

DNA extraction method affects microbial community profiles from soils and sediment

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Abstract To evaluate whether different deoxyribonucleic acid (DNA) extraction procedures can affect estimates of bacterial community composition, based on the 16S ribosomal ribonucleic acid gene denaturing gradient gel electrophoresis (DGGE) profiles, we compared four in situ lysis procedures using three soils and one marine sediment. Analysis of DGGE profiles, generated by polymerase chain reaction of purified DNA extracts, demonstrated that the choice of DNA extraction method significantly influenced the bacterial community profiles generated. This was re-

flected both in the number of bands or ribotypes detected from each sample and in subsequent principle coordinate analysis and unweighted-pair group method using arithmetic average analyses. The methods also differed significantly in their robustness, i.e. reproducibility across multiple analyses. Two methods, both based on bead beating, were demonstrated to be suitable for comparative studies of a range of soil and sediment types.

Keywords Soil · Sediment · DNA extraction · DGGE · Lysis efficiency · Microbial diversity

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Introduction

Microbial communities play a key role in soil processes, such as organic matter transformation and nutrient cycling, and may potentially be the earliest predictor of soil quality changes (Scow et al. 1998; Griffiths et al. 2002; Ibekwe et al. 2002; Nielsen and Winding 2002; Torsvik and Øvreås 2002). These diverse functions are mediated by a multitude of interacting, genotypically diverse microorganisms, and a number of studies have provided evidence that link changes in soil microbial community structure with the alteration of functional capabilities (Grayston et al. 1996; Torsvik and Øvreås 2002; Nannipieri et al. 2003). Yet, despite these facts, the microbiological status of soil is often not measured in studies of soil processes, such as nutrient leaching and greenhouse gas emissions. Furthermore, there is only sparse knowledge of the number and type of species present in different soils and even less on the functions and interactions of these complex microbial communities. It is thought that less than 1% of microorganisms, present in soil, are culturable using conventional techniques (Amann

et al. 1995; Smit et al. 2001; Schloss and Handelsman 2003) and this has limited our knowledge of the microbial ecology of soil. However, the application of nucleic acid-based methodologies has provided a means to overcome this limitation, allowing for the monitoring of organisms or particular genes directly from environmental samples (Olsen et al. 1986; Amann et al. 1995).

While detailed and reliable, the amount of time and resources required for the classical cloning–sequencing approach were deemed inefficient for the large-scale soil survey being undertaken in this study (>1,000 samples); therefore, the relatively rapid and cost-effective denaturing gradient gel electrophoresis (DGGE) approach was used. Indeed, many previous studies have relied on similar techniques as part of evaluations of deoxyribonucleic acid (DNA) extraction protocols for use with environmental samples (e.g. Krsek and Wellington 1999; Griffiths et al. 2000; Robe et al. 2003). The principle of DGGE is to separate small variable regions of the 16S ribosomal ribonucleic acid (rRNA) gene on a denaturing gradient based on nucleotide differences (Muyzer et al. 1993). It is, therefore, possible to discriminate between different organisms or ribotypes within a DGGE profile of the whole community. A complicating factor, however, is variation in rRNA gene copy numbers and slight sequence variation within single species, allowing for the possibility of individual organisms producing a number of different bands on a gel (Hill et al. 2000). Furthermore, a single band frequently comprises several different species in profiles of mixed environmental communities (Kisand and Wikner 2003). Despite these drawbacks, these techniques, although only semi-quantitative at best, remain the most efficient for the measurement of, for example, relative differences or temporal changes in microbial community structure in environmental samples. In any case, the application of quantitative or real-time polymerase chain reaction (PCR) can facilitate the quantification of constituent ribotypes or genes in microbial communities.

The most commonly applied approach for DNA extraction from soil involves the in situ lysis of cells (Trevors 1992; Roose-Amsaleg et al. 2001) through chemical and/or enzymatic disruption and/or mechanical lysis using, for example, freeze–thaw (Kuske et al. 1998; Luna et al. 2006), bead-beating, freeze-grind or sonication (Robe et al. 2003). Many studies on soils and sediments have focused on the advantages and disadvantages of using various combinations of these techniques (e.g. Moré et al. 1994; Leff et al. 1995; Gabor et al. 2003; Robe et al. 2003). Nevertheless, it is clear that the application of in situ lysis techniques provides both increased DNA yields and facilitates proportionally representative recovery of different microbial species compared to protocols based on indirect cell lysis (LaMontagne et al. 2002; Gabor et al. 2003; Luna et al. 2006).

The lysis efficiency of any nucleic acid extraction procedure is critical in determining its success, such that an accurate representation of the microbial community can be achieved (O'Donnell and Gorres 1999; Bürgmann et al. 2001; Robe et al. 2003; de Liphay et al. 2004). Issues such as failure to lyse certain cell types (Bürgmann et al. 2001), distribution of microorganisms within soil pores (Nunan et al. 2003) and microbe adherence to soil particles can all affect consequent availability of these cells for lysis. Furthermore, the presence of extra-cellular DNA, which may already be bound to soil particles, can also affect the composition of retrieved microbial community DNA (Lornez and Wackernagel 1987; Ogram 2000; Demanéche et al. 2001; Robe et al. 2003).

