APPLICATION OF ANALYTICAL MICROBIAL ECOLOGY TO THE ANAEROBIC CONVERSION OF BIOMASS TO METHANE

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ABSTRACT

Lipid biomarker and radiotracer analysis, microcosms, and the inhibitors oxygen and chloroform were used to monitor the operation of a unique design of high-solids high-productivity methanogenic reactor. The 2 reactors examined gave an example of a microbial community adapted to the batch feeding schedule and very high methane production. The second reactor had been damaged by overfeeding. It had adapted to high acetate and ammonia concentrations, low pH and productivity. Under oxygen inhibition, methanogens were out-competed by facultative anaerobes, rather than killed. While chloroform inhibition abolished radiolabeled acetate incorporation into methane, label was incorporated into archaeal ether lipids, possibly indicating an unknown methanogen adaptation to toxic stress. The utility of analytical microbial ecology to practical problems is discussed.

ABBREVIATIONS


KEYWORDS

Biomarkers, ether lipids, microcosms, polar lipid fatty acids, radiotracer analysis.

INTRODUCTION

Analytical microbial ecology is the application of analytical chemistry and molecular biology methods to the study of bacteria, archaea (i.e. archaebacteria), and micro-eukaryotes in diverse environments. It has been applied to environments as varied as estuarine sediments,1,2 deep subsurface aquifers,3,4 and hydrothermal vent microbiota;5,6 and to ecological problems as diverse as microbially influenced corrosion of metals,7 in situ biodegradation of groundwater pollutants,8 and the production of methane from biomass as an energy resource.9-15

The anaerobic conversion of biomass to methane requires a complex ecosystem including fermentative and hydrolytic bacteria, acetogens fermenting volatile fatty acids (and alcohols in inhibited systems) to acetate, and the archaeal methanogens converting acetate and carbon dioxide/hydrogen to methane.16 Areas of active research include digester failure due to over-loading, the role of trace metals in digester stability, and production of biogas with higher methane content. In order to comprehend and eventually predict this intersection of engineering and microbiology, there is a need for methods to monitor the
microbial biomass, community structure, metabolic status, and activities. On-line methods to detect the coenzymes NAD(P)H and Fd by fluorescence, antibody probes for specific subpopulations of methanogens, and immuno-gold microscopic techniques to detect specific organisms and their spatial organization have been investigated under GRI sponsorship.

Our work has centered on the development of radiotracer and lipid biomarker methods for anaerobic biomass reactors. The in situ metabolic activities of a microbial community can be detected by the use of radiotracer techniques. In this work, acetate labeled on the methyl carbon (14C-2-acetate) was used to probe microbial synthesis in a high-solids high-yield methanogenic digester. Membrane lipid analyses are valuable for estimating the in situ viable microbial biomass, community structure, and metabolic status. The polar fraction of the total lipid extract contains the bacterial and eukaryote membrane polar lipid fatty acids (PLFA). PLFA are an accurate measure of viable microbial biomass, and have a rapid turnover in natural environments. Membrane polar lipid ethers (PLEth) are characteristic of the archaeal methanogens, a key component of the methanogenic community. Community structure is defined as the proportions of distinguishable groups of organisms within the viable biomass. The pattern of membrane lipids provides a "fingerprint" of the microbial community structure of the environment sampled. Specific lipid markers are also known which reflect changes in the metabolic status of members of the microbial community. For example, the ratio of trans to cis mono-unsaturated PLFA increases under conditions of unbalanced growth and the amount of cyclopropyl PLFA increases in the stationary phase of bacterial growth.

The application of analytical microbial ecology to the study of two experimental methanogenic digesters at the Department of Agricultural and Biological Engineering, Cornell University is described. These reactors were chosen because of their innovative design and potential for industrial application. They were operated at unusually high temperature (thermophilic, 55°C), percent solids (23%), pH (7.8), and productivity (6 L·kg⁻¹·day⁻¹, on a liters of methane per kg reactor contents basis). This digester design has achieved sustained methane production rates of 7.5 L·kg⁻¹·day⁻¹, which is approximately 4 to 5 times the maximum production rates found in low solids digesters (1.5 L·kg⁻¹·day⁻¹ and 1.8 L·kg⁻¹·day⁻¹).

