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Critical evaluation of solid waste sample processing for DNA-based microbial community analysis

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Abstract Landfills represent a unique microbial ecosystem and play a significant role in global biogeochemical processes. The study of complex ecosystems such as landfills using DNA-based techniques can be advantageous since they allow for analysis of uncultured organisms and offer higher resolution in measuring demographic and metabolic (functional) diversity. However, sample acquisition and processing from refuse is challenging due to material heterogeneity. Decomposed refuse was used to evaluate the effect of seven sample processing methods on *Bacteria* and *Archaea* community structure using T-RFLP. Bias was assessed using measured richness and by comparing community structure using multi-dimensional scaling (MDS). Generally, direct methods were found to be most biased while indirect methods (i.e., removal of cellular material from the refuse matrix before DNA extraction) were least biased. An indirect method using PO_4 buffer gave consistently high bacterial and archaeal richness and also resulted in 28 and 34% recovery of *R. albus* and *M. formicicum* spiked into refuse, respectively. However, the highest recovery of less abundant T-RFs was achieved using multiple processing methods. Results indicate differences in measured T-RF diversity from studies of landfill ecosystems could be caused by methodological (i.e., processing method) variation rather than refuse heterogeneity or true divergence in community structure.

Keywords Landfill · Solid waste · Refuse · T-RFLP · Sample processing · Bias

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Introduction

The relationship between community structure and microbial function in landfills is important as landfills significantly impact global biogeochemical processes (e.g., greenhouse gas emissions, carbon sequestration). Landfilled refuse is the second largest global source of anthropogenic methane (CH_4), which is a potent greenhouse gas (Denman et al. 2007). Refuse decomposition is microbially mediated and the diversity and metabolic function of microbial communities act as mechanistic controls on the rate of greenhouse gas emissions, extent of decomposition, and carbon sequestration capacity. There is a pressing need for improved technologies to enhance solid waste decomposition. Unfortunately optimization of these technologies is limited by a poor understanding

of microbial interactions during refuse degradation which are due, in part, to the heterogeneous and unstructured nature of the wastes.

Compared to culture-based techniques, the study of complex microbial ecosystems using molecular techniques can be advantageous since they allow for analysis of uncultured organisms and offer higher resolution in measuring demographic (number of species) and metabolic (functional) diversity. However, accurate ecological assessments are predicated on extracting nucleic acids that are representative of the microbial community. The importance of sample processing procedures prior to, or, as part of nucleic acid extraction when assessing microbial diversity has been recognized and it has been suggested that using multiple methods may give rise to the least biased DNA pool in sediments (Luna et al. 2006). Previous studies on soil and activated sludge have shown that sample processing can impose a bias because microbial community structure in the extracted nucleic acids is different from that in the sample (Keith et al. 2005; Sessitsch et al. 2002). This suggests that methods used to process samples from complex or heterogeneous materials may be equally or more important than the extraction procedure. Heterogeneous materials can be defined as having variable consistency, particle size, and composition and include organic-rich soils/sediments, humus, leaf litter, manure, rumen/cecum contents, peat, compost and refuse.

The sample mass from which nucleic acids are extracted using commercial kits and many laboratory protocols typically ranges from 0.25 to 1 g with lower and upper limits of roughly 0.1 and 10 g, respectively. This range is governed by a number of factors including available tube size, reagent volume:sample mass ratio and cell lysing efficiency. Such small masses, along with large particle sizes, often preclude direct placement of heterogeneous materials into extraction tubes. For example, particle size in municipal refuse can vary from millimeters to meters depending on the composition and extent of degradation (Hull et al. 2005). Additionally, refuse contains varying fractions of paper, plastic, metals, food scraps and other components (USEPA 2005), making it extremely heterogeneous from a chemical and structural perspective. Thus, traditional protocols used for soils, sediments, wastewater and other comparatively homogeneous matrices cannot be employed. Rather, heterogeneous materials must be processed prior to extraction to reduce particle size and ensure a reasonable degree of sample homogeneity.

Most sample processing protocols utilize one or more types of physical, chemical or enzymatic methods to make the sample material more amenable for subsequent nucleic acid recovery. Direct methods, where cells are lysed within the sample matrix, have been compared to indirect methods, where cells are first separated from non-cellular debris prior to lysing (Gabor et al. 2003). The direct method assumes the material placed into extraction tubes is representative of the sampled ecosystem; however, this assumption is tenuous for heterogeneous materials. Thus, indirect methods may prove beneficial for heterogeneous samples since separation from the material matrix may serve to concentrate cells from larger sample masses.

The objective of this study was to develop a sample processing method for municipal refuse for DNA extraction that minimizes bias while ensuring quantity and quality are sufficient for sensitive downstream molecular applications such as qPCR. Previous molecular studies evaluating landfill microbiology have relied on aqueous phase (leachate) samples or have extracted DNA without consideration of sample processing bias (Calli and Girgin 2005; Uz et al. 2003). Seven processing methods, comprising both direct and indirect protocols, were modified from previously published studies and compared using terminal restriction fragment length polymorphism (T-RFLP). Most studies evaluating sample processing and DNA/RNA extraction methods have used yield and extract purity as metrics for assessing effectiveness (Table 1). Only a few have evaluated how a particular method affects community structure using fingerprinting methods (e.g., DGGE, T-RFLP) or clone libraries (Luna et al. 2006; Sessitsch et al. 2002; Gabor et al. 2003). Although T-RFLP is not effective at estimating absolute diversity or detecting very rare species, it has been shown to be effective for comparing differences in community structure between treatments (Dunbar et al. 2001; Hartmann and Widmer 2006, 2008).

