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Composition of the Landfill Microbial Community as Determined by Application of Domain- and Group-Specific 16S and 18S rRNA-Targeted Oligonucleotide Probes[▽]

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The microbial community composition of colonized cotton and leachate samples from a landfill was quantified using small subunit (SSU) rRNA probes (quantitative rRNA hybridization). Relative quantification of bacteria, eukaryotes, and archaea revealed variations in the landfill microbial community between samples from different areas of the landfill site and indicated the presence of potentially novel archaea. Anaerobic fungi were quantified in rumen fluid samples but were not sufficiently abundant for direct detection in the landfill samples.

Molecular ecological studies should now be focused on assigning functions to the recognized microbial assemblages in any given environment. However, with respect to landfill microbiology, even the identity and abundance of the indigenous microbiota are barely understood. This is in part due to the unfeasibility of obtaining representative samples of the highly heterogeneous solid waste fraction, together with the inability to retrieve waste samples other than leachate from capped landfill sites (2). PCR-based molecular ecological studies of landfill leachate have nevertheless enabled the direct detection of species known to be involved in primary degradation of cellulose, the main carbon source in landfill (29), and more recently, taxa that had previously been thought to occur only in the herbivore gastrointestinal tract, i.e., anaerobic fungi (16) and fibrobacters (20). Landfill microcosms or leachate bioreactors have also been used to provide source material for DNA and culture-based analyses (3, 4), but the composition of these microbial communities has not been directly compared to those of the landfill sites themselves. In the field of molecular microbial ecology, the overreliance on analysis of sequences generated by PCR amplification of environmental DNA extracts has recently been questioned (13), primarily on the basis of the recognized variation in amplification efficiency with different DNA templates, a shortcoming which also applies to quantitative PCR techniques. Furthermore, there is evidence that probe-based methodologies for the detection of rRNA genes in environmental microarrays reveal a greater diversity of microbial taxa than does the traditional sequencing of clones from PCR amplification products (9).

In this study, domain- and group-specific oligonucleotide probes were applied in slot blot hybridization experiments to quantify different obligately anaerobic microbial groups associated with cellulose degradation in rumen fluid, landfill leachate, and landfill microcosm RNA samples. The identity and molecular ecology of microorganisms capable of cellulose hydrolysis in the rumen are relatively well understood, and these samples therefore provided a reference point for evaluation of this approach as well as for providing fresh information on the abundance of anaerobic fungi in rumen fluid. Two landfill leachate microcosms were constructed with material from different waste cells within the same landfill site. Dewaxed cotton string (19) was incubated in each microcosm, and the RNA extracted from the cotton biofilm and the planktonic leachate-associated community was subjected to quantitative hybridization with domain-targeted probes and probes specific for particular subgroups of cellulolytic microorganisms.

The almost entire small subunit (SSU) (16S/18S) rRNA gene in DNA extracted from each bacterial, archaeal, and eukaryotic control strain was amplified using the primer sets pA and pH′, 1Af and 1404R, NS1-Euk and Univ 1390, respectively (10, 21, 30, 33), and Phusion high-fidelity (HF) DNA polymerase (Finnzymes). PCRs were performed in 50-μl volumes containing the following: 0.2 mM each primer, 0.2 mM each deoxynucleoside triphosphate (dNTP), 1× Phusion HF buffer (Finnzymes), 3% dimethyl sulfoxide (DMSO), 1× bovine serum albumin (BSA), 1 unit Phusion HF DNA polymerase (Finnzymes) and double-distilled water (ddH_2O). PCR cycling conditions were as follows: 98°C for 45 s, 30 cycles of 98°C for 10 s, 30 s at the specific annealing temperature for each primer set, 72°C for 20 s, and a final extension of 72°C for 8 min. PCR amplification products were cloned into *Escherichia coli* JM109 cells (Promega) by using the pGEM-T Easy vector system I (Promega) according to the manufacturer's protocol. Plasmid DNA was extracted and sequenced in both orientations by Macrogen, Inc. (South Korea). Cloned DNA sequences were assembled into contigs by using PreGap 4 and Gap 4 software (25), and base calling was visually checked using the sequencing traces. Reference rRNAs were synthesized from linearized plasmid DNA (3 μg) by using either T7 or SP6 RNA polymerase (Promega), and plasmid DNA was digested using a Turbo DNA-free kit (Ambion).

