Measurement of methanotroph and methanogen signature phospholipids for use in assessment of biomass and community structure in model systems*

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Abstract—Methanotrophic biomass and community structure were assessed for a soil column enriched with natural gas. An increase in microbial biomass, based on phospholipid ester-linked fatty acids (PLFA), was apparent for the natural gas-enriched column relative to a control column and untreated surface soil. Following GC–MS analyses of the derivatized monounsaturated fatty acids, the major component (22% of the PLFA) of the natural gas-enriched column was identified as 18:1Δ10c. This relatively novel fatty acid has only been previously reported as a major component in methanotrophs. Its presence in the soil, together with other supportive evidence, implies that this microbial metabolic group makes a large contribution to the column flora. Other microbial groups were also recognized and differences compared between the soils analysed. A recently developed HPLC method for the separation and characterisation of archaebacterial phospholipid-derived signature di- and tetra-ether lipids was used to examine methane-producing digesters. With this technique, methanogenic biomasses of approximately 10¹⁰ bacteria per g dry weight of digestor material were determined. Differences between ratios of diether to tetraether phospholipids were apparent for the digestors analysed, though the causes are at this stage unknown. Taken together, these two methods can be used to estimate methanotrophic and methanogenic contributions in both model systems and environmental samples.

Key words: archaebacteria, methanogens, methanotrophs, phospholipids, fatty acids, ether lipids, GC–MS, HPLC, FT-IR, biomass, community structure

INTRODUCTION

The interactions of aerobic and anaerobic bacteria in aquatic environments are extremely complex (Jones, 1982). Methane-oxidizing bacteria occupy an important niche in biogeochemical cycling, and the methanogenic bacteria, which are ubiquitous in most anaerobic sediments (Zeikus, 1977), are responsible for one of the major end products from the decomposition of organic matter (Wolfe, 1971; Zehnder, 1978). Consequently, methods of identifying and quantifying the presence of methane-oxidizing and methanogenic bacteria would be extremely useful to both organic geochemists and microbial ecologists. Not only are these two groups of organisms significant in the biogeochemical carbon cycle, but an increasing number of reports have documented the biodegradation of chlorinated ethenes and other toxic substances under either methanogenic or methanotrophic conditions (Fogel et al., 1986; Vogel and McCarty, 1985; Wilson and Wilson, 1983). Techniques of directly measuring these organisms would, therefore, also be of use in studies of recent sediments and projects aiming to optimize biotransformation conditions.

As certain fatty acids are specific to certain bacteria and different groups of bacteria may have different fatty acid compositions (Goodfellow and Minnikin, 1985; Lechevalier, 1977), it has been the practice to determine microbial community structure through the use of fatty acid profiles (e.g. Bobbie and White 1980; Gillan et al., 1983; Harvey et al., 1984; Perry et al., 1979). The advantages of biochemical procedures over classical enumeration procedures have been reviewed—analysis of fatty acids derived from phospholipids of microorganisms associated with biofilms, soils and sediments provides a reproducible and quantitative measure of the biomass and community structure of microbial assemblies (White, 1983). Some investigators have analysed sedimentary fatty acids derived from the total lipid extract (Gillan and Hogg, 1984; Parkes and Taylor, 1983; Perry et al., 1979; Volkman and Johns, 1977; Volkman et al., 1980). Other workers have examined the fatty acids of individual lipid classes, such as the phospholipids, rather than the total lipid (Gillan and Sandstrom, 1985; Guckert et al., 1985; White, 1983). Measurement of microbial biomass using the phospholipid approach have been shown to correlate well with measurements by other methods [e.g. muramic acid levels and total adenosine triphosphate (White, 1983)]. In addition, results obtained using the fatty
Acid techniques have proven to be free of the distortions encountered using methods that either require quantitative removal of organisms from surfaces, or introduce selectivity when organisms are grown on artificial substrates.

Archaeabacteria lack the ester-linked phospholipid fatty acids found in the cellular membranes of eubacteria (Kates, 1978; Langworthy, 1977; Tornabene and Langworthy, 1979). Membrane lipids of archaeabacteria, including the methanogens, consist of two isoprenoid-branched, hydrocarbon side chains that are ether-linked to the glycerol phosphate backbone (Kates, 1972; Kates et al., 1965). In some cases, the side chains of two diether lipids are linked head-to-head to form tetraether lipids (Langworthy et al., 1981).

