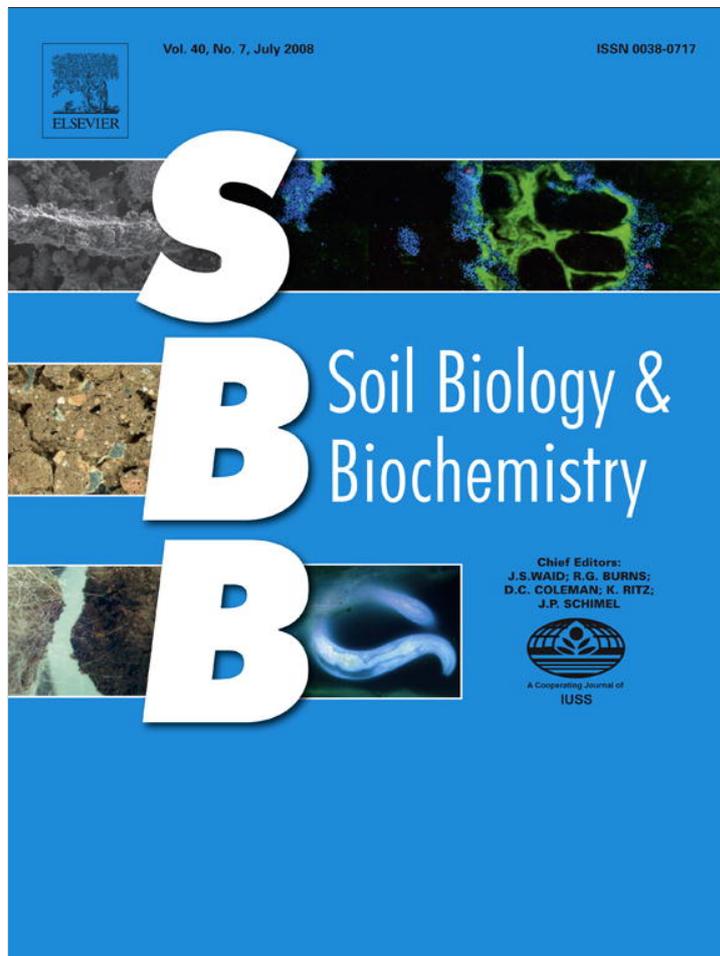


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Extraction of soil bacteria from a Ferralsol

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ABSTRACT

Extraction of intact bacteria from soil by dispersion and density gradient centrifugation may facilitate analyses of soil bacterial communities which are otherwise hampered by soil particles. Reasonable cell yield and representative fractions with sufficient purity can be extracted from most soils, but highly weathered, clayey and acidic tropical soils like Ferralsols represent a challenge to the method due to low cell yields. At an early stage of our studies with Ferralsols we also found substantial contamination of the extracted bacterial fractions by soil material, measured as total Al and Fe. We have adapted the method to a Ferralsol (pH 4.9, 64% clay, 21% silt, 15% sand) by factorial combinations of pH modification and salt (NaCl) concentration during soil dispersion, and evaluated the yield (microscopic cell counts), purity (optical density and Al + Fe content), and compared the composition of the extracted bacteria (phospholipid fatty acid analysis and 16S rDNA-denaturing gradient gel electrophoresis) versus that of the intact soil community. The cell yield was increased with a factor 2–3 by increasing the pH to 7.5, while it was decreased to a similar extent by adding NaCl (8 g l⁻¹). However, NaCl removed more than 99% of the Al + Fe contamination of the bacterial extracts, and the combination of modified pH and NaCl addition secured reasonable cell yield (4.6% of total number) and low contamination. The observed effects of pH and NaCl are probably due to changes in variable charge (by pH) and ion distribution (NaCl) around interacting particles (soil and bacteria), thus affecting their flocculation. Phospholipid fatty acid and denaturing gradient gel electrophoresis analysis indicate that the bias of the bacterial extracts compared to direct soil extracts increases with the addition of NaCl as well as by pH manipulation. Nevertheless, representativeness was acceptable as indicated by a Bray–Curtis similarity index (bacterial extracts versus soil) of 70% and 87% for phospholipid fatty acid profiles and denaturing gradient gel electrophoresis, respectively. Overall, the results reveal a trade-off among yield, purity and representativeness. Thus, depending on application and analyses, future users can choose the right treatment according to their specific purpose.

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1. Introduction

Analyses of soil bacteria are often hampered by soil particles, since most soil bacteria are attached to soil surfaces and/or trapped within soil aggregates (Mills, 2003). The extraction of bacteria from soil by density gradient centrifugation as described by Bakken and Lindahl (1995) is a way to overcome this obstacle. The method has already been used for a wide range of purposes, e.g. to study heavy metals in and adsorbed onto soil bacteria (Sitaula et al., 1999), Zn and Cd tolerance of soil bacteria (Almas et al., 2004) as well as for determination of cell size by flow cytometric analysis (Christensen et al., 1994) and for detection of microorganisms by fluorescence in situ hybridization (Caracciolo et al., 2005; Bertaux et al., 2007). In

addition, it can be used to obtain nucleic acids of soil bacteria for molecular genetic analysis (Bakken and Lindahl, 1995; Courtois et al., 2001; Bakken and Frostegård, 2006).