Materials and methods

Refuse sample preparation and reactor monitoring

Roughly 1.4 m^3 of municipal refuse was obtained from a residential area collection vehicle at the Holly

Table 1 Occurrence of sample processing and DNA extraction techniques used in previous solid waste, rumen and soil studies

Sample processing methods	Solid waste	Rumen	Soil	Total	% of category	References[c]
Physical methods						
Bead beat	4	3	12	19	18.1	1–19
Filtration[a]	1	11	3	15	14.3	11, 20–34
Blending	2	6	6	14	13.3	4, 9, 12, 15, 17, 23, 25–28, 31, 34–36
Freeze–thaw	3	2	7	12	11.4	1, 3, 7, 9, 12–14, 16, 35, 37–39
Centrifugation[b]	0	5	6	11	10.5	2, 17, 20, 25, 29, 30, 31, 35, 36, 40, 41
Shaking	1	2	6	9	8.6	2, 9, 17, 28, 29, 32, 35, 38, 42
Chill	1	7	1	9	8.6	12, 15, 22, 23, 25, 27, 28, 29, 33
Grind	0	1	6	7	6.7	11, 34, 37, 38, 40, 42, 43
Heat	0	2	4	6	5.7	1, 3, 16, 29, 35, 38
Sonication	0	1	2	3	2.9	12, 32, 34
Total for category				105	100	
Chemical methods						
SDS	5	4	17	26	33.3	1–3, 6–17, 34–40, 43, 44, 46, 47
CTAB	0	3	10	13	16.7	5, 7, 8. 11, 12, 17, 36–38, 41–43, 47
PVPP	0	1	8	9	11.5	6, 8, 11, 12, 34–38
Methylcellulose	0	7	0	7	9.0	28, 29, 33, 44, 48, 49, 50
Tween 80	0	4	1	5	6.4	22, 23, 28, 32, 33
Formaldehyde	0	3	1	4	5.1	22, 28, 32, 50
Guanidine thiocyanate	1	1	2	4	5.1	7, 9, 16, 39
Carboxymethyl cellulose	0	3	0	3	3.8	48, 50, 51
Methanol	0	3	0	3	3.8	28, 33, 44
Tertiary butanol	0	2	0	2	2.6	29, 33
Sodium oleate	0	1	0	1	1.3	26
Dextrin	0	1	0	1	1.3	48
Total for category				78	100	
Enzymatic methods						
Lysozyme	3	0	9	12	52.2	7, 9, 15, 17, 19, 31, 35, 34, 39, 42, 45, 46
Proteinase K	2	1	8	11	47.8	7, 8, 17, 19, 35, 36, 42, 43, 45–47
Total for category				23	100	

Number of times the method was used in 51 studies. In cases where the method was used multiple times in a single study, each method was counted once per study to avoid artificially inflating the method's frequency of use

SDS sodium dodecyl sulfate, *CTAB* cetyltrimethylammonium bromide, *PVPP* polyvinyl polypyrrolidone

[a] Performed by either hand squeezing or sieving

[b] Centrifugation procedure denotes differential centrifugation was performed for cell separation

[c] Numbers in this table are designated for cited references as follows: 1 (Cullen and Hirsch 1998), 2 (Duarte et al. 1998), 3 (Kuske et al. 1998), 4 (Reilly and Attwood 1998), 5 (Burgmann et al. 2001), 6 (Martin-Laurent et al. 2001), 7 (Niemi et al. 2001), 8 (Stach et al. 2001), 9 (LaMontagne et al. 2002), 10 (Lehman and O'Connell 2002), 11 (Sessitsch et al. 2002), 12 (Anderson and Lebepe-Mazur 2003), 13 (Chen et al. 2003a, b), 14 (Chen et al. 2003a, b), 15 (Costa and de Oliveira 2003), 16 (Fus et al. 2003), 17 (Gabor et al. 2003), 18 (Uz et al. 2003), 19 (Webster et al. 2003), 20 (Grubb and Dehority 1976), 21 (Miller et al. 1986), 22 (Craig et al. 1987), 23 (Leedle et al. 1987), 24 (Leedle and Butine 1987), 25 (Olubobokun et al. 1987), 26 (Barsuhn et al. 1988), 27 (Barlaz et al. 1989a, b), 28 (Whitehouse et al. 1994), 29 (Martin-Orue et al. 1998), 30 (Yanagita et al. 2000), 31 (Courtois et al. 2001), 32 (Bockelmann et al. 2003), 33 (Ranilla and Carro 2003), 34 (Frostegard et al. 1999), 35 (Tien et al. 1999), 36 (Luna et al. 2006), 37 (Wikstrom et al. 1996), 38 (Zhou et al. 1996), 39 (Miller et al. 1999), 40 (Volossiouk et al. 1995), 41 (Krause et al. 2001), 42 (Ranjard et al. 1998), 43 (Sharma et al. 2003), 44 (Cheng et al. 1991), 45 (Huang et al. 2003), 46 (Huang et al. 2004), 47 (Fortin et al. 2004), 48 (Minato and Suto 1978), 49 (Kudo et al. 1987), 50 (Rasmussen et al. 1989), 51 (Minato and Suto 1981)

Springs (NC) Transfer Station and appeared consistent with refuse composition given by the USEPA (2005). The refuse was shredded the same day in a slow speed, high torque shredder to a maximum particle size of approximately 2 cm × 6 cm. After shredding, refuse was mixed on a clean plastic sheet using hand tools and then stored at 4°C in plastic trash bags prior to compacting a portion of the shredded refuse (~20% of the initial volume) in a 208-L reactor for anaerobic degradation. The reactor was maintained at 37°C and operated with leachate recycle and neutralization to accelerate decomposition, as described previously (Staley et al. 2006). Gas was collected in tedlar gas bags (PMC, Inc; Oak Park, IL) and volume was measured by evacuation into a container of known volume (Sanin et al. 2000). CH_4, CO_2, O_2, and N_2 were analyzed using a GOW-MAC 580 gas chromatograph with a thermal conductivity detector (Price et al. 2003). Samples were removed from reactors during the accelerated methane and decelerated methane (also referred to as well-decomposed) phases of decomposition (Barlaz et al. 1989a, b). For accelerated methane phase sampling, refuse was removed when the fraction of CH_4 in the biogas reached 50% and while the gas production rate was increasing exponentially (~31 days after reactor initiation). The CH_4 production rate at the time of sampling was 0.76 ml day^{-1} dry g refuse^{-1}. For the well-decomposed phase, refuse was allowed to decompose for approximately 6 months and the CH_4 production rate at the time of sampling was 0.004 ml day^{-1} dry g refuse^{-1}.

