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The impact of bioaugmentation on metal cyanide degradation and soil bacteria community structure

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Abstract

Metal cyanides are significant contaminants of many soils found at the site of former industrial activity. In this study we isolated bacteria capable of degrading ferric ferrocyanide and $K_2Ni(CN)_4$. One of these bacteria a *Rhodococcus* spp. was subsequently used to bioaugment a minimal medium broth, spiked with $K_2Ni(CN)_4$, containing 1 g of either an uncontaminated topsoil or a former coke works site soil. Degradation of the $K_2Ni(CN)_4$ was observed in both soils, however, bioaugmentation did not significantly impact the rate or degree of $K_2Ni(CN)_4$ removal. Statistical analysis of denaturing gradient gel electrophoresis profiles showed that the topsoil bacterial community had a higher biodiversity, and its structure was not significantly affected by either $K_2Ni(CN)_4$ or bioaugmentation. In contrast, profiles from the coke works site indicated significant changes in the bacterial community in response to these additions. Moreover, in both soils although bioaugmentation did not affect rates of biodegradation the *Rhodococcus* spp. did become established in the communities in broths containing both top and coke works soil. We conclude that bacterial communities from contaminated soils with low biodiversity are much more readily perturbed through interventions such as contamination events or bioaugmentation treatments and discuss the implications of these findings for bioremediation studies.

Introduction

Many derelict industrial sites such as former manufactured gas plants, coking works and electroplating plants are highly contaminated with polyaromatic hydrocarbons (PAH), heavy metals and cyanide compounds (Thomas & Lester 1994). During the manufacturing process, contaminants, such as tars, hydrogen sulphide and cyanides were removed using bog iron ore that when exhausted, was used as an on-site fill (Ghosh et al. 1999). As bog iron ore is acidic, the soils at these sites often have a low pH (2–5), contain large quantities of sulphur and may be blue in colour, due to presence of ferric ferrocyanide $Fe_4(Fe(CN)_6)_3$ ('Prussian blue') (Kjeldsen 1999; Young & Theis 1991). Metal-cyanide compounds in these soils can be present at concentrations ranging between 10 and 5000 mg/kg, therefore, remediation of these contaminants as well as PAH and heavy metals is required before these sites can be redeveloped (Meeussen et al. 1992; Thomas et al. 1991).

There have been relatively few studies on the bacterial degradation of metal-cyanide complexes. Several strains of *Pseudomonas fluorescens* are capable of degrading metal-cyanides. For example, ferrocyanide ions by *P. fluorescens* (P70) and zinc, copper, silver and iron metal–cyanide complexes by *P. fluorescens* BKM B-5040 (Dursun et al. 1999; Shpak et al. 1995). *Pseudomonas fluorescens* NCIMB 17764 could utilise tetracyanonickelate (TCN) as a sole nitrogen source (Rollinson et al. 1987), initially this activity was thought to be catalysed by a cyanide oxygenase (Dorr & Knowles 1989),

however, a more recent study suggests that this enzyme only acts on the free cyanide liberated as metal–cyanide complexes dissociate (Kunz et al. 2001). Silva-Avalos et al. (1990) isolated seven pseudomonads, from soil, fresh water and sewage sludge with no previous known exposure to TCN that could grow using this compound as the sole nitrogen source. Studies of TCN utilisation by one of these bacteria, *Pseudomonas putida* BCN3, demonstrated that degradation proceeds via the formation of $Ni(CN)_2$ as a metabolite. *Escherichia coli* BCN6 has also been shown to use iron, zinc and copper cyanide complexes as a nitrogen source, however, each metal–cyanide complex was observed to have a different effect on growth, most likely due to the differing toxicities of the metals used (Figueira et al. 1996). Also an *Acinetobacter* sp. strain RBPI isolated from gold mine effluents was capable of degrading gold, silver, cadmium, zinc, copper, cobalt and iron cyanide complexes. This bacterium appeared to have a novel cyanide degrading enzyme system as a single, purified, cyanide degrading complex was shown to be capable of degrading metal–cyanides, simple cyanides and nitriles, unlike other types of cyanide and nitrile degrading enzymes studied (Finnegan et al. 1991).

Here we study the effect of $K_2Ni(CN)_4$ on soil bacterial communities and investigate the impact of bioaugmentation with a $K_2Ni(CN)_4$ degrading isolate on biodegradaion and community structure.

Materials and methods

Soil and soil preparation

Uncontaminated topsoil (pasture soil, Washington, Tyne & Wear, UK) and soil from a former coke works site (Lambton, Tyne & Wear, UK) were used in this study. Soil samples were obtained from the top 30 cm of the sample sites, placed in black polythene bags during transport to the laboratory and subsequently stored at 4 °C until required. Prior to use the soil was sieved (2 mm) and air-dried overnight at 25 °C. When required soils were sterilised by autoclaving the soil (121 °C, 15 min) on three consecutive days with incubations at 37 °C in between and a final incubation at 100 °C for 30 min. to dry.

Preparation of broth cultures and monitoring of $K_2Ni(CN)_4$ degradation

Iron cyanide complexes such as Prussian blue are common contaminants of former industrial sites, such as coke works and manufactured gas plants often giving a blue colouration to the soil (Kjeldsen 1999). While relatively stable at low pH, iron cyanide complexes are soluble at pH > 6, liberating free cyanide into soil solutions (Meeussen et al. 1992). In contrast, $K_2Ni(CN)_4$ is much more toxic due to its higher solubility and because it more readily dissociates (Ghosh et al. 1999). We, therefore, used $K_2Ni(CN)_4$ as a model metal cyanide compound in this study.

