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Response of an agricultural soil to phenanthrene and pentachlorophenol pollution and to different bioremediation strategies

Ph.D. Dissertation
by Rosalia Scelza

Tutor
Prof. Liliana Gianfreda

Coordinator
Prof. Matteo Lorito

Chi non ha mai commesso un errore
non ha mai tentato qualcosa di nuovo.

Albert Einstein

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Here I am at the end of this long adventure. All the people I have met during my PhD contributed, in one way or another, to realise this work and to make this time of my life something to remember. That is why all of them deserve to be mentioned here.

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I hope I have not forgotten anyone…

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Chapter 1

Introduction

Soil is a living dynamic, non-renewable, resource and its conditions influence food production, environmental efficiency and global balance (Doran and Parkin, 1994; Dick, 1997; Doran and Zeiss, 2000). The quality of soil depends in part on its natural composition, and also on the changes caused by human use and management (Pierce and Larson, 1993). Unusual management of soil, such as intensive cultivation without crop rotation (Reeves, 1997), or accidental/deliberate contamination by municipal and industrial wastes (Edwards, 2002), are major causes of land degradation and reduced soil productivity.

Conventional physical and chemical approaches (*e.g.* land-filling, recycling, pyrolysis and incineration) to the remediation of contaminated sites are inefficient and costly and can also lead to the formation of toxic intermediates (Spain et al., 2000; Dua et al., 2002). Thus, biological decontamination (*bioremediation*) methods are preferable to conventional approaches in terms of both costs and environmental impact. Bioremediation is the use of living organisms to remove contaminants from soil, air and water. The main agents of bioremediation are microorganisms, plants and enzymatic proteins (Gianfreda and Nannipieri, 2001), able to degrade numerous pollutants without producing toxic intermediates (Pieper and Reineke, 2000; Furukawa, 2003). The introduction of microorganisms, proven to degrade the target pollutant (Sarkar et al., 2005), and/or of additional nutrients to increase the endogenous microbial population and enhance its degradative capability (Pankrantz, 2001) into a contaminated system, is often pursued to improve the effectiveness of bioremediation processes.

To assess the results of biological decontamination it is not sufficient to measure the remaining pollutant content in soil or its transformation in non-toxic end-products but it is necessary to monitor whether and how soil biological functions are affected by and during the process. There is evidence that soil biological parameters (such as soil respiration, biomass, enzyme activities, microbial counts) may hold potential as early and sensitive indicators of soil ecological stress or restoration (Dick, 1992; Dick and Tabatabai, 1992).

1.1. Environmental contamination

The environment is continuously polluted by a large array of hazardous chemicals, released from several anthropogenic sources and with different structures and different toxicity levels. Three main sources of pollution can be identified: industrial activities, munitions waste and agricultural practices (Fig. 1.1). The explosive development of chemical industries has produced a large variety of chemical compounds that include pesticides, fuels, solvents, alkanes, polycyclic aromatic hydrocarbons (PAHs), explosives, dyes and more.

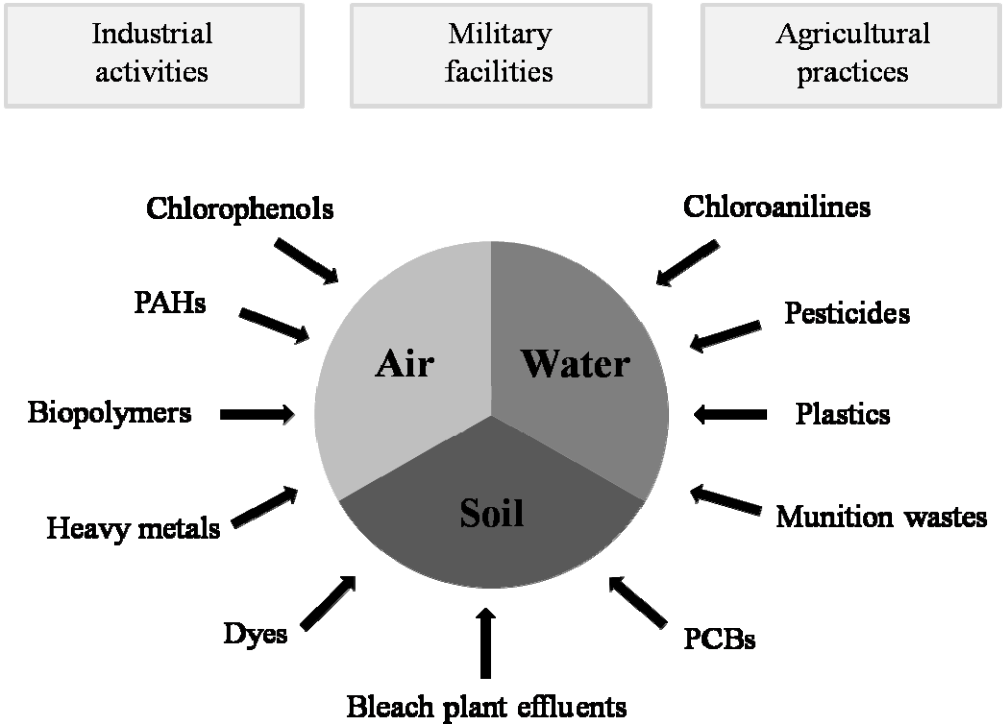

Fig. 1.1. Pollution of the environment by inorganic and organic compounds (from Gianfreda and Rao, 2004).

Although these compounds have contributed to modernize our lifestyle, several of them may accumulate in one or all of the environmental compartments. Chemicals released into the environment undergo various

dissipation pathways, and their persistence varies widely. Depending on their behaviour in the environment, contaminants are often classified as biodegradable, persistent, or recalcitrant. Factors affecting the local concentration of a contaminant include the amount of compound released, the rate at which the compound is released, its stability in the environment under various conditions, the extent of its dilution, its mobility in a particular environment, and its rate of biological or non-biological degradation (Harayama, 1997; Ellis, 2000; Janssen et al., 2001). Both organic and inorganic contaminants in soil and groundwater can be degraded or immobilized by naturally occurring processes, and the toxicity, mass and/or mobility of the contaminants can be reduced without human intervention when suitable conditions prevail.

The environment has a unique innate capability to resist pollution and remediate itself. Indeed, naturally occurring processes are involved in the attenuation of pollutants in the environment, including chemical, physical and biological processes such as sorption, dilution, dispersion, volatilization, hydrolysis, ion exchange, abiotic transformation, and biological degradation by intrinsic organisms (Fig. 1.2) (Christensen et al., 2001).

Among these natural processes, abiotic oxidation, hydrolysis and biodegradation are the only effective attenuation mechanisms, since they are capable to destroy the contaminants and transform them into innocuous end products.

In particular, microorganisms that are ubiquitous in the natural environments are considered the principal mediators of the natural attenuation of many pollutants, such as organic molecules, inorganic compounds, and metals (Christensen et al., 2001; Lovley, 2001). Therefore, degradation of pollutants by microorganisms has been considered as a

major pathway, among natural processes, by which various industrial compounds in the environment are attenuated.

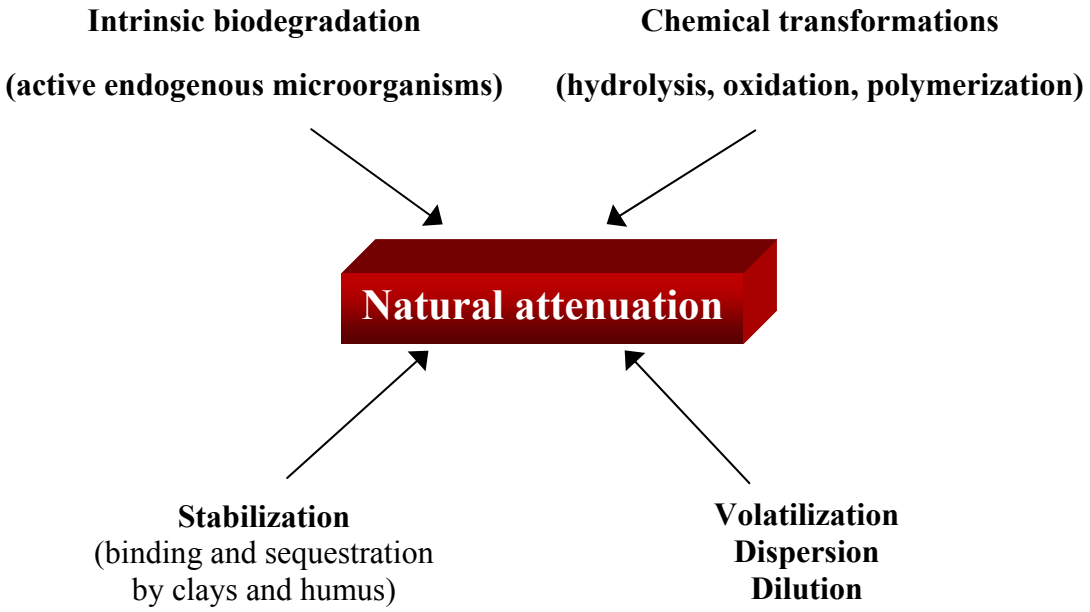

Fig. 1.2. Natural attenuation phenomena of organic pollutants in soil (from Gianfreda and Rao, 2004).

When the migration rate of contaminants exceeds their naturally occurring degradation rate, it is necessary to resort to the use of engineered remediation processes that require human intervention to enhance or accelerate the degradative power of the selected remediating agents. Several strategies have been developed to remediate and restore polluted environments: physical and chemical methods and biological approaches, requiring the involvement of biological agents.

These techniques may be utilized *in situ*, i.e. in the contaminated place itself, offering numerous advantages over *ex situ* technologies. The first ones can be done on site, eliminating transportation costs, are less expensive, can be applied to diluted and widely diffused contaminants, and minimize dangerous manipulations of the environment. While in *ex situ*

techniques, the treatments removing the contaminants occur at a separate treatment facility (Iwamoto and Nasu, 2001).

1.2. Soil contamination

Soil is one of the three major natural resources, alongside air and water. For a long time it was considered to be simply the loose fraction of the earth's crust. It wasn't until the late 1800s that soil was recognized as a natural body, worthy of study in its own right. Soil is a complex mixture of air (25%), water (25%), minerals that come from rocks below or nearby (45%), and organic matter (5%) which is the remains of plants and animals that use the soil and the living organisms that reside in the soil (Fig. 1.3). The proportion of each of these components is important in determining the type of soil that is present. But other factors such as climate, vegetation, time, the surrounding terrain, and even human activities (*e.g.* farming, grazing, gardening, etc.), are also important in influencing how soil is formed and the types of soil that occur in a particular landscape.

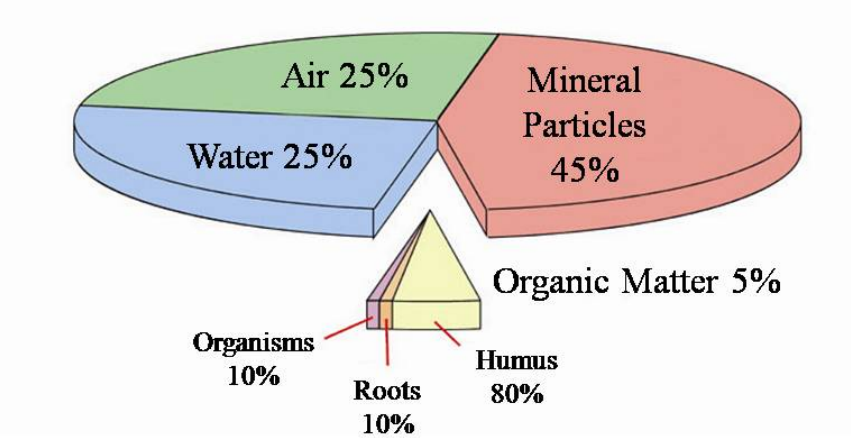

Fig. 1.3. Soil contains four basic components: mineral particles, water, air, and organic matter. Organic matter can be further sub-divided into humus, roots, and living organisms.

Soil provides the structural support and the source of water and nutrients for plants used in agriculture; therefore it is of great importance to preserve its quality.

Soil quality depends in part on its natural composition, and also on the changes caused by human use and management (Pierce and Larson, 1993). Natural events and anthropogenic activities continuously affect the quality of soil. The main anthropogenic contamination sources arise from the rupture of underground storage tanks, application of pesticides, percolation of contaminated surface water to subsurface strata, oil and fuel dumping, leaching of wastes from landfills or direct discharge of industrial wastes to the soil (Fig. 1.4).

Fig. 1.4. Soil anthropogenic contamination sources.

Soil can reduce the negative effects of the contaminants because of its auto-depuration capacity. Thanks to soil absorbent power, contaminants can be partially detracted from circulating solution, thus reducing the evaporation and the lisciviation processes and protecting the other environmental compartments. Nevertheless, the excess of contaminants, due to the overtaking of the holding capacity or environmental conditions changes (for example pH variations), results in loss of soil quality and release of contaminants.

1.3. Fate of organic contaminants in the soil environment

After its arrival to the soil, an organic contaminant (OC) may be lost by both biological and physical-chemical pathways. Biological transformations (biodegradation) are usually carried out by living organisms and/or their associated enzymatic complement, whereas physical-chemical pathways are the consequences of abiotic processes such as leaching or volatilization, accumulation within the soil biota or sequestration within the soil mineral and organic matter fractions (Fig. 1.5). Microbiological metabolic processes are, however, considered the principal mechanism of biological transformation of the toxic organic compounds.

The biological transformation may occur either through direct metabolic process such as mineralization, cometabolism, polymerization, or by indirect effects of the microbiological activity, such as changes of the soil pH and redox conditions (Bollag and Liu, 1990). Biodegradation has been described by Neilson et al. (1987) as the mineralization of a substrate to CO_2, H_2O, SO_2^{-4}, PO_2^{-4}, or NH^{+4} by microorganisms, providing final, non-toxic end-products and resulting in the formation of new biomass.

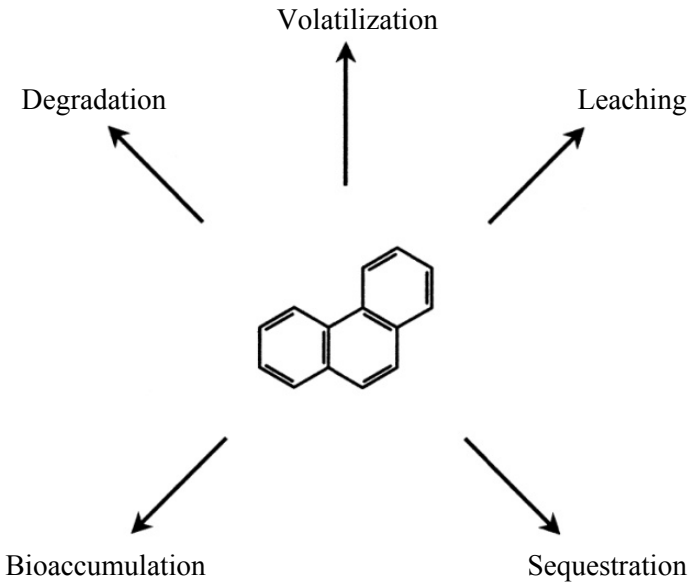

Fig. 1.5. Assumed fate and behaviour of a model organic contaminant (phenanthrene) in soil (from Semple et al., 2003).

Biotransformation can be described as small modifications in the substrate structure, such as the introduction of a hydroxyl group, o-methylation of a phenol, or oxidation of a thiol to a sulfoxide, but not as complete mineralization.

The ability of the soil microbial community to degrade organic compounds is fundamental to soil health and fertility. One of the principal mechanisms that accounts for the removal of OCs from soils is the catabolic activity of the microbes (Pritchard and Bourquin, 1984). Soil microflora has a diverse capacity for attacking OCs. This catabolic ability is due primarily to the co-evolution of soil microflora and naturally occurring organic compounds, which contain chemical structures analogous to those of OCs (Dagley, 1975). The rate of microbial decomposition of OCs in soils is a function of several factors, either singly or in combination (Macleod et al., 2001):

1. the availability of the contaminants to the microorganisms that have the catabolic ability to degrade them;
2. the numbers of degrading microorganisms present in the soil;

3. the activity of degrading microorganisms;
4. the molecular structure of the contaminant, and
5. the water solubility of the contaminant.

For sparingly soluble contaminants, biodegradation is generally slower than for more soluble contaminants, as the chemicals will partition more readily with the solid phases of the soil (Bosma et al., 1997).

However, the processes that control the evolution of catabolic activity in soils are not well understood. The catabolic activity can develop by adaptation, by the following processes:

1. the induction or depression of specific enzymes;
2. the development of new metabolic capabilities through genetic changes, such as plasmid transfer or mutation, and
3. selective enrichment of organisms able to transform the target contaminant(s) (Spain and van Veld, 1983; Pritchard and Bourquin, 1984).

Adaptation is thought to be controlled by the concentration of the OC interacting with the microflora, as well as the length of time the chemical is in contact with the soil (Bosma et al., 1997; Alexander, 2000; Macleod et al., 2001). For example, Macleod and Semple (2002) investigated the development of pyrene catabolic activity in two soils (pasture and woodland) with disparate amounts of organic matter amended with 100 mg pyrene kg^{-1}. Pyrene mineralization was observed in the pasture soil after 8 weeks of incubation, whereas it took 76 weeks in the woodland soil. Degradative investigations on the woodland soil showed that pyrene was bioavailable but that the microbial community in the woodland soil could not mineralize the pyrene. The observers thought the disparity in catabolic activity was due to the slower transfer of pyrene from the soil to the

microorganisms in the woodland soil caused by its larger organic matter content.

Microorganisms can utilize contaminants in the liquid phase by direct contact of cells with the organic contaminant, or with submicrometric particles dispersed in the aqueous phase (Nakahara et al., 1977). Microbial interaction with OCs involves two processes (Bosma et al., 1997):

1. a physical or chemical component involving the movement of the chemical in the physical environment, in relation to the degrading microorganisms, and
2. a biological component involving the metabolism of the chemical.

The relative importance of these mechanisms depends on how strongly the contaminant is sequestered as well as the rate of degradation. The rate at which a sequestered OC becomes available is influenced by the ability of microorganisms to reduce the concentration in the aqueous phase and the tendency of organisms to adhere to the sorbent (Calvillo and Alexander, 1996). Increased contact time reduces the magnitude of the rapidly desorbing phase and extent of biodegradation (Hatzinger and Alexander, 1995; Pignatello and Xing, 1996; Cornelissen et al., 1998).

The term 'bioavailability' refers to the fraction of a chemical in a soil that can be taken up or transformed by living organisms. Two important factors determine the amount of a chemical that is bioavailable: (i) the rate of transfer of the compound from the soil to the living cell (mass transfer) and (ii) the rate of uptake and metabolism (the intrinsic activity of the cell). Bioavailability has also been defined as the degree to which a compound is free to move into or on to an organism, and as such the term is best used in the context of a specific organism(s) because it is known that bioavailability differs between organisms and even species (Reid et al., 2000a).

It is well established that sequestration of organic contaminants in soil reduces the bioavailability of organic chemicals and results in a non-degraded residue in the soil (Fig. 1.6), even if some evidences confirm that the association with dissolved organic matter can increase water solubility and mobility of the contaminants (Kögel-Knabner and Totsche, 1998; Marschner, 1998).

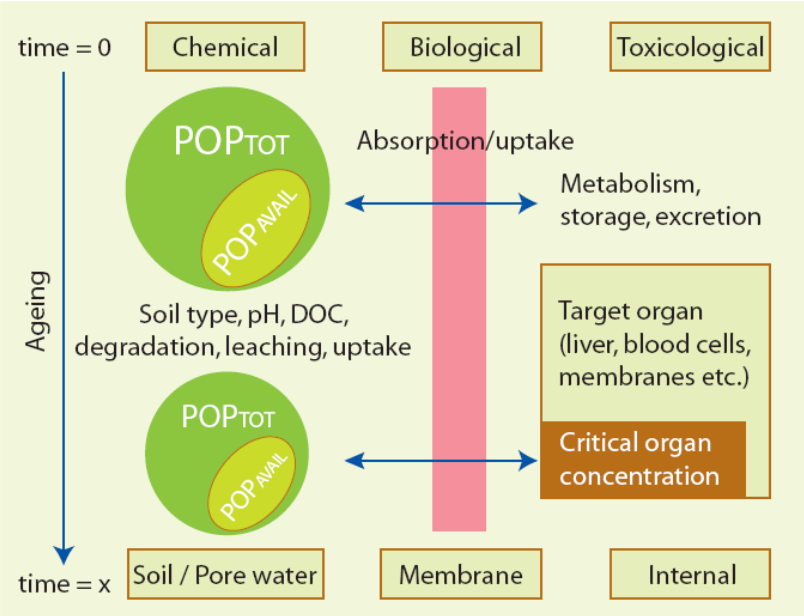

Fig. 1.6. Examples of processes governing the concept of bioavailability and ageing: chemical availability, including processes controlled by substance and soil specific parameters; biological availability, including processes controlled by species-specific parameters; toxicological availability, including processes controlled by organism-specific parameters. POP = persistent organic pollutant.

Normally, as the time of contact between contaminant and soil increases there is a decrease in chemical and biological availability, a process termed *ageing* (Hatzinger and Alexander, 1995). Contaminants that have aged in soil are not available for degradation even though freshly added compounds are still degradable (Alexander, 1995). Fig. 1.7 shows the influence of contact time on the extractability and bioavailability of OCs in

soil. Over time, the readily available fraction (easily extractable or bioavailable fraction) diminishes in a biphasic manner, i.e. some is degraded or lost from the soil and some is transformed into the recalcitrant fraction.

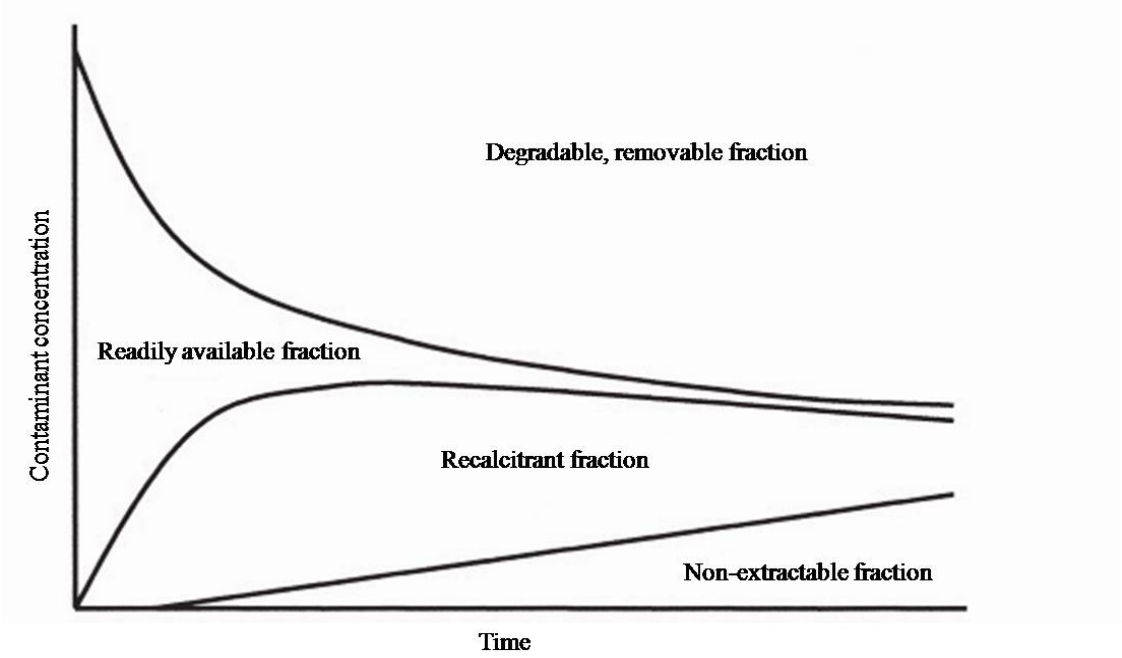

Fig. 1.7. The influence of contact time on the extractability and bioavailability of a contaminant (from Semple et al., 2003).

There is an increase in the recalcitrant fraction, which can be accessed only by specific and sometimes aggressive extractions, followed by a slower increase in a fraction deemed to be non-extractable (Macleod and Semple, 2000).

Sorption is the major factor involved in the sequestration of hydrocarbons in soil (Bosma et al., 1997). Slow sorption results in a fraction of the OC becoming resistant to desorption and in increased persistence within the soil matrix (Hatzinger and Alexander, 1995). The following hypotheses have been suggested as explanations for ageing:

1. the aged fraction results from the slow diffusion of the organic compounds within the solid organic matter fraction of soil, possibly the lipid fraction (Alexander, 2000);
2. the contaminant slowly diffuses through the soil and becomes sorbed and entrapped within nano- and micro-pores within the soil (Hatzinger and Alexander, 1995).

Of course, contaminants may become sequestered by a combination of both the above mechanisms (Fig. 1.8).

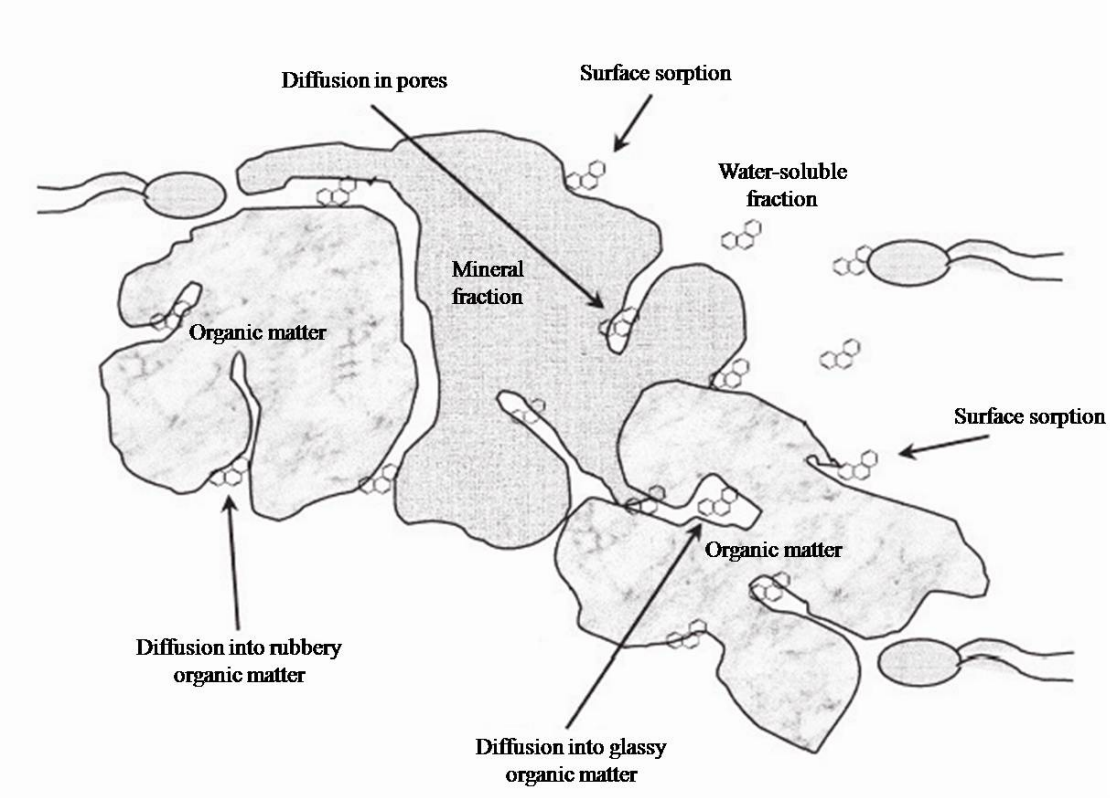

Fig. 1.8. Physical behaviour of a contaminant within the soil (from Semple et al., 2003).

Evidence for the sequestration of contaminants includes (i) laboratory and field investigations, which demonstrate a decreasing availability to organisms (Chung and Alexander, 1998); (ii) investigations into the extractability of aged OCs and the kinetics of sorption and desorption (Hatzinger and Alexander, 1995); (iii) temporal changes in the rate and extent of contaminant mineralization (Hatzinger and Alexander, 1995; Reid

et al., 2000b), and (iv) the assessment of toxicity. This last is very important for decisions regarding risk and environmental regulations; however, the evidence is based on only a few studies by Salanitro et al. (1997) and Saterbak et al. (1999, 2000). Simplistically, ageing may be associated with the continuous diffusion of OCs into small pores where the organic molecules are retained by sorption. This explains the decreases in solvent extractability and bioavailability of OCs. It also means that toxic organic chemicals that have been in contact with the soil matrix for a long time are unlikely to be available to humans, animals or plants (Alexander, 1995). However, we do not know yet how long this fraction will remain in this state or whether the contaminant(s) will remobilize and so become extractable and bioavailable.

1.4. Persistent organic pollutants

Of all the pollutants released into the environment every year by human activity, persistent organic pollutants (POPs) are among the most dangerous. POPs are used as pesticides, consumed by industry, or generated unintentionally as by-products of various industrial/combustion processes. They are highly toxic and cause an array of adverse effects, notably death, disease, and birth defects among humans and animals. Specific effects can include cancer, allergies and hypersensitivity, damage to the central and peripheral nervous systems, reproductive disorders, and disruption of the immune system. Some POPs are also considered to be endocrine disrupters which, by altering the hormonal system, can damage the reproductive and immune systems of exposed individuals as well as their offspring; they can also have developmental and carcinogenic effects.

These highly stable compounds can last for years or decades before breaking down. They circulate globally through a process known as the 'grasshopper effect'. POPs released in one part of the world can, through a repeated (and often seasonal) process of evaporation and deposit, be transported through the atmosphere to regions far away from the original source. In addition, POPs can bioaccumulate in living organisms. They are associated with the manufacture and use of certain chemicals, with spills and leaks and with the combustion of both fuels and wastes (http://web.worldbank.org/).

Two main classes of POPs are polycyclic aromatic hydrocarbons and chlorophenols.

1.4.1. Polycyclic aromatic hydrocarbons

1.4.1.1. General properties

Polycyclic aromatic hydrocarbons (PAHs) are non-polar organic compounds made up of two or more fused benzene rings, arranged in linear, angular or clustered structures (Fig. 1.9).

PAHs are hydrophobic compounds and their persistence in the environment is chiefly due to their low water solubility (Cerniglia, 1992) and high octanol-water partition coefficient (K_{ow}). Generally, PAHs solubility and volatility decreases and hydrophobicity increases with an increase in number of fused benzene rings (Wilson and Jones, 1993). PAHs are classified according to the number of rings, the type of ring and the atom composition. The low molecular weight (LMW) PAHs contain two or three aromatic rings and the high molecular weight (HMW) ones more than three. Many PAHs are carcinogenic and they are, therefore, of significant concern as environmental contaminants.

Numerous studies have indicated that one-, two- and three-ring compounds are acutely toxic (Sims and Overcash, 1983), while higher molecular weight PAHs are considered to be genotoxic (Phillips, 1983; Lijinsky, 1991; Mersch-Sundermann et al., 1992; Nylund et al., 1992). PAHs are highly lipid soluble and thus readily adsorbed by the gastrointestinal tract of mammals (Cerniglia, 1984). They are rapidly distributed in a wide variety of tissues with a marked tendency for localization in body fat.

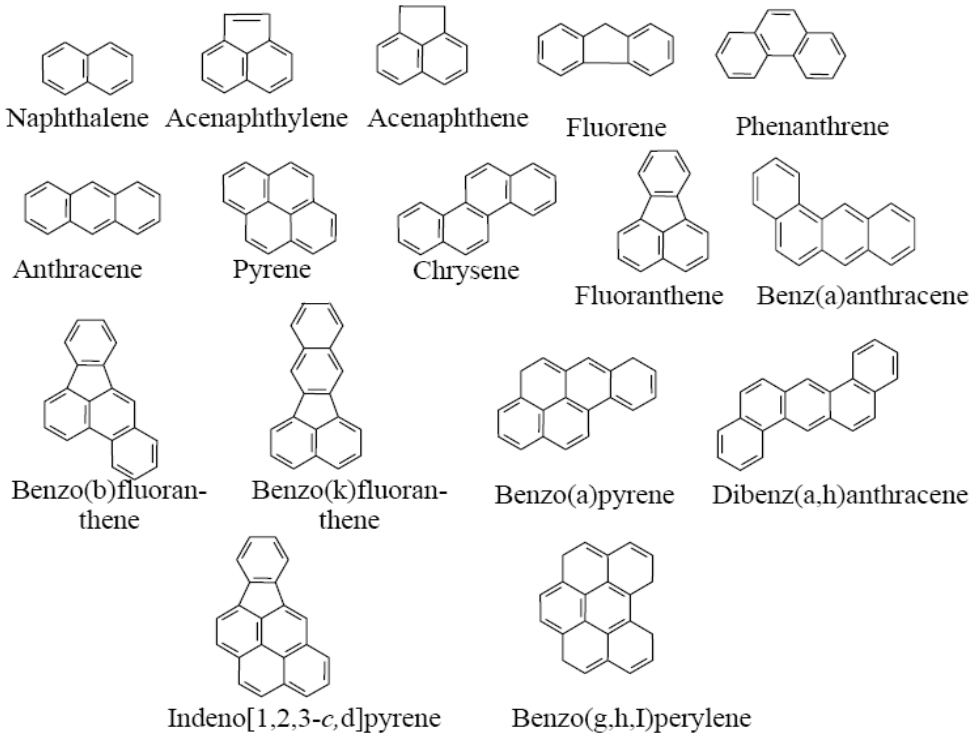

Fig. 1.9. Structure of some abundant polycyclic aromatic hydrocarbons (PAHs) in the environment.

Metabolism of PAHs occurs via the cytochrome P450-mediated mixed function oxidase system with oxidation or hydroxylation as the first step (Stegeman et al., 2001). The resultant epoxides or phenols might get detoxified in a reaction to produce glucoronides, sulfates or glutathione conjugates. Some of the epoxides might metabolize into dihydrodiols, which, in turn, could undergo conjugation to form soluble detoxification

products or be oxidized to diol-epoxides. Many PAHs contain a 'bay-region' as well as 'K-region', both of which allow metabolic formation of bay- and K-region epoxides, which are highly reactive. K-region is represented by carbons 9 and 10 of the phenanthrene ring system and it seems to be the reactive spot in the various hydrocarbon carcinogen. A common bay-region is the site on benzo(a)pyrene, an indirect carcinogen that is metabolically activated by the P450 system. Carcinogenicity has been demonstrated by some of these epoxides (Goldman et al., 2001) (Fig. 1.10).

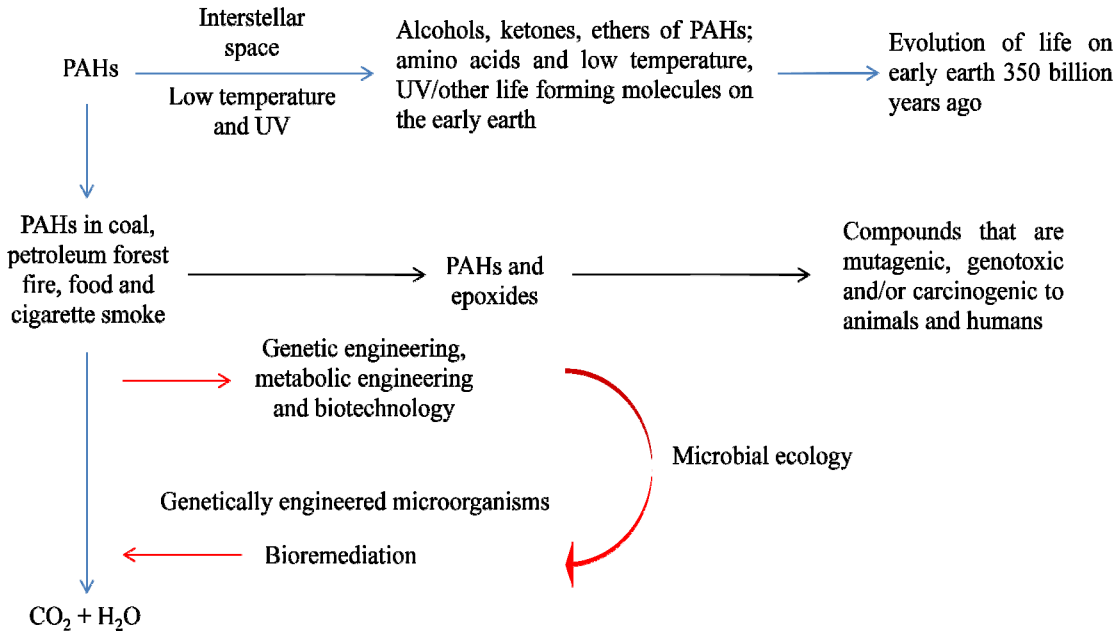

Fig. 1.10. Fate, toxicity and remediation of polycyclic aromatic hydrocarbons (PAHs) in the environment. A wide variety of PAHs are abundant in nature owing to incomplete combustion of organic matters. The PAHs from extraterrestrial matter are also oxidized and reduced owing to prevalent astrophysical conditions and resulting in the formation of various organic molecules, which are the basis of early life on primitive earth. The microorganisms (naturally occurring or genetically engineered) can mineralize toxic PAHs into CO_2 and H_2O (from Samanta et al., 2002).

Therefore, many PAHs are considered to be environmental pollutants that can have a harmful effect on the flora and fauna of affected habitats, resulting in the uptake and accumulation of toxic chemicals in food chains

and, in some instances, in serious health problems and/or genetic defects in humans. Consequently, the United States Environmental Protection Agency (U.S. EPA) and the European Community have listed 16 PAHs as priority environmental pollutants (Wattiau, 2002).

Naphthalene, the first member of the PAHs group, is a common micropollutant in potable water. The toxicity of naphthalene has been well documented and cataractogenic activity has been reported in laboratory animals (Mastrangela et al., 1997; Goldman et al., 2001).

Naphthalene binds covalently to molecules in liver, kidney and lung tissues, thereby enhancing its toxicity; it is also an inhibitor of mitochondrial respiration (Falahatpisheh et al., 2001). Acute naphthalene poisoning in humans can lead to haemolytic anaemia and nephrotoxicity. In addition, dermal and ophthalmological changes have been observed in workers occupationally exposed to naphthalene.

Phenanthrene is known to be a photosensitizer of human skin, a mild allergen and mutagenic to bacterial systems under specific conditions (Mastrangela et al., 1997). It is a weak inducer of sister chromatid exchanges and a potent inhibitor of gap junctional intercellular communication (Weis et al., 1998).

Interestingly, because phenanthrene is the smallest PAH to have a bay-region and a K-region, it is often used as a model substrate for studies on the metabolism of carcinogenic PAHs (Bücker et al., 1979). Little information is available for other PAHs such as acenaphthene, fluoranthene and flourene with respect to their toxicity in mammals.

However, the toxicity of benzo(a)pyrene, benzo(a)anthracene, benzo(b)fluoranthene, benzo(k)fluranthene, dibenz(a,h)anthracene and indeno(1,2,3-c,d)pyrene has been studied and there is sufficient

experimental evidence to show that they are carcinogenic (Mastrangela et al., 1997; Šrám et al., 1999; Liu et al., 2001).

1.4.1.2. PAHs in the environment: sources and fate

The release of PAHs into the environment is widespread since these compounds are ubiquitous products of incomplete combustion and uncontrolled emissions (Wilson and Jones, 1993). PAHs have been detected in a wide variety of environmental samples, including air (Greenberg et al., 1985; Sexton et al., 1985; Freeman and Cattell, 1990), soil (Jones et al., 1989a,b,c; Wilson and Jones, 1993), sediments (Youngblood and Blumer, 1975; Laflamme and Hites, 1978; Shiaris and Jambard-Sweet, 1986), water (Cerniglia and Heitkamp, 1989), oils, tars (Nishioka et al., 1986) and foodstuffs (Dipple and Bigger, 1991; Lijinsky, 1991). Industrial activities, such as processing, combustion and disposal of fossil fuels, are usually associated with the presence of PAHs at highly contaminated sites. PAHs contamination in industrial sites is commonly associated with spills and leaks from storage tanks and with the conveyance, processing, use and disposal of these fuel/oil products (Wilson and Jones, 1993). PAHs are also a major constituent of creosote (approximately 85% PAH by weight) and anthracene oil, which are commonly used as pesticides for wood treatment (Bos et al., 1984; Bumpus, 1989; Walter et al., 1991). As such, PAH contamination is frequently associated with wood treatment activities (Sims and Overcash, 1983; Mueller et al., 1993; Vanneck et al., 1995) and wood-preservative production (Wilson and Jones, 1993).

