A diversified approach to evaluate biostimulation and bioaugmentation strategies for heavy-oil-contaminated soil.

S. Lladó[a], A.M. Solanas[a], J. de Lapuente[c], M. Borras[c] and M. Viñas[b,]

[a]Department of Microbiology, University of Barcelona, Diagonal 645,

E-08028 Barcelona, Spain. vadorllado21@ub.edu, asolanas@ub.edu

[b]IRTA. GIRO Joint Research Unit IRTA-UPC.Torre Marimon, E-08140 Caldes de Montbui, Barcelona, Spain.
marc.vinas@irta.cat

[c]Unitat de Toxicologia Experimental i Ecotoxicologia, Parc Científic de Barcelona, Josep

Samitier 1–5, 08028 Barcelona, Spain. jlapuente@pcb.ub.cat, mborras@pcb.ub.cat

Corresponding Author:

Marc Viñas Canals

[1]IRTA. GIRO Joint Research Unit IRTA-UPC. Torre Marimon, E-08140 Caldes de Montbui, Barcelona, Spain.
Tel: (+34) 93 467 4040

FAX: (+34) 93 467 4042

marc.vinas@irta.cat

To be submitted to Science of the Total Environment

ABSTRACT

A diversified approach involving chemical, microbiological and ecotoxicity assessment of soil polluted by heavy mineral oil was adopted, in order to improve our understanding of the biodegradability of pollutants, microbial community dynamics and ecotoxicological effects of various bioremediation strategies.

With the aim of improving hydrocarbon degradation, the following bioremediation treatments were assayed: *i)* addition of inorganic nutrients; *ii)* addition of the rhamnolipid-based biosurfactant MAT10; *iii)* inoculation of an aliphatic hydrocarbon-degrading microbial consortium (TD); and *iv)* inoculation of a known hydrocarbon-degrading white-rot fungus strain of *Trametes versicolor*.

After 200 days, all the bioremediation assays achieved between 30% and 50% total petroleum hydrocarbon (TPH) biodegradation, with the *T. versicolor* inoculation degrading it the most. Biostimulation and *T. versicolor* inoculation promoted the *Brevundimonas* genus concurrently with other α-proteobacteria, β-proteobacteria and Cytophaga-Flexibacter-Bacteroides (CFB) as well as Actinobacteria groups. However, *T. versicolor* inoculation, which produced the highest hydrocarbon degradation in soil, also promoted autochthonous Gram-positive bacterial groups, such as Firmicutes and Actinobacteria. An acute toxicity test using *Eisenia fetida* confirmed the improvement in the quality of the soil after all biostimulation and bioaugmentation strategies.

Keywords: Soil bioremediation, *Trametes versicolor*, bioaugmentation, mycoremediation, mineral oil, *Eisenia fetida*, DGGE, hydrocarbons.

1 INTRODUCTION

The application of bioremediation technologies to soils contaminated by light oil products, such as petrol or diesel, is feasible. However, decontaminating soils polluted with mineral oils that comprise the heaviest hydrocarbon fractions is still a challenge because of the low bioavailability and complex chemical composition of these products (Lee et al., 2008; Sabaté et al., 2004). In addition, an excessive residual concentration of hydrocarbons and possible oxidative metabolites with unacceptable human health risks may remain in the soil after bioremediation (Nocentini et al., 2000).

The aliphatic fraction of an oil product is formed mainly of alkanes, branched alkanes and isoprenoids, and to a lesser extent by cycloalkanes. Alkanes are more easily biodegraded than branched alkanes and biodegradability decreases with an increase in the number of carbon atoms. This pattern of hydrocarbon biodegradation has been described for bacterial and fungus metabolism (Colombo et al., 1996). Heavy-oil products have a considerable fraction of the so-called unresolved complex mixture (UCM) on the basis of its chromatographic profile. In fact, little is known about the composition of the UCM despite it being the main component of fuel oils (Wang and Fingas, 2003) that harbour branched and cyclic aliphatic and aromatic hydrocarbons, characterized by high resistance to biodegradation (Nievas et al., 2008). Furthermore, increases in the UCM after oil biodegradation processes have been reported in several studies (Ross et al., 2010).

Given this biodegradability pattern, the residual hydrocarbons in a soil contaminated with a heavy-oil product after bioremediation is a complex mixture rich in high-molecular-weight (HMW) hydrocarbons with a substantial proportion of a UCM. Because of this and as it is particularly difficult to decrease the concentration of total

petroleum hydrocarbons (TPH) below the limits established by legislation in soils contaminated with heavy-oil products, efforts should be made to minimize the presence of such compounds and to better understand their effect on soil ecotoxicity. To improve understanding and efficacy, both chemical biodegradation and the predominant microbial populations need to be assessed during bioremediation processes. Previous studies have focused on microbial communities responsible for degrading heavy fuel in marine environments (Alonso-Gutierrez et al., 2009) but little is known about oil-degrading communities in industrially polluted soils (MacNaughton et al., 1999; Mishra et al., 2001; Zucchi et al., 2003).

Ecotoxicological tests have successfully been used as a complementary tool to monitor bioremediation efficiency in soil, which is important to assess ecological risks at polluted sites (Wang et al., 2010). However, very few studies combine these toxicological tests with a detailed study of the microbial communities in historically oil-polluted soils (Liu et al., 2010; Sheppard et al., 2011). To ensure proper risk assessment of contaminated sites and the monitoring of bioremediation processes, toxicity assays, chemical analyses and molecular microbial ecology studies of the microbial populations in polluted areas should be combined (Plaza et al., 2010).

Here we evaluated the feasibility of several biostimulation and bioaugmentation agents in soil contaminated with a heavy mineral oil (C_{15}-C_{35}). To this end, we tested the following strategies: *i)* addition of the biosurfactant M_{AT10}, obtained by cultivating the strain *Pseudomonas aeruginosa* AT10 (Abalos et al., 2004); *ii)* addition of glucose; *iii)* inoculation of a microbial consortium (TD) that is specialized in the biodegradation of the aliphatic fraction of crude oil (Vinas et al., 2002); and *iv)* inoculation of a hydrocarbon-degrading strain of the ligninolytic fungus *Trametes versicolor* (Borràs et al., 2010). In addition, to better understand potential metabolic strategies and their final

effects on soil toxicity, we studied toxicity and characterized the microbial community during biodegradation by means of multiple culture-independent techniques.

2 MATERIAL AND METHODS

2.1 Soil analysis.

Oil-contaminated soil was sampled from a former screw manufacturing metallurgic facility in the city of Barcelona (Spain) which was decommissioned in 1990. The soil has been subjected to contamination during a period of 20 years. A cutting oil-contaminated soil from a former screw manufacturing metallurgic facility in the city of Barcelona (Spain) was affected by a previous period pollution of 20 years which was decommissioned in 1990. The upper part of the soil (1,5 m) was excavated and disposed into a landfill in 2005. In the present study a composite soil sample (50 kg) was obtained from the top soil layer (0-20 cm) and sieved (< 6 mm) after soil excavation. Inorganic nutrients were determined by ion chromatography in a 1:5 (w/w) soil:water slurry with double deionised water. Nitrite, nitrate and phosphate were measured in a chromatographic system equipped with a Waters 515 pumping system, a Waters IC-PAK Anion column (Waters Corporate, Milford, USA), a UV/V Kontron model 332 detector (Kontron Instruments, Milan, Italy) and a Wescan conductivity meter (Wedan Instruments, Santa Clara, USA). The ammonium concentration was assessed using the automated phenate method (Standard Method 4500-NH3 H, American Public Health Association, 1992) in a Technicon Autoanalyzer II (Bran and Luebbe Analyzing Technologies Inc., Elmsford, USA). The pH was measured in a 1:2.5 (w/v) soil:water slurry with a Crison micro pH 2000 meter (Crison, Barcelona, Spain). Conductivity was determined with a Crison conductimeter model 522 in a 1:10 (w/v)

soil:water slurry. Other physicochemical parameters such as soil moisture and water-holding capacity (WHC) were determined as described elsewhere (Sabaté et al., 2004).

