Environmental Microbiology (1999) 1(3), 231–241

Microbial characterization of a JP-4 fuel-contaminated site using a combined lipid biomarker/polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE)-based approach

John R. Stephen,[1] Yun-Juan Chang,[1]
Ying Dong Gan,[1] Aaron Peacock,[1] Susan M. Pfiffner,[1]
Michael J. Barcelona,[2] David C. White[1,3] and
Sarah J. Macnaughton[1]*

[1]*Center for Environmental Biotechnology, The University of Tennessee, 10515 Research Drive, Suite 300, Knoxville, TN 37932, USA.*
[2]*National Center for Integrated Bioremediation Research and Development, Department of Civil and Environmental Engineering, University of Michigan, MI 48109, USA.*
[3]*Environmental Sciences Division, Oak Ridge National Laboratory, Oak Ridge, TN 37831, USA.*

Summary

The impact of pollution on soil microbial communities and subsequent bioremediation can be measured quantitatively *in situ* using direct, non-culture-dependent techniques. Such techniques have advantages over culture-based methods, which often account for less than 1% of the extant microbial community. In 1988, a JP-4 fuel spill contaminated the glacio-fluvial aquifer at Wurtsmith Air Force Base, Michigan, USA. In this study, lipid biomarker characterization of the bacterial and eukaryotic communities was combined with polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) analysis of the eubacterial community to evaluate correlation between contaminant (JP-4 fuel) concentration and community structure shifts. Vadose, capillary fringe and saturated zone samples were taken from cores within and up- and down-gradient from the contaminant plume. Lipid biomarker analysis indicated that samples from within the plume contained increased biomass, with large proportions of typically Gram-negative bacteria. Outside the plume, lipid profiles indicated low-biomass microbial communities compared with those within the initial spill site. 16S rDNA sequences derived from DGGE profiles from within the initial spill site suggested dominance of the eubacterial community by a limited number of phylogenetically diverse organisms. Used in tandem with pollutant quantification, these molecular techniques should facilitate significant improvements over current assessment procedures for the determination of remediation end-points.

Received 4 September, 1998; revised 8 January, 1999; accepted 1 February, 1999. *For correspondence. E-mail sjmac@utk.edu; Tel. (+1) 423 974 8005; Fax (+1) 423 974 8027.

© 1999 Blackwell Science Ltd

Introduction

Accidental spillage of JP-4 jet fuel has been a significant source of hydrocarbon contamination in soils and groundwaters (Fang *et al.*, 1997). Microorganisms have been shown to use many of the components of such fuels, including the 'BTEX' compounds (benzene, toluene, ethylbenzene and xylenes), as electron donors, thereby facilitating contaminant remediation (Weidemeier *et al.*, 1995). One of the major concerns in the field of environmental remediation is in the establishment of protocols for the determination of defensible end-points. Current practices often monitor the disappearance of pollutants and their by-products to regulatory levels or use single-species toxicity tests (Rand and Petrocelli, 1985). However, shifts in the microbial community can also provide a measure of bioremediation effectiveness (Pfiffner *et al.*, 1997; White *et al.*, 1998). Indeed, the microbial community response may prove to be a more comprehensive assessment indicator of residual toxicity, because it may provide a more sensitive measure of biologically available contaminants than current toxicity tests. In this role, microbial community analysis could serve as a useful complement to current methods for measuring the disappearance or sequestration of hazardous contaminants.

Traditionally, methods used to monitor microorganisms require *ex situ* culture analysis. However, it has been documented repeatedly that such culture techniques account for only between 0.1% and 10% of the community numerically detectable by direct counting (Skinner *et al.*, 1952; White, 1983; Bakken, 1985; Tunlid and White, 1992; White *et al.*, 1993). Equally, current culture-based methods have now been shown to overlook many of the *in situ* numerically dominant species completely (e.g. Amann *et al.*, 1995, and references therein). Signature lipid biomarkers (SLBs) can be used to determine shifts

in microbial biomass, nutritional or physiological stress responses and community diversity *in situ*. Total cellular phospholipid fatty acids (PLFAs) are not stored and are turned over rapidly. They can therefore be used as indicators of viable biomass (Vestal and White, 1989). Increases in the ratios of *trans/cis* monoenoic PLFAs in cells are indicative of the effects of toxic/sublethal stress on bacterial communities and of the growth phase of cells respectively (Guckert *et al.*, 1986; Heipieper *et al.*, 1992; White *et al.*, 1996a,b). Also, bacteria make poly-β-hydroxyalkanoic acid (PHA) as endogenous storage lipids (Findlay and White, 1983; Doi, 1990), and the relative proportion of this lipid to PLFAs can provide a measure of cellular nutritional status. Specific PLFA biomarkers can be used as indicators of microbial community composition, e.g. eubacteria, fungi, algae, Gram-negative and Gram-positive organisms, sphingomonads, actinomycetes and sulphate-reducing bacteria (Vestal and White, 1989; White *et al.*, 1996a,b). Plasmalogen-derived dimethylacetals (DMAs) are formed after mild acid methanolysis, and an increased ratio of DMA to PLFA indicates increased proportions of facultative and obligate anaerobic metabolism (White *et al.*, 1996b). Despite its versatility, PLFA analysis does have limitations for the analysis of the Gram-negative bacteria community structure. Generally, the PLFA profiles of Gram-negative bacteria are dominated by monoenoic, saturated and cyclopropane fatty acids (Wilkinson, 1988; Zelles, 1997), the vast majority of which are broadly distributed and, as such, uninformative in subdividing the Gram-negative community structure.

To overcome this, we have used a complementary nucleic acid-based analysis. The polymerase chain reaction–denaturing gradient gel electrophoresis (PCR–DGGE) approach used employed primers that recognize the 16S rDNA targets of all known and inferred (from 16S rDNA clone libraries) eubacterial species (Muyzer *et al.*, 1993). PCR products of approximately equal length are separated on the basis of their melting behaviour in an acrylamide gel matrix. The banding patterns and relative intensities of the recovered bands can provide a measure of changes in the eubacterial community between sampling sites and over time. The major limitation of this technique lies in low sensitivity; it is likely that any given 16S rDNA sequence type must comprise at least 1% of the total target organisms in a sample to remain above the background level of minor bacterial amplification products (Muyzer *et al.*, 1993; Heuer and Smalla, 1997; Stephen *et al.*, 1998). Excision and sequence analysis of individual bands can be used to provide fine-scale biomarkers and loosely infer the identity of the source organisms using database searches and phylogenetic methods.

These two technologies applied in tandem should permit broad and specific observations of whole-community succession and subpopulation dynamics in contaminated soils, as well as enabling the partial characterization of bioremediative communities *in situ*. The aim of this study was to provide a detailed microbial community structure characterization using lipid/PCR–DGGE-based analysis of a hydrocarbon-contaminated site. We describe a multifaceted microbiological analysis using SLB and PCR–DGGE of total eubacterial 16S rDNA to measure microbial biomass, community diversity and nutritional/physiological status (community stress indicators) at a highly contaminated and at uncontaminated locations in a JP-4 spill site for which the organic contaminants have been comprehensively documented previously (Fang *et al.*, 1997).