Possible fates of PAHs in the environment include volatilization, photo-oxidation, chemical oxidation, bioaccumulation, interaction with the soil matrix and biodegradation (Cerniglia, 1992); the importance of these

processes depending on the environment, i.e. atmosphere, soil or water. In soils, PAHs can undergo abiotic reactions (photo-oxidation and chemical oxidation) and some, i.e. naphthalene and alkyl naphthalene, are partly lost by volatilization (Park et al., 1990). However, the main transformation is the result of microbial degradation (Cerniglia, 1992) and a relevant fate is the adsorption to the soil matrix.

1.4.2. Chlorophenols

1.4.2.1. General properties

Chlorophenols (CPs) are organic chemicals formed from phenol (1-hydroxybenzene) by substitution in the phenol ring with one or more atoms of chlorine. Nineteen congeners are possible, ranging from monochlorophenols to the fully chlorinated pentachlorophenol (PCP) (Fig. 1.11.).

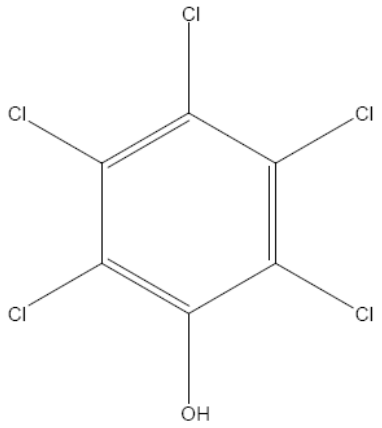

Fig. 1.11. Pentachlorophenol molecule.

Chlorophenols, particularly trichlorophenols, tetrachloro-phenols and PCP, are also available as sodium or potassium salts. The aqueous solubility of chlorophenols is low, but their sodium or potassium salts are up to four

orders of magnitude more soluble in water than the parent compounds. The changes in solubility may reflect in increases or decreases of bioavailability of the compound to the microbial attack. Usually, increased water solubility results in greater bioavailability and in turn in higher levels of degradation by the microbial agent. The acidity of chlorophenols increases as the number of chlorine substitutions increases. The *n*-octanol/water partition coefficients of chlorophenols increase with chlorination, indicating a tendency for the higher chlorophenols to bioaccumulate.

Because of their broad-spectrum antimicrobial properties, chlorophenols have been used as preservative agents for wood, paints, vegetable fibres and leather and as disinfectants. In addition, they are used as herbicides, fungicides and insecticides and as intermediates in the production of pharmaceuticals and dyes.

The toxicity of chlorophenols depends upon the degree of chlorination, the position of the chlorine atoms and the purity of the sample. Chlorophenols have an irritating effect on eyes and on respiratory tract. Toxic doses of chlorophenols cause convulsions, shortness of breath, coma and finally death. Chlorophenols can be adsorbed through the lungs, the gastro-intestinal tract and the skin. A high concentration of CPs in the affected organisms can develop long-term negative effects including teratogenicity and mutagenicity (Kogevinas et al., 1997; Farah et al., 2004).

1.4.2.2. Chlorophenols in the environment: sources and fate

The most important source of these compounds is the chlorine bleaching process of pulp and paper mills, which discharges large volumes of brown-coloured effluents (Rubilar et al., 2008) (Table 1.1). The toxicity of this effluent has primarily been attributed to wood resins, chlorinated phenols, and tannins, while the brown colour results from the presence of lignin or

polymerized tannins in the wastewater (Kookana and Rogers, 1995; Diez et al., 1999).

Therefore, the occurrence of organochlorinated compounds in nature is generally ascribed to anthropogenic activities (Annachhatre and Gheewala, 1996). However, 2000 chlorinated and otherwise halogenated chemicals are discharged into our biosphere by plants, marine organisms, insects, bacteria, fungi, mammals, and enzymatic, thermal, and other natural processes. These are constantly occurring in many ecosystems-surface waters and groundwater, sediments, atmospheric air, and soils and lead to the formation of chlorinated chemicals, including dioxins and chlorofluorocarbons that previously were thought to result only from the actions of humans (Gribble, 1994).

Table 1.1. Main chlorinated phenolic compounds in pulp-mill effluents after bleaching with hypochlorite and chlorine dioxide (from Rubilar et al., 2008).

Compounds	Conventional Cl beach process (g ton^{-1} pulp)	50-60% ClO_2 substitution (g ton^{-1} pulp)
Chlorinated phenols	1.4-4.8	0.4
2,4-Dichlorophenol	0.3-0.5	0.4
2,4,6-Trichlorophenol	0.8-1.1	0.6-0.7
2,3,4,6-Tetrachlorophenol	2.1-2.2	0.4
Pentachlorophenol	1.0-1.3	1.4

The majority of chlorophenols released into the environment go into water, with very little entering the air. The compounds that seem most likely to go into the air are the mono- and dichlorophenols because of their volatility (that is, have the greatest tendency to form vapours or gases). Once in the air, sunlight helps destroy these compounds and rain washes them out of the air. CPs stick to soil and to sediments at the bottom of lakes, rivers, or streams.

In the natural environment, chlorophenols may be transformed by several natural attenuation processes such as biodegradation, chemical and photochemical degradation, volatilization, dispersion, and stabilization by adsorption on soil constituents (Czaplicka, 2004). However, such natural processes can occur at various efficiencies and rate and they can be so slow that pollutants may persist for years. For instance, their susceptibility to biodegradation may change drastically, depending on several factors related to the chemical and physical properties of both the chemical and the environment in which they are present (Rubilar et al., 2008).

1.5. Soil remediation

The production, distribution, use, misuse, disposal, or accidental spills of many chemicals have polluted some environments to levels that threaten the health of humans, livestock, wildlife and, indeed, whole ecosystems. Most of these chemicals are produced and used in efforts to improve human health, standards of living and safety through advancements in manufacturing, agriculture and agribusiness, medicine, and to strengthen national defence. Ironically, their unplanned intrusions into the environment can reverse the same standards of living that they are intended to foster. Nowadays there is the need to remedy the contaminated sites, especially soils, either as a response to the risk of adverse health or environmental effects caused by contamination or to enable the soil to be redeveloped for use.

There is a very wide range of remediation methods available to tackle contamination although two broad approaches can be distinguished (Wood, 1997):

- engineering approaches - these are primarily the traditional methods of excavation and disposal to landfill, or the use of appropriate containment systems;
- process based techniques include physical, chemical, biological, stabilization/solidification, and thermal processes.

1.5.1. Engineering approaches

Contaminated sites are frequently remediated by excavation of the contaminated material and subsequent disposal of this to a controlled landfill. The approach represents a rapid method of dealing with a contaminated site but it has been criticized as it represents only a transfer of the contaminated material from one location to another rather than a final solution. Additionally, it is very difficult and increasingly expensive to find new landfill sites for the final disposal of the material. The breakdown and/or stabilization of many pollutants cannot be guaranteed under landfill conditions; in fact, for some compounds, breakdown or stabilization processes are retarded in landfills. Contaminated material disposed to landfill must be prevented from causing any further environmental damage. The principal approaches that contribute towards prevention are (Armishaw et al., 1992; Wood, 1997):

- containment,
- attenuation.

The concept of containment as a method for dealing with contaminated ground is based on the use of low-permeability barriers to isolate the contaminated material, or any associated leachate or gaseous products, from the environment. The barriers can be constructed from natural or synthetic materials, or a combination of both, and can be placed over, under or around a contaminated area or pollution source. The technique can be

used to isolate existing hazards such as a contamination source, to prevent the spread of contaminants from a disposal site such as landfill, or to isolate specially designed mono-disposal sites for contaminated soil. The effective design and installation of a containment system requires extensive geological and hydrological investigation, modelling and monitoring. Although low permeability is a necessary characteristic of containment materials complete impermeability is rarely attained in practice. However, any materials used for containment may also act as a substrate for attenuation mechanisms. A further degree of containment can be achieved if the contaminated material is subjected to stabilization/solidification techniques prior to disposal.

Remediation of many contaminated sites has been achieved by covering the surface with clean material incorporating a low-permeability layer. Whereas this may reduce infiltration and form a physical barrier to the contamination it may not necessarily control adequately the movement of contaminants. In order to provide adequate control it may be necessary to use such cover systems in conjunction with vertical and horizontal in-ground barriers or cut-offs to achieve partial or total isolation of the site. A *cover system* consists of a single layer, or succession of layers, of selected, suitable non-contaminated material that covers the area of contamination.

In-ground barriers can be used to isolate, usually by physical means, a contaminated mass of ground from the surrounding environmental or other targets. Low-permeability material may be introduced around or under the contaminated site, or methods incorporating some sort of physical, biological or chemical control of contaminant migration can be used. In-ground barriers can be placed around, above and below a contaminant source to achieve complete isolation.

The effectiveness and applicability of barrier methods vary according to the types and nature of contaminants present, physical conditions of the site and the design life of the barrier.

As stated above (Fig. 1.1), natural attenuation refers to the decrease in the mass and/or concentration of a contaminant due to physical, chemical or biological mechanisms and intrinsic bioremediation, *i.e.* natural bioremediation by indigenous microorganisms, is becoming a favoured treatment technology for contaminated sites. Indeed natural processes have been found to be satisfactory for removal of many pollutants (Rügge et al., 1995; Semprini et al., 1995), and other more aggressive treatments still do not totally eliminate contaminants or do not result in expected removal or destruction rates (Bredehoeft, 1992; Valkenburg, 1994; Uhlman, 1995).

1.5.2. Process based techniques

1.5.2.1. Physical methods

Soils that contain a wide range of contaminants and contaminant mixtures can be treated by physical methods. Physical processes separate contaminants from uncontaminated material by exploiting differences in their physical properties (*e.g.* density, particle size, volatility) by applying some external force (*e.g.* abrasion) or by altering some physical characteristic to enable separation to occur (*e.g.* flotation). Depending on the nature and distribution of the contamination within the soil, physical processes may result in the segregation of differentially contaminated fractions (for example, a relatively uncontaminated material and a contaminant concentrate based on a size separation) or separation of the contaminants (for example oil or metal particles) from the soil particles. The range of physical processes includes a diverse variety of methods that

include both *in situ* and *ex situ* approaches. This variation has been classified into two main groups (Barber et al., 1994):

- *Washing and sorting treatments* which are commonly referred to as soil separation and washing. The main aim of the processes is to concentrate the contaminants into a relatively small volume so that the costs associated with disposal and further treatment are related only to the reduced volume of process residues.
- *Extraction treatments* which involve processes that remove the contaminants from soils by involving a mobilizing and/or releasing process to remove the contaminant from the soil matrix. Three main categories of extraction treatments are soil vapour extraction, electro-remediation and soil flushing and chemical extraction.

1.5.2.2. Chemical methods

Chemical treatments for the remediation of contaminated soils are designed either to destroy contaminants or to convert them into less environmentally hazardous form. Chemical reagents are added to the soil to bring about the appropriate reaction. In general, excess reagents may need to be added to ensure that the treatment is complete. This in turn may result in excessive quantities of un-reacted reagents remaining in the soil following treatment. Heat and mixing may also be necessary to support the chemical reaction. Chemical processes can also concentrate contaminants in a manner similar to physical processes. A range of chemical remediation processes is at various stages of development, both for *in situ* and *ex situ* applications. Many of these are based on the treatment of waste water or other hazardous waste. However, the range of processes that have been widely used at full scale is restricted. Major types include:

- oxidation-reduction,

- dechlorination,
- extraction,
- hydrolysis,
- pH adjustment.

Redox reactions can be applied to soil remediation to achieve a reduction of toxicity or a reduction in solubility. Oxidation and reduction processes can treat a range of contaminants including organic compounds and heavy metals. Oxidizing agents that can be used include oxygen, ozone, ultraviolet light, hydrogen peroxide, chlorine gas and various chlorine compounds. Reduction agents that can be used include aluminium, sodium and zinc metals, alkaline polyethylene glycols, and some specific iron compounds.

Chemical dechlorination processes use reduction reagents to remove chlorine atoms from hazardous chlorinated molecules to leave less hazardous compounds. Dechlorination can be used to treat soils and waste contaminated with volatile halogenated hydrocarbons, polychlorinated biphenyls, and organochlorine pesticides.

Extraction techniques that can be used for the treatment of contaminated soils include organic solvent extraction, supercritical extraction, and metal extraction using acids. The methods are applicable to soils, wastes, sludge and liquids. The extraction liquid containing the contaminant has to be collected for treatment.

Hydrolysis refers to the displacement of a functional group on an organic molecule with a hydroxide group derived from water. A restricted range of organic contaminants is potentially treatable by hydrolysis, although hydrolysis products may be as hazardous, or even more hazardous, than the original contaminant.

pH adjustment refers to the application of weak acidic or basic materials to the soil or groundwater to adjust the pH to acceptable levels. A common example is the addition of lime to neutralize acidic agricultural soils. Neutralization can also be used to affect the mobility or availability of contaminants such as metals by enhancing their precipitation as hydroxides.

1.5.2.3. Stabilization/Solidification

Stabilization/solidification methods operate by solidifying contaminated material, converting contaminants into a less mobile chemical form and/or by binding them within an insoluble matrix offering low leaching characteristics. These processes can be adopted to treat soils, wastes, sludge and even liquids, and a variety of contaminant types. However, the treatment of organic contaminants is generally more difficult and more expensive. An added benefit is the improved handling and geotechnical properties of the treated product that might result compared with the original contaminated material. Stabilization/solidification processes have been applied both *in situ* and *ex situ*, the latter being both on and off site. With an *ex situ* approach it may be necessary to landfill the stabilized product if an alternative use or disposal option is not possible. A disadvantage here is that the volume of the stabilized product can be considerably greater than the original contaminated material because of the quantities of stabilization materials that have been added.

1.5.2.4. Thermal methods

The number of technologies that are commercially available is considerably restricted. Techniques under development and commercially available can

be either *in situ* or *ex situ*. Three *ex-situ* techniques that operate in different temperature regimes will be outlined (Barber et al., 1994; Wood, 1997):

- thermal desorption,
- incineration,
- vitrification.

Thermal desorption involves the excavation of the contaminated soil following by heating to temperature in the region of 600 °C. At these temperatures the volatile contaminants are evaporated and subsequently removed from the exhaust gasses by condensation, scrubbing, filtration or destruction at higher temperatures. Following treatment it may be possible to re-use the soil depending on the temperatures used and the concentration of any residual contamination. Thermal desorption has its primary use in the treatment of organic contamination although it has also been used for the treatment of mercury-contaminated soils.

Incineration involves the heating (either directly or indirectly) of excavated soil to temperatures of 880-1200 °C to destroy or detoxify contaminants. Incineration can also be used for the treatment of contaminated liquids and sludge. Incineration results in the destruction of the soil texture and removes all natural humic components. Residues may also have high heavy metal contents. Exhaust gasses need to be treated to remove particulates and any harmful combustion products. A range of methods of incineration are available although the use of rotary kilns is probably the most widespread. Costs of treatment are heavily dependent on the water content of the material being treated and any calorific value that the material may have. Vitrification involves the heating of excavated soil to temperatures in the region of 1000-1700 °C. At these temperatures vitrification of the soil occurs forming amonolithic solid glassy product. Contaminants will either be destroyed or trapped in the glassy product. The technology works by

melting the alumino-silicate minerals in the soil which, on cooling, solidify to form the glass. In soils or wastes where there are insufficient alumino-silicates these can be added in the form of glass or clay. The product from vitrification may have very low leaching characteristics. Exhaust gases require treatment for the removal of any volatile metals or hazardous combustion products. Vitrification is an expensive process and likely to be restricted in use for particularly hazardous contaminants that are not readily treated by other methods.

1.6. Biological methods

Biological remediation, or *bioremediation*, is a process in which indigenous or inoculated microorganisms, for instance fungi, bacteria and other microbes, degrade (*metabolize*) organic contaminants found in soil (and groundwater), converting them to innocuous end-products.

It uses naturally occurring bacteria and fungi or plants to degrade or detoxify substances hazardous to human health and/or the environment. The microorganisms may be indigenous to a contaminated area or they may be isolated from elsewhere and brought to the contaminated site. Contaminant compounds are transformed by living organisms through reactions that take place as a part of their aerobic or anaerobic metabolism. Usually, aerobic biodegradation is much more efficient than the anaerobic process and it is widely used in relation to the chemical nature of the contaminant. Both processes can be applied in series to reduce the complexity and toxicity of the contaminant. Biodegradation of a compound is often a result of the actions of multiple organisms. For bioremediation to be effective, microorganisms must enzymatically attack the pollutants and convert them to harmless products. The control and optimization of

bioremediation processes is a complex system of many factors. These factors include: the existence of a microbial population capable of degrading the pollutants; the availability of contaminants to the microbial population; the environment factors (type of soil, temperature, pH, the presence of oxygen or other electron acceptors, and nutrients). Bioremediation technologies can be broadly classified as *in situ* and *ex situ*. *In situ* techniques involve treatment of the contaminated material in place. By contrast, e*x situ* techniques are those treatments which involve the physical removal of the contaminated material for treatment.

1.6.1. *In situ* bioremediation

These techniques (U.S. EPA, 2001, 2002) are generally the most desirable options due to lower cost and less disturbance since they provide the treatment in place avoiding excavation and transport of contaminants. *In situ* treatment is limited by the depth of the soil that can be effectively treated.

In many soils effective oxygen diffusion for desirable rates of bioremediation extends from a range of only a few centimetres to about 30 cm into the soil, although depths of 60 cm and greater have been effectively treated in some cases.

The most important land treatments are as follows:

- *Bioventing,* the most common *in situ* treatment that involves supplying air and nutrients through wells to contaminated soil to stimulate the indigenous bacteria. Bioventing employs low air flow rates and provides only the amount of oxygen necessary for the biodegradation while minimizing volatilization and release of contaminants to the atmosphere. It works for simple hydrocarbons and can be used where the contamination is deep under the surface.

- *Biosparging* that involves the injection of air under pressure below the water table to increase groundwater oxygen concentrations and enhance the rate of biological degradation of contaminants by naturally occurring bacteria. Biosparging increases the mixing in the saturated zone and thereby increases the contact between soil and groundwater. The ease and low cost of installing small-diameter air injection points allows considerable flexibility in the design and construction of the system.
- *Bioaugmentation*, that involves the addition of microorganisms indigenous or exogenous to the contaminated sites. Two factors limit the use of added microbial cultures in a land treatment unit: 1) non-indigenous cultures rarely compete well enough with an indigenous population to develop and sustain useful population levels, and 2) most soils with long-term exposure to biodegradable waste have indigenous microorganisms that are effective degraders if the land treatment unit is well managed.
- *Biostimulation*, that involves some stimulation of the numbers and activities of natural populations, usually bacteria or fungi, so they can better break down pollutants into harmless products. Biostimulation is based on the assumption that a polluted medium (soil, water, etc.) already contains microbes that are capable of destroying or detoxifying particular pollutants in that medium. The reason for the persistence of a pollutant, therefore, may be due to one or more of the following: a) unbalanced and/or inappropriate levels of nutrients or aeration; b) strong binding of the pollutant to the medium (adsorption) that prevents the availability of the pollutant for destruction or transformation by microbes; or c) inactivity of the native microbes caused by excessively high (toxic) concentrations of

pollutants. Accordingly, provision of appropriate nutrient and environmental conditions, including alleviation of toxicity problems, should allow natural pollutant cleanup or stabilization to proceed.

1.6.2. *Ex situ* bioremediation

These techniques involve the excavation or removal of contaminated soil from ground. Some examples are reported below:

- *Landfarming* is a simple technique in which contaminated soil is excavated and spread over a prepared bed and periodically tilled until pollutants are degraded. The goal is to stimulate indigenous biodegradative microorganisms and facilitate their aerobic degradation of contaminants. In general, the practice is limited to the treatment of superficial 10-35 cm of soil. Since landfarming has the potential to reduce monitoring and maintenance costs, as well as clean-up liabilities, it has received much attention as a disposal alternative.
- *Biopiles* are a hybrid of landfarming and composting. Essentially, engineered cells are constructed as aerated composted piles. Typically used for treatment of surface contamination with petroleum hydrocarbons they are a refined version of landfarming that tend to control physical losses of the contaminants by leaching and volatilization. Biopiles provide a favourable environment for indigenous aerobic and anaerobic microorganisms (von Fahnestock et al., 1998).
- *Bioreactors.* Slurry reactors or aqueous reactors are used for *ex situ* treatment of contaminated soil and water pumped up from a contaminated plume. Bioremediation in reactors involves the processing of contaminated solid material (soil, sediment, sludge) or

water through an engineered containment system. A slurry bioreactor may be defined as a containment vessel and apparatus used to create a three-phase (solid, liquid, and gas) mixing condition to increase the bioremediation rate of soil-bound and water-soluble pollutants as a water slurry of the contaminated soil and biomass (usually indigenous microorganisms) capable of degrading target contaminants. In general, the rate and extent of biodegradation are greater in a bioreactor system than *in situ* or in solid-phase systems because the contained environment is more manageable and hence more controllable and predictable. Despite the advantages of reactor systems, there are some disadvantages. The contaminated soil requires pre-treatment (*e.g.* excavation) or alternatively the contaminant can be stripped from the soil via soil washing or physical extraction (*e.g.* vacuum extraction) before being placed in a bioreactor.

1.6.3. Advantages and disadvantages of bioremediation

Bioremediation may present either advantages or disadvantages. The main advantages can be summarized as follows (Vidali, 2001):

- Bioremediation is a natural process and is therefore perceived by the public as an acceptable waste treatment process for contaminated material such as soil. Microbes able to degrade the contaminant increase in numbers when the contaminant is present; when the contaminant is degraded, the biodegradative population declines. The residues from the treatment are usually harmless products and include carbon dioxide, water, and cell biomass.
- Theoretically, bioremediation is useful for the complete destruction of a wide variety of contaminants. Many compounds that are legally

considered to be hazardous can be transformed to harmless products. This eliminates the chance of future liability associated with treatment and disposal of contaminated material.

- Instead of transferring contaminants from one environmental medium to another, for example, from land to water or air, the complete destruction of target pollutants is possible.
- Bioremediation can often be carried out on site, often without causing a major disruption of normal activities. This also eliminates the need to transport quantities of waste off site and the potential threats to human health and the environment that can arise during transportation. A reduction of exposure risks for clean-up personnel can also result.
- Bioremediation techniques are typically more economical than traditional methods.

The main disadvantages are:

- Bioremediation is limited to those compounds that are biodegradable. Some compounds, such as chlorinated organic or high aromatic hydrocarbons are resistant to microbial attack. They are degraded either slowly or not at all, hence it is not easy to predict the rates of clean-up for a bioremediation exercise.
- There are some concerns that the products of biodegradation may be more persistent or toxic than the parent compound.
- Biological processes are often highly specific. Important site factors required for success include the presence of metabolically capable microbial populations, suitable environmental growth conditions, and appropriate levels of nutrients and contaminants.
- It is difficult to extrapolate from bench and pilot-scale studies to full-scale field operations.

- Research is needed to develop and engineer bioremediation technologies that are appropriate for sites with complex mixtures of contaminants that are not evenly dispersed in the environment. Contaminants may be present as solids, liquids, and gases.
- Bioremediation often takes longer than other treatment options, such as excavation and removal of soil or incineration.
- Regulatory uncertainty remains regarding acceptable performance criteria for bioremediation.

1.7. Soil quality indicators

It is important to distinguish soil quality from soil health, which often are used interchangeably (Doran and Safley, 1997). Doran et al. (1996) defined soil health as 'the continued capacity of soil to function as a vital living system, within natural or managed ecosystem boundaries to sustain biological productivity, maintain environmental quality, and promote plant, animal and human health'. Soil quality is the end product of soil degradative or conserving processes and is a combination of the physical, chemical and biological properties (Fig. 1.12) that are essential for plant growth, regulating and partitioning of surface to ground water, and buffering, detoxifying and scrubbing of hazardous chemicals. It is rather dynamic and can affect the sustainability and productivity of land use, furthermore it is increasingly proposed as an integrative indicator of environmental quality (National Research Council, 1993; Monreal et al., 1998), food security (Lal, 1999) and economic viability (Hillel, 1991).

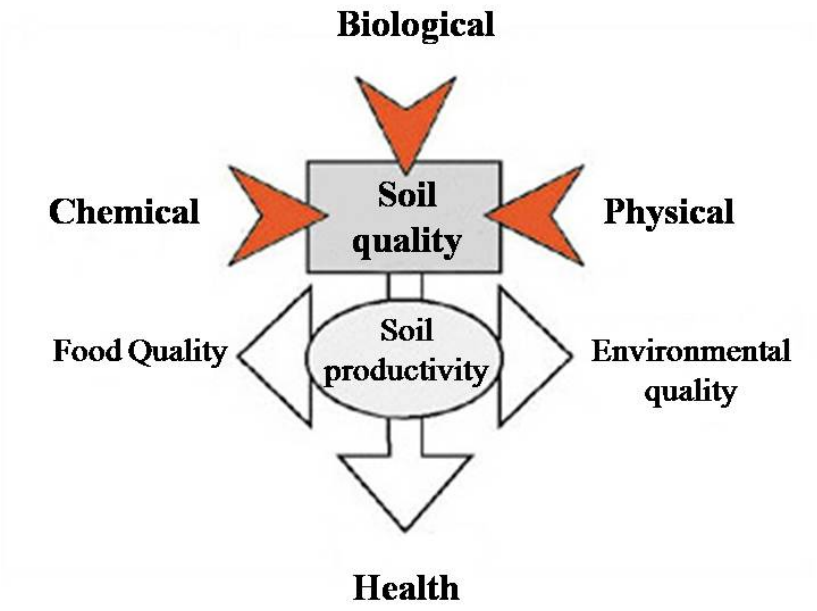

Fig. 1.12. Soil quality as affected by soil properties.

Basic soil quality indicators should (1) well correlate with ecosystem functions such as C and N cycling (Visser and Parkinson, 1992); (2) integrate soil physical, chemical, and biological properties and processes and serve as basic inputs needed for estimation of soil properties or functions which are more difficult to measure directly; (3) be measurable by as many users as possible and not limited to a select group of research scientists; (4) be applicable to field conditions, *i.e.* they should describe the major ecological processes in soil and ensure that measurements made reflect conditions as they exist in the field under a given management system; (5) be sensitive to variations in management and climate; and (6) be components of existing soil data bases where possible (Doran and Parkin, 1994). Quantifying these variables through long-term monitoring may lead to an understanding about the effects of land management practices and natural or human-caused disturbances on the soil component of ecosystems (Knoepp et al., 2000).

It is often difficult to separate soil functions into chemical, physical, and biological processes because of the dynamic, interactive nature of these processes (Schoenholtz et al., 2000). Because of this interactions, soil indices are extremely variable.

Many soil chemical properties directly influence microbiological processes (*e.g.* via nutrient and carbon supply), and these processes, together with soil physical-chemical processes, determine the capacity of soils to hold and supply nutrients cycles (including carbon), and the movement and availability of water. Therefore soil chemical indicators are used mostly in the context of nutrient relations and may also be referred to as 'indices of nutrient supply' (Powers et al., 1998).

By contrast, biological and biochemical properties, including soil respiration, microbial biomass and the activities of soil enzymes, are most useful for detecting the deterioration of soil quality (Visser and Parkinson, 1992) because of their importance in cycling of organic matter and regulating active nutrient pools in soils (Caravaca and Roldán, 2003).

The identification of biological indicators of soil quality is important because soil quality is strongly influenced by microorganism mediated processes (nutrient cycling, nutrient capacity, aggregate stability), whereby the key is to identifying those components that rapidly respond to changes in soil quality (Doran and Parkin, 1994). Nevertheless, there is the problem of knowing which indicator responds to a specific soil treatment or contaminant. Therefore, the use of multiple biological and biochemical properties is often suggested (Ros et al., 2006). General biochemical properties such as microbial biomass carbon (Brookes, 1995), or ecophysiological quotients such as qCO_2 and qD (Anderson and Domsch, 1993), as well as specific biochemical properties such as hydrolytic soil enzymes related to C, N and P cycles (Nannipieri et al., 1990) are

suggested. Some examples of bioindicators that have been tested as potential monitoring tools, especially for contaminants removal, are reported in Table 1.2.

One limitation in using biological assays for soil quality indication is the lack of standard methodology. Considerable variation exists among assay procedures used by various researchers, making actual activity comparisons between sites difficult. It was thus emphasized that if bioassays are to be used as soil quality indicators, soil sample pre-treatment, assay procedures and units of measurement must be standardized (Dick, 1994).

Table 1.2. Some examples of bioindicators used in monitoring hydrocarbons removal (adapted from Maila and Cloete, 2005).

Bioindicator	Pollutant specificity	Sensitivity and range tested	References
Enzymes			
Soil lipase	Diesel oil, mineral oil	Sensitive; up to 1 mg m^{-1} soil	Margesin et al., 1999, 2000;
Soil dehydrogenase	Crude oil and refined petroleum products	Moderately sensitive; 20-60% (w/w) oil/dry soil	Frankenberger and Johanson, 1983;
Urease and catalase	Mineral oil	Less sensitive; detectable at high TPH concentration (5000 mg kg^{-1} soil)	Margesin et al., 2000
Seed germination			
Prairie grass (Canada blue grass and slender wheatgrass)	Aromatics (Halogenated)	Sensitive; 13-133 µg kg^{-1} soil	Wang and Freemark, 1995; Siciliano et al., 1997
L. sativum	PAHs	Moderately sensitive, 50-100 mg kg^{-1} soil	Maila and Cloete, 2002
Microbial biomass	Oil contaminated soil	Moderately sensitive	Kandeler et al., 1994
Batteries of bioindicators			
Microbial bioluminescence, earthworm and seed germination	Creosote, heavy, medium and light crude oils	Moderately sensitive. Earthworm>seed germination>bioluminescence 25-17, 400 µg g^{-1} soil	Wang and Freemark, 1995; Dorn et al., 1998; Marwood et al., 1998; Phillips et al., 2000; Shakir et al., 2002

1.7.1. Soil enzymes

Nutrient cycling in soils involves biochemical, chemical and physico-chemical reactions, with biochemical processes being mediated by microorganisms, plant roots, and soil animals. It is well known that all biochemical reactions are catalysed by enzymes, which are proteins that act as catalysts without undergoing permanent alteration and causing chemical reactions to proceed at faster rates. In addition, they are specific for the types of chemical reactions in which they participate (Tabatabai, 1994).

Burns (1982) classified soil enzymes according to their location in soil (Fig. 1.13). Three main enzyme categories (termed *biotic enzymes*) are associated with viable proliferating cells. They are located: (i) intracellularly in cell cytoplasm, (ii) in the periplasmic space, and (iii) at the outer cell surfaces. Enzymes in the soil solution are generally short-lived because they are readily inactivated by physical adsorption, denaturation or degradation (Sarkar and Burns, 1984). The remaining categories are broadly characterized as *abiontic* (Skujinš, 1976). Abiontic enzymes are those exclusive of live cells that include enzymes excreted by living cells during cell growth and division from extant or lysed cells but whose original functional location was on or within the cell. Additionally, abiontic enzymes can exist as stabilized enzymes in two locations: adsorbed to internal or external clay surfaces, and complexed with humic colloids through adsorption, entrapment, or copolymerization during humic matter genesis (Boyd and Mortland, 1990).

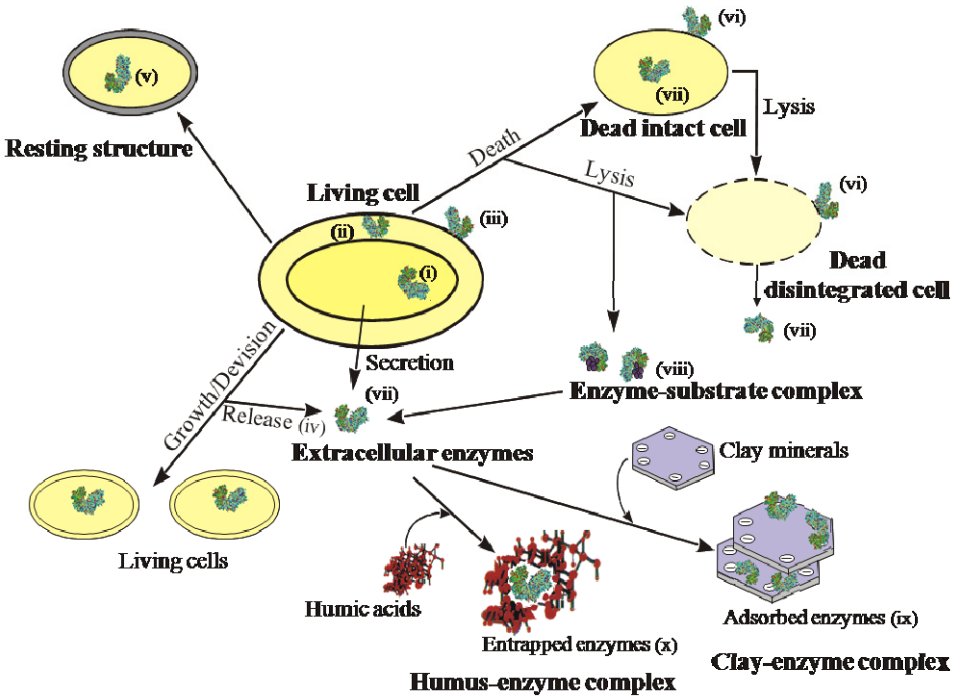

Fig. 1.13. Soil enzymes location. (i) Intracellular enzymes, (ii) periplasmic enzymes, (iii) enzymes attached to outer surface of cell membranes, (iv) enzymes released during cell growth and division, (v) enzymes within non-proliferating cells (spores, cysts, seeds, endospores), (vi) enzymes attached to dead cells and cell debris, (vii) enzymes leaking from intact cells or released from lysed cells (viii), enzymes temporarily associated in enzyme-substrate complexes, (ix) enzymes adsorbed to surfaces of clay minerals, (x) enzymes complexed with humic colloids (according to Burns, 1982 and Nannipieri, 1994).

Enzymes associated with humic substances and to a lesser extent with clay particulates are protected against thermal denaturation, proteolysis, dehydration or decomposition and are part of a persistent extracellular enzyme pool that is independent of the existing microbiota (Burns, 1982; Sarkar and Burns, 1984; Miller and Dick, 1995). The humic-enzyme fractions retain the original properties of the enzymes (Busto and Perez-Mateos, 1995) as stable enzyme-organic matter complexes and they were found to allow diffusion of substrates to the active enzyme site (Burns, 1982). Therefore soil can be considered as a sink and source of indigenous and persistent enzymatic capacity which is independent of current or recent

microbial and plant activity (Galstian, 1974; Burns, 1986; Lähdesmäki and Piispanen, 1992; Busto and Perez-Mateos, 1995). Moreover, the enzymatic activity of a soil is conditioned by land use history since enzymes are produced by living organisms which contribute to the biological soil formation.

The activity and stability of enzymes in soil is regulated by pH (Frankenberger and Johanson, 1983; Trasar-Cepeda and Gil-Sotres, 1987; Dick et al., 1988), microbial biomass (Häussling and Marschner, 1989; Saffigna et al., 1989; Carter, 1991; Srivastava and Singh, 1991), vegetation (Juma and Tabatabai, 1978; Harrison, 1983; Perucci et al., 1984; Helal and Sauerbeck, 1987; Tarafdar and Jungk, 1987), soil and crop management practices (Perucci and Scarponi, 1985; Beck, 1990; Martens et al., 1992; Kandeler and Eder, 1993), soil organic matter (Juma and Tabatabai, 1978; Chhonkar and Tarafdar, 1984; Sparling et al., 1986), clay minerals (Makboul and Ottow, 1979; Huang et al., 1995) and to the soil moisture content (Harrison, 1983; West et al., 1988a,b).

Enzyme activities are an important index of the biological activity of a soil because they are involved in the dynamics of soil nutrient cycling and energy transfer. Indeed, they reflect the intensity and direction of biochemical processes in the soil matrix. Hence, their activity indicates the biological capacity of a soil to carry out the biochemical processes which are important to maintaining the soil fertility (Galstian, 1974; Dkhar and Mishra, 1983; Burns, 1986; Garcia et al., 1994) as soil fertility depends not only on nutrient status and availability but also on the turnover of N, P and other nutrients (Lopez-Hernandez et al., 1989). Actually, enzymatic processes are closely associated with soil fertility as they mediate the conversion of unavailable forms of nutrients to forms that are readily assimilable by plants and microbial biomass (Sarathchandra et al., 1984;

Dick et al., 1988; Sarkar et al., 1989; Dick, 1992; Martens et al., 1992; Sinsabaugh, 1994).

Soil enzymes also participate in the decomposition and synthesis of organic substances and are important for the formation of recalcitrant organic molecules (Galstian, 1974; Martens et al., 1992).

As enzymes do not react readily to environmental changes like the soil microbial biomass, their activity is a more stable indicator of biological processes (Galstian, 1974).

1.7.1.1. Soil enzymes as indicators of pollution

Soil enzymes are the catalysts not only of important metabolic process functions but also of decomposition of organic inputs and detoxification of xenobiotics. Such compounds are of crucial concern in the soil environment as they could affect many biological and biochemical reactions in soils (Dick, 1997).

Pollution indicators should possess the following attributes (Doran and Parkin, 1994; Elliott, 1997):

1. sensitivity to the presence of pollutant;
2. ability to reflect different levels of pollution;
3. constancy in the response to any given pollutant;
4. sensitivity to the greatest possible number of pollutants;
5. discriminating between the effect of the pollutant and any prior degradation of the polluted soil;
6. differentiating among pollutants according to the different degrees of soil degradation they cause.

Although their use has been confined to laboratory studies, many enzymes have been tested for their potential to monitor pollutants removal, such as

pesticides, heavy metals, hydrocarbons and other industrial and agricultural chemicals.

Pesticides including herbicides, fungicides, etc., introduced into the soil environment have potential to affect non-target organisms and soil biochemical processes (Dick, 1997). In soils, pesticides are subjected to (i) biodegradation, (ii) cometabolism, (iii) polymerization and (iv) accumulation in microorganisms (Bollag and Liu, 1990). They can be also sorbed by clay and metal oxide surface and by humic substances, undergoing to abiontic reactions.

Several investigations have been devoted to study the effects of various pesticides on the activities of enzymes in soils from different origin (Gianfreda and Rao, 2008). If recommended field application rates are used, inhibitory results are temporary, and enzyme activities return to levels similar to those in untreated soils in a few weeks or months. When pesticides are applied to soils at very high concentrations such as when there is an accidental spill, enzymes activities are significantly affected.

Heavy metals can have toxic effects on soil biology and soil biochemical processes. Enzyme reactions are inhibited by metals (i) through complexation on the substrate, (ii) by combining with the protein-active groups of the enzymes, or (iii) by reacting with the enzyme-substrate complex. The oxidation state of the metal (Ross et al., 1981) and the soil type (Speir et al., 1992) are important factors in determining the toxicity of heavy metals on soil enzymes.

Soil enzyme activities hold potential also for assessing the impact of hydrocarbons on soils and the effectiveness of remediation processes (Dick, 1997; Maila and Cloete, 2005) because of their central role in the degradation of these molecules in water and carbon dioxide.

Therefore, as indicators of soil quality enzymes can provide information about the progress of remediation operations or the sustainability of particular types of land management (Schloter et al., 2003).

