

Survival of bacterial DNA and culturable bacteria in archived soils from the Rothamsted Broadbalk experiment

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Abstract

Dried soil samples from many sources have been stored in archives world-wide over the years, but there has been little research on their value for studying microbial populations. Samples collected since 1843 from the Broadbalk field experiment on crop nutrition at Rothamsted have been used to document changes in the structure and composition of soils as agricultural practices evolve, also offering an invaluable record of environmental changes from the pre- to post-industrial era in the UK. To date, the microbial communities of these soils have not been studied, in part due to the well-documented drop in bacterial culturability in dried soils. However, modern molecular methods based on PCR amplification of DNA extracted directly from soil do not require bacterial cells to be viable or intact and may allow investigations into the legacy of bacteria that were present at the time of sample collection.

In a preliminary study, to establish if dried soils can provide a historical record of bacterial communities, samples from the Broadbalk soil archive dating back to 1868 were investigated and plots treated with either farmyard manure (FYM) or inorganic fertilizer (NPK) were compared. As anticipated, the processes of air-drying and milling greatly reduced bacterial viability whilst DNA yields declined less and may be preserved by desiccation. A higher proportion of culturable bacteria survived the archiving process in the FYM soil, possibly protected by the increased soil organic matter. The majority of surviving bacteria were firmicutes, whether collected in 2003 or in 1914, but a wide range of genera was detected in DNA extracted from the samples using PCR and DGGE of 16S rRNA genes. Analysis of DGGE band profiles indicated that the two plots maintained divergent populations. Sequence analysis of bands excised from DGGE gels, from a sample collected in 1914, revealed DNA from α - and β -proteobacteria as well as firmicutes. PCR using primers specific for ammonia oxidizing bacteria showed similar band profiles across the two treatments in recently collected samples, however older samples from the NPK plot showed greater divergence. Primers specific for the genus *Pseudomonas* were designed and used in real-time quantitative PCR to indicate that archived soil collected in 1868 contained 10-fold less pseudomonad DNA than fresh soil, representing around 10^5 genomes g^{-1} soil. Prior to milling, dramatically less pseudomonad DNA was extracted from recently collected air-dried soil from the NPK compared to the FYM plot; otherwise, the two plots followed similar trends. Overall bacterial abundance, diversity and survival during the archiving process differed in the two soils, possibly due to differences in clay and soil organic matter content. Nevertheless, the results demonstrate that air-dried soils can protect microbial DNA for more than 150 years and offer an invaluable resource for future research.

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1. Introduction

Agricultural inputs to soil are assumed to affect soil microbial populations but the large numbers of bacteria in temperate regions, estimated at 10^{10} bacterial cells and up to 10^4 species per cm^3 soil (Torsvik et al., 2002) together

with the heterogeneity of the soil environment makes meaningful comparisons difficult. Biogeographic studies indicate that soil pH is the most important factor predicting bacterial population diversity, but other soil properties including parent material, texture, moisture deficit, % organic C, C:N ratio, vegetation and land management are also implicated (Fierer and Jackson, 2006; Ulrich and Becker, 2006; Cookson et al., 2007). Soil microbial populations vary spatially at a range of scales and

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temporally (Horner-Devine et al., 2004) with seasonal changes influencing bacterial growth directly (e.g. soil temperature; Cookson et al., 2007) and indirectly (plant growth stage, fertilizer additions, Wieland et al., 2001; Green et al., 2006). In similar types of soil with contrasting treatments, major impacts on the soil biota may only become apparent when treatments are continued over a long period.

The Broadbalk winter wheat experiment at Rothamsted Research was initiated in 1843 to investigate the impact of different fertilizer combinations on wheat, and has continued since that time with appropriate modifications reflecting contemporary agricultural practices such as the introduction of short-straw wheat cultivars in 1968 and the use of crop rotations in some sections (Poulton, 2006). Samples of grain and straw have been retained in an archive from most treatments and years; soil samples have also been archived less frequently but still constitute an extensive archive. Annual crop yield data, together with some soil analyses, have been collated since the field trials began. This provides a unique record of an arable agricultural soil from the pre- to post-industrial age and analysis of stored soil samples has been used to monitor, for example, atmospheric deposition of N (Goulding et al., 1998), S (Zhao et al., 2003), polycyclic aromatic hydrocarbons (PAHs) (Jones et al., 1989) and polychlorinated biphenyls (PCBs) (Alcock et al., 1993). Similarly, stored wheat samples have provided evidence of population shifts in wheat pathogenic fungi over 160 years (Bearehell et al., 2005). The experiment was designed before the development of modern statistical methods and concepts of experimental design and so it is not replicated, but nonetheless it has proved a valuable resource for many investigations (Rasmussen et al., 1998; Tilman, 1998).

The Rothamsted Sample Archive, the oldest in the world, contains >300,000 crop and soil samples taken from Broadbalk and other long-term “Classical” field experiments set up to look at crop yields in relation to inorganic and organic fertilizer inputs. Storage of soil samples appears to have resulted in some increase in exchangeable cations but not to significant changes in the C and N content (Blake et al., 2000). The process of archiving involves air-drying and milling soils which are then stored in sealed jars at ambient temperature. Each of these steps may result in loss of bacterial viability and cellular integrity with concomitant loss of genetic material, although bacteria may possess one or more mechanisms that provide tolerance to desiccation, including spore and cyst formation (Moreno et al., 1986; Roszak and Colwell, 1987; Potts, 1994) enabling some groups to survive long periods in dry soil. Recently, De Nobili et al. (2006) described the recovery of activity by the microbial biomass on re-wetting air-dried Broadbalk soil stored for 2 years. The ATP levels (indicating microbial activity) in dried soils were less than half of those measured in the same samples when freshly collected, indicating a loss of viability. The ability of soil ATP levels to recover on re-wetting

deteriorated over time during sample storage. Fresh and archived soils from plots in another experiment receiving farmyard manure (FYM), resulting in a high organic matter content, had increased microbial biomass compared to plots receiving inorganic N, P and K fertilizer (NPK). For both treatments, the drop in biomass ATP in stored samples, as a proportion of that in fresh samples, was similar when measured 2 d after re-wetting but the FYM samples regained much greater activity, almost to that of fresh soil, at 12 d (De Nobili et al., 2006). This may indicate qualitative differences in the microbial populations and/or the C substrates available for microbial growth in the two treatments. To date, soil from the Rothamsted Archive has not been exploited to study microbial population diversity.

The purpose of this preliminary study was to establish the potential of the Rothamsted Archive as a resource of material for studying soil bacteria and to investigate the effect of soil organic matter on bacterial community diversity and dynamics over a 150-year period. It included investigation of the survival of bacterial cells and DNA during the archiving process and over time in two soils with different long-term fertilizer treatments using culturing and culture-independent methods. Additionally, we specifically investigated the survival of DNA from the genus *Pseudomonas* (considered important in plant health) as we had previously found dramatic reductions in culturable cells in surface soils during periods of drought. We also investigated a group that is very difficult to culture, the ammonia oxidizing bacteria, which play a key role in nitrification. Two plots were chosen for this study that had comparable wheat yields over the entire length of the 160-year field trial but which had contrasting fertilizer treatments, inorganic NPK and organic FYM since 1852. Yields on both treatments dramatically increased with the introduction of modern cultivars and fungicides in 1968 (Rasmussen et al., 1998). In the FYM plots, soil organic matter has increased markedly since 1843 whilst remaining relatively static in the NPK plots, resulting in differences in soil structure and composition (Watts et al., 2006), but there has been little information concerning impacts on the soil microbial communities in the two treatments.