Experimental design

For each phase of decomposition, roughly 2 kg anaerobically decomposed refuse was removed from the reactor and mixed well prior to separation into 50 g aliquots (~50 cm^3 volume), which were then frozen until sample processing. Triplicate aliquots from the same refuse sample were processed with each of seven processing methods described below. Each aliquot was processed individually and triplicate DNA extractions were performed for each aliquot, resulting in 9 total DNA extractions per processing method. The variability due to the heterogeneous nature of the refuse material was assessed using T-RFLP for three processing methods FT-D, DG-D and PO_4-C (described below). The richness and Bray-Curtis similarities using multi-dimensional scaling (MDS) analysis (see below) were compared for the triplicate aliquots. The results showed that in general, the variation due to refuse heterogeneity was lower than the differences between sample processing methods. Thus, in subsequent comparisons for all methods, equal DNA masses from each of the nine extracts were combined to create a pooled DNA sample for T-RFLP analyses. This pooling served to minimize both sample-to sample as well as extraction variability for each processing method. Parallel aliquots were used for moisture content analysis.

Processing methods

A number of methods identified from the literature (Table 1) were highly cited but were not incorporated into sample processing protocols for this study. For example, bead beating was a component of the DNA extraction kit used and thus was not considered for sample processing. Differential centrifugation was not selected as an indirect protocol given its potential to select against attached microorganisms (Robe et al. 2003). CTAB is a cationic detergent typically used in DNA extraction to precipitate polysaccharides and proteins and does not aid in either cell detachment or lysis (Mygind et al. 2003). Similarly, PVPP aids humic acid removal but is ineffective for cell lysis (Robe et al. 2003); thus, neither were used. Refuse samples were subjected to the following direct or indirect sample processing techniques prior to DNA extraction.

Direct methods

Direct phosphate buffer method (PO_4-D) (Barlaz et al. 1989a, b): Each aliquot was combined with 250 ml chilled 23.7 mM PO_4 buffer at pH 6.73 (31.87 g $Na_2HPO_4{\cdot}7H_2O$ l^{-1} and 16.1 g KH_2PO_4 l^{-1}) and homogenized in a sterile Waring blender (Torrington, CT) for 1 min. Then 200 ml of the mixture was transferred immediately after blending to four sterile 50 ml tubes and centrifuged at 3,220×*g* for 5 min. In supplemental work, the effect of two centrifuge speeds, 3,220×*g* and 16,000×*g* (Burgmann et al. 2001) was compared and there were no significant differences ($P > 0.05$) in richness and total sample fluorescence using T-RFLP (data not shown). Supernatant was decanted and pellets were combined into a single tube. Residual in emptied tubes was suspended with deionized (DI) water, combined with the pellet

and centrifuged for 5 min at 3,220×*g*. The resulting combined pellet was well-mixed by hand using a sterile spatula.

Freeze–Thaw method (FT-D) (Miller et al. 1999): Aliquots were subjected to three freeze–thaw cycles, which exclude the initial freezing step noted in the Refuse sample preparation and reactor monitoring section. Each cycle included freezing at −80°C for more than 4 h followed by thawing at 60°C until the aliquot reached 25°C. Each aliquot was then combined with 250 ml chilled PO_4 buffer containing 27% sodium dodecyl sulfate (SDS) (20% w/v) and 5.5% lysozyme (50 mg ml^{-1}) by volume (Gabor et al. 2003). Aliquots were homogenized in a Waring blender for 1 min and then pelletized as described in the PO_4-D method.

SDS/lysozyme method (SDS-D) (Gabor et al. 2003): This method is identical to method FT-D except there were no freeze/thaw cycles prior to SDS and lysozyme addition.

Dry and grind method (DG-D): The DG-D method was developed as part of this study. Aliquots were placed in sterile mason jars and dried at 65°C for approximately 48 h. Two layers of absorbent wipes were used to cover the opening while drying and preliminary work indicated no cross-contamination between aliquots during drying. Aliquots were then ground in a wiley mill to pass a 1 mm screen. The wiley mill was wiped with ethanol between aliquots. The dry, ground material was transferred to a 50 ml centrifuge tube and mixed using a sterile spatula.

Indirect (cell separation) methods

Indirect phosphate buffer method (PO_4-C) (Barlaz et al. 1989a, b): Aliquots were combined with 250 ml chilled PO_4 buffer and homogenized in a Waring blender for 1 min. To separate microbial cells, the blended mixture was poured into a 3.8 l 75-μm nylon paint strainer bag (Trima Co.) and hand-squeezed. Supernatant was collected in a 1.9 l sterile plastic container, transferred to six sterile 50 ml tubes, centrifuged and combined to generate a single pellet as described in method PO_4-D.

Indirect methylcellulose method (M-C): This method is the same as PO_4-C except that the PO_4 buffer included 1% (w/v) methylcellulose and the mixture was allowed to stand for 10 min after blending (Kudo et al. 1987).

Methylcellulose/tween 80 method (MT-C): This method is identical to the M-C method except that 0.1% Tween 80 (Sigma-Aldrich) by volume was also included in the phosphate buffer (Whitehouse et al. 1994).

DNA quantitation and extraction

In preliminary work, two DNA quantitation methods were compared: (a) spectrophotometry at 260 nm and (b) fluorometry using SYBR Green I as described (Zipper et al. 2003). Quantification using spectrophotometry gave higher but statistically similar ($P > 0.05$) values compared to fluorometry using SYBR Green I (data not shown), suggesting any impurities carried over during extraction did not translate into significant quantitation error using spectrophotometry. Thus, spectrophotometry was used in the main study for DNA quantitation and to assess purity (A_{260}/A_{280}) (Sambrook and Russell 2001).

The centrifuged pellet (300 mg) or dried, ground material (50 mg) generated by the processing methods were used for DNA extraction, which was performed following the protocol supplied by the extraction kit manufacturer. Initial work was performed to compare two commercial kits: MoBio PowerSoil (Carlsbad, CA) and Epicentre SoilMaster (Madison, WI) and the MoBio kit was selected for use in the main experiment (see "Results and discussion" section). Comparisons between the two kits were made by processing aliquots of the well-decomposed refuse sample using the PO_4-D and PO_4-C methods. For each kit, five replicate extractions were performed on each aliquot of processed material, resulting in 10 extractions per kit.

