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Microbial biomass and community structure of a phase-separated methanogenic reactor determined by lipid analysis

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SUMMARY

An anaerobic phase-separation biomass reactor was established on cellulose with the hydrolysis and fermentation steps occurring in the first stage, and acetogenesis and methanogenesis in the second stage. Based upon lipid biomarker analysis, eubacterial and eukaryotic cells accounted for approximately 6% of the volatile solids of the first stage and 17% of the second, while methanogens were approximately 1% of the volatile solids in the first stage and 9% of the second. Clustering the polar lipid fatty acids into groups based upon their distributions between the two stages of the reactor clarified the differences in community structure caused by phase-separated operation. Although inoculated from the same source, the two stages maintained very different microbial communities. Signature fatty acids known as indicators of unbalanced growth in eubacteria were significantly higher in the first stage of the reactor.

INTRODUCTION

The use of biomass and cellulosic wastes for methane and chemical production is limited by substrate resistance to hydrolysis and the economics of physical and chemical pretreatment. Subsequently, there is considerable interest in increasing the solubilization rates and efficiencies of biological processes such as anaerobic fermentation. The anaerobic conversion of biomass to methane and carbon dioxide can be conceptually divided into two phases: (i) the hydrolytic and fermentative stage where biological polymers are hydrolysed and fermented to volatile fatty acids (acetate, propionate, butyrate), carbon dioxide and other minor products; and (ii) the acetogenic and methanogenic stage where the higher volatile fatty acids are degraded to acetate, acetate to methane and carbon dioxide, and hydrogen and carbon dioxide are combined to form methane [18]. The potential benefits from the physical separation of these stages include [7]: (i) increased rate and stability of the process due to separate optimization of the two stages; (ii) lower capital costs due to the increased rate; (iii) lower cost of carbon dioxide removal from methane; and (iv) the volatile fatty acids produced by the first stage may be utilized as chemical feedstocks [15].

Phase-separation has been attempted by kinetic suppression of the slower growing methanogens, inhibition by pH control, oxygen addition, or methanogen poisoning. This has been difficult in practice, as the fermentative bacteria are also inhibited. There is anecdotal evidence that the inhibition of fermenters by low pH might be lessened at a lower temperature of operation (Dr. Tom White, unpublished observations). A combination of kinetic suppression and pH control with psychrophilic operation was utilized to achieve phase separation in this study.

The biomass, community structure and metabolic state of microbial communities can be determined by analysis of their lipids. Polar lipid fatty acids (PLFA) are a quantitative and reproducible measure of viable eubacterial biomass, while details of the eubacterial com-

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munity structure are found in the pattern of PLFA [22]. Specific PLFA have been shown to reliably indicate the metabolic status of eubacteria: the ratio of *trans* to *cis* mono-unsaturated PLFA [10] increases when bacteria are stressed by starvation, low pH or a toxic environment. The polar lipid ethers (PLEth) are used to determine the archaebacterial (methanogenic) biomass [12].

Phase-separated reactor operation was found to maintain very different microbial communities in the two stages of the system. The eubacteria in the first stage were metabolically stressed relative to those in the second. Methanogens made up a higher proportion of the biomass and were more active in the second stage.

MATERIALS AND METHODS

Phase-separation reactor

Microcrystalline cellulose (Sigmacell, Type 50, Sigma Chem. Co., St. Louis, MO) was 98% total solids (percent of wet weight), the total solids were 100% volatile solids, the chemical oxygen demand (COD) was 1.18 g $O_2 \cdot$(g volatile solids)$^{-1}$, and it was 100% biodegradable. The feed solution contained 3–6 g $\cdot l^{-1}$ $NaHCO_3$ (for pH control), and the following nutrients, expressed as g $\cdot$ g COD^{-1}: 0.28 NH_4Cl, 0.09 KH_2PO_4, 0.04 K_2HPO_4, and 0.004 yeast extract. Feed and buffer were delivered to the first stage hydrolytic/fermentative reactor (R1) on a semi-continuous basis from the feed tank at a flow of 0.5 l $\cdot$ day^{-1} (see Fig. 1). First stage effluent was passed through a clarifier for liquid-solids separation before second stage digestion. All solids collected in the clarifier were returned to the fermenter by the clarifier recycle pump. The clarifier recycle pump and the second stage recycle pump operated together returned a portion of the second stage effluent to the first stage reactor to help buffer the pH. Dilution rates for the fermenter were adjusted to one reactor volume per day based on feed plus effluent recycle flow.

