

Marquette University
e-Publications@Marquette

Biological Sciences Faculty Research and
Publications

Biological Sciences, Department of

9-1-2016

Effect of Benzene and Ethylbenzene on the Transcription of methyl-*tert*-butyl Ether Degradation Genes of *Methylibium petroleiphilum* PM1

Geetika Joshi

University of California - Davis

Radomir Schmidt

University of California - Davis

Kate M. Scow

University of California - Davis

Michael S. Denison

University of California - Davis

Krassimira R. Hristova

Marquette University, krassimira.hristova@marquette.edu

Accepted version. *Microbiology*, Vol. 162 (September 2016): 1563-1571. DOI. © 2016 Society for
General Microbiology. Used with permission.

Effect of Benzene and Ethylbenzene on the Transcription of Methyl-*Tert*-Butyl Ether Degradation Genes of *Methylibium petroleiphilum* PM1

Geetika Joshi

*Department of Land, Air and Water Resources,
University of California,
Davis, CA*

Radomir Schmidt

*Department of Land, Air and Water Resources,
University of California,
Davis, CA*

Kate M. Scow

*Department of Land, Air and Water Resources,
University of California,
Davis, CA*

Michael S. Denison

*Department of Environmental Toxicology,
University of California,
Davis, CA*

Krassimira R. Hristova

*Department of Land, Air and Water Resources,
University of California,
Davis, CA*

*Department of Biological Sciences, Marquette University,
Milwaukee, WI*

Abstract: Methyl-*tert*-butyl ether (MTBE) and its degradation by-product, *tert*-butyl alcohol (TBA), are widespread contaminants detected frequently in groundwater in California. Since MTBE was used as a fuel oxygenate for almost two decades, leaking underground fuel storage tanks are an important source of contamination. Gasoline components such as BTEX (benzene, toluene, ethylbenzene and xylenes) are often present in mixtures with MTBE and TBA. Investigations of interactions between BTEX and MTBE degradation have not yielded consistent trends, and the molecular mechanisms of BTEX compounds' impact on MTBE degradation are not well understood. We investigated trends in transcription of biodegradation genes in the MTBE-degrading bacterium, *Methylibium petroleiphilum* PM1 upon exposure to MTBE, TBA, ethylbenzene and benzene as individual compounds or in mixtures. We designed real-time quantitative PCR assays to target functional genes of strain PM1 and provide evidence for induction of genes *mdpA* (MTBE monooxygenase), *mdpJ* (TBA hydroxylase) and *bmoA* (benzene monooxygenase) in response to MTBE, TBA and benzene, respectively. Delayed induction of *mdpA* and *mdpJ* transcription occurred with mixtures of benzene and MTBE or TBA, respectively. *bmoA* transcription was similar in the presence of MTBE or TBA with benzene as in their absence. Our results also indicate that ethylbenzene, previously proposed as an inhibitor of MTBE degradation in some bacteria, inhibits transcription of *mdpA*, *mdpJ* and *bmoA* genes in strain PM1.

Keyword(s): strain PM1, groundwater, BTEX, functional gene transcription, MTBE biodegradation

Abbreviations:

BTEX benzene, toluene, ethylbenzene and xylenes

MTBE methyl-*tert*-butyl ether

RT-qPCR real-time quantitative polymerase chain reaction

TBA *tert*-butyl alcohol

TSB tryptic soy broth

Introduction

Methyl- *tert*-butyl ether (MTBE) was used as oxygen enhancer for gasoline to replace tetra ethyl lead after 1979 ([USEPA, 2004–05](#)). Over the years, MTBE has resulted in pollution by entering groundwater via accidental spills during manufacture, distribution and storage of blended fuel, especially through leaking underground (fuel) storage tanks ([Deeb *et al.*, 2003](#)), in California and many other regions in the world. MTBE was banned from use in California in 2003, and several of the contaminant plumes have exhibited shrinkage over the past decade ([McDade *et al.*, 2015](#) ; [McHugh *et al.*, 2015](#)).

MTBE often co-occurs with various components of gasoline, including the mono-aromatic compounds collectively known as BTEX (benzene, toluene, ethylbenzene and *o*-, *m*- and *p*-xylene). As potential alternative carbon sources, these compounds are likely to affect microbial regulatory networks and degradation pathways in degrading bacteria. There is a need to understand the nature of these interactions at the molecular scale in order to develop more effective bioremediation strategies for gasoline spills.

The effect of BTEX compounds on MTBE biodegradation rates has been evaluated in several studies with varying results ([Deeb & Alvarez-Cohen, 2000](#) ; [Deeb *et al.*, 2001](#) ; [Lee & Cho, 2009](#) ; [Lin *et al.*, 2007](#) ; [Pruden *et al.*, 2001](#) , [2003](#) ; [Pruden & Suidan, 2004](#) ; [Raynal & Pruden, 2008](#) ; [Sedran *et al.*, 2002](#)). MTBE degradation in *Pseudomonas aeruginosa* was inhibited in the presence of BTEX compounds ([Lin *et al.*, 2007](#)). In a bioreactor study with a mixed culture consortium enriched on either MTBE or benzene prior to inoculation, MTBE degradation was significantly negatively impacted in the presence of all four BTEX compounds, and the microbial community composition of this consortium associated with MTBE degradation was significantly different in the presence versus absence of BTEX ([Raynal & Pruden, 2008](#)). At a field site where the activity of aquifer microorganisms was stimulated using oxygen release compounds, MTBE degradation occurred only after a significant reduction in BTEX concentrations ([Koenigsberg *et al.*, 1999](#)). A pure culture designated PEL-B201 co-metabolized MTBE following growth on benzene; however, MTBE degradation was severely inhibited in the presence of benzene at concentrations as low as 0.15 mg l⁻¹

presumably due to competitive inhibition ([Koenigsberg et al., 1999](#)). Benzene and ethylbenzene have also been shown to inhibit MTBE oxidation in an alkane-grown pure culture of *Mycobacterium austroafricanum* JOB5 ([House & Hyman, 2010](#)). In contrast, other investigations of interactions between BTEX and MTBE in continuous reactors and mixed bacterial consortia showed no impacts of BTEX compounds on MTBE degradation ([Pruden et al., 2001](#) ; [Sedran et al., 2002](#)). In a porous pot reactor system with the bacterial strain UC1, which has 99 % 16S rDNA sequence similarity with bacterial strain PM1, a mixture of BTEX produced no decrease in either the rate or lag period of MTBE or *tert*-butyl alcohol (TBA) degradation ([Pruden & Suidan, 2004](#)). The presence of MTBE at concentrations comparable to those detected in groundwater at gasoline-contaminated sites has not been shown to significantly alter BTEX degradation rates by bacterial cultures that may be capable of MTBE degradation or mineralization ([Deeb & Alvarez-Cohen, 2000](#) ; [Lee & Cho, 2009](#)).