2.2 Soil microcosm experiments.

Initially the soil was treated with water and aerated by means of mechanical mixing with a glass rod twice a week for 100 days. Afterwards, the soil was subjected to different treatments for an additional 180 days. For each treatment, three independent replicates (200-ml glass receptacles covered with perforated parafilm) were prepared as microcosms, each containing 60 g of sieved (< 6 mm) soil. In all the treatments, the water content was adjusted to 60% of WHC. Twice a week, the microcosm contents were mixed and the soil water content was restored by controlling the weight.

Seven different treatments were applied in triplicate:

1) *Basic treatment (H):* soil was aerated by mixing every week and water added to maintain at 60% of the WHC. This basic treatment (H) was applied to all the samples except the air dried control.

2) *Inorganic nutrient treatment (H+N)*: NH_4NO_3 and K_2HPO_4 were added during the first 30 days, to produce a final C:N:P molar concentration equivalent to 300:10:1.

3) *Easily biodegradable substrate (H+N+G):* inorganic nutrients and 0.2% w/w glucose were added.

4) *Bioaugmentation I: (H+N+TD):* nutrients and the bacterial consortium TD, as a gas-oil degrading inoculum, were inoculated into the soil to reach 10^8 microorganisms $\cdot$ g^{-1} of soil (Abalos et al., 2004). Consortium TD is capable of extensively degrading Casablanca crude oil by using both the linear aliphatic fraction and the branched alkanes

to a high degree (Vinas et al., 2002). The mixture has been maintained using diesel as the sole carbon and energy source for 10 years.

5) *Bioaugmentation II: (H+N+F):* the ligninolytic fungus *Trametes versicolo*r strain ATCC#42530 pre-grown on 3.5g of rice straw, previously described as a PAH-degrading inoculum (Borràs et al., 2010), was inoculated into the soil. The fungus was previously grown with the rice straw for seven days. Once the mycelium colonized the straw, the mycelium and the straw were crashed together and mixed with the soil to generate many different points of fungal colonization.

6) *Biosurfactant treatment (H+N+BS):* nutrients and the biosurfactant M_{AT10} were added to the soil in two different concentrations: 10 and 100 times above its critical micelle concentration (CMC) defined as 39 mg/l (Abalos et al., 2004). M_{AT10} rhamnolipids were harvested from the supernatant of a cell culture of *Pseudomonas aeruginosa* AT10 grown in a mineral medium with soybean oil, as previously described (Abalos et al., 2004).

7) *Air dried soil* (1% (w/w) water content) was used as a biodegradation control.

2.3 Analysis of TPH.

At days 0, 100, 190, and 280, a 30-g soil sample was taken from each microcosm in triplicate. The samples were sieved (2-mm grid) and dried for 16 h at room temperature. Organic pollutants were extracted from 10 g of the soil. Before the extraction, *o*-terphenyl (50 µg) was added in acetone solution as a surrogate internal standard. The acetone was allowed to evaporate and 10 g of anhydrous Na_2SO_4 was

added and mixed. Soxhlet extraction was performed on this mixture with dichloromethane:acetone (1:1 (v/v)) for 6h. The extract was dehydrated through a Na_2SO_4 column and concentrated to 1 ml with a rotary evaporator. The TPH fraction was obtained with an alumina chromatographic column following the EPA3611 method (U.S. Environmental Protection Agency). The TPH fraction was analysed by gas chromatography with flame ionization detection (Llado et al., 2009). The TPH content was calculated from the total area compared to that of an aliphatic standard (AccuStandard, New Haven, USA) calibration curve.

2.4 Counting of total heterotrophic and hydrocarbon-degrading microbial populations

Heterotrophic and alkane-degrading microbial populations were enumerated throughout the microcosm experiments by the miniaturized most-probable-number (MPN) technique (Wrenn and Venosa, 1996). The heterotrophic microbial populations were enumerated on Trypticase Soy Broth. The mineral medium with the aliphatic saturated fraction (F1) of Casablanca crude oil was used as the sole source of carbon and energy for the alkane degraders (Aceves et al., 1988).

2.5 Microbial community characterization by means of denaturing gradient gel electrophoresis (DGGE).

2.5.1 DNA extraction

Soil samples were collected from each microcosm for DNA extraction at days 0, 100 and 280 in sterile Eppendorf tubes and stored at –20°C prior to analysis. To ascertain the repeatability of the DNA extraction process and PCR protocols, a set of replicates was analysed by means of DGGE. This showed a high degree of repeatability of the sampling and molecular protocols (DNA extraction and PCR) among replicates

(Figure 3B). Hence, DNA was extracted from a composite 0.75-g sample containing 0.25 g from each microcosm replicate. Total community DNA was extracted from the soil microcosms following a bead beating protocol using the Power Soil DNA extraction kit (MoBio Laboratories, Solano Beach, USA), according to the manufacturer's instructions. A further clean-up step was necessary to avoid PCR inhibition; we performed this using Clean DNA Wizard kit (Promega, Madison, USA).

PCR: The V3-V5 hypervariable regions of the 16S rRNA gene were amplified from total community DNA by PCR using primers F341-GC and R907 (Yu and Morrison, 2004). The primer F341-GC included a GC clamp at the 5' end (5'-CGCCCGCCGCGC CCCGCGCCCGTCCCGCCGCCCCCGCCCG-3'). All PCR reactions were performed in a Mastercycler personal thermocycler (Eppendorf, Hamburg, Germany). Fifty ml of the PCR mixture contained 2.5 U Takara Ex Taq DNA Polymerase (Takara Bio, Otsu, Shiga, Japan), 25 mM TAPS (pH 9.3), 50 mM KCl, 2 mM $MgCl_2$, 200 µM of each deoxynucleoside triphosphate, 0.5 µM of each primer, and 100 ng of template DNA quantified by means of the Low DNA Mass Ladder (Gibco BRL, Rockville, USA). After 9 min of initial denaturation at 95ºC, a touchdown thermal profile protocol was performed and the annealing temperature was decreased by 1ºC per cycle from 65ºC to 55ºC, at which temperature 20 additional cycles were carried out. Amplification was carried out with 1 min of denaturation at 94ºC, 1 min of primer annealing and 1.5 min of primer extension at 72ºC. The last step involved a 10-min extension at 72ºC.

2.5.2 DGGE gel.

Approximately 800 ng of purified PCR-16SrRNA amplicon product was loaded onto a 6% (wt/vol) polyacrylamide gel, 0.75 mm thick (to obtain better resolution) with denaturing chemical gradients of formamide and urea ranging from 40% to 60% (100%

denaturant contains 7 M urea and 40% formamide). The Low DNA Mass Ladder was used for quantification. DGGE was performed in 1X TAE buffer (40 mM Tris, 20 mM sodium acetate, 1 mM EDTA, pH 7.4) using a DGGE-2001 System (CBS Scientific Company, Del Mar, USA) at 100 V and 60ºC for 16 hours.

The gels were stained for 45 min in 1X TAE buffer containing SybrGold (Molecular probes, Inc., Eugene, USA), then scanned using a Bio-Rad molecular imager FX Pro Plus multi-imaging system (Bio-Rad Laboratories, Hercules, USA) in DNA stain gel mode for SybrGold at medium sample intensity. Images of the DGGE gels were digitalized and the DGGE bands were processed using Quantity-one image analysis software, version 4.1 (Bio-Rad Laboratories) and corrected manually.

