Protocol Development for Marine Oil Spill Bioremediation

Bin Xie

Abstract

Marine oil spill mainly comes from the leak or wreckage of petroleum cargo tanks, and is severe compared to other types of oil spill for the huge amount of oil pollution to the marine, beach and shore ecosystem. Marine oil spills devastate local environment and ecosystems not only in the water bodies, but also affects mammals and birds since oil would be deposited on the beach and shore. Bioremediation is a relatively new methodology to clean up marine petroleum oil spill compared to conventional treatment relying on manual labor or chemical agents. Bioremediation uses and promotes microorganisms as consumer of hydrocarbons, thus is preferred over conventional methods for its environmental significance of minimized toxic effects. Since the passage of 1990 Oil Pollution Act (OPA-90), the science of oil spill bioremediation has gained momentum. The bioremediation technology has been successfully implemented in several severe marine oil spill cases including the 1989 (39k ton) Exxon Valdez oil spill in Alaska, the 1993 (85k ton) Braer oil spill in Sheland Island, and the 1996 (72k ton) Sea Empress oil spill in eastern Atlantic Ocean. Due to the variant composition of the petroleum and complexity of each oil spill case, a protocol must be designed for the optimal bioremediation strategy. Based on the known literatures in the past fifteen years, this literature review focuses on the development of a five-tier protocol for a successful implementation of marine oil spill bioremediation.

Key words

Protocol for Oil Spill Bioremediation, Bio stimulation, Optimal Nutrition Application

Introduction

Millions of gallons of oil are lost in marine environment which severely harms the environment by killing fish, birds, wildlife and biota, destroying habitat and food, producing toxic effects in organisms and ecosystems, contaminating surface, ground and drinking water, wasting nonrenewable resources, and costing the economy multi millions of dollars to clean up (USEPA, 2001). The composition of the oil spills differs from one to another, and the accurate risk and toxicity assessment is always case based. However, oil spill share in one common point is that the toxicity imposed on local ecosystem. For example, oiled wildlife dies immediately because oil fouls fur and feathers and destroys their insulation value, and birds and mammals in extreme north and south would die of hypothermia (coldness); oil impaired marine species will die gradually, because the oil caused organ damage or blindness would make them uncompetitive for food or predators. Oil also alter the physical environment by forming hard, cement-like pavements, which cover and smother the vegetations and aquatic invertebrates which are the water, food source and shelter for a lot of plants and animals, therefore a lot of organisms can no longer survive in their old habitat (NOAA, 2000).

Protocol Development

Characterized remediation protocol is developed for each specific oil spill case. The bioremediation method proves to be a promising way to clean up oil hydrocarbons with relatively much less interruption on the surrounding ecosystem compared to traditional physical and chemical remediation methods. Hydrocarbons, due to their different chemical structures and physical properties, degrade more or less in natural environment by indigenous microorganism groups. The difference lies in the biodegradation kinetics rate: an effective biodegradation could clean up the oil spill within weeks, while ineffective biodegradation would allow the residual recalcitrant hydrocarbons, such as polycyclic aromatic hydrocarbons (PAH) which can inhibit bacteria growth and probably be toxic to living beings, to linger in the environment and ecosystem for up to years, even decades. Therefore, bioremediation is the enhancement of the natural biodegradation of crude oil chemicals by speeding up the biodegradation kinetics rate.

Bioremediation is mainly approached by two methods: bio augmentation (seeding) and bio stimulation (aeration and fertilization).

Bio augmentation

Bio augmentation is the application of new oil-degrading microbial group into the oil-polluted land or water body, it is also known as seeding. Typically, the seeded microbes are suspended by a stabilizing agent and lie dormant in a spore until activated in solution and applied together with micronutrients and bio stimulants. Microorganisms developed, bioengineered, and enriched *in vitro* are applied *in vivo* to an oil polluted site to stimulate rapid biodegradation (Ecochem®, 1998).

Bio stimulation

Bio stimulation is the changing of background environment to promote indigenous microbial growth thus increasing the oil spill biodegradation. Bio stimulation involves aeration and the addition of micronutrients such as nitrogen and phosphorus. Bio stimulation is proved to enhance biodegradation effectiveness when indigenous microbial population is present in the substrate and high enough to degrade the contaminants (Ecochem®, 1998). In many cases, indigenous oil-degrading microbial is ubiquitous, and the presence of nitrogen and phosphorus are frequently proved to be the limiting factors for oil biodegradation (Atlas et al., 1973).

Bioremediation Protocol Development

The effective bioremediation could only be carried out upon carefully designed protocols. The US Environmental Protection Agency (USEPA) and National Environmental Technology Application Center (NETAC) have developed the five-tier (I to IV) protocol system for the effective and economical bioremediation application for oil spill cases. I have combined the EPA developed pretreatment protocol with this four-tier protocol and present as follows the complete structure of a *five-tier bioremediation protocol* in this literature review. The protocol is presented in terms of decision tree as in Figure 1:

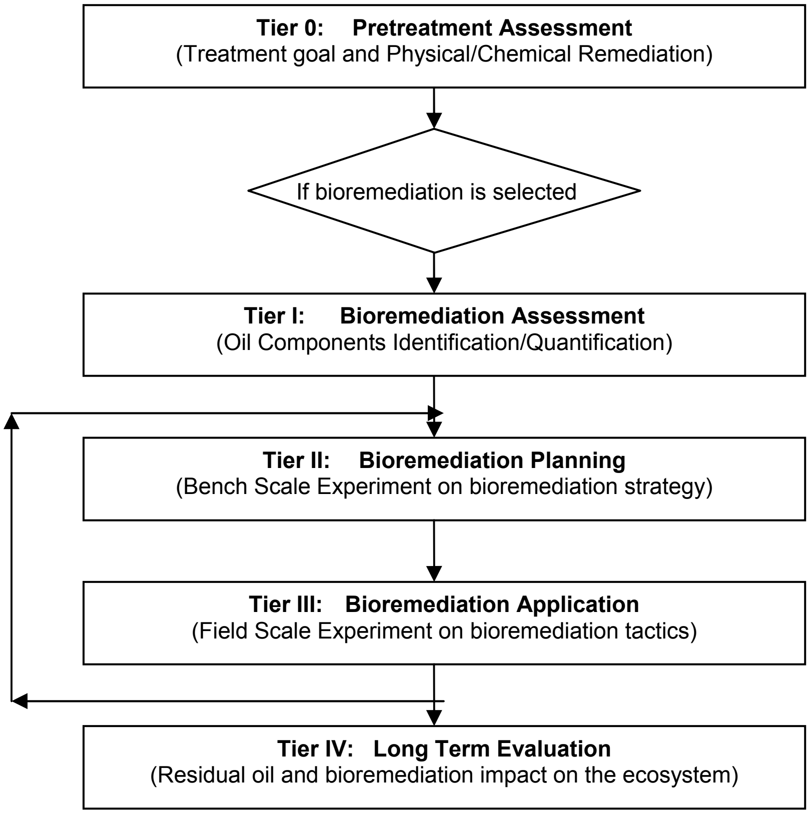


Figure 1. Decision Tree of Five-Tier Protocol on Oil Spill Bioremediation
(Modified from Venosa, 2001)

Protocol Tier 0: Pretreatment Assessment

Before the bioremediation methods are allowed to be carried out, the oil spill treatment strategy must be assessed in *Tier 0* according to the severity and specialty of each case. Oil spills are usually first pretreated with *in situ* and/or *ex situ* physical and chemical methods. The treatment of oil spill can be *in situ* and *ex situ*, where *in situ* remediation occurs right on the contaminated spot, and *ex situ* treatment requires the transportation of oil clumps to other places for remediation. *Ex situ* treatment is applied to soil contamination, and *in situ* treatment is applied to marine and beach contamination (Boopathy, 2000). The treatment methods depends on the oil thickness and oil mobility in the spilled environment which is governed by both the wind-wave height, wind speed and oil components' physical properties. The physical properties of the oil hydrocarbons include: surface tension is the measure of attraction between surface molecules of a liquid, viscosity is the measure of liquid's resistance to flow, and a higher surface tension and a larger viscosity will lead to a decreased mobility of the oil; specific gravity is the density of a substance compared to the water, as most hydrocarbon would float on top of water yet heavier oils such as vegetable oils and animal fats may sink to the bottom (USEPA, 1999). The various treatment applied on beach oil spill according to the oil thickness, wind-wave height and wind speed is described in Figure 2. When oil thickness surpasses a certain degree, *in situ* burning can be performed after certain restriction. Besides that, common *in situ* physical recovery pretreatment by includes booming (a floating physical barrier placed on the water to contain, divert or exclude oil), skimming (the use of weir, centrifuge or submersion plane to skim oil from the surface), barriers (a physical barrier other than a boom is placed across an area to prevent the oil from passing), physical herding (trapped oil is dislodged from ecologically important area), flooding (flood trapped oil out of the boom by water flow at high pressures and recover the flushed oil by skimmers), etc. The purpose of *in situ* physical pretreatment is mainly to confine the mobility of the spill oil into one specified geological section by mechanical cleanup, followed by *ex situ* chemical or biological treatment. When oil is disposed at a higher wind-wave height (greater than 4ft) which indicates a potential stronger mobility, *in situ* chemical dispersants are sometimes employed to reduce the oil/water interfacial tension thus make it easier for the oil droplets to break into smaller particles. For those falls out of range of physical and chemical in situ remediation, biological remediation comes into the picture. Bioremediation is preferred over natural weathering and dispersion for its enhanced oil biodegradation rate. (USEPA, 2001).

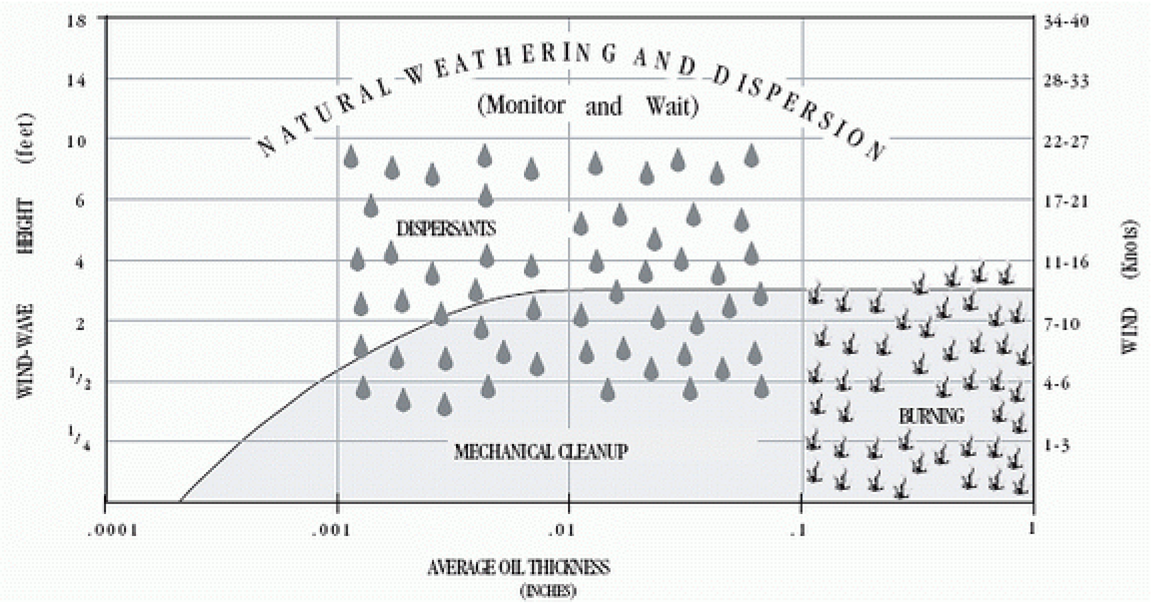


Figure 2. Primary response options of on beach oil spill under various wind/sea conditions and oil film thickness (Adapted from USEPA, 2001)

