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From Functional Potential of Soil Bacterial Communities Towards Petroleum Hydrocarbons Bioremediation

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Abstract
Molecular ecology researches are rapidly advancing the knowledge of microorganisms associated with petroleum hydrocarbon degradation, one of the major large-scale pollutants in terrestrial ecosystems. The design and monitoring of bioremediation techniques for hydrocarbons rely on a thorough understanding of the diversity of enzymes involved in the processes of hydrocarbon degradation and the microbes that harbor their allocated genes. This review describes the impact of hydrocarbon pollution on soil microbial communities, the state of the art of detecting functional genes, and functional groups. We will focus on i) the structure, function and succession behavior of microbial communities exposed to hydrocarbons, ii) key genes and pathways, iii) future prospect into bioremediation of petroleum hydrocarbons in aerobic environments. The aim is to get a fundamental insight in these issues to ultimately improve petroleum hydrocarbons bioremediation.
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1. Introduction

Petroleum hydrocarbon compounds can impact soil and groundwater mainly due to spills during exploration, production and distribution of oil and gas and derived petroleum products or as a result of natural seepage (Das & Chandran 2010). Hydrocarbons include a large array of linear, branched, cyclic and aromatic components (Brown *et al*. 2017) all of which different in terms of volatility, bioavailability, toxicity, degradability, and persistence. Alkanes with carbon chain lengths longer than C_8, for instance, can cause hardening and, as a result, limit wetting of soils (Liu *et al*. 2015). Polycyclic aromatic hydrocarbons (PAHs) are dangerous to human health as they cause mutagenesis and carcinogenesis even at low levels of exposure (Hesham *et al*. 2014). As a consequence, sixteen PAHs have been listed by the US Environmental Protection Agency as priority contaminants in ecosystems (Habe & Omori 2003; Keith & Telliard 1979).

However, biodegradation of hydrocarbons occurs naturally and is found in most types of soils (Pinholt *et al*. 1979; Whitby & Skovhus 2009). In fact, microbes which have the potential to degrade hydrocarbons are widely distributed in the environment (Bamforth & Singleton 2005; Head *et al*. 2006; Whitby & Skovhus 2009; Fukuhara *et al*. 2013; Boon *et al*. 2014) and exposure to hydrocarbons result in changes in the microbial community structure. During this process, genes encoding for a variety of metabolizing enzyme are critical for the ability of microorganisms to biodegrade hydrocarbons (Liu *et al*. 2015).

Technologies based on abilities of certain microorganisms to enzymatically degrade petroleum hydrocarbons can often be very effective for remediation because they are minimally invasive, require little disturbance of soils, are often cost-effective and result in minimal secondary contamination (Balba *et al*. 1998; Das & Chandran 2010; Fuentes *et al*. 2014; Wilson & Jones 1993). Abiotic methods used for the soil remediation such as excavation followed by incineration, soil washing, removal of hydrocarbons by thermal desorption are expensive and can lead to incomplete decomposition of contaminants (Das & Chandran 2010; Morelli *et al*. 2013; Thakur 2014). The most promising and energy efficient process for removing petroleum hydrocarbons from soil environments is aerobic bioremediation that can result in the complete mineralization of hydrocarbons (Bailey *et al*. 1973; Das & Chandran 2010; Fuentes *et al*. 2014).

Despite soil microbial communities having great potential for bioremediation of petroleum hydrocarbons and the considerable amount of literature on microbial hydrocarbon degradation, the scientific community is yet to arrive at a consensus on the key bacteria species and molecular mechanisms involved, and how they can contribute to oil bioremediation in polluted soils. Information on community composition of bacteria, their catabolic genes involved in hydrocarbon degradation along with their mutual interactions will enable a more efficient implementation of remedial strategies that rely on monitored natural attenuation as well as remedies that aim at stimulating natural attenuation through addition of nutrients, electron acceptors, and specific microorganisms (Head *et al*. 2006; Adams *et al*. 2015).

Previous reviews on the microbiology of hydrocarbon degradation initially emphasized i) the environmental factors that contribute to the biodegradation rate (Leahy & Colwell 1990), ii) the diversity of alkane oxygenase

systems (Beilen & Funhoff 2005; Das & Chandran 2010; Varjani 2017), iii) the enzymes for aerobic biodegradation of PAHs and their apparent functional redundancies (Fuentes *et al*. 2014; Head *et al*. 2006; Vila *et al*. 2015), and iv) microbial communities modeling approaches for hydrocarbon degradation (Röling *et al*. 2014a). In spite of all this information, the relationship between a repertoire of genes, encoded enzymes and functional groups existing in a microbial community is still not well understood.

The aim of this review is to organize a guideline of the state of the art of the species composition and genes associated with hydrocarbon degradation in microbial communities, their interactions and succession and, ultimately, how the control of the overall degradation process is divided over different functional groups. To achieve this, three topics will be discussed: i) the succession of soil microbial communities upon hydrocarbon pollution, ii) genes and pathways associated with hydrocarbon biodegradation, iii) future prospect of bioremediation of petroleum hydrocarbons in oxic environments, with an emphasis on soil systems.

2. Succession of soil microbial communities upon hydrocarbon pollution

Degradation of petroleum hydrocarbons generally requires evolution of the existing microbial community to a new organizational state that usually constitute a small number of phylotypes with overall broad enzymatic capacities (Bordenave *et al*. 2007; Hesham *et al*. 2014; Leahy & Colwell 1990). Patel *et al*. (2016) showed that exposure to petroleum hydrocarbons causes a significant reduction on microbial diversity which can be attributed to a combination of hydrocarbon compounds being toxic to some microbes while providing novel types of carbon and free energy sources to other more specialized microbes.

Various researchers have demonstrated that the indigenous microbial communities can adapt within hours, with an increase in hydrocarbon-degrading bacteria along with their hydrocarbon-degrading genes (Atlas & Bartha 1997; Juck *et al*. 2000; Kostka *et al*. 2011; Lindstrom *et al*. 1999; Röling *et al*. 2002). Some of the genes involved in catabolism of hydrocarbons are expressed constitutively (Cappelletti *et al*. 2011; Tani *et al*. 2001). However most of them are ordered in inducible operons located on chromosomes or plasmids. There are three interconnected mechanisms to start biodegradation. These are i) selective enrichment of organisms able to transform the compounds of interest, ii) induction of specific enzymes in these species, and iii) genetic changes which result in new metabolic capabilities and enhanced rates of growth on oil components (Leahy & Colwell 1990; MacNaughton *et al*. 1999; Ogino *et al*. 2001; Röling, Milner, *et al*. 2004). Those mechanisms generate a series of successional changes in the structure of the microbial community (Roy *et al*. 2018). In general terms, *Archaea* are no longer detected because they are usually hydrocarbon sensitive (Röling *et al*. 2004), while growth of alkane-degrading specialists sometimes occurs within hours. Following the depletion of alkanes, certain specialists in aromatic hydrocarbon degradation are able to dominate the bacterial community (Habe & Omori 2003; Head *et al*. 2006; Röling *et al*. 2014a). Thereby, hydrocarbon-utilizing bacteria are able to reflect the degree of contamination of the soil using specific catabolic genes markers (Evans *et al*. 2004; Leahy & Colwell 1990).

