

Degradation of Petroleum Hydrocarbon in the Wetlands by Estuarine Biofilms

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Abstract: As a result of increased generation of energy from fossil fuel in recent times, there is increased contamination of sensitive ecosystems (such as the estuaries) worldwide. The human population is not exposed to the severity of the impact of pollutants due largely to the activities of microorganisms. The survival of mankind from increasing pollution inevitably depends on the constant monitoring of the environment. The estuaries exhibit interesting features as choice human habitats in which pollutants are circulated continuously. The biota (especially the microalgal and bacterial biofilms) in such ecosystems become hardy and are very valuable agents in pollutant biodegradation. This paper reviews the peculiar nature of the estuaries in recycling pollutants; the interaction between the resilient estuarine biofilms and recalcitrant PHC in the wetlands and the field evaluation of in situ bioremediation.

Key words: Petroleum hydrocarbon, estuary, biofilm, pollution, biodegradation, toxicity.

Introduction

The threat to the environment globally arising from pollution, led to the convergence of 153 countries on the United Nations Framework Convention on Climate Change (Markham, 1995). Indications were that the rate of climatic change was more in the last century than in any other. These changes were mostly as a result of human activities, primarily, from fossil fuel use, land-use and agriculture (IPCC Report, 1990). The use of PHC for energy source has exacerbated over the decades; this was accompanied by increased contamination of sensitive ecosystems (such as the estuaries) worldwide. Energy generation is probably the most ubiquitous cause of pollution worldwide (Allen, 1992). Much of the energy consumed comes from fossil fuels. The world production and consumption of petroleum increased within the range of respectively, 21-24 and 64-72 million barrels per day in 1960 and 1996 indicating an increase of over 300%

(Davis, 2000). Petroleum constitutes an environmental nuisance as a result of the large amounts that are extracted from the geological strata in which it is contained and allowed to contaminate the earth's ecosystems in large amounts (Muller, 1992). The cities and urban centres of the world, with their high population densities, consume a lot of energy for various activities and pollute the air, water and land, especially the low-energy ecosystems such as the estuaries and wetlands (Alloway and Ayres, 1993).

Due largely to the activities of microorganisms, the human population is not exposed to the severity of the impact of pollutants. The survival of mankind from unexpected increasing pollution inevitably depends on the constant monitoring of the environment. It is therefore strategic to enhance the innate bioremediative activity of the microorganisms in order to ensure environmental sustainable utilization (Alloway and Ayres, 1993). The estuaries exhibit interesting features as human habitats

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in which pollutants are circulated continuously. The biota (especially the microalgal and bacterial biofilms) in such ecosystems become hardy and are suspected to be very valuable agents in their biodegradation.

In situ bioremediation is fast gaining ground as one of the most eco-friendly method for the remediation of oil-polluted ecosystems. Especially for widespread oil pollution of the wetlands, this appears to be the only reliable approach in the intriguing relationship that exists between the recalcitrant and toxic petroleum hydrocarbon (PHC) and the resilient biofilms in the estuaries. PHC occurs in the estuaries from anthropogenic sources while the estuarine biofilms are part of the epipellic microbial forms that also occur in the estuaries. In the in situ bioremediation of polluted wetlands, the issue of enhancing the innate biodegradative potential of the biofilms therefore requires continuous review. In view of the resilience of the biofilms and their co-occurrence with the recalcitrant and refractile weathered PHC in the same estuarine environment, this paper reviews the peculiar nature of the estuaries in recycling pollutants; the interaction between the resilient biofilms and PHC in the wetlands; the field evaluation of in situ bioremediation and PHC biodegradation with respect to the physiological kinetics of the estuarine biofilms.

Petroleum Hydrocarbon

Physicochemical Properties

Fossil fuel (petroleum) arises from the thermal diagenesis of ancient plant materials in fine-grained sedimentary rocks (Lin-Yen and Engol, 1981). Crude oil is an extremely complex and highly viscous mixture of hydrocarbons made up of mainly hydrocarbons (about 97% of its content). Other elements that are present include nitrogen, sulphur and oxygen. Sometimes mineral

salts as well as trace elements (nickel, vanadium and chromium) are found. Structurally the hydrocarbons could be characterized as saturates; the olefins (found in refined products) which are unsaturated; aromatics and polar compounds. The defining characteristics of the PHC are well described by Clark et al., 1997. The saturates include alkanes, cycloalkanes and the waxes; the aromatics (which could account for 1-20% of total hydrocarbons) include benzene, and alkylbenzenes with one or two methyl or ethyl groups (i.e. toluene, xylenes, ethylbenzene) or the BTEX compounds; and the Polycyclic aromatic hydrocarbons (PAH) which make up to 0.2-7% of crude oils (National Research Council, 2003). By comparison, the aromatics are more toxic than the aliphatics; the middle molecular weight components are more toxic than the high molecular weight tars. Most of the lighter toxic components of spilled crude oil are lost through volatilization soon after a spillage and exhibit a 'hit and run' lethal effect on sensitive biota (Clark et al., 1997).

