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Bioremediation of diesel oil in a co-contaminated soil by bioaugmentation with a microbial formula tailored with native strains selected for heavy metals resistance

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A B S T R A C T

The aim of the work is to assess the feasibility of bioremediation of a soil, containing heavy metals and spiked with diesel oil (DO), through a bioaugmentation strategy based on the use of a microbial formula tailored with selected native strains. The soil originated from the metallurgic area of Bagnoli (Naples, Italy). The formula, named *ENEA-LAM*, combines ten bacterial strains selected for multiple resistance to heavy metals among the native microbial community. The biodegradation process of diesel oil was assessed in biometer flasks by monitoring the following parameters: DO composition by GC-MS, CO_2 evolution rate, microbial load and composition of the community by T-RFLP, physiological profile in Biolog® ECOplates and ecotoxicity of the system. The application of this microbial formula allowed to obtain, in the presence of heavy metals, the complete degradation of n-$C_{12–20}$, the total disappearance of phenantrene, a 60% reduction of isoprenoids and an overall reduction of about 75% of the total diesel hydrocarbons in 42 days. Concurrently with the increase of metabolic activity at community level and the microbial load, the gradual abatement of the ecotoxicity was observed. The T-RFLP analysis highlighted that most of the *ENEA-LAM* strains survived and some minor native strains, undetectable in the soil at the beginning of the experiment, developed. Such a bioaugmentation approach allows the newly established microbial community to strike a balance between the introduced and the naturally present microorganisms. The results indicate that the use of a tailored microbial formula may efficiently facilitate and speed up the bioremediation of matrices co-contaminated with hydrocarbons and heavy metals. The study represents the first step for the scale up of the system and should be verified at a larger scale. In this view, this bioaugmentation strategy may contribute to overcome a critical bottleneck of the bioremediation technology.

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1. Introduction

As successful bioremediation programs require the application of strategies tailored to the specific environmental parameters of the contaminated site, feasibility studies are a prerequisite for any planned intervention. In particular, co-contaminated matrices represent a problem in the bioremediation processes because high metal concentrations can inhibit the biodegradation of organic pollutants, imposing a double stress on the microbial populations (Roane et al., 2001). Intervention strategies for a successful bioremediation have recently focused on the study of the multi-component systems like a consortium of microorganisms, which represent a model closer than the single-component systems, to the real situations of the natural environment (Ledin, 2000). These studies represent a step forward to bridge the existing gap between pure cultures and “in situ” studies, exploiting the richer metabolic network available in a microbial consortium for complete biodegradation processes.

The activation of natural degradation potentials in environmental media is currently the challenge in the environmental research addressed to remediation methods. Ways to activate these potentials must consider that most degradation potentials are widely distributed among microorganisms (Alexander, 1999) but indigenous microbes are usually present in a very small number. Moreover, the degradative metabolism towards specific pollutants needs often to be induced and the presence of heavy metals often inhibits the biodegradation processes in co-contaminated matrices. Possible ways to overcome these limitations include the changing of physicochemical parameters (pH, T, electron donors or acceptor, etc.) as well as a “niche adjustment” by the inoculation of competent microorganisms into these systems (bioaugmentation).

Bioaugmentation offers a way to provide specific microbes in sufficient number to complete the biodegradation. Over the last few years, contaminating compounds as insecticides, petroleum compounds and a growing number of toxic organic chemicals have been

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successfully remediated using the bioaugmentation (Kanaly et al., 2000; Lendvay et al., 2003; Silva et al., 2004). A recent review (Gentry et al., 2004) provides an extensive survey of new bioaugmentation strategies. Bioaugmentation has several advantages over other techniques: when a specific microbial population is injected the degradation process can start immediately, while biostimulation, for instance, involves a delay after injection of nutrients as the microbial population propagates and also nutrient are not specific, so that all microbes will potentially propagate, diluting the effect of the nutrients (Weston and Balba, 2003). Studies on the comparison of natural attenuation, biostimulation and bioaugmentation on the degradation of total petroleum hydrocarbons (TPH) in soils contaminated with diesel oil come to heterogeneous results, also due to the site-specific feature of bioremediation technology. Hamdi et al. (2007) observed that the bioremediation efficacy was more likely to rely on the selectivity and specialisation of added microorganisms rather than on nutrient load. Bento et al. (2003) concluded that the best approach for bioremediation of diesel oil was the bioaugmentation performed by inoculating microorganisms pre-selected from their own environment. Using native soils has the advantage that the microbes are more likely to survive and propagate when reintroduced into the site. Van der Gast et al. (2004) demonstrated that the strategy of using tailor-made consortia, which links functionally the *in situ* microbial community structure with the contaminants revealed by chemical analyses, seems to be a promising avenue towards rational selection of effective inocula for bioremediation applications.

Based on these considerations, the achievement of a deeper understanding of microorganisms involved in the respective ecosystems, their abilities, together with their metabolic networks as well as their cellular resistance and adaptation mechanisms, will bring out a variety of appropriate “microorganisms formula”. Commercial companies argue that, as soon as these formulas become more and more commercially available, bioaugmentation will become the standard for rapid and precise cleanup of a variety of contaminated soil and waste water situations (Weston and Balba, 2003). At present, bioaugmentation remains debatable as a scientific and as a technological endeavour and the current decade will be crucial in giving clear-cut answers on the real potential of bioaugmentation but, under such circumstances, bioaugmentation could emerge as one of only a few environmentally friendly techniques for pollution abatement (El Fantroussi and Agathos, 2005).

The present study aimed to determine the effectiveness for the bioremediation of diesel-contaminated soil of a microbial formula, tailor-made with native bacteria specifically selected for their multiple resistance to heavy metals. The trials were conducted in biometers and the degradation of diesel hydrocarbons was assessed by GC-MS analyses and by measuring the CO_2 evolution rate. During the experiment, the soil toxicity was evaluated by ecotoxicological test battery and the evolution of the microbial population was followed by plate count, T-RFLP and Biolog® analyses.

2. Materials and methods

2.1. Soil samples

Soils from the dismissed industrial site of Bagnoli (Naples, Italy) were extensively studied by Buondonno et al. (1988) which describe a high variability in the physical and chemical properties due to the human activity in the site: as general characteristics, these soils are sandy (66–94%) with pH ranging from 5.3 to 7.0 in natural soils and from 7.0 to 12.6 in industrial soils.

