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Challenges with bioaugmentation and field-scale application of bioremediation processes for petroleum-contaminated sites: A review

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ABSTRACT

Bioremediation is a promising method for cleaning up sites contaminated with petroleum hydrocarbons (PHCs), with bioaugmentation being a common strategy that involves the use of microbial consortia to treat these sites. However, using a single bacterium is not effective in treating PHC contamination due to the range of compounds present. The use of different strains as a consortium can improve treatment, but bioaugmentation often fails due to various factors, especially in large-scale applications. The number and survival of introduced microorganisms are critical during bioremediation, and immobilization by using carrier materials such as biochar can protect added microorganisms from adverse impacts. Advanced composting methods can also be effective in treating petroleum-polluted sites in commercial-scale applications. By adjusting environmental factors and adding compost, native microorganisms can be biostimulated. This review aims to critically analyze the challenges associated with bioaugmentation and field-scale applications of bioremediation for petroleum-contaminated sites, specifically identifying key variables that impact the success of bioremediation efforts and evaluating the effectiveness of different bioremediation strategies in field conditions. The review addresses the primary factors contributing to the failure of bioaugmentation in large-scale applications, how the survival and activity of introduced microbial consortia can be enhanced in contaminated environments, and the most effective strategies for field-scale bioremediation of petroleum-contaminated sites. The strategies discussed are evaluated based on their ability to enhance microbial survival, their practicality in large-scale applications, their environmental impact, and their overall effectiveness in reducing PHC levels. By providing a comprehensive analysis of these aspects, this review aims to offer insights into optimizing bioremediation processes for field-scale applications.

Keywords: Petroleum hydrocarbons, Bioaugmentation challenges, Biotic and abiotic factors, Biochar immobilization, Advanced composting methods

1. Introduction

Various natural resources are contaminated with petroleum hydrocarbons (PHCs) due to accidents, leaks, and spills that occur during crude oil exploration, production, well drilling, transport, and storage, resulting in the release of significant amounts of these materials into the environment (Fetisov et al., 2023). The pollution of the environment by petroleum compounds is a global issue that poses a serious threat to all living organisms (Kumari et al., 2023). Additionally, these substances are now classified as emerging priority contaminants (Caetano et al., 2023). Petroleum products can be broadly categorized into several types, including: crude oil, gasoline, diesel fuel, kerosene, liquefied petroleum gas (LPG), asphalt and bitumen, paraffin wax, and petroleum coke (Alfares, 2023). Polycyclic aromatic hydrocarbons (PAHs) are an example of compounds that can cause moderate to high acute toxicity effects in aquatic and terrestrial organisms. Exposure to these substances can lead to serious health problems, such as lung cancer and issues related to the reproductive system and immunity, as well as an increased risk of tumor development (Udom et al., 2023).

Bioremediation is a green, economical and environmentally benign method for treatment of contaminated soil or sludge and it can mineralize pollutants to the end products: CO_2 and H_2O as compared with other chemically-based techniques leaving toxic secondary by-products (Koolivand et al., 2017; Mishra et al., 2001b; Samarghandi et al., 2018; Shahsavari et al., 2017; Yarahmadi et al., 2016). One of the most widely used bioremediation processes is bioaugmentation, in which acclimatized microorganisms capable of degrading petroleum hydrocarbons are inoculated to purify polluted sites. Although these microorganisms may be well adapted to the target contaminants, the results have often been unsatisfying, particularly in field-scale applications (Fernández-Luqueño et al., 2011). Several significant factors can lead to microbial inoculation failure, including the absence of appropriate strains that are well-suited to the environment, poor selection of strains, inadequate survival and tolerance of the strains, insufficient population size of the strain consortium during bioremediation, and other related factors. The crucial aspect of any bioremediation strategy is scaling up from the pilot phase to the field (Frutos et al., 2012). The difficulties encountered during field-scale tests, which include changes in groundwater table height, the presence of free-phase petroleum hydrocarbons, and other factors, indicate the necessity of conducting a pilot-scale test in a 3D soil tank to verify the effectiveness of the treatment before proceeding with a full field trial. This highlights the significance of comprehending the factors that influence biodegradation at different scales and taking into account environmental fluctuations to use laboratory-scale data effectively in the field (Miri et al., 2023). A major question is: why does not the bioaugmentation process mainly reach a sufficient efficiency in treating polluted sites containing high quantities of petroleum products, especially in actual-scale applications? Furthermore, what reasonable strategies exhibiting an appropriate performance can be utilized? Therefore, the prime objective of this review is to discuss the variables causing failures in microbial inoculation for the treatment of petroleum-contaminated sites and suggest solutions for overcoming the problems. Moreover, since there are few articles, in which a bioremediation process has been used to clean up actual environments, we argue the issues associated with large-scale utilization of bioremediation strategies. Further, environmental factors effective in bioremediation of petroleum products are tackled.

2. Scaling up bioaugmentation: overcoming challenges for effective large-scale applications

It seems that there is no direct correlation between laboratory-scale and field-scale applications when it comes to addressing petroleum compound pollution. Interestingly, smaller-scale approaches have been found to result in a much greater amount of biodegradation of these compounds compared to larger-scale trials. For example, in small-scale conditions, phenanthrene, dibenzothiophene, and n-alkanes were effectively treated in a short period of time. However, this level of success was not observed in field trials of the same process (Röling et al., 2004); one reason for this insufficient performance in large-scale applications is the lack of having controlled conditions like temperature. Undoubtedly, a strategy of bioremediation may be entirely unsuccessful when it is applied in actual environments as it is expected to see various changes like high salt and heavy metal levels, or TPH pollution over 10%, which inhibit microbial activity, in sites polluted with PHCs (Huesemann, 1994).

In order to improve the bioremediation rate of PHCs in field practices, particularly in the case of highly polluted sites, air sparging (biosparging), by which air is injected to all polluted strata, is a feasible and innovative strategy; in a project conducted on an actual site contaminated, it was found that there is a close relationship between biodegradation rate and air sparging and, in turn, 75% of pollution was treated (Machackova et al., 2012); apparently, oxygenation through air sparging can act as a biostimulation factor (Machackova et al., 2012) as oxygen is a main electron acceptor in bioreactions and petroleum compounds act as the electron donor (Li et al., 2004). Of course, before starting a bioremediation process, the most important step is the measurement and adjustment of nutrient contents. Also, unlike small-scale conditions, in large-scale approaches, the low diffusion of oxygen, substrates and nutrients in inner layers of contaminated soil is a big issue, thereby declining microbial activity and bioremediation rate (Scow and Hutson, 1992).

In actual petroleum-polluted sites with cold weather, lower temperature leads to lower microbial activity; therefore, besides aeration, other influencing factors should be closely optimized (Thomassin-Lacroix et al., 2002). Naturally, covering biopiles can contribute to keep heat. In addition, construction of a greenhouse can effectively minimize the inhibitory impacts of temperature changes (Ko et al., 2007). For example, it was reported that, in comparison with uncovered places, temperatures in greenhouse conditions were approximately 10 °C more, which caused the degradation efficiency of oil and grease to increase sharply (Liu et al., 2009).