2.6 Sequencing and phylogenetic analysis

Predominant DGGE bands were excised with a sterile razor blade, resuspended in 50 µL sterilized MilliQ water and stored at 4ºC overnight. An aliquot of the supernatant (2 µL) was used to reamplify the DGGE bands with primers F341, without the GC clamp, and R907, under the same conditions. Band-PCR products were further purified for sequencing using a Wizard SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's instructions. The DNA sequencing reaction was carried out in a thermocycler (Mastercycler) using an ABI Big Dye Terminatior v3.1 Cycle Sequencing Kit (Applied Biosystems) as specified by the manufacturer. The primers used were F341 and R907 and the conditions of the amplification were as follows: an initial denaturing step of 1 min at 96°C, followed by 25 cycles of 10 s at 96°C, 5 s at 55°C and 4 min at 60°C. The sequencing reaction was analysed by the Scientific-Technical Services of the University of Barcelona (SCT-UB) using an ABI Prism 3700 DNA Analyzer (Applied Biosystems). .

Raw sequence data were checked and analysed with the BioEdit (version 7.0) software package (Ibis Biosciences, Carlsbad, USA), inspected for the presence of ambiguous base assignments and subjected to the Chimera check with Bellerophon version 3 (Huber et al., 2004). Sequences were compared with those deposited in the GenBank (NCBI) database using alignment tool comparison software (BLASTn and RDP) to find the closest sequence match and taxonomic affiliation.

The 18 nucleotide sequences (DGGE bands 1-18) identified in this study were deposited in the GenBank database under accession numbers JN795892 to JN795909.

2.7 Acute toxicity test in Eisenia fetida.

Worms were selected from a lab-reared population destined for experimentation. The individuals were more than 3 months old, with well-developed clitellum and a weight of from 0.25 to 0.4 g per animal.

Toxicity testing was performed according to Organisation for Economic Cooperation and Development (OECD) guideline 207 for acute soil toxicity testing in its "artificial soil" modality (OECD, 1984). The artificial soil test yields toxicity data that is more representative of natural earthworm exposure to chemicals than the "simple contact" test, which is easier to perform. The OECD guideline uses an artificial soil both as a control in the toxicity assays and also to obtain the polluted soil dilutions for the assay.

Ten earthworms were cultivated in 200 g of each treatment and in control soils for 14 days. The dilutions of the experimental soil were carried out using dry weight of test artificial soil according to OECD guideline 207 (70% sand, 20% kaolin clay and 10% sphagnum peat). The soil moisture was adjusted every three days to 35% with deionized water. The temperature of the assay was 21ºC ± 3ºC and the photoperiod was

of 16h light:8h dark.A range-finding test using 37.5%, 50%, 75%, 87.5% and 100% of polluted soil was initially performed to determine the concentrations at which 0% and 100% mortality occurred, and to establish lethal concentration 50 (LC_{50}). Two full assays were then carried out at 100 and 190 days with the treatments described previously in Section 2.6. In each period of exposure, a parallel negative control of the artificial soil was performed (OECD, 1984). After 7 and 14 days, the weight of each earthworm was recorded as well as the number of casualties and any comments.

2.8 Comet assay.

After exposure to the treatment soils, coelomocytes were obtained from the surviving earthworms using the extrusion method as previously described (Eyambe et al., 1991). Genotoxicity was determined using the Comet Assay (Singh et al., 1988). The cell suspensions from each animal, in each exposure, were included in low-melting-point agarose and extended on coded slides previously treated with a layer of agarose of normal melting point. After solidification at 4ºC for 10 minutes, the cover slides were removed and the slides were immersed in lysis buffer (4ºC, 2.5 M NaCl, 100 mM disodium EDTA and 10 mM Tris; and 1% Triton X-100 just before use, pH 10) for 2 hours. The slides were placed in an electrophoresis tank with electrophoresis buffer (4ºC, 1 mM disodium EDTA and 300 mm NaOH, pH>13) for 20 minutes to facilitate the unwinding of the cell DNA. The electrophoresis was then run for 20 minutes at 25 V and 300 mA. After the electrophoresis, the DNA was fixed with 0.4 M Tris buffer pH 7.5 with 3 changes of 5 minutes each at 4ºC. The samples were stained with DAPI (4',6-diamidino-2-phenylindole) and 50 cells from each individual were analysed, whenever possible, with Analysis ® software.

2.9 Statistical analysis.

The statistical significance of the TPH data from the biodegradation experiments was evaluated by analysis of variance (ANOVA) and Tukey's multiple comparison test. The data were considered to be significantly different if $P \leq 0,05$. The effect of the main biostimulation and bioaugmentation treatments on the microbial diversity of the soil was assessed by comparing the DGGE profiles using a similarity cluster analysis. A dendogram was constructed, using the group average method with the Pearson product-moment correlation coefficient. Version 5.1 of Statgraphics Plus (Statistical Graphics Corp.) was used for all chemical and microbiological assay statistical analysis.

The comet assay was statistically evaluated using the SPSS 15.0 statistical package (SPSS Inc., Chicago,USA). Each encoded sample was considered as independent and duplicates were performed.

3 RESULTS and DISCUSSION

3.1 Soil description.

The soil used (sandy-loam texture) was from the site of a former screw plant, which had been operating for several decades before this study. Thus, information about the kind of contaminating products present was obtained from the chromatographic profile. The TPH profile was of a heavy-oil product (mineral oil), in the hydrocarbon range of C_{15}-C_{35}, with a considerable UCM, which might well correspond to a heavy mineral oil, such as drilling/cutting oil (Figure 1). First, to establish the feasibility of applying bioremediation technology to this soil, we performed a bio-feasibility assay, as previously described (Sabaté et al., 2004). Also, the optimum water content of the soil for the microcosm experiments was defined as 60% of WHC. Table 1 shows the main physical, chemical and microbiological characteristics of the soil studied. It contained a

significant amount and proportion of an alkane (saturated) fraction-degrading population (0.2%), thereby indicating that biostimulation and bioaugmentation strategies were suitable for this matrix.

3.2 Biostimulation and bioaugmentation microcosm assays.

Microcosm experiments were carried out for 280 days. During the first 100 days, biostimulation was only by means of aeration at optimal humidity (60% WHC). This process caused a 15% depletion of the soil TPH content.

After biostimulation and bioaugmentation strategies applied for the following 180 days, TPH biodegradation ranging from 30% to 50% was achieved, depending on the treatment (Figure 2). Neither the nutrient additions nor the nutrient additions plus the TD consortium improved the hydrocarbon degradation achieved by the autochthonous microbial population biostimulated by optimal soil water content. This finding is consistent with other studies reporting no benefit from bacterial inocula in hydrocarbon-contaminated soil (Jorgensen et al., 2000). It is important to point out that the highest TPH degradation was reached after *T. versicolor* inoculation, with a reduction of 50% of TPH ($p<0.05$) accompanied by a considerable decrease in the UCM and a significant shift in the microbial population's diversity (Figure 3), promoting hydrocarbon-degrading microbial populations (Figure 4). Ligninolytic fungi have traditionally been used to enhance the biodegradation of recalcitrant compounds with structural similarities to lignin, such as polycyclic aromatic hydrocarbons (PAHs) (Chupungars et al., 2009). Nevertheless, the degradation of TPH by *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Coriolus versicolor* has also been reported (Yateem et al., 1997). Several studies have shown degradation of TPH in crude oil by *T. versicolor*, but only in liquid biodegradation assays (Colombo et al., 1996).