Results

Geochemical and volatile organic compound (VOC) 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 downgradient of the site. These findings were contrary to those of Fang *et al.* (1997), who detected trace amounts of VOCs down-gradient rather than up-gradient of the crash site. The major compounds detected within the crash site included the *m* and *p* xylenes (203 $\mu g\,kg^{-1}$), 1,2,4-trimethylbenzine (91 $\mu g\,kg^{-1}$), various methylbenzines (24–44 $\mu g\,kg^{-1}$), naphthalene (22 $\mu g\,kg^{-1}$) and methylnaphthalene (20 $\mu g\,kg^{-1}$).

Biomass content

Biomass contents varied considerably between boreholes (Table 1, Fig. 1). The biomass content of the samples from the crash site was significantly higher than that of samples taken from either up- or down-gradient of the site ($P<0.05$), as determined using analysis of variance (ANOVA). Bacterial cell numbers were calculated based on 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 g 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 depending on both borehole location and depth (Fig. 2). Figure 2 shows different structural groups of PLFA plotted as a percentage of total PLFA. The microbial

Table 1. Total biomarker (PLFA, PHA and DMA) and culturable anaerobe/sulphate-reducing bacteria contents of the soil boreholes.

Site	UG-V	UG-CF	UG-S	CS-V	CS-CF	CS-S	[a]DG-V	DG-CF	DG-S
PLFA ($pmol\,g^{-1}$)	187 (32)	29 (8.2)	27 (0.8)	1114 (13)	1990 (163)	410 (92)	736	210 (130)	71 (2.7)
Bacterial PLFA ($pmol\,g^{-1}$)	141 (25)	27 (7.4)	26.2 (0.8)	1079 (12)	1934 (158)	403 (91)	368	183 (114)	53 (5.2)
[b]Bacterial cell number ($\times 10^6$)	2.8 (0.5)	0.54 (0.14)	0.52 (0.02)	22 (0.002)	39 (3.2)	8.1 (1.8)	7.4	3.6 (2.2)	1.1 (1)
[c]ω7t/ω7c	0.09	0.2	0.11	0.22	0.24	0.38	0.06	0.17	0.09
[d]Cy/ω7c	0.6	1.78	1.28	4.07	3.99	1.25	0.5	1.47	1.48
PHA/PLFA	ND	ND	ND	0.0025 (0.0007)	0.015 (0.0012)	0.024 (0.009)	ND	ND	ND
DMA ($pmol\,g^{-1}$)	1.13 (0.5)	0.33 (0.04)	0.22 (0.01)	159 (25)	176 (54)	28 (14)	2.15	2.58(0.3)	ND
[e]DMA/PLFA	NC	NC	NC	0.143	0.088	0.068	NC	NC	NC

UG, upgradient; CS, crash site, DG, downgradient; V, vadose, CF, capillary fringe, S, saturated. ND, not detected; NA, not analysed. Standard deviations are in brackets ($n = 2$).
a. No standard deviations.
b. Cell equivalent number (Balkwill *et al.*, 1988).
c. Represents Gram-negative metabolic stress (16:1ω7t/16:1ωc + 18:1ω7t/18:1ω7c); higher number indicates higher stress.
d. Represents Gram-negative growth phase/turnover rate (cy17:0/16:1ω7c + cy19:0/18:1ω7c); higher number indicates slower turnover rate.
e. DMA/PLFA was only determined where $>5\,pmol\,g^{-1}$ DMA was detected.

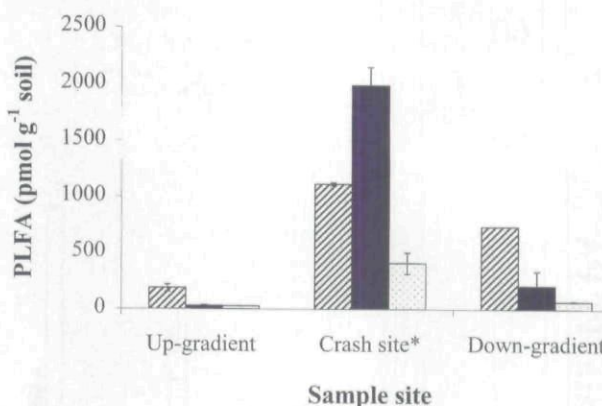

Fig. 1. Total biomass measured as PLFA g^{-1} dry weight soil. (▨) represents the vadose zone; (■) represents the capillary fringe; and (▤) represents the saturated zone. $n = 2$, error bars represent standard deviation. Downgradient vadose represents a single data point. Crash site samples (*) contained significantly more total biomass than did samples from up- and downgradient.

communities from the crash site samples contained significantly more monoenoic PLFA ($P < 0.05$), indicative of Gram-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 sulphate-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 sulphate-reducing bacteria were significantly higher ($P < 0.05$) in the capillary fringe and saturated zones (Fig. 2). In all samples, the relative proportions of terminally branched saturated PLFAs, such as i15:0, i17:0 and cy17:0, which were taken in this case to be indicative of anaerobic Gram-negative bacteria (Wilkinson, 1988), increased with zone depth ($P < 0.05$). Conversely, relative proportions of biomarkers typical of eukaryote PLFAs (e.g. 18:2ω6 and 18:3) decreased with depth.

A hierarchical cluster analysis (HCA) of the bacterial PLFA profiles (arcsine-transformed mol percentage data) showed the relatedness between samples, as indicated in Fig. 3. The bacterial PLFAs comprised the total PLFAs minus the polyenoic and normal saturated PLFAs above 18 carbons in chain length, both of which are generally associated with eukaryote biomass (White *et al.*, 1996b), although polyenoic fatty acids have been detected in large amounts in some *Shewanella* spp. (Nichols *et al.*, 1997; Watanabe *et al.*, 1997). From the HCA, it was apparent that the bacterial populations from within the crash site were dissimilar to one another and from the up- and down-gradient samples, whereas the PLFA profiles from up- and down-gradient showed a higher

