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# Cultivation-independent *in situ* molecular analysis of bacteria involved in degradation of pentachlorophenol in soil

**Shahid Mahmood,[1] Graeme I. Paton[2] and James I. Prosser[1]***

[1]*School of Medical Sciences, Institute of Medical Sciences, and* [2]*School of Biological Sciences, University of Aberdeen, Aberdeen, UK.*

## Summary

**The central aim of this study was to determine which components of an indigenous bacterial community in pristine grassland soil were capable of degrading pentachlorophenol (PCP) using two cultivation-independent, *in situ*, molecular techniques. The first involved polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) amplification of 16S rRNA genes from DNA and RNA, respectively, extracted from PCP-amended soil. The second involved stable isotope probing (SIP), with incubation of soil with $^{13}$C-PCP and molecular analysis of $^{13}$C-labelled RNA, derived from cells incorporating PCP or its breakdown products, after separation from $^{12}$C-RNA by ultracentrifugation. Bacterial communities were characterized by denaturing gradient gel electrophoresis (DGGE) analysis of amplification products. PCP was degraded at an approximate rate of 1.18 ± 0.25 (SEM) mg kg$^{-1}$ day$^{-1}$ and 39% of the measurable PCP fraction was degraded after incubation for 63 days. PCP degradation was associated with significant changes in bacterial community structure, leading to the appearance of seven bands in both DNA- and RNA-based DGGE profiles, the latter providing clearer evidence of qualitative shifts in community structure. The majority of novel bands increased in relative intensity during the first 35 days and subsequently decreased in relative intensity as incubation continued. Sequence and phylogenetic analysis of six of these bands indicated most to have closest database relatives that were uncultured bacteria with sequence homologies to reported hydrocarbon degraders. No band could be detected in RNA-SIP-DGGE profiles derived from $^{13}$C-RNA fractions at day 0 but several faint bands appeared in these fractions after incubation of soil for 4 days, indicating assimilation of PCP or its degradation products. These bands increased in intensity during subsequent incubation for 21 days and decreased with further incubation. With one exception, RNA-SIP-DGGE and RNA-DGGE profiles were similar, indicating that RNA-targeted DGGE, in this case, provided a good indication of the metabolically active microbial community.**

Received 22 November, 2004; accepted 15 March, 2005. *For correspondence. E-mail j.prosser@abdn.ac.uk; Tel. (+44) 1224 555848; Fax (+44) 1224 555844.

## Introduction

Pentachlorophenol (PCP) is a broad-spectrum biocide that has been used globally in fungicides, herbicides, insecticides and general disinfectant products. Its frequent and widespread use has led to contamination of aquatic and terrestrial ecosystems (Jensen, 1996). There is now a complete ban on PCP production within the European Union and import of PCP and its derivatives is regulated under the Council Directives 99/51/EEC, 91/173/EEC and 76/769/EEC. Due to persistence of PCP in soil and water environments, both the European and US Environment Protection Agencies have classified PCP as a 'priority pollutant' and have recommended restricted use to minimize its further accumulation and to circumvent toxicity of the ecosystem.

PCP residues are widespread in humans and other living organisms, including those with no obvious direct exposure (Eisler, 1989; Fisher, 1991). In addition to its acute effects, exposure to PCP causes cancer and birth defects in laboratory animals, and chromosome abnormalities, blood disorders and nerve damage in humans (Fisher, 1991) and affects fish populations (Eisler, 1989). PCPs are also found in waste materials and effluents and Alcock and Jones (1997) reviewed the sources of PCP to sewage sludge (biosolids) in the UK. The recent ban on disposal of waste water sludge into the sea and public awareness of the negative consequences of landfill have increased interest in the sustainable use of biosolids in agriculture and forestry to recover essential nutrients such as N, P and base cations. However, biosolids containing

hazardous pollutants such as PCP may reduce the long-term sustainability and environmental acceptability of this practice.

Microorganisms are responsible for the biodegradation of waste material and toxic pollutants in natural environments and PCP degradation has been studied thoroughly in the soil bacterium *Sphingomonas chlorophenolica*, isolated from a PCP-contaminated site in Minnesota (Saber and Crawford, 1985). PCP 4-monooxygenase (*pcpB* gene) converts PCP to tetrachlorohydroquinone (Xun and Orser, 1991). Reductive dehalogenase (*pcpC* gene) replaces chlorine atoms by hydrogen atoms to form trichlorohydroquinone and subsequently 2,6-dichlorohydroquinone (DCHQ) (Xun *et al*., 1992), which is then converted, by DCHQ 1,2-dioxygenase (*pcpA* gene), to 2-chloromaleylacetate (Ohtsubo *et al*., 1999). Previous studies, based mostly on pure cultures, have indicated a range of bacteria with the potential to mineralize PCP, including *Burkholderia*, *Flavobacterium*, *Mycobacterium*, *Nocardioides*, *Pseudomonas*, *Ralstonia*, *Sphingomonas* (Häggblom and Valo, 1995; McAllister *et al*., 1996; Puhakka and Melin, 1996; Männistö *et al*., 1999) and fungi, such as *Gloeophyllum striatum*, *Gloeophyllum trabeum*, *Trametes versicolor*, *Phanerochaete chrysosporium*, *Inonotus dryophilus*, *Abortiporus biennis*, *Cerrena unicolor* (Alleman *et al*., 1995; Fahr *et al*., 1999; Cho *et al*., 2001). *In situ* bioremediation of PCP-contaminated soils has been investigated following inoculation with culturable bacteria, fungi or consortia (Colores and Schmidt, 1999; Combrisson and Monrozier, 1999; Tuomela *et al*., 1999; McGrath and Singleton, 2000; Kao *et al*., 2004). These studies suggest effective biodegradation by inoculated microorganisms but little is known of their persistence. Identification of members of the indigenous soil microbial community actively degrading PCP is limited by the inability to detect assimilation of PCP, and its breakdown products, by microorganisms that have yet to be cultivated in the laboratory.

The central aim of this study was to determine which components of a native bacterial community in pristine grassland soil were capable of degrading PCP, using cultivation-independent, *in situ* molecular techniques. Two approaches were employed, based on sequence analysis of 16S rRNA genes. In the first, changes in community structure were determined by analysis of 16S rRNA gene sequences amplified from DNA and RNA extracted from PCP-amended soils. Targeting of RNA increases sensitivity but may also provide a better indication of active members of the soil community (Felske and Akkermans, 1998; Nicol *et al*., 2004). In the second approach, stable isotope probing (SIP; Radajewski *et al*., 2000), soil was amended with $^{13}$C-PCP and molecular analysis was carried out on $^{13}$C-labelled RNA, derived from cells incorporating PCP or its breakdown products, after separation from $^{12}$C-RNA by ultracentrifugation. In all cases, amplification products were analysed by denaturing gradient gel electrophoresis (DGGE).

