EFFECT OF PETROLEUM HYDROCARBON ON ESTUARINE MICROALGAL BIOFILMS IN THE WETLAND: GENERAL INTRODUCTION, LITERATURE REVIEW, AND METHODS

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A. GENERAL INTRODUCTION

1.0 Introduction

The effect of numerous and varied types of oil spillages on estuarine wetlands over the years has been a source of concern. This is because there has been scarcity of studies on *in situ* field experiments between the microphytobenthos and petroleum hydrocarbon (PHC) in wetlands due largely to constraints in environmental protection regulations. The interaction of estuarine microalgal biofilms with fractionated crude oil in a mesocosm was assessed in our study. Fractionated crude was used in the study due to the difficulty in assessing quantitative and qualitative parameters of fresh crude oil (which is volatile). In the first paper, we determined if there was any biodegradation or loss of petroleum hydrocarbons (PHC) arising from the mesocosm-based experiments. In our second paper we assessed the state of health of the biofilms (i.e. their fecundity) as determined by their photochemical efficiency. The effect of PHC on biofilms ability to synthesize chlorophyll-*a* / phaeopigment and carbohydrates were reported in our third and fourth papers respectively. Earlier studies on microbial interactions with crude oil (especially those studies that were based on pure

culture isolates) were divergent in their findings probably due to the fact that the biofilms gave varied responses in the presence of varying amounts of crude oil (whether fresh or weathered) and this was also affected by the type and amount of nutrient and the type of biota present in the ecosystems. In this study, these responses, using fractionated crude oil (i.e. stabilized crude oil) have been classified as three distinct responses. In response I, the baseline status of the biofilms (i.e. the effect of attenuated sedimentary PHC in the environment) was assessed. In response II, the biofilms ability to cope with spiked oil in the absence of added nutrient was assessed. While in response III, the biofilms biosynthetic ability after spiking with oil in the presence of added nutrients was assessed. The effect of PHC at varying concentrations, under graded nutrient enrichment in the light and in the dark, were assessed based on these responses.

{**Citation:** Lovet T. Kigigha, Graham J. C. Underwood. Effect of Petroleum hydrocarbon on estuarine microalgal biofilms in the wetland: general introduction, literature review, and methods. American Journal of Research Communication, 2013, 1(6): 136-173} www.usa-journals.com, ISSN: 2325-4076.

B. GENERAL LITERATURE REVIEW

2.1 The Biofilms

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. The concept of biofilms is often given variable descriptions among scientists. According to Wimpenny (2000) biofilms form at a phase boundary generally, but not always, at a liquid/solid interface; spatially and temporally heterogeneous; may have specific mechanisms for attachment to surfaces; generates exopolymer polysaccharide for adhesion, protection and to facilitate community interactions. 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); 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; Costerton et al., 1985; Hamilton, 1985; Stal and de Brouwer, 2003). The two basic defining characteristics of biofilms are that they occur on surfaces and that they are aggregates of a mixture of organisms enclosed in their exopolysaccharide metabolic products.

2.2 Biofilms Study

Interest in biofilms studies could be traced to the recognition of the inability to control infestations by microbial communities using the classic pure culture antimicrobial methods in various aspects of the environment. They are shown to be highly resistant to all forms of chemical applications aimed at their eradication; this indicated the difference, physiologically between the isolated organisms from the community. The development of the study of microbiology for the past 150 years following the Koch's Postulates according to Costerton (2000) was a digression which was not in consonance with the ecological approach adopted in other fields of study in Biology. The study of biofilms requires the introduction of separate conceptual and experimental approaches that are wide departures from the classic microbiological techniques. This requires the *in situ* study of live aggregates of mixed species as they carry out their physiological role in the ecosystem. A different principle of microscopy is used which allows the examination of living and fully hydrated organisms. For example, the Confocal Scanning Laser Microscope (CSLM; Lawrence *et al*., 1991) or the High Resolution Imagery Technique (developed at the University of Essex, U.K). These methods operate by subtracting out-of-focus planes from the image, thereby making it possible to examine both the surface and the bulk fluid components, including the slime-enclosed biofilms (Geesey et al., 1977). Also, these novel imaging techniques (with resolutions of less than 5µm) are

coupled with an array of physical and chemical probes making it possible to collect data on factors such as oligonucleotide sequence (for the direct identification of species), pH, dissolved oxygen etc.

2.3 Resilience of biofilms

The mode of growth of microorganisms in their natural environment appears to be in the form of cell aggregates. It has enabled several bacterial species to colonize harsh environments especially in the medical field and the industry. In particular, biofilms are found to be involved in chronic infections and infections involving the use of various implantations of medical devices (Costerton et al., 1987). They play very active roles in bio-fouling and bio-corrosion of pipelines of heat exchangers etc (Characklis, 1990; Little et al., 1990). Biofilms are involved in increasing frictional resistance to fluid flow on ships, water conduits; in 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 have reported on the broad spectrum and extent of resistance of the biofilms to antibiotics, biocides, antiseptics etc. This is in the region of 100-1000 times that of the free living forms (Allison and Gilbert, 1995; Costerton et al., 1987).

