Molecular Characterization of Microbial Communities in a JP-4 Fuel Contaminated Soil

David C. White, [1,42] John R. Stephen,' Yun-Juan Chang,[1] Ying Dong Gan[1] Aaron Peacock,' Michael J. Barcelona,[2] Sarah J. Macnaughton'

[1]UT/CEB, 10515 Research Dr., Suite 300, Knoxville, TN 37932
[2]National Center for Integrated Bioremediation Research and Development, Dept. of Civil and Environmental Engineering, University of Michigan, MI 48 109

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MOLECULAR CHARACTERIZATION OF MICROBIAL COMMUNITIES IN A J-P-4 FUEL CONTAMINATED SOIL

David C. White[†2], John R. Stephen[†], Yun-Juan Chang[†], Ying Dong Gan[†], Aaron Peacock[†], Susan M. Pfiffner[2], Michael J. Barcelona[3], Sarah J. Macnaughton[†]

[†] Center for Environmental Biotechnology, The University of Tennessee, Knoxville, TN 37932
[2]Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831[1]
[3]National Center for Integrated Bioremediation Research and Development, Dept. of Civil and Environmental Engineering, University of Michigan, MI 48 109.

ABSTRACT: In this study, lipid biomarker characterization of the bacterial and eukaryotic communities was combined with PCR-DGGE analysis of the eubacterial community to evaluate correlation between JP-4 fuel concentration and community structure shifts. Vadose, capillary fringe and saturated- soils were taken from cores within, up- and down-gradient of the contaminant plume. Significant differences in biomass and proportion of Gram negative bacteria were found inside and outside the plume. Sequence analysis of DGGE bands from within the spill site suggested dominance by a limited number of phylogenetically diverse bacteria. Used in tandem with pollutant quantification,' these molecular techniques should facilitate significant improvements over current assessment procedures for determination of remediation end points.

INTRODUCTION

Shifts in microbial community structure provide a sensitive target for assay of the progress of bioremediation. The dominant organisms of contaminated sites are likely to be active in remediation of the contaminant. By combining PLFA analysis with PCR-DGGE analysis of the bacterial community we document herein shifts in a field population structure resulting from contamination with JP-4 fuel.

METHODS AND MATERIALS

Field site and sampling. The contaminated area was located at the KC-135 crash site at Wurtsmith Airforce Base (WAFB), Oscoda, Michigan. Soil samples were obtained using a Geoprobe piston corer. Cores were taken from 3 bore-holes, up-gradient, within, and down-gradient of the initial crash site. Each core was sectioned into vadose (-2.13 m), capillary fringe (-2.43 m) and saturated zones (-2.74 m). Samples were split for, a) lipid biomarker analysis (75 g cone line sections), b) DNA extraction and subsequent PCR (-1.5 g; in sterile whirlpacks), and c) volatile organic compound analysis (VOCs; 7.0 g; EPA vials). Samples for VOC analysis were preserved with 5 mL 40 % $NaHSO_4$ aqueous solution. Lipid and DNA samples were preserved on dry ice and shipped overnight to the University of Tennessee, Knoxville.

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Volatile Organic Compounds. Samples were analyzed for VOCs on a HP-5890 series II Gas Chromatograph (GC) with an HP 5972 mass selective (MS) detector as described by Fang *et al.*, (1997). Separation was accomplished using an HP-624 GC column: 60 m x 0.25 mm i.d. (film thickness d_f = 1.8 μm; Hewlett-Packard). For all 43 compounds detected, calibration curves were linear between 1.0 μg/L to 200 μg/L. Compounds were identified based on relative retention time and verified by mass spectra. Concentrations of VOCs were calculated using the internal standard method, and are reported as μg kg^{-1} (Fang *et al.*, 1997).