Furthermore, the filamentous fungus *Penicilium simplicissimum* YK degrades long-chain alkanes comprising up to 50 carbon atoms (Yamada-Onodera et al., 2002). While most previous *Trametes* bioaugmentation studies of polluted soils focus mainly on PAH biodegradation, its effect on a non-sterile industrial mineral-oil-polluted soil including active autochthonous microbial populations has rarely been reported (Yateem et al., 1997). Yateem et al. (1997) described significant enhancement of heavy-oil biodegradation, but, as in other fungal bioaugmentation studies of industrially polluted soils, reported no information about its effect on either the autochthonous microbial community or soil ecotoxicity. In contrast, among the biostimulation agents, the addition of the rhamnolipids produced by the strain AT10 from *P. aeruginosa* did not improve the biodegradation achieved by the treatments. In a previous paper we described, in a liquid culture, a considerable improvement in the biodegradation of a crude oil by a microbial consortium specializing in degrading polycyclic aromatic hydrocarbons in the presence of the same biosurfactant as that used in the present study (Abalos et al., 2004). The interactions between the surfactant, the solid matrix, the contaminant and the microbial populations in a soil are highly complex and give rise to a lot of controversy (Elliot et al., 2010; Whang et al., 2008). The preferential use of surfactants as a carbon source by hydrocarbon degraders could explain the inhibited biodegradation of the pollutants (Deschenes et al., 1996).

3.3 Monitoring of heterotrophic and hydrocarbon-degrading microbial populations.

The MPN results show that, from day 100, the presence of heterotrophic populations decreased due to almost all the treatments (Figure 4). This finding suggests a reduction in organic matter that can be easily assimilated during incubation. In contrast, the population of aliphatic hydrocarbon degraders increased from one-fold to

five-fold in all the biostimulation and bioaugmentation treatments, with the highest values reached when *T. versicolor* was inoculated. A similar phenomenon has been described in other historically polluted soils, which suggests that it is a common trend in bioremediation processes for this matrix (Liu et al., 2010). This is consistent with the gradual depletion of TPH detected in the soil.

In the treatment with *T. versicolor*, the hydrocarbon-degrading population was higher than in the other treatments, reaching 100% of the heterotrophic population after 280 days. This increase in the specialized population as a consequence of fungal bioaugmentation, which was concomitant with a marked change in the eubacterial diversity detected by PCR-DGGE analyses (Figure 3), may explain the TPH biodegradation efficiency. The change in the eubacterial community could be explained by the presence of the ligninolytic substrate in the soil, the use of fungal exudates as a nutrient source (Boer et al., 2005) or the antimicrobial compounds produced by the inoculated fungus (Vázquez et al., 2000). Furthermore, the heterotrophic population was also approximately double that in the other treatments. A significant part of this bacterial growth could be attributed to the presence of the fungal ligninolytic substrate in the microcosms, as well as changes in the microbial population (Federici et al., 2007). Nonetheless, mycoremediation was enhanced by the presence of active autochthonous microbial populations. Positive and negative interactions between the indigenous microbial populations and inoculated fungi have been described. Thus, fungi could participate in the transformation of some HMW hydrocarbons into readily biodegradable substrates by bacteria. In keeping with this, an increase in heterotrophic cultivable bacteria in soils inoculated with *Irpex lacteus* and *Pleorotus ostreatus* has been reported (Leonardi et al., 2008). In contrast, the growth of certain white-rot fungus

is commonly suppressed by indigenous soil microbes and by abiotic features of soil compounds (Tucker et al., 1995).

3.4 Microbial community assessment.

To analyse the initial microbial population in the soil and its response to different bioremediation treatments, we performed a PCR-DGGE analysis (Figure 3).

A DGGE profile of the initial polluted soil showed little diversity, which is a common result of the DGGE technique and is also common in polluted environments. Two predominant DGGE bands were detected. Band B1 was found in all the treatments. On the basis of partial *16SrRNA* gene sequences, band B1 was found to be very similar to the *Brevundimonas* genus, while band B2 was very similar to the *Dietzia* genus. Although *Brevundimonas* and *Dietzia* are microbial genera commonly found in pristine soil environments, some members isolated from polluted environments show aliphatic hydrocarbon-degrading capability as well (Bodtker et al., 2009; Xiao et al., 2010).

The DGGE profiles from the first 100 days of biostimulation (Figure 3; lanes 2 and 3) were not very different. The addition of rice straw on day 100 did not alter the soil population substantially, either. However, during the following 180 days of treatment, biodiversity increased considerably in the three profiles (basic biostimulation, inorganic nutrients and *T. versicolor* inoculation). This finding could be attributable to the late growth of bacterial species that are adapted to the use of more recalcitrant hydrocarbons as a carbon source.

Soil biostimulation with water or water plus nutrients for 280 days resulted in similar DGGE profiles and TPH degradation rates (Lanes 4 and 5 in Figure 3). However, other studies report that the DGGE profiles for a hydrocarbon-polluted soil biostimulated with water or water plus nutrients differ greatly (Wu et al., 2008). These

distinct diversity patterns suggest that similar biostimulation treatments produce population changes that differ, depending on the polluted soil matrix and the microbial community involved.

At the end of the bioaugmentation experiments involving *T. versicolor* inoculation, five additional bands (B14, B15, B16, B17 and B18) appeared in the 16SrRNA-DGGE. B14 corresponded to *Herbaspirillum sp.*, B18 to *Streptomyces sp.*, and B15 and B16 to *Bacillus sp.* All these genera have been associated with recalcitrant hydrocarbon biodegradation (Chaudhary et al., 2011; Das and Mukherjee, 2007; Ross et al., 2010). Finally, B17 corresponded to the genus *Arthrobacter*, which produces extracellular emulsifier factors with the capacity to emulsify light petroleum oil, diesel oil and a variety of crude oils and gas oils (Rosenberg et al., 1979).

These results confirm that the presence of *T. versicolor* and its ligninolytic substrate in the soil substantially changed the bacterial biodiversity over the 180 days of incubation, promoting the enrichment of Gram-positive bacteria belonging to the *Actinobacteria* and *Bacillus* groups. It is important to point out that microbial diversity changes promoted after *T. versicolor* inoculation were concomitant with both the high proportion of hydrocarbon degraders encountered in the MPN assays and the higher TPH biodegradation observed in the white-rot fungus bioaugmentation treatment.

3.5 Acute toxicity test in Eisenia fetida.

Filtering organisms in ecosystems reflect the health of the environment; in particular, *E. fetida* is one of the clearest cases of this. This is why the organism has been used as an indicator of pollution in many studies and is the experimental system of choice in the Organisation for Economic Cooperation and Development guidelines for soil assessment (OECD, 1984).

No *E. fetida* mortality was observed in the range finding test (section 2.7), at any polluted soil dilution tested. Therefore no LC_{50} could be established for the contaminated soil.

Undiluted soil was used for the subsequent worm weight assessment and acute toxicity tests for the most significant bioremediation treatments (Figure 5). No lethality was observed at day 100 or in three of the assays at day 190 (H, H+N and H+N+F); none of the exposure patterns tested affected *E. fetida* mortality. This finding could be explained by the low bioavailability of the pollutant. However, bioremediation treatments altered worm weight during the incubation period in relation to controls. Other studies have reported decreasing toxicity in polluted soils during bioremediation treatments (Liu et al., 2010). At day 190, the individuals in all three of the treatment groups showed lower weight losses than after 0 or 100 days, and there was even a weight increase in the H+N+F group. This finding suggests positive correlation between the length of treatment and the health of the organisms (expressed as weight). At 190 days, the treatments increased soil quality in the order: H+N+F > H+N > H. The increased eubacterial biodiversity in the degrading population detected through DGGE in the bioaugmentation with *T. versicolor* may be related to the increased detoxifying potential.