Reasons have been adduced for the observed 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 antimicrobial agents 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 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 biofilms. In dense biofilms, 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 antimicrobials 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 biofilms specific phenotypes are induced through cellular attachment to surfaces. (Allison et al, 2000).

2.4 Mechanism of Biofilms response to pollutants

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 (Rothemund et al., 1996; Mason et al., 2003). 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. It is important therefore to understand the mechanisms of microbial communal responses to pollutants. Recent publications based on studies of pure culture biofilms have indicated a number of possible mechanisms: First, from the study of the interactions between two organisms with respect to syntrophic cooperation or antagonism/inhibition or production of bacteriocins; Karthikeyan *et al.,* 1999, showed a situation in which one organism depended on another in the biofilms for protection from high concentrations of an inhibitory substance. The observed co-culture phenomenon was also reported in which an anaerobic heterotroph and a hydrogenotrophic methanogen grew together without the need for the supply of hydrogen and CO_2 for the methanogenic species. Secondly, in flow systems it has been suggested that interacting organisms established a diffusion-dominated stable microenvironment through the production of extracellular polymeric substances (EPS).

Boult et al., 1997, have suggested that diffusion properties and charged reactive groups in the EPS may be responsible for the steady state in the 'heterogeneous organization of cells.' Decho (1999), reviewing the role of EPS has suggested that the high density of cells in the biofilms could create a diffusion barrier that could localize chemicals released by the cells. Further, that such an assemblage could lead to the formation of auto inducer-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). Geesey and Costerton, 1986, suggested that there is a greater likelihood of utilization and breakdown of recalcitrant compounds (such as PHC) by the consortia activities in the biofilms than in the pure culture.

2.5. Biofilms response to 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 their size, composition and metabolic activities (Pfaender and Buckley III, 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. The incidents of oil spill especially in the aquatic environment in the later part of the last century provided the basis for much research work on the response of microbial communities to petroleum.

A survey of previous research works on the response of benthic microbial communities to PHC in the United Kingdom and other parts of the world 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. They observed 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*., (1976a), on marine sediments from the Atlantic Ocean using 1% mixed hydrocarbon components showed a decrease in the TCFU.

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 Hudson 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. Reports on the effect of petroleum on the metabolic activities of microbial communities also showed variable responses (Griffiths et al., 1981a).

2.6. Bioremediation potentialities of biofilms

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. An effective bioremediation of polluted sites (especially with hydrophobic pollutants like petroleum) requires an understanding of the characteristics of the pollutant with respect to: its bioavailability for degradation; sorption/desorption kinetics with the ecosystem and the presence of surface active agents. These have been reported to be the reason for the persistence of certain pollutants in the soil, sediment, aquifers etc (Jafvert and Weber, 1992; Coates and Elzerman, 1986; Rao *et al*., 1993). Petroleum hydrocarbons are made up largely of hydrophobic components especially the poly aromatic hydrocarbons (PAH) being very low in solubility in the aqueous phase (Weber *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 because they naturally produce hydrocarbons and thus have the biochemical capability to degrade them (O'Brien and Dixon, 1976; Ellis, 1977; Cerniglia and co-workers, 1980; Walker *et al*., 1975b).

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 (Admiraal, 1984; 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; loss of stability was indicated only when the perturbations were high. Steinberg *et al*., 1987, showed that entrapment of organic chemicals in the intra-particulate micro-pores of soils or sediments were a source of residual materials as a result of 'irreversible'sorption.

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 (Edwards *et al*., 1991; Liu *et al*., 1991); nevertheless, their usefulness has been overtaken by the controversy relating to their innate toxicity and persistence in the environment (Robichaux and Myrick, 1972). 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). The oil degrading biofilms are also known to be associated with large plasmids whose presence has been correlated with the ability to produce surface active agents (Floodgate, 1984; Widada *et al*., 2002).

2.6. Petroleum hydrocarbon

Estuarine wetlands are continually exposed to chronic and accidental spillages of petroleum hydrocarbons (PHC). As low-energy ecosystems wetlands accumulate and enhance the deposition of PHC (Little, 1987; Kigigha and Underwood, 2009). Wetland sediments are efficient in sequestering PHC; and once contaminated with PHCs, they take decades to bioremediate naturally without some form of intervention to enhance their breakdown in the environment (Catallo, 1993). This is compounded by the complex nature of PHC (Pfaender and Buckley III, 1984; Clark et al.,

1997) and the poorly understood sequestration-bioavailability mechanisms between PHC and sediments in the wetlands (Alexander, 1999).

Furthermore, the biofilms (bacterial and/or microalgal) have their complex and least understood interaction with various contaminants and ions in their immediate environment (Karthikeyan et al., 1999).They respond to any given contaminant according to their stored information on previous encounter with the contaminant (Carman *et al*., 2000). Thus, deductive conclusions about PHC and biofilms interactions in the wetlands from previous works are rarely precise. Emphasis therefore is on observable overview trends and departures in the results of manipulated and conditioned experiments often carried out in microcosms or mesocosms.