Methylibium petroleiphilum strain PM1 (ATCC BAA-1232D-5) is a Gram-negative bacterium, member of the class *Betaproteobacteria* and one of the few pure bacterial strains capable of mineralizing MTBE to carbon dioxide whilst using it as a growth substrate ([Nakatsu et al., 2006](#)). The PM1 genome includes an ~600 kb megaplasmid that houses the genes responsible for MTBE degradation. PM1 chromosome also contains genes encoding for aromatic and alkane degradation ([Kane et al., 2007](#)). The gene *mdpA* on the megaplasmid has been demonstrated to be the first gene involved in the breakdown of MTBE via the enzyme MTBE monooxygenase ([Schmidt et al., 2008](#)). Degradation of TBA generated during MTBE metabolism most likely occurs via the enzyme TBA hydroxylase, encoded by genes *mdpJ* and *mdpK* in strain PM1 ([Kane et al., 2007](#)). A comparative transcriptomics microarray study conducted on strain PM1 grown on MTBE or ethanol indicated that expression of genes *mdpA* and *mdpJ* was 1.5 and 11.4 times higher, respectively, in cells grown on MTBE than ethanol ([Hristova et al., 2007](#)). A detailed analysis of strain PM1 cells grown on MTBE or benzene as sole carbon source and then exposed to MTBE or BTEX in mixtures showed that PM1 was unable to degrade ethylbenzene and two xylene isomers at concentrations of 20 mg l⁻¹ following culture growth on MTBE. In addition, the presence of ethylbenzene or the xylenes in mixtures with MTBE completely inhibited MTBE degradation ([Deeb et al., 2001](#)).

Whilst inferences related to MTBE degradation mechanisms have been made from such studies, the understanding of the interaction of MTBE degradation and BTEX degradation pathways at the genetic level is limited. In this study, we examined the transcription of MTBE degradation genes in strain PM1 cells exposed to MTBE, TBA, benzene and ethylbenzene individually and as mixtures to determine if MTBE, TBA and benzene degradation genes are induced in the presence of their corresponding substrates in these cells, and if the presence of benzene and ethylbenzene affect the induction of these genes of strain PM1.

Methods

Culture growth and preparation.

M. petroleiphilum PM1 cultures were routinely grown in 0.33× tryptic soy broth (TSB) at 28 °C with rotary shaking at 150 r.p.m. or on 0.33× TSB agar at 28 °C. Prior to microcosm set-up, cultures were inoculated into 50 ml mineral salts medium [KH_2PO_4 (51 mM), K_2HPO_4 (49 mM), $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ (0.027 mM), MgSO_4 (1 mM), NaHCO_3 (4 mM), NH_4Cl (5.4 mM), CoCl_2 (25 μM), CuSO_4 (3 μM), H_3BO_3 (50 μM), ZnSO_4 (8 μM), FeCl_3 (40 μM), MnCl_2 (10 μM) and Na_2MoO_4 (1 μM)] supplemented with 500 mg l⁻¹ pyruvate in 250 ml glass bottles and allowed to grow to mid-log phase ($\text{OD}_{595\text{ nm}}=0.25\text{--}0.4$). Two millilitres of culture was inoculated into 48 ml MSM with 500 mg l⁻¹ pyruvate, and cultures were grown to mid-log phase. Finally, these cultures were used to inoculate up to 3 l MSM with 500 mg l⁻¹ pyruvate to achieve initial $\text{OD}_{595\text{ nm}}=0.01$ and grown to mid-log phase.

Resting-cell microcosm experiments.

Pyruvate-grown PM1 cultures were harvested by centrifugation at 6000 r.p.m. for 10 min, washed twice in MSM with no carbon source and finally re-suspended in 10 ml (initial experiments with MTBE, TBA exposure or background pyruvate as carbon source) or 50 ml MSM, supplemented with MTBE (50 mg l⁻¹ or 0.57 mM), TBA (52.4 mg l⁻¹ or 0.71 mM), benzene (36.9 mg l⁻¹ or 0.47 mM), ethylbenzene (36.9 mg l⁻¹ or 0.35 mM) and pyruvate (83.1 mg l⁻¹ or 0.94 mM), individually or in mixtures, providing an equivalent amount of carbon; each treatment

was replicated three times. Substrate concentration of 50 mg l⁻¹ MTBE (or equivalent carbon from other substrates) was chosen to enable a molecular response in a short period of time in order to study the functional role of the genes, rather than to mimic environmental conditions directly. Experiments were conducted in 50 ml or 250 ml sterile glass bottles fitted with Teflon-lined Mininert valve caps (Restek). Final aqueous concentrations of volatile compounds in MSM were calculated using dimensionless Henry's constant, reactor liquid and gas volumes as described previously ([Deeb & Alvarez-Cohen, 1999](#)). Microcosm bottles with MSM and appropriate amounts of carbon source were incubated in the dark at 28 °C, 150 r.p.m. for 3 h prior to addition of washed PM1 cells in order to facilitate equilibration of aqueous partitioning of volatile compounds. Inoculum volume was calculated such that final volume in microcosm bottles was 50 ml and OD_{595 nm} was 0.9–1.0 (OD_{595 nm} of 1.0 corresponded to 5×10⁹ cells ml⁻¹). Microcosms were incubated for up to 48 h. Samples (1 ml) for RNA extraction and analysis by gas chromatography were collected aseptically every 3 h using Micro-Mate glass syringes (Cadence) fitted with 20-G, 6-inch stainless steel deflected point septum penetration needles with Luer hub (Cadence). Samples for RNA extraction were preserved with RNAProtect Bacteria Reagent (Qiagen) according to manufacturer's instructions and stored at –70 °C for no longer than 2 weeks prior to extraction. Samples for gas chromatography were stored in 10 ml headspace vials preserved with sodium phosphate tribasic dodecahydrate at a concentration of 1 % by weight and sealed with 20 mm Teflon-lined septa and aluminium crimp caps. Samples were stored for no longer than 1 week at 4 °C prior to analysis. Abiotic controls for each microcosm were also set up with no PM1 cells added and were sampled for analysis by GC.

RNA extraction, reverse transcription and real-time quantitative PCR (RT-qPCR).