MATERIALS AND METHODS

High-solids methanogenic reactors

The Cornell University Department of Agricultural and Biological Engineering constructed the high-solids reactors from 20 L polypropylene carboys and attached low-flow meters. Each reactor contained 5 kg of reactor material statically incubated at 55°C. Reactors were fed 3 times weekly (Monday, Wednesday, and Friday) by replacing 5 to 15% of the reactor contents with a 1:1 mixture of α-cellulose (Sigma Chemical Co.) and milled dried sorghum (Sorghum bicolor, Stanford Seeds variety X9204). High-Solids Reactor #1 (HS1) had been operated under stable operating conditions for efficient conversion of biomass to methane for 60 days at the time of sampling, with an organic loading rate of 18 grams volatile solids per kilogram wet weight reactor contents per day (gVS·kg⁻¹·day⁻¹). Since the reactor contents were composed of moist solids, it was quantified by wet weight rather than the more common volume units. Calculations of solid and hydraulic retention times are also found in reference 27. High-Solids Reactor #2 (HS2) had been operated similarly to HS1, except that it had an organic loading rate of 24 gVS·kg⁻¹·day⁻¹ for 36 days which lead to reactor failure. HS2 had displayed instability due to the overfeeding, as indicated by variable and falling gas production, and increasing volatile fatty acid and ammonia concentrations. Feeding for HS2 were then suspended for 10 days, but were resumed the day before sampling at the higher rate. The "Condition 2 Digester" described in reference 26 was the same reactor as the overfed HS2 in this paper, when stable operation had been reattained months later.

The "healthy" condition of HS1 is defined as the point of maximum measured productivity at 24 hours after feeding; "disturbed" — 6 hours after removal of reactor contents and the disturbance of feeding; and "starved" at 48 hours. The "overfed" condition is defined as HS2 24 hours after feeding.
Anaerobic conversion of biomass to methane

Lipid analyses

Lyophilized samples of reactor contents were ground in a mortar and pestle, and 250 to 300 mg weighed for extraction. The total lipid was extracted, fractionated, and derivatized as previously described. All subsequent steps were carried out with the polar lipid (PL) fraction. Mild alkaline transesterification was used to cleave and methylate the PL ester-linked fatty acids. The lipid recovered from the mild alkaline transesterification was again fractionated by silicic acid column chromatography to obtain the PLFA methyl esters in the neutral lipid fraction and alkali-stable polar lipids, including the archaeal ether lipids, in the PL fraction. The archaeal PLEth were then released by strong acid methanolysis.

The PLFA were analyzed by capillary gas chromatography, and the peak identities verified by gas chromatography-mass spectroscopy. The gas chromatograph used was a Schimadzu GC-9A equipped with an flame ionization detector and an HP 1 50 m non-polar column, 0.2 mm I.D. and 0.11 µm film thickness. Gas chromatography-mass spectroscopy was performed on a Hewlett-Packard 5995A system with the same column and operating conditions. A supercritical fluid chromatograph was constructed from a Varian 3700 gas chromatograph (Varian Instrument Group, Palo Alto, CA), an ISCO LC 2600 syringe pump (Lincoln, NE), a Commodore 64 computer to control the pump, and other readily available components.

The PLFA nomenclature used was as follows: the number of carbons, "C:" (colon), the number of unsaturations, "o" (omega), the distance of the first unsaturation from the methyl end of the fatty acid, and "c" or "t" for the cis or trans geometric isomers of the unsaturation. The prefix "Cy" indicates a cyclopropyl moiety.

Radiotracer analysis

Approximately 100 g (wet weight) of digester contents was placed in a small plastic container under a headspace of nitrogen. Two mL of material was gently packed into the open end of a 3 mL plastic syringe with the Luer-lock end cut off. Care was taken not to pack the material so tightly that liquid was expressed and to avoid unnecessary exposure to oxygen. The sample was immediately placed in a pre-weighed pressure tube (Bellco Glass Inc., Vineland, NJ) with 0.74 µCi of 14C-2-acetate (50 mCi.mmol⁻¹, New England Nuclear, Boston, MA) in 1 mL sterile, anaerobic, deionized water, and then vigorously shaken. The precise weight of the sample was determined by difference. Tubes were incubated in triplicate with radiolabel for 0.5, 3, and 24 hours at 55°C before inhibiting biological activity with 5 mL formalin. After inhibition, the tubes were immediately frozen until analysis.

Total and labeled methane and carbon dioxide were determined with a Shimadzu GC-BA gas chromatograph equipped with a Carbosieve 8000 column and a thermal conductivity detector, and a Packard Model 894 gas proportional counter collected to the gas chromatograph effluent as described. Immediately after gas chromatographic analysis, the pressure tubes were opened and the pH measured in order to calculate the partition coefficient for carbon dioxide between aqueous and gas phases. The pressure tubes were then refrozen until analysis of labeled lipids.