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TABLE 1. SSU (16S/18S) rRNA oligonucleotide hybridization probes used in this study and their wash temperatures (T_w)

Probe	Target site[a]	Sequence (5′–3′)[b]	Target group[c]	T_w (°C)	Reference
Univ 1390	1,390–1,407	GACGGGCGGTGTGTACAA	All known organisms	44	33
Eub 338	338–355	GCTGCCTCCCGTAGGAGT	Domain *Bacteria*	57	1
Cther 1352	1,352–1,370	GRCAGTATDCTGACCTRCC	*Clostridium* III	63	29
Erec 482	482–500	GCTTCTTAGTCARGTACCG	*Clostridium* XIVab	57	11
Euk 516	502–517	ACCAGACTTGCCCTCC	Domain *Eukarya*	51	1
Chyt 719	719–738	CAGTACACACAATGAAGTGC	Chytridiomycetes	44	16
Arc 915	915–934	GTGCTCCCCCGCCAATTCCT	Domain *Archaea*	64	26

[a] Probe position according to *Escherichia coli* 16S rRNA gene numbering.
[b] Ambiguities: K = G or T; S = G or C; W = A or T; Y = C or T; H = A, C, or T; R = A or G; M = A or C; D = G, A, or T; V = A, C, or G.
[c] Roman numerals refer to phylogenetic cluster of the *Clostridiaceae* as previously designated (6).

To determine the specificity of an oligonucleotide probe for its target group, each probe was hybridized against a panel of 32 reference rRNAs from control strains, which included representatives of phylogenetic groups indigenous to landfill and rumen environments, and control rRNAs possessing 1- to 4-bp mismatches within the probe target site. Stock reference rRNAs (100 ng μl^{-1}) were denatured by adding 3 volumes of 2% (vol/vol) glutaraldehyde in 50 mM NaH_2PO_4 (pH 7.0), followed by incubation for 10 min at room temperature. The rRNA was then diluted to 2 ng μl^{-1} by using a hybridization sample buffer (RNase-free water containing 1 $\mu g\ ml^{-1}$ poly[A] [Sigma] and 0.004% bromophenol blue). A 100-μl volume of each reference rRNA (200 ng) was blotted onto positively charged nylon membranes (Hybond) by using a slot blot device (Bio-Rad) under slight vacuum. Membranes were air dried, and rRNA was fixed to membranes by using a Stratalinker 2400 UV cross-linker (Stratagene). DNA oligonucleotide probes were 5′-end labeled with ^{32}P by using a T4 polynucleotide kinase (New England Biolabs) and [γ-^{32}P]ATP (Perkin Elmer) (8). Membranes were placed in glass hybridization tubes (Hybaid) and 100 μl hybridization buffer (0.9 M NaCl, 50 mM NaH_2PO_4 [pH 7.0], 5 mM EDTA, 10× Denhardt's solution, 0.5% SDS, and 0.1 mg poly[A] ml^{-1}) added per cm^2 of membrane. Membranes were prehybridized for 2 h at 40°C. A labeled probe (400 μl) was subsequently added and hybridized at 40°C for 16 to 18 h in a Techne hybridizer HB-1 (Techne) and then washed for 15 min at 40°C in wash buffer (1× SSC [0.15 M NaCl plus 0.0015 M sodium citrate], 1% SDS) and washed two subsequent times for 30 min each at the optimum wash temperature (T_w) for each probe (Table 1). Phosphor screens (Molecular Dynamics) were used to visualize membranes, and hybridization signals were determined using a Storm 860 scanner (Molecular Dynamics) and quantified using TotalLab TL100 software (Nonlinear Dynamics).

All probes were checked using the Ribosomal Database Project (RDP) probe match function (5) and the Probebase website (17) and found to be specific for their target group (Table 1). Probe specificity was initially tested using the published T_w for each respective probe. If hybridization with nontarget reference rRNAs was observed after washing at the published T_w, the membrane was subsequently washed with increasing temperatures of 1 to 2°C until hybridization with nontarget reference rRNAs could not be observed. The T_ws of the new probes, Chyt 719 and Cther 1352, were determined in this manner (Table 1). The universal and domain-specific hybridization probes performed as expected, and hybridization was observed only with target organisms (Fig. 1). The bacterial probe (Eub 338) did not hybridize to the *Clostridium pasteurianum* reference rRNA (Eub 338 membrane, position 11 [Fig. 1]) because the probe target site had a 1-bp mismatch with the Eub 338 probe, and this observation therefore confirmed the absolute specificity of probe Eub 338 under the hybridization conditions applied. Although signal intensities varied (Fig. 1), this is not unexpected in quantitative RNA probing (22, 24) and is obviated here by our inclusion of additional controls in which known mixtures of control RNAs were used to demonstrate that quantitation was nevertheless achievable (Fig. 2).