Biodegradability and Toxicity

The alkanes are easily degraded (Davies and Hughes, 1968); this takes place by a mono-terminal oxidation leading to the formation of primary alcohols, followed by aldehyde and then mono-carboxylic acids (Foster, 1962). Further degradation of the carboxylic acids takes place through β -oxidation in which two carbon units are removed sequentially with the eventual giving off of CO_2 (Figure 1). The biodegradation of the aromatic hydrocarbons had been reviewed extensively (Cripps and Watkinson, 1978; Gibson, 1968). The pathway in bacteria involves the formation of diol followed by cleavage and formation of di-acids while trans-diols are formed in the eukaryotes. Cerniglia et al. (1980) investigated the metabolism of naphthalene by cyanobacteria; it was

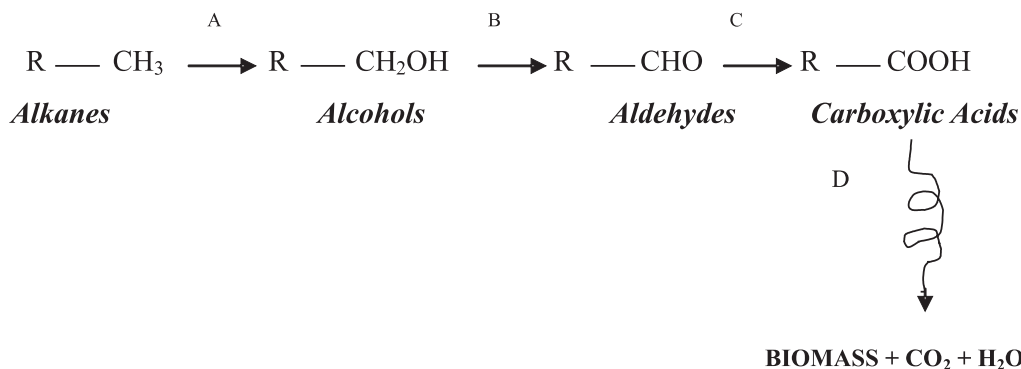


Figure 1: Pathway for bacterial degradation of alkanes: A. Hydroxylation; B. Enzymatic mono-oxidation; C. Further oxidation (+ H_2O); D. β -oxidation (+ Co-Enzyme-A). Adapted from Müller, 1992.

reported that oxidation took place in the light but not in the dark. These organisms are of interest as they exhibit a variety of mechanisms for the initiation of naphthalene oxidation (Masters and Zajic, 1971).

Effect of PHC on Primary Productivity (Microalgae)

The microalgae constitute the major primary producers of the oceans; deleterious interactions with petroleum could consequently affect other organisms higher in the food web. The generally held view is that the effect of accidental oil spills on phytoplankton was not detrimental (O'Brien and Dixon, 1976). Ellis (1977) showed that certain freshwater algae decomposed phenol and catechol; Cerniglia et al. (1980) demonstrated the production of a number of metabolites from naphthalene by several species of marine microalgae. Furthermore, when grown on crude oil, the resin and asphaltene fractions were more reduced. A number of research works have shown that petroleum affects the growth, respiration and photosynthesis in the algae. The microalgae appear to play active role in the degradation of the more aged and refractory residual hydrocarbons sequestered in the sediment. Various authors have reported that at high concentrations hydrocarbons exhibit negative effects on respiration and photosynthesis of organisms. However, low oil concentrations have been reported to stimulate photosynthesis particularly in the microalgae (Gordon and Prouse, 1973; Karydis, 1979). Armstrong and Calder (1978) have suggested that the primary effect of petroleum interaction with the microalgae is on the electron transport system. Usually when oil was well dispersed, as in emulsions, there was more toxic effect and primary production was found to be sensitive to less than 30 µl of oil per litre of seawater. For oil floating as a 'slick,' there was less toxicity requiring up to 80 µl of oil to inhibit primary production.

It was further reported that the detergent Coretex (used as oil dispersant) accentuated the toxicity effect of crude oil. Of particular interest is the report by Bott et al. (1978) on cyanobacteria; which, when challenged with Nigerian crude, No.2 Fuel and used crankcase oil, switched from a photosynthetic to heterotrophic mode of metabolism; showing a reversal as oil was removed.

Effect of PHC on Primary Productivity (Bacterial)

PHCs occur in the environment from natural and anthropogenic sources; the potentiality for their degradation is thus widespread in several bacterial species in the ecosystem especially in the aerobic forms. Under aerobic conditions, alkane degradation pathways have

been identified in *Pseudomonas putida* (formerly *P. oleovorans*) (van Beilen et al., 2001); *Burkholderia cepacia* (Marin et al., 2001), *Acinetobacter* sp. (Geissdorfer et al., 1999; Ratafczak et al., 1998); *Nocardiodes* sp. (Hamamura et al., 2001), *Rhodococcus* sp. (Koike et al., 1999), and *Alcanivorax* sp. (Dutta and Haramaya, 2001). Polyaromatic hydrocarbon biodegradation pathways had been much studied in plasmids (NAH7) associated with *Pseudomonas putida*; later studies had also indicated pathways in *Nocardia*, *Rhodococcus* and *Mycobacterium* spp. (Dean-Ross et al., 2001; Rehmann et al., 2001). The last decade had witnessed much research work in anaerobic biodegradation of PHC involving microbial consortia (Widdel and Rabus, 2001). Toluene biodegradation had been linked to denitrifying bacteria processes in *Azoarcus* sp., and *Thauera aromatica* (Achong et al., 2001; Beller et al., 1998). Species of *Dechloromonas* have been associated with benzene biodegradation (Coates et al., 2001).