For analysis of the labeled acetate incorporated into lipids, the tubes were thawed and the contents extracted by the method of Bligh and Dyer. Ten percent of the total lipid extract was reserved for liquid scintillation counting. The lipid extract was separated into neutral lipid, glycolipid, and polar lipid (PL) fractions by silicic acid column chromatography. The PL fraction was further separated into the diether (DE), tetraether (TE), and PLFA by treating the PL with a strong acid hydrolysis, freeing the PLFA as free fatty acids and the PLEth as ether-alcohols. The PLFA, DE, and TE were then separated by thin layer chromatography. TE purified from Methanobacterium formicum, 1,2-di-O-hexadecylglycerol, and stearic acid were used as retention standards.

The radiolabeled poly-β-hydroxyalkanoate (PHA) in the glycolipid fraction was determined by the method of Findlay, et al. The glycolipid was dried onto a 1 cm x 4 cm piece of filter paper, and fixed in a drying oven at 80°C for 30 min. Other lipids were then eluted with alternating diethyl ether and ethanol washes.
The triglyceride was separated from the neutral lipid fraction by thin layer chromatography with hexane:ethyl ether:acetic acid (80:20:2) as the mobile phase on Whatman Linear K plates. Tristearin was used as the retention standard. Liquid scintillation counting was done with a LKB 1212 Rackbeta (Gaithersburg, MD) using the channels ratio correction method and Ecolume scintillation fluid (ICN Biomedicals, Irvine, CA).

Inhibition experiments

Inhibitors (oxygen and chloroform) were added to the pressure tubes (prepared as for radiolabel analysis) before the reactor sample. Each sampling included controls inhibited at the time of sampling, controls incubated without chloroform or oxygen, and treatments incubated with either chloroform or oxygen. All were incubated for 24 hours. Oxygen addition was performed by equilibration of the pressure tubes with room air for 30 minutes, followed by inoculation, injection of 30 mL air, and 5 hours later injection of an additional 10 mL room air. This provided 9.9 mg O₂ (308 µmol) per gram reactor material. Chloroform treatments were with either 4 or 100 L chloroform, to yield 3.1 or 76 mg chloroform (26 or 630 µmol) per gram wet weight, respectively.

RESULTS

Methane production rates for the high-solids reactors are shown in Fig. 1A. There was very good agreement between the methane production rates measured on the reactors and those measured in the pressure tube microcosms, except for the sampling 48 hours after feeding. At 48 hours, the in vitro measurement showed a decline in rate, in agreement with observations of digester performance in previous feeding cycles. Methane and carbon dioxide production rates in the starved condition of HS1 at 48 hours after feeding and in the overfed condition of HS2 24 hours after feeding were statistically significantly less than the healthy condition of HS1 (Tables 1 and 2).

The pH and the ammonia concentration in HS1 (Fig. 1B and 1C) rose slightly in the first 6 hours after feeding. The acetate concentration (Fig. 1D) was relatively stable. The variation in these parameters was small, and on the same order as the standard deviations in other measured parameters (Fig. 1) for which replication was available, indicating that the environment in HS1 as measured by these parameters was stable over the feeding cycle. HS2 had a much lower pH than HS1 (Fig. 1B), and much higher ammonia (Fig. 1C), acetate (Fig. 1D), and propionate concentrations (data not shown).

Supercritical fluid chromatograph performance

The sensitivity of the SFC was calculated from a 0.2 pL injection of 20 µg·mL⁻¹ synthetic diether standard, 1,2-di-O-hexadecylglycerol. Given the convention that the minimum detectable peak is 2 times as high as the width of the noise, the minimum detectable quantity corresponded to approximately 0.6 ng of DE. A minimum of 20 µL of solvent is required to make the 0.2 µL injection, so the practical limit of detection is 60 ng per sample. This represents approximately 7.5 µg dry weight cells or 150 million methanogen cells, based upon conversion factors of 8 mg ether lipid per gram dry weight and 2x10¹⁵ prokaryotic cells per gram dry weight cells.

The reproducibility of quantification was estimated from 22 injections of the standard SDE over 11 days of analyzing routine samples. The first injection of each day had been eliminated from calculations due to low peak area, as is often observed for aging chromatography columns. The relative standard deviations for injections of 20, 200, and 2000 µg·mL⁻¹ were 9.3%, 6.5%, and 6.0% of the mean, respectively.