Another challenge with the large-scale utilization of the bioaugmentation process for the clean-up of contaminated sites is the lack of sufficient indigenous microorganisms, particularly when aeration as a biostimulating factor is not conducted. Furthermore, it has been documented that the petroleum-degrading microorganisms make up only approximately 10% of all flora exist in soil (Cerniglia, 1993; Simarro et al., 2013). In this state, over bioremediation processes, inoculation is required; also the survival of microorganisms added, as a consortium, is of primary importance. Regular reinoculation, every 30 d, is a good solution (Beškoski et al., 2011) because the population number of microorganisms decline on account of low survival and, in addition this, after a while the bacterial community is aged. In the case of freshly polluted sites, there are usually lower numbers of microorganisms and hence the biodiversity of native microorganisms is not high (Lladó et al., 2013). Moreover, when pollutants have a toxic impact on the native microorganisms, the bioaugmentation of the polluted site with allochthonous microorganisms may be considered as a necessity (Polyak et al., 2018). On the other hand, in the case of historically polluted soils, bioavailability of pollutants is a limiting factor (Lladó et al., 2013); in these conditions, sole inoculation does not work and the application of mobilizing agents like surfactants and biosurfactants should be considered to boost the solubility of petroleum substances (Cameotra and Bollag, 2003; Lladó et al., 2013).

In order to scale up bioremediation strategies, the following parameters should be carefully taken into account: spatial distribution of sorbed hydrocarbons and microbial count in soil matrix, soil field moisture level, diffusion and the sorption of pollutants, amount and availability of nutrients and oxygen, pH, and soil type (Ko et al., 2007). To support this claim, it was found that a greater proportion of PHCs is treated when the amount of sand in polluted soil is high and, in this case, the lab-scale experiment can be better scaled up (Khan et al., 2015). Also, a few parameters like volatilization, off-site migration, sorption onto soil particles and abiotic transformation cause the performance of bioremediation processes to decline in the field (Sturman et al., 1995).

Two other major challenges with inoculation are adaptation and how to deliver appropriate microorganisms to the desired sites. In order to resolve these problems, the use of bulking agents, surfactants, foams and adapted strains resistant to adhesion may be considered according to local situations (Mrozik and Piotrowska-Seget, 2010). Furthermore, spatial heterogeneities and the presence of competing microorganisms are the two main obstacles, resulting in a failure in large-scale bioremediation processes (Liu et al., 2008; Sturman et al., 1995).

3. Application of biochar in immobilization of PHCs

Agricultural fertilizers or amendments like biochar, cow dungs, chicken excrement, rice husk, peat, sewage sludge and corncob powder can enhance bioremediation processes for large-scale sites polluted with PHCs by maintaining adequate moisture, aeration, and pH levels (Brown et al., 2017). Biochar, in particular, has gained attention as a carbon-based substance derived from the pyrolysis of biomass. It serves as an excellent carrier material for nutrients and microbial inoculants due to its significant pore volume and surface area, which create an ideal environment for microorganisms and enhance soil water retention (Bolan et al., 2023). Additionally, biochar can have a beneficial impact on microbial growth by raising the soil pH, promoting better aeration, and retaining nutrients. Furthermore, biochar is useful for cleaning up soils that have been contaminated with environmental pollutants because it can either immobilize the pollutants directly within the soil or indirectly influence soil metabolic activities to break them down. The observations of a study indicated that the combination of bioaugmentation and biochar was notably more effective than individual treatments. This approach resulted in over 20% diesel degradation in both scenarios within twelve weeks. Additionally, there was an observed increase in bacterial diversity during this period. Thus, the synergy between biochar and bioaugmentation points to a highly efficient and eco-friendly bioremediation process (Guirado et al., 2021). Immobilization, which restricts the mobility of microbial cells and their enzymes while preserving their biodegradation capabilities, is a promising method to enhance the survival of oil-degrading microorganisms (Dzionek et al., 2016; Guzik et al., 2014; Kourkoutas et al., 2004). Among the four primary immobilization techniques—adsorption, binding on a surface, entrapment in a porous matrix, and encapsulation—the latter two are more practical for large-scale applications due to their use of inexpensive, readily available natural materials like plant fibers, sugarcane bagasse, sawdust, corncob, expanded perlite, tezontle, and alginate. It should be noted that the main upside of immobilization is the protection of microorganisms from adverse factors; this lowers bioremediation time because of decreased lag phase of bacteria. Furthermore, encapsulation of bacteria can protect them from adverse factors and, besides, bioremediation time lessens because of the decreased lag phase of bacteria. To our best knowledge, most of immobilization studies are culture- and bioreactor-based experiments (Hazaimeh et al., 2014; Obuekwe and Al-Muttawa, 2001). More studies are needed to investigate well the application of immobilization measures in large-scale trials. Therefore, at first, all key parameters effective in large-scale applications should be well studied and then optimized.

Biochar immobilization microbe (BIM) is a promising remediation strategy that involves impregnating contaminant-degrading microorganisms with biochar to enhance the mass transfer of contaminants to the degrading microbial community and enrich degrading bacteria to form biofilms. The porous structure of biochar provides space for the growth and reproduction of microorganisms, and can improve soil fertility and biological community composition. BIM can be useful in treating contaminated soils and increasing biosorption by reducing the inhibition of excessive pollutant concentrations on the growth of microorganisms (Wu et al., 2022). The effectiveness of biochar in sorbing pollutants depends on its properties, such as porosity, aromaticity, basicity, specific surface area, and surface functional groups, as well as those of the organic pollutants and soil. Increasing the pyrolysis temperature increases the surface area and the number of micropores of biochar, which improves the sorption of organic pollutants by pore filling and hydrophobic interaction. BIM can face challenges such as operational complexity, biochar stability, environmental complexity, mass transfer constraints from biochar to immobilized microbes, and the formation of thick biofilms that can block pores. Although BIM is a promising approach, it is not without exceptions (Wu et al., 2022). Various methods are used for immobilizing microorganisms by means of biochar, including adsorption, entrapping, covalent bonding, and crosslinking. The most commonly used methods for preparing biocatalyst immobilization matrices are adsorption and entrapping. It should be pointed out that the interactions between microorganisms and substrates are weak and unstable, and microorganisms can easily detach from the fixed matrix. Adsorption is a popular immobilization technique due to its low cost, ease of operation, and minimal impact on cell activity. Thus, adsorption is best suited for immobilizing living cell organisms that require minimal contact with the immobilization matrix. While the fixed matrix of adsorption can be reused, the technique's weak interactions make it less suitable for long-term stabilization of microorganisms. Biochar has been shown to regulate bacterial community dynamics in composting, particularly in the thermophilic and maturation stages. The primary contributors to the bacterial communities were Firmicutes, Bacteroidota, and Proteobacteria, with the genera Solibacillus, Fermentimonas, and Taibaiella being particularly important. The addition of 7.5% and 10% biochar was found to provide a nutrient-rich substrate and facilitate the formation of more complex co-occurrence networks among various bacterial communities, leading to greater stability and environmental resistance. The addition of biochar was also found to stimulate bacterial activity, enhance resource cascades, and improve the interaction and stability of bacterial communities (Duan et al., 2023). Studies suggest that biochar is suitable for enhancing the effectiveness and shelf life of microbial inoculants in soil. Lignocellulosic biomass-derived biochar has increased surface area and porosity that are beneficial for housing soil microbes. However, biochars from non-lignocellulosic biomass can have high levels of volatile organic matter and environmentally persistent free radicals that can be toxic to soil microbiota. The feedstock type and pyrolysis temperature can also affect the properties of the resulting biochar, which can impact its effectiveness as a soil amendment and inoculant carrier (Wu et al., 2022; Xiang et al., 2022). Also, biochar modification can significantly enhance its effectiveness in hydrocarbon bioremediation by altering its properties, such as surface area, pH, and nutrient content. Although various modification methods have been extensively reviewed, the impact on hydrocarbon removal is rarely reported, except for studies involving microorganism-immobilized biochar (Dike et al., 2022). For instance, biochar modified with NaOH showed a 28% higher phenanthrene dissipation compared to unmodified biochar, likely due to improved microbial community structure and higher bioavailable nitrogen (Ding et al., 2021). The modification also increased the abundance of certain hydrocarbon-degrading bacteria, making it more effective in bioremediation (Dike et al., 2022).

