Impact of Yeast Extract and Basal Salts Medium on 1,4-Dioxane Biodegradation Rates and the Microorganisms Involved in Carbon Uptake from 1,4-Dioxane

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Abstract

Conventional physical and chemical treatment technologies for 1,4-dioxane can be ineffective and consequently attention has focused on bioremediation. Towards this, the current research investigated the impact of basal salts medium (BSM) and yeast extract on 1,4-dioxane biodegradation rates in microcosms with different soil or sediment (agricultural soil, wetland sediment, sediment from an impacted site). Phylotypes responsible for carbon uptake from 1,4-dioxane were determined using stable isotope probing (SIP), both with and without BSM and yeast extract. Further, putative functional genes were investigated using 1) soluble di-iron monooxygenase (SDIMO) based amplicon sequencing, 2) qPCR targeting propane monooxygenase (large subunit, *prmA*) and 3) a predictive approach (PICRUSt2). The addition of BSM and yeast extract significantly enhanced 1,4-dioxane removal rates the agricultural soil and impacted site sediment microcosms. The phylotypes associated with carbon uptake varied across treatments and inocula. *Gemmatimonas* was important in the heavy SIP fractions of the wetland sediment microcosms. Unclassified *Solirubacteraceae*, *Solirubrobacter*, *Pseudonocardia* and *RB4* were dominant in the heavy SIP fractions of the agricultural soil microcosms. The heavy SIP fractions of the impacted site microcosms were dominated by only two phylotypes, unclassified *Burkholderiaceae* and *oc3299*. SDIMO based amplicon sequencing detected three genes previously associated with 1,4-dioxane. The predicted functional gene analysis suggested the importance of propane monooxygenases associated with *Solirubrobacter* and *Pseudonocardia.* Overall, more microorganisms were involved in carbon uptake from 1,4-dioxane in both the wetland and agricultural soil microcosms compared to the impacted site sediment microcosms. Many of these microorganisms have not previously been associated with 1,4-dioxane removal.

Keywords: 1,4-Dioxane, stable isotope probing, *Gemmatimonas, Solirubrobacter*, *Pseudonocardia,* propane monooxygenase

1. Introduction

1,4-Dioxane, a probable human carcinogen, commonly used as a solvent and stabilizer for the chlorinated solvents, has been detected in both surface water and groundwater (Adamson et al.,

2017; Dang et al., 2018; Derosa et al., 1996; ITRC; Karges et al., 2018; USEPA, 2013). The characteristics of 1,4-dioxane (high water solubility and low Henry's Law constant) pose challenges for remediation using conventional treatment techniques, such as air stripping or activated carbon (Godri Pollitt et al., 2019; Kikani et al., 2022; Steffan et al., 2007; Zenker et al., 2003; Zhang et al., 2017). In the past decade, biologically-mediated 1,4-dioxane removal has been used as alternative approach to clean up 1,4-dioxane contaminated sites (Bell et al., 2022; Divine et al., 2024; Horst et al., 2019; Lippincott et al., 2015).

Numerous microorganisms have been associated with metabolic or co-metabolic 1,4-dioxane biodegradation. *Pseudonocardia dioxanivorans* CB1190 (Mahendra and Alvarez-Cohen, 2006; Parales et al., 1994), *Pseudonocardia* sp. D17 (Sei et al., 2013), *Pseudonocardia* sp. N23 (Yamamoto et al., 2018), *Pseudonocardia benzenivorans* B5 (Mahendra and Alvarez-Cohen, 2006), *Xanthobacter flavus* DT8 (Chen et al., 2016), *Mycobacterium* sp. PH-06 (Kim et al., 2009), *Acinetobacter baumannii* DD1 (Huang et al. 2014) and *Rhodanobacter* AYS5 (Pugazhendi et al., 2015) utilize 1,4-dioxane as a sole carbon source. Others degrade 1,4-dioxane co-metabolically, including: *Pseudonocardia* sp. ENV478 (Vainberg et al., 2006), *Pseudonocardia tetrahydrofuranoxydans* sp. K1 (Kohlweyer et al., 2000) and *Rhodococcus* sp. YYL (Yao et al., 2009b) induced by tetrahydrofuran; *Rhodococcus* sp. RR1, *Burkholderia cepacia* G4, *Ralstonia pickettii* PKO1 and *Pseudomonas mendocina* KR1 (Mahendra and Alvarez-Cohen, 2006) induced by toluene; and *Rhodococcus ruber* ENV425 (Lippincott et al., 2015), *Mycobacterium vaccae* JOB5 (Mahendra and Alvarez-Cohen, 2006) and *Rhodococcus jostii* RHA1 (Hand et al., 2015) induced by propane. A full list of pure culture species and strains capable of degrading 1,4-dioxane was recently generated (Divine et al., 2024).

The biochemical pathway for 1,4-dioxane biodegradation is initiated by soluble di-iron monooxygenases (SDIMOs). In general, SDIMOs have been classified into seven groups based on their substrate specificity and function (Coleman et al., 2006; Notomista et al., 2003; Yang et al., 2024). SDIMOs associated with the co-metabolic and metabolic biodegradation of 1,4-dioxane were previously summarized, being primarily in SDIMO groups 1, 2, 3, 5 and 6 (He et al., 2017). To date, the majority of 1,4-dioxane focused research has involved groups 5 and 6 SDIMOs, such as propane monooxygenase from *Mycobacterium dioxanotrophicus* PH-06 (group

6) (Deng et al., 2018) and tetrahydrofuran monooxygenase from *Pseudonocardia dioxanivorans* CB1190 (group 5) (Sales et al., 2013; Sales et al., 2011), *Pseudonocardia* sp. strain ENV478 (group 5) (Masuda et al., 2012) and *Pseudonocardia tetrahydrofuranoxydans* K1 (group 5) (Sales et al., 2013). Propane monooxygenase subunit sequences similar to *Rhodococcus* sp. RR1 *prmA* (group 5) were also linked to 1,4-dioxane biodegradation in mixed microbial communities (Eshghdoostkhatami and Cupples, 2024).

