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Sustainable remediation – The application of bioremediated soil for use in the degradation of TNT chips

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ABSTRACT

Environmental contamination by TNT (2,4,6 trinitrotoluene), historically used in civilian industries and the military as an explosive is of great concern due to its toxicity. Scientific studies have however shown that TNT is susceptible to microbial transformation. The aim of this study was to assess the potential of a previously bioremediated hydrocarbon contaminated soil (PBR) to increase TNT degradation rates. This was investigated by adding TNT chips to PBR and uncontaminated soils (PNC) in laboratory based studies (up to 16 weeks). Residual TNT chip analysis showed greater TNT degradation in PBR soils (70%) and significantly higher metabolic rates (4.5 fold increase in cumulative CO_2 levels) than in PNC soils (30%). Molecular analysis (PCR-DGGE-cluster analysis) showed substantial shifts in soil microbial communities associated with TNT contamination between day 0 and week 4 especially in PBR soils. Bacterial communities appeared to be more sensitive to TNT contamination than fungal communities in both soils. Quantitative PCR analysis showed ~3 fold increase in the abundance of nitroreductase genes (*pnrA*) in PBR soils with a gradual reduction in community evenness (Pareto-Lorenz curves) in contrast to PNC soils. These results suggest that microbial response to TNT contamination was dependent on the history of soil use. The results also confirm that the microbial potential of waste soils such as PBR soil (usually disposed of via landfill) can be successfully used for accelerated TNT chip degradation. This promotes sustainable re-use of waste soils extending the life span of landfill sites.

1. Introduction

TNT (2,4,6 trinitrotoluene) has been historically used in civilian industries and the military as an explosive. A review of literature by El Fantroussi and Agathos (2005) has shown that the use of TNT can cause contamination of both soil and aquatic (groundwater) environments with TNT contamination levels of up to 700,000 mg kg^{-1} being detected in military sites in the US and Europe (Boopathy et al., 1998a; Conder et al., 2004; Elovitz and Weber, 1999; Green et al., 1999; Hovatter et al., 1997). Typical locations of sites contaminated with TNT include manufacturing plants, munitions storage locations, ordnance disposal and military locations (Esteve-Nunez et al., 2001; Manning, 1999). Environmental contamination with TNT is of concern due to its toxicity to living systems and persistence in the environment. TNT contamination has been linked to mutagenicity in organisms at different trophic levels with associated toxic effects on their reproductive systems, immune systems, reduction in biomass and alterations in microbial diversity (Best et al., 2008; Johnson et al., 2000; Letzel et al., 2003; Travis et al., 2008). Therefore, detoxification of TNT contaminated environments is crucial before such environments can be used for other purposes.

Studies have shown that TNT is susceptible to biotransformation under aerobic and anaerobic conditions (Esteve-Nunez et al., 2001; In et al., 2008). This susceptibility to biological (microbial) degradation can be exploited for decontamination purposes. TNT degradation involves a variety of enzymes such as type 1 nitroreductases, hydrogenases and ring hydroxylating dioxygenases (Smets et al., 2007). Nitroreductase enzymes (genes) (Bryant et al., 1981) are especially important because of their crucial role in the detoxification of nitro-aromatic compounds and use in assessing TNT degradation potential in bioremediation (Caballero

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et al., 2005; Cho et al., 2009; Hannink et al., 2001). Different bacterial species such as *Pseudomonas* strains can produce nitroreductase (PnrA and PnrB). Other microbial enzymes such as PETN reductase (French et al., 1998) and NsfA and NsfB (Caballero et al., 2005; Salazar-Salinas, 2010) are also involved in TNT degradation.

The application of biological methods such as bioremediation for TNT detoxification offers a number of advantages, namely lower labour, infrastructure and production costs (Louisa Wessels, 2010). There are other additional benefits to waste management processes as waste products such as manure and sewage sludge can be used in treating TNT contaminated soils (In et al., 2007; Rezaei et al., 2010). Bioremediation of contaminated environments (compared to physical and chemical methods) is more environmentally friendly and economical; for example a comparison between windrow composting and incineration cost found windrow composting was 40–50% cheaper at 1200–30,000 ton scale (Craig et al., 1995). These benefits therefore make bioremediation a highly competitive remediation strategy and a range of bioremediation methodologies has been successfully applied to TNT contaminated soil. These include the use of slurry (Boopathy et al., 1998b,c), composting (Rezaei et al., 2010), land farming (Clark and Boopathy, 2007), bioaugmentation and biostimulation methods and the use of transgenic plants (van Dillewijn et al., 2008; Wang et al., 2009).