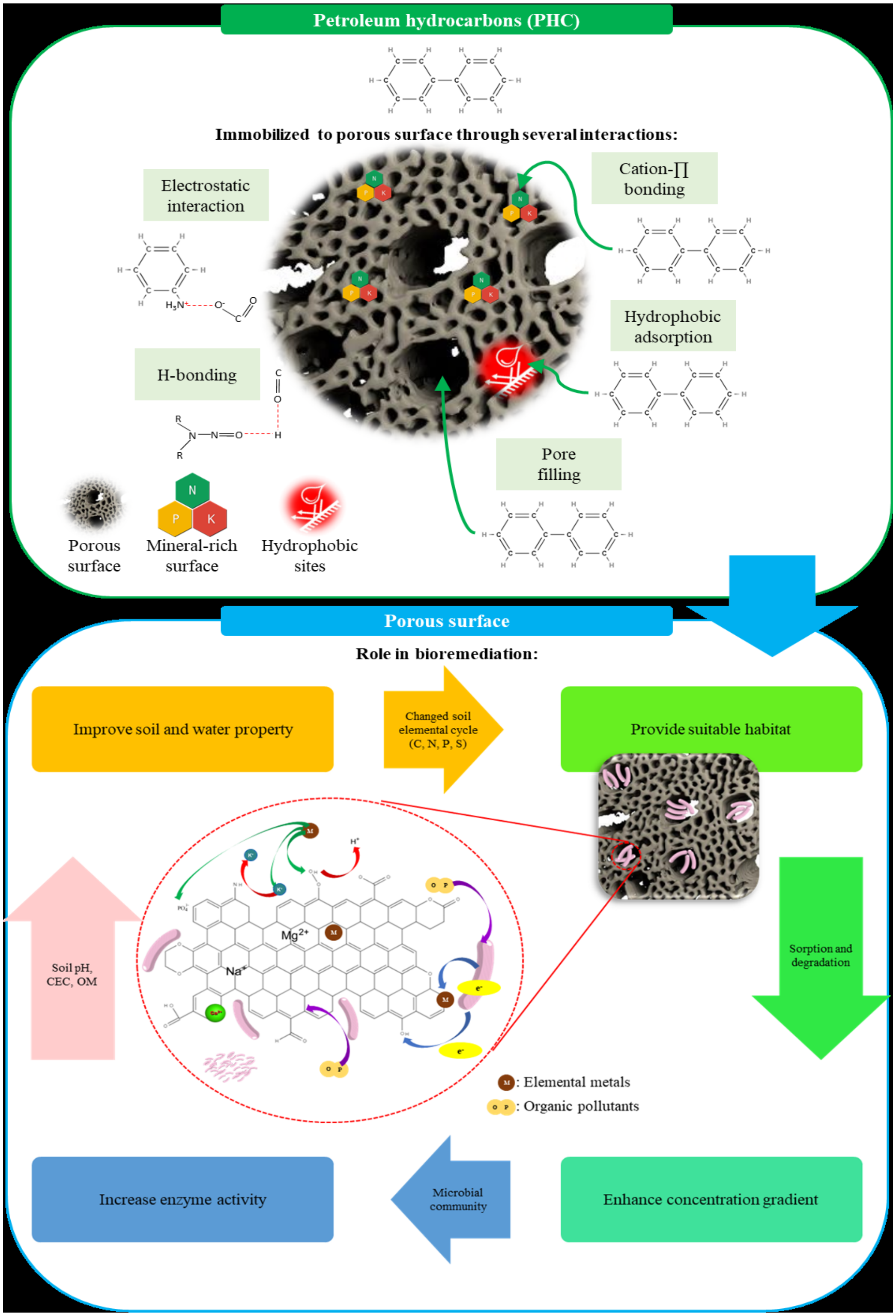

Figure 1: The suggested immobilization methods by means of biochar (Wu et al., 2022; Xiang et al., 2022)

4. Factors affecting PHC bioremediation in bioaugmentation process

4.1. Biotic factors: challenges with microbial inoculation and strain selection

PHs are comprised of various compounds which cannot be degraded by a single microorganism (Raju et al., 2017). Moreover, when the application of bioaugmentation is inevitable, the use of a mixture of multiple genera, which are capable of decomposing PHCs, is of the greatest importance; for instance, in a study it was stated that *Bacillus*. *cereus* ACE4 could degrade effectively n-alkanes (C10-C20). In addition, other researchers have reported that *Bacillus* strains can also degrade crude oil and oily sludge (Ijah and Antai, 2003; Verma et al., 2006) and they can survive even in harsh environment containing 30-40% of hydrocarbons (Ijah and Antai, 2003). Thus, the application of *Bacillus* strains in a consortium is nearly essential even though their capacity in removal of PHCs should be tested in laboratory.

This issue is very challenging in natural contaminated sites as they are wholly heterogenic and there are many polluters; this necessitates utilizing a microbial consortium caring a diverse substrate of substrates used as carbon and energy sources by which co-oxidation and commensalism can happen in such environment (Gojgic-Cvijovic et al., 2012). Besides, it has synergistic and co-metabolism characteristics and increases the adaptation and survival of microorganisms (Gojgic-Cvijovic et al., 2012; Mishra et al., 2001a; Rahman et al., 2003). At this stage, the selection of potent strains to be used in a consortium is very important. It should be noted that the bacterial strains affiliated to the Gamma subclass of the *Proteobacteria* and *Firmicutes* can generate catabolic enzymes beneficial in biodegradation of PHCs and they can also adapt in different extreme environments such as oil sludge or highly contaminated soils (Nnamchi et al., 2006) (Table 1). Some of petroleum-degrading microorganisms are as follows: *Alcaligenes*, *Acinetobacter*, *Arthrobacter*, *Bacillus*, *Citrobacter*, *Micrococcus*, *Pseudomonas*, *Rhodococcus*, *Serratia* and so forth (Raju et al., 2017) (Table 1); all of these bacteria have been isolated and identified from petroleum-contaminated environments because they have the trait of growing in harsh environments and degrading petroleum hydrocarbons (Godini et al., 2018; Raju et al., 2017). Furthermore, it has been documented that these strains have the ability to adapt via coincidental induction of different catabolic pathways, resulting in gaining new ways for biodegradation of various contaminants (Ornston and Yeh, 1982). For instance, Whyte et al. stated that a transfer of biodegradative pathways between mesophiles and psychrotrophs in nature may be the cause of the similar ALK pathways (C5-C12 n-alkanes) of mesophilic *P. oleovorans* and two hydrocarbon-degrading psychrotrophic *Pseudomonas* spp. strains, purified from crude oil-polluted Arctic soil (Whyte et al., 1996). Another important consideration that should be given in the issue of factory-scale applications of bioremediation processes is the survival of microorganisms used in consortium. Indigenous microbial communities often have a diverse range of metabolic pathways that can degrade a wide variety of contaminants. Introduced strains in bioaugmentation may lack this diversity, limiting their effectiveness (Adams et al., 2015). As mentioned above, one of the most important issues in bioaugmentation is to use sufficiently-adapted microorganisms to improve the survival of added microorganisms and, if microorganisms are not adapted to the polluted soil, a pre-adaptation should be performed before the bioremediation application (Megharaj et al., 2011). In a field-scale process, *Comamomonas testosteroni*, *Bacillus* sp., *Pseudomonas alcaligenes*, *Sphingomonas paucimobilis* and *Achromobacter xylosoxidans* were added to the contaminated site as a consortium, it was found that only *Bacillus* sp. survived (Poi et al., 2017). Also, Ghazali et al. used two consortia: consortium 1 (*Pseudomonas aeruginosa* strains S4.1 and S5 3 and *Bacillus* sp. Strain S3.2) and consortium 2 (consortium 1 plus *Bacillus* sp. strains 113i and O63 and *Micrococcus* sp. strain S) to purify diesel-polluted soil; they reported that the second consortium could better degrade alkanes because it contained more strains of *Bacillus* strains capable of extending bioremediation, thereby increasing bioremediation rate (Ghazali et al., 2004). Here, a method for the selection of bacteria able to degrade effectively PHCs is vital; the selective enrichment method has been introduced as a powerful way to purify hydrocarbon-degrading microorganisms (Thompson et al., 2005). In this strategy, petroleum compounds are used as the only sources of carbon and energy for microorganisms (Godini et al., 2018). In this situation, only bacteria that can grow in culture containing high levels of these pollutants and use them as the only carbon and energy sources are isolated. The isolates can be used for augmenting the polluted site.

