Microbial Bioremediation of Polycyclic Aromatic Hydrocarbons (PAHs) in Oily Sludge Wastes

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ABSTRACT

Polycyclic Aromatic Hydrocarbons (PAHs) are fused-ring hydrocarbon compounds that are highly recalcitrant under normal conditions due to their structural complexity and strong molecular bonds. These groups of petro-chemicals are mainly found in petroleum-refining plants, accidental oil spills and pipe leakages, and rainwater run-off from roadway. Improperly managed and disposed PAHs can cause environmental pollution as they accumulate in the surrounding soil sediment. PAH contamination is highly unwanted as they are hazardous to human health due to their carcinogenic, mutagenic and potentially immunotoxicants properties. Although in the natural environments they are readily degraded by indigenous microbial communities, these processes are very time-consuming. Various physical and chemical applications are currently employed to remediate the problems caused by PAHs pollution. However, these forms of treatments are either unsound economically or may in fact cause downstream complications. Therefore, the intent of this review is to present the applications of microbial bioremediation of PAHs in oily sludge wastes. Among other things, this review will cover a broad overview of the constituents of PAHs, the microbial dynamics of hydrocarbon catabolism, factors (biological, chemical and physical) effecting microbial degradation and the various strategies available for the enhancement of microbial elimination of PAHs, such as bioaugmentation and biostimulation.

KEYWORDS

Bioaugmentation, biostimulation, petroleum hydrocarbon, oily sludge waste, aerobic/anaerobic degradation, biodegradation quantification.

INTRODUCTION

The world today is very much dependent on oil, either to fuel the vast majority of its mechanized transportation equipment or as the primary feedstock for many of the petro-chemical industries. In the year 2003, crude oil production volumes surpassed 82.3 million barrels per day and this volume is estimated to increased to 94.3 barrels per day in 2010 and 101.6 barrels per day in 2015 (US DOE/EIA, 2006). Oil or petroleum hydrocarbons, therefore, represent high-volume global materials (Ward et al., 2003). Dubbed as the bloodline of modern civilization, petroleum-hydrocarbon compositions vary greatly in its complex mixture of hydrocarbons and other organic and inorganic compounds, which contribute to the diversity in its physical properties (van Hamme et al., 2003). Petroleum hydrocarbons are generally classified into four main groups, namely, the saturates, the aromatics, the resins and the asphaltenes (Table 1) (Leahy and Colwell, 1990). The aromatics and asphaltenes are also termed the Polycyclic Aromatic Hydrocarbons (PAHs). PAHs are fused-ring compounds that are structurally complex. They are highly recalcitrant under normal conditions because of their strong molecular bonds. These groups of petro-chemicals are mainly found in the areas surrounding petroleum-refining plants, accidental oil spills and pipe leakages, and rainwater runoff from roadways (Soriano and Pereira, 1998; Angelidaki et al., 2000; Bach et al., 2005)

Improper management and disposal of oily sludge wastes may cause environmental pollution, particularly to the soil and groundwater systems, due to their low volatility and aqueous solubility. PAHs are also recalcitrant in nature and they have high affinity for soil material and particulate matter. Overtime, they will accumulate to the extend that they are harder to eliminate. It is also important to note that many of the constituents of PAHs are not only carcinogenic and mutagenic, but they are also potent immunotoxicants (Mishra et al., 2001, and Bach et al., 2005). There have even been reports of their impacts on critical habitats such as the benthic ecosystems, which may ultimately get into the marine food chain (Bach et al., 2005).

Table 1. List of typical hydrocarbon fractions from various refineries (Ward et al., 2003)

Location of refinery	Sludge TPH (%)	Hydrocarbon fractions (% of total)			
		Saturates	Aromatics	Resins	Asphaltenes
Ontario (A)	18.8	49.6	32.7	10.3	7.4
Ontario (B)	15.8	42.0	42.0	6.9	9.1
Ontario (C)	13.2	40.4	40.4	7.1	7.6
Quebec	9.3	48.7	25.6	10.2	15.5
Western Canada	20.2	21.2	47.8	9.6	21.4
Eastern Canada	20.9	46.4	33.5	10.8	9.3
Western USA	17.1	45.4	37.8	3.9	12.9
Eastern USA	15.5	44.3	43.7	6.7	5.4
Latin America (A)	15.1	51.3	18.9	14.9	14.9
Latin America (B)	21.3	41.2	35.6	9.7	13.5
South East Asia	33.7	44.7	40.8	6.5	8.0
Middle East	8.3	38.3	45.5	6.9	9.3

Generally, PAHs and other hydrocarbons compounds are readily biodegraded and eliminated from the environmental by indigenous microorganisms, such as bacteria and fungi. In fact, a large number of bacterial species have the ability to degrade the majority of natural hydrocarbon components from oily sludge wastes, especially low-molecular-weight PAHs (Ward, 2003). Although these associations have long been acknowledged, it was only after high profile incidences like the Exxon Valdez oil spill (1989) that government agencies like the United States Environmental Protection Agency (EPA) was finally forced to establish all out researches to determine the viability of bioremediation, particularly microbial applications technologies for the cleanup of future catastrophic oil spills (van Hammes et al., 2003, and Haines et al., 2005).

