

FINAL REPORT

The Development of Anaerobic Bioremediation Approaches for
Chlorinated Solvent
and 1,4-Dioxane Co-Contaminated Sites

SERDP Project ER-2712

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Alison Cupples
Michigan State University

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Table of Contents

Abstract	
Introduction and Objectives	1
Technical Approach	1
Results	1
Benefits.....	1
Graphic Depiction	2
Executive Summary	3
Introduction	3
Objectives.....	3
Technical Approach	4
Results and Discussion.....	4
Implications for Future Research and Benefits	6
1,4-Dioxane Biodegradation In Microcosms Inoculated with Contaminated or Uncontaminated Soils and Sediments and Various Electron Acceptors	7
Abstract	7
Introduction	8
Methods	11
Chemicals and Inocula.....	11
Experimental Setup.....	11
1,4-Dioxane Analysis	13
Compound Specific Isotope Analysis (CSIA).....	14
DNA Extraction and High Throughput Amplicon Sequencing.....	14
Analysis of Sequencing Data.....	15
Statistical Analysis	15
Results	16
1,4-Dioxane Removal Trends.....	16
Compound Specific Isotope Analysis (CSIA).....	18
Putative 1,4-Dioxane Degraders.....	18
Discussion	20
References	23
Enrichment of Novel Actinomycetales and Detection of Monooxygenases during Aerobic 1,4- Dioxane Biodegradation with Uncontaminated and Contaminated Inocula.....	38
Abstract	38
Introduction	39
Methods	42
Chemicals and Inocula.....	42
Experimental Setup, DNA Extraction, 1,4-Dioxane Analysis	42
Library Preparation, Sequencing, MG-RAST and DIAMOND analysis	44
Results	46
Biodegradation of 1,4-Dioxane and General Microbial Community Composition	46
Genera Associated with 1,4-Dioxane Degradation	47
Genes Associated with 1,4-Dioxane Degradation	48
Discussion	49
References	52
Conclusions and Implications for Future Research	73

List of Tables

Table 1.1. Experimental design of the five microcosm studies.

Table 1.2. Percent removal in samples compared to the controls at the last time point (when the difference was statistically significant, determined with student's t-test for independent variables with equal variance, $p < 0.05$).

Table 2.1. Summary of sequencing information processed by MG-RAST.

Table 2.2. Classification of genera statistically significantly enriched ($p < 0.05$) in the samples compared to the controls (no 1,4-dioxane) following the degradation of 1,4-dioxane in all soils collectively and when the soils were analyzed individually. The last column also illustrates the difference in means between the controls and the samples for each genera. Genera in bold were identified in the BLASTP search as containing genes similar to *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. *prmA* (as discussed in the functional gene analysis results).

List of Figures

Figure 1.1. 1,4-Dioxane concentrations in sample and abiotic control microcosms of three soils amended with EDTA/iron and humic acid. The values and bars represent averages and standard deviations from triplicates. The circles represent a significant difference (two-tailed t-test, $p < 0.05$) between the samples and abiotic controls (when abiotic controls > samples).

Figure 1.2. 1,4-Dioxane concentrations in sample and control microcosms of three soils with different electron acceptor amendments (A-D). The values and bars represent averages and standard deviations from triplicates (except soil G, $n = 2$, for three cases). The circles represent a significant different (two-tailed t-test, $p < 0.05$) between the samples and abiotic controls (when controls > samples). The arrows indicate the samples subject to DNA extraction.

Figure 1.3. 1,4-Dioxane concentrations in Sediment J and H sample and control microcosms over time with different amendments. The points illustrate average values from triplicates (except nitrate amended controls, sediment H, $n=1$) and the bars illustrate standard deviations. The circles represent a significant different (two-tailed t-test, $p < 0.05$) between the samples and abiotic controls. The arrow indicate the samples subject to DNA extraction.

Figure 1.4. 1,4-Dioxane concentrations in Maine (Auger) and California (10A) sample and control microcosms over time with different amendments. The points illustrate average values from triplicates (except sulfate amended Maine sample, $n=2$ and sulfate amended CA control, $n=2$) and the bars illustrate standard deviations. The circles represent a significant different (two-tailed t-test, $p < 0.05$) between the samples and abiotic controls (controls > samples). The arrows indicate the samples subject to DNA extraction.

Figure 1.5. 1,4-Dioxane concentrations in sample and control microcosms over time with different inocula. The points illustrate average values from triplicates (except Soil E and KBS Soil 2 samples, which are replicates) and the bars illustrate standard deviations. The circles

represent a significant difference (two-tailed t-test, $p < 0.05$) between the samples and abiotic controls. The arrows indicate the samples subject to DNA extraction.

Figure 1.6. Comparison of $\delta^{13}\text{C}$ (A) and $\delta^2\text{H}$ (H) values in samples and abiotic controls of a sub-set of the experimental microcosms. The error bars represent standard deviations from triplicate samples and control microcosms and the p values were derived from the student's t-test (two tailed, independent variables, equal variance).

Figure 1.7. Phylotypes with a statistically significant difference (Welch's t-test, two sided, $p < 0.05$) between the 1,4-dioxane degrading samples ($n=3$) compared to the live controls ($n=3$). The comparisons are shown for the Contaminated Site (CA) microcosms (A), the Sediment H microcosms (B) and Soil 3 microcosms (C). No differences were noted for KBS Soil 3. The data points to the left of the dashed line indicate phylotypes more abundant in the 1,4-dioxane degrading samples compared to the controls and those to the right indicate the reverse. For each, the x-axis have different scales.

Figure 1.8. Relative abundance (%) of genera previously associated with 1,4-dioxane biodegradation in replicates of 1,4-dioxane degrading microcosms or live controls (C, no 1,4-dioxane) inoculated with different soils. The inserts have a different axis to better illustrate the relative abundance of *Rhodanobacter*.

Figure 1.9. Relative abundance (%) of genera previously associated with 1,4-dioxane biodegradation in replicates of 1,4-dioxane degrading microcosms or live controls (C, no 1,4-dioxane) inoculated with different sediments (A, B, C). The inserts have a different axis to better illustrate the relative abundance of *Pseudomonas*.

Figure 2.1. Average 1,4-dioxane concentrations (mg/L) in triplicate samples and abiotic controls with different inocula (bars represent standard deviations). 1,4-dioxane was reamended to the samples microcosms twice (arrows).

Figure 2.2. Extended error bar plot illustrating genera statistically significantly different in relative abundance (Welch's two sided t-test, $p < 0.05$) in the samples ($n=9$) compared to the live controls (no 1, 4-dioxane, $n=8$) following 1,4-dioxane degradation (A). The symbols to the left of the dashed line (yellow) indicate a higher relative abundance in the samples compared to the controls and the symbols to the right (blue) indicate the reverse. A comparison of the relative abundance values (%) for the genera enriched in the samples is also shown in a box plot format (B). The inset illustrates the relative abundance of these enriched genera in the contaminated site sample (C7A) with a different scale on the y-axis.

Figure 2.3. Extended error bar plot illustrating genera statistically significantly different in relative abundance (Welch's two sided t-test, $p < 0.05$) in the samples compared to the live controls following 1,4-dioxane degradation in soil 1 (A) and 2 (B). The symbols to the left of the dashed line (in yellow) indicate a higher relative abundance in the samples compared to the controls and the symbols to the right (in blue) indicate the reverse.

Figure 2.4. Extended error bar plot illustrating genera statistically significantly different in relative abundance (Welch's two sided t-test, $p < 0.05$) in the samples compared to the live controls following 1,4-dioxane degradation in soil F (A) and G (B). The symbols to the left of the dashed line (in yellow) indicate a higher relative abundance in the samples compared to the controls and the symbols to the right (in blue) indicate the reverse.

Figure 2.5. Summary of the relative abundance of statistically significantly enriched genera in the samples compared to the controls (no 1,4-dioxane) for soils 1, 2 and F (there were no enriched genera in soil G). The inset illustrates the relative abundance of these genera in the contaminated site sample (C7A) with a different scale on the y-axis.

Figure 2.6. Relative abundance (%) of genera associated with metabolic and co-metabolic degradation of 1,4-dioxane in live controls ($n=8$) and samples ($n=9$) in four soils and one contaminated site sample (C7A). The value "a" indicates a significant difference ($p < 0.05$) in a two tailed student's t-test between the samples and controls. The insert illustrates the same data with a different y-axis.

Figure 2.7. Relative abundance (%) of reads aligning ($\geq 60\%$ identity for ≥ 49 amino acids) to genes previously associated with the metabolic and co-metabolic degradation of 1,4- dioxane in Soil F and C7A (A), Soil G (B), Soil 1 (C) and Soil 2 (D).

Figure 2.8. Phylogenetic tree of *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. *prmA* and BLASTP results ($>94.8\%$ similar to the two query sequences). Only genera that were enriched following 1,4-dioxane degradation (compared to the controls) are shown (Table 2). The evolutionary history was inferred by using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model. The tree with the highest log likelihood (-2731.06) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 48 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 439 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.

Supplementary Figures

Supplementary Figure 2.1. Phylogram (created with MEGAN6, version 6.11.7) illustrating the relative abundance and classification (Class Level) of all bacteria across all metagenomes (samples and controls).

Supplementary Figure 2.2. Phylogram (created with MEGAN6, version 6.11.7) illustrating the most abundant genera (ranked by average relative abundance, then selected if average relative abundance $>0.5\%$) across all metagenomes (samples and controls).

Supplementary Figure 2.3. The twenty-five most common genera (by relative abundance, %), ranked by the averages of the samples and controls, in soil 1 (A), soil 2 (B), soil F (C), soil G (D) and the contaminated site sediment 7A (E).

List of Acronyms

1,1,1-TCA: 1,1,1-trichloroethane
EPA: Environmental Protection Agency
DoD: Department of Defense
PCE: Tetrachloroethene
TCE: Trichloroethene
RDX: Royal Demolition Explosive
MTBE: Methyl tert butyl ether
GC FID: Gas chromatograph flame ionization detector
DNA: Deoxyribonucleic acid
THF: Tetrahydrofuran
EDTA: Ethylenediaminetetraacetic acid
HMX: Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
1,1-DCA: 1,1-dichloroethane
CA: Monochloroethane
Koc: Organic Carbon Water Partition Coefficient
PCR: Polymerase chain reaction

Keywords

1,4-dioxane, *Actinomycetales*, soluble di-iron monooxygenases

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Abstract

Introduction and Objectives

1,4-dioxane was commonly used as a stabilizer in 1,1,1-trichloroethane formulations and is now frequently detected at sites where the chlorinated solvents are present. A major challenge to 1,4-dioxane remediation concerns chemical characteristics that result in migration and persistence. Given the limitations associated with traditional remediation methods, interest has turned to bioremediation to address 1,4-dioxane contamination. The overall objective here was to determine the susceptibility of 1,4-dioxane to biodegradation over a range of redox conditions.

Technical Approach

The potential for 1,4-dioxane biodegradation was examined using multiple inocula sources and electron acceptor amendments. The inocula included uncontaminated agricultural soils and river sediments as well as sediments from two 1,4-dioxane contaminated sites. Compound specific isotope analysis (CSIA) was used to investigate biodegradation in a subset of the microcosms. Also, DNA was extracted from microcosms exhibiting 1,4-dioxane biodegradation for microbial community analysis using 16S rRNA gene amplicon and shotgun sequencing.

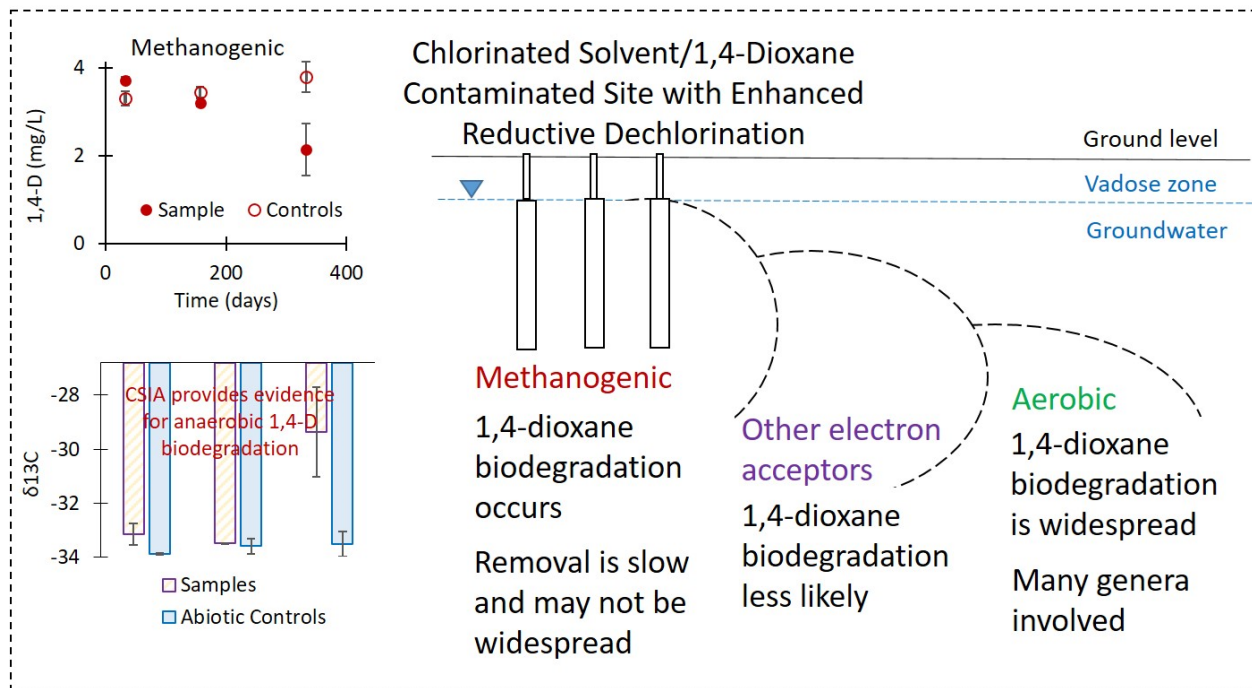
Results

The iron/EDTA/humic acid or sulfate amendments did not result in 1,4-dioxane biodegradation in the majority of cases. However, 1,4-dioxane biodegradation was noted in a subset of the nitrate amended and no electron acceptor treatments (although slow biodegradation rates were observed). In two of the three cases examined, CSIA provided additional evidence for 1,4-dioxane biodegradation. Microbial community analysis indicated unclassified *Comamonadaceae* and 3 *genus incertae sedis* were obtaining a growth benefit during anaerobic 1,4-dioxane degradation. Under aerobic conditions, 1,4-dioxane biodegradation was observed with all six inocula. A comparison of live microcosms and live controls (no 1,4-dioxane) indicated seventeen genera were enriched following exposure to 1,4-dioxane, suggesting a growth benefit for 1,4-dioxane degradation. The three most enriched were *Mycobacterium*, *Nocardioideae*, *Kribbella*. The three most common functional genes detected were those aligning to *Rhodococcus jostii* RHA1 *prmA*, *Rhodococcus* sp. RR1 *prmA* and *Burkholderia cepacia* G4 *tomA3*.

Benefits

The current study indicates 1,4-dioxane biodegradation under anaerobic (likely methanogenic) conditions is feasible. Therefore, natural attenuation of 1,4-dioxane may occur over a wider range of conditions, notably methanogenic areas where highly reducing conditions exist either naturally or as a result of enhanced reductive dechlorination. The work is novel as it is the first to document the frequency of 1,4-dioxane biodegradation over a range redox conditions and inocula types and provides evidence for the feasibility of anaerobic 1,4-dioxane remediation. Under aerobic conditions, 1,4-dioxane degradation was observed with all inocula types, indicating 1,4-dioxane degraders are widespread. Further, a large number of genera were enriched following 1,4-dioxane degradation. The aerobic study is innovative as it combines taxonomic and functional data to generate a more complete picture of the multiple microorganisms and genes linked to 1,4-dioxane degradation in mixed communities. Additional research is needed to determine the occurrence of the microorganisms putatively linked to anaerobic 1,4-dioxane biodegradation at contaminated sites.

Graphic Depiction



Executive Summary

Introduction

1,4-dioxane, a probable human carcinogen (DeRosa et al., 1996), was commonly used as a stabilizer in 1,1,1-trichloroethane formulations and is now frequently detected at sites where the chlorinated solvents are present (Adamson et al., 2015; Adamson et al., 2014; ATSDR, 2012; Mohr et al., 2010). No federal maximum contaminant level for 1,4-dioxane in drinking water has been established (EPA, 2017), however, several states have set low advisory action levels (e.g. California, Florida, Michigan and North Carolina have levels <5 ppb). A major challenge to 1,4-dioxane remediation concerns chemical characteristics that result in migration and persistence (Adamson et al., 2015; Mohr et al., 2010). A low organic carbon partition coefficient ($\log K_{OC} = 1.23$) and Henry's Law Constant ($5 \times 10^{-6} \text{ atm} \cdot \text{m}^3 \text{mol}^{-1}$) make traditional remediation methods such as air stripping or activated carbon largely ineffective (Mahendra and Alvarez-Cohen, 2006; Steffan et al., 2007; Zenker et al., 2003). *Ex situ* oxidation methods including ozone and hydrogen peroxide (Adams et al., 1994) or hydrogen peroxide and ultraviolet light (Stefan and Bolton, 1998) have been commercially applied, however these can be expensive at high concentrations (Steffan et al., 2007). Given the limitations associated with traditional remediation methods, interest has turned to bioremediation to address 1,4-dioxane contamination. Numerous bacteria have been associated with aerobic metabolic and co-metabolic degradation of this chemical. The initiation of aerobic 1,4-dioxane biodegradation has been associated with various groups of soluble di-iron monooxygenases (SDIMOs). In contrast, little is known about the potential for 1,4-dioxane biodegradation under anaerobic conditions. The lack of information on the susceptibility of 1,4-dioxane to biodegradation under such conditions is a significant knowledge gap. Additional research gaps concern the relative abundance of SDIMOs and genera associated with aerobic 1,4-dioxane biodegradation across mixed microbial communities.

Objectives

The overall objective of the current study was to determine the susceptibility of 1,4-dioxane to biodegradation over a range of redox conditions using inocula from contaminated and

uncontaminated sites. The first project focused on the susceptibility of 1,4-dioxane to degradation under anaerobic conditions and the second study focused on aerobic conditions. The specific objectives of the first study were 1) to determine the susceptibility of 1,4-dioxane to degradation under anaerobic conditions (nitrate, iron, sulfate amended or no amendments) and 2) identify which phylotypes are enriched during 1,4-dioxane biodegradation. The specific objectives of the second study were 1) to identify which aerobic 1,4-dioxane degrading functional genes (SDIMOs) are present across different microbial communities and 2) to determine which genera may be using 1,4-dioxane and/or metabolites under aerobic conditions to support growth.

Technical Approach

In the first study, the potential for 1,4-dioxane biodegradation was examined using multiple inocula sources and electron acceptor amendments (nitrate, iron/EDTA/humic acid, sulfate, no amendment). The inocula included uncontaminated agricultural soils and river sediments as well as sediments from two 1,4-dioxane contaminated sites. Five separate experiments included the examination of triplicate live microcosms and abiotic controls for approximately one year. Compound specific isotope analysis (CSIA) was used to investigate biodegradation in a subset of the microcosms. Also, DNA was extracted from microcosms exhibiting 1,4-dioxane biodegradation for microbial community analysis using 16S rRNA gene amplicon high throughput sequencing. In the second study, laboratory microcosms and abiotic control microcosms were inoculated with four uncontaminated soils and sediments from two contaminated sites. The sample microcosms were amended with 1,4-dioxane three times over approximately 250 days. Live control microcosms were treated in the same manner, except 1,4-dioxane was not added. The microbial communities present and the relative abundance of 1,4-dioxane degrading functional genes (SDIMOs) were examined using shotgun sequencing.

Results and Discussion

In the first study, given the long incubation periods, it is likely that electron acceptor depletion occurred and methanogenic conditions eventually dominated (confirmed in one case). The iron/EDTA/humic acid or sulfate amendments did not result in 1,4-dioxane biodegradation in the majority of cases. In contrast, 1,4-dioxane biodegradation was noted in a subset of the nitrate amended treatments and no electron acceptor treatments. Notably, both contaminated site

sediments illustrated significant removal in the samples compared to the abiotic controls in the no electron acceptor treatment. However, it is important to note that slow degradation rates were observed, with concentration statistically significant reductions occurring over approximately one year. In two of the three cases examined, CSIA provided additional evidence for 1,4-dioxane biodegradation. In one case, the reduction in 1,4-dioxane in the samples compared the controls was likely too low for the method to detect a significant $^{13}\text{C}/^{12}\text{C}$ enrichment. Further research is required to determine the value of measuring $^2\text{H}/^1\text{H}$ for generating evidence for the biodegradation of this chemical. The analysis of the microbial communities indicated the phylotypes unclassified *Comamonadaceae* and 3 *genus incertae sedis* were obtaining a growth benefit during 1,4-dioxane degradation in microcosms inoculated with contaminated and uncontaminated site sediment, respectively. The soil and sediment microcosms contained genera previously associated with 1,4-dioxane degradation. The soil microcosms were dominated primarily by *Rhodanobacter* with lower relative abundance values for *Pseudomonas*, *Mycobacterium* and *Acinetobacter*. The sediment communities were dominated by *Pseudomonas* and *Rhodanobacter*. Additional research is needed to confirm the role of these phylotypes in 1,4-dioxane biodegradation.

In the second study, 1,4-dioxane decreased in live microcosms with all six inocula, but not in the abiotic controls, suggesting biodegradation occurred. A comparison of live microcosms and live controls (no 1,4-dioxane) indicated seventeen genera were enriched following exposure to 1,4-dioxane, suggesting a growth benefit for 1,4-dioxane degradation. The three most enriched were *Mycobacterium*, *Nocardioides*, *Kribbella* (classifying as *Actinomycetales*). There was also a higher level of enrichment for *Arthrobacter*, *Nocardia* and *Gordonia* (also *Actinomycetales*) in one soil and *Hyphomicrobium* (*Rhizobiales*) in two soils. Although *Arthrobacter*, *Mycobacterium* and *Nocardia* have previously been linked to 1,4-dioxane degradation, *Nocardioides*, *Gordonia* and *Kribbella* are potentially novel degraders. Eleven of the twelve functional genes (all except *Mycobacterium dioxanotrophicus* PH-06 *prmA*) associated with 1,4-dioxane were found in the soil and sediment metagenomes. Reads aligning to *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA* illustrated the highest relative abundance values and were present in all eighteen metagenomes. The third most abundant was *Burkholderia cepacia* G4 *tomA3* and this was also present in all eighteen metagenomes. The contaminated site

sample generated the same trend for the three most abundant genes and the values were greater compared to those in the soil metagenomes.

Implications for Future Research and Benefits

Overall, the current study indicates 1,4-dioxane biodegradation under anaerobic (likely methanogenic) conditions is feasible. Therefore, natural attenuation of 1,4-dioxane may occur over wider range of conditions, notably methanogenic areas where highly reducing conditions occur either naturally or as a result of enhanced reductive dechlorination. The work is novel as it is the first to document the frequency of 1,4-dioxane biodegradation over a range redox conditions and inocula types and provides critical evidence for the feasibility of 1,4-dioxane remediation through natural attenuation. Under aerobic conditions, 1,4-dioxane degradation was observed with all inocula types, indicating 1,4-dioxane degraders are widespread. Further, a large number of genera were enriched following 1,4-dioxane degradation. The second study is innovative as it combines taxonomic and functional data to generate a more complete picture of the multiple microorganisms and genes linked to 1,4-dioxane degradation in mixed communities.

