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MINI-REVIEW

Bioremediation and monitoring of aromatic-polluted habitats

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Abstract Bioremediation may restore contaminated soils through the broad biodegradative capabilities evolved by microorganisms towards undesirable organic compounds. Understanding bioremediation and its effectiveness is rapidly advancing, bringing available molecular approaches for examining the presence and expression of the key genes involved in microbial processes. These methods are continuously improving and require further development and validation of primer- and probe-based analyses and expansion of databases for alternative microbial markers. Phylogenetic marker approaches provide tools to determine which organisms are present or generally active in a community; functional gene markers provide only information concerning the distribution or transcript levels (deoxyribonucleic acid [DNA]- or messenger ribonucleic acid [mRNA]-based approaches) of specific gene populations across environmental gradients. Stable isotope probing methods offer great potential to identify microorganisms that metabolize and assimilate specific substrates in environmental samples, incorporating usually a rare isotope (i.e., ^{13}C) into their DNA and RNA. DNA and RNA in situ characterization allows the determination of the species actually involved in the processes being measured. DNA microarrays may analyze the expression of thousands of genes in a soil simultaneously. A global analysis of which genes are being expressed under various conditions in contaminated soils will reveal the metabolic status of microorganisms and indicate environmental modifications accelerating bioremediation.

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Introduction

In the recent years, a high number of polluting compounds has been released into the environment because of several industrial and/or agricultural activities. In particular, the rapid industrialization of agriculture, expansions in the chemical industry, and the need to generate cheap forms of energy have all resulted in an ever-increasing reliance on anthropogenic organic chemicals and caused the contamination of a significant number of soil environments by xenobiotic compounds (Reid et al. 2000) with negative, irreversible effects on environmental quality and health.

Over the last decades, there has been an increasing interest in biological methodologies, collectively indicated as *bioremediation* that may help reduce the risk of organic pollutants in soil and effectively restore polluted sites. These methodologies, usually considered environment-friendly treatments, constitute essentially a managed or spontaneous process mediated by living organisms (mainly microorganisms), which degrade or transform contaminants to less toxic or nontoxic products, with mitigation or elimination of environmental contamination.

To evaluate the status of a contaminated site in terms of its capability of successfully responding to a bioremediation approach, to design and implement suitable microbial detoxifying strategies, and finally to monitor the effectiveness of the

bioremediation approach, not only a detailed understanding of the main metabolic and genetic features of contaminant microbial degradation but also the advantages and bottlenecks of the available monitoring methodologies are needed.

This is a brief survey of some aspects, with particular reference to soil, concerning (a) the pollution of the environment by two classes of aromatic pollutants namely, polycyclic aromatic hydrocarbons (PAHs) and volatiles aromatics collectively indicated as BTEX (benzene, toluene, ethylbenzene, xylene), (b) the main metabolic pathways and the genetic bases of their microbial degradation, (c) the biological strategies to reduce or to eliminate their contamination, and (d) the more advanced monitoring techniques to evaluate the effectiveness of a bioremediation process.

Fate of PAHs and BTEX in soil

Soils and groundwater are preferred sinks for complex contamination. As possible consequence, various chemical, biological, and biochemical soil properties have been profoundly altered, and their main effect has been the continuous lost of soil functions in sustaining the survival of living organisms.

PAHs (i.e., naphthalene, phenanthrene, anthracene, etc.) and BTEX, occurring ubiquitously in the environment as complex mixtures, are particularly abundant and have been recognized as an environmental contamination problem of worldwide magnitude. PAHs, listed by the US Environmental Protection Agency and the European Community as priority pollutants (Samanta et al. 2002), are hydrophobic compounds occurring in soil, sediment, water, air, and plants as the result of the incomplete combustion of coal, oil, petrol, and wood or of petrochemical industries. The majority of these substances are highly persistent within ecosystems in unaltered, less degradable chemical forms because of their low water solubility, their intrinsic chemical stability, and high recalcitrance to degradation. Therefore, hydrocarbon-polluted sites may represent a long-term source of pollution and pose a severe risk to environmental health.

BTEX enter the environment primarily through processes associated with gasoline and petroleum fuels, leakage of underground petroleum storage thanks, and spills at petroleum wells but also from industrial effluents, wood processing, and manufacturing of pesticides, detergents, chemicals, paints, and varnishes. They are highly soluble and volatile toxic substances, thus forming one of the main groundwater and health-risk contaminant groups.

The fate and behavior of PAHs and BTEX in soil is governed by several different factors linked to soil properties, compound characteristics, and environmental factors (Fig. 1).

In general, when an organic chemical enters the soil, it can be subjected to two basic processes (Fig. 1a): (1) transfer processes that relocate the substances without altering their structure and (2) degradation processes that alter their chemical structures, by the splitting in different products.

In all the processes depicted in Fig. 1a, a main role is played by the interactions occurring at interfaces between organic and inorganic soil colloids and chemicals through sorption/desorption mechanisms (Stotzky 1986). These interactions may strongly affect the movement of chemicals, their availability for plant or microbial uptake, and their transformation by abiotic or biotic agents.

Moreover, the concentration of an organic contaminant in the liquid phase, regulating consequently its accessibility to target organisms, is mainly governed by adsorption whose extent depends on both the properties of the adsorbent matrix and of the compound.

If the organic pollutant is highly hydrophobic, i.e., with a very low solubility in water, the most important process affecting the rate of all above mentioned processes in soil is its partitioning between soil components: air, soil solution, and soil matrix. In particular, the higher is the hydrophobicity of the compound, the greater is its persistence in the environment, with deleterious, often irreversible effects on environmental health.

The persistence of an organic contaminant is, however, not only due to its intrinsic molecular property, but it is much more a result of environmental microhabitats that affect both the mass transfer of the xenobiotic to microorganism and its degradative activity (Huesemann et al. 2004).

The PAH bioavailability is further complicated when PAHs interact with nonaqueous phase liquids (NAPL) and soil colloids, thus resulting less or completely unavailable for microorganisms (Efroymson and Alexander 1995, Birman and Alexander 1996).

An important process affecting the concentration of an organic compound in soil and consequently its availability is the “aging,” (Fig. 1b) during which the organic contaminant can form stronger bonds with the soil or can be incorporated or sequestered in structural micropores of mineral lattices or in hydrophobic remote areas of the soil organic matrix (Alexander 2000; Gevao et al. 2000). This can occur at a so greater extent that desorption becomes very slow, thus limiting the flux of the contaminant to the aqueous phase, and the contaminant becomes not readily bioavailable. The combination of the above phenomena leads to a different distribution and partitioning of the contaminants in soil (Weissenfels et al. 1992), rendering them less readily bioavailable, resistant to biodegradation, and thus more persistent in the soil (Barraclough et al. 2005).

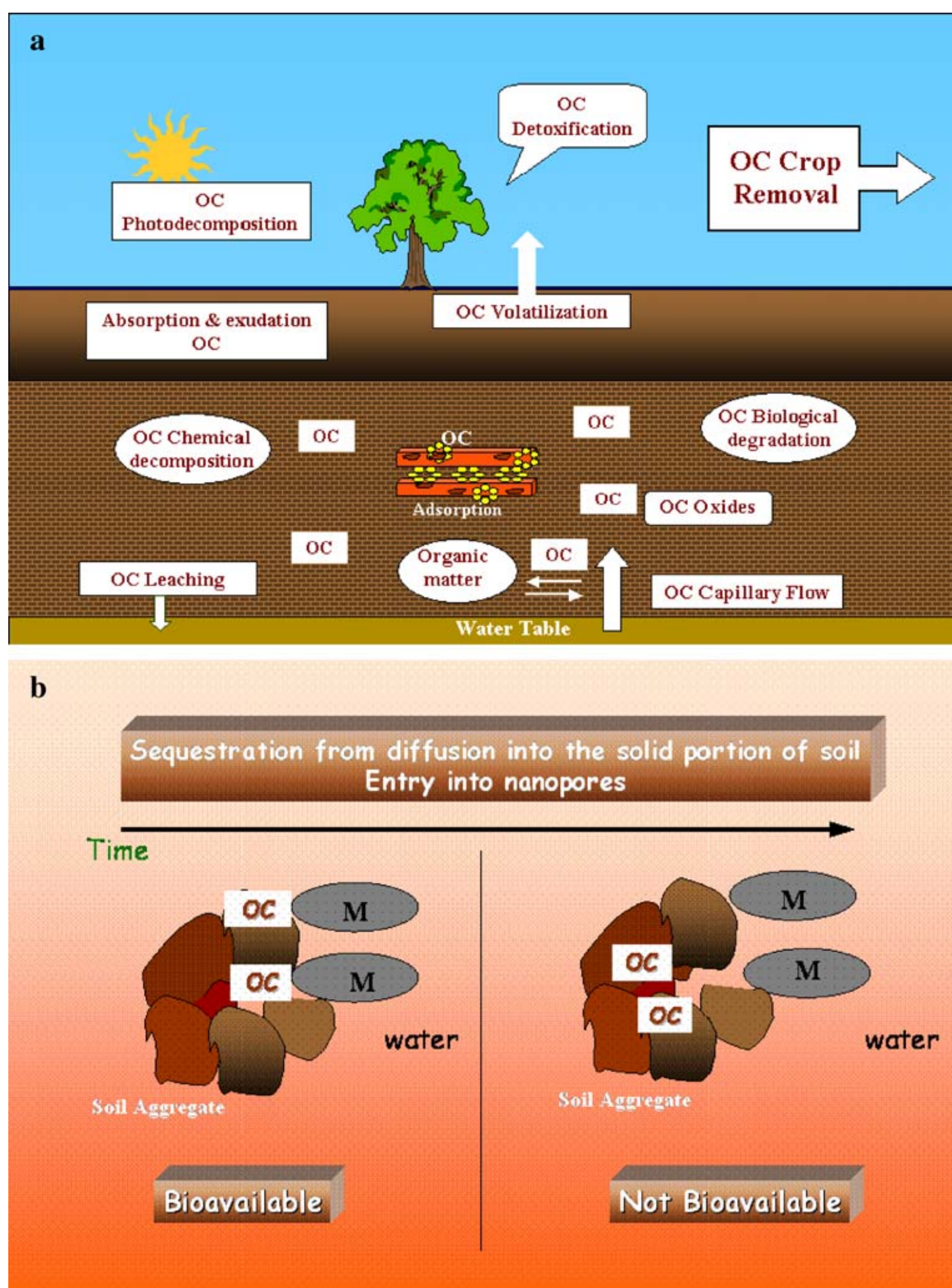


Fig. 1 Main processes (**a**) and aging (**b**) affecting the fate of an organic chemical in soil. *OC* Organic chemical, *M* microorganisms

Similarly to other volatile organic compounds, BTEX can adsorb into soil organic matter and on soil mineral surfaces. Their behavior in soil as well as their removal by bioremediation approaches has been illustrated by several authors, resorting often the use of model systems and claiming the importance of diffusion mechanisms in the heterogeneous soil matrix (Stapleton et al. 1998; Margesin et al. 2003a; Scullion 2006). Soil has been often considered a biphorous sorbent model, and the contribution of soil organic matter has been negligible to describe the adsorption kinetics of toluene (Arocha et al. 1996).

