

FINAL REPORT

Developing and Field-Testing Genetic Catabolic Probes for
Monitored Natural Attenuation of 1,4-Dioxane

SERDP Project ER-2301

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14. ABSTRACT Remediation of aquifers contaminated with 1,4-dioxane (dioxane) is a difficult task due to its extremely hydrophilic nature. Monitored natural attenuation (MNA), which relies primarily on biodegradation, is often the most cost-effective approach to manage large and dilute groundwater plumes of primary pollutants, such as those formed by dioxane. However, improved forensic tools are needed to ensure biodegradation is occurring at impacted sites. During this one-year project, catabolic biomarkers of high selectivity and sensitivity were developed to target (until now elusive) dioxane/tetrahydrofuran soluble di-iron monooxygenase genes using Taqman chemistry. Further, dioxane degradation activity observed in microcosms prepared with groundwater samples and aquifer materials from multiple sites was found to be significantly correlated with the abundance of <i>thmA/dxmA</i> genes, suggesting the usefulness of this probe to assess feasibility of MNA/bioremediation and estimate degradation rates.					
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EXECUTIVE SUMMARY

Objectives. Remediation of aquifers contaminated with 1,4-dioxane can be complex because dioxane is highly mobile in groundwater, recalcitrant to biodegradation, and is not easily removed by volatilization or adsorption. Monitored natural attenuation (MNA), which relies primarily on contaminant's removal by biodegradation, is often the most cost-effective approach to manage large and dilute groundwater plumes such as those encountered in sites impacted by dioxane contamination. However, the burden of proof that MNA is an appropriate solution lies on the proponent, which requires site-specific demonstration of contaminant's removal with strong evidences to support the presence of expression of biodegradation capabilities.

In this project, we *developed and validated catabolic gene probe(s) to quantify the presence and expression of dioxane biodegradation genes*, to support decisions to select or reject MNA and assess its performance at dioxane-impacted sites. Specific tasks included:

1. Identification of genes responsible for initiating dioxane biodegradation, development of a primer/probe biomarker to target the identified genes, and assessment of reliability (selectivity/sensitivity) of gene biomarker to evaluate the presence and activity of dioxane degraders at a broad range of contaminated sites;
2. Statistical analyze to determine how the abundance of this catabolic biomarker (and associated degradation activity) is influenced by geochemical factors and contamination scenarios;
3. Identification (and perhaps isolation) of dioxane degraders from these sites, using enrichment and dilution to extinction or fluorescence activated flow cytometry with cell sorting techniques; and
4. Characterization of catabolic gene sequences involved in dioxane metabolism in isolated or sorted cells using DNA and mRNA sequencing approaches to obtain more sequences for possible adjustment/optimization of our previous biomarker design.

Technical Approach. This project required a multidisciplinary multi-scale approach. The first step was to identify genes responsible for initiating dioxane biodegradation. We identified the *dxmADBC* operon in the archetype dioxane metabolizer, *Pseudonocardia dioxanivorans* CB1190, using bioinformatics and transcriptomic analyses. Then, we developed a primer/probe set to quantify a putative dioxane monooxygenase gene using PCR and *Taqman* chemistry. This process involved multiple sequence alignment of the four *thmA/dxmA* genes available on the NCBI database. Then we used our developed *thmA/dxmA* biomarker and qPCR assays to conduct a broad survey of the presence of dioxane degraders and assess their activity at numerous, diverse dioxane-impacted sites (Task 1). This was critical to determine if our biomarker was selective, sensitive as well as reliable as a tool to assess dioxane biodegradation potential with potential to infer on site-specific biodegradation rates. The data obtained during Task 1 was statistically evaluated (multivariate ANOVA) in conjunction with site-specific geochemical characteristics and contaminant concentrations to heuristically discern general conditions that are amenable for MNA, which served to identify factors that may broadly affect biodegradation

activity (Task 2). Furthermore, we developed a method to isolate and identify the phylogenetic and metabolic diversity of indigenous dioxane degraders using gene probes to target mRNA from catabolic genes (i.e., actively degrading bacteria) and fluorescence activated flow cytometry with cell sorting (Task 3). The catabolic genes in isolated bacteria were sequenced and annotated to obtain additional information about dioxane metabolic pathways, and to optimize the design of catabolic biomarkers for further evaluation of the performance of dioxane MNA and bioremediation (Task 4).

Results.

Through multiple alignment analyses, we found that critical nucleotide sequences of key catabolic genes coding for the active site of the enzymes are highly conserved. These sequences were used to develop the *thmA/dxmA* probe capable of targeting putative dioxane degrading monooxygenase. We demonstrated that this primer-probe set was both highly selective (no false positives) and sensitive (7,000 ~ 8,000 copies/g soil) when tested against a dioxane-degrading reference strain (e.g., CB1190). A significant correlation between biodegradation rates and the abundance of *thmA/dxmA* genes were observed. Contrariwise, a nonspecific 16S rRNA gene copy numbers were neither sensitive nor reliable as indicator of dioxane biodegradation activity (Task 1).

While many geochemical factors (pH, temperature, nutrients) and the abundance of specific degraders may be important for dioxane biodegradation, these factors may not exert as strong of an influence on the potential biodegradation activity as the concentration of dioxane. Unequivocally, *in situ* dioxane concentrations significantly influenced the observed dioxane biodegradation rates with higher dioxane concentrations. Whereas auxiliary carbon sources may temporarily enhance dioxane biodegradation, they may also exert counterproductive long-term consequences. Non-inducing growth substrates can boost the overall microbial biomass but inhibit the indigenous dioxane degraders (Task 2).

Fluorescently labeled oligonucleotide probes and flow cytometry is a cultivation-free strategy to separate and concentrate genus-specific subpopulations of interest. This will expedite discovering novel *Pseudonocardia* strains and discerning the molecular basis for specific metabolic traits of interest. Besides, using dilution to extinction method, two dioxane degrading consortia were enriched from uncontaminated environments. These have comparable dioxane degradation capabilities with two archetype dioxane degraders. *Mycobacterium* was the dominant genus and group 5 and group 6 soluble diiron monooxygenase (SDIMOs) was prevalent after enrichment (Task 3).

Through whole-genome sequencing, RNA sequencing and RT-qPCR, a group-6 propane monooxygenase (*prmABCD*) was identified in *Mycobacterium dioxanotrophicus* PH-06. Accordingly, a primer/probe set was developed and validated through four dioxane-degrading consortia and strong correlation was found between dioxane degradation rates and the abundance of biomarkers. This new biomarker is highly selective and sensitive and would minimize the false negatives of our previous biomarker (*thmA/dxmA*). Furthermore, the preliminary results of construction of a reporter strain to monitor dioxane degradation activities would facilitate future development (Task 4).

Benefits. This project supports the rational selection of MNA over other remediation approaches that are usually more complex and costly (e.g., pump-and-treat with advanced chemical oxidation treatment). It also provides a scientific basis for the interruption of remediation strategies when these are no longer removing dioxane faster than MNA. Together, these findings could aid decision makers to more appropriately select for cost-effective clean up strategies saving the industry, state and federal government from multi-billion-dollar risk penalties. This work also responds to urgent calls for more reliable risk assessment and management approaches for hazardous wastes that have come from environmental advocacy groups, industry and the regulatory community.

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Nomenclature

Dioxane	1,4-Dioxane
MNA	Monitored Natural Attenuation
SDIMO	Soluble Di-iron Monooxygenase
MO	Monooxygenase
THF	Tetrahydrofuran
PCR	Polymerase Chain Reaction
GC/MS	Gas Chromatograph / Mass Spectrometer

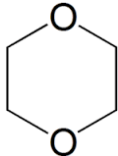
Chapter 1

Objectives

1.1 Introduction

1,4-Dioxane (dioxane) (Table 1.1) is a widespread groundwater contaminant of emerging concerns,¹ and was classified as a probable human carcinogen (Class B2) by the United States Environmental Protection Agency.² Dioxane was widely used as a stabilizer for chlorinated solvents and is found as a co-contaminant at thousands of solvent-contaminated sites.³ Removal of dioxane from such sites is usually challenging due to its relative recalcitrance to biodegradation and high migration potential with low tendency to be retarded by sorption.⁴

Table 1. 1 Physicochemical property of dioxane.

Property	Value	Reference
Chemical Structure		-
Molecular weight	88.1 g/mol	4
Density	1.03 g/cm ³	4
Solubility	Fully miscible	4
Log Kow	-0.27	5
Henry's constant	4.88×10^{-6} atm m ³ /mol	5

Remediation of dioxane-impacted groundwater often relies on pump and treat technologies using advanced oxidation processes (e.g., Pall-Gelman Sciences site in Ann Arbor, MI; Hoechst Celanese site in Spartanburg, SC; and Gloucester Landfill site in Ontario, Canada use UV and hydrogen peroxide hydrogen peroxide).^{6, 7} *In situ* chemical oxidation (ISCO) of dioxane (e.g., with permanganate or persulfate) has also been tested at various sites.^{8, 9} Whereas ISCO can treat source zones, including co-occurring chlorinated solvent DNAPLs, it also suffers from site-specific inefficient production of hydroxyl radicals, scavenging of radicals by aquifer minerals or organic matter, and aesthetic impacts to the groundwater (e.g., pink coloring by permanganate).¹⁰ Furthermore, neither pump and treat nor ISCO are cost effective for the remediation of large and dilute dioxane plumes.

Monitored natural attenuation (MNA), which relies primarily on intrinsic bioremediation, is often the most cost-effective approach to manage trace levels of priority pollutants.¹¹ However, the burden of proof that MNA is an appropriate solution lies on the proponent, and MNA has not been widely used at dioxane-impacted sites because (1) the presence and expression of dioxane biodegradation capabilities are not ubiquitous, and (2) our ability to assess

the feasibility and efficacy of intrinsic bioremediation is precluded by our very limited understanding of the diversity and spatial distribution of microorganisms that degrade dioxane. Thus, advancing fundamental understanding of dioxane biodegradation and developing appropriate forensic tools to quantify the presence and expression of relevant catabolic capacities are of critical importance to determine the (site-specific) feasibility of MNA and (when appropriate) enhance its acceptability to both the regulatory and public communities. This should be a research priority because the relatively large and dilute dioxane plumes (often longer than two miles) are not amenable for treatment by more aggressive remediation techniques such as *in situ* chemical oxidation.¹ Furthermore, recent findings by our lab and others suggest that indigenous bacteria that can degrade dioxane might be more widespread than previously assumed. Since many dioxane plumes could be effectively managed by MNA at a small fraction of alternative treatment costs, it is important to develop simple and reliable forensic approaches that enable site-specific decisions to select or reject MNA, and to enhance performance assessment.

1.2 Objectives and Hypothesis

1.2.1 Objectives

We seek to advance our genetic and molecular understanding of dioxane biodegradation and develop a simple molecular biology approach to rapidly quantify the presence and assess the activity of indigenous dioxane degrading bacteria. This will allow us to unequivocally assess the feasibility and performance of MNA or bioremediation at dioxane-impacted sites. We will focus on aerobic processes that prevail in the more dilute regions of the plume (e.g., at the leading edge)¹² and enable MNA even under oligotrophic conditions.¹³ Specific tasks for this project included to:

1. Assess the reliability of *thmA/dxmA* gene biomarker to evaluate the presence and activity of dioxane degraders at a broad range of contaminated sites. This is important to assess the temporal and spatial variability of dioxane degradation capabilities and delineate the capability and limitations of this biomarker as a forensic tool (e.g., assess the potential for false positives/negatives, and demonstrate adequate sensitivity).
2. Statistically analyze how the abundance of this catabolic biomarker (and associated activity) is influenced by site-specific geochemical factors (e.g., dissolved oxygen, pH, temperature, nutrient and electron acceptor concentrations, redox conditions, and concentrations of chlorinated solvents and background organic matter) and contamination scenario (e.g., presence and concentration of additional pollutants). This heuristic exercise would allow us to broadly discern general conditions that are amenable for (or hinder) MNA;
3. Identify (and perhaps isolate) new indigenous dioxane degraders, using (i) enrichment and dilution to extinction approaches, and (ii) gene probes that target mRNA from catabolic genes (i.e., actively degrading bacteria) with the help of fluorescence activated flow cytometry and cell sorting techniques. This would advance our understanding of the phylogenetic and metabolic diversity of indigenous dioxane degraders; and

4. Characterize catabolic gene sequences involved in dioxane metabolism in these isolates or sorted cells, using DNA and mRNA sequencing technologies. This would allow us to investigate how conserved are the critical dioxane degrading genes (e.g., *thmA/dxmA*), and obtain more sequences for possible adjustment/optimization of our previous biomarker design.

As we developed functional gene probes to assess the feasibility and performance of MNA at dioxane-impacted sites, several outstanding questions of broader significance were addressed, such as:

- Can our functional gene biomarkers be used to unequivocally establish the presence and quantify the concentration of the “important” specific degraders? (i.e., the most abundant and/or most active microorganisms that initiate dioxane degradation).
- Are these biomarkers selective enough to avoid false positives, and are the associated analytical methods sufficiently sensitive to ensure “adequate” quantification (for example, biomarker detection in areas where the plume is dilute and dioxane concentrations are lower than 100 µg/L)?
- Are the concentrations of these biomarkers correlated to dioxane biodegradation activity so that bioremediation rates could be reasonably estimated based on biomarker measurements?
- If so, which types of biomarkers (DNA-based catabolic genes or mRNA produced by their expression) are best correlated with degradation rates in different matrices?
- Do we need to base such correlations on analysis of aquifer material (because most subsurface microorganisms live attached to surfaces) or can we reliably estimate biodegradation rates based on biomarker concentrations in groundwater samples (which are easier to collect and analyze)?
- What types of site-specific geochemical characteristics or contamination scenarios enable a more reliable use of these catabolic gene biomarkers as an MNA forensic approach?
- Besides the known dioxane degrading bacteria, whether we can isolate novel degraders and identify corresponding dioxane degrading gene clusters?
- Can fluorescence activated cell sorting with probes that target our *thmA/dxmA* biomarker be used to identify uncultivable dioxane degraders?
- Can genetic information from these novel degraders (obtained by DNA and mRNA sequencing) be used to adjust our *thmA/dxmA* biomarker to ensure detection of a broader range of dioxane degraders and a more reliable quantitative assessment of MNA?

1.2.2 Hypothesis

To reliably use functional gene biomarkers for multiple purposes (e.g., quantify the presence of dioxane degraders, enable single-cell sorting analysis to identify uncultivable strains, and construct plasmids for gene bioaugmentation), we first need to understand the molecular

basis of dioxane biodegradation. A first step is to recognize (syllogistically) that (1) aerobic degradation of dioxane is initiated by THF/dioxane monooxygenases,¹⁴ gene sequences for these monooxygenases generally have conserved regions (common to multiple species) that could be targeted by probes,¹⁵ and (3) bioinformatics and transcriptomic analyses of putative dioxane monooxygenase genes from several bacterial strains can be used to identify key catabolic gene sequences (i.e., conserved regions coding for the active site of the enzymes that initiate dioxane biodegradation) and design probes that reliably detect a wide variety of dioxane degraders.^{16, 17}

Note that several multi-component soluble di-iron monooxygenases (SDIMOs) are known to degrade dioxane by co-metabolic pathways, including phenol hydroxylase, toluene-2-monooxygenase, toluene-3-monooxygenase, toluene-4-monooxygenase, and soluble methane monooxygenase.^{11, 18} However, only tetrahydrofuran/dioxane monooxygenases are known to initiate the degradation of dioxane and other cyclic ethers as their primary substrate through more sustainable and more efficient metabolic pathways.^{14, 19} Of specific interest is the dioxane monooxygenase (*dxmADBC*) in CB1190 with high sequence similarity to, as well as the same component arrangement as, the tetrahydrofuran monooxygenases (THF MO) in *Pseudonocardia tetrahydrofuranoxydans* K1,²⁰ *Pseudonocardia* sp. ENV478,²¹ and *Rhodococcus* sp. YYL.²² The active sites of these SDIMOs are located near a binuclear iron center on the α -subunit of the hydroxylase component within the L110 to I239 region.²³ Hydrophobic amino acids in this region play a key role in substrate recognition and 'gating'.^{24, 25} Thus, with the help of primary sequence alignments, we should be able to identify conserved nucleotide regions coding for the active sites of these enzymes. This would enable the design of gene probes that would be validated with known isolates and field samples exhibiting dioxane degradation activity.

We also postulate that dioxane degrading bacteria (and associated catabolic biomarkers) will be enriched in the (aerobic) leading edge of many dioxane plumes relative to uncontaminated areas, and higher biomarker concentrations (due to growth of specific degraders) will correspond to higher biodegradation activity, thus providing evidence of intrinsic bioremediation.

Whereas catabolic DNA biomarkers should be present in samples exhibiting biodegradation activity, their presence would not guarantee activity because the presence of a gene does not guarantee its expression. Instantaneous biodegradation activity would be better inferred by the presence of the corresponding mRNA, but this is a much more difficult analytical task due to the instability of mRNA. Yet, we postulate that mRNA analysis is not required to infer degradation activity over the large temporal scales that are relevant to MNA because the catabolic biomarkers will experience long-term enrichment as a result of bacterial growth on dioxane. Thus, easier-to-quantify DNA-based biomarkers would be appropriate for forensic analysis of MNA and to study the spatial variability of degradation activity.

Chapter 2

Background

2.1 Introduction of dioxane

Dioxane is a synthetic cyclic ether which was widely used as a solvent and chlorinated solvents stabilizer, in particular, 1,1,1-trichloroethane (TCA).²⁶ Although TCA was eventually phased out of use in the U.S. in the 1990s, dioxane has continued to be used as a solvent for paints, oils, polishing compositions, coatings, and personal care products and cosmetics.^{2, 26} Consequently, dioxane is a groundwater contaminant of emerging concern that is commonly found as a co-contaminant at thousands of solvent-contaminated sites.³ However, unlike those chlorinated solvents, dioxane has very special physiochemical properties and is highly hydrophilic (Table 1.1) and experiences extraordinary mobility in the groundwater, leading to much larger regions of influence and longer plumes.³

Dioxane was classified by the International Agency for Research on Cancer (IARC) as a Group 2B carcinogen in 1999.²⁷ It was also included in the Final Third Drinking Water Contaminant Candidate List by U.S. EPA in 2013, due to its probable human carcinogenicity.² Currently, dioxane is not strictly regulated in the US, but several states have established notification levels and guidelines (Table 2.1), and the acceptable concentrations of this contaminant are trending lower. EPA risk assessments indicate that drinking water concentration representing a 1×10^{-6} cancer risk level for 1,4-dioxane is 0.35 $\mu\text{g/L}$.²⁸

Table 2. 1 Dioxane guidelines of various states for drinking water or groundwater.
(Adapted from EPA (2013))²⁸

State	Guideline (µg/L)	Source
Alaska	77	AL DEC 2016
California	1.0	Cal/EPA 2011
Colorado	0.35	CDPHE 2017
Connecticut	3.0	CTDPH 2013
Delaware	6.0	DE DNR 1999
Florida	3.2	FDEP 2005
Indiana	7.8	IDEM 2015
Maine	4.0	MEDEP 2016
Massachusetts	0.3	MADEP 2004
Mississippi	6.09	MS DEQ 2002
New Hampshire	0.25	NH DES 2011
New Jersey	0.4	NJDEP 2015
North Carolina	3.0	NCDENR 2015
Pennsylvania	6.4	PADEP 2011
Texas	9.1	TCEQ 2016
Vermont	3.0	VTDEP 2016
Washington	0.438	WA ECY 2015
West Virginia	6.1	WV DEP 2009

The historical usage, improper storage, and disposal of dioxane-containing solvent wastes have caused the co-contamination with other common environmental pollutants in groundwater and drinking water. Andreson et al.(2012) reported an association between dioxane and various chlorinated solvents where approximately 94% of groundwater monitoring wells with detectable levels of dioxane also contained TCA and/or trichloroethylene(TCE).³ Though dioxane was initially used as a stabilizer for TCA but not for TCE in the records,²⁶ 64% of dioxane detections in monitoring wells were independent of TCA.²⁹ A perfect match was also found between the concentrations of dioxane (ranged from 0.15 µg /L to 152 µg /L) and TCE in the vertical and horizontal distribution profiles at sites with known 1,1,1-trichloroethane (TCA) and TCE contaminations, in landfill leachates, and at sites of detergent production in Western Germany.³⁰ This finding implies the necessity of investigating dioxane contamination in previously identified TCE plumes and other mixed chlorinated solvent plumes. For instance, dioxane (1.1 µg /L to 340 µg /L) was mixed with TCE (0.72 µg /L to 650 µg /L) at the United States Air Force Plant No.44 (AFP 44) aquifers.³¹ Furthermore, dioxane was also found co-existed with poly- and per-fluorinated compounds.³² Dioxane was one of the 28 unregulated contaminants that were monitored during the three-year reporting period for unregulated contaminant monitoring rule (UCMR3). Dioxane was detected in samples from 21% of 4,864 public water systems (Figure

2.1), and 6.9% of these systems were in exceedance of the health-based reference concentration ($0.35 \mu\text{g/L}$). Although much of the focus on dioxane has been its role as a groundwater contaminant, the detection frequency of dioxane in surface water was only marginally lower than in groundwater.³³

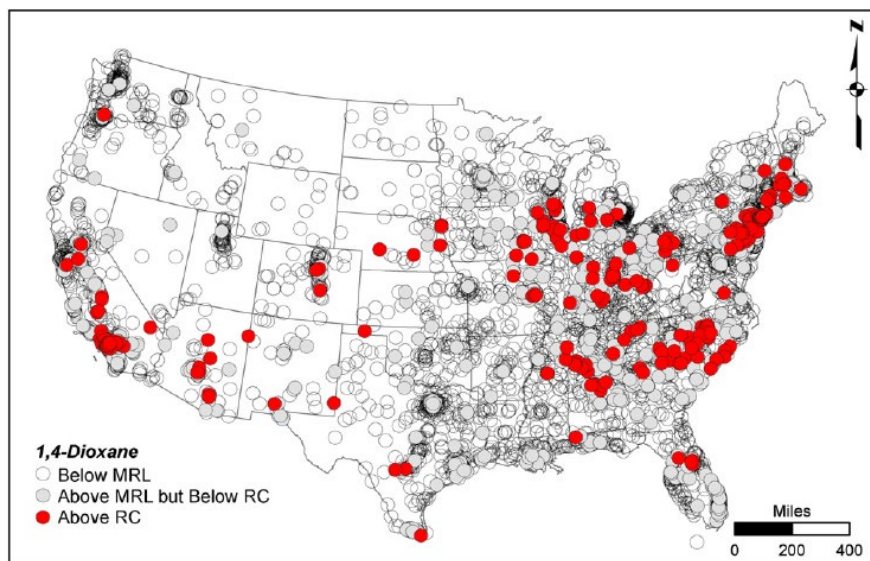


Figure 2. 1 Geographic distribution of dioxane occurrence based on UCMR3 results.

Each symbol represents data for a single public water system(PWS). MRL = minimum reporting limit ($0.07 \mu\text{g/L}$ for dioxane). RC = Reference concentration($0.35 \mu\text{g/L}$ for dioxane). Results for continental United States (48 states) are shown. (Adapted from Adamson et.al 2017)³³

2.2 Treatment of dioxane

Conventional physical-chemical treatment methods are marginally effective to remove dioxane from impacted sites. Due to its low Henry's Law constant and extremely hydrophilic nature (Table 1.1), dioxane is neither sufficiently volatile for air sparging nor efficiently adsorbed onto activated carbon. Advanced chemical oxidation and photocatalytic processes, including use of ozone,³⁴ hydrogen peroxide,³⁵ titanium dioxide,³⁶ sonication,³⁷ and UV irradiation³⁵ can degrade dioxane effectively, but such techniques can be prohibitively expensive, and the contaminated groundwater needs to be pumped out from the subsurface for effective treatment. On the other hand, the products of advanced oxidation processes (AOPs) are usually low molecular weight fatty acids, such as formic acid, oxalic acids, which can be biotransformed through conventional biological methods.³⁸ These findings suggest a combined AOPs and biological treatment approach would be considered as a viable remediation strategy for sites contaminated by high concentrations of dioxane (like source zone).

Dioxane was initially considered as a non-biodegradable industrial chemical. However, in the past decade, a number of strains have been isolated,³⁹⁻⁴² suggesting a greater potential of bioremediation for dioxane-impacted sites. Up to date, almost all of the biodegradation of

dioxane happened in an aerobic condition. Little evidence of anaerobic dioxane biodegradation has been reported other than a single study about an iron-reducing bacterium capable of growing on dioxane.³⁸ Among the bacteria capable of degrading dioxane, most of them are gram-positive bacteria that belong to *Pseudonocardia*, *Mycobacteria*, and *Rhodococcus* genera.

2.3 Soluble di-iron monooxygenases and dioxane biodegradation

Soluble di-iron monooxygenases (SDIMOs) are multi-component bacterial enzymes that incorporate one oxygen atom from O₂ into various substrates such as chlorinated solvents, aromatic hydrocarbons, alkanes and alkenes to initiate catabolism.^{23, 43} SDIMOs are composed of four to six subunits coded by a single gene cluster, including a hydroxylase component, a reductase, and a coupling protein.^{23, 43} The hydroxylase component is the site of hydrocarbon oxidation. It is comprised of two or three subunits in dimeric configuration and contains the very highly conserved amino acid sequence (i.e., DE*RH), which is the active center responsible for interacting with substrates. The reductase component transfers electrons to the hydroxylase via a small effector (or coupling protein). In some SDIMOs, including phenol hydroxylases and several soluble methane monooxygenases, they have another protein called accessory protein, which is neither involved in catalytic activity nor required for the assembly of the hydroxylase. A study showed that this component may be responsible for enhancing iron incorporation.⁴⁴

Phylogenetic studies reveal that most SDIMO-harboring bacteria belong to the class of α -*Proteobacteria*, β -*Proteobacteria*, γ -*Proteobacteria* or *Actinobacteria*. These bacteria were prevailing in all types of environments.^{23, 43} In addition, these SDIMOs are found present not only in the chromosome, but also in plasmids. The distribution pattern of SDIMOs indicates that it is likely that some of the SDIMOs were acquired by horizontal gene transfer. This thought can also get support from lower GC base composition of the SDIMOs genes compared with average GC base composition for the genome.^{23, 43}

Based on the component arrangement, substrate specificity and sequence similarity, SDIMOs are divided into six groups,^{15, 23, 43} including aromatic/alkene monooxygenases (group 1), phenol monooxygenases (group 2), soluble methane monooxygenases (group 3), alkene monooxygenases (group 4), THF monooxygenases (group 5) and propane monooxygenases (group 6).^{15, 23, 45}

Before, the only well-characterized gene cluster responsible for the initiation of dioxane degradation is a THF monooxygenase gene cluster (*thmADBC*) that was identified in two dioxane metabolizers (i.e., *Pseudonocardia dioxanivorans* CB1190⁴⁶ and *Pseudonocardia* sp. N23⁴⁷) and several dioxane cometabolizers, including *Pseudonocardia tetrahydrofuranoxydans* K1,¹⁹ *Rhodococcus* sp. YYL,⁴⁸ and *Pseudonocardia* sp. ENV478.⁴⁹ This THF monooxygenase belongs to group-5 SDIMOs, and it has four subunits with the order as follows: hydroxylase alpha subunit (*thmA*), reductase protein (*thmD*), hydroxylase beta subunit (*thmB*) and coupling protein (*thmC*). A biomarker (primer/probe set) was designed to target the hydroxylase alpha subunit (*thmA*). The abundance of this biomarker was correlated well with the dioxane degradation rates in many dioxane impacted sites.¹⁶ Besides, though the specific genes were not identified, monooxygenase genes are thought to be involved in dioxane degradation by many degraders,⁴⁰ especially the family of SDIMOs, which are most likely involved in the scission of the C-O bond in dioxane.⁴⁶ Analysis of SDIMOs in dioxane/THF degrading strains revealed that

propane monooxygenase-like SDIMOs can be involved in dioxane degradation.⁵⁰ Besides, the study of cometabolic biotransformation of dioxane by a series of monooxygenase-expressing (including soluble methane monooxygenase, propane monooxygenase, toluene monooxygenase and tetrahydrofuran monooxygenase) bacteria revealed the importance and involvement of monooxygenase in dioxane biodegradation.¹¹

As discussed before, dioxane is almost always present with chlorinated solvents, such as 1,1,1-trichloroethane (TCA), trichloroethene (TCE), and 1,1-dichloroethene (DCE). Many studies about how these chlorinated solvents would affect the biodegradation of dioxane were conducted. One study about the chlorinated solvents on aerobic dioxane biodegradation by CB1190 demonstrated that 1,1-dichloroethene (1,1-DCE) was the strongest inhibitor of dioxane biodegradation in pure cultures as well as in environmental samples. The inhibition was attributed to delayed ATP production and down-regulation of dioxane monooxygenase genes.⁵¹ A similar study conducted in packed column flow-through systems showed that degradation of dioxane is inhibited by co-contaminants 1,1-DCE and Cu^{2+} . However, the inhibitory effects of co-contaminants are much less severe in the column than in planktonic systems, which may result from decreased bioavailability of 1,1-DCE and /or increased tolerance of microbes to these co-contaminants in soil columns.⁵² A recently isolated bacterium, *Azoarcus* sp. DD4, however, can sustain the concurrent co-oxidation of dioxane and 1,1-DCE using propane as the primary substrate without observable formation of clumps.⁵³

Another feature of dioxane degraders is that they all grow very slowly, for example, the generation time of CB1190 is 30 h at 30 °C.³⁹ To get sufficient bacterial culture for bioaugmentation, another rich substrate (e.g. R2A) would be used for cultivation. However, as mentioned before, all the known dioxane degrading gene clusters are in a plasmid. Therefore, the loss of the plasmid would result in the loss of dioxane degrading capabilities. Long-term cultivation of CB1190 in rich media, such as R2A, would cause the loss of the plasmid.⁵⁴ Also, in some sites, the concentration of dioxane degradation is very low, and cannot provide sufficient carbon source for dioxane degraders. One strategy is biostimulation by adding another substrate, auxiliary substrate (which only provide the growth carbon and energy source) or inducing substrate (which can also induce the expression of the functional gene). However, both options have their own advantages and disadvantages: auxiliary carbon sources can temporarily enhance dioxane degradation, but have counterproductive long-term consequences, while inducing substrate support growth of degraders but exert competitive inhibition.⁵⁴ These results indicate a better way to evaluate and monitor the biostimulation performance is needed.

2.4 Evidence of dioxane natural attenuation

2.4.1 Microcosm studies mimicking natural attenuation

The very first evidence indicating the presence of dioxane natural attenuation in environmental samples was observed in our lab in 2010.¹³ Dioxane biodegradation was investigated in microcosms prepared with groundwater and soil from an impacted site in Alaska. Natural attenuation microcosms (not biostimulated with nutrients) showed significant degradation when the initial dioxane concentration was 500 µg/L (Figure 2.2). Biodegradation followed zero-order kinetics (i.e., linear decrease in C versus t) indicating lack of significant microbial growth (as expected given the low concentration of dioxane available) and saturated

enzymes kinetics that are characteristic of oligotrophic bacteria with high affinity for dioxane.⁵⁵ However, no significant dioxane degradation was observed in microcosms spiked with dioxane as high as 50 mg/L compared with the autoclaved control. This study indicated that indigenous bacteria may consume dioxane in the diluted area of the plume even in oligotrophic ecosystems.

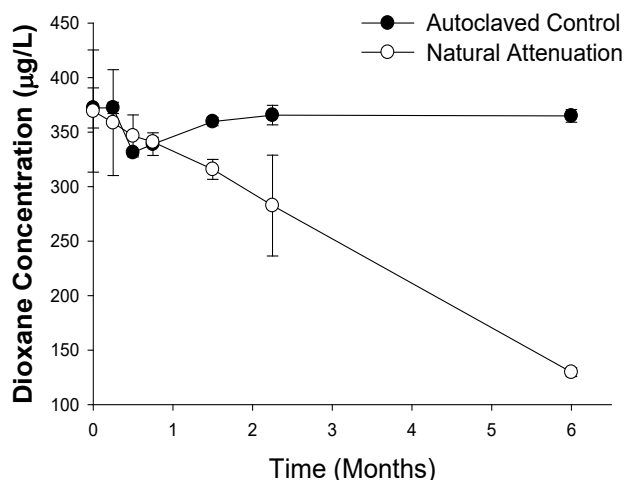


Figure 2. 2 Dioxane degradation in natural attenuation microcosms at 14 °C.¹³

Moreover, Sei and his coworkers surveyed the dioxane degradation potentials in various environmental samples collected in Japan, including four river water samples, three activated sludge samples, six drainage soil samples, and seven garden soil samples, when they are spiked with an initial dioxane concentration of 100 mg/L.⁵⁶ Dioxane was fully depleted in five of the drainage soil samples without addition of THF. One activated sludge sample exhibited co-metabolism of dioxane with the presence of equal amount of THF. This research demonstrates that different environmental samples may contain different types of dioxane degraders, but the dioxane degradation capability is not ubiquitous in nature.

2.4.2 Molecular evidence suggesting the presence of dioxane natural attenuation

Recent studies in our lab demonstrated the spatial distribution of SDIMO and other functional genes at a dioxane-impacted site on the north slope of Alaska.¹⁷ Functional gene array (i.e., GeoChip) analysis of Arctic groundwater revealed that various dioxane-degrading SDIMO genes were widespread (Figure 2.3), and PCR-DGGE analysis showed that group-5 SDIMOs were present in every tested sample, including background groundwater with no known dioxane exposure history (Figure 2.4). A group-5 *thmA*-like gene was enriched (2.4-fold over background, $p < 0.05$) in source-zone samples with higher dioxane concentrations, suggesting selective pressure by dioxane. Microcosm assays with ¹⁴C-labeled dioxane showed that the highest mineralization capacity ($6.4 \pm 0.1\%$ ¹⁴CO₂ recovery during 15 days, representing over 60% of the amount degraded) corresponded to the source area, which was presumably more acclimated and contained a higher abundance of SDIMO genes. Dioxane mineralization ceased after 7 days and was resumed by adding acetate (0.24 mM) as an auxiliary substrate to replenish NADH, a key coenzyme for the functioning of monooxygenases. Acetylene inactivation tests further corroborated the vital role of monooxygenases in dioxane degradation. This is the first

report of the prevalence of oxygenase genes that are likely involved in dioxane degradation and suggests their usefulness as biomarkers of dioxane natural attenuation.

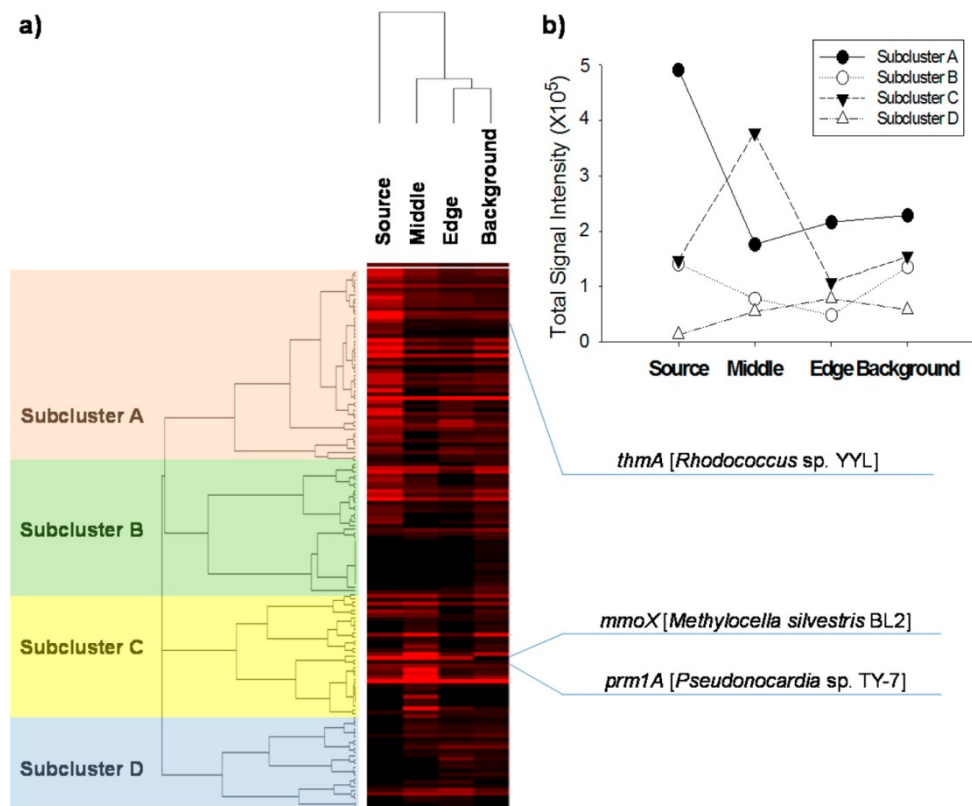


Figure 2.3 Hierarchical cluster analysis of SDIMO genes detected by GeoChip 4.0.

Red indicates positive averaged signal intensities of the replicates at each sampling location, and black indicates intensities below background. Representative organisms for selected genes are depicted on the right of the heat map. SDIMO genes were divided into four subclusters based on correlations of their relative abundance distribution patterns among different sampling locations (a). The total signal intensities in each subcluster are provided for each sampling location (b). Subcluster A genes, which included *thmA*, were enriched at the source, while Subcluster C genes were dominate in the middle of the plume. (Adapted from Li et al. 2013)¹⁷

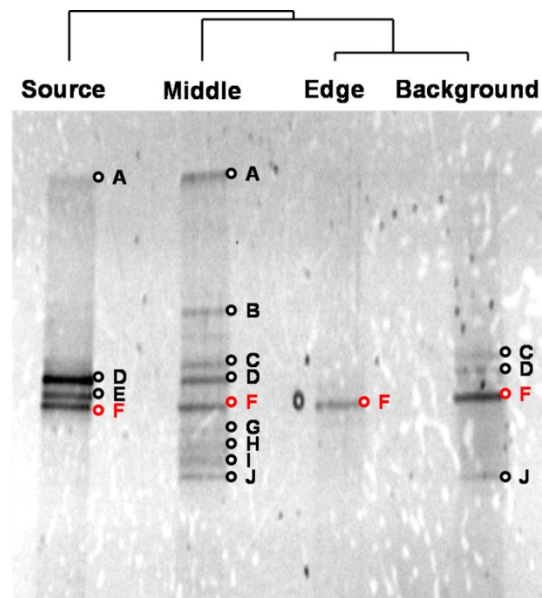


Figure 2. 4 DGGE gel photograph showing the heterogeneous presence of SDIMOs by nested PCR. (NVC58 and NVC65 as the primers for the first PCR set and then NVC57 + GC clamps and NVC66 as the primers for the second PCR set). Band F (highlighted in red) was found at all four sampling locations and corresponds to a *thmA*-like gene.

2.5 Reporter strains

Reporter strains have proven to be powerful tools to study bacterial physiology. Transcriptional and translational reporter strains are engineered by fusing a reporter gene, encoding a fluorescent or luminescent protein, downstream of the promoter of a gene of interest. When the reporter is expressed, it generates a signal that acts as a synthetic phenotype, enabling the study of physiologies that acts as a synthetic phenotype, enabling the study of physiologies that might have otherwise been hidden.⁵⁷ Reporter strain is a low-cost alternative for real-time analysis of gene expression. In fluorescent approaches, an external source of light is required for excitation of the protein. In contrast, bioluminescent reporter proteins can produce light by using appropriate substrates.⁵⁸

Fluorescent reporter proteins have been used to investigate microbial activities, and to screen microbial population dynamics *in situ* for years.⁵⁹ Green fluorescent protein (GFP) was first isolated in 1960 from jellyfish *Aequorea victoria* and first utilized to report gene expression.⁶⁰ GFP has excitation and emission maxima of 395 nm and 509 nm, respectively. However, wild-type GFP misfolds when expressed in *E. coli*. In 2006, a very robustly folded version of GFP, superfolder GFP (sfGFP) was generated. This protein does not have the drawbacks of other fluorescence proteins, such as the tendency of aggregating and the irreversibility of their denaturation. sfGFP has very high efficiency of folding, and it is capable of folding even when fused to a poorly folded partner peptide. Furthermore, in such a fusion construct, sfGFP can increase the folding efficiency of its partner, thus it can be used for the expression of poorly-soluble recombinant proteins and peptides.⁶¹

Another commonly used fluorescent reporter, similar to GFP, is *DsRed*, originally cloned from the coral *Discosoma striata*.⁶² Compared with GFP, the excitation and emission maxima of *DsRed* can be red-shifted to 558 nm and 583 nm, respectively. This allows for long-term excitation, as well as a reduction in auto-fluorescence.⁶³ However, the *DsRed* is not an ideal reporter due to its strong oligomerization and slow maturation.⁶² Mutant derivatives of *DsRed* have been generated to overcome these problems, like *mCherry*⁶⁴ and *Turbo-635*.⁶⁵ These optimized red or far-red fluorescent proteins ensure less auto-fluorescence background, higher fluorescence resonance energy transfer efficiency, and much more effective light penetration, allowing them for more applications.⁶⁶

Fluorescent reporter proteins have been extensively used in different areas, including bioremediation. For example, sfGFP was used to validate diverse new mRNA targets of the classic porin regulator, MicF RNA.⁶⁷ In another study, sfGFP was used to construct stable fluorescent reporter plasmids for dynamic imaging studies in *Staphylococcus aureus*.⁶⁸ GFP was used to monitor the survival and activity of *Arthrobacter chlorophenolicus* A6 cells during the degradation of 4-chlorophenol in the soil.⁶⁹ The fluorescent protein *mCherry* was inserted into the chromosome of *Aromatoleum aromaticum*, which is a highly versatile degrader of many aromatic compounds, to monitor the catabolic operon expression levels.⁷⁰

Another commonly used reporter is a luciferase reporter system. Two types of luciferase protein, firefly and bacterial, are commonly used. Firefly luciferase uses luciferin as a substrate, oxidizing it to oxyluciferin in a reaction that utilizes molecular oxygen and ATP, and releases lights at 560 nm.⁷¹ Bacterial luciferase is encoded by the *luxCDABE* operon. The luciferase protein is a heterodimer formed by the *luxA* and *luxB* gene products. The *luxC*, *luxD*, and *luxE* gene products encode for a reductase, transferase, and synthase respectively. Together with molecular oxygen, these components can produce a bioluminescent signal. Some marine species have additional genes (*luxI* and *luxR*) that govern the expression of the remainder of the operon.⁷²

The *lux* reporting system has been used in the visualization of gene expression, cellular population monitoring, and exogenous target detection. In bioremediation, *lux* reporting system has also been used extensively. For example, *lux* gene-marked bacterial biosensor was used to identify constraints to site remediation of BTEX.⁷³ Besides, a novel fiber optic detection system that employs a *lux* bioreporter organism was used to monitor real-time, *in situ* microbial activity in porous media.⁷⁴

Chapter 3

Untangling the Multiple Soluble Di-iron Monooxygenases Harbored by *Pseudonocardia dioxanivorans* CB1190

3.1 Introduction

Pseudonocardia dioxanivorans CB1190 is the first isolated bacterial strain that is capable of growing on dioxane as sole carbon and energy source.³⁹ Although an increasing number of microbes have been identified as their ability to transform dioxane,⁴⁰ CB1190 still serves as the model bacterium, since it can mineralize dioxane to CO₂ as well as assimilate it into biomass.^{39, 75} Such metabolic process is proposed to be initiated by monooxygenases. Metabolites of dioxane can be further catabolized and enter TCA cycle through the glyoxylate carboligase pathway to support the growth of CB1190.⁴⁶

A significant amount of energy is required to break the C-O bond in cyclic ethers, which greatly inhibits their decomposition by microorganisms. Therefore, the research in this chapter focuses on identifying the essential genes in the genome of CB1190 that encode the catabolic enzymes able to initiate the oxidation of cyclic ethers, including dioxane and THF. This is of great importance for the design and development of forensic tools to evaluate dioxane biodegradation in both pure cultures and complex environment.

3.2 Materials and Methods

3.2.1 Chemicals

All chemicals used in the experiments were of ACS grade or better. 1,4-Dioxane (99.9%, stabilized with 10 mg/L sodium diethyldithiocarbonate) was purchased from EM Science, Cherry Hill, NJ. 1,4-Dioxane-d₈ (99.9%) and tetrahydrofuran (99%) was obtained from Sigma Aldrich, St. Louis, MO. Dimethylene chloride (99.9%) and 1-butanol were obtained from Fisher Scientific, Fair Lawn, NJ. Anhydrous sodium sulfate and anhydrous mercury chloride were purchased from Thermo Fisher Scientific, Waltham, MA.

3.2.2 Reference strain

CB1190 (ATCC #55486) was grown in ammonium mineral salts (AMS) medium fed with dioxane as sole carbon and energy source at 24 °C shaking at 120 rpm. Cells were harvested at stationary phase by centrifugation at 8000 rpm for 15 min. The supernatant was decanted, and the pellets were washed three times with phosphorous buffer to remove all the organic residues. Then, the cell pellets were resuspended with 10 mL AMS medium for further treatment.

One liter of ammonium mineral salts (AMS) medium contained 100 mL of 10x salts solution, 1.0 mL of AMS trace elements, 1.0 mL of stock A, and 20 mL of 1.0 M phosphate buffer (added after sterilization). The AMS 10 × salt solution contained 6.6g of $(\text{NH}_4)_2\text{SO}_4$, 10.0 g of $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 0.15g of $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$. The AMS trace elements contained, per liter, 0.5 g of $\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$, 0.4g of $\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$, 0.02g of $\text{MnSO}_4 \cdot \text{H}_2\text{O}$, 0.015g of H_3BO_3 , 0.01g of $\text{NiCl}_2 \cdot 6\text{H}_2\text{O}$, 0.05g of $\text{CoCl}_2 \cdot 2\text{H}_2\text{O}$, and 0.25 of EDTA. AMS stock A contained, per liter, 5.0g of Fe-Na EDTA and 2.0g of $\text{NaMoO}_4 \cdot 2\text{H}_2\text{O}$. The 1M phosphate buffer contained 113.0 g of K_2HPO_4 and 47.0 g of KH_2PO_4 .

3.2.3 Culture growth and RNA extraction

Triplicate microcosms were prepared with 100 mL AMS medium inoculated with 1 mL concentrated CB1190 culture solution in 250 mL amber bottles. Then different treatments were separately amended with dioxane (100 mg/L), THF (100 mg/L), glucose (1 g/L), or acetate (1 g/L) as sole carbon source and incubated at room temperature while shaking at 120 rpm. The concentrations of amended organic chemicals in the microcosms were monitored everyday by Agilent 5890 Chromatograph (GC) with a Flame Ionization Detector (FID).¹³

CB1190 tends to form clumps on the surface of the aqueous phase. Therefore, when more than half of the added substrates were depleted (1 to 5 days), bacterial cells were harvested by filtering through 0.45 μm HA membrane filters (HAWP02500, Millipore, Billerica, MA) assembled with Swinnex 25 mm filter holders (Millipore, Billerica, MA). The biomass along with the filter was then immediately dissolved in the lysozyme solution (10 mg/mL) in a 15 mL centrifuge tube. After adding SDS solution and lysis buffer, the cell lysate was homogenized using an ultrasonic homogenizer Sonic Ruptor 250 (Omni International, Kennesaw, GA) at the power output of 80 for 2 min. Then the solution was transfer to a 1.7 mL sterile tube by pipetting, and then purified using PureLink RNA Mini kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, followed by On-column PureLink DNase Treatment (Invitrogen, Carlsbad, CA) to remove the interference from genomic DNA. RNA concentrations were determined by Nanodrop ND-1000 from Nanodrop products Inc. (Wilmington, NE). cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, CA) added with RNase inhibitor (Applied Biosystems, Carlsbad, CA), and then purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) following the manufacturer's instruction. The DNA elution (50 μL) was diluted to 1ng/ μL with DNA/RNase free water.

3.2.4 Reverse transcription - quantitative PCR (RT-qPCR)

To determine expression of all seven putative SDIMO genes harbored by CB1190, paired primers (Table 3.1) with the length of 18 to 22 bp were designed to target the α -subunits of these MOs using the PrimerQuest software from IDTDNA (<http://www.idtdna.com/Primerquest/Home/Index>). The expected amplicon size ranged from 80 to 120 bp with the annealing temperatures set as 58 to 62 °C for all primers.

Table 3. 1 Primers used in RT-qPCR.

Targeted Gene	Primer	Sequence (5' - 3')
<i>prmA</i>	Psed_0629_F	AAC ATG GGT CGC CTG GTC GG
	Psed_0629_R	TTG CCG TCG TCC CGA ACG AA
<i>dmpN</i>	Psed_0768_F	CAT GTC GCC GTT GAA ACT
	Psed_0768_R	CGA GTT CCT GAT CTC CAT CT
<i>tmo1A</i>	Psed_0815_F	TTC CCG CCG TAG GAC AGG GA
	Psed_0815_R	GTT GCC GTG GTT GTG CAG CA
<i>tmo3A</i>	Psed_1155_F	CTC TCC GAG TAC GCC GCC TG
	Psed_1155_R	GCC ATG TCG GAG CTC GTC GA
<i>tmo2A</i>	Psed_1436_F	CTC TGC AGC CTG TGC CAC CT
	Psed_1436_R	CCC GTT GTG GGT GAG CGA GT
<i>tmo4A</i>	Psed_6062_F	GCT CCA TGA ACT GCT TGA
	Psed_6062_R	GGT CTG TCG ATG GAC TAC TA
<i>dxmA</i>	Psed_6976_F	GGA CCG TGC ACG CAT TCG TC
	Psed_6976_R	CGT GGT CCA GCT TTC CGG GT
16S rRNA	CB1190_16S_F	TGG GTT TGA CAT GCA CCA GAC A
	CB1190_16S_R	ATA ACC CGC TGG CAA CAT GGA A

Quantitative PCR was performed using a 7500 real time PCR system from Applied Biosystems (Carlsbad, CA) in 15 μ L of reaction mixture composed of 1 μ L cDNA (1ng/ μ L), Power SYBR Green PCR Master Mix (7.5 μ L), 0.3 μ M of each primer and DNA/RNase free water. The reaction temperature program included 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The method was used to quantify differential gene expression, and the results were analyzed with the calculation formulae below for the expression fold change.

$$\Delta\Delta C_T, \text{Target gene} = (C_{T, \text{Target gene}} - C_{T, \text{Housekeeping gene}})_{\text{Treatment}} - (C_{T, \text{Target gene}} - C_{T, \text{Housekeeping gene}})_{\text{Control}}$$

In this case, target genes are the genes encoding α subunits of different SDIMOs possessed by CB1190. Specific primers targeting the 16S rRNA gene of CB1190 were designed and used as the housekeeping gene to normalize experimental variances.

3.3 Results and Discussion

3.3.1 Unveiling the seven SDIMO genes harbored by CB1190

As the genome of CB1190 has been recently sequenced by the U.S. Department of Energy Joint Genome Institute (JGI) in 2011,⁷⁶ seven putative gene clusters encoding SDIMOs were annotated by searching the monooxygenase component *mmoB/dmpM* against the whole CB1190 genome, which included one putative phenol hydroxylase (*dmpKLMNOP*), four putative toluene-4-monooxygenases (*tmo1ABDECF*, *tmo2ABDECF*, *tmo3ABCDE+F?*, and *tmo4ABCDEFGF*), one putative propane monooxygenase (*prmABCD*), and one putative THF/dioxane monooxygenase (*dxmADBC*). The proposed components of each gene cluster were listed in Table 3.2, and their arrangements were visualized in Figure 3.1. It is notable that all the

predicted monooxygenases other than the putative THF/dioxane monooxygenase are located in the chromosome of CB1190.

Table 3. 2 Genetic components of seven putative SDIMOs in CB1190.

Paired gene names were assigned according to their sequence matches and positions in the gene clusters. The genes encoding the α subunits of SDIMOs are highlighted in yellow.

Gene ID (Psd)	0629	0630	0631	0632		
Annotation in GenBank	methane monooxygenase	ferredoxin--NAD(+) reductase	methane/phenol/toluene hydroxylase	monooxygenase component MmoB/DmpM		
Paired Putative Genes	<i>prmA</i>	<i>prmB</i>	<i>prmC</i>	<i>prmD</i>		
Gene ID (Psd)	0766	0767	0768	0769	0770	0771
Annotation in GenBank	Phenol 2-monooxygenase	Phenol hydroxylase conserved region	Phenol 2-monooxygenase	monooxygenase component MmoB/DmpM	Phenol 2-monooxygenase	hypothetical protein
Paired Putative Genes	<i>dmpP</i>	<i>dmpO</i>	<i>dmpN</i>	<i>dmpM</i>	<i>dmpL</i>	<i>dmpK</i>
Gene ID (Psd)	0810	0811	0812	0813	0814	0815
Annotation in GenBank	ferredoxin--NAD(+) reductase	Rieske (2Fe-2S) iron-sulfur domain-containing protein	methane/phenol/toluene hydroxylase	monooxygenase component MmoB/DmpM	Toluene-4-monooxygenase system B	methane/phenol/toluene hydroxylase
Paired Putative Genes	<i>tmo1F</i>	<i>tmo1C</i>	<i>tmo1E</i>	<i>tmo1D</i>	<i>tmo1B</i>	<i>tmo1A</i>
Gene ID (Psd)	1155	1156	1157	1158	1159	1160
Annotation in GenBank	Phenol 2-monooxygenase	hypothetical protein	Rieske (2Fe-2S) iron-sulfur domain-containing protein	monooxygenase component MmoB/DmpM	methane/phenol/toluene hydroxylase	transcriptional regulator CdaR
Paired Putative Genes	<i>tmo3A</i>	<i>tmo3B</i>	<i>tmo3C</i>	<i>tmo3D</i>	<i>tmo3E</i>	<i>tmo3F?</i>
Gene ID (Psd)	1436	1437	1438	1439	1440	1441
Annotation in GenBank	methane/phenol/toluene hydroxylase	Toluene-4-monooxygenase system B	monooxygenase component MmoB/DmpM	methane/phenol/toluene hydroxylase	Rieske (2Fe-2S) iron-sulfur domain-containing protein	ferredoxin--NAD(+) reductase
Paired Putative Genes	<i>tmo2A</i>	<i>tmo2B</i>	<i>tmo2D</i>	<i>tmo2E</i>	<i>tmo2C</i>	<i>tmo2F</i>
Gene ID (Psd)	6056	6057	6058	6059	6060	6061
Annotation in GenBank	ferredoxin--NAD(+) reductase	alcohol dehydrogenase GroES domain-containing protein	methane/phenol/toluene hydroxylase	monooxygenase component MmoB/DmpM	hypothetical protein	Toluene-4-monooxygenase system B
Paired Putative Genes	<i>tmo4F</i>	<i>tmo4G?</i>	<i>tmo4E</i>	<i>tmo4D</i>	<i>tmo4C</i>	<i>tmo4B</i>
Gene ID (Psd)	6976	6977	6978	6979		
Annotation in GenBank	hypothetical protein	Ferredoxin--NAD(+) reductase	methane/phenol/toluene hydroxylase	monooxygenase component MmoB/DmpM		
Paired Putative Genes	<i>thmA</i>	<i>thmD</i>	<i>thmB</i>	<i>thmC</i>		
Gene ID (Psd)						
Annotation in GenBank						
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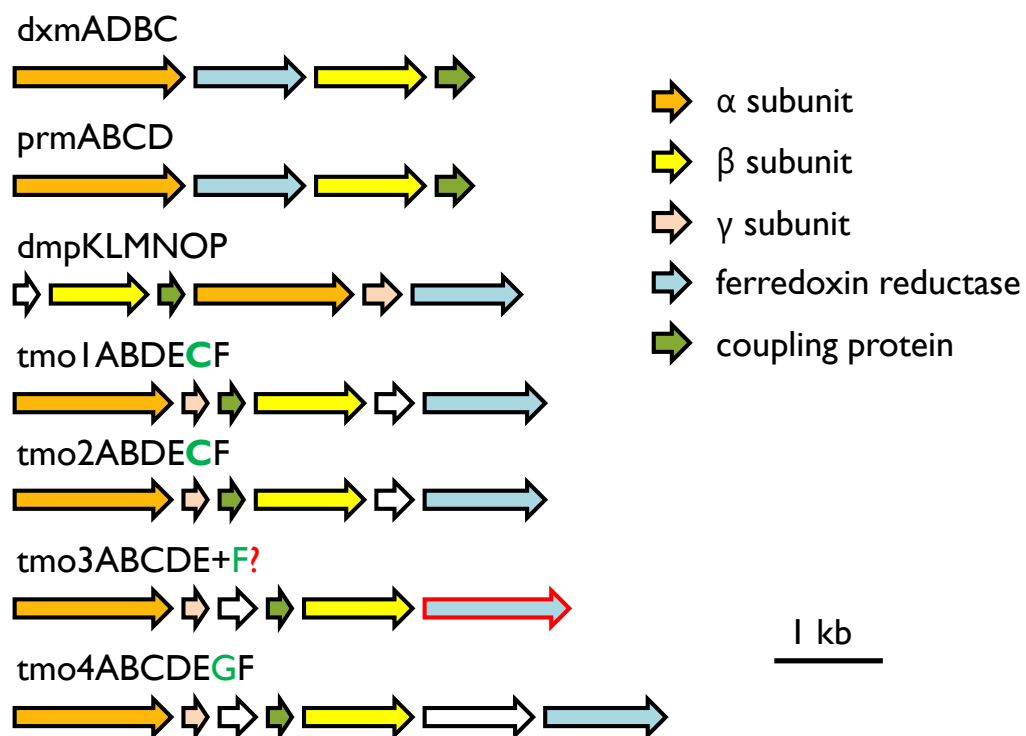


Figure 3. 1 Organization of seven monooxygenase gene clusters.

Functioning ORFs are indicated by block arrows (sized to scale) below their predicted operon name. Key components of SDIMOs are highlighted in different colors.

Table 3. 3 Amino acid sequence analysis of the α subunit of SDIMOs possessed by CB1190.

Protein Accession	Gene Name	Best GenBank Match	Maximum Identity
YP_004330880	<i>dmpN</i>	putative phenol hydroxylase component [<i>Gordonia amarae</i> NBRC 15530]	86%
YP_004330924	<i>tmo1A</i>	putative methane/phenol/toluene monooxygenase [<i>Gordonia polyisoprenivorans</i> VH2]	82%
YP_004331528	<i>tmo2A</i>	putative methane/phenol/toluene monooxygenase [<i>Gordonia polyisoprenivorans</i> VH2]	82%
YP_004331255	<i>tmo3A</i>	methane/phenol/toluene hydroxylase [<i>Streptomyces</i> sp. AA4]	60%
YP_004336024	<i>tmo4A</i>	methane/phenol/toluene hydroxylase [<i>Kyrpidia tusciae</i> DSM 2912]	71%
YP_004330745	<i>prmA</i>	methane monooxygenase [<i>Pseudonocardia</i> sp. P1]	92%
CP002597*	<i>dxmA</i>	alpha-subunit of multicomponent tetrahydrofuran monooxygenase [<i>Pseudonocardia</i> sp. K1]	99%

* No protein accession number available.

Note that the gene cluster of this putative dioxane MO is highly identical in both arrangement and sequence with the putative THF MOs from three isolated THF degraders, including *Pseudonocardia* sp. K1, *Pseudonocardia* sp. ENV478, and *Rhodococcus* sp. YYL.^{19, 22} According to blastp analysis, the maximum identities between their amino acid sequences are 99%, 99%, 95%, respectively. In addition, Kim and his colleagues isolated four cyclic ether-utilizing strains from activated sewage sludge enriched with THF. All four strains harbor *thmA*-like genes revealed by restriction fragment analysis.⁷⁷ It is noteworthy that this gene cluster is located on a medium-sized plasmid pPSED02 (66,907 bp, accession number: NC_016601). This elicits the possibility and feasibility for indigenous microorganisms to obtain dioxane degradative capability via horizontal gene transfer under the pressure of high concentration of dioxane in the source zone at field sites.

