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Microbial community response to a release of neat ethanol onto residual hydrocarbons in a pilot-scale aquifer tank

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Summary

The microbial community response to a neat ethanol release (E100, 76 l) onto residual hydrocarbons in sandy soil was evaluated in a continuous-flow 8 m^3 pilot-scale aquifer tank, simulating a release at a bulk fuel terminal. Microbial genotypic shifts were assessed using quantitative real-time PCR analysis. High ethanol concentrations in the capillary fringe at potentially toxic levels, exceeding 100 000 mg l^{-1}, were tolerated by the microbial community. The high biochemical oxygen demand exerted by ethanol rapidly induced anaerobic conditions, and both methane production (up to 1.2 mg l^{-1}) and growth of putative methanogenic *Archaea* (up to 10^6 gene copies per g of soil) were observed in shallow groundwater and soil samples 75 cm down gradient from the source. Aerobic conditions returned after ethanol was flushed out of the system, approximately 45 days after the spill (less than 7.5 pore volumes flushed). Total *Bacteria* growth coincided with ethanol migration and availability, which was restricted to a relatively thin layer at the capillary fringe and water table interface. The concentrations of bacteria harbouring the aerobic catabolic genes *dmpN* (coding for phenol hydroxylase) and *todC1* (coding for toluene dioxygenase) increased (up to 100×) down gradient from the source, likely as a result of both fortuitous growth on ethanol and on aromatic hydrocarbons mobilized by ethanol. Growth of hydrocarbon degraders was corroborated by denaturing gradient gel electrophoresis analysis showing proliferation of *Azospirillum* and *Brevundimonas* spp., which are bacteria commonly associated with microaerophilic hydrocarbon degradation. Nevertheless, the relative abundance of hydrocarbon-specific degraders (as a fraction of total *Bacteria*) decreased as other bacteria grew to a higher extent. Overall, the observed growth of hydrocarbon degraders suggests a potential enhancement in aerobic natural attenuation in shallow aquifers after ethanol and its degradation by-products are degraded or flushed from sites impacted by ethanol-blended fuels.

Introduction

The potential for groundwater contamination by ethanol-blended fuels is rapidly increasing with the increased reliance on ethanol to meet renewable fuel and Clean Air Act requirements (Deeb *et al*., 2003; Farrell *et al*., 2006). As biodegradation is a critical natural attenuation mechanism following a fuel release, a better understanding is needed about how the presence of ethanol affects microorganisms associated with the biodegradation of fuel hydrocarbons. In particular, it is important to understand how ethanol affects the abundance and activity of bacteria that degrade benzene, toluene, and xylenes (BTX), which are often the fuel hydrocarbons of greatest concern in groundwater.

Ethanol is a favourable bacterial growth substrate that can be easily metabolized, and bacteria that can feed on ethanol are more ubiquitous in the environment than those that degrade BTX compounds (Powers *et al*., 2001a,b). However, ethanol toxicity studies with indigenous organisms in soil matrices have shown that concentrations above 10 g l^{-1} inhibit bacterial growth (Ingram and Buttke, 1984) and concentrations exceeding 40–100 g l^{-1} exert a bactericidal effect (Ingram and Buttke, 1984; Hunt *et al*., 1997; Araujo *et al*., 1998). The presence of ethanol can also stimulate metabolic shifts

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that hinder BTX degradation (e.g. metabolic flux dilution and catabolic gene repression) (Lovanh *et al*., 2002; Lovanh and Alvarez, 2004), and accelerate the depletion of electron acceptors and nutrients that are consumed during its degradation (Corseuil *et al*., 1998; Da Silva and Alvarez, 2002; Ruiz-Aguilar *et al*., 2002; 2003; Mackay *et al*., 2006; 2007).

Whereas the effect of ethanol on individual bacteria that degrade BTX has been previously investigated (Lovanh *et al*., 2002; Lovanh and Alvarez, 2004), the overall effect on the microbial community, particularly on the concentration and relative abundance of bacteria that participate in BTX bioremediation, has received limited attention. Understanding the effect of ethanol on microbial communities is important because of the connections among community structures, biodegradation and source zone longevity, which ultimately influence dissolved contaminant plume lengths and proximity to receptors (Chiang *et al*., 1989; Deeb *et al*., 2002; Molsen *et al*., 2002; Ruiz-Aguilar *et al*., 2003; Gomez *et al*., 2007).

In this study, the microbial community in a pilot-scale aquifer tank filled with sandy soil was monitored following a release of neat ethanol onto a residual hydrocarbon source to evaluate the potential impacts on bacteria associated with BTX degradation. Specifically, the concentration and relative abundance of bacteria harbouring the anaerobic catabolic gene *bssA* (coding for benzylsuccinate synthase) (Beller and Edwards, 2000; Beller *et al*., 2002), and the aerobic catabolic genes *dmpN* (coding for phenol hydroxylase) (Nordlund *et al*., 1990; Baldwin *et al*., 2003) and *todC1* (coding for toluene dioxygenase) (Gibson *et al*., 1968; 1995; Zylstra and Gibson, 1989) were quantified. The study was conducted to better understand and quantify the response of the microbial community following a release of highly concentrated ethanol onto pre-existing fuel (i.e. BTX)-contaminated soil. This scenario simulates potential and historic (Buscheck *et al*., 2001) releases of highly concentrated ethanol at bulk terminals.

Results and discussion

Genotypic response following ethanol release

Aqueous samples collected directly beneath the water table and within the capillary zone consistently exhibited the highest concentrations of both ethanol and bacteria (one to two orders of magnitude higher than samples collected 30 and 45 cm deeper), corroborating that the water table interface was the primary region of ethanol migration and thus bioavailability (Cápiro *et al*., 2007). Two representative locations were selected for microbial community analysis using quantitative real-time PCR (qPCR). Sampling location A was within the capillary fringe 0.15 m down gradient from the source and sampling location B was located just beneath the water table 0.75 m down gradient from the source (Fig. 1). Cross-sections up gradient of the source, and those from the opposite side of the tank were not selected for more intensive analysis because these alternative sampling locations were exposed to relatively low concentrations of ethanol (Stafford, 2007) and experienced relatively low increases in microbial concentrations (i.e. less than one order of magnitude, $P > 0.05$, data not shown).