Microbial degradation of PAHs and BTEX

PAHs and BTEX are biodegradable under both aerobic (Gibson and Parales 2000; Habe and Omori 2003) and anaerobic conditions (Gibson and Harwood 2002; Chakraborty and Coates 2004).

Under aerobic conditions, bacteria and fungi utilize oxygen for both ring activation and cleavage of the aromatic nucleus and as the electron acceptor for its complete degradation.

The biodegradation of PAHs can serve as carbon and energy sources for the degrading organisms (assimilative

biodegradation) or for intracellular detoxification. The intracellular hydroxylation of PAHs in bacteria is an initial step in preparing ring fission and assimilation, whereas in fungi, it is an initial step in detoxification (Cerniglia 1993).

PAHs with low molecular weights are more rapidly degraded than less soluble and higher molecular weights compounds. Indeed, only a very limited number of bacteria can grow in pure cultures on PAHs with five or more aromatic rings. Environmental bacterial isolates often degrade only a narrow range of PAHs, and the patterns of the simultaneous degradation of PAH mixtures are complex. Often, they are cooperative processes that involve a consortium of strains with complementary capacities (Bouchez et al. 1995).

PAH bacterial metabolism usually occurs via the initial incorporation of molecular oxygen by an initial dioxygenase three-component enzymatic system (Habe and Omori 2003) and forming a *cis*-dihydrodiol. Figure 2 summarizes the initial attack of low molecular PAHs. Naphthalene, phenanthrene, anthracene, and pyrene are biodegraded through similar steps (Fig. 2a). The initial oxygenolytic attack produces for all the PAHs the dihydroxylated derivatives. Afterwards, these latter are further metabolized to few intermediates, including catechol and gentisic and protocathecuic acids (Fig. 2), which are channelled into the tricarboxylic acid cycle intermediates by their dioxygenase-mediated ring cleavage in either *meta* or *para* position.

The initial oxidative attack of BTEX consists of direct oxidation of the aromatic ring via a mono-oxygenase (Khan et al. 2001), a dioxygenase attack, or oxidation of the alkyl side chain, catalyzed by mono-oxygenase, through the typical toluene mono-oxygenase, toluene dioxygenase,

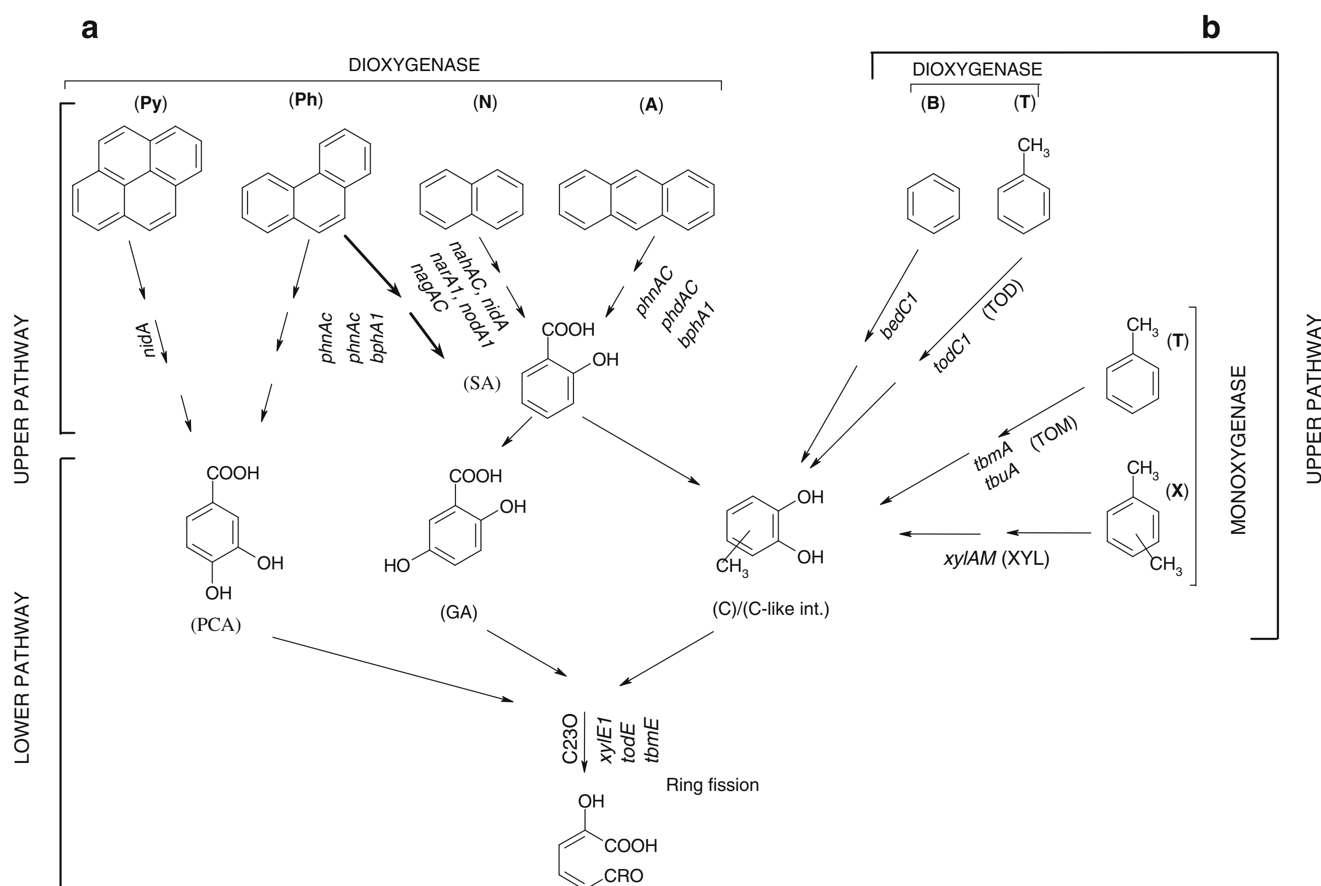


Fig. 2 Schematic presentation of initial microbial attack and ring cleavage and genes involved in the respective reactions for PAHs (**a**) and for BTEX (**b**). **a**, Naphthalene (*N*) is initially oxygenated by a naphthalene dioxygenase to yield 1,2-dihydroxynaphthalene, which is further degraded to salicylate (*SA*). SA is then metabolized via catechol (*C*) or gentisic acid (*GA*). Phenanthrene (*Ph*) is mostly oxygenated by a phenanthrene dioxygenase to yield 3,4-dihydroxyphenanthrene, which is further metabolized through two pathways: one follows the N biodegradation pathway with the formation of SA, and the other leads to protochatechuic acid (*PCA*). Anthracene (*A*) is dioxygenated at the 1,2 position to yield 1,2-dihydroxyanthracene, which is later metabolized to SA and C. Pyrene (*Py*) is mostly dioxygenated at the 1,2 position. A successive derivative, 4-phenanthroic acid, undergoes a second dioxygenase reaction and further metabolized via catabolic pathways similar to those of Ph (for detailed reactions, see Habe and Omori 2003). **b**, Toluene (*T*) and xylenes (*X*) are degraded via different routes to catechol-like intermediates (*C-like int*), catechol (*C*), and protocatechuic acid (*PCA*); benzene (*B*) is degraded to catechol (*C*) (adapted from Williams and Sayers 1994; Habe and Omori 2003)

and toluene pathways (Fig. 2b; Williams and Sayers 1994; Gibson and Parales 2000). All these pathways converge in the formation of (substituted) catechol intermediates (Reineke 1998), which are cleaved by *ortho*- or, as in the majority of the already described pathways, by *meta*-cleavage dioxygenases.

The biodegradability of PAHs and BTEX in the absence of oxygen by a diversity of organisms (Gibson and Harwood 2002; Chakraborty and Coates 2004) makes possible beneficial remediation under anaerobic conditions.

Anaerobic zones frequently develop in soils during compaction, as well as in soils and sediments contaminated with hydrocarbons, where the indigenous aerobic microbial population is stimulated and a rapid depletion of the dissolved oxygen content of the groundwater may occur (Lovley 1997). Water entering the contaminated environment carries only small amount of dissolved oxygen because of its low solubility, and O_2 replenishment from the atmosphere is slow.

The microbial utilization of aromatic compounds in the absence of oxygen occurs without the perturbation of the benzene nucleus. Anaerobic degradation of naphthalene and, in some instances, of phenanthrene has been measured in microbial communities in soil and sediments under condition of denitrification (Al-Bashir et al. 1990) or sulphate reduction (Zhang et al. 2000; Meckenstock et al. 2000). With the identification of a sequence of metabolites during naphthalene and phenanthrene degradation by consortia, a pathway for these PAHs has been depicted (Fig. 3). As confirmed with a pure culture of a sulphate-reducing bacterium, strain NaphS2 (Galushko et al. 1999), phenanthrene is carboxylated by the addition of an external bicarbonate molecule to yield phenanthrene carboxylic acid (Annweiler et al. 2002).