Probes Cther 1352 (*Clostridium* cluster III), Erec 482 (*Clostridium* cluster XIV), and Chyt 719 (Neocallimastigales) hybridized only with their target reference rRNAs (data not shown). Dewaxed cotton string (32) in nylon mesh bags was suspended from nylon ropes inside the necks of two 10-liter carboys (Nalgene). The carboys were transported to the Bromborough Dock landfill site (Wirral, United Kingdom) where they were filled to the top with fresh landfill leachate pumped directly from risers 3 and 5. Once filled to the brim, the microcosms were immediately sealed and transported to the laboratory, where they were incubated at ambient temperature. Nylon mesh bags containing cotton were retrieved after 6 weeks of incubation in the microcosm. One bovine and two ovine rumen fluid samples were kindly provided by Richard Kemp (University of Liverpool). For RNA extraction, 500 μl of rumen fluid containing fibrous plant material, a small piece of colonized cotton (~0.5 g) from landfill microcosms, or an entire 0.2- and 0.7-μm-pore-diameter filter membrane through which 2 liters of leachate had been filtered was processed according to the method of Griffiths et al. (12) and DNase treated with a Turbo DNA-free kit (Ambion). RNA extracts at a concentration of 100 ng μl^{-1} were denatured by adding 3 volumes of 2% (vol/vol) glutaraldehyde in 50 mM NaH_2PO_4 (pH 7.0), incubated for 10 min at room temperature, and diluted in a hybridization sample buffer to a final concentration of 4 ng μl^{-1}. A 100-μl volume of sample (400 ng RNA) was applied in triplicate to positively charged nylon membranes and hybridized as described above. A dilution series of known RNA quantities from an appropriate reference rRNA positive control recognized by the oligonucleotide probe target group was included on each membrane as a standard. Following hybridization, each membrane was washed at the optimum wash temperature (T_w) for each probe (Table 1). To determine rRNA abundance for each oligonucleotide probe target group, a standard curve was constructed using linear regression from