The understanding of the microbial-community dynamics during bioremediation is still in its infancy. Because an individual bacterium can metabolize only a limited range of hydrocarbons (Adams *et al*. 2015; Chikere *et al*. 2011; Paniagua-Michel & Fathepure 2018), the breakdown intermediates can be used by the same bacteria or by other members of the community forming part of a microbial degradation network. The microbial interactions during hydrocarbon degradation involves antagonistic interactions such as competition for limiting nutrients (mostly N and P), antibiotic production by competing organisms, and predation by bacteria, protozoa and bacteriophages (Gentry *et al*. 2004). The positive interactions include i) production of biosurfactant that reduce the surface tension and disperse hydrocarbons into small droplets (commensalism), ii) cross-feeding or pathway completion where microorganisms are engaged in exchange of metabolites (mutualism) and iii) horizontal gene transfer reported as one of the major mechanisms responsible for the evolution of enhanced hydrocarbon degradation rates (Obayori & Salam 2010). All those interactions are influenced by the element fluxes of metabolites and nutrients on the one hand and environmental conditions of the spatial-temporal dynamic of the community on the other hand.

The identification of the primary degraders and their catalytic potential as a first critical step in this ecological network is important for understanding, evaluating and developing in situ petroleum hydrocarbons bioremediation strategies.

3. Genes and pathways associated with aerobic hydrocarbon degradation

The initial intracellular attack of hydrocarbons involves the activation as well as incorporation of oxygen in an enzymatic key reaction catalyzed by oxygenases. These are usually monooxygenases for breakdown of aliphatic compounds and dioxygenases for that of aromatic compounds. The general pathway is shown in figure 1. The oxygenases are part of different enzyme systems that are distributed mainly among *Proteobacteria*, *Actinobacteria* and *Firmicutes* (Fuentes *et al*. 2014; Popp *et al*. 2006). Alkanes are highly reduced molecules with single bonds that are less difficult to break down. Homocyclic aromatic compounds on the other hand are

particularly difficult to degrade due to the even distribution of electrons around the aromatic ring which makes them relatively more recalcitrant to reduction or oxidation reactions required for degradation. Thus, hydrocarbons differ in their susceptibility to enzymatic attack according to their chemical reactivity. The easiest ones to degrade are n-alkanes, followed by branched alkanes, low molecular weight aromatic cyclic alkanes and finally high molecular weight aromatics.

3.1 Alkane-degrading enzymes

Monooxygenases can catalyze a variety of reactions including the hydroxylation of linear and branched aliphatic, alicyclic, and alkylaromatic compounds (Ji *et al*. 2013). The aerobic degradation starts by converting alkanes to the corresponding alcohols, aldehydes, carboxylic acids, and acyl-coenzyme A's (CoAs), which then enter the β-oxidation pathway. Based on the type of cofactor and cellular location, five families of bacterial alkane monooxygenase can be distinguished (Coleman *et al*. 2011; Torres Pazmiño *et al*. 2010). These are i) particulate alkane hydroxylases (pAH/AlkB) (Kok *et al*. 1989; van Beilen *et al*. 2002), ii) cytochromes P450 (CYP) (Bernhardt 2006; Maier *et al*. 2001), iii) flavin-dependent monooxygenases (van Berkel *et al*. 2006; Throne-Holst *et al*. 2007), iv) particulate copper-containing alkane monooxygenases (pMMO-family) (Lieberman & Rosenzweig 2004) and v) soluble diiron alkane monooxygenases (sMMO-family) (Leahy *et al*. 2003; Notomista *et al*. 2003). A summary of the key genes, composition of enzymes, substrate preferences and hydrocarbon degraders in soils is shown in Table 1.

3.1.1 Particulate alkane hydroxylases (pAH/AlkB)

The particulate (or membrane-associated) non-heme iron alkane hydroxylases oxidize substrates with chain lengths from C_3 to C_{16} (Beilen *et al*. 2003; Smith *et al*. 2013). They are encoded by three genes, *alkB* for the catalytically active alkane hydroxylase, and *alkG* and *alkT* for rubredoxin and rubredoxin reductase, respectively (Cappelletti *et al*. 2011; Smits *et al*. 2002; Staijen *et al*. 2000). The alkane hydroxylase gene (*alkB*) and its promoter were first identified in *Pseudomonas putida* (Baptist *et al*. 1963) where they were shown to be located on a so-called OCT-plasmid (Kok *et al*. 1989). *As such, the gene cluster* may well be spread amongst community members via horizontal gene transfer (van Beilen *et al*. 2001).

The *alkB* gene sequences contain sufficient conservation for the design of broad-spectrum PCR primers that are used to amplify the *alkB* gene from environmental samples as a functional marker for tracking the abundance and diversity of alkane degrading communities in a variety of polluted soils (Gielnik *et al*. 2019; Kloos *et al*. 2006; Pérez-de-Mora *et al*. 2011; Whyte *et al*. 2002). Those studies showed that richness and diversity of *alkB* genes was higher in polluted soils compared to unpolluted soils.

3.1.2 Cytochrome P450 (CYP)

The soluble cytochrome P450 alkane hydroxylases constitute a super family of ubiquitous heme-thiolate monooxygenases (Das & Chandran 2010). Phylogenetic analyses grouped them in more than 100 families (Rojo 2009), only 10–15% of which are found in bacteria (Beilen & Funhoff 2005). They incorporate oxygen into alkanes with chain lengths from C_5 to C_{16}. Electrons for these reactions are provided by ferredoxins or rubredoxins. Reduction of these iron-sulphur proteins is catalyzed by a ferredoxin reductase that uses NAD(P)H as the initial electron donor.

The first member of this family characterized in bacteria was CYP153A1 from *Acinetobacter sp.* (Maier *et al*. 2001). After that discovery, the abundance and expression of the *CYP* gene in soils was studied in much more detail (Afzal *et al*. 2011; Arslan *et al*. 2014; Kubota *et al*. 2005). Cytochrome P450 alkane hydroxylases have since then been described in members of the *Actinobacteria* such as *Mycobacterium* and *Rhodococcus* as well as in other phyla (van Beilen *et al*. 2005, 2006; Sekine *et al*. 2006). Bacteria with CYP genes have great interest since their enzymes are not only involved in alkane hydroxylation but also in dehalogenation of aromatic compounds. As such, they may be exploited for future biotechnological needs in bioremediation of soils polluted with these kinds of compound.

3.1.3 Flavin-dependent monooxygenases (AlmA and LadA)

Flavin dependent monooxygenases have either flavin mononucleotide (FMN) or flavin adenine dinucleotide (FAD) as cofactor. These enzymes are relevant for the biodegradation of long chain alkanes. In contrast to cytochrome P450, genes encoding flavin-dependent monooxygenases are more abundant in prokaryotic genomes (Torres Pazmiño *et al*. 2010). We will describe two principal enzymes AlmA and LadA, respectively.

AlmA is an integral membrane enzyme involved in the metabolism of alkanes from C_{10} to C_{32}, and longer. The enzyme is a member of the subclass of flavin-containing monooxygenase (FMOs) (van Berkel *et al*. 2006; Ziegler 2002). The *almA* gene was first identified in *Acinetobacter sp.* DSM 17874 (Throne-Holst *et al*. 2007). It was then recognized in many other *Acinetobacter* species capable of degrading long chain alkanes (Wentzel *et al*. 2007) as well as in in marine microbes belonging to the genus *Pseudomonas*, *Alcanivorax, Marinobacter, Acinetobacter, Salinisphaera* and *Parvibaculum* (Liu *et al*. 2011; Wang & Shao 2012). Little is known about the environmental distribution of this gene type in contaminated soils. The only putative AlmA-like oxygenase reported in contaminated soil is from *Pseudomonas aeruginosa* (Liu *et al*. 2014).