At the sampling location A near the source, changes in microbial concentrations were observed in response to the depletion of dissolved oxygen. High concentrations of ethanol (above 100 000 mg l^{-1}) in the capillary fringe led

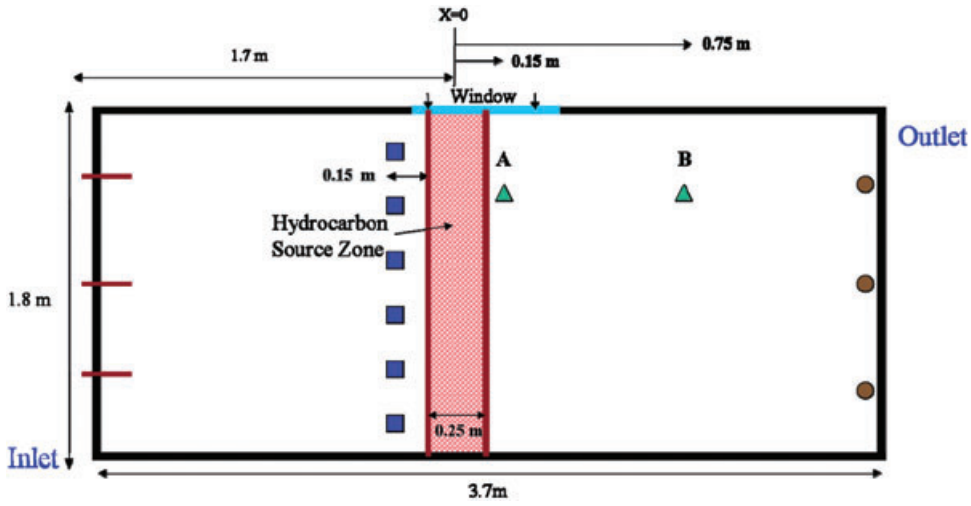

Fig. 1. Sampling locations (plan view). Microbial sampling locations are designated by the triangles labelled 'A' and 'B', respectively 0.15 and 0.75 m from the centre of the hydrocarbon source. The six E100 source injection locations 0.3 m up gradient of the mid-point of the emplaced hydrocarbon source zone are represented with squares, and the circles represent outlet well locations.

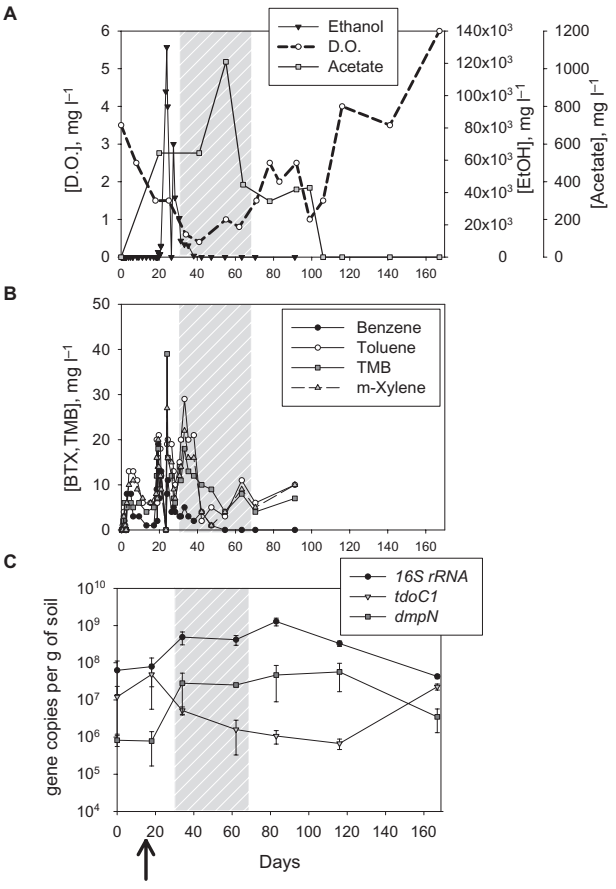

Fig. 2. Measurements from within the capillary fringe 0.15 m down gradient of the BTX source mid-point (location A, Fig. 1). Arrow indicates E100 release (day 18).
A. Aqueous concentrations [ethanol, dissolved oxygen (D.O.) and acetate] with anaerobic conditions depicted as a shadow.
B. BTX and trimethylbenzene (TMB) breakthrough.
C. Genotypic variation.

to the rapid consumption of oxygen and the formation of acetate, which is a common by-product of anaerobic ethanol degradation (Powers *et al*., 2001b) (Fig. 2A). Aerobic BTX degraders harbouring *dmpN* and *todC1* genes have been shown to survive anaerobiosis and degrade BTX under oxygen-limiting conditions, as low as 0.05 mg l^{-1} (Yershalmi *et al*., 1999; 2001; 2002). Thus, it is not surprising that these genes were detected throughout the sampling period, although growth of these specific degraders was unlikely during the anaerobic stage that developed in the presence of relatively high ethanol concentrations (days 25–65) (Fig. 2A and C).

Ethanol eventually migrated through location A or was diluted and degraded, and oxygen returned through the shallow 0.5 m vadose zone (day 70, Fig. 2A). At the same time that ethanol arrived, BTX compounds had also migrated into this sampling location (Fig. 2B). This stimulated a two order of magnitude increase in total *Bacteria* and BTX degraders harbouring the *dmpN* gene (day 83, Fig. 2C). On the other hand, BTX degraders harbouring the *todC1* gene were only able to rebound to initial concentrations after an extended period (day 162, Fig. 2C). It is unknown why bacteria harbouring the *dmpN* monooxygenase gene fared better under oxygen-limited conditions than those harbouring the *todC1* dioxygenase gene, although similar results have been observed by other researchers who demonstrated that bacteria utilizing monooxygenases to initiate toluene catabolism outcompeted those relying on dioxygenases in toluene-fed hypoxic chemostats (Duetz *et al*., 1994; Fang *et al*., 2000).

The relative abundance of BTX degraders harbouring these aerobic *todC1* catabolic genes (relative to the total *Bacteria* population) decreased with the transient exposure to ethanol (days 34–116). The *todC1* gene concentration was high (10^7 gene copies per g of soil) prior to the ethanol release, decreased upon exposure to high ethanol concentrations and rebounded to initial concentrations as ethanol was flushed out. The rebound of *todC1* was further enabled by the higher concentrations of BTX that were mobilized by the ethanol to the sampling point, and the return of aerobic conditions (Fig. 2). On the other hand, *dmpN* gene concentrations followed the overall trend of total *Bacteria* (16S rRNA gene), which grew with increased ethanol availability (Fig. 2C).

