

FINAL REPORT – PHASE I

Developing and Field-Testing Genetic Catabolic Probes
for Monitored Natural Attenuation of 1,4-Dioxane
with a One-Year Timeframe

SERDP Project ER-2300

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Pedro Alvarez
Mengyan Li
Jacques Mathieu
Rice University

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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 (dioxane) is a difficult task because dioxane can be recalcitrant to biodegradation, is not easily removed by volatilization or adsorption, and is highly mobile in groundwater. Monitored natural attenuation (MNA), which relies primarily on biodegradation, is often the most cost-effective approach to manage large and dilute groundwater plumes of priority pollutants, such as those formed by dioxane. However, the burden of proof that MNA is an appropriate solution lies on the proponent, which requires demonstration of the presence and expression of relevant biodegradation capabilities. For this one-year “Limited Scope” SERDP project, we seek to *develop catabolic gene probe(s) to quantify the presence and expression of dioxane biodegradation capacity* to support decisions to select or reject MNA at dioxane-impacted sites.

Approach. This project required a multidisciplinary multi-scale approach. We discerned biomarkers for dioxane-degrading genes at the molecular scale and developed probes to quantify them and assess MNA at the field scale. The first step was to identify genes responsible for initiating dioxane biodegradation. We identified the *dxmADBC* operon in the archetype dioxane metabolizer, *Pseudonocardia sp.* CB1190, using bioinformatics and transcriptomic analyses. Then, we developed a primer/probe set to target a putative dioxane monooxygenase gene and measure its concentration using *Taqman* chemistry. This process involved multiple sequence alignment of the four *thmA/dxmA* genes available on the NCBI database.

Results. Our serendipitous discovery that the critical nucleotide sequence of the key catabolic genes (coding for the active site of the enzymes) is highly conserved enables detection of multiple dioxane degraders. This allowed us to: 1) demonstrate that dioxane degraders are widespread in contaminated sites, 2) show that dioxane degradation activity in microcosms correlates with gene biomarker concentrations, and 3) prove that the concentration of catabolic biomarkers (and their relative abundance) increases when dioxane is consumed due to growth of specific degraders.

Real-time PCR using reference strain genomic DNA demonstrated the high selectivity (no false positives) and sensitivity of this probe (7,000 ~ 8,000 copies/g soil). Microcosm tests prepared with groundwater samples from 16 monitoring wells at five different dioxane-impacted sites showed that enrichment of this catabolic gene (up to 114-fold) was significantly correlated to the amount of dioxane degraded. A significant correlation was also found between biodegradation rates and the abundance of *thmA/dxmA* genes. In contrast, 16S rRNA gene copy numbers (a measure of total bacteria) were neither sensitive nor reliable indicators of dioxane biodegradation activity. Overall, these results suggest that this novel catabolic biomarker (*thmA/dxmA*) has great potential to rapidly assess the performance of natural attenuation or bioremediation of dioxane plumes.

Benefits. This project supports the rational selection of MNA over current, much more expensive treatment approaches (commonly pump and treat followed with advanced chemical oxidation) and provides a scientific basis for early cessation of active treatment when it is no longer removing dioxane faster than MNA. Use of this approach could result in a more cost-effective way to protect public and environmental health, as well as in multi-billion dollar savings to industry and state and federal governments. This work also responds to 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
1,1,1-TCA	1,1,1-Trichloroethane
TCE	Trichloroethene
ISCO	<i>In situ</i> Chemical Oxidation
DO	Dissolved Oxygen
TOC	Total Organic Carbon
THF	Tetrahydrofuran
DCM	Dichloromethane
GC/MS	Gas Chromatograph/Mass Spectrometer
PCR	Polymerase Chain Reaction
DGGE	Denaturing Gradient Gel Electrophoresis
LSC	Liquid Scintillation Counter
MTBE	Methyl Tertiary Butyl Ether
HEAA	2-Hydroxyethoxyacetic Acid

Chapter 1

Objectives

1.1. Introduction

1,4-Dioxane (dioxane) is a cyclic ether that is widely used as a stabilizer for chlorinated solvents (mainly 1,1,1-trichloroethane [TCA]) [1]. Consequently, dioxane is a common co-contaminant in thousands of sites impacted by chlorinated solvents in North America [2]. This is of significant concern because dioxane is a probable human carcinogen [3] and is subject to a stringent drinking water advisory level of 0.35 $\mu\text{g/L}$, corresponding to the 10^{-6} lifetime cancer risk [4]. Clean-up of dioxane-contaminated sites is a difficult task because of its potential recalcitrance to biodegradation, and its physicochemical properties preclude effective removal by volatilization or adsorption. Furthermore, dioxane exhibits very high mobility in groundwater and tends to migrate further and impact larger areas than the co-occurring chlorinated solvents [1]. This underscores the need for cost effective alternatives to manage large and dilute dioxane plumes.

Remediation of dioxane-impacted groundwater often relies on pump and treat approaches 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) [5-7]. However, in addition to the relatively high cost of such approaches, turbidity of the aqueous streams and hydroxyl radical scavengers significantly inhibit removal efficiencies [8]. *In situ* chemical oxidation (ISCO) of dioxane (e.g., with permanganate or persulfate) has also been tested at various sites [9-11]. 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) [12]. 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 [13].

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 [8, 14]. Furthermore, recent findings by our lab and others suggest that indigenous bacteria that can degrade dioxane might be more widespread than previously assumed [15-18]. 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. Hypothesis and Objectives

1.2.1. Objectives

This aim of this project was to advance our enzymatic and molecular understanding of dioxane biodegradation and *develop functional gene probe(s) to quantify the presence and expression of catabolic capacity* to support the assessment of the feasibility of MNA at dioxane-impacted sites. This study focused on aerobic processes that prevail along the diluted plume (e.g., near the source zone and at the leading edge) [8] and enable MNA even under oligotrophic conditions [15]. Specific tasks for this proposed work included to:

- 1. Discern genes that code for enzymes that initiate dioxane biodegradation in the model bacterium, *Pseudonocardia dioxanivorans* CB1190 (CB1190);***
- 2. Design probes to target conserved regions of gene sequences that code for these enzymes (likely monooxygenases); this would ultimately enable quantification of similar genes in other bacteria capable of dioxane metabolism or co-metabolism;***
- 3. Test these degenerate functional probes with reference strains that metabolize and co-metabolize dioxane (including negative controls) to determine probe selectivity and sensitivity; and***
- 4. Validate the probes with samples from multiple sites exhibiting dioxane degradation capabilities (verified by microcosm assays). This effort would involve determining whether (a) degradation activity correlates with***

genetic biomarker copy numbers, (b) these copy numbers increase when dioxane is consumed, and (c) the relative abundance of these copy numbers (determined as % of the total population) also increases relative to background samples due to the selective pressure exerted by dioxane.

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

- What are the catabolic genes associated with dioxane degradation by CB1190, which is the only annotated strain capable of mineralizing dioxane?
- Are there conserved regions on these genes (particularly in sequences coding for the active sites of enzymes that catalyze the initial biotransformation steps) that would also present in many other bacteria that degrade dioxane?
- Can such 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?
- Are the concentrations of these biomarkers significantly correlated to dioxane biodegradation activity so that intrinsic bioremediation rates could be reasonably estimated based on biomarker measurements?
- Do we need to base such correlations on analyses of aquifer material (because most subsurface microorganisms live attached to surfaces) or can we also reliably estimate biodegradation rates based on biomarker concentrations in groundwater samples (which are easier to collect and analyze)?

1.2.2. Hypothesis

To identify appropriate functional gene biomarkers and develop appropriate probes, we first need to understand the molecular and enzymatic basis of dioxane biodegradation and recognize (syllogistically) that (1) aerobic biodegradation of dioxane is likely initiated by oxygenases [16, 19, 20], (2) gene sequences coding for oxygenase subunits containing the “active sites” generally have conserved regions (common to multiple species) that could be targeted by degenerate probes [21], and (3) such degenerate probes based on dioxane oxygenase genes from a well-characterized indigenous strain are likely to also detect other dioxane degraders [22-24]. Thus, focusing on oxygenase genes from the model dioxane degrader *Pseudonocardia dioxanivorans* CB1190, whose genome has been sequenced [25], is a logical first step.

Note that several multi-component soluble di-iron monooxygenases (SDIMOs) are known to initiate dioxane degradation through co-metabolic pathways, including tetrahydrofuran monooxygenase (THF MO), toluene-2-MO, toluene-3-MO, toluene-4-MO, and soluble methane MO [13, 18]. Moreover, a putative dioxane monooxygenase (*dxm*) in CB1190 that possesses extremely high sequence similarity to the tetrahydrofuran monooxygenase (THF MO) in *Pseudonocardia sp.* K1 was recently annotated [25-27]. 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 [28]. Some of the hydrophilic amino acid residues in this region play an important role in substrate recognition and 'gating' [29, 30]. Thus, with the help of primary sequence alignments, specific active sites involved in dioxane biodegradation will be identified and degenerate probes will be designed and tested with known isolates and field samples.