Analysis of the polar phospholipids derived from eubacteria and archaeabacteria as described briefly above has been used primarily by microbial ecologists. Such information is also relevant in organic geochemical studies, since polar lipids represent the major portion of the lipid content of most bacteria (Harwood and Russell, 1984), and would therefore be expected to contribute substantially to bacterial lipid input to recent sediments.

Polar phospholipid data will be presented here for two environments: one enriched for methanotrophs and the other known to contain methanogens. For the first, phospholipid fatty acid profiles and other data were obtained for a soil column enriched for methanotrophs and other organisms capable of oxidizing minor alkane components present in natural gas. These data are part of a larger program that has been examining biotransformation of organic low-molecular-weight pollutants in soil. Wilson and Wilson (1985) used natural gas in a soil column to enrich a population of bacteria capable of degrading trichloroethylene. It follows that methanotrophic bacteria could be useful in removing halogenated one- or two-carbon compounds from contaminated environments (Henson et al., 1985). Techniques of estimating methanotrophic biomass in natural systems could, therefore, also be useful in monitoring and optimizing conditions for their growth and activity in systems designed for the biotransformation of contaminants. To our knowledge, prior to this study methods for the direct measurements of methanotrophic bacteria in microcosm or field samples did not exist. Data obtained from the application of a recently developed high performance liquid chromatography technique, that separates and measures archaeabacterial di- and tetra-ether lipids without side-chain cleavage, to environmental samples will also be reported. The biogenesis of methane under controlled conditions has the potential to be a significant source of renewable energy. Application of the signature phospholipid approach to anaerobic digestion systems can be used to further understanding of the microbial ecology of these fermentation systems. The overall goal of this study is to demonstrate the potential of using phospholipid components to study microbial consortia that produce or consume methane, a fundamental molecule in the biogeochemical carbon cycle.

MATERIALS AND METHODS

Sample Description

Freeze dried bacteria

Methanotrophic bacteria were obtained from Dr R. S. Hanson, Gray Freshwater Biological Institute, MN, U.S.A. and Dr L. L. Jahnke, NASA-Ames Research Centre, CA, U.S.A. Lyophilized cells of Methanobacterium thermoautotrophicum strain Hveragerdi were a gift from J. P. Kaiser and Dr K. Hanselman of the University of Zurich, Zurich, Switzerland. Lyophilized cells of Methanosarcina barkeri strain Jolich were obtained from Dr P. A. Scherer of Scherpunkt Biotechnologie, Fachhochsule Weihenstephan Lowentorgebaude, Friesing, F.R.G.

Culture conditions for the methanotrophic and methanogenic bacteria were as described elsewhere (Nichols et al., 1985; Jahnke and Nichols, 1986; Binder et al., 1981; Scherer et al., 1986).

Soil and natural gas-enriched column

Soil columns were prepared with Lincoln Fine Sand obtained near Ada, OK, U.S.A. as described previously (Wilson et al., 1981; Wilson and Wilson, 1985; Henson et al., 1985). Column A was exposed to a headspace of 0.6% natural gas (77% methane) in air and additionally received a mixture of halogenated hydrocarbons. Results for the degradation of these compounds are reported elsewhere (Henson et al., 1985). Column B, the control column, was inhibited by the addition of 0.1% sodium azide to the water and was not exposed to natural gas. After three months of operation at 22-25°C, the columns were unpacked and increments from the top 0-10 cm and bottom 148-150 cm were obtained for subsequent lipid analysis. Soil sample C, from 0 to 10 cm, was acquired from the same site near Ada and was untreated prior to analysis. For samples from all depths, pH ranged from 6.1 to 6.6; cation exchange capacity from 4.8 to 2.3 meq/100 g; sand from 89 to 95%; silt from 4.0 to 8.8% and clay from 1.5 to 3.5%. Organic carbon contents of the 0-10 cm and 148-150 cm section were 0.2 and 0.02% respectively. All samples were lyophilised prior to lipid extraction.

Digestors

Anaerobic sewage sludge was supplied by W. C. Leseman, Laboratory Director, Waste Water Treatment Plant, Tallahassee, FL. Additional specimens from various levels of an anaerobic digester fed by water hyacinth were obtained from Dr D. Cenoweth, Agricultural Engineering Department, Uni-
Univrsity of Florida, Gainesville, FL. Samples were freeze-dried prior to extraction.

**Lipid extraction and fractionation**

A schematic illustration of the lipid fractionation procedure is shown in Fig. 1. Lipids were quantitatively extracted with the modified one-phase chloroform methanol Bligh and Dyer extraction (White et al., 1979a). After separation of phases, the lipids were recovered in the chloroform layer, the solvents removed in vacuo, and the lipids stored under nitrogen at -20°C. Replicate extractions of each bacteria and sediment sample were performed.