## Results

### *Biodegradation of PCP*

Degradation of PCP was determined by high-performance liquid chromatography (HPLC) analysis following methanol/chlorophenol extraction from soil. Immediately after amendment of soil, 85% of added PCP was recovered (Fig. 1) and degradation of PCP then occurred with no apparent lag phase (Fig. 1). Degradation occurred in two phases during incubation for 63 days. Degradation occurred at a rate of 1.43 mg kg$^{-1}$ day$^{-1}$ during the first phase (days 0–35) and then, in the second phase (days 35–63) decreased to 0.38 and 0.70 mg kg$^{-1}$ day$^{-1}$ at 49 and 63 days respectively. At the end of the incubation period (63 days), 39% of the PCP measurable immediately after amendment (85%) had been degraded (Fig. 1). The overall rate of PCP degradation throughout the incubation period was $1.18 \pm 0.25$ (SEM) mg kg$^{-1}$ day$^{-1}$.

### *DNA- and RNA-based DGGE analysis of the bacterial community*

The influence of PCP amendment on soil bacterial communities was determined by DGGE analysis of 16S rRNA

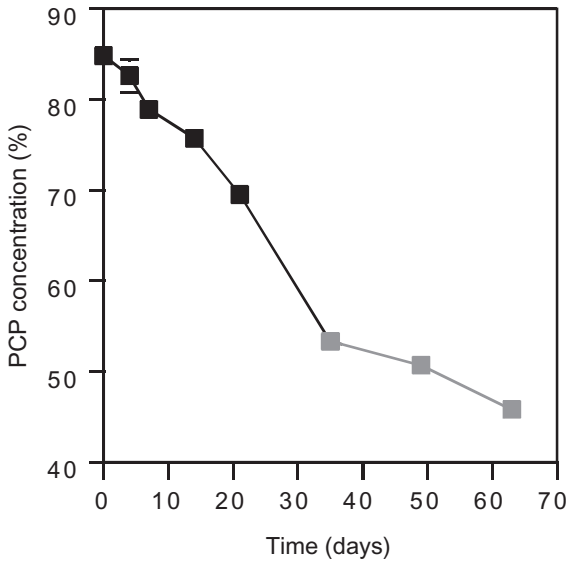


**Fig. 1.** Pentachlorophenol (PCP) biodegradation in a pristine grassland soil. PCP residual concentration (%) in soil microcosms during incubation for 63 days suggests two phases of PCP degradation: phase I, days 0–35 (black line) and phase II, days 35–63 (grey line). The experiment was performed in a microcosm system with soil amended with PCP or left unamended (control). Triplicate microcosms were harvested destructively on each sampling occasion. Vertical bars represent SEM.

gene fragments amplified by polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) from extracted DNA and RNA respectively. DNA-DGGE indicates changes in relative abundance of all organisms present while RNA-DGGE provides greater sensitivity and also, potentially, targets active members of the community. PCP application significantly influenced community structure of relatively abundant and active soil bacteria (Figs 2 and 3). There was no detectable difference between DGGE profiles of control and PCP-treated soils at day 0, immediately after amendment. At the next sampling time (day 4) both RNA- and DNA-DGGE profiles of PCP-amended soils samples differed from controls, with the appearance of several new bands (RNA-DGGE profiles) or an increase in relative intensities of existing bands (DNA-DGGE profiles) in PCP-amended soils (Figs 2 and 3). These differences persisted throughout the incubation period. DNA-DGGE analysis of control soils exhibited persistent banding profiles with little or no change in band intensities during incubation, while there was a general increase in relative intensities of novel bands in RNA-DGGE profiles until day 21, followed by a decrease until the end of the incubation period (Figs 2 and 3). Although DGGE profiles from PCP-amended and control soils differed, DNA- and RNA-DGGE profiles within these two treatments were similar at all time intervals, with differences only in relative intensities of different bands and the disappearance of a small number of bands. Seven bands were detected in RNA-DGGE profiles derived from PCP-treated soil only, and were most prominent in the DGGE profile for samples taken at 35 days (Fig. 3). The majority of these novel bands increased in relative intensity during the first 35 days and subsequently decreased in relative intensity as incubation continued (day 63). For example, the relative intensity of band 6 was high until day 35, and then decreased. This indicates an increase in the activity of this particular strain, and a potential role in PCP degradation. In DNA-DGGE profiles, six major bands (bands 2–7, Fig. 2) increased in relative intensity in PCP-amended soils compared with profiles of the respective controls during the incubation period. There was little evidence of a significant toxic effect of 200 mg PCP per kg soil on the abundance and activity of the bacterial community, as the DGGE patterns of major bands were persistent, although three relatively faint bands (bands a–c) became undetectable and profiles of minor bands exhibited dynamic changes (Figs 2 and 3). This could, however, have resulted from increases in relative abundance of the remainder of the community, rather than death of the strains generating these bands. Sequencing and phylogenetic analysis of six of the seven bands which appeared in the DGGE profiles of PCP-amended soils indicated that most were uncultured bacteria with sequence homologies with reported hydrocarbon degraders (Table 1). Despite repeated attempts at purification, the seventh band generated a mixed sequence that could not be analysed.

### *Comparison of DNA- and RNA-derived DGGE profiles*

Differences were observed between RNA- or DNA-based DGGE profiles of the bacterial community (Figs 2 and 3). In particular, the RNA-targeted approach indicated much

**Table 1.** Closest matches between 16S rRNA gene sequences of bands[a] excised from DGGE gels and sequences from the GenBank databases obtained using the BLASTN search tool.

| Band No. | Percentage similarity[b] | Closest relative in GenBank[c] | GenBank accession No. | Reference |
|---|---|---|---|---|
| Band 1 | 98% | *Pseudomonas mandelii* | AY179326 | Soares *et al*. (2003) |
| | 98% | *Pseudomonas* sp. LAB-23 | AF051699 | Futamata *et al*. (2001) |
| Band 2 | 97% | Uncultured gamma proteobacterium clone MB10gamma-k4 | AF526527 | D. Seghers, K. Verthe, D. Reheul, R. Bulcke, S.D. Siciliano, W. Verstraete and E.M. Top, unpublished |
| | 95% | Uncultured gamma proteobacterium clone LTUG07456 | AY144255 | Kaplan and Kitts (2004) |
| Band 3 | 95% | *Collimonas* sp. CTO 300 | AY281145 | De Boer *et al*. (2004) |
| | 95% | Uncultured beta proteobacterium | AB011726 | S. Shikano and H. Mitsui, unpublished |
| Band 4 | 99% | Uncultured *Burkholderia* sp. clone Ba04 | AF407355 | Salles *et al*. (2002) |
| | 98% | Uncultured eubacterium WD220 | AJ292665 | Nogales *et al*. (2001) |
| Band 5 | 97% | Uncultured *Sphingomonas* sp. DGGE band B2-1 | AY758563 | M. Vinas, J. Sabate and A.M. Solanas, unpublished |
| | 97% | Uncultured bacterium clone MNM-ML-91 | AY309175 | L.C. Schultz, M.D. Zwolinski, B.M. Miller and S.C. Nold, unpublished |
| Band 6 | 100% | Unidentified gamma proteobacterium | AB011751 | S. Shikano and H. Mitsui, unpublished |

