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USE OF MOLECULAR TOOLS TO MONITOR MICROBIAL COMMUNITIES DURING THE BIOREMEDIATION OF POLYCYCLIC AROMATIC HYDROCARBON-CONTAMINATED SOILS

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ABSTRACT

One of the priority environmental pollutants, because of its high toxicity and persistence, are the polycyclic aromatic hydrocarbons (PAHs). PAHs are recalcitrant compounds exhibiting high hydrophobicity and therefore readily absorbed in the gastrointestinal tract of mammals, also having a rapid distribution in a variety of tissues with a marked tendency to fatty deposits. Because of its importance as environmental pollutants, soil contamination with PAHs is a priority environmental issue. In recent years, there have been great advances in the understanding of the mechanisms of degradation of PAHs and the techniques for the monitoring of these processes in polluted soils. However, the validation and performance of a bioremediation strategy should be based not only on the effect of the microorganisms in soil (biodegradation of the contaminant), but also in the detection and monitoring of the inoculated microorganisms. This review presents an overview of the strategies for the bioremediation of soils contaminated with PAHs, focusing on the molecular biology methods that can be used for the monitoring of these soils in the field.

INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a group of chemically similar organic compounds containing two or more fused aromatic rings, produced as a result of the incomplete combustion and pyrolysis of organic matter. Several natural and anthropogenic sources contribute to the release of PAHs to the environment such as forest fires, volcanic eruptions, vehicle emissions, oil refining and industrial combustion of fossil fuels. Due to its toxicity, mutagenicity and carcinogenicity, many PAHs have been identified as priority pollutants by regulatory authorities including the Environmental Protection Agency of the United States (US-EPA, 2008). PAHs are highly hydrophobic compounds, and as the molecular weight of PAHs increases their solubility in water and volatility decreases (Haritash and Kaushik, 2009). Chemical and photochemical reactivity, as well as many physical and biological characteristics, are greatly influenced by structural aspects like the degree of saturation, number of rings and spatial configuration of PAHs (Mukherji and Ghosh, 2012). Moreover, as the association of a contaminant with the soil organic matter is directly related with its hydrophobicity, PAHs are highly recalcitrant compounds having high octanol-water partitioning coefficients (Kow) (Accardi-Dey and Gschwend, 2003).

The bioremediation of PAH-polluted soils has become an increasing environmental priority. For years, mechanisms on PAH biodegradation and the use of native and introduced organisms in polluted soils, either as single organisms or as consortia, have been studied. Many microbial species including bacteria, fungi and algae have been described as capable of partially or completely metabolize low molecular weight (LMW) or high molecular weight (HMW) PAHs under aerobic or anaerobic conditions (Seo, *et al*., 2009; Cerniglia and Sutherland, 2010). Once in soil, PAH-degrading organisms face a series of biotic and abiotic factors that permanently affects their adaption as well as permanence

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and survival. In addition, the validation and performance of a bioremediation strategy should be based not only on the effect of the microorganisms in soil (biodegradation of the contaminant), but also in the detection and monitoring of the inoculated microorganisms. Thus, the monitoring of degrading populations is a key aspect to control PAH bioremediation in soils. However, the use of conventional microbiological tools are often insufficient to properly monitor the shits in microbial communities and survival of PAH degraders in soils and does not allow to detect most of non-cultivable populations involved in the metabolism of PAHs. Since only a small proportion of the microorganisms involved in PAH-degradation in soil are cultivable, it becomes necessary to use more informative techniques to monitor the fate of microorganisms in soil. Molecular biology tools allow the direct analysis of PAH-degrading microorganisms without isolating them, providing also a way to solve the problems mentioned above. This review presents an overview of the strategies for the bioremediation of soils contaminated with PAHs, with special focus on the molecular biology methods that can be used for the monitoring of these soils in the field.

