Enumeration of fermentative and hydrolytic micro-organisms from three sanitary landfills

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(Received 14 April 1992; revised 7 September 1992; accepted 21 October 1992)

Fermentative bacteria were isolated from refuse excavated from municipal solid waste landfills in New York, Florida and Arizona. Anaerobic bacteria cultured on enriched solid media ranged from $10^5$ to $10^8$ c.f.u. (g dry wt of refuse)$^{-1}$. A significant correlation ($P < 0.03$) was found between numbers of anaerobes cultured at 37 °C and moisture content of refuse. Bacteria hydrolysing starch and protein represented 0–15% of the total anaerobes cultured; no anaerobic bacteria hydrolysing ball-milled cellulose were isolated. Aerobic bacteria isolated on enriched medium ranged between $10^4$ and $10^7$ c.f.u. (g dry wt)$^{-1}$. Direct microscopic counts of total bacteria associated with refuse were in the order of $10^8$ bacteria (g dry wt)$^{-1}$. These data suggest that, despite relatively high densities of bacteria in landfills, polymer hydrolysis is mediated by a small percentage of the fermentative population.

Introduction

Public concern with the disposal of municipal solid waste (MSW) in landfills has escalated in recent years as existing landfills close and new landfills become difficult to site (Carra & Cossu, 1990). In the US, an estimated 73% of MSW (118 million metric tons) was landfilled in 1988 (USEPA, 1990). Materials generated in the MSW stream in the US were of approximately 40% paper and paperboard, 17.6% yard waste, 7% glass, 8.5% metals, 7.4% food wastes, 8% plastics, and 11.6% other refuse, by weight (USEPA, 1990).

Components of the organic fraction of refuse may serve as substrates for micro-organisms in landfill environments (Grainger et al., 1984; Archer & Robertson, 1986; Sleat et al., 1987). The chemical composition of refuse includes polymers such as cellulose, starch, protein and lignin (Jones & Grainger, 1983). Aerobic bacteria may have a minor role in refuse decomposition prior to burial and the establishment of anoxic conditions. Anaerobic consortia within landfills mediate the processes of polymer hydrolysis, fermentation to organic acids, and mineralization by methanogenesis (Senior & Balba, 1990). Methanogenesis, the final step of decomposition of refuse in landfills, has received considerable attention (Barlaz et al., 1987; Archer & Peck, 1989) driven in part by interest in methane as an alternative fuel source (Campbell et al., 1985). For high molecular mass polymeric materials in MSW, however, polymer breakdown to monomers and their subsequent fermentation to organic acids must occur prior to methanogenesis.

Laboratory scale landfills or lysimeters have been widely used to study the degradation of refuse by anaerobic microbial consortia (Bookter & Ham, 1982; Barlaz et al., 1989a, b; Pohland et al., 1985). These laboratory models have proven useful for hypothesis testing about landfill processes, however, extrapolation of models to full-scale facilities can be limited, because of diversity among and heterogeneity within landfills (Parkes & Senior, 1988). To date, microbiological data from full-scale, operating landfills are few (Campbell et al., 1985) owing to the logistical difficulties inherent in their excavation.

The purpose of this study was (i) to culture and enumerate populations of fermentative anaerobic and aerobic bacteria; and (ii) to estimate populations of polymer-degrading micro-organisms in refuse excavated from three MSW landfills.

Methods

Study sites. Fresh Kills Landfill in Staten Island, New York was excavated 6–10 October, 1989 (Sulfta et al., 1992); the area typically receives about 109 cm of rain and 63 cm of snow annually. Upper
layers of refuse were relatively dry, however, refuse collected from below the water table was saturated, creating a dark grey slime. Los Reales Landfill in Tucson, AZ was excavated on 21 November, 1989; Tucson receives an average of 28 cm of rain per year. The Naples Landfill in Naples, FL, which receives about 135 cm of rain annually, was excavated on 12–14 March, 1990. All landfill excavations were conducted in collaboration with The Garbage Project, University of Arizona.

