Bench-scale and field-scale evaluation of catechol 2,3-dioxygenase specific primers for monitoring BTX bioremediation

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Abstract

The objective of this work was to test a molecular genetic method for in situ monitoring of aerobic benzene, toluene, and xylene (BTX) biodegrading microorganisms. Catechol 2,3-dioxygenase (C23DO) genes occur in bacteria that biodegrade benzene, toluene, xylenes, phenol, biphenyl, and naphthalene. A competitive quantitative polymerase chain reaction (QC-PCR) technique using a single set of primers specific for an entire subfamily of C23DO genes was recently developed. To determine whether bacteria containing these C23DO genes actually exist in environments contaminated by BTX, aerobic microcosms containing previously uncontaminated soil were amended with different aromatic hydrocarbons and DNA extracts were analyzed by QC-PCR for C23DO genes. Anaerobic microcosms were established to confirm that oxygen was also necessary for the enrichment of C23DO genes. Field testing was done at two sites undergoing monitored natural attenuation. In microcosm experiments naphthalene, m-xylene, and p-xylene strongly enriched for C23DO genes while benzene, toluene, and o-xylene produced only transient, weakly detectable genes. In the field study, C23DO genes were detected in groundwater samples contaminated with either xylenes or naphthalene. The results of this study demonstrated that molecular genetic techniques can provide an accurate and rapid method to detect microorganisms capable of aromatic hydrocarbon biodegradation. Such a technique would be useful for monitoring the effectiveness of aeration technologies and for documenting microbial processes for monitored natural attenuation.

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1. Introduction

Aerobic bioremediation and monitored natural attenuation (MNA) are frequently used treatment technologies for the remediation of gasoline spills [1]. The gasoline constituents BTX are of greatest toxicological and regulatory concern; however, no accurate and direct method exists to monitor aerobic BTX degraders.

Therefore, the effectiveness of bioventing, air sparging, or peroxide amendments for increasing the population of aerobic BTX degraders cannot be directly monitored. Molecular genetic techniques for measuring the abundance of specific aerobic BTX degraders could be used as part of the required three-part strategy to provide direct field evidence of biodegradation and document the effectiveness of natural attenuation [2]. In order to determine whether MNA is feasible the USEPA recommends collecting data that directly demonstrate the types of attenuation mechanisms active onsite [3]. Cultivation-based methods detect less than 10% of soil
microbial populations [4]. Direct analysis of DNA eliminates the need to cultivate cells. The objective of this work was to test a molecular genetic method that would be an effective direct technique for monitoring aerobic BTX biodegrading microorganisms.

Catabolic genes determine the biodegradation potential of the microbial community. Nucleotide sequences of catabolic genes for the microbial degradation of many environmental pollutants are known. This information has been used to create gene probes for direct detection of toluene, methane, alkane, PCB, and naphthalene catabolic genes (e.g. [5]). PCR can be used to amplify genes allowing more sensitive detection than with direct probing [6]. Hybridizing gene probes to the products of PCR amplification further improves the PCR detection limit [7].

QC-PCR techniques have been developed to enumerate genes. QC-PCR uses, as an internal standard, a competing DNA sequence that is amplified by the same primers that are used to amplify the target sequence [8]. QC-PCR has been used to measure the viral load in humans, numbers of plant pathogens, fungal populations, 4-chlorobiphenyl degraders, and uncultivated bacterial strains in soils (e.g. [9]). However there has been relatively little research using QC-PCR to monitor bioremediation.

To monitor BTX bioremediation an appropriate target gene must be selected. Meta cleavage of catechol-like metabolites from a wide range of aromatic compounds is mediated by C23DO enzymes. C23DO genes are known to occur in bacteria that catabolize benzene, toluene, xylene, phenol, biphenyl, naphthalene, and other aromatic compounds (Fig. 1) [10]. Several C23DO primers have previously been developed [11–14]. However, our design is the only one capable of detecting an entire subfamily of C23DO genes with a single set of PCR primers. Our QC-PCR technique was recently tested on several pure cultures known to contain I.2.A subfamily C23DO genes, as defined by Eltis and Bolin [10]. The detection limit of our technique is $10^2$–$10^3$ C23DO genes by PCR alone, which is improved to $10^0$–$10^1$ C23DO genes if hybridization with a gene specific probe is performed [15]. The quantitation limit is $10^4$ genes.