Although much is known about the enzymes, genes and microorganisms associated with 1,4-dioxane biodegradation in pure culture, less is known about removal mechanisms in mixed microbial cultures. A valuable approach for examining contaminant biodegradation in mixed communities is known as stable isotope probing (SIP). SIP is a cultivation-independent method, tracking the incorporation of a stable isotope from a labeled chemical into DNA or RNA (Cupples, 2016; Kim et al., 2023; Radajewski et al., 2000). This approach has been applied to characterize active degraders for various chemicals, such as ethane and propane (Farhan Ul Haque et al., 2022), *n*-hexadecane (Liu et al., 2019), phenanthrene (Bao et al., 2022; Thomas et al., 2019), vinyl chloride (Paes et al., 2015), hexahydro-1,3,5-trinitro-1,3,5-triazine (Cho et al., 2013; Jayamani and Cupples, 2015), methyl *tert*-butyl ether (Sun et al., 2012) and *cis*-dichloroethene (Dang and Cupples, 2021). Previous studies used this approach to identify 1,4-dioxane degraders in sludge (Aoyagi et al., 2018), groundwater (Bell et al., 2016) and soils (Dang and Cupples, 2021).

Optimizing bioremediation at contaminated sites often involves the addition of carbon sources to support *in situ* microbial communities. Various carbon sources have been evaluated as substrates to enhance 1,4-dioxane biodegradation, including tetrahydrofuran, 1,3,5-trioxane, ethylene glycol, diethylene glycol, 1,4-butanediol, butanone, acetone, 1-butanol, 2-butanol, phenol, propanol, acetate, ethane, propane, methane and lactate (Dang and Cupples, 2021; Hatzinger et al., 2017; Inoue et al., 2022; Inoue et al., 2020; Miao et al., 2021; Sei et al., 2010; Tawfik et al., 2022; Xiong et al., 2020; Xiong et al., 2019). The current study examined the impact of adding yeast extract and basal salts medium (BSM) on 1,4-dioxane biodegradation rates. As yeast extract contains multiple growth factors, it has the potential to be beneficial to numerous microorganisms potentially linked to 1,4-dioxane biodegradation. Although yeast extract has

previously been shown to enhance 1,4-dioxane biodegradation in pure cultures (Chen et al., 2016; Pugazhendi et al., 2015), little is known about the impact in mixed communities. Also, in those studies, high yeast extract concentrations were used (20 mg/L and 100 mg/L) (Chen et al., 2016; Pugazhendi et al., 2015), which is unlikely to be suitable at contaminated sites because of biofouling. Therefore, the current research examined the impact of lower yeast extract concentrations (60 µg/L) on 1,4-dioxane biodegradation rates. The objectives were to 1) examine the impact of BSM and yeast extract on 1,4-dioxane degradation rates in microcosms amended with different inocula (agricultural soil, wetland sediment and impacted site sediments), 2) identify the phylotypes involved in carbon uptake from 1,4-dioxane using SIP, and 3) determine the functional genes putatively associated with 1,4-dioxane biodegradation.

2. Methods

2.1. Chemicals, Inocula and Microcosm Setup

Unlabeled 1,4-dioxane (≥99.5%) and 1,4-dioxane-d_8 (≥99% isotopic purity) were purchased from Sigma-Aldrich (MO, USA). Labeled ^{13}C-1,4-dioxane ($(^{13}C)_4H_8O_2$, 99% isotopic purity) was purchased from Santa Cruz Biotechnology (TX, USA). The biodegradation of 1,4-dioxane was examined using three inocula, including wetland sediment (Lake Lansing, MI), sediment from an impacted site in California (West Coast Naval Station) and agricultural soil. Three microbial communities from diverse environments were selected to provide a potentially wide range of functional genes and active microorganisms. The basic soil characteristics are shown in Supplementary Table 1. The agricultural soil was collected from six replicate plots of the Main Cropping System Experiment at the Kellogg Biological Station Long-Term Ecological Research, in southwest Michigan. This treatment receives no chemical inputs, compost or manure. More details of this treatment can be found at https://lter.kbs.msu.edu/research/long-term-experiments/main-cropping-system-experiment/. All soils and sediments were stored at 4 °C in the dark before use. Laboratory microcosms were established with soil or sediment (10 g wet weight) and 30 mL of liquid (reverse osmosis purified, non-sterile water or BSM with yeast extract) in 160 mL serum bottles. The BSM was modified from a previous recipe (Pugazhendi et al., 2015) and contained NH_4Cl (1.0 g/L), K_2HPO_4 (3.24 g/L), NaH_2PO_4 (1.0 g/L), $MgSO_4$ (0.20 g/L), $FeSO_4$ (0.012 g/L), $MnSO_4$ (0.003 g/L), $ZnSO_4$ (0.003 g/L) and $CoCl_2$ (0.001 g/L). The final pH of the BSM was adjusted to 7.4 with 0.1N NaOH. The media also contained 60 µg/L

yeast extract (Sigma-Aldrich, USA). For each inocula type and treatment, the experimental design included triplicate live microcosms amended with ^{13}C labeled 1,4-dioxane, triplicate live microcosms amended with ^{12}C 1,4-dioxane and triplicate abiotic microcosms (abiotic controls) amended with ^{12}C 1,4-dioxane. For each inocula type, the nine microcosms were either amended with water or were amended with BSM and yeast extract (eighteen microcosms for each in total). For each inocula type, six abiotic controls were included (three with water and three with BSM with yeast extract) and were amended with unlabeled 1,4-dioxane. For all treatments, the abiotic controls were autoclaved daily for three consecutive days. All microcosms, sealed with a rubber stopper and aluminum crimp, were incubated at room temperature on a rotary bench-top shaker. The microcosms were opened for 0.5 hr every five days for aeration. The initial concentrations of 1,4-dioxane were ~2 mg/L in the live sample microcosms and abiotic controls. This initial concentration was used to ensure a sufficient level of label uptake for SIP. 1,4-Dioxane concentrations were measured over 50 days (due to cost limitations associated with the analysis).

2.2. 1,4-Dioxane Analysis

A triple quadrupole Agilent 7010B GC/MS system (Agilent Technologies, CA, USA) equipped with a VF-5ms column (length 30 m, inner diameter 0.25 mm, film thickness 0.25 μm) and solid phase micro extraction (SPME) (Sigma-Aldrich, MO, USA) was used to measure 1,4-dioxane concentrations in the liquid phase of the microcosms. The SPME fiber was assembled with 30 μm carboxen/polydimethylsiloxane layer, 50 μm divinylbenzene layer and a 24 Ga needle. At each sampling time-point, 1 mL of each sample was collected using a sterile syringe (3 mL) and needle (22 Ga 1.5 in.) and then filtered (0.22 μm nylon filter) (Biomed Scientific). An aliquot (500 μL) of the filtered samples or series of diluted external standards were added into amber glass vials (40 mL). Also, 500 μL of 200 μg/L 1,4-dioxane-d_8 was added into each vial as an internal standard. The vials were maintained at 40°C before the measurement. The SPME fiber was conditioned at 270 °C for 30 mins before each sequence run. For each sample, the fiber was inserted into the vials and exposed to the analytes for 2 mins. The analytes in the headspace adsorbed onto the fiber and then the fiber was exposed to the inlet. The initial oven temperature time was 40 °C and this was maintained for 4 mins. The oven temperature was then programmed to increase to 250 °C at a rate of 40 °C/min. The flow rate of carrier gas (helium) was 1.2 mL/min in constant flow mode. The limit of detection was 1.72 μg/L and the limit of

quantification was 5.21 µg/L. R squared values for standard curves were typically greater than 0.98. Although recovery rates were not measured, all initial values were within approximately 15% of that expected. Triplicate samples (or controls) generally produced similar concentrations (as can be seen from the resulting graphs)."