RNA was extracted from preserved cell pellets using the RNeasy Mini Kit (Qiagen). DNA removal from extracted RNA was performed using the Ambion TURBO DNA- free Kit (Life Technologies). RNA was quantified using the Qubit RNA Assay Kit (Life Technologies) and converted into single-stranded cDNA using the SuperScript III First-Strand Synthesis SuperMix for RT-qPCR (Life Technologies). cDNA (8

ng per reaction) was used as template for various quantitative PCR assays designed to detect MTBE, TBA and benzene degradation genes of strain PM1. In addition, eight housekeeping genes were also quantified to serve as internal standards using the geNorm approach (Vandesompele *et al.*, 2002). Primers were designed using Primer3 (Koressaar & Remm, 2007; Untergasser *et al.*, 2012) based on the PM1 genome sequence. Primers were used in PCR reactions and reaction product specificity was determined by gel electrophoresis and sequencing. Optimum annealing temperatures for primers were also determined during this step. Details of primers and PCR conditions for each assay are described in Table 1. In RT-qPCR assays, dissociation curves were checked to confirm a single peak corresponding to the desired reaction product. Briefly, 0.5 mM of each primer was used in 25 µl PCR reactions prepared with SYBR GreenER qPCR SuperMix for ABI PRISM (Life Technologies) with MicroAmp optical 96-well reaction plates and MicroAmp optical adhesive film (Applied Biosystems) on an Applied Biosystems 7300 Real-Time PCR System.

Table 1. RT-qPCR assays designed for use in this study

Primer name	Targeted gene	Function in assay	Amplicon size (bp)	Primer Sequence (5' - 3')	PCR conditions			Number of cycles
					Initial denaturation	Denaturation	Annealing	
mtpA 211F	MTBE monooxygenase	Gene of interest	211	TCATCATCGGATGTTGCTA ACOSTAGAAAGACCAGCGAGA	95 °C, 10 min	95 °C, 15 s	58 °C, 1 min	40
mtpA 211R								
mtpJ 245F	TBA hydroxylase	Gene of interest	245	TCTCCAATGTCTTCSACTGC GATTCGGATCCAGACTTCGT	95 °C, 10 min	95 °C, 15 s	58 °C, 1 min	40
mtpJ 245R								
bmoA F	Benzene monooxygenase	Gene of interest	278	GAGAGAACGATGGCGCTAC ATCCAGCCTGGGTCAGC	95 °C, 10 min	95 °C, 15 s	63 °C, 1 min	40
bmoA R								
pykF F	Pyruvate kinase	Internal standard	177	GAGCTTCAGTGGGAGTACC TCGCTAGCCTGAGGATCTG	95 °C, 10 min	95 °C, 15 s	58 °C, 1 min	40
pykF R								
gyrA F	DNA gyrase subunit A	Internal standard	237	GACAAGAGCTGGGATTDGTC CGCCATCACCTCCTTGATT	95 °C, 10 min	95 °C, 15 s	58 °C, 1 min	40
gyrA R								
gyrB F	DNA gyrase subunit B	Internal standard	202	AATACAACCCGACAAGCTG CGCTCCTCAGGTAAGTCTC	95 °C, 10 min	95 °C, 15 s	56 °C, 1 min	40
gyrB R								
glyA F	Serine hydroxymethyl transferase	Internal standard	178	CAGCAACAGGTGGTGAAGAA TCTTGTTCAGGTCATGTGC	95 °C, 10 min	95 °C, 15 s	62 °C, 1 min	40
glyA R								
rho F	RNA polymerase rho subunit	Internal standard	190	GGGCTGAGTAGATGTDSTC CCTTGAGATCGAGAACAGC	95 °C, 10 min	95 °C, 15 s	58 °C, 1 min	40
rho R								
gap1 F	Glyceraldehyde-3- phosphate dehydrogenase	Internal standard	247	CAAGAAGCACGACATCCAGA TTCTTGGTCTGSAAGAAGC	95 °C, 10 min	95 °C, 15 s	62 °C, 1 min	40
gap1 R								
gap2 F	Glyceraldehyde-3- phosphate dehydrogenase	Internal standard	150	TGAGTTCGGGTAGATCAGC AGATCACCACGATCCAGAT	95 °C, 10 min	95 °C, 15 s	58 °C, 1 min	40
gap2 R								
gap3 F	Glyceraldehyde-3- phosphate dehydrogenase	Internal standard	202	ATEGACATCGTCATGGAGTG GCCAGTTGGTGGTACAGGAG	95 °C, 10 min	95 °C, 15 s	58 °C, 1 min	40
gap3 R								

All genes are specific to strain PM1.

Gas chromatography

MTBE, TBA, benzene and ethylbenzene were quantified on an Agilent 6890N gas chromatograph equipped with a flame ionization detector and an HP 7694 headspace autosampler. Organic compounds were separated using an Agilent HP1 capillary column (60 m by 1 μ m by 0.320 μ m) using methods described previously ([Mackay *et al.*, 2006](#); [Schmidt *et al.*, 2008](#)).

Total protein analysis.

Total protein content was determined using 1 ml samples collected at the beginning and end of incubations. The cell suspension was centrifuged at 10 000 **g** for 2 min and resulting pellets were frozen at -20°C until analysis. Pellets were thawed and treated with Benzonase Nuclease and BugBuster Protein Extraction Reagent (EMD Millipore, Merck KGaA) to extract total protein that was then quantified using the Bio-Rad Protein Assay Kit (Bio-Rad Laboratories) according to manufacturer's protocols in each case.

Data analysis.

Bacterial gene transcription data were normalized by dividing gene quantity values determined by RT-qPCR by the geometric mean of gene quantities of the most stable genes as determined by the geNorm package for Microsoft Excel ([Vandesompele *et al.*, 2002](#)). The minimum number of normalizing genes was selected based on the pairwise variation between two sequential normalization factors containing an increasing number of genes; if pairwise variation for each new gene tested was below 0.15, then an additional internal standard gene was not included. Most stable reference genes determined by the geNorm approach used for normalization of gene transcription under various conditions are listed in Table S1 (available in the online Supplementary Material). The output from the gas chromatograph was analysed using ChemStation revision A.10.02 software (Agilent). Two-sample *t* test was used to determine effect of specific substrates on functional gene transcription in pairwise comparisons amongst different treatments; *P* value <0.05 was considered significant (Table S2).