We hypothesized that CB1190 and many other dioxane degraders use monooxygenases to initiate catabolism. Such bacteria would be enriched in the (aerobic) source area of many dioxane plumes relative to uncontaminated areas, providing evidence of intrinsic bioremediation. Furthermore, gene sequences coding for these monooxygenases have conserved regions that facilitate the design of reliable gene probes for monitoring natural attenuation. We also postulated that dioxane degradation rates, averaged over large spatial and temporal scales that are relevant to MNA, will be correlated to the concentration of specific degraders, which can be determined by targeting such functional gene biomarkers.

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

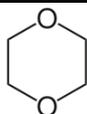
Chapter 2

Background

2.1. Introduction

1,4-Dioxane (dioxane) is a cyclic ether widely used as a stabilizer for chlorinated solvents, mainly 1,1,1-TCA [31]. Consequently, dioxane is a groundwater contaminant of emerging concern that is commonly found at sites impacted by chlorinated solvent spills [14]. Moreover, co-occurrence of this emerging contaminant with chlorinated solvents (~ 17% of probability) may delay the closure of on-going remedial projects or call for revisiting existing infrastructure.[32] However, unlike chlorinated solvents, dioxane is highly hydrophilic (Table 2.1) and experiences extraordinary mobility in the groundwater, leading to much larger regions of influence. Recently dioxane was included in the Final Third Drinking Water Contaminant Candidate List by U.S. EPA in September 2009 [33], due to its probable impact as human carcinogen (B2), as classified by the International Agency for Research on Cancer [34].

Table 2.1 Physicochemical properties of dioxane.

Property	Value	Reference
Chemical structure		-
Molecular weight	88.1 g/mol	[35]
Density	1.03 g/cm ³	[35]
Solubility	Fully miscible	[35]
log K _{ow}	-0.27	[36]
Henry's constant	4.88×10 ⁻⁶ atm m ³ /mol	[36]

Conventional physical-chemical treatment methods are marginally effective at removing dioxane from impacted sites. Because of its low Henry's Law constant (5×10^{-6} atm m³ mol⁻¹ at 20

°C) and highly hydrophilic nature ($\log K_{ow} = -0.27$) [35], dioxane is neither sufficiently volatile for air sparging nor efficiently absorbed onto activated carbon. Moreover, due to its small molecular weight of 88 g mol^{-1} , low-pressure reverse osmosis membrane may not be able to retain dioxane [37]. Advanced chemical oxidation (e.g., Fenton's process) and photocatalytic processes, utilizing hydrogen peroxide [38], zero-valent iron [39], titanium dioxide [40], ozone, electrolysis [41], and sonication [42] with or without UV irradiation can degrade dioxane in aqueous solution, but such techniques can be prohibitively expensive, and the contaminated groundwater needs to be pumped out from the subsurface for efficient treatment. Although plants such as hybrid poplars can assimilate and evapotranspire dioxane from aqueous solutions [43], the depth of contaminated groundwater typically exceeds root penetration and hinders the feasibility of phytoremediation. Furthermore, dioxane's heterocyclic ether structure makes it recalcitrant to biodegradation.

Monitored natural attenuation (MNA), which relies primarily on intrinsic bioremediation, is often a cost-effective approach to manage trace levels of priority pollutants [13]. 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 generally perceived to be rare in the environment, 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. This underscores the importance to assess the distribution of dioxane degradation capabilities in different environments.

2.2. Biodegradation of Dioxane

2.2.1. *Pseudonocardia dioxanivorans* CB1190

As one of the best characterized and researched dioxane metabolizer, *Pseudonocardia dioxanivorans* CB1190 is an Actinomycete first isolated from dioxane-contaminated industrial sludge by Dr. Parales and her colleagues in 1994 [27]. This strain is capable of sustaining growth with dioxane and other cyclic ethers compounds (e.g., 1,3-dioxane, THF, and tetrahydropyran [THP]) as sole carbon and energy source under aerobic condition [27]. Previous batch studies fed with radiolabeled and non-radiolabeled dioxane both determine that CB1190 can assimilate dioxane into biomass, as well as mineralize it to CO_2 (around 60% of degraded dioxane) [27, 44]. The cell yield for CB1190 is estimated as low as 0.01 to 0.09 mg protein/mg dioxane [13, 27, 45, 46]. And its optimum growth temperature is $30 \text{ }^\circ\text{C}$ [46].

Kinetic studies for dioxane degradation by CB1190 demonstrate a great fit with the Monod equation, with a maximum dioxane degradation rate (k_{max}) and half saturation concentration (K_s) calculated as $1.1 \pm 0.01 \text{ mg dioxane/mg protein/h}$ and $160 \pm 44 \text{ mg/L}$. [13] However, presence of chlorinated solvents (e.g., 1,1,1-TCA, and its abiotic breakdown product, 1,1-dichloroethene [1,1-DCE]) significantly diminishes and retards the dioxane transformation by CB1190 cells, although neither 1,1,1-TCA nor 1,1-DCE could serve as a carbon source for CB1190. However, such inhibitory effects are found to be reversible and positively correlated with the amount of exposed chemicals. Therefore, after removing these chlorinated compounds, dioxane degradation rebounded back as those from un-poisoned cells [47]. Moreover, brief

CB1190 has been used to augment phytoremediation and bioremediation in bench-scale studies. Kelly *et al.* observed significant enhancement of dioxane degradation and mineralization in soils planted with hybrid poplar trees. Such biodegradation process was further stimulated by adding THF, 1-butanol or root-extract, but neither glucose nor soil extract. The stimulation from THF is due to the enzymatic induction for monooxygenases that initialize cleavage of cyclic ethers, while 1-butanol is attributed to the growth of CB1190 as carbon and energy source [45]. Accordingly, dioxane degradation in microcosms prepared with groundwater and soil sampled collected at an Arctic site was also greatly boosted with amendment of CB1190, 1-butanol, and mineral salts. A simultaneous growth in biomass was observed at the same time. The degradation rate was fastest (0.16 ± 0.04 mg dioxane/mg protein/d) at the incubation temperature of 14 °C, and decreased drastically at 4 °C with a three-month lag period, suggesting that adaptation to cold weather is possible for CB1190 [51].

Following the development of advanced 454 and Illumina techniques, the genome of CB1190 was fully sequenced by the U.S. Department of Energy Joint Genome Institute (JGI) in 2011 [25]. The size of the genome is 7.44 Mb with an average G+C content of 73.1%, containing the circular chromosome (7.1 Mb), and three plasmids (192 kb, 137 kb, and 15 kb) [52]. More than 6,700 candidate protein-encoding genes were retrieved and predicted by searching against various protein databases, such as Pfam, KEGG, and COG. Replication of tRNA and rRNA genes was identified with copies of 46 and 3, respectively [25]. It is notable that several gene clusters encoding the bacterial multi-component monooxygenases are present in the genome of CB1190. The discovery of these monooxygenases is of great importance due to their potential roles in initiating degradation of xenobiotic compounds, especially dioxane and THF.

2.2.2. Other isolated dioxane bacterial degraders

Several other bacterial isolates (Table 2.3) have been identified as capable of using dioxane as their sole carbon and energy source, such as *Rhodococcus ruber* 219 [50, 53] and *Mycobacterium* sp. PH-06 [54]. Some of them exhibit desirable dioxane degradation characteristics relative to CB1190. For instance, the cell yield for *Pseudonocardia* sp. D17 is 0.22 mg protein/mg dioxane, which is 2.4 times as high as that observed for CB1190, suggesting D17 can more efficiently transform the carbon into biomass. Moreover, the half saturation concentration for D17 is 59.7 mg/L, which is only about one third of that for CB1190, indicating a higher affinity for dioxane attacking. In addition, D17 exhibits constitutive dioxane degradation ability even after grown on rich medium. However, dioxane degradation by CB1190 is greatly delayed if pre-grown in medium with carbon sources other than its inducers (e.g., THF and dioxane) [13, 55].

2.3. Soluble Di-iron Monooxygenases and Dioxane Degradation

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 [28]. According to the available genetic and enzymatic structural information to date, all SDIMOs are transcribed from a single cluster that is composed of four to six genes encoding functioning components. The four essential components include a large and a small subunit assembling into a high molecular

hydroxylating complex, an NADH-dependent reductase, and a small coupling cofactor (Figure 2.2) [28, 56]. Both genetic and enzymatic studies indicate the ligands of the carboxylate-bridged binuclear iron center interacting with substrates are all allocated at the large subunit of the hydroxylases. The carboxylate-bridged di-iron centers at the active site are highly conserved in the amino acid sequence (i.e., DE*RH).



Figure 2.2 Schematic view of the operon structure of *dxmADBC/thmADBC* gene clusters. A represents the *dxmA/thmA* genes encoding the large subunit of the hydroxylase; B represents the *dxmB/thmB* genes encoding the small subunit of the hydroxylase; C represents the *dxmC/thmC* genes encoding the coupling protein; D represents the *dxmD/thmD* genes encoding the NADH-dependent reductase.

In general, SDIMOs are divided into five groups, based on component arrangement, substrate specificity, and sequence similarity. These include phenol hydroxylases (group 1), toluene/benzene monooxygenases (group 2), methane/butane monooxygenases (group 3), alkene monooxygenases (group 4), and THF/propane monooxygenases (group 5) [28, 57]. Several SDIMOs (Table 2.4) are likely involved in the scission of the C-O bond for cyclic ethers such as dioxane and its structural analog, THF [13, 18, 26, 58]. Their relationship with dioxane biodegradation is discussed in detail below.