Even though (putative methanogenic) *Archaea* were detected at location A, at 10^4 gene copies per g of soil during the oxygen-limited period (data not shown), no significant growth ($P > 0.05$) was measured and no methane was detected. The fact that this sampling location was located in the capillary zone, where re-aeration may be faster than in the saturated zone, may have precluded the development of strongly anaerobic conditions needed for methanogens to thrive.

Beneath the water table at location A, the ethanol concentration was on average less than 60 mg l^{-1} during the 40 day plume migration (Stafford, 2007) and microbial growth was not significant ($P > 0.05$). However, further down gradient from the source (75 cm away) at location B (Fig. 1), microbial growth just below the water table was now evident as ethanol concentrations were 10 times higher than those found beneath the water table near the source (Stafford, 2007). Similar to the capillary fringe at location A, the biochemical oxygen demand exerted by ethanol rapidly consumed the dissolved oxygen, even though lower (two orders of magnitude) ethanol concentrations reached this depth. Unlike location A, oxygen consumption appeared to be faster than reaeration, and strongly anaerobic conditions were maintained for approximately 50 days (Fig. 3A). This was evident by the detection of methane (up to 0.8 mg l^{-1}), and corroborated by qPCR data showing a two orders of magnitude concomitant growth of (putative methanogenic) *Archaea*

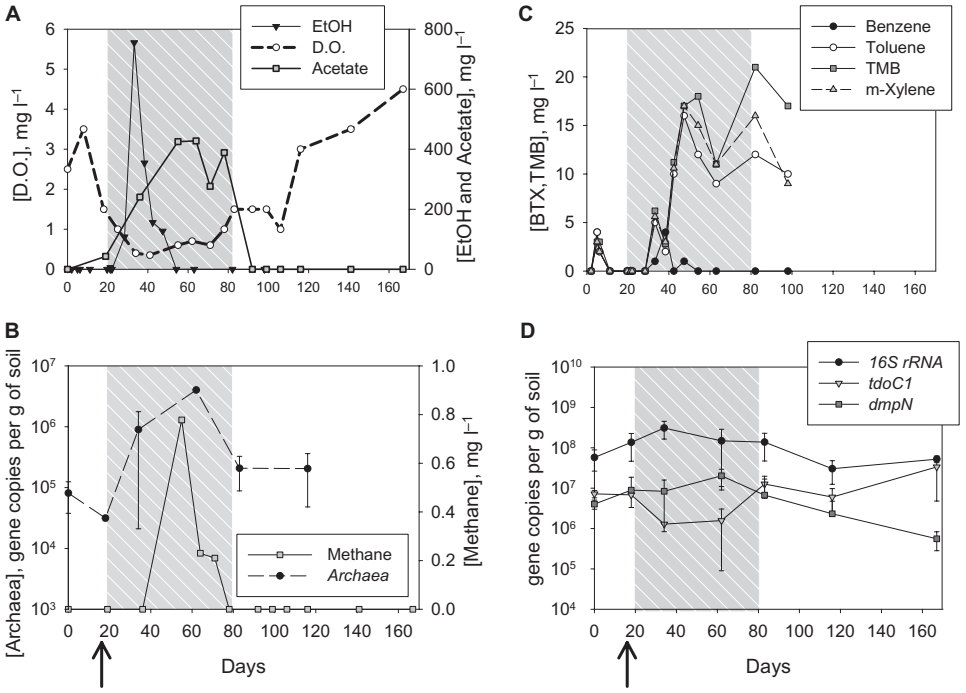

Fig. 3. Measurements from within the saturated zone 0.75 m down gradient of source (location B, Fig. 1). Arrow indicates day of E100 addition (day 18).
A. Aqueous concentrations (ethanol, dissolved oxygen and acetate) with anaerobic conditions depicted as a shadow.
B. Development of methanogenic conditions.
C. BTX breakthrough.
D. Genotypic variation.

(Fig. 3B), which were not detected after ethanol was flushed and aerobic conditions returned.

The introduction of ethanol enhanced the breakthrough of BTX through location B (Fig. 3C), possibly as a result of a combination of mobilization by its cosolvent effect and decreased biodegradation as a result of oxygen depletion and preferential utilization of ethanol (Powers *et al.*, 2001b). BTX breakthrough persisted after ethanol was flushed and aerobic conditions returned, suggesting that ethanol also mobilized the BTX nonaqueous phase liquid (NAPL) closer to this sampling point. Total *Bacteria* grew significantly ($P < 0.05$) as ethanol broke through (day 34, Fig. 3D), whereas significant ($P < 0.05$) growth of BTX degraders harbouring *dmpN* took longer and coincided mainly with BTX availability (day 62, Fig. 3D). Similar to location A, the concentrations of strains harbouring *todC1* decreased significantly ($P < 0.05$) when anaerobic and hypoxic conditions prevailed. Although, unlike location A, *dmpN* reached significantly higher than initial values ($P < 0.05$) after aerobic conditions returned (day 83, Fig. 3D), and eventually increased by one order of magnitude (day 162, Fig. 3D). However, while ethanol was present, the relative abundance of both *todC1* and *dmpN* (compared with total *Bacteria*) decreased by up to two orders of magnitude, reflecting that ethanol promotes the growth BTX degraders to a smaller extent than other bacteria. Even though the high biochemical oxygen demand exerted by ethanol led to strongly anaerobic (methanogenic) conditions, bacteria harbouring *bssA* genes (associated with anaerobic toluene degradation) were not detected (qPCR detection limit $\geq 10^2$ gene copies per g of soil). Apparently, longer acclimation times are required for the development of anaerobic BTX degradation activity.

Microbial community structure

Denaturing gradient gel electrophoresis (DGGE) analysis was conducted on soil-DNA samples collected from the capillary fringe at location B (Fig. 1). Samples were collected prior to the emplacement of the residual BTX source (day 0) and during exposure to ethanol (day 34) (Fig. 4). We recognize that phylogenetic identification of dominant species does not necessarily reflect biodegradation activity, but this information can yield valuable (circumstantial) insight on catabolic potential.