In this study, laboratory and field experiments were conducted to determine whether C23DO genes can be detected in ‘wild-type’ indigenous organisms and if detection and enumeration of C23DO genes is a reliable measurement of aerobic BTX bioremediation potential. C23DO genes were monitored in aerobic microcosms in which uncontaminated soil was exposed to different aromatic hydrocarbons. Control microcosms were either anaerobic or no substrate was added. Culturable aromatic hydrocarbon-degraders were also isolated from the microcosms and tested for the presence of the target C23DO genes. In addition, QC-PCR for C23DO genes was performed on DNA extracts from environmental samples obtained from two field sites undergoing MNA. Since DNA is known to strongly adsorb to clay, DNA spiking experiments were done to determine whether clay fines at one of the sites influenced DNA recovery. Heterotrophic and benzoate plate counts were performed to estimate the total culturable community size and the size of the C23DO utilizing population. Results from these assays were compared to BTX and naphthalene concentrations in groundwater samples. If aerobic BTX biodegradation was active, our QC-PCR assay should detect enrichment of C23DO genes resulting from exposure to aromatic hydrocarbons.

2. Materials and methods

2.1. DNA extraction and PCR

PCR was performed with DEG-F and DEG-R primers to detect C23DO genes as described in detail previously [15]. Briefly, DNA was extracted from 0.5 g aquifer solids or soil using the FastPrep soil DNA extraction protocol (QBIogene, CA). Extracts (5 μl) were run on a 0.7% agarose gel to check DNA purity. Concentrations were measured with a DNA fluorometer calibrated with calf thymus DNA. DNA isolation from cells was performed by lysing cells in PCR buffer mix at 97°C for 10 min. Bovine serum albumin (1 μg μl$^{-1}$) and Taq DNA polymerase were added to each tube and PCR was performed. QC-PCR was performed on extracts containing C23DO genes above the quantitation limit. All PCR products were separated on agarose gels, transferred to nylon membranes, and hybridized under low stringency conditions with a
238-BP digoxigenin-labeled C23DO probe created from *P. putida* HS1. PCR and hybridization were always performed with both positive and negative controls. Because DNA concentrations were very low, PCR was also performed on Linton samples using universal 16S rDNA primers as a positive control [16].

### 2.2. Microcosm study

In order to mimic conditions of a petroleum-release, 5 g of uncontaminated sandy loam soil lacking detectable C23DO genes were added to sterile 120-ml glass serum vials containing 20 ml sterile minimal media [17]. Each microcosm was prepared in duplicate. The vials were spiked with an aromatic hydrocarbon over a range of concentrations (Table 1) and sealed with Teflon-lined rubber septa. Due to their lower solubility, naphthalene, biphenyl, and phenanthrene were added as solids. Two microcosms with needles piercing the septa were placed in a sealed can containing a 2 ml vial filled with unleaded gasoline. Anaerobic naphthalene-fed control microcosms were purged with nitrogen. Aerobic controls contained no additional carbon. All microcosms (except gasoline) were stored upside down in the dark at room temperature and gently shaken daily. Microcosms were sampled once per week by shaking the soil slurry, uncappping and withdrawing 0.5 ml with a sterile glass pipette, then adding substrate and recapping. Anaerobic microcosms were re-purged.

### 2.3. Isolation and characterization of hydrocarbon-degraders

After 4 weeks, aliquots (0.1 ml) of microcosms were plated onto minimal media plates vapor-fed with the substrate used in the microcosm and incubated at room temperature. Morphologically distinct colonies were picked, re-streaked and grown to confirm substrate utilization. Specific hydrocarbon-degraders were not isolated from the gasoline-fed microcosms. PCR was performed on 15–20 of the isolates from each substrate.

### 2.4. Field site history and characterization

Groundwater samples were obtained from sites in Linton and Winamac, IN. Various consulting firms contracted by the Indiana Department of Transportation conducted leaking underground storage tank (LUST) site investigations and remedial activities. Both sites were undergoing MNA at the time of sampling.