Toxicity and environmental fate of PAHs

While there are more than 100 PAHs identified, only 16 of them have been classified as priority pollutants by the US-EPA (US-EPA, 2008). A large number of PAHs possess toxic, mutagenic and/or carcinogenic properties (Goldman, *et al.*, 2001). PAHs are poorly hydrosoluble and therefore are readily absorbed in the gastrointestinal tract of mammals, being rapidly distributed in a variety of tissues with a marked tendency towards fatty tissues. That is why PAHs are considered persistent environmental contaminants that can have a detrimental effect on the flora and fauna of affected habitats, resulting in the absorption and accumulation of toxic chemicals in food chains and, in some cases, serious health problems or genetic defects in humans. PAH absorption in mammals can take place by inhalation, ingestion or direct contact. In some cases, there is a metabolic activation by monooxygenase enzymes (mainly cytochrome P450) to form toxic intermediates as phenols, trans-dihydrodiols, quinones, and occasionally diol epoxides (Wattiau, *et al.*, 2002). Production of diol epoxides are directly associated with carcinogenicity; diol epoxides generated in the bay region of a PAH molecule are highly reactive and mutagenic and can produce covalent interactions with DNA, forming PAH-DNA adducts (Weis, *et al.*, 1998). The formation of PAH-DNA adducts plays a key role in the initiation of cancer, especially when they occur at a critical site for the growth or differentiation of cells. Thus, DNA adducts can be measured and used as markers of exposure and PAH-specific genetic damage. While some of 2 and 3-ringed PAHs have not been found to be carcinogens for mammals, genotoxicity tests have revealed a great amount of 3 to 6 rings PAHs having probable toxic, teratogenic and mutagenic effects in exposed humans (WHO-IARC, 2010). PAH genotoxicity increases as molecule size augment to 4 or 5 rings (Kanaly and Harayama, 2000). According to the World Health Organization (WHO) international agency for research of cancer (IARC) classification, benzo[a]pyrene, dibenzo [a,h] anthracene and benzo[a]anthracene are probable human carcinogens (2A category) while benzo[a] fluoranthene, benzo[k] fluoranthene and indene [a,2,3-cd]pyrene are possible carcinogens (2B category). These compounds are also included in the US-EPA list of 16 priority PAHs.

Pollutants such as PAHs can be adsorbed in soil material or even dissolve in small amounts, but most of them are adsorbed to soil particles. Once in soil PAHs can be transformed by physical or chemical agents, sediment, bioaccumulate or can eventually be degraded by soil microbial communities (Juhasz and Naidu, 2000). Because of their low vapor pressures, PAHs remain in solid form and thus volatilization is limited to only few low molecular compounds (e.g. naphthalene, fluorene). In addition, a high content of organic matter negatively affects the volatilization and increases the adsorption extent (Alexander, 1999). PAHs can also be oxidized by absorbing electromagnetic radiations from sunlight, a process dependent on variables such as soil texture, temperature, salinity and pH (Kong and Ferry, 2003). On mangrove contamination with oil, photochemical oxidation was found to have an important role in PAH degradation as reported by (Ke, *et al.*, 2002). However, microbial degradation is considered the main natural form of degradation of PAHs (Juhasz and Naidu, 2000).

Strategies for PAH bioremediation in soil

Several physical, chemical, thermal and biological technologies are available to remove or reduce to its minimum the degree of contamination in soils, each one having its own advantages and disadvantages. The selection of the method of remediation greatly depends on factors such as the extent of the contamination, type and concentration of the contaminants, physicochemical characteristics of the soil, soil availability and cost, among others. Integrated soil remediation technologies, which

combine separation and destruction of PAHs appear to be a good choice in the technical field, allowing PAH removal with improved efficiency. However, it is not a secret that the cost of implementing a remediation technology plays a decisive role in choosing a method. In this sense, bioremediation has large advantages over other technologies as well as being environmentally friendly and producing good results in terms of removal (Zafra, 2014). Biological methods possess advantages over physicochemical and thermal methods, such as its cost-effectiveness, the possibility of fully mineralize pollutants and the little need for further treatment.