Due to the same operon structure and component assembly, both THF/dioxane MOs and propane MOs are included in the Group 5 of SDIMOs.¹⁵ Phylogenetic analysis (Figure 3.2) revealed a large number of *Actinomycetales* possessing similar propane MOs as CB1190. Among them, the *prm*-like MO sequence in CB1190 is highly homologous to the gene cluster *prm1ABCD* from *Pseudonocardia* sp. TY-7 (PID: BAF34304) with identity of 85% for their α subunits. Northern blot analysis and RT-qPCR results suggested that the whole gene cluster of *prm1ABCD* was induced by gaseous n-alkane except methane (i.e., ethane, propane, and butane), and the main metabolic pathway was confirmed as subterminal oxidation in *Pseudonocardia* sp. TY-7 by assessing its growth substrates in whole-cell assays.⁷⁸ Moreover, *prmA*-like genes were present in several Gram-positive *Rhodococcus* and *Gordonia* strains, which were proved to be greatly correlated with their degradation activities for linear alkyl ethers and dibenzyl ether.⁷⁷ Hence, both *thm*-like and *prm*-like MOs may be in charge of the initial hydroxylation of small molecule alkane and ether compounds at an internal carbon position where the electron density is relatively low.⁷⁷

Phenol hydroxylase is one of the key enzymes to break phenolic compounds and form corresponding catechols. This is identified as the rate-limiting step of the degradation pathway.⁷⁹

There exists a six-component MO gene cluster in the chromosome of CB1190, whose obtained sequences are putative orthologues that constitute non-heme phenol hydroxylases. The amino acid sequence of its α subunit is closely related to *Gordonia amarae* NBRC 15530 (Table 3.3). A series of research conducted in Prof. Watanabe's lab has established the relationship between the kinetic trait of phenol consumption and the phylogeny of the harbored phenol hydroxylase for phenol-degrading bacteria isolated from activated sludge and TCE-impacted aquifer. Multi-component phenol hydroxylases were divided to three genotypes, corresponding to the three kinetic groups (i.e., low, medium, and high affinity constant K_s for phenol).⁸⁰ We included the sequence from the putative phenol hydroxylase of CB1190, and rebuilt the phylogenetic tree based on amino acid sequences of their α subunits.

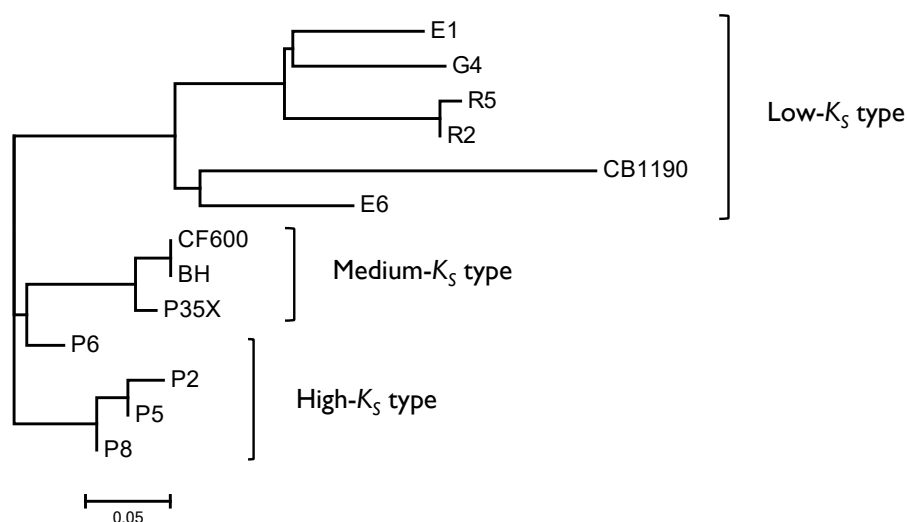


Figure 3.2 Phylogenetic analysis of CB1190 and several phenol-degrading bacteria according to the amino acid sequences of their α subunits.

The bar represents 0.05 substitution per site.

Figure 3.2 shows that the putative phenol hydroxylase of CB1190 belongs to the low- K_s phenotype, indicating its growth on phenol may be relatively slow. Meanwhile, those low- K_s type of phenol degraders generally exhibited very high activities to fortuitously degrade TCE while using phenol as the substrate inducer.⁸¹ Therefore, it is possible that CB1190 may be capable of transforming TCE after growth with phenol compared to other low- K_s type of phenol-degrading bacteria.

Besides the three SDIMO gene clusters mentioned above, all the other four gene clusters contain *tmo*-like genes encoding toluene-4-MOs. Toluene-4-MOs are able to hydroxylase toluene and generate *p*-cresol, and the gene cluster *tmoABCDEF* from *Pseudomonas mendocina* KR1 is the first identified archetypal member of Group 2 of SDIMO genes.⁸²⁻⁸⁴ This group of SDIMOs is distinct from other groups, because the enzymes in this group contain a Rieske-type ferredoxin protein (*tmoC*) functioning as the iron-sulfur ferredoxins from aromatic ring dioxygenases.⁸⁵ However, the position of this *tmoC* gene in both gene cluster *tmo1* and *tmo2* from CB1190 is greatly different from that from *Pseudomonas mendocina* KR1, locating between the coupling protein (*tmoE*) and the NADH reductase (*tmoF*). Blastp also revealed that the amino acid sequences of *tmo1* and *tmo2* are highly identical (82% for α subunits). Duplicated

copies of a similar gene cluster may avoid the loss of essential functional genes caused by mutations. Functional redundancy is common for bacteria possessing SDIMOs,⁸⁶ but it is hard to discern such genetic duplication for catabolic genes, because small difference in their genes can alter their substrate specificities or functioning conditions.⁸⁷ For *tmo3* in Figure 3.1, the *tmoF* gene encoding the NADH reductase is absent, and substituted by a gene encoding transcriptional regulator CdaR. The gene cluster *tmo4* is the only one containing all essential genes with the same arrangement order as that from *Pseudomonas mendocina* KR1. However, there is an insertion of an alcohol dehydrogenase GroES domain-containing protein (tmoG?) between *tmo4E* and *tmo4F*. Overall, in the chromosome of CB1190, none of the four putative toluene-4-MO genes are fully identical to the previously-well-studied SDIMO genes in Group 2 based on their component arrangement and structure. It was reported that such *tmo*-like genes were rarely detected in BTEX degraders isolated from an oil-refinery site.⁸⁸ This is the first evidence of an Actinomycetale strain harboring four *tmo*-like genes, illustrating a competitive advantage for CB1190 in adapting to various polluted environments.

The cofounding of diverse content of monooxygenase genes possessed by CB1190 implies its broad metabolic versatility. CB1190 may be able to degrade diverse contaminants other than ethers (e.g., dioxane, THF, 2-methyl-1,3-dioxolane and butyl methyl ether), alcohols, and benzene.³⁹ Moreover, it also provides evidence that propane, toluene, phenol, or other similar compounds may be suitable as auxiliary substrate candidates for *in situ* bio-augmented remediation of dioxane or chlorinated solvents, which sustain the degrading bacteria and stimulate related MOs at the same time.

3.3.2 Up-regulation of the *dxmA* gene during dioxane, and THF metabolism

To gain the insights into the dominant SDIMOs involved in dioxane metabolism, reverse transcription quantitative PCR (RT-qPCR) was used to assess the expression levels of the α subunit of each SDIMOs in CB1190 (highlighted in yellow in Table 3.2) in the dioxane, THF, and glucose treatment compared with the acetate treatment as experimental control.

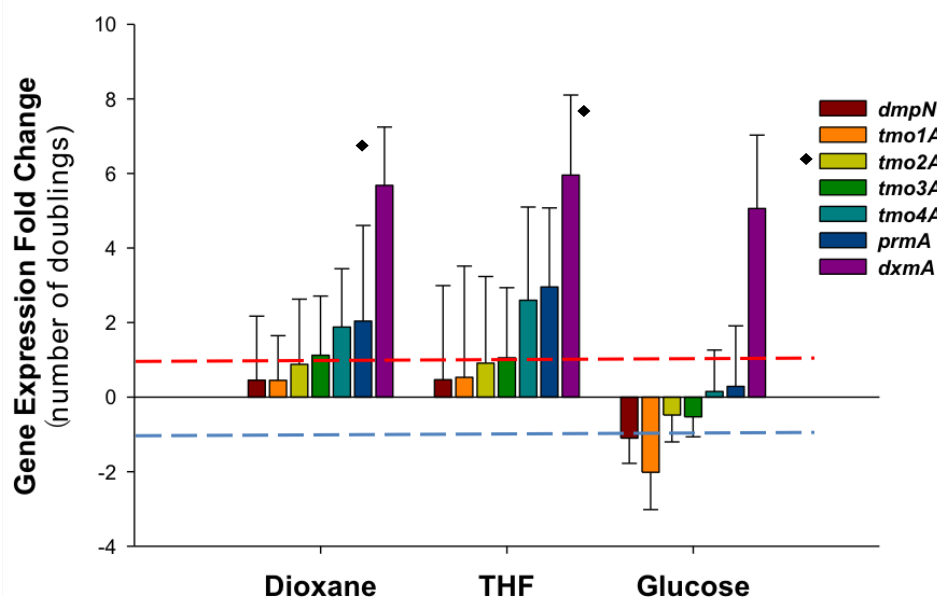


Figure 3. 3 Expression of genes encoding α subunits of the seven putative SDIMOs by CB1190 fed with various substrates.

The $2^{-\Delta\Delta CT}$ method was used to quantify differential gene expression.⁸⁹ Results were normalized to the control treatment (fed sodium acetate as sole carbon source). Error bars represent $\pm$ one standard deviation from the mean of triplicate measurements. The dotted red line represents 2-fold up-regulation, and the dotted blue line represents 2-fold down-regulation. The diamonds indicate significant ($p < 0.05$) up-regulation and down-regulation compared to the acetate-fed control.

Among the seven SDIMOs of CB1190, only *dxmA* was identified to be significantly up-regulated (Figure 3.3) during both dioxane and THF metabolism compared with the acetate control ($p < 0.05$). And the expression doubling fold changes were 5.7 ± 1.6 and 6.0 ± 2.1 , respectively. These results are in accordance with former transcription tests and protein assays for *thm* genes from THF-degrading bacteria.⁴⁶ Moreover, all four components of this putative dioxane monooxygenase cluster were confirmed be up-regulated after amendment with dioxane compared with pyruvate control according to both transcription microarray (*dxmD*, *dxmB*, and *dxmC*) and RT-qPCR (*dxmA*).⁴⁶ Surprisingly, strong RT-PCR products were obtained for this gene even when fed with glucose as sole carbon source.

To some extent, *tmo4A* and *prmA* genes exhibited up-regulations induced by both dioxane and THF, but not glucose. The cometabolism of dioxane was observed for toluene-4-MO containing strain *Pseudomonas mendocina* KR1 with toluene as the growth substrate. Further, the catabolic capability of toluene-4-MOs to transform dioxane was confirmed by using *Escherichia coli* strain TG1/pBS(Kan) containing constitutively expressed toluene-4-MO.¹¹ Therefore, the up-regulation of the expression of *tmo4A* demonstrated that this toluene-4-MO was induced by cyclic ethers and might participate in their degradation processes. For propane MOs, although no direct research have been conducted to prove its transformation capabilities on cyclic ethers, the presence of this gene in several bacterial strains was correlated with their degradation activities on linear alkyl ethers and dibenzyl ether.⁷⁷ We hypothesize that this

propane MO may be partially induced by the linear ether intermediates (e.g., 2-Hydroxyethoxyacetaldehyde) once the dioxane MO break the cycle.⁷⁵

Overall, our results support our hypothesis that the dioxane monooxygenase (*dxmADB*) played an essential role in transformation of cyclic ethers (e.g., dioxane and THF) by CB1190, and suggested the *dxmA* gene might be a great candidate for developing catabolic biomarkers to monitor intrinsic dioxane degradation.

Chapter 4

Development and Validation of Catabolic Biomarkers for Monitored Natural Attenuation of Dioxane

4.1 Introduction

Monitored natural attenuation (MNA) is among the most cost-effective approaches to manage groundwater contamination by organic pollutants at low concentrations.¹¹ However, the feasibility of MNA requires demonstration of site-specific biodegradation capabilities. Recent findings by our lab and others suggest that indigenous bacteria that can degrade dioxane might be more widespread than previously assumed.¹⁷ However, these studies relied on advanced microbial molecular techniques, such as cloning, microarray, restriction fragment length polymorphism (RFLP), and phospholipid fatty acid analysis associated with stable isotope probes (PLFA-SIP), which can be labor-intensive and may not provide unequivocal evidence to link the abundance of the indigenous degraders to the intrinsic biodegradation activity.

Numerous catabolic and phylogenetic biomarkers have been tested to assess biodegradation of different contaminants (e.g., *bssA* for anaerobic toluene degradation, and *tceA* for reductive dechlorination of trichloroethylene).^{80, 80, 90-97} Although selectivity and sensitivity can be highly variable for different probes and matrices, biomarkers offer a relatively straightforward approach to delineate *in situ* biodegradation potential.

Multiple lines of circumstantial evidence suggest that *thmA/dxmA* genes, encoding the large hydroxylase subunits of tetrahydrofuran (THF)/dioxane monooxygenases, would be excellent candidates as biomarkers for dioxane biodegradation. Here, we develop a genetic primer/probe set targeting *thmA/dxmA* genes, assess its sensitivity and selectivity, and explore the correlation between the abundance of this catabolic biomarker and dioxane degradation activity at various contaminated sites. This effort enables the determination of the (site-specific) feasibility of MNA for dioxane plumes and enhances performance assessment.

4.2 Materials and Methods

4.2.1 Primer and probe design

Multiple sequence alignment (Clustal X 2.1)⁹⁸ was used to identify homologous regions between the four *thmA/dxmA* genes available on NCBI and avoid overlap with other soluble di-iron monooxygenase (SDIMO) genes that do not share the same primary substrate range. The phylogenetic tree based on amino acid sequences was then visualized using MEGA 5.1.⁹⁹

DNA residues 217 and 587 from the putative *dxmA* gene of CB1190 were used as the input sequence for Primer Quest (Integrated DNA Technologies, Coralville, IA) to generate a series of possible primer/probe sets which satisfied the design criteria for TaqMan assays. After manual comparison and adjustment, the final set (Table 4.1) was chosen allowing a nucleotide

mismatch not greater than one, including the forward primer, 5'- CTG TAT GGG CAT GCT TGT -3', the reverse primer, 5'-CCA GCG ATA CAG GTT CAT C -3', and the probe, 5'- (6-FAM) - ACG CCT ATT - (ZEN) - ACA TCC AGC AGC TCG A - (IABkFQ) -3'. The amplicons were approximately 115 bp in length. All primers and probes were synthesized by Integrated DNA Technologies, and a novel internal quencher ZEN was integrated to reduce background noise.

Table 4. 1 Properties of the primers and probe targeting *thmA/dxmA* genes.

Probe/Primer	Name	Sequence (5'-3')	Size	GC	Tm
<i>Forward Primer</i>	<i>thmA</i> TqFWD330	CTG TAT GGG CAT GCT TGT	18	50	59.8
<i>Reverse Primer</i>	<i>thmA</i> TqREV444	CCA GCG ATA CAG GTT CAT C	19	52.6	59.7
<i>Taqman Probe</i>	<i>thmA</i> TqPRB377	/6-FAM/ACG CCT ATT /ZEN/ACA TCC AGC AGC TCG A/IABkFQ/	25	52	68.9

4.2.2 Specificity and coverage tests with bacterial genomic DNA

To evaluate the specificity and selectivity of the *thmA/dxmA* probe and primer set, qPCR was conducted with the genomic DNA isolated from reference strains (Table 4.3). After growth in LB or R2A media at room temperature for 1 to 7 days, cells were harvested by centrifugation, and their genomic DNA was extracted using an UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA). The final DNA concentrations were measured by UV spectroscopy using an ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE).

4.2.3 Microcosm studies mimicking dioxane natural attenuation

To assess the efficacy of the catabolic biomarker in enhancing the forensic analysis of MNA, aquifer materials and groundwater samples were collected from 20 monitoring wells from five different dioxane-impacted sites in the U.S. (3 in CA, 1 in AK, and 1 in TX). Triplicate microcosms were prepared with dioxane-impacted groundwater (100 to 150 mL with initial dioxane concentrations reaching up to 46,000 µg/L) and aquifer materials (50 g) and incubated at room temperature under aerobic conditions. To distinguish abiotic losses of dioxane, sterile controls were prepared with autoclaved samples and poisoned with HgCl₂ (200 mg/L). Dioxane concentrations were monitored for 12 to 20 weeks using a frozen micro-extraction method followed by GC/MS.¹⁰⁰

At the beginning and the termination of the microcosm experiments, 10 mL of sample mixture was transferred into a 15 mL centrifugation tube. Aquifer materials together with biomass were separated by centrifugation at × 10,000 g for 20 min. Total microbial genomic DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA). The eluted

DNA (100 μ L) was further purified and concentrated to 16 μ L using a Genomic DNA Clean & Concentrator Kit (Zymo Resesarch, Irvine, CA). The DNA Extraction efficiency and PCR inhibition factor were determined by recovery of bacteriophage λ DNA (Sigma-Aldrich, St. Louis, MO), which was added as internal standard.⁹²

4.2.4 Quantitative PCR

qPCR assisted with Taqman assays was used to quantify *thmA/dxmA* genes from dioxane-degrading bacteria as well as total Bacteria.¹⁰¹ The PCR reaction mixture contained 1 μ L of undiluted DNA (or 1 ng/ μ L diluted bacterial genomic DNA), 300 nM of the forward and reverse primers, 150 nM of the fluorogenic probe, 10 μ L of TaqMan universal master mix II (Applied Biosystems, Foster City, CA), and DNA-free water to reach a total volume of 20 μ L. The qPCR was performed with a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) using the following temperature program: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions (10^{-4} - 10^1 ng DNA/ μ L) of the extracted genomic DNA of CB1190 were utilized to prepare the calibration curves for both *dxmA* (1 copy/genome) and 16S rDNA (3 copies/genome) genes. Assuming a genome size of 7.44 Mb^{76, 102} and 9.124×10^{14} bp/ μ g (i.e., $[6.022 \times 10^{17} \text{ Da}/\mu\text{g of DNA}]/[660 \text{ Da/bp}]$) for CB1190, the gene copy numbers were calculated based on the equation below.

$$\frac{\text{gene copies}}{\mu\text{L}} = \left(\frac{\frac{\mu\text{g of DNA}}{\mu\text{L}}}{\frac{7.44\text{Mb}}{\text{genome}}} \right) \left(\frac{9.124 \times 10^{14} \text{ bp}}{\mu\text{g of DNA}} \right) \left(\frac{\text{gene copies}}{\text{genome}} \right)$$

The calibration curves (Figure 4.1) were generated using series dilution of standard CB1190 DNA samples (10^{-4} - 10^1 ng genomic DNA/ μ L) corresponding to a known gene copy number over six orders of magnitude (i.e., 12 - 1.23×10^6 for *thmA/dxmA* and 37 - 3.68×10^6 for 16S rRNA). High amplification efficiency of 95% was obtained in *thmA/dxmA* quantification, with an R^2 value of 0.998 and a slope of -3.45. Similarly, efficiency of 92% was obtained for quantification of 16S rRNA genes, with an R^2 value of 0.996 and a slope of -3.52.

The qPCR instrument MDL is identified as the minimum detectable copy number when seven sequential analyses were successful. The overall MDLs (in copy numbers/gram of aquifer materials in microcosms) are calculated as the instrument qPCR MDLs (in copy numbers/reaction mixture) adjusted with the DNA recoveries of these seven quantifications and the proportion (F) of the DNA used as the template in the qPCR.

$$\text{Overall MCL} = \frac{\text{qPCR instrument MDL}}{\text{DNA Recovery}} \times F$$

Method detection limits (MDLs) were 7,000 ~ 8,000 copies of *thmA/dxmA* genes/g soil and 2,000 ~ 3,000 copies of 16S rRNA genes/g soil (Table 4.2) DNA extraction recoveries ranged from 2.3 to 48.9 %. Similar recovery ranges are commonly reported for soil DNA extractions,^{92, 96, 103} with the lower values reflecting sequential elution and residual impurities that hinder *Taq* polymerase reactions.¹⁰⁴

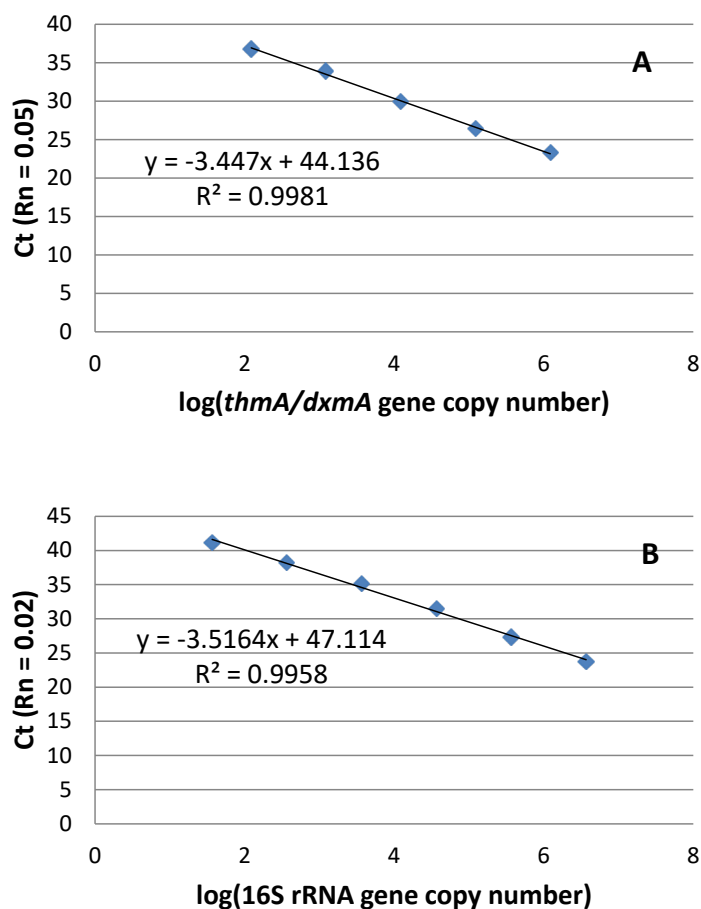


Figure 4. 1 Q-PCR calibration curves for *thmA/dxmA* genes (A) and 16S rRNA (B).

Table 4. 2 Method detection limits (MDLs) for *thmA/dxmA* and 16S rRNA genes.

Parameter	<i>thmA/dxmA</i>	16S rRNA
qPCR instrument MDL (copy numbers/reaction mixture)	123	37
Overall MDL ^a (copy numbers/g soil)	7,203-7,984	2,324-2,573

^a Including DNA recovery and an F of 64.

4.2. 5 Clone library construction

PCR was performed in 50 μ L samples with 1 μ L concentrated genomic DNA, 0.2 mM dNTPs, 2.5 mM MgCl₂, 0.8 mM of each primer (Table 4.1). 1 $\times$ Green GoTaq Flexi buffer, and 1.25 U GoTaq Hot Start polymerase (Promega, Madison, WI). Thermocycling conditions for the PCR

reaction were as follows: initial denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C for 45 s, 50 °C for 45 s, and 73 °C for 30 s, and a final elongation at 73 °C for 5 min. The final PCR products (approximately 115 bp) were checked by gel electrophoresis.

The PCR products were then purified and concentrated using DNA Clean & Concentrator-5 kit (Zymo Research, Irvine, CA), and TOPO cloned into the pCR4-TOPO TA vector using the TOPO TA Cloning kit for Sequencing (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The TOPO reaction mixture was transformed into TOP10 competent cells (Invitrogen, Carlsbad, CA), which were grown on LB agar plates containing the antibiotic ampicillin. A total of 96 colonies were picked and cultured in LB medium with 50 µg/mL ampicillin overnight. Plasmid DNA was prepped using a proprietary alkaline lysis protocol followed by ethanol precipitation. DNA cycle sequencing was performed using BigDye Terminator v3.1 chemistry in conjunction with the M13F universal primer. Sequencing reactions were cleaned up on Sephadex. Sequence delineation and basecalling were performed using an ABI model 3730 XL automated fluorescent DNA sequencer by SeqWright Genomic Services (Houston, TX).

4.3 Results and Discussion

4.3.1 The *thmA/dxmA* probe is selective.

Biochemical, structural, and evolutionary studies indicate that the large hydroxylases of all the enzymes belonging to this SDIMO family contain a highly conserved carboxylate-bridged di-iron center (i.e., DE*RH motif) that serves as the active site for hydroxylation or peroxidation reactions.^{23, 43} However, different groups of SDIMOs exhibit different substrate specificity. Substrate recognition and binding may be primarily associated with the hydrophobic residues that surround the di-iron center, because these are conserved within each SDIMO group.^{23, 105-107} Since only THF/dioxane monooxygenases are of interest in this effort, the criteria for the *thmA/dxmA* biomarker design consisted of i) avoiding the di-iron centers conserved by all SDIMOs, and ii) targeting the surrounding hydrophobic residues only shared by THF/dioxane monooxygenases.

Figure 4.2 illustrates that the amino acid residues targeted by the *thmA/dxmA* primers/probe set are identical among all four known THF/dioxane monooxygenases, but significantly different from other SDIMOs. The q-PCR analysis indicated that both *dxmA* from CB1190 and *thmA* from K1 (which were the positive controls we had readily available) were detected with comparable sensitivity (C_T values approximately 25 for 1 ng genomic DNA). Negative controls, using genomic DNA from bacteria with other types of SDIMOs, (e.g., bacteria with dioxygenases such as *Pseudomonas putida* F1, and bacteria without SDIMOs such as *Escherichia coli* K12), and bacteriophage λ, were used to assess the potential for false positives. None of these negative controls (Table 4.3) were detected by this *thmA/dxmA* probe and primer set, but previously designed primer sets in our lab using the SYBR Green system had yielded false positives for other SDIMO genes (e.g., *tmo*), indicating that the use of TaqMan probes significantly reduces the possibility of hybridization with non-specific templates. These results corroborate that the *thmA* probe and primer set we developed for the TaqMan system

enables sensitive detection of *thm/dxm* genes and avoids false positives from other oxygenase genes that bear a close evolutionary relationship.

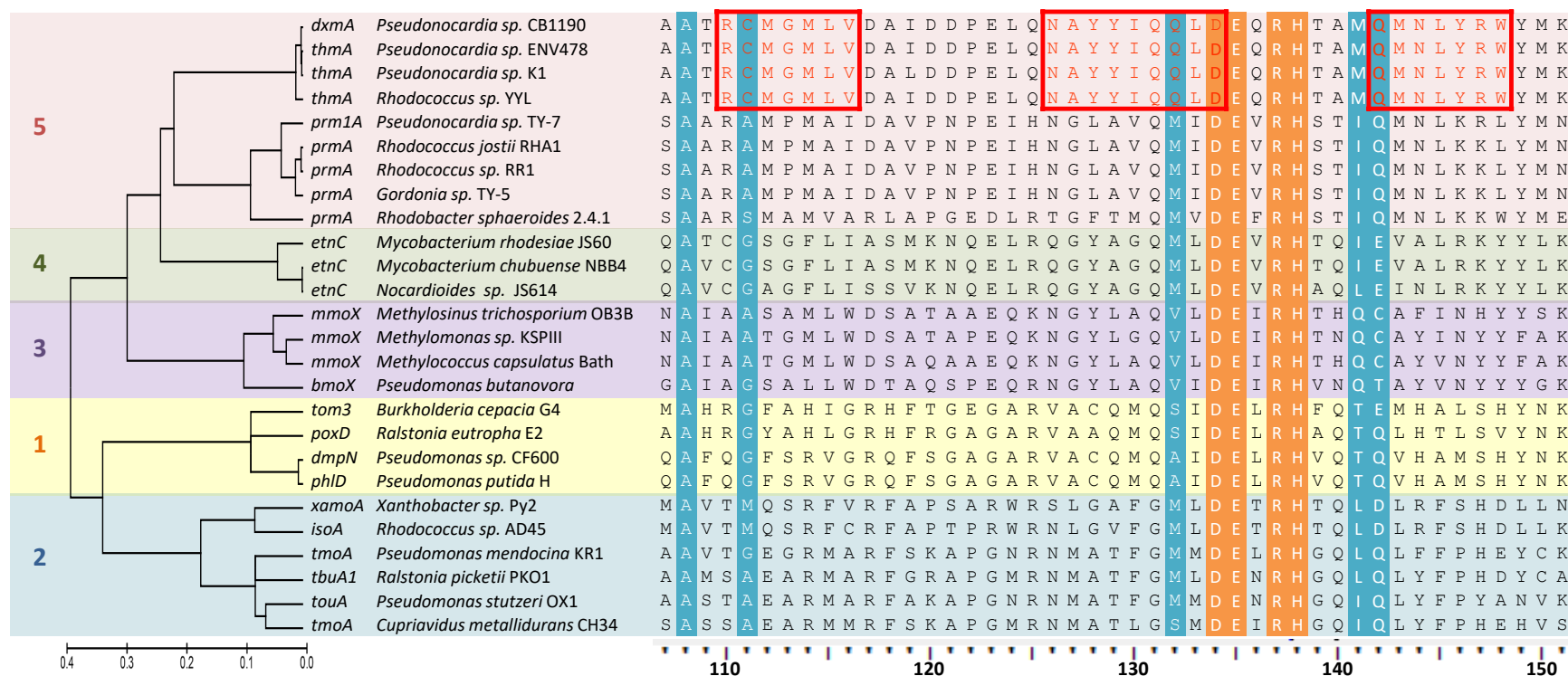


Figure 4. 2 Alignment of the deduced amino acid sequences corresponding to the large hydroxylase of SDIMOs clustered by subfamily. The amino acid residues selected for biomarker design are highlighted in red and circled with rectangular. The conserved di-iron center (i.e., DE*RH) was highlighted in orange, and the hydrophobic residues surrounding the diiron center were highlighted in blue. Numbers below the sequences correspond to the numbering for *dxmA* from CB1190. Numbers in front of the subclusters represent the group numbers of SDIMOs.

Table 4. 3 Specificity and coverage tests for the designed *thmA/dxmA* biomarker.

Gene Name	Encoding Enzymes ^a	SDIMO Group	Microorganism Strain	Biomarker Detection ^b	
				<i>thmA</i>	16S rRNA
<i>dxm</i>	Dioxane MO	5	<i>Pseudonocardia dioxanivorans</i> CB1190	+	+
<i>thm</i>	Tetrahydrofuran MO	5	<i>Pseudonocardia tetrahydrofuranooxydans</i> K1	+	+
<i>tmo</i>	Toluene-4-MO	2	<i>Pseudomonas mendocina</i> KR1	–	+
<i>tbu</i>	Toluene-3-MO	2	<i>Ralstonia pickettii</i> PKO1	–	+
<i>tom</i>	Toluene-2-MO	1	<i>Burkholderia cepacia</i> G4	–	+
<i>dmp</i>	Phenol HD	1	<i>Pseudomonas putida</i> CF600	–	+
<i>prm</i>	Propane MO	5	<i>Rhodococcus jostii</i> RHA1	–	+
<i>mmo</i>	Soluble methane MO	3	<i>Methylomonas methanica</i> MC09	–	+
-	-	-	<i>Escherichia coli</i> K12	–	+
-	-	-	Bacteriophage λ	–	–
<i>amo</i>	Ammonia MO	-	<i>Nitrosomonas europaea</i> Winogradsky	–	+
<i>tod</i>	Toluene DO	-	<i>Pseudomonas putida</i> F1	–	+
<i>xyl</i>	Toluene 1,2-DO	-	<i>Pseudomonas aeruginosa</i> PAO1	–	+

^a MO = monooxygenase; HD = hydroxylase; DO = dioxygenase.

^b + indicates a positive detection was obtained above the detection limit by using the primers/probe set in qPCR; – indicates no positive detection was obtained above the detection limit by using the primers/probe set in qPCR.

4.3.2 Microcosms revealed naturally occurring dioxane biodegradation

After three to five months of incubation, considerable dioxane removal was observed in 16 of the 20 microcosms prepared with aquifer materials collected at different locations at all five sites compared to the sterile controls (Figure 4.3).¹⁰⁸ This indicated that the presence of indigenous microbes that are capable of degrading dioxane. The fitted zero-order decay rates varied greatly from 10^{-1} to 10^3 $\mu\text{g/L/week}$ (Table 4.4), suggesting the enzymatic saturation of dioxane degrading oxygenases at the sites. For instance, the microcosms prepared with the source zone samples collected at Site 1 in California experienced the fastest dioxane degradation rate as high as 3448.7 ± 459.3 $\mu\text{g/L/week}$, which is the highest dioxane degradation rate reported in environmental samples. In addition, the dioxane degradation rates (11.1 ± 0.7 $\mu\text{g/L/week}$) in microcosms prepared with the source zone samples at the Alaska site were comparable with the ones previously reported by our group (approximately 9.8 $\mu\text{g/L/week}$),¹⁰⁹ indicating that dioxane degradation capability by the indigenous microbes at the Arctic site was consistent.

It is also notable that dioxane degradation activity decreased with increasing distance from the contaminant source zone, presumably due to less acclimation. Further, ^{14}C -labeled dioxane was amended in microcosms prepared with samples collected in California to track the transformation of dioxane. Percentage of mineralization to CO_2 and incorporation into biomass were significantly correlated with dioxane degradation activities observed in microcosms by monitoring the partitioning of ^{14}C radioactivity in air and solid phases, respectively. All these results indicate long term of dioxane exposure at high concentrations may promote the indigenous microbes to adapt and acclimate dioxane degradation capability.

Table 4. 4 Microcosm preparation and the observed dioxane degradation rates fitted with zero-order decay.

Site	Sampling Locations	Distance from the Source (ft)	Initial Dioxane Concentration ($\mu\text{g/L}$)	Zero-order Dioxane Degradation Rate ($\mu\text{g/L/week}$)
CA1	1-1S	0	46049.8 ± 2429.7	3448.7 ± 459.3
	1-1M	0	30906.1 ± 804.7	1548.7 ± 132.9
	1-1D	0	14214 ± 920.2	654.2 ± 49.5
	1-2S	200	1540.3 ± 103.9	69.6 ± 2.2
	1-2M	200	12034.8 ± 319.4	584.2 ± 14.7
	1-2D	200	19289.8 ± 1056.9	848.6 ± 51.6
	1-4	1550	412.7 ± 24.8	-
	1-5	3900	203.8 ± 12.8	-
	1-6	NA	ND (≤ 1.6)	-
CA2	2-1	NA	248.2 ± 7.8	9.9 ± 0.8
	2-2	NA	7.5 ± 0.2	0.3 ± 0.1
CA3	3-1	0	7150.7 ± 203.6	326.5 ± 8.5
	3-2	200	2261.5 ± 78.2	112.1 ± 5.6
	3-3	1000	876 ± 13.9	17.4 ± 3.2
	3-4	1350	582.9 ± 38.7	7.2 ± 0.9
	3-5	NA	32.4 ± 0.8	-
AK	A-201	0	516.8 ± 23.7	11.1 ± 0.7
	A-11	195	15.2 ± 0.9	0.4 ± 0.1
TX	T-S	0	238.6 ± 7.1	5.6 ± 0.2
	T-M	1700	109.7 ± 1.5	2.6 ± 0.1

4.3.3 Dioxane biodegradation activity was significantly correlated to *thmA* abundance

Growth of dioxane degraders in these microcosms was evident by an increase in *thmA* copy numbers, up to 114-fold (Figure 4.3). This increase was significantly correlated ($p < 0.05$, $R^2 = 0.72$) to the amount of consumed dioxane (Figure 4.5). However, *thmA/dxmA* genes were

not detected in killed controls or in microcosms prepared with background samples that did not experience dioxane removal (e.g., 1-6 and 3-5 in Table 4.4). Assuming a dry cell weight of 10^{-12} g and protein composition of 55%,¹¹⁰ the cell yield coefficient (Y) for the indigenous dioxane degraders was calculated as 0.14 mg protein/mg dioxane (i.e., $Y = \Delta X / \Delta S$ = regression line slope in Figure 4.6A), which is comparable with reported yield coefficients for CB1190 (0.01 ~ 0.09 mg protein/mg dioxane¹¹ and other dioxane metabolizers, such as *Mycobacterium* sp. D11 (0.18 mg protein/mg dioxane⁴¹). A significant correlation ($p < 0.05$, $R^2 = 0.70$) was also observed between the final *thmA* copy numbers and dioxane degradation rates (Figure 4.7A). In contrast, copy numbers of 16S rRNA genes (a phylogenetic biomarker that is commonly used to enumerate total bacteria) were not significantly correlated ($p = 0.44$) to dioxane biodegradation activity (Figure 4.7B), corroborating the selectivity of this *thmA/dxmA* probe.

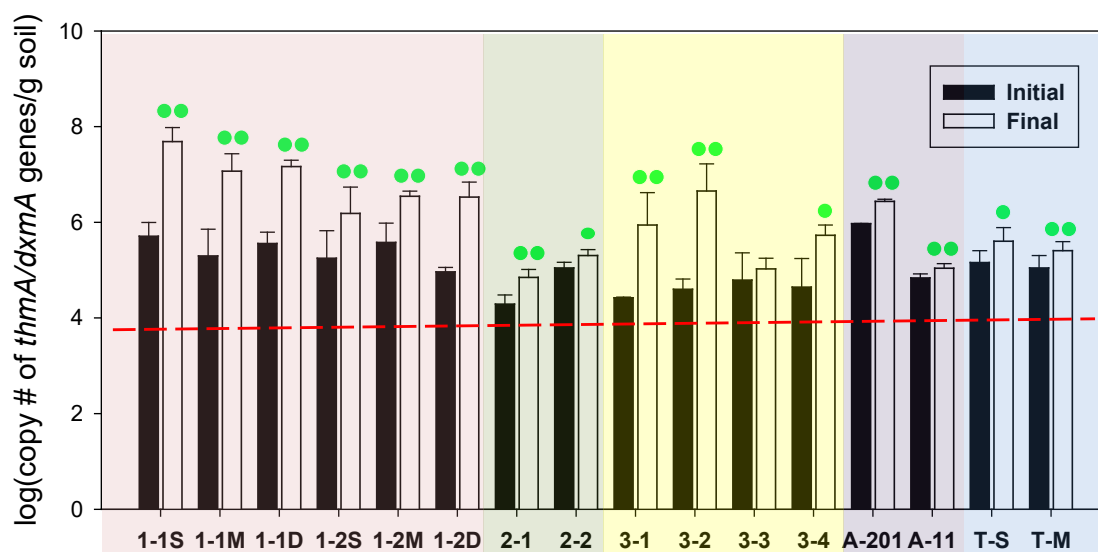


Figure 4. 3 Detection of copy numbers of *thmA/dxmA* genes in microcosms over three to five months' incubation.

The red dot line represents the estimated value of the MDL. Double green dots indicate an increase with p value lower than 0.05, and a single green dot indicates an increase with p value lower than 0.1.

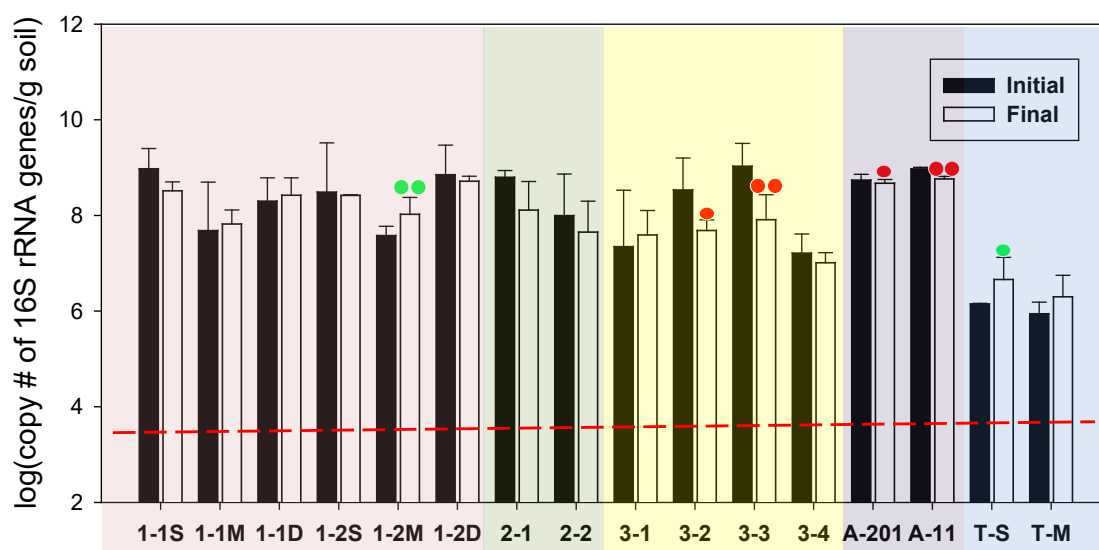


Figure 4. 4 Detection of copy numbers of 16S rRNA genes in microcosms over three to five months' incubation.

The red dot line represents the estimated value of the MDL. Double green dots indicate an increase with p value lower than 0.05, and a single green dot indicates an increase with p value lower than 0.1. In contrast, double red dots indicate a decrease with p value lower than 0.05, and a single red dot indicates a decrease with p value lower than 0.1.

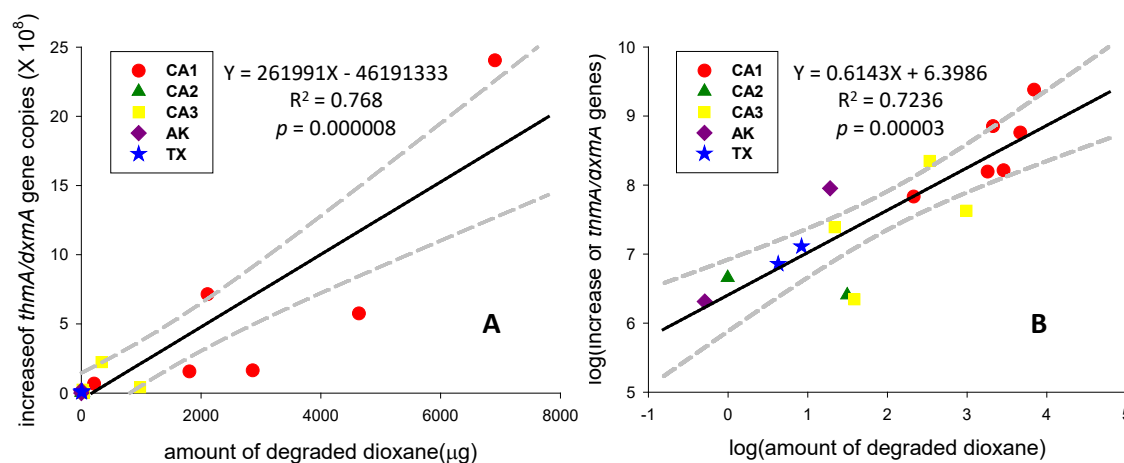


Figure 4. 5 Correlation between the amount of consumed dioxane (μg) and the increase of *thmA/dxmA* gene copy numbers in microcosms on a normal (A) and logarithmic (B) scale. The slope of the regression line of the left graph (A) was used to estimate the cell yield of dioxane by indigenous microbial degraders. The solid line represents the least square regression; the dashed lines represent the 95% confidence envelope.

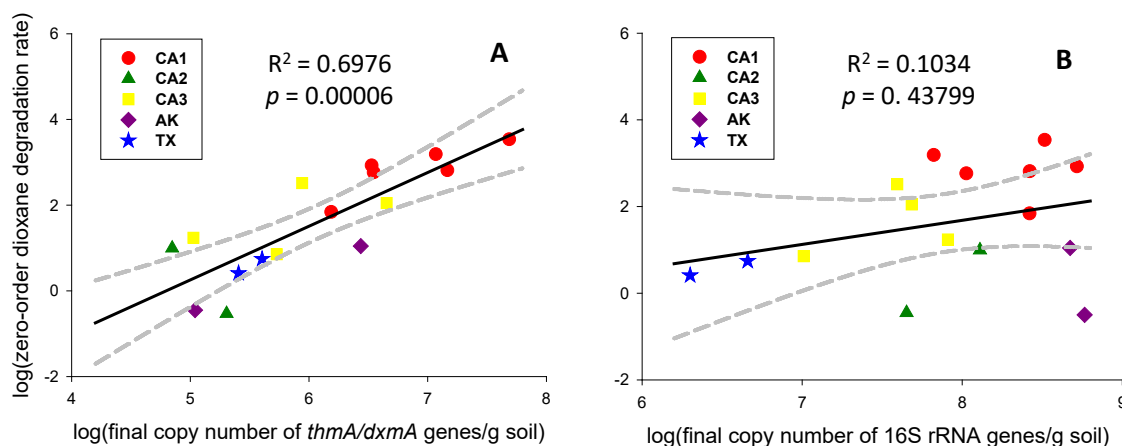


Figure 4. 6 Correlation between zero-order dioxane degradation rates ($\mu\text{g/L/week}$) and final copy numbers of *thmA/dxmA* (A) but not 16S rRNA (B) genes observed per gram of aquifer materials added in microcosms for various sites.

The solid lines represent the least square regression; the dashed lines represent the 95% confidence envelope.

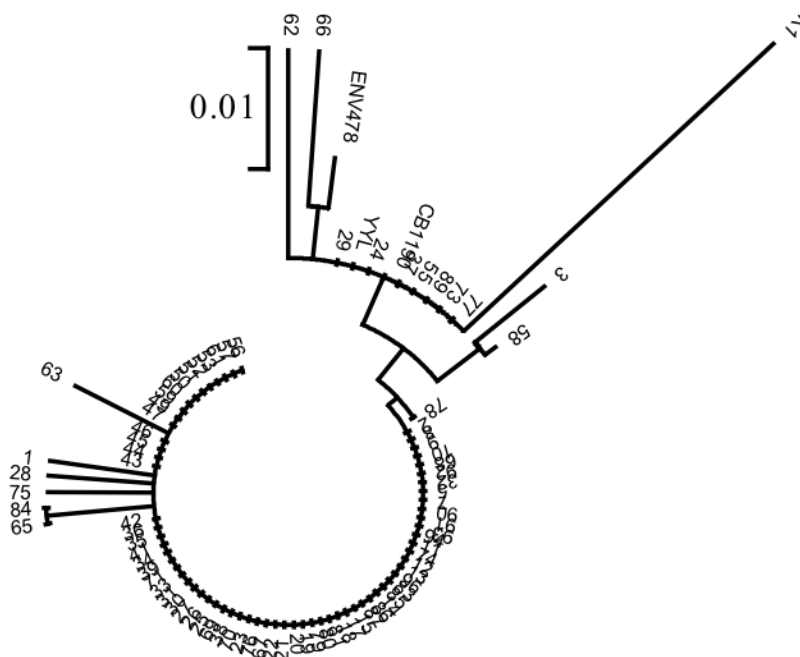


Figure 4. 7 Phylogenetic tree (constructed and visualized using MEGA 5.1) based on partial *thmA/dxmA* gene sequences from clone libraries constructed from soil samples collected in Microcosm 1-1S at week 20.

Trimmed sequences of four known *thmA/dxmA* genes were aligned to depict the evolutionary relationship, including *dxmA* from *Pseudonocardia dioxanivorans* CB1190, *thmA* from *Pseudonocardia tetrahydrofuranoxydans* K1, *thmA* from *Pseudonocardia* sp. ENV478, and *thmA* from *Rhodococcus* sp. YYL. The figure shows a high similarity between all DNA fragments amplified using our *thmA/dxmA* primer set.

To further verify that amplification products from the complex environmental samples were fragments of the intended *thmA/dxmA* genes, a clone library was constructed with genomic DNA isolated from Microcosm 1-1S, generating a total of 86 valid clones that were sequenced and aligned (Figure 4.7). All sequences exhibited high identity with previously reported *thmA/dxmA* genes ($\geq 95\%$), and no more than one nucleotide mismatch was found between our Taqman probe and its targeted sequences in the clone library, which provides further evidence for the reliability of our primer/probe set.

We recognize that numerous site-specific factors could confound the correlation between biodegradation activity and *thmA/dxmA* abundance. These include nutrient and electron acceptor influx, redox conditions, pH, temperature and presence of inhibitory compounds. However, such confounding factors are likely to affect similarly both degradation rates and biomarker enrichment (through microbial growth or decay) over the large temporal scales that are relevant to MNA. Thus, these results suggest that *thmA/dxmA* can be a valuable biomarker to help determine the feasibility and assess the performance of MNA at dioxane-impacted sites.

Chapter 5

Bench-Scale Biodegradation Tests to Assess Natural Attenuation Potential of 1,4-Dioxane at Three Sites in California

5.1 Introduction

Monitored natural attenuation (MNA), which relies primarily on intrinsic bioremediation, is often the most cost-effective approach to manage trace levels of priority pollutants.¹¹¹ Previous research in our lab demonstrated the presence of dioxane degradation and the potential for natural attenuation in microcosms prepared with groundwater and soil from an impacted site in Alaska.¹⁰⁹ Previous studies have also surveyed the dioxane degradation potential in various environmental compartments, such as river water, activated sludge, drainage soil, and garden soil samples.⁵⁶

In this chapter, we conducted microcosm studies that mimic *in situ* conditions at various monitoring wells from three sites in California (i.e., using local groundwater and aquifer material, and incubated in the dark at site-specific pH) to determine the potential for the indigenous microorganisms to degrade dioxane and assess the spatial variability of degradation kinetics and patterns along the dioxane plume. This is one of the few available studies to illustrate natural attenuation/biodegradation of dioxane in groundwater at a wide range of concentrations (10 to 10,000 µg/L).

5.2 Materials and Methods

5.2.1 Site information and sample collection

In June 2012, soil cores and groundwater samples were collected from three industrial sites in California (Figure 5.1). Site 1 was impacted by 1,1,1-TCA and 1,1-dichloroethene (1,1-DCE) (up to 58 and 4.5 mg/L, respectively). Groundwater dissolved oxygen (DO) ranged from non-detect to 2.9 mg/L. Site 2 was exposed to dioxane from an up-gradient unknown source and contained a small dioxane plume. Downhole DO was 2.3 mg/L. Site 3 aquifer contained a 0.2 mile-long-plume (Figure 5.1) in a sandy/silty layer. Downhole DO ranged from 0.1 to 3.1 mg/L. No discernible relationship between DO and distance from the contaminant source was observed at all three sites. Groundwater at all sites had neutral pH.

Groundwater samples were collected using new sterile disposable bailers, and then stored in 5-gallon clean, sterile plastic cubes at 4°C with headspace minimized. Soil samples were obtained either during well installation by driving a split spoon sampler lined with brass tubes into undisturbed soil or by collection of well bottom sediment during groundwater sample collection.

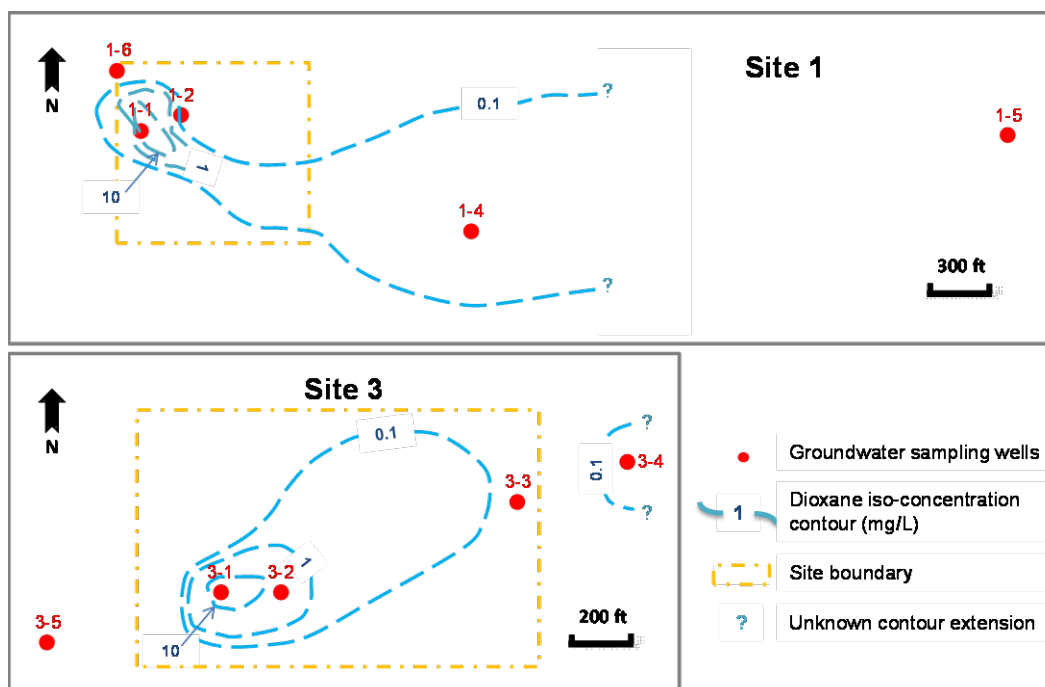


Figure 5.1 Site maps with groundwater dioxane iso-concentration contour.

Site map is unavailable for Site 2 due to the uncertainty of its contamination source. The distance between monitoring well 2-1 and 2-2 is approximately 550 ft.

5.2.2 Microcosm studies

A significant decrease in dioxane concentrations in the groundwater at Site 1 has been observed over last few decades. However, the extent to which biodegradation (rather than dilution or migration) contributes to these decreases is unknown. Moreover, the other two sites have shown relatively stable dioxane concentrations in their monitoring wells. To verify that biodegradation was occurring and assess the associated patterns, the following microcosm sets were prepared.

- 1) Biologically active microcosms prepared with groundwater from monitoring wells near the source and in the leading edge of the plume, with dioxane concentrations ranging from 8 to 46,000 $\mu\text{g/L}$;
- 2) Background microcosms (i.e., sample 1-6 and 3-5) prepared with samples from locations up-gradient of the source area with minimal or no impact by dioxane, to bench-mark biodegradation patterns for microcosms prepared with plume samples, which might be acclimated and degrade dioxane faster or with a shorter lag time;
- 3) Microcosms prepared with samples from near-source wells of each site and inoculated with the archetype dioxane degrader *Pseudonocardia dioxanivorans* CB1190³⁹ to serve as positive controls; and
- 4) Sterile negative controls to discern potential dioxane abiotic losses and to provide a baseline for mineralization studies.

For each sampling location, triplicate microcosms were prepared in 250-mL amber glass bottles amended with 150 mL of groundwater plus 50 g of aquifer material (saturated soil or suspended sediments). For monitoring wells 1-1 and 1-2, three layers of soil core samples were obtained, including an upper sand layer (S), middle silt layer (M), and lower sand layer (D). However, groundwater samples were only collected in the upper and lower layers. No groundwater was obtained from the middle layers. Hence, 75 mL groundwater from both the S and D layers were added into these microcosms mimicking dioxane natural attenuation at the middle layers (designated as 1-1M and 1-2M). Two up-gradient background microcosms (i.e., 1-6 and 3-5) only received groundwater with no solid mass amended. For all other monitoring well locations, suspended sediments were obtained from the slurry bottom in the plastic cubes and condensed by centrifugation. However, microcosms 1-5 were only amended with 10 g of sediment per bottle due to the limited amount obtained. For discussion purposes, the area of highest known groundwater concentrations was assumed to be the source areas, the authors have no specific knowledge of the location of sources.

Negative (sterile) controls were prepared using samples from the monitoring well experiencing the highest contamination level for each site, including 1-1D, 2-1, and 3-1, respectively. Samples were autoclaved separately at 121 °C for 20 min and poisoned with HgCl₂ (200 mg/L), to discern biodegradation from potential abiotic losses. *P. dioxanivorans* CB1190 is a well-characterized Gram-positive actinomycete that is capable of degrading dioxane as sole carbon and energy source.³⁹ Therefore, positive controls were prepared with aquifer material (50 g) and groundwater (120 mL) from the same monitoring wells as negative controls, and inoculated with 30 mL of ammonia mineral salt (AMS) media¹⁰⁹ containing CB1190 (10 mg/L as total protein concentration) to enhance the biodegradation of dioxane and evaluate whether degradation inhibitors are present at the sites.

All microcosms were incubated for 28 weeks in the dark at the room temperature (i.e., 24 ± 3 °C) under quiescent conditions. At preselected times (e.g., monthly), 0.5-mL liquid samples were removed from the microcosms using 1-mL gas-tight syringes tipped with 25-gauge 1.5-inch needles (Becton Dickson and Co.). Samples were filtered with a 0.2-µm syringe nylon filter (Fisher Scientific, Inc.) and stored at 4 °C until analyzed by GC/MS-SIM combining a novel frozen micro-extraction method developed in our lab.¹⁰⁰ A series dilution with a range from 12.5 to 800 µg/L was used for each calibration. During sample preparation for analysis, samples of predicted concentrations exceeding 1000 µg/L were diluted 10- or 100-fold to achieve a more precise mass analysis. The detection limit was approximately 1 µg/L.

The zero-order degradation rate was calculated as the slope of the linear regression between incubation time and monitored dioxane concentration data above the detection limit. Similarly, the exponential decay constant by CB1190 in positive controls was estimated by fitting with first-order biodegradation kinetic regime.¹¹² Statistical significance among treatments was evaluated by student *t*-test.

5.2.3 Fate of ¹⁴C-dioxane

For improved determination of the fate of dioxane (e.g., % mineralization to CO₂ and % conversion to bound residue, including possible incorporation into biomass), 100 µL of uniformly ¹⁴C-labeled 1,4-dioxane (10-µCi/mL stock solution) were added in the aqueous solution prior to capping the microcosms, resulting in 1 µCi as total radioactivity and 12.8 µg/L

as the spiked concentration of ^{14}C labeled dioxane per microcosm. The ^{14}C -labeled 1,4-dioxane was custom-synthesized in August 2010 by ChemDepo, Inc. (Camarillo, CA) with the purity greater than 99%. The diluted stock solution was kept in dark at -80°C to minimize the potential for unintended chemical transformation and decomposition. As shown in Figure 2A, a test tube containing 1 mL of 1 N NaOH was placed inside the microcosms to trap $^{14}\text{CO}_2$ in the headspace, as previously described¹¹³. These test tubes were replaced with new sterile ones containing fresh NaOH during every sampling period of this study. Teflon tapes were wrapped around the screw-top threading to tighten the seal and minimize gas release.

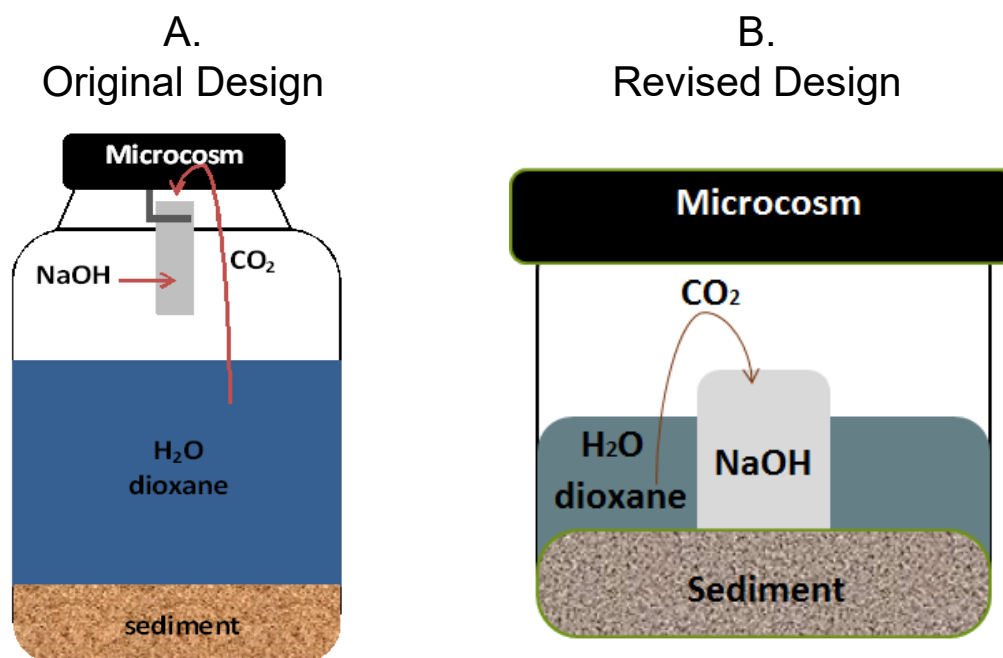


Figure 5. 2 Microcosm design.

The major difference between these two designs is their capacity to carry sufficient NaOH solution (1 mL for Design A and at least 10 mL for Design B) to scavenge CO₂ generated via microbial aerobic respiration inside the microcosm.

