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Biological Degradation of Polycyclic Aromatic Compounds (PAHs) in Soil: a Current Perspective

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Abstract

Purpose of Review Here we examine recent research on the degradation of polyaromatic hydrocarbons (PAHs) by fungi and bacteria. In addition, we provide information regarding the role that omics tools (next-generation sequencing) can play in the future development of bioremediation of PAHs.

Recent Findings The toxicity of petrogenic wastes containing PAHs to biotic communities, including humans, is well established. Bioremediation strategies based on the use of microorganisms represent an economic and environmentally friendly approach (compared with other remediation methods) which is increasingly being applied for the treatment of PAH-contaminated soils.

Summary Biological treatments or bioremediation exploits the hydrocarbon-degrading abilities of microorganisms, resulting in destruction of the contaminants and significant detoxification of the contaminated material. To further develop this approach as a consistent commercial technology, it is important to understand the microbial ecology of the remediation process, determining the key microorganisms which drive the underlying PAH degradation processes.

Keywords Bioremediation · PAHs · PAH degradation · Microbial community · Metagenomics

Introduction

Oil pollution through the accidental release both to land and marine ecosystems results in adverse effects to humans, plants and animal life and the environment [15]. Oil spills mainly occur because of human activity (exploration and transport of oil, refining and storage, road run off, burning of fuels), although naturally occurring oil seeps also contribute to the presence of crude oil in the environment. Some of the most infamous oil spills have occurred in the marine environment but have also affected the land when the oil is washed ashore. One of these spills was the *Exxon Valdez* disaster where a reported 37,000 tonnes of crude oil from the stricken ship washed ashore at Prince William Sound, Alaska, in 1989 [65]. This disaster was also the first high-profile field demonstration of bioremediation [16]. A more recent spill was the *BP Deepwater Horizon* accident in 2010 where 3.19 million barrels (506×10^6 L) of crude oil was released into the ocean after an explosion on an oil rig operating in the Gulf of Mexico [40].

Crude oil is a complex mixture of hydrocarbons, many of which are known to be carcinogenic, teratogenic and mutagenic [39, 51]. It also contains small quantities of oxygen-, sulphur- and nitrogen-containing compounds along with trace amounts of organometallic compounds [25]. Components of crude oil can be grouped into four classes according to their differing solubility in organic solvents and water. The classes are the saturates or aliphatics (n- and branched alkanes and cycloparaffins), the aromatics (mono-, di- and polycyclic aromatic compounds containing one or more benzene rings), the resins (aggregates with various building blocks such as pyridines, quinolines, sulfoxides and amides) and the least soluble of all fractions, the asphaltenes (aggregates of molecules with condensed aromatic and naphthenic rings connected by paraffin chains). Weathered oil, which is generally found as a contaminant in the environment, is dominated by complex mixtures of the aliphatic and polycyclic aromatic hydrocarbons (PAHs) and thus are a major environmental concern. Figure 1 shows the chemical structures of aliphatic, aromatic and asphaltene compounds found in crude oil.

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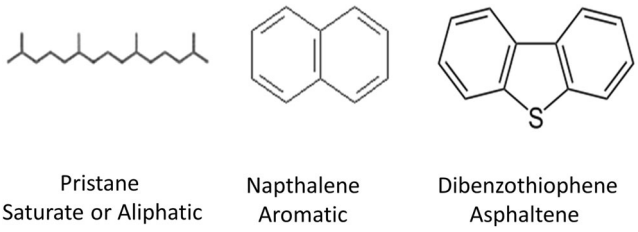

Fig. 1 Representative chemical structures of aliphatic, aromatic and asphaltene fractions of crude oil

Polycyclic aromatic hydrocarbons (PAHs) are a class of toxic-fused ring aromatic compounds consisting of hydrocarbon molecules of two or more fused benzene or aromatic rings produced naturally and from anthropogenic sources [54]. PAHs exist as a complex mixture in many different petroleum-based products such as tar and creosote and as such are widespread pollutants in the environment. Common areas of pollution are soils and waters surrounding gas plants, oil refineries, air bases, petrol stations and chemical-manufacturing facilities [30, 53]. Of most concern are the higher molecular weight PAHs (HMW-PAHs) as they present a significant threat to human health due to their mutagenic and carcinogenic properties. Sixteen PAH compounds are recognized as priority pollutants by the US EPA and the EU [34]. PAHs are persistent pollutants in the environment due to their hydrophobicity, low water solubility and strong tendency to absorb to the soil matrix. All of these factors contribute to low PAH bioavailability and thus low biodegradation rate [33].