The extraction of bacteria by density gradient centrifugation comprises two steps. The first step is to detach bacteria from soil particles by physical or chemical dispersion or by a combination of both. The second step is the separation of detached bacteria from soil by high speed centrifugation over Nycodenz, a nonionic density gradient medium. Due to differences in buoyant densities, soil particles will sink through the Nycodenz to the bottom of the centrifugation tube, whereas bacteria will float on top of the Nycodenz and can be harvested by siphoning off the supernatant. Bacteria attached to soil particles will sink together with soil, hence an imperfect detachment of bacterial cells from soil results in low cell yield and representativeness. The extent of dispersion and detachment of bacterial cells depends on soil type and texture, with lower yields generally observed on soils with higher clay content

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(Bakken, 1985; Bakken and Lindahl, 1995). Yields in most studies vary between 10 and 20% of the total number of bacteria in the soil (Bakken and Frostegård, 2006). For example, Maron et al. (2006) were able to extract 9% of all soil bacteria on a sandy soil from France (48% sand, 35% silt, 17% clay), but only 0.5% of the cells from a clayey, acidic, tropical forest soil (24% sand, 6% silt, 70% clay). The difficulties in extracting cells from such highly weathered, clayey, tropical soils are probably due to a combination of low pH and high contents of metal oxides and clay. According to Stotzky (1985) and Mills (2003), electrostatic interaction (caused by surface charges) and van der Waals forces play a key role in the attachment of bacteria to soil surfaces. Furthermore, at low pH, surfaces with variable charge carry a net positive charge while with increasing pH the net charge turns negative. The pH at which the total number of positive charges equals the number of negative charges is referred to as the point of zero charge (PZC). Bacterial cells have a PZC in the region of pH 1.75–4.15 (Harden and Harris, 1953; Wicken, 1985). Therefore, the positive charge of some clays and metal oxides causes bacterial sorption (Mills, 2003).

A wide range of physical and chemical dispersion techniques have been used to promote bacterial detachment and thus to increase the yield of extracted bacteria. As for the physical dispersion, treatment in a Waring blender for 3 × 1 min appears to provide reasonable cell yields, while minimizing mechanical cell damage (Lindahl and Bakken, 1995; Courtois et al., 2001). Chemical agents to promote dispersion include ligand exchangers and chelating agents such as oxalate, citrate and EDTA (Katayama et al., 1997), Tris buffer (Niepold et al., 1979; Hopkins and O'Donnell, 1992), pyrophosphate (Lindahl, 1996) and the use of cation exchange resins (Hopkins and O'Donnell, 1992). The intended function of these chemical agents is to mask positive charges of clay minerals and to chelate multivalent cations in order to increase net repulsion, thus enhancing detachment of cells from soil particles (Niepold et al., 1979; Hopkins and O'Donnell, 1992) and hindering reattachment of cells already released (Bakken and Lindahl, 1995.).

The fact that Ferralsols, like other highly weathered, tropical soils, have a large proportion of variable charge on the mineral particles (Parfitt, 1980) could open for an alternative approach. Instead of masking positive surface charges with ligand exchangers, it is possible to change variable charges from positive to negative by increasing the surrounding pH (Parfitt, 1980). Thus, electrostatic repulsion between bacteria and soil particles should be increased by increasing the pH during soil dispersion in the blender.

As a second factor, the ion density during soil dispersion could be modified. The ion density determines the thickness of the diffuse layer of ions that surrounds a charged surface. A low ion density increases the thickness of the diffusive double layer, making an attachment due to van der Waals forces less likely to occur (Loeb, 1985).

The aim of our study was to find an improved protocol for extraction of bacteria from highly weathered, clayey and acidic tropical soils. Our tentative criteria for an acceptable protocol were high cell yields, combined with acceptable purity and representativeness to allow a broad range of applications. In preliminary experiments, we tried some of the mentioned chemical dispersion agents, i.e. oxalate, citrate and EDTA (at 10 mM each) during dispersion of our Ferralsol in a Waring blender. None of the agents improved the cell yield significantly compared to the use of distilled water. The experiments further demonstrated that the suspensions of cells extracted from the Ferralsol were heavily contaminated with iron oxides. We hypothesized a differential effect of pH and ion concentrations on the repulsion between soil colloids versus that between bacterial cells and soil colloids, which would open for a selective enhancement of bacterial dispersion versus that of soil colloids. In total, four different dispersion treatments with a factorial combination of pH modification and ion density were tested.

The estimated yields of extracted bacteria were based on epifluorescence direct counts. The purity was estimated by measuring the optical density and total Al and Fe content in the extracted bacterial suspension. Representativeness was assessed by analyses of phospholipid fatty acid (PLFA) and 16S rDNA-denaturing gradient gel electrophoresis (DGGE) patterns of the various cell extracts versus those of intact soil.