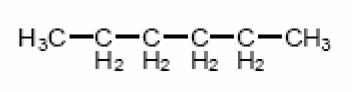
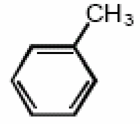
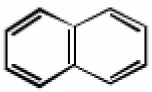
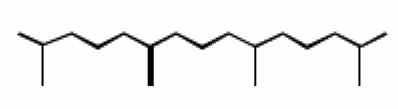
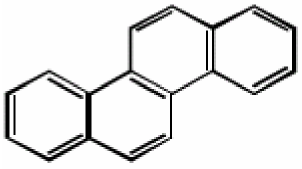
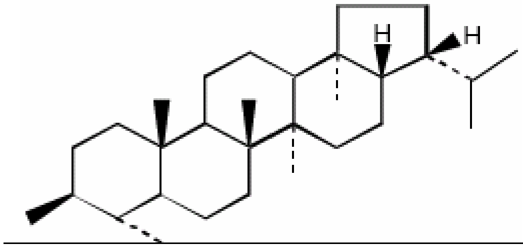
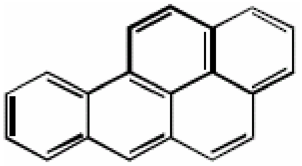
Protocol Tier I: Bioremediation Assessment

The toxic, carcinogenic and hazardous substances contained in the crude oil spill is identified and further quantified in *Tier I* for bioremediation assessment. *Tier I* provides the basis for a preliminary evaluation for bioremediation agents' safe and effective application.

Crude oil (petroleum) and refined oil (gasoline) consist of several hundreds individual hydrocarbons accounting for up to 98% of total composition, and non-hydrocarbon compounds (Atlas & Bartha, 1993). The structural difference of each component leads to the differences in physical attributes such as boiling point, sorption affinity, mobility, viscosity, etc. Furthermore, the biodegradability is determined by the chemical structure of the compounds. Therefore, an analysis of the spilled petroleum composition is necessary for the successful implementation of bioremediation strategy and protocol (Atlas & Bartha, 1993). Oil can be are categorized into four major groups due to their chemical structures, from simple to complex are: saturates, aromatics, resins, and asphaltenes. The saturates are saturated hydrocarbons (alkanes) which could be acyclic (C_nH_{2n+2}), also called aliphatics, and cyclic (C_nH_{2n}), also called alicyclics. Saturates range from chain length in carbon number from 1 to over 40, and make up of the majority of the oil. Common saturates can be normal or branched n-hexane (n-C_6H_{14}), n-heptadecane (n-$C_{17}H_{36}$), n-pristane(n-$C_{19}H_{40}$), and hopane ($C_{24}H_{48}$). The aromatics include monocyclic aromatics such as benzene, toluene and xylenes, and polycyclic aromatic hydrocarbon (PAH) which have two or more fused aromatic rings. PAH is well known for its recalcitrance and potential toxic and carcinogenic effect to the environment. Resins include polar compounds containing elements other than C and H, such as N, S, and O, therefore resins are also referred to as NSO compounds. Asphaltenes is poorly characterized compounds with high molecular weight and often associated with heavy metals (Zhu et al., 2001). The chemical structures of several hydrocarbons are presented in Table 1.

There is no single "super power" bacterium capable of degrading all the oil components. Representative hydrocarbon degrading bacteria are: *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Brevibacterium, Cornybacterium, Flavobacterium, Nocardia, Pseudimonas, Pseudimonas, Vibrio,* etc.; and prepresentative hydrocarbon degrading yeast and fungi are: *Aspergillus, Candida, Cladosporium, Penicillium, Rhodotorula, Sporobolomyces, Trichoderma,* etc (Leahy and Colwell, 1990). The biodegradability of hydrocarbons decreases in the following order: saturates > aromatics > resins > asphaltenes. The n-alkanes with carbon number ranging from 1 to 44 are demonstrated to be readily biodegradable under aerobic conditions (Haines and Alexander, 1974). The branched alkane isomers are less readily biodegradable as the biodegradability decreases with the increase of branches, and those alkanes with carbon number from 1 to 26 have been shown to be biodegradable (Atlas, 1981). However, the cycloalkanes are relatively resistant to biodegradation and complex alicyclics such as hopanes and steranes are considered the most persistent compounds of petroleum (Atlas, 1981). The biodegradation pathway of saturates involves terminal oxidation to the corresponding alcohol, aldehydes, or fatty acid functional group (NAS, 1985). The aromatics biodegradability depends on the number of fused aromatic rings. Monoaromatics and PAH with less than 4 aromatics rings can be biodegraded gradually by anaerobes at low concentrations; while PAH with more than 5 aromatic rings can only be biodegraded through microorganism co-metabolism where PAH cannot serve as primary carbon source. The biodegradation pathway of aromatics involves aromatic ring cleavage which is one ring at one time, and the formation of carboxylic acid (Atlas 1981; Cerniglia, 1992; Prince, 1993). Very little is known about the biodegradation of resins and asphaltenes due to their complex structures. Generally, the biodegradation ability decrease from easy to difficult is: n-alkanes > branched alkanes > alkylbenzenes > phenanthrenes > dibenzothiophenes > benzofluoranthene > homohopanes > tricyclic termpanes > aromatic steranes > resins > asphaltenes (Moldowan et al., 1992). For those who are not so familiar with organic chemistry, there is one rule to roughly judge the biodegradability from the hydrocarbon's chemical structure: the more rings (aromatic or cyclic) and branches the hydrocarbon has, the more difficult to be degraded.