Sample collection. Refuse samples from Fresh Kills and Naples Landfill were collected with a bucket auger (Kellett’s Well Boring, Simpsonville, SC). Refuse samples from the Los Reales Landfill were collected with a back hoe. All samples were collected from depths >3 m to avoid contamination with surface refuse. Samples were dated from newspapers found in the excavated refuse. Prior to microbiological analysis, refuse samples were passed through a 5 cm mesh screen to remove larger debris. Exposure of samples to air was minimized as much as possible during sample collection.

Immediately upon collection, refuse samples from each site (Fresh Kills Landfill, 5 samples; Los Reales Landfill, 2 samples; Naples Landfill, 5 samples) were placed in sterile Petri dishes, then enclosed in anaerobic bags (Gas Pak, BBL, Becton Dickinson) with an atmosphere of 5–10% (v/v) CO₂. The anaerobic bags were kept cold on blue ice during transport to the Environmental Safety Department where they were placed in an anaerobic chamber (Forma Scientific Model 1024, Marietta, OH) with an atmosphere of 85% N₂, 10% H₂, and 5% CO₂, by vol.

Sample preparation. Inside an anaerobic chamber, 10 g of refuse was removed from the centre of each sample and added to a stomacher bag (Tekmar, Cincinnati, OH) containing 300 ml of pre-reduced, anaerobically sterilized (PRAS) buffer (Scott Laboratories) and mixed for approximately 1 min. This extract was serially diluted in PRAS buffer prior to inoculation.

Viable counts of fermentative and hydrolytic anaerobes. To culture fermentative bacteria, 0.1 ml diluted extract was inoculated under a continuous stream of N₂ into each medium using a VPI Anaerobic Chamber (Forma Scientific). Roll tubes were prepared immediately upon inoculation on a Belico tube spinner. Parallel sets of roll tube cultures were incubated at 22 °C and 37 °C for approximately 1–2 weeks or until the number of c.f.u. remained unchanged. C.f.u. were normalized per gram dry weight of refuse samples.

Fermentative anaerobes were isolated on Medium 10 (Carr Scarborough), which is a non-selective, enriched medium for isolation and enumeration of carbohydrate-fermenting bacteria (Caldwell & Bryant, 1966). Briefly, 11 of Medium 10 contained: glucose, 0.05 g; cellobiose, 0.05 g; soluble starch, 0.05 g; yeast extract, 0.05 g; trypticase, 0.2 g; haemin; mineral salts; a mixture of volatile fatty acids (acetic acid, propionic acid, n-butyric acid, n-valeric acid, isovaleric acid, isobutyric acid, DL-α-methylbutyric acid), resazurin, and cystine sulphide.

Polymer-degrading anaerobes from Fresh Kills and Tucson Landfills were cultured on peptone yeast glucose (PYG) agar amended with the polymer of interest. Differential staining or clearing of the medium identified polymer-degrading microbes. Anaerobic starch-degrading bacteria were identified by adding approximately 0.5 ml saturated KI solution to colonies growing on PYG medium with 0.2% soluble starch. Starch hydrolysis was indicated by clear zones around colonies. These samples were then stained with acridine orange and filtered on to 0.2 μm porosity Nuclepore filters; the bacteria were counted as described by Hobbie et al. (1977), and bacterial numbers were normalized to dry weight of refuse.

Results

Refuse samples used for culturing bacteria from Fresh Kills Landfill dated from 1962/1965 (the sample contained a mixture of refuse from two years) to 1988 (Table 1). Moisture levels for these samples ranged from 23±4% in the 1980 sample to 74±7% in the 1971 sample. In situ temperatures were lowest for older refuse samples, ranging from 18±3 °C (1962/1965) to 43±3 °C (1988) at five Fresh Kills Landfill sites sampled for culturing bacteria. All Fresh Kills Landfill sites were characterized by relatively high densities of bacteria, as determined by direct microscopic counts (1.1–8×10⁶ bacteria (g dry wt)⁻¹) using acridine orange stain. Despite predominantly anaerobic conditions in the landfill, aerobic bacteria were culturable at numbers comparable to anaerobes. Anaerobic bacteria ranged from 0.2–32.8×10⁶ c.f.u. (g dry wt)⁻¹ at 22 °C and 0.5–542×10⁶ c.f.u. (g dry wt)⁻¹ at 37 °C; aerobic bacteria ranged from 0.5–23.2×10⁶ c.f.u. (g dry wt)⁻¹ at 22 °C and 0.2–174×10⁶ c.f.u. (g dry wt)⁻¹ at 37 °C. The lowest numbers of anaerobic and aerobic bacteria were found in the oldest refuse (1962/1965). A positive correlation was found between total numbers of anaerobic bacteria cultured at 37 °C and percent moisture (r = 0.92; P < 0.03; Systat). No significant correlation, however, was found between moisture and anaerobes cultured at 22 °C, or moisture and aerobes cultured at 22 °C or 37 °C.

