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Validation of signature polarlipid fatty acid biomarkers for alkane-utilizing bacteria in soils and subsurface aquifer materials

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1. SUMMARY

Extractable cell membrane-derived polarlipid ester-linked fatty acids (PLFA) obtained from aerated soils gassed with methane or propane and from methane- and propane-oxidizing bacteria isolated from the soils were analyzed by capillary gas chromatography/mass spectrometry. Exposure of aerated soils to methane resulted in the formation of a high proportion of an unusual 18-carbon mono-unsaturated PLFA, 18 : lw8c. High proportions of this fatty acid biomarker are found in monocultures from this soil grown in minimal media with methane. This PLFA has been previously established as associated with authentic type II methane-oxidizing bacteria. The microbiota in aerated soils exposed to hydrocarbons containing propane, formed a suite of PLFA characterized by high proportions of a 16-carbon mono-unsaturated acid, 16 : lw6c, and an 18-carbon saturated fatty acid with an additional methyl branch at the 10 position, 10 Me 18 : 0. This PLFA pattern has been detected in several monocultures enriched from the soil with propane-amended minimal media. The correspondence of high proportions of these unusual mono-unsaturated PLFA in the isolated monocultures and in situ in the soils after stimulation with the appropriate hydrocarbon is a strong validation of the utility of these biomarkers in defining the community structure of the surface soil microbial community.

2. INTRODUCTION

The quantitative definition of the biomass and community structure of the microbiota of surface soils is one of the most pressing challenges of

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microbial ecology. Polarlipids, intercellular adenosine nucleotides, and cell wall amino-sugars are biochemical components of cells that have been utilized to estimate microbial biomass [1]. Smith et al. [2] have shown that there appears to be a unique microbial community in uncontaminated subsurface materials from below the root zone with a sparsely distributed microbiota which have an identical cocco-bacillary morphology. In these subsurface sediments the biomass and cell numbers estimated from direct cell count after acridine orange staining agree with the numbers and biomass estimated from the extractable polarlipid phosphate and total fatty acids, the total adenosine triphosphate, the fatty acids from the lipopolysaccharide lipid A, and the cell wall muramic acid content [3].

The ester-linked fatty acids in the polarlipids (PLFA) are presently one of the more sensitive and useful chemical measures of microbial biomass and community structure [4–6]. The use of fatty acids that are specifically ester-linked in the polarlipid fraction of the total lipid extract greatly increases the selectivity of this assay as many of the anthropogenic contaminants as well as the endogenous storage lipids are found in the neutral or glycolipid fractions of the lipids. For example, by isolating the polarlipid fraction for fatty acid analysis it has proved possible to detect specific bacteria in the sludge of crude oil tanks (unpublished data).

Validations of the use of PLFA in defining microbial community structure have been reviewed [7]. These validations involved: modifying the microenvironment to induce changes that can often be predicted (changing nutrients or electron acceptors, adding antibiotics etc.), shifting the community nutritional status and observing the effects on formation of polymers and endogenous storage components, observing the effects on biofilms by grazers with known specificity, and isolating specific monocultures for PLFA biomarker analysis and showing the appropriate response in situ when that specific group of organism is induced or repressed.

In this study, PLFA biomarker techniques were applied to aerated subsurface materials or surface mucks (soils with high organic content) which were gassed with methane or propane containing hydrocarbons. The gassing induced the formation of microbial consortia with significantly increased proportions of specific PLFA. These specific PLFA were found to be characteristic of monocultures recovered from these experimental systems by selection for growth with either methane- or propane-oxidation. The overall goal of the present study was to extend the range of microbial environments for which the biomarker techniques have been validated to include surface and subsurface soils in which methane- and propane-oxidizing bacteria are enriched.

3. MATERIALS AND METHODS

3.1. Materials

Solvents were highest quality distilled in glass and were of residue analysis grade or better (J.T. Baker Chemical Co., Phillipsburg, NJ). Standards and derivatizing agents were purchased from Supelco, Inc. (Bellefonte, PA), Applied Science (State College, PA), Aldrich, Inc. (Milwaukee, WI), Sigma Chemical Co. (St. Louis, MO), and Pierce Chemical Co. (Rockford, IL).

3.2. Soil and microcosm samples

Soil was recovered from a shallow semi-confined alluvial aquifer at the Stanford University field demonstration site at Moffett Naval Air Station, Mountain View, CA, [8]. The aquifer zone consists of a layer of poorly sorted silty sand and gravel approximately 1.18 to 1.8 m thick bounded above and below by clay. A series of injection and extraction wells were drilled and the sediments recovered for microcosm studies. This core material was transferred to the R.S. Kerr Laboratory, Ada, OK, where it was packed into glass columns (3.7 cm diameter × 22 cm long), perfused with Moffett Field site water, sparged with oxygen, amended with an aqueous solution of both trichloroethylene (TCE) and 1,1,1,-thrichloroethane, and an aqueous solution saturated with either methane or propane. A treatment cycle consisted of perfusion of two pore volumes (150 ml) of solution and incubation for 10 days followed by flushing with two pore volumes. After eleven cycles

the sediment was lyophilized and sent to Knoxville for analysis. Control samples of untreated Moffett Field subsurface aquifer material similar to that used in the microcosm experiments were also obtained.