Microbial biomass

In reactor HS1, the measures of bacterial and archaeal biomass – PLFA and PLEth – decreased between before feeding and 6 hours after feeding (disturbed) (Table 1). At 24 (healthy) and 48 hours (starved) after feeding, these biomass markers had risen to approximately their initial level. Both PLFA and PLEth were lower in the overfed HS2 samples than in the healthy HS1 samples at 24 hours after feeding. The PLFA content was statistically significantly different between the disturbed and healthy conditions, and PLEth was significantly different between the overfed and healthy conditions.
Table 1. Gas Production and Biomass Variation over a Feeding Cycle of High Solids Reactor 1 and Reactor 2 at 24 Hours after Feeding. Methane and carbon dioxide productions are expressed as L·kg⁻¹·dy⁻¹, the lipid biomass measures, ethers and polar lipid fatty acids (PLFA) as mg·kg⁻¹, the gas production to biomass measure ratios as L·mg⁻¹·dy⁻¹, Cy:7:0 as mole % of total PLFA, and the trans/cis ratios as mol·mol⁻¹. Modified from Refs 9 and 10.

<table>
<thead>
<tr>
<th></th>
<th>High Solids Reactor 1</th>
<th>Reactor 2</th>
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<tbody>
<tr>
<td></td>
<td>Before Feeding</td>
<td>Disturbed</td>
</tr>
<tr>
<td></td>
<td>0 hr</td>
<td>6 hr</td>
</tr>
<tr>
<td><strong>Gas Production</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Methane</td>
<td>5.8 (1.2)</td>
<td>5.7 (0.7)</td>
</tr>
<tr>
<td>Carbon Dioxide</td>
<td>21 (4)</td>
<td>26 (3)</td>
</tr>
<tr>
<td><strong>Microbial Biomass</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethers</td>
<td>4.0 (0.7)</td>
<td>2.9 (0.4)</td>
</tr>
<tr>
<td>PLFA</td>
<td>76 (4)</td>
<td>52 (17)</td>
</tr>
<tr>
<td><strong>Gas Production per Biomass</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>CH₄/PLEth</td>
<td>1.5 (0.4)</td>
<td>1.9 (0.3)</td>
</tr>
<tr>
<td>CO₂/PLFA</td>
<td>0.3 (0.1)</td>
<td>0.5 (0.3)</td>
</tr>
<tr>
<td><strong>Metabolic Status</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cy17:0</td>
<td>0.09 (0.01)</td>
<td>0.13 (0.08)</td>
</tr>
<tr>
<td>trans/cis</td>
<td></td>
<td></td>
</tr>
<tr>
<td>16:1</td>
<td>0.55 (0.03)</td>
<td>0.77 (0.10)</td>
</tr>
<tr>
<td>18:1</td>
<td>0.34 (0.02)</td>
<td>0.31 (0.09)</td>
</tr>
</tbody>
</table>
Fig. 1. Digester performance parameters measured on the high solids digesters: methane production rate, pH, and ammonia and acetate concentrations. The horizontal axis is the time after feeding in hours. Figure 1A: Black bars represent methane production of digester HS1 measured by continuous volumetry and gas chromatography; the diagonally striped bar represents methane produced by the overfed digester HS2. Figures 1A, 1B, 1C, and 1D: Open squares represent the parameters measured in the radiolabel incubation tubes for HS1 and filled circles HS2. From Ref. 9.

Table 2. Statistical significance of shifts in measures of activity, biomass, community structure, and metabolic status, relative to the Healthy state of HS1. Symbols: $\uparrow\uparrow =$ significantly greater than in the Healthy reactor, $\downarrow\downarrow =$ significantly less than, $\uparrow =$ greater than, $\downarrow =$ less than, and $\approx =$ approximately equal to the Healthy reactor, $-$ data not available. Significance of differences was tested for at the $P = 0.05$ level. Modified from references 9 and 10.

<table>
<thead>
<tr>
<th></th>
<th>Disturbed</th>
<th>Starved</th>
<th>Overfed</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>PLFA (bacteria)</strong></td>
<td>$\uparrow$</td>
<td>$\approx$</td>
<td>$\uparrow$</td>
</tr>
<tr>
<td><strong>PLFeth (methanogens)</strong></td>
<td>$\uparrow$</td>
<td>$\approx$</td>
<td>$\uparrow$</td>
</tr>
<tr>
<td><strong>Cyl17:0</strong></td>
<td>$\uparrow$</td>
<td>$\approx$</td>
<td>$\uparrow\uparrow$</td>
</tr>
<tr>
<td><strong>trans/cis 16:1w7</strong></td>
<td>$\uparrow\uparrow$</td>
<td>$\uparrow\uparrow$</td>
<td>$\uparrow$</td>
</tr>
<tr>
<td><strong>trans/cis 18:1w7</strong></td>
<td>$\approx$</td>
<td>$\approx$</td>
<td>$\uparrow\uparrow$</td>
</tr>
<tr>
<td><strong>Methane Production</strong></td>
<td>$\approx$</td>
<td>$\uparrow\uparrow$</td>
<td>$\uparrow\uparrow$</td>
</tr>
<tr>
<td><strong>Carbon Dioxide</strong></td>
<td>$\approx$</td>
<td>$\uparrow\uparrow$</td>
<td>$\uparrow\uparrow$</td>
</tr>
</tbody>
</table>