1,4-Dioxane Biodegradation In Microcosms Inoculated with Contaminated or Uncontaminated Soils and Sediments and Various Electron Acceptors

Abstract

1,4-dioxane is a probable human carcinogen and a co-contaminant at many chlorinated solvent contaminated sites. Although numerous 1,4-dioxane degrading aerobic bacteria have been isolated, almost no information exists on the microorganisms able to degrade this chemical under anaerobic conditions. Only one report has previously documented anaerobic 1,4-dioxane biodegradation (iron/EDTA/humic acid amendments). In the current study, the potential for 1,4-dioxane biodegradation was examined using multiple inocula sources and electron acceptor amendments. The inocula included uncontaminated agricultural soils and river sediments as well as sediments from two 1,4-dioxane contaminated sites. Five separate experiments included the examination of triplicate live microcosms and abiotic controls for approximately one year. Compound specific isotope analysis (CSIA) was used to investigate biodegradation in a subset of the microcosms. Also, DNA was extracted from microcosms exhibiting 1,4-dioxane biodegradation for microbial community analysis using 16S rRNA gene amplicon high throughput sequencing.

Given the long incubation periods, it is likely that electron acceptor depletion occurred and methanogenic conditions eventually dominated (confirmed in one case). The iron/EDTA/humic acid or sulfate amendments did not result in 1,4-dioxane biodegradation in the majority of cases. 1,4-dioxane biodegradation was most commonly observed in the nitrate amended and no electron acceptor treatments. Notably, both contaminated site sediments illustrated removal in the samples compared to the abiotic controls in the no electron acceptor treatment. However, it is important to note that the degradation was slow (with statistically significant concentration reductions occurring over approximately one year). In two of the three cases examined, CSIA provided additional evidence for 1,4-dioxane biodegradation. In one case, the reduction in 1,4-dioxane in the samples compared the controls was likely too low for the method to detect a significant $^{13}\text{C}/^{12}\text{C}$ enrichment. Further research is required to determine the value of measuring $^2\text{H}/^1\text{H}$ for generating evidence for the biodegradation of this chemical.

The microbial community analysis indicated the phylotypes unclassified *Comamonadaceae* and 3 *genus incertae sedis* were more abundant in 1,4-dioxane degrading microcosms compared to the live controls (no 1,4-dioxane) in microcosms inoculated with contaminated and uncontaminated sediment, respectively. The relative abundance of known 1,4-dioxane degraders was also investigated at the genus level. The soil microcosms were dominated primarily by *Rhodanobacter* with lower relative abundance values for *Pseudomonas*, *Mycobacterium* and *Acinetobacter*. The sediment communities were dominated by *Pseudomonas* and *Rhodanobacter*. Overall, the current study indicates 1,4-dioxane biodegradation under anaerobic, and likely methanogenic conditions, is feasible. Therefore, natural attenuation of 1,4-dioxane may occur over a wider range of conditions, notably methanogenic areas where highly reducing conditions exist either naturally or as a result of enhanced reductive dechlorination.

Introduction

There is a critical need to develop management strategies for the emerging contaminant 1,4-dioxane due to its widespread occurrence (Adamson et al., 2015). This chemical, a probable human carcinogen (DeRosa et al., 1996), was commonly used as a stabilizer in 1,1,1-trichloroethane (1,1,1-TCA) formulations and is now frequently detected at sites where the chlorinated solvents are present (Adamson et al., 2015; Adamson et al., 2014; Mohr et al., 2010). As 1,4-dioxane was typically not on the EPA's target compound lists, it is likely that closed sites will require re-opening to address contamination. In fact, a multisite survey aimed at examining the extent of the 1,4-dioxane problem, indicated a primary risk is the large number of sites where this chemical is likely to be present but has yet to be identified (Adamson et al., 2014).

A major challenge in 1,4-dioxane remediation concerns chemical characteristics that result in movement and persistence (Adamson et al., 2015; Mohr et al., 2010). Traditional methods such as air stripping or activated carbon can be ineffective because of the chemical's low organic carbon partition coefficient ($\log K_{oc} = 1.23$) and low Henry's Law Constant (5×10^{-6} atm. $\text{m}^3\text{mol}^{-1}$) (Mahendra and Alvarez-Cohen, 2006; Steffan et al., 2007; Zenker et al., 2003). Although *ex situ* oxidation methods such as ozone and hydrogen peroxide (Adams et al., 1994) or hydrogen peroxide and ultraviolet light (Stefan and Bolton, 1998) have been commercially applied, these approaches can be expensive at high concentrations (Steffan et al., 2007). Given

the limitations associated with traditional remediation methods, interest has turned to the use of microorganisms to biodegrade this problematic contaminant (Chu et al., 2009; Li et al., 2010; Lippincott et al., 2015).

Numerous bacteria have been associated with the biodegradation (co-metabolic or metabolic) of 1,4-dioxane. Those linked to aerobic metabolic biodegradation classify within the genera *Pseudonocardia* (Kampfer and Kroppenstedt, 2004; Mahendra and Alvarez-Cohen, 2005; Mahendra and Alvarez-Cohen, 2006; Matsui et al., 2016; Parales et al., 1994; Sei et al., 2013a; Yamamoto et al., 2018), *Mycobacterium* (Kim et al., 2009; Sei et al., 2013a), *Afipia* (Isaka et al., 2016; Sei et al., 2013a), *Xanthobacter* (Chen et al., 2016), *Acinetobacter* (Huang et al., 2014), *Rhodococcus* (Bernhardt and Diekmann, 1991; Inoue et al., 2016; Inoue et al., 2018) and *Rhodanobacter* (Pugazhendi et al., 2015).

Genera associated with aerobic co-metabolic 1,4-dioxane degradation include *Pseudonocardia* (Kohlweyer et al., 2000; Mahendra and Alvarez-Cohen, 2006; Vainberg et al., 2006)], *Mycobacterium* (Lan et al., 2013; Masuda, 2009) and *Rhodococcus* (Hand et al., 2015; Lippincott et al., 2015; Mahendra and Alvarez-Cohen, 2006; Sei et al., 2013b; Steffan et al., 1997; Stringfellow and Alvarez-Cohen, 1999), *Flavobacterium* (Sun et al., 2011), *Burkholderia* (Mahendra and Alvarez-Cohen, 2006), *Nocardia* (Masuda, 2009), *Ralstonia* (Mahendra and Alvarez-Cohen, 2006), *Pseudomonas* (Mahendra and Alvarez-Cohen, 2006), *Methylosinus* (Mahendra and Alvarez-Cohen, 2006; Whittenbury et al., 1970) and *Azoarcus* (Deng et al., 2018b). However, it is unlikely that these aerobic 1,4-dioxane degraders will be effective at chlorinated solvent sites, as these sites are typically highly reducing. Under such conditions, tetrachloroethene and trichloroethene undergo sequential reductive dechlorination to *cis*-1,2-dichloroethene and vinyl chloride, finally forming the non-toxic end product, ethene (Freedman and Gossett, 1989). Reduction is commonly associated with microbial taxa such as *Dehalococcoides mccartyi* and *Dehalobacter* (Cupples, 2008; Cupples et al., 2003; He et al., 2003; Holliger et al., 1998; Löffler et al., 2013; Maymó-Gatell et al., 1997; Sung et al., 2006). Commercially available reductive dechlorinating mixed cultures containing such strains are frequently used for bioaugmenting contaminated groundwater aquifers (Major et al., 2002; Steffan and Vainberg, 2013; Vainberg et al., 2009). The lack of information on the susceptibility

of the chlorinated solvent co-contaminant 1,4-dioxane to biodegradation under anaerobic conditions is a significant knowledge gap.

No anaerobic 1,4-dioxane degrading isolates have been identified and, to our knowledge, only two studies have examined 1,4-dioxane biodegradation under anaerobic conditions. One project investigated 1,4-dioxane degradation over a range of redox conditions (aerobic, nitrate reducing, iron reducing, sulfate reducing and methanogenic) (Steffan, 2007). The work involved microcosm experiments with soil and groundwater from a site heavily contaminated with the chlorinated solvents and 1,4-dioxane. In these tests, nitrate, nitrite, sulfate and ferric iron were added as electron acceptors. In another set of experiments, samples from across a vegetable oil biobarrier were investigated, without the addition of electron acceptors, as it was expected that the biobarrier had resulted in a range of redox conditions. Notably, 1,4-dioxane was not degraded in any of the anaerobic microcosms during >400 days.

Another study produced more promising results, documenting 1,4-dioxane biodegradation under iron reducing conditions using an enrichment originating from wastewater treatment plant sludge (Shen et al., 2008). The researchers found that when Fe(III) was supplied as Fe(III)-EDTA, biodegradation was stimulated. Also, they reported that humic acids stimulated the activity of the Fe(III) reducing bacteria and 1,4-dioxane biodegradation. The authors hypothesized that humic acids promoted electron shuttling to Fe(III) in a catalytic manner. Others have also reported stimulation of bacterial growth and biodegradation (cyclic nitroamines RDX and HMX) in the presence of humic acids and Fe(III) (Bhushan et al., 2006).

The overall objective of the current study was to determine the susceptibility of 1,4-dioxane to biodegradation under anaerobic conditions using inocula from contaminated and uncontaminated sites. For this, microcosms were established with different inocula (soils or sediments) and various electron acceptor amendments (nitrate, iron-EDTA/humic acid, sulfate and no amendment). Additionally, compound specific isotope analysis (CSIA) was used to determine changes in $^{13}\text{C}/^{12}\text{C}$ ratios in a subset of the samples. The work is novel as it is the first to document the frequency of 1,4-dioxane biodegradation over a range redox conditions and

inocula types and provides critical evidence for the feasibility of 1,4-dioxane remediation through natural attenuation .

Methods

Chemicals and Inocula

1,4-dioxane was purchased from Alfa Aesar (MA, USA, 99+ % purity) and Sigma-Aldrich (MO, USA, 99.8% purity). Ethylenediaminetetraacetic acid (EDTA), iron (III) sodium salt, sodium sulfate, sodium nitrate and humic acid were purchased from Sigma-Aldrich (MO, USA). All stock solutions and dilutions were prepared using DI water. The uncontaminated agricultural soil samples were collected from two locations on the campus of Michigan State University (MSU), East Lansing, MI (herein called Soils E, F and G) and two locations at the Kellogg Biological Station (MSU), Hickory Corners, MI (KBS Soils 1, 2 and 3). The uncontaminated river sediment samples (sediments H and J) were collected from Red Cedar River, Okemos, MI and from a river leading to Lake Lansing in Haslett, MI. The contaminated site samples were sent to MSU from California (contaminated with trichloroethene, 1,1-dichloroethene and 1,4-dioxane) and Maine (contaminated with traces of 1,4-dioxane). All of the samples were stored in the dark at 6 °C until use.

Experimental Setup

Five sets of experiments were performed to examine the susceptibility of 1,4-dioxane to biodegradation under different potential electron acceptors (Table 1.1). All five experiments contained triplicates of live samples and abiotic controls (autoclaved daily for three consecutive days). The final set of experiments (experiment 5) also included live control microcosms (no 1,4-dioxane) to enable comparisons to the 1,4-dioxane degrading microbial communities.

The first set of set of experiments was designed based on the positive results generated previously for 1,4-dioxane under iron reducing conditions (Shen et al., 2008). For this, 18 microcosms (70 mL serum bottles) were each inoculated with one of three agricultural soils (Soils E, F and G, 20 g wet weight), and 55 mL of a solution consisting of EDTA iron (III) sodium salt (10 mM), sodium lactate (5 mM) and humic acid (0.5 g/L). The triplicate

microcosms and abiotic controls were amended with approximately 5 mg/L of 1,4-dioxane (Alfa Aesar).

The second set of microcosms contained the same three agricultural soils (Soils E,F and G) under four redox conditions (nitrate, iron, sulfate amended and no amendment). Here, 72 microcosms were established (3 soils, 4 treatments) containing soil (5 g wet weight), 10 mL of a solution with either NaNO₃ (10mM), NaSO₄ (10mM), EDTA iron(III) sodium salt (10mM) or DI water (no amendment) in 30 mL glass serum bottles. The microcosms and abiotic controls were amended with approximately 5 mg/L of 1,4-dioxane (Alfa Aesar). The third set of microcosms contained each of the uncontaminated river sediment samples (Sediments H and J) using the same experimental setup (four redox conditions). Sodium lactate was not added for both sets of experiments to encourage the metabolic degradation of 1,4-dioxane.

The fourth set of microcosms (48 bottles including 2 sediments, 4 treatments) contained each of the contaminated sediment samples (from CA and MN) also under four redox conditions (nitrate, iron, sulfate amended and no amendment). In this case, a media solution was added containing NH₄Cl (1.5 g/L), NaH₂PO₄ (0.6 g/L), CaCl₂·2H₂O (0.1 g/L), KCl (0.1 g/L), MgCl₂·6H₂O (0.002 g/L) and sodium lactate (5 mM). The electron acceptor and 1,4-dioxane amendments were as described above, however 1,4-dioxane from Sigma Aldrich was used.

The final set of microcosms contained no electron acceptor amendment with the aim of creating methanogenic conditions (based on positive results from some of the microcosms described above). The 72 microcosms included 8 different inocula (Soils E, F, G, KBS Soils 1, 2 and 3, Sediment H and the CA Contaminated Sediment) with triplicates of sample microcosms and abiotic controls. Additionally, triplicate live control microcosms were included and these were treated in the same manner as the sample microcosms except no 1,4-dioxane was added. As stated above, this treatment was included to enable comparisons to the microbial communities exposed to 1,4-dioxane. The microcosms contained 5 g (wet weight) of soil or sediment, 25 mL of a solution containing sodium lactate (5mM), NH₄Cl (1.5 g/L), NaH₂PO₄ (0.6 g/L), CaCl₂·2H₂O (0.1 g/L), KCl (0.1 g/L) and MgCl₂·6H₂O (0.002 g/L) in 30 mL amber glass serum

bottles. 1,4-dioxane (Sigma Aldrich, between 5-14 mg/L) was added the sample microcosms and abiotic controls.

All solutions were purged under a stream of nitrogen before being introduced into the anaerobic chamber. The solutions and soils/sediments were added to each serum bottle in the anaerobic chamber and, after approximately 3 days, 1,4-dioxane was added. The microcosms, closed with septa, were incubated in the anaerobic chamber at 20 °C. The anaerobic chamber was maintained with gaseous mix of approximately 5% H₂, 90% N₂ and 5% CO₂. The vials were sealed using BiMetal vial crimp with PTFE/silicone septas to maintain the cultures at anaerobic conditions. All microcosms were transferred on a shaker at 200 rpm and maintained at 20 °C. The nitrate amended microcosms were tested for methane after 200 days of incubation using a GC (Hewlett Packard 5890).

1,4-Dioxane Analysis

A GC/MS with Agilent 5975 GC/single quadrupole MS (Agilent Technologies, CA, USA) equipped with a CTC Combi Pal autosampler was used to determine 1,4-dioxane concentrations. Sterile syringes (1 mL) and needles (22 Ga 1.5 in.) were used to collect samples (0.7 mL) from each microcosm. The samples were filtered (0.22 µm nylon filter) before being injected into an amber glass vial (40 mL) for GC/MS analysis. A method was developed to analyze 1,4-dioxane using solid phase micro extraction (SPME). The SPME fiber was inserted in the headspace of the vial and exposed to the analyte for 1 minute before being injected into the GC for thermal desorption. The fiber coating can adsorb the analytes in the vapor phase. Splitless injection was executed and the vials were maintained at 40 °C. The SPME fiber assembly involved 50/30µm Divinylbenzene/ Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS) and 24 Ga needle for injection. The initial oven temperature was 35 °C and was programmed to increase at a rate of 20 °C/min to 120 °C. Once it reached 120 °C, it increased at a rate of 40 °C/min to 250 °C, which was maintained for 3 min. A VF5MS column was used with helium as the carrier gas in constant flow mode at a flow rate of 1 ml/min. The conditioning of the SPME fiber was at 270 °C for 60 min at the beginning of each sequence.

Compound Specific Isotope Analysis (CSIA)

At the last sampling point, three sets of samples and abiotic controls were submitted for CSIA analysis, including Soil F (nitrate amended, experiment two), and the two contaminated site samples with no electron acceptor amendment (experiment four). CSIA was performed at the University of Waterloo Environmental Isotope Laboratory (UWEIL), Ontario, Canada. The ratios of $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ were measured using a recently developed method. For this, the dilute 1,4-dioxane samples were concentrated on to a sorbent and subjected to thermal desorption in a GC coupled with an isotope ratio mass spectrometer (Bennett et al., 2018). All three sets of samples were submit to $^{13}\text{C}/^{12}\text{C}$ analysis, however only Soil F was subject to $^2\text{H}/^1\text{H}$.

DNA Extraction and High Throughput Amplicon Sequencing

DNA was extracted (using QIAGEN DNeasy PowerSoil kit) from the sample microcosms and live control microcosms (2 mL liquid and 0.6 mg soil/sediment) of the fifth set of microcosms (no electron acceptor amendment) that illustrated 1,4-dioxane degradation (Soil F, CA Contaminated Site, Sediment H and KBS Soil 3, 24 extracts). Additionally, DNA was extracted from 31 other microcosms that illustrated 1,4-dioxane biodegradation (from the other four experiments). The concentration of DNA was determined using QUBIT dsDNA HS kit. The DNA extracts were submitted for high throughput 16S rRNA gene amplicon sequencing following previously described protocols (Caporaso et al., 2012; Caporaso et al., 2011) at the Research Technology Support Facility at Michigan State University. Specifically, 55 samples of microbial metagenomic DNA were submitted for NGS amplicon library preparation and sequencing. The V4 hypervariable region of the 16S rRNA gene was amplified using the Illumina compatible, dual indexed primers 515f/806r. The primers and the library construction protocol developed in Patrick Schloss' lab were previously described (Kozich et al., 2013). PCR products were batch normalized using Invitrogen SequalPrep DNA Normalization plates and product recovered from the plate pooled. This pool was cleaned up and concentrated using AmpureXP magnetic beads. The pool was 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 then loaded onto an Illumina MiSeq v2 Standard flow cell and sequencing was performed in a 2x250bp paired end format using a MiSeq v2 500 cycle reagent kit. Custom sequencing and index primers complementary to the 515f/806r oligos were added to

appropriate wells of the reagent cartridge as previously described (Kozich et al., 2013). Base calling was performed by Illumina Real Time Analysis (RTA) v1.18.54 and the output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.

Analysis of Sequencing Data

The amplicon sequencing data in the fastq file format was analyzed on Mothur (version 1.41.3) from Patrick D. Schloss Laboratory (Schloss, 2009) using the MiSeq standard operating procedure (Schloss, 2013). The steps involved the removal of barcode information and contiguous sequences were created using the forward and reverse reads. These were analyzed for errors and then classified. This involved checking the samples for the proper read length (<275 bp), ambiguous bases and homopolymer length greater than 8 to eliminate such sequences. The sequences were then aligned with the SILVA bacteria database (Pruesse et al., 2007) for the V4 region. Chimeras, mitochondrial and chloroplast lineage sequences were removed and then the sequences were classified into OTU's. The summary file created by mothur was analyzed with Microsoft Excel 2016 and STAMP (Statistical Analyses of Metagenomic Profiles, software version 2.1.3.) (Parks et al., 2014). STAMP was used to detect differences in the relative proportions of the taxonomic profiles between the live controls (no 1,4-dioxane) and the samples for each of the four inocula in the fifth set of experiments (Soil F, Contaminated Site 10A, Sediment H and KBS Soil 3). This analysis included Welch's two sided t-test for two groups (samples and live controls) ($p < 0.05$) to generate extended error bar figures for each soil.

Statistical Analysis

1,4-dioxane concentrations in the samples and controls were compared using the student's t-test for independent variables with equal variance. Normality of the 1,4-dioxane concentration data was confirmed using the Shapiro-Wilk test ($p > 0.05$) and the assumption of equal variance was confirmed using Levene's tests ($p > 0.05$). The same tests were used to confirm equal variance and normality for the CSIA carbon data. The CSIA hydrogen data were not normal, therefore the Mann Whitney was used. These statistical tests were performed in XLSTAT software for statistical analysis in Excel (2019, <http://www.xlstat.com>).

Results

1,4-Dioxane Removal Trends

1,4-dioxane concentrations in samples and abiotic controls were monitored for approximately one year in each of the five sets of experiments. Given the long incubation period, it is likely that the electron acceptors (nitrate, iron, sulfate) were depleted for each treatment. This was confirmed in one case, by the production of methane in the nitrate amended, agricultural soil inoculated microcosms. The first set of microcosms (inoculated with three agricultural soils and an EDTA iron/humic acid amendment) illustrated no significant difference ($p < 0.05$) between the samples and abiotic controls for the majority of sampling times, suggesting no biological 1,4-dioxane degradation occurred with this treatment for these soils (Figure 1.1).

The second set of microcosms (agricultural soils and four electron acceptor treatments) produced a variety of trends between the soils and treatments (Figure 1.2). Comparing the treatments, the most consistent removal was in the nitrate amended microcosms (Figure 1.2A). For all three soils, there was a statistically significant difference ($p < 0.05$) between the samples and abiotic controls at the last sampling point (day 295 to 316). Similar to the first set of microcosms, no significant removal was noted in the iron amended microcosms for all three soils (Figure 1.2B). In the sulfate amended microcosms, only soil E resulted in a significant difference between the samples and controls (Figure 1.2C). When no electron acceptor was added, soils E and F illustrated reduced concentrations in the samples compared to the abiotic controls (Figure 1.2D).

In the third set of experiments (uncontaminated sediments and four treatments) (Figure 1.3), a difference between the samples and abiotic controls was only noted for Sediment H with no electron acceptor amendment. This was one of the most dramatic reductions in 1,4-dioxane in the live sample microcosms compared to the abiotic controls over all treatments and inocula types (Figure 1.3D).

The fourth set of experiments (inoculated with sediments from two contaminated sites over four treatments) illustrated similar trends as those from the agricultural soils (Figure 1.4). Specifically, in one case for each of the nitrate amended and sulfate amended microcosms, there was a significant difference between the samples and controls (CA and MN, respectively)

(Figure 1.4A and C). Further, no differences were observed between the samples and controls under the iron amended treatment (Figure 1.4B). Finally, in both cases a substantial decrease (~21% and 50% for CA and MN) was observed in the samples compared to the abiotic controls when no electron acceptor was added (Figure 1.4D).

A summary of the results described in the four sets of experiments is provided, focusing only on the last sampling point for each (Table 1.2). In the nitrate amended treatments, four of the six inocula types (or 57%) produced a significant reduction in 1,4-dioxane in the samples compared to the abiotic controls. In contrast, no differences were noted between the samples and abiotic controls under any of the iron amendment treatments. Only two (Soil E and the MN contaminated site sediments) of the seven cases (or 29%) illustrated biodegradation under the sulfate amendment treatment. Finally, the most frequent (five of the seven inocula types or 71%) reduction of 1,4-dioxane was noted under the no amendment treatment. The average removal (again, only at the last time point) was greatest under the no amendment treatment ($22 \pm 19 \%$), followed by the nitrate amended samples ($16 \pm 16 \%$) and the sulfate amended samples ($7 \pm 12 \%$). Overall, it appears the likelihood of 1,4-dioxane biodegradation is the greatest with no amendments, when the microbial populations are likely under methanogenic redox conditions.