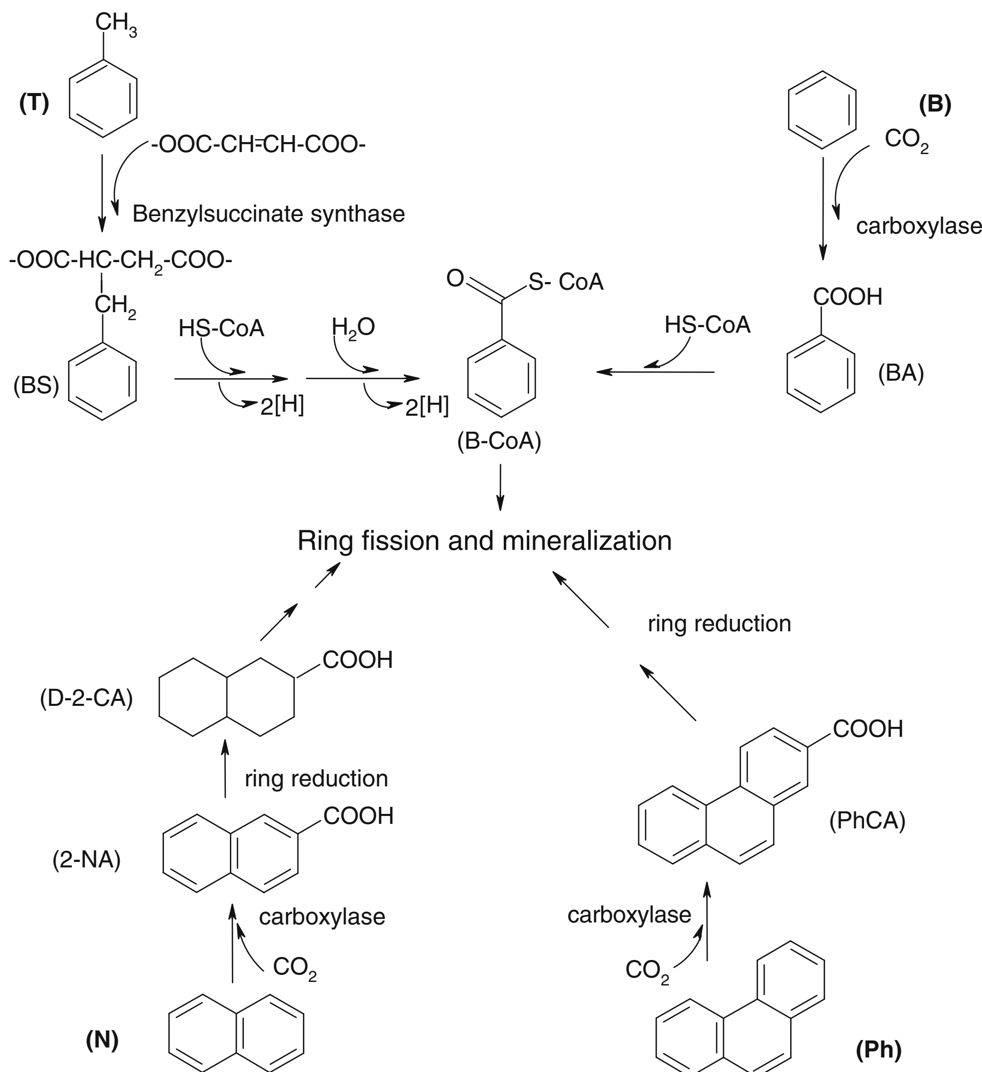


Fig. 3 Schematic presentation for the anaerobic degradation of toluene (*T*), benzene (*B*), naphthalene (*N*), and phenanthrene (*Ph*). Initial reaction involved: For T, addition of fumarate at the methyl group of toluene to form benzylsuccinate (*BS*). After activation with CoA, BS is oxidized through the formation of phenylitaconate to benzoyl CoA (*B-CoA*) as a central intermediate (for detailed reactions from BS to B-CoA, see Spormann and Widdel 2000); for B, addition of CO_2 to benzoic acid (*BA*) and subsequent transformation to B-CoA; for N, addition of CO_2 to yield 2-naphtoic acid (*2-NA*), which is further degraded by sequential reduction steps to decalin-2-carboxylic acid (*D-2-CA*); Ph is also directly carboxylated to phenanthrene carboxylic acid (*PhCA*) as the initial step towards mineralization (adapted from Young and Phelps 2005; Chakraborty and Coates 2004)

Among BTEX, the anaerobic biodegradation of toluene has been the most investigated. It can be degraded under many reducing conditions (Andersen et al. 1995; Coates et al. 1996). The first step in the anaerobic metabolism of toluene and xylene, as well as of *m*- and *p*-cresol and methylnaphthalene, is mediated by an unusual enzymatic reaction that is the addition of fumarate to their methyl group to form benzylsuccinate (Fig. 3) through a benzylsuccinate synthase (BBS) and not involving a net redox reaction. Benzylsuccinate has been identified in strains of *Thauera* and *Azoarcus*, in sulphate-reducing strains, and in methanogenic sediments (Krieger et al. 1999; Elshahed et al. 2001).

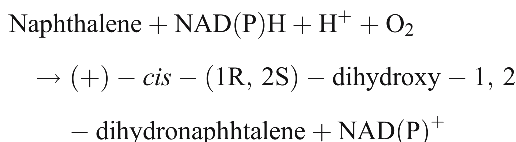
Anaerobic benzene degradation can include initial carboxylation (Fig. 3), hydroxylation, methylation, or reduction of the aromatic ring to form benzoyl-CoA (Chakraborty and Coates 2004). It is noteworthy that benzoyl-CoA for benzene, benzylsuccinate, and *e*-phenylitaconate for toluene, methylbenzylsuccinate for dimethylbenzene and xylene, are the unique extracellular intermediates identified during the anaerobic degradation of BTEX (Young and Phelps 2005).

The two intermediates of the naphthalene pathway, 2-naphthoic acid and decalin-2-carboxylic acid, can serve to assess an actual naphthalene degradation occurring in field sites. Similarly, benzylsuccinate-like metabolites, which are not anthropogenic compounds, can be used as indicators of the presence in the environment of an active microbial population utilizing BTEX. Detailed studies performed with Seal Beach groundwater in California confirmed that the concentration of such biomarkers decreased over time with a decrease in the concentration of BTEX (Beller et al. 1995).

Genetic bases of PAH and BTEX microbial degradation activity

The design of molecular tools for the detection of PAH and BTEX degradation actually occurring at a contaminated site requires to have clear insights into the genetic bases of their microbial degradation activity.

A broad range of oxygenases, differing in structure, mechanism, and cofactor requirement, are distributed among the microorganisms that grow on PAHs and BTEX. Aromatic-ring-hydroxylating dioxygenases generally consist of a terminal dioxygenase (an iron sulfur protein) and a reductase chain, which transfers electrons from NAD(P)H to the terminal dioxygenase. The reduced terminal dioxygenase catalyzes the direct insertion of molecular oxygen into the substrate to form *cis*-arene diols. Some terminal dioxygenases are homomultimers, whereas others are heteromultimers consisting of a large subunit (α) and a small subunit (β) (Furukawa et al. 1993). For example, naphthalene 1,2-dioxygenase catalyzes the reaction:



$$\mathrm{Naphthalene} + \mathrm{NAD(P)H} + \mathrm{H}^{+} + \mathrm{O}_2 \rightarrow (+) - cis - (1\mathrm{R}, 2\mathrm{S}) - \mathrm{dihydroxy} - 1,2 - \mathrm{dihydronaphhtalene} + \mathrm{NAD(P)}^{+}$$

The large subunits with the same reported substrate specificity are in general closely related, and their deoxyribonucleic acid (DNA) sequences are conserved. Current knowledge on the α-subunits of the terminal oxygenases indicates two major lineages of importance for PAH degradation. Phylogenetic studies of amino acid sequences of the proteins, involved in the initial oxidative attack of PAHs and BTEX and in their ring cleavage, show significant sequence homology, indicating a common ancestry that allowed the design of group-specific primer sets for detection by polymerase chain reaction (PCR) (Eltis and Bolin 1996; Meyer et al. 1999; Baldwin et al. 2003).

Among the Gram-negative PAH-degrading bacteria, several groups of genes for the initial naphthalene oxygenation, which are often borne on quite large and self-transmissible NAH plasmids, are known: *ndo* gene from *P. putida* NCIB9816, *nah* genes from *P. putida* G7 and NCIB 9816-4, *dox* genes derived from *Pseudomonas* sp. C18, and *pah* genes cloned from *P. putida* OUS82 and *P. aeruginosa* PaK1. Each gene name is referred to the substrate used by the strains: *nah* for naphthalene degradation, *ndo* for naphthalene dioxygenation (equivalent to *nah*), *dox* for dibenzothiophene oxidation, and *pah* for phenanthrene degradation. These genes are grouped as the "classical *nah*-like genes" for their gene organization and sequence similarity (about 90%) to that of the *nah* genes of strain G7.

Other phenanthrene and naphthalene upper catabolic genes are instead evolutionarily different from the *nah*-like genes. They have been characterized from both Gram-negative bacteria other than *Pseudomonas* species and from Gram-positive bacteria. They are *phd* genes from *Comamonas testosteroni* strain GZ39, *nag* genes from *Ralstonia* sp. U2, *phn* genes from *Burkholderia* sp. RP007, *Alcaligenes faecalis* AFK2, and *Sphingomonas aromaticovorans* 177 and its related species (Habe and Omori 2003). The *phn* genes, although isofunctional to the classical *nah*-like genes, show very low homology, being significantly different in sequence and gene order from the previously characterized genes for PAH degradation. Strain RP007 utilizes both phenanthrene and naphthalene (Laurie and Lloyd-Jones 1998); strain AFK2 utilizes phenanthrene but not naphthalene (Kiyohara et al. 1982); differently, *Sphingomonas* F199 can grow on various monocyclic aromatic hydrocarbons and PAHs, on biphenyl

and dibenzothiophene (Fredrickson et al. 1999). Dioxygenases involved in PAH degradation by Gram-positive bacteria are distantly related to PAH dioxygenases of Gram-negative strains. *Nar* genes and *phd* genes have been identified in *Rhodococcus* sp. strain NCIMB12038 (Larkin et al. 1999) and in *Nocardioides* sp. KP7 (Saito et al. 1999), respectively. Similar dioxygenase system (*nid*) genes which encode a novel polycyclic aromatic-ring dioxygenase have been reported from *Mycobacterium* sp. PYR-1 and *Mycobacterium* sp. 6PY (Khan et al. 2001). In particular, PYR-1 can mineralize pyrene, 1-nitropyrene, phenanthrene, anthracene, fluoranthene, and benzo(α)pyrene and has either mono- or dioxygenases to catalyze the initial attack of PAHs.