**a.** The prominent bands appearing only in the DGGE profiles of PCP-treated soil were excised and sequenced.
**b.** Percentage similarity between the sequence of a band excised from DGGE gel and the closest match in GenBank.
**c.** Identities are based on closest NCBI sequences of phylogenetically related bacteria.

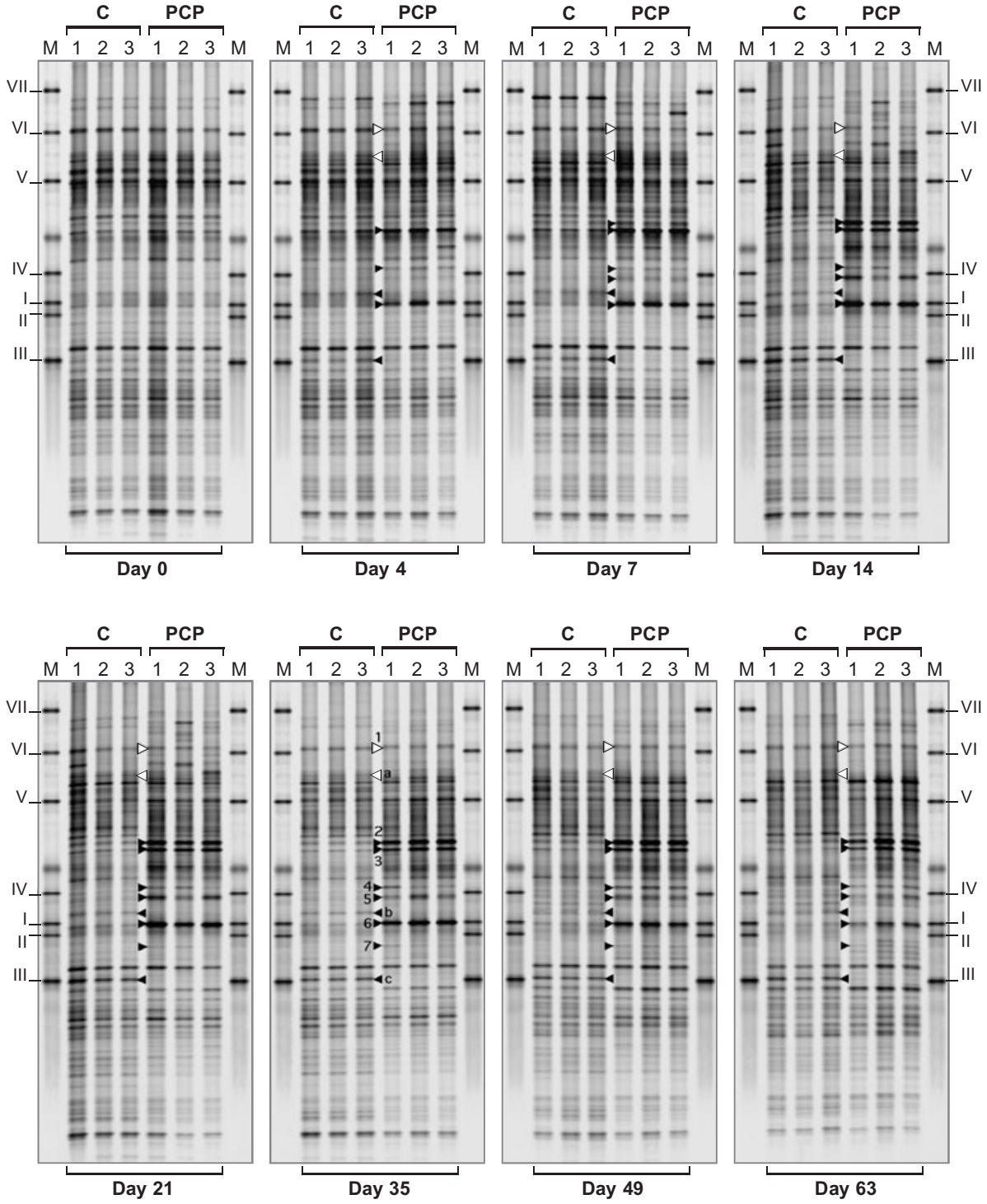


**Fig. 2.** Denaturing gradient gel electrophoresis (DGGE) banding profiles of bacterial 16S rRNA genes PCR-amplified from control (C) and pentachlorophenol (PCP)-amended soils. Lanes 1–3 relate to samples from triplicate microcosms of control or PCP treatments that were harvested destructively on each sampling occasion. Black arrowheads indicate bands that increased in relative intensity (right-directing arrowheads) or disappeared (left-directing arrowheads) in response to PCP treatment. White arrowheads designate bands that did not change significantly in relative intensity in response to PCP treatment compared with corresponding bands in RNA-DGGE profiles (see Fig. 3). The bands 1–6 (see day 35 gel) were excised and sequenced (for identities, see Table 1). Markers in lane M consisted of seven unrelated bacteria with known migratory positions or melting behaviour on DGGE gels.

