Hydrocarbon contaminated soil treated by bioremediation technology: microbiological and toxicological preliminary findings

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ABSTRACT

Soil contamination is a significant factor in the general degradation of the environment. Remedial intervention on contaminated soils may be carried out by means of specific and different technologies, including chemical, physical and biological methodologies. Of these, the latter are the least invasive and bioremediation intervention can be carried out both *in situ* and *ex situ*. The object of this study was a clay soil highly contaminated by heavy hydrocarbons and polycyclic aromatic hydrocarbons (PAHs). The soil was subjected to remediation treatment using a bioremediation technique, applied by inoculating a nutritive and enzymatic-bacterial mixture. A qualitative-quantitative microbial characterization of the soil matrix was undertaken, both before, and thirty days after, the treatment. The percentage abatement in hydrocarbon concentration and the overall microbial concentration present in the soil were measured, and the eco-toxicological impact was estimated. The eco-toxicological aspect was assessed by applying the ostracod test and the phytotoxicity test. The microbial component was estimated by the Direct Count method on plates: this method highlighted the vital countable cells of telluric and degrader mesophilic bacteria, of the Pseudomonadaceae, of the actinomycetes and total mycetes (moulds and yeasts). This study was focused on evaluating biological remedial technology combining chemical analysis with microbiological assessment and bioassays, because chemical analysis alone cannot provide a full picture of the bioremediation process and its effectiveness. The data obtained indicated that the bioremediation technique applied gave optimal results in terms of percentage abatement of the hydrocarbon concentration, microbial enrichment of the soil matrix and reduction of toxicity found in the soil before the clean-up treatment.

INTRODUCTION

Remediation work on a contaminated site, besides being an instrument for environmental and human health protection, is an important vector for social and economic development as it encourages the transformation of entire areas from non-productive zones to zones of environmental, urban and economic redevelopment.

The suitability of different remedial technologies must be assessed on the basis of a series of parameters connected with the pollutant type and its relative concentration, the characteristics of the contaminated matrix and the site typology, the specific environmental matrix, the chemical-physical characteristics of the pollutants, the age of the pollution and, finally, with the matrix microbial activity (Davini et al. 2001; Mariotti and Merlo 2003).

This study was carried out on an abandoned petrochemical industrial site in Northern Italy, contaminated by petroleum compounds and, thus, by C>12 hydrocarbons and by polycyclic aromatic hydrocarbons (PAHs).

Soil pollution is a significant factor in general environmental degradation and contamination by heavy hydrocarbons and PAHs in particular, is one of the most dangerous and worrying aspects for human health. These are highly heterogeneous pollutants, principally arising from the incomplete combustion of organic materials such as fossil fuels, i.e. carbon or petroleum. PAHs have well documented, toxic, mutagen and carcinogen properties (Alexandrie et al. 2000; Buckley et al. 1995; Faustioni et al. 2000; Goldman et al. 2001; Maier et al. 1998; Mastrangelo et al. 1996; Perera et al. 2005; Rybicki et al. 2004; Wang et al. 2008).

The mutagenic and carcinogenic activity of PAHs is a consequence of metabolic transformations, activated in human organisms in order to facilitate elimination of these compounds. As a result of PAH metabolism in the human body, epoxide compounds arise, which have mutagenic and carcinogenic properties. Cases of lung, intestinal, liver, pancreatic and skin cancer, have been reported (Samanta et al. 2002).

PAH exposure occurs by inhalation, ingestion and dermal contact and, as they are highly lipid-soluble, they are quickly absorbed through the gastrointestinal tract in mammals (Faustioni et al. 2000; Sciarra et al. 2000).

Benzo(*a*)pyrene (B*a*P) is a qualitative and quantitative indicator of the presence of PAHs. B*a*P is a carcinogenic mutagenic substance that is toxic to reproduction with unacceptable risks to human health or the environment. As such B*a*P is assigned to category 2 in the framework of Directive 67/548/CEE (European Directive 67/548/CEE).

Dispersion of PAHs in the environment is closely linked to their degree of solubility in water and to vapour pressure. The more soluble compounds have a greater migration capacity than those with high molecular weight which have a greater tendency to be retained, creating a noxious and widespread soil component. Exposure may result from inhalation of these compounds, as they are dispersed in the air and by contact with the polluted soil.

Most contaminated sites are in industrialised countries and result from numerous industrial complexes, closed and abandoned, or transferred without necessary remedial work being carried out. But dispersal of PAHs, as above, means that contamination is also an issue in rural and forested areas with a significant negative impact on underground water sources as well the soil.

For a long time, this ecosystem was wrongly thought to have an intrinsic, and infinite capacity for self-healing, making human protection and intervention unnecessary; this led to a dangerous underestimation of the complexity of the problem of PAH contamination.

In Italy, the problem of soil and water contamination was only acknowledged in all its gravity from 1997, legislative measures were introduced intended to deal with the problems of waste management, protection of water resources and remediation of contaminated sites. Under current legislation, when the established values for risk concentration limits are exceeded, the law imposes the application of specific remediation plans (Italia: Decreto Legislativo 22/97, Decreto Legislativo 36/03, Decreto Legislativo 152/06, Decreto Ministeriale 471/99; Musmeci et al. 2007).