Biological Remediation Techniques (Bioremediation)

As a result of the toxicity associated with oil pollution, there is a need to detoxify or remediate contaminated environments. Remediation methods are generally divided into two categories: in situ and ex situ remediation methods. Traditionally, the treatment of polluted soils has involved physical methods where polluted soils were either excavated and disposed to landfill or isolated in situ using various barriers to prevent movement of pollutants off-site or contact between humans and the pollutants.

The most common treatment is disposal to landfill; for example, in South Australia, an estimated 87,000 tonnes per annum of polluted soil is disposed off to landfill sites [61]. This is becoming a non-viable option due to gradual changes in disposal regulations, which have resulted in increased fees and liabilities for landfill disposal. In some countries, the in situ containment of contaminants is considered as waste disposal and therefore subject to the same stringent regulations, permitting processes and liabilities [52]. Thus, these practises are becoming less prevalent, increasing the demand to develop alternate, more sustainable techniques. In contrast, many physical and chemical remediation techniques require high energy input and are not cost-effective nor environmentally friendly or sustainable.

Bioremediation is the use of living microorganisms to degrade environmental pollution or the application of a biological treatment to clean-up hazardous chemicals through natural biological systems. During various bioremediation processes, organic molecules undergo transformations involving enzymes resulting in the complete conversion of an organic molecule to inorganic products. There are many organic contaminants that are amenable to bioremediation (Fig. 2); however, the effectiveness of bioremediation is dependent on the contaminant, its bioavailability and the microbial capacity of the natural environment that has been contaminated [3]. The major advantage of bioremediation is that it can be conducted in situ, which removes the cost and liability of transport and minimizes site disruption. It also eliminates the need to find an area where the removed soil can be treated [57].

The specific bioremediation processes that are used depends on the contaminant type and characteristics of the environment studied. For example, hydrocarbon-degrading organisms are present in most soils; they may be as low as 0.1% of soil microbiota in pristine ecosystems, whereas they can dominate oil contaminated ecosystems [22]. Bioremediation strategies can involve any of the following techniques, whether it be in situ or in a bioreactor:

- *Natural attenuation* is generally a 'hands-off' process, which allows the endogenous microbes to degrade pollutant without any addition of exogenous macronutrients or microbes.
- *Biostimulation* accelerates the rate of bioremediation by promotion of the growth conditions of the endogenous microbes by addition of exogenous macronutrients which are often limited in contaminated environments, namely nitrogen and phosphorus [42, 57]. Organic carbon has also been used to biostimulate the degradation of TCE [50]. Biostimulation often results in a more rapid onset of

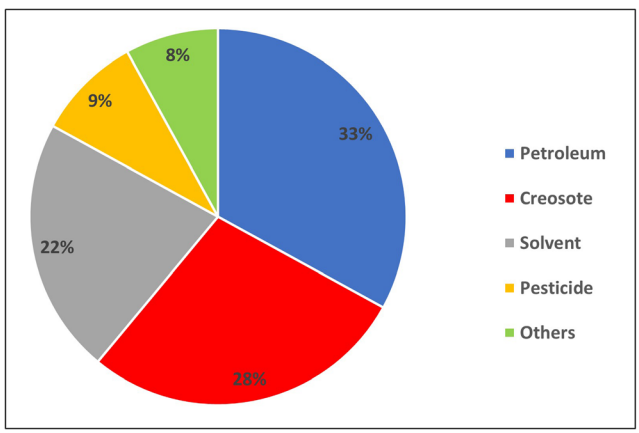

Fig. 2 The major types of waste chemicals amenable to bioremediation. Adapted from Ball [8]

degradation, although some studies have found that degradation rates converge with time, with no marked improvement in overall treatment compared with natural attenuation [55].