Crude oil contains 0.5-2.1% of nitrogen which occurs as pyrroles, indoles, and carbazole; a number of denitrifying bacterial species (e.g. *Alcaligenes*, *Bacillus*, *Beijerinckia*, etc.) have been identified with the capability to utilize these along with their alkyl derivatives (Aislabie et al., 1998). Sulphur which constitutes 0.05-5% of crude oil had to be removed at extra cost from oil during refining; desulphurization species of bacteria especially *Rhodococcus erythropolis* (Kilban and Bielaga, 1990) and others (e.g. *Nocardia*, *Agrobacterium*, *Mycobacterium* etc.) have been known to remove sulphur from oil (Nekodzuka et al., 1997). Areas of chronic oil pollution such as the oil shipping lanes, seaports, offshore oil terminals, and estuaries show increase in bacterial populations that are hydrocarbonoclastic (Gunkel and Gassman, 1980). Atlas (1981) concluded that in non-polluted sites less than 0.1% of the total heterotrophic population has the capacity to degrade oil. Austin et al. (1977) also found differences between planktonic and benthic communities, while Westlake and Cook (1980) found significant shifts in the composition of intertidal and pelagic populations when challenged with Prudhoe Bay oil.

Effect of Physicochemical Factors on PHC Degradation

There are a number of physicochemical factors, which affect the degradability of petroleum hydrocarbons in a given environment. The chemical and physical nature of oil varies from one type of crude to the other; even for the same crude, variations in different batches occur.

Physical effects such as temperature and light intensity that are seasonally dependent have been reported to be important factors regulating toxicity (Shields et al., 1973). Surface seawater temperatures vary from 35 °C in enclosed areas in the tropics to -2 °C in the Polar regions. Most marine microbes have been shown to be active within this range of temperature. Volatilization, especially in the tropics, could alter the composition of oil. Hsiao (1978) studied the effect of temperature on phytoplankton production; toxicity increased with increasing temperature from 0°C to 10°C and this was related to the release of the soluble fractions of petroleum. Earlier investigators had reported on the non-availability of microbial species that could degrade oil at low temperatures (Grunkel, 1967; Gibbs and Davies, 1976). Atlas and Bartha (1972), demonstrated that temperature affected chemical composition and low temperatures depressed volatility of the low molecular weight components (these are often associated with toxicity); thus microbial oxidative activity was also depressed as a result of increased toxicity.

The chemical characteristics of seawater, especially its iron content, can contribute to the rate of oil breakdown (Dibble and Bartha, 1976); this has been attributed to the stimulatory effect of iron on the growth of phytoplankton (Tranter and Newell, 1963). In oil oxidation processes nitrogen and phosphorus are found to be limiting factors. These are not available in petroleum and are also limiting in the seawater; though oil increases the carbon and energy sources. The nitrogen and phosphorus status of the seawater is expressed as its

C/N and C/P ratios. A number of authors have reported on the stimulatory effect of nitrogen and phosphorus in the degradation of petroleum (Atlas and Bartha, 1972b; Reisfeld et al., 1972). The effect of fertilizer application to provide these inorganic nutrients in the field is limited by their migration through leaching out of the treatment site. To control this effect oleophilic fertilizers are used (Röling et al., 2002). The degradation of petroleum involves the direct incorporation of molecular oxygen. In the open sea, however, oxygen is not limiting.

Estuaries

Physical Features

Estuaries are coastal bodies of water that are semi-enclosed and have free connection with the open ocean and within which seawater is measurably diluted by freshwater derived from land drainage (Lauff, 1967). They are characterized by high productivity as a result of their water circulatory pattern. The less dense freshwater flows from the head of the estuary to the mouth along the surface while the more dense seawater moves in and upwards with the tide at the bottom of the estuary. There is therefore a continuous mixing of seawater into the freshwater. Consequently, this results in a net inflow and upward mixing of seawater at the bottom of the estuary. Plankton that drift out on the surface current may be sunk or eaten and then excreted offshore regenerating nutrients which tend to be swept back by the net influx of bottom water (Figure 2).

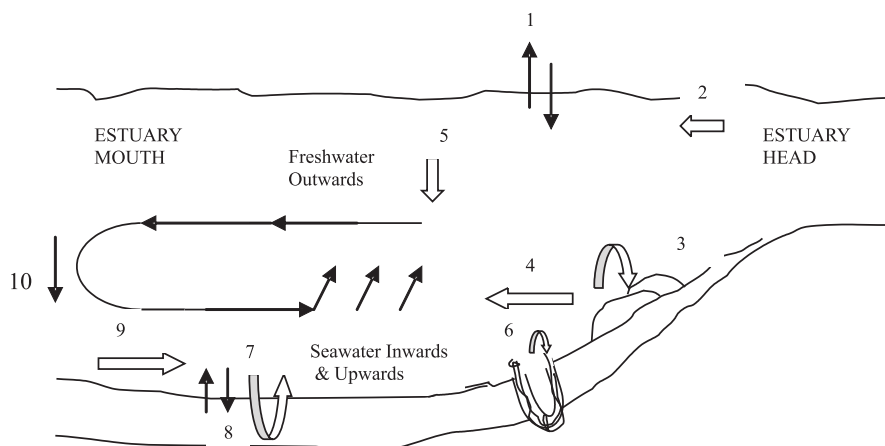


Figure 2: Estuarine contaminant circulation. 1. Air-water Diffusion. 2. Riverine Input. 3. Mussel bed filtration 4. Biodeposition, Elimination with faeces. 5. Vertical settling of matter. 6. Filtration and biodeposition by infauna. 7. Bioresuspension. 8. Sediment burial/resuspension. 9. Lateral Advection 10. Sinking of Detritus and Pollutants. Adapted from Laws (1993).

