterium hodleri* strain B, enrichment culture clones and the genera *Rhodococcus* and *Bradyrhizobium*. It is not known if the enzymes encoded by these genes are capable of 1,4-dioxane biodegradation. However, future studies could target these sequences to determine if gene copy numbers are correlated to 1,4-dioxane removal.

5. Conclusions

Monooxygenase targeted amplicon based sequencing highlighted the importance of group 5 SDIMOs in three mixed microbial communities. Building on these results, two qPCR assays were developed to quantify these genes. The assays targeted *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA,* both found within SDIMO subcluster 5A. Gene copies of *Rhodococcus* RR1 *prmA* were detected in all three mixed communities examined, while gene copies of *Rhodococcus jostii* RHA1 *prmA* were detected in two of the three of the three sample types (except impacted site sediment). There was a statistically significant increase in RR1 *prmA* gene copies in the microcosms inoculated with impacted site sediment following 1,4-dioxane biodegradation compared to the control microcosms (no 1,4-dioxane) or to the initial copy numbers before incubation. Such a trend is perhaps indicative of growth of the microorganism harboring RR1 *prmA* on 1,4-dioxane, however, more research is needed to confirm this. The newly designed qPCR assays should be of interest to those involved in the remediation of 1,4-dioxane contaminated sites.

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CRediT authorship contribution statement

ZE: Writing – original draft, Formal analysis, Investigation, Methodology, Data curation.

AMC: Writing – review & editing, Supervision, Funding acquisition, Conceptualization

Declaration of Competing Interest

The authors declare that they have no conflicts of interests.

Data Availability

NCBI BioSample accession numbers: SAMN38186925 to SAMN38186933.

References

Adams, C.D., Scanlan, P.A., Secrist, N.D., 1994. Oxidation and biodegradability enhancement of 1,4-dioxane using hydrogen peroxide and ozone. Environmental Science & Technology. 28, 1812-1818.

Adamson, D.T., Anderson, R.H., Mahendra, S., Newell, C.J., 2015. Evidence of 1,4-dioxane attenuation at groundwater sites contaminated with chlorinated solvents and 1,4-dioxane. Environmental Science & Technology. 49, 6510-6518.

Adamson, D.T., Mahendra, S., Walker, K.L., Rauch, S.R., Sengupta, S., Newell, C.J., 2014. A multisite survey to identify the scale of the 1,4-dioxane problem atcontaminated groundwater sites. Environ Sci Tech Let. 1, 254-258.

Altschul, S.F., Gish, W., Miller, W., Myers, E.W., Lipman, D.J., 1990. Basic local alignment search tool. J Mol Biol. 215, 403-410.

Bell, C.H., Wong, J., Parsons, K., Semel, W., McDonough, J., Gerber, K., 2022. First Full-Scale In Situ Propane Biosparging for Co-Metabolic Bioremediation of 1, 4-Dioxane. Groundwater Monitoring & Remediation. 42, 54-66.

Bernhardt, D., Diekmann, H., 1991. Degradation of dioxane, tetrahydrofuran and other cyclic ethers by an environmental *Rhodococcus* strain. Appl Microbiol Biot. 36, 120-123.

Burback, B.L., Perry, J.J., 1993. Biodegradation and biotransformation of groundwater pollutant mixtures by *Mycobacterium vaccae*. Applied and Environmental Microbiology. 59, 1025-1029.

Bustin, S.A., Benes, V., Garson, J.A., Hellemans, J., Huggett, J., Kubista, M., Mueller, R., Nolan, T., Pfaffl, M.W., Shipley, G.L., 2009. The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments, Oxford University Press.

Chen, D.Z., Jin, X.J., Chen, J., Ye, J.X., Jiang, N.X., Chen, J.M., 2016. Intermediates and substrate interaction of 1,4-dioxane degradation by the effective metabolizer *Xanthobacter flavus* DT8. Int Biodeter Biodegr. 106, 133-140.