The fifth set of experiments were established to compare the microbial communities between the live microcosms and the live control microcosms (no 1,4-dioxane amendment) and focused only on the no amendment treatment. For this, six agricultural soils, one contaminated and one uncontaminated sediment sample were used as inocula (Figure 1.5). A significant reduction in 1,4-dioxane in the samples compared to the abiotic controls was only noted for two of six agricultural soils (Figure 1.5B, H). Consistent with the results described above (third and fourth set of experiments), a significant reduction in 1,4-dioxane was noted in the samples compared to the controls in sediment H (Figure 1.5E) and the contaminated samples from CA (Figure 1.5D). In this set of experiments, only 50% of the inocula types illustrated detectable levels of 1,4-dioxane biodegradation.

Compound Specific Isotope Analysis (CSIA)

CSIA was used to further investigate biodegradation in three sets of anaerobic 1,4-dioxane degrading microcosms. For this, subsamples were collected from sample microcosms and abiotic controls from the Soil F/nitrate treatment (second set of experiments) and from the CA and MN contaminated site sediments/no electron acceptor treatment (fourth set of experiments) and these were sent to UWEIL for CSIA. This laboratory has already developed the methodology to measure $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values for 1,4-dioxane (Bennett et al., 2018). All three sets of samples (six for each treatment, eighteen in total) were subject to $^{13}\text{C}/^{12}\text{C}$ analysis, but only the six Soil F/nitrate microcosms were subject to $^2\text{H}/^1\text{H}$ analysis. 1,4-dioxane degradation should result in more positive $^{13}\text{C}/^{12}\text{C}$ and $^2\text{H}/^1\text{H}$ ratios (or more positive $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values) because bonds involving heavier isotopes are more difficult to break, and so bonds consisting of lighter isotopes are preferentially degraded, causing the residual, non-degraded contaminant to be heavy isotope enriched. In all cases, more positive $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values were found in the live samples compared to the abiotic controls (Figure 1.6). However, the differences in $\delta^{13}\text{C}$ values were only statistically significantly different between the samples and abiotic controls for Soil F/nitrate and contaminated site MN/no electron acceptor. It is likely that the decrease in 1,4-dioxane concentrations were too low for this method to detect a difference in $\delta^{13}\text{C}$ values for the contaminated site CA/no electron acceptor. Specifically, for this treatment the average reduction in 1,4-dioxane concentration in the samples compared to the controls was 17% compared to 25% and 50% reductions in the other two treatments. The $\delta^2\text{H}$ values were not significantly different between the samples and controls for Soil F (Figure 1.6B).

Putative 1,4-Dioxane Degraders

To determine if any microorganism could obtain a growth benefit from 1,4-dioxane degradation, sample microcosms were supplied with media and 1,4-dioxane and the live control microcosms were supplied with the same media, but no 1,4-dioxane (fifth set of microcosms). Therefore, an increase in the relative abundance of any microorganism between the samples and live controls could be attributed to the presence of 1,4-dioxane. From this, a reasonable hypothesis would be that the enriched microorganisms are being exposed to growth supporting substrates from 1,4-dioxane degradation. This comparison focused on the four inocula that illustrated 1,4-dioxane degradation (Figure 1.5B, D, E, H). Significant phylotypes enrichments ($p < 0.05$) were noted for

the microcosms inoculated with CA contaminated site sediments, Sediment F and KBS Soil 3 (Figure 1.7). Only one phylotypes, unclassified *Comamonadaceae*, (phylum *Proteobacteria*, class *Betaproteobacteria*, order *Burkholderiales*) was enriched in the CA contaminated site microcosms (Figure 1.7A). Nine phylotypes were enriched in the uncontaminated site (Sediment H) microcosms, with 3 *genus incertae sedis* (phylum *Verrucomicrobia*, class *Subdivision3*) being dominant (Figure 1.7B). There was a small (yet significant) enrichment of one phylotype, *Pseudoxanthomonas*, (phylum *Proteobacteria*, class *Gammaproteobacteria*, order *Xanthomonadales*, family *Xanthomonadaceae*) in the KBS Soil 3 microcosms (Figure 1.7C) and no enrichment of any phylotype in the Soil F microcosms.

The sequencing data sets were also analyzed to determine the relative abundance of previously identified 1,4-dioxane degraders (as listed in the introduction). It is important to note that the analysis could only be performed at the genus level, therefore it is not possible to determine if particular species or strains are present. As stated above, the 55 DNA extracts were from the fifth set of microcosms that illustrated 1,4-dioxane degradation (Soil F, Contaminated Site 10A, Sediment H and KBS Soil 3) and from 31 other microcosms that illustrated 1,4-dioxane biodegradation (from the other four experiments) (Table 1.1). Interestingly, all four soil inoculated microcosms contained a high relative abundance (~5-45%) of the genus *Rhodanobacter* (Figure 1.8, inserts). All four soils contained the genera *Pseudomonas*, *Mycobacterium*, *Acinetobacter*, although the relative abundance values were much lower (< 0.15%) than *Rhodanobacter* (Figure 1.8). The microcosms inoculated with KBS Soil 3 illustrated the largest number of previously identified 1,4-dioxane degraders (11 from 15 genera) (Figure 1.8A). The Soils E, F and G inoculated microcosms primarily contained 4, 8 and 5 of these genera. The well-studied *Pseudonocardia* and *Rhodococcus* genera were only common in KBS Soil 3 microcosms (Figure 1.8A). The microcosms inoculated with contaminated site (CA) and uncontaminated site (Sediment H) sediments contained lower relative abundance values (<0.04%) for *Rhodanobacter* (Figure 1.9A and B). The CA contaminated site microcosms illustrated high levels of *Pseudomonas* (~20-70%) and lower levels of *Rhodanobacter* and *Acinetobacter* (Figure 1.9A). *Flavobacterium* and *Rhodococcus* were only present in two from the twelve CA microcosms examined. The Sediment H microcosms contained lower levels (<0.011%) of *Pseudomonas*, along with *Rhodanobacter* and four other genera (Figure 1.9B). The

KBS Soil 3 microcosms illustrated higher values for *Rhodanobacter* (in one case, *Pseudomonas*) and lower levels of seven other genera (Figure 1.9C). *Rhodococcus* was present at low levels with all three inocula, but only in a small number of microcosms. *Pseudonocardia* was absent from the CA microcosms, present at low levels in both a subset of the Sediment H microcosms and in all of the KBS Soil 3 microcosms. The average relative abundance values of the two enriched phylotypes discussed above (unclassified *Comamonadaceae* at 4.9% and 3 *genus incertae sedis* at 2.7%) were markedly higher than all of the previously identified dioxane degraders, except for *Rhodanobacter* and *Pseudomonas*.

Discussion

Although much is known about the microorganisms and functional genes associated with 1,4-dioxane degradation under aerobic conditions, limited research has addressed the susceptibility of 1,4-dioxane to biodegradation under anaerobic conditions (Shen et al., 2008; Steffan, 2007). This research area is particularly important because 1,4-dioxane is a frequent co-contaminant at chlorinated solvent sites, which may be subject to remediation under highly reducing conditions (i.e. sulfate reducing or methanogenic). As discussed above, previous research reported no 1,4-dioxane biodegradation over a range of redox conditions (Steffan, 2007) with biodegradation occurring under iron reducing conditions with EDTA/humic acid amendments (Shen et al., 2008). In contrast, in the current work, the iron/EDTA/humic acid amended microcosms did not result in any significant removal in the live samples compared to the abiotic controls. One hypothesis for the conflicting results could be the different sources of inocula (anaerobic wastewater sludge compared to soils and sediments in the current study) for the two studies, resulting in microbial communities with varying degradative abilities. It is important to note that in the current study, the contaminated site microcosms did not illustrate any degradation under these conditions (iron/EDTA/humic acid), therefore it is unlikely that *in situ* iron amendments would be a successful 1,4-dioxane remediation approach.

An important limitation to the current study concerns the lack of data on electron acceptor concentrations. Given the long incubation periods, it is likely that electron acceptor depletion occurred and methanogenic conditions eventually dominated (which was confirmed in one case). Therefore, caution should be taken interpreting the impact of the addition of the various electron

acceptors. However, it is reasonable to conclude that iron/EDTA/humic acid or sulfate amendments did not facilitate 1,4-dioxane biodegradation in the majority of cases and therefore these treatments would be ineffective *in situ* remediation techniques.

1,4-dioxane biodegradation most commonly occurred in the nitrate amended treatments and no electron acceptor treatments. Four and five (from seven) inocula sources examined in the first four sets of experiments produced significant reductions in 1,4-dioxane concentrations in the samples compared to the abiotic controls in the nitrate and no electron acceptor treatments, respectively. Further, both contaminated site sediments illustrated significant removal in the samples compared to the controls in the no electron acceptor treatment. However, the biodegradation rates were slow, with significant concentration reductions occurring over approximately one year. In the other treatments, it is possible that the presence of other electron acceptors delayed the onset of methanogenesis and therefore 1,4-dioxane biodegradation. It is unclear why the nitrate amended treatments did not have this effect. Taken together, the data indicate *in situ* 1,4-dioxane bioremediation under methanogenic conditions may be a feasible remediation approach.

CSIA has been applied in previous studies to examine aerobic 1,4-dioxane biodegradation (Bennett et al., 2018; Gedalanga et al., 2016). Recently, CSIA enabled the discrimination between the activities of the bacteria *Rhodococcus rhodochrous* ATCC 21198 and *Pseudonocardia tetrahydrofuranoxidans* K1 during 1,4-dioxane biodegradation (Bennett et al., 2018). Also, CSIA provided additional evidence of 1,4-dioxane biodegradation in a groundwater plume that contained both metabolic and co-metabolic 1,4-dioxane degrading bacteria (Gedalanga et al., 2016).

To our knowledge, this is the first application of CSIA to investigate anaerobic 1,4-dioxane biodegradation. In two of the three cases examined, CSIA provided additional evidence for 1,4-dioxane biodegradation. In one case, the reduction in 1,4-dioxane in the samples compared the controls was likely too low for the method to detect a significant $^{13}\text{C}/^{12}\text{C}$ enrichment. The changes in $^{13}\text{C}/^{12}\text{C}$ ratios in the current study were much less than those reported previously for aerobic 1,4-dioxane biodegradation (Bennett et al., 2018), however, the changes in 1,4-dioxane

concentrations were also much less in the current study. The preliminary data set generated here indicate the value and limitations for $^{13}\text{C}/^{12}\text{C}$ analysis for documenting anaerobic 1,4-dioxane biodegradation. Further research is required to determine the value of measuring $^2\text{H}/^1\text{H}$ for generating evidence for the biodegradation of this chemical.

The greatest 1,4-dioxane decreases were observed in the microcosms inoculated with river sediment (sediment H) and the contaminated sediment from Maine. Therefore, future research should examine the capabilities of other sediments to biodegrade this contaminant. The current research included experiments both with and without the addition of sodium lactate as an electron donor. Unfortunately, no clear trend was apparent as to the value of adding this substrate. Many chlorinated solvent sites are amended with an electron donor (e.g. vegetable soil, hydrogen release compound) so at least this particular substrate did not have any negative impact on 1,4-dioxane biodegradation. Additional research will be needed before any conclusions can be reached on the value of adding lactate to enhance biodegradation.

The analysis of the microbial communities from 1,4-dioxane degrading microcosms indicated unclassified *Comamonadaceae* were obtaining a growth benefit in microcosms inoculated with sediment from the CA contaminated site. *Comamonadaceae* is a large and diverse family (over 100 species in 29 genera) that illustrate an impressive level of phenotypic diversity, including aerobic organotrophs, anaerobic denitrifiers, iron reducing bacteria, hydrogen oxidizers, photoautotrophic and photoheterotrophic bacteria, and fermentative bacteria (Willems, 2014). Additional research will be needed to determine which particular species within this family were enriched during 1,4-dioxane degradation.

For the uncontaminated sediment (Sediment H), nine phylotypes were enriched in the 1,4-dioxane degrading microcosms compared to the controls with 3 *genus incertae sedis* (phylum *Verrucomicrobia*, class *Subdivision3*) being more highly enriched compared to the others (average 2.7% relative abundance). This phylotype also exhibited a high relative abundance (average 2.9%) in the MN contaminated sediment microcosms that illustrated 1,4-dioxane degradation. *Verrucomicrobia* is a rarely cultivated phylum that is generally poorly studied

(Naumoff and Dedysh, 2018; Tanaka et al., 2017). Again, additional research is needed to confirm the role of these phylotypes in 1,4-dioxane biodegradation.

The 1,4-dioxane degrading communities also contained sequences that classified in the genera of previously reported 1,4-dioxane degraders. The soil microcosms were dominated primarily by *Rhodanobacter* with lower abundance values for *Pseudomonas*, *Mycobacterium* and *Acinetobacter*. The sediment communities were dominated by *Pseudomonas* and *Rhodanobacter*. However, as stated above, it is important to note the classification is only to the genus level, therefore it is unknown if specific strains or species are present. Again, additional research is needed to determine the importance of these genera in 1,4-dioxane degradation under these experimental conditions.

Overall, the current study indicates 1,4-dioxane biodegradation under anaerobic, and likely highly reducing conditions, is feasible. Therefore, natural attenuation of 1,4-dioxane may occur over a wider range of conditions, notably methanogenic areas where highly reducing conditions exist either naturally or as a result of enhanced reductive dechlorination. Future research should target sediments for other contaminated sites along with additional CSIA measurements to confirm biodegradation. Also, other substrates could be tested for their potential to enhance biodegradation rates.

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Table 1.1. Experimental design of the five microcosm studies.

Experiment	1	2	3	4	5
Soils E, F, G	X	X			X
KBS Soils 1, 2, 3					X
River Sediments H, J			X		H only
Contaminated Sites				CA, MN	CA only
Iron/EDTA/humic acid	X	X	X	X	
Nitrate		X	X	X	
Sulfate		X	X	X	
No electron acceptor		X	X	X	
Lactate	X			X	X
Basal salts media				X	X
Community analysis		17	3	11	24

Table 1.2. Percent removal in samples compared to the controls at the last time point (when the difference was statistically significant, determined with student's t-test for independent variables with equal variance, $p < 0.05$).

	Nitrate	Iron	Sulfate	No Amendment
Soil E	29	0	21	23
Soil F	25	0	0	24
Soil G	41	0	0	0
Sediment H	0	0	0	43
Sediment J	0	0	0	0
Maine	0	0	29	50
California	16	0	0	16
% of sample types with decreases in samples compared to the controls	57	0	29	71
% average removal w/ std deviation	16 ± 16	0	7 ± 12	22 ± 19

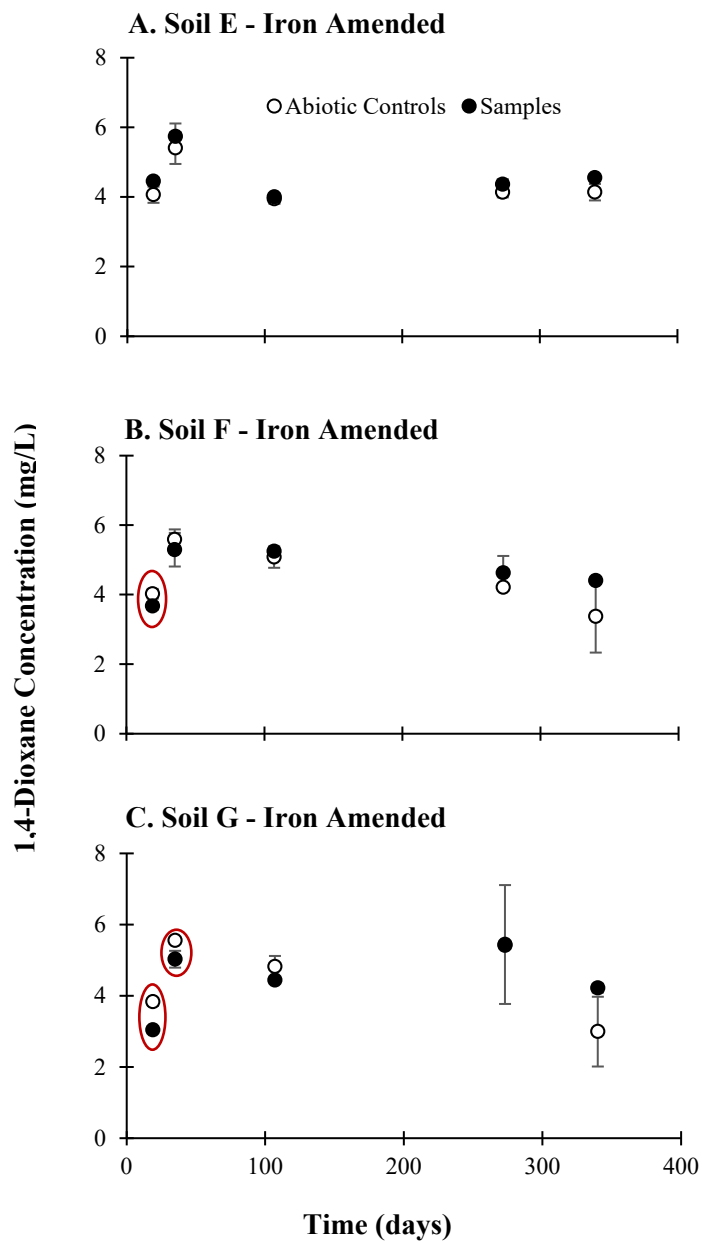


Figure 1.1. 1,4-Dioxane concentrations in sample and abiotic control microcosms of three soils amended with EDTA/iron and humic acid. The values and bars represent averages and standard deviations from triplicates. The circles represent a significant difference (two-tailed t-test, $p < 0.05$) between the samples and abiotic controls (when abiotic controls $>$ samples).

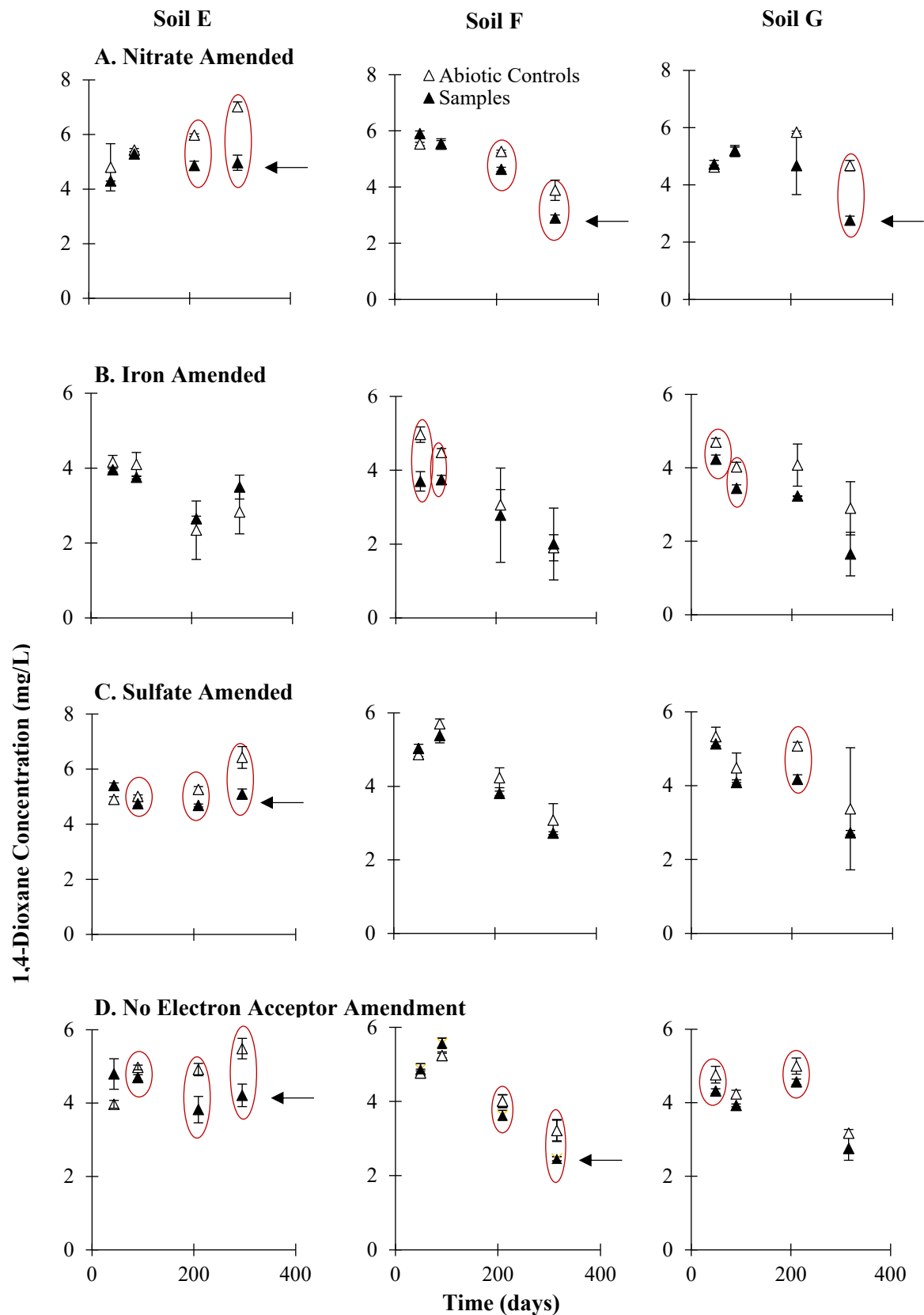


Figure 1.2. 1,4-Dioxane concentrations in sample and control microcosms of three soils with different electron acceptor amendments (A-D). The values and bars represent averages and standard deviations from triplicates (except soil G, $n = 2$ for three cases). The circles represent a significant different (two-tailed t-test, $p < 0.05$) between the samples and abiotic controls (when controls > samples). The arrows indicate the samples subject to DNA extraction.