Contaminant Circulation

A negative consequence of the circulatory pattern of the estuaries is that pollutants are also circulated. These pollutants are not easily washed out to the sea but rather are recycled repeatedly and in the process some become trapped in the sediment. Estuaries thus exhibit exacerbated impact of pollutants discharged into them. They are eutrophic systems as a result of the high efficiency with which nutrients are being recycled. Estuaries are very important ecosystems as they serve as breeding and nursery grounds for many migratory species. These are choice human habitats by virtue of their fisheries potentials, easy and cheap water transportation between the hinterland and the coast etc. Consequently, large populations and industrial centres are found to develop along them. Incidentally wastes are dumped in the estuaries with the assumption that they would be carried to the sea and diluted into extinction; rather they remain for several years and slowly undergo anaerobic biodegradation.

Exopolysaccharide (EPS) Stabilization of Sediment

Sediment in natural waters is usually associated with much biological activity. This is shown by the presence of much Extracellular Polymeric Substances (EPS) exuded by the diatoms among many other organisms (Craig et al., 1990; Underwood, 1994; Smith and Underwood, 1998). It has been reported that the presence of microbial EPS is responsible for the stability of sediments (Dade et al., 1996; Decho, 1994). The production of EPS by diatoms is shown also to be influenced by the amount of metal concentration. In the estuary, the EPS is shown to act as a collating agent that could sequester pollutants and xenobiotic compounds; thereby providing some protection for sensitive organisms against toxicants and also account for the persistence of pollutants (Miles, 1994).

Physicochemical Variability in the Estuarine Ecosystem

Estuarine biota is exposed to a number of sudden changes. While the nutrient rich freshwater can directly stimulate primary production, it poses a salinity stress on the marine forms resulting in their gradual replacement by the freshwater species (Kromkamp and Peene, 1995). Temperature effects could arise in estuaries depending on the source of the freshwater; this may affect enzyme systems involved in photosynthesis in the phytoplankton. In the case of the microphytobenthos temperature effects on the rate of photosynthesis is usually very severe

(Blanchard and Guarini, 1999). Many workers have reported on the effect of CO₂ availability (especially under high pH and O₂ super saturation) on limitation of photosynthesis in the microphytobenthos biofilms (Admiraal et al., 1982; Kromkamp et al., 1998).

The Biofilms

Composition and Occurrence

Microbial aggregates occur in various forms in the environment. They include: bacterial colony, effluent treatment floc, anaerobic digester granules, food associated assemblages, marine snow, mycelial balls, 'wolf packs,' pellicles, alga mat communities and biofilms (Wimpenny, 2000). They are found on the surfaces of stones and particulate matter in aqueous environments; on soils and sediments; on particles in trickling filters; on leaves, roots, germinating seeds; in dental plaque; on intestinal and rumen epithelial tissues; on medical prostheses (catheters); on human artificial joints on ship hulls and external surfaces of offshore oil production platforms; on the internal surfaces of chemical processing equipments etc. (Atkinson and Fowler, 1974; Wimpenny et al., 1983; Costerton et al., 1985; Hamilton, 1985). The two basic defining features of biofilms are that they occur on surfaces and that they are aggregates of one species or a mixture of organisms enclosed in their extracellular polysaccharide metabolic products.

The Intertidal Estuarine Biofilms

The estuarine intertidal sediments are dominated by the microalgal biofilms especially the epipellic diatoms (Admiraal, 1984; Underwood, 1994; MacIntyre et al., 1996; Underwood and Kromkamp, 1999). These organisms play a vital role in the primary productivity and dynamics of the estuarine ecosystem (Paterson, 1994; Underwood and Kromkamp, 1999). Underwood and Kromkamp (1999), in a review highlighted a number of factors that could affect the productivity of the phytoplankton and the microphytobenthos in estuarine ecosystems. The formation of algal blooms, for example, which is a feature of eutrophic estuaries, occurs as a result of the interplay of the processes of bottom-up control of photosynthesis and the top-down control of biomass production. Secondly, the rate of photosynthesis in the phytoplankton in the euphotic zone when nutrient is not limiting, affects primary production (Wofsy, 1983; Cole and Cloern, 1987; Pennock, 1985; Cole et al., 1986). With the microphytobenthos, spatial and temporal gradients of light occur within the sediment, coupled with the varying patterns of diel illumination periods; thus they

have to assume varying positions in order to photosynthesize (Brotas and Catarino, 1995; Guarini et al., 1997; Edgar and Pickett-Heaps, 1984; Smith and Underwood, 1998). In terms of nutrient limitation, phytoplanktons are affected both in their photosynthesis i.e. the Blackman type of limitation and in biomass production i.e. the Liebig type of limitation (Kolber and Falkowski, 1993; Falkowski et al., 1992). In the microphytobenthos, there is a correlation between nutrient gradients and biomass; this has been attributed to the fact that fine cohesive estuarine sediments are rich in organic matter coupled with the intensive bacterial mineralization activities that occur (Underwood et al., 1998; Admiraal, 1984; Heip et al., 1995).