2. Materials and methods

2.1. Soil

Soil was collected in August 2006 from 0 to 20 cm depth of a field trial site in Nyabeda, Western Kenya (altitude: 1420 m, latitude: 0°06' N, longitude 34°34' E) under a maize–soybean crop rotation. The soil was stored at 4 °C in moist condition (0.25 ml water g⁻¹ soil dry weight) for 2 months before use. It contains 64% clay, 21% silt and 15% sand, has a pH (H₂O) of 4.9 and is classified as a Ferralsol (FAO/ISRIC/ISSS, 1998). Organic C content measured with a CNS Analyzer (FlashEA 1112, Thermo Electron Corporation) is 22 g kg⁻¹.

According to X-ray diffraction (XRD) and Rietveld analyses (Jenkins, 2000), the soil contained 40% kaolinite, 18% quartz, 17% goethite, 3% potash feldspar, 2% hematite, 2% anatase, and 1% crandallite. Amorphous compounds accounted for 18%.

Oxalate extractable Fe and Al (McKeague and Day, 1966) are 2.6 g kg⁻¹ (±0.04) and 1.3 g kg⁻¹ (±0.01), respectively. Dithionite–citrate–bicarbonate extractable Fe and Al (Mehra and Jackson, 1960) are 51.7 g kg⁻¹ (±0.51) and 3.8 g kg⁻¹ (±0.12), respectively. Regarding the soil properties, the Ferralsol being used in our study is typical for a highly weathered, clayey and acidic tropical soil.

2.2. Extraction of bacteria from soil

Bacteria were extracted from soil by high speed centrifugation over Nycodenz (Nycodenz Pharma, Norway), a nonionic density gradient medium, as described by Bakken and Lindahl (1995). Ten grams of soil (fresh weight) and 200 ml of liquid were dispersed using a Waring Commercial Blender with 1 l glass containers, run at full speed (18 000 rev min⁻¹) for 3 × 1 min, with intermittent cooling on ice for 1–2 min to sustain low temperature. Aliquots of the soil suspension were then transferred to centrifugation tubes and the Nycodenz solution was placed underneath using a syringe with a 15 cm steel needle.

For centrifugation two different volumes were used. For the determination of yield as well as for the DGGE and PLFA analysis, 250 ml centrifugation tubes with 200 ml of soil suspension and 40 ml Nycodenz solution (0.8 g ml⁻¹ H₂O) were used. The samples were centrifuged for 2 h (4 °C) at 10 000 g (Avanti J-25 Centrifuge, JS-7.5 swinging bucket rotor, both Beckmann Coulter International). For purity measurements, 50 ml centrifugation tubes with 30 ml of soil suspension and 8 ml of Nycodenz solution were used. This time centrifugation for 2 h at 4 °C was performed at 15 000 g with a JS-13.1 swinging bucket rotor (Beckmann Coulter International). The different g-forces (10 000 g with 250 ml tubes, 15 000 g with 50 ml tubes) did not affect the yield (data not shown), yet using the 50 ml centrifugation tubes proved to be more practical since the separation between phases was more distinct and reliable.

After centrifugation, we siphoned off and discarded the supernatant down to approximately 1 cm above the Nycodenz cushion. The remaining supernatant with bacteria floating on top of the Nycodenz phase was then harvested by siphoning off and the extracted bacterial suspension was stored at 4 °C. For PLFA and DGGE analysis the extracted bacteria were collected by filtering through polycarbonate filters (pore size 0.2 µm). Filters with bacteria were then stored at –20 °C.

Four different soil dispersion treatments, a factorial combination of pH modification and salt concentration in the soil suspension (Table 1), were tested. In the water (W)-treatments (Wnon and Wmod), filter-sterilized ultrapure water was used as dispersion liquid, whereas in the salt (S)-treatments (Snon and Smod) a 0.8% NaCl solution was used. The pH in soil suspension remained non-modified in treatments Wnon and Snon while it was modified to pH 7.5 by adding 1 M NaOH in treatments Wmod and Smod. The amount of 1 M NaOH needed was determined in a separate titration experiment where the pH response after 10 min was measured in the soil suspension. This time span was chosen since it is approximately the time the dispersion of the soil in the Waring Blender takes. The extraction was performed in triplicate for each treatment.

2.3. Bacterial counts

The numbers of bacterial cells in the soil and in the extracted bacterial suspension were quantified by acridine orange direct counts as described by Hobbie et al. (1977). The cell yield was calculated as number of extracted bacteria as percent of the total number of bacteria in the soil.

2.4. Determination of purity

Purity was assessed by determining the optical density (OD), which provides an estimate of soil particle concentration in a suspension (Hesterberg and Page, 1990), and by measuring the Fe and Al content of the extracted bacterial suspension. The OD was determined as % absorbance at 530 nm wavelength compared to pure water using a spectrophotometer (UV-1601, Shimadzu). Fe and Al were measured by ICP-MS analyses after sodium hydroxide-persulfate digestion according to Ebina et al. (1983).