Chiang, S.Y., Anderson, R., Wilken, M., Walecka-Hutchison, C., 2016. Practical perspectives of 1, 4-dioxane investigation and remediation. Remediation Journal. 27, 7-27.

Coleman, N.V., Bui, N.B., Holmes, A.J., 2006. Soluble di-iron monooxygenase gene diversity in soils, sediments and ethene enrichments. Environ Microbiol. 8, 1228-1239.

Coleman, N.V., Bui, N.B., Holmes, A.J., 2006. Soluble di-iron monooxygenase gene diversity in soils, sediments and ethene enrichments. Environmental Microbiology. 8, 1228-1239.

Deng, D., Li, F., Li, M., 2018. A novel propane monooxygenase initiating degradation of 1,4-dioxane by *Mycobacterium dioxanotrophicus* PH-06. Environmental Science and Technolology Letters. 5, 86-91.

Deng, D., Li, F., Li, M., 2018. A novel propane monooxygenase initiating degradation of 1, 4-dioxane by Mycobacterium dioxanotrophicus PH-06. Environmental Science & Technology Letters. 5, 86-91.

Deng, D., Ngoc Pham, D., Li, F., Lia, M., 2020. Discovery of an inducible toluene monooxygenase that pooxidizes 1,4-dioxane and 1,1-dichloroethylene in propanotrophic *Azoarcus* sp. Strain DD4. Applied and Environmental Microbiology. 86, e01163-01120

Deng, D.Y., Pham, D.N., Li, M.Y., 2022. Emerging investigator series: environment-specific auxiliary substrates tailored for effective cometabolic bioremediation of 1,4-dioxane. Environmental Science: Water Research & Technology. 8, 2521-2530.

Edgar, R.C., 2010. Search and clustering orders of magnitude faster than BLAST. Bioinformatics 26, 2460-2461.

Edgar, R.C., 2013. UPARSE: highly accurate OTU sequences from microbial amplicon reads. Nat Methods. 10, 996-998.

EPA, E.P.A., 2013. 1,4-Dioxane. www.clu-in.org/contaminantfocus/default.focus/sec/1,4-Dioxane/cat/Overview/.

EPA, E.P.A., 2014. Technical fact sheet –1,4-dioxane. Office of Solid Waste and Emergency Response (5106P). EPA 505-F-14-011.

Fingerhut L., I., C., 2021. ampir Predict Antimicrobial Peptides_. R package version 1.1.0. <https://CRAN.R-project.org/package=ampir>.

Gedalanga, P.B., Pornwongthong, P., Mora, R., Chiang, S.Y., Baldwin, B., Ogles, D., Mahendra, S., 2014. Identification of biomarker genes to predict biodegradation of 1,4-dioxane. Appl Environ Microbiol. 80, 3209-3218.

Gedalanga, P.B., Pornwongthong, P., Mora, R., Chiang, S.Y.D., Baldwin, B., Ogles, D., Mahendra, S., 2014. Identification of Biomarker Genes To Predict Biodegradation of 1,4-Dioxane. Applied and Environmental Microbiology. 80, 3209-3218.

Hand, S., Wang, B., Chu, K.H., 2015. Biodegradation of 1,4-dioxane: effects of enzyme inducers and trichloroethylene. Sci Total Environ. 520, 154-159.

Hand, S., Wang, B.X., Chu, K.H., 2015. Biodegradation of 1,4-dioxane: Effects of enzyme inducers and trichloroethylene. Science of the Total Environment. 520, 154-159.

Hatzinger, P.B., Banerjee, R., Rezes, R., Streger, S.H., McClay, K., Schaefer, C.E., 2017. Potential for cometabolic biodegradation of 1, 4-dioxane in aquifers with methane or ethane as primary substrates. Biodegradation. 28, 453-468.

He, Y., Mathieu, J., da Silva, M.L.B., Li, M., Alvarez, P.J.J., 2018. 1,4-Dioxane-degrading consortia can be enriched from uncontaminated soils: prevalence of *Mycobacterium* and soluble di-iron monooxygenase genes. Microbial Biotechnology. 11, 189-198.