The lipid was transferred in a minimum volume of chloroform to a silicic acid column (Unisil, 100–200 mesh, Clarkson Chemical Co., Inc., Williamsport, PA) and fractionated into neutral lipids, glycolipids, and phospholipids by elution with chloroform, acetone, and methanol respectively (Guckert et al., 1985). The fractions were collected in test tubes fitted with Teflon-lined, screw-caps and dried under a stream of nitrogen.

**Diacylphospholipid and soil-residue-bound lipids**

The mild alkaline methanolysis procedure (White et al., 1979a) was applied to the phospholipid fraction. The technique was modified slightly by using hexane:chloroform (4:1, v/v), rather than chloroform, to extract the resulting fatty acid methyl esters (FAME). Bound lipids, which include lipopolysaccharide (LPS) derived normal and hydroxy fatty acids, were recovered by acidification of the sediment residue in 50 ml of 1N HCl. After being refluxed at 100°C for 3 hr and cooled, the contents were transferred to a separatory funnel with washes of 25 ml and 2 x 5 ml of chloroform. The two phases were allowed to separate overnight, the chloroform phase recovered, and the solvent removed under a stream of nitrogen. The fatty acids were then methylated and converted to their corresponding trimethylsilyl (TMSi) ethers (Pierce Chemical Co., Rockford, IL).

**Ether lipids**

Phospholipid fractions were hydrolysed by adding 1 ml of chloroform-methanol-conc. HCl 1:10:1 (v/v/v) and by heating to 100°C for 2 hr. Glycerol diethers and tetraethers were formed by acidic hydrolysis of the phospholipids and were extracted after the addition of 1 ml of hexane and 1 ml of water. The pooled organic fractions from two extractions were dried under a stream of nitrogen. Digestor samples were first subjected to mild alkaline methanolysis and thin-layer chromatography (Mancuso et al., 1986a). The origin of the thin-layer plate was scraped. The phospho-ether lipids were eluted with CHCL3:MeOH (1:2, v/v) and dried under a stream of nitrogen. This fraction was then acid-hydrolysed as described above.

**Gas chromatography of diacyl phospholipid FAME and LPS hydroxy FAME**

FAME samples were dissolved in hexane with methylnonadecanoate (19:0) as the internal injection standard. Separation of individual normal and hydroxy fatty acids was performed by high resolution gas chromatography, using a Hewlett-Packard 5880A gas chromatograph equipped with a flame ionization detector. Samples were injected at 50°C in the splitless mode with a 0.5-min venting time, using a Hewlett-Packard 7672 automatic sampler and a non-
polar cross-linked methyl silicone capillary column (50 m x 0.2 mm i.d., Hewlett-Packard). The oven temperature was programmed from 50 to 160°C at 10°C per min, then at 4°C per min to 300°C. Hydrogen was used as the carrier gas (1 ml/min). The injector and detector were maintained at 300°C.

Tentative peak identification, prior to GC–MS analysis, was based on comparison of retention times with those obtained for standards from Supelco Inc. (Bellefonte, PA) and Applied Science Laboratories Inc. (State College, PA) and previously identified laboratory standards. Peak areas were quantified with a Hewlett-Packard 3350 series programmable laboratory data system operated in an internal standard program. Data on the fatty acid composition of these samples are the means of replicate samples for the three soil types; each replicate was extracted independently (column A, n = 10; column B, n = 2; untreated soil C, n = 2). Standard deviations for individual fatty acids were generally in the range 0–30%, typically <10%.

Gas chromatography–mass spectrometry (GC–MS)

GC–MS analyses of FAME and OH-FAME were performed on a Hewlett-Packard 5996A system fitted with a direct capillary inlet. The same column type described above was used for analyses. Samples were injected in the splitless mode at 100°C with a 0.5-min venting time, after which the oven temperature was programmed to 300°C at either 3 or 4°C per min. Helium was used as the carrier gas. MS operating parameters were: electron multiplier between 1300 and 1400 V, transfer line 300°C, source and analyser 250°C, autotune file DFTPP normalized, optics tuned at m/z 502, electron impact energy 70 eV. Mass spectral data were acquired and processed with a Hewlett-Packard RTE-6/VM data system.