All experiments were performed in 100 ml of minimal media (MM) broth as described by Hartmans et al. (1989), except that filter sterilised $K_2Ni(CN)_4$ was the sole source of N, the pH was 7.0. Broths were inoculated with 1 g of topsoil or coke works soil, 0.1 mM $K_2Ni(CN)_4$ and incubated at 25 °C in an orbital shaker at 100 rpm. Experiments performed using topsoil were both with and without glucose (5 mM) addition. All those experiments inoculated with coke works soil required glucose addition, in order to obtain any $K_2Ni(CN)_4$ degradation. Controls were inoculated with the appropriate sterile soil.

The degradation of $K_2Ni(CN)_4$ was measured by following the change in the absorbance at 267 nm in the supernatant (Rollinson et al. 1987). Samples (1 ml) of the broths were centrifuged briefly ($13{,}000 \times g$) to pellet cell and soil matter, the supernatant was then processed with C_{18} Sep pak Cartridges (Waters) to remove humic substances that could interfere with the measurement of $K_2Ni(CN)_4$. Losses of $K_2Ni(CN)_4$ during the extraction were less than 5%.

Isolation of metal-cyanide degrading organisms from coke works soil

Enrichments cultures were prepared using MM containing 0.25 mM $K_2Ni(CN)_4$ and 20 mM glucose that were inoculated with 1 g of coke works soil. They were sub-cultured each week into fresh medium for 4 weeks and pure cultures were obtained by repeated streaking onto minimal media agar plates supplemented with 0.25 mM $K_2Ni(CN)_4$ and 20 mM glucose. To ensure that metal-cyanide degrading isolates were capable of

degrading Prussian Blue as well as $K_2Ni(CN)_4$, isolates were streaked out onto minimal media agar plates supplemented with 0.5 mM $Fe_4[Fe(CN)_6]_3$ (Prussian Blue) and 20 mM glucose. Degradation of the metal-cyanide complex was visualised by decolourisation of the medium, forming halos around the colonies.

Degradation of $Ni(CN)_4^{2-}$ by metal-cyanide degrading organisms isolated from coke works soil

Three purified metal-cyanide degrading isolates (B, E and H) were grown in MM with 20 mM glucose and 20 mM $(NH_4)_2SO_4$. Cells were harvested by centrifugation ($11{,}000 \times g$, r_{av} 9.5 cm, 20 min) washed twice in 1% PBS and were resuspended to an optical density at 540_{nm} of 1.0. Isolates were then added as a 2% inoculum ($\sim 1.9 \times 10^8$ cfu/ml) to MM containing 0.25 mM $K_2Ni(CN)_4$ and 20 mM glucose as the sole nitrogen and carbon sources, respectively. The degradation of $K_2Ni(CN)_4$ was measured by following the change in the absorbance at 267 nm in the culture supernatant as described previously. Bacterial growth was followed by the change in optical density at 540 nm. To assess the efficacy of bioaugmentation on $K_2Ni(CN)_4$ degradation, isolate E was added, as above, to topsoil and coke works soil containing 0.1 mM $K_2Ni(CN)_4$.

Characterisation of metal-cyanide degrading organisms isolated from coke works soil

Chromosomal DNA was extracted from 1 ml of late exponential culture of isolate B using a Puregene DNA isolation kit (Gentra). Chromosomal DNA was extracted from 1.5 ml of late exponential culture of isolates E and H using an adaptation of the methods of Assaf and Dick (1993), to improve DNA yield from actinomycetes (Heath et al. 1986) and a Puregene DNA isolation kit (Gentra).

The 16S rDNA of isolates B, E and H was amplified by PCR using the following primers 16S1 forward AGAGTTTGATCCTGGCTCAG and 16 S3 reverse GGHTACCTTGTTACGACTT (Weisburg et al. 1991). The PCR reaction conditions in each tube and the conditions for thermal cycling were as described by Humphry et al. (2003).The 1.5-kb amplified 16S rDNA product was purified using a SpinPrep PCR clean-up kit (Novagen). The purified product was ligated into the pGEM-T Easy (Promega) cloning vector and transformed into *E. coli* XL-1 Blue (Stratagene). Recombinants were detected by blue-white selection and recombinant plasmids were extracted using a SpinPrep plasmid isolation kit (Merck).

DNA extraction and PCR amplification of 16S rRNA genes from soil enrichments

DNA was extracted from bacteria with an UltraClean Soil DNA kit (MoBio) and stored at −20 °C. PCR amplification of V3 region of the bacterial 16S rRNA gene was performed using Primer 1 (5′-CCTACGGGAGGCAGCAG-3′) and primer 2 (5′-CGCCCGCCGCGCGCGGCG-GGCGGGGCGGGGGCACGGGGGGCCT-3′) (Muyzer et al. 1993). Amplification of soil enrichment DNA was performed with 0.5 μM each primer, 1× *Pfx* amplification buffer, 1 μl template DNA, 0.3 mM each dNTP, 1 mM $MgSO_4$, 1× PCR enhancement solution and 1.25 U Platinum *Pfx* DNA polymerase (Invitrogen) in a 50 μl PCR reaction mixture. The thermocycling conditions used were as follows: 5 min at 94 °C (1 cycle), 94 °C for 1 min. (20 cycles) 65 °C for 1 min (with reduction of 0.5 °C every cycle), 68 °C for 3 min (20 cycles), then 94 °C for 1 min, 55 °C for 1 min., 68 °C for 3 min (10 cycles) with a final extension step at 68 °C for 10 min (1 cycle). All reactions were carried out using the Eppendorf Mastercycler Gradient PCR machine. PCR products were analysed by agarose (1.5% w/v) gel electrophoresis and ethidium bromide staining.

Denaturing gradient gel electrophoresis

DGGE analysis was performed using the D-Code DGGE system (Bio-Rad). PCR products were loaded on to polyacrylamide gels (12%) with a denaturant gradient ranging from 35 to 55% denaturant (with 100% denaturant corresponding to 7 M urea plus 40% v/v formamide). Gels were run at 60 °C for 4.5 h at 200 V and were stained in SYBR Green I (1:10,000 dilution in 1× TAE buffer; Molecular Probes) for 30 min. Stained gels were viewed with UV transillumination and photographed using the Gel Doc 2000 gel documentation system (Bio-Rad).