2.7. Bioavailability of dispersed oil

An important property of non-aqueous phase compounds such as PHC is their sorption on to surfaces and particles, and sequestration in sediment. Contaminants have been known to be variously rendered non-available for biological degradation by these phenomena (Loehr and Webster, 1997). The detailed understanding of how these phenomena could affect bioavailability of contaminants is outside the scope of this study nonetheless, the issue about how bioavailability of PHC could vary under diel conditions is of interest. Various reasons have been adduced to explain the occurrence of non-availability of contaminants. Firstly, that background small populations of contaminant degradative organisms need a period of increase in number to substantially degrade the contaminant. This view is supported by the observed increase in bacterial population (especially in a low nutrient ecosystem) from the addition of nutrients (Hekmat *et al.,* 2004). Secondly that the presence of toxic substances in a contaminant, such as the volatile components in PHC (in this study; this factor had been minimized by the fractionation of the crude oil before use in the mesocosms) could extend the lag phase period before biodegradative organisms begin to become dominant (Atlas and Bartha, 1972). Other factors that could contribute to extended bioavailability include the effect of predators

on the degradative organisms and the delayed appearance of contaminant degradative genotypes where they may be lacking in the ecosystem. Of particular interest to this study is the explanation that contaminants could be non-available for biodegradation as a result of diauxie factor or the preferential breakdown of one compound to a second one (Harder et al., 1983).

Various authors have estimated the period required for bioavailability of different contaminants. For example, 4-Nitrophenol breakdown in water-sediment interphase required 40-80 h and the breakdown of chlorinated benzenes by bacterial biofilms 10 days – 5 months. There is no certainty therefore with regards to when a contaminant could be available for biodegradation as it results from the interplay of a number of biological, chemical and ecological factors (Alexander, 1999).

2.8. Toxicity of petroleum hydrocarbon

Petroleum hydrocarbons occur in the environment from natural and diagenic sources; anthropogenic activities are responsible for their widespread environmental contamination. The potentiality for PHC degradation is thus widespread in several bacterial species in the ecosystem especially the aerobic forms. Some of the frequently isolated genera include: *Pseudomonas, Achromobater, Flavobacterium, Acinetobacter, Vibrio, Bacillus, Arthrobacter, Norcardia, Corynebacterium* and *Micrococcus.* Some genera are only occasionally involved, these include: *Enterobacteria, Brevibacterium, Azotomonas, Protaminobacterium, Mycococcus* and *Aeromonas* species (Das and Chandran 2011).

There is abundant evidence supporting the observation that indigenous bacteria respond to increased oil concentration in the environment by a corresponding increase in oil-degradative capacity. This is brought about by either a selection of oil-degrading strains or by induction of enzymatic mechanisms within the organism or even by a contribution of both processes. Areas of chronic oil pollution such as the oil shipping lanes, seaports, offshore oil terminal, and

estuaries etc show increase in bacterial populations that are hydrocarbonoclastic (Gunkel and Gassman, 1980). Atlas, (1981a) concluded that in non-polluted sites less than 0.1% of the total heterotrophic population has the capacity to degrade oil.

Generally the water-soluble components of petroleum (fresh, weathered, refined or fractionated products) are toxic to a wide spectrum of plants and animals. By comparison, the aromatics are more toxic than the aliphatics; the middle molecular weight components are more toxic than the high molecular weight tars. The low molecular weight compounds are usually lost through evaporation into the atmosphere. Most of the lighter toxic components of spilled crude oil are lost through volatilization soon after a spillage. They exhibit a '*hit and run'* lethal effect on sensitive organisms (Clark et al., 1997). In this study crude oil was fractionated to avoid the difficulty in assessing the effect of the volatile components of PHC on the biofilms.

Under aerobic conditions, alkane degradation pathways have been identified in *Pseudomonas putida (formerly P. oleovorans)* (van Beilen et al 1994; 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 (PAH) 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). Research in anaerobic biodegradation of PHC involving microbial consortia was reported in the review by Widdel and Rabus, 2001 and Jani *et al*, 2004; toluene biodegradation had been linked to denitrifying bacteria processes in *Azoarcus* sp, and *Thauera aromatica* (Achong *et al*., 2001; Beller *et al*., 1997, 1998). Species of *Dechloromonas* have been associated with benzene biodegradation (Coates *et al*., 2001).

The Microalgae constitute the major primary producers of the oceans; interactions with petroleum capable of causing serious effects on these organisms could consequently affect other

organisms higher in the food web. There is much interest in determining the response of these organisms in oil-polluted environments. In a review by O'Brien and Dixon (1976), it was concluded that, the effects of accidental oil spills on phytoplankton yielded no observations particularly incriminating to oil or its residue. 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. Walker et al., (1975c) showed that a particular strain of achlorophyllous algae, *Prototheca zopfi*, isolated from the polluted sediments from Colgate creek, Baltimore Harbour, was able to degrade 10% of motor oil and 40% of crude oil. The degradation potentiality was comparable to that of bacteria (Walker et al., 1975b). Interestingly, this strain degraded more of the aromatics than saturates when grown on fuel oil. Furthermore, when grown on crude oil, the resin and asphaltene fractions were more reduced. Much of the research work that related to algae petroleum interactions has been on the effects on growth, respiration and photosynthesis. Various authors have reported on the toxicity of hydrocarbons as regards adverse effects on respiration and photosynthesis at high concentrations (Karydis, 1979). Adekunle and Adebambo (2007), reported on the utilization of PHC by fungi isolated from *Detarium senegalis* (J.F.Gmelin) Seeds.