In considering soil enzymes as an indicator of soil quality, which enzymes are important? The soil enzymes most frequently studied are oxidoreductases, transferases and hydrolases. Among oxidoreductases, dehydrogenase is the most studied partly because of its apparent role in the oxidation of organic matter where it transfers hydrogen from substrates to acceptors. Although dehydrogenase activity depends on the total metabolic activity of the viable microbial populations, and should exist only in integral parts of intact cells, it has not always reflected total numbers of viable microorganisms isolated on a particular medium or with oxygen consumption or CO_2 evolution (Skujinš, 1976). Lots of compounds can act as alternative hydrogen acceptors instead of the oxygen, for example the extracellular phenol oxidase existing in soil (Howard, 1972), some anions in soil, such as nitrate (Bremner and Tabatabai, 1973), and Cu, naturally present in soils or in contaminated amendments (Chander and Brookes, 1991). Some hydrolases and transferases have been extensively studied because of their role in decomposition of various organic compounds, and thus are important in nutrient cycling and formation of soil organic matter. These would include enzymes involved in the C cycle (amylases, cellulases, lipases, glucosidases, and invertase), the N cycle (proteases, amidases, urease, and deaminases), the P cycle (phosphatases) and the S cycle (arylsulphatase) (Dick, 1994).

1.7.2. Soil microbial biomass

The soil microbial biomass (MB) can be defined as organisms living in soil that are generally smaller than approximately 10 μm (Schloter et al., 2003).

It is made up of bacteria, fungi, actinomycetes, algae, protozoa and some nematodes, and is estimated to contribute about ¼ of the total biomass on earth (Pankhurst et al., 1995; Roper and Gupta, 1995). Measurements of the carbon (C), nitrogen (N), phosphorus (P), and sulphur (S) contained in the soil microbial biomass provide a basis for studies of the formation and turnover of soil organic matter, as the microbial biomass is one of the key definable fractions (Brookes et al., 1990). The data can be used for assessing changes in soil organic matter caused by soil management (Powlson et al., 1987) and tillage practices (Spedding et al., 2004), for assessing the impact of management on soil strength and porosity, soil structure and aggregate stability (Hernández-Hernández and López-Hernandez, 2002), and for assessing soil N fertility status (Elliot et al., 1996). Because it is such a sensitive indicator of changing soil conditions, the soil microbial biomass as an 'early warning' of effects of stresses on the soil ecosystem and contributes to the maintenance of soil fertility and soil quality in both natural and managed terrestrial ecosystems (Turco et al., 1994; Elliott et al., 1996).

Soil microbial biomass measurements have been used for determining the effects of environmental contaminants like heavy metals (Renella et al., 2004), pesticides (Harden et al., 1993), and antibiotics (Castro et al., 2002) on the soil ecosystem, and to monitor bioremediation of oil-contaminated soils (Plante and Voroney, 1998).

Microbial biomass content is an integrative signal of the microbial significance in soils because it is one of the few fractions of soil organic matter (SOM), biologically meaningful, sensitive to management or pollution and finally measurable (Powlson, 1994).

The quality and quantity of the organic matter of soils normally changes at slow rates which are difficult to detect in the short term because of the

large pool-size of organic matter and the spatial variability of soils. However, the soil microbial biomass, as active fraction of the organic matter, responds much more rapidly than soil organic matter as a whole to changes in management, climate, etc. For that reason, soil microbial biomass and the ratio between microbial biomass and SOM has been proposed as an indicator of the state and changes of total soil organic matter (Dick, 1992; Powlson, 1994; Pankhurst and Lynch, 1995; Pankhurst et al., 1995). Soil microorganisms are continually changing and adapting to changes in the environment. This dynamic nature makes them a sensitive indicator to assess changes and to predict long-term effects of changes in soil resulting from management practices (Kennedy and Papendick, 1995; Kennedy and Smith, 1995).

Soil microorganisms control many key processes in soils, thus contributing to the maintenance of soil quality, and are involved in the decomposition and accumulation of SOM, nearly all mineral nutrient transformations in soils related to plant nutrition and soil fertility (Apsimon et al., 1990; Kennedy and Papendick, 1995; Pankhurst et al., 1995).

Soil microbial biomass also serves as a source and sink for mineral nutrients and organic substrates in the short term, and as a catalyst to convert plant nutrients from stable organic forms to available mineral forms over longer periods (McGill et al., 1986). Finally, the microbial biomass is releasing and containing enzymes which are responsible for nutrient cycling (Saffigna et al., 1989; Carter, 1991; Ocio et al., 1991; Srivastava and Singh, 1991).

The size and activity of the microbial biomass is regulated by the soil organic matter quantity and quality and has been related to climatic conditions (Insam, 1990), soil moisture content (Van Veen et al., 1985; Doran et al., 1990; Van Gestel et al., 1996), soil temperature (Joergensen et

al., 1990), soil pH (Jenkinson and Powlson, 1976; Roper and Gupta, 1995), soil structure and texture (Jocteur-Monrozier et al., 1992; Ladd, 1992) and to soil and crop management practices (Aoyama and Nozawa, 1990; Ocio et al., 1991; Amato and Ladd, 1992; Mueller et al., 1992; Ritz et al., 1992; Srivastava and Lal, 1994).

1.7.3. Microbial activity

Soil microbial activity leads to the liberation of nutrients available for plants but also to the mineralization and mobilization of pollutants and xenobiotics. Thus microbial activity is of crucial importance in biogeochemical cycling. Microbial activities are regulated by nutritional conditions, temperature and water availability as well as by proton concentrations and oxygen supply.

There is some concern with the use of microorganisms as bioindicators. Indeed, changes in bacterial numbers might be indicative of a stimulated biodegradation process, but they do not necessarily represent an accurate measurement of the actual biodegradation. Microbial processes have been used in monitoring of both hydrocarbons and pesticides removal from soils (Wünsche et al., 1995; Margesin et al., 1999; Top et al., 1999). Respiration is the most widely used in detecting biotoxicity and biodegradation of contaminants (Martin et al., 1978; Weissenfels et al., 1992; Margesin et al., 2000). However, this process cannot be reliably used to monitor hydrocarbons removal, as it is difficult to distinguish biological hydrocarbons removal from decomposition of other soil organic compounds simultaneously present in the soil (Maila and Cloete, 2005). Moreover, it suffers in separating the activity of microorganisms and other organisms such as plants, which vary significantly in different systems and throughout the season (Dilly et al., 2000). However, soil microbial activity

can be estimated using two groups of microbiological approaches. At first, experiments in the field that often require long periods of incubation (Hatch et al., 1991; Alves et al., 1993) before significant changes of product concentrations are detected, i.e. 4-8 weeks for the estimation of net N mineralization. In this case, variations of soil conditions during the experiment are inevitable, i.e. aeration, and may influence the results (Madsen, 1996). By contrast short-term laboratory procedures are usually carried out with sieved samples at standardized temperature, water content and pH value. Short-term designs of 2-5 h minimize changes in biomass structure during the experiments (Brock and Madigan, 1991). Laboratory methods have the advantage in standardizing environmental factors and, thus, allowing the comparison of soils from different geographical locations and environmental conditions and also results from different laboratories. Laboratory results refer to microbial capabilities, as they are determined under optimized conditions of one or more factors, such as temperature, water availability and/or substrate (Schloter et al., 2003).

1.7.4. C_{mic}/C_{org} ratio

The ratio of microbial biomass-C to soil organic-C (C_{mic}/C_{org}) is the microbial-C content per unit of soil carbon (Anderson and Domsch, 1989; Sparling, 1992). The ratio has been proved to be a sensitive indicator of quantitative changes in soil organic matter due to the changing of management conditions and climate (Anderson and Domsch, 1989; Insam et al., 1989). Soils that exhibit a ratio higher or lower than proposed equilibrium values, ranging from 0.27 to over 7.0% (Anderson and Domsch, 1980; Adams and Laughlin, 1981; Brookes et al., 1984; Woods and Schuman, 1986; Sørensen, 1987; Insam et al., 1989), would therefore be accumulating or loosing C, respectively (Anderson and Domsch, 1989).

Thus, the ratio of biomass-C to total organic C (C_{mic}/C_{org}) will increase for a time if the input of organic matter to a soil is increased and decreases for a time if the input is decreased (Anderson and Domsch, 1989). Constancy of the C_{mic}/C_{org} ratio is thus an indication of a system at a new equilibrium. However, to establish whether the C_{mic}/C_{org} ratio of a soil is in equilibrium, thus whether a soil has achieved equilibrium in organic matter status, it will be necessary to establish a baseline or reference values for each soil and a set of conditions to which the tested soil can be compared (Sparling, 1992). One problem associated with the C_{mic}/C_{org} ratio is that both components have a common origin, and are not independent of each other. Also, changes in organic carbon will impact more on the ratio than changes in microbial biomass since the former is quantitatively much more abundant.

1.7.5. Seed germination

Various authors have reported from time to time different biomonitoring indices based on germination and seedling growth to indicate both heavy metals and organic contamination (Abdul-Baki and Anderson, 1973; Chou et al., 1978; Mhatre and Chaphekar, 1982). Seed germination assays are sensitive to changes in soil toxicity during bioremediation of soil contaminated with polycyclic aromatic hydrocarbons (PAHs) or petroleum hydrocarbons (Athey et al., 1989; Dorn et al., 1998; Marwood et al., 1998; Henner et al., 1999; Knoke et al., 1999; Rezek et al., 2008). Bioindicators response to organic pollutants usually varies in different plant species. Consequently the use of tests with more than one vegetal species could be recommended.

Because of their simple methodology and potential for use both *in situ* and *ex situ* they are useful as bioindicator response endpoints. Nevertheless, the

application of these tests as potential bioindicators has been confined to laboratory-scale studies (Maila and Cloete, 2005).

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Chapter 2

Aims

Contamination of soils, groundwater, sediments, surface water, and air with hazardous and toxic chemicals is one of the major problems facing the industrialized world today. The need to remediate these sites has led to the development of new technologies that emphasize the destruction of the pollutants rather than the conventional approach of disposal.

Bioremediation, i.e. the use of microorganisms or microbial processes to degrade environmental contaminants, is among these new technologies. Bioremediation has numerous applications, including clean-up of groundwater, soils, lagoons, sludge, and process-waste streams. One important characteristic of bioremediation is that it is carried out in non-sterile open environments that contain a variety of organisms. Of these, bacteria, such as those capable of degrading pollutants, usually have central roles in bioremediation, whereas other organisms (*e.g.* fungi and grazing protozoa) also affect the process.

Several microbial and chemical transformations may occur during bioremediation, thereby producing a variety of breakdown products. Identification and analysis of these products can be difficult and time-consuming and may not provide any indication on the impact of either the presence of the contaminant or of its transformation products on soil health and quality. By contrast, soil biological and biochemical properties are highly sensitive to changes caused by management practices and environmental stress, and may provide an early warning of soil quality changes. Therefore determination of the quality-related properties of soil may serve, along with other specific indicators, to assess soil status, quality

and productivity and provide an estimate of successful soil reclamation processes.

The present work has had two different aims:

1) The response of an agricultural soil to fresh organic contamination has been studied in terms of effects of the selected contaminants on the main chemical, biochemical and biological soil properties, in a long-term experiment.

The contaminants studied have been chosen to better represent the class of POPs, known as recalcitrant and hazardous compounds. In particular, PAHs and chlorophenols have been investigated, and phenanthrene and pentachlorophenol have been, respectively, selected as their representative model compounds.

2) Various bioremediation techniques have been tested to remediate the artificially contaminated soil. These processes have been approached by using both inoculation of microorganisms, able to degrade the target contaminant (*bioaugmentation*), and addition of nutrients, such as compost, to enhance the attenuation process naturally occurring in the soil (*biostimulation*), and of dissolved organic matter, to improve the desorption and solubility of the organic contaminants.

Chapter 3

Strategies for bioremediation of an artificially Phe-contaminated soil[1]

Polycyclic aromatic hydrocarbons (PAHs) are highly recalcitrant widespread environmental pollutants. Bioremediation, accomplished by the introduction of PAH-degrading microorganisms (*bioaugmentation*) and/or by applying additional nutrients (*biostimulation*) into a contaminated system is a valuable alternative to traditional chemical and physical treatments for the decontamination of PAH-contaminated soils.

The work reported in this Chapter has been focused on the fate of phenanthrene (Phe), selected to represent PAHs, when added to a fresh, agricultural soil with no history of PAH contamination. The relative effect of compost (C), applied at two different doses, and the efficiency of a Phe-degrading bacterial culture inoculated into the soil (S) and soil-compost (S-C1 and S-C2) systems have been investigated.

Changes in various functionally related properties such as microbial biomass, basal respiration, and soil hydrolases and oxido-reductases activities were measured over time. The variations of the main physical and chemical properties have been also monitored.

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[1]A version of this Chapter has been published as:

Scelza R., Rao M.A., Gianfreda L., 2007. Effects of compost and of bacterial cells on the decontamination and the chemical and biological properties of an agricultural soil artificially contaminated with phenanthrene. *Soil Biology & Biochemistry* 39, 1303-1317.

The soil has showed an intrinsic capability for degrading Phe, enhanced and stimulated by the lower compost dose. A simultaneous, rapid increase of soil respiration and microbial biomass, and higher phosphatase and arylsulphatase activities were measured, suggesting that microbial growth and activity had increased. Phe degradation was accelerated immediately after inoculum with Phe-degrading culture. Several of the soil properties showed differentiated responses to the presence of the Phe, the compost and/or the exogenous culture. In particular, soil systems with and without the inoculated cells showed similar trends for several of the measured enzymatic properties (*e.g.* phosphatase, arylsulphatase, β-glucosidase and urease activities), indicating that the intrinsic soil enzymatic activity was not affected by the exogenous microorganisms. Temporary and permanent changes were observed for several of the properties investigated, thereby providing useful information on the impact of Phe on soil metabolic activity.

3.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are ubiquitous, organic soil contaminants with hydrophobic and carcinogenic properties (Belkin et al., 1994). One of the main reasons for the prolonged persistence of hydrophobic hydrocarbons in the environments is their low water solubility which increases their sorption to soil particles and limits their availability to biodegrading microorganisms (Cerniglia, 1993). The decontamination of PAH polluted sites is mandatory because many PAH compounds are known or suspected to be toxic, mutagenic or carcinogenic (Patnaik, 1992). As widely described in Chapter 1, possible fates of PAHs in the environment include volatilization, photo-oxidation, chemical oxidation, bioaccumulation, adsorption to soil particles, leaching and microbial degradation (Fig. 3.1).

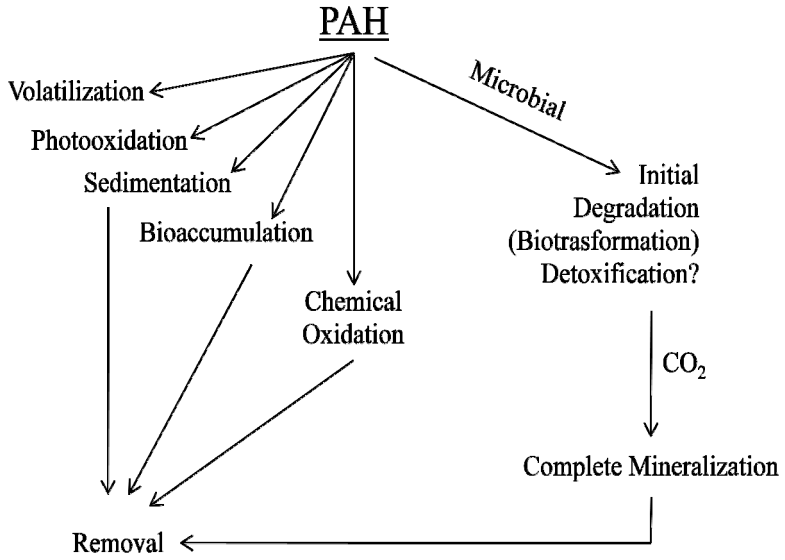

Fig. 3.1. Schematic representation of the environmental fate of polycyclic aromatic hydrocarbons (from Cerniglia, 1992).

However, microbiological degradation is the major process that results in the decontamination of sediment and surface soil (Sims et al., 1990). PAHs can be totally degraded (mineralized) or be partially transformed by either a

community of microorganisms or by a single microorganism (Cerniglia, 1984; Gibson and Subramanian, 1984; Cerniglia and Heitkamp, 1989). A wide variety of fungi, algae and especially bacteria (Table 3.1) have the ability to metabolize PAHs. Generally, the rate of degradation of PAHs is inversely proportional to the number of rings in the PAH molecule. Thus, the lower weight PAHs are biodegraded more rapidly than the higher weight compounds (Cerniglia and Heitkamp, 1989). The microbial degradation of PAHs such as naphthalene, phenanthrene, anthracene and acenaphthene has been well documented and the biochemical pathways have been elucidated (Cerniglia, 1984; Gibson and Subramanian, 1984; Schocken and Gibson, 1984; Pothuluri et al., 1992).

Table 3.1. PAHs oxidized by different species of bacteria (adapted from Cerniglia, 1992).

Compound	Organisms	Reference
Naphthalene	*Acinetobacter calcoaceticus, Alcaligenes denitrificans, Mycobacterium* sp., *Pseudomonas* sp., *Pseudomonas putida, Pseudornonas fluorescens, Pseudomonas paucimobitis, Pseudomonas vesicularis, Pseudornonas cepacia, Pseudomonas testosteroni, Rhodococcus* sp., *Corynebacteriurn renale, Moraxella* sp., *Streptornyces* sp., *Bacillus cereus*	Ryu et al., 1989; Weissenfels et al., 1990, 1991; Kelley et al., 1991; Dunn and Gunsalus, 1973; Davies and Evans, 1964; Foght and Westlake, 1988; Jeffrey et al., 1975; Mueller et al., 1990; Kuhm et al., 1991; Walter et al., 1991; Dua and Meera, 1981; Tagger et al., 1990; Garcia-Valdes et al., 1988; Trower et al., 1988; Grund et al., 1992; Cerniglia et al., 1984; Barnsley, 1983.
Phenanthrene	*Aeromonas* sp., *Alcaligenes faecalis*, *Alcaligenes denitrificans*, *Arthrobacter polychromogenes*, *Beijerinckia* sp., *Micrococcus* sp., *Mycobacterium* sp., *Pseudomonas putida*, *Pseudomonas paucimobilis*, *Rhodococcus* sp., *Vibrio* sp., *Nocardia* sp., *Flavobacterium* sp., *Streptomyces* sp., *Streptomyces griseus*, *Acinetobacter* sp.	Kiyohara et al., 1976, 1982, 1990; Weissenfels et al., 1990, 1991; Keuth and Rehm, 1991; Jerina et al., 1976; Colla et al., 1959; West et al., 1984; Kiyohara and Nagao, 1978; Heitkamp and Cerniglia, 1988; Guerin and Jones, 1988, 1989; Treccani et al., 1954; Evans et al., 1965; Foght and Westlake, 1988; Mueller et al., 1990; Sutherland et al., 1990; Ghosh and Mishra, 1983; Savino and Lollini, 1977; Trower et al., 1988; Barnsley, 1983.
Benzo[a]pyrene	*Beijerinckia* sp., *Mycobacterium* sp.	Gibson et al., 1975; Heitkamp and Cerniglia, 1988; Grosser et al., 1991.

Biodegradation mechanisms require the presence of molecular oxygen to initiate enzymatic attack on the PAH rings (Gibson et al., 1968; Dagley,

1971, 1975; Chapman, 1979; Gibson and Subramanian, 1984). Both atoms of oxygen molecule are incorporated into the aromatic ring to form *cis*-dihydrodiol. This initial hydroxylation step of unsubstituted PAHs is catalysed by a dioxygenase (Fig. 3.2). Since PAHs, such as phenanthrene, pyrene, benzo[a]pyrene and benz[a]anthracene, are complex fused ring structures, bacteria metabolize PAHs at multiple sites to form isomeric *cis*-dihydrodiols (Mueller et al., 1996). Monooxygenases have also been shown to be involved in oxidation to form *trans*-dihydrodiols (Heitkamp et al., 1988; Kelley et al., 1991). The *cis*-dihydrodiols undergo re-aromatization by dehydrogenases to form dihydroxylated intermediates (Patel and Gibson, 1974).

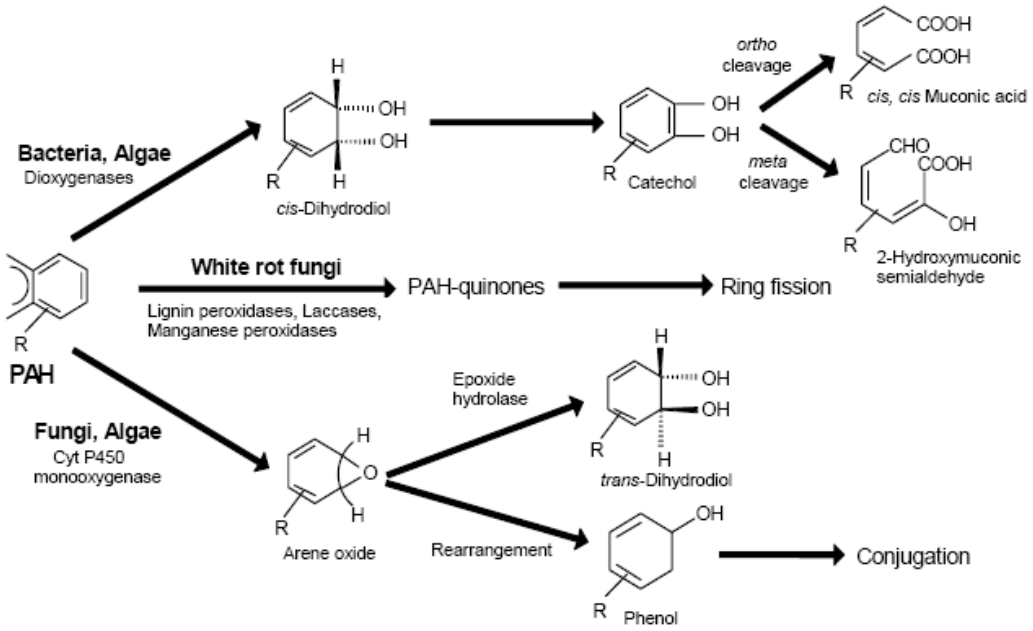

Fig. 3.2. Major pathways involved in the metabolism of polycyclic aromatic hydrocarbons by bacteria, fungi and algae (adapted from Mueller et al., 1996).

Further, catabolism involves ring cleavage by dioxygenases to form aliphatic intermediates. Cleavage of these *ortho*-dihydroxylated intermediates occurs either between the two hydroxyl groups (intradiol or

ortho-fission) or adjacent to one of the hydroxyl groups (extradiol or *meta* fission) (Mueller et al., 1996). There are different enzymes for different ring fission substrates, each forming a different aliphatic product. The aromatic ring dioxygenases are multi-component enzymes which consist of a reductase, a ferredoxin and a third component consisting of two proteins, large and small iron sulfur protein subunits (Ensley and Gibson, 1983; Suen and Gibson, 1993; Suen et al., 1996).

Bacterial genera, capable of degrading PAHs commonly, include species of *Pseudomonas*, *Alcaligenes*, *Rhodococcus*, *Sphingomonas,* and *Mycobacterium* (Labana et al., 2005). Most of these bacteria have been enriched based on their ability to grow on low molecular PAHs such as naphthalene, phenanthrene, fluorene, anthracene and acenaphthene.

The rate and extent of biodegradation of PAHs in soils and sediments is affected by multiple factors (Table 3.2).

Table 3.2. Factors affecting bioremediation of PAH-contaminated sites (from Labana et al., 2005).

Physico-chemical factors	Biological factors	Environmental factors
Physical/chemical properties of PAHs (number of rings, log K_{ow})	Characteristics of the microbial population (diversity, genetic/catabolic potential)	Temperature
Organic content of soil		Moisture
Structure/particle size of soil		pH
Presence of contaminants		Sorption
		Degree of contamination

The major factor limiting the bioremediation of soils and sediments contaminated with PAHs is the poor availability of these hydrophobic contaminants to microorganisms (Mihelcic et al., 1993; Hughes et al., 1997). Bioavailability may be the most important factor in determining the feasibility of bioremediation of PAHs.

Generally, the natural biodegradation of contaminants in soil is slow and there is a requirement to accelerate its rate. This can be accomplished by two main techniques: *bioaugmentation* and *biostimulation*.

3.2. Bioaugmentation

Bioaugmentation (Fig. 3.3) is defined as the addition of indigenous, exogenous or genetically modified organisms, generally microorganisms, to polluted sites in order to accelerate the removal of the target toxic molecules (Odokuma and Dickson, 2003). There have been numerous reports on feasibility and field application of bioaugmentation as a remediation technology (Vogel, 1996).

Indigenous microorganisms are those that occur naturally at a site. They are usually present in very small quantities. They are often better distributed, in general, than added microorganisms, although not necessarily with regard to the target pollutant.

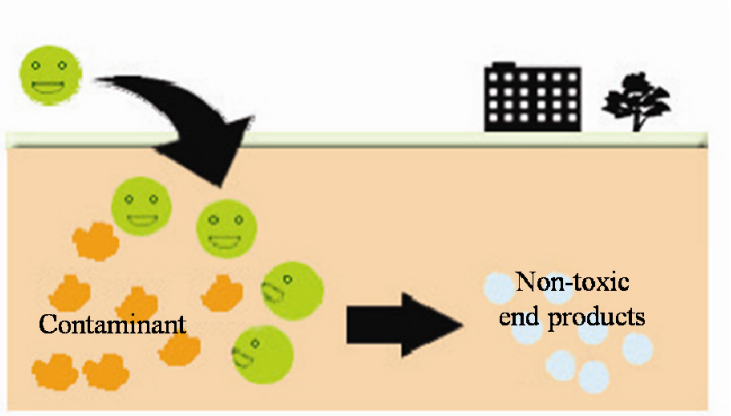

Fig. 3.3. Bioaugmentation.

Microbial exogenous inocula are prepared in the laboratory from soil or groundwater either from the site where they are to be used or from another site where the biodegradation of the chemicals of interest is known to be

occurring. Microbes from the soil or groundwater are isolated and are added to media containing the chemicals to be degraded. Only microbes capable of metabolizing the chemicals will grow on the media. This process isolates the microbial population of interest, which may contain several different strains of microbes. The isolated microbes can then be propagated in a nutrient medium and concentrated to produce an inoculum.

Using native soils has the advantage that the microbes are more likely to survive and propagate when re-injected at the site (http://www.craworld.com). The direct enrichment has several advantages over isolating and culturing microorganisms, in fact, many species, which are not cultivable but may be of importance in degradation of chemicals in natural environment, can be enriched.

While the use of pure culture of isolated strains can be associated with the accumulation of partial degradation products which might be more toxic than parent materials, mixed consortia are more likely to completely degrade the target compounds. Mixed consortia have also been known to have the advantage of being more resistant to natural environmental conditions and predation (Fewson, 1988), compared to pure cultures which often fail to generate the desired activities when released to the environments.

Using microbes from a different site has the advantage that they are known to biodegrade the chemicals of concern. However, there is a possibility that these microbes will not be able to adapt to their new environment and will not propagate. Typically the microbes will adapt if the new environment is similar to their native environment.

Genetic engineering has been used to confer new functions to microorganisms and to enhance their catabolic activities. The microorganisms constructed are called genetically engineered

microorganisms (GEMs). It also provides microorganisms with multiple metabolic activities in single strain, which is often required for degradation of specific pollutants. Bioaugmentation of contaminated sites with microbes that are genetically engineered for degradation of specific compounds is an area that is currently being explored as a cleanup option.

Successful application of bioaugmentation techniques is dependent on the identification and isolation of appropriate microbial strains, and their subsequent survival and activity, once released into the target habitat, on the nature of the xenobiotics, the physico-chemical conditions and the metabolic potential of the microflora.

A key factor involved in the lack of success in bioaugmentation is the rapid decline of the population size of the introduced cells (Ramos et al., 1991; Thiem et al., 1994). Factors governing the fate of introduced microorganisms in various environments include physical-chemical parameters (Evans et al., 1993; Shonnard et al., 1994), nutrient availability (Goldstein et al., 1985; Fujita et al., 1994; Wilson and Lindow, 1995; Watanabe et al., 1998) and the existence of microniches (Postma et al., 1990). The introduced strain may face intense competition, predation or parasitism in sewage, natural water or soil. According to Blasco et al. (1997) accumulation of toxic intermediates or end-products of pollutants by members of indigenous bacteria also has negative effects on the survival of introduced microorganisms. The competition can be controlled by adding specific nutrients that inoculants can utilize (Ogunseitan et al., 1991; van Veen et al., 1997) or by changing operation parameters (Fujita et al., 1994; Blumenroth and Wagner-Döbber, 1998). In addition, it has been reported that survival of bacteria added to soil was improved by pre-adaptation of the strains on a minimal medium with soil extract (Timmis, 1997) or by starvation in an inorganic medium (van Elsas et al., 1994; Watanabe et al.,

2000). However, good survival and growing capabilities of the incorporated strains do not always insure the breakdown of the xenobiotic (Lewis et al., 1986; McClure et al., 1991).

3.3. Biostimulation

Biostimulation (Fig. 3.4), i.e. the artificial creation of an environment that promotes the growth of naturally occurring microorganisms capable of degrading the target contaminants, is the method in which biodegradation by indigenous microorganisms is stimulated and the reaction rates are increased.

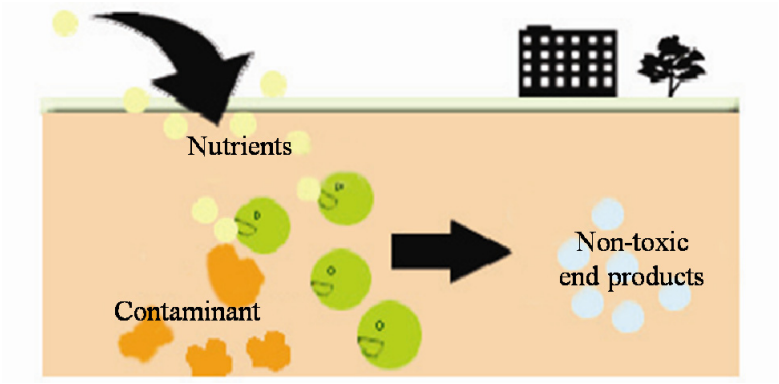

Fig. 3.4. Biostimulation.

This option is adopted when there are indigenous microbes with degradation capacity but natural degradation does not occur or the degradation is too slow. Biostimulation includes supplying the environment with nutrients such as carbon, nitrogen, phosphorus, or other substrates. Various additional nutrient sources such as inorganic fertilizers, urea, sawdust, compost, manure, and biosolids have been used (Rosenberg et al., 1992; Walworth and Reynolds, 1995; Cho et al., 1997; Williams et al., 1999; Namkoong et al., 2002). In particular, composts have enormous potential for bioremediation, as they are capable of sustaining diverse

populations of microorganisms, all with the potential to degrade a variety of organic contaminants, including polycyclic aromatic hydrocarbons (Kästner et al., 1995; Kästner and Mahro, 1996; Namkoong et al., 2002).

Several studies (Kästner and Mahro, 1996; Puglisi et al., 2007) demonstrated that bioavailability of phenanthrene is significantly reduced in soils amended with compost, but, at the same time, the degradation of the contaminant is enhanced by the microflora present in the compost, and this process is kinetically more important. Thus, compost addition is an important means by which the degradation of easily degradable compounds in soils may be induced, providing that suitable bacteria are already present. Alternatively, for more recalcitrant PAHs, compost may represent a way to reduce their bioavailability by retaining them in the aged fraction, since sorption of organic compounds increase with the content of organic matter (Means et al., 1980; Hassett and Banwart, 1989).

Organic amendments activate the autochthonous microorganisms of the soil, and indirectly stimulate the biogeochemical cycles therein (Pascual et al., 1997). Moreover, they provide various minerals (*e.g.* N, P, and S) essential for plant nutrition. They also increase the soil organic matter content and microbial biomass, and influence soil structure and many other related physical, chemical and biological properties (Perucci, 1992; Jörgensen et al., 1996; Ros et al., 2006; López-Piñeiro et al., 2007), as well as soil fertility (Clark et al., 2007). Therefore composts are considered also soil ameliorants, especially those obtained from food and vegetable residues (Adani et al., 2006), because they have been shown to be very effective in changing physico-chemical parameters of soil, such as pH, moisture content, and soil structure. Furthermore, land application of products from organic wastes, such as composts and bio-fertilizers, is gaining importance all over Europe, as integrated and biological agriculture

are becoming increasingly popular. This is because such products are often considered beneficial for the soil and at the same time the problem of organic waste streams is alleviated, resulting in an environmentally acceptable way of recycling waste materials (Lalande et al., 2000; Masciandaro et al., 2000).

Biostimulation can occur simultaneously to the bioaugmentation process in case of amendments using active organic residues that may contain microbial strains capable of metabolizing pollutants. In this respect, the presence of biopolymers (cellulose, hemicellulose and lignin) in sewage sludge composts (Li et al., 2001) and decaying rice straw (Glissmann and Conrad, 2000) may pave the way for a possible induced degradation of PAHs in soil.

3.4. Bioaugmentation vs. Biostimulation

Bioaugmentation has several advantages over biostimulation. A concentrated and specialized population of specific microbes is injected and can begin degrading contaminants immediately. Biostimulation is dependent on appropriate indigenous microbial population and organic material present and, furthermore, there is a delay after injection of nutrients as the microbial population propagates. Also, the nutrients are not specific and all microbes present at the site will potentially propagate, diluting the effect of the nutrients.

Bioaugmentation can be applied with minimal cost, disruption and time, while testing the ability of indigenous microbes can require complex, potentially costly, analytical methods to measure density and nutrient content.

Biostimulation modifies the environment to enhance the growth of indigenous microbes and, differently from bioaugmentation, sites cannot

always be cleaned and closed rapidly within budget amounts and under target dates (www.obio.com).

3.5. Case study

This study reports the decontamination and the chemical and biochemical properties of an agricultural soil artificially contaminated with phenanthrene, with and without biostimulation and bioaugmentation treatments.

Phenanthrene has been selected as representative of polycyclic aromatic hydrocarbons. Phenanthrene, a three ringed PAH, is an ideal model system to study various aspects of microbial metabolism and physiology. Furthermore, since it is the smallest aromatic hydrocarbon to have a 'bay-region' and a 'K-region' (Cerniglia and Yang, 1984), phenanthrene is often used as a model substrate for studies on the metabolism of other PAHs with carcinogenic properties and on the bioavailability and transformation of PAHs in soil (Smith et al., 1997; Ortega-Calvo and Saiz-Jimenez, 1998; Nam and Alexander, 2001). Moreover, it is suitable for laboratory experiments because it is easier to handle and is safer than its higher congeners because of its relatively low toxicity levels.

The effectiveness of biostimulation and bioaugmentation processes in the removal of the phenanthrene has been monitored in a long-term experiment. In particular, in the bioaugmentation process, a mixed Phe-degrading microbial culture has been used as an exogenous microbial inoculum. The microbial culture was isolated from a Belgian soil (Andreoni et al., 2004), characterized by a medium-term (< 3 years) exposure to PAHs. Andreoni et al. (2004) identified in the culture different strains, such as *Achromobacter xylosoxidans*, *Methylobacterium* sp., *Alcaligenes* sp., *Rhizobium galegae*, *Rhodococcus aetherovorans*,

Aquamicrobium defluvium, and *Stenotrophomonas acidaminiphila*, all able to grow and to degrade phenanthrene, as demonstrate by the decrease of phenanthrene content in the same Belgian soil after a biotreatment in the presence of the culture enrichments. Furthermore, the Phe-degrading microbial culture also contained bacteria that did not use phenanthrene, suggesting that the Phe-degraders themselves may be associated with bacteria using metabolites of phenanthrene (Andreoni et al., 2004).

This culture was also used in model systems simulating different Phe bioavailability conditions (Russo et al., 2005; Cavalca et al., 2008) and its capability to degrade the contaminant was demonstrated.

In the current study, the mixed culture has been inoculated in an agricultural soil, with no history of PAHs contamination, to prove its capability to degrade the contaminant in a real system.

In the biostimulation process, a compost from urban solid waste, supplemented at two different doses has been used as a nutrient source.

To better monitor the effectiveness of both bioremediation processes, the dynamics of chemical, biochemical and biological properties that reflect soil quality and functioning of the Phe-contaminated soil amended or not amended with compost and/or cell cultures has been monitored.

3.6. Materials and Methods

3.6.1. Chemicals

Reagent-grade Phe (>99% purity) and HPLC-grade solvents were purchased from Sigma Aldrich (Germany). All other chemicals, reagent grade, were supplied by Analar, BDH (Germany), unless otherwise stated.

3.6.2. Phe-degrading cultures

A Phe-degrading mixed bacterial culture, isolated from a PAH-polluted soil (Andreoni et al., 2004), was kindly provided by Prof. Andreoni of the University of Milan, Italy. Cultures were stored at 4 °C in a liquid mineral medium, M9 (Kunz and Chapman, 1981), supplemented with Phe (300 mg l^{-1}). For longer preservation (about 5 months), the culture was stored at -18 °C in tryptone soya broth (TSB) medium and glycerol.

TSB composition (g l^{-1}) was 17.0 g pancreatic digest of casein, 3.0 g papaic digest of soybean meal, 5.0 g sodium chloride, 2.5 g dibasic potassium phosphate, and 2.5 g glucose. TSB and glycerol were sterilized separately in an autoclave at 121 °C for 15 min and then mixed (75:25, respectively) under sterile conditions. The culture, grown in M9 mineral medium, was centrifuged at 12000 *g* for 10 min, the supernatant was removed and the pellet was re-suspended in 1.5 ml of TSB+glycerol solution. For the inoculation in soil systems, a microbial pre-culture was used. A suitable amount (usually 500 ml) of the freshly prepared-phenanthrene stock solution in acetone (30 mg ml^{-1}) was left to evaporate, under sterile conditions, at the bottom of 100 ml Erlenmeyer flasks, then sterile M9 (50 ml) was added and inoculated with 10% (v:v) of the culture stored in a refrigerator. The flasks were closed with Teflon-lined stoppers and incubated at 30 °C in darkness for 24 h on an orbital shaker at 100 rev min^{-1}. The cell density of the culture was determined by appropriate 10-fold

dilutions in sterile phosphate buffer (pH 7). The several dilutions were plated in duplicate on plate count agar medium (PCA composition per litre of distilled water: tryptone 10.0 g; yeast extract 5.0 g; dextrose 2.0 g; agar 15.0 g), incubated at 30 °C for 24 h and then counted. A culture containing $1 \cdot 10^7$ CFU (colony forming units) ml^{-1} was used for soil inoculation.

3.6.3. Degradation of Phe in a soil slurry reactor

Preliminary experiments were performed by adding 1 g of soil suspended in 50 ml of sterile M9 into 100 ml Erlenmeyer flasks supplemented with 10 mg of Phe (as described above, a suitable amount of the freshly prepared-Phe stock solution in acetone was left to evaporate in the flask under sterile conditions) and inoculated with 10% (v:v) of the culture stored in a refrigerator. The flasks were closed with Teflon-lined stoppers and incubated at 30 °C for 24 h on an orbital shaker at 100 rev min^{-1} in darkness. Before Phe addition, the soil was sterilized three times by autoclaving at 121 °C for 20 min every 24 h. This would also ensure that bacterial spores should be destroyed (Shaw et al., 1999). The soil sterility was monitored, using the spread-plate method, by determining at increasing incubation times the growth of bacterial cells. Four flasks containing Phe, soil, and M9 were prepared, inoculated with the bacterial pre-culture, and incubated at 25 °C in darkness with agitation on an orbital shaker at 180 rev min^{-1}. Bottles containing only Phe or soil served as controls. At different incubation times the controls and two inoculated flasks were processed for the quantification of Phe, and two for determining the protein content, according to the Bradford method (Bradford, 1976).