Statistical analysis

All growth experiments and $K_2Ni(CN)_4$ degradation data in soil were determined from triplicate samples derived from three independent experiments. The DGGE gels were derived from pooled DNA extracted from three independent cultures. Banding reproducibility was confirmed by repeating DGGE in triplicate.

DGGE profiles were analysed using Quantity One 4.1.1 software rolling disc algorithm and the lanes were normalised to ensure all lanes contained the same amount of total signal. Bands were assigned and matched automatically and band assignments were manually checked. Band patterns were analysed using two methods. (i) Correspondence analysis was performed to visualise the relationships among band patterns using Statistica 6.0 software (StatSoft Inc.). Correspondence analysis is a multivariate analysis technique that is used to transform complex data into a new perspective by constructing a new set of variables that are linear combinations of the original variables in the dataset, in this case 16S rRNA gene bands and enrichment treatment (ii) Band pattern data was analysed using Raup and Crick's index of similarity and calculated using the PAST statistical analysis software. The Raup and Crick index of similarity was used to test if similarities observed between soil cultures profile patterns were significantly higher or lower than would be observed by chance (Rowan et al. 2003).

Partial sequencing of the cloned bacterial 16S rDNA fragments

Selected bands that were assessed to have been affected by the different treatments were excised, taking care that only the centre parts of the bands were transferred, and eluted in 50 μl of molecular biology grade water overnight. The eluted DNA was reamplified as described above and electrophoresed with DGGE alongside the original sample to ensure the correct band had been excised. Band excision, PCR and DGGE were repeated until a single band with the correct gel mobility was present. Once this was achieved, the PCR products generated from the DGGE bands were ligated into pCR-blunt vector (Invitrogen) and transformed into One Shot TOP 10 competent cells (Invitrogen). Colonies were screened to ensure clones contained inserts of the correct size and subjected to sequencing. Sequencing was performed using a PE Biosystems sequencer (Lark Technologies, UK). The derived sequences were compared to 16S rRNA gene sequences in the National Centre for Biotechnology Information database using BLAST-N (Altschul et al. 1997).

Results and discussion

Isolation and characterisation of metal cyanide degrading bacteria as candidates for bioaugmentation studies

Bacteria capable of degrading Prussian blue were isolated and purified on self-flagging MM agar. Subsequently, three isolates E, B, and H were selected on the basis of growth rate and their ability to utilise $K_2Ni(CN)_4$ as the sole source of nitrogen. The 16S rDNA gene of each bacterium was sequenced and the organisms were putatively identified, E as a *Rhodococcus* spp., B as a *Pseudomonas* spp. and H as a *Microbacter* spp. Growth rates for E, B and H in MM with glucose and $K_2Ni(CN)_4$ as the sole N source were 0.054, 0.03 and 0.055 h^{-1}, respectively. The most rapid degradation of $K_2Ni(CN)_4$ was observed with the *Rhodococcus* spp. and this bacterium was chosen to be used in the in bioaugmentation experiments.

Previous studies have shown that microorganisms capable of degrading cyanide compounds are common in soil (Barclay et al. 1998; Dhillon & Shivaraman 1999). For example, *Pseudomonas* spp. can degrade a range of metal cyanides including $K_2Ni(CN)_4$ and *Rhodococcus* species degrade a number of organic cyanide compounds can be readily isolated (Brandão et al. 2002; Silva-Avlos et al. 1990).

Degradation of $K_2Ni(CN)_4$ in broth cultures with added soil

The degradation of $K_2Ni(CN)_4$ in an uncontaminated topsoil, without glucose addition, commenced after 70 h. However, providing glucose to the culture reduced the lag phase before the onset of degradation to 30 h. The overall level of degradation was comparable in both treatments (Figure 1). A second experiment used soil obtained from a disused coke works site (Lambton, Tyne &

Wear, UK). Degradation of $K_2Ni(CN)_4$ was observed in this soil after a lag of 50 h but only when 5 mM glucose was added to the culture. Without glucose, no degradation of $K_2Ni(CN)_4$ was observed, even after incubation for several weeks (data not shown).

Glucose has been shown to exert abiotic effects on potassium cyanide in solution under anaerobic conditions. The degradation of cyanide in the presence of a growing culture of a strain of *Klebsiella planticola* was shown to be due to a chemical process dependent upon the presence of a reducing sugar in the medium. The conversion of cyanide to ammonia was independent of any biological factors under these conditions (Hope & Knowles 1991). However, in this study experiments were performed in aerated conditions, and sterile controls containing glucose showed no significant reduction in $K_2Ni(CN)_4$ during the course of the experiments (Figure 1). These observations are in agreement with aerobic studies using other metal cyanide compounds that do not seem to complex with glucose under these conditions (Patil & Paknikar 2000).

The effect of non-specific binding of $K_2Ni(CN)_4$ to the soil was determined using sterile controls. After addition of the $K_2Ni(CN)_4$ to the enrichments there was a very rapid decrease in the concentration of $K_2Ni(CN)_4$ in solution. However, within 60 min the concentration of $K_2Ni(CN)_4$ in solution had stabilised and did not decrease significantly in the controls during the experimental period (Figure 1).

The effect of bioaugmentation on the degradation of $K_2Ni(CN)_4$

The $K_2Ni(CN)_4$ degrading isolate putatively identified by 16S rDNA sequence analysis as a *Rhodococcus* spp. was used to bioaugment enrichment cultures from both top soil and coke work soil. In topsoil the effect of bioaugmentation was not significant in affecting the onset or speed of degradation (Figure 2a) in comparison with non-bioaugmented cultures (Figure 1). When the soil was sterilised prior to the addition of the *Rhodococcus* spp. and/or glucose, it was clear that the bacterium was capable of achieving comparable levels and rates of $K_2Ni(CN)_4$ degradation to those observed in the non-sterile treatments (Figure 2a).