**Microbial Activities**

<table>
<thead>
<tr>
<th></th>
<th>Disturbed</th>
<th>Starved</th>
<th>Overfed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Rate of Label Incorporation into:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total Lipid</td>
<td>$\uparrow\uparrow$</td>
<td>$\uparrow\uparrow$</td>
<td>$\uparrow\uparrow$</td>
</tr>
<tr>
<td>Methane</td>
<td>$\uparrow\uparrow$</td>
<td>$\uparrow\uparrow$</td>
<td>$\uparrow\uparrow$</td>
</tr>
<tr>
<td>Carbon Dioxide</td>
<td>$\uparrow\uparrow$</td>
<td>$\uparrow\uparrow$</td>
<td>$\uparrow\uparrow$</td>
</tr>
</tbody>
</table>

| Ratio of Label into:   |
| Fatty Acids to Ethers  | $\uparrow\uparrow$ | $\uparrow\uparrow$ | $\uparrow\uparrow$ |
| PHA to Polar Lipid     | $\approx$           | $\approx$           | $\uparrow$           |
| TG to Polar Lipid      | $\approx$           | $\approx$           | $\approx$           |
Lipid stress markers

Cyclopropyl fatty acids accumulate in many bacteria during the stationary phase of growth and under stressed conditions. The mole % of Cy17:0 was much more variable in the disturbed samples (Table 1) and was significantly greater in the overfed HS2 than in the healthy HS1 (Table 2).

The ratio of trans to cis geometrical isomers has been used as a marker for bacterial stress. The trans/cis ratio for 16:1o7 was significantly greater for the disturbed and starved conditions than for the healthy state, while trans/cis for 18:1 7 was significantly greater in the overfed HS2 than the healthy HS1 (Table 2).

Microbial metabolic status measures

The ratio of radiolabel incorporated into bacterial and eukaryotic PLFA to that into archaeal PLEth (PLFA/PLEth, Table 3) showed a significant difference between the healthy and starved and between the healthy and overfed conditions (Table 2). PHA label divided by polar lipid label (PHA/PL, Table 3) showed no significant difference between the treatments (Table 2). For the ratio of triglyceride label to polar lipid label (TG/PL, Table 2), both the differences were significant.

Table 3. Lipid measures of microbial metabolic status within the methanogenic digester. Radiolabel incorporated from acetate into lipid components expressed as ratios, each expressed as average (standard deviation), n = 3. Modified from Ref 9.

<table>
<thead>
<tr>
<th>Metabolic state:</th>
<th>Healthy</th>
<th>Starved</th>
<th>Overfed</th>
</tr>
</thead>
<tbody>
<tr>
<td>Digester: HS1 @ 24 hr</td>
<td>HS1 @ 48 hr</td>
<td>HS2 @ 24 hr</td>
<td></td>
</tr>
<tr>
<td>PLFA/PLEth</td>
<td>14.5 (2.4)</td>
<td>22.4 (4.7)</td>
<td>1.7 (0.5)</td>
</tr>
<tr>
<td>PHA/PL</td>
<td>0.30 (0.11)</td>
<td>0.28 (0.07)</td>
<td>0.42 (0.08)</td>
</tr>
<tr>
<td>TG/PL</td>
<td>0.18 (0.03)</td>
<td>0.06 (0.01)</td>
<td>0.04 (0.04)</td>
</tr>
</tbody>
</table>

Abbreviations:
- PLFA/PLETH = polar lipid fatty acids to ethers.
- PHA/PL = poly-β-hydroxyalkanoate to total polar lipid.
- TG/PL = triglyceride to total polar lipid.

Oxygen treatment

The introduction of oxygen decreased the production of total methane by 43% versus controls, but it was not a statistically significant difference due to the high variability in measurement (Table 4). The percent of label incorporated into methane versus controls only decreased by 7%, significant at the 95% confidence level. The production of carbon dioxide was stimulated 87%, while the percent of label incorporated into carbon dioxide was increased by 100%, both differences highly significant. The label incorporated into DE increased significantly (166%), decreased significantly into TE (45%), and was not significantly different for PLFA. Oxygen treatment significantly increased the fraction of label incorporated into PHA and significantly decreased the fraction incorporated into triglyceride.