Experimental verification of biodegradability of TCE with concomitant use of hydrocarbons was performed as described [8]. Controls for microbial activity were poisoned with 0.1% sodium azide in the perfusate.

In other experiments a prototype soil reactor was constructed at Racine, WI, using Rollin muck soil (6.5% organic matter, 45% clay with a pH of 7.4) through which a mixture of air with 0.2% of hydrocarbons with 27 mol% propane, 29 mol% iso-butane, and 44 mol% *n*-butane flowed at 3 cm^3 per cm^2 surface area per min [9]. In the soil reactor, the soil was 90 cm thick and covered an area of 190 m^3. The pressure drop across the 90 cm thick bed was 85 cm water and the residence time of the perfused gases in the soil was approximately 15 min. Samples were recovered at levels of 15, 59, and 88 cm below the surface, packed in ice, and sent to Knoxville for analysis. The soil reactor has been exposed to the hydrocarbons for three years. Control samples were recovered from Rollin muck soil not exposed to the hydrocarbons.

The removal of TCE in acclimated Rollin muck soil was examined by adding 46 g of muck soil at 30% moisture content to replicate 160 ml serum bottles with Teflon-faced silicone septa and aluminum crimp caps. The air-filled space in the bottle was 60-times greater than the air-filled pores of the soil in the prototype bioreactor. This dilution slowed the rate of TCE biodegradation sufficiently to provide accurate estimations. Each measurement represented a microcosm sampled for alkanes in the head space, adding water to form a slurry with the soil and then collecting the TCE for gas chromatographic analysis by the purge and trap technique [9].

3.3. Organisms and cultural conditions

Propane and methane-oxidizing organisms were isolated from the Rollin muck soil by enrichments in 150 ml serum bottles with 2 g soil plus 50 ml of mineral salts medium with a headspace containing roughly 50% methane or propane and air. The bottles were shaken at 100 r.p.m. After about a month at room temperature 0.1 ml was plated on mineral salts medium on Nobel agar (Difco Inc., Detroit, MI). Isolates were restreaked for purity and single colony isolates grown for analysis of the PLFA. The mineral salts medium contained in g/l: 2.0 $NaNO_3$, 0.2 g $MgSO_4 \cdot 7H_2O$, 0.04 g KCL, 0.015 g $CaCl_2$ vitamins and 2 ml of trace minerals. The trace mineral solution contained in g/l: 1.5 g nitrilotriacetic acid, 0.1 g $FeSO_4 \cdot 7H_2O$, 0.1 g $MnCl_2 \cdot 4\ H_2O$, 0.17 g $CoCl_2 \cdot 2H_2O$, 0.1 g $CaCl \cdot 2H_2O$, 0.1 g $ZnCl_2$, 0.02 g $CuCl_2 \cdot 2H_2O$, 0.01 g H_3BO_3, 0.01 g Na Molybdate, 1.0 g NaCl, 0.017 g Na_2SeO_3, 0.026 g $NiSO_4 \cdot 6H_2O$. The vitamin solution in g/l contains 0.002 g biotin, 0.002 g folic acid, 0.01 g pyridoxine · HCl, 0.005 g thiamine · HCl, 0.005 g riboflavin, 0.005 g pantothenic acid, 0.0001 g cyanocobalamin, 0.005 g *p*-aminobenzoic acid, and 0.005 g lipoic acid.

Organisms were harvested by centrifugation at $10\,000 \times g$ for 15 min at 4°C, washed twice in phosphate buffer or growth medium minus energy source, and lyophilized.

3.4. Extraction

The analytical sequence utilized for PLFA analysis involves extraction, fractionation on silicic acid, derivatization, and analysis by capillary gas chromatography (GC) with mass spectral structural confirmation. The modified one-phase Bligh and Dyer extraction was utilized for all samples [10]. Duplicate samples were extracted separately and all data is expressed as the mean of two determinations unless otherwise stated. After the overnight separation of the lipid and aqueous phases in the second stage of the extraction, the organic fraction was filtered through fluted Whatman 2V filters pre-extracted with chloroform and reduced in volume using a rotating evaporator. The samples were transferred to teflon lined screw capped test tubes and dried under a stream of nitrogen at room temperature.

3.5. Polar lipid isolation

Silicic acid columns were prepared using 1 g Unisil (100–200 mesh, Clarkson Chemical Co., Inc, Williamsport, PA) activated at 100°C for 60

min and pre-extracted with chloroform. Columns were prepared with the approximate ratios of 50 : 1 stationary phase to lipid (dry weight) and 1.7 : 1 stationary phase column bed height : cross sectional area. Total lipid was applied to the top of the columns in a minimal volume of chloroform. Sequential washes of 10 ml of chloroform, acetone, and methanol eluted the neutral, glyco- and polar lipids. The polar lipid fraction was dried under a stream of nitrogen.