2.3. DNA Extraction, Fractioning and MiSeq Illumina Sequencing

DNA was extracted, in triplicate, from the live ^{12}C 1,4-dioxane and ^{13}C labeled 1,4-dioxane amendment microcosms using the DNA extraction kit (DNeasy PowerLyzer PowerSoil Kit, Mo Bio, USA) according to the manual protocol. The concentration of DNA in each extract was quantified using the Quant-iT™ dsDNA High-Sensitivity Assay Kit. Ultracentrifugation and fractioning were performed as previously described (Dang and Cupples, 2021; Li et al., 2024). For each of the labeled and unlabeled 1,4-dioxane DNA extracts, twelve tubes were ultracentrifuged, including DNA from duplicate microcosms for both the H_2O treatment and the BSM with yeast extract treatment, for impacted site, agricultural soil, and wetland sediment. In total, twenty-four tubes were ultracentrifuged (2 isotopes [^{12}C and ^{13}C] × 2 microcosms replicates × 2 treatments × 3 soil/sediment types). Two of the three triplicates were randomly selected for SIP due to limitations on sequencing costs. For each of the twenty-four ultracentrifugation runs, three heavy fractions (buoyant density ~1.73 to ~1.75 g/mL) and one light fraction (buoyant density ~1.7 g/mL) were selected. Although fractions of heavier buoyant density were collected, they did not meet the minimum DNA concentration required for 16S rRNA gene amplicon sequencing. In total, three 96-well plates (4 fractions, 3 replicates for each fraction, 2 isotopes, 2 microcosms replicates, 2 treatments, 3 soil types) were submitted to the Genomic Cores at the Research Technology Support Facility (RTSF) at Michigan State University (MSU).

The V4 region of 16S rRNA gene was targeted for amplification using primers 515f (5'-GTGCCAGCMGCCGCGGTAA-3') and 806r (5'-GGACTACHVGGGTWTCTAAT-3') following a previously described protocol (Kozich et al., 2013). PCR products were batch normalized using Invitrogen SequalPrep DNA Normalization plates and the products recovered from the plates pooled. The pool was cleaned and concentrated using AmpureXP magnetic beads; then QC'd and quantified using a combination of Qubit dsDNA HS, Agilent 4200

TapeStation HS DNA1000, and Kapa Illumina Library Quantification qPCR assays. The pool was loaded onto an Illumina MiSeq v2 standard flow cell and sequencing was performed in a 2 × 250 bp paired end format using a MiSeq v2 500 cycle reagent cartridge. Custom sequencing and index primers were added to appropriate wells of the reagent cartridge. Base calling was performed by Illumina Real Time Analysis (RTA) v1.18.54 and RTA output demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1. The raw sequences were summitted to NCBI under Bioproject PRJNA1073031 (accession numbers SAMN39784393 to SAMN39784676).

2.4. Microbial Community Analyses & the Identification of Phylotypes Incorporating ^{13}C

Raw amplicon sequences in the fastq format were combined, trimmed, aligned and quality controlled using Mothur (Schloss et al., 2009) on the High Performance Computing Cluster (HPCC) at MSU. The SILVA bacteria database (Release 138) for the V4 region (Pruesse et al., 2007) was used for the alignments and the sequences were then classified into operational taxonomic units (OTUs) at 97% similarity. The classification of OTUs into taxonomic levels and downstream analysis were conducted with two Mothur files (shared file and taxonomy file) with R (Version 4.2.1) (R Core Team, 2018) in RStudio (Version 2022.12.0) (RStudio_Team, 2020). The packages phyloseq (version 1.34.0) (McMurdie and Holmes, 2013) and microbiome (version 1.12.0) (Lahti and Shetty, 2012-2019) were used to 1) determine the relative abundance at the phyla level in the fractions, 2) generate barcharts for the most abundant families in the three soil samples, 3) perform the alpha diversity analysis (Chao1, ACE, Shannon's values, Simpson, Inverse of Simpson, and Fisher indices), and 4) conduct the Principal Coordinate Analysis (PCoA).

The statistically enriched phylotypes in the heavy fractions of the ^{13}C 1,4-dioxane amended samples (those responsible for carbon uptake from 1,4-dioxane) were determined using the R packages dplyr (version 1.1.3) (Wickham et al., 2023a), tidyr (version 1.3.0) (Wickham et al., 2023b), ggpubr (version 0.6.0) (Kassambara, 2023a) and rstatix (version 0.7.2) (Kassambara, 2023b). For this, the Wilcoxon Test (function wilcox_test) (one sided, $p < 0.05$) was used to determine which phylotypes exhibited a greater relative abundance in the ^{13}C 1,4-dioxane

amended heavy fractions compared to the corresponding ^{12}C 1,4-dioxane amended heavy fractions. Following this, phylotypes statistically enriched in the light ^{13}C 1,4-dioxane amended fractions compared to the corresponding ^{12}C 1,4-dioxane amended light fractions were removed from the list generated above to avoid possible false positives. The R packages ggplot2 (version 3.3.5) (Wickham, 2016a) and forcats (version 1.0.0) (Wickham, 2023) were used to generate the boxplots for the top ten most abundant statistically enriched phylotypes. The packages dplyr (version 1.1.3) (Wickham et al., 2023a) and ggplot2 (version 3.3.5) (Wickham, 2016a) were used to illustrate the number of enriched OTUs and families in the three soil types.