Chloroform treatment

The effects of chloroform were very different from that of oxygen (Table 4). The amount of methane found in the chloroform poisoned tubes was on the same order as those tubes inhibited at zero hours—the methane entrapped in the material at sampling. Production of methane and incorporation of label into methane was entirely prevented by treatment with chloroform, compared to being decreased with oxygen. The production of carbon dioxide and the incorporation of label into it were decreased relative to controls, rather than increased as with oxygen treatment. The differences in all 4 of the biogas measures at both levels of chloroform addition were found to be statistically significant. The percent of
label incorporated into both DE and TE decreased significantly at both levels of chloroform addition. The fraction of label incorporated into PLFA was not significantly changed by the low level of chloroform addition, but was by the high level chloroform addition. Label incorporation into PHA was not significantly affected, and into triglyceride was significantly decreased by both chloroform treatments.

Table 4. Effects of oxygen and low and high concentrations of chloroform on microbial activities in the high-solids anaerobic biomass reactor, expressed as percent difference from control and the standard deviation, ((control - treatment)/control) x 100. Number of replicates = 3. Statistical significance was tested by the Student's t-test at the 95% confidence level. ↑ = Significantly greater than control. ↓ = Significantly less than control. Modified from Ref 11.

<table>
<thead>
<tr>
<th></th>
<th>Oxygen</th>
<th>Chloroform Low</th>
<th>Chloroform High</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Gas Production</td>
<td></td>
</tr>
<tr>
<td>Methane</td>
<td>-43 (47)</td>
<td>-99 (41)↑</td>
<td>-99 (44)↑</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>87 (42)↑</td>
<td>-69 (39)↑</td>
<td>-82 (33)↑</td>
</tr>
<tr>
<td>Methane</td>
<td>-7 (5)↑</td>
<td>-100 (3)↑</td>
<td>-100 (1)↑</td>
</tr>
<tr>
<td>Carbon dioxide</td>
<td>100 (14)↑</td>
<td>-54 (35)↑</td>
<td>-92 (10)↑</td>
</tr>
<tr>
<td>Fatty Acids</td>
<td>-28 (37)↑</td>
<td>-45 (53)↑</td>
<td>-99 (31)↑</td>
</tr>
<tr>
<td>Diether</td>
<td>166 (132)↑</td>
<td>-89 (88)↑</td>
<td>-97 (37)↑</td>
</tr>
<tr>
<td>Tetraether</td>
<td>-45 (34)↑</td>
<td>-96 (27)↑</td>
<td>-100 (26)↑</td>
</tr>
</tbody>
</table>

**DISCUSSION**

**Biomass measures and biogas production**

Over the feeding cycle of HS1, the methanogen and bacterial biomass measured as PLEth and PLFA, respectively, were decreased by the removal of reactor contents at feeding (Table 1). Starvation, however, did not affect the reactor's biomass. The biomass per wet weight of HS2 was much lower than the healthy condition. Biogas production was decreased under starvation and overfeeding, but not by the disturbance of feeding. The methanogen and bacterial specific activities (the ratios of biogas production to biomarker for biomass, CH4/PLEth and CO2/PLFA, respectively) offer another view of the reactor's ecology (Table 1). The specific gas production was highest at 6 hour after feeding when the high starch and soluble sugar content of the sorghum feed was being utilized. Reactor HS1 in the starved condition had the lowest specific activity of any of the conditions. While the CO2/PLFA ratios for HS1 and HS2 each at 24 hr after feeding were equal, CH4/PLEth for HS1 was approximately 1.5 times as great as that for HS2. The feeding regime for HS2 and the resulting high volatile fatty acid and ammonia contents had inhibited methanogen growth and activity. It is likely that the poisonous conditions in HS2 had selected for a resistant bacterial population, to be able to maintain as high a specific activity (CO2/PLFA) as the healthy HS1.

**Lipid markers of metabolic status**

High values for the lipid measures of metabolic stress (Cy17:0, and the trans/cis ratios for 16:1ω7 and 18:1ω7, Table 1) indicate bacteria in a state of unbalanced growth. That is, cell division limited by a toxic chemical, inhibitory pH, or the lack of some nutrient. Trans/cis 16:1ω7 was significantly greater in the disturbed and starved conditions, and Cy17:0 and trans/cis 18:1ω7 were significantly greater in the overfed condition than in the healthy HS1 (Table 2). This indicates that the lipid measures of metabolic
stress responded to changes in reactor conditions, that the response observed depended upon the type of change, and that different groups of bacteria responded differently. The mole % of Cy17:0 in the PLFA of the disturbed condition of HS1 was highest of all the time points tested, but was not found to be significant due to its high variability. For all 3 lipid metabolic status markers, the standard deviation of the disturbed condition was much greater than the healthy or starved condition. This variability may have been due to rapid colonization of the fresh feed material leading to localized areas of starvation for a nutrient such as nitrogen, phosphorous, or a trace metal.