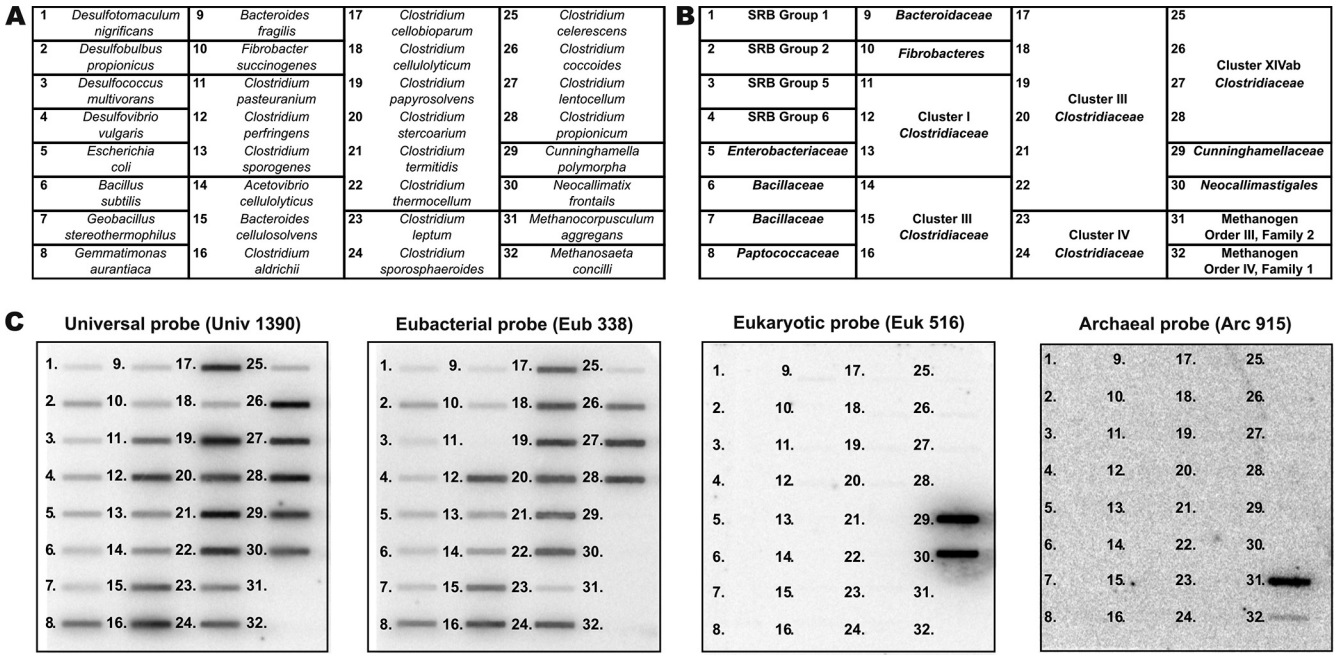

FIG. 1. Hybridization probe specificity tests. (A) Membrane layout of reference SSU rRNAs generated from 32 target and nontarget species. (B) Phylogenetic affiliation of reference rRNA species. (C) Hybridization of universal and domain-specific probes to target and nontarget SSU reference rRNAs. The membrane layout was as shown in panel A. Sulfate-reducing bacterium (SRB) groups as defined by Daly et al. (7); *Clostridiaceae* clusters as described by Collins et al. (6). The universal probe did not hybridize with the archaeal control rRNAs (position 31 and 32 on the universal membrane), as the archaeal control RNAs were generated from cloned sequences containing an incomplete 16S rRNA gene product. Therefore, the probe binding site for Univ 1390 was absent from the rRNA molecules.

the known quantities of rRNA standards applied to each membrane. The relative abundance of each target group was calculated as a percentage of the total SSU rRNA abundance as determined by hybridization of each sample with a universal probe (Univ 1390). Abundances of target groups relative to total bacterial 16S rRNA and eukaryotic 18S rRNA were determined by comparison with data from application of the general bacterial and general eukaryotic probes, Eub 338 and Euk 516, respectively (1). Quantitative hybridization of the spiked rRNA mixture also demonstrated that all seven of the probes applied in this study were stringent and capable of accurate quantification of the mixed rRNA template sample (Fig. 2).

Community RNA preparations from bovine and ovine rumen fluid samples were hybridized with the suite of rRNA probes described and validated above. Quantitative data were obtained for bacteria, eukaryotes, and archaea (Fig. 3), in addition to *Clostridium* cluster XIV and Neocallimastigales (Table 2). Although the *Clostridium* cluster III probe was suc-

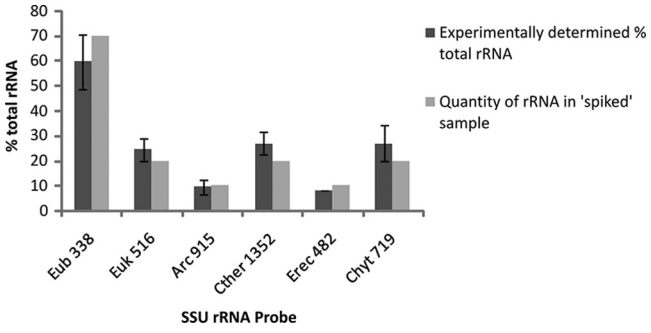

FIG. 2. Quantification of SSU rRNA in a "spiked" mixture containing known quantities of reference rRNA, using domain- and group-specific hybridization probes. Error bars represent standard deviations; samples were blotted in triplicate on each membrane; and results represent the average of triplicate blots. For probe targets and specificity, see Table 1.

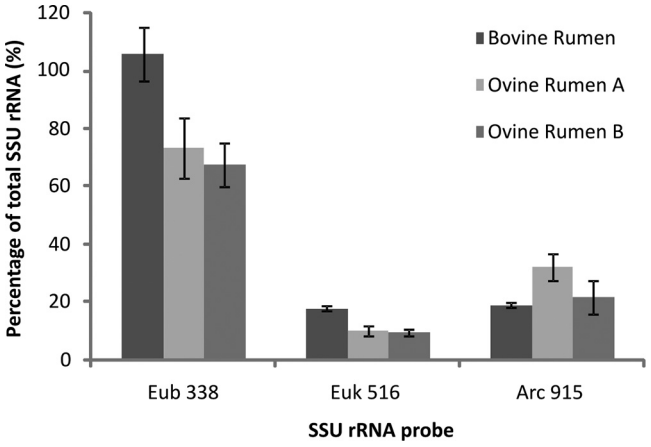

FIG. 3. Quantification of SSU rRNA in bovine and ovine rumen fluid community RNA samples using domain-specific hybridization probes. Error bars represent standard deviations; samples were blotted in triplicate on each membrane; and results represent the average of triplicate blots. For probe targets and specificity, see Table 1.