2. Materials and methods

2.1. Choice of long-term plots

Grain yield data taken from the Broadbalk continuous wheat field trial were assumed to be a proxy measurement of plant growth and hence of nutrient inputs to soil that might influence soil and rhizosphere bacteria. To assess the long-term effects of fertilizer treatment, wheat grain yields from plots receiving no fertilizer, FYM, or a range of N inputs with P and K were compared using data from the electronic Rothamsted Archive (e-RA, <http://www.rothamsted.bbsrc.ac.uk/eRA/index.php>). From this information (data not shown), the NPK plot 081 that receives 144 kg N, 35 kg P and 90 kg K ha⁻¹ y⁻¹ and plot 221 treated

with FYM at $35 \text{ t ha}^{-1} \text{ y}^{-1}$ showed the greatest similarity in yields over the 150 years of the field trial, consistent with the analysis made by Poulton (2006), and were chosen for sampling.

2.2. Soil sampling, milling and storage

The standard procedure for archiving soil samples at Rothamsted involves sieving field-collected cores through a 5 mm mesh, air-drying at ambient temperature then milling to $<2 \text{ mm}$ and storing in sealed jars at ambient temperature in the Rothamsted Archive. Fresh soil samples were collected in June 2003 from NPK plot 081 or FYM plot 221. The soil is a flinty clay loam over chalk, Batcome series, with ca. 33% clay, 1.13% soil organic C, pH 7.30 in plot 081 and ca. 23% clay, 2.83% soil organic C, pH 7.82 in plot 221 (data collected autumn 2000, Paul Poulton, personal communication). Ten soil cores were taken on a “W” transect across each plot, pooled, sieved through a 2 mm mesh and thoroughly mixed then divided into four parts. These were either: frozen at -80°C within 1 h of sampling for subsequent DNA extraction; stored overnight at 4°C for analyses of culturable bacteria; air-dried and stored at ambient temperature; or air-dried and milled to $<2 \text{ mm}$ to replicate the archiving process. Previous investigation had revealed that there was no significant increase in colony counts when fresh field soil samples were stored overnight at 4°C . Soil samples from the same plots, dated 1868, 1893, 1914, 1944 and 2002, were taken from the archive for comparison. The historical samples were chosen because relatively large amounts of soil were collected and archived in those years. Soil-handling equipment (augers, sieves, spatulas, etc.) were sterilised before use and where soil was handled, sterile gloves were used and sterile technique observed although such precautions had not been undertaken during the archiving of historical soil samples prior to 2002. However, the standard practice for removing samples from the archive avoids any direct handling of the soil and makes it unlikely that previous adventitious contamination could introduce culturable bacteria from contemporary sources after the drying and milling steps.

As the treatments in the Broadbalk experiment are not replicated, three pseudoreplicates from each mixed soil sample were taken for all subsequent analyses, to account for within-sample heterogeneity. The moisture content of all soil samples was estimated from sub-samples oven-dried at 80°C for 3 d.

2.3. Survival of culturable bacteria

Selective media were used to determine particular culturable bacterial populations in samples taken in 2003 (fresh, air dried or air dried/milled) and archived soils (dried/milled) from 1868, 1914, 1944 and 2002. For each soil sample, three 1 g samples were resuspended in 10 ml phosphate buffered saline (PBS) pH 7.0, shaken vigorously

for 7 min at 4°C then serially diluted in PBS to 10^{-6} . Dilutions were plated in triplicate and incubated at 28°C , colonies were counted daily for 5 d and colony forming units per gram oven-dried soil (CFU g soil^{-1}) estimated. Media used were: MCA (MacConkey agar; Oxoid CM7) to select enterobacteria and other Gram-negative bacteria; PSA (*Pseudomonas* selective agar; Oxoid *Pseudomonas* agar base CM559 with C-F-C supplement SR0103) for *Pseudomonas* spp. and related pseudomonads; and TSA (1/10th tryptic soya agar; Oxoid tryptic soya broth CM129 solidified with 15 g l^{-1} agar and 100 mg l^{-1} cycloheximide) for copiotrophic bacteria. Heat-resistant spore-forming copiotrophic bacteria were selected by incubating soil dilutions at 80°C for 30 min prior to inoculating TSA plates and incubation at 28°C .

To assess the effect of rehydrating archived soil on bacterial viability and CFU, 1 g samples were resuspended in 10 ml PBS and incubated overnight at 4°C before shaking, diluting and plating as described above.

2.4. Soil DNA extraction and purification

Soil DNA was extracted from archived soils dated 1868, 1893, 1914, 1944 and 2002 and from samples collected and processed in 2003 using an adapted protocol for the UltracleanTM soil DNA isolation kit (MO BIO Laboratories, Inc.) including bead beating at 5 ms^{-1} for 30 s (FastPrep[®] Instrument). Prior to DNA extraction, the average wet weight of archived and fresh samples was determined (2.4% and 20.7%, respectively) and the water content for archived soils was adjusted to 20% by adding sterile PCR-grade water and incubating at 4°C for 20 min prior to DNA extraction. This rehydration step was found to improve both the quality and quantity of DNA extracted from archived soils (data not shown). DNA was extracted from three individual 0.25 g soil subsamples per treatment, utilizing the alternative UltracleanTM protocol (for maximum yields) as described by the manufacturer, with the addition of three washes of 300 μl S4 solution. Aliquots of the eluted DNA solution were stored at -80°C as prior experience had shown that soil-extracted DNA samples stored at -20°C would degrade over time. Serial dilutions of the extracts were prepared and the efficiency of PCR amplification and the quality of denaturing gradient gel electrophoresis (DGGE) profiles were tested at each dilution.

2.5. Diversity of 16S rRNA genes in soil samples

The bacterial communities represented in the soil-extracted DNA were assessed by profiling genes encoding 16S rRNA, using PCR amplification followed by DGGE (Muyzer et al., 1993). For improved DGGE band profiles, a nested PCR approach was used, initially with the universal eubacterial primers fd1, rd1 (Weisburg et al., 1991) followed by primer 2 (MuyR) and the GC clamped primer 3 (GCMuyF) for V3 variable region which corresponds to

position 341 to position 534 in the 16S rRNA gene of *Escherichia coli* (Muyzer et al., 1993). The first round PCR consisted of 1 µl soil DNA (at 100-fold dilution) added to 20 µl PCR mix containing 1 × Roche Taq PCR buffer, 0.2 µM each fD1 and rD1 primer, 200 µM each dNTP, 2 mM MgCl₂ (final concentration) and 0.08 µl (0.4 U) Roche Taq polymerase. All reactions were kept on ice during set up then added to a 94 °C preheated PCR block. PCR amplification was performed using 94 °C 30 s denaturing, 55 °C 30 s annealing and 72 °C 2 min extension for 20 cycles with a final extension at 72 °C for 20 min. Second round nested PCR was as above but 1 µl first round PCR product was used with the primers MuyR and GCMuyF and denaturing and annealing times were reduced to 15 s with the annealing temperature 53 °C, extension time was 30 s with a final extension of 30 min. Successful amplification was assessed on a 1.5% (w/v) agarose gel stained with ethidium bromide and visualised on a UV light transilluminator. PCR reactions were performed in triplicate and pooled for each soil DNA extraction. Nested PCR reactions (8 µl) were run on DGGE gels (8% w/v, acrylamide/bis-acrylamide with a denaturing gradient of 30–55% where 100% denaturant is defined as 7 M urea and 40% v/v, formamide) at 60 °C, 1 × TAE buffer at 200 V for 5 h. Gels were stained for 15 min with 10,000 dilution of Sybr[®] Gold and visualised with UV light as above. Band profiles were compared using TotalLab software (Nonlinear dynamics, Newcastle upon Tyne, England). Bands of interest for sequencing were cut out from gels, reamplified with MuyR and GCMuyF primers and cloned into pCR[®] 2.1-TOPO[®] (TOPO TA Cloning[®] Kit, Invitrogen[™]).