LadA is member of a subclass of bacterial luciferases and can degrade alkanes ranging from C_{15} to C_{36} (Feng *et al*. 2007). The LadA homologue from the soil bacteria *Geobacillus thermodenitrificans*, is characterized as a thermophilic extracellular and soluble long-chain alkane monooxygenase. The gene *LadA*, isolated from a deep-subsurface oil reservoir after genome sequencing and confirmed by in vivo and in vitro experiments (Feng *et al*. 2007), shows no detectable similarity to other alkane oxidizing enzymes from soils or other environments. It is the first long-chain n-alkane monooxygenase to be cloned and structurally characterized (Li *et al*. 2008). The crystal structure of LadA reveals a two-component flavin-dependent oxygenase with a large hydrophobic pocket to accommodate the FMN cofactor, O_2, and terminal parts of a long-chain n-alkane in order to produce primary alcohols (Feng *et al*. 2007). This thermophilic LadA is an ideal candidate for treatment of environmental oil pollutions and biosynthesis of complex molecules.

3.1.4 Copper-containing enzymes (pMMOs)

The particulate methane monooxygenase (pMMOs) are found in bacteria that aerobically grow on methane (Torres Pazmiño *et al*. 2010), but these enzymes also act on alkanes from C_2 to C_5 as well as on several other hydrophobic compounds (Elliott *et al*. 1997). An example of these monooxygenases is pMMO from *Methylococcus capsulatus* whose crystal structure shows three subunits α, β, and γ, and three metal-binding centers, two of which are copper centers (mono- and dinuclear, respectively), and the third contains zinc (Balasubramanian *et al*. 2010; Culpepper & Rosenzweig 2012; Kitmitto *et al*. 2005; Lieberman & Rosenzweig 2005; Smith *et al*. 2011). The pMMO gene has been used as a phylogenetic marker for identifying methanotroph-specific DNA sequences in soils (Baani & Liesack 2008; Hoffmann *et al*. 2002; R & J.Colin 1997; Roey & Ralf 2009), but it may identify alkane degrading capacities as well.

3.1.5 Soluble diiron monooxygenases (sMMO)

Another type of MMO is a cytoplasmic diiron monooxygenases (sMMO). They can oxidize alkanes from C_1 to C_8 as well as a broad range of substrates and as such they are relevant for biodegradation of alkanes and mono-aromatic hydrocarbons (Beilen & Funhoff 2007; Green & Dalton 1989). Its crystal structure reveals three components: hydroxylase, reductase and a regulatory protein. The hydroxylase component is a dimer with three subunits namely $(\alpha\beta\gamma)_2$ (Grosse *et al*. 1999; Rosenzweig *et al*. 1993). Structural and functional analysis has revealed that the α-subunit contains the catalytic binuclear iron center that is required for the oxidation reaction (Merkx *et al*. 2001). It turns out that sMMO is less prevalent than pMMO (Rojo 2009). If a strain contains both pMMO and sMMO, expression of sMMO occurs only under conditions of low copper availability (Hakemian & Rosenzweig 2007; Lieberman & Rosenzweig 2004).

3.2 Aromatic hydrocarbons degrading enzymes

The catabolism of aromatic hydrocarbons in bacteria is extremely diverse and complex because of i) the large variety in PAHs (660 reported by (Reed 2011)), ii) the considerable amount of energy required to change an aromatic compound into a nonaromatic and iii) the number of genes and enzymes involved in the complete breakdown of these compounds. For instance, in *Mycobacterium vanbaalenii* PYR-1, the degradation of pyrene needs the sequential action of 27 enzymes (Kim *et al*. 2007). Furthermore, the genetic organization and function of aromatic catabolic genes are still not fully understood since many proposed gene functions need to be assessed experimentally. In addition, the activation of an aromatic hydrocarbon may produce molecules with higher toxicity, leading to persistent products (Lundstedt *et al*. 2003).

Evidence for biodegradation of high-molecular-weight PAHs, containing more than four aromatic rings such as chrysene, benzo[a]pyrene and benz[a]anthracene is still limited. This is in contrast to the bacterial degradation of monoaromatic compounds and low-molecular-weight PAHs (containing two or three aromatic rings) such as naphthalene, acenaphthene, fluorene, phenanthrene and anthracene, since the biochemical pathways for their breakdown have been well studied and reviewed (Fuentes *et al*. 2014; Habe & Omori 2003; Jones *et al*. 2011; Ladino-Orjuela *et al*. 2016; Lu *et al*. 2011; Peng *et al*. 2008; Seo *et al*. 2009; Wick *et al*. 2003). The biodegradation process of these relatively simpler compounds is commonly separated into peripheral and central pathways. Peripheral pathways include the incorporation of molecular oxygen into the aromatic nucleus forming cis-dihydrodiol (Carredano *et al*. 2000; Ferraro *et al*. 2006). These compounds are rearomatized through a cis-diol dehydrogenase to yield dehydroxylated intermediates such as catechol, salicylate, gentisate, homogentisate, protocatechuate or phthalate (Fuentes *et al*. 2014). In the central pathway, the covalent bond of the aromatic ring of these dihydroxylated intermediates are cleaved by enzymes between two adjacent carbon atoms with hydroxyl groups (meta-pathway) or between a carbon with a hydroxyl group and its adjacent carbon with a carboxyl group (ortho-pathway) (Seo *et al*. 2006), which then are further degraded into TCA cycle intermediates (Cerniglia 1992; Habe & Omori 2003). Most of the enzymes necessary in the central pathway can be common between community members.

The incorporation of molecular oxygen into an aromatic hydrocarbon is the most difficult yet important catalytic step (Jouanneau *et al*. 2011). It is catalyzed by a ring hydroxylating oxygenase (RHO) belonging to the family of Rieske non-heme iron oxygenases (Gibson & Parales 2000) that have been studied in many different

microorganisms. RHOs are multi-component enzymes consisting of an oxygenase and an electron transport chain. The oxygenase is the catalytic portion composed of large and small subunits (α and β, respectively) that are either homo- (αn) or hetero-oligomers (αnβn) (Butler & Mason 1997; Kweon *et al*. 2008; Parales *et al*. 1998). The electron transport chain can be a flavoprotein reductase or a flavoprotein reductase and a ferredoxin (Figure 2). The electron transport chain transfers reducing equivalents from NAD(P)H to the oxygenase components. Finally, the oxygenase transfers electrons from the electron donor to break down the aromatic hydrocarbon electron acceptor.

To date, over a thousand of RHOs have been identified and compiled in the GenBank as well as in the ring-hydroxylating oxygenase database (Chakraborty *et al*. 2014). Based on substrate specificities and functional evolutionary behavior of the enzyme components, a classification of RHOs into five type-groups has been proposed (Chakraborty *et al*. 2012; Kweon *et al*. 2008; Nam *et al*. 2001). Type I comprises broad range monooxygenases including monooxygenases like sMMO. The other four types contain typical dioxygenases (RHD). RHOs have low substrate specificity as a common feature (Jouanneau *et al*. 2011), allowing bacteria to initiate the degradation of a wide range of aromatic hydrocarbons. Type II enzymes such as benzoate and toluate dioxygenases activate the catabolism of heterocyclic compounds. Types III, IV and V include enzyme systems for the activation of aromatic hydrocarbons. Figure 3 summarize the pathways proposed for each type.

3.2.1 Type III naphthalene/PAH dioxygenases

Type III enzymes make part of three-component systems that consist of the oxygenase, a [2Fe-2S]-type ferredoxin and a ferredoxin-$NADP^+$ reductase (FNR) typically present in Gram negative bacteria. The three-dimensional structure of the catalytic component of naphthalene dioxygenase of *Pseudomonas sp*. NCIB9816-4 (NDO) has been characterized. It have revealed that NDO oxidizes bi- and tri-cyclic PAH substrates, such as naphthalene, phenanthrene and anthracene (Kauppi *et al*. 1998).