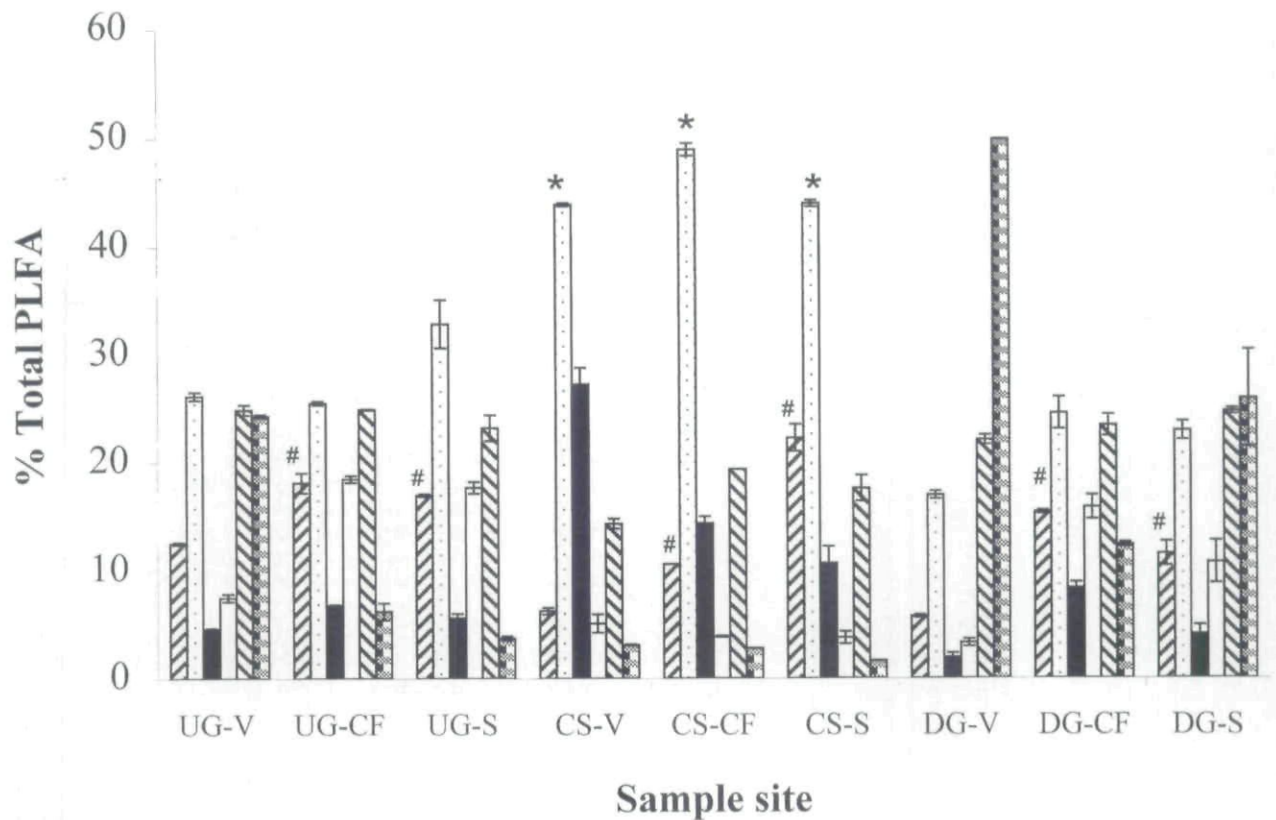

Fig. 2. A comparison of the relative percentages of total PLFA functional groups from boreholes located upgradient, within the crash site and downgradient of the contaminant plume. Functional groups are defined according to PLFA molecular structure, which is related to fatty acid biosynthesis. Terminally branched saturates (▨) are attributed to Gram-positive and, in this case, anaerobic Gram-negative bacteria; monoenoic unsaturates (▦) are attributed to Gram-negative bacteria; branched monoenoic unsaturates (■) and mid-chain branched saturates (□) are attributed to actinomycetes and sulphate-reducing bacteria; normal saturates (▧) are ubiquitous; and polyenoics (▩) are attributed to eukaryotes. UG, upgradient; CS, crash site; DG, downgradient; V, vadose zone, CF, capillary fringe; S, saturated zone. $n = 2$, error bars represent standard deviation, DG-V represents a single data point. Asterisks denote the significantly ($P < 0.05$) higher mole percentage of monoenoic PLFAs present in the crash site samples compared with those from the upgradient and downgradient sites. The # sign denotes the significantly higher mole percentage of terminally branched saturated PLFAs present in the capillary fringe and saturated zones compared with the vadose zones.

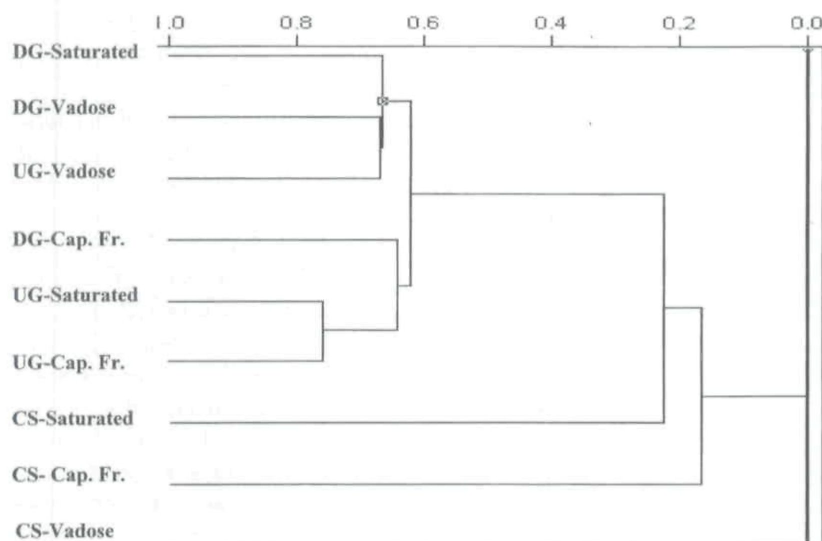

Fig. 3. A dendrogram representation of a hierarchical cluster analysis (single linkage based on Euclidean distance) for the bacterial PLFA profiles described in Fig. 1. DG, downgradient; CS, crash site; UG, upgradient.

level of relatedness, with the two vadose zones clustering together. The crash site vadose zone contained the most unique community, resulting in the main from the higher relative proportion of 2me13:0, a fatty acid of unknown origin that was also detected in the crash site capillary fringe and saturated zones. A principal component 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 (data not shown). Principal component 1 was most strongly influenced by 2me13:0, cy17 and cy19 and accounted for the diffuse grouping of the crash site samples. Of these PLFAs, cy17:0 and cy19:0 are common components of Gram-negative bacteria. Principal component 2 was most strongly influenced by 10me16:0, representative of the sulphate-reducing bacteria *Desulfobacter* (Dowling *et al.*, 1986), 16:0, 18:1ω9c and a15:0. Representative of the presence of anaerobic bacteria, DMAs were detected in all samples, but were present in significantly greater quantities in the samples taken from the crash site. In these samples, the relative DMA concentrations (DMA/PLFA) followed the order, capillary fringe > saturated > vadose zone. The relative DMA concentration followed no discernible order in the remaining boreholes.

Physiological status

The lipid composition of microorganisms is a product of metabolic pathways and thus reflects the phenotypical response of the organism to the environment encountered in a specific sample (White *et al.*, 1996b). Poly-β-hydroxyalkanoic acid was detected in sediment from the crash site core (the ratio of PHA/PLFA following the decreasing order: saturated > capillary fringe > vadose; Table 1), indicative of unbalanced growth on excess carbon. The physiological status of Gram-negative communities can be assessed from the ratios of specific PLFAs. Gram-negative bacteria make *trans* fatty acids to modify their cell membranes as protection against environmental stresses (Guckert *et al.*, 1986; Keift *et al.*, 1994; Sikkema *et al.*, 1995), with the higher ratio of *trans/cis* fatty acids indicating greater levels of environmental stress. Ratios of 0.05 or less are representative of healthy non-stressed communities (White *et al.*, 1996b). The *trans* to *cis* ratio for 16:1ω7 and 18:1ω7 was higher within the crash site samples than in the samples taken from up-gradient and down-gradient of the site, where VOCs were either undetectable or present only in trace amounts (Table 1).

