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# Use of omic tools to assess methyl *tert*-butyl ether (MTBE) degradation in groundwater

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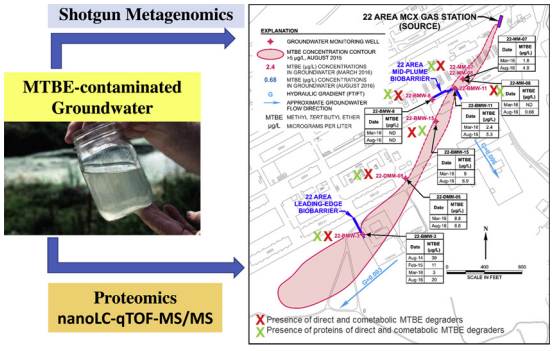
## GRAPHICAL ABSTRACT

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## ABSTRACT

This study employed innovative technologies to evaluate multiple lines of evidence for natural attenuation (NA) of methyl *tertiary*-butyl ether (MTBE) in groundwater at the 22 Area of Marine Corps Base (MCB) Camp Pendleton after decommissioning of a biobarrier system. For comparison, data from the 13 Area Gas Station where active treatment of MTBE is occurring was used to evaluate the effectiveness of omic techniques in assessing biodegradation. Overall, the 22 Area Gas Station appeared to be anoxic. MTBE was detected in large portion of the plume. In comparison, concentrations of MTBE at the 13 Area Gas Station were much higher (42,000 µg/L to 2800 µg/L); however, none of the oxygenates were detected. Metagenomic analysis of the indigenous groundwater microbial community revealed the presence of bacterial strains known to aerobically and anaerobically degrade MTBE at both sites. While proteomic analysis at the 22 Area Gas Station showed the presence of proteins of MTBE degrading microorganisms, the MTBE degradative proteins were only found at the 13 Area Gas Station. Taken together, these results provide evidence for previous NA of MTBE in the groundwater at 22 Area Gas Station and demonstrate the effectiveness of innovative–omic technologies to assist monitored NA assessments.

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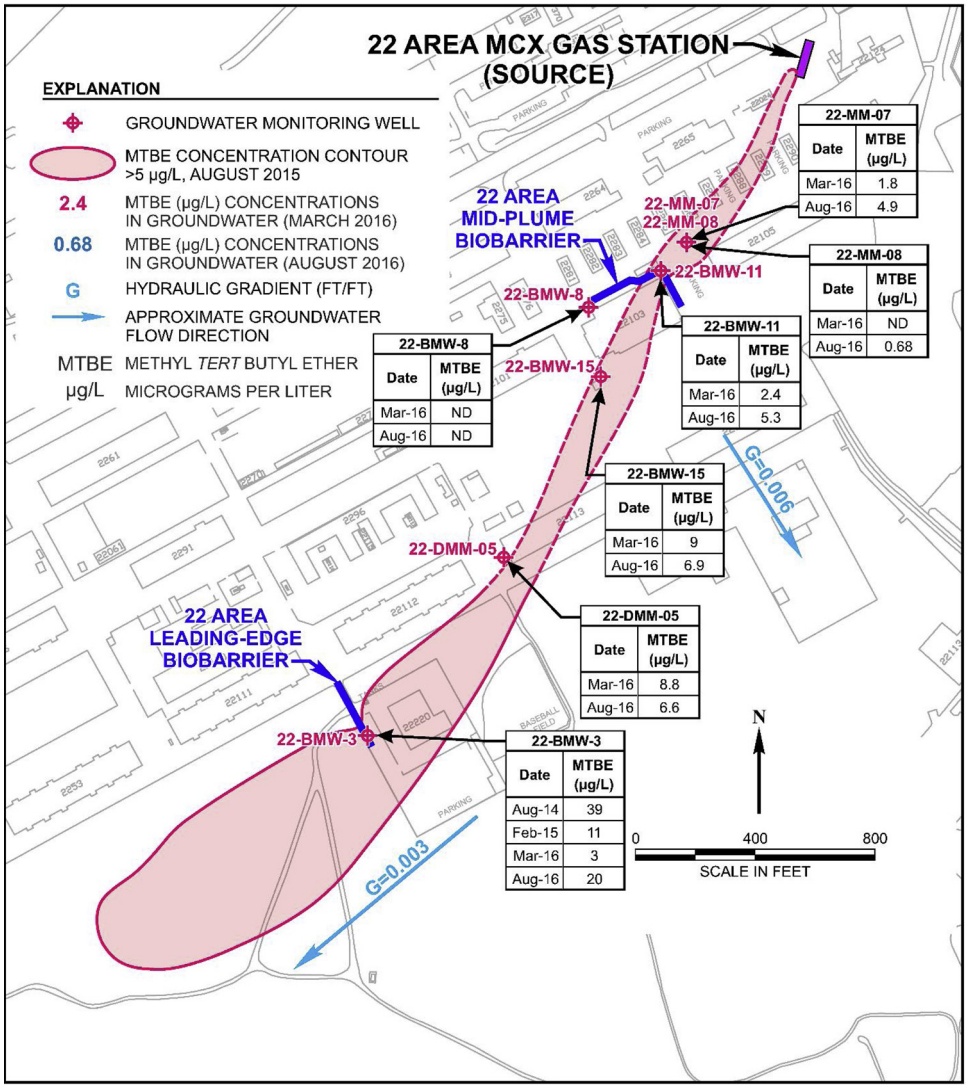


**Fig. 1.** MTBE Concentrations in Groundwater at the 22 Area Gas Station Site.

## 1. Introduction

The fuel oxygenate, methyl *tert*-butyl ether (MTBE) has been used as a gasoline additive and is a widespread groundwater contaminant in the United States [1]. It is extremely water soluble, rapidly moves through soil column and has the potential to pollute aquifers impacted by gasoline releases from leaking tanks [2]. As a result, these leaks have created large MTBE groundwater plumes that are a concern for human health [2]. Significant efforts have been made to remove MTBE from soil [3] and groundwater [4] through technologies such as soil vapor extraction, air sparging and biosparing. However, the treatment and subsequent long-term monitoring of MTBE-contaminated sites is still ongoing [5].