In a previous work (Sprocati et al., 2006b) different areas of the industrial site were investigated for the presence of pollutants and for a comparative study of the native microbial communities. Based on these results, the co-contaminated millwork area (LAM) was chosen for this bioremediation study. Seven samples of soil from the top 30 cm were collected in sterile plastic bags; the samples were kept refrigerated and transported to the laboratory, where they were oven-dried at 40 °C o/n, sieved (<2 mm) and pooled. The soil had a pH of 9.6 (measured according to the Official Methods, 1999), 2.02% of total carbon (1.08% organic C), and less than 0.1% of total nitrogen. The soil was analysed for its content in heavy metals (Mn 1044 mg kg^{-1}, Zn 115 mg kg^{-1}, Cr 12 mg kg^{-1}, As 20 mg kg^{-1}, Pb 48 mg kg^{-1},Cd 0.12 mg kg^{-1}, Cu 13.90 mg kg^{-1}) and organic pollutants (total PHAs 0.18 mg kg^{-1} and paraffins C19–C33 2.55 mg kg^{-1}).

2.2. Selection of microorganisms for heavy metals resistance

Microorganisms employed in this study were isolated in 2004 from a previous sampling (Sprocati et al., 2006b). The heterotrophic microbial community was extracted from 30 g of fresh soil mixture, added with 80 ml of pyrophosphate and stirred for 1 h with glass beads. The slurry was centrifuged at 300 rpm and the supernatant containing the microorganisms was recovered in sterile tubes. The growth of culturable microorganisms was carried out on both Mineral Medium (Schmidt and Schlegel, 1989) and Tryptic Soy Broth (TSB) at 28 °C in orbiting shaker (180 rpm). Heterotrophic bacteria were isolated on Tryptic Soy Agar (TSA) plates (28 °C, 60% of humidity) and were selected for their resistance to heavy metals on Mineral Medium agar plates amended with heavy metals salts: $Pb(NO_3)_2$, $ZnSO_4$, $CuSO_4$, $NiSO_4$, $CoSO_4$, $K_2Cr_2O_7$, and $CdCl_2$ were added at increasing concentrations (ranging from 0.1 mM up to 8 mM) to determine the MIC (Minimum Inhibitory Concentration). MIC defines the lowest concentration that causes the total growth inhibition. In order to discriminate among resistant and sensitive strains and to evaluate the resistance levels, two reference strains have been used: *E. coli* (heavy metals-sensitive) and *Ralstonia metallidurans* (highly resistant). All growth media were purchased from Liofilchem (Teramo, Italy).

2.3. Identification of bacterial strains

The selected strains were identified by means of 16 S r-DNA sequencing. Single-colony 16S r-DNA amplification was performed with a GeneAmp PCR System 9700 thermocycler (Perkin Elmer, Norwalk, CT, USA), using the universal Eubacteria primers P0 (5′-GAG AGT TTG ATC CGT GCT CAG- 3′) and P6 (5′-CTA CGG CTA CCT TGT TAC GA- 3′), yielding a fragment of about 1500 bp. Each PCR reaction (50 µl) contained 2 µl of cell lysate (obtained from a single colony of each isolate, according to the procedure described by Di Cello et al., 1997), 25 µl 2× Master Mix (Fermentas, Burlington, Ontario, Canada), 100 pmol of each primer and 0.5 µl formamide. The PCR thermal cycling scheme consisted of 1 min at 95 °C, followed by 30 s at 95 °C, 30 s at 60 °C, 4 min at 72 °C (5 cycles); then 30 s at 95 °C, 30 s at 55 °C, 4 min at 72 °C (5 cycles); then 30 s at 95 °C, 30 s at 50 °C, 4 min at 72 °C (25 cycles), followed by a final extension at 72 °C (10 min) and at 60 °C (10 min). PCR products were purified and concentrated with Microcon 100 (Millipore, Biollerica, MA, USA), following the manufacturer's instructions. The sequencing was performed by Biofab (Pomezia, Italy) using the same pair of primers.

The sequences obtained were compared to data-base sequences using the BLAST system (http://www.ncbi.nlm.nih.gov/BLAST) and deposited in the GenBank® genetic sequence database (Nucleic Acids Research 2007 Jan. 35; Database issue D21-5), with the Accession Numbers from EU019982 to EU019991 (Table 1). All primers were purchased from Metabion (Martinsried, Germany).

Table 1
Experimental conditions

Sample	Crude oil (%)	Medium	Microorganism	Surfactant
Spiked soil	1%	MM	ENEA-LAM	0.2%
Abiotic control	1%	MM	–	0.2%
Biotic control	–	MM	ENEA-LAM	–

2.4. Set up of the microbial formula for the bioaugmentation

Bioaugmentation was performed using the following strains isolated from the 2004 sampling and selected on purpose for multiple resistance to heavy metals: LAM 1, LAM 9, LAM 11, LAM 19, LAM 22, LAM 18, LAM 23, LAM 29, LAM 30 and LAM 33. Each strain was grown as a pure culture in TSA plates at 28 °C for 3 days. The cells were then harvested and suspended in sterile phosphate buffer up to a density of 0.09 OD_{600}. The individual suspensions were then pooled in equal proportions to setup the formula for bioaugmentation, inoculated at a final concentration of 5×10^7 CFU ml^{-1}.

2.5. Experimental setup

10 g of sieved soil (<2 mm) from sample *LAM 2006* were added with 40 ml of Mineral Medium (pH 7) in 250 ml biometer flasks (Bartha and Pramer, 1965; EPA, 1996). The slurries obtained were spiked with 0.5 g of diesel oil (DO) simulating a soil contamination corresponding to 50,000 mg kg^{-1} soil. The surfactant 10-ethoxylated nonylphenol (Sigma-Aldrich, St. Louis, MO, USA) was used at final concentration of 2 g L^{-1} to emulsify the oil in the aqueous phase. Bioaugmentation was carried out inoculating the bacterial pool, prepared as described above, at a final concentration of 5×10^7 CFU ml^{-1}, determined by the plate count method. Biometer flasks were incubated in a rotating shaker at 150 rpm and 28 °C, in the dark. Abiotic controls were performed by the addition of mercuric chloride (Sigma-Aldrich, St. Louis, MO, USA) 50 mg L^{-1}, in order to inhibit the activity of indigenous soil microorganisms and evaluate the abiotic CO_2 production (Wolf et al., 1989). Biotic controls were performed by adding to the soil the bacterial inoculum, in order to monitor the basal capacity of the soil to sustain the bacterial growth. The details of the experimental conditions are shown in Table 1.

Chemical, microbiological and ecotoxicological analyses were performed using the whole content of two biometer flasks for each experimental condition after 0, 15 and 42 days of incubation; CO_2 evolution was measured every two days.

2.6. Chemical analyses

A commercial diesel oil (DO) was used in this study. DO is a distillate containing different components including n-alkanes (from C_{11} to C_{25}), branched alkanes, cycloalkanes and aromatic hydrocarbons. Besides, the DO was enriched in phenantrene (up to a concentration of 27.6 mg kg^{-1} soil) in order to evaluate the biodegradation of polycyclic aromatic hydrocarbons.