Results

Induction of MTBE degradation genes in the presence of MTBE and TBA

PM1 cells were exposed to MTBE, TBA or pyruvate to determine time-dependent changes in transcription of MTBE and TBA degradation genes *mdpA* and *mdpJ*. When PM1 cells grown on pyruvate were washed and exposed to 50 mg l⁻¹ MTBE, *mdpA* was induced up to 2.3-fold ($P=0.0002$) of initial level by 3 h, reaching as high as 7.6-fold ($P<0.0001$) of the initial level by 18 h (Fig. 1). Exposure to MTBE also resulted in induction of gene *mdpJ* up to 1.9-fold ($P<0.0001$) within 3 h, reaching as high as 27.6-fold ($P<0.0001$) initial level by 18 h (Fig. 1). Of the initial MTBE concentration, 36.3±1.8 % was degraded after 48 h.

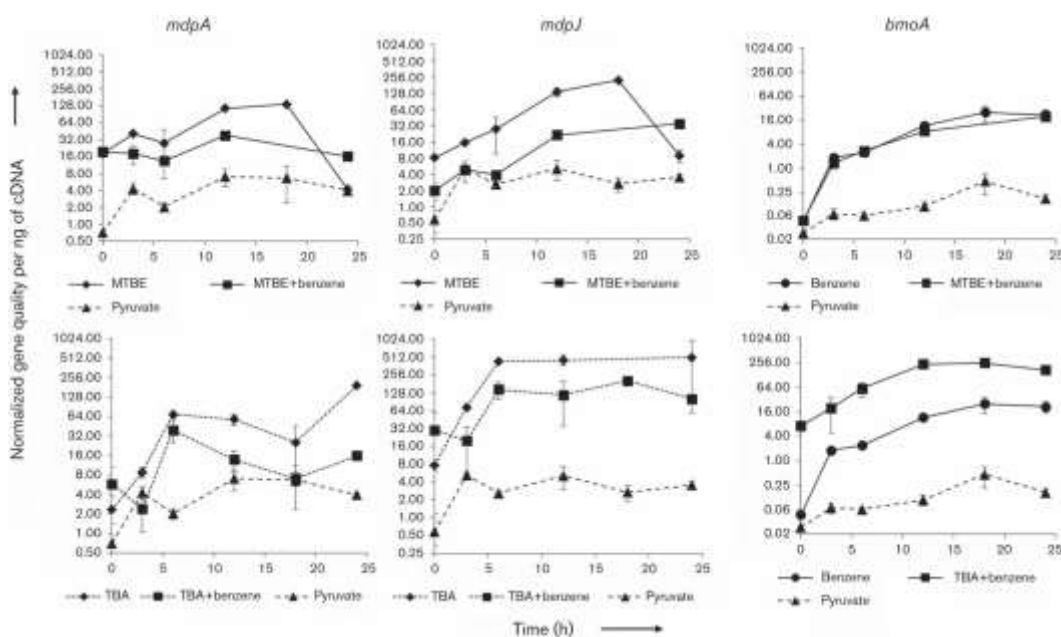


Fig. 1. Strain PM1 gene transcription profile for genes *mdpA* (MTBE monooxygenase), *mdpJ* (TBA hydroxylase) and *bmoA* (benzene monooxygenase) after exposure of pyruvate grown cells to 50 mg l⁻¹ MTBE, 52.4 mg l⁻¹ TBA, 36.9 mg l⁻¹ benzene and 83.1 mg l⁻¹ pyruvate (control) and combinations of MTBE or TBA+benzene. Transcription levels for genes of interest were normalized with geometric means of transcription of housekeeping genes *gyrA*, *gyrB*, *pykF* and *glyA* monitored simultaneously. The y-axis was transformed logarithmically (base 2). Error bars represent the standard deviation of the mean value of three replicates.

Washed PM1 cells grown on pyruvate were also exposed to 52.4 mg l⁻¹ TBA ([Fig. 1](#)). Exposure to TBA resulted in the induction of gene *mdpJ* transcription, which encodes the enzyme TBA hydroxylase, up to 9.5-fold (*P*=0.0002) within 3 h of exposure, reaching as high as 66.4-fold (*P*=0.0006) of the initial level at 24 h ([Fig. 1](#)). Exposure to TBA also resulted in induction of *mdpA* transcription up to 3.8-fold (*P*=0.007) within 3 h, reaching as high as 83.1-fold (*P*<0.0001) after 24 h ([Fig. 1](#)). After 48 h, 37.0±29.5 % of the initial TBA concentration had been degraded.

To compare induction with a baseline level of transcription, we also studied resting PM1 cells exposed to 83.1 mg l⁻¹ pyruvate ([Fig. 1](#)). In this case, there was no significant change in *mdpA* and *mdpJ* transcripts throughout the course of the incubation (Table S2).

Transcription profile of MTBE and benzene degradation genes in the presence of benzene and ethylbenzene

In the next series of experiments, washed PM1 cells were exposed to 50 mg l⁻¹ MTBE, 52.4 mg l⁻¹ TBA, 36.9 mg l⁻¹ benzene and ethylbenzene, or 83.1 mg l⁻¹ pyruvate, either alone or in the following mixtures: (MTBE or TBA) + benzene, (MTBE or TBA) + ethylbenzene, or (MTBE or TBA) + benzene + ethylbenzene ([Figs 1](#) and [2](#)). Concentrations of compounds were selected to ensure equivalent amounts of carbon as 50 mg l⁻¹ MTBE.