Resilience of Biofilms

The mode of growth of microorganisms in their natural environment is in the form of similar or dissimilar cell aggregates. This mode of existence has enabled the biofilms to colonize harsh environments especially in the medical field and in the industry. They play very active roles in bio-fouling and bio-corrosion of pipelines of heat exchangers etc. (Characklis, 1990; Little et al., 1990); increase frictional resistance to fluid flow on ships, water conduits; the corrosion of steel pipelines and tanks in the oil industry; and spoilage of foods (Holah et al., 1994; Eginton et al., 1998).

A number of researchers from different fields of study have reported on the resistance of the biofilms to chemicals, desiccation, temperature extremes etc. This is between 100 and 1000 times that of the free living forms (Allison and Gilbert, 1995; Costerton et al., 1987; Nickel et al., 1985). Reasons have been adduced to explain the response of biofilms to adverse conditions in the environment. The most cited factor is the extracellular polymer matrix, also called glycocalyx. The mechanism of protection is attributed to the restriction to access of antimicrobials to the inner portions of the biofilms; the glycocalyx acting as a substrate for chemically reactive agents or through non-specific binding of highly charged antimicrobial compounds. Also the matrix binds to the extracellular enzymes such as β -lactamases and formaldehyde lyases, which interfere with the diffusibility of antimicrobial agents and destroy them. Another factor relates to the closeness of cells in a biofilm. In a dense biofilm, there is a graded distribution of nutrients, oxygen etc.; consequently a gradient of growth rates occur to the effect that the peripheral cells would metabolize faster. When challenged with toxic substances, the core slow-growing cells, which are highly dormant, have been shown to over-express non-specific defenses such as

shock proteins, multi-drug efflux pump (*arcAB*) and also extracellular polymers. A third factor relates to quorum-sensing mechanisms, in which biofilm specific phenotypes are induced through cellular attachment to surfaces (Allison et al., 2000).

The efficiency with which organic and inorganic contaminants are attenuated, mineralized and transported in the sediments has been attributed to the activities of microbial communities (Rothmund et al., 1996). Especially the biofilms formed by microalgae have been known to accumulate metals at levels more than four orders higher than the concentration in the aquatic ecosystem (Sillitoe et al., 1994; Liehr et al., 1994). In the interaction between microbial communities and pollutants an array of possible responses is encountered; these responses represent not only the diversity and composition of such communities but also indicate the physical and chemical nature of the pollutants. Decho (1990), reviewing the role of EPS, has suggested that the high density of cells in the biofilm could create a diffusion barrier that could localize chemicals released by the cells. Further, that such an assemblage could lead to the formation of autoinducer-receptor complexes. These phenomena have been demonstrated in the attenuation of metals and the concentration of organic substrates in biofilms (Lawrence et al., 1998; Wolfraardt et al., 1995).

Use of Biofilms in In Situ Bioremediation

The biofilms can readily be developed for bioremediation purposes as the different members of the community exhibit syntrophic mode of association when exposed to either starvation or poisonous and inhibitory substances. The biofilms are also known to be associated with large plasmids whose presence has been correlated with the ability to produce surface-active agents (Hada and Sizemore, 1981; Floodgate, 1984). Three main types of approach are usually adopted for the assessment of the impact of PHC in the environment: The *post-hoc* monitoring of acute and chronic oil spillages (Elmgreen et al., 1983; Nance, 1991); laboratory studies in which selected organisms are exposed to PHC under specified conditions (McElroy, 1990) and use of microcosm or mesocosm experiments in which the pollutant, nutrients, bioventing, temperature etc. are maintained at predetermined levels in order to optimize biodegradation. Generally all such experiments in their various modifications and combinations relate very poorly to what actually takes place in the natural environment because they do not give an explanation about the type of interactions that occur in situ between the microbial communities and the pollutants (Catallo, 1993).

Experimentation in mesocosms is probably the closest approach to what takes place in the ecosystem; it allows for a control of experimentation conditions while the effect on the biofilm is being manipulated (Carman et al., 1995; Greer et al., 2002).

Aspects that could be investigated, therefore, taking into consideration the peculiar known responses of the biofilms, would include: The background status of the estuary as regards the environmental conditions of existence of the biofilms prior to impactation with oil. In this assessment, the degradation of often refractory and innocuous PHC interaction with biofilm is assessed as the baseline value with which other treatments could be compared. This will include assessments on the effect on the physiological characteristics of the biofilms. The bioremediation status of the estuarine biofilms from oil impactation in the absence of any form of enhancements indicating loss or otherwise of PHC including assessments on the effect on the physiological characteristics. The bioremediation status of the estuarine biofilms from oil impactation in the presence of some form of enhancements for example added nutrient indicating loss or otherwise of PHC, including assessments on the effect on the physiological characteristics.