Bioremediation uses the metabolic versatility of microorganisms to degrade different hazardous pollutants. The main objective of bioremediation is to transform organic pollutants into non-toxic metabolites or mineralize them to carbon dioxide (CO_2) and water (Haritash and Kaushik, 2009). A viable soil remediation technology requires microorganisms capable of a rapid adaptation and tolerance to pollutants (Zafra, *et al*., 2015). Many factors influence the microorganisms for using pollutants as substrates; therefore, understanding the mechanisms, catabolic pathways and responsible enzymes for pollutant degradation is a key point to define the most relevant factors for implementing a bioremediation technology at field scale. Two main strategies for soil bioremediation are commonly used: (1) biostimulation, which consists in the addition of fertilizers, texturizing agents and aeration to improving the growth conditions of soil native degrading populations, and (2) the addition of specific pollutant-degrading microorganisms, or so called bioaugmentation. These two approaches can be applied through *in situ* techniques such as land farming, composting and biopiles for the degradation of PAHs and other hydrocarbons in soil, while more advanced methods *ex-situ*, as the use of bioreactors provide better control temperature and pressure to improve the degradation processes but lacks in versatility.

Bioaugmentation

Bioaugmentation is an effective strategy in soils highly contaminated with PAHs and native microbial populations with low proportion of PAH-degrading microorganisms (<105 CFU/g), which results in lower biodegradation rates. PAH biodegradation through bioaugmentation with autochthonous aromatic degrading bacteria and fungi have been described and successfully used (Cerniglia and Sutherland, 2010; Mrozik and Piotrowska-Seget, 2010; Sayara, *et al*., 2011; Zafra and Cortes-Espinosa, 2015; Zafra, *et al*., 2015), although there is controversy about reproducibility, operating conditions and scaling efficiency. Autochthonous populations composing soil microbial communities are responsible for a biotic balance that acts to prevent the establishment of foreign species. This balance allows a community to resist biotic and abiotic environmental changes, even though important abiotic disturbances (such as an episode of extensive contamination) can produce appreciable changes in the community. Native soil microbial populations established in a polluted environment possess a high degree of stability and resilience towards introduced microorganisms, being an extremely important factor in the long-term success of a bioaugmentation process. Introducing different exogenous species is rarely enough to permanently modify soil microbial communities. Because of this, bioaugmentation should not only face the possibility to degrade a pollutant, but also to address the main factors associated with the homeostasis of the microbial community. Therefore, the microorganism(s) selected for bioaugmentation should ideally be one common member of the native community, but if that is not possible, should at least have a great adaptation and growth capability under conditions of competition, predation and parasitism (Zafra, *et al*., 2015). The isolation and reintroduction of organisms from a contaminated site increases the survival of the inoculum, their growth and degradation capability (Gentry, *et al*., 2004; Silva, *et al*., 2009).

Biostimulation

Biostimulation consists in the addition of nutrients, texturizing agents and the improvement of aeration of soils in order to increase the amount of substrates used by the microorganisms and promote cometabolism between native soil microorganisms. PAH contaminated sites often possess low-diversity native microbiota, but have adapted organisms capable of degrading hydrocarbons of different molecular weight (Cunliffe and Kertesz, 2006). Since these microorganisms exist in very low amounts (in the order of 1×10^3 CFU/gram soil) is necessary to increase their number and improve conditions to accelerate the degradation processes. Thus, biostimulation involves the addition of aqueous solutions (containing nutrients and/or oxygen) and vegetal residues to contaminated soils to stimulate the activity of indigenous degrading microorganisms and accelerate the biodegradation of organic pollutants as PAHs, favored also by the increase of compounds such as N, P, K, S and Cu (de Lorenzo, 2008). Substrates such as sugarcane bagasse, corn, wheat and oat residues, sludges, dried blood, vermicompost and compost have