As MTBE is susceptible to biodegradation, there has been extensive research examining MTBE degradation by pure microbial cultures and mixed consortia. MTBE-utilizing bacteria include aerobes, such as *Methylibium petroleiphilum* PM1 [6], *Aquincola tertiaricarbonis* L108 [7], *Hydrogenophaga flava* ENV 735 [8], and anaerobes such as *Aquincola* [7] and *Cupriavidus* species. Only a few pure strains of aerobes have been cultured to date and have been observed to grow on MTBE at a relatively slow rate [4]. Thus, in the case of MTBE degradation, the value of conventional molecular biological tools (MBTs) is limited due to a wide variety of microorganisms which perform MTBE biotransformation reactions [6,9–11]. During MTBE direct aerobic degradation (Figure S1) the intermediate product, tertiary butyl alcohol (TBA), often accumulates and increases the toxicity of the aquifer. Therefore, before an MTBE bioremediation strategy can be used, an assessment of the risks associated with the accumulation of its breakdown products is essential. In addition to TBA, intermediate products of direct metabolism or co-metabolism of MTBE include tertiary butyl formate (TBF), hydroxyisobutyraldehyde (HIBA), 2-methyl-2-hydroxyl-1-propanol and acetone [12]. Persistence of these intermediate species can be variable and depends on the rate-limiting step in their production and degradation, geochemical conditions, and the composition of the in situ microbial community [7,13].

To date, only a subset of qPCR assays has been designed to enumerate specific MTBE-degrading microorganisms such as *M. petroleiphilum* PM1 or ETBE degradation gene *ethB*. While *ethB* gene copy numbers provide useful abundance information, they do not inform on the MTBE expressed degradation activity. New advanced 'omic' tools with metagenome sequencing offer an ability to characterize the microbial MTBE-degrading community. Coupled with proteomics to detect the enzymes responsible of MTBE breakdown as evidence of biodegradation activity, omic-based monitoring has the potential to provide culture-independent data on the potential for MTBE biodegradation in contaminated groundwater.

This study evaluated long-term monitoring data at the 22 Area Gas Station site where natural attenuation of MTBE is used as a polishing

step following in situ bioremediation. In addition to data collected with traditional monitoring techniques, metagenomics and metaproteomics were applied to improve the understanding of long-term impacts of the remedy on biodegradation at the site. Sampling and analysis at the 13 Area Gas Station site, at which a soil vapor extraction and biosparging system is currently in operation to treat high concentrations of MTBE, was used as a positive control.

## 2. Materials and methods

### 2.1. MTBE Plume at the Marine Corps Base (MCB) Camp Pendleton

Two commercial gasoline service station sites located at the Marine Corps Base (MCB) Camp Pendleton, California were selected for this demonstration. At the 22 Area Gas Station, an estimated 51,255 lbs of total petroleum hydrocarbon (TPH) mass was removed from the source area during the implementation of in situ air sparge/soil vapor extraction. Downgradient contamination consisted solely of MTBE. The resultant dissolved-phase MTBE plume was treated with a two-stage biobarrier system consisting of a mid-plume and a leading-edge biobarrier (Fig. 1). During their operation MTBE concentrations in groundwater declined significantly and only dilute levels of MTBE (i.e., 5 µg/L to 40 µg/L) remained. At cessation of biobarriers, low-level dissolved-phase MTBE still existed at concentrations exceeding the MTBE secondary maximum contaminant level (5 µg/L). Thus, the site was transitioned to long term monitoring.

At the positive control site, 13 Area Gas Station (Fig. 2), the remediation system remains operational and as of December 2016, an estimated 462,497 lbs of TPH have been removed from the subsurface via soil vapor extraction and biosparging. As of July 2015, MTBE concentrations at the site were as high as 23,000 µg/L [14].

### 2.2. Groundwater sampling and analytical methods

Groundwater monitoring wells were sampled twice (Table S1) in 2018. Field parameters (e.g., oxidation reduction potential [ORP], and dissolved oxygen [DO]) were measured for each well. Groundwater was analyzed for volatile organic compounds (VOCs), collected and shipped on ice for traditional MNA and advanced MBT analyses.

### 2.3. DNA extraction and metagenome sequencing

Approximately 1 L of groundwater from each well was filtered through 0.2-µm membrane filters. DNA was extracted using MoBio Laboratories PowerWater DNA Isolation Kit®. DNA was quantified by fluorimetry (Qubit 2.0) and qPCR was run using adaptor flanked primers targeting the 16S rDNA [15]. The amplified products were tagged with a sample-specific index sequence and sequenced using an Illumina MiSeq. To perform analyses of microbial organisms, a list of common direct and cometabolic MTBE degraders was compiled (Table S2) and used together with a custom 16S reference database. The identified microorganisms served as a foundation to build a database for mass spectrometry spectral searching. For each metagenome, organisms were grouped into four categories: aerobic/anaerobic, direct and cometabolic [13] MTBE metabolizers. The number of reads associated with the groups were totaled and compared across each of the samples using a Dirichlet- multinomial model [16].