DGGE analysis of microbial diversity

DGGE analysis of triplicate subsamples from the three depths at each of the three sampling sites demonstrated that the low-biomass up-gradient and down-gradient samples carried complex communities in which few discernible major bands were observed (data not shown). However, analysis of triplicate subsamples taken within the heavily JP-4-impacted zone showed strong and reproducible banding and stratification at all depths (Fig. 4A). The banding pattern is schematized in Fig. 4B to simplify labelling. Two sequences (A and G) were recovered from all three depths and represented unknown organisms from the β-subgroup of the class proteobacteria. Bands C, H, M and N were absent from the saturated zone; sequence H showed affinity to the α-subgroup of proteobacteria, whereas the others were classified as β-proteobacteria. Sequences E, I and K were unique to the capillary fringe and represented a novel bacterium associated with the *Flexibacter–Cytophaga–Bacteroides* phylum, a β-proteobacterium and an α-proteobacterium respectively. Sequence D was the only major sequence recovered from both the capillary and the saturated zones, representing a member of the *Cytophaga* subgroup. Sequences B, F, J, L and O were only recovered from the saturated zone and represented members of the ϵ-subgroup of proteobacteria, *Flexibacter–Cytophaga–Bacteroides* phylum, Gram-positive phylum, δ-subgroup of proteobacteria and the α-proteobacteria respectively. Less conservative placements of the derived sequences within the RDP scheme are given in Table 2. Bands E and F (both related to the *Saprospira* group) co-migrated, as did bands K and L (representing α- and β-subdivision proteobacteria respectively) under the DGGE conditions used, reflecting the difficulty in interpreting DGGE banding patterns without the use of a secondary analytical technique. Results from sequence analysis of clones were confirmed by hybridization of digoxygenin (DIG)-labelled DNA to a transfer membrane prepared from a duplicate gel (results not shown). The nucleotide sequences for A to O were deposited sequentially into GenBank as accession numbers AFO87798 to AFO87811 and AF107769.

Discussion

A microbial community response to the JP-4 fuel contamination at the KC-135 crash site at Wurtsmith Airforce Base was detectable from the results of the combined lipid biomarker/PCR–DGGE analysis. Within the contaminated site, the increased microbial biomass and the shift in the community lipid profiles towards domination by Gram-negative communities were related to the increased VOC concentration within the crash site compared with samples taken up- and down-gradient of the site (Table 1; Fang *et al.*, 1997). Moreover, the PLFA profiles of the samples taken up- and down-gradient of the contamination indicated greater similarity between microbial biomass at these sites than with those obtained from samples taken from the crash site (Fig. 3).

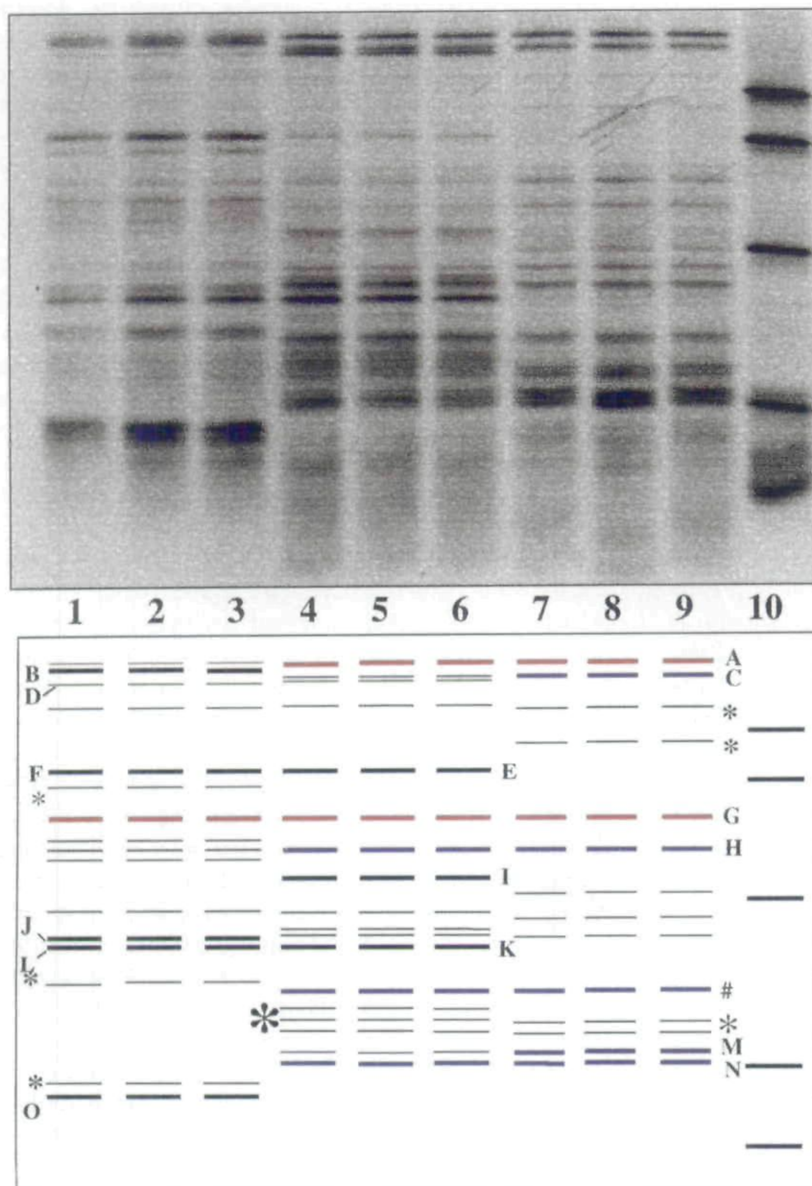

Fig. 4. A. DGGE eubacterial community profile of soil from within the crash site. The portion of the gel shown represents the range 30–52% denaturant, in which all visible bands were found. Lanes 1–3, saturated zone. Lanes 4–6, capillary fringe. Lanes 7–9, vadose zone. Lane 10, migration standards, from top: *Pseudomonas aeruginosa* FRD-1, *Shewanella putrifaciens* sp. 200, *Sphingomonas aromaticivorans* B0695, *Alcaligenes eutrophus* CH34 and (diffuse double band) *Desulfovibrio vulgaris* Hildenborough.
B. Schematic of (A). Colour scheme of labelled bands: red, sequences common to all three soil zones; blue, sequences recovered from both vadose zone and capillary fringe; green, sequences common to capillary fringe and saturated zone; black, sequences only recovered from a single zone. Note: co-migrating bands are not necessarily identical. Letters refer to sequence comparisons given in Table 2. Thin black lines refer to sequences that were not identified. The blue band marked # contained numerous sequence types (as defined by *Msp*I digestion patterns of clones), which were not sequenced. Migration standards are indicated by black lines on the right. Only the base of the *D. vulgaris* product is marked.