The use of treated waste soils for TNT bioremediation is a biological method that has not been extensively utilised in TNT detoxification. Treated or previously bioremediated waste soil can have an enhanced microbial community for the degradation of the original contaminant (such as hydrocarbon) in comparison to a non-contaminated soil. Harnessing this capacity to successfully treat new hydrocarbon contaminants has been reported (Aleer et al., 2011; Makadia et al., 2011) although this same principle has not been widely applied to TNT contamination. However, it is possible that the enhanced capacity to degrade hydrocarbons found in treated hydrocarbon contaminated waste soils can be harnessed to treat TNT contamination because some microbial groups that degrade hydrocarbons can also bio transform TNT (Boopathy, 2009). For example *Stenotrophomonas maltophilia* and *Sphingomonas sanguinis* can degrade TNT (Boopathy, 2009; Oh and Kim, 1998; Rahal and Moussa, 2011) with *Stenotrophomonas* and other members of the *Sphingomonas* group being able to degrade hydrocarbon as well (Snellinx et al., 2003; Zhang et al., 2010). *Escherichia coli* cultures which have been used for the bioremediation of hydrocarbons are also capable of using TNT as a nitrogen source if glucose is present (González-Pérez et al., 2007; Zhang et al., 2010).

Since prior contact with hydrocarbon contaminants can increase the waste soils' hydrocarbon degrading capacity, the use of such soils for treating new toxic contaminants is desirable as it allows the beneficial re-use of the enhanced microbial capacities for new contaminant degradation. With hydrocarbon contaminated waste soils being more readily available in Australia (than those with TNT), it makes more economic sense to use them provided that this enhanced microbial capacity can be successfully utilised for TNT removal. Re-use of waste soils (instead of putting them in landfills) also fits into the Australian Environmental Protection Authority (Waste to Resources) Policy (2010) which advocates waste minimisation and discourages landfill disposal.

The molecular analysis of the soil is important in order to determine the effectiveness of any bioremediation strategy and also to assess its effect on the microbial community (Ciric et al., 2010; Muyzer and Smalla, 1998). Different molecular tools such as PCR, Quantitative PCR, molecular fingerprinting (such as Denaturing Gradient Gel Electrophoresis), cloning and sequencing can be used to assess these changes or effects (Muyzer and Smalla, 1998; Ning et al., 2009). For example, DGGE analysis of bacterial communities in TNT contaminated soil has shown that a shift towards degraders of TNT occurred over time (George et al., 2008). However, comparatively little research has been conducted on changes in the fungal community in TNT contaminated soil using DGGE. Fungi can also play major roles in the degradation of TNT and understanding changes in the fungal community can enhance the understanding of how the fungal community responds to TNT in soil (Bayman and Radkar, 1997; Gao et al., 2010).

This study is therefore aimed at investigating the suitability of using the enhanced potential in bioremediated soil to degrade TNT. This will involve assessing the rate of TNT degradation in waste soils by monitoring the soil metabolic rates (CO_2) and the residual amount of TNT in soil in laboratory based experiments. In addition, changes in the diversity of the soil microbial (bacterial and fungal) community during TNT degradation will be assessed through the use of DGGE and Quantitative PCR.

2. Materials and methods

2.1. Soil collection, analysis and pre-treatment

Two types of soils were used in this study. The first type was a bioremediated hydrocarbon contaminated soil (PBR, treated waste soil) which was obtained from an eight month old soil pile in South Australia. The soil was part of a completed bioremediation project where biostimulation was employed in lowering the TPH level (to 6000 mg kg^{-1}) suitable for landfill disposal. The soil (50 kg) was collected and stored at 4 °C prior to the start of the experiments. The second soil which was similar to the first soil type was obtained from a site that had no history of hydrocarbon contamination (PNC). The collected soils were sieved (0.5 cm) to remove stones and large rocks. Preliminary analyses of the sieved soils were then carried out to determine soil pH and soil characteristics such as moisture content, and organic matter content using standard methods.

2.2. Microcosm set up (PNC and PBR soils)

Soil microcosms (3 L sealed containers) containing soil (1.5 kg) were set up in replicates at 40% Soil Water Holding Capacity to compare the degradation rate of TNT (applied as chips >3 mm in length) (Fig. 1a) between clean (PNC) and bioremediated (PBR) soils. The Redfield ratio (C/N/P) was also calculated in order to determine the amount of soil nutrient supplementation that would be required (Cleveland and Liptzin, 2007). Based on this, sulphur phosphate (14 g kg^{-1} soil) was added to each vessel to act as phosphorus source, limiting additional nitrogen and carbon sources to the TNT present in the soil. Each soil was then spiked with TNT chips at 10 g kg^{-1} of soil (dry wt) with the soil being thoroughly mixed with the chips. Control microcosms were set up in the same way without the addition of TNT for both clean and bioremediated soils (Table 1). All the microcosms were mixed periodically and water added as needed to maintain the desired soil moisture content. The rate of CO_2 evolution was then measured in the microcosms throughout the experimental period and samples were obtained from the microcosms at pre-determined time intervals (day 0, weeks 4, 8, 12 and 16) for molecular analysis. Replicate microcosms made up of clean and bioremediated soils were also set up as previously described to monitor TNT chips degradation and these were only sampled on day 0 and week 16 for residual TNT chip analysis.