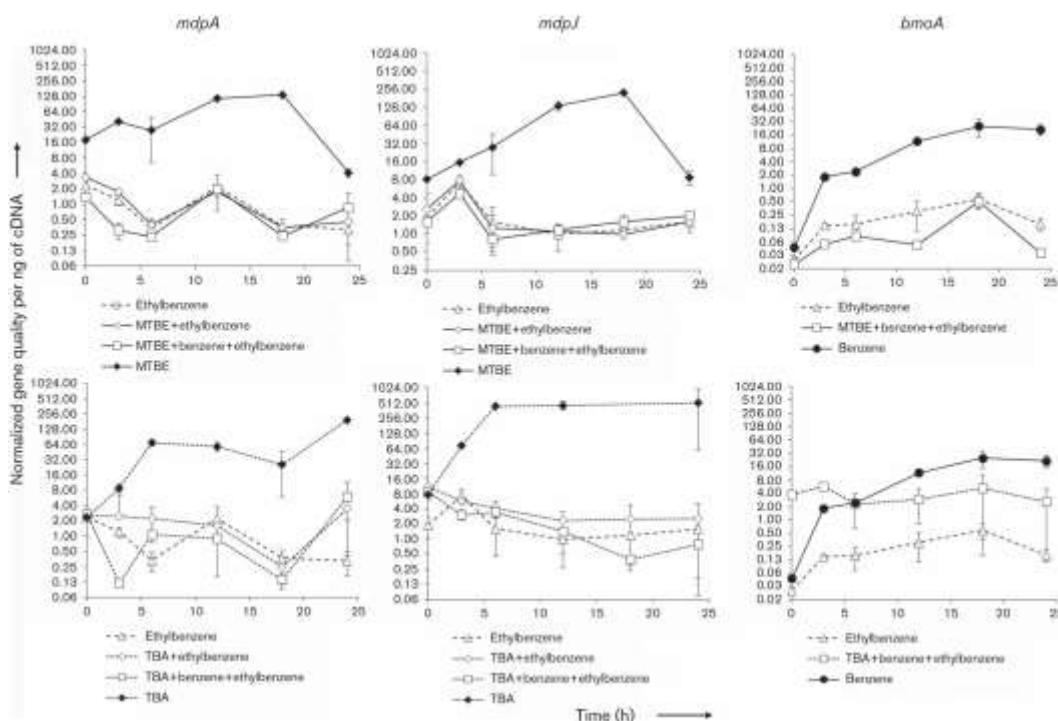


Fig. 2. Strain PM1 gene transcription profile for genes *mdpA* (MTBE monooxygenase), *mdpJ* (TBA hydroxylase) and *bmoA* (benzene monooxygenase) after exposure of pyruvate grown cells to 50 mg l⁻¹ MTBE, 52.4 mg l⁻¹ TBA, 36.9 mg l⁻¹ benzene and 36.9 mg l⁻¹ ethylbenzene and combinations of MTBE/TBA with benzene and ethylbenzene. Transcription levels for genes of interest were normalized with geometric means of transcription of housekeeping genes *gyrA*, *gyrB*, *pykF* and *glyA* monitored simultaneously. The y-axis was transformed logarithmically (base 2). Error bars represent the standard deviation of the mean value of three replicates.

As previously noted, *mdpA* was induced in the presence of both MTBE and TBA relative to that in the presence of pyruvate (Fig. 1). No induction was observed in the presence of benzene (Fig. S1), ethylbenzene (Fig. 2) or pyruvate (Fig. 1). In the presence of MTBE and benzene mixture, *mdpA* induction was slower, increasing up to 2-fold ($P < 0.0001$) in 12 h (Fig. 1) from the original level. In the presence of TBA and benzene mixture, *mdpA* induction was observed after 6 h, up to 6.8-fold ($P = 0.0158$) of original level (Fig. 1). In the presence of ethylbenzene, alone or in mixture with TBA, or TBA and benzene, *mdpA* induction was not observed up to 24 h of exposure (Fig. 2). Overall, *mdpA* transcription was lower in the presence of benzene than with TBA alone (Fig. 1), and there was no induction in the presence of ethylbenzene (Fig. 2).

As noted previously, *mdpJ* was induced in the presence of both MTBE and TBA ([Fig. 1](#)). There was no significant change in transcription of *mdpJ* when PM1 cells were exposed to benzene (Fig. S1), ethylbenzene ([Fig. 2](#)) or pyruvate ([Fig. 1](#)). When MTBE or TBA and benzene were present together, *mdpJ* was induced ([Fig. 1](#)). However, in the presence of ethylbenzene (individually or in mixtures), *mdpJ* transcription significantly decreased compared to the initial gene copy numbers after 3 h. *mdpJ* transcription was 1.9-fold ($P=0.0036$) and 2.8-fold ($P=0.006$) less than initial levels in the presence of mixtures of ethylbenzene with TBA or with TBA+benzene, respectively ([Fig. 2](#)).

In summary, *mdpA* and *mdpJ* transcription was induced after exposure to MTBE and TBA and also when benzene was co-present; however, the presence of ethylbenzene, whether alone or in the presence of inducing compounds, inhibited induction of these genes.

The gene encoding benzene monooxygenase in PM1, *bmoA*, was induced after 3 h up to 36.4-fold ($P<0.0001$) of initial level in response to benzene, rising to as high as 499.6-fold after 24 h ($P=0.0146$) of exposure. Transcription of *bmoA* was not induced in the presence of MTBE, TBA, pyruvate (Fig. S1) or ethylbenzene ([Fig. 2](#)) during the 24 h incubation. When MTBE and benzene were present together, *bmoA* was induced at levels similar to those in the presence of benzene alone, 26.8-fold ($P=0.0054$) in 3 h, increasing up to 391-fold ($P=0.0002$) after 18 h of exposure ([Fig. 1](#)) indicating that MTBE is not an inhibitor of *bmoA* induction. *bmoA* was also induced in the TBA+benzene mixture, up to 8.2-fold ($P=0.0154$) within 6 h and as high as 35.1-fold ($P<0.0001$) after 18 h of exposure ([Fig. 1](#)). When ethylbenzene was present, alone or in combinations with MTBE, TBA or benzene, *bmoA* was not induced in the 24 h of incubation ([Fig. 2](#)). Background or constitutive level of *bmoA* in samples was close to the detection limit of the qPCR assay.

Degradation of substrates by strain PM1

There was little degradation detected for the tested substrates after incubation with PM1 resting cells in 24 or 48 h; however, in microcosms with MTBE, TBA (as a product of MTBE oxidation) started to appear within 5 h of incubation ([Fig. 3](#)). Despite the lower

degradation rates observed (Table S3) in the short incubation time in our experiments, the trends observed in *mdpA* and *mdpJ* transcription were consistent across experiments, with induction occurring upon exposure to both MTBE and TBA within 3 h and increasing to a maximum level by 18 or 24 h. Subsequently, microcosms with TBA combinations with benzene were incubated up to 168 h to measure biodegradation. In this case, >98 % of TBA and benzene were degraded after 168 h (data not shown).