The previous study (Fang *et al.*, 1997), which characterized the organic contamination and the geochemical features at this site extensively, used PLFA analysis to a limited extent by analysing samples from one borehole (within the crash site) but to a greater depth than was done here. The results presented in the study by Fang *et al.* (1997) suggested a significant anaerobic response in the community adaptation to the presence of hydrocarbons. In our study, however, microbial community responses within the crash site were shown to occur in all three zones, all of which were principally centred on the Gram-negative population. Throughout all the capillary fringe and saturated zones, there were increases in the relative proportions of PLFA indicative of anaerobic bacteria as well as of DMAs. All these data provide further evidence of increasing anaerobic activity with depth. Relative proportions of biomarkers indicative of sulphate reducers (10me16:0, i17:1ω7c) tended to increase with depth, particularly in the cores taken from up- and down-gradient of the contamination. These biomarkers were recovered at very low abundance from the borehole within the most highly contaminated sampling site (Fig. 2, CS), which is in agreement with the fact that no 16S rDNA sequences clearly related to known sulphate-reducing lineages were recovered from this site. The level of each sulphate-reducing species was therefore likely to have been below the detection limit of PCR–DGGE (≈ 1% of the eubacterial population; Muyzer *et al.*, 1993; Stephen *et al.*, 1998). In all boreholes, the relative proportion of eukaryote-type PLFAs (18:2ω6, 18:3) decreased with

Table 2. Comparison of sequences derived from DGGE bands with databases.

Band	Closest match	Percentage identity	RDP affiliation	RDP group name
A	*Nevskia ramosa*	91	2.14.2	β-Subdivision proteobacteria
B	*Wollinella succinogenes*	86[a]	2.14. 5	ϵ-Subdivision proteobacteria
C	*Neisseria flavescens*	92[b]	2.14.2	β-Subdivision proteobacteria
D	*Bacteroides* spb-17BO	89	2.7.1.2	*Cytophaga* subgroup
E	*Flexibacter elegans*	87	2.7.2.3	*Saprospira* group
F	*Flexibacter ferrugineum*	89	2.7.2.3	*Saprospira* group
G	Unknown proteobacterium	92	2.14.2	β-Subdivision proteobacteria
H	*Rasbobacterium*	98	2.14.1.9	*Rhizobium–Agrobacterium* subgroup
I	*Thiobacillus thioparus*	95	2.14.2	β-Subdivision proteobacteria
J	*Clavibacter xyli*	98	2.16.1.7	*Arthobacter* group
K	*Hirschia baltica*	92	2.14.1	α-Subdivision proteobacteria
L	*Polyangium* sp.	88	2.14.4	δ-Subdivision proteobacteria
M	*Thiobacillus thioparus*	95	2.14.2	β-Subdivision proteobacteria
N	*Thiobacillus thioparus*	91	2.14.2	β-Subdivision proteobacteria
O	*Sphingomonas* sp. BAL 48	90	2.14.1	α-Subdivision proteobacteria

Closest matches were derived from searches of GenBank. RDP classification is based on 'similarity rank' analysis service provided by the RDP (Maidak *et al.*, 1997). Accession numbers are provided for unpublished sequences.
a. 90% to unknown ϵ-proteobacteria BD4-8.
b. 99% to clone sequence D84619.

increasing depth, indicating an increased sparseness of eukaryote biomass. Little or no polyenoic PLFAs above 19 carbons in chain length were detected in the core samples, a finding that has been found to be characteristic of such subsurface sediments (Smith *et al.*, 1986).

The increased *trans*/*cis* ratios detected in the contaminated samples indicated increased bacterial adaptation to metabolic stress. Although soil is a complex system, and care should be taken when interpreting changes in the *trans*/*cis* ratios (Frostegård *et al.*, 1996), such shifts have been detected previously in and correlated with organic solvent contamination of hydrocarbon-contaminated groundwater and sediments (Cox *et al.*, 1994; Pfiffner *et al.*, 1997), hydrocarbon-contaminated soil in laboratory-based microcosm studies (White *et al.*, 1998) and metal-polluted soils (Frostegård *et al.*, 1993; 1996), as well as in pure culture studies using solvent treatments (Pinkart *et al.*, 1996). For the contaminated soils analysed here, the *trans*/*cis* ratios in soil taken from the crash site were higher than those from samples taken from upgradient and downgradient of the site. Furthermore, the *trans*/*cis* ratios from the crash site followed the decreasing order, saturated zone > capillary fringe > vadose zone, an order that corresponded with that of the PHA:PLFA ratio (Table 1), indicative of unbalanced growth (Findlay and White, 1983; Doi, 1990). Increases in the PHA:PLFA ratio have been detected previously in soils contaminated with organic solvents (Cox *et al.*, 1994; White *et al.*, 1998). Taken with the shift in the *trans*/*cis* ratio, these data indicate an adaptation of the microbial communities at this site to growth in metabolically 'stressful' conditions with abundant available carbon.

The soils up- and down-gradient of the crash site were shown to contain similar PLFA profiles (Fig. 3) with low biomass, about one order of magnitude less than comparable samples taken from within the crash site. Similarly, PCR–DGGE analysis of samples from these sites generated complex profiles indicative of an even distribution of numerous bacterial species. None of the major bands from these sites co-migrated with strong bands from the crash site (data not shown). PCR–DGGE of samples from within the crash site generated strong amplification products, and the patterns were indicative of communities dominated by a limited number of species. Given the probable heterogeneity of the geochemistry within the contaminant plume, it is clear that a far more extensive site analysis is required before the contribution of the species detected to natural bioremediation can be fully evaluated.

In conclusion, shifts in biomass content and community structure throughout the JP-4-contaminated soil samples were detected, which seemed to be clearly related to the increased VOC concentration. Measured as PLFAs, the highest viable biomass levels, comprising bacteria that were actively responding to metabolically stressful conditions and growing on excess carbon, were detected in the most highly contaminated site. The PLFA and DGGE profiles of samples taken from outside the plume (both up- and down-gradient) indicated little or no ongoing bioremediation, with only trace or non-detectable VOC contamination. DGGE analysis of the contaminated site demonstrated that the vadose, capillary fringe and saturated zones were dominated by distinct populations of bacteria, few if any of which could be assigned to cultured genera. The sequences of these bands are of limited use in species identification in the absence of cultured organisms from the same site. However, they will be of value in the compilation of molecular biomarkers indicative of active bioremediation as more data become available

from this and similar sites. It is fair to speculate that these sequences represent at least some of the major eubacterial species active in bioremediation at the site of sampling.

