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The Abundance of Tetrahydrofuran/Dioxane Monooxygenase Genes (*thmA*/*dxmA*) and 1,4-Dioxane Degradation Activity Are Significantly Correlated at Various Impacted Aquifers

Mengyan Li,[†] Jacques Mathieu,[†] Yuanyuan Liu,[‡] E. Tess Van Orden,[†] Yu Yang,[†] Stephanie Fiorenza,[§] and Pedro J. J. Alvarez*[,†]

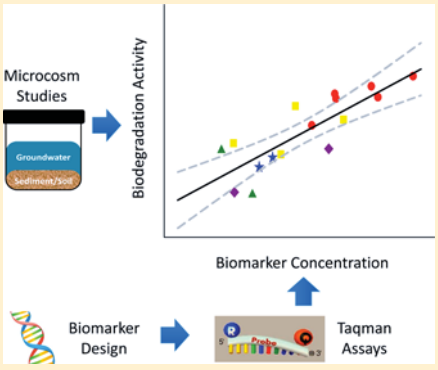
[†]Department of Civil and Environmental Engineering, Rice University, Houston, Texas 77005, United States

[‡]College of Environmental Science and Engineering, Key Laboratory of Environmental Biology and Pollution Control, Hunan University, Changsha, Hunan, P. R. China

[§]Remediation Engineering and Technology, BP America, Houston, Texas 77079, United States

Supporting Information

ABSTRACT: 1,4-Dioxane (dioxane) is a cocontaminant of emerging concern at thousands of sites impacted by chlorinated solvents, and there is an urgent need to assess site-specific dioxane biodegradation capabilities. In this study, 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 National Center for Biotechnology Information database. The probe targets conserved regions surrounding the active site, thus allowing detection of multiple dioxane degraders. Real-time polymerase chain reaction using reference strain genomic DNA demonstrated the high selectivity (no false positives) and sensitivity of this probe (7000−8000 copies/g of 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 with 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 for the rapid assessment of the performance of natural attenuation or bioremediation of dioxane plumes.



INTRODUCTION

1,4-Dioxane (dioxane) is a groundwater contaminant of emerging concern because of its recently discovered widespread occurrence at thousands of sites impacted by chlorinated solvent releases,[1,2] as well as its potential carcinogenicity.[3,4] Dioxane was commonly used as a stabilizer for industrial solvents, typically 1,1,1-trichloroethane (1,1,1-TCA), thus explaining this common co-occurrence. Because of its recalcitrant cyclic ether structure and high mobility in aquifers, dioxane tends to impact large areas with low levels of contamination.

Monitored natural attenuation (MNA) is among the most cost-effective approaches to manage groundwater contamination by organic pollutants at low concentrations.[5] However, the feasibility of MNA requires the 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.[6−11] However, these studies relied on complex molecular biological techniques, such as cloning, microarray, restriction fragment length polymorphism (RFLP), and phospholipid fatty acid analysis associated with stable isotope probing, 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 the biodegradation of different contaminants (e.g., *bssA* for anaerobic toluene degradation and *tceA* for reductive dechlorination of trichloroethylene).[12−20] Although selectivity and sensitivity can be highly variable for different probes and matrices, catabolic 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

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Table 1. Specificity and Coverage Tests for the Designed *thmA*/*dxmA* Biomarker

gene name	encoding enzyme[a]	SDIMO group	microorganism strain	biomarker detection[b]	
				thmA/*dxmA*	16S rRNA
dxm	dioxane MO	5	*P. dioxanivorans* CB1190	+	+
thm	tetrahydrofuran MO	5	*P. tetrahydrofuranoxydans* K1	+	+
tmo	toluene 4-MO	2	*Pseudomonas mendocina* KR1	−	+
tbu	toluene 3-MO	2	*Ralstonia pickettii* PKO1	−	+
tom	toluene 2-MO	1	*Burkholderia cepacia* G4	−	+
dmp	phenol HD	1	*Pseudomonas putida* CF600	−	+
prm	propane MO	5	*Rhodococcus jostii* RHA1	−	+
mmo	soluble methane MO	3	*Methylomonas methanica* MC09	−	+
−	−	−	*Escherichia coli* K12	−	+
−	−	−	Bacteriophage λ	−	−
amo	ammonia MO	−	*Nitrosomonas europaea* Winogradsky	−	+
tod	toluene DO	−	*Pseudomonas putida* F1	−	+
xyl	toluate 1,2-DO	−	*Pseudomonas aeruginosa* PAO1	−	+