Table 2.2 Monooxygenases implicated in dioxane degradation

Gene Name	Monooxygenase (MO)	SDIMO Group	Representative Bacterial Strain	GenBank Accession No.
<i>Thm</i>	Tetrahydrofuran MO	5	<i>Pseudonocardia</i> sp. K1	AJ296087
<i>Prm</i>	Propane MO	5	<i>Rhodococcus</i> sp. RR1	HM209445
<i>Tom</i>	Toluene-2-MO	1	<i>Burkholderia cepacia</i> G4	AF319657
<i>Tbu</i>	Toluene-3-MO	2	<i>Ralstonia pickettii</i> PKO1	U04052
<i>Tmo</i>	Toluene-4-MO	2	<i>Pseudomonas mendocina</i> KR1	M65106
<i>Mmo</i>	Soluble Methane MO	3	<i>Methylococcus capsulatus</i> Bath	M90050

2.3.1. THF/Dioxane monooxygenases

To date, a total of four gene clusters encoding THF/dioxane monooxygenases have been identified (Table 2.5). All four gene clusters share the same arrangement for four gene components coding for a large hydroxylase subunit (62.5kDa), an NADH-dependent reductase (33.9 kDa), a small hydroxylase subunit (39.3 kDa), and a coupling cofactor (12.5 kDa) in sequence (Figure 2.2) [26].

Notably, a putative dioxane monooxygenase gene cluster (designated as *dxmADBC* in this dissertation) located in plasmid pPSED02 of the dioxane metabolizer *Pseudonocardia dioxanivorans* CB1190 has been identified [25-27]. This *dxm* cluster is highly homologous to the putative THF monooxygenase gene clusters for *Pseudonocardia sp.* K1, *Pseudonocardia sp.* ENV478 and *Rhodococcus sp.* YYL, based on the nucleotide sequence identity of each gene (e.g., > 97% for α subunit [Table 2.5]) as well as the arrangement of genetic components [26, 58, 59]. Moreover, among the eight predicted monooxygenases in CB1190, only the genes encoding this putative dioxane monooxygenase cluster showed significant up-regulation after amendment with dioxane compared to the controls fed with pyruvate [49]. Recently the gene clusters, *dxmADBC* from CB1190 and *thmADBC* from K1, were successfully cloned into a thiostreptin-inducible expression vector (pTip-QC2), and expressed in a heterologous host, *Rhodococcus* RHA1. Oxidation activity on both dioxane and THF was identified in the two RHA1 clones, confirming the essential role of THF/dioxane monooxygenases. However, HEAA accumulates as a terminal metabolite in these reactions, suggesting a different enzyme from CB1190 is responsible for the transformation of HEAA [60]. Additional studies using Northern blot [26], colorimetric naphthol assays [13], and RNA antisense knockout [61] have all implicated THF and dioxane monooxygenases as key enzymes that initiate dioxane catabolism through 2-hydroxylation.

2.3.2. Propane monooxygenases

Propane monooxygenases bear a close evolutionary relationship with THF/dioxane monooxygenases, and are grouped in the same subdivision (group 5), although they do not share the same substrate range. However, dioxane cometabolism has been observed with bacteria containing propane monooxygenases. For instance, propane-grown *Rhodococcus ruber* ENV425, and toluene-grown *Rhodococcus sp.* RR1 can rapidly remove dioxane in aqueous solution with degradation rates of 0.01 mg/hr/mg TSS, and 0.38 mg/hr/mg protein, respectively [13, 58]. The degradation rate for the latter strain was twice as high as that of the metabolizer CB1190 (0.19 mg/hr/mg protein) [13].

A putative propane monooxygenase gene *prmA* was recently identified in RR1, whose nucleotide sequence and propane-inducible transcription are highly consistent with *prmA* from *Rhodococcus jostii* RHA1. Notably, an RHA1 *prmA* knockout mutant lacked the ability to metabolize propane [62]. Furthermore, a cultured propanotroph SL-D degraded at least 10 mg/L dioxane after the primary growth substrate (i.e., propane) was fully depleted [63]. Collectively, these reports implicate the potential of propane monooxygenases to initiate dioxane cometabolism. Hence, it is likely that Group-5 SDIMOs (e.g., THF/dioxane and propane monooxygenases) play a crucial role in dioxane biodegradation. However, whether bacteria harboring such SDIMOs are widely enriched at various dioxane-impacted sites is unknown.

2.3.3. Toluene monooxygenases

Co-metabolic dioxane oxidation activity has been demonstrated in three bacterial strains after growth with toluene, including *Burkholderia cepacia* G4, *Ralstonia pickettii* PKO1, *Pseudomonas mendocina* KR1. These strains are known for their capability to hydroxylase the benzene ring of toluene by three different toluene monooxygenases through *ortho*-, *meta*-, and *para*-oxidation, respectively [13]. Although with much lower transformation rates, dioxane

degradation was also observed by recombinant *E. coli* strains expressing these toluene monooxygenases, confirming their catabolic capacity of initializing the oxidation of dioxane. It is also very interesting that bacteria harboring both toluene-3 and toluene-4-monooxygenase genes exhibited above two times higher degradation activities compared to the ones expressing toluene-2-monooxygenases [13]. This is possibly linked with the substrate preference or affinity for different groups of SDIMOs, because the toluene-2-monooxygenase from G4 actually belongs to the group of phenol hydroxylases (group 1), while the other two toluene monooxygenases are group-2 SDIMOs. Therefore, their components, substrate range, and enzymatic activities varied greatly from each other.

2.3.4. Soluble methane monooxygenases

Soluble methane monooxygenases (sMMOs), other than particulate methane monooxygenases (pMMOs), are known for their capability of fortuitously transforming a wide range of organic compounds, such as *n*-alkanes, *n*-alkenes, aromatic and alicyclic compounds, and chlorinated solvents (e.g., trichloroethylene [TCE]) [64-66]. In the absence of copper, *Methylosinus trichosporium* OB3b is able to express soluble methane monooxygenase and degrade more than 60% of dioxane (500 µg/L as the initial concentration) within 40 min after pre-grown with methane. [13]

Methanotrophic bacteria are ubiquitous in environment as the key players in local and global carbon cycling. It is plausible to use methane as a cost-efficient auxiliary substrate to biostimulate the methanotrophs to mitigate a large diluted dioxane plume. This proposed biostimulation treatment can be further facilitated by various biological molecular tools available to monitor abundance of both type I and type II methanotrophs in complex environmental samples, including qPCR, fluorescence in situ hybridization (FISH) and flow cytometry [67-69]. However, the risk of explosion and inhibition from copper may prohibit the efficiency of the biostimulation of sMMOs in environment, requiring additional attention and consideration before implementation of any *in situ* remediation strategies.

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 [51]. 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 2.4). 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 [70]. 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.

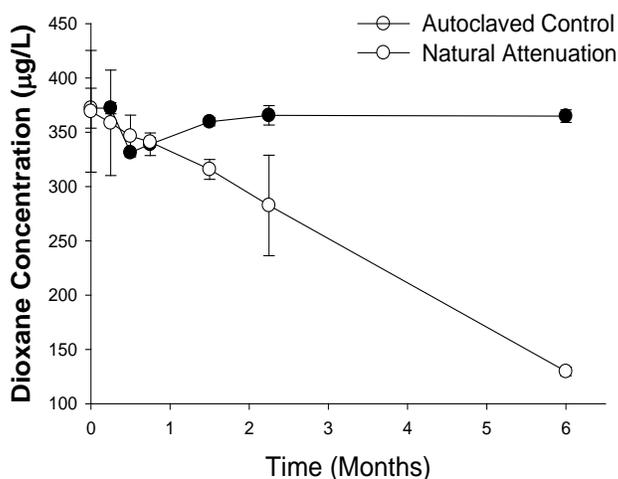


Figure 2.3 Dioxane degradation in natural attenuation microcosms at 14 °C. [51]

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 [71]. 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 [72]. Functional gene array (i.e., GeoChip) analysis of Arctic groundwater revealed that various dioxane-degrading SDIMO genes were widespread (Figure 2.4), 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 2.5). 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 ^{14}C -labeled dioxane showed that the highest mineralization capacity ($6.4 \pm 0.1\%$ $^{14}\text{CO}_2$ 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.

