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Note

A novel and in situ technique for the quantitative detection of MTBE and benzene degrading bacteria in contaminated matrices

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Abstract

A novel and in situ technique is presented here as a better alternative to culture-dependent and PCR-based techniques for the quantitative detection of predominant bacterial species involved in the bioremediation of contaminants. It allowed rapid, specific and in situ identification of Biosep®-immobilized eubacteria from MTBE- and benzene-contaminated matrices.

Keywords: MTBE; Fuel oxygenate; Bioremediation; FISH; Spectral imaging

Gasoline and other petroleum fuels are commonly stored in aboveground (ASTs) and underground storage tanks (USTs) at a wide range of facilities (EPA, 2004). They contain methyl tert-butyl ether (MTBE), tert-butyl alcohol (TBA) and benzene. MTBE has been the main fuel oxygenate in U.S. gasoline since 1979 (Swain, 1999) and a significant proportion of the more than 100 billion gallons of gasoline used in the U.S. annually has contained MTBE and other oxygenates at concentrations greater than 10% per volume (EPA, 2004). However, MTBE has become a cause of environmental concern due to leaking USTs (LUSTs). In recent years, a high frequency of LUSTs has been reported (Squillace et al., 1996), which resulted in nationwide detection of MTBE in soils and drinking water sources (GAO, 2002). It has become a widespread contaminant with an estimated 3000 plumes in California alone (Keller et al., 1998) and is the most commonly detected contaminant in urban groundwater in the U.S. (Johnson et al., 2000; EPA, 1999). Several technologies are used to remediate ground water, soil and drinking water contaminated with MTBE and other petroleum derivatives. As an alternative to more labor intensive and costly approaches (e.g. soil vapor extraction, in situ chemical oxidation, pump-and-treat), bioremediation is increasingly used for treating such contamination and has shown MTBE concentration reductions greater than 99%, with no waste products (EPA, 2004). Out of 323 MTBE remediation projects listed on the EPA's MTBE Treatment Profile website, 73 used bioremediation primarily (EPA, 2004). In this approach, isolating the most suitable bacteria for the contaminant degradation is crucial to amend the soils with the most efficient microbial consortia. Recently, Biosep® beads (porous, adsorptive polymeric biocatalyst) have been used to concentrate and extract the predominant bacterial species from such matrices (Kim et al., 2004). However, identification of Biosep®-immobilized bacteria has been most commonly carried out 1) by physical separation of the bacteria from the Biosep® material and 2) by characterization using culture-dependent techniques or complex quantitative real-time polymerase chain reaction (qPCR). The use of an in situ identification tool such as fluorescence in situ hybridization (FISH), which has proven a rapid, direct, accurate and highly specific technique for identification

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Table 1
Sample description

Sample designation	Sites/contamination	Well #	Contaminants/concentrations	Biosep® bead bait
035CH8	Orange County, CA	1	MTBE up to 250 mg/L	MTBE (1.5 mg/L)
035CH10	Gasoline contaminated soil	2	TBA up to 390 mg/L	
054CH4	Ft Lupton, CO	1	BTEX < 50 µg/L	Benzene (1.5 mg/L)
054CH5	Gas condensate contaminated aquifer	1		
054CH6		1		
054CH10		2		
054CH11		2		
054CH12		2		

MTBE: Methyl tert-butyl ether; TBA: tert-butyl alcohol; BTEX: Sum of benzene, toluene, ethyl benzene and xylenes.