The metabolism of naphthalene has been studied in *Pseudomonas putida* strain G7. In this strains, the transmissible plasmid coding for naphthalene catabolism was isolated (Dunn & Gunsalus 1973). This plasmid contains the naphthalene catabolic genes (*nah*) organized into two operons: upper and lower encoding enzymes for the peripheral and central pathways, respectively. The upper operon *nahABFCED* encodes six enzymes for the conversion of naphthalene into salicylate (Habe & Omori 2003). Enzymes encoded by the lower operon *nahGTHINLOMKJ* transform salicylate via meta-cleavage into pyruvate and acetaldehyde (Simon *et al*. 1993). Both upper and lower operons are regulated by a trans-acting positive control regulator encoded by the *nahR* gene, which is sandwiched by the two operons. NahR is needed for high-level expression of the *nah* genes and their induction by salicylate. In effect, binding of salicylate to NahR at the promoters induces a conformational change in DNA-bound NahR that enables transcription to occurs.

Bacteria with RHD Type III enzymes like *Pseudomonas* are ubiquitous in soil environments particularly after hydrocarbon pollutions (Ahn *et al*. 1999; Lloyd-Jones *et al*. 1999; Martin *et al*. 2013; Tuomi *et al*. 2004; Widada *et al*. 2002). The genetic organization of the naphthalene dioxygenase system present in *P. putida* resembles those in other different *Pseudomonas* strains as well as in other *Proteobacteria* like *Comamonas testeroni* strain GZ42 (Goyal & Zylstra 1996), and *Burkholderia sp*. strain RP007 (Laurie & Lloyd-Jones 1999). Yet other strains like *Ralstonia sp*. U2 and *Polaromonas naphthalenivorans* CJ2 have so-called *nag* genes to convert naphthalene to gentisate rather than the *Pseudomonas*-type *nah* genes where naphthalene is converted in salicylate (Jeon *et al*. 2006; Zhou *et al*. 2001). A third group of species, like *Alcaligenes faecalis* AFK2 and *Acidovorax* NA3, have *phn* genes to degrade phenanthrene through protocatechuate with some unique accessory genes (Singleton *et al*. 2009).

3.2.2 Type IV benzene/toluene/biphenyl dioxygenases

The type IV enzymes make part of yet another three-component system that consist of an oxygenase, a [2Fe-2S]-type ferredoxin and a glutathione reductase (GR). This type is the largest group for the known RHD enzyme systems that includes the biphenyl, toluene and PAH metabolic enzymes from both Gram negative and positive bacteria (Takeda *et al*. 1998). The three dimensional structure of the components of biphenyl dioxygenase from *Sphingobium yanoikuyae* B1 was characterized (Ferraro *et al*. 2007; Yu *et al*. 2007). Later, also those from *Sphingomonas* CHY-1 (Jakoncic *et al*. 2007) and *Rhodococcus sp.* RHA1 were determined (Furusawa *et al*. 2004). The biphenyl dioxygenase system (BDO) is able to oxidize a broad range of substrates, ranging from one to four-ring PAHs (Ferraro *et al*. 2007; Kimura *et al*. 1996; Yu *et al*. 2007).

The biphenyl biodegradation pathway by the enzymes encoded from the genes *bph* has been studied in *Pseudomonas pseudoalcaligenes* KF707 (Furukawa & Miyazaki 1986) and *Burkholderia xenovorans* LB400 (Mondello 1989). Typically, the genes *bph* are located in two clusters. The first cluster called *bphABCD* is common in many aerobic biphenyl degraders and consists of seven genes. Enzymes encoded by this operon are responsible for the transformation of biphenyl into benzoates and aliphatic acids (biphenyl upper pathway). The second cluster of genes is responsible for metabolism of benzoates and aliphatic acids into pyruvate and acetaldehyde via the catechol pathway (Seeger & Pieper 2010), which can then be further oxidized via the TCA cycle (biphenyl lower pathway). Besides, the biodegradation of monocyclic aromatic hydrocarbon initiated by

RHD like toluene dioxygenase (TDO) in general follow the similar upper and lower pathways described in this functional group including the catechol pathway.

The diversity and versatility of RHD Type IV enzymes were assessed from different soils contaminated with BTEX and PAH (Cárcer *et al*. 2007; Furukawa *et al*. 2004; Seeger & Pieper 2010). Furthermore, key enzymes in the common pathway, such as catechol 1,2- dioxygenase and catechol 2,3-dioxygenase, were used for detecting biphenyl and toluene metabolic enzymes in soils (Mesarch *et al*. 2000).

Similar genetic organization of the biphenyl dioxygenase genes described in *Burkholderia xenovorans* LB400 appear in *Ralstonia eutropha* H850 (Bedard *et al*. 1987), *Achromobacter xylosoxydans* KF701 (Furukawa *et al*. 1989) *Pseudomonas putida* KF715 (Hayase *et al*. 1990) and *Burkholderia sp.* LB400 (Bartels *et al*. 1999). Those results could suggest that certain *bph* gene clusters were transferred among soil bacteria and have evolved from a common ancestor.

On the other hand, a heterogeneous genetic organization of the biphenyl dioxygenase system is present in *Sphingomonads* (that includes the genera *Sphingomonas, Novosphingobium, Sphingopyxis and Sphingobium*) which are able to degrade a wide range of PAHs such as naphthalene, fluorine and phenanthrene because of a large substrate-binding pocket. In *Sphingomonads* the catalytic genes for breakdown of aromatic hydrocarbons are often localized separately. There is no evident biochemical function of functional operons. All the *Sphingomonads* genes for degradation of aromatic hydrocarbons are carried on a single plasmid (pNL1) and they encode α and β subunits of seven distinct RHOs (Jouanneau *et al*. 2011; Romine *et al*. 1999). All these RHOs may receive their electrons from a single set of ferredoxin and ferredoxin reductase. This gives *Sphingomonads* flexibility and means to adapt easily to new environmental conditions since their RHOs can catalyze the oxidation of many types of aromatic hydrocarbons and simultaneously share the same electron transport chain (Jouanneau *et al*. 2011; Khara *et al*. 2014). Therefore, *Sphingomonads* evolved as an independent subgroup with a possible restriction of gene transfer to other bacteria.

3.2.3 Type V phthalate/phenanthrene dioxygenases

Type V enzymes make part of yet other three-component systems that consist of a dioxygenase, a [3Fe-4S]-type ferredoxin and of a glutathione reductase (GR), typically found in Gram positive bacteria. The first well-known example in this group is phenanthrene dioxygenase (encoded by the *phdABCD* operon) from *Nocardioides sp*. KP7 that transforms phenanthrene to 1-hydroxy-2-naphthoate (Saito *et al*. 1999). Later on, PhdC was shown to be a novel type of [3Fe-4s]-type ferredoxin as typical electron carrier present in *Mycobacterium* and *Rhodococcus* strains (Kweon *et al*. 2008).

The ability of the genus *Mycobacterium* to degrade different types of high molecular weight PAHs, including pyrene, fluoranthene, and benzo[a]pyrene has been documented (Cheung & Kinkle 2001; Kim *et al*. 2005; Schneider *et al*. 1996). In *Mycobacterium* PYR-1, the genes encoding the hydroxylase component of a pyrene dioxygenase were first called *nidBA* (nid for naphthalene induced) (Khan *et al*., 2001). Based on system biology analysis of genomic and proteomic data, 27 enzymes were identified to be necessary for constructing a complete pathway for pyrene degradation to central intermediates through o-phthalate and the β-ketoadipate pathway in *M. vanbaalenii* PYR-1, (Kim *et al*. 2007). In another pyrene degrader *Mycobacterium* 6PY1, a thorough proteomic analysis of pyrene- and phenanthrene-induced polypeptides identified 23 proteins, four of which were subunits of two gram-positive RHDs named Pdo1 and Pdo2 (Krivobok *et al*. 2003). Amino acid sequence comparison indicated that Pdo1 and Pdo2 are similar to the pyrene dioxygenase from strain PYR-1, and phenanthrene dioxygenase from strain KP7, respectively (Krivobok *et al*., 2003). Additionally, Other *Mycobacterium* strains able to degrade pyrene or fluoranthene were found to express RHDs homologous to Pdo1 or Pdo2 (Kim *et al*. 2005; Roey & Ralf 2009; Seo *et al*. 2006).