**Activity measurements on microcosms**

The successful reproduction of the *in situ* methane production rates in the pressure tube microcosms supports the contention that the microcosms are a valid model of the reactor's behavior (Fig. 1A). The apparent difference between the rates measured at 48 hours after feeding HS1 was an artifact due to the different time resolution of the 2 techniques. The methane production measured on the reactor was averaged between 24 and 48 hours after feeding, while the rate in the pressure tube was measured between 48 and 51 hours after feeding.

Over 60 days of operation of HS1, by 48 hours after each feeding the starved condition of HS1 was attained – the feed was nearly exhausted and methane production much decreased, as was found in this experiment. The pH, and the ammonia and acetate concentrations, however, were relatively unchanged (Fig. 1b and 1d), indicating that the digester design maintained a stable environment for the microbial community during feedstock limitation. The lower ammonia concentration is presumably due to nitrogen utilization for cell growth.

The comparison of the overfed with the healthy digester is much different from the comparison of the starved and healthy (Table 2). In the overfed digester, the pH was lower, due to non-utilization of volatile fatty acids released by fermentation (e.g. acetate, Fig. 1D). In addition to the high, toxic concentrations of acetate and ammonia (Fig 1C, 1D), the overfed HS2 contained 5.5 g propionate per kg digester contents. All of the rates measured were lower in the overfed digester than in the healthy. The PLFA/PLEth ratio showed a much lower proportion of the labeled acetate being incorporated into fatty acids in HS2. The general inhibition of metabolic activity in HS2 left more of the preferred bacterial substrates available for lipid synthesis and less of the bacterial lipid derived from labeled acetate.

The increase in PHA/PL seen in the overfed digester is due to the lower incorporation of label into PL, rather than an increase into PHA. Lipid synthesis for growth was inhibited more than PHA synthesis. TG/PL was much less in the overfed digester, probably due to the effects of the toxic conditions on the micro-eukaryotes responsible for its synthesis.

**The effects of oxygen addition**

Oxygen addition decreased methane production (Table 4), for which there are at least 2 possible mechanisms: oxygen could have directly inhibited the methanogens, or the facultative anaerobes could have out-competed the methanogens for reducing equivalents when provided with a high-energy terminal electron acceptor. Both of these mechanisms predict a decrease in methane production, an increase in carbon dioxide production, and an increase in labeled acetate incorporation into carbon dioxide, as was observed. However, the oxygen toxicity mechanism predicts that label incorporation into methane should decrease by approximately the same amount as methane production. Label incorporation into methane was only decreased by 7%, while methane production was decreased by 43% (Table 4), relative to the controls. Radiolabeled acetate consumption increased with oxygen addition, but not enough to account for the only 7% decrease in labeled methane by a change in the acetate pool size. Kuenz and Leisinger found that the plating efficiencies of *Methanobacterium thermoautotrophicum*, *Methanobrevibacter arboriphilus*, and *Methanosarcina barkeri* were not affected by from 10 to 30 hours of exposure to air-equilibrated media, so viable methanogens are possible under these conditions.

Further evidence for the viability of the methanogens in the oxygen-inhibited microcosms is seen in radiolabeled acetate incorporation into the archaeal DE and TE (Table 4). The increase in label incorporated into DE and the decrease into TE may be due to the known differences in oxygen susceptibility between sub-groups of methanogens, or to an unknown adaptive reaction to oxygen.
toxicity. In a different thermophilic anaerobic biomass reactor system Henson et al.\textsuperscript{13} found that supplementation with nitrate, another high-energy electron acceptor, increased viable microbial biomass and decreased methane production. Rivard et al.\textsuperscript{38} observed that the decrease in methane production in a nitrate-amended digester was due to the demand for electrons for nitrate reduction competing with carbon dioxide reduction to methane.

Addition of oxygen greatly increased the amount of carbon dioxide produced and the incorporation of radiolabeled acetate into carbon dioxide, but did not significantly change the proportion of label incorporated into fatty acids. The facultative anaerobes in the high-solids reactor were able to take advantage of the availability of oxygen for growth. The 92% increase in the amount of labeled acetate incorporated into PHA could be due to the facultative aerobic bacteria rapidly growing by oxygen respiration, and becoming limited by the lack of some nutrient. Similarly, in a study of the effect of nitrate on a thermophilic digester's performance, the amount of PHA produced increased.\textsuperscript{13}

The 83% decrease in acetate incorporated into triglyceride indicates that the eukaryotic components of the microbial community were adversely affected by the presence of oxygen. The fungi\textsuperscript{70} and the protozoans with methanogen symbionts\textsuperscript{40} found in methanogenic systems could have been poisoned by oxygen toxicity or overgrown by the aerobic bacterial bloom.