The second stage acetogenic/methanogenic reactor (R2), fed the effluent from R1, was a 3-l anaerobic expanded bed system with MP-79 diatomaceous earth (Eagle-Picher Minerals, Inc., Reno, NV) as support media. It was constructed from 9.5 cm internal diameter acrylic tube fitted with a conical bottom constructed from a settling cone (Fisher Scientific, Inc.). Separate ports were provided to facilitate gas separation, effluent flow, and internal recycle. Bed expansion was maintained at approximately 15% using recycle flow. A schematic of the two-stage system is presented in Fig. 1.

Reactor performance analyses

Biogas from each reactor was collected and monitored separately using 1- and 8-l traps for R1 and R2, respectively. Each trap contained an acidified brine solution (9:1 water to NaCl-saturated H_2SO_4) and food coloring for ease of reading. Hydraulic retention times for the reactors were calculated on the basis of feedstock and water additions. Both systems were maintained under psychrophilic conditions, at 20 °C in a constant temperature room.

Daily measurements of reactor performance parameters were made over a 6-month period in order to determine the system's stability, efficiency, and products. Reactor performance was tested under different hydraulic retention time, organic loading rate and pH regimes. Only the values at the time of sampling are reported here (Table 1). Biogas volume and composition, total solids and total volatile solids, total and soluble COD, and volatile fatty acids (VFA) were determined daily to monitor reactor performance. Except for biogas and volatile fatty acid analyses, all tests were performed according to standard methods [1]. Methane and carbon dioxide contents of the biogas were determined with a Gow Mac Series 580 gas chromatograph with thermal conductivity detection. Dry gas volumes were obtained by subtracting the water volume fractions (calculated from water vapor density data and the general gas law) from the measured biogas volumes before conversion to standard temperature and pressure. Volatile fatty acids were analysed with a Gow Mac Series 740P gas chromatograph equipped with a flame ionization detector and a 15-m, 0.53-mm internal diameter Nukol capillary column (Supelco, Inc., St. Louis, MO). Samples were adjusted to pH ≤ 2 with concentrated H_3PO_4, shaken, centrifuged, and the supernant was filtered (Whatman GF/C or 934AH). 1-μl aliquots were injected with appropriate external standards bracketing the observed concentrations.

Lipid analysis

At the time of sampling, five 40-ml samples of reactor contents were obtained from R1, and four from R2. Samples were taken from the center of each stage of the reactor. They were both stirred to apparent homogeneity, the first stage with the stir bar and the second by recycling the fluids. Samples were immediately frozen and stored at −20 °C until lyophilization. High recoveries and reproducibilities of PLFA profiles are obtained when care is taken to prevent thawing of sample before lyophilization (unpublished data). Sample extraction on site or preservation in formaldehyde were not possible due to the need to transport the samples by commercial airline. Eight 5-ml samples were collected from each stage for determination of dry weight and volatile solids (VS). Dry weight was determined by heating at 100 °C to constant weight; VS at 450 °C for 12 h.