Refuse collected from Los Reales and Naples Landfills dated from the 1980s. Two refuse samples excavated from Los Reales Landfill were characterized by lower moisture than Fresh Kills Landfill; in situ temperature data were not collected from Los Reales Landfill. Despite lower moisture, direct microscopic counts for Los Reales Landfill (2.82–4.17×10⁶ bacteria (g dry wt)⁻¹) were similar in magnitude for counts from Fresh Kills Landfill,
and numbers of culturable fermentative bacteria were within the range found for samples from Fresh Kills Landfill. The Naples Landfill was characterized by moderate moisture (12.3–35.4%) and higher temperatures (49.4–62.8 °C). Refuse samples from Naples Landfill were only cultured at 37 °C because of the higher in situ temperatures. Both direct microscopic counts and c.f.u. per g dry wt of refuse were, in general, within the range found for refuse from Fresh Kills Landfill.

Bacteria from refuse samples were cultured anaerobically on media enriched with specific polymers to screen for hydrolytic activity (Table 2). Proteolytic colonies represented 0.6 to 15% and 0 to 4% of the

<table>
<thead>
<tr>
<th>Landfill Site Year</th>
<th>Water (%)</th>
<th>Site temperature (°C)</th>
<th>AODC [10^-10 x (g dry wt)-1]</th>
<th>Anaerobes [10^-6 x c.f.u. (g dry wt)-1]*</th>
<th>Aerobes [10^-6 x c.f.u. (g dry wt)-1]*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh Kills 8-2 1988</td>
<td>40</td>
<td>43</td>
<td>4.6 ± 3.0</td>
<td>20.5 ± 18</td>
<td>96.8 ± 0.15</td>
</tr>
<tr>
<td>Fresh Kills 1-2 1988</td>
<td>29</td>
<td>49</td>
<td>10.0 ± 10*</td>
<td>5.0 ± 0</td>
<td>4.9 ± 14</td>
</tr>
<tr>
<td>Fresh Kills 3-2 1980</td>
<td>23.4</td>
<td>21.6</td>
<td>58.9 ± 9*</td>
<td>65.0 ± 0.06†</td>
<td>7.9 ± 0.22†</td>
</tr>
<tr>
<td>Fresh Kills 9-2 1971</td>
<td>21.6</td>
<td>21.6</td>
<td>51 ± 2.7*</td>
<td>3.5 ± 1.5†</td>
<td>84.2 ± 24†</td>
</tr>
<tr>
<td>Fresh Kills 5-3 1962</td>
<td>43.3</td>
<td>18.3</td>
<td>24.9 ± 0.9*</td>
<td>0.2 ± 0.3</td>
<td>19.0 ± 0.31</td>
</tr>
<tr>
<td>Los Reales 1 1986</td>
<td>26.2</td>
<td>ND</td>
<td>2.8</td>
<td>3.1 ± 0.4†</td>
<td>7.2 ± 0.15†</td>
</tr>
<tr>
<td>Los Reales 2 1986</td>
<td>6.1</td>
<td>ND</td>
<td>4.17</td>
<td>2.29 ± 0.038†</td>
<td>2.86 ± 0.13†</td>
</tr>
<tr>
<td>Naples 1-2 1987</td>
<td>16.1</td>
<td>49.4</td>
<td>4.88</td>
<td>ND</td>
<td>50.3 ± 0.9</td>
</tr>
<tr>
<td>Naples 2-4 1987</td>
<td>35.4</td>
<td>57.2</td>
<td>2.81</td>
<td>0.0789 ± 0.0080</td>
<td>0.020 ± 0.008</td>
</tr>
<tr>
<td>Naples 2-6 1987</td>
<td>22.0</td>
<td>60.0</td>
<td>3.00</td>
<td>0.032 ± 0.002</td>
<td>0.063 ± 0.007</td>
</tr>
<tr>
<td>Naples 1-3 1987</td>
<td>12.3</td>
<td>62.8</td>
<td>3.05</td>
<td>ND</td>
<td>0.26 ± 0.01</td>
</tr>
<tr>
<td>Naples 2-8 1982</td>
<td>29.5</td>
<td>54.4</td>
<td>4.05</td>
<td>ND</td>
<td>4.70 ± 0.38</td>
</tr>
</tbody>
</table>