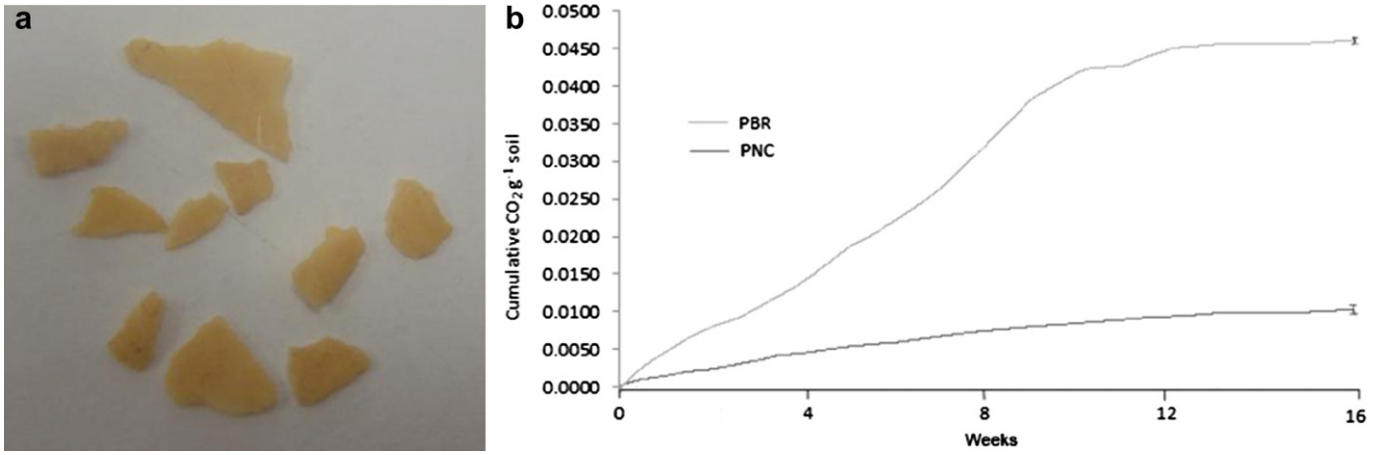

Fig. 1. Samples of TNT chips (a) and a comparison of cumulative CO_2 levels in bioremediated (PBR) and clean (PNC) soils over 16 weeks (b). For (b), $n = 3$.

2.3. Measurement of TNT degradation in soil

Two methods were used for the assessment of TNT biodegradation over 16 weeks. The first method involved CO_2 measurement while the second method involved determining the residual weight of TNT chips in soil. The CO_2 produced from each vessel was used as a measure of the activity of the soil microbial community in terms of TNT degradation. CO_2 concentration (mg kg^{-1}) was recorded on an hourly basis using a WMPA4 infrared gas analyser (PP Systems, Hitchin, UK). Throughout the degradation experiment, the background CO_2 level was also determined in controls set up with phosphate supplemented bioremediated and non-contaminated (clean) soils. A multi-channel switch was connected between the vessel and WMPA4 and used to switch between each vessel to allow for hourly measurement from each chamber. The data was logged using Summerbreeze 1.04 Software (written specifically for the system) which saved each hourly reading from the WMPA4. The air intake (1.2 L per a minute) was passed through a sofonolime filter to adsorb the CO_2 prior to passage through the mesocosm; this filter was changed fortnightly. The readings obtained from the control microcosms were deducted from the CO_2 readings from the respective bioremediated and clean soil microcosms (with TNT chips). The normalised values from bioremediated (PBR) and clean (PNC) soils were then plotted over time. The total amount of carbon added to the soil by the addition of TNT was also calculated and compared with the total amount of C released as CO_2 from each microcosm over the 16 week incubation. The second method involved the removal of TNT chips from soil. Aliquots of homogenised soil (100 g) was sieved using a 2 mm sieve, this was repeated three times for each soil. The soil was removed from the sieved TNT chips by shaking the soil-TNT mixture in 100 ml of water. The supernatant was discarded and the wash was repeated three times. TNT chips were then manually separated from soil particles and dried to constant weight at 37 °C before being weighed. The weight of the TNT chips at week 16 were compared to the initial weight on day 0 and expressed as a percentage of total TNT chips recovered.

Table 1
Description of soil microcosms used for TNT degradation assays.

Soil treatment	TNT	Phosphate
Non previously contaminated soil (PNC)	+	+
Bioremediated soil (PBR)	+	+
Control, Non previously contaminated soil	−	+
Control, Bioremediated soil	−	+

2.4. DNA extraction, polymerase chain reaction and DGGE

DNA was extracted from the soil (0.25 g) using a MoBio Power Soil DNA extraction kit according to the manufacturer's instructions (MoBio Laboratories Inc, Carlsbad, CA, USA). Bacterial and fungal primers were then used for PCR of the extracted DNA. The bacterial community was analysed using primers 341F GC and 518R (Muyzer et al., 1993) using a thermocycling program consisting of 5 min of denaturation at 95 °C and 30 cycles of 2 min at 95 °C, 1 min at 55 °C and 1 min at 72 °C, followed by an extension step at 72 °C for 10 min. Primers ITS1, ITS1F-GC, ITS2 and ITS4 (Anderson and Parkin, 2007) were used to analyse the fungal community. A nested PCR involving the use of ITS1 and ITS 4 PCR products as amplicons for ITS1F-GC and ITS2 reactions was then carried out (Anderson and Parkin, 2007). DGGE analysis was conducted on a Universal Mutation Detection System Dcode system using a 9% polyacrylamide gel (Bio-Rad Laboratories). Bacterial PCR amplicons were ran on a 40%–60% gradient, and fungal PCR amplicons on a 40%–55% gradient. The DGGE gels were ran at 60 V at 60 °C for 20 h and then silver stained (Girvan et al., 2003). After staining, DGGE gels where then scanned and analysed using TotalLab TL120 (Phoretix Ltd, UK).