A second type of aromatic dioxygenases is composed of biphenyl and monoaromatic dioxygenases. The toluene/biphenyl family includes enzymes for the degradation of toluene (*tod* genes), benzene (*bed* genes), isopropylbenzene (*ipb* genes), chlorobenzene (*tcb* genes) and biphenyl (*bph* genes) from both Gram-negative and Gram-positive bacteria (Gibson and Parales 2000; Baldwin et al. 2003). The dioxygenases for toluene, benzene, and chlorobenzene degradation are closely related to each other and can be used to deduce toluene-dioxygenase-specific primers (TOD primers).

By aligning the nucleotide sequences of the large subunits of toluene monooxygenase genes, the existence of two types of monooxygenases, differing in their mode of attack, have been found. They are divided into families on the basis of substrate specificity: two families of aromatic hydrocarbon monooxygenases (*tmo* genes in *Pseudomonas mendocina* KR1 and *tbm* genes in *Pseudomonas* sp. strain JS150) and one family of phenol hydroxylase (*phh* genes in *Pseudomonas putida* P35X and *dmp* genes in *P. putida* CF600) and the alkyl-group-hydroxylating monooxygenases, (*xyl* genes in *P. putida* mt-2). The *tbm* operon from strain JS150 has a strong sequence identity to the phenol hydroxylase encoded by the *dmp* and *phh* genes. On the basis of the apparent phylogeny of these monooxygenases, four primer sets have been identified by Baldwin et al. 2003 to detect these genes.

Finally, the catechol 2,3-dioxygenase genes comprise a diverse family of genes (Eltis and Bolin 1996) that codes for a group of enzymes with aromatic ring fission activity toward a wide range of aromatic pollutants. The gene phylogeny of these very closely related sequences does not follow strictly a taxonomical relation with the bacterial hosts because these genes are mainly found on plasmids and their evolution and conservation rates are heavily affected by traits like selection pressures, horizontal transfer, and mobile genetic elements (Williams et al. 2002).

Collectively, these features make the α-subunits of the iron sulfur protein, components of dioxygenases, amenable to retrieval by PCR and allow for the identification of retrieved fragments in the context of existing databases.

In recent years, substantial progress has been made in understanding the catabolic gene associated with the first step of anaerobic metabolism of toluene and xylene degradation in *Thauera aromatica* and *Azoarcus* strains. The target gene, *bssA*, codes for the α-subunit of BBS, which is distributed across a wide range of phylogenetically and physiologically diverse bacteria (Leuthner et al. 1998).

Microbial diversity of polluted and unpolluted environments

Organic pollution has been reported to reduce diversity in microbial communities in a variety of environments (Juck et al. 2000; Roling et al. 2002). In many cases, enrichment of specific microbial population also occurs. In unpolluted ecosystems, hydrocarbon-degrading organisms usually represent less than 0.1% of the cultivable heterotrophic microbial communities, whereas in contaminated environments, they constitute up to 100% of the viable microbial population. Studies on pristine soils and soils with a known history of PAH contamination revealed that pristine soils did not yield PAH degraders, whereas contaminated soils harbored closely related PAH-degrading bacteria to at least 1.5×10^7 cells (g dry wt.)$^{-1}$, several orders of magnitude more than in comparable noncontaminated sites (Mueller et al. 1994). *Mycobacterium* spp., *Sphingomonas* spp., *Rhodococcus* spp., *Arthrobacter* spp., and *Nocardia* spp. populations have been often found to be selectively stimulated in soil contaminated by PAHs (Kanaly et al. 2000; Daane et al. 2001).

Catabolic genotypes involved in the microbial degradation pathways of representative fractions of petroleum hydrocarbons, including *n*-alkanes, aromatics, and PAHs, are widespread in Arctic soils (Whyte et al. 1999) and Alaskan sediments (Sotsky et al. 1994). For example, *Rhodococcus* spp. were found to be the most abundant alkane-degrading genotypes in pristine and contaminated Arctic and Antarctic soils, while *Pseudomonas* spp. were enriched after contamination events, and *Acinetobacter* spp. were never found to be predominant members of polar alkane-degrading communities (Whyte et al. 2002).

Similar trends were observed by Margesin et al. 2003b) in both contaminated and pristine Alpine soils from Tyrol (Austria). They determined the genotypes involved in the degradation of *n*-alkanes and PAHs by PCR hybridization analyses of total soil community DNA, using oligonucleotide primers and DNA probes specific for each genotype. A significantly higher percentage of genotypes containing genes from Gram-negative bacteria (*P. putida AlkB*, *xyl E*,

and *ndoB* and *Acinetobacter AlkM*; 50–75%) than in the pristine (0–12.5%) soils indicated that these organisms were enriched after contamination. Moreover, genotypes containing genes from Gram-positive bacteria (*Rhodococccus AlkB1* and *AlkB2* and *Mycobacterium nidA*) were detected at high frequency in both contaminated (41.7–75%) and pristine (37.5–50%) soils, indicating that they were already present before a contamination event.

Actinobacteria (including mainly *Rhodococcus* and *Arthrobacter* strains) and *Proteobacteria*, including mainly γ-*Proteobacteria*, and more specifically *Pseudomonas*, were found to be the resident cultivable BTEX degraders both in BTEX polluted soils (Stapleton and Sayler 2000; Hendrickx et al. 2006) and aquifers (Cavalca et al. 2004). The proliferation of BTEX-degrading *Proteobacteria* therefore seems to be a major characteristic of adaptation in BTEX-contaminated sites. A proliferation of minor phylotypes within a fuel-contaminated aquifer upon toluene exposure was observed by Shi et al. 1999, when comparing the biodiversity of BTEX-polluted and BTEX-unpolluted aquifers.

The prevalence of certain genotypes in pristine or contaminated soils may be explained by the r–K scheme, which assumes that evolution favors either adaptation to high rates of reproduction (*r* strategists) or utilization of environmental resources (*K* strategists). *P. putida* and *Acinetobacter*, which rapidly colonize and grow on hydrocarbon contaminants, are *r* strategists; *Rhodococccus* and *Mycobacterium*, which tend to be more successful in nutrient limited situations, are *K* strategists. Population of *K* strategists is usually more stable and permanent members of the community (Atlas and Bartha 1998).

It has been rarely shown whether organic pollution causes communities to diverge from each other. Massol-Deya et al. 1997 reported that groundwater microbial communities treated with aromatic hydrocarbons converged in a fixed-film bioreactor where the environmental parameters were kept constant. In contrast, Bundy et al. 2002 noted that three soil communities amended with diesel were distinct from the controls and from each other, while Roling et al. 2002 observed large changes in the community structure in a simulated beach oil spill but also a low similarity between replicates and controls. In a study performed to analyze the direct and indirect effects of benzene pollution on microbial community diversity and structure, Fahy et al. 2005, by combining nucleic acid-based community profiling with multivariate statistical analysis, found that the environmental changes caused by benzene metabolism greatly affected the structure of microbial communities rather than the toxic and stressful effects of benzene that only moderately influenced it.

In situ strategies to remediate polluted environments

Remediation techniques, whichever physical, chemical, or biological treatments suitable for partial or total restoration of a polluted system (Bouwer and Zehnder 1993; Cerniglia 1993; Adriano et al. 1999; Gevao et al. 2000; Gianfreda and Nannipieri 2001; Iwamoto and Nasu 2001), may be utilized in situ, i.e., in the contaminated place itself, offering numerous advantages over ex situ technologies.

A complete remediation program usually requires more than one step, including: (a) knowledge of the past history of the polluted area and activities leading to the contamination of the site, (b) examination and quantification of the severity of the contamination problem, (c) development of the remediation action program to target the specific contaminant or group of contaminants, (d) development of treatments and a treatment sequence suited for the wastes and the site, and (e) an effective monitoring program to evaluate the effectiveness of the adopted bioremediation plan.

Bioremediation, either as an intrinsic degradation or as an engineered process, usually refers to the use of microorganisms (mainly) but also of plants, enzymes, and plant–microorganisms associations that transform pollutants into innocuous products (Dec and Bollag 1994; Siciliano and Germida 1998; Pointing 2001; Gianfreda and Bollag 2002; Torres et al. 2003).

As claimed by Adriano et al. 1999, the purpose of soil bioremediation is “not only to enhance the timely degradation, transformation, remediation or detoxification of pollutants by biological means, but also to protect soil quality.” Soil quality has been defined by Doran and Parkin 1994 as the capacity of a soil to function within ecosystem boundaries to (1) sustain biological productivity, (2) maintain environmental quality, and (3) promote plant and animal health.

Intrinsic or natural attenuation

Natural attenuation of contaminated sites is getting more and more interesting because it seems to enable bioremediation with a minimized cost, avoiding land disruption and human exposure.

Biodegradation, chemical transformation, stabilization (i.e., binding and sequestration by clays and humus), volatilization, dispersion, dissolution, and dilution are all natural attenuation processes of organic contaminants in soil and groundwater. However, such natural processes can be very slow, and consequently, certain chemicals 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.

Methods to assess microbial natural attenuation of BTEX and PAHs include analysis of the subsurface geology and hydrology of the site, qualitative and quantitative pollutant biochemical profiles, pollutant bioavailability, composition and activity of the microflora, and microcosm studies. An appropriate evaluation of natural attenuation, however, requires the demonstration that the transformation processes are taking place at a rate that is protective of human health and environment.

Intrinsic contaminant biodegradability, contaminant concentration, environmental pH and temperature, and availability of electron acceptors are the main factors that affect the biochemistry of pollutant degradation.

Appropriate redox conditions determine which redox regime occurs and, in turn, which kind of substance is degraded and the rate of biodegradation. Although the rate of aerobic biodegradation is higher than that of the anaerobic one, anaerobic processes may be more dominant when the rate of microbial respiration exceeds the rate of oxygen input. At a low dissolved oxygen amount, degradation may even occur by a definite sequence of oxy-anions as alternate electron acceptors (aerobic → denitrification → Mn(IV), Fe(III) reduction → sulphate reduction → methanogenesis; Bouwer and Zehnder 1993) used by microbial species, active in a given condition.

In the soil, microorganisms may develop various mechanisms to access sorbed compounds after sorption and aging processes on soil particles and sediments as well as to utilize water-insoluble pollutants. Indeed, microorganisms may wait for a new equilibrium state, create concentration gradients, bring about microenvironmental pH shifts, produce surfactants, solvents, and chelators, secrete extracellular enzymes, and degrade exposed substituents. In addition, they may produce emulsifiers and surfactants that usually facilitate the partitioning of the chemical from the NAPL to the water phase (Chung and Alexander 2002; Gevao et al. 2000).