Prior to the ethanol release, the dominant bands corresponded to uncultured gamma proteobacteria clone I3K-0534 (similarity index, SI = 100%, NCBI #AY868004),

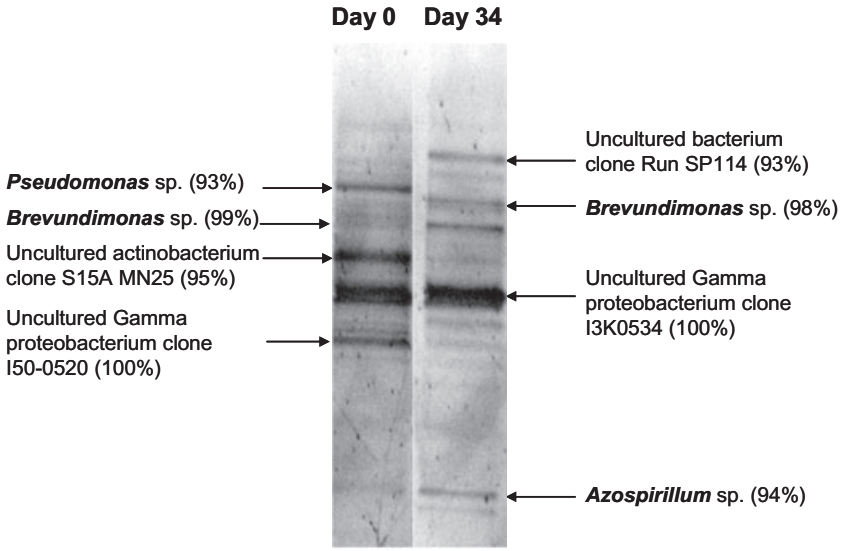

Fig. 4. DGGE image of 16S rRNA gene extracted from the capillary fringe, 0.75 m down gradient from the source zone (location B, Fig. 1). Samples analysed prior to emplacement of the residual BTX source (day 0) and during exposure to ethanol (day 34). Putative BTX degraders are depicted in bold letters. The SI is shown in parenthesis.

Brevundimonas sp. (SI = 98%, NCBI #DQ833394), *Brevundimonas* sp. (SI = 99%, NCBI #DQ833394) and *Pseudomonas* sp. (SI = 93%, NCBI #DQ337603) (Fig. 4). The presence of the putative BTX degraders *Brevundimonas* sp. and *Pseudomonas* sp. (Applegate *et al*., 1998; Shim and Yang, 1999; Greene *et al*., 2000; Leys *et al*., 2005; Witzig *et al*., 2006) is consistent with prior exposure to BTX as a result of a previous E95 release (Cápiro *et al*., 2007), which likely resulted in the presence of residual hydrocarbons prior to the emplacement of the source zone and the release of ethanol.

Sixteen days following the ethanol release, an increase in the populations of putative BTX degraders was indicated by the increase in thickness of these bands relative to the background sample (Fig. 4). Putative BTX degraders that proliferated include *Brevundimonas* sp. (SI = 98%, NCBI #DQ833394), *Brevundimonas* sp. (SI = 99%, NCBI #DQ833394) and *Azospirillum* sp. (SI = 94%, NCBI #DQ438998) (Greene *et al*., 2000; Ortega-Calvo *et al*., 2003; Leys *et al*., 2005), which are microaerophilic bacteria (Grifoni *et al*., 1995; Buczolits *et al*., 2001). Their growth was likely supported by the presence of both BTX and ethanol. Overall, the DGGE analysis corroborated the qPCR results suggesting that ethanol can support the growth of different bacteria, including species that can degrade BTX.

In summary, cell growth coincided with ethanol availability, and concentrations of ethanol in excess of 100 000 mg l^{-1} were tolerated by the bacteria, which grew by up to two orders of magnitude after high concentrations of ethanol passed through. Some BTX degraders grew on ethanol and possibly also on BTX that appears to have been mobilized by the cosolvent effect exerted by ethanol. The DGGE analysis showed an increase in the concentration of putative microaerophilic BTX degraders (e.g. *Azospirillum* and *Brevundimonas* spp.) following ethanol breakthrough, concurring with qPCR measurements of the catabolic genes *dmpN* (coding for phenol hydroxylase) and *todC1* (coding for toluene dioxygenase). Overall, this pilot-scale tank study demonstrates that spills of highly concentrated ethanol may promote microbial population shifts that can decrease the relative abundance of some BTX degraders. However, the fortuitous growth of BTX degraders on ethanol would potentially enhance the rate of aerobic natural attenuation in shallow aquifers after ethanol and its degradation by-products are degraded or flushed out.

Experimental procedures

Experimental system design

The ethanol release experiment was conducted in an 8 m^3 continuous-flow tank packed with fine grain masonry sand (Circle Sand, Houston, TX), which was used as received without inoculation with microorganisms. The metal experimental tank (3.7 m × 1.8 m × 1.2 m; Fig. 1) was covered by a canopy and open to the atmosphere (volatilization losses were not monitored). The influent water (Houston tap water) had a pH of 7.5 ± 0.4, a dissolved oxygen concentration of 3 mg l^{-1}, and an ionic strength of 6–12 mM. An extensive grid of sampling ports (21 in total, not shown) was installed downstream from the source zone to monitor the plume above and below the water table, in addition to routine effluent measurements (Fig. 1). Comprehensive descriptions of the experimental tank system and operational procedures can be found elsewhere (Cápiro *et al*., 2007; Stafford, 2007).

After the completion of an earlier fuel-grade ethanol spill (E95, 95% v/v ethanol, 5% v/v hydrocarbon mixture as a denaturant) (Cápiro *et al*., 2007), the highly contaminated areas of the tank were excavated and repacked with clean sand prior to the emplacement of a surrogate light non-

aqueous phase liquid (LNAPL) mixture and the injection of the neat ethanol. Even though aqueous hydrocarbons concentrations were not detected (detection limit approximately 1 mg l^{-1}) following the sand replacement, residual trace amounts of gasoline hydrocarbons are likely to have remained in the system. These trapped trace level hydrocarbons served as the background conditions from which initial microbial concentrations were measured at the locations shown in Fig. 1. A bromide tracer analysis was performed according the previously described methods (Cápiro *et al.*, 2007) to evaluate the flow field after repacking the tank (Cápiro *et al.*, 2007). Tracer breakthrough curve data yielded an average groundwater seepage velocity of 60 cm day^{-1}, which represents approximately a 6 day residence time (i.e. duration for one pore volumes) from inlet to outlet.