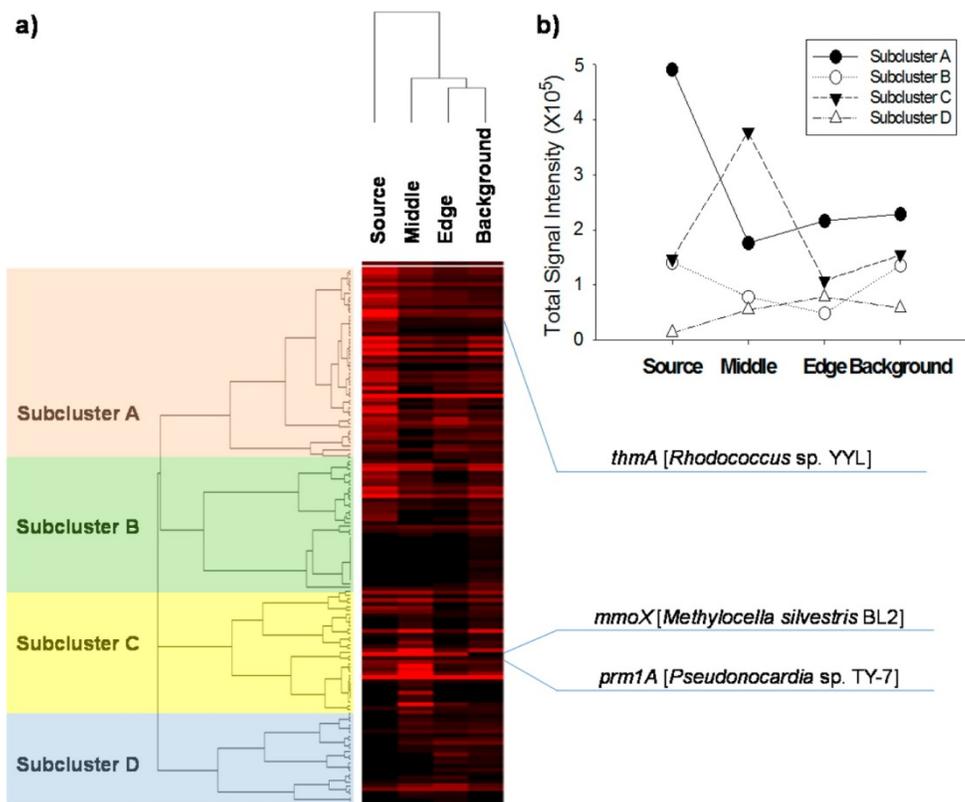


Figure 2.4 Hierarchical cluster analysis of SDIMO genes detected by GeoChip 4.0. Red indicates positive averaged signal intensities of the replicates at each sampling location, and black indicates intensities below background. Representative organisms for selected genes are depicted on the right of the 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 [72])

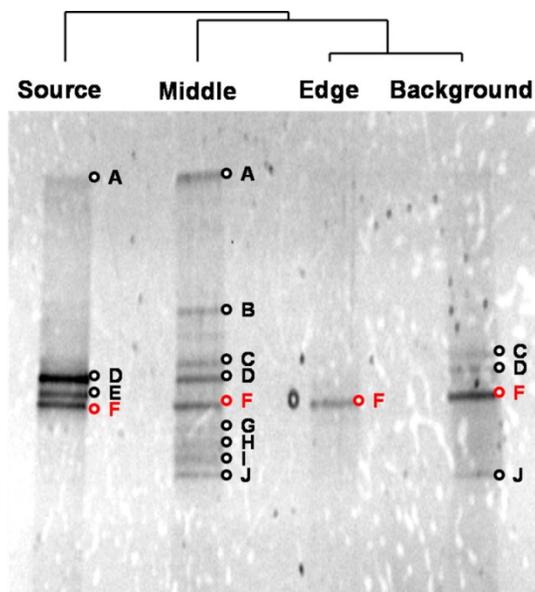


Figure 2.5 DGGE gel photograph showing the heterogeneous presence of SDIMOs by nested PCR (NVC58 and NVC65 as the primers for the first PCR set and then NVC57 + GC clamps and NVC66 as the primers for the second PCR set). Band F (highlighted in red) was found at all four sampling locations and corresponds to a *thmA*-like gene.

A recent research by Chiang and her coworkers studied the potential of natural attenuation of dioxane and TCE at a site located in Arizona using various ecological molecular tools [73]. Phospholipid fatty acid analysis associated with stable isotope probes (PLFA-SIP) demonstrated a significant amount of ^{13}C from dioxane incorporated into biomass in the samples heavily impacted by dioxane (340 $\mu\text{g/L}$). However, attempts to prove a clear correlation between SDIMO genes and dioxane or TCE degradation activity were failed, since only genes encoding monooxygenases that co-metabolize dioxane or TCE, such as phenol hydroxylases and toluene monooxygenases, were investigated in their study. This urges the needs to develop specific genetic biomarkers targeting the essential THF/dioxane monooxygenases in environmental samples, and provide unequivocal evidence to link the abundance of the indigenous degraders to the intrinsic biodegradation activity in environment.

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 [27]. Although an increasing number of microbes have been identified as their ability to transform dioxane [13, 16, 50, 54, 74], CB1190 still serves as the model bacterium, since it can mineralize dioxane to CO₂ as well as assimilate it into biomass [27, 44]. 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 [44, 49].

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) [51].

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/RNANase 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 4.1) 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 4.1 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 [25], 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 *tmo4ABCDEGF*), one putative propane monooxygenase (*prmABCD*), and one putative THF/dioxane monooxygenase (*dxmADBC*). The proposed components of each gene cluster were listed in Table 4.2, and their arrangements were visualized in Figure 4.1. It is notable that all the predicted monooxygenases other than the putative THF/dioxane monooxygenase are located in the chromosome of CB1190.

Table 4.2 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 the α subunits of SDIMOs are highlighted in yellow.

Gene ID (Psed_)	0629	0630	0631	0632			
Annotation in GenBank	methane monooxygenase	ferredoxin--NAD(+) reductase	methane/phenol/toluene hydroxylase	monooxygenase component MmoB/DmpM			
Paired Putative Genes Gene ID (Psed_)	<i>prmA</i> 0766	<i>prmB</i> 0767	<i>prmC</i> 0768	<i>prmD</i> 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 Gene ID (Psed_)	<i>dmpP</i> 0810	<i>dmpO</i> 0811	<i>dmpN</i> 0812	<i>dmpM</i> 0813	<i>dmpL</i> 0814	<i>dmpK</i> 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 Gene ID (Psed_)	<i>tmo1F</i> 1155	<i>tmo1C</i> 1156	<i>tmo1E</i> 1157	<i>tom1D</i> 1158	<i>tmo1B</i> 1159	<i>tmo1A</i> 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 Gene ID (Psed_)	<i>tmo3A</i> 1436	<i>tmo3B</i> 1437	<i>tmo3C</i> 1438	<i>tmo3D</i> 1439	<i>tmo3E</i> 1440	<i>tmo3F?</i> 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 Gene ID (Psed_)	<i>tmo2A</i> 6056	<i>tmo2B</i> 6057	<i>tmo2D</i> 6058	<i>tmo2E</i> 6059	<i>tmo2C</i> 6060	<i>tmo2F</i> 6061	6062
Annotation in GenBank	ferredoxin--NAD(+) reductase	dehydrogenase GroES domain-containing protein	methane/phenol/toluene hydroxylase	monooxygenase component MmoB/DmpM	hypothetical protein	Toluene-4-monooxygenase system B	Phenol 2-monooxygenase
Paired Putative Genes Gene ID (Psed_)	<i>tmo4F</i> 6976	<i>tmo4G?</i> 6977	<i>tmo4E</i> 6978	<i>tmo4D</i> 6979	<i>tmo4C</i>	<i>tmo4B</i>	<i>tmo4A</i>
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>			