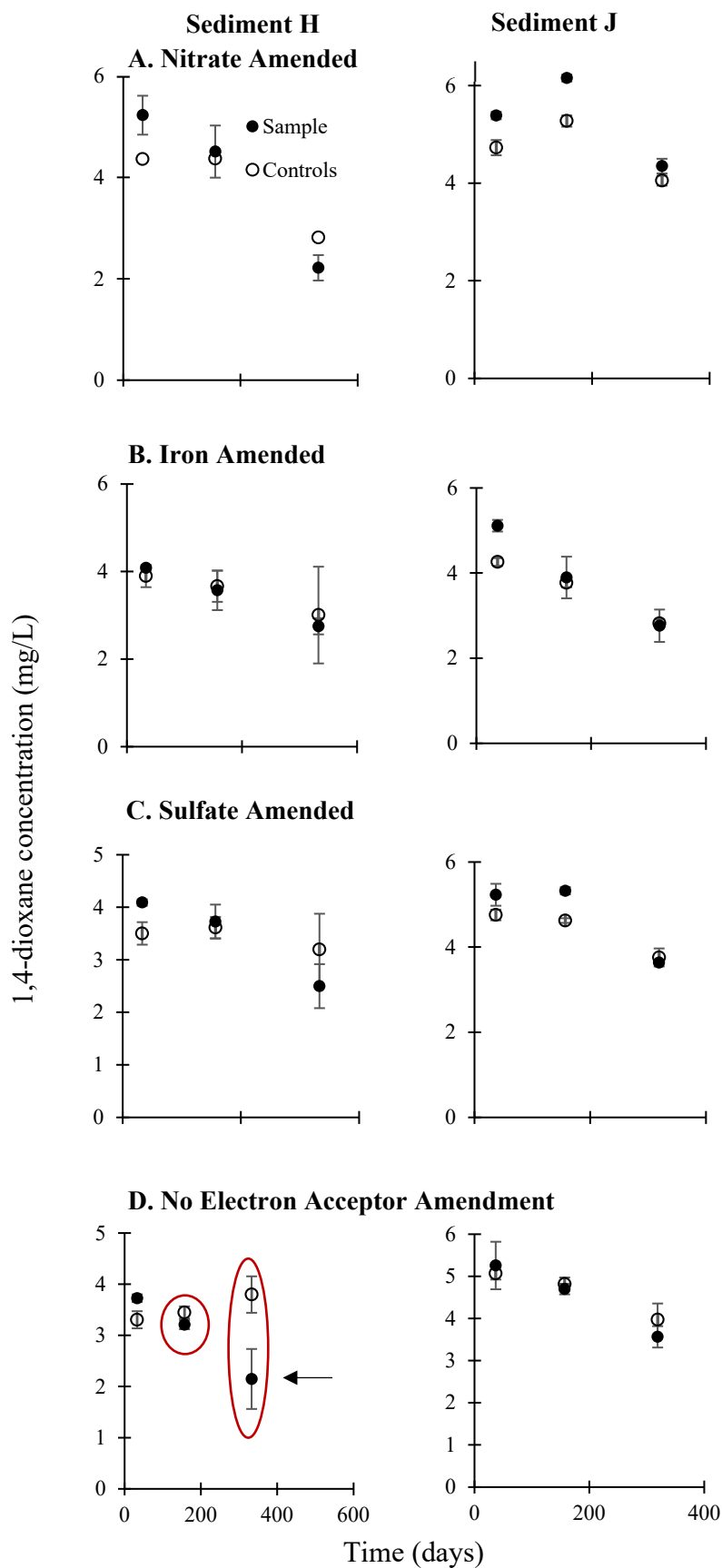


Figure 1.3. 1,4-Dioxane concentrations in Sediment J and H sample and control microcosms over time with different amendments. The points illustrate average values from triplicates (except nitrate amended controls, sediment H, $n=1$) and the bars illustrate standard deviations. The circles represent a significant different (two-tailed t-test, $p<0.05$) between the samples and abiotic controls. The arrow indicate the samples subject to DNA extraction.

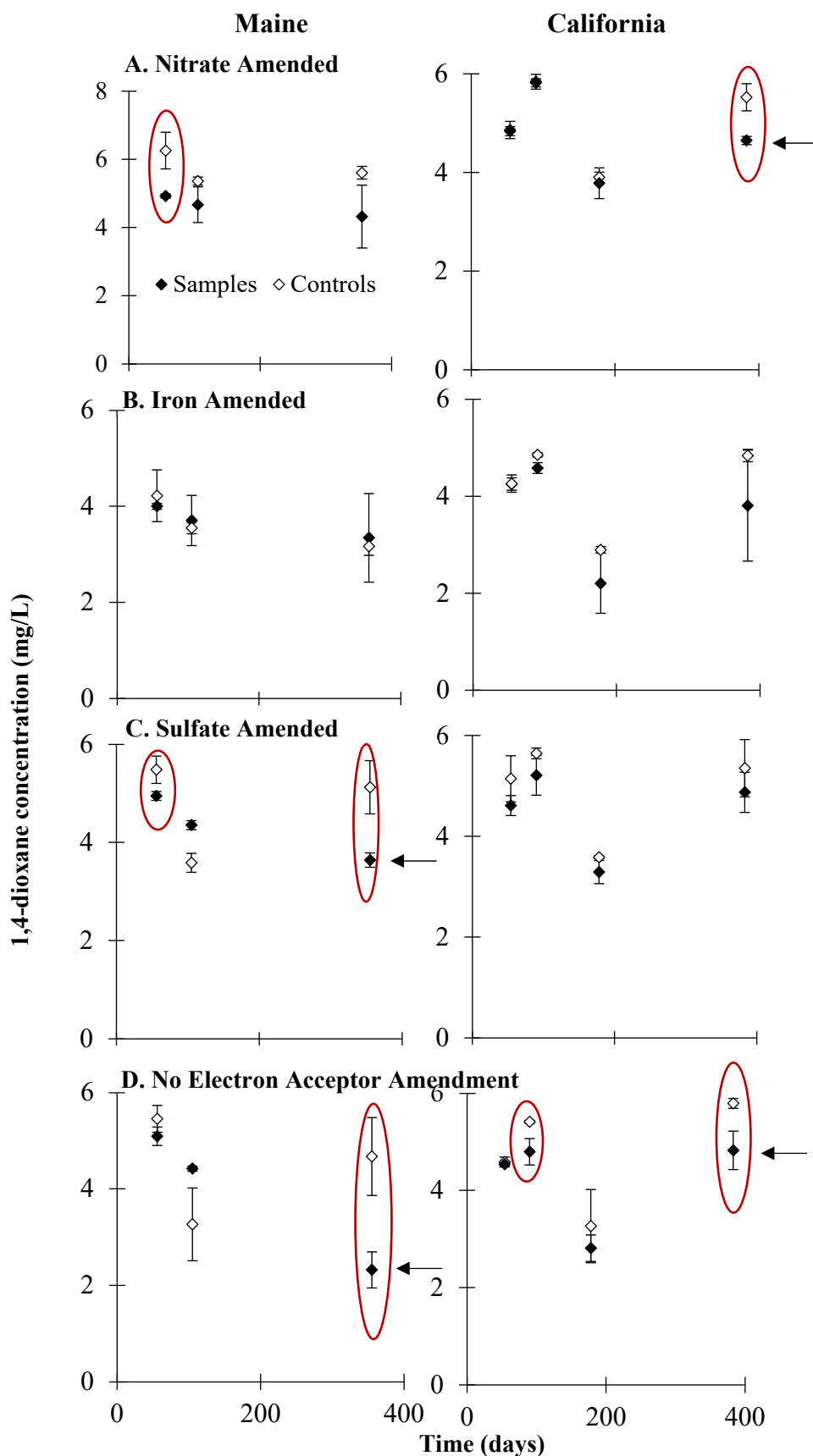


Figure 1.4. 1,4-Dioxane concentrations in Maine and California sample and abiotic control microcosms over time with different amendments. The points illustrate average values from triplicates (except sulfate amended Maine sample, $n=2$ and sulfate amended CA control, $n=2$) and the bars illustrate standard deviations. The circles represent a significant different (two-tailed t-test, $p<0.05$) between the samples and abiotic controls (controls > samples). The arrows indicate the samples subject to DNA extraction. ³²

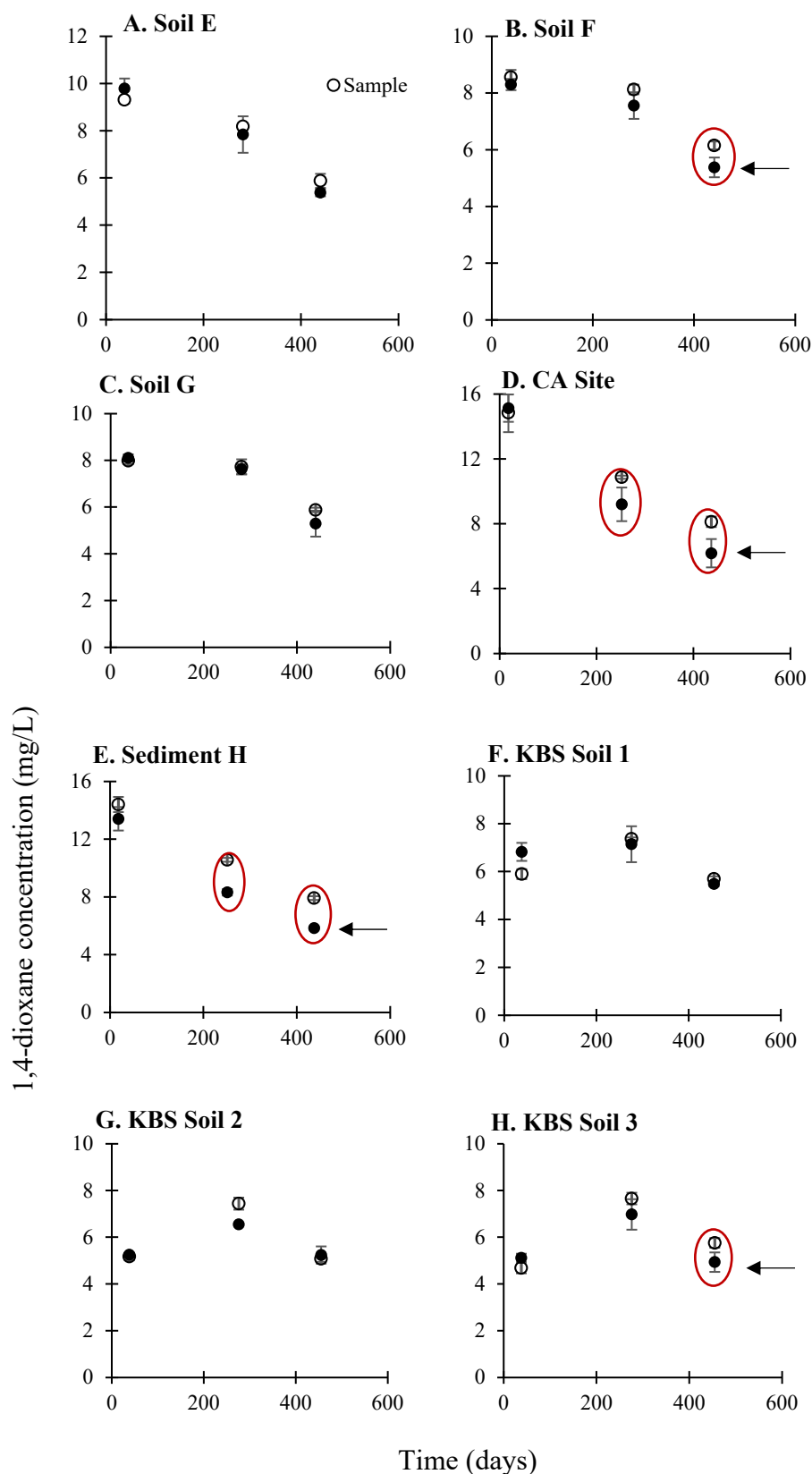


Figure 1.5. 1,4-Dioxane concentrations in sample and control microcosms over time with different inocula. The points illustrate average values from triplicates (except Soil E and KBS Soil 2 samples, which are replicates) and the bars illustrate standard deviations. The circles represent a significant different (two-tailed t-test, $p < 0.05$) between the samples and abiotic controls. The arrows indicate the samples subject to DNA extraction.

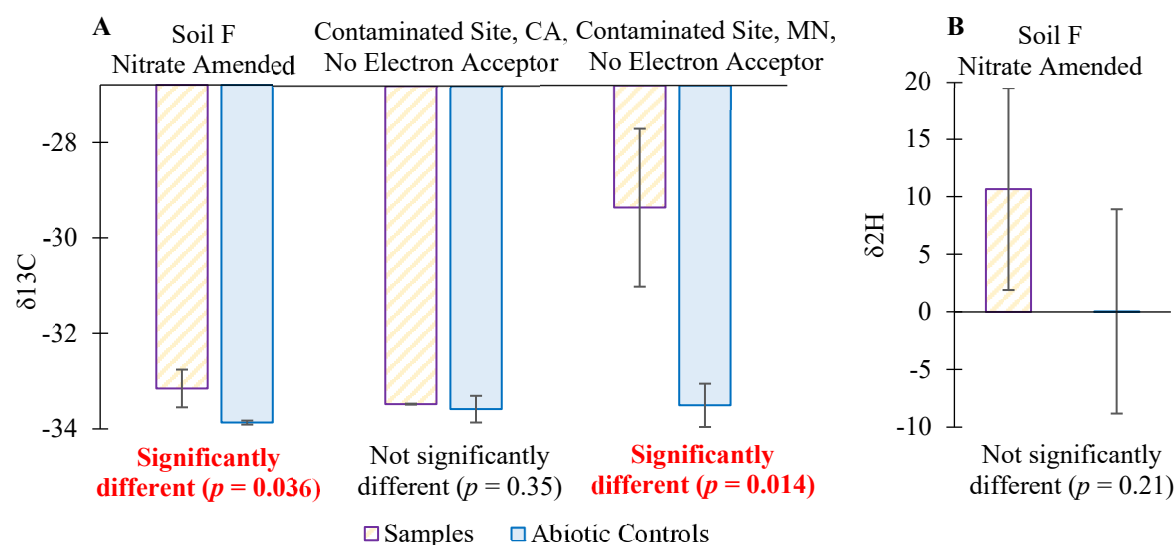


Figure 1.6. Comparison of $\delta^{13}\text{C}$ (A) and $\delta^2\text{H}$ (H) values in samples and abiotic controls of a sub-set of the experimental microcosms. The error bars represent standard deviations from triplicate samples and control microcosms and the p values were derived from the student's t-test (two tailed, independent variables, equal variance).

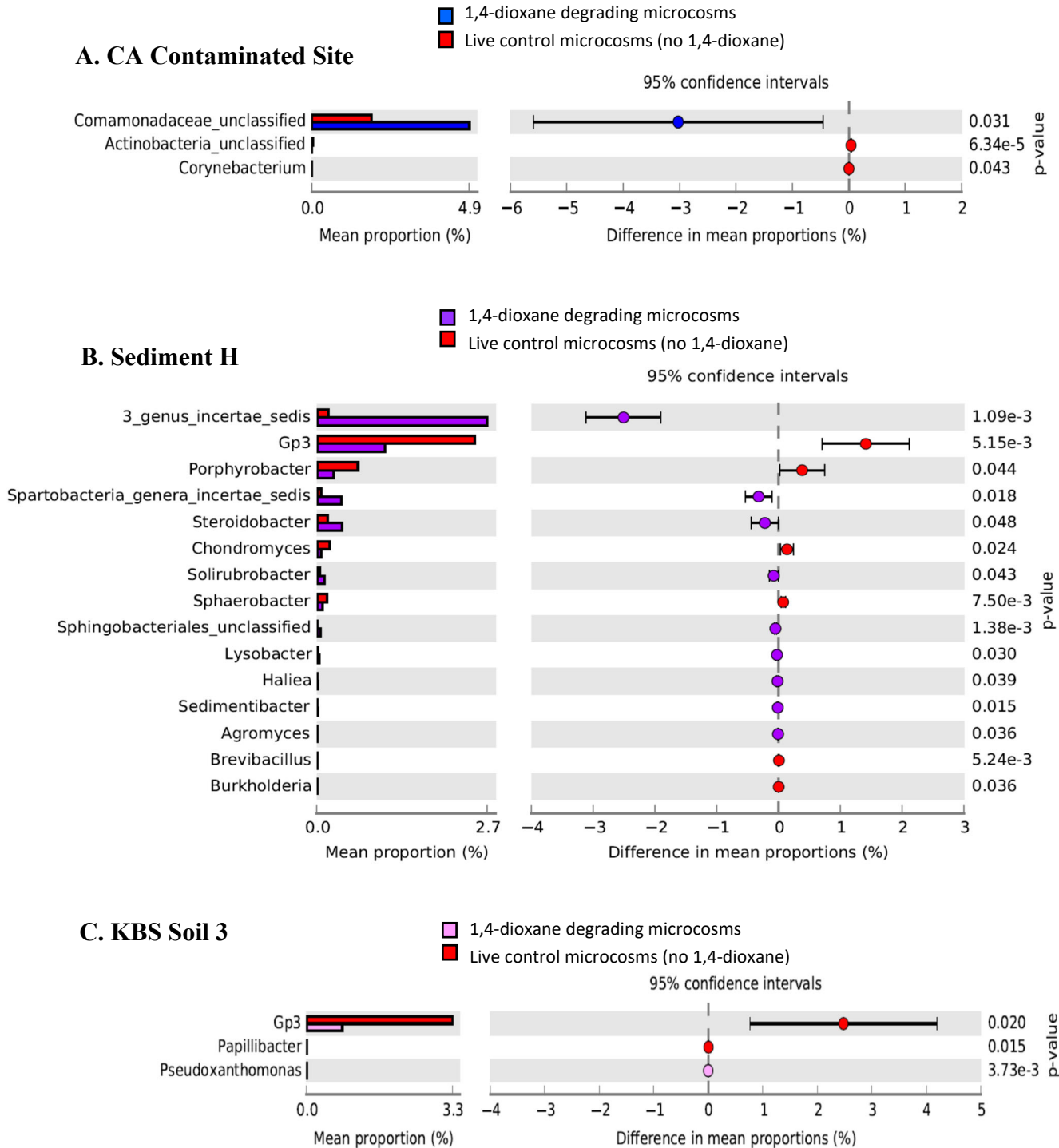
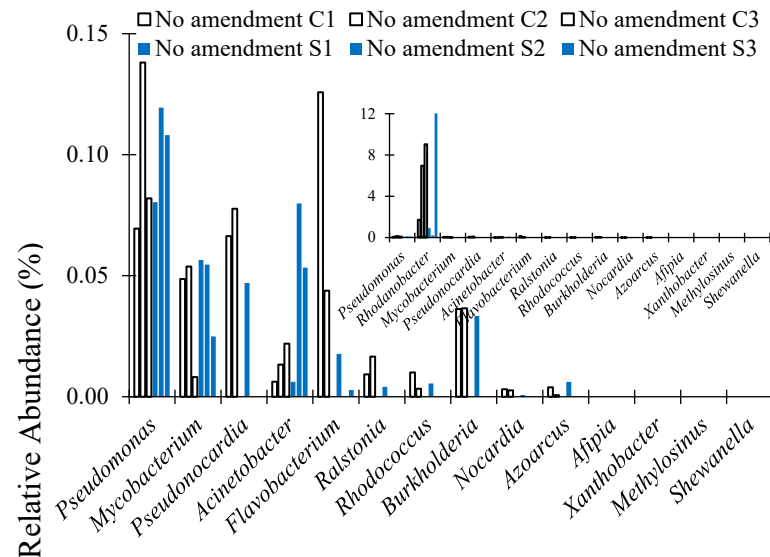
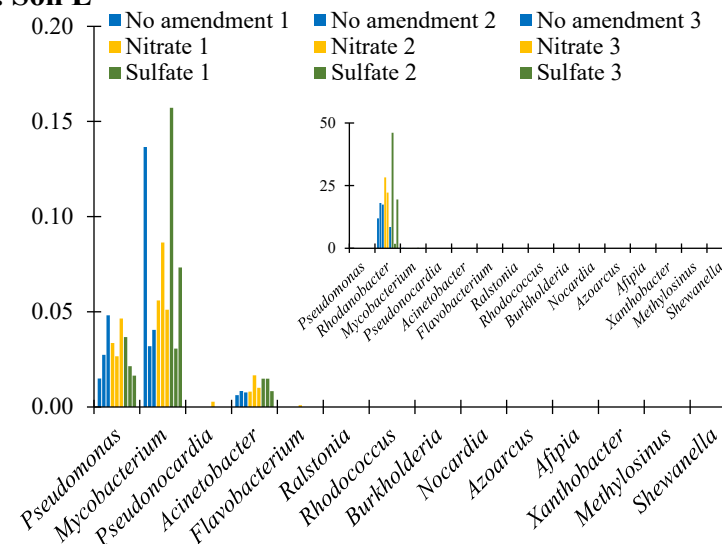


Figure 1.7. Phylotypes with a statistically significant difference (Welch's t-test, two sided, $p < 0.05$) between the 1,4-dioxane degrading samples ($n=3$) compared to the live controls ($n=3$). The comparisons are shown for the Contaminated Site (CA) microcosms (A), the Sediment H microcosms (B) and KBS Soil 3 microcosms (C). No differences were noted for Soil F. The data points to the left of the dashed line indicate phylotypes more abundant in the 1,4-dioxane degrading samples compared to the controls and those to the right indicate the reverse. For each, the x-axis have different scales.