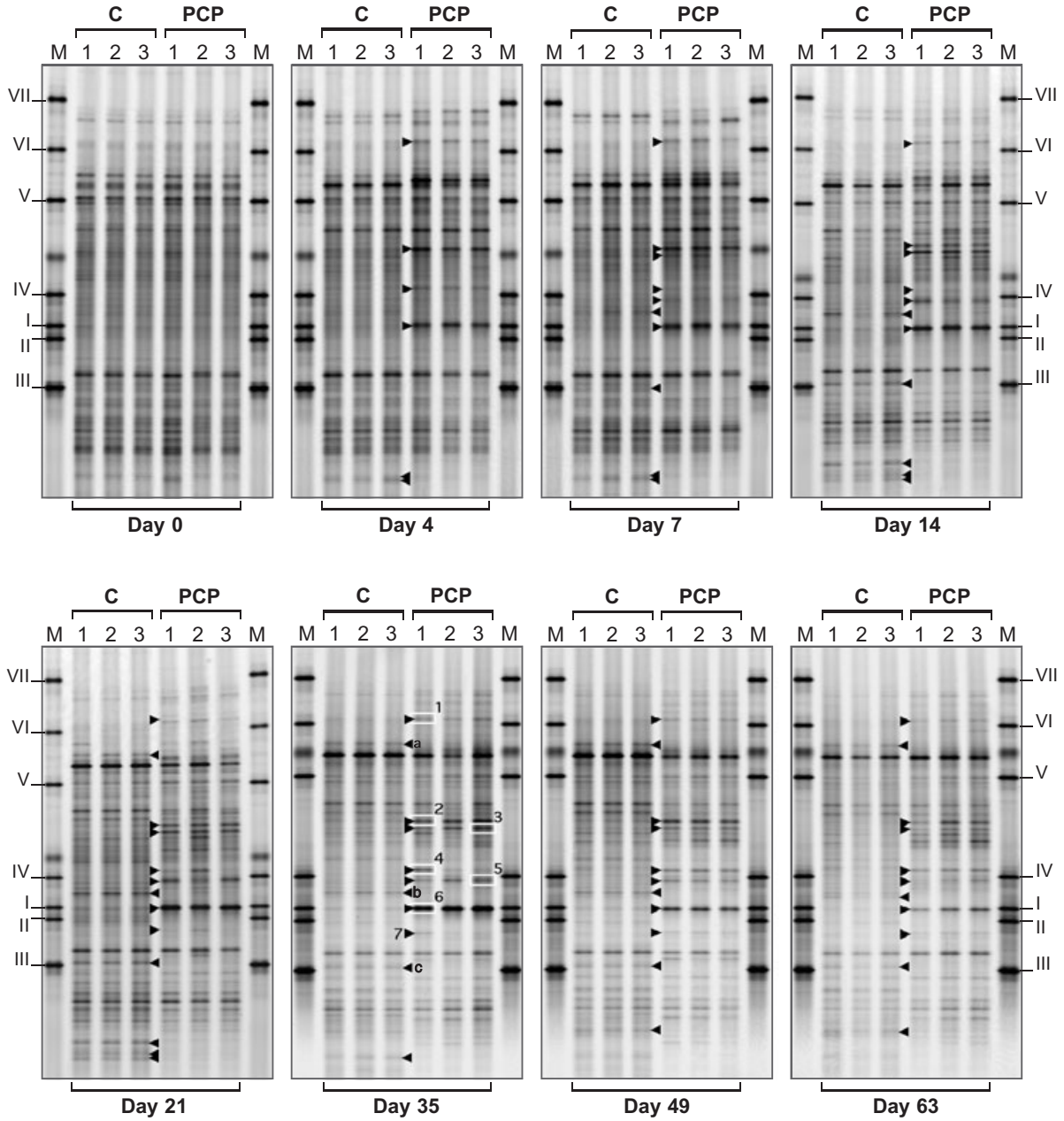


**Fig. 3.** Denaturing gradient gel electrophoresis (DGGE) banding profiles of bacterial 16S rRNA genes RT-PCR amplified from control (C) and pentachlorophenol (PCP)-amended soils. Lanes 1–3 relate to samples from triplicate microcosms of control or PCP treatment that were harvested destructively on each sampling occasion. Black arrowheads indicate bands that increased in relative intensity (right-directing arrowheads) or disappeared (left-directing arrowheads) in response to PCP treatment. Bands 1–6 (see day 35 gel, white rectangles) were excised and sequenced (for identities, see Table 1). Markers in lane M consisted of seven unrelated bacteria with known migratory positions or melting behaviour on DGGE gels.

clearer qualitative shifts in bacterial community in response to PCP amendment of soil. Although the seven bands that appeared in RNA-DGGE profiles of PCP-treated soil could also be detected in the DNA-DGGE gels, there were qualitative differences in the banding profiles and relative intensities. For instance, band 1 was present at the same relative intensity in DNA-DGGE profiles of control and PCP-treated soils (Fig. 2), while RNA-DGGE profiles showed a significant increase in relative intensity of this band in PCP-treated soils, but not in controls (Fig. 3). Similarly, band 'a' was distinctly visible in RNA-DGGE profiles of control soils but was barely detectable in DNA-DGGE profiles. RNA-DGGE profiles clearly showed disappearance of band 'a' in PCP-amended soils

of days 21–63, but resolution of this band in DNA-DGGE was low. Bands 2–7 in the DNA-DGGE profiles also showed an increase in relative band intensity in response to PCP amendment (indicating an increase in relative abundance of these particular bacterial strains), but the quantitative shifts in bacterial community structure were of lower magnitude than those in RNA-based DGGE profiles. The dynamic changes in bacterial communities in control and PCP-treated soils with time were also more pronounced in RNA-based DGGE profiles than in the DNA-DGGE gels (Figs 2 and 3).

### *RNA-SIP-DGGE profiles*

The nature of the bacterial community involved in assimilation of PCP and its degradation products was determined by SIP of extracted RNA following incubation with $^{13}$C-labelled PCP. 16S rRNA genes were RT-PCR amplified from fractions taken from a gradient of $^{12}$C-RNA–$^{13}$C-RNA obtained by density gradient centrifugation of extracted RNA and PCR products were analysed by DGGE. RNA-SIP-DGGE profiles from $^{12}$C-RNA fractions (fractions 7–12), obtained immediately after PCP amendment (day 0), were typical for soil bacterial communities, with complex banding patterns similar to those observed using the standard RNA-DGGE approach described above. No band could be detected in day 0 profiles from the high buoyant density, $^{13}$C-RNA gradient fractions (fractions 3–6) (Fig. 4). Profiles from $^{12}$C-RNA fractions from soil sampled at subsequent time points were similar to those described above for standard RNA-DGGE analysis, with seven bands increasing in relative intensity at migration positions equivalent to those illustrated in Fig. 3.