[a]Abbreviations: MO, monooxygenase; HD, hydroxylase; DO, dioxygenase. [b]A plus sign indicates positive detection was obtained above the detection limit by using the primer/probe set via qPCR. A minus sign indicates no positive detection was obtained above the detection limit by using the primer/probe set via qPCR.

excellent candidates as biomarkers for dioxane biodegradation. First, both genetic and enzymatic studies have indicated the vital role of THF/dioxane monooxygenases during the initial oxidation of cyclic ethers by bacteria.[21−23] Second, the large hydroxylase subunits of THF/dioxane monooxygenases, which contain the active site, were found to be highly conserved (>97% identity)[9] for the four bacteria known to metabolize the cyclic ethers THF and/or dioxane (i.e., *Pseudonocardia dioxanivorans* CB1190,[24] *Pseudonocardia tetrahydrofuranoxydans* K1,[25] *Pseudonocardia* sp. ENV478,[26] and *Rhodococcus* sp. YYL[27]). Third, the activity of THF/dioxane monooxygenases from CB1190 and K1 toward dioxane and THF has been verified by transformation and expression in a heterologous host, *Rhodococcus jostii* RHA1.[28] Furthermore, microarray and denaturing gradient gel electrophoresis (DGGE) analyses demonstrated the enrichment of *thmA*-like genes near the source zone of an Arctic dioxane-contaminated site, where the highest dioxane degradation activity was observed.[9]

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 allows the determination of the (site-specific) feasibility of MNA for dioxane plumes and enhances performance assessment.

MATERIALS AND METHODS

Primer and Probe Design. Multiple-sequence alignment (Clustal X 2.1)[29] was used to identify homologous regions among the four *thmA*/*dxmA* genes available on the National Center for Biotechnology Information database and prevent 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 version 5.2.[30]

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 that satisfied the design criteria for TaqMan assays. After manual comparison and adjustment, the final set (Table S1 of the Supporting Information) 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.

Specificity and Coverage Tests with Bacterial Genomic DNA. To evaluate the specificity and selectivity of the *thmA*/*dxmA* probe and primer set, quantitative polymerase chain reaction (qPCR) was conducted with the genomic DNA isolated from reference strains (Table 1). After being grown in LB or R2A medium at room temperature for 1−7 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).

Microcosm Studies. To assess the efficacy of the catabolic biomarker in enhancing the forensic analysis of MNA, aquifer materials and groundwater samples were collected from 20 monitoring wells from five different dioxane-impacted sites in the United States (three in California, one in Alaska, and one in Texas). Triplicate microcosms were prepared with dioxane-impacted groundwater (100−150 mL with initial dioxane concentrations reaching up to 46000 μg/L) and aquifer materials (50 g) and incubated at room temperature under aerobic conditions. To distinguish abiotic losses of dioxane, sterile controls were prepared with autoclaved samples and poisoned with $HgCl_2$ (200 mg/L). Dioxane concentrations were monitored for 12−20 weeks using a frozen micro-extraction method followed by gas chromatography/mass spectrometry.[31]

At the beginning and termination of the microcosm experiments, 10 mL of the sample mixture was transferred into a 15 mL centrifugation tube. Aquifer materials together with biomass were separated by centrifugation at 10000 *g* for 20 min. Total microbial genomic DNA was extracted using a PowerSoil DNA Isolation Kit (MoBio). The eluted DNA (100 μL) was further purified and concentrated to 16 μL using a Genomic DNA Clean & Concentrator Kit (Zymo Resesarch,