Multiple pathways of pyrene degradation operate in *Mycobacterium vanbaalenii* PYR 1 strain (Brezna *et al*. 2006; Heitkamp & Cerniglia 1988). The general pathway of pyrene degradation is dioxygenation at the 4,5-positions to produce *trans*-4,5-pyrenedihydrodiol. Rearomatization of the dihydrodiol and subsequent *ortho*-cleavage lead to the formation of 4,5-dicarboxyphenanthrene, which is further decarboxylated to 4-phenanthroate. Following another dioxygenation reaction, 4-phenanthroate forms *cis*-3,4-dihydroxyphenanthrene-4-carboxylate. Rearomatization of the metabolite yields 3,4-dihydroxyphenanthrene, which is further metabolized to 1-hydroxy-2-naphthoate. The subsequent enzymatic reactions, including intradiol ring cleavage dioxygenation, result in the production of *o*-phthalate. Then phthalate is further metabolized via the β-ketoadipate pathway and via the TCA cycle, successively (Kim *et al*. 2003; Wang *et al*. 2000).

RHD genes from *Mycobacterium* and *Rhodococcus* were reported in soil environments by (Debruyn *et al*. 2009; Hall *et al*. 2005; Marcos *et al*. 2009; Peng *et al*. 2010; Ren *et al*. 2015; Shahsavari *et al*. 2016). Therefore, the abundance of *nidA* gene serves as a biomarker for pyrene and other PAH degradation processes. The prevalence of *Mycobacterium nidA* genotypes corroborated previous studies indicating that PAH-degrading Mycobacteria have a cosmopolitan distribution and suggests they play an important role in natural attenuation of PAHs.

3.3 Conclusion

Although none of the enzyme systems is completely specific, a broad correlation between the grouping in alkanes and aromatics-degrading enzymes subfamilies and the native substrates oxidized by the subfamily members can be perceived. It is unlikely that PCR primers that were used to genotype particular environments will reliably cover the huge diversity of homologous genes within each sub-group. Besides the first catalytic steps that lead the degradation pathways, the complete metabolism of hydrocarbons depends upon the collective activities of peripheral and central pathway enzymes. We may then conclude that diversity of hydrocarbons-degrading genes and enzymes is unexpectedly high, which may support the hypothesis that natural attenuation of petroleum hydrocarbons occurs widely in nature by functional redundancies in enzymes that overlap in substrates and metabolites. The full range of genes involved, their genetic regulation and organization as well as the interaction of all those enzymes in the microbial degradation network is still far from being understood. Therefore, the application of omics approaches is crucial to study the functional potential of microbial communities that we are going to discuss in the next section.

4. Future prospect into bioremediation of petroleum hydrocarbons in soils

Traditional analytical chemistry and molecular tools have identified individual functional genes, metabolic pathways and key players. For example, culturable bacteria such as *Pseudomonas, Sphingobium* and *Acinetobacter* have been isolated and provide suitable, tractable systems to perform controlled biodegradation experiments (Röling *et al*. 2014a). Genome and transcriptome sequencing of these organisms have been carried out. Databases such as GenBank and Uniprot support sequencing query and are applied for prediction of gene function of unknown key-players. However, the catabolic machinery of a complex microbial community is still far from being completely understood. The recent applications of cultivation-independent methods such as molecular meta-omics technologies (e.g. meta-barcoding, genomics, transcriptomics, proteomics and environmental metabolomics) can provide a deeper insight into the active microbial processes during hydrocarbon degradation in soils. The implementation of culture-independent methods enables further exploration of the environmental diversity of PAH-degrading bacteria as well as of alkane-degrading enzymes and RHOs.

4.1 Metabarcoding and metagenomics

Next-generation sequencing of DNA has revolutionized the study of microbial communities. Metabarcoding, a technology based on the amplicon sequencing of 16S rRNA gene, enabled studying the structure and diversity of microbial communities and may create predictive models in hydrocarbon contaminated soils (Fowler *et al*. 2016; Jiao *et al*. 2016; Yang *et al*. 2016). The output of metabarcoding analysis is a table containing the relative abundance of each Operational Taxonomic Unit (OTU), which represent species abundances (16S reads) in a community sample. Using statistical and network analysis-based tools, it is possible to compute the correlation coefficients among species abundances (Ma *et al*. 2016). In this way we may identify bacterial interactions (based on correlation) that possibly drive hydrocarbon degradation. This technology can also be applied to specific catabolic genes markers in a targeted functional gene analysis.

Metagenomics, a technology based on shotgun sequencing, allows the study of total genomes from a microbial community. The assemblage of functional groups is essential to predict for example pathways that mediate key reactions in the hydrocarbon biodegradation process (Duarte *et al*. 2017; Sharpton 2014). Metabarcoding and metagenomics analyses can be combined with targeted functional gene analyses to characterize the metabolic potential of uncultured organisms. This new technology uses labelled ^{13}C hydrocarbons that can be assimilated into cellular biomass (e. g. heavy DNA) of hydrocarbon-degrading bacteria. The resulting labeled DNA is amplified by PCR using stable isotope probing (DNA-SIP) for further metabarcoding or metagenomics sequencing to retrieve the genomes of uncultured hydrocarbon degraders (Grob *et al*. 2015). Similar SIP based technologies are starting to be applied to characterize RNA (Lueders 2015) and proteins (Bergen *et al*. 2013).

4.2 Metatranscriptomics

A limitation of genetic profiling of hydrocarbon degradation is that genes can be present but not expressed. Metagenomics coupled with metatranscriptomics allows elucidation of the active metabolic processes by quantification of gene expression in an existing microbial community. Only a few metatranscriptomics studies have been applied in hydrocarbon degradation in soils and sediments so far (Gonzalez *et al*. 2015; de Menezes *et al*. 2012; Reid *et al*. 2018). The studies show increases in RHO gene expression and widespread changes in the microbial community structure of soils contaminated with phenanthrene. (Gonzalez *et al*. 2015) found *Bacillus* and *Klebsiella* in greater abundance in trees cultivated on hydrocarbon contaminated soil. (Reid *et al*. 2018) observed abundance of alkane-degrading and RHO gene expression that suggest considerable natural degradation in hydrocarbon rich sediments. Although most of the dominant species were unclassified, which

revealed novel lineages of microbes potentially involved in hydrocarbon degradation. Furthermore, highly expressed sequences in their metatranscriptomic data encode proteins with still unknown function. In effect, metatranscriptomics requires mapping of expressed sequences to reference genomes but if absent they remain unexplored and vice versa (González *et al*. 2016; Hanson *et al*. 2009). Therefore, it is better to focus on the complete metatranscriptome of an environmental sample with the hypothesis that they serve as genetic fingerprints for contamination by hydrocarbons (Pérez-Pantoja & Tamames 2015). Consequently, processing data collected from metatranscriptomics studies may well lead to prediction of novel genes important for the degradation of hydrocarbons.

4.3 Metaproteomics

While proteomics has been stablished as a robust and reliable technique to study the functional network in single organisms, metaproteomics can be used to understand complex community interactions associated with in situ bioremediation of soil, but it is still in its infancy (Siggins *et al*. 2012). Metaproteomics analyses involves sample preparation, high throughput mass spectrometry analysis and bioinformatics data processing (Christie-Oleza *et al*. 2015). Current bottlenecks for this technology applied to contaminated soils include: the lack of complete genome data for all the bacteria, the difficulty in assigning peptides that are highly conserved, the complexity of the peptide mixture, the size of mass spectrometer data generated, and the purification of proteins from contaminated soils that contain interfering humic acids. As a result, there is a small subset of the diversity of proteins expected in soils (Williams *et al*. 2010). Metaproteomics in combination with protein-stable isotope probing revealed enzymes for the naphthalene degradation pathway in laboratory microcosm that fail in situ (Herbst *et al*. 2013).