3.6 Comet assay in coelomocytes of E. fetida.

We performed a comet assay using coelomocytes from surviving worms from the different biotreated soil samples after the acute toxicity tests. DNA degradation, ranging from 33% to 47%, was observed in all the treatments. However, no significant differences on the basis of DNA fragmentation was observed between treatments over time compared to their respective controls ($P>0.05$). This result suggests that the

aliphatic compounds present in the polluted soil were not genotoxic. This notion is supported by the lack of evidence in the literature of genotoxicity caused by aliphatic hydrocarbons. However, a genotoxicity evaluation should be performed because, although the parental compounds are non-genotoxic, intermediate metabolites produced by the microbial metabolism could contribute to increased soil genotoxicity (Cao et al., 2009).

4 CONCLUSIONS

This study confirms that mycoremediation by means of allochthonous bioaugmentation with a white-rot fungus such as *T. Versicolor* is an effective remediation and detoxifying strategy, not only for PAH-polluted soils, but also for soils contaminated with heavy mineral oil.

The study also highlights the importance of carrying out an in-depth microbiological assessment through bioremediation experiments involving historically polluted soils, in order to gain insight into bacteria–fungi interactions. Here we report that the use of an external fungal inoculum produces a significant increase and shift in the detectable biodiversity of the autochthonous bacterial community, promoting more hydrocarbon-degrading microbial populations in the soil than other biostimulation treatments do.

Finally, we recommend a diversified approach in bioremediation tests at the bench scale by combining TPH degradation, microbial ecology, acute toxicity and genotoxicity assessment in order to clarify biodegradation processes and ensure reliable risk assessment throughout the bioremediation of industrially polluted soils.

ACKNOWLEDGEMENTS

This study was supported financially by the Spanish Ministry of Science and Technology (CTM2007-61097/TECNO) and by the Spanish Ministry of the Environment (094/PC08/3-01.1).

FIGURE LEGENDS

Figure 1. GC-FID chromatographic profile of the TPH content of the original heavy-oil-polluted soil.

Figure 2. Residual concentration of TPH after bioremediation treatments.●, control (air-dried soil); ○, basic (H); ▼, nutrients (H+N); △, nutrients and glucose (H+N+G); ■, nutrients and TD consortium (H+N+TD); □, nutrients and Trametes versicolor (H+N+F); ◆, nutrients and surfactant (H+N+BS) at 10 times its critical micelle concentration (CMC); ◇, nutrients and surfactant (H+N+BS) at 100 times its CMC. Different letters in brackets indicate significant differences among the treatments ($P < 0.05$). Vertical bars represent the standard deviation of three independent replicates (n=3)

Figure 3. A) Denaturing gradient gel electrophoresis (40% to 60% denaturant) profiles and cluster analysis (group average method; squared Euclidean distance) of eubacterial biodiversity from the original and five treated soils. From left to right: Lane 1, 0 days; Lane 2, 100 days; Lane 3, 100 days plus rice straw; Lane 4, basic treatment at 280 days; Lane 5, nutrient treatment at 280 days; Lane 6, nutrient and *Trametes versicolor* treatment at 280 days. Numbered DGGE bands were successfully excised

and sequenced and are shown in Table 2. B) DGGE (20% to 80% denaturant) from a set of independent samples in triplicate (sample: 100 days plus rice straw addition).

Figure 4. Heterotrophic (A) and F1-degrading (B) populations in soil treatments over the 280 days of incubation in microcosms.●, control (air-dried soil); ○, basic (H); ▼, nutrients (H+N); △, nutrients and glucose (H+N+G); ■, nutrients and TD consortium (H+N+TD); □, nutrients and *Trametes versicolor* (H+N+G); ◇, nutrients and surfactant (H+N+BS) at 10 times its CMC; ▲, nutrients and surfactant (H+N+BS) at 100 times its CMC. Figure B shows the percentage of the heterotrophic population represented by the aliphatic (F1)-degrading population.

Figure 5. Evolution of *Eisenia fetida* weight during the soil experiment. From left to right: S(0), soil at 0 days; S+H(100d), soil + humidity at 100 days ; S+H(190d), soil + humidity at 190 days; S+H+N(190d), soil + humidity + nutrients at 190 days; S+H+N+F(190d), soil + humidity + nutrients + fungus at 190 days. Different letters in brackets indicate significant differences between the treatments ($P < 0.05$). Vertical bars represent the standard deviation (n=10)

REFERENCES

Abalos A, Vinas M, Sabate J, Manresa MA, Solanas AM. Enhanced biodegradation of Casablanca crude oil by a microbial consortium in presence of a rhamnolipid produced by Pseudomonas aeruginosa AT10. Biodegradation 2004; 15: 249-60.

Aceves M, Grimalt J, Albaiges J, Broto F, Comellas L, Gassiot M. Analysis of hydrocarbons in aquatic sediments. II. Evaluation of common preparative

procedures for petroleum and chlorinated hydrocarbons. J Chromatogr 1988; 436: 503-9.

Alonso-Gutierrez J, Figueras A, Albaiges J, Jimenez N, Vinas M, Solanas AM, et al. Bacterial communities from shoreline environments (costa da morte, northwestern Spain) affected by the prestige oil spill. Appl Environ Microbiol 2009; 75: 3407-18.

Bodtker G, Hvidsten IV, Barth T, Torsvik T. Hydrocarbon degradation by Dietzia sp. A14101 isolated from an oil reservoir model column. Antonie Van Leeuwenhoek 2009; 96: 459-69.

Boer W, Folman LB, Summerbell RC, Boddy L. Living in a fungal world: impact of fungi on soil bacterial niche development. FEMS Microbiol Rev 2005; 29: 795-811.

Borràs E, Caminal G, Sarrà M, Novotný C. Effect of soil bacteria on the ability of polycyclic aromatic hydrocarbons (PAHs) removal by Trametes versicolor and Irpex lacteus from contaminated soil. Soil Biology & Biochemistry 2010; 42: 2087-2093.

Cao B, Nagarajan K, Loh KC. Biodegradation of aromatic compounds: current status and opportunities for biomolecular approaches. Appl Microbiol Biotechnol 2009; 85: 207-28.

Colombo JC, Cabello M, Arambarri AM. Biodegradation of aliphatic and aromatic hydrocarbons by natural soil microflora and pure cultures of imperfect and lignolitic fungi. Environ Pollut 1996; 94: 355-62.

Chaudhary P, Sharma R, Singh SB, Nain L. Bioremediation of PAH by Streptomyces sp. Bull Environ Contam Toxicol 2011; 86: 268-71.

Chupungars K, Rerngsamran P, S. T. Polycyclic aromatic hydrocarbons degradation by Agrocybe sp. CU-43 and its fluorene transformation. International Biodeterioration & Biodegradation 2009; 63: 93-99.

Das K, Mukherjee AK. Crude petroleum-oil biodegradation efficiency of Bacillus subtilis and Pseudomonas aeruginosa strains isolated from a petroleum-oil contaminated soil from North-East India. Bioresour Technol 2007; 98: 1339-45.

Deschenes L, Lafrance P, Villeneuve JP, Samson R. Adding sodium dodecyl sulfate and Pseudomonas aeruginosa UG2 biosurfactants inhibits polycyclic aromatic hydrocarbon biodegradation in a weathered creosote-contaminated soil. Appl Microbiol Biotechnol 1996; 46: 638-46.