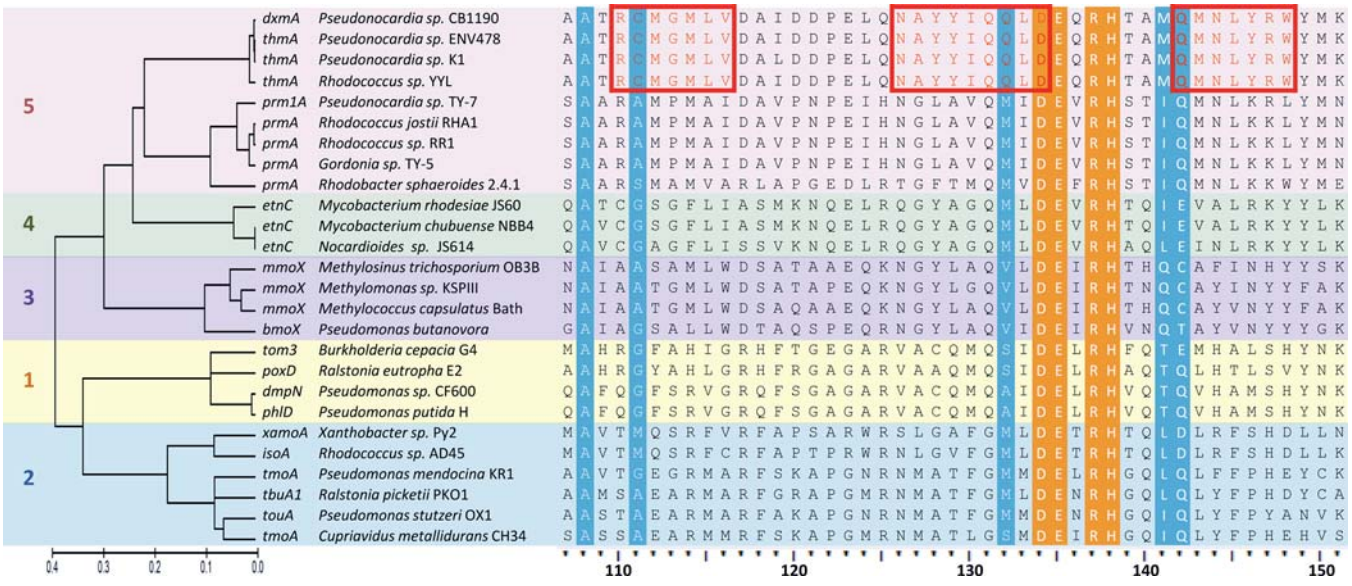

Figure 1. Alignment of the deduced amino acid sequences corresponding to the large hydroxylase of SDIMOs clustered by subfamily. The amino acid residues selected for biomarker design are colored red and boxed. The conserved di-iron center (i.e., DE*RH) is highlighted in orange, and the hydrophobic residues surrounding the di-iron center are highlighted in blue. Numbers below the sequences correpsond to the numbering for *dxmA* from CB1190. Numbers in front of the subclusters represent the group numbers of SDIMOs. The bootstrapping neighbor-joining tree was generated with MEGA version 5.2 using ClustalW as the computing algorithm.

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 an internal standard.[15]

Quantitative PCR. qPCR assisted by TaqMan assays was used to quantify *thmA*/*dxmA* genes from dioxane-degrading bacteria as well as total bacteria.[32] The PCR mixture contained 1 μL of undiluted DNA (or 1 ng/μL diluted bacterial genomic DNA), 300 nM forward and reverse primers, 150 nM fluorogenic probe, 10 μL of TaqMan universal master mix II (Applied Biosystems, Foster City, CA), and DNA-free water, yielding a total volume of 20 μL. qPCR was performed with a 7500 Real-Time PCR system (Applied Biosystems) using the following temperature program: 50 °C for 2 min, 95 °C for 10 min, and 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Serial dilutions (10^{-4} to 10 ng of DNA/μL) of the extracted genomic DNA of CB1190 were utilized to prepare the calibration curves for both *thmA*/*dxmA* (one copy per genome) and 16S rRNA (three copies per genome) genes (Figure S1 of the Supporting Information). Assuming a genome size of 7.44 Mb[33,34] and 9.124×10^{14} bp/μg [i.e., $(6.022 \times 10^{17}$ Da/μg of DNA)/(660 Da/bp)] for CB1190, the gene copy numbers were calculated on the basis of the equation

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

Method detection limits (MDLs) were 7000−8000 copies of *thmA*/*dxmA* genes/g of soil and 2000−3000 copies of 16S rRNA genes/g of soil (Table S2 of the Supporting Information). DNA extraction recoveries ranged from 2.3 to 48.9%. Similar recovery ranges are commonly reported for soil DNA extractions,[15,19,35] with the lower values reflecting sequential elution and residual impurities that hinder *Taq* polymerase reactions.[36]

RESULTS AND DISCUSSION

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 1).[37,38] However, different groups of SDIMOs exhibit different substrate specificities. 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.[37,39−41] Because 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 shared by only THF/dioxane monooxygenases.