In coke works soil enrichments, glucose addition was essential for any degradative activity. Bioaugmentation gave no discernable effect on the rapidity or level of $K_2Ni(CN)_4$ removal compared to non-augmented cultures. When the soil was sterilised prior to the addition of the *Rhodococcus* spp. the rapidity of $K_2Ni(CN)_4$ removal was significantly reduced compared to the unsterilised cultures (Figure 2b). This suggests that the

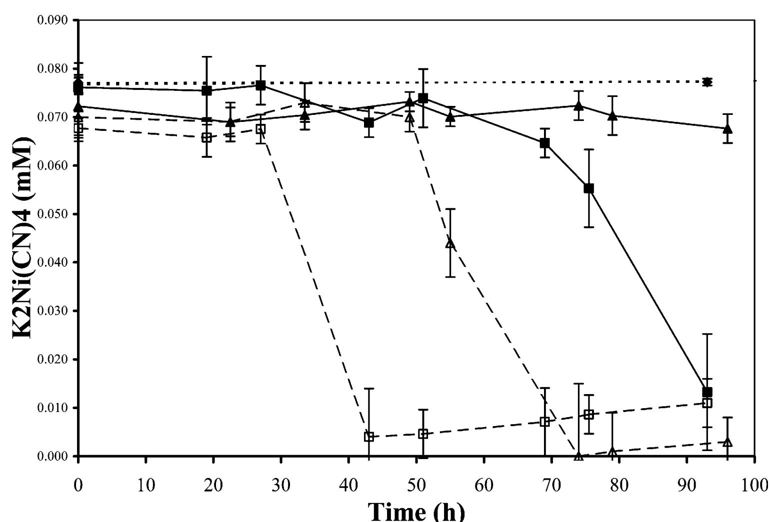

Figure 1. A comparison of the degradation of $K_2Ni(CN)_4$ over time in coke works soil with (Δ) and without (▲) 5 mM glucose; uncontaminated topsoil with (□) and without glucose (■). Sterile controls containing 5 mM glucose for topsoil (◆) and coke works soil (() were also included to identify any abiotic effects.

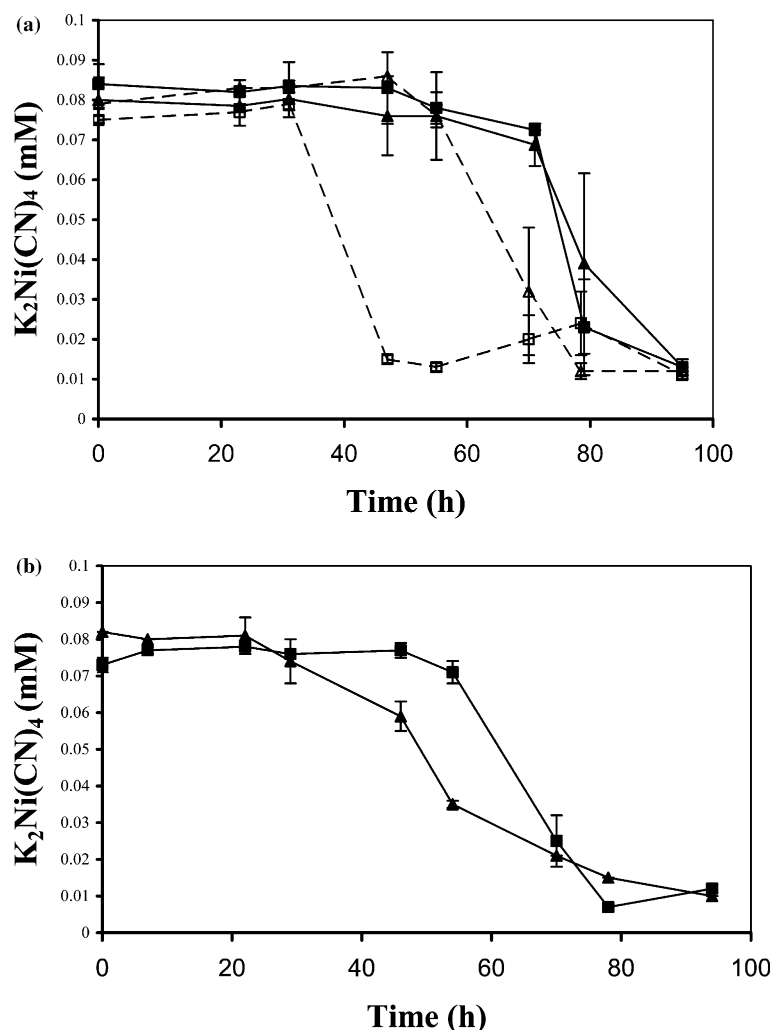

Figure 2. The impact of bioaugmentation of soil with *Rhodococcus* spp on degradation of $K_2Ni(CN)_4$. (a) Showing $K_2Ni(CN)_4$ degradation in bioaugmented topsoil enrichments with (□) and without additional glucose (■). In addition, sterilised topsoil treatments that were subsequently bioaugmented, with (Δ) and without (▲) additional glucose are also shown. (b) Degradation of $K_2Ni(CN)_4$ in non-sterile (■), and sterile (▲) coke soil enrichments.

Rhodococcus spp. activity was less efficient than that of the total bacterial community at $K_2Ni(CN)_4$ degradation in these broth cultures.

