GUIDELINES FOR THE BIOREMEDIATION OF MARINE SHORELINES AND FRESHWATER WETLANDS

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EXECUTIVE SUMMARY

The objective of this document is to present a detailed technical guidance document for use by spill responders for the bioremediation of marine shorelines and freshwater wetlands contaminated with oil and oil products. Technical personnel who are responsible for designing and operating field bioremediation processes as well as consultants and equipment manufacturers will also find it useful. This manual presents a rational approach for the design of bioremediation processes pertinent to cleanup of oil-contaminated marine shorelines and freshwater wetlands. This document evaluates current practices and state-of-the-art research results pertaining to bioremediation of hydrocarbon contamination relative to types and amounts of amendments used, frequency of application, assessment of the extent of bioremediation, sampling, and analysis. The scope of the document is limited to marine shorelines and freshwater wetlands because of definitive results from recently completed, EPA-sponsored field studies. The final product is presented in a report form that is understandable by responders, on-scene coordinators, and remediation specialists. This report includes a thorough review and critique of the literature and theories pertinent to oil biodegradation, nutrient dynamics in shorelines, and analytical chemistry of oil and remediation nutrients.

A planning approach to site identification, evaluation, and selection along with information on field investigations is also presented. The manual includes examples of bioremediation options and case studies of bioremediation applied to marine shorelines and freshwater wetland environments.

The contents of this document are arranged in a logical sequence first to provide basic information for the evaluation of bioremediation as a spill response option followed by guidelines for application that includes methods to monitor its effectiveness. Thus, Chapter 1 presents an overall introduction and background discussion of bioremediation including occurrence of oil spills, response methodologies, and a summary of the scope, organization, and objective of the manual. Chapter 2 covers the basic information about oil, shorelines, mechanisms of oil biodegradation, and a state-of-the-art review of controlled laboratory experiments and field trials of oil biodegradation and nutrient dynamics in shoreline environments. For additional background information, Chapter 3 provides a more thorough review and critique of current analytical methods used to monitor and verify oil spill bioremediation success. Chapter 4 summarizes major biostimulatory and bioaugmenting amendment methods and their application strategies. Chapter 5 is the heart of the document and presents the actual guidelines for designing, planning, and implementing oil bioremediation in the field, including site characterization, evaluation of appropriate bioremediation technologies, and the selection of the most appropriate technology for a specific site. Finally, Chapter 6 provides guidelines for assessment and interpretation of field results and provides help in assessing endpoints of bioremediation (i.e., when treatment is considered complete).

The overall conclusions reached by the guidance manual are as follows. First, with respect to marine sandy shorelines, natural attenuation may be appropriate if background nutrient concentrations were high enough that intrinsic biodegradation would take place at close to the expected maximum rate. The Delaware study proved this clearly. Certainly in nutrient-limited places like Prince William Sound, Alaska, nutrient addition should accelerate cleanup rates

many-fold. However, the decision to use the natural attenuation approach may be tempered by the need to protect a certain habitat or vital resource from the impact of oil. For example, using Delaware as the model, every spring season, horseshoe crabs migrate to the shoreline of Delaware for their annual mating season. Millions of eggs are laid and buried a few mm below the surface of the sand. Migrating birds making their way from South America to Arctic Canada fly by this area and feed upon these eggs to provide energy to continue their long flight. If an oil spill occurred in February or March, it would certainly be appropriate to institute bioremediation to accelerate the disappearance of the oil prior to the horseshoe crab mating season despite the expected high natural attenuation rate. So, even in the case where background nutrients are high enough to support rapid biodegradation, addition of more nutrients would help protect such a vital resource. If the spill occurred during the summer, and no vital natural resources were threatened by the spill, then reliance on natural attenuation might be the wisest course of action. Of course, removal of free product and high concentrations of oil should still be conducted by conventional means even if a no bioremediation action is warranted by the circumstances.

With respect to freshwater wetlands, the St. Lawrence River study demonstrated that, if significant penetration of oil takes place into the subsurface, biodegradation would take place very slowly and ineffectively. This is because of the anaerobic conditions that quickly occur in these types of saturated environments, and anaerobic biodegradation of petroleum oils is much slower and less complete than under aerobic conditions. One of the objectives of the St. Lawrence River experimental design was to determine the amenability of wetlands to biodegradation when oil has penetrated into the sediment. The oil was artificially raked into the sediment to mimic such an occurrence. Consequently, no significant treatment effects were observed because all the nutrients in the world would not stimulate biodegradation if oxygen were the primary limiting material. If penetration did not take place beyond a few mm, then bioremediation might be an appropriate cleanup technology, since more oxygen would be available near the surface. It is clear that whatever oxygen gets transported to the root zone by the plants is only sufficient to support plant growth and insufficient to support the rhizosphere microorganisms to degrade contaminating oil.

However, if ecosystem restoration is the primary goal rather than oil cleanup, the St. Lawrence River study strongly suggested that nutrient addition would accelerate and greatly enhance restoration of the site. Abundant plant growth took place in the nutrient-treated plots despite the lack of oil disappearance from the extra nutrients. Furthermore, the stimulation lasted more than one growing season even though nutrients were never added after the first year. Clearly, the plants took up and stored the extra nitrogen for use in subsequent growing seasons, so restoration of the site was abundantly evident in a few short months.

Thus, in conclusion, the decision to bioremediate a site is dependent on cleanup, restoration, and habitat protection objectives and whatever factors that are present that would have an impact on success. Responders must take into consideration the oxygen and nutrient balance at the site. If the circumstances are such that no amount of nutrients will accelerate biodegradation, then the decision should be made on the need to accelerate oil disappearance to protect a vital living resource or simply to speed up restoration of the ecosystem. If there is no immediate need to protect a vital resource or restore the ecosystem, then natural attenuation may be the appropriate response action. These decisions are clearly influenced by the circumstances of the spill.

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Chapter 1 INTRODUCTION

1.1 Occurrence of Oil Spills

Modern society continues to rely on the use of petroleum hydrocarbons for its energy needs. Despite recent technological advances, accidental spills of crude oil and its refined products occur on a frequent basis during routine operations of extraction, transportation, storage, refining and distribution. It is estimated that between 1.7 and 8.8 million metric tons of oil are released into the world's water every year (NAS, 1985), of which more than 90% is directly related to human activities including deliberate waste disposal. Contrary to popular perception, only one eighth of the oil released into the aquatic environment is from tanker accidents. It is also estimated that about 30% of the spilled oil enters freshwater systems (Cooney, 1984). These figures are rather uncertain and can vary greatly from year to year depending on sources of estimation and spill incidents. Table 1.1 summarizes the number of oil spills and the quantities of release from 1970 to 1999 based on International Tanker Owners Pollution Federation's oil spill database (ITOPF, 2001). These data include oil spills of over seven tons from tankers, combined carriers, and barges. Although the data suggest a reduction in oil spills, the trend may only represent a temporary downward fluctuation that is part of erratic cycling over the long term (Etkin and Welch, 1997).

Marine shorelines are important public and ecological resources that serve as a home to a variety of wildlife and provide public recreation. Marine oil spills, particularly large scale spill accidents, have posed great threats and cause extensive damage to the marine coastal environments. For example, the spill of 37,000 metric tons (11 million gallons) of North Slope crude oil into Prince William Sound, Alaska, from the *Exxon Valdez* in 1989 led to the mortality of thousands of seabirds and marine mammals, a significant reduction in population of many intertidal and subtidal organisms, and many long term environmental impacts (Spies *et al*., 1996). In 1996, the *Sea Empress* released approximately 72,000 tons of Forties crude oil and 360 tons of heavy fuel oil at Milford Haven in South Wales and posed a considerable threat to local fisheries, wildlife and tourism (Edwards and White, 1999; Harris; 1997).

Compared to marine oil spills, inland oil spills have received much less attention. However, freshwater spills are very common, with more than 2000 oil spills, on average, taking place each year in the inland waters of the continental United States (Owens *et al*., 1993). Although freshwater spills tend to be of a smaller volume than their marine counterparts (Stalcup, *et al*., 1997), they have a greater potential to endanger public health and the environment because they often occur within populated areas and may directly contaminate surface water and groundwater supplies. For example, in 1988, an Ashland Oil Company storage tank in Pittsburgh ruptured and spilled about 2,500 tons (750,000 gallons) of diesel oil in the Monongahela River, which contaminated drinking water intakes and led to downstream water shortages as far as 200 miles (Miklaucic and Saseen, 1989).

These catastrophic accidents, especially the *Exxon Valdez* spill, have increased public awareness about the risks involved in the storage and transportation of oil and oil products and have prompted more stringent regulations, such as the enactment of the 1990 Oil Pollution Act by

Congress. However, because oil is so widely used, despite all the precautions, it is almost certain that oil spills and leakage will continue to occur. Thus, it is essential that we have effective countermeasures to deal with the problem.

Table 1.1 Oil spills from tankers and other vessels into world water (adapted from ITOPF's Oil Spill Database, http://www.itopf.com/stats.html)

Year	Number of Oil Spills		Quantities of Oil Spills ($\times 10^3$ metric tons)
	< 700 metric tons	> 700 metric tons	
1970	6	29	301
1971	18	14	167
1972	49	24	311
1973	25	32	166
1974	91	26	222
1975	97	19	342
1976	67	25	369
1977	65	16	298
1978	54	23	395
1979	59	34	608
1980	51	13	206
1981	49	6	44
1982	44	3	11
1983	52	11	384
1984	25	8	28
1985	29	8	88
1986	25	7	19
1987	27	10	30
1988	11	10	198
1989	32	13	178
1990	50	13	61
1991	27	8	435
1992	31	9	162
1993	30	11	144
1994	27	7	105
1995	21	2	9
1996	20	3	79
1997	27	10	67
1998	22	4	10
1999	19	5	24

1.2 Response to Oil Spills in Marine Shorelines and Freshwater Environments

Strategies for cleaning up an oil spill are greatly affected by a variety of factors, such as the type of oil, the characteristics of the spill site, and occasionally political considerations. A number of approaches and technologies have been developed for controlling oil spills in marine shorelines and freshwater environments. These methods have been reviewed and described extensively in a number of technical documents, such as: *Shoreline Countermeasure Manual* (NOAA, 1992), *Options for Minimizing Environmental Impacts of Freshwater Spill Response* (NOAA and API, 1994), *Understanding Oil Spills and Oil Spill Response* (U.S. EPA, 1999), and *Oil Spill Response in the Marine Environment* (Doerffer, 1992). The most commonly used shoreline cleanup options (Table 1.2) are briefly described in the following text.

Table 1.2. Conventional Shoreline Clean-up Options

Category of Response Options	Example Technology
Natural method	Natural attenuation
Physical method	Booming Skimming Manual removal (Wiping) Mechanical removal Washing Sediment relocation/Surf-washing Tilling *In-situ* burning
Chemical method	Dispersants Demulsifiers Solidifiers Surface film chemicals

1.2.1 Natural methods

Natural attenuation or natural recovery is basically a no-action option that allows oil to be removed and degraded by natural means. For some spills, it is probably more cost-effective and ecologically sound to leave an oil-contaminated site to recover naturally than to attempt to intervene. Examples of such cases are spills at remote or inaccessible locations when natural removal rates are fast, or spills at sensitive sites where cleanup actions may cause more harm than good. It should also be noted that when natural attenuation is used as a clean up method, a monitoring program is still required to assess the performance of natural attenuation. Major natural processes that result in the removal of oils include:

- Evaporation: Evaporation is the most important natural cleansing process during the early stages of an oil spill, and it results in the removal of lighter-weight components in oil. Depending on the composition of the oil spilled, up to 50 percent of the more toxic, lighter weight components of an oil may evaporate within the first 12 hours following a spill (U.S. EPA, 1999).

- Photooxidation: Photooxidation occurs when oxygen under sunlight reacts with oil components. Photooxidation leads to the breakdown of more complex compounds into simpler compounds that tend to be lighter in weight and more soluble in water, allowing them to be removed further through other processes.

- Biodegradation: Various types of microorganisms that are capable of oxidizing petroleum hydrocarbons are widespread in nature. Biodegradation is a particularly important mechanism for removing the non-volatile components of oil from the environment. This is a relatively slow process and may require months to years for microorganisms to degrade a significant fraction of an oil stranded within the sediments of marine and/or freshwater environments.

1.2.2 Physical methods

Physical containment and recovery of bulk or free oil is the primary response option of choice in the United States for the cleanup of oil spills in marine and freshwater shoreline environments. Commonly used physical methods include:

- Booming and skimming: Use of booms to contain and control the movement of floating oil and use of skimmers to recover it. The environmental impact of this method is minimal if traffic of the cleanup work force is controlled.

- Wiping with absorbent materials: Use of hydrophobic materials to wipe up oil from the contaminated surface. While the disposal of contaminated waste is an issue, the environmental effect of this method is also limited if traffic of cleanup crew and waste generation is controlled.

- Mechanical removal: Collection and removal of oiled surface sediments by using mechanical equipment. This method should be used only when limited amounts of oiled materials have to be removed. It should not be considered for cleanup of sensitive habitats or where beach erosion may result.

- Washing: washing of the oil adhering along the shorelines to the water's edge for collection. Washing strategies range from low-pressure cold water flushing to high-pressure hot water flushing. This method, especially using high-pressure or hot water, should be avoided for wetlands or other sensitive habitats.

- Sediment relocation and tilling: Movement of oiled sediment from one section of the beach to another or tilling and mixing the contaminated sediment to enhance natural cleansing

processes by facilitating the dispersion of oil into the water column and promoting the interaction between oil and mineral fines. Tilling may cause oil penetration deep into the shoreline sediments. The potential environmental impacts from the release of oil and oiled sediment into adjacent water bodies should also be considered.

- *In-situ* burning: Oil on the shoreline is burned usually when it is on a combustible substrate such as vegetation, logs, and other debris. This method may cause significant air pollution and destruction of plants and animals.

1.2.3 Chemical methods

Chemical methods, particularly dispersants, have been routinely used in many countries as a response option. For some countries, such as the United Kingdom, where rough coastal conditions may make mechanical response problematic, dispersants are the primary choice (Lessard and Demarco, 2000). However, chemical methods have not been extensively used in the United States due to the disagreement about their effectiveness and the concerns of their toxicity and long-term environmental effects (U.S. EPA, 1999). Major existing chemical agents include:

- Dispersants: dispersing agents, which contain surfactants, are used to remove floating oil from the water surface to disperse it into the water column before the oil reaches and contaminates the shoreline. This is done to reduce toxicity effects by dilution to benign concentrations and accelerate oil biodegradation rates by increasing its effective surface area.

- Demulsifiers: Used to break oil-in-water emulsions and to enhance natural dispersion.

- Solidifiers: Chemicals that enhance the polymerization of oil can be used to stabilize the oil, to minimize spreading, and to increase the effectiveness of physical recovery operations.

- Surface film chemicals: Film-forming agents can be used to prevent oil from adhering to shoreline substrates and to enhance the removal of oil adhering to surfaces in pressure washing operations.

1.3 Bioremediation as an Oil Spill Cleanup Technology

Although conventional methods, such as physical removal, are the first response option, they rarely achieve complete cleanup of oil spills. According to the Office of Technology Assessment (OTA, 1990), current mechanical methods typically recover no more than 10-15 percent of the oil after a major spill. Bioremediation has emerged as one of the most promising secondary treatment options for oil removal since its successful application after the 1989 *Exxon Valdez* spill (Bragg *et al*., 1994; Prince *et. al*., 1994). Bioremediation has been defined as "the act of adding materials to contaminated environments to cause an acceleration of the natural biodegradation processes" (OTA, 1991). This technology is based on the premise that a large percentage of oil components are readily biodegradable in nature (Atlas, 1984, 1981; Prince, 1993). The success of oil spill bioremediation depends on our ability to establish and maintain

conditions that favor enhanced oil biodegradation rates in the contaminated environment. There are two main approaches to oil spill bioremediation:

- *bioaugmentation,* in which known oil-degrading bacteria are added to supplement the existing microbial population, and

- *biostimulation*, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting cosubstrates, and/or by alterations in environmental conditions (e.g. surf-washing, oxygen addition by plant growth, etc.).

Both laboratory studies and field tests have shown that bioremediation, biostimulation in particular, can enhance oil biodegradation on contaminated shorelines (Prince, 1993; Swannell, *et al.*, 1996). Recent field studies have also demonstrated that biostimulation is a more effective approach because the addition of hydrocarbon degrading microorganisms will not enhance oil degradation more than simple nutrient addition (Lee *et al*, 1997a; Venosa *et al*., 1996; see Chapter 2 in detail). Bioremediation has several advantages over conventional technologies. First, the application of bioremediation is relatively inexpensive. For example, during the cleanup of the *Exxon Valdez* spill, the cost of bioremediating 120 km of shoreline was less than one day's costs for physical washing (Atlas, 1995). Bioremediation is also a more environmentally benign technology since it involves the eventual degradation of oil to mineral products (such as carbon dioxide and water), while physical and chemical methods typically transfer the contaminant from one environmental compartment to another. Since it is based on natural processes and is less intrusive and disruptive to the contaminated site, this "green technology" may also be more acceptable to the general public.

Bioremediation like other technologies also has its limitations. Bioremediation involves highly heterogeneous and complex processes. The success of oil bioremediation depends on having the appropriate microorganisms in place under suitable environmental conditions. Its operational use can be limited by the composition of the oil spilled. Bioremediation is also a relatively slow process, requiring weeks to months to take effect, which may not be feasible when immediate cleanup is demanded. Concerns also arise about potential adverse effects associated with the application of bioremediation agents. These include the toxicity of bioremediation agents themselves and metabolic by-products of oil degradation and possible eutrophic effects associated with nutrient enrichment (Swannell *et al*., 1996). Bioremediation has been proven to be a cost-effective treatment tool, if used properly, in cleaning certain oil-contaminated environments. Few detrimental treatment effects have been observed in actual field operations.

Currently, one of the major challenges in the application of oil bioremediation is the lack of guidelines regarding when and how to use this technology. Although extensive research has been conducted on oil bioremediation during the last decade, most existing studies have concentrated on either evaluating the feasibility of bioremediation for dealing with oil contamination, or testing favored products and methods (Mearns, 1997). Only limited number of pilot-scale and field trials, which may provide the most convincing demonstrations of this technology, have been carried out. To make matters worse, many field tests have not been properly designed, well controlled or correctly analyzed, leading to skepticism and confusion

among the user community (Venosa, 1998). There is an urgent need for a detailed and workable set of guidelines for the application of this technology for oil spill responders that answers questions such as when to use bioremediation, what bioremediation agents should be used, how to apply them, and how to monitor and evaluate the results. Scientific data for the support of an operational guidelines document has recently been provided from laboratory studies and fields trials carried out by the U.S. Environmental Protection Agency (EPA), University of Cincinnati, and Fisheries and Oceans Canada.

1.4 Scope of This Document

1.4.1 Objectives

The objective of this manual is to provide detailed and applicable technical guidelines for use by spill responders for the bioremediation of marine shorelines and freshwater wetlands that are contaminated with crude oil and its refined products. The document will also provide guidance to scientists, other technical personnel, and manufacturers with an interest in the design and implementation of field bioremediation processes. The document evaluates current practices and state-of-the-art research results pertaining to bioremediation of hydrocarbon contamination and presents a rational procedure for the design of bioremediation processes pertinent to clean-up of oil stranded within sediments of shoreline environments.

The scope of this document is limited to marine shorelines and freshwater wetlands where the effectiveness of bioremediation has been quantified in controlled field and case studies following spill incidents. To date, there is no conclusive evidence of successful oil spill clean up operations in the open sea by the application of bioremediation strategies (Atlas, 1995; Swannell *et al*., 1996). With respect to freshwater environments, an oil spill is most likely to have the greatest impact on sensitive wetlands or marshes rather than running rivers. To provide insight into the feasibility of bioremediation strategies in these habitats, the information presented in this manual is based on experimental oil spills conducted in the 1990s jointly by EPA, the University of Cincinnati, and Fisheries and Oceans-Canada. The first study was a field experiment on a sandy beach in Delaware in 1994. In 1999 and 2000, a similar field investigation was conducted on a freshwater wetland along the shoreline of the St. Lawrence River, Quebec, Canada. Another field study was conduced in Dartmouth, Nova Scotia in 2000 on a salt marsh dominated by *Spartina alterniflora*. This document will provide oil bioremediation guidelines based on current practices and research with the emphasis on the findings of these fields studies. Because the study conducted on the shoreline of Nova Scotia has not been concluded at the time of this writing, guidelines of oil bioremediation in salt marshes will be available as a supplementary document upon the conclusion of this investigation.