2.5. PLFA analysis

PLFA composition of the soil and extracted bacteria was analyzed to assess the representativeness of the extracted soil bacteria for the bacterial community in the soil. The PLFAs were extracted from soil and from extracted bacteria in three independent replicates for each treatment. Lipid extraction and preparation of fatty acid methyl esters followed the method described by Frostegård et al. (1993). Conditions for gas chromatography were as described by Jia et al. (2006).

The PLFA 18:2 ω 6 is considered a fungal biomarker (Klamer and Bååth, 2004), whereas a15:0, i16:0, 16:1 ω 7t, 16:1 ω 5, i17:0, a17:0, 18:1 ω 7 and cy19:0 are all considered to be of bacterial origin (Tunlid and White, 1992; Frostegård and Bååth, 1996). Accordingly, the fungal:bacterial ratio was calculated by dividing the amount of 18:2 ω 6 by the sum of the bacterial fatty acids. Furthermore the PLFAs cy17:0 and 18:1 ω 7 were used as indicators for Gram-negative bacteria (Wilkinson, 1988; Joner et al., 2005) and the anteiso- and iso-branched PLFAs a15:0, a17:0, i16:0, i17:0 and br18:0 as well as br17:0 were considered as indicators for Gram-positive bacteria (O'Leary and Wilkinson, 1988). In addition, 10Me17:0 and 10Me18:0 were used as indicators for actinomycetes (Kroppenstedt, 1992).

Table 1

Modifications of the dispersion step in extraction of soil bacteria from a Ferralsol: treatment abbreviations

Treatment	Dispersion medium	pH modification
Wnon	H ₂ O	None
Snon	0.8% NaCl	None
Wmod	H ₂ O	Raised to 7.5
Smod	0.8% NaCl	Raised to 7.5

2.6. DNA extraction, amplification and DGGE fingerprinting

DNA was extracted in three independent replicates from 0.5 g of soil and from extracted cells collected on polycarbonate filters using a soil DNA extraction and purification kit (FastDNA SPIN for Soil Kit from Q-BIOgene). DNA concentrations were measured with a NanoDrop ND 1000 spectrophotometer (NanoDrop Technologies, Inc.). Partial 16S rRNA gene was amplified by PCR using the eubacterial universal forward primers 8F-GC clamp (5'-cgcccgcgcgcgcggcgggcgggcgggggcagcggggg AGA GTT TGA TCC TGG CTC AG-3') and 518R (5'-ATT ACC GCG GCT GCT GG-3') (Hicks et al., 1992; Muyzer et al., 1993). A 25 μ l reaction mixture contained 1 U of Promega Taq DNA polymerase (Promega Co.), 1 \times PCR buffer (Mg²⁺ free), 2 mM MgCl₂, 10 pmol of each primer, 200 μ M of each deoxynucleoside triphosphate (dNTP), and 20 ng of DNA. The samples were amplified in a thermocycler PCR system (GeneAmp PCR System, Applied Biosystems). PCR products were checked by agarose gel electrophoresis (0.8% wt/vol) stained by ethidium bromide and photographed by Bio-Rad Gel Doc 2000 (Bio-Rad Laboratories, Hercules).

PCR amplicons were separated on 8% (wt/vol) polyacrylamide gels with a denaturing gradient from 40 to 58% (100% denaturant corresponds to 7 M urea and 40% deionized formamide) using a Dcode System apparatus (Bio-Rad, Hercules). DGGE was run in 1 \times TAE buffer at constant voltage (160 V) and temperature (60 °C) for 240 min. Gels were stained by SYBR green (Amresco, Solon) for 1 h and photographed by Bio-Rad Gel Doc 2000 (Bio-Rad Laboratories, Hercules). The DGGE gels were analyzed by Quantity One (version 4.4.0, Bio-Rad Laboratories, Hercules) using all the bands.

2.7. Statistical analysis

To analyze differences in microbial community composition between the treatments, principal component analyses (PCAs) were performed on the PLFA and the DGGE data sets.

The PLFA composition was analyzed based on the relative abundance of individual PLFAs (mol% values). The PLFA 18:2 ω 6 was disregarded for PCA since it is considered to be a fungal biomarker (Klamer and Bååth, 2004). For the DGGE data, the relative intensity of individual bands (% of the summed intensity of all selected bands in a treatment) was used. The % values both for the PLFA and DGGE data sets were standardized before PCA.

Similarity of the PLFA and DGGE profiles was analyzed using the Bray–Curtis coefficient. The Bray–Curtis coefficient is a proportion coefficient for abundance data and was calculated as follows:

$$BC_{jk} = 1 - \frac{\sum_{i=1}^n |y_{ij} - y_{ik}|}{\sum_{i=1}^n (y_{ij} + y_{ik})}$$

where y is the relative band density (DGGE) or lipid concentration (PLFA), while i denotes the band/lipid identity across j and k samples (Bray and Curtis, 1957; McCune and Grace, 2002). The remaining statistical analyses were done using SYSTAT 11 (SYSTAT Software, Inc., 2004). The impact of pH, NaCl and their interaction were analysed by a two-way ANOVA followed by Tukey's multiple comparison test whenever significant differences ($P < 0.05$) were indicated.