The introduction of the new contaminant source was conducted during the summer months with outdoor temperatures of 25–40°C. The source zone was created by mixing a total of 200 kg sand with 5 l of a surrogate LNAPL fuel mixture. This six-component mixture contained the following hydrocarbons (% by weight): benzene (2.0), toluene (5.6), *m*-xylene (11.7), *iso*-octane (51.1), trimethylbenzene (TMB, 29.4) and naphthalene (0.2). The contaminated sand was added into a 25-cm-wide trench that stretched the 180 cm width of the tank. The emplaced source zone occupied a region 20 cm above and 10 cm below the water table and had a pore saturation of approximately 10% residual NAPL. Following the monitoring of hydrocarbon concentrations for 18 days, 76 l of neat ethanol (100%, anhydrous) (AAPER Alcohol and Chemical, Shelbyville, KY) was injected 15 cm up gradient of the residual hydrocarbon source. Peristaltic Masterflex pumps (Easy-load L/S Cole Parmer 7518-10, Vernon Hills, IL) were used to meter in the ethanol [70 ml $(min\text{-}well)^{-1}$] through six 0.64 cm (0.25 inch) injection wells situated within the capillary fringe (20 cm above the water table).

Aqueous sample collection and analysis

Samples were collected from groundwater and capillary fringe ports (one to six times per day) to assess the extent and location of gasoline hydrocarbons and ethanol near the water table. The groundwater sampling protocol for within the saturated zone was described in previous work (Cápiro *et al.*, 2007). Capillary zone samples were collected using a 30 cm (12 inch) needle (Popper and Sons) attached to a 5 ml plastic syringe (Fisher Scientific, Hampton, NH). The needle was then placed into the designated capillary zone monitoring locations 5–10 cm above the water table (near microbial sampling ports in Fig. 1) and dependent upon the height of the capillary fringe which changed throughout the experiment (Stafford, 2007). Three samples were pulled from each location to determine an average concentration that was reported for the region, each sample was filtered through a 2 μm membrane attachable syringe filter (Whatman 6872-2502, Fisher Scientific), and placed into 2 ml glass vial (SUN SRI). The samples were then stored at 4°C prior to analysis. A detailed description of gas chromatography methods for ethanol and BTX measurements is provided elsewhere (Stafford, 2007). Briefly, aqueous samples were analysed by direct aqueous injection of 2 μl into a Hewlett Packard model 6890 gas chromatograph (GC) equipped with a Supelco (SPB-5) capillary column and a flame ionization detector (FID, OI Analytical) with detection limits of approximately 1 mg l^{-1} for the hydrocarbons and 10 mg l^{-1} for ethanol. Additional aqueous samples were collected from each sampling port located at the water table on a weekly basis. These included: (i) 50 ml samples in 125 ml glass serum bottles previously capped with a Teflon-lined septa and aluminum crimps, for methane analysis, (ii) 25 ml samples for immediate pH and dissolved oxygen analysis and (iii) 100 ml samples for analysis of degradation products.

A GC (Hewlett-Packard 5890) equipped with a FID and a packed column (6 feet × 0.125 inches outer diameter) containing 60/80 Carbopack B/1% SP-1000 (Supelco) was used to measure methane concentrations (detection limit 0.1 mg l^{-1}). Using a pressure-locking syringe, headspace samples (100 μl) were collected and injected directly into the GC.

Samples for acetate analysis were prepared by first filtering 2.7 ml of the aqueous samples through a syringe filter (0.2 μm) into a 10 ml screw-cap vial. To this sample, 0.3 M oxalic acid (0.3 ml) was added to yield a final concentration of 0.03 M oxalic acid. One microlitre of this sample was injected directly into a GC equipped with a FID and glass column (2 m × 2 mm inner diameter) containing 80/120 Carbopack B-DA*/4% Carbowax 20 M (Supelco) with a detection limit of 1.0 mg l^{-1}.

The pH levels were measured using an Accumet AP84 (Fisher Scientific; Waltham, MA) and dissolved oxygen was measured using CHEMets Kits K-7512 (1–12 mg l^{-1}) and K-7501 (0–1 mg l^{-1}) (CHEMetrics; Calverton, VA).

Soil sample collection and DNA extraction

Soil cores were collected throughout the course of the experiment, typically every 28 days (or on average every 4.5 pore volumes) at distances of 45 cm away from both sidewalls of the tank. This distance away from the walls was selected to avoid interfering with capillary zone sampling taking place 60 cm from the walls. These sets of soil cores were taken at distances 15 cm (location A) and 75 cm (location B) down gradient of source (Fig. 1) at depths within the capillary zone down to the water table.

The soil core sampling was performed using 1.2 cm (0.5 inch) PVC piping. Each pipe was hammered into the soil using a rubber mallet from the sand surface down to the bottom of the tank. The top of the pipe was sealed with duct tape, the pipe was then extracted by hand and the bottom of the pipe was also taped. The sealed pipes were then cut into three 25–30 cm segments. The three pipe segments consisted of the locations above the water table (upper capillary zone), at the water table interface down to ~6 cm and 36–60 cm below the water table (the bottom 15 cm was typically lost during the core extraction). Samples were drained and dried in weighing boats (Fisher Scientific) at room temperature (22°C) prior to DNA extraction. Samples that could not be extracted on the following day were placed in 15 ml centrifuge tubes and stored at −80°C.

Once the soil was dry, the samples were homogenized and 0.25 g was transferred into a PowerBead spin tube (Mo Bio Laboratories; Carlsbad, CA, USA) in accordance with the procedure outlined by the manufacture of PowerSoil DNA

Isolation Kit (Mo Bio Laboratories). The collected DNA (100 μl) was placed in a 1.5 ml Eppendorf tube and stored in a freezer at –80°C until analyses, according to the kit procedure.