Between 1990 and 1996 one 1000 gallon diesel fuel UST, 8000 gallon gasoline UST, 1000 gallon heating oil UST and 550 gallon waste oil UST were removed from the Winamac site (Fig. 2A). The soil is silty clay grading to sand and gravel at approximately 7 ft below grade. Twelve groundwater monitoring wells were installed between 1996 and 1998 that are screened from 5 to 15 ft. Groundwater flows primarily west.

### Table 1

Detection of C23DO genes in microcosms

<table>
<thead>
<tr>
<th>Microcosm</th>
<th>Substrate (μg l⁻¹)</th>
<th>Week 1</th>
<th>Week 2</th>
<th>Week 3</th>
<th>Week 4</th>
<th>Week 5</th>
<th>Substrate plate count (Log cfu ml⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>p</em>-xylene</td>
<td>210</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>5.93</td>
</tr>
<tr>
<td><em>m</em>-xylene</td>
<td>7100</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>5.63</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>&lt;31,500b</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>7.96</td>
</tr>
<tr>
<td>(aerobic)</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gasoline</td>
<td>Saturated vapor</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>6.79</td>
</tr>
<tr>
<td>Toluene (high)</td>
<td>7500</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>6.74</td>
</tr>
<tr>
<td>Benzene</td>
<td>330</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>6.74</td>
</tr>
<tr>
<td><em>o</em>-xylene</td>
<td>225</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>7.34</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>&lt;7000b</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>7.32</td>
</tr>
<tr>
<td>Toluene (low)</td>
<td>250</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>Nt</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>&lt;1100b</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>7.38</td>
</tr>
<tr>
<td>Naphthalene (anaerobic)</td>
<td>&lt;31,500b</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>Nt</td>
</tr>
<tr>
<td>Unamended</td>
<td></td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>O</td>
<td>Nt</td>
</tr>
</tbody>
</table>

○—C23DO genes not detected in either microcosm.
○—C23DO genes detected in one of two microcosms.
●—C23DO genes detected in both microcosms.
Nt—not tested.
aConcentrations were calculated for a three-phase system (air, water, soil).
bNumber is solubility limit. Pure crystals were present at all times.
cSubstrate concentrations were increased by 10× in benzene and *o*-xylene fed microcosms after 4 weeks.
The Linton site (Fig. 2B) contained one 10,000 gallon diesel fuel UST and 6000 gallon gasoline UST which were replaced in 1987. Soil staining and gasoline odors were detected in 1995 during final removal. The groundwater flows west to northwest at 8–11.5 ft below grade and the soil is clay down to bedrock at 16 ft. Six groundwater monitoring wells, screened from 5 to 15 ft below grade, were installed in 1995.

2.5. Field sampling

Groundwater was sampled from the Linton site in August 1999 and from the Winamac site in April 2000 using sterile disposable bailers. Three well volumes were purged prior to sampling. Contractors shipped two 40-ml samples on ice to certified laboratories for BTX and semi-volatiles (SVOC) analysis. Two-liter groundwater samples from Linton and 1 l groundwater samples from Winamac were collected in sterile glass bottles and kept on ice for microbiological and genetic analysis at Purdue University. At the Linton site four soil borings (SB) were taken from 0 to 4 ft and 4 to 8 ft for microbiological and genetic analysis at Purdue University then composited, and subsampled for total petroleum hydrocarbons (TPH) analysis at certified laboratories.

2.6. Sample processing

Within 12 h of sampling, solids were collected from groundwater samples by centrifugation at 10,000 g for 30 min. Supernatants collected after centrifugation were clear, with the exception of W-7 at the Winamac site. Supernatant from W-7 was filtered through a 0.45 μm filter and the filtrate combined with the solids.

2.7. Spiking experiment

Due to low DNA recovery from clay soils, the extractability of DNA from the Linton samples was tested. Groundwater solids were sterilized by autoclaving three times on alternate days. A control soil (Chalmers silty loam) was prepared in the same manner. Duplicate 0.5 g samples of soils were spiked with 10^7 Pseudomonas sp. strain PpG7 cells containing the C23DO gene (in a volume of 100 μl) and allowed to incubate for 30 min and 24 h before extracting the DNA and performing QC-PCR.