2.5. Band excision, cloning and sequencing

Bands of interest on the bacterial community profile were excised and incubated in sterile MilliQ water overnight at 50 °C for DNA elution. Two microlitres of the eluted DNA was used as template for PCR reaction in a total volume of 25 μL consisting of 12.5 μL Gotaq master mix (Promega®; Australia); 1 μL (10 μM) each of forward (314F) and reverse primer (518R); 10.5 μL nuclease free water (Promega®; Australia) using the previously described thermocycling condition. PCR products was checked for specificity by electrophoresis in agarose gel and 1 μL of the product cloned into PCR 4 TOPO® vector supplied with the TOPO TA Cloning® kit for sequencing (Invitrogen™ Australia) according to the manufacturer's instruction. Positive clones were selected on nutrient agar (Oxoid; Australia) plates containing 100 μg L^{-1} ampicilin and cloned inserts verified by PCR according to instructions supplied with the cloning kit. The PCR amplicons were purified using the Wizard® SV Gel and PCR Clean-Up System (Promega® Australia) and sequenced. Retrieved sequences were analysed using the

BLASTN algorithm of the National Center for Biotechnology Information (NCBI) database to determine their putative identities.

2.6. Quantitative PCR

Quantitative PCR was performed to assess the effects of TNT contamination on the abundance of *pnrA* genes in PBR and PNC soils. This was carried out on a Bio-Rad iQ5 real time PCR detection system (Bio-Rad, USA, 2000). 16S rRNA genes were analysed with primers 341F and 518R (Muyzer et al., 1993) while *pnrA* genes were analysed with primers PnrART52 and PnrART3 (Caballero et al., 2005) using SYBR Green supermix from Bio-Rad. The optimized reaction condition used for 341F and 518R was 95 °C for 5 min, 40 cycles of 95 °C for 10 s, 55 °C for 30 s and 72 °C for 30 s, and then 95 °C for 1 min, 55 °C for 1 min, 81 cycles from 55 to 95 °C for 1 s with an increase of 0.5 °C after every second cycle for generating melting curve in order to check that non-specific fragments were not amplified. The program for amplifying *pnrA* was 95 °C for 7 min, 40 cycles of 95 °C for 15 s, 55 °C for 30 s, 72 °C for 20 s followed by the melting curve cycle earlier described. The amplification efficiencies of the standard curves of both 16S rRNA and pnrA genes were close (97–99%).The *pnrA* target was therefore normalized to the 16S rRNA target of the same sample using the formula; $\Delta C(t)_{\text{sample}} = \text{average } C(t)_{\text{pnrA}} - \text{average } C(t)_{\text{16S rRNA}}$. For the $2^{-\Delta\Delta C(t)}$ analysis, the normalized samples were referenced to time 0 values (calibrator) in order to deduce the fold increase of target *pnrA* using the formula $\Delta\Delta C(t)_{\text{sample}} = \Delta C(t)_{\text{sample}} - \Delta C(t)_{\text{time 0}}$ and the ratio of *pnrA* genes relative to time 0 estimated with $2^{-\Delta\Delta C(t)}$ (Livak and Schmittgen, 2001) (Treusch et al., 2005) (Table S1 in supplementary information).

2.7. Statistical analysis

Digitized DGGE gels were analysed using TotalLab TL120 in order to assess changes in microbial communities associated with TNT contamination. The similarities between microbial communities in clean and bioremediated soils were expressed as similarity clusters by means of the unweighted pair group method with mathematical averages (UPGMA). Changes in community evenness and functional organization were determined with Pareto–Lorenz (PL) distribution curves curve (Marzorati et al., 2008). For PL curves, DGGE gels were analysed using TL120 and band intensities were ranked from high to low based on their Gaussian values and the normalized cumulative intensities of these bands plotted on *y*–axis. The cumulative normalized band numbers were plotted on *x* axis. The community was analysed at 20% (0.2 × axis intercept) of the population to determine the proportional cumulative intensities with a line of perfect evenness drawn at 45% (Marzorati et al., 2008).

3. Results

3.1. Soil characteristics

The physicochemical characteristic of the clean (PNC) and bioremediated (PBR) soils showed that they were sandy loam soils (76% sand and 24% loam), pH (8) and showed similar moisture content (15–16%) at the time of sampling. The organic matter content of the two soils was varied (4% for PNC and 3% for PBR).