This legislation led to the development of numerous remedial methodologies with technological, chemical, physical and biological efforts intended to deliver, at sustainable costs, both the limits required by sector rules and the maintenance of the original characteristics of the site to be recovered.

Among the soil reclamation and recovery techniques, the least invasive are those of a biological type such as bioremediation and bio-augmentation. These respectively implement the autochthonous microbial flora and introduce selected microbes to the contaminated matrices in order to augment the intrinsic bio-transformation activity and promote the transformation of pollutants into non-noxious substances.

For sites contaminated by organic compounds, bioremediation techniques are based on stimulation of the catabolic activity of microorganisms capable of using the organic pollutants as sources of carbon and energy. The pollutants are completely degraded to carbon dioxide and water. This is done through a complex series of reactions with consequent continuous transformation of the organic contaminants and the bacterial colonies. They may also be mineralised or bio-transformed into less toxic compounds (Atlas and Bartha 1997; Marta et al. 2006).

Bioaugmentation may be carried out by using pure cultures or, more frequently, microbial consortia which are generally more efficacious for the bio-degradation synergies which may derive from the simultaneous presence of diverse microbial groups. Bioaugmentation is also appropriate and effective where the native microbial population lacks suitable catabolic abilities.

In addition to integrating the performance of the native microflora, bio-augmentation may also accelerate the degradation process, or reduce the period of adaptation to the xenobiotic to be treated (Alexander 1994; Mulligan 2001, Rogers et al. 2002; Yong and Mulligan 2006).

Studies of bioaugmentation, carried out on sites polluted by aliphatic hydrocarbons, demonstrated that the degradation kinetics of these pollutants, studied in vitro, in aqueous and solid phases, were accelerated by a combination of enzymes and/or surfactants treatment (Bardi et al. 2000; Steffan et al. 2001). But, synthetic surfactants, used to increase contaminant solubility are often toxic thus constituting an additional source of contamination (Cha 2000).

Microbial naturally produced biosurfactants or surface active compounds have similar properties but are less toxic, biodegradable, and can be produced *in situ*, at the contaminated site (Cha 2000). For these reasons, biosurfactants have gained increased attention. They can be produced from cheap raw materials and are effective under extreme conditions (Bodour and Maier 2002; Mulligan 2005; Nitschke and Pastore 2006; Plaza et al. 2006; Ron and Rosemberg 2002; Sanchez et al. 2007; Youssef et al. 2004).

Many microorganisms, including bacteria, yeasts and moulds, can produce extracellular or membrane-associated surface-active compounds which are used to assist the enhancement of emulsification and dispersion of water-insoluble compounds. These biosurfactants can reduce surface tension at the air-water interface and they can assemble into a wide variety of morphologically different structures. Most of them are of lipid nature: glycolipids, lipoaminoacids and lipopeptides. Rhamnolipids, for example, are a group of biosurfactants of glycolipid nature; the type and the portion of the rhamnolipids produced depends on the bacterial strain, the carbon source used and the culture conditions (Sorbeon-Chavez et al. 2005). This production can be growth associated because they can either use the emulsification of the substrate (extracellular) or facilitate the passage of the substrate through the membrane (cell membrane associated).

The growth of bacteria on hydrocarbon contaminated soil is usually accompanied by the production of biosurfactants that helps cell adherence to oil droplets spread in soils (Rosemberg and Rosemberg 1981). Studies of contaminated soil using culture and molecular techniques were reported.

Results showed that Gram negative strains were found in hydrocarbon or mixed contaminant soils while Gram positive biosurfactant-producers were found in heavy metal contaminated and uncontaminated soil (Bodur et al. 2003).

Microorganisms capable of emulsifying and solubilising hydrophobic contaminants *in situ* may have a distinct advantage over competitors in contaminated areas and therefore isolates and samples from such sites are often rich in microorganisms with the desired characteristics for both *in situ* and *ex situ* bioremediation processes (Batista et al. 2006; Nasr et al. 2009).

The area studied was subjected to in field remedial treatment by bioaugmentation using an enzymatic-bacterial consortium selected to test its effectiveness in degrading PAHs in soil. The treatment applied was land-farming typology, which involves the removal and sitting of the soil to be treated in a suitable area identified on the same location (*ex situ on site* treatment).

The soil-microorganisms complex should be considered an integrated self-organizing system that can remodel its state in response to environmental change. In this study, an aerobic and anaerobic microbial characterization was made on the soil before the bioaugmentation treatment. Microbial communities were assessed using a plate technique. This allowed the direct quantification and isolation of bacteria which degrade polycyclic aromatic hydrocarbons. This enabled the valuation of potent and versatile autochthonous mineralizing microflora in PAH-contaminated soil.