Physical effects such as temperature and light intensity that are seasonally dependent have also been reported to be important factors regulating toxicity (Shield et al., 1973). However, low oil concentrations have been reported to stimulate photosynthesis particularly in the microalgae (Gordon and Prouse, 1973; Karydis, 1979). Other workers, Prouse et al., (1976) have used the growth of algal cell to measure the effect of petroleum on the environment. In concentrations of less than 1 mg l^{-1} of Venezuela and Kuwait crude and No. 2 fuel oil, either no response was reported or there was a slight stimulation of growth. Armstrong and Calder (1978) have suggested that the primary effect of petroleum interaction with the microalgae is on the electron transport system. Two regimes of fractionated oil concentrations were assessed in this study to ascertain the effects of low

and high oil toxicity on the biofilms. The effect of fractionated crude oil on the physiological attributes of the biofilms such as the photochemical efficiency, ability to synthesize chlorophyll – *a* and phaeopigment and production of carbohydrates were also assessed.

2.9. Factors affecting microbial productivity

The estuarine intertidal sediments are dominated by the microalgal biofilms especially the epipelic diatoms (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, 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 mineral nutrient is not limiting, affects primary production (Pennock, 1985; Cole et al., 1986). For 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 (Smith and Underwood, 1998). In terms of nutrient limitation, phytoplankton are affected both in their photosynthesis (the Blackman type of limitation), and in biomass production (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; Heip et al., 1995).

The increase in bacterial population by 3-5 times from the addition of inorganic nitrogen and phosphate (i.e. N-P inorganic fertilizers) during bioremediation of oil spillages in marine and estuarine ecosystems is cited by a number of authors. PHC biodegradation in the marine ecosystems is limited by the availability of oxygen, nitrogen and phosphorous (Lessard et al., 1995; Yehuda 2002). Fries et al., (1994) and Bregnard et al, (1997) indicated the use of nitrates as alternate electron donors under anaerobic conditions to enhance PHC biodegradation. Caldwell *et al*., (1998) indicated that sulphates also act as alternate electron donors. Thus, it could be inferred that nitrates, sulphates and probably phosphates are useful not only as nutrients but also as electron donors when oxygen gradients may be low as in the oil-contaminated wetlands. In oil oxidation processes Nitrogen and Phosphorus are found to be limiting factors. These factors are not available in petroleum and are also limiting in the seawater; though oil increases the carbon and energy sources (Ran and Jeffrey 2003). 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 Nitrates and Phosphorus in the degradation of petroleum (Reisfeld et al., 1972, LePetit and N'Guyen, 1976).

The degradation of petroleum involves the direct incorporation of molecular Oxygen. In the open sea, oxygen is never limiting. According to Floodgate (1976b), the *Michaelis Constant* of oxygenases indicates that the enzymes have a high affinity for oxygen and will therefore continue to function even where the concentration of dissolved oxygen is low. Even though some mineralization of alkanes under anaerobic condition takes place in the sediment (Hambrick *et al*., 1980), it has been demonstrated using radio labelled hydrocarbons, that much of the degradative activity is aerobic and that this occurs in the upper 5 cm of sediment (Ward *et al*., 1980; Jani et al, 2004). The effect of fertilization from these inorganic nutrients in the field is limited by their leaching out of the treatment site. To control this effect oleophilic fertilizers are often used. In experiments inorganic nutrients could be proportionately added and mixed thoroughly with the oil before application to achieve oleophilic conditions in order to reduce leaching-out of nutrients (Röling *et al*, 2002). In this study,

the effect of variation in the enrichment of phosphate and nitrate at three different levels mixed with the fractionated crude were assessed.

2.10. Assessment of the response of bacteria to PHC

The response of bacteria to petroleum hydrocarbon can be assessed with respect to changes in biomass, community structure, biochemical capabilities of the communities and changes in the physiology of individual strains. Cobet and Guard (1973) studied the effects of a series of compounds representing several chemical classes derived from petroleum on a number of bacterial strains; a variety of responses were observed. Batch-cultures of *Serratia marinarubra* and *Vibrio parahaemolytica* in the study produced less growth in the presence of dissolved aromatic hydrocarbons in magnitudes that were proportional to the concentration and toxicity of the hydrocarbons.

Also, partial oxidation products of naphthalene (as found in weathered oil) were found to be more toxic than the parent compound. In a similar study by Griffins and Calder (1977) the toxic effects of the water-soluble fractions of three crude and two refined oils were tested on *Serratia marinarubra*. It was found that growth rate and maximum cell density were reduced in the batch-culture. The indication was that artificial weathering increased the toxicity of the water-soluble fraction (Obire *et al,* 2008). This inhibition was partly overcome by the addition of yeast extracts. Walker et al., (1975e) found that there was a decrease in proteolytic, chitinolytic and cellulolytic activity in a number of physiological groups of oil-degraders when challenged with South Louisiana crude and No. 2 fuel oil. Griffiths and Morita (1980) in a similar study also recorded reduction in cellulose and chitin breakdown, but then there was a stimulation of starch and algin breakdown.