* Means ± SD of 3 subsamples.
† Significant difference between two temperatures at P < 0.005.
‡ Dated year of sample.
§ AODC, acridine orange direct count.
ND, no data.

Table 2. Fermentative bacteria degrading protein or starch in refuse excavated from Fresh Kills and Los Reales landfills

Figures are mean percentages ± SD from 3 replicates. Absolute figures [10^-6 x c.f.u. (g dry wt)-1] representing 100% are in parentheses.

<table>
<thead>
<tr>
<th>Landfill Site Year</th>
<th>22 °C</th>
<th>37 °C</th>
<th>22 °C</th>
<th>37 °C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Fresh kills 8-2 1988</td>
<td>15 ± 4</td>
<td>0.5 ± 0.8</td>
<td>12 ± 7</td>
<td>2.2 ± 0.5</td>
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<tr>
<td>Fresh kills 1-2 1984</td>
<td>11 ± 4</td>
<td>2.1 ± 0.2</td>
<td>4.0 ± 0.5</td>
<td>2.9 ± 0.1</td>
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<tr>
<td>Fresh kills 3-2 1980</td>
<td>2.0 ± 0.5</td>
<td>2.0 ± 0.1</td>
<td>(22.1 ± 1.8)</td>
<td>(0.04 ± 0.03)</td>
</tr>
<tr>
<td>Fresh kills 9-2 1971</td>
<td>10 ± 3</td>
<td>0.9 ± 0.3</td>
<td>3.3 ± 0.5</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Fresh kills 5-3 1965</td>
<td>7 ± 2</td>
<td>0 ± 0</td>
<td>7.08 ± 1.5</td>
<td>2.1 ± 0.2</td>
</tr>
<tr>
<td>Los Reales 1 1986</td>
<td>0.6 ± 0.3</td>
<td>0.2 ± 0.4</td>
<td>0.2 ± 0.2</td>
<td>0.7 ± 0.8</td>
</tr>
<tr>
<td>Los Reales 2 1986</td>
<td>5.5 ± 1.2</td>
<td>3.4 ± 1.2</td>
<td>13 ± 7.2</td>
<td>5.3 ± 1.0</td>
</tr>
</tbody>
</table>

* Dated year of sample.
fermentative populations in refuse samples from Fresh Kills and Los Reales Landfills incubated at 22 °C and 37 °C, respectively. Amylolytic bacteria comprised 0-2-14% and 0-7-5.3% of the fermentative population cultured from refuse samples incubated at 22 °C and 37 °C, respectively. No consistent trend in the percentage of hydrolytic bacteria was evident with age of refuse. Cellulolytic organisms, which were to be identified by clearing of ball-milled cellulose in an agar matrix, were not cultured from samples from Fresh Kills, Los Reales or Naples Landfills.

**Discussion**

Although relatively high numbers of fermentative bacteria \[10^5-10^9\text{c.f.u. (g dry wt)}^{-1}\] were cultured from landfilled refuse, only a small percentage (0-15%) were capable of producing extracellular hydrolytic enzymes for starch or protein degradation. While hydrolysis is a prerequisite for utilization of complex biopolymers by micro-organisms, initial enzymic attack may depend on a select few organisms (Colberg, 1988). Enumeration of c.f.u. on solid media is undoubtedly a very conservative estimate of the total number of fermentative bacteria in a landfill, since only some bacteria will be culturable on the medium provided. Moreover, the isolation of a viable organism from a landfill does not imply that the organism is active *in situ*; it may be merely surviving under landfill conditions. By contrast, the direct microscopic counts of acidine orange stained bacteria \[10^{10}\text{bacteria (g dry wt)}^{-1}\] may be an overestimate of viable bacteria, because non-viable cells may be inadvertently counted by this method.