Microbial biodegradation is an effective and inexpensive approach to degrade and remove PAHs and other hydrocarbon compounds from contaminated soils, as long as the correct population of microorganisms is employed and the oily sludge wastes are conducive to the biodegradation of the contaminants (Philips et al., 2000). Furthermore, with recent developments and applications of state of the art molecular techniques, the processes of hydrocarbon catabolisms have advanced substantially. Following this, many novel catalytic mechanisms have been understood and characterized such as the cellular and other physiological adaptations of microbes to the presence of hydrocarbons, as well as the biochemical mechanisms involved in hydrocarbon accession and uptake. The applications of genetically engineered and enhanced microbes for bioremediation has also been developed and considered (van Hamme et al., 2003). However, amidst all these recent advances, according to Ward and colleagues (2003) presently, there is no clear application of microbial processes for the biodegradation of refinery wastes in the United States. Although some hydrocarbon wastes are treated chemically or via physical methods such as thermal treatments in cement kilns or thermal desorbers, most of the accumulated oily wastes are disposed of in landfills, without proper practices that are environmentally acceptable (Ward, 2003, Piskonen and Itävaara, 2004).

Therefore, the intent of this review is to present the applications of microbial bioremediation of Polycyclic Aromatic Hydrocarbons (PAHs) in oily sludge wastes. Among other things, this article will cover a broad overview of the constituents of PAHs, the microbial dynamics of hydrocarbon degradation, factors (biological, chemical and physical) which are important in determining the rate at which and extent to which PAHs are removed from the oily sludge wastes. The highlights of this review will be on the various strategies available for microbial applications of PAHs degradations and how to quantify the efficiency of the treatments.

POLYCYCLIC AROMATIC HYDROCARBONS (PAHs)

Polycyclic aromatic hydrocarbons or PAHs as they are fondly called, are chemical compounds made of two or more fused benzene rings (Figure 1). They are known soil and aquatic contaminants. Either naturally occurring or formed during the incomplete combustion of fossil fuels, low concentrations can usually be found just about everywhere. They are also associated with industrial activities and around wood preservation stations where creosote oils have been used (Piskonen and Itävaara 2004). PAH-contaminated areas pose a health risk to humans since these pollutants exert toxic, mutagenic, carcinogenic effects and potential endocrine-disrupting properties (Lee and Hosomi 2001, and Sabate et al., 2006). If left unchecked, they may infiltrate the ground

water systems and eventually into contaminating the drinking water supplies. According to Philips (2000), (PAHs) are among the list of US EPA priority pollutants.

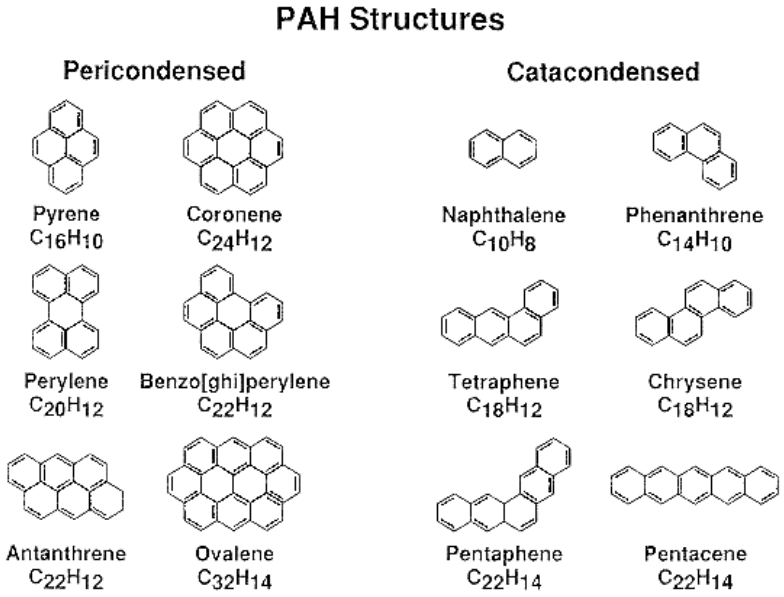

Figure 1. PAHs representatives and their chemical structures (NASA Ames Research Centre, 2005)

PAHs contaminate the soil come from various sources, which includes leakage from storage tank bottoms, oil–water separators, dissolved air floatation units and drilling operations. These contaminated solid vary in hydrocarbon composition (Table 2) and are considered hazardous by the United States EPA (Ward, 2003). Table 3 shows the standards of pollution level for PAHs.