DNA mass was normalized per dry g of material based on the centrifuged pellet or dried material remaining after processing. For both direct and indirect methods, normalization per dry g of centrifuged pellet or dried material was computed by dividing total extracted DNA (μg) by the pellet dry mass. For direct methods, this is identical to the DNA mass per dry mass refuse. For indirect methods, DNA mass collected in the supernatant reflects the total DNA harvested from the 50 g refuse aliquot and the DNA mass per dry g refuse was normalized by taking into account the total mass of pellet generated from hand squeezing as follows:

$$\text{DNA mass}\left(\mu\text{g DNA dry g refuse}^{-1}\right) = \frac{\left(\text{DNA conc., } \mu\text{g ml}^{-1}\right)(\text{Elution vol., ml})(\text{Total pellet mass, dry g})}{(\text{Pellet mass sub} - \text{sample used for extraction, dry g})(\text{Refuse aliquot mass, dry g})}$$

Dry masses were calculated using the moisture content. All statistical analyses related to DNA quantitation were performed using an analysis of variance (ANOVA) Tukey's test using Statistical Analysis Software, Proc-GLM (SAS, Cary, NC).

PCR conditions

Polymerase chain reaction (PCR) on pooled DNA (from the 9 DNA extractions noted in the "Experimental design" section unless noted otherwise) was performed using 25 µl of FailSafe PCR system reaction mix F (Epicentre; Madison, WI), 0.6 µl FailSafe enzyme mix (Epicentre), 0.25 µM of each primer, ~15 ng DNA and sterile pure water added to a total volume of 50 µl. The template mass used was based on preliminary PCR optimizations to achieve approximately equal final masses of amplified product (Blackwood et al. 2003). The primers used were specific for conserved 16S RNA gene sequences targeting the bacterial and archaeal domains, respectively. Bacterial primers used were 8f (5′-AGAGTTTGATCCTGGCTCAG) and 1492r (5′-GGTTACCTTGTTACGACTT) (Klappenbach et al. 2000). Archaeal primers used were ARC-8f (5′-TCCGGTTGATCCTGCC) and ARC-1492r (5′-GGCTACCTTGTTACGACTT) (Banning et al. 2005). Forward primers for both primer sets were labeled at the 5′ end with 6-FAM for T-RFLP analysis. PCR product concentrations were computed by loading equal product volumes on a 1% agarose gel and estimating mass using Gel-Pro analyzer software (Media Cybernetics; Silver Spring, MD).

PCR was performed in an Eppendorf thermocycler programmed as follows: initial denaturing step at 94°C for 5 min, followed by 30 cycles of denaturation at 95°C for 1 min, annealing at 55°C for 1 min, extension at 72°C for 1.5 min, and final extension at 72°C for 45 min. Duplicate PCR reactions were performed and subsequently combined prior to quantification.

T-RFLP analysis

Amplicons (100 µl) were purified using QIAquick PCR purification (Qiagen, Valencia, CA) per the manufacturer's protocol. Purified PCR products were quantified by spectrophotometry and approximately 300 ng were digested separately with restriction endonucleases *Hha*I, *Msp*I and *Rsa*I (New England Biolabs; Ipswich, MA) for *Bacteria* and *Hha*I, *Hae*III and *Taq*I (New England Biolabs; Ipswich, MA) for *Archaea*. The restriction enzymes for *Archaea* were selected based on previous studies (Banning et al. 2005; Chan et al. 2005). Restriction digestion was performed in 20 µl reactions containing: 1.5 µl restriction enzyme, 2 µl 10× buffer, a volume equivalent to 300 ng PCR product, and DI water. The same enzyme volume was used for all reactions resulting in activities ranging from 15 to 30 U per reaction depending on the enzyme used. Terminal restriction fragments were analyzed by capillary electrophoresis at the Michigan State University Research Technology Support Facility using a 3130 Genetic Analyzer (Applied Biosystems; Foster City, CA). T-RFLP profiles were analyzed using Genescan™ (Applied Biosystems) and GenescanView software (CRIBI, Italy).

Analysis of T-RFLP profiles

Electropherograms were visually inspected and numerical output from each fragment pattern was exported to Microsoft Excel. To minimize analytical error, comparable loads of PCR product for T-RFLP analysis were verified using the total fluorescence. Fluorescence was verified as being within a factor of 2 compared to that of other samples or was re-analyzed. Terminal restriction fragment (T-RF) areas were standardized to eliminate noise and identify true peaks via an automated algorithm using a standard deviation from the baseline of 3 and a bin

size of ±0.5 bp (Abdo et al. 2006). To compare community structure between processing replicates and methods, the number of T-RFs, referred to as richness (S), and the Bray-Curtis similarity index were computed using Community Analysis Package software 4.0 (Pisces Conservation Ltd).

Enzymes yielding higher numbers of T-RFs offer greater resolution between OTUs. Of the three enzymes used, *Msp*I and *Hha*I gave the highest resolution for *Bacteria* and *Archaea*, respectively, and thus were used to compare treatments (data not shown).

Comparison of methods using MDS analysis of Bray-Curtis similarities

T-RFLP results from each method were compared using non-metric MDS scaling using Bray-Curtis similarity. Bray-Curtis similarity was used as it has been shown as an effective metric in comparing microbial communities using T-RFLP (Rees et al. 2004). A datum to compare the performance of individual methods was created by pooling T-RF profiles from all methods, resulting in a compiled set of accumulated T-RFs recovered from all methods. Establishing a datum in this manner is analogous to that used in ecology studies, in which aggregated diversity from multiple capture methods is used to compare performance of a particular individual method (Magurran 2004). Non-metric multidimensional scaling was computed from T-RF profiles using Community Analysis Package software (Seaby and Henderson 2007).

Pure culture spiking and quantitative PCR

To determine the recovery of processing methods, pure cultures of a cellulolytic bacterium (*Ruminococcus albus* Hungate, ATCC 27210) and methanogenic archaeon (*Methanobacterium formicicum* MF, ATCC 33274) were grown in liquid media as recommended by the supplier (ATCC; Manassas, VA) then spiked into refuse 10 min prior to processing. Spiked and unspiked aliquots were processed using PO_4-C, MT-C and DG-D methods and frozen until DNA extraction could be performed. Triplicate aliquots were used per method and triplicate DNA extractions were performed from each aliquot. To determine the volume of live cells to add based on DNA quantity, pure culture DNA was extracted from triplicate 1.8 ml ($O.D._{600} = 0.87$) culture samples and pooled to establish a ratio between extracted pure culture DNA and inoculum volume (ng DNA/ml inoculum). Then live whole cells were added based on the DNA mass per mL of pure culture inoculum at a pure culture DNA:total DNA ratio of 2.5 ± 0.8%. Spiking ratios of 0.4–46% were tested in preliminary work using the spiking protocol noted above, and recovery measured using qPCR showed a spiking ratio of 2.5% gave the highest recovery. Pure culture DNA recovery was computed by subtracting the pure culture:total DNA ratio measured in unspiked aliquots from the same ratio measured in spiked aliquots to normalize for DNA native to the refuse that might be targeted with the PCR primers. The result was then divided by the pure culture:total DNA ratio initially spiked into the refuse.