The lipids were extracted in separatory funnels by a modified method of Bligh and Dyer [24]. The lipid extract

was fractionated into neutral lipid, glycolipid, and polar lipid by column chromatography [9]. The PLFA were released as fatty acid methyl esters by mild alkaline methanolysis [3], and the lipids were extracted from the methanolysis mixture with chloroform. The methanolysed lipid was then re-fractionated on a silicic acid column as described [9] except that the acetone elution was omitted. The PLFA were retrieved in the neutral lipid fraction as their methyl esters and the unchanged PLEth in the polar lipid fraction. The PLFA were identified and quantified by capillary gas chromatography and identification verified by capillary gas chromatography–mass spectroscopy [23]. The PLEth were cleaved from their phosphate functional groups by strong acid methanolysis [12]. The amounts and identities of the PLEth were determined by supercritical fluid chromatography (SFC).

The PLFA were named according to the standard form: number of carbon atoms, colon (:), number of unsaturations, omega (ω), distance of the unsaturation from the methyl terminus. The prefix 'i' indicates an iso-branched fatty acid; 'a' an anteiso-branched; the suffix 'c' the *cis* geometric isomer of the unsaturation; 't' the *trans* geometric isomer.

Cluster analysis of the PLFA profiles were performed using a 1-Pearson correlation coefficient clustering rule (Systat statistical program, Systat, Inc., San Diego, CA) with complete linkage on the arcsin of the square root [25] of the mol fraction data. The significance of differences was tested using the Student's t-test at an $\alpha = 0.05$ overall confidence level [5].

RESULTS

Reactor performance

Reactor performance parameters for the hydrolytic/fermentative R1 and acetogenic/methanogenic R2 phases are presented in Table 1. The long 41-day solids retention time for R1 relative to its 1-day hydraulic retention time was due to the solids recycle feature (Fig. 1). High VFA production was seen in R1 (50% of the applied COD) while only 2% of the COD applied to R2 appeared as VFA in the effluent. The methane appearing in R1 accounted for only 9% of the biodegradable COD fed R1, while R2 had a 73% efficiency for conversion of the applied COD to methane.

Microbial biomass

The measures of biomass, PLFA and PLEth, were expressed as micrograms per gram volatile solids ($\mu g \cdot g\ VS^{-1}$) rather than per gram dry weight (Table 2). R2 was significantly higher than R1 for both biomass measures.

TABLE 1

Operating conditions and effluent characteristics of the phase-separation reactor at the time of sampling

Processes	Hydrolytic/ fermentative	Acetogenic/ methanogenic
Reactor	R1	R2
Feed	Cellulose	R1 effluent
Hydraulic retention time (days)	1	2.5
Solids retention time (days)	41	NA[a]
pH	5.90	6.76
Efficiency (percent of applied COD)		
Solubilization	91%	NA[a]
VFA Production	50%	2%
CH_4 Removal	8.5%	73%

[a] NA, not applicable. Only very low levels of solids were applied to or removed from R2, consisting of bacterial flocs.