Both soils used in the study contained bacteria with catabolic activity against $K_2Ni(CN)_4$. In the highly contaminated soil obtained from the former coke works site, the capacity to degrade cyanide is limited by the availability of a readily utilisable source of carbon. Stimulation of degradation was achieved in both soils by the addition of glucose. Although the coke work soil contains high levels of PAH (data not shown), the failure of $K_2Ni(CN)_4$ to degrade in cultures containing this soil without the addition of glucose (Figure 1) probably reflects how these compounds become sequestered over time and are no longer bioavailable (Nam & Alexander 2001).

The effect of bioaugmenting the soils with a *Rhodococcus* species with demonstrable $K_2Ni(CN)_4$ mineralising activity did not enhance $K_2Ni(CN)_4$ degradation. Such findings are in agreement with the study by Rosseaux et al. (2003) who demonstrated that when the indigenous flora are capable of degrading a atrazine, bioaugmentation will not necessarily enhance the rate of mineralisation. However, other studies have dem-

onstrated that bioaugmentation, with either an individual organism, or a consortium of bacteria can have a positive effect on degradation of pollutants in soil (Dejonghe et al. 2001).

The impact of $K_2Ni(CN)_4$ and bioaugmentation on bacterial diversity and community structure

The DGGE analyses were performed to demonstrate the impact of $K_2Ni(CN)_4$ on the microbial community present in the two soils. The DGGE profile of topsoil enrichments (Figure 3) demonstrated a greater number of bands (30.5 ± 4.1) than those derived from coke works soil (19 ± 3.6). The Shannon–Weaver diversity index (H) was calculated for each treatment, in topsoil enrichments, the diversity was significantly ($p = 0.05$) higher ($H = 3.31 \pm 0.15$) than in coke work soil enrichments ($H = 2.5 \pm 0.26$).

The banding pattern produced by the DGGE profile typically reflect only the most abundant 16S rDNA in the community because low abundance sequences may not be sufficiently amplified to be visible, some partial sequences do not allow two or more species to be resolved and some bacteria carry multiple copies of the gene (Boon et al. 2002). Accepting these limitations two statistical tests were employed, correspondence analysis (CA) and Raup and Crick analysis to help understand the impact of amending the soils with $K_2Ni(CN)_4$, glucose and the *Rhodococcus* spp.

Changes in the level of expression in a number of bands were observed as a result of different treatments. For example, in the coke work soil enrichment all the treatments were augmented with glucose, however, the addition of $K_2Ni(CN)_4$ and both $K_2Ni(CN)_4$ and *Rhodococcus* spp. produced changes in the DGGE profile compared to the control (Figure 3b). Each treatment gave unique bands, for example, bands 10 to 12 and 15 to 18 in the control and 8 and 14 in the bioaugmented culture.

The most significant finding of the DGGE analysis indicated that the *Rhodococcus* spp. established itself in the bacterial community of broths containing both top and coke works soil (Figure 3a and b). Although its impact on cyanide degradation was not marked, it had an impact on the composition of both the bacterial communities.

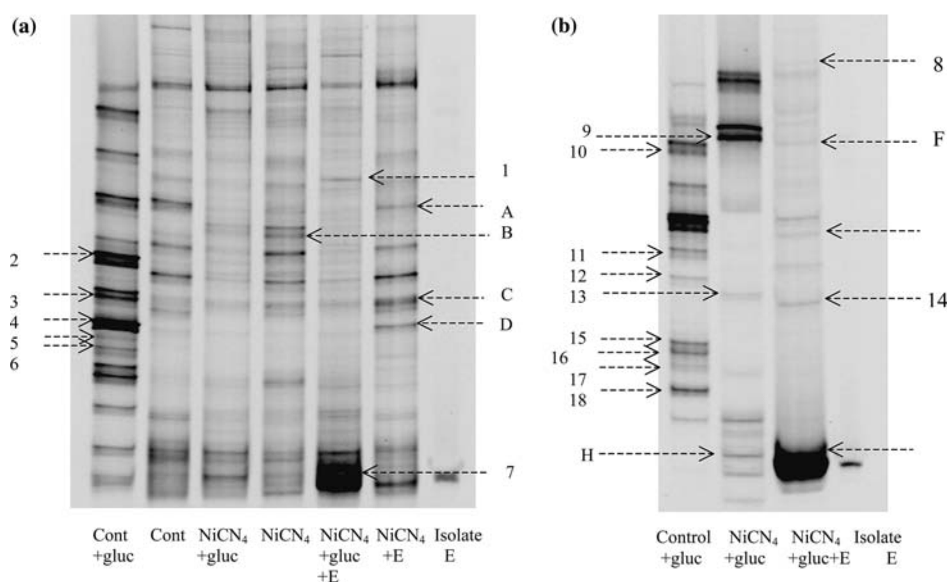

Figure 3. DGGE profiles of bacterial communities found in topsoil (a) and coke work soil (b) enrichments. Lanes labelled Cont. indicate control lanes; $NiCN_4$ indicates those enrichments spiked with 0.1 mM $K_2Ni(CN)_4$; addition of 5 mM glucose is indicated by +gluc; E indicates bioaugmentation with *Rhodococcus* spp. In topsoil enrichments (a) sequenced bands A, B and C were identified as *Pseudomonas* spp. D as a *Frateuria* spp. In coke soil enrichments band F was identified as a *Burkholderia* spp. G as a *Pseudomonas* spp. and H as a *Bacillus* spp. Bands indicated by numbers were identified as being of significance in the correspondence analysis (Figures 4a and 5).