Table 1. Chemical structures of typical hydrocarbons (saturates and aromatics)

Saturates		**Aromatics**	
Chemical Structure	Name	Chemical Structure	Name
 $H_3C-\underset{H_2}{C}-\underset{H_2}{C}-\underset{H_2}{C}-\underset{H_2}{C}-CH_3$	n-hexane		toluene
	n-heptadecane		naphthalene
	pristane		chrysene
	17α, 21β-hopane		benzo-pyrene

Protocol Tier II: Bioremediation Planning

The microorganism is evaluated for its biodegradation efficacy in the *Tier II* bench scale batch study usually performed in a shaker flask. *Tier II* evaluation provides primary data for the selected microorganism oil degradation effectiveness and efficiency under different environments, such as various application of fertilizer, pH, temperature, etc.

The laboratory scale study report must include these key information as follows

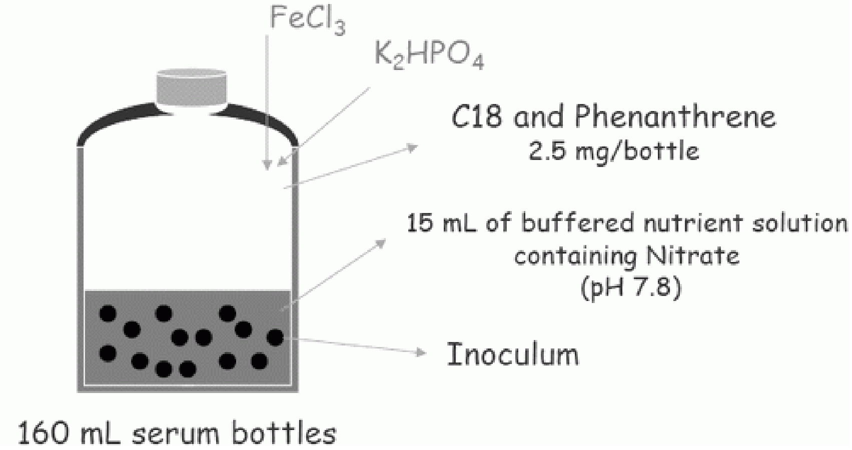
1. Identification or development of bioremediation agents.

As mentioned before, bioremediation can be approached by bio augmentation, bio stimulation, or the combination of both. Hydrocarbon degraders are ubiquitous in nature, when an oil spill occurs, the influx of oil will cause an immediate increased response in the hydrocarbon-degrading populations (USEPA, 2001). Microbial ecologist have demonstrated that the number of oil-degrading bacteria within sediments (Venosa et al., 1997), open waters (Atlas, 1993), and ice sea (Delille et al., 1997) increased quickly following the exposure to oil contamination. Thus, there appears to be little or no need to add microorganisms to oil contaminated ecosystems. Furthermore, studies have shown that the addition of a commercial mixture of oil-degrading marine bacteria (Lee & Levy, 1987) and/or lab-enriched cultures of indigenous oil-degrading bacteria (Fayad et al., 1992) did not significantly enhance rates of oil biodegradation in the natural environment of in situ oil spill bioremediation over that achieved by nutrient enrichment alone (Venosa et al.,1996). Despite the negative results of field trial experiments (USEPA, 2001), commercial vendors continue to promote bioremediation agents containing microbial additives, as they promote the concept of a genetically engineered “super-bug” capable of degrading crude oil single handedly (Ecochem®, 1998). However, this idea till now has not been applicable due to the measurement of biodegraded hydrocarbon contributions. Sterile controls in shaker flask efficacy tests of commercial products containing microbial inocula demonstrated that indigenous oil-degrading populations performed most, if not all, of the biodegrading activity (Venosa et al., 1992). Furthermore, the considerable metabolic potential required to deal with the diverse array of chemicals, and the burden of maintaining all of these versatile chemical degrading genes is likely to be too great to make the recombinant strains noncompetitive compared to indigenous bacteria (Lee & Merlin, 1999). Indigenous bacteria naturally occurring at the oil spill spot are sampled at sites and identified in laboratory to test their hydrocarbon biodegradation capacity. If they go through the laboratory efficacy test and are proved efficient for *in situ* oil spill bioremediation, usually the bioremediation strategy would focus on bio stimulation to exert the bacteria growth and oil biodegradation. If not, then laboratory mixed bacteria consortium extracted from natural environment are sometimes introduced to augment the biodegradation.