Nucleic acid-based methodologies for the monitoring of organisms or particular genes also require nucleic acid extracts that are sufficiently free from inhibitory compounds, such as proteins, phenolic compounds, humic acids and heavy metals, to allow reliable PCR amplification, enzyme digestion, hybridisation and/or reverse transcription (Zhou et al. 1996; Menking et al. 1999; Ogram 2000; Niemi et al. 2001). To this end, a number of nucleic acid purification strategies have been devised (for reviews, see Roose-Amsaleg et al. 2001; Robe et al. 2003); however, the application of these strategies can reduce nucleic acid yield and, consequently, devalue the nucleic acids for the study of microbial diversity or environmental genomic analyses.

Four DNA extraction methods were selected for evaluation purposes during the current study. Each method was tested against three soils and one marine sediment with respect to: (1) cell lysis efficiency, (2) DNA yield, (3) PCR amplification of isolated DNA, and (4) reproducibility of community profiles generated by DGGE fingerprinting.

Materials and methods

Soil sampling

A total of three Irish soils (collected from the top 0–10 cm) and one marine sediment were obtained, as follows: (1) Grey Brown Podzolic (Silvermines, Tipperary 52°47'N 08°13'W), (2) Gley (Corrib, Galway 53°16'N 09°03'W; both sampled using a Dutch auger), (3) *Sphagnum* peat (Inverin, Galway 57°17'N 09°25'W) sampled using a Russian Peat corer (0.5 m length×80 mm diameter; Duncan & Associates, UK) and (4) a marine sediment sample (*PAP*, Porcupine Abyssal Plain site 48°50'N 16°30'W), which was taken from a depth of 4,800 m (Eardly et al. 2001) in September 1998 using a multi-corer (inside diameter 9 cm; depth penetration>20 cm Ocean Scientific, UK). All three soil samples were collected from the top 10 cm of the soil

profile and refrigerated (4°C) immediately on return to the laboratory. The sediment sample was frozen (in liquid nitrogen) immediately upon recovery, and the 0–1-cm-depth section was used in this study. The sediment sample was used in this study as it had been well quantified with respect to DNA yield and lysis efficiency previously at the National University of Ireland, Galway, and thus provided an internal standard for method evaluation.

DNA extraction

Four extraction procedures, method 1 to method 4 (M1–M4), were employed; all were based on the direct lysis of cells in the sample, with subsequent recovery and purification of nucleic acids. Before extraction, all solutions were rendered DNase-free by treatment with 0.1% diethyl pyrocarbonate (DEPC). Method 1 (M1) was modified from DeLong et al. (1993).

- Method 1 (M1): liquid nitrogen (approx. 10 ml) was mixed with 250 mg of each biomass sample (wet weight from pellet) in a mortar, ground and transferred to a micro-centrifuge tube (Eppendorf, Germany), and 1 ml of cetyl trimethylammonium bromide (CTAB) extraction buffer (Griffiths et al. 2000) was added, followed by vortexing for 30 s. After the addition of 500 µl of lysis buffer (50 µM Tris–HCl [pH 8]; 40 µM ethylene diamine tetraacetic acid [EDTA; pH 8]; 750 µM filter-sterilised sucrose) and 20 µl of lysozyme (10 mg ml⁻¹; Sigma-Aldrich, Germany), mixtures were briefly vortexed (30 s) and incubated at 37°C for 30 min. Sodium dodecyl sulphate was added to a final concentration of 2%; the samples were again vortexed and then incubated at 70°C for 1 h. After this, 6 µl of proteinase K (Sigma-Aldrich) were added. Samples were then vortexed and incubated at 50°C for a further 30 min followed by centrifugation for 15 min (10,000×g). The supernatants were transferred to fresh micro-centrifuge tubes, and the aqueous phase was extracted by mixing an equal volume of chloroform–isoamyl alcohol (24:1) followed by centrifugation (10,000×g) for 10 min. Total nucleic acids were then precipitated from the extracted aqueous layer with 0.6 vol of isopropanol overnight, at room temperature, followed by centrifugation (10,000×g) for 15 min. The pelleted nucleic acids were washed in 70% (v/v) ice-cold ethanol and air dried before re-suspension in 50 µl DEPC-treated water.
- Method 2 (M2): Soil or sediment of 250 mg and 1 ml of 1% CTAB were beaten for 2 min with 250 mg of zirconia/silica beads (1.0, 0.5 and 0.1 mm; Biospec Products, USA), in the Mini Beadbeater-8 (Biospec Products) at the median speed setting. A 500-µl aliquot

of lysis buffer was added to the mixture, and the remainder of the extraction protocol was continued as described for M1.