been used to accelerate PAH degradation in soils (Fernandez-Luqueno, *et al*., 2011; Zafra, *et al*., 2014). Biostimulation possesses limitations, particularly in clay, highly stratified or too heterogeneous soils as a consequence of poor oxygen transfer rates. Other factors restricting its application, includes soils with extreme conditions limiting microbial growth or an increase in contaminant mobility. While nutritional requirements of microorganisms are approximately the same as the composition of their cells, the exception to this is carbon, which is required in high amounts (Suthersan, 1999). Based on this, the optimum ratio of carbon/nitrogen/phosphorous recommended for bioremediation processes is approximately of 100:10:1. If carbon source is easily and quickly converted into CO_2, then more carbon is required to support microbial soil communities. In addition to carbon, nitrogen and phosphorous nutrients are commonly added in the biostimulation process. Since each supplemental nutrient can have a different redox potential depending on the terminal electron acceptor, especial care must be taken to determine the type and amount of nutrients to be added in the process of biostimulation, in order to maintain an optimal redox potential. The specific ratio will depend on the rate and extent of degradation of PAHs, bioavailability of nutrients, soil type, and irrigation among others (Yannopoulos, *et al*., 2015).

Molecular biology tools for the monitoring of soil bioremediation

Monitoring tests can provide relevant information of the bioremediation process and usually include the application of laboratory methods (either microbiological, analytical or molecular) to measure the status and effectiveness of bioremediation under certain conditions. Many of these tests are directed to monitor the fate of both introduced and autochthonous microorganisms. In this sense, in many cases it is important to determine the survival of introduced exogenous microorganisms and the native populations that were already present in the soil, as well as enumerate them or quantify genes of interest for the biodegradation process.

The characterization of microbial communities presents limitations due to the lack of sensitive detection methods. Traditional microbiological approaches, such as isolation of pure cultures and further study of their physiological and biochemical properties are not always well suited to study microbial communities and their behavior. In fact, more than 99% of the microorganisms present in soils cannot be isolated due to a lack of knowledge of their physiological needs. Therefore, molecular techniques have been developed to compensate the disadvantages inherent to traditional culture methods. Molecular techniques based on the polymerase chain reaction (PCR) of 16s rRNA, RT-PCR and real-time PCR are becoming standard tools for the detection and quantification of microorganisms previously added to soils. Similarly, other less conventional methods such as FISH (fluorescent *in situ* hybridization), DGGE (denaturing gradient gel electrophoresis), TGGE (temperature gradient gel electrophoresis) and metagenomics are being tested and implemented to study and better comprehend the microbial degradation of PAHs (Table 1).

Polymerase chain reaction-based strategies

DNA amplification by the PCR has been used in many studies to detect, characterize and identify soil degrading microbial populations as well as those introduced during the bioremediation process. PCR detection of genes encoding for microbial monooxygenases and dioxygenases such as nahA, phnAc, nidA and narB, among others, have showed to be useful for the detection of PAH-degrading microbial populations in soils and sediments (Lu, *et al*., 2011). However, one of the most commonly used approach for the detection and identification of microorganisms in soils is the PCR amplification of microbial ribosomal RNA (rRNA) genes (e.g. 16s, 18s and 23s rRNA). The rRNA genes are the basis for microbial phylogenetic analyses, having several million sequences published in the GenBank database. During soil bioaugmentation treatments, rRNA of introduced microorganisms can be easily amplified by PCR and detected by gel electrophoresis. In most cases it is necessary to analyze the rRNA amplification products by additional techniques, such as the terminal-restriction fragment length polymorphism (T-RFLP) or fully sequencing the amplified product, to increase the specificity of detection and identification (Gu, *et al*., 2004).