3. Results

3.1. Yield of soil bacteria

Extraction efficiencies (Table 2) are expressed as percent of the bacterial numbers in the dispersed soil suspension, which was 3.7×10^9 g⁻¹ fresh soil with a standard deviation (SD) of 1.3×10^8 .

Table 2

Treatment effects on yield of extracted soil bacteria and on optical density (OD, 530 nm wavelength) and sum of Fe and Al in the extracted bacterial suspension^a

Treatment	Yield (% of total soil bacteria)	OD ₅₃₀ (%)	Sum of Fe and Al (mg l ⁻¹)
Wnon	4.94 (0.07) b	9.41 (4.23) a	34.06 (12.3) b
Snon	1.56 (0.14) c	0.12 (0.06) b	0.04 (0.07) c
Wmod	10.65 (0.19) a	9.33 (1.53) a	65.13 (6.12) a
Smod	4.61 (0.30) b	0.33 (0.22) b	0.05 (0.05) c
ANOVA			
pH	***	n.s.	**
NaCl	***	***	***
pH × NaCl	***	n.s.	**

*** Indicates significance at $P < 0.001$.

** Indicates significance at $P < 0.01$.

n.s. No significance ($P \geq 0.05$).

^a All values are means of three replicates, SD in brackets, values within a column followed by the same letter do not differ significantly ($P < 0.05$) according to Tukey's test.

The highest yield (10.7%) was obtained with high pH and no NaCl added (Wmod). The yields of the Wnon and Smod treatments did not differ significantly and were just below 5%. The Snon treatment had the lowest yield with extraction of 1.6% of total bacteria in soil. Thus, raising the pH to 7.5 increased the yield, whereas the addition of NaCl to the dispersion liquid had a lowering effect.

3.2. Estimation of impurities

We observed that extracted bacterial suspensions of the W-treatments had a brown-reddish color while the extracted bacterial suspensions of both S-treatments were practically colorless. Around 9% of the light was absorbed in the W-treatments compared to less than 0.5% in the S-treatments (Table 2). The bacterial suspensions from the S-treatments contained low amounts of impurities (sum of Fe and Al below 0.1 mg l⁻¹), while the suspensions from the Wnon and Wmod treatments contained 34 and 65 mg l⁻¹, respectively. Thus, the amount of Fe and Al in the extracted bacterial suspension was grossly reduced by NaCl, while it was increased by raising the pH.

3.3. Representativeness of extracted bacterial communities

A PCA was performed using the relative abundance of the selected PLFAs to compare the PLFA composition of the four dispersion treatments and the soil (Fig. 1). The fungal PLFA 18:2ω6 was excluded from this analysis. The first principal component (PC1) explained 40.1% and the second principal component (PC2) explained 27.6% of the total variation. The PLFA profile of the Wmod treatment was most similar to that of the soil while the salt treatments, Smod and Snon, were the least similar to the soil samples, as seen by the separation along PC2. The PLFA patterns did not reveal consistent treatment effect on broad bacterial groups such as Gram negatives and actinomycetes, as judged by their signature lipids. For example, the PLFA 18:1w7, which is representative of Gram-negative bacteria, showed the highest relative abundance in the Smod treatment while the opposite was true for cy17:0, which is another PLFA representative of Gram-negative bacteria (Fig. 1b). Similarly, 10Me18:0, which is specific to actinomycetes, had the highest relative abundance in the Smod treatment, while 10Me17:0, also specific to actinomycetes, was most abundant in the samples from whole soil, Wnon and Wmod. PLFAs representative of Gram-positive bacteria were also scattered in the loading plot, e.g. i16:0, i17:0 and br18:0 showed the highest relative abundance in the whole soil and in the Wmod treatments, while a15:0, a17:0 and br17:0 were most abundant in the Wnon and Snon.

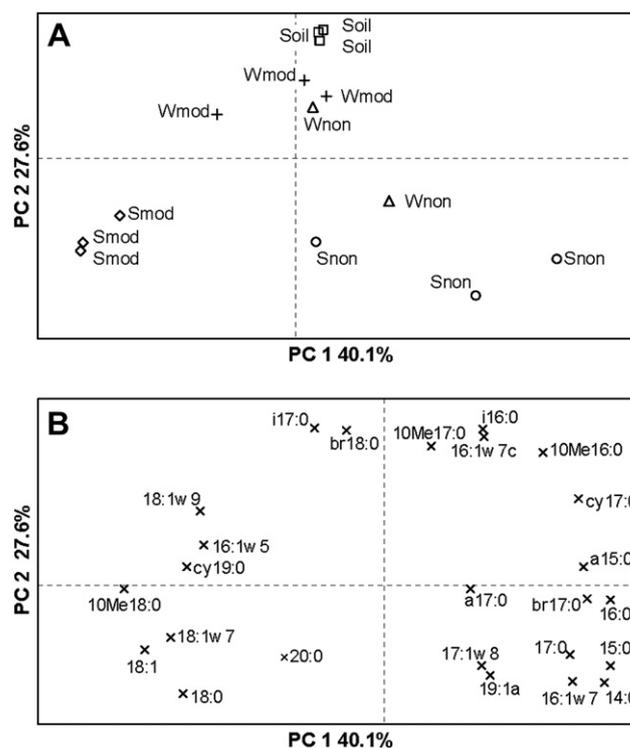


Fig. 1. PCA generated from PLFA profiles from soil and from extracted bacteria. Profiles were obtained from three replicates except for the Wnon treatment (duplicates).