3.6. Mild alkaline methanolysis

The mild alkaline methanolysis procedure [10] was utilized to prepare methyl esters of the ester-linked fatty acids of the polar lipids.

3.7. Determination of mono-unsaturated PLFA double bond position and configuration

The dimethyl disulfide (DMDS) adducts of mono-unsaturated PLFA were formed to determine double bond position and geometry using methods previously described [11–13]. Samples were analyzed by capillary GC and GC/MS as described below. These derivatives increase the resolution between *cis* and *trans* geometrical isomers in capillary GC.

3.8. Gas chromatography (GC)

Dry PLFA methyl esters were dissolved in isooctane containing the internal standard of methyl nonadecanoate. Samples of 1.0 μl were injected onto a 50 m nonpolar, cross-linked methyl silicone fused silica capillary column (0.2 mm i.d., Hewlett Packard) in a Shimadzu GC-9A GC. A 30 s splitless injection with the injection temperature at 270 °C was used. Hydrogen at a linear velocity of 35 cm/s was the carrier gas with a temperature program starting with an initial temperature of 80 °C. After a one minute delay at 80 °C, the temperature was programmed at a 10 °C/min rise to 150 °C, followed by a 3 °C/min rise to 240 °C, followed by a 5 °/min rise to 280 °C with a terminal isothermal period of 5 min at 280 °C. Detection was by hydrogen flame (F.I.D.) using a 30 ml/min nitrogen make up gas at a temperature of 270 °C. An equal detector response was assumed for all components. Peak areas were quantified with a programmable laboratory data system (Nelson Analytical 3000 Series Chromatography Data System (Revision 3.6)).

3.9. Gas chromatography / mass spectrometry (GC / MS)

Tentative component identification prior to GC/MS analysis was based on comparison of the retention time data or co-elution with authentic standards. GC/MS analysis was performed on a Hewlett Packard 5996A GC/MS fitted with a direct capillary inlet utilizing the same chromatographic system except for use of a helium carrier gas and the temperature program which was begun at 100 °C and increased to 280 °C at 3 °C/min for a total analysis time of 60 min. The electron multiplier voltage was between 1800 and 2000 V, the transfer line maintained at 300 °C, the source 280 °C and analyzer 250 °C, and the GC/MS was autotuned with DFTPP (decafluorotriphenylphosphine) at m/z 502 with an ionization energy of 70 eV. The data was acquired and manipulated using the Hewlett Packard RTE 6/VM data system. Other conditions were as described previously [14].

3.10 Fatty acid nomenclature

Fatty acids are designated as total number of carbon atoms: number of double bonds with the position of the double bond nearest to the aliphatic (w) end of the molecule indicated. This is followed by the suffix c for *cis* and t for *trans* configuration of monoenoic fatty acids. The prefixes i, a, or br indicate iso, anteiso, or branched (position undetermined). Mid-chain branching is indicated by the number of carbon atoms from the carboxyl end of the molecule and Me for the methyl group (10Me16 : 0 is a 17-carbon PLFA). Cyclopropane rings are indicated with the prefix cy with the ring position indicated from the Aliphatic end of the molecule.

4. RESULTS

4.1. Effect of amending subsurface materials with propane and a mixture of methane and propane

The subsurface sediments from the aquifer at the Moffett Field demonstration site showed a

PLFA profile typical of subsurface sediments with high levels of saturated, branched, cyclopropane, and monoenoic PLFA (Table 1, first column). Exposing the sediments to propane and air and then sealing the system for 10 days through a series of 13 cycles depressed the total biomass by a factor of 10 and profoundly shifted the proportions of PLFA (Table 1, second column). There was a 7-fold increase in the proportion of 18 : 2w6, a 4-fold increase in 18 : 1w9c, but little change in the proportions of 16 : 0. There was a 7-fold drop in the proportions of 10Mel6 : 0. The most striking change was the appearance of an unusual PLFA, 16 : 1w6c, which became the most prominent component of the polar lipids. Two other unusual PLFA also increased in their proportions, 10Me18 : 0, and 16 : 1w5c. Exposing the sediments to methane for 12 cycles but with an inadvertent exposure to propane for one cycle showed a higher total biomass (roughly half the starting material) and decreases in 16 : 1w6c and 10Me18 : 0 over amounts found in the propane exposed material. Methane exposure also resulted in the appearance of the PLFA, 18 : 1w8c which has been shown to be characteristic of authentic type II methane-oxidizing bacteria [14]. The data given in Table 1, the second and third columns, show no standard deviations as the total combined sample was utilized to provide enough material for mass spectral confirmation of the structure. In separate experiments the standard deviation ranged between 10 and 50% of the mean ($n = 3$) in these samples. In these experiments the oxygen utilization in the microcosms acclimated to propane removed 54–64% of the theoretical oxygen demand of the propane removed from the perfusion water [8]. The oxygen depletion in the columns acclimated to methane was 91–107% of the theoretical oxygen demand [8].