Table 2
16S rRNA oligonucleotide probes used for identification of all eubacteria[a] and δ-Proteobacteria subclass

Probe	Probe sequence (5′-3′)	Fluorescent label	Specificity	Reference
EUB338	GCT GCC TCC CGT AGG AGT	Texas Red®-X	All Eubacteria[a]	Daims et al. (1999)
SRB385	CGG CGT CGC TGC GTC AGG	Alexa® 647	δ-Proteobacteria	Amann et al. (1990b)

[a] With exceptions defined by Bouvier and del Giorgio (2003) and Daims et al. (1999).

of microorganisms without need for cultivation (Kalmbach et al., 2000; Manz et al., 1993; Amann et al., 1990a,b), would circumvent the several drawbacks of the previous approach (e.g. physical separation, variability of culture-dependent techniques, time-consuming DNA extraction for qPCR). The combination of bacterial concentration and extraction by Biosep® beads and in situ identification by FISH would constitute a novel and in situ approach for rapid characterization of predominant bacterial species in contaminated matrices. Therefore, the aim of this study was to design a technique for specific identification of Biosep®-immobilized MTBE and benzene degrading eubacteria from two contaminated sites using this approach, focusing primarily on the δ-subclass of Proteobacteria for its bioremediation potential. Table 1 presents a description of the samples that were analyzed, indicating the sites, contaminants present and compounds used to bait the Biosep® beads. Colonized Biosep® beads were received and sliced in half using a sterile surgical scalpel to optimize penetration of reagents and probes. FISH was carried out as follows. Each bead half was transferred to a 600 µL sterile microcentrifuge tube and immersed in a 1:3 (v/v) fixative solution of 4% paraformaldehyde in phosphate buffer saline (PBS). Tubes were incubated in the dark at 4 °C for 2 h. The fixative was carefully pipetted out and the beads were rinsed with PBS twice. Hybridization was performed based on the protocol of Manz et al. (1993). Fixed Biosep®-immobilized eubacteria were hybridized by immersing the colonized bead halves in a mixture of 500 µL of hybridization buffer (containing 0.9 M NaCl, 20 mM Tris–HCl, 30% formamide,

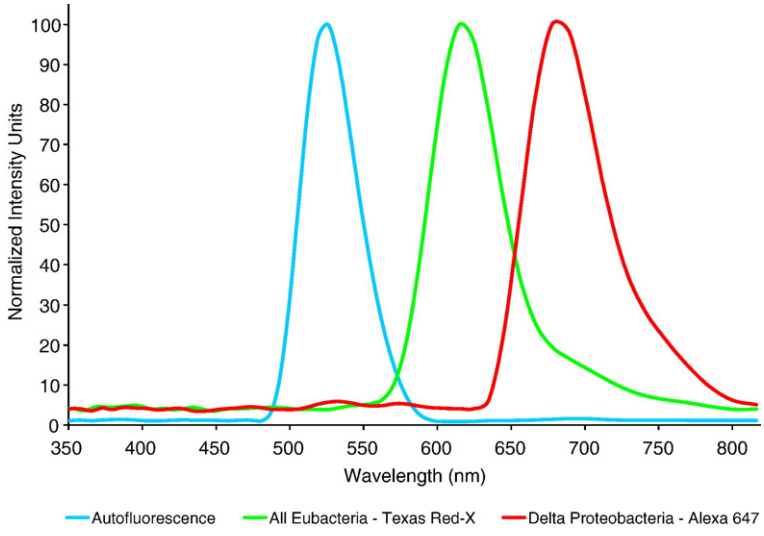

Fig. 1. Normalized fluorescence spectra of specific 16S rRNA oligonucleotide probes and Biosep® bead autofluorescence, obtained with interferometer-based ASI imaging system. False-painting was performed to improve contrast between the different images. Green: EUB338 (all eubacteria, with exceptions defined by Bouvier and del Giorgio, 2003; Daims et al., 1999) labeled Texas Red; Red: SRB385 (δ-Proteobacteria subclass) labeled Alexa®647; Blue: Autofluorescence of Biosep® bead material.