Elliot R, Singhal N, Swift S. Surfactants and Bacterial Bioremediation of Polycyclic Aromatic Hydrocarbon Contaminated Soil—Unlocking the Targets. Critical Reviews in Environmental Science and Technology 2010; 41: 78-124.

Eyambe GS, Goven AJ, Fitzpatrick LC, Venables BJ, Cooper EL. A non-invasive technique for sequential collection of earthworm (Lumbricus terrestris) leukocytes during subchronic immunotoxicity studies. Lab Anim 1991; 25: 61-7.

Federici E, Leonardi V, Giubilei MA, Quaratino D, Spaccapelo R, D'Annibale A, et al. Addition of allochthonous fungi to a historically contaminated soil affects both remediation efficiency and bacterial diversity. Appl Microbiol Biotechnol 2007; 77: 203-11.

Huber T, Faulkner G, Hugenholtz P. Bellerophon: a program to detect chimeric sequences in multiple sequence alignments. Bioinformatics 2004; 20: 2317-9.

Jorgensen KS, Puustinen J, Suortti AM. Bioremediation of petroleum hydrocarbon-contaminated soil by composting in biopiles. Environ Pollut 2000; 107: 245-54.

Lee S-H, Oh B-I, Kim J-g. Effect of various amendments on heavy mineral oil bioremediation and soil microbial activity. Bioresource Technology 2008; 99: 2578-2587.

Leonardi V, Giubilei MA, Federici E, Spaccapelo R, Sasek V, Novotny C, et al. Mobilizing agents enhance fungal degradation of polycyclic aromatic hydrocarbons and affect diversity of indigenous bacteria in soil. Biotechnol Bioeng 2008; 101: 273-85.

Liu W, Luo Y, Teng Y, Li Z, Ma LQ. Bioremediation of oily sludge-contaminated soil by stimulating indigenous microbes. Environ Geochem Health 2010; 32: 23-9.

Llado S, Jimenez N, Vinas M, Solanas AM. Microbial populations related to PAH biodegradation in an aged biostimulated creosote-contaminated soil. Biodegradation 2009; 20: 593-601.

MacNaughton SJ, Stephen JR, Venosa AD, Davis GA, Chang YJ, White DC. Microbial population changes during bioremediation of an experimental oil spill. Appl Environ Microbiol 1999; 65: 3566-74.

Mishra S, Jyot J, Kuhad RC, Lal B. Evaluation of inoculum addition to stimulate in situ bioremediation of oily-sludge-contaminated soil. Appl Environ Microbiol 2001; 67: 1675-81.

Nievas ML, Commendatore MG, Esteves JL, Bucala V. Biodegradation pattern of hydrocarbons from a fuel oil-type complex residue by an emulsifier-producing microbial consortium. J Hazard Mater 2008; 154: 96-104.

Nocentini M, Pinelli D, Fava F. Bioremediation of a soil contaminated by hydrocarbon mixtures: the residual concentration problem. Chemosphere 2000; 41: 1115-23.

OECD. Guideline for Testing Chemicals. Earthworm acute toxicity tests Paris. Organisation for Economic Cooperation and Development 207. 1984.

Plaza GA, Nalecz-Jawecki G, Pinyakong O, Illmer P, Margesin R. Ecotoxicological and microbiological characterization of soils from heavy-metal- and hydrocarbon-contaminated sites. Environ Monit Assess 2010; 163: 477-88.

Rosenberg E, Zuckerberg A, Rubinovitz C, Gutnick DL. Emulsifier of Arthrobacter RAG-1: isolation and emulsifying properties. Appl Environ Microbiol 1979; 37: 402-8.

Ross AS, Farrimond P, Erdmann M, Larter SR. Geochemical compositional gradients in a mixed oil reservoir indicative of ongoing biodegradation. Organic Geochemistry 2010; 41: 307-320.

Sabaté J, Viñas M, Solanas A. Laboratory-scale bioremediation experiments on hydrocarbon-contaminated soils. International Biodeterioration & Biodegradation 2004; 54: 19-25.

Sheppard P, Adetutu E, Makadia T, Ball A. Microbial community and ecotoxicity analysis of bioremediated, weathered hydrocarbon-contaminated soil. Soil Research 2011; 49: 261-269.

Singh N, McCoy M, Tice R, Schneider E. A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 1988; 175: 184-191.

Tucker B, Radtke C, Kwon I, Anderson A. Suppression of bioremediation by Phanerochaete chrysosporium by soil factors. Journal of Hazardous Materials 1995; 41: 251-265.

Vázquez MM, César S, Azcón R, Barea JM. Interactions between arbuscular mycorrhizal fungi and other microbial inoculants (Azospirillum, Pseudomonas, Trichoderma) and their effects on microbial population and enzyme activities in the rhizosphere of maize plants. Applied Soil Ecology 2000; 15: 261-272.

Vinas M, Grifoll M, Sabate J, Solanas AM. Biodegradation of a crude oil by three microbial consortia of different origins and metabolic capabilities. J Ind Microbiol Biotechnol 2002; 28: 252-60.

Wang SJ, Yan ZG, Guo GL, Lu GL, Wang QH, Li FS. Ecotoxicity assessment of aged petroleum sludge using a suite of effects-based end points in earthworm Eisenia fetida. Environ Monit Assess 2010; 169: 417-28.

Wang Z, Fingas MF. Development of oil hydrocarbon fingerprinting and identification techniques. Mar Pollut Bull 2003; 47: 423-52.

Whang LM, Liu PW, Ma CC, Cheng SS. Application of biosurfactants, rhamnolipid, and surfactin, for enhanced biodegradation of diesel-contaminated water and soil. J Hazard Mater 2008; 151: 155-63.

Wrenn BA, Venosa AD. Selective enumeration of aromatic and aliphatic hydrocarbon degrading bacteria by a most-probable-number procedure. Can J Microbiol 1996; 42: 252-8.

Wu Y, Luo Y, Zou D, Ni J, Liu W, Teng Y, et al. Bioremediation of polycyclic aromatic hydrocarbons contaminated soil with Monilinia sp.: degradation and microbial community analysis. Biodegradation 2008; 19: 247-57.

Xiao J, Guo L, Wang S, Lu Y. Comparative impact of cadmium on two phenanthrene-degrading bacteria isolated from cadmium and phenanthrene co-contaminated soil in China. J Hazard Mater 2010; 174: 818-23.

Yamada-Onodera K, Mukumoto H, Katsuyama Y, Tani Y. Degradation of long-chain alkanes by a polyethylene-degrading fungus, Penicillium simplicissimum YK. Enzyme and Microbial Technology 2002; 30: 828-831.

Yateem A, Balba M, Al-Awadhi N, El-Nawawy A. White rot fungi and their role in remediating oil-contaminated soil. Environmental International 1997; 24: 181-187.

Yu Z, Morrison M. Comparisons of different hypervariable regions of rrs genes for use in fingerprinting of microbial communities by PCR-denaturing gradient gel electrophoresis. Appl Environ Microbiol 2004; 70: 4800-6.

Zucchi M, Angiolini L, Borin S, Brusetti L, Dietrich N, Gigliotti C, et al. Response of bacterial community during bioremediation of an oil-polluted soil. J Appl Microbiol 2003; 94: 248-57.

Table 1

Click here to download Table: Table 1 july 2012.docx

Table 1. Physical, chemical and microbiological characteristics of the contaminated soil.