3.2. Measurement of TNT degradation

Fig. 1 show a sample of the TNT chips (Fig. 1a) and the cumulative CO_2 levels obtained from both clean (PNC) and bioremediated (PBR) soils (Fig. 1b). PBR soils showed a consistently higher cumulative CO_2 evolution levels than PNC soils. By week 16, PBR soils had a cumulative CO_2 level of 0.046 g CO_2 per g $soil^{-1}$ compared to a level of 0.010 g CO_2 per g $soil^{-1}$ in PNC soils (Fig. 1a). This showed that PBR soils inoculated with TNT chips produced approximately 4.5 fold more CO_2, compared to PNC soils. This difference was confirmed to be statistically significant (T-TEST; $P \leq 0.05$). In PNC soils the amount of C produced expressed as a percentage of the C added as TNT chips was 20%; in PBR soils, this value was 40%. The conversion of carbon in the TNT to CO_2 has been shown in other studies in the ranges of 1%–23% (Boopathy et al., 1998a; Bumpus and Tatarko, 1994; Esteve-Nuñez et al., 2000; Fernando and Aust Steven, 1991). TNT degradation was also assessed directly through determination of the residual TNT chips at the end of the experiment. Analysis revealed that approximately 70% of the TNT chips had been degraded through incubation in bioremediated soil (PBR), compared with a value of approximately 30% in the clean soil (PNC) (data not shown). There was therefore significant reduction in residual TNT chips at week 16 in PBR soils (*T*-TEST; $P \leq 0.05$).

3.3. Microbial community and TNT contamination

Changes in microbial communities associated with TNT contamination were assessed in both bacterial and fungal communities during the experimental period. UPGMA dendrogram analysis of the bacterial community showed that the bacterial community in PNC and PBR soils were substantially different at the start of the experimental period (day 0; 28% similarity level) (Fig. 2a). Weeks 4–16 PBR samples formed a cluster which was distinct (22% similarity level) from weeks 8–16 PNC cluster. Analysis of the PBR and PNC communities showed that the introduction of TNT caused a substantial shift in bacterial community from the original population on day 0 (PBR and PNC; day 0–16% similar (84% dissimilar) to other PBR and PNC samples). These differences in the bacterial banding patterns continued within each soil type (PBR and PNC) till week 16 (Fig. 2a). A similar shift in fungal community was observed in both PBR and PNC soils as a result of TNT introduction between day 0 and weeks 4–16 (Fig. 2b). The variability in replicate samples were high in both soil types between week 4 and week 16 and it was not possible to detect a consistent trend in fungal banding patterns at these periods. Consequently, as greater differentiation between PBR and PNC soils was observed in the bacterial community profile, some dominant bands on this profile were excised (Fig. 2c). The putative identities of bands whose identities could be resolved are shown in Table 2. These include sequences similar to *Moraxella*, *Actinobacillus*, *Xanthomonas* and *Streptobacillus* sp.

Fig. 3a shows the Pareto–Lorenz (PL) distribution curves for the bacterial population. The range of the bacterial community distribution PL values or percentages was between 0.42 and 0.67 (43–67%) in PBR soils (medium evenness) indicating medium functional organization in this community. The PL values increased from day 0 to week 16 in PBR soils (day 0–42%, week 4–51%, week 8–52%, week 12–58% and week 16–67%) suggesting decreasing community evenness (Fig. 3a). The range of PL values or percentages in PNC soils was 0.28–0.47 (28–47%) but unlike in PBR, the addition of TNT chips did not cause a substantial increase in PL values (only fluctuations) over 16 weeks (Fig. 3b). The fungal community had a PL value range of 0.19–0.33 (19–33%) for PBR and 0.25 to 0.34 (25–34%) for PNC (both show high evenness) indicating low functional organization in these fungal communities (data not shown). Overall TNT chips caused comparatively less change in the fungal community. Fig. 4 shows that while the abundance of *pnrA* genes in both PBR and PNC soils was largely the same on day 0, TNT spiking resulted in approximately a 3 fold increase in the abundance of *pnrA* genes in PBR soils compared to PNC soils by week 16.