4.4 Environmental metabolomics

In contrast to proteomics, metabolomics focuses on identification of metabolites and serve to predict enzymatic activity in an individual organism. These findings may then be extrapolated to environmental metabolomics at the community level to study the functional potential of soils. The microbial degradation of a complex hydrocarbon mixtures generates mixture of oxygenated metabolites which are relatively difficult to detect. The identification of hydrocarbons metabolites require gas chromatography coupled with mass spectrometry and can be theoretically divided in targeted and untargeted approaches (Bonifay *et al*. 2016). Targeted analyses focus on a specific small number of compounds that are diagnostic for a particular pathway and the original substrate can be identified by the initial activation of the hydrocarbon by the oxygenase. In contrast, an untargeted metabolic analysis qualitatively measures generally unknown metabolites that result from all catabolic pathways in the microbial community (Bargiela *et al*. 2015). Unraveling such a metabolic fingerprint aimed at the identification of combined catabolic pathways is challenging and may not even be practically achievable.

4.5 Systems biology modelling approaches

Mathematical approaches are needed to get a more fundamental understanding of how microbial communities function (Succurro & Ebenhöh 2018; Wade *et al*. 2016; Xiao *et al*. 2017). Systems biology can integrate information on genes, transcripts, proteins, and interacting biological networks in single cells. This approach may be extended to populations and communities in order to characterize a community phenotype. Figure 4 is a scheme that connects different omics approaches in hydrocarbon degradation the integration of which is carried out with modelling approaches. For example, Ecological Regulation Analysis (ERA), is a systems biology approach that quantifies fluxes through a microbial network at the level of individual species or functional groups. Data on cells numbers of species and fluxes through those cells (e.g. substrate consumption, product formation or respiration) in at least two different (near) steady-state conditions enables the calculation of regulation coefficients of each individual species or functional groups (Röling *et al*. 2014b).

Metabolic flux models investigate the functional and microbial capacity either in top-down or in bottom-up approaches. The top-down approach focuses on ecological significances of microbial community, where the species provide the building blocks for the model. They include interaction of species and metabolites, but they lack details at the gene level. Conversely, the bottom-up approach focuses on metabolic pathways and biochemical interactions of the entire community. Those models may provide a high level of prediction, but its complexity is extremely challenging.

Final remarks

Although decades of microbial research into hydrocarbon degradation processes have generated a comprehensive overview of which hydrocarbons can be biodegraded and which bacteria and genes are involved, there are still many gaps in our understanding about the main drivers for degradation of hydrocarbons by the microbial communities. Despite novel emerging technologies, it is still difficult to develop a comprehensive understanding on sites contaminated by hydrocarbons whether which microbes are essential and how to enhance

the biodegradation.

Based on the present review, it may be concluded that the microbial communities play a critical role in the cleaning up process of oil spills in soils. In order to effectively monitor, manage or stimulate a remedial strategy that relies on biodegradation three aspects remain essential: i) to reveal the dynamics and interaction of the indigenous microbial communities, ii) to detect functional metabolic capabilities to monitor and optimize bioremediation and iii) to provide appropriate environmental conditions. Consequently, further research is required to enhance these new technologies to elucidate the metabolic potential and interaction between non-culturable bacteria specialist in hydrocarbon degradation.

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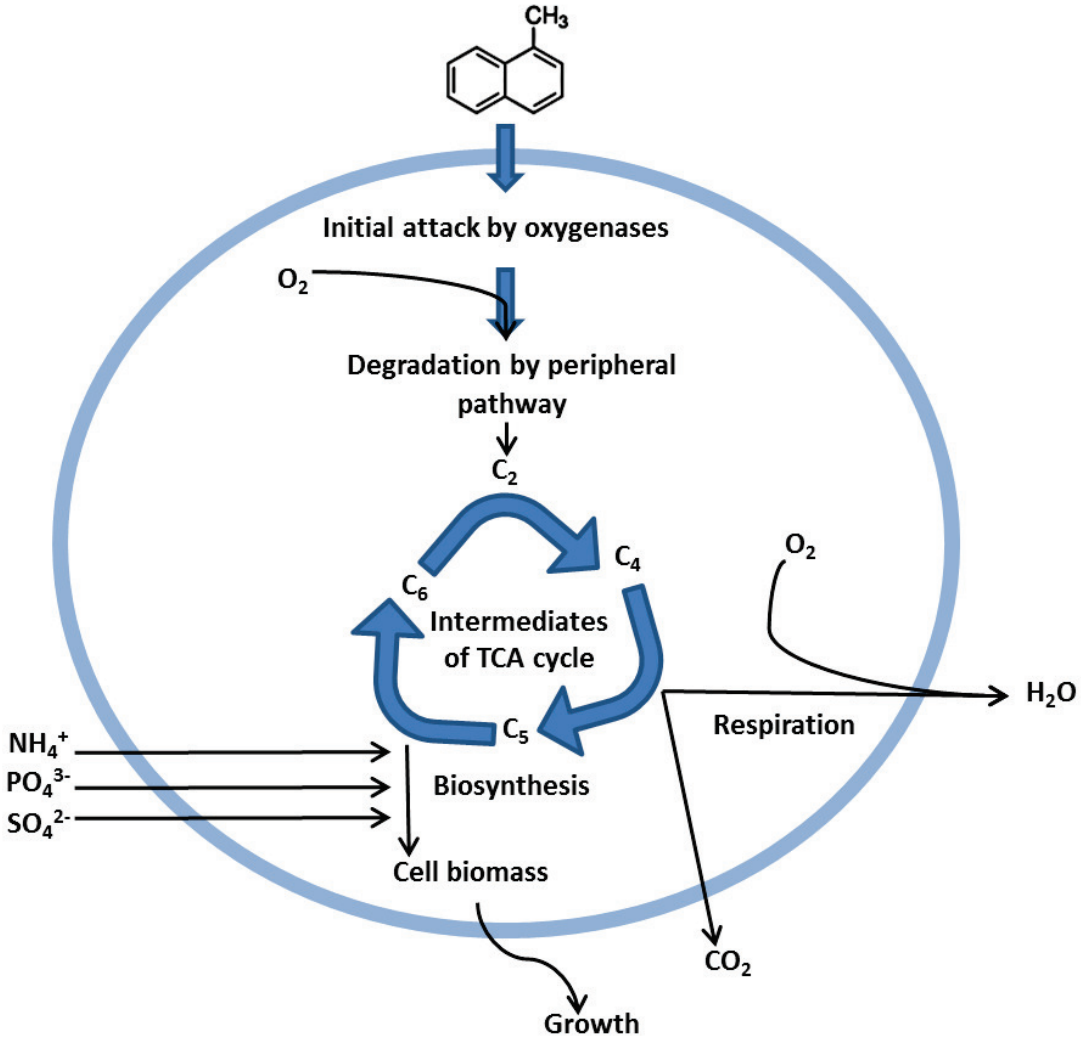

Figure 1. Principle of aerobic degradation of hydrocarbons for growth associated processes. Adapted from Fritsche & Hofrichter (2000)