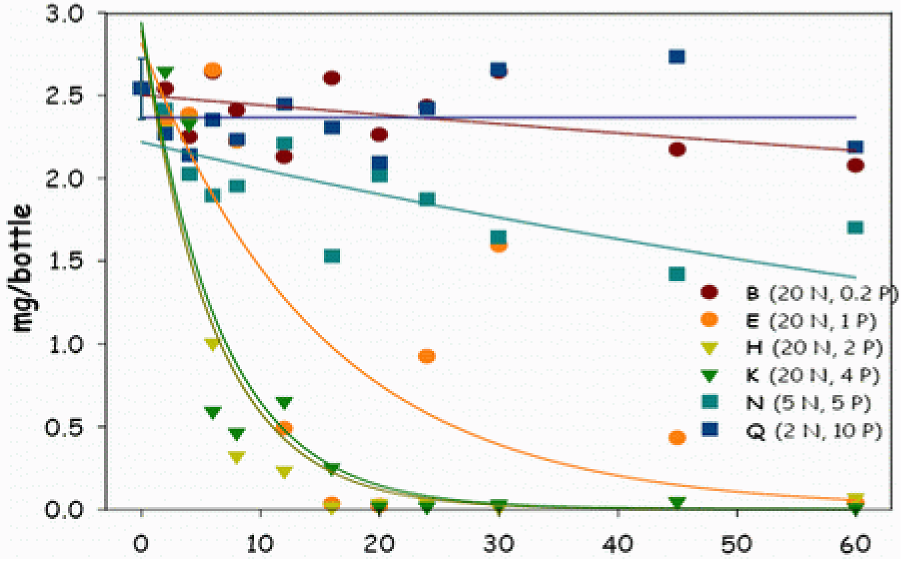
2. Oil chemicals' composition changes under different bioremediation strategies.

The biodegradation effectiveness or extent is obtained by thermodynamics test which compares the equilibrium hydrocarbon amount at the maximum oil degrading moment; the biodegradation efficiency or rate is obtained by kinetics test which records the residual hydrocarbon during the change of time. The biodegradation kinetics are usually fit into the zero, first or second kinetics order curve. Experimental studies have shown that fertilization with nitrogen and phosphorus is very promising, and the column tested fertilizer nutrients mass ratios of carbon, nitrogen, and phosphorus have been defined for different hydrocarbons (Santa et al., 1999). Therefore, the application of individual or combination of nutrition species, for example, inorganic nitrogen nutrient, nitrate as alternate electron acceptor, inorganic phosphorus nutrient, etc., is evaluated in *Tier II* lab scale experiment. The concept of nutrition as a limiting factor of oil biodegradation has been widely accepted; and biodegradation is regarded as a function of pore water retained nitrogen concentration (Brag et al., 1994). The application of nutrition would enhance the biodegradation rate greatly compared to no action control: nutrition addition accelerates the biodegradation with a factor of five or more. Surprisingly, although great enhancement was observed under all cases of nutrient application, however, alternate nutrition differs little in the improvement (Venosa et al., 1996). The nutrients can be of the following types: (a). Oleophilic fertilizer (Bragg et al., 1994); (b). Dissolved water-soluble fertilizer (Pritchard and Costa, 1991); (c). Slow-release (granular) inorganic fertilizer formulations (Bragg et al., 1994); (d). Microbial inocula (Venosa et al., 1992).

In one typical shaker flask experiment, different bio stimulation strategies were tested for their impact on the biodegradation of two common hydrocarbons, phenanthrene (C10) and octadecane (C18) (Garcia-Blanco et al., 1999). The adopted bio stimulation strategies were a series of inorganic nutrient N, P supply at various ratios and total amounts. It was hypothesized that change in the relative N : P supply ratio will cause significant changes in the microbial community thus change the rate and extent of biodegradation, also the changes in absolute nutrient supply at a constant supply ratio will alter the total hydrocarbon biodegrading biomass thus consequently change the rates of biodegradation (Tillman, 1982). The goal of this bench scale test is to find out the optimal N : P ratio and the minimized total nutrient amount (N + P) to achieve satisfactory biodegradation rate which is supposed to be at least second order kinetics decay. The bench scale experiment was carried out in a 160ml shaker flask, filled with buffer solutions maintaining pH at 7.8, the target alkanes applied at an initial amount of 2.5mg per bottle, and the microbial were implanted in inoculum applied to the flasks at the same amount. The added inorganic N is NO_3 and inorganic P is HPO_4, both of them measured by Ion Exchange Chromatography. The changes in the amount of target alkanes were analyzed by GC/MS following Standard Methods 8270C. At a fixed total amount of 100mg/L to 10mg/L, the N : P ratio was tested at 100:1, 20:1, 10:1, 5:1, 1:1 and 1:5 on both alkanes; at a fixed N : P ratio, the total nutrient amount is tested at 100mg/L, 50mg/L, 10mg/L and 2mg/L on both alkanes. The results show that for C10 biodegradation, N is the limiting factor and P does not have a significant effect, and the optimal N : P ratio is reached at 20: 1, 10 : 1 and 5 : 1. For C18 biodegradation, when N is highest (greater than 100mg/L) and lowest (less than 2mg/L), P has no effect on biodegradation as greater than 100mg/L N supply guarantees a quick biodegradation of the total 2.5mg C18 within 10 days, and less than 2mg/L N, C18 is not decaying at all; at intermediate N level ranging from 10mg/L to 20mg/L, the biodegradation rate increases with the increase of P concentration, as P level lower than 0.5mg/L the biodegradation ceases, and P level greater than 1mg/L revives the biodegradation process at a second order kinetics. The biodegradation rate increases with more total nutrients applied, however, the difference is not significant when N supply surpasses 10mg/L for C10, also not significant when N supply surpasses 50mg/L for C18. The changes in absolute nutrient supply levels at a constant supply ratio affect hydrocarbon biodegrading biomass thus influence degradation rate, and the trend is more dramatic for C18 than C10 as shown in the experiment. It can be concluded that N plays a much more important role in hydrocarbon degradation especially for C10: with abundant N supply (greater than 100mg/L) the biodegradation occurs readily regardless of P supply; with intermediate N supply (10mg/L to 50mg/L) the biodegradation rate increases with the increase of P supply (1mg/L to 5mg/L); with low N supply (lower than 2mg/L) the biodegradation ceases even at a high P supply. Also, background P at environmental concentration is usually enough to support C10 degradation and N should be applied at N : P ratio of 30:1. No C18 degradation could be observed for N<2mg/L and P<0.5mg/L, and the applied ratios should be C : N = 150:1 and C : P = 600:1. The overall nutrient supply strategy developed for simultaneous degradation of C10 and C18 is 100mg/L N and 10mg/L P, and the suggested C : N : P ratio should be 30:10:1 to reach more than 95% degradation after 6 days. The typical bench-scale experimental design and results are shown in Figure 3.