Table 1

Effect of propane and methane + a single exposure to propane amendment on phospholipid ester-linked fatty acid (PLFA) profile of the microbiota in sub-surface materials from the Moffett Field, CA field demonstration site

PLFA	Mol %			
	A		B	C
	Control [b]	(S.D.)	Propane [c]	Propane and Methane [d]
14:0 [a]	hd [g]	–	nd	0.8
i15:0	1.6	0.0	nd	3.0
a15:0	1.6	0.07	nd	2.4
15:0	0.8	0.14	1.5	0.8
i16:0	4.3	0.14	nd	2.0
16:1w7c	3.5	–	nd	1.5
16:1w6c	nd	–	21.9	17.7
16:1w5c	1.7	0.21	3.2	2.3
16:1w5t	nd	–	nd	6.4
16:0	15.2	0.21	16.6	15.9
i17:1w7c	1.9	0.07	2.2	2.1
10Me16:0 [a]	13.3	0.0	1.2	7.0
i17:0	4.4	0.21	nd	1.7
a17:0/17:1	2.9	0.07	4.4	1.9
cy17:(7,8)	2.0	0.42	nd	0.5
17:0	1.0	0.0	3.2	0.9
18:2w6 [a]	0.9	0.14	7.2	1.1
18:1w9c	4.5	0.42	18.4	3.3
18:1w8c	nd	–	nd	11.7
18:1w7c	6.3	0.84	nd	0.2
18:1w5c [a]	1.0	0.14	nd	0.3
18:0	4.9	0.21	5.3	2.2
10Me18:0	2.7	0.07	8.6	1.7
19:1 [a]	nd	–	1.7	0.7
cy19:0	5.6	0.07	3.3	9.6
i20:0 [a]	nd	–	nd	0.2
20:1 [a]	nd	–	nd	0.2
20:1 [a]	nd	–	nd	0.1
20:0 [a]	1.7	1.1	1.2	1.9
22:0 [a]	1.5	0.71	nd	0.0
pmol/g dwt [e]	556 [f]	67.9	57	253

[a] Identification based on retention time on capillary gas chromatography only.
[b] Subsurface materials from Moffett Field demonstration site. Data given as a mean (S.D.) $n = 3$.
[c] Material from Column A exposed to propane.
[d] Material from Column A exposed to methane + propane.
[e] Total PLFA pmol/g dry wt.
[f] Additional PLFA (not shown) accounts for missing mol %.
[g] nd indicates not detected.

4.2. Effect of exposing Rollin muck soil to a mixture of hydrocarbons

Table 2 gives the mean and standard deviation of the proportions of PLFA found in the control and the Rollin muck soil exposed to a mixture of propane, *iso*-butane, and butane over a period of 2 years. The gassed soils show a consistently higher biomass based on the total PLFA/g dry wt and a higher proportion of the endogenous storage lipid PHA relative to the PLFA recovered from the soil. Highest levels were found at depths of 14.7 and 59

Table 2

Proportions of phospholipid ester-linked acids (PLFA), total biomass, and poly beta-hydroxyalkanote (PHA) from the microbiota induced by exposure to short chain hydrocarbons in a soil bed reactor; air was injected at a depth of 90 cm