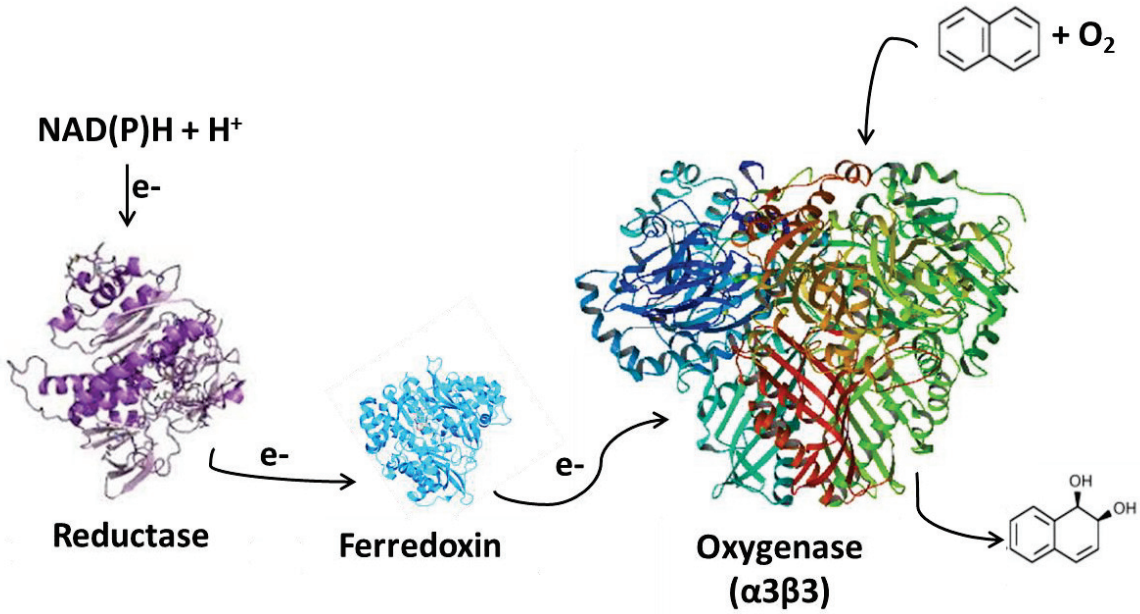

Figure 2. Composition of and electron flow within a Rieske non-heme iron oxygenases. Adapted from Chakraborty *et al*. (2014).

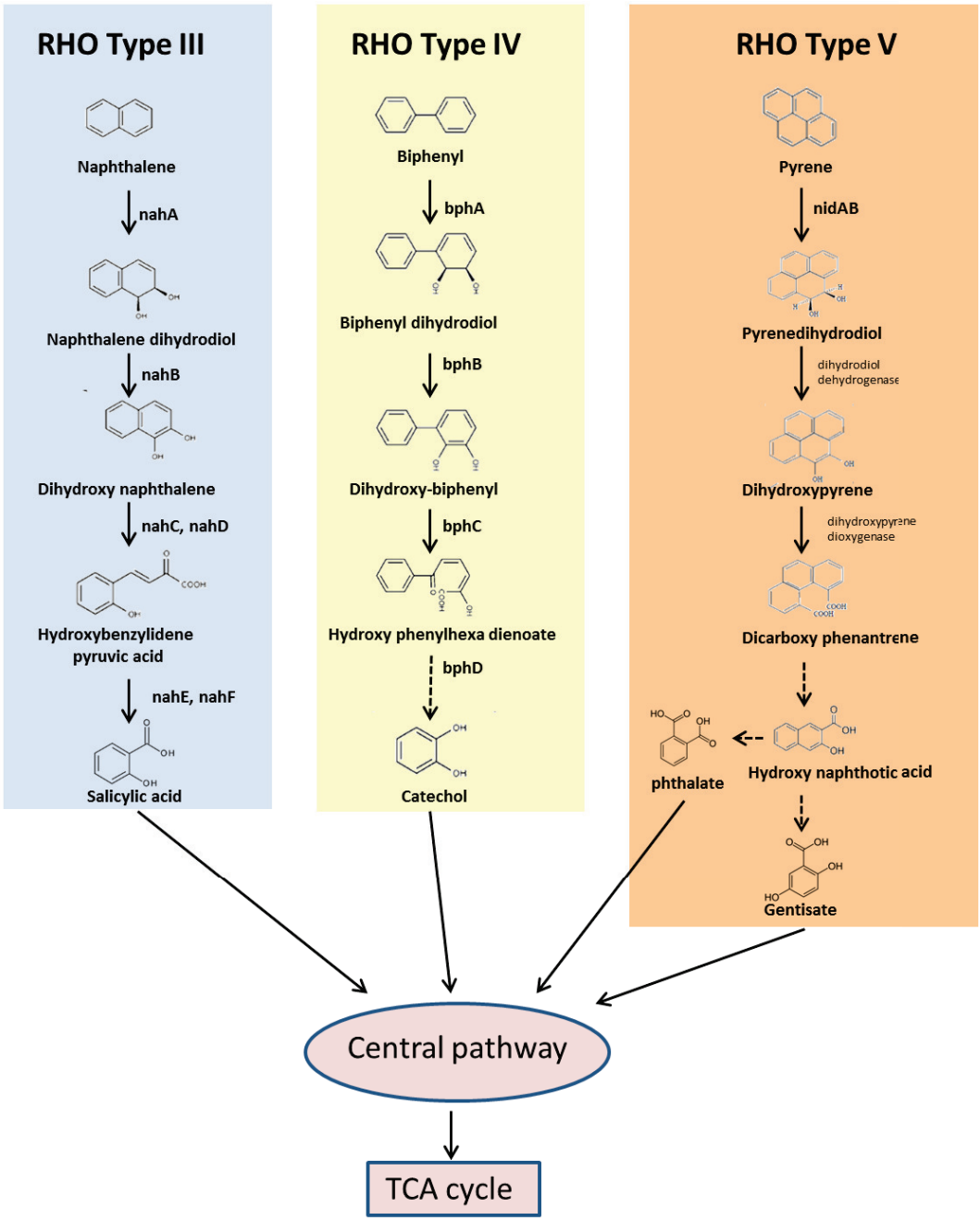

Figure 3. Pathways attributed to each sub family of ring hydroxylating oxygenase (RHO) for aromatic hydrocarbon degradation. i) RHO type III genes are part of operon *nahABFCED* that encodes six enzymes for the conversion of naphthalene into salicylic acid in *Pseudomonas putida* G. ii) RHO type IV genes are part of the *bphABCD* operon that encodes seven enzymes to transform biphenyl into catechol in *Burkholderia xenovorans* LB400. iii) RHO type V genes are present in several complex gene clusters that encode enzymes to break down pyrene into phthalate or gentisate in *Mycobacterium* PYR-1.

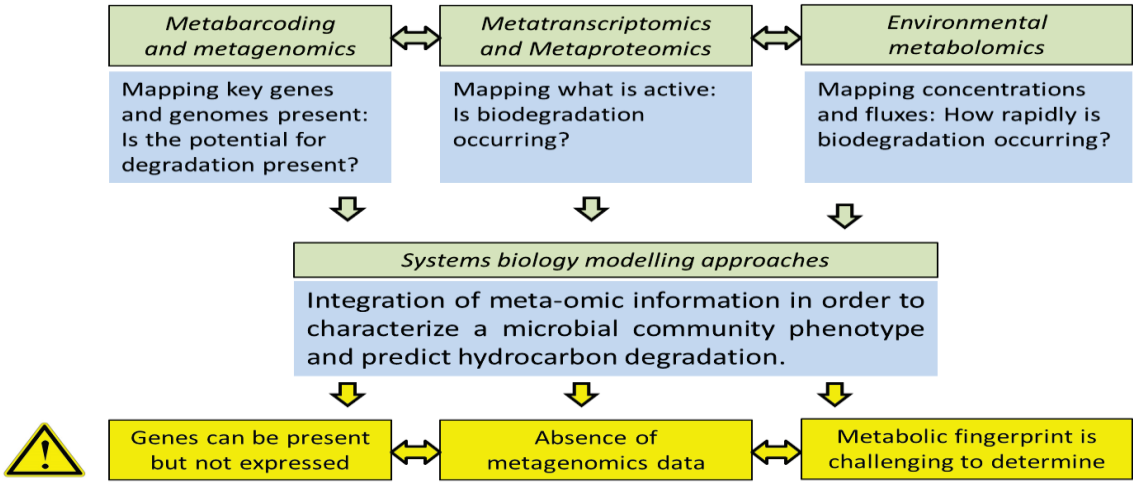

Figure 4. Flow chart of recent technologies for bioremediation of petroleum hydrocarbons and principal limitations.