The Bray–Curtis coefficient (Table 3) showed that the Wmod treatment was closest to the soil (83% similarity) while the Smod treatment was the least similar (70%), thus corroborating the results from the PCA.

The relative abundance of the fungal fatty acid 18:2ω6 was highest in both S-treatments with more than 4% (Table 4). However, the relative abundance of 18:2ω6 did not differ significantly between the different PLFA profiles due to the high variation. Likewise, the calculated fungal:bacterial ratio did not differ significantly between treatments, although there was a tendency for the ratios to be higher in the S-treatments.

A photograph of the DGGE gel is shown in Fig. 2. The number of detectable bands was 22 in both S-treatments, 24 in the Wmod and 27 in the Wnon treatments (all with a SD = 0). The results indicate that modifying the pH as well as using a 0.8% NaCl solution reduced the number of detectable bands. The number of detectable bands was significantly higher for the Wnon treatment than for DNA extracted directly from soil (average = 25.7 bands, SD = 0.58), whereas the number of bands delivered by the other treatments was significantly lower.

The results of the PCA of the DGGE data are shown in Fig. 3. The first axis, which explained 42.0% of the variation, highlighted the

Table 3

Bray–Curtis similarity coefficients calculated for the PLFA profiles of the soil and of extracted bacteria (using four different extraction treatments)^a

	Soil	Wnon	Snon	Wmod	Smod
Soil	0.99	0.77	0.73	0.83	0.70
Wnon		0.83	0.79	0.77	0.66
Snon			0.90	0.73	0.65
Wmod				0.93	0.72
Smod					0.90

^a Coefficients were calculated on the basis of three replicates except for the Wnon treatment (duplicates).

Table 4
Relative abundance of the fungal signature fatty acid 18:2 ω 6 and fungal:bacterial ratio in the extracted bacterial suspensions and in soil^a

Treatment	18:2 ω 6 (%)	Fungal:bacterial ratio
Soil	1.48 (0.57) a	0.03 (0.01) a
Wnon	1.43 (2.02) ^b a	0.06 ^b a
Snon	4.50 (0.94) a	0.11 (0.04) a
Wmod	1.15 (0.08) a	0.02 (0.00) a
Smod	4.03 (4.15) a	0.09 (0.10) a
ANOVA		
Treatment	n.s.	n.s.

n.s. No significance ($P \geq 0.05$).

^a All values are means of three replicates except for the Wnon treatment (duplicates), SD in brackets, values within a row followed by the same letter do not differ significantly ($P < 0.05$) according to Tukey's test.

^b 18:2 ω 6 was detected only in one of the two replicates of the Wnon treatment.

difference between the Wmod treatment and the other treatments. The second axis (25.2% of variation) captured the effect of NaCl in the dispersion liquid (treatments Smod and Snon). The DGGE profile of the Wnon treatment was the one most similar to that of the soil bacterial community.

The Bray–Curtis coefficients showed similar trends as the PCA (Table 5). The Wnon treatment was most similar to the soil (91%), whereas the Snon treatment was least similar. Nevertheless, similarity between Snon and soil was rather high (86%).

4. Discussion

4.1. Yield and purity

Electrostatic attraction caused by surface charges and van der Waals forces plays a key role in the attachment of bacteria to soil surfaces. A variable charge on soil particles arises from the presence of amphoteric constituents, like crystalline and amorphous Fe-, Al- and Ti-oxides (Bolan et al., 1999). In the investigated Ferralsol, crystalline minerals containing iron (goethite and hematite), aluminum (kaolinite and crandallite) and titanium (anatase) account for 62% of all soil minerals. Additionally, the soil contained significant amounts of amorphous iron and aluminum oxides and

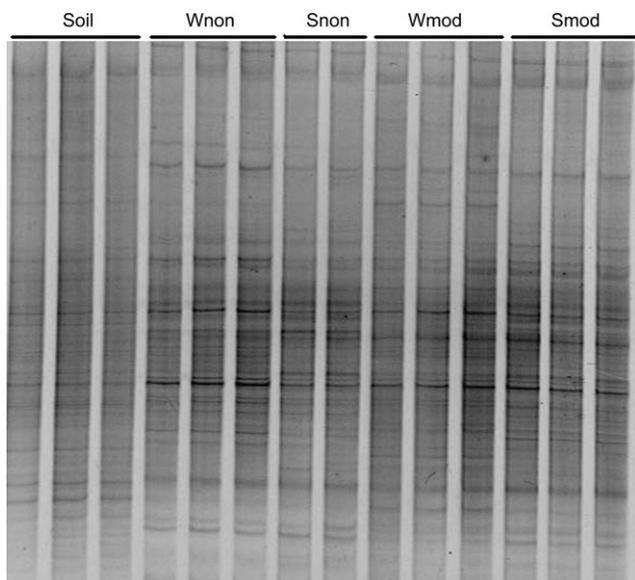


Fig. 2. Photograph of the DGGE gel, showing three lanes for each treatment except for the Snon treatment (duplicates).