1.4.2 Organization of the guidance document

For ease of use, the contents of this document are arranged in a logical sequence first to provide basic information for the evaluation of bioremediation as a spill response option followed by guidelines for application that includes methods to monitor its effectiveness. Thus, Chapter 2 covers the basic information about oil, shorelines, mechanisms of oil biodegradation, and a state-

of-art review of controlled laboratory experiments and field trials of oil biodegradation and nutrient dynamics in shoreline environments. For additional background information, Chapter 3 provides a more thorough review and critique of current analytical methods used to monitor and verify oil spill bioremediation success. Chapter 4 summarizes major biostimulatory and bioaugmenting amendment methods and their application strategies. Chapter 5 presents guidelines for designing, planning, and implementing oil bioremediation in the field, including site characterization, evaluation of appropriate bioremediation technologies, and the selection of the most appropriate technology for a specific site. Finally, Chapter 6 provides guidelines for assessment and interpretation of field results.

Chapter 2 FACTORS AFFECTING NATURAL OIL BIODEGRADATION AND BIOREMEDIATION SUCCESS

Oil bioremediation is a complex process involving interactions of oil and microorganisms under the conditions of the prevailing environment. To understand the scope and strategies of oil bioremediation, it is essential to first understand the properties of oil, the environment of concern (e.g., marine shorelines and freshwater wetlands), the fate of oil in that environment, the mechanisms of oil biodegradation and the factors that control its rate.

2.1 Physical-Chemical Properties of Crude Oil and Oil Products

Crude oil and petroleum products are very complex and variable mixtures of thousands of individual compounds that exhibit a wide range of physical properties. Understanding these properties is important in determining behavior of spilled oil and the appropriate response option. The composition and properties of various petroleum hydrocarbons have been described in detail by Clark and Brown (1977) and the National Academy of Sciences (1985). Large oil property databases also exist such as the one posted on the Internet by Environment Canada (www.etcenttre.org/spills), which contains information on over 400 oils (Jokuty, *et al*., 2000).

2.1.1 Chemical composition of crude oils and oil products

Crude Oil

Crude oil is comprised of both hydrocarbon compounds (accounting for 50–98% of total composition) and non-hydrocarbon compounds (containing sulfur, nitrogen, oxygen, and various trace metals) in a wide array of combinations (Clark and Brown, 1977). The chemical composition and physical characteristics of several crude oils is illustrated in Table 2.1. Some representative organic compounds found in crude oil are illustrated in Figure 2.1. Petroleum components may be classified into four major groups based on their differential solubility in organic solvents (Leahy and Colwell, 1990).

1. Saturated hydrocarbons: Include normal and branched alkanes with structures of C_nH_{2n+2} (aliphatics) and cyclic alkanes with structures of C_nH_{2n} (alicyclics), which range in chain length from one carbon to over 40 carbons. Saturates usually are the most abundant constituents in crude oils.

2. Aromatic hydrocarbons: Include monocyclic aromatics (e.g., benzene, toluene, and xylenes) and polycyclic aromatic hydrocarbons (PAHs) (e.g., naphthalene, anthracene, and phenanthrene), which have two or more fused aromatic rings. PAHs are of particular environmental concern because they are potential carcinogens or may be transformed into carcinogens by microbial metabolism.

3. Resins: Include polar compounds containing nitrogen, sulfur, and oxygen (e.g., pyridines and thiophenes). They are often referred to as NSO compounds.

4. Asphaltenes: Consist of poorly characterized high molecular weight compounds that include both high molecular weight and poorly characterized hydrocarbons and NSOs. Metals such as nickel, vanadium, and iron are also associated with asphaltenes.

Table 2.1 Chemical composition and physical properties of representative crude oils (adapted from Clark and Brown, 1977)

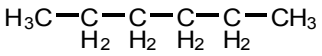
Characteristic or Component	Prudhoe Bay	South Louisiana	Kuwait
API gravity (20°C)	27.8	34.5	31.4
Sulfur (wt%)	0.94	0.25	2.44
Nitrogen (wt%)	0.23	0.69	0.14
Nickel (ppm)	10	2.2	7.7
Vanadium (ppm)	20	1.9	28
Naphtha fraction (wt%)[a]	23.2	18.6	28.0
Saturates	19.9	16.5	20.3
Aromatics	3.2	2.1	2.4
Resins & Asphaltenes	--	--	--
High-boiling fraction (wt%)[b]	76.8	81.4	77.3
Saturates	47.7	56.3	34.0
Aromatics	25.0	16.5	21.9
Resins & Asphaltenes	4.1	8.6	21.4

These analyses represent values for one typical crude oil from three distinct geographical regions; variations in composition can be expected for oils produced from different formations or fields within each region.

a Fraction boiling from 20° to 205°C

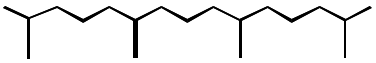
b Fraction boiling above 205°C

SATURATES

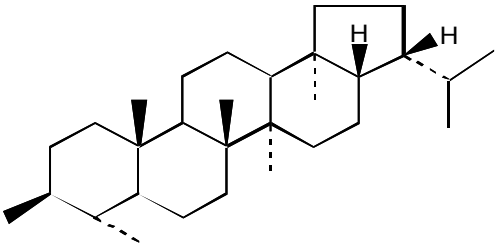


n-hexane

n-heptadecane (n-$C_{17}H_{36}$)

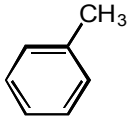


pristane ($C_{19}H_{40}$)



17α(H),21β(H)-hopane

AROMATICS



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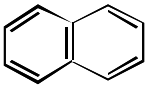
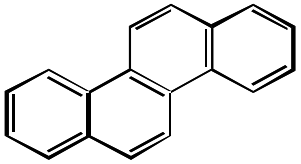
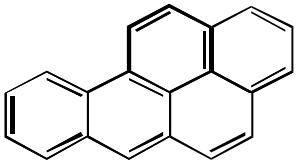


naphthalene



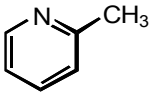
chrysene



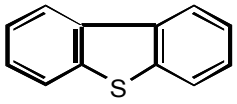
benzo[a]pyrene

RESINS

2-methylpyridine



dibenzo-
thiophene



ASPHALTENES

$(C_{79}H_{92}N_2S_2O)_3$

Figure 2.1 Representative organic compounds found in crude oils

Refined oil products

Refined petroleum products, such as gasoline, kerosene, jet fuels, fuel oils, and lubricating oils, are derived from crude oils through processes such as catalytic cracking and fractional distillation. These products have physical and chemical characteristics that differ according to the type of crude oil and subsequent refining processes. They contain components of crude oil covering a narrow range of boiling points. In addition, during catalytic cracking operations, unsaturated compounds, or olefins (alkenes and cycloalkenes), which are not present in crude oils, can be formed. The concentrations of olefins are as high as 30% in gasoline and about 1% in jet fuel (NAS, 1985). A list of chemical compositions of the fractions of crude oils and the refined products is shown in Table 2.2.

Table 2.2 Chemical compositions of refined petroleum products (adapted from Clark and Brown, 1977)

Distillation Fraction	Hydrocarbon Types	Range of Carbon Atoms	Typical Refined Products
Gasoline & naphtha	Saturates Olefins Aromatics	4-12	Gasoline
Middle distillate	Saturates Olefins Aromatics	10-20	Kerosene Jet fuel Heating oils Diesel oils
Wide-cut gas oil	Saturates Aromatics	18-45	Wax Lubricating oil
Residum	Resins Asphaltenes	>40	Residual oils Asphalt

2.1.2 Physical properties of oil

Important physical properties of oil that affect its behavior in the environment and spill cleanup responses include:

1. Density: Two types of density expressions for oils are often used: specific gravity and American Petroleum Institute (API) gravity. Specific gravity is the ratio of the mass of a substance to the mass of the equivalent volume of water at a specified temperature. The API gravity arbitrarily assigns a value of 10° to pure water at 10°C (60°F). The API gravity can be calculated from the specific gravity using the formula:

$$API\ Gravity\left(^{o}\right)=\frac{141.5}{Specific\ Gavity\ (16\ ^{o}C/60\ ^{o}F)}-131.5 \qquad (2.1)$$

Oils with low densities or low specify gravities have high API gravities. Crude oils have specific gravities in the range of 0.79 to 1.00 (equivalent to API Gravities of 10 to 48) (Clark and Brown, 1977). Oil density is an important index of oil composition that is frequently used to predict its fate in water.

2. Viscosity: Viscosity is the property of a fluid that describes how it resists a change in shape or movement. The lower the viscosity a fluid has, the more easily it flows. The viscosity of petroleum is related to oil compositions and the ambient temperature. It is an important index of the spreading rate of a spilled oil.

3. Pour Point: The pour point of an oil is the temperature at which it becomes semi-solid or stops flowing. The pour point of crude oils varies from –57°C to 32°C. It is another important characteristic with respect to oil fate and cleanup strategies.

4. Solubility in water: The solubility of oil in water is extremely low and depends on the chemical composition of the petroleum hydrocarbon in question and temperature. For a typical crude oil, solubility is around 30 mg/L (NAS, 1985). The most soluble oil components are the low molecular weight aromatics such as benzene, toluene and xylene. This property is important with respect to oil fate, oil toxicity and bioremediation processes.

Other important physical properties of oils include flash point, vapor pressure, surface tension, and adhesion.

2.2 Behavior of Oil in the Environment

2.2.1 Weathering processes

When oil is introduced into the environment, it immediately goes through a variety of physical, chemical and biological changes (Figure 2.2). These weathering processes will alter oil composition and properties in ways that may affect spill response strategies. Bioremediation is a relatively slow process that is often used as a polishing step after conventional cleanup options have been applied. Thus the residual target oil may be extensively weathered prior to the deployment of bioremediation strategies.

Weathering processes, including biodegradation, have been reviewed and described extensively in the literature (Clark and MacLeod, 1977; Jordan, R.E. and Payne, J.R., 1980; National Academy of Sciences, 1985). Major physical and chemical fates of oil are briefly summarized in this section and the biological fate will be discussed in section 2.3.

Spreading
The spreading of oil on water is one of the most important processes during the first hours of a spill, provided that the oil pour point is lower than the ambient temperature. The principal forces influencing the spreading of oil include gravity, inertia, friction, viscosity and surface tension. This process increases the overall surface area of the spill, thus enhancing mass transfer via evaporation, dissolution, and later biodegradation.

Evaporation
In terms of environmental impacts, evaporation is the most important weathering process during the early stages of an oil spill in that it can be responsible for the removal of a large fraction of the oil including the more toxic, lower molecular weight components. For oil on water, evaporation removes virtually all the normal alkanes smaller than C_{15} within 1 to 10 days. Volatile aromatic compounds, such as benzene and toluene, can also be rapidly removed from an oil slick through evaporation. However, these volatile oil components may be more persistent when oil is stranded in sediments. The volatile components make up 20-50% of most crude oils, about 75% of No. 2 fuel oil, and about 100% of gasoline and kerosene. As a result, the physical properties of the remaining slick change significantly (e.g., increased density and viscosity). Major factors influencing the rate of evaporation include composition and physical properties of the oil, wave action, wind velocity, and water temperature (Clark and MacLeod, 1977; Jordan, R.E. and Payne, J.R., 1980).

Dissolution
Although dissolution is less important from the viewpoint of mass loss during an oil spill, dissolved hydrocarbon concentrations in water are particularly important due to their potential influence on the success of bioremediation and the effect of toxicity on biological systems. The extent of dissolution depends on the solubility of the spilled oil, weather conditions, and the characteristics of the spill site. The low molecular weight aromatics are the most soluble oil components, and they are also the most toxic components in crude and refined oils. Although many of them may be removed through evaporation, their impact on the environment is much greater than simple mass balance considerations would imply (NAS, 1985). Dissolution rates are also influenced by photochemical and biological processes.

Photooxidation
Photooxidation is another weathering process that may have important biological consequences. In the presence of oxygen, natural sunlight has sufficient energy to transform many complex petroleum compounds such as high molecular weight aromatics and polar compounds into simpler compounds through a series of free-radical chain reactions. This process may increase the solubility of oil in water, due to the formation of polar compounds such as hydroperoxides, aldehydes, ketones, phenols, and carboxylic acids. Detrimental effects may be associated with this increase in the solubility of oil in water (i.e., bioavailability) and the formation of toxic compounds mediated by photooxidation. On the other hand, the formation of polar compounds may increase the rate of biodegradation of petroleum, particularly at lower concentrations where acute toxicity effects are limited (Nicodem *et al*. 1997).

Dispersion

Dispersion, or formation of oil-in-water emulsions, involves incorporating small droplets of oil into the water column, resulting in an increase in surface area of the oil. In general, oil-in-water emulsions are not stable. However, they can be maintained by continuous agitation, interaction with suspended particulates, and the addition of chemical dispersants. Dispersion may influence oil biodegradation rates by increasing the contact between oil and microorganisms and/or by increasing the dissolution rates of the more soluble oil components.

Emulsification

The process of emulsification of oils involves a change of state from an oil-on-water slick or an oil-in-water dispersion to a water-in-oil emulsion, with the eventual possible formation of a thick, sticky mixture that may contain up to 80% water, commonly called "chocolate mousse". The formation and stability of emulsions are primarily related to the chemical composition of the oils and are enhanced by wax and asphaltic materials. Surface-active materials generated through photochemical and biological processes are also involved in formation of the emulsions. The formation of emulsions makes oil clean-up operations more difficult by decreasing the effectiveness of physical oil spill recovery procedures and suppressing the natural rates of oil biodegradation.

Other important physical and chemical weathering processes that influence the rates of oil degradation include adsorption onto suspended particulate materials, sinking and sedimentation, and tar ball formation.

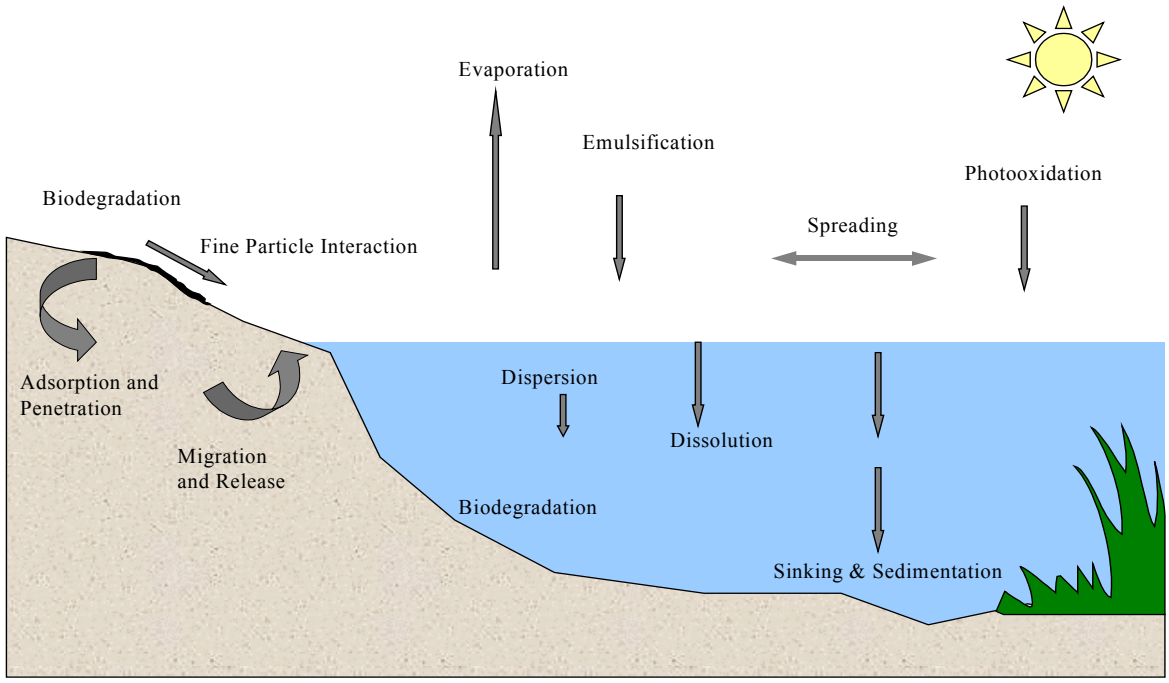


Figure 2.2 Major weathering processes after an oil spill

2.2.2 Oil and shoreline interactions

When oil spills occur in marine or freshwater shoreline (e.g., freshwater wetlands) environments, interactions between the spilled oil and the shore further complicate the weathering processes. It is very important to understand these interactions in determining the scope and limitations of oil spill bioremediation.

The behavior of spilled oil in shoreline environments is primarily dependent on the properties of the shoreline, such as the porosity of the substrate and the energy of the waves acting on a shoreline. Higher wave exposure enhances both physical removal and weathering processes. Wave-swept rocky shores tend to recover from oil spills within a matter of months whereas mangroves and marshes may act as a petroleum sink for many years. However, tidal pumping is also a factor promoting oil penetration into the sediments. The rate and depth of oil penetration depend primarily on the porosity of the substrate. On coarse-grained shorelines like cobble and sandy beaches, oil can penetrate deeper and remain longer (when it is trapped below the limit of wave action), compared to finer grained sediments such as silts and clay. However, oil is more easily removed by water flushing from coarse-grained sediments. Interactions of oil with tidal action, waves, and shoreline substrate may also form asphalt-like oil-sediment mats that are resistant to further biological and photochemical weathering.

Recent studies have shown that the interactions between oil and fine mineral particles also play an important role in natural oil cleansing in marine shorelines (Bragg and Owens, 1995; Lee *et al.,* 1997b). This process of oil and fine-particle interaction reduces the adhesion of oil to intertidal shoreline substrates through the formation of oil-mineral fine flocs that are easily dispersed by tidal action and currents. More importantly, oil-mineral fine flocs enhance the availability of oil for biodegradation, and thus oil biodegradation rates are accelerated by this process (Lee *et al*., 1997c).

2.2.3 Shoreline sensitivities

Marine shorelines and freshwater environments have a wide range of sensitivities to oil and clean-up activities. The National Oceanic & Atmospheric Administration and the American Petroleum Institute have developed the Environmental Sensitivity Index (ESI) to classify shoreline types for spill response (NOAA, 1992; NOAA and API, 1994). This classification scheme (e.g., Table 2.3) has been used in oil spill contingency planning and spill response operations (Hayes *et al*., 1995). Major factors considered in ranking habitat sensitivity include shoreline type (substrate, grain size, tidal elevation), exposure to wave and tidal energy, biological productivity and sensitivity, and ease of cleanup. Bioremediation may be effective and cause the least damage on both the moderately and the most sensitive shoreline types.

The Environmental Sensitivity Index for freshwater shorelines is shown in Table 2.4, based on NOAA & API (1994) and Hayes *et al*. (1995 & 1997). Major factors considered in ranking ESI for these habitats include degree of exposure to natural removal processes, biological

productivity and sensitivity, human use of the habitat, and ease of oil removal. Bioremediation may be feasible and cause the least damage on both the moderately sensitive and the most sensitive shoreline types, although its effectiveness is still uncertain due to the lack of sufficient research and field efficacy demonstrations.

Table 2.3 Shoreline ESI ranking for habitats in marine shorelines (where 1 is least sensitive and 10 is most sensitive to oil and clean up actions)

Environmental Sensitivity Index (ESI)	Shoreline Type
1	Exposed rocky shores
	Sea walls and piers
2	Exposed wave-cut platforms
3	Fine-grained sand beaches
4	Coarse-grained sand beaches
5	Mixed sand and gravel beaches
6	Gravel beaches and riprap
7	Exposed tidal flats
8	Sheltered rocky shores
9	Sheltered tidal flats
10	Salt marshes and Mangroves

2.3 Biodegradation of Oil

Biodegradation of oil is one of the most important processes involved in weathering and the eventual removal of petroleum from the environment, particularly for the nonvolatile components of petroleum. Numerous scientific review articles have covered various aspects of this process and the environmental factors that influence the rate of biodegradation (Zobell, 1946 & 1973; Atlas, 1981 & 1984; NAS, 1985; Focht and Westlake, 1987; Leahy and Colwell, 1990).