Quantification and determination of monounsaturated fatty acid double-bond configuration

The dimethyl disulfide (DMDS) adducts of monounsaturated FAME were formed to determine double-bond position and geometry, using methods previously described (Dunkelblum et al., 1985; Nichols et al., 1986a). Samples were analysed by GC and GC–MS as described above. Both the monounsaturated FAME isomers and DMDS adducts were separated under these chromatographic conditions, allowing direct quantitation of isomers from chromatograms.

Separation, quantification and identification of ether lipids

Ether lipids were separated by high performance liquid chromatography (HPLC) with an isocratic solvent system of hexane: n-propanol (99:1) on an amino-bonded silica column with a refractive index detector (Mancuso et al., 1986, b). The diether internal injection standard, 1,2-O-hexadecyl-rac-glycerol, was purchased from Sigma (MI). Authentic tetraether standard was a gift of G. Pauly and Dr E. S. Van Vleet of the University of South Florida, FL. Peak areas were determined by triangulation. Peak areas for dilutions of ether lipids correlated well with both diether (r = 0.93) and tetraether (r = 0.99) standards, giving a linear response in the concentration range used. An equal response factor for the diether and tetraether was determined. Identification of ether lipids was confirmed with Fourier transform-infrared spectrometry, using diffuse reflectance of separated components as described in Mancuso et al. (1986, b).

Fatty acid nomenclature

Fatty acids are designated by total number of carbon atoms: number of double bonds, followed by the position of the double bond from the Δ (carboxylic) end of the molecule. The suffixes c and t indicate cis and trans geometry. The prefixes i and a refer to iso and anteiso branching respectively, and the prefix OH indicates a hydroxy group at the position indicated. Other methyl-branching is indicated as position of the additional methyl carbon from the carboxylic (Δ) end, i.e. 10-methyl 16:0. Cyclopropane fatty acids are designated with the prefix cy, with the ring position relative to the carboxylic end of the molecule in parentheses.

RESULTS AND DISCUSSION

Natural Gas-Enriched Soil

Microbial biomass

Conversion of phospholipid ester-linked fatty acid (PLFA) concentration data (Table 1) into number of cells per gram of sediment can be performed using the following approximations (White et al., 1979a): average bacteria the size of E. coli contain 100 μmol PLFA/g (dry weight) and 1 g of bacteria is equivalent to 5.9 x 10^12 cells (dry weight). Using these conversion factors the following microbial cell concentrations (number of cells/g dry weight of sediment) were determined for the upper 10 cm of the natural gas-enriched column A, the azide-inhibited column B and untreated surface soil C respectively: A 3.2 x 10^9; B 5.3 x 10^8 and C 1.4 x 10^9. Enrichment of the soil with natural gas was thus accompanied by an increase in microbial biomass. Microbial biomass in the soil from the bottom of the two columns was at least two orders of magnitude less than in the upper layers.

The validity of the PLFA approach may be questioned on the grounds that bacterial cell composition may vary with growth conditions (White et al., 1979b). Under conditions expected in natural communities, however, the bacteria contain a relatively constant proportion of their biomass as phospholipids (White et al., 1979a, b). In this study, the PLFA approach produced similar results to those obtained by the acridine orange direct count method at the nearby Lula, Oklahoma site (7.0–8.2 x 10^9 cells/g wet weight; Baulkwill, personal commu-
Table 1. Composition of phospholipid ester-linked fatty acids from: a natural gas-enriched soil column A, a sodium azide-treated soil column not exposed to natural gas B, and untreated surface soil C