Walsh and Mitchell (1973) reported on the interference with the chemoreception mechanism when challenged with Kuwait crude oil, toluene, phenol and several pure hydrocarbons. Holloway et al., (1980), observed that oil from Buccaneer field did not affect either the growth or the

attachment mechanisms of isolated strains. Nitrogen fixation biological processes in the marine ecosystem were neither stimulated nor inhibited by a series of low molecular weight hydrocarbons. However the presence of carbohydrates stimulated Nitrogen fixation (Knowles and Wishart, 1977). In contrast, however, Griffiths et al., (1981a, b) found inhibition by low concentrations of crude showing reduction in denitrification and phosphatase activity. Generally, the synthetic ability and transport mechanisms appear to be adversely affected by hydrocarbons (Griffiths *et al*., 1981b).

Comparatively, there is a paucity of published work on how the genome of a strain of microorganisms is expressed in the randomly mixed populations or microbial community or biofilms as they occur in the natural ecosystems. Meyer *et al*., (1975), studied the dynamics of mixed bacterial populations with a number of metabolites. The model, which they developed, predicted a steady state over a range of small perturbations with a loss of stability if the disturbances were large. Soli (1973); Soli and Bens (1972) challenged randomly selected isolates from seawater with 28 different hydrocarbons. The results showed that the n-alkanes were the most readily degraded, next were the iso-alkanes; the cycloalkanes; the olefins, tended to be toxic; the aromatics at low concentrations readily served as substrates. This was in agreement with the observation of Horowitz *et al*., (1975). They tested the sequential growth of several strains on crude oil; it was observed that one strain removed two-thirds of the petroleum; the residue supported the growth of two further strains, which resulted in the production of new paraffin compounds (Walker and Colwell 1976c). Jobson et al., (1979) studied the relationship between aerobic and anaerobic bacteria with respect to petroleum degradation; while the pure nor mixed cultures of sulphate reducers could not grow on oil anaerobically, such strains could easily grow on the residues from aerobic attack on oil.

The stability of the genotype of hydrocarbonoclastic bacteria has been a subject of some curiosity. Hada and Sizemore (1981); Zajic and Westlake (1976); Gutwick and Rosenberg (1977); had observed that the area close to an oil field had more plasmid–bearing strains than an area that is not contaminated by oil. It was also observed that there was a corresponding increase in the

ability to produce surface active materials (some form of biosurfactants important in petroleum degradation in the environment). The speculation is that the rapid degradation rates shown by oil-degrading strains was the result of a genome that imparts a wide specificity degradative capacity possibly due to the presence of plasmids, and the ability of such strains to produce surface–active agents (Floodgate, 1984; Jonathan *et al* 2003).

2.11. Sediment stabilization in the estuaries

Sediment in natural waters (including those in the ocean bed) is usually associated with biological activity. This is shown by the presence of extracellular polymeric substances (EPS) exuded by the diatoms among many other organisms (Craig *et al*., 1990; 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). The production of EPS by diatoms is shown also to be influenced by the amount of mineral 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; Mason *et al* 2003).

2.12. Mesocosm experimentation

Manipulated field experimentation had been recommended for the study of interactions between environmental contaminants and biological systems (Underwood & Peterson, 1988; Madsen, 1991). The method of enhancement of indigenous organisms' remediation of contaminants or bioremediation had been adjudged cost effective compared to evacuation and or incineration of oily wastes in wetlands (*Natl. Acad. Sci*., 1985). There is scarcity of study on field experiments between the microphytobenthos and contaminants in wetlands due largely to constraints in environmental protection regulations.

Mesocosm experimentation allows for a control of conditions while the effect on the organisms can be simulated (Kuiper *et al*., 1984). In an earlier study, Carman and co-researchers used microcosm experimentation to compare the effect of exposure to diesel contamination on two benthic salt-marsh communities. They suggested that spatial and temporal consistency of diesel impactation resulted in predictable community responses (Carman *et al*., 2000). Much research work on the effect of PHC on microbial community size and composition had been carried out in the past. Byrom *et al.,* (1970), found increases in the total colony forming units (TCFU) and shifts in the predominant genera when sediment from the English estuary was spiked with Kuwait crude. Walker *et al.,* (1976a), reported decreases in TCFU when marine sediment from the Atlantic Ocean was spiked with mixed hydrocarbons. A number of reasons have been adduced for the varied observations on response of microbial communities to PHC; the magnitude of response was found to be a factor of the habitat and nature of organism and the amount of impacting PHC (Caperello and LaRock, 1975; Westlake et al., 1980).

There are no guidelines on the amount of nutrient supplementation during bioremediation neither were there specifications on the nature or pre-treatment of the oil that was used (Head & Swannell, 1999). There is scarce published work on diurnal effect on the innate physiological attributes of the microbial communities during bioremediation. The mesocosm experiments described in this study were therefore designed to investigate the effect of diurnal variation and nutrient enrichment on the enhancement of natural bioremediation capabilities of the estuarine biofilms. The following hypotheses were tested:

1. That there was no loss of PHC arising from the mesocosm treatments under diurnal conditions.
2. That there were no variations arising from the effect of PHC on the physiological variables of the biofilms (i.e. chlorophyll-*a* and phaeopigment, photochemical efficiency and carbohydrates) arising from the mesocosm treatments under diurnal conditions.