2.3.1 Mechanism of oil biodegradation: a microbiological perspective

Distribution of hydrocarbon-degrading microorganisms

Microorganisms capable of degrading petroleum hydrocarbons and related compounds are ubiquitous in marine, freshwater, and soil habitats. Over 200 species of bacteria, yeasts and fungi have been shown to degrade hydrocarbons ranging from methane to compounds of over 40 carbon atoms (Zobell, 1973). In the marine environment, bacteria are considered to be the predominant hydrocarbon-degraders with a distribution range that even covers extreme cold Antarctic and Arctic environments (Floodgate, 1984; Jordan and Payne, 1980). In the freshwater environment, yeast and fungi may also play a significant role in degrading petroleum hydrocarbons (Cooney, 1984). Some of the most important hydrocarbon-degrading microorganisms in both marine and freshwater environments are listed in Table 2.5.

Table 2.4 Shoreline ESI ranking for habitats in freshwater shorelines (where 1 is least sensitive and 10 is most sensitive to oil and clean up actions)

ESI	Lacustrine [a]	Large Rivers [a]	Small Rivers [b]
1	Exposed rocky cliffs Exposed man-made structures	Exposed rocky banks Vertical, solid revetments	Quiet pools with low-sensitive banks
2	Shelving bedrock shores	Rocky shoals, bedrock Ledges	Small, nonnavigable channel with moderate currents and low-sensitive banks
3	Eroding scarps in unconsolidated sediments	Exposed, eroding banks in Unconsolidated sediments	Navigable channel with moderate currents and low-sensitive banks
4	Sand beaches	Sandy bars and gently Sloping banks	Small, nonnavigable channel with rapids over bedrock
5	Mixed sand and gravel beaches	Mixed sand and gravel bars	Navigable channel with rapids over bedrock
6	Gravel beaches and riprap	Gravel bars and riprap	Channel with associated low-vulnerable upper bottomland hardwoods
7	Exposed flats	Not present	Navigable streams with associated wide swamps on one side
8	Sheltered rocky shores Sheltered man-made structures	Vegetated, steeply sloping Bluffs Sheltered man-made Structures	Navigable streams with associated wide swamps on both sides
9	Sheltered vegetated low banks	Vegetated low banks Muddy substrates (unvegetated)	Meandering channel with abundant leakage points into associated swamps and ox-bows
10	Sheltered sand flats Freshwater marshes and swamps	Freshwater marshes and Swamps	Navigable anastomosing channel with abundant leakage points into associated swamps

a ESI adapted from Hayes *et al.* (1995)
b RSI (Reach Sensitivity Index) adapted from Hayes *et al.* (1997)

Table 2.5 Representative microorganisms capable of degrading petroleum hydrocarbons (Based on Atlas, 1984; Focht and Westlake, 1987; Jordan and Payne, 1980; Leahy and Colwell, 1990)

Bacteria	Yeast and Fungi
Achromobacter	*Aspergillus*
Acinetobacter	*Candida*
Alcaligenes	*Cladosporium*
Arthrobacter	*Penicillium*
Bacillus	*Rhodotorula*
Brevibacterium	*Sporobolomyces*
Cornybacterium	*Trichoderma*
Flavobacterium	
Nocardia	
Pseudimonas	
Vibrio	

The distribution of hydrocarbon-utilizing microorganisms is also related to the historical exposure of the environment to hydrocarbons. Those environments with a recent or chronic oil contamination will have a higher percentage of hydrocarbon degraders than unpolluted areas. In "pristine" ecosystems, hydrocarbon utilizers may make up less than 0.1% of the microbial community; and in oil-polluted environments, they can constitute up to 100% of the viable microorganisms (Atlas, 1981).

It should be noted that there is no single strain of bacteria with the metabolic capacity to degrade all the components found within crude oil. In nature, biodegradation of a crude oil typically involves a succession of species within the consortia of microbes present. Microorganisms classified as non-hydrocarbon utilizers may also play an important role in the eventual removal of petroleum from the environment. Degradation of petroleum involves progressive or sequential reactions, in which certain organisms may carry out the initial attack on the petroleum constituent; this produces intermediate compounds that are subsequently utilized by a different group of organisms, in the process that results in further degradation (Karrick, 1977).

Biodegradation of oil components

As described earlier, petroleum components can be classified into four major groups: saturates, aromatics, resins, and asphaltenes. Major metabolic pathways for many of these compounds have been well studied and documented (Atlas, 1981 & 1984; Cerniglia, 1992; Watkinson and Morgon, 1990) to explain their differences in susceptibility to biodegradation.

Saturates In general, the *n*-alkanes are the most readily degraded components in a petroleum mixture (Zobell, 1946; Atlas, 1981). Biodegradation of *n*-alkanes with molecular weights up to C_{44} has been demonstrated (Haines and Alexander, 1974). Alkanes in the C_{10} to C_{26} range are considered the most readily and frequently utilized hydrocarbons (Atlas 1995b;

NAS, 1985). The predominant mechanism of n-alkane degradation involves terminal oxidation to the corresponding alcohol, aldehydes, or fatty acid functional group. Branched alkanes are less readily degraded in comparison to *n*-alkanes. Methyl branching increases the resistance to microbial attack because fewer alkane degraders can overcome the blockage of beta-oxidation (NAS, 1985). Highly branched isoprenoid alkanes, such as pristane and phytane, which were earlier thought to be resistant to biodegradation, have also been shown to be readily biodegradable. Cycloalkanes, however, are particularly resistant to biodegradation. Complex alicyclic compounds such as hopanes and steranes are among the most persistent compounds of petroleum spills in the environment (Atlas, 1981).

Aromatics Although the aromatics are generally more resistant to biodegradation, some low molecular weight aromatics such as naphthalene may actually be oxidized before many saturates (Focht and Westlake, 1987). Monoaromatic hydrocarbons are toxic to some microorganisms due to their solvent action on cell membranes, but in low concentrations they are easily biodegradable under aerobic conditions. PAHs with 2-4 rings are less toxic and biodegradable at rates that decrease with the level of complexity. PAHs with five or more rings can only be degraded through co-metabolism, in which microorganisms fortuitously transform non-growth substrates while metabolizing simpler hydrocarbons or other primary substrates in the oil. Alkylated aromatics are degraded less rapidly than their parent compounds; the more highly alkylated groups are degraded less rapidly than less alkylated ones. The metabolic pathways for the biodegradation of aromatic compounds have been the subject of extensive study (Atlas, 1981; Prince, 1993; Cerniglia, 1992). The bacterial degradation of aromatics normally involves the formation of a diol, followed by ring cleavage and formation of a di-carboxylic acid. Fungi and other eukaryotes normally oxidize aromatics using mono-oxygenases, forming a trans-diol.

Resins and asphaltenes Compared to saturates and aromatics, very little is known about biodegradation of resins and asphaltenes; this is due to their complex structures, which are difficult to analyze. Resins and asphaltenes have previously been considered to be refractory to degradation. However, there is recent evidence of asphaltene degradation through cometabolism (Leahy and Colwell, 1990). Some resins, particularly low-molecular-weight resin fractions, can also be biodegraded at low concentrations (NAS, 1985). Further research is still needed to understand the biodegradation of these compounds.

In summary, the susceptibility of petroleum hydrocarbons to microbial degradation is generally in the following order: n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes. However, this pattern is not universal (Perry, 1984). The compositional heterogeneity among different oils greatly affects the biodegradation rate of their constituents. The degradation rate for the same oil constituents may vary significantly for different oils. Cometabolism also plays an important role in oil biodegradation. Many complex branched, cyclic, and aromatic hydrocarbons, which otherwise would not be biodegraded individually, can be oxidized through cometabolism in an oil mixture due to the abundance of other substrates that can be metabolized easily within the oil (Atlas, 1981). The biological fate of oil components in an oil mixture still requires further research. Particularly, effort should be made to establish a database regarding the biodegradability of different types of oils and petroleum products.

2.3.2 Environmental factors affecting oil biodegradation

When oil spills occur in the environment, the rate of oil biodegradation is also greatly influenced by the characteristics of the contaminated environment. Major environmental factors affecting oil biodegradation include weathering processes, temperature, availability and concentration of nutrients, availability and concentration of oxygen, and pH.

Weathering processes

The weathering processes described in section 2.2.1 have profound effects on oil biodegradation. Evaporation of volatile oil components may benefit microorganisms by removing more toxic low-molecular-weight components such as benzene and smaller *n*-alkanes. However, this process also leads to a lower biodegradable percentage of oil, since these components in general are readily biodegraded (Atlas, 1981; NAS, 1985). The oil surface area is important because growth of oil degraders occurs almost exclusively at the oil-water interface (Atlas and Bartha, 1992). Formation of water-in-oil emulsions or mousses reduces the surface area, therefore decreasing biodegradation. Tarballs, which are large aggregates of weathered and undegraded oil, also restrict access to microorganisms because of their limited surface area (Leahy and Colwell, 1990). Dispersion of hydrocarbons in the water column in the form of oil-in-water emulsions increases the surface area of the oil and thus its availability for microbial attack. The formation of oil-in-water emulsions through the microbial production and release of biosurfactants has also been found to be an important process in the uptake of hydrocarbons by bacteria and fungi (Singer and Finnerty, 1984). In contrast, the application of chemical dispersants has produced mixed results and has not been shown to be an effective way to enhance oil biodegradation. Photooxidation leads to the formation of more soluble compounds, which are often more biodegradable. However, the effect of photooxidation processes on biodegradation is still not well understood (Nicodem *et al*., 1997).

Biodegradation rates are also influenced by concentrations of individual oil constituents, which may be affected by various weathering processes. For example, microbes may attack very low concentrations of pollutants in the environment inefficiently (Focht and Westlake, 1987). However, high concentrations of hydrocarbons may cause inhibition of biodegradation by nutrient or oxygen limitations or toxic effects. There would seem to be, for many hydrocarbons, an optimum concentration range for metabolism below which degradation is not stimulated and above which inhibition occurs. Weathering processes will affect the ultimate concentrations of petroleum hydrocarbons in the environment in different ways. Evaporation may reduce the concentrations of volatile compounds but concentrate some other constituents. Sorption and emulsification may concentrate the pollutants, while dispersion and dissolution tend to dilute them.

Temperature

The ambient temperature of an environment affects both the properties of spilled oil and the activity or population of microorganisms. At low temperatures, the viscosity of the oil increases, while the volatility of toxic low-molecular-weight hydrocarbons is reduced, delaying the onset of

biodegradation (Atlas, 1981). Some hydrocarbons are more soluble at lower temperatures (e.g., short-chain alkanes), and some low-molecular-weight aromatics are more soluble at the higher temperature (Focht and Westlake, 1987). Although hydrocarbon biodegradation can occur over a wide range of temperatures, the rate of biodegradation generally decreases with decreasing temperature. Highest degradation rates generally occur in the range of 30 to 40°C in soil environments, 20 to 30°C in some freshwater environments, and 15 to 20°C in marine environments (Bossert and Bartha, 1984; Cooney, 1984; Jordan and Payne, 1980).

The effect of temperature is also complicated by other factors such as the composition of the microbial population. In environments where a psychrophilic population has been established, degradation can occur at significant rates under cold conditions. Hydrocarbon biodegradation has been observed at temperature as low as 0-2°C in seawater and –1.1°C in a soil. Colwell *et al.* (1978) reported greater degradation of Metula crude oil at 3°C than at 22°C with a mixed culture in beach sand samples. Westlake *et al.* (1974) also found that bacteria capable of degradation at 4°C would metabolize oil at 30°C, but those populations that developed at 30°C had a limited activity at 4°C.

Oxygen

Aerobic conditions are generally considered necessary for extensive degradation of oil hydrocarbons in the environment since major degradative pathways for both saturates and aromatics involve oxygenases (Atlas, 1981; NAS, 1984; Cerniglia, 1992). Many studies have shown that oxygen depletion leads to sharply reduced biodegradation activities in marine sediments and in soils (Atlas, 1981; Bossert and Bartha, 1984; Hambrick III *et al*., 1980). Conditions of oxygen limitation normally do not exist in the upper levels of the water column in marine and freshwater environments and in the surface layer of most beach environments. It may become limiting in subsurface sediments, anoxic zones of water columns, and most fine-grained marine shorelines, freshwater wetlands, mudflats, and salt marshes. Factors affecting the availability of oxygen also include the action of wave and water flow, the physical state of the oil, and the amount of available substrates.

Anaerobic oil degradation has been shown in some studies to occur only at negligible rates, as reviewed by Atlas (1981), leading to the conclusion that the environmental importance of anaerobic hydrocarbon degradation can be discounted. However, recent studies have shown that anaerobic hydrocarbon metabolism may be an important process in certain conditions (Head and Swannell, 1999). The biodegradation of some aromatic hydrocarbons, such as BTEX compounds, has been clearly demonstrated to occur under a variety of anaerobic conditions (Krumholz *et al*., 1996; Leathy and Colwell, 1990). Studies have also demonstrated that in some marine sediments, PAHs and alkanes can be degraded under sulfate-reducing conditions at similar rates to those under aerobic conditions (Caldwell *et al.*, 1998; Coates *et al*., 1997). The importance of anaerobic biodegradation of oil in the environment still requires further studies.

Nutrients

In theory, approximately 150 mg of nitrogen and 30 mg of phosphorus are utilized in the conversion of 1 g of hydrocarbon to cell materials (Rosenberg and Ron, 1996). When a major

oil spill occurs in marine and freshwater environments the supply of carbon is dramatically increased and the availability of nitrogen and phosphorus generally becomes the limiting factor for oil degradation (Atlas, 1984; Leahy and Colwell, 1990). In marine environments, nutrient limitation is generally correlated to the low background levels of nitrogen and phosphorus in seawater (Floodgate, 1984). Nutrient concentrations are more variable in freshwater systems where lakes and wetlands range from oligotrophic to eutrophic; rivers can be nutrient-poor at the source, but generally become nutrient-rich downstream after receiving industrial and domestic effluents and agricultural runoff (Cooney, 1984). Freshwater wetlands are typically considered to be nutrient limited, due to heavy demand for nutrients by the plants. They are also viewed as being nutrient traps, as a substantial amount of nutrients may be bound in biomass (Mitsch and Gosselink, 1993). Both freshwater lakes and wetlands may also exhibit seasonal variations in nutrient levels, which will affect the performance of oil biodegradation. Ward and Brock (1976) found that in an oil-contaminated lake, oil biodegradation was at the highest rate during early spring when the nutrient content (i.e., N and P) was also high. As N and P levels decreased in the summer (probably due to algal productivity) oil biodegradation also decreased. Another potential limiting nutrient is iron, which was found to limit oil degradation in clean offshore seawater, but is not likely to be limiting in freshwater (Focht and Westlake, 1987).

Other factors

Other important factors affecting biodegradation of petroleum hydrocarbons include pH and salinity. The pH of seawater is generally stable and slightly alkaline (Bossert and Bartha, 1984). In contrast, the pH of freshwater and soil environments can vary widely. Organic soils in wetlands are often acidic, while mineral soils have more neutral and alkaline conditions. Most heterotrophic bacteria and fungi favor a neutral pH, with fungi being more tolerant of acidic conditions. Studies have shown that degradation of oil increases with increasing pH, and that optimum degradation occurs under slightly alkaline conditions (Dibble and Bartha, 1979; Focht and Westlake, 1987).

Changes in salinity may affect oil biodegradation through alteration of the microbial population. Dramatic variation in salinity may occur in estuarine environments where marine organisms mingle with freshwater forms. Many freshwater organisms can survive for long periods in seawater although few can reproduce. In contrast, most marine species have an optimum salinity range of 2.5 to 3.5% and grow poorly or not at all at salinity lower than 1.5 to 2% (Zobell, 1973). In a study of hypersaline salt evaporation ponds, Ward and Brock (1978) showed that rates of hydrocarbon metabolism decreased with increasing salinity in the range of 3.3 to 28.4%. More studies are required to understand the effect of salinity on oil biodegradation.

2.3.3 Evaluation of oil biodegradation: application of biomarkers

The evaluation of oil biodegradation is a difficult task, especially in the field, due to the complication of weathering processes and the heterogeneity of contaminated sites. As described earlier, physical and chemical weathering can significantly affect the composition and concentrations of oils. Oil contaminated sites are often highly heterogeneous, where oil concentrations can vary greatly within a small area. Consequently, variability associated with

field studies can be so high as to preclude or interfere with one's ability to discern significant treatment differences. Non-biodegradable or slowly biodegradable components in oil - often called biomarkers - have been used successfully to mitigate the high variability associated with field studies (Bragg *et al*., 1994; Venosa *et al*., 1996; Lee *et al*., 1997a). This approach estimates the extent of biodegradation by evaluating the ratios of target hydrocarbon concentrations relative to the concentration of these recalcitrant biomarkers. Studies have shown that use of biomarkers reduces spatial variability of oil data when compared to other mass balance approaches and allows biodegradation to be monitored effectively by reducing the number of samples required (Douglas *et al.*, 1994).

Commonly used biomarkers for evaluation of biodegradation of crude oils include the isoprenoids pristane and phytane, steranes, and the pentacyclic triterpanes such as the hopanoids (Peters and Moldowan, 1993). While the isoprenoids pristane and phytane are somewhat more resistant to biodegradation than *n*-alkanes with similar boiling points (C_{17}, C_{18}), they should only be used to monitor the earliest stages of a biodegradation treatment program, as they are known to be biodegradable under natural conditions (Prince *et al*., 1994b). Hopanes have become the biomarker of choice as they are much more resistant to microbial biodegradation (Atlas, 1981; Peters and Moldowan, 1993). The compound 17α(H), 21β(H)-hopane was successfully used to determine the efficacy of bioremediation field trials coordinated with the *Exxon Valdez* spill clean up operations (Douglas *et al*., 1994; Mearns, 1997; Prince, *et al*., 1994 a&b). However, caution must be taken with their use as biomarkers since they are also very resistant to physical and chemical weathering processes that affect many alkanes and aromatics. Therefore, hopane normalization is more useful in reducing the variability associated with heterogeneous oil distribution or in cases where the effects of physical and chemical weathering are negligible. Biodegradation can also be verified as the main removal mechanism by determining the relative degradation rates for homologous series of alkylated PAHs (Elmendorf *et al*., 1994; Venosa *et al.*, 1997a).

2.4 Laboratory Studies on Bioremediation of Oil

Biodegradation as a natural process may proceed slowly, depending on the type of oil (i.e., light crude oils degrade faster than heavier oils). Bioremediation strategies are based on the application of various methodologies to increase the rate or extent of the biodegradation process. The success of oil spill bioremediation depends on our ability to optimize various physical, chemical, and biological conditions in the contaminated environment. As described in previous sections, the most important requirement is the presence of microorganisms with the appropriate metabolic capabilities. If these microorganisms are present, then optimal rates of growth and hydrocarbon biodegradation can be sustained by ensuring that adequate concentrations of nutrients and oxygen are present and that the pH is between 6 and 9 (Atlas and Bartha, 1992). The physical and chemical characteristics of the oil and oil surface area are also important determinants of bioremediation success. Obviously, some of these factors can be manipulated more easily than others. For example, on an operational scale, there is nothing that can be done to alter the chemical composition of the oil.

There are two main approaches to oil spill bioremediation. 1) *bioaugmentation,* in which oil-degrading microorganisms are added to supplement the existing microbial population, and 2) *biostimulation*, in which the growth of indigenous oil degraders is stimulated by the addition of nutrients or other growth-limiting cosubstrates and/or habitat alteration. Both these approaches have been extensively studied, on a laboratory scale as well as in the bioremediation of oil contaminated shorelines.

2.4.1 Bioaugmentation

Although hydrocarbon-degrading microorganisms are widespread in nature, bioaugmentation has been considered as a potential strategy for oil-bioremediation since the 1970s. The rationale for adding oil-degrading microorganisms is that indigenous microbial populations may not be capable of degrading the wide range of potential substrates present in complex mixtures such as petroleum (Leahy and Colwell, 1990). Other conditions under which bioaugmentation may be considered are when the indigenous hydrocarbon-degrading population is low, the speed of decontamination is the primary factor, and when seeding may reduce the lag period to start the bioremediation process (Forsyth et al., 1995).