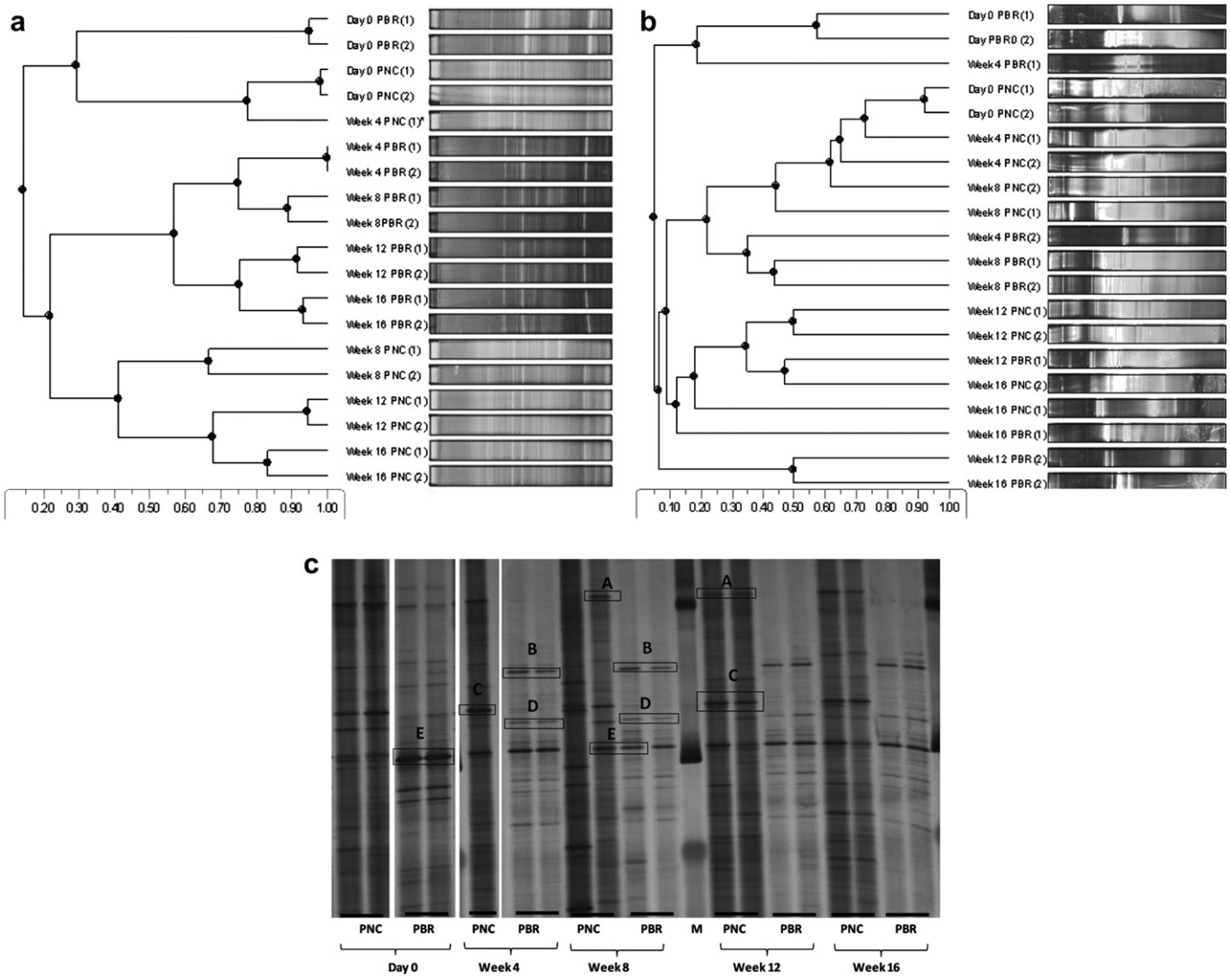

Fig. 2. Changes in bacterial (a) and fungal (b) communities in TNT chips contaminated bioremediated (PBR) and clean (PNC) soils over 16 weeks. Scale is indicative of similarity levels. The locations of excised and sequenced bands on the bacterial community profile are shown in boxes (c). Note that for (a), amplicons could only be generated from one replicate sample for week 4 PNC.

4. Discussion

4.1. TNT degradation

The metabolic rate, as determined by soil respiratory activity was significantly higher in the bioremediated (PBR) soil in comparison to the PNC soil over 16 weeks. The final difference between the cumulative CO_2 by week 16 was 0.036 g CO_2 g-soil of CO_2 (Fig. 1a). It is likely that the increased CO_2 respiration was related to the degradation of TNT in the previously bioremediated soil. Respiration has long been used as an effective indicator of remediating activity (Meyns et al., 2002), although a priming effect associated with the introduction of a compound into a soil has been noted previously. Priming of the microbial activity by the introduction of an organic compound can lead to increased microbial activity (CO_2) not associated with the degradation of the introduced compound (Sayer et al., 2007). Therefore a secondary method to assess TNT degradation is a useful check. HPLC has often been used as an assessment tool, but only when TNT was added homogeneously to a soil (generally in a liquid or powdery form ensuring homogeneity). However, TNT was added as chips in this study as this is the frequent form of TNT contamination in soils. Under these conditions the weight of chips remaining at the end of the experiment could be a useful indicator of direct degradation. The results confirmed that significantly more chips had been degraded in the previously bioremediated soil compared to the control soil (70% compared to 30% respectively). This suggests that the PBR soil has a higher potential to degrade TNT in

Table 2
Putative identities of bands excised and cloned from the bacterial community's DGGE profile.