Biofilm Physiological Interaction with PHC

Petroleum has a complex composition and it is found in the environment at varying degrees of weathering and biodegradation. The response of biofilms to petroleum is diverse, especially with respect to the effects on size, composition and metabolic activities (Pfaender and Buckley III, 1984; Atlas 1984). Generally, while some members of the microbial community are enriched by the presence of petroleum, others are inhibited and the whole episode is dependent on the chemical composition of the oil and the characteristics of the ecosystem. A survey of previous research works on the response of benthic microbial communities to petroleum indicated a variable pattern. Bryom et al. (1970) tested the effect of Kuwait crude on benthic species in the marine sediment from the English Estuary. There was an increase in the Total Colony Forming Unit (TCFU) but with shift in the population of the dominant species. A similar test by Walker et al. (1976) on marine sediments from the Atlantic Ocean using 1% mixed hydrocarbon components showed a decrease in the TCFU. Observations by Stewart and Mark (1978) on the marine sediments of Chedabucto Bay, Nova Scotia during the Arrow spill with Bunker C oil, showed increase in the TCFU at the impacted sites. Gunkel et al. (1980) reported increase in the population

of hydrogen utilizing species when surface water of the North Sea was impacted with Baseline Oil. A similar work by Hodson et al. (1977) on the surface water of Saanich Inlet, British Columbia using Loussiana crude; Kuwait crude; No.2 fuel oil and Bunker C oil, showed no response. Effect of petroleum on the metabolic activities of microbial communities also showed variable responses (Hodson et al., 1977; Griffiths et al., 1981a).

The study of the interaction of PHC with the biofilms would involve not only the assessment of the loss of PHC through biodegradation but also the elucidation of the impact on the composition, productivity, and especially the physiological state of the biofilms. While many studies have established that PHC in high concentrations is toxic to organisms (Neff and Anderson, 1981), impair physiological output (Capuzzo, 1987), alter DNA synthesis (National Research Council, 1985) etc., these studies often do not predict effects on the microbial communal functional interactions and between trophic levels (Elmgren et al., 1980).

Kinetics of Biofilm and PHC Interactions

An effective bioremediation of polluted sites (especially with hydrophobic pollutants like petroleum) requires an understanding of the characteristics of the pollutant with respect to: bioavailability for biodegradation; sorption/desorption kinetics with the ecosystem and the presence of surface active agents. These have been reported to be the cause of the persistence of certain pollutants in the soil, sediment and aquifers (Jafvert and Weber, 1992; Rao et al., 1993). In a given environment, bioavailability of a pollutant depends on the soil type, texture and the content of organic matter. Benthic microbial communities (especially the microalgal population) show resilience towards petroleum hydrocarbon toxicity; these organisms produce hydrocarbons and have the biochemical capacity to degrade them (Cerniglia et al., 1980). Microalgal forms have been cited by a number of authors for their abundance, productivity, mineralization and influence over the dynamics of the estuarine intertidal sediments (Underwood, 1994; MacIntyre et al., 1996). Meyer et al. (1975) presented a model for the interaction of mixed microbial populations with various metabolites; a steady state was predicted over a range of small perturbations; and loss of stability was indicated only when the perturbations were high. The presence of Surface Active Agents is very important for the removal of any sorbed components of petroleum. This is required for the degradation of the non-aqueous phase liquids and for the subsequent increase of bioavailability and thus degradation of the pollutant. A number of synthetic

surfactants are available (Kile and Chion, 1990; Liu et al., 1991), nevertheless, their usefulness has been overtaken by the controversy relating to their toxicity and persistence in the environment (Robichaux and Myrick, 1972) and in their place, the biosurfactants are now being used. Other factors needed for bioremediation of the polluted sites include the monitoring of the physicochemical parameters such as oxygen gradient, nutrient availability, temperature, pH, salinity etc. (Foght and Westlake, 1987).

Bioremediation

Relevance

Bioremediation is defined as the recovery of an environment from the effects of a contaminant by the use of living organisms, usually microorganisms. According to Prince (1992), it involves encouraging natural processes of biodegradation to clean up the environmental problems of anthropogenic origin. Organic wastes are degraded under controlled conditions to an innocuous state or to pre-determined levels by regulatory authorities (Mueller et al., 1996). There are technically, two natural processes of pollution control: the abiotic and biotic processes. The abiotic processes result from action of the physical environment. Volatilization involves the evaporation and loss of the light molecular weight compounds of pollutants; in crude oils these constitute much of the toxic components (Atlas, 1975). Photolysis is the breakdown of compounds by electromagnetic radiation (such as occurs in sunlight). Surface oil slicks especially the Poly Aromatic Hydrocarbons (PAH) are much weathered by this process (Floodgate, 1984). It has been reported that for some recalcitrant compounds (for example, anthracene) this appears to be the major route for their breakdown (Barthel et al., 1981). Other processes include sorption unto particulate matter and dissolution in water; mechanical dispersion and emulsification, which reduce the pollutants into droplets. The biotic processes involve the interaction of a number of organisms with the pollutant leading to their biodegradation. Especially for the PHC, there are a number of mixed microbial populations, which are genetically, and biochemically adaptable for the breakdown of the various components (Atlas, 1981; Cooney, 1984). Gunkel and Gassman (1980) stated that environments that have been exposed to oil have a higher proportion of oil-degrading species than pristine areas; this was supported by the work of Carman et al. (2000). The part played by plasmids in enhancing oil breakdown in some bacterial species is less understood. Glassman

and McNicol (1980) reported that a larger proportion of bacteria from oil-polluted sites carried multiple large plasmids compared to bacteria from sites that are not polluted whose plasmids are single and smaller.