After incubation for 4 days, several faint bands (see arrowheads, Fig. 4) appeared in $^{13}$C-RNA fractions, indicating assimilation of PCP or its degradation products, and increased in relative intensity during subsequent

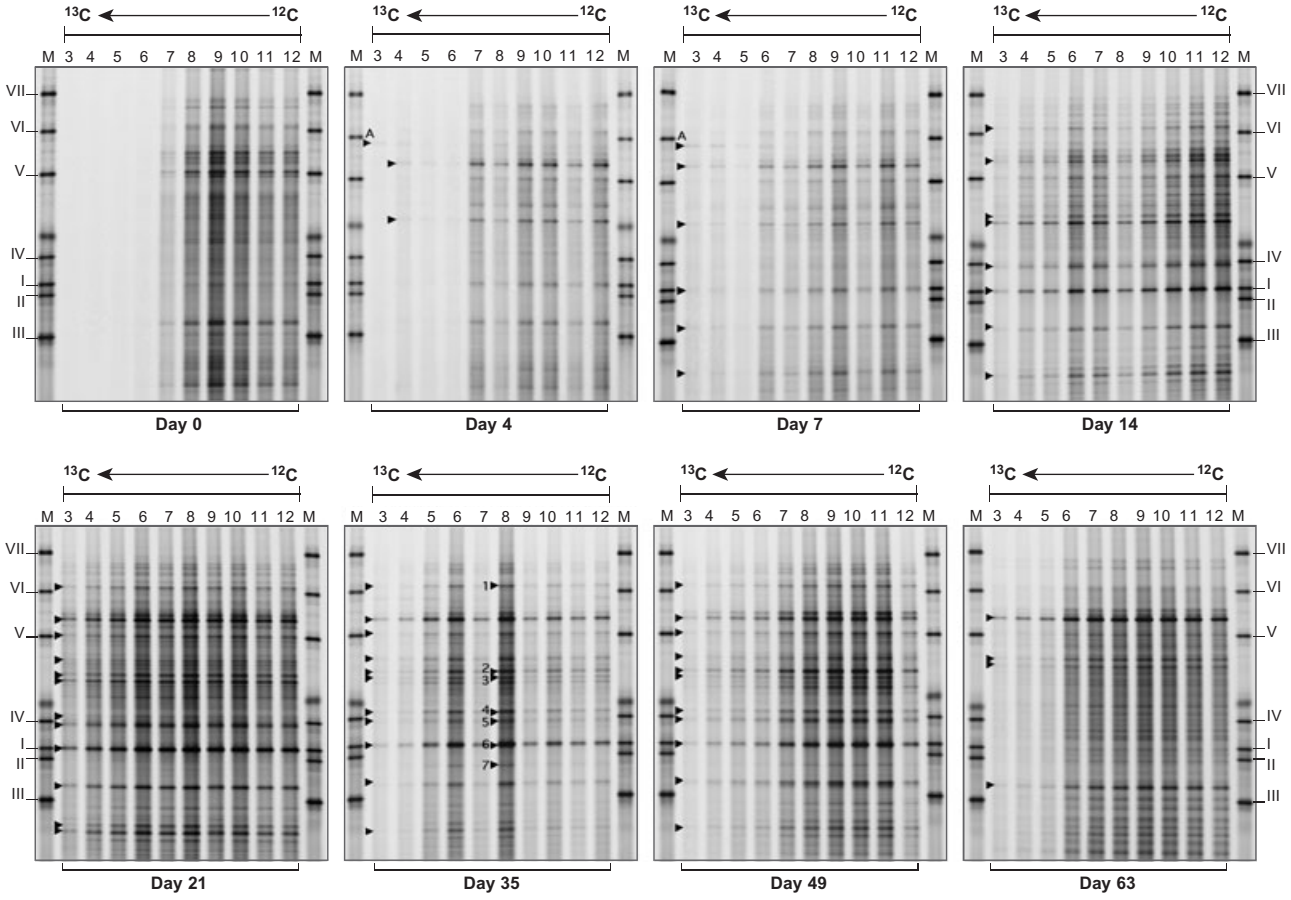


**Fig. 4.** Stable isotope probing (SIP)-based DGGE banding profiles of bacterial 16S rRNA genes RT-PCR amplified from [$^{13}$C]-pentachlorophenol (PCP)-amended soils. Extracted RNA was subjected to density gradient centrifugation to separate $^{13}$C- and $^{12}$C-RNA. Lane numbers 3–12 correspond to density gradient fractions (heavy $^{13}$C- to light $^{12}$C-labelled RNA). Black arrowheads indicate the bands that appeared in the heavy fractions. Band A (see arrowhead marked 'A' in days 4 and 7 gels) appeared only in the heavy fractions and could not be seen in the lighter fractions or in the later samplings. The bands 1–6 migrated to the same positions as equivalent bands in Figs 2 and 3 (for identities, see Table 1). Markers in lane M consisted of seven unrelated bacteria with known migratory positions or melting behaviour on DGGE gels.

incubation. With the exception of a single band (A), all the bands that appeared in these heavy fractions were also visible in the lighter ($^{12}C$) RNA fractions. The relative intensity of band A was highest in the heavy fractions from day 7 samples but declined in the lighter fractions and could not be detected beyond fraction 9 or in subsequent harvests of 14–63 days. Although this band was excised and re-amplified, it was not possible to obtain sequence data of sufficient quality for phylogenetic analysis. The overall relative intensities of bands in the heavy fractions were greatest in samples taken after incubation for 21 days. Relative intensity in subsequent samples decreased, reaching lowest values in samples taken at 63 days. With the exception of band A, which was observed only in heavy fractions at days 4 and 7, DGGE profiles in the heavy and light fractions were qualitatively similar, with increases in relative intensity of identical bands. There were, however, some differences in the relative intensities of the bands, which were generally greater in the lighter fractions than in the $^{13}C$-RNA fractions. Total band intensities were greatest at day 21 for both fractions (Fig. 4).

## Discussion

This study demonstrates degradation of PCP by the indigenous bacterial community of a grassland soil with no prior history of exposure to PCP or other organic pollutants. Similar findings have been reported in other studies. For example, McGrath and Singleton (2000) found a decrease in PCP concentration from 250 to 2 mg $kg^{-1}$ (PCP extraction efficiency 90%) after incubation of a clay loam soil (pH 6.4) for 6 weeks at 25°C. This rate of degradation is greater than observed in this study, in which PCP concentration decreased from 200 to 92 mg $kg^{-1}$ (PCP extraction efficiency 85%) after incubation for 9 weeks. The lower rate of degradation most likely results from differences in soil type and incubation temperature. Loss of PCP due to volatilization is likely to have been negligible at 15°C and in closed microcosms, as in this study. In a second example, Chaudri and colleagues (2000) investigated the influence of different PCP concentrations (25–200 mg $kg^{-1}$) on soil microbial biomass C and concentrations of total aerobic heterotrophic bacteria after incubation of soil for 6 months. Unexpectedly, the lower concentrations of PCP were more detrimental to soil microbial biomass C, with a reduction of only 22% at 200 mg PCP $kg^{-1}$, and there was no detectable influence on total aerobic heterotrophic bacterial counts at this PCP concentration. The present study employed a PCP concentration of 200 mg $kg^{-1}$, and there was little evidence of any toxic effect on the bacterial community.