<table>
<thead>
<tr>
<th>Fatty acid</th>
<th>A 0-10 cm&lt;sup&gt;*&lt;/sup&gt;</th>
<th>B 0-10 cm&lt;sup&gt;*&lt;/sup&gt;</th>
<th>C 0-10 cm&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Proposed source&lt;sup&gt;x&lt;/sup&gt;</th>
<th>Fatty acid</th>
<th>A 0-10 cm&lt;sup&gt;*&lt;/sup&gt;</th>
<th>B 0-10 cm&lt;sup&gt;*&lt;/sup&gt;</th>
<th>C 0-10 cm&lt;sup&gt;*&lt;/sup&gt;</th>
<th>Proposed source&lt;sup&gt;x&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>12:0</td>
<td>TR&lt;sup&gt;+&lt;/sup&gt;</td>
<td>--</td>
<td>--</td>
<td>17:0</td>
<td></td>
<td>0.86</td>
<td>0.25</td>
<td>0.76</td>
<td>S, A</td>
</tr>
<tr>
<td>13:0</td>
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<td>--</td>
<td>--</td>
<td>17:0</td>
<td></td>
<td>1.1</td>
<td>0.43</td>
<td>0.53</td>
<td>S, A</td>
</tr>
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<td>TR&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.04</td>
<td>17:0</td>
<td></td>
<td>0.21</td>
<td>0.07</td>
<td>0.15</td>
<td>S, A</td>
</tr>
<tr>
<td>15:0</td>
<td>0.75</td>
<td>0.08</td>
<td>0.08</td>
<td>17:0</td>
<td></td>
<td>0.24</td>
<td>0.10</td>
<td>0.25</td>
<td>A</td>
</tr>
<tr>
<td>15:1&lt;sup&gt;y&lt;/sup&gt;</td>
<td>0.12</td>
<td>TR</td>
<td>TR</td>
<td>S</td>
<td>18:4&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1.1</td>
<td>0.03</td>
<td>0.05</td>
<td>E</td>
</tr>
<tr>
<td>16:0</td>
<td>1.5</td>
<td>0.48</td>
<td>1.4</td>
<td>18:2&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>0.59</td>
<td>0.08</td>
<td>1.1</td>
<td>E</td>
</tr>
<tr>
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<td>18:1&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
<td>1.6</td>
<td>0.51</td>
<td>1.6</td>
<td>M</td>
</tr>
<tr>
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<td></td>
<td>12.0</td>
<td>--</td>
<td>--</td>
<td>M</td>
</tr>
<tr>
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<td>0.11</td>
<td>18:1&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>9.2</td>
<td>0.60</td>
<td>2.1</td>
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<td></td>
<td>1.0</td>
<td>0.28</td>
<td>0.60</td>
<td>M, A</td>
</tr>
<tr>
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<td>--</td>
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<td></td>
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<td>5.6</td>
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<td>0.81</td>
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<td></td>
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<td>1.9</td>
<td>S, A</td>
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<td>20:4&lt;sup&gt;c&lt;/sup&gt;</td>
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<td>0.78</td>
<td>0.03</td>
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<td>E</td>
</tr>
<tr>
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<td>2.3</td>
<td>0.47</td>
<td>1.2</td>
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<td></td>
<td>0.12</td>
<td>TR&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0.08</td>
<td>E</td>
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<tr>
<td>18:0</td>
<td>0.07</td>
<td>--</td>
<td>--</td>
<td>20:3</td>
<td></td>
<td>0.12</td>
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<td>0.17</td>
<td>E</td>
</tr>
<tr>
<td>18:0</td>
<td>4.6</td>
<td>1.2</td>
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<td></td>
<td>0.03</td>
<td>--</td>
<td>0.07</td>
<td>A</td>
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<tr>
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<td>0.70</td>
<td>0.19</td>
<td>0.62</td>
<td>S</td>
<td>20:0</td>
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<td>0.08</td>
<td>0.09</td>
<td>M, A</td>
</tr>
<tr>
<td>10Me16:0</td>
<td>1.10</td>
<td>0.48</td>
<td>1.3</td>
<td>S, A</td>
<td>others</td>
<td>1.0</td>
<td>0.53</td>
<td>1.7</td>
<td>A, E</td>
</tr>
<tr>
<td>17:0</td>
<td>0.59</td>
<td>0.25</td>
<td>0.67</td>
<td>Total FAMES&lt;sup&gt;+&lt;/sup&gt;</td>
<td>53.8</td>
<td>8.9</td>
<td>23.0</td>
<td>(12.2)</td>
<td>(0.14)</td>
</tr>
</tbody>
</table>

<sup>*</sup>Sediment depth.

<sup>x</sup>Proposed source of fatty acid in sediments: M = methanotroph; S = sulphate-reducing bacteria; A = Actinomycetes; E = eukaryotes.

<sup>y</sup>TR; trace <0.03 nmol/g.

<sup>+</sup>Not detected.

<sup>+</sup>Position of unsaturation not determined due to insufficient sample. Component coelutes with 15:1<sup>c</sup>.

16:1<sup>c</sup> also present.

<sup>+</sup>Mean and (standard deviation).
The agreement between biomass assessments obtained using the PLFA and AODC methods add further support to the use of PLFA as a biomass assessment tool for surface soils (this study) and subsurface aquifer sediments (Balkwill et al., 1987). The use of this approach has been previously reported and validated for estuarine and benthic marine sediments (White, 1983).