Using correspondence analysis to analyse how the banding pattern between soil treatments varied we found that in the topsoil enrichments the most significant effect on the DGGE profile was the addition of glucose. When added to the control treatment the banding pattern was the most distinct from the other treatment profiles in the first dimension of the CA analysis (Figure 4a). The remaining profiles were separated along the second dimension, the bands responsible for producing the clusters observed in the CA are identified (Figures 3a and 4a). The bioaug-

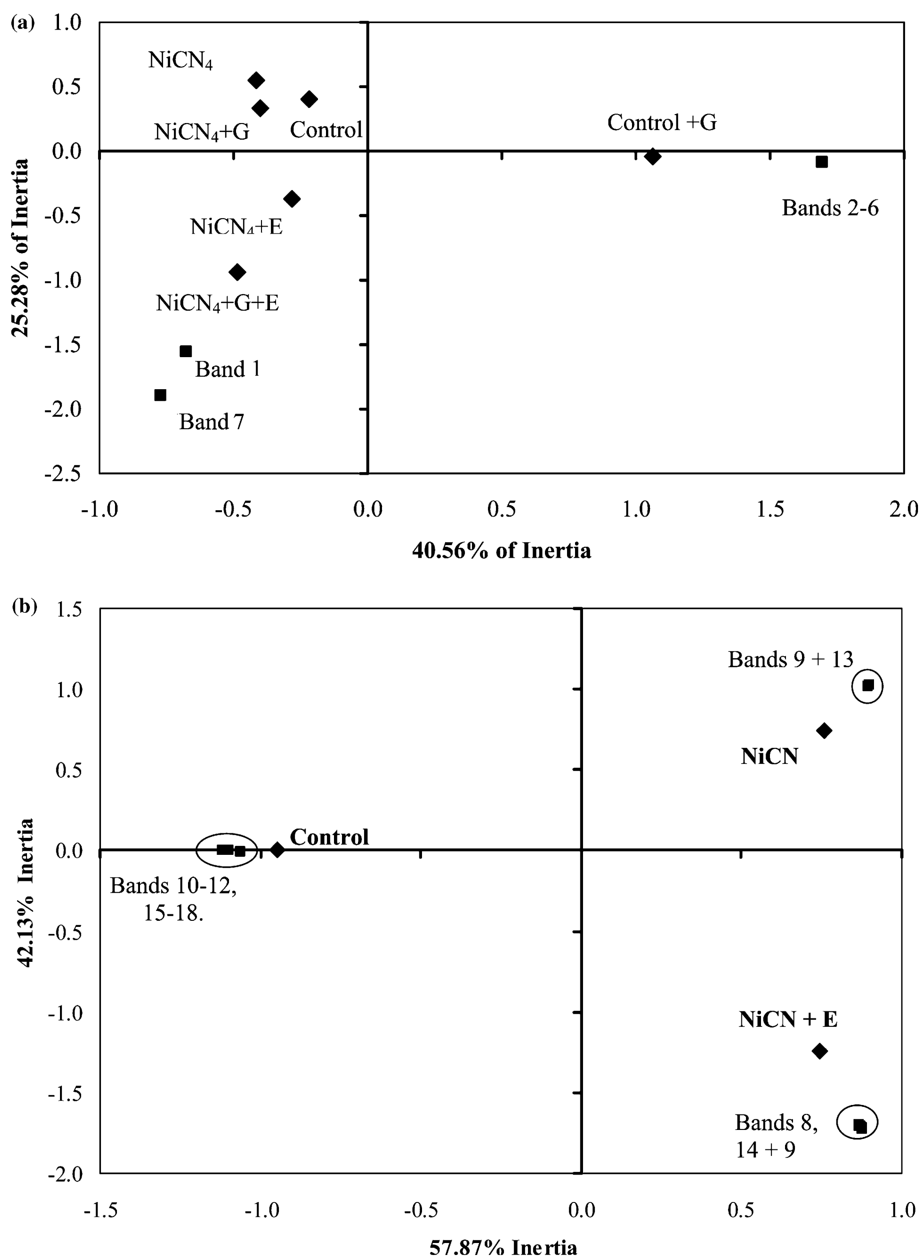

Figure 4. (a) The correspondence analysis of the community profiles from topsoil enrichments. (b) The correspondence analysis of the coke soil enrichment profiles. $NiCN_4$ indicates those enrichments spiked with 0.1 mM $K_2Ni(CN)_4$; addition of 5 mM glucose is indicated by +gluc; E indicates bioaugmentation with *Rhodococcus* spp. The bands responsible for the variance giving the patterns observed are indicated.

mented culture profiles formed a distinct cluster along this axis.

The coke work soil enrichment was also analysed using CA, the first dimension explained ~58% of the inertia and separated the banding pattern of the control enrichment augmented with glucose from the soil cultures containing cyanide (Figure 4b). The cyanide containing soil demonstrated patterns that were separated in the second dimension that explained 42% of the inertia, the bands responsible for the distribution seen in the analysis were plotted and it is clear that the bands responsible for the inertia cluster into three distinct groups that were unique to the treatment that the samples were subjected to (Figure 3b).

In order to determine whether the pattern of bands observed in the enrichment cultures were statistically significant the Raup and Crick similarity index was determined, to enable evaluation of the DGGE profiles (Table 1.). Essentially, this analysis indicates whether the DGGE data correspond to populations that have arisen randomly or whether the similarities or dissimilarities are more than can be accounted for by chance (Rowan et al. 2003). The results of these analyses in the topsoil showed that in those treatments that had not been supplemented with glucose the profiles were not significantly different from each other irrespective of other additions (Table I). In other words, the Raup and Crick analysis indicated the addition of cyanide and or bioaugmentation had no significant effect on the bacterial community development in the topsoil. Moreover, the addition of glucose to soil did not result in significant changes between their profiles and those of other treatments in all but three cases, specifically the soil amended with $K_2Ni(CN)_4$ and $K_2Ni(CN)_4$ plus the *Rhodococcus* spp. and their counterparts that had been supplemented with glucose these were significantly similar. The only treatments that had a significantly different profile, were those that contained additional $K_2Ni(CN)_4$ compared to control broths that had had glucose added.

The coke works soil enrichment DGGE profiles were also subjected to analysis using the Raup and Crick similarity index (Table I). The analyses indicated that in soils to which cyanide was added and those additionally bioaugmented with *Rhodococcus* spp. the population of bacteria present could have arisen by chance matching of the bands. However, both these treatments had population profiles that were significantly different from that of the control treatment (Table 1).