**The effects of chloroform**

Chloroform is specifically toxic to methanogenesis\textsuperscript{41} and has non-specific membrane effects on all cells.\textsuperscript{42} The addition of the low and high concentrations of chloroform decreased total and labeled methane production by 99 and 100%, respectively (Table 3), and the proportion of label into DE and TE by 89 and 100%. There were many differences between the 2 treatments, however. In the low chloroform treatment, total and labeled carbon dioxide production was decreased relative to controls, but not as much as in the high chloroform treatment, indicating that degradation of feedstock and mineralization of labeled acetate continued with the low treatment. The incorporation of label into PLFA and PHA was not significantly changed by the low chloroform treatment, indicating that there was still significant bacterial metabolic activity, while the high chloroform treatment reduced incorporation to nearly zero. The percent of label incorporated into archaeal DE and TE was significantly less than control for the low and the high chloroform treatments, indicating a specific effect on the methanogens. Radiolabel was still incorporated into DE in the low chloroform treatment in the complete absence of methanogenesis.

The amount of chloroform used in the low level treatment, 26 \(\mu\text{mol}\cdot\text{g}^{-1}\), correcting for the approximately 29% total solids, gave an aqueous concentration of 4.4 mg L\(^{-1}\). Due to the extremely high solids content complexing the water and lowering the amount of free liquid, the effective solution concentration of chloroform could have been much higher. Yang and Speece\textsuperscript{41} found gas production from an acetate-fed methanogenic culture (CSTR, 50 day SRT) completely inhibited by 2.5 mg L\(^{-1}\) chloroform. The lower sensitivity to chloroform of this high-solids system than the completely liquid one of Yang and Speece may have been due to a protective effect of digester solids, as has been reported for heavy metals poisoning.\textsuperscript{43}

**CONCLUSIONS**

**High-solids reactor performance**

Differences between the disturbed, healthy, starved, and overfed reactor conditions have been found in their metabolic activities, PLFA profiles, and metabolic state markers, as well as indications of the micro-heterogeneity of the reactor contents in the disturbed condition 6 hours after feeding but not in the starved condition.

The reactor HS1 presents an example of a microbial community selected to efficiently utilize the iterative batch feeding cycle. The biomass was mechanically removed at feeding, as observed in the lower biomass per wet weight in the disturbed condition. However, the biomass was not less in the...
starved condition relative to the healthy. The microbial community was not dying off in the starved condition, but waiting to be fed again.

The overfed reactor HS2 was much less active, had a slightly lower biomass, a different community structure, and higher stress markers than the healthy condition of HS1 (Table 2). How the toxic overfed condition selected for a community that could survive such conditions warrants further study. Further, the overfed reactor was very difficult to bring back to a highly productive condition. Reduced feeding and ammonia supplementation rates, replacement of 30% of the reactor contents with healthy inoculum, and trace mineral supplementation did not restore performance for months. It should be determined whether members of the community were eliminated that were required for rapid fermentation and conversion of products to methane; or whether another community had been selected to survive and maintain the high acetate, propionate, and ammonia concentrations, the low pH and low productivity environment.

**Analytical microbial ecology**

Lipid biomarker analysis provides a "snapshot" of the amount, composition, and metabolic status of the viable microbial community. Radiotracer analysis allows specific in situ metabolic activities to be quantitated. The use of appropriate microcosms of the ecology studied allows the addition of radioactive or toxic compounds. The use of these methods in concert gave much more information than the sum of them applied individually. The relevant question in planning microbial ecology study is not whether the techniques of molecular biology, microscopy, lipid analysis, radiotracers, or microcosms are better, but which techniques answer the specific questions posed, and what combination of methods will give a synergistic increase in understanding.

The methane and carbon dioxide production rates and the pHs observed in the control pressure tubes were the same as those measured in the reactor itself, indicating that the anaerobic pressure tube microcosms were a suitable model system for evaluation of the high solids bioreactor activities in situ.

The use of lipid analysis, pressure tube microcosms, and radiolabel tracers to determine the effects of added oxygen or chloroform gave detailed information on shifts in the activities of specific populations within the reactor. One of the most interesting results was the increase in radiolabeled acetate incorporation into archael DE with exposure to oxygen. Further experiments with pure cultures and samples from bioreactors are planned to determine whether this is due to a differential effect on different groups of methanogens, or an adaptive response to the presence of oxygen.

The effects of a possibly toxic compound on a municipal solid waste treatment plant can not be tested on the plant itself. The detailed analysis of the effects of the toxicant on an appropriate microcosm system can be used to determine its mechanism of action and the possible efficacy of prophylactic measures.

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**REFERENCES**


Anaerobic conversion of biomass to methane