^{14}C in the aqueous solution and $^{14}\text{CO}_2$ in the headspace (trapped in NaOH) were analyzed by mixing 1 mL of filtered water sample/NaOH solution with 10 mL of ^{14}C -scintillation cocktail (UN2924, R.J. Harvey Instrument, NY) and measured by Liquid Scintillation Counter (LSC) using a Beckman LS 6500 (Beckman Instrument Inc., CA). This ^{14}C -scintillation cocktail used in the experiment is compatible with basic solutions. At week 28, one replicate of each treatment was sacrificed, and the biomass together with the aquifer materials was acquired by centrifugation at 5000 rpm for 5 min. The solids were further rinsed with sterile Millipore water three times to remove unbound ^{14}C -labeled compounds. Then, 1 g of the centrifuged wet solids was combusted at 915°C for 2 min by an OX600 Biological Oxidizer (R.J. Harvey Instrument, NY) in duplicates, and the effluent was carried out by N₂ gas and effervesced through a glass U-tube containing 15 mL of scintillation cocktail. The recoveries of the total ^{14}C by such combustion method were determined to be $95.2 \pm 2.8\%$ by spike tests ($n = 5$). Similar recovery ranges are commonly reported for biological oxidizer-liquid scintillation (BO-LS) analysis.^{114, 115}

After combustion, the radioactivity in the cocktails was then measured by LSC and referred as the ^{14}C bound residue.^{116, 117}

5.2.4 Replication of 1-1S microcosms with new microcosm design

In order to confirm our observation that relatively fast dioxane degradation occurred in these microcosms, groundwater samples were collected again from sampling location 1-1S in March 2013. To achieve a better recovery of ^{14}C , a new microcosm design (Figure 2B) was selected that utilized a larger NaOH trap attached to the inside-bottom of a glass jar with a lid capable of sealing gas generating media and eliminating the leakage of generated $^{14}\text{CO}_2$. The trap is a 20 mL capless glass vial that accommodates 10 mL of 1 N NaOH solution and offers easy access for sampling and NaOH replacement during each sampling.

Selected microcosm assays exhibiting relatively fast dioxane biodegradation were repeated for confirmatory purposes. These microcosms were prepared with 75 mL of the newly collected groundwater, and 25 g of aquifer material from sealed brass tubes, which are half of the amount we added in previously prepared microcosms. Then, 1 μCi of ^{14}C -labeled dioxane was spiked into the aqueous phase.

5.3 Results and Discussion

5.3.1 Evidence of dioxane biodegradation

Significant dioxane removal in biologically active microcosms, but not in sterile controls, demonstrates dioxane biodegradation (Figure 3). Within 28 weeks of incubation, 12 of 16 microcosms mimicking natural attenuation exhibited significant dioxane removal. This was corroborated in a separate study quantifying the abundance of *thmA/dxmA* genes, which encode for the active site (i.e., the large α hydroxylase subunit) of THF/dioxane monooxygenases.¹⁶ Previous molecular and enzymatic studies demonstrated the crucial role of this type of oxygenases in initiating dioxane biodegradation.¹⁶

For Site 1, dioxane was fully depleted by the indigenous microorganisms in all six microcosms prepared with samples from three different geological layers of monitoring well 1-1 and 1-2 till week 24 (Figure 3). Notably, source-zone microcosm set 1-1S experienced the highest dioxane degradation rates ($3449 \pm 459 \mu\text{g/L/week}$), suggesting the high abundance of indigenous dioxane degraders. This was evident by qPCR assays with *thmA/dxmA* biomarkers to enumerate dioxane degrading microorganisms harboring THF/dioxane monooxygenases.¹⁶ Before microcosm 1-1S were terminated, the concentrations of *thmA/dxmA* genes reached $10^{7.7 \pm 0.3}$ copies/g soil, which represents approximately 15% of the total bacteria (assessed by total 16S-rRNA) in the microcosms. This is consistent with the high dioxane biodegradation activity observed in these microcosms. In contrast, no dioxane removal was detected in microcosm sets 1-4, 1-5 and 1-6 (background control), suggesting the absence or low population of dioxane degraders down gradient from the source zone. The absence of dioxane degradation activity in these microcosms was corroborated by catabolic gene biomarker analysis, since the concentration of *thmA/dxmA* genes were lower than the method detection limit (i.e., 7,000 ~ 8,000 copies of *thmA/dxmA* genes/g soil).¹⁶

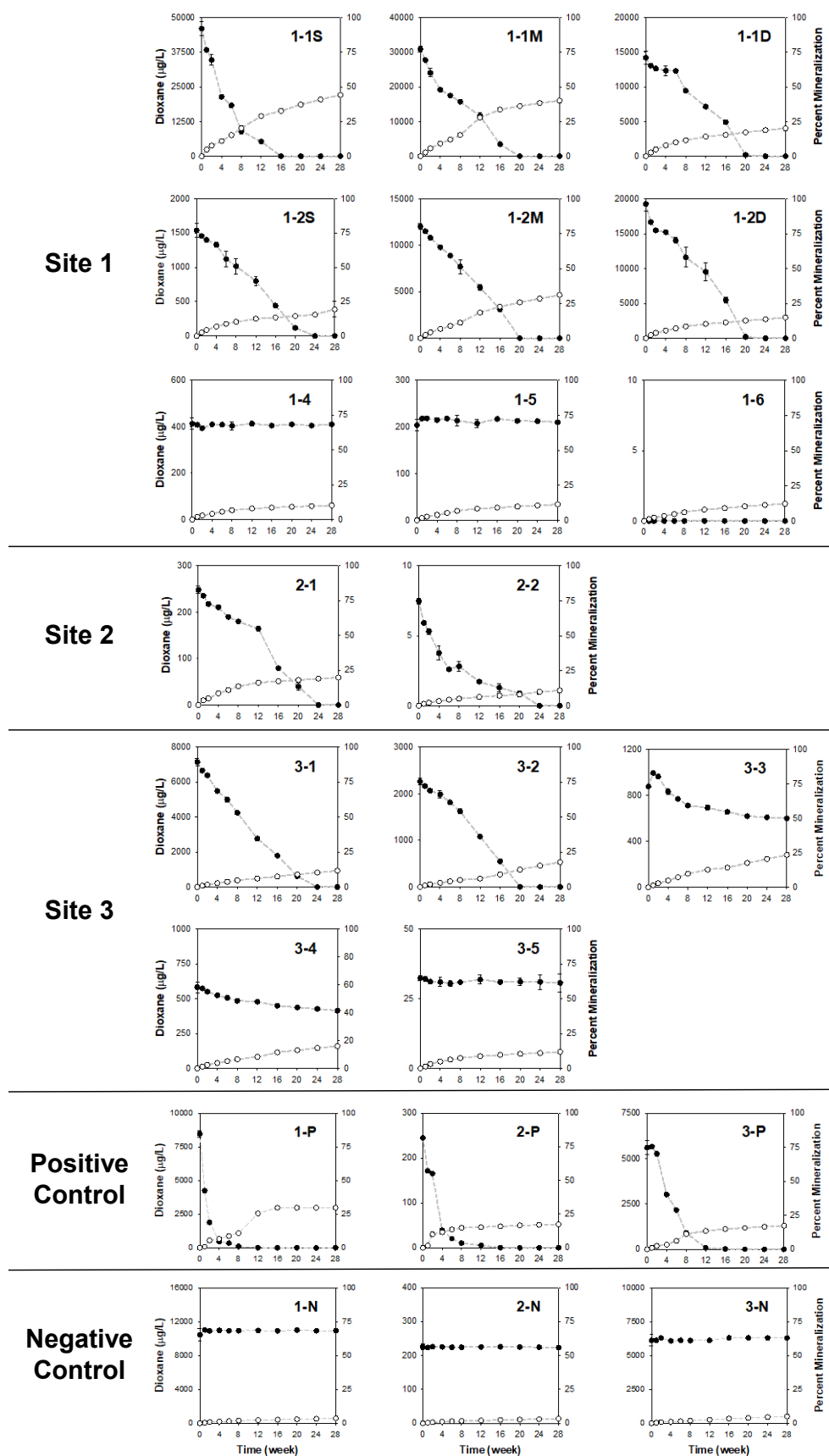


Figure 5. 3 Degradation (close circles) and accumulative mineralization (open circles) of dioxane in microcosms mimicking natural attenuation and positive and negative controls.

Dioxane concentrations lower than the detection limit (i.e., 1 µg/L) were denoted as 0 in the graphs.

Although the contamination source is uncertain, dioxane was completely degraded by week 24 in both microcosms prepared with samples from Site 2. Similarly, significant dioxane degradation was observed in Site-3 microcosms prepared with samples from the source zone (i.e., 3-1 and 3-2) and diluted down-gradient area (i.e., 3-3 and 3-4), but not in up-gradient controls (i.e., 3-5). In addition, dioxane degradation rates greatly decreased as the increase of the distance between the sampling location and the source of Site 3 (Table 5.1). The greatest dioxane degradation potential at site 3 was observed at the source zone microcosms 3-1 with degradation rates as high as 326.5 ± 8.5 µg/L/week, which is more than one order of magnitude lower than the rates observed in microcosms 1-1S. Comparably, the abundance of dioxane degraders in microcosms 3-1 was $10^{5.9 \pm 0.7}$ copies of *thmA/dxmA* genes/g soil, which is approximately 62 times fewer than that in microcosms 1-1S.¹⁶

Zero-order decay was observed in all natural attenuation microcosms experiencing dioxane biodegradation, with $R^2 > 0.80$ (Table 5.1). The estimated degradation rates ranged widely from 0.3 ± 0.1 µg/L/week to 3449 ± 459 µg/L/week. Previous microcosm studies using materials collected in north slope of Alaska exhibited a slow dioxane degradation rate of 9.8 ± 0.1 µg/L/week at a lower incubation temperature of 14 °C when the initial dioxane concentration was 500 µg/L.¹³ These degradation trends suggest saturated enzyme kinetics (low Monod K_s value) and little microbial growth.¹¹²

First-order degradation kinetics was observed in positive controls (inoculated with CB1190, which apparently exhibits higher K_s values¹¹) with $R^2 > 0.95$. The exponential decay rates were estimated as 0.53 ± 0.05 , 0.35 ± 0.03 , and 0.45 ± 0.04 week⁻¹ for Site 1, 2, and 3, respectively. No degradation lags were observed in positive controls for Site 1 and 2, suggesting the absence of inhibitory compounds at the both sites. No significant abiotic dioxane loss was observed in all three negative controls (Figure 5.3).

In order to verify the fast biodegradation observed in microcosm sets 1-1D and 3-1, these microcosms were respiked with dioxane after complete dioxane removal had occurred. The respiked dioxane was degraded even faster, and no dioxane was detected after 8 weeks (Figure 5.4). The zero-order decay rates were 668 and 355 µg/L/week for the respiked microcosm 1-1D and 3-1, respectively. This suggests that the microcosms had become more acclimated to dioxane after the first stage (Table 5.1), possibly due to the proliferation of indigenous dioxane degraders.

Table 5. 1 Microcosm preparation and the observed dioxane degradation rates fitted with zero-order decay.

Site	Sampling Locations	Distance from the Source (ft)	Initial Dioxane Concentration ($\mu\text{g/L}$)	Zero-order Dioxane Degradation Rate ($\mu\text{g/L/week}$)	R^2
Site 1	1-1S	0	46050 ± 2430	3448.7 ± 459.3	0.919
	1-1M	0	30906 ± 805	1548.7 ± 132.9	0.958
	1-1D	0	14214 ± 920	654.2 ± 49.5	0.962
	1-2S	200	1540 ± 104	69.6 ± 2.2	0.993
	1-2M	200	12035 ± 319	584.2 ± 14.7	0.996
	1-2D	200	19290 ± 1057	848.6 ± 51.6	0.975
	1-4	1550	413 ± 25	-	-
	1-5	3900	204 ± 13	-	-
	1-6	-250 ^a	NA ^c	-	-
Site 2	2-1	Unknown	248 ± 8	9.9 ± 0.8	0.958
	2-2	550 ^b	7.5 ± 0.2	0.3 ± 0.1	0.819
Site 3	3-1	0	7151 ± 204	326.5 ± 8.5	0.995
	3-2	200	2264 ± 78	112.1 ± 5.6	0.983
	3-3	1000	876 ± 14	17.4 ± 3.2	0.804
	3-4	1350	583 ± 39	7.2 ± 0.9	0.914
	3-5	-550 ^a	32.4 ± 0.8	-	-

^a indicates an up-gradient distance away from the source.

^b This is the distance between 2-1 and 2-2. The contamination source for site 2 is unknown.

^c This is below the detection limits of our analysis method (i.e., 1 $\mu\text{g/L}$).

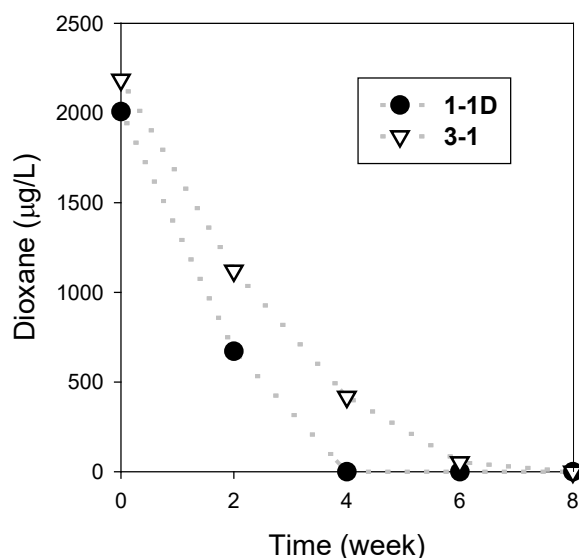


Figure 5. 4 Dioxane loss over time for the respiked microcosms.

5.3.2 The presence of indigenous dioxane degraders is further suggested by conversion to ^{14}C -labeled bound residue

Sediment and biomass-associated ^{14}C was recovered by analysis of the suspended solids (including bacteria) using a biological oxidizer with LSC at the end of the experiment. Since dioxane ($\log K_{ow} = -0.27$; Schwarzenbach et al. 2003) and its main metabolites (e.g., 2-hydroxyethoxyacetic acid [HEAA], ethylene glycol, glycolate, and oxalate,⁷⁵ are hydrophilic and have limited tendency to adsorb to aquifer materials, most of this ^{14}C amount was likely associated with biomass growth (Table 5.2). This represents an additional line of circumstantial evidence of the presence of indigenous dioxane degraders at these sites. Note that incorporation into bound residue was only observed in microcosms exhibiting significant dioxane biodegradation over 28 weeks, while no suspended-solids-associated ^{14}C was detected in microcosms experiencing no dioxane degradation (Table 5.2), which confirms limited adsorption and supports the hypothesis that most of the ^{14}C recovered by BO-LS was associated with biomass. In source zone microcosms for Site 1, 2.8 to 7.9% of the spiked ^{14}C from ^{14}C -labeled dioxane has been converted into bound residue after 7 months' incubation. Site 2 and Site 3 experienced a maximum of 6.9 % determined by solid combustion (Table 5.2).

Proliferation of dioxane degrading microbes was also corroborated by our previous biomarker study.¹⁶ Significant enrichment of *thmA/dxmA* genes was observed ($p < 0.1$) in all microcosms exhibiting dioxane degradation activities except microcosms 3-3, supporting our assumption that the indigenous microbes harboring THF/dioxane monooxygenase genes could utilize dioxane as carbon and energy source to sustain their activity and growth. Other than THF/dioxane monooxygenases, previous studies have indicated that a large variety of soluble di-iron monooxygenases (SDIMOs) can degrade dioxane via cometabolism, such as soluble methane monooxygenases, toluene-2-monooxygenases, toluene-3-monooxygenases, and toluene-

4-monooxygenases.¹¹ However, dioxane degradation by these SDIMOs is not efficient in environment compared to THF/dioxane monooxygenases, since they tend to primarily attack their substrates, such as methane and toluene, rather than dioxane. It is possible that cometabolism of dioxane is occurring in the microcosms prepared with field samples. However, it is not the dominant biodegradation process due to the high abundance of THF/dioxane monooxygenase genes revealed by qPCR assays.¹⁶

Table 5. 2 Mass Balances of uninoculated microcosms and negative controls.

Percentages are calculated as fraction of total ^{14}C added (initially as radiolabeled dioxane). Carbon sinks are categorized as: incorporation of ^{14}C into bound residue, mineralization as $^{14}\text{CO}_2$, and remaining radioactivity in the aqueous phase.

Treatment	Incorporated to bound residue	Mineralized as $^{14}\text{CO}_2$	Remaining in solution	Total
1-N	- ^b	$3.7 \pm 0.05\%$	$94.5 \pm 1.29\%$	$98.2 \pm 0.91\%$
2-N	-	$3.4 \pm 0.02\%$	$88.8 \pm 0.65\%$	$92.2 \pm 0.46\%$
3-N	-	$5.0 \pm 0.05\%$	$102.5 \pm 2.41\%$	$107.7 \pm 1.70\%$
1-1S	$7.9 \pm 0.47\%$	$44.2 \pm 0.49\%$	-	$52.1 \pm 0.48\%$
1-1M	$7.4 \pm 0.14\%$	$40.1 \pm 0.98\%$	-	$47.5 \pm 0.70\%$
1-1D	$7.5 \pm 0.04\%$	$20.0 \pm 0.41\%$	-	$27.5 \pm 0.29\%$
1-2S	$2.8 \pm 0.09\%$	$19.5 \pm 0.58\%$	-	$22.3 \pm 0.42\%$
1-2M	$3.0 \pm 0.22\%$	$31.0 \pm 0.09\%$	-	$34.0 \pm 0.17\%$
1-2D	$6.7 \pm 0.41\%$	$14.8 \pm 0.27\%$	-	$21.5 \pm 0.35\%$
1-4	-	$10.1 \pm 0.11\%$	$87.6 \pm 0.49\%$	$97.7 \pm 0.36\%$
1-5	-	$11.2 \pm 0.36\%$	$89.2 \pm 3.25\%$	$100.4 \pm 2.31\%$
1-6	-	$12.2 \pm 0.33\%$	$92.1 \pm 0.45\%$	$104.3 \pm 0.39\%$
2-1	$6.5 \pm 0.53\%$	$19.7 \pm 0.20\%$	-	$27.2 \pm 0.40\%$
2-2	$1.5 \pm 0.34\%$	$10.8 \pm 0.09\%$	-	$12.3 \pm 0.25\%$
3-1	$6.9 \pm 0.29\%$	$11.6 \pm 0.58\%$	-	$18.5 \pm 0.46\%$
3-2	$5.0 \pm 0.04\%$	$17.7 \pm 0.19\%$	-	$22.5 \pm 0.14\%$
3-3	$3.8 \pm 0.17\%$	$23.4 \pm 0.16\%$	$84.6 \pm 4.61\%$	$111.8 \pm 2.66\%$
3-4	$2.6 \pm 0.20\%$	$16.0 \pm 0.28\%$	$29.4 \pm 0.85\%$	$48.0 \pm 0.53\%$
3-5	-	$11.8 \pm 0.29\%$	$90.3 \pm 1.70\%$	$102.1 \pm 1.22\%$
1-1S Replicate ^a	$6.3 \pm 0.07\%$	$53.4 \pm 0.13\%$	$34.7 \pm 0.27\%$	$94.4 \pm 0.18\%$

^a Microcosms were prepared with a new design to enhance ^{14}C recovery.

^b – indicates no radioactivity was detected above 30 DPM by scintillation counter.

5.3.3 ^{14}C -Dioxane putatively assimilated by biomass was correlated with the observed biodegradation activity.

A significant ($p < 0.05$) correlation was observed between the degradation rates and the extent of ^{14}C accumulation in bound residue (Figure 5.5A), further supporting the notion that

dioxane was assimilated by the indigenous microorganisms. Student *t*-test analysis indicated significant ^{14}C accumulation in bound residue (and thus possible biomass growth) in microcosms that exhibited a degradation rate higher than $5\text{ }\mu\text{g/L/week}$ (Figure 5.5A). Putative biomass assimilation was also significantly higher in microcosms prepared with samples from the source zone compared to those with leading-edge or background samples (Figure 5.5A). For instance, microcosms 1-1, 1-2D, and 3-1, which contain source zone materials, experienced the greatest conversion to bound residue at 6.9 to 7.9% of the total amount of the spiked ^{14}C (Table 5. 2). The apparent biomass yield found in this study is comparable with that reported in a previously study that found 5% of radiolabeled dioxane was incorporated into CB1190 biomass.⁷⁵ This suggests that the cell yield for these indigenous dioxane degrading consortia is relatively low (i.e., less than $0.1\text{ mg protein/mg dioxane}$) in general.

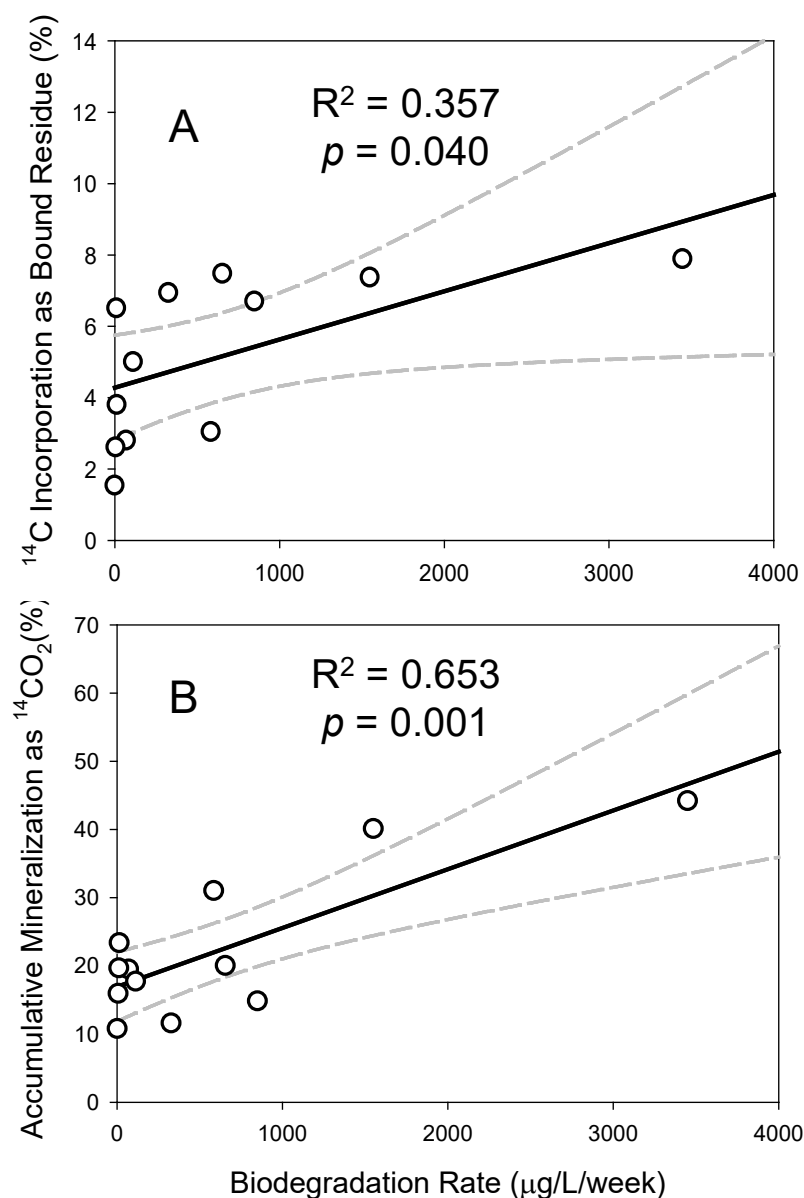


Figure 5. 5 Correlation between zero-order dioxane degradation rate and (A) ^{14}C incorporation as bound residue or (B) cumulative mineralization as $^{14}\text{CO}_2$.

5.3.4 Mineralization activity correlated to degradation rates

To confirm the biodegradation of dioxane observed in uninoculated microcosms mimicking natural attenuation, accumulation of $^{14}\text{CO}_2$ was also monitored during incubation. The overall mineralization observed in the biologically active microcosms (positive controls and degrading) is significantly greater ($p < 0.05$) than that of the inactive microcosms (negative controls and those exhibiting no degradation) (Table 5.2). For instance, mineralization extents of $44 \pm 0.5\%$, $38 \pm 1.0\%$, and $20 \pm 0.4\%$ of the spiked ^{14}C were observed for microcosms 1-1S, 1-

1M, and 1-1D, respectively. However, the cumulative $^{14}\text{CO}_2$ trapped by basic solutions was less than 5% in all control (killed) microcosms (Figure 3). In addition, the cumulative mineralization as $^{14}\text{CO}_2$ was significantly correlated ($p < 0.05$) with the zero-order dioxane decay rates observed in microcosms mimicking natural attenuation (Figure 5B), corroborating the capability of indigenous microorganisms to decompose dioxane into CO_2 and utilize this xenobiotic compound as their carbon and energy source. However, the total mass recovery was relatively low with this experimental setup, especially for microcosms exhibiting fast dioxane degradation (e.g., 1-1S, 2-1, and 3-1). In this microcosm design (Figure 5.2A), the total volume of NaOH solution was merely 1mL providing limited interfacial surface area that might prohibit complete reactions with CO_2 generated in these microcosms. Further, long incubation time and possible gas leakage while sampling were also likely to cause the low ^{14}C recoveries observed in this experiment.

5.3.5 Degradation rates were significantly higher in source-zone samples, possibly reflecting higher acclimation

With the exception of samples from well 3-3 and 3-4, the well samples demonstrating biodegradation are all within approximately 200 ft of the dioxane source for Site 1 and 3. Student t -tests indicated that dioxane biodegradation rates were significantly higher ($p < 0.05$) near the source-zone samples than down-gradient diluted area (i.e., more than 200 ft distance away from the source in this study). For example, microcosm sets 1-4 and 1-5 exhibited no degradation at the same site where the highest degradation, 1-1S ($3449 \pm 459 \mu\text{g/L/week}$), was observed in the source zone samples. It has been previously reported that long term exposure to high concentrations of xenobiotic compounds results in a higher acclimation and more rapid metabolic response over time.^{118, 119} Such acclimation effects were also evident in other dioxane degradation studies using various lines of evidence. For instance, both functional microarray and denaturing gradient gel electrophoresis (DGGE) analysis indicated monooxygenase genes that are likely involved in initiating dioxane oxidation were significantly enriched in source-zone area at an Arctic site, suggesting selective pressure by dioxane.¹⁷ For another site in Arizona, compound specific isotope analysis (CSIA) demonstrated a significant amount of ^{13}C from dioxane incorporated into biomass in the samples heavily impacted by dioxane, but not in the areas with no previous exposure history.³¹ These previous studies concluded that MNA might be a feasible component of the remedial response.

Therefore, a new microcosm design utilizing a larger mineralization trap and an improved (tighter) microcosm cap (Figure 5.2B) was used to prepare a new set of 1-1S microcosms which ultimately yielded a more complete mass balance. In addition, freshly sampled groundwater from monitoring well 1-1 was used in this experiment to confirm the observed fast degradation rates. Within 12 weeks, dioxane removal reached $64 \pm 0.2\%$ with $53 \pm 0.2\%$ mineralization of the spiked ^{14}C (Figure 5.6). No dioxane loss and low $^{14}\text{CO}_2$ recovery ($< 15\%$) were observed in the 1-1S Replicate negative control. Better mass balance recovery of the overall ^{14}C was obtained with this new design, ranging from 92 to 98 % (Table 5.2). This suggests that insufficient interfacial surface area with the NaOH trap to capture the emitted CO_2 in the original microcosm design (Figure 5.2A) was one of the key reasons for low ^{14}C recoveries.

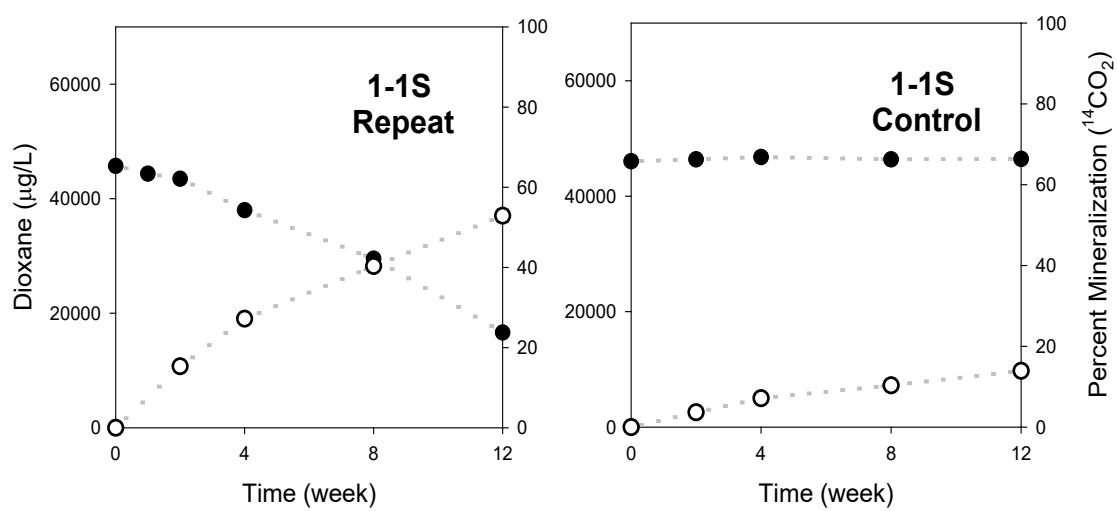


Figure 5. 6 Dioxane degradation and CO₂ mineralization for the 1-1S replicates and control.

Chapter 6

Associating Potential Dioxane Biodegradation Activity with Groundwater Geochemical Parameters at Four Different Contaminated Sites

6.1 Introduction

Studies have shown that bacteria capable of degrading dioxane might be more widespread than previously assumed,^{39, 120} which has increased interest in bioremediation and monitored natural attenuation as potentially cost-effective alternatives to manage large dioxane plumes. Thus, assessing the potential biodegradation activity at contaminated sites is important for the selection (or rejection) of such remedial strategies.

Natural attenuation rates of dioxane at source zones have been previously estimated by evaluating temporal concentration changes within comprehensive field datasets, and linear discriminant analyses have been used to determine how site-specific conditions influence the estimated rates.³ However, statistical analyses of field data cannot discern the contribution of biodegradation to the observed removal rate relative to all other natural attenuation processes (e.g., advection, dilution, dispersion). Moreover, correlations based on prolonged microbial exposure under a set of geochemical conditions cannot infer the potential biodegradation activity in an environment where (for instance) oxygen is no longer a major limiting factor.

Biodegradation is a very important attenuation pathway for dioxane¹⁷ and rates can be influenced by site-specific conditions, such as microorganisms with specific catabolic potential, presence of sufficient dissolved oxygen concentrations for sustained microbial growth and/or activity,³ and the presence of co-occurring contaminants that enhance or inhibit biodegradation of the priority pollutant (e.g. dioxane).¹²¹ We postulate that potential biodegradation activity can be determined in microcosms under controlled favorable settings (e.g., sufficient oxygen availability) to assess how geochemical conditions affect the potential for bioremediation. Such an evaluation would need to consider major environmental factors that generally affect biodegradation activity, such as dioxane and nutrient concentrations, presence of bacteria harboring pertinent catabolic genes, and groundwater pH and temperature.

In this chapter, ordinary least squares regression as well as Pearson and Spearman correlation coefficients were used to assess possible associations between groundwater geochemical parameters (measured at 16 monitoring wells from four different contaminated sites) and the corresponding potential dioxane biodegradation activity, which was determined in microcosm under unlimited oxygen conditions. These heuristic relationships may provide valuable insight on dioxane contamination scenarios that are amenable to remediation by monitored natural attenuation.

6.2 Materials and Methods

6.2.1 Groundwater monitoring data

Groundwater geochemical data were compiled from various groundwater monitoring wells (MWs) installed at four separate contaminated sites in the US. Two MWs were located in Alaska and 14 MWs were located in three independent sites in Southern California. Aquifer material used for determination of potential dioxane biodegradation activity in microcosms was representative of the time groundwater was collected for geochemical analysis. Possible correlations between dioxane biodegradation rates were tested against most typical geochemical variables to predict biodegradation such as nutrients (N-NO₃/NO₂, N-NH₃ and P-PO₄²⁻), temperature, pH and conductivity. It is important to recognize that other co-contaminants were also reported [e.g., tetrachloroethylene (PCE), trichloroethylene (TCE), 1,1-dichloroethylene (1,1-DCE), trichloroethane (TCA), and 1,1-dichloroethane (DCA), methyl-tert-butyl ether (MTBE), benzene, toluene, ethyl benzene and isomers of xylene (BTEX), 1,2,4-trimethylbenzene, 1,3,5-trimethylbenzene, *sec*-butylbenzene, *tert*-butylbenzene, *n*-butylbenzene, isopropylbenzene, *n*-propylbenzene, *p*-isopropyl toluene, naphthalene, 2-methylnaphthalene, chloroform, and trichloropropane]. Other non-volatile co-contaminants were also found at trace levels and in only a few of the MW's considered. Analysis of these parameters was not statistically dependable due to insufficient number of data and therefore not explored here.

6.2.2 Determination of potential dioxane biodegradation activity

Dioxane biodegradation rates were determined using microcosms prepared with aquifer materials (10-50 g) and groundwater (50-150 mL) samples collected from four contaminated sites.^{13, 17} Microcosms were maintained at controlled temperatures that matched site-specific groundwater temperatures. Autoclaved and poisoned (200 mg/L of HgCl₂) microcosms served as controls to discern dioxane biodegradation from abiotic losses. The concentrations of dioxane were monitored over time using a frozen microextraction method followed by gas chromatography/mass spectrometry¹⁰⁰ (detection limit of ≥ 1 ppb). In each microcosm, the dioxane biodegradation rate was calculated as the average of the removal rate (concentration versus time slope) corrected for the losses in autoclaved controls.

6.2.3 Determination of *thmA*/*dxmA* genes concentrations

At the end of microcosm experiments, aquifer material was collected from microcosms and the bacteria DNA was extracted using commercially available kits (PowerSoil DNA Isolation Kit-MoBio). Real-time quantitative PCR was performed using a previously developed set of primers and probes to target *thmA*/*dxmA* genes coding for soluble di-iron monooxygenase (SDIMO) known to be involved in the initiation of dioxane and tetrahydrofuran metabolism.¹⁷

6.2.4 Statistical analyses

Associations between dioxane biodegradation rates (k' ; $\mu\text{g/L/d}$) and groundwater physical-chemical parameters were assessed using linear regression analyses and Pearson and Spearman correlation coefficients. The Kruskal-Wallis test for dioxane concentrations from the 4 sites yielded a p -value of 0.12 while the test for biodegradation rate yielded a p -value of 0.35. This implies that dioxane concentration and biodegradation rate distributions are statistically

undiscernible at the 95% confidence level and can be aggregated for further analyses. Linear regression analyses were performed using normally distributed biodegradation rates. Because the observed biodegradation rates violated the normality assumption by being non-symmetric and right-skewed, a logarithmic transformation was used for correction. To ensure that any positive correlations were not driven by outliers, 16 $\log_{10}$ dioxane concentrations were binned by 25th, 50th, and 75th percentiles into 4 clusters to compare $\log_{10}$ biodegradation rates. For each cluster of $\log_{10}$ dioxane concentrations, a boxplot of $\log_{10}$ biodegradation rates were generated to verify a positive trend. The detection limit for observed dioxane biodegradation rates introduces zero values, which are undefined under a logarithmic transformation. Thus, dioxane biodegradation rates were translated by +0.01, corresponding to the detection limit. Any small, positive value less than the detection limit would be suitable, but +0.01 was chosen for simplicity and numerical convenience. Chemical parameters and contaminants were also translated and $\log_{10}$ transformed in situations where the quantity of interest was right skewed with values below the detection limit (e.g., PCE, TCE, DCE, TCA, and DCA).

Additional regression analyses were performed to test for linear relationships between initial concentrations of dioxane and chlorinated solvents. Due to missing values for various chemical parameters three groups ($n=16$, $n=14$, $n=12$) were considered for correlation analyses: *thmA* copy numbers ($n=16$); dissolved oxygen, temperature, pH, and conductivity ($n=14$); and the concentrations of TOC, nitrate, and phosphorus ($n=12$).

Pearson and Spearman correlation coefficients were also calculated for the three groups to determine strength of linear relationships and non-linear, monotonic relationships, respectively. Significance of Pearson and Spearman correlation coefficients was assessed using test statistics based on the t and F distributions, respectively. More specifically, the test statistics check if the correlation value (R) is not equal to zero at the 95% confidence level. Thus, p -values ≤ 0.05 denote significant correlations.

The Kruskal-Wallis test, regression analysis, and correlations were performed in R, a statistical software package (<https://cran.r-project.org/>) using built in functions for Kruskal-Wallis tests and linear regressions; Harrell Miscellaneous (Hmisc) package version 4.0-1 for correlations (<https://cran.r-project.org/web/packages/Hmisc/index.html>); and ggplot2 (<http://ggplot2.org>) for graphics. R markdown and Knitr (<http://rmarkdown.rstudio.com>) were used to prepare the supporting information.

6.3 Results and Discussion

Dioxane concentrations showed positive associations with the concentrations of commonly found co-contaminants, i.e., TCA ($p<0.01$; $R=0.87$), TCE ($p=0.02$; $R=0.58$), and their dechlorinated byproduct DCE ($p=0.04$; $R=0.53$) (Table 6.1). The strongest association was observed with TCA, which might reflect the fact that dioxane was primarily used as a stabilizer for this specific chlorinated solvent.²⁶

Dioxane biodegradation rate was significantly associated with the concentrations of dioxane present in the monitoring wells at the time of sampling ($p<0.01$; $R=0.70$). This is paradoxical because groundwater from high-concentration monitoring wells should experience faster degradation rates that rapidly decrease dioxane concentrations, which is not always observed. However, quantitative interpretation of natural attenuation can be confounded by

poorly-understood source dynamics, such as release of dioxane that had been entrapped in clay pockets that serve as a sustained source for groundwater contamination.¹²² Replenishment of dioxane from such overlooked sources would offset removal by biodegradation. Our microcosm experiments avoided such confounding factors and quantitatively discerned potential biodegradation activity. Furthermore, this statistical inference is consistent with the law of mass action; i.e., degradation rates are faster with higher (non-toxic) reactant concentrations¹²¹ and trace contaminant concentrations may pose a challenge to meet enzyme induction thresholds¹²³ or minimum cell maintenance energy requirements.^{123, 124}

Biodegradation rates were also significantly associated with the copy numbers of the catabolic *thmA* gene, which codes for dioxane monooxygenase ($p < 0.01$; $R = 0.80$) (Table 6. 2). The correlation between *thmA* gene copy numbers and dioxane biodegradation rate was discussed elsewhere.¹⁷ The potential involvement of other important monooxygenases, dehydrogenases, and or hydroxylases that are also known to participate in co-metabolic biodegradation of dioxane should not be ruled out.¹¹ Nonetheless, the strong association between *thmA* abundance and dioxane biodegradation rates suggested that *thmA* was a relevant biomarker to infer on biodegradation at these sites.

Dioxane biodegradation rates ranged from 0.03 to 493 $\mu\text{g/L/d}$ (average of 68.2 ± 129.1 $\mu\text{g/L/d}$) and followed zero-order kinetics. Adamson et al. (2015), have previously evaluated data from large number of contaminated sites and reported the importance of dissolved oxygen on the natural attenuation of dioxane.¹²⁵ Therefore, the influence of oxygen concentrations on dioxane biodegradation is already well established. In this work, dioxane biodegradation rates were measured in microcosms under unlimited oxygen conditions as a measure of potential biodegradation activity. Accordingly, a non-significant association was observed between degradation rates and the dissolved oxygen concentration (Table 6. 2) present at these sites at average concentrations of 2.5 ± 1.8 mg/L. The presence of nitrogen ($\text{NO}_3 + \text{NO}_2$) and phosphorus (PO_4) as nutrients in groundwater at average concentrations of 15.3 ± 21.1 mg/L and 10.4 ± 22.9 mg/L, respectively, did not significantly affect potential dioxane biodegradation activity. Other physical-chemical parameters such as pH (ranging from 6.1 to 7.1), conductivity (ranging from 0.2 to 2.5 mS/cm) and temperature (varying from 5.1 to 25°C) also were not significantly associated with the measured degradation activity.

Table 6. 1 Associations between dioxane and chlorinated solvent concentrations per Pearson correlation analysis.Significant associations were assumed for p -values ≤ 0.05 , as depicted by gray shaded areas. Sample size $n=16$ unless otherwise noted.

	Log ₁₀ PCE		Log ₁₀ TCE		Log ₁₀ DCE		Log ₁₀ TCA		Log ₁₀ DCA	
	p^a	R ^b	p	R	p	R	p	R	p	R
Log ₁₀ Dioxane	0.507	-0.18	0.02	0.58	0.04	0.53	<0.01	0.87	0.236	-0.31
Log ₁₀ TCE	0.230	0.32	-	-	-	-	-	-	-	-
Log ₁₀ DCE	0.315	0.27	<0.01	0.79	-	-	-	-	-	-
Log ₁₀ TCA	0.534	-0.17	0.09	0.43	0.357	0.25	-	-	-	-
Log ₁₀ DCA	0.01	0.624	0.71	-0.10	0.728	-0.09	0.654	-0.12	-	-

^a p -values for Pearson correlation^b Pearson correlation**Table 6. 2 Associations between dioxane biodegradation rates (k') and geochemical data per Pearson's or Spearman's correlation analyses.**Significant associations were assumed for p -values ≤ 0.05 , as depicted by gray shaded areas. Sample size $n=14$ unless otherwise noted.

Pearson												Spearman ^d				
	Log Dioxane (µg/L)		Log ₁₀ <i>thmA</i> ^c (gene copy numbers/g-soil)		DO (mg/L)		pH		Groundwater (°C)		Log ₁₀ Conductivity (mS/cm)		NO ₃ + NO ₂ as N (mg/L)		Log ₁₀ PO ₄ as P (mg/L)	
	<i>p</i> ^a	R ^b	<i>p</i>	R	<i>p</i>	R	<i>p</i>	R	<i>p</i>	R	<i>p</i>	R	<i>p</i>	R	<i>p</i>	R
Log ₁₀ (k') (µg/L/d)	<0.01	0.70	<0.01	0.80	0.483	0.21	0.248	-0.33	0.259	0.32	0.239	-0.34	0.372	-0.28	0.712	-0.12

^a p -values for Pearson or Spearman correlations^b Pearson correlation^c $n = 16$ ^d $n = 12$

Most volatile organic compounds present in the groundwater may have been lost through volatilization during sample collection and/ or transportation, as well as during microcosm preparation and samplings. Therefore, determination of dependable associations between dioxane biodegradation rates and the concentrations of volatile compounds present at the time of sampling could be misleading and were not explored here. Note that while some chlorinated solvents hinder dioxane biodegradation by some bacteria (e.g., *Pseudonocardia dioxanivorans* CB1190),⁵¹ an inhibitory effect was not observed for other dioxane degraders using different enzymes [e.g., *Pseudomonas mendocina* KR1 and *Escherichia coli* TG1 (T4MO)].¹²⁶ It is unclear whether potential inhibitory effects mainly take place immediately after microorganisms are first exposed to chlorinated solvents, or if inhibition occurs to a lesser extent (or not at all) for acclimated bacteria with exposure history.

Determining the variation in lithology among the 16 different contaminated sites was beyond the scope of this study. In general, these sites were characterized by unconsolidated sediments (e.g., sands, silts, and clays) and were thus comparable. An evaluation of how the site specific sediment composition¹²⁷ and background organic matter type and concentration¹²⁸ as well as associated hydrogeologic characteristics may impact dioxane biodegradation rates and correlations would be valuable. However, such an evaluation was not possible in this study due to the relatively limited number of sites available, all with similar lithology and monitoring well construction (i.e., fully submerged well screens). Epidemiologists often account for such confounding factors by considering very large databases that randomize them, which was impossible in this study.

Overall, these results suggest that while many geochemical factors (pH, temperature, nutrients) and the abundance of specific degraders may be important for dioxane biodegradation, these factors may not exert as strong of an influence on the potential biodegradation activity as the concentration of dioxane. Unequivocally, *in situ* dioxane concentrations significantly influenced the observed dioxane biodegradation rates (Figure 6. 1) with higher dioxane concentrations possibly selecting for a thriving microbial population, enhancing the sustenance of bacteria metabolic capabilities⁴⁶ and/or inducing specific enzymes.¹⁴ This finding provides valuable insight into site-specific scenarios where significant dioxane biodegradation might be expected. Specifically, while sites with higher dioxane concentrations are more likely to select for and sustain dioxane degraders, sites with relatively low dioxane concentrations would be more challenging to attenuate naturally and may require implementation of alternative remediation strategies. Whether there is a site-specific threshold dioxane concentration of regulatory concern below which sustained biodegradation is unlikely to proceed remains to be determined.

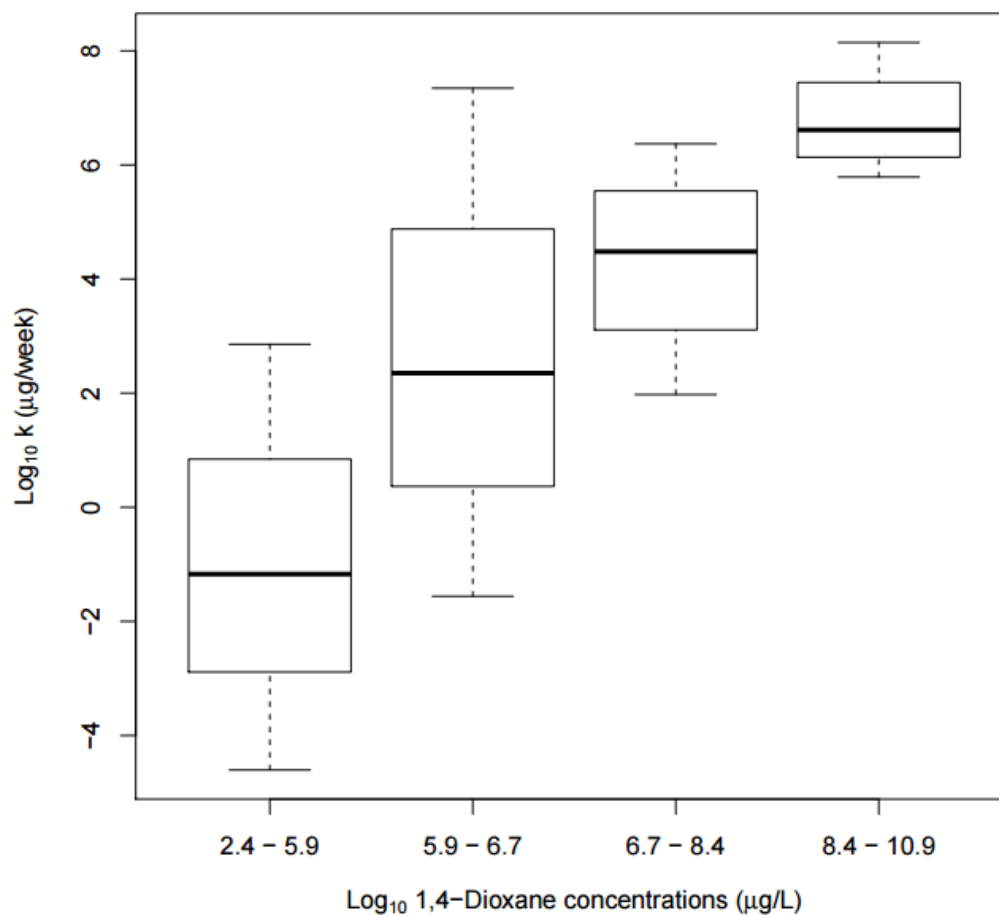


Figure 6. 1 Boxplots of logarithmically transformed biodegradation rates and dioxane concentrations from 16 monitoring wells.

Dioxane concentrations were binned into quartiles resulting in data from 4 wells per bin. For each bin, a boxplot was constructed using log biodegradation rates for the respective MW.

Chapter 7

Hindrance of 1,4-Dioxane Biodegradation in Microcosms Biostimulated with Inducing or Non-Inducing Auxiliary Substrates

7.1 Introduction

Recent research has demonstrated that indigenous bacteria that can degrade dioxane might be more widespread than previously assumed.¹⁷ However, the suitability of bioremediation must be determined site-specifically because the presence and expression of dioxane biodegradation capabilities are not ubiquitous. Furthermore, the potential to stimulate dioxane biodegradation by adding auxiliary carbon sources has received limited attention in the literature. Such auxiliary substrates might be beneficial when dioxane is present at trace concentrations that are insufficient to induce or sustain specific degraders.

Auxiliary substrates can be arbitrarily divided into two groups: (1) inducing substrates that induce catabolic enzymes (e.g., tetrahydrofuran [THF], which induces dioxane monooxygenase (MO) in various actinomycetes),⁴⁹ and (2) non-inducing substrates that fortuitously support the growth of dioxane degraders (e.g., 1-butanol [1-BuOH]²¹). Nevertheless, it is unclear if such substrates are ubiquitously applicable for stimulating dioxane biodegradation at sites harboring dioxane degraders, and what the unintended consequences of such biostimulation approaches might be.

Activation of dioxane metabolism is commonly initiated by plasmid-encoded enzymes. The rate-limiting step for the biodegradation of dioxane and its structural analogue THF is generally the oxidation of the carbon atom adjacent to the oxygen atom in both compounds (i.e., 2-hydroxylation). The known gene clusters encoding multi-component dioxane/THF monooxygenase (MOs) (i.e., *dxmADBC* and *thmADBC*) are located in plasmids.^{14, 19, 22} The localization and relatively low GC content of these *dxm/thm* gene clusters suggested that horizontal gene transfer might be the dominant venue by which these bacteria obtained these plasmids and gained the capacity to degrade dioxane and THF. In polluted environments, such plasmids may confer their hosts improved ecological fitness and broader substrate range. However, maintenance and expression of these catabolic plasmids contribute to additional metabolic burden that needs to be compensated by the host cells.¹²⁹

In this chapter, we compare the merits and limitations of biostimulation of dioxane degradation with a non-inducing growth substrate (1-BuOH) versus an inducing substrate (THF). Using microcosms prepared with samples from a contaminated site, we consider the dynamics of the catabolic biomarker *thmA/dxmA* as well as microbial population shifts. To further investigate the molecular basis of how auxiliary substrates influence *dxmADBC/thmADBC* bearing dioxane degraders, the archetype *Pseudonocardia dioxanivorans* CB1190 was selected as a model strain. Experiments in controlled laboratory setups were conducted with pure cultures of CB1190 to discern how these auxiliary substrates affect the induction or repression of essential genes involved in dioxane degradation, as well as the stability of pertinent catabolic plasmids under

different substrate conditions. Therefore, this chapter advances mechanistic understanding of how different types of auxiliary or co-occurring substrates affect the expression and maintenance of dioxane biodegradation capabilities.

7.2 Materials and Methods

7.2.1 Site description and sample collection

In March 2013, groundwater and aquifer materials were collected from an oil and gas operation site located in the Southwestern US. This site was mainly used for chemical storage and equipment repair, and past activities resulted in releases of dioxane and chlorinated volatile organic compounds (CVOCs) to the subsurface. Hydraulic modeling predicted the leading edge of the dioxane plume was approximately 6,000 ft downgradient of the site using the Texas Commission on Environmental Quality PCL (i.e., $9.1 \mu\text{g/L}$) to define the contamination boundary. The plume width was estimated to be 1,600 ft at the $9.1 \mu\text{g/L}$ contour. The maximum dioxane concentration was $395 \mu\text{g/L}$. Four monitoring wells representing different dioxane contamination levels were selected for sample collection, including the source zone, the middle, and the edge of the plume, as well as the uncontaminated upstream region (Figure 7.1 and Table 7.1).

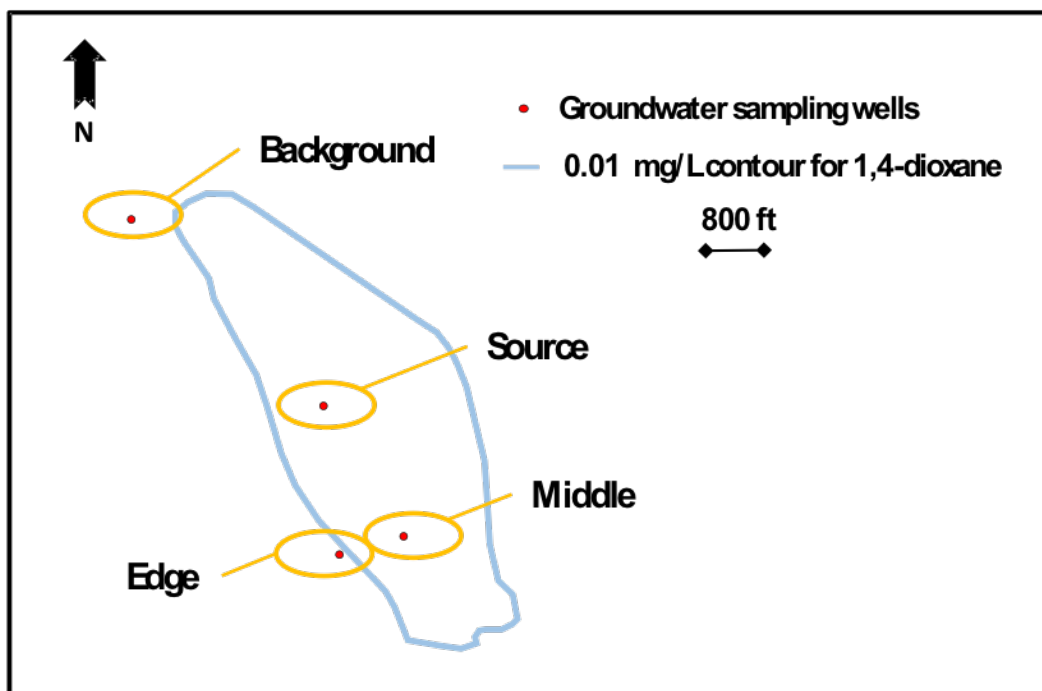


Figure 7. 1 Site map with groundwater dioxane iso-concentration contour.
The four sampling locations with different dioxane contamination levels are depicted.

Table 7. 1 . Concentrations of dioxane in the four collected groundwater samples.

Monitoring Well	Sampling Location Description	Dioxane	
		P&T ^a	FME ^b
Source	Plume center with maximum dioxane concentration	172	206 ± 3.1
Middle	Down-gradient of the plume center with dioxane concentration greater than 20 µg/L ^d	118	93.0 ± 23.3
Edge	Leading edge of the dioxane with trace level of dioxane (< 20 µg/L)	6.03	5.8 ± 0.1
Background	Away of the dioxane plume without contamination	ND ^c (≤ 3)	ND (≤ 1.6)

^a P&T = Purge and trap. Dioxane concentrations in this column were analyzed in a commercial lab using EPA method 8260 followed by GC/MS;

^b FME = Frozen micro-extraction. Dioxane concentrations in this column were measured in our lab using frozen micro-extraction followed by GC/MS-SIM;

^c ND = Not detected. The value in parentheses is the method detection limit for this measurement.

^d The local groundwater action limit.

7.2.2. Benchtop microcosm assays mimicking natural attenuation and biostimulation

To investigate the potential for natural attenuation, microcosms were prepared in 250 mL amber glass bottles using 100 mL of groundwater and 50 g of aquifer material collected at different site locations.^{13, 120} We focused on aerobic processes that prevailed at the aquifer under consideration near the source zone (i.e., dissolved oxygen > 2 mg/L, ORP > 100 mV). Furthermore, increased surface area of the aquifer material and contact with air during sample handling and microcosm preparation would also contribute to aerobic conditions. Whereas these handling may result in faster dioxane degradation in microcosms than *in situ*, this would not affect the substrate interactions studied here because all microcosm treatments and controls were handled similarly. Microcosms were incubated in the dark under quiescent condition at 24 ± 2 °C, which was similar to field conditions (Table 7.2).

Table 7. 2 Physiochemical characteristics of the collected groundwater at the field.

Field Parameters	Source Zone	Middle of Plume
pH	6.7	6.5
Conductivity (mS/cm)	2.67	3.11
Temperature (°C)	21.4	20.2
Turbidity (NTU)	19.8	8.5
Dissolved Oxygen (mg/L)	4.9	2.18
ORP (mV)	113	224

In parallel, biostimulation assays were conducted in microcosms with source-zone samples to assess whether dioxane removal could be enhanced. 1-BuOH and THF were used as the auxiliary substrates for amendment, with spiked dosages of 100 mg/L and 300 µg/L, respectively. To discern biodegradation from abiotic removal, killed control treatments were prepared with materials that were separately autoclaved and poisoned with HgCl₂ (200 mg/L). Triplicates were prepared for each treatment.

Aqueous microcosm samples were taken and analyzed weekly or biweekly. Dioxane and THF concentrations were measured by GC/MS using the frozen microextraction method developed in our lab.¹⁰⁰ The concentrations of 1-BuOH were measured by Agilent 5890 Chromatograph (GC) with a Flame Ionization Detector (FID) using direct water injection after filtration by 0.2-µm Nylon syringe filters to remove the biomass and suspended particles.¹³

A first-order (exponential) decay model was used to fit the dioxane degradation data. This is appropriate according to Monod's model, since the initial dioxane concentrations (6 to 300 µg/L) were at least two orders of magnitude lower than the Monod half-saturation constants (21 to 330 mg/L) reported for dioxane-degrading isolates.¹¹

At selected time points, 10 mL of water-sediment mixture was removed from each microcosm. The total biomass and solids were harvested by centrifugation at 10,000 × g for 10 min. A total of 0.25 gram of biomass/solid mixture was transferred to PowerBead tubes (Mo Bio, Carlsbad, CA) and spiked with 5 µL of bacteriophage λ DNA (0.2 ng/µL, Sigma-Aldrich, St. Louis, MO) to track the extraction efficiency. Total microbial genomic DNA (gDNA) was then extracted using a PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA) following the manufacturer's instruction. The eluted DNA was further purified and concentrated using a Genomic DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA). Copy numbers of the *thmA/dxmA* genes encoding for large hydroxylases of the dioxane-degrading enzymes (i.e., THF/dioxane MOs) were quantified by qPCR using a specific probe/primers set developed in our group.¹³⁰ Total bacterial biomass was quantified by enumerating the copy numbers of 16S rRNA genes.¹⁰¹ For qPCR analysis, detailed information regarding the instrument setup and calibration is provided in.¹³⁰

7.2.3. Bacterial cultures

Pseudonocardia dioxanivorans CB1190 (ATCC #55486)³⁹ was selected as the archetypic model to investigate the molecular impacts of both inducing and non-inducing compounds. CB1190 was grown in AMS medium amended with dioxane (500 mg/L) as the sole carbon and energy source at 24 ± 2 °C with shaking (120 rpm). Cells were harvested at late exponential phase by centrifugation at 10,000 × g for 15 min. The supernatant was decanted and the pellets were washed three times with 1 × phosphorous buffered saline (PBS) to remove organic residues. Then, the cell pellets were resuspended and concentrated with AMS medium (approximately 10 mg/L as total protein concentration) for subsequent gene expression and plasmid retention analysis.

7.2.4. Gene expression analysis

To evaluate changes in gene expression induced by various substrate compounds, triplicate treatments were prepared in 250 mL amber glass bottles containing 100 mL of AMS

medium amended with dioxane (100 mg/L), THF (100 mg/L), 1-butanol (100 mg/L), or acetate (100 mg/L) as sole carbon source or 100 mL of the nutrient-rich R2A medium. Treatments were then inoculated with 1 mL of concentrated CB1190 culture stock and incubated at 24 ± 2 °C while shaking at 120 rpm. The concentrations of amended organic chemicals in the microcosms were monitored daily by Agilent 5890 GC-FID by direct injection of 1 μ L of the filtered aqueous phase.¹³

When more than one-half of the added substrates were depleted (2 to 4 days), cells were harvested by manually filtering through 0.45 μ m sterile HA membrane filters (Millipore, Billerica, MA) assembled with Swinnex 25 mm filter holders (Millipore, Billerica, MA). The biomass along with the filter was then immediately immersed in 100 μ L lysozyme solution (10 mg/mL) in a 15 mL sterile centrifuge tube, and the cell pellets were resuspended by vortexing. After the addition of 0.5 μ L 10% SDS solution and 350 μ L lysis buffer with 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO), the cell lysate was homogenized using an ultrasonic homogenizer Sonic Ruptor 250 (Omni International, Kennesaw, GA) at a power output of 80 watts for 2 min. Then, the solution was transferred to a 1.7 mL sterile tube by pipetting, and the total RNA in the solution was extracted using a PureLink RNA Mini kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, in combination with an on-column PureLink DNase Treatment (Invitrogen, Carlsbad, CA) to eliminate interference from DNA. cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Carlsbad, CA) with RNase inhibitor (Applied Biosystems, Carlsbad, CA) added, and then purified using a Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI) following the manufacturer's protocol. The cDNA concentration in the elution (50 μ L) was determined by Nanodrop ND-1000 (Nanodrop products Inc., Wilmington, NE), and subsequently diluted to 1 ng/ μ L with DNA/RNase free water for reverse transcription-quantitative PCR analysis (RT-qPCR).