Lipid analysis. Duplicate sub-samples of each zone from each soil core (six sub-samples per core) were extracted using the modified Bligh/Dyer as described previously by White *et al.*, (1979). The total lipids obtained were then fractionated into glyco-, neutral- and polar-lipids (Guckert *et al.*, 1985) with polar lipid then subjected to a sequential saponification/acid hydrolysis/esterification (Mayberry and Lane, 1993). The PLFA and dimethyl acetals @MA) methyl esters were recovered. The PLFA and DMAs were separated, quantified and identified by gas chromatography-mass spectrometry (GC-MS; Ringelberg *et al.*, 1994). Fatty acids were identified by relative retention times, comparison with authentic standards (Matreya Inc., Pleasant Gap, Pa) and by the mass spectra (collected at an electron energy of 70 mV) Ringelberg *et al.*, (1989). Fatty acid is as described by Kates (1986).

The glycolipid fraction was subjected to ethanolysis and the β-hydroxy acids from the PHA extracted and analysed by GC-MS (Findlay and White, 1983). The GC was programmed from an initial temperature of 60°C to 280°C at 10°C per min, and then held at this temperature for 3 min. The injector and housing temperatures were maintained at 270°C and 290°C respectively. Mass spectra were collected as described above. PHA was identified by relative retention time and mass spectra (Findlay and White, 1983).

DNA analysis. Nucleic acid was extracted directly from triplicate 0.5 g sub-samples from each zone from each soil core (9 sub-samples per core) using the method described in Stephen *et al.* (1999). PCR amplification and DGGE were carried out as described in Muyzer *et al.*, (1993) using a D-Code 16/16 cm gel system

Sequence analysis. PCR products from excised bands and cloned products were sequenced using the primer 5 16r (Lane *et al.*, 1985) and an ABI-Prism model 373 automatic sequencer with dye terminators. Sequences were compared to the GenBank database by use of the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov). Sequences were classified using the RDP release of 3 1 -July- 1998 (Maidak *et al.*, 1997).

Statistical analysis Analysis of variance (ANOVA) was used to determine whether there were significant differences between the lipid biomarker data obtained from the crash site (n=3) and that obtained from up- and down-gradient of the site (n=6). ANOVA was also used to determine significant difference between lipid biomarker data obtained from each zone (n=3 for each zone). ANOVA was performed on the means of the duplicate sub-samples using Statistica Version 5.1 for Windows software.

RESULTS AND DISCUSSION

Geochemical and volatile organic compound analyses. Total VOCs from within and up-gradient of the crash site were detected in the saturated zone at 732, and 46 $\mu g\ kg^{-1}$, respectively. The VOCs were below detection limits in all vadose and capillary fringe zones and at all levels down-gradient of the site. These findings were contrary to those of Fang *et al.* (1997) in which the trace amounts of VOCs were detected down-gradient rather than up-gradient of the crash site.

Biomass content. Biomass contents varied considerably between bore-holes. The crash site biomass content was significantly higher than that of samples taken from either up- or down-gradient of the site ($P<0.05$). Bacterial cell numbers were calculated based from PLFA recovery data (Balkwill *et al.*, 1988). It is important to remember that with any conversion factor, the number of cells can vary by up to an order of magnitude (Findlay and Dobbs, 1993). Bacterial cell numbers for these samples per gram wet weight ranged from $5.2 \pm 0.2 \times 10^5$ in the saturated zone from the up-gradient sample, to $3.9 \pm 0.3 \times 10^7$ in the sample taken from the capillary fringe of the crash site sample.

Community structure. The microbial community structures of the samples differed dependent on both bore-hole location and depth (Figure 1). The microbial communities from the crash site samples contained significantly more monoenoic PLFA ($P<0.05$) indicative of Cram negative bacteria (Wilkinson, 1988), than did samples from up- and down-gradient of the site. Although containing significantly less biomass than samples taken from within the crash site($P<0.05$), samples from up-gradient and down-gradient contained significantly higher relative proportions of PLFA ($P<0.05$) indicative of sulfate-reducing bacteria (10me16:0, i17:1ω7c, Dowling *et al.*, 1986 and Edlund *et al.*, 1985, respectively). At all sites, the relative proportions of the biomarkers indicative of sulfate-reducing bacteria were significantly higher ($P<0.05$) in the capillary fringe and saturated zones. In all samples, the relative proportions of terminally-branched saturated PLFA, such as i15:0, i17:0 and cy17:0, indicative of anaerobic Cram negative bacteria (Wilkinson, 1988), increased with zone depth ($P<0.05$). Conversely, relative proportions of biomarkers typical of eukaryote PLFA (e.g. 18:2ω6 and 18:3) decreased with depth.