2.8. Plate counts

Heterotrophic plate counts were performed to enumerate the aerobic heterotrophic bacteria. Aquifer solids or soils were serially diluted in sterile phosphate buffer and plated in triplicate on either 10% Tryptic Soy Broth (Linton site) or Peptone-Tryptone-Yeast extract-Medium (125, 125, 250 mg l^{-1}) (Winamac site) and incubated in the dark at room temperature for 2 weeks. Benzoate is a low-volatility aromatic compound that can easily be incorporated into agar and is an intermediate in known degradation pathways utilizing C23DO genes [18]. Benzoate plate counts (250 mg l^{-1} benzoate) were incubated for 3 weeks to enumerate bacteria capable of degrading aromatic compounds [17]. Naphthalene plate counts were performed only on Linton samples.
3. Results

3.1. Detection of catechol 2,3-dioxygenase genes in microcosms

C23DO genes were detected in aerobic m-xylene, p-xylene, and naphthalene microcosms in each of the 4 weeks of testing (Table 1). Within a 1-week period gene copy numbers increased from non-detectable levels to greater than $10^7$ genes ml$^{-1}$ in naphthalene microcosms and to greater than $10^6$ genes ml$^{-1}$ in m-xylene microcosms (Fig. 3). High concentrations of m-xylene (7100 mg l$^{-1}$) resulted in enrichment of quantifiable C23DO genes every week (Fig. 3), while low concentrations of p-xylene (210 mg l$^{-1}$) produced consistent signals of C23DO genes below the quantitation limit (Table 1). No C23DO genes were detected at low concentrations of o-xylene or benzene through 4 weeks. However, when the concentration of o-xylene and benzene was increased 10-fold C23DO genes were detected in one of each pair of microcosms (Table 1). Similarly, in the toluene microcosms no C23DO genes were detected with low concentrations while C23DO genes were initially detected at higher concentrations (Table 1). Gasoline produced a consistent C23DO signal in week 2 and 3.

Throughout the 4 week test duration no subfamily I.2.A C23DO genes were detected in any of the DNA extracts from the biphenyl or phenanthrene amended microcosms, nor in control microcosms without added substrate. No C23DO genes were ever detected in anaerobic naphthalene amended microcosms.

3.2. Cultivation and detection of catechol 2,3-dioxygenase genes in isolates

An abundant number of degraders were cultivated from the hydrocarbon amended microcosms (Table 1). There were lower numbers of degraders in the m-xylene and p-xylene microcosms. The proportion of isolates containing C23DO genes was highest among the naphthalene (19 of 20), m-xylene (14 of 15), and p-xylene (11 of 20) degrading isolates, and lower in isolates from the o-xylene (1 of 20) and toluene (1 of 20) microcosms. None of the 20 biphenyl, benzene or phenanthrene isolates tested possessed a C23DO gene detectable by our primers. The gasoline isolates were not tested for the presence of C23DO genes because the primary substrate(s) used by these microorganisms could not be determined.

3.3. Winamac field site

Five of the 11 wells sampled at the Winamac site contained aromatic compounds (Table 2). DNA extracted from Winamac was minimally sheared and concentrations ranged from 1.5 to 21.5 ng DNA ml$^{-1}$ extract (Table 2). Three of the five contaminated wells and three uncontaminated wells contained C23DO genes (Table 2, Fig. 2A). QC-PCR showed elevated numbers of C23DO genes in W-7, W-8, and W-11 extracts (Table 2). Two contaminated wells (W-5 and W-12) did not contain any C23DO genes. There was no significant difference ($P = 0.01$) between total heterotrophs or benzoate-degraders in contaminated and uncontaminated samples from the Winamac site, nor was there any correlation between heterotrophs and BTX+SVOC concentrations ($r = -0.34$) or benzoate-degraders and BTX+SVOC concentrations ($r = -0.25$).