Experimental procedures

Field site and sampling

The contaminated area was located at the KC-135 crash site at Wurtsmith Air Force Base (WAFB), Oscoda, MI, USA (Fig. 5). Sediment samples were obtained using a Geoprobe piston corer (Geoprobe Systems). Cores were taken from three boreholes, up-gradient, within and down-gradient of the initial crash site (Fig. 5). Each core was sectioned into vadose (≈ 2.13 m), capillary fringe (≈ 2.43 m) and saturated zones (≈ 2.74 m). Samples were split for (i) lipid biomarker analysis (75 g cone line sections); (ii) DNA extraction and subsequent PCR (≈ 1.5 g; in sterile whirlpacks); and (iii) 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.

Volatile organic compounds

Samples were analysed 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). Before analysis, samples were spiked with two internal standards (d10-xylene and d8-napthalene) and two spike compounds (1,2-difluorobenzene and d5-chlorobenzene) at 20 $\mu g\,l^{-1}$. Separation was accomplished using an HP-624 GC column: 60 m ×0.25 mm internal diameter (film thickness d.f. = 1.8 μm; Hewlett-Packard). For all 43 compounds detected, calibration curves were linear between 1.0 $mg\,l^{-1}$ and 200 $\mu g\,l^{-1}$. 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 $\mu g\,kg^{-1}$ (Fang *et al.*, 1997).

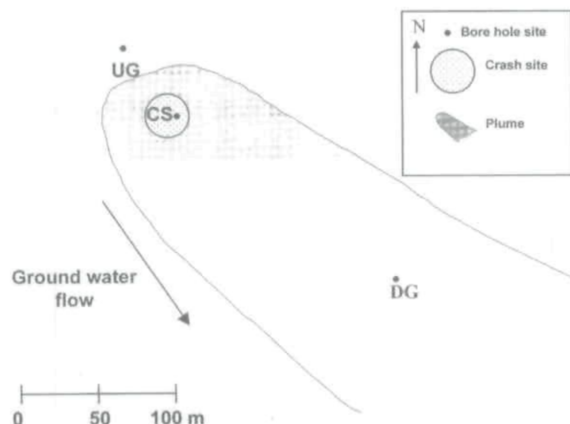

Fig. 5. Schematic map of the site at Wurtsmith AFB showing the area covered by the plume and the locations of boreholes and monitoring wells.

Lipid analysis

All solvents used were of GC grade and were obtained from Fisher Scientific. Duplicate subsamples of each zone from each soil core (six subsamples per core) were extracted using the modified Bligh/Dyer technique 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). The polar lipid was subjected to a sequential saponification/acid hydrolysis/esterification (Mayberry and Lane, 1993). The PLFA and dimethylacetal (DMA) 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) and by the mass spectra (collected at an electron energy of 70 mV; Ringelberg *et al.*, 1989). Fatty acid nomenclature is in the form of 'A:BωC', where 'A' designates the total number of carbons, 'B' the number of double bonds and 'C' the distance of the closest unsaturation from the aliphatic end of the molecule. The suffixes 'c' for *cis* and 't' for *trans* refer to geometric isomers. The prefixes 'i', 'a' and 'me' refer to iso and anteiso methyl branching and mid-chain methyl branching, respectively, with cyclopropyl rings indicated by 'cy' (Kates, 1986).

The glycolipid fraction was subjected to ethanolysis, and the β-hydroxy acids from the PHA were 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 subsamples from each zone from each soil core (nine subsamples per core) according to the method of Borneman *et al.* (1996), with modifications described in Stephen *et al.* (1998). PCR amplification of the 16S rDNA fragments before DGGE was performed as described by Stephen *et al.* (1998). Briefly, thermocycling consisted of 35 cycles of 92°C for 45 s, 55°C for 30 s and 68°C for 45 s, using 1.25 units of Expand HF polymerase (Boehringer) and 10 pmol each of the primers described by Muyzer *et al.* (1993) (the forward primer carried the 40 bp GC clamp) in a total volume of 25 μl. Thermocycling was performed using a 'Robocyler' PCR block (Stratagene). The primers targeted eubacterial 16S regions corresponding to *Escherichia coli* positions 341–534 (Brosius *et al.*, 1981). A portion (20%) of each PCR product was analysed by agarose gel electrophoresis (1.5% agarose, 1× TAE buffer) and ethidium bromide fluorescence. The amount of DNA used for DGGE analysis was standardized to 600 ng by comparison with molecular weight standards (1 kb+ ladder; Gibco BRL) using ALPHA-IMAGER software (Alpha Innotech).

DGGE was performed using a D-Code 16/16 cm gel system with 1.5 mm gel width (Bio-Rad) maintained at a constant temperature of 60°C in 6 l of 0.5×TAE buffer (20 mM Tris acetate, 0.5 mM EDTA, pH 8.0). Gradients were formed

between 20% and 55% denaturant with 100% denaturant defined as 7 M urea plus 40% (v/v) formamide. Gels were run at 35 V for 16 h. Gels were stained in purified water (Milli-Ro; Millipore) containing ethidium bromide (0.5 mg l^{-1}) and destained twice in 0.5×TAE for 15 min each. Images were captured using the ALPHA-IMAGER software (Alpha Innotech).

Extraction of DNA from acrylamide gels

The central 1 mm^2 portion of strong DGGE bands were excised using a razor blade (American Safety Razor Company) and soaked in 50 μl of purified water (Milli-Ro; Millipore) overnight. A portion (15 μl) was removed and used as the template in a PCR reaction as above. The products were purified by electrophoresis through a 1.2% agarose/TAE gel followed by glass-milk extraction (Gene-Clean kit; BIO-101).

Cloning of PCR-amplified products

Amplification products that failed to generate legible sequence directly were cloned into the PCR-TOPO 2.1 cloning vector (Invitrogen) according to the manufacturer's instructions. Recombinant (white) colonies were screened by a two-stage procedure to ensure recovery of the DGGE band of interest. First, plasmid inserts ($n = 12$ for each band) were reamplified by PCR using vector-specific primers (M13 reverse and T7; Invitrogen). The products were digested with restriction endonuclease *Msp*I and analysed by agarose gel electrophoresis (2% agarose, 1× TAE buffer). Two products from each digestion pattern group were reamplified using the 16S-specific PCR primers described above (Muyzer *et al.*, 1993) and subjected to DGGE analysis to select sequences that co-migrated with the original band of interest. Sequences that were of high frequency in clone libraries (as defined by digestion pattern) and co-migrated with the original environmental band were selected for sequence analysis (two clones $band^{-1}$) and used as probes in confirmatory membrane hybridization analysis.

Membrane transfer and hybridization analysis

DNA was transferred electrophoretically from DGGE gels to positively charged nylon support hybridization membranes (Boehringer Mannheim) using a model SD electroblotter (Bio-Rad) at 40 mA for 1 h. The transfer buffer was 0.5×TAE. Hybridization analysis used DIG-labelled PCR products (PCR DIG probe synthesis kit; Boehringer Mannheim) and a DIG nucleic acids detection system (Boehringer Mannheim) according to the manufacturer's instructions. Hybridization was at 68°C overnight in a rotary oven (Personal Hyb; Stratagene) in the manufacturer's 'standard buffer'. Washing and staining were as recommended by the manufacturer.