Biostimulation and bioaugmentation

The absence of natural biodegradation in a contaminated site may indicate that both localized environmental conditions and physical and biological constraints may limit partially or totally the degradation of pollutants and negatively affect the bioactivity of the process. Bioremediation technologies usually help natural biodegradation processes work faster, or they may provide additional, exogenous biological agents to polluted systems and improve the transformation processes.

Several strategies may be adopted to efficiently improve the process and overcome the above constraints: bioaugmentation, i.e., the addition of strains with desired degradative capacities, and biostimulation, i.e., the addition of supplementing carbon sources or other nutrients to stimulate the activity of indigenous or inoculated degrading strains.

The utility of bioaugmentation is supported by studies showing the incapacity of indigenous microorganisms in some cases and the apparent enhanced bioremediation rate after the addition of competent microorganisms (Edgehill 1999).

The use of pure cultures into multi-substrate-polluted systems, such as soil or groundwater, has had variable results (Bouchez et al. 2000). However, bioaugmentation using strains enriched from sites containing the target contaminant, where the population has acquired the catabolic ability, has had success (Schwartz et al. 2000).

Despite bioaugmentation is still a source of controversy within environmental microbiology, it is considered as a way of enlarging the genetic capacity present in a given site, thus corresponding to an increase in the gene pool and in the genetic diversity of that site (Dejonghe et al. 2001). In this contest, the long-term survival of the introduced donor strain does not represent the major bottleneck in bioaugmentation processes.

Recently, Thomson et al. 2005 pointed out technical advances to perform a strain selection based on an in situ understanding of organism abundance, functional activity, and population dynamics in the habitat from which they are derived. It seems logical that a strain, derived from a population, temporally and spatially prevalent in a specific habitat, more likely persists as an inoculum when reintroduced, than one that is transient or alien to such a habitat. Once an abundant population has been identified, the second phase of the selection procedure is to identify strains that are capable of degrading the contaminant(s).

Investigations of microbial populations and their activities have increasingly necessitated the development and use of culture-independent methods, such as those involving PCR-amplified ribosomal ribonucleic acid (rRNA) genes. With rapid molecular procedures, such as denaturing or temperature gradient gel electrophoresis (DGGE or TGGE; Muyzer et al. 1993), terminal restriction fragment length polymorphism (Liu et al. 1997), and length-heterogeneity PCR (Suzuki et al. 1998), together with other complementary techniques, such as fluorescence in situ hybridization (FISH; Yang and Zeyer 2003) and stable isotopic probing (SIP; Jeon et al. 2003), it may be possible to obtain a more comprehensive assessment of the composition and structure of bacterial communities and

the responses of the bacteria to different soil conditions and to determine exactly which organisms assimilate specific contaminants.

Determination of the potential success of bioaugmentation, however, requires an understanding of the bioavailability of the pollutant, the survival and activity of the added microorganism/s or its genetic material, and the general environmental conditions that control soil bioremediation rates. With available genetic techniques, the comprehension of the fate of added microorganisms and of the critical parameters for the design of bioaugmentation processes can be achieved.

For instance, *Pseudomonas stutzeri* KC was selected for site inoculation because of its ability to mineralize carbon tetrachloride under anoxic conditions (Dybas et al. 1998). By manipulating the pH in the field to pH 8, which lowered iron availability, the strain persisted and actively degraded the contaminant, by out-competing indigenous populations that were unable to obtain adequate concentrations of iron.

To overcome chemical and environmental constraints, delivery systems may be designed to provide nutrients (nitrogen and phosphorous), oxygen, and other electron acceptors to stimulate and maintain the activity of microbial degraders. Similarly, environmental conditions can be improved to get optimal values of pH, moisture, etc. for the microbial degradation of the organic pollutant. Substances more amenable to biodegradation than the target contaminant can be added to the soil to stimulate the microbial cometabolic transformation of the pollutants, otherwise not degraded. The addition of nutrients can lead to an increased development of indigenous microorganisms, which themselves either biostimulate the process or hinder the process by consuming the added nutrient or carbon source. In cases where cometabolism is desired, the consumption of added substrates by indigenous microorganisms, incapable of cometabolizing the pollutant, can hinder the growth of the added microorganisms (Bewley 1996; Adriano et al. 1999, Boopathy 2000; Gianfreda and Nannipieri 2001; Margesin et al. 2003a,b; Scullion 2006).

Monitoring in situ bioremediation

When the bioremediation process is designed and implemented, its effectiveness has to be demonstrated by a continuous monitoring through chemical, biological, microbial, and environmental indicators.

Some general criteria and tools to verify them can be also adopted (Table 1). They should establish whether the cleanup of the site has been effectively achieved and the pollution completely removed. As a result, the evaluation of the effective removal and disappearance of the pollutant is the most mandatory. After the treatment, an improvement of the polluted area in terms of both visual and sensory aspects as well as the absence of any eco-toxicological risk should be also assessed.

The assessment of environmental hazards of remediated sites is mostly established on chemical analyses, based on the determination of either fine (i.e., pollutant consumption, intermediates/final products) or lumped (i.e., biochemical oxygen demand, chemical oxygen demand, total organic carbon) parameters.

Different approaches for monitoring bioremediation of contaminated soils/aquifers and evaluating the efficiency of bioremediation in lowering long-term environmental toxicity have been proposed. They include measurements of distinctive metabolites or stable carbon isotope ratios of dissolved inorganic carbon or residual contaminants. Another approach is to show that the indigenous microbial communities are capable of degrading the contaminants and, ideally, that these bacteria are enriched in contaminated zones. Nucleic-acid-based techniques are preferred for such monitoring because they preclude isolation and cultivation of bacteria. Preferably, molecular techniques focus on catabolic genes that code for specific pollutant-degrading enzymes.

Table 1 Criteria and tools for bioremediation process efficacy evaluation

Aspects to be considered	Criteria to meet	Available tools
Chemical and engineering aspects	Were the target cleanup goals achieved? Could mass balances and predictive models be established?	Chemical and analytical determinations
Biological nature of the process	Was the biological nature of the process demonstrated? What were the relative contributions of abiotic processes?	Microorganism presence, variation of microbial structure and communities
Eco-toxicological aspects	Was the pollution completely detoxified? Did the treatment pose a human or ecological risk?	Toxicity assays (genotoxicity and general toxicity)
Aesthetic aspects	Does the treatment result in visual or sensory improvement of the polluted site?	Social and general response

In the following, the main aspects of some of these methodologies, their drawbacks, and advantages will be addressed.

Chemical and biological monitoring

The primary goal in managing contaminated sites is to render the site environmentally acceptable through management or remediation so the site can be used for some acceptable purposes. This requires to select some soil cleanup criteria and these latter must be adopted under a risk-based approach. Answering "how clean is clean" in a meaningful way requires making a determination of what concentration of a chemical is environmentally acceptable at a specific site, i.e., to determine what constitutes an environmental acceptable endpoint. An efficient instrumental procedure should be designed to determine both the total quantity of pollutants and their compositions before and after the bioremediation process.

Several chemical methods are available to evaluate the concentration of a pollutant and its depletion in a contaminated site. They include gas chromatography (GC), GC/mass spectrometry (MS), GC/flame ionization detector (FID), infrared (IR), fluorescence spectroscopy, luminescence techniques, and others. Each of them may present advantages or constraints and may be more or less suitable and applicable to a particular type of soil (low or high organic matter content, low or high clay content, etc.) or contaminant mixtures. For example, GC/FID (as also GC/MS) is suitable for the identification and determination of contaminants and has the advantage that FID response is linear in a wide range of concentrations and not influenced by temperature changes. However, this methodology can have poor sensitivity because some carbon compounds may be oxidable (Korda et al. 1997). Similarly, luminescence techniques are very sensitive and present a reasonable selectivity to detect microbial activity and the aromatic content of complex mixtures of hydrocarbons. They, however, have a limited application in multispecies oil-degrading microbial communities and under field conditions because of the severe and not quantifiable quenching of light emission in soil slurries or soil media (Korda et al. 1997).

Among the analytical methods, fluorescence spectroscopy, although generally accepted as a powerful and sensitive analytical tool to determine aromatic pollutants such as PAHs (Lombardi and Jardim 1999; Nakashima et al. 2000), has, however, limited applicability because the spectra of complex mixtures cannot be often resolved adequately (Lombardi and Jardim 1999).

Gomez et al. 2004 employed a derivative technique such as synchronous luminescence to classify the pollutants on a routine basis and isolate the specific compounds from polluted soil. By applying IR, GC, and synchronous fluorescence spectroscopy, they successfully determined many PAHs in contaminated soil.

Usually, an exhaustive chemical extraction of the remediated soil should be performed to evaluate the residual concentration of the pollutant. Indeed, the final concentration of the pollutant has to be lower than that determined by this method because the contamination levels established by laws and regulations are frequently governed by this requirement.

This approach, however, could be misleading. For instance, the residual soil concentration of PAHs detected by an exhaustive extraction method after bioremediation treatment could represent the nonbioavailable fraction. Because nonbioavailable fractions of pollutants are usually considered of minor hazard (Alexander 2000), several methodologies to estimate only the remaining bioavailable fraction of pollutants have been developed. They include chemical tests capable of measuring the desorbing fraction, based on nonexhaustive methodologies and utilizing various extractants such as solvent mixtures, surfactants, cyclodextrins, and soil-phase extraction (Sabatè et al. 2006).

In a feasibility study performed on a biostimulated creosote-contaminated soil, Sabatè et al. 2006 established a theoretical biodegradation limit based on the desorbing fraction using a mild extraction technique involving cyclodextrins. No metabolite accumulation was detected, indicating that biostimulation conditions permitted ample metabolic cooperation among the microbial populations present in the creosote-contaminated soil. No toxic intermediate products and a negligible formation of bound residues were also determined.

A wide variety of organic pollutants is degradable by white-rot fungi through the action of their aspecific enzymatic systems (Gianfreda and Rao 2004). Therefore, they can be helpful when dealing with a site contaminated by a mixture of different xenobiotic pollutants. To monitor and control the effectiveness of the bioremediation process, the fungal biomass has usually to be quantified in situ. Indirect methods capable of estimate fungal biomass have been developed to overcome the incapability of measuring directly the amount of fungal biomass. These indirect techniques include biochemical methods that rely on the chemical assay of fungus-specific cell components such as chitin, phospholipids fatty acids, or ergosterol. This latter is considered a useful indicator for fungal biomass in polluted soils as well as a tool for monitoring bioremediation processes, as its content is independent of the presence or the absence of pollutants (Barajas-Acheve et al. 2002).