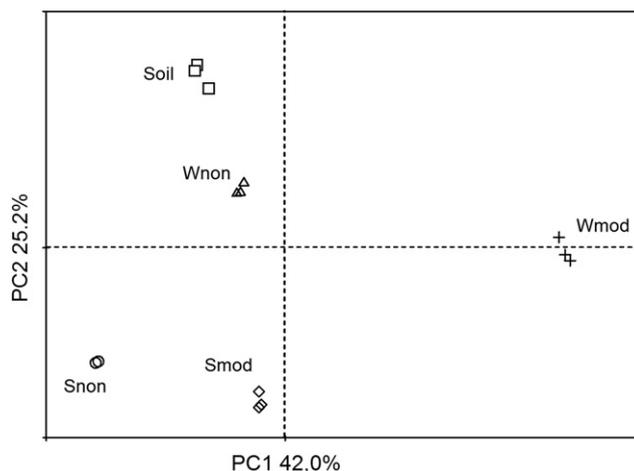


Fig. 3. PCA generated from DGGE profiles from DNA extracted directly from soil and from extracted bacteria. Profiles were obtained from three replicates except for the Snon treatment (duplicates).

hydroxides, as assessed by oxalate extraction. Amorphous compounds belong to the most reactive oxides in soil due to their high surface area (Loeppert and Inskeep, 1996). Apart from soil particles, bacteria are known to have a variable charge as well (Harden and Harris, 1953). Since the main factor determining the variable charge is the pH (Parfitt, 1980), the significant effect of pH modification on bacterial yield in this study was probably caused by changing the variable surface charge of interacting particles (soil and bacteria). The PZC for bacteria ranges from pH 1.75 to 4.15 (Harden and Harris, 1953), for quartz it is around pH 2 (Bolan et al., 1999) and for kaolinite it ranges between 4.6 and 4.8 (Kretzschmar et al., 1997; Bolan et al., 1999). Therefore, it can be assumed that bacteria, quartz and kaolinite had a negative net charge in all treatments, no matter whether the pH was modified or not. Nevertheless the negative charge was most likely increased by raising the pH to 7.5.

Parfitt (1980) reported a PZC of 8.1 for a goethite. Therefore, it can be assumed that goethite had a positive net charge that decreased substantially when the pH was raised to 7.5. A similar effect can be expected for amorphous Fe- and Al-oxides and hydroxides, since Stumm and Morgan (1996) report a PZC of 8.5 for an amorphous Fe(OH)₃ and Hendershot and Lavkulich (1983) report a positive surface charge for Al-hydroxides at pH 6.0.

Thus, raising the pH to 7.5 will result in stronger electrostatic repulsion between bacteria and quartz and kaolinite, and in weaker electrostatic attraction between bacteria and goethite and amorphous Fe- and Al-oxides and hydroxides. This would explain why we observed higher cell yields in response to pH 7.5. The same mechanism probably caused increased electrostatic repulsion between soil particles, which therefore enhanced soil dispersion and hence the detachment of soil bacteria trapped inside soil aggregates. It is thus not surprising that both cell yield and impurities

Table 5

Bray–Curtis similarity coefficients calculated for the DGGE profiles of DNA extracted directly from soil and of DNA from extracted bacteria (using four different extraction treatments)^a

	Soil	Wnon	Snon	Wmod	Smod
Soil	0.98	0.91	0.86	0.87	0.87
Wnon		0.98	0.85	0.88	0.85
Snon			1.00	0.83	0.91
Wmod				0.97	0.88
Smod					0.98

^a Coefficients were calculated on the basis of three replicates except for the Snon treatment (duplicates).

increased after pH modification. Similarly, Frenkel et al. (1992) found an increased dispersion and decreased flocculation of kaolinite clay when increasing the pH.

The addition of NaCl to the dispersion liquid led to a moderate reduction of cell yield and a great reduction in impurities. This was most likely due to the higher ion strength leading to thinner diffuse double layers, hence enhancing flocculation of soil particles as well as reattachment of bacteria due to van der Waals forces (Bolan et al., 1999). During centrifugation these aggregates sank through the Nycodenz layer, sedimented at the bottom of the tube, and led to a decrease in yield and impurity.

Frenkel et al. (1992) observed the same effect of ion concentration on the dispersion and flocculation of clayey soils. Mills et al. (1994) and Gross et al. (1995) found a similar effect of ion density on the attachment of bacteria to particles, whereas Courtois et al. (2001) reported that cell yield was not affected by ion density in their study.