Anthropogenic activities such as pollution, industrialization and urbanization can alter the structure of soil microbial communities and, thus, alter the capacity of the soil to contribute effectively to the global ecosystem. By interaction between physical, chemical and biological components the soil system changes over time. As up to 90% of microorganisms are thought to be associated with solid surfaces, minerals and organic matter also have an important role in determining microbial diversity and community structure in soils (O'Donnel et al. 2007). Many authors have demonstrated that past events and contemporary disturbances had a similar influence on bacterial diversity in soil (Ge et al. 2008).

Moreover, an important characteristic of microbial life in soil is that microorganisms can change their chemical environment through the production of metabolites such as acids, bases and ligands which interact both directly and indirectly with soil minerals altering their surface properties and modifying microbial interaction with the surface (O'Donnel et al. 2007).

This work was principally aimed at evaluating soil quality before and after remedial treatment from a mainly microbiological and toxicological point of view. Bioremediation is usually monitored by observing the concentration of the targeted contaminants; but the reduction in contamination is not always accompanied by reduced soil toxicity. Incomplete degradation and the formation of intermediary metabolites might indeed lead to increased soil toxicity (Mphekgo et al. 2005; Philips et al. 2000).

The fundamental aspects of the characterization were therefore assessed to define their state of health. The microbiological condition was assessed by identification of suitable microbial communities, the transformers of the pollutant substances originally present in the soil. The eco-toxicological aspect was assessed by evaluation of intrinsic and/or residual acute and chronic toxicity, as well as the percentage abatement of the hydrocarbons.

Data about the exact mechanisms of the production of the biosurfactants, their chemical composition and PAHs degradation percentage was not shown in this paper. The data reported thus far should be considered preliminary.

MATERIAL AND METHODS

Soil treatment

As required by the land-farming technique, the soil was drawn and placed in a suitable area located at the same site. The soil to be remediated was then stratified and inoculated with the enzymatic-bacterial bio-activator called MICROPAN ALFA-POBs, applied together with a pool of synergising nutrients, named MICROPAN BETA-POBs, both produced by Eurovix S.p.A. and available on the market in powder form (Maggioni and Brignoli 2004). Distribution of the MICROPAN BETA-POBs synergising nutrient, spread in granular form, was effected in the measure of $1.5kg·m^{-3}$ of soil; this consisted of stabilised organic matrix as well as amino acids and oligopeptides, carbohydrates, natural growth factors, mineral salts, mineral nutrients of organic origin (N 3%, P 6% and K 1%), mineral bio-catalysers rich in oligo-elements, vegetable extracts and selected microorganisms and yeasts.

The MICROPAN ALFA-POBs enzymatic-bacterial bio-activator is principally composed of a pool of carefully selected enzymes, selected microorganisms mainly from the genus *Bacillus*, culture agar medium, carbohydrates and active molecules. After being dissolved in water, the bio-activator inoculation was effected by spraying the contaminated soil at a rate of $0.5kg·m^{-3}$.

In order to favour greater oxygenation during the bioremediation process, the soil was periodically turned over mechanically to support the microbial-degrading activity with the production of biosurfactants in general, and rhamnolipids in particular which are inefficient under oxygen-limited conditions (Chayabutra et al. 2001; Wei et al. 2005).

Soil sampling

Soil samples were collected before bioaugmentation and after the remedial activity. Before the remedial operations, the soil samples were taken at three different points, geo-referenced precisely via Global Positioning System (GPS). The site was excavated at each point to a depth of three metres using a mechanical digger. Greater depth of excavation was impossible because, at all points, at 3 metres there was water infiltration at the bottom, caused by groundwater levels.

The samples were taken, using a sterile bone spoon, along the excavation vertical from 0 to 3 metres, taking a triple sample from each layer at a depth of 0-75cm; 75cm-150cm; 150cm-300cm. By means of a penetrating probe, the temperature relative to the three different depths and the pH were measured. The temperature and humidity of the external air were also measured using a multi-parametric probe.

The soil samples were placed in sterile glass containers in order to retain a sufficiently representative quantity and then carried to the laboratory, refrigerated and conserved at +4℃.

An identical sampling procedure was applied to the soil subjected to bioaugmentation treatment although, given the procedure used, it was not necessary to excavate to the same depth; sample quotas were again taken at three points, according to the quartering method, but at a maximum depth of 30cm (IRSA 1985).

Sample preparation

One kilogram of composite sample was obtained mixing identical soil quantities taken at the three different geo-referenced sampled points. Then a manual mixing and homogenization was carried out and the pH was measured. Finally, an aliquot of the same sample was placed in a dry oven at 105℃ for 24h to determine the dry weight before being subjected to chemical, microbiological and toxicological investigation.

Total petroleum hydrocarbons and PAHs were quantified according to the Canada Wide Standard (CCME 2001) (Data not shown).

Microbiological analysis

The soil matrix was analysed microbiologically in order to estimate, as far as possible, the countable vital bacterial flora concentration. All micro-organisms are highly tolerant to stress but to grow and establish a population one organism must be able to compete alone with the local population which may be better adapted to the specific conditions. Furthermore, microbial life is determined by genetic differentiations due to mutation, selection or genetic drift arisen by mixing of individuals. There are also overwhelming spatial differences in taxonomic abundance due to ecological interactions or to ecological drift (Bell 2000; Hubbel 2001).