RT-qPCR was performed using a 7500 real time PCR system from Applied Biosystems (Carlsbad, CA) in 15 μ L of reaction mixture composed of 1 μ L diluted cDNA (1 ng/ μ L), Power SYBR Green PCR Master Mix (7.5 μ L), 0.3 μ M of each primer and DNA/RNase free water. The thermocycler program was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The $2^{-\Delta\Delta C_T}$ method was used to quantify differential gene expression,¹³¹ and the expression fold change were estimated with the calculation formula below (Eq. 1).

$$\Delta\Delta C_T, \text{ Target gene} = (C_{T, \text{ Target gene}} - C_{T, \text{ Housekeeping gene}})_{\text{Treatment}} - (C_{T, \text{ Target gene}} - C_{T, \text{ Housekeeping gene}})_{\text{Control}}$$

--- Eq. 1

For this assay, the target gene was *dxmA*, which encodes the α subunit of the CB1190 dioxane MO. The CB1190 16S rRNA gene was selected as the housekeeping gene to normalize experimental variance. Specific primers targeting the *dxmA* and 16S rRNA gene of CB1190 were designed and indicated in the supplementary information (Table 7.3). Treatments fed with acetate as the sole carbon source were used as the control for expression normalization and statistical analysis.

Table 7. 3 Primers used in RT-qPCR to quantify the relative gene expression levels with the presence of different substrates.

Targeted Gene	Primer	Sequence (5' - 3')
<i>dxmA</i>	Psed_6976_F	GGA CCG TGC ACG CAT TCG TC
	Psed_6976_R	CGT GGT CCA GCT TTC CGG GT
16S rRNA	CB1190_16S_F	TGG GTT TGA CAT GCA CCA GAC A
	CB1190_16S_R	ATA ACC CGC TGG CAA CAT GGA A

7.2.5 Plasmid retention assay

The genome of CB1190 contains three copies of the 16S rRNA gene on the chromosome and one copy of *dxmA* on the plasmid pPSED02.⁷⁶ Thus, the copy number ratio between *dxmA* and 16S rRNA genes is useful to predict the per cell retention rate of pPSED02 under selective conditions (i.e., in the presence of substrates that induce the transcription of the *dxmA* gene) or non-selective conditions (when such substrate induction is absent). To create such metabolic pressure, concentrated CB1190 (1 mL) was inoculated into 125 mL glass flasks sealed with semi-permeable 0.2 µm membranes, and supplemented with 50 mL of five types of growth medium, including: (i) AMS amended with 500 mg/L of dioxane, (ii) AMS amended with 500 mg/L of 1-BuOH, (iii) AMS amended with 500 mg/L of THF, (iv) R2A, and (v) AMS amended with 500 mg/L acetate. All treatments were kept at room temperature while shaking at 150 rpm. The cell pellets were harvested weekly from 10 mL of the culture solution in each treatment. After washing with 1 × PBS buffer for three times, the harvested cells were resuspended and inoculated into glass flasks containing 50 mL of newly-prepared medium for each treatment. After four weeks of cultivation, the cells in different treatments were collected by centrifugation for gDNA isolation. Total gDNA was extracted using an Ultra Clean Microbial DNA Isolation Kit (Mo Bio, Carlsbad, CA) based on the manufacturer's protocol. qPCR using TaqMan chemistry was used for absolute quantification of 16S rRNA and *dxmA* gene copy numbers based on a method modified from Li *et al.*¹³⁰ The plasmid retention percentage was calculated using the equation below (Eq. 2) based on the comparison of the ratio between the abundance of *dxmA* and 16S rRNA genes at Time 0 and 4 weeks.

$$\text{Percent Retention of Plasmid} = \frac{(C_{dxmA,T}/C_{16S\ rRNA,T})}{(C_{dxmA,0}/C_{16S\ rRNA,0})} \times 100 \%$$

--- Eq. 2

7.2.6 Whole genome sequencing of CB1190 mutants

After successive subculturing in R2A medium for over six months, CB1190 cells were found to lose their capability to degrade dioxane. The gDNA of these CB1190 cells were then extracted using Ultra Clean Microbial Isolation Kit (Mo Bio, Carlsbad, CA) and sent to an external sequencing facility (MR DNA, Shallowater, TX) for whole genome sequencing. Details regarding the library preparation and sequencing procedures by MiSeq (Illumina, San Diego, CA) are included in the Supplementary Information.

The paired Illumina reads were aligned against the reference genome of CB1190⁷⁶ using the BWA-MEM algorithm (version: 0.7.5a-r405).^{132, 133} For each individual DNA molecule

possessed by CB1190 (i.e., one chromosome and three plasmids), its average sequence coverage was estimated as the total sequencing read length normalized with its original genome size, representing the detection frequency of a given DNA molecule in the reference genome. Further, genomic structural variants such as gene insertions and deletions were examined with BreakDancer (version: 1.1.2) based on the aligned bam.¹³⁴

7.2.7 Statistical analysis

Statistical significance of differences between experimental treatments was assessed using Student's *t*-test at the 95% confidence level. Paired *t*-tests were used when samples originated from the same microcosm set.

7.3 Results and Discussion

7.3.1 Addition of 1-BuOH temporarily enhanced dioxane biodegradation, but such stimulatory effects cannot be sustained by repeated amendments

Microcosms were prepared with source zone samples, which exhibited the fastest attenuation rate and highest initial abundance of indigenous dioxane degraders (Supplementary Information). The auxiliary substrate 1-BuOH, which is known to support the growth of some dioxane degraders,^{39, 135} was tested to enhance dioxane degradation by the indigenous consortium. The addition of 1-BuOH initially improved dioxane removal compared to unamended (natural attenuation) microcosms. This temporary enhancement was small but statistically significant ($p < 0.05$) (Figure 7.2), with the first-order dioxane degradation rate coefficient significantly increasing from $0.020 \pm 0.001 \text{ week}^{-1}$ to $0.027 \pm 0.003 \text{ week}^{-1}$ (Table 7.4). Analysis by qPCR (Figure 7.2B) showed that both the abundance of dioxane degraders and overall microbial populations increased compared to the beginning of the incubation ($p < 0.05$). However, at Week 16, the abundance of dioxane degraders (indicated by *dxmA/thmA* gene counts) did not increase significantly compared to the natural attenuation treatment (Figure 7.3B). Thus, addition of 1-BuOH was not directly linked to the proliferation of dioxane degradation genes.

Table 7. 4 1st order kinetic constants for dioxane biodegradation.

Treatment	1st order kinetic constants (week⁻¹)	R²
Natural Attenuation	0.020 ± 0.001	0.975
Single 1-BuOH Amendment	0.027 ± 0.003	0.943
Double 1-BuOH Amendments (Week 0 to 12)	0.028 ± 0.007	0.857
Double 1-BuOH Amendments (Week 12 to 16)	0.012 ± 0.002	0.967
THF Amendment	0.027 ± 0.013	0.808
THF Biodegradation*	0.037 ± 0.004	0.950

* This 1st order kinetic constant represents the degradation rate of THF (not dioxane) observed in microcosms amended with THF.

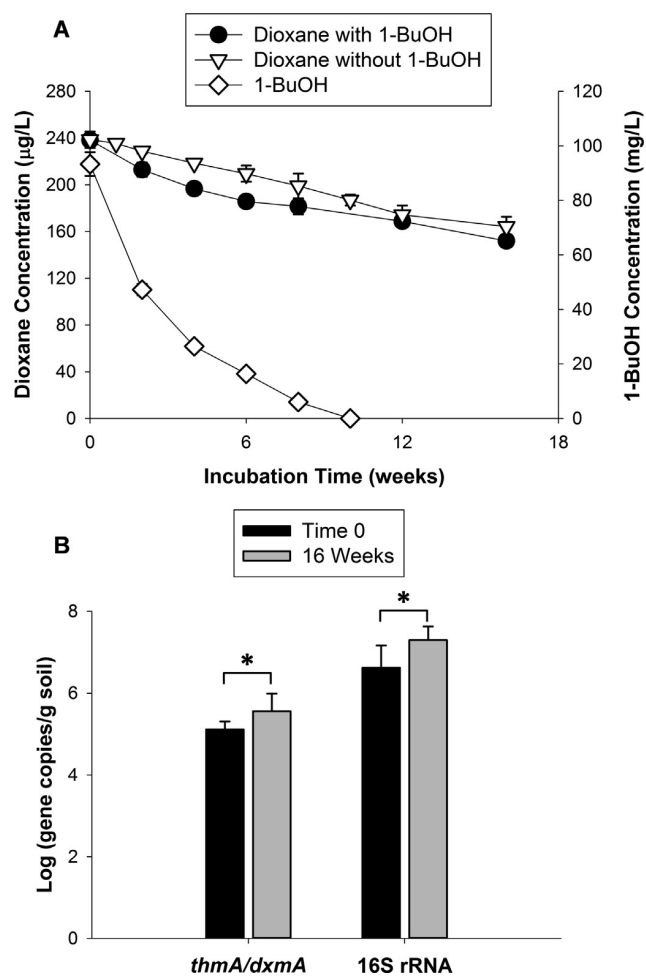


Figure 7. 2 Slight temporary enhancement of dioxane biodegradation by 1-butanol addition (relative to natural attenuation) in microcosms prepared with source zone materials (A) and abundance of *thmA/dxmA* and 16S rRNA genes at different sampling time points (B). Asterisks indicate the p values less than 0.05 based on the student t test.

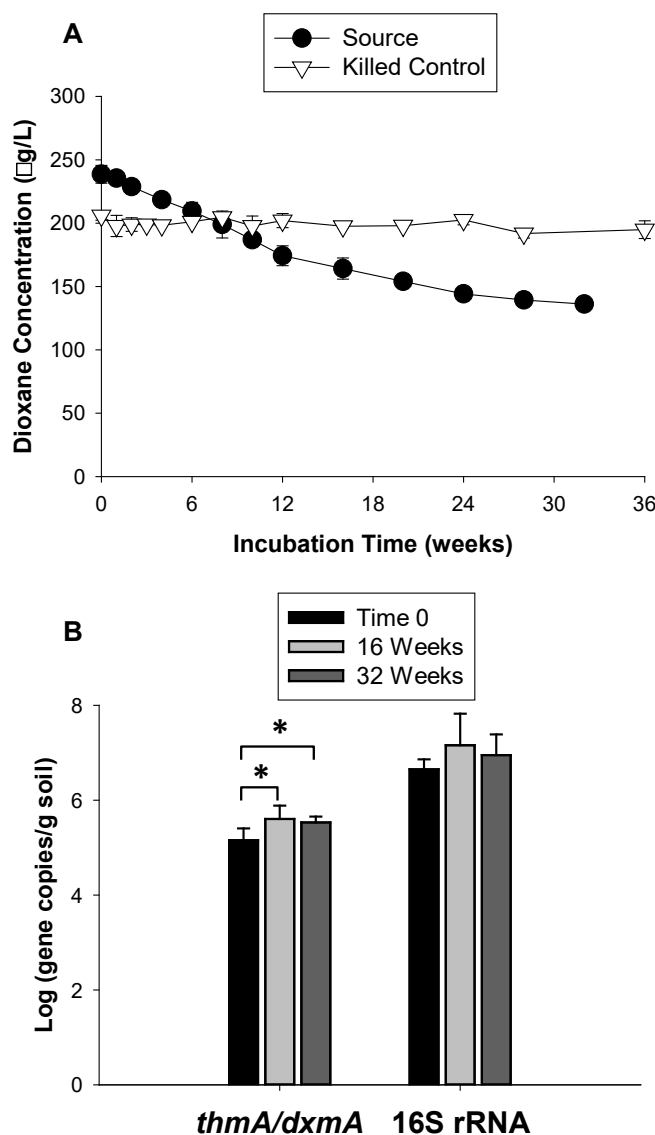


Figure 7.3 Degradation of dioxane in natural attenuation microcosms prepared with source zone materials (A) and abundance of *thmA/dxmA* and 16S rRNA genes at different sampling time points (B).

Asterisks indicate the *p* values less than 0.05 based on the student *t*-test.

To assess the effects of repeated biostimulation, 1-BuOH (100 mg/L) was respiked immediately after it was completely consumed. Similar to observations in the microcosms with a single 1-BuOH amendment, this first amendment of 1-BuOH also enhanced dioxane removal as well as the growth of dioxane degraders and total biomass (Figure 7.4). As predicted by enhanced acclimation, the second 1-BuOH amendment was consumed much faster (i.e., less than 4 weeks) compared to the first amendment. However, unlike the first amendment, this second 1-BuOH amendment did not enhance dioxane degradation. On the contrary, the estimated first-order degradation rate coefficient for dioxane significantly decreased from $0.028 \pm 0.007 \text{ week}^{-1}$ to $0.012 \pm 0.002 \text{ week}^{-1}$, which was slower than observed in microcosms mimicking natural attenuation ($0.020 \pm 0.001 \text{ week}^{-1}$). This decrease in dioxane removal rate was also corroborated by our qPCR analysis results, which showed a significant decrease of dioxane degrading

microorganisms harboring the *thmA/dxmA* genes compared to Week 12 (Figure 7.4B), though total biomass increased. Our results also show that 1-BuOH was preferentially degraded over dioxane in these microcosms.

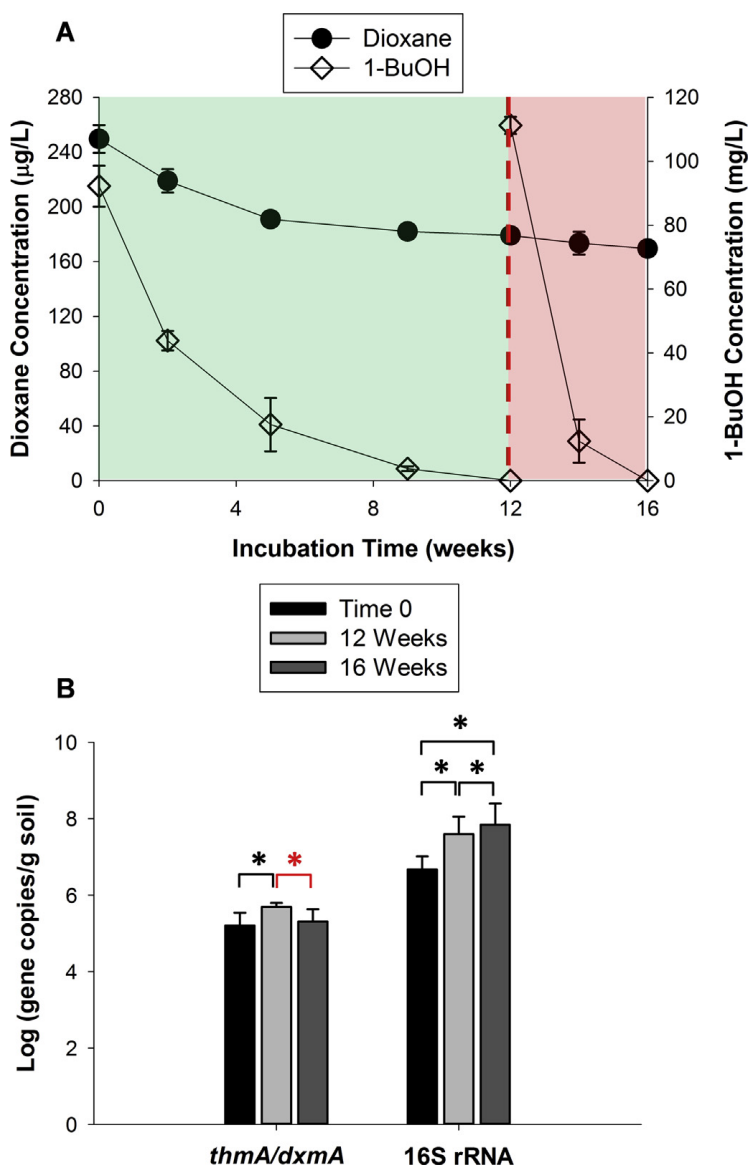


Figure 7. 4 Untenable biodegradation of dioxane in microcosms prepared with source zone material by repeated amendment of 1-BuOH at Week 12 (A) and abundance of *thmA/dxmA* and 16S rRNA genes at different sampling time points (B).

Asterisks indicate the *p* values less than 0.05 based on the student *t*-test.

The stimulation of growth by 1-BuOH amendment for both total biomass and dioxane degraders (represented by the ^{13}C enriched biomass) was also observed in the *in situ* microcosm deployed at the source zone monitoring well (Table 7.5). However, dioxane decomposition and mineralization were not significantly improved compared to the natural attenuation controls. This

corroborates our finding that amendment with 1-BuOH can temporarily enhance the growth of dioxane degraders but is not capable of sustaining induction of the necessary catabolic enzymes.

Table 7. 5 Stable isotope probe analysis indicating the dioxane removal and incorporation of ^{13}C into biomass and dissolved inorganic carbon (DIC) in Bio-Trap samplers baited with ^{13}C -labeled dioxane.

Sampling Location Description	Source Zone		Middle of Plume	
	MNA	1-BuOH	MNA	1-BuOH
^{13}C 1,4-dioxane Pre-deployment ($\mu\text{g}/\text{bead}$)	42 ± 6	42 ± 6	42 ± 6	42 ± 6
^{13}C 1,4-dioxane Post-deployment ($\mu\text{g}/\text{bead}$)	7 ± 1	6 ± 2	2 ± 0.2	0.8 ± 0.2
^{13}C 1,4-dioxane Loss ^a (%)	59.3	61.7	71.2	74.1
Total Biomass (cells/bead)	1.01E+06	1.77E+06	5.13E+06	9.90E+05
^{13}C Enriched Biomass (cells/bead)	1.42E+04	2.26E+04	5.49E+04	1.28E+04
^{13}C Incorporation (%)	1.41	1.28	1.07	1.29
Average PLFA $\delta^{13}\text{C}$ (‰)	410	200	35	232
Maximum PLFA $\delta^{13}\text{C}$ (‰)	1,571	484	127	711
DIC $\delta^{13}\text{C}$ ^b (‰)	29	23	2.7	15

^a The loss of ^{13}C 1,4-dioxane was corrected for loss due to desorption based on pre-leach studies

^b The average background $\delta^{13}\text{C}$ value for DIC would be about -25 ‰.

Three possible mechanisms may explain the decline of dioxane degraders after the second amendment of 1-BuOH. The first possibility is that 1-BuOH or its metabolites are toxic to the indigenous degraders. This possibility can be ruled out because 1-BuOH and its metabolites (e.g., butyraldehyde and butyric acid) are typically non-toxic below 100 mg/L (which was the initial concentration of the parent compound, 1-BuOH). In addition, significant growth of dioxane degraders and total bacteria was observed after the first 1-BuOH amendment.

The second possibility is that dioxane degraders might be outcompeted by other microorganisms in the enriched consortium. Though dioxane degraders, such as CB1190, are known to metabolize 1-BuOH,²¹ numerous other bacteria that also grow on 1-BuOH cannot degrade dioxane. The percentage of dioxane degraders decreased from 3.4 % to 1.2 % at the first 1-BuOH amendment and continued to decrease to 0.3 % after the second amendment at Week 16. This is indicative of genotypic dilution by the proliferation of non-competent strains.¹³⁶ Extensive proliferation of the non-dioxane degraders might lead to an unfavorable environment for dioxane degraders due to competition for nutrients (N and P), trace elements, and electron acceptors (i.e., oxygen), as well as potential amensalism, which might contribute to the decline of the absolute abundance of dioxane degraders after the second 1-BuOH amendment.

The third (non-exclusive) possibility is that the continuous metabolic flux of 1-BuOH might lead to effects at the gene expression level, including catabolite repression and metabolic flux dilution (discussed in Section 3.3). As the essential genes for dioxane biodegradation (e.g., *thmA**DBC*/*dxmA**DBC* genes) are typically located on plasmids⁷⁶ lack of induction of dioxane-degrading enzymes may cause the curing of the catabolic plasmids. Due to the difficulty of tracking deletion or deamplification of genetic elements in mixed consortia, additional experiments were conducted to discern the molecular impacts of non-inducing substrates using a monoculture of the archetype dioxane degrader CB1190. Results that support this hypothesis are discussed below (Section 3.4). Overall, these microcosm experiments suggest that long term, repeated amendment of a non-inducing growth substrate (especially at large dosages) may hinder dioxane biodegradation. Hindrance mechanisms likely include higher microbial competition and genotypic dilution, and (in some cases) loss of intrinsic biodegradation capacity.

7.3.2 Addition of THF stimulated the growth of dioxane degraders, but hindered dioxane removal

In parallel to 1-BuOH, THF was tested as an inducing substrate for dioxane biostimulation due to its similar cyclic ether structure to dioxane. Surprisingly, a similar dosage of THF (300 µg/L) completely suppressed dioxane biodegradation for the first 16 weeks (Figure 7.5). During the incubation, THF was preferentially degraded over dioxane. Dioxane degradation began between Week 16 and 23 after the THF concentration decreased below 186.5 ± 27.5 . Though this lag was experienced, dioxane degradation proceeded at a faster pace (0.027 ± 0.013 week⁻¹) than the natural attenuation microcosms. Accordingly, a significant increase in the abundance of dioxane degrading genes was measured by qPCR analysis (Figure 7.5B). No significant growth of total biomass (indicated by 16S rRNA genes) was observed.

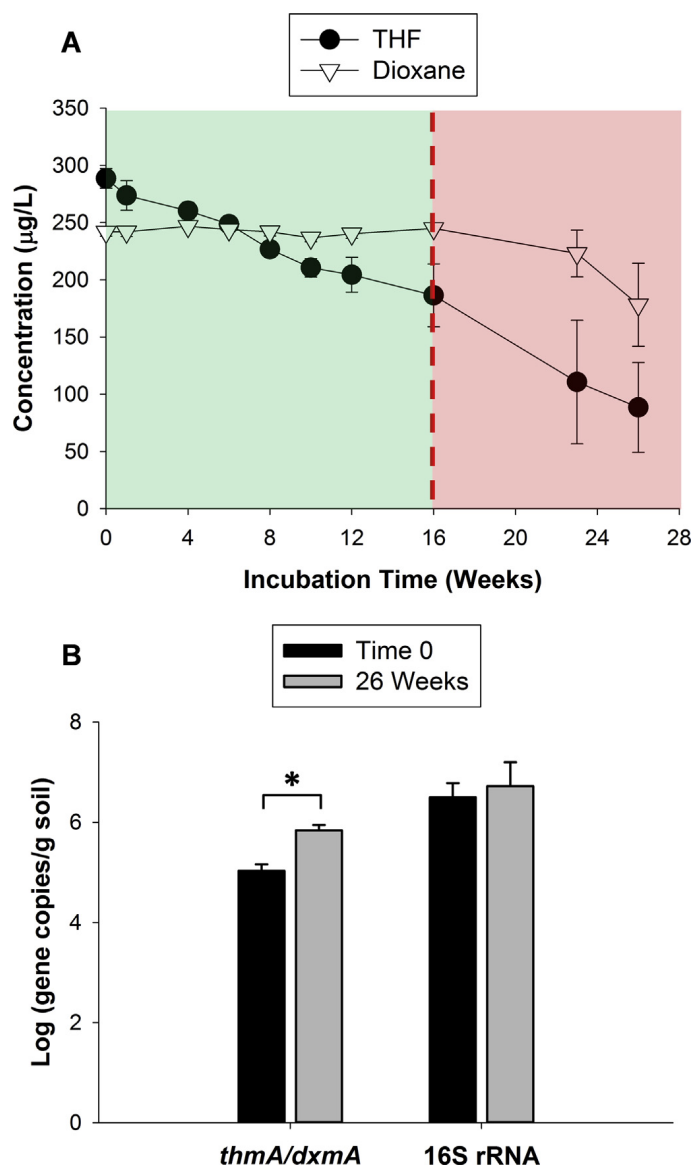


Figure 7.5 Delayed removal of dioxane in microcosms prepared with source zone materials and amended with comparable amount of THF (300 µg/L) at the beginning of the experiments (A) and abundance of *thmA/dxmA* and 16S rRNA genes at different sampling time points (B).

Significant decrease of dioxane concentration occurred after Week 16. Asterisks indicate the p values less than 0.05 based on the student t -test.

The initial inhibitory effect of THF on dioxane degradation is probably due to competitive inhibition, when both compounds are degraded by the same enzyme and bind to its active site. Competitive inhibition can be pronounced when i) the affinity to the inhibitor (i.e., THF) is greater than the substrate (i.e., dioxane) or ii) the substrate concentration is low compared to its Monod half saturation constant (K_s). Previous studies observed a higher affinity of THF/dioxane MO towards THF compared to dioxane.³⁹ For some cometabolic reactions, pure strains or enriched consortia require THF as primary substrate and enzyme inducer to degrade dioxane.²¹ In addition, kinetic studies using isolated dioxane degrading strains (e.g., CB1190 and *Mycobacterium* sp. D6) showed that their K_s values towards dioxane are typical large, between

20.6 and 330 mg/L,¹¹ which is indicative of relatively low affinity. The initial dioxane concentration ($241.9 \pm 3.7 \mu\text{g/L}$) in the prepared microcosms was two orders of magnitude lower than these K_s values, which is conducive to significant competitive inhibition by alternative substrates that bind with the active site of the enzyme. This is predominantly observed in cometabolic biostimulation treatments where the primary substrate binds to the enzyme and inhibits degradation of the target pollutant. For instance, cometabolism of trichloroethylene (TCE) by methane monooxygenases can be significantly inhibited by methane, as well as by co-occurring chlorinated aliphatic hydrocarbons, such as chloroform.^{137, 138}

Accelerated dioxane removal and increased abundance of *thmA/dxmA* (but not 16S rRNA) genes were observed after THF concentration decreased below approximately 160 $\mu\text{g/L}$. Apparently, THF selectively stimulated the growth and activity of dioxane degraders, as discussed below in Section 3.3. Overall, THF may serve as both inducer and competitor for the enzymes that initiate dioxane degradation. Though addition of THF can enhance the growth and activity of indigenous dioxane degraders and stimulate biodegradation in the long-term, a measurable delay or lag phase can occur due to competitive inhibition exacerbated by low dioxane concentrations commonly found at thousands of sites impacted by chlorinated solvents.^{3, 125}

7.3.3 Expression of *dxmA* was induced by THF, but not by 1-BuOH

To investigate the effect of different substrates on the induction of dioxane MO, RT-qPCR was conducted using total RNA extracts from the archetype dioxane degrader *Pseudonocardia dioxanivorans* CB1190 after growth in AMS with a single carbon source (e.g., dioxane, 1-BuOH, THF, or acetate as control) as well as complex R2A medium. As expected, dioxane MO was induced at relatively high expression levels by dioxane and its structural analogue, THF (Figure 7.6). Compared to acetate, the expression of *dxmA* was significantly up-regulated by dioxane (51-fold higher) and THF (62-fold higher) (Figure 7.6). This corroborates the role of this MO in the metabolism of small cyclic ethers, as demonstrated by other studies using advanced molecular techniques, such as transcription microarray, Northern blot, and SDS-PAGE.⁴⁶ The inducibility of dioxane MO by THF in CB1190 is consistent with the stimulatory effects of THF in dioxane degradation observed in the microcosm assays prepared with field samples (Figure 7.5). Conversely, the presence of easily biodegradable substrates (e.g., 1-BuOH), repressed induction of dioxane MO (Figure 7.6). No significant up-regulation of the *dxmA* gene was observed in treatments fed with 1-BuOH or R2A compared to the acetate control (Figure 7.6).

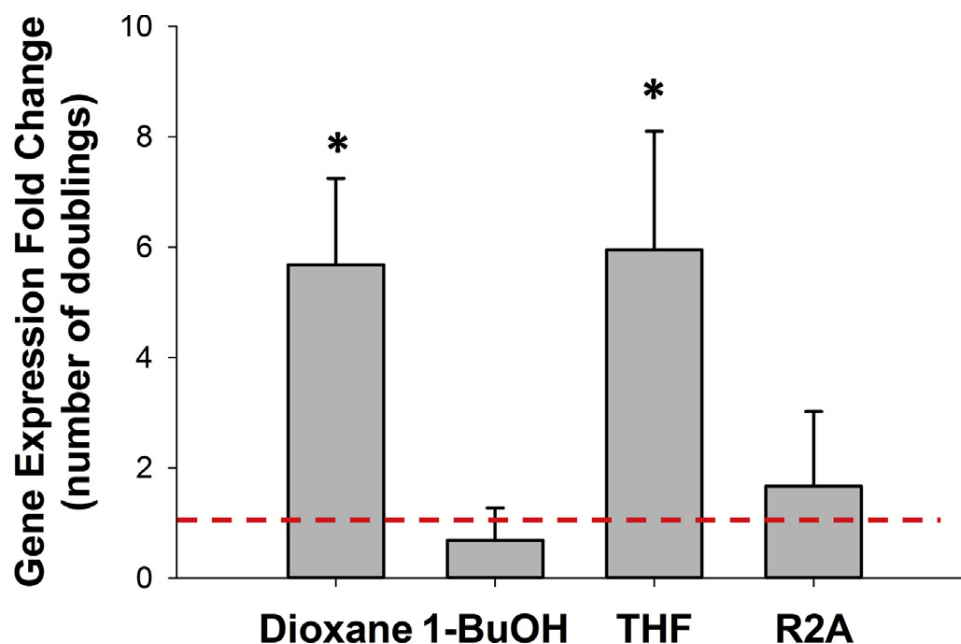


Figure 7. 6 Expression of *dxmA* genes in CB1190 after fed with various substrates for two to four days.

Data were normalized to the treatment fed with acetate as sole carbon and energy source. The $2^{-\Delta\Delta CT}$ method was used to quantify differential gene expression. Asterisks indicate significant ($p < 0.05$) up-regulation compared to the acetate-fed control. The dotted red line represents 2-fold up-regulation.

Acetate, 1-BuOH, and the major constituents of R2A (e.g., pyruvate and casamino acids) are readily biodegradable by constitutive enzymes through central metabolic pathways (e.g., Krebs' cycle and β -oxidation).¹¹² In contrast, biodegradation of recalcitrant contaminants, such as dioxane and THF, is often initiated by specific catabolic enzymes (e.g., inducible monooxygenases) that transform the compounds into metabolites that subsequently enter the central metabolic pathways. Thus, exposure to large concentrations of the readily biodegradable substrates may dilute the metabolic flux of dioxane through these pathways, resulting in slower dioxane degradation rates.¹³⁹

High concentrations of easily degradable substrates may also hinder expression of genes involved in dioxane degradation via catabolite repression. As part of the global control system in microorganisms, catabolite repression facilitates enzymatic adaption to accelerate and prioritize the metabolism of preferred carbon and energy source (e.g., 1-BuOH or acetate) by restraining the synthesis of other enzymes that are not needed (e.g., dioxane MO). Previous studies have shown that substrates that are degraded via central metabolic pathways by constitutive enzymes can inhibit the synthesis of inducible catabolic enzymes that are involved in biotransformation of xenobiotics.^{140, 141} Pronounced catabolite repression is associated with the high concentration of the preferred substrates, fast microbial proliferation, and limited availability of nutrients.^{142, 143} These conditions are well aligned with our microcosm study of repeated 1-BuOH amendment, including the repeated large dosage of 1-BuOH as the rapidly metabolizable substrate supporting significant growth of total biomass. Thus, catabolite repression also likely contributed to the observed hindrance of dioxane degradation in 1-BuOH-amended microcosms.

7.3.4 Long-term, successive culturing in non-selective medium resulted in pPSED02 plasmid curing and loss of *dxmADBC*.

The long-term effects on dioxane degradation capacity caused by amendment of auxiliary substrates were explored using the model strain CB1190. The amended substrate for each treatment was kept in excess by refreshing the medium weekly. After four weeks of incubation, the relative *dxmA* abundance in treatments fed with dioxane and THF did not differ significantly from concentrations measured at the beginning of the experiment (Figure 7.7), indicating persistence of the plasmid pPSED02 under inducing conditions. However, significant loss of this catabolic plasmid was observed in treatments fed with 1-BuOH or acetate, which do not induce or require dioxane MO. Compared with the *dxmA*/16S rRNA gene ratio measured in the initial inoculum, only 9.5 ± 7.4 % and 26.6 ± 6.2 % of the plasmids were retained per cell after growth on 1-BuOH and acetate, respectively, for four weeks. The low retention rate of *dxmADBC* in CB1190 after long-term growth on 1-BuOH corroborates our hypothesis that repeated amendments of non-inducing substrates may lead to the loss or deamplification of mobile genetic elements containing the dioxane degrading genes. This also explains the significant decrease in relative abundance of dioxane degrading genes and dioxane degradation rates observed in the microcosm experiments (Figure 7.4). After growth in complex R2A medium, the relative abundance of plasmid pPSED02 also decreased to 69.0 ± 13.6 %. Since total DNA extractions were used to obtain both chromosomal and plasmid DNA, our results lessen the possibility that the lost *dxmA* gene was integrated into the chromosome.

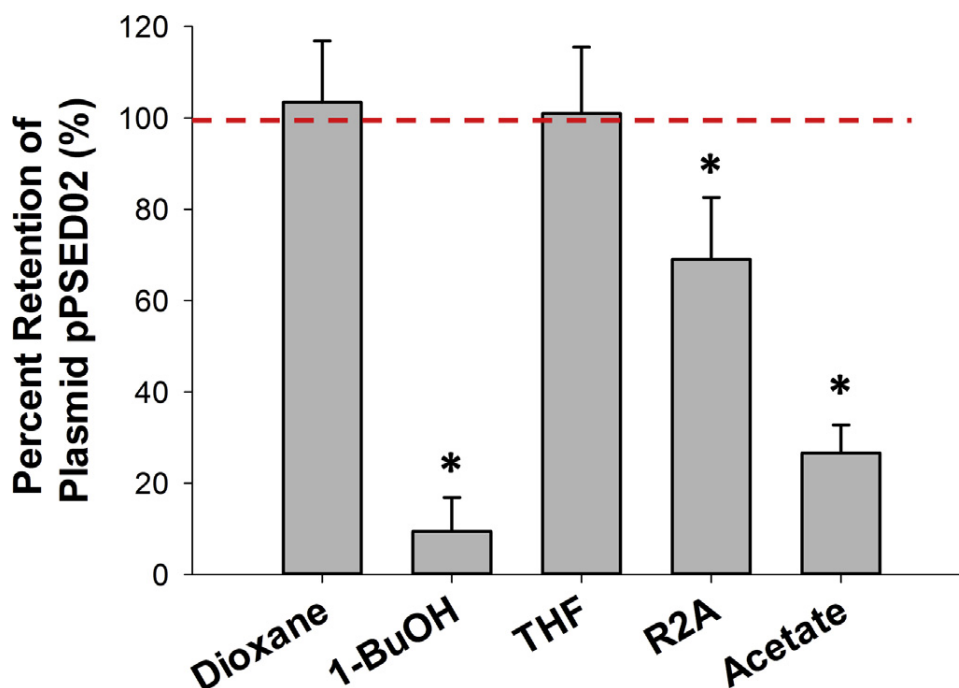


Figure 7. 7 Percent retention of the plasmid pPSED02 in CB1190 segregants after growth on different substrates for four weeks.

Data were normalized to the initial *dxmA*/16S rRNA gene ratio in stock cultures fed with dioxane.

Asterisks indicate the *p* values less than 0.05 based on the student *t*-test. The dotted red line represents the 100% retention level, implying no plasmid loss.

In long-term studies, complete curing of pPSED02 was observed in CB1190 under non-selective conditions (i.e., in R2A medium). Genome sequencing (Table 7.6) showed that the average sequence coverage, an indicator of the detection frequency, was 3 X, 0.09 X and 41 X for the three CB1190-bearing plasmids, pPSED01, pPSED02 and pPSED03, respectively. However, the average sequence coverage for the chromosome in the obtained CB1190 cells was as high as 100 X, representing the detection frequency when no curing of genetic constituents occurs. The considerably low detection frequencies of plasmids pPSED01 and pPSED02 suggests the complete loss of these two plasmids in most CB1190 cells obtained after successive subculturing in nutrient-rich medium R2A for over 6 months. The relative low abundance of the smallest pPSED03 implies the occurrence of partial loss, assuming the extraction efficiencies for the chromosomal and plasmid DNA were not significantly different. In addition, genomic structural variants such as gene insertions and deletions were not detected on either the chromosome or the plasmid pPSED03, suggesting their stability during subculturing.

Table 7. 6 Average sequence coverage of the chromosome and three plasmids of the CB1190 mutant culture after growth in R2A for over 6 months.

DNA Molecules	Length	Average Sequence Coverage
chromosome	7.1 Mb	100X
pPSED01	192 kb	3X
pPSED02	137 kb	0.09X
pPSED03	15 kb	41X

Plasmid loss in non-selective environments is possibly due to segregational instability during cell division. For instance, absence of antibiotics (for resistance plasmids) or inducing substrates (for catabolic plasmids) can impact segregational stability or result in plasmid deamplification which subsequently reduce the plasmid abundance or retention.¹⁴⁴ Even under nutrient-rich conditions, maintenance and replication of the catabolic plasmids represents an energy cost. Since the encoded enzymes from the plasmid are not needed for growth when the easily-degradable non-inducing substrates are present, the advantage in maintaining non-essential plasmids is diminished. In addition, the large size of these two cured plasmids (i.e., 192 kb and 137 kb) represents a significant energy and resource burden during replication, which may be reduced by segregational instability in which random loss and distribution of plasmids occurs during cell divisions.¹⁴⁵ For plasmid pPSED03, the relatively high retention ratio was probably attributed to its small size whose maintenance requires less energy.

A number of previous studies have reported that plasmids are more stable in nutrient-rich media, which provides sufficient energetic content to meet the metabolic burden for plasmid reproduction and maintenance.^{146, 147} However, for plasmids bearing specific metabolic functions, such as pPSED02 that contains the gene cluster encoding dioxane degradation enzymes, the stability of such plasmids can be highly dependent on the selective pressure exerted by the substrates or metabolic inducers. When dioxane or THF is absent, pPSED02 might become less advantageous for the cell to maintain, which possibly contributes to plasmid curing though grown in a nutrient-rich environment.

Note that incomplete or complete plasmid loss is the result of natural selection and genetic mutations, which increase host fitness in a specific environment. Under certain conditions with a non-inducing carbon source, derivative (“cured”) segregants may evolve with distinct metabolic advantages for the available substrates and outcompete the wild-type over time.¹⁴⁸ Plasmid-free derivative strains also tend to grow faster than the wild-type due to a reduced cell size and an enhanced surface-to-volume ratio.¹⁴⁹ Thus, it is plausible to conclude the CB1190 segregants without pPSED01 and pPSED02 have a higher fitness in R2A medium than the wild-type strain. The propagation of CB1190 segregants that lack dioxane degradation capabilities likely contributes to the lengthy dioxane degradation lag commonly observed using CB1190 cells grown in rich medium, such as R2A and LB.

Potential negative effects from amending non-inducing substrates at large dosages should also be considered in the context of bioaugmentation; if dioxane concentrations are too low to induce and sustain specific degraders, or if more easily degradable contaminants are readily available, the possibility of plasmid curing or inhibition of dioxane degradation may be higher.

Chapter 8

Detection and Cell Sorting of *Pseudonocardia* Species by Fluorescence *in situ* Hybridization and Flow Cytometry Using 16S rRNA-Targeted Oligonucleotide Probes

8.1 Introduction

Pseudonocardia is a genus of *Actinobacteria* that has received significant attention due to its biotechnological, pharmaceutical, agricultural, and environmental remediation applications, as well as for its unique ecological roles. Similar to other actinomycetes, some members of *Pseudonocardia* produce bioactive secondary metabolites, including antibiotics¹⁵⁰⁻¹⁵³ and immune-modulating agents.¹⁵⁴ For instance, *Pseudonocardia* is renowned for its commensal relationship with fungus-farming leaf-cutting ants^{155, 156} and produces target-specific antibiotics (e.g., dentigerumycin) to remove competing microbes or parasites.^{151, 157} Since leaf-cutting ants are serious pests and contribute to tremendous crop reduction (20 ~ 30 %) in tropical and subtropical America, detection and control of mutualistic *Pseudonocardia* may leverage the ant management and thus promote crop yield.¹⁵⁸

Pseudonocardia spp. are found in diverse environments such as soils, water, activated sludge, and plant roots,^{13, 20, 159} and are widely recognized for their broad metabolic range that enables adaptation to different environmental niches. Though some isolates exhibit autotrophic growth via fixation of atmospheric carbon dioxide/monoxide,^{160, 161} most *Pseudonocardia* spp. live heterotrophically under aerobic or microaerophilic conditions. Members of *Pseudonocardia* are known to degrade various priority pollutants, including aromatic hydrocarbons (e.g., benzene and toluene),²⁰ methyl sulfides,¹⁶² and halogenated compounds (e.g., chloroethene, 1,2,3,5-tetrachlorobenzene, and monofluorophenols).¹⁶³⁻¹⁶⁵

Notably, *Pseudonocardia* species are predominantly associated with the biodegradation of recalcitrant cyclic ether pollutants, particularly dioxane and tetrahydrofuran (THF), which are contaminants of emerging concern.²⁶ To date many bacterial strains have been isolated from various environments based on their capability to degrade dioxane and/or THF (Figure 8.1), and the majority of these bacteria are actinomycetes. It is remarkable that more than one-half (18 out of 34) of the isolates belong to the *Pseudonocardia* genus. A recent study randomly selected 13 *Pseudonocardia* strains from global cell culture resource facilities, and 11 of these strains (~ 85%) were able to grow on dioxane and/or THF as the sole carbon and energy source.⁵⁰ Two archetypic degraders of dioxane and THF are also *Pseudonocardia* species. *Pseudonocardia dioxanivorans* CB1190 is so far the best characterized strain capable of utilizing dioxane as a sole carbon and energy source.^{39, 166} Recently, its genome was sequenced, revealing a broad diversity of metabolic traits, including nitrogen fixation and autotrophic growth.⁷⁶

Pseudonocardia tetrahydrofuranoxydans K1 is the first-isolated THF degrader,²⁰ and can also fortuitously degrade dioxane via cometabolism when fed with THF as the primary substrate.¹¹ Thus, converging lines of evidence point to a strong link between *Pseudonocardia* spp. and their potential to degrade cyclic ethers. Although not all *Pseudonocardia* spp. degrade dioxane or THF, it is very plausible that *Pseudonocardia* spp. that grow and thrive at dioxane/THF contaminated sites are likely directly participating in the bioremediation process.

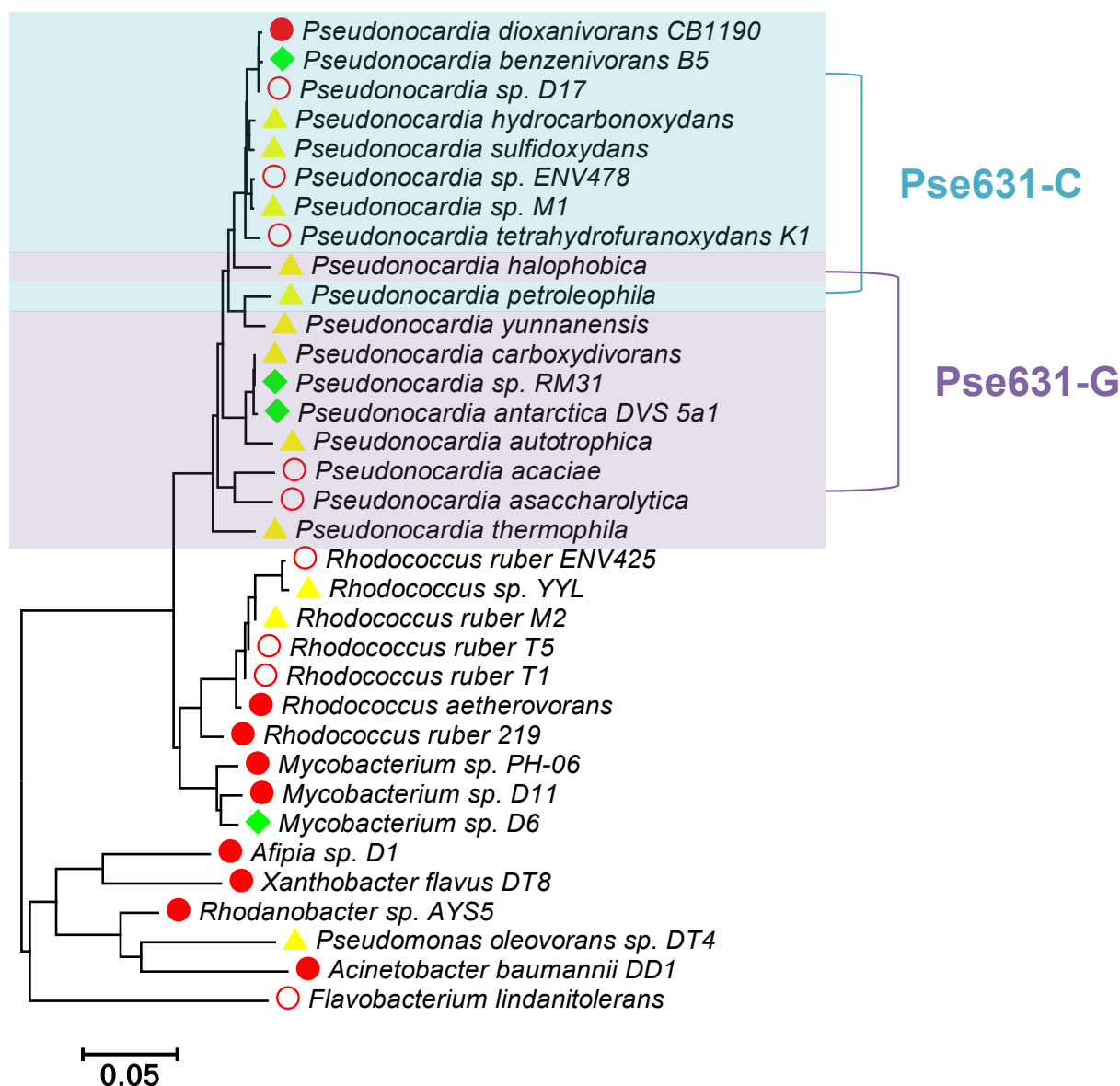


Figure 8. 1 Phylogenetic relationship of known dioxane and THF degraders and the coverage of designed probes (Pse631-C and Pse631-G) targeting the genus of *Pseudonocardia*.

Bacterial strains capable of metabolizing both dioxane and THF are indicated with solid red circle. Strains that can metabolize THF and cometabolize dioxane with THF as the primary substrate are indicated by hollow red circle. Dioxane degraders whose ability to degrade THF is unknown or absent are indicated with solid green diamond. THF degraders whose ability to degrade dioxane is unknown or absent are indicated by a solid yellow triangle. *Pseudonocardia* species targeted by the probes Pse631-C and

Pse631-G are shadowed in blue and purple, respectively. The tree was generated from the alignment of the full 16S rRNA sequences (approximately 1,400 nucleotides in length) retrieved from NCBI database using the maximum-likelihood algorithm by MEGA.

Given the significance of *Pseudonocardia* in industrial, agricultural, and bioremediation practices, expediting the identification and characterization of novel *Pseudonocardia* strains is important to advance understanding of their metabolic and physiological idiosyncrasies. However, traditional isolation techniques (e.g., enrichment and serial dilution for separating colonies on plates) are challenged by the fastidious nature of *Pseudonocardia* due to their slow growth rates, low cell yields, dissimilar colony morphologies, and dormant stages of their life cycles (e.g., spore formation).¹⁵³ For instance, the cell yield of the dioxane-degrading CB1190 is lower than 0.1 mg protein/mg dioxane even under optimal growth conditions.³⁹ Though many *Pseudonocardia* strains have been isolated, recent studies employed culture-independent techniques (e.g., 454-pyrosequencing) and uncovered discrepancy between the results from culture-dependent and culture-independent approaches, as well as isolation biases when screening for *Pseudonocardia*.^{167, 168} For example, other common genera (e.g., *Bacillus* and *Streptomyces*) grow faster under nutrient-rich conditions and outcompete *Pseudonocardia* by stripping nutrients or amensalistically generating antibiotics or acids.¹⁶⁹ In contrast, the use of hybridization probes in conjunction with modern flow cytometry technology avoids these cultivation biases and enables *in situ* detection and identification of *Pseudonocardia* species.¹⁷⁰⁻¹⁷²

In this chapter, we present a novel fluorescently-labelled rRNA-targeted oligonucleotide probe for imaging, separating, and enumerating *Pseudonocardia* cells. Coverage and specificity of this probe are evaluated based on empirical and computational assessment. In combination with high-throughput flow cytometry, this effort enables synchronized quantification and cell sorting of *Pseudonocardia* species from complex microbial mixtures recovered from target environments. This approach would facilitate molecular analysis of sorted cells to advance understanding of their ecological traits and potentiate applications ranging from antibiotic synthesis to cyclic ether bioremediation.

8.2 Materials and Methods

8.2.1 Oligonucleotide probe design and synthesis

To design genus-specific probes targeting *Pseudonocardia* species, multiple sequence alignment by ClustalX 2.1⁹⁸ was employed using the 16S rRNA genes retrieved from the National Center for Biotechnology Information (NCBI) database for a wide phylogenetic variety of bacterial strains, including all cyclic ether degrading bacteria listed in Table 8.1 and Figure 8.1. First, locations of the nine hypervariable regions in their 16S rRNA sequences were discerned using *Escherichia coli* K-12 as the reference.¹⁷³ Within each hypervariable region, sequence fragments that are only conserved among *Pseudonocardia* species but distinct from the other genera were selected for further probe development. The nucleotide fragments that are shorter than 18 bp were eliminated to ensure a margin of specificity.¹⁷⁴ To refine the probe-targeting nucleotide sequences, the starting and end positions of each probe candidate were manually adjusted to gratify the design criteria for oligonucleotide probes from Keller and Manak (1989). Probes were then designed as the reverse complementary sequence of the target nucleotides.

Table 8. 1 Bacteria capable of degrading dioxane and/or THF and the source materials where they were isolated from.

Bacterial Strain	Dioxane	THF	Isolation Source	Ref
<i>Rhodococcus ruber</i> 219	+	+	Effluent samples, Germany	175
<i>Pseudonocardia dioxanivorans</i> CB1190	+	+	Industrial sludge from a site at Darlington, SC, USA	39
<i>Pseudonocardia benzenivorans</i> B5	+	NA	Contaminated soil from Bitterfeld, Germany	176
<i>Mycobacterium</i> sp. PH-06	+	+	River sediment from Nakdong River, South Korea	40
<i>Pseudonocardia antarctica</i> DVS 5a1	+	NA	Moraine sample from the McMurdo Dry Valleys region of Antarctica	177
<i>Afipia</i> sp. D1	+	+	Drainage surface soil samples near a chemical factory producing dioxane in Japan	41
<i>Mycobacterium</i> sp. D6	+	-	Same as above	41
<i>Mycobacterium</i> sp. D11	+	+	Same as above	41
<i>Pseudonocardia</i> sp. D17	+	+	Same as above	41
<i>Acinetobacter baumannii</i> DD1	+	+	Mixture of activated sludge obtained from Qige urban sewage treatment plant, Hangzhou, Zhejiang, China	42
<i>Rhodanobacter</i> sp. AYS5	+	+	Industrial sludge, Saudi Arabia	178
<i>Xanthobacter flavus</i> DT8	+	+	Activated sludge of pharmaceutical plants, Zhejiang, China	179
<i>Pseudonocardia</i> sp. RM31	+	NA	Surface seawater, Okinawa Islands, Japan	180
<i>Rhodococcus aetherivorans</i> JCM 14343	+	+	Petrochemical biotreater sludge in Deer Park, TX, USA	50
<i>Pseudonocardia tetrahydrofuranoxydans</i> sp. K1	*	+	Sludge from a wastewater plant in Gottingen, Germany	20
<i>Pseudonocardia</i> sp. ENV478	*	+	Industrial wastewater treatment system in New Jersey	21
<i>Rhodococcus</i> sp. YYL	NA	+	Activated sludge samples from wastewater and solid waste treatment plants, chemical and pharmaceutical factories in Zhejiang, China.	22
<i>Rhodococcus ruber</i> ENV425	*	+	Uncontaminated turf soil enriched with propane	21
<i>Flavobacterium</i> sp.	*	+	Soil from a dioxane-contaminated groundwater plume in Canada	181
<i>Pseudomonas oleovorans</i> sp. DT4	NA	+	Activated sludge from a pharmaceutical factory in Zhejiang, China	182
<i>Pseudonocardia</i> sp. M1	NA	+	Mixed aqueous samples from an industrial wastewater treatment plant in U.S.	183
<i>Rhodococcus ruber</i> M2	NA	+	Same as above	183
<i>Rhodococcus ruber</i> T1	*	+	Landfill soil in Japan	184
<i>Rhodococcus ruber</i> T5	*	+	Activated sludge from a wastewater treatment plant in Osaka, Japan	184
<i>Pseudonocardia acaciae</i> JCM 16707	*	+	Roots of <i>Acacia auriculiformis</i> A. Cunn. ex Benth	50

<i>Pseudonocardia asaccharolytica</i> JCM 10410	*	+	Biofilters filled with tree-bark compost	50
<i>Pseudonocardia autotrophica</i> JCM 4348	–	+	Flood-plain marsh in the Moscow region	50
<i>Pseudonocardia carboxydvorans</i> JCM 14827	–	+	A soil sample collected from a roadside in Seoul, Korea	50
<i>Pseudonocardia halophobica</i> JCM 9421	–	+	Soil in the Moscow region	50
<i>Pseudonocardia hydrocarbonoxydans</i> JCM 3392	–	+	Aerial contamination on a silica gel plate	50
<i>Pseudonocardia petroleophila</i> JCM 3378	–	+	Soil, Göttingen, German	50
<i>Pseudonocardia sulfidoxydans</i> JCM 10411	–	+	Biofilters filled with tree-bark compost	50
<i>Pseudonocardia thermophila</i> JCM 3095	–	+	Fresh horse manure, Berlin, Germany	50
<i>Pseudonocardia yunnanensis</i> JCM 9330	–	+	Soil, Weixin, Yunnan, China	50

+ indicates the bacterial strain is able to metabolize dioxane or THF as sole carbon and energy source;

* indicates the bacterial strain can degrade dioxane only when pre-grown with auxiliary substrates, such as THF and propane;

- indicates the bacterial cannot utilize the substrates;

NA indicates no information was available whether dioxane/THF was biodegradable by the bacterial strain.

All the obtained oligonucleotide probe candidates were then evaluated based on their coverage and specificity using two freely accessible web-based platforms, ProbeMatch¹⁸⁵ from RDP database (<http://rdp.cme.msu.edu/probematch>) and probeCheck¹⁸⁶ from the Silva database (<http://www.microbial-ecology.net/probecheck>). Using the two equations listed below, coverage and specificity of each designed probes were estimated based on the number of probe hits (perfect match and one-nucleotide mismatch) and the total number of available 16S rRNA sequences belonging to the targeted genus and the domain of Bacteria in the RDP database. To determine the binding stabilities of probe-target hybrids, probeCheck was also used to estimate their free energy (ΔG) of hybridization. Sequence, target site, and other properties of our designed probes are listed in Table 8.2. Probe Pse631 with the highest coverage, specificity, and binding energy were chosen for the genus of *Pseudonocardia*. Coverage and specificity were calculated as follows:

$$\text{Coverage} = \frac{\text{number of target strains detected}}{\text{total number of target strains tested}} \times 100\%$$

$$\text{Specificity} = \frac{\text{number of non - target strains undetected}}{\text{total number of non - target strains tested}} \times 100\%$$

For the optimization of hybridization and empirical assessment of coverage and specificity by epifluorescence microscopy, oligonucleotide probes Pse631-C, Pse631-G, and EUB338 with 5' TYETM 563 label was synthesized and purified by Integrated DNA Technologies, Inc. (Coralville, IA). For the application of flow cytometry, Pse631 (mixture with equal amount of Pse631-C and Pse631-G) was synthesized with Alexa Fluor®-488 dye at the 5' end with endured brightness and photostability.

Table 8. 2 Properties of the genus-specific probes targeting the 16S rRNA genes of *Pseudonocardia*.

Probe Characteristics	
Probe Name	Pse631
Target Genus	<i>Pseudonocardia</i>
Hypervariable Region	V4
<i>E. Coli</i> Positions ^a	631-652
ΔG^b (kcal/mol)	-26.43
GC%	59.1
Length (bp)	22
Sequence	5'-AGTSATGCCCGTATCGACCGCA-3' (5'-AGTCATGCCCGTATCGACCGCA-3' for Pse631-C) (5'-AGTGATGCCCGTATCGACCGCA-3' for Pse631-G)

^a Position numbering was based on the *E. coli* system of nomenclature.¹⁷³

^b Free energy (ΔG) for intact matched probe-target hybrids was calculated using two-state hybridization algorithm by probeCheck.

8.2.2 Bacterial strains and culture conditions

The bacterial strains used in this study are listed in Table 8.3. Most strains were obtained from either the Leibniz Institute - German Collection of Microorganisms and Cell Cultures (DSMZ) or the American Type Culture Collection (ATCC). *Mycobacterium* sp. PH-06, *Rhodococcus jostii* RHA1, and *Methylosinus trichosporium* OB3b were kindly provided by Dr. Yoon-Seok Chang (POSTECH, Pohang, South Korea), Dr. William Mohn (University of British Columbia, Vancouver, Canada), and Dr. Jeremy Semrau (University of Michigan, Ann Arbor, MI), respectively.

Table 8. 3 Specificity and coverage tests for the designed phylogenetic probes.

Microorganism Strain	Strain Number	Taxonomy (Family/Phylum)	Gram Stain	Phylogenetic Probe Hybridization ^a	
				EUB338 ^b	Pse631
<i>Pseudonocardia dioxanivorans</i> CB1190	DSM-44775	<i>Pseudonocardineae/Actinobacteria</i>	+	+	+
<i>Pseudonocardia tetrahydrofuranoxydans</i> K1	DSM-44239	<i>Pseudonocardineae/Actinobacteria</i>	+	+	+
<i>Pseudonocardia antarctica</i> DVS 5a1	DSM-44749	<i>Pseudonocardineae/Actinobacteria</i>	+	+	+
<i>Mycobacterium</i> sp. PH-06	NA ^c	<i>Mycobacteriaceae/Actinobacteria</i>	+	+	–
<i>Escherichia coli</i> K12	ATCC- 10798	<i>Enterobacteriaceae/Gamma-Proteobacteria</i>	–	+	–
<i>Pseudomonas aeruginosa</i> PAO1	ATCC-15692	<i>Pseudomonadaceae/Gamma-Proteobacteria</i>	–	+	–
<i>Burkholderia cepacia</i> G4	DSM-11737	<i>Burkholderiaceae/Beta-Proteobacteria</i>	–	+	–
<i>Methylosinus trichosporium</i> OB3b	ATCC- 35070	<i>Methylocystaceae/Alpha-Proteobacteria</i>	–	+	–
<i>Desulfovibrio vulgaris</i> Hildenborough	DSM-644	<i>Desulfovibrionaceae/Delta-Proteobacteria</i>	–	+	–
<i>Rhodococcus jostii</i> RHA1	NA	<i>Nocardiaceae/Actinobacteria</i>	+	+	–
<i>Bacillus subtilis</i>	DSM-10	<i>Bacillaceae/Firmicutes</i>	+	+	–
<i>Halanaerobium congolense</i>	DSM-11287	<i>Halanaerobiaceae/Firmicutes</i>	–	+	–

^a + indicates a positive visualization was obtained after hybridization with the indicated phylogenetic probe; – indicates no positive visualization was obtained after hybridization with the indicated phylogenetic probe.