- *Bioaugmentation* is used if there is a lack of adapted microorganisms for pollutant degradation (i.e. hydrocarbon) or insufficient microbial capacity for degradation. The endogenous community is augmented by seeding hydrocarbon-degrading microorganisms into the contaminated environment (often as well as nutrient addition), so that biodegradation is created and stimulated. However, the survival of the exogenous inoculums is a limitation to this process [72]. The introduced microbes may not be adapted to thrive in the specific conditions by either incompatible conditions or competition from the endogenous community, thus resulting in slow or no bioremediation. This can sometimes be overcome by isolation and culture of indigenous microbes with the capacity to degrade the contaminant with subsequent re-introduction at increased concentrations [57].

Bioremediation results in pollutants being permanently eliminated by conversion to harmless substances such as carbon dioxide, water and ethane, which makes bioremediation environmentally safe and therefore is generally well accepted by the public [28]. These advantages all contribute towards bioremediation being a low-cost and low-energy method for degrading organic contaminants in soil, groundwater and shorelines.

PAH Degradation

PAHs once exposed to the environment can be degraded via biotic and abiotic mechanisms, with the chief process for the natural elimination of PAHs from the contaminated environment being microbial degradation. A wide variety of organisms are known to metabolize PAHs. Contaminated environments typically contain a wide variety of bacteria, fungi and algae capable of PAH degradation which all have different metabolic pathways and substrate ranges [47].

Abiotic PAH Degradation

There are several processes that can occur to reduce the concentration of PAHs in the environment that do not involve microbial degradation:

- *Transfer processes* cause the relocation of PAHs without altering their structure via volatilization, absorption, leaching or erosion. The tendency for loss of PAHs through these methods decreases as the molecular weight of the compound increases [4].
- *Chemical degradation* alters the structure of the compounds to generally less toxic compounds, through naturally occurring chemical processes such as oxidation-reduction or photochemical exposure [73].

Biotic PAH Degradation

A wide variety of bacteria and fungi have been observed to be capable of PAH degradation using varying metabolic pathways and substrate ranges under both aerobic and anaerobic conditions (Table 1). Historically, most degradation studies have been conducted in aerobic environments as these reactions are the more favoured and often more rapid [23, 58, 67]. However, anaerobic conditions are often promoted when the degree of contamination is very high, thereby limiting oxygen flow due to soil pore saturation or clogging [20].

The rate of PAH degradation is indirectly proportional to the number of aromatic/benzene rings present in the molecule; low molecular weight PAHs are more readily biodegradable than higher weight compounds. For bacterial degradation, this is usually due their inability to incorporate the higher molecular weight (HMW)-PAH into the cell due to their large size. The degradation rate of HMW-PAHs is also controlled by desorption kinetics, which over time reduce due to the hydrophobic PAHs being sequestered into the soil matrix [42]. Other factors that affect PAH biodegradation rates are temperature, pH, soil type, aeration, nutrients, depth, diffusion, microbial adaptations or capacity, bioavailability, previous chemical exposure, water availability, sediment toxicity, physicochemical properties of the PAH, concentration of the PAH and seasonal factors [42, 73]. Biodegradation of PAHs is highly regio- and stereoselective with the specific pathway involved highly dependent on the molecular weight of the PAH and the type of microorganisms involved. The degradation pathway for aromatic compounds also depends on whether the fungi or bacteria are mineralising the compound (Fig. 3).

Bacterial PAH Degradation

Initial bacterial degradation involves the incorporation of molecular oxygen into the aromatic nucleus/ring. This reaction is catalysed by multicomponent dioxygenase enzymes (also known as ring-hydroxylating dioxygenase or RHD) to form *cis*-dihydrodiol [5, 12, 29, 49, 60]. This initial ring oxidation is usually the rate-limiting step in the biodegradation of PAHs. The enzyme *cis*-dihydriol dehydrogenase then re-aromatises the aromatic nucleus of the *cis*-dihydrodiol to form dihydroxylated intermediates; further oxidization of the intermediates leads to the formation of catechol. The next step in bacterial metabolism is confirmation-dependent aromatic ring fission. If the hydroxyl groups of the dihydroxylated

Table 1 Example of microbial genera associated with PAH degradation. Adapted from Cerniglia [12], Juhasz and Naidu [29], Seo et al. [53] and Fathepure [17] with permission