N-alkanes and isoprenoid hydrocarbons were detected individually; total determination was performed integrating the unresolved-mass complex (UMC) to the baseline from time 8.7 to time 40 of the chromatogram, including C_{13} and C_{25} n-alkanes. To evaluate possible losses due to evaporation, abiotic control flasks were analysed at the start and at the end of the experiment.

The whole content of the biometer flasks was decanted to separate the liquid phase from the solid phase. The liquid phase was extracted with hexane (liquid–liquid extraction). The solid phase was extracted with acetone, acetone/hexane (1:1) and hexane, in three successive steps. All the extracts were dehydrated on anhydrous sodium sulphate and pooled together. α-Androstane was used as internal standard. The analyses were performed by gas chromatography with mass selective detector (GC-MSD) using a Thermo Trace GC 2000 gas chromatograph coupled to a Trace MS detector, split–splitless injector and AS 2000 auto sampler (all from Thermo Electron Corporation, USA). A fused silica capillary column DB-5 MS (Agilent Technologies, USA), 60 m by 0.25 mm i.d., and film thickness 0.25 µm was used. The injector and interface temperatures were 250 °C and 280 °C respectively. The carrier gas was helium at a flow rate of 1 ml min^{-1}. One µl of sample was injected in splitless mode (60 s) and the oven temperature was programmed as follows: 60 °C for 1 min, $60^{-1}60$ °C at 20 °C min^{-1}, 160 °C for 1 min, 160–320 °C at 3 °C min^{-1}, 320 °C for 5 min. The mass spectrometer was operated in scansion mode for the determination of hydrocarbons and of the unresolved-mass complex (UMC), while single ion monitoring (SIM) was applied for the determination of phenantrene. Removal efficiency was expressed as the ratio of the mean concentration of each hydrocarbon in the spiked DO soil sample to the same hydrocarbon in the abiotic control.

2.7. Soil respiration

The CO_2 produced during the experimental process was trapped in the side arm of the biometer flasks (EPA, 1996), containing 10 ml of 0.1 N KOH solution. The amount of CO_2 produced was periodically measured by Warder titration (Benedetti-Pichler and Cefola, 1939) using 0.05 N HCl solution. Metabolic CO_2 was calculated by the difference between the treated samples and the abiotic control. Biometer flasks were provided with Ascarite filters to avoid the input of atmospheric CO_2 during KOH sampling.

2.8. Microbial community analysis: Biolog® ECOPlates and T-RFLP

Total count of the heterotrophic microbial population was determined at the beginning of the experiment (time zero) and after 15 and 42 days of incubation, by the plate count method on TSA agar plates.

2.8.1. Biolog® ECOPlates

The metabolic profiles of the microbial communities were generated by means of the Biolog® Microstation System 4.2 (Biolog Inc., Hayward, CA, USA) using ECOPlates, specifically designed for community analysis and microbial ecological studies. The ECOPlate contains 31 of the most useful carbon sources for soil community analysis, repeated 3 times to give more replicates of the data. The whole content of the biometer flasks (two for each experimental condition: spiked, abiotic and biotic control) at time zero and after 15 and 42 days of incubation, was stirred with glass beads for 1 h. In order to extract the microbial community, the slurry was centrifuged at 300 rpm, the supernatant was recovered in sterile tubes and centrifuged at 13,000 rpm for 20 min: the resulting pellet was suspended in sterile physiological solution. The cell concentration was adjusted to 20% of transmittance and inoculated into Biolog® ECOPlates according to manufacturer's instructions. Then, plates were incubated at 28 °C in the dark and analysed by the Microplate Reader (dual wavelength data: OD_{590}–OD_{750}) after 2, 3 and 7 days. The inoculum density and incubation times were standardized in order to obtain a correct estimate of the quantitative metabolic profile of the communities (Garland, 1997). Kinetic analysis has been performed using AWCD (Average Well Colour Development) as parameter that enables to capture an integral picture of differences in carbon sources utilisation. AWCD was calculated as the arithmetic mean of the OD values of all of the positive wells in the plate per reading time (Garland, 1996).

2.8.2. Microbial community DNA extraction

Total genomic DNAs from each sample were extracted from the bacterial pellets obtained as described in the Biolog® section. Aliquots (200 µl) of bacterial pellets were suspended in 500 µl of lysis buffer (100 mM Tris–HCl pH 8, 100 mM EDTA, 2% SDS). Lysozyme (1 mg ml^{-1}) was added and the suspensions were incubated 2 h at 37 °C. After the addition of proteinase K (0.1 mg ml^{-1}) the suspensions were further incubated 2 h at 37 °C. CTAB and NaCl were added at a final concentration of 1% and 1.5 M, respectively, and the suspensions were incubated at 50 °C overnight. After centrifugation, the supernatants were extracted in an equal volume of chloroform:isoamyl alcohol