The following general parameters were investigated: total mesophilic bacteria (Total Bacterial Count at 37℃); total telluric bacteria (Total Bacterial Count at 22℃) and Total Gram negative bacteria. Primary degraders such as total *Pseudomonadaceae*, Moulds, Yeasts and Actinomycetes were also investigated.

Although most degrading biosurfactant-producing organisms are aerobic, some examples of anaerobic product exist (Mulligan 2005). The concentrations of the facultative and obligatory anaerobic microorganisms were evaluated exclusively on the soil samples taken before the bioaugmentation treatment. The evaluation involved the incubation of replications of the seeded plates also in anaerobic conditions.

In addition to the microorganisms cited above we also investigated parameters such as sulphite-reducing Clostridia (spores) and total anaerobic sporigen and non sporigen bacteria.

Next, a quantity of the sample equal to 10g was suspended in 90ml of buffered sterile physiological solution, mechanically homogenised in a Stomacher circulator 500 at 200 RPM for 15 minutes. Suitable serial dilutions were prepared and known aliquots of the same were triple seeded by the direct count method on plates using Seeding on Agar Surface technique. Specific and/or selective cultural agar media were used relative to the microbial groups to be counted.

The culture media, temperatures and incubation times used were: Trypticase Soya Agar for TBC at 22℃ and at 37℃, with incubation at 21 ± 1℃, up to 7 days and at 36 ± 1℃ for 48-72h, respectively; Eosin Methylene Blue Agar for total Gram negative bacteria, incubated at 36 ± 1℃ for 24-48h; Pseudomonas Isolation Agar and Pseudomonas Agar Base C-N selective supplement for *Pseudomonas* spp.; incubated at temperatures of 27 ± 1℃ and 42 ± 1℃ for 24-48h (registering the non fluorescent and fluorescent colonies, presumed *Pseudomonas aeruginosa*, counted using Woods' light at double wavelength); Rose Bengal Chloranmphenicol Agar to enumerate the Moulds and Yeasts colonies at 21 ± 1℃ up to 7 days; Actinomyces Isolation Agar at 21 ± 1℃ and at 36 ± 1℃, up to 7-14 days, for psychrophilic and mesophilic Actinomyces, respectively; Sulphite Polymixin Sulphadiazine Agar for the sulphite-reducing Clostridia spores and for total anaerobic sporigen bacteria; Wilkins-Chalgren Anaerobe Agar for anaerobic non sporigen bacteria, both incubated in conditions of anaerobiosis at 36 ± 1℃ from 24h up to 7 days. At the end of the incubation period, the colonies developed were counted, a mean of the values obtained was calculated and the results were expressed as Colony Forming Unit on dry matter gram ($CFU \cdot g_{dm}^{-1}$).

To identify and characterize the microorganisms of bacterial genus in the study, multiple isolations of different and recurrent morphologies colonies were carried out on maintenance nutrient media and Gram stain was made in addition to the principal screening tests, the specific biochemical differentiation tests, fermentative or oxidative metabolism tests, growth tests on different selective nutrient substrata. Finally, species level identification was carried out by miniaturised biochemical kits: API 20 NE, API 20E; API STAF, API 20 STREP, API 20 A (Biomerieux).

Identification of moulds and yeasts was carried out by means of isolation and maintenance of the cells grown, which were then fixed on slides and stained by Lactophenol blue solution, under microscopic observation of the structural elements to enable definitive identification. With reference to the yeasts isolated and maintained in culture; yeast cells were identified by the miniaturised API 20 CAUX biochemical kits (Biomerieux).

The identification of fungi will be dealt with in a forthcoming paper.

Toxicological investigation

Soil eco-toxicity tests can be performed either as contact (direct) tests or as tests on soil elutriate.

Toxicity tests are "species specific" and there is no test species which is "the most sensitive" for all chemicals. So, unless the most appropriate test for a particular type of investigation has already been identified through prior analyses, a test battery has to be applied. The test battery should be composed of one or more test species representative of the different trophic levels of the biological chain; production (plants) – consumption (animals) – decomposition (bacteria).

In this study eco-toxicological analyses were conducted directly on soil samples using Ostracod contact tests and phytotoxicity tests, as diverse studies have demonstrated that these are much more sensitive than the tests carried out on aqueous or eluate extracts which are merely an approximation of the effective degree of the problem (Hubalck et al. 2007).

The Ostracod test (OSTRACODTOXKIT F^{TM}) was purchased from MicroBioTests Inc., Nazareth, Belgium. This test was originally developed for sediment testing, but it was tested in this study with PAH-contaminated soil.

The Ostracod test requires the use of *Heterocypris incongruens*, a benthonic organism mainly localized in the interfaces between water and sediment which, feeding on solid particles, is particularly sensitive to toxicity deriving from pollutants of organic and inorganic origin, released by the substratum (Plaza et al. 2005).