**Fatty acid profiles**

The PLFA profile obtained for a soil column A, enriched for methanotrophs and other organisms capable of oxidizing other minor alkane components present in natural gas, was compared to the profiles of sodium azide-treated soil not exposed to natural gas (column B) and untreated surface soil (C). A higher proportion of C₁₆ saturated and monounsaturated PLFA (49% of total fatty acids) was observed for the natural gas-enriched soil (column A) than for column (18%) or the untreated soil (25%) (Table I and Fig. 2). Methanotrophic bacteria are grouped into two types based on differences in intracytoplasmic membrane organization and carbon metabolism (Anthony, 1982; Higgins et al., 1981). Type I bacteria contain fatty acids, predominantly 16-carbons in length, with saturated and monounsatur-
ated acids present, whilst type II methanotrophic bacteria have monounsaturated 18-carbon chain-length (18:1) fatty acids as the predominant PLFA (Makula, 1978; Urakami and Komagata, 1984). The higher proportion of C_{16} relative to C_{18} PLFA in column A indicates that type II methanotrophs are more abundant than type I organisms in this column (Fig. 2).

Precise determination of double-bond position and geometry using the DMDS procedure makes it possible to distinguish signature fatty acids specific to methanotrophic bacteria. The major component (22% of total PLFA) in the natural gas-enriched soil column A was the relatively novel monounsaturated PLFA 18:1A10c (Table 1). This fatty acid was not detected in either control column B or the untreated surface soil C. It has only been reported as a major component in type II methanotrophic bacteria, including *Methylosinus trichosporium* (Makula, 1978; Nicols *et al.*, 1985). To our knowledge, this is the first report of the presence of 18:1A10c as the major fatty acid in a soil sample. The high observed abundance of this component in the methane-enriched soil is consistent with a substantial contribution of *M. trichosporium* or related organisms to the microbial assembly.

The second most abundant component detected in the methane-enriched column A, 18:1A11c, was the dominant component (84–89% of the total PLFA) in two strains of the type II methanotroph *Methylo bacterium organophilum* (Nichols *et al.*, 1985). Thus, it appears that *M. organophilum* or related type II methanotrophs may also contribute a substantial proportion of 18:1A11c, and therefore to the overall microbial biomass in soil column A. This acid (18:1A11c) is the most commonly detected bacterial C_{18} monounsaturated fatty acid in many environments (Gillian and Hogg, 1984; Guckert *et al.*, 1985), and thus sources additional to type II methanotrophic bacteria may also be possible. In the present case, however, the significant increases in both the absolute and relative proportions of 18:1A11c, when the natural gas-enriched soil column A is compared to the control column and untreated surface soil, are consistent with a large increase in the biomass of *M. organophilum* or related type II methanotrophs.

Several other observations further support the view that methanotrophic, in particular type II, organisms contribute significant portions of the microbial biomass to the natural gas-enriched column. Poly-β-hydroxybutyrate (prokaryotic endogenous storage polymer) to PLFA ratios observed for the natural gas-enriched soil column were close to ratios determined for type II methanotrophs (unpublished data). The obligate methanotroph *Methylococcus capsulatus* and other type I methanotrophic bacteria have not been shown to form this polymer (Anthony, 1982). Concurrent microbiological culturing experiments have resulted in the isolation of pure type II methanotrophs and methylo troph enrichment cultures from soil material analysed in this study (Henson, personal communication). Preliminary analyses of PLFA from the culture material has shown the presence of high relative levels of 18:1A10c and 18:1A11c.

Analysis of the bound lipids, which include lipo polysaccharide derived β-hydroxy fatty acids, also showed a profile similar to those observed for pure and mixed cultures of methanotrophs. Seven β-OH acids were detected, with even-carbon numbered components predominating. β-OH 14:0, β-OH 16:0 and β-OH 18:0 generally accounted for approximately 80% of the total OH acid content, as previously reported for methanotrophs (Nichols *et al.*, 1985). This β-hydroxy acid profile is not unique to methanotrophs. Taken together with PLFA and PHB data, and preliminary results from culturing experiments, the observed hydroxy acid pattern is, however, consistent with methanotrophs making a major contribution to the soil microbial community within column A.

In addition to the large community of methanotrophic bacteria in the natural gas-enriched column A, other microbial populations were distinguished through PLFA compositional data. The presence of a series of 10-methyl-branched fatty acids in all soil samples analysed (Table 1) is consistent with the presence of members of the Actinomycetes (O’Donnell *et al.*, 1982). The relative proportion of these components is significantly lower in column A than in either column B or the untreated surface soil. A number of fatty acids reported in sulphate-reducing bacteria were present in all samples (Table 1) (Boon *et al.*, 1977; Edlund *et al.*, 1985; Taylor and Parkes, 1983).