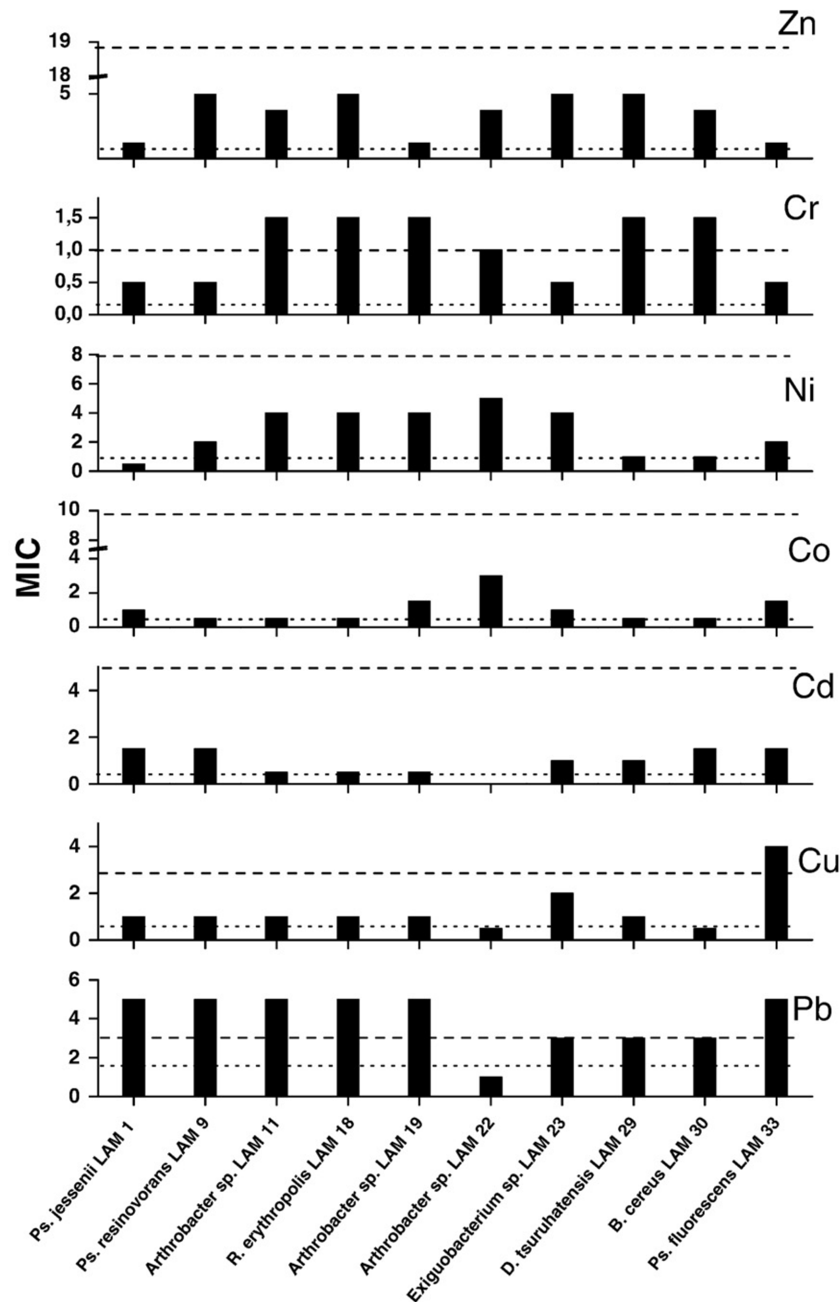

Fig. 1. Minimal inhibitory concentration (MIC) of seven heavy metals to ten selected bacterial strains. Dotted lines: *E. coli* MIC values. Dashed lines: *R. metallidurans* MIC values.

(24:1), the aqueous phases were added with 0.5 vol. of 5 M ammonium acetate and incubated at 4 °C for 1 h. After centrifugation, DNA in the supernatants was recovered by the addition of 0.55 vol. of isopropanol followed by centrifugation at 13,000 rpm for 15 min. The pellets were washed with 70% ethanol, dried and suspended in 50 µl of sterile water.

2.8.3. T-RFLP amplification and analysis

16S rDNAs from bacterial community DNA and from the ten inoculated strains were amplified with the oligonucleotide primers 63F (5′-CAG GCC TAA CAC ATG CAA GTC-3′) and 1389R (5′-ACG GGC GGT GTG TAC AAG-3′). The PCR products were again amplified with the same primers 63F and 1389R, labelled at the 5′ end with the phosphoramidite dyes 6-FAM and HEX, respectively, according to the method of Osborn et al. (2000), and purified using CentriSep columns to remove unincorporated nucleotides. Labeled PCR products were digested with 20U of *AluI* (Fermentas, Burlington, Ontario, Canada) in a total volume of 15 µl at 37 °C for 3 h. The restriction digestions (2 µl) were mixed with 2 µl of deionized formamide and sent to Bio-Fab Research (Pomezia, Italy) for electrophoretical analysis in a 3730 DNA Analyzer (Applied Biosystems, Carlsbad, CA, USA), 36 cm capillary array, POP-7 Performance Optimized Polymer. T-RFLP profiles were analysed using GeneMapper software (version 4.0). The size of terminal restriction fragments (T-RFs) was estimated by reference to the internal standard (Gene Scan G500, Liz). Baseline threshold was set at 100 fluorescence units. The fluorescence integrated under any peak is referred as the area of that peak, and the total area for any profile is the sum of the areas of all of the peaks, excluding those generated by fragments of less than 35 nucleotides or greater than 500 nucleotides (Osborne et al., 2006). Chemicals and enzymes were purchased from Sigma-Aldrich (St. Louis, MO, USA) unless otherwise specified.

2.9. Ecotoxicity tests

Ecotoxicity tests were performed on the liquid phase of slurry from two flasks for each experimental condition at time zero, 15 and 42 days of incubation. The liquid phase was recovered after centrifugation (4000 rpm, 5 min). The toxicity was expressed in Toxic Units ($TU=100/EC_{50}$), where EC_{50} is defined as the sample concentration that induces an effect on target organisms of 50% with respect to the control. Sample dilutions were performed in order to obtain the concentration–response curve for each sample. Concentration–response curves of DO and surfactant for all the test organisms were also determined. The EC_{50} was calculated using the Linear Interpolation Method.

2.9.1. Vibrio fischeri bioluminescence test

The test is based on the inhibition of natural bioluminescence of *V. fischeri* (ISO, 1998). The test was performed using the Microtox System (Microbics Corporation, Carlsbad, CA), according to the manufacturer's instructions. The inhibition of bioluminescence was measured after 5–15–30 min of exposure (15 °C) to the matrix using the Microtox Model 500 Analyzer. The results were expressed as the sample concentration that caused a 50% reduction of bioluminescence respect to the control (EC_{50}).

2.9.2. Daphnia magna acute immobilisation test

The toxicity test with the cladoceran *D. magna* was carried out according to OECD (2004). Serial dilutions of the sample were tested in triplicate exposing 5 daphnids (aged less than 24 h) in each Petri dish containing 10 ml of sample. The number of animals, considered immobilised after a 15 s observation, was recorded after 24 and 48 h of incubation (20 ± 1 °C, in the dark). The results were expressed as EC_{50}, defined as the sample concentration estimated to immobilise 50% of the daphnids.

2.9.3. Selenastrum capricornutum growth inhibition test

According to EPA (1985) a population of the green microalga *S. capricornutum*, from ENEA-Portici cultures, was exposed to sample dilutions. Cell density was measured after 96 h incubation (25 ± 1 °C, 4000 lx) both in test solutions and control. The percentage inhibition of algal growth with respect to the control was determined by cell count and the results expressed as EC_{50} (the sample concentration that inhibits cell growth of 50%).

3. Results

3.1. Heavy metals resistances

In order to select microorganisms resistant to heavy metals, to be employed in bioremediation of co-contaminated matrices, the culturable strains isolated from the community native to the area LAM were grown on solid agar containing increasing concentrations of different heavy metals: $Pb(NO_3)_2$, $ZnSO_4$, $CuSO_4$, $NiSO_4$, $CoSO_4$, $K_2Cr_2O_7$, and $CdCl_2$. The distribution of the resistances observed reflects the quantitative presence of heavy metals detected in that area, in particular for Cr and Pb and partly for Zn (Sprocati et al., 2006b). The bacterial strains showing multiple heavy metal resistances are shown in Fig. 1: the strain LAM 33 is resistant to all the metals tested; all the strains are resistant to Cr, Pb and Zn, with the exception of strain LAM 22, sensible to lead but highly resistant to Ni and Co.