^b EUB338 is an universal probe targeting the Prokaryotic domain.

^c Strain source is indicated in the MATERIALS AND METHODS.

Pseudonocardia dioxanivorans CB1190 was cultured in ammonia mineral salt (AMS) medium amended with dioxane (100 mg/L) as the carbon and energy source. Other actinomycetes (*Pseudonocardia tetrahydrofuranoxydans* K1, *Pseudonocardia antarctica* DVS 5a1, *Mycobacterium* sp. PH-06, and *Rhodococcus jostii* RHA1) were grown in R2A medium. Proteobacteria (*Escherichia coli* K12, *Pseudomonas aeruginosa* PAO1, *Burkholderia cepacia* G4) and *Bacillus subtilis* were grown in Luria-Bertani (LB) broth. *Methylosinus trichosporium* OB3b was grown in nitrate mineral salt (NMS) medium amended with methane (20%, v/v) in the headspace as the sole carbon source.¹⁸⁷ *Desulfovibrio vulgaris* Hildenborough and *Halanaerobium congolense* were grown as recommended by DSMZ in the anaerobic chamber (Coy Lab, Grass Lake, MI) with sulfate and thiosulfate as the sole electron acceptors, respectively.

8.2.3 Cell fixation

All bacterial cells were harvested during logarithmic growth by centrifugation of an aliquot (ca. 2 mL) in microcentrifuge (10,000 × g, 10 min). The cells pellets were washed three times with 1 × phosphate buffered saline (PBS, Thermo Scientific, Waltham, MA). For Gram-positive bacteria, washed cells were fixed in 1 mL of ethanol/PBS (1:1, v/v) and incubated at room temperature for 2 h.¹⁸⁸ For Gram-negative bacteria, cells were resuspended thoroughly in 1 mL of 4% paraformaldehyde fixative at 4°C for 3 h.¹⁷⁰ After fixation, all cells were washed twice with 500 µL 1 × PBS and stored at -20°C prior to further analysis.

A consortium was obtained from the enrichment of a groundwater sample collected at the source zone of a historically contaminated site in San Antonio, TX. 16S rRNA metagenomic sequencing analysis discovered the two dominant genera of the consortium are *Mycobacterium* (Gram positive) and *Sediminibacterium* (Gram negative) (data not shown). Thus, we adapted the fixation method for sediment samples.¹⁸⁹ Briefly, collected cells were fixed with 4% paraformaldehyde fixative at 4°C for up to 24 h. After washed twice with 500 µL 1 × PBS, cells were then resuspended in 1 mL of ethanol/PBS (1:1, v/v) for storage.

8.2.4 Fluorescence *in situ* hybridization (FISH)

An efficient in-solution hybridization approach was developed based on previously standardized protocols.^{190, 191} Briefly, the cell pellets were washed with 1 × PBS, and resuspended by adding 90 µL hybridization buffer (0.9 M NaCl, 20 mM Tris-HCl [pH 8], 0.01% [wt/vol] sodium dodecyl sulfate [SDS], 20 or 25 % [wt/vol] formamide depending on the probes) and 10 µL 50 ng/µL fluorescent probe stock (final concentration of ~500 nM) in 2 mL centrifuge tubes. After incubation for 2 h at 46 °C, 1.5 mL of pre-warmed (48 °C) washing buffer (225 or 159 mM NaCl depending on the formamide concentration in the hybridization buffer, 5 mM EDTA, 20 mM Tris-HCl [pH 8], 0.01% [wt/vol] SDS) prior to centrifugation (10,000 × g, 2 min). The cell pellets were rinsed again to remove unbounded probes with 0.5 mL of washing buffer and incubated at 48 °C for 15 min. Finally, the cell suspension was kept on ice and filtered with a 0.22 µm polycarbonate membrane for microscopic observation of staining. The filtered membrane was carefully cut into two 1 cm×1 cm membrane squares, which were further stained with 50 µL of 1 µg/mL 4',6-diamidino-2-phenylindole (DAPI, Thermo Scientific, Waltham,

MA) and then gently rinsed with sterilized $1 \times$ PBS to remove unbound stains. Samples were then mounted on glass slides and examined using an Olympus IX71 inverted fluorescence microscope (Olympus, Center Valley, PA) equipped with a $100 \times$ oil immersion objective. DAPI- and probe-labeled images were obtained separately from the same field of view (exposure time for 100 ms) using the accordant filter sets. Probe/dye-conferred fluorescence of each imaged cell was quantified as the mean pixel intensity by ImageJ.¹⁹²

The optimal formamide concentrations for probes Pse631-C and Pse631-G was determined by changing its concentration in the hybridization buffer with an increment of 5 or 10 % from 0 to 60 % at one fixed incubation temperature (i.e., 46 °C). Concentrations of NaCl in washing buffer was changed accordingly to eliminate the undesirable mismatched probe-target hybrids. Two *Pseudonocardia* strains, *P. dioxanivorans* CB1190 and *P. antarctica* DVS 5a1, were used as model strains to experimentally evaluate probe-target dissociation under the varying hybridization and washing conditions. Two web-based tools, ProbeMelt from DECIPHER¹⁹³ (<http://decipher.cee.wisc.edu/ProbeMelt.html>) and Formamide Curve Generator from mathFISH¹⁹⁴ (<http://mathfish.cee.wisc.edu/formamide.html>), were employed to simulate designed probe hybridization efficiencies with the tested strains at different concentrations of the denaturant formamide.

8.2.5 Flow cytometry and cell sorting

Flow cytometry was performed using an FACS AriaII Flow Cytometer (BD Biosciences, San Jose, CA) equipped with a red laser (wavelength of 640 nm), a violet laser (wavelength of 405 nm), yellow-green laser (wavelength of 561 nm), a blue laser (488 nm wavelength) and an UV laser (wavelength of 355 nm) controlled by a computer equipped with the software BD FACS Diva 8.0 (BD Bioscience, San Jose, CA). Sheath pressure was set to 70 psi, and nozzle size was 70 μ m. Forward scatter (FSC), side scatter (SSC), blue (450 ± 25 nm, DAPI) and green fluorescence (530 ± 15 nm, Alexa Fluor 488) signals were measured and analyzed in logarithmic amplification mode. The bacteria exhibiting fluorescence signals above the cutoff of DAPI were gated using the scattergrams of SSC versus DAPI, and Pse631-labelled cells (gated by AF488+) were set similarly in the scattergrams of SSC versus Alexa Fluor 488. Plots displayed 10,000 - 50,000 events, and target gates were defined according to all negative control samples (e.g., non-Pse631-labeled *Pseudonocardia* cells and Pse631-labeled cells that are not *Pseudonocardia*). Cell sorting was performed on the same flow cytometer with the drop drive frequency set at 88.7 kHz/s. Each event falling within the gate AF488+ was sorted in a sort envelope of 1.2 drops at a rate of 1×10^2 to 1×10^3 events/s. In order to achieve high purity and recovery, the purity precision mode was used. Sorted cells were collected in sterile microcentrifuge tubes (Eppendorf, Hamburg, Germany).

To further assess the sensitivity of the cell sorting method, fixed CB1190 cells were mixed with consortium cells to achieve a serial dilution of percentage abundance (10%, 3%, 1%, 0.3% and 0.1%), and then in-solution hybridization was employed to the cell mixtures with probe Pse631. The total bacteria in the mixture were enumerated as the positive DAPI-stained cells detected by flow cytometry. The relative abundance of the hybridized cells was estimated as the ratio of Pse631-stained cells detected by flow cytometry (i.e., the events gated by AF488+) to the total bacteria. Detection efficiency of the method was calculated as the ratio of the relative

abundance of the detected CB1190 cells to the amendment percentage prior to hybridization and flow cytometric analysis.

8.2.6 Quantitative PCR (qPCR)

To evaluate the effectiveness of our cell sorting method, qPCR using TaqMan chemistry was employed to quantify the CB1190 cells (enumerated as the abundance of *thmA* genes coding for THF/dioxane monooxygenases)¹⁶ and the total bacteria (enumerated by 16S rRNA genes)¹⁰¹ in the sorted cells. The sorted cells were spun down at 10,000 rpm for 10 mins and washed three times with autoclaved distilled water. The cells were re-suspended in 20 μ L DNA-free water, and then subject to three boiling-freeze cycles (95°C for 10 min and -70°C for 10 min) to release the genetic materials. The qPCR reaction mixture (a total volume of 20 μ L) consisted of 1 μ L of the obtained DNA, 300 nM forward and reverse primers, 150 nM fluorogenic probe, 10 μ L of TaqMan universal master mix II (Applied Biosystems, Foster City, CA), and DNA-free water. qPCR was performed with a CFX 96™ Real-Time System (Bio-Rad, Hercules, CA) using the following temperature program: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions (10^{-4} to 100 ng of DNA/ μ L) of the extracted genomic DNA of CB1190 were utilized to prepare the calibration curves for both *thmA* (one copy per genome) and 16S rRNA (three copies per genome) genes. The calculation of copy numbers and relative abundance of CB1190 cells have been previously described.¹⁶

8.3 Results

8.3.1 16S rRNA-targeted probe design

Pse631 is a degenerate probe consisting of two oligomers (Pse631-C and Pse631-G). The singular degenerate site on the 16S rRNA is located at the 649th base from the 5' end (G or C) based on the *E. coli* system of nomenclature. As shown in Figure 8.1, Pse631-C targets the two archetype cyclic ether degraders (CB1190 and K1) and a number of *Pseudonocardia* species (e.g., *P. benzenivorans* B5, *P. hydrocarbonoxydans*, *P. sulfidoxydans*, and *P. petroleophila*) that are known for their capability to degrade a wide range of priority organic pollutants. For instance, THF-grown ENV478 can degrade a variety of ethers, including dioxane and its related solvent 1,3-dioxolane, the plasticizer bis-2-chloroethyl ether (BCEE), and the gasoline additive methyl *tert*-butyl ether (MTBE).²¹ However, Probe Pse631-C only covers approximately 12% of the *Pseudonocardia* species with a perfect match based on the 1873 16S rRNA gene sequences retrieved from RDP (Table 8.4). The other probe, Pse631-G, is complementary to 16S rRNA genes from the majority of the rest species (~ 74%) belonging to the genus of *Pseudonocardia*. Among these species, there are a few strains that thrive under harsh conditions (e.g., cold temperature and high salinity), including *P. halophobica*, *P. antarctica*, and *P. thermophila*, suggesting the potential to target, separate and characterize extremophilic strains.

Table 8. 4 Theoretical coverage and specificity estimation of phylogenetic oligonucleotide probes targeting *Pseudonocardia* and other genera in the order of Actinomycetales^a.

Probe Name	Target Genus	Coverage		Specificity		Reference
		no mismatch	1 mismatch	no mismatch	1 mismatch	
Pse631	<i>Pseudonocardia</i>	86.01 %	91.40 %	99.99 %	99.95 %	this study
Pse631-C	<i>Pseudonocardia</i> (Partial)	12.22 %	87.56 %	< 100 % ^c	99.96 %	this study
Pse631-G	<i>Pseudonocardia</i> (Partial)	73.79 %	90.12 %	99.99 %	99.98 %	this study
Myb736b	<i>Mycobacterium</i>	75.85 %	79.05 %	98.86 %	97.44 %	195
Myc657	<i>Mycobacterium</i>	75.65 %	84.78 %	99.61 %	99.38 %	196
Gor596	<i>Gordonia</i>	87.57 %	92.61 %	< 100 %	< 100 %	195
Modesto	<i>Modestobacter</i>	62.26 %	70.35 %	< 100 %	99.98 %	197
Geo/Blasto	<i>Geodermatophilus</i>	38.46 %	53.58 %	< 100 %	< 100 %	197
APYO183	<i>Trueperella</i>	63.39 %	72.68 %	100 %	< 100 %	198
MNP1 ^b	<i>Corynebacterineae</i>	75.06 %	92.47 %	99.30 %	98.94 %	199

^a Calculation of probe coverage and specificity were based on the 16S rRNA sequence collection retrieved from the RDP database updated in March 2017.

^b MNP1 was designed to probe nocardioform actinomycetes. Thus, sequences obtained in the suborder *Corynebacterineae* was used for coverage and specificity estimation.

^c < 100% indicates a specificity between 99.99 % and 100 %.

The theoretical estimation using ProbeMatch (Table 8.4) shows that Pse631 can effectively hybridize with the majority (> 86 %) of the *Pseudonocardia* species. Such high coverage of probes did not sacrifice their ability to preclude false positives. As indicated in Table 8.4, specificity of our designed probes was greater than 99.9 %. Formation of mismatched probe-target hybrids can be greatly eliminated, but not completely precluded, via optimizing the hybridization condition. By enabling a matching distance of 1 (i.e., one mismatch, insert, or delete is allowable when matching a probe to a sequence), our probe design exhibited an improved coverage (> 91 %) and maintained high specificity (> 99.9 %), suggesting the high resilience of our probe to occasional mismatching during *in situ* hybridization. Compared to our probe, oligonucleotide probes targeting other genera from the order of Actinomycetales offer comparable or less coverage and specificity based on the ProbeMatch analysis (Table 8.4).

8.3.2 Optimization of Hybridization (Laboratory and Computation Tests)

To optimize the hybridization system, probe-conferred fluorescence was examined under varying hybridization stringencies. Pure cultures of CB1190 and *P. antarctica* DVS 5a1 were used as the perfect targets for probe Pse631-C and Pse631-G, respectively. Figure 8.2 shows that the average signals per cell remained at comparable levels until the added formamide concentration reached 25% for both probes Pse631-C and Pse631-G.

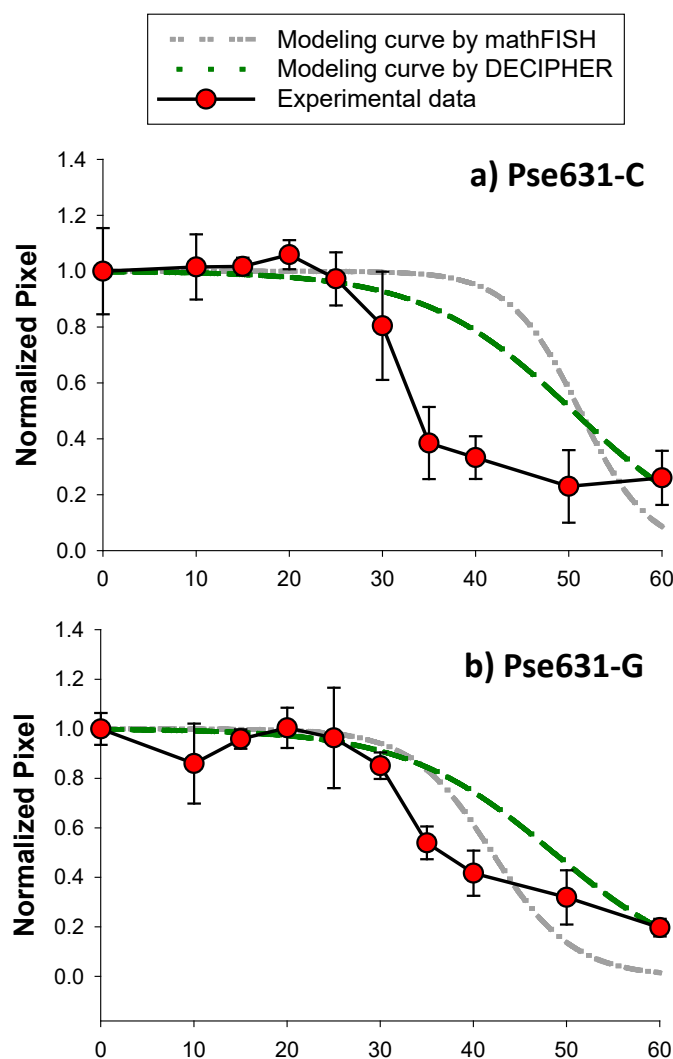


Figure 8. 2 Probe dissociation curve under various formamide concentrations for *in situ* hybridization of (a) Pse631-C with *Pseudonocardia dioxanivorans* CB1190, and (b) Pse631-G with *Pseudonocardia antarctica* DVS 5a1.

Each experimental data point represents the average value of fluorescence intensity of approximately 50 cells normalized with the one obtained without the presence of formamide in the hybridization solution. Error bars indicate the standard deviations. Hybridization efficiency curves simulated by two theoretical models were based on two web tools, ProbeMelt from DECIPHER¹⁹³ and Formamide Curve Generator from mathFISH.¹⁹⁴

In addition to the empirical tests, mathematical models DECIPHER and mathFISH were used to provide theoretical guidance on the thermodynamic feasibility and mismatch discrimination for *in situ* hybridization experiments. Results from both models corroborated favorable equilibrium thermodynamics for both designed probes with sufficient hybridization affinity but low risk to produce false positives or false negatives. In general, our experimental data fit better with the hybridization curves simulated by DECIPHER, which recommended formamide concentrations ranging between 20 to 30 % (Figure 8.2). Therefore, considering

results from both empirical tests and theoretical computation, 25 % formamide was chosen in the later hybridization for Pse631, a mixture of Pse631-C and Pse631-G.

8.3.3 Specificity and coverage of Pse631 with pure and mixed bacterial strains

To validate the coverage and specificity of the designed probes, FISH analysis (Table 8.3 and Figure 8.3) indicated that our designed fluorescence probe Pse631 was capable of efficiently hybridizing with various strains in the genus of *Pseudonocardia*, including *P. dioxanivorans* CB1190, *P. tetrahydrofuranoxydans* K1, and *P. antarctica* DVS 5a1. Negative controls, using pure and mixed bacterial strains from other genera that are phylogenetically close or distant to *Pseudonocardia* (e.g., *Rhodococcus jostii* RHA1, *Mycobacterium* sp. PH-06, *Burkholderia cepacia* G4, *Halanaerobium congolense*, and *Pseudomonas aeruginosa* PAO1) were used to evaluate the potential for false positive hybridization. None of these negative controls were visualized under the same hybridization and exposure condition by this Pse631 probe. Our method can effectively exclude non-specific hybridization with negative control strains that share very high 16S rRNA sequence identities with *Pseudonocardia* species (e.g., 93% as identities for both RHA1 and PH-06 compared to CB1190). These results confirmed the specificity of our newly developed probe, implying its usefulness to target the *Pseudonocardia* species that are ‘non-cultivable’ or difficult for culturing with the help of fluorescence assisted cell sorting.

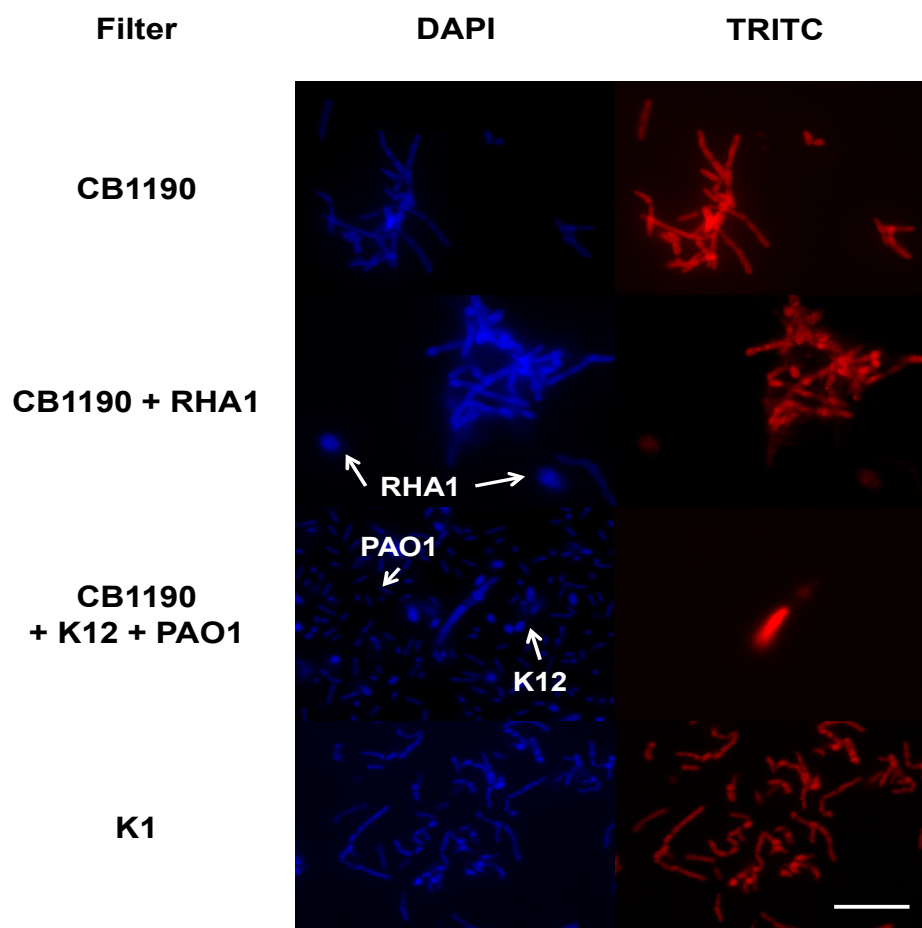


Figure 8. 3 Reference bacterial strains visualized by epifluorescence microscopy after hybridization with probe Pse631 (visualized with the TRITC filter), as well as DAPI, a fluorescent dye used to stain both live and fixed cells.

The scale bar at the bottom right indicates a length of 10 μm .

7.3.4 Flow cytometry for efficient cell sorting

As illustrated in Figure 8.4, 35.6 ± 4.2 % of CB1190 cells (enumerated as the events in the defined gate AF488+) were labeled by probe Pse631 and quantified by flow cytometry using our phylogenetic probe. Similar labeling and detection efficiencies (30 to 55%) are commonly reported using fluorescence-conjugated oligonucleotide probes targeting *actinomycetes* and other bacteria after *in situ* hybridization.^{190, 200, 201} The specificity of probe Pse631 was also verified with non-labeled CB1190 cells. Minimal false negatives were observed in Gram-positive and Gram-negative bacterial cells hybridized with Pse631, including *Escherichia coli* K12 (0.0 %), *Bacillus subtilis* (0.0 %) and *Mycobacterium* sp. PH-06 (0.1 %). Staining with DAPI was used to estimate the total bacterial count.

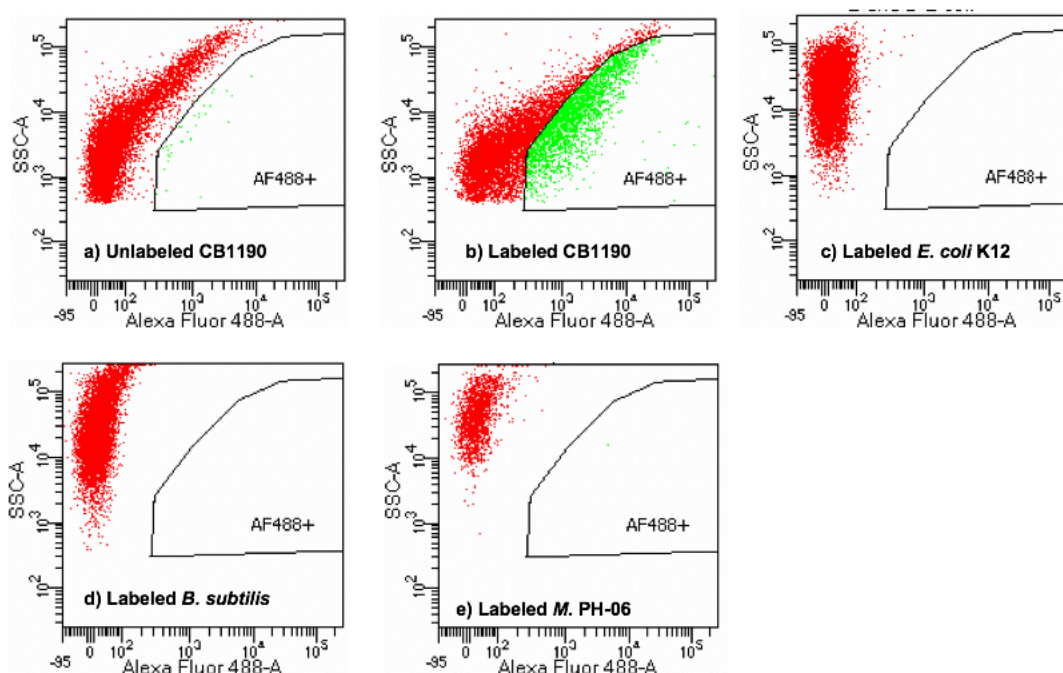


Figure 8. 4 The performance of probe Pse631 verified by flow cytometry in scattergrams of SSC (90° sidelight scatter) vs. Alexa Fluor 488 (green fluorescence).

Red dots indicate the total bacteria labeled by DAPI (the events with high DAPI fluorescence) and green dots indicate bacteria labeled by the Pse631 probe (the events with high Alexa Fluor-488 signals in AF488+ gate). The strains *M. PH-06*, *E. coli* K12, *B. subtilis* and unlabeled CB1190 were considered as negative control here.

The performance of our cell sorting method was verified using a consortium enriched from a field sample. Our 16S rRNA metagenomic sequencing data showed that *Pseudonocardia* spp. were absent in the consortium (data not shown). Thus, a series of concentrations of CB1190 cells was diluted with the consortium cells to assess probe sensitivity and efficiency of our cell sorting method. As shown in Figure 8.5 and Figure 8.6, the detection efficiency was $36.7 \pm 4.1\%$ for mixed samples with 10 % of CB1190, which is comparable to that of pure CB1190 cells (i.e., $35.6 \pm 4.2\%$). The detection efficiency of our approach increased with decreasing relative abundance of CB1190 (Figure 8.5) probably due to the greater ratio of our probes to the target cells. Though probe design is of the primary significance for effective hybridization, probe-to-target ratio also affects the success of hybridization and detection especially in complex environmental matrices mimicked by our experiment. The optimum concentration of *Pseudonocardia* cells for our method was 0.3 % given its overall efficiency was as high as 89 % (Figure 8.5). Thus, for enriched samples with relatively high abundance of *Pseudonocardia* cells, appropriate dilution prior to staining is recommended to improve the overall detection efficiency for cell counting or sorting purposes. Our approach also exhibited reliable sensitivity when the percentage of CB1190 cells in the mixed consortium was as low as 0.1 %, and no significant false positives occurred for the control containing consortium cells without CB1190 (Figures 8.5, 8.6).

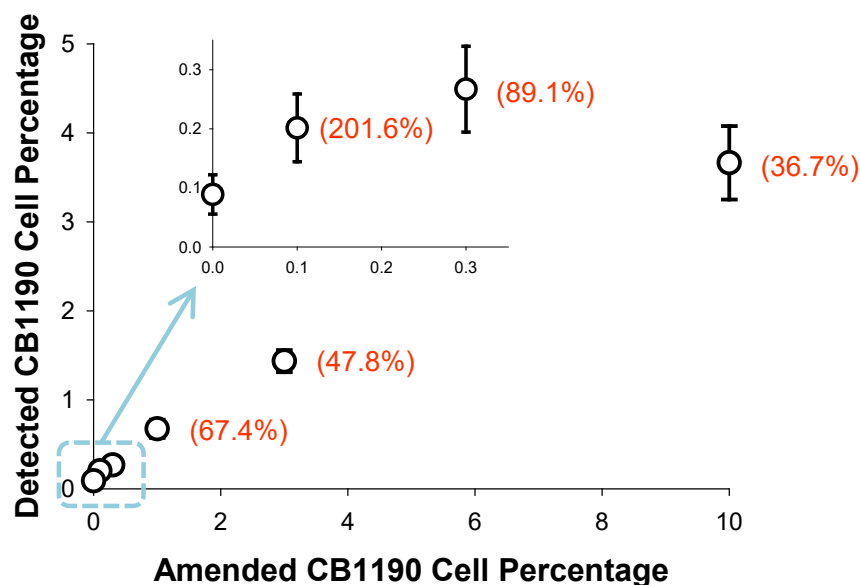


Figure 8. 5 Relative abundance of the hybridized CB1190 cells detected by flow cytometry in relation to the amendment percentage of CB1190 cells when mixed with the enriched consortium. Detection efficiency is indicated as the percentage in the parentheses next to the respective data symbol.

To further verify the specificity of our method, the relative abundance of CB1190 cells in the sorted cells and original consortium samples mixed with 10 % of CB1190 was quantified and compared using Taqman qPCR assays. Theoretically, in the genome of CB1190, the ratio of *thmA* to 16S rRNA gene is 0.33.⁷⁶ Comparably, this *thmA*/16S rRNA ratio was determined by qPCR analysis to be 0.27 ± 0.05 in the genomic DNA of pure CB1190 cultures (Table 8.5). After cell sorting, the *thmA*/16S rRNA ratio in sorted cells remained 0.20 ± 0.06 , which is statistically indiscernible from the ratio in pure CB1190 cells. Thus, our method can specifically sort *Pseudonocardia* cells from a mixed consortium. The *thmA*/16S RNA ratio in sorted cells was more than 10 times greater than that detected in the original cell mixture before the sorting (0.02 ± 0.01). This demonstrates that most of the sorted cells were CB1190 and no significant false positive cells was observed. Overall, our results suggest high sensitivity and reliable specificity of our Pse631 probe in complex environmental samples.

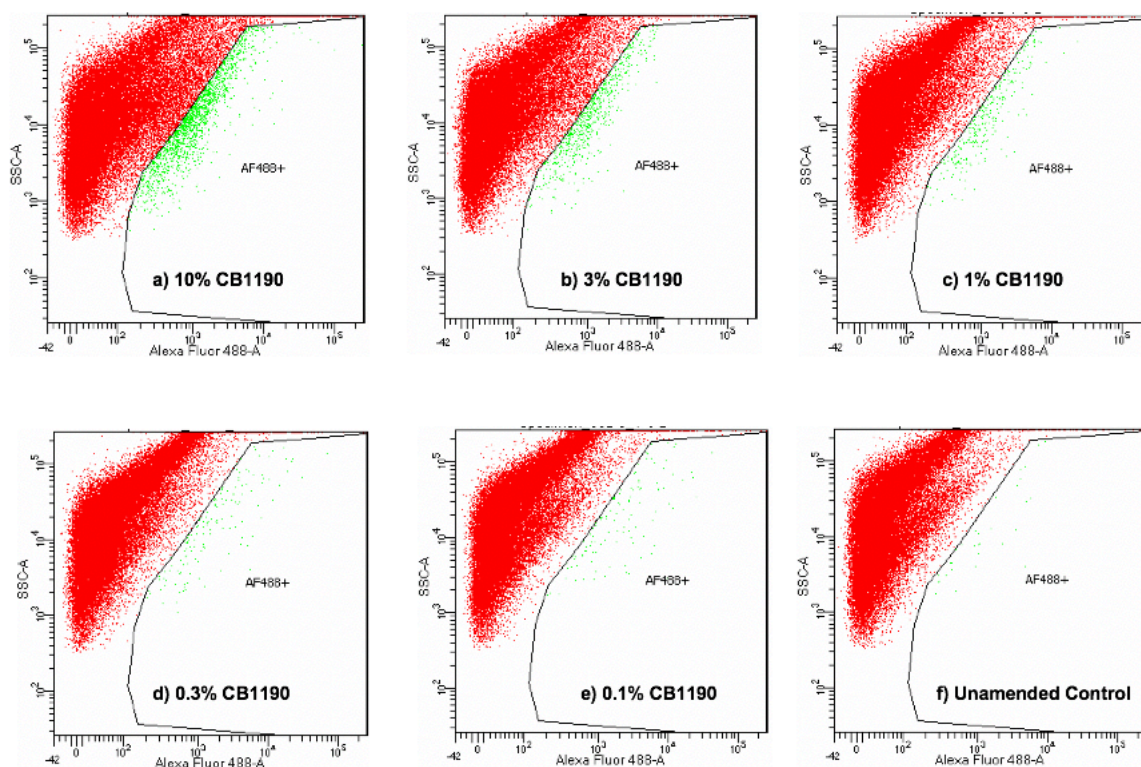


Figure 8.6 Performance of probe Pse631 to sort CB1190 cells from the mixture with an enriched consortium in dot plots of SSC (90° sidelight scatter vs. Alexa Fluor 488 (green fluorescence). Red dots indicate total bacteria labeled by DAPI (the events with high DAPI fluorescence) and green dots indicate the bacteria labeled by the Pse631 probe (the events with high Alexa Fluor-488 signals in AF488+ gate).

Table 8. 5 Quantification of *thmA*/16S rRNA ratios by qPCR.

Sample	<i>thmA</i> /16S rRNA Ratio	Relative Abundance of CB1190 Cells ^a
CB1190	0.27 ± 0.05	81 ± 15%
Sorted cells	0.20 ± 0.06	60 ± 18%
Enriched consortium	NA ^b	0%
Enriched consortium with 10% of CB1190	0.02 ± 0.01*	8.5 ± 4.2 %

^a Each CB1190 genome contains three copies of 16S rRNA genes. An average of 4.2 copies per genome was assumed for the microbial mixtures.²⁰²

^b The abundance of *thmA* genes in the enriched consortium was below our method detection limit (approximately 7,000 copies per gram of biomass).

* indicates significant increases compared to CB1190 ($p < 0.05$).

8.4 Discussion

Isolating and separating difficult-to-cultivate *Pseudonocardia* populations from complex communities facilitate subsequent molecular analyses to discover new species and understand their metabolic and physiological characteristics and functional niches. Here we designed a 16S rRNA-based probe and used (culture-independent) FISH-flow cytometry to efficiently detect and separate microbial populations of interest that potentiate molecular analysis to align their genetic identities with metabolic potentials. Note that Pse631, similar to many other well-designed probes (e.g., Myc657 and Gor596), targets the nucleotide sequences within the 16S rRNA hypervariable region V4 (i.e., *E. coli* 16S rRNA positions 576–682) (Table 8.2). As highlighted in Figure 8.1, all cyclic ether degrading *Pseudonocardia* can be accurately targeted by either Pse631-C or Pse631-G except *P. asaccharolytica*. The 16S rRNA gene of this strain has one mismatch with Pse631-G at the position of 631 with G rather than T, and this mismatch is located at the 3' end of the probe. Hybridization probably occurs between the probe and this single-mismatched target, since defects towards either end of the probes usually lead to minor signal reduction (10 – 20 %).²⁰³⁻²⁰⁵ Therefore, all known cyclic ether degrading *Pseudonocardia* isolates can be efficiently targeted by Pse631 (Figure 8.1).

With this designed phylogenetic probe, a high-throughput culture-independent approach was developed and optimized to integrate flow cytometry and fluorescence activated cell sorting (FACS). 16S rRNA is an ideal target for FACS to isolate the dominant bacteria from environmental samples or enriched consortia. The ubiquity of 16S rRNA and its relatively high copy numbers ($10^4 - 10^5$ per cell) in prokaryotic cells are conducive to strong signals,²⁰⁶ while targeting functional genes can be more technically challenging and requires signal amplification. Our approach eliminates the use of potentially biased cultivation and DNA extraction methods, both of which are time-consuming and laborious. Additionally, sorted cells remain accessible for molecular analysis, since fluorescent probe-stained and sorted cells have been successfully used as templates for PCR amplicon sequencing.²⁰⁷

Despite the hybridization limitation caused by cell permeabilization and molecular binding between probes and targets, the detection efficiency of FACS is tightly associated with the heterogeneity of fluorescent signals from labeled cells and the stringency to preclude false positives. Our hybridized CB1190 cells possessed a wide range of fluorescence intensities and

sizes (Figure 8.4b), suggesting the applicability of our method to target cells with different morphological properties and at different growth stages. To ensure the accuracy and exclude false positives, 1) the gate AF488+ was set at high stringency that excludes background signal from autofluorescence; 2) negative and positive controls, such as pure culture or spiked environmental samples, is always prepared to achieve correct gate for accurate analysis.

The high sensitivity and specificity of our method – demonstrated with pure and mixed cultures – corroborate its feasibility to detect and sort *Pseudonocardia* cells from various environments. This could help identify novel cyclic ether degraders and associated genes from field samples collected from contaminated sites. Since our method can reliably sort out CB1190 cells from a mixed culture when CB1190 makes up as few as 0.1 % of the total population, it should be able to target other *Pseudonocardia* cells that would be enriched in systems exhibiting cyclic ether degradation activity. Our method may also facilitate monitoring and enumeration of CB1190 and other *Pseudonocardia* strains when used for *in situ* bioaugmentation of dioxane contaminated sites.

In summary, this chapter presents a cultivation-free strategy to separate and concentrate genus-specific subpopulations by using our fluorescently labeled oligonucleotide probes and flow cytometry. This will expedite discovering novel *Pseudonocardia* strains and discerning the molecular basis for specific metabolic traits of interest (e.g., antibiotic production, biodegradation of cyclic ethers, and C₁ autotrophic fixation). The obtained genetic sequences and metabolic information will be valuable to expand the current database of reference genes or genomes for the development of closed-format molecular approaches (e.g., qPCR and microarray) and reroute the open-format next-generation sequencing resources to increase the portion of informative reads (e.g., bypassing community-focused metagenomics to single cell analysis).²⁰⁸

Chapter 9

Isolation and investigation of novel dioxane degraders from soils with no previous exposure to dioxane

9.1 Introduction

Although dioxane is relatively recalcitrant, a number of strains capable of degrading dioxane have been isolated. Most of these strains degrade dioxane cometabolically (i.e. dioxane degradation is induced by another substrate, and it does not serve as carbon or energy source to support bacterial growth). Nearly all of these strains were isolated from wastewater treatment plants or dioxane-impacted sites. However, the potential for encountering dioxane degraders in uncontaminated environments, which is important to assess the distribution and potential ubiquity of dioxane degraders and the associated natural attenuation potential, has not been reported in the literature.

Interestingly, all reported dioxane-degrading bacteria can also degrade other cyclic ethers (e.g. THF)¹⁴ which have the similar structure as dioxane. Besides, natural products containing cyclic ethers are commonly encountered in nature (e.g. brevetoxin A)²⁰⁹ and are used for different biochemistry processes (e.g. synthesis of certain antibiotic).²¹⁰ This raises the possibility that dioxane degrading bacteria may exist or can be cultured in environments with no previous exposure to dioxane.

In this chapter, we describe the enrichment of two consortia from garden soils with no known previous exposure to dioxane. We compare their dioxane degradation capabilities with those of two archetype degraders (*Pseudonocardia dioxanivorans* CB1190 and *Mycobacterium dioxanotrophicus* PH-06). Both high (500 mg/L) and low (300 µg/L) initial dioxane concentrations are considered to assess the performance of these consortia over a broad range of scenarios at impacted sites. We also examine the microbial communities before and after enrichment with dioxane through phylogenetic gene sequencing analyses and investigate the suspected enzymes that initiate the degradation process using acetylene inactivation tests, qPCR assays, and SDIMO sequence analyses.

9.2 Materials and Methods

9.2.1 Enrichment of two dioxane degrading bacteria consortia

Soil samples were obtained at a depth of 5 cm from the soil surface. Two samples (corresponding to the two consortia) were collected at two different spots (10 m away) at different times (in September 2015 and March 2016). A dark soil sample collected from an area near bushes and trees was used for the enrichment of Consortium A, while a lighter yellowish soil sample from a grassy area was used to enrich Consortium B. No dioxane was detected in these soils (detection limit $< 5 \mu\text{g/L}$).

Soil samples (5 g of soil in 10 mL deionized water) were vortexed vigorously on a Vortex-Genie[®] 2 (MO BIO Laboratories, Inc. Carlsbad, CA, USA) at the maximum speed for 10 min, and the aqueous phase was used as the initial inoculum of the enrichment. A series of ten-fold dilutions²¹¹ were performed for each sample each week, using ammonium mineral salts (AMS) medium³⁹ amended with 100 mg/L of dioxane as the sole source of carbon. Amphotericin B solution (2.5 mg/L) (Sigma-Aldrich, St. Louis, MO, USA) was added to the medium to avoid the growth of fungus, which could confound assessment of dioxane biodegradation by bacteria. All samples were kept at 30 °C while shaking on a Digital Linear Shaker (Scilogex, Rocky Hill, CT, USA) at 150 rpm.

After 12 cycles of enrichment for approximately three months, rapid dioxane biodegradation was sustained at relatively constant rates by both consortia. However, isolation of pure strains that metabolize dioxane was unsuccessful using agar plates prepared with 1.5 % molecular grade agar and AMS amended with 100 mg/L dioxane. The failure to isolate dioxane degraders was partially because some bacteria in the enriched consortia grew on the plates using agar, rather than dioxane, as carbon source. Thus, the enriched consortia were used for further studies.

9.2.2 Dioxane degradation rates and bioaugmentation studies

Dioxane degradation capabilities by these consortia were benchmarked against those of two archetype degraders: CB1190 and PH-06. Both reference strains and the two consortia were grown in AMS medium with dioxane as the only carbon source and were harvested in the exponential stage by centrifuging and washing with AMS medium for three times. Batch biodegradation assays were conducted in 250-mL bottles amended with 100-mL AMS medium with an initial dioxane concentration of 500 mg/L while shaking at 150 rpm.

Microcosms prepared with dioxane-impacted groundwater from a site in Seattle, WA, the USA that was not exhibiting intrinsic biodegradation capabilities. A low initial concentration of dioxane (300 $\mu\text{g/L}$), was used to assess the potential of these consortia for bioaugmentation of contaminated sites where dioxane might be present at insufficient concentrations to induce and/or sustain an active degrading indigenous community. This study was conducted at 15 °C to mimic the actual average groundwater temperature.

9.2.3 Dioxane and biomass analytical procedures

To measure the dioxane concentrations in the original soil samples, 5 g of soil were mixed with 10 ml of deionized water and vortexed vigorously on a Vortex-Genie[®] 2 (MO BIO

Laboratories, Inc. Carlsbad, CA, USA) at the maximum speed for 10 min. The extracted dioxane concentration in the aqueous phase was measured as detailed below, and the total dioxane mass contained in the 10 ml of extract was divided by the initial 5 g of soil to calculate the soil concentration.

Liquid samples were filtered through 0.22 μ m syringe filters and dioxane was extracted by the liquid/liquid frozen micro-extraction method using dichloromethane as the solvent.¹⁰⁰ Dioxane concentrations were measured using an Agilent 7820A gas chromatograph equipped with a 5977E mass spectrum detector.

Total biomass was quantified as the total protein concentration using the PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Serial dilutions of the bovine serum albumin (BSA) standard were prepared for calibration.

Bacterial growth on dioxane was assessed per increase in optical density at 600 nm (OD₆₀₀). Even though both consortia clumped to a much lower extent than the two archetype dioxane degraders (CB1190 and PH-06), the bacteria suspensions were not as uniform as that of other non-aggregating bacteria. Thus, before the measurement of OD₆₀₀, the bacterial cultures were put into a 1.5 ml centrifuge tube and sonicated for 10 min in a 5510 Branson Ultrasonic Cleaner (Sigma-Aldrich, St. Louis, MO, USA) at 40 kHz. The OD₆₀₀ was then measured with a UltrospecTM 2100 *pro* UV/Visible Spectrophotometer (GE Healthcare, Little Chalfont, United Kingdom).

9.2.4 Microbial community analyses

16S rRNA gene sequencing was used to determine the microbial community composition of the original soil samples and the enriched two consortia. DNA from the original soil samples was extracted using PowerSoil[®] DNA Isolation Kit (MO BIO Laboratories, Inc. Carlsbad, CA, USA). DNA from bacteria was extracted from bacteria biomass harvested in the exponential growth phase when half or more of the added dioxane (100 mg/L) was consumed. The extraction kit was the UltraClean[®] Microbial DNA Isolation Kit (MO BIO, Carlsbad, CA, USA). The V4 region of the 16S rRNA gene was amplified by PCR using the forward 515F and reverse 806R primers.²¹² Sequencing was performed at MR DNA (www.mrdnalab.com, Shallowater, TX, USA) by Illumina MiSeq paired-end sequencing (approximately 2×300 bp as the read length). Sequence data were processed using MR DNA analysis pipeline. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity).²¹³ Final OTUs were taxonomically classified using BLASTn against the RDPII (<http://rdp.cme.msu.edu>) and NCBI (www.ncbi.nlm.nih.gov) databases.

9.2.5 Real-time quantitative PCR (qPCR)

qPCR analysis of the bacterial DNA was conducted to determine the presence and abundance of 16S rRNA and *thmA**DBC* genes.¹⁶ 16S rRNA was used as a positive control to show amplification of genes unrelated to degradation of dioxane. qPCR mixture contained 10 ng of genomic DNA, 300 nM of forward and reverse primers, 150 nM of fluorogenic probe, 10 μ L of TaqMan universal master mix II (Applied Biosystems, Foster City, CA, USA), and DNA-free water, to a total volume of 20 μ L. qPCR was performed in a CFX 96TM Real-Time System (Bio-Rad, Hercules, CA, USA) with the following temperature set up: 50 °C for 2 min, 95 °C for 10

min, and 40 cycles of 95 °C for 15 s and 60 °C for 10 min. Serial dilutions (10^{-4} to 10 ng of DNA/ μ L) of the extracted genomic DNA of CB1190 were used to prepare the calibration curves and determine the amplification efficiency for the tested genes.

9.2.6 Analysis of soluble di-iron monooxygenase (SDIMO) genes

Previously designed degenerate primers NVC57, NVC58, NVC65 and NVC66 to target conserved regions in the soluble di-iron monooxygenases (SDIMO) alpha subunit gene¹⁵ were used to examine the presence and diversity of SDIMO genes in the original soil samples and the two consortia. A nested PCR strategy was used to increase the PCR product yield. In the first run, the PCR mixture contained 1 μ L of NVC65 and NVC58 primer mixture (10 μ M), 20 ng of the extracted genomic DNA, 12.5 μ L of KAPA HiFi HotStart ReadyMix (2X) (KAPA Biosystems, Wilmington, MA, USA), and nuclease-free water to yield a total volume of 25 μ L. PCR was performed in a Bio-Rad T100™ Thermal Cycler (Bio-Rad, Hercules, CA, USA) with the following temperature profile: initial denaturation (94°C, 5 min), then 29 amplification cycles (94°C for 30 s, 55°C for 30 s, 72°C for 1 min per kb) and a final extension (72°C for 5 min).¹⁵ The length of the PCR products in the first run was checked by 1% agarose gel and DNA bands of the correct size (1100 bp) were excised and purified. 20 ng of the purified PCR product was used as the DNA template in the second run, with the second set of primers (NVC57 and NVC66). The purified product (420 bp) from the second PCR was sent to MR DNA (www.mrdnalab.com, Shallowater, TX, USA) for Illumina MiSeq paired-end sequencing (approximately 2 $\times$ 300 bp as the read length). Sequence data were processed using MR DNA analysis pipeline. Operational taxonomic units (OTUs) were defined by clustering at 3% divergence (97% similarity).²¹³ A database including all of the currently reported SDIMO genes on NCBI was created and used to taxonomically classify the final OTUs.

9.3 Results and Discussion

9.3.1 Dioxane degradation rates for both consortia were similar to those of two archetype degraders.

Using surface soil samples (5 g) with no known history of dioxane exposure as bacterial seeds, two consortia (Figure 9.1) were enriched over three months following 12 weekly cycles of 10-fold serial dilution and growth in 100 mL of ammonium mineral salts (AMS) medium³⁹ amended with 100 mg/L dioxane as the sole carbon source. These two consortia were capable of degrading 500 mg/L dioxane (in AMS medium) as sole source of carbon and energy to non-detect levels (< 5 μ g/L) within one week (Figure 9.2), with a doubling time of approximately 24 h at 30 °C and pH = 7. After normalizing rates to the biomass concentrations, the specific degradation rates were 7.12 ± 0.18 g-dioxane/g-protein/day for Consortium A and 5.68 ± 0.07 g-dioxane/g-protein/day for Consortium B. These rates are comparable to those of two archetype dioxane degraders, *Pseudonocardia dioxanivorans* CB1190 (6.21 ± 0.68 g-dioxane/g-protein/day) and *Mycobacterium dioxanotrophicus* PH-06 (7.35 ± 0.20 g-dioxane/g-protein/day) (Figure 9.2). For both consortia, dioxane removal was coupled to bacterial growth (cell yield coefficients were 0.11 g-protein /g-dioxane for Consortium A and 0.09 g-protein /g-dioxane for Consortium B) (Figure 9.2), indicating metabolic (rather than cometabolic) degradation of dioxane as sole carbon and energy source.

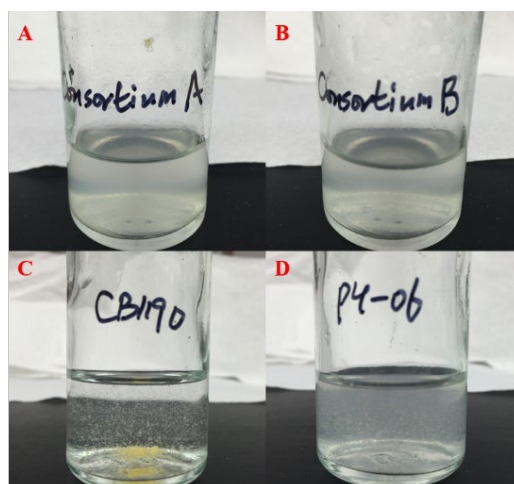


Figure 9. 1 The two consortia enriched from uncontaminated soil were less prone to aggregate than two dioxane-degrading archetypes.

The pictures show that Consortium A (A) and Consortium B (B) did not clump as much as *Pseudonocardia dioxanivorans* CB1190 (C) and *Mycobacterium dioxanotrophicus* PH-06 (D) after grown in AMS medium amended with initial 500 mg/L dioxane for one week. T=30 °C, pH=7. At the end of the experiments, the total biomass (quantified by protein) concentrations were 38 ± 4.8 mg/L for Consortium A, 27 ± 1.9 mg/L for Consortium B, 44 ± 4.2 mg/L for CB1190, and 49 ± 2.3 mg/L for PH-06.

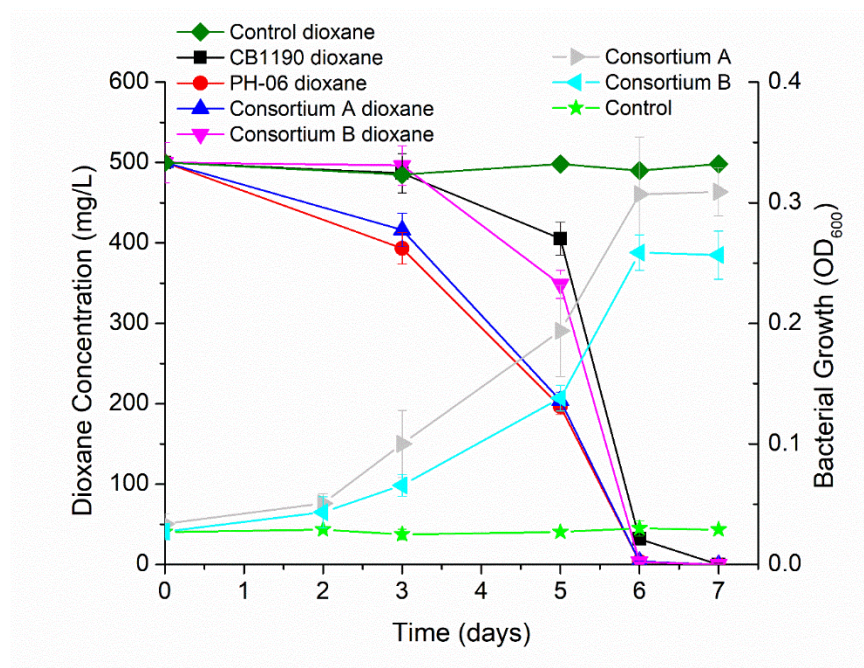


Figure 9. 2 Dioxane degradation by enriched consortia and two archetypes (left) and the concurrent growth of consortia (right).

Experiments were conducted using AMS medium with an initial dioxane concentration of 500 mg/L. The initial biomass densities (measured as protein content) were 25 mg/L for CB1190, 32 mg/L for PH-06, 19 mg/L for Consortium A, and 36 mg/L for Consortium B. The controls contained autoclaved bacteria.

Dioxane impacted groundwater from a site in Seattle, WA, that was not exhibiting intrinsic biodegradation capabilities was used to assess the potential of these consortia for bioaugmentation. Addition of both consortia (48 mg/L for Consortium A and 18 mg/L for Consortium B) resulted in dioxane removal below detection levels ($< 5 \mu\text{g/L}$) within three days, even when the initial dioxane concentration was low (i.e., $300 \mu\text{g/L}$) (Figure 9.3). This is an encouraging result because such low dioxane concentrations may represent a bioremediation challenge at impacted sites, as they might be insufficient to induce and/or sustain an active degrading indigenous population.³ The specific dioxane biodegradation rates of these two consortia (0.062 ± 0.003 g-dioxane/g-protein/day for Consortium A, and 0.056 ± 0.003 g-dioxane/g-protein/day for Consortium B) are comparable with those of two archetype degraders (0.052 ± 0.002 g-dioxane/g-protein/day for CB1190 and 0.10 ± 0.005 g-dioxane/g-protein/day for PH-06) when the initial dioxane concentration was similarly low.

A key challenge to consider in bioaugmentation is the subsurface distribution of injected bacteria, which is hindered by filtration through the aquifer material – especially when the cultures aggregate. This is an important limitation for bioaugmentation with the archetype dioxane degraders CB1190 and PH-06, which form clumps in suspension.⁴⁵ Interestingly, these two consortia do not clump as much as CB1190 and PH-06 (Figure 9.1), which could make them superior candidates for *in situ* bioaugmentation (i.e., easier perfusion through porous media) at sites where the indigenous dioxane biodegradation capacity is insufficient. Furthermore, this unprecedented enrichment of two consortia from uncontaminated soil based on their capability to grow on dioxane as sole carbon source indicates that pristine environments may harbor dioxane degraders and exhibit associated natural attenuation capacity.

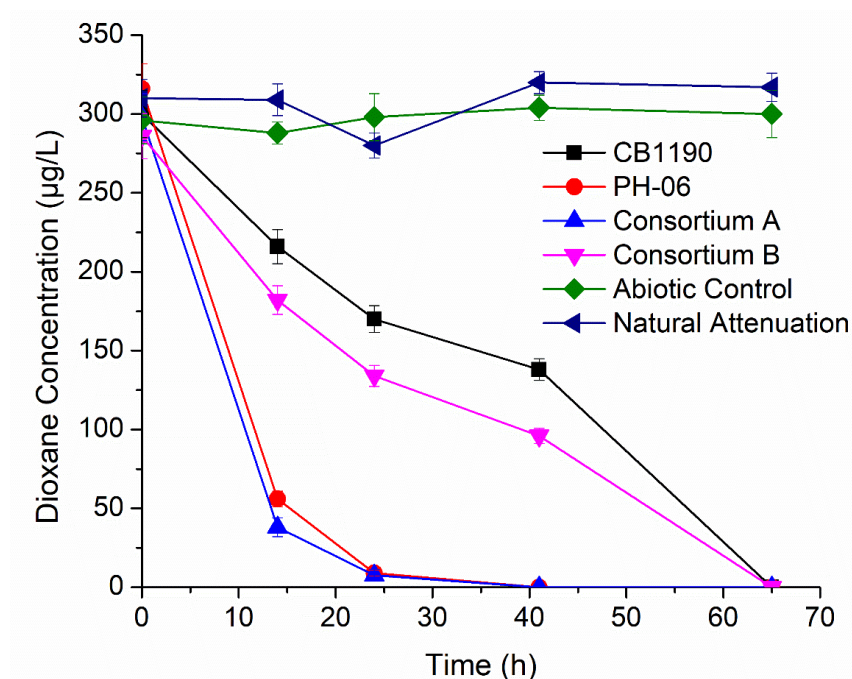


Figure 9. 3 Dioxane degradation in various bioaugmented microcosms.

Experiments were conducted using groundwater (initial dioxane concentration was 300 $\mu\text{g/L}$) from a dioxane impacted site in Seattle, Washington, USA, $T=15^\circ\text{C}$. The added bacterial biomass measured as protein content were 20 mg/L for CB1190, 32 mg/L for PH-06, 48 mg/L for Consortium A and 18 mg/L for Consortium B.

Dioxane is a synthetic organic (rather than naturally occurring) compound to which the original soil biota had no known previous exposure. Thus, the observed metabolic capacity may seem counterintuitive. Nevertheless, all reported dioxane-degrading bacteria can also degrade other cyclic ethers such as tetrahydrofuran (THF) and some naturally occurring compounds. For example, cyclic ethers containing 5- and 6-membered rings are widespread in nature and are used in the biosynthesis of certain antibiotics,²¹⁰ and the capacity to degrade these antibiotics may serve a protective role.²¹⁴ Additionally, lactones, which can be intermediates of both dioxane and THF biodegradation¹⁴ (Figure 8.4), are also commonly encountered in nature as chemical signals for bacterial communication (i.e., quorum sensing).²¹⁵ Many bacteria produce lactonases to degrade these signals and interrupt quorum-sensing (Figure 9.4), which enhances their ability to compete against the bacteria that produce them.²¹⁶ This raises the possibility that biodegradation of simple cyclic ethers such as THF and dioxane (which are lactone precursors) may be a fortuitous result of backward evolution of catabolic pathways.²¹⁷ Specifically, we cannot rule out the possibility that if an organism is capable of growing on lactones that became depleted, this could impose selective pressure to transform other compounds (e.g., dioxane) into a lactone for enhanced survival.

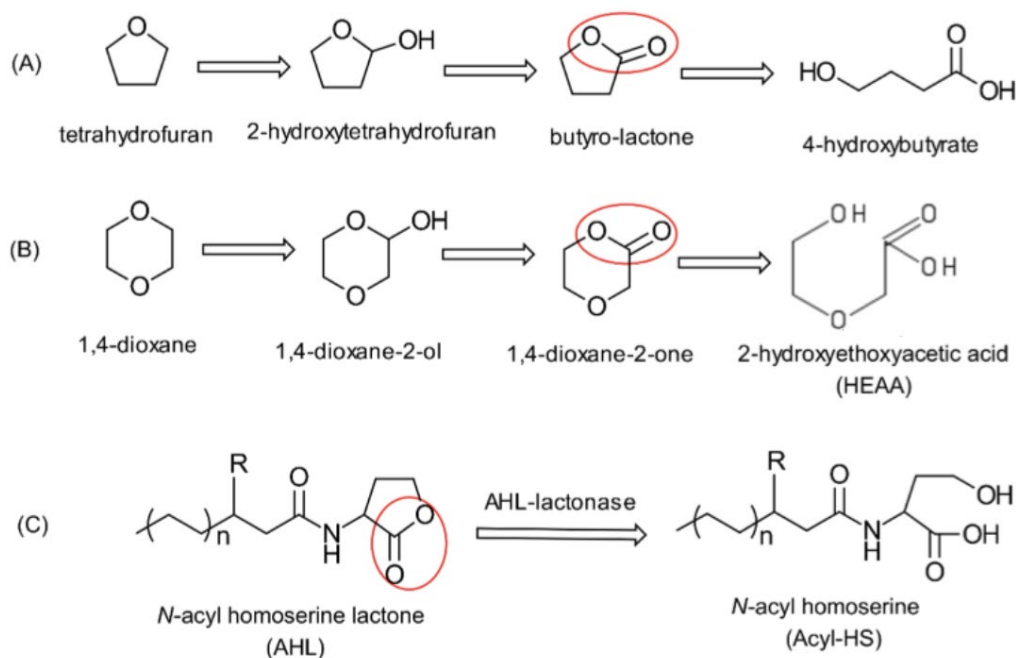


Figure 9. 4 Lactones can be the intermediates of both THF (A) and dioxane (B) biodegradation intermediates, as well as quorum sensing molecules (C). Some bacteria can produce lactonases to degrade these quorum sensing molecules.

9.3.2 Microbial characterization in both dioxane-degrading consortia.

Phylogenetic analysis by 16S rRNA sequences revealed a decrease in microbial diversity after enrichment on dioxane. For consortium A, the number of genera present decreased from 288 in the original soil samples to 47 genera after enrichment. The corresponding decrease in consortium B was from 340 to 50. The dominant genus in the original soils samples was *Acidobacterium* (33 % in Consortium A and 30% in Consortium B) (Table 9.1). After enrichment, the dominant genus in both consortia is *Mycobacterium* (56% in Consortium A and 49% in Consortium B) (Figure 9.5). Enrichment significantly changed the microbial community structure. For example, the top 3 genera in the original soil samples disappeared, while the top 3 genera in enriched consortia were minor populations in the original soil samples (Table 9.1). The observed population shift with a clear predominance of *Mycobacterium* spp., suggests that some species from this genus were involved in dioxane biodegradation.