2.6. Diversity of cultured bacteria

DNA was extracted from individual bacterial colonies on TSA plates from 1914, 1944 and 2003 archived and fresh soil samples using a rapid DNA extraction method (Klimyuk et al., 1993). In essence, bacterial colonies were resuspended in 40 µl 0.25 M NaOH, heated to 95 °C for 30 s, neutralised with 40 µl 0.25 M HCl and 20 µl 0.5 M Tris-HCl pH 8.0 containing 0.25% (w/v) Nonidet P-40 and heated 95 °C for 2 min. This crude DNA solution (1 µl undiluted or diluted 10-fold in SDW) was sufficient for amplifying 16S rRNA genes with MuyR and MuyF primers as described above. PCR products were cloned into pCR[®] 2.1-TOPO[®] (TOPO TA Cloning[®] Kit, Invitrogen[™]) prior to sequencing for identification.

These DNA extracts were also fingerprinted using ERIC primers as described by Versalovic et al. (1991). The rationale for this was to establish if the culturable population was diverse, or dominated by only a few types with very similar ERIC profiles. PCR amplification was performed in a final volume of 25 µl using 50 pM of each primer (ERIC2: 5'-AAG TAA GTG ACT GGG GTG AGC G-3' and R1CIRE: 5'-CAC TTA GGG GTC CTC GAA TGT A-3'), 2.5 mM MgCl₂, 200 µM each dNTP and

0.1 µl (0.5 U) Roche Taq polymerase. PCR cycle conditions were 95 °C hotstart followed by 30 cycles of 94, 42 and 72 °C each for 1 min with a final extension at 72 °C for 10 min. ERIC profiles were visualised on 1.5% agarose gels and converted into binary data using TotalLab as described above, then analysed using FreeTree (<http://www.natur.cuni.cz/~flegel/freetree.php>; Hampl et al., 2001) creating a similarity matrix using Jaccard's coefficient.

2.7. Phylogenetic analysis of 16S rRNA genes

The phylogenetic affiliations of 16S rRNA gene fragment sequences cloned from selected DGGE bands and cultured bacteria were performed using the maximum likelihood program DNAML (<http://evolution.gs.washington.edu/phyliip.html>; Felsenstein, 2004) with 1000 bootstraps and a transition/transversion parameter of 0.98 as determined using PUZZLE (Strimmer and von Haeseler, 1996) and the resulting tree was viewed in TreeView. Reference sequences from groups identified in a preliminary screen (BLAST) related to the cloned fragments were obtained from the GenBank[®] database via the National Centre for Biotechnology Information (<http://www.ncbi.nlm.nih.gov/>).

2.8. Survival of bacterial DNA during the archiving process and storage

To develop primers that would specifically amplify *Pseudomonas* spp., and allow quantification of DNA from this group within the soil samples, 16S rRNA gene sequences (>1200 bp in length) were obtained from the Ribosomal Database Project II (RDPII, <http://rdp.cme.msu.edu/>; Cole et al., 2003) and the NCBI website (<http://www.ncbi.nlm.nih.gov/>) covering a wide range of bacterial species within the phylum *Proteobacteria* including multiple pseudomonad sequences. Multiple sequence alignment was performed using ClustalW within the Bioedit program (Hall, 1999) and regions potentially specific for the detection of pseudomonads identified. Oligonucleotides designed for these regions were assessed for their specificity by checking them against the RDP II using the probe matching facility (Table 1) and PseuF1 and PseuR1 were chosen as the best primer set giving a PCR product of 249 bp. The primers were 100% identical to the corresponding region of the 16S sequences of a few strains not classified in the RDPII as the genus *Pseudomonas*; however, the genera *Flavimonas* and *Chryseomonas* are now considered synonyms of *Pseudomonas* (Anzai et al., 1997). PCR conditions were optimised on a thermal gradient thermocycler and tested against a range of diverse bacterial isolates including 37 cultured bacteria collected from all soil samples which included firmicutes, actinobacteria, bacteriodes and γ-proteobacteria (incorporating five *Pseudomonas* spp. cultured from fresh FYM and NPK soil). In addition, the primers were tested against culture collection strains including: firmicutes (*Enterococcus fae-*

Table 1
Pseudomonas genus-specific primer design

| PseuF1 reverse complement 5'-TGATAGCGCAAGGCCCGAAG-3' | | Hits | Total | | | Hits | Total | |
|--|---------------------|------|---------|---|--------------------------|--------------------------|-------|------|
| Domain | Bacteria | 2275 | 107,136 | | | | | |
| Class | Gammaproteobacteria | 2275 | 17,587 | | | | | |
| Order | Chromatiales | 0 | 469 | } | Family | Pseudomonadaceae | 2274 | 3041 |
| | Acidithiobacillales | 0 | 230 | | Genus | Pseudomonas | 2244 | 2939 |
| | Xanthomonadales | 0 | 1082 | | | Azomonas | 0 | 0 |
| | Cardiobacteriales | 0 | 14 | | | Azotobacter | 0 | 0 |
| | Thiotrichales | 0 | 303 | | | Cellvibrio | 0 | 20 |
| | Legionellales | 0 | 210 | | | Chryseomonas | 0 | 6 |
| | Methylococcales | 0 | 183 | | | Flavimonas | 6 | 10 |
| | Oceanospirillales | 0 | 1176 | | | Mesophilobacter | 0 | 0 |
| | Pseudomonadales | 2274 | 3935 | | | Rhizobacter | 0 | 0 |
| | Alteromonadales | 0 | 2072 | | | Rugamonas | 0 | 0 |
| | Vibrionales | 0 | 1172 | | | Serpens | 0 | 0 |
| | Aeromonadales | 0 | 929 | | | Unclassified pseudomonas | 24 | 66 |
| | Enterobacteriales | 0 | 3619 | | Family | Moraxellaceae | 0 | 863 |
| | Pasteurellales | 0 | 1106 | | Family | Incertae sedis | 0 | 28 |
| | Salinisphaerales | 0 | 8 | | Unclassified pseudomonas | | 0 | 3 |
| Unclassified gammaproteobacteria | | 24 | 1079 | | | | | |
| PseuR1 reverse complement 5'-GCCCTTCCTCCCAACTTAA-3' | | Hits | Total | | | Hits | Total | |
| Domain | Bacteria | 2314 | 107,136 | | | | | |
| Class | Gammaproteobacteria | 2314 | 17,587 | | | | | |
| Order | Chromatiales | 0 | 469 | } | Family | Pseudomonadaceae | 2314 | 3041 |
| | Acidithiobacillales | 0 | 230 | | Genus | Pseudomonas | 2269 | 2939 |
| | Xanthomonadales | 2 | 1082 | | | Azomonas | 0 | 0 |
| | Cardiobacteriales | 0 | 14 | | | Azotobacter | 0 | 0 |
| | Thiotrichales | 0 | 303 | | | Cellvibrio | 0 | 20 |
| | Legionellales | 0 | 210 | | | Chryseomonas | 1 | 6 |
| | Methylococcales | 0 | 183 | | | Flavimonas | 5 | 10 |
| | Oceanospirillales | 1 | 1176 | | | Mesophilobacter | 0 | 0 |
| | Pseudomonadales | 2314 | 3935 | | | Rhizobacter | 0 | 0 |
| | Alteromonadales | 0 | 2072 | | | Rugamonas | 0 | 0 |
| | Vibrionales | 0 | 1172 | | | Serpens | 0 | 0 |
| | Aeromonadales | 0 | 929 | | | Unclassified pseudomonas | 39 | 66 |
| | Enterobacteriales | 0 | 3619 | | Family | Moraxellaceae | 0 | 863 |
| | Pasteurellales | 0 | 1106 | | Family | Incertae sedis | 6 | 28 |
| | Salinisphaerales | 0 | 8 | | Unclassified pseudomonas | | 0 | 3 |
| Unclassified gammaproteobacteria | | 0 | 1079 | | | | | |