DGGE Band Label	Phylum	Nearest match	Accession number	Sequence similarity (%)
A	Actinobacteria	*Actinobacillus* sp	GU226323.1	95
	Pseudomonadales	*Moraxella* sp	AF 005189.1	100
B	Pasteurellaes	*Actinobacillus* sp	FJ435959.1	96
	Fusobacteriales	*Fusobacterium* sp	GU797848.1	100
C	Rhodobacterales	*Thalassobius* sp	JF895519.1	100
	Oceanospirillales	*Alcanivorax* sp	EU732747.1	99
D	Xanthomonadales	*Xanthomonas* sp	JN648097.1	99
	Xanthomonadales	*Xanthomonas* sp	JF835910.1	98
E	Fusobacteriales	*Streptobacillus* sp	AB330760.1	96
	Neisseriales	*Simonsiella* sp	AY370189.1	95

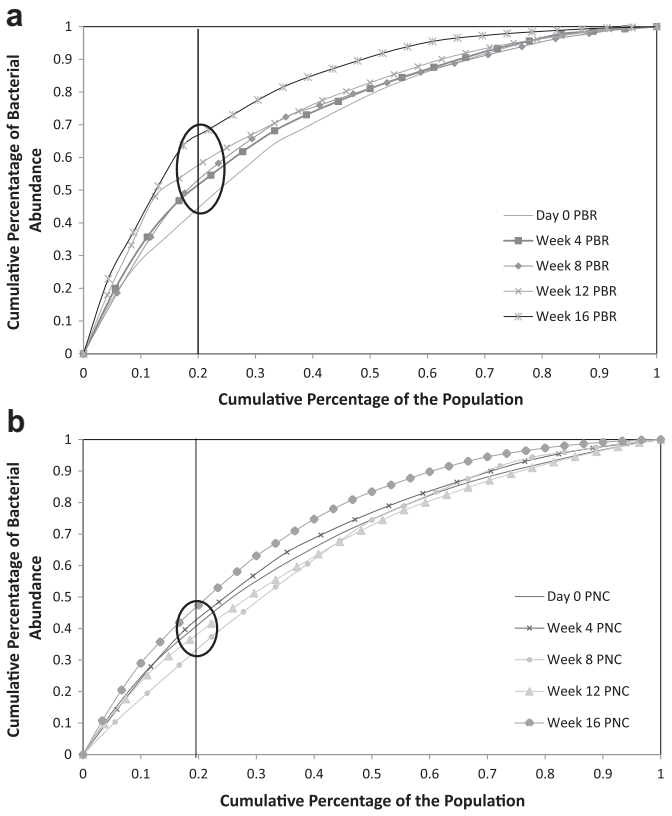

Fig. 3. Pareto–Lorenz curve of the bacterial population of bioremediated (PBR) (a) and clean (PNC) (b) soils over 16 weeks.

comparison to PNC soil and that the use of PBR soil is a more effective method for the bioremediation of TNT. The differences in the soils' respiratory rates and recovered TNT chips might also be due to soil use history. PBR soils with higher respiratory rates were originally contaminated with hydrocarbons before being bioremediated unlike clean PNC soils. Therefore PBR soils had higher residual soil TPH levels and were likely to contain more hydrocarbon degrading bacterial groups than clean soils. The hydrocarbon and TNT degrading activities of these bacterial groups could have accounted for the observed increase in soil respiration and reduction in TNT levels in PBR as some hydrocarbon degrading organisms can also degrade TNT (Boopathy, 2009; Popesku et al., 2004; Rahal and Moussa, 2011).

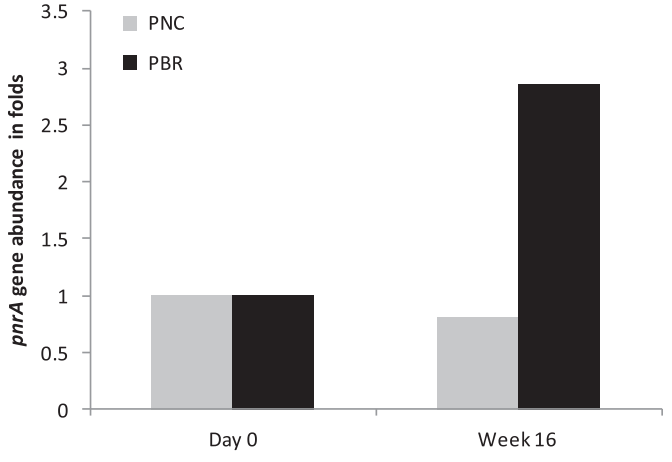

Fig. 4. The abundance of *pnrA* gene copies in PNC and PBR soils over 16 weeks. The fold increase in abundance was calculated with normalized sample values and referenced to the time 0 values.