Table 9. 1 Three most abundant genera before and after enrichment.

Consortium A			Consortium B		
Genus name	Percentage (%)		Genus name	Percentage (%)	
	Before	After		Before	After
<i>Acidobacterium</i>	31.9	0.00	<i>Acidobacterium</i>	29.81	0.00
<i>Pirellula</i>	3.6	0.00	<i>Chloroflexus</i>	6.67	0.00
<i>Holophaga</i>	3.6	0.00	<i>Dongia</i>	2.56	0.00
<i>Mycobacterium</i>	0.1	56.0	<i>Mycobacterium</i>	0.2	48.8
<i>Aminobacter</i>	0.04	10.5	<i>Chitinophaga</i>	0.01	15.5
<i>Ralstonia</i>	0.08	5.9	<i>Aminobacter</i>	0.03	9.2

Various *Mycobacterium* strains capable of degrading dioxane metabolically or cometabolically have been previously isolated from environmental samples with known dioxane exposure history (Table 9.2). The predominance of *Mycobacterium* spp, in these consortia, supports the notion that this is an important and commonly encountered genus of dioxane degraders. Furthermore, we show that *Mycobacterium* may be more prevalent in some environments than the more widely studied dioxane-degrading actinomycetes belonging to *Pseudonocardia* and *Rhodococcus* genera.

Among other genera present that make at least 2% of these consortia (Figure 9.5) (i.e., *Aminobacter* (10%), *Ralstonia* (6%), *Chitinophaga* (4%), *Cupriavidus* (4%), *Afipia* (3%), *Ensifer* (3%) and *Sediminibacterium* (2%) in Consortium A; *Chitinophaga* (16%), *Aminobacter* (9%), *Terrimonas* (3%), *Acidovorax* (3%), *Variovorax*(3%), *Brevundimonas* (2%) and *Sediminibacterium* (2%) in Consortium B), only *Afipia* encompasses a strain (i.e., *Afipia* sp. D1) that was reported to degrade dioxane as sole carbon and energy source.⁴¹ *Aminobacter* and *Sediminibacterium* were the only genera that were enriched in both consortia, with *Aminobacter* reaching much higher abundance. It is still uncertain if and how the other genera identified in these consortia contribute to the biodegradation of dioxane and/or its potential byproducts. Our unsuccessful efforts to isolate dioxane-degrading strains using the conventional enrichment and dilution to extinction approach²¹¹ suggests that the dominant *Mycobacteria* and other coexisting bacterial species might constitute complex syntrophic communities.

Table 9. 2 Bacteria capable of metabolizing or co-metabolizing dioxane.

Bacterial strain	Dioxane metabolism ^a	Co-substrate	Inoculum source	Reference
<i>Rhodococcus ruber</i> 219	+	-	WWTP ^b effluent	175
<i>Pseudonocardia dioxanivorans</i> CB1190	+	-	Industrial sludge from a dioxane contaminated site	39
<i>Pseudonocardia benzenivorans</i> B5	+	-	Soil contaminated by various chlorinated, aromatic compounds	163
<i>Mycobacterium dioxanotrophicus</i> PH-06	+	-	River sediment contaminated by dioxane	40
<i>Afipia</i> sp. D1	+	-	Soil samples near a dioxane producing factory	41
<i>Mycobacterium</i> sp. D6	+	-	Same as above	41
<i>Mycobacterium</i> sp. D11	+	-	Same as above	41
<i>Pseudonocardia</i> sp. D17	+	-	Same as above	41
<i>Acinetobacter baumannii</i> DD1	+	-	WWTP sludge	42
<i>Rhodanbacter</i> AYS5	+	-	Industrial sludge	178
<i>Xanthobacter flavus</i> DT8	+	-	Activated sludge of pharmaceutical plants	179
<i>Pseudonocardia</i> sp.N23	+	-	Groundwater contaminated by dioxane	218
<i>Rhodococcus aetherivorans</i> JCM 14343	+	-	A chemical effluent treatment plant	219
<i>Pseudonocardia tetrahydrofuranoxydans</i> sp. K1	-	Tetrahydrofuran	WWTP sludge	20
<i>Pseudonocardia</i> sp. ENV478	-	Tetrahydrofuran	Industrial wastewater treatment system	21
<i>Rhodococcus ruber</i> T1	-	Tetrahydrofuran	Landfill soil	184
<i>Rhodococcus ruber</i> T5	-	Tetrahydrofuran	WWTP Sludge	184
<i>Flavobacterium</i> sp.	-	Tetrahydrofuran	A contaminated groundwater plume	181
<i>Mycobacterium</i> sp. JOB5	-	Propane	Soil samples with hydrocarbons	220
<i>Mycobacterium</i> sp. ENV421	-	Propane	Turf soil enriched with propane	221
<i>Rhodococcus ruber</i> ENV 425	-	Propane	Turf soil samples enriched with propane	222
<i>Azoarcus</i> sp. DD4	-	Propane	Activated Sludge sample	53
<i>Pseudomonas mendocina</i> KR1	-	Toluene	Algal-bacterial mat from Colorado River in Austin	223
<i>Rhodococcus</i> RR1	-	Toluene	Gasoline contaminated aquifer	224
<i>Ralstonia pickettii</i> PKO1	-	Toluene	Soil microcosms amended with BTEX ^c	225
<i>Burkholderia cepacia</i> G4	-	Toluene	Water and soil samples	226
<i>Methylosinus trichosporium</i> OB3b	-	Methane	Mud, river and stream water, soil samples	187

^a “+” and “-” indicate this condition is applicable and not applicable, respectively.

^b WWTP: wastewater treatment plant; ^c BTEX: Benzene, Toluene, Ethylbenzene, and Xylenes.

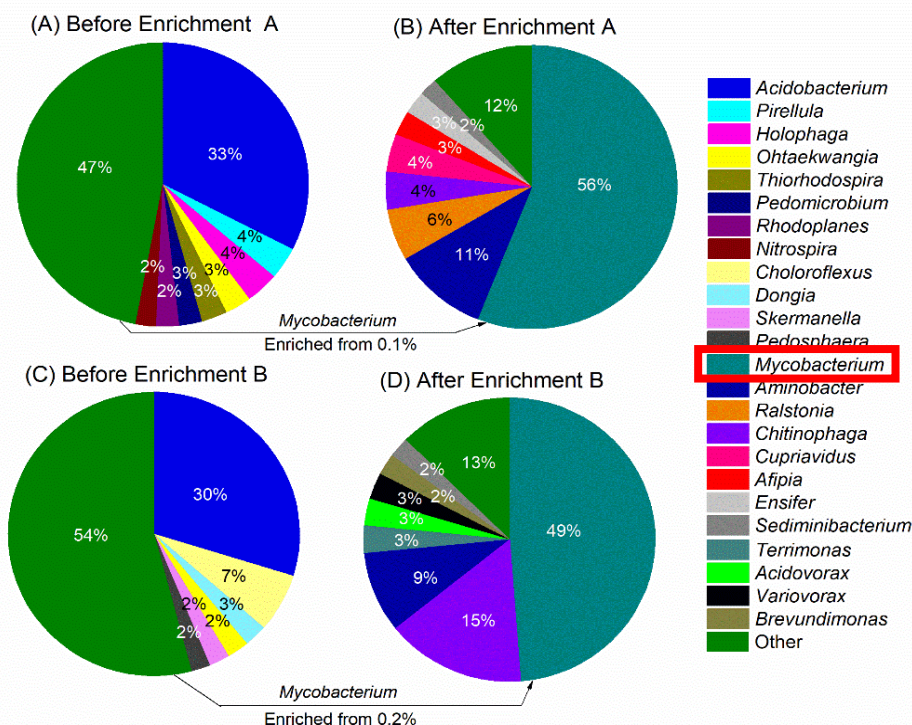


Figure 9. 5 16S rRNA sequencing analyses of the two original soil samples and enriched consortia
The sum of all bacteria genera representing less than 2% of the total community is indicated as “Other”.
These sequence data were submitted to the GenBank database under accession number SRP103870.

9.3.3 Acetylene inhibition test indicates the involvement of monooxygenases.

Acetylene, a known monooxygenase inhibitor,²²⁷ was used to determine whether monooxygenases could play a role in dioxane degradation by the two consortia. When exposed to 8% (v/v) of acetylene in headspace, dioxane removal significantly decreased (by > 90% over seven days) compared to the untreated controls (Figure 9.6). These results suggest that monooxygenases played a crucial role in dioxane degradation, likely catalyzing the initial biotransformation steps. This corroborates previous reports about the importance of monooxygenases in initiating the aerobic degradation of dioxane.¹¹ Therefore, information on the responsible monooxygenase genes can advance understanding and assessment of dioxane biodegradation and natural attenuation potential at contaminated sites.

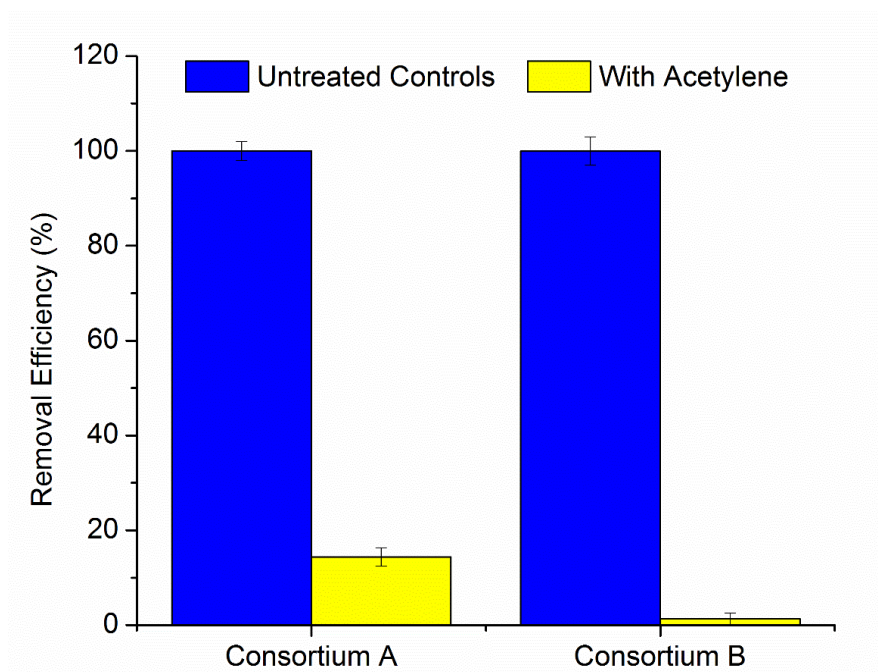


Figure 9. 6 Inhibition of dioxane removal by acetylene (8% in the headspace).

Experiments were conducted for seven days with 100 mg/L dioxane as the only carbon source in AMS medium. The initial total biomass concentration was 20 mg-protein/L.

9.3.4 The *thmA* biomarker was not detected in these two consortia.

The acetylene inhibition test indicates the involvement of monooxygenases, although it is still unclear which specific monooxygenases initiated dioxane catabolism. Up to date, despite the successful isolation of numerous dioxane-degrading bacteria in previous work (Table 9.2), little is known about the genetic basis for dioxane biodegradation. In fact, only one enzyme responsible for initiating dioxane metabolism has been discovered. This tetrahydrofuran monooxygenase is encoded by the gene cluster *thmADBC*, which is located on plasmid pPSED02 in *Pseudonocardia dioxanivorans* CB1190.¹⁴ A primer/probe set for the *thmA* biomarker designed to target the nucleotide sequence coding for the hydroxylase α - subunit (i.e., the enzyme's active site) was shown to be an excellent indicator of dioxane degradation capacity,¹⁶ and no false positives have been observed. However, this *thmA* biomarker was not detected in either of our two consortia using qPCR analysis (limit of detection = 12 copies/ng genomic DNA; 16S rRNA from the consortia and *thmA* from CB1190 were used as positive controls, Figure 9.7). Therefore, other monooxygenase genes were likely responsible for dioxane degradation in these consortia.

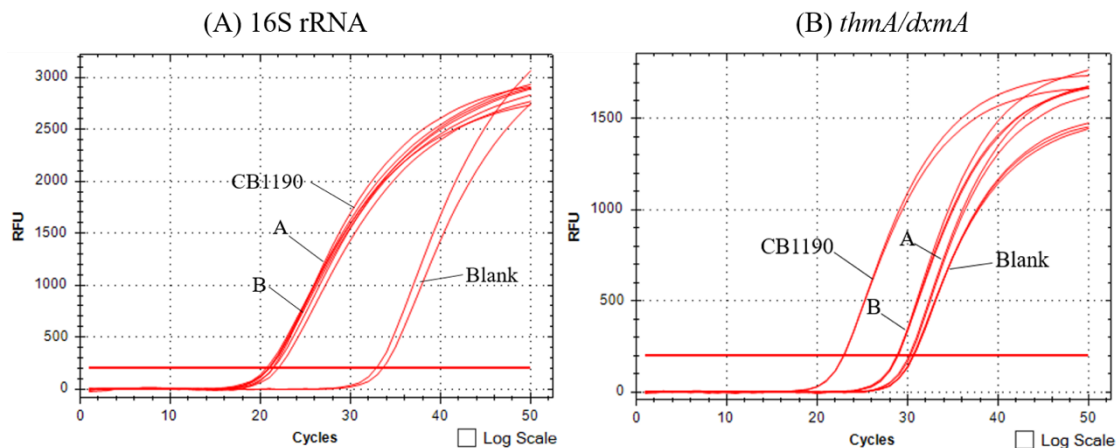


Figure 9. 7 Real-time qPCR assay to test the existence of 16S rRNA (A) and *thmA/dxmA* (B) genes using genomic DNA extracted from both consortia, *Pseudonocardia dioxanivorans* CB1190 (positive control) and Nuclease-free water (blank control).

All of the qPCR assays were conducted using an equal amount of DNA and in triplicate. Quantification cycle (Cq value) of *thmA/dxmA* for strain CB1190, Consortium A, B, and Blank control were 22.94 ± 0.06 , 30.22 ± 0.10 , 28.87 ± 0.07 and 30.62 ± 0.05 , respectively, while the baseline threshold value of Relative Fluorescence Units (RFU) was set to 200.

9.3.5 The role of soluble di-iron monooxygenase (SDIMO) on dioxane biodegradation.

A nested PCR analysis using two degenerate primers to target the hydroxylase α -subunit of SDIMOs¹⁵ was performed to gain insights into which enzymes were responsible for dioxane degradation by these consortia. SDIMOs were present in both consortia as indicated by agarose gel electrophoresis (Figure 9.8).

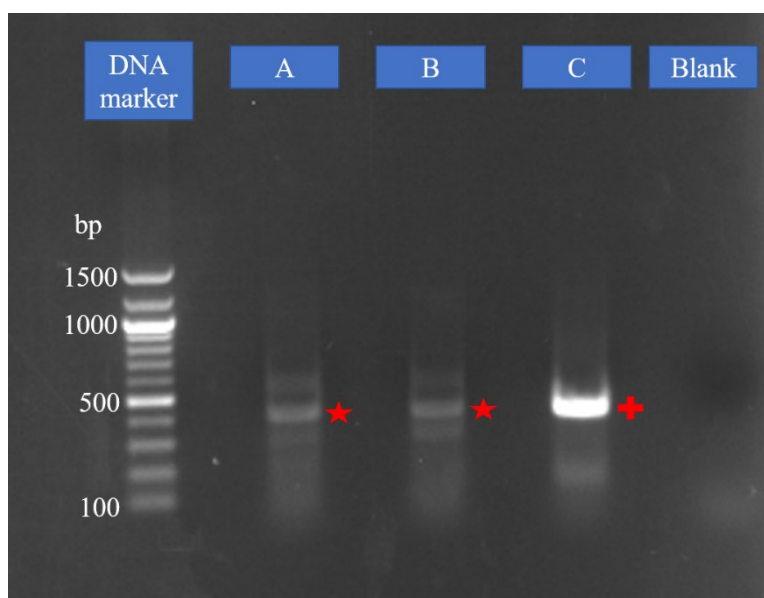


Figure 9. 8 Agarose gel electrophoresis showing PCR products obtained with primers NVC57 and NVC66.

The presence of SDIMO is represented by the 420 bp DNA fragment in Consortium A (A) and B (B). DNA of *Pseudonocardia dioxanivorans* CB1190 (C) and nuclease-free water as a template were used as positive and negative controls, respectively.

SDIMOs are divided into six groups, based on their subunit organization and composition, substrate specificity, and sequence similarity,¹⁵ and the only sequenced dioxane monooxygenase gene cluster to date (i.e., *thmADBC*) belongs to group 5.⁴⁶ To gain insight about the specific SDIMOs harbored by these two consortia, the purified products obtained from the second PCR run were sequenced and compared to genes databases (NCBI) encompassing all of the currently reported SDIMOs. The diversity of SDIMO genes in both consortia decreased compared with the original soil samples. The dominant SDIMO genes in Consortium A corresponded to a group 6 SDIMO (98.81%); while in Consortium B, SDIMO genes from both groups 5 (47.27%) and 6 (51.88%) were observed. These represent 3- to 7- fold increases, respectively, relative to the original soil samples (Figure 9.9). In both consortia, the relative abundance of *thmA* gene was negligible (0.03%), which is consistent with the negative amplification of these genes by qPCR (Figure 9.7).

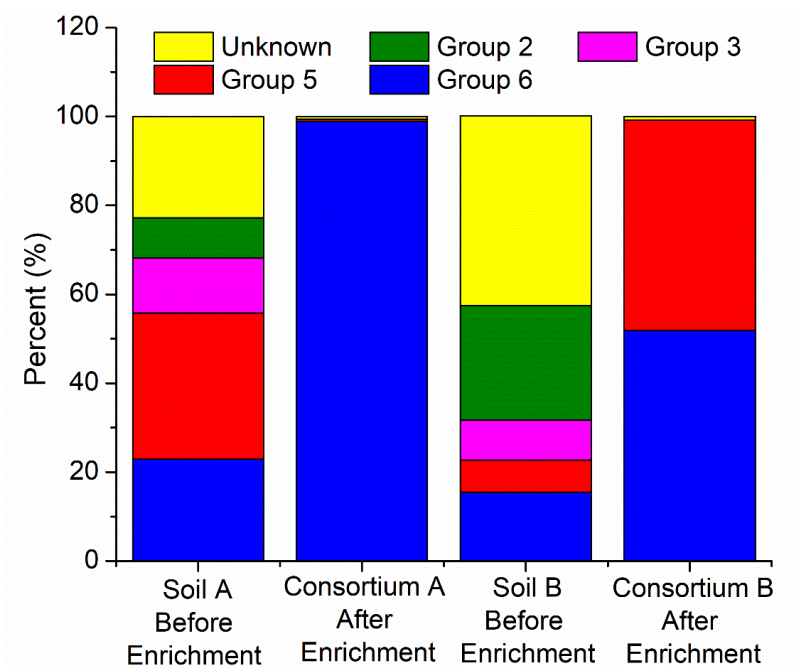


Figure 9. 9 SDIMO gene sequencing of nested PCR products from the original soil samples and enriched consortia.

“Group 2”, “Group 3”, “Group 5”, and “Group 6” indicate a high similarity (>93%) with groups 2, 3, 5 and 6 soluble diiron monooxygenase hydroxylase (SDIMOs) alpha subunit genes, respectively. “Unknown” indicates no significant similarities with any SDIMO gene sequences available on NCBI. The percentages bars represent the fractions of different SDIMO gene groups in the two consortia. These sequence data were submitted to the GenBank database under accession number SRP103872.

The prevalence of such SDIMOs is consistent with the observed enrichment of *Mycobacterium* spp., since previous studies have reported that groups 5 and 6 SDIMO genes are harbored by *Mycobacterium* spp. These include group 5 SDIMO genes in *Mycobacterium semegmatis* MC² 155 and *Mycobacterium goodii* strain 12523,²²⁸ and group 6 SDIMO genes in *Mycobacterium chubuense* NBB4,¹⁵ *Mycobacterium* sp. TY-6⁷⁸ and *Mycobacterium* sp. ENV421.²²⁹ This underscores the need for further research on SDIMO genes and enzymes involved in dioxane biodegradation to develop novel biomarkers that can be useful for forensic analysis and performance assessment of bioremediation and natural attenuation at dioxane-impacted sites.

Chapter 10

Identification of new catabolic genes responsible for dioxane degradation

10.1 Introduction

Several pure strains or consortia capable of degrading dioxane have been isolated which suggests that bioremediation and monitored natural attenuation may be a promising strategy to manage dioxane contamination at some sites. When applicable, such biodegradation approaches (including intrinsic bioremediation associated with monitored natural attenuation [MNA]) are usually more economical and environmentally friendly than more aggressive remediation alternatives such as advanced oxidation, especially for managing large and dilute dioxane plumes. However, the burden of proof that MNA is an appropriate solution lies on the proponent. Thus, there is a growing need for molecular tools to quantitatively assess the presence and activity of dioxane degraders to support decisions to accept or reject MNA. This could result in significant economic benefits associated with overcoming the current underutilization of MNA, and advance understanding of the diversity of the associated degradation pathways.

Soluble di-iron monooxygenases (SDIMOs) play a key role in dioxane biodegradation.¹¹ Based on the number and arrangement of subunits, substrate specificity, and sequence similarity, SDIMOs are categorized into six groups.⁴³ Recent genomic and molecular studies with *Pseudonocardia dioxanivorans* CB1190 helped elucidate the role of the group-5 SDIMO gene cluster *thmADBC* (located on a plasmid), which codes for a monooxygenase associated with the initial hydroxylation of dioxane at the α carbon position, leading to the cleavage of the high-energy C-O bond.⁴⁶ Gene clusters with high sequence similarities have been identified in bacteria capable of degrading tetrahydrofuran (THF), which is a structural analog of dioxane.⁴⁹ In addition, group-5 SDIMO genes were enriched at a number of geographically distinct, dioxane-impacted sites.¹⁷ However, little is known about the importance of other SDIMO groups dioxane bioremediation.

As discovered in Chapter 9, the two dioxane degrading consortia, which were enriched from soil samples and are dominated by *Mycobacterium* spp, don't harbor *thmADBC*, while there is a significant increase in group-6 SDIMO genes after enrichment.²³⁰ This indicates that probing for this gene using the biomarker designed based on *thmADBC* gene information would give false negatives regarding dioxane degradation potential. Moreover, a dioxane-degrading isolate *Mycobacterium dioxanotrophicus* PH-06 lacks *thmADBC*, which was confirmed by PCR, qPCR (data not shown), and whole genome sequencing.²³¹ PH-06 exhibits higher affinity for dioxane ($K_s = 78 \pm 10$ mg/L) and greater cell yield ($Y = 0.16$ g protein /g dioxane) than the archetype dioxane degrader CB1190 ($K_s = 145 \pm 17$ mg/L, $Y = 0.11$ g protein /g dioxane) which suggests that it could be a more suitable candidate for bioaugmentation (Figure 10.1).

Thus, in this chapter, we identify and characterize a novel plasmid-encoded group-6 SDIMO gene cluster responsible for dioxane degradation by PH-06, which is phylogenetically disparate from other well-studied dioxane degraders.

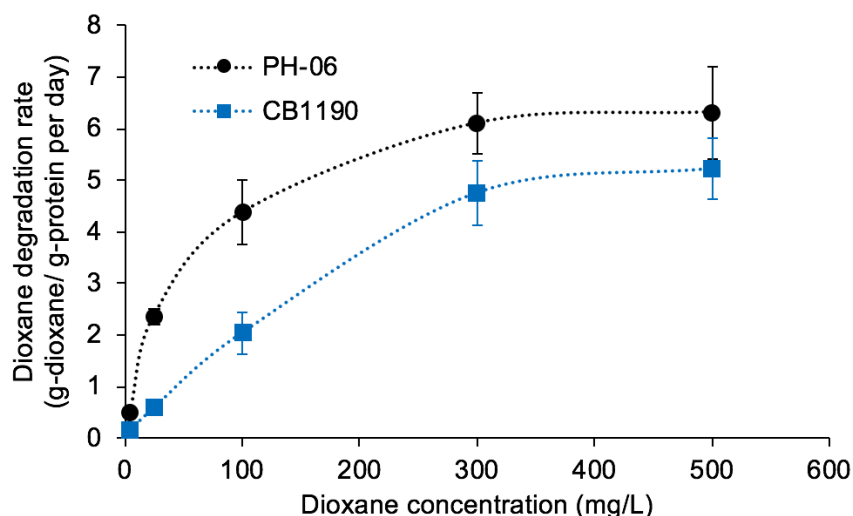


Figure 10. 1 Monod plot for the dioxane degradation by *Mycobacterium dioxanotrophicus* PH-06 ($K_s = 78 \pm 10$ mg/L, $Y = 0.16$ g protein/g dioxane) and *Pseudonocardia dioxanivorans* CB1190 ($K_s = 145 \pm 17$ mg/L, $Y = 0.11$ g protein/ g dioxane). Our data for CB1190 are consistent with the previously reported values ($K_s = 160 \pm 44$ mg/L, $Y = 0.09$ g protein/ g dioxane).¹¹

10.2 Materials and Methods

10.2.1 Bacterial strains and culture conditions

Mycobacterium dioxanotrophicus PH-06⁴⁰ was provided by Dr. Yoon-Seok Chang (POSTECH, South Korean). PH-06 was grown in AMS medium. Growth substrates (dioxane, THF, glucose or sodium acetate) were added to the culture medium to achieve a final concentration of 500 mg/L. Glucose and acetate are used as control substrates for transcriptomic studies because they are readily biodegradable without the need for SDIMOs. Whereas dioxane concentrations at contaminated sites are commonly lower than 1 mg/L,³ these relatively high concentrations were used to easily observe growth and obtain sufficient biomass for transcriptomic and other analyses. All cultures were incubated aerobically while shaking at 150 rpm at 30 °C.

10.2.2 Whole genome sequencing

Whole genome sequencing was conducted using both PacBio RS II (Yale Center for Genome Analysis, <http://ycga.yale.edu/>) and Illumina HiSeq 4000 (Beijing Genomic Institute (BGI), <http://www.bgi.com/>) platforms. The whole genome was assembled and annotated in collaboration with BGI. The following steps were performed: 1) Prior to assembly, K-mer analysis was used to evaluate genome size, heterogeneity and repeat information based on the data obtained by Illumina;²³² 2) PacBio RS II reads were assembled using RS_HGAP Assembly

of SMRT Analysis v 2.3.0 (<https://github.com/PacificBiosciences/SMRT-Analysis>) to obtain the main contig with length close to the estimated genome size, and Illumina reads were used to correct and optimize assembly results; 3) Contigs' bases were corrected by Quiver, Pilon, SOAPsnp, SOAPindel, and GATK; 4) Contig circle analysis was completed by verifying overlap regions; 5) Glimmer,²³³ TRF,²³⁴ RNAmmer v1.2,²³⁵ tRNAscan-SE v1.3.1,²³⁶ Infernal,²³⁷ Rfam²³⁸ and BLAST were used to predict genes, repeat sequences, rRNA, tRNA, and ncRNA; 6) Predicted genes were searched against the GO,²³⁹ KEGG,²⁴⁰ COG,²⁴¹ NR, Swiss-Prot,²⁴² PHI,²⁴³ VFDB,²⁴⁴ ARDB,²⁴⁵ and CAZy²⁴⁶ databases to annotate gene function and identify metabolic pathways, pathogenicity and drug resistance.

10.2.3 Total RNA extraction

PH-06 cells in the exponential growth phase (after half of the added substrates were consumed) were used to extract total RNA for RNA sequencing (RNA-Seq) and reverse transcription quantitative polymerase chain reaction (RT-qPCR). RNAProtect Bacteria Reagent (Qiagen, Hilden, Germany) was added to each culture prior to extraction according to the manufacturer's instructions, and cultures were then incubated at room temperature (25 °C) for 5 min to stabilize the RNA before further processing. The stabilized bacterial cultures were centrifuged for 10 min at 5000 × g. The supernatant was decanted, and the pellets were immersed and lysed in an appropriate volume of Buffer RLT (RNase Mini Kit, Qiagen, Hilden, Germany) with 2-mercaptoethanol (Sigma-Aldrich, St. Louis, MO, USA). After incubation for 5 min at room temperature, the lysed biomass in Buffer RLT was transferred into MicroRNA Bead Tubes (UltraClean® Microbial RNA Isolation Kit, MO BIO, Carlsbad, CA, USA) containing 0.25 g of beads and vortexed at maximum speed for 5 min using a Vortex-Genie® 2 (MO BIO, Carlsbad, CA, USA) to further homogenize the cell lysate. Then the lysis solution was centrifuged at maximum speed for 30 s and the supernatant was transferred into a new tube and the total RNA was purified using an RNase Mini Kit (Qiagen, Hilden, Germany). On-column DNA digestion was performed during the purification using an RNase-Free DNase Set (Qiagen, Hilden, Germany) to get high quality of RNA.

10.2.4 RNA Sequencing

RNA sequencing was used to investigate gene expression levels of PH-06 during growth on two different carbon sources (dioxane and glucose). RNA samples were sent to an external sequencing facility (LC Sciences, Houston, TX, USA) for mRNA sequencing. Total RNA quality control was performed with a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Products Inc., ThermoFisher Scientific, Waltham, USA) and Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) to make sure both UV absorbance parameters A_{260}/A_{230} and A_{260}/A_{280} were 1.8 - 2.2 and RNA Integrity Number (RIN) was larger than 8. Then, mRNA libraries were prepared following Illumina mRNA-Seq Sample Prep Kit and sequencing was performed using an Illumina HiSeq 2500 system with a Single-End (SE) rapid-run mode. Raw reads were mapped to the whole genome sequencing of PH-06²³¹ and the relative gene expression levels were estimated using EDGE-pro v 1.3.1 (<http://ccb.jhu.edu/software/EDGE-pro/>).²⁴⁷ These sequence data were submitted to the GenBank database under accession number SRP116048.

10.2.5 Reverse transcription quantitative PCR (RT-qPCR)

Total RNA was extracted from PH-06 cells after growth on dioxane, THF, glucose, and sodium acetate. The cDNA was synthesized using High-Capacity cDNA Reverse Transcription Kits with the addition of RNase inhibitor (Applied Biosystems, Foster City, CA, USA). The synthesized cDNA was purified with Wizard SV Gel and PCR Clean-Up System (Promega, Madison, WI, USA) following the manufacturer's protocol. The cDNA concentration in the elution (30 μ L) was determined by NanoDrop® ND-1000 and subsequently diluted to 10 ng/ μ L with Nuclease-Free water for RT-qPCR analysis. RT-qPCR mixture contained 1 μ L of diluted cDNA (10 ng/ μ L), 7.5 μ L of 2X SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA), 0.3 μ M of each forward and reverse primers and DNA-free water, to a total volume of 15 μ L. RT-qPCR was performed in a CFX 96™ Real-Time System (Bio-Rad, Hercules, CA, USA) with the following temperature set up: 95 °C for 10 min, and 40 cycles of 95 °C 15 s and 60 °C for 1 min. Specific primers (Table 10.1) targeting each subunit of the monooxygenase gene cluster were designed by the Primer Quest Tool (Integrated DNA Technologies, Coralville, IA, USA).

Table 9. 3 Primers used in RT-qPCR to determine the relative gene expression levels of *prmABCD* under two different treatments.

Targeted Gene	Forward Primer (5'-3')	Reverse Primer (5'-3')
<i>prmA</i>	TTT CCA GGG CTC GAC TTA	TCC AAC CGC TCG ATG TA
<i>prmB</i>	GCA GCG TCC TTA CAT CAA	CGC CAA CGC TTC GTA ATA
<i>prmC</i>	CCA GGC GAC CGT GCA TTA	GTC TCG AGC AGG AAG AAC GTG
<i>prmD</i>	GGC GTC AAC CTG CAA TA	TCC TCG GAA TCG CAT AGA
16S rRNA	CAG GAC GCT GGT AGA GAT A	CGG GAC TTA ACC CAA CAT C

10.2.6 Quantification of gene expression

The $2^{-\Delta\Delta C_T}$ method was used to quantify differential gene expression, and the results were analyzed with SDS 1.3.1.¹³¹ The calculation equation is:

$$\Delta\Delta C_{T, \text{Target gene}} = (C_{T, \text{Target gene}} - C_{T, \text{Housekeeping gene}})_{\text{Treatment}} - (C_{T, \text{Target gene}} - C_{T, \text{Housekeeping gene}})_{\text{Control}}$$

C_T is the cycle threshold, treatment is dioxane or THF, and control is glucose or acetate. This value is used to calculate the expression fold change of the target gene, as follows:

$$\text{Expression fold change} = 2^{-\Delta\Delta C_T}$$

Here, a housekeeping gene (16S rRNA of PH-06) was used as an internal standard for RT-qPCR. Treatments with glucose or sodium acetate as the sole carbon source were used as controls to calculate the expression level of the target genes in the presence of dioxane or THF.

10.2.7 Analytical procedures

Biomass was quantified through total protein concentration using a PierceTM BCA Protein Assay Kit (Thermo Fisher Scientific, Rockford, IL, USA). Dioxane and THF were measured using Agilent 7820A gas chromatograph (GC) equipped with a 5977E mass spectrum detector (MSD) after filtration through 0.22- μ m 13-mm syringe filters and extraction by a liquid/liquid frozen micro-extraction method using dichloromethane as the solvent.¹⁰⁰ Glucose and sodium acetate concentrations were determined by an Agilent 5890 gas chromatograph (GC) with a Flame Ionization Detector (FID) after derivation.²⁴⁸

10.3 Results and Discussion

10.3.1 Whole-genome sequencing identified only one SDIMO gene cluster in PH-06.

PH-06 genome consists of four contigs, including the chromosome (circular, 7.6 Mb), Plasmid_1 (circular, 156kb), Plasmid_2 (circular, 153kb), Plasmid_3 (linear, 106kb) and Plasmid_4 (linear, 70kb), and has an average G+C content of 66.46%. A total of 7,339 protein-encoding genes, 83 tRNA, 9 rRNA, and 4 ncRNA are present. KEGG database analysis revealed genes encoding the complete citric acid and pentose phosphate pathways. Furthermore, 1071 genes appear to be involved in xenobiotics metabolism. Pathogenicity analysis indicates that PH-06 harbors no known toxins or pathogenicity islands, suggesting it may be safe for bioaugmentation.²⁴³

Dioxane degradation by PH-06 is likely initiated by a monooxygenase, as suggested by a previous isotope labeling study⁴⁰ and supported by our acetylene inhibition test (Figure 10.2), which is a common approach to determine the participation of monooxygenases.²²⁷ The PH-06 genome contains only one SDIMO gene cluster (Figure 10.3) located in Plasmid_3.²³¹ Because another SDIMO has been reported to play a key role in dioxane biodegradation by CB1190,¹¹ we explored its association with dioxane degradation by PH-06. This SDIMO gene cluster has four subunits (a hydroxylase with large and small subunits, a coupling protein, and a reductase) with a total size of approximately 4 kb (Figure 10.3). NCBI BLAST results show that this gene cluster has high similarity with propane monooxygenase genes of *Rhodococcus wratislaviensis* IFP 2016 (89%), *Mycobacterium chubuense* NBB4 (86%) and *Mycobacterium* sp. ENV421 (89%).^{15, 86, 229} Thus, we named this SDIMO gene cluster as propane monooxygenase *prmABCD*. Upregulation of this gene cluster by propane was verified (Figure 10.4). The presence of a transposase gene immediately upstream or downstream of *prmABCD* (Figure 10.3), and its lower G+C content (62.9 %) compared with the chromosome (66.5%),²³¹ suggest that it may have originated from horizontal gene transfer.

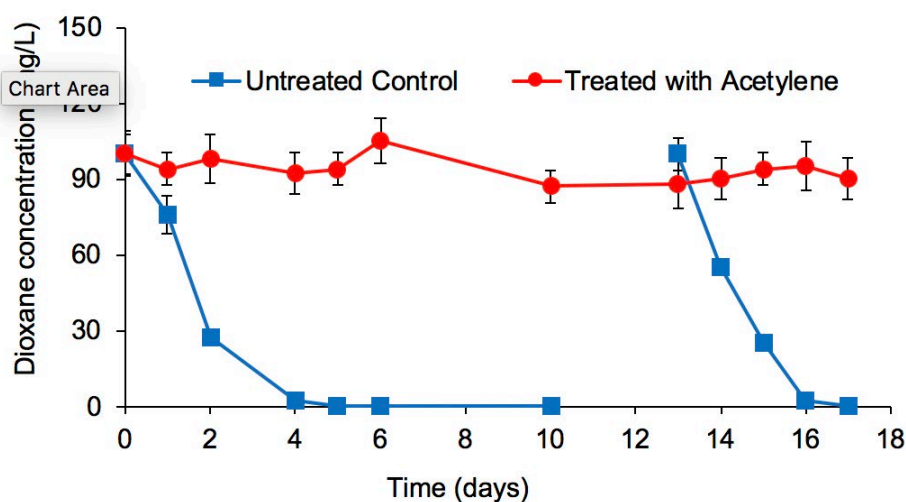


Figure 10. 2 Inhibition by acetylene on biodegradation of dioxane by *Mycobacterium dioxanotrophicus* PH-06.

The degradation capability was significantly inhibited by acetylene (8% in headspace) and was not recovered after the removal of acetylene. The initial dioxane and biomass concentration (measured as protein density) were 100 mg/L and 20 mg/L, respectively. On Day 13, acetylene was removed and 100 mg/L of dioxane was added into the control. Error bars represent $\pm$ one standard deviation from the mean of triplicate measurements.

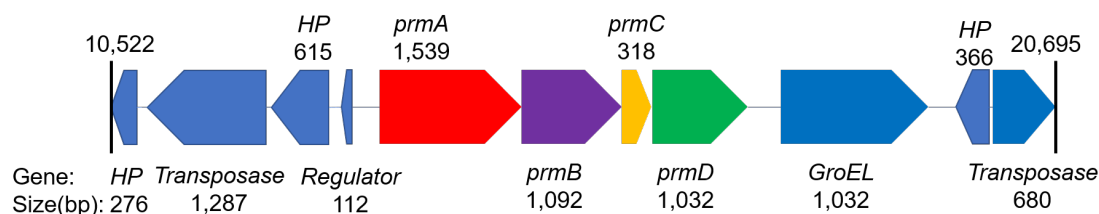


Figure 10. 3 Scheme of the propane monooxygenase (*prmABCD*) gene cluster.

The numbers on the above left and right sides indicate their locations in Plasmid_3. The arrows indicate genes and the strings between them indicate the gaps between different genes. The length of the strings and the arrows is proportional to their relative sizes. The right or left direction of the arrows indicates a positive or negative strand. Gene name abbreviation: HP (hypothetical protein); Regulator (two-component system response regulator); *prmA* (propane monooxygenase alpha subunit); *prmB* (propane monooxygenase beta subunit); *prmC* (propane monooxygenase coupling protein); *prmD* (propane monooxygenase reductase protein); *GroEL* (chaperonin *GroEL*).

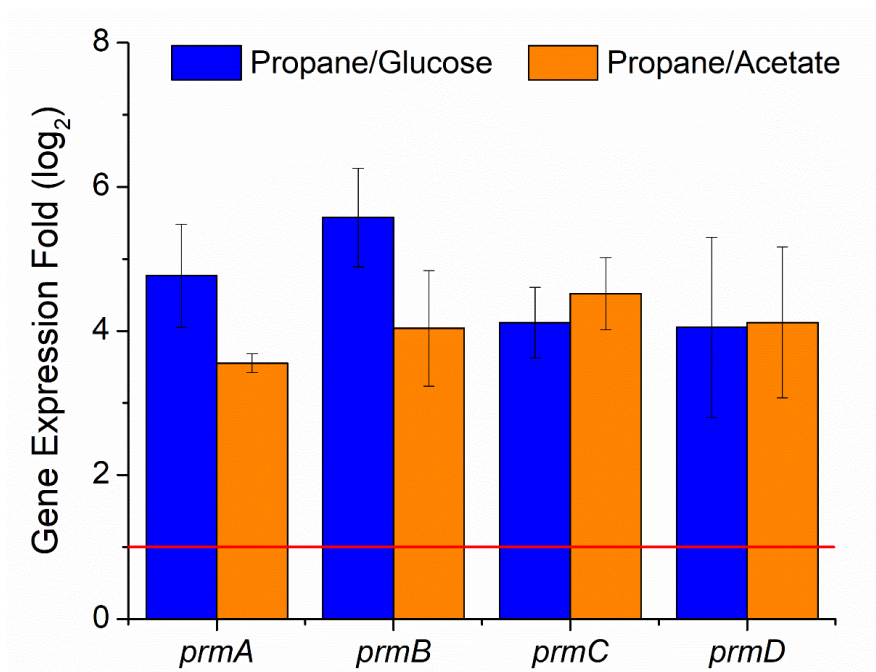


Figure 10. 4 Gene expression fold of *prmABCD* in PH-06 pure cultures grown on propane relative to glucose or acetate.

16S rRNA gene of PH-06 was used as the housekeeping gene for error control among samples. The red horizontal lines indicated upregulation criteria (two-fold gene expression change).

10.3.2 RNA-seq and RT-qPCR demonstrated the upregulation of *prmABCD* by dioxane.

To determine whether *prmABCD* is involved in dioxane degradation, RNA sequencing (RNA-Seq) was used to quantify gene expression levels during growth on dioxane or glucose. Dioxane (but not glucose) significantly upregulated *prmABCD*. The expression fold-changes were 6.5, 8.1, 9.7, and 10.7 for the four subunits, respectively (Table 10.2). Another multicomponent monooxygenase gene cluster (i.e., ammonia monooxygenase) located in Plasmid_3 and several different monooxygenase genes located in the chromosome were also upregulated (Table 10.2). However, the functions of these encoded enzymes exclude their involvement in dioxane degradation. For example, ammonia monooxygenase catalyzes the oxidation of ammonia to hydroxylamine,²⁴⁹ while cyclohexanone monooxygenase catalyzes an oxygen insertion on cyclohexanone to form a cyclic lactone.²⁵⁰ Therefore, *prmABCD* was chosen for further studies and confirmation of its involvement in dioxane degradation.

Table 10. 1 Monooxygenase genes upregulated during growth on dioxane relative to glucose shown by RNA-seq^a.

Gene ID ^b	Gene product ^c	Replicon ^d	Expression Fold Change
BTO20_RS38210	hydroxylase alpha chain (<i>prmA</i>)	P3	6.5
BTO20_RS38215	hydroxylase beta chain (<i>prmB</i>)	P3	8.1
BTO20_RS38220	coupling protein (<i>prmC</i>)	P3	9.7
BTO20_RS38225	reductase protein (<i>prmD</i>)	P3	10.7
BTO20_RS38395	ammonia monooxygenase subunit C	P3	5.5
BTO20_RS38400	ammonia monooxygenase subunit A	P3	7.5
BTO20_RS38400	ammonia monooxygenase subunit B	P3	13.3
BTO20_RS22865	cyclohexanone monooxygenase	C	13.9
BTO20_RS00055	antibiotic biosynthesis monooxygenase	C	8.9
BTO20_RS27410	4-hydroxyacetophenone monooxygenase	C	8.6
BTO20_RS11965	FAD-binding monooxygenase	C	6.8
BTO20_RS26085	alkane 1-monooxygenase	C	5.8
BTO20_RS22540	alkanesulfonate monooxygenase	C	4.7
BTO20_RS08645	nitronate monooxygenase	C	4.2
BTO20_RS31515	steroid C27-monooxygenase	C	4.2
BTO20_RS24595	FMNH ₂ -dependent monooxygenase	C	4.0
BTO20_RS24130	phenol 2-monooxygenase	C	4.0
BTO20_RS25380	dimethylaniline monooxygenase	C	3.3
BTO20_RS16950	phenylalanine 4-monooxygenase	C	3.3

^a RNA sequence data were submitted to the GenBank database under accession number SRP116048.

^b Gene ID: locus_tag on GenBank.

^c Gene product: The upregulated four subunits of *prmABCD* were highlighted in bold.

^d Replicon: C, Chromosome; P3, Plasmid_3.

RT-qPCR experiments confirmed upregulation of the four subunits when treated with dioxane or its analog THF in comparison with glucose and acetate (Figure 10.5). Additionally, THF induced higher expression of *prmABCD* (1.5-2 times) than dioxane (Figure 10.5), which is consistent with the preferential consumption of THF when THF and dioxane were fed concurrently (Figure 10.6). This preferential utilization was also corroborated by an aquifer microcosm study.⁵⁴

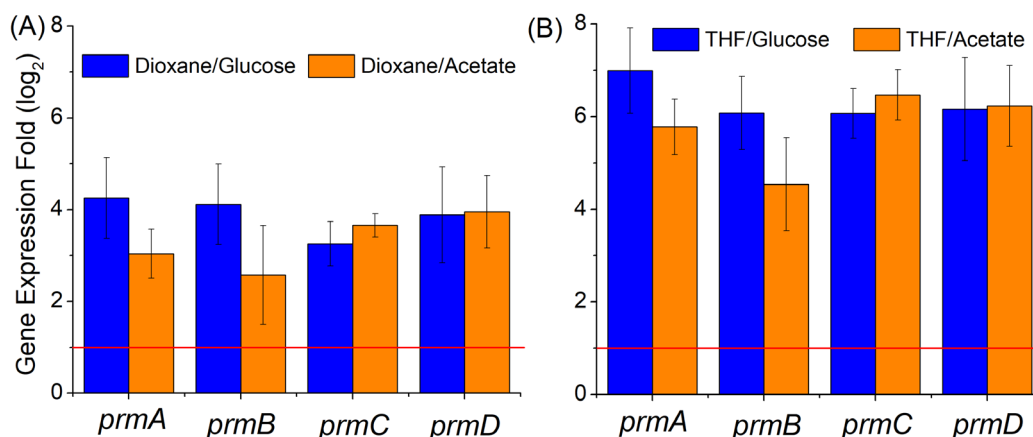


Figure 10. 5 Upregulation of *prmABCD* in PH-06 pure cultures grown on dioxane (A) and THF (B) relative to glucose or acetate.

16S rRNA gene of PH-06 was used as the housekeeping gene for error control among samples. The red horizontal lines indicate significant upregulation criteria (two-fold gene expression change).

10.3.3 *prmABCD* is the first identified group-6 SDIMO associated with dioxane degradation.

According to the classification rules of SDIMOs, which are based on the number and arrangement of subunits, substrate specificity, and sequence similarity,⁴³ *prmABCD* is a group-6 SDIMO. The amino acid sequence alignment of the large hydroxylase subunit (*prmA*) with other SDIMOs shows that this gene cluster is significantly different and phylogenetically distant from the *thmADBC* gene cluster (Figure 10.7). This explains the absence of the *thmA* biomarker¹⁶ in *Mycobacterium dioxanotrophicus* PH-06 by qPCR (data not shown). Though another *Mycobacterium* (i.e., ENV421) also harbors a group-6 SDIMO (Figure 10.7), the association of this SDIMO with dioxane degradation was not confirmed by heterologous host gene studies.²²⁹ To our best knowledge, *prmABCD* is the only identified group-6 SDIMO associated with dioxane degradation in a pure bacterial strain. This suggests that dioxane degrading genes are more diverse than previously appreciated, and it provides a basis for minimizing false negatives when looking for them at contaminated sites.

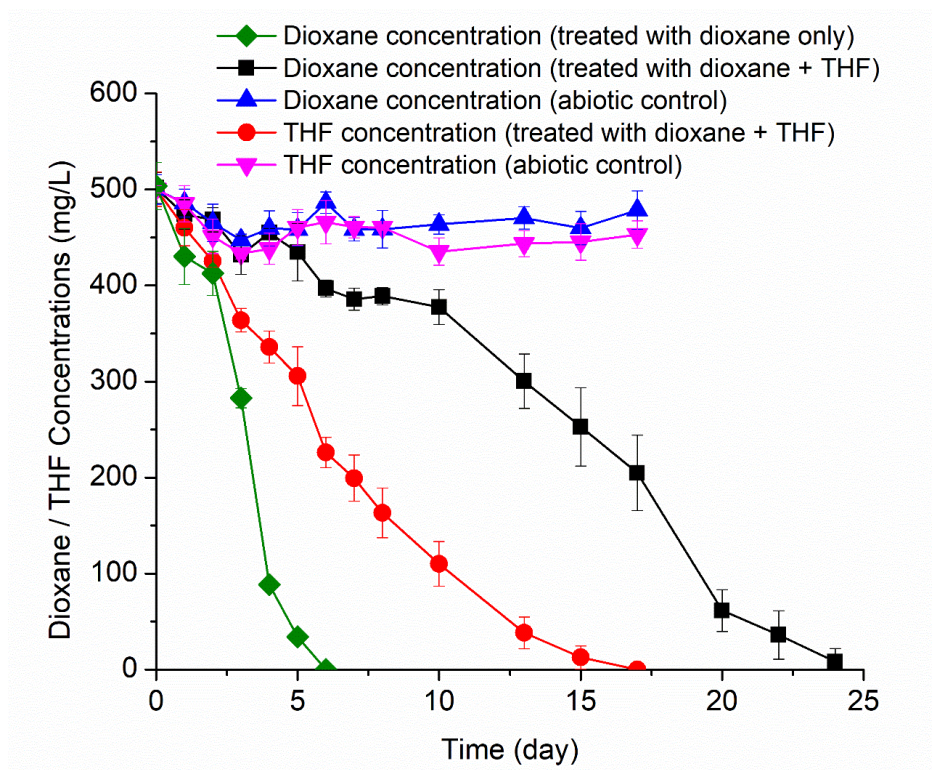


Figure 10. 6 Inhibition of dioxane degradation by THF.

Dioxane was degraded much faster when present alone than with THF. When dioxane and THF were added concurrently, the degradation of dioxane was inhibited initially, but after THF was consumed (80% degraded to 100 mg/L), dioxane was more rapidly degraded.

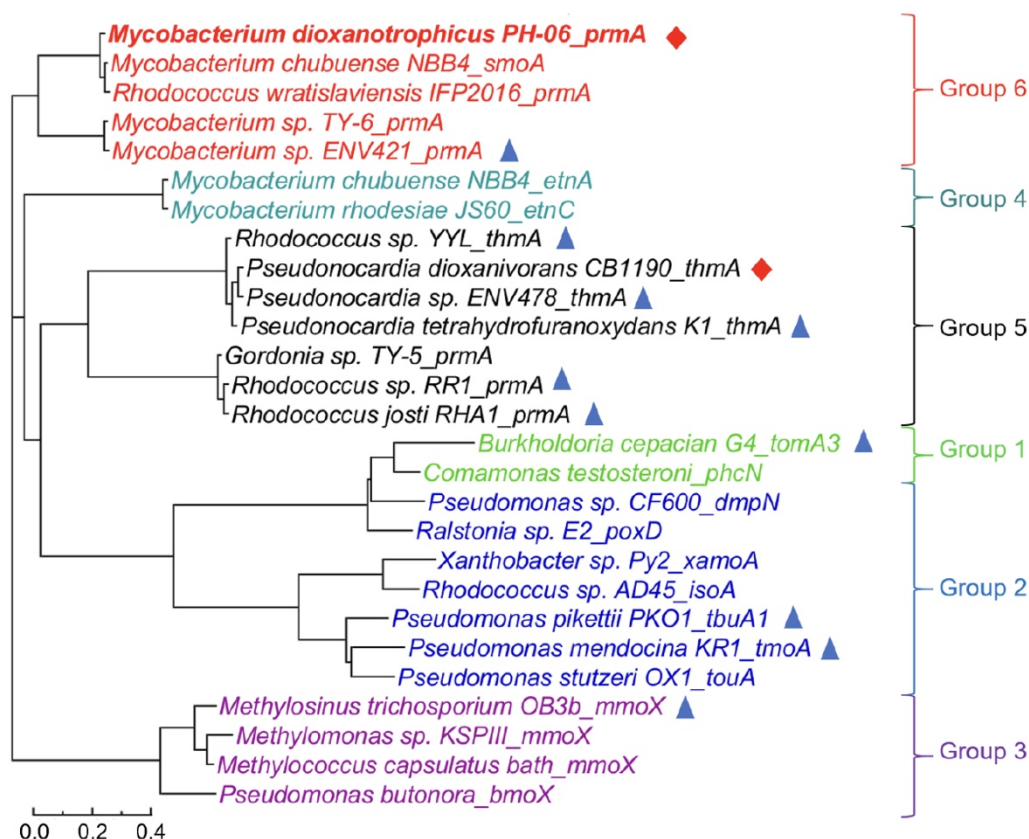


Figure 10. 7 Neighbor-joining tree showing the phylogenetic relationship of the large hydroxylase subunit of *prmABCD* with other SDIMOs.

The tree was generated on TreeGraph2 based on the alignment of amino acid sequences. Only the strains marked with a red diamond or a blue triangle are confirmed to degrade dioxane metabolically or cometabolically, respectively. The scale bar indicates the average number of amino acid substitutions per site. Group numbers to which these SDIMOs belong to are indicated on the right.

Chapter 11

Identification of a new biomarker probe to assess bioremediation and natural attenuation potential in dioxane contaminated sites

11.1 Introduction

Monitored natural attenuation (MNA) is among the most cost-effective approaches to manage groundwater contamination by organic pollutants at low concentrations. However, the feasibility of MNA requires demonstration of site-specific biodegradation capabilities. Previous studies suggest that indigenous bacteria that can degrade dioxane might be more widespread than previously assumed, and the enrichment of two consortia in our study also suggested the widespread presence of dioxane degraders.¹⁷ Monitoring dioxane concentrations to show consistent removal over time can be a strategy to indicate the potential efficacy of MNA, although this represents a lengthy and labor intensive process that may be confounded by dioxane dispersion and dilution. Other microbial molecular techniques to assess expression of dioxane degradation capabilities, such as cloning, microarray can also be labor intensive and (because of complex mass transfer limitations) may not provide unequivocal evidence to link the abundance of the indigenous degraders to the intrinsic biodegradation activity.

Numerous catabolic and phylogenetic biomarkers have been tested to assess biodegradation of different contaminants (e.g., aromatic oxygenase, phenol hydroxylase, anaerobic toluene monooxygenase).⁹⁰ Though selectivity and sensitivity can be highly variable for different probes and matrices, biomarkers offer a relatively straightforward approach to delineate *in situ* biodegradation potential.

Previously, a genetic primer/probe set biomarker (*thmA*) was developed to target the large hydroxylase subunit of tetrahydrofuran (THF) monooxygenase. It was found that there was a good correlation between the copy numbers of that *thmA* biomarker with the dioxane degradation rates in several dioxane impacted sites.¹⁶ Though this biomarker does not give false positive results as far as we know, it may give false negatives. For example, it cannot be used to detect the dioxane degradation potential of PH-06 and our two enriched consortia.^{40, 230} Also, a study shows that propane-like monooxygenase besides this THF monooxygenase can also be involved in dioxane degradation.⁵⁰ With the identification of a new catabolic gene cluster in PH-06, a new biomarker (primer/probe set) can be designed to assess dioxane natural attenuation potential to minimize false negatives by our previous *thmA* biomarker.

Here, we use the sequence information of our newly identified gene cluster to develop a new primer/probe biomarker set targeting *prmA* gene, assess its sensitivity and selectivity, and explore the correlation between the abundance of this catabolic biomarker and dioxane degradation activity in several enrichments. This effort enables the determination of the site-specific feasibility of MNA for dioxane plumes and enhances performance assessment.

11.2 Materials and Methods

11.2.1 Design of the *prmA* primer/probe set

Multiple sequence alignment (Clustal X 2.1) was used to align the amino acid sequences of the large hydroxylase subunits of this *prmABCD* and other SDIMOs. The phylogenetic tree based on amino acid sequences was then visualized using MEGA 5.1.⁹⁹ The primer/probe set targeting the hydroxylase subunit (*prmA*) was designed based on the following criteria: 1) avoid the di-iron centers (i.e. DE*RH motif) conserved by all SDIMOs because they are the active site for hydroxylation or peroxidation reactions; and 2) target the surrounding hydrophobic residues because they may be associated with the substrate recognition and binding.⁴³ The amino acid residues selected for this new biomarker design were marked in Figure 11.1. Partial DNA sequence (from 200 to 600) of the *prmA* gene of PH-06 was used as the input sequence in Primer Quest (Integrated DNA Technologies, Coraville, IA) to generate a series of possible primer/probe sets that satisfied the design criteria for TaqMan assays. After manual comparison and adjustment, the final primer/probe set was chosen (Table 11.1). The amplicon was 103 bp in length. All primers and probes were synthesized by Integrated DNA Technologies, and a novel internal quencher ZEN was integrated to reduce background noise.

Group	Bacterial strain	Gene	
1	<i>Comamonas testosteroni</i>	<i>phcN</i>	P L E Y Y A H R G F A H A G R H F T G A G A R V A A Q M Q S I D E L R H F Q T E T H A L S N Y
	<i>Burkholderia cepacia</i> G4	<i>tomA3</i>	P L E Y M A H R G F A H I G R H F T G E G A R V A C Q M Q S I D E L R H F Q T E M H A L S H Y
	<i>Ralstonia</i> sp E2	<i>poxD</i>	P L E Y A A H R G Y A H L G R H F R G A G A R V A A Q M Q S I D E L R H A Q T Q L H T L S V Y
2	<i>Pseudomonas</i> sp. CF600	<i>dmpN</i>	P L E Y Q A F Q G G F S R V G R Q F S G A G A R V A C Q M Q A I D E L R H V Q T Q V H A M S H Y
	<i>Xanthobacter</i> sp. Py2	<i>xamoA</i>	M V E H M A V T M Q S R F V R F A P S A R W R S L G A F G M L D E T R H T Q L D L R F S H D L
	<i>Rhodococcus</i> sp. AD45	<i>isoA</i>	M V E H M A V T M Q S R F C R F A P T P R W R N L G V F G M L D E T R H T Q L D L R F S H D L
	<i>Pseudomonas mendocina</i> KR1	<i>tmoA</i>	V G E Y A A V T G E G R M A R F S K A P G N R N M A T F G M M D E L R H G Q L Q L F F P H E Y
	<i>Pseudomonas pikettii</i> PKO1	<i>tbaA1</i>	L G E Y A A M S A E A R M A R F G R A P G M R N M A T F G M L D E N R H G Q L Q L Y F P H D Y
	<i>Pseudomonas stutzeri</i> OX1	<i>tauA</i>	L E E Y A A S T A E A R M A R F A K A P G N R N M A T F G M M D E N R H G Q I Q L Y F P Y A N
3	<i>Methylosinus trichosporium</i> OB3b	<i>mmoX</i>	V G E Y N A I A A S A M L W D S A T A A E Q K N G Y L A Q V L D E L R H T H Q C A F I N H Y Y
	<i>Methylomonas</i> sp. KSP111	<i>mmoX</i>	V G E Y N A I A A T G M L W D S A T A P E Q K N G Y L G Q V L D E L R H T N Q C A Y I N Y Y F
	<i>Methylococcus capsulatus</i> bath	<i>mmoX</i>	V G E Y N A I A A T G M L W D S A Q A A E Q K N G Y L A Q V L D E L R H T H Q C A Y V N Y Y F
	<i>Pseudomonas butanora</i>	<i>bmoX</i>	T G E Y G A I A G S A L L W D T A Q S P E Q R N G Y L A Q V I D E L R H V N Q T A Y V N Y Y Y
4	<i>Mycobacterium chubuense</i> NBB4	<i>etnC</i>	D A E Y Q A V C G S G F L I A S M K N Q E L R Q G Y A G Q M L D E V R H T Q I E V A L R K Y Y
	<i>Mycobacterium rhodesiae</i> JS60	<i>etnC</i>	D A E Y Q A T C G S G F L I A S M K N Q E L R Q G Y A G Q M L D E V R H T Q I E V A L R K Y Y
	<i>Pseudonocardia dioxanivorans</i> CB1190	<i>thmA</i>	S A E A A A T R C M G M L V D A L D D P E L Q N A Y Y I Q Q L D E O R H T A M Q M N L Y R W Y
5	<i>Pseudonocardia</i> sp. ENV478	<i>thmA</i>	S A E A A A T R C M G M L V D A I D D P E L Q N A Y Y I Q Q L D E O R H T A M Q M N L Y R W Y
	<i>Rhodococcus</i> sp. YYL	<i>thmA</i>	S A E A A A T R C M G M L V D A I D D P E L Q N A Y Y I Q Q L D E O R H T A M Q M N L Y R W Y
	<i>Pseudonocardia tetrahydrofuranoxydans</i> K1	<i>thmA</i>	S A E A A A T R C M G M L V D A L D D P E L Q N A Y Y I Q Q L D E O R H T A M Q M N L Y R W Y
	<i>Rhodococcus</i> sp. RR1	<i>prmA</i>	F P E I S A A R A M P M A I D A V P N P E I H N G L A V Q M I D E V R H S T I Q M N L K K L Y
	<i>Rhodococcus josti</i> RHA1	<i>prmA</i>	F P E I S A A R A M P M A I D A V P N P E I H N G L A V Q M I D E V R H S T I Q M N L K K L Y
	<i>Gordonia</i> sp. TY-5	<i>prmA</i>	F P E I S A A R A M P M A I D A V P N P E I H N G L A V Q M I D E V R H S T I Q M N L K K L Y
6	<i>Mycobacterium</i> sp. TY-6	<i>prmA</i>	F G E Y A A M K C C G Q L V D T V N N A E L R Q G Y M A Q M I D E V R H T N Q E L Y L N R Y F
	<i>Mycobacterium</i> sp. ENV421	<i>prmA</i>	F G E Y A A M K C C G Q L V D T V N A E L R Q G Y M A Q M I D E V R H T N Q E L Y L N R Y F
	<i>Mycobacterium chubuense</i> NBB4	<i>smaA</i>	F G E Y A A M K C T A M L V D T V N P E L R Q G Y L A Q M I D E V R H T N Q E A Y L M R Y F
	<i>Rhodococcus wratislaviensis</i> IFP 2016	<i>prmA</i>	F G E Y A A M K C T A M L V D T V N P E L R Q G Y L A Q M I D E V R H T N Q E A Y L M R Y F
	<i>Mycobacterium dioxanotrophicus</i> PH-06	<i>prmA</i>	F A E Y A A M K C T A M L V D T V N P E L R Q G F L A Q M I D E V R H T N Q E A Y L M R Y F

Figure 11. 1 Alignment of the amino acid sequences of the alpha hydroxylase subunits for different groups of SDIMO.