Quantitative real-time PCR analysis

Concentrations of *Bacteria* (16S rRNA gene) (Suzuki *et al.*, 2000), *Archaea* (Suzuki *et al.*, 2000), anaerobic BTX-degrading bacteria harbouring benzylsuccinate synthase (*bssA*) (Beller *et al.*, 2002) and aerobic BTX-degrading bacteria harbouring toluene dioxygenase (*todC1*) (Baldwin *et al.*, 2003) and phenol monooxygenase (*dmpN*) (Baldwin *et al.*, 2003) were estimated using qPCR. The primers and probes used were obtained from IDT Technologies (Coralville, IA). Bacteriophage λ (500 bp) (Beller *et al.*, 2002) was used as an internal standard for the determination of DNA recovery efficiency. Prior to DNA extraction, the phage was added to all experimental samples and to triplicate samples set up as controls using sterile glass beads in lieu of soil. Recoveries from soil samples lower than 100% were normalized to the average fraction recovered from the controls. It was assumed that any losses encountered when using glass beads instead of soil material were due to the extraction process itself, as the beads are highly unlikely to exhibit interference from sample impurities. Wide variations in DNA recoveries (e.g. 0.6–126%) are commonly reported (Zhou et al., 1996; Mygind et al., 2003) and low recoveries are probably due to the binding of sample impurities (e.g. humic acids) that interfere with the activity of *Taq* polymerase during PCR analysis (Porteous et al., 1997).

For Taqman PCR reactions, the mixture contained 1× Taqman PCR Master Mix (Applied Biosystems; Foster City, CA, USA), 0.5 μM forward (0.25 μM of each forward *Archaea* primer) and reverse primers, 0.25 μM probe, 2 μl of sample DNA and sterile DNAase-free water to make up a final volume of 25 μl. For SYBR green PCR reactions, the mixture contained 1× SYBR Green (Applied Biosystems), 0.5 μM of each forward primer and reverse primer, 2 μl of sample DNA and sterile DNAase-free water to make up a final volume of 25 μl. This mixture was placed into a 96-well optical plate (Applied Biosystems) and covered with an optical adhesive (Applied Biosystems) before being loaded for analysis. A Sequence Detector (Model ABI 7500, Applied Biosystems) was used to perform qPCR reactions with the following temperature conditions: 50°C for 2 min, followed by 95°C for 10 min and 40 cycles at 95°C for 15 s, and 60°C for 1 min. The bacterial genotype concentration in each sample was estimated based on the following equation:

$$\text{Gene copies } \mu l^{-1} \text{ (or per g of soil)} = [(\mu g \text{ DNA } \mu l^{-1})/(\text{X bp per genome})] \times (9.1257 \times 10^{14} \text{ bp per } \mu g \text{ of DNA}) \times (\text{Y genes per genome})$$

A bacterial genome size (X) of 6.2×10^6 bp, which is the genome size of *Pseudomonas putida* F1 strain (NCBI# CP000712), was used to estimate *todC1*, *dmpN* and *Bacteria* concentrations (http://www.genomesonline.org). A 1.66×10^6 bp per genome (*Methanococcus maripaludis*, NCBI #BX950229) was used to estimate *Archaea* concentrations (http://www.genomesonline.org). There are approximately 9.12576×10^{14} bp per μg of DNA. The number of gene copies per genome (Y) was 7 for *P. putida* and 1 for *Methanococcus* (http://rrndb.cme.msu.edu). Calibration curves (10^1–10^8 gene copies per g of soil) were prepared for all genes under consideration ($r^2 \geq 0.99$). The above assumptions were not used to quantify bacteriophage λ because, in this case, the solutions contained DNA fragments of identical length to those used in the standards.

Denaturing gradient gel electrophoresis analysis

Extracted soil DNA from two sampling events was used to characterize microbial community structure and determine dominant bacteria profiles using DGGE. The first samples represented initial conditions prior to the addition of E100 and the emplacement of the residual BTX source (day 0), and the second sampling event represented the onset of E100 breakthrough just before the anoxic conditions developed (day 34). Samples were evaluated from within the capillary fringe at a location 0.75 m down gradient of the source (location B, Fig. 1).

The PCR-based DGGE was conducted using a Biometra thermocycler (Model T-gradient, Goettingen, Germany). The PCR reaction mix consisted of 1× Qiagen Taq PCR buffer (Qiagen, Valencia, CA, USA), the bacterial forward (5′-ATGGCTGTCGTCAGCT-3′) and reverse (5′-CGCCC GCCGCGCCCCGCGCCCGGCCCGCCGCCCCCGCCCCA CGGGCGGTGTGTAC-3′) primers (0.5 μM each) (Ferris *et al.*, 1996), 8 μl of the sample DNA and DNAase-free water to make up a final volume of 100 μl. The pair of primers used amplified a 323 bp section of the 16S rRNA genes of the members of the domain *Bacteria*. The PCR temperature conditions for this set of primers were: 94°C 5 min, 94°C 1 min, 53–43°C (–1°C cycle^{-1}, total of 10 cycles), 72°C 3 min, 94°C 1 min, 43°C 1 min (total of 20 cycles), 72°C 3 min and a final extension of 72°C for 10 min. Once the PCR reaction was finished, the presence of PCR products was verified on an agarose gel prior to DGGE analysis to ensure sufficient amplified DNA. The PCR gel consisted of 1% agarose containing 1× TAE running buffer and the dye ethidium bromide (0.5 μg ml^{-1}). The presence of PCR products were visualized under UV light (EpiChemi UVP BioImaging System, Upland, CA, USA).

DGGE was performed using a BIO-RAD DCODE Universal mutation detection system. The acrylamide gel consisted of a high-denaturant (80%) and low-denaturant (30%) phase. The DGGE was run for 14 h at 46 V. Fragments of DNA (bands) in the polyacrylamide gel were excised and sequenced (Lone Star Laboratories, Houston, TX, USA). Phylogenetic affiliations were determined by comparing the DNA sequences retrieved to known bacterial sequences in the Ribosomal Database Project II (http://rdp.cme.msu.edu) (Cole *et al.*, 2005) and the BLAST facility of the National Center for Biotechnology Information (including GenBank database) (http://ncbi.nlm.nih.gov) (Benson *et al.*, 2007). Briefly, these alignment tools approximate random gene sequences based on a comparison of local similarities algorithm to conserved regions that results on the probability of finding the best sequence SI (Lynch, 1990). The results are presented as a score of the average fraction of shared restriction fragments between the unknown sequence and the database (for review see Altschul *et al.*, 1990).

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References

Altschul, S.F., Warren Gish, Miller, W., Myers, E.W., and Lipman, D.J. (1990) Basic local alignment search tool. *J Mol Biol* **215:** 403–410.

Applegate, B.M., Kehrmeyer, S.R., and Sayler, G.S. (1998) A chromosomally based *tod-luxCDABE* whole-cell reporter for benzene, toluene, ethybenzene, and xylene (BTEX) sensing. *Appl Environ Microbiol* **64:** 2730–2735.