Table 2. Characteristics of a typical PAH-contaminated soil (Piskonen and Itävaara, 2004)

Parameter	Characteristic
Type	Sandy loam
pH	7.7
Ash content (%)	9
WHC (ml g−1)	0.85
Total PAHs (μgkg−1)	222,000
Naphthalene	2,410
Acenaphthalene	14,300
Acenaphthene	2,030
Fluorene	5,150
Phenanthrene	20,200
Anthracene	15,200
Fluoranthene	27,000
Pyrene	30,700
Benz(a)anthracene	18,700
Chrysene	17,600
Benzo(b)flouranthene	10,200
Benzo(k)flouranthene	16,000
BaP	21,200
Indeno(1,2,3-c,d)pyrene	10,800
Dibenz(a,h)anthracene	1,590
Benzo(g,h,i)perylene	9,100

Table 3. Standard limiting PAH content (μg kg^{-1}) in the soil surface layer (Malawska and Wilkomirski, 2001)

Total PAH content	Pollution Class	Soil Assessment
< 200	0	Unpolluted (natural content)
200 – 600	I	Unpolluted (increased content)
600 – 1000	II	Slightly polluted
1000 – 5000	III	Polluted
5000 – 10000	IV	Heavily polluted
> 10000	V	Very heavily polluted

Although PAHs with lower-molecular-weight (two to four ringed compounds) such as naphthalene, acenaphthylene and fluorene are relatively easy to degrade, in general, the rate of degradation is rather slow in the environment, particularly in the aquatic systems (Han et al., 2003). A large number of microbial strains able to eliminate these compounds has been described, whereas very little have been documented on the microorganisms capable of utilizing five-ring (or more) PAHs, such as benzo (a)pyrene (Whyte 1997; Bastieans 2000). Their stable and complex molecular structures, and the ability to adsorp onto sediments are among the factors contributing to this phenomena (Bach et al., 2005). Moreover, due to their limited water solubility in soil environments, they are vitually low in their bioavailability (Bastieans et al., 2000). They are ranked as below in their susceptibility to microbial attack in the following order of decreasing susceptibility: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes (Leahy and Colwell. 1990, and Soriano and Pereira, 1998).

BIOREMEDIATION STRATEGIES/MICROBIAL TREATMENT OF PAHs

Soiled contaminated by petroleum hydrocarbons may be treated using various means and applications. There are reports of physical treatment via thermal or chemical process (Piskonen and Itävaara, 2004). However, these treatments are not only unsound economically, they are also prone to prolonged cycle time (Leah and Colwell, 1990, and Ward et al., 2003). The next choice of treatment would be to involve microbiological applications as according to Phillips (2000), "biodegradation can be an effective and inexpensive approach to remediating soils which contain PAHs and other hydrocarbon compounds" Table 4 below demonstrates the effectiveness of microbial degradation of oil in several sludge samples.

Table 4. Effectiveness of microbial degradation of oil in various sludge samples (Ward et al., 2003)

Oily waste	Initial oil concentration (ppm)	Oil degradation (%)	Time (days)
Drilling oil	50,000	99.0	7
Drilling mud	50,000	90.0	14
Steel mill scale oily sludge	41,000	80.5	24
Metal plating oily sludge	15,500	89.3	14
Paint solvent sludge	128,000	96.0	14
Lubricant oily sludge	50,000	85.0	10
Wastewater oily biosolids	26,000	92.3	10
Oily clay fines	52,000	91.8	14
Coker catcher fines	63,000	89.5	21

To successfully exploit the microbial degradation of PAHs, it is imperative that we understand and master the mechanisms needed in order to manipulate the microbial activities. Microbial bioremediation of PAHs from oily sludge wastes are very much dependent on these three factors:

1. Physical characteristics of the PAH constituents.
   According to Kanaly and Harayama (2000), "the fate of PAHs in the soil depends on the molecular size and topology of the compound". For low molecular weight (4-ring or below) PAHs, removal through evaporation is the first line of elimination. However, as the molecular sizes increase and when exposure to soil particles is prolonged, bioavailability is reduced greatly and, biodegradation rates become slower. In order to enhance the biodegradation processes and making it economically realistic and rapid, it is imperative that the bioavailability of PAHs in soil be increased (Piskonen and Itävaara, 2004). Another characteristics of the sludge that has to be considered is the total concentrations of the hydrocarbon present. Documented recommended concentration is around 5% (Ward et al., 2003). The same literature also stated

that maximum metabolic activities are typically observed in the upper soil layer of between 10 to 15 cm deep.