The amino acid residues selected for primers and probe design are marked in red and green boxes, respectively. The highlighted columns in black were 100% identity, including the di-iron center (i.e., DE*RH). Numbers below the sequences correspond to the numbering for PH-06.

Table 11. 1 Properties of the designed *prmA* primer/probe set.

Probe/Primer	Name	Sequence (5'-3')	Size	G+C (%)	T _m
Forward primer	<i>prmA</i> _307F	ACTGCGATGCTGGTTGAC	18	55.6	62
Reverse primer	<i>prmA</i> _410R	TCAGGTACGCCTCCTGATT	19	52.6	62
Taqman Probe	<i>prmA</i> _355P	/56-FAM/TTCCTCGCG/ZEN/ CAGATGATCGACG/3IABkFQ/	22	59	68

11.2.2 Specificity test with bacterial genomic DNA

Quantitative PCR (qPCR) by TaqMan assays was conducted on the genomic DNA (including chromosomal and plasmid DNA) extracted from reference strains (Table 10.2) to testify the specificity of this primer/probe set. After grown in AMS media with the addition of 500 mg/L 1-propanol at 30 °C with shaking at 150 rpm for 5 days, pure cultures of these strains were harvested by centrifugation, and their genomic DNA was extracted using an UltraClean Microbial DNA Isolation Kit (MoBio, Carlsbad, CA). The final DNA concentrations were measured by UV spectroscopy using an ND-1000 Spectrophotometer (NanoDrop, Wilmington, DE). The PCR mixture contained 1 µL of 1 ng/µL DNA (or a series of diluted DNA), 300 nM forward and reverse primers, 150 nM probe, 10 µL of TaqMan universal master mix II (Applied Biosystems, Foster City, CA), and DNA-free water, to a total volume of 20 µL. qPCR was performed in a CFX 96™ Real-Time System (Bio-Rad, Hercules, CA, USA) with the following temperature set up: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C 15 s and 60 °C for 1 min.

11.2.3 Dioxane degradation rates and sensitivity test

To assess the efficacy of the catabolic biomarker in enhancing the forensic analysis of MNA, we investigated the correlation of the abundance of this biomarker with the dioxane degradation rates of four consortia that were enriched from different origins. The dioxane degradation experiments with four consortia (Table 10.3) were conducted in 250-mL bottles amended with 100-mL AMS medium with an initial dioxane concentration of 500 mg/L, while shaking on a Digital Linear Shaker (Scilogex, Rocky Hill, CT, USA) at 150 rpm (T=30 °C, pH=7). For the dioxane impacted aquifer, it was a natural attenuation experiment (no nutrients were added). The aquifer is from a dioxane impacted site in Seattle, WA. The dioxane concentration was about 300 µg/L. The natural attenuation experiment was conducted at 15 °C to mimic the actual average groundwater temperature at this site. The genomic DNA from these consortia were used in qPCR assays processed as described above.

Table 11. 2 Specificity and coverage tests for the designed *prmA* primer/probe set.

Bacterial Strain	Dioxane degrader ^a	Gene name	Encoding enzyme ^b	SDIMO group	<i>prmA</i> detection ^c
<i>Mycobacterium dioxanotrophicus</i> PH-06	+	<i>prm</i>	Dioxane MO	6	+
<i>Rhodococcus wratislaviensis</i> IFP 2016	-	<i>prm</i>	Propane MO	6	-
<i>Mycobacterium</i> sp. ENV421	+	<i>prm</i>	Propane MO	6	-
<i>Mycobacterium chubuense</i> NBB4	-	<i>smo</i>	Propane MO	6	-
<i>Pseudonocardia tetrahydrofuranoxydans</i> K1	+	<i>thm</i>	Tetrahydrofuran MO	5	-
<i>Pseudonocardia dioxanivorans</i> CB1190	+	<i>thm</i>	Tetrahydrofuran MO	5	-
<i>Rhodococcus jostii</i> RHA1	+	<i>prm</i>	Propane MO	5	-
<i>Mycobacterium chubuense</i> NBB4	-	<i>etn</i>	Ethene MO	4	-
<i>Mycobacterium chubuense</i> NBB4	-	<i>smo</i>	Soluble methane MO	3	-
<i>Pseudomonas putida</i> CF600	-	<i>dmp</i>	Phenol HD	2	-
<i>Pseudonocardia dioxanivorans</i> CB1190	+	<i>phm</i>	Phenol HD	1	-
<i>Mycobacterium vanbaalenii</i> PYR-1	-	<i>nid</i>	PAH DO	-	-
<i>Pseudomonas putida</i> F1	-	<i>tod</i>	Toluene DO	-	-
<i>Escherichia coli</i> K-12	-	-	-	-	-
Bacteriophage λ	-	-	-	-	-

^a Plus and minus signs indicate whether a specific strain can degrade dioxane or not, respectively, including metabolism and cometabolism.

^bAbbreviations: MO, monooxygenase; HD, hydroxylase; DO, dioxygenase; PAH, polycyclic aromatic hydrocarbon.

^c Plus and minus signs indicate positive and negative detection, respectively.

Table 11. 3 Properties of the enriched consortia and dioxane impacted aquifer.

Name	Enriched source	Dioxane degrading rate (g dioxane/g protein/day)
Consortium A	Uncontaminated garden soil	7.1
Consortium B	Uncontaminated garden soil	5.7
Consortium C	Dioxane impacted site	3.02
Consortium D	Wastewater sludge	3.8
Dioxane impacted aquifer	-	0

11.2.4 Quantification of the *prmA* gene copies.

The genome size of PH-06 is 8.08 Mb²³¹ and DNA molecule weight is 9.12× 10¹¹ bp /ng of DNA [i.e., (6.022 × 10¹⁴ Da/ng of DNA)/(660 Da/bp)]. The gene copy numbers were calculated using the following equation:

$$\frac{\text{gene copies}}{\mu\text{L}} = \left(\frac{\text{ng of DNA}}{\mu\text{L}} \right) \times \left(\frac{9.12 \times 10^{11} \text{ bp}}{\text{ng of DNA}} \right) \times \left(\frac{\text{genome}}{8.08 \times 10^6 \text{ bp}} \right) \times \left(\frac{\text{gene copies}}{\text{genome}} \right)$$

The overall detection limit (DL) of *prmA* biomarker (in copy numbers/ ml of bacterial culture or aquifer materials) was determined by the following equation:

$$\text{Overall DL} = \frac{\text{qPCR instrument DL}}{\text{DNA recovery}} \times \text{Proportion of DNA used in qPCR reaction}$$

qPCR instrument DL is identified as the minimum detectable copy number when seven sequential analyses are successful.

Serial dilutions of the extracted genomic DNA of PH-06 were utilized to prepare the calibration curves (Figure 11.2) for *prmA* and 16S rRNA. The sequences (5'-3') of universal 16S rRNA primer/probe set are: Forward primer (CGGTGAATACGTTTCYCGG), Reverse primer (GGWTACCTTGTTACGACTT), and Probe (FAM-CTTGTACACACCGCCCGTC-BHQ1).²⁵¹

11.2.5 Statistical analysis

All treatments were conducted in triplicate. Statistical significance of differences between experimental treatments were assessed using two-tailed unpaired Student's *t*-test at the 95% confidence level.

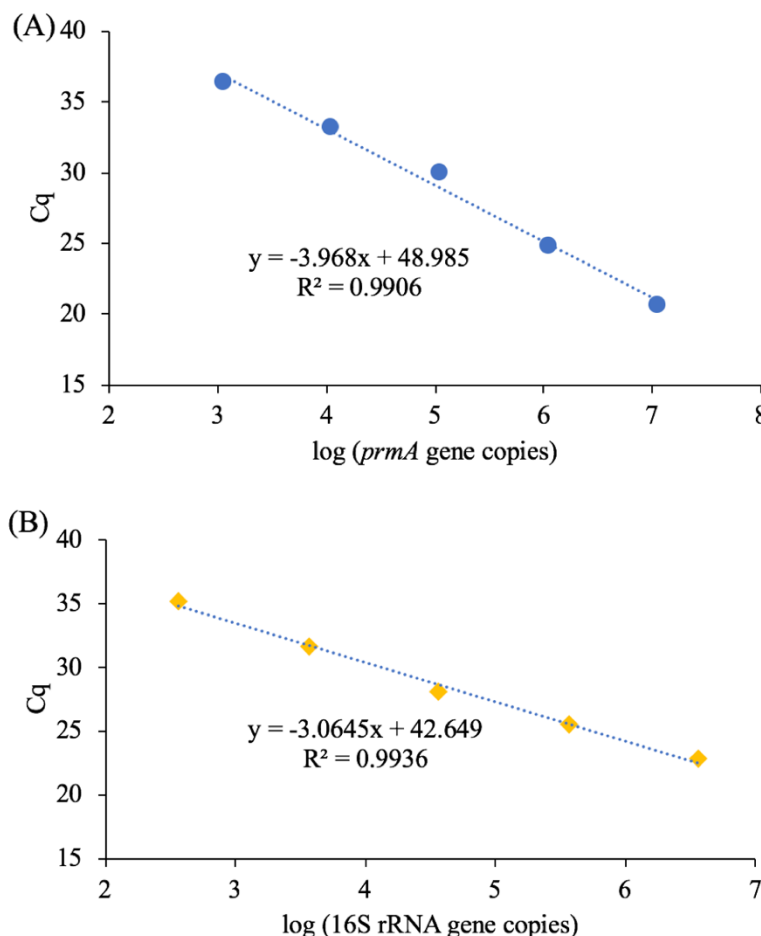


Figure 11. 2 qPCR calibration curves for *prmA* biomarker (A) and 16S rRNA genes.

11.3 Results and Discussion

11.3.1 The *prmA* primer/probe set is highly specific.

Biochemical, structural, and evolutionary studies indicate that the large hydroxylases of all the enzymes belonging to SDIMO family contain a highly conserved carboxylate-bridged di-iron center (i.e. DE*RH motif) that serves as the active site for the hydroxylation or peroxidation reactions.⁴³ However, different groups of SDIMOs exhibit different substrate specificity. Substrate recognition and binding may be primarily associated with the hydrophobic residues that surround the di-iron center because these are conserved within each SDIMO group.⁴³ Thus, the design of the primer/probe set targeting the large hydroxylase is based on two criteria: avoid the di-iron centers shared by all SDIMOs and targeting the surrounding hydrophobic residues only shared by *prmABCD* monooxygenase (Figure 11.1).

To make sure our designed primer/probe set not give false positives, we used qPCR to perform the specificity test. The negative controls include genomic DNA of bacteria containing different types of SDIMOs, bacteria with dioxygenase, and bacteriophage λ (Table 11.2). None

of the negative controls were detected by this *prmA* primer/probe set, indicating the high specificity of this biomarker.

11.3.2 The abundance of *prmA* is significantly correlated with the degradation activity of enriched consortia.

The performance of our primer/probe set was also tested with four dioxane degrading consortia (Table 11.3) where *Mycobacterium spp.* were dominant²³⁰. The specific degradation rates of these consortia were significantly correlated ($p < 0.05$) with the abundance of this *prmA* biomarker (indicative of the concentration of specific degraders harboring group-6 SDIMO genes), but not with 16S rRNA (indicative of total bacteria concentration) (Figure 11.3). Whereas caution should be exercised about correlations based on only four data points, the depicted 95% predictive intervals clearly suggest that this *prmA* biomarker (but not 16S rRNA) can be a valuable indicator of potential dioxane degradation activity. A sample from aquifer material (Table 11.3) impacted by dioxane but not exhibiting biodegradation activity was included as the negative control; consistently, the *prmA* biomarker was not detected (i.e., no false positive) (detection limit = 6000 – 7000 gene copies/ml aquifer).

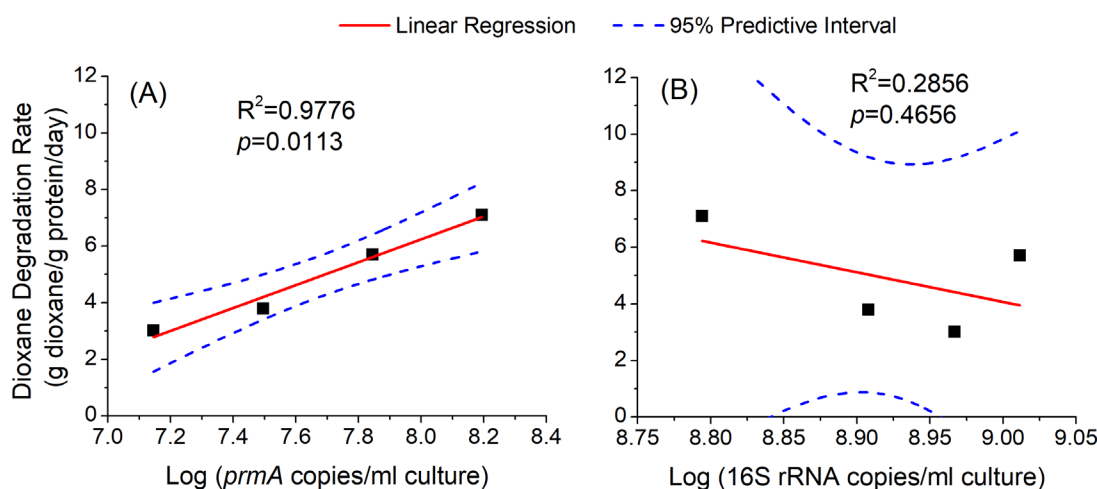


Figure 11. 3 Correlation between dioxane degradation rate and abundance of *prmA* (A) but not 16S rRNA (B) gene copies.

Experiments were conducted with microbial consortia enriched from soil or activated sludge.

While the abundance of the group-5 SDIMO *thmA* biomarker can also be positively correlated with dioxane biodegradation at some sites,¹⁶ this analysis may suffer from false negatives if the predominant dioxane degraders are *Mycobacterium* species, which we have found may be more widespread than previously appreciated.²³⁰ Thus, the new *prmA* biomarker would help reduce the occurrence of false negatives when assessing dioxane natural attenuation.

11.3.3 The *prmA* primer/probe set is highly sensitive.

This primer/probe set exhibited high sensitivity (detection limit = 3000 – 4000 gene copies/ml culture, which is within the commonly reported range for catabolic genes (Table 11.4), and no false positives, even with other oxygenase genes that bear a close evolutionary

relationship (Table 11.2). This indicates that we can use this biomarker to assess the dioxane degradation potentials of impacted sites.

Table 11. 4 Detection limit of various catabolic genes.

Biomarker	Targeted gene	Detection limit (reported)	Detection limit (converted) ^a	Reference
<i>prmA</i>	propane monooxygenase	$3 - 4 \times 10^3$ copies/ml	$3 - 4 \times 10^3$ copies/ml	This study
<i>dxmB</i>	dioxane monooxygenase	2×10^6 copies/ L	2×10^3 copies/ ml	252
<i>poxD</i>	phenol-2-monooxygenase	2×10^6 copies/ L	2×10^3 copies/ ml	252
<i>thmA</i>	dioxane monooxygenase	$7 - 8 \times 10^3$ copies/g of soil	$2.4 - 2.7 \times 10^4$ copies/ ml	16
<i>pmoA</i>	particulate methane monooxygenase	1.9×10^4 copies/g of soil	6.5×10^4 copies/ml	253
PAH- RHDα	PAH-ring hydroxylating dioxygenase	2×10^3 copies/g of soil	6.8×10^3 copies/ml	254
<i>nahAc</i>	naphthalene dioxygenase	5×10^3 copies/g of soil	1.7×10^4 copies/ml	255

^a Gene copies per g-soil were converted to copies per mL of pore volume by multiplying the former by the soil or aquifer material bulk density and dividing by its porosity. Assuming a bulk density of 1.7 g/ ml²⁵⁶ and a porosity of 0.5,²⁵⁷ the conversion factor is 3.4 g of soil/ ml of pore water.

Chapter 12

Insights to construct a reporter strain to assess dioxane degradation activity: Lessons learned and strategies to overcome technical challenges

12.1 Introduction

One common limitation to bioremediation and MNA is that dioxane concentrations at many impacted sites are relatively low ($\mu\text{g/L}$ level) and insufficient to induce and sustain dioxane degraders.²⁹ A common strategy to enhance bioremediation at such sites is biostimulation: adding substrates to stimulate the growth of degraders.^{258, 259} However, biostimulation may fail to enhance dioxane bioremediation if the added auxiliary substrates are preferentially degraded or results in catabolic plasmid curing.⁵⁴ Furthermore, some co-substrates (e.g., 1-butanol and THF) exert competitive inhibition on the required monooxygenases, which decreases dioxane degradation rates.⁵⁴ Thus, it is very important to select and optimize appropriate biostimulation strategies on a case by case basis, which would be facilitated by reporter strains that emit a signal when the appropriate catabolic genes are expressed.

Up to date, we have known two physiologically distinct mechanisms of two aerobic dioxane degraders; i.e., *Pseudonocardia dioxanivorans* CB1190 and *Mycobacterium dioxanotrophicus* PH-06 and the corresponding biomarkers were developed to target the genes responsible for the dioxane degradation.^{45, 46} However, to use the probes to monitor the gene expression levels, we have to extract the RNA and then conduct reverse transcription qPCR assays. The process of RNA extraction needs very special caution because it is very easy to degrade RNA during extraction and make RNA contaminated, and the RNA integrity will affect the assessment of following gene expression levels by qPCR.^{260, 261} For gram-positive bacteria, such as CB1190 and PH-06, the difficulty of RNA extraction would be much higher because of their thick cell-walls and the trend of growing to form clumps, which would inhibit the lysis of cells further during the first step of RNA extraction.³⁹ Usually, this reverse transcription qPCR method can only give us the snap-shot data, it would be very hard to monitor the real-time gene expression change. However, in many scenarios, we want to know how the dioxane degraders react with the environmental matrix timely and then take corresponding measures, which would require more convenient monitoring tools.

In contrast, monitoring gene expression and bacterial activities by a reporter strain is much more convenient.⁶⁰ Reporter strains are engineered by fusing a reporter gene, encoding a fluorescent or luminescent protein, downstream of the promoter of a gene of interest. When the reporter is expressed, it generates a signal that acts as a synthetic phenotype, enabling the study of physiologies that acts as a synthetic phenotype, enabling the study of physiologies that might have otherwise been hidden.⁵⁷ Reporter strain is a low-cost alternative for real-time analysis of gene expression. In fluorescent approaches, an external source of light is required for excitation

of the protein. In contrast, bioluminescent reporter proteins can produce light by using appropriate substrates.⁵⁸

In this chapter, we describe a strategy to quantify and monitor the expression of degradation activities by constructing a reporter strain. Briefly, we describe the construction of a gene cassette which contains the promoter of the dioxane degrading gene cluster,^{45, 46} followed by a reporter protein gene (e.g. GFP).⁶¹ This cassette would be inserted into dioxane degraders by transformation.²⁶² When the promoter of dioxane degrading gene cluster is induced, it should initiate the transcription of both the dioxane degrading gene cluster (leading eventually to dioxane degradation) and the reporter gene (leading to the emission of fluorescence), which can then be used to monitor gene expression levels in real-time.

Although this strain was not fully constructed, this effort advanced understanding of pertinent physiological challenges and provided insight into potentially feasible strategies to overcome them. Accordingly, we summarize the process of constructing the gene cassettes containing the essential parts to build the reporter strain and the challenges faced to integrate these components, along with strategies and preliminary results that encourage future efforts.

12.2 Materials and Methods

12.2.1 Bacterial strains, plasmids and culture conditions

Two archetype dioxane degraders, *Mycobacterium dioxanotrophicus* PH-06 and *Pseudonocardia dioxanivorans* CB1190³⁹ are used as the host to construct the report strains. Both PH-06 and CB1190 were grown in an Ammonium Mineral Salts (AMS) medium at 30 °C, pH =7, amended with dioxane as the carbon and energy source to achieve a final concentration of 500 mg/L. *Mycobacterium smegmatis* MC²-155 (ATCC 700084)²⁶³ and *E. coli* 10G chemically competent cells (Lucigen) were used to construct and test the gene cassettes. Both bacteria are grown in LB medium at 37 °C, pH =7. All bacterial cultures were incubated aerobically, while shaking at 120 rpm.

The plasmids used in this study included three *Mycobacteria-E.coli* shuttle vectors, pCharge3 (6309 bp), pUS116 (6635 bp), and pALACE (8221bp), bought from Addgene (www.addgene.org). Specifically, pCharge3 is a low copy number plasmid and uses Hygromycin as the selection marker, and the other two are high copy number plasmids and use Kanamycin and Hygromycin as the selection markers, respectively. Additionally, pUC19 plasmid, which harbors a sfGFP gene⁶¹ and a tetracycline promoter²⁶⁴ and uses ampicillin as the selection marker, was also used during our study.

12.2.2 Bioinformatic analysis and selected genetic regions

The promoters of two dioxane degrading gene clusters, i.e., tetrahydrofuran/dioxane monooxygenase (*thmADBC*) from CB1190 and propane/ dioxane monooxygenase (*prmABCD*) from PH-06)^{45, 46} were used to construct promoter-reporter gene cassettes. For *thmADBC*, the inter-genetic region (613 bp) between Psed_6973 gene (encoding Hypothetical Protein) and Psed_6974 gene (encoding Ethyl tert-butyl ether degradation *EthD*) is amplified by PCR as a whole to be used as the promoter of *thmADBC* (Figure 11.1). A promoter prediction platform,

BPROM platform from Softberry (Softberry, Inc., Mt. Kisco, NY <http://www.softberry.com>), indicated the existence of two promoters in this intergenetic region (Figure 11.1). In the following, *thmP* will be used to represent the promoter of *thmADBC*. For *prmABCD*, the intergenetic region (303 bp) between BT020_RS38205 gene (encoding a two-component system response regulator, complement) and BT020_RS38210 gene (*prmA* gene) is amplified by PCR as a whole to be used as the promoter of *prmABCD*. Though the BPROM promoter prediction platform does not specify a specific promoter, it ensures that this region contains the promoter of *prmABCD* because the two genes in the upstream and downstream of this intergenic segment are in two different directions. In the following, *prmP* will be used to represent the promoter of *prmABCD*. Besides, a constitutive promoter (*thiC*, 200 bp), which is the promoter of Psed_6168 gene (encoding phosphomethylpyrimidine synthase *thiC*) in CB1190, and has been used as a housekeeping gene reference in another study,⁴⁶ is used as the positive control in our study.

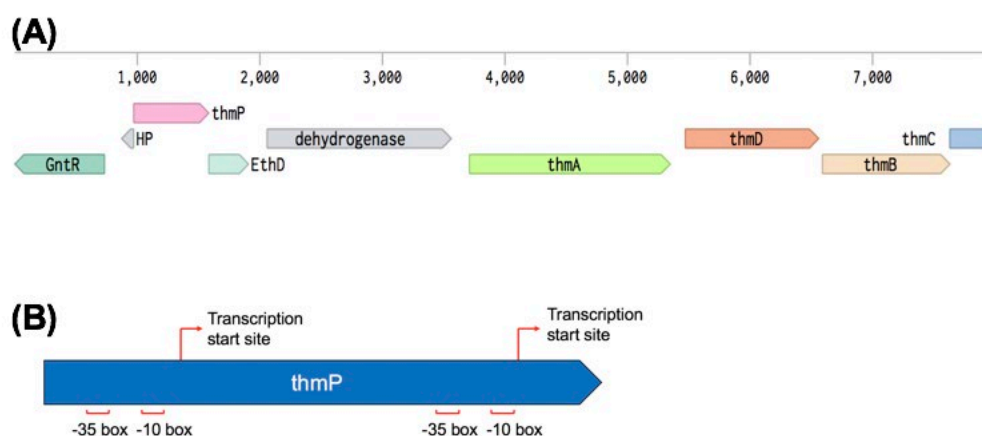


Figure 11. 4 Scheme of *thmADBC* gene cluster (A) and the predicted transcription start sites (B). Abbreviation (from left to right): *GntR* (Psed_6972 gene *GntR* transcription factor); *HP* (Psed_6973 gene Hypothetical Protein); *thmP* (Promoter of *thmADBC*); *EthD* (Psed_6974 gene Ethyl tert-butyl ether degradation *EthD*); dehydrogenase (Psed_6975 gene Betaine-aldehyde dehydrogenase); *thmA* (Psed_6976 gene *thmA* monooxygenase); *thmD* (Psed_6977 gene *thmD* monooxygenase); *thmB* (Psed_6978 gene *thmB* monooxygenase); *thmC* (Psed_6979 gene *thmC* monooxygenase). -10 box and -35 box represent the consensus sequence in the upstream of transcription start site, which is important in the initiation of transcription.

12.2.3 Cloning methods

Restriction cloning is used to construct the promoter-reporter gene cassettes. Briefly, our interested gene segments were amplified separately with primers containing restriction enzyme sites at two ends. The details of main genes, restriction enzymes, and primers used in this study is shown in Table 12.1. After amplification, the PCR products were checked by electrophoresis and the correct bands were cut to be purified by a gel extraction kit (OMEGA). Then the purified PCR products and the corresponding plasmids were digested separately by restriction enzymes following the manufacturer's protocol (New England Biolabs, www.neb.com). After digestion, electrophoresis was used to check the correct bands. The correct bands were cut and purified again as before. Then, T4 DNA ligase (New England Biolabs) was used to join the

purified inserts and vectors. *E. coli* 10G chemically competent cells (Lucigen) were used as the cloning host. The transformation method is the same as the manufacturer's protocol (Lucigen). After incubation overnight, Colony PCR checking and sequencing were used to verify the correctness of cloning. The correct clone was grown in LB medium with selective antibiotics and plasmids were extracted using a plasmid extraction kit (ZR Plasmid Miniprep™, Zymo Research) in the exponential growth phase. The extracted plasmids were used to transform into *Mycobacterium* hosts (i.e. *Mycobacterium dioxanotrophicus* PH-06 and *Mycobacterium smegmatis* mc² 155) by electroporation²⁶² or to build the transposons to further introduce into *Pseudonocardia dioxanivorans* CB1190 through transposon mutagenesis.²⁶⁵

Table 12. 1 Main genes, primers and restriction enzymes used in this study.

Gene	Primers and Restriction Enzymes
<i>thmP</i>	F: 5'-TTATCAGGATCCCACCTGCCCTCCGTTGCGACTT-3' (BamHI) R: 5'- ATACATGGTACCTTTCTCCTCTTTAATGAATTCAGTCCAGCCTCCGAGTCTCC-3' (KpnI)
<i>sfGFP</i>	F: 5'-ATGCAAGGTACCATGCGTAAA-3' (KpnI) R: 5'-TTGCATGAGCTCTCATCATTTG-3' (SacI)
<i>thicP</i>	F: 5'-TTATCAGGATCCTGGGAGCTGCGCGCAGGAGT-3' (BamHI) R: 5'-ATACATGGTACCTTTCTCCTCTTTAATGAATTCGTGACGTCCCTCCCTCGCC- 3'(KpnI)
<i>GntR</i>	F: 5'-AAGTCTGGTACCATGAAGGGCGTTTCGA-3'(KpnI) R: 5'-TGCATTGCTAGCTCAACCGTCGACGCCTG-3'(NheI)
<i>prmP</i>	F: 5'-ATGCTCTAGACTGCACGCGCGCGCGCCTTC-3' (XbaI) R: 5'- GTAGGATCCTTGGGTTGTGACGCCGGTGCCGA-3' (BamHI)

Note:

F represents forward primer, and R represents reverse primer;

Restriction enzymes are highlighted by underline and their names are appended in the end of each primer in parentheses.

12.2.4 Transformation strategies

As there is no *Pseudonocardia-E.coli* shuttle vector available, we used another strategy called transposon mutagenesis to attempt to introduce the promoter-reporter gene cassette into CB1190 cells. Briefly, we were following the protocols of EZ-Tn5™ Transposase (Cat.No. TN92110, Lucigen).²⁶⁵ First, we amplified the cassettes with forward and reverse primers containing ME (Mosaic Ends) sequences (5'-CTG TCT CTT ATA CAC ATC T - 3') in the end. To optimize the transposition efficiency, we added a 5'-phosphate to both PCR primers. Then we incubated the PCR DNA with EZ-Tn5™ Transposase and glycerol for 30 min at room temperature. Then 1µL of the incubated solution was used for electroporation into competent cells. The specific electroporation process and the preparation of the competent cells were the same as described in the literature.²²¹

The introduction of the plasmids containing the promoter-reporter gene cassettes into *Mycobacterium dioxanotrophicus* PH-06 and *Mycobacterium smegmatis* MC²-155 is through

electroporation following the typical *Mycobacterium* electroporation protocols.²⁶² Briefly, cells were grown to the exponential phase in AMS medium amended with 500 mg/L dioxane (for PH-06) or in LB medium (for MC²-155) and harvested by centrifugation for 10 min at room temperature. Then cells were washed in ice-cold 10% glycerol for three times. 0.5–5 µg of plasmid DNA in no more than 5 µL volume was added to 0.2 mL cell suspension and left on ice for 10 min. The incubated solution was transferred to a 0.2 cm electrode gap electroporation cuvette. The electroporation was conducted with a single pulse of 2.5 kV, 25 µF, with the pulse-controller resistance set a 1000 Ω resistance. Then cell suspension was transferred to LB medium and incubated at 37 °C for 2 to 3 h. Then the cells were harvested by centrifugation at 3000 × g for 10 min and plated out on LB agar with appropriate antibiotics. The plates were incubated at 37 °C until colonies become visible. Colony PCR and sequencing were used to check the correctness of transformation.

12.2.5 Fluorescence measurement

The fluorescence signal was measured by two methods: Fluorescent Activated Cell Sorting (FACS) and Microplate readers. FACS was performed using an FACS ArialII Flow Cytometer (BD Biosciences, San Joes, CA) equipped with red laser (wavelength of 640 nm), a violet laser (wavelength of 405 nm), yellow-green laser (wavelength of 561 nm), a blue laser (488 nm wavelength), and an UV laser (wavelength of 355 nm) controlled by a computer equipped with the software BD FACS Diva 8.0 (BD Bioscience, San Jose, CA). Sheath pressure was set to 70 psi, and nozzle size was 70 µm. Forward scatter (FSC), side scatter (SSC), and blue (450 ± 25 nm, DAPI) and green fluorescence (530 ± 15 nm, Alexa Fluor 488) signals were measured and analyzed in logarithmic amplification mode. Cell sorting was performed on the same flow cytometer with the drop drive frequency set at 88.7 kHz/s. Each event falling within the gate AF488+ was sorted in a sort envelope of 1.2 drops at a rate of 1×10^2 to 1×10^3 events/s.

A Microplate reader (TECAN) was also used to test the fluorescence intensity in the solution. The excitation and emission wavelengths are 485 ± 9 nm and 510 ± 20 nm, respectively. Also, a fluorescence microscope (Olympus, Center Valley, PA) equipped with a ×100 oil immersion objective was also used to visually check the fluorescence.

12.3 Results and Discussion

12.3.1 The challenging transformation of the promoter-reporter cassette into CB1190 and PH-06 may be due to the cells' restriction-modification (R-M) self-defense systems.

The promoter-reporter cassettes (*thmp-sfGFP* and *thicp-sfGFP*) were constructed using pUC19 plasmid through restriction cloning. Primers with ME (Mosaic Ends) ends were used to amplify the cassettes to obtain PCR for transposon mutagenesis in CB1190 following the transposon mutagenesis protocol.²⁶⁵ After electroporation, the transformed cells were grown in LB medium (with no dioxane) and AMS medium with the addition of 100 mg/L dioxane (pH = 7, T = 30 °C), respectively. After five days of culturing, the cells were analyzed by FACS. However, there was no significant fluorescence difference between the transformed CB1190 cells and controls (Figure 11.2). At the same time, PCR assays targeting the promoter-reporter cassettes were performed to verify the introduction of the cassettes. However, PCR sequencing

indicated that there was no successful introduction of any promoter-reporter gene cassettes into CB1190 cells. Though different protocols of electroporation^{221, 266, 267} and the preconstructed EZ-Tn5™ <KAN-2> (Lucigen)²⁶⁵ were attempted, no any successful transformation of CB1190 cells was observed.

The challenge of the electroporation of CB1190 is widely recognized. Alvarez-Cohen et al. performed electroporation of CB1190 using seven different gram-positive plasmids containing different antibiotic resistance genes, but no any antibiotic resistant transformant of CB1190 was obtained after many attempts.²⁶⁷ Plasmid pNV18, which was successfully transformed into *Pseudonocardia* sp. ENV 478,²²¹ was also tried to introduce into CB1190, but still no any successful transformation of CB1190 was observed. However, when the same procedure of electroporation was performed in *E. coli*, successful transformation was observed, and plasmid contains *thiCP-sfGFP* cassette (which was used as a positive control) expressed the green fluorescence (Figure 11.2).

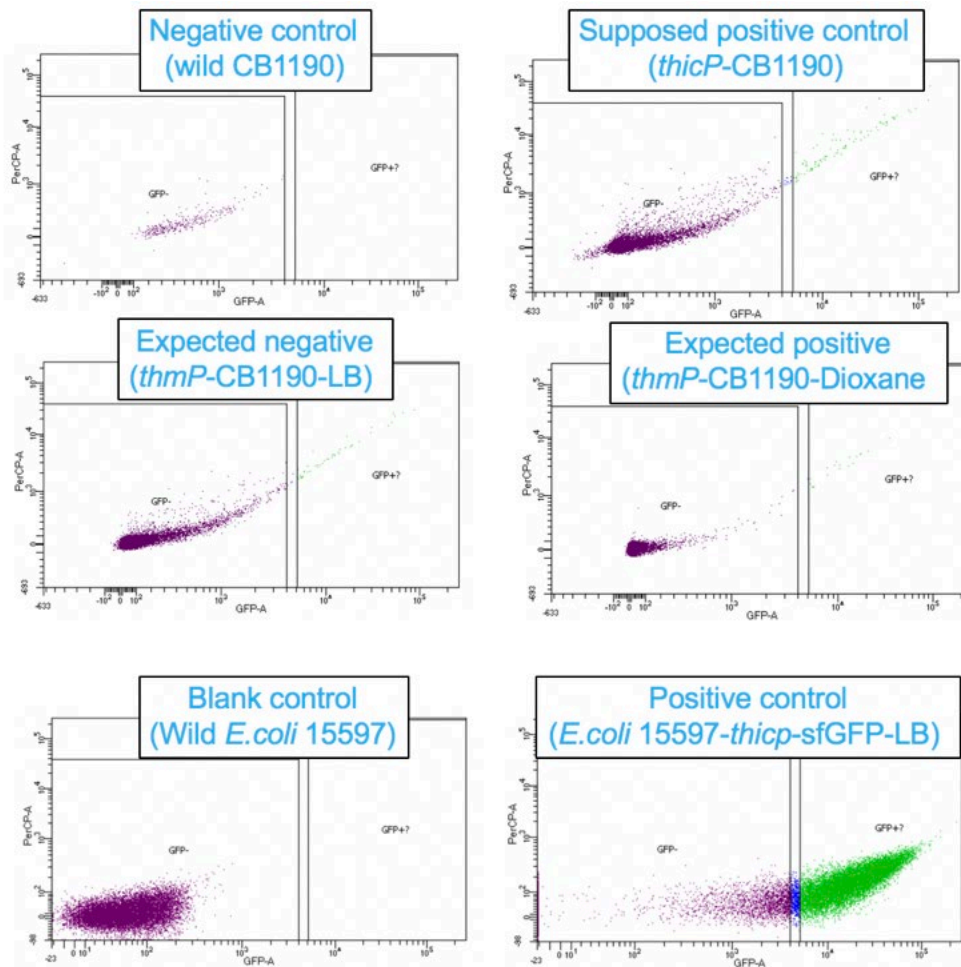


Figure 12. 1 Flow cytometry and sorting results for CB1190 transformed with the promoter-reporter gene cassette.

The results show that there is no significant fluorescence difference between the negative control, positive control, and experiments. The negative control is the wild CB1190 with no transformation, and the supposed positive control is CB1190 transformed with the *thiCp-sfGFP* cassette. The expected negative result is CB1190 transformed with the *thmP-sfGFP* cassette but was grown in LB medium and with no dioxane addition. The expected positive result is CB1190 transformed with the *thmP-sfGFP* cassette but was grown in AMS medium and with dioxane as the sole carbon source. However, when we perform the same electroporation in *E. coli*, we can get successful transformation and the *sfGFP* was expressed in LB with the existence of promoter *thiCp*.

The challenge to transform CB1190 with foreign DNA may be due to two reasons. First, CB1190 tends to aggregate in growth, and the clumping hinders the insertion of DNA into the cells further.²⁶⁸ Second, CB1190 may have some defense systems to prevent the introduction of foreign DNA. For example, some bacteria use a restriction-modification (R-M) system to degrade foreign DNA.²⁶⁹ R-M systems are ubiquitous and are often considered primitive immune systems in bacteria. They comprise two contrasting enzymatic activities: a restriction endonuclease (REase) and a methyltransferase (MTase). The REase recognizes and cleaves foreign DNA sequences at specific sites, while MTase ensures discrimination between self and nonself DNA, by transferring methyl groups to the same specific DNA sequence within the host's genome.²⁶⁹ R-M systems are classified mainly into four different types based on their subunit composition, sequence recognition, cleavage position, cofactor requirements, and substrate specificity.²⁷⁰ Among them, Type II enzymes are the most widely studied and are also extensively utilized nucleases in genetic engineering.²⁶⁹ By analyzing the genomic sequences of CB1190 *in silico* on REBASE (a database for DNA restriction and modification, <http://tools.neb.com/genomes/>),²⁷¹ three putative R-M operons were found in the chromosome and one putative R-M operon were found in plasmid pPSED02 (Figure 12.3 and Table 12.2), including three Type II MTase and one Type IV MTase. Though these bioinformatic results cannot identify the methylated positions, they help to explain the challenge of transformation foreign DNA into CB1190, both from us and other people. As far as we know, this is the first time to show the putative R-M systems in CB1190 and their effects on gene engineering.

Through analyzing the genomic data on REBASE (<http://tools.neb.com/genomes/>), the putative R-M operons in *Mycobacterium dioxanotrophicus* PH-06 (A), *Pseudonocardia dioxanivorans* CB1190 (B) and *Mycobacterium smegmatis* MC²-155 (C) were obtained. PH-06 contains a total of 14 R-M operons, including 11 in the chromosome, and two in Plasmid 1 and one in Plasmid 4; CB1190 contains two R-M operons in the chromosome, and one R-M operons in the plasmid pPSED02; MC²-155 only contains one R-M operon in the chromosome.

Table 12. 2 Restriction-Modification System in three bacterial strains.

<i>Mycobacterium dioxanotrophicus</i> PH-06				
Name ^a	Position ^b	ORFs ^c	Gene Product ^d	Type ^e
RM6670	C	BTO20_06675	Restriction endonuclease subunit R	II
		BTO20_06680	Restriction endonuclease subunit M	
RM6720	C	BTO20_06720	Very short patch repair endonuclease	II
		BTO20_06725	DNA cytosine methyltransferase	
RM6750	C	BTO20_06750	DNA (cytosine-5-)-methyltransferase	II
		BTO20_06760	Restriction endonuclease subunit R	
RM6770	C	BTO20_06770	Restriction endonuclease	II
RM10070	C	BTO20_10070	Restriction endonuclease subunit R	I
RM10125	C	BTO20_10120	Restriction endonuclease subunit S	I
		BTO20_10125	Restriction endonuclease subunit M	
RM11305	C	BTO20_11305	DNA cytosine methyltransferase	II
RM11315	C	BTO20_11315	DNA methylase	II
RM17845	C	BTO20_17840	Restriction endonuclease subunit R	II
		BTO20_17845	SAM-dependent methyltransferase	
RM20020	C	BTO20_20020	site-specific DNA-methyltransferase	III
		BTO20_20025	Restriction endonuclease subunit R	
RM20965	C	BTO20_20965	DNA adenine methylase	II
RM36760	P1	BTO20_36760	Restriction endonuclease subunit M	II
RM36795	P1	BTO20_36795	Restriction endonuclease subunit M	II
RM38685	P4	BTO20_38685	DNA methyltransferase	II
<i>Pseudonocardia dioxanivorans</i> CB1190				
Name	Position	ORFs	Gene Product	Type
RM103	C	Psed_0103	adenine-specific DNA methyltransferase	II
RM4066	C	Psed_4066	Restriction endonuclease subunit R	IV
RM4811	C	Psed_4811	DNA methylase	II
RM6888	pPSED02	Psed_6888	C-5 cytosine methyltransferase	II
<i>Mycobacterium smegmatis</i> MC ² -155				
Name	Position	ORFs	Gene Product	Type
RM3213	C	MSMEG_3213	SAM-dependent methyltransferase	II

Note:

a: Named based on the ORF ID;

b: C represents Chromosome, P represents Plasmid;

c: ORF ID in GenBank;

d: gene products based on GenBank annotation and NCBI blast;

e: four types of Restriction-Modification (RM) System.

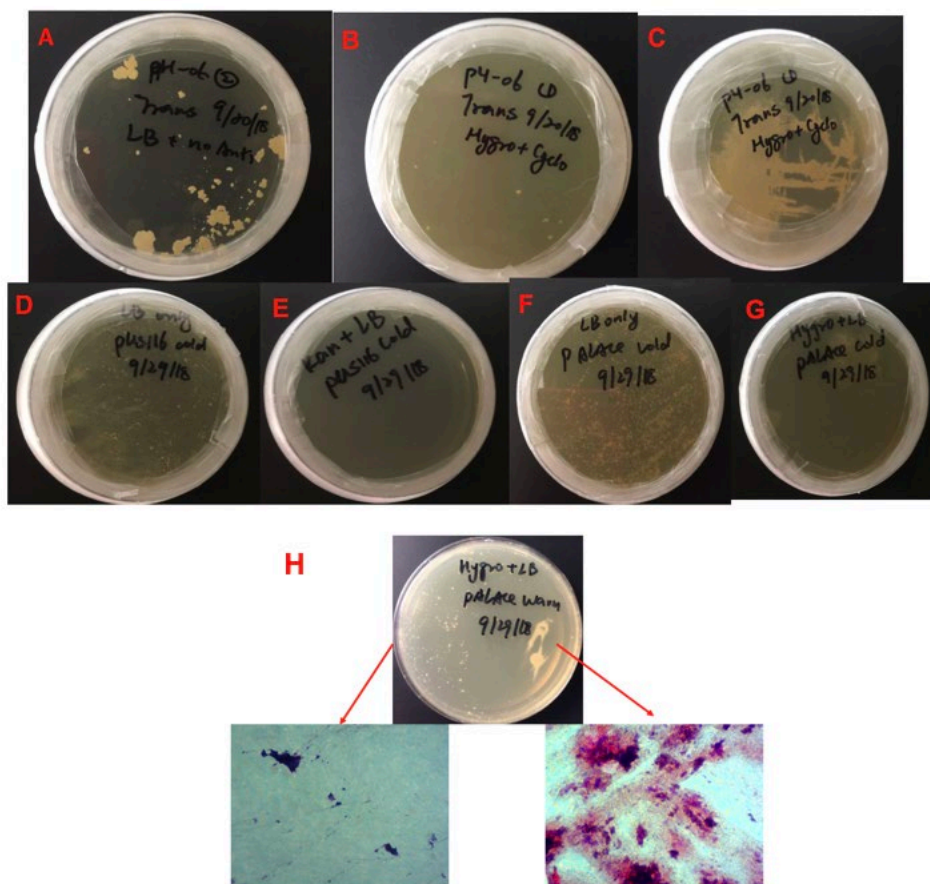


Figure 12. 3 Transformation of PH-06 with different plasmids.

Transformation of PH-06 with plasmid pCharge3: (A) plates with no antibiotics (control), after 10 days of transformation. The bacteria grew well after electroporation means that electroporation did not kill all cells; (B) plates with hygromycin as the selection marker, no any colony visible after 10 days; (C) plates with hygromycin as the selection marker, contaminated by gram-negative bacteria after 15 days of transformation. Transformation with plasmid pUS116 after six days with no antibiotics (control) (D) and with kanamycin as the selection marker (E). Transformation with plasmid pALACE after 6 days with no antibiotics (control) (F) and with Hygromycin as the selection marker (G). Transformation with plasmid pALACE after 20 days with Hygromycin as the selection marker (H), the left side was still PH-06 verified by 16S rRNA sequencing, while the right side was contaminated by gram-negative bacteria.

Unlike CB1190, PH-06 is a *Mycobacterium*, and several *Mycobacteria-E. coli* shuttle vectors are available for genetic engineering use. Three vectors (pCharge3, pUS116, and pALACE) were bought from Addgene. The typical electroporation protocol of *Mycobacteria* was used to conduct transformation experiments.²⁶² However, it is a great challenge to introduce these vectors into PH-06 cells (Figure 12.4). The protocols says it may take three to five days to see the colonies,²⁶² but no colony was observed even after 10 days on plates with antibiotics as the selection markers. In contrast, colonies appeared in the control plates with no antibiotics after five days (Figure 12.4). It is observed that the plates were often dominated by gram-negative bacteria after long time incubation. The same phenomenon was also reported as a common thing in other studies. And this is because that antibiotics may lose their effects after long time incubation.²⁶² Interestingly, after more than 20 days, several colonies were observed on the left

side of the plate transformed with pALACE plasmid. However, the right side of the same plate was still dominated by some gram-negative bacteria. Despite many attempts, the successful transformation result cannot be replicated.

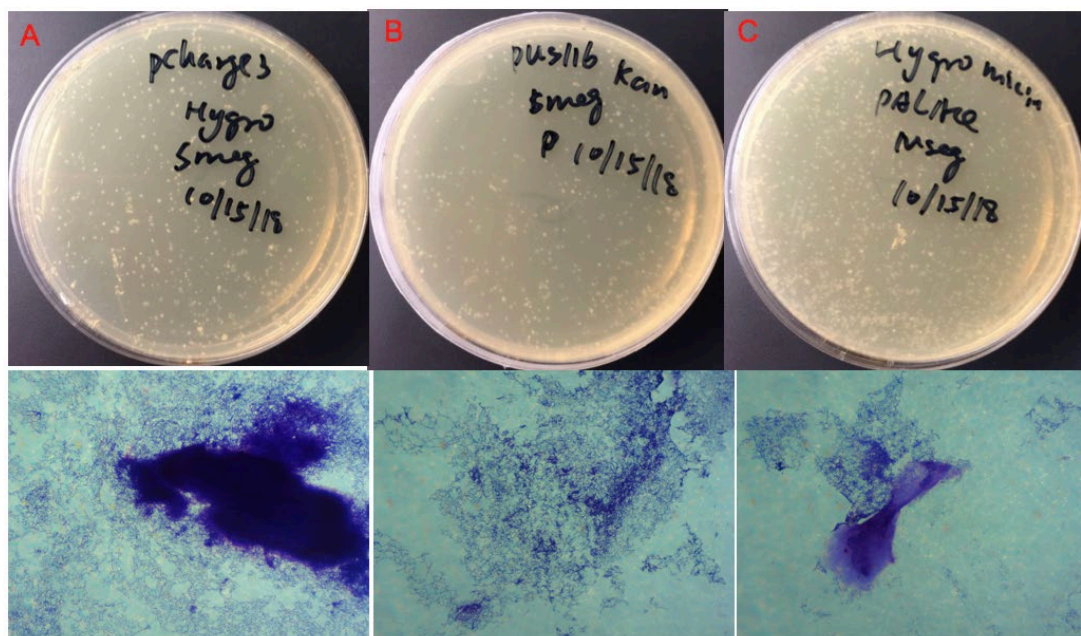


Figure 12. 4 Transformation of *Mycobacterium smegmatis* MC²-155 with three plasmids: pCharge3(A), pUS116(B) and pALACE(C).

The transformation was conducted exactly the same as PH-06. After six days, many visible colonies were present on the plates containing appropriate antibiotics. The Microscopy and 16S rRNA sequencing both confirmed the successful transformation of plasmids into *Mycobacterium smegmatis* MC²-155.

In contrast, the transformation of another *Mycobacterium* strain, *Mycobacterium smegmatis* MC²-155, was much easier and very successful, as verified by Microscopy and 16S rRNA sequencing (Figure 11.5). The transformation efficiency difference between these two *Mycobacteria* strains may be due to four reasons: 1) PH-06 aggregates much more than MC²-155 in growth, which makes it harder for electroporation; 2) PH-06 grows much more slowly and needs long time to see the colonies (the generation time of PH-06 is 24 h at 30°C, pH = 7 in R2A medium,²³⁰ while the generation time of MC²-155 is 3 to 4 h.²⁷² But the long-time incubation in the process of transformation leads the loss of antibiotic selection effect and other bacteria especially gram-negative bacteria will dominate; and 3) PH-06 has four plasmids while MC²-155 does not contain any plasmid. The plasmid incompatibility may affect the transformation of a foreign plasmid;²⁷³ 4) PH-06 has other defense systems (e.g. Restriction Modification (R-M) system) to prevent the invasion of foreign DNA.²⁷⁴ Similar to CB1190, the R-M systems of PH-06 and MC²-155 were analyzed on REBASE database (Figure 12.3 and Table 12.2). 14 putative R-M operons are found in PH-06 (11 in chromosome, and two in Plasmid 1 and one in Plasmid 4, including Type I, II and III), while only one putative Type II R-M operon is found in MC²-155. This considerable difference between PH-06 and MC²-155 would help to explain their difference of transformation efficiency.

Interestingly, uptake of plasmid pCharge3 containing the *thicP-sfGFP* cassette into MC²-155 led to the detection of fluorescence compared with transformation of plasmid pCharge3 that does not contain the *thicP-sfGFP* cassette (Figure 11.6). Understanding the regulation of the promoter of dioxane degrading gene cluster would facilitate cloning the dioxane degradation gene cluster and the promoter-reporter gene cassette into MC²-155 to develop a reporter strain for dioxane degradation. Accordingly, *Mycobacterium smegmatis* mc²-155 may be a good candidate to build a reporter strain.

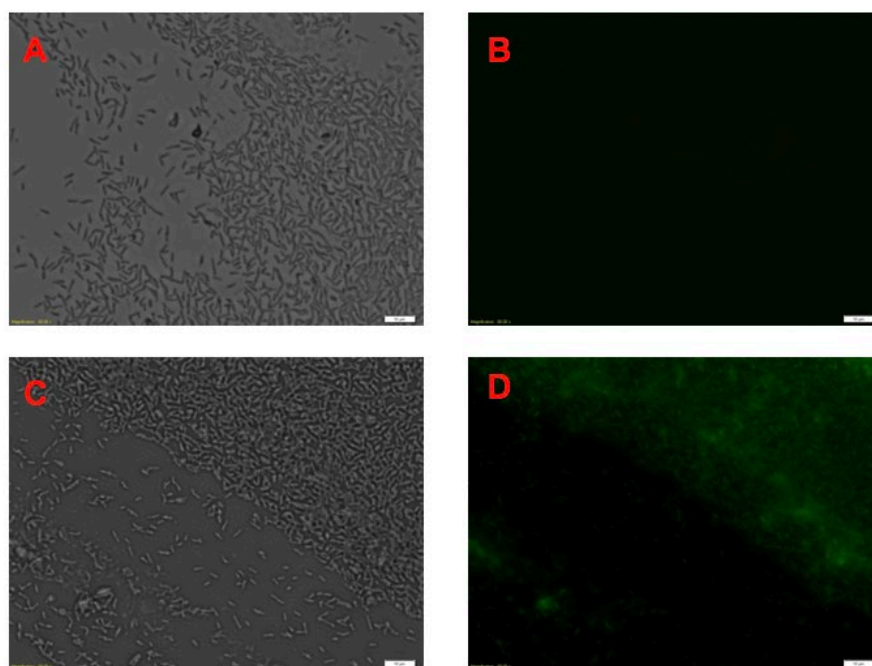


Figure 12. 5 Transformation of pCharge3 containing the *thicP-sfGFP* cassette into *Mycobacterium smegmatis* MC²-155 led to the expression of fluorescence.

The transformation of pCharge3 containing the cassette (the constitutive promoter *thicP* followed by a *sfGFP* gene) into *Mycobacterium smegmatis* MC²-155 led to the expression of fluorescence as shown under microscopy (D), and (C) is the white field. Controls: Transformation with pCharge3 containing no *thicP-sfGFP* did not show any fluorescence(B) and (A) is the corresponding white fields.

12.3.2 The promoters of *thmADBC* and *prmABCD* may be regulated by two different systems.

As the introduction of the promoter-reporter gene cassettes into dioxane degraders proved to be rather difficult, it is important to understand how the dioxane degrading gene cluster is regulated and induced, which would help us to build the reporter strains into other easily transformed strains. Therefore, the promoter-reporter gene cluster was transformed into *E. coli*, which is a common research platform for synthetic biology, and the expression levels of fluorescence were measured. We found that *thmP* promoter was not induced in *E. coli* when cultured in LB medium, but the constitutive *thicP* promoter was functioning well and it initiated the expression of *sfGFP* (Figure 11.7 and Figure 11.8). Interestingly, when *thicP* was put in the front of *thmP*, *sfGFP* gene can be expressed normally though the fluorescence intensity was not

as high as the cassette containing only *thiC*P as the promoter (Figure 11.9). This indicates that *thmP* was repressed in *E. coli* when grown in LB medium, but when *thmP* is in CB1190 cells in the presence of dioxane, the repressor can be released and the gene cluster *thmP* can be induced.

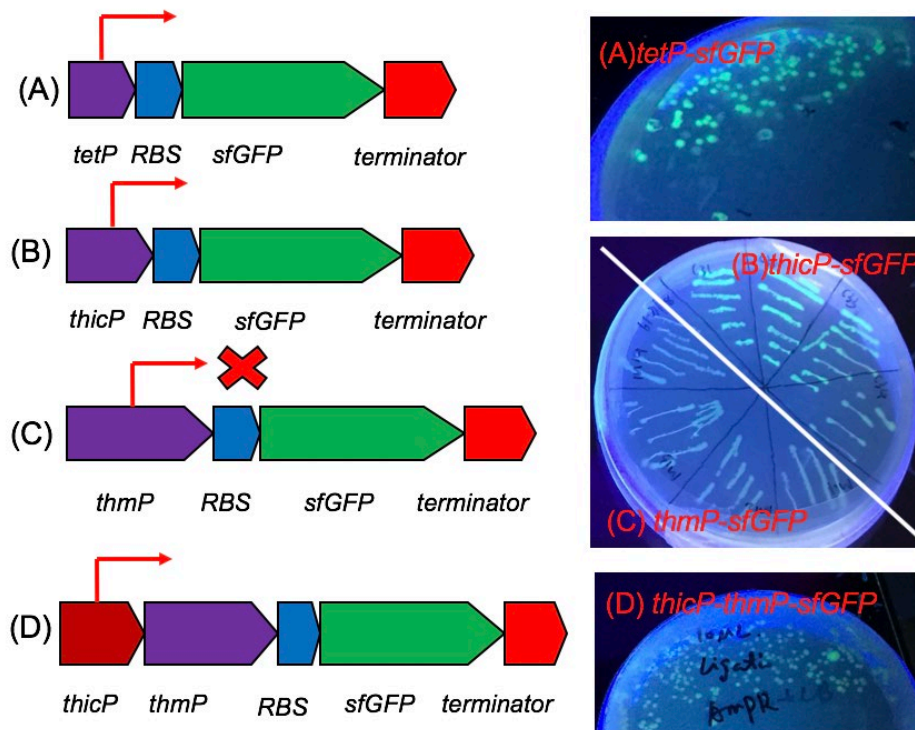


Figure 12. 6 Different promoter-reporter gene cassettes in plasmid pUC19 and the expression of *sfGFP* gene in *E. coli*.

Abbreviations: *tetP*(tetracycline promoter); *RBS*(Ribosome binding site); *sfGFP*(superfolder green fluorescence protein); *terminator*: transcription terminator; *thiC*P(the promoter of *thiC* gene); *thmP*(the promoter of *thmADBC* gene cluster). The photos in the right were taken under UV 302 nm. (A) *tetP-sfGFP*: positive control; (B) *thiC-sfGFP*: *thiC*P can be induced even in *E. coli*. (C) *thmP-sfGFP*: *thmP* cannot be induced in *E. coli*. (D) *thiC-thmP-sfGFP*: with the addition of the constitutive promoter in the front of *thmP*, *sfGFP* can be expressed, which means that *thmP* is repressed in *E. coli* grown in LB medium.

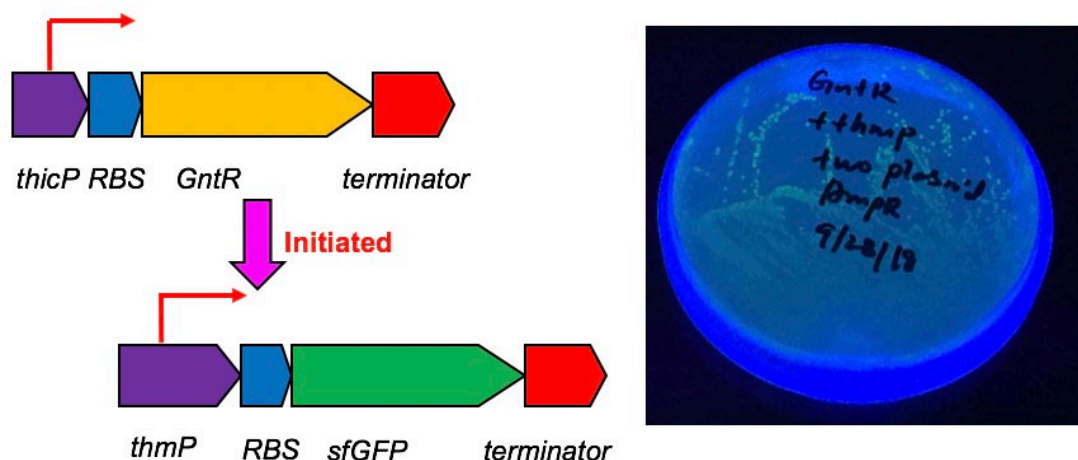


Figure 12. 7 Psed_6972 *GntR* transcription factor is the regulator governing the initiation of *thmP* promoter.

The co-transformation of two cassettes containing *thicP-GntR* and *thmP-sfGFP* into *E. coli* led to the expression of *sfGFP* gene. The photo on the right side was taken under UV 302 nm.