PLFA	Mol % [a]					
	Gassed			Not Gassed		
	88 cm	59 cm	14 cm	88 cm	59 cm	14 cm
i15:1	0.3 (0.1)	0.7 (0.0)	0.6 (0.0)	0.8 (0.0)	0.7 (0.2)	0.8 (0.2)
i15:0	3.6 (1.0)	3.4 (0.0)	4.4 (0.0)	6.6 (0.0)	6.3 (0.9)	6.0 (0.4)
a15:0	2.9 (0.7)	2.8 (0.1)	3.2 (0.1)	5.2 (0.3)	5.0 (0.8)	6.8 (0.4)
15:1w6	0.2 (0.1)	0.3 (0.0)	0.2 (0.0)	0.2 (0.0)	0.2 (0.0)	0.5 (0.0)
Br16:0	0.3 (0.0)	0.2 (0.0)	0.4 (0.0)	0.7 (0.1)	0.6 (0.0)	0.8 (0.1)
il6:1w6	0.5 (0.1)	0.6 (0.0)	0.7 (0.0)	0.6 (0.0)	0.8 (0.0)	0.7 (0.0)
10Me15:0	0.3 (0.0)	0.2 (0.0)	0.3 (0.0)	0.3 (0.0)	0.5 (0.0)	0.3 (0.1)
il6:0	2.4 (0.0)	2.1 (0.0)	2.1 (0.0)	2.4 (0.0)	2.6 (0.3)	2.2 (0.0)
16:1wll,w9	2.0 (0.2)	2.5 (0.0)	2.1 (0.1)	1.7 (0.0)	1.9 (0.1)	1.6 (0.2)
16:1w7c	7.1 (0.1)	6.8 (0.0)	9.3 (0.4)	8.1 (0.1)	6.6 (0.1)	7.3 (0.2)
16:1w6c	2.9 (0.1)	4.8 (0.4)	1.6 (0.1)	0.4 (0.0)	0.4 (0.0)	0.3 (0.1)
16:1w5c	6.3 (0.6)	14.3 (0.0)	4.3 (0.2)	4.5 (0.2)	4.2 (0.2)	4.4 (0.05)
16:0	13.1 (1.1)	11.5 (1.0)	10.1 (0.3)	10.5 (0.1)	10.1 (0.8)	8.7 (0.02)
Br17:0	0.9 (0.1)	0.5 (0.1)	1.0 (0.1)	1.1 (0.1)	1.6 (0.4)	1.2 (0.02)
il7:1w7	2.6 (0.2)	1.8 (0.1)	3.3 (0.1)	4.3 (0.1)	4.4 (0.0)	5.6 (0.02)
10me16:0	5.4 (0.1)	3.3 (0.2)	6.5 (0.3)	10.9 (0.2)	13.3 (1.4)	9.6 (0.04)
i17:0	1.3 (0.1)	0.9 (0.0)	1.3 (0.0)	1.7 (0.0)	1.7 (0.1)	1.8 (0.1)
a17:0/17:1w8	2.1 (0.1)	1.7 (0.0)	2.1 (0.0)	2.5 (0.0)	2.5 (0.1)	2.3 (0.0)
17:1w6(cyl7:0w7,8)	3.3 (0.1)	2.8 (0.1)	4.9 (0.3)	4.4 (0.1)	4.3 (0.5)	4.0 (0.1)
cyl7:0	1.0 (0.1)	2.5 (0.0)	0.4 (0.0)	0.4 (0.1)	0.5 (0.1)	0.4 (0.0)
17:0	0.8 (0.1)	0.8 (0.0)	0.6 (0.0)	0.6 (0.0)	0.5 (0.0)	0.5 (0.0)
5me17:0	0.9 (0.1)	0.5 (0.0)	1.3 (0.0)	1.7 (0.1)	1.8 (0.2)	2.5 (0.3)
10me17:0	0.8 (0.1)	0.5 (0.0)	0.9 (0.1)	0.8 (0.0)	0.8 (0.0)	1.2 (0.1)
Br18:1	0.4 (0.0)	0.7 (0.1)	0.8 (0.2)	0.3 (0.1)	0.3 (0.0)	0.3 (0.0)
18:1w7c/18:1w8c	10.0 (0.4)	7.9 (0.3)	10.5 (0.1)	6.1 (0.1)	5.5 (0.7)	7.4 (0.3)
18:0	2.0 (0.4)	1.2 (0.0)	1.8 (0.0)	2.2 (0.1)	2.3 (0.1)	1.9 (0.1)
11me18:1w6	1.0 (0.2)	0.6 (0.0)	0.9 (0.0)	0.4 (0.0)	0.4 (0.1)	1.6 (0.0)
10me18:0	5.2 (1.0)	5.8 (0.4)	2.6 (0.3)	1.6 (0.1)	1.5 (0.1)	1.6 (0.1)
20:4w6	0.6 (0.1)	0.5 (0.0)	0.1 (0.0)	0.2 (0.1)	0.2 (0.1)	0.4 (0.0)
20:1w9	0.6 (0.0)	0.4 (0.0)	0.8 (0.0)	0.6 (0.0)	0.8 (0.1)	0.6 (0.1)
20:0	0.5 (0.2)	0.3 (0.0)	0.5 (0.0)	0.8 (0.1)	0.8 (0.1)	0.7 (0.2)
21:0	0.6 (0.2)	0.3 (0.0)	0.5 (0.0)	1.0 (0.0)	0.7 (0.3)	0.9 (0.1)
PLFA [b]	42.4 (3.4)	78.6 (4.6)	48.9 (10.8)	31.2 (4.9)	28.7 (4.4)	23.7 (2.0)
PHA [c]	85 (120)	705 (742)	55 (7)	10 (14)	10 (14)	20 (28)

[a] Data are given as mean mol % (S.D.), $n = 5$, with PLFA indicated as defined in the text. Only those PLFA showing significant difference ($P > 0.05$) by ANOVA are given in the table.

[b] Total nmoles PLFA/g dry wt.