### 2.4. Proteomic characterization of groundwater

Proteins were extracted from lyophilized groundwater using MoBio NoviPure Microbial Protein Kit (Quiagen), reduced, alkylated, trypsin-Lys-C digested, and subjected to liquid chromatography mass spectrometry (LC–MS/MS) using a Nano 415 LC system in line with an ABI Sciex Triple TOF 5600 high resolution MS instrument as previously reported [17]. The protein and peptide concentrations were calculated using published methods [18]. Protein identification is described in detail in the SI section.

## 3. Results and discussion

### 3.1. Evidence of MTBE degradation based on MTBE/TBA concentrations trends

Results have shown that while MTBE was detected in groundwater of all sampled wells at 22 Area Gas Station with concentrations ranging from 1.8 µg/L (at 22-MM-07) to 9.0 µg/L (at 22-BMW-15), no other oxygenates were found. The wells with the highest MTBE concentrations were located between the biobarriers and in the leading-edge biobarrier (Fig. 1).

Prior to active treatment with the biobarriers at the 22 Area Gas Station, MTBE concentrations ranged from 119 to 1420 µg/L [22,23]

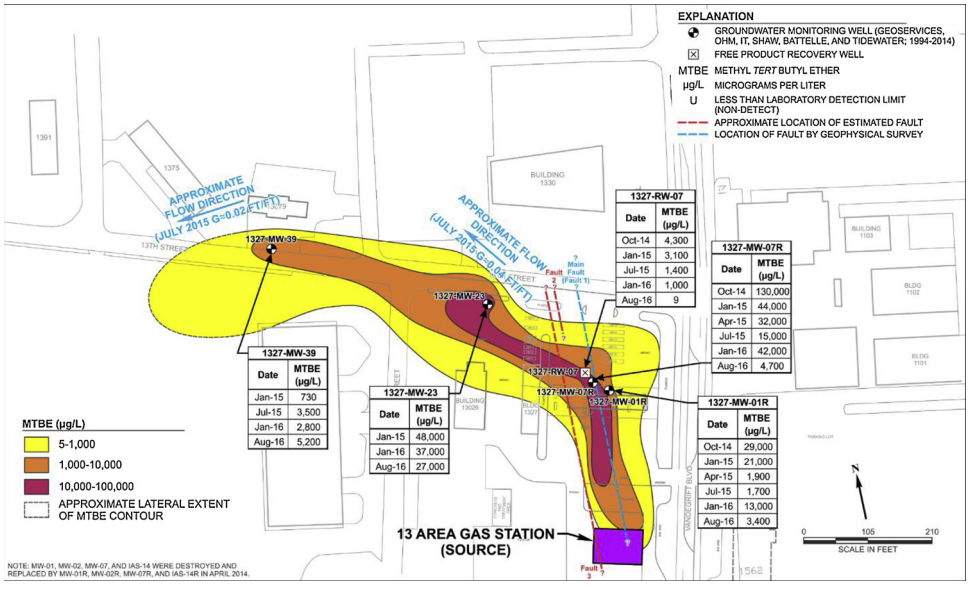


**Fig. 2.** MTBE Concentrations in Groundwater at 13 Area Gas Station MCB Camp Pendleton Site.

**Table 1**
Summary of MTBE Degradation Trends in Site Historical Data and Summary of MTBE and TBA Results from 2016 Sampling at the 22 Area Gas Station.

| Location | MTBE and TBA Analytical Results | | | | Mann-Kendall Statistics and MTBE Historical Degradation Trends | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Monitoring Well | Sampling Date | MTBE (µg/L) | TBA[1] (µg/L) | Time | Phase | COV[2] | Mann Kendall Statistic (S) | CF | MTBE Trend |
| Upgradient | 22-MM-07 | 3/8/2016 | 1.8 | ND | 2002-2016 | N/A | Insufficient data collected | | | |
| | | 8/17/2016 | 4.9 | ND | | | | | | |
| | 22-MM-08 | 3/8/2016 | ND | ND | 2002-2016 | N/A | 1.9 | −439 | > 99.9% | Decreasing |
| | | 8/17/2016 | 0.68 | ND | | | | | | |
| Within Mid-Plume Biobarrier | 22-BMW-11 | 3/9/2016 | 2.4 | ND | 2005-2016 | Overall | 1.81 | −148 | 98.2% | Decreasing |
| | | 8/17/2016 | 5.3 | ND | 2005-2010 | Active | 1.69 | −101 | 99.4% | Decreasing |
| | | | | | 2010-2016 | MNA | 0.48 | −6 | 64.8% | Stable |
| | 22-BMW-08 | 3/8/2016 | ND | ND | 2002-2016 | Overall | 4.58 | −59 | 91.10% | Probably Decreasing |
| | | 8/17/2016 | ND | ND | 2005-2010 | Active | 2.05 | −91 | 99.9% | Decreasing |
| | | | | | 2010-2016 | MNA | Insufficient data collected | | | |
| Between Biobarriers | 22-BMW-15 | 3/8/2016 | 9.0 | ND | 2005-2016 | Overall | 1.60 | −170 | 98.70% | Decreasing |
| | | | | | 2005-2010 | Active | 1.43 | −207 | > 99.9% | Decreasing |
| | | 8/17/2016 | 6.9 | ND | 2010-2016 | MNA | 0.47 | 15 | 92.50% | Probably Increasing |
| | 22-DMM-05 | 3/8/2016 | 8.8 | ND | 2005-2016 | Overall | 1.7 | −474 | > 99.9% | Decreasing |
| | | 8/17/2016 | 6.6 | ND | 2005-2010 | Active | 1.42 | −287 | > 99.9% | Decreasing |
| | | | | | 2010-2016 | MNA | 1.15 | 2 | 53.50% | No Trend |
| Leading-Edge Biobarrier | 22-BMW-3 | 3/8/2016 | 3 | ND | 2004-2016 | Overall | 1.29 | −227 | 99.60% | Decreasing |
| | | 8/17/2016 | 20 | ND | 2004-2012 | Active | 1.19 | −112 | 97.60% | Decreasing |
| | | | | | 2012-2016 | MNA | 0.76 | −10 | 78.40% | Stable |