This study has shown that the addition of $K_2Ni(CN)_4$, glucose and bioaugmentation with the *Rhodococcus* spp. can lead to qualitative and quantitative changes in the bacterial communities in the two soils studied. Analysing how these populations change using correspondence analysis, that indicates variance of the DGGE banding pattern in a treatment from the 'average' pattern, illustrates that $K_2Ni(CN)_4$ addition, bioaugmentation or the addition of glucose can produce patterns that cluster on the basis of the presence of $K_2Ni(CN)_4$ or the *Rhodococcus* spp. (Figure 4a). However, the Raup and Crick analysis suggests

Table 1. A comparison of similarity values (S_{RC}) for the topsoil and coke works soil DGGE data

Topsoil	Control + G	Control	NiCN + G	NiCN	NiCN + G + E	NiCN + E
Control + G	1	0.5275	0.2575	0.005	0.2925	0.705
Control		1	0.81	0.85	0.51	0.925
NiCN + G			1	0.9575	0.5125	0.6125
CN				1	0.4125	0.5825
NiCN + G + E					1	0.9975
NiCN + E						1
Coke work soil						
Control + G	1	ND	0.02	ND	0.02	ND
NiCN + G		ND	1	ND	0.28	ND
NiCN + G + E		ND		ND	1	ND

NiCN = $K_2Ni(CN)_4$; G = glucose; and E = isolate E (*Rhodococcus* spp.); ND = not determined. If 0.95 < S_{RC} < 0.05 similarity no greater than expected by chance (null hypothesis upheld), S_{RC} < 0.05 significant dissimilarity, S_{RC} < 0.95 significant similarity (null hypothesis fails).

that this variance is not significant in most cases. Indeed, it would appear that in topsoil the bacterial population development is particularly robust. We have found that in the absence of added glucose neither $K_2Ni(CN)_4$ or *Rhodococcus* spp. had any significant impact in changing the bacterial population. Moreover, even adding glucose only reinforced the similarities in all but one comparison of experimental treatments where a significant effect was observed (Table 1). In contrast, in coke works soil the addition of $K_2Ni(CN)_4$ and/or *Rhodococcus* spp. gave significantly different population profiles to the controls. This differential effect of contamination or bioaugmentation may reflect that conditions in stressful environments, such as the coke works soil, result in communities that are unstable. The lack of diversity compared to the topsoil cultures may result in environmental perturbations having a much more profound effect on community structure. Similar studies looking at 4-chlorophenol contaminated soils noted changes in community structure after the soil had been inoculated with a 4-chlorophenol degrading *Arthrobacter*, that may have reflected adjustments in the community structure favouring the populations that are better at coping with the stress caused by the contaminant (Jernberg & Jansson 2002).

Identification of bacterial isolates in the soil enrichments

The composition of the bacterial community in the different enrichments was also investigated. The majority of bacteria identified from the topsoil belonged to the γ proteobacteria, three of which (A, B and C) were identified as *Pseudomonas* spp. In bioaugmented treatments the *Rhodococcus* spp. became established and could be identified as an abundant member of the community (Figure 3a). In addition, a band corresponding to that of the *Rhodococcus* spp. was identified in the treatments subjected to cyanide addition but not bioaugmented (Figure 3a lane 2 and 3) indicating that the organism may be an indigenous component of the soil flora. In the coke works soil those bacteria identified belonged to the γ proteobacteria (F and G) as well as a *Bacillus* spp (H) (Figure 3b). The bioaugmented *Rhodococcus* spp. also established itself in these soils, however, it was not detected in non-bioaugmented treatments, suggesting that it is not a dominant member of the flora in such soils.

Conclusions

The results of this work indicate that in a uncontaminated soil with a high biodiversity the addition of $K_2Ni(CN)_4$ and/or *Rhodococcus* spp. did not significantly affect the bacterial community development. In contrast, in the coke works soil the community structure was subject to significant change by the addition of $K_2Ni(CN)_4$ and *Rhodococcus* spp. It would appear that in the contaminated coke works soil the bacterial community structure is much less robust than that observed in uncontaminated topsoil. Despite having no significant effect on the rate of $K_2Ni(CN)_4$ degradation the inoculated *Rhodococcus* spp. became a significant member of the community in both soils, particularly in those cultures supplemented with glucose. Therefore, in this limited sense, bioaugmentation was successful in both soils, given that the aim of such intervention is to manipulate the dominant organisms in order to establish the cyanide degrading activity as part of the active flora (Dejonghe et al. 2001). This suggests that such a strategy may be effective in manipulating the bacterial community in cyanide-contaminated soils. More detailed soil microcosm experiments to investigate the role of bioaugmenting soils contaminated with $K_2Ni(CN)_4$ are currently being undertaken.

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References

Altschul SF, Madden TL, Schaffer AA, Zhang JH, Zhang Z, Miller W & Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic Acids Res. 25: 3389–3402

Assaf NA & Dick WA (1993) Spheroplast formation and plasmid isolation from *Rhodococcus* spp. Biotechniques 15: 1010–1014

Barclay M, Hart A, Knowles CJ, Meeussen JCL & Tett VA (1998) Biodegradation of metal cyanides by mixed and pure cultures of fungi. Enzyme Microb. Technol. 22: 223–231

Boon N, Windt WDe, Verstaete W & Top EM (2002) Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. FEMS Microbiol. Ecol. 39: 101–112

Brandão PFB, Clapp JP & Bull AT (2002) Discrimination and taxonomy of geographically diverse strains of nitrile-metabolizing actinomycetes using chemometric and molecular sequence techniques. Environ. Microbiol. 4: 262–276