This test is a 6 day chronic assay based on two distinct effects criteria: mortality of the test organisms or growth inhibition, resulting from direct contact with (non-diluted) sediment.

Heterocypris incongruens assay can be raised at low cost and minimal equipment is needed in the test performance; furthermore, the use of dormant eggs (cysts), easily hatched on demand, allows the obtaining, for each analysis, of individuals with the same genetic characteristics.

Newborn individuals were used for this test taken from opened cysts in Standard Freshwater at a temperature of $25\pm2°C$ for 52h at 3000lux and administering, at 48h, pre-feeding constituted by algae of the *Spirulina* genus. Then, 10 newborn ostracods, $200\mu m$ in length, were triple transferred to 6-well multi-plates containing: Standard Freshwater *Scenedesmus* sp. alga (as nutrient suspension) and soil sample or substratum for reference. After incubation in the dark at $25\pm2°C$ for 6 days, the non vital organisms were counted and the percentage mortality rate (acute toxicity) was calculated, when measured at incidences below 30%. Finally, the growth inhibition percentage was calculated (chronic toxicity), measuring the vital organisms after fixing in Lugol. The test was considered valid when, in the control, the percentage mortality rate was below 20% and growth in the organisms saw an increment of no less than $300\mu m$.

The phytotoxicity test is based on the use of seeds from different vegetable species to evaluate the potential toxicity of liquid and solid samples, examining germination and radical growth with respect to a matrix of reference. So, three species of rapidly growing plant seeds were used: two dicotyledons such as *Sinapis alba* and *Lepidium sativum*, and one monocotyledon *Sorghum saccharatum*.

Before the test a saturation test was done to determine the quantity of water to be added to the sample in order to obtain 100% humidity. The test was carried out in three replications using, for each vegetable species, 10 seeds placed in Petri capsules containing either the soil sample or the substratum of reference and incubated in the dark at $25\pm2°C$. After 72 hours the germinated seeds were counted against the matrix of reference to calculate the percentage germination as an evaluation of acute toxicity.

The percentage inhibition of radical growth was also determined (chronic toxicity) measuring the length of the roots network, again relative to the substratum of reference.

RESULTS

The microbiological and toxicological analytical results obtained from analysis of the soil samples taken before and after the bioaugmentation operations are reported in Tables 1-5.

Table 1. Aerobic and anaerobic microbial flora concentrations before bioaugmentation treatment (CFU·g$_{dm}$$^{-1}$).

Parameters	Aerobic microorganisms	Anaerobic microorganisms
Total telluric bacteria	$4.2\cdot10^6$	$4.5\cdot10^5$
Total mesophilic bacteria	$6.8\cdot10^6$	$1.7\cdot10^6$
Total Gram-negative bacteria	$8.1\cdot10^4$	$1.2\cdot10^5$
Pseudomonas aeruginosa	$1.6\cdot10^3$	$5.8\cdot10^2$
Moulds	$1.6\cdot10^3$	–
Yeasts	$1.2\cdot10^2$	–
Psycrophilic Actinomycetes	$1.2\cdot10^5$	$1.2\cdot10^4$
Mesophilic Actinomycetes	$6.9\cdot10^4$	$2.3\cdot10^4$
Clostridium spp. (spores)	–	$2.8\cdot10^2$
Total anaerobic non sporigen bacteria	–	$3.5\cdot10^5$
Total anaerobic sporigen bacteria	–	$6.9\cdot10^3$

Table 2. Microbial species before bioaugmentation treatment and recovered in aerobiosis and anaerobiosis culture conditions.

Species	Aerobiosis	Anaerobiosis
Acinetobacter spp.	+	−
Acinetobacter iwoffii	+	−
Actynomices spp.	+	+
Actynomices viscosus 1	−	+
Actynomices viscosus 2	−	+
Agrobacterium radiobacter	+	−
Alcaligenes faecalis	−	+
Alcaligenes xylosoxidans subsp. denitrificans	+	+
Alcaligenes xylosoxidans subsp. xylosius	+	+
Bacillus spp.	+	+
Candida spp.	+	−
Capnocytophaga spp.	+	+
Clostridium spp.	−	+
Clostridium perfringens	−	+
Cryseomonas luteola	−	+
Comomonas acidovorans	+	+
Enterobacter agglomerans	+	−
Erwinia spp.	+	−
Flavobacterium spp.	+	−
Flavobacterium oryzihabitans	+	−
Micrococcus spp.	−	+
Moraxella spp.	+	−
Plesiomonas shigelloides	+	−
Pseudomonas spp.	+	+
Pseudomanas acidovorans	+	−
Pseudomonas aeruginosa	+	+
Pseudomonas aureofaciens	+	+
Pseudomonas cepacia	+	−
Pseudomonas chlororaphis	+	+
Pseudomonas diminuta	+	−
Pseudomonas fluorescens	+	+
Pseudomonas putida	+	−
Pseudomonas pseudomallei	+	−
Pseudomonas stutzeri	+	−
Rhodotorula glutinis	+	−
Rhodotorula spp.	+	−
Shewanella putrefaciens	+	−
Serratia marcescens	+	+
Serratia odorifera	+	+
Sphingomonas paucimobilis	+	−
Staphylococcus epidermidis	−	+
Staphylococcus hominis	−	+
Staphylococcus lentus	+	−
Staphylococcus warneri	−	+

Table 1 lists the concentration of aerobic and anaerobic microbial flora in the soil samples taken and analysed before the bioaugmentation treatment. This table indicates that, for almost all the various microorganisms investigated, the concentration of vital countable cells shows higher values at the aerobic level than anaerobic.