In bioaugmentation, a big challenge is the number of introduced microorganisms because in a study it was reported that, when the number of bacteria is less than 10^2 CFU ml^{-1}, the degradation rate declined dramatically (Gojgic-Cvijovic et al., 2012; Ruberto et al., 2010). Furthermore, for a potent land farming, the count of microbial population to treat petroleum hydrocarbons should be at least 10^3 CFU/g of polluted soil (Manual, 2004). In a field-scale work, it was explained that the number of indigenous hydrocarbon-degrading bacteria ranging between 10^3 and 10^4 CFU/g of soil is still low for bioremediation (Mishra et al., 2001a). Also, Mishra et al. claimed that bioremediation of TPHs is very low if the number of microorganisms able to degrade petroleum compounds is less than 10^5 CFU/g (Mishra et al., 2001b). In a study, approximately 82% of TPHs was degraded by using a consortium with the population number of 10^8 CFU/g during a 141-day period (Grace Liu et al., 2011). Microorganisms capable of degrading contaminants are mainly present in the environment (particularly in soils, sediments and aquifers) and biotreatment often take places, but the removal efficiency is very low because of the lack of the sufficient population of microbial degraders (Helmy et al., 2015); in this state, all agents inhibiting the microorganisms to reach a suitable growth rate should be investigated. For example, when the number of microorganisms is less than 10^3, it may mean that there are toxic organic or inorganic compounds at a concentration that has an inhibitory impact on the growth and activity of bacteria (Manual, 2004). In order to overcome this problem and lessen the concentration of growth inhibitors, the polluted soil can be mixed with clean soil.

It is imperative that well-adapted microorganisms be utilized as a consortium for beneficial biodegradation, because exogenous strains or new species used as a consortium may compete with natural microorganisms or may be predated by protozoa and bacteriophages (Mrozik and Piotrowska-Seget, 2010). It should be pointed out that, when the population number of indigenous microorganisms is at the suitable range, the employment of bioaugmentation is nearly illogical (Ruberto et al., 2010). Moreover, one drawback is that it has been reported that less than 1% of microorganisms found in nature can be grown in a lab. Previous research has shown that microbes that are good at breaking down pollutants in a controlled lab setting may not perform as well in larger-scale bioremediation efforts. Su et al. also found evidence of bacteria that are alive but cannot be cultured in polluted environments. As a result, efforts have been made to identify and select "culturable" organisms that can work well with non-culturable communities at the site needing remediation (Xiang et al., 2022). More importantly, many researchers have stated that in most cases the addition of new species is a waste of time and money (Jørgensen et al., 2000; Neralla and Weaver, 1997; Poi et al., 2017), as the influence of inoculation can be hindered by both abiotic and biotic factors (El Fantroussi and Agathos, 2005). In a study, it was claimed that abiotic factors are more effective than biotic factors, because when the soil samples was sterilized, a dramatic decrease was seen in the number of added strains (Ruberto et al., 2010). Thus, it is concluded that ecological variables far outweigh inoculation. Nonetheless, biotic factors should not be ignored in the application of bioremediation processes.

Table 1. An overview of some field- and pilot-scale application of bioremediation process

Bioremediation Type & microbial consortium	**Pollutant**	**Scale**	**Findings**	**Reference**
22 adapted bacterial strain including *Bacillus* sp., *Pseudomonas* sp., *Arthrobacter* sp., *Acinetobacter* sp., *Alcaligenes* sp., *Brevibacillus* sp.	TPHs	Field	Reduction of concentrations from 26240, 622657 and 978399 mg/kg to under 1000 mg/kg	(Poi et al., 2017)
Two species of *Bacillus*, *B. thuringiensis* B3 and *B. cereus* B6 (isolated from crude oil-polluted sites)	Diesel, crude oil and spent lubricating oil	Field	28-84% reduction	(Raju et al., 2017)
Five strains: two diesel-degrading strains (*Gordonia alkanivorans* CC-JG39 and *Rhodococcus erythropolis* isolated, respectively, from a gas station oil storage tank and a campus soil sample) and three strains of fuel-oil decomposer (*Acinetobacter junii* CC-FH2, *Exiguobacterium aurantiacum* CC-LSH-4, and *Serratia marcescens* KH1 isolated from an ex-situ oil bioremediation site)	TPHs	Batch experiments	More than 80%	(Grace Liu et al., 2011)
Zymogenous microorganisms	Mazut	Ex-situ field	Nearly 95%	(Beškoski et al., 2011)
Acinetobacter baumannii (S19, S26, S30), *Burkholderia cepacia* (P20), and *Pseudomonas* sp. (S24). A carrier-based bed for the strains was prepared.	TPHs	Field	More than 90%	(Mishra et al., 2001b)
Biostimulation (indigenous microorganisms)	C10-C25 n-alkane components	Pilot	60%	(Kahraman et al., 2017)
Biostimulation (indigenous microorganisms)	TPHs	Field (large scale landfarming)	53%	(Brown et al., 2017)
Two hydrocarbonoclastic bacteria, *Pseudomonas* spp. (4M12) and *Pseudomonas xanthomarina* under biostimulation-bioaugmentation treatment	Crude oil	Pilot	96-97%	(Mansur et al., 2016)
Bacillus cereus BL01, *Pseudomonas stutzeri* BL02 and *Acinetobacter* sp. BL03 and *Bacillus* sp. BL04	Aged petroleum oil	Field	Reduction of 46 g TPH per Kg (initial TPHs concentration: 320 g/kg)	(Helmy et al., 2015)

Bioaugmentation/biostimulation of indigenous microflora	TPHs	Field	Reduction of pollutants from 196 to lower than 10 mg/kg of soil	(Kinoshita et al., 2001)
Biostimulation via water hyacinth compost	Crude oil	Pilot	93%	(Udume et al., 2023)