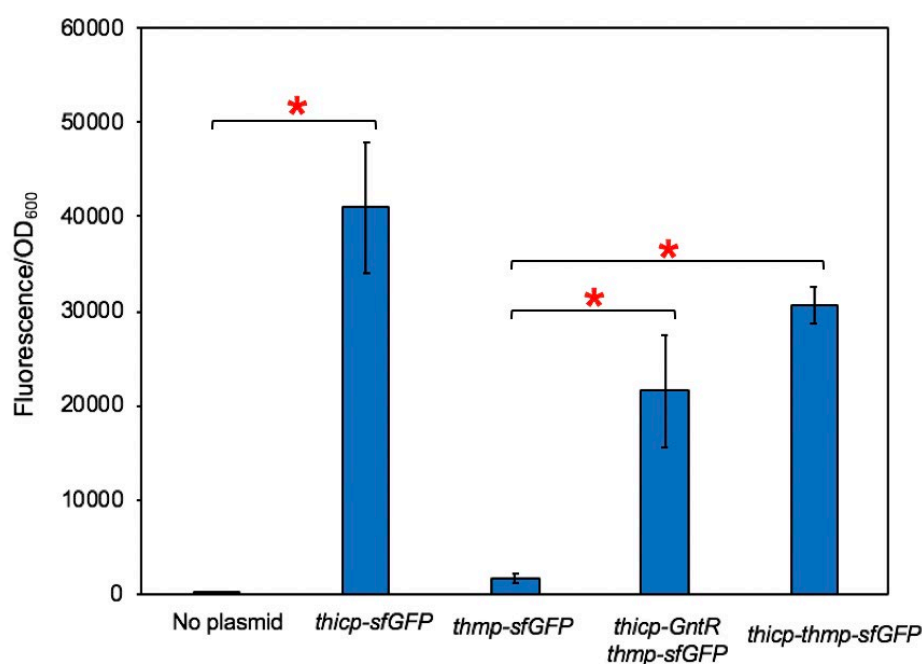


Figure 12. 8 Fluorescence intensity measurement for *E. coli* containing different plasmids.

E. coli cells transformed with pUC19 plasmid containing different cassettes were grown in LB medium for 18 hours and the fluorescence measurement was performed on a Microplate Reader (TECAN) with the excitation wavelength at 485 ± 9 nm and emission wavelength at 510 ± 20 nm. Fluorescence intensity was normalized by bacterial density indicated by OD₆₀₀. (*) represents statistically significant difference ($p < 0.05$).

Table 12. 3 Upregulated transcription factors by dioxane in CB1190 with pyruvate as the controls.
Adapted from Grostern et al. (2012)

Gene ID	Gene name	Position	Expression ratio (dioxane/pyruvate)
Psed_6972	<i>GntR</i>	Plasmid 2	3.4
Psed_7002	<i>WhiB</i>	Plasmid 2	2.7
Psed_3935	<i>GntR</i>	Chromosome	2.5
Psed_0038	<i>ArsR</i>	Chromosome	2.0

According to a previous study by Grostern et al. (2012),⁴⁶ several transcription factors are upregulated (Table 12.3) when grown on dioxane compared with pyruvate. Thus, cassettes containing the constitutive promoter *thiC*P followed by different transcription factor (TF) genes were built and transformed into plasmid pUC19 to detect the effect of these transcription factors on the transcription of dioxane degrading gene. We found that only *GntR* (Psed_6972) transcription factor located in the same plasmid as *thmADBC* gene cluster can induce the expression of *sfGFP* (Figure 12.8), though the fluorescence intensity was about half of that directly induced by the constitutive promoter *thiC*P (Figure 12.9). This indicates that Psed_6972 *GntR* is the transcription regulator governing the initiation of *thmP* promoter.

For *prmABCD*, bioinformatic analysis suggests that regulation may involve a two-component system (Figure 12.10 and Figure 12.11). Through the NCBI BLAST of *prmA* gene, we found that there are four *Mycobacterium* strains that contain a two-component regulatory system in the upstream of the *prmA* gene. The two-component system contains a sensor kinase gene which encodes a protein to sense the changes of environment and transfers the signal to another component encoded by a response regulator protein gene. The signal would cause the change of response regulator protein and make it bind to DNA to induce the corresponding transcription and expression of related genes.²⁷⁵ We did not find the two-component system in the upstream of *prmABCD* as other *Mycobacteria*, but we found the similar (88% similarity) two-component regulatory system in another plasmid in PH-06 (Figure 12.10). These findings would guide us to elucidate the regulation and initiation of the dioxane degrading gene cluster.

❖ *Mycobacterium chubuense* NBB4 plasmid pMYCCH.01



❖ *Mycobacterium dioxanotrophicus* PH-06



Plasmid 3



Plasmid 2



88%
similarity

Figure 12. 9 Schematic representation of the candidate two-component regulatory system in PH-06 based on the bioinformatic analysis.

Abbreviations: SK (sensor kinase); RR(response regulator). Based on NCBI BLAST, we found a two-component system in four *Mycobacteria* strains just in the upstream of *prmA* gene. This figure just shows the one in plasmid pMYCCH.01 in *Mycobacterium chubuense* NBB4. However, in PH-06, the candidate two-component regulatory system is located in another plasmid.

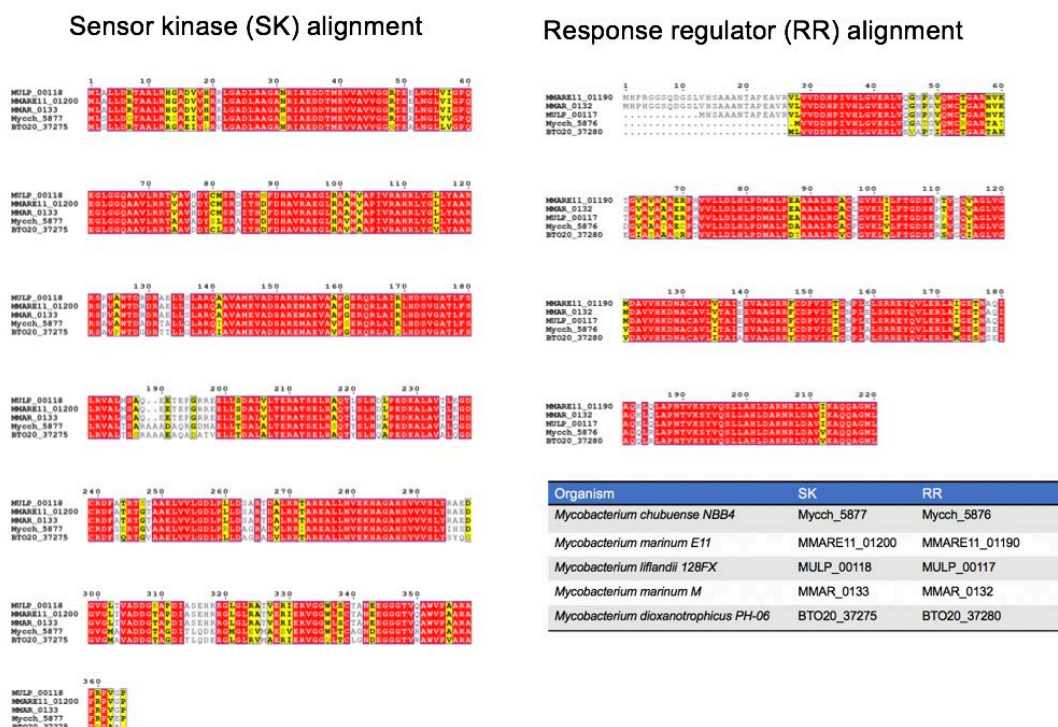


Figure 12.10 Sensor Kinase (SK) and Response Regulator (RR) alignment (amino acid sequences).

First, we searched against *prmA* gene of PH-06 in database and found four strains contains *prmA* gene with high similarity (88%) as that of PH-06. Then, we obtained the Sensor Kinase and Response Regulator components in the upstream of *prmA* gene in the three strains except PH-06. Then we searched the two components in the whole genome of PH-06 and found them in Plasmid_2. Left part of this figure shows the alignment of sensor kinase alignment and right part shows the alignment of response regulator alignment. The table in the right bottom presents the locations of these two components.

To investigate the regulation of the candidate two-component regulatory system in PH-06, we built two plasmids containing the sensor kinase gene and response regulator gene separately (Figure 12.12). The sensor kinase gene will be induced by IPTG (Isopropyl β -D-1-thiogalactopyranoside) and the response regulator gene will be induced by aTc (anhydrotetracycline). The construction process of the two plasmids and the test of fluorescence intensity were the same as previously described.²⁷⁶ The preliminary results did not give significant fluorescence signal but maybe some modifications about the two plasmids and experiment parameters would help verify the regulation of *prmABCD* by the two component system we identified through bioinformatic analysis.

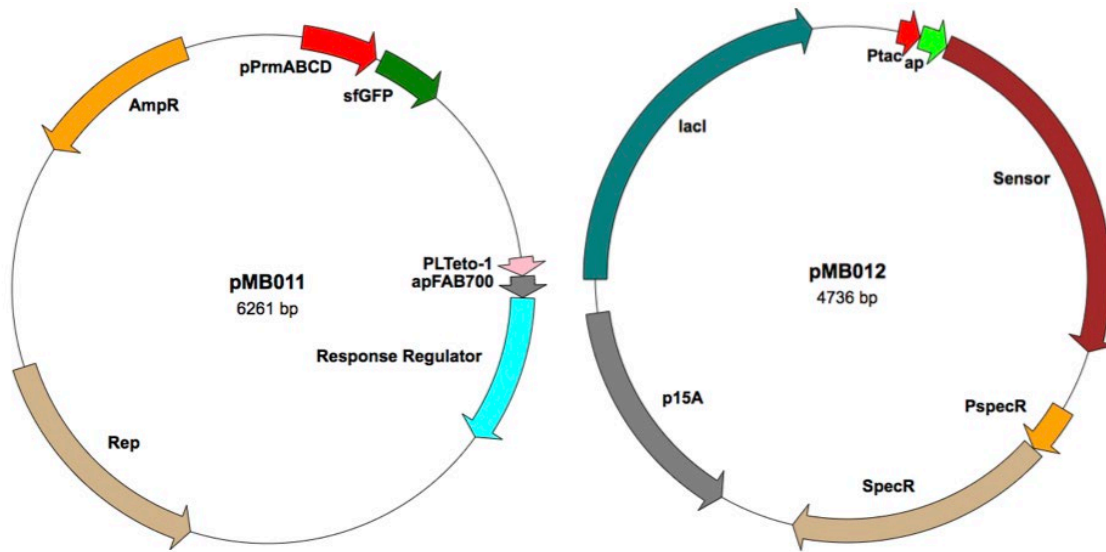


Figure 12. 11 Two plasmids containing the sensor gene and the response regulator gene respectively.

Plasmid pMB011 contains the response regulator gene promoted by promoter PLTteto (induced by aTc), and the *sfGFP* gene using the promoter of *prmABCD* as the promoter. Plasmid pMB012 contains the sensor kinase gene promoted by Ptac (induced by IPTG).

Chapter 13

Enhanced attenuation of 1,4-dioxane in bioaugmented flow-through aquifer columns

13.1 Introduction

Monitored natural attenuation, which primarily relies on biodegradation, may be the most cost-effective approach for managing dioxane plumes. However, natural attenuation is not always observed. It was reported that only 22 out of 193 sites (from California GeoTracker) and 131 out of 441 wells (from Air Force data set)¹²⁵ exhibited significant attenuation rates. In the absence of natural attenuation, biostimulation or bioaugmentation approaches may be the next most cost-effective strategies, particularly for large dilute plumes. Biodegradation potential by native soil bacteria has been demonstrated^{230, 277} and it has been reported that several bacterial isolates or consortia can degrade dioxane metabolically^{40, 42, 45, 277, 278} or cometabolically.^{11, 53, 279} Therefore, the implementation of bioaugmentation strategies in contaminated sites where biodegradation activity potential by indigenous bacteria is absent could help accelerate dioxane removal.^{11, 45, 46, 49}

Recently, dioxane biodegradation was successfully demonstrated at field scale after the introduction of *Rhodococcus ruber* ENV425, which is a bacterium capable of breaking down dioxane cometabolically in the presence of a primary substrate (e.g., propane).²⁵⁹ Nonetheless, supplementation with auxiliary co-substrates increases complexity and costs. Also, adequate distribution of the primary substrate can be challenging, and continuous injection of auxiliary substrates can lead to unintended consequences such as catabolic plasmid loss.⁵⁴ Therefore, bioaugmentation using bacteria capable of metabolically degrading dioxane may be preferable.

Successful bioaugmentation using metabolic strains has been reported in microcosm studies^{230, 280} or small flow-through columns (e.g., 10 cm).²⁸¹ However, these experimental approaches are unlikely to realistically simulate temporal changes and natural gradients in environmental oxidation-reduction potential that occur at contaminated sites, and provide scarce (if any) information on bacteria transport and distribution after injection. Accordingly, in this work we used large flow-through aquifer columns (120-cm length, 5-cm i.d.) filled with uncontaminated soil. These columns were monitored for over two years to assess long term natural attenuation of dioxane and its subsequently enhanced biodegradation after bioaugmentation with the archetype metabolizer *Pseudonocardia dioxanivorans* CB1190. Experiments were conducted using either low (200 µg/L) and high (5 mg/L) dioxane concentrations to assess the perseverance of the added strain (and the relative abundance of the pertinent catabolic gene, *thmA*), as well as its effectiveness in the removal of dioxane from both diluted contamination plumes and source zones.

13.2 Materials and Methods

13.2.1 Aquifer columns

Three flow through aquifer columns (120-cm length, 5-cm diameter, 19.63 cm² cross sectional area) equipped with eight sampling ports (at 2.5, 7.6, 14, 20, 40, 60, 80, and 100 cm from the inlet) were used to investigate the natural attenuation of dioxane and its enhanced biodegradation through aerobic bioaugmentation. Columns were filled with sandy aquifer material. The physical-chemical characteristics of the soil was (in mg/L): N-NO₃⁻ (8), P-PO₄²⁻ (35), K (115), Ca²⁺ (3,886), Mg²⁺ (161), S (23), Na (11), pH of 7.9, conductivity of 144 µmho/cm, and an organic matter of 0.83% (Soil, Water and Forage Testing Laboratory, Department of Soil and Crops Sciences, College Station, TX). The columns were kept in the dark at room temperature (22°C) and fed continuously with a carbonate-buffered synthetic groundwater for over one year in an up-flow mode. The mineral composition of synthetic groundwater (in mg/L) was as follows: NO₃⁻ (5.0); SO₄²⁻ (4.0); CaCO₃ (1,000); NH₄⁺ (5.5); Mg²⁺ (1.5); PO₄³⁻ (0.06); and Ni(II), Cu(II), Zn(II), Co(II), and Mo(IV) (0.002 each) (modified from ²⁸²). The groundwater was initially fed at flow rate of 7.2 mL/h using a peristaltic pump (Masterflex model 7519-15). Over the course of the experiment, the flow rate was decreased to 2 mL/h (using a syringe pump, Harvard Apparatus model 22) to reduce column's seepage velocity and increase residence time for improved biodegradation. The hydraulic characteristics of the columns were estimated by fitting bromide tracer data to the one-dimensional advection-dispersion equation as described previously.²⁸³ Two flow-through aquifer columns were bioaugmented and a third column was not bioaugmented and served as negative control (natural attenuation alone). Columns were fed with groundwater amended with 200 µg/L or 5 mg/L of dioxane. These two dioxane concentrations were selected to represent the typical dioxane levels of in contaminated plumes and source zones, respectively.¹²⁵ A 120-mL of stock bacteria inoculum (~10⁹ cells/ mL) was injected into the port vial located at 40 cm from the column's inlet. This location was selected to assess the potential spatial distribution of the introduced bacteria through quantification of *thmA* catabolic genes along the column's length months after inoculation. Then, after 3 months of monitoring, one of the bioaugmented columns was reinoculated (120 mL, ~10⁹ cells/ mL) near the column's influent (port located at 2.5 cm from the column's inlet) where the concentration of dissolved oxygen was higher.

13.2.2 Bacteria inoculum

The well-characterized *Pseudonocardia dioxanivorans* CB1190,²⁷⁸ capable of using dioxane as sole carbon and energy source, was utilized in this work as the inoculum. CB1190 was obtained directly from the American Type Culture Collection (ATCC 55486). Cells were grown in 2 L batch reactors containing Ammonium Mineral Salts (AMS) medium²⁷⁸ amended with 800 mg/L dioxane. Batch reactors were kept at room temperature (23°C) under continuous aeration (using 0.22 µm inline sterile filters to avoid contamination) using aquarium pumps. After 18 days, the grown cells were harvested by centrifugation (Heraeus MegaFuge8, Thermo Scientific), washed three times in phosphate buffer, and sonicated in a water bath (Ultrasonic Cleaner Branson 5510R-MT) for 40 seconds to disaggregate cell clumps. Bacteria concentration was estimated after sonication by optical density at 600 nm (OD₆₀₀ of 1 = 8×10⁸ cells/ mL) using a Ultrospec™ 2100 pro UV/Visible Spectrophotometer (GE Healthcare, Little

Chalfont, UK). After sonication, cells were serially diluted and plated on Reasoner's 2A (R2A) plates to assess bacteria count (colony forming units, CFU) and viability.

Prior to inoculation of the columns, microcosms studies were performed to assess the metabolic activity of the harvested bacteria. 120 mL glass batch reactors were filled with 50 mL groundwater collected from a dioxane-contaminated site and capped with Teflon septa. Two reactors were prepared using groundwater from two independent sampling wells. Groundwater contained dioxane (1.5 – 4.5 mg/L) as well as chlorinated solvents present as co-contaminants (in mg/L): trichloroethene (2 – 3), 1,1-dichloroethene (3 – 5), and vinyl chloride (0.1). Microcosms were inoculated with approximately 1.6×10^8 cells/mL (final concentration). Two other microcosms, not inoculated (negative controls), served to distinguish the effects of bioaugmentation compared to natural attenuation alone. Microcosms were kept at room temperature (22 °C) and sampled to assess changes in dioxane concentrations over time. Dioxane was only degraded in the bioaugmented microcosms within 3 – 7 days, indicating the metabolic activity of CB1190.

13.2.3 Biosparging

Air was injected directly in the bioaugmented column (using a Harvard Apparatus 22) at flow rate of 5 mL/hr in attempt to overcome the rapid depletion of dissolved oxygen inside the columns and to enhance dioxane removal by the inoculated bacteria. We found that higher air flow rate could increase oxygen and enhance biological activity, but due to limitations in column's dimensions, any further increase in air flow could result in the formation of air pockets and changes in hydraulic characteristics. A 60 mL plastic syringe filled with air and connected to a Tygon® tube was used to pump air directly into the column inlet. The syringe was refilled with air daily until the end of the experiments.

13.2.4 Soil DNA extraction and quantification of bacteria 16S rRNA and *thmA* catabolic gene

Column's aquifer materials were collected for bacterial genes analyses. About 2 g of soil was taken from the port vials located at 2.5, 40, and 80 cm from the columns' inlet. Soil samples were transferred into a lysing matrix tube for DNA extraction by using a QIAGEN DNEASY POWERSOIL kit according to the manufacturer's protocols. A bead-beating device (MINI Beadbeater) was utilized for soil lysis. A 50- μ L soil DNA sample was collected in a 1.5-ml Eppendorf vial and stored in a freezer (IsoTemp® basic) at –75°C for further analysis.

Real-time quantitative PCR (qPCR) analysis was used to quantify the concentration of total bacteria (based on 16S rRNA) and dioxane-degraders based on a previously developed primers/probe set targeting genes encoding the large hydroxylase subunit (*thmA*) of a putative tetrahydrofuran/dioxane monooxygenase (an enzyme proposed to initiate dioxane catabolism).¹⁶ Another primers/ probe set previously designed to target *prmA* gene encoding the large hydroxylase subunit (*prmA*) of a propane monooxygenase enzyme that may be also involved in dioxane degradation by indigenous soil bacteria belonging to *Mycobacterium* spp. were also evaluated as biomarker.^{45, 284} qPCR mixture contained 10 ng of genomic DNA, 300 nM of forward and reverse primers, 150 nM of fluorogenic probe, 10 μ L of TaqMan universal master mix II (Applied Biosystems, Foster City, CA, USA) and DNA-free water, to a total volume of 20 μ L. The primers and probe used (Integrated DNA Technologies, Inc.) to target total bacteria 16S

rRNA were: 5'-CGGTGAATACGTTTCYCGG-3' (forward primer), 5'-GGWTACCTTGTTACGACTT-3' (reverse primer), and FAM-5'-CTTGATACACACCGCCCGTC3'-BHQ-1 (probe). The primers and probe to target *thmA* was: 5'-CTGTATGGGCATGCTTGT-3' (forward primer), 5'-CCAGCGATACAGGTTCAT C-3' (reverse primer), and 5'-6-FAM-ACGCCTATT/ZEN/ACATCCAGCAGCTCGA-IABkFQ-3' (probe). The primers and probe to target *prmA* were: forward 5'-ACTGCGATGCTGGTTGAC-3', reverse 5'-TCAGGTACGCCTCCTGATT-3', and probe 5'-6-FAM/TTCCTCGCG/ZEN/CAGATGATCGACG-IABkFQ-3'. qPCR was performed in a CFX 96 real-time system (Bio-Rad, Hercules, CA, USA) with the following temperature program: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60°C for 1 min. Serial dilutions (10^{-4} - 10^1 ng DNA/ μ L) of the extracted genomic DNA of CB1190 and *Mycobacterium dioxanotrophicus* PH-06 were utilized to prepare the calibration curves for *thmA* (1 copy/genome) and *prmA* genes (1 copy/ genome), respectively. CB1190 DNA was also used to prepare calibration curves for 16S rDNA (3 copies/genome) gene. Assuming a genome size of 7.44 Mb²⁸⁵ and 9.124×10^{14} bp/ μ g [i.e., (6.022×10^{17} Da/ μ g of DNA/660 Da/bp)], the gene copy numbers were calculated based on the equation below.

$$\frac{\text{gene copies}}{\mu\text{L}} = \left(\frac{\frac{\mu\text{g of DNA}}{\mu\text{L}}}{\frac{7.44\text{Mb}}{\text{genome}}} \right) \left(\frac{9.124 \times 10^{14} \text{ bp}}{\mu\text{g of DNA}} \right) \left(\frac{\text{gene copies}}{\text{genome}} \right)$$

13.2.4 Analytical procedures

Groundwater samples were withdrawn from column's lateral sampling ports using a 1-mL glass type syringe (Hamilton). Dioxane was measured using an Agilent 7820A gas chromatograph (GC) equipped with a 5977E mass spectrum detector (MSD) after filtration through 0.22 μ m syringe filters and extraction by a liquid/liquid frozen microextraction method with dichloromethane as the solvent.¹⁰⁰ GC operational temperatures were set at 175°C for the injector, 250°C for the detector, and 150°C for the oven. Detection limit for dioxane was 5 μ g/L. The concentration of dissolved oxygen was measured directly from the columns sampling ports using a micro dissolved oxygen probe (Microsensor PreSens, PM-PSt1). Bromide used as a tracer (1g/L added in the groundwater feeding solution) was measured over time in the effluent of the columns by conductivity (Orion Versastar Pro, Thermo scientific). All chemicals used were analytical grade (>99.8 purity or higher).

Statistical differences between two data sets were determined by comparison of means using T test at the 95% confidence level ($p < 0.05$). Data were presented as average $\pm$ standard deviation.

13.3 Results and Discussion

13.3.1 Bioaugmentation to remediate low levels (μ g/L) of dioxane

The column's hydraulic parameters (with values in parentheses) were as follows: groundwater flow ($Q = 7.2$ mL/h), dispersion ($D = 0.4$ - 2.5 cm²/h), effective porosity ($\eta_e = 0.4$ - 0.50) and seepage velocity ($V = 0.75$ - 0.93 cm/h). The obtained hydraulic conductivity (i.e., $K =$

$10^{-7} - 10^{-6}$ cm/s) and η_e were within typical values reported for sandy aquifers.²⁸⁶ Approximately five days were required to exchange one pore volume.

Figure 13.1 shows dioxane concentration profiles in the control (natural attenuation alone) and bioaugmented columns fed synthetic groundwater amended with 200 $\mu\text{g/L}$ dioxane. Dioxane was not removed in the control column even after 2 years of acclimation (146 pore volumes exchanged), implying the absence of dioxane degradation potential. Whereas some studies successfully demonstrated biodegradation in microcosms^{230, 277} and small flow through columns²⁸¹ experiments simulating natural attenuation, dioxane has been reported to be persistent in many contaminated sites.¹²⁵ This implies that indigenous microorganisms may not always readily degrade this contaminant and that bioaugmentation may be justified in such cases to shorten otherwise longer acclimation periods, enhancing degradation rates.

The observed recalcitrance of dioxane motivated us to investigate whether bioaugmentation with the metabolic dioxane degrader CB1190 could enhance sustained biodegradation of dioxane present at low ($\mu\text{g/L}$) levels. Bioaugmentation has a great probability of success when the added microorganism can fill a metabolic niche that is not being exploited by the indigenous microflora.²⁸⁷ Thus, after six months, one of the acclimated columns was inoculated with CB1190 at the sampling port located 40 cm from the column's inlet. The effects of bioaugmentation was then measured by comparing the dioxane concentration profiles between the inoculated and unamended control columns.

In the bioaugmented column, dioxane decreased approximately $34\% \pm 5.6\%$ after 10 days (~ 2 pore volumes exchanged) and for up to three months (~ 18 pore volumes exchanged) following inoculation, suggesting the robustness of bioaugmentation and its potential to offer long-term enhanced biodegradation capabilities. Despite the observed decrease in concentration, however, dioxane concentrations downgradient from the inoculation point remained above established regulatory drinking water levels that range from 0.3 to 85 $\mu\text{g/L}$ depending on the state.² Biological removal of dioxane at these $\mu\text{g/L}$ levels seems difficult to achieve, apparently because of insufficient substrate concentration needed to induce enzyme activity and/or sustain bacterial growth. Other studies reported that complete degradation of dioxane at ≤ 1 mg/L may still be possible in the presence of additional co-substrates that support dioxane cometabolism²⁸⁸. For instance, bioaugmentation studies conducted at the field-scale using the propanotroph *Rhodococcus ruber* ENV425a showed dioxane degradation to < 2 $\mu\text{g/L}$ in the presence of a co-substrate (e.g., propane).²⁵⁹ Nonetheless, it is very important to select and optimize appropriate biostimulation strategies on a case by case basis scenario. This is because cometabolism may hinder degradation of dioxane as result of preferential degradation of the added substrate and the accompanied depletion of nutrients and electron acceptors (e.g., oxygen) that would be otherwise utilized for dioxane degradation. Long-term exposure to co-substrates could also cause the loss of the catabolic plasmid.⁵⁴ Moreover, some co-substrates (e.g., 1-butanol and tetrahydrofuran (THF)) may exert competitive inhibition on the required monooxygenases, decreasing dioxane degradation rates. The adequate *in situ* distribution of some primary substrate such as propane can also be quite challenging but of paramount importance to obtain the desired degradation rates.

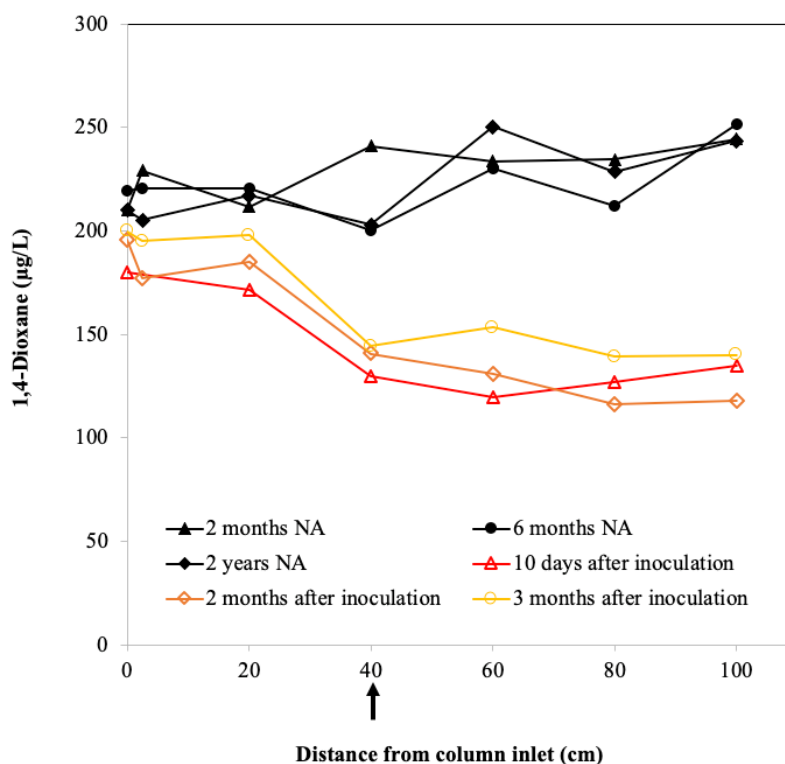


Figure 13.1 Dioxane concentration profiles in control column (not bioaugmented) under natural attenuation (NA) and in the column inoculated with CB1190.

Dioxane was not biodegraded in the control column, even after 2 years of acclimation. Bacteria was inoculated at sampling port vial located 40 cm from the column inlet (indicated with an arrow). Samples were taken after stopping the flow for 2 days then immediately resumed.

13.3.2 Bioaugmentation to remediate dioxane at higher (source-zone-like) concentrations

Dioxane was not removed in the natural attenuation control column (not bioaugmented) even after 6 months of acclimation (Figure 13.2). Biodegradation of dioxane by native soil bacteria, encountered in many sites previously exposed to dioxane or not, is clearly not ubiquitous. The results showed a consistent dioxane removal of 92% only in the bioaugmented column (Figure 13.2A). Significant degradation activity occurred only at the inoculation port located at 40 cm from column's inlet.

Removal of higher concentrations of dioxane (5 mg/L) was comparatively higher than previous tests conducted to simulate bioaugmentation in low levels (200 µg/L) (Figure 13.1) since this low concentration cannot sustain bacteria metabolic activity. Without stopping the groundwater flow for 48h prior sampling, however, negligible removal of dioxane was observed, suggesting that the seepage velocity was too high to ensure sufficient residence times for biodegradation to occur. To assess the potential for continuous dioxane removal downgradient from the inoculation port, the groundwater flow was then reduced from 7.2 to 2 mL/h (V decreased from 0.93 to 0.36 cm/h) to increase contact time for biodegradation to proceed. Notably, stopping the flow for longer periods of time from 48 up to 120 h did not lead to complete removal, and dioxane persisted at 0.5 ± 0.10 mg/L. With the reduction in groundwater flow, the concentration of dissolved oxygen (DO) downgradient from the column's inlet also

diminished and anaerobic conditions that hinder dioxane biodegradation were established near the inoculation point ($\text{DO} = 0.0 \text{ mg/L}$; Figure 13.2B). At this point, increasing groundwater flow to previous seepage velocity neither improved dissolved oxygen concentrations nor recovered biodegradation activity (Figure 13.2B). Soil samples collected along the column's length further demonstrated the formation of black precipitates typically associated with precipitation of metals in anaerobic conditions. Hence, we investigated whether inoculation near the column's inlet where DO was still present could sustain biodegradation activity thus simulating traditional push and pull bioaugmentation strategies.

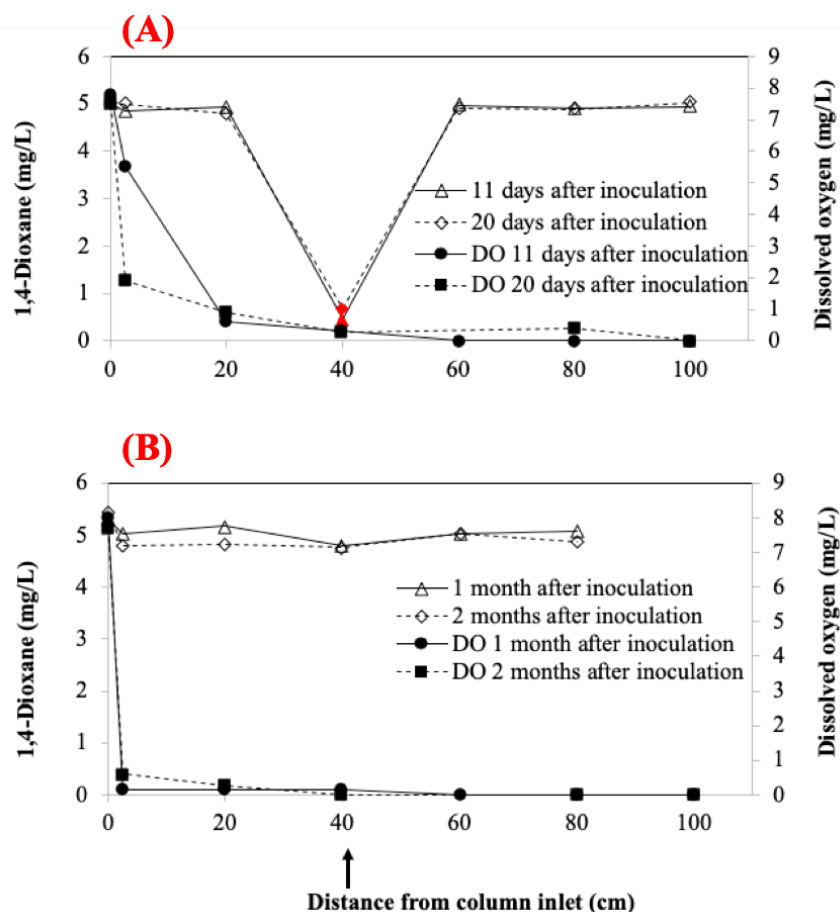


Figure 13. 2 Dioxane and dissolved oxygen concentration profiles in the column inoculated with CB1190. Bacteria was inoculated at sampling port located 40 cm from the column inlet (indicated with an arrow).

Biodegradation of dioxane (approximately 92%) was only observed at the location where bacteria was inoculated (A). The decrease in flow rates from 7 mL/h ($V=1.5 \text{ cm/h}$) to 2 mL/h ($V=0.3 \text{ cm/h}$) led to anaerobic conditions and the loss of biodegradation activity (B). Increasing flow rate ($V=1.5 \text{ cm/h}$) neither recovered aerobic conditions nor biodegradation activity. Arrow indicates the location where bacteria was inoculated. Samples were taken after stopping the flow for 2 days then immediately resumed.

Following inoculation, dioxane removal ($24\% \pm 3.5\%$) was consistently observed for over 18 days (Figure 13.3). This relatively low removal efficiency was likely attributed to

insufficient concentration of dissolved oxygen measured at the column inlet (0.17 ± 0.01 mg/L). To test this hypothesis, we injected air (5 mL/h) directly and in parallel with groundwater in the column's inlet in an attempt to offset the rapid depletion of oxygen inside the columns as result of the presence of 0.8% organic matter content and associated microbial biodegradation. Prior to use biosparging, hydrogen peroxide was tried alternatively as possible oxygen delivering strategy (aimed at 100 mg/L DO); however, degradation of dioxane by up to 50% was observed in the groundwater stock feeding solution within three days probably by Fenton-like reactions.²⁸⁹ After beginning biosparging, the concentration of dissolved oxygen increased to 1.13 ± 0.01 mg/L and removal efficiency was significantly ($p < 0.05$) improved to $42\% \pm 1.8\%$ for the remaining 18 days of monitoring (Figure 13.3). The increase in dioxane removal after biosparging was attributed to biodegradation processes and not to volatilization because dioxane has a high solubility and low Henry's constant which makes removal by air sparging unlikely.

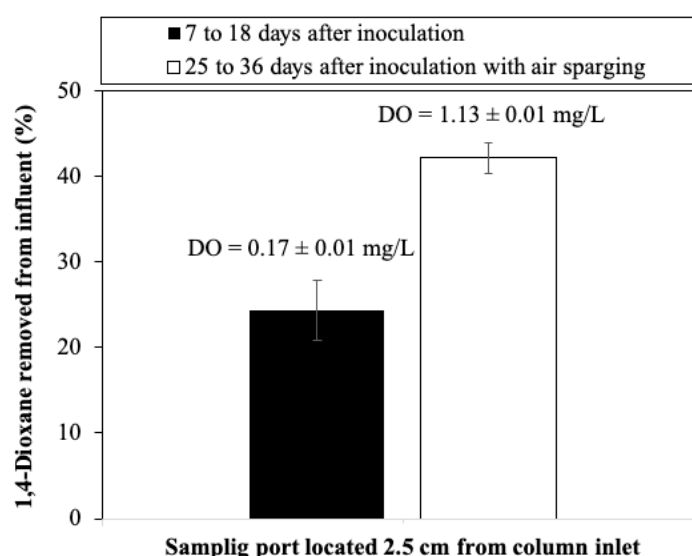


Figure 13. 3 Dioxane removal in the column's inlet after re-inoculation with CB1190. Biosparging increased dissolved oxygen concentration significantly ($p < 0.05$), improving dioxane removal.

The positive association between dissolved oxygen concentrations and removal efficiency suggests that additional injection of air or oxygen would be needed to enhance biodegradation activity and removal rates. However, as explained previously, increasing air flow rate above 5 mL/h could result in hydraulics disruption due to entrapped air bubbles causing groundwater preferential flow. Field experiments conducted using biosparging to remove benzene, toluene and xylenes (BTX) from shallow aquifer showed that 80% -100% of hydrocarbons present at concentrations ranging from 0.12-16 mg/L were degraded with half-lives of 5-20 days when the dissolved oxygen was > 2 mg/L.²⁹⁰ In contrast, oxygen concentration of ~ 2 mg/L resulted in half-life of 20 to 60 days and little or no degradation were observed at dissolved oxygen ≤ 0.5 mg/L. Batch simulations showed that dioxane biodegradation can be significantly impaired when dissolved oxygen concentrations are less than 2 mg/L.²⁸⁸ This could explain why dioxane was not completely removed in the bioaugmented column. Nonetheless, the demonstrated capabilities of *Pseudonocardia* spp. to thrive in the column's microaerophilic

conditions, suggests the potential of CB1190 for bioaugmentation where oxygen availability is usually a common limiting factor for the metabolism of dioxane in situ.

13.3.3 Molecular analysis

The survival of the added microorganisms and/or low bacterial transport through the aquifer material can pose limitations to bioaugmentation.²⁹¹ Therefore, soil DNA analysis was performed in the column's aquifer material after three months of inoculation to determine the concentration of the injected bacteria and to investigate if bacterial cells were distributed spatially along the column's length. Distribution of the injected bacteria would be associated with groundwater transport because CB1190 is a non-motile bacterium.²⁹²

The *thmA* gene that encodes an enzyme that initiates dioxane degradation in CB1190 was used as biomarker. The concentration of bacteria did not vary along the column's length ($\sim 10^4$ cells g of soil⁻¹), except at the inoculation port where the concentration was expectedly higher ($\sim 10^6$ cells g of soil⁻¹) as a result of inoculation (Figure 13.4). The *thmA* gene was only detected at the inoculation port ($\sim 10^6$ cells/ g of soil⁻¹) corroborating with the only location where biodegradation was determined (Figure 13.4). The concentration of CB1190 measured by quantification of *thmA* copy numbers is in agreement with theoretical estimations of $\sim 7 \times 10^5$ cells/ mL assuming CB1190 yield [0.18 mg-cells / mg-dioxane¹³] the concentration of dioxane consumed (~ 4 mg/L) (Figure 13.2A), and 10^{-9} cells/ mg-dry weight. We also tried another set of primers and probe that target *prmA* gene that has been previously encountered in dioxane degraders belonging to *Mycobacteria* genus. However, *prmA* gene was not detected in the column's soil corroborating with absence of measurable biodegradation of dioxane by native soil bacteria in the control columns.

Detection of *thmA* in the soil collected three months after inoculation suggests the robustness of bioaugmentation and its potential to offer long-term enhanced biodegradation capabilities. Insignificant transport and dispersion of the introduced bacteria beyond the injection port was determined as indicated by the absence of *thmA* used as biomarker to trace distribution of CB1190 along the column's length. This may be attributed to CB1190 characteristics to form cell aggregates that can be filtered by the soil. Mobility and dispersion of bacteria through soil is generally preferable for bioremediation.²⁹¹ However, while bacterial aggregation may pose distribution challenges through bioaugmented aquifers, restricting the spread of the catabolic potential beyond the injection well radius of influence, these bacteria may be useful for the establishment of biobarriers.

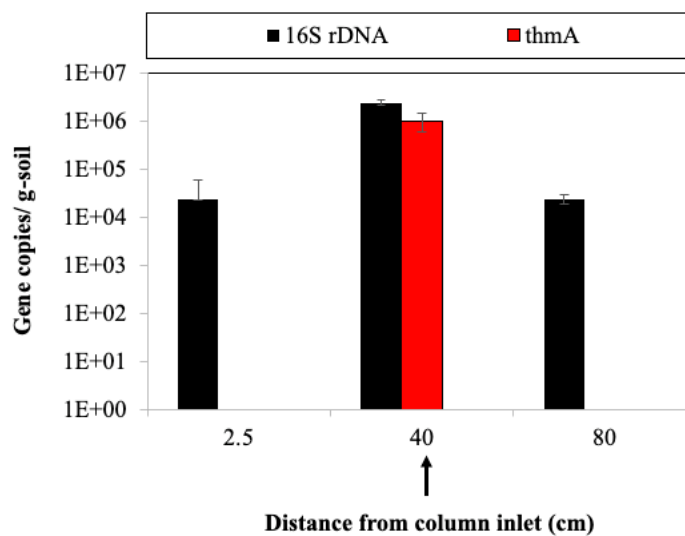


Figure 13. 4 Total bacteria (16S rRNA) and *Pseudonocardia dioxanivorans* CB1190 (*thmA*) concentration profile along the column's length.

Migration of the introduced bacteria and its catabolic gene *thmA* beyond the injection port (arrow) was not observed even after 102 days.

Chapter 14

Summary and Conclusions

14.1 Summary and Conclusions

Volatilization or adsorption that are commonly used strategies to remediate groundwater pollutants are inefficient to treat dioxane due to its high hydrophilic nature. Biodegradation is also not widely demonstrated in laboratory or field scale, yet a few newly isolated dioxane degraders from dioxane-contaminated samples imply the possibility of natural attenuation. Nonetheless, the employment of MNA as one of the most cost-efficient approaches to manage large and dilute groundwater plumes of priority pollutants, such as those formed by dioxane still relies on demonstration of the presence and expression of relevant biodegradation capabilities. Because dioxane is found as a co-contaminant at thousands of sites impacted by chlorinated solvents, there is an urgent need for tools to assess site-specific dioxane natural attenuation potential.

The primary objective of this project research was to identify the key enzymes that are involved in dioxane degradation and design and validate catabolic biomarkers to quantify the presence and expression of dioxane biodegradation capacity to support decisions to select or reject MNA at dioxane-impacted sites. Soluble di-iron monooxygenases (SDIMOs), especially group-5 SDIMOs (e.g., THF/dioxane monooxygenases), are of significant interest due to their potential role in the initializing the cleavage of cyclic ethers. Consequently, seven gene clusters encoding SDIMOs were discovered and annotated in the genome of *Pseudonocardia dioxanivorans* CB1190, a well-characterized bacterial dioxane degrader, including one putative THF/dioxane monooxygenase (*dxmADBC*). This gene cluster encoding the putative THF/dioxane monooxygenase was found to possess highly similarity to previous annotated *thmADBC* gene clusters from THF degraders (> 97% for the large hydroxylase in nucleotide sequence). RT-qPCR analysis revealed that only the *dxmA* gene encoding the large hydroxylase of the putative dioxane monooxygenase was significantly up-regulated when fed with dioxane and its structural analog, THF, compared to non-inducible substrate acetate ($p < 0.05$). The expression doubling and fold change were 5.7 ± 1.6 and 6.0 ± 2.1 , respectively. These results provided strong evidences to support the main role of this dioxane monooxygenase in dioxane degradation.

A primer/probe set was thus developed to target bacterial genes encoding the large hydroxylase subunit of a putative tetrahydrofuran/dioxane monooxygenase (an enzyme known to initiate dioxane catabolism), using *Taqman* (5'-nuclease) chemistry. Multiple sequence alignment of the four *thmA/dxmA* genes available on the NCBI database were used to construct a probe that targets conserved regions surrounding the active site of dioxane degradation activity in several dioxane degraders. The probe showed high selectivity (no false positives) and sensitivity (7,000-8,000 copies/g soil). Microcosms prepared with groundwater samples from 16 monitoring wells from five different dioxane-impacted sites showed that enrichment of this

catabolic gene (up to 114-fold) was significantly correlated with the amount of dioxane degraded. A significant correlation was also found between biodegradation rates and the abundance of *thmA* genes. In contrast, the universal 16S rRNA gene copy numbers were neither sensitive nor reliable indicators of dioxane biodegradation activity suggesting this novel catabolic biomarker (*thmA/dxmA*) holds promises to rapidly assess the performance of natural attenuation or bioremediation of dioxane plumes.

To assess dioxane natural attenuation/ biodegradation potential, microcosms studies were performed. Three independent lines of evidence indicated dioxane biodegradation when tested as a sole carbon source: (1) removal was observed in biologically active microcosms but not in sterile controls; (2) ^{14}C -labeled dioxane was oxidized to $^{14}\text{CO}_2$ and a higher extent of mineralization was observed for (presumably more acclimated) source-zone samples exhibiting higher degradation activity; and (3) ^{14}C was converted to bound residue (including possible incorporation into biomass) only in microcosms where dioxane was biodegraded. Both re-spiked and replicated microcosm sets confirmed the relatively rapid dioxane degradation (i.e., 100% removal within 20 weeks). Therefore, it appears that MNA could be an interesting remediation option for these contaminated sites, but long-term efforts to monitor the water quality and contamination levels are warranted. Moreover, the enrichment of two dioxane degrading consortia from uncontaminated environments with no known previous exposure suggests that dioxane degraders may exist in environments we did not expect even in uncontaminated environments. This understanding is important for our future decisions of bioremediation strategies (i.e., natural attenuation or bioaugmentation). The prevalence of groups 5 and 6 SDIMOs in these two consortia suggests the importance of monooxygenases in dioxane degradation. However, the absence of *thmADBC* indicates other monooxygenases are involved in dioxane biodegradation. A novel dioxane degrading gene cluster (*prmABCD*) that is phylogenetically distant from previously characterized one (*thmADBC*) was further discovered. This implied that dioxane degrading gene clusters are more diverse than our previous understanding and it is worthwhile discovering new catabolic genes as it would contribute our further genetic research and serve corresponding bioremediation. Moreover, based on what we have known about dioxane degraders and considering the characteristics of dioxane impacted sites, it is helpful to have a reporter strain to signal the expression of catabolic genes and associated dioxane degradation activity. Our preliminary results showed that the challenging transformation of the promoter-reporter cassette into CB1190 and PH-06 may be due to the cells' restriction-modification (R-M) self-defense systems. Furthermore, the promoters of *thmADBC* and *prmABCD* may be regulated by two different systems.

While many geochemical factors (pH, temperature, nutrients) and the abundance of specific degraders may be important for dioxane biodegradation, these factors may not exert as strong of an influence on the potential biodegradation activity as the concentration of dioxane. Unequivocally, *in situ* dioxane concentrations significantly influenced the observed dioxane biodegradation rates with higher dioxane concentrations possibly selecting for a thriving microbial population, enhancing the sustenance of bacteria metabolic capabilities and/or inducing specific enzymes. This finding provides valuable insight into site-specific scenarios where significant dioxane biodegradation might be expected. Specifically, while sites with higher dioxane concentrations are more likely to select for and sustain dioxane degraders, sites with relatively low dioxane concentrations would be more challenging to attenuate naturally and may require implementation of alternative remediation strategies. Whether there is a site-specific

threshold dioxane concentration of regulatory concern below which sustained biodegradation is unlikely to proceed remains to be determined.

The use of auxiliary (biostimulation using growth-supporting or inducing) carbon sources may temporarily enhance dioxane biodegradation. However, these external substrates may also exert counterproductive long-term consequences. Non-inducing growth substrates, such as 1-BuOH, can boost the overall microbial biomass, but inhibit the indigenous dioxane degraders possibly by exerting catabolite repression and metabolic flux dilution, as well as promoting the growth of incompetent species that increase interspecies competition. Furthermore, long-term exposure to high concentrations of easily degradable non-inducing substrates can contribute to the loss or curing of catabolic plasmids, eliminating the intrinsic dioxane biodegradation capability. Therefore, when the catabolic genes are harbored in plasmids, the effects of non-inducing substrates need to be evaluated carefully. Furthermore, though the inducing substrate THF effectively stimulates enzyme (dioxane MO) expression, supports the growth of dioxane degraders, and maintains the catabolic plasmids, it may exert competitive inhibition that increases the lag period for dioxane removal. This underscores the need for further development of effective bioremediation techniques to accelerate dioxane removal at trace concentrations.

We demonstrated a cultivation-free strategy to separate and concentrate genus-specific subpopulations of potential dioxane degraders by using our fluorescently labeled oligonucleotide probes and flow cytometry. This will expedite discovering novel *Pseudonocardia* strains and discerning the molecular basis for specific metabolic traits of interest (e.g., antibiotic production, biodegradation of cyclic ethers, and C₁ autotrophic fixation). The obtained genetic sequences and metabolic information will be valuable to expand the current database of reference genes or genomes for the development of closed-format molecular approaches (e.g., qPCR and microarray) and reroute the open-format next-generation sequencing resources to increase the portion of informative reads (e.g., bypassing community-focused metagenomics to single cell analysis).

In conclusion, groundwater contamination by dioxane is an emerging challenge with important implications for the management of chlorinated solvent plumes, including the need to revisit capture zones, monitoring networks, and treatment technology. Theoretical considerations and preliminary results suggest the practicality of MNA to manage large, dilute dioxane plumes. Thus, genetic biomarkers that reliably detect dioxane degradation potential and activity are needed to easily determine when MNA is an appropriate option, and to assess its performance. *This research provides us significant novel insight into the enzymatic and molecular basis of dioxane biodegradation, as well as selective and reliable functional gene probes to unequivocally assess dioxane biodegradation potential and quantify expression (i.e., activity). This is highly valuable to support site-specific decisions to select or reject MNA as a remedial activity and assess its performance.* Furthermore, the study about the effects of geographical parameters and biostimulants would provide valuable insight into site-specific scenarios where significant dioxane biodegradation might be expected. Our fluorescently labeled oligonucleotide probes and flow cytometry can be a cultivation-free strategy to separate and concentrate genus-specific subpopulations. The enrichment of two consortia and the identification of new dioxane degrading gene cluster suggests the widespread occurrence of dioxane degraders and responsible genes. At last, the new biomarker would minimize the false negatives of our previous one and

the preliminary results about the construction of reporter strain would facilitate future efforts to monitor dioxane degradation activities in a more convenient way.

14.2 Implications for Future Research/Implementation

14.2.1 Continue to investigate the distribution of dioxane degraders and dioxane degrading gene clusters.

In this project, we developed catabolic probes to target dioxane genes and found strong correlations with dioxane degradation rates. At the same time, we have shown the possibility of isolating dioxane degraders from environments with no previous exposure to dioxane. Therefore, it is very likely that more dioxane degraders exist in different environments considering the similar chemical structures of dioxane with other naturally occurring compounds in natural environments. Besides, after enrichment, dioxane degrading gene cluster became prevalent in our two consortia. Furthermore, almost all the identified gene clusters are in a plasmid. These imply the possibility that dioxane degrading genes may come from horizontal gene transfer. If proven, this could have an important scientific value for studying the distribution of gene clusters.

14.2.2 Utilize bioinformatic and machine learning tools to study dioxane degradation mechanisms.

With more and more dioxane degraders isolated and more genetic information of these degraders available, it is possible to study dioxane degradation in a high throughput way. For example, we could use bioinformatic tools to analyze these gene clusters to find the relationship of these gene clusters and their regulatory mechanisms. When more data is available for use, we may adapt machine learning tools to predict and to detect the potentials of some microorganisms to degrade dioxane. At that time, it will be much more efficient to run computational analysis first and then use the biological experiment to check. Also, this kind of analysis would be helpful for further genetic engineering work.

14.2.3 Use molecular biology tools to engineer dioxane degraders to gain better dioxane degradation.

The genetic information of dioxane degraders and advanced analyzing and predicting tools can make it possible to engineer dioxane degraders for faster and robust dioxane degradation. Many failures of current genetic engineering work are due to the lack of enough genetic information, especially about the regulation system. However, if we gather enough information on why dioxane degraders grow so slowly and the cells aggregate so much and how dioxane is degraded and regulated in bacteria metabolism, we could manipulate bacteria to make them more useful in bioaugmentation.

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Appendices

A. List of Scientific/Technical Publications

Articles in peer-reviewed journals

1. He, Y., Mathieu, J., Schwarz, C., Sun, R., & Alvarez, P. J. (2019). Insights to construct a reporter strain to assess dioxane degradation activity: Lessons learned and strategies to overcome technical challenges. (*In preparation*).
2. Da Silva, M.L.B., He, Y., Alvarez, P.J.J. (2019). Enhanced Natural Attenuation of 1,4-Dioxane in Flow Through Aquifer Columns Inoculated with *Pseudonocardia dioxivorans* CB1190. (*In preparation*).
3. Li, M., Yang, Y., He, Y., Mathieu, J., Yu, C., Li, Q., & Alvarez, P. J. (2018). Detection and cell sorting of *Pseudonocardia* species by fluorescence in situ hybridization and flow cytometry using 16S rRNA-targeted oligonucleotide probes. *Applied Microbiology and Biotechnology*, 102(7), 3375-3386.
4. He, Y., Mathieu, J., Da Silva, M. L., Li, M., & Alvarez, P. J. (2018). 1,4-Dioxane-degrading consortia can be enriched from uncontaminated soils: prevalence of *Mycobacterium* and soluble di-iron monooxygenase genes. *Microbial Biotechnology*, 11(1), 189-198.
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6. He, Y., Mathieu, J., Yang, Y., Yu, P., Da Silva, M. L., & Alvarez, P. J. (2017). 1, 4-Dioxane biodegradation by *Mycobacterium dioxanotrophicus* PH-06 is associated with a group-6 soluble di-iron monooxygenase. *Environmental Science & Technology Letters*, 4(11), 494-499.
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Conference or symposium abstracts

1. He, Y., Mathieu, J., & Alvarez, P. (2018). Isolation of novel 1, 4-dioxane degraders and investigation of responsible catabolic genes. *American Chemical Society (ACS) 256th National Meeting & Exposition*, August 19 - 23, 2018, Boston, MA. (Oral Presentation)
2. Yang, Y., Li, M., He, Y., Mathieu, J., & Alvarez, P. (2018). Discovering and sorting potential 1, 4-dioxane degrading bacteria by fluorescence *in situ* hybridization and flow cytometry using phylogenetic or functional oligonucleotide probes. *American Chemical Society (ACS) 256th National Meeting & Exposition*, August 19 - 23, 2018, Boston, MA. (Oral Presentation)
3. He, Y., Mathieu, J., & Alvarez, P. J. (2017) 1,4-dioxane biodegradation by *Mycobacterium dioxanotrophicus* PH-06 is associated with a group-6 soluble di-iron monooxygenase *SERDP-ESTCP Symposium*, November 28 - 30, 2017, Washington, D.C. (Poster Presentation)
4. He, Y., Mathieu, J., & Alvarez, P. J. (2017) 1,4-Dioxane-degrading consortia can be enriched from uncontaminated soils. *SERDP-ESTCP Symposium*, November 28 - 30, 2017, Washington, D.C. (Poster Presentation)
5. He, Y., Mathieu, J., Yang, Y., Yu, P., & Alvarez, P. J. (2017) A novel putative propane monooxygenase initiating metabolism of 1,4-dioxane. *American Society for Microbiology (ASM) Microbe Conference*, June 1-5, 2017, New Orleans, LA. (Invited Poster Talk)
6. He, Y., Liu, Y., Alvarez, P., & Li, M. (2017) Hindrance of 1,4-dioxane biodegradation in microcosms biostimulated with inducing or non-inducing auxiliary substrates. *American*

Society for Microbiology (ASM) Microbe Conference, June 1-5, 2017, New Orleans, LA. (Poster Presentation)

7. He, Y., Mathieu, J., da Silva, M. L., Li, M., & Alvarez, P. J. (2017) Microbial characterization of two 1,4-dioxane degrading bacteria consortia isolated from uncontaminated environments. The Fourth International Symposium on Bioremediation and Sustainable Environmental Technologies, May 22-25, 2017, in Miami, FL. (Poster Presentation)
8. He, Y., Mathieu, J., & Alvarez, P. J. (2017) Characterization of a novel putative propane monooxygenase initiating degradation of 1,4-dioxane. Remediation Technology Summit (RemTEC), March 7-9, 2017, Denver, CO. (Poster Presentation)
9. Li, M., Liu, Y., He, Y., Yang, Y., Mathieu, J., & Alvarez, P. (2016). Catabolic biomarkers for sensitive and fast quantification of 1, 4-dioxane biodegradation activities at impacted aquifers. American Chemical Society (ACS) 252nd National Meeting & Exposition, August 21-25, 2016, Philadelphia, PA. (Oral Presentation)
10. He, Y., Mathieu, J., Li, M., & Alvarez, P. J. (2016) Bench-scale Monitored Natural Attenuation (MNA) and bioaugmentation of 1,4-dioxane at a contaminated site. Emerging Contaminants Summit. March 1-2, 2016, Westminster, CO. (Poster Presentation)
11. Li, M., Ma, J., Mathieu, J., and Alvarez, P. J. (2014). Catabolic biomarkers as efficient forensic tools for monitoring natural attenuation and estimating bioremediation rates. Gordon Research Seminar: Environmental Sciences: Water. June 21, Holderness, NH. (Oral Presentation)
12. Li, M., Mathieu, J., and Alvarez, P. J. (2014). Catabolic biomarker design and validation for monitored natural attenuation and bioremediation of 1,4-dioxane in environment. The 9th International Conference on Remediation of Chlorinated and Recalcitrant Compounds. May 21, Monterey, CA. (Student-Award Winner Presentation)
13. Li, M., He, Z., Deng, Y., Mathieu, J., Yang, Y., Fiorenza, S., and Alvarez, P. J. (2013). Distribution of soluble di-iron monooxygenase (SDIMO) Genes in Arctic groundwater impacted by 1,4-dioxane. The 2nd International Symposium on Bioremediation and Sustainable Environmental Technologies. June 12, Jacksonville, FL. (Oral Presentation)
14. Van Orden, E. T., Li, M., and Alvarez, P. J. J (2013). Microcosm assessment of aerobic intrinsic bioremediation and mineralization potential for three 1,4-dioxane-impacted sites. The 2nd International Symposium on Bioremediation and Sustainable Environmental Technologies. June Jacksonville, FL. (Poster Presentation)

B. Other Supporting Materials

Patents

- Li, M., Mathieu, J., & Alvarez, P. J. (2015). "Monitoring of 1, 4-dioxane biodegradation in various environments." *U.S. Patent Application No. 14/562,517*.

Dissertations and Theses

- Ya He, Ph.D. Dissertation, Rice University, 2019. "Isolation of 1,4-dioxane degraders and investigation of responsible catabolic genes."
- Mengyan Li, Ph.D. Dissertation, Rice University, 2013. "Genetic catabolic probes to assess the natural attenuation of 1,4-dioxane."
- E. Tess Van Orden, M.S. Thesis, Rice University, 2013. "Microcosm assessment of aerobic intrinsic bioremediation and mineralization potential for three 1,4-dioxane impacted sites."
- Mengyan Li, M.S. Dissertation, Rice University, 2010. "1,4-Dioxane biodegradation at low temperatures in Arctic groundwater samples."

Scientific or technical awards or honors

- **Ya He**, Student Paper Competition Winner for the Fourth International Symposium on Bioremediation and Sustainable Environmental Technologies (2017)
- **Mengyan Li**, Honor Award for the Excellence in Environmental Engineering and Science™ Competition in the category of University Research (2014), American Academy of Environmental Engineering and Sciences (AAEES)
- **Mengyan Li**, Student Paper Winner for the Ninth International Conference on Remediation of Chlorinated and Recalcitrant Compounds (2014)
- **Mengyan Li**, Geosyntec™ Student Paper Competition, 3rd place (2013)