and decreased on average of two orders of magnitude during the active treatment. After active treatment, MTBE concentrations ranged from 5.3–11 µg/L indicating that the biobarrier served to reduce contaminant concentrations [24]. Based on the overall trend of the historical data, during the active remediation and MNA, the MTBE concentrations showed a decreasing trend (confidence factor [CF] > 95%) (Table S3). Mann-Kendall trend analyses demonstrated a significant decrease in MTBE concentrations (Table 1) at every well, indicating successful implementation of the remedy [5].

To evaluate contaminant concentration trends during the MNA phase of the remedy, Mann-Kendall trend analysis was performed on data collected after discontinuing biobarrier operation. Within the mid-plume biobarrier, the analysis showed MTBE concentrations at 22-BMW-11 were stable – neither increasing nor decreasing. Between the biobarriers, at wells 22-BMW-15 and 22-DMM-05, the analysis revealed no trend in the data. At the leading-edge biobarrier well, 22-BMW-3, the analysis indicated a stable MTBE trend after system shutdown in 2012. Well-by-well Mann-Kendal results after system shutdown showed no comprehensive statistically significant trend across the site.

At the positive control site, groundwater was analyzed for MTBE and other oxygenates (Table S4). In the first sampling event, MTBE was detected in the monitoring wells with concentrations ranging from 2800 to 42,000 µg/L. The highest MTBE concentrations were located in the source area well 1327-MW-07R and mid-plume at 1327-MW-23, respectively (Fig. 2). In addition to MTBE, the intermediate TBA and oxygenates ETBE and TAME were detected. Mann-Kendall (Table 2) analysis results showed statistically significant MTBE decreasing trends at most wells. These results are a strong line of evidence that treatment activities are effectively reducing MTBE concentrations.

### 3.2. Evidence of degradation based on the site geochemistry

Geochemical data, shown in Tables S3 and S4, were collected at 22 Area Gas Station to delineate biogeochemical processes and to infer microbial MTBE biodegradation [5]. The data from the representative well 22-DMM-05 is shown in Figure S2.

Based on DO readings, the site was predominately anoxic (DO = 0 mg/L). The exceptions were the wells between the biobarriers, which showed oxic conditions during the first sampling event (DO 5.17–5.29 mg/L). The ORP varied spatially and temporally. Based on ORP data alone, second sampling event indicated the site was anoxic except for wells 22-DMM-05 (10–39 mV) and 22-BMW-3 (80–201 mV). However, the DO measured in these wells was zero, exemplifying conflicting data. Overall, ORP data did not indicate whether the wells were predominately oxic or anoxic because there was too much variation in data.

Nitrate and ferrous iron concentrations at 22 Area Gas Station were below their detection limits (< 0.25 mg/L and < 0.05 mg/L) and the levels of sulfate did not indicate sulfate reduction has or is occurring. Methane levels were at or below the detection limit of 0.010 mg/L. Thus, the geochemical data do not indicate which specific biogeochemical processes are occurring and microbial activity cannot be inferred for MTBE biodegradation [25].

The geochemical parameters at the positive control site were used to evaluate the biosparging system performance with DO as the main parameter. Since the site is under active remediation, monitoring wells were sampled 24 h after the biosparge system was temporarily shut down (Table S4**).** It appears that some areas in the treatment zone were rapidly depleted of oxygen where at other locations oxygenated groundwater remained for several days after system shutdown. In the mid-plume and at the leading edge of the plume, oxygenation by the biosparge system was limited, and overall the ORP levels indicated anoxic conditions.