It emerged that the obligatory anaerobic microorganisms were much fewer than the facultative aerobics as the facultative aerobics were able to grow in both environmental conditions. However the vital, countable, facultative anaerobic Gram negative bacteria cells, measured in this study, were larger by an order of magnitude. Similar studies carried out by cultural and molecular techniques reported that Gram negative strains were found in hydrocarbon or mixed contaminant soils, while Gram positive biosurfactant-producers were found in heavy metal and uncontaminated soil (Bodur et al. 2003).

The primary obligatory aerobic degraders such as moulds and yeasts showed low concentration titles.

Table 2 lists the microorganisms, identified at species level by biochemical typing, present in the soil samples taken and analysed before the bioaugmentation treatment and recovered in aerobiosis and anaerobiosis culture conditions.

As to the bacterial cells grown, a total of 305 isolations of microorganisms with diverse morphologies were carried out, 70% of which were identified with the miniaturised biochemical kits used, 20% of the colonies were unidentifiable using the systems cited above, while the remaining 10% did not produce vital cells after the isolation.

The concentration of aerobic microorganisms found before and after biological remedial treatment, expressed as $CFU \cdot g_{dm}^{-1}$ is at Table 3. In general, this table indicates an increase by illustrated two orders of magnitude in the levels of microbial concentration in the soil after treatment with respect to the same analysis before the bioremediation operations. After remediation we observed increased concentrations for total psychrophilic bacteria, up to values of 10^9 $CFU \cdot g_{dm}^{-1}$; *Pseudomonas* spp. increased by an order of magnitude, while *Pseudomonas aeruginosa* and mesophilic *Actinomyces* did not change.

The results from eco-toxicological tests on contaminated soil samples before and after biological remedial treatment are reported at Tables 4 and 5.

Table 4 shows the results of the phytotoxicity test, expressed as a percentage inhibition of germination and radical growth. The phytotoxicity test did not allow an acute toxicity assessment, in particular, among the three species selected, *Sinapis alba* showed greater sensitivity to the contaminants present in the soil. No acute toxic effect, however, was found for *Lepidium sativum* which, in pre-remedial phase, budded. Clearer results were obtained for chronic toxicity, the three species showed a notable reduction in growth at the radical apex after treatment: *Sinapis alba* was the most sensitive species. The data showed a net abatement of acute toxicity and a distinct reduction in chronic toxicity after the bioremediation treatment.

Table 3. Microbial concentrations before and after bioaugmentation treatment ($CFU \cdot g_{dm}^{-1}$).

Parameters	Pre-treatment	Post-treatment
Total telluric bacteria	$4.2 \cdot 10^6$	$2.4 \cdot 10^9$
Total mesophilic bacteria	$6.8 \cdot 10^6$	$1.0 \cdot 10^8$
Total Gram-negative bacteria	$8.1 \cdot 10^4$	$1.2 \cdot 10^5$
Pseudomonas spp.	$4.6 \cdot 10^4$	$1.3 \cdot 10^5$
Pseudomonas aeruginosa	$1.6 \cdot 10^3$	$3.1 \cdot 10^3$
Moulds	$1.6 \cdot 10^3$	$5.3 \cdot 10^5$
Yeasts	$1.2 \cdot 10^2$	$4.1 \cdot 10^4$
Psycrophilic Actinomycetes	$1.2 \cdot 10^5$	$1.2 \cdot 10^4$
Mesophilic Actinomycetes	$6.9 \cdot 10^4$	$2.3 \cdot 10^4$

Table 4. Results of the phytotoxicity test before and after bioaugmentation treatment, expressed as a percentage inhibition of germination and radical growth.

Test species	Pre-treatment		Post-treatment	
	Inhibition (%)			
	Germination	Growth	Germination	Growt
Lepidium sativum	0.0	32.6	0.0	3.8
Sinapis alba	23.0	53.9	0.0	8.6
Sorghum saccaratum	11.0	40.6	0.0	15.8

Table 5. Results of the ecotoxicity test on *Heterocypris incongruens* before and after bioaugmentation treatment, expressed as a percentage of mortality and of growth inhibition.