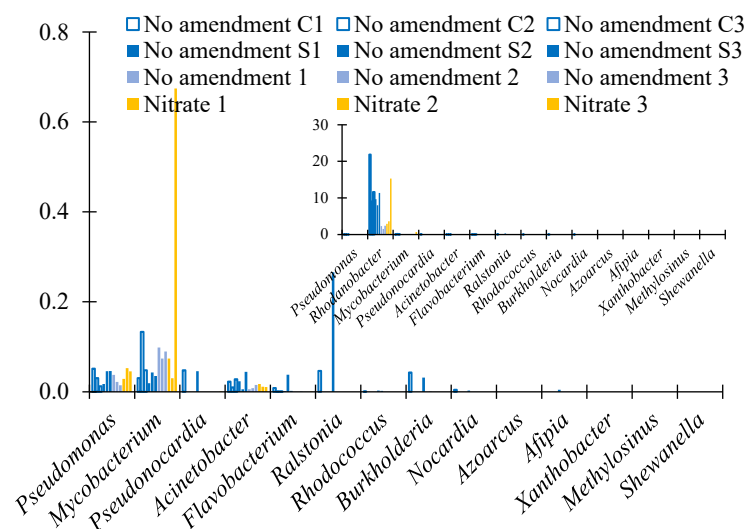
A. KBS Soil 3



B. Soil E



C. Soil F



D. Soil G

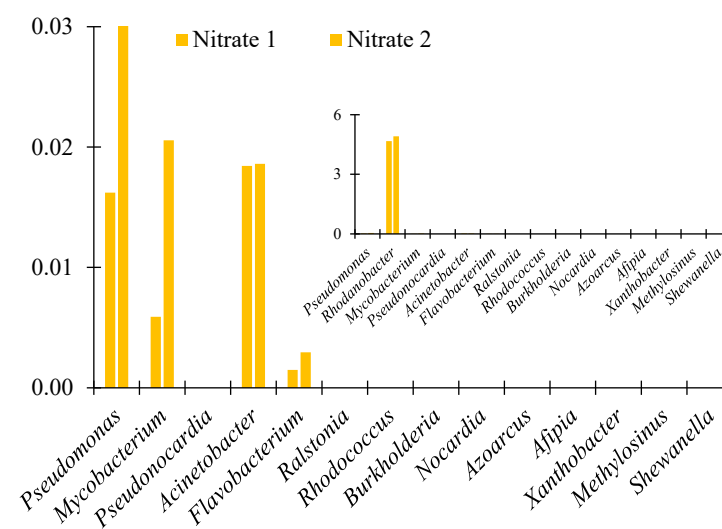
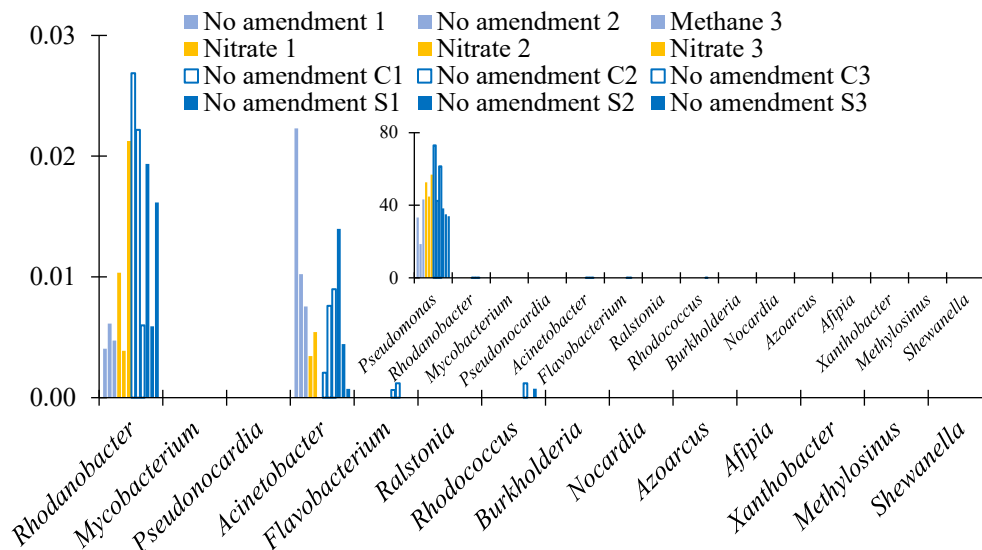
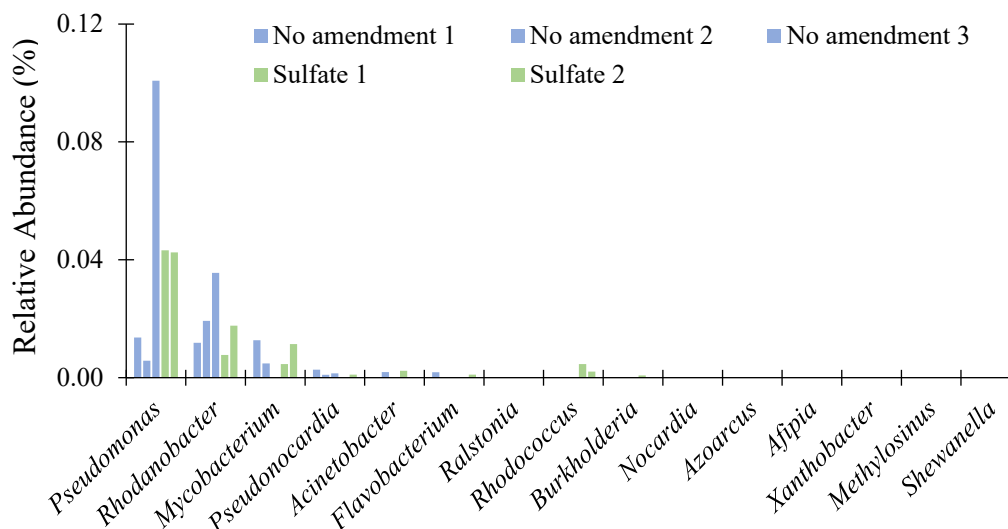


Figure 1.8. Relative abundance (%) of genera previously associated with 1,4-dioxane biodegradation in replicates of 1,4-dioxane degrading microcosms or live controls (C, no 1,4-dioxane) inoculated with different soils. The inserts have a different axis to better illustrate the relative abundance of *Rhodanobacter*.

A. CA Contaminated Site



B. MN Contaminated Site



C. Sediment H

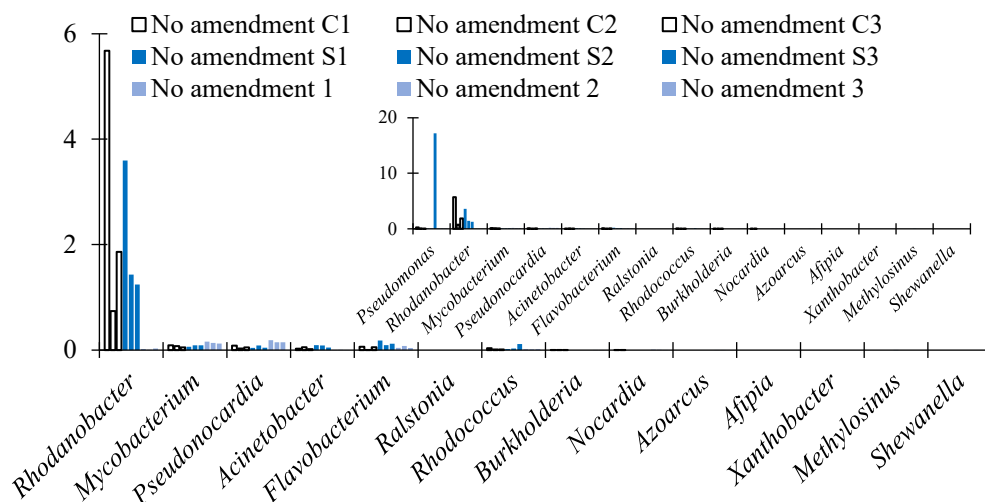


Figure 1.9. Relative abundance (%) of genera previously associated with 1,4-dioxane biodegradation in replicates of 1,4-dioxane degrading microcosms or live controls (C, no 1,4-dioxane) inoculated with different sediments (A, B, C). The inserts have a different axis to better illustrate the relative abundance of *Pseudomonas*.

Enrichment of Novel Actinomycetales and Detection of Monooxygenases during Aerobic 1,4-Dioxane Biodegradation with Uncontaminated and Contaminated Inocula

Abstract

1,4-dioxane, a co-contaminant at many chlorinated solvent sites, is a problematic groundwater pollutant because of risks to human health and characteristics which make remediation challenging. *In situ* 1,4-dioxane bioremediation has recently been shown to be an effective remediation strategy. However, the presence/abundance of 1,4-dioxane degrading species across different environmental samples is generally unknown. Here, the objectives were to identify which 1,4-dioxane degrading functional genes are present and which genera may be using 1,4-dioxane and/or metabolites to support growth across different microbial communities. Laboratory sample microcosms and abiotic control microcosms (containing media) were inoculated with four uncontaminated soils and sediments from two contaminated sites. The sample microcosms were amended with 1,4-dioxane three times. Live control microcosms were treated in the same manner, except 1,4-dioxane was not added.

1,4-dioxane decreased in live microcosms with all six inocula, but not in the abiotic controls, suggesting biodegradation occurred. A comparison of live microcosms and live controls (no 1,4-dioxane) indicated seventeen genera were enriched following exposure to 1,4-dioxane, suggesting a growth benefit for 1,4-dioxane degradation. The three most enriched were *Mycobacterium*, *Nocardioides*, *Kribbella* (classifying as *Actinomycetales*). There was also a higher level of enrichment for *Arthrobacter*, *Nocardia* and *Gordonia* (also *Actinomycetales*) in one soil and *Hyphomicrobium* (*Rhizobiales*) in two soils. Although *Arthrobacter*, *Mycobacterium* and *Nocardia* have previously been linked to 1,4-dioxane degradation, *Nocardioides*, *Gordonia* and *Kribbella* are potentially novel degraders.

Eleven of the twelve functional genes (all except *Mycobacterium dioxanotrophicus* PH-06 *prmA*) associated with 1,4-dioxane were found in the soil and sediment metagenomes. Reads aligning to

Rhodococcus jostii RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA* illustrated the highest relative abundance values and were present in all eighteen metagenomes. The third most abundant was *Burkholderia cepacia* G4 *tomA3* and this was also present in all eighteen metagenomes. The contaminated site sample generated the same trend for the three most abundant genes and the values were greater compared to those in the soil metagenomes. The work provides valuable and novel insights into the identity of the multiple genera and functional genes associated with 1,4-dioxane in mixed communities.

Introduction

1,4-dioxane, a probable human carcinogen (DeRosa et al., 1996), was commonly used as a stabilizer in 1,1,1-trichloroethane formulations and is now frequently detected at sites where the chlorinated solvents are present (Adamson et al., 2015; Adamson et al., 2014; ATSDR, 2012; Mohr et al., 2010). For example, 1,4-dioxane was found at 195 sites in California with 95% containing one or more of the chlorinated solvents (Adamson et al., 2014). No federal maximum contaminant level for 1,4-dioxane in drinking water has been established (EPA, 2017), however, several states have set low advisory action levels (e.g. California, Florida, Michigan and North Carolina have levels <5 ppb). A major challenge to 1,4-dioxane remediation concerns chemical characteristics that result in migration and persistence (Adamson et al., 2015; Mohr et al., 2010). A low organic carbon partition coefficient ($\log K_{oc} = 1.23$) and Henry's Law Constant (5×10^{-6} atm. $\text{m}^3\text{mol}^{-1}$), make traditional remediation methods such as air stripping or activated carbon largely ineffective (Mahendra and Alvarez-Cohen, 2006; Steffan et al., 2007; Zenker et al., 2003). *Ex situ* oxidation methods including ozone and hydrogen peroxide (Adams et al., 1994) or hydrogen peroxide and ultraviolet light (Stefan and Bolton, 1998) have been commercially applied, however these can be expensive at high concentrations (Steffan et al., 2007).

Given the limitations associated with traditional remediation methods, interest has turned to bioremediation to address 1,4-dioxane contamination. An increasingly popular approach involves biostimulation with propane to facilitate aerobic cometabolic degradation. A successful example of this included propane biosparging and bioaugmentation with the propanotroph *Rhodococcus ruber* ENV425 to promote *in situ* 1,4-dioxane biodegradation at Vandenberg Air Force Base, Ca (Lippincott et al., 2015). More recently, *in situ* biostimulation of native

microorganisms was demonstrated for the concurrent degradation of 1,4-dioxane, trichloroethene and 1,2-dichloroethene through intermittent, alternating exposure to propane and oxygen (Chu et al., 2018). Given the flexibility in co-substrate use for cometabolic 1,4-dioxane degradation (Burbach and Perry, 1993; Hand et al., 2015; Kohlweyer et al., 2000; Mahendra and Alvarez-Cohen, 2006; Vainberg et al., 2006) it is likely that other substrates will also be explored for *in situ* 1,4-dioxane remediation.

Numerous bacteria have been associated with the (co-metabolic or metabolic) biodegradation of 1,4-dioxane. Those linked to the metabolic biodegradation classify within the genera *Pseudonocardia* [*P. dioxanivorans* CB1190 (Mahendra and Alvarez-Cohen, 2005; Parales et al., 1994), *P. benzenivorans* B5 (Kampfer and Kroppenstedt, 2004; Mahendra and Alvarez-Cohen, 2006), *P. carboxydvorans* RM-31 (Matsui et al., 2016), *P. sp.* D17 (Sei et al., 2013a), *P. sp.* N23 (Yamamoto et al., 2018)], *Mycobacterium* [*M. sp.* PH-06 (Kim et al., 2009), *sp.* D6 and *sp.* D11 (Sei et al., 2013a)], *Afipia* [*A. sp.* D1 (Isaka et al., 2016; Sei et al., 2013a)], *Xanthobacter* [*X. flavus* DT8 (Chen et al., 2016)], *Acinetobacter* [*A. baumannii* DD1 (Huang et al., 2014)], *Rhodococcus* [*R. rubber* 219 (Bernhardt and Diekmann, 1991), *R. aetherivorans* JCM 14343 (Inoue et al., 2016; Inoue et al., 2018)] and *Rhodanobacter* [*R. sp.* AYS5 (Pugazhendi et al., 2015)].

Three of the above genera also contain isolates capable of co-metabolic 1,4-dioxane biodegradation; specifically, *Pseudonocardia* [*P. sp.* strain ENV478 (Vainberg et al., 2006), *P. sp.* K1 (Kohlweyer et al., 2000; Mahendra and Alvarez-Cohen, 2006)], *Mycobacterium* [*M. vaccae* JOB5 (Lan et al., 2013), *M. sp.* ENV421 (Masuda, 2009)] and *Rhodococcus* [*R. ruber* T1, T5, ENV425 (Lippincott et al., 2015; Sei et al., 2013b; Steffan et al., 1997), *R. jostii* RHA1 (Hand et al., 2015), *R. sp.* RR1 (Mahendra and Alvarez-Cohen, 2006; Stringfellow and Alvarez-Cohen, 1999)]. Additional microorganisms capable of co-metabolic 1,4-dioxane biodegradation include *Flavobacterium* (Sun et al., 2011), *Burkholderia cepacia* G4 (Mahendra and Alvarez-Cohen, 2006), *Nocardia sp.* ENV425 (Masuda, 2009), *Ralstonia pickettii* PK01 (Mahendra and Alvarez-Cohen, 2006), *Pseudomonas mendocina* KR1 (Mahendra and Alvarez-Cohen, 2006), *Methylosinus trichosporium* OB3b (Mahendra and Alvarez-Cohen, 2006; Whittenbury et al., 1970) and *Azoarcus sp.* DD4 (Deng et al., 2018b). Co-metabolic 1,4-dioxane degradation has

previously been reported with growth supporting substrates such as tetrahydrofuran, methane, propane, toluene, ethanol, sucrose, lactate, yeast extract and 2-propanol (Burbach and Perry, 1993; Hand et al., 2015; Kohlweyer et al., 2000; Mahendra and Alvarez-Cohen, 2006; Vainberg et al., 2006).

The initiation of 1,4-dioxane biodegradation has been associated with various groups of soluble di-iron monooxygenases (SDIMOs) (He et al., 2017), including *Burkholderia cepacia* G4 *tomA3* (Group 1) (Mahendra and Alvarez-Cohen, 2006; Newman and Wackett, 1995), *Pseudomonas pickettii* PKO1 *tbuA1* (Group 2) (Fishman et al., 2004; Mahendra and Alvarez-Cohen, 2006), *Pseudomonas mendocina* KR1 *tmoA* (Group 2) (Mahendra and Alvarez-Cohen, 2006; Yen et al., 1991), *Methylosinus trichosporium* OB3b *mmoX* (Group 3) (Mahendra and Alvarez-Cohen, 2006; Oldenhuis et al., 1989), *Pseudonocardia dioxanivorans* CB1190 *prmA* (Group 5) (Parales et al., 1994; Sales et al., 2013a; Sales et al., 2011), *Pseudonocardia tetrahydrofuranooxydans* K1 *thmA* (Group 5) (Kampfer et al., 2006; Thiemer et al., 2003), *Pseudonocardia* sp. strain ENV478 *thmA* (Group 5) (Masuda et al., 2012), *Rhodococcus* sp. strain YYL *thmA* (Group 5) (Yao et al., 2009), *Rhodococcus jostii* RHA1 *prmA* (Group 5) (Hand et al., 2015; Sharp et al., 2007), *Rhodococcus* sp. RR1 *prmA* (Group 5) (Sharp et al., 2007), *Mycobacterium* sp. ENV421 *prmA* (Group 6) (Masuda, 2009) and *Mycobacterium dioxanotrophicus* PH-06 *prmA* (Group 6) (He et al., 2017).

As the success of natural attenuation or biostimulation often depends on the population of native degraders present at the contaminated site, several studies have developed methods targeting these functional genes (Gedalanga et al., 2014; He et al., 2018; Li et al., 2013a; Li et al., 2013b). For example, methods have been developed for the functional genes associated with *Pseudonocardia* and *Mycobacterium* (Deng et al., 2018a; Gedalanga et al., 2014; He et al., 2017). Another study focused specifically on detecting functional genes of four 1,4-dioxane degraders (*Pseudonocardia dioxanivorans* CB1190, *Pseudonocardia* sp. strain ENV478, *Pseudonocardia tetrahydrofuranooxydans* K1, *Rhodococcus* sp. strain YYL). A larger number of functional genes were investigated with microarray-based technology (GeoChip 4.0) and denaturing gradient gel electrophoresis (Li et al., 2013b). More recently, high throughput shotgun sequencing was used to evaluate the presence of the functional genes listed above in

impacted and non-impacted groundwater (Dang et al., 2018). This approach has the added advantage of enabling taxonomic as well as functional analysis of microbial communities. The current study adopted a similar approach to examine the microbial communities involved in 1,4-dioxane degradation in contaminated and uncontaminated samples.

In the current study, the objectives were 1) to identify which 1,4-dioxane degrading functional genes are present across different microbial communities and 2) to determine which genera may be using 1,4-dioxane and/or metabolites to support growth. The research focused on laboratory microcosms inoculated with four uncontaminated soils and sediment samples from two 1,4-dioxane contaminated sites. The media selected for the experiments followed the approach used to enrich *Pseudonocardia dioxanivorans* CB1190 from industrial sludge (Parales et al., 1994). The work is novel as it combines taxonomic and functional data to generate a more complete picture of the multiple microorganisms and genes linked to 1,4-dioxane degradation in mixed communities.

Methods

Chemicals and Inocula

1,4-dioxane was purchased from Sigma-Aldrich (MO, USA) with 99.8% purity. All stock solutions and dilutions were prepared using DI water. The agricultural samples were collected from two locations on campus, East Lansing, Michigan (herein called soils F and G) and two locations at the Kellogg Biological Station, Hickory Corners, Michigan (soils 1 and 2). The contaminated site samples were obtained from California (contaminated with trichloroethene, 1,1-dichloroethene and 1,4-dioxane, herein called C7A) and Maine (contaminated with traces of 1,4-dioxane, herein called M10A). All samples were stored in the dark at 6 °C until use.

Experimental Setup, DNA Extraction, 1,4-Dioxane Analysis

Laboratory microcosms were established with soil or sediment (5g wet weight) and 25 mL of media in 30 mL serum bottles. For each of the six inocula (four uncontaminated soils or two contaminated sediments), the experiment design included triplicate sample microcosms, triplicate live control microcosms and triplicate abiotic control microcosms (autoclaved daily for

three consecutive days). The triplicate live control microcosms were treated in the same manner as the sample microcosms except no 1,4-dioxane was added. This treatment was included to enable comparisons to the microbial communities exposed to 1,4-dioxane. Following the approach used to enrich *Pseudonocardia dioxanivorans* CB1190 from industrial sludge, each liter of the final media contained 100 mL of a buffer stock [K_2HPO_4 (32.4 g/L), KH_2PO_4 (10 g/L), NH_4Cl (20 g/L)] and 100 mL of a trace metal stock [nitrilotriacetic acid (disodium salt) (1.23 g/L), $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ (2 g/L), $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$ (0.12 g/L), $\text{MnSO}_4 \cdot \text{H}_2\text{O}$ (0.03 g/L), $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$ (0.03 g/L) and $\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$ (0.01 g/L)] (Parales et al., 1994). The nitrilotriacetic acid (within the trace metal stock solution) represents an additional carbon source. The live sample microcosms were re-spiked with 1,4-dioxane two additional times.

A GC/MS with Agilent 5975 GC/single quadrupole MS (Agilent Technologies, CA, USA) equipped with a CTC Combi Pal autosampler was used to determine 1,4-dioxane concentrations. Sterile syringes (1 mL) and needles (22 Ga 1.5 in.) were used to collect samples (0.7 mL) from each microcosm. The samples were filtered (0.22 μm nylon filter) before being injected into an amber glass vial (40 mL) for GC/MS analysis. A method was developed to analyze 1,4-dioxane using solid phase micro extraction (SPME). The SPME fiber was inserted in the headspace of the vial and exposed to the analyte for 1 minute before being injected into the GC for thermal desorption. The fiber coating can adsorb the analytes in the vapor phase. Splitless injection was executed and the vials were maintained at 40 °C. The SPME fiber assembly involved 50/30 μm Divinylbenzene/ Carboxen/ Polydimethylsiloxane (DVB/CAR/PDMS) and 24 Ga needle for injection. The initial oven temperature was 35 °C and was programmed to increase at a rate of 20 °C/min to 120 °C. Once it reached 120 °C, it increased at a rate of 40 °C/min to 250 °C, which was maintained for 3 min. A VF5MS column was used with helium as the carrier gas in constant flow mode at a flow rate of 1 mL/min. The conditioning of the SPME fiber was at 270 °C for 60 min at the beginning of each sequence.

DNA was extracted from the soil inoculated sample microcosms and live control microcosms (1.2 mL and 0.4 g soil) using QIAGEN DNeasy PowerSoil kit as per the manufacturer's instructions. The QIAGEN DNeasy Powermax Soil kit was used to extract DNA from the microcosms inoculated with the two contaminated sediments. For this, the entire content of each

microcosm was sacrificed for DNA extraction. The DNA concentrations were determined using QUBIT dsDNA HS kit. The DNA extracts with the highest DNA yields were selected for shotgun sequencing.

Library Preparation, Sequencing, MG-RAST and DIAMOND analysis

Twenty-six samples were submitted for library generation and shotgun sequencing to the Research Technology Support Facility Genomics Core at Michigan State University (MSU). Libraries were prepared using the Takara SMARTer ThruPLEX DNA Library Preparation Kit following manufacturer's recommendations. Completed libraries were QC'd and quantified using a combination of Qubit dsDNA HS and Agilent 4200 TapeStation HS DNA1000 assays. Eight samples did not generate libraries of sufficient concentration for sequencing and were removed from further analysis. The remaining eighteen libraries were pooled in equimolar amounts for multiplexed sequencing. The pool was quantified using the Kapa Biosystems Illumina Library Quantification qPCR kit and loaded onto one lane of an Illumina HiSeq 4000 flow cell. Sequencing was performed in a 2x150 bp paired end format using HiSeq 4000 SBS reagents. Base calling was done by Illumina Real Time Analysis (RTA) v2.7.7 and output of RTA was demultiplexed and converted to FastQ format with Illumina Bcl2fastq v2.19.1.

The Meta Genome Rapid Annotation using Subsystem Technology (MG-RAST) (Meyer et al., 2008) version 4.0.2. was used for the taxonomic analysis of the metagenomes. The processing pipeline involved merging paired end reads, SolexaQA (Cox et al., 2010) to trim low-quality regions and dereplication to remove the artificial duplicate reads. Gene calling was performed using FragGeneScan (Rho et al., 2010). For the taxonomic profiles, the best hit classification at a maximum e-value of $1e^{-5}$, a minimum identity of 60% and a minimum alignment length of 15 against the RefSeq database (Pruitt et al., 2005) were used. MG-RAST ID numbers and sequencing data have been summarized (Table 2.1) and the datasets are publicly available on MG-RAST. The number of sequences generated post quality control per sample was 4.7 ± 2.0 million (ranging from ~1.2 to ~11 million) and the average length was 237.3 ± 2.9 bp (averages ranging from 233 to 243 bp).

The MG-RAST data files were downloaded and analyzed in Microsoft Excel 2016, STAMP (Statistical Analyses of Metagenomic Profiles, software version 2.1.3.) (Parks et al., 2014) and MEGAN6 (version 6.11.7) (Huson et al., 2016). STAMP was used to detect differences in the relative proportions of the taxonomic profiles between the live controls (no 1,4-dioxane) and the samples for each soil. This analysis included Welch's two sided t-test for two groups (samples and live controls) ($p < 0.05$) to generate extended error bar figures for each soil. The same Welch's test was performed to compare the profiles of all samples ($n = 9$) to all live controls ($n = 8$). MEGAN6 was used to generate two phylograms. One phylogram illustrates the eighteen metagenomes classified to the Class Level. The other phylogram represents the most common genera (ranked by average relative abundance, then selected if average values $> 0.5\%$) across all metagenomes.