Terminal electron acceptors for anaerobic processes in the active treatment zone of 13 Area Gas Station showed aerobic processes were predominant and reflected the impact of the ongoing biosparging

**Table 2**
Summary of MTBE and TBA Results from 2016 Sampling at the 13 Area Gas Station.

| Location | Monitoring Well | Sampling Date | MTBE (µg/L) | TBA (µg/L) | DIPE (µg/L) | ETBE (µg/L) | TAME (µg/L) |
|---|---|---|---|---|---|---|---|
| Source Area | 1327-MW-01R | 1/27/2016 | 13,000 | 2,100 | ND | 5.8 | ND |
| | | 8/18/2016 | 3,400 | 450 | ND | ND | ND |
| | 1327-RW-07 | 1/27/2016 | 1,000 | 36,000 | ND | ND | ND |
| | | 8/18/2016 | 8.6 | ND | ND | ND | ND |
| | 1327-MW-07R | 1/27/2016 | 42,000 | 11,000 | ND | ND | 140 |
| | | 8/18/2016 | 4,700 | 4,400 | ND | ND | ND |
| Mid Plume | 1327-MW-23 | 1/27/2016 | 37,000 | 2,100 | ND | ND | ND |
| | | 8/18/2016 | 27,000 | 2,100 | ND | ND | ND |
| Leading Edge | 1327-MW-39 | 1/27/2016 | 2800 | 270 | ND | ND | 19 |
| | | 8/18/2016 | 5,200 | 1,300 | ND | ND | ND |

ND – non-detect.

process. As such, no detectable concentrations of ferrous iron and methane were observed, and sulfate concentrations were not depleted. In contrast, outside of the active treatment zone, the wells indicated that natural attenuation may be occurring via anaerobic biodegradation. Concentrations of ferrous iron was observed in wells in the downgradient plume, mid-plume and leading edge, and methane was detected at the leading-edge well (Table S4). Additionally, sulfate concentrations were depleted downgradient of the active treatment zone. The electron acceptors in the mid-plume and leading-edge wells indicated that NA of MTBE may be contributing to contaminant decreases outside of the source area (active biosparge treatment zone).

### 3.3. Evidence of MTBE degradation based on metagenomics

Data from the 22 Area Gas Station was compared to the data from the 13 Area Gas Station and a significant difference ($p = 0.0028$) was found with respect to composition of MTBE-degrading microorganisms (Fig. 3).

### 3.4. 22 Area gas station

Most microorganisms detected were aerobic with a small percentage of anaerobic or facultative anaerobic MTBE co-metabolizing species (Fig. 3). At the locations upgradient from the biobarrier, aerobic species of *Acinetobacter, Pseudoxanthomonas* [26] and *Sphingomonas* were dominant and a small percentage of anaerobic species of *Rhodoferax* and *Pseudomonas* were detected. Similarly, the mid-plume biobarrier and the area between the biobarriers were characteristic of an abundance of aerobic species with only a small percentage of anaerobic or facultative anaerobic MTBE degraders. At the leading-edge biobarrier, the presence of both aerobic and anaerobic MTBE degraders with a dominance of cometabolic *Cupriavidus, Rhodoferax* and *Variovorax* were detected suggesting MTBE cometabolism as a main degradation mechanism [20,29,30].

Species known to be MTBE direct mineralizers were detected in all samples. Additionally, species known to support cometabolic MTBE degraders were present in all sampled locations with highest relative abundance detected in the leading-edge biobarrier samples. Upgradient of the biobarrier and the mid-plume biobarrier were dominated by the abundance of species from genus *Cupriavidus* (˜7%), *Variovorax* (˜8%) and *Pseudoxanthomonas* (10–20%) known to degrade MTBE to TBA [26] and equipped with most enzymes, to support MTBE mineralization [13]. The cometabolic bacteria detected in these two areas were predominantly of genus: *Acidobacteria* (ETBE degradation to TBA) [11], *Pseudomonas* (MTBE and BTEX degradation when grown on pentane, partial MTBE degradation to HIBA grown on pentane, cometabolic MTBE degradation when grown on $C_5$ – $C_8$ n-alkanes) [20,21,31], and *Sphingomonas* (partial MTBE degradation).

The area between the two biobarriers had the highest abundance of direct mineralizers, signifying the highest potential for complete MTBE mineralization (Figure S3). Presence of *M. petroleiphilum* PM1 [10,32], *Hydrogenophaga flava* ENV735 [8], *Mycobacterium austroafricanum* and *Pseudoxanthomonas* spp. [12] further supported this observation. This elevated relative abundance of MTBE degraders in between biobarriers is most likely linked to the oxygen injection activities at the site during the past remedy implementation phase. These results suggested that the biobarrier installation and oxygen sparging activities impacted the microbiology of the site, enriching the MTBE aerobic population. The cometabolic microorganisms present were classified into genus *Acinetobacter* -partial oxidation of alkyl ethers [11]; *Nocardioides* - partial MTBE degradation using propane as a carbon source[35]; and *Sphingomonas* - partial MTBE degradation.

The mid-plume biobarrier and leading-edge biobarrier sampling locations showed higher relative abundance of cometabolic MTBE degraders from the genera *Pseudomonas* and *Sphingomonas* (Figure S4) in comparison to the direct mineralizers. This suggested preferential metabolism of $C_5$ to $C_8$ n-alkanes versus utilization of MTBE as a carbon source. The presence of propane [31] degraders (*Nocardioides* sp., *Xhanthobacter, Mycobacterium* sp.), as well as species utilizing butane [31] (*Arthrobacter*), ethanol (*Gordonia terrae*), pentene (*Rhodococcus* sp., *Pseudomonas aeruginosa*) and hydrocarbon mixtures [33,34] (*Pseudomonas* sp.) [20,35] demonstrated existence of a robust microbial population capable of degrading mixed gasoline components.