Dejonghe W, Boon N, Seghers D, Top EM & Verstraete W (2001) Bioaugmentation of soils by increasing microbial richness: missing links. Environ. Microbiol. 3: 649–657

Dhillon JK & Shivaraman N (1999) Biodegradation of cyanide compounds by a *Pseudomonas* species (S1). Can. J. Microbiol. 45: 201–208

Dorr PK & Knowles CJ (1989) Cyanide oxygenase and cyanase activities of *Pseudomonas fluorescens* NCIMB 11764. FEMS Microbiol. Lett. 60: 289–294

Dursun AY, Calik A & Aksu Z (1999) Degradation of ferrous(II) cyanide complex ions by *Pseudomonas fluorescens*. Proc. Biochem. 34: 901–908

Figueira MM, Ciminelli VST, Andrade MCde & Linardi VR (1996) Cyanide degradation by an *Escherichia coli* strain. Can. J. Microbiol. 42: 519–523

Finnegan I, Toerien S, Abbot L, Smit F & Raubenheimer HG (1991) Identification and characterisation of an *Acinetobacter* spp. capable of assimilation of a range of cyano-metal complexes, free cyanide ions and simple organic nitriles. Appl. Microbiol. Biotechnol. 36: 142–144

Ghosh RS, Dzombak DA, Luthy RG & Nakles DV (1999) Subsurface fate and transport of cyanide species at a manufactured-gas plant site. Water Environ. Res. 71: 1205–1216

Hartmans S, Smits JP, Werf MJVan de, Volkering F & Bont JAMde (1989) Metabolism of styrene oxide and 2-phenylethanol in the styrene-degrading *Xanthobacter* strain 124X. Appl. Environ. Microbiol. 55: 2850–2855

Heath LS, Sloan GL & Heath HE (1986) A simple and generally applicable procedure for releasing DNA from bacterial cells. Appl. Environ. Microbiol. 51: 1138–1140

Hope KM & Knowles CJ (1991) The anaerobic utilisation of cyanide in the presence of sugars by microbial cultures can involve an abiotic process. FEMS Microbiol. Lett. 80: 217–220

Humphry DR, Black GW & Cummings SP (2003) Reclassification of *Pseudomonas fluorescens* subspecies *cellulosa* NCIMB 10462 (Ueda et al, 1952) as *Cellvibrio japonicus* T sp. nov. and revival of *Cellvibrio vulgaris* sp. nov., nom. rev. and *Cellvibrio fulvus* sp. nov., nom. rev. Int. J. Syst. Evol. Microbiol. 53: 393–400

Jernberg C & Jansson JK (2002) Impact of 4-chlorophenol contamination and/or inoculation with the 4-chlorophenol-degrading strain, *Arthrobacter chlorophenolicus* A6L, on soil bacterial community structure. FEMS Microbiol. Ecol. 42: 387–397

Kjeldsen P (1999) Behaviour of cyanides in soil and groundwater: a review. Water Air Soil Pollut. 115: 279–307

Kunz DA, Fernandez RF & Parab P (2001) Evidence that bacterial cyanide oxygenase is a pterin-dependant hydroxylase. Biochem. Biophys. Res. Commun. 287: 514–518

Meeussen JCL, Keizer MG, Vanreimsdijk WH & Dehaan FAM (1992) Dissolution behaviour of iron cyanide (prussian blue) in contaminated soils. Environ. Sci. Technol. 26: 1832–1838

Muyzer G, Dewaal EC & Uitterlinden AG (1993) Profiling of complex microbial populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction amplified genes coding for 16S ribosomal RNA. Appl. Environ. Microbiol. 59: 695–700

Nam K & Alexander M (2001) Relationship between biodegradation rate and the percentage of a compound that becomes sequestered in soil. Soil Biol. Biochem. 33: 787–792

Patil YB & Paknikar KM (2000) Biotoxification of silver-cyanide from electroplating industry wastewater. Lett. Appl. Microbiol. 30: 33–37

Rollinson G, Jones R, Meadows MP, Harris RE & Knowles CJ (1987) The growth of a cyanide-utilising strain of *Pseudomonas fluorescens* in liquid culture on nickel cyanide as a source of nitrogen. FEMS Microbiol. Lett. 40: 199–205

Rousseaux S, Hartmann A, Lagacherie B, Piutti S, Andreux F & Soulas G (2003) Inoculation of an atrazine-degrading strain, *Chelobacter heintzii* Cit1, in four different soils: effects of different inoculum densities. Chemosphere 51: 569–576

Rowan AK, Snape JR, Fearnside D, Barer MR, Curtis TP & Head IM (2003) Composition and diversity of ammonia-oxidising bacterial communities in wastewater treatment reactors of different design treating identical wastewater. FEMS Microbiol. Ecol. 43: 195–206

Shpak VE, Podolskaya VI, Ulberg ZR & Shpak EA (1995) Degradation of metal-cyanide complexes in microbe dispersions. Colloid J. 57: 102–105

Silva-Avalos J, Richmond MG, Nagappan O & Kunz DA (1990) Degradation of the metal-cyano complex tetracyanonickelate(II) by cyanide-utilizing bacterial isolates. Appl. Environ. Microbiol. 56: 3664–3670

Thomas AO, Johnston PM & Lester JN (1991) The characterization of the subsurface at former gasworks sites in respect of *in situ* microbiology, chemistry and physical structure. Hazard. Waste Hazard. Mater. 8: 341–365

Thomas AO & Lester JN (1994) The reclamation of disused gasworks sites: New solutions to an old problem. Sci. Tot. Environ. 152: 239–260

Weisburg WG, Barns SM, Pelletier DA & Lane DJ (1991) 16S Ribosomal DNA amplification for phylogenetic study. J. Bacteriol. 173: 697–703

Young YC & Theis TL (1991) Determination of cyanide in manufactured-gas plant purifier wastes. Environ. Technol. 12: 1063–1069