2.5. PICRUSt2 Monooxygenase Gene Predictions

PICRUSt2 (Douglas et al., 2020) was utilized to analyze the Mothur generated files on the HPCC at MSU. The inputs to PICRUSt2 involved a fasta file and a biom file. PICRUSt2 predicts the functional potential of microbial communities based on marker gene (16S rRNA gene) sequencing profiles. PICRUSt2 was applied with EPA-NG (Barbera et al., 2019) and gappa (Czech et al., 2020) for phylogenetic placement of reads, castor (Louca and Doebeli, 2018) for hidden state prediction and MinPath (Ye and Doak, 2009) for pathway inference. The PICRUSt2 generated files (pred_metagenome_contrib.tsv and pred_metagenome_contrib.tsv) were investigated (primarily using the R packages tidyr and dplyr) for the presence of genes associated with monooxygenases (from the KEGG database (Kanehisa 2002)) as well as the phylotypes associated with each monooxygenase. More information on the data within each file can be found in the following tutorial (https://github.com/picrust/picrust2/wiki/PICRUSt2-Tutorial-(v2.5.0)). Functional genes investigated (KEGG number in parenthesis) included: *prmA* propane 2-monooxygenase large subunit (K18223), *prmB* propane monooxygenase reductase component (K18225), *prmC* propane 2-monooxygenase small subunit (K18224), *prmD* (K18226) propane monooxygenase coupling protein, *pmoA-amoA* methane/ammonia monooxygenase subunit A (K10944), *pmoB-amoB* methane/ammonia monooxygenase subunit B (K10945), *pmoC-amoC* methane/ammonia monooxygenase subunit C (K10946), *mmoX* methane monooxygenase component A alpha chain (K16157), *mmoY* methane monooxygenase component A beta chain (K16158), *mmoZ* methane monooxygenase component A gamma chain (K16159), *mmoB* methane monooxygenase regulatory protein B (K16160), *mmoC* methane monooxygenase

component C (K16161), *mmoD* methane monooxygenase component D (K16162), *tmoA, tbuA1, touA* toluene monooxygenase system protein A (K15760), *tmoB, tbuU, touB* toluene monooxygenase system protein B (K15761), *tmoC, tbuB, touC* toluene monooxygenase system ferredoxin subunit (K15762), *tmoD, tbuV, touD* toluene monooxygenase system protein D (K15763), *tmoE, tbuA2, touE* toluene monooxygenase system protein E (K15764), *tmoF, tbuC, touF* toluene monooxygenase electron transfer component (K15765), *dmpK/poxA/tomA0* phenol/toluene 2-monooxygenase (NADH) P0/A0 (K16249), *dmpL/poxB/tomA1* phenol/toluene 2-monooxygenase (NADH) P1/A1 (K16243), *dmpM/ poxC/tomA2* phenol/toluene 2-monooxygenase (NADH) P2/A2 (K16244), *dmpN/poxD/tomA3* phenol/toluene 2-monooxygenase (NADH) P3/A3 (K16242), *dmpO/poxE/ tomA4* phenol/toluene 2-monooxygenase (NADH) P4/A4 (K16245) and *dmpP/poxF/tomA5* phenol/toluene 2-monooxygenase (NADH) P5/A5 (K16246).

RStudio on the HPCC at MSU was used to generate a file that contained which gene subunits and phylotypes were present using the PICRUSt2 output file pred_metagenome_contrib.tsv (unzipped). The approach involved combining this file with 1) a file containing gene numbers and descriptions and 2) a taxonomy file (from Mothur), using the R packages data.table (version 1.14.8) (Dowle and Srinivasan, 2023), dplyr (version 1.1.3) (Wickham et al., 2023a), tidyr (version 1.3.0) (Wickham et al., 2023b), ggplot2 (Wickham, 2016b) and patchwork (version 1.1.3) (Pedersen, 2023). Bar charts were generated for each monooxygenase, faceted by the sample type and the gene subunits.

2.6. SDIMO Gene Amplicon Sequencing

A two-step library preparation was completed for sequencing, first involving PCR with target-specific primers with tags on the 5 prime ends (Fluidigm common oligos CS1/CS2) to facilitate the second PCR for barcoding. The target-specific primers included two degenerate primers previously designed to target conserved regions in the SDIMO alpha subunit gene (called NVC57 and NVC66, target size 420 bp, Supplementary Table 2) (Coleman et al., 2006). The following steps were performed by the Genomics Core at RTSF at MSU. PCR amplicons were batch-normalized using Invitrogen SequalPrep DNA Normalization plates and the recovered product was pooled. The pool was QC'd and quantified using a combination of Qubit dsDNA

HS, Agilent 4200 TapeStation HS DNA1000 and Invitrogen Collibri Library Quantification qPCR assays. This pool was loaded onto one (1) Illumina MiSeq v2 Standard flow cell and sequencing was carried out in a 2x250bp paired end format using a MiSeq v2 500 cycle reagent 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. The raw sequences were summitted to NCBI under Bioproject PRJNA1073036 (accession numbers SAMN39784693 to SAMN39784716).

2.7. SDIMO Sequences Processing and Analysis

The amplicon sequencing files were processed on the HPCC at MSU using usearchv11 (Edgar, 2010). The steps included an inspection of data quality and using the commands -fastx_info and fastq_eestats2. Sequences were then pooled using -fastq_mergepairs. Quality filtering was achieved using -fastq_filter, with a maximum expected error threshold set to 1.0. Following this, sequences were dereplicated using -fastx_uniques. The command cluster_otus was used to complete 97% OTU clustering using the UPARSE-OTU (Edgar, 2013) algorithm and to filter chimeras. The -otutab command was used to generate OTU tables with OTU abundance values.

To enable a comparison of the OTUs to genes previously associated with 1,4-dioxane metabolism and co-metabolism, twelve gene sequences previously associated with 1,4-dioxane biodegradation as summarized in (He et al., 2017) were obtained from NCBI. Each of the twelve gene sequence were then uploaded for a nucleotide-nucleotide blastn search to find highly similar sequences to create a blast database for each (Altschul et al., 1990). To ensure only highly similar sequences were selected, the resulting databases were filtered using a percent identity and query length threshold of greater than or equal to 95%. The occurrence of the gene sequences in each database was investigated in the usearch files generated by using blastn (BLAST/2.10.0-Linux_x86_64 on HPCC).

The results from the blastn search were downloaded from HPCC and were examined using R (Version 4.2.1) (R Core Team, 2018) in RStudio (Version 2022.12.0) (RStudio_Team, 2020).

Specifically, the results 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 numbers of OTUs aligning to each gene database for each sample were determined and the datasets were used to construct phylogenetic trees (as described below). As only three (*Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA*, *Pseudonocardia dioxanivorans* CB1190 plasmid pPSED02 Psed_6976) of the twelve genes were detected, only three trees were generated. Data manipulation, data analysis and the generation of figures was completed 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. and I., 2021), writexl (Version 1.4.2) (Ooms, 2023), readxl (Version 1.4.2) (Wickham and Bryan, 2023), writexl (Ooms, 2023), ggplot2 (Wickham, 2016b) and phylotools (Version 0.2.2) (Zhang, 2017).