TABLE 2. Quantification of members of *Clostridium* cluster XIVab and anaerobic fungi (Neocallimastigales) compared to total SSU rRNA and total bacterial and eukaryotic rRNA, respectively, in ovine and bovine rumen samples

Sample	% Total of indicated rRNA in:			
	Clostridiaceae cluster XIVab		Anaerobic fungi (Neocallimastigales)	
	SSU	Bacterial	SSU	Eukaryotic
Bovine rumen	26.5 ± 2.9	25.1 ± 0.5	13.8 ± 1.1	78.4 ± 1.5
Ovine rumen A	15.3 ± 1.1	21.1 ± 2.4	6.8 ± 1.0	69.7 ± 1.7
Ovine rumen B	8.3 ± 1.2	12.4 ± 0.9	5.1 ± 3.0	58.5 ± 1.8

cessfully applied to the spiked rRNA mixture (Fig. 2), a very low hybridization signal was obtained for rumen fluid samples. *Clostridium* cluster III has been quantified in equine colonic samples, and the relative abundance varied from 1.3% to 3.0% of the total rRNA (8). It may be, therefore, that the relative proportion of cluster III clostridia is also low in the rumen fluid samples analyzed here and abundances of 1 to 3% are below the threshold for accurate quantification. Similar abundances of bacteria, archaea, and eukaryotes were observed across the three rumen fluid samples. The total abundances for the three domain probes combined were 142%, 115%, and 98% for each of the rumen samples (bovine, ovine A, and ovine B), respectively, indicating that the universal probe does not bind to all SSU rRNA molecules. The relative abundance of bacteria in the rumen samples varied from 67% to 106% (Fig. 3), indicating that bacteria are the most abundant microbial group in the rumen. Archaea were the second most abundant group, with relative abundance values ranging from 19% to 32%, and the relative abundance of eukaryotes varied from 9.4% to 17.6% (Fig. 3). A previous study using the same probe (Eub 338) by Lin et al. (15) assessed the microbial community structure in samples from domestic animals (including bovine and ovine samples) and also reported bacteria as the most abundant group, with relative abundances similar to those obtained in this study.

The relative abundance of *Clostridium* cluster XIV, as determined using probe Erec 482, varied from 8 to 27% for the rumen samples (Table 2). These data are in agreement with the study of Daly and Shirazi-Beechey (8), in which the abundance of cluster XIV as determined with the same probe (Erec 482) was found to represent the highest proportion of rRNA found in any one of the equine intestinal samples studied (28%). The abundance of cluster XIV clostridia was found to vary greatly in their study, and this was also the case here. Quantitative data for the anaerobic fungi also compared favorably with previously published data. The average abundance of Neocallimastigales in the rumen samples was 9% (range, 5 to 14%) (Table 2), and it has been suggested that anaerobic fungi represent about 8% of the rumen biomass based on quantification of lipid biomarkers (14). The data reported here therefore suggest that anaerobic fungi account for between 59 and 78% of the eukaryotic rRNA in ovine and bovine rumen samples (Table 2), further supporting their importance in cellulose hydrolysis in rumen environments (27). Members of this fungal order possess the most-potent cellulase systems known in the biological world (31) and are recognized as major colonizers of plant biomass (27).

It was not possible to obtain quantitative data for any of the *Clostridium* clusters or the Neocallimastigales in the landfill leachate or colonized cotton samples because only low levels of hybridization were observed. However, domain-level relative abundances of the samples were obtained and demonstrated marked differences in the microbial community composition between the two microcosm experiments. No eukaryotic rRNA was detected in community RNA from Bromborough Dock riser 3 leachate or colonized cotton samples that had been suspended in leachate samples taken from riser 3 (Fig. 4). However, eukaryotes were detected in the riser 5 samples, and their relative abundances were 7% (±0.5%) and 17% (±0.5%) for the leachate and colonized cotton samples, respectively (Fig. 4). These differences in the detection levels for eukaryotes are probably a reflection of the inherent heterogeneity of the microbial community in samples drawn from different areas of the same landfill site.