Compound degraded	Bacterial species	Fungal species
Naphthalene	*Acinetobacter*, *Alcaligenes*, *Marinobacter*, *Brevundimonas*, *Burkholderia*, *Cycloclasticus*, *Pseudomonas*, *Rhodococcus*, *Sphingomonas*	*Aspergillus*, *Candida*, *Cunninghamella*, *Gilbertella*, *Linderina*, *Panaeolus*, *Penicillium*, *Rhizophlyctis*, *Thannidium*, *Zygorhynchus*
Anthracene	*Alcaligenes*, *Beijernickia*, *Comamonas*, *Cycloclasticus*, *Janibacter*, *Mycobacterium*, *Rhodococcus*, *Sphingomonas*	*Bjerkandera*, *Cunninghamella*, *Cladosporium*, *Daedaela*, *Penicullium*, *Phanerochaete*, *Ramaria*, *Rhizopus*, *Trametes*
Phenanthrene	*Acidovorax*, *Acinetobacter*, *Arthrobacter*, *Burkholderia*, *Cycloclasticus*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Nocardioides*, *Pseudomonas*, *Streptomyces*, *Staphylococcus*, *Sphingomonas*,	*Aspergillus*, *Bjerkandera*, *Cunninghamella*, *Curvularia*, *Penicillium*, *Phanerochaete*, *Pleurotus*, *Syncephalastrum*, *Trametes*
Fluoranthene	*Acidovorax*, *Arthrobacter*, *Janibacter*, *Mycobacterium*, *Pseudomonas*, *Sphingomonas*, *Stenotrophomonas*, *Terrabacter*	*Aspergillus*, *Bjerkandera*, *Cryptococcus*, *Cunninghamella*, *Flamulina*, *Laetiporus*, *Penicillium*, *Pleurotus*,
Pyrene	*Acidovorax*, *Bacillus*, *Burkholderia*, *Mycobacterium*, *Pseudomonas*, *Rhodococcus*, *Xanthamonas*	*Agrocybe*, *Cunninghamella*, *Kuehneromyces*, *Penicillium*, *Phanerochaete*, *Syncephalastrum*, *Trammetes*,

intermediate are in the *ortho*-position (the groups are in positions 1 and 2 of the aromatic ring), then, oxygenolytic cleavage occurs between the two hydroxyl groups by intradiol (*ortho*) cleaving dioxygenase resulting in the formation of *cis*,*cis*-muconic acid [29]. If the hydroxyl groups are in the *meta*-position (groups are on positions 1 and 3), cleavage occurs adjacent to the hydroxyl groups catalysed by the enzyme extradiol (*meta*) cleaving dioxygenase forming 2-hydroxymuconic semi-aldehyde (see Fig. 3) [12, 21, 55].

This entire process is referred to as the upper catabolic pathway of PAH degradation [12, 24]. Once the first aromatic ring of the PAH molecule is degraded, the second ring is attacked in the same manner and so on [6]. Degradation via the upper degradation pathway (ring cleavage) results in the production of succinic, fumaric, pyruvic and acetic acids and aldehydes, and the by-products of this reaction are carbon dioxide and water. The cleavage products are utilized by microorganisms for the synthesis of cellular constituents and energy [24].

The catabolic enzymes involved in the degradation of various PAHs have been well studied; the first hydroxylation step is performed mainly by aerobic bacteria that contain the PAH ring-hydroxylating dioxygenase (PAH-RHD) system [11]. Homologous PAH-RHD enzymes are encoded by specific genes present in both Gram-positive (GP) and Gram-negative (GN) bacterial species, with the arrangement of these genes varying with the type of bacteria [24, 78].

Anaerobic Degradation of PAHs

The anaerobic degradation of aromatic hydrocarbons including PAHs has been observed in situ [1, 46] and recently reviewed [2, 32, 66, 76]. In anoxic conditions, maximum PAH degradation occurs under sulfidogenic conditions followed by methanogenic and nitrate-reducing conditions (Fig. 3) [13].