3.6.4. Degradation of Phe in solid-state cultures: experimental design

Experiments to evaluate the Phe removal in solid-state cultures were performed with four types of microcosms. Fresh soil (S) was air-dried to 14% moisture content and passed through a 2-mm sieve. Then it was placed (100 g) in closed 1-l glass jars. The compost was added at two different doses, 0.27% (C1) and 0.83% (C2). The two amounts correspond to a field rate of 10 and 30 t ha^{-1} of compost, respectively. Three samples were obtained: S, S-C1, and S-C2 and these represented the control microcosm (M_1) (Table 3.3).

Similar samples were supplemented with microbial cultures (10 ml of the Phe-degrading cultures in 100 g of soil) and named M_2. Other samples were prepared by using the soil previously spiked with Phe (150 g kg^{-1} of soil, as described below), supplemented with the two compost doses and formed the microcosm M_3, and others, supplemented with both the Phe and the microbial culture, formed the microcosm M_4. As summarized in Table 3.3, the total number of samples were 12 and duplicates were performed for each microcosm. The microcosms were placed in the dark in a climatic, humidity-controlled chamber set at 25 °C. Periodically, in particular after 0, 15, 45, 140, and 280 d of incubation, two sacrificial replicates were taken and split into sub-samples for phenanthrene determination and for all physical-chemical and biochemical analyses. Some sub-samples were kept at 4 °C and biochemical activities were determined within 5 d from the soil collection, while other sub-samples were air dried and used for the determination of physical-chemical properties.

Table 3.3. Investigated microcosms.

M_1	***S***	***S-C1***[a]	***S-C2***[b]
	Soil	Soil +Compost	Soil +Compost
M_2	***S-Cells***	***S-C1-Cells***	***S-C2-Cells***
	Soil +Phe[c]-degrading culture[d]	Soil +Compost +Phe-degrading culture	Soil +Compost +Phe-degrading culture
M_3	***S-Phe***	***S-C1-Phe***	***S-C2-Phe***
	Soil +Phenanthrene[e]	Soil +Compost +Phenanthrene	Soil +Compost +Phenanthrene
M_4	***S-Phe-Cells***	***S-C1-Phe-Cells***	***S-C2-Phe-Cells***
	Soil +Phenanthrene +Phe-degrading culture	Soil +Compost +Phenanthrene +Phe-degrading culture	Soil +Compost +Phenanthrene +Phe-degrading culture

[a]Compost amount = 0.27%.
[b]Compost amount = 0.83%.
[c]Phe = Phenanthrene.
[d]Phenanthrene-degrading culture = 10 ml of culture 100 g^{-1} soil.
[e]Phenanthrene =150 mg kg^{-1} of soil.

3.6.5. Phe spiked into soil

A stock solution of Phe (15 g l^{-1}) was prepared in acetone and stored under refrigeration at 4 °C. Soil samples were rewet to a moisture content of 14%, and homogenized with a stainless steel spatula. Approximately 10 g of the rewet soil was spiked with 7 ml of acetone and 1 ml of the Phe-stock solution in order to obtain a final concentration of 150 mg kg^{-1} soil (on the basis of 100 g, the total amount of soil). The soil was placed in a 1-l glass jar and mixed manually with a stainless steel spatula. Soil was gradually added to the glass jars in 10 g aliquots and extensively mixed with the spiked soil. This procedure was repeated until the entire amount of soil (100 g) was added and mixed. The jars containing the spiked soil were

covered with aluminium foil, hermetically closed and left overnight to shake for inversion. Then the acetone was left to evaporate for about 2 h under a flow hood and the soil was immediately used.

3.6.6. Physical and chemical properties of soil

The soil investigated was kindly provided by the Dipartimento di Biologia e Chimica Agroforestale e Ambientale, University of Bari, Italy, and stored at 10 °C. It was surface sampled (0 ±20 cm) from an agricultural area in the South of Italy (Bari), and had no history of PAH contamination. Physical-chemical analyses were performed on air-dried and sieved (> 2 mm) soil samples according to standard techniques (Sparks, 1996). The major physical-chemical properties of the soil are shown in Table 3.4. According to USDA (Soil Survey Staff, 1975) the soil was classified as a sandy clay loam soil (clay 32%, sand 45%, and silt 23%). Matured compost obtained from solid urban wastes was provided and characterized by the Dipartimento di Valorizzazione e Protezione delle Risorse Agroforestali, University of Torino, Italy. Some properties of the compost are shown in Table 3.4.

Table 3.4. Main physical-chemical properties of the soil and compost.

Soil Property		Compost	
pH (H_2O)	7.8 (±0.0)[a]	pH (H_2O)	9.0 (±0.02)[a]
Olsen P (mg kg^{-1})	19.0 (± 0.8)	Volatile compounds (% dry matter)	67.7 (±1.1)
Moisture (%)	25.0 (±0.1)	Moisture (%)	33.7 (±0.8)
TN (g kg^{-1})	2.4 (±0.1)	TN (g kg^{-1})	2.8 (±1.1)
TOC (g kg^{-1})	24.9 (±0.2)	TOC (g kg^{-1})	31.6 (±6)
C/N	10.4 (±0.7)	C/N	11.4 (±0.6)
EC (dS m^{-1})	0.36 (±0.03)	EC (dS m^{-1})	3.2 (±0.1)
Ca (meq 100 g^{-1})	28.4 (±0.4)	Ash (% dry matter)	32.3 (±0.9)
Mg (meq 100 g^{-1})	1.4 (±0.1)	Heavy metals (mg kg^{-1} dry matter)	
Na (meq 100 g^{-1})	1.1 (±0.3)	Cu	146.8 (±2.6)
K (meq 100 g^{-1})	1.6 (±0.1)	Pb	110.7 (±1.8)
Clay (g kg^{-1})	380 (±2)	Ni	46.3 (±1.1)
Sand (g kg^{-1})	284 (±3)	Cr	36.1 (±1.4)
Lime (g kg^{-1})	336 (±3)	Cd	< 2

[a] Values in parentheses show standard deviation.

3.6.7. Soil biochemical analyses

A fumigation-extraction method was used to estimate microbial biomass C (MB-C) with extractable C converted to microbial C using standard factors (Brookes et al., 1985; Vance et al., 1987). Organic C in soil extracts was determined as described by Vance et al. (1987). Basal respiration was determined, according to Stotzky (1965) with some modifications (Piotrowska et al., 2006), after 1, 3, 8, 11, 16, 22, 29, 35, 45, and 53 d of incubation. All determinations were made in triplicate and data were corrected to oven-dry (16 h at 105 °C) moisture content. Enzyme activities were determined on fresh, moist, sieved (> 2 mm) soils as described in detail by Rao et al. (2003). Substrates, i.e. *p*-nitrophenyl-β-D-

glucopyranoside, *p*-nitrophenylphosphate, and *p*-nitrophenylsulphate, for β-D-glucosidase (GLU), phosphatase (PHO), and arylsulphatase (ARYL) were used, respectively. Specific buffers and pH values were used as reported in Rao et al. (2003).

The activity of urease (UR) was determined as described by Kandeler and Gerber (1988). Dehydrogenase (DH) activity was measured with tetrazolium salts (TTC) as the substrate, according to Trevors (1984).

Control tests with autoclaved soils were carried out to evaluate the spontaneous or abiotic transformations of substrates.

The soil and/or the compost (or some of the compost components) were tested for possible interference with the analytical methods adopted for evaluating enzymatic product concentration and/or adsorption of the product released by the enzymatic action (i.e., increase or decrease of optical absorbance as a function of soil and/or soil+compost presence). Therefore, each analytical method was calibrated in the presence of soil with and without the two compost doses. An example is given (Fig. 3.5) of the calibration curve obtained for given amounts of *p*-nitrophenol (*p*-NP), the product produced by GLU, PHO, and ARYL reactions. It is evident that different extinction coefficients were obtained, ranging from 18.35 for pure *p*-NP to 15.96 for *p*-NP+soil and for *p*-NP+soil+compost. Similar responses were obtained for the other enzymatic assays. Appropriate extinction coefficients were used for the calculation of enzyme activity units.

Each value is the mean of two determinations. All SDs were smaller than the symbol sizes.

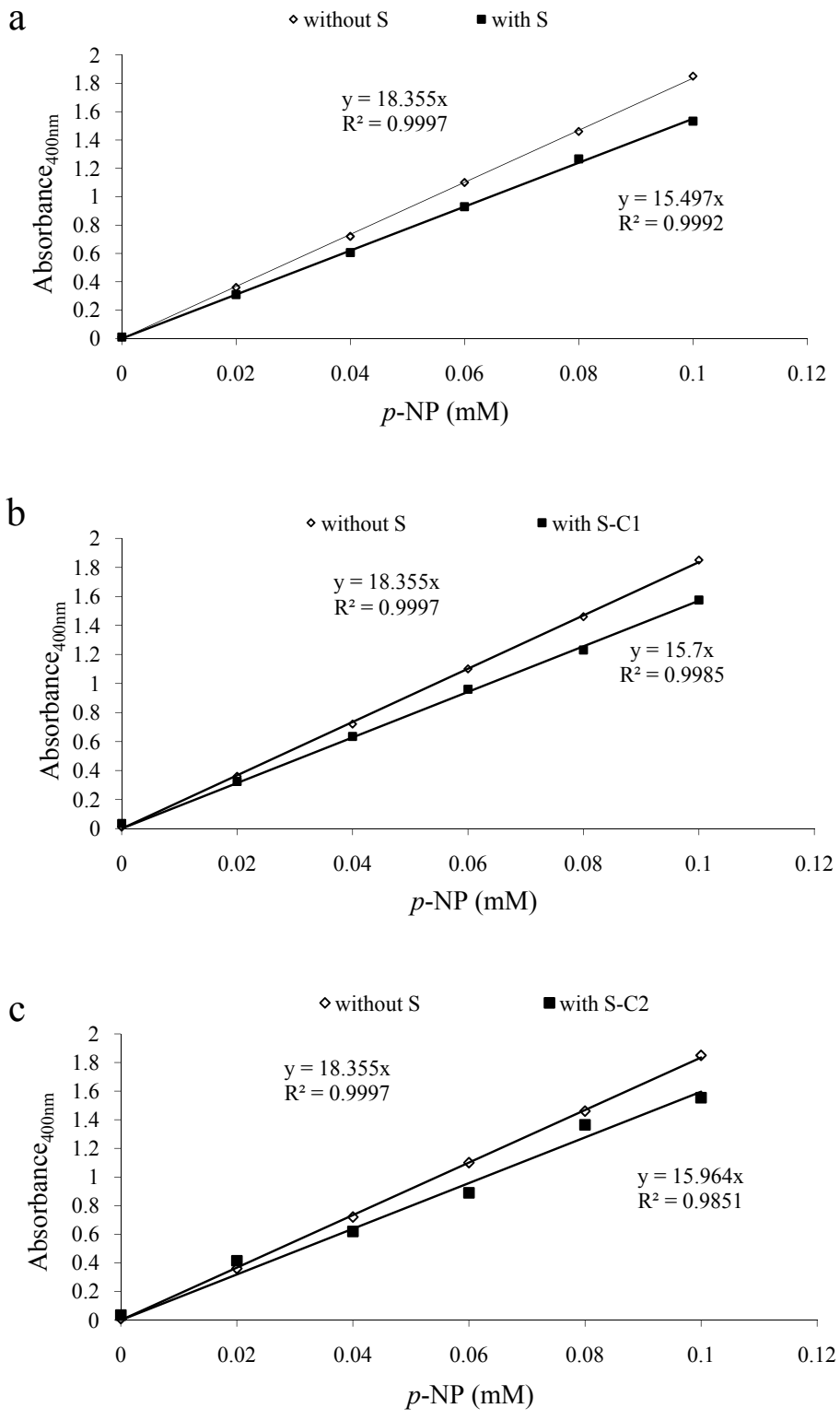

Fig. 3.5. Calibration curve of *p*-nithrophenol (*p*-NP) with and without 1 g of soil, soil+0.27% compost (S-C1), and soil+0.83% of compost (S-C2).

One unit of enzyme activity was defined as the number of μmol (for GLU, PHO, and ARYL) or μg (for DH and UR) of product released at 30 °C (or 37 °C for UR) h^{-1} by 1 g of dried soil.

Triplicates were performed for each activity assay.

Total heterotrophic microbial counts for soil, compost, and soil+compost (at the two different rates), before and after the addition of the Phe-degrading culture, were determined by adding 27 ml of a sterile extracting solution ($Na_4P_2O_7$, 2g l^{-1}) to 3 g of soil, compost, or soil+compost in 250-ml flasks and then incubating on an orbital shaker for 1 h at 150 rpm. After a 10-min sedimentation period, appropriate 10-fold dilutions in phosphate buffer (pH 7) were plated in duplicate on PCA medium. The plates were incubated at 30 °C for 24 h and then counted.

3.6.8. Phe extraction and detection

Ethanol (12 ml) was added to duplicate soil samples (0.6 g of dry weight at 25 °C). The tubes were capped and agitated for 30 min, then centrifuged at 3000 *g* for 15 min, and the supernatants were removed. The sediments were re-extracted with 12 ml of ethanol/*n*-hexane (75:25, v:v), and the two extracts were combined and concentrated by evaporation under vacuum. Each concentrate was resuspended in 5ml of acetonitrile and the Phe was quantified by high-performance liquid chromatography (HPLC) using a C-18 column and a diode-array detector, as described in detail by Russo et al. (2005). Methanol and water (86:14, v:v) were the mobile phase, and the flow rate was 1.0 ml min^{-1}. The retention time for Phe was about 9.5 min. Detection was carried out at 254 nm. Experimental runs were carried out with Phe-soil and/or Phe-soil+compost mixtures at different Phe-soil (soil+compost) ratios to evaluate the efficiency of the extraction method. Phe extraction of 100% was achieved for all the mixtures with the method

adopted. All the experiments were carried out at least in duplicate and standard deviations were calculated.

3.6.9. Statistic analysis

All data were subjected to analysis of variance by using SPSS for Windows, Version 15.0. The assumptions of normality and homogeneity of variances were tested by the Kolmogoroff-Smirnoff test and the Levene test. The significant differences between means at $P < 0.05$ were assessed according to Tukey's multiple comparison test.

3.7. Results

3.7.1. Degradation of Phe in a soil slurry reactor

A preliminary experiment was carried out in a soil slurry reactor in order to get indications about the efficiency of the bacterial culture towards Phe utilization in the presence of soil.

Fig. 3.6 shows the disappearance of the Phe by the cell culture, and the protein content (a measure of the cell growth) with and without soil.

The amount of extractable Phe at zero incubation time was 10% lower than the amount added, indicating either a fairly small initial adsorption of Phe on soil, or an additional microbial Phe degradation effect by the soil.

The shape of the Phe degradation curve by the bacterial culture was not substantially affected by the soil, although the degradation kinetic constant was decreased with respect to that measured with Phe ($k = 0.21\ d^{-1}$ with soil against $0.36\ d^{-1}$ for the control). After incubation for 21 d, no more Phe was measured in both systems. A fairly similar behaviour of protein increase was also detected, although at lower levels in the presence of soil (Fig. 3.6).

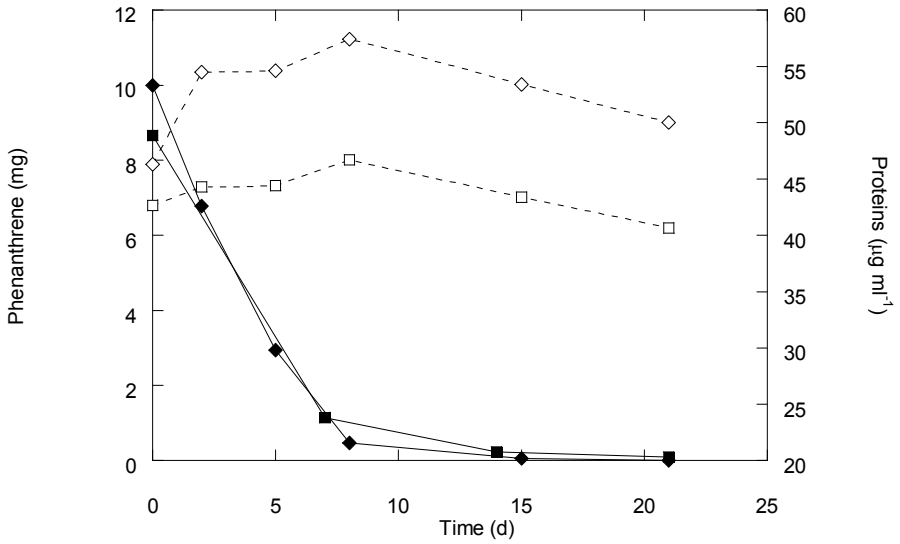

Fig. 3.6. Phenanthrene (Phe) degradation in a soil slurry reactor by the Phe-degrading bacterial culture. Disappearance of: solid Phe (—♦—); solid Phe+soil (—■—); corresponding cell growth on solid Phe (---◊---); and solid Phe+soil (---□---). Each value is the mean of two determinations. All SDs were smaller than the symbol sizes.

3.7.2. Degradation of Phe in solid-state cultures

3.7.2.1. Residual Phe

The evolution with time of extractable Phe from M_3 and M_4 microcosms is shown in Fig. 3.7. At the time zero of incubation (corresponding to 24 h of contact between soil and Phe), a 30% reduction of the extractable Phe was measured in M_3 samples containing only Phe, and no effect was observed for the two compost treatments. Conversely, when the Phe-degrading bacterial culture was present in the microcosms, the initial extractable Phe was further diminished to 40-10% of that initially added.

In the case of M_3, a lower decline of the residual extractable Phe occurred with time for both S and S-C2, whereas in the presence of the lower compost dose, the S-C1 sample, the amount of the residual Phe decreased from 70% to about 50% within 45 d. Thereafter it decreased slowly. At the

end of the incubation period, residual Phe ranged from 33% to 45% of the initial amount in the three M_3 samples.

A completely different behaviour was observed in the microcosm containing the Phe-degrading cells M_4. Most of the Phe losses occurred within the first 15 d (values proximate to 10% in S and S-C2, and to zero in S-C1, respectively). Losses slowed thereafter with little further change recorded by the end of the experiment.

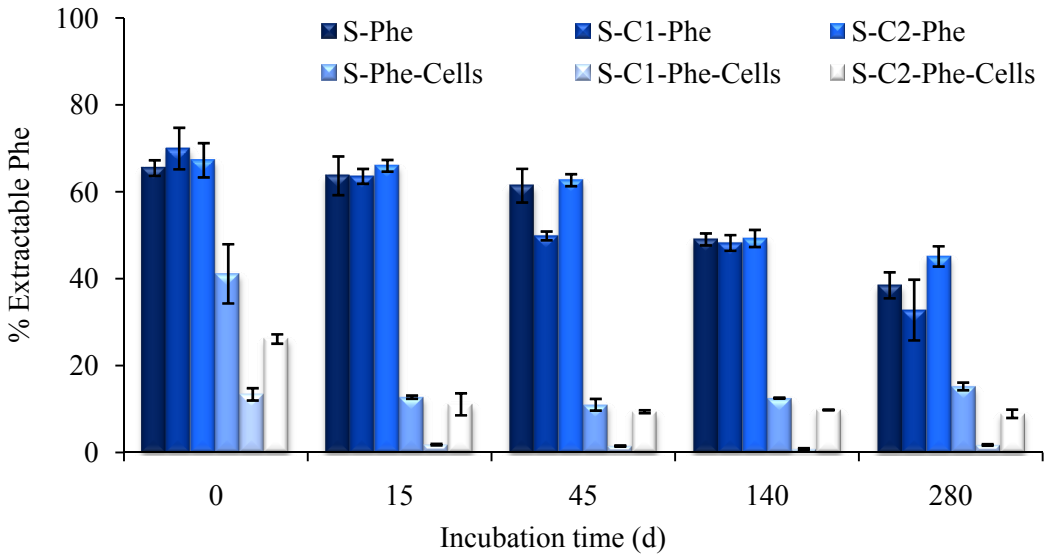

Fig. 3.7. Extractable phenanthrene (Phe) (%) with time at 25 °C in M_3 and M_4. The microcosms analysed are described in detail in Table 3.3. Each value is the mean of two determinations.

The final residual amounts of Phe in M_3 and M_4 microcosms were also measured using the Soxhlet extraction procedure. Values were obtained similar (0 ±1%) to those measured using the ethanol/*n*-hexane extraction.

The kinetics of Phe disappearance was estimated by a time course analysis of data shown in Fig. 3.7. When the data were plotted in a semilog scale, one-slope behaviour was observed for the M_3 microcosm, whereas a typical two slope was observed for M_4, *i.e.* for soils with cells. The kinetics was

best fitted by single and two coupled first order exponential equations, according to the following expressions: $Xt = X_0\ e^{-kt}$, and $Xt = X_{0,1}\ e^{-k1t} + X_{0,2}\ e^{-k2t}$ where Xt (mg l^{-1}), the concentration of Phe at time t, is described by X_0 for single and $X_{0,1} + X_{0,2}$ (mg l^{-1}) for coupled equations, and the disappearance constants k for single and k_1 and k_2 for coupled equations. This could imply that in M_3 the whole Phe removal process is dominated by a single, straightforward key step, whereas for microcosms M_4 a more complex mechanism, involving a faster intermediate step, occurred.

Table 3.5 gives the disappearance constants calculated by means of a non-linear regression routine applied to the Phe disappearance data.

Table 3.5. Values of phenanthrene disappearance constants for S, S-C1, and S-C2 samples in the microcosms investigated, as described in Table 3.3.

Microcosm	Sample	k_1 (d^{-1})	k_2 (d^{-1})	R^{2b}
M_3	S	0.0019	-	0.95
	S-C1	0.0024	-	0.99
	S-C2	0.0015	-	0.99
M_4	S-Cells	0.082	0.0012	0.98
	S-C1-Cells	0.133	0.0011	0.99
	S-C2-Cells	0.057	0.0010	0.97

[b]R^2 is the correlation coefficient.

The one-step kinetics occurring for M_3 microcosms was characterized by very low constants, suggesting a slower utilization/removal of Phe by/from soil or soil+compost. By contrast, in microcosms M_4 the Phe was almost completely removed (more than 90% of the amount detected at zero time) within 15 d, with disappearance constants two orders of magnitude higher than for M_3. Longer times were required for a complete removal. It is

noteworthy that: (a) in both cases the constants are in the order S-C1>S>S-C2; (b) those calculated for the second step in M_4 are very similar to those obtained for M_3 (Table 3.5). That indicates that after the first rapid removal of Phe, similar phenomena probably occurred in both M_3 and M_4.

3.7.3. Physical and chemical properties

The addition of the two doses of compost had different effects on some of the physical-chemical properties of the soil and their variations with time (Tables 3.6a, b, c). Some, such as pH, moisture, CEC (data not shown) did not change significantly, whereas detectable increases were measured for TOC, phosphorus (P_2O_5), and the alkaline bivalent cations Ca and Mg (Tables 3.6a, b, c). The increase of TOC, however, was less than that expected on the basis of the total organic carbon supplied by the compost.

As expected, the addition of the bacterial cells (M_2) did not influence these properties and their changes except for phosphorus which sharply increased from values ranging from 30-50 up to 202-213 mg kg^{-1}, followed by a constant decrease with time. By the end of the incubation time the P contents were in the range 110-124 mg kg^{-1} in M_2 and M_4 and 33-47 mg kg^{-1} soil in M_1 and M_3. No influence was measured from the addition of Phe except for the EC that was reduced on average by more than 50%. No variations of EC values with time were, however, observed for any of the microcosms investigated.

With time, a slight decrease of TOC was measured in all the microcosms, and after 280 d, in the case of M_3 (*i.e.* the microcosms containing only Phe), the TOC values were quite similar to the initial values. Moisture, pH, CEC, and TN did not change (data not shown), whereas moderate increases of Ca^{2+} and Mg^{2+} were measured at longer incubation times (Tables 3.6a, b, c). By the end of the experiment, Ca^{2+} values were higher than the initial

ones for all the microcosms, except for the control S, in which Ca^{2+} concentrations diminished from 31.6 to 25.0 meq 100 g^{-1}, even though temporary increases occurred at 45 and 140 d. The same behaviour was observed for Mg^{2+} values, but the final amounts in all the microcosms were higher than the initial amounts. After 280 d of incubation a decrease of K^{+} was observed in M_1 and M_3, while no significant differences were detected for Na^{+}.

Table 3.6a. Major chemical properties of S, S-C1, and S-C2 samples in the microcosms investigated, as described in Table 3.3.

Property	Sample		Incubation times (d)			
			0	45	140	280
TOC (g kg^{-1})	M_1	S	28.3 (±1.0)[a]	27.2 (±0.3)	26.6 (±0.4)	25.9 (±1.4)
		S-C1	29.4 (±0.4)	28.9 (±0.0)	28.0 (±1.2)	25.9 (±0.3)
		S-C2	30.4 (±1.0)	29.6 (±0.0)	28.9 (±1.0)	27.4 (±0.8)
	M_2	S	28.7 (±0.1)	27.3 (±0.5)	27.0 (±0.0)	25.4 (±0.4)
		S-C1	29.4 (±0.6)	28.8 (±0.1)	27.6 (±0.2)	27.3 (±1.0)
		S-C2	30.9 (±0.0)	29.9 (±0.6)	29.0 (±0.2)	27.6 (±0.2)
	M_3	S	26.7 (±1.3)	25.6 (±0.2)	25.1 (±0.7)	26.2 (±0.3)
		S-C1	27.5 (±0.0)	25.2 (±0.4)	24.9 (±0.2)	26.6 (±0.9)
		S-C2	28.3 (±0.4)	27.6 (±0.3)	26.9 (±0.8)	28.0 (±0.2)
	M_4	S	26.6 (±0.3)	26.0 (±0.5)	25.4 (±0.8)	25.4 (±0.1)
		S-C1	27.7 (±0.1)	26.0 (±0.0)	25.6 (±0.0)	26.3 (±0.1)
		S-C2	29.8 (±0.5)	28.5 (±0.1)	27.9 (±0.0)	28.2 (±0.6)
P_2O_5 (mg kg^{-1})	M_1	S	38.9 (±0.9)	46.8 (±2.7)	33.2 (±1.8)	29.3 (±3.1)
		S-C1	40.7 (±1.1)	45.1 (±0.3)	39.5 (±0.9)	35.6 (±4.6)
		S-C2	49.1 (±9.8)	57.0 (±0.6)	54.8 (±17.1)	33.5 (±0.3)
	M_2	S	192.3 (±3.8)	178.3 (±18.9)	155.2 (±24.1)	108.3 (±11.6)
		S-C1	216.1 (±0.7)	83.9 (±15.9)	163.4 (±14.3)	113.3 (±1.5)
		S-C2	198.2 (±13.3)	207.7 (±15.3)	177.6 (±2.1)	111.8 (±11.6)
	M3	S	44.0 (±5.6)	45.3 (±6.7)	36.0 (±5.8)	64.3 (±0.4)
		S-C1	43.4 (±0.1)	42.9 (±6.4)	30.2 (±3.7)	46.2 (±0.1)
		S-C2	54.8 (±12.2)	41.7 (±13.1)	49.9 (±22.3)	32.4 (±0.2)
	M4	S	178.8 (±18.1)	102.3 (±18.9)	150.2 (±8.5)	114.2 (±8.9)
		S-C1	229.9 (±28.3)	204.6 (±22.0)	214.3 (±41.2)	127.1 (±9.5)
		S-C2	228.8 (±19.5)	240.4 (±58.0)	163.4 (±2.7)	130.6 (±22.9)

[a]Values in parentheses show standard deviations.

Table 3.6b. Major chemical properties of S, S-C1, and S-C2 samples in the microcosms investigated, as described in Table 3.3.

Property	Sample		Incubation times (d)			
			0	45	140	280
Ca (meq 100 g^{-1})	M_1	S	31.6 (±0.2)[a]	45.8 (±0.5)	48.0 (±0.7)	25.0 (±1.0)
		S-C1	32.6 (±0.7)	33.2 (±0.4)	34.0 (±0.1)	49.6 (±1.2)
		S-C2	36.2 (±0.0)	50.5 (±1.0)	52.0 (±0.2)	61.3 (±0.4)
	M_2	S	30.5 (±0.7)	45.3 (±0.0)	47.0 (±0.0)	53.6 (±0.1)
		S-C1	33.7 (±1.2)	47.1 (±0.6)	50.0 (±0.9)	53.0 (±0.1)
		S-C2	32.4 (±0.0)	46.7 (±0.1)	48.0 (±0.0)	54.4 (±0.2)
	M_3	S	30.4 (±0.1)	41.7 (±0.4)	45.0 (±0.9)	59.5 (±0.2)
		S-C1	30.3 (±0.2)	39.1 (±0.6)	41.0 (±0.5)	52.5 (±0.1)
		S-C2	32.5 (±0.7)	47.1 (±0.1)	51.9 (±0.6)	55.2 (±0.6)
	M_4	S	35.3 (±0.3)	44.4 (±0.9)	45.6 (±0.2)	51.5 (±0.4)
		S-C1	32.5 (±0.3)	43.9 (±1.0)	44.3 (±0.6)	48.9 (±0.5)
		S-C2	34.2 (±0.2)	33.9 (±0.4)	35.9 (±0.2)	50.9 (±0.7)
Mg (meq 100 g^{-1})	M_1	S	1.2 (±0.1)	2.2 (±0.3)	2.2 (±0.1)	1.4 (±0.1)
		S-C1	1.8 (±0.1)	1.8 (±0.3)	1.8 (±0.0)	2.0 (±0.0)
		S-C2	2.2 (±0.1)	2.7 (±0.2)	2.8 (±0.1)	3.6 (±0.1)
	M_2	S	1.0 (±0.1)	2.3 (±0.2)	2.3 (±0.1)	2.6 (±0.1)
		S-C1	2.7 (±0.2)	2.4 (±0.1)	2.4 (±0.1)	2.5 (±0.0)
		S-C2	1.5 (±0.1)	2.7 (±0.1)	2.8 (±0.1)	3.1 (±0.1)
	M_3	S	1.0 (±0.0)	1.9 (±0.1)	2.0 (±0.1)	3.9 (±0.1)
		S-C1	1.3 (±0.2)	3.1 (±0.1)	3.2 (±0.0)	2.3 (±0.1)
		S-C2	0.9 (±0.1)	3.1 (±0.1)	3.2 (±0.5)	3.7 (±0.0)
	M_4	S	2.4 (±0.1)	2.7 (±0.1)	2.7 (±0.2)	3.6 (±0.0)
		S-C1	1.7 (±0.1)	3.6 (±0.0)	3.7 (±0.1)	2.1 (±0.1)
		S-C2	3.1 (±0.3)	2.0 (±0.0)	2.0 (±0.1)	3.3 (±0.1)

[a]Values in parentheses show standard deviations.

Table 3.6c. Major chemical properties of S, S-C1, and S-C2 samples in the microcosms investigated, as described in Table 3.3.

Property	Sample		Incubation times (d)			
			0	45	140	280
Na (meq 100 g^{-1})	M_1	S	1.05 (±0.2)[a]	1.10(±0.5)	1.4 (±0.7)	1.6 (±1.0)
		S-C1	1.06 (±0.7)	1.25 (±0.4)	1.43 (±0.1)	1.62 (±1.2)
		S-C2	1.18 (±0.0)	1.21 (±1.0)	1.45 (±0.2)	1.7 (±0.4)
	M_2	S	2.00 (±0.7)	1.90 (±0.0)	1.78 (±0.0)	1.67 (±0.1)
		S-C1	2.09 (±1.2)	2.13 (±0.6)	1.96 (±0.9)	1.8 (±0.1)
		S-C2	2.08 (±0.0)	2.16 (±0.1)	1.99 (±0.0)	1.82 (±0.2)
	M_3	S	1.04 (±0.1)	1.21 (±0.4)	1.43 (±0.9)	1.65 (±0.2)
		S-C1	1.07 (±0.2)	1.15 (±0.6)	1.4 (±0.5)	1.61 (±0.1)
		S-C2	0.83 (±0.7)	1.24 (±0.1)	1.5 (±0.6)	1.7 (±0.6)
	M_4	S	1.8 (±0.3)	1.92 (±0.9)	1.82 (±0.2)	1.73 (±0.4)
		S-C1	1.89 (±0.3)	1.71 (±1.0)	1.73 (±0.6)	1.75 (±0.5)
		S-C2	1.88 (±0.2)	2.08 (±0.4)	2.82 (±0.2)	1.83 (±0.7)
K (meq 100 g^{-1})	M_1	S	1.6 (±0.1)	1.43 (±0.3)	1.0 (±0.1)	0.56 (±0.1)
		S-C1	1.61 (±0.1)	1.62 (±0.3)	1.12 (±0.0)	0.61 (±0.0)
		S-C2	1.72 (±0.1)	1.71 (±0.2)	1.2 (±0.1)	0.66 (±0.1)
	M_2	S	1.71 (±0.1)	1.7 (±0.2)	1.53 (±0.1)	1.37 (±0.1)
		S-C1	1.76 (±0.2)	1.88 (±0.1)	1.66 (±0.1)	1.44 (±0.0)
		S-C2	1.8 (±0.1)	1.98 (±0.1)	1.72 (±0.1)	1.46 (±0.1)
	M_3	S	1.62 (±0.0)	1.81 (±0.1)	1.2 (±0.1)	0.57 (±0.1)
		S-C1	1.68 (±0.2)	1.76 (±0.1)	1.14 (±0.0)	0.53 (±0.1)
		S-C2	1.8 (±0.1)	1.82 (±0.1)	1.23 (±0.5)	0.65 (±0.0)
	M_4	S	1.75 (±0.1)	1.72 (±0.1)	1.53 (±0.2)	1.34 (±0.0)
		S-C1	1.87 (±0.1)	1.67 (±0.0)	1.5 (±0.1)	1.34 (±0.1)
		S-C2	2.03 (±0.3)	2.0 (±0.0)	1.7 (±0.1)	1.4 (±0.1)

[a]Values in parentheses show standard deviations.

3.7.4. Biochemical properties

The addition of compost having 10^9 CFU g^{-1} did not influence the total heterotrophic microbial count of the soil (CFU values of $1 \cdot 10^7$ g^{-1}). After the addition of the bacterial cells, an increase up to $2.25 \cdot 10^7$ CFU g^{-1} was measured for the soil alone, whereas the values decreased to 3.0 and $4.5 \cdot 10^6$ CFU g^{-1} in the cases of S-C1 and S-C2, respectively. That indicated a possible inhibitory effect by the endogenous microflora of the compost.

Accumulated CO_2 evolution showed significant differences in the four microcosms (Fig. 3.8). At zero time (after 1 d of incubation) the values were similar for M_1 and M_2, about 23 mg CO_2 100 g^{-1} of dry weight soil (100 g dw^{-1}), while in the presence of Phe (M_3 and M_4) the CO_2 release was 2-fold higher, with no differences between the compost amended and non-amended samples. After 3 d, a very strong increase was detected in M_2, M_3, and M_4, followed by a rapid decline until day 10 of incubation. In particular, the increase was more evident only for S in M_3 and for S-C1 in M_2, while it was marked for both S and S-C1 in M_4. Another increase of S respiration was observed after 16 d of incubation in the case of M_3 only. The behaviour of soil respiration in the four microcosms was very similar at longer incubation times and showed relatively constant values by the end of the incubation (about 27 mg CO_2 100 g dw^{-1}).

The behaviour of biomass-C did not reflect that of soil respiration (Fig. 3.9). Some differences of MB-C values were already seen at zero time. In M_1, for S, and S-C2 the same initial values of MB-C were observed, as was a similar behaviour throughout the incubation period. This was characterized by a small increase after 45 d and a slow decline at the end of the incubation but with higher final values of MB-C for S. By contrast, MB-C showed higher values at zero time for S-C1. These slowly declined after 15 d, and levelled off to values similar to those for S and for S-C2.

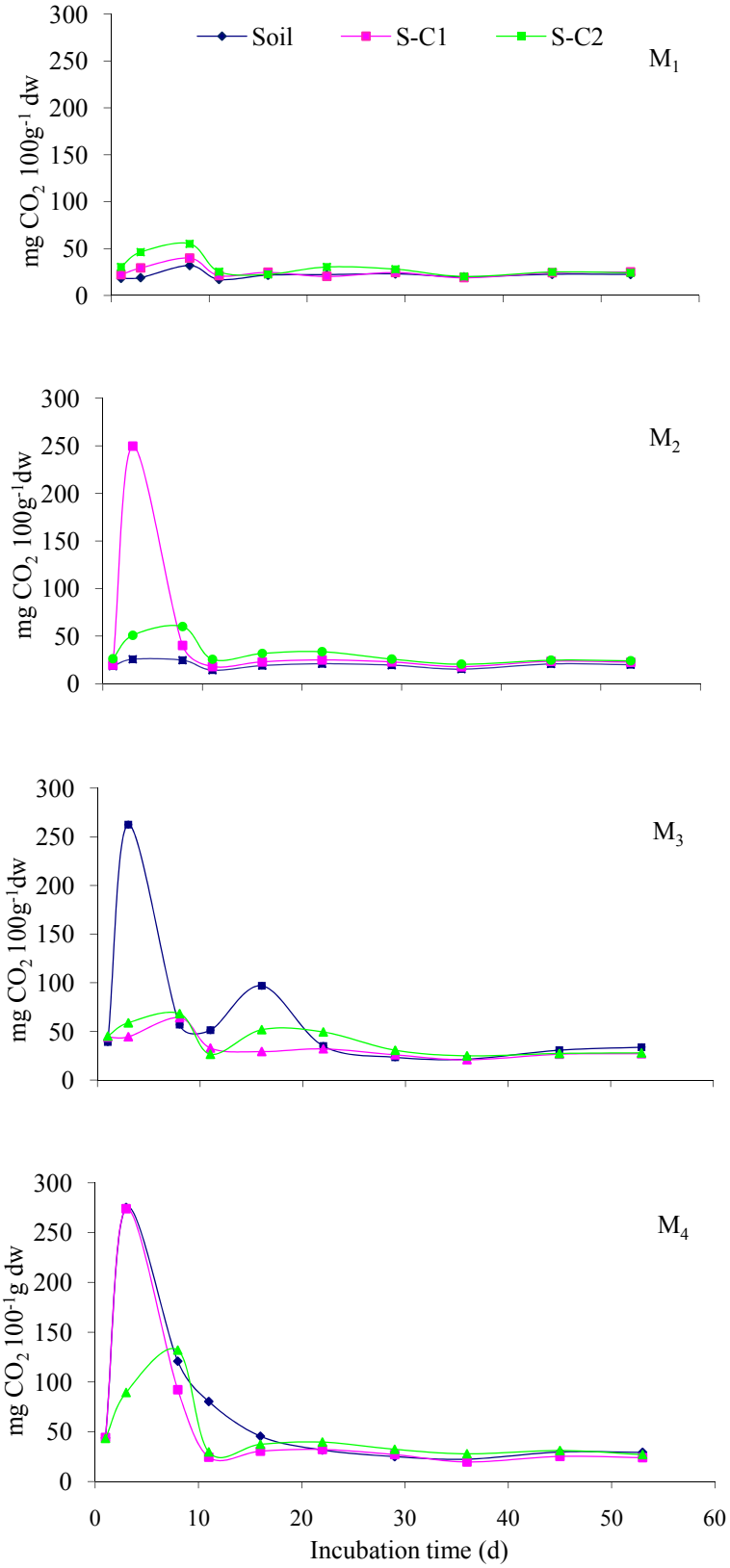

Fig. 3.8. Basal respiration of S (—♦—), S-C1 (—■—), and S-C2 (—▲—) samples in M_1, M_2, M_3, and M_4 (for details see Table 3.3). All SDs were smaller than the symbol sizes.