This microbial set, offering a combination of resistances to many heavy metals, should give mutual benefit to the single components and should be then suitable for bioremediation of co-contaminated soils.

Table 2
Accession number and closest match of 16S rDNA sequences of the ten bacterial strains in the consortium ENEA-LAM

GenBank accession number	Isolate	Closest match (%)
EU019982	LAM 1	*Pseudomonas jessenii* (99%)
EU019983	LAM 9	*Pseudomonas resinovorans* (98%)
EU019984	LAM 11	*Arthrobacter* sp. (98%)
EU019985	LAM 18	*Rhodococcus erythropolis* (99%)
EU019986	LAM 19	*Arthrobacter* sp. (97%)
EU019987	LAM 22	*Arthrobacter* sp. (98%)
EU019988	LAM 23	*Exiguobacterium* sp. (99%)
EU019989	LAM 29	*Delftia tsuruhatensis* (99%)
EU019990	LAM 30	*Bacillus cereus* (99%)
EU019991	LAM 33	*Pseudomonas fluorescens* (99%)

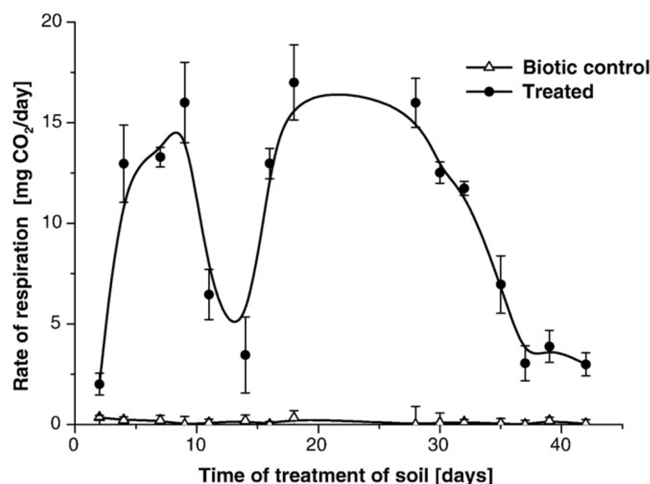

Fig. 2. Respiration rate of microbial community in the biometers spiked with diesel oil (black circles) and biotic control (open triangles), expressed as the daily production of CO_2 during 42 days of treatment.

3.2. Identification of bacterial strains

16S rDNA PCR amplification and subsequent sequencing yielded a DNA fragment of about 1.5 kbp, consistent with the expected length of the amplification product. For each strain, a fragment of about 1450 unambiguous bases was used in Ribosomal Database Project and Genbank® database searches: both resulted in sequence homologies from 97 to 99%. The identification of these strains by sequencing of 16rDNA fragments and their Genbank® accession numbers are shown in Table 2: the strains LAM 11, LAM 19, LAM 22 were close to the genus *Arthrobacter*; LAM 1, LAM 9 and LAM 33 were identified as different species of the genus *Pseudomonas*, LAM 18 was identified as *Rhodococcus erythropolis*, LAM 30 as *Bacillus cereus*, LAM 23 as *Exiguobacterium* sp. and LAM 29 as *Delftia tsuruhatensis*.

3.3. Respiration rate and bacterial growth

The production of CO_2 is a measure of the respiration rate and indicates indirectly the capability of the bacterial community to use the DO as a carbon source. The respiration rate (Fig. 2) in flasks spiked with the DO showed a biphasic curve with two peaks at 8 and 16 days of incubation; after 28 days, the rate of CO_2 production progressively decreased. The biotic control showed a respiration rate very low compared with DO spiked soil. The plate count on agar was in agreement with the respirometric data: the total heterotrophic flora in the soil spiked with the DO was subjected to little quantitative changes (10^7–10^8 CFU ml^{-1}) due to the capability of the microorganisms to metabolise the DO as carbon source. In the biotic control the plate count dropped to 10^2 CFU ml^{-1} after 15 days and remained stable until the end of the experiment.

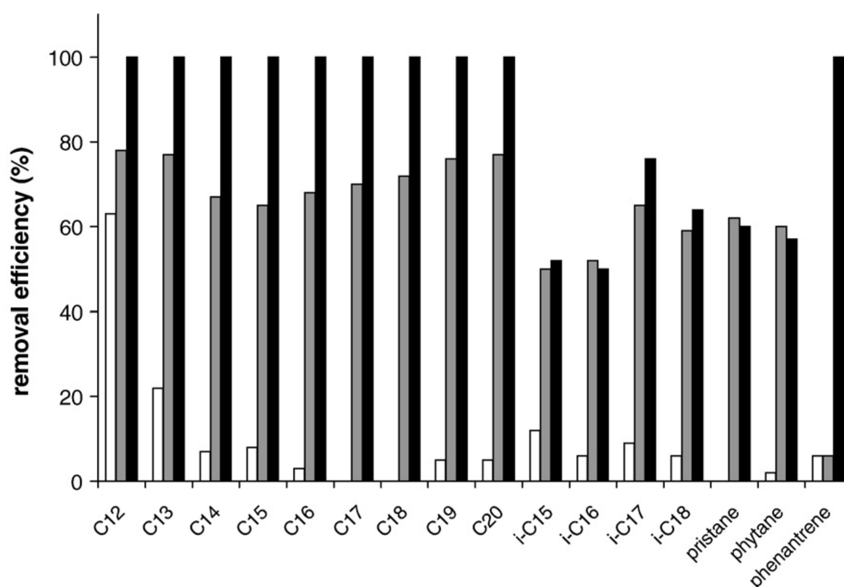

Fig. 3. Removal efficiency (%) of diesel oil hydrocarbons. Open bars: abiotic control after 42 days. Grey bars: spiked soil after 15 days. Black bars: spiked soil after 42 days.

3.4. Diesel oil biodegradation

The DO degradation was followed by MS-GC; the analysis of the chemical composition of hexane extracts (Fig. 3) shows that in the abiotic control after 42 days of incubation, as expected, the alkanes with low molecular masses suffered from evaporation: dodecane (C_{12}) was lost at 60% and tridecane (C_{13}) at 20%, while isoprenoid alkanes and phenantrene were practically not affected by evaporation losses. After 15 days of incubation the microorganisms degraded 65–75% of the initial amount of linear hydrocarbons (C_{12}–C_{20}) and at the end of the experiment they were totally degraded. The most recalcitrant isoprenoid alkanes (i-C_{15} to i-C_{18}) after 15 days were biodegraded to 50–60% of the initial level, and were not further degraded by the end of the experiment. The polycyclic aromatic hydrocarbon phenantrene was not degraded after 15 days, but disappeared completely at the end of the experiment.