A strategy for monitoring in situ biodegradation of a compound is the method known as SIP that relies on the changes in stable isotope composition of the molecule of interest. It plays a role in attributing the assimilation of

specific elements from certain compounds to microorganisms within complex microbial communities. In practice, the application of SIP has almost exclusively been restricted to the use of ^{13}C, and the microbial community should have a sufficient number of labeled atoms to allow biomarker separation and detection. The use of this technique presents two limitations: (1) the separation based on density requires the substrate to be labeled at close to 100%, and this is expensive and sometimes unavailable in addition to requiring long incubation times, and (2) the labeled substrate is used predominantly for growth to surmount diluted label issues (Wellington et al. 2003).

Hydrocarbons enter the subsurface with a distinct isotopic composition or $^{13}C/^{12}C$ ratio. Microbial processes make small but significant changes in isotopic compositions by preferentially utilizing the lighter isotopes (Richnow et al. 2003) and leaving the residual substrate fraction enriched of the heavier isotopes.

SIP involves tracking stable isotope atoms from particular substrates into components of microbial cells, referred to as biomarkers. The informative biomarkers used in environmental microbiology are DNA, RNA and phospholipid fatty acids (PLFAs); each of them has its strengths and weaknesses (Dumont and Murrell 2005; Manefield et al. 2002).

Because SIP is founded on assimilatory processes, its utility is restricted to chemical transformations that microorganisms perform, at least in part, for anabolic purposes. Nonassimilatory processes (co-oxidation) fall outside the applicability of SIP. DNA- and RNA-SIP are usually aimed at discovering 16 S rRNA gene sequences, hence, taxa.

SIP can be used to describe in situ biodegradation of pollutants in both qualitative and quantitative terms. In a field study, BTEX and PAHs of a polluted, anoxic aquifer exhibited a significant concentration gradient along an 800-m groundwater flow path downstream of the source of contamination. Richnow et al. 2003 determined the concentrations and the isotopic compositions of contaminants to examine in situ biodegradation at the test site by applying laboratory-derived fractionation factors and the relative Rayleigh equation for closed systems (Hoefs 1997). On the basis of isotope values and on the laboratory-derived isotope fractionation factors for toluene and *o*-xylene in the monitoring wells, a significant biodegradation along the groundwater flow path was found, and in the wells, at the end of the plume, toluene and *o*-xylene were almost completely reduced by in situ microbial degradation.

The most remarkable advantage to use PLFAs as biomarkers in SIP is the rapidity with which PFLAs become detectably labeled and the quantitative information generated from the analysis of PLFAs with isotope ratio MS. The primary limitation of this approach is that the phylogenetic resolution offered by this biomarker is inferior to that offered by nucleic-acid-based biomarkers and that it requires signature PLFAs to be identified from close cultivable relatives.

PLFA-SIP has been applied to link the degradation of toluene to genera within the order of *Actinomycetales* (Hanson et al. 1999) and to identify sulphate-reducing, toluene degrading microorganisms in the sediment of a petroleum hydrocarbon-contaminated aquifer (Pelz et al. 2001).

Jeon et al. 2003 released ^{13}C-labeled naphthalene in a contaminated site to trace the flow of pollutant carbon into the naturally occurring microbial community. They succeeded in (a) documenting $^{13}CO_2$ evolution, (b) creating a library of 16 S rRNA gene clones from ^{13}C-labeled sediment DNA, (c) identifying a taxonomic cluster (92–95 clones) involved in metabolism of the added naphthalene, and (d) isolating a previously undescribed bacterium (strain CJ2) closely related to *Polaromonas vacuolata*. This latter hosts the sequence of a dioxygenase gene (*nah*Ac) responsible for in situ naphthalene biodegradation. Naphthalene utilization by strain CJ2 was then confirmed in mineral salts media analyzed by GC/MS.

Because DNA-SIP relies on the isolation of labeled DNA by density centrifugation, the degree of isotopic enrichment is crucial. The DNA synthesis in situ, which reflects organism replication rates, limits the enrichment of this biomarker and hence the utility of DNA-SIP. By comparison, RNA synthesis rates are higher than those of DNA, being a function of copy number and the turnover of RNA. For this reason, RNA will be labeled more rapidly, making it a more responsive biomarker with a high degree of phylogenetic resolution. The potential limitation inherent to RNA-SIP is the ability to extract high quality RNA from environmental samples. Advances in nucleic acid extraction methodologies suggest, however, that this will not impede the broad application of this technique.

Genetic monitoring

Detection of specific nucleic acid sequences and nucleic acid hybridization, using specific probes for a biodegradative gene or gene message, is today an indispensable approach for the identification of microorganisms in environmental samples. The detection of phylogenetic and catabolic genes in environmental samples is based on a set of different genomic approaches (Fig. 4).

Hybridization- or PCR-based detection systems involve recognition of a specific nucleic acid fragment or different electrophoretic patterns (fingerprints) (Sayler and Layton 1990; Steffan and Atlas 1991).

The probes may be employed to determine the overall genetic diversity, the dominant and active gene pool, and the density and frequency of specific gene lines required to degrade a target compound at a site.

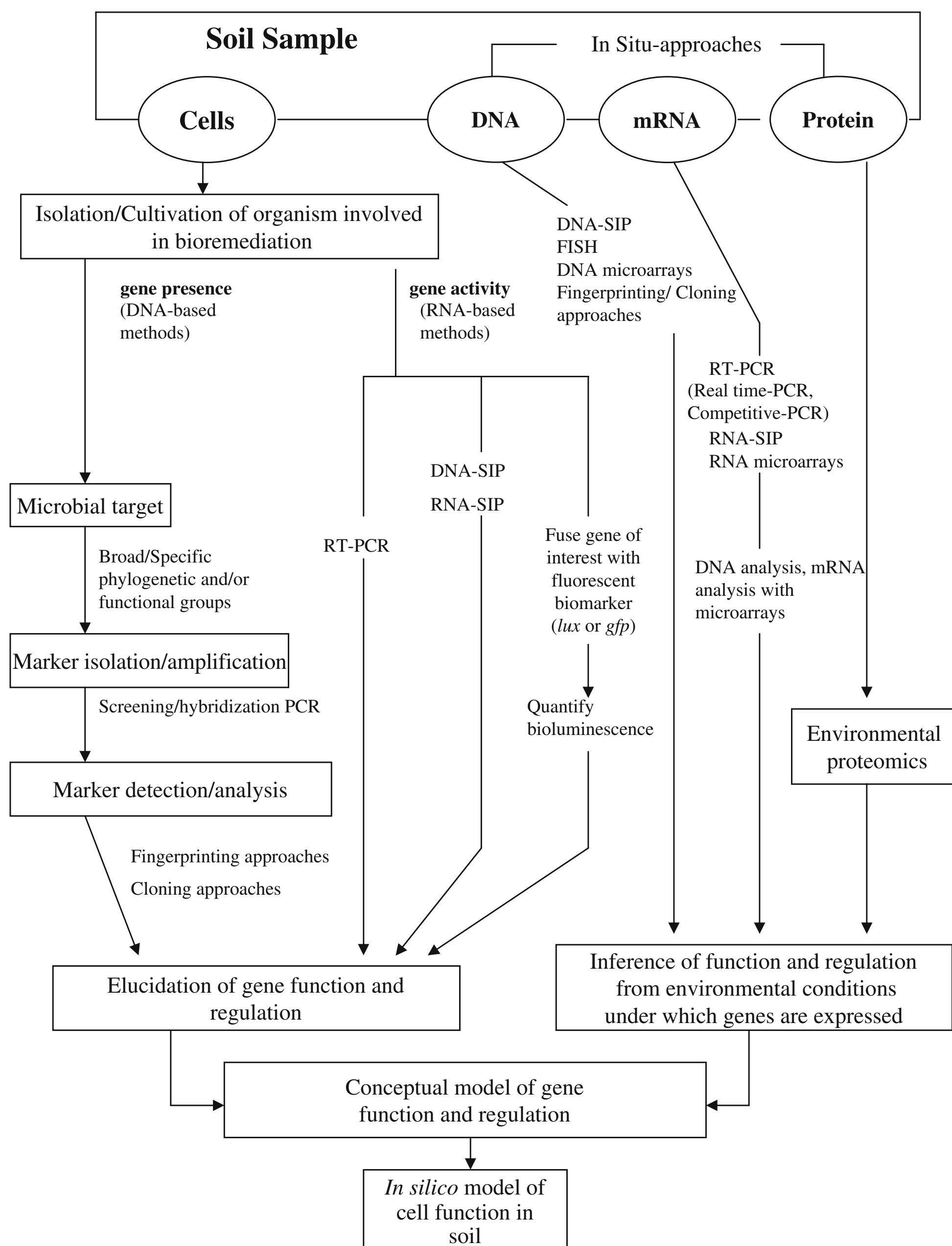


Fig. 4 Different genomic approaches available to examine microbial communities of polluted soils. Microorganisms isolated from the soil provide information on their physiology and on the gene composition usable for the analysis of mRNA and proteins that are extracted directly from the soil. Genomic DNA extracted from soil furnishes data on the genetic potential of microbial communities. mRNA and proteins provide information on gene expression under different environmental conditions. Comparison of data from pure culture and mixed communities yields data for the development of models of microbial function in the soil (adapted from Lovley 2003)

On account of the key position of the initial- and ring-oxygenases during aerobic PAH and BTEX degradation, as well as of BBS for anaerobic toluene/xylene degradation, the genes encoding these enzymes have been used as targets to detect the presence of hydrocarbon degraders at the DNA level and to construct specific gene probes.

Once a probe has been developed, the probe can be expanded for many uses including quantitative PCR or a second generation PCR, i.e., real-time PCR, and functional gene probe arrays. Real-time PCR uses a fluorescent dual-labeled probe-based detection system (TaqMan probe) allowing PCR amplification and detection within closed tube systems.

The development in the years of several primers has allowed detecting and quantifying in environmental samples the presence of specific genotypes encoding the key steps in PAH and BTEX biodegradation and in their messenger RNA (mRNA) (Meyer et al. 1999; Ringelberg et al. 2001; Baldwin et al. 2003; Junca and Pieper 2003; Hall et al. 2005; Hendrickx et al. 2006).