Quantitative PCR was performed using a SYBR Green I assay with the Bio-RAD iQ5 thermocycler (Hercules, CA). Reactions were prepared in 25 µl volumes using 12.5 µl SYBR Green Supermix, 0.25 µM of each primer, 1 µl DNA extract and 10.5 µl sterile pure water. A serial dilution was performed on extracted pure culture DNA and triplicates from each dilution were subjected to qPCR to generate a 7 point standard curve. 16S RNA gene PCR primers were designed using Primer 3 software (Rozen and Skaletsky 1998) for *R. albus* (15f: 5′-CACATGCAAGTCGAACGAGCGAAA, 101r: 5′-CGAAAGGCAGATTGCTCACGTGTT) and *M. formicicum* (246f: 5′-TGCCCACCAAGCCAGTAATCTGTA, 366r: 5′-ACTTTCGTGCATTGCGGAGGTTTC). Primer specificity was verified in silico via a BLAST search (NCBI) and in the laboratory via PCR on DNA from *R. albus*, *M. formicicum*, and mixed refuse. Primers were optimized for annealing temperature at the concentrations stated above and PCR was performed as follows: initial denaturing step at 95°C for 5 min, followed by 40 cycles of denaturation at 95°C for 1 min, annealing at 58°C for 45 s, extension at 72°C for 1 min. qPCR results were analyzed using iQ5 Optical System software (Bio-Rad).

Results and discussion

DNA extraction and yield

Initial work was performed to ensure the commercial kit used for the main study had the ability to recover measurable DNA yields from decomposed

refuse. MoBio PowerSoil and Epicentre SoilMaster DNA extraction kits were evaluated because they used different approaches to isolate DNA from environmental samples. DNA yields from the MoBio kit were significantly greater ($P < 0.05$) and averaged 3.5 times higher than the Epicentre kit. DNA yields were also less variable with the MoBio kit (Coefficient of Variation (CV) = 13%) compared to the Epicentre kit (CV = 91%). This is most likely because a DNA binding column is used with the MoBio kit, whereas the Epicentre kit uses ethanol precipitation to isolate the DNA pellet. It is suspected that ethanol precipitation and the subsequent decanting step introduces more variability. Based on these results, the MoBio PowerSoil kit was used for all DNA extractions in the main study.

DNA yields from all sample processing methods are shown in Fig. 1. The M-C and DG-D methods resulted in the highest yields for well-decomposed refuse and were statistically similar ($P > 0.05$). The PO_4-C method had the highest yield for accelerated methane phase refuse and was statistically similar to the DG-D method ($P > 0.05$). DNA yield from well-decomposed refuse had CVs ranging from 11% (SDS-D) to 50% (M-C) with the majority being less than 20%. Accelerated methane phase DNA yields were variable and CVs ranged from 12% (FT-D) to 64% (DG-D). Roughly one-third of the methods had CVs less than 20% for this substrate. Purity ratios (A_{260}/A_{280}) exhibited some variability between methods (Table 2), however; no trend was evident indicating a particular processing method resulted in a higher or lower purity ratio, or reduced variability, using well-decomposed and accelerated CH_4 phase refuse. DNA from all treatments was successfully PCR amplified.

Table 2 Purity (A_{260}/A_{280}) ratios of extracted DNA

	Well-decomposed refuse	Accelerated CH_4 phase refuse
Direct		
PO_4-D	1.60 (0.09)	1.62 (0.06)
FT-D	1.60 (0.14)	1.67 (0.24)
SDS-D	1.65 (0.20)	1.66 (0.17)
DG-D	1.52 (0.07)	1.73 (0.14)
Cell separation		
PO_4-C	1.61 (0.16)	1.73 (0.03)
M-C	1.68 (0.05)	1.57 (0.15)
MT-C	1.52 (0.16)	1.49 (0.08)

Standard deviation is given in parentheses based on nine extraction replicates. All samples were successfully amplified using PCR

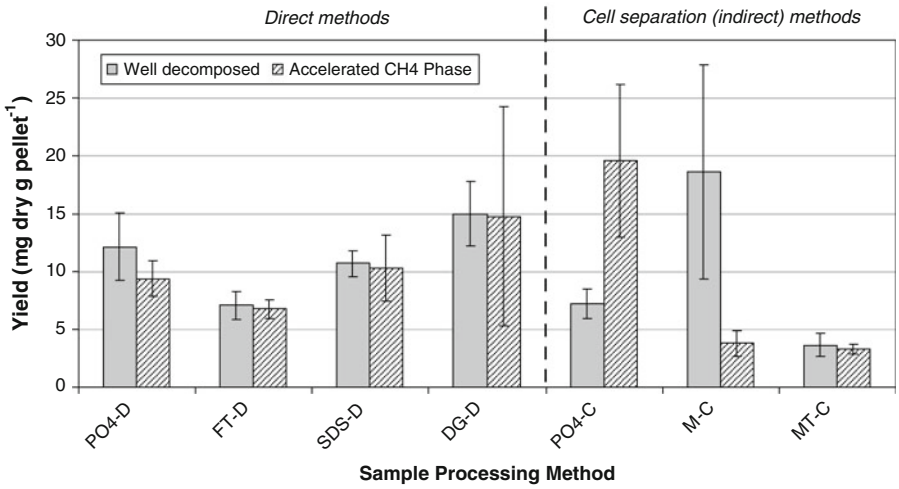

Fig. 1 Effect of sample processing methods on DNA yield. Abbreviations: *PO_4-D* direct phosphate buffer method, *FT-D*, direct freeze–thaw method, *SDS-D* direct sodium dodecyl sulfate method, *DG-D* direct dry and grind method, *PO_4-C* cell separation phosphate buffer method, *M-C* cell separation methylcellulose method, *MT-C* cell separation methylcellulose/Tween 80 method. *Error bars* indicate standard deviation, n = 9

The DNA yields obtained from indirect methods (M-C for well-decomposed, PO_4-C for accelerated CH_4 phase refuse) were not always significantly lower than direct methods, which contrasts to previous work (Jacobsen and Rasmussen 1992; Robe et al. 2003; Tien et al. 1999). It is hypothesized that cell separation via hand-squeezing serves to consolidate a larger mass of material and does not remove particulates to the same extent as the more common differential centrifugation technique (Faegri et al. 1977; Robe et al. 2003; Steffan et al. 1988; van Elsas et al. 1998). For cell separation methods, roughly 30% (well-decomposed phase) and 8.3% (accelerated CH_4 phase) of the aliquot dry mass was recovered from the hand-squeezed supernatant. This inefficiency in particulate removal appears advantageous and is consistent with previous work which suggests a significant fraction of the cells are likely attached to the particulates (Corinaldesi et al. 2005).