### 3.5. 13 Area gas station

The majority of species known to degrade MTBE were aerobic with a small percentage of anaerobic or facultative anaerobic MTBE cometabolizers (Fig. 3). Within the active treatment area and mid-plume locations (within the ROI of biosparging), aerobic direct degraders species were the most abundant with a small percentage of anaerobes also present. The mid-plume well, located inside of the radius of influence of biosparging system, was characteristic of high abundance of aerobes from genera *Bacillus* and *Rhodobacter* detected during the first sampling event. A high relative abundance of *Mycobacterium* genus (86%) specifically, *Mycobacterium austroafricanum* [36] was observed in the samples collected during second sampling event. In the leading edge of the plume (within the MNA zone) 1.7% of total microorganisms were MTBE-degraders of which 0.8% were aerobes of genera: *Bacillus, Hydrogenophaga, Methylobium* and *Mycobacterium.*

Direct MTBE mineralizers were present in all samples with lowest relative abundance, approximately 0.3%, in the leading edge of the plume. Cometabolic MTBE degraders were present in all sampled locations with highest relative abundance in the active treatment zone (up to 24%) and leading edge of the plume (MNA zone) (up to 30%). The source zone was dominated by genera *Bacillus* (˜6%), *Hydrogenophaga* (˜7.5%), *Methylobium* (35%), *Variovorax* (˜8%) and *Sphingopyxis* (15%) capable of MTBE biodegradation to TBA. Figure S5 illustrates the microbial community composition in source zone sample 1327-MW-01R. The cometabolic bacteria in source zone wells were predominantly of genera: *Mycobacterium* (found to grow on MTBE and TBA) [37], *Nocardioides, Rhodoferax* and *Rhodobacter*, which perform

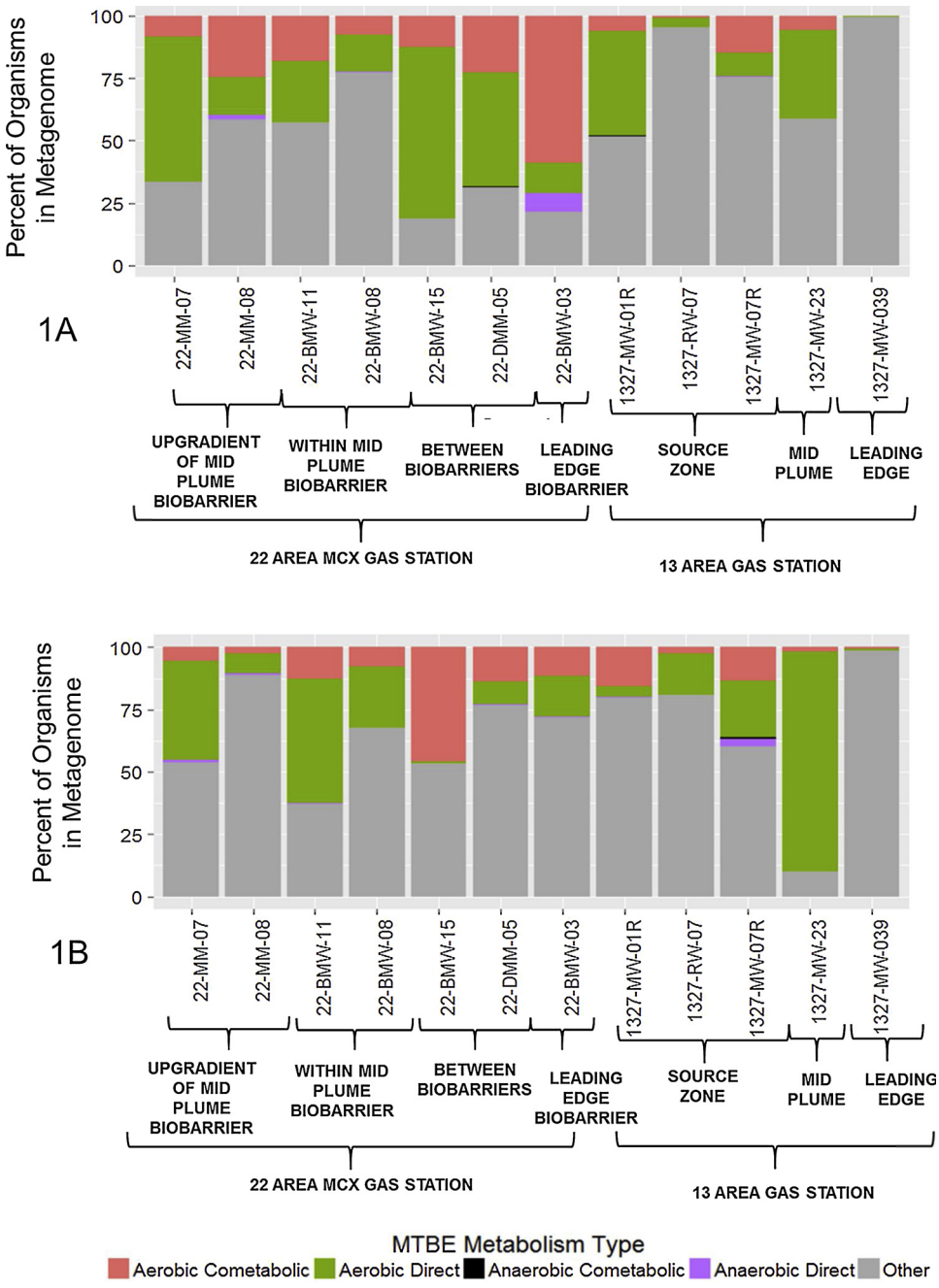


**Fig. 3.** Percent Abundance of Microorganisms in Samples from the 22 Area MCX Gas Station and the 13 Area Gas Station where 1A is the First Sampling Event and 1B is the Second Sampling Event. MTBE-degrading Microorganisms were Categorized Depending on Aerobic, Anaerobic, Direct and Cometabolic Degradation.

partial MTBE degradation with cyclohexane. The mid-plume sample was rich in direct MTBE metabolizers (30% of total MTBE degraders) and the dominant cometabolic fraction of the microbial population was represented by species of genera: *Mycobacterium, Nocardioides* and *Rhodobacter*. The leading-edge population showed the least percentage abundance of direct MTBE species (0.3%) and cometabolic (0.3%) species.