Primers designed to be specific for the genus *Pseudomonas* were compared using “probe matching” with all 16S rRNA gene sequences longer than 1200 bp and defined as good quality according to Seqmatch and Pintail held on the RDPII database. Positive results are those with 100% identity over the full length of the primers: all were accessions classified as *Pseudomonas* or synonymous genera. The reverse complement of the forward primer PseuF1 (5'-CTTCGGGCCTTGCGCTATCA-3') was used for probe matching.

calis NCTC 12201 and *E. faecium* NCTC 12202); α -proteobacteria (*Rhizobium leguminosarum* RSM2004); β -proteobacteria (uncultured *Nitrospira* 16S-5 16S rRNA gene clone AJ238202) and γ -proteobacteria (*E. coli* JM109, *Serratia* sp. RCA1, *Pseudomonas* sp. RSM4002, *Pseudomonas fluorescens* SBW25). Positive PCR products derived from PseuF1 and PseuR1 primers were only detected from DNA extracted from pseudomonad isolates. Quantitative PCR was performed using an ABI 7700 Sequence Detection System (PE Applied Biosystems), with SYBR[®] Green for detection and primer concentrations were optimised at 300 nM. Reactions contained 1 μ l soil DNA (at 100-fold dilution) in a total volume of 25 μ l with SYBR[®] Green Jumpstart[™] Taq ReadyMix[™] (Sigma) and 60 nM ROX dye. PCR amplifications were performed in triplicate for all standards and soil samples using 40 cycles with 94 °C for 15 s denaturing, 63 °C for 15 s annealing and 72 °C for 30 s extension. Standard curves were generated using genomic DNA from *P. fluorescens* isolate RSM4002 ranging from 0.1 to 100,000 genome equivalents and gave a correlation coefficient of $R^2 = 0.9929$, and standard trend line $y = -1.4017 \ln(x) + 12.56$. Spiking of soil samples with DNA standards derived from *P. fluorescens* isolate RSM4002 genomic DNA showed no inhibition of PCR.

To detect DNA in fresh and archived soil samples from ammonia oxidizing bacteria (selected as an example of a group that is considered very difficult to grow in laboratory culture), specific 16S gene primers were used (Kowalchuk et al., 1997, 1998). These were: CTO189f-GC (CCGCCGCGCGGCGGGCGGGGCGGGGACGGGGGGAGRAAAGYAGGGGATCG) and CTO654r (CTAGCYTTGYAGTTTCAAACGC). PCRs were performed in a final volume of 20 μ l using 5 pM of each, 2.5 mM MgCl₂ and 0.08 μ l (0.4 U) Roche Taq polymerase with 0.5 μ l soil DNA. PCR cycle conditions were a 95 °C hot start followed by 35 cycles of 95 °C for 30 s, 63 °C for 15 s and 72 °C for 1 min with a final extension of 72 °C for 10 min. PCR products were run on DGGE gels, as described above.

3. Results

3.1. Soil properties

The Broadbalk data archive showed that the soil N and organic C content of the FYM plot had both continued to increase since the start of the experiment and organic C was, at 2.83%, more than twice that of the NPK plot (1.13%), which had remained relatively stable since 1843. The moisture content of all archived soil samples was, on average, 2.4% ($\pm 0.27\%$). For the fresh FYM soil moisture content was 23.5% but for the fresh NPK soil it was lower, 17.9%. This may reflect the higher soil organic matter content (directly proportional to the soil organic C) in the FYM compared to NPK soil, although there was no significant difference in moisture content in the archived samples.

3.2. Survival of culturable bacteria

The addition of an overnight rehydration step for soil samples (1868, 1944, 2002 archived soils and air dried and dried/milled soils from 2003) before shaking, diluting and plating out made no significant difference to the average number of CFU obtained so this step was omitted. There was a tendency, most apparent on TSA, for more CFU from both fresh and archived FYM soils, compared to NPK (Fig. 1) consistent with the higher microbial biomass reported in the FYM soil (Brookes, 2001). In freshly sampled soil, the heat-resistant spore-formers appeared to comprise approximately 10% of the total copiotroph population selected on TSA, with the number of CFU similar to those selected on PSA or MCA (Fig. 1). In general, the drying and milling process of archiving soil samples resulted in a reduction in the culturable counts of bacteria compared to those observed in fresh soil immediately after sampling. This was particularly noticeable for the populations selected on PSA and MCA, where CFU dropped more than 10-fold although numbers appeared to recover somewhat after milling for both soils on MCA and for NPK on PSA. No culturable bacteria were detected in archived soils when plated on MCA. The number of CFU on PSA were greatly reduced, with 2–3 orders of magnitude fewer in the 2002 soil compared with the numbers found in the 2003 archived sample; otherwise, only the FYM 1914 samples yielded a few colonies (three colonies on one plate). In contrast, for both heat-resistant spore-formers and the total copiotroph population selected on TSA, the maximum reduction observed was a 10-fold drop in the 1914 NPK soil compared with the other archived and fresh soil samples.

3.3. Diversity of bacteria in soil and culture

There were obvious differences in banding patterns observed from 16S DGGE, using universal eubacterial primers, between fresh soils from the two sites, samples from the same site during the archiving process, and in samples from different years (Fig. 2). The numerous bands of similar intensities in the fresh and recently archived samples indicate that many species that are represented with similar abundance. The apparently brighter and clearer bands in the DGGE gels of 1914 and 1944 samples may indicate a reduction in the overall number and diversity of intact 16S templates present, or a change in the relative abundance of different types due to better survival of particular groups. The problems in interpreting DGGE results, where the number of bands may depend on the evenness with which types occur in a community and thus provide a biased estimate of diversity and abundance, have been discussed at length in the literature (Forney et al., 2004).