2. The choice of microbial consortium.
   Many microbial strains are capable of degrading only specific hydrocarbon compounds. However, oily sludge wastes are complex mixtures of different PAHs members, not to mention, the alkanes, NSO (nitrogen-, sulfur-, and oxygen-containing compounds) and resins fractions (MacNaughton et al., 1999). A single bacterial species has only limited capacity to degrade all the fractions of hydrocarbons presents (Loser et al., 1998). Hence, a mixture of outside bacterial armies that can degrade a broad range of the hydrocarbon constituents of the oily sludge waste should be employed. However, steps must be taken to ensure that the original indigenous bacterial communities be part of the regiment. A study done by Mishra and colleagues (2001) suggested that indigenous microorganisms isolated from a contaminated site will assist in overcoming this problem, as the microorganisms can degrade the constituents and have a higher tolerance to toxicity that may wipe off introduced outside species.

3. Factors affecting the biodegradation mechanism.
   There are many factors (physical, chemical and biological) that will ultimately determine the effectives of strategies of choice for microbial bioremediation of PAHs. According to van Hamme et al. (2003), these factors include:
   - **i.** ***Biosurfactant.*** According to Leahy and Colwell (1990), biosurfactants are important agents in the effective uptake of PAHs by bacteria and fungi. The formation of emulsions in the presence of biosurfactants is reported to be in 96% of hydrocarbon metabolizing freshwater bacteria Broderick and Coone (1982). In addition, the contaminated sludge may be augmented with additives and bulking agents, to enhance overall hydrocarbon catabolism (Ward et al., 2003). Bulking agents such as compost will enhance metabolism of organic contaminants because they provide extra nutrients, additional carbon sources and assist in retaining moisture content of the pile (Namkoong et al., 2002). (Sim and Ward, 1997) also reported that commercial chemical surfactant may also be used to boost microbial degradation of hydrocarbon, although different types of surfactant would have different effect (Table 5).

     Table 5. Effects of different type of chemical surfactants on Total Petroleum Hydrocarbon (TPH) degradation (Ward et al., 2000)

Surfactant	Chemical class	TPH degradation (%)
Control (none)	-	46
Biosoft EN 600	Alcohol ethoxylate	63
Igepal CO-630	Alkyl phenol ethoxylate	66
Marlipal 013/120	Oxoalcohol polyglycol ether	45
Sorbax PMO-20	Fatty acid ethoxylate	42
Witcomul 4016	Complex alkylate	42

   - **ii.** ***pH.*** Most important PAHs degrading heterotrophic bacteria and fungi perform best when pH is neutral. However, fungi are known to be more tolerant of acidic conditions (Al-Daher et al., 1998). At pH 7, the mineralization of oily sludge in soil is also improved, thus, enhancing the overall biodegradation process (van Hamme et al., 2003).
   - **ii.** ***Nutrients.*** van Hamme and colleagues (2003) also reported that nitrogen and phosphorus contents greatly effect the microbial degradation of hydrocarbons. He further stated that adjustment of the ratios of these two elements ratios by the addition of nitrogen and phosphorus in the form of slow releasing fertilizers stimulated the biodegradation of crude oil and individual PAHs. Studies done elsewhere also supported the stimulated degradation of PAHs in the top soil and the aquifer sand following the addition of inorganic nitrogen and phosphorous (Breedveld and Sparrevik, 2000). In fact according to Huesemann (1994), it a normal practice in many countries to spray fertilizer onto the oily sludge wastes to enhance the metabolic activities of the microbial community for PAHs degradation.
   - **iii.** ***Salinity.*** Studies have shown that there are generally positive correlations between salinity and rates of mineralization of PAHs such as phenanthrene and naphthalene as reported by Leahy and Colwell (1990). However, hypersalinity will result in the decrease in microbial metabolic rates.
   - **iv.** ***Oxygen.*** Aerobic biodegradation is the most effective pathway for bioremediation, which means the presence and concentration of oxygen is the rate-limiting parameter in the biodegradation and

catabolism of cyclic and aromatic hydrocarbons by bacteria and fungi (van Hamme et al., 2003). This is because PAHs break-down processes involve the utilization by oxygenases, for which molecular oxygen is required. This is also documented by a study done by Ward and colleagues (2003) that showed greater efficiency of natural microbial hydrocarbon degradation when oxygen is available. Although anaerobic degradation of PAHs by microorganisms has been shown to occur, the rates are somewhat negligible and limited to halogenated aromatic compounds such as the halobenzoates, chlorophenols and alkyl-substituted aromatics (Sulfita, et al. 1982; Boyd and Shelton, 1984; Angelidaki et al., 2000).