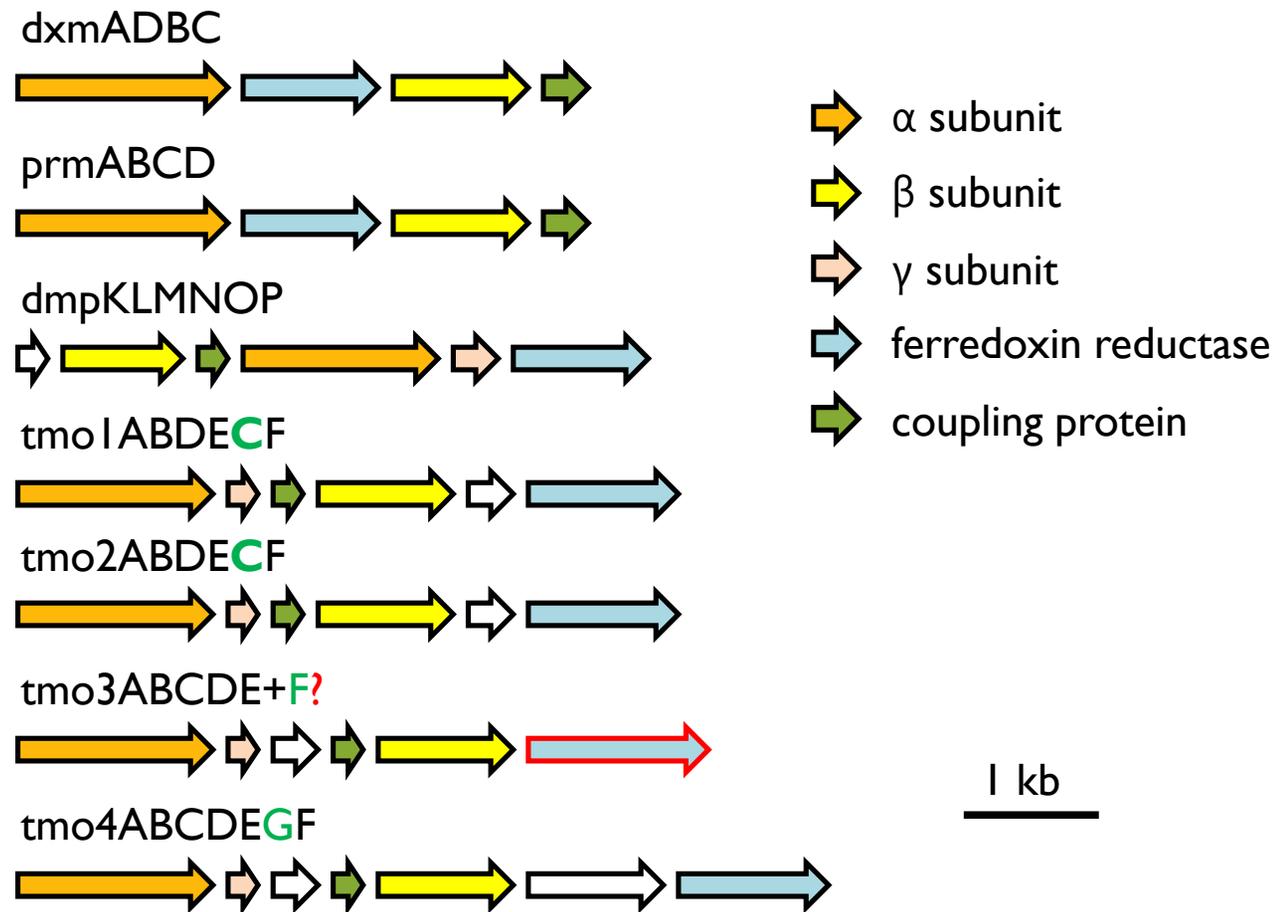


Figure 4.1 Organization of seven monooxygenase gene clusters. Functioning ORFs are indicated by block arrows (sized to scale) below their predicted operon name. Key components of SDIMOs are highlighted in different colors.

Table 4.3 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 [16, 26, 75]. 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 [17]. 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 [21, 56]. Phylogenetic analysis (Figure 2.3) 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 *prmIABCD* 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 *prmIABCD* 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 [76]. 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 [17]. 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 [17].

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 [77]. 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 4.3). 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) [78]. 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.

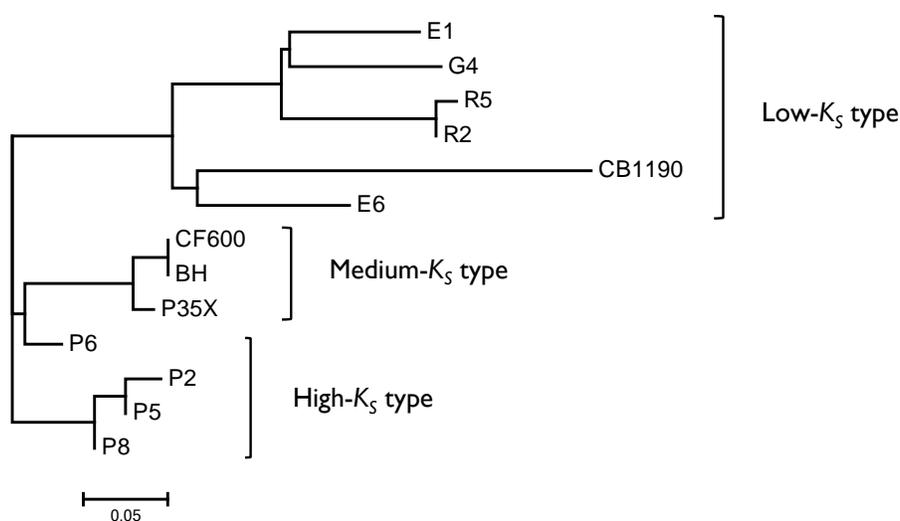


Figure 4.2 Phylogenetic analysis of CB1190 and several phenol-degrading bacteria according to the amino acid sequences of their α subunits. The bar represents 0.05 substitution per site.

Figure 4.2 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 [79]. 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 [80-82]. 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 [83]. 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 [25, 84-86], 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 [86]. For *tmo3* in Figure 4.1, 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 [87]. 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 [27]. 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 4.2) in the dioxane, THF, and glucose treatment compared with the acetate treatment as experimental control.

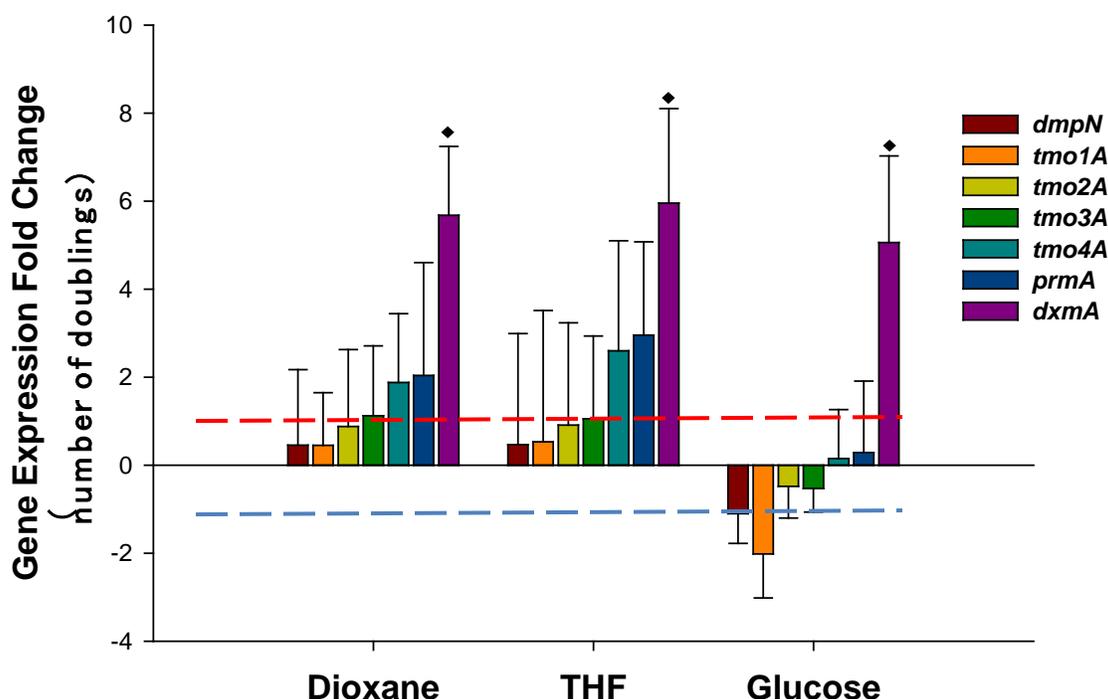


Figure 4.3 Expression of genes encoding α subunits of the seven putative SDIMOs by CB1190 fed with various substrates. The $2^{-\Delta\Delta CT}$ method was used to quantify differential gene expression [88]. Results were normalized to the control treatment (fed sodium acetate as sole carbon source). 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 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 [16, 26, 49]. Moreover, all four components of this putative dioxane monooxygenase cluster were confirmed to be up-regulated after amendment with dioxane compared with pyruvate control according to both transcription microarray (*dxmD*, *dxmB*, and *dxmC*) and RT-qPCR (*dxmA*) [49]. 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 [13]. 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 [17]. 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 [44].

Overall, our results support our hypothesis that the dioxane monooxygenase (*dxmADBC*) 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 [13]. 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, 51, 71-73]. 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) [89-97]. 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 6.1) was chosen allowing a nucleotide mismatch not greater than 1, 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 6.1 Properties of the primers and probe targeting *thmA/dxmA* genes.

Probe/Primer	Name	Sequence (5'-3')	Size	GC Content	T _m
<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 6.2). 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 5 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 $\mu\text{g/L}$) 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 HgCl_2 (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 $\times 10,000$ g for 20 min. Total microbial genomic DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio, Carlsbad, CA). The eluted DNA (100 μL) was further purified and concentrated to 16 μL 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 μL of undiluted DNA (or 1 ng/ μL diluted bacterial genomic DNA), 300 nM of the forward and reverse primers, 150 nM of the fluorogenic probe, 10 μL of TaqMan universal master mix II (Applied Biosystems, Foster City, CA), and DNA-free water to reach a total volume of 20 μL . The qPCR was performed with a 7500 Real-Time PCR system (Applied Biosystems, Foster City, CA) using the following temperature program: 50 $^\circ\text{C}$ for 2 min, 95 $^\circ\text{C}$ for 10 min, and 40 cycles of 95 $^\circ\text{C}$ for 15 s and 60 $^\circ\text{C}$ for 1 min. Serial dilutions (10^{-4} - 10^1 ng DNA/ μL) 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 [25, 52] and 9.124×10^{14} bp/ μg (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 6.1) were generated using series dilution of standard CB1190 DNA samples (10^{-4} - 10^1 ng genomic DNA/ μL) 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 6.2) DNA extraction recoveries ranged from 2.3 to 48.9 %. Similar recovery ranges are commonly reported for soil DNA extractions [92, 96, 102], with the lower values reflecting sequential elution and residual impurities that hinder *Taq* polymerase reactions [103].