An encouraging observation is that there appears to be a close correlation between the abundances of bacteria, archaea, and eukaryotes in landfill leachate and the colonized cotton samples in leachate microcosms. These data therefore support the use of leachate-based laboratory simulators of landfill microbiology, at least in terms of comparable relative abundances of bacteria, archaea, and eukaryotes. The relative abundance of archaea in the riser 3 microcosm samples was, however, surprising. Abundances of 225% (±42%) and 215% (±34%)

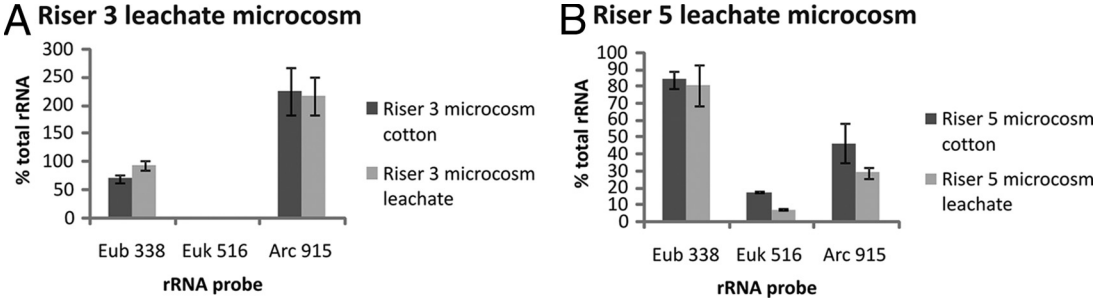

FIG. 4. Quantification of bacteria, archaea, and eukaryotes in community RNA samples extracted from colonized cotton and leachate from the riser 3 (A) and riser 5 (B) landfill leachate microcosms, using domain-specific hybridization probes targeting the SSU rRNA gene. Error bars represent standard deviations; samples were blotted in triplicate on each membrane; and results represent the average of triplicate blots. For probe targets and specificity, see Table 1.

were obtained for the colonized cotton and leachate samples, respectively. As the Arc 915 probe performed so effectively in quantifying the spiked RNA and rumen samples (Fig. 2 and 3) and in probe specificity tests (Fig. 1), it is unlikely that this is erroneous. The most likely explanation is the presence of archaea that have sequence diversity within the probe target site for the universal probe (Univ 1390) and are therefore not detected by that probe. This implies the existence of as-yet-uncharacterized members of the *Archaea* in the landfill microbial community. *Bacteria* were the most abundant group in the riser 5 microcosm samples (Fig. 4) and represented 84% (±5%) and 80% (±12%) of the total rRNA in the colonized cotton and leachate samples, respectively.

A major concern in the study of landfill site microbiology is the question of whether a leachate sample is representative of the microbial community that colonizes biodegradable organic material within the site. Furthermore, the heterogeneity of landfill waste across a given site means that obtaining a representative sample of the waste fraction is impossible (23). A network of leachate collection pipes and pumps (or risers) removes leachate from the base of the waste cell to a treatment plant, and thus, leachate is the only sample type available for direct analysis. As leachate results from the percolation of liquid through the site, it potentially provides a comprehensive sample of the landfill microbiota. Lab-scale landfill bioreactors have also been used in previous studies of landfill microbiology (3, 4). In this study, the construction of laboratory-based landfill leachate microcosms enabled the comparison of the microbial community composition of colonized cellulosic material and leachate samples derived from the same area of a landfill site. For the first time, these data have highlighted two important observations: (i) the composition of microbial populations in different areas of the same landfill site can be significantly different (Fig. 4); and (ii) at the domain level, the relative abundances of bacteria, archaea, and eukaryotes in colonized cotton substrates from microcosm experiments and landfill leachate samples from the same region of the landfill site in most cases are not significantly different. To our knowledge, there have been no previous reports on the relative contributions of bacteria, archaea, and eukaryotes to the total microbial community in leachate or colonized cellulosic substrates in landfill, and this study therefore provides an indication of the importance of each microbial domain *in situ*.

A limitation of the slot blot hybridization method used here is the sensitivity of detection that can be achieved, and this method has now almost entirely been replaced by quantitative PCR methodologies that offer up to 1,000-fold greater sensitivity of detection than do RNA hybridization experiments (18). Single probes may, however, be more capable of detecting a greater diversity of sequences in SSU rRNA than that which would be detected by amplification with a PCR primer pair (28). This has certainly been the case for the phylogenetic microarray, the PhyloChip (9), where the application of 16S rRNA gene probes identified a greater diversity of microbial taxa in environmental samples than that of cloned and sequenced universal PCR amplification products from the same samples.

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