2.13. The approach to the study

The focus of this study was on the interaction of fractionated oil with estuarine microbial communities (biofilms). This involved the elucidation of the impact of oil on the composition, productivity, and physiological state of the biofilms. While many studies have established that the PHC in high concentrations is toxic to organisms (Neff and Anderson, 1981); impair physiological output through reduction in their physiological capability (Capuzzo, 1987; Cowles, 1983b); alter DNA synthesis etc these studies do not predict effects on the microbial communal functional interactions and between trophic levels (Elmgren *et al*., 1980).

Three main types of approach are usually adopted for the assessment of the impact of oil 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 degradation. Generally all such experiments in their various modifications and combinations relate very poorly to what actually takes place in the natural environment for the fact that 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, one of the closest approaches to what takes place in the ecosystem, was designed for this study (Kuiper *et al*., 1984; Carman *et al*., 1995; Greer *et al*., 2003).

2.14. Aims and objectives of study

The study was designed to investigate the following:

1. The background status of the estuary as regards the environmental condition of existence of the biofilms prior to impactation with oil (Biofilms Response I).

2. The bioremediation status of the estuarine biofilms from oil impactation without nutrient enrichment (Biofilms Response II).

3. The bioremediation status of the estuarine biofilms from oil impactation with nutrient enrichment (Biofilms Response III).

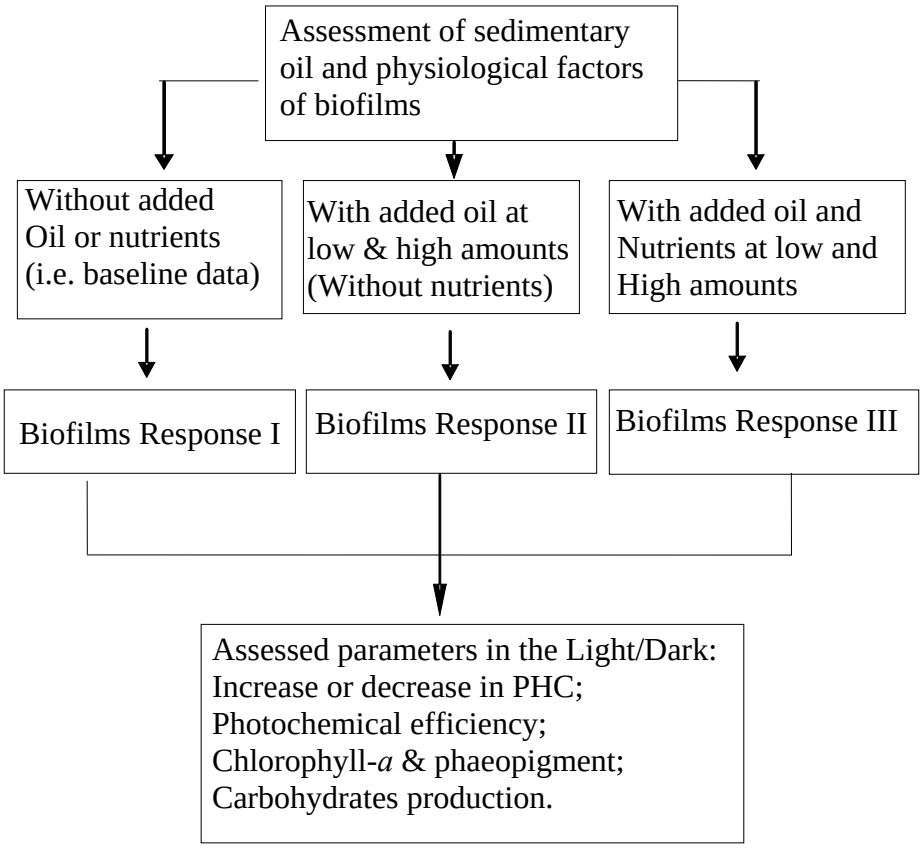

Fig 1: Biofilms oil bioremediation and enrichment strategy in the wetland.

The exploratory study was initiated to elucidate the oil bioremediative potential of the estuarine biofilms as shown in Fig 1. To the extent of the probable limitations and constraints, the study was designed to achieve the stated objectives and hypotheses. Based on the known characteristics of the biofilms (especially their interaction with contaminants in the ecosystems) three responses I, II and III were conceptualized and investigated and assessed as probable occurrences requiring appropriate interpretation of results.