Fungal PAH Degradation

Like bacteria, the initial step of fungal PAH metabolism involves the introduction of atmospheric oxygen to the aromatic nucleus. Non-ligninolytic fungi tend to utilize cytochrome P-450 monooxygenase enzymes to incorporate oxygen, resulting in arene oxide intermediates (Fig. 3). These intermediates can either undergo further metabolism by epoxide hydrolase to form *trans*-dihydrodiols, or undergo non-enzymatic rearrangement to form phenol which is then conjugated with sulphate, glucuronic acid or glucose [26]. Ligninolytic fungi produce lignin peroxidases and manganese-dependent peroxidases that degrade both lignin-related compounds and catalyse the oxidation of PAHs to quinines [37]. The metabolites from fungal metabolism are generally less mutagenic than the parent compound but are not fully degraded; at this point, bacteria continue the metabolism. Literature suggests that fungal extracellular enzymes initiate the degradation of HMW-PAHs, removing the need to incorporate the pollutant into the cell, producing smaller metabolites which are then further metabolized by bacteria [7, 21, 58]. Extracellular enzymes also catalyse the decomposition of plant residues, releasing nutrients into the soil that help sustain and stimulate microbial growth. Decomposition also breaks down organic matter that pollutants have sorbed to, thus releasing the pollutants for microbial degradation [72]. Moreover, fungal hyphae penetrate contaminated soil, reaching pollutants, giving fungi a significant advantage over bacteria [18, 31]. Even though

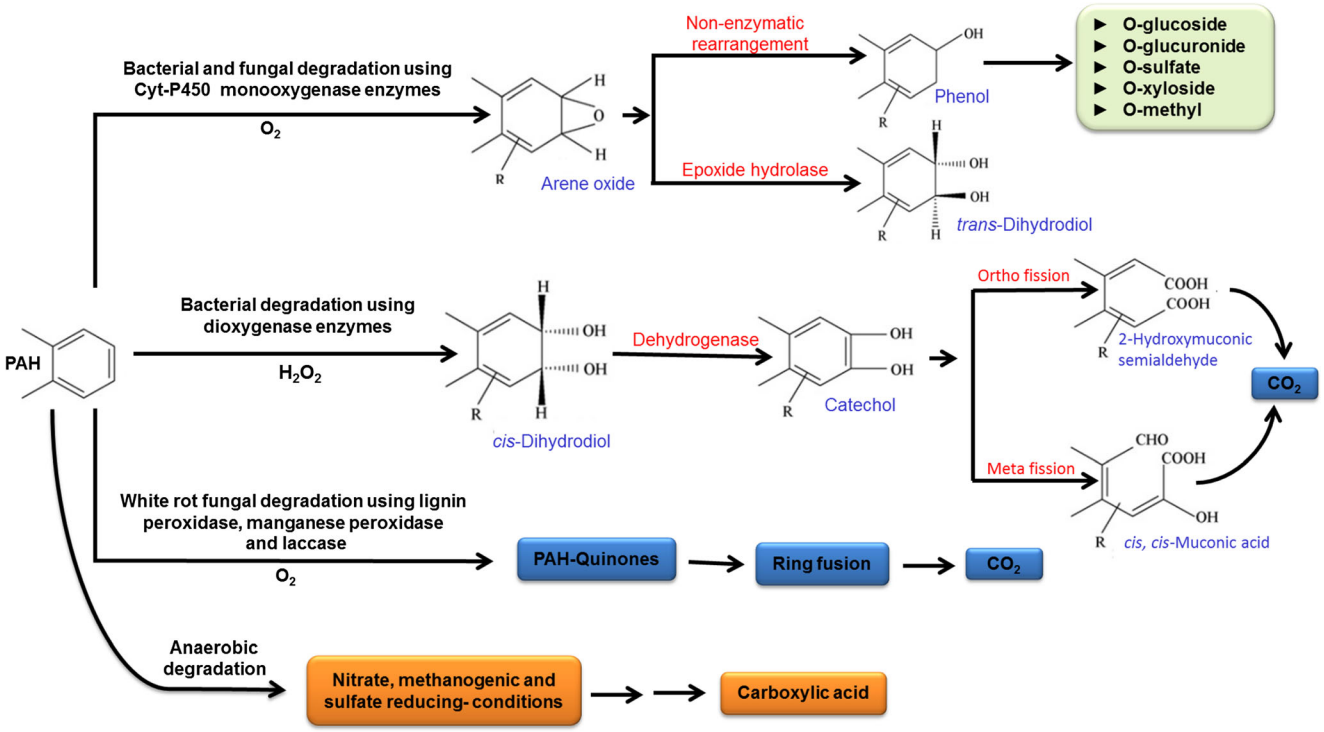

Fig. 3 Pathways for the microbial degradation of polycyclic aromatic hydrocarbons. Reprinted from Shahsavari et al. [55]