0.01% SDS) and 100 µL of probe solution (containing 25 ng of each probe of interest) for 2 h at 46 °C. Specific, fluorescently-labeled 16S rRNA oligonucleotide probes (Sigma-Genosys, The Woodlands, TX) were used to target all eubacteria (with exceptions defined by Bouvier and del Giorgio, 2003; Daims et 2al., 1999; and this mention should be understood throughout the text within the boundaries of the said limitations) and δ-Proteobacteria (Table 2, Fig. 1). Bead halves were then incubated in prewarmed washing buffer (containing 0.1 M NaCl, 20 mM Tris–HCl, 0.01% SDS) for 30 min. at 48 °C, rinsed with ice-cold sterile deionized water and air-dried in a biosafety cabinet for 30 min. Additionally, in situ observation of stained bacteria within the beads required sectioning of the beads. Thin frozen sections of bead halves (30 µm thick) from the middle section of the bead were cut using a cooled Cryostat microtome (Leica Microsystems, Nussloch, Germany), spotted onto a microscope slide, air-dried in a biosafety cabinet for 15 min., mounted in ProLong® Gold antifading agent (Invitrogen, Carlsbad, CA) and finally covered with a coverslip and sealed with nail polish. Subsequently, slides of bead sections were analyzed with an Applied Spectral Imaging platform (ASI, Migdal Ha'Emek, Israel) at 600× total magnification (60× objective) to allow for separation of bacterial colonies from the bead material based on morphological aspects (round shaped colonies, Fig. 2) in agreement with the electron micrograph of Kim et al. (2004). This platform allowed acquisition of the spectral signature of each pixel in the image and targeting of specific fluorescent signals. Preliminary work on unstained and uncolonized beads revealed that, under the excitation/emission conditions described in this paper, the bead material exhibited endogenous fluorescence with a single peak in the green range (maximum at 526 nm, Figs. 1 and 2A) with no contribution in the red or blue ranges, in absence of hybridization. This precluded the choice of red fluorescent labels (i.e. Texas Red®-