2.8. Phylogenetic Trees

Sequences were first 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 generated (using the Neighbor-Joining method and Jukes-Cantor model) were then exported in Newick format and uploaded to the Interactive Tree of Life (https://itol.embl.de) (Letunic and Bork, 2021) (Version 6.7.2). The OTU abundance values for each sample were added using the datasets function called multi value bar chart.

2.9. Quantitative PCR on SIP Fractions

Gene copies of *Rhodococcus* sp. RR1 *prmA* were determined the SIP fractions using a previously developed qPCR assay (Eshghdoostkhatami and Cupples, 2024) (Supplementary Table 2). Quantitative PCR was performed with the CFX96™ Real-Time PCR System (Bio-Rad, Hercules, CA), using 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). Bovine serum albumin was added as it has been shown to limit inhibition in environmental samples (Gedalanga et al., 2014; Kreader,

1996; Wang et al., 2007). The thermal cycler program involved an initial activation at 95°C for 10 minutes, followed by 40 cycles of denaturation at 95°C for 15 seconds, and annealing at 60°C for 1 minute. The target gene (*prmA*) 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 3), as well as data concerning the qPCR assays (as suggested by MIQE guidelines) (Bustin et al., 2009) (Supplementary Table 4) has been summarized.

2.10. Statistical Analysis

Differences in the 1,4-dioxane degradation rates (determined via linear regression) and microbial alpha diversity values between inocula and treatments were investigated using one-way analysis of variance (ANOVA) and Welch's two-sided *t*-tests. If the *p*-value from the one-way ANOVA was smaller than 0.05, *t*-tests were used to compare the differences between inocula or treatments. The results of ANOVA and *t*-tests are provided (Supplementary Tables 5-12).

3. Results

3.1. 1,4-Dioxane Biodegradation Rates

1,4-Dioxane concentrations in all live and control microcosms for all treatments were monitored over 50 days (Figure 1). For all live microcosms, the 95% confidence intervals (CIs) for the regression lines of ^{12}C 1,4-dioxane and ^{13}C 1,4-dioxane amended live samples overlapped entirely over the incubation, indicating the label did not impact removal rates. In contrast, the 95% CIs differed between the live samples and abiotic controls. The removal slopes were also significantly different between the live samples and corresponding abiotic controls ($p < 0.05$) (Supplementary Tables 5-8), indicating decreases in 1,4-dioxane concentrations were due to biodegradation. The decrease in 1,4-dioxane concentration in the autoclaved controls may have been a result of abiotic processes.

1,4-Dioxane removal rates were significantly different between the microcosms with the three inocula types (one-way ANOVA, $p < 0.05$) (Supplementary Table 5). Treatment (water vs. BSM and yeast extract) differences between 1,4-dioxane removal rates also varied between the three

inocula. Although the 1,4-dioxane removal rate was higher in the BSM and yeast extract treatment compared to the water treatment in the wetland sediment microcosms, the difference was not significant (t-tests, $p > 0.05$) (Supplementary Table 6). However, the addition of BSM and yeast extract significantly increased 1,4-dioxane removal rates, compared to the water treatments, in both the agricultural soil and impacted site sediment microcosms (t-tests, $p < 0.05$) (Supplementary Tables 7 & 8). The lack of effect of yeast extract on the wetland sediment microcosms may be related to the high % organic matter (34.6 % and 38.1 %) in the wetland sediments compared to the other two inocula types (site sediment: 0.3 %, 0.2%, agricultural soil: 1.6%, 1.5%) (Supplementary Table 1). It is important to note that all microcosms were well aerated and removal rates may be lowered under reduced dissolved oxygen conditions (Lee et al., 2014).

3.2. Microbial Community Analyses

PCoA analysis indicated the microbial communities differed between the agricultural soils, wetland sediments and site sediments microcosms (Supplementary Figure 1). Greater differentiations were observed between heavy and light fractions for the soil and wetland sediments microcosms (Supplementary Figure 1B & 1C), compared to the site sediments microcosms (Supplementary Figure 1D). The alpha diversity indices in the microcosms were significantly different (one-way ANOVA and *t*-tests) between the three soil/sediment types (Supplementary Tables 9-11). For both the water and BSM and yeast extract treatments, the soil microcosms illustrated the highest alpha diversity and richness indices, followed by wetland sediment microcosms, then the impacted site sediment microcosms (Figure 2). For the soil microcosms, all the richness (Chao1, ACE) and diversity (Shannon, Simpson, Inverse of Simpson and Fisher) indices were significantly higher in the no yeast (water only) compared to the BSM and yeast extract treatment ($p < 0.05$; Supplementary Table 12). To speculate, the reduced diversity and richness in the BSM and yeast extract treatment may be related to a smaller group of microorganisms being favored compared to the water only treatment. Four indices (Chao1, ACE, Inverse of Simpson and Fisher) and three indices (Chao1, ACE and Fisher) were significantly higher in the treatments with water compared to those with BSM and yeast extract for wetland and impacted site sediment microcosms, respectively ($p < 0.05$; Supplementary Table 12).

435

436 **3.3. Phyla and Phylotypes Responsible for Carbon Uptake from 1,4-Dioxane**

437 The relative abundance of phyla in the ^{12}C and ^{13}C amended heavy and lights fractions were
438 identified and compared (Figure 3). As in all other SIP studies, it is important to note that carbon
439 uptake could be from the primary substrate or from degradation products. For the soil and
440 wetland sediment microcosms, different phyla dominated in the heavy fractions compared to the
441 light fractions. Specifically, *Firmicutes* and *Bacteroides* were dominant in the light fractions,
442 while *Actinobacteria* and *Proteobacteria* were dominant in the heavy fractions. For impacted site
443 sediment microcosms, the light and heavy fractions illustrated similar trends at the phylum level,
444 with both being dominated by *Proteobacteria*.

445

446 The ten most abundant phylotypes statistically enriched in the heavy fractions of ^{13}C 1,4-dioxane
447 amended samples compared to the ^{12}C 1,4-dioxane amended samples were determined using the
448 Wilcoxon Rank test ($p < 0.05$) (Figure 4). The phylotypes associated with carbon uptake from
449 1,4-dioxane varied both across treatments (water vs. BSM and yeast extract) and inocula types
450 (Figure 4). In the wetland microcosms with water only, the dominant phylotypes included an
451 uncultured strain, *Gemmatimonas*, *Gemmata* and an unclassified *Alphaproteobacteria*. In the
452 wetland microcosms with BSM and yeast extract, dominant phylotypes included *Massilia*,
453 unclassified *Rhizobiales* as well as two *Gemmatimonas* strains. In the soil microcosms with
454 water, the enriched phylotypes were dominated by *RB4, Udaeobacter, Subgroup 6* and *Ellin*.
455 Whereas, in the soil microcosms with BSM and yeast extract, *Solirubacteraceae*,
456 *Pseudonocardia*, *Solirubrobacter*, *Acidothermus* and *Gaiella* were primarily associated with
457 label uptake. In contrast, the enriched phylotypes in the site microcosms were dominated by only
458 one phylotype in each treatment, an unclassified *Burkholderiaceae* (water treatment) and *oc3299*
459 (BSM and yeast extract treatment).