Many vendors offer microbial agents claiming to enhance oil biodegradation (Prince, (1993). However, laboratory studies on bioaugmentation have produced mixed results. Aldrett *et al.* (1997) tested 12 commercial microbial cultures for bioremediation of Alaska North Slope crude oil in the lab. After 28 days, four products showed an enhancement of oil biodegradation with significantly higher degradation rates of alkanes and aromatics when compared to a nutrient control. In another shaker-flask experiment, Hozumi *et al*. (2000) investigated the effectiveness of a microbial product in treating a heavy oil spilled from *Nakhodka* using the thin layer chromatography-flame ionization detection (TLC-FID) analysis. They found that approximately 35% of the oil was degraded with addition of the microbial product compared to no oil loss for a control during a three-week test period. Surprisingly, the asphaltene fraction showed the highest loss among the four major oil components, which raises the question whether this oil loss was actually due to biodegradation rather than some quality control problem with the chemical analysis. Some laboratory studies found that microbial seeding may enhance oil degradation in seawater but not in freshwater environments (Leahy and Colwell, 1990). To examine whether microbial products can compete with the indigenous populations, Venosa *et al.* (1991) tested 10 different commercial microbial products using weathered Alaskan crude oil in shaker flask microcosms. Although two products showed enhancement compared to a nutrient control, better degradation was observed in every case when the commercial products were first sterilized, suggesting that indigenous Alaskan microorganisms were primarily responsible for the oil biodegradation and seeded microorganisms seemed to compete poorly with the indigenous population in the closed flask environment. Thus, bioaugmentation may be effective in bench-scale studies where environmental conditions are well controlled, but this will not guarantee its effectiveness in the field.

Creation of a "superbug" that combines the genetic information from many organisms and the ability to degrade a variety of different types of hydrocarbons has also been considered. Friello

et al. (1976) successfully produced a multiplasmid-containing *Pseudomonas* strain capable of oxidizing aliphatic, aromatic, terpenic, and polyaromatic hydrocarbons. Thibault and Elliot (1980) also developed a multiplasmid *P. putida* strain that can simultaneously degrade some lighter alkanes and aromatics. However, the survival of such a strain in the environment could is questionable. More importantly, the issues of safety, containment, and the potential for ecological damage must be fully resolved before field testing of these organisms can be conducted (Leahy and Colwell, 1990). There is also the problem of public perception over the release of "foreign" and especially "genetically engineered" microorganisms into the environment.

2.4.2 Biostimulation

Biostimulation involves the addition of rate-limiting nutrients to accelerate the biodegradation process. In most shoreline ecosystems that have been heavily contaminated with hydrocarbons, nutrients are likely the limiting factors in oil biodegradation. The main purpose of bench-scale treatability studies is to determine the type, concentration, and frequency of addition of amendments needed for maximum stimulation in the field (Venosa, 1998).

Most laboratory experiments have shown that addition of growth limiting nutrients, namely nitrogen and phosphorus, has enhanced the rate of oil biodegradation. However, the optimal nutrient types and concentrations vary widely depending on the oil properties and the environmental conditions. Wrenn *et al.* (1994) studied the effects of different forms of nitrogen on biodegradation of light Arabian crude oil in respirometers. They found that in poorly buffered seawater, nitrate is a better nitrogen source than ammonia because acid production associated with ammonia metabolism may inhibit oil biodegradation. When the culture pH was controlled, the performance of oil biodegradation was similar for both amendments with a shorter lag time for ammonia addition. Ramstad and Sveum (1995) also compared the effect of nitrate, ammonia, and an organic nitrogen-containing nutrient on biodegradation of topped Statfjord crude oil in a continuous-flow seawater column system. With no control of pH in this study, nitrate was found to have the most pronounced effect in stimulating oil degradation when using pristane as a biomarker. However, in a microcosm study, Jackson and Pardue (1999) found that addition of ammonia appeared to be more effective than nitrate in stimulating degradation of crude oil in salt marsh soils. On a weight basis, the amount of ammonia required to achieve the same increase in biodegradation as nitrate was only about 20%. This was attributed to the fact that ammonia is less likely to be lost from the system by washout due to its higher adsorptive capacity to organic matter.

Oil biodegradation largely takes place at the interface between oil and water. Therefore, the effectiveness of biostimulation depends on the nutrient concentration in the interstitial pore water of oily sediments (Atlas and Bartha, 1992; Bragg *et al*., 1994). The nutrient concentration should be maintained at a level high enough to facilitate bacterial growth. However, caution must be exercised as excessive concentrations of nutrients, such as ammonia, induce toxic responses in many marine species (Pritchard *et al*., 1991). Using nitrate as a biostimulation agent, Venosa *et al.* (1994) determined that approximately 1.5 to 2.0 mg N/L supported near

maximal biodegradation of heptadecane immobilized onto sand particles in a microcosm study. Du *et al.* (1999) investigated the optimal nitrogen concentration for the biodegradation of Alaska North Slope crude oil in continuous flow beach microcosms at a loading of 5g-oil/kg sand. The results showed that nitrate concentrations below approximately 10 mgN/L limited the rate of oil biodegradation. The higher nutrient requirement was attributed to the more complex substrate (crude oil). Ahn (1999) further studied the effect of nitrate concentrations under tidal flow conditions using the same microcosms, oil, and oil loading as Du *et al.* (1999). Nitrate concentrations ranging from 6.25 to 400 mg N/L were supplied semi-diurnally to simulate tide flow. The results from both oil analysis (hopane as a biomarker) and microbial growth (phospholipid analysis) showed that the optimal nitrate concentration fed under these conditions was approximately 25 mgN/L. However, the data showed that at the end of the test (after one month), an approximately 80% degradation of all target alkanes and PAHs was achieved in all test cases that covered a range of nitrate concentrations in the test solutions added. This result suggested that nitrate concentration might only affect the rates and not the extent of oil degradation. Further research in this regard is required to optimize bioremediation strategies.

One of the main challenges associated with biostimulation in oil-contaminated coastal areas is maintaining optimal nutrient concentrations in contact with the oil. Oil from offshore spills usually contaminates the intertidal zone, where the washout rate for water-soluble nutrients can be very high, and this can adversely impact the effectiveness of biostimulation. Many attempts have been made in the design of nutrient delivery systems that overcome the washout problems characteristic of intertidal environments (Prince, 1993). These include oleophilic and slow-release fertilizer formulations, as well as systems that rely on the subsurface flow of water through the beach (Wise *et al*., 1994). Several papers have compared the effectiveness of these nutrient products to stimulate hydrocarbon biodegradation rates. Croft *et al.* (1995) tested the efficiency of an oleophilic organic (Inipol EAP22) and a slow-release inorganic fertilizer (Max Bac) and found that the oleophilic fertilizer was much more effective at stimulating oil degradation on sand than the slow-release product. Sveum and Ramstad (1995) also found that organic products such as fish meal and stick water (a fish meal by-product) were more effective than a slow-release fertilizer (Mac Bac). The failure for this slow-release fertilizer was attributed to the nutrient release rate being too slow to affect oil biodegradation. However, in some other studies, application of organic fertilizers such as Inipol EAP22 also failed to stimulate oil biodegradation (Sveum and Ladousse, 1989; Safferman, 1991). Safferman (1991) investigated the rates of nutrient release from several slow-release products and found that Inipol EAP22 was rapidly washed out from oiled cobble before becoming available to hydrocarbon-degrading bacteria. However, no attempt was made in most of these studies to estimate the steady-state nutrient concentrations that would result in an intertidal environment. The variable results from the laboratory studies indicate that the performance of these products greatly depends on the nutrient release rates and the prevailing environmental conditions.

Although much research has been carried out on the bioremediation of oil-contaminated marine shorelines, few studies have been conducted on oil bioremediation in wetland environments. Purandare *et al.* (1999) conducted the only reported microcosm study on biostimulation in freshwater wetland. They investigated different inorganic mineral nutrients for their ability to

enhance biodegradation of a crude oil. Aquaria of 10-gallon capacity, filled with wetland soil and planted with species of emergent wetland plants, were used to simulate natural wetlands. Two levels of water coverage were studied: (1) water level even with soil surface, and (2) water level 10 cm above the soil surface. Six treatments were evaluated for each water level: unoiled, no-nutrient control; oiled + no nutrient control; oiled + nitrate addition; oiled + nitrate + phosphate addition; oiled + ammonia addition; and oiled + ammonia + phosphate addition. Approximately 14g of weathered light Arabian crude oil was added to each column to form about a 2mm oil layer. The results showed that for both flooded and unflooded wetland conditions, the addition of nitrate and phosphate seemed to enhance the degradation of oil above the natural attenuation rate. The highest biodegradation rates of alkanes and PAHs occurred in the high water level microcosms receiving nitrate and phosphorus, in which a 90% alkane reduction and 50% PAH removal was observed, compared to only 50% alkane reduction and 40% PAH removal in the control microcosms. Higher degradation of alkanes and PAHs in the high water level relative to that in the low water level suggested an increased hydrocarbon bioavailability. However, a microcosm study conducted by Garcia-Blanco *et al*. (2001a) in a simulated tidal salt marsh found that addition of nutrients did not stimulate the biodegradation of a No. 2 fuel oil. Low oxygen availability was suggested to be the limiting factor for oil degradation in salt marshes.

In summary, laboratory studies have shown that biostimulation and, in some cases bioaugmentation, can enhance the rates of oil biodegradation, particularly in marine environments. Oxygen may become a limiting factor in oil biodegradation under certain circumstances, such as salt mashes and freshwater wetlands. However, these conclusions still need to be verified through field evaluations.

2.5 Demonstrations of Oil Bioremediation Under Field Conditions

Field studies can provide the most convincing demonstration of the effectiveness of oil bioremediation since laboratory studies are not always able to account for numerous real world conditions such as spatial heterogeneity, biological interactions, and mass transfer limitations. Compared to laboratory investigations, relatively few tests have been carried out to evaluate the effectiveness of oil bioremediation in the field because such trials are both difficult and expensive to conduct. Swannell *et al.* (1996) conducted the most extensive review available on field evaluations of oil bioremediation in marine environments. Venosa (1998) presented an in-depth critical review emphasizing problems in the design and control of the existing field tests. A review by Lee (2000) addressed the potential significance of enhancing plant growth (i.e. phytoremediation) and oil mineral aggregate formation as biostimulation treatments. Other reviews are also available (Prince, 1993; Leahy and Colwell, 1990). This section will summarize the latest findings from recent field studies on marine shorelines and freshwater wetlands, as well as major points identified in the previous reviews.

2.5.1 Mesocosm studies

Mesocosms or pilot-scale systems can help to simulate actual conditions at relatively low cost, and are frequently used as bridges between microcosms and field systems. Mesocosms have been used to evaluate the effectiveness of numerous bioremediation strategies.

Bioaugmentation

Unlike results from bench-scale tests, numerous mesocosm studies have demonstrated the ineffectiveness of bioaugmentation treatments. For example, Tagger *et al*. (1983) overlaid two mesocosms with crude oil. One was inoculated with an acclimated culture, while only indigenous populations were used in the other. Five months after inoculation, no statistically significant change in oil composition occurred between the two treatments. Neralla *et al.* (1995) investigated the effect of seeding in salt marsh conditions. The greenhouse experiment was conducted with 19-L buckets filled with marsh sediments and actively growing *Spartina alterniflora*. There were 10 duplicated treatments in a total of 20 mesocosms. Results showed that the addition of bioaugmentation products did not enhance the degradation of weathered Arabian Lube crude oil. The conclusion was not surprising since the soil used in the experiment came from microcosms used in a similar study, and a large population of hydrocarbon-degrading microorganisms was already present in the sediment. These results again indicate that oil biodegradation is unlikely limited by availability of hydrocarbon degraders and that seeded microorganisms could not compete with indigent populations.

Biostimulation

Contrary to the mesocosm bioaugmentation studies, the addition of nutrients has proven to be an effective strategy for oil bioremediation. Basseres *et al.* (1993) conducted a mesocosm trial on bioremediation of light Arabian crude oil using two 600-liter tanks filled with sandy beach materials at a site near the Mediterranean. Animal meal containing 60% protein was added at 10 % w/w to one tank. A second was left untreated as a control. Over the 60-day period of the test, 40% of the aliphatic fraction in the treated oil was degraded whereas only 25% was degraded in the control. The number of hydrocarbon degraders was found to be higher in the meal-treated mesocosm than in the control. However, no replicate treatments and nutrient measurements were performed during the study, so it was not possible to determine if the observed differences were statistically significant.

Mendelssohn *et al.* (1995) conducted a greenhouse mesocosm study to determine the effect of oil bioremediation products on salt marsh ecosystems. A randomized block design was used with three treatments (fertilizer, microbial products and control) at two levels of oil dosage. Each treatment combination was replicated five times for a total of 30 sods of marsh. The results demonstrated that the addition of a fertilizer product significantly increased the growth response of a salt marsh grass (*Spartina alterniflora)* and the rate of soil respiration, while the microbial products did not significantly affect either of these processes, suggesting the bioremediation products had neither toxic nor stimulatory effects on the salt marsh environments.

Purandare (1999) conducted a mesocosm study following the microcosm study described earlier (Purandare *et al*., 1999) to further test the effectiveness of bioremediation of an oil-contaminated freshwater wetland. Outdoor mesocosms each measuring 6 m x 4.5 m, filled with wetland sediments, and planted with three species of emergent wetland plants were used in the study to investigate the effect of different inorganic mineral nutrients on biodegradation of a Louisiana crude oil. A total of 12 of these mesocosms were used (3 replicates of 4 treatments). The four treatment strategies included a no-nutrient control, phosphate addition, nitrate + phosphate addition, and ammonia + phosphate addition. Biodegradation rates were computed from hopane-normalized analyte data. The results showed that addition of nutrients seemed to enhance oil biodegradation initially. However, beyond 12 weeks, the untreated control achieved a comparable degree of biodegradation. No conclusion as to which nutrients were actually limiting the biodegradation process was reached due to the high variability in the data. The study also found that addition of nutrients led to better plant and root growth, which suggested that, although biostimulation may not significantly enhance oil degradation in freshwater wetlands, it may encourage a faster recovery of the ecosystem. It also suggested that the wetland plants may have out-competed the oil-degrading microorganisms for nutrients and may have used substrates other than oil hydrocarbons for their growth (soil organic matter).

2.5.2 Field Demonstrations

Bioremediation field studies are reviewed here, first with respect to bioaugmentation and biostimulation, then by a more detailed discussion of four case studies that cover a wide range of shoreline types.

Bioaugmentation

The effectiveness of seeding has been studied in only a few field trials. Venosa *et al*. (1992) conducted a field test in Prince William Sound following the *Exxon Valdez* spill to investigate the effectiveness of two commercial microbial products vis-à-vis natural attenuation and nutrient addition alone. These products were selected based on a previous laboratory study (Venosa *et al.*, 1991). This field trial failed to demonstrate enhanced oil biodegradation by these products. No biostimulation occurred in the nutrient control plots either. There were no significant differences between the treatment and control plots during the 27-day trial period. However, the site where the project took place (Disk Island) was characterized as having highly weathered (degraded) oil and very calm waters, so dissolved oxygen may have been limiting, thus precluding effective biodegradation by any means.

Other studies (Lee and Levy, 1987; Tagger *et al*., 1983) suggested that exogenous microbial inocula are not able to compete successfully with indigenous populations. One approach in overcoming this competition has been proposed by Rosenberg *et al*. (1992). They developed a product that combined a polymerized ureaformaldehyde (F-1) with a selected oil-degrading culture capable of using this fertilizer as a nitrogen source. Thus, the bacteria had a selective advantage over the indigenous population unable to utilize F-1 as nutrient source. A field trial conducted at an Israeli beach showed that this approach seemed to be successful in enhancing oil

biodegradation. However, conclusions were confounded by the lack of adequate controls in the study (Swannell *et al*., 1996; Venosa, 1998).

Studies comparing the performance of bioaugmentation and biostimulation have suggested that nutrient addition alone had a greater effect on oil biodegradation than did the addition of microbial products (Lee *et al*., 1997a; Venosa *et al.*, 1996).). This is probably because the microbial population is rarely a limiting factor as compared to the nutrients since the size of the hydrocarbon-degrading bacterial population usually increases rapidly in response to oil contamination. Lee *et al*. (1997a) conducted a 129-day field trial to compare the effect of four treatments on biodegradation of weathered Venture Condensate in a sandy beach. The four treatments included: inorganic nutrient and oil, a microbial product and oil, inorganic nutrient, a microbial product and oil, and oiled control. C2-chrysenes was used as a biomarker due to the low concentration of hopane in the condensate. The results showed that periodic addition of inorganic nutrients was the most effective strategy for enhancing oil degradation and reducing sediment toxicity, and that the full potential of the microbial product was limited by nutrient availability. A similar study conducted in a wetland by Simon *et al*. (1999) also show that addition of bioaugmentation agents did not enhance biodegradation of an Arabian medium crude oil. However, nutrient addition did not demonstrate any significant effect in their study either, suggesting other factors, such as oxygen, were limiting oil degradation.

Several other possible reasons for the failure of inocula in degrading contaminants in nature were summarized by Goldstein *et al*. (1985), which include: (1) the concentration of the contaminant may be too low to support the growth of the inoculated species, (2) the natural environment may contain substances inhibiting growth or activity of the inocula, (3) the growth rate of the inoculated species may be limited by predation such as protozoa, (4) the added species may use other substrates in nature rather than the targeted contaminants, and (5) the seeded microorganisms may be unable to move through the pores of the sediment to the contaminants.

A few field trials did claim success in demonstrating the effectiveness of oil bioaugmentation, such as using Alpha BioSea^{TM} to treat the Angolan Palanca crude oil spilled from *Mega Borg* off Texas coast (Mauro and Wynne, 1990) and using Terra-Zyme^{TM} in enhancing biodegradation of a heavy oil spilled from *Nakhodka* in Japan (Tsutsumi *et al*., 2000). However, the success of these studies was based on either visual observation (i.e. the *Mega Borg* study) or digital photographic image analysis (i.e., the *Nakhodka* study). No comprehensive monitoring program was used to verify the oil was indeed removed through enhanced biodegradation. The two products basically contains the same formula of bacteria cultures and nutrients (Hozumi *et al*., 2000). The observed visual effects may have been due to physical or chemical processes such as surfactant action associated with the products (Swannell *et al*., 1996).

It seems that in most environments, indigenous oil-degrading microorganisms are more than sufficient to carry out oil biodegradation if nutrient levels and other adverse environmental conditions do not limit them. Future research on oil bioaugmentation should focus on investigating which ecosystems may be deficient in oil degrading microorganisms and what types of oils or important oil components indigenous bacteria may be incapable of degrading.

Biostimulation

Although laboratory and pilot-scale studies have shown that biostimulation is a promising approach in enhancing oil biodegradation, the effectiveness of various types of nutrients and delivery strategies still require field demonstration.

Sveum and Ladousse (1989) investigated the performance of Inipol EAP 22 in different types of sediments. The results showed that the oleophilic fertilizer enhanced oil biodegradation in coarse-grained sediments but not in fine-grained sediments.

Researchers from Fisheries and Oceans-Canada (Lee and Levy, 1987; Lee and Levy, 1989; Lee and Levy, 1991; Lee and Trembley, 1993; Lee *et al*., 1995a; and Lee *et al*., 1997a) conducted a series of field tests to investigate the effect of different types of fertilizer and different deliver strategies in a low energy, sandy beach or in a salt marsh. Their studies demonstrated that biostimulation using periodic addition of inorganic fertilizers (ammonium nitrate and triple super phosphate) increased the rate of oil removal from beaches as measured by changes in oil composition relative to conserved biomarkers such as C_2-chrysenes and/or the decline in the *n*-C_{17}/pristane and *n*-C_{18}/phytane ratios. In contrast, the addition of the oleophilic fertilizer Inipol EAP 22 did not enhance oil degradation in a sandy beach (Lee and Levy, 1987 and 1989). Another study involved periodic addition of water-soluble fertilizer granules (ammonium nitrate and triple super phosphate) in an attempt to enhance biodegradation of waxy crude oil in a low-energy, sandy beach and in a salt marsh (Lee and Levy, 1991). Two concentrations of the NH_4NO_3 were tested (0.34 and 1.36 g/L sediment). The oil used was Terra Nova crude at two different levels (0.3 and 3.0%). Results from the sandy beach showed that at the lower level of oil contamination, no enhancement by fertilizer was achieved. However, at the higher oil contamination level, substantial oil degradation occurred in the fertilized plots compared to the unfertilized ones. Results in the salt marsh were the exact opposite. Enhancement by fertilizer was significant at the 0.3% contamination level, but no enhancement occurred at the 3% oil contamination, which was attributed to the penetration of oil into the anaerobic zone where little degradation is expected. Studies on the utility and efficacy of various slow-release fertilizer formulations also were evaluated (Lee *et al*., 1993). They demonstrated that the effects of environmental factors controlling nutrient delivery from the various formulations under review (e.g., sulfur-coated urea) were the key to bioremediation success. Another field study conducted by Lee *et al.* (1995a) compared the performance of inorganic nutrients with organic fish bone-meal fertilizer. These results showed that the organic fertilizer had the greatest effect on microbial growth and activity, while the inorganic nutrients were much more effective in crude oil degradation.