A hierarchical cluster analysis (HCA) of the bacterial PLFA profiles (arc sine transformed mol % data) showed the relatedness between samples (Figure 1). The bacterial PLFA comprised the total PLFA minus the polyenoic and normal saturate PLFA above 18 carbons in chain length, both of which are generally associated with eukaryote biomass. From the HCA it was apparent that the bacterial populations from within the crash site were dissimilar from one another and the up- and down-gradient samples, while the PLFA profiles from up- and down-gradient showed a higher level of relatedness, with the two vadose zones clustering together. The crash site vadose zone contained the most unique community, mainly due to the, higher relative proportion of 2me13:0. A principle components analysis of the same data gave similar results, with two principal components derived, accounting for, sequentially, 76 and 15% of the variance inherent in the data set. Principle component 1 was most strongly influenced by 2me13:0, cy17 and cy19 and accounted for the diffise grouping of the crash-site samples. Of these PLFA, cy17:0 and cy19:0 are common components of Gram negative bacteria. Principle component 2 was most strongly influenced by 10me16:0, representative of the sulfate-reducing bacteria *Desulfobacter* (Dowling *et al.*, 1986), 16:0, 18:1w9c and a15:0. Representative of the presence of anaerobic bacteria, DMAs were present in significantly greater quantities in the samples taken from the crash site. In these samples the relative DMA concentrations