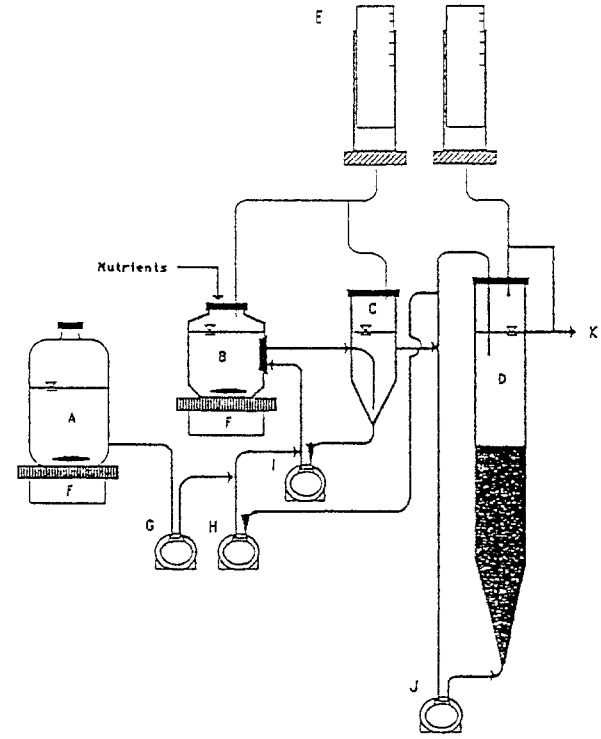

Fig. 1. Schematic of the phase-separation reactor. (A) Feed tank; (B) R1, the hydrolytic/fermentative reactor; (C) clarifier; (D) R2, the expanded-bed acetogenic/methanogenic reactor; (E) gas traps; (F) magnetic stirrers; (G) feed pump; (H) second stage recycle pump; (I) clarifier recycle pump; (J) expanded bed recycle pump; (K) system effluent.

Community structure

Six measures of the community structure of R1 and R2 were derived from the lipid data (Table 2), and all but one showed a statistically significant difference between R1 and R2. The ratio of polar lipid ethers to polar lipid fatty acids (Eth/FA), representing the ratio of methanogens to eubacteria, was almost six times greater in R2 than in R1. The ratio of tetraether to diether (TE/DE) was almost twice as great in R1 as R2.

Cluster analysis was used in this study to combine the 30 PLFA into a smaller number of groups which preserve most of the information content of the raw data. The lengths of the horizontal lines in Fig. 2 are proportional to Pearson's *r* correlation coefficient calculated as for a linear regression. The averages and standard deviations of the mol percent PLFA and the statistical significance of the difference between R1 and R2 for each cluster is presented in Table 2. Cluster 1 contained all of the fatty