The relative abundance of 1,4-dioxane degrading functional genes was determined using the alignment tool DIAMOND (double index alignment of next-generation sequencing data) (Buchfink et al., 2015). Specifically, reads aligning to the twelve genes previously associated with aerobic degradation (metabolic and co-metabolic) of 1,4-dioxane, as summarized previously (He et al., 2017), were determined. First, low quality sequences and Illumina adapters sequences were removed using Trimmomatic in the paired end mode (Bolger et al., 2014). The two paired output files were used for gene alignments in DIAMOND. Following alignment, the DIAMOND files were analyzed within Excel, which included combining the data from the two paired files and deleting duplicated data. The sort function was used to select reads that exhibited an identity of $\geq 60\%$ and an alignment length ≥ 49 amino acids. For each gene, the relative abundance values were calculated using the number of aligned reads divided by the total number of sequences for each sample (determined by Trimmomatic).

The above analysis indicated two functional genes (*Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. *prmA*) were dominant in the soil and sediment metagenomes. Therefore, BLASTP 2.9.0+ (Altschul et al., 1997) (protein-protein BLAST) from the NCBI website was used to search for similar protein sequences to these two genes. The sequences obtained ($> 94.8\%$ similar to the two query sequences) were used to create a phylogenetic tree in MEGAN7 using the Maximum Likelihood method (Jones et al., 1992; Kumar et al., 2016).

Results

Biodegradation of 1,4-Dioxane and General Microbial Community Composition

The concentration of 1,4-dioxane declined in all of the live microcosms (inoculated with all four uncontaminated soils and with two contaminated site sediments), but not in the abiotic controls, indicating biological removal (Figure 2.1). Decreases in 1,4-dioxane concentrations were observed after two additional amendments of 1,4-dioxane. However, only limited biodegradation (only one replicate decreased) was observed for soil F after the last amendment. Between approximately 220 and 245 days was required to reduce the majority of amended 1,4-dioxane.

Although DNA was extracted from all microcosms and live controls (no 1,4-dioxane), in some cases insufficient DNA was extracted (and was not submitted for library generation) or did not generate libraries of sufficient concentration for sequencing. Unfortunately, this included all DNA extracts for the microcosms inoculated with sediment from one of the two 1,4-dioxane contaminated sites (M10A three samples and three live controls). Also, only one sample from the other contaminated site (C7A) generated enough DNA for sequencing. Overall, eighteen libraries were sequenced, which included two samples and two live controls for each soil (except soil F which included three samples) and one sample from one contaminated site (C7A).

The phylogenetic analysis of the eighteen soil and sediment metagenomes indicated the majority of the microorganisms classified within the classes *Acidobacteria*, *Alpha-*, *Beta-*, *Gamma-*, *Deltaproteobacteria*, *Actinobacteria*, *Bacilli* and *Clostridia* (Supplementary Figure 2.1). The most abundant genera, averaged across all metagenomes, included *Candidatus Solibacter*, *Bradyrhizobium*, *Mesorhizobium*, *Burkholderia*, *Pseudomonas*, *Stenotrophomonas*, *Xanthomonas*, *Mycobacterium* and *Streptomyces* (Supplementary Figure 2.2). The relative abundance (%) of the most abundant genera (25 most abundant) for each soil analyzed separately is also shown (Supplementary Figure 2.3). The most abundant genera in all four soils were similar and included *Xanthomonas*, *Streptomyces*, *Mesorhizobium*, *Bradyrhizobium* and *Burkholderia*. In contrast, *Pseudomonas*, *Rhodococcus*, *Arthrobacter*, *Mycobacterium* and *Corynebacterium* were the most abundant genera in the contaminated site microcosms.

Genera Associated with 1,4-Dioxane Degradation

The metagenomes of the samples were compared to the live controls (no 1,4-dioxane) to determine which genera were positively influenced by 1,4-dioxane degradation. First, all of the samples ($n=9$) were compared to all of the live controls ($n=8$) (Figure 2.2). Overall, seventeen genera were statistically significantly enriched in the live samples compared to the controls. The greatest differences between the means were noted for *Mycobacterium* (0.307%, $p=0.0028$), followed by *Nocardioidea* (0.125%, $p=0.026$), and *Kribbella* (0.079%, $p=0.016$). The trends suggest these genera are obtaining a growth benefit from the presence of 1,4-dioxane. The relative abundance of these genera in the contaminated site microcosm is also shown (Figure 2.2B, inset). Except for *Ureaplasma*, the enriched genera all classify within the order *Actinomycetales* (Table 2.2).

The metagenomes of the samples and controls were also compared for each soil individually. One genus (*Hyphomicrobium*) and seven genera (*Arthrobacter*, *Nocardia*, *Gordonia*, *Kocuria*, *Brevibacterium*, *Rothia*, *Erysipelothrix*) were enriched in soils 1 and 2, respectively (Figure 2.3). Four genera (*Hyphomicrobium*, *Acetobacter*, *Veillonella*, *Natronomonas*) and no genera were enriched in soils F and G, respectively (Figure 2.4). Six of the eleven listed above classify within the *Actinomycetales* (Table 2.2). The differences between the means (for the individual soil analysis) were the highest ($\geq 0.049\%$) for *Arthrobacter*, *Hyphomicrobium* and *Nocardia* (Table 2.2). The relative abundance of the eleven enriched genera in the samples compared to the controls (for the individual soil analysis) and for the contaminated site sample is shown (Figure 2.5). Both *Arthrobacter* and *Nocardia* have relative abundance values of $>1\%$ in the contaminated site sample.

The metagenomes were also investigated to determine the relative abundance (%) of fifteen genera previously associated with metabolic or co-metabolic 1,4-dioxane degradation (Figure 2.6). All except *Pseudonocardia* and *Rhodanbacter* were present in the samples and controls. *Burkholderia*, *Mycobacterium*, *Pseudomonas* and *Rhodococcus* were present at the highest relative abundance levels (0.84-2.46%). Only *Mycobacterium* was statistically significantly ($p<0.05$) enriched in the samples compared to the live controls. *Pseudonocardia* and *Rhodanbacter* were also absent in the contaminated site sample (Figure 2.6, insert). In the

contaminated site metagenome, the four most abundant genera were *Pseudomonas* (49.0%), *Rhodococcus* (5.9%), *Mycobacterium* (3.9%) and *Nocardia* (1.2%).

Genes Associated with 1,4-Dioxane Degradation

The data produced by DIAMOND was used to determine the relative abundance (%) of the reads that aligned to known 1,4-dioxane degrading genes (Figure 2.7). No reads in any metagenome aligned with *Mycobacterium dioxanotrophicus* PH-06 *prmA*. Only six and eleven metagenomes contained reads aligning with *Methylosinus trichosporium* OB3b *touA* and *Pseudomonas pickettii* PKO1 *tbuA1*, respectively. Six functional genes (*Pseudomonas mendocina* KR1 *tmoA*, *Rhodococcus* sp. YYL *thmA*, *Pseudonocardia* sp. ENV478 *thmA*, *Mycobacterium* sp. ENV421 *prmA*, *Pseudonocardia tetrahydrofuranoxydans* *thmA*, *Pseudonocardia dioxanivorans* CB1190 *thmA*) were present in between fourteen and sixteen metagenomes. Reads aligning to *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA* illustrated the highest relative abundance values and were present in all eighteen metagenomes. The third most abundant was *Burkholderia cepacia* G4 *tomA3* and this was also present in all eighteen metagenomes. All four soils generated similar trends for the functional genes and no statistically significant differences were noted between the live controls and samples. The contaminated site sample generated the same trend for the three most abundant genes and the values were greater compared to those in the soil metagenomes (Figure 2.7A).

Following the discovery of the dominance of *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA* in the soil metagenomes, a BLASTP search was performed to find the closest matching sequences in the NCBI database. The matching protein sequences, with number of microorganisms shown in parenthesis, belonged to the genera *Rhodococcus* (60), *Kribbella* (16), *Gordonia* (10), *Mycolicibacterium* (10), *Mycobacterium* (8), *Nocardia* (7), *Nocardioides* (6), *Hoyosella* (3), *Intrasporangium* (2), *Millisia* (1), *Cryptosporangium* (1) and *Acidobacteria* (1). Interestingly, five of these genera (*Mycobacterium*, *Nocardioides*, *Kribbella*, *Gordonia* and *Nocardia*) were enriched in the samples compared to the live controls (as discussed above, Table 2.2). A phylogenetic tree was generated to illustrate the evolutionary relationships between the two query sequences and the enriched genera (Figure 2.8). *Rhodococcus jostii* RHA1 *prmA*

clustered closest to *Nocardia* sequences and *Rhodococcus* sp. RR1 *prmA* clustered closest to *Kribbella* sequences.

Discussion

An objective of the current work was to determine if any microorganism could obtain a growth benefit from 1,4-dioxane degradation. For this, the sample microcosms were supplied with media and 1,4-dioxane and the live control microcosms were supplied with the same media, but no 1,4-dioxane. Consequently, an increase in the relative abundance of any microorganism between the samples and live controls could be attributed to the presence of 1,4-dioxane. From this, a reasonable hypothesis would be that the enriched microorganisms are being exposed to growth supporting substrates from 1,4-dioxane degradation. Here, seventeen genera increased in abundance following 1,4-dioxane degradation compared to the live controls (no 1,4-dioxane). The three most enriched across all four soils were *Mycobacterium*, *Nocardioides*, *Kribbella* (all classifying as *Actinomycetales*). There was also a higher level of enrichment for *Arthrobacter*, *Nocardia* and *Gordonia* (also *Actinomycetales*) in one soil and *Hyphomicrobium* (*Rhizobiales*) in two soils.

There are at least two hypotheses on why these genera increased in abundance in 1,4-dioxane amended samples compared to the live controls. One hypothesis being that these microorganisms are obtaining a growth benefit from consuming 1,4-dioxane biodegradation products. Several studies have examined 1,4-dioxane biodegradation pathways (Grostern et al., 2012; Huang et al., 2014; Kim et al., 2009; Mahendra et al., 2007; Sales et al., 2013b; Vainberg et al., 2006). A study with *Pseudonocardia dioxanivorans* CB1190 provided evidence that carbon from 1,4-dioxane enters central metabolism via glyoxlate (Grostern et al., 2012). In contrast, *Pseudonocardia* sp. strain ENV478 produces 2-hydroxyethoxyacetic acid (HEAA) as a terminal product of 1,4-dioxane biodegradation (Vainberg et al., 2006). Conversely, 1,4-dioxane biodegradation by *Pseudonocardia dioxanivorans* CB1190 (metabolic 1,4-dioxane degrader), *Mycobacterium vaccae* JOB5, *Pseudomonas mendocina* KR1, *Pseudonocardia tetrahydrofuranoxydans* K1 (co-metabolic 1,4-dioxane degraders) produced HEAA transiently, but the chemical did not accumulate. They identified ethylene glycol, glycolic acid, glyoxylic acid and oxalic acid as 1,4-dioxane biodegradation intermediates by these isolates (Mahendra et al., 2007). Others have also identified ethylene glycol (Huang et al., 2014; Kim et al., 2009) and

oxalic acid during 1,4-dioxane degradation (Huang et al., 2014). The enriched genera may have benefited from funneling these degradation intermediates into central metabolism.

A second hypothesis being that the enriched genera are responsible for both the initial attack on 1,4-dioxane and for the consumption of degradation products. Evidence for this concerns the similarity of genes belonging to the enriched genera (*Mycobacterium*, *Nocardioides*, *Kribbella*, *Nocardia* and *Gordonia*) to *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA* (as shown in the phylogenetic tree). Although *Rhodococcus jostii* RHA1 and *Rhodococcus* sp. RR1 co-metabolically degrade 1,4-dioxane, the enriched genera may also contain genes downstream in the pathway enabling growth on 1,4-dioxane. *Arthrobacter* did not contain genes similar to the *Rhodococcus* strains, although others have reported that *Arthrobacter* (ATCC 27779) can co-metabolically degrade 1,4-dioxane (Chu et al., 2009). *Arthrobacter*, *Mycobacterium* and *Nocardia* have previously been linked to 1,4-dioxane degradation (Chu et al., 2009; Lan et al., 2013; Masuda, 2009), whereas *Nocardioides*, *Gordonia* and *Kribbella* are potentially novel degraders. Similar to the current work, 1,4-dioxane degrading consortia derived from uncontaminated soil were enriched in *Mycobacterium* (He et al., 2018). Overall, both hypotheses suggest that many genera (almost all classifying with the *Actinomycetales*) are likely involved in the degradation of 1,4-dioxane and/or 1,4-dioxane metabolites in the soil microcosms studied.

In the current study, reads from all of the 1,4-dioxane degrading function genes, except *Mycobacterium dioxanotrophicus* PH-06 *prmA*, were observed in soil metagenomes. Others have detected this gene in 1,4-dioxane enrichments from soil and activated sludge using quantitative PCR (He et al., 2017). This suggests either the occurrence of this gene is not uniform across all soils, or the method employed in the current study was not sensitive enough to detect it. Another functional gene study noted a correlation between *dxmA/thmA* (designed based on *Rhodococcus* sp. YYL *thmA*, *Pseudonocardia* sp. ENV478 *thmA*, *Pseudonocardia tetrahydrofuranoxydans* K1 *thmA* and *Pseudonocardia dioxanivorans* CB1190 *thmA*) and the amount of 1,4-dioxane degraded in groundwater inoculated microcosms (Li et al., 2013a). These genes were also present in the soil metagenomes (between fourteen and sixteen) of the current study.

Consistent with the current study, others have detected SDIMOs from the majority (five from six groups) of SDIMO groups (Li et al., 2013b). In that research, the authors examined Arctic groundwater impacted by 1,4-dioxane using high-throughput microarrays and denaturing gradient gel electrophoresis and found an enrichment of *thmA*-like genes near the source zone (Li et al., 2013b). Also similar to the current work, a 1,4-dioxane degrading consortia contained a high percentage of group five SDIMOs (*Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA* are group five SDIMOs), although the specific genes were not determined (He et al., 2018).

Recently, shotgun sequencing was used to examine 1,4-dioxane degrading genes in groundwater from multiple chlorinated solvent sites (previously bioaugmented with SDC-9) (Dang et al., 2018). From the twelve genes examined, only six were found in the groundwater metagenomes. The six included the three most abundant genes in the current study; *Rhodococcus* sp. RR1 *prmA*, *Rhodococcus jostii* RHA1 *prmA* and *Burkholderia cepacia* G4 *tomA3*. From these, the *Rhodococcus* genes were both found in a only small number of metagenomes (~18%) and *B. cepacia* G4 *tomA3* was found in the majority (~68%). The occurrence of the three genes in both studies could suggest their importance across different environments (soil vs. groundwater, aerobic vs. oxygen depleted). Unlike the current study, the groundwater metagenomes contained high relative abundance values for *Methylosinus trichosporium* OB3b *touA* (up to 0.0031%) followed by *Pseudomonas mendocina* KR1 *tmoA* (up to 0.00022%) and *Pseudomonas pickettii* PKO1 *tbuA1* (up to 0.0013%). The different results between the two studies are likely due to variations in the conditions (redox potential, carbon availability, nutrient availability, soil vs. groundwater) from which the samples were obtained.

In summary, several key findings highly relevant for 1,4-dioxane bioremediation were generated in the current study. Under aerobic conditions, 1,4-dioxane degradation was observed with all inocula types, indicating 1,4-dioxane degraders are widespread. Further, a large number of genera were enriched following 1,4-dioxane degradation. Shotgun sequencing enabled both taxonomic and functional analyses to be performed on multiple mixed microbial communities. Multiple genera classifying (including novel and previously identified degraders) within the *Actinomycetales* were enriched during 1,4-dioxane degradation and may be associated with

growth linked 1,4-dioxane degradation. The three most enriched were *Mycobacterium*, *Nocardioides*, *Kribbella* (classifying as *Actinomycetales*). There was also a higher level of enrichment for *Arthrobacter*, *Nocardia* and *Gordonia* (also *Actinomycetales*) in one soil and *Hyphomicrobium* (*Rhizobiales*) in two soils. Although *Arthrobacter*, *Mycobacterium* and *Nocardia* have previously been linked to 1,4-dioxane degradation, *Nocardioides*, *Gordonia* and *Kribbella* are potentially novel degraders. However, it is unknown if these microorganisms are benefiting from the complete degradation of the chemical or from the consumption of 1,4-dioxane degradation products, such as HEAA, ethylene glycol, glycolic acid, glyoxylic acid or oxalic acid. Finally, the majority of the functional genes (all except *Mycobacterium dioxanotrophicus* PH-06 *prmA*) associated with 1,4-dioxane were found in the soil and sediment metagenomes. Reads aligning to *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. RR1 *prmA* illustrated the highest relative abundance values and were present in all eighteen metagenomes. Future research should be directed towards similar molecular analyses of groundwater and sediment samples from 1,4-dioxane contaminated sites as well as comparisons to 1,4-dioxane removal rates for propane amended samples.

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Table 2.1. Summary of sequencing information processed by MG-RAST.

ID	Name	Upload: bp Count	Upload: Sequences Count	Artificial Duplicate Reads: Sequence Count	Post QC: bp Count	Post QC: Sequences Count	Post QC: Mean Sequence Length bp
mgm4846244.3	C7A 6 S10 L001 R	928,461,523	3,828,893	730,886	745,308,161	3,067,261	243 ± 33
mgm4842040.3	SF 2 S3 L001 R	1,249,466,781	5,278,200	741,761	1,057,029,663	4,469,282	237 ± 36
mgm4846245.3	SF 3 S6 L001 R	1,497,947,613	6,267,333	1,045,636	1,227,596,223	5,142,267	239 ± 35
mgm4846246.3	SF 4 S8 L001 R	698,047,178	2,986,100	484,606	572,423,699	2,454,542	233 ± 36
mgm4846247.3	SF 5 S11 L001 R	1,455,181,423	6,244,496	875,408	1,226,035,914	5,268,011	233 ± 37
mgm4846248.3	SF 6 S13 L001 R	1,276,229,786	5,446,704	791,730	1,069,530,685	4,570,187	234 ± 36
mgm4846291.3	SG 1 S15 L001 R	362,019,482	1,517,474	232,203	300,578,338	1,261,641	238 ± 35
mgm4841972.3	SG 3 S17 L001 R	1,237,320,023	5,151,224	739,217	1,042,837,451	4,346,084	240 ± 34
mgm4841973.3	SG 4 S1 L001 R	1,183,825,731	4,906,054	779,630	975,862,599	4,048,734	241 ± 34
mgm4842102.3	SG 6 S4 L001 R	1,097,936,207	4,598,102	612,987	933,115,160	3,911,767	239 ± 35
mgm4841974.3	ST1 2 S7 L001 R	1,722,580,822	7,248,808	1,205,707	1,409,132,207	5,932,625	238 ± 35
mgm4846290.3	ST1 3 S9 L001 R	687,193,817	2,855,664	394,713	580,636,546	2,415,127	240 ± 35
mgm4842023.3	ST1 4 S12 L001 R	1,720,695,830	7,269,781	1,124,424	1,433,388,134	6,053,609	237 ± 35
mgm4842024.3	ST1 6 S14 L001 R	1,165,586,999	4,879,142	683,336	983,135,037	4,119,069	239 ± 35
mgm4842104.3	ST2 1 S16 L001 R	3,144,582,833	13,429,024	2,172,387	2,579,894,248	11,026,268	234 ± 36
mgm4842103.3	ST2 3 S18 L001 R	1,274,658,549	5,369,262	727,123	1,080,887,053	4,557,307	237 ± 35
mgm4842107.3	ST2 5 S2 L001 R	1,622,482,462	6,854,685	982,861	1,362,403,381	5,762,840	236 ± 35
mgm4842106.3	ST2 6 S5 L001 R	1,463,433,476	6,249,417	846,173	1,252,082,629	5,349,932	234 ± 36

QC – Quality Control

Table 2.2. Classification of genera statistically significantly enriched ($p < 0.05$) in the samples compared to the controls (no 1,4-dioxane) following the degradation of 1,4-dioxane in all soils collectively and when the soils were analyzed individually. The last column also illustrates the difference in means between the controls and the samples for each genera. Genera in bold were identified in the BLASTP search as containing genes similar to *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. *prmA* (as discussed in the functional gene analysis results).

Phylum	Class	Order	Family	Genus	Difference in Means (%)
All Soils: All samples (n=9) compared to all controls (n=8)					
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Mycobacteriaceae</i>	<i>Mycobacterium</i>	0.307
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Nocardiodiaceae</i>	<i>Nocardioideis</i>	0.125
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Nocardiodiaceae</i>	<i>Kribbella</i>	0.079
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Pseudonocardiaceae</i>	<i>Amycolatopsis</i>	0.042
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Cellulomonadaceae</i>	<i>Cellulomonas</i>	0.035
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Actinosynnemataceae</i>	<i>Actinosynnema</i>	0.028
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Beutenbergiaceae</i>	<i>Beutenbergia</i>	0.025
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Sanguibacteraceae</i>	<i>Sanguibacter</i>	0.024
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Pseudonocardiaceae</i>	<i>Saccharopolyspora</i>	0.019
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Promicromonosporaceae</i>	<i>Xylanimonas</i>	0.018
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Glycomycetaceae</i>	<i>Stacebrandtia</i>	0.016
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Gordoniaceae</i>	<i>Gordonia</i>	0.014
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Tsukamurellaceae</i>	<i>Tsukamurella</i>	0.008
<i>Tenericutes</i>	<i>Mollicutes</i>	<i>Mycoplasmatales</i>	<i>Mycoplasmataceae</i>	<i>Ureaplasma</i>	0.0002
Soil 1: Samples (n=3) compared to controls (n=2)					
<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Hyphomicrobiaceae</i>	<i>Hyphomicrobium</i>	0.075
Soil 2: Samples (n=2) compared to controls (n=2)					
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Micrococcaceae</i>	<i>Arthrobacter</i>	0.276
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Nocardiaceae</i>	<i>Nocardia</i>	0.049
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Gordoniaceae</i>	<i>Gordonia</i>	0.019
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Micrococcaceae</i>	<i>Kocuria</i>	0.017
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Brevibacteriaceae</i>	<i>Brevibacterium</i>	0.015
<i>Actinobacteria</i>	<i>Actinobacteria</i>	<i>Actinomycetales</i>	<i>Micrococcaceae</i>	<i>Rothia</i>	0.006
<i>Firmicutes</i>	<i>Erysipelotrichi</i>	<i>Erysipelotrichales</i>	<i>Erysipelotrichaceae</i>	<i>Erysipelothrix</i>	0.0005
Soil F: Samples (n=2) compared to controls (n=2)					
<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhizobiales</i>	<i>Hyphomicrobiaceae</i>	<i>Hyphomicrobium</i>	0.034
<i>Proteobacteria</i>	<i>Alphaproteobacteria</i>	<i>Rhodospirillales</i>	<i>Acetobacteraceae</i>	<i>Acetobacter</i>	0.003
<i>Firmicutes</i>	<i>Negativicutes</i>	<i>Selenomonadales</i>	<i>Veillonellaceae</i>	<i>Veillonella</i>	0.003
<i>Euryarchaeota</i>	<i>Halobacteria</i>	<i>Halobacteriales</i>	<i>Halobacteriaceae</i>	<i>Natronomonas</i>	0.001
Soil G: Samples (n=2) compared to controls (n=2) – no enriched genera					

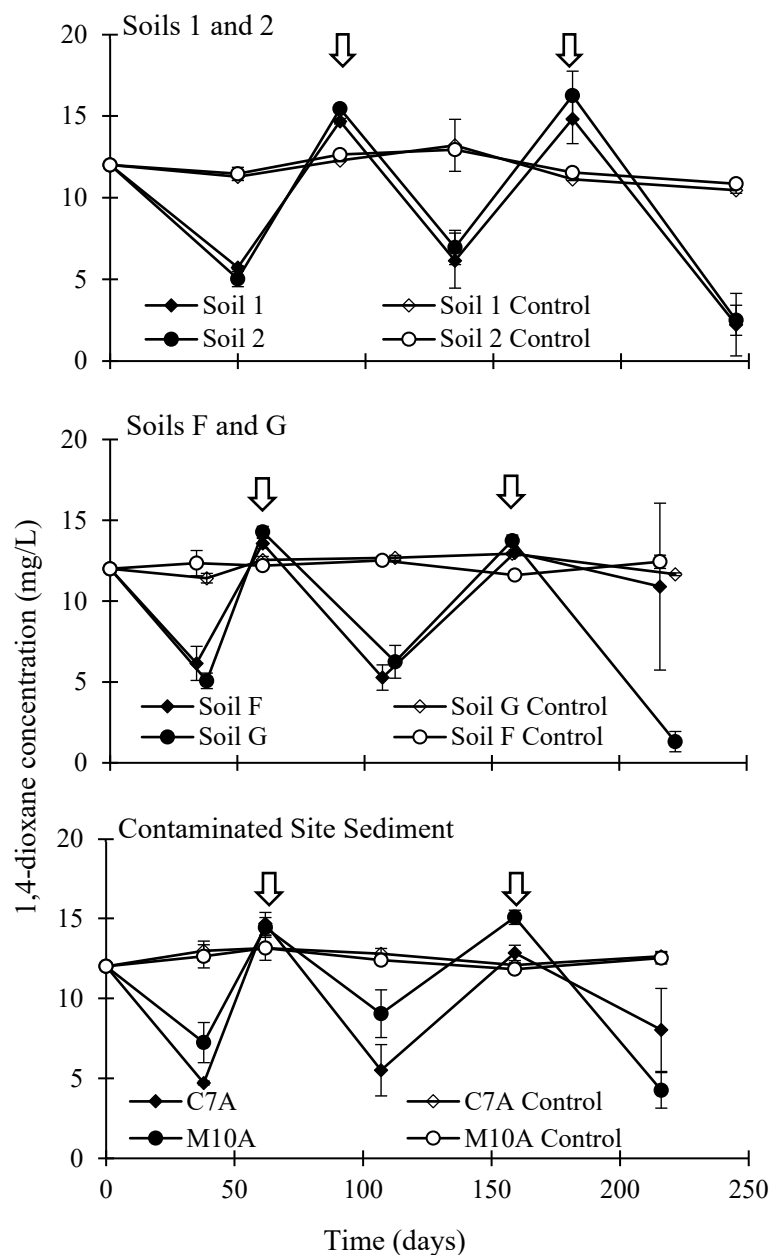


Figure 2.1. Average 1,4-dioxane concentrations (mg/L) in triplicate samples and abiotic controls with different inocula (bars represent standard deviations). 1,4-dioxane was reamended to the samples microcosms twice (arrows).