460

461 The datasets were also summarized to illustrate enrichment patterns for all statistically enriched
462 phylotypes across treatments and sample types (Figure 5). The largest number of OTUs and
463 families were enriched in the wetland communities, followed by the agricultural soil, then the
464 impacted site sediment (Figure 5A & 5B). In the comparison between BSM and yeast extract and
465 water treatments, the number of statistically enriched OTUs and families were similar for the

impacted site sediments. However, for both soil and wetland sediments, the numbers of enriched families and OTUs were greater in the BSM and yeast extract treatment compared to the water treatment.

3.4. SDIMO Amplicon Sequencing and *prmA* Quantitative PCR Assay

The BLAST analysis compared the SDIMO amplicon sequencing OTUs to genes previously associated with 1,4-dioxane metabolism or co-metabolism. Three genes (*Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA*, *Pseudonocardia dioxanivorans* CB1190 plasmid pPSED02 Psed_6976) were detected and aligned in the three soil/sediment types (Figure 6). The majority of alignments to both *Rhodococcus prmA* databases involved methane monooxygenases or propane monooxygenases from other *Rhodococcus* species. The numbers of alignments to both databases were the greatest for the wetland sediments, followed by the soil, then the impacted site sediments. The majority of the alignments to the *Pseudonocardia dioxanivorans* CB1190 plasmid pPSED02 Psed_6976 database were from the impacted site sediment microcosms (Figure 6C). The alignments were associated with genes encoding for tetrahydrofuran monooxygenase alpha subunit (*thmA*) from *Pseudonocardia*, *Rhodococcus*, *Arthrobacter* and *Acinetobacter*.

Gene copies of *Rhodococcus* sp. RR1 *prmA* were further investigated using qPCR in the ^{12}C and ^{13}C gradient fractions for the wetland sediments, soil and impacted site sediment microcosms. Only the fractions from the wetland microcosms illustrated an increase in buoyant density in the heavy fractions of the ^{13}C amended samples compared to the heavy fractions of the ^{12}C controls (Figure 7). The trends were similar for both replicates of both treatments (with and without yeast). In the no yeast treatment (Figure 7A), ^{13}C-labeled *prmA* genes peaked at heavier buoyant densities (BDs) (1.7382 and 1.7371 g/mL) compared to those of ^{12}C-labeled fractions (1.7360 and 1.7306 g/mL). In the yeast treatment (Figure 7B), ^{13}C-labeled *prmA* genes also peaked at heavier BDs (1.7393 and 1.7349 g/mL) compared to those of ^{12}C-labeled fractions (1.7328 and 1.7306 g/mL).

3.5. Prediction of ^{13}C Enriched Monooxygenase Genes

PICRUSt2 predicted the phylotypes associated with monooxygenase genes for the three soil/sediment types (Figure 8, Supplementary Figures 2-5). A number of microorganisms were associated with propane monooxygenase in the three sample types, however, only a small number were linked to all four subunits (*prmA*, *prmB*, *prmC* and *prmD*) (Figure 8). Specifically, in all three sample types, *Pseudonocardia*, unclassified *Pseudonocardiaceae, Solirubrobacter* and unclassified *Solirubrobacteraceae* were primarily associated with all four subunits. A number of phylotypes were linked to all six subunits of phenol/toluene 2-monooxygenase in all three samples types (Supplementary Figure 2). The most dominant for the impacted site samples included unclassified *Burkholderiaceae*. The most dominant for the soil samples included *IS-44*, *oc32* (*Nitrosomonadaceae*), *Pseudomonas* and *SC-I_84*. For the wetland samples, *Acinetobacter* was associated with all six subunits (although the levels for three subunits were lower), as was *IS-44, MND1, oc32* and *SC-I_84*.

Dominant patterns for the other functional genes included *Labrys* (*Rhizobiales*) for the six subunits of toluene monooxygenase in the impacted site sediments (Supplementary Figure 3), unclassified *Rhizobiales* for the three subunits of ammonia/particulate methane monooxygenase for all three sample types (Supplementary Figure 4) and *Mycobacterium* for the five subunits of soluble methane monooxygenase for all three sample types (Supplementary Figure 5).

4. Discussion

This study examined the phylotypes and functional genes associated with 1,4-dioxane biodegradation in three mixed microbial communities. The impact of BSM and yeast extract on 1,4-dioxane biodegradation was also investigated as an easily available and non-hazardous amendment to potentially enhance removal rates *in situ*. Multiple molecular methods were utilized to ascertain the key biomarkers. The phylotypes responsible for the carbon uptake from 1,4-dioxane were identified using DNA-based SIP. The genes encoding for putative 1,4-dioxane degradative enzymes were investigated using 1) SDIMO based amplicon sequencing, 2) qPCR targeting *Rhodococcus* sp. RR1 *prmA* in the SIP fractions and 3) a predictive method (PICRUSt2) for the occurrence of oxygenase genes (Douglas et al., 2020).

The impact of BSM and yeast extract on 1,4-dioxane biodegradation rates differed between the three microbial communities. The addition of BSM and yeast extract enhanced removal rates in all three inocula types, however, differences were only significant for the agricultural soil and impacted site sediment microcosms. The lack of effect of yeast extract on the wetland sediment microcosms may be related to the high % organic matter already present in these samples. A number of previous studies have added yeast extract while examining 1,4-dioxane biodegradation. One group added yeast extract to laboratory incubations with four river water samples, however, no 1,4-dioxane biodegradation was observed within the study period (29 days) (Sei et al., 2010). Others have reported yeast extract accelerates 1,4-dioxane degradation rates by pure cultures (Chen et al., 2016; Pugazhendi et al., 2015). *Rhodanobacter* AYS5 completely degraded 100 mg/L 1,4-dioxane in 4 days with 20 mg/L of yeast extract as an additional substrate (Pugazhendi et al., 2015). *Xanthobacter flavus* DT8 degraded 100 mg/L 1,4-dioxane in less than 25 h with 100 mg/L of yeast extract (Chen et al., 2016). The biodegradation of tetrahydrofuran (a structural analog of 1,4-dioxane) by *Rhodococcus* strain YYL was also improved by the addition of yeast extract (Yao et al., 2009a). *Rhodococcus ruber* 219 sustained the degradation of low concentrations of 1,4-dioxane (<100 μg/L) to below health advisory levels (0.35 μg/L) when supplied with thiamine (Simmer et al., 2021). The researchers suggest that *in situ* biostimulation with growth supplements might result in efficient removal of 1,4-dioxane (Simmer et al., 2021). In the current study, it is important to clarify we can only conclude it was the combination of BSM and yeast extract that impacted removal rates (and not yeast extract alone). It is possible that the differences noted between treatments may have been a consequence of BSM modifying the alkalinity and pH of the microcosms.