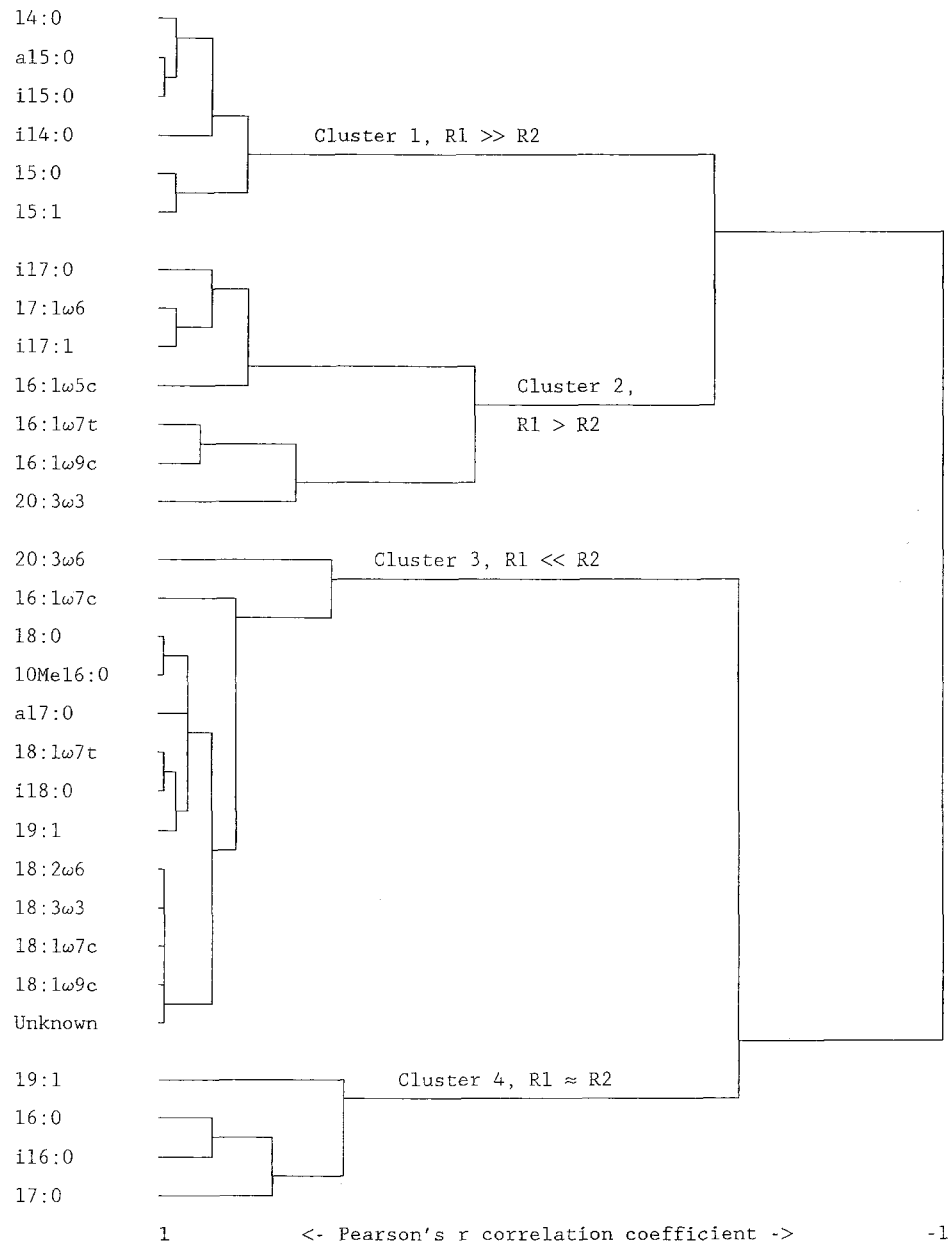

Fig. 2. Cluster analysis of the polar lipid fatty acids from the two stages of the phase separation reactor. The fatty acids were clustered using the 1-Pearson's correlation coefficient distance metric and complete linkage. The relative contributions to the profiles of R1 and R2 are indicated.

TABLE 2

Lipid measures of the viable biomass, community structure, and metabolic status of the phase-separation reactor microbiota

	Hydrolytic/fermentative R1 (Average (SD))	Acetogenic/methanogenic R2 (Average (SD))	Significant difference?
Biomass measures (μg/g VS)			
PLFA	1691 (542)	5024 (638)	Yes
PLEth	61 (11)	1045 (103)	Yes
Community structure (weight ratio)			
Eth/FA	0.04 (0.01)	0.23 (0.13)	Yes
TE/DE	0.42 (0.11)	0.22 (0.03)	Yes
Fatty acid clusters (mol %)			
Cluster 1	46.3 (7.8)	20.1 (2.0)	Yes
Cluster 2	16.2 (3.4)	11.1 (0.8)	Yes
Cluster 3	13.6 (2.4)	40.2 (1.2)	Yes
Cluster 4	21.2 (2.7)	23.2 (3.9)	No
Metabolic status			
trans/cis 16:1ω7	0.17 (0.01)	0.14 (0.01)	Yes
trans/cis 18:1ω7	0.18 (0.02)	0.08 (0.01)	Yes

Abbreviations: PLFA, polar lipid fatty acids; PLEth, polar lipid ethers; and TE/DE, the ratio of tetraether to diether.

acids with less than 16 carbons, and made a significantly much larger contribution to R1 than to R2, 46.3 $\pm$ 7.8% and 20.1 $\pm$ 2.0% (average $\pm$ SD), respectively. Cluster 2 contained 16 and 17 carbon monounsaturates, i17:0, and 20:3ω3, and also made up a significantly larger fraction of the PLFA of R1 (16.2 $\pm$ 3.4%) than R2 (11.1 $\pm$ 0.8%), but not as great a difference as in Cluster 1. Cluster 3 contained 13 PLFA: 5 mono-unsaturates, 3 poly-unsaturates, 18:0, a17:0, i18:0, 10Me16:0, and an unknown fatty acid. It was significantly greater in R2 (40.2 $\pm$ 1.2%) than in R1 (13.6 $\pm$ 2.4%). Cluster 4 contained 16:0, 17:0, i16:0, and 19:1. It did not differ in its contribution to R1 and R2's PLFA.