Three approaches were used to assess the influence of PCP on the soil bacterial community. The first, DNA-targeted amplification of 16S rRNA genes, followed by DGGE analysis (DNA-DGGE), has been used widely to determine changes in bacterial species composition, using relative intensity of DGGE bands as an indication of the relative abundance of specific community members (Muyzer, 1999; Nicolaisen and Ramsing, 2002). DNA-DGGE gel profiles in both control and PCP-amended soils changed during incubation but PCP amendment led to increases in relative intensity of several bands not evident in control soils. DGGE assesses relative abundance and increasing relative intensity of specific bands could therefore result from an increase in abundance of a particular strain, potentially a PCP-degrading strain, or a decrease in abundance of other strains, for example, due to PCP toxicity. Changes in DNA-DGGE profiles will result from bacterial growth or death and do not enable distinction between active and dormant organisms. The second approach, RNA-targeted DGGE, provides two advantages. The first is greater sensitivity, as cellular ribosome number will be several orders of magnitude greater than rRNA gene copy number. The second is the potential ability to target active organisms, which will possess higher cellular RNA content. While there is evidence for this in a number of organisms (Wagner, 1994; Felske and Akkermans, 1998; Kerkhof and Kemp, 1999), there is also evidence that cellular RNA content in some organisms does not decrease rapidly when active cells are starved (Wagner *et al*., 1995). RNA-DGGE profiles derived from PCP-amended soils indicated changes in the bacterial community similar to those determined using DNA-DGGE but RNA-DGGE profiles changed more rapidly and provided better resolution of qualitative shifts in the bacterial community than in DNA-DGGE profiles.

The third approach, SIP, has targeted both DNA and RNA (Radajewski *et al*., 2000; Manefield *et al*., 2002; Lueders *et al*., 2004). RNA-SIP was employed in this study to increase sensitivity, as $^{13}C$ incorporation into RNA will be greater, will occur earlier than into DNA and will detect cells which are active but not growing. SIP also provides a much more direct indication of activity following addition of the $^{13}C$-labelled substrate, as it measures assimilation of the substrate or its breakdown products. The appearance of a band therefore results from growth or activity of a particular organism and changes in DGGE profiles will characterize only those members of the community active in substrate degradation. The absence of bands in $^{13}C$-fractions from samples at day 0 confirms the efficiency of the $^{12}C$-$^{13}C$-fractionation method, as also demonstrated in other studies (Manefield *et al*., 2002; Griffiths *et al*., 2004; Rangel-Castro *et al*., 2005). Several faint bands appeared in the heavy fractions after incubation with PCP for 4 days, at which point PCP was actively being degraded. With one exception (band A), bands appearing in profiles from heavy fractions in this and

subsequent samples migrated to positions similar to those observed in the lighter fractions and in standard DNA-DGGE and RNA-DGGE gels. Thus, RNA-SIP-DGGE data provide strong evidence that changes in DNA- and, in particular, RNA-DGGE analyses resulted from differences in activities of particular bacterial groups, rather than decreases in other groups due to PCP toxicity. Band A increased in relative intensity at day 7, but could not be detected in lighter fractions or in DNA-DGGE or RNA-DGGE profiles. This suggests that this is a primary PCP utilizer that is present at low relative abundance within the total and active bacterial communities, but at high relative abundance in the PCP-degrading community. Although only one band was found to exhibit this behaviour, it demonstrates the potential of SIP approaches to detect substrate utilization by less abundant bacteria in complex, natural communities. The disappearance of band A from profiles at subsequent sampling points could result from sensitivity of this strain to PCP or its degradation products and/or its inability to compete with other members of the microbial community under the incubation conditions employed.

When using techniques involving substrate amendment, it is difficult to determine whether increases in activity or growth result from assimilation of the substrate by primary utilizers, or utilization of degradation products by secondary utilizers. However, appearance of bands during the early stages of degradation and similarities between sequences of excised bands and known degraders suggests that, in this case, RNA-DGGE and RNA-SIP-DGGE identified primary utilizers of PCP until day 35. The majority of sequences of bands that appeared or increased in relative intensity in RNA-DGGE gels following PCP amendment were most closely related to organisms isolated from contaminated environments or to sequences of uncultured organisms from such sites. Contaminants and bioremediation systems included nonylphenol (Soares *et al*., 2003), trichloroethylene (Futamata *et al*., 2001), herbicides (D. Seghers, K. Verthe, D. Reheul, R. Bulcke, S.D. Siciliano, W. Verstraete and E.M. Top, unpublished), petroleum (Kaplan and Kitts, 2004), polychlorinated biphenyl- (Nogales *et al*., 2001) and creosote- (M. Vinas, J. Sabate and A.M. Solanas, unpublished) contaminated soils respectively. The most closely related cultivated organisms were strains of *Pseudomonas*, *Burkholderia* and *Sphingomonas*. Pseudomonads are known to mineralize PCP (Radehaus and Schmidt, 1992; Lee *et al*., 1998) and Kao and colleagues (2004) recently isolated a strain of *Pseudomonas mendocina* NSYSU from PCP-contaminated soils in Taiwan capable of utilizing PCP as a sole source of carbon and energy, which completely removed PCP. *Burkholderia* spp., particularly strain PS14, can utilize 1,2,4,5-tetrachlo-, 1,2,4-trichloro- and three isomeric dichloro-benzenes (1,2-DCB, 1,3-DCB and 1,4-DCB) as the source of carbon and energy (Beil *et al*., 1997; Rapp and Timmis, 1999). Similarly, *Sphingomonas* spp. degrade high concentrations of PCP in soil and survive for several months in PCP-contaminated soils (van Elsas *et al*., 1998; Colores and Schmidt, 1999; Ohtsubo *et al*., 1999). Although these data suggest that bands 1–6 may be primary PCP degraders, care must be taken in inferring function from RNA phylogeny.

SIP gradients were loaded with equal amounts of RNA (500 ng) at each sampling point and were treated identically and there was a noticeable difference in the total intensity of bands from $^{13}C$ fractions with incubation time until day 21, with a subsequent decrease as incubation proceeded. Quantification of abundance on the basis of band intensities from PCR amplification products is dangerous, but the changes in band intensities of heavy fractions are consistent with an increase in activity of bacteria capable of assimilating $^{13}C$ from PCP and its degradation products, thereby leading to $^{13}C$ enrichment in the template RNA. Changes in total band intensity coincided with increases and decreases in the rate of PCP degradation. The decrease in degradation rate after 35 days could result from reduction in bioavailable PCP to growth-limiting concentrations. PCP adsorbs strongly to soil (Kenaga, 1980), particularly under acidic conditions (Callahan *et al*., 1979), and the pH of soil employed in this study was 4.9. This could explain reduced incorporation of $^{13}C$ into RNA, which could also have resulted from dilution of the label through respiration of PCP and its degradation products.