C. GENERAL MATETRIAL AND METHODS

3.1. Location

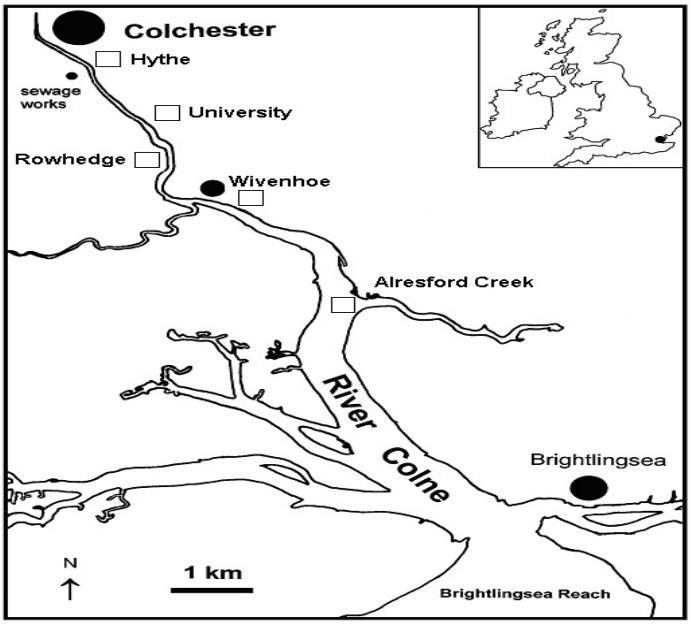

Fig 1. Map of River Colne estuary (Essex, U.K.) showing five sampling locations

HYT (Hythe); UNI (University); RHG (Rowhedge); WIV (Wivenhoe) and ALR (Alresford).

3.2. Experimentation

3.2.1. Selection of site for sediment and water for mesocosms

Five wetland locations (at the Hythe, University of Essex, Rowhedge, Wivenhoe and Alresford sailing boat sites) on the River Colne estuary in the United Kingdom were used for the preliminary evaluation of best site for sediment collection for the mesocosm study (as shown in Fig 1). All the sites were located on tidal mudflats and rich in algal blooms. They were selected for the study, as they were locations on the River Colne estuary with a high anthropogenic activity in the vicinity (including high vehicular movements, sewage treatment plant, agricultural production etc) and were close to the University of Essex, and suspected to be rich in hydrocarbonoclastic organisms (Carman et al., 2000).

3.2.2. Sediment collection for mesocosms setup

Sediment collection was carried out separately for the five locations. Samples were collected at low-tide on the same day and taken to the laboratory within one hour after collection. Corers of poly vinyl chloride PVC (i.d = 7.0 cm; depth = 12.0 cm) five for each location were used to collect core sediment with intact diatom-mat surfaces. The cores were carefully stored in plastic boxes in an up-right position for analysis. The baseline characteristics of River Colne estuary at Wivenhoe, Rowhedge, University of Essex and Alresford waterfronts, determined in a preliminary experiment however indicated that among the five locations assessed, it was the Wivenhoe location that was the most suitable for use in bioremediation trials. The site was the lowest in spatially distributed PHC in the sediment and river water (thus it would be more amenable to further impactation with oil); high in carbohydrates, and low in inorganic nutrients (and so it could be more amenable to further nutrient enrichment trials).

3.2.3. Mesocosm setups

Four different mesocosm experiments (A-D) were run over a period of four months. Estuarine water was collected in washed 30 litre PVC jerry-cans and transferred to the lower tanks of the mesocosm units. Each set of mesocosm system (consisting of an upper and lower tank) in which the upper tank received ten sediment cores and the lower tank received 26 litres of water. Water was immediately circulated between the upper and lower tanks of the mesocosm using submerged aquarium pumps for aeration. A drilled hole on the upper tank allowed water to pour back into the lower tank during the low tide. Sediment cores were collected from the Wivenhoe waterfront with the algal-mat surface area facing upwards in each of the mesocosm upper tanks.

Each experiment (A-D) consisted of 18 independent mesocosms grouped in treatment replicates of three (i.e. $n = 3$). Each mesocosm was made up of two plastic tanks (30 litres capacity each). Nine mesocosms were kept unshielded from natural daylight while another set of nine were

covered with dark polythene (with few perforations for aeration) to provide darkening continually for the period of the experiment (Fig 2 (a & b)). The upper tanks contained 10 cores of sediment each. Each core had four diametrically cut 5 cm slits to allow draining of the core surface during ebb. The lower tank in each of the mesocosm was fitted with an aquarium pump and contained 26 litres of water from the estuary. The flow and ebb in all the mesocosms were synchronised and maintained at 6h intervals to mimic a natural estuarine tidal flow using a digital timer. Experiments were carried out at predetermined times at fixed temperature, humidity and light intensity in an automated green house. Temperature and light intensity were maintained at 18-25 ^{o}C and daylight respectively.

The addition of nutrients into each mesocosm was carried out as shown in Table 1. Nitrate was added with respect to the amount of PHC: at the rate of 3% (i.e. n), 6% (i.e. N) and 9% (i.e. 3N) for nitrate (w/v of the measured amount of oil). Phosphate was added with respect to the amount of PHC: at the rate of 0.3% (i.e. p), 0.6% (i.e. P) and 0.9% (i.e. 3P) for phosphate (w/v of the measured amount of oil) (see Röling *et al*., 2002). The nutrients were measured accordingly and added in aqueous solution to the fractionated crude oil and thoroughly emulsified for 15 min in a shaker before addition to the mesocosms (in mg l^{-1} of water in each Mesocosm unit) to reduce loss of nutrients through leaching out.

The oil had been stabilized through fractionation by boiling in a fractionating column until the temperature range was between 174-180 ^{o}C. The fractionation was carried out under reduced pressure in a round-bottomed flask containing glass bumpers on which was attached the fractionating column in an atmosphere of N_2 gas. At this range of extraction the volatile components with less than eleven C- carbon atoms were removed, in order to reduce PHC losses from volatilization, photo-oxidation etc. during mesocosm experimentation.