The branched chain monoenoic fatty acids, i17:1A9c and i15:1A9c, are common to *Desulfovibrio* spp., and 10-methyl 16:0 is a major component in *Desulfo bacter*. Iso 17:1A9c has also been detected recently in several *Flexibacter* (Nichols *et al.*, 1986b). In the *Flexibacter* analysed, i17:1A11 was also present in proportions similar to those of i17:1A9c. As i17:1A11 was not detected in this study, the contribution from *Flexibacter* is probably minimal.

A minor contribution from eukaryotic organisms is occurring in all samples, as the C_{16} polyunsaturated fatty acids 20:4Δ5 and 20:5Δ5 are specific to eukaryotes (Bobbie and White, 1980; Erwin, 1973; Volkman *et al.*, 1980).

It is possible to use differences in PLFA profile data noted above and average eubacterial PLFA compositional data to estimate and contrast the contribution of methanotrophic, actinomycetes, sulphate-reducing, eukaryotic and other organisms in the three soils analysed (Fig. 3). These estimations are based on the following approximations. For example:

1. In methanotrophic type II organisms containing 18:1A10c, this acid represents between
37-51% of the extractable fatty acids (Makulla, 1978; Nichols et al., 1985);
(2) In *Desulfovibrio* spp., i17:1t7c is present at between 20 and 41% of the total fatty acids (Boon et al., 1977; Edlund et al., 1985; Taylor and Parkes, 1983);
(3) The C20 PUFA typically represent ~20% of the total fatty acids in micro-eukaryotes, including protozoa (e.g. Volkman et al., 1980; Phillips, 1984);
(4) In actinomycetes that contain 10-methyl-branched fatty acids, these components account for 4.6-36.3% of the total fatty acids (O'Donnell et al., 1982).
(5) The group designated “other organisms” will include those bacteria with 18:1Δ11c as the major component without the presence of 18:1Δ10c. Although type II methanotrophs such as *M. organophilum* may be the major source of 18:1Δ11c in column A, their contribution has not been calculated because 18:1Δ11c is ubiquitous in bacteria.
Calculations using these approximations do not take into account contributions of these components from other organisms. Similarly, changes in fatty acid profiles due to differences in nutrient or growth conditions may contribute to the observed differences. Nonetheless, we believe, in spite of these considerations, that such an approach at this stage will highlight PLFA differences in terms of the contributing microbial community.
Unidentified cyclic components were detected by GC and GC-MS of the neutral lipid fractions of methanotrophic bacteria analysed in this study. Further work is planned to determine the structure of these components and to assess their potential to act as signatures for methanotrophs in both manipulated microcosms and environmental samples.

**Methanogenic Cultures and Digestors**

**Pure cultures**
HPLC analysis of lipids from methanogenic monocultures has made possible the quantification of glycerol-ether lipids (Mancuso et al., 1986a). Figure 4 illustrates the ability of this technique to resolve glycerol diethers and tetræthers isolated from the phospholipid fraction of *M. thermoautotrophicum*.
strain Hveragerdi. Data for this analysis and for M. barkeri strain Jolich are summarized in Table 2. The molar ratio of diether to tetraether was 1:7 for M. thermoautotrophicum and 1:0 for M. barkeri. The latter archaebacterium is known to lack the tetraether lipid (Langworthy et al., 1981). The total amount of glycerol-ether phospholipid measured was 2.3 and 2.6 (μmol/g dry wt) for M. barkeri and M. thermoautotrophicum, respectively. These results, combined with analyses of other methanogenic monocultures obtained from a variety of sources (unpublished data), yielded an average value of 2.5 μmol glycerol-ether phospholipid per g dry weight of methanogenic bacterial cells.

**Digestor Samples**

The total glycerol-ether phospholipid ranged from 0.05 (top) to 0.03 (bottom) μmol/g dry wt. These values converted to give corresponding biomass of 1.2 × 10^11 (top) and 7.1 × 10^10 cells/g (bottom) dry wt for these samples. Methane production rates (unpublished data) determined for the top and bottom layers of the digestor correlate well with these biomass values. A comparison of the sewage sludge and the water hyacinth digestors revealed a 10-fold difference in methanogenic biomass in addition to differences in the diether to tetraether ratio, which is discussed below.