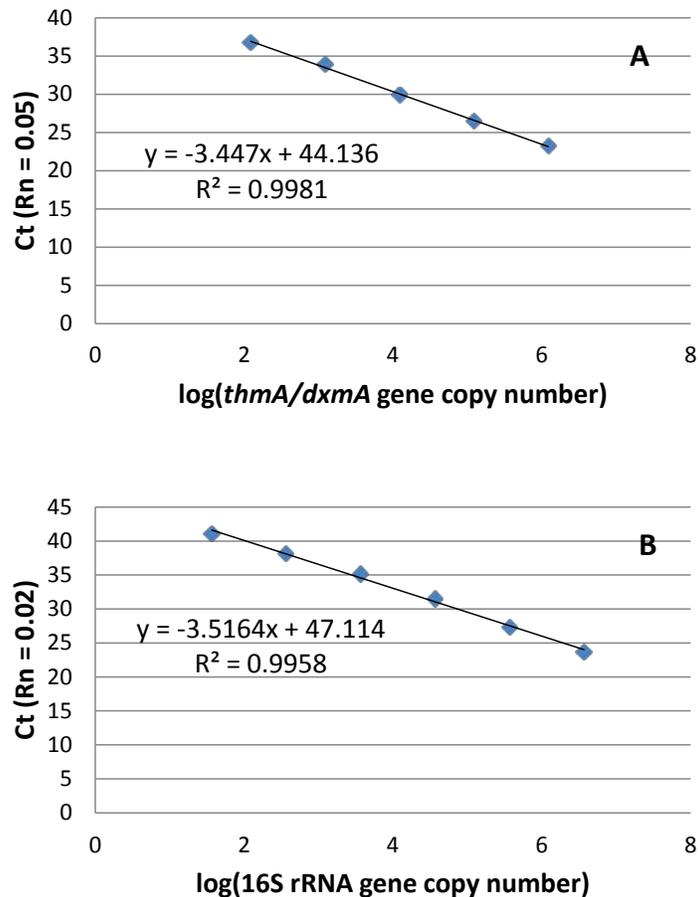


Figure 4.1. Q-PCR calibration curves for *thmA/dxmA* genes (A) and 16S rRNA (B).

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 S1), 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 μ g/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 (Figure 6.1) [28, 56]. 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 [28, 104-106]. 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 6.3) 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.

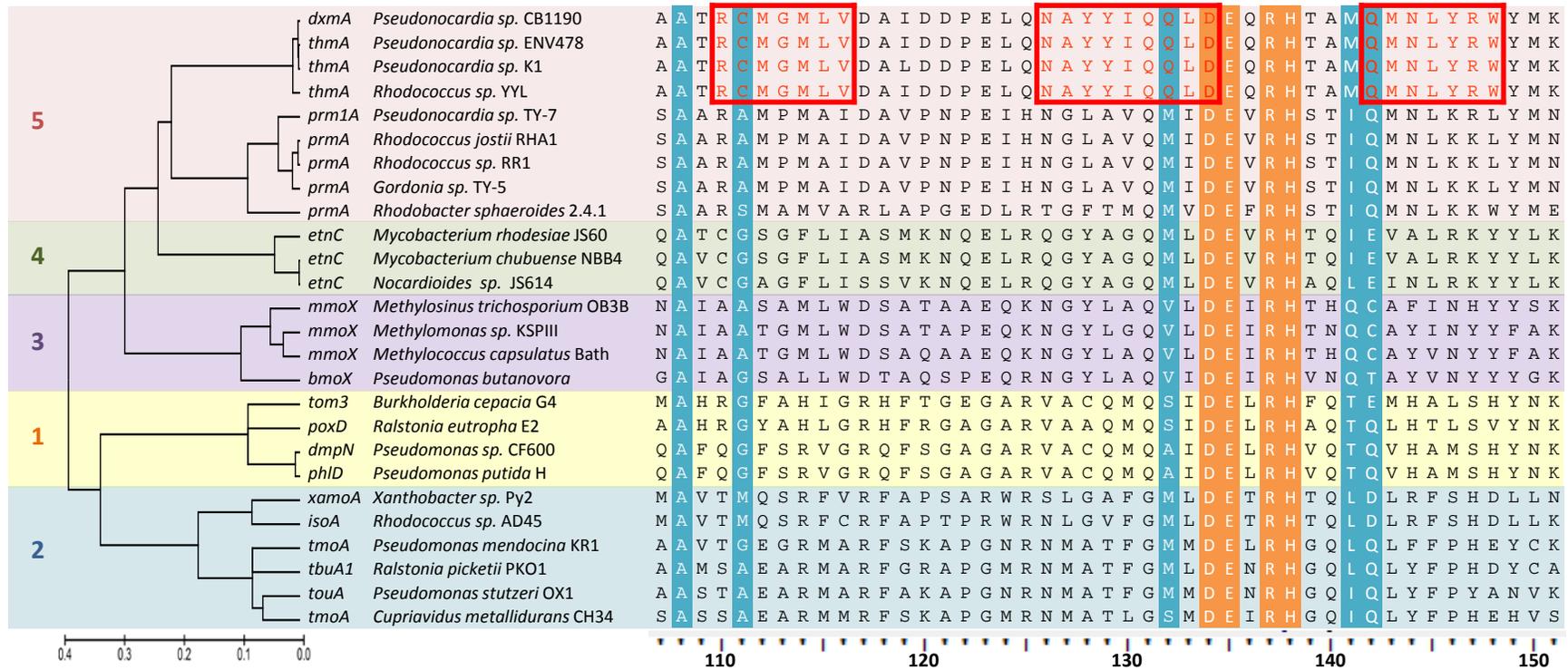


Figure 4.2 Alignment of the deduced amino acid sequences corresponding to the large hydroxylase of SDIMOs clustered by subfamily. The amino acid residues selected for biomarker design are highlighted in red and circled with rectangular. The conserved di-iron center (i.e., DE*RH) was highlighted in orange, 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 tetrahydrofuranoxydans</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).[107] 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 ($11.1 \pm 0.7 \mu\text{g/L/week}$) in microcosms prepared with the source zone samples at the Alaska site were comparable with the ones previously reported by our group (approximately $9.8 \mu\text{g/L/week}$) [51], 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% [108], 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 dioxane[13, 27, 45]) and other dioxane metabolizers, such as *Mycobacterium* sp. D11 (0.18 mg protein/mg dioxane [55]). 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/dhmA* probe.

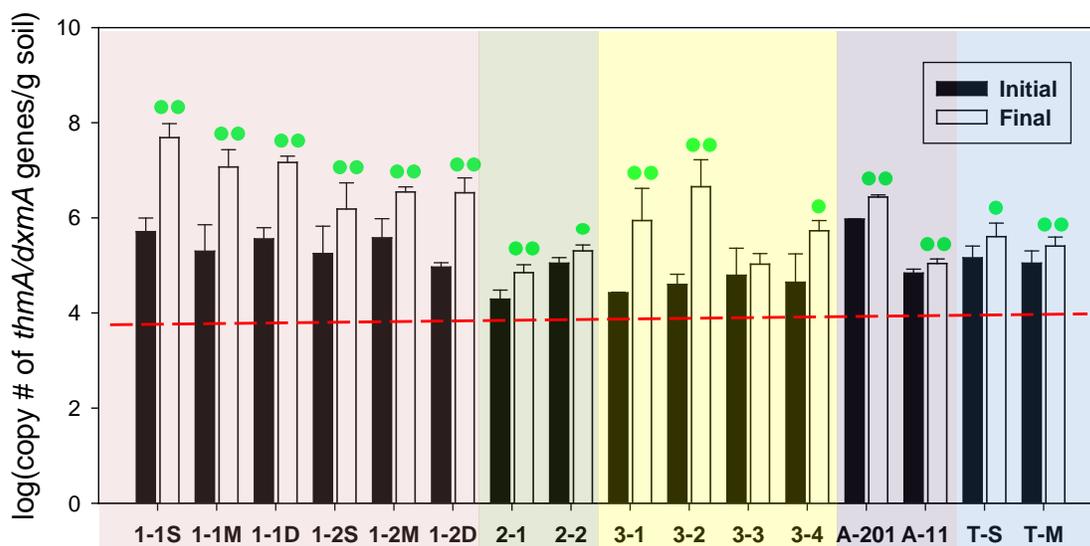


Figure 4.3 Detection of copy numbers of *thmA/dxmA* genes in microcosms over three to five months' incubation. The red dot line represents the estimated value of the MDL. Double green dots indicate an increase with p value lower than 0.05, and a single green dot indicates an increase with p value lower than 0.1.