- Method 3 (M3): Briefly, 500 mg of the soil or sediment samples were added to 0.5 ml of CTAB extraction buffer and 0.5 ml of phenol–chloroform–isoamyl alcohol (25:24:1; pH 8.0) and lysed for 30 s with 250 mg of zirconia/silica beads (1.0, 0.5 and 0.1 mm), in the Mini Beadbeater-8 at the median speed setting. The aqueous phase, containing the nucleic acids, was separated by centrifugation at 10,000×g for 5 min and removed to respective fresh micro-centrifuge tubes. The aqueous phase was extracted, and phenol was removed by addition of an equal volume of chloroform–isoamyl alcohol (24:1) followed by centrifugation for 5 min (10,000×g). Two volumes of polyethylene glycol (PEG)–1.6 M NaCl (30% w/v) were used to precipitate total nucleic acids at room temperature, which were then washed with ice-cold 70% (v/v) ethanol and air dried before re-suspension in 50 µl of DEPC-treated water (Griffiths et al. 2000).
- Method 4 (M4): The MoBio Ultraclean™ soil DNA kit (Cambio, Cambridge, UK). DNA was extracted from 250 mg of soil or sediment according to the manufacturer's instructions.

To inspect the quality of extracted DNA, 5-µl aliquots of crude extract were run on Tris–acetate–EDTA (TAE) agarose gels (1%) containing ethidium bromide (1 ng ml⁻¹; Maniatis et al. 1982) for DNA staining and visualisation, with Lambda DNA/*Hind*III molecular size marker (Promega, USA). Gel images were captured using a UV transillumination table and the AlphaDigiDoc 1201 system (Alpha Innotech, USA).

Cell counts and lysis efficiency determination

Total cell counts and cell lysis efficiencies for soil samples were determined using epifluorescent microscopy. For total microbial count determinations (pre-extraction), 1 g of soil was added to 9 ml of sterile saline, and samples were sonicated for three 20-s bursts, at 16 µm wave amplitude using a Soniprep 150 (MSE Scientific Instruments, Crawley, Sussex, England) thereby freeing cells from the matrix allowing a more accurate count (Weinbauer et al. 1998). The rest of the procedure was carried out as described by Eardly et al. (2001) with some modifications. The soil suspensions were serially diluted to an appropriate range (i.e. <100 cells/field), and 2 ml of each suspension was added to respective filtration columns, which were then incubated for 5 min along with 200 µl of Sybr Gold™ (10× stock solution in Tris–EDTA [TE] buffer, pH 8). At least 50 random fields were counted on every filter. For lysis efficiency determina-

tion, the post-DNA extraction pellets were added to 9 ml of saline, without sonication, and cell numbers were counted as described above. Cell lysis efficiencies (%) for each method were calculated in relation to total counts observed for each sample.

Quantification of soil DNA yield

The quantity of extracted DNA was estimated using the PicoGreen[®] dsDNA quantitation kit (Molecular Probes, USA; Sandaa et al. 1998). Briefly, samples were diluted 400-fold in 1× TE, and 100 µl of these dilutions were added to 100 µl of a 200-fold dilution of PicoGreen (Molecular Probes). These were reacted in the dark, in black microtitre plates (Corning, USA) for 5 min at room temperature. Fluorescence was measured using the GENios System 7 (Tecan, Austria) at an excitation wavelength of 485 nm and emission at 535 nm. TE buffer was used as a blank sample, and DNA standards were prepared from bacteriophage λ DNA stocks (both provided by the manufacturer). The level of DNA recovery was expressed as a percentage (%) of DNA in the crude extract.

Nucleic acid purification

Crude DNA extracts were purified to facilitate successful PCR amplification. Silvermines and Corrib samples were cleaned using 2% (w/v) low-melting-point agarose (Cambio) and the GELase[™] Agarose Gel-Digesting Preparation (Epicentre Biotechnologies, USA), according to the manufacturer's instructions, while PAP and Inverin samples were subject to 10⁻¹ dilution using DEPC-treated water.

PCR and DGGE

The V3 region of the eubacterial 16S rRNA gene was PCR amplified using primers EBGC 341f (5'-CCT ACG GGA GGC AGC AG; Muyzer et al. 1993) and UN517r (5'-ATT ACC GCG GCT GCT GG; Muyzer et al. 1993). A 40-base GC clamp was attached to the 5' end of the forward primer to increase the separation of DGGE bands for DGGE analysis (Muyzer et al. 1993). PCR was performed using an Eppendorf Mastercycler Gradient (Eppendorf) in a final volume of 50 µl. Each reaction containing 25 pmol of each DNA primer (MWG-Biotech, Germany), 1× ammonium (NH₄) buffer (Bioline, U.K.), 3 mM MgCl₂ (Bioline), each deoxyribonucleoside triphosphate at a concentration of 200 µM (Biogene, UK), 0.5 µl of bovine serum albumin (1 mg ml⁻¹; Promega), 1 U *Taq* DNA polymerase (Bioline) and 1 µl of template DNA. Negative controls were included to screen for contaminant amplification both pre- and during preparation of reaction mixtures, and genomic DNA from pure cultures of *Escherichia coli* were used to