Glycerol-ether phospholipid fractions isolated from anaerobic sewage sludge digestors were also analysed by HPLC. A representative chromatogram is shown in Fig. 4. Additional peaks were present on chromatograms obtained for the sludge material compared to methanogenic pure cultures. FT-IR analysis of these isolated fractions revealed the presence of strongly absorbing carbonyl bands (1750–1710 cm⁻¹), indicating that these components were not ether lipids. The ratio of the glycerol diether to glycerol tetraether in the sewage-sludge digestor was 3:1, and the amount of total glycerol-ether phospholipids was 0.29 μmol/g dry wt sewage sludge (Table 2). The conversion factors of 2.5 μmol glycerol ether phospholipid per g dry wt methanogenic cells, as determined by pure culture analysis, and a cell mass of 5.9 × 10^12 cells/g dry wt (White et al., 1979a) were used to estimate a biomass of 6.8 × 10^11 methanogenic cells/g dry wt in this anaerobic environment.

Methanogenic biomass in the past has been determined by the "most probable number technique". After sewage sludge samples were serially diluted, the highest dilution of cells still showing methane production was used to calculate methanogenic biomass (Zehnder, 1978). Pure culture isolation of these strict anaerobes, in order to identify a resident population, is a complex procedure (Zeikus, 1977; Wolfe, 1971) and may underestimate the biomass (Brock et al., 1985). Biological activity measurements or methane evolution rates are used by microbial ecologists to assess the role of methanogens in such systems (Conrad et al., 1985; Phelps and Zeikus, 1984; King, 1984). Measurements of the biomass of methanogenic archaebacteria by quantification of glycerol-ether phospholipids, as performed in this study, provides a more direct method without the need for tedious manipulations of the anaerobic culture.

Analysis of samples taken from the top and bottom of an anaerobic, water hyacinth fed digestor showed slightly different results from those of the sewage sludge digestor. The ratio of glycerol diethers to glycerol tetraether was 1:1 for both samples compared with 3:1 noted for the sewage sludge digestor (Table 2). Pauly and Van Vleet (1986) recently reported variations in glycerol diether to tetraether ratios for a swamp sediment depth profile. They speculated that changes in growth substrate, Eh or pH may cause a change in the predominant methanogenic species or that differences in growth phase could account for the observed changes. Differences in the diether to tetraether ratios (Table 2) of the two anaerobic methane-producing digestors analysed here may be similarly explained. Further work, including manipulation experiments with cultured methanogens, is needed to understand the variations in the diether to tetraether ratio in natural systems.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Total glycerol-ether phospholipids (μmol/g dry wt)</th>
<th>DE:TE ratio (molar basis)</th>
<th>Biomass estimation (cell/g dry wt)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pure cultures</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. barkeri strain Jolich</td>
<td>2.30</td>
<td>1:0</td>
<td>—</td>
</tr>
<tr>
<td>M. thermoautotrophicum strain Hveragerdi</td>
<td>2.60</td>
<td>1:7</td>
<td>—</td>
</tr>
<tr>
<td>Anaerobic digestors</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sewage sludge digestor</td>
<td>0.29</td>
<td>3:1</td>
<td>6.8 × 10^11</td>
</tr>
<tr>
<td>Water hyacinth fed digestor</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(top)</td>
<td>0.05</td>
<td>1:1</td>
<td>1.2 × 10^11</td>
</tr>
<tr>
<td>(bottom)</td>
<td>0.03</td>
<td>1:1</td>
<td>7.1 × 10^10</td>
</tr>
</tbody>
</table>

*Conversion factors: 2.5 μmol glycerol ether phospholipid/g dry wt methanogenic bacteria; 5.9 × 10^12 bacteria/g dry wt (White et al., 1979).

*Not determined.
Such information would be pertinent to organic geochemical studies.

Henson et al. (1985b) examined the phospholipid fatty acids of thermophilic methane-producing digestors fed with Bermuda grass and cattle feed. Eubacterial biomass, estimated by measurement of total extractable lipid phosphates, was $2.5 \times 10^{11}$ bacteria per g dry wt. Variations in added substrate caused changes in microbial community structure. The combination of analyses for eubacterial and archaebacterial membrane constituents will provide a more complete picture of the microbial community structure in these anaerobic digestors.

CONCLUSION

Methanogens are ubiquitous in most anaerobic sediments, playing an important role in the decomposition of organic matter as they anaerobically produce methane. Methylotrophs are widespread in soil and water, where they oxidize methane under aerobic conditions. These two groups of bacteria thus represent important links in the biogeochemical carbon cycle. In this study we have demonstrated that the biomass of these two groups can be assessed using signature phospholipids, and these data can be used to characterise the microbial community structure in sediment from various environments.

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