For example, *nid* gene sequences encoding for the large (*nidA*) and the small (*nidB*) subunits of the dioxygenase system have been used to create a gene probe for the detection of PAH-degrading mycobacteria in soils undergoing bioremediation (Hall et al. 2005).

With an in situ mesocosm study, Hendrickx et al. 2005 examined the effect of a BTEX-contaminated groundwater plume on the in situ dynamics of a bacterial aquifer community and on the dynamics of specific BTEX-catabolic genotypes within the community. A rapid colonization of downstream aquifer material by *Pseudomonas* spp. from the contaminated upstream area occurred, and the new community remained stable over an extended time period. Changes in the BTEX catabolic genotype structure reflected the community change; in particular, the community in the contaminated area was especially characterized by the appearance of *xylM*/*xylE1*-(C23O)-like genotypes, while in the uncontaminated area, only *tmoA*-like genotypes were detected. Successively, a successful design of eight new primer sets evidenced that *tmoA*-, *xylM*-, and *xylE1*-like genes were the most frequently recovered in both soil DNA and isolates from subsurface soils sampled at different locations of the BTEX-contaminated site. While *xylM* and *xylE1* were only recovered from the contaminated samples, *tmoA* was detected from both the contaminated and noncontaminated samples (Hendricks et al. 2006). *tmoA* and *xylE* genes were instead found significantly higher in contaminated subsurface samples collected along a BTEX concentration gradient at a fuel-oil-contaminated site than in noncontaminated samples (Guo et al. 1997; Cavalca et al. 2004).

Competitive quantitative PCR techniques that used as PCR targets the conserved regions of the C23O genes allowed to enumerate their gene copy number and to correlate their relative abundances to pollutant levels. C23O genes were enumerated by this technique with a 163-bp competitor in a petroleum-amended soil using the same primer. As a prerequisite, target and competitor should have had identical amplification kinetics (Mesarch et al. 2000).

The intrinsic biodegradative potential of harbor sediments contaminated with PAHs was examined in bioslurry microcosms, during a 4-month treatment, by correlating the microbial community structure, through PLFA analyses, to catabolic gene presence and to PAH loss. PLFA analysis revealed a threefold increase in total microbial biomass and a dynamic microbial community composition, strongly correlated with changes in the PAH chemistry. The copies of genes associated with PAH degradation increased by four orders of magnitude and shifts in gene copy numbers correlated with shifts in specific subsets of extant microbial community. Declines in the concentrations of phenanthrene correlated with PLFA indicative of *Rhodococcus* spp. and/or actinomycetes and genes encoding for naphtahalene-, biphenyl-, and C23O-degradative enzymes (Ringelberg et al. 2001).

Therefore, the greater numbers of catabolic gene copies within a contaminated area, in comparison to those in uncontaminated soils, can be used as evidence of natural attenuation or of the effectiveness of engineered bioremediation.

With a real-time PCR method, anaerobic toluene-degrading bacteria were monitored by quantifying the *bssA* gene with a primer–probe set in microcosms inoculated with aquifer sediments and incubated anaerobically with BTEX and nitrate in the presence and absence of ethanol (Beller et al. 2002). Population trends were consistent with observed toluene degrading activity: the microcosms with the most rapid toluene degradation had the largest numbers of *bssA* copies, where they increased from 100 up to 1,000 folds over the first 4 days of incubation, when most of the toluene had been consumed.

DNA microarrays provide a mean for the simultaneous analysis of many genes. Based on the types of probes arrayed, microarrays for environmental studies can be phylogenetic oligonucleotides, functional genes, and community genoma arrays. The last type is constructed using whole genomic or even large fragments of genomic DNA originating from metagenomic libraries (Wu et al. 2004).

Although several phylogenetic microarray platforms show great promise, the technical challenges of such complex hybridization experiments, their limited quantitative capabilities and the incomplete knowledge of extant microbial diversity, still limit their widespread use (Zhou 2003). Both PCR-amplified fragments and nucleotides derived from functional genes can be used to fabricate functional gene arrays.

Recently, Rhee et al. 2004 developed a comprehensive 50-mer-based oligonucleotide microarray containing 1,657 probes from all 2,402 known genes involved in PAH and BTEX biodegradation and metal reduction and used it to monitor the biodegradation potential and activity of enrichment cultures and soil microcosms supplemented with naphthalene. With the developed 50-mer functional gene array, they successfully detected changes in the microbial community structures in enrichment and soil microcosms depending on the incubation conditions. While the naphthalene-degrading genes from *Rhodococcus*-type bacteria were dominant in naphthalene enrichments, the genes involved in naphthalene and PAH degradation from Gram-negative bacteria like *Ralstonia*, *Comamonas,* and *Burkholderia* were most abundant in soil microcosms. Naphthalene-degrading genes from *Pseudomonas* were never detected (Rhee et al. 2004). These results strengthen the potentiality of this technology in monitoring bioremediation processes in situ, although much work is needed to improve detection sensitivity.

Expression of functional genes in the soil can be monitored by detection of their transcription directly as mRNA or via a reporter gene fused to the target gene. Indeed, despite technical difficulties to extract purified RNA at a sufficient yield from environmental samples for

subsequent manipulation and analyses, a variety of methods developed for mRNA extraction are now available to detect the gene expression.

Numerous environmental factors can activate or repress gene expression and thereby modulate microbial activity. The influence of agents increasing PAH bioavailability on the expression of the catabolic gene *nahAC* has been studied by using a real-time PCR assay and to evaluate the regulation of the *nahAC* gene during phenanthrene degradation (Marlowe et al. 2002). Gene expression was successfully determined by extraction of bacterial mRNA followed by real-time PCR amplification of the *nahAC* gene and of an internal housekeeping gene (*rpoD*). The *rpoD* gene, which encodes a housekeeping sigma factor for transcription initiation, served as a baseline control for evaluation/interpretation of catabolic gene expression. With this gene expression assay, temporal changes in *nahAC* expression were shown when phenanthrene was degraded. Thus, monitoring gene expression may allow detection of subtle changes in the expression of degradative genes, because of temporal changes in cell physiology or changes in toxicity associated with accumulation of intermediates.

An alternative molecular tool is to use biomarkers as specific tags for the identification of specific bacteria or gene activity. A biomarker is defined as a DNA sequence, introduced in an organism, which confers a distinct genotype or phenotype to enable monitoring in a given environment. Bioluminescent, fluorescent, and chromogenic marker genes, such as *luxAB* (bacterial luciferase), *gfp* (green fluorescent protein, GFP), and *xylE* (catechol 2,3 oxygenase) genes, as well as reporter genes are examples of promising biomarkers (Jansson 2003).

The choice of the biomarker and monitoring system depends on the particular site, bacterial strain, and sensitivity, and specificity of detection is required. Recently, *gfp* was used to tag different environmental *Mycobacterium* isolates able to degrade different PAHs. Confocal laser microscopy was used to analyze both GFP- and PAH-derived fluorescence signals and allowed the bacteria to be visualized in association with crystalline substrates (Wattiau et al. 2002).

Reporter bacteria, also referred as bioreporters, are equipped with reporter genes that encode a product, which is easily assayed and related to metabolic activity or specific gene expression of the host cell. Most of the bioreporters use an environmentally or metabolically responsive promoter, which is fused to a suitable reporter gene and introduced into a microbial host. Host cells then react to environmental stimuli by production of an easily detectable reporter protein, thus informing on the conditions that the cells encounter or on the status of the cells themselves (Jansson 2003).

Environmental biosensors represent a breakthrough for the monitoring of pollutants in contaminated matrices because they have the unique ability to measure the interaction of specific compounds with biological systems through highly sensitive recognition processes.

One of the best known examples of a biosensor is the use of a bioluminescent pseudomonad to monitor naphthalene. When this strain was inoculated into lysimeters contaminated with naphthalene, light emission was detected using light pipes at different depths (Sayler et al. 2001).

In another study, a GFP-based *Pseudomonas fluorescens* strain A506 biosensor was constructed for the detection of BTEX in aqueous solutions (Stiner and Halverson 2002). The biosensor was constructed by creating a transcriptional fusion between *gfp* and toluene–benzene utilization (*tbu*) pathway promoter of *Ralstonia pickettii* PKO1cells. The biosensor cells were readily induced, and fluorescence emission after a 3-h period of induction correlated well with BTEX concentrations.

When not much is known about the genetics and regulatory circuits involved in pollutant degradation, a random mutant library can be made to screen for cells that produce a response to a chemical from an indigenous promoter. According to this strategy, the promoter probe mini-Tn5-l*uxAB*-*tet* was used to create a *luxAB* transcriptional fusion responding to fluoreine in the fluorine-utilizing bacterium *Sphingomonas* sp. LB126 (Bastiaens et al. 2001). The mutant strain, named L-132, was impaired in fluorene utilization and strongly emitted light upon addition of fluorene to the growth medium.

FISH with rRNA-targeted nucleic acid probes (Lee et al. 1999; Ginige et al. 2004) or the combination of 16 S rRNA FISH and ^{13}C isotope data (Orphan et al. 2001) can be used to directly identify active bacteria within complex samples in a few hours.

To link results obtained using the catabolic-gene approach to those of the rRNA approach, FISH with tyramide signal amplification (TSA) has been used to detect intracellular mRNA in microbial mats in high alpine lakes (Schonhuber et al. 1999) A TSA-FISH method was developed and applied to *P. putida* NCB9816-4 cells actively transcribing naphthalene dyioxygenase (*nahAC*) and related genes in environmental samples from a coal-tar-contaminated site, thus documenting the in situ expression of specific genes in individual cells (Bakermans and Madsen 2002). The widespread use of TSA-FISH is still limited by the databases usable to design probes and by differences in cell permeabilization efficiency.