positively control PCR reactions. A touchdown PCR protocol was performed, with denaturation at 95°C for 10 min followed by 10 cycles of 95°C for 45 s, an initial annealing temperature of 65°C for 45 seconds and an extension at 72°C for 1 min. The annealing temperature was dropped by 1°C per cycle until it reached 55°C and 15 further amplification cycles were carried out at this annealing temperature. A final extension step was performed at 72°C for 10 min. PCR amplicons were resolved on 1X TAE agarose gels (1%) as described previously. DGGE was performed using the D-Code system (BioRad Laboratories, USA). Polyacrylamide gels were prepared with denaturing gradients ranging from 35 to 70% (where 100% denaturant contained urea [7 M] and 40% [v/v] formamide). Gels were run at 60°C at 60 V over 15 h and were stained after electrophoresis with Sybr-Gold[™] nucleic acid stain for 20 min, followed by de-staining in sterile DEPC-treated water for 10 min. Gel images were captured as described in "DNA extraction" and were analysed using the AlphaEaseFC Software Version 3.3.0 (Alpha Innotech).

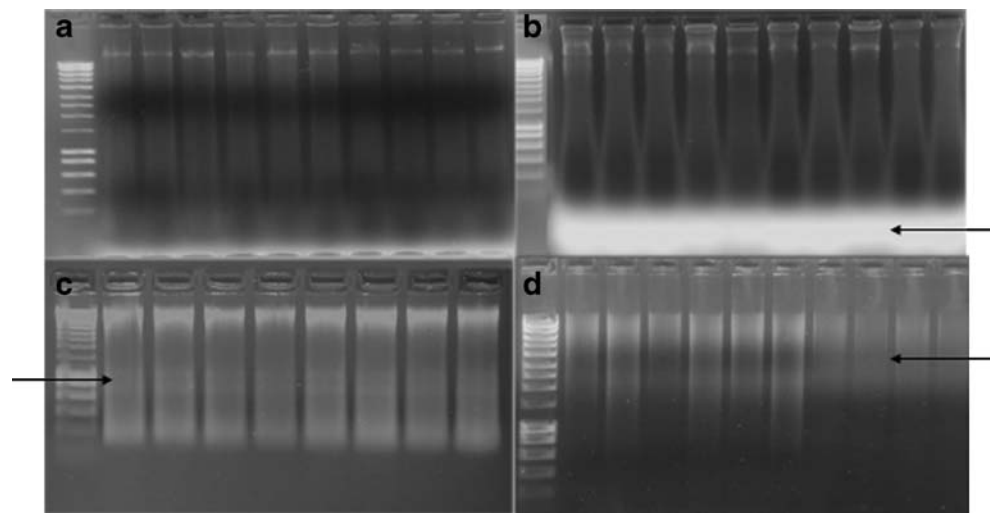
Statistical analyses

Ten replicate extractions were carried out for each soil/sediment type with each DNA extraction method. Three random samples from each set of ten were used for lysis efficiency estimation; each of these three samples was further sub-sampled resulting in nine individual filters counted. Six replicated samples ($n=12$) were used to calculate DNA yields. DGGE profiles were created using PCR products derived from two of the ten samples extracted from each soil/sediment type to examine the differences in DGGE profiles ($n=3$). Further to this and to examine the robustness of the methodologies, five of the ten samples from the Silvermines and Corrib soils were PCR amplified in duplicate and run on DGGE gels ($n=10$). Statistical analysis of the data sets was carried out using Excel (Microsoft, USA), and two-factor analysis of variance (ANOVA) tests were used to calculate the effects of soil type, extraction method and their interaction (Excel, Microsoft).

DGGE gels were visually inspected, and binary matrices were created whereby the presence or absence of bands, throughout sample sets, was scored with the numeric values (1) or (0), respectively. Dendrograms and scatter plots were constructed by the unweighted-pair group method using arithmetic averages (UPGMA) incorporating Jaccard's coefficient of similarity and by principle coordinate analysis (PCO) using Euclidean distance, respectively, using the MultiVariate Statistical Package (MVSP) version 3.12 h (1999; Kovach Computing Services, U.K.).

Fig. 1 Ethidium bromide stained 1.0% agarose gel image displaying genomic DNA extracted from the Silvermines soil samples run with λ DNA cut with *Hind*III.

a M1, **b** M2, *arrow* indicating humic substances, **c** M3, *arrow* indicating rRNA bands, **d** M4, *arrow* indicating sheared DNA



Results

DNA quality, cell lysis efficiency and DNA yield achieved by M1–M4

All four methods successfully extracted DNA, which was visible on agarose gels from the three soils tested (Fig. 1), while for the marine sediment, M2 was the sole method with which DNA was observed at this stage. In almost all cases, the DNA was sized at least at the 23-kb marker point, except in the case of M4, where shearing of the DNA into smaller fragments was consistently evident for the Inverin

(peat) and Corrib (Gley) soils. M3 also co-extracted 16S and 23S rRNA from the soils, as reported by Griffiths et al. (2000; Fig. 1).