The unresolved-mass complex UMC was decreased to 45% of the initial area. The ratios C18/phytane and C17/pristane, currently used as indicators of biodegradation, decreased from 2.5 to 1.8 in the first part of the experiment (0–15 days), and after 42 days the ratios were reduced to zero.

3.5. Microbial community analysis: T-RFLP and Biolog® ECOPlates

The T-RFLP data obtained after the digestion with *AluI* were analysed, as shown in Table 3 and Fig. 4, in terms of biodiversity (i.e. the number of fragments) and abundance (i.e. the total peak area as fluorescence units). In the soil spiked with DO, the number of fragments and the bacterial abundance (Table 3) during the time-course of the experiment decreased after 15 days, to increase again at the end of the experiment, indicating a raise in diversity of bacterial species, while in the biotic control both the parameters showed a gradual decline. After 42 days, the T-RFLP profile (Fig. 4) of the spiked soil community showed the T-RFs corresponding to the fragments of *Pseudomonas*, *Arthrobacter*, *Exiguobacterium* and *Delftia* strains inoculated with the microbial formula, while the T-RFs corresponding to the fragments of the other inoculated strains, *B. cereus* and *R. erythropolis*, were not detected. Furthermore, several new unidentified fragments appeared.

Table 3
T-RFLP fragments and total peak area (as fluorescence units, FU) obtained from the electrophoretic profiles

Time (days)	Biotic control		Spiked soil	
	No. of fragments	Total area (FU × 10^3)	No. of fragments	Total area (FU × 10^3)
T0	21	360	21	360
T15	14	191	17	190
T42	10	180	31	277

The average well colour development (AWCD) of the microbial community of the soil spiked with DO, in comparison with the AWCD of the biotic control is shown in Fig. 5. The AWCD measure gives a general indication on the metabolic capacity of the community and on the degree of activity with respect to 31 different substrates. The physiological state of the cells, as well as the inoculum density, plays a role in the rate of colour formation, indicating that the rate of O_2 consumption influences the rate of colour development; the type of organisms present in the sample may influence the rate of AWCD as well (Garland, 1996). For these reasons, the inoculum density and incubation times were standardized in order to follow the dynamics in composition of the bacterial community. The wider and the highest metabolic activity was recorded within the community recovered in the spiked samples after 15 days, that was able to metabolise 28 out of 31 substrates, while the biotic control only 22 and showed a much higher metabolic activity, reaching AWCD values of 1.2 OD in 168 h of incubation in ECOPlates; after 42 days in the presence of DO, the AWCD showed values around 0.8 OD. The biotic control showed AWCD values lower than 0.2 OD even after 172 h of plate incubation, indicating a microbial activity nearly at basal metabolic rate.

The two communities showed a metabolic diversification in the carbon sources oxidation (Table 4). The community in the biotic control was unable to utilise: D-galactonic acid lactone, L-serine, α-cyclodextrin, γ-hydroxybutyric acid, glycyl-L-glutamic acid, α-keto-butyric acid, phenylethylamine and D-malic acid, while the community in the spiked sample was unable to utilise L-threonine, glycogen, D-glucosaminic acid and dextrin. The only substrate that both the communities were unable to metabolise was 2-hydroxybenzoic acid.

3.6. Ecotoxicity tests

The toxicity of liquid phase of the slurry at zero, 15 and 42 days for the spiked soil was reported in Fig. 6. The toxicity decreased with time both for *V. fischeri* and *D. magna*, while for *S. capricornutum* at 15 days the toxicity was greater (approximately 70%) than at the start of the

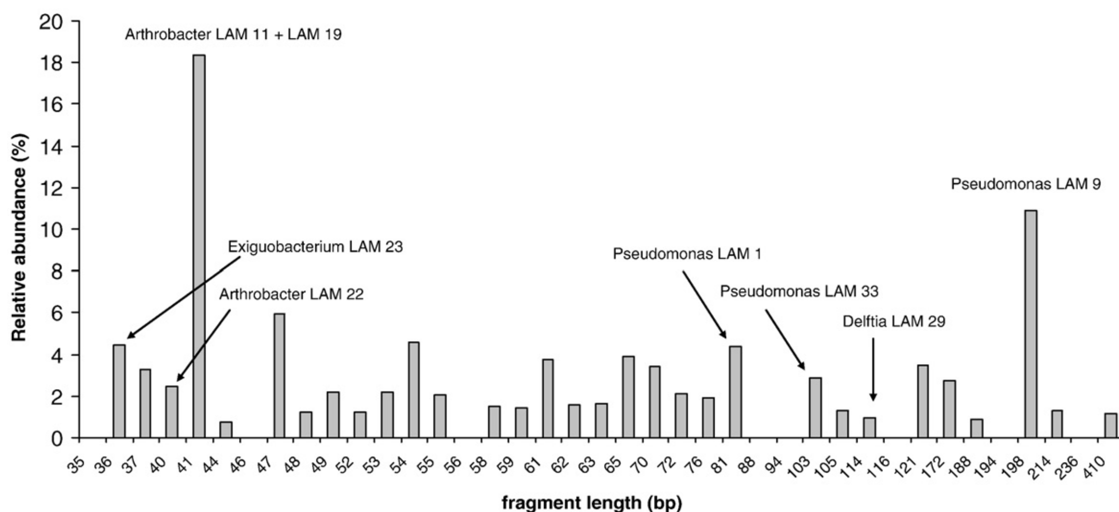

Fig. 4. T-RFLP profile of bacterial community DNA from spiked soil incubated for 42 days. Bars represent the T-RFs (in bp) vs their relative abundance (%); the T-RFs of the inoculated strains are shown on the correspondent bars.

experiment. Nevertheless, the toxicity was strongly decreased at the end of the experiment for all the tested organisms.

The abiotic control poisoned with $HgCl_2$ showed a very high toxicity level (data not shown) on all used organisms. The biotic control showed no detectable toxicity on all used organisms. The DO toxicity was comparable for *V. fischeri* and *S. capricornutum* (EC_{50}=2.5 mg l^{-1} e 2.7 mg l^{-1} respectively) while for *D. magna* it was lower (EC50>4 mg l^{-1}). The surfactant was less toxic for *V. fischeri* (EC_{50}=7300 mg l^{-1}) than for *D. magna* and *S. capricornutum* (EC_{50}=25.5 mg l^{-1}, EC_{50}=36.3 mg l^{-1} respectively). Moreover, the surfactant caused a biostimulation growth (40%) in *S. capricornutum* population at low concentrations (<30 mg l^{-1}). The toxicity of solid phase also decreases at 42 day upon *V. fischeri*, after a slight increase (approximately 20%) at 15 days of the experiment (data not shown).