v. ***Temperature.*** Temperature is another important variable that influences petroleum biodegradation. Optimum temperature dictates the rate of PAHs metabolism by microorganisms and also the pattern of the microbial community. Temperature also has direct effect on the physical nature and chemical composition of the PAHs constituents (Atlas, 1981). When temperatures are low, PAHs tend to be more viscous and their water solubility is greatly reduced (Leahy and Colwell, 1990). Low temperature will also effect microbial growth and propagation, and under normal circumstances, rates of degradation decrease accordingly (Gibb et al., 2001). This is a result primarily of decreased rates of enzymatic activity. The optimum temperature is typically in the range of 30 to 40°C. At temperature above this norm, enzymatic activities are inhibited as proteins denature (Leahy and Colwell, 1990).

vi. ***Pressure.*** Leahy and Colwell (1990) reported that pressure may have positive impacts on the break-down of certain hydrocarbons. For instance, they reported that "at 4°C, 94% of the hexadecane was utilized only after a 40-week incubation under conditions of high pressure, as compared to 8 weeks at 1 atm".

vii. ***Water activities/moisture contents.*** The rates at which PAHs are degraded are also determined by moisture level, according to Vinas et al., (2005). The reason is simple, that water is needed for microbial growth and enzymatic/biochemical activities (Leahy and Colwell, 1990).

viii. ***Genetic enhancement/mechanisms.*** Genetic compatibility and readiness is probably one of the most important determining factor in the success of microbial catabolism of PAHs. Bacterial species with either chromosomal or plasmid-borne genes capable of PAHs of hydrocarbon metabolisms are well documented. The most extensively characterized gene is encoded by the *Pseudomonas putida Gpo1* (van Hamme et al, 2003). Two other related gene, from the distinct monooxygenase classes, a Cu-containing monooxygenase and an integral-membrane, binuclear-iron monooxygenase have also been described in *Nocardiodes* by Hamamura and colleagues (2001). For the overall and broad PAH-degrading capabilities in many bacterial species, the presence of multiple oxygenases have been extensively studied elsewhere (Story et al. 2001). This gene has also been reported to be present in *Sphingomonas aromaticivorans* strain F199 (van Hamme et al., 2003). The role of plasmid is also well documented in the bacteria communities, especially in the *Pseudomonads*. According to Chakrabarty (1976), the metabolic pathways for compounds such as naphthalene, salicylate, camphor, octane, xylene, and toluene have been shown to be encoded on plasmids in *Pseudomonas spp*. Generally, exposure of indigenous microbial communities to pollutants may favor species harboring the necessary survival plasmids (i.e. *OCT*, *NAH*, and *TOL*) (Sayler, 1990). This will ultimately result in an overall increase in the plasmid-carrying members in the community (Whyte, 1997).

After determining the factors that are involved in the microbial biodegradation process, the next step is in choosing the right strategies. Currently, there are a few methods being utilized and according to several other reports (van Hamme et al. 2003, Ward et al., (2003); and Haines et al., 2005), these include:

1. Bioaugmentation
2. Biostimulation
3. Passive bioremediation processes (Biopiling and/or biofarming of oily wastes)
4. Bioreactor-based processes
5. Bioventing - biofiltration of Volatile Organic Compounds
6. Removal of H_2S and SO_X

In this review, a general approach that combines the biopiling of oily waste with natural attenuation plus several biostimulations and bioaugmentation techniques will be discussed.

Biostimulation and bioaugmentation of biopiles is an alluring method of microbial bioremediation as it is not only effective, but also low cost and causes minimal environment impact (Kaplan and Kitts, 2004). Contaminated oily sludge wastes can be transported an isolated area. A confinement made of concrete may be utilized for virgin sludge to prevent excessive run-off or absorption into the soil. This method can have capacities to handle as much as 10,000 m^3 oily sludge per year (Ward et al., 2003).