All these results suggest that the success of bioremediation is case specific, depending on oil properties, the nature of the bioremediation products and the characteristics of the contaminated environments. Fortunately, recent studies have shown that the oil biodegradation rate depends on the nutrient concentrations in the pore water of the sediments, which could provide important guidance for nutrient applications (Bragg *et al*., 1994, Venosa, 1996). This finding may also explain why some earlier trials have failed to demonstrate the effectiveness of nutrient

application since the nutrient concentrations in the interstitial pore water had not been monitored and controlled in most of these studies. Venosa *et al.* (1996) found that maintenance of a threshold nitrogen concentration of 1 - 2 mg N/L in the interstitial pore water would result in close to maximum hydrocarbon biodegradation in a sandy beach (this will be discussed in detail in the section on case studies). Future research on biostimulation probably should focus on the determination of the optimal interstitial nutrient concentration and the best strategies to maintain this concentration for various environments whenever the degradation is limited by nutrient availability.

In addition to the demonstration of the efficacy of oil degradation, it is also necessary to demonstrate that bioremediation does not produce any undesired environmental and ecological effects. There have been concerns that enhanced microbial degradation of oil might produce toxic metabolic by-products. To address this concern, Lee *et al*. (1995b) conducted a field study using different fertilizers to investigate the effect of bioremediation on the toxicity of Venture condensate stranded on sandy beach sediments. The toxicity of the sediments was monitored using the Microtox® Solid-Phase Test. The results indicated that sediment toxicity was not significantly affected by the addition of an inorganic fertilizer (ammonium nitrate and triple-superphosphate). However, they did observe a slowing of the decrease in toxicity when the organic fertilizer (fishbone meal) was applied repeatedly, which was attributed to rapid biodegradation of the fertilizer and the production of ammonia that exceeded toxicity threshold limits.

Case studies

Four representative field studies are described in more detail here. Three types of marine shorelines and one freshwater wetland are covered.

Exxon Valdez

Following the grounding of the supertanker *Exxon Valdez* on Bligh Reef in Prince William Sound in 1989, U.S. EPA, in conjunction with the Exxon Corporation and the state of Alaska, embarked on the largest oil spill bioremediation project ever attempted in the field. Extensive field trials at various sites were conducted, which have been well documented in the literature (Bragg *et al.*, 1994; Prince, 1993; Prince *et al*., 1994; Pritchard and Costa, 1991; Swannell *et al.*, 1996; Venosa, 1998). Important findings and lessons learned from these studies are summarized as follows.

- Seeding of bioaugmentation products failed to demonstrate enhanced oil biodegradation. It was found that oil biodegradation on the shoreline of the Prince Williams Sound was limited by the concentration of nutrients, particularly nitrogen, and not by the absence of hydrocarbon-degrading microorganisms (Pritchard and Costa, 1991; Venosa *et al*., 1992).

- Three types of nutrients or fertilizers were tested in the field: a water-soluble inorganic fertilizer (23:2 N:P garden fertilizer formulation), a slow-release inorganic fertilizer (Customblen), and an oleophilic fertilizer (Inipol EAP 22). Each was shown to be variably

effective. Inipol EAP22 and Customblen were chosen as bioremediation agents, and approximately 50,000 kg of nitrogen and 5,000 kg of phosphorus were applied over 120 km of the oil contaminated shorelines during 1989 and 1992. Within two weeks after the fertilizer application, the area of cobble beach treated with Inipol EAP22 and Customblen appeared to be visibly cleaner than the untreated area (Pritchard and Costa, 1991; Pritchard *et al*., 1992). However, it was later found that the oil coating the surfaces of the cobble had been lifted and re-deposited within the interstices in the beach surface.

- Heterogeneous oil distribution on the contaminated beaches made it difficult to determine the rates of oil biodegradation using established methods. The changes in the ratios of a hydrocarbon component to a conserved internal standard or biomarker were used as the basis for determining the oil degradation rate. They also found that previously traditional biomarkers such as pristane and phytane degraded rapidly in Alaskan beaches. This observation rendered use of such biomarkers ineffective in permitting firm conclusions on bioremediation effectiveness. 17α(H),21β(H)-hopane, the pentacyclic triterpane containing 30 carbon atoms, was used instead. Using hopane as the biomarker, Bragg *et al*. (1994) showed that fertilizer application accelerated the rate of oil removal by a factor of approximately five-fold compared to natural attenuation. This observation was made, however, from samples repeatedly collected from only one site, so the statistical basis supporting this conclusion is tenuous.

- Oil biodegradation rate appeared to be dependent on the nitrogen concentration in the pore water of the intertidal sediments, suggesting that on-site monitoring of nutrients in the sediment pore waters could provide practical guidance for nutrient applications (Bragg *et al*., 1994).

- According to the EPA/Exxon/State of Alaska joint monitoring program, bioremediation was an environmentally sound remediation technique. This was based on the results of testing the toxicity of nearshore water to sensitive marine species such as Mysid shrimp, analyzing ammonium and nitrate concentrations, evaluating the potential of algal growth, and monitoring oil release into nearshore water after the application of fertilizers (Prince *et al*, 1994).

- The results of the fertilizer application following the *Exxon Valdez* spill generally demonstrate that bioremediation may enhance oil biodegradation on certain marine shorelines. However, conclusions on the effectiveness of bioremediation in the Exxon Valdez study are somewhat questionable, in part because the experimental design was not entirely based on sound statistical principles (Venosa, 1998). Major flaws included the lack of replication and the attempt to determine too many factors in a limited number of tests, resulting in the confounding of different effects. The lessons learned from the Exxon Valdez project led to the replacement of "post *Exxon Valdez* excitement" with more scientifically-valid approaches (Mearns, 1997).

Delaware Field Study

The main purposes of this field study were to obtain credible statistical evidence in determining if bioremediation with inorganic mineral nutrients and/or microbial inoculation enhances the removal of crude oil, to compute the rate at which such enhancement takes place, and to establish engineering guidelines on how to bioremediate an oil-contaminated sandy shoreline.

The trial was conducted on a medium- to coarse-grained sandy beach (environmental sensitivity index = 4) at Fowler Beach, Delaware (located midway between Dover and Rehoboth Beach). A randomized complete block design was used in the study. Twenty 4 x 9 m plots were set up in five replicate blocks. Each block contained four treatments in random order, which included: an unoiled control plot, a no-nutrient control plot (natural attenuation), a plot receiving water-soluble nutrients, and a plot receiving water-soluble nutrients supplemented with a natural microbial inoculum from the site. Weathered Nigerian Bonny Light crude oil was intentionally released onto 15 plots. Nutrients ($NaNO_3$ and $Na_5P_3O_{10}$) were applied daily through a sprinkler system at a rate designed to achieve a target of 1.5 mgN/L average interstitial pore water concentrations. Once a week, 30 L of a suspended mixed population of hydrocarbon-degrading bacteria was also added to the inoculum plots. Sand samples were collected every 14 days from the 15 oiled plots for oil analysis, and all analytes were normalized to hopane. Nitrate concentrations in the interstitial pore water of oiled plots were measured each day.

Figure 2.3a shows the hopane-normalized concentrations of total target alkanes (i.e., the sum of all alkane analytes from *n*-C_{10} to *n*-$C_{35,}$ plus pristane and phytane), while Figure 2.3b shows the total target aromatics (i.e., the sum of all groups of PAHs and sulfur heterocyclics analyzable by GC/MS and their alkyl-substituted homologues) in the nutrient-treated, inoculum-treated, and control plots, all as a function of time. Although substantial hydrocarbon biodegradation occurred in the untreated plots, statistically significant differences between treated and untreated plots were observed in the biodegradation rates of total alkanes and total aromatic hydrocarbons. The results also show that bioaugmentation, even with indigenous organisms, does not stimulate further degradation of hydrocarbons beyond simple nutrient addition. The studies further demonstrated that maintenance of a threshold nitrogen concentration of 1 - 2 mg N/L in the interstitial pore water would permit close to maximum hydrocarbon biodegradation.

Another important conclusion from this study is that background nutrient concentrations at the contaminated site should be a determining factor in the decision to apply bioremediation. The background nitrogen concentration at Fowler Beach was high enough to permit close to maximum hydrocarbon biodegradation without the need to apply additional fertilizer despite the enhancement observed from nutrient addition. The enhanced effect, although statistically significant, was not substantial enough to have warranted a decision to implement bioremediation on a full-scale basis had there been a real spill at this site. This demonstrates that assisted bioremediation might not always be necessary if sufficient nutrients are naturally present at a spill site in high enough concentrations to perform natural cleanup. For coastlines having low natural input levels of nutrients, bioremediation would be appropriate as an alternative cleanup option.

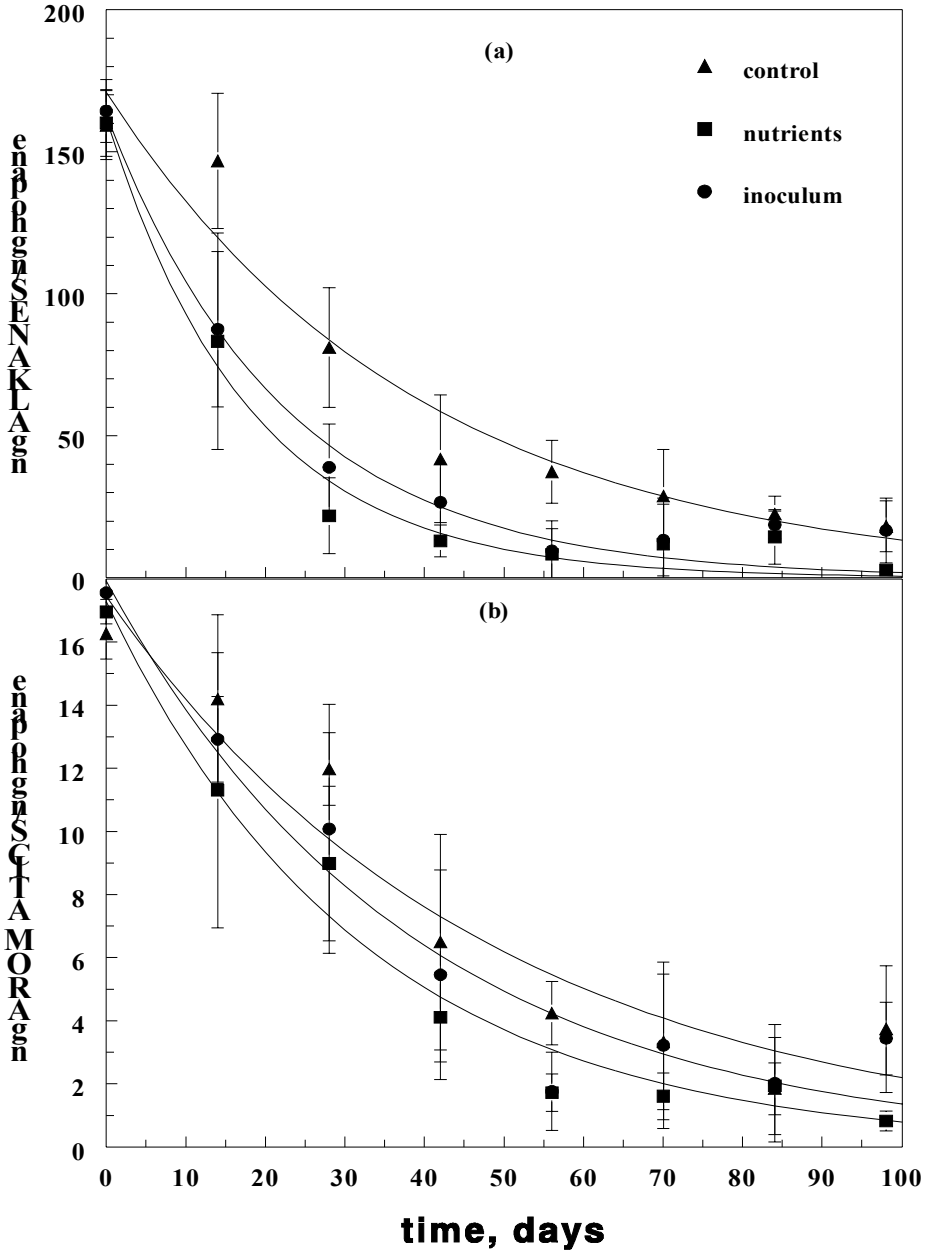


Figure 2.3. Results of the Delaware field study: first-order declines in (a) total target alkanes and (b) total target aromatics

Bioremediation of a Fine Sediment in England

The previous two cases have demonstrated that bioremediation can be effective for cobble and sandy shorelines, although the extent of the enhancement in effectiveness is determined by the natural presence of nutrients. However, much less attention has been given to fine sediment marine shorelines such as mudflats. A recent study conducted by Swannell *et al.* (1999a) was intended to fill this gap. One of the objectives of this study was to investigate the potential of bioremediation in treating buried oil.

The field site was located within the Stert Flats on the southwest coast of England. Twelve plots were set up on an 80-m stretch of sand composed of 3.2 % mud and 80% particles in the range of 125-180μm. The experimental area was divided into three replicate blocks. Each block consisted of four randomly assigned treatments that consisted of an unoiled control plot, an unoiled plot treated with fertilizer alone, an oiled plot with no amendments added, and an oiled plot treated with fertilizer. The sediment in each plot was retained in mesh enclosures (0.4 m x 0.4 m x 0.05m) and buried at a depth of 15 cm. Weathered and emulsified Arabian Light crude oil was applied to the appropriate enclosures at 3.7 kg/m^2. Inorganic fertilizer ($NaNO_3$ and KH_2PO_4) was applied using a sprinkling device at rates of 2% of N and 0.2% of P by weight of the initial oil concentration, and at a frequency of once a week for the first four weeks and then every two weeks thereafter. Samples were taken on Day 7, 49, and 108 for oil analysis, and hopane was used as a biomarker.

The results showed that more oil was degraded in 2 of the 3 fertilized plots than in the controls after 108 days. In blocks 1 and 3, the mean total GC resolvable hydrocarbons (TGCRH)/hopane ratio decreased by 58.4% and 48.4% respectively, compared to 23.0% and 4.4% in the two oiled controls respectively. In block 2, oil degradation was slower, with only 14% decrease of TGCRH/hopane ratios in the treated plot vs. no removal in the control plot. Statistical analysis demonstrated that differences in the ratio of TGCRH and TPH against hopane between the fertilized plots and the controls were highly significant ($p<0.0001$). Microbiological analyses also showed that nutrient addition increased the numbers of hydrocarbon-degraders on the oiled plots by ten-fold. This study suggested that bioremediation by nutrient enrichment for the treatment of buried oil in fine sediments may be feasible after an oil spill incident. However, because of the third replicate failing to confirm the results of the other two, more definitive conclusions cannot be made from this study.

One deficiency of this study was that by aerating the subsurface sediments during the sediment burying and periodic excavation of the site due to nutrient application, the potential problem of oxygen limitation in this environment could not be evaluated. Oxygen limitation is a major concern for application of oil bioremediation in subsurface sediments, anoxic zones of a water column, and most fine-grained marine shorelines (Head and Swannell, 1999). Because this experimental site was a high-energy beach with a tidal bore, oxygen may not have been a limiting factor for this specific fine sediment beach. Conclusions from this study, therefore, should only be confined to the Stert Flats and not extrapolated to similar environments. Further

research is still required to determine the effectiveness of bioremediation in other types of fine sediments.

Bioremediation of a Freshwater Wetland in Canada

A field study was conducted by the U.S. Environmental Protection Agency, University of Cincinnati, and Fisheries and Oceans Canada at a freshwater wetland site situated along the St. Lawrence River, Ste. Croix, Quebec, Canada. The objective of this study was to determine the effectiveness of biostimulation strategies in accelerating restoration of an oil-contaminated freshwater wetland site. (Garcia-Blanco *et al.*, 2001b, Venosa *et al*., 2002). Strategies that were evaluated were bioremediation by nutrient enrichment in the presence and absence of vegetative growth of the dominant plant species, *Scirpus pungens*. Twenty 5m x 4m plots were set up in the upper intertidal zone of a study site located along the St. Lawrence River, where the water was far enough away from the Atlantic to be still fresh, yet was tidally influenced. These plots were divided into four replicate blocks. Each block consisted of five randomly assigned treatments, which were: (1) oiled, no added nutrients, with intact plants (natural attenuation control), (2) oiled, NH_4NO_3 + $Ca(H_2PO_4)_2 \cdot H_2O$ added, with all vegetative growth cut back to ground surface daily to suppress plant growth, (3) oiled, NH_4NO_3 + $Ca(H_2PO_4)_2 \cdot H_2O$ added, with intact *Scirpus pungens*, (4) oiled, $NaNO_3$ + $Ca(H_2PO_4)_2 \cdot H_2O$ added, with intact *Scirpus pungens*, and (5) unoiled, NH_4NO_3 + $Ca(H_2PO_4)_2 \cdot H_2O$ added, with intact *Scirpus pungens* (background control). Weathered Mesa light crude oil was released onto each plot earmarked for oiling. The amount of oil released was 12 L per plot. Composite core samples were collected after 0, 1, 2, 4, 6, 8, 12, 16, and 21 weeks for quantification of the remaining oil constituents by gas chromatography/mass spectroscopy (GC/MS) operating in the Selected Ion Monitoring mode or SIM. To account for differences due to physical washout, all oil constituents were normalized to hopane.

Figure 2.4 illustrates results from the hopane-normalized concentrations of total target alkanes and total target PAHs for the four treatments as a function of time. Although the bioremediation and phytoremediation treatments achieved slightly better degradation of hydrocarbons than natural attenuation, no statistically significant evidence of stimulation through addition of nutrients or biodegradation enhancement by vegetation was observed. After 21 weeks, reduction of target parent and alkyl-substituted polycyclic aromatic hydrocarbons (PAHs) averaged 32% in all treatments. Reduction of total target alkanes was of a similar magnitude. The pattern of disappearance of hydrocarbons was characteristic of biodegradation; namely, the lower molecular weight alkanes declined to a greater extent than the higher carbon-number alkanes, as did the lower molecular weight PAHs compared to the higher molecular weight PAHs. Since there was little evidence to support enhancement of biodegradation by nutrient addition with and without vegetation, it was suggested that oxygen limitation was most likely the dominant cause of the persistence of oil hydrocarbons on the oil-contaminated plots.