4.2. Abiotic factors

4.2.1. Sources of nitrogen, phosphors and potassium

An important issue in biodegradation of PHCs is co-metabolism as compounds like aromatic hydrocarbons and asphaltene and resin are less biodegraded than n-alkanes and, at the second stage of the process, bacteria require other sources of carbon saturated hydrocarbons and so forth, which are biodegraded easier, to be able to grow in soil or sludge and decompose these pollutants; therefore, the addition of new sources of carbon may be essential (Li et al., 2002). For this purpose, Vasconcelos et al. used raw glycerol as a co-substrate for bioremediation of aromatic hydrocarbons and they reported that the results were successful (Vasconcelos et al., 2013). Furthermore, compost has been shown as another satisfying source of co-substrate; it is also rich in N and P, mesophilic and thermophilic bacteria and lignin-degrading fungi (Taccari et al., 2012). Additionally, when compost, which is high humic materials in content, was used as a biostimulating factor, hydrophobic organic components are desorbed more from soil, leading to higher rates of PHC bioremediation (Taccari et al., 2012). Accordingly, Rezaei Kalantary et al. demonstrated that humic compounds can decrease the bond between the soil and phenanthrene and then increases the bioavailability of the pollutant (Kalantary et al., 2013). In the study by Koolivand et al. employing a two-stage composting process: windrow-in-vessel composting, it was claimed that the system used could remove TPH pollution as high as 95%; the main reason lies behind this may be the addition of immature compost in both phases that this improves the microbial populations, particularly in the second stage in which there is a decrease in microbial growth and then the rate of co-metabolism (Koolivand et al., 2017). However, in industrial approaches the application of compost may be limited because it may be comprised of pathogens or other pollutants; in this case, the disadvantages of using compost may outweigh the advantages (Gentry et al., 2004). Fava et al. investigated the impact of the addition of biogenic materials: soya lecithin or humic materials (at 1.5% w/w), utilized as an easier degradable carbon source by native soil microorganisms, in biological treatment of aged contaminated soil with PAHs; they found that these materials dramatically increased PAH bioavailability in soil (Fava et al., 2004). These natural materials have similar results in comparison with commonly used chemical surfactants employed in large-scale and they are cheaper as well (Fava et al., 2004). In general, the addition of nitrogen and phosphors is of the greatest importance and should be considered carefully because petroleum products are often high in carbon and, accordingly, low in these nutrients (Fallgren et al., 2010; Kahraman et al., 2017; Margesin et al., 2007; Ronen et al., 2000). Roy et al. reported that biostimulation with both N and P had the highest biodegradation rate in oil refinery sludge and this process led to an enhancement in the abundance of fermentative, sulfate-reducing, hydrocarbon degrading, syntrophic and methanogenic populations (Roy et al., 2018).

When there are higher amounts of nutrients in environment, the growth of bacteria and other microorganisms involved in bioremediation is adversely affected because of high ionic conditions (Zappi et al., 1999). More importantly, the selection of a suitable source of nitrogen is entirely important owing to its biostimulative effects on bacteria. Ammonium salts, nitrate salts and urea have widely been used to provide the required content of N (Kahraman et al., 2017). The results of this study confirmed that, among the nitrogen sources studied, optimum nitrogen source for biodegradation of n-alkane components in diesel fuel contaminated soil was $(NH_4)2SO_4$. Since urea changes the pH value of soil or sludge, its application has been limited (Kahraman et al., 2017). Also, Na_3PO_4 has been introduced as a good source for providing phosphors (Kinoshita et al., 2001). It should be noted that the ratio of C/N/P should be adjusted at the optimum level over the bioremediation process; the US EPA suggested 100/10/1 for biopile measures and 100/15/1 for ex-situ processes (Manual, 2004). A very important point to make here is that, in addition to the C_{TPH}/N/P ratio, the C/N/P ratio should be closely taken into account during all periods of bioremediation, particularly at the second stage. What's more, Beškoski et al. used a modified C/N/P ratio (C/N/P/K) with considering the level of potassium; they concluded that the optimum ratio was 100/10/1/0.1 for ex-situ biodegradation of a place polluted with heavy residual fuel oil (Beškoski et al., 2011). Rezaei Kalantary et al., who studied the performance of an adapted bacterial consortium in phenanthrene removal, found the following order of some elements: N > K > P > Cl > Na > Mg (Kalantary et al., 2014). Of course, only the adjustment of C/N/P ratio at the appropriate level is not sufficient in the case of bioremediation of industrial polluted sites with PHCs because, if there are higher amounts of carbon sources in the environment, they may be targeted and preferred by the microorganism, leading to a decrease in biodegradation rate. Thus, the kinds of hydrocarbons and content of easier biodegradable carbon sources should be examined in laboratory and suitable levels of them should be applied in large-scale experiments.

4.2.2. Soil moisture

Soil moisture is one of the most important environmental parameters affecting the bioremediation process. It has been demonstrated that the highest microbial activity occurs at moisture content levels between 50 and 80% (Silva-Castro et al., 2016). Another study suggests that the optimal moisture content ranges from 40 to 85% of the soil's water-holding capacity (field capacity), or approximately 12 to 30% by weight (Manual, 2004). Water-holding capacity, a characteristic dependent on soil gradation and structure, significantly influences microbial activity and the bioremediation rate (Beškoski et al., 2011). This capacity also affects other soil properties that are influential in biodegradation, potentially having synergistic or diminishing impacts. For instance, appropriate moisture levels can enhance the availability of nutrients and surfactants for microorganisms (Chang et al., 2013). Optimal soil moisture levels facilitate microbial activity by providing the necessary water for microbial metabolism and nutrient transport. Microbes thrive in moist environments where they can access nutrients and degrade contaminants more effectively (Cho et al., 2000). Adequate moisture helps dissolve nutrients and surfactants, making them more accessible to microorganisms. This enhances the biodegradation process as microbes can efficiently utilize these nutrients for growth and contaminant degradation (Alori et al., 2022). Soil moisture influences the oxygen availability in the soil. In well-aerated soils with optimal moisture, oxygen can easily diffuse, supporting aerobic microbial processes essential for the degradation of many contaminants (Alkorta et al., 2017). Some kinds of soil have complex porous channels and dead-end pores capable of trapping water; in this case, microbes are prevented from drying and they have longer survival and tolerate the impacts of toxic materials in environment (Chang et al., 2013). For example, Ronen et al. (Ronen et al., 2000) studied the survival of *Achromobacter piechaundii* TBPZ and decomposition of tribromophenol; the findings showed that the biodegradation declined when the water content was 10%. Also, it has been reported that water potential is very important in bacterial survival (Mrozik and Piotrowska-Seget, 2010). On the other hand, in the state of excessive soil moisture, soil areas are saturated with water and air cannot move through the subsurface of soil; in this case, anoxic conditions are dominant in soil and oxygen, which is also necessary for aerobic bacterial metabolic processes, cannot reach bacteria (Kinoshita et al., 2001; Manual, 2004). So as to stimulate bioremediation, drainage can be a good solution (Kinoshita et al., 2001).