The broad range of substrates and metabolites present in PAHs-contaminated soils provides an environment for the development of a quite complex microbial community. Bacteria which metabolize the various components of petroleum hydrocarbons such as polynuclear aromatic hydrocarbons (PAHs) are readily isolated from oil sludge wastes (Whyte et al., 1997). According to Zucchi and colleagues, (2003) "bacterial communities in contaminated soils tend to be dominated by the strains that can survive toxicity and are able to utilize the contaminant itself for growth. As a response to bioremediation treatment, these populations may begin to actively degrade the pollutants and detoxify the soil, allowing other quiescent/starving populations to increase their numbers, leading to an increase of the bacterial community within the soil". As reported by Ward (2003), the best degradation was observed with the mixed-cultures. This is also demonstrated in Table 6.

Table 6. Comparison between biodegradation of different composition(s) of bacterial species (van Hamme and Ward, 2000)

Culture	Maximum degradation rate (μg/h)			
	n-C8	n-C9	n-C10	n-C11
Mixed culture	4.1	2.0	0.5	0.1
Pseudomonas auruginosa	0.8	0.5	0.2	0.1
Rhodococcus globerulus	0.6	0.8	0.4	0.2
P. aeriginosa + *R. globerulus*	0.7	0.8	0.3	0.1

Normally, during the biodegradation stages, there is generally a shift in the bacterial communities' dynamics (Vinas et al., 2005):

1. The genera *Sphingomonas* and *Azospirillum* – during all stages of treatments.
2. The genus *Xanthomonas*, the genus *Sphingomonas* and the *Cytophaga-Flexibacter-Bacteroides* group – during treatment whereby no supplementary nutrient is added.
3. The genus *Xanthomonas*, the genera *Alcaligenes* and *Achromobacter* and the genus *Sphingomonas* - during the treatment when extra nutrient is added.

In the simplest form of this type of bioremediation system, little or no microbiological expertise is needed, as the only concern would be to monitor and ensure that the contaminant concentration in oily sludge wastes is kept in check to assure that natural processes of contaminant degradation are active. However, with biostimulation and bioaugmentation, the indigenous bacterial communities will be teamed up with foreign allochthonous PAHs degrading bugs. According to Atlas (1981), these introduced microbes should be chosen based on their abilities to degrade a wide range of PAHs and other petroleum hydrocarbon components, possesses genetic stability, maintain viability during prolonged storage, rapid and non-fastidious growth following storage, have high degrees of enzymatic activity, the ability to exist with indigenous microorganisms, nonpathogenic and do not produce toxic metabolites. This new consortia of degraders are then ensured with a favorable environment (usually with extra aeration and nutrients such as Nitrogen and Phosphorus) in which they can effectively perform their metabolism and catabolism of PAHs (Salanitro et al., 1997, van Hamme et al., 2003). A recent study done by Zucchi and colleagues (2003) suggested a mineral solution comprised of the following to boost the biodegradation rates and increase the PAHs availability to supply the following (per kg of soil): 0.05% v/v Tween 80, NH_4Cl 3.55 g, $(NH_4)_2SO_4$ 2.22 g, K_2HPO_4 0.79 g, KH_2PO_4 0.61 g to reach a C : N : P ratio of 100 : 10 : 1 (Zucchi et al., 2003).

To optimize this method of biodegradation, the break-down of PAHs under anaerobic conditions must also be fully exploited, as certain two- and three-ring PAHs, such as naphthalene, may also be metabolized anaerobically by sulfate-reducing (Morasch et al., 2001) and denitrifying (Salanitro et al., 1997) bacteria. Aromatic, halogenated aromatic compounds such as benzoate, halobenzoates and polychlorinated biphenyls are also reported to be metabolized under anaerobic conditions (Suflita et al., 1982 and Chen et al., 1988). To accomplish this purpose, sediment samples may be amended with biostimulating agents alone and nitrogen and phosphorus in the form of slow-release fertilizer (SRF), lactate, yeast extract (YE), and Tween 80. According to Bach et al. (2005), the addition of these agents showed marked improvement of up to 8.2 times more than control in the metabolism of "PAHs, including naphthalene, acenaphthene, anthracene, fluorene, phenanthrene, fluoranthene, pyrene, chrysene, and benzo[a]pyrene". The addition of yeast extract and lactate was also documented by other studies (Chang et al., 2002). According to their studies, the yeast extract was chosen to supply much needed amino acids, vitamins and trace elements for the rapidly growing population of

microorganisms, and lactate was highly utilized by the sulfate-reducing bacteria under anaerobic conditions. In the same study, they also recommend the use of dextrin (as amending agents) and other co-substrates such as acetate and glucose.