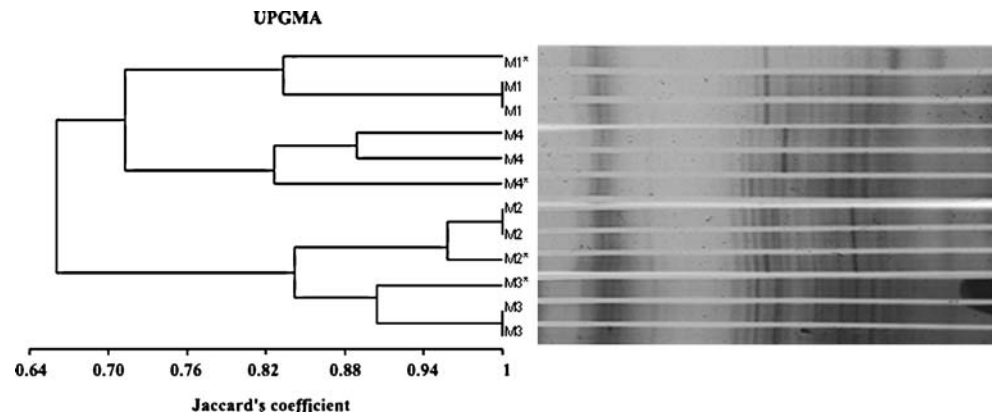
Post-extraction counts of Corrib, Silvermines and Inverin soils were considerably lower than pre-extraction counts, thus indicating high lysis efficiencies (Table 1). However, differences were apparent with respect to the lysis efficiencies achieved by the various methods. When two-factor ANOVA analysis was carried out, soil type was found to have a significant impact on the lysis efficiencies (%), with cell lysis most retarded by the Silvermines soil and least with the Inverin peat ($p \leq 0.05$). ANOVA also showed that

Table 1 Cell counts, lysis efficiencies, crude and final DNA yields and band numbers (± 1 standard deviation in parentheses) with respect to each of the DNA extraction methods tested, against all samples

Site location	Initial count (cells g ⁻¹)	Method	Lysis efficiency (% \pm SD)	Crude DNA yield, μ g g ^{-1a} (\pm SD)	Final DNA yield, μ g g ^{-1a} (\pm SD)	Crude DNA recovery (mean %)	Number of bands (\pm SD)
Corrib	$1.19 \pm 0.25 \times 10^9$	1	82 (± 4.5)	6.2 (± 3.3)	1.4 (± 1.2)	22	31.0 (n/a)
		2	95 (± 1.1)	5.7 (± 0.8)	1.8 (± 0.1)	32	46.0 (± 0.0)
		3	91 (± 4.9)	10.4 (± 1.6)	5.2 (± 1.7)	50	44.0 (± 0.0)
		4	91 (± 2.1)	2.9 (± 0.5)	1.4 (± 0.5)	49	36.7 (± 1.5)
Silvermines	$9.1 \pm 1.8 \times 10^8$	1	81 (± 2.5)	13.0 (± 1.2)	0.6 (± 0.3)	5	16.3 (± 0.6)
		2	90 (± 3.0)	5.0 (± 1.1)	2.5 (± 0.5)	49	23.6 (± 0.6)
		3	80 (± 1.6)	6.5 (± 2.1)	4.3 (± 3.5)	65	20.3 (± 1.2)
		4	77 (± 2.1)	2.0 (± 0.7)	0.9 (± 0.3)	42	16.0 (± 2.0)
Inverin	$6.8 \pm 1.1 \times 10^9$	1	99 (± 0.05)	6.4 (± 1.5)	2.94 (± 0.5)	n/a	12.0 (± 1.4)
		2	98 (± 0.56)	10.4 (± 2.2)	5.1 (± 0.9)	n/a	9.6 (± 0.6)
		3	99 (± 1.1)	2.1 (± 1.5)	1.1 (± 0.03)	n/a	13.6 (± 2.5)
		4	97 (± 1.7)	3.1 (± 0.7)	1.46 (± 3.7)	n/a	15.0 (± 2.0)
PAP	n/a	1	n/a	1.1 (± 0.1)	0.7 (± 0.1)	n/a	10.0 (± 3.0)
		2	n/a	0.5 (± 0.1)	0.4 (± 0.04)	n/a	15.6 (± 0.6)
		3	n/a	0.9 (± 0.1)	0.5 (± 0.02)	n/a	5.7 (± 4.7)
		4	n/a	0.1 (± 0.02)	0.1 (± 0.02)	n/a	8.3 (± 1.5)

^a Wet weight

Fig. 2 DGGE gel and UPGMA dendrogram constructed from similarity matching data produced from the DGGE profiles generated by using MVSP version 3.12 h, illustrating the differences between bacterial community profiles of Silvermines Grey Brown Podzolic soil obtained with M1–M4. Two replicate DGGE analysis from a single DNA extract and one DGGE profile generated from a separate DNA extraction shown (represented by *asterisk*)



the choice of method had an effect, where M2 lysed most cells (93.8%), while M1 was least successful (87.7%). M3 and M4 were very similar with regards to their lysis efficiencies, and consequently, a further one-factor ANOVA was carried out. No significant difference was noted, however, between M3 and M4 with respect to lysis efficiencies across all soil types ($p \geq 0.05$).