4.2. Changes in microbial community

Fig. 2 shows a dendogram for the bacterial communities of PBR and PNC soils. The introduction of TNT caused substantial shifts in the bacterial community of both PBR and PNC soils from day 0 to week 4. Changes in bacterial community have been shown in TNT contaminated soils (George et al., 2008) and are probably related to TNT toxicity. TNT contamination might lead to selection of microorganisms capable of tolerating or degrading TNT. PNC bacterial community also seemed to have experienced a longer lag phase prior to forming a stable community (from week 8) in comparison to the PBR soil (from week 4) (Fig. 2a). Significant changes in the structure, abundance of the total microbial community have been shown to occur in contaminated soils (Wikström et al., 2000). The detection of some bacterial groups putatively identified as *Xanthomonas* sp in the bacterial community profiles is not unusual as these have also been detected in TNT contaminated environments and are thought to be involved in TNT degradation (George et al., 2008). They could have played similar roles in this study although this was not specifically investigated. TNT contamination can sometimes cause a decrease in bacterial population and a decrease in the amount of extracted DNA (George et al., 2008) with TNT introduction into pristine soils causing a clear shift towards a narrower range of bacterial species. Quantitative PCR analysis in this study showed that TNT spiking caused ~3 fold increase in the abundance of *pnrA* genes in PBR soils at week 16 compared to time 0 unlike in PNC soils. This indicated that the introduction of TNT to PBR soil caused an increase in *pnrA* gene copies which could have been because the community was becoming more selective towards microbes which could tolerate or transform TNT. *Pnr* genes (A and B) are bacterial nitroreductases which are known to code for proteins that reduce 2,4,6-trinitrotoluene to dinitrotoluene (Caballero et al., 2005; Roldán et al., 2008). They are therefore important in TNT biotransformation in the natural environment. Increase in *pnrB* gene copy numbers have been correlated with significant reduction in TNT levels during bioremediation (Cho et al., 2009). Therefore, the 3 fold increase in *pnrA* gene abundance observed in PBR soils might have contributed to the significant degradation of TNT chips observed in these soils at week 16. The difference in nitroreductase gene abundance between PBR and PNC soils might also be related to the history of soil use with PBR soils having a higher contaminant degrading potential than PNC.

The Pareto-Lorenz curve gives an indication of the evenness of a microbial community and how well the community can deal with stress. The evenness of the bacterial community as shown by the Pareto-Lorenz curve for PBR (Fig. 3a) decreased over the 16 weeks (that is 20% of the population became responsible for more cumulative intensities over time). The PL values of the bacterial community of PBR were found to be similar to those of a medium functionally organized community. Microbial community of a medium functional organization type indicates that it can respond to stressors such as TNT better (Marzorati et al., 2008). The Pareto-Lorenz curve evenness for the bacterial community in PNC soil did not show this trend (Fig. 3b) indicating a different response by the bacterial community in clean soils. The PNC bacterial community displayed low functional organization which indicates that it will likely have a different response (slow response) to the introduction of a stressor (Marzorati et al., 2008).

There was a similar change in fungal community (dendrogram) over the sixteen weeks in the dendrogram with TNT introduction causing a substantial shift in fungal community between day 0 and week 4 in both PBR and PNC. Soil contamination with TNT has been reported to cause changes in fungal communities (Perchet et al., 2008). The fungal community was not as similar from week to week compared to the bacterial community (low similarity values), which indicated that there was high variation in replicate fungal communities from each sample analysed. The high replicate sample variation in the fungal communities from week to week maybe due to the fact that DNA extraction from the soil was not optimised for fungal DNA isolation. Fungi can exist in both mycelia (active stage) and spores (resting or dispersal stage) in soil which may require different extraction processes. It is therefore difficult to know whether the DNA amplified by fungal primers in this study is from spores or mycelia or from both (van Elsas et al., 2000). This then makes it hard to determine if the DNA that is extracted is from a functional organism. The Pareto–Lorenz distribution curve analysis of the PBR and the PNC fungal communities showed that these communities showed greater evenness and fewer changes than bacterial communities over 16 weeks. This means that TNT contamination in PBR and PNC caused comparatively fewer alterations in fungal communities (weeks 4–16), unlike in the bacterial community.

5. Conclusion

This investigation has shown that the enhanced microbial potential for contaminant removal in PBR soils can be used for TNT chips degradation. This is because PBR soils had higher rates of CO_2 evolution, greater abundance of nitroreductase genes but lower residual TNT chips than clean PNC soils. The initial introduction of TNT chips also caused substantial shifts in both bacterial and fungal communities although greater effects of TNT (reduced evenness) were observed in bacterial communities (PBR) than in fungal communities. The history of soil use seemed to play a role in microbial response to contaminants as bacteria in hydrocarbon contaminated soils responded differently to TNT contamination when compared to those in clean soils. However, further research in this area needs to be carried out especially concerning the use of bioremediated TNT contaminated soil for treating new TNT wastes rather than using bioremediated hydrocarbon contaminated soils. The use of such bioremediated TNT contaminated soils could lead to greater and faster TNT degradation than observed in this study.

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Supplementary data

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Table S1
Determination of the fold increase in *pnr*A genes normalized to 16S rDNA relative to time 0.

Sample	Avg $C(T)_{pnrA} \pm$ S.E.	Avg $C(T)_{16S} \pm$ S.E.	$\Delta C(T)$ avg $C(T)_{pnrA}$ − avg $\Delta C(T)$16s	$\Delta\Delta C(T)$ avg $\Delta C(T)$ − avg $\Delta C(T)_{sampleDay0}$	$2^{-\Delta\Delta C(T)}$
Day 0 Treated (PBR)	33.11 ± 0.60	28.73 ± 0.11	4.38	0	1
Day 0 Control (PNC)	32.62 ± 0.72	27.68 ± 0.32	4.94	0	1
Week 16 Treated (PBR)	31.35 ± 0.18	28.49 ± 0.66	2.86	−1.52	2.86
Week 16 Control (PNC)	31.83 ± 0.05	26.60 ± 0.89	5.23	0.29	0.81

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