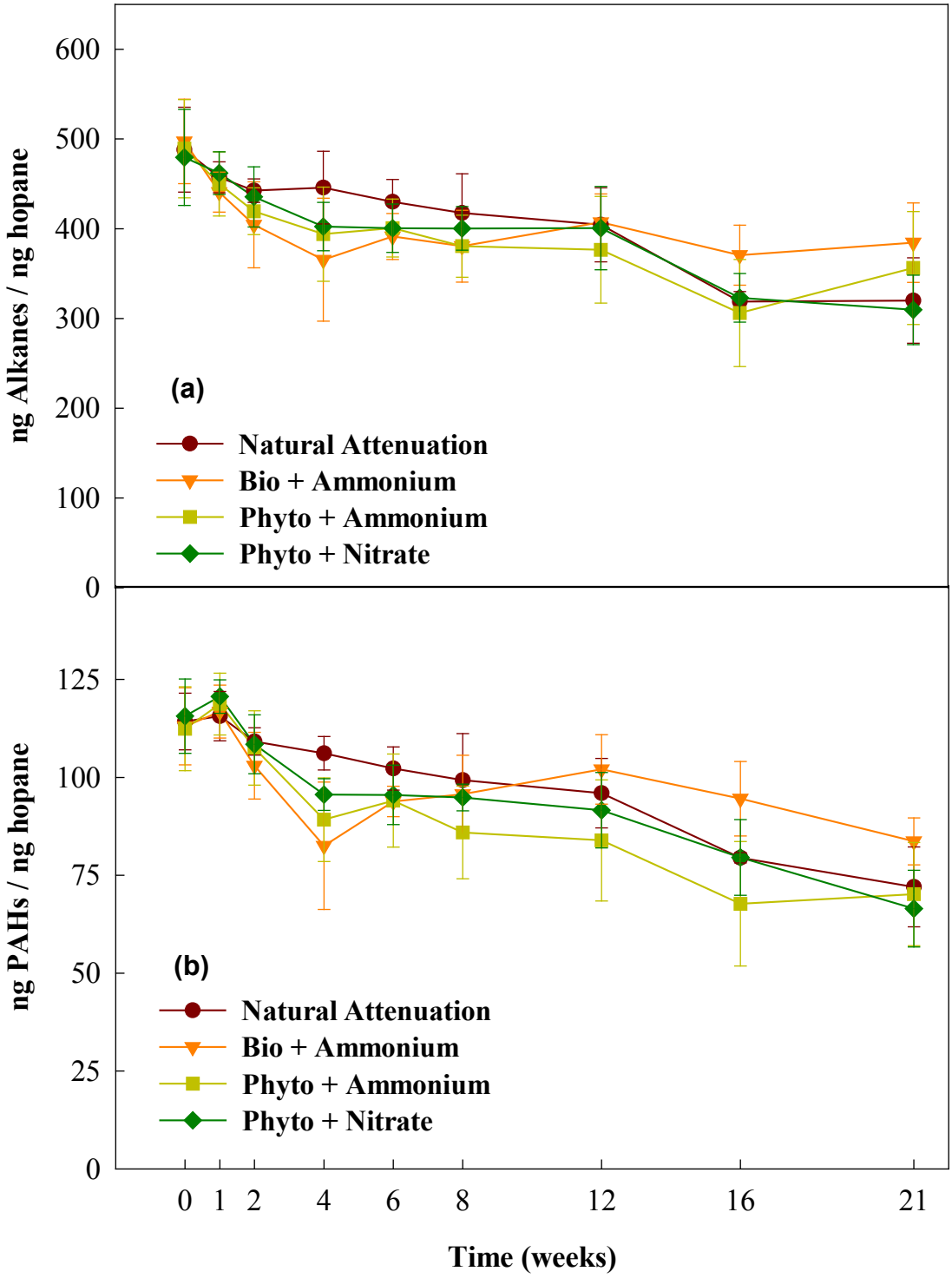


Figure 2.4. Results of St. Lawrence River field study: concentration declines in (a) total target alkanes and (b) total target PAHs

While the Ste. Croix results indicate that biostimulation might not be an effective strategy to mediate the removal of residual oil from the sediments, significant changes in biological measures of habitat were observed (Lee *et al*., 2001a). For example, *S. pungens*, the dominant plant species, was tolerant to the oil, and its growth was significantly enhanced above that of the unoiled control by the addition of nutrients. Other biotest organisms (bacteria, *Vibrio sp*., invertebrates, Daphnia, Hyalella, and *Viviparus sp*.) provided additional evidence of both enhanced recovery and potential detrimental effects. The study indicates that on an operational scale, natural attenuation may be the most practical treatment option for oxygen-limited freshwater wetlands.

2.5.3 Kinetics of oil bioremediation

Knowledge of the kinetics of oil biodegradation under different environmental conditions is important for assessing the potential fate of targeted compounds, evaluating the efficacy of bioremediation, and determining appropriate strategies to enhance oil biodegradation. Oil biodegradation rates are difficult to predict due to the complexity of the environment. The rates of biodegradation vary greatly among the various components of crude oils and petroleum products. The presence of other substrates may affect the degradation rates of the compounds of interest. Environmental factors such as temperature, nutrient concentrations, and oxygen tension also influence the kinetics of oil degradation. The heterogeneity of oil distribution on shorelines or wetland sediments makes kinetics studies even more difficult.

Very few kinetic studies on oil degradation under field conditions have been conducted. The *Exxon Valdez* monitoring program developed a multiple regression model based on field studies conducted by researchers from Exxon (Prince *et al*., 1994). The best-fitting model was expressed as:

$$C_h(t) = \alpha[1-p(t)]^{\gamma} e^{\delta r(t)+\omega t} \varepsilon \qquad (2.2)$$

where $C_h(t)$ is the time-varying hopane-normalized concentration of an analyte, p is the polar fraction of the oil, r is the ratio of the average residual nitrogen concentration to oil loading, and ε is the assumed multiplicative error term, while α, δ, γ, and ω are fitting parameters determined from the multiple regression analysis. This model matched the experimental results in Alaska well when the parameters are chosen to fit the data. However, its potential for process understanding and prediction is limited because the data set used for the regression was limited to only one small, non-replicated area in the field.

Venosa *et al.* (1996) developed from field data first-order biodegradation rate constants for resolvable alkanes and important two- and three-ring PAH groups present in light crude oil. The first order relationship was expressed as:

$$\left(\frac{A}{H}\right) = \left(\frac{A}{H}\right)_0 e^{-kt} \qquad (2.3)$$

where (A/H) is the time-varying hopane-normalized concentration of an analyte, $(A/H)_0$ is that quantity at time zero, and k is the first-order biodegradation rate constant for an analyte.

For the field study conducted in Delaware, first-order biodegradation rate coefficients ranged from 0.026 to 0.056 day^{-1} for total resolvable alkanes and from 0.021 to 0.031 day^{-1} for total resolvable PAHs (Venosa *et al*., 1996).

Actual 1st-order biodegradation rates are not constant, however. Instead, they are a function of the residual nutrient concentration:

$$k_{obs} = k_{\max}\left(\frac{N}{K_n + N}\right) \tag{2.4}$$

where k_{obs} and k_{max} (T^{-1}) are the observed and maximum first-order hydrocarbon biodegradation rates, respectively, K_n (M_nL^{-3}) is the half-saturation concentration for a specific nutrient, and N (M_nL^{-3}) is the interstitial pore water residual nutrient concentration. Experiments conducted at the University of Cincinnati showed that the K_n for nitrate is approximately 0.5 mg N/L (unpublished). The model that incorporates Equation 2.3 and 2.4 will be very useful in the experimental design and the performance prediction for the bioremediation of oil contaminated shorelines.

Studies have also been conducted to compare oil biodegradation rates obtained in laboratory tests to those calculated from the Delaware field study (Venosa *et al*., 1996, 1997a; Holder *et al*., 1999). Venosa *et al*. (1996, 1997a) found that the degradation rates of all target alkanes and aromatics in a light crude oil were close to an order of magnitude lower in the field compared to results from the laboratory. However, when the rate data of PAHs were normalized to the highest alkyl-substituted homologue in each given PAH series, the first order rate constants in the field were nearly identical to rate constants from the lab. These relationships were consistent even with different microbial consortia isolated from eight different marine shorelines of the United States. Similar results were also observed in a laboratory study using 14 different marine and freshwater consortia (Holder *et al*., 1999).

Simon *et al*. (1999) reported some kinetic data derived from a study conducted in a coastal wetland contaminated with Arabian light crude oil. First-order biodegradation rate coefficients ranging from 0.017 to 0.061 day^{-1} for total target saturates and from 0.009 to 0.027 day^{-1} for total target aromatics were reported. These rate coefficients were similar to those of Venosa *et al*. (1996). Further research is still required to develop more state-of-art models and to establish a database of kinetic parameters for different types of oil under various marine shoreline and freshwater wetland environments.

2.6 Nutrient Hydrodynamics

Since nutrient addition has been found to be the most effective bioremediation strategy in aerobic environments, particularly for marine shorelines, a full understanding of the fate of water soluble

nutrients on marine beaches and the hydrodynamics controlling their transport and persistence is necessary. One of the main challenges associated with biostimulation in oil-contaminated coastal areas is maintaining optimum nutrient concentrations in contact with the oil and the degrading microorganisms. Oil from offshore spills usually contaminates the upper third of the intertidal zone, where the washout rate for water-soluble nutrients can be very high. Various oleophilic and slow-release nutrient formulations have been developed to improve the contact between oil and nutrients within the environment. However, most slow-release and many oleophilic fertilizers rely on dissolution of the nutrients into the aqueous phase before they can be used by hydrocarbon degraders (Safferman, 1991). Thus, design of effective oil bioremediation strategies and nutrient delivery systems requires an understanding of the transport of water-soluble fertilizers in a beach ecosystem.

Dissolved nutrients are expected to move with the water in the beach sand. Water flow through the porous matrix of a beach is driven by a combination of three main factors (Boufadel *et al.*, 1999b; Wrenn *et al*., 1997): (1) tides that result in rise and fall of beach groundwater level (typically, the water level in a beach tracks the level of the rising tide with only a slight lag, but the beach drains much more slowly when the tide ebbs because of resistance from the porous matrix), (2) wave action that operates through two main mechanisms (at wave runup, water enters the beach and percolates vertically through the unsaturated zone until it reaches the water table; at wave rundown, water moves in a predominantly horizontal direction and exits at the water line), and (3) flow of fresh groundwater from coastal aquifers, which causes continuous horizontal advective flow from the beach face at or near the water line (Glover, 1959). This type of groundwater flow can interact with tidal fluctuations to produce complex variations in the groundwater level within the beach (See Section 2.6.1).

2.6.1 Nutrient transport in beaches: a mesocosm study

Beach hydraulics and nutrient hydrodynamics were investigated through both theoretical and experimental approaches by Boufadel *et al*. (1999 a and b, 1998, 1997). A two-dimensional finite element model for water flow and salt transport in saturated and unsaturated porous media was developed. The model also considers the effects of salt concentration on water density and water viscosity. An experimental wave tank was used to validate the model and to investigate cases that cannot be simulated by the numerical model. Tracer tests were carried out to investigate the separate and combined effects of tide, waves, and buoyancy on the transport of soluble inorganic nutrients in sand beaches. The major findings from these studies were:

- In the absence of regional seaward groundwater flow, the tide generated a predominantly downward and seaward hydraulic gradient that caused the washout to the sea of nutrients applied to the top section of beaches. The presence of waves under these conditions accelerated the washout, the rate of which was found to increase by about 30% when waves were superimposed on the tide.

- Beach geometry plays a major role in beach hydraulics and hydrodynamics because the flow lines are perpendicular to the beach surface. Under tidal action, seawater enters the beach

from above, flowing vertically downward, and may cause the entrapment of less saline water in the beach. This finding is very important because systems that rely on continuous or intermittent injection of nutrient solutions through trenches or horizontal wells installed in the supratidal zone rely on subsurface flow to carry the nutrients through the contaminated area (Wise *et al*., 1994). The approach that was proposed by Wise *et al*. (1994) assumes that nutrients dissolved in the freshwater plume will be brought into contact with the oiled beach material periodically by the rising tide, because the freshwater plume should float on top of the saltwater. The finding of freshwater trapped between two saltwater wedges indicates that subsurface injection of nutrients may not be an effective method for providing nutrients to the bioremediation zone when a freshwater plum exists because the impact zone will never be exposed to the nutrients.

- Different nutrient application strategies at low tide were investigated in the mesocosm beach since application of nutrients at the beach surface at low tide (Venosa *et al.*, 1996) appears to be the best application strategy for biostimulation. The results showed that applying nutrients at the beach surface pre-dissolved in water resulted in generally longer residence times and larger spreading of the nutrient plume in the top section of the beach compared to applying nutrients in granular form at the beach surface and hosing them in with a water spray. However, it should be noted that in practice, addition of granular fertilizer may be prudent in cases where it is not possible to sprinkle pre-dissolved nutrients as the application method.

2.6.2 Nutrient transport in beaches: field trials

To verify the findings from the mesocosm studies and to further investigate nutrient transport under field conditions, tracer studies were conducted in the intertidal zone of three different marine beaches in Delaware and Maine (Suidan and Wrenn; 2001; Wrenn *et al*.,1997a & b).

The Delaware Tracer Study

The purpose of this study was to characterize the transport of water-soluble nutrients in the intertidal region and to estimate their washout rates from the bioremediation zone (i.e., the oil-contaminated area). The study was conducted on a moderate-energy, sandy beach (Slaughter Beach) on Delaware Bay. The typical wave heights at this beach were between 15 to 30 cm. A conservative tracer ($LiNO_3$) was applied to eight replicate 5 m x 10 m plots in the upper intertidal zone at low tide during full moon spring tide and the last-quarter moon neap tide. The tops of the plots were placed approximately at the spring high tide line. Sand samples were collected for tracer analysis.

This study showed that the rate of tracer washout from the bioremediation zone was more rapid when the tracer was applied at spring tide (when the tidal amplitude is largest) than at neap tide. When the conservative tracer ($LiNO_3$) was applied to the beach surface in the upper intertidal zone at the full moon spring tide, it was completely removed within one day. When it was applied at neap tide, however, the tracer persisted in the bioremediation zone for several days. The results indicated that the amount of nutrient remaining in the bioremediation zone was highly correlated with the maximum extent to which the treated area had previously been

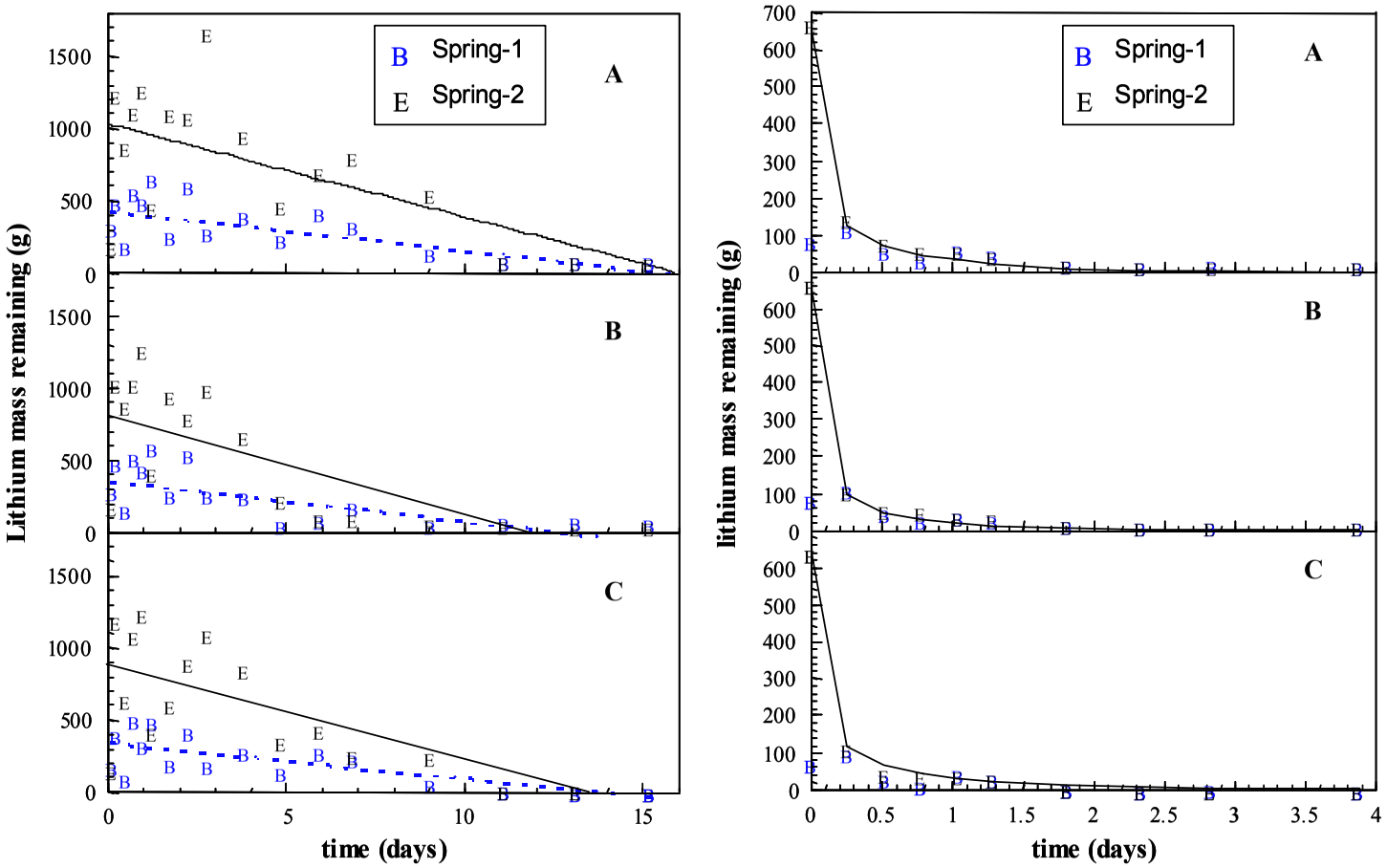
submerged by water at high tide; submergence resulted in nearly complete removal of dissolved compounds from the bioremediation zone. Therefore, the fertilizer release rates should be designed to achieve optimal nutrient concentrations while the tide is out. The Delaware tracer study indicates that nutrient transport in sandy beaches is driven by tidally influenced hydraulic gradients and wave activity. However, it is impossible to clearly separate the influence of tide and wave action in this study.

The Maine Tracer Study

The main purpose of this field study was to separately evaluate the effect of tide and wave action on nutrient transport since the results of the Slaughter Beach field studies could not distinguish between these two processes. This was accomplished by comparing the transport rate and characteristics of a dissolved conservative tracer (lithium nitrate) on a high-energy beach to those on a low-energy beach to determine how waves affect solute transport (Suidan and Wrenn, 2001; Wrenn et al., 1997b). The two beaches are located in the town of Scarborough, in southern Maine. Scarborough Beach is a high-energy beach that faces the Atlantic Ocean with average heights of breaking waves between 0.3 to 1 m during the course of this study, whereas Ferry Beach faces a protected harbor with the typical wave height of less than 3 cm. The tidal range at both beaches was essentially the same. Dissolved tracer ($LiNO_3$) was applied to four replicate plots on each beach at low tide during full moon spring tide and the last-quarter moon neap tide. Both water and sand samples were collected for tracer analysis.

Washout of lithium from the upper intertidal zone during the spring-tide experiment is shown in Figure 2.5. The differences between the two beaches are very clear. Whereas lithium was completely removed from the entire experimental domain within two days on the high-energy beach, more than two weeks were required to achieve the same degree of washout from the low-energy beach. Washout during the neap-tide experiment was much slower than that observed during the spring-tide experiment. Lithium was completely removed from the experimental domain on Scarborough Beach within one week, whereas the total mass of lithium was essentially unchanged for more than two weeks at Ferry Beach. Slower washout was expected during the neap-tide experiment, because only the bottoms of the plots were covered by water at high tide during most of the first five or six days. Since plot coverage by the tide was essentially the same on the two beaches during the first week of this experiment, the differences in washout rate must have been due primarily to wave activity.

The Maine field study clearly shows that the washout rate of nutrients from the bioremediation zone is strongly affected by the wave activity of the contaminated beach. Wave action in the upper intertidal zone may cause nutrients from the surface layers of the beach to be diluted directly into the water column, resulting in their immediate loss from the bioremediation zone. On the other hand, washout due to tidal activity alone is relatively slow, and nutrients will probably remain in contact with oiled beach material long enough to effectively stimulate oil biodegradation on low-energy beaches.



(a) Ferry Beach

(b) Scarborough Beach

Figure 2.5 Washout of Lithium from the upper intertidal zone during the spring-tide experiment at (a) Ferry Beach and (b) Scarborough Beach The mass remaining is shown for (A) in the total experimental domain (B) inside the plot boundaries, and (C) in the bioremediation zone (i.e., within 30 cm of the surface).

Salinity distribution was also examined in the Maine study. In both beaches, the "sandwiching phenomenon" (a layer of lower salinity water located between two higher salinity water layers) was observed, which was confirmed by Boufadel *et al*. (1999b) in the mesocosm study. Both these mesocosm and field studies are very helpful in providing guidelines for the optimal applications of nutrients on marine beaches, which will be discussed later in this document.

Although many questions remain unanswered, we have made tremendous progress in understanding various aspects of oil bioremediation in the last decade. The development of an operational guideline for bioremediation of oil contaminated marine shorelines and freshwater wetlands is not only possible but also necessary in ensuring that full-scale cleanup in the future proceeds rapidly and efficiently.

Chapter 3 Methods Used in Monitoring Oil Bioremediation

In order to demonstrate that biodegradation is taking place in the field, the chemistry or microbial population must be shown to change in ways that would be predicted if bioremediation were occurring (NRC, 1993). Environmental conditions, particularly nutrient concentrations, should also be monitored for evaluating the effects of bioremediation. Some of the most important methods used in monitoring oil bioremediation will be overviewed in this chapter. References will be given for detailed descriptions of methods.

3.1 Analytical Methods

3.1.1 Microbiological analysis

Existing methods for microbial analysis can be classified into cultured-based techniques and culture-independent techniques. Commonly used microbial analysis methods in monitoring oil bioremediation are summarized in this section and table 3.1.