It is remarkable that NaCl addition reduced the contamination of soil particles in the extracted bacterial suspension to a greater extent than it reduced the cell yield. Especially at the high pH, the impurities were practically eliminated (OD reduced by 96%, content of Al and Fe by 99%) by NaCl addition, whereas cell yield was only reduced by 57%. Frenkel et al. (1978) reported that the ion density affects the dispersion and flocculation of different clay minerals differently. In a subsequent study Frenkel et al. (1992) showed that different clay minerals have different critical flocculation concentrations (defined as the NaCl concentration required to sediment 80% of the clay after standing for 24 h). Regarding our results, the physiological NaCl concentration used in the S-treatments caused mineral soil particles to flocculate almost entirely, whereas a considerable amount of soil bacteria evidently remained detached. Thus, in our study, the addition of NaCl promoted a selective removal of soil particles from the extracted bacterial suspension, as hypothesized. This represents a great improvement of the method of cell extraction from the Ferralsol, and is probably applicable for a range of other highly weathered tropical soils.

4.2. Representativeness

The PLFA profile of the Wmod treatment (11% yield) was the one most similar to the PLFA profile of the soil. In contrast, the DGGE profile of the Wnon treatment (5% yield) was the one most similar to the DGGE profile of the soil. Similarly, Maron et al. (2006) found that yield and representativeness are not necessarily related when describing representativeness by automated-ribosomal inter-genetic spacer analysis.

One reason for the difference between results based on PLFA and DNA biomarkers could be that PLFA analyses are not specific to bacteria. They rather represent the entire microbial community. This was true also for the present investigation since several of the PLFAs that were included in the PCA exist in both bacteria and fungi. In contrast, 16S rDNA based DGGE analyses target only bacteria. Direct extraction of DNA from soil is, however, afflicted with several biases, and therefore differences in DGGE profiles between soil DNA and DNA from extracted bacteria do not necessarily reflect a bias of the cell extraction. For example, some bacteria are difficult to lyse either due to morphological characteristics or because they are protected within cavities or soil aggregates. Furthermore, the DNA extracted from intact soil may contain extracellular DNA or be biased by selective adsorption of DNA to soil particles (Frostegård et al., 1999; Bakken and Frostegård, 2006). Drawbacks depend on the soil type and are usually more severe on highly weathered tropical soils with a high clay content (Frostegård et al., 1999; Maron et al., 2006).

Combining all obtained information given by PLFA and DGGE, the S-treatments were the ones with the lowest representativeness

according to the PCAs and Bray–Curtis coefficients as well as the information given by the number of detectable bands. The Wmod treatment was the one closest to the soil with regard to the PLFA pattern, yet this was not the case for the DGGE profiles. Therefore, the Wnon treatment seems to be the recommendable treatment regarding representativeness of the bacterial community since its DGGE profile is the one most similar to that of the soil.

DGGE analyses detect only dominating species (i.e. those that represent $\geq 1\%$ of the total community) (Muyzer et al., 1993). A higher number of detected bands for the Wnon treatment than for soil DNA is thus reflecting somewhat higher evenness. Furthermore, amplicons from more than one species may be hidden behind a single band and, conversely, the same species may be represented by different bands (Heuer et al., 2001). Given these shortcomings of the DGGE analyses, they can only be expected to detect grossly biased cell extraction methods. The fact that neither the present nor previous studies have revealed such biased cell extraction by the Nycodenz methodology (Mayr et al., 1999; Courtois et al., 2001) suggests a reasonably representative extraction of dominating broad bacterial groups. It is no proof, however, of the absence of bias for specific groups, as demonstrated for methane oxidizing (Prieme et al., 1996) and ammonia oxidizing bacteria (Aakra et al., 2000).

The extraction by density centrifugation focuses on the extraction of bacteria, since recovery of fungal biomass is expected to be rather low (Bakken and Frostegård, 2006). Probably, the fungal hyphal structure is difficult to detach from soil particles. Therefore, the high relative abundance of 18:2 ω 6 in the extracted bacterial suspensions and the fact that the fungal:bacterial ratio did not differ significantly between the extracted bacterial suspensions and the soil is rather surprising. An explanation could be that significant amounts of single-cell fungal structures like yeasts and spores are recovered from the soil during the extraction. Nevertheless, the relative abundance of 18:2 ω 6 and the calculated fungal:bacterial ratio in the soil are similar to values found by Frostegård and Bååth (1996) in soils with low organic matter content, and Bünemann et al. (2004) found a comparable relative abundance of 18:2 ω 6 in a Kenyan Ferralsol.

4.3. Conclusions

Overall, the extraction of bacteria has been successfully adapted to a Ferralsol. However, there was a trade-off between the three main objectives yield, purity and representativeness. The Wmod treatment delivered the highest yield of soil bacteria and was most similar to the PLFA profile of the soil. The Wnon treatment provided a bacterial community with the highest apparent species richness (measured as the number of DGGE bands) and a DGGE profile closest to that of the soil. This treatment would probably be preferred for studies of bacterial community composition. The Smod treatment, however, still provided sufficient yield and representativeness in combination with a great improvement of purity. Accordingly, it might be preferred for studies on nutrient composition of soil bacteria. The choice of extraction protocol clearly depends on the purpose of the study.

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