Araujo, D.B., Bulter, B., and Mayfield, C. (1998) Effects of gasoline and ethanol mixtures on aquifer microorganisms. In *Annual Meeting of the Canadian Society of Microbiologist*. Guelph, Ontario, Canada.

Baldwin, B.R., Nakatsu, C.H., and Nies, L. (2003) Detection and enumeration of aromatic oxygenase genes by multiplex and real-time PCR. *Appl Environ Microbiol* **69:** 3350–3358.

Beller, H.R., and Edwards, E.A. (2000) Anaerobic toluene activation by benzylsuccinate synthase in a highly enriched methanogenic culture. *Appl Environ Microbiol* **66:** 5503–5505.

Beller, H.R., Kane, S.R., Legler, T.C., and Alvarez, P.J.J. (2002) A real-time polymerase chain reaction method for monitoring anaerobic, hydrocarbon-degrading bacteria based on a catabolic gene. *Environ Sci Technol* **36:** 3977–3984.

Benson, D.A., Karsch-Mizrachi, I., Lipman, D.J., Ostell, J., and Wheeler, D.L. (2007) GenBank. *Nucleic Acids Res* **35:** D21–D25.

Buczolits, S., Rosengarten, R., Hirt, R., and Busse, H.-J. (2001) Classification of a *Brevundimonas* strain detectable after PCR with a *Helicobacter*-specific primer pair. *Syst Appl Microbiol* **24:** 368–376.

Buscheck, T.E., O'Reilly, K., Koschal, G., and O'Regan, G. (2001) Ethanol in groundwater at a northwest terminal. In *Bioremediation of MTBE, Alcohols, and Ethers; Proceedings of the Sixth International In Situ and On-Site Bioremediation Symposium*. Magar, V.S., Gibbs, J.T., O'Reilly, K.T., Hyman, M.R. and Leeson, A. (eds). San Diego, CA: Battelle Press, pp. 203–210.

Cápiro, N.L., Stafford, B.P., Rixey, W.G., Bedient, P.B., and Alvarez, P.J.J. (2007) Fuel-grade ethanol transport and impacts to groundwater in a pilot-scale aquifer tank. *Water Res* **41:** 656–664.

Chiang, C., Salanitro, J., Chai, E., Colthart, J., and Klein, C. (1989) Aerobic biodegradation of benzene, toluene, and xylene in a sandy aquifer: data analysis and computer modeling. *Ground Water* **27:** 823–834.

Cole, J.R., Chai, B., Farris, R.J., Wang, Q., Kulam, S.A., McGarrell, D.M., *et al.* (2005) The Ribosomal Database Project (RDP-II): sequences and tools for high-throughput rRNA analysis. *Nucleic Acids Res* **33:** D294–D296.

Corseuil, H.X., Hunt, C.S., Santos, R.C.F.D., and Alvarez, P.J.J. (1998) The influence of the gasoline oxygenate ethanol on aerobic and anaerobic BTEX biodegradation. *Water Res* **32:** 2065–2072.

Da Silva, M.L.B., and Alvarez, P.J.J. (2002) Effects of ethanol versus MTBE on benzene, toluene, ethylbenzene, and xylene natural attenuation in aquifer columns. *ASCE J Environ Eng* **128:** 862–867.

Deeb, R.A., Sharp, J.O., Stocking, A., McDonald, S., West, K.A., Laugier, M., *et al.* (2002) Impact of ethanol on benzene plume lengths: microbial and modeling studies. *ASCE J Environ Eng* **128:** 868–875.

Deeb, R.A., Chu, K.-H., Shih, T., Linder, S., Suffet, I., Kavanaugh, M.C., and Alvarez-Cohen, L. (2003) MTBE and other oxygenates: environmental sources, analysis, occurrence, and treatment. *Environ Sci Eng* **20:** 433–447.

Duetz, W.A., De Jong, C., Williams, P.A., and Van Andel, J.G. (1994) Competition in chemostat culture between *Pseudomonas* strains that use different pathways for the degradation of toluene. *Appl Environ Microbiol* **60:** 2858–2863.

Fang, J., Barcelona, M.J., and Alvarez, P.J. (2000) Phospholipids of five pseudomonad archetypes for different toluene degradation pathways. *Bioremediation J* **4:** 181–185.

Farrell, A.E., Plevin, R.J., Turner, B.T., Jones, A.D., O'Hare, M., and Kammen, D.M. (2006) Ethanol can contribute to energy and environmental goals. *Science* **311:** 506–508.

Ferris, M.J., Muyzer, G., and Ward, D.M. (1996) Denaturing gradient gel electrophoresis profiles of 16S rRNA-defined populations inhabiting a hot spring microbial mat community. *Appl Environ Microbiol* **62:** 340–346.

Gibson, D.T., Koch, J.R., and Kallio, R.E. (1968) Oxidative degradation of aromatic hydrocarbons by microorganisms. I. Enzymatic formation of catechol from benzene. *Biochemistry* **7:** 2653–2662.

Gibson, D.T., Resnick, S.M., Lee, K., Brand, J.M., Torok, D.S., Wackett, L.P., *et al.* (1995) Desaturation, dioxygenation, and monooxygenation reactions catalyzed by napthalene dioxygenase from *Pseudomonas* sp. strain 9816-4. *J Bacteriol* **177:** 2615–2621.

Gomez, D., Leblanc, P., Bedient, P., Rixey, W., and Alvarez, P.J.J. (2007) Simulation of effects of ethanol on benzene plume length using RT3D with a general substrate interaction module. *Water Resour Res* (in press). doi:10.1029/2007WR006184.

Greene, E.A., Kay, J.G., Jaber, K., Stehmeier, L.G., and Voordouw, G. (2000) Composition of soil microbial communities enriched on a mixture of aromatic hydrocarbons. *Appl Environ Microbiol* **66:** 5282–5289.

Grifoni, A., Bazzicalupo, M., Serio, C.D., Fancelli, S., and Fani, R. (1995) Identification of *Azospirillum* strains by restriction fragment length polymorphism of the 16S rDNA and of the histidine operon. *FEMS Microbiol Lett* **127:** 85–91.