1 **(DMA/PLFA)** followed the order, capillary **fringe> saturated>vadose** zone. The relative DMA
2 concentration followed no discernable order in the remaining bore-holes.
3
4 **DGGE analysis of microbial diversity..DGGE** analysis of triplicate sub-samples taken within
5 the heavily JP-4 impacted zone showed strong and reproducible banding and stratification at all
6 depths (Figure 2). Two sequences were recovered from all three depths and represented unknown
7 organisms **from** the P-subgroup proteobacteria. Four bands were absent **from** the saturated zone,
8 representing an a-and 3 **β-proteobacterial** sequences. The capillary fringe displayed 3 unique
9 bands, representing an uncultured bacterium associated with the **Flexibacter-Cytophaga-**
10 Bacteroides phylum, an α and a **β-proteobacterium.** A member of the Cytophaga-subgroup was
11 found in both capillary and saturated soil. Five sequences were recovered **from** only the saturated
12 zone, and represented members of the a, p-and e-subgroup proteobacteria, **Flexibacter-**
13 Cytophaga-Bacteroides- phylum and the Cram-positive phylum. None of these bands were
14 visible outside the plume, suggesting that these organisms were active in remediation of the JP-4.
15
16 **CONCLUSION**
17 **Shifts** in biomass content and community structure throughout the JP-4 contaminated soil
18 samples were detected related to the increased VOC concentration. Measured as **PLFA,** the
19 highest viable biomass levels were detected in the most highly contaminated site.
20
21 **ACKNOWLEDGEMENTS**
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23 Development Program (SERDP contract number 1 **1XSY887Y).**
24
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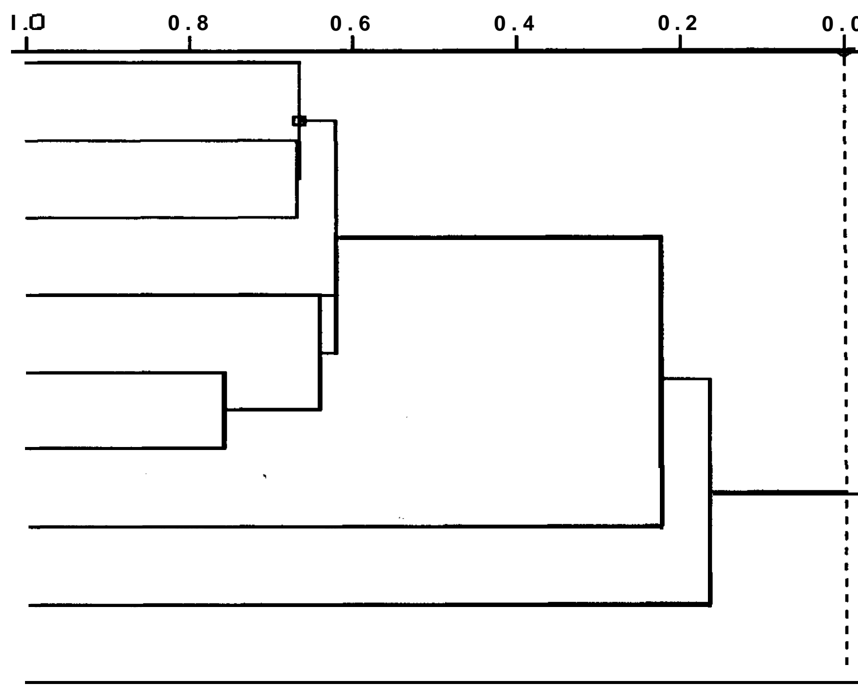
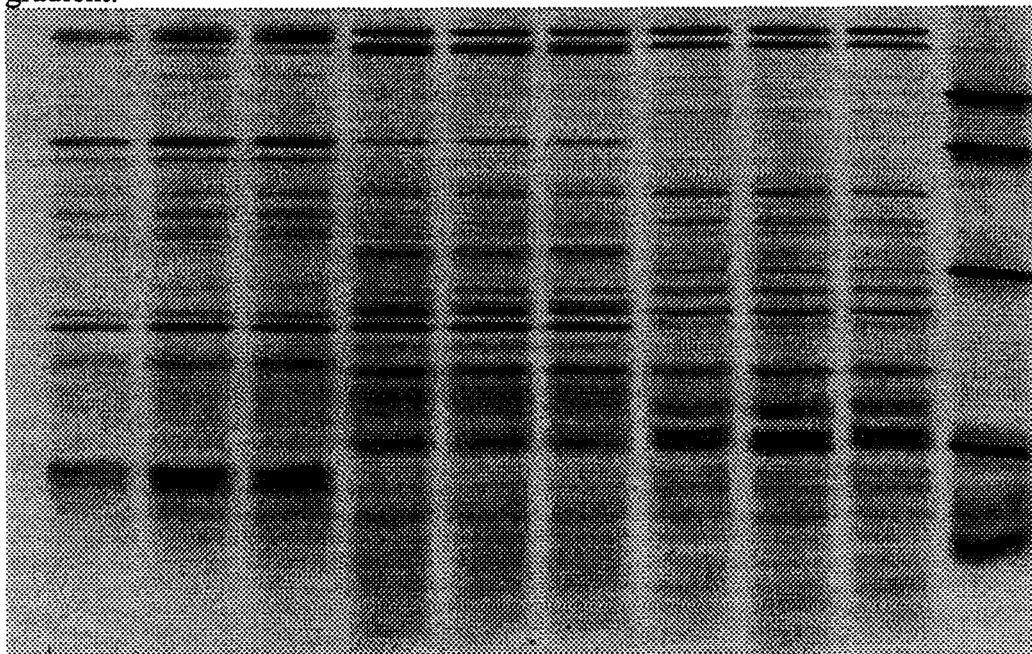


2
3 Figure 1: A dendogram representation of a hierarchical cluster analysis (single linkage based on
4 euclidean distance) for the bacterial PLFA profiles. DG, down-gradient; CS, crash site; UG, up-
5 gradient.



6
7 Figure 2: DGGE-eubacterial community profile of soil from within the crash-site. The portion of
8 the gel shown represents the range 30-52 % denaturant, in which all visible bands were found.
9 Lanes 1-3: saturated zone. Lanes 4-6: capillary fringe. Lanes 7-9: vadose zone. Lane 10:
10 Migration standards.

Vadose

CF

Sat