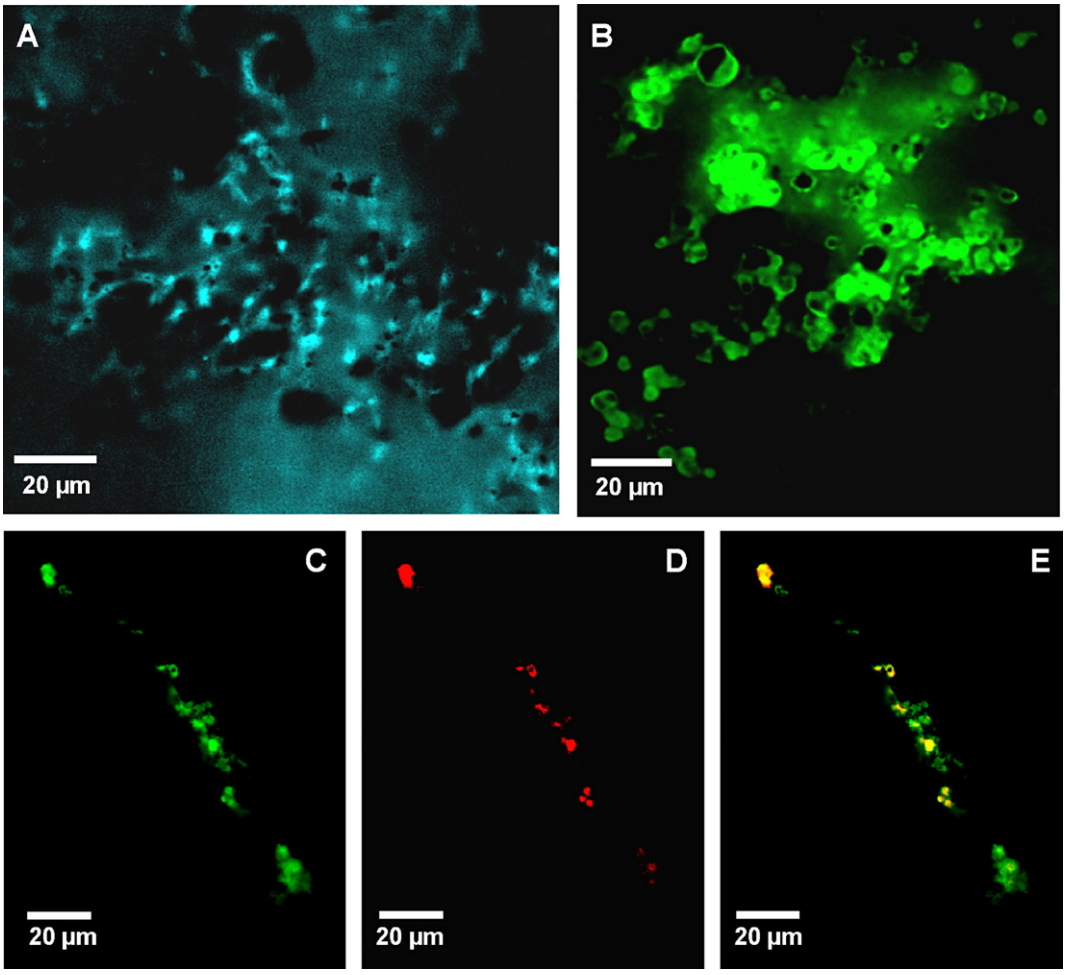

Fig. 2. Images of 30 µm cryosection of a Biosep® bead, at 600× magnification. False-painting was performed to improve contrast between the different images. A. Uncolonized and unstained bead treated with 70% isopropyl alcohol exhibiting green autofluorescence, false-painted in blue (maximum emission: 526 nm); (B–E). Beads FISH-stained with EUB338 labeled with Texas Red (max emission: 615 nm) targeting all eubacteria (with exceptions defined by Bouvier and del Giorgio, 2003; Daims et al., 1999) and SRB385 labeled with Alexa® 647 (max emission: 678 nm) targeting δ-Proteobacteria; B. Sample 035CH8, image corresponding to Texas Red®-X signal, i.e. all eubacteria, false-painted in green, showing extensive colonization in clusters; (C–E). Sample 054CH10, images in the same field of view; C. Image corresponding to Texas Red®-X signal, i.e. all eubacteria, false-painted in green; D. Image corresponding to Alexa® 647 signal, i.e. δ-Proteobacteria, false-painted in red; E. Composite image of C and D showing δ-Proteobacteria in orange and other eubacteria in red. This image corresponds to a ratio of δ-Proteobacteria to all eubacteria of 56.2% for this field of view.

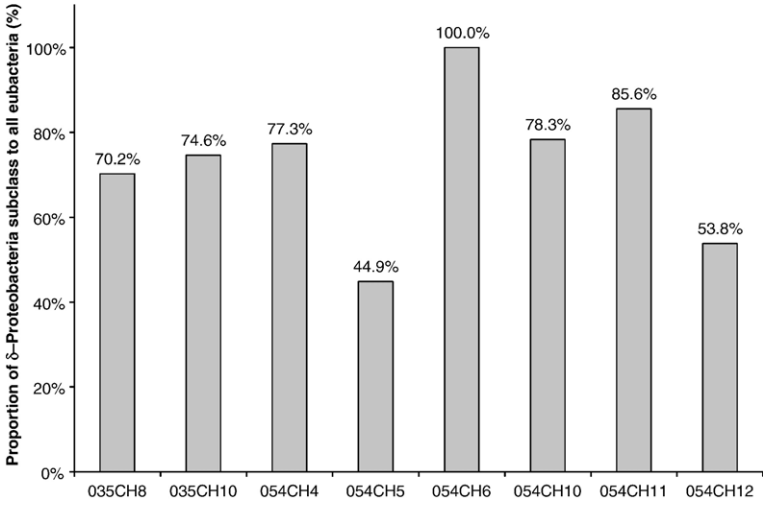

Fig. 3. Median values of proportions of δ-Proteobacteria subclass to all eubacteria (with exceptions defined by Bouvier and del Giorgio, 2003; Daims et al., 1999) per sample.