Microbial community structure bias

Most studies evaluating sample processing and DNA/RNA extraction methods have used yield and extract purity as metrics for assessing effectiveness (Forney et al. 2004). Only a few have evaluated how a particular method affects community structure using molecular fingerprinting methods or clone libraries (Gabor et al. 2003; LaMontagne et al. 2002; Luna et al. 2006; Martin-Laurent et al. 2001; Sessitsch et al. 2002). Differences in measured community structure can be especially important if a particular method is biased against ubiquitous or functionally important microbial taxa. Bias, as defined in this study, refers only to measured T-RF richness (rather than absolute species richness), and Bray-Curtis similarities based on T-RFLP analysis. It is well known that T-RFLP is not effective at estimating absolute diversity or detecting rare species (Hartmann and Widmer 2008). However, previous work has shown T-RFLP to be very effective in comparing community structure differences between treatments, making its use in this study particularly applicable (Dunbar et al. 2001; Hartmann and Widmer 2006, 2008). Additionally, taxonomic assessment of rare species usually requires libraries consisting of thousands of clones which can be cost prohibitive (Dunbar et al. 2002).

Variation induced by refuse heterogeneity

Variability in T-RF richness between triplicate aliquots of well-decomposed refuse processed by the same method was generally lower than the observed differences between sample processing methods FT-D, DG-D and PO_4-C. Two-tailed *t*-tests (assuming unequal variance) performed using T-RF richness from triplicate aliquots showed differences between processing methods for *Bacteria* at the 92% confidence level ($P < 0.08$). *t*-Tests between methods for *Archaea* were different between the PO_4-C/DG-D and PO_4-C/FT-D methods ($P < 0.13$). There was no difference between the DG-D/FT-D methods ($P = 0.56$) but this was because aliquot T-RF richness was similar between methods (data not shown). MDS analysis showed the community structure between replicates processed by the same method clustered together while the community structure recovered by separate processing methods clustered separately (Fig. 2). For *Archaea*, differences between DG-D replicates were substantially larger than that observed from the PO_4-C and FT-D methods (Fig. 2b). However, the PO_4-C and FT-D methods clustered together for *Archaea* (Fig. 2b) and the DG-D replicates for *Bacteria* clustered together (Fig. 2a). Additionally, the T-RFLP and qPCR results comparing all methods indicated the DG-D method did not result in similar archaeal community structure relative to other methods (see subsequent sections). Taken together, the results from this experiment suggest that differences between replicates were not induced by refuse heterogeneity but, rather, by the DG-D method itself. As a result, the DG-D method was excluded from subsequent comparisons of sample processing methods. Collectively, these results show differences between processing methods were not attributed to variation induced by refuse heterogeneity. As a result, equal DNA masses from each of the nine extracts were combined to create a pooled DNA sample for T-RFLP analyses in subsequent comparisons of all seven sample processing methods. This pooling served to minimize both aliquot to aliquot and extraction variability for each processing method.

Comparison of sample processing methods

Generally, T-RFs with the highest capture frequency were most abundant. Capture frequency, as used in

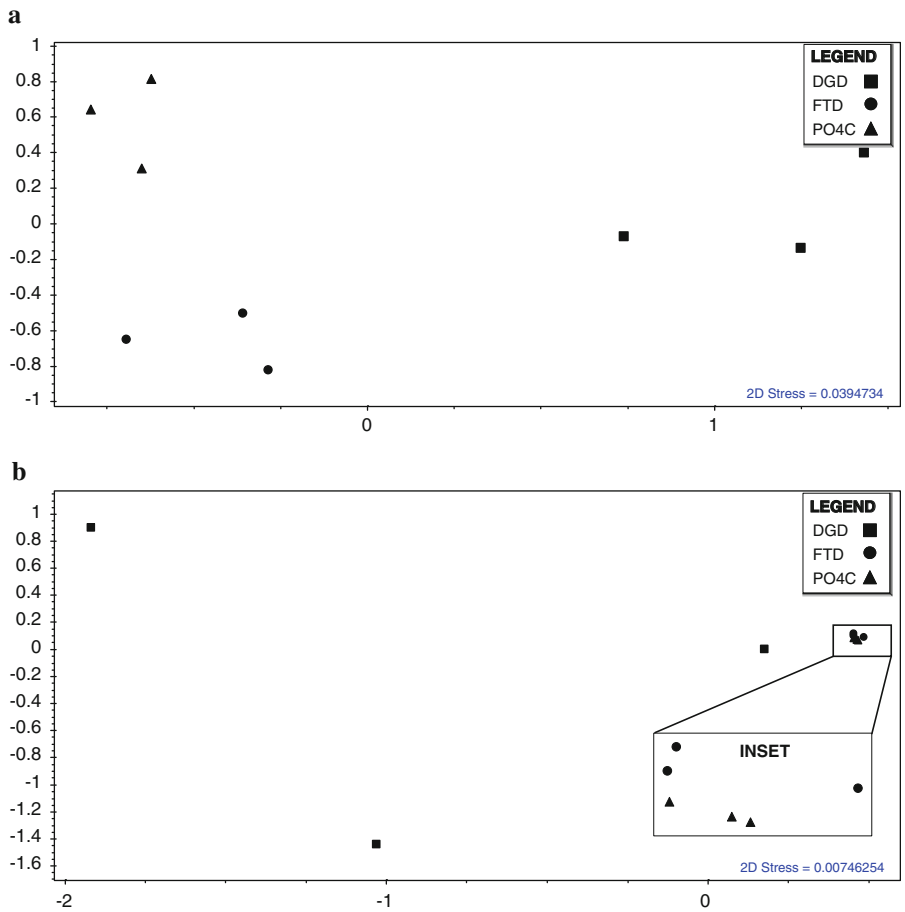

Fig. 2 MDS using Bray-Curtis similarity for triplicate aliquots of sample processing methods FT-D, DG-D and PO_4-C for **a** *Bacteria* and **b** *Archaea* using well-decomposed refuse. *Inset* for *Archaea* shows differences between methods PO_4-C and FT-D not evident at original scale. Analysis indicates relative impact of refuse heterogeneity on differences between sample processing methods