Metabolic status

The two measures of eubacterial metabolic stress, the *trans/cis* ratios for 16:1ω7 and 18:1ω7, were significantly greater in R1 than in R2.

DISCUSSION

Phase separation

A major objective of this study was demonstration of acid-phase digestion using low pH, psychrophilic fermentors. The degree of phase separation achieved in this system compares favorably with those reported in the literature [7]. When the phase-separation bioreactor was first started, R1 had produced methane, which decreased as the pH lowered. The PLEth content and methane production of R1 showed that there were viable methanogens, but that they were a smaller fraction of the microbial community and much less active than in R2. Thus, temperature depression with pH control appears useful as a technique for phase separation.

Biomass measures

The measures of microbial biomass were expressed as microgram of PLFA or PLEth per gram of VS in order to eliminate the contribution of the diatomaceous earth support material in R2 to the dry weight of the sample. By using the conversion factors of 100 μmol PLFA per g dry weight of eubacterial cells [2], 256 g per mol fatty acid (as for palmitate), and that the dry weight of a bacterial cell is 90% VS, the percentages of VS as viable eubacterial cells for R1 and R2 can be estimated as 5.9% and 17%, respectively. The balance of the VS in R1 was recalcitrant cellulose particles and bacterial flocks, while since R2 received no cellulose, the balance would consist of bacterial flocks. This can be compared with 25% cells in a bench-scale completely stirred tank reactor fed Bermuda grass/cattle feed (3:1) and with a 20-day retention time [13].

The amounts of methanogen ether lipid found in R1 and R2, 61 and 1045 $\mu g \cdot g\ VS^{-1}$, (Table 2) bracket the 236 $\mu g \cdot g\ VS^{-1}$ found in a municipal sewage sludge [17]. An estimate of the relative activities of the methanogenic populations of R1 and R2 was calculated from the methane production of each stage of the reactor divided by the total PLEth in each. R1 produced approximately 5 kg COD $\cdot$ g $PLEth^{-1} \cdot day^{-1}$ of methane and R2 90 kg

COD · g PLEth^{-1} · day^{-1}. Therefore, there are not only more methanogens per gram VS in R2 than in R1, but they were over 17 times more active.

Community structure

The community structure of the first and second stages were significantly different in Eth/FA, TE/DE, and three of the four PLFA clusters. Both R1 and R2 were originally inoculated from the same biomass reactor, and due to the flow from R1 to R2 and the recycled effluent from R2 to R1 used to buffer the system, they were continually re-inoculated from each other. Therefore, the phase separation strategy adopted for this system maintained the very different community structures in R1 and R2.

The weight ratio of tetraether to diethers, TE/DE, was significantly higher in R1 than in R2, 0.42 ($\pm$ 0.11) versus 0.22 ($\pm$ 0.03). In studies of pure cultures, TE/DE varied from 0 for *Methanosarcina barkeri* to 14 for *Methanobacterium thermoautotrophicum* strain Hveragerdi [17]. The same report gave ratios of 0.6 for a municipal sewage sludge and 2 for a water hyacinth-fed continuously stirred tank reactor. Several species with intermediate values have been reported [21]. The toxic conditions (lower pH and higher VFA content) of R1 as opposed to R2 is either selecting for a different population of methanogens, and/or the methanogens present are synthesizing a different pattern of lipids in response to the different environment. Due to the solids recycle feature of the phase-separation reactor, any methanogens added to R1 in the inoculum or from the low-level inoculation from R2 would leave R1 at a very low rate.