Principal component analysis of the diversity of bacteria based on these banding patterns showed that FYM and NPK soils were separated by the first axis (which, however,

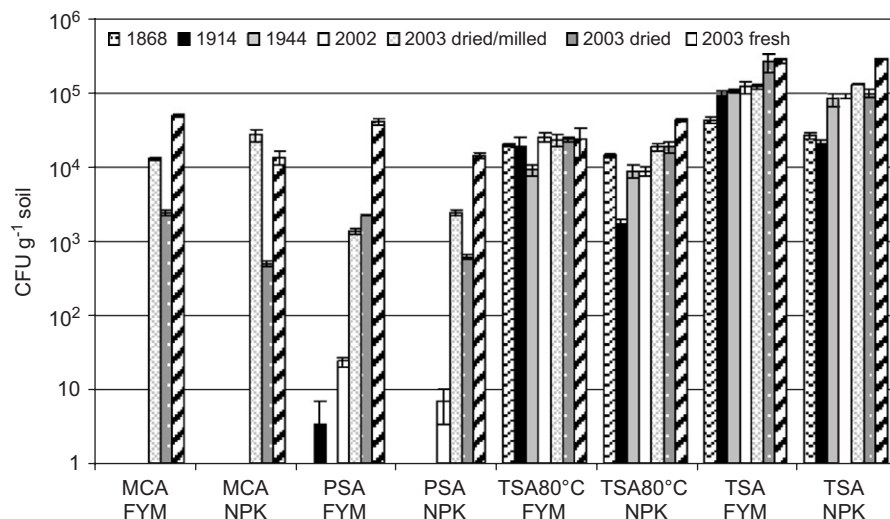


Fig. 1. Survival of culturable bacteria in fresh, air-dried and archived soil samples dating back to 1868, plated on different media. FYM, plot receiving farmyard manure; NPK, NPK fertilizer; TSA, 1/10th tryptone soy agar; TSA 80°C, samples heated at 80°C prior to plating on 1/10th TSA; PSA, *Pseudomonas* selective agar; MCA, McConkey agar. Standard errors of the mean are indicated.

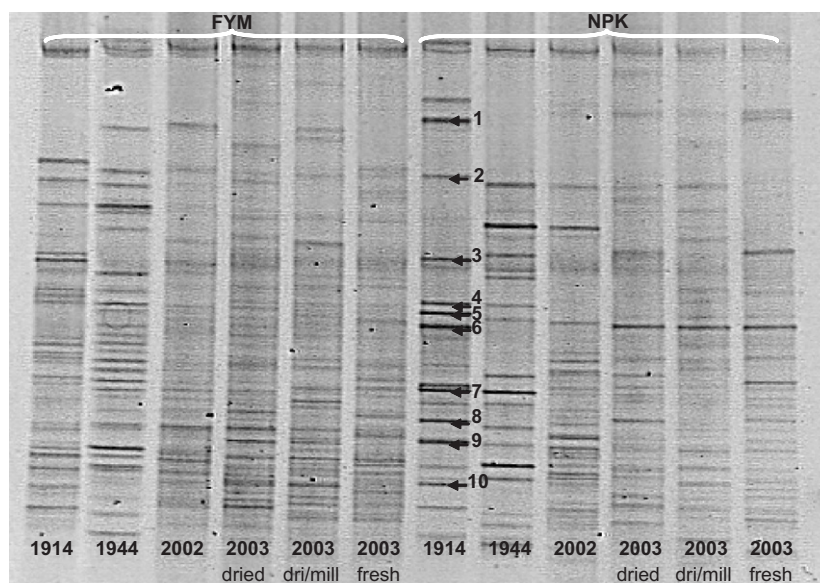


Fig. 2. DGGE profiles of fresh and archived soil samples dating back to 1914. PCR products were amplified using universal eubacterial 16S rRNA gene primers. FYM, plot receiving farmyard manure; NPK, NPK fertilizer. For soil collected in 2003, DNA was extracted from samples that were fresh, air-dried or air-dried and milled (dri-mill) before archiving.

accounted for less than 18% of the difference) and that the NPK soils were more similar to each other than were the FYM soils, where the archived samples grouped separately (Fig. 3).

The 10 most prominent bands were excised from the 1914 NPK lane on the DGGE gel (indicated in Fig. 2), and the DNA cloned and sequenced. Three of the bands contained more than one different sequence. Of the sequences analysed, three showed identity to α -proteobacteria, two to β -proteobacteria, one to the verrucomicrobia and nine to firmicutes including *Bacillus* and *Paenibacillus* (Fig. 4). Representative colonies picked from the TSA culture plates

from the fresh 2003, 1944 and 1914 FYM and NPK samples were sequenced for comparison, and of these 27 grouped with other firmicutes, two with actinobacteria, one with bacterioidetes and seven with γ -proteobacteria including five from fresh soil that were similar to *Pseudomonas* and one from 1944 NPK soil that was similar to *E. coli* (Fig. 4). The ERIC profiles (10–15 isolates per treatment) of these bacteria isolated on TSA showed no grouping according to year (1914, 1944 or 2003), plot (FYM or NPK) or if they were fresh or archived (data not shown).

DNA fingerprinting using ERIC or other arbitrary primers can identify bacterial isolates with identical profiles

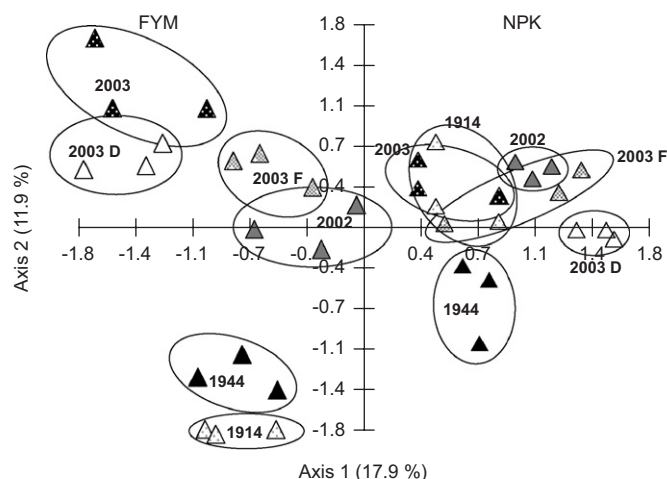


Fig. 3. Principal component analysis of bacterial DNA diversity in fresh and archived soil samples dating back to 1914 assessed by 16S DGGE. FYM, plot receiving farmyard manure; NPK, NPK fertilizer. For soil collected in 2003, DNA was extracted from samples that were fresh (F), air-dried (D) or air-dried and milled before archiving.

that are probably very closely related but it is contentious to make assumptions about the relationships between isolates with similar but not identical profiles. Here, ERIC fingerprinting of cultured isolates was to determine whether archived soils contained diverse culturable bacteria, or were dominated by only one or two types. The former was demonstrated, as the majority of isolates had different ERIC profiles, even when they had identical 16S rRNA sequences, and no profile group had more than two representatives. Pairs of isolates with identical profiles were obtained from, for example, archived samples from 1914 FYM and 2003 NPK. These had almost identical 16S sequences, similar to *Paenibacillus* spp. In contrast, another pair of isolates with apparently identical ERIC profiles from fresh and archived 2003 NPK soil had different 16S sequences, one similar to *Paenibacillus amolyticus* and the other to *Sporosarcina macmurdoensis* (Fig. 4), highlighting the limitation of DNA fingerprint analyses for assessing community diversity.