[c] Total nmoles PHA/g dry wt.

cm below the surface. Maximum rates of destruction of the alkane occurred at the 52 cm depth which was just above the water level. The lowest increase in biomass occurred at the 88 cm layer which was very close to the level at which the gases and air were injected into the soil bioreactor. Only the PLFA with a statistical difference by ANOVA $P > 0.05$ are listed in the table. Subjecting the data of Table 2 to be a Tukey's significance difference test shows significant increases in the proportions of 16:1w6c as consistently higher in all the soils exposed to propane, *iso*-butane, and butane (Table 3). The combined 18:1w7c + 18:1w8c and 10Me18:0 PLFA showed signifi-

Table 3

Tukey's Significant Difference Maps generated from Tukey's HSD test (SPSS), with the within-experiment, family-wise error rate set at alpha = 0.05 for logarithmically-transformed data from Table 2. Treatment means for each PLFA increase left to right, and those means connected by a common line segment are not significantly different for this test. Abbreviations as in Table 1.

i15:1w10	1A [a]	3A	2B	2A	1B	3B
i15:0	2A	1A	3A	3B	2B	1B
a15:0	2A	1A	3A	3B	2B	1B
15:1w6	3B	2B	3A	1A	1B	2A
br16:0	2A	1A	3A	2B	1B	3B
i16:1w6	1A	1B	2A	3B	3A	2B
10Me15:0	2A	1B	1A	3B	3A	2B
i16:0	3A	2A	3B	1B	1A	2B
16:1w11c/16:1w9c	3B	1B	2B	1A	3A	2A
16:1w7c	2B	2A	1A	3B	1B	3A
16:1w6c	3B	2B	1B	3A	1A	2A
16:1w5c	2B	3A	3B	1B	1A	2A
16:0	3B	3A	2B	1B	2A	1A
br17:0	2A	1A	3A	1B	3B	2B
i17:1w7c	2A	1A	3A	1B	2B	3B
10Me16:0	2A	1A	3A	3B	1B	2B
i17:0	2A	1A	3A	2B	1B	3B
a17:0/17:1w8c	2A	1A	3A	3B	1B	2B
17:1w6/cy17:0(7,8)	2A	1A	3B	2B	1B	3A
cy17:0(5,6)	3A	1B	3B	2B	1A	2A
17:0	2B	3B	3A	1B	2A	1A
10Me17:0	2A	1A	2B	1B	3A	3B
br18:1	2B	3B	1B	1A	2A	3A
18:2w6	1A	2A	2B	1B	3B	3A
18:1w7c+18:1w8c	2B	1B	3B	2A	1A	3A
18:0	2A	3A	3B	1A	1B	2B
11Me18:1w6	2B	1B	2A	3B	3A	1A
10Me18:0	2B	1B	3B	3A	1A	2A
20:4w6	2B	1B	3B	2A	1A	3A
20:1w9	2A	1A	3B	1B	2B	3A
20:0	2A	3A	1A	3B	1B	2B
21:0	2A	3A	1A	2B	3B	1B

[a] 1A, 2A, 3A indicate samples exposed to propane, butane and isobutane recovered from 88 cm, 59 cm, and 14.7 cm below the surface of the soil bed bioreactor. 1B, 2B and 3B are from the same depths in a soil sample not exposed to the hydrocarbons.

cantly higher proportions in two of the gassed soils (Table 3).

Microcosms formed of Rollin muck soils exposed to butane showed concomitant degradation of TCE and butane that was not detected in the control (Figure 1). The control had no oxygen in the headspace.

4.3. PLFA patterns of monocultures isolated with propane or methane on mineral-salts agar

Table 4 shows the PLFA pattern of 4 monocultures grown from single colony isolates grown with propane and 2 grown with methane recovered from the Rollin muck soil. The propane isolates formed colored colonies with the microscopic appearance of Actinomycetes. These monocultures all showed high proportions of the unusual PLFA (16:1w6c) seen in both soils exposed to propane. These bacteria also showed high proportions of tuberculostearic acid, 10Me18:0 a characteristic PLFA of many Actinomycetes [15]. The cyclopropane PLFA cy17:0 or cy19:0 were not detected in organisms grown with propane. The isolates also showed high proportions of

17 : 1w8c which was not detected as a significant PLFA in the subsurface aquifer material or muck soil exposed to propane.

When methane was utilized as carbon source in mineral salts agar, pink colonies typical of type II methane oxidizers were isolated. These monocultures displayed high proportions of the unusual PLFA, 18 : 1w8c which to date has only been found in type II methane-oxidizing bacteria [14–16] and that has been found consistently when soils are exposed to air and methane [17]. The PLFA representative of propane-utilizing isolates were not detected in these methane-utilizing organisms (Table 4).

4.4. Mass spectral identification of characteristic PLFA of methane and propane utilizing bacteria

The characteristic ion fragments of the adducts formed by reaction of DMDS with the 'signature' PLFA found in the propane- and methane-utilizing bacteria stimulated in subsurface materials and muck are given in Table 5. Iso or mid-chain

Table 4

Phospholipid ester-linked fatty acid patterns of monocultures grown with propane (Columns A–D) or methane (Columns E, F) isolated from Rollin Muck soil