4.2.3. Soil properties

Soil properties significantly influence bacterial activity and the overall rate of bioremediation, making it crucial to consider these properties in the context of specific soil compositions and pollutant types. For instance, while high clay content generally reduces bioremediation efficiency due to decreased oxygen diffusion and microbial activity (Ko et al., 2007; Korda et al., 1997; Mueller et al., 1991); the extent of this impact can vary depending on the specific pollutants involved and the presence of other soil components. In soils contaminated with aged petroleum compounds, clay and organic matter may strongly adsorb these substances, reducing their bioavailability and the subsequent bioremediation rate (Grace Liu et al., 2013; Khan et al., 2015; Li et al., 2002). However, the degree of adsorption can vary with different types of organic pollutants and the specific composition of organic matter in the soil. In this state, two main solutions should be considered: adding substrates (at a suitable concentration) for co-metabolism and using surfactants (Li et al., 2002). Another important factor is the amount of organic matter in soil because of its priming effect on microbial communities (Grace Liu et al., 2013). Organic matter in soil can entrap hydrophobic compounds, declining the bioavailability of these compounds (Grace Liu et al., 2013). In the case of bioaugmentation, high contents of organic matters in soil lessen the survival of introduced strains, as inoculation; thus, the amount of desorption declines and, in turn, bacterial growth decreases. On the other hand, some degrees of organic materials are needed because they may contain some substances, which can be considered as a nutrient source for bacteria to grow faster (Mrozik and Piotrowska-Seget, 2010). Naturally, a decrease in biodegradation happens when there are more levels of organic matters in soil because they are used as their substrates easier than pollutants (Guo et al., 2012; Khan et al., 2015). In general, it can be said that the role of organic matter in bioremediation of different contaminants is not alike in different circumstances (Sabaté et al., 2004).

Another property that is effective in bioremediation performance is the size of soil's micropores (Chang et al., 2010). For instance, Noordman et al. found that hexadecane was biodegraded more when it was immobilized in silica gel with the size of 6 nm than larger sizes (Noordman et al., 2002). Organic pollutants are also attracted to organic polymers present in soil because of nonpolar gel structure of the polymers and covalent or hydrogen bonding occurs between them (Pignatello, 1989). It is essential to tailor bioremediation strategies based on the specific soil composition and pollutant types. This may involve adjusting the levels of organic matter, using specific microbial strains, or employing physical and chemical amendments to optimize the bioremediation process.

4.2.4. Soil temperature

Temperature, as a function of regional geography, season, sunlight and surface albedo at the site, is an effective parameter affecting microbial metabolism and growth of microorganisms (Thomassin-Lacroix et al., 2002). Optimal temperature ranges vary for different bioremediation processes, with composting processes typically requiring temperatures between 40 and 70 °C (Dzionek et al., 2016). Higher temperatures generally enhance microbial activity by increasing the rate of biochemical reactions. As temperature rises, the adsorption rate of pollutants decreases, leading to a higher rate of diffusion and advection, thereby enhancing the availability of these substances for biodegradation (Liu et al., 2009; Sturman et al., 1995). Generally, it is expected to see a declined bioremediation rate in cold climate like Arctic. This challenge can be resolved by using cold adapted hydrocarbon-degrading microorganisms. Many studies have claimed that consortia containing these kinds of bacteria can biodegrade petroleum hydrocarbons at temperatures above 10 °C (Chang et al., 2010; Delille et al., 2007; Ferguson et al., 2008; Walworth et al., 2001; Whyte et al., 1998). For instance, Mohn and Stewart concluded that hydrocarbon biodegradation improved dramatically when temperature increased between 7 and 15 °C in remediation of Arctic soils contaminated with petroleum hydrocarbons (Mohn and Stewart, 2000). Also, in a field-scale approach of land farming, it was reported that, when the soil's temperature ranged 15-25 °C, the applied in-situ system had an acceptable performance in both summer and winter (Ko et al., 2007).

3.2.5. Soil pH

In most PHC bioremediation studies, it has been concluded that the suitable range for pH is between 6 and 8, because it results in an enhancement in microbial growth and activity (Atagana, 2004; Ko et al., 2007; Ma et al., 2016; Manual, 2004). Zargar et al. expressed that when the pH value ranged between 7.5 and 8.5, the process used for bioremediation of oil contaminated soil was not inhibited (Zargar et al., 2014). Hong et al. claimed that slightly alkaline conditions yield the best results in fenitrothion bioremediation (Hong et al., 2007). A rise in pH may improve solubility due to enhanced dissociation of acid functional groups (Andersson et al., 2000). A very important point to make in composting process is NH_3 volatilization, in which nitrogen is lost at high levels of pH and temperatures (Koolivand et al., 2017). Also, over composting, pH may go up, which is because of ammonium release (Nwankwegu et al., 2016). In this case, the optimal C/N/P ratio may be impaired. On the other hand, in the case of pH values outside the suitable range, microbial activities are not supported, which leads to a decrease in PHC removal (Ko et al., 2007; Koolivand et al., 2017). At low pHs, unlike manganese the availability of aluminum, which is toxic to microorganisms, in soil is enhanced (Aciego Pietri and Brookes, 2008; Flis et al., 1993). Of course, when soil has a calcareous nature, it has a high buffering capacity, avoiding pH changes during bioremediation (Kahraman et al., 2017). If the soil is too acidic (pH <5), lime may be added to the soil to increase the pH to the required optimum range. If the soil is too alkaline (pH >9), elemental sulfur or ammonium/aluminum sulfate may be added to lower the pH (Huesemann, 1994). Therefore, it is suggested that initial neutral pH is adjusted for starting bioremediation processes and pH should closely be monitored and kept in the suitable range over the process.

5. Types of bioremediation strategies

All bioremediation strategies are divided into two main categories: in-situ and ex-situ remediation. In-situ (or on-site) bioremediation is purification of polluted soil in place. Additionally, the latter one refers to processes in which polluted soil, sludge or sediment are excavated and transported to another place for treatment (Cenčič Predikaka et al., 2023). All in all, the main downside of ex-situ strategies is more expensive on account of the related excavation and transportation costs. In the case of landfarming, contaminated soil is excavated and spread over a prepared bed and periodically tilled to biostimulate microorganisms (Vidali, 2001). When polluted soil or sludge is piled and nutrients and air are introduced into the piles, it is named biopile. Different amendments (explained above) may be added to biopiles. A composting process describes the situation in which organic matters are added to biopiles (Mudhoo and Sharma, 2010). In terms of time and cost, a method like landfarming may be long in field-scale applications even though the costs may be lower than measures in which aeration is done artificially. Since composting benefits from cometabolism properties, it would be preferable to be employed in soil without containing sufficient amounts of organic matters. The results of a review article revealed that the composting system can effectively treat TPH contamination (up to 380000 mg/kg) in clay, silt, and sandy soils, with most studies reporting a removal efficiency of over 70% and a maximum of 99%(Tran et al., 2021). For example, a field-scale study investigated the application of co-composting for bioremediation of oil-polluted soil in Iran. It focused on 1200 m³ of saline soil contaminated with heavy oil, with initial TPH levels between 6.9 and 17.1 g/kg. Organic waste from a local sugarcane factory, mixed with urea, sugar, and compost, was added to the soil. Over three months, irrigation and aeration reduced TPH levels significantly. The remediated soil, analyzed via gas chromatography, was then reintegrated into the environment to support ecosystem growth. This method shows potential for application in other areas with similar conditions (**Fig. 2**) (Parnian et al., 2022).)