4. Discussion

The aim of the trial was to assess the effectiveness of a microbial formula, made up with native selected strains, as a bioaugmentation agent for the bioremediation of a co-contaminated soil. The microbial formula, named *ENEA-LAM*, has been tailored by combining ten bacterial strains selected from the native microbial community for their multiple resistances to heavy metals (Fig. 1, Table 2). Some of these strains belong to genera known to play a role in bioremediation: i.e. *Pseudomonas fluorescens* and *Arthrobacter* sp. were successfully employed in the clean up of soils polluted by atrazine, PCBs and PHAs (El Fantroussi and Agathos, 2005 and references therein). Different biodegradation pathways have been also described for these microorganisms: organomercury, trinitrotoluene, α-pinene, 4-hydroxyacetophenone, triphenyltin, and nitroglycerin for *P. fluorescens*; 2,4-dichlorophenoxyacetic, organosilicone, tyrosine, 2,4-dichlorobenzoate,1,3-dichloro-2-propanol, fluorene, nitrophenol family (an/aerobic), endosulfan, iprodione pyrrole-2-carboxylate, vanillin, and 2,4-dichlorobenzoate, for *Arthrobacter* sp. (http://umbbd.msi.umn.edu/index.html). Strains of *R. erythropolis* have been described to be effective in degrading several types of mineral oil

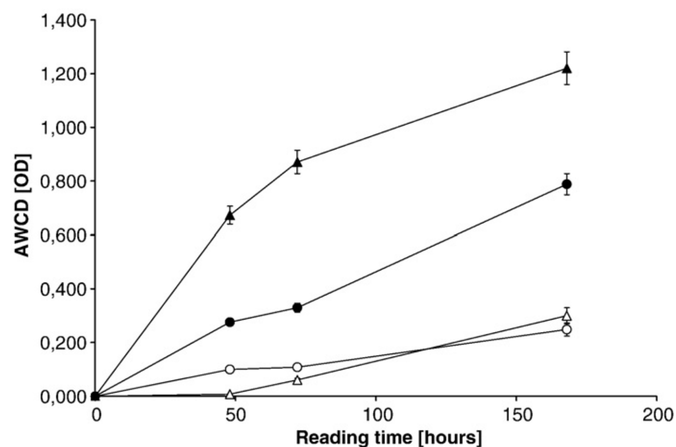

Fig. 5. Metabolic activity of microbial community in the soil spiked with diesel oil (black triangles: 15 days; black circles: 42 days) and biotic control (open triangles: 15 days; open circles: 42 days), expressed as AWCD in ECOPlates incubated up to 168 h.

Table 4
Biolog ECOPlates substrates utilisation by the microbial communities from spiked soil and biotic control after 42 days

ECOPlates	Spiked T 42	Control T 42
β-methyl-d-glucoside	+	+
D-galactonic acid lactone	+	−
L-arginine	+	+
Pyruvic acid methylester	+	+
D-xylose	+	+
D-galacturonic acid	+	+
L-asparagine	+	+
Tween 40	+	+
i-erythritol	+	+
2-hydroxybenzoic acid	−	−
L-phenylalanine	+	+
Tween 80	+	+
D-mannitol	+	+
4-hydroxybenzoic acid	+	+
L-serine	+	−
α-cyclodextrin	+	−
N-acetyl-d-glucosamine	+	+
γ-hydroxybutyric acid	+	−
L-threonine	−	+
Glycogen	−	+
D-glucosaminic acid	−	+
Itaconic Acid	+	+
Glycyl-l-glutamic acid	+	−
D-cellobiose	+	+
α-d-glucose-1-phosphate	+	+
α-ketobutyric acid	+	−
Phenylethylamine	+	−
α-d-lactose	+	+
Dextrin	−	+
D-malic Acid	+	−
Putrescine	+	+

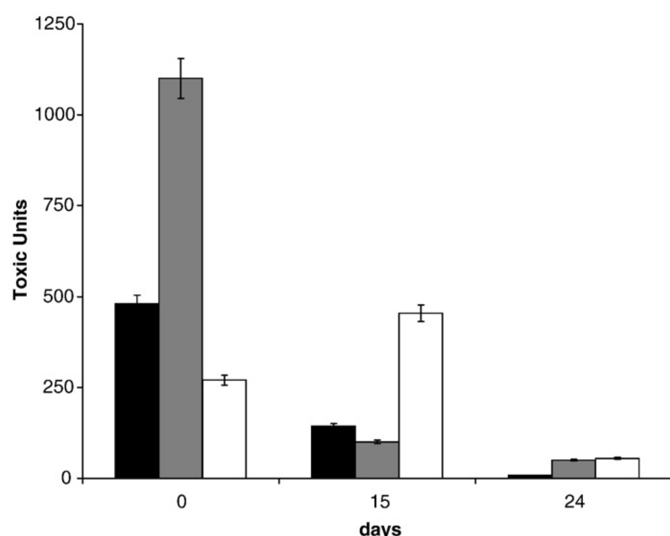

Fig. 6. Toxicity of the spiked soil at 0, 15 and 42 days of treatment, measured on three different organisms: black bars: *Vibrio fischeri*, grey bars: *Daphnia magna*, white bars: *Selenastrum capricornutum*.

(Aoshima et al., 2007), halogenate compounds (Erable et al., 2005) and hydrocarbons; a recently discovered strain of *D. tsuruhatensis* has been studied for its capability to degrade aniline, phthalate and amide compounds (Shigematsu et al., 2003; Zheng et al., 2007). *Exiguobacterium* sp. has been described to be able to survive in highly contaminated or extreme environments (Wyborski et al., 2005) and *B. cereus* was described in relation to heavy metals resistance and accumulation (Hu et al., 2007; Zhang et al., 2007). The combination of these metabolic characteristics offers a large bioremediation potential.

While some authors (Doelman et al., 1994) speculated that in soils contaminated with heavy metals the resistances to these may reduce the bacterial bioremediation capability towards chlorinated aromatics and polyaromatic hydrocarbons, others have proven the capacity of metal-resistant strains to play a role in improving bioremediation of organic pollutants (Roane et al., 2001). In principle we expect that native strains of co-contaminated matrices, already shaped by selective pressure, could take advantage with respect to sensitive strains in accomplishing biodegradation, when heavy metals are present. This way, they could help to overcome an important limitation in bioremediation applications, namely the co-occurrence of heavy metals, which inhibit respiratory processes in microorganisms.