Microbiological data from full-scale landfills is very limited, and many earlier studies focused on aerobic rather than anaerobic populations (Cook et al., 1967). This emphasis on aerobic populations is somewhat surprising, since aerobic micro-organisms undoubtedly play only a minor role in refuse decomposition and gas production in landfills. In the present study, approximately \[10^4\text{}\text{to}10^7\text{aerobic bacteria (g dry wt)}^{-1}\] were cultured from landfilled refuse samples on solid media. Campbell et al. (1985) suggested that aerobes may contribute to heat production prior to the development of anaerobic conditions in buried refuse. Cook et al. (1967) isolated aerobic fungi, streptomycetes, and photosynthetic bacteria from a sanitary landfill, fresh household refuse, and seepage from the landfill. Public health considerations were the impetus for several studies of landfill microbiology which focused on the isolation of indicator organisms such as coliforms and faecal streptococci from model and full scale landfills which received both municipal and hospital waste (Donnelly & Scarpino, 1984; Pahren, 1987). MSW that had been incubated for 20 months in a model landfill contained an estimated \[10^5\text{}\text{to}10^9\text{fungi and}10^6\text{actinomycetes by plate counts, and}10^2\text{}\text{to}10^6\text{aerobic proteolytic bacteria (g dry wt)}^{-1}\] by MPN counts using liquid media (Filip & Kuster, 1979).

Our screening of colonies for polymer hydrolysis suggested that only a small proportion (<15%) of the fermentative population produced extracellular enzymes capable of hydrolyzing protein and starch. Jones et al. (1983) examined hydrolytic bacteria in refuse from Aveley Landfill, Essex, Great Britain, as a function of depth. Proteolytic and amylolytic bacteria were each approximately 1% of the total anaerobic population. In a separate lysimeter study, Jones & Grainger (1983) used MPN counts to estimate changes in bacterial numbers during a 120 d laboratory incubation of water-saturated, freshly pulverized domestic refuse. Proteolytic organisms decreased from approximately \[10^8\text{}\text{to}10^6\text{organisms (g dry wt)}^{-1}\] during the course of the incubation while amylolytic organisms were approximately \[10^6\text{}\text{(g dry wt)}^{-1}\].

To date, very few cellulolytic bacteria from landfills have been isolated and characterized (Bagnara et al., 1985; Westlake, 1989). Our inability to isolate cellulolytic bacteria may be the result of (i) their low abundance in landfills (Jones et al. 1983); (ii) difficulty in extracting bacteria from refuse; (iii) inadequacy of cellulase detection (Robson & Chambliss, 1989); or most likely, (iv) their inability to grow on the solid media provided. Since <1% of the total bacteria determined by microscopic counts were culturable, cellulose-degrading bacteria may have easily been missed.

Logistic constraints resulted in analysis of relatively few refuse samples from the very heterogeneous landfill environment. Clearly, it is not appropriate to generalize from three sites to the approximately 7000 landfills in the United States which vary considerably in mode and efficiency of operation. However, we believe that our data on landfill microbiology will provide a baseline for future studies on a relatively poorly understood community of decomposers. An understanding of the establishment and maintenance of anaerobic communities in landfills is critical to improving leachate quality and thereby reducing the risk of environmental impacts of landfills such as groundwater contamination (Stegmann, 1983; Harper & Pohland, 1988).

Dr W. L. Rathje and The Garbage Project, Dr C. P. Gerba, Dr R. K. Ham, Dr J. S. Robinson, Dr J. M. Suflita, Mr W. Hughes, Mr L. W. King, and Mr B. Kellett collaborated on the Fresh Kills Landfill excavation. Moisture data were supplied by Dr J. M. Suflita. The research program was funded in its entirety by contributions from the Council for Solid Waste Solutions, E. I. Dupont de Nemours & Co., Exxon Chemical Co., Hercules, Inc., Hoechst Celanese, Jefferson Smurfit/Container Corporation of America, National Council of the Paper Industry on Air and Stream Improvement, The Perseco Co., The Procter & Gamble Co., Scott Paper Co., and Sonoco Products Co. The
authors thank Dr T W. Federle and Ms B A. Nuck for helpful discussions.

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