The addition of cells (M_2) resulted in lower values of MB-C for S-C1 at zero time, followed by a small increase at 45 d, and then a slow decline as in M_1. In S and S-C2 samples, the MB-C declined with time and at the end reached the same values as S-C1 (Fig. 3.9). Addition of Phe (M_3) strongly affected the initial MB-C value of the S sample, which increased 1.75-fold with respect to the control (M_1) (Fig. 3.9). Furthermore, the effects of Phe addition were more visible within 45 d of incubation. In both S and S-C2 samples the initial MB-C values decreased significantly (by more than 70% and 99%, respectively). By contrast, those of the S-C1 sample showed an increase at 15 d followed by a decrease to values very close to those of the S-C2 sample. The presence of both cells and Phe (M_4) resulted in no significant differences in MB-C values between S-C1 and S-C2. Lower initial values were, however, detected in S-C2. In the case of S, MB-C increased at 45 d of incubation, but then declined to zero after 140 d.

Measurements of enzyme activities (Figs. 3.10, 3.11, 3.12 and 3.13) showed different patterns for the four microcosms. With the exception of M_4, which contained both Phe and microbial cells, the values for dehydrogenase activity ranged between 1.8 and 2.5 mg TPF g^{-1} h^{-1} in all the microcosms (Fig. 3.10). In the case of M_4, lower values were observed for the two amounts of compost used. After 15 d a decline of DH activity was observed in M_1, M_2, and M_3, with no significant differences for C1 and C2. This decline was more marked in M_3 where the DH activity levelled off to nearly zero and then slightly increased with time. After 150 d of incubation, a similar behaviour was observed for M_1 and M_2. In fact, an increase of DH activity occurred, followed by a decline at the end of incubation.

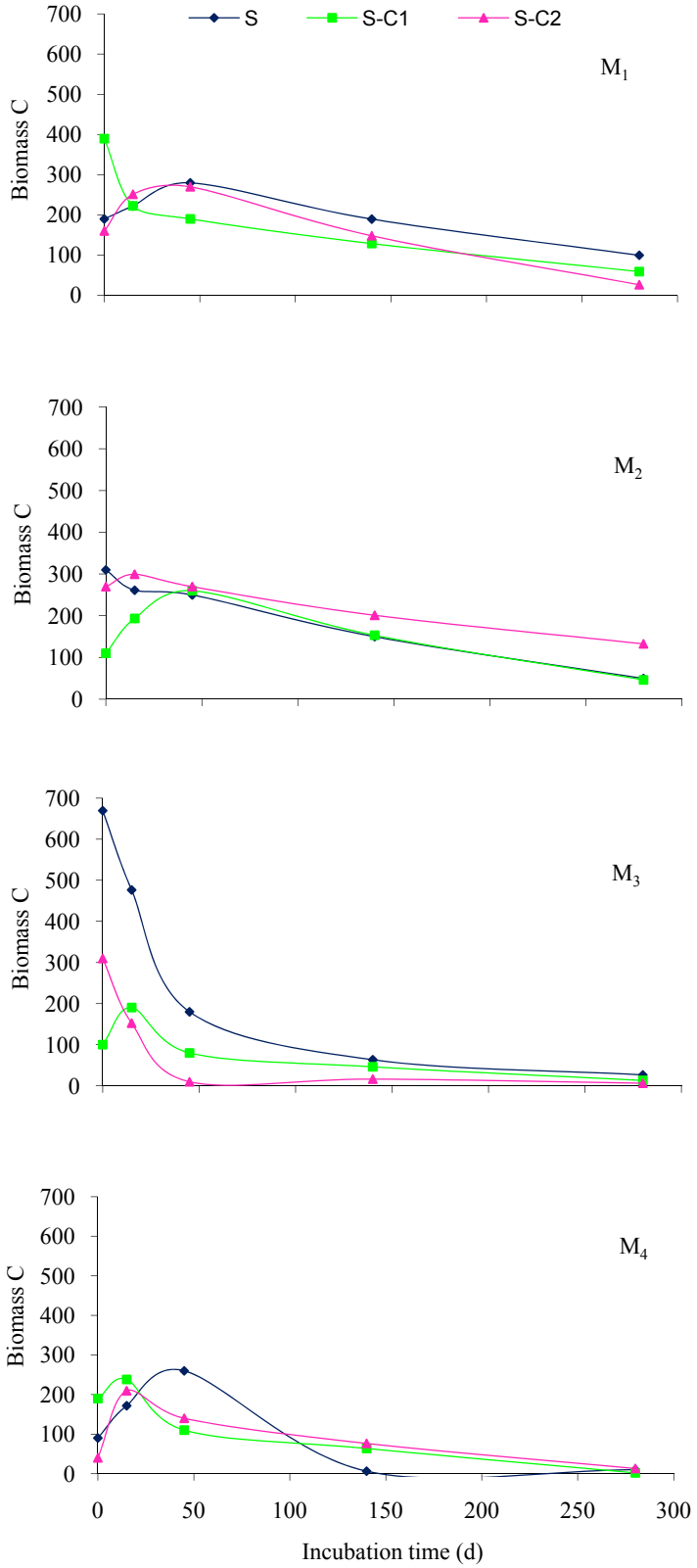

Fig. 3.9. Microbial biomass of S (—♦—), S-C1 (—■—), and S-C2 (—▲—) samples in M_1, M_2, M_3, and M_4, (for details see Table 3.3). All SDs were smaller than the symbol sizes.

In the cell inoculated samples (M_2) the increase was higher in S, while a trend constant with time was observed for S-C2. The DH activity showed completely different temporal pattern in M_4. After 15 d of incubation there was a big increase in the DH activity in the S sample followed by a decline. The values for S-C1 were constant with time, while an increase was measured in the case of S-C2 at the end of incubation (Fig. 3.10).

The GLU activity was quite similar in microcosms M_1, M_2, and M_3 (Fig. 3.11). It showed little differences at zero time, then it slowly declined with time. No significant effects were observed for both compost doses. The presence of Phe (M_3) apparently inhibited the initial values of GLU activity. A different trend was observed in M_4 where a rapid decline of GLU activity occurred in the first 15 d of incubation, followed by a small recovery, especially for the sample containing the lower amount of compost, and that was followed by a constant decline up to 150 d of incubation. At the end of incubation, all the samples had reached quite similar values (about 0.9 μmol *p*-NP g^{-1} h^{-1}) of GLU activity.

Neither the initial activity values of soil PHO activity nor its trend with time differed significantly in M_1 and M_2 (Fig. 3.12). The main effects were observed for M_3, particularly in the first 15 d of incubation. The presence of the Phe gave rise to very high values of PHO activity, even at zero time when compared to the control samples M_1; the PHO activity still increased after 15 d, then it rapidly declined to lower values, and after that it remained constant with time. By contrast, the presence of both Phe and cells resulted in lower values of PHO activity at all incubation times (Fig. 3.12).

An initial ARYL activity of about 0.6 μmol *p*-NP g^{-1} h^{-1} was measured in M_3 and M_4 (both with Phe), while M_1 and M_2 showed lower values, about 0.35 μmol *p*-NP g^{-1} h^{-1} (Fig. 3.13). After 15 d of incubation, ARYL activity

had increased up to 0.5 μmol *p*-NP g^{-1} h^{-1} in M_1 whereas it had rapidly declined in the other microcosms, especially in M_3 and M_4. All the microcosms presented a peak of activity at 150 d of incubation, and this was much higher in M_2 for the S-C1 sample. In contrast, this effect was more evident in M_3 for the S sample. After this period, a decline of activity occurred in all the samples, and reached values close to zero for the microcosms containing Phe (M_3 and M_4).

In the presence of Phe (M_3 and M_4) negative values of UR activity were measured in some samples for several incubation times (data not shown). In M1 the three samples S, S-C1, and S-C2 presented an initial UR activity of 3.0 μg NH_4-N g^{-1} h^{-1}. This slightly increased at 15 d and then declined up to 150 d of incubation. In the case of C1, a strong increase of UR activity was observed, to values close to those reached after 15 d of incubation. Then there was a constant decline for the higher amounts of compost, and a small increase was observed to intermediate activity values for the non-amended sample. In the case of M_2, the response of UR activity was similar in the three samples: initial values ranged between 3 and 3.5 μg NH_4-N g^{-1} h^{-1}, and a rapid decline occurred in the first 50 d to activities that fell by 66%. This type of response was much more evident for S-C2.

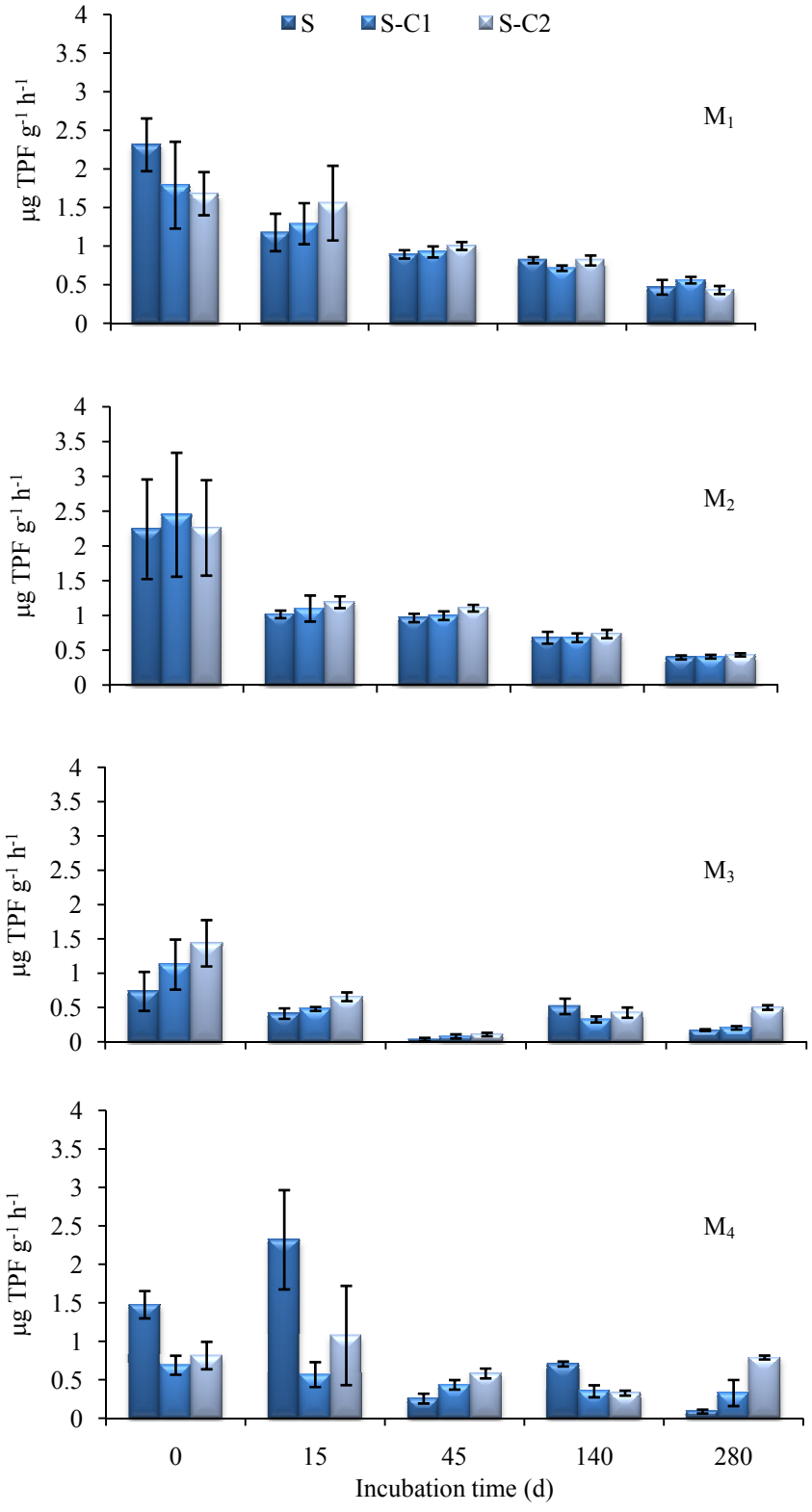

Fig. 3.10. Dehydrogenase activity (µg TPF g^{-1} h^{-1}) in M_1, M_2, M_3 and M_4, (for details see Table 3.3).

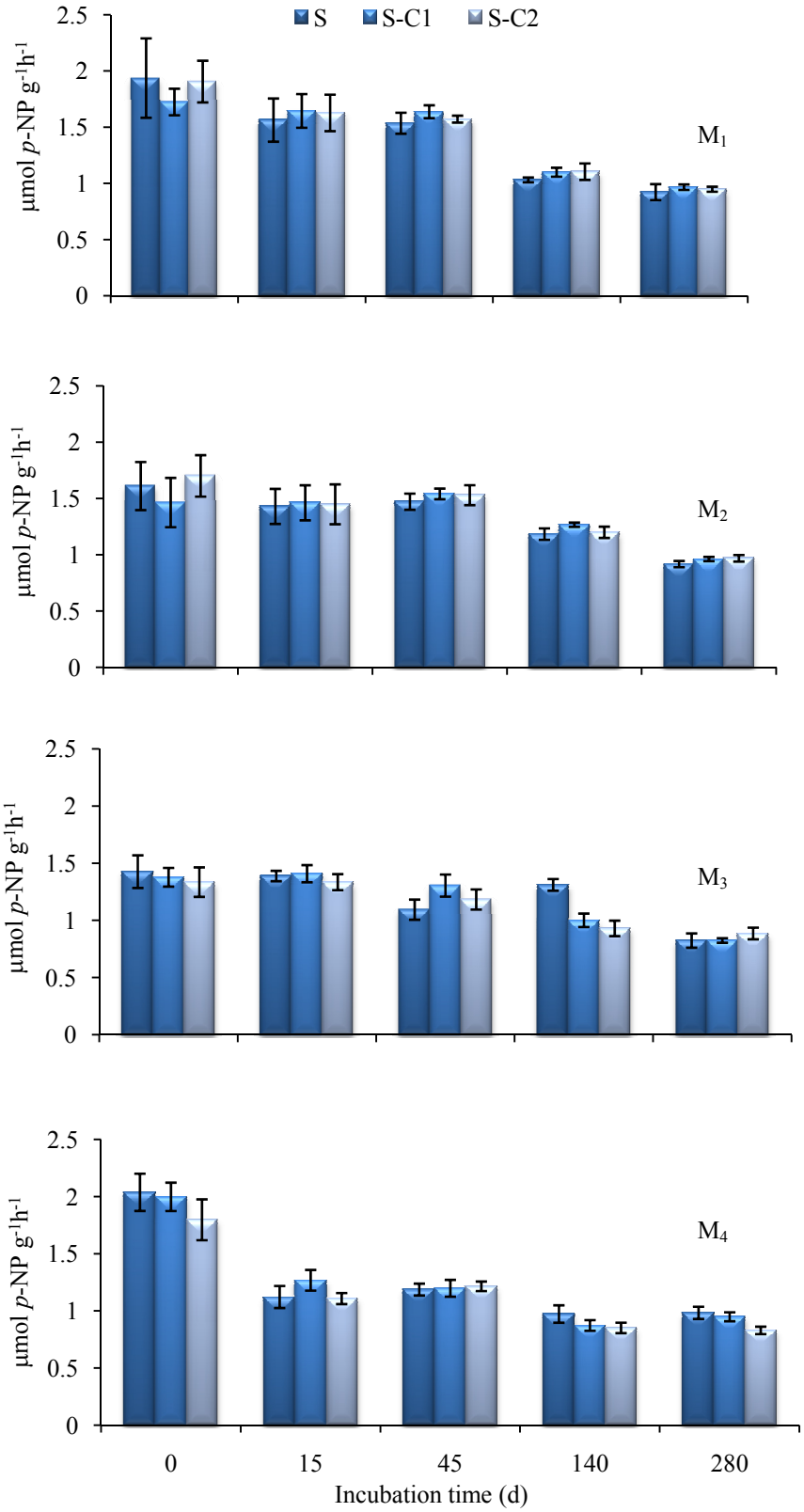

Fig. 3.11. β-glucosidase activity (µmol *p*-NP $g^{-1}h^{-1}$) in M_1, M_2, M_3 and M_4, (for details see Table 3.3).

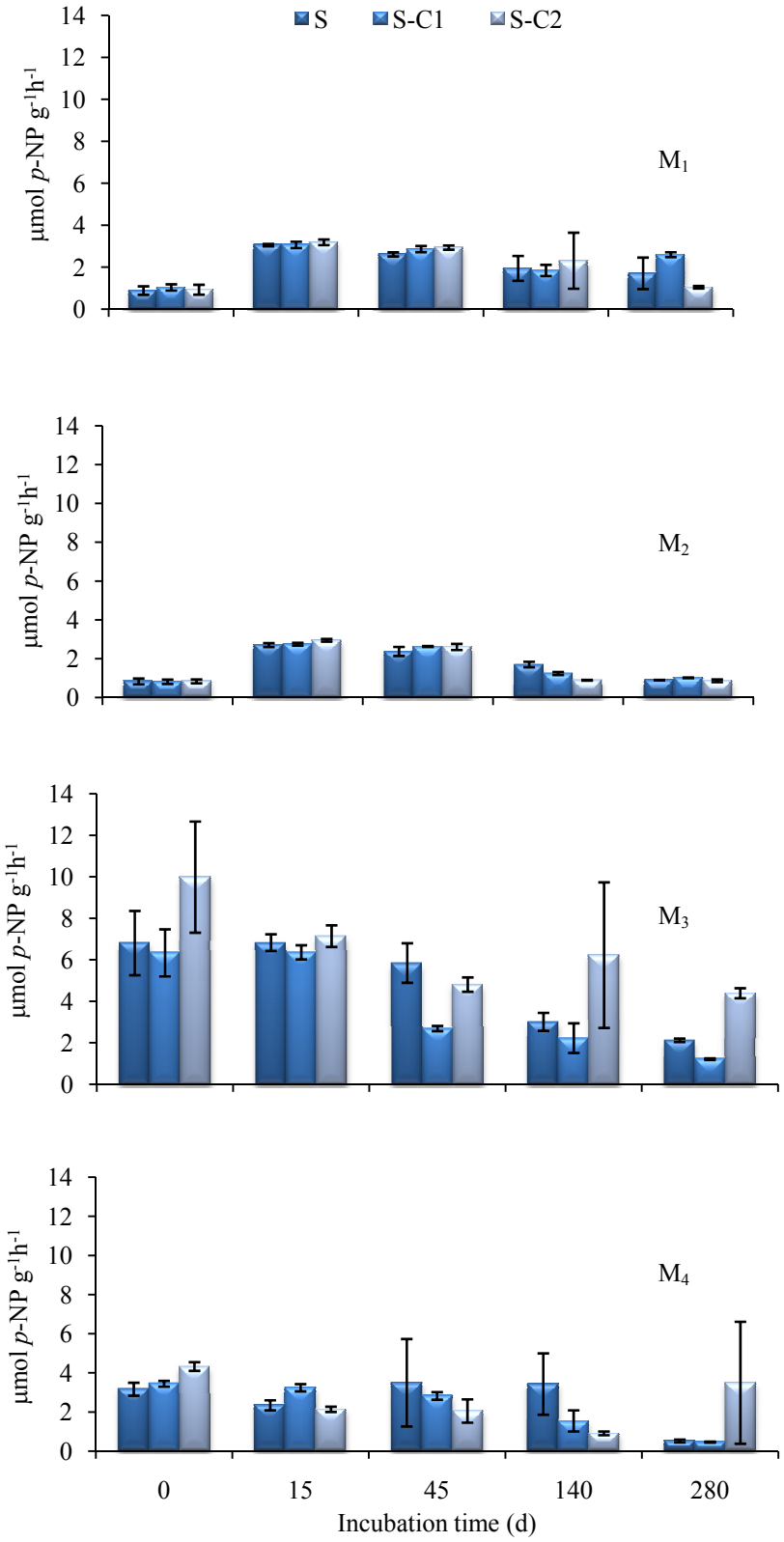

Fig. 3.12. Phosphatase activity (µmol *p*-NP $g^{-1}h^{-1}$) in M_1, M_2, M_3 and M_4, (for details see Table 3.3).

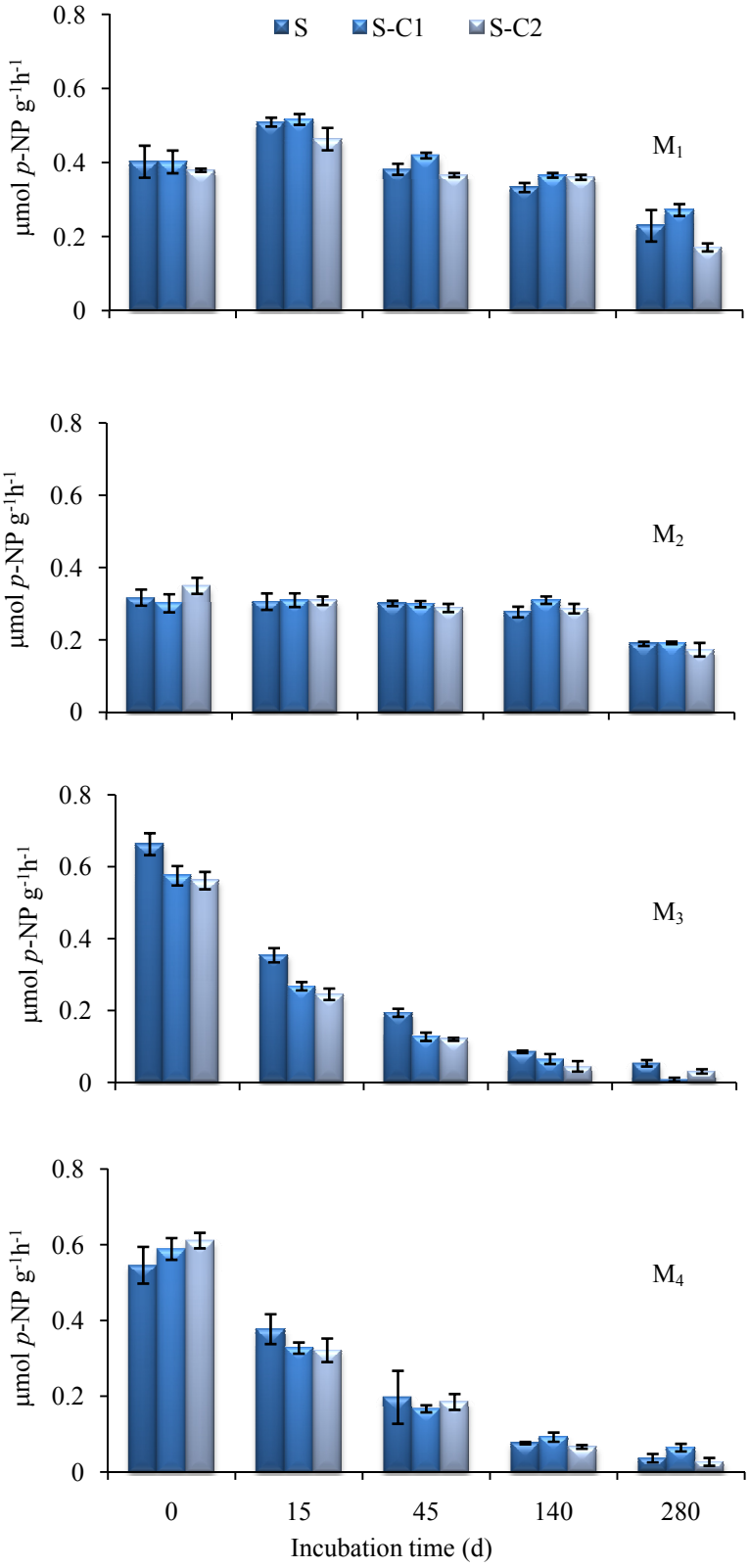

Fig. 3.13. Arylsulphatase activity (µmol *p*-NP $g^{-1}h^{-1}$) in M_1, M_2, M_3 and M_4, (for details see Table 3.3).

3.8. Discussion

3.8.1. Depletion of Phe in the investigated microcosms

The depletion of the Phe measured in the M_3 samples (Fig. 3.7) might be accounted for by real microbial degradation or ageing phenomena. Ageing processes usually lead to the adsorption and/or sequestration of organic compounds, including Phe (Nam and Alexander, 2001), into the soil matrix. The organic compounds become less, or even not bioavailable, and can be recovered only by exhaustive extraction procedures. In fresh, non-sterilized soil, transformations can be carried out by indigenous microorganisms provided conditions are appropriate for growth.

Evidence from several experiments would support the concept that microbial Phe degradation rather than a physical ageing phenomenon occurred in the M_3 microcosm (Fig. 3.7). The soil investigated very likely had an indigenous microbial Phe-degradative capacity. This was enhanced and stimulated by the addition of the lower amount of compost (there was a higher Phe disappearance constant in the S-C1 sample, Table 3.5). The addition of Phe to soil and to soil+compost samples led to a rapid increase of soil respiration and of microbial biomass (Figs. 3.8 and 3.9). Both properties are indicative of an increased microbial growth and activity. Similarly, the activities of PHO and ARY (Figs. 3.12 and 3.13), involved in the cycles of P and S (two nutrients linked to the growth of microorganisms), were higher in M_3 than in the control M_1.

It might be hypothesized that the indigenous bacteria of the soil revived their activity in the presence of a new carbon source during the incubation (and much more when an additional, more available carbon source was supplied with the compost) since the soil had not been sterilized. On the other hand, Cavalca et al. (2005) demonstrated that Phe-degrading strains were enriched by canonical procedures from the same agricultural soil.

Moreover, the possible adsorption and/or sequestration of the Phe in the soil matrix, that increased with ageing, seems to be negligible as the milder ethanol/*n*-hexane extraction gave values of the extractable Phe equal to those obtained with the Soxhlet extraction. The latter is considered to be an exhaustive, vigorous extraction capable of extracting PAH even if strongly immobilized in the solid matrices.

The inoculation of the soil systems with a Phe-degrading bacterial culture strongly accelerated the Phe degradation. The Phe-degrading bacterial culture used in this study was proven to be capable of degrading Phe, not only in batch liquid systems (Andreoni et al., 2004), but also when the Phe was entrapped in synthetic organic-matter complexes (Russo et al., 2005), or adsorbed to an organo-mineral soil colloid (Cavalca et al., 2008). The data in Fig. 3.6 further support the capability of the cell culture to degrade the Phe when a more complex solid matrix such soil is present in the batch system. This degradative capability was also maintained by the culture in the solid-state experiments.

The kinetics of the Phe degradation in the M_4 microcosm was characterized by a fast initial degradation phase (with respect to M_3 the disappearance constants were higher by two orders of magnitude, Table 3.5) and a subsequent second phase of a slower and diminishing degradation rate. Again, a beneficial influence was detected for C1. It is noteworthy that the time (15 d) to obtain the complete degradation of the Phe was very similar to that occurring in the soil slurry reactor (Fig. 3.6), as well as in liquid batch experiments with only Phe (Andreoni et al., 2004). A biphasic process was often observed for the degradation of numerous organic chemicals and described by single or multiple differential rate equations (Thiele-Bruhn and Brummer, 2005). Furthermore, apparent increases in

total Phe concentration observed during the experiment (Fig. 3.7) could be explained by changing Phe binding strength and extractability.

Although increasing amounts of compost usually enhance the degradation of PAHs (Kästner and Mahro, 1996), no apparent effects were observed with the higher compost dose in either the M_3 or M_4 microcosms. These results could be attributed to a possible inhibition towards the indigenous (and/or exogenous) soil microbial population by the higher concentrations of some compost components (*e.g.* heavy metals, salts). Probably, the higher dose of compost puts the microbial population under stress, thereby counteracting the beneficial effects of the organic substrate supply.

Moreover, the possible degradation of carbon compounds other than Phe, stimulated by the addition of higher concentrations of nutrients can be hypothesized (Johnson and Scow, 1999). The added nutrients could have induced shifts in the metabolism of Phe-degrading microorganisms, or favoured the growth of microorganisms not degrading Phe but competing with the Phe-degraders for available nutrients. A repression of Phe degradation may have resulted.

3.8.2. Responses of chemical and biochemical properties

The response of the chemical and biochemical properties was very diverse in the four microcosms. Furthermore, at the end of the investigation permanent changes were observed, mainly biochemical, for several of the properties investigated.

As a general response, microcosms with or without the bacterial cells (see M_2 vs. M_1 and M_4 vs. M_3) showed similar trends for several of the measured enzymatic activities (*e.g.* PHO, ARYL, GLU, and UR) during the course of the experiment. That would indicate that the intrinsic enzymatic

activity of the soil (and very often also for the soil+C1/C2) was not affected by the simultaneous presence of the Phe-degrading cells.

By contrast, the properties linked to microbial activity, such as respiration and dehydrogenase activity (its initial values) (Figs. 3.8 and 3.10) were generally enhanced by the presence of the cells, although differently in the presence or absence of the compost. Initial contrasting effects by the added cells occurred for the microbial biomass (Fig. 3.9). Moreover, the peaks observed for the evolved CO_2 during the first few days of incubation (again much higher in S-C1, Fig. 3.9) confirm the intrinsic capability of the soil to display a microbial activity, in this case enhanced by the addition of exogenous cells.

The similar response exhibited by most of the enzyme activities with or without the inoculated microbial cells is not unexpected. Various intracellular and extracellular enzymatic forms contribute quantitatively and qualitatively to the overall enzymatic activity of soil (Gianfreda and Bollag, 1996; Gianfreda and Ruggiero, 2006). Enzymatic categories may present different features and their relative composition in terms of both origins and locations may change with time and space. Furthermore, the response of each component to a given factor will probably differ and the final result will be the combination of different, individual changes (Gianfreda and Bollag, 1996; Gianfreda and Ruggiero, 2006).

Probably, the addition of the exogenous cells (and of the compost) might have influenced differently the activities of the different categories and enzymatic fractions and their relative composition. No significant changes might have resulted of the whole activity of a given soil enzyme.

3.9. Conclusions

In conclusion, the results presented here demonstrate that a natural attenuation process occurred in the soil investigated, which showed an intrinsic capability of degrading Phe. The addition of a limited dose of compost, as well as the inoculation with a Phe-degrading bacterial culture strongly stimulated and enhanced the attenuation process. Furthermore, several of the soil properties showed differentiated responses to the presence of the Phe, the compost, and/or the exogenous culture. Temporary and permanent changes occurred showing that soil biological investigations (such as soil respiration, biomass, and enzyme activities) can give information about the intensity and the kind and duration of the effects of pollutants on the metabolic activity of soil. Such investigations are thus well suited for measuring the effects of pollution on soil health and to act as a monitoring tool for the decontamination process of a polluted soil.

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Chapter 4

Influence of ageing on bioremediation of phenanthrene[1]

In the previous Chapter, the effects of fresh spiked phenanthrene on the main properties of an agricultural soil were studied. The results showed different responses due to the presence of the contaminant, particularly during the first stage of incubation.

Furthermore, the addition of compost and microbial culture able to degrade phenanthrene appeared to be very efficient in enhancing the natural attenuation process occurred in the soil system.

If a contaminant persists in soil for long time it is subjected to the ageing process. This will affect not only the properties of the contaminant but also the response of the soil to remediation approaches. Moreover, the status of the soil, as a whole in terms of chemical, biological and biochemical properties, will be also affected.

As persistent rather than fresh PAH-contamination of soil is more common in polluted sites, it appeared interesting to investigate the effects of aged phenanthrene on soil properties and the capability of the compost and the microbial culture on its remediation.

[1]A version of this Chapter has been prepared for publication as:

Scelza R., Rao M.A., Gianfreda L.. Properties of an aged phenanthrene-contaminated soil and its response to bioremediation processes.

This Chapter is dedicated to establish, in a long-term experiment under laboratory conditions, the effect of ageing on the main biochemical and chemical properties of an agricultural soil, artificially contaminated with phenanthrene and aged for two years, and the efficiency of both the phenanthrene-degrading bacterial culture and of the compost on the disappearance of the compound.

Functionally related properties such as several soil enzyme activities (hydrolases and oxido-reductases) involved in the cycles of the main biological nutrients C, N, S and P were tested before and after ageing. The variations of the main physical-chemical properties (*i.e.* pH, total organic C and N, phosphorous) were also monitored.

4.1. Introduction

The sorption of organic compounds to soils and sediments is an important process controlling their environmental fate and effects. Organic compounds that persist in soil exhibit declining extractability and bioavailability to microorganisms and other soil organisms (including earthworms and invertebrates), with increasing contact time or 'ageing' (Hatzinger and Alexander, 1995; White and Alexander, 1996; Kelsey et al., 1997).

In the past it was assumed that these observations were due to the degradation of contaminants by microbial processes in soil. However, studies utilizing isotopically labelled compounds have demonstrated that significant amounts of compound are retained in soil as non-available and non-extractable sequestered residues (Northcott and Jones, 2000) (Fig. 4.1).

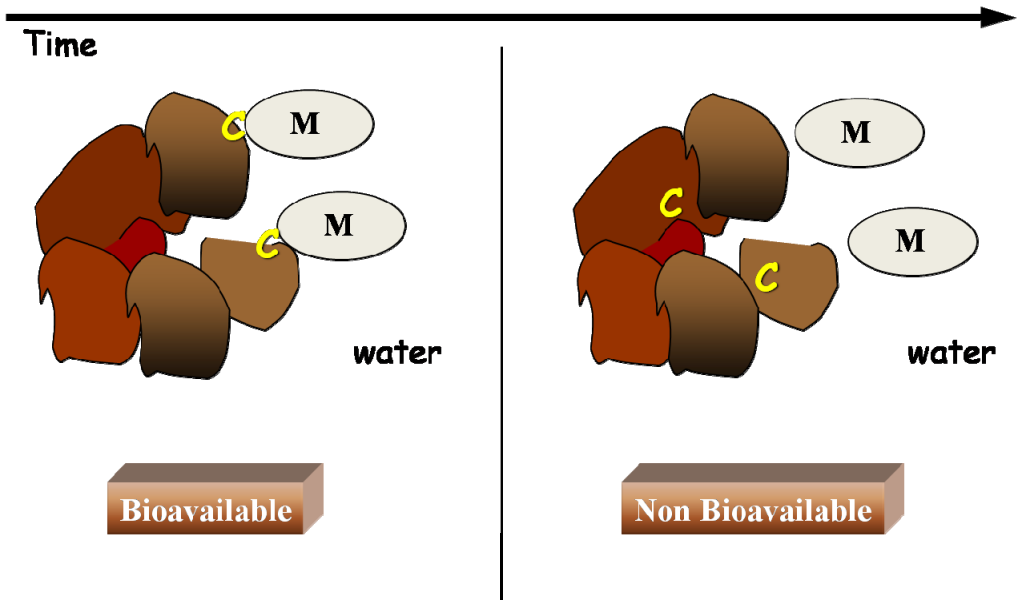


Fig. 4.1. Sequestration from diffusion into the solid portion of soil. C = Contaminant, M = Microorganisms.

Although the ageing phenomenon does exist in the environment, it is still not clear which mechanisms are involved in it (Nam et al., 2003). Among the possible mechanisms are the association of organic compounds with natural organic matter (Carroll et al., 1994) and the penetration of contaminants into small pores in soil (Wu and Gschwend, 1986).

Some experimental observations showed different competitive effects in the sorption of organic contaminants (Xing et al., 1996). According to this model, natural organic matter has two different sorptive domains that interact with organic contaminants: partitioning domain and hydrophobic hole domain. The hydrophobic hole domain exhibits competitive sorption behaviour and may be responsible for the desorption and extraction-resistant fractions of aged contaminants. This concept is consistent with the findings that small pores with hydrophobic surfaces are responsible for resistant desorption (Werth and Reinhard, 1997) and declined bioavailability of contaminants to bacteria (Nam and Alexander, 1998).

Weber and Huang (1996) proposed that the hydrophobic hole domain is located between loose, amorphous humic materials and mineral surfaces and is composed of highly compact humic materials, which is typical to humin. Earlier studies have demonstrated that humin has macromolecular aliphatic chains as major constituents (Almendros and Gonzalez-Vila, 1987; Almendros and Sanz, 1991) and has significant amounts of small pores on its surface (Malekani et al., 1997). In these regards, it seems reasonable to hypothesize that the hydrophobic hole domain (which is proposed to be responsible for persistence of organic compounds) may exist in humin fraction of soil organic matter.

Ageing is toxicologically significant because the assimilation and acute and chronic toxicity of harmful compounds decline as they persist and become increasingly sequestered with time (Alexander, 2000). Although ageing

reduces toxicity, it does not eliminate exposure and risk. A time-dependent decline in bioavailability does not always occur. This may be related to properties of the soil or of the compound.

The extent of ageing differs between soils (Hatzinger and Alexander, 1995; Chung and Alexander, 1998, 2002) and may also be affected by environmental factors such as drought (White et al., 1997). However, the soil organic carbon content has been found to be the major determinant for ageing of organic substances (Nam et al., 1998).

Nam et al. (1998) found that the bioavailability of phenanthrene to microorganisms was reduced after 200 d for soils with an organic carbon content higher than 2%, whereas no such ageing effects were evident in soils with an organic carbon content less than 2%. The degree of ageing may also depend on the concentration of the contaminant in the soil (Chung and Alexander, 1999).

As stated above, organic compounds become sequestered as they age or persist in soil. This sequestration results in a reduced bioavailability of contaminants to bacteria (Nam et al., 1998) and higher organisms such as plants (Bowmer, 1991). From a microbial perspective, soils containing an aged contamination can be considered oligotrophic (Wick et al., 2003) and harsh environments for microbes to proliferate. Nevertheless, some microorganisms are able to degrade aged compounds, especially PAHs.

Uyttebroek et al. (2006) found that *Mycobacterium* strains were specialized in proliferating in the oligotrophic environment of PAH-contaminated soil and in degrading sorbed PAHs; in particular *Mycobacterium* is associated with the clay fraction of contaminated soils; this fraction provides food sources and nutrient (Kandeler et al., 2000; Sessitsch et al., 2001) to the microorganisms and help them to remain active and competitive in the oligotrophic environment (Wick et al., 2003). Anyway, the behaviour of

aged compounds is much different from that of freshly added chemicals (Loehr and Webster, 1996) and bioavailability is one of the main factor limiting their biodegradation.

An important aspect of ageing is its possible mitigation or even elimination of the negative effects that an organic pollutant may have on the biological, biochemical and phytotoxic properties of the contaminated soil. Several findings have demonstrated that the addition of pollutants and mainly of recalcitrant compounds such as PAHs might induce temporary and permanent changes in several chemical and biochemical soil properties (i.e. biomass, enzyme activity) (Andreoni et al., 2004) as well as in soil phytotoxicity, as assessed by germination tests (Henner et al., 1999). Additionally, the copresence into the soil of compost, capable of sustaining diverse populations of microorganisms (Kästner et al., 1995; Kästner and Mahro, 1996) and acting as soil ameliorant (Semple et al., 2001) may not only influence the distribution and behaviour of the pollutant but also the soil properties. With time the soil system will reorganize itself and will try to return after disturbance to its original or to a new dynamic equilibrium.

Laboratory tests suffer from several problems associated with the interpretation of the data in terms of issues in the field because it is not yet clear how ageing in nature should be simulated in the laboratory and because of the possibility that additions of pollutants in a convenient solvent may introduce artefacts (Alexander, 2000). Typically, the bioremediation of soils containing PAHs, although reducing the concentration of many individual compounds, does not rid the treated site of PAHs because the microorganisms are present, the environmental conditions are conducive to their activity, but somehow the compounds are inaccessible.

The view that the contaminants became sequestered as they reside in the field gains credence in light of the finding that biodegradation of the seemingly resistant PAHs takes place if they are extracted and then added back to soil (Alexander, 2000).

4.2. Case study

Chapter 3 has been dedicated to the response of an agricultural soil to a fresh phenanthrene contamination. The capability of a mixed microbial culture and of a compost to degrade the contaminant have been evaluated. Positive results were obtained in both investigated bioremediation processes. In this Chapter the research is addressed to test the potential of the same microbial culture and of the compost to remediate a soil contaminated with phenanthrene and subjected to a 2-year ageing.

As for the fresh Phe-contaminated soil the main biochemical and chemical properties of soil have been measured before and after the ageing process. The capability of the soil to sustain the seed germination of two plants, *Cucumis sativus L.* and *Lepidium sativum L.*, with different sensitivity to the presence of contaminants has been evaluated, as well.