He, Y., Mathieu, J., Yang, Y., Yu, P., da Silva , M.L.B., Alvarez, P.J.J., 2017. 1,4-Dioxane biodegradation by *Mycobacterium dioxanotrophicus* PH-06 is associated with a group-6 soluble di-iron monooxygenase. Environmental Science and Technolology Letters. 4, 494–499.
He, Y., Mathieu, J., Yang, Y., Yu, P.F., da Silva, M.L.B., Alvarez, P.J.J., 2017. 1,4-Dioxane Biodegradation by Mycobacterium dioxanotrophicus PH-06 Is Associated with a Group-6 Soluble Di-Iron Monooxygenase. Environ Sci Tech Let. 4, 494-499.
He, Y., Wei, K., Si, K., Mathieu, J., Li, M., Alvarez, P.J.J., 2017. Whole-genome sequence of the 1, 4-dioxane-degrading bacterium Mycobacterium dioxanotrophicus PH-06. Genome Announcements. 5, 10-1128.
Inoue, D., Hisada, K., Ike, M., 2022. Effectiveness of tetrahydrofuran at enhancing the 1,4-dioxane degradation ability of activated sludge lacking prior exposure to 1,4-dioxane. Water Science and Technology. 86, 1707-1718.
Inoue, D., Hisada, K., Okumura, T., Yabuki, Y., Yoshida, G., Kuroda, M., Ike, M., 2020. Carbon sources that enable enrichment of 1,4-dioxane-degrading bacteria in landfill leachate. Biodegradation. 31, 23-34.
Inoue, D., Tsunoda, T., Sawada, K., Yamamoto, N., Saito, Y., Sei, K., Ike, M., 2016. 1,4-Dioxane degradation potential of members of the genera *Pseudonocardia* and *Rhodococcus*. Biodegradation. 27, 277-286.
Isaka, K., Udagawa, M., Sei, K., Ike, M., 2016. Pilot test of biological removal of 1,4-dioxane from a chemical factory wastewater by gel carrier entrapping *Afipia* sp strain D1. J Hazard Mater. 304, 251-258.
Katoh, K., Rozewicki, J., Yamada, K.D., 2019. MAFFT online service: multiple sequence alignment, interactive sequence choice and visualization. Brief Bioinform. 20, 1160-1166.
Kim, Y.M., Jeon, J.R., Murugesan, K., Kim, E.J., Chang, Y.S., 2009. Biodegradation of 1,4-dioxane and transformation of related cyclic compounds by a newly isolated *Mycobacterium* sp PH-06. Biodegradation. 20, 511-519.
Kim, Y.M., Jeon, J.R., Murugesan, K., Kim, E.J., Chang, Y.S., 2009. Biodegradation of 1,4-dioxane and transformation of related cyclic compounds by a newly isolated *Mycobacterium* sp. PH-06. Biodegradation. 20, 511-519.
Kohlweyer, U., Thiemer, B., Schrader, T., Andreesen, J.R., 2000. Tetrahydrofuran degradation by a newly isolated culture of *Pseudonocardia* sp strain K1. Fems Microbiol Lett. 186, 301-306.
Kreader, C.A., 1996. Relief of amplification inhibition in PCR with bovine serum albumin or T4 gene 32 protein. Applied and Environmental Microbiology. 62, 1102-1106.
Kumar, S., Stecher, G., Li, M., Knyaz, C., Tamura, K., 2018. MEGA X: molecular evolutionary genetics analysis across computing platforms. Mol Biol Evol. 35, 1547-1549.
Letunic, I., Bork, P., 2021. Interactive Tree Of Life (iTOL) v5: an online tool for phylogenetic tree display and annotation. Nucleic Acids Res. 49, W293-W296.
Li, M., Liu, Y., He, Y., Mathieu, J., Hatton, J., DiGuiseppi, W., Alvarez, P.J.J., 2017. Hindrance of 1, 4-dioxane biodegradation in microcosms biostimulated with inducing or non-inducing auxiliary substrates. Water research. 112, 217-225.
Li, M., Mathieu, J., Liu, Y., Tess Van Orden, E., Yang, Y., Fiorenza, S., Alvarez, P.J.J., 2014. The abundance of tetrahydrofuran/dioxane monooxygenase genes (*thmA/dxmA*) and 1,4-dioxane degradation activity are significantly correlated at various impacted aquifers. Environmental Science and Technolology Letters. 1, 122-127.
Mahendra, S., Alvarez-Cohen, L., 2005. *Pseudonocardia dioxanivorans* sp nov., a novel actinomycete that grows on 1,4-dioxane. Int J Syst Evol Micr. 55, 593-598.
Mahendra, S., Alvarez-Cohen, L., 2006. Kinetics of 1,4-dioxane biodegradation by monooxygenase-expressing bacteria. Environ Sci Technol. 40, 5435-5442.
Mahendra, S., Alvarez-Cohen, L., 2006. Kinetics of 1,4-dioxane biodegradation by monooxygenase-expressing bacteria. Environ Sci Technol. 40, 5435-5442.