3.4. Survival of bacterial DNA during the archiving process and over time

The viability of pseudomonads defined by their ability to grow on PSA was greatly reduced in archived soils (Fig. 1) but quantitative PCR on DNA extracted from these samples and then amplified with 16S rRNA gene primers specific for the genus *Pseudomonas* indicated that DNA was present in all samples tested (Fig. 5). In fresh soil, qPCR estimated 10^6 pseudomonad genome equivalents g^{-1} soil, approximately 100-fold greater than the culturable population on PSA. This fell to 10^4 – 10^5 genome equivalents in the 2002 archived samples, 3–4 orders of magnitude greater than the culturable population estimated by CFU and 10^4 and 10^5 genome equivalents were detected in the 1944 and 1868 soils, respectively, where no culturable

bacteria had been obtained on PSA. In the oldest samples tested, dating from the 19th century, *Pseudomonas* DNA could be detected at levels 10-fold lower than those in the 2003 archived sample.

In the archived samples, there were on average three-fold more *Pseudomonas* genome equivalents g^{-1} NPK soil compared to FYM in contrast to the fresh soils, where the genome equivalents in the NPK soil were half those in FYM. An apparent decrease in the amount of *Pseudomonas* DNA from 10^6 genome equivalents in fresh NPK soil to 10^2 in the dried soil was reversed after milling, reflecting changes in the number of CFU in the same samples. These showed a 20-fold drop in dried soil from 10^4 CFU g^{-1} fresh soil and a four-fold increase after milling. This may reflect structural differences, with higher organic matter leading to a more friable soil in the FYM plot. The higher proportion of clay in the NPK soil resulted in the formation of small hard aggregates after sieving and drying, which may have rendered any bacteria captured inside inaccessible (despite the bead beating step of the DNA extraction protocol) until milling exposed them to the extraction processes. The higher clay content in the NPK soil could offer more protection to DNA released after milling, although it appears that the higher organic matter in FYM samples may harbour or protect more culturable cells.

The presence of another group of bacteria that are difficult to grow in culture, the ammonia oxidizing bacteria, was investigated using PCR with primers specific for this group. DGGE revealed multiple bands and a similar pattern in the 2003 fresh and archived (dried and milled samples) for both FYM and NPK soils although older samples in the NPK soils showed more differences in banding patterns than FYM soils (Fig. 6).

4. Discussion

4.1. Survival of culturable bacteria

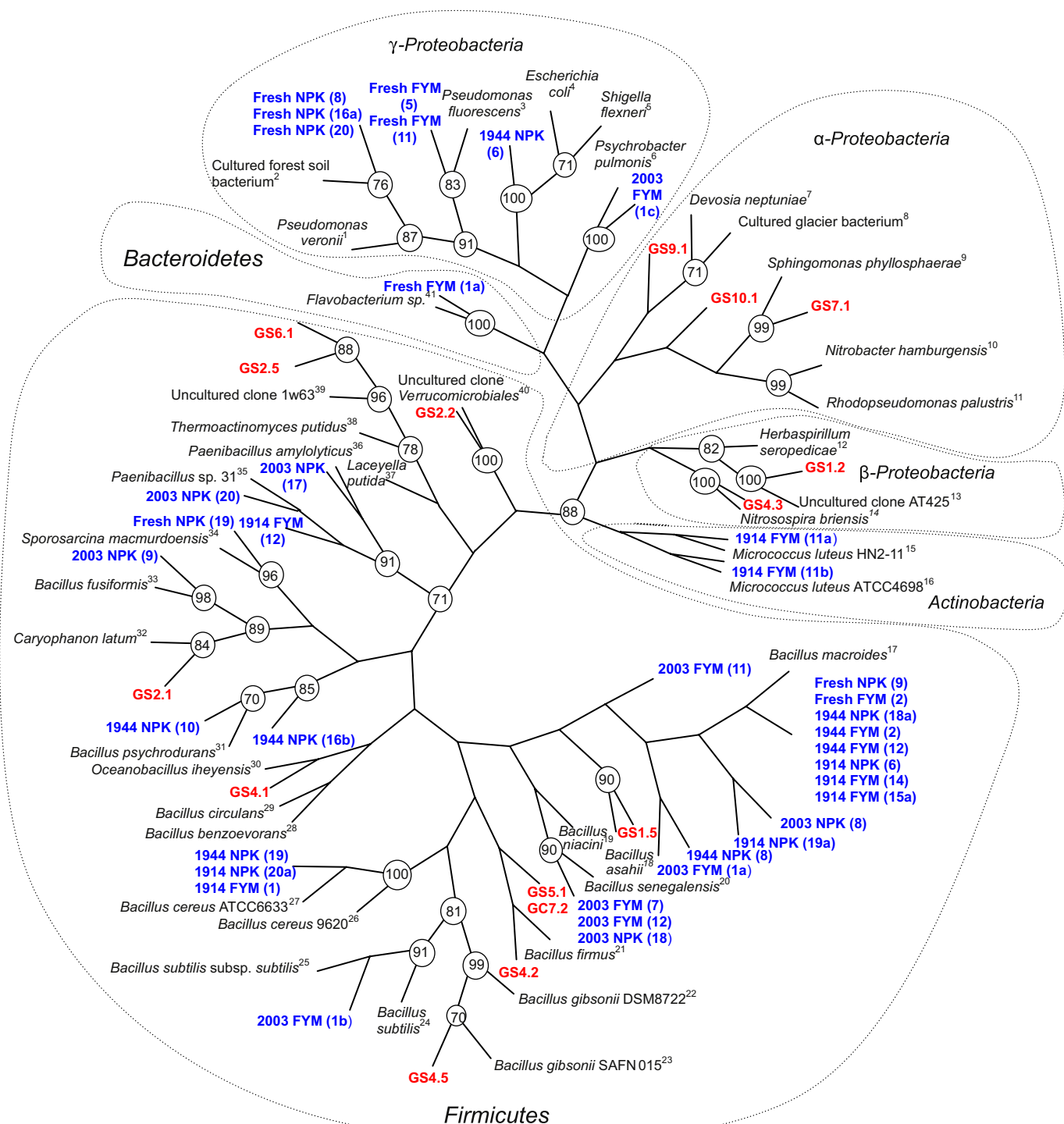
It is unsurprising that the majority of culturable bacteria surviving air-drying and storage were spore-forming firmicutes, since spores are adapted for long-term survival in adverse, dry, conditions. Although heat treatment of soil suspensions prior to plating on TSA resulted in a 10-fold drop in the number of CFU compared to untreated samples, 73% (29/37) of the colonies from the latter had 16S sequences similar to firmicutes, the majority from the well known spore-forming soil bacterial genera *Bacillus* and *Paenibacillus*. Not all spores are heat resistant, and the treatment adds an extra stress that may reduce survival, so it is likely that spore-forming Firmicutes dominate the culturable population when archived soils are resuspended and plated, with or without heat selection. In contrast, the proportion of firmicutes recovered from fresh soil (3/9) was lower than that of pseudomonads on TSA (5/9 colonies sequenced). Plate counts on PSA indicate that few

Pseudomonas survive the air-drying process that is the first step in archiving soil. However, the *Pseudomonas* population estimated from plate counts, at 10^4 – 10^5 CFU g^{-1} fresh soil, is 100-fold less than the estimate of *Pseudomonas* genome-equivalents estimated by qPCR, indicating that in fresh soil only 1% of cells of this genus that are present can form colonies on PSA selective agar. This illustrates the limitations of culture-based investigations of soil microbial communities and reinforces a view

that studies on culturable bacteria are likely to be of limited use when investigating archived soil samples.