this study, refers to the number of processing methods that recovered a particular T-RF, which is analogous to the way in which trapping methods are compared in ecological diversity studies (Magurran 2004). Roughly 96 ± 5 and 91 ± 7% of total relative abundance was from T-RFs occurring in 3 or more methods (i.e. capture efficiency ≥ 3) for *Archaea* and *Bacteria*, respectively. For this reason, richness at a capture efficiency ≥ 3 was compared in addition to all T-RFs recovered by a particular method (Table 3). However, the relative abundance for individual T-RFs occurring in 3 or more methods ranged from ~0.3 to 65%, indicating T-RFs with a high capture frequency were not limited to those with high relative abundances. It should be noted that T-RF abundances were used in this study comparatively and do not necessarily reflect abundances from the native ecosystem, primarily due to limitations in T-RF resolution, extraction efficiency and PCR amplification bias (Dunbar et al. 2001; Hartmann and Widmer 2008).

Richness from individual processing methods was generally comparable, with the difference averaging ~5 T-RFs between methods at a capture frequency ≥ 3 (Table 3). Exceptions to this were the DG-D method for *Bacteria* and the MT-C method for *Archaea*, both of which had a richness roughly 10 T-RFs lower than other methods. Trends were similar when all T-RFs were included (capture frequency ≥ 1) but richness was expectedly higher since this reflects capture of less ubiquitous T-RFs (Table 3).

Although different methods provided varying DNA yields, there was no correlation between yield (Fig. 1)

Table 3 Effect of sample processing method on bacterial and archaeal richness at capture frequencies (F) of ≥1 and ≥3

	Well-decomposed refuse		Accelerated CH_4 phase refuse	
	F ≥ 1	F ≥ 3	F ≥ 1	F ≥ 3
Bacteria				
Direct				
PO_4-D	30	26	34	28
FT-D	30	22	34	19
SDS-D	32	22	27	19
DG-D	21	14	11	6
Cell separation				
PO_4-C	28	27	29	27
M-C	28	28	32	27
MT-C	37	26	28	26
All methods	61	31	67	33
Archaea				
Direct				
PO_4-D	33	29	23	16
FT-D	41	35	29	15
SDS-D	36	33	18	15
DG-D	38	32	36	15
Cell separation				
PO_4-C	36	35	20	18
M-C	39	36	18	17
MT-C	23	23	22	17
All methods	59	40	63	20

Bacteria and *Archaea* data shown for *Msp*I and *Hha*I restriction enzymes, respectively. Abbreviations are defined in the "Materials and methods" section. Capture frequency refers to the total number of methods that recovered a particular T-RF

and T-RF richness (Table 3) ($R^2 = 0.26$), which is similar to the results of other studies (Frostegard et al. 1999; Luna et al. 2006). Despite the inability of T-RFLP to detect less abundant ribotypes (Luna et al. 2006), methods resulting in significantly larger DNA yields should have increased detection of T-RFs at abundances near T-RFLP detection limits if a correlation between yield and richness existed. Rank abundance curves exhibited a 'long tail' of less abundant T-RFs (data not shown), which is typical of organism distributions in most ecosystems (Magurran 2004). This suggests no anomalous distributions of taxa were inherent in the refuse ecosystem that would have skewed a yield-richness correlation. Thus, taxa capture efficiency by the processing method appears to play a larger role than extracted DNA yield in detecting dominant T-RFs.

MDS plots show community structure varied between methods (Fig. 3). For *Bacteria*, methods SDS-D and FT-D were consistently biased and were not clustered with other sample processing methods in both types of refuse (Fig. 3a, b). In the well-decomposed refuse, methods PO_4-D and MT-C also exhibited bias relative to the pooled T-RF profile for all methods (Fig. 3a). Collectively, this indicates that the least biased methods for *Bacteria* are the PO_4-C and M-C methods. MDS plots for *Archaea* show all methods except the SDS-D method clustered centrally with other sample processing methods for the well-decomposed refuse treatment but were somewhat skewed relative to the pooled T-RF profile for all methods (Fig. 3c). For accelerated CH_4 refuse, the PO4-C profile was clearly the least biased method relative to *Archaea* profiles recovered by the other methods (Fig. 3d). These results show that relatively unbiased recovery of *Archaea* was achieved with multiple methods; however, the PO_4-C method appeared to recover an archaeal community structure similar to (Fig. 3c) or more diverse than (Fig. 3d) other processing methods.

Pure culture spiking and recovery using quantitative PCR

Recovery of spiked pure cultures was quantified using qPCR since the technique has a detection limit several orders of magnitude lower than T-RFLP based on published T-RFLP detection limits (Luna et al. 2006) and qPCR instrumentation specifications (Bio-Rad 2003). Variation in measured pure culture abundance was eliminated since cultures were spiked in known quantities and unspiked aliquots were used as controls. Methods PO_4-C, MT-C and DG-D were evaluated because they ranged from most effective to least effective, respectively, based on the T-RFLP comparisons of richness and MDS of Bray-Curtis similarities (Table 3; Fig. 3).

While *R. albus* recoveries were higher using PO_4-C compared to MT-C, all methods were statistically similar from one another at the 95% confidence level ($P < 0.05$) (Table 4). The DG-D method gave the highest average recoveries of *R. albus*; however, results were highly variable (CV = 57%) compared to the PO_4-C (CV = 13%) and MT-C (CV = 39%) methods.