(a) The typical experiment design



(b) The typical results fit into zero, first and second order curves

Figure 3. The typical shaker-flask batch test for evaluation of best nutrient application plans (Adapted from Garcia-Blanco et al., 1999)

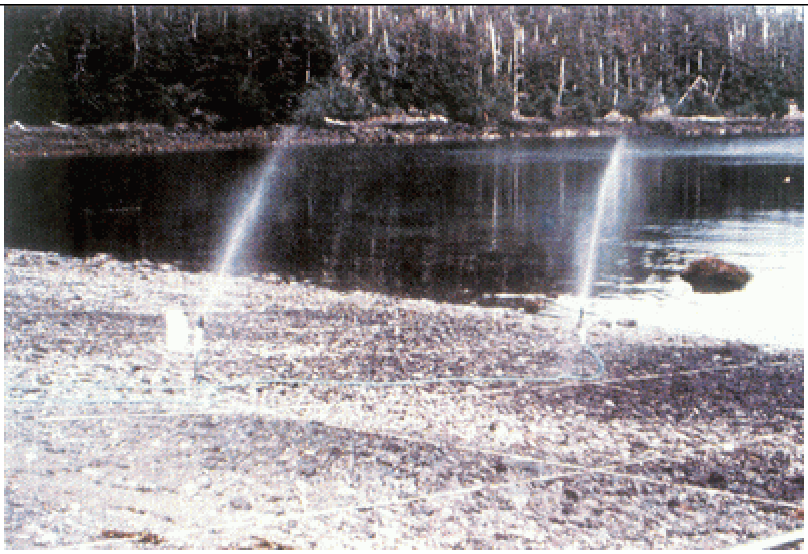
3. (Optional) The microorganism community structure change over time.

The great advancement in the gene sequencing and analysis enables the in depth study of microbial diversity and community dynamics. The microorganism community would change over time during incubation process under different oil and nutritional conditions. The understanding of the change of microbial population during oil bioremediation would provide us insight into the viable biomass and community structure affected by nutritional status, and help us better understand and further design the biological remediation strategy. There are several ways to analyze the microorganism samples. Most probable number (MPN) technique is for quantification of the alkane and aromatic hydrocarbon-degrading population changes over time. Phospholipid fatty acid (PLFA) analysis shows the fractions of lipid biomarkers changes over time (Machnaughton et al., 1999). Recently developed cloning and sequencing of PCR-amplified 16S ribosomal DNA (rDNA) monitored by denaturing gradient gel electrophoresis (DGGE) elucidates the bacterial community dynamics shown as DGGE profiles, as different samples are compared with Dice or Pearson similarity coefficient, the phylogenetic tree can be revealed coupled with gene library (Röling et al., 2002). During one experimental oil bioremediation process, the microbial communities within contaminated ecosystems were found to be dominated by the competitive organisms capable of utilizing the nutrients and surviving the toxic environment. The communities' complexity was increased by the present chemicals, then over some time decreased as the microbial shifted to these survived bacteria groups (Machnaughton et al., 1999). The biodegradation results as described in step 2, coupled with the microorganism community structure observed, would provide thought provoking results. For example, in an experiment one group of samples were applied to oil without nutrition supply, as a result no significant oil biodegradation occurred over time as measured by the residual hydrocarbon amount. However, the samples' DGGE profiles differed from the DGGE profiles of no oil added control samples, suggesting that the bacterial communities changed considerably under the toxic inhibition of the oil. This was explained by the release of nitrogen and phosphorus from those bacteria who were killed by the oil, in turn to support the growth of those bacteria who survived the oil toxicity. The survivals were identified most likely to be *Alcanivorax* and *Fundibacter* (Röling et al., 2002). The predominant bacterium species at the peak of biodegradation rate were identified to be *Alcanivorax* and *Pseudomonas stutzeri* with a 99.7% probability (Röling et al., 2004).

Protocol Tier III: Bioremediation Application

Tier III is the bioremediation application strategy where the method, amount and frequency of the bioremediation application are defined, in some specific cases, the bioremediation procedures are monitored by periodical sampling, and the strategy might go through minor adjustment due to monitoring results.

The bioremediation strategies developed in the laboratory may be limited by the geological or climate conditions at the specific oil spill spots. In the laboratory bench scale evaluation, the nutrients C : N : P about 100 : 5 : 1 can be applied at a fix ratio; however, maintaining a fixed ratio of nutrients or keeping the total nutritional amount for biodegradation could be mission impossible for the practical application of bioremediation strategies. In the marine environment, where the oil polluted local ecosystem must be restored, the nutrition required for bacteria growth can be easily diluted and washed away in open water by tides or waves. The kinetics of nutrients loss rate is expected to reversely correlate with the size of beach granulates, while the larger beach particles would lead to a better retaining of the nutrients. The retention time of the nutrition in these natural media is supposed to be: Clay > Cobble > Gravel > Slit > Sand. For example, Inipol EAP22, a microbial inocula developed in France, has been tested successful in Exxon Valdez spill bioremediation because the Prince William beach is mainly made up of the cobble, however, Inipol EAP22 did not reach satisfactory result in Florida Tampa oil-contaminated sand beach sites, due to the rapid nutrient loss and unweathered condensate acute toxic products kills nature micro flora (Pritchard and Costa, 1991). It has been pointed out by Brag et al. (1994) that the effectiveness of bioremediation large depends on the maintenance of the limiting nutrient(s) level within the pore water at an optimal range. When the nutrient(s) level falls short of a certain threshold, the bioremediation would fail, which explains why the inocula Inipol EAP22 would work for Exxon cobble beach but not for Florida Tampa sand beach. Furthermore, this crucial threshold value for the nutrient(s), mainly nitrogen, was pointed out by Venosa et al. (1996) that, 1 – 2mg/L Nitrogen per liter in the interstitial pore water would result in almost optimal hydrocarbon biodegradation in a sandy beach. Prior to the field application, the background nutrient level is always tested, as the background nutrient level is enough to support close to maximum biodegradation, then it is not necessary to apply artificial nutrients (Venosa et al., 1996).