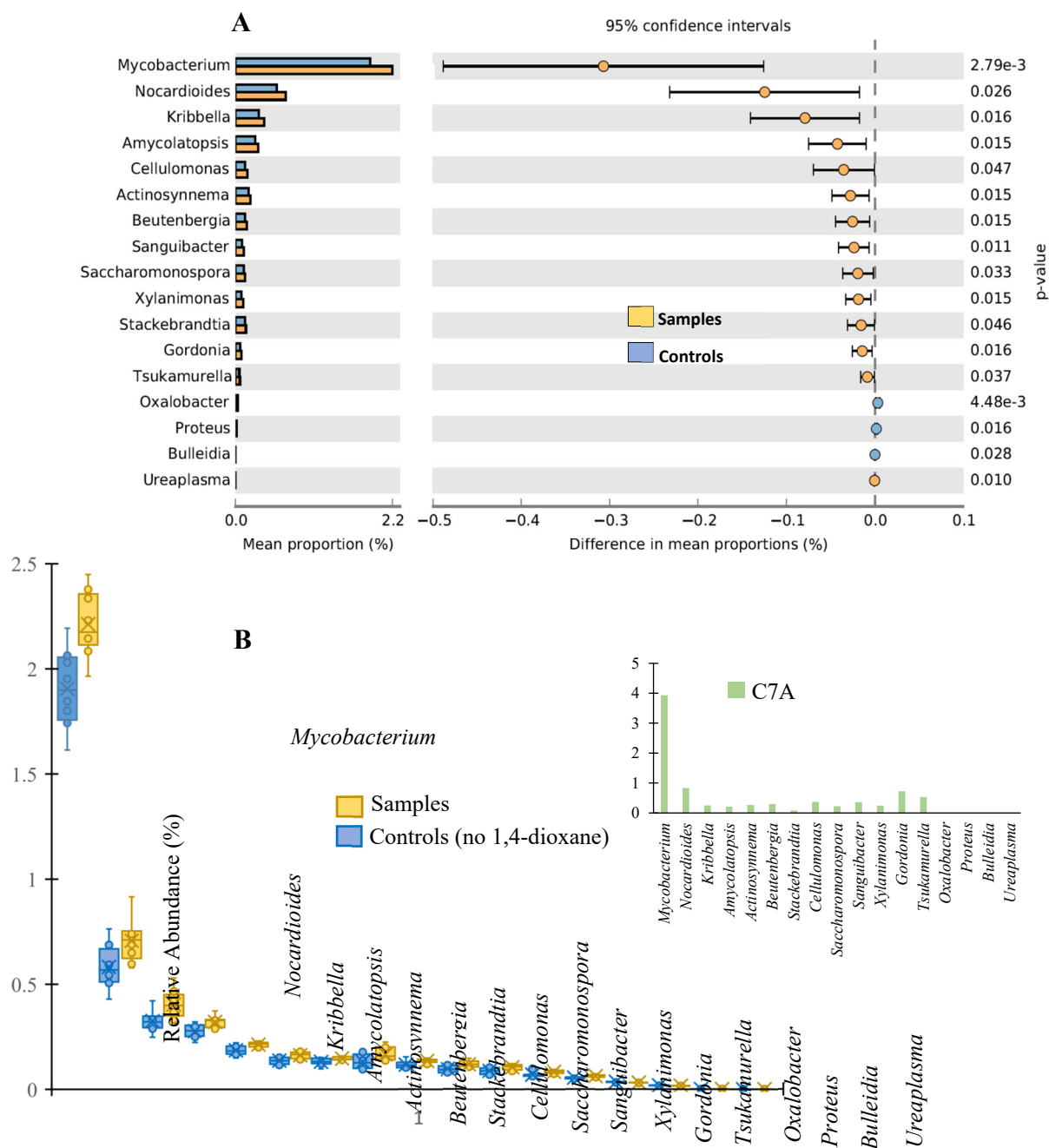


Figure 2.2. Extended error bar plot illustrating genera statistically significantly different in relative abundance (Welch's two sided t-test, $p < 0.05$) in the samples ($n=9$) compared to the live controls (no 1, 4-dioxane, $n=8$) following 1,4-dioxane degradation (A). The symbols to the left of the dashed line (yellow) indicate a higher relative abundance in the samples compared to the controls and the symbols to the right (blue) indicate the reverse. A comparison of the relative abundance values (%) for the genera enriched in the samples is also shown in a box plot format (B). The insert illustrates the relative abundance of these enriched genera in the contaminated site sample (C7A) with a different scale on the y-axis.

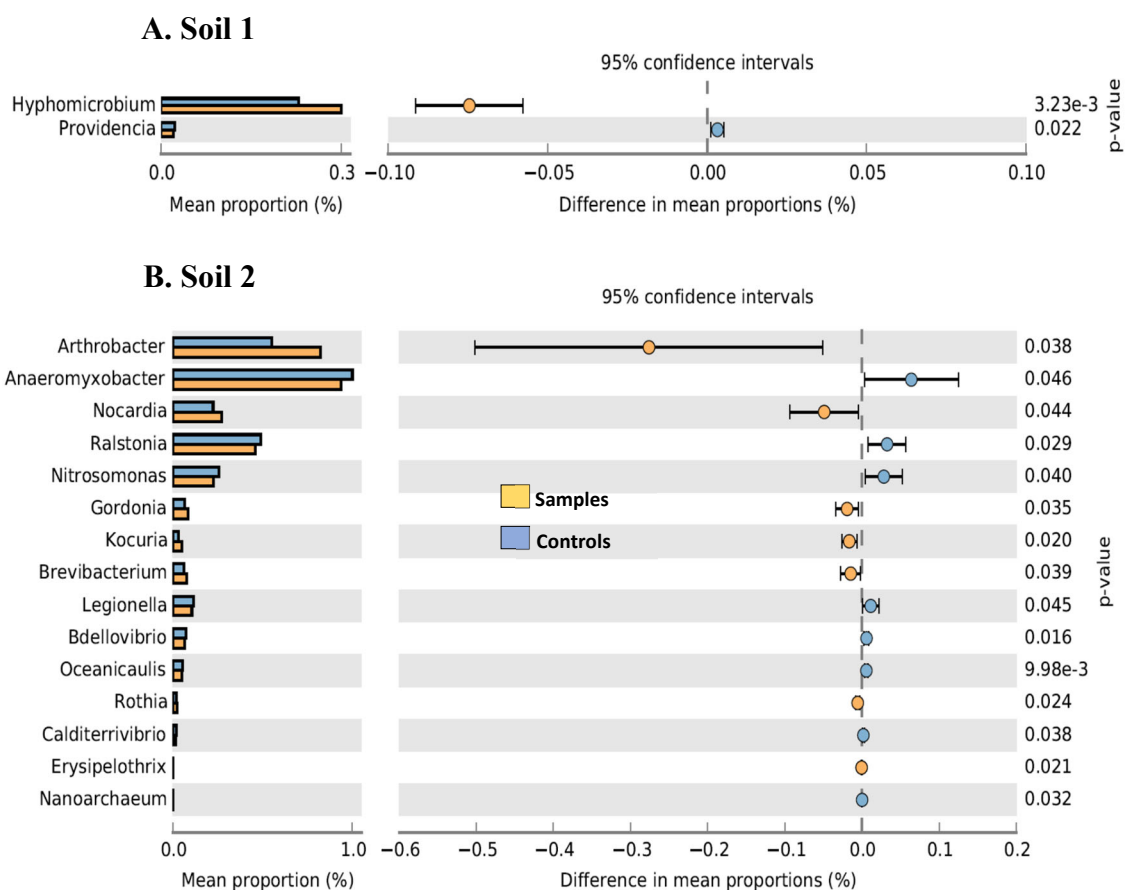


Figure 2.3. Extended error bar plot illustrating genera statistically significantly different in relative abundance (Welch's two sided t-test, $p < 0.05$) in the samples compared to the live controls following 1,4-dioxane degradation in soil 1 (A) and 2 (B). The symbols to the left of the dashed line (in yellow) indicate a higher relative abundance in the samples compared to the controls and the symbols to the right (in blue) indicate the reverse.

A. Soil F

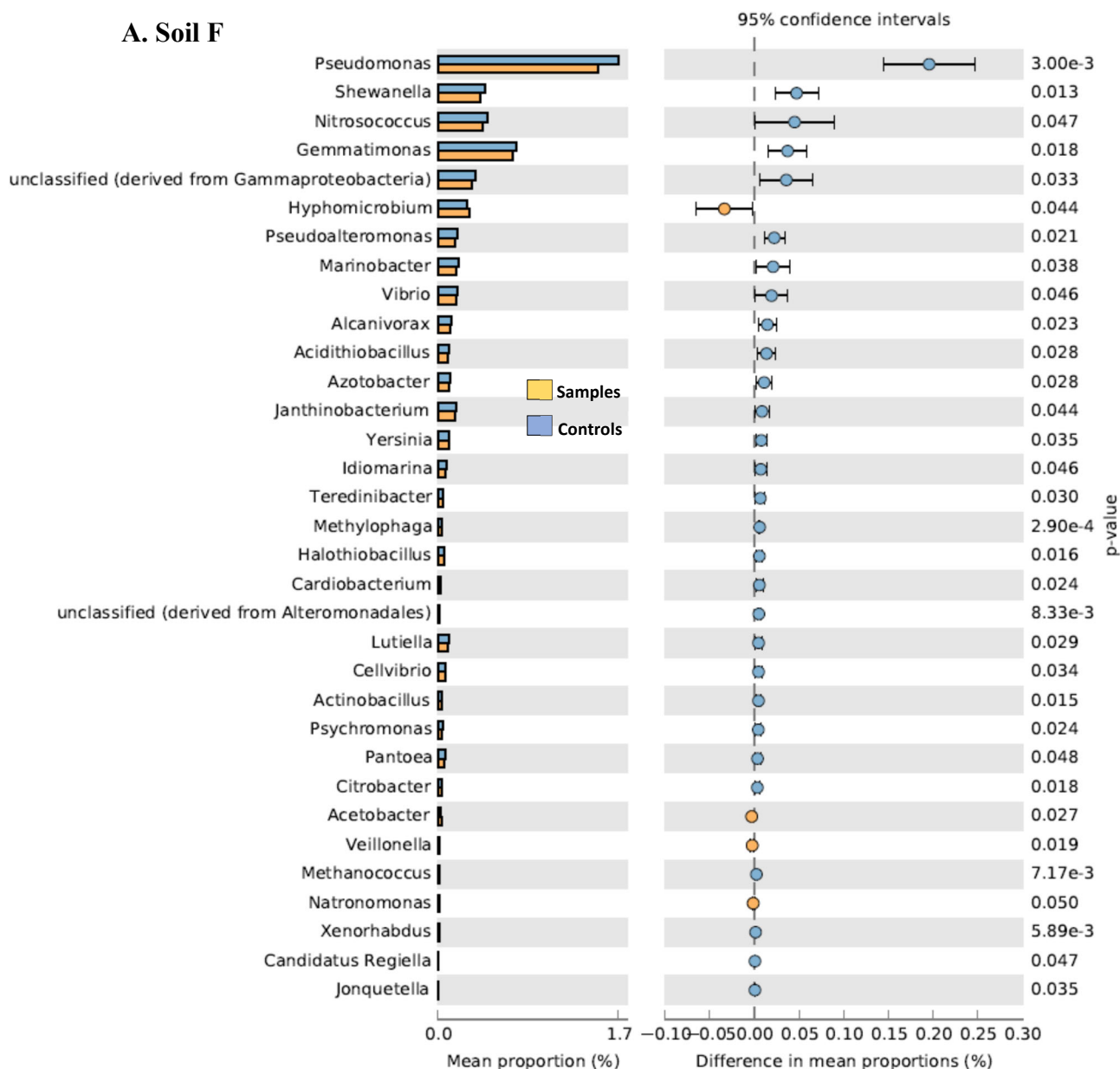
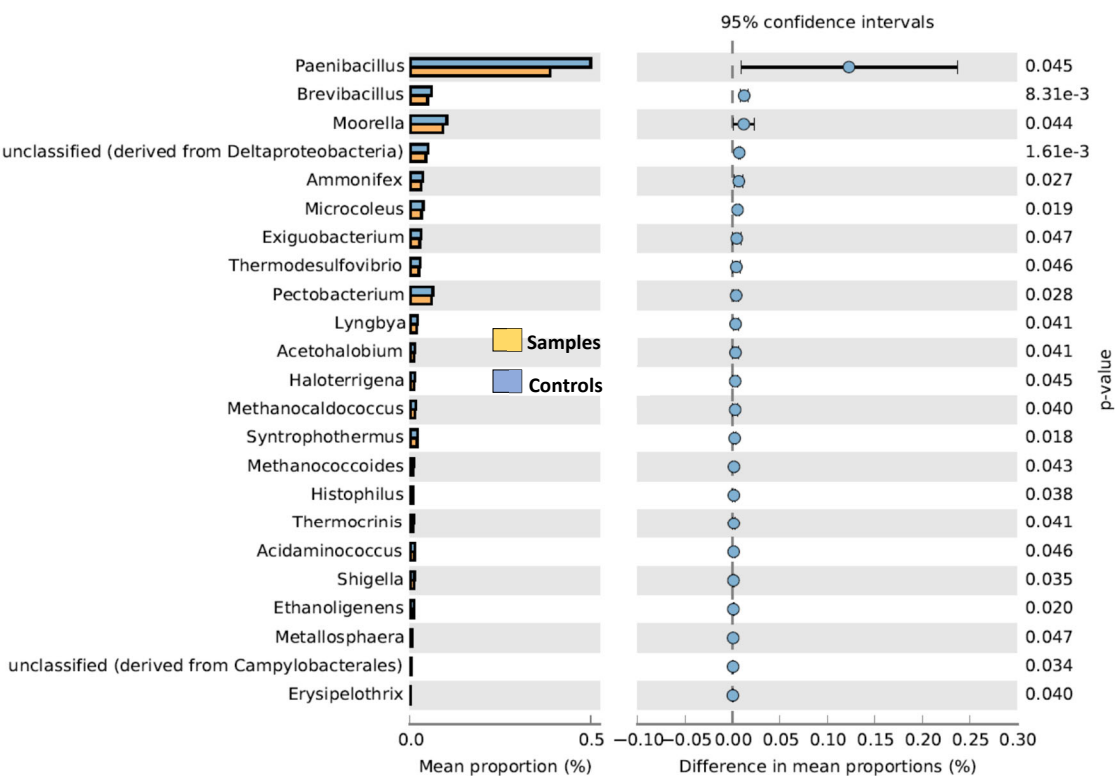


Figure 2.4. Extended error bar plot illustrating genera statistically significantly different in relative abundance (Welch's two sided t-test, $p < 0.05$) in the samples compared to the live controls following 1,4-dioxane degradation in soil F (A) and G (B). The symbols to the left of the dashed line (in yellow) indicate a higher relative abundance in the samples compared to the controls and the symbols to the right (in blue) indicate the reverse.

B. Soil G



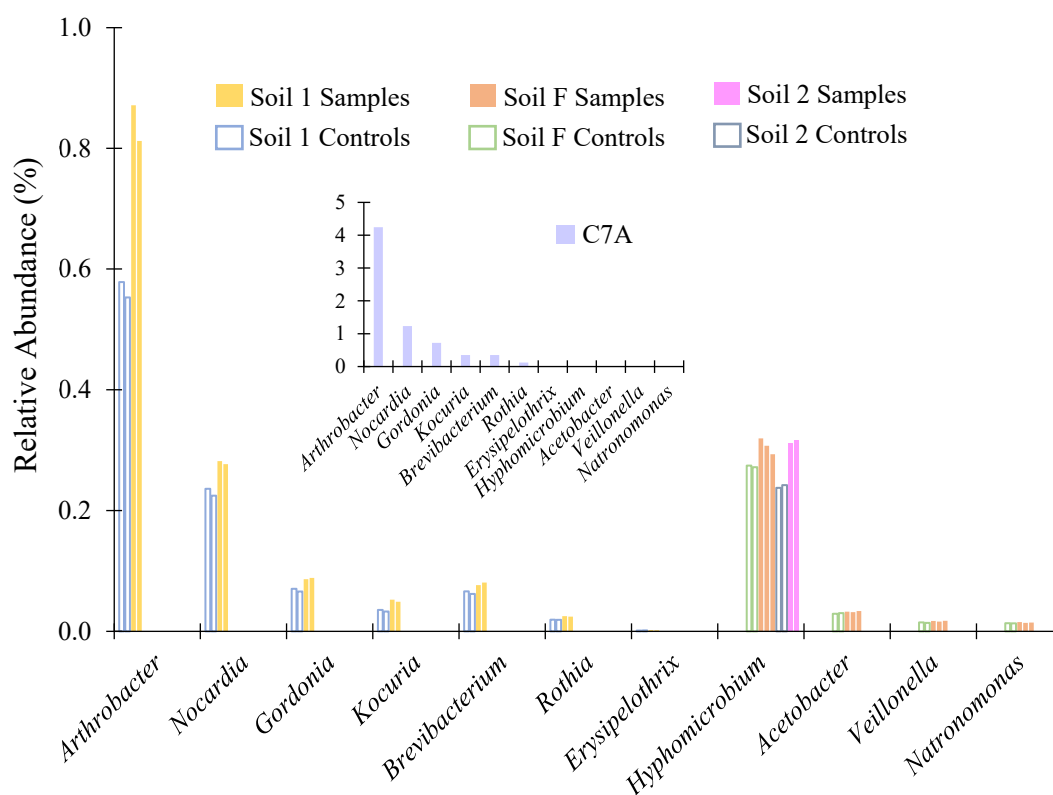


Figure 2.5. Summary of the relative abundance of statistically significantly enriched genera in the samples compared to the controls (no 1,4-dioxane) for soils 1, 2 and F (there were no enriched genera in soil G). The inset illustrates the relative abundance of these genera in the contaminated site sample (C7A) with a different scale on the y-axis.

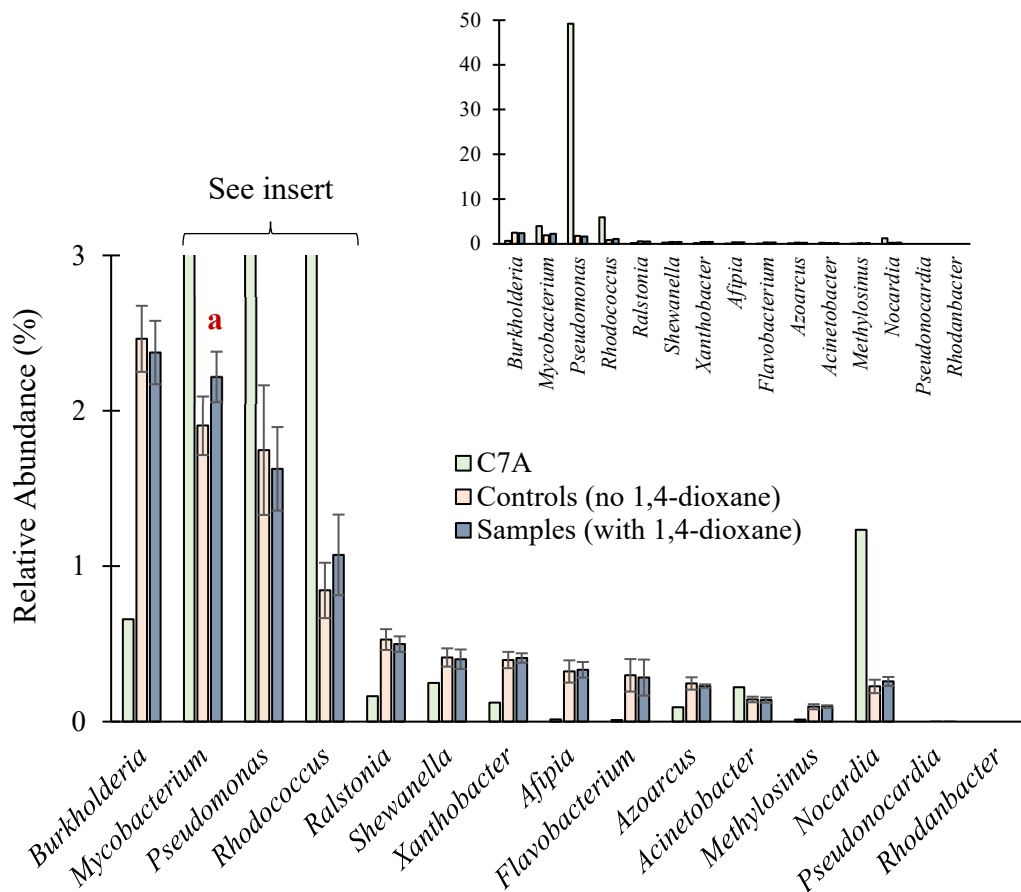


Figure 2.6. Relative abundance (%) of genera associated with metabolic and co-metabolic degradation of 1,4-dioxane in live controls ($n=8$) and samples ($n=9$) in four soils and one contaminated site sample (C7A). The value "a" indicates a significant difference ($p<0.05$) in a two tailed student's t-test between the samples and controls. The insert illustrates the same data with a different y-axis.