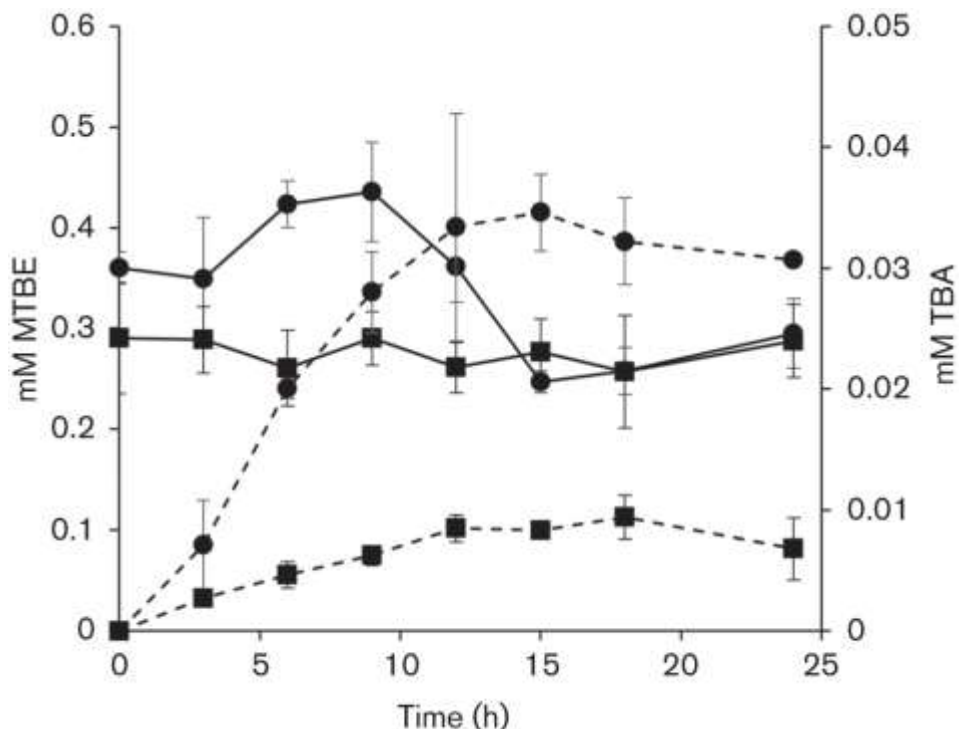


Fig. 3. Appearance of TBA (dashed line) during incubation of strain PM1 cells exposed to MTBE (circles) or MTBE+benzene (squares). Solid line represents MTBE concentration on the primary y-axis. Dashed line represents TBA concentration on the secondary y-axis. Error bars represent standard deviation of the mean value of three replicates.

MTBE degradation rate was not significantly different in the presence of benzene in mixture compared to the MTBE-only treatment. Benzene was degraded at rates similar to or greater, in mixtures with MTBE or TBA, respectively, than in their absence. TBA degradation rate was higher in the presence of benzene in mixture than when present alone (Table S3). When ethylbenzene was present in mixtures with MTBE, TBA and/or benzene, none of the four substrates was degraded throughout the entire incubation period, up to 168 h. PM1 was not able

to degrade ethylbenzene for the entire duration of incubation. No significant reduction in concentrations of MTBE, TBA, benzene and ethylbenzene was observed in abiotic controls (no PM1 cells added), up to 24 or 168 h. There was no significant change in total protein content after 48 h of incubation in all treatments ($0.05 \pm 0.02 \text{ mg l}^{-1}$ at 0 h and $0.04 \pm 0.02 \text{ mg l}^{-1}$ at 48 h).

Discussion

In this study, we found that *mdpA* and *mdpJ* genes of strain PM1 were induced upon exposure to MTBE and TBA, respectively; their induction was inhibited in the presence of ethylbenzene using specifically designed RT-qPCR assays to determine the response of MTBE degradation genes to the presence of the chemicals MTBE, TBA, benzene and ethylbenzene, both individually and in mixtures. We performed resting-cell experiments to specifically observe the transcription of MTBE degradation genes independently of growth by the organism, similar to a previous study with this strain ([Schmidt et al., 2008](#)).

Our study provides conclusive genetic evidence that *mdpA* is constitutively expressed at low levels that increase significantly upon exposure to MTBE ([Fig. 1](#)). In a previous study ([Schmidt et al., 2008](#)), MTBE degradation in resting PM1 cells occurred in the presence of chloramphenicol, an inhibitor of protein synthesis, but at a rate 6- to 8-fold lower than chloramphenicol-absent controls. Thus based on both findings, the presence of MTBE likely highly induced *mdpA* gene transcription compared to the lower background level. MTBE degradation reported in this study is lower than in earlier studies, such as one where up to 10 mg l^{-1} MTBE was degraded by PM1 cells grown on ethanol or MTBE within 2–4 h of exposure to MTBE ([Schmidt et al., 2008](#)). In this study, we grew cells on pyruvate, a compound with no known influence on the regulation of oxygenate or aromatic hydrocarbon degradation pathways, in order to ensure accurate transcription induction data. We thus observed longer lag phases and slower degradation rates compared to studies where MTBE, TBA or ethanol was the carbon source for culture growth. We rejected ethanol as a growth substrate when preliminary studies showed that the background transcription of *mdpA* in the presence of

ethanol exceeded that on pyruvate and thus the cellular response to MTBE exposure was quicker. TBA appeared in MTBE-treated microcosms at 6 h, reaching a maximum by 12 h, which is consistent with the highest level of *mdpJ* transcription in these microcosms within the 6–12 h time frame ([Figs 1](#) and [3](#)). The slower rate of MTBE removal up to 24 h ([Fig. 3](#)) may be due to potential downstream regulatory mechanisms affecting MdpA translation, folding, transport and membrane insertion, or activation. These mechanisms were not investigated in this study.

The induction of *mdpJ* in response to TBA in strain PM1 followed a trend similar to that of *mdpA* in response to MTBE: induction occurred within 3 h and steadily increased subsequently. This is similar to other bacteria, such as the TBA degrader *Aquicola tertiaricarbonis* L108 where MdpJ protein is induced upon exposure to TBA ([Schaefer et al., 2007](#)).

Whilst TBA is a downstream metabolite of MTBE degradation and substrate for MdpJ, *mdpA* was induced by TBA up to 3.8-fold of the original level within 3 h ([Fig. 1](#)). It is unclear at this point if the induction of both *mdpA* and *mdpJ* by both MTBE and TBA is due to a single regulatory system that interacts with both MTBE and TBA or separate molecules that sense MTBE and TBA and induce both enzymes. It is known that *mdpA* and *mdpJ* induction is regulated by a transcriptional activator MdpC; however, a separate mechanism also likely regulates *mdpJ* induction ([Joshi et al., 2015](#)).