most research points to the fact that fungi initiate the metabolism of HMW-PAHs, Gram-positive bacteria have been seen to dominate communities in older PAH-polluted sites [10]. It has also been shown that Gram-positive bacteria are able to increase PAH bioavailability in aged contaminated soils due to biosurfactant and biofilm formation, which together enable these bacteria to initiate PAH degradation. This all provides strength to the argument that when devising remediation strategies, especially those of older PAH-polluted sites, both fungal and bacterial community dynamics should be investigated and promoted. For example, the cell-free extract of *Phanerochaete chrysosporium* was used to enhance the degradation of the PAHs in biosolids intended for agricultural use [63]. In addition, the halotolerant bacteria *Corynebacterium variabile* (with biochar as biocarrier) has been used as novel strategy for the bioremediation of PAHs [77].

Microbial Ecology: Methods for Investigation/Characterization

It is well known that microbes that have hydrocarbon-degrading ability are ubiquitous within the soil environment; generally, HMW-PAHs are degraded by fungi, while lower molecular mass compounds are predominately degraded by bacteria [52], making the total microbial community of interest for study in terms of the bioremediation of PAHs. It is well recognized that less than 1% of the microbial diversity of soil can be cultured; further, estimating numbers of fungi present via methods such as plate counting can be misleading due to the presence of spores [19, 42, 62]. Culture-dependent techniques are also laborious, time-consuming and most importantly, selective and biased for the growth of specific microorganisms. The introduction of molecular microbial ecological approaches such as polymerase chain reaction (PCR)–based community profiling has, to some extent, overcome these limitations [48]. Many of these techniques exploit the 16S rRNA gene in prokaryotes and the 28S rRNA gene in eukaryotes, which encode for the small subunit of the ribosome that is critical to the function of all organisms. For example, a popular method for separation and identification of species detected from environmental samples is denaturing gradient gel electrophoresis (DGGE) or temperature gradient gel electrophoresis (TGGE). These methods allow the separation of the same sized DNA fragments based on sequence. DNA fragments are separated by electrophoresis in polyacrylamide gels containing a gradient of denaturing substances. In DGGE, a chemical gradient is created using urea and formamide, whereas TGGE creates a temperature gradient. The use of sequence separation was adapted to microbial ecology by Muyzer et al. *[43]* using the V3 variable region of the 16S rRNA with a GC clamp to prevent total denaturation. Since then, countless studies have utilized DGGE and to a lesser extent TGGE, to profile various communities including the Archaea and Eukaryotes as well as role-specific communities such as sulphur-reducers or nitrogen-fixing species.

A significant benefit of DGGE and TGGE is that the gels can be scanned to analyse the pattern of bands for further

comparative analysis. Furthermore, electrophoresed fragments can be directly excised from the gel, amplified and sequenced, thereby bypassing cloning, making identification much quicker. While metagenomic based on next-generation sequencing (NGS) methods gives a clearer picture of microbial communities, these tools remain useful, relevant technologies for assessing changes in the dominant microbial community.

The application of molecular ecological techniques such as DGGE and TGGE has resulted in a significant increase in the knowledge of microbial community dynamics and the existence of formerly unknown microorganisms. Culture-independent descriptions of microbial communities now dominate the literature in all areas of microbial ecology. Advances in a procedure called stable isotope probing (SIP) has further improved understanding of the active portion of the soil microbial community [14, 27, 44, 51, 56]. Stable isotope probing offers great potential for wide application in microbial ecology, offering a culture-independent means of investigating the effect of changes in environmental conditions on the microbiota. SIP is based on the premise that physiologically active organisms will incorporate carbon and nitrogen from stable isotopically labelled substrates into its biomarkers when the labelled substrate is supplied as the sole energy source [38, 44].