4.2. Diversity of bacterial populations

The ability to extract bacterial DNA and investigate community diversity using PCR and DGGE illustrates one potential use of archived soil samples, although it is not possible to be certain that survival of bacterial DNA is



equivalent in different soils. Comparison of DGGE band patterns (Figs. 2 and 3) indicates that different bacterial populations are present in fresh FYM and NPK soil, and that a different complement of bacterial DNA survives over time in the two soil treatments, although the distinction between bands seen on DGGE gels by PCA may not be statistically significant. Nevertheless, it is interesting to note that there is little to separate NPK samples taken at different times whereas there appears to be a shift over time in the microbial community from the older archived samples from the FYM treatments compared to the more recent 2002 and 2003 samples. However, the results should be treated with caution as they could reflect either a change in the organic matter in the FYM plot over time, or differential survival of bacterial DNA, or both: soil organic matter and N have been slowly increasing in the FYM plot but have remained static in the NPK. Dolfing et al. (2004) reported that microbial 16S rRNA gene diversity assessed by DGGE discriminated between plots receiving inorganic fertiliser and manure in archived soils dating back to 1956.

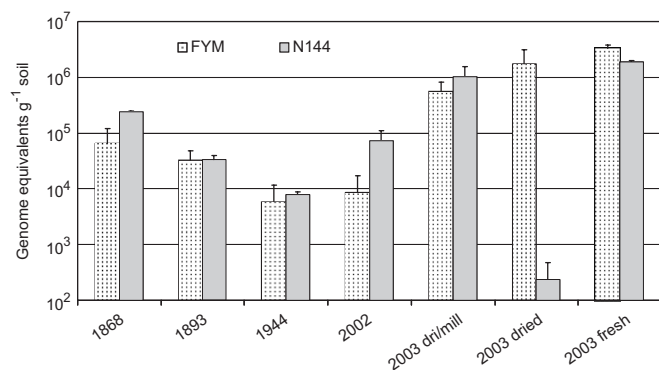


Fig. 5. Estimation of *Pseudomonas* DNA in fresh and archived soil samples dating back to 1868, from quantitative PCR with genus-specific 16S rRNA gene primers. FYM, plot receiving farmyard manure; NPK, NPK fertilizer. Standard errors of the mean are indicated.

4.3. Survival of bacterial DNA during the archiving process and over time

Not all the DNA surviving the archiving process can be attributed to culturable bacterial populations but results do not reveal whether the remainder survived within viable but non-culturable cells, non-viable cells, extracellularly, or a combination of all three. A recent review (Janssen, 2006) of the relative abundance of different phyla in a variety of soil bacterial communities concluded that these are dominated by proteobacteria, which comprise about 50% of 16S rRNA genes in fresh soils. Firmicutes only represented a mean of 2% over a range of these soil types although the difficulties in lysing firmicute cells and spores may have led to under-representation.

The prevalence of firmicute 16S rRNA gene products in the 1914 soil sample contrasts with the consensus findings in fresh soil reported by Janssen (2006) and indicates that genomic DNA from these spore formers may survive better over long periods than DNA from other bacterial phyla. However, the relatively large number of 16S rRNA gene repeats found in *Bacillus* spp. compared with *Pseudomonas* spp. (approximately 10 and 4, respectively, listed on the Ribosomal RNA Operon Copy Number Database (rrndb) website <http://rrndb.cme.msu.edu/>, Klappenbach et al., 2001; Acinas et al., 2004) may also contribute to this finding.

The results reported here indicate that bacterial DNA survives the archiving process much better than culturable bacteria. Nevertheless, qPCR of *Pseudomonas* DNA indicates that it may be subject to some degradation during archiving. It is not clear if the downward trend from 2003, with 10-fold fewer genome equivalents in 2002 and 100-fold fewer in the 1944 NPK soil, represents a loss of DNA or natural fluctuations in the *Pseudomonas* population size as a result of environmental stresses, as the older samples (1893, 1868) indicate larger populations, closer to those in the 2002 archived samples. The slightly higher *Pseudomonas* genome equivalents detected in fresh FYM soil (approximately twice that in the fresh

Fig. 4. Sequence comparison of 16S rRNA genes from bacteria cultured from fresh and archived soil samples dating back to 1914, and bands excised from the 1914 NPK soil-extracted DNA 16S DGGE gel, with known bacterial species in the GenBank database. Cultured bacteria are denoted in bold: “**Fresh NPK (8)**”; gel bands as GS1.2, etc.; known species from GenBank in *italics*. Results are shown as an unrooted tree with bootstrap values greater than 70% indicated. All sequences have been placed on the GenBank database with accession numbers EU273297–EU273348. Cultured isolates: 1914 FYM(12)—EU273297; 1944 NPK(16b)—EU273298; 1914 FYM(1)—EU273299; 1914 FYM(11a)—EU273300; 1914 FYM(11b)—EU273301; 1914 FYM(14)—EU273302; 1914 FYM(19a)—EU273302; 1914 NPK(20a)—EU273304; 1914 NPK(6)—EU273305; 1914 FYM(15a)—EU273306; 1944 FYM(12)—EU273307; 1944 FYM(2)—EU273308; 1944 NPK(10)—EU273309; 1944 NPK(18a)—EU273310; 1944 NPK(19)—EU273311; 1944 NPK(6)—EU273312; 1944 NPK(8)—EU273313; 2003 FYM(11)—EU273314; 2003 FYM(12)—EU273315; 2003 FYM(1a)—EU273316; 2003 FYM(1b)—EU273317; 2003 FYM(1c)—EU273318; 2003 FYM(7)—EU273319; Fresh FYM(11)—EU273320; Fresh FYM(1a)—EU273321; Fresh FYM(2)—EU273322; Fresh FYM(5)—EU273323; 2003 NPK(17)—EU273324; 2003 NPK(18)—EU273325; 2003 NPK(20)—EU273326; 2003 NPK(8)—EU273327; 2003 NPK(9)—EU273328; Fresh NPK(16a)—EU273329; Fresh NPK(19)—EU273330; Fresh NPK(20)—EU273331; Fresh NPK(8)—EU273332; Fresh NPK(9)—EU273333. Gel bands: GS10.1—EU273334; GS1.2—EU273335; GS1.5—EU273336; GS2.1—EU273337; GS2.2—EU273338; GS2.5—EU273339; GS4.1—EU273340; GS4.2—EU273341; GS4.3—EU273342; GS4.5—EU273343; GS5.1—EU273344; GS6.1—EU273345; GS7.1—EU273346; GS7.2—EU273347; GS9.1—EU273348. Reference sequences from the GenBank database used in the construction of the phylogram were: ¹AY144583; ²AY043579; ³AF094725; ⁴U00006; ⁵AE015391; ⁶AJ437696; ⁷AF469072; ⁸AY315165; ⁹AY453855; ¹⁰L35502; ¹¹D89811; ¹²AF164062; ¹³AY053477; ¹⁴AJ298741; ¹⁵AF057289; ¹⁶AF542073; ¹⁷AY167822; ¹⁸AB109209; ¹⁹AY167811; ²⁰AF519468; ²¹D78314; ²²x76446; ²³AY167815; ²⁴AB018486; ²⁵Z99108; ²⁶AF155952; ²⁷Z84581; ²⁸AY167808; ²⁹AY043084; ³⁰AP004600; ³¹AJ277984; ³²x70319; ³³AJ310083; ³⁴AJ514408; ³⁵AY504457; ³⁶D85396; ³⁷AB091323; ³⁸AJ251776; ³⁹AY154558; ⁴⁰AF141391; ⁴¹AF493646.