The potential for monitoring in situ microbial gene expression in soils appears feasible. Metagenomics, i.e., the genomic analysis of entire microbial communities, seems to be a powerful approach to study the potential functions of uncultivated microorganisms. Genomic DNA extracted

from soil can be cloned, for example, into large cloning vectors, based on the *Escherichia coli* F factor (99.2 kb) and referred to as Bacterial Artificial Chromosomes (BACs), to make libraries of environmental genomic DNA (termed metagenomic libraries; Handelsman et al. 2002). The advantage of BAC vectors is that they may maintain very large DNA inserts (greater than 600 kb) stably in *E. coli*, facilitating the cloning of large fragments of DNA. Moreover, the *E. coli* F factor replicon offers strict copy number control that limits the number of BACs to one to two copies per cell. Several authors have demonstrated the utility of capturing community DNA in the form of BAC libraries and of linking phylogenetic and functional information within specific BAC clones, thus gaining new perspectives on the microbial ecology of natural environments (Rondon et al. 2000; Bèjà et al. 2002; Liles et al. 2003). For instance, for a particular soil microbial community, Rondon et al. 2000 successfully constructed BAC libraries with average insert sizes of 27- and 44.5-kb BAC libraries, thus linking the phylogeny of uncultured soil microoorganisms to their physiological and genetic activities encoded on metagenomic fragments captured in BAC libraries; Bèjà et al. 2002, instead, by characterizing BAC clones from a natural microbial population containing identical or near-identical rRNA genes from planktonic crenarcheotes, found the existence of a considerable functional diversity within species that have an identical rRNA sequence. Potential biases affecting the identification of BAC clones containing rRNA genes include preferential amplification from the host rDNA template, an inability to differentiate between host and nonhost RFLP patterns and potential toxicity of heterologously expressed rRNA gene operons.

Environmental DNA microarrays produced from the genomic data could be used to analyze simultaneously the expression of numerous genes in a given soil. However, the use of microarrays has been limited to 16 S rRNA markers or a relatively small set of functional genes, and no practical approach has been developed to specifically target the uncultivable majority of the species in the environment (Sebat et al. 2003). Functional genomic and expression data allow determining when a cell uses particular genes and when it does not. However, the prediction of the functioning of individual microorganisms in a complex environment requires to have a holistic view of metabolism “in silico models” (a computer representations of their in vivo counterparts) that can describe the interactions of these microorganisms with others of the community (Palsson 2000).

Monitoring of soil ecotoxicology and quality

As reported above, one of the main questions remaining after a bioremediation process is to assess that the toxicity at the contaminated site has been lowered and the site has regained its initial biological activity and productivity. Therefore, toxicity tests and biological activity measurements may be used as potential monitoring tools or bioindicators during and after bioremediation of contaminated soils. They have been incorporated in the program for ecological assessment of bioremediation at hazardous waste sites and for supporting management decisions for remediation (Hankard et al. 2004; Maila et al. 2005; Płaza et al. 2005). As chemical data alone are not sufficient to evaluate the biological effects, ecotoxicological tests have been recently used as supplementary tools to monitor bioremediation of hydrocarbons, both in laboratory and field studies, and to appropriately define acceptable cleanup standards (Saterbak et al. 1999; Dorn and Salanitro 2000).

In a recent study by Płaza et al. 2005, the bioassays Spirotox, Microtox, Ostracodtoxkit FTM, umu-test with S-9 activation, and plant assays were applied and compared to evaluate different bioremediation processes in heavily petroleum-contaminated soils. They were shown to be sensitive indicators for the assessment of the bioremediation effectiveness and the evaluation of soil quality. Six higher plant species (*Secale cereale* L., *Lactuca sativa* L., *Zea mays* L., *Lepidium sativum* L., *Triticumvulgare* L., and *Brassica oleracea* L.) were used for bioassay tests based on seed germination and root elongation. The test species demonstrated varying sensitivity to soils after the different bioremediation processes, and the effects on test organisms well correlated with the soil contaminant concentration.

A different approach was utilized by Hankard et al. 2004 who investigated the response of two chemical-sensitive earthworm biomarkers, lysosomal membrane stability measured using the neutral red retention assay (NRR-T) and the total immune activity (TIA) assay, after in situ exposure to complexly contaminated field soils at three industrial sites as well as urban and rural controls. Significantly lower NRR-T and TIA were found in the contaminated soils when compared to the two uncontaminated soil controls, thus demonstrating their capability to successfully identify significant exposure and biological effects caused by the mixture of chemicals present.

The effect of PAHs and heavy metals on the soil microbial activity and on some plants at an early stage of their development was investigated in pot and laboratory experiments by Maliszewska-Kordybach and Smreczak 2003. The results indicated that the dehydrogenase activity appeared to be the most sensitive parameter to PAH contamination (Maliszewska-Kordybach and Smreczak 2003). A high applicability of the dehydrogenase activity for soil ecotoxicological testing and as a sensitive index for measuring the toxicity of heavy metals and PAHs on microbial communities present in soils was also suggested by other authors (Rossel et al. 1997; Ihra et al. 2003).

Scelza et al. 2007 investigated on a laboratory scale the fate of phenanthrene, when added to a fresh, agricultural soil with no history of PAH contamination and its influence on various functionally enzymatic and biological related properties. The biostimulation effect of compost and the bioaugmentation efficiency of a phenanthrene-degrading bacterial culture were also investigated. As a general response, the intrinsic soil's enzymatic activity was not affected by exogenous microorganisms, whereas temporary and permanent changes were observed for several of the properties investigated in the presence of phenanthrene.

In a screening for total petroleum hydrocarbons (TPH) and their toxicity to soil algal populations, microbial biomass, and soil enzymes (dehydrogenase and urease) in a long-term TPH-polluted site with reference to an adjacent unpolluted site, a severe inhibition was observed in the soil with a medium level of pollution as the result of a combined effect of more than one chemical on the tested parameters (Megharaj et al. 2000). The particular sensitivity to the presence of a pollutant of algal species suggested these latter as a useful pollution bioindicator.

Very low levels of enzyme activities and a different bacterial diversity as assessed by DGGE profiles of the 16 S rDNA genes were found in soils heavily polluted by PAHs as compared to an uncontaminated soil (Andreoni et al. 2004).

In a recent review, Maila et al. 2005 have examined the potential, the performance, the variability, and the failure of several bioindicators including enzyme activities, seed germination, earthworm survival, and microorganisms or microbial bioluminescence. They concluded that at this stage, there is no general guarantee of successful utilization of biological activities as monitoring tools and these latter should be complemented by existing traditional monitoring tools. Indeed, "a number of methods can be used to assess the extent of hydrocarbon contamination in soil. However, the uniqueness or heterogeneity of the soil, the formation of toxic metabolites and the influence of technology contribute towards 'poor' bioindicator response of the different biological activities. Chemical and toxicity data do not always corroborate one another, and the results of each toxicity test in a battery do not always agree, due to each soil being unique in the response that it induces and each toxicity test unique in its ability to detect different contaminant levels in different soils" (Maila et al. 2005).

When a qualitative and quantitative assessment of soil quality has to be determined for a relatively unknown situation, several properties must be measured. Usually, a minimum data set (MDS) of soil properties or indicators are selected that should be responsive, affordable, interpretable, internationally accepted, and ecologically significant (Doran and Parkin 1994).

Criteria for including particular soil properties in the MDS depend on their relevance to the soil under examination. The choice can be made from international literature, and preliminary studies are needed to validate the selected MDS and to standardize the sampling method. According to the concept of soil quality, soil attributes that influence the capacity of soil to perform crop production or environmental functions and are sensitive to change in land use, management, or conservation practices must be included in MDS and evaluated simultaneously, using statistical procedures that account for correlation among soil attributes.

As also underlined above, a critical aspect to be considered is the influence of soil components on the bioavailability of the organic contaminant to soil biota. A battery of bioindicators showing different sensitivity to contaminants could be used, and their response in a contaminated soil as compared to an uncontaminated one could be evaluated. Very often, the choice of the uncontaminated control site may affect the reliability of the investigation. Indeed, it can be difficult to recognize a real control soil, which should be the same soil lacking in only the addition of contaminants.

Practical application of bioremediation technologies

Because the scope of bioremediation is to decrease the concentration of organic pollutants to levels undetectable or, if measurable, lower than the limits established as safe or tolerable by regulatory agencies, several criteria must be met for it to be seriously considered as a practical method for treatment.

These criteria can be summarized as follows: (a) microorganisms must exist, and they must have the necessary catabolic activity, (b) those organisms must have the capacity to transform the compound at reasonable rates and bring the concentrations to levels that meet regulatory standards, (c) products that are toxic at the concentrations likely to be achieved during remediation must not be produced, (d) chemicals that are markedly inhibitory to the biodegrading species must be absent at the site, (e) the target compound(s) must be bioavailable, (f) conditions at the site must exist or be made appropriate to sustain microbial growth or activity, and (g) the technology must be less or, at the worst, no more expensive than other alternative technologies.

None of these criteria is negligible. If any one is not met, either the biodegradative approach could be rejected, or the cleanup goals could not be achieved.

To meet all of these criteria and successfully implement a bioremediation technology, a multidisciplinary approach and basic knowledge in microbiology, biochemistry, physiology, ecology, and genetics are required. Moreover, much

information about the factors controlling the growth and metabolism of microorganism in polluted environments is necessary because several of the above criteria are highly empirical rather than knowledge based.

As outlined by Lovley 2003, “for bioremediation to advance as a science, there is a strong need for the comprehensive understanding of physiological properties that genome-enabled approaches can provide.” The continued generation of new information on the biochemistry, genetics, and molecular biology of microbial degradative pathways should expand opportunities for molecular environmental analysis. Examining the presence and the expression of the key genes involved in bioremediation can yield more information on microbial processes than analysis of 16 S rRNA sequences. However, the genes for bioremediation can be present but not expressed. Often, increased mRNA concentrations can be, at least, qualitatively associated with high rates of contaminant degradation. Highly sensitive methods that can detect mRNA for key bioremediation genes in cells are now available, and evaluation of the metabolic state of degrading microorganisms could help to identify modifications in the contaminated environments that might promote bioremediation.

On the other hand, contributions by environmental chemists, engineers, hydrogeologists, and soil scientists are necessary to design a system well tailored to the contamination problem and possessing optimal environmental conditions for the action of the decontaminating organism. Furthermore, an economical evaluation of the overall bioremediation program by economists is also required to assess the use of the remediation technique as a competitive and alternative decontamination strategy.

Once a bioremediation program has been designed, its feasibility can be evaluated by considering: (a) *the applicability*, strictly related to the properties of the contaminants and the environmental, biological, and hydrogeochemical features of the contaminated site, (b) *the treatability studies* to determine the potential for bioremediation, to define required operating and management practices, to design and implement the bioremediation plan, and to establish a suitable and effective monitoring program, (c) *the possible limitations and drawbacks*, and (d) *the advantages* linked to its potential of harnessing naturally occurring biogeochemical processes, immobilizing or destroying, partially or completely, contaminants.

Only when all these conditions are met, a successful, productive, not-deleterious of soil quality, and costly convenient bioremediation process will occur.

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