Cluster 1 (Fig. 2) made a much larger contribution to the biomass of R1 than R2 and contained all of the 14 and 15 carbon PLFA. These PLFA have been associated with Gram-positive and other eubacterial anaerobes by a cluster analysis of estuarine sediments undergoing disturbance [8] and by comparison with lipid profiles of pure cultures [6,14]. The contribution of the Cluster 2 PLFA to R1 was significantly greater than to R2, but not as great a difference as Cluster 1. Six of the seven PLFA in cluster 2 were characteristic of eubacterial input, being iso-branched or mono-unsaturated, but the presence of 20:3ω3, a eukaryotic fatty acid [11], in cluster 2 was anomalous (see below). The third cluster contributed significantly more to the PLFA of R2 than to R1 and was made up of both distinctly eubacterial (10Me16:0 and a17:0, for example), and eukaryotic (poly-unsaturated 18 and 20 carbon PLFA [4]) biomarkers. Reports of eukaryotes in anaerobic biomass-degrading communities include yeast in a fixed-bed methanogenic reactor [19], fungi in the rumen [16], and protozoans associated with methanogenic bacteria in anaerobic biomass reactors, fresh and marine sediments, and the rumen [20]. Cluster 4 contains the universally distributed 16:0, and low levels of three eubacterial PLFA, in equal proportions in the two reactors.

Metabolic status

Many eubacteria produce PLFA with the *trans* geometric isomer of unsaturation under conditions of metabolic stress such as unbalanced growth (lack of a trace nutrient), starvation, or toxicity (pH, solvents) [8,10,14]. The lipid biomarkers *trans/cis* 16:1ω7 and 18:1ω7 (Table 2) were both significantly higher in R1 than in R2, however, neither were in the range found in a severely stressed *Vibrio* culture starved for 30 days — *trans/cis* ratio of over 1 for 16:1&ohgr;7 [10]. The higher *trans/cis* ratio observed in R1 was probably due to the more acidic conditions in the first stage, as had been found for *Clostridium acetobutylicum* grown at different pH values [14].

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REFERENCES

1 American Public Health Association. 1971. Standard Methods for the Examination of Water and Wastewater, 14th edn., American Public Health Association, Washington, DC.
2 Balkwill, D.L., F.R. Leach, J.T. Wilson, J.F. McNabb and D.C. White. 1988. Equivalence of microbial biomass measures based on membrane lipid and cell wall components, adenosine triphosphate, and direct counts in subsurface aquifer sediments. Microb. Ecol. 16: 73–84.
3 Bobbie, R.J. and D.C. White. 1977. Characterization of benthic microbial community structure by high resolution gas chromatography of fatty acid methyl esters. Appl. Environ. Microbiol. 39: 1212–1222.
4 Dewey, V.C. 1967. Lipid composition, nutrition, and metabolism. In: Chemical Zoology, Volume I: Protozoa (Kidder, G.V., Ed.), pp. 161–274, Academic Press, New York, NY.
5 Downie, N.M. and R.W. Heath. 1974. Basic Statistical Methods, 4th edn., pp. 165–174, Harper & Row, New York, NY.
6 Edlund, A., P.D. Nichols, R. Roffey, and D.C. White. 1985. Extractable and lipopolysaccharide fatty acid profiles from *Desulfovibrio* species. J. Lipid Res. 26: 982–988.