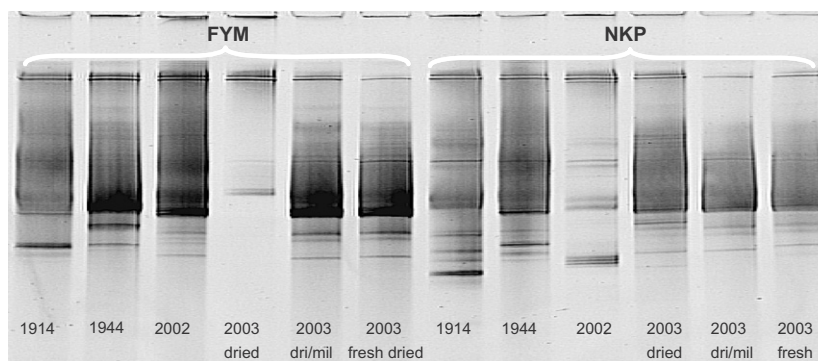


Fig. 6. DGGE profiles of ammonia oxidizing bacterial DNA in fresh and archived soil samples dating back to 1914, assessed by 16S DGGE with group-specific primers. FYM, farmyard manure; NPK, NPK fertilizer. For soil collected in 2003, DNA was extracted from samples that were fresh, air-dried or air-dried and milled (dri-mill) before archiving.

NPK soil) is consistent with the increased CFU on PSA in fresh FYM (approximately three times that in fresh NPK soil) and higher microbial biomass reported previously (Brookes, 2001; De Nobili et al., 2006). In the fresh soil, there was a slightly higher estimate of *Pseudomonas* genome equivalents in the FYM than in the corresponding NPK soil but the trend was reversed in archived samples. DNA survival and/or recovery from *Pseudomonas* cells may be less efficient in FYM soils once archived indicating an effect of soil organic matter but the mechanisms remain unclear. Soil properties such as soil pH can result in differential survival of DNA, but in this case the two Broadbalk plots are maintained at similar, neutral pH. One factor could be the higher proportion of clay, reported to bind and protect extracellular DNA (Levy-Booth et al., 2007) in the NPK soil. In the FYM soil, there is a third less clay, partly due to dilution by the increased organic matter. Differential recovery of DNA might also be due to the irreversible binding of DNA to soil organic matter in the FYM soil. However, different 16S rRNA gene profiles in the two treatments at all time points indicate that the two soils maintain divergent microbial communities. Further research is needed on the nature of any biases due to clay and soil organic matter, and also to establish if the *Pseudomonas* species composition differs between samples and treatments.

The survival of DNA from nitrifying bacteria may also offer the possibility of monitoring populations over time in each of the plots. Although there may be a disparity in DNA survival rates in different treatments, and organic matter and N have accumulated in the FYM plots over time, comparative studies within plots are possible. Previously Mendum and Hirsch (2002) detected differences in the nitrifier populations on Broadbalk plots treated with NPK or FYM using 16S PCR with group-specific gene probes. Band patterns were identical in the samples collected in 2003 on the two plots and analysed by DGGE although the older samples did reveal differences. Whether this represents differential survival of DNA in the two soils, an artefact due to the different analytical methods in

the two studies, or convergence of nitrifier population diversity, remains to be investigated.

4.4. Implications for other investigations

Most culturable bacteria surviving the archiving process were spore-forming firmicutes, which may help to explain the observations of De Nobili et al. (2006), in particular the reduction in biomass ATP content of soils following air-drying, the decrease in the ability of biomass ATP to return to the levels in fresh soil on re-wetting as the age of samples increased, and the differences in this when FYM and NPK soils were compared. Spores have relatively low ATP content but on germination they will regain the levels of other vegetative bacterial cells. The reduction in ATP biomass when soil is dried indicates that spores dominate the surviving propagules, and that other cells lose viability although their DNA may remain in the soil. The FYM soil may develop a larger active biomass after a period of incubation because there is more available C, a larger pool of dead bacteria that remain in the soil and possibly also there are qualitative differences in the firmicute population, although we did not find evidence for this in isolates from the 1914 samples. Many 16S sequences from NPK and FYM soils grouped together.

There are biases inherent in all indirect sampling methods whether based on culture or molecular genetics. It is not possible to extrapolate the results from fresh soil to those from archived soils stored for 140 years but it is reasonable to assume that if appropriate controls are in place, useful data can be collected from soils pre-dating the industrial revolution in the UK. It is clear that although firmicutes dominate both the culturable propagules and extractable DNA in archived samples, substantial amounts of genomic DNA from other bacterial groups remain at a relatively stable level after an initial drop which would, for example, enable comparison of species diversity within defined phyla over time within any one treatment. More uncertainty would be introduced if two treatments that result in substantial changes to soil properties are compared. For example, although the *Pseudomonas* qPCR

estimates in the FYM and NPK soils showed the same trend over time, without knowledge of the initial population size, DNA degradation rates cannot be assumed to be equivalent. To establish this, it will be necessary to compare DNA extraction profiles from archived and frozen soils (kept at -80°C or under liquid nitrogen) from the same sampling date, over extended periods of time, using a range of group-specific and universal primers.

There are other field experiments represented in the Rothamsted Soil Archive, offering potential for studies on, for example, soil microbial community dynamics related to changes in vegetation and soil pH over time. Our preliminary studies show that the same methods can also be applied to archived samples from these experiments. Additionally, there are other soil archives in research institutes around the world, which although started more recently than the Rothamsted Archive, could be used to investigate microbial populations in soils (Dolfing et al., 2004), monitoring, for example, long-term effects of climate change, farming practices, xenobiotic pollution and other environmental influences. Taking into account the uncertainties of differential survival and decay of bacterial cells and DNA in contrasting soils discussed above, it should be possible to design experiments to examine the dynamics of specific populations over time.

Information on survival of bacteria in dry soil is relevant also to studies in biogeography epidemiology and evolution. Not only may bacteria signify previous plant cover or land use, but also large numbers of soil bacteria are present in the dust plumes that can cross oceans and continents and are associated in some cases with bacterial infections (Griffin and Kellogg, 2004). Our results demonstrate that although culturable cells may be restricted to certain groups, diverse DNA from soil bacteria is likely to survive in such conditions with implications for horizontal gene transfer to any recipient populations. Our findings also indicate the potential of DNA-based microbiological analysis of ancient soils if they have remained dry.

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