Previous applications of SIP have demonstrated its ability to identify organisms capable of degrading organic compounds, including xenobiotics (Manefield *et al*., 2002; Padmanabhan *et al*., 2003) and root exudates (Rangel-Castro *et al*., 2005), but we believe that this is the first application of RNA-SIP technique to a soil system involving biodegradation of a priority pollutant. The study demonstrates the ability of RNA-SIP to link functional components of a natural soil bacterial community capable of degrading and assimilating PCP and its by-products to phylogeny. The study focussed on degradation by bacteria but is equally capable of identifying PCP-degrading soil fungi, which are likely to contribute to degradation (Alleman *et al*., 1995; Fahr *et al*., 1999; Cho *et al*., 2001). Molecular analysis could also be extended to functional genes. In characterizing the active community, SIP provides an important new tool for investigating members of microbial communities that are directly involved in biodegradation of pollutants, in contrast to established molecular analysis which does not distinguish between active and dormant organisms. It therefore provides significant potential for more accurate determination of links between microbial diversity and ecosystem function.

## Experimental procedures

### *Soil microcosms*

Laboratory microcosms were established containing grassland soil with no prior history of exposure to PCP or other organic pollutants. The soil was collected in May 2003 from an unimproved plot at the Sourhope Research Station located in the Borders region of Scotland (map reference NT 850 205). The vegetation cover was dominated by *Agrostis capillaris*, *Festuca ovina* and *Galium saxatile* and further details of the site can be found in McCaig and colleagues (1999). After sampling, the soil was stored in a cold room at 4°C for 3 days and passed through a 3.35 mm sieve to remove roots and stones. Microcosms consisted of 10 g soil in 28 ml, glass Universal bottles with screw caps (Fisher Scientific, Loughborough, UK). PCP degradation was investigated by amending soil with [$^{13}$C]-pentachlorophenol (PCP, $^{13}C_6$, 99%, Cambridge Isotope Laboratories, Andover, USA) using a modification of the method described by Brinch and colleagues (2002). Briefly, 2.5 g of soil was transferred to a microcosm and amended with 500 μl of ethanol containing 2.0 mg of [$^{13}$C]-PCP. Control microcosms were treated only with 500 μl of ethanol (no PCP addition). The screw cap was closed for 5 min to enable dispersion of the solvent, which was then allowed to evaporate for 16 h with the cap removed. The remaining 7.5 g of soil was added and mixed well with a sterile spatula for 2 min. Preliminary investigation of DGGE profiles of the experimental control (2.5 g of soil + ethanol (overnight evaporation) + 7.5 g of soil, no PCP) and untreated soil (no ethanol) did not reveal any significant difference in bacterial community.

After fitting the screw caps loosely, microcosms were incubated in the dark at 15°C and triplicate microcosms for control and PCP treatments were harvested and sampled destructively after 0, 4, 7, 14, 21, 35, 49 and 63 days. At each harvest, soil samples were stored at −80°C until extraction of nucleic acids and determination of PCP concentration.

### *DNA/RNA extraction*

Nucleic acids were extracted from 0.5 g of soil samples according to Griffiths and colleagues (2000). In brief, cells were lysed for two cycles of 20 s with a Ribolyser cell disruptor (Hybaid, Ashford, UK) at 4 m s$^{-1}$ in 2 ml Blue Matrix Ribolyser tubes (Hybaid) containing 0.5 ml of hexadecyltrimethylammonium bromide extraction buffer, 0.5 ml of phenol–chloroform–isoamyl alcohol (25:24:1 v/v, pH 8.0) and ceramic and silica beads. Further extraction and precipitation of nucleic acids involved removal of phenol with an equal volume of chloroform–isoamyl alcohol (24:1) followed by precipitation from the aqueous layer with two volumes of 30% (w/v) polyethylene glycol 6000–1.6 M NaCl. Pelleted nucleic acids were washed in ice-cold 70% (v/v) ethanol, air-dried and resuspended in 50 μl of RNase-free sterile water.

### *PCR amplification of 16S rRNA genes*

16S rRNA gene amplifications of extracted soil DNA were achieved by a primary PCR using universal bacterial primers 27f and Pf1053r (Edwards *et al*., 1989; Lane, 1991) and a secondary PCR with 357f-GC and 518r primers (Muyzer *et al*., 1993) to generate products for DGGE analysis. The nested PCR amplification approach yielded 100% PCR success, which was not found for one-step amplification with the 357f-GC-518r primer set, and there was no detectable difference in DGGE banding profiles of PCR products generated using either one or two primer sets for amplification. PCR amplification was carried out in 50 μl reaction volumes with a PCR Express thermal cycler (Hybaid, Middlesex, UK). The reaction mixture contained 20 ng of template DNA, 0.2 μM of each primer, 1× PCR buffer (Bioline, London, UK), 250 μM each deoxynucleoside triphosphate, 1.5 mM $MgCl_2$ (20 ng of bovine serum albumin was added to the primary PCR only) and 1 U of Biotaq™ DNA polymerase (Bioline). The thermocycling conditions for both primer sets were as follows: 95°C for 5 min, followed by 10 cycles of 94°C for 30 s, 55°C for 30 s and 72°C for 30 s, 25 cycles of 92°C for 30 s, 55°C for 30 s and 72°C for 45 s, and a final incubation at 72°C for 10 min (McCaig *et al*., 2001). The primers 27f-Pf1053r and 357f-GC-518r amplified 1 kb and 161 bp fragments respectively. PCR amplification products were analysed by electrophoresis on 1% (w/v) agarose gels and detected by staining with ethidium bromide (Sambrook *et al*., 1989).

### *Reverse transcription polymerase chain reaction (RT-PCR) amplification of 16S rRNA genes*

DNA was removed from crude nucleic acid extracts by treatment with DNase for 1 h at 37°C according to the manufacturer's recommendations (Promega, USA). Reverse transcription of RNA to complementary DNA (cDNA) was performed according to Griffiths and colleagues (2000) with modifications. The RNA secondary structure was melted by incubating the RNA samples with reverse 16S rRNA primer (518r, 5 pmol μl$^{-1}$) at 70°C for 10 min. Samples of annealed primer template were then chilled on ice and 8 μl of reverse transcription reaction mixture [containing: SuperScript RNase H$^-$ Reverse Transcriptase, 5× First-Strand Buffer, 0.1 M dithiothreitol (DTT)] was added as specified by the manufacturer (Invitrogen, Paisley, UK). Control reactions (without reverse transcription) were run to check for DNA contamination of RNA samples. Reverse transcription was carried out at 42°C for 50 min and the enzyme was subsequently heat-inactivated for 10 min at 70°C. PCR amplification of cDNA template was performed in a 50 μl reaction volume with 1 μl of cDNA template and eubacterial primers 357f-GC and 518r (Muyzer *et al*., 1993). The concentration of reagents in the PCR reaction mixture and thermocycling conditions were as described above. PCR amplification products were analysed by electrophoresis on 1% (w/v) agarose gels and detected by staining with ethidium bromide (Sambrook *et al*., 1989).