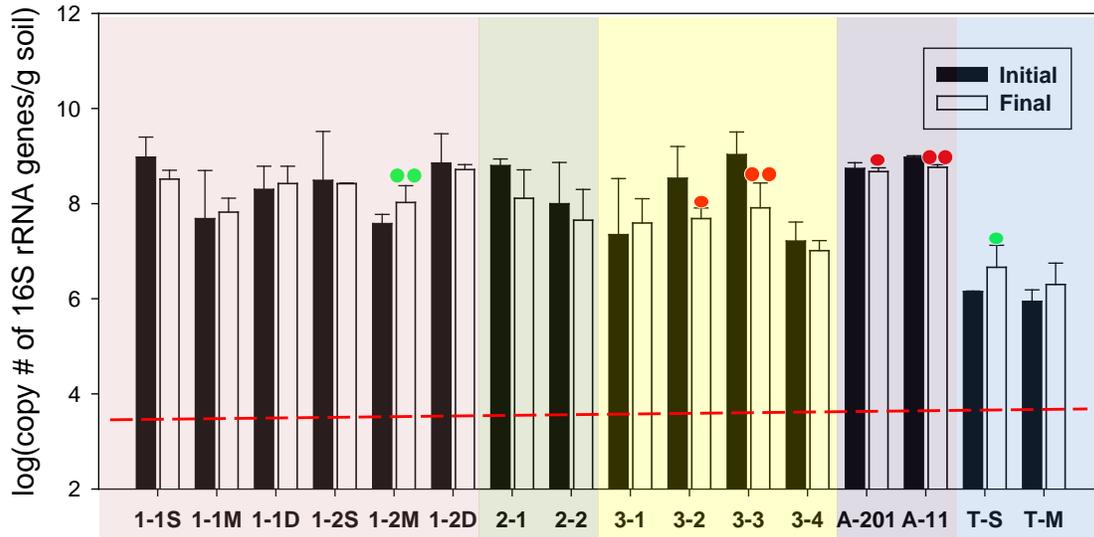


Figure 4.4 Detection of copy numbers of 16S rRNA genes in microcosms over three to five months' incubation. The red dot line represents the estimated value of the MDL. Double green dots indicate an increase with p value lower than 0.05, and a single green dot indicates an increase with p value lower 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.

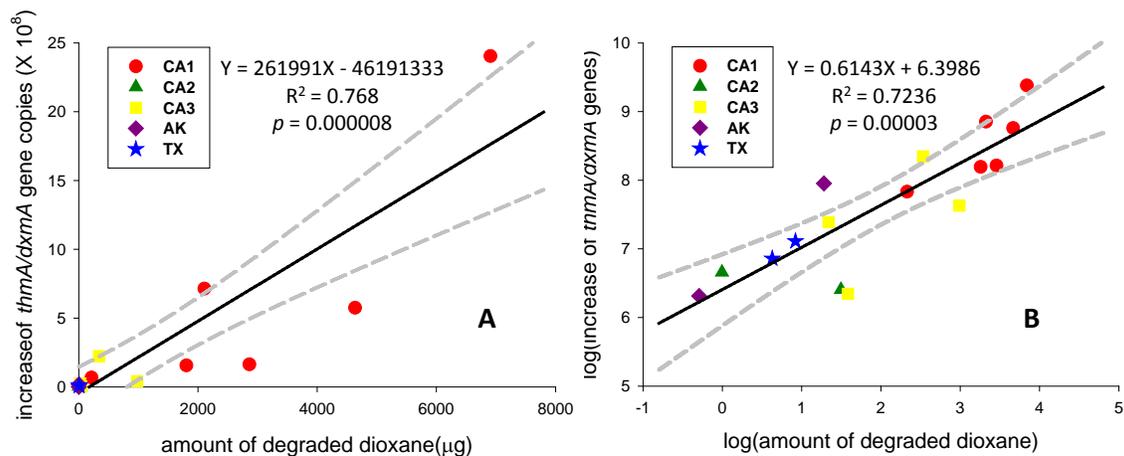


Figure 4.5 Correlation between the amount of consumed dioxane (μg) and the increase of *thmA/dxmA* gene copy numbers in microcosms on a normal (A) and logarithmic (B) scale. The slope of the regression line of the left graph (A) was used to estimate the cell yield of dioxane by indigenous microbial degraders. The solid line represents the least square regression; the dashed lines represent the 95% confidence envelope.

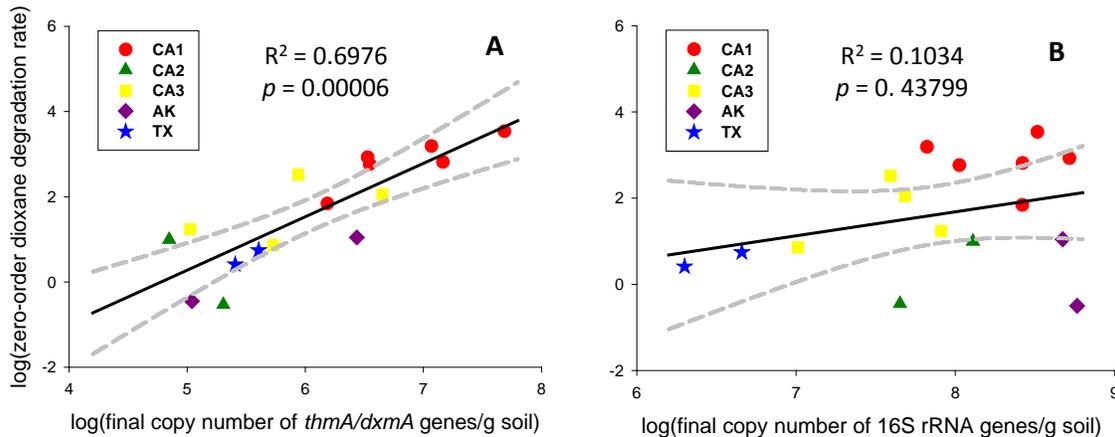
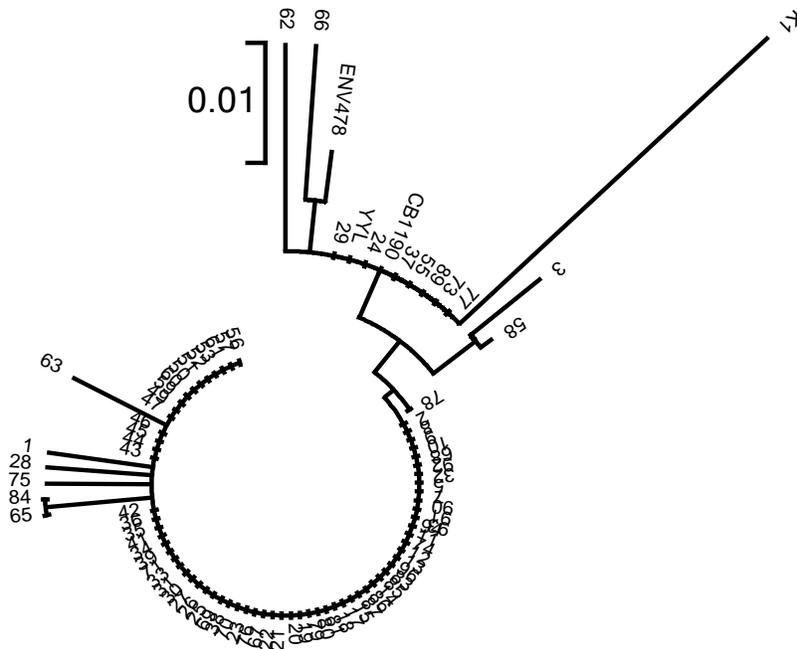


Figure 4.6. Correlation between zero-order dioxane degradation rates ($\mu\text{g/L/week}$) and final copy numbers of *thmA/dxmA* (A) but not 16S rRNA (B) genes observed per gram of aquifer materials added in microcosms for various sites. The solid lines represent the least square regression; the dashed lines represent the 95% confidence envelope.



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To further verify that amplification products from the complex environmental samples were actually 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 6.8). All sequences exhibited high identity with previously reported *thmA/dxmA* genes ($\geq 95\%$) [16, 25, 26, 59], 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

Summary and Conclusions

Dioxane is highly recalcitrant to bioremediation, and its hydrophilic nature greatly inhibits efficient removal using traditional treatments, such as volatilization or adsorption. Yet, newly-isolated dioxane degraders from dioxane-contaminated samples imply the possibility of natural attenuation of this xenobiotic compound. As one of the most cost-efficient approaches to manage large and dilute groundwater plumes of priority pollutants, such as those formed by dioxane, MNA relies on demonstration of the presence and expression of relevant biodegradation capabilities. As dioxane is 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). Reverse transcription quantitative polymerase chain reaction (RT-qPCR) results 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 controls fed with acetate ($p < 0.05$). And the expression doubling fold changes were 5.7 ± 1.6 and 6.0 ± 2.1 , respectively. These results corroborate the essential role of this dioxane monooxygenase in dioxane degradation.

Thus, a primer/probe set was developed to target bacterial genes encoding the large hydroxylase subunit of a putative tetrahydrofuran/dioxane monooxygenase (an enzyme proposed to initiate dioxane catabolism), using *Taqman* (5'-nuclease) chemistry. This effort relied on multiple sequence alignment of the four *thmA/dxmA* genes available on the NCBI database. The probe targets conserved regions surrounding the active site, thus enabling detection of multiple dioxane degraders. Real-time PCR using reference strain genomic DNA demonstrated the high selectivity (no false positives) and sensitivity of this probe (7,000-8,000 copies/g soil). Microcosm tests prepared with groundwater samples from 16 monitoring wells at five different dioxane-impacted sites showed that enrichment of this catabolic gene (up to 114-fold) was significantly correlated to the amount of dioxane degraded. A significant correlation was also found between biodegradation rates and the abundance of *thmA* genes. In contrast, 16S rRNA gene copy numbers were neither sensitive nor reliable indicators of dioxane biodegradation activity. Overall, these results suggest that this novel catabolic biomarker (*thmA/dxmA*) has great potential to rapidly assess the performance of natural attenuation or bioremediation of dioxane plumes.