### 3.6. Evidence of MTBE degradation based on metaproteomics

Shotgun proteomics data was compiled in Table S5 and served as indicators to determine: 1) presence of MTBE degradative proteins, and 2) presence of proteins from known MTBE-degrading microorganisms. Presence of MTBE degradative proteins provides direct evidence of activity of the degradation processes, while detection of proteins from known MTBE-degrading microorganisms serves as indirect evidence of degradation [13,38]. At the 22 Area, no MTBE degradative proteins were identified. Thus, no direct evidence of active natural attenuation was provided with metaproteomics. However, a few proteins from co-metabolic MTBE-degrading microorganisms were detected. In contrast, both groups of protein indicators were found at the 13 Area Gas Station confirming that this site can serve a positive control for metaproteomic analysis.

A comprehensive review of proteomic results at 22 Area Gas Station is shown in Table 3 with the number of peptides identified from MTBE direct, cometabolic, anaerobic- or aerobic microorganisms for each sampling well. During the first sampling event, the peptides detected at all sampling locations were derived from aerobic, cometabolic MTBE-degrading microorganisms. For example, proteins from the aerobic cometabolic MTBE metabolizer, *Nocardioides,* were found in the

**Table 3**
Number of Peptides from MTBE-Degrading Microorganisms Identified in Samples from the 22 and 13 Area Gas Stations during Sampling Events 1 and 2.

| | | | Aerobic | Anaerobic | Direct | Cometabolic |
|---|---|---|---|---|---|---|
| **22 Area Gas Station** | | | | | | |
| **Sampling Event 1** | Upgradient of Mid-Plume Biobarrier | 22-MM-7 | 5 | 0 | 0 | 5 |
| | | 22-MM-8 | 4 | 0 | 0 | 4 |
| | Within Mid Plume Biobarrier | 22-BMW-11 | 1 | 0 | 0 | 1 |
| | | 22-BMW-8 | 4 | 0 | 0 | 4 |
| | Between Biobarriers | 22-BMW-15 | 1 | 0 | 0 | 1 |
| | | 22-DMM-05 | 8 | 0 | 0 | 8 |
| | Within Leading-Edge Biobarrier | 22-BMW- 3 | 2 | 0 | 0 | 2 |
| **Sampling Event 2** | Upgradient of Mid-Plume Biobarrier | 22-MM-7 | 5 | 0 | 1 | 4 |
| | | 22-MM-8 | 2 | 0 | 0 | 2 |
| | Within Mid Plume Biobarrier | 22-BMW-11 | 0 | 0 | 0 | 0 |
| | | 22-BMW-8 | 6 | 0 | 2 | 4 |
| | Between Biobarriers | 22-BMW-15 | 4 | 0 | 1 | 3 |
| | | 22-DMM-05 | 4 | 0 | 0 | 4 |
| | Within Leading-Edge Biobarrier | 22-BMW- 3 | 6 | 4 | 4 | 6 |
| **13 Area Gas Station** | | | | | | |
| **Sampling Event 1** | Source Zone | 1327-MW-01R | 519 | 0 | 502 | 17 |
| | | 1327-RW-07 | 26 | 0 | 26 | 0 |
| | | 1327-MW-07R | 16 | 0 | 13 | 3 |
| | Mid Plume | 1327-MW-23 | 49 | 0 | 45 | 4 |
| | Leading Edge | 1327-MW-39 | 1 | 0 | 1 | 0 |
| **Sampling Event 2** | Source Zone | 1327-MW-01R | 1 | 0 | 1 | 0 |
| | | 1327-RW-07 | 6 | 0 | 5 | 1 |
| | | 1327-MW-07R | 0 | 1 | 1 | 0 |
| | Mid Plume | 1327-MW-23 | 0 | 0 | 0 | 0 |
| | Leading Edge | 1327-MW-39 | 12 | 1 | 10 | 3 |

upgradient and leading-edge biobarrier wells and suggested the potential for cometabolic degradation. Proteins from the aerobic cometabolic MTBE-degrader, *Pseudomonas* spp., including a dehydrogenase, an aldehyde dehydrogenase and monooxygenase [13] were also present. However, the number of identified peptides was small, which suggested limited to negligible MTBE degradation could be occurring. During the second sampling event, a few peptides derived from aerobic direct MTBE-degraders were detected in addition to the peptides from aerobic cometabolic MTBE-degrading microorganisms. Both types of peptides were detected in all locations except for the mid-plume biobarrier. A few membrane structural proteins from the genera *Pseudomonas, Methylibium* and *Cupriavidus* were identified and suggested potential for either aerobic direct or cometabolic degradation [20,39]. Overall, although a few proteins from MTBE degraders were identified at the site, no MTBE degradative peptides were detected. These results were due to either the lack of ongoing MTBE degradation at the site or peptides concentration falling below the method detection limit.