### *Stable isotope probing (SIP) of RNA*

$^{12}$C- and $^{13}$C-labelled RNA were separated by density gradient ultracentrifugation using caesium trifluoroacetate (CsTFA) gradients as described by Manefield and colleagues (2002). The gradient mixture consisted of 1.86 ml of a 1.99 g ml$^{-1}$ CsTFA solution (Amersham Pharmacia Biotech,

Uppsala, Sweden), 75 μl of deionized formamide (Amersham Pharmacia Biotech) and 375 μl of PCR grade sterile $H_2O$. After adding 500 ng of total RNA to CsTFA gradient mixture in Beckman pollyallomer bell-top Quick-seal centrifuge tubes (11 × 32 mm), the tubes were sealed using a Beckman tube sealer and spun in a Beckman TVL-100 rotor in a TL-100 ultracentrifuge (Beckman) at 140 800 *g*, at 20°C for 40 h. A Beckman Fraction Recovery System was used to fractionate gradients by piercing the tubes from below and controlling the flow rate from the top of the gradient by displacement with PCR grade sterile $H_2O$ using a peristaltic pump (Gilson, Villiers Le Bel, France). To precipitate RNA, isopropanol (100 μl) was added to each gradient fraction, incubated at –20°C for 2 h and centrifuged for 30 min at 4°C. After further purification with isopropanol, the RNA pellet was air-dried and resuspended in 20 μl of $H_2O$. Reverse transcription of RNA to cDNA and PCR amplification were performed as described above.

### *Denaturing gradient gel electrophoresis (DGGE) analysis*

DGGE analysis of PCR products was carried out using the D-Code Universal Mutation Detection System (Bio-Rad, USA). Briefly, 8% polyacrylamide gels containing a gradient of 30–60% denaturant were prepared using a gradient maker (Fisher Scientific, UK), with 100% denaturing solution defined as 7 M urea and 40% formamide (McCaig *et al*., 2001). Gels were run for 16 h at 75 V in 1× TAE buffer at a constant temperature of 60°C. Following electrophoresis, the gels were silver-stained and scanned as described by McCaig and colleagues (2001). To enable gel–gel comparison, all gels included two marker lanes, consisting of 16S rRNA gene fragments from seven unrelated ammonia oxidizing bacteria: (i) EnvB1-8 (*Nitrosospira*); (ii) pH 4.2 A/27 (*Nitrosospira*); (iii) pH 4.2 A/4 (*Nitrosospira*); (iv), pH 7B/C3 (*Nitrosospira*); (v) EnvA1-21 (*Nitrosomonas*); (vi), EnvC1-19 (*Nitrosomonas*); and (vii) *N. europaea* (*Nitrosomonas*). An additional artefactual band, with inconsistent migration behaviour, was sometimes observed in the marker lane.

### *Recovery and purification of DNA template from DGGE bands*

Representative, prominent bands appearing only in the DGGE profiles of PCP-treated soil were excised and sequenced. To facilitate comparisons between banding profiles from the three approaches (DNA-, RNA- or RNA-SIP-DGGE), selected samples were run on a single DGGE gel and migratory positions of bands of interest were analysed in relation to the DGGE control markers (with known mobility behaviour on the gel). The bands that migrated to a similar position on the gel were presumed to have similar sequences and therefore only representative bands (with identical migratory positions) were excised for subsequent DNA sequence analysis. It should be noted that most of the bands excised in this study were unambiguously strong and segregated well. DGGE gels were run as described above, except that they were stained with a 1:10 000 dilution of SYBR gold nucleic acid stain (Molecular Probes, Eugene, USA) in 1× TAE for 20 min. The bands of interest were excised with a sterile razor while the gels were illuminated on a Dark Reader (Clare Chemical Research, Detroit, USA). DNA was eluted from excised bands by incubation of the crushed band in 25 μl of sterile $ddH_2O$ at 65°C for 30 min followed by centrifugation at 5000 *g* for 1 min. The eluted DNA (1 μl) was used as template for PCR amplification under the conditions described above and the resultant amplified products were analysed on an agarose gel to estimate product concentration before DGGE analysis to check purity of the product. In most cases, the PCR products yielded a number of bands in addition to the band of interest, necessitating further (typically 2–3) rounds of band excision, PCR amplification and DGGE analysis to check purity. The melting behaviour and correct migration of PCR products of purified bands were confirmed by DGGE analysis of these products and environmental PCR products on the same gel. Purified PCR products were subjected to a final cleaning step as described by Boyle and Lew (1995).

### *Sequencing and phylogenetic analysis*

The purified PCR products were sequenced with the 518r primer (Muyzer *et al*., 1993) using the BigDye Terminator cycle-sequencing kit (PE Biosystems, UK) and sequences were analysed using an ABI377 automated sequencer (PE Biosystems, UK). The BLASTN search tool (Altschul *et al*., 1990) was used to find sequence homology and to determine the most similar sequences in the GenBank database. All sequences from this study were deposited to GenBank under the Accession Nos AY816331–AY816336.

### *Pentachlorophenol (PCP) analysis*

PCP was extracted from 2 g of soil samples in 20 ml of Rathburn HPLC Grade methanol. Before chlorophenol determination, the supernatant was cleaned using solid phase extraction columns (100 mg of C18 Bond Elut, capacity 1 ml; Varian, Middelburg, the Netherlands). Before analysis, the C18 column was conditioned with 1 ml of methanol. One millilitre of soil extract was loaded onto the column with a flow rate of 1 ml $min^{-1}$ and the column was then washed with 1 ml of deionized water. For the final step, 1 ml of methanol was passed through the column to elute chlorophenols for further analysis. Extract solutions were analysed by reverse phase HPLC (Thermo Separation Product, FL, USA). The HPLC system consisted of a binary pump (P2000), an autosampler (AS3000), UV-VIS detector (UV1000) and an integrator (SN4000). Chlorophenols were separated on an ODS-IK5 column (15 cm × 4.6 mm inner diameter, ODS2-interpak material packing, 5 μm particle diameter) by elution with 70% acetonitrile and 30% of 0.1% (v/v) acetic acid in deionized water with a flow rate 1 ml $min^{-1}$. Detector wavelength was set at 230 nm and 2,4-dibromophenol (10 mg $l^{-1}$) was used as the internal standard.

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