Hunt, C.S., Ferreira, D.S., Corseuil, H.X., and Alvarez, P.J.J. (1997) Effect of ethanol on aerobic BTEX degradation. In *Natural Attenuation of Chlorinated Solvents, Petroleum Hydrocarbons, and Other Organic Compounds: Proceedings of the Fifth International in-Situ and on-Site Bioremediation Symposium*. Alleman, B.C., and

Leeson, A. (eds). New Orleans, LA, USA: Battelle Press, pp. 135–140.

Ingram, L.O., and Buttke, T.M. (1984) Effects of alcohols on microorganisms. *Adv Microb Physiol* **25:** 253–301.

Leys, N.M., Ryngaert, A., Bastiaens, L., Wattiau, P., Top, E.M., Verstraete, W., and Springael, D. (2005) Occurrence and community composition of fast-growing *Mycobacterium* in soils contaminated with polycyclic aromatic hydrocarbons. *FEMS Microbiol Ecol* **51:** 375–388.

Lovanh, N., and Alvarez, P.J.J. (2004) Effect of ethanol, acetate, and phenol on toluene degradation activity and *tod-lux* expression in *Pseudomonas putida* TOD102: evaluation of the metabolic flux dilution model. *Biotechnol Bioeng* **86:** 801–808.

Lovanh, N., Hunt, C.S., and Alvarez, P.J. (2002) Effect of ethanol on BTEX biodegradation kinetics: aerobic continuous culture experiments. *Water Res* **36:** 3739–3746.

Lynch, M. (1990) The similarity index and DNA fingerprinting. *Mol Biol Evol* **7:** 478–484.

Mackay, D., deSieyes, N., Einarson, M., Feris, K., Pappas, A., Wood, I., *et al.* (2007) Impact of ethanol on the natural attenuation of MTBE in a normally sulfate-reducing aquifer. *Environ Sci Technol* **41:** 2015–2021.

Mackay, D.M., deSieyes, N.R., Einarson, M.D., Feris, K.P., Pappas, A.A., Wood, I.A., *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.

Molsen, J.W., Barker, J.F., Frind, E.O., and Schirmer, M. (2002) Modeling the impact of ethanol on the persistence of benzene in gasoline-contaminated groundwater. *Water Resour Res* **38:** 4–1–4-12.

Mygind, T., Ostergaard, L., Birkelund, S., Lindholt, J.S., and Christansen, G. (2003) Evaluation of five DNA extraction methods for purification of DNA from atherosclerotic tissue and estimation of prevalence of *Chlamydia pneumoniae* in tissue from a Danish population undergoing vascular repair. *BMC Microbiol* **3:** 19.

Nordlund, I., Powlowski, J., and Shingler, V. (1990) Complete nucleotide sequence and polypeptide analysis of multicomponent phenol hydroxylase from *Pseudomonas* sp. strain CF600. *J Bacteriol* **172:** 6826–6833.

Ortega-Calvo, J.J., Marchenko, A.I., Vorobyov, A.V., and Borovick, R.V. (2003) Chemotaxis in polycyclic aromatic hydrocarbon-degrading bacteria isolated from coal-tar- and oil-polluted rhizospheres. *FEMS Microbiol Ecol* **44:** 373–381.

Porteous, L.A., Seidler R.J., and Watrud, L.S. (1997) An improved method for purifying DNA from soil for polymerase chain reaction amplification and molecular ecology applications. *Mol Ecol* **6:** 787–791.

Powers, S.E., Rice, D., Dooher, B., and Alvarez, P.J.J. (2001a) Will ethanol-blended gasoline affect groundwater quality? *Environ Sci Technol News* **35:** 24A–30A.

Powers, S.E., Hunt, C.S., Heermann, S.E., Corseuil, H.X., Rice, D., and Alvarez, P.J.J. (2001b) The transport and fate of ethanol and BTEX in groundwater contaminated by gasohol. *Crit Rev Environ Sci Technol* **31:** 79–123.

Ruiz-Aguilar, G.M.L., Fernandez-Sanchez, J.M., Kane, S.R., Kim, D., and Alvarez, P.J.J. (2002) Effect of ethanol and methyl-*tert*-butyl ether on monoaromatic hydrocarbon biodegradation: response varability for different aquifer materials under various electron-accepting conditions. *Environ Toxicol Chem* **21:** 2631–2639.

Ruiz-Aguilar, G.M.L., O'Reilly, K., and Alvarez, P.J.J. (2003) A comparison of benzene and toluene plume lengths for sites contaminated with regular vs. ethanol-amended gasoline. *Ground Water Monit Remedia* **22:** 1–6.

Shim, H., and Yang, S.T. (1999) Biodegradation of benzene, toluene, ethylbenzene, and *o*-xylene by a coculture of *Pseudomonas putida* and *Pseudomonas fluorescens* immobilized in a fibrous-bed bioreactor. *J Biotechnol* **67:** 99–112.

Stafford, B.P. (2007) Impacts to ground water from releases of fuel grade ethanol: source behavior. PhD Dissertation. Department of Civil and Environmental Engineering. Houston, TX: University of Houston.

Suzuki, M.T., Taylor, L.T., and DeLong, E.F. (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5′ – nuclease assays. *Appl Environ Microbiol* **66:** 4605–4614.

Witzig, R., Junca, H., Hecht, H.J., and Pieper, D.H. (2006) Assessment of toluene/biphenyl dioxygenase gene diversity in benzene-polluted soils: links between benzene biodegradation and genes similar to those encoding isopropylbenzene dioxygenases. *Appl Environ Microbiol* **72:** 3504–3514.

Yershalmi, L., Manuel, M.F., and Guiot, S.R. (1999) Biodegradation of gasoline in a microaerophilic biobarrier. *Biodegradation* **10:** 341–352.

Yershalmi, L., Lascourreges, J.-F., Rhofir, C., and Guiot, S.R. (2001) Detection of intermediate metabolites of benzene under microaerophilic conditions. *Biodegradation* **12:** 379–391.

Yershalmi, L., Lascourreges, J.-F., and Guiot, S.R. (2002) Kinetics of benzene biotransformation under microaerophilic oxygen-limited conditions. *Biotechnol Bioeng* **79:** 347–355.

Zhou, J.Z., Bruns, M.A., and Tiedje, J.M. (1996) DNA recovery from soils of diverse composition. *Appl Environ Microbiol* **62:** 316–322.

Zylstra, G.J., and Gibson, D.T. (1989) Toluene degradation by *Pseudomonas putida* F1. Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli*. *J Biol Chem* **264:** 14940–14946.