DNA yields were determined before and after DNA purification using the PicoGreen assay (Sandaa et al. 1998). Significant differences were also observed between the four methods with respect to DNA yields; for example, for the Corrib soil, crude DNA yields varied from 2.9 (M4) to 10.4 (M2) $\mu\text{g g}^{-1}$ wet weight (Table 1). Some anomalies were observed between the results obtained with the agarose gel images and the calculated DNA yields ($\mu\text{g g}^{-1}$) for particular samples, an example can be seen in Fig. 1b, where very little extracted DNA can be seen on the gel; this may be the result of ethidium bromide uptake by humic substances, leaving little for DNA staining, while PicoGreen analysis suggests the presence of a much higher DNA yield comparable to that of M3 (Fig. 1c; Table 1) in this case. When two-factor ANOVA was carried out on the crude DNA yields, significant differences were found between soil types ($p < 0.001$) and methodology ($p < 0.001$); however, the very low DNA yields achieved with the PAP sample and the problems associated with humic contamination of the crude extracts may be factors implicated in these differences; despite this, however, a mean of 9.5 $\mu\text{g g}^{-1}$ of crude DNA was achieved with M4, significantly less than all other methods, which ranged from 26.2 to 34.6 $\mu\text{g g}^{-1}$ of crude DNA.

Surprisingly, humic acid co-extraction was not problematic for the Inverin peat samples, regardless of extraction method employed, possibly because of the material being well humified. However, the crude extracts of the Silvermines and Corrib samples, from M1 and M2, were contaminated with humic acids (dark brown appearance), despite the incorporation in these protocols of the CTAB (high salt) buffer, intended to limit humic acid co-extraction

(LaMontagne et al. 2002). Conversely, very little co-extracted humics were observed when DNA was extracted from the Silvermines and Corrib samples using M3 and M4 (Fig. 1).

Purification of isolated DNA

Although generally suitable for direct PCR, all crude extracts were further purified by either 10^{-1} dilution in DEPC-treated water (PAP and Inverin) or GELase enzyme digestion (Corrib and Silvermines), throughout this study. The gel digestion approach successfully removed any contaminating substances and brown colour from crude DNA extracts.

Reproducibility and robustness of bacterial community structure profiles

Analysis of DGGE profiles, generated by PCR of purified DNA extracts, demonstrated that the choice of the DNA extraction method significantly influenced the bacterial community profiles generated. This was reflected in the number of bands or ribotypes detected from each sample (Fig. 2; Table 1). For example, two-factor ANOVA found significant differences in the apparent number of ribotypes present in DGGE profiles of all four biomass types ($p < 0.001$; Table 1) and with each of the four methods suggesting a significant impact both by sample type and method chosen. Indeed, Corrib and Silvermines soils, based on M2 and M3 extracts, contained more bands than those based on M1 and M4 extracts. Duplicate DGGE profiles generated from M2 or M3 extracts were, in all cases, with all soils tested, characterised by a greater than 85% similarity based on cluster analysis (Jaccard's co-efficient), usually corresponding to the presence/absence of a single band or ribotype (e.g. Fig. 2). In general, the profiles generated based on M2 and M3 were reproducible across multiple analyses, i.e. when the same soil sample was used for multiple DNA extractions, followed by DGGE, or when