Sequence analysis

PCR products from excised bands and cloned products were sequenced using the primer 516r (GWATTACCGCGGC-KGCTG; W = A or T, K = G or T; Lane *et al.*, 1985) and an ABI-Prism model 373 automatic sequencer with dye terminators (Perkin-Elmer). Sequences were compared with the GenBank database using the BLASTN facility of the National Center for Biotechnology Information (http://ncbi.nlm.nih.gov). Sequences were classified using the RDP release of 31 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 differences between lipid biomarker data obtained from each zone ($n = 3$ for each zone). ANOVA was performed on the means of the duplicate subsamples (pseudoreplicates) using STATISTICA version 5.1 for Windows software (Statsoft). Groupings for ANOVA were assigned a posteriori. Chromatographic peaks with zero area were replaced with half the minimum integrated area for each injection before calculation of mole percentages. Plots of log (average) versus log (variance) were used to determine the appropriate transformation of the variables (Downing, 1979). The square root transformation was chosen. A hierarchical cluster analysis (complete linkage method) based on Euclidean distance was performed on the transformed data. Hierarchical and principal component analyses were performed using the statistical package EINSIGHT (Infometrix).

Acknowledgements

The authors thank Barry Kinsall of ORNL, and Mark Henry of the NCIBRD, University of Michigan, for valuable-assistance in field sampling. This work was supported by a grant from the Strategic Environmental Research and Development Program (SERDP contract number 11XSY887Y).

References

Amann, R.I., Ludwig, W., and Schleifer, K.-H. (1995) Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol Rev* **59:** 143–169.

Bakken, L.R. (1985) Separation and purification of bacteria from soil. *Appl Environ Microbiol* **49:** 1188–1195.

Balkwill, D.L., Leach, F.R., Wilson, J.T., McNabb, J.F., and White, D.C. (1988) Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface sediments. *Microbial Ecol* **16:** 73–84.

Borneman, J., Skroch, P.W., O'Sullivan, K.M., Palus, J.A., Rumjanek, N.G., Jansen, J.L., *et al.* (1996) Molecular microbial diversity of an agricultural soil in Wisconsin. *Appl Environ Microbiol* **62:** 1935–1943.

Brosius, J., Dull, T.L., Sleeter, D.D., and Noller, H.F. (1981) Gene organisation and primary structure of the ribosomal RNA operon in *Escherichia coli*. *J Mol Biol* **148:** 107–127.

Cox, E.E., Major, D.W., Acton, D.W., Phelps, T.J., and White, D.C. (1994) Evaluating trichloroethylene biodegradation by

measuring the *in situ* status and activities of microbial populations. In *Bioremediation of Chlorinated Polycyclic Aromatic Compounds.* Hinchee, R.E., Leeson, A., Semprini, L., and Ong, S.K. (eds). Ann Arbor: Lewis Publications, pp. 37–49.

Doi, Y. (1990) *Microbial Polyesters.* New York: VCH Publishers, pp. 1–8.

Dowling, N.J.E., Widdel, F., and White, D.C. (1986) Phospholipid ester-linked fatty acid biomarkers of acetate-oxidizing sulphate-reducers and other sulphide-forming bacteria. *J Gen Microbiol* **132:** 1815–1825.

Downing, J.A. (1979) Aggregation, transformation and the design of benthic sampling programs. *J Fish Res Board Can* **36:** 1454–1463.

Edlund, A., Nichols, P.D., Roffey, R., and White, D.C. (1985) Extractable and lipopolysaccharide fatty acid and hydroxy acid profiles from *Desulfovobrio* species. *J Lipid Res* **26:** 982–988.

Fang, J., Barcelona, M.J., and West, C. (1997) The use of aromatic acids and phospholipid ester linked fatty acids for delineation of processes affecting an aquifer contaminated with JP-4 fuel. In *Molecular Markers in Environmental Geochemistry.* Egenhouse, R.E. (ed.). American Chemical Society Symposium 671. Washington DC: American Chemical Society, pp. 65–76.

Findlay, R.H., and Dobbs, F.C. (1993) Quantitative description of microbial communities using lipid analysis. In *Handbook of Methods in Aquatic Microbial Ecology.* Kemp, P.F., Sherr, B.F., Sherr, E.B., and Cole, J.J. (eds). Boca Raton, FL: Lewis Publishers, pp. 271–284.

Findlay, R.H., and White, D.C. (1983) Polymeric beta hydroxyalkanoates from environmental samples and *Bacillus megatarium. Appl Environ Microbiol* **45:** 71–78.

Frostegård, A., Tunlid, A., and Bååth, E. (1993) Phospholipid fatty acid composition, biomass, and activity of microbial communities from two soil types experimentally exposed to different heavy metals. *Appl Environ Microbiol* **59:** 3605–3617.

Frostegård, A., Tunlid, A., and Bååth, E. (1996) Changes in microbial community structure during long-term incubation in two soils experimentally contaminated with metals. *Soil Biol Biochem* **28:** 55–63.

Guckert, J.B., Antworth, C.P., Nichols, P.D., and White, D.C. (1985) Phospholipid ester-linked fatty acid profiles as reproducible assays for changes in prokaryotic community structure of estuarine sediments. *FEMS Microbiol Ecol* **31:** 147–158.

Guckert, J.B., Hood, M.A., and White, D.C. (1986) Phospholipid, ester-linked fatty acid profile changes during nutrient depletion of *Vibrio cholerae*: increases in the *trans/cis* and proportions of cyclopropyl fatty acids. *Appl Environ Microbiol* **52:** 794–801.

Heipieper, H.-J., Diefenbach, R., and Kewoloh, H. (1992) Conversion of *cis* unsaturated fatty acids to *trans*, a possible mechanism for the protection of phenol-degrading *Pseudomonas putida* P8 from substrate toxicity. *Appl Environ Microbiol* **58:** 1847–1852.

Heuer, H., and Smalla, K. (1997) Application of denaturing gel electrophoresis and temperature gradient gel electrophoresis for studying soil microbial communities. In *Modern Soil Microbiology.* Van Elsas, J.D., Trevors, J.T., and Wellington, E.M.H. (eds). New York: Marcel Dekker, pp. 353–374.

Kates, M. (1986) *Techniques in Lipidology: Isolation, Analysis and Identification of Lipids*, 2nd edn. Amsterdam: Elsevier Press.

Keift, T.L., Ringelberg, D.B., and White, D.C. (1994) Changes in ester-linked phospholipid fatty acid profiles of subsurface bacteria during starvation and desiccation of a porous medium. *Appl Environ Microbiol* **60:** 3292–3299.

Lane, D., Pace, B., Olsen, G.J., Stahl, D.A., Sogin, M.L., and Pace, N.R. (1985) Rapid determination of 16S ribosomal RNA sequences for phylogenetic analyses. *Proc Natl Acad Sci USA* **82:** 6955–6959.