3.4. Linton field site

At the Linton site only L-2 and L-3 contained BTX compounds above the detection limit (Table 2). All soil cores contained levels of TPH above 100 mg kg$^{-1}$. Components of TPH include BTX. All Linton DNA concentrations were below the fluorometer detection limit of 1 ng DNA ml$^{-1}$ extract. However, DNA amplification did occur in all samples using universal 16S rDNA primers indicating that there was DNA present in all extracts and that it was of sufficient quality to allow PCR amplification. No target C23DO genes were detected in extracts from L-2 or L-3 (Table 2). Weak amplification of C23DO genes was found in the uncontaminated sample from L-1, and from TPH contaminated SB-1 and SB-4. The Linton site contained fewer culturable aerobic bacteria than the Winamac site. There was no correlation between heterotrophs and BTX+SVOC concentrations at the Linton site ($r = -0.27$). None of the Linton...
samples contained culturable aerobic benzoate-degrading or naphthalene-degrading bacteria. In contrast to the Linton aquifer solids which contained culturable heterotrophs, no bacteria could be cultured from the TPH contaminated soil from SBs.

Clay content has been shown to decrease the size and activity of microbial communities [19]. High clay content results in low water potential and low hydraulic conductivity, limiting both nutrient and oxygen transport to subsurface bacteria. It has been established that DNA adsorbs strongly to clay [20]. Linton clay that was spiked with Pseudomonas sp. strain PpG7 cells resulted in weak C23DO amplification. More than 99% of the spiked DNA was not recovered after 30 min and 24 h, suggesting that adsorption was rapid, and significant cell growth did not occur. In contrast, 10^7 PpG7 cells spiked into Chalmers soil were present at 2.3 \times 10^7 C23DO copies g^{-1} soil after 30 min and 7.3 \times 10^7 C23DO copies g^{-1} soil after 24 h.

### 4. Discussion

#### 4.1. Microcosm experiments

Although subfamily I.2.A C23DO genes are known to catabolize a wide variety of aromatic substrates, not all of the substrates we tested enriched for these specific C23DO genes in microcosms and environmental samples. Naphthalene and m-xylene strongly selected for microorganisms containing subfamily I.2.A C23DO genes in microcosms as the C23DO gene copy number increased immediately and remained at high levels throughout the 4 week experiment (Fig. 3). Microcosms fed p-xylene at levels more than an order of magnitude lower than m-xylene resulted in the enrichment of consistently detectable C23DO genes. Weak and transient elevations in C23DO gene copy numbers were detected in benzene, o-xylene, and toluene-fed microcosms. Since sites are generally contaminated with petroleum mixtures that contain m-xylene, p-xylene, and naphthalene, our C23DO primers would be suitable for general monitoring of aerobic bioremediation.

The detection of C23DO genes in almost all of the cultivated naphthalene isolates was consistent with the existence of one primary aerobic naphthalene biodegradation pathway that includes I.2.A subfamily C23DO enzymes [21]. However, no C23DO genes were detected in anaerobic naphthalene microcosms nor in no-substrate controls through 4 weeks. Since dioxygenase enzymes require molecular oxygen in order to function [22], this clearly demonstrates that C23DO gene detection confirms the presence of both oxygen and an aromatic substrate.
Out of five aerobic toluene biodegradation pathways described in the literature, only bacteria that use the TOL pathway carry the C23DO gene detected by our primers [23]. TOL organisms are also capable of degrading \( m \)- and \( p \)-xylene, two significant environmental pollutants, which likely explains the widespread occurrence of TOL plasmid organisms in the environment. Toluene degraders using the TOL pathway have been shown to be less competitive [23]. High toluene concentrations have been reported to decrease numbers of aerobic toluene-degrading bacteria due to toluene toxicity [24]. Higher substrate concentrations have also been shown to decrease the diversity of bacteria capable of degrading a given substrate (e.g. [25]). The C23DO genes detected only in weeks 1 and 2 in the high toluene concentration microcosms may reflect the greater diversity of toluene-degraders that would exist prior to the enrichment of a more homogeneous population lacking the C23DO containing TOL pathway.

Aerobic benzene degradation is thought to more commonly occur via the ortho cleavage pathway which yields more energy than the meta cleavage pathway. Organisms containing both ortho and meta cleavage enzymes use the meta cleavage pathway when parent compounds such as toluene or xylenses are present, or when catechol accumulates due to the slow kinetics of catechol 1,2-dioxygenase [26]. Thus, at high substrate concentrations, both ortho and meta cleavage pathways may be active. This is consistent with the detection of C23DO genes in one microcosm after increasing the benzene concentration tenfold.