Another key element to consider when attempting the bioremediation of PAHs contaminated sludge using microbial communities is the adaptation-effect of exposure of the introduced microbes to the potentially toxic pollutants. Prior exposure of a microbial community to hydrocarbons and how rapidly subsequent hydrocarbon inputs can be biodegraded is known as adaptation (Spain et al., 1990). The consortia of choice must adapt well to the presence of pollutants to prevent death or inhibition.

The beauty in the application of microbial bioremediation doesn't just lie in the direct metabolism of the pollutants. The utilization of mixed culture used in this type bioremediation may also assist the following processes:

1. Elimination of Volatile Organic Compounds (VOC)
   According to Ward et al. (2003), consortia of microbes "exhibited a capacity for high-rate degradation of volatile organic carbons and the potential use of the culture as a liquid biofilter". He further noted that even single species innocula such as *Rhodococcus spp*. and *Pseudomonas spp*. Had the ability to degrade the volatile fraction 45% and 55% in 2 days and 4 days, respectively. The same method for biological oxidation of volatile organic carbon vapors by microorganisms was also reported elsewhere with BTEX removal efficiencies of up to 99% (Lu et al., 1999 and van Hamme et al., 2003)

2. Desulphurization of the hydrocarbon materials
   Sulfurized hydrocarbons are highly undesirable. However, sulfur is the third most abundant element in crude oil. Usually refineries would use expensive physicochemical methods to amend this problem (Shennan, 1996.). However, aerobically *Rhodococcus spp.* was found to remove the sulfur from compounds such as dibenzothiophene (DBT) a sole source of sulfur. Other aerobic selective desulfurizing. Some thermophillic species such as *Paenibacillus* may perform the task as well (Konishi et al., 1997)

3. Denitrification of nitrogenous compounds.
   Crude oil contains up to 2.1% nitrogen and nitrogenous hydrocarbons that are both toxic and mutagenic. Furthermore, they contribute to the formation of air polluting nitric oxides (van Hamme et al., 2003). Just as desulphurization of crude oil, nitrogenous compounds are generally eliminated from petroleum by expensive hydrotreatment under high temperatures and pressures. However, utilization of bacterial species such as *Azoarcus, Bacillus*, *Brevibacterium*, and *Corynebacterium* may provide a more economically sound solution (Rhee et al., 1997).

What has been discussed on the use of microbial communities, foreign or indigenous, so far may have been only the tip of the ice-berg for the potential use of this type of treatment. With recent progress in modern microbiology, molecular biology, and genetic engineering, improved and synergistic biodegraders and biocatalysts (microbes and enzymes) for bioremediation are just around the corner (Timmis and Pieper, 1999). In the very near future, new tools to collect information on microbial populations in oily wastes contaminated sites will be available to aid in the evaluation and formulation of strategies for effective microbial bioremediation (Watanabe, 2001). In fact, there have been reports on the field applications of a genetically engineered *Pseudomonas fluorescens* HK44, containing plasmid pUTK21 for the naphthalene metabolic genes (Sayler and Rip, 2000).

QUANTIFYING BIOREMEDIATION

Quantifying bioremediation is not an easy process. According to Phillips et al. (2000), PAHs "bioremediation is often monitored by following target contaminant concentrations, reductions of which are not always indicative of decreased soil toxicity". Incomplete degradation may occur and as a result, toxic intermediary constituents will be formed (Heitzer and Sayler, 1993). Neither single chemical analysis nor biological assay will ever suffice in the comprehensive and total assessment of PAHs bioremediation from contaminated sludge. A battery of chemical analysis, for target contaminant levels, and toxicity testing for measuring soil toxicity, are highly recommended (Philips et al., 2000; Mishra et al., 2001; and Zucchi et al., 2003). In this part of our review, no one test will be discussed in length but a simple list of methods used by previous studies will be briefly introduced:

1. Soil toxicity test by performing the response of Sheep Red Blood Cells (SRBC), lettuce seed germination and earthworm survival assays were performed by several researchers (Knoke et al., 1999 and Philips et al., 2000) on PAHs spiked soil to monitor biodegradation. According to Knoke and colleagues (1999), "bioassays to monitor changes in soil toxicity during bioremediation are often recommended to complement chemical analysis of contaminants, particularly where complex mixtures of soil contaminants are present and possible biodegradation products have not been characterized".