In view of the fact that the natural environment has a recognizable capacity to breakdown and absorb PHC pollutants, the need for bioremediation may not be seen as necessary. The urgency for the remediation of contaminated environments by the generality of the populace is very high. As a result of the magnitude, rate of impactation and damaging effects of PHC on the environment especially its aesthetic values, coupled with the pressure from increasing population growth which demands for more sustainable use of limited land resource, all contribute in increasing the urgency for clean-up of oil-contaminated land. Thus bioremediation is needed in order to hasten the rather slow rate of biodegradation under natural conditions. Slow degradation of oil could result from cold temperatures. Generally biodegradation is slower under anaerobic condition; thereby making bioventing a necessity for biodegradation systems. In some environments, nutrients and co-substrates may be limiting; for example, in oil polluted sites this could be in the form of shortage of nitrogen and phosphorus for which reason fertilizers are used to enhance biodegradation. Also, as with oil pollution, the contaminant could be hydrophobic and thus unavailable for biodegradation; this is taken care of by the application of detergents and surfactants. Sometimes, the potentiality for biodegradation may be lacking in the indigenous microbial species, for which reason, bioaugmentation is applied.

Bioremediation methods can be broadly classified into two types viz. the *ex situ* and *in situ* techniques. In the *ex situ* bioremediation, the pollutant is removed or excavated from the site to a treatment site. The process involves: slurry phase remediation (in which water is added and mixed with the pollutant); treatment-bed remediation (in which nutrients are added and agitated); and bio-piling (during which time waste is piled up and aerated). The process is called composting if organic matter is added to enhance the biodegradation. Variations of this process are the landfill and the solid-phase bioremediation techniques. Composting has been used for the treatment of oily wastes from petroleum (Samson et al., 1994; Filauro et al., 1998; Koning et al., 1998). The main limitation of the *ex-situ* bioremediation as observed by a number of researchers is the partial biodegradation of persistence chemicals due to poor bioavailability and mass transfer problems (Bossert et al., 1984; Morgan and Watkinson, 1989; Wang et al., 1990).

In situ bioremediation, on the other hand, involves the treatment of pollutants where they occur. It is a less expensive technology than the ex situ method. It has been successfully applied in the treatment of underground water aquifers (Buermann, 1992; Herrling et al., 1992) and treatment of refined fuel products (Hutchins et al., 1991; Morgan and Watkinson, 1990). Recent improvements have led to the application of this technology to the treatment of creosote (which are not readily biodegradable), crude oil (Ensley and DeFlaun, 1993; Pitchard and Costa, 1992) and the chlorinated solvents (Broholm et al., 1991; Mueller et al., 1992). There are situations when in situ bioremediation represents the only option for treatment for example when widespread and large amounts of pollution of the environment are involved. The main setbacks include the need for more treatment time, complicated sampling and analysis problems (U.S Patent Office, 1992a, b, c, d).

Bioaugmentation

The use of indigenous microbes for oil bioremediation is based on the assumption that they have adapted to the environmental physicochemical parameters and that they have, from previous exposure to pollutants, acquired the genetic make-up for their biodegradation. There are instances, however, where such indigenous inocula have failed to biodegrade efficiently (Ismailov, 1985; Mueller et al., 1993, 1992). Sourcing for inocula for efficient bioremediation is therefore considered justifiable. This is carried out either by enrichment in the immediate environment of the pollutant for specific biodegradative species or by actually adding more efficient exogenous microorganisms. The later alternative could involve the 'construction' in the laboratory of species through chemical treatments or genetic engineering of organisms for specific degradative ability. An ambiguity pointed out by a number of investigators is that there is a wide margin between the success stories of laboratory simulations and what is achieved on the field practically (Grosser et al., 1991; Kelly and Cerniglia, 1991). According to Mueller et al. (1993a): *"one is quite challenged to find a single report in the peer-review scientific literature that unequivocally documents the successful use of inocula in the field."* The uses of inocula in the bioreactors (i.e. under controlled environments) appear to be the only exception outside the laboratory (Mueller et al., 1991, 1992, 1993a). The field scenario appears to indicate that as soon as the strains are applied in the open environment, they lose their presence; they are confronted by chemical and physical variables and even biotic antagonism from the indigenous, often more resilient microbial communities (biofilms); and outright predation. The end

result is that the 'trained' strains show rapid die-off enhanced by a myriad of physiological shocks (Mueller et al., 1993b).