Figure 1 illustrates that the amino acid residues targeted by the *thmA*/*dxmA* primer/probe set are identical among all four known THF/dioxane monooxygenases but significantly different from those of other SDIMOs. qPCR analysis (Table 1) 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 of approximately 25 for 1 ng of genomic DNA). Negative controls, using genomic DNA from bacteria with other types of SDIMOs (e.g., bacteria with dioxygenases such as *P. putida* F1 and bacteria without SDIMOs such as *E. coli* K12) and bacteriophage λ, were used to assess the potential for false positives. None of these negative controls were detected by this *thmA*/*dxmA* primer/probe 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

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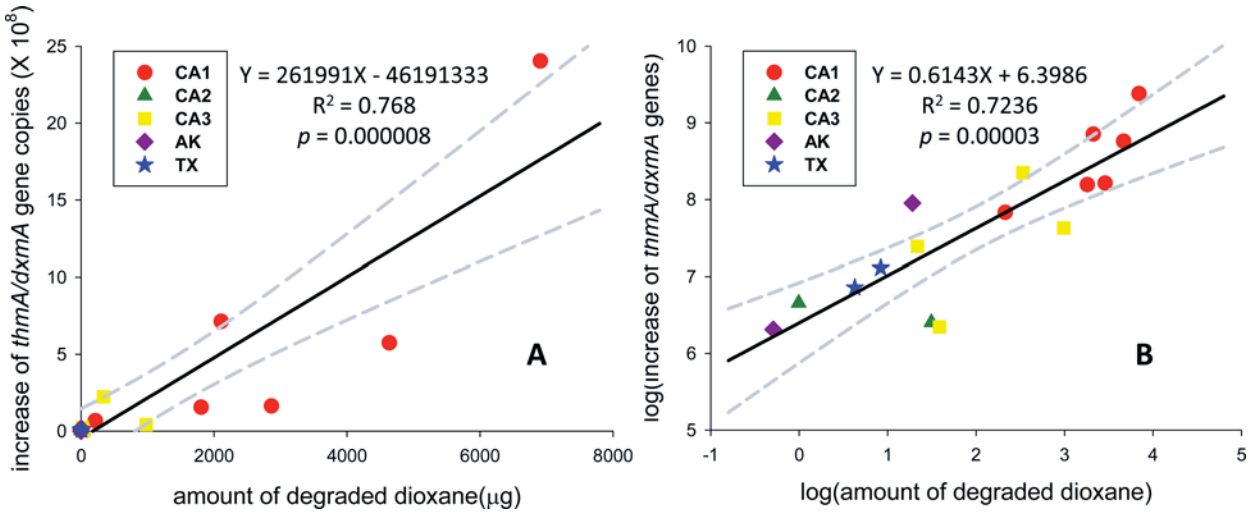

Figure 2. Correlation between the amounts of consumed dioxane (micrograms) and the increases in *thmA*/*dxmA* gene copy numbers in microcosms on a normal (A) and logarithmic (B) scale. The slope of the regression line of panel A was used to estimate the cell yield of dioxane for indigenous microbial degraders. The solid line represents the least-squares regression; the dashed lines represent the 95% confidence envelope.

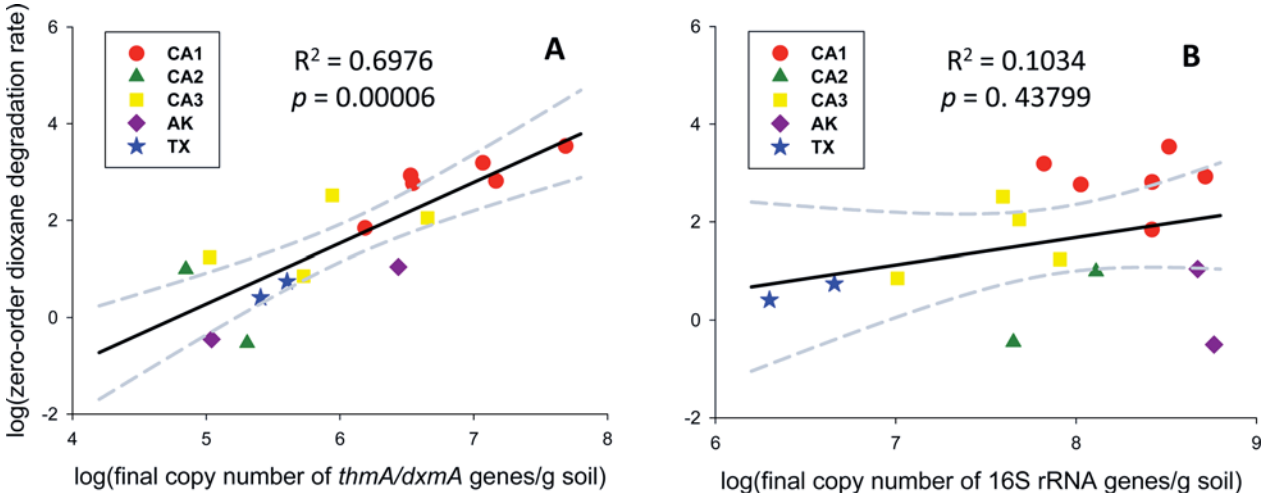

Figure 3. Correlation between zero-order dioxane degradation rates (micrograms per liter per week) and final copy numbers of *thmA*/*dxmA* (A) but not 16S rRNA (B) genes in microcosms for various sites. The solid lines represent the least-squares regression; the dashed lines represent the 95% confidence envelope.

hybridization with nonspecific templates. These results corroborate that the *thmA*/*dxmA* primer/probe set we developed for the TaqMan system allows the sensitive detection of *thm*/*dxm* genes and prevents false positives from other oxygenase genes that bear a close evolutionary relationship.

Dioxane Biodegradation Activity Was Significantly Correlated with the Abundance of *thmA*/*dxmA* Genes. After incubation for 3−5 months, considerable dioxane removal was observed in 16 of the 20 microcosms compared to the sterile controls, indicating the presence of dioxane degraders at these sites (Figure S2 of the Supporting Information).[42] The fitted zero-order decay rates varied from 10^{-1} to 10^{3} μg L^{-1} $week^{-1}$ (Table S3 of the Supporting Information). Growth of dioxane degraders was evident by increases in *thmA*/*dxmA* copy numbers, up to 114-fold (Figure S3 of the Supporting Information). This increase was significantly correlated ($p < 0.05$; $R^2 = 0.72$) to the amount of consumed dioxane (Figure 2). 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 S3 of the Supporting Information). Assuming a dry cell weight of 10^{-12} g and a protein composition of 55%,[43] the cell yield coefficient (Y) for the indigenous dioxane degraders was estimated to be 0.14 mg of protein/mg of dioxane (i.e., $Y = \Delta X/\Delta S$ = the regression line slope in Figure 2A), which is comparable with reported yield coefficients for CB1190 (0.01−0.09 mg of protein/mg of dioxane[5,24,44]) and other dioxane metabolizers, such as *Mycobacterium* sp. D11 (0.18 mg of protein/mg of dioxane[45]). A significant correlation ($p < 0.05$; $R^2 = 0.70$) was also observed between the final *thmA*/*dxmA* copy numbers and dioxane degradation rates (Figure 3A). In contrast, copy numbers of 16S rRNA genes (a phylogenetic biomarker that is commonly used to enumerate total bacteria) were not significantly correlated ($p = 0.44$) with dioxane biodegradation activity (Figure 3B), corroborating the selectivity of this *thmA*/*dxmA* probe.

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

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with genomic DNA isolated from microcosm 1-1S (Supporting Information), generating a total of 86 valid clones that were sequenced and aligned (Figure S5 of the Supporting Information). All sequences exhibited a high level of identity with previously reported *thmA*/*dxmA* genes (≥95%),[21,26,27,34] 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 of 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 the 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. As more *thm*/*dxm* genes are discovered, our biomarker may require revision and reevaluation. Nevertheless, the current understanding that these genes are highly conserved suggests that such optimization efforts would require minimal sequence adjustments.

■ ASSOCIATED CONTENT

Supporting Information

Additional methods, Tables S1−S3, and Figures S1−S5. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author

*E-mail: alvarez@rice.edu.

Notes

The authors declare no competing financial interest.

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