(a) Spraying system for water-soluble agricultural fertilizers

(b) Granulated form of slow-release agricultural fertilizers

Figure 4. Two different forms of common agricultural fertilizers for beach bioremediation (Adapted from Pritchard and Costa, 1991)

In the case of artificial nutrients are necessary for successful bioremediation application, there are two approaches, the common agricultural fertilizer, and the commercial fertilizer. The common agricultural fertilizers would be the first choice for nutrient amendment because they are cheap and readily available. The agricultural fertilizers are usually inorganic nitrogen containing chemicals, such as, potassium/sodium/ammonia nitrate, potassium diphosphate, etc. The agricultural fertilizers are usually applied through spraying in terms of solutions onto the oil-contaminated field. As the sand beach does not have a long hydraulic retention time, water-soluble nutrient can be lost through dissipation easily, therefore frequent nutrient applications of water- soluble fertilizers are required for the maintenance of interstitial pore nutrient level, which would need be labor-intensive and potentially costly (Figure 4, (a)). Therefore, a form of slow-release fertilizers are designed on basis of water soluble agricultural fertilizers to overcome washout problems and provide continuous sources of nutrients (Figure 4, (b)). The comparison field test applied to the oil-contaminated artic field includes four treatment methods, (a) natural attenuation with no nutrient amendment; (b) water-soluble nutrient amendment with N : P = 20 : 1 applied every two weeks; (c) slow-release nutrient amendment with N : P = 18 : 1 applied every two months; (d) both water-soluble at every two weeks and slow-release nutrient at every two months. (d) achieved the maximum hydrocarbon degradation effect as expected, and (c) achieved almost the same biodegradation result as (d) (Prince et al., 1999). The greatest challenge of designing slow-release fertilizer is the nutrient release speed is hard to control: The field trials on one of the Prince William shorelines of a slow-release fertilizer, Customblen granules, released the nutrient too quick therefore the nutrient was washed away before it became a lasting source (Swannell et al., 1996); another slow-release fertilizer, Max Bac, however, released the nutrient too slow thus the applied fertilizer cannot support the rapid oil biodegradation rate (Sveum and Ramstad, 1995). Due to the limitations of common agricultural fertilizers as the water-soluble ones need frequent application and slow-release ones are difficult to design, the commercial fertilizers are developed and available as bioremediation agents. Most of the commercial fertilizers are oleophilic nutrient products, whose design is based on the principle that oil biodegradation mainly occurs at oil-water interface, and oleophilic fertilizers are able to adhere to oil surface and provide nutrients at oil-water interface, thus enhancing the biodegradation without increasing the bulk pore water nutrient level. For example, Inipol EAP22, listed as one of the hundreds National Pollution Contingency Plan (NPCP) recognized hydrocarbon degraders, is composed of micro emulsified urea as N source, sodium phosphate as P source, 2-butoxyl-1-ethanol as surfactant, and oleic acid to attain oleophicity. In the 1989 Exxon Valdez oil spill, Inipol EAP22 was lucky enough to be chosen as the bioremediation agent for at that time it was the only commercially available hydrocarbon biodegrader with large production capacity. A huge amount of Inipol EAP22 containing approximately 50 ton nitrogen and 5 ton of phosphorus were applied over 120km oil-contaminated shoreline of Prince William. Inipol EAP22 gained its fame through the official recorded effectiveness bioremediation during 1989 and 1990 field application (Pritchard and Costa, 1991).

Protocol Tier IV: Long Term Evaluation

Bioremediation can enhance the biodegradation extent and rate, however, no matter how efficient the bioremediation strategy works, as long as the oil spill occurs, there will be harm done to the environment. Bioremediation is to minimize the harm, yet in most marine oil spill cases the harm cannot be minimized to zero, and long term monitoring and evaluation on the oil polluted ecosystem is the task for *Tier IV*.

There has been concerns that the nutrients applied to oil field might form toxic byproduct under bacteria enzymatic effect. Incomplete biodegradation from large molecule PAH into small molecule aromatics may increase the toxicity, as carbon number from 1 ~ 20, the toxicity decreases with the increase of carbon number, as benzene is acutely toxic, the PAH with 5 or more rings is only suspected to be carcinogenic. The toxicity of oil buried in shoreline sediments is limited by low accessibility and bioavailability, as PAH and oily particles may partition or be washed from oiled sediment into pore water and overlying water. The solid to liquid partition of oil is restricted by oil/water interface, however during the natural oil weathering process, the restriction is gradually lifted as the viscosity of oil increases, the oil is limited and sealed from the surface “skin” and kept away from bioremediation. Moreover, laboratory biodegradation cannot extrapolate to field application, and bioremediation results obtained in bench scale cannot necessarily predict the field scale application result. In conclusion, there should be a monitoring system to record the contaminant residual even after the bioremediation operational endpoints (Lee and Merlin, 1999).