Evaluation of In Situ Bioremediation

The central issue in the evaluation of the progress and success of in situ bioremediation is to ascertain that any loss of oil is accounted for by the biodegradative activity of microorganisms in order to confirm that oil loss was not as a result of one or more of the abiotic factors. According to the recommendations of the Committee on In Situ Bioremediation, a case for bioremediation can be inferred based on the premise of convergent lines of independent evidence of three types of information viz., (a) loss of contaminant from the site (or plot); (b) laboratory assays indicating that microorganisms from the site have the potential to transform the contaminant; and (c) one or more pieces of information to show that biodegradation potential was realizable in the field (or plot). It is an established fact that no particular method is sufficient to make a fool-proof case in in situ bioremediation assessments. This is as a result of the complex issue of interpretation of cause-and-effect involving contaminant mixtures, hydrogeochemical factors and the competing abiotic factors for contaminant loss in the field (National Research Centre, 1993).

In mesocosm experimentation in the laboratory, which is a modified field in situ bioremediation setup, different physicochemical factors could be assessed, for example, the effect of varying amounts of oil and nutrients on bioremediation. Oil could be used as weathered or fresh or pretreated (as fractionated oil). In this respect, therefore, the mesocosm setup is constitutionally very different from field experimentation. The use of indicator compounds such as hopane etc. to estimate loss of PHC as a result of spillage or inability to determine nutrients losses due to migration from site are not encountered (National Research Centre, 1993). There are different approaches for the setting up of controls to justify the inference of biodegradation of PHC: (a) for pure culture experiments in which known microorganisms are used for biodegradation and for which there is known predetermined lethal dosage, negative controls could be setup using killed organisms through autoclaving or inhibited with chemicals. This option however is technically not amenable for mesocosm experimentation in which mixed population of organisms are involved. This is for the obvious reason that biofilm consortia are hardy and may not be completely sterilized as they could exhibit over 1000 times resistance compared to the pure culture. Moreover there could be the problem of secondary colonization of a sterilized mesocosm with

more resistant species or consortia leading to unreliable assessment of PHC, which could be misleading. Other control options, which could be considered, include use of carbon-labeled PHC or use of artificial sediment. (b) For field in situ bioremediation experiments, an appropriate control could involve comparing the rate of disappearance of linear alkanes (17-18 carbon atoms) with highly branched alkanes of similar molecular weights such as pristane and phytane. Hopane, which is less degradable than pristane and phytane, could also be used (Lessard et al., 1995). Other methods make use of the ratio of stable carbon isotopes (^{13}C and ^{12}C) in carbon dioxide arising from biological mineralization to that associated with abiotic processes as these take place at different rates (Aggarwal and Hinshee, 1991). For the mesocosm experiments a common approach that could be adapted, which is different from the control for field in situ bioremediation, is to setup controls in which the estuarine biofilms were allowed to degrade residual PHC under natural conditions as a positive control. In a second control, oil could be spiked without enhancement from added nutrients. Treatments and effects could be assessed using these controls as the status quo for non-impacted and impacted controls respectively. This is similar to the practice of setting up of an appropriate pristine location or using an initial assessment of parameters as baseline values with which to compare future assessments (Treweek, 1999).

Assessment of PHC Biodegradation

Assessment and monitoring of spatially dispersed oil in wetlands is tedious and expensive (Albaiges et al., 1984). For the analysis of a large number of samples (as could be generated in a mesocosm experiment) and for continuous real-time monitoring under field conditions simpler methods, such as spectrofluorimetric methods, have been recommended in preference to gas chromatography, mass spectroscopy, high performance liquid chromatography and thin layer chromatography (Picer and Hocenski, 1994). These simpler methods also have the advantage of indicating sequestration of oil (especially if PHC could be assessed at ambient temperature) as an indication of chemical non-availability of PHC, which probably compares with the fraction of the PHC that could be metabolized (i.e. bioavailability of soluble oil fraction).

Conclusion

The increase in the world demand for PHC is indicative of the increased pollution of the earth's ecosystems,

especially of the sensitive and productive estuaries. The estuarine biofilms exhibit an appreciable biodegradative potential for PHC under aerobic and anaerobic conditions. In this capacity, different biodegradative activities have been elucidated accounting for the breakdown of alkanes, aromatics, sulphur and nitrogen-rich PHC. This is by the various microalgae and bacterial consortia such as the diatoms and the denitrifying and desulphurization bacteria under various conditions of physicochemical conditions. The involvement of these consortia appears to be the main thrust of present day in situ oil bioremediation strategies in the wetlands as the method is probably the most eco-friendly and cheapest.

The high level of pollution of estuaries especially from PHC is well documented. The survival of the teeming population of plants and animals in such ecosystems (which constitute the primary producers) depend very largely on the elaborated EPS by the estuarine biofilms. This is the interesting inter-relationship between the complex and least understood PHC and the resilient estuarine biofilms. This is fast becoming the main thrust of many studies in recent times, in the global control of pollution from fossilized hydrocarbons. The process of control of oil-pollution using indigenous consortia, such as the biofilms, of microalgae and bacteria origin, is gaining grounds especially in the Third World. The method is cost-effective and quite amenable to manipulations (in microcosms and macrocosms) for various studies.

Constitutionally the biofilms are more likely to utilize and breakdown recalcitrant compounds in view of their complex consortial activities than the pure cultures. The response of biofilms to PHC, appear to indicate variable responses. The future prospect of more research in in situ bioremediation as the method of choice in the remediation of widespread oil-polluted wetlands is very high. This is especially so when other factors that could affect the kinetics of bioremediation such as nutrient balance and the presence of large plasmid associated with oil-degradative species and nutrients are also available.

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