3.1.1.1 Enumeration of hydrocarbon-degrading microorganisms: culture based techniques

Microbial counts are often used to monitor the bioremediation process. In general, the more microbes, the more quickly the contaminants will be degraded. Correlating an increase in the number of contaminant-degrading bacteria above normal field conditions is one indicator that bioremediation is taking place. Analysis of the microbial communities that take part in *in-situ* hydrocarbon biodegradation activities has been a challenge to microbiologists (Macnaughton *et al*., 1999). The reason for this is that most (~90 to 99%) of the species making up competent degrading communities do not form colonies when current laboratory-based culture techniques are used (Rollins and Colwell, 1986; Rozsak and Colwell, 1987; Wilkinson, 1988). The techniques are briefly described below.

Plate count

Plate count is a traditional technique, which quantifies the number of bacteria capable of growing on a prescribed set of nutrients and substrates in a solid medium, by counting the colonies formed (National Research Council, 1993). The general procedure involves (1) making the solid medium or gel from a liquid solution with appropriate nutrients and substrates, using a solidifying agent like agar, (2) Spreading a sample containing the bacteria of interest thinly over the surface of the gel in plates, (3) Incubating the plates, (4) counting the bacterial colonies formed. Each colony is assumed to have arisen from a single bacterial cell.

A number of studies have used hydrocarbon incorporated into either agar-based or silica-based media to enumerate of hydrocarbon-degrading microorganisms (Horowitz and Atlas, 1978; Sexstone and Atlas, 1977; Walker and Colwell, 1976). However, other researchers reported that plate counts are unsuitable for enumerating hydrocarbon-utilizing microorganisms because many marine bacteria can grow and produce micro-colonies on small amounts of organic matter existing in the solid media, resulting in the counting of non-hydrocarbon utilizers (Atlas, 1981; Higashihara, *et al.*, 1978). Plate counts also underestimate the number and diversity of bacteria because of the difficulty in enriching viable colonies from environmental samples. Culturable

techniques have been found to be inferior to techniques that do not rely on viable culturing for enumeration (Macnaughton et al., 1999).

Most-probable-number (MPN) procedures

MPN procedures have been viewed as a more reliable method for enumerating hydrocarbon-utilizing microorganisms because such procedures eliminate the need for a solidifying agent and permit direct assessment of the ability to actually utilize hydrocarbons (Atlas, 1981; Wrenn and Venosa, 1996). MPN procedures use liquid nutrient media in test tubes or microtiter plates and hydrocarbons as the sole carbon source. The enumeration is carried out through a statistical analysis based on the numbers of a series of diluted liquid samples that show evidence of bacterial growth. This evidence of bacterial growth can be established based on turbidity, release of $^{14}CO_2$ from radiolabeled hydrocarbons, disruption of oil sheen, and reduction of dyes (Rice and Hemmingsen, 1997). Either statistical tables (Eaton *et al*., 1995) or a computer program (Klee, 1993) can be used to determine the MPN.

Most existing MPN procedures use crude oil or a refined petroleum product as the selected hydrocarbons, which can not distinguish different groups of hydrocarbon degraders. For example, the Sheen Screen Method uses dispersion or emulsification of the crude oil substrate to identify positive wells (Brown and Braddock, 1990). But these effects are associated primarily with growth on aliphatic hydrocarbons (Hommel, 1990). Aliphatic hydrocarbons are of less environmental concern than PAHs, however, because they are less toxic and are biodegraded more rapidly. Wrenn and Venosa (1996) recently developed an MPN procedure that can separately enumerate aliphatic and aromatic hydrocarbon-degrading bacteria. The size of the two populations is estimated using separate 96-well microtiter plates. The alkane-degrader MPN method uses hexadecane as the selective growth substrate, and positive wells are detected by reduction of iodonitrotetrazolium violet, which is added after incubation for 2 weeks at 20^{o}C. PAH degraders are grown on a mixture of PAHs in separate plates. Positive wells turn yellow to greenish brown from accumulation of the partial oxidation products of the aromatic substrates after 3 weeks incubation. This method is simple enough for use in the field and provides reliable estimates for the density and composition of specific hydrocarbon-degrading populations.

For a detailed description of existing MPN procedures, readers can refer to Rice and Hemmingsen (1997), and Wrenn and Venosa (1996).

3.1.1.2 Culture-independent population/community techniques

The main challenge for accurate analysis of hydrocarbon-degraders using existing culture-based techniques is that most these species are not able to be cultured (Atlas & Bartha, 1987, Macnaughton *et al*., 1999). The emergent culture-independent molecular techniques have made it possible to identify the diversity and composition of uncultivated microbial communities and to enumerate bacteria in more precise ways.

Phospholipid fatty acid (PLFA) analysis

Phospholipid fatty acid (PLFA) analysis is based on the characteristic "signature" of fatty acids present in the membranes of all cells (National Research Council, 1993). The distribution of fatty acids is unique and stable. Therefore, it can be used as an identifying index. Determination of

biomass through analysis of the extractable lipids avoids culture bias. This technique also provides a quantitative means to measure viable biomass, community composition, and nutritional stature (White *et al*., 1998).

Phospholipids can be extracted from the sample and the phosphate can be measured by colorimetric techniques (Findlay *et al*., 1989). The results can represent the amounts of viable cells and biological activities in the sample. A more powerful PLFA method involves extraction and separation of lipid classes into neutral-, glyco-, and polar-lipid fractions, followed by quantitative analysis using gas chromatography/mass spectrometry (GC/MS) (Macnaughton *et al*., 1999; White *et al*., 1998). This procedure can quantitatively determine the characteristics of microbial communities. However, PLFA analysis cannot identify species composition.

Nucleic acid-based molecular techniques

Nucleic acid-based molecular techniques can identify bacterial species by the unique sequence of molecular codes in their genes. One of most useful methods for determining the diversity of bacterial communities is denaturing gradient gel electrophoresis (DGGE) (Muyzer et al., 1993). The method provides a means of separating the PCR (polymerase chain reaction) products from mixed cultures based on the melting properties of the DNA. Usually the 16s rDNA portion of the bacterial genome is targeted for PCR amplification, since this region is commonly used for bacterial identification. PCR primers can be designed to detect a broad range of bacteria (universal) or can be designed just for a specific group of interest.

The DGGE gel is made of acrylamide, and contains a gradient of formamide and urea, which both act to denature, or pull the strands of the DNA apart. The PCR products are loaded onto the gel, and a voltage is established across the gel for several hours. As DNA, which carries a net negative charge, is carried through the gel, it encounters an increasing gradient of denaturant, which causes the DNA chains to separate and the effective size of the molecules increases, causing movement through the gel to cease. The end result is a DNA banding pattern, where DNA requiring more chemical potential to denature travels farther, and DNA requiring less chemical potential to denature stays near the top of the gel. Each DNA band approximately corresponds to the presence of one kind of organism in the mixed culture. This banding pattern is sometimes referred to as a "community fingerprint," and allows for a quick approximation of number of bacterial species (diversity) present. Bands can be excised for sequencing analysis, and sequences can be compared to the Ribosomal Database Project (Maidak et al., 2000) containing the 16S rDNA sequences of currently known organisms.

The use of DGGE for quantitative purposes is still not well established. However, it can be used in conjunction with other quantitative methods such as PLFA analysis to provide insight into microbial species distribution. The PLFA-DGGE techniques were successfully used in determination of microbial population changes during the Delaware field study on oil bioremediation (Macnaughton *et al*., 1999).

Table 3.1 Commonly used microbial analysis methods in monitoring oil bioremediation

Method	Advantages	Disadvantages
Plate counts	Well-established and easy to perform	Counts only organisms viable on solid-media and may allow growth of non-hydrocarbon degraders
Most probable number (MPN) techniques	More reliable method for enumerating hydrocarbon-utilizing microorganisms since hydrocarbons are used as the sole carbon source. Some of the procedures can separately enumerate aliphatic and aromatic hydrocarbon-degrading bacteria	Relatively labor-intensive (large number of incubations) and time consuming, and may still be subject to culture bias
Phospholipid fatty acid (PLFA) analysis	Eliminate culture bias and quantitatively determine viable biomass, community composition, and nutritional stature	Require specified knowledge and equipment, and can not identify species composition
Denaturing gradient gel electrophoresis (DGGE)	Identify species distribution without culture bias	Also require more specified knowledge and equipment, and quantitative analysis is still not well-established

3.1.2. Chemical analysis of nutrients

Since oil biodegradation is limited by availability of nutrients in most marine shorelines, monitoring nutrients, particularly the nutrient concentrations in pore water, is critical in developing proper bioremediation strategies and assessing the effect of oil bioremediation (Bragg *et al*., 1994; Venosa *et al*., 1996). Important nutrient analyses include measurements of ammonium, nitrate, nitrite, and phosphorus. Commonly used methods for these nutrient analyses are summarized as follows.

Sample preparation

Analysis of nutrients in sediments can either be conducted on site or be frozen and shipped to a lab for measurements. Before the analysis, available nitrogen and phosphorus species can be extracted from sediments using a 2M solution of KCl or an acidified 0.1% NaCl solution (Page *et al.*, 1986; Tan, 1996). Total nitrogen and phosphorus can be liberated from sediment samples by persulfate digestion at 121°C.

Ammonia analysis

The most commonly used methods for ammonia analysis are automated colorimetric methods due to their high sensitivity and ease of use. Two major automated colorimetric methods are available (1) Automated phenate method (4500-NH_3 H, Eaton *et al*., 1995) (2) Automated salicylate-hypochlorite method. The latter was recently developed due to the environmental concerns associated with the phenol used by the phenate method (Tan, 1996). The extracted ammonia nitrogen can also be measured in the field using an ammonia-selective electrode (4500-NH_3 F, Eaton *et al*., 1995) or some commercial kits, such as a Chemetrics kit (Chemetrics Inc., Calverton, VA) although these methods are less sensitive and more susceptible to interference than colorimetric methods.

Nitrate and nitrite analysis

Commonly used techniques for nitrate analysis include the ultraviolet spectrophotometric method (4500-NO_3^- B, Eaton *et al*., 1995), automated cadmium reduction method (4500-NO_3^- F, Eaton *et al*., 1995) and nitrate electrode method (4500-NO_3^- D, Eaton *et al*., 1995). The ultraviolet spectrophotometric method is suitable for rapid measurement or screening samples that have low organic matter contents. The automated cadmium reduction method is more sensitive and suitable for nitrate analysis in various types of water and wastewater. When using this method, nitrate is reduced to nitrite by passing through a Cu-Cd reduction column. The nitrite is then determined using a colorimetric procedure. Therefore, without the reduction step, this method can be used for nitrite analysis. Total nitrogen can also be measured as nitrate by the automated cadmium reduction method following oxidation of urea, ammonia, and nitrite by potassium persulfate at 121°C (4500-N_{org} D, Eaton *et al*., 1995). The nitrate electrode method can be used in the field for rapid nitrate analysis although it is less reliable than cadmium reduction method.

Phosphorus analysis

Phosphorus analysis involves two general procedures: (1) conversion of the phosphorus form of interest to dissolved orthophosphate and (2) colorimetric determination of dissolved orthophosphate (Eaton *et al*., 1995). Total phosphorus analysis usually uses persulfate digestion procedures (method (4500-P B, Eaton *et al*., 1995). Various digestion procedures have been developed for analysis of *Available Phosphorus* (Tan, 1996), a variable concept that reflects the amount of phosphorus available to plants or microorganisms. The ascorbic acid method (4500-P E, Eaton *et al*., 1995) is recommended for analysis of phosphate in the concentration range of 0.01 to 6 mg P/L. Commercial kits, such as Hach® phosphate analysis procedures (Hach Company, Loveland, CO), are also available for use in the field.

3.1.3 Chemical analysis of oil and oil constituents

One of the primary measures of the success of bioremediation treatments is reduction in the concentrations of spilled oils and target oil constituents in particular. Various techniques have been developed and used in petroleum hydrocarbon analysis, which include gravimetric methods, infrared spectroscopy (IR), gas chromatography/flame ionization detection (GC/FID), gas chromatography-mass spectrometry (GC/MS), and thin-layer chromatography-flame ionization detection (TLC-FID). Oil analysis methods can be generally classified into two

categories: nonspecific methods to measure total petroleum hydrocarbons (TPHs), and specific methods using various chromatographic techniques to quantify target oil constituents. Commonly used oil analysis methods in monitoring oil bioremediation are summarized in this section and table 3.2.

3.1.3.1 Total petroleum hydrocarbon (TPH) techniques

TPH techniques mainly include gravimetric and infrared spectroscopic methods. These techniques are widely accepted methods to rapidly quantify the oil due to their simplicity and low costs. However, these methods provide little information about oil components, exhibit high detection limits and are susceptible to various interferences (Douglas *et al* 1991, Xie *et al*., 1999). Furthermore, they are unable to distinguish between abiotic and biotic losses.

Gravimetric analysis involves solvent (e.g., dichloromethane) extraction, evaporation, and gravimetric measurement (EPA 413.1 and EPA 9071). This method does not distinguish between petroleum hydrocarbons and naturally occurring biogenic compounds (such as plant lipids) and may result in overestimating TPH. Infrared spectroscopy (IR) involves solvent extraction normally using trichlorotrifluoroethane (Freon 113). TPH is subsequently measured by comparing the infrared absorption of the extraction liquid against that of a defined hydrocarbon mixture (e.g., EPA 418.1 (U.S. EPA, 1992)). Although this technique is a more sensitive measure of hydrocarbons than gravimetric methods, it may also overestimate or underestimate TPH for a variety of reasons (Xie *et al*., 1999). Environmental concerns regarding the use of Freon as a solvent is also a potential problem for its application (Romero and Ferrer, 1999). Fully halogenated solvents such as tetrachloroethene or tetrachloromethane can be substituted for Freon.

Commercial kits, such as Petroflag® test kit (Dexsil, Hamden, CT) and Hach® DR/2000 test kit (Hach Company, Loveland, CO), are also available for use in the field with limited reliability (Lambert *et al*., 1999a&b). All TPH techniques are severely affected by the spatial heterogeneity. A larger quantity of oil at one spot could give a misleading high TPH value. They do not distinguish between abiotic and biotic losses that are important in correctly interpreting the data for the fate of the petroleum in the environment. So, one has to be careful in carrying out these tests as well as in interpreting the data. A sufficiently large number of samples may help to overcome some of the variability.

3.1.3.2 Analysis of specific oil constituents

To assess the effect of oil bioremediation, identification and quantification of individual oil components and compounds, particularly those constituents that are of significant environmental concern , is required. Various chromatographic techniques, particularly GC/FID and GC/MS, are widely used to provide specific and sensitive analysis of oil constituents (Douglas *et al.*, 1994; Wang *et al*. 1997).

Gas chromatography/flame ionization detection (GC/FID)
GC/FID combines chromatographic separation of hydrocarbon fractions on a capillary GC column with quantification by FID (e.g., EPA 8100(U.S. EPA, 1992)). Pretreatment of oiled

sediment samples involves drying of samples, addition of surrogates, and solvent extraction (e.g., EPA 3540c (U.S. EPA, 1992)). This method has been mainly used for detection of aliphatic hydrocarbons such as individual C_{10} to C_{35} n-alkanes, and isoprenoid hydrocarbons (Table 3.3). GC/FID can also be used to determine TPHs by the method of internal standards (Douglas *et al*, 1994). Total GC-detectable hydrocarbons make up more than 50% of the oil components (Prince *et al*., 1994). However, GC/FID may not be used to identify and quantify PAHs and biomarker compounds because it can not clearly separate many of these compounds, especially the alkylated PAHs. Since PAHs are of most environmental concerns and biomarker compounds are critical in distinguishing between biodegradation from physical weathering processes and reducing spatial variability of oil data, measurement of these compounds is very important in monitoring oil bioremediation.

Gas chromatography-mass spectrometry (GC/MS)

GC/MS, which combines chemical separation by GC and spectral resolution by MS, is the choice of methods for specific compound determination, especially for identification and quantification of PAHs and biomarkers (Wang *et al*., 1997). The mass spectrometer is often operated in the selected ion monitoring mode (SIM) to further increase sensitivity and selectivity relative to conventional full-scan GC/MS. For a detailed description of GC/MS analytical procedures, readers can refer to Douglas *et al*. (1994), EPA Method 8270 (US EPA,1989), and Venosa *et al*. (1996). Typical target alkanes and PAHs analyzed using GC/MS in recent USEPA-sponsored projects are listed in Table 3.3 and 3.4 (Purandare, 1999; Venosa *et al*., 1996). Because the distribution of oil on shorelines contaminated by offshore spills can be highly heterogeneous (Bragg *et al*., 1994), the concentrations of all target analytes are often reported relative to a conservative biomarker such as 17α(H),21β(H)-hopane (Douglas *et al*., 1994). Detailed discussions on biomarkers will be presented in Section 3.2.

Thin-layer chromatography flame ionization detection (TLC-FID)

Thin layer chromatography-flame ionization detection (TLC-FID), which uses a special instrument called the Iatroscan, separates hydrocarbons on a Chromarod thin layer based on characteristic chemical types or fractions such as aliphatic, aromatic, polar, and asphaltene compounds (Goto *et al*., 1994). This method has advantages in measuring high-boiling-point hydrocarbons such as higher molecular weight saturates, aromatics, resins, and asphaltenes, some of which may not be detectable by GC or HPLC. Unlike the other analytical techniques, which are either too gross (e.g. TPH techniques) or very specific (e.g. GC based methods), the TLC-FID can measure the relative percentages of the four major fractions of petroleum in a short period of time. This method has been successfully used for monitoring oil bioremediation in a wetland environment (Stephens *et al*., 1999). However, TLC-FID can not identify specific compounds and may only be used as a screening tool. Controversy also exists concerning the reliability of the methods due to some confusing results, and modifications of the technique have been suggested (Cebolla *et al.*, 1998).

Table 3.2 Commonly used methods for petroleum hydrocarbon analysis

Method	Advantages	Disadvantages	References
TPH techniques	Inexpensive and easy to perform. Used as quick screen tools	Low sensitivity and selectivity; can not be used for identification of oil components. Not recommended.	USEPA, 1992
GC/FID	A specific method used for detection of aliphatic and a limited number of aromatic hydrocarbons.	Unable to identify and quantify alkylated PAHs and biomarkers	Douglas *et al.*, 1994; Wang *et al.*,1997
GC/MS	Highly sensitive and selective method for identification and quantification of a wide range of hydrocarbons, including PAHs and biomarkers	Expensive equipment and complicated procedures	Douglas *et al.*, 1994; USEPA, 1992; Venosa *et al.*, 1996; Wang *et al.*,1997
TLC/FID	Quick detection of a wide range of oil components, including high molecular weight saturates, aromatics, resin, and asphaltenes; can be used as an effective screening tool.	Unable to identify specific compounds; quantitative analysis is still not well-established	Stephens *et al.*, 1999; Cebolla *et al.*, 1998

Table 3.3 Target alkanes list (Purandare, 1999)

Compound Name	**Misc. Info.**	**QIon**	**Response Factors Reference Compounds**	**Internal Standards**
D26 n-dodecane	surrogate	66	D26 n-dodecane	D22 n-decane QIon: 66
*n*C10		57	*n*C10	
*n*C11		57	*n*C11	
*n*C12		57	*n*C12	
*n*C13		57	*n*C13	
*n*C14		57	*n*C14	
*n*C15		57	*n*C15	
D36 n-heptadecane	surrogate	66	D36 n-heptadecane	D34 n-hexadecane QIon: 66
*n*C17		57	*n*C17	
Pristane		57	Pristane	
*n*C18		57	*n*C18	
Phytane		57	Phytane	
*n*C19		57	*n*C19	
D50 n-tetracosane	surrogate	66	D50 n-tetracosane	D42 n-eicosane QIon: 66
*n*C20		57	*n*C20	
*n*C21		57	*n*C21	
*n*C22		57	*n*C22	
*n*C23		57	*n*C23	
*n*C24		57	*n*C24	
*n*C25		57	*n*C25	
*n*C26		57	*n*C26	
*n*C27		57	*n*C27	
D66 n-dotriacontane	surrogate	66	D66-dotriacontane	D62 n-triacontane Qion: 66
*n*C28		57	*n*C28	
*n*C29		57	*n*C29	
*n*C30		57	*n*C30	
*n*C31		57	*n*C31	
*n*C32		57	*n*C32	
*n*C33		57	*n*C33	
*n*C34		57	*n*C34	
*n*C35		57	*n*C35	
5 –cholestane	surrogate	217	5 -cholestane	5 -antrostane QIon: 245
Hopane	alkane	191	Hopane	

Table 3.4 Target PAHs analyzed by GC/MS (Purandare, 1999)

Compound Name	**Misc. Info.**	**QIon**	**Response Factors Reference Compounds**	**Internal Standards**
D10 1-methyl naphthalene	surrogate	152	D10 1-methyl naphthalene	D8-naphthalene QIon: 136
Naphthalene	2-ring PAH	128	Naphthalene	
C1 naphthalene	2-ring alkyl PAHs	142	Naphthalene	
C2 naphthalene		156	Naphthalene	
C3 naphthalene		170	Naphthalene	
C4 naphthalene		184	Naphthalene	
Fluorene	3 ring PAH	166	Fluorene	D10-anthracene QIon: 188
C1 fluorenes	3 ring alkyl PAHs	180	Fluorene	
C2 fluorenes		194	Fluorene	
C3 fluorene		208	Fluorene	
Dibenzothiophene	3 ring PAH	184	Dibenzothiophene	
C1 dibenzothiophene	3 ring alkyl PAHs	198	Dibenzothiophene	
C2 dibenzothiophene		212	Dibenzothiophene	
C3 dibenzothiophene		226	Dibenzothiophene	
D10 phenanthrene	surrogate	188	D10 phenanthrene	
Phenanthrene	3-ring PAHs	178	Phenanthrene	
Anthracene		178	Anthracene	
C1 phenanthrenes	3 ring alkyl PAHs	192	Phenanthrene	
C2 phenanthrenes		206	Phenanthrene	
C3 phenanthrenes		220	Phenanthrene	
C4 phenanthrenes		234	Phenanthrene	
Naphthobenzothiophene	4 ring PAH	234	Dibenzothiophene	
C1 naphthobenzothiophene	4 ring alkyl PAHs	248	Dibenzothiophene	
C2 naphthobenzothiophene		262	Dibenzothiophene	
C3 naphthobenzothiophene		276	Dibenzothiophene	
Fluoranthene	4-ring PAH	202	Fluoranthene	D12-chrysene QIon: 240
D10 pyrene	surrogate	212	D10 pyrene	
Pyrene	4-ring PAH	202	Pyrene	
C1 pyrenes	4 ring alky PAHs	216	Pyrene	
C2 pyrenes		230	Pyrene	

Table 3.4. Target PAHs (Contd.)