To our knowledge, it has not been reported previously that ethylbenzene blocks transcription of *mdpA* ([Fig. 2](#)) and consequently the degradation of MTBE and TBA in *M. petroleiphilum* PM1 (Table S3). Induction of *mdpA*, *mdpJ* and *bmoA* was inhibited in the presence of ethylbenzene as the sole carbon source. Furthermore, no induction was observed when respective inducers of these genes were present along with ethylbenzene, indicating that ethylbenzene likely acts as a regulatory inhibitor of MTBE, TBA and benzene degradation pathways in strain PM1. This finding is consistent with the earlier finding ([Deeb et al., 2001](#)) where no degradation of MTBE was seen in the presence of 20 mg l⁻¹ ethylbenzene. The absence of gene induction is a clear indicator of inhibition of transcription, rather than a negative effect on translation or enzyme inhibition by ethylbenzene. The transcription of

genes involved in central cellular mechanisms, such as *pykF* (pyruvate kinase), was not significantly different in the presence or absence of ethylbenzene; therefore, it may be inferred that the latter functioned as an inhibitor of pathways rather than as a toxin resulting in cell death (Fig. S2).

In contrast to ethylbenzene, benzene had a minor effect on transcription of *mdpA* and *mdpJ*. Benzene also had no significant impact on MTBE degradation, whilst degradation of benzene ($P=0.0034$) and TBA ($P=0.0012$) was enhanced if present in a mixture (Table S3). Decreased transcription of *mdpA* and enhanced transcription of *bmoA* when both MTBE and benzene were present in a mixture might explain earlier findings, where benzene degradation rates rose from $1.1 \text{ mg l}^{-1} \text{ h}^{-1}$ for benzene alone to $3.5 \text{ mg l}^{-1} \text{ h}^{-1}$ when MTBE and benzene were present together ([Deeb et al., 2001](#)).

In this study, whilst *mdpJ* (TBA hydroxylase) induction was lower in the presence of benzene in mixture than with TBA alone ([Fig. 1](#)), the overall rate of TBA degradation was higher in the presence of benzene in mixture ($P=0.0012$) (Table S3). In a study conducted with an MTBE-grown culture UC1, which has >99 % sequence similarity to that of the 16S rRNA gene of PM1, degradation of $6\text{--}45 \text{ mg l}^{-1}$ MTBE was not impacted by the presence of BTEX at 0.3–0.6 times the molar mass of MTBE (or TBA), whilst TBA degradation was enhanced ~ 1.5 -fold in the presence of benzene ([Pruden & Suidan, 2004](#)). In the presence of a mixed culture amongst which PM1-like bacteria were also present, BTEX had no effect on MTBE degradation in a batch reactor, but a lag in TBA degradation was observed ([Sedran et al., 2002](#)). In our study, in addition to enhanced TBA degradation in the mixture, benzene degradation rate was also faster in the presence of TBA than its absence, based on the rate of substrate removal from the mixture (Table S3). *bmoA* induction was similar in benzene-only and benzene/MTBE mixtures but was more rapid and greater in the presence of TBA ([Fig. 1](#)). Substrate degradation by enzymes in the second pathway might be suggested as a possible mechanism for these observations, though no supporting evidence is available to date. The mechanism for higher benzene degradation was not investigated in this study.

Our data also provide clear genetic evidence for the inducible nature of the benzene degradation pathway in strain PM1. The independent, substrate-specific induction of *bmoA* and *mdpA* transcription in response to benzene and MTBE, respectively, provides direct confirmation for previous reports that benzene degradation pathway regulation is separate from that for MTBE in strain PM1 (Deeb et al., 2001 ; Kane et al., 2007). The presence of benzene delayed *mdpJ* transcription and the higher increase occurred between 6 and 12 h, rather than within 3 h as in the presence of MTBE or TBA alone (Fig. 2). In resting-cell experiments conducted with MTBE or MTBE+benzene, TBA was first detected after 3 h of exposure, reaching a plateau after 12 h (Fig. 3). This trend is expected as TBA is a downstream metabolite of MTBE degradation. The low concentrations of TBA detected relative to MTBE and the high *mdpJ* induction levels are consistent with simultaneous removal of TBA by MdpJ in the system.

Implications: Previous field studies have found that *M. petroleiphilum* strain PM1 is present in MTBE and TBA-contaminated aquifers and may play an important role in bioremediation especially under oxygenated conditions, as indicated by larger population sizes in contaminated aquifers undergoing oxygenation (Hristova et al., 2003 ; North et al., 2012). Biostimulation via oxygenation or bioaugmentation using strain PM1 has also been shown to be an effective bioremediation strategy (Smith et al., 2005). Our result that ethylbenzene inhibits transcription of MTBE and benzene degradation genes of strain PM1 shows that, in addition to oxygen availability and presence of strain PM1 in native population, the concentration of co-occurring contaminants, especially ethylbenzene, is likely to be an important factor in determining the success of bioremediation efforts involving strain PM1. Along with ethylbenzene inhibition of MTBE and TBA degradation, the likelihood of benzene to be utilized as a preferred carbon source and greater extent of *bmoA* induction in the presence of TBA could additionally have practical implications at contaminated sites that are dominated by strain PM1 (and related organisms) in explaining delay in MTBE degradation until BTEX is degraded or MTBE plume migrates away from the source zone.

Acknowledgements

The authors would like to thank Johnny Hsia and Kevin Nicholson of UC Davis for assistance with RT-qPCR analyses. This project was supported in part by (i) Grant Number T32-GM008799 from National Institute of General Medical Sciences (NIGMS), National Institutes of Health (NIH) and (ii) National Institute of Environmental Health Sciences (NIEHS) of the NIH under Award Number P42ES004699 and (iii) by an industry/campus supported fellowship under the Training Program in Biomolecular Technology (T32-GM008799) at the University of California, Davis. Its contents are solely the responsibility of the authors and do not necessarily represent the official views of the NIGMS, NIEHS or NIH.

References

- Deeb R. A., Alvarez-Cohen L. (1999). Temperature effects and substrate interactions during the aerobic biotransformation of BTEX mixtures by toluene-enriched consortia and *Rhodococcus rhodochrous*. *Biotechnol Bioeng* 62 526–536.
- Deeb R. A., Alvarez-Cohen L. (2000). Aerobic biotransformation of gasoline aromatics in multi component mixtures. *Bioremed J* 4 171–179.
- Deeb R. A., Hu H.-Y., Hanson J. R., Scow K. M., Alvarez-Cohen L. (2001). Substrate interactions in BTEX and MTBE mixtures by an MTBE-degrading isolate. *Environ Sci Technol* 35 312–317.
- Deeb R. A., Chu K.-H., Shih T., Linder S., Suffet I., Kavanaugh M. C., Alvarez-Cohen L. (2003). MTBE and other oxygenates: environmental sources, analysis, occurrence, and treatment. *Environ Eng Sci* 20 433–447.
- House A. J., Hyman M. R. (2010). Effects of gasoline components on MTBE and TBA cometabolism by *Mycobacterium austroafricanum* JOB5. *Biodegradation* 21 525–541.
- Hristova K., Gebreyesus B., Mackay D., Scow K. M. (2003). Naturally occurring bacteria similar to the methyl *tert*-butyl ether (MTBE)-degrading strain PM1 are present in MTBE-contaminated groundwater. *Appl Environ Microbiol* 69 2616–2623.
- Hristova K. R., Schmidt R., Chakicherla A. Y., Legler T. C., Wu J., Chain P. S., Scow K. M., Kane S. R. (2007). Comparative transcriptome analysis of *Methylibium petroleiphilum* PM1 exposed to the fuel oxygenates methyl *tert*-butyl ether and ethanol. *Appl Environ Microbiol* 73 7347–7357.
- Joshi G., Schmidt R., Scow K. M., Denison M. S., Hristova K. R. (2015). Gene *mdpC* plays a regulatory role in the methyl *tert*-butyl ether