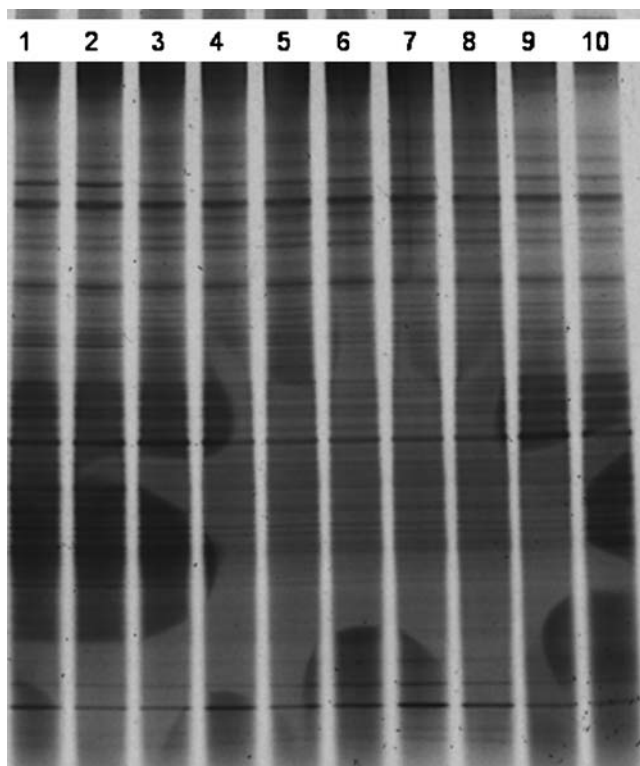


Fig. 3 DGGE gel illustrating reproducibility of M2-based DGGE profiles from Silvermines Grey Brown Podzolic; *Lanes 1–5*: replicate DGGE analysis from a single DNA extract; *lanes 6–10*: DGGE profiles generated from five separate extractions

multiple DGGE experiments were carried out on the same extract (Fig. 3). Greater variations were, however, observed between replicate DGGE profiles generated with M1 and M4, demonstrating that a lesser degree of reproducibility was achieved with these methods. This was particularly evident for the Inverin peat and PAP sediment samples (Table 1). Aside from the differences in ribotype richness between differing protocols, a number of variations in relative representation of certain DNA bands were apparent (e.g. Fig. 2). de Liphay et al. (2004), Kozdróz and van Elsas (2000) and Westergaard et al. (2001) have made similar observations.

Discussion

Numerous studies in the literature have evaluated a number of DNA extraction protocols using a variety of soil types (Steffan et al. 1988; Frostegård et al. 1999; Miller et al. 1999; Bürgmann et al. 2001; Lloyd-Jones and Hunter 2001; Niemi et al. 2001; de Liphay et al. 2004). The criteria employed by this study for the evaluation of a suitable extraction technique, which is compatible with a wide range of soils, included high cell lysis efficiency and DNA yield,

and—perhaps more importantly—maximum detection of microbial community members by PCR-DGGE.

Two methods are available for the estimation of lysis efficiency, those being the recovery of DNA from seeded samples or the use of a fluorochrome to compare DNA recovery to direct counts (Miller et al. 1999). In this study, we used the latter, as there are many known complications with the former, including lack of the full diversity of indigenous organisms and the fact that these seeded samples do not account for the effect the soil matrix may have on the results (Miller et al. 1999). Many studies of DNA extraction techniques have reported lysis efficiencies that concur with the range observed in this study (Table 1), including Howeler et al. (2003) who reported a lysis efficiency of $95.3 \pm 2.3\%$ of microbial cells from compost, while Zhou et al. (1996) reported lysis efficiencies of 67–92% from soils of different composition. M2 achieved, in almost every case, significantly higher cell lysis than the other three methods; however, there was considerable variation in the lysis efficiencies between different soil types, indicating that soil texture has a substantial impact on these measurements (Table 1).

DNA yields were found to vary depending on the method used. However, in a method similar to that of M1 and M2 used in this study, Tsai and Olson (1991) found DNA yields of $12 \mu\text{g g}^{-1}$ of soil, which is quite similar to what was found in this study (Table 1). Martin-Laurent et al. (2001) found $0.1\text{--}2.5 \mu\text{g g}^{-1}$ of soil in their study of three physicochemically contrasting soils. Other studies have reported DNA yields of 10 to $20 \mu\text{g DNA g}^{-1}$ of brown forest soil (Griffiths et al. 2002) and 13 to $136 \mu\text{g DNA g}^{-1}$ of Gartenacker soil (Bürgmann et al. 2001). Discrepancies were found between the DNA yields as seen on the agarose gels and those found with the PicoGreen assay. PicoGreen DNA yields appeared to be somewhat inflated, while concomitant masking and underestimation of DNA yields were experienced with ethidium bromide stained agarose gels. We posit that interference, caused by the presence of humic acids, was the principal factor contributing to the discrepancies, as these contaminants and DNA are known to fluoresce at similar wavelengths (Sandaa et al. 1998; Zipper et al. 2003), while humic acids are thought to sequester ethidium bromide thereby reducing the amount available for DNA intercalation (Rochelle et al. 1992). To this end, the main anomalous results were from DNA extracts obtained with M1 and M2 and resulted in artificially high estimates of initial DNA yields, based on PicoGreen analysis. This resulted in exaggerated DNA losses upon purification, e.g. recoveries of 22 (Corrib) and 5% (Silvermines) for M1 and 32% (Corrib) recovery for M2, respectively. However, for the majority of samples and indeed those less affected by humic acid co-extraction,