Table 1. Alkane hydroxylases in microbial communities

Enzyme system	Composition and cofactors	Substrate range	Reference
Particulate alkane hydroxylases (pAH/AlkB)	Membrane hydroxylase: binuclear iron Rubredoxin: iron Rubredoxin reductase: FAD, NADH	C_3 to C_{16}	*Pseudomonas putida* (Baptist *et al*. 1963) *Acinetobacter* (Ratajczak *et al*. 1998) *Alcanivorax, Burkholderia, Mycobacterium, Pseudomonas and Rhodococcus* (Beilen *et al*. 2003).
Cytochrome P450 (CYP)	P450 oxygenase: P450 heme ferredoxin: iron–sulfur ferredoxin reductase: FAD, NADH	C_5 to C_{16}	*Acinetobacter sp.* (Maier *et al*. 2001) *Mycobacterium* (van Beilen *et al*. 2005) *Rhodococcus* (Sekine *et al*. 2006)
Flavin-dependent monooxygenases (almA)	αβ external monooxygenase FAD NAD(P)H	C_{22} to C_{36}	*Acinetobacter sp.* (Wentzel *et al*. 2007) *Alcanivorax*, *Marinobacter*, *Salinisphaera* & *Parvibaculum*, (Wang & Shao 2012). *Pseudomonas aeruginosa* (Liu *et al*. 2014)
Flavin-dependent monooxygenases (ladA)	αβ external oxygenase FMN / NAD(P)H	C_{15} to C_{36}	*Geobacillus thermodenitrificans* (Wang *et al*. 2006)
Copper-containing monooxygenases (pMMO)	αβγ hydroxylase; copper, iron Quinone reductase: FAD, NADH	C_1 to C_5	*Methylococcus, Methylosinus, Methylocystis, Methylobacter, Methylomonas, Methylomicrobium* (McDonald *et al*. 2006) *Methylocystis echinoides* (Pieja *et al*. 2011)
Soluble diiron monooxygenases (sMMO)	α2β2γ2 hydroxylase; binuclear iron reductase, [2Fe–2S], FAD, NADH regulatory subunit	C_1 to C_8	*M. capsulatus* (Bath) (Green & Dalton 1989) *Methylocystis* sp. strain M (Nakajima *et al*. 1992), and *M. trichosporium* OB3b (Fox *et al*. 1989)

Table 2. Aromatic hydrocarbon degrading dioxygenases

Enzyme system	Composition and cofactors	Substrate range	Strain, gene and reference
Type III naphthalene dioxygenase and PAH dioxygenases	α3β3 oxygenase FNR_N-type reductase [2Fe-2S]-type ferredoxin	Low-MW PAH (naphthalene, phenanthrene and anthracene)	*Pseudomonas sp*. NCIB9816-4 (*ndo*) (Kauppi *et al*. 1998) *Pseudomonas putida* G7 (*nah*) (Simon *et al*. 1993) *Comamonas testeroni* GZ42 (*phn)* (Goyal & Zylstra 1996) *Burkholderia sp*. RP007, (*phn)* (Laurie & Lloyd-Jones 1999) *Ralstonia sp*. U2 (*nag)* (Zhou *et al*. 2001) *Polaromonas naphthalenivorans* CJ2 (*nag)* (Jeon *et al*. 2006) *Alcaligenes faecalis* AFK2 (*phn)* (Kiyohara *et al*. 1982) *Acidovorax* NA3 (*phn)* (Singleton *et al*. 2009).
Type IV toluene dioxygenase and biphenyl dioxygenase	α3β3 oxygenase GR-type reductase [2Fe-2S]-type ferredoxin	BTEX, biphenyl, naphthalene, fluorene, phenanthrene and pyrene	*Pseudomonas putida* Fl (tol) (Zylstra & Gibson 1989) *Pseudomonas pseudoalcaligenes* KF707*(bph)* (Furukawa & Miyazaki 1986) *Burkholderia xenovorans.* LB400 *(bph)* (Mondello 1989) *Rhodococcus sp.* RHA1 *(bph)* (Furusawa *et al*. 2004) *Sphingobium yanoikuyae* B1 *(bph)* (Ferraro *et al*. 2007) *Sphingomonas* CHY-1*(bph)* (Jakoncic *et al*. 2007) *Novosphingobium pentaromativorans sp. nov.*(*bph*) (Sohn *et al*. 2004)
Type V phenanthrene and phthalate dioxygenase	α3β3 oxygenase, GR-type reductase [3Fe-4S]-type ferredoxin	phenanthrene and high-MW PAH	*Nocardioides sp*. KP7 (*phd*) (Saito *et al*. 1999) *Mycobacterium vanbaalenii* PYR-1 (*pht*) (Kim *et al*. 2007) *Mycobacterium sp.* (*pht*) 6PY1 (Krivobok *et al*. 2003) *Terrabacter sp.* strain DBF63 (*pht*) (Habe *et al*. 2003) *Rhodococcus sp.* UW1 (*pht*) (Walter *et al*. 1991)

Glossary

Alkanes. Saturated hydrocarbons, they can be linear (n-alkanes), cyclic (cyclo-alkanes) or branched (iso-alkanes) (Wentzel *et al*. 2007)

Biodegradation. Process by which organic substances are broken down by enzymes produced by living microbial organisms (mainly by aerobic bacteria but also reported under anaerobic conditions) into simpler substances such as carbon dioxide, water and ammonia (OECD, 1997).

Hydroxylases. Group of enzymes that catalyze oxidation reactions in which one of the two atoms of molecular oxygen is incorporated into the substrate and the other is used to oxidize NADH or NADPH (Massart & Vercauteren 1959).

Metatranscriptomics. Science that studies gene expression profiling of complex microbial communities within natural environments (Aguiar-Pulido *et al*. 2016).

Metabolomics. Science of chemical processes involving metabolites, the small molecule intermediates and products of metabolism in a biological system (Aguiar-Pulido *et al*. 2016).

Microbial communities. Multi-species assemblages, in which organisms live together in a contiguous environment and interact with each other (Boon *et al*. 2014; Konopka 2009).

Microbial bioremediation. Technology treatment of naturally occurring organisms and their products in

different biotechnological applications to break down hazardous substances into less toxic or non-toxic substances (Vitorino & Bessa 2017)

Petroleum hydrocarbons. Organic compounds from oil that contain only carbon and hydrogen (Atlas, 1981).

PAHs. Polycyclic aromatic hydrocarbons are a group of chemicals that contain two or more fused aromatic rings in linear, angular, or cluster arrangements (Cerniglia 1992).

Proteomics. Large-scale study of proteins in a biological system aimed at understanding of gene function (Pandey & Mann 2000).

RHO and RHD. Rieske, non-heme iron-type aromatic ring-hydroxylating oxygenases are multicomponent enzymes that catalyze the insertion of molecular oxygen into benzene rings, a common first step in the bacterial degradation of aromatic compounds. They can be monooxygenases (RHO) or dioxygenases (RHD) when one or both atoms of dioxygen are inserted into the substrate, respectively (Ferraro *et al*. 2005).