At the 13 Area Gas Station, most peptides identified during the first sampling event (Table 3) were associated with aerobic direct MTBE-degraders, including MTBE degradative proteins and only a few peptides derived from cometabolic species. Numerous proteins associated with MTBE-degradation pathways [10,13,21,40] were detected in the source zone (Table S6). Most MTBE degradative proteins were detected in the 1327-MW-01R well and proteins derived from direct aerobic MTBE degraders such as *M. petroleiphilum* PM1, *A. tertiaricarbonis* and *M. austroafricanum* were found. Seven out of ten known proteins from the *M. petroleiphilum* PM1 MTBE degradation pathway (Figure S1) were detected. During the first sampling event, the remaining two source zone area wells, 1327-RW-07 and 1327-MW-07R, showed presence of proteins from *M. petroleiphilum* PM1. However, no Mdp proteins from the MTBE degradation pathway were identified but membrane proteins (i.e., porins or membrane transporters) were found that are known to be involved in phenol degradation [39] and transport of both ions and small molecules across a cellular membrane were found. Their role (if any) in direct MTBE degradation is unknown. Moreover, in the source zone phenol degrading proteins and cytochrome P450 that catalyzes hydroxylation of methoxy and ethoxy residues in fuel oxygenates [39] were detected, suggesting a potential for BTEX degradation [4]. Presence of proteins of known cometabolic MTBE degraders such as *Nocardioides* [41] also suggested some MTBE cometabolic degradation could be occurring.

The mid-plume and leading-edge samples collected during the first sampling event, showed a low number of proteins from MTBE-degrading microorganisms. No MTBE proteins related to the Mdp or cometabolic pathways were found, but a handful of *Aquinicola* sp. and *Methylibium* sp. membrane proteins and porins were detected. It is worth noting that the mid-plume and leading-edge wells are located outside the radius of influence of the biosparge system. Thus, the degradation of MTBE and diversity of microbial community differed significantly ($p = 0.016$) in comparison to the source zone location. In the second sampling event, the total number of proteins detected throughout the site were lower in comparison to the first sampling event. Although no proteins associated with the MTBE degradation pathway were detected, proteomic data showed general cellular metabolism and structural proteins from direct aerobic MTBE degraders *Mycobacterium* spp, *M. petroleiphilum* PM1 and *A. tertiaricarbonis.* A variety of proteins from *Methanosaeta concilii,* which is a known obligate anaerobic archaea [42] populating sites with gasoline contamination, were also found.

The difference found in the number of detected proteins at the 13 Area Gas Station between the two sampling events may be due to the fluctuations in groundwater levels. The changes in groundwater level may affect the concentration of planktonic microbial biomass available for collection. It is possible that with a decrease in the groundwater level, the bulk of the microbial biomass is tightly bound to the sediment or present within the sediment porous spaces. However, to explicitly prove that this was the case, additional rounds of sampling and data analysis should be performed.

### 3.7. Concordance between lines of evidence

The natural attenuation capacity at 22 Area Gas Station, and a control site 13 Area Gas Station was evaluated using a combination of conventional contaminant concentration data, geochemistry trend analyses and advanced MBTs, including metaproteomics and metagenomics. To our knowledge, the use of metaproteomics to assess the

occurrence of natural attenuation has not been previously utilized at MTBE contaminated sites. The collected data indicate that MTBE has been or is capable of being degraded when DO is available at the 22 Area Gas Station. In comparison, geochemical parameters at the 13 Area Gas Station supported aerobic conditions and active MTBE degradation within the source zone which was consistent with ongoing biosparging and the decreasing MTBE concentrations directly indicated ongoing biodegradation.

Metagenomic data from both sites showed the presence of a broad diversity of MTBE degrading bacteria with varied relative abundance of species known to be direct MTBE metabolizers to cometabolic MTBE degraders. Most of the species known to be MTBE degraders identified at both sites were aerobic, which indicated the *potential* for aerobic direct or cometabolic degradation processes. The highest relative abundance of these species was present in the active treatment zone at the 13 Area Gas Station where *M. petroleiphilum* PM1 was detected. Metaproteomic data from the 22 Area Gas Station showed the presence of proteins from species known to be MTBE-degrading microorganisms, but no proteins involved in MTBE degradation were detected. This finding supports the COC and geochemistry evaluation results indicating negligible MTBE degradation at the 22 Area Gas Station. However, it is unclear if lack of protein detection is caused by the negligible degradation or detection limits for MTBE-degrading peptides targeted in the proteomic analyses. Similar findings were shown within the MNA zone at the 13 Area Gas Station.

Overall, the advanced MBTs show that there is evidence for potential of MTBE degradation at the 22 Area Gas Station and confirm MTBE biodegradation in the active treatment zone at the 13 Area Gas Station. These positive indicators of bioremediation at the 13 Area Gas Station demonstrate that both metagenomics and metaproteomics provide a direct indication of potential and occurrence of molecular processes involved in MTBE degradation. The data generated by these two tools can thus inform on the potential for MTBE degradation and provide a tertiary line of evidence on the ongoing MNA. Thus, the proposed integrated approach can provide necessary information about the status of the aquifer microbiome and generate predictive understanding about the trajectory of a contaminant plume. Such information is crucial for decision making and for identifying sites where contaminant containment and cleanup goals cannot be achieved without more aggressive treatment. This consolidated knowledge will enhance our understanding of active degradation processes and provide a compelling line of evidence to transition from active to passive treatment.

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## Appendix A. Supplementary data

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