628 Mohr, T., 2010. Environmental Investigation and Remediation 1,4 Dioxane and Other Solvent Stabilizers
629 Introduction.
630 Mohr, T., Stickney, J., DiGuiseppi, W., 2010. Environmental Investigation and Remediation: 1,4-Dioxane
631 and Other Solvent Stabilizers, CRC Press, Boca Raton, ML.
632 Mohr, T.K.G., 2001. 1, 4-Dioxane and other solvent stabilizers. White Paper. Santa Clara Valley Water
633 District.
634 Myers, M.A., Johnson, N.W., Marin, E.Z., Pornwongthong, P., Liu, Y., Gedalanga, P.B., Mahendra, S.,
635 2018. Abiotic and bioaugmented granular activated carbon for the treatment of 1,4-dioxane-
636 contaminated water. Environmental Pollution. 240, 916-924.
637 Ooms, J., 2023. writexl: Export Data Frames to Excel 'xlsx' Format_. R package version 1.4.2.
638 <https://CRAN.R-project.org/package=writexl>.
639 Parales, R.E., Adamus, J.E., White, N., May, H.D., 1994. Degradation of 1,4-dioxane by an actinomycete
640 in pure culture. Applied and Environmental Microbiology. 60, 4527-4530.
641 Pugazhendi, A., Banu, J.R., Dhavamani, J., Yeom, I.T., 2015. Biodegradation of 1,4-dioxane by
642 *Rhodanobacter* AYS5 and the role of additional substrates. Ann Microbiol. 65, 2201-2208.
643 R Core Team, 2018. R: A language and environment for statistical computing, R Foundation for Statistical
644 Computing, Vienna, Austria. .
645 Ramalingam, V., Cupples, A.M., 2020. Anaerobic 1,4-dioxane biodegradation and microbial community
646 analysis in microcosms inoculated with soils or sediments and different electron acceptors. Applied
647 Microbiology and Biotechnology. 104, 4155-4170.
648 Ramalingam, V., Cupples, A.M., 2020. Enrichment of novel *Actinomycetales* and the detection of
649 monooxygenases during aerobic 1,4-dioxane biodegradation with uncontaminated and contaminated
650 inocula. Appl Microbiol Biotechnol. 104, 2255-2269.
651 Rolston, H.M., Hyman, M.R., Semprini, L., 2019. Aerobic cometabolism of 1, 4-dioxane by isobutane-
652 utilizing microorganisms including Rhodococcus rhodochrous strain 21198 in aquifer microcosms:
653 Experimental and modeling study. Science of The Total Environment. 694, 133688.
654 RStudio_Team, 2020. RStudio: Integrated Development for R. RStudio, PBC, Boston, MA pp. URL
655 http://www.rstudio.com/.
656 Sei, K., Miyagaki, K., Kakinoki, T., Fukugasako, K., Inoue, D., Ike, M., 2013. Isolation and characterization
657 of bacterial strains that have high ability to degrade 1,4-dioxane as a sole carbon and energy source.
658 Biodegradation. 24, 665-674.
659 Sharp, J.O., Sales, C.M., Alvarez-Cohen, L., 2010. Functional characterization of propane-enhanced N-
660 nitrosodimethylamine degradation by two actinomycetales. Biotechnol Bioeng. 107, 924-932.
661 Sharp, J.O., Sales, C.M., LeBlanc, J.C., Liu, J., Wood, T.K., Eltis, L.D., Mohn, W.W., Alvarez-Cohen, L., 2007.
662 An inducible propane monooxygenase is responsible for n-nitrosodimethylamine degradation by
663 *Rhodococcus* sp. strain RHA1. Applied and Environmental Microbiology. 73, 6930-6938.
664 Stefan, M.I., Bolton, J.R., 1998. Mechanism of the degradation of 1,4-dioxane in dilute aqueous solution
665 using the UV hydrogen peroxide process. Environmental Science & Technology. 32, 1588-1595.
666 Steffan, R.J., McClay, K.R., Masuda, H., Zylstra, G.J., 2007. Biodegradatdion of 1,4-dioxane. Strategic
667 Environmental Research and Development Program: ER-1422 Final Report.
668 Vainberg, S., McClay, K., Masuda, H., Root, D., Condee, C., Zylstra, G.J., Steffan, R.J., 2006.
669 Biodegradation of ether pollutants by *Pseudonocardia* sp strain ENV478. Applied and Environmental
670 Microbiology. 72, 5218-5224.
671 Wang, M.Y., Olson, B.H., Chang, J.S., 2007. Improving PCR and qPCR detection of hydrogenase A (hydA)
672 associated with Clostridia in pure cultures and environmental sludges using bovine serum albumin. Appl
673 Microbiol Biot. 77, 645-656.
674 Wickham, H., 2016. ggplot2: Elegant Graphics for Data Analysis. Springer-Verlag New York, 2016.
675 https://ggplot2.tidyverse.org.