In the current study, the different trends between the three microbial communities may be related to the nutritional requirements of the degradative microorganisms involved and/or the nutritional resources already present in the wetland sediment compared to the other two sample types. Given the practical implications, the most important trend is the enhancement of 1,4-dioxane biodegradation rates in the impacted site microcosms due to the addition of BSM and yeast extract. It is notable the effect was significant at an order of magnitude lower yeast extract concentration (60 μg/L) compared to the concentrations used in the pure culture studies.

SIP revealed different phylotypes were responsible for carbon uptake from 1,4-dioxane between the three mixed communities. *Gemmatimonas* was notably enriched in both wetland treatments. This genus belongs to the phylum *Gemmatimonadetes* and members of this phylum are widely distributed across various natural environments (DeBruyn et al., 2011; Hanada and Sekiguchi, 2014; Zhang et al., 2003). However, the physiology and environmental role of the members are largely unknown due to the limited number of cultivated species (Zeng et al., 2015). To our knowledge, members of this genus have not been previously associated with carbon uptake from 1,4-dioxane. However, *Gemmatimonas* was previously associated with benzoate biodegradation (Zhang et al., 2003) and was dominant in hydrocarbon-polluted soil (Sampaio et al., 2017). *Gemmatimonas* was also associated with pyrene (Wang et al., 2018) and phenanthrene degradation in soil (Dou et al., 2021; Elyamine and Hu, 2020; Wang et al., 2021).

Xanthobacteraceae (*Rhizobiales* order) and unclassified *Rhizobiales* were also responsible for carbon uptake from 1,4-dioxane in the wetland sediment microcosms. *Xanthobacteraceae* was previously linked to carbon uptake from 1,4-dioxane in soil microcosms (Dang and Cupples, 2021). *Xanthobacteraceae* has also been associated with 1,4-dioxane biodegradation in activated sludge (Chen et al., 2021; Samadi et al., 2023). Further, the 1,4-dioxane degraders *Xanthobacter flavus* DT8 (Chen et al., 2016) and *Xanthobacter* sp. YN2 (Ma et al., 2021) classify within the same family. Also consistent with the current study, genera classifying within the order *Rhizobiales* (*Hyphomicrobium* and *Chelativorans*) were enriched following 1,4-dioxane biodegradation compared to control microcosms (no 1,4-dioxane) in agricultural soil microcosms (Ramalingam and Cupples, 2020).

In the agricultural soil microcosms amended with BSM and yeast extract, both the genus *Solirubrobacter* and the family *Solirubacteraceae* were linked to carbon uptake from 1,4-dioxane. *Solirubrobacter* (*Solirubacteraceae* family) commonly exists in agricultural soil rhizospheres (Aguiar et al., 2020; Cordero Elvia et al., 2021; Lee et al., 2021). Members of this genus are difficult to cultivate and isolate due to their slow growth and the lack of specific media (Seki et al., 2015). *Solirubrobacter* has previously been associated with the degradation of various substrates, such as 4-nonylphenol (Hung et al., 2022), coal (Wang et al., 2019), organic matter (Bukin et al., 2016) and petroleum hydrocarbons (Peng et al., 2015). To date, no 1,4-

dioxane degrading *Solirubrobacter* isolate has been reported. *Pseudonocardia* was also associated with carbon uptake from 1,4-dioxane in the agricultural soil microcosms amended with BSM and yeast extract. This genus contains many well-known 1,4-dioxane degraders. For example, *Pseudonocardia dioxanivorans* CB1190 (Mahendra and Alvarez-Cohen, 2006; Parales et al., 1994), *Pseudonocardia* sp. D17 (Sei et al., 2013), *Pseudonocardia* sp. N23 (Yamamoto et al., 2018) and *Pseudonocardia benzenivorans* B5 (Mahendra and Alvarez-Cohen, 2006) can degrade 1,4-dioxane metabolically. *Pseudonocardia asaccharolytica* JCM 14343 (Inoue et al., 2016), *Pseudonocardia* sp. ENV478 (Vainberg et al., 2006) and *Pseudonocardia tetrahydrofuranoxydans* sp. K1 (Kohlweyer et al., 2000) can degrade 1,4-dioxane co-metabolically when induced with tetrahydrofuran.

RB4 (*Pyrinomonadaceae* family) was notably enriched in the water only treatment of the agricultural soil microcosms. This is the first report of carbon uptake from 1,4-dioxane by this phylotype. Members of the same family were linked to phenanthrene degradation in oil field soil with ryegrass root exudates (Li et al., 2019), with the degradation of cellulose, starch and xylan (Wüst et al., 2016) and with the degradation of benzo [α] pyrene in soil (Lu et al., 2022).

Carbon uptake in the impacted site microcosms was dominated by two phylotypes, *oc3299* in the BSM and yeast extract treatment and *Burkholderiacae* in the water only treatment. *oc3299* classifies within a family (*Nitrosomonadaceae*) known to contain microorganisms with ammonia monooxygenases (Clark et al., 2021; Cupples and Thelusmond, 2022). This enzyme has been linked to the biodegradation of many environmental contaminants, such as 17 alpha-ethinylestradiol (Wang and Li, 2023), 2-chlorophenol (Perez-Alfaro et al., 2023), micropollutants (Yu et al., 2018) and trichloroethene (Alpaslan Kocamemi and Cecen, 2007). Similar to the wetland sediment microcosms, *Gemmatimonas* was also responsible for carbon uptake in the impacted site microcosms amended with BSM and yeast extract. The family *Burkholderiacae* contains the genus *Burkholderia* which been associated with the biodegradation of many chemicals (Morya et al., 2020), such as hexadecane (Wu, Dang et al. 2011), phenol (Huang, Shao et al. 2022), naphthalene and phenanthrene (Kim, Lee et al. 2003), methyl parathion (Fernández-López, Popoca-Ursino et al. 2017, Castrejón-Godínez, Tovar-Sánchez et al. 2022) and polychlorinated biphenyls (Tillmann, Strömpl et al. 2005, Ponce, Latorre et al. 2011). Further,

Burkholderia cepacia G4 degrades 1,4-dioxane co-metabolically when induced by toluene (Mahendra and Alvarez-Cohen, 2006).