7 Fannin, K.F., V.J. Srivastava and H. Isaacson. 1987. Improving product gas composition by two-phase digestion of biomass. In: Energy From Biomass and Wastes (Klass, D.L., Ed.), pp. 1045–1056, Elsevier Applied Science, London.
8 Findlay, R.H., M.B. Trexler, J.B. Guckert and D.C. White. 1990. Laboratory study of disturbance in marine sediments: response of a microbial community. Mar. Ecol. Progr. Ser. 62: 121–133.
9 Findlay, R.H. and D.C. White. 1987. A simplified method for bacterial nutritional status based on the simultaneous determination of phospholipid and endogenous storage lipid poly-beta-hydroxyalkanoate. J. Microbiol. Methods 2: 275–293.
10 Guckert, J.B., M.A. Hood and D.C. White. 1986. Phospholipid ester-linked fatty acid profile changes during nutrient deprivation of *Vibrio cholerae*: increases in the *trans/cis* ratio and proportions of cyclopropyl fatty acids. Appl. Environ. Microbiol. 52: 794–801.
11 Harwood, J.C. and N.J. Russell. 1984. Lipids in Plants and Microbes, George Allen and Unwin, London.
12 Hedrick, D.B., J.B. Guckert and D.C. White. 1991. Archaebacterial ether lipid diversity: analysis by supercritical fluid chromatography. J. Lipid Res. 81: 659–666.
13 Henson, J.M., P.H. Smith and D.C. White. 1985. Examination of thermophilic methane-producing digesters by analysis of bacterial lipids. Appl. Environ. Microbiol. 50: 1428–1433.
14 Lapage, C., F. Fayolle, M. Hermann, and J.-P. Vandecasteele. 1987. Changes in membrane lipid composition of *Clostridium acetobutylicum* during acetone-butanol fermentation: effects of solvents, growth temperature, and pH. J. Gen. Microbiol. 133: 103–110.
15 Ljungdahl, L.G. 1983. Formation of acetate using homoacetate fermenting anaerobic bacteria. In: Organic Chemicals from Biomass (Wise, D.L., Ed.), pp. 219–248, Benjamin Cummings Publishing, Menlo Park.
16 Mountfort, D.O. 1986. Role of anaerobic fungi in the rumen ecosystem. In: Perspectives in Microbial Ecology, Proceedings of the Fourth International Symposium on Microbial Ecology, pp. 176–179. Slovene Society for Microbiology, Ljubljana, Yugoslavia.
17 Nichols, P.D., C.A. Mancuso and D.C. White. 1987. Measurement of methanotroph and methanogen signature phospholipids for use in assessment of biomass and community structure in model systems. Org. Geochem. 11: 451–461.
18 Peck, H.D. Jr. and M. Odom. 1981. Anaerobic fermentations of cellulose to methane. In: Trends in the Biology of Fermentation for Fuels and Chemicals (Hollaender, A., Ed.), pp. 375–395, Plenum Press, New York, NY.
19 Robinson, R.W., D.E. Akin, R.A. Nordstedt, M.V. Thomas and H.C. Aldrich. 1984. Light and electron microscopic examinations of methane-producing biofilms from anaerobic fixed-bed reactors. Appl. Environ. Microbiol. 48: 127–136.
20 Stumm, C.K., K.B. Zwart, J.J.A. Von Bruggen and G.D. Vogels. 1986. Methanogenic bacteria as endosymbionts of anaerobic protozoa. In: Perspectives in Microbial Ecology, Proceedings of the Fourth International Symposium on Microbial Ecology, pp. 169–175, Slovene Society for Microbiology, Ljubljana, Yugoslavia.
21 Tornebene, T.G. and T.A. Langworthy. 1978. Diphytanyl and dibiphytanyl glycerol ether lipids of methanogenic archaebacteria. Science 203: 51–53.
22 Vestal, R.J. and D.C. White. 1989. Lipid analysis in microbial ecology. Bioscience 39: 535–541.
23 White, D.C., R.J. Bobbie, J.D. King, J.S. Nickels and P. Amoe. 1979. Lipid analysis of sediments for microbial biomass and community structure. In: Methodology for Biomass Determinations and Microbial Activities in Sediments, ASTM 673 (Litchfield, C.D. and Seyfried, P.L., Eds.), pp. 87–103, American Society for Testing Materials, Washington, DC.
24 White, D.C, W.M. Davis, J.S. Nickels, J.D. King and R.J. Bobbie. 1979. Determination of sedimentary microbial biomass by extractable lipid phosphate. Oecologia 40: 51–60.
25 Winer, B.J. 1971. Statistical Principles in Experimental Design, p. 208, McGraw-Hill, New York, NY.