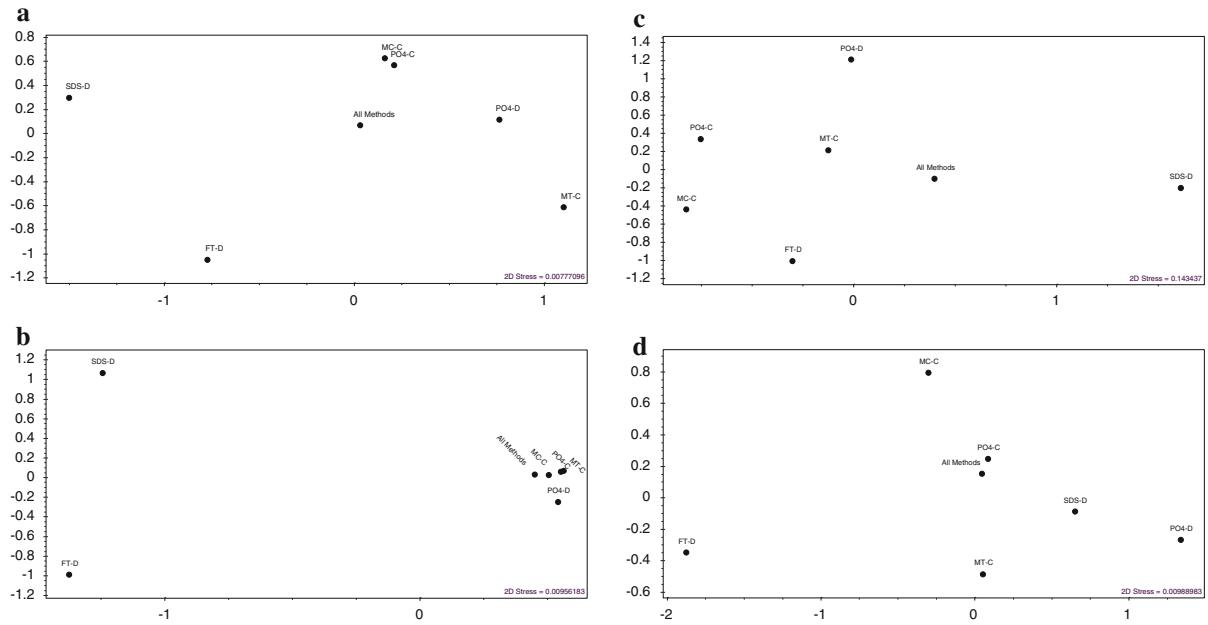

Fig. 3 MDS using Bray-Curtis similarity for sample processing methods for *Bacteria* in **a** well-decomposed and **b** accelerated CH_4 phase refuse and for *Archaea* in **c** well-decomposed and **d** accelerated CH_4 phase refuse. The 'All Methods' treatment refers to the aggregate T-RF profile compiled from all sample processing methods. The DGD-D method has been excluded due to issues with this method (noted in the "Results and discussion" section). Abbreviations shown are given the "Materials and methods" section

The PO_4-C and DG-D methods gave the highest *M. formicicum* recovery and were statistically similar (ANOVA, $P > 0.05$). However, recoveries using the DG-D method were again more variable (CV = 46%) compared to the PO_4-C method (CV = 13%). The MT-C method gave lower *M. formicicum* recoveries compared to the other method yet were statistically similar using ANOVA ($P > 0.05$).

The PO_4-C method, which consistently exhibited low bias relative to other methods (Fig. 3), gave consistent pure culture recoveries and was less variable (Table 4). In contrast, the DG-D method performed reasonably well for *M. formicium* (*Archaea*) but exhibited large variation in recovering *R. albus* (*Bacteria*). This is consistent with the measured richness and MDS plots (Table 3; Fig. 3), which showed the DG-D was consistently biased compared to other methods.

Recommendations

This study confirms that observed bias is most likely caused by the sample processing method rather than an analytical artifact or sample variation. It has been previously reported that recovery of rare taxa is difficult unless countered by a massive sampling effort and that higher richness can be obtained using multiple methods since unique T-RFs are captured by different methods (Luna et al. 2006; Magurran 2004). This was confirmed here since the richness from all seven methods combined was ~2 times higher on average ($S = 63 \pm 3$) than richness from a single method alone ($S = 29 \pm 7$). Thus, if the goal is to capture less abundant taxa, then multiple processing methods and replicates must be used. Despite the recommendation to employ multiple methods, use of a single protocol still prevails in many microbial ecology studies for reasons of convenience, increased sample throughput, reduced labor and lower cost. Thus, if it is necessary to use a single method, the PO_4-C method offers the least bias against more ubiquitous taxa and may provide an acceptable number of less abundant taxa depending on the experimental goal(s). From a practical standpoint, the end material generated by cell separation (hand squeezing) is less fibrous and

Table 4 Recovery of pure cultures spiked into refuse and processed with selected methods measured using qPCR

Pure culture/ method	Ratio of pure culture DNA to total DNA[a]		% Recovery
	Initial Spike (%)	Recovered[b] (%)	
Ruminococcus albus			
PO_4-C	3.7 (0.2)	1.0 (0.1)	28 (3.6)^{x,y}
MT-C	1.7 (0.1)	0.2 (0.1)	12 (4.7)^{y}
DG-D	2.2 (0.4)	2.6 (1.6)	109 (63)^{x}
Methanobacterium formicicum			
PO_4-C	1.9 (0.1)	0.6 (0.1)	34 (4.5)^{x}
MT-C	1.7 (0.1)	0.1 (0.1)	6.5 (6.5)^{x}
DG-D	2.2 (0.4)	1.0 (0.6)	42 (19)^{x}

Data presented are averages and standard deviation is given in parentheses. Whole cells were spiked into refuse at a level corresponding to a known DNA mass per volume of pure culture. Abbreviations shown are defined in Fig. 1 and the "Materials and methods" section

[a] Total DNA is inclusive of the pure culture DNA added to the sample

[b] Recovered values were corrected for *R. albus* or *M. formicicum* DNA existing in the sample prior to spiking. Different superscripts indicate that, for a particular pure culture, methods were statistically different ($P < 0.05$)

results in lower pellet volumes after centrifugation, making the material less difficult to work with compared to direct methods.

These results show the sample processing method used can have a substantial effect on the number of T-RFs captured and the microbial community structure observed. This implies that differences between microbial ecology studies could be attributed, in whole or in part, to methodological differences rather than environmental selection mechanisms and suggests caution must be used when interpreting microbial ecology data sets where different methods were used to process samples.

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