2. Effluent toxicity test can be assessed by monitoring biological responses of aquatic protozoans. One such organism used is *Daphnia similis.* In a version of this test, effluent toxicity was evaluated by comparing the responses of *D. similis* in medium with no oily sludge and in the oily sludge medium pre and post biotreatment and also in the final discharge (Soriano and Pereira, 1998). *D. similis* is an indicator organism of choice basically because of its rapid behavioral and physiological response to possible hazardous substances (Burton, 1998).

3. Microbial respiratory activity is a more common biological assay. Used by many research groups, respirometry is highly recommended as an excellent measure of CO_2 production rates arising from microbial activity in hydrocarbon contaminated soils (Zucchi et al., 2003). For this method, direct measurements of microbial activity are recorded. Since CO_2 production is proportional to microbial activity, measuring its release can provide accurate data and proof biodegradation is occurring. Hydrocarbon degradation rates calculated from CO_2 production rates can provide an accurate estimate of biodegradation time and provide data for continuous application. Oxygen uptake, either global (OUR) or specific (SOUR) are, good indicators for monitoring microbial activity. According to Soriano and Pereira (1998), "the amount of activity per cell or per gram of biomass can vary widely". This means measuring biodegradation according to microbial population will not be correct if performance is to be determined.

4. Resting-cells assay is also a common technique performed routinely for the quantification of PAHs degradation (Stringfellow and Aitken, 1995, Bastieans et al., 2000, Goris et al., 2004). In an assay mentioned by Bastieans et al. (2000), cells grown in the presence of PAHs and control were analyzed via HPLC upon inactivation with 0.07% perchloric acid. A similar approach by Stringfellow and Aitken (1995) were performed by using the cells of *Pseudomonas stutzeri* P-16 and *P. saccharophila* P-15 isolated from a creosote-contaminated soil.

5. Chemical analysis of PAH compounds are normally performed by using Gas Chromatography Mass Spectrometer (GC-MS) and Flame Ionization Detector (FID). These standard chemical tests were reported elsewhere (Angelidaki et al., 2000, Breedveld and Sparrevik, 2000 and Bach et al., 2005). Bach and colleague (2005) recommended that in order to evaluate the anaerobic degradation of PAHs in oily sludge wastes, GC-detectable concentrations of PAHs were tested upon 120 days of biotreatment. Measurement by this often provides data with high variability, as it depends on the constituents of the PAHs. A more detailed description of the chemical analysis was described by Mishra and colleagues (2001). According to their methods, the properties and chemical properties of the treated soil samples were taken from the top 25 cm layer. Once dried and pulverized, the samples were then analyzed for total organism carbon, nitrogen, phosphorus, potassium, moisture level and pH. In a similar study done by Malawska and Wilkomirski (2001), the following PAHs can be determined: acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, Indeno(123-cd)pyrene, dibenzo(ah)anthracene and benzo(ghi)perylene. An additional analysis of the presence of heavy metals can also be performed (Malawska and Wilkomirski, 2001).

CONCLUSIONS

The biodegradation of PAHs and other petroleum hydrocarbons in the environment is a complex process. Microorganisms such as bacteria and fungi are the key agents of bioremediation, with "bacteria assuming the dominant role in marine ecosystems and fungi becoming more important in freshwater and terrestrial environments" (Leahy and Colwell, 1990). However, factors such as the characteristics, content and concentration of the PAHs present, the physical and chemical environmental conditions and the composition of the microbial consortia, dictate the rate of the overall microbial degradation processes. Zucchi et al (2003) also stated that the duration of treatment is of major importance for the overall break-down of the contaminants by successive activation phases.

Although in the natural environment, adequate PAHs biodegradation may be achieved through natural attenuation. the relatively long timescales required for conventional bioremediation and natural attenuation processes warrants human intervention, especially when contaminations and pollution is a large scale (Phillips et al. , 2000; Ward et al., 2003). Probably the most significant contributions mankind has to offer come in the emergence and recent developments of molecular biology and genetic engineering. These technologies allowed the discovery of genes encoding the metabolism and catabolism of various constituents of PAHs that would otherwise be impossible by traditional culture techniques. Furthermore, as mentioned earlier (Sayler and Rip, 2000), the marriage of modern Recombinant DNA technology and the petroleum industries may produce new strains capable of not only broad hydrocarbon metabolism, but also adaptability to contaminated environments (van Hamme et al., 2003).

Indeed there is great future for the application of microbial biodegradation for oily sludge wastes contaminated with PAHs. Simply put, this method is cheaper, requires low start-up capital, and needs few expensive high-tech machinery and non-labor intensive. Furthermore, candidate microbes or bugs are either easily isolated from the natural environment or may even be purchase from commercial supplier. The hindrance is the full support from the petroleum industries and the related government authorities.

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