- degradation pathway of *Methylibium petroleiphilum* strain PM1. *FEMS Microbiol Lett* 362 fnv029.
- Kane S. R., Chakicherla A. Y., Chain P. S., Schmidt R., Shin M. W., Legler T. C., Scow K. M., Larimer F. W., Lucas S. M. et al. (2007). Whole-genome analysis of the methyl *tert*-butyl ether-degrading *beta*-proteobacterium *Methylibium petroleiphilum* PM1. *J Bacteriol* 189 1931–1945.
- Koenigsberg S., Sandefur C., Mahaffey W., Deshusses M., Fortin N. (1999). *Peroxygen mediated bioremediation of MTBE. In In Situ Bioremediation of Petroleum Hydrocarbon and Other Organic Compounds*, pp. 13–18. Edited by Alleman B. C., Leeson. Columbus A. OH: Battelle Press.
- Koressaar T., Remm M. (2007). Enhancements and modifications of primer design program Primer3. *Bioinformatics* 23 1289–1291.
- Lee E.-H., Cho K.-S. (2009). Effect of substrate interaction on the degradation of methyl- *tert*-butyl ether, benzene, toluene, ethylbenzene, and xylene by *Rhodococcus* sp. *J Hazard Mater* 167 669–674.
- Lin C.-W., Cheng Y.-W., Tsai S.-L. (2007). Multi-substrate biodegradation kinetics of MTBE and BTEX mixtures by *Pseudomonas aeruginosa*. *Process Biochem* 42 1211–1217.
- Mackay D. M., de Sieyes N. R., Einarson M. D., Feris K. P., Pappas A. A., Wood I. A., Jacobson L., Justice L. G., Noske M. N. et al. (2006). Impact of ethanol on the natural attenuation of benzene, toluene, and *o*-xylene in a normally sulfate-reducing aquifer. *Environ Sci Technol* 40 6123–6130.
- McDade J. M., Connor J. A., Paquette S. M., Small J. M. (2015). Exceptionally long MTBE plumes of the past have greatly diminished. *Groundwater* 53 515–524.
- McHugh T. E., Rauch S. R., Paquette S. M., Connor J. A., Daus A. D. (2015). Life cycle of methyl- *tert*-butyl ether in California public water supply wells. *Environ Sci Technol Lett* 2 7–11.
- Nakatsu C. H., Hristova K., Hanada S., Meng X. Y., Hanson J. R., Scow K. M., Kamagata Y. (2006). *Methylibium petroleiphilum* gen. nov., sp. nov., a novel methyl- *tert*-butyl ether-degrading methylotroph of the betaproteobacteria. *Int J Syst Evol Microbiol* 56 983–989.
- North K. P., Mackay D. M., Kayne J. S., Petersen D., Rasa E., Rastegarzadeh L., Holland R. B., Scow K. M. (2012). *In situ* biotreatment of TBA with recirculation/oxygenation. *Ground Water Monit Remediat* 32 52–62.
- Pruden A., Suidan M. T., Venosa A. D., Wilson G. J. (2001). Biodegradation of methyl-butyl ether under various substrate conditions. *Environ Sci Technol* 35 4235–4241.

- Pruden A., Sedran M., Suidan M., Venosa A. (2003). Biodegradation of MTBE and BTEX in an aerobic fluidized bed reactor. *Water Sci Technol* 47 123–128.
- Pruden A., Suidan M. (2004). Effect of benzene, toluene, ethylbenzene, and *p*-xylene (BTEX) mixture on biodegradation of methyl- *tert*-butyl ether (MTBE) and *tert*-butyl alcohol (TBA) by pure culture UC1. *Biodegradation* 15 213–227.
- Raynal M., Pruden A. (2008). Aerobic MTBE biodegradation in the presence of BTEX by two consortia under batch and semi-batch conditions. *Biodegradation* 19 269–282.
- Schäfer F., Breuer U., Benndorf D., von Bergen M., Harms H., Müller R. H. (2007). Growth of *Aquicola tertiaricarbonis* L108 on *tert*-butyl alcohol leads to the induction of a phthalate dioxygenase-related protein and its associated oxidoreductase subunit. *Eng Life Sci* 7 512–519.
- Schmidt R., Battaglia V., Scow K., Kane S., Hristova K. R. (2008). Involvement of a novel enzyme MdpA, in methyl- *tert*-butyl ether degradation in *Methylibium petroleiphilum* PM1. *Appl Environ Microbiol* 74 6631–6638.
- Sedran M. A., Pruden A., Wilson G. J., Suidan M. T., Venosa A. D. (2002). Effect of BTEX on degradation of MTBE and TBA by mixed bacterial consortium. *J Environ Eng* 128 830–835.
- Smith A. E., Hristova K., Wood I., Mackay D. M., Lory E., Lorenzana D., Scow K. M. (2005). Comparison of biostimulation versus bioaugmentation with bacterial strain PM1 for treatment of groundwater contaminated with methyl tertiary butyl ether (MTBE). *Environ Health Perspect* 113 317–322.
- Untergasser A., Cutcutache I., Koressaar T., Ye J., Faircloth B. C., Remm M., Rozen S. G. (2012). Primer3—new capabilities and interfaces. *Nucleic Acids Res* 40 e115.
- USEPA (2004-05). Focus on: the oxygenate MTBE. In *Technical Support Times*, pp. 1–6. EPA.
- Vandesompele J., De Preter K., Pattyn F., Poppe B., Van Roy N., De Paepe A., Speleman F. (2002). Accurate normalisation of real-time quantitative RT-PCR data by geometric averaging of multiple internal control genes. *Genome Biol* 3 research0034.