Another significant advance in the investigation of soil microbial communities has come with the advent of NGS platforms and associated bioinformatics tools which have enabled the use of high-throughput sequencing for rapid, cultivation-independent and relatively low-cost investigations of the metagenome (the study of the collective microbial genomes) of a community [35, 64, 71, 75]. Metagenomics has allowed the assessment and exploitation of the taxonomic and metabolic diversity of varying microbial communities on an ecosystem level. The development of metagenomics has also permitted the identification of the most frequently represented functional genes and metabolic pathways that are relevant in a given ecosystem and has allowed for comparison of systems (comparative metagenomics).

Recently, research has begun to link the process of SIP with next-generation sequencing and metagenomics, enabling an effective alternative to large-scale whole-community metagenomic studies by specifically targeting the organisms or biochemical transformations of interest, thereby reducing the sequencing effort and time-consuming bioinformatic analyses of large datasets [38, 41, 68, 70].

Improvements in NGS have also seen a boom in other 'omics' technologies including metatranscriptomics, metaproteomics and metabolomics. Omics refer to any advanced technique for identifying genes (genomics), mRNA (transcriptomics), proteins (proteomics) and metabolites (metabolomics) in a living organism. The formation of metagenomic complementary DNA (cDNA) libraries from messenger RNA (metatranscriptomics) has allowed identification of the expressed biological signatures in complex ecosystems [45]; however, this is still rare due to the difficulties associated with processing environmental RNA samples. Metaproteomics aims at assessing the immediate catalytic potential of a microbial community [59] although this technique is challenged by uneven species distribution, broad-ranging protein expression levels within microorganisms and the large genetic heterogeneity within microbial communities. Metabolomics is the application of techniques to analyse the interactions of organisms with their environment, such as identifying the stress from abiotic (such as xenobiotic exposure or temperature) and biotic stressors (such as competition) [36]. Increasingly, researchers have found that a combination of all 'omics' technologies is necessary to gain a comprehensive understanding of the complex microbial communities [9].

Future Directions for the Remediation of PAH-Contaminated Soils

There are some issues regarding the degradation of PAH in the soils. Among them, major challenges of biological remediation of PAHs in soil are:

- The continuous generation of novel recalcitrant pollutants
- Rapid industrialization in countries without a robust regulatory framework
- Low microbial adaptability
- Low bioavailability of pollutants

Therefore, new technologies for degradation of PAHs are needed. Future directions for the remediation of PAH-contaminated soils have been discussed by Kuppusamy et al. (2017) [34]. Some of the techniques that are in a developmental stage are electrokinetic remediation, vermiremediation and biocatalyst-assisted remediation. Also, the authors have proposed mixed cell culture system, biosurfactant flushing, transgenic approaches and nanoremediation for the successful remediation of long-term PAH-contaminated soils.

For example, electrokinetic remediation has been used where other techniques such as natural attenuation is unsuitable. Low-intensity direct current is applied through the soil to transport ionic pollutants by electromigration. Although slow desorption rates and hydrophobicity make PAHs difficult to remove subsurface environments, the addition of surfactants, cyclodextrins and co-solvents enhance the method's efficiency. Enzyme-mediated remediation is another method which can be used for PAH-contaminated soil. Free laccase from *Trametes* sp. has been used to transform 15 priority PAH-contaminated field soils in the presence of a redox mediator [74]. Transformation of PAHs was observed in reaction

mixtures and soil suggesting that the enzyme may have potential for the efficient and safe clean-up of PAH-contaminated soils. Similarly, nanoremediation also represents a useful method for the degradation of PAH in the soil, as nanoparticles can be distributed more widely in situ, allowing the efficient remediation of soils contaminated to large depths; the technique has also been reported to be compatible with bioremediation [69].

Conclusion

Bioremediation of PAH-contaminated sites using bacteria and fungi offers a simple, inexpensive and environmentally friendly technology which can be performed using different bioremediation strategies (e.g. bioaugmentation). The approach proposed for the degradation of PAHs is a holistic approach that integrates physical, chemical and biological measurements. The application of 'omics' technologies for real-time measurement of PAH-degradative processes using key genes, encoding the key degradative enzymes during bioremediation, offers significant opportunities to understand the bioremediation process, thereby improving the success of its application.

Compliance with Ethical Standards

Conflict of Interest There is no conflict of interest for this paper.

Human and Animal Rights and Informed Consent This article does not contain any studies with human or animal subjects performed by any of the authors.

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