Gasoline contains the aromatic compounds \( m \)-xylene, naphthalene and \( p \)-xylene. In addition, gasoline contains a large proportion of easily metabolized alkanes. The gasoline used in this study also contained ethanol, which is preferentially oxidized by bacteria [27]. A single vial of gasoline was used during the 4-week experiment. Thus, the volatile aromatic compounds would be depleted over the course of the experiment. Early preferential substrate utilization (e.g. ethanol) and later depletion of xylenses may account for detection of C23DO genes in gasoline microcosms only in weeks 2 and 3.

4.2. Field experiments

Based on knowledge from the microcosm experiments and site hydrology, C23DO genes were found where expected in samples from the Winamac site in downgradient and contaminated wells containing xylenses or naphthalene. The highest levels of C23DO genes occurred in W-7, which was previously contaminated with xylenses. W-7 is just downgradient from where a 1000 gallon tank had been removed (Fig. 2A). C23DO genes were also detected in W-3 and W-9. Xylenses were previously detected in W-3. W-9 is less than 50 ft downgradient from W-2, which contains elevated BTX and C23DO levels. The presence of C23DO genes is probably due to its close proximity to the contaminant plume and a flux of aromatics through this soil sufficient to maintain a C23DO-containing population. Hybridization of a C23DO-specific probe confirmed that detection of C23DO genes in downgradient monitoring wells was not a spurious result. C23DO genes were not detected in contaminated wells W-5 and W-12. However, these wells contained only benzene (W-12) or benzene and ethylbenzene (W-5). In microcosms benzene concentrations higher than those found in W-5 and W-12 failed to strongly select for bacteria containing C23DO genes. In addition, W-5 and W-12 occur roughly in the center of the contaminant plume which are often anaerobic because oxygen utilization rates are greater than oxygen recharge rates. No C23DO genes were detected in W-4, W-6 or W-10. BTX has never been detected in these ‘sentinel’ wells dating back to 1996. The pattern of C23DO detection at the Winamac site is consistent with our microcosm studies and strongly suggests that aerobic bioremediation is occurring.

The low level of C23DO genes in Linton aquifer samples, in combination with the absence of culturable benzoate- or naphthalene-degrading bacteria, strongly suggests that significant aerobic bioremediation was not occurring. The occurrence of C23DO genes in L-1, SB-1, and SB-4 may indicate the aerobic fringe of the contaminant plume. Ribosomal genes (16S rDNA) were detected in DNA extracts from all Linton aquifer solids indicating that the DNA was of sufficient quality and quantity for PCR amplification. However, as indicated by the poor DNA recovery from both the DNA spiking experiment and the Linton field samples, the clay soil compromises the C23DO detection limit by as much as three orders of magnitude. Modifications of the DNA extraction procedure could possibly alleviate this problem.

5. Summary and conclusions

The use of PCR to monitor microcosms and two field sites provided insight that was not attainable using standard culture techniques. Plate count data showed no correlation to the occurrence of BTX, which is in agreement with previous studies [28]. In contrast, there is a very strong correlation between the presence of xylenses and naphthalene to enrichment of C23DO genes. Microcosm studies confirmed that C23DO genes are indicators of oxygen as well. More research comparing pollutant flux, rather than concentration, to gene copy number is still needed.

While the primers developed in this work proved to be very satisfactory for detecting naphthalene, \( m \)-xylene, and \( p \)-xylene degrading bacteria, more work must be done in order to fully realize the potential of molecular genetic methods as tools for monitoring bioremediation.
The development of PCR primers for other catabolic genes would be useful. In addition, rapid progress is being made in developing quantitative PCR methods that can be completed more quickly than the QC-PCR technique described here. The specificity of molecular genetic techniques provides critical information about the in situ microbial community that would be useful for monitoring the effects of subsurface aeration technology or oxygen amendments used for in situ bioremediation. The ability to perform accurate direct microbial analysis combined with the short time required to process samples, poises molecular genetic techniques at the threshold of future in situ bioremediation monitoring.

References