This study comprehensively evaluated the essential roles of THF/dioxane monooxygenases in dioxane degradation in dioxane-degrading bacterial strains and environment samples. The development of the novel catabolic biomarker (*thmA/dxmA*) is of great research and engineering value to unequivocally assess both dioxane biodegradation potential and activity for enhanced MNA forensics.

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.*

Implications for Future Research/Implementation

This study expanded our current understanding of dioxane biodegradation and provided a simple and rapid forensic tool for the assessment of dioxane natural attenuation and bioremediation. However, validation and delineation of the capabilities of this approach (e.g., selectivity and sensitivity) and ability to infer biodegradation rates is still required. Potential directions for future research include:

A) Dioxane attenuation survey with designed *thmA/dxmA* biomarker

Till now, our designed biomarker has been successfully used to determine the abundance of *thmA/dxmA* genes in microcosms prepared with groundwater and soil/sediment samples from more than 20 monitoring wells from five dioxane-impacted sites located in three different US states. However, this is very limited compared to thousands of sites impacted by chlorinated solvents in US where dioxane might co-exist. Therefore, a dioxane attenuation survey with a larger scale of sampling locations is needed to evaluate the spatial and temporal distributions of dioxane degrading genes and estimate how site-specific factors (e.g., nutrient and electron acceptor influx, redox conditions, pH, and temperature) affect intrinsic bioremediation of dioxane at various sites.

Further, our catabolic biomarkers were designed based on nucleotide sequences from only four *thmA/dxmA* genes available on NCBI database. Although the *thmA/dxmA* genes are highly conserved, as additional *thm/dxm* genes are discovered, this designed probe/primer set may need to be reevaluated and adjusted (optimized) to incorporate new information.

B) Isolation of non-cultivable dioxane degraders using fluorescence activated cell sorting

Isolating and characterizing bacteria capable of degrading dioxane is important to understand their metabolic and physiological idiosyncrasies and enhance biodegradation processes. However, this is not an easy task if one relies on traditional isolation techniques (e.g., enrichment and serial dilution for separating colonies on plates) due to the fastidious nature of many indigenous organisms. In general, less than 5% of known bacteria can grow on plates. We can overcome this critical limitation by using fluorescence activated cell sorting (a way to separate bacteria based on their optical properties) in conjunction with fluorescent rRNA and mRNA probes as well as FISH and FISH-labeling techniques [109, 110].

C) Gene bioaugmentation

Bioremediation can be a cost-effective and practical approach for removing persistent organic compounds, xenobiotics, and emerging contaminants from soil or groundwater, however the indigenous microbial population at a given site may not possess the appropriate catabolic enzymes to effectively degrade the contaminants. To facilitate degradation in such circumstances, contaminated sites may be inoculated with an exogenous microbial strain or consortium that harbors genes involved in catabolism of the contaminant. This strategy, known as bioaugmentation, has been demonstrated to enhance the biodegradation of dioxane at various tested sites based on our microcosm studies. However, the initial rapid dioxane degradation rates might not be sustained for long term duration. For instance, the observed degradation rates in microcosms prepared with samples collected in the source zone of the Midland site considerably slowed down 1 month post-inoculation. This can be explained by the low affinity the amended bacteria, *Pseudonocardia dioxivorans* CB1190, has for dioxane. The K_S value (i.e., the dioxane concentration at which the specific dioxane degradation rate is one-half of its maximum) of this bacterial strain CB1190 is as high as 160 mg/L [13], which is 3200 times greater than the highest dioxane concentration (i.e., ~ 200 μ g/L) detected at the site. Thus, CB1190 may not be able to

sustain growth and degradation activity at such low levels of dioxane. In addition, as an actinomycete, CB1190 tends to form clumps in the nutrient media. It would be very challenging to effectively distribute this microorganism into the subsurface.

Gene bioaugmentation may circumvent the problems associated with cell bioaugmentation. This approach involves the introduction of catabolic genes into the environment via mobile genetic elements. This is now possible since we have identified a highly conserved gene sequence for the active site of the enzyme initiating dioxane biodegradation. A basic premise of its application is that indigenous microbial soil/groundwater species that uptake the introduced genes have already undergone strong selective pressures to optimize survivability in their ecological niche, so should be more fit than an exogenous species. However, lab-scale efforts are required to synthesize conjugative plasmids that contain the essential genes involved in dioxane degradation and assess efficacies of gene bioaugmentation in several transformation circumstances (e.g., chemically and electronically) to enhance the gene uptake and transfer frequencies that are sufficient to increase contaminant catabolism a significant extent.

D) Bio-stimulation of dioxane biodegradation by short-chain alkanes

As common biostimulators of oxygenase-harboring bacteria, methane and propane have been widely used as the auxiliary carbon source for *in situ* remediation of TCE and other organics [111]. Several previous studies provide evidence demonstrating the capability of some methanotrophs and propanotrophs on dioxane co-metabolism. However, several issues remain unclear, such as i) the roles of methanotrophs and propanotrophs in mitigating dioxane plume expansion at various sites, ii) the exposure factors (e.g., amount and duration) to optimize treatment efficiency, and iii) the influence of environmental variables on the co-metabolic degradation (e.g., the inhibitory effects from copper on soluble methane monooxygenases). Forensic tools facilitated with advanced microbial molecular techniques are required to iron out these site-specific concerns.

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Appendices

A. Supporting data

Nucleotide sequences of the partial *thmA/dxmA* genes from clone libraries constructed using the genomic DNA isolated from soil samples collected in Microcosm 1-1S at week 20.

Transformed clones are designated with numbers from 1 to 96. Short sequences and untransformed clones are not included.

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B. List of Scientific/Technical Publications

Articles in peer-reviewed journals

1. Li, M., and P. J. J. Alvarez. "Untangling the multiple soluble di-iron monooxygenase genes and their degradation capabilities in *Pseudonocardia dioxanivorans* CB1190." (in preparation)
2. Liu, Y., M. Li, and P. J. J. Alvarez. "Evaluation of various auxiliary substrates on 1,4-dioxane biodegradation in groundwater at a site in Texas." (in preparation)
3. Li, M., Y. Yang, and P. J. J. Alvarez. "Shifting of microbial community structures at various sampling locations experiencing different levels of groundwater contamination by 1,4-dioxane, chlorinated solvents, and hydrocarbons." (in preparation)
4. Li, M., E. T. Van Orden, D. DeVries, X. Zhong, R. Hinchee, and P. J. J. Alvarez (2014). "Bench-scale biodegradation tests to assess natural attenuation potential of 1,4-dioxane at three California sites." (under revision)
5. Li, M., J. Mathieu, Y. Liu, E. T. Van Orden, Y. Yang, S. Fiorenza, and P. J. J. Alvarez (2014). "The abundance of tetrahydrofuran/dioxane monooxygenase genes (*thmA/dxmA*) and 1,4-dioxane degradation activity are significantly correlated at various impacted aquifers." Environmental Science and Technology Letters. 1 (1): 122–127.
6. Li, M., J. Mathieu, Y. Yang, S. Fiorenza, Y. Deng, Z. He, J. Zhou and P. J. J. Alvarez (2013). "Widespread distribution of soluble di-iron monooxygenase (SDIMO) genes in Arctic groundwater impacted by 1,4-dioxane." Environmental Science and Technology. 47(17): 9950–9958.
7. Li, M., P. Conlon, S. Fiorenza, R. J. Vitale and P. J. J. Alvarez (2011). "Rapid analysis of 1,4-dioxane in groundwater by Frozen Micro-Extraction with Gas Chromatography/Mass Spectrometry." Ground Water Monitoring and Remediation 31(4): 70-76.
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Conference or symposium abstracts

1. Li, M., J. Ma, J. Mathieu, and P. J. J. Alvarez (Jun 21, 2014). Catabolic biomarkers as efficient forensic tools for monitoring natural attenuation and estimating bioremediation rates. Gordon Research Seminar: Environmental Sciences: Water. Holderness, NH. (Talk)
2. Li, M., J. Mathieu, and P. J. J. Alvarez (May 21, 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. Monterey, CA. (Student-Award Winning Talk)

3. Li, M., Z. He, Y. Deng, J. Mathieu, Y. Yang, S. Fiorenza, J. Zhou and P. J. J. Alvarez (Jun 12, 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. Jacksonville, FL. (Talk)
4. Van Orden, E. T., M. Li and P. J. J. Alvarez (Jun 11, 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. Jacksonville, FL. (Poster)

C. Other Supporting Materials

Patents

- *Li, M., J. Mathieu, and P. J. J. Alvarez. "A catabolic gene probe for assessing the feasibility of monitored natural attenuation at 1,4-dioxane-impacted sites." US Patent Application Number 61912304 (Filed in Dec 2013)*

Dissertations and Theses

- *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

- *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)*
- *Student Paper Winner for the Ninth International Conference on Remediation of Chlorinated and Recalcitrant Compounds (2014)*
- *Geosyntec™ Student Paper Competition, 3rd place (2013)*