The post-bioremediation monitoring of Exxon Valdez oil spill, which was invested $100K, shows that no evident bioremediation byproduct has formed residual hazardous substances. However, 12 years has passed after the 1989 through 1990 bioremediation, 10 years' natural weathering and biodegradation, there is still residual oil impact on the isolated oil polluted area. This can be explained as: if the oil has high accessibility, it will be easily washed into the water, leave the shore, and does not harm the living beings on the beach any more; if the oil has low accessibility, it is mainly because of the high viscosity and oil would form a thin surface skin. While the skin make the oil persistent from bioremediation, it also isolates the oil from direct exposure to the beach ecosystem. Therefore, although in the 2001 survey, 53 sites out of the monitored 91 sites along the Prince William shoreline still has oil inside, the major ecological balance after the 1989 oil spill has been gradually reached in 1995 (Zhu et al., 2004).

Conclusions

1. In marine oil spill cases, the oil was first burned and removed physically, and sometimes followed by chemical dispersant and sorption. The oil contaminated beach due to the large impacted area, could only be treated by in-situ methods.
2. Bioremediation as the enhance of biodegradation is preferred over chemical remediation for its environmental significance. The nutrient levels especially the nitrogen concentration in the pore water is the limiting factor for bacteria growth, thus the bio stimulation is often the choice of bioremediation.
3. The optimal nutrient application is determined in lab scale experiment to find out the best C : N : P ratio for different types of hydrocarbons.
4. The difficulty of marine oil spill field application of agriculture fertilizers lies in the maintenance of the nutrient from being washed out by marine waves and tides. Several approaches such as slow-release agricultural fertilizers and commercial fertilizers are introduced as solution.
5. Long term monitoring is required for the assessment of successful bioremediation and residual toxicity effect.

Reference

Brag, J. R., Prince, R. C., Harner, E. J., and Atlas, R. M. (1994). Effectiveness of Bioremediation for the Exxon Valdez Oil Spill. *Nature*, **368**, 413.

Lee, K. and Merlin, F. X. (1999). Bioremediation of Oil on Shoreline Environments: Development of Techniques and Guidelines. *Pure Appl. Chem.*, **71**, 161.

Macnaughton, S. J., Stephen, J. R., Venosa, A. D., Davis, G. A., Chang, Y, J., and White, D. C. (1999). Microbial Population Changes during Bioremediation of an Experimented Oil Spill. *App. Environ. Microbiol.*, **65**, 3566.

National Ocean Service Education (NOAA) document. (2001). Prince William's Oily Mess: A Tale of Recovery. http://oceanservice.noaa.gov/education/stories/oilymess/welcome.html

Prince, R. C., Bare, R. E., Garrett, R. M., Grossmann, M. J., Haith, C. E., Keim, L. G., Lee, K., Holtom, G. J., Lambert, P., Sergy, G. A., Owens, E. H., and Guenette, C. C. (1999). Bioremediation of a Marine Oil Spill in the Arctic. *In-Situ Bioremediation of Petroleum Hydrocarbon and Other Organic Compounds,* Battelle Press, Columbus, OH.

Pritchard, P. H., and Costa, C. F. (1991). EPA's Alaska Oil Spill Bioremediation Project: Final Part of a Five-part Series. *Environ. Sci. Technol.*, **25**, 372.

USEPA document. (1999). Understanding Oil spills in Freshwater Environments. *EPA 540-K-99-007*. Office of Emergency And Remedial Response, USEPA, Cincinnati, OH.

USEPA document. (2001). Oil Spill Bioremediation Research. http://www.epa.gov/ORD/NRMRL/lrpcd/esm/oil_spill_bioremediation_researc.htm

Venosa, A. D., Suidan, M. T., Wrenn, B. A., Strohmeier, K. L., Haines, J. R., Eberhart, B. L., King, D., and Holder, E. L. (1996). Bioremediation of an Experimental Oil Spill on the Shoreline of Delaware Bay. *Environ. Sci. Technol.*, **30**, 1764.

Venosa, A. D. (2001). Guidelines for the Bioremediation of Freshwater Wetlands. *Freshwater Spills Symposia*. http://www.epa.gov/oilspill/pdfs/venosapresent2.pdf

Röling W. F., Milner, M. G., Jones, D, M., Lee, K., Daniel, F., Swannell, R. J. P., and Head, I. M. (2002). Robust Hydrocarbon Degradation and Dynamics of Bacterial Communities during Nutrient – Enhanced Oil Spill Bioremediation. *App. Environ. Microbiol.*, **68**, 5537.

Röling W. F., Milner, M. G., Jones, D, M., Fratepietro, F., Swannell, R. J. P., Daniel, F. and Head, I. M. (2004). Bacterial Community Dynamics and Hydrocarbon Degradation during a Field-Scale Evaluation of Bioremediation on a Mudflat Beach Contaminated with Buried Oil. *App. Environ. Microbiol.*, **70**, 2603.

Swannell, R. P. J., Lee, K., and McDonagh, M. (1996). Field Evaluation of Marine Oil Spill Bioremediation. *Microbiol. Reviews*, **60**, 342.

Sveum, P., and Ramstad, S. (1995). Bioremediation of Oil-Contaminated Shorelines with Organic and Inorganic Nutrients. *Applied Bioremediation of Petroleum Hydrocarbons*, Battelle Press, Columbus, OH.

Zhu, X., Venosa, A. D., and Suidan, M. T. (2004). Literature Review on the Use of Commercial Bioremediation Agents for Cleanup of Oil-Contaminated Estuarine Environments. *EPA/600/R-04/075*. Office of Research and Development, USEPA, Cincinnati, OH.