4.3. Materials and Methods

4.3.1. Chemicals

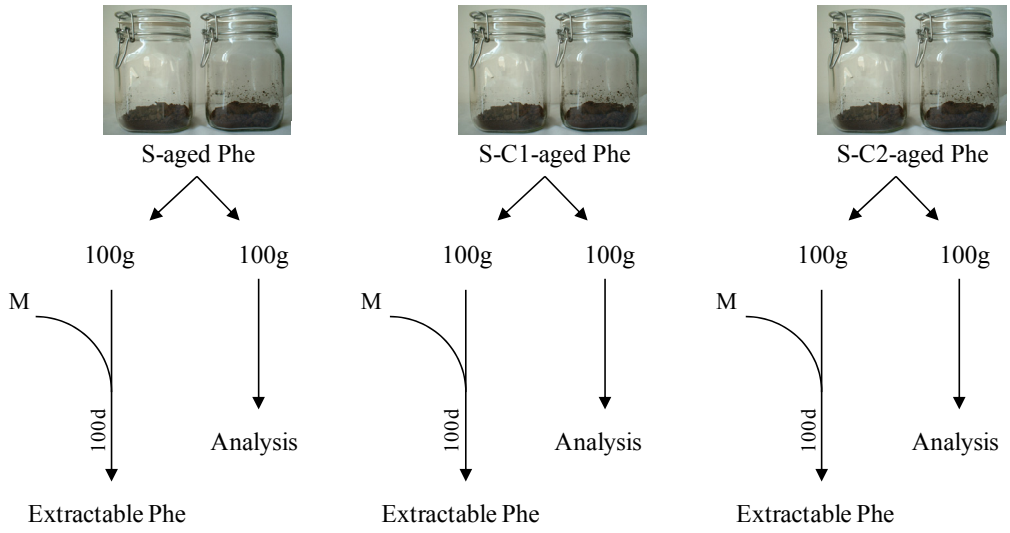
Reagent-grade Phe (>99% purity) and HPLC-grade solvents were purchased from Sigma Aldrich (Germany). All other chemicals, reagent grade, were supplied by Analar, BDH (Germany), unless otherwise stated.

4.3.2. Experimental design

Fresh soil (S) was air-dried to 14% moisture content and passed through a 2-mm sieve. Then it was placed (100 g) in closed 1 l glass jars. The compost was added at two different doses, 0.27% (C1) and 0.83% (C2), corresponding to a field rate of 10 and 30 t ha^{-1} of compost, respectively. Phenanthrene was spiked into the soil as described in details in Chapter 3.

Three samples were obtained: S-Phe, S-C1-Phe, and S-C2-Phe. Two replicates were performed for each sample. The samples were placed in the dark in a climatic, moisture-controlled chamber set at 25 °C. One set of samples was suddenly analysed and this was considered the zero time of incubation. After 650 days of incubation, the remaining samples were analysed for chemical and biochemical analyses and phenanthrene extraction, as well. Then they were mixed and split again into two replicates of 100 g each (Scheme 4.1). One replicate was subjected to biochemical analyses, the other one was further split in two samples, 50 g per each and only one was seeded with the phenanthrene-degrading culture, already described in Chapter 3.

Seeded samples (S-B, S-C1-B, S-C2-B) were incubated for 100 days under conditions previously described (see above) and periodically, little amounts (<1g to not disturb the system) of soil were taken (no destructive replicates) for phenanthrene detection.



Scheme 4.1. Experimental design. M = Microbial culture.

4.3.3. Soil chemical and biochemical properties

The characterization of the soil and compost has been described in Chapter 3 (see Table 3.4).

Chemical analyses were performed in triplicate on air-dried and sieved (<2 mm) soil samples as described in Chapter 3. Total organic C (TOC) and total N (TN) were measured by the ash combustion procedure with a Fisons 1108 Elemental Analyzer, calibrated with appropriate standards (acetanilide). To obtain homogeneous samples, soils (25-30 mg) were air-dried, pounded and sieved at 0.5 mm prior to analysis. Accuracy (<0.05%) and recovery of C and N (for both instrument detection limit 10 mg kg^{-1}) were checked by analyzing a sample of the standard material after each set of eight sample analyses.

Activities of arylsulphatase, β-glucosidase, phosphatase, urease enzymes and dehydrogenase were detected as described in detail in Chapter 3.

4.3.4. Germination tests

Germination tests were performed on contaminated soil amended or not with compost (APAT, 2004), before and after 650 days of incubation. *C. sativus L.* and *L. sativum L.* seeds were incubated for 72 h at 25 ±2 °C in the dark on 10x90 mm Petri dishes, equipped with soil and soil+compost (10 g dw) contaminated with phenanthrene. Control tests were carried out with distilled water and uncontaminated soil. A primary root >2 mm was considered as the end germination point. Experiments were performed in 4 replicates. The relative germination R.G. = $100 \cdot (G_s/G_c)$ and the germination index G.I. = $100 \cdot (G_s/G_c)$ (L_s/L_c) were calculated for each treatment where G_s and G_c are the numbers of roots germinated in the sample and control, respectively, and L_s and L_c are the roots length in the sample and control, respectively.

4.3.5. Fractionation of humic substances

After ageing of phenanthrene for 650 days, the soil and soil-compost samples were fractionated into fulvic acid (FA), humic acid (HA), and humin-mineral (HU) fraction, as described by Nam and Kim (2002), and the Phe was then extracted from each fraction. Briefly, 10 g of the phenanthrene-aged soil was transferred to a 250-ml Teflon centrifuge bottle and 100 ml of 0.1 N NaOH solution was added to the bottle. The suspension was shaken on a horizontal shaker (200 rpm) for 24 h at room temperature. The dark brown coloured supernatant containing humic and fulvic acids was separated from the residual soil solid by centrifugation (12860 *g* for 20 min). The precipitated solid was considered as a humin-mineral fraction. The solid was recovered and washed with distilled water until its pH reached about 7. The supernatant was acidified with

concentrated hydrochloric acid (pH <1) to precipitate humic acid fraction. The resulting solution contained fulvic acid.

4.3.6. Phenanthrene extraction and detection

Phenanthrene from the soil and soil-compost samples was extracted and detected as described in Chapter 3.

Phenanthrene was extracted from the three humic fractions according to Nam and Kim (2002). The acidified solution containing fulvic acid was mixed with 20 ml of *n*-hexane in a 250-ml Teflon centrifuge bottle, and the mixture was shaken on a horizontal shaker (200 rpm) at room temperature. After shaking for 24 h, 10 ml of *n*-hexane layer was recovered and concentrated to less than 1 ml by using a rotary evaporator.

Phe from humic acid fraction was recovered using the mixture of hexane and *n*-butanol. Twenty millilitres of *n*-hexane and 5 ml of *n*-butanol were mixed with the phenanthrene-humic acid fraction, and the suspension was shaken for 16 h at room temperature on a horizontal shaker (200 rpm). After shaking, the solvent mixture was recovered from the humic acid fraction by centrifugation (18600 *g* for 20 min) and the solvent was concentrated to less than 1 ml by evaporation as described above.

For extraction of phenanthrene from humin-mineral fraction, 20 ml of *n*-butanol was added to a 50-ml Teflon centrifuge tube containing the solid, and the suspension was mixed with a vortex mixer for 1 min. The solvent-soil mixture was then shaken vigorously on a horizontal shaker (200 rpm) for 16 h at room temperature and centrifuged at 18600 *g* for 20 min.

The extract was concentrated to less than 1 ml by evaporation as described above. Solvent extracts from each component of humic substances were analysed by high-pressure liquid chromatography under the same operative conditions described in Chapter 3.

4.3.7. Statistic analysis

All data were subjected to analysis of variance by using SPSS for Windows, Version 15.0. The assumptions of normality and homogeneity of variances were tested by the Kolmogoroff-Smirnoff test and the Levene test. The significant differences between means at $P<0.05$ were assessed according to Tukey's multiple comparison test.

4.4. Results

4.4.1. Phenanthrene removal

According to what observed for the fresh spiked phenanthrene experiment, the amount of the extractable contaminant decreased with time. At the beginning of the experiment (zero time) a natural attenuation was registered (Fig. 4.1). The best recovery of Phe was observed with the higher amount of compost, about 83% of the initial amount as respect to 76% and 68% of the control and the soil amended with the lower compost amount. A 26% reduction (on average) of extractable Phe was, therefore, observed immediately after Phe spiking and confirmed what already observed in Chapter 3.

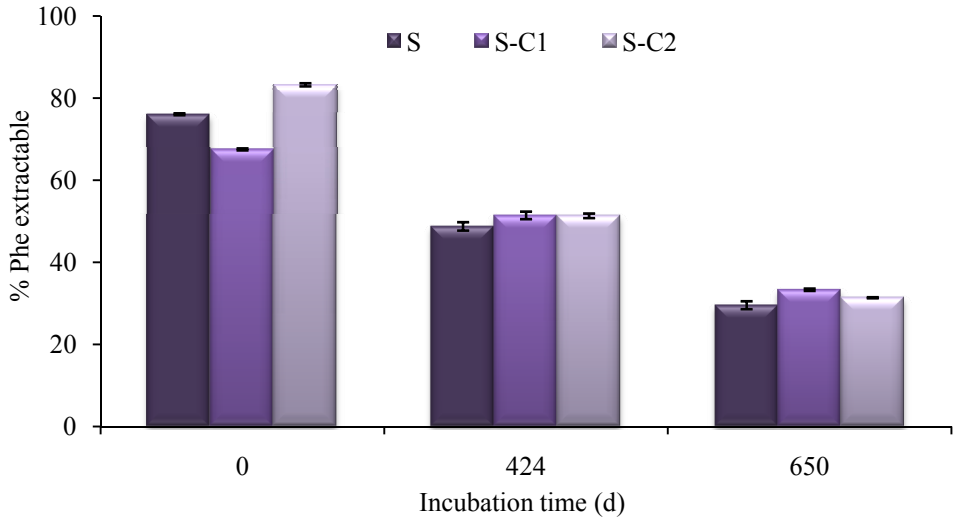


Fig. 4.1. Extractable phenanthrene in S, S-C1 and S-C2.

During the time, all the samples showed the same trend, characterized by a slow decline reaching values close to 30-35% of extractable phenanthrene after 650 d of incubation. No differences were observed for both compost amounts. After 650 d, soil samples were seeded with the phenanthrene-degrading culture and the extractable phenanthrene was monitored for further 100 d (Fig. 4.2).

A strong decrease to values close to zero was observed during the 100 d incubation period. The samples showed, however, a different trend with time. In particular, during the first 14 d, quite constant values were observed for all the samples except for S-C2-B where the amount of extractable Phe decreased to 15% after 14 d. A strong decline was observed for all the samples but at different times, in particular for S, S-C1 and S-C2 after 14 d, for S-B after 21 d, for S-C1-B after 50 d and for S-C2-B after 7 d of incubation. After further 7 d incubation (21 d) the values strongly decreased for S, S-C2 and S-C2-B, whereas they remained quite constant for S-B, S-C1 and S-C2-B. In any case, all samples reached the same value after 100 d of incubation.

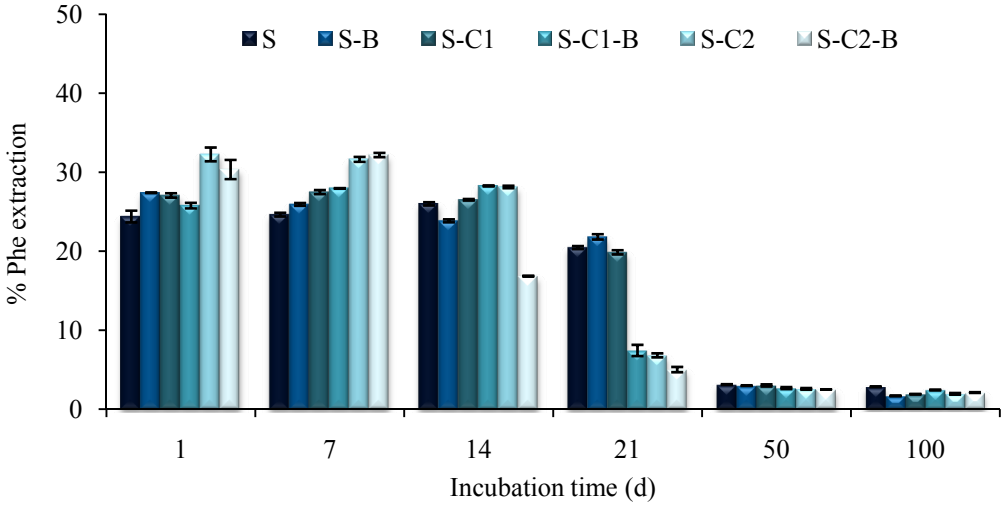


Fig. 4.2. Extractable phenanthrene after microbial culture (B) addition.

4.4.2. Fractionation

Fig. 4.3a shows the concentrations (mg l^{-1}) of Phe extracted from each humic fraction of the three analysed samples. The amounts of Phe extracted from the three humic fractions significantly differed from each other. In particular, the highest amount of Phe was recovered from HU whereas the lowest resulted in FA for all the samples with amounts close to zero. Fig. 4.3b reports the comparison of the amounts of Phe extracted from the 650 d aged unfractionated samples (Fig. 4.1) and the total of Phe amounts recovered in the three fractions (sum of the values shown in Fig. 4.3a).

Higher concentrations of Phe were measured after extraction from humic fractions as compared to unfractionated samples. In particular, the extracted Phe ranged from 6 to 8-fold recovered from unfractionated samples. And the humin fraction contributed more than 78% to this amount.

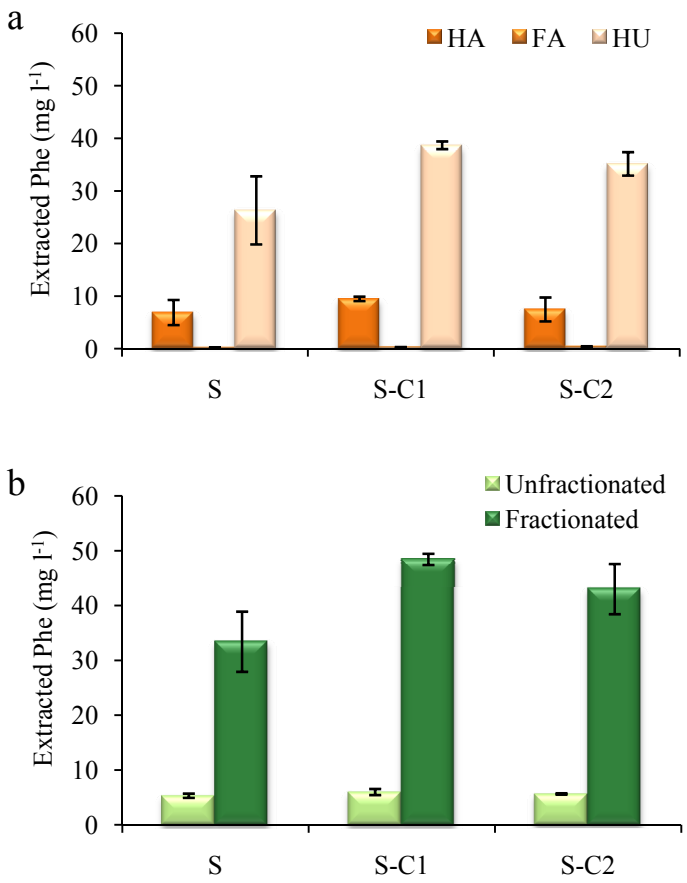


Fig. 4.3. Extracted phenanthrene (Phe) (mg l^{-1}) in 650 d aged samples. Each value is the mean of two replicates. (a) Distribution of the extractable Phe in the three humic fractions, fulvic acids (FA), humic acids (HA) and humin (HU). (b) Amounts of the extractable Phe in the fractionated and unfractionated samples.

4.4.3. Chemical properties

The main chemical properties were measured and no significant differences were observed during the incubation time (Table 4.1). In detail, pH values were so close for all the samples, at zero time as after 650 d, even if a little increase of pH was observed at the end of the experiment. Total organic carbon was not significantly affected by compost addition, even if S-C1 showed higher values at zero time as respect to S and S-C2, while a little increase due to ageing process was detected for the amended samples. Phosphorus values strongly increased during ageing ranging from 61.3% for S, 67.3% for SC1 and 77.9% for SC2. Also total nitrogen values slightly increased after ageing period especially for amended samples.

Table 4.1. Chemical properties before and after ageing process.

Property								
	pH		TOC ($g\ kg^{-1}$)		TN ($g\ kg^{-1}$)		P_2O_5 ($mg\ kg^{-1}$)	
d→	0	650	0	650	0	650	0	650
S	7.96±0.1	8.19±0.0	26.18±0.2	24.38±0.2	2.49±0.0	2.42±0.0	22.88±0.6	37.33±0.3
S-C1	7.92±0.0	8.29±0.0	27.27±0.2	28.55±0.3	2.56±0.0	2.70±0.0	23.31±0.6	34.64±0.5
S-C2	7.90±0.0	8.26±0.0	25.72±0.1	27.22±0.1	2.41±0.0	2.63±0.0	28.71±3.4	36.84±0.9

4.4.4. Enzymes activities

Ageing process did not appreciably affect enzyme activities (Figs. 4.4 and 4.5). No significant differences due to compost addition were observed for phosphatase activity at the zero time of incubation. After 650 days of incubation, the activity was higher in non-amended soil (4 μmol *p*-NP g^{-1} h^{-1}) and a little difference was observed for S-C1 and S-C2 samples, in particular S-C1 showed higher values of activity (3.36 μmol *p*-NP $g^{-1}h^{-1}$).

Differences between samples neither time-dependent nor due to compost amounts were observed for GLU activity (values on average of 1.090 μmol *p*-NP $g^{-1}h^{-1}$).

All samples showed similar ARYL activities. A strong increase of the activity was however observed for all the samples after ageing process (2.7 μmol *p*-NP $g^{-1}h^{-1}$ after 650 d on average against 1.2 μmol *p*-NP $g^{-1}h^{-1}$ measured at the zero time of incubation).

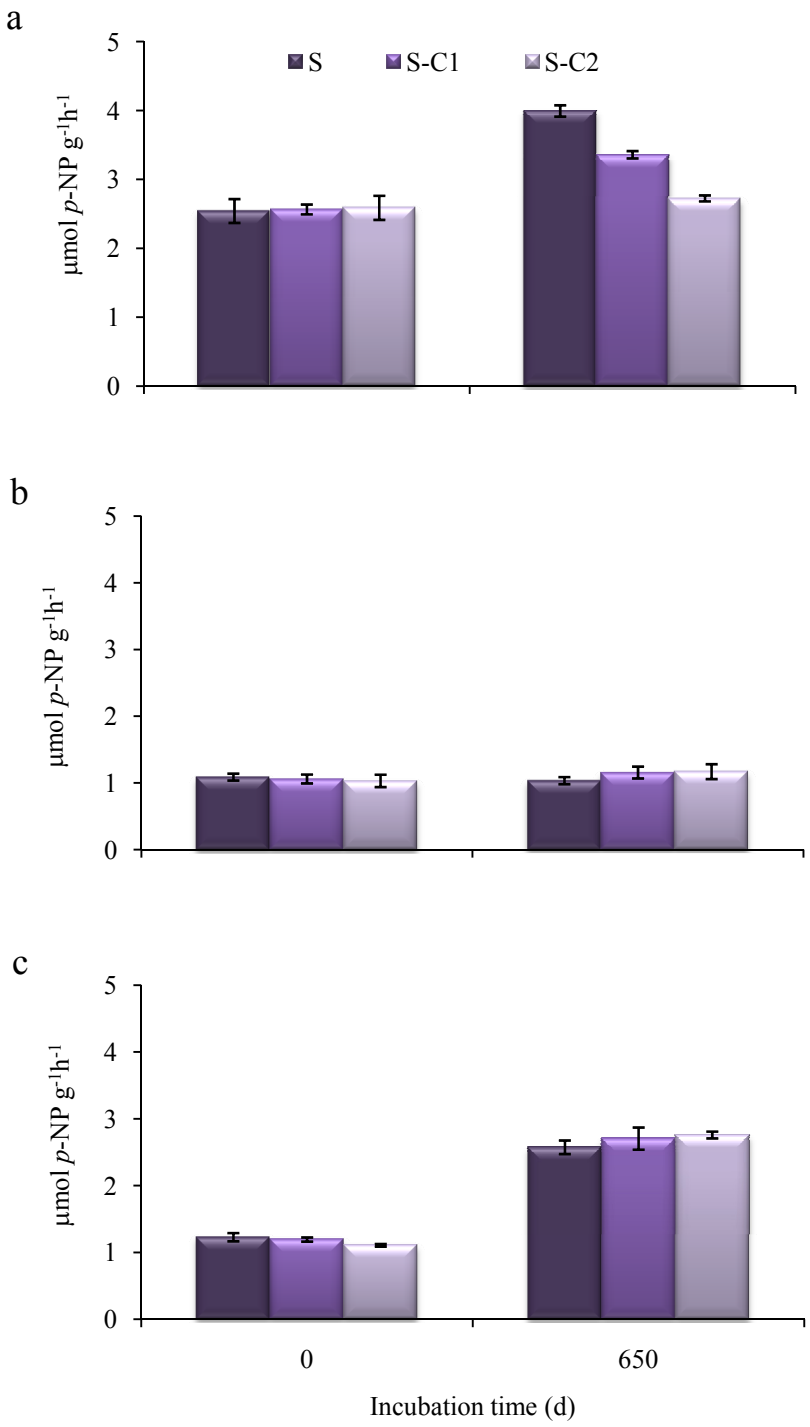


Fig. 4.4. Phosphatase (a), β-glucosidase (b) and arylsulphatase (c) activities (µmol *p*-NP $g^{-1}h^{-1}$) before and after ageing process.

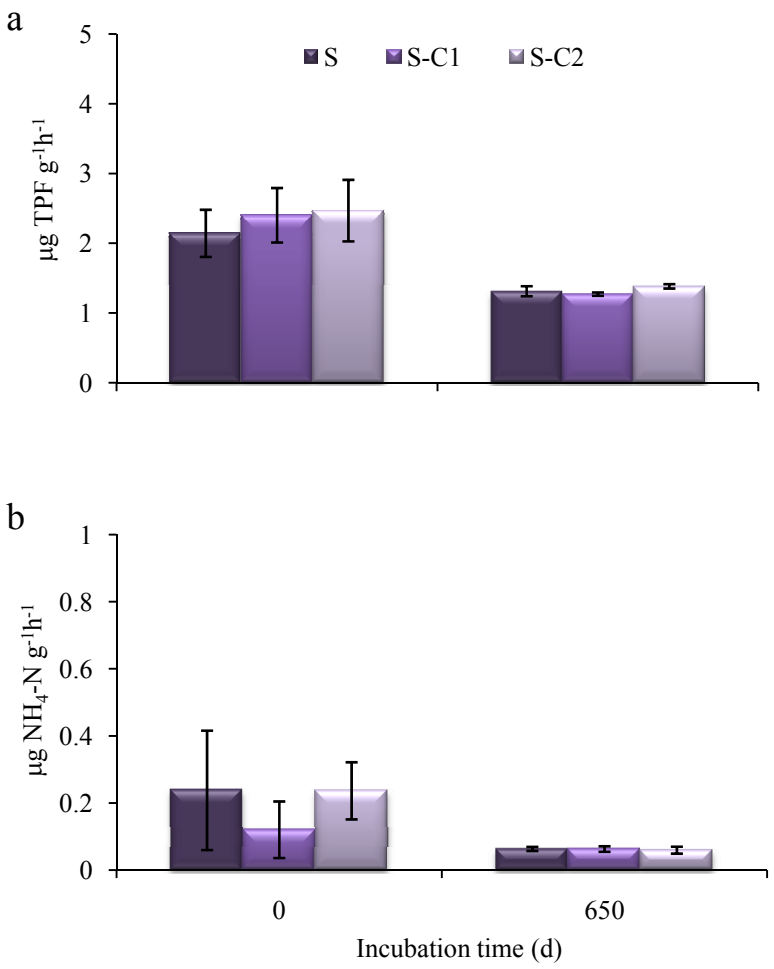


Fig. 4.5. Dehydrogenase (µg TPF $g^{-1}h^{-1}$) (a) and urease (µg NH_4-N $g^{-1}h^{-1}$) (b) activities before and after ageing process.

A little decrease of dehydrogenase activity (by 43% on average) was observed during the time with no differences between the samples (Fig. 4.5a).

Similar values of UR activity were obtained at zero time for all the samples (Fig. 4.5b). After 650 d of incubation a no significant decrease was observed, with no differences among the samples.

4.4.5. Germination tests

As respect to non-contaminated samples, the presence of aged phenanthrene drastically reduced the relative germination (R.G.) in soil from 94.9% to 50% for *C. sativus* seeds and completely annulled that for *L. sativum*. Compost addition, especially the higher amount C2, enhanced this negative effect (Table 4.2).

Correspondly, lower or null values of G.I. were measured with *C. sativus* and *L. sativum* respectively.

Tests performed with fresh Phe-contaminated soils gave similar results, although the effect of Phe was more marked. Indeed, slightly lower R.G. and G.I. values were calculated as respect to controls (data not shown).

These results clearly indicate that aged phenathrene was very phytotoxic, in particular for *L. sativum*, more sensitive to the contaminant effect (Fig. 4.6). The presence of a little compost amount appeared to slightly reduce the phenanthrene phytotoxcity, whereas higher amounts greatly enhanced it.

Table 4.2. Relative germination percentage (R.G.) and germination index (G.I.) of aged-soil samples before and after ageing process.

	Cucumis sativus L.		*Lepidium sativum L.*	
	R.G. (%)	G.I. (%)	R.G. (%)	G.I. (%)
S	94.9	177.8	100	271.6
S-C1	95.1	178.1	100	273.8
S-C2	93.8	175.0	100	266.3
S-aged Phe	50.0	24.2	0.0	0.0
S-C1-aged Phe	43.3	33.5	2.9	0.2
S-C2-aged Phe	30.0	19.5	0.0	0.0

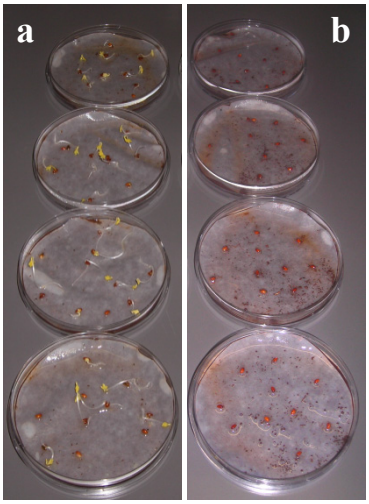


Fig. 4.6. Seeds of *C. sativus* in non-contaminated soil (a) and Phe-contaminated soil (b).

4.5. Discussion

The depletion of phenanthrene after 650 d of incubation, before the inoculum with the Phe-degrading culture, could be attributed to the presence of two concomitant processes: a natural attenuation process and ageing. The initial Phe concentration (Fig. 4.1) was about 13.7 mg kg^{-1} in S, 12 mg kg^{-1} in S-C1 and 15 mg kg^{-1} in S-C2. After 650 d losses by approximately 46.5%, 34.2% and 52.0% were measured, respectively. As the soil was non-sterile, they could be attributed to Phe degradation by active indigenous microorganisms. Nevertheless, an ageing phenomenon can not be excluded. Indeed, it is well known that persistent organic compounds show a declining availability to microorganisms, invertebrates, and plants with increased residence time in soil.

The extent of this sequestration varies among soils. PAH sorption onto soils is highly dependent on the soil organic fraction, especially the composition of the organic matter, which, according to Gaboriau and Saada (2001), is the primary factor controlling phenanthrene retention by the soil. Soil used in this experiment had 2.5% of organic carbon. According to

Nam et al. (1998), the decline in Phe-extractability with increasing time of persistence would be more rapid in soils with >2.0% organic C.

The higher recovery of Phe after fractionation of humic substances (Fig. 4.3b) seems to support this hypothesis. Higher Phe amounts were sequestered in humic fractions; in particular they were associated with the humin (Fig. 4.3a) in all the samples. This is not surprising since the large surface area of humin phase allows for greater sorption of the contaminant than in the other fractions of the soil (Kamath et al., 2005).

Indigenous microorganisms screened from polluted soils were, often, more effective to metabolize PAHs than organisms obtained from elsewhere in bioremediation (Chapter 3). The microbial Phe-degrading culture used to seed the soil samples had been isolated from a PAH-contaminated soil (Andreoni et al., 2004) and its capability to degrade the contaminant, under different bioavailability conditions, had been detected. In the Chapter 3, it was demonstrated that the culture was very efficient when inoculated in a fresh Phe-spiked soil, and its efficiency manifested suddenly after its inoculation into the contaminated samples. This to indicate that no a lag acclimatization phase was necessary to the culture to start Phe degradation.

In the case of the Phe-aged soil, the culture seems to have not the same capabilities (Fig. 4.2). In fact, in the first 14 d of incubation, no declining of extractable Phe was observed neither in the seeded nor in the non-seeded samples. After 21 d a strong reduction of extractable Phe occurred and its amount remained practically constant until the end of the observation period. Probably, the culture needed a period to acclimatize itself to the system conditions or to implement suitable strategies to access the less available Phe, and thereafter to start Phe utilization. Indeed, microorganisms have developed a range of strategies to access sorbed or sequestered compounds. They can be summarized as follows: waiting for a

new equilibrium state; creating concentration gradients; causing microenvironmental pH shifts; producing surfactant, solvents, and chelators; secreting extracellular enzymes; and degrading exposed substituents. All of them require a certain brief or long lapse of time before being effective.

It is also interesting to highlight that as the incubation proceeded (*i.e.* at 50 and 100 d of incubation) a reduction of the extractable Phe was noted also in non-seeded samples (S, S-C1 and S-C2 in Fig. 4.2). These results could be explained by two phenomena possibly occurring in a soil when endogenous (resident) and exogenous microorganisms are simultaneously present: the limited persistence and effectiveness of exogenous microorganisms as compared to the indigenous microflora, due to competitive effects by resident microbes (Allard et al., 2000), and alternatively the capability of the endogenous microflora to regain its degradative activity towards the aged contaminant, once its transformation has occurred by the action of exogenous microbes with consequent production of less complex organic products, and very likely more accessible as carbon sources. The possible use of biomass of the microbial inoculation for resident microorganism nutrition could be also hypothesized.

Aged Phe did not strongly affect soil enzymatic activities. Some of them, in particular arylsulphatase and phosphatase activities, showed higher values after 650 d of incubation in the presence of Phe. This can be explained by the reduction of toxicity of an aged compound due to its not complete availability, being very likely sequestered in non accessible sites of the soil. Therefore, the indigenous bacteria of the soil could have expressed their activity, and in turn some of their enzymatic activities, such as when the contaminant was absent. This hypothesis seems, however, to be

contradicted by the detectable decrease measured for dehydrogenase and urease in the presence of the aged Phe. In Chapter 3, the two enzymes were shown to be very sensitive to Phe presence. Even negative, not measurable, values of urease activity were detected in the microcosms contaminated with Phe (M_3 and M_4). Conversely, the lower but still measurable values of both activities in the aged soil indicate that a detectable microbial activity is present when the contaminant is under a less available status.

In contrast to what Henner et al. (1999) observed, germination tests showed that even if not available, Phe can strongly inhibit seed germination. Data obtained with fresh Phe solutions and fresh Phe-spiked soils have demonstrated that Phe was toxic at very low concentrations to both the two plant species used. Evidently, the amount of Phe still present as available after the ageing period, was high enough to show its phytotoxicity.

4.6. Conclusions

In conclusion, the results reported in this Chapter still support that complex phenomena occur in a soil when a contaminant is present and persist for long time in it. As respect to fresh contamination, an aged contaminated soil will behave differently only if the contaminant will undergo an ageing phenomenon. The higher amounts of Phe extracted by the humic fractions, and in particular from humin, strongly indicate that ageing of Phe actually occurred and was favoured by the presence of the high level of organic matter in the soil.

Moreover, the effects of an aged contaminant will be probably less evident depending on the parameters tested. For instance, the biochemical parameters such as the activity of some enzymes appeared less influenced when Phe was aged for 2 years as respect to their response to fresh Phe-contamination. Conversely, germination tests were more sensitive to the

contamination and negatively responded to the presence of the aged compound. This to highlight that the choice of the biochemical parameter to use as indicator of soil quality might be crucial for obtaining correct and easily interpretable results. The use of more than one indicator is recommended.

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Chapter 5

Biostimulation of an artificially pentachlorophenol-contaminated soil[1]

Pentachlorophenol (PCP) is a highly chlorinated organic compound that has been extensively used as a broad-spectrum biocide, particularly in the wood preservation industry. Due to its stable aromatic ring structure and high chlorine content, PCP is persistent in the environment, and it has become one of the most widespread contaminants in soil and water.

The response of a fresh, agricultural soil when contaminated with pentachlorophenol (PCP) and supplemented with compost (C) or dissolved organic matter (DOM) was studied in the laboratory. The concentration of PCP and the changes in various functionally related properties (i.e. microbial biomass, basal respiration, and soil hydrolase and oxidoreductase activity) were measured over 150 days. Variations in the main physical and chemical properties of the soils were also monitored.

[1]A version of this Chapter has been published as:

Scelza R., Rao M.A., Gianfreda L., 2008. Response of an agricultural soil to pentachlorophenol (PCP) contamination and the addition of compost or dissolved organic matter. *Soil Biology & Biochemistry* 40, 2162-2169.

Two different doses of compost (C1 = 0.27% and C2 = 0.83%, corresponding to 10 and 30 t ha^{-1}, respectively,) or DOM (D1 = 0.07% and D2 = 0.2%) equivalent to the carbon content of the two compost doses C1 and C2, were used and the following five systems were investigated: soil (S), soil-compost (S-C1 and S-C2) and soil-DOM (S-D1 and S-D2). PCP concentrations declined progressively and significantly with time. This effect was most pronounced for the soils amended with the lower compost dose C1 (S-C1) and with the two DOM (S-D1 and S-D2) amounts. Significant reduced amounts of PCP were extracted after its 500-d residence in the various systems. Higher amounts of the residual PCP were extracted from the humic acids (HA), fulvic acids (FA) and humin-mineral (HU) fractions of the 500 d aged samples than from the same un-fractionated samples, indicating that the residual PCP preferentially accumulated in the organic fractions of soil.

The soil showed an endogenous microbial activity as indicated by basal respiration, microbial biomass and all the enzymatic activities tested (dehydrogenase, β-glucosidase, phosphatase, arylsulphatase, urease). Addition of the PCP severely depressed some of the tested biochemical properties suggesting an inhibitory effect on microbial activity. Conversely, higher basal respiration, and similar β-glucosidase and phosphatase activities were measured in comparison with the controls. No significant effects were observed following the addition of two doses of the compost or the DOM. Fungal colonies belonging to the taxonomic group of *Ascomycetes* and identified as *Byssochlamys fulva* developed with time in all the PCP-contaminated samples. Growth of *B. fulva* in vitro in the presence of PCP showed that the isolate was tolerant to 12.5 and 25 mg l^{-1} PCP and degraded 20% of its initial concentration in 8 d. Overall, the results indicate that many complex processes occurred in the contaminated

soils and combinations of these determined the response to PCP contamination. The sorption of PCP to the soil matrix (which increased with time) and its degradation/transformation by indigenous soil microbial activity, were likely involved. Both the processes appeared to be favoured by the presence of dissolved organic matter.

5.1. Introduction

Since its commercial introduction in 1936, pentachlorophenol (PCP) has found world-wide application, *e.g.*, in commercial wood treatment (as a preservative, insecticide and microbiocide), for paper production (for reduction of slime), in leather industry (as a preservative and fungicide), and in agriculture (as an herbicide and insecticide) (Crosby et al., 1981; Needham et al., 1981; Secchieri et al., 1991). Nowadays, although it has been banned in several countries, the extensive number of soil and water contaminated sites, contamination levels, and toxicity have resulted in PCP being listed as one of the priority pollutants (U.S. EPA, 2004).

In soil, PCP may undergo several processes, of which adsorption on soil particles is the most common. Indeed, PCP is a weak acid (pK_a 4.75); therefore it can be present in the environment as both neutral (phenol) and charged (phenolate ion) forms. Its adsorption on soil is a combination of hydrophobic and electrostatic interactions, the relative importance of each being dependent on the environmental conditions, particularly on pH. Higher PCP adsorption was usually observed at lower pH (Lee et al., 1990; Lafrance et al., 1994) at which the PCP will be expected to exist mostly as the neutral phenol. Therefore, PCP adsorption via hydrophobic interaction is probably stronger than that via electrostatic interaction. This is not surprising, considering the significant hydrophobic character of the PCP

molecule, shown by its low solubility in water at neutral pH even when it exists as the phenolate ion (Tam et al., 1999).

Several studies report that PCP adsorption is enhanced by the presence of organic matter and the extent of enhancement appeared to increase with the amount of organic matter present in the test mixtures (Lagas, 1988; Banerji et al., 1993; Lafrance et al., 1994; Tam et al., 1999).

Currently, increasing interest is addressed to the use of mobile sorbents, such as dissolved organic matter, to reduce the sorption to the solid phase enhancing mobility and solubility of organic contaminants (McCarthy and Zachara, 1989).

PCP is a strong biocide, therefore it is the most resistant chlorophenol to biological degradation (McAllister et al., 1996).

The biodegradation of PCP has been studied in both aerobic and anaerobic systems. Reductive dechlorination has been suggested as the primary PCP biodegradation mechanism (Wang et al., 1998; Vallecillo et al., 1999; Tartakovsky et al., 1999; Tartakovsky et al., 2001). Under anaerobic conditions, chlorine can be removed from the aromatic ring by reductive dechlorination resulting in partially or fully dechlorinated product which is then more susceptible to either aerobic or anaerobic attack (Vallecillo et al., 1999). The aromatic ring is thus totally dechlorinated prior to ring cleavage. Further degradation results in the production of methane and carbon dioxide (Vallecillo et al., 1999). Under aerobic conditions, the biodegradation pathways of PCP are more diverse than under anaerobic conditions. Ring cleavage can occur either before or after removal of the chlorine substituents, giving rise to a whole array of intermediates of varying toxicity (Vallecillo et al., 1999; Reddy and Gold, 2000).

Although a large number of bacterial and fungal strains, such as *Flavobacterium* sp. (Crawford and Mohn, 1985; Martinson et al., 1986),

Mycobacterium sp. (Briglia et al., 1994), *Arthrobacter* sp. (Edgehill, 1996), *Phanerochaete* sp. (Chung and Aust, 1995), have been reported to be capable of degrading PCP in both soil and water, microbial degradation can be limited by different factors, for example sub-optimal nutrient levels, temperatures and pH (McAllister et al., 1996; Miller et al., 2004a).

Furthermore, an important consideration is that PCP can be sequestered in soil aggregates where reduced accessibility to enzymatic processes limits degradation (Warith et al., 1993), and this question of bioavailability is an important impediment to the credible application of bioremediation treatments in general (Head, 1998).

Composting and the use of composted materials as supplemental nutrients were successfully applied to the bioremediation of PCP-contaminated soils (Laine and Jorgensen, 1997; Miller et al., 2004b) with evidence that mineralization of the xenobiotic was achieved (Laine and Jorgensen, 1996).

5.2. Dissolved organic matter

In soils, dissolved organic matter (DOM) is probably the most bioavailable fraction of soil organic matter. The size limit, which is used to differentiate DOM from particulate organic is somewhat arbitrary, but there is an almost universal consensus that it is around 0.45 μm (Zsolnay, 2003).