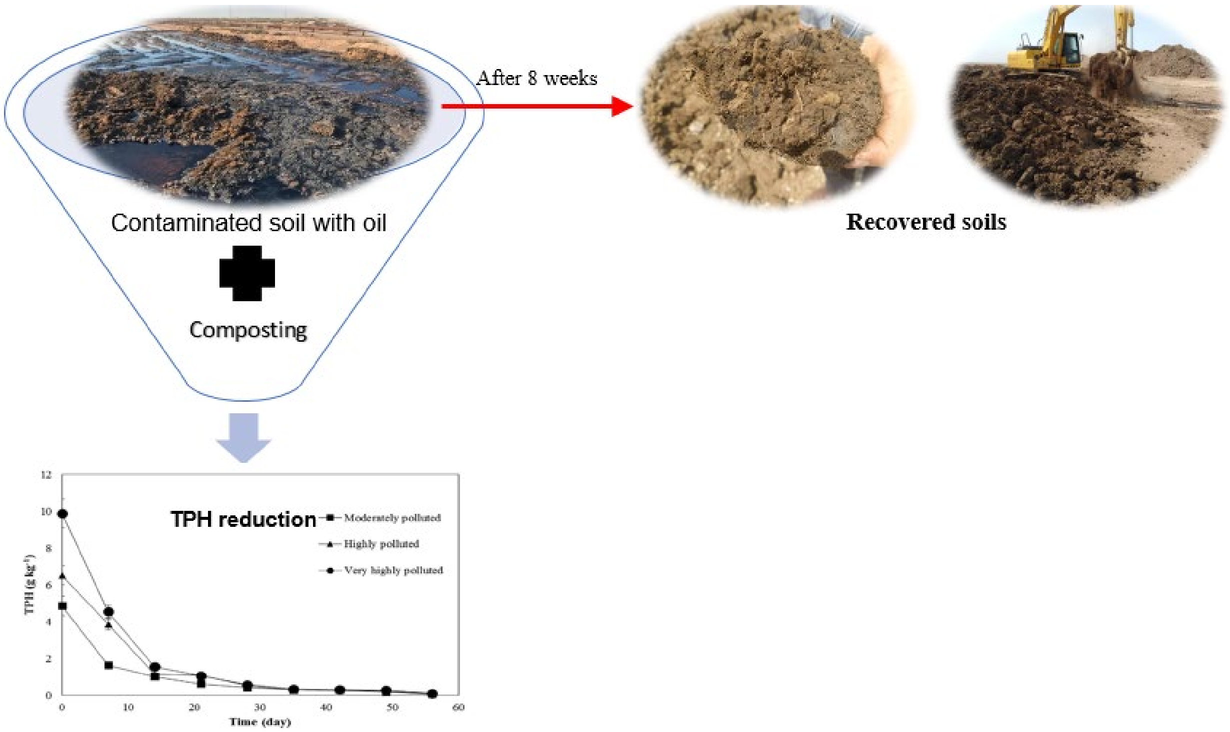

Figure 2. The remediation potential of the composting process at large-scale application (Parnian et al., 2022)

It should be noted that advanced systems of the composting system have reached excellent findings; for instance, Koolivand et al. used a two-phase composting process: the windrow-invessel composting and they reported that over 90% of TPHs of oily sludge was biotreated during an 80-day period (Koolivand et al., 2017). Besides, when it comes to treatment of polluted soil in cold climate conditions, a simple biopile may not reach successful findings, but a drum composter can be considered as a suitable alternative (Gojgic-Cvijovic et al., 2012). In each highly-polluted soil with changing environmental conditions, bioaugmentation in concert with biostimulation should be closely taken into account in all types of methods at large; for this purpose, slurry bioreactors, which benefit from controlled operating parameters and consequently provide a suitable environment for degrading microorganisms to grow and decompose pollutant more, can be a satisfying resolution (Dzionek et al., 2016; Golodyaev et al., 2009; Tomei and Daugulis, 2013; Xu and Lu, 2010). In summary, while bioaugmentation can be highly effective in freshly polluted sites due to favorable conditions and reduced competition, biostimulation tends to perform better in aged sites by enhancing the activity of well-adapted indigenous microbes. Understanding these dynamics helps in selecting the most appropriate bioremediation strategy for different contamination scenarios.

6. Types of contaminants

Petroleum compounds contain various complex combination like crude oil (Fig. 3), as well as asphaltenes and resins (Grace Liu et al., 2011). All these contaminants have been situated on the US EPA priority pollutant list since they threaten human health and the environment (Agency, 1986).

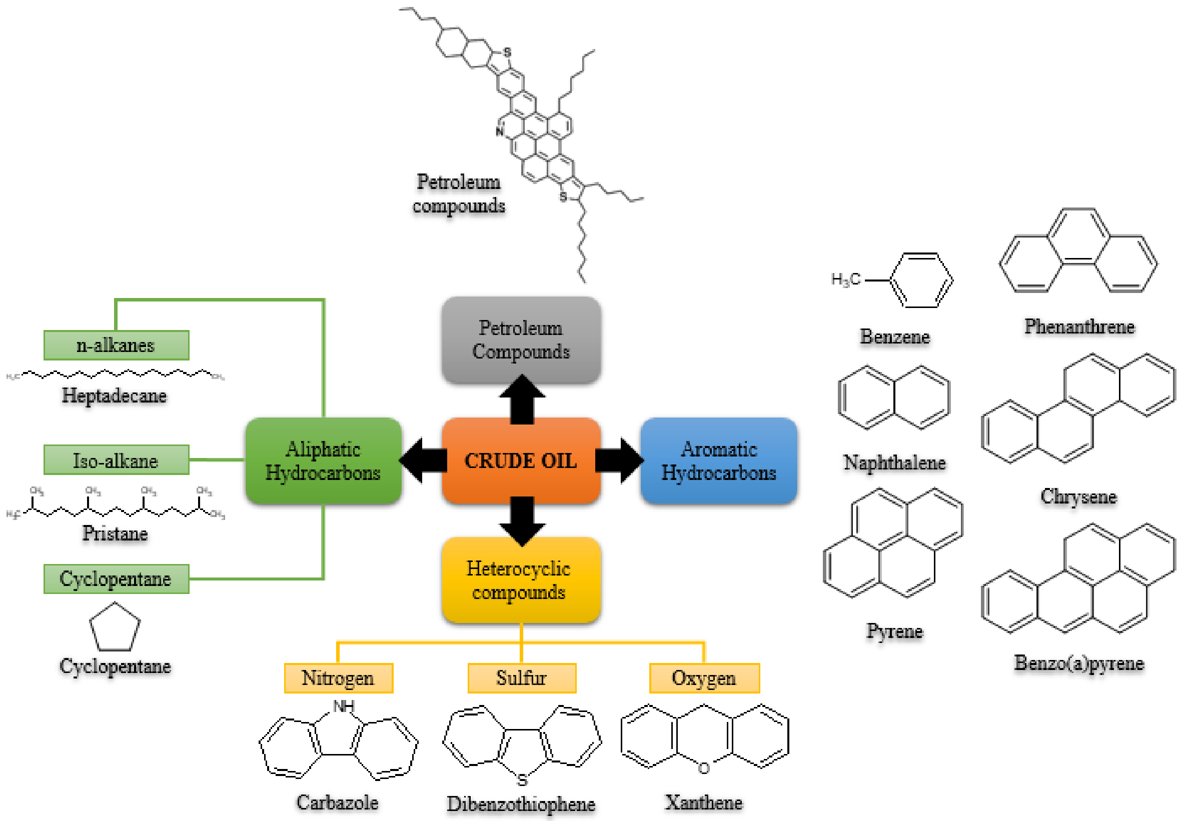

Fig 3. Various components of petroleum products (Lopez-Chavez et al., 2007; Mahjoubi et al., 2018)

To date, many researchers have investigated the application of different bioremediation processes for these pollutants in soil, sediment and sludge. Nonetheless, few studies have tackled the biodegradation of heavy petroleum substances (compounds containing more than 30 C atoms and branched-chain hydrocarbons) (Kunihiro et al., 2005; Wentzel et al., 2007). It has been claimed that in an environment like soil comprised of different kinds of these pollutants, microorganisms prefer hydrocarbons that C atoms range from 14 to 30 (Grace Liu et al., 2013). Saturated hydrocarbons, for example, have the highest degradation rate. On the other hand, asphaltene and resin cannot effectively be degraded by bacteria even in comparison with aromatic compounds (Li et al., 2002) (Fig. 4). And, n-alkanes are the most preferable carbon and energy source for bacteria, which is followed by isoalkanes, low molecular weight aromatics and naphthenes (Gojgic-Cvijovic et al., 2012; Perry, 1984).