Main characteristics	Values
TPH (mg · kg^{-1})	1727
pH	7.5
Conductivity (μS · cm-1)	322
WHC (% Humidity w/w)[a]	33.7
Humidity (% WHC)	58.8
N-NH_4 (mg · kg-1)	45.8
N-(NO_3+NO_2) (mg · kg-1)	1.7
Heterotrophs (MPN · kg^{-1})[b]	$8.0 \cdot 10^8$
F1 degraders (MPN · kg^{-1})[c]	$2.1 \cdot 10^6$

[a]WHC: Water Holding Capacity

[b]MPN: Most Probably Number.

[c]F1: Aliphatic saturated fraction of the Casablanca crude oil [13].

Table 2

Click here to download Table: Table 2 july 2012.doc

Table 2: Properties of DGGE bands: designations and accession numbers for the band sequences and levels of similarity to related organisms

Band	Band detection[a] L1	L2	L3	L4	L5	L6	Closest organism in GenBank database (accession no.)	% similarity[b]	Phylogenetic group[c]
B1=B3=B4=B13	+	+	+	+	+	+	*Brevundimonas vesicularis* (JN084130)	96%	*Caulobacteraceae* (α)
B2	+	-	-	-	-	-	*Dietzia maris* (JF505994)	100%	*Corynebacterineae* (Actinobacteria)
B5	-	-	+	-	-	-	*Rhizobium sp.* (Y12350)	90%	*Rhizobiaceae* (α)
B6	-	-	+	-	-	-	*Flavobacterium sp.* (EU037956)	99%	*Flavobacteriaceae* (CFB group)
B7	-	-	-	+	+	-	*Altererythrobacter sp.* (FN397680)	94%	*Erythrobacteraceae* (α)
B8	-	-	-	+	+	-	*Parasegitibacter luojiensis* (NR_044576)	97%	*Chitinophagaceae* (CFB group)
B9	-	-	+	+	+	+	Uncultured *Sphingobacteriales*(AM934931)	98%	*Sphingobacteriales* (CFB group)
B10	-	-	-	+	+	+	*Comamonadaceae* bacterium (GQ454852)	95%	*Comamonadaceae* (β)
B11	+	+	+	+	+	-	Uncultured *Sphingobacteriales* (AM936239)	88%	*Sphingobacteriales* (CFB group)
B12	-	-	-	+	+	+	*Ramlibacter sp.* (AM411936)	97%	*Comamonadaceae* (β)
B14	-	-	-	-	-	+	*Herbaspirillum sp.* (AB545652)	94%	*Oxalobacteraceae* (β)
B15=B16	-	-	-	-	-	+	*Bacillus selenatarsenatis* (JN624922)	100%	*Bacillaceae* (Firmicutes)
B17	-	-	-	+	+	+	*Arthrobacter sulfonivorans*(HQ824849)	99%	*Micrococcaceae* (Actinobacteria)
B18	-	-	-	-	-	+	*Streptomyces sp.* (JN572690)	98%	*Streptomycetaceae* (Actinobacteria)

[a]Band detection (+) above 1% of relative intensity.
[b]Sequences were aligned against the GenBank database with the BLAST search alignment tool.
[c]Phylogenetic groups were defined by using the Ribosomal Data Project (RDP) Naive Bayesian Classifier (Wang et al., 2007). Family is represented.α, β, represent α-proteobacteria and β-proteobacteria, respectively.