On the other hand, the technology of quantitative PCR or real-time PCR has been also used to quantify microorganisms after its introduction to different environmental matrices (Kikuchi, *et al*., 2002). One of the most specific and widely way to perform qPCR is with the use of Taqman probes. In this technology, Taq polymerase cleave a fluorogenic Taqman probe that binds to an internal site within the sequence being amplified, during the extension step. This results in the release of a fluorescent molecule (fluorophore), resulting in fluorescence. The cycle threshold value (Ct) is determined at the point where a significant increase in the fluorescence emission

Table 1. Use of molecular tools to monitor microbial populations in PAH-polluted environmental matrices

Method	Target	References
PCR and qPCR	Naphthalene hydroxylating dioxygenase (*nah*Ac) gene	Han, *et al.*, 2014; Layton, *et al.*, 2012; Nyyssonen, *et al.*, 2006
	PAH-ring hydroxylating dioxygenase (PAH-RHDa) genes	Cebron, *et al.*, 2008; Han, *et al.*, 2014; Yergeau, *et al.*, 2009
	Bacterial luciferase (l*ux*A) gene	Layton, *et al.*, 2012
	Catechol 2,3-dioxygenase (C23O) gene	Hesham, *et al.*, 2012
	16s rRNA gene	Han, *et al.*, 2014
qRT-PCR	Cd_1-containing nitrite reductase (*nir*S)	Yergeau *et al.*, 2009
	Alkane monooxygenase (*Alk*B) gene	Yergeau, *et al.*, 2009
	PAH-ring hydroxylating dioxygenase (PAH-RHDa) genes	Yergeau, *et al.*, 2009
DGGE/TGGE	Bacterial RNA polymerase, β subunit (*rpoB*) gene	Dahllof, *et al.*, 2000; Zafra, 2014
	Cd_1-containing nitrite reductase (*nir*S) gene	Throback, *et al.*, 2004
	Cu-containing nitrite reductase (*nir*K) gene	Throback, *et al.*, 2004
	16s rRNA gene	Cunliffe and Kertesz, 2006; Geets, *et al.*, 2006; Hesham, *et al.*, 2012; Li, *et al.*, 2006; Muhling, *et al.*, 2008; Wakase, *et al.*, 2008; Wang and Tam, 2011; Zafra, 2014)
T-RFLP	16s rRNA gene	Vazquez, *et al.*, 2009
Microarrays	Functional gene array containing *Alk*B, C23O, benzyl alcohol dehydrogenase and anthranilate dioxygenase genes	Yergeau, *et al.*, 2009
	Functional gene array containing 200,393 coding sequences	Kappell, *et al.*, 2014
	16s rRNA gene	Yergeau, *et al.*, 2009
FISH	16s and 23s rRNA genes	Chang, *et al.*, 2014; Hesham, *et al.*, 2012; van Herwijnen, *et al.*, 2006
	Naphthalene dioxygenase	Bakermans and Madsen, 2002
	Toluene dioxygenase	Galvao, *et al.*, 2005
NGS	Whole genome shotgun sequencing (WGS)	Kappell, *et al.*, 2014; Layton, *et al.*, 2012; Loviso, *et al.*, 2015; Mason, *et al.*, 2014; Zafra, 2014
NGS	Amplicon-based (16s rRNA)	Layton, *et al.*, 2012; Mason, *et al.*, 2014

occurs, as compared with a background base line. A larger initial concentration of target DNA results in a lower Ct value. qPCR eliminates the use of gels and allows the sample to be analyzed in real time, in less time than conventional PCR. qPCR has been used in bioremediation studies to calculate the copy number of benzyl succinate synthase gene (bssA) and naphthalene dioxygenase (nahAc) in hydrocarbon-contaminated soils, bioaugmentated with degrading microbial consortia (da Silva and Alvarez, 2004; Nyyssonen, *et al*., 2006; Cebron, *et al*. 2008) reported the use of qPCR to detect and quantify PAH-ring hydroxylating dioxygenases (PAH-RHDα) in soil and sediment samples contaminated with PAHs. The results of these studies highlighted a positive correlation among the PAH-degrading genes copy levels and microbial biodegradation potential, as well as the contamination levels in the studied soils.