PLFA	Mol %					
	Propane-utilizing				Methane-utilizing	
	A	B	C	D	E	F
14 : 0	0.8	0.4	0.7	0.6	nd	nd
15 : 1	0.2	0.2	0.2	0.2	nd	nd
15 : 1w5c	0.1	0.1	nd	Trace	nd	nd
15 : 1	0.7	0.9	nd	0.7	nd	nd
15 : 0	5.8	4.5	15.7	5.3	nd	nd
16 : 1w9c	0.5	0.3	nd	0.3	nd	nd
16 : 1w7c	0.7	17.4	0.7	0.7	0.6	2.9
16 : 1w6c	8.1	7.4	9.4	7.9	nd	nd
16 : 1w5c [a]	nd	Trace	0.2	nd	nd	nd
16 : 0	23.6	20.7	11.4	25.4	0.2	0.2
i17 : 1 [a]	nd	nd	nd	nd	nd	Trace
10Me16 : 0 [a]	Trace	Trace	1	Trace	nd	nd
17 : 1w8	13.2	9.5	13.6	11.2	0.2	0.2
17 : 1w7	2.9	2	4.7	2.5	0.1	0.1
i17 : 0	nd	nd	nd	nd	0.1	0.1
17 : 1w6 [a]	nd	0.1	0.1	0.1	nd	nd
cy17 : 0	Trace	1	0.6	0.1	nd	nd
17 : 0	4.6	3.9	6.2	4.8	0.1	Trace
i18 : 0	3.5	1.7	24.9	3.1	nd	nd
18 : 1w9c	21.9	13.3	2.4	20.2	nd	2.8
18 : 1w8c	nd	Trace	0.4	nd	22.9	18.9
18 : 1w7c	0.1	9.9	0.1	1.7	75.4	74.6
18 : 1w5c [a]	nd	Trace	nd	nd	0.1	0.1
18 : 0	1.1	1.5	Trace	1.3	0.1	Trace
br19 : 1	nd	nd	nd	0.9	nd	nd
10Me18 : 0	10.5	4.4	6.8	11.5	nd	nd
19 : 1	1.3	0.6	0.9	1	nd	nd
cy19 : 0	nd	nd	nd	0.1	nd	nd
Total PLFA	78.9	98.6	35.8	72.7	179.8	208.0

[a] Identification based on GC retention time only. nmol/g dry wt.

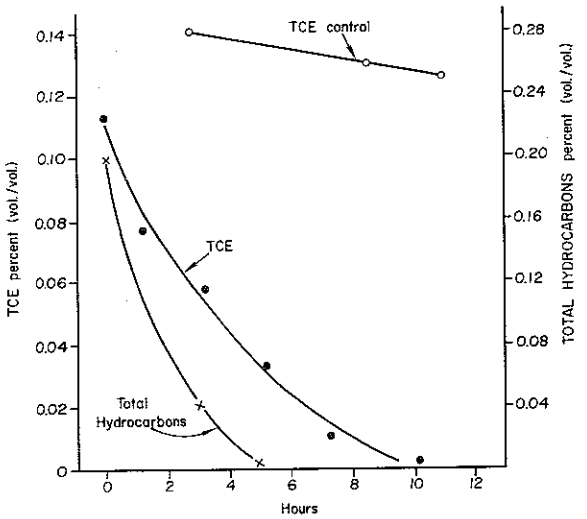

Fig. 1. Concomitant removal of the vapors of trichlorethylene and a mixture of propane, *n*-butane and isobutane in serum bottle microcosms constructed with Rollin muck soil. Conditions of the experiment are as described [9].

Table 5

Structural characterization of 'signature' PLFA from methane-(M) and propane-(P) utilizing bacteria by electron impact mass spectrometry

Mono-unsaturated fatty acids [a]	M+	w-fragment [b]	Δ-fragment [c]	Type
15:1w5	348(26.7) [d]	117(88.9)	231(100)	P
16:1w9	362(28.8)	173(74.2)	189(46.7)	P
16:1w6	362(22.9)	131(89.2)	231(100)	P
17:1w8	376(20.6)	159(82.8)	217(100)	P
17:1w7	376(43.9)	145(64.5)	231(99.9)	P
18:1w8	390(16.4)	159(78.0)	231(100)	M
18:1w7	390(11.0)	145(60.2)	245(83.0)	M

Other fatty acids [e]	M+	diagnostic ions	Type
i18:0	298 (8.5)	225(22.9), 43(60.7)	P
10Me18:0	312(12.3)	171 (7.8), 199(15.3)	P

[a] Identified as dimethyl disulfide adduct.
[b] w-fragment indicates fragment including aliphatic end of molecule.
[c] Δ-fragment indicates fragment including carboxylic end of molecule.
[d] Fragment masses given as Mass (data given as M/Z abundance).
[e] Identified as fatty acid methyl esters.

branching is defined by the fragmentation patterns given in the lower section of Table 5.

5. DISCUSSION

5.1. Usefulness of Biomarkers to define shifts in microbial community structure

Assessment of the microbial community found in soils and sediments presents complex assay problems. Determination of biomass by classical viable counts methods require that microbes be quantitatively recovered from the substratum and that the growth medium be universal. Direct microscopic estimation of biomass are difficult, particularly in the surface soils where there is great diversity in the microbial morphology and assumptions of biovolumes can be complex. Use of biomarker components to estimate biomass requires only that the component be universally distributed, be reproducibly related to the cell biomass, and be quantitatively recoverable. The biomarker components universally distributed in microbes such as intercellular ATP, cell wall muramic acid and polarlipid phosphate and ester-linked fatty acids have been shown to give identical biomass and numerical estimations in uncontaminated subsurface soils as the direct microscopic counts [3]. In the soil materials analyzed in this study, the microbiota consisted of uniformly sized small coccibacillary bacteria and the biovolume was readily estimated.