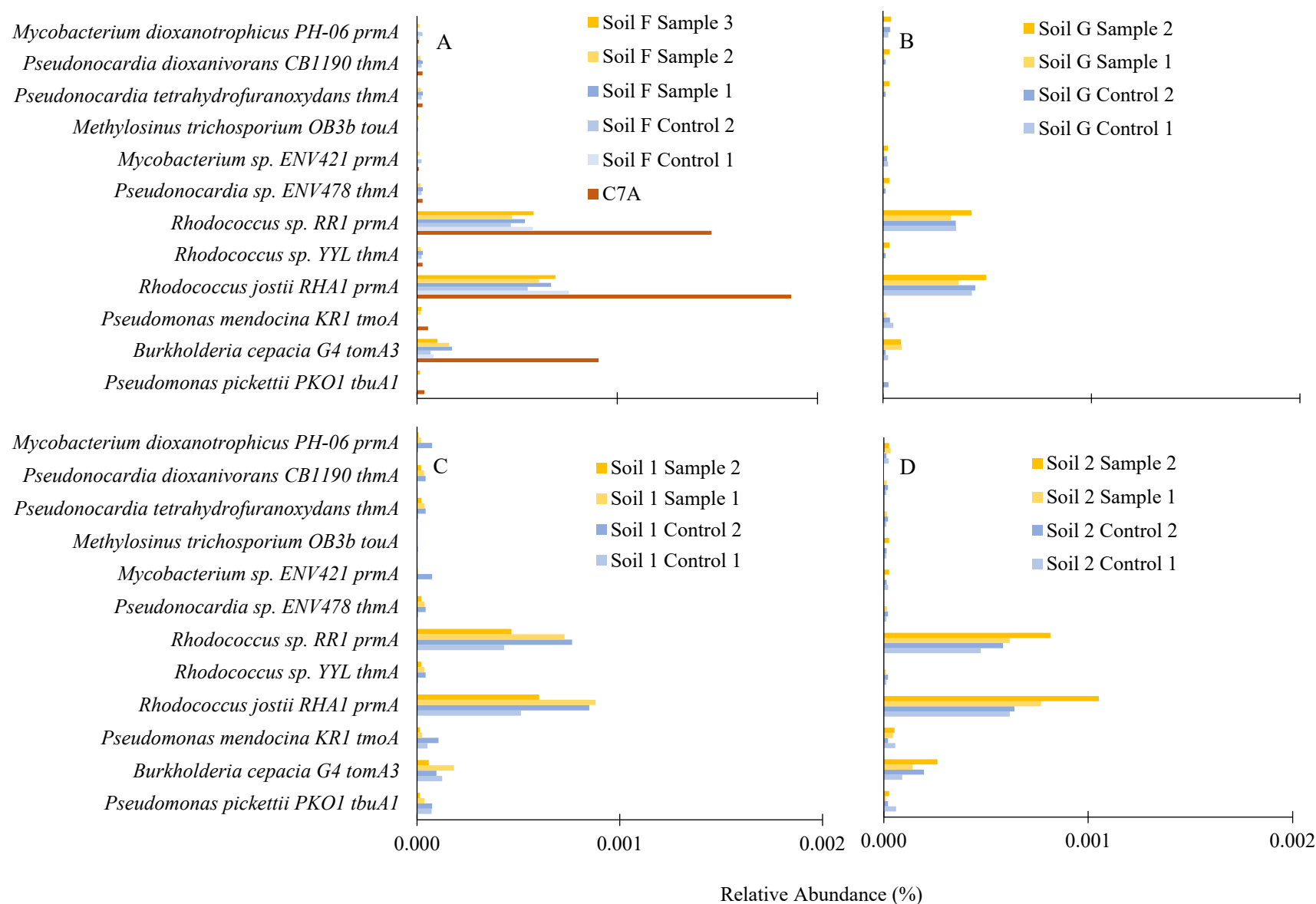


Figure 2.7. Relative abundance (%) of reads aligning ($\geq 60\%$ identity for ≥ 49 amino acids) to genes previously associated with the metabolic and co-metabolic degradation of 1,4- dioxane in Soil F and C7A (A), Soil G (B), Soil 1 (C) and Soil 2 (D).

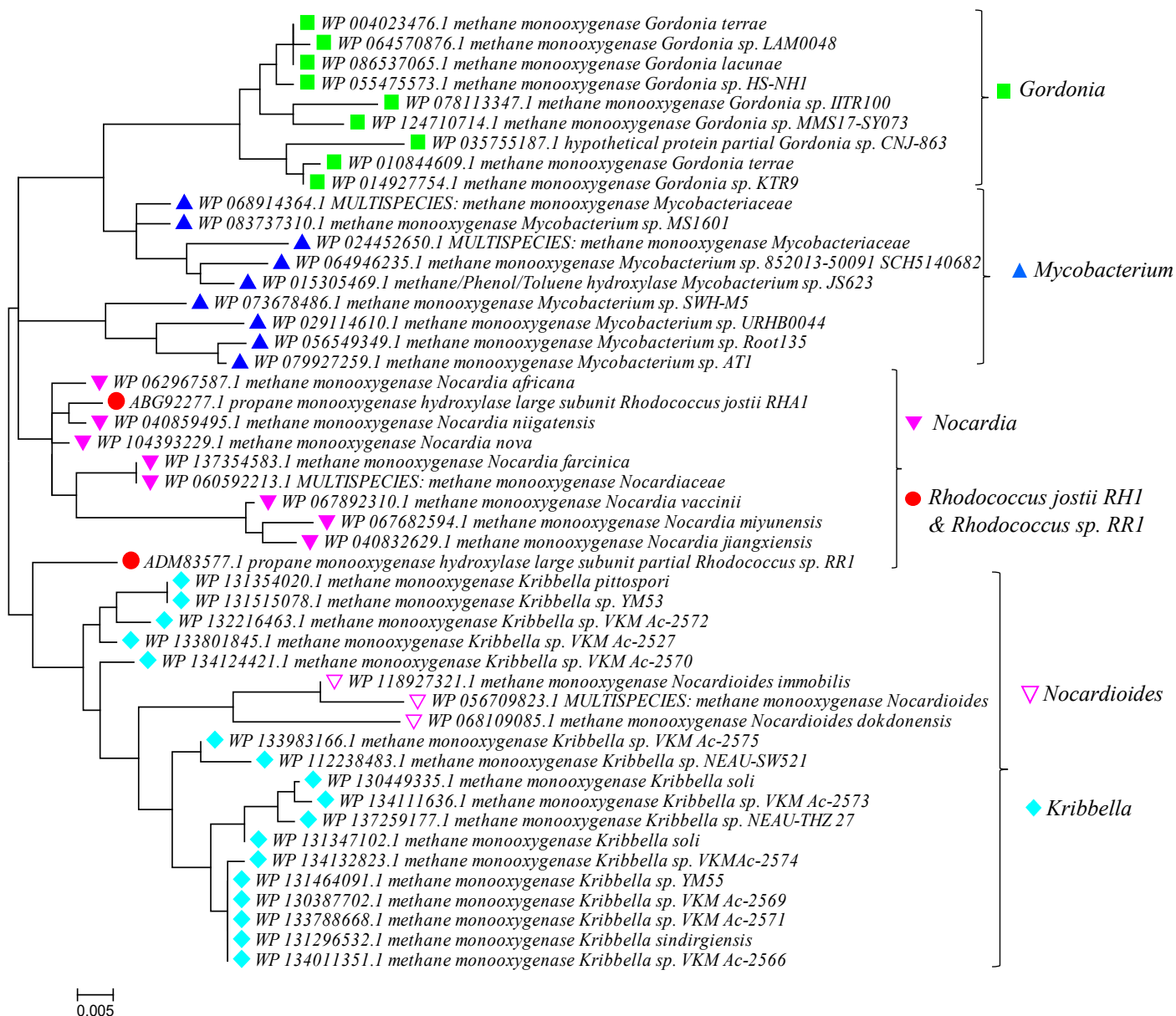
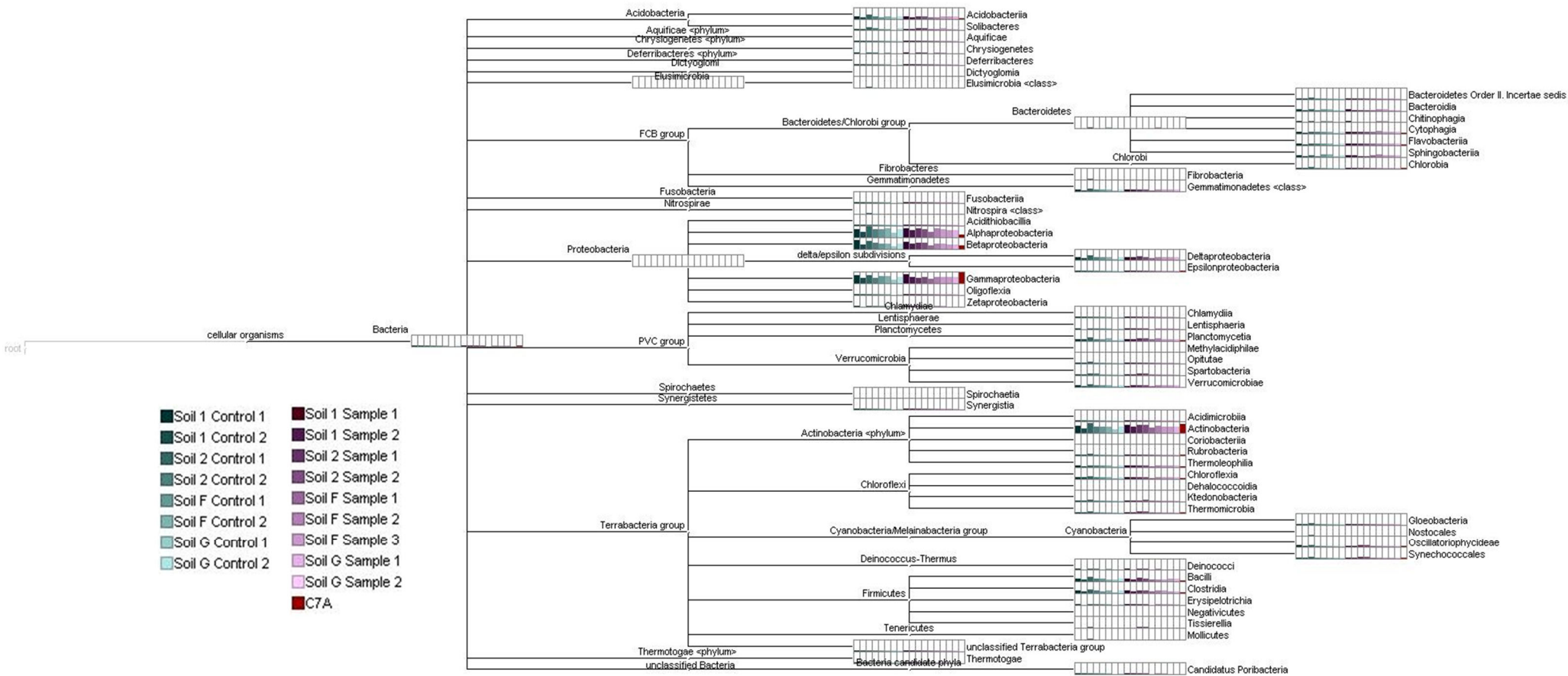
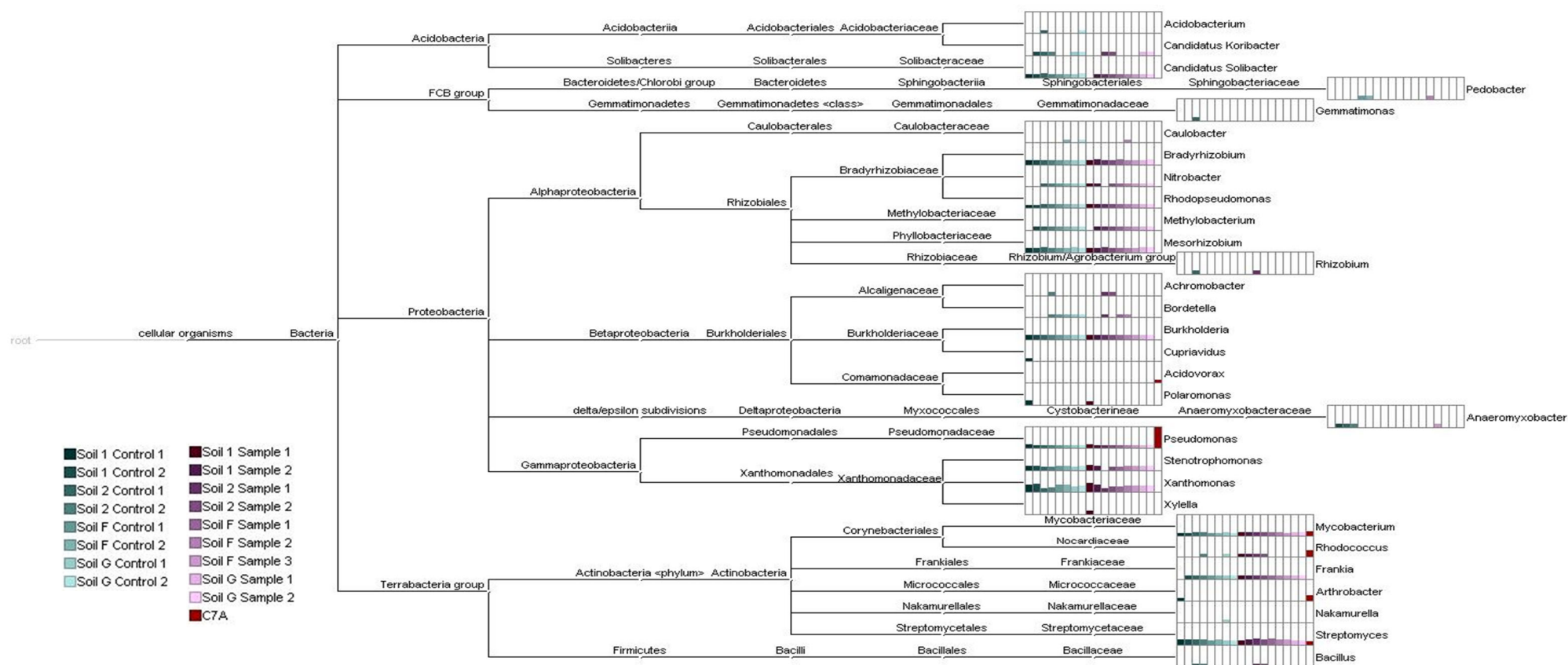


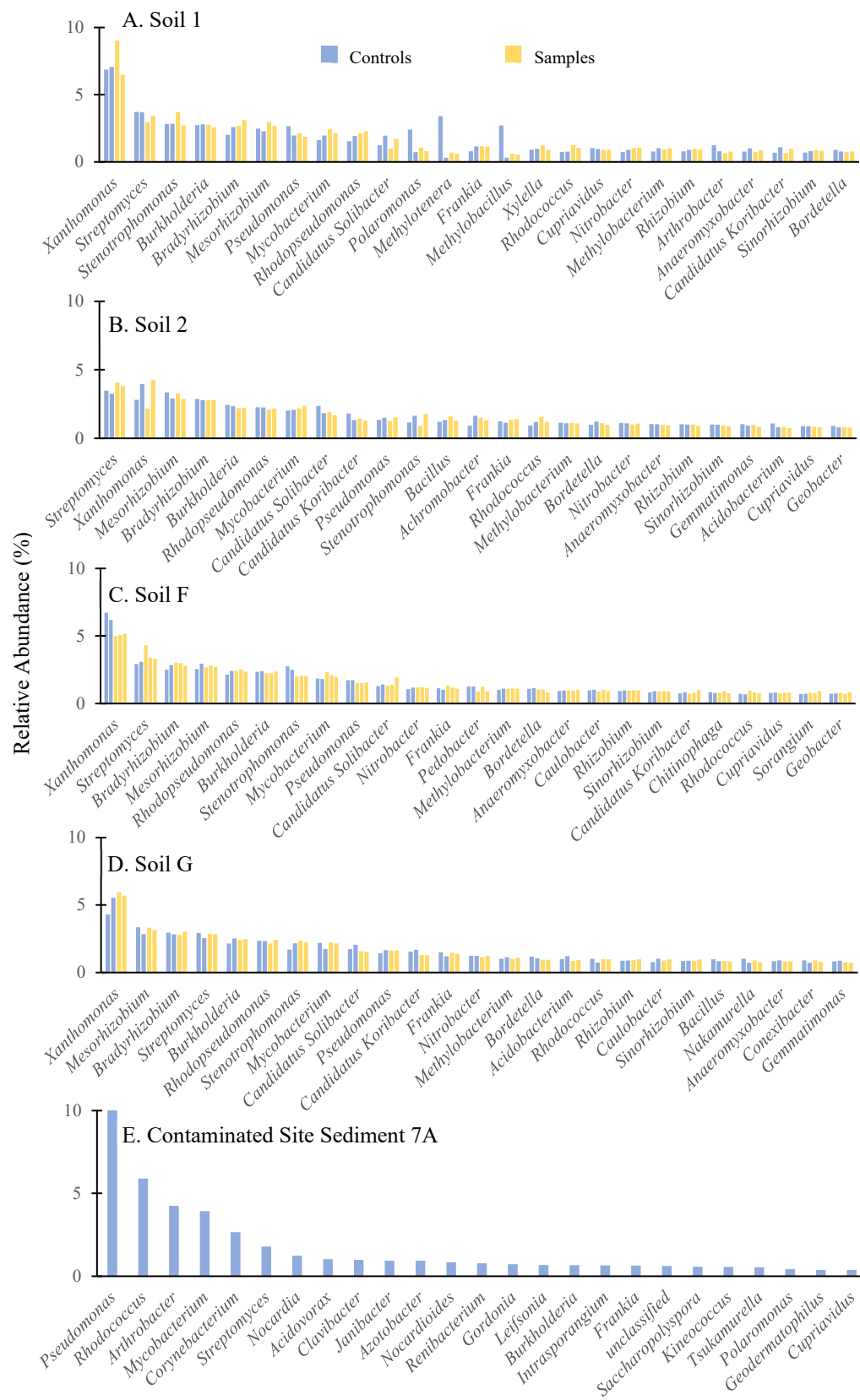
Figure 2.8. Phylogenetic tree of *Rhodococcus jostii* RHA1 *prmA* and *Rhodococcus* sp. *prmA* and BLASTP results (>94.8% similar to the two query sequences). Only genera that were enriched following 1,4-dioxane degradation (compared to the controls) are shown (Table 2). The evolutionary history was inferred by using the Maximum Likelihood method based on the Jones-Taylor-Thornton (JTT) matrix-based model. The tree with the highest log likelihood (-2731.06) is shown. Initial tree(s) for the heuristic search were obtained automatically by applying Neighbor-Join and BioNJ algorithms to a matrix of pairwise distances estimated using a JTT model, and then selecting the topology with superior log likelihood value. The tree is drawn to scale, with branch lengths measured in the number of substitutions per site. The analysis involved 48 amino acid sequences. All positions containing gaps and missing data were eliminated. There were a total of 439 positions in the final dataset. Evolutionary analyses were conducted in MEGA7.



Supplementary Figure 2.1. Phylogram (created with MEGAN6, version 6.11.7) illustrating the relative abundance and classification (Class Level) of all bacteria across all metagenomes (samples and controls).



Supplementary Figure 2.2. Phylogram (created with MEGAN6, version 6.11.7) illustrating the most abundant genera (ranked by average relative abundance, then selected if average relative abundance >0.5%) across all metagenomes (samples and controls).



Supplementary Figure 2.3. The twenty-five most common genera (by relative abundance, %), ranked by the averages of the samples and controls, in soil 1 (A), soil 2 (B), soil F (C), soil G (D) and the contaminated site sediment 7A (E).

Conclusions and Implications for Future Research

This research was initiated to address the feasibility of 1,4-dioxane biodegradation over a range of redox conditions. This issue is important because the electron accepting conditions may vary across one site or between different sites. For example, anaerobic conditions are often actively created through injecting substrates into the subsurface at sites contaminated with the chlorinated solvents (common co-contaminants with 1,4-dioxane). Although much is known about aerobic 1,4-dioxane biodegradation, limited research has addressed the susceptibility of 1,4-dioxane to biodegradation under anaerobic conditions. Previous research has indicated no removal under such conditions or removal only under iron reducing conditions.

The current research provides several valuable contributions to improve the management opinions at 1,4-dioxane contaminated sites. Specifically, 1,4-dioxane biodegradation was observed under methanogenic conditions. However, limited biodegradation was noted in the iron or sulfate amended treatments. Although 1,4-dioxane removal was observed in the nitrate amended samples, these microcosms had likely already transited to methanogenic conditions (because of the long incubation times). The biodegradation of this chemical under methanogenic conditions indicates natural attenuation of 1,4-dioxane may occur in areas where highly reducing conditions occur either naturally or as a result of enhanced reductive dechlorination. In the methanogenic samples, it is important to note that removal did not occur in all of the samples tested, therefore the microorganisms responsible for removal under these conditions may not be widespread. This information will be important at sites co-contaminated with the chlorinated solvents, as these sites are often driven to anaerobic conditions through enhanced reductive dechlorination.

Other significant contributions were provided from the aerobic biodegradation studies. Under aerobic conditions, 1,4-dioxane degradation was observed with all inocula types, indicating aerobic 1,4-dioxane degraders are widespread. Further, a large number of genera were enriched following 1,4-dioxane degradation. Such information suggests that natural attenuation of 1,4-dioxane at aerobic sites is likely.

Future research should provide stronger evidence for the phylotypes associated with anaerobic 1,4-dioxane biodegradation. This study indicated two phylotypes were enriched during the degradation of this chemical under methanogenic conditions. However, additional evidence is needed to support this conclusion (e.g. through the use of stable isotope probing). Another important issue for future research concerns the impact of enhanced reductive dechlorination substrates on 1,4-dioxane biodegradation. It is likely that these substrates will generate methanogenic conditions more rapidly and this could result in faster removal of 1,4-dioxane. This approach would be advantageous as it could result in the removal of both the chlorinated solvents and 1,4-dioxane. Clearly, additional research is needed to provide evidence for this hypothesis. A final important area for future research involves the potential for CSIA as a diagnostic tool for quantifying anaerobic 1,4-dioxane biodegradation in the field.