The current work investigated SDIMOs via amplicon-based sequencing. When the OTUs generated in the current work were compared to twelve genes previously associated with 1,4-dioxane metabolism and co-metabolism (as summarized (He et al., 2017)), three genes (*Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA*, *Pseudonocardia dioxanivorans* CB1190 plasmid pPSED02 Psed_6976) were detected in the three soil/sediment types. A similar trend of the dominance of the two *prmA* sequences in mixed microbial communities was also observed in previous work (Eshghdoostkhatami and Cupples, 2024). Notably, in the current study, in all three mixed communities, the SIP results did not associate *Rhodococcus* with carbon uptake from 1,4-dioxane. Carbon uptake from microorganisms harboring *Rhodococcus* sp. RR1 *prmA*-like genes only occurred in the wetland sediments microcosms. The lack of *Rhodococcus* in the wetland SIP results could suggest other microorganisms may harbor similar genes.

The current study also revealed genes encoding for tetrahydrofuran monooxygenase alpha subunit *thmA* from *Pseudonocardia* were present in the impacted site sediments. However, SIP did not identify *Pseudonocardia* as a carbon consumer in the impacted site microcosms. The pattern suggests either these genes were not active, or biodegradation was co-metabolic and did not involve carbon uptake. The biomarker *thmA* has been associated with cometabolic 1,4-dioxane degradation by *Pseudonocardia tetrahydrofuran* K1, *Pseudonocardia* sp. ENV478 and *Rhodococcus* sp. YYL (Mahendra and Alvarez-Cohen, 2006; Masuda et al., 2012; Thiemer et al., 2003; Yao et al., 2009b). The current research suggests BSM and yeast extract could stimulate the co-metabolism of 1,4-dioxane in the impacted site sediments via tetrahydrofuran monooxygenase.

PICRUSt2 predicted the phylotypes and the functional genes associated with the 1,4-dioxane degradation. The identified degraders *Solirubrobacter* and *Pseudonocardia* (as discussed above) were predicted to be associated with all four subunits of propane monooxygenase (*prmA*, *prmB*, *prmC* and *prmD*) in all three soil types. Consistent with this, a NCBI search indicated

Solirubrobacter pauli strain DSM 14954 contained the four propane monooxygenase subunits (all located together and with the correct predicted length for each subunit). *Labrys* (*Rhizobiales*) and unclassified *Rhizobiales* were predicted to be associated with toluene monooxygenase (*tmo*/*tbu*/*tou*) and ammonia/particulate methane monooxygenase genes (*pmo*/*amo*) in all three samples. This order was also predicted to be a major phylotype associated with *pmo*/*amo* KEGG group in other soils (Cupples et al., 2022). PICRUSt2 predicted *Mycobacterium* was associated with soluble methane monooxygenase genes in three soil types, however, this genus was not associated with carbon uptake from 1,4-dioxane. The trend indicates either sMMO was not involved in 1,4-dioxane degradation in the current study or the transformation did not result in any carbon uptake.

5. Conclusion

This research provides insight into the impact of BSM and yeast extract on 1,4-dioxane biodegradation rates as well as the microorganisms involved in carbon uptake during biodegradation. The addition of BSM and yeast extract enhanced removal rates in all three inocula types, however, differences were only significant for the agricultural soil and impacted site sediment microcosms. Numerous phylotypes were associated with carbon uptake across the three communities and two treatments. *Gemmatimonas* was particularly important in the heavy fractions of both treatments of the wetland sediment microcosms. Unclassified *Solirubacteraceae*, *Solirubrobacter*, *Pseudonocardia* and *RB4* were the dominant enriched phylotypes in the agricultural soil microcosms. The impacted site microcosms were dominated by only two phylotypes, unclassified *Burkholderiaceae* (water treatment) and *oc3299* (BSM and yeast extract treatment). To our knowledge, *Gemmatimonas*, *Solirubacteraceae*, *Solirubrobacter*, *RB4* and *oc3299* have not previously been linked to carbon uptake from 1,4-dioxane.

The SDIMO based amplicon sequencing detected three genes (*Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA*, *Pseudonocardia dioxanivorans* CB1190 plasmid pPSED02 Psed_6976) in the mixed microbial communities. Although the genes were present, *prmA* was only linked to 1,4-dioxane biodegradation in one set of samples. The predicted functional gene analysis suggested the importance of propane monooxygenases associated with *Solirubrobacter*

and *Pseudonocardia.* Overall, it is likely that a community of microorganisms is involved in 1,4-dioxane biodegradation in both the wetland and agricultural soil microcosms. In contrast, the carbon from 1,4-dioxane in the impacted site microcosms was largely restricted to two phylotypes. The results suggest that amending with BSM and yeast extract, even at low levels, could be a promising approach for the enhancement of 1,4-dioxane biodegradation.However, it is important to note that there may be challenges, not addressed here, associated with adding these amendments to aquifers.

CRediT Authorship Contribution Statement

ZL was responsible for all laboratory work, methodology, formal analysis and the preparation of the manuscript. AMC was responsible for supervision, conceptualization, formal analysis, reviewing and editing.

Declaration of Competing Interest

The authors declare no known competing financial or personal interests.

Data Availability

The 16S rRNA gene sequences were summitted to NCBI under Bioproject PRJNA1073031 (accession numbers SAMN39784393 to SAMN39784676). The SDIMO sequences were summitted to NCBI under Bioproject PRJNA1073036 (accession numbers SAMN39784693 to SAMN39784716).

Acknowledgements

Thanks to Stacey VanderWulp (MSU) for providing the agricultural soil samples from Kellogg Biological Station (KBS, MSU). Thanks to Casey Johnny, James O'Keefe, and Anthony Schilmiller at the Mass Spectrometry Laboratory at the RTSF (MSU) for 1,4-dioxane analytical method support. Thanks to the genomics facility for their support in DNA sequencing. Also, thanks to Anthony Danko and Michael Pound (NAVFAC) for providing the sediment samples from the contaminated site. This research was supported by a grant from NSF (Award Number 1902250). Support was also provided by the NSF Long-term Ecological Research Program (DEB 1832042) at the KBS and by MSU AgBioResearch.

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