X and Alexa® 647, Invitrogen, Carlsbad, CA) for the probes used in this study, to avoid interference between probe and autofluorescence signals (Fig. 1). An ASI triple bandpass filter was used to capture fields of view for a given stained slide. This filter produced excitation light in the ranges 455–480 nm, 555–575 nm and 625–655 nm and collected emitted light in the ranges 500–550 nm, 580–620 nm and 660–740 nm. It permitted simultaneous acquisition and separation of the fluorescent signals of the bead material, Texas Red®-X labeled EUB338 probe, and Alexa® 647 labeled SRB385 probe (Table 2). A scan of the entire bead slice was performed and twenty fields of view representing the densest colonized areas on the slice were imaged for a given sample. These areas were chosen visually at 600× total magnification by morphological identification of the bacterial colonies (Fig. 2B, C and D), to avoid the bias of selecting either type of bacteria (δ-Proteobacteria or not). Upon image acquisition, each picture was spectrally analyzed. The fluorescent signals of the two fluorescently-labeled probes were retained in distinct channels while the autofluorescence signal of the bead material was eliminated from the image. Each remaining channel was treated using the ASI SpectraView software to retain only fluorescent signals corresponding to morphologically distinct bacterial colonies. Since δ-Proteobacteria were dually stained by both probes (Table 2), while other eubacteria were only stained by EUB338, δ-Proteobacteria colonies exhibited colocalized signals in both channels, while other eubacteria did not (orange yellow colonies in Fig. 2D). Each spectrally treated channel was saved as a separate image file. Each of these images was treated using the Image-Pro® Plus image analysis software (Media Cybernetics Inc., Silver Spring, MD) to calculate by thresholding the surface area in μm^2 occupied by each fluorescent probe label in each channel for each field of view. The ratio of the surface area corresponding to Alexa® 647 labeled SRB385 probe to the surface area corresponding to Texas Red®-X labeled EUB338 probe was calculated, revealing the proportion of δ-Proteobacteria to all eubacteria in a given field of view (Fig. 3). Statistical analysis was carried out on this dataset using SPSS statistical analysis software (SPSS Inc., Chicago, IL). For each sample (i.e. 20 fields of view) the population of proportions was tested for normality and outliers using the Shapiro–Wilk test and Q–Q normality plots. Since 50% of the dataset did not come from a normal distribution ($p<0.05$), it was decided to use medians and percentiles (25% and 75%) as comparison tools rather than means and standard deviations (Table 3). Finally a Kruskal–Wallis Analysis of Ranks test revealed that the median values were statistically different from each other ($p<0.05$). This newly designed technique yielded proportions of δ-Proteobacteria above 40% for all samples and above 70% for six samples out of eight, showing a significant colonization of δ-Proteobacteria in the Biosep® beads incubated at these sites (Fig. 3). It can be also noted that all control samples that were provided exhibited undetectable bacterial colonization. Our results demonstrated that this novel technique offers several advantages over culture-dependent or PCR-based techniques for detecting Biosep®-immobilized bacteria: i) the ability for in situ staining of bacteria, preventing disruption of the architecture of bacterial communities within the beads or loss of bacterial inventories through physical extraction; ii) simultaneous

Table 3
Descriptive statistics (median, 25th and 75th percentiles) for proportions of δ-Proteobacteria subclass to all eubacteria[a] in Biosep® bead samples

Sample	Percentiles		
	25th	50th — Median	75th
035CH8	0.270	0.702	0.869
035CH10	0.650	0.746	0.897
054CH4	0.668	0.773	0.860
054CH5	0.197	0.449	1.000
054CH6	0.443	1.000	1.000
054CH10	0.559	0.783	1.000
054CH11	0.784	0.856	1.000
054CH12	0.401	0.538	0.745

[a] With exceptions defined by Bouvier and del Giorgio (2003) and Daims et al. (1999).

identification of different types of stained bacteria due to the ability of producing thin frozen bead sections and spectral detection of fluorescent labels; iii) much more rapid and specific than conventional bacterial identification assays (which involve time-consuming extraction, purification and incubation steps), yielding shorter processing time per sample and faster acquisition of additional relevant data. This method will be further refined to simultaneously identify several groups or species of Biosep®-immobilized bacteria involved in the biodegradation of MTBE and benzene in contaminated matrices.

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