If specific biomarker component patterns can be related to specific subsets of the microbial community, then the patterns can be used to define the microbial community structure. PLFA are presently the most sensitive and useful chemical measures of community structure [17]. The validation of the use of biomarkers as 'signatures' for particular groups of organisms has been based on the induction of shifts in microbial community structure by altering the microenvironment with resulting changes that can often be predicted (such as the formation of fungus 'heaven' and 'hell' on biofilms suspended in a subtropical estuary by manipulation of nutrients and inhibitors) [18]. A second validation rests on the alteration of community structure by predator grazing with known

specificity (such as selective removal of non-photosynthetic microeukaryotes from sandy sediments by sand dollars) [19]. Possibly the best validation is the detection of biomarkers for specific microbes in the environment that can be found in specific isolates from that environment. The present study extends this validation to the alkane-oxidizing bacteria in surface soils and aquifer materials.

In these studies the position and confirmation of unsaturation of the monoenoic PLFA have been important. Gillan and Hogg [20] used the position of unsaturation in monounsaturated FAME to classify sedimentary microorganisms into 'chemotypes'. Nichols et al. [14,21] found unusual patterns of unsaturation in the monoenoic PLFA of the methane-oxidizing bacteria and the pathogen *Francisella tularensis.* With both the acid-forming *Thiobacilli* [22], and the sulfate-reducing bacteria [23–26], the monoenic PLFA analysis was very important. These 'signature' patterns were sufficiently unique for detection of these organisms in environmental samples [27,28]. Guckert et al. [5] used changes in the proportions of monoenoic PLFA with defined positions of unsaturation to indicate the shifts in the benthic marine microbiota from aerobic to anaerobic growth.

The PLFA analysis not only provides insight into the microbial community structure but also the nutritional status. Shifts in PLFA pattern occur in some bacteria with culture conditions. Cyclopropane rings are formed from monoenoic esters of specific phospholipids in the membranes of bacteria [29]. Their formation with the concomitant decrease in monoenoic PLFA occurs in monocultures that undergo metabolic stress such as stationary phase growth [30]. The same phenomenon has been observed for the benthic marine microbiota [31]. Guckert et al. [32] showed that accumulation of *trans* monoenoic PLFA correlated with the starvation response in *Vibrio cholerae.* Similar accumulations of *trans* monoenoic PLFA occurred in subsurface bacteria grown under conditions of starvation (Pfiffner, unpublished observations). Shifts in PLFA in some bacteria also have been detected when the major carbon source is changed. Carbon sources with odd numbered chains can increase the proportions of odd numbered PLFA [25,26,33] and the soils and organisms exposed to propane generally show higher proportions of odd numbered PLFA.

The use of patterns of PLFA to define the community structure of microbial consortia have been utilized to show detrial succession, the effects of disturbance, or predation in marine sediments, the response to subsurface aquifer pollution and in environment effects testing [2,17,34–36]. The effects of shifts in microbiota biofouling and corrosion studies have also been reviewed [37,38].

5.2. Structural identification of unusual PLFA from propane- and methane-utilizing bacteria

The unusual fatty acids from the phospholipids of the methane- and propane-utilizing bacteria were tentatively identified from their retention times when compared to authentic standards. To define the structure of the PLFA, derivatives were made and the structures confirmed based on interpretation of their fragmentation patterns obtained with electron ionization MS (Table 5).

The position of unsaturation in monoenoic PLFA was the key to defining the 'signature' PLFA biomarkers for the methane- and propane-utilizing bacteria that were induced by exposure of soils to the hydrocarbons. Unsaturations at the w6 position in the 16 : 1w6c found in high proportions in the propane-utilizing bacteria and at the w8 position in the 18 : 1w8c of the methane-oxidizing bacteria are sufficiently unusual to be diagnostic in environments as complex as surface soils and aquifer materials. High proportions of 16 : 1w6c in the PLFA are not unique to propane-oxidizing bacteria. This PLFA is also found in type I methane-oxidizing bacteria [14,16,39]. However, the high proportion of 10Me18 : 0 PLFA (characteristic of many Actinomycetes) [15] and 17 : 1 isomer present in the propane-oxidizing bacteria found in this environment are not found in the type I methane-oxidizing bacteria. Based on the differences in PLFA profiles like those observed in this study, it should be possible to use these methods to monitor shifts in microbial biomass and community structure in aquifers where indigenous bacteria are stimulated to transform pollutant compounds.

DISCLAIMER

Although the research in this article has been funded wholly or in part by the United States Environmental Protection Agency, it has not been subjected to the Agency's peer and administrative review and therefore may not necessarily reflect the views of the Agency and no official endorsement should be inferred.

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