Much progress has been made in the understanding of dissolved organic matter (DOM) functions and dynamics in soils. Today, it is commonly acknowledged that DOM can enhance the solubility and mobility of metals and organic compounds (McCarthy and Jimenez, 1985; Blaser, 1994; Piccolo, 1994; Zsolnay, 1996; Marschner, 1999; Graber et al., 2001; Zsolnay, 2003; Song et al., 2008) and thus contributes to pollutants transport or to micronutrients availability. In the presence of DOM, weathering rates can be accelerated (Raulund-Rasmussen et al., 1998), and

DOM plays a central role during podsolisation (Lundström et al., 1995). Furthermore, DOM contains organically bound nutrients such as N, P and S, and DOM dynamics will therefore also affect their mobility and availability (Kalbitz et al., 2000; Kaiser et al., 2001).

DOM is also a substrate for microorganisms. In soils, DOM may be the most important C source since soil microorganisms are basically aquatic and all microbial uptake mechanisms require a water environment (Metting, 1993). Moreover, the soluble state is presumably a prerequisite for the diffusion of substrates through microbial cell membranes so that the degradation of solid phase organic matter or large molecules can only occur after dissolution or hydrolysis by exoenzymes.

Although DOM enhances the water solubility and mobility of highly hydrophobic contaminants, due to its hydrophobicity, it can be also sorbed onto soil organic material, thus increasing the sorption and reducing the mobility of contaminants that may be associated with the DOM (Kile and Chiou, 1989). Therefore, as a mobile phase, DOM can enhance the mobility of hydrophobic organic compounds such as PAHs, or as a sorbed phase, it can increase PAH sorption and decrease mobility (Chiou et al., 1987; McCarthy and Zachara, 1989; Magee et al., 1991; Liu and Amy, 1993; Johnson and Amy, 1995).

As mobile sorbent, DOM can bind contaminants and thus accelerate their transport through porous media (McCarthy and Zachara, 1989; Johnson et al., 1995; Johnson and Amy, 1995; Kim and Corapcioglu, 2002; Moon et al., 2003).

DOM interacts with organic pollutants through hydrophobic binding, forming humic-solute complexes in the aqueous phase (Sabbah et al., 2004). Humic and fulvic acids (HA, FA) are classes of DOM that, being naturally occurring organic material, will interact with organic

contaminants in the environment and may also serve as environmentally friendly decontamination agents for site remediation as shown by Rebhun et al. (1992, 1996), Molson et al. (2002), and Van Stempvoort et al. (2002). The binding of organic xenobiotics to DOM and the resulting effects on their bioavailability are mainly determined by the hydrophobicity of the pollutant and the origins, quantities and properties of DOM (Haitzer et al., 1999). In general, increasing concentrations of DOM decrease the bioavailability of xenobiotics (Haitzer et al., 1998), thus rendering ineffective the application of microorganisms to degrade them.

5.3. Transformation of chlorinated phenolic compounds by fungi

The ability to degrade PCP has been demonstrated by a variety of microorganisms. It is degraded aerobically by a number of bacterial isolates including *Flavobacterium* sp. (Topp and Hanson, 1990).

Among microorganisms able to degrade polychlorinated phenols, white-rot fungi play a predominant role (Rubilar et al., 2008). White rot fungi are a group of organisms very suitable for the removal of chlorinated phenolic compounds from the environment. Indeed, they are robust, ubiquitous organisms and may survive also in the presence of high concentrations of various pollutants, even with a low bioavailability. White rot fungi possess the lignin-degrading enzyme system (LDS) that confers them broad substrate specificity and ability to oxidize several environmental pollutants. Besides the lignin-degrading systems, white rot fungi contain other non-ligninolytic enzymes that may participate in the transformation of polluting substances. Furthermore, enzymes that form extracellular hydrogen peroxide from molecular oxygen are also produced; these enzymes utilize

glyoxal glucose and other products from cellulose and lignin degradation as substrates for the production of H_2O_2 (Kirk and Farrel, 1987).

Phanerochaete chrysosporium is one of the most widely studied fungi. It has been shown to have non specific ability to degrade many persistent toxic organic chemicals, including PCB, PCP, DDT and several polycyclic aromatic hydrocarbons (Lin and Wang, 1990; Bumpus and Aust, 1995). For this fungus intermediary products and reactions involved in the degradation of chlorophenols have been identified (Rubilar et al., 2008 and references therein). Extracellular laccases and peroxidases carry out the first productive step in the oxidation of chlorophenols, forming *para*-quinones and consequently releasing a chlorine atom. Further degradative steps involving several enzymes and highly reactive, non-specific redox mediators produced by the fungus render it capable of efficiently degrading several toxic compounds. Experimental evidences demonstrated that *Phanerochaete chrysosporium* was able to degrade high levels of PCP in PCP-contaminated soils (McGrath and Singleton, 2000). However, other white-rot fungi, such as *Trametes versicolor*, have shown potential as PCP degraders (Seigle-Murandi et al., 1991, 1993; Alleman et al., 1992; Lamar and Dietrich, 1992; Ricotta et al., 1996; Walter et al., 2005).

5.4. Case study

The previous Chapters (3-4) have been widely dedicated to the effects of phenanthrene on the main properties of an agricultural soil and on the effectiveness of some bioremediation strategies on its restoration.

Different responses for the fresh spiked or aged phenanthrene have been detected, confirming that the behaviour of aged compounds is much different from that of freshly added chemicals.

As they persist in soil, organic compounds become progressively less available for uptake by organisms, for exerting toxic effects, and for biodegradation and bioremediation.

In recent years growing attention has been given to the effect of mobile sorbents, i.e., dissolved or colloidal-size aqueous phase components, on the behaviour of polycyclic aromatic hydrocarbons (PAHs) and other hydrophobic pollutants in soils and sediments (Kögel-Knabner and Totsche, 1998). Several types of materials were identified as mobile sorbents and shown to increase the water solubility of organic and inorganic pollutants: inorganic colloids, such as clay and silt minerals or iron oxides, and mobile organic colloids (dissolved organic matter, DOM) (Nakayama et al., 1986; Short et al., 1988; Chiou, 1989; McCarthy and Zachara, 1989). DOM has been shown to specifically enhance the mobility of organic contaminants in aquifers and soils.

The present Chapter is focused on another persistent organic pollutant, pentachlorophenol (PCP), different from phenanthrene in terms of structure, properties and behaviour in soil, indeed PCP can more strongly adsorb to soil particles thus becoming not available for biodegradation.

In particular, the effects of this chemical on the main soil properties have been studied in a long-term experiment. Processes of both biostimulation (using compost as nutrient source) and enhancing mobility (using dissolved organic matter) of PCP have been carried on.

To assess the effect of ageing on PCP behaviour, a fractionation of humic substances and subsequently PCP extraction from each single fraction (humic acids, fulvic acids and humin), have been performed on soil samples incubated for longer time.

Furthermore, during the experiment, the development of fungal colonies on PCP-contaminated soil samples, induced to isolate and identify the specie and, preliminarily, to evaluate its potential to degrade pentachlorophenol.

5.5. Materials and Methods

5.5.1. Chemicals

Reagent-grade PCP (>99% purity) and HPLC-grade solvents were purchased from Sigma Aldrich (Germany). All other chemicals, reagent grade, were supplied by Analar, BDH (Germany), unless otherwise stated.

5.5.2. Disappearance of PCP in solid-state systems: experimental design

Fresh soil (S) was air-dried to 18% moisture content and 2-mm sieved. Then it was placed (100 g) in closed 1-l glass jars. The compost was added at two different doses, 0.27% (C1) and 0.83% (C2), corresponding to a field rate of 10 and 30 t ha^{-1} of compost, respectively. DOM was added at two rates, D1 (0.07%) and D2 (0.2%), equivalent to the carbon content of the two compost doses C1 and C2, respectively. Five samples were thus obtained: S (only soil), S-C1 (soil+0.27% compost), S-C2 (soil+0.83% compost), S-D1 (soil+0.07% DOM) and S-D2 (soil+0.2% DOM). These represented the controls. Similar samples were prepared by using the soil previously spiked with PCP (50 mg kg^{-1} of soil, as described below), supplemented with the two compost doses or DOM amounts, and they formed the PCP-contaminated samples. For both controls and PCP-contaminated samples, five sets (S, S-C1, S-C2, S-D1 and S-D2), one for each incubation time, were prepared in duplicate. The samples (50 controls and 50 PCP-contaminated samples in all) were placed in the dark in a climatic chamber set at 25 °C. Periodically, in particular after 0, 20, 65, 150 d of incubation, two sacrificial sets of replicates, one for controls and one for PCP-contaminated samples, were taken and split into sub-samples (two per replicate) for pentachlorophenol determination and for all physical, chemical and biochemical analyses. Soil samples for biochemical analyses

were kept at 4 °C and measurements were made within 5 d of soil collection, while physical and chemical properties were determined on air-dried soil samples. For both controls and PCP-samples, one set of two replicates was incubated until 500 d, and then each replicate was split into two sub-samples: one was analysed for residual PCP and the other was subjected to fractionation of humic substances and analysed to quantify PCP contents (see below).

A stock solution of PCP (5 g l^{-1}) was prepared in acetone and stored under refrigeration at 4 °C. Soil samples were rewetted to a moisture content of 18%, and homogenized with a stainless steel spatula. Approximately 10 g of the rewetted soil was spiked with 7 ml of acetone and 1 ml of the PCP-stock solution in order to obtain a final concentration of 50 mg kg^{-1} soil. The soil was placed in a 1-l glass jar and manually mixed with a stainless steel spatula. Soil was gradually added to the glass jars in 10 g aliquots and extensively mixed with the spiked soil. This procedure was repeated until the entire amount of soil (100 g) was added and mixed. The jars containing the spiked soil were covered with aluminium foil, hermetically closed and left overnight to shake for inversion. Then the acetone was left to evaporate for about 2 h under a flow hood and the soil was immediately used.

The control samples were treated with the same amount of acetone (without PCP) and subjected to the same experimental procedure. Preliminary experiments with acetone-treated samples demonstrated that the addition of acetone did not affect the chemical and biochemical properties of soil, soil+compost and soil+DOM mixtures or their variations with time.

5.5.3. Physical and chemical properties of soil and compost

Soil and compost used for PCP experiment have been described in Chapter 3 (see Table 3.4).

Physical and chemical analyses were performed in triplicate on air-dried and sieved (<2 mm) soil samples as described in Chapter 3. Total organic C (TOC) and total N (TN) were measured by the ash combustion procedure with a Fisons 1108 Elemental Analyser, calibrated with appropriate standards (acetanilide). To obtain homogeneous samples, soils (25-30 mg) were air-dried, pounded and sieved at 0.5 mm prior to analysis. Accuracy (<0.05%) and recovery of C and N (for both instrument detection limit 10 mg kg^{-1}) were checked by analysing a sample of the standard material after each set of eight sample analyses.

5.5.4. Soil biochemical analyses

Microbial biomass C (MB-C), basal respiration and enzyme activities methods have been described in detail in Chapter 3. As previously described, the soil, compost and also DOM were tested for possible interference with the analytical methods adopted for evaluating enzymatic product concentration and/or adsorption of the product released by the enzymatic action. Appropriate extinction coefficients were used for the calculation of enzyme activity units as determined by calibration curves obtained in the presence of soil, soil+compost, soil+DOM.

Enzymatic Units are those defined in Chapter 3.

5.5.5. Fractionation of humic substances

The fractionation of humic substances, i.e., humic acids (HA), fulvic acids (FA) and humin-mineral fractions (HU), was performed only on the 500 d incubated samples, according to the method described by Nieman et al. (2005). Ten g of spiked soil were placed in 40 ml Teflon centrifuge tubes and tumbled with 15 ml of 0.5 N NaOH for 17 h. The samples were then centrifuged at 10000 *g* for 10 min, and the supernatant containing humic

and fulvic acids was removed. The soil was extracted two more times, once with 15 ml and once with 10 ml of 0.5 M NaOH for 4.5 and 6 h respectively, and these extracts were added to the previous one. The residue obtained thereby represented the humin-mineral fraction. The extract was then acidified to pH <2.0 with 2 ml of HCl (37%) and centrifuged to separate humic (insoluble) and fulvic acid fractions.

Pentachlorophenol was extracted from the three fractions, and concentrations of the compound were determined by high-pressure liquid chromatography (HPLC) as described below.

5.5.6. PCP extraction and detection

The extraction of PCP from spiked soil, soil+compost and soil+DOM systems was performed using a water-ethanol mixture as described by Khodadoust et al. (1999). Briefly, 1 g of moist soil was extracted with 20 ml of water-ethanol (50:50, v:v) on a horizontal shaker (190 rev min^{-1}) for 1 h. The supernatant was separated from the residual soil by centrifugation at 3000 *g* for 15 min and concentrated by evaporation under vacuum. Each concentrate was re-suspended in 2 ml of methyl alcohol for high-performance liquid chromatography (HPLC) analysis.

Pentachlorophenol was extracted from humin and humic fractions as described by Nieman et al. (2005) with some modifications. The humin-mineral fraction was solvent extracted by shaking (200 rev min^{-1}) with 20 ml acetone and *n*-hexane (1:1, v:v) for 2 h. The solvent was decanted after centrifugation at 10000 *g* for 10 min. Isolated humic acid samples were allowed to dry at 30 °C for 24 h and subsequently extracted with 10 ml acetone and *n*-hexane (1:1, v:v) for 10 min followed by 10 min of centrifugation at 10000 *g* (Nieman et al., 2005). Solvent extracts were dried under vacuum and redissolved in acetonitrile for HPLC analysis.

PCP extraction from fulvic acid fractions was performed according to the extraction method of Nam and Kim (2002) described for phenanthrene. The acidified solution (6 ml) containing fulvic acid was mixed with 30 ml of *n*-hexane (5:1, v:v) in a Teflon centrifuge tube and shaken on a horizontal shaker (200 rev min^{-1}) at room temperature. After shaking for 24 h, an appropriate volume (3 ml) was recovered and concentrated by using a rotary evaporator.

The residual PCP in all the samples was quantified by HPLC using an Agilent Technologies R1100 instrument with a pump and a diode-array detector. A Phenomenex 250 x 4.6 mm C-18 column with 4 μm particle size and a Phenomenex C-18 (4.6 x 30 mm) guard column were used. Analysis was conducted using 68% of acetonitrile and 32% of buffered water (1% acetic acid) as mobile phase and the 1.0 ml min^{-1} flow rate. Detection was carried out at 220 nm. The retention time for PCP was about 10 min.

Preliminary tests performed with PCP-soil, PCP-soil+compost, and PCP-soil+DOM mixtures at different PCP-solid phase ratios showed a 100% efficiency of the adopted extraction procedures. Four determinations were performed for PCP analysis.

5.5.7. Growth and isolation of fungal species

Samples showing white spots on the surface, were analysed for total fungal count using a fungi-specific substrate, Potato Dextrose Agar (PDA) with the addition of chloramphenicol (10%). PDA composition (per litre) was 4.0 g potato extract, 20.0 g dextrose and 15.0 g agar. Colonies were isolated and cultivated separately using the same solid medium for several days (about 10 d) at room temperature. Identification of colonies was carried out by optical microscopy according to general principles of fungal

classification (Samson et al., 2000). Experiments to evaluate the ability of the isolated fungal strains to degrade PCP were carried out in Erlenmeyer flasks containing 100 ml of Potato Dextrose Broth (PDB, with the same composition of PDA without agar), at 22 °C in a rotary shaker (85 rpm) supplement with initial PCP concentrations of 12.5 and 25 mg l^{-1}. Controls were cultured without PCP. Five replicated samples were periodically analysed for residual PCP as reported above.

5.5.8. Statistic analysis

All data were subjected to analysis of variance by using SPSS for Windows, Version 15.0. The assumptions of normality and homogeneity of variances were tested by the Kolmogoroff-Smirnoff test and the Levene test. The significant differences between means at $P<0.05$ were assessed according to Tukey's multiple comparison test.

5.6. Results

5.6.1. Disappearance of PCP in soil, soil+compost, and soil+DOM systems

PCP depletion showed different trends in the investigated samples (Fig. 5.1). At the zero time of the incubation (corresponding to 24 h of contact between soil and PCP) a 20% reduction of the extractable PCP was measured in all samples. The extracted PCP amounts were significantly ($P<0.05$) affected by incubation time or soil treatment as the sole source of variation. By contrast, treatment x incubation time interaction had no significant effect. Moreover, the addition of DOM significantly decreased the amount of extractable PCP at any time (Fig. 5.1).

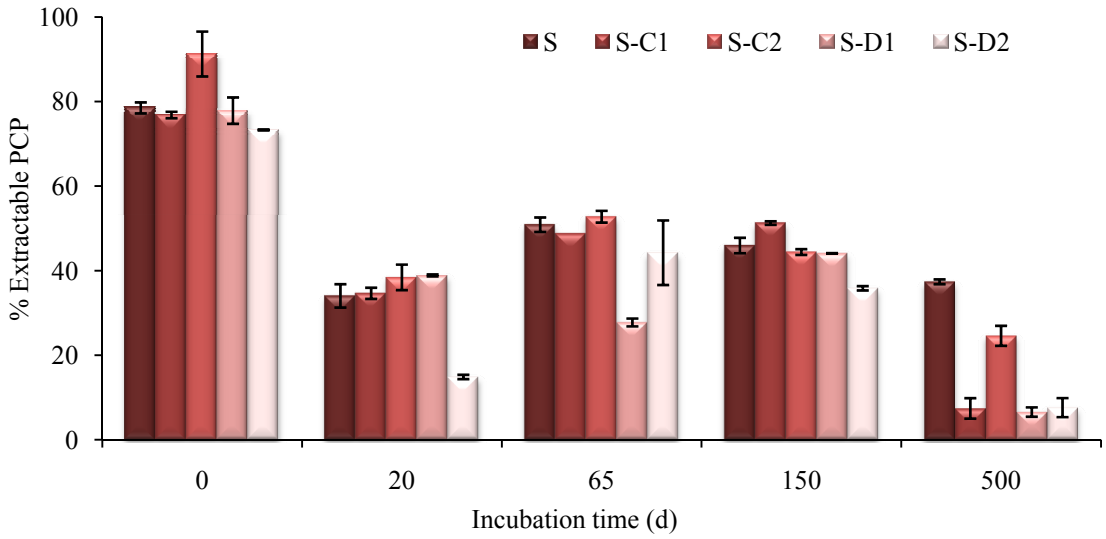

Fig. 5.1. Extracted pentachlorophenol (PCP) (%) with time at 25 °C. S (soil), S-C1 (soil+0.27% compost), S-C2 (soil+0.83% compost), S-D1 (soil+0.07% DOM) and S-D2 (soil+0.2% DOM). Each value is the mean of two replicates.

The 500 d-aged samples were also subjected to the fractionation of humic substances followed by the extraction of PCP from each single fraction HA, FA and HU. Fig. 5.2a shows the concentrations (mg l^{-1}) of PCP extracted from each humic fraction of the five analysed samples. The amounts of PCP extracted from the three humic fractions significantly differed from each other (Fig. 5.2a) independently of the addition of compost or DOM.

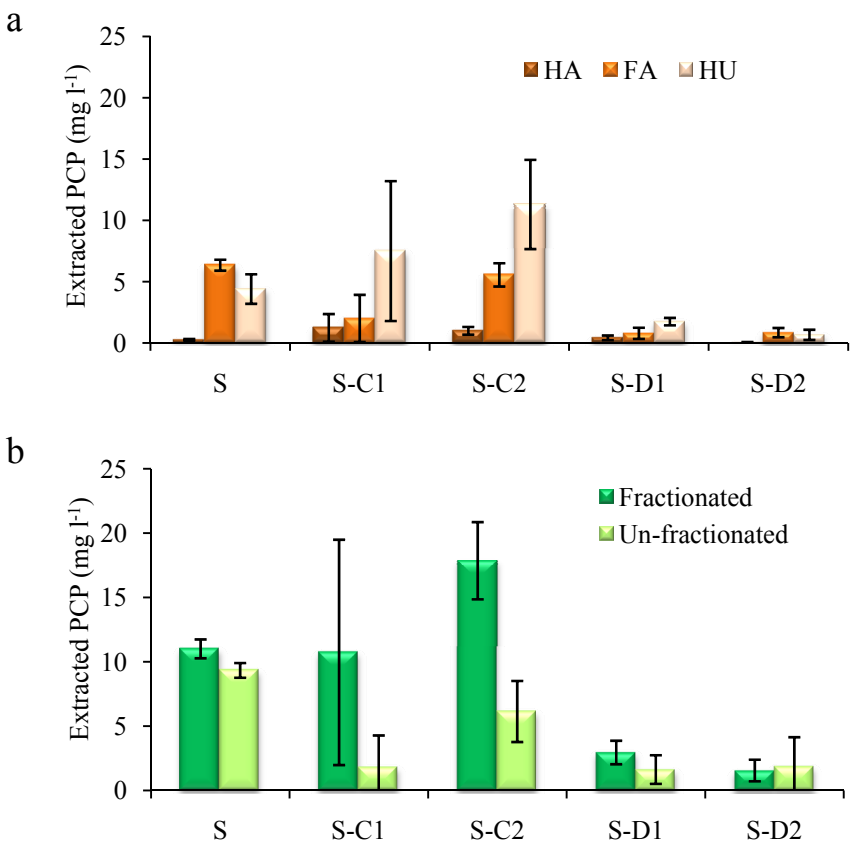

Fig. 5.2. Extracted pentachlorophenol (PCP) (mg l^{-1}) in 500 d aged samples (for details see legend Fig. 5.1). Each value is the mean of two replicates. (a) Distribution of the extractable PCP in the three humic fractions, fulvic acids (FA), humic acids (HA) and humin (HU). (b) Amounts of the extractable PCP in the fractionated and unfractionated samples.

In particular, in S the highest amount of PCP was recovered from FA whereas the lowest resulted in HA. In S-C2 the extractable PCP was higher in the humin fraction HU and also in this case the lowest PCP concentration was recovered from the HA fraction. S-D1 and S-D2 differed little but significantly in PCP distribution, PCP values being higher in HU for S-D1 and in FA for S-D2.

Fig. 5.2b reports for comparison the amounts of PCP extracted from the 500 d-aged unfractionated samples (Fig. 5.1) and the total of PCP amounts recovered in the three fractions (sum of the values shown in Fig. 5.2a). Higher concentrations of PCP were measured after extraction from humic

fractions as compared to unfractionated samples and the treatment (i.e., the presence of additional organic matter and/or compost) significantly influenced the measured amounts (Fig. 5.2b). Moreover, these results indicate that all residual PCP extracted from soil was located in its organic fractions.

5.6.2. Physical and chemical properties

The addition of the two doses of compost and DOM had different effects on some of the physical and chemical properties of the soil and their variations with time (Tables 5.1a and b). Some such as moisture, Na, K and the alkaline bivalent cations Ca and Mg did not change significantly, whereas decreases were generally measured for pH, TOC, and TN. A significant decrease in pH (initial values 7.83 - 7.95) (Fig. 5.1a) with time was measured in all the control samples, and after 150 d the pH values decreased by 0.3 pH units (on average). Conversely, the content of phosphorus (P_2O_5) (Fig. 5.1b) was significantly affected by the treatment. The value increased from 18.9 mg kg^{-1} up to 38.6 mg kg^{-1} and 26.8 mg kg^{-1} with the addition of the two compost doses and the lower DOM amount, respectively. The increase (on average 10%) in TOC observed after compost or DOM addition was expected, additional organic carbon being supplied by either the compost or DOM (as already demonstrated in Chapter 3).

In PCP-contaminated samples, fluctuating, non-significant changes in pH occurred on addition of PCP. By contrast, both TOC and TN parameters were significantly affected by PCP addition x treatment x incubation time interaction (P=0.005 and P<0.0001 for TOC and TN, respectively). Consistent decreases of both occurred in S, partially annulled by the addition of the compost and DOM. Immediately after PCP addition, the

TOC and TN decreased from 24.5 g kg^{-1} to 17.0 g kg^{-1} and from 2.2 g kg^{-1} to 1.5 g kg^{-1}, respectively. Both parameters significantly increased in compost- and DOM-amended samples, reaching for S-D2 the values measured in S. By the end of the experiment and even at 500 d incubation, equal values of TN and quite similar amounts of TOC were measured for both the microcosms. Consequently, no significant variations occurred in the C/N ratios.

Table 5.1a. Chemical properties of S, S-C1, S-C2, S-D1 and S-D2 samples contaminated (+PCP) or not contaminated (-PCP).

Property		Sample	Incubation times (d)			
			0	20	65	150
pH	-PCP	S	7.95 (±0.0)[a]	8.04 (±0.0)	7.62 (±0.0)	7.60 (±0.0)
		S-C1	7.83 (±0.0)	8.00 (±0.0)	7.61 (±0.0)	7.53 (±0.0)
		S-C2	7.84 (±0.1)	7.95 (±0.0)	7.60 (±0.0)	7.55 (±0.0)
		S-D1	7.84 (±0.0)	7.92 (±0.0)	7.61 (±0.0)	7.56 (±0.0)
		S-D2	7.90 (±0.0)	7.92 (±0.0)	7.61 (±0.0)	7.52 (±0.0)
	+PCP	S	7.95 (±0.0)	7.97 (±0.0)	7.73 (±0.1)	8.04 (±0.0)
		S-C1	7.96 (±0.0)	7.92 (±0.1)	7.74 (±0.0)	7.99 (±0.0)
		S-C2	7.97 (±0.0)	7.93 (±0.0)	7.86 (±0.1)	7.99 (±0.0)
		S-D1	7.88 (±0.0)	7.98 (±0.0)	7.87 (±0.0)	8.05 (±0.1)
		S-D2	7.94 (±0.1)	8.03 (±0.0)	7.83 (±0.0)	7.92 (±0.0)
TOC (g kg^{-1})	-PCP	S	24.5 (±0.2)	19.8 (±0.3)	18.6 (±0.1)	23.7 (±0.1)
		S-C1	21.7 (±0.3)	20.1 (±0.1)	23.3 (±0.2)	22.6 (±0.1)
		S-C2	23.5 (±0.3)	21.1 (±0.2)	23.2 (±0.1)	23.2 (±0.1)
		S-D1	20.8 (±0.4)	20.3 (±0.3)	23.3 (±0.2)	22.7 (±0.1)
		S-D2	21.3 (±0.3)	19.5 (±0.4)	22.4 (±0.1)	23.3 (±0.1)
	+PCP	S	17.0 (±0.2)	22.2 (±0.3)	23.6 (±0.2)	22.7 (±0.2)
		S-C1	20.7 (±0.3)	23.1 (±0.2)	26.3 (±0.2)	23.0 (±0.2)
		S-C2	19.5 (±0.1)	23.2 (±0.2)	23.7 (±0.2)	22.9 (±0.1)
		S-D1	19.1 (±0.2)	19.7 (±0.3)	23.0 (±0.2)	22.7 (±0.2)
		S-D2	21.8 (±0.3)	23.3 (±0.3)	24.1 (±0.1)	23.4 (±0.2)

[a]Values in parentheses show standard deviations

Table 5.1b. Chemical properties of S, S-C1, S-C2, S-D1 and S-D2 samples contaminated (+PCP) or not contaminated (-PCP).

Property		Sample	Incubation times (d)			
			0	20	65	150
TN (g kg^{-1})	-PCP	S	2.2 (±0.0)	1.9 (±0.0)	1.9 (±0.0)	2.3 (±0.0)
		S-C1	2.0 (±0.0)	1.9 (±0.0)	2.3 (±0.0)	2.2 (±0.0)
		S-C2	2.2 (±0.0)	2.0 (±0.0)	2.3 (±0.0)	2.3 (±0.0)
		S-D1	1.9 (±0.0)	2.0 (±0.0)	2.3 (±0.0)	2.3 (±0.0)
		S-D2	1.9 (±0.0)	1.9 (±0.0)	2.2 (±0.0)	2.3 (±0.0)
	+PCP	S	1.5 (±0.0)	2.2 (±0.0)	2.3 (±0.0)	2.2 (±0.0)
		S-C1	2.0 (±0.0)	2.3 (±0.0)	2.5 (±0.0)	2.2 (±0.0)
		S-C2	1.9 (±0.0)	2.2 (±0.0)	2.3 (±0.0)	2.2 (±0.0)
		S-D1	1.8 (±0.0)	2.0 (±0.0)	2.2 (±0.0)	2.2 (±0.0)
		S-D2	2.2 (±0.0)	2.2 (±0.0)	2.4 (±0.0)	2.2 (±0.0)
C/N	-PCP	S	10.9	10.2	10.0	10.2
		S-C1	10.7	10.4	9.9	10.1
		S-C2	10.7	10.5	10.1	10.0
		S-D1	11.0	10.2	10.1	10.0
		S-D2	11.4	10.3	10.1	10.1
	+PCP	S	11.4	10.3	10.3	10.2
		S-C1	10.4	10.1	10.7	10.3
		S-C2	10.4	10.4	10.3	10.3
		S-D1	10.7	9.8	10.3	10.4
		S-D2	10.1	10.4	10.2	10.5
P_2O_5 (mg kg^{-1})	-PCP	S	18.9 (±0.3)	30.9 (±0.0)	20.1 (±2.8)	16.5 (±1.7)
		S-C1	27.5 (±13.9)	34.0 (±0.0)	25.9 (±5.2)	22.4 (±4.7)
		S-C2	38.6 (±3.6)	35.8 (±0.0)	23.5 (±4.1)	20.7 (±5.9)
		S-D1	26.8 (±4.6)	44.2 (±0.0)	32.0 (±14.5)	34.7 (±19.5)
		S-D2	19.4 (±0.4)	37.1 (±0.0)	22.4 (±0.3)	18.0 (±0.2)
	+PCP	S	23.0 (±6.6)	42.2(±0.0)	22.5 (±5.1)	16.4 (±3.6)
		S-C1	25.9 (±1.8)	51.9 (±0.0)	26.2 (±0.0)	21.2 (±3.1)
		S-C2	27.4 (±0.6)	53.3 (±0.0)	25.1 (±4.8)	26.0 (±0.4)
		S-D1	23.5 (±5.8)	40.8 (±0.0)	22.5 (±3.5)	22.9 (±1.8)
		S-D2	26.5 (±3.9)	57.1 (±0.0)	21.6 (±0.1)	24.2 (±1.2)

[a]Values in parentheses show standard deviations

A temporary, significant increase in P contents (Fig. 5.1b), much greater in PCP-contaminated samples, took place at 20 d incubation where they were in the range 34-44 mg kg^{-1} in the controls and 41-57 mg kg^{-1} in the PCP-contaminated samples. A decrease in P with time was detected in all samples, and after 150 d the P values were quite similar to the initial ones.

5.6.3. Biochemical properties

Some enzymatic activities, involved in the cycles of the main biological nutrients C, N, S and P, were evaluated, and different patterns were observed in the controls and PCP-contaminated samples. Control samples showed the same trend of β-glucosidase activity (GLU) (Fig. 5.3), an important enzyme in carbon cycling, during the entire incubation period.

With respect to an average initial value of 0.8 μmol *p*-NP g^{-1} h^{-1}, a slight significant increase was registered in the first 20 d of incubation, followed by a small decrease until 65 d, after which constant values of activity were measured by the end of incubation. At that time, S, S-C1 and S-C2 showed significant higher values of GLU activity (0.84 μmol *p*-NP g^{-1} h^{-1}) than DOM-amended samples (0.71 μmol *p*-NP g^{-1} h^{-1}). The addition of PCP did not significantly affect the values or patterns of GLU activity.

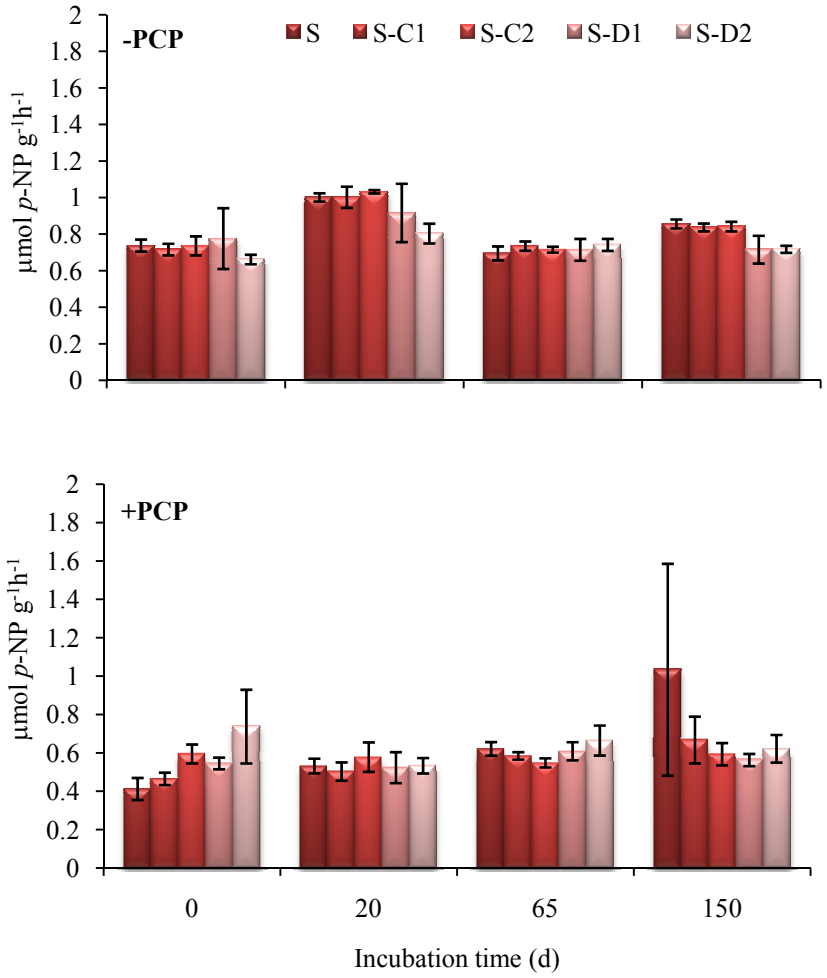

Fig. 5.3. β-glucosidase activity (µmol *p*-NP g^{-1} h^{-1}) in controls (-PCP) and PCP-contaminated samples (+PCP). S (soil), SC1 (soil+0.27% compost), S-C2 (soil+0.83% compost), S-D1 (soil+0.07% DOM) and SD2 (soil+0.2% DOM). Each value is the mean of two replicates.

By contrast, the values and behaviour of dehydrogenase activity (DH), another key enzyme in the C cycle, were very different in the samples with or without PCP and they significantly differed with incubation time (Fig. 5.4). The presence of PCP severely and significantly depressed DH activity and no significant differences were observed between amended or non-amended samples. Values close to zero were measured at each time.

In the absence of PCP (Fig. 5.4) DH recorded initial values of 0.8 µg TPF g^{-1} h^{-1} until 20 d of incubation; after which a sharp increase was noted for

all the samples, the rise being constant with time. At 150 d of incubation about 8 µg TPF g^{-1} h^{-1} were measured, 10-fold higher than those measured at the zero time of incubation.

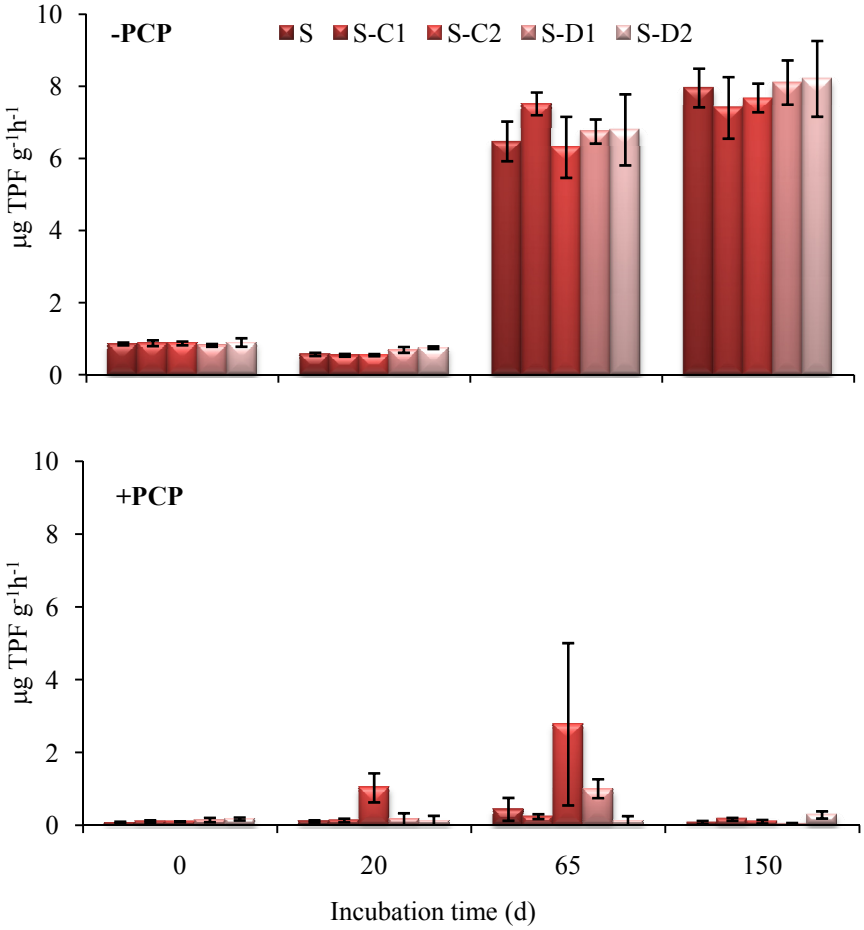

Fig. 5.4. Dehydrogenase activity (µg TPF g^{-1} h^{-1}) in controls (-PCP) and PCP-contaminated samples (+PCP). S (soil), SC1 (soil+0.27% compost), S-C2 (soil+0.83% compost), S-D1 (soil+0.07% DOM) and SD2 (soil+0.2% DOM). Each value is the mean of two replicates.

Values of arylsulphatase activity (ARYL) of the tested samples are shown in Fig. 5.5. ARYL values were significantly (P=0.003) affected by PCP addition x treatment x incubation time interaction. In particular, for all the treatments the addition of PCP severely depressed ARYL activity and

lower values were measured in all samples when compared to the controls (Fig. 5.5).

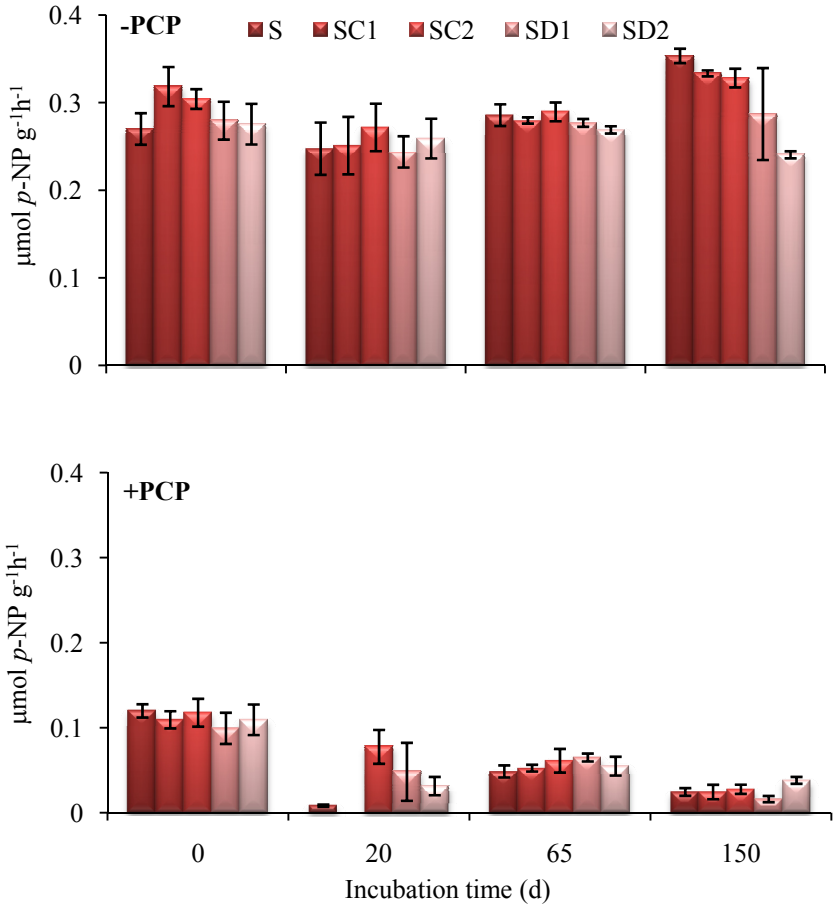

Fig. 5.5. Arylsulphatase activity (µmol *p*-NP g^{-1} h^{-1}) in controls (-PCP) and PCP-contaminated samples (+PCP). S (soil), SC1 (soil+0.27% compost), S-C2 (soil+0.83% compost), S-D1 (soil+0.07% DOM) and SD2 (soil+0.2% DOM). Each value is the mean of two replicates.