676 Wickham, H., , , Bryan, J., 2023. readxl: Read Excel Files_. R package version 1.4.2,. <https://CRAN.R-
677 project.org/package=readxl>.
678 Wickham, H., Averick, M., Bryan, J., Chang, W., McGowan, L.D.A., François, R., Grolemund, G., Hayes, A.,
679 Henry, L., Hester, J., Kuhn, M., Pedersen, T.L., Miller, E., Bache, S.M., Müller, K., Ooms, J., Robinson, D.,
680 Seidel, D.P., Spinu, V., Takahashi, K., Vaughan, D., Wilke, C., Woo, K., Yutani, H., 2019. Welcome to the
681 Tidyverse. Journal of Open Source Software. 4(43), 1686,.
682 Yamamoto, N., Inoue, D., Sei, K., Saito, Y., Ike, M., 2018. Field test of on-site treatment of 1,4-dioxane-
683 contaminated groundwater using *Pseudonocardia* sp. D17. Journal of Water and Environment
684 Technology. 16, 256-268.
685 Ye, J., Coulouris, G., Zaretskaya, I., Cutcutache, I., Rozen, S., Madden, T.L., 2012. Primer-BLAST: a tool to
686 design target-specific primers for polymerase chain reaction BMC Bioinformatics. 18, 134.
687 Zenker, M.J., Borden, R.C., Barlaz, M.A., 2003. Occurrence and treatment of 1, 4-dioxane in aqueous
688 environments. Environmental Engineering Science. 20, 423-432.
689 Zhang, J., 2017. phylotools: Phylogenetic Tools for Eco-Phylogenetics_. R package version 0.2.2.
690 <https://CRAN.R-project.org/package=phylotools>.

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