Maidak, B.L., Olsen, G.J., Larsen, N., Overbeek, R., McCaughey, M.J., and Woese, C.R. (1997) The RDP (Ribosomal Database Project). *Nucleic Acids Res* **25:** 109–111.

Mayberry, W.R., and Lane, J.R. (1993) Sequential alkaline saponification/acid hydrolysis/esterification: a one tube method with enhanced recovery of both cyclopropane and hydroxylated fatty acids. *J Microbiol Methods* **18:** 21–32.

Muyzer, G., de Waal, E.C., and Uitterlinden, A.G. (1993) Profiling of microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S rRNA. *Appl Environ Microbiol* **59:** 695–700.

Nichols, D.S., Nichols, P.D., Russell, N.J., Davies, N.W., and McMeekin, T.A. (1997) Polyunsaturated fatty acids in the psychrophilic bacterium *Shewanella gelidimarina* ACAM 456T: molecular species analysis of major phospholipids and biosythesis of eicosapentaenoic acid. *Biochim Biophys Acta* **1347:** 164–176.

Pfiffner, S.M., Palumbo, A.V., Gibon, T., Ringelberg, D.B., and McCarthy, J.F. (1997) Relating groundwater and sediment chemistry to microbial Characterisation at a BTEX-contaminated site. *Appl Biochem Biotech* **63–65:** 775–788.

Pinkart, H.C., Wolfram, J.W., Rogers, R., and White, D.C. (1996) Cell envelope changes in solvent-tolerant and solvent-sensitive *Pseudomonas puitida* strains following exposure to *O*-xylene. *Appl Environ Microbiol* **62:** 1127–1131.

Rand, G.M., and Petrocelli, S.R. (1985) *Fundamentals of Aquatic Toxicology: Methods and Applications.* New York: Hemisphere Publishing Corporation.

Ringelberg, D.B., Davis, J.D., Smith, G.A., Pfiffner, S.M., Nichols, P.D., Nickels, J.B., *et al.* (1989) Validation of signature polarlipid fatty acid biomarkers for alkane-utilizing bacteria in soils and subsurface aquifer materials. *FEMS Microbiol Ecol* **62:** 39–50.

Ringelberg, D.B., Townsend, G.T., DeWeerd, K.A., Sulita, J.M., and White, D.C. (1994) Detection of the anaerobic dechlorinating microorganism *Desulfomonile tiedjei* in environmental matrices by its signature lipopolysaccharide branch-long-chain hydroxy fatty acids. *FEMS Microbiol Ecol* **14:** 9–18.

Sikkema, J., deBont, J.A.M., and Poolman, B. (1995) Mechanisms of membrane toxicity of hydrocarbons. *Microbiol Rev* **59:** 201–222.

Skinner, F.A., Jones, P.C.T., and Mollison, J.E. (1952) A comparison of a direct and a plate counting technique for the quantitative estimation of soil microorganisms. *J Gen Microbiol* **6:** 261–271.

Smith, G.A., Nickels, J.S., Kerger, B.D., Davis, J.D., Collins, S.P., Wilson, J.T., *et al.* (1986) Quantitative characterisation of microbial biomass and community structure in subsurface material: a prokaryotic consortium responsive to organic contamination. *Can J Microbiol* **32:** 104–111.

Stephen, J.R., Chang, Y.-J., Macnaughton, S.J., Whitaker, S.L., Hicks, C., Leung, K.T., *et al.* (1999) Fate of a metal-resistant inoculum in contaminated and pristine soils assessed by denaturing gradient gel electrophoresis. *Environ Toxicol Chem* **18** (in press).

Tunlid, A., and White, D.C. (1992) Biochemical analysis of biomass, community structure, nutritional status and metabolic activity of the microbial community in soil. In *Soil Biochemistry*, Vol. 7. Bollag, J.-M., and Stotzky, G. (eds). New York: Marcel Dekker, pp. 229–262.

Vestal, R.J., and White, D.C. (1989) Lipid analysis in microbial ecology. *Bioscience* **39:** 535–541.

Watanabe, K., Yazawa, K., Kondo, K., and Kawagushi, A. (1997) Fatty acid synthesis of an eicosapentaenoic acid-producing bacterium: *de novo* synthesis, chain elongation, and desaturation systems. *J Biochem* **122:** 467–473.

Weidemeier, T.H., Swanson, M.A., Wilson, J.T., Kampbell, D.H., Miller, R.N., and Hansen, J.E. (1995) Patterns of intrinsic bioremediation at two US Airforce bases. In *Intrinsic Bioremediation*. Hinchee, R.H., Wilson, J.T., and Downey, D.C. (eds). Columbus: Batelle Press, pp. 31–51.

White, D.C. (1983) Analysis of microorganisms in terms of quantity and activity in natural environments. In *Society for General Microbiology*, Vol. 34. *Microbes in their Natural Environments*. Slater, J.H., Whittenbury, R., and Wimpenny, J.W.T. (eds). London: Cambridge University Press, pp. 37–66.

White, D.C., Davis, W.M., Nickels, J.S., King, J.D., and Bobbie, R.J. (1979) Determination of the sedimentary microbial biomass by extractable lipid phosphate. *Oecologia* **40:** 51–62.

White, D.C., Ringelberg, D.B., Hedrick, D.B., and Nivens, D.E. (1993) Rapid identification of microbes from clinical and environmental matrices by mass spectrometry. In *Identification of Microorganisms by Mass Spectrometry*. Fenselau,C. (ed.). American Chemical Society Symposium series 541. Washington, DC: American Chemical Society, pp. 8–17.

White, D.C., Pinkart, H.C., and Ringelberg, D.B. (1996a) Biomass measurements: biochemical approaches. In *Manual of Environmental Microbiology*. 1st edn. Hurst, C.J., Knudsen, G.R., McInerney, M.J., Stetzenbach, L.D., and Walter, M.V. (eds). Washington DC: American Society for Microbiology Press, pp. 91–101.

White, D.C., Stair, J.O., and Ringelberg, D.B. (1996b) Quantitative comparisons of *in situ* microbial biodiversity by signature biomarker analysis. *J Ind Microbiol* **17:** 185–196.

White, D.C., Flemming, C.A., Leung, K.T., and Macnaughton, S.J. (1998) *In situ* microbial ecology for quantitative appraisal, monitoring, and risk assessment of pollution remediation in soils, the subsurface, the rhizosphere, and in biofilms. *J Microb Methods* **32:** 93–105.

Wilkinson, S.G. (1988) Gram-negative bacteria. In *Microbial Lipids*. Ratledge, C., and Wilkinson, S.G. (eds). London: Academic Press, pp. 299–488.

Zelles, L. (1997) Phospholipid fatty acid profiles in selected members of soil microbial communities *Chemosphere* **35:** 275–294.

Copyright of Environmental Microbiology is the property of Blackwell Publishing Limited and its content may not be copied or emailed to multiple sites or posted to a listserv without the copyright holder's express written permission. However, users may print, download, or email articles for individual use.