DGGE and TGGE

As mentioned before, one of the more frequently molecular techniques used in soils is the analysis of 16s rRNA sequences by PCR. PCR allows the identification of microorganisms composing soil microbial communities after sequencing of PCR-amplified fragments and comparison with other sequences. However, the evolution of the microbial community composition over time cannot be easily monitored using this technique. An alternative is the use of DGGE and TGGE (Muyzer, 1999). In DGGE systems, fragments of double stranded DNA are run through a polyacrylamide gel containing a linear gradient of denaturing agents (usually urea and formamide) at a constant temperature (from 60°C to 80°C). During migration in the gel, the fragment remains double chain until reaching a concentration of denaturing agents equivalent to a DNA denaturation temperature, allowing a portion of the DNA molecule to denature. At this point, the fragment changes from a helical structure to a partially denatured molecule, virtually stopping its migration through the gel. In TGGE systems, the chemical denaturing gradient

is replaced by a temperature gradient but the same principle applies for DNA migration through the gel. Thus, two fragments in which an AT is replaced by a GC may be separated and visualized. However, it is estimated that the DGGE detects only 50% of all changes in fragments of about 50 to several hundred base pairs. To solve this problem, the fragments to be separated can be previously amplified using a pair of modified primers. This modification consists of insertion of a GC-rich sequence (approximately 40 base pairs) in the 5' end of one of the primers. This prevents the complete dissociation of the fragments during migration in the gel, and therefore, the loss of migration in a sequence-dependent manner.

By using DGGE/TGGE, complex communities of bacteria and fungi can be analyzed after PCR amplification using primers designed to specifically bind to conserved regions of bacterial 16s rRNA genes or fungal 18s rRNA. By using group-specific primers, is also possible to analyze selected communities, such as actinomycetes or Archaea. Although the 16s and 18s rRNA genes are the genes most often used. Other genes such as the β-subunit of bacterial RNA polymerase (rpoB) can also be used to analyze the microbial diversity and survival of inoculated microorganisms in soil (Dahllof, *et al*., 2000). DGGE has been shown to be useful in the monitoring of the bioremediation of freshly and aged PAH-contaminated soils (Cunliffe and Kertesz, 2006), allowing the monitoring of the survival of inoculated *Sphingobium yanoikuyae* as well as monitoring changes in the native bacterial communities over time. Studies by (Zhou, *et al*., 2009; Wang and Tam, 2011; HuiJie, *et al*., 2011) have also used DGGE to study microbial community dynamics and biodegradation during PAH biodegradation in soils and sediments.

Fluorescence *in situ* hybridization

The FISH is a rapid and sensitive technique that allows the direct visualization and identification of environmental microorganisms, without culturing them (Bakermans and Madsen, 2002). In this method, the microbial cells are hybridized with a DNA probe which is labeled with a fluorescent molecule allowing its microscopic detection. FISH is a valuable tool for the counting, monitoring and examination of changes in specific microbial communities present in environmental matrices. FISH probes are commonly designed on the basis of ribosomal DNA sequences, but can also be designed based on functional genes to monitor the abundance and expression of PAH-catabolic genes such as naphthalene dioxygenase (Bakermans and Madsen, 2002) and toluene dioxygenase (Galvao, *et al*., 2005). Simultaneous detection and quantitation of multiple microbial groups (e.g. bacteria and archaea) can also be achieved using several probes at the same time.

For years, FISH has been used to investigate the microbial community composition in PAH-contaminated soils, mainly detecting the 16s and 23s rDNA gene as reported by several authors (van Herwijnen, *et al*., 2006; Hesham, *et al*., 2012; Chang, *et al*., 2015). However, special care must be taken at using FISH directly on soil or sediment samples. Reported issues, such as the high autofluorescence of soil particles and the high background by probe unspecific adsorption onto soil (Moter and Gôbel, 2000), makes this technique sometimes difficult to use in soils polluted with PAHs. Alternatives to FISH, such as the catalyzed reporter deposition-fluorescence *in situ* hybridization (CARD-FISH) and the fluorescence *in situ* hybridization-microautoradiography (FISH-MAR) are being used to overcome these issues and measuring the potential for PAH degradation in soils (Teira, *et al*., 2007; Lekunberri, *et al*., 2010).