Table 1: Mesocosm treatment setup. Experiment: A (Treatments: 1-8); Experiment: B (Treatments: 9-16); Experiment C (Treatments: 17-24); and Experiment: D (Treatments: 25-32). L = Light; D = Dark; *Crb* = control without oil or nutrients added (a); *Cra* = control with added oil without added nutrients (b); NO_3^-: *n = 0.77 mg l^{-1}; N = 2.31 mg l^{-1}; 3N = 6.92 mg l^{-1}; PO_4^{3-}: p = 0.077 mg l^{-1}; P = 0.231 mg l^{-1}; 3P = 0.692 mg l^{-1}; Low oil: *I = 21.5 mg l^{-1}; High oil: II = 64.5 mg l^{-1}; M = Mesocosm. Treatments were designed to overlap with stepwise increments in oil and nutrients enrichment to allow for comparison.

Treat. No	Treat. Descript.	Expt.	NO_3^-	PO_4^{-3}	L/D	Treat. Code
1.	Low oil (I) + nutrients	A	n	p	L	L I np
2.	Low oil+ nutrients	A	n	P	L	L I nP
3.	Low oil; no nutrients	A	Nil	Nil	L	L I A Cra
4.	No oil; no nutrients	A	Nil	Nil	L	L Crb#
5.	Low oil+ nutrients	A	n	p	D	D I np
6.	Low oil + nutrients	A	n	P	D	D I nP
7.	Low oil; no nutrients	A	Nil	Nil	D	D I A Cra
8.	No oil & nutrients	A	Nil	Nil	D	D Crb#
9.	Low oil+ nutrients	B	N	p	L	L I Np
10.	Low oil+ nutrients	B	N	P	L	L I NP
11.	Low oil; no nutrients	B	Nil	Nil	L	L I B Cra
12.	Low oil + nutrients	B	Nil	Nil	L	L Crb
13.	Low oil + nutrients	B	N	p	D	D I Np
14.	Low oil + nutrients	B	N	P	D	D I NP
15.	Low oil no nutrients	B	Nil	Nil	D	D I B Cra
16.	No oil; no nutrients	B	Nil	Nil	D	D Crb
17.	High oil (II) + nutrients	C	N	P	L	L II NP
18.	High oil + nutrients	C	N	3P	L	L II N3P
19.	High oil; no nutrients	C	Nil	Nil	L	L II C Cra
20.	No oil; no nutrients	C	Nil	Nil	L	L Crb
21.	High oil + nutrients	C	N	P	D	D II NP
22.	High oil + nutrients	C	N	3P	D	D II N3P
23.	High oil; no nutrients	C	Nil	Nil	D	D II C Cra
24.	No oil; no nutrients	C	Nil	Nil	D	D Crb
25.	High oil + nutrients	D	3N	P	L	L II 3NP
26.	High oil + nutrients	D	3N	3P	L	L II 3N3P
27.	High oil no nutrients	D	Nil	Nil	L	L II D Cra
28.	No oil; no nutrients	D	Nil	Nil	L	L Crb
29.	High oil + nutrients	D	3N	P	D	D II 3NP
30.	High oil + nutrients	D	3N	3P	D	D II 3N3P
31.	High oil no nutrients	D	Nil	Nil	D	D II D Cra
32.	No oil; no nutrients	D	Nil	Nil	D	D Crb

*I: Oil was diluted in n-hexane; *n: nutrients were diluted with water before being emulsified with oil. L Crb# and D Crb# were separately determined in triplicates and were the same values for all the Mesocosm Experiments.

Two controls were included in each mesocosm experiment: in the first control (*Crb*), no nutrients and oil were added (i.e. an estimation of the sedimentary residual oil prior to oil spiking). In the second control (*Cra*), oil was added without nutrients (i.e. assessment of the bioremediation potential of the unaided estuarine biofilms from spiked oil). Six ebb and flow periods were allowed for the physical and chemical equilibration of the mesocosms before baseline data was measured. After the addition of the oil and nutrients at high tide, one ebb and flow period was allowed (about 6h) before 0 hour reading was taken. This was to ensure impactation of sediment from the added oil and nutrients. Two cores were taken out for analysis of Chlorophyll-*a* and Phaeopigment, Photochemical efficiency and Carbohydrates, each time from each mesocosm. Samples were collected at 5 intervals: baseline (before oil was added); 0 hour, day 3, 7 and 14.

3.3. Statistical analysis

Using the *Zigma Stat 32* statistical package, data were tested for normality of distribution and equality of variance (respectively the *Kolmogorow-Smirnov* and the *Levine Median tests*). Data that departed from normality or homescedasticity were analyzed using the *Kruskal-Wallis* ANOVA on ranks with *post hoc* analysis by *Dunn's method*. Where normality and homescedasticity were satisfied, data from each day and treatment were analyzed using *One-Way Analysis of Variance* (ANOVA) or by *Two-Way Analysis of Variance* ANOVA when data from each day and treatment for light and darkness were compared. *Post-hoc* Tukey tests were used to identify significantly different groups. All the effects were considered significant at the P= 0.05 or lower.

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