The bioaugmentation with the microbial formula *ENEA-LAM* led to a DO degradation of about 75% in 42 days, in the presence of heavy metals. The microbial community carried out the DO biodegradation process in two phases, as can be observed comparing data on respiration rate (Fig. 2), DO removal efficiency (Fig. 3), metabolism (Fig. 5) and composition of the microbial community (Table 3, Fig. 4): during the first 15 days, most of the linear hydrocarbons and a few of the isoprenoid alkanes were degraded by those bacterial strains prompter to develop in the presence of DO. After this period, a lull in the microbial activity occurred, as shown by the declining of CO_2 production. The microbial community needs this temporary and brief phase in order to shift dynamics: soon after, CO_2 production recovered and population diversity increased, as shown by the final increase of the total fragments number in T-RFLP analysis. The partial DO biodegradation and the subsequent availability of intermediate metabolic compounds allowed the development of a succession of microbial strains which played a role in the biodegradation of DO and phenantrene (Figs. 3 and 4). These indigenous strains, not detected at the beginning of the trial probably because of their low abundance, may emerge once new carbon sources, derived from DO transformation, become available. At the end of the experimental time, the ecotoxicity of the system (Fig. 6) drastically decreased for all the tested organisms, whereas on *S. capricornutum* a toxic effect was detected after 15 days, greater than the toxicity measured at the start of the experiment. This could probably due to the highest sensitivity of the green algae to the intermediate compounds produced during the DO biodegradation.

Phenantrene completely disappeared during the interval between 15 and 42 days and could have been oxidized as co-substrate of the intermediate metabolites of linear hydrocarbons biodegradation, given that hydrocarbons with fused benzene rings are believed to be biodegraded throughout the process of co-oxidation (Hurst et al., 1995).

The microbial activity, in terms of CO_2 production, was only supported by the addition of DO as a readily available carbon source (50,000 mg kg^{-1} soil). This DO amount maintained the bacterial load around the value of 10^8 CFU ml^{-1} for the whole time of the trial. Neither nitrogen or phosphorus were added to sustain the bacterial community, in order to least alter the chemical environment of the native soil microbial population, considering that Margesin et al. (2007) relate that biostimulation with inorganic fertilisers significantly increases Gram-negative bacterial population. Inoculation of soil with native degrading microorganisms, provided in a sufficient number to enhance the biodegradation process, has been proposed as a generic bioremediation approach to ameliorate the clean-up of polluted areas. Previous studies have demonstrated that, under certain conditions, inoculation accelerates the rate and the degree of diesel oil biodegradation (Mueller et al., 1992; Bento et al., 2003). As in a previous work we observed that the form of consortium confers protection to the single components with respect to metal tolerance (Sprocati et al., 2006a), the approach assumed in this study was based on performing the bioaugmentation by strengthening a portion of the native microbial community with those strains provided with multiple resistances to heavy metals, in order to establish a microbial formula (*ENEA-LAM*) that could operate either directly on biodegradation process and/or indirectly by giving protection from metal toxicity to the metal sensitive organic-degrading strains. The persistence in the spiked sample of most of the strains used in the microbial formula (*ENEA-LAM*) and the increase in the final total number of T-RFs (Fig. 4 and Table 3), suggest that this bioaugmentation approach allows the newly established microbial community to strike a balance between the introduced and the naturally present microorganisms. Among the latter, the development of minor strains, undetectable at the beginning of the experiment, was observed. This is supported by the physiological profiling data of biotic control (Fig. 5 and Table 4) that show a very low but wide-ranging metabolic activity. The molecular along with the physiological data seem to suggest that this basal soil community is characterised by a low abundance and moderate species richness. It is also remarkable that the unique substrate that both microbial communities are unable to metabolise is 2-hydroxybenzoic acid (salicylic acid). The degradation of salicylic acid and the genetic basis of the metabolism have been investigated for decades with various bacteria and it is believed that a majority of the bacteria able to use salicylic acid, which is an intermediate in the naphtalene pathway, possess the enzyme required for the degradation of naphthalene or certain tricyclic aromatic hydrocarbons (Liu et al., 1995). In our case we observe that even if the microbial community is not able to use salicylic acid in ECOPlates, it is able to metabolise phenantrene in biometers. This leads to presume that either the biodegradation of phenantrene is limited to the upper catabolic pathway, accumulating intermediates and probably salicylic acid in the soil, or the community found an alternative pathway. This will be matter for a deeper investigation.

Concerning the degree of biodegradation, the results obtained by the bioaugmentation with the microbial formula *ENEA-LAM* seems quite promising, compared to other studies. For instance, Siddiqui and Adams (2001) followed the fate of diesel hydrocarbons added in two different soils, artificially spiked with diesel oil, amended or not with N and P as fertilisers, with and without previous hydrocarbon contamination

histories, and observed that the best response was obtained with the soil with a previous hydrocarbon contamination and amended with N and P as well. Similarly to that soil, we observed a 2-days-lag phase before CO_2 evolution, while 6 days were required by the microbial community of the soil without hydrocarbon history to become active. The subsequent phases of the respiration curve are instead quite different: while in that soil the respiration declines after 19 days, in our trial it follows a diauxic behaviour, extending the active biodegradation period up to 37 days. This way we have obtained, in comparison, a more efficient biodegradation process with respect to both time and degree.

During 42 days we observed a 100% abatement of n-$C_{12–20}$ and phenantrene and a 60% of pristane, phytane and i-$C_{15–18}$. The residue of isoprenoids, consistently with other studies, should not be due to an inadequacy of the bioaugmentation formula, but it depends rather on the time. For instance, Kennicut (1988) studying the effect of biodegradation on crude oil bulk observed that isoprenoids persisted up to 90 days around the initial levels then suddenly disappeared. Consequently, the isoprenoids biodegradation of 60% we have obtained in 42 days seems to be a promising starting point for the development of further applications.

In conclusion, the tailor-made microbial formula allowed to obtain, in the presence of heavy metals, the complete degradation of n-$C_{12–20}$, the disappearance of phenantrene and a reduction of 60% of isoprenoids, and an overall reduction of about 75% of the total hydrocarbons in 42 days. Concurrently, the complete abatement of the ecotoxicity was observed. These results seem quite encouraging and allow us to identify some adjustment that could be introduced for the scale up of the experimentation, for instance strengthening the bacterial formula with biosurfactant-producers, in order to avoid the introduction in the environment of further chemicals, and increasing the abundance of *Pseudomonas* strains at the time of inoculation or in a further step. Finally, the study indicates that the use of a tailored microbial formula may efficiently facilitate and speed up the bioremediation of matrices co-contaminated with hydrocarbons and heavy metals. This bioaugmentation strategy may contribute to overcome a critical bottle neck of the bioremediation technology.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.scitotenv.2009.01.011.

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