Figure 1

Click here to download Figure: Figure 1 july 2012.docx

FIGURE 1

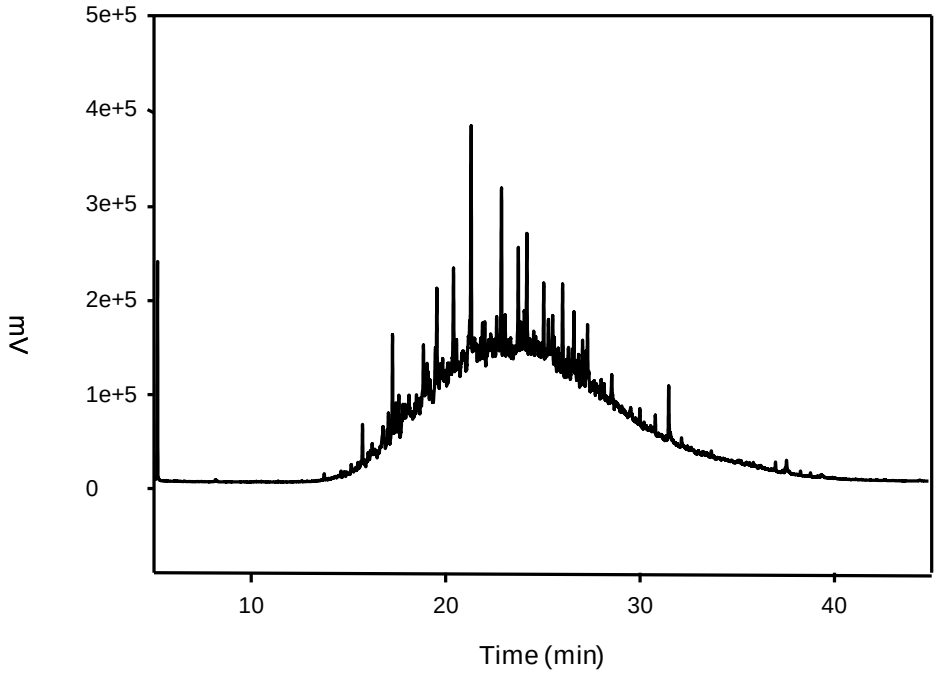

Figure 2
Click here to download Figure: Figure 2 july 2012.docx

Figure 2

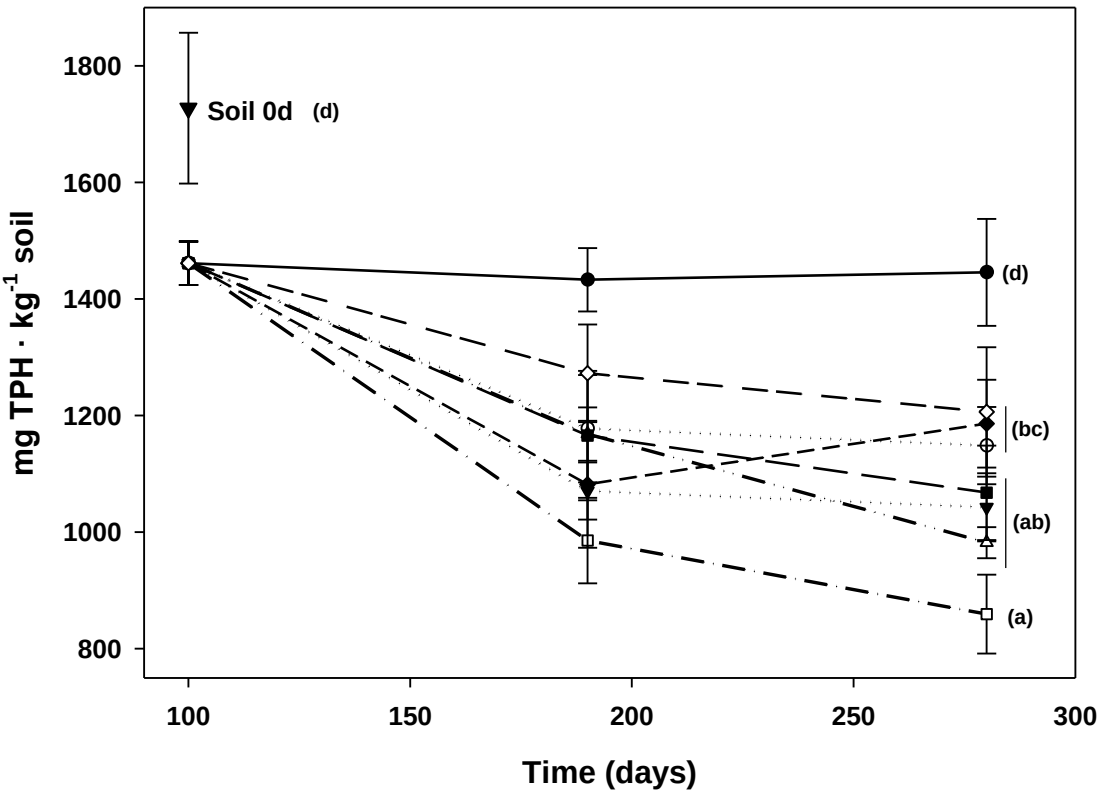

Figure 3

Click here to download Figure: Figure 3 july 2012.docx

FIGURE 3

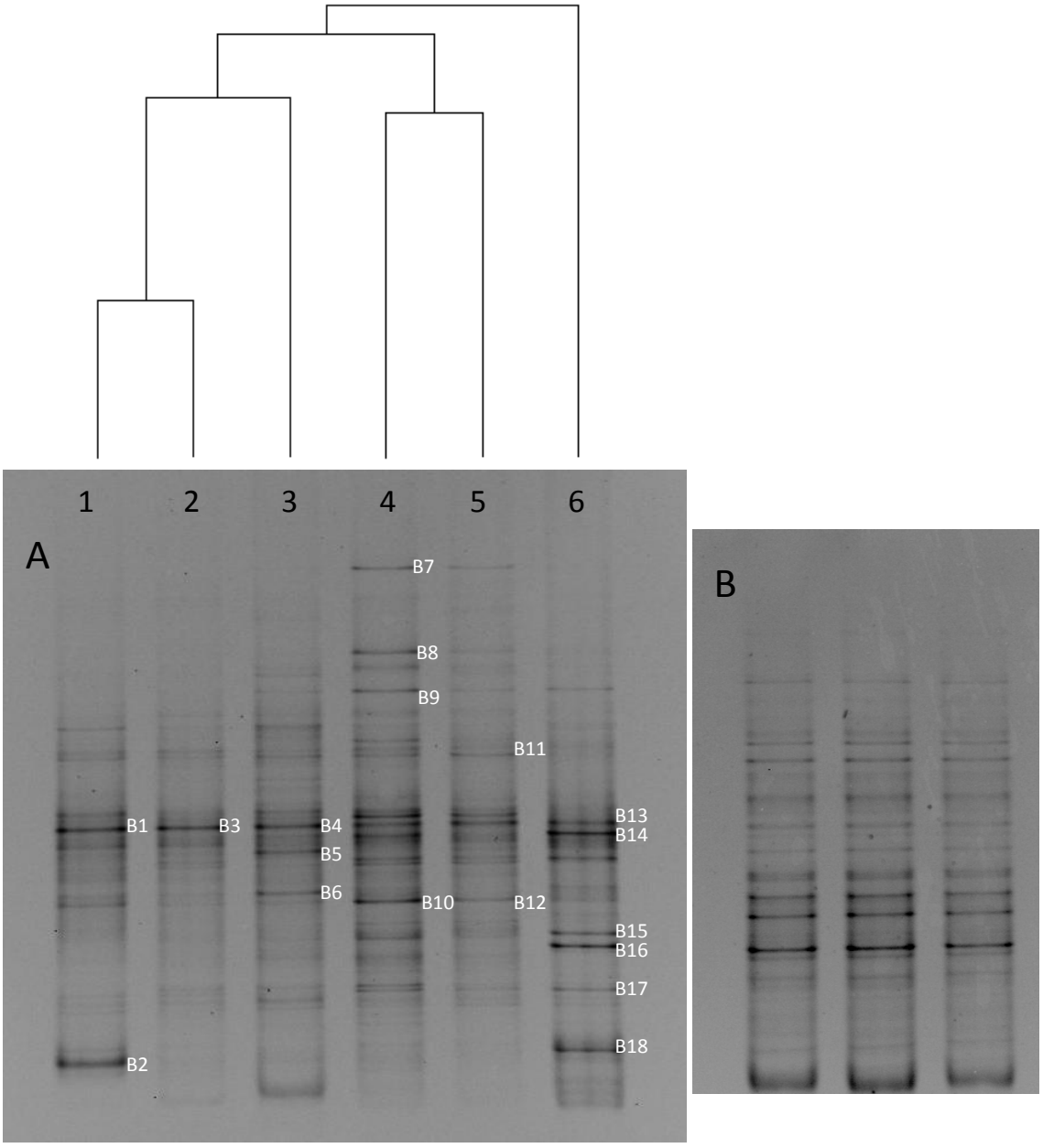

Figure 4

Click here to download Figure: Figure 4 july 2012.docx

Figure 4

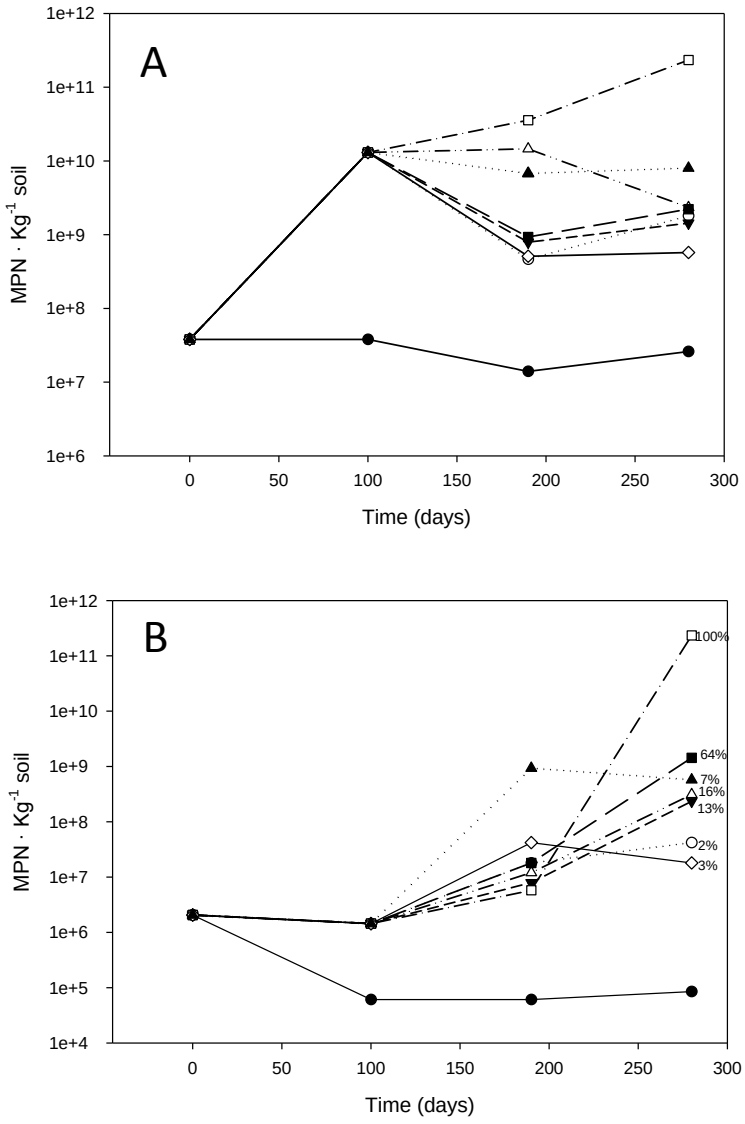

Figure 5
Click here to download Figure: Figure 5 june 2012.docx

FIGURE 5