Moreover, ARYL activity was also significantly influenced by incubation time and treatment as single factors (P=0.001) and their interactions (P<0.01). Initial ARYL activity of 0.1 µmol *p*-NP g^{-1} h^{-1}, on average, was measured in PCP-contaminated samples against 0.3 µmol *p*-NP g^{-1} h^{-1} detected for the controls. A detectable decline of ARYL activity occurred in the first 20 d of incubation, more evident for S and S-C1, followed by a

slight increase at 65 d. At 150 d incubation all samples reached values close to zero.

In the control samples phosphatase activity (PHO), a crucial enzyme for the transformation of organic P compounds, was significantly (P>0.3) affected neither by the sampling time nor by the addition of compost or DOM (Fig. 5.6). Indeed, PHO activity of about 2.0 µmol *p*-NP g^{-1} h^{-1} was measured for all the samples, with no differences among compost- or DOM-amended samples.

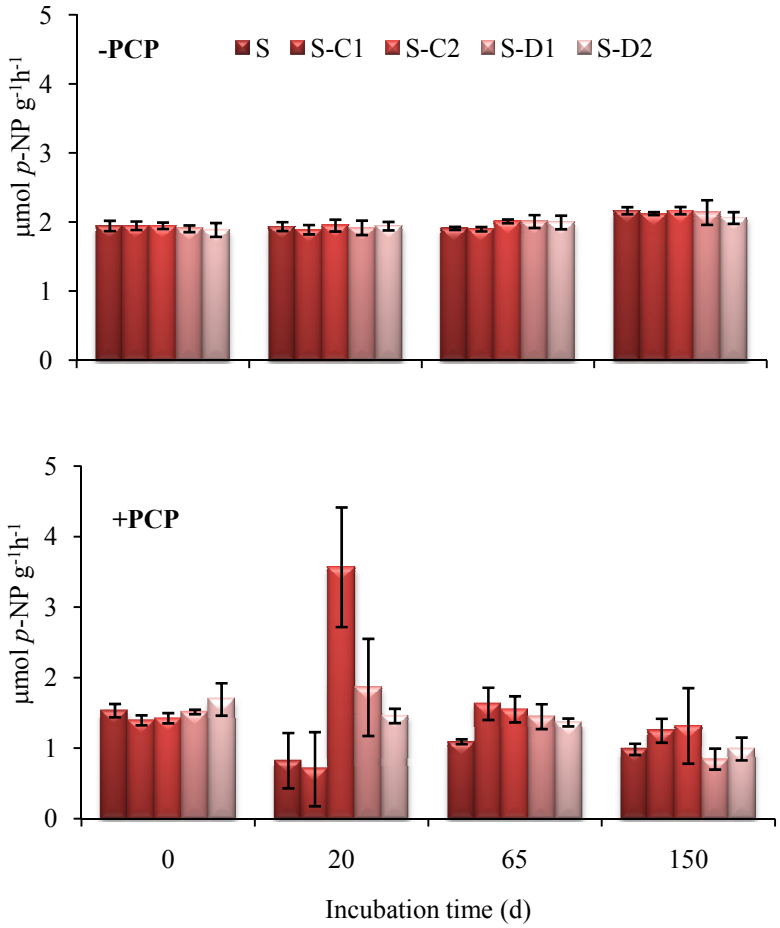

Fig. 5.6. Phosphatase activity (µmol *p*-NP g^{-1} h^{-1}) in controls (-PCP) and PCP-contaminated samples (+PCP). S (soil), SC1 (soil+0.27% compost), S-C2 (soil+0.83% compost), S-D1 (soil+0.07% DOM) and SD2 (soil+0.2% DOM). Each value is the mean of two replicates.

The addition of PCP significantly affected PHO activity only in the first 65 d of incubation: at zero time no differences in activity were observed when compared to the control samples; after 20 d of incubation substantial decreases in activity (46 and 50%) occurred in S and S-C1, respectively, while a great increase (activity of 3.5 μmol p-NP g^{-1} h^{-1}) was observed in S-C2. This opposite trend was suddenly annulled at 65 d of incubation, when all the samples levelled off to similar values. This behaviour was constant with time. The PHO activities of DOM-amended samples were not significantly affected by the presence of PCP all over the monitoring period with respect to the control samples.

A more dramatic effect of the presence of PCP was observed for urease activity. Negative values of UR were measured at each incubation time and for the majority of samples, thus indicating a possible strong interference of the PCP on the activity assay (data not shown). By contrast, controls showed detectable UR activity values. All the samples presented an initial UR activity of 2 μg NH_4-N g^{-1} h^{-1}. A severe decline for compost-amended samples and for S occurred after 20 d of incubation, followed by an increase until 65 d to values ranging from 3.3 ±0.4 μg NH_4-N g^{-1} h^{-1} for S-D2 up to 5.5 ±0.4 μg NH_4-N g^{-1} h^{-1} for S-C2. Afterwards, a decline to very similar values (on average 3.5 μg NH_4-N g^{-1} h^{-1}) occurred for all the samples.

Soil respiration was significantly and strongly affected by each factor considered and also by each of their interactions at $P<0.0001$ (Fig. 5.7). The presence of PCP increased soil respiration. The effect was evident and significant already at zero time (after 1 d of incubation) when the CO_2 produced in PCP-contaminated systems was ~ 4-fold higher than that of the controls (on average 17 mg *vs*. 4 mg of CO_2 for 100 g^{-1} of dry wt soil, 100 g dw^{-1}). For both controls and PCP-contaminated samples, the evolved CO_2

increased significantly as the incubation time increased, with similar trends in all the samples. At 31 d incubation, however, the increase in the samples with PCP was more evident for S-D1 where 100 mg of CO_2 100 g dw^{-1} were measured. At the end of the incubation all samples reached relatively similar values of evolved CO_2 (about 40 mg of CO_2 100 g dw^{-1}).

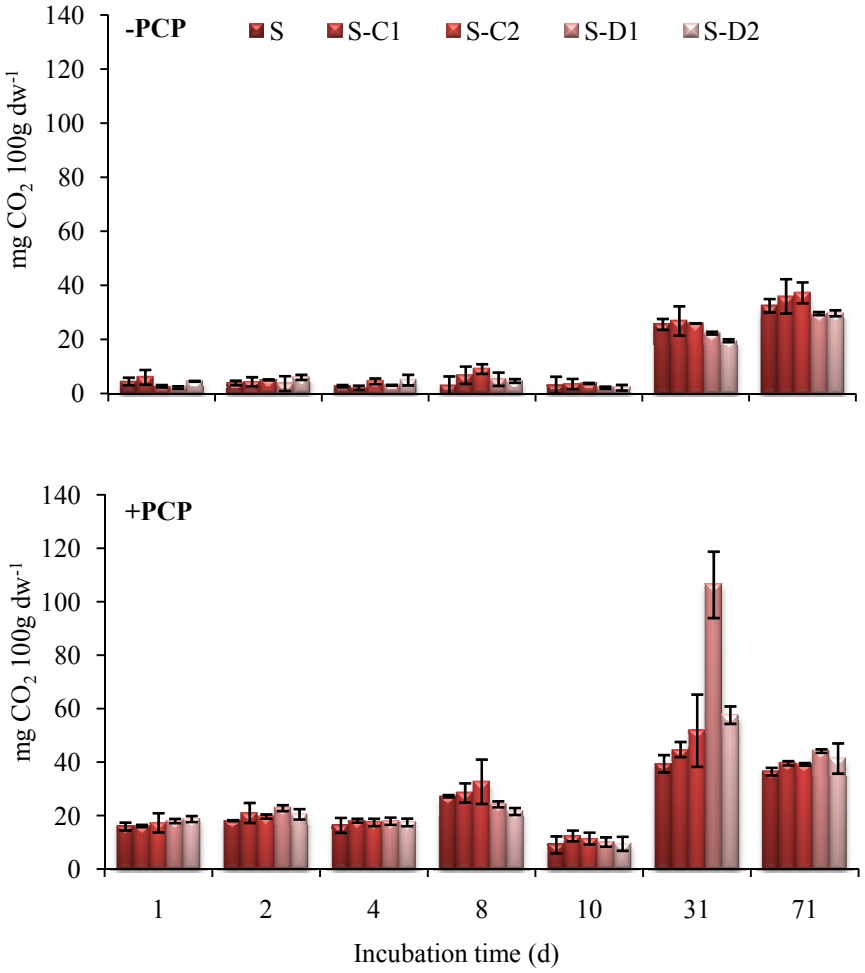

Fig. 5.7. Basal respiration of controls (-PCP) and PCP-contaminated samples (+PCP). S (soil), SC1 (soil+0.27% compost), S-C2 (soil+0.83% compost), S-D1 (soil+0.07% DOM) and SD2 (soil+0.2% DOM). Each value is the mean of two replicates. Each value is the mean of two replicates.

In the absence of PCP a similar behaviour was detected for all the samples, with no differences among S and compost- and DOM-S samples. Also in

this case the final values (ranging from 29 to 37 mg of CO_2 100 g dw^{-1}) were higher than the initial ones.

The trend of biomass-C (MB-C) was quite similar in samples with or without PCP, and reflected the behaviour of DH activity (Fig. 5.8).

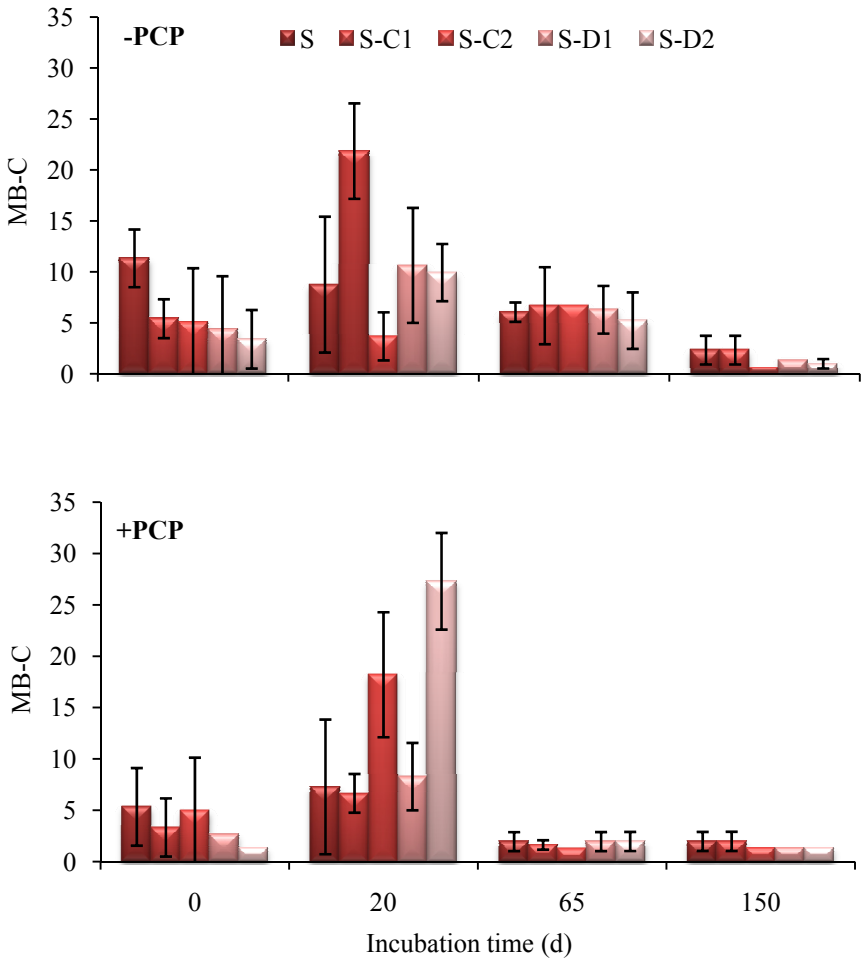

Fig. 5.8. Microbial biomass C (MB-C) of controls (-PCP) and PCP-contaminated samples (+PCP). S (soil), SC1 (soil+0.27% compost), S-C2 (soil+0.83% compost), S-D1 (soil+0.07% DOM) and SD2 (soil+0.2% DOM). Each value is the mean of two replicates. Each value is the mean of two replicates.

PCP addition x treatment x incubation time interaction significantly (P=0.001) affected MB-C values that differed from each other, whereas no significant differences were detected for PCP or treatment as single factors. In the controls a small decrease was observed for S with no significant

differences in time. The amended samples showed similar values at all the incubation times, except at 20 d when a sharp increase was observed for S-C1. At 150 d all samples reached values of MB-C close to zero. The addition of PCP resulted in lower values of biomass C already at zero time. After 20 d of incubation, a temporary positive effect was observed for S-C2 and S-D2, i.e. the soils amended with the higher dose of compost and DOM, respectively. Both showed higher values of MB-C. At 65 and 150 d the biomass was close to zero for all the samples, irrespective of the presence of amendments.

5.6.4. Growth of fungi during incubation

White spots, easily visible with the naked eye and resembling fungal mycelium, started to develop on the surfaces of the PCP-contaminated samples at 65 d incubation and intensified during the experiment. The phenomenon was much more evident for the S-DOM samples. No spots were observed on control samples (Fig. 5.9). The growth of fungi was assumed to be induced by the presence of PCP. The biofilm was removed from the 150 and 500 d incubated samples and subjected to total fungal count ($4 \cdot 10^5$ CFU on average) using a fungi-specific substrate. Two fungal colonies differing in morphology were isolated and cultivated separately. Both were identified as fungi belonging to the *Byssochlamys fulva* strain of the taxonomic group of *Ascomycetes*. *Byssochlamys fulva* is a soil fungus which produces heat-resistant spores (Doyle et al., 1998). This species is distributed world-wide in bottled fruit, harvested grapes and soil, especially in orchards. It has been implicated in the spoilage of canned and bottled fruit. It produces pectinolytic (Reid, 1951; Chu and Chang, 1973) and ligninolytic (Furukawa et al., 1999) enzymes. In addition, Doyle et al.

(1998) analysed a α-amylase from *B. fulva* capable to produce industrially significant levels of maltose (55%, w/w) on hydrolysis of starch.

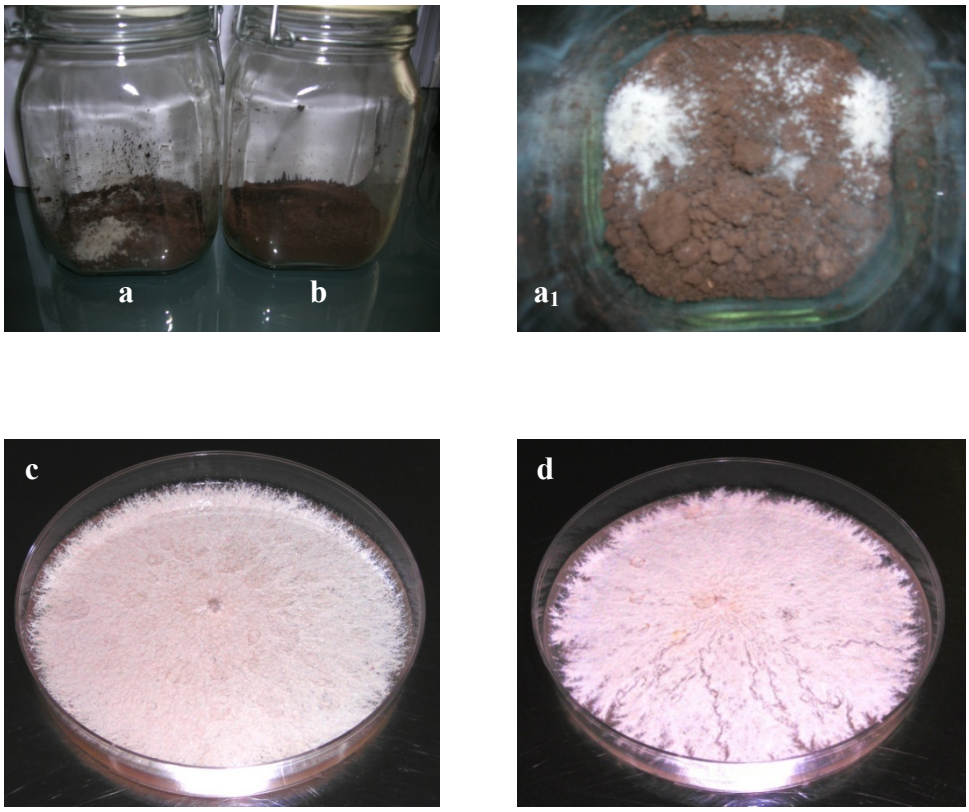

Figure 5.9. Photographs of PCP-contaminated samples at 150 d incubation and the isolated fungal colonies. White spots on the surface of the S-D1 PCP-contaminates sample, as seen (a) from the front and (a_1) from the upper side, (b) S-D1 control sample as seen from the front side; (c) and (d) colonies of *Byssochlamys fulva*, belonging to taxonomic group of *Ascomycetes*.

Experiments were performed to establish whether the isolated strain could transform PCP. When the fungal isolate was grown on PCP, 20% of the initial PCP concentration was degraded in 8 days, thus indicating that the isolated fungal species was tolerant to PCP and able to degrade it.

5.7. Discussion

When PCP is added to a fresh non-contaminated soil several processes may simultaneously take place and concur to determine the response of the soil to PCP contamination: i) adsorption of PCP onto the soil that increases with ageing and causes its sequestration in the soil matrix, ii) inhibition or even repression of endogenous soil microbial activity, iii) degradation/transformation of PCP by endogenous microbial organisms possibly stimulated by its presence, and iv) subsequent production of PCP transformation products, very often more toxic than their parent precursor. These processes may be strongly influenced by the addition of nutrient sources such as compost or by additional organic matter such as DOM.

Overall the results reported herein seem to indicate that all the cited processes very likely occurred in the investigated systems, thus determining their response to PCP contamination. PCP concentration actually decreased significantly in the investigated samples (Fig. 5.1). In particular, its residence for 500 d in the various systems led to a significant reduction in its extractable amount, mainly from the samples amended with the lowest compost dose C1 and with the two DOM amounts (Fig. 5.1). The fractionation of samples in the humic fractions indicated that PCP was in practice recovered only from the organic soil fractions (Fig. 5.2).

Adsorption of PCP on soil is a combination of hydrophobic and electrostatic interactions, and their relative importance is dependent on the environmental conditions, particularly on pH and soil organic matter. At the soil pH values (7-8) in this case-study PCP, a weak acid with a pK_a of 4.75, is present as a phenolate ion (Tam et al., 1999; Park and Bielefeldt, 2003) and as such it is to a lesser extent adsorbed to soil, or not at all. PCP can, however, form irreversible bonds with soil (Chen et al., 2004) even at high pH values (Abramovitch and Capracotta, 2003). Indeed, it forms a

non-extractable complex with soil, probably because it may be trapped in the clay lamella, and becomes impossible to extract even with exhaustive extraction methods. Moreover, PCP is a hydrophobic molecule with low solubility in water at neutral pH even when it exists as a phenolate ion (Tam et al., 1999). The presence of organic matter may enhance the sorption of PCP to soil (Park and Bielefeldt, 2003), and the extent of enhancement appears to increase with the amount of organic matter (Banerji et al., 1993). By increasing the organic matter of the soil (higher TOC values in the compost- and DOM-amended soils) compost or DOM addition evidently favoured the sorption of the compound to the soil matrix. Additionally, the presence of fungi detected in the PCP-contaminated systems could also have favoured the entrapment of PCP in the humic material, thus rendering PCP hard to extract (Rüttimann-Johnson and Lamar, 1997).

The higher amounts of PCP extracted from the humin fraction (HU), with respect to the FA and HA fractions, as well as its lower amounts measured in DOM-amended soil samples (Fig. 5.2), seem to support the previous hypotheses.

The investigated agricultural soil showed an intrinsic microbial activity as indicated by the increase and/or the constant values of most biochemical properties, observed as the incubation time increased. Live microbial cells very likely existed in it and their activity was expressed after a first acclimation period to the new environmental (laboratory) conditions.

The lower values of DH activity (Fig. 5.4) and microbial biomass, both indicative of microbial growth and activity, and of ARYL activity (Fig. 5.5) indicate that PCP actually exerted a depressing effect on soil microbial activity. Moreover, the absence of significant increases in biomass-C and the previously cited enzymatic activities measured in the presence of

compost or DOM (Fig. 5.8) clearly indicates that the PCP effect was so toxic as to annul any stimulating effect on soil microbial activity by both amendments acting as additional carbon sources.

Pentachlorophenol as a biocide may impact negatively on microflora diversity and soil activity. Chaudri et al. (2000) showed that 50 mg kg^{-1} of PCP, the same amount utilized in this study, was deleterious to the soil microbial biomass and lower biomass-C values were measured with respect to soil not spiked with PCP. Also, smaller concentrations of PCP applied to soil significantly decreased the soil microbial biomass (Schönborn and Dumpert, 1990). McGrath and Singleton (2000) found that soil DH activity dramatically decreased in the presence of 250 mg kg^{-1} of PCP and did not recover throughout the experiment (6 weeks) although soil PCP levels dropped to 2 mg kg^{-1}.

In PCP-contaminated soils the higher respiratory activity (Fig. 5.7), the relatively similar, constant activity values of both PHO and GLU, an enzyme mainly produced by fungi (Perucci, 1992), the increase in TOC and TN during incubation, the constant values of the microbial C/N ratios, indicative of high proportions of fungi over bacteria,, and especially the detectable growth of fungi, displaying PCP degradative capability, suggest that soil microbial activity possibly revived and degradation/transformation of PCP occurred with the resulting formation of its metabolites.

Although no attempts were made to ascertain the presence of PCP degradation products in the investigated systems, the presence of its metabolites may be hypothesized. Several authors (Sato, 1983; Augustin-Beckers et al., 1994) have demonstrated that PCP is moderately persistent in the soil environment, with a reported half-life of 50 d, and then after this period PCP metabolites are formed. These metabolites may be more toxic than the parent compound to the indigenous soil microflora (McGrath and

Singleton, 2000). The lower biomass-C and DH activity, often used as an index of PCP metabolite toxicity (McGrath and Singleton, 2000), as well as the reduced ARYL and UR activities, measured at 65 and 150 d of incubation, even though PCP concentrations were apparently very low, seem to suggest that toxic PCP transformation products were formed.

5.8. Conclusions

In conclusion, our results appear to confirm that PCP has contrasting effects on the properties of a fresh, agricultural soil, caused by several complex processes occurring simultaneously in the systems concerned. In our experiment, PCP considerably reduced the levels of some biochemical properties that diminished with time, thus suggesting a depressing effect on the soil microflora which failed to recover from its initial toxic response to PCP. Conversely, the presence of the contaminant promoted the development of fungal colonies, possibly contributing to its degradation and consequent production of PCP metabolites, considered more toxic than the parent compound. Indeed, PCP disappeared from the systems and the presence of dissolved organic matter improved its depletion. However, an ageing phenomenon, partly resulting from the presence of the dissolved organic matter and leading to a decrease in extractable PCP, cannot be ruled out.

Although the experiments presented herein are limited by the controlled laboratory conditions adopted (i.e., absence of soil fauna and leaching), they may be suitable for providing information on the intensity and nature of the response of soil to an applied disturbance. Furthermore, such investigations may be helpful for further studies aimed at validating and extrapolating the data to natural situations.

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Chapter 6

General conclusions

The purpose of the present research was to study the response of an agricultural soil to the presence of two persistent organic contaminants, phenanthrene and pentachlorophenol, belonging to the group of polycyclic aromatic hydrocarbons and chlorinated compounds, respectively.

The efficiency of two strategies, biostimulation and bioaugmentation, was also evaluated for the bioremediation of the contaminated soils.

In particular, biostimulation was explored by using a matured compost from solid urban wastes. A Phe-degrading culture was used in bioaugmentation experiments. It was proven to be competent in degrading phenanthrene under different environmental conditions.

The efficiency of the two bioremediating approaches was also evaluated against a Phe-aged (2 years) contaminated soil.

The obtained results demonstrated that two complex processes occurred simultaneously in the contaminated soil: natural attenuation and ageing. The soil showed an intrinsic capability in degrading both the added contaminants, thus confirming that natural attenuation processes actually occurred. On the other hand, an ageing phenomenon, favoured by the presence of the high soil organic carbon content or enhanced by the supply of additional organic matter also occurred. Experiments performed on the soil contaminated with phenanthrene and incubated for 2 years supported the occurrence of the ageing process.

The addition of compost as well as the inoculation with the Phe-degrading bacterial culture strongly stimulated and enhanced the attenuation process toward phenanthrene detoxification in the fresh contaminated soil. Furthermore, several of the soil properties showed differentiated responses

to the presence of the Phe, the compost, and/or the exogenous culture. As respect to fresh contamination, the aged Phe-contaminated soil behaved differently because high amounts of Phe were sequestered into the humic fractions. For instance, a detectable acclimatization period was needed to the Phe-degrading culture to be able to transform the aged phenanthrene.

When PCP was the contaminant, the levels of some biochemical properties diminished with time, thus suggesting a depressing effect on the soil microflora which failed to recover from its initial toxic response to PCP. Conversely, fungal colonies, possibly contributing to PCP degradation and consequent production of its metabolites, considered more toxic than the parent compound, developed in the PCP-contaminated soils.

The temporary and permanent changes observed in several of the tested biochemical properties, in response to the presence of the two contaminants, strengthen the concept that soil biological investigations (such as soil respiration, biomass, and enzyme activities) can give useful information on the status of soil quality, and on soil resilience to anthropogenic influences.

Although the experiments presented herein are limited by the controlled laboratory conditions adopted (i.e., absence of soil fauna and leaching), they can well act as a monitoring tool for the decontamination process of a polluted soil. Furthermore, such investigations may be helpful for further studies aimed at validating and extrapolating the data to natural situations.

Appendix

Proteomics as tool to monitor soil contamination

Environmental proteomics concerns the study of proteins and peptides found in water, sediments, soils, etc.

In the soil environment, in particular, proteins are released after the death and disruption of the cells of organisms, or as extracellular enzymes, which are excreted by a number of microorganisms (Skujinš, 1976). Proteins are also secreted from plant roots (Brenner et al., 1998).

Although the extracellular proteins present in soil are quickly decomposed into small polypeptide fragments by indigenous soil microbes, a small portion is considered to be resistant to microbial decomposition by binding with clay minerals and humic substances (Boyd and Mortland, 1990).

Nowadays, proteomics is rapidly becoming an essential component of biological research such as health, environmental and agricultural sciences.

Analysis of proteins extracted from environmental samples may help to characterise the response of microbial communities to stressful conditions such as contamination with toxic chemicals (Blom et al., 1992), starvation (Matin, 1990), heat (Neidhardt et al., 1984), or oxygen levels (Morgan et al., 1986; Spector et al., 1986). Study of proteins can be utilized as a 'fingerprint' to type the diversity in the sample (Wright, 1992) and as an index for monitoring the progress of a biocatalytic reaction in situ (Ogunseitan, 1993).

Proteomics can be used as tool to monitor biological effects of potentially toxic contaminants on soil ecosystems as alternative to the traditional study of soil enzyme activities and other soil quality indicators. These measurements often bear no relation between total levels of pollutants and their actual toxicity due to decreased pollutant bioavailability in the

complex soil ecosystem and it is extremely important to determine if pollutants are affecting soil functions or microbial populations (Singleton et al., 2003).

Soil is a very complex system and because of its high humic matter contents it is very difficult to obtain clear protein extracts. Although several protein extraction methods are improving (Singleton et al., 2003; Ogunseitan, 2006; Benndorf et al., 2007; Solaiman et al., 2007; Masciandaro et al., 2008), there is not a standardization of these methods.

We are now interested in developing a reproducible protein extraction method from soil, thus identifying biomarkers of environmental stress, in particular the aim of this part of the research is addressed to identify proteins as indicators of organic contamination by phenanthrene and pentachlorophenol in soil.

The preliminary study here reported was performed at The University of Warwick, Coventry (UK) under the supervision of Prof. Elizabeth M.H. Wellington.

Materials and Methods

Three different methods were selected to extract proteins from Phe- and PCP-contaminated soils. A non-contaminated soil was used as control.

The first method, described in detail by Masciandaro et al. (2008), was used to extract extracellular proteins from soil with some modifications. Potassium sulphate 0.5 M pH 6.6. was added to soil (1:3, w:v) and the extraction was carried out at room temperature for 1 h in an orbital shaker at 200 rpm. Soil extracts were centrifuged at 10000 rpm for 15 min at 4 °C and filtered through a 0.22 μm millipore membrane to remove bacterial cells. Filtrated samples were dialysed against distilled water until reaching electrical conductivity values of less than 0.5 dS m^{-1}, as salts could interfere

with further assays, such as SDS-PAGE. Then the dialysed extracts were concentrated with an Amicon PM-10 diaflomembrane (molecular cut-off 10.000) under a nitrogen atmosphere.

All the extractions were done in duplicate using different amounts of soil, in particular 30, 60 and 100 g.

The protein extract was precipitated by the TCA-DOC (Na-deoxycholate detergent) method. To one volume of protein extracts 1/100 volume of 2% DOC were added and allowed to stand for 30 min at 4 °C. Then 1/10 (v/v) of trichloroacetic acid (TCA) 100% (454 ml H_2O kg^{-1} TCA) was added and protein samples were precipitated overnight at -20 °C.

The samples were centrifuged at 14600 *g* for 15 min at 4 °C and after the supernatant was carefully removed, the TCA insoluble fraction was washed twice with one volume of cold acetone. After centrifugation, the supernatant was removed and discarded and the pellet was air-dried.

The second method was described by Benndorf et al. (2007). 5 g of soil were treated with 10 ml 0.1 M NaOH for 30 min at room temperature. The suspension was centrifuged 10 min at 16000 *g* at 20 °C. About 6 ml of supernatant were mixed with 16 ml phenol solution and 10 ml water and shaken for 1 h at 20 °C. Afterwards, the phases were separated by centrifugation (10 min at 14000 *g*). About 15 ml of the lower phenol phase were collected and washed by mixing with 15 ml water, followed by 5 min shaking and subsequent centrifugation (10 min at 14000 *g*). The proteins in the phenol phase (15 ml) were precipitated with the 5-fold volume of 0.1 M ammonium acetate in methanol at -20 °C overnight. Then, the sample was centrifuged (10 min at 16000 *g* at 0 °C), the pellet was suspended by sonication in 10 ml 0.1 M ammonium acetate in methanol, incubated 15 min at -20 °C and centrifuged again (10 min at 16000 *g* at 0 °C). The pellet was successively washed in 2 ml 0.1 M ammonium acetate in methanol,

2ml 80% acetone, 2 ml 70% ethanol, each washing step including 15 min incubation at -20 °C and subsequent centrifugation for 10 min at 16000 *g* at 0 °C.

The third method (not published) was performed for the first time at University of Milan, Department of Plant Productions under the supervision of Prof. Luca Espen.

Soil was treated with Tris-HCl buffer (1:5, w:v), 100 mM, pH 7.8 for 2 h at room temperature in an side-arm shaker.

The suspension was centrifuged 30 min at 13000 *g* at room temperature. Supernatant was removed and stored at 4 °C. The pellet was washed with further 15 ml of Tris buffer and shaked until resuspended. After centrifugation for 30 min at 13000 *g* (4 °C), supernatant was removed and combined with the first one. Phenol solution was added to the combined supernatants (1:1, v:v) and shaked for 30 min at 4 °C. After centrifugation for 30 min at 4000 *g* (4 °C), supernatant was discarded and ammonium acetate 0.1 M was added to the pellet (1:5, v:w) and incubated overnight at -20 °C. The pellet was successively washed four time in 2 ml 0.1 M ammonium acetate in methanol, three times in 0.5 ml 80% acetone, each washing step including 15 min incubation at -20 °C and subsequent centrifugation for 30 min at 13000 *g* at 0 °C.

Proteins concentrations was determined by Lowry colorimetric method.

All the dried pellets were resuspended in 25 µl of sample buffer for SDS-PAGE (Laemmli, 1970) and heated at 100 °C for 3 min. After a fast centrifugation, proteins were loaded on a sodiumdodecylsulphate-polyacrylamide electrophoresis gel (4% stacking/10% resolving) at 200 V, until the tracking dye was near the bottom of the gel. After electrophoresis, gels were fixed using the Silver Stain procedure. The relative molecular

masses of proteins in the gels were estimated by co-electrophoresis with standard protein markers (Sigma-Aldrich, Italy).

Results

Detectable results were obtained neither with Benndorf et al. (2007) method nor with the method performed in Milan.

By contrast, Masciandaro et al. (2008) method gave positive results but only using 100 g of soil. Fig. 1 shows the SDS-PAGE of proteins extracted from soil control (S1 and S2), soil+lysozyme (0.1%) (S3 and S4) and soil+biochitin (5%) (S5 and S6).

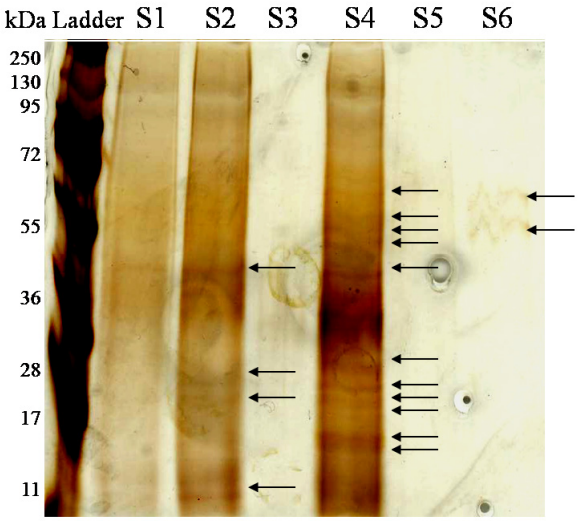

Fig. 1. SDS-PAGE of proteins.

Although visible protein bands were obtained, extracted samples were not clear probably because of the presence of humic substances. A calibration curve of BSA in the presence of different concentrations of humic acids was carried out (Fig. 2). 50 mg ml^{-1}, 100 mg ml^{-1} and 200 mg ml^{-1} of humic acids were added to BSA solution (1 mg ml^{-1}) to perform the calibration curve.

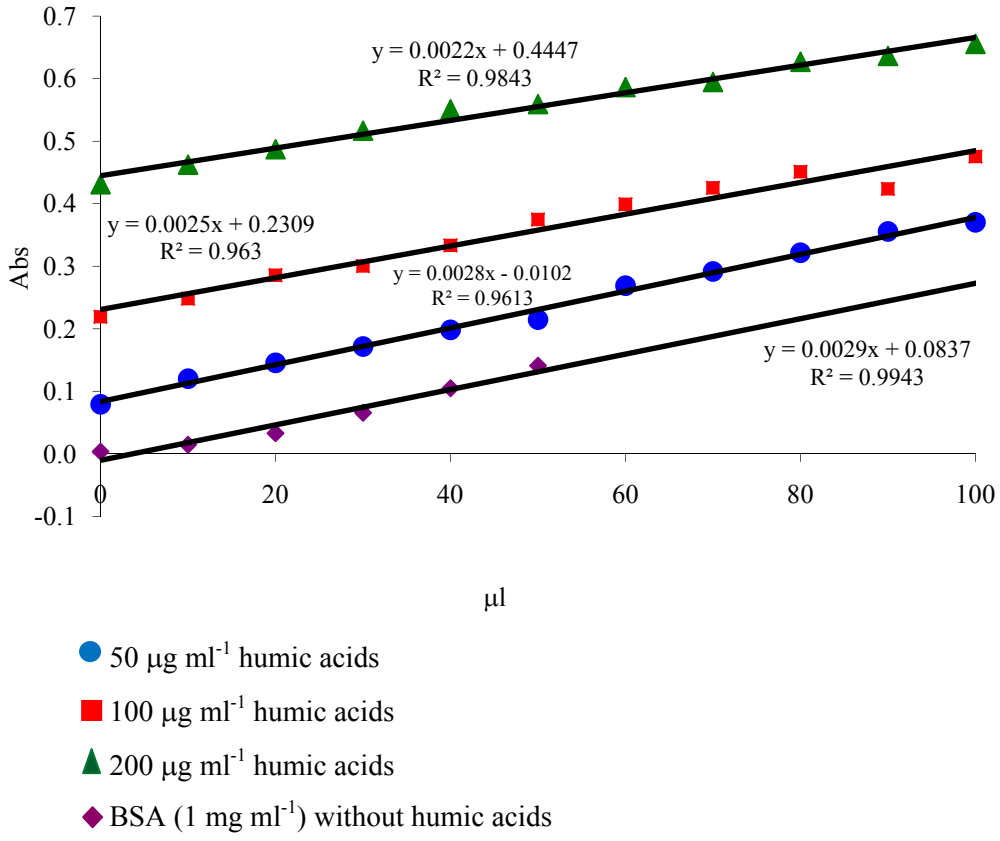

Fig. 2. BSA calibration curves with or without humic acids

The presence of humic acid evidently results in an overestimation of the proteins quantification.

These preliminary results seem optimistic and future studies will be devoted to optimizing the protein extraction by using purification steps to decrease the presence of interfering compounds that affect protein bands and to identify proteins by using innovative techniques such as 2-DE and LC-MS analysis.

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Curriculum Vitae

Personal data

Name Rosalia Scelza

Residence Via Alcide de Gasperi 180, 80053 C/mare di Stabia (Naples) Italy

Date of birth July 26, 1977

Place of birth C/mare di Stabia (Naples) Italy

Nationality Italian

Education

11 . 2005 - 10 . 2008 Ph.D. student in Agrobiology and Agrochemistry, University of Naples Federico II, Faculty of Agriculture, Naples, Italy.

09 . 2003 Degree in Food Sciences and Technologies, mark 105/110, University of Naples Federico II. Thesis entitled 'Selection of microbial cultures able to bio-preserve mozzarella cheese' Tutor: Prof. Salvatore Coppola.

Languages

Proficient in English.

Research experiences

04 . 2004 – 12. 2004 Research Fellowship at the Department of Soil, Plant and Environmental Sciences, University of Naples Federico II, Naples, Italy.

01 . 2005 – 12 . 2005 Research Fellowship at the Department of Soil, Plant and Environmental Sciences, University of Naples Federico II, Naples, Italy.

12 . 2005 – 04 . 2006 Research Fellowship at the Department of Soil, Plant and Environmental Sciences, University of Naples Federico II, Naples, Italy.

06 . 2006 – 11 . 2007 Research Fellowship at the Department of Soil, Plant and Environmental Sciences, University of Naples Federico II, Naples, Italy.

03 . 2008 – 07 . 2008 Research Fellowship at the Department of Soil, Plant and Environmental Sciences, University of Naples Federico II, Naples, Italy.

07 . 2008 – 04 . 2009 Research Fellowship at the Department of Soil, Plant and Environmental Sciences, University of Naples Federico II, Naples, Italy.

Stages

05. 2006 – 06. 2006 Department of Food and Microbiology Sciences and Technologies and Department of Plant Productions of the University of Milan, Italy.

- Techniques of molecular biology (DNA extraction from complex matrices, amplification via PCR, analysis of microbial DNA with DGGE technique, protein extraction and following two dimensional electrophoresis).

04 . 2008 – 08 . 2008 Department of Biological Sciences of The University of Warwick, Coventry, United Kingdom.

- Molecular biology techniques for DNA and RNA extraction from soil and microbial cultures. Synthesis of cDNA via PCR.
- Direct extraction of proteins from soil followed by SDS-PAGE analysis.

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