Metagenomic studies

As previously mentioned, most of the microorganisms present in nature cannot be cultivated under standard laboratory conditions, which is a serious limitation to studying the diversity and function of environmental microbial communities. This is particularly relevant in soil, which is probably one of the most complex environments due to its extremely high microbial diversity and heterogeneous nature. Metagenomics refers to the study of all the genomes contained in an environmental or biological matrix directly from DNA samples without isolating or culturing microorganisms (Tringe and Rubin, 2005), thus allowing the study not only of the taxonomic diversity (e.g. species richness, microbial structure and distribution) of the soil, but also of the real metabolic potential of soil microorganisms.

The power of metagenomic studies relies on the use of high-throughput automated sequencing methods, introduced during the last decade into the study of microbial ecology and capable of revealing the taxonomic and functional aspects of microbial communities at a new level of resolution (Sogin, *et al*., 2006). Traditional automated Sanger sequencing is considered a 'first generation' technology, while next-generation sequencing (NGS) are newer methods broadly grouped as template preparation, sequencing, imaging, and data analysis. NGS technologies are also very sensitive compared with

Sanger sequencing, with approximately 99.9% accuracy in <200 bp amplicons and 99% for <400 bp amplicons (Metzker, 2010). One of the major advantages of NGS is the production of massive quantities of data quickly and cheaply, which have opened the possibility to perform large-scale comparative studies. Commercially available technologies include Roche 454 platforms, Illumina/ Solexa, Life technologies/Ion platforms, Applied biosystems/SOLiD and Helicos bio sciences systems, among others. Meta genomics may provide valuable information about the entire functional gene composition of microbial communities, overcoming limitations associated with the use of only one gene (e.g. the 16s rRNA gene), as well as genetic information on novel enzymes, functional profiles and potential metabolic pathways (Thomas, *et al*., 2012). With sequencing prices falling and a large list of bioinformatic tools available for the sequence assembly, gene prediction and taxonomic binning of metagenomic data (Prakash and Taylor, 2012), metagenomics is one of the most valuable and fastest growing scientific disciplines applicable to the study of microbial ecology.

Recent studies using NGS during hydrocarbon bioremediation processes have shown the great potential of these technologies to identify, monitor and estimate proportions of crude oil (Coulon, *et al*., 2012; dos Santos, *et al*., 2011) and diesel degrading populations (Yergeau, *et al*., 2012) present in soils and bioreactors, making possible the discovery of novel degradation pathways (Sierra-García, *et al*., 2014). Important evidence about the regulation of PAH degradation processes has also been found by the metagenomic study of pure bacterial strains (Uchiyama and Miyazaki, 2013), increasing the understanding of the function of important transcriptional regulators. However, there is still scarce information about the metagenomics aspects of microbial communities involved in PAH degradation in soils. In an interesting study using a genetically engineered microorganism (GEM), metagenomics has been used to analyze the microbial diversity of soil communities and the survival of a bacterial GEM strain after 14 years (Layton, *et al*., 2012). Other studies have also used metagenomics to monitor the population dynamics and survival of degrading microorganisms in PAH-polluted soils (Hesham, *et al*., 2012; Zafra, 2014; Mason, *et al*., 2014; Loviso, *et al*., 2015;). The results of these studies reveals that over the past years, research on the biodegradation of PAHs has changed from conventional microbiological methods to molecular biology strategies aiming to give insight into the effectiveness of bioremediation and the fate of the PAH-degrading microorganisms.

CONCLUSIONS

This review provides an overview of the bioremediation strategies of PAH-polluted soils and the most commonly used molecular biology methods to monitor degrading microbial populations in soils. However, while molecular biology techniques are powerful tools for monitoring bioremediation processes, in recent years these have been evolving into more comprehensive studies aimed at understanding the molecular mechanisms involved in the metabolism of hydrocarbons, and particularly, the interactions among soil microorganisms responsible. As PAH-degrading populations are highly diverse and their taxonomic and functional variation depends on both biotic and abiotic interactions, the molecular analysis of soil microbial communities directly involved in PAH degradation can provide important insights regarding the metabolic pathways and specific enzymes involved in the bioremediation process.

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