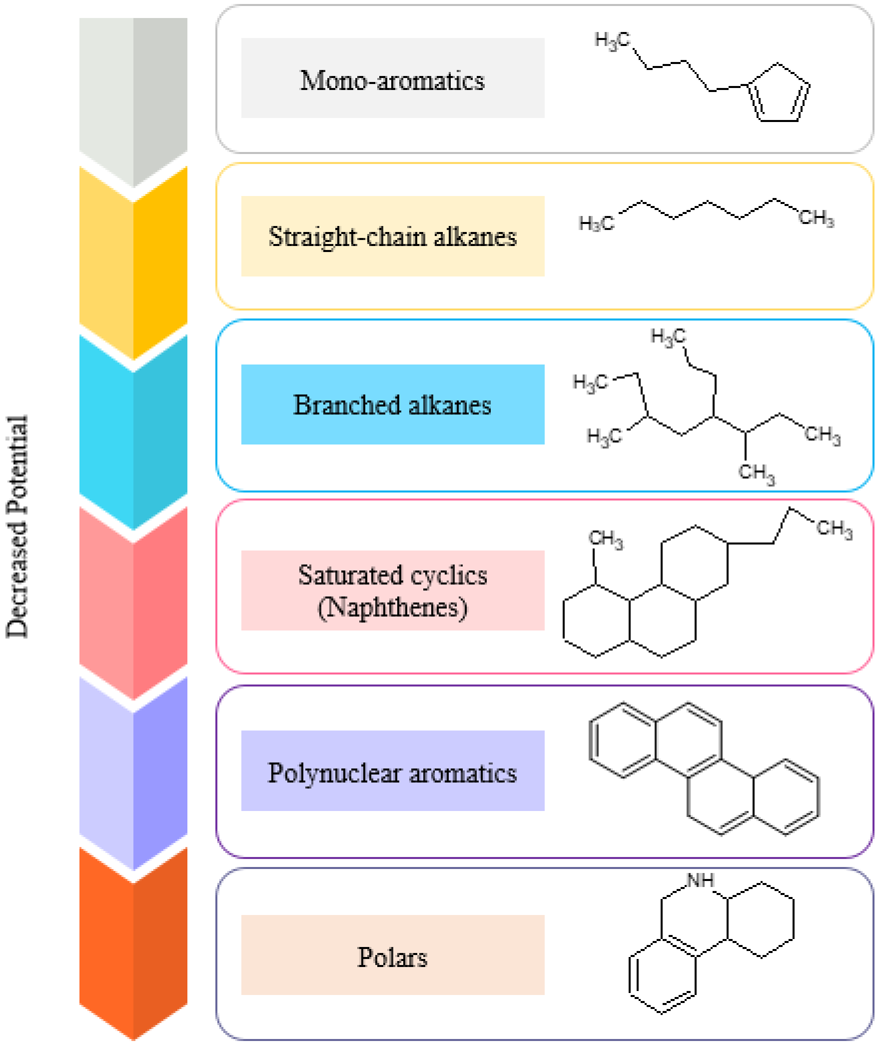

Fig. 4 Biotreatibility rate of PHCs (Huesemann, 1994)

For instance, Gough et al. reported that compounds from n-C27 to n-C36 are less biodegradable (Gouch et al., 1992). As mentioned before, easily biodegradable combinations are degraded in the early stage and intermediate products or metabolites, which are often hydrocarbon polar fractions-resins, nitrogen-, sulfur-, and oxygen-heterocyclics, and asphaltenes (Huesemann, 1994), are accumulated in the later stage and, in turn, cause the performance of bioremediation in PHCs degradation to decline. It should be noted that the polar fraction compounds are greatly resistant to bioremediation. In general, two reasons have been addressed for the formation of these compounds: 1) biodegradation of hydrocarbons with single bacteria (this issue has been closely discussed before) and 2) application of low-nutrient environments (Grace Liu et al., 2011). Of course, Grace claimed that this problem can be resolved by adding kitchen waste, as a composting material, in the second stage of bioremediation when intermediates have been accumulated (Grace Liu et al., 2011). But, having said that, petroleum products are rich in sulfur and trace metals, as well as nickel and vanadium, which can be used as the source of micronutrients required for bacterial growth (Whiteside, 1993). Therefore, in order to treat contaminants effectively in the case of large-scale polluted sites, the measurement of the kinds and contents of PHCs should be performed in during all bioremediation stages.

7. Conclusions and recommendations

Bioremediation is a complex process that requires careful consideration of multiple factors to achieve optimal results. Based on the findings, the following specific guidelines are recommended for implementing bioremediation processes:

- Microbial consortia: A single bacterium is insufficient for treating sites with diverse petroleum contaminants. Instead, a well-characterized microbial consortium that includes microorganisms with complementary metabolic pathways should be selected and optimized for the specific types of hydrocarbons present. For instance, *Pseudomonas* species could be used for degrading alkanes, while Mycobacterium species might be more effective for aromatic hydrocarbons. It is also crucial to maintain microbial populations above a threshold of 10^3-10^5 CFU/g of soil throughout the bioremediation process to ensure sustained activity.

- Tailoring bioaugmentation and biostimulation: bioaugmentation should be employed in freshly contaminated sites where indigenous microbial populations are insufficient to initiate degradation. The selection of microbial strains should be tailored to the specific pollutants and site conditions, such as pH, temperature, and moisture content. In contrast, biostimulation should be prioritized in sites where native microbial populations are present but require enhancement. For example, adding specific nutrients like nitrogen and phosphorus can be crucial in carbon-rich environments to promote microbial growth. However, the nutrient levels must be carefully controlled to avoid inhibiting microbial activity due to high ionic strength or imbalances in the C/N/P ratio.
- Advanced composting for PHC-Contaminated Soils: For large-scale bioremediation, advanced composting processes are recommended due to their effectiveness in degrading PHCs under varying environmental conditions. However, the choice of composting system should be based on site-specific factors such as soil composition, pollutant types, and climate. For instance, windrow composting may be more suitable for sites with limited access to mechanical aeration, while in-vessel composting could be used for more controlled environments.
- Site-specific pilot studies: before scaling up bioremediation efforts, conducting pilot studies tailored to the specific site conditions is essential. These studies should optimize key parameters, such as microbial inoculum size, nutrient amendments, and environmental controls (e.g., aeration, moisture management). Field-scale implementation should follow

only after successful pilot studies, ensuring that the bioremediation approach is both effective and economically viable.

Despite the advances in bioremediation, several areas require further study:

- Microbial interaction and synergy: research is needed to better understand the interactions between different microbial species within consortia, including the mechanisms of synergy and competition. This could lead to the development of more effective microbial consortia tailored to specific contaminants.

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