Compound Name	Misc. Info.	QIon	Response Factors Reference Compounds	Internal Standards
Chrysene	4 ring PAH	228	Chrysene	D12-chrysene QIon: 240
C1 chrysenes	4 ring alkyl PAHs	242	Chrysene	
C2 chrysenes		256	Chrysene	
C3 chrysenes		270	Chrysene	
C4 chrysenes		284	Chrysene	
benzo(b)fluranthene	5-ring PAHs	252	Benzo(b)fluoranthene	D12-perylene QIon: 264
benzo(k)fluoranthene		252	Benzo(k)fluoranthene	
benzo(e)pyrene		252	Benzo(e)pyrene	
benzo(a)pyrene		252	Benzo(a)pyrene	
indeno(1, 2, 3-cd) pyrene	6-ring PAHs	276	Indeno (1, 2, 3-cd) pyrene	
dibenzo(a,h)anthracene	5-ring PAHs	278	Dibenzo (a,h)anthracene	
benzo(g,h,i)perylene	6-ring PAHs	276	Benzo(g,h,i)perylene	

3.2 Biomarkers

As mentioned in Chapter 2, internal biomarkers have been widely used to distinguish between biodegradation and the physical or chemical loss of oil from treated plots in bioremediation field studies (Bragg *et al*., 1994; Venosa *et al*., 1996; Lee *et al*., 1997b). An ideal biomarker should (1) provide source specific information, (2) not be formed during physical, chemical weathering and biological processes, (3) be non-biodegradable or relatively resistant to biodegradation on time scales relevant to the study or cleanup, (4) be extracted from the sample with similar efficiency to the other associated compounds (Douglas *et al*, 1994; Prince *et al*., 1994a,b). The ideal biomarker would also be subject to the same physical and chemical removal mechanisms as the target analytes so that any differences could be attributed to biodegradation. Unfortunately, existing biomarkers can rarely meet all these criteria. An overview of commonly used biomarkers in monitoring oil bioremediation is given here. Issues to be aware of when using biomarkers will be discussed.

3.2.1 Commonly used biomarkers

Pristane and phytane

Hydrocarbon degrading microorganisms usually degrade branched alkanes or isoprenoid compounds such as pristane and phytane at lower rates than straight-chain alkanes. Pristane and phytane are also subject to the same physical and chemical removal mechanisms as their

corresponding straight-chain alkanes (Wang *et al*., 1998). Therefore, n-C17:pristane and n-C18:phytane ratios have been traditionally used to interpret the extent of biodegradation (Gaudlach E.R *et al*., 1983; Lee and Levy, 1987,1989,1991). However, it was later found in Prince William Sound that these compounds were resistant to degradation in the initial time period, but they degraded rather rapidly over longer periods of time (Prince 1993, Bragg, *et al*., 1994). This phenomenon was also observed in a study on bioremediation of Light Arabian crude oil in a freshwater wetland environment as shown in Figure 3.1 (Purandare, 1999). The study examined the dry weight normalized pristane, phytane, and hopane concentrations over the 32-week experimental period. It can be seen that the pristane and phytane showed significant degradation, but hopane remained constant throughout the course of the study. These results show that the n-alkane:isoprenoid ratio may be useful indications of biodegradation in very short term or the earliest part of a study. However, they may substantially underestimate the extent of biodegradation in a long run.

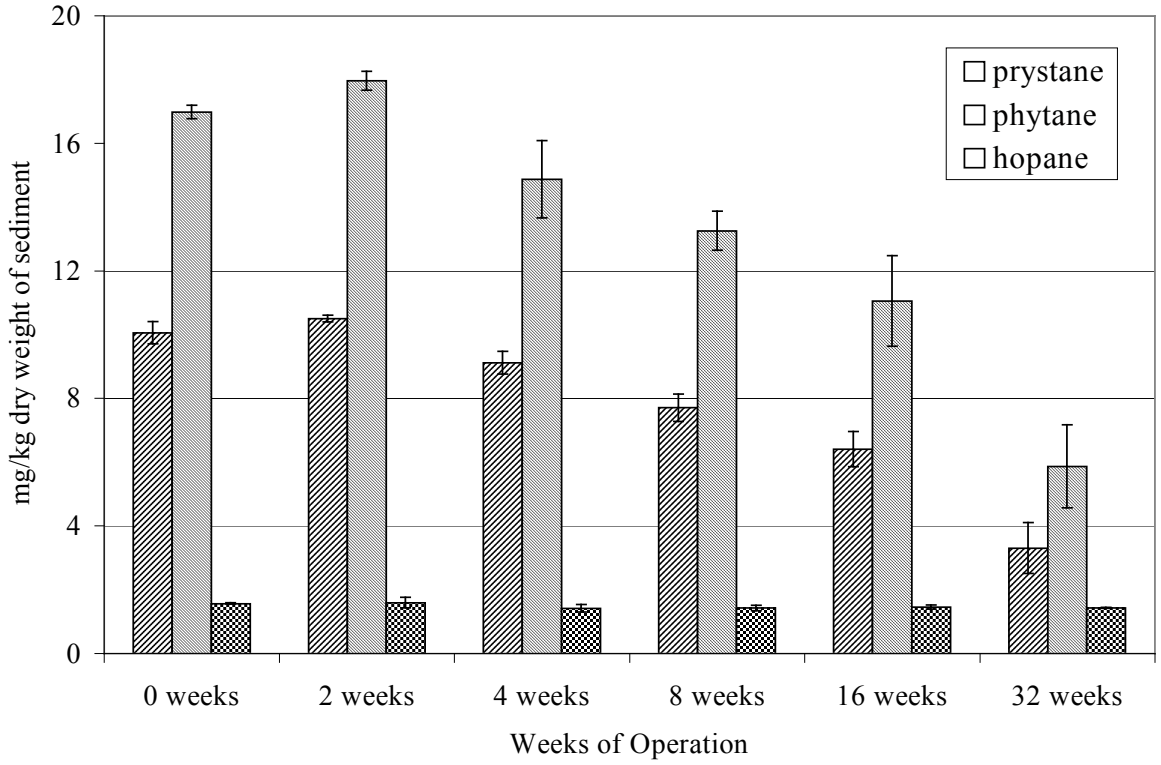


Figure 3.1 Change of pristane, phytane and hopane in a freshwater wetland

Hopanes

Hopanes, derived from the molecular fossils of prokaryotic and eukaryotic membranes (Peters and Moldowan, 1993), are very resistant to biodegradation as shown in Figure 3.1. 17α(H),21β(H)-hopane (Figure 3.2) has been found to be neither generated nor biodegraded during the biodegradation of crude oil on time scales relevant to estimating the cleaning of oil spills and therefore has appropriate characteristics to serve as an internal standard for monitoring

biodegradation of both specific petroleum compounds and total oil in crude oil in the environment (Prince *et al*., 1994). Hopanes have been viewed as the biomarkers of choice (Mearns, 1997) since the successful application of 17α(H),21β(H)-hopane for evaluating oil bioremediation after the *Exxon Valdez* spill (Douglas *et al*., 1994; Prince, *et al*., 1994a&b). Many recent studies have also chosen this hopane as the biomarker (Garcia-Blanco *et al*., 2001b; Purandare, 1999; Swannell *et al*., 1999a; Venosa *et al.*, 1996).

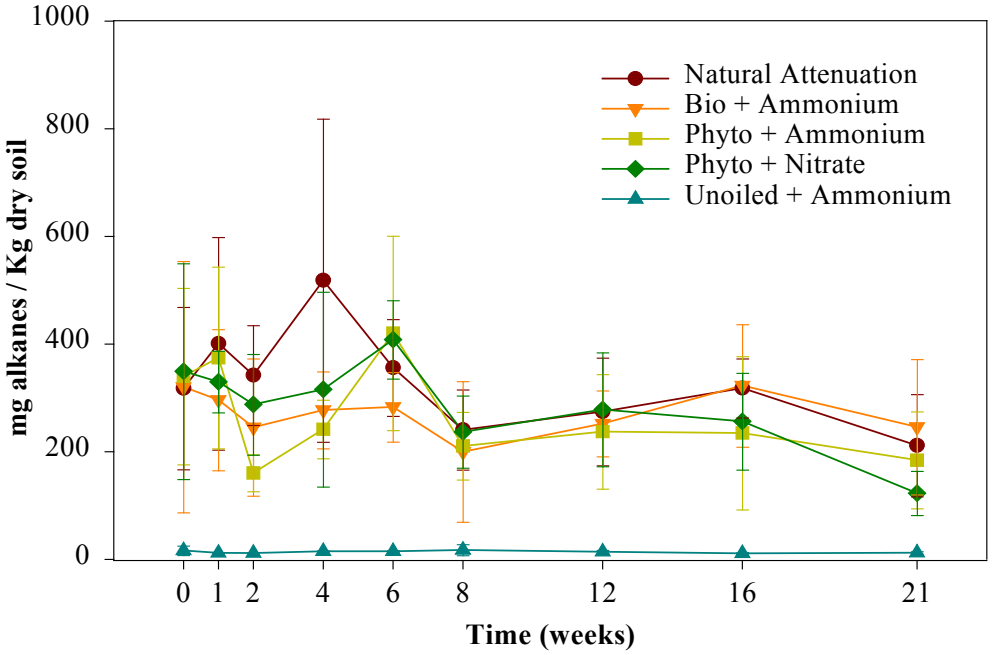
Figure 3.2 shows alkane analysis results in soil core samples during the first 21 weeks of the St. Lawrence River field study using dry weight soil normalization and hopane normalization (Garcia-Blanco *et al*., 2001b). Due to the substantial heterogeneity of oil distribution in the freshwater wetland sediments, large standard deviations (17-72% of the mean concentrations) were obtained when using dry weight soil normalization. No convincing conclusions were able to be reached based on these data. However, the standard deviation of oil concentrations in the sediment samples were much lower (2-15% of the mean oil concentrations) when using hopane normalization, which enabled evaluation of oil biodegradation with high levels of statistical confidence.

However, it should be noted that hopanes are also very resistant to those physical and chemical weathering processes that affect most target alkanes and aromatics (e.g., dissolution, volatilization, and photooxidation). Therefore, although hopane normalization is very useful in reducing the variability associated with heterogeneous oil distribution, changes in the hopane-normalized analyte concentrations alone may not be used to verify that biodegradation is the primary removal mechanism. Use of hopane normalization to distinguish biodegradation from physical loss of oil is valid mostly when effects of dissolution and volatilization are negligible (Prince, 1993; Venosa *et al*., 1996), which is not always true. Other means of verifying biodegradation are needed such as the use of alkylated PAH isomers (see next section).

Alkylated PAH isomers

Biodegradation can be verified as a removal mechanism by determining the relative degradation rates for homologous series of alkylated PAHs. Preferential biodegradation of aliphatic and aromatic hydrocarbons based on molecular structure has long been recognized (Jobson et al., 1972; Walker et al., 1976; Roubal and Atlas, 1978; Fedorak and Westlake, 1981; Elmendorf et al., 1994, Wang et al, 1998). This is particularly true for alkylated PAHs, which are biologically transformed more slowly as the extent of alkyl substitution increases (Elmendorf et al., 1994; Venosa et al., 1997). Biodegradation results in unique characteristic changes in the distributions of homologous series of alkylated PAHs. On the other hand, physical weathering does not cause the same types of alterations in their relative distributions (Wang et al., 1998). In other words, physical weathering causes equal losses in all homologues irrespective of the extent of alkyl substitutions. Recent research has also shown that the relative biodegradation rates for alkylated homologs of the 2- and 3-ring PAHs were remarkably similar for mixed cultures of hydrocarbon-degrading bacteria isolated from a wide variety of sources (Venosa et al., 1997), and they were also very similar in the field, despite the much higher absolute rates that were observed in the laboratory (Venosa et al., 1996). Therefore, the distribution patterns of alkylated PAHs, when used in conjunction with other oil analysis data, can be very useful in accurate assessment of the extent and progress of oil biodegradation.

a) Dry soil weight normalized



b) Hopane normalized

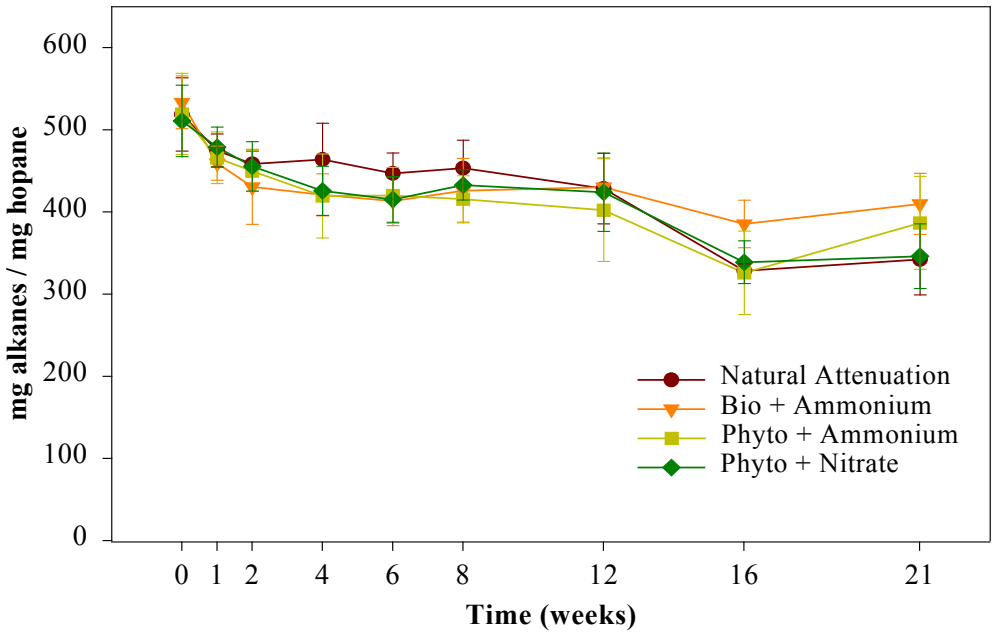


Figure 3.2 Comparison of alkanes analysis results in soil core samples from St. Lawrence field study using (a) dry soil weight normalization and (b) hopane normalization

Other Biomarkers

For refined petroleum products such as diesel fuel and fuel oil #2 that do not contain the hopane, C_4-phenanthrenes/anthracenes may be substituted (Douglas *et al*., 1994). These compounds are degraded, but very slowly. Lee *et al*. (1997b) used C2-chrysenes as biomarkers in their studies on bioremediation of weathered Venture Condensate because hopane concentration in the oil was at or near the detection limits of their instruments and C2-chrysenes are also considered highly degradation resistant. C4-chrysenes can also be used if their concentrations are high enough to be detectable.

Table 3.5 Biomarkers and their characteristics

Biomarkers	Advantages	Limitations	Applicable to	References
Pristane & Phytane		Biodegradable	Monitoring early stages of biodegradation	Lee *et al.* 1987&1989
Hopanes	Resistant to biodegradation	Can not distinguish biodegradation from abiotic weathering processes	Reducing spatial variability	Prince *et al.*, 1994; Douglas *et al.,* 1994
Alkylated PAHs isomers	Loss pattern characteristic of biodegradation		Verification of biodegradation	Venosa *et al*., 1997a; Wang *et al*., 1997&1998
Phenanthrenes, Anthracenes, and Chrysenes			Refined petroleum products	Douglas *et al*., 1994

3.2.2 The effect of contaminant redistribution on observed remediation rates

Loss of oil due to physical washout and sand redistribution can be significant in an oiled beach. Hopane normalization is an effective way to distinguish biodegradation from the effects of the physical washout and sediment exchange between the inside and outside of experimental plots when all of the oil is initially present inside of the plots and most of the beach is clean, such as in a study involving intentional oiling (Venosa *et al*., 1996). However, it will not work well in a study involving small plots set up on a beach contaminated by a "spill of opportunity" or a real oil spill. The reason is that when a study is carried out on a beach completely contaminated by an oil spill, oil or oiled sand will transport between relatively small treated areas (i.e., experimental plots) and large untreated but oiled areas. Since hopane is a conservative biomarker, its concentration will be the same inside and outside of the plots (assuming that the beaches were

uniformly oiled initially). Therefore, oiled sand coming into the plots will have the same amount of hopane as the sand leaving the plots (assuming that treatment does not result in physical removal of bulk oil from sand inside the plots), but the concentrations of target analytes will be higher, because the biodegradation rates will be lower in untreated areas. Since the hopane concentration inside the plots will not be affected by sand transport, it does not allow us to quantify the rate of sand exchange between the inside and outside of the plots.

A theoretical analysis conducted by Wrenn *et al*. (1999) illustrates how physical exchange of sand between treated and untreated areas of the beach affects the observed biodegradation rate of target analytes when small plots are set up on a large beach. For the nutrient-treated plots, the rate of change in the analyte concentrations, A_{treat} , is given by:

$$\frac{dA_{treat}}{dt} = \frac{A_{treat}}{T}\frac{dT}{dt} - k_{treat}A_{treat} + \left(\frac{1}{H}\frac{dH}{dt} - \frac{1}{T}\frac{dT}{dt}\right)A_{treat} \tag{3.1}$$

For the untreated control plots and in the untreated areas of the beach, the rate of analyte, A_{con}, disappearance is:

$$\frac{dA_{con}}{dt} = \frac{A_{con}}{H}\left(\frac{dH}{dt}\right) - k_{con}A_{con} \tag{3.2}$$

Where k_{treat} and k_{con} are the first-order biodegradation rate coefficients in the presence and absence of nutrients; H is the concentration of hopane; and T is the concentration of a hypothetical nonbiodegradable tracer that is present in the oil inside of the plots but not outside of the plots (e.g., a hydrophobic fluorescent dye that is added to the oiled sand inside the plots at the start of treatment). Equation (3.1) describes the rate of change of the analyte concentration inside the treated plots due to transport of treated oiled sand out of the plots, biodegradation, and transport of untreated oiled sand into the plots. Assuming the rates of physical loss of treated oiled sand from inside the plots and loss of oiled sand from the beach to be first-order processes, the hopane-normalