



Hindrance of 1,4-dioxane biodegradation in microcosms biostimulated with inducing or non-inducing auxiliary substrates



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ABSTRACT

A microcosm study was conducted to assess two biostimulation strategies (relative to natural attenuation) to bioremediate 1,4-dioxane contamination at a site in west Texas. Dioxane concentrations were relatively low (<300 µg/L), which represents a potential challenge to sustain and induce specific degraders. Thus, biostimulation was attempted with an auxiliary substrate known to induce dioxane-degrading monooxygenases (i.e., tetrahydrofuran [THF]) or with a non-inducing growth substrate (1-butanol [1-BuOH]). Amendment of 1-BuOH (100 mg/L) to microcosms that were not oxygen-limited temporarily enhanced dioxane biodegradation by the indigenous microorganisms. However, this stimulatory effect was not sustained by repeated amendments, which might be attributed to i) the inability of 1-BuOH to induce dioxane-degrading enzymes, ii) curing of catabolic plasmids, iii) metabolic flux dilution and catabolite repression, and iv) increased competition by commensal bacteria that do not degrade dioxane. Experiments with the archetype dioxane degrader *Pseudonocardia dioxanivorans* CB1190 repeatedly amended with 1-BuOH (500 mg/L added weekly for 4 weeks) corroborated the partial curing of catabolic plasmids (9.5 ± 7.4% was the plasmid retention ratio) and proliferation of derivative segments that lost their ability to degrade dioxane. Addition of THF (300 µg/L) also had limited benefit due to competitive inhibition; significant dioxane degradation occurred only when the THF concentration decreased below approximately 160 µg/L. Overall, these results illustrate the importance of considering the possibility of unintentional hindrance of catabolism associated with the addition of auxiliary carbon sources to bioremediate aquifers impacted with trace concentrations of dioxane.

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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]) (Mohr et al., 2010). Consequently, dioxane is commonly found at sites impacted by chlorinated solvent releases. Groundwater contamination by dioxane is a significant concern due to its potential carcinogenicity, resulting in a stringent Environmental Protection Agency (EPA) drinking water advisory level of 0.35 µg/L for a 10⁻⁶ lifetime cancer risk (EPA, 2010), as well as its high

mobility in groundwater, resulting in relatively large plumes with a median length of 269 m for 2000 sites (Adamson et al., 2014). Dioxane may also enter water systems via accidental or incidental release from various industrial operations, including its direct use in membrane and pharmaceutical manufacture, or its unintended generation as a byproduct of textile processing, polyester preparation, and synthesis of ethoxylated surfactants, which are common ingredients of soaps and sundries (Mohr et al., 2010). Thus, dioxane is also a contaminant of emerging concern to water utilities. Among the twenty-one emerging organic contaminants monitored by U.S. EPA's Unregulated Contaminant Monitoring Rule 3 (UCMR3), dioxane was found with the highest detection frequency (approximately 12%) in over 34,000 water samples collected in water treatment facilities between 2013 and 2015 (EPA, 2016).

In situ bioremediation, when applicable, is economically

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advantageous for treating large and dilute dioxane plumes compared to commonly used pump and treat processes (DiGuseppi and Whitesides, 2007; EPA, 2006; Zenker et al., 2003). Although dioxane is relatively recalcitrant to microbial degradation, recent research has demonstrated that indigenous bacteria that can degrade dioxane might be more widespread than previously assumed (Chiang et al., 2012; Kim et al., 2009; Li et al., 2010, 2013, 2014, 2015; Mahendra and Alvarez-Cohen, 2006; Sei et al., 2010). However, the suitability of bioremediation must be determined site-specifically because the presence and expression of dioxane biodegradation capabilities are not ubiquitous. Furthermore, the potential to stimulate dioxane biodegradation by adding auxiliary carbon sources has received limited attention in the literature. Such auxiliary substrates might be beneficial when dioxane is present at trace concentrations that are insufficient to induce or sustain specific degraders.

Auxiliary substrates can be arbitrarily divided into two groups: (1) inducing substrates that induce catabolic enzymes (e.g., tetrahydrofuran [THF], which induces dioxane monooxygenase (MO) in various actinomycetes (Masuda et al., 2012; Sales et al., 2013)), and (2) non-inducing substrates that fortuitously support the growth of dioxane degraders (e.g., 1-butanol [1-BuOH] (Parales et al., 1994; Vainberg et al., 2006)). Previous microcosm studies have shown that addition of 1-BuOH with Ammonia Mineral Salt Medium (AMS) can stimulate dioxane biodegradation by boosting the overall biomass in nutrient-limited samples from a contaminated site in Alaska (Li et al., 2010). Furthermore, separate studies have shown that THF can induce dioxane biodegradation pathways in both dioxane metabolizers and cometabolizers. THF is known to sustain most isolated dioxane metabolizers as a sole carbon and energy source (Parales et al., 1994; Sei et al., 2013), as well as to stimulate dioxane cometabolism (Kohlweyer et al., 2000; Sei et al., 2010; Vainberg et al., 2006; Zenker et al., 2000). In addition, the relatively low toxicity, mild bioaccumulation tendency, and short half-life in the environment (Fowles et al., 2013) make THF a potential auxiliary substrate for dioxane biostimulation. Nevertheless, it is unclear if such substrates are ubiquitously applicable for stimulating dioxane biodegradation at sites harboring dioxane degraders, and what the unintended consequences of such biostimulation approaches might be.

Activation of dioxane metabolism is commonly initiated by plasmid-encoded enzymes. The rate-limiting step for the biodegradation of dioxane and its structural analogue THF is generally the oxidation of the carbon atom adjacent to the oxygen atom in both compounds (i.e., 2-hydroxylation). This critical step that precedes cleavage of cyclic ethers is catalyzed by soluble di-iron monooxygenases (SDIMOs) named dioxane/THF monooxygenases (dioxane/THF MOs) (Masuda, 2009; Sales et al., 2013; Thiemer et al., 2003; Yao et al., 2009). The gene clusters encoding these multi-component dioxane/THF MOs (i.e., *dxmADBC* and *thmADBC*) were identified in the archetype dioxane and THF degraders *Pseudonocardia dioxanivorans* CB1190 and *Pseudonocardia tetrahydrofuranoxydans* K1, respectively (Sales et al., 2013; Thiemer et al., 2003). Both *dxmADBC* and *thmADBC* gene clusters are located in plasmids, as determined by Southern hybridization assays and the next-generation sequencing (Sales et al., 2011; Thiemer et al., 2003). Notably, the GC contents of the four dioxane/THF MO gene clusters available in the NCBI database are about 61%, which is lower than a typical GC content range (65–75%) of the genomes of *Pseudonocardia* and *Rhodococcus* species (Masuda, 2009; Sales et al., 2013; Thiemer et al., 2003; Yao et al., 2009). The localization and relatively low GC content of these *dxm/thm* gene clusters suggested that horizontal gene transfer might be the dominant venue by which these bacteria obtained these plasmids and gained the capacity to degrade dioxane and THF. In polluted environments, such

plasmids may confer their hosts improved ecological fitness and broader substrate range. However, maintenance and expression of these catabolic plasmids contribute to additional metabolic burden that needs to be compensated by the host cells (Sayler et al., 1990).

In this substrate interactions study, we compared the merits and limitations of biostimulation of dioxane degradation with a non-inducing growth substrate (1-BuOH) versus an inducing substrate (THF). We focused on the degradation of dioxane via 2-hydroxylation because of its crucial role for dioxane decomposition. Using microcosms prepared with samples from a contaminated site, we considered the dynamics of the catabolic biomarker *thmA/dxmA* (coding for the active site of THF/dioxane MO (Li et al., 2014)) as well as microbial population shifts. To further investigate the molecular basis of how auxiliary substrates influence *dxmADBC/thmADBC* bearing dioxane degraders, the archetype *Pseudonocardia dioxanivorans* CB1190 was selected as a model strain. CB1190 is the best characterized dioxane-degrading strain, with a completely sequenced genome (Sales et al., 2011) and a known dioxane biodegradation pathway (Grostern et al., 2012; Mahendra et al., 2007). Experiments in controlled laboratory setups were conducted with pure cultures of CB1190 to discern how these auxiliary substrates affect the induction or repression of essential genes involved in dioxane degradation, as well as the stability of pertinent catabolic plasmids under different substrate conditions. Therefore, this paper advances mechanistic understanding of how different types of auxiliary or co-occurring substrates affect the expression and maintenance of dioxane biodegradation capabilities.

2. Material and methods

2.1. Site description and sample collection

In March 2013, groundwater and aquifer materials were collected from an oil and gas operation site located in the Southwestern US. This site was mainly used for chemical storage and equipment repair, and past activities resulted in releases of dioxane and chlorinated volatile organic compounds (CVOCs) to the subsurface. Hydraulic modeling predicted the leading edge of the dioxane plume was approximately 6000 ft downgradient of the site using the Texas Commission on Environmental Quality PCL (i.e., 9.1 $\mu\text{g/L}$) to define the contamination boundary. The plume width was estimated to be 1600 ft at the 9.1 $\mu\text{g/L}$ contour. The maximum dioxane concentration was 395 $\mu\text{g/L}$. Four monitoring wells representing different dioxane contamination levels were selected for sample collection, including the source zone, the middle, and the edge of the plume, as well as the uncontaminated upstream region (Fig. S1 and Table S1).

2.2. Benchtop microcosm assays mimicking natural attenuation and biostimulation

To investigate the potential for natural attenuation, microcosms were prepared in 250 mL amber glass bottles using 100 mL of groundwater and 50 g of aquifer material collected at different site locations (Li et al., 2010, 2015). We focused on aerobic processes that prevailed at the aquifer under consideration near the source zone (i.e., dissolved oxygen > 2 mg/L, ORP > 100 mV). Furthermore, increased surface area of the aquifer material and contact with air during sample handling and microcosm preparation would also contribute to aerobic conditions. Whereas these handling may result in faster dioxane degradation in microcosms than *in situ*, this would not affect the substrate interactions studied here because all microcosm treatments and controls were handled similarly. Microcosms were incubated in the dark under quiescent condition at 24 ± 2 °C, which was similar to field conditions (Table S2).

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

Aqueous microcosm samples were taken and analyzed weekly or biweekly. Dioxane and THF concentrations were measured by GC/MS using the frozen microextraction method developed in our lab (Li et al., 2011). The concentrations of 1-BuOH were measured by Agilent 5890 Chromatograph (GC) with a Flame Ionization Detector (FID) using direct water injection after filtration by 0.2-µm Nylon syringe filters to remove the biomass and suspended particles (Li et al., 2010). A first-order (exponential) decay model was used to fit the dioxane degradation data. This is appropriate according to Monod's model, since the initial dioxane concentrations (6–300 µg/L) were at least two orders of magnitude lower than the Monod half-saturation constants (21–330 mg/L) reported for dioxane-degrading isolates (Mahendra and Alvarez-Cohen, 2006; Sei et al., 2013).

At each time point, 10 mL of water-sediment mixture was removed from each microcosm. The total biomass and solids were harvested by centrifugation at 10,000×g for 10 min. A total of 0.25 g of biomass/solid mixture was transferred to PowerBead tubes (Mo Bio, Carlsbad, CA) and spiked with 5 µL of bacteriophage λ DNA (0.2 ng/µL, Sigma-Aldrich, St. Louis, MO) to track the extraction efficiency. Total microbial genomic DNA (gDNA) was then extracted using a PowerSoil DNA Isolation Kit (Mo Bio, Carlsbad, CA) following the manufacturer's instruction. The eluted DNA was further purified and concentrated using a Genomic DNA Clean & Concentrator Kit (Zymo Research, Irvine, CA). Copy numbers of the *thmA/dxmA* genes encoding for large hydroxylases of the dioxane-degrading enzymes (i.e., THF/dioxane MOs) were quantified by qPCR using a specific probe/primers set developed in our group (Li et al., 2014). Total bacterial biomass was quantified by enumerating the copy numbers of 16S rRNA genes (Nadkarni et al., 2002). For qPCR analysis, detailed information regarding the instrument setup and calibration is provided in (Li et al., 2014).

2.3. Bacterial cultures

Pseudonocardia dioxanivorans CB1190 (ATCC #55486) (Parales et al., 1994) was selected as the archetypic model to investigate the molecular impacts of both inducing and non-inducing compounds. CB1190 was grown in AMS medium amended with dioxane (500 mg/L) as the sole carbon and energy source at 24 ± 2 °C with shaking (120 rpm). Cells were harvested at late exponential phase by centrifugation at 10,000 × g for 15 min. The supernatant was decanted and the pellets were washed three times with 1 × phosphorous buffered saline (PBS) to remove organic residues. Then, the cell pellets were resuspended and concentrated with AMS medium (approximately 10 mg/L as total protein concentration) for subsequent gene expression and plasmid retention analysis.

2.4. Gene expression analysis

To evaluate changes in gene expression induced by various substrate compounds, triplicate treatments were prepared in 250 mL amber glass bottles containing 100 mL of AMS medium amended with dioxane (100 mg/L), THF (100 mg/L), 1-butanol (100 mg/L), or acetate (100 mg/L) as sole carbon source or 100 mL of the nutrient-rich R2A medium. Treatments were then

inoculated with 1 mL of concentrated CB1190 culture stock and incubated at 24 ± 2 °C while shaking at 120 rpm. The concentrations of amended organic chemicals in the microcosms were monitored daily by Agilent 5890 GC-FID by direct injection of 1 µL of the filtered aqueous phase (Li et al., 2010).

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

RT-qPCR was performed using a 7500 real time PCR system from Applied Biosystems (Carlsbad, CA) in 15 µL of reaction mixture composed of 1 µL diluted cDNA (1 ng/µL), Power SYBR Green PCR Master Mix (7.5 µL), 0.3 µM of each primer and DNA/RNase free water. The thermocycler program was 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. The 2^{-ΔΔCT} method was used to quantify differential gene expression (Livak and Schmittgen, 2001; Yang et al., 2011), and the expression fold change were estimated with the calculation formula below (Eq. (1)).

$$\Delta\Delta C_{T, \text{Target gene}} = \left(C_{T, \text{Target gene}} - C_{T, \text{Housekeeping gene}} \right)_{\text{Treatment}} - \left(C_{T, \text{Target gene}} - C_{T, \text{Housekeeping gene}} \right)_{\text{Control}} \quad (1)$$

For this assay, the target gene was *dxmA*, which encodes the α subunit of the CB1190 dioxane MO. The CB1190 16S rRNA gene was selected as the housekeeping gene to normalize experimental variance. Specific primers targeting the *dxmA* and 16S rRNA gene of CB1190 were designed and indicated in the supplementary information (Table S3). Treatments fed with acetate as the sole carbon source were used as the control for expression normalization and statistical analysis.

2.5. Plasmid retention assay

The genome of CB1190 contains three copies of the 16S rRNA gene on the chromosome and one copy of *dxmA* on the plasmid pPSED02 (Sales et al., 2011). Thus, the copy number ratio between *dxmA* and 16S rRNA genes is useful to predict the per cell retention rate of pPSED02 under selective conditions (i.e., in the presence of substrates that induce the transcription of the *dxmA* gene) or non-

selective conditions (when such substrate induction is absent). To create such metabolic pressure, concentrated CB1190 (1 mL) was inoculated into 125 mL glass flasks sealed with semi-permeable 0.2 μm membranes, and supplemented with 50 mL of five types of growth medium, including: (i) AMS amended with 500 mg/L of dioxane, (ii) AMS amended with 500 mg/L of 1-BuOH, (iii) AMS amended with 500 mg/L of THF, (iv) R2A, and (v) AMS amended with 500 mg/L acetate. All treatments were kept at room temperature while shaking at 150 rpm. The cell pellets were harvested weekly from 10 mL of the culture solution in each treatment. After washing with $1 \times$ PBS buffer for three times, the harvested cells were resuspended and inoculated into glass flasks containing 50 mL of newly-prepared medium for each treatment. After four weeks of cultivation, the cells in different treatments were collected by centrifugation for gDNA isolation. Total gDNA was extracted using an Ultra Clean Microbial DNA Isolation Kit (Mo Bio, Carlsbad, CA) based on the manufacturer's protocol. qPCR using TaqMan chemistry was used for absolute quantification of 16S rRNA and *dxmA* gene copy numbers based on a method modified from Li et al., (2014). The plasmid retention percentage was calculated using the equation below (Eq. (2)) based on the comparison of the ratio between the abundance of *dxmA* and 16S rRNA genes at Time 0 and 4 weeks.

$$\text{Percent Retention of Plasmid} = \frac{(C_{dxmA, T} / C_{16S\ rRNA, T})}{(C_{dxmA, 0} / C_{16S\ rRNA, 0})} \times 100\% \quad (2)$$

2.6. Whole genome sequencing of CB1190 mutants

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

The paired Illumina reads were aligned against the reference genome of CB1190 (Sales et al., 2011) using the BWA-MEM algorithm (version: 0.7.5a-r405) (Li and Durbin, 2009; Li, 2013). For each individual DNA molecule possessed by CB1190 (i.e., one chromosome and three plasmids), its average sequence coverage was estimated as the total sequencing read length normalized with its original genome size, representing the detection frequency of a given DNA molecule in the reference genome. Further, genomic structural variants such as gene insertions and deletions were examined with BreakDancer (version: 1.1.2) based on the aligned bam (Chen et al., 2009; Fan et al., 2014).

2.7. Statistical analysis

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

3. Results and discussion

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

Microcosms were prepared with source zone samples, which

exhibited the fastest attenuation rate and highest initial abundance of indigenous dioxane degraders (Supplementary Information). The auxiliary substrate 1-BuOH, which is known to support the growth of some dioxane degraders (Kelley et al., 2001; Li et al., 2010; Parales et al., 1994; Vainberg et al., 2006), was tested to enhance dioxane degradation by the indigenous consortium. The addition of 1-BuOH initially improved dioxane removal compared to unamended (natural attenuation) microcosms. This temporary enhancement was small but statistically significant ($p < 0.05$) (Fig. 1), with the first-order dioxane degradation rate coefficient significantly increasing from $0.020 \pm 0.001 \text{ week}^{-1}$ to $0.027 \pm 0.003 \text{ week}^{-1}$ (Table 1). Analysis by qPCR (Fig. 1B) showed that both the abundance of dioxane degraders and overall microbial populations increased compared to the beginning of the incubation ($p < 0.05$). However, at Week 16, the abundance of dioxane degraders (indicated by *dxmA/thmA* gene counts) did not increase significantly compared to the natural attenuation treatment (Fig. S2B). Thus, addition of 1-BuOH was not directly linked to the proliferation of dioxane degradation genes.

To assess the effects of repeated biostimulation, 1-BuOH (100 mg/L) was respiked immediately after it was completely consumed. Similar to observations in the microcosms with a single

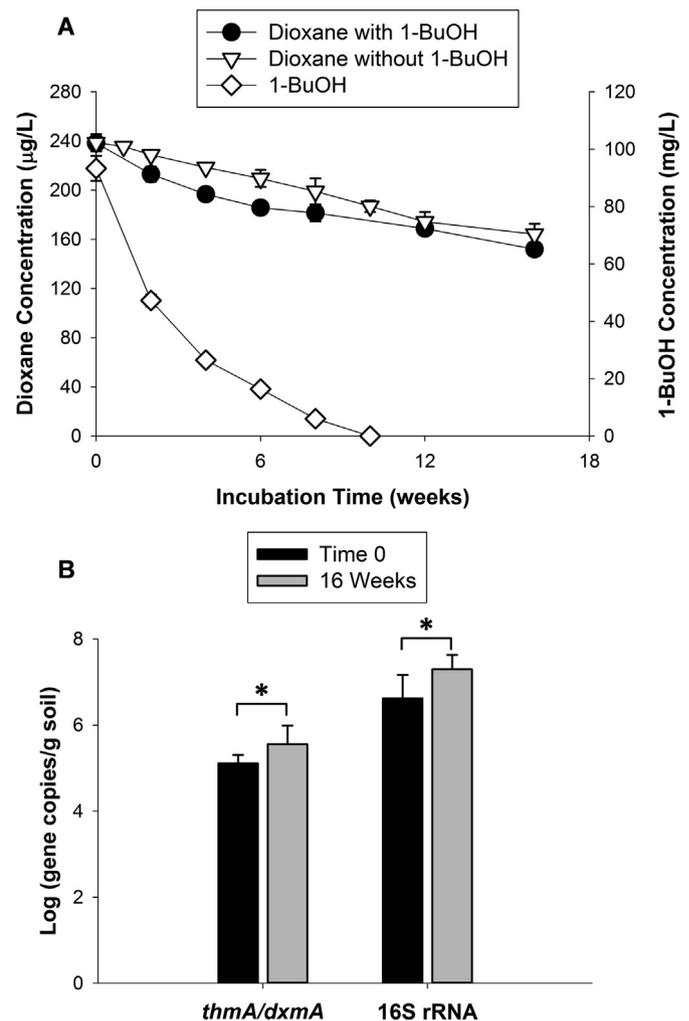


Fig. 1. Slight temporary enhancement of dioxane biodegradation by 1-butanol addition (relative to natural attenuation) in microcosms prepared with source zone materials (A) and abundance of *thmA/dxMA* and 16S rRNA genes at different sampling time points (B). Asterisks indicate the *p* values less than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

Table 1
1st order kinetic constants for dioxane biodegradation.

Treatment	1st order kinetic constants (week ⁻¹)	R ²
Natural attenuation	0.020 ± 0.001	0.975
Single 1-BuOH amendment	0.027 ± 0.003	0.943
Double 1-BuOH amendments (Week 0–12)	0.028 ± 0.007	0.857
Double 1-BuOH amendments (Week 12–16)	0.012 ± 0.002	0.967
THF amendment	0.027 ± 0.013	0.808
THF biodegradation ^a	0.037 ± 0.004	0.950

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

1-BuOH amendment, this first amendment of 1-BuOH also enhanced dioxane removal as well as the growth of dioxane degraders and total biomass (Fig. 2). As predicted by enhanced acclimation, the second 1-BuOH amendment was consumed much faster (i.e., less than 4 weeks) compared to the first amendment. However, unlike the first amendment, this second 1-BuOH amendment did not enhance dioxane degradation. On the contrary, the estimated first-order degradation rate coefficient for dioxane significantly decreased from $0.028 \pm 0.007 \text{ week}^{-1}$ to $0.012 \pm 0.002 \text{ week}^{-1}$, which was slower than observed in microcosms mimicking natural attenuation ($0.020 \pm 0.001 \text{ week}^{-1}$). This

decrease in dioxane removal rate was also corroborated by our qPCR analysis results, which showed a significant decrease of dioxane degrading microorganisms harboring the *thmA/dxmA* genes compared to Week 12 (Fig. 2B), though total biomass increased. Our results also show that 1-BuOH was preferentially degraded over dioxane in these microcosms.

The stimulation of growth by 1-BuOH amendment for both total biomass and dioxane degraders (represented by the ¹³C enriched biomass) was also observed in the *in situ* microcosm deployed at the source zone monitoring well (Table S4). However, dioxane decomposition and mineralization were not significantly improved compared to the natural attenuation controls. This corroborates our finding that amendment with 1-BuOH can temporarily enhance the growth of dioxane degraders but is not capable of sustaining induction of the necessary catabolic enzymes.

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

The second possibility is that dioxane degraders might be out-competed by other microorganisms in the enriched consortium. Though dioxane degraders, such as CB1190, are known to metabolize 1-BuOH (Parales et al., 1994; Vainberg et al., 2006), numerous other bacteria that also grow on 1-BuOH cannot degrade dioxane. The percentage of dioxane degraders decreased from 3.4% to 1.2% at the first 1-BuOH amendment, and continued to decrease to 0.3% after the second amendment at Week 16. This is indicative of genotypic dilution by the proliferation of non-competent strains (Da Silva and Alvarez, 2002). Extensive proliferation of the non-dioxane degraders might lead to an unfavorable environment for dioxane degraders due to competition for nutrients (N and P), trace elements, and electron acceptors (i.e., oxygen), as well as potential amensalism, which might contribute to the decline of the absolute abundance of dioxane degraders after the second 1-BuOH amendment.

The third (non-exclusive) possibility is that the continuous metabolic flux of 1-BuOH might lead to effects at the gene expression level, including catabolite repression and metabolic flux dilution (discussed in Section 3.3). As the essential genes for dioxane biodegradation (e.g., *thmADBC/dxmADBC* genes) are typically located on plasmids (Sales et al., 2011; Thiemer et al., 2003), lack of induction of dioxane-degrading enzymes may cause the curing of the catabolic plasmids. Due to the difficulty of tracking deletion or deamplification of genetic elements in mixed consortia, additional experiments were conducted to discern the molecular impacts of non-inducing substrates using a monoculture of the archetype dioxane degrader CB1190. Results that support this hypothesis are discussed below (Section 3.4). Overall, these

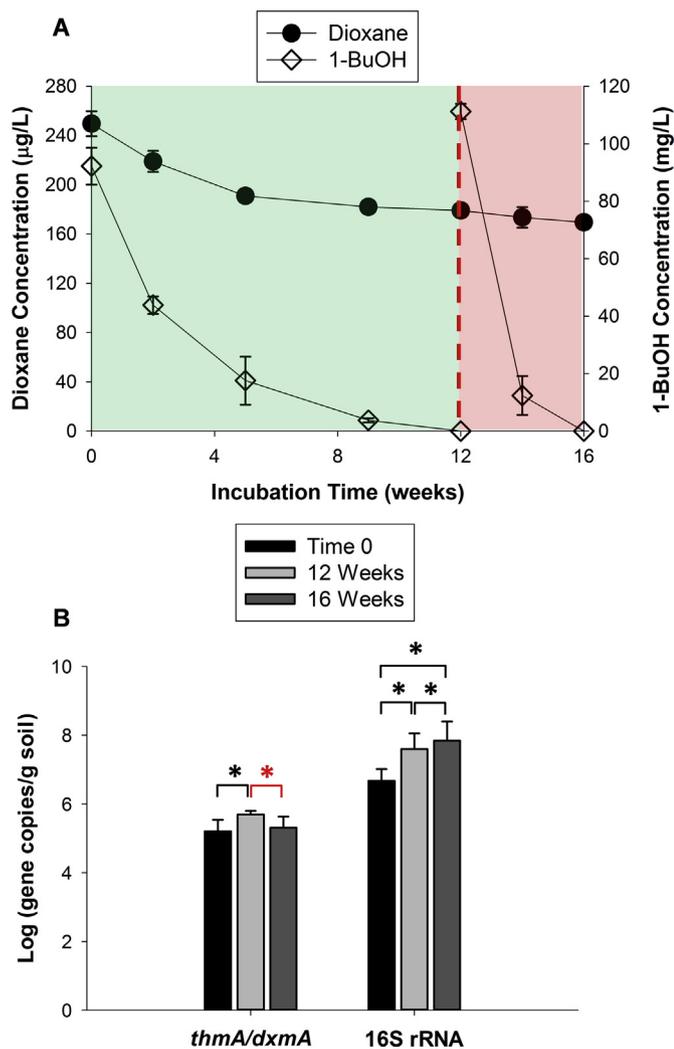


Fig. 2. Untenable biodegradation of dioxane in microcosms prepared with source zone material by repeated amendment of 1-BuOH at Week 12 (A) and abundance of *thmA/dxmA* and 16S rRNA genes at different sampling time points (B). Asterisks indicate the *p* values less than 0.05 based on the student *t*-test.

microcosm experiments suggest that long term, repeated amendment of a non-inducing growth substrate (especially at large dosages) may hinder dioxane biodegradation. Hindrance mechanisms likely include higher microbial competition and genotypic dilution, and (in some cases) loss of intrinsic biodegradation capacity.

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

In parallel to 1-BuOH, THF was tested as an inducing substrate for dioxane biostimulation due to its similar cyclic ether structure to dioxane. Surprisingly, a similar dosage of THF (300 µg/L) completely suppressed dioxane biodegradation for the first 16 weeks (Fig. 3). During the incubation, THF was preferentially degraded over dioxane. Dioxane degradation began between Week 16 and 23 after the THF concentration decreased below 186.5 ± 27.5 . Though this lag was experienced, dioxane degradation proceeded at a faster pace ($0.027 \pm 0.013 \text{ week}^{-1}$) than the natural attenuation microcosms. Accordingly, a significant increase in the abundance of dioxane degrading genes was measured by qPCR analysis (Fig. 3B). No significant growth of total biomass (indicated by 16S rRNA genes) was observed.

The initial inhibitory effect of THF on dioxane degradation is probably due to competitive inhibition, when both compounds are degraded by the same enzyme and bind to its active site. Competitive inhibition can be pronounced when i) the affinity to the inhibitor (i.e., THF) is greater than the substrate (i.e., dioxane) or ii) the substrate concentration is low compared to its Monod half saturation constant (K_S). Previous studies observed a higher affinity of THF/dioxane MO towards THF compared to dioxane (Parales et al., 1994). For some cometabolic reactions, pure strains or enriched consortia require THF as primary substrate and enzyme inducer to degrade dioxane (Sei et al., 2010; Vainberg et al., 2006). In addition, kinetic studies using isolated dioxane degrading strains (e.g., CB1190 and *Mycobacterium* sp. D6) showed that their K_S values towards dioxane are typically large, between 20.6 and 330 mg/L (Mahendra and Alvarez-Cohen, 2006; Sei et al., 2013), which is indicative of relatively low affinity. The initial dioxane concentration ($241.9 \pm 3.7 \text{ µg/L}$) in the prepared microcosms was two orders of magnitude lower than these K_S values, which is conducive to significant competitive inhibition by alternative substrates that bind with the active site of the enzyme. This is predominantly observed in cometabolic biostimulation treatments where the primary substrate binds to the enzyme and inhibits degradation of the target pollutant. For instance, cometabolism of trichloroethylene (TCE) by methane monooxygenases can be significantly inhibited by methane, as well as by co-occurring chlorinated aliphatic hydrocarbons, such as chloroform (Alvarez-Cohen and McCarty, 1991; Suttinun et al., 2013).

Accelerated dioxane removal and increased abundance of *thmA/dxmA* (but not 16S rRNA) genes were observed after THF concentration decreased below approximately 160 µg/L. Apparently, THF selectively stimulated the growth and activity of dioxane degraders, as discussed below in Section 3.3. Overall, THF may serve as both inducer and competitor for the enzymes that initiate dioxane degradation. Though addition of THF can enhance the growth and activity of indigenous dioxane degraders and stimulate biodegradation in the long-term, a measurable delay or lag phase can occur due to competitive inhibition exacerbated by low dioxane concentrations commonly found at thousands of sites impacted by chlorinated solvents (Adamson et al., 2014, 2015).

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

To investigate the effect of different substrates on the induction

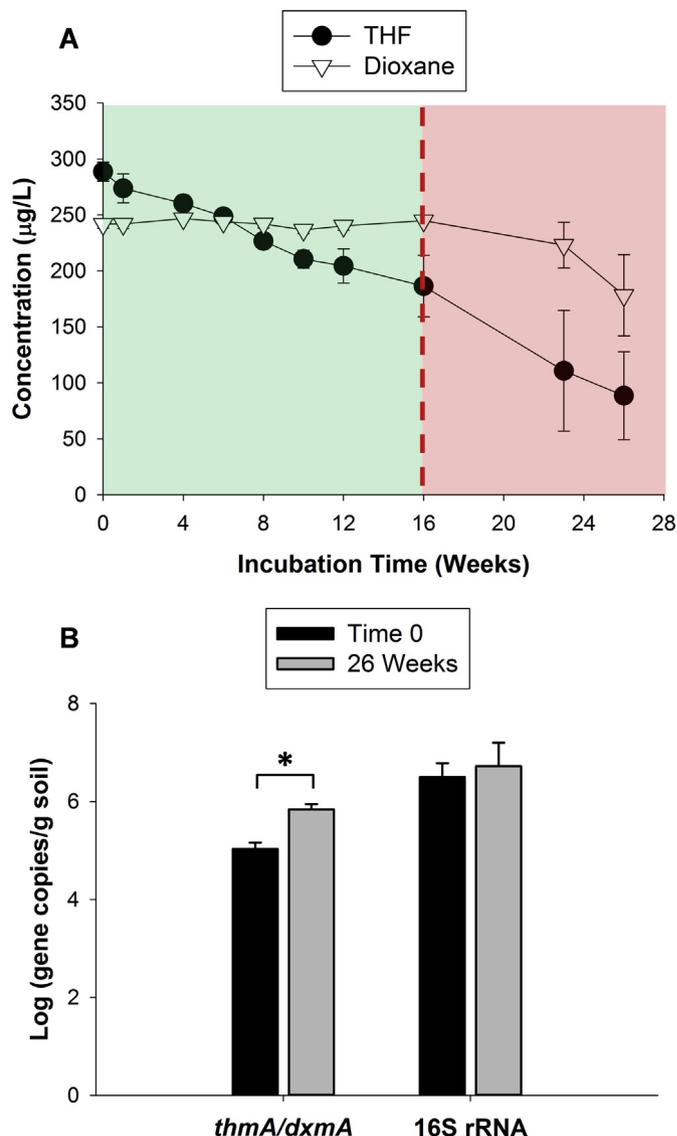


Fig. 3. Delayed removal of dioxane in microcosms prepared with source zone materials and amended with comparable amount of THF (300 µg/L) at the beginning of the experiments (A) and abundance of *thmA/dxmA* and 16S rRNA genes at different sampling time points (B). Significant decrease of dioxane concentration occurred after Week 16. Asterisks indicate the p values less than 0.05 based on the student t -test.

of dioxane MO, RT-qPCR was conducted using total RNA extracts from the archetype dioxane degrader *Pseudonocardia dioxanivorans* CB1190 after growth in AMS with a single carbon source (e.g., dioxane, 1-BuOH, THF, or acetate as control) as well as complex R2A medium. As expected, dioxane MO was induced at relatively high expression levels by dioxane and its structural analogue, THF (Fig. 4). Compared to acetate, the expression of *dxmA* was significantly up-regulated by dioxane (51-fold higher) and THF (62-fold higher) (Fig. 4). This corroborates the role of this MO in the metabolism of small cyclic ethers, as demonstrated by other studies using advanced molecular techniques, such as transcription microarray, Northern blot, and SDS-PAGE (Grosterm et al., 2012; Thiemer et al., 2003; Vainberg et al., 2006). The inducibility of dioxane MO by THF in CB1190 is consistent with the stimulatory effects of THF in dioxane degradation observed in the microcosm assays prepared with field samples (Fig. 3). Conversely, the presence of easily biodegradable substrates (e.g. 1-BuOH), repressed

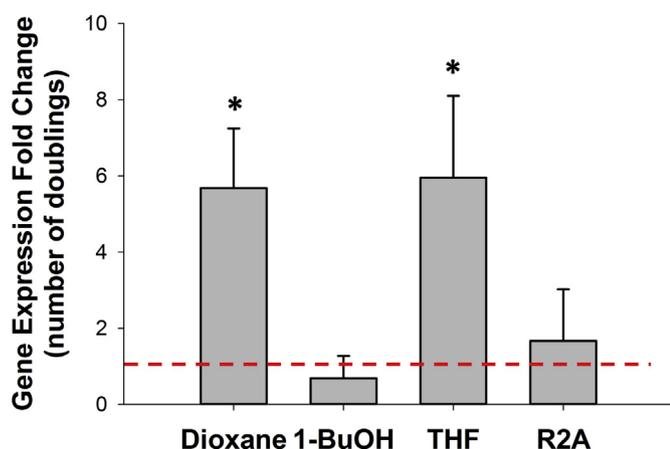


Fig. 4. Expression of *dxmA* genes in CB1190 after fed with various substrates for two to four days. Data were normalized to the treatment fed with acetate as sole carbon and energy source. The $2^{-\Delta\Delta CT}$ method was used to quantify differential gene expression. Asterisks indicate significant ($p < 0.05$) up-regulation compared to the acetate-fed control. The dotted red line represents 2-fold up-regulation. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

induction of dioxane MO (Fig. 4). No significant up-regulation of the *dxmA* gene was observed in treatments fed with 1-BuOH or R2A compared to the acetate control (Fig. 4).

Acetate, 1-BuOH, and the major constituents of R2A (e.g., pyruvate and casamino acids) are readily biodegradable by constitutive enzymes through central metabolic pathways (e.g., Krebs' cycle and β -oxidation) (Alvarez and Illman, 2006). In contrast, biodegradation of recalcitrant contaminants, such as dioxane and THF, is often initiated by specific catabolic enzymes (e.g., inducible monooxygenases) that transform the compounds into metabolites that subsequently enter the central metabolic pathways. Thus, exposure to large concentrations of the readily biodegradable substrates may dilute the metabolic flux of dioxane through these pathways, resulting in slower dioxane degradation rates (Lovanh and Alvarez, 2004).

High concentrations of easily degradable substrates may also hinder expression of genes involved in dioxane degradation via catabolite repression. As part of the global control system in microorganisms, catabolite repression facilitates enzymatic adaption to accelerate and prioritize the metabolism of preferred carbon and energy source (e.g., 1-BuOH or acetate) by restraining the synthesis of other enzymes that are not needed (e.g., dioxane MO). Previous studies have shown that substrates that are degraded via central metabolic pathways by constitutive enzymes can inhibit the synthesis of inducible catabolic enzymes that are involved in biotransformation of xenobiotics (Kamath et al., 2004; Mason, 1994). Pronounced catabolite repression is associated with the high concentration of the preferred substrates, fast microbial proliferation, and limited availability of nutrients (Duetz et al., 1994, 1996). These conditions are well aligned with our microcosm study of repeated 1-BuOH amendment, including the repeated large dosage of 1-BuOH as the rapidly metabolizable substrate supporting significant growth of total biomass. Thus, catabolite repression also likely contributed to the observed hindrance of dioxane degradation in 1-BuOH-amended microcosms.

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

The long-term effects on dioxane degradation capacity caused by amendment of auxiliary substrates were explored using the

model strain CB1190. The amended substrate for each treatment was kept in excess by refreshing the medium weekly. After four weeks of incubation, the relative *dxmA* abundance in treatments fed with dioxane and THF did not differ significantly from concentrations measured at the beginning of the experiment (Fig. 5), indicating persistence of the plasmid *pPSED02* under inducing conditions. However, significant loss of this catabolic plasmid was observed in treatments fed with 1-BuOH or acetate, which do not induce or require dioxane MO. Compared with the *dxmA*/16S rRNA gene ratio measured in the initial inoculum, only $9.5 \pm 7.4\%$ and $26.6 \pm 6.2\%$ of the plasmids were retained per cell after growth on 1-BuOH and acetate, respectively, for four weeks. The low retention rate of *dxmADBC* in CB1190 after long-term growth on 1-BuOH corroborates our hypothesis that repeated amendments of non-inducing substrates may lead to the loss or deamplification of mobile genetic elements containing the dioxane degrading genes. This also explains the significant decrease in relative abundance of dioxane degrading genes and dioxane degradation rates observed in the microcosm experiments (Fig. 2). After growth in complex R2A medium, the relative abundance of plasmid *pPSED02* also decreased to $69.0 \pm 13.6\%$. Since total DNA extractions were used to obtain both chromosomal and plasmid DNA, our results lessen the possibility that the lost *dxmA* gene was integrated into the chromosome.

In long-term studies, complete curing of *pPSED02* was observed in CB1190 under non-selective conditions (i.e., in R2A medium). Genome sequencing (Table 2) showed that the average sequence coverage, an indicator of the detection frequency, was 3 X, 0.09 X and 41 X for the three CB1190-bearing plasmids, *pPSED01*, *pPSED02* and *pPSED03*, respectively. However, the average sequence coverage for the chromosome in the obtained CB1190 cells was as high as 100 X, representing the detection frequency when no curing of genetic constituents occurs. The considerably low detection frequencies of plasmids *pPSED01* and *pPSED02* suggests the complete loss of these two plasmids in most CB1190 cells obtained after successive subculturing in nutrient-rich medium R2A for over 6 months. The relative low abundance of the smallest *pPSED03* implies the occurrence of partial loss, assuming the extraction efficiencies for the chromosomal and plasmid DNA were not significantly different. In addition, genomic structural variants such

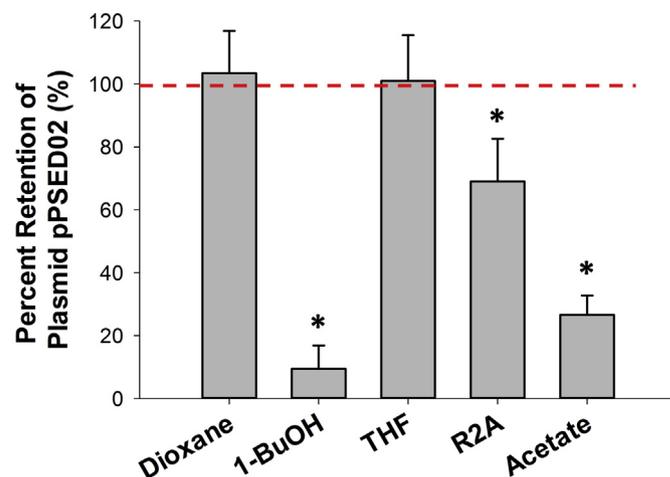


Fig. 5. Percent retention of the plasmid *pPSED02* in CB1190 segregants after growth on different substrates for four weeks. Data were normalized to the initial *dxmA*/16S rRNA gene ratio in stock cultures fed with dioxane. Asterisks indicate the p values less than 0.05 based on the student t -test. The dotted red line represents the 100% retention level, implying no plasmid loss. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 2

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

DNA molecules	Length	Average sequence coverage
Chromosome	7.1 Mb	100X
pPSED01	192 kb	3X
pPSED02	137 kb	0.09X
pPSED03	15 kb	41X

as gene insertions and deletions were not detected on either the chromosome or the plasmid pPSED03, suggesting their stability during subculturing.

Plasmid loss in non-selective environments is possibly due to segregational instability during cell division. For instance, absence of antibiotics (for resistance plasmids) or inducing substrates (for catabolic plasmids) can impact segregational stability or result in plasmid deamplification which subsequently reduce the plasmid abundance or retention (Smith and Bidochka, 1998). Even under nutrient-rich conditions, maintenance and replication of the catabolic plasmids represents an energy cost. Since the encoded enzymes from the plasmid are not needed for growth when the easily-degradable non-inducing substrates are present, the advantage in maintaining non-essential plasmids is diminished. In addition, the large size of these two cured plasmids (i.e., 192 kb and 137 kb) represents a significant energy and resource burden during replication, which may be reduced by segregational instability in which random loss and distribution of plasmids occurs during cell divisions (Austin, 1988). For plasmid pPSED03, the relatively high retention ratio was probably attributed to its small size whose maintenance requires less energy.

A number of previous studies have reported that plasmids are more stable in nutrient-rich media, which provides sufficient energetic content to meet the metabolic burden for plasmid reproduction and maintenance (Fleming and Patching, 1994; Rysz et al., 2013). However, for plasmids bearing specific metabolic functions, such as pPSED02 that contains the gene cluster encoding dioxane degradation enzymes, the stability of such plasmids can be highly dependent on the selective pressure exerted by the substrates or metabolic inducers. When dioxane or THF is absent, pPSED02 might become less advantageous for the cell to maintain, which possibly contributes to plasmid curing though grown in a nutrient-rich environment.

Note that incomplete or complete plasmid loss is the result of natural selection and genetic mutations, which increase host fitness in a specific environment. Under certain conditions with a non-inducing carbon source, derivative (“cured”) segregants may evolve with distinct metabolic advantages for the available substrates and outcompete the wild-type over time (Williams et al., 1988). Plasmid-free derivative strains also tend to grow faster than the wild-type due to a reduced cell size and an enhanced surface-to-volume ratio (Duetz and van Andel, 1991). Thus, it is plausible to conclude the CB1190 segregants without pPSED01 and pPSED02 have a higher fitness in R2A medium than the wild-type strain. The propagation of CB1190 segregants that lack dioxane degradation capabilities likely contributes to the lengthy dioxane degradation lag commonly observed using CB1190 cells grown in rich medium, such as R2A and LB.

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

4. Conclusions

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

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Appendix A. Supplementary data

Supplementary data related to this article can be found at <http://dx.doi.org/10.1016/j.watres.2017.01.047>.

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