Toxic effects	Pre-treatment	Post-treatment
Mortality (%) (acute toxicity)	100.0	6.67
Growth inhibition (%) (chronic toxicity)	no data	15.74

Table 5 shows the results of the ecotoxicity test carried out on *Heterocypris incongruens* before and after bioaugmentation treatment, expressed as a percentage of mortality and of growth inhibition. The test demonstrated that, after the treatment, there was a significant reduction in acute toxic effects; indeed the elevated initial toxicity found (expressed as a percentage mortality rate of 100%) subsequently decreased to 6.67%. Also chronic toxicity, expressed as a percentage of growth inhibition, seems to show values of significant importance after the biological remedial treatment.

Further, the chemical analysis carried out on the soil samples taken after bioremediation treatment indicated an abatement of the concentration of both hydrocarbons C>12 and of PAHs of 70-80 % thus achieving values below the limits established by Italian Legislative Decree No. 152/06 - Enclosure 5: Table 1, column A, for PAHs and column B for hydrocarbons C>12 (Italia Decreto Legislativo 152/06). (Data not shown)

DISCUSSION

Integration of analytical, microbiological, toxicological and bioremediation data is necessary to assess the human health and ecological risk of bioremediation processes properly.

A significant decrease in toxicity was found and, at the microbiological level, there was a significant increase in the concentration of certain degrader microbial groups, as well as of the basal mesophilic and psychrophilic flora. Thus, the soil was rich and biologically active in the presence of bacterial strains producing biosurfactants (tensioactive substances of natural origin, which make the pollutant easily available and attackable, and encourage its biodegradation, without causing problems in the environment). A large natural production of biosurfactants is observed when the C/N ratio is in the range 16:1 to 18:1 and the principal factors which condition the production are pH, oxygenation and salts concentrations, that in this study were optimal (Maggioni and Brignoli 2004).

A microbial quantification was performed after 30 days of clean-up operations to assess both active countable and cultivable microflora, and soil fertility. In fact, some microbial taxa could possess the combination of traits that allows them to colonize on a global scale such as *Bacillus* spp., which was recovered as autochthonous (*Bacillus licheniformis* would be well suited for *in situ* studies for enhanced oil recovery or soil decontamination) (Mulligan 2005; Nasr et al. 2009).

Many microorganisms might have short dispersal and restricted geographic distributions but there is little information about the prevalence and geographical distribution of various hydrocarbon-degrading populations in soil (Martiny et al. 2006).

The presence of *Pseudomonas aeruginosa*, found at both the aerobic and anaerobic levels, is important, because of their ability to adapt to extreme environmental conditions and to metabolise many different substrata. Further, its presence indicates the intrinsic production of biosurfactants and its ability to assimilate hydrocarbons for rhamnolipid production.

PAH degradation in the presence of biosurfactant production by *P. aeruginosa* has been widely reported (Garcia-Jungo et al. 2003; Hwang and Cutright 2002; Santos et al. 2008; Straube et al. 2003). This bacteria is able to produce six types of rhamnolipids with similar chemical structure and surface activity, for this reason it was detected as autochthonous microflora in this study. Moreover, its characteristics enable application of the *P. aeruginosa* strains in bioremediation of petroleum pollution (Cha et al. 2008; Maier and Soberon-Chevez 2000; Nitschke et al. 2005; Wei et al. 2005).

Others studies have reported that bacteria such as *Pseudomonas* and members of the genus *Acinetobacter*, found as autochthonous in the present investigation, can rapidly colonize and grow on nutrient rich material, in this case the nutrients were represented by the hydrocarbon contamination. Other microorganisms, such as streptomycetes, corynebacteria and similar soil bacteria tend to be more successful in resource limited situations and are usually more permanent and stable members of the soil community (Margesin et al. 2003). These were also found in the studied soil.

The presence of cultivable microorganisms recovered from the soil before bioaugmentation treatment and reported in Table 2, have demonstrated the great importance of the regulatory agencies and entities responsible for natural attenuation and for the biological site remediation. Most of the taxa found were identified only to genus level because of the limited possibilities of the biochemical miniaturized identification test. There are many species that can emulsify hydrocarbon substrates. *Pseudomonas* spp, in general, and *P. fluorescens*, in particular, can emulsify toluene, texane, kerosene and other poly-aromatic substrates such as naphthalene and phenanthrene and utilizes short as well long chain n-alkans (Barathi and Vasudevan 2001). *Acinetobacter* spp. in general and *A. calcoaceticus*, in particular, can produce a protein associated with the polymers, which is required for emulsification activity (Bodour and Maier 2002).

Biosurfactant-producing by *Flavobacterium* spp. strain is widely available in the soils. Among the many classes of biosurfactants, lipopeptides from *Bacillus* spp., and especially from *B. subtilis*, are particularly interesting because of their high

surface activity, surfactin production and therapeutic potential (Banat 1995; Mulligan et al. 2001).

Polymeric biosurfactants with emulsification abilities are produced not only by a number of bacteria but also by archea and yeasts (Bodour and Maier 2002). The presence of *Candida* spp. as autochthonous in the soil is a very important factor because *C. bombicola*, for example, is one of few yeasts to produce biosurfactants and moreover, other *Candida* strains can produce sulphurous lipids (Mulligan 2005).