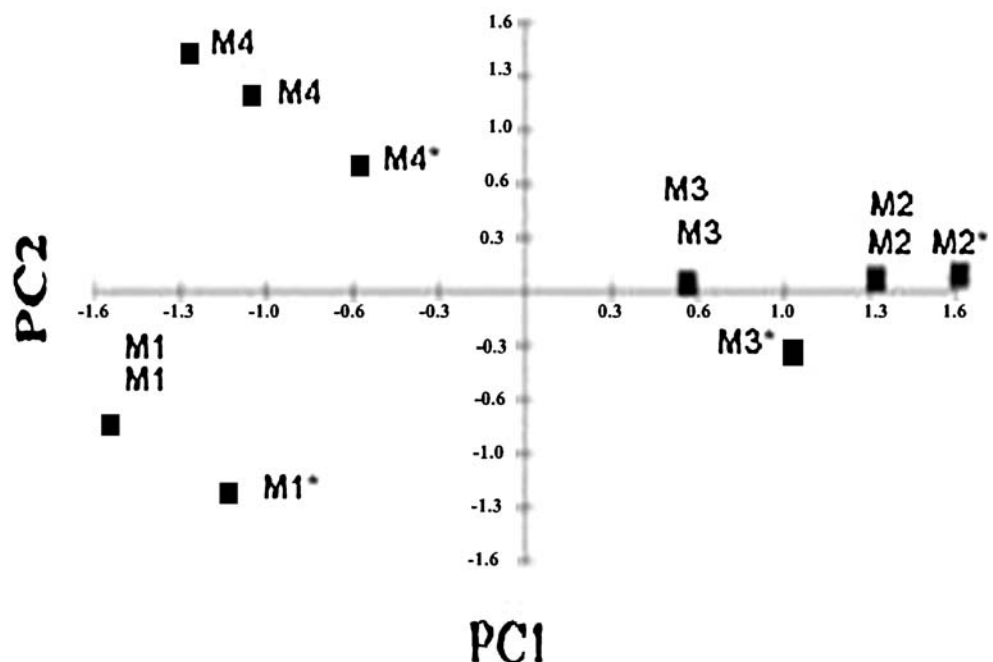
DNA recovery, i.e. when soil crude DNA was purified, was found to be $51 \pm 8.5\%$, which is within the expected range, based on literature values (Table 1; Robe et al. 2003), and indeed were higher than those found by Miller et al. (1999) who achieved DNA recovery of $29 \pm 17\%$ with a similar gel-based purification method. The degree of contamination with humic acids in some samples also necessitated the use of additional methods of DNA purification aside from those inherent in the protocols. Humic acid contamination was negligible with M3, possibly because of the absence of a heating step in the protocol or the incorporation of a PEG precipitation, which is noted for its role in reducing humic acid co-extraction (Bürgmann et al. 2001; Roose-Amsaleg et al. 2001).

Analysis of DGGE profiles, generated by PCR of purified DNA extracts, demonstrated that the choice of DNA extraction method significantly influenced the bacterial community profiles generated from all soils tested suggesting that for comparative analysis of soils, a single method must be selected. This was reflected in the number of bands or ribotypes detected from each sample (Fig. 2; Table 1). DGGE was found to be both reproducible and robust through the concurrent testing of replicate samples from the same DNA extraction and analysis of PCR amplicons from multiple extractions. In almost every case, UPGMA analysis separated the various methods (Fig. 2). While M2 and M3 were found to be similar because of the similar number of ribotypes they contained, when PCO analysis was applied, a greater degree of separation was found (Silvermines; Fig. 4) because of differing speciation in the DNA banding profiles.

The reproducibility and resolution of PCR-derived fingerprint analysis of soil microbial communities has been evaluated by several other studies (Ferris and Ward 1997; Tiedje et al. 1999; Osborn et al. 2000; Dunbar et al. 2001; Fromin et al. 2002). However, the choice of statistical analysis of the profiles is important for the discriminating power, as well as the number of errors when replicate DGGE profiles are clustered into different groups (Blackwood and Paul 2003). In this study, there was a general agreement between the cluster-based UPGMA and multivariate PCO approaches for comparison of DGGE profiles, an observation supported by a number of authors (Fromin et al. 2002; Terashima et al. 2002).

The results of this comprehensive evaluation of nucleic acid extraction methods suggested that M2 and M3 were both suitable for use in a large-scale study involving the direct comparative analysis of multiple soil types. The application of M2—in almost all cases—resulted in the resolution of greater diversity than did M3 despite using only half the volume of soil sample. The possibility of a simultaneous analysis of DNA and RNA fractions is an attractive feature of M3 (Griffiths et al. 2000), as this approach could provide the basis for discrimination between the active and non-active fractions of the soil microbial community and also the possibility for incorporation of quantitative reverse transcriptase and/or real-time PCR assays into soil research. However, this will require further research to evaluate the efficiency of RNA recovery from soils and the stability of RNA during field sampling and transport and to optimise the experimental protocols for quantitative PCR analysis of multiple soil types.

Fig. 4 Principle coordinate analysis of similarity matching data produced from the DGGE profiles of Silvermines Grey Brown Podzolic. M1–M4 represent methods 1–4



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