The fungal community was detected not only for its degradation abilities but also because the presence of fungal communities in the soil can improve the degradation of PAH. Fungal hyphae, in fact, may act as vectors to immobilize bacteria upon fungal growth and the creation of voids and provision of continuous surfaces by fungal hyphae could facilitate the displacement of bacteria in the soil (Li et al. 2008).

Microbial consortia are more effective in the degradation of recalcitrant compounds because of the potential to treat a larger number of PAHs and a higher degradation and mineralization rate in vitro and in soil. Bioremediation efficiency relates to the ability of the inoculated microbial degraders to remain active in the natural environment. Nevertheless a soil's chemical, physical and biological complexity can reduce the introduced allochthonous population by an antagonistic relationship, biotic factors (predation and competition from the autochthonous populations) as well as by physiological stresses caused by abiotic factors (pH, availability of water and bioavailability of carbon and energy sources) (Jacques et al. 2008).

Previous laboratory studies reported, for example, that bioaugmentation eliminated all anthracene added to the soil at rates 10 times higher and 7 times faster than the degradation produced by a soil autochthonous microbial population (Kästener et al. 1998).

Bacterial cells sense their population density through a sophisticated cell-cell communication system and expression of particular genes are triggered when the population density reaches a certain threshold (Dong et al. 2001), the chemical transmissions of one species can be detected by another and these signals may be used for inter species as well as intra species communications (Henke and Bassler 2004). By this process, called "quorum sensing", bacteria can measure the concentration of these signalling molecules in order to assess the size of the bacterial population and/or direct detection of eukaryotic target cells (Coburn et al. 2004; Lazdunski et al. 2004).

Because microbial communities occupy a confined space, over time concentrations of extracellular signalling molecules accumulate, providing stimulus or unique and varied cellular responses as well as protection from competing microbial communities. In addition to regulating intraspecies survival and differentiation in bacterial communities, quorum sensing also relates interspecies information between symbionts and competitors (Diggle et al. 2007; March and Bentley 2004).

However, in spite of the theoretically favourable, environmental conditions, the microbial populations do not always have the complete enzymatic sequence to produce those chemical reactions transforming the base compound into an energetically utilizable product. If this transformation is interrupted, the xenobiotic is only partially degraded and the resulting metabolic intermediates can cause accumulation and/or toxicity problems.

This study demonstrated that, as regards the ecotoxicological tests applied, the use of the ostracod *Heterocypris incongruens* was particularly effective in the pollutants assessed in the soil. But the phytotoxicity test did not indicate a significant correlation between seed germination percentage and PAHs concentration, as in several similar studies that revealed no relationship between the level of hydrocarbons in soil and seed germination rate (Mendonca and Picado 2002; Smith et al. 2005).

Conversely, other authors observed good correlations between the level of PAHs in contaminated sites and dicotyledonous seed germinability (Hamdi et al. 2007; Potter et al. 1999). This disparity in results points to the fact that seed germination and plant development are complex mechanisms involving a wide range of influencing factors (soil texture, pH, salinity, PAH bioavailability and the presence of metals or inherent phytotoxic compounds that are often underestimated). However, within the present study, the endpoint inhibition of root growth allowed a more reliable estimation of the toxicity compared to inhibition of seed germination.

The ostracod test was demonstrated to be highly effective, in that the contaminants come into direct contact with the digestive apparatus of the test organism and toxicity estimation is not, thus influenced by phenomena of chemical-physical interaction of the contaminant with the matrix, as happens with the phytotoxicity test.

The efficiency of this test had already been expressed in other studies which compared it with other test typologies, demonstrating it to be an available alternative test to endobenthic assays (Chial and Persoone 2002; Chial et al. 2003; Franzosi et al. 2004). The present data, reported in Table 5, suggest that Ostracod growth can be an appropriate parameter for monitoring biological remediation processes (Joner et al. 2004). Moreover, other studies have proven the sensitivity of *Heterocypris incongruens* species to PAHs and their usefulness for detecting them, especially anthracene, pyrene and benzo[*a*]pyrene. Recently an Ostracod bioassay for soil ecotoxicity was developed. In fact, the Ostracod test is a valuable bioassay for soil direct toxicity evaluation especially when assessing hydrophobic compounds such as PAHs (Hamdi et al. 2007).

In conclusion the use of the two bioassays chosen in the present experiment to assess soil toxicity and to reflect also PAH degradation, were effective because both are rapid, sensitive and relatively inexpensive and they demand small sample volumes compared to a traditional soil test with, for example, earthworms.

Microbiological results obtained in the present study showed that the inoculation with microbial consortia used to degrade PAHs in petroleum contaminated soil could be

considered as a useful method of remediation. The selection and identification of all microorganisms responsible for biodegradation is difficult when using traditional microbiological methods. But in further studies the total community DNA will be extracted from the soil in order to determine the prevalence of various hydrocarbon degrading genotypes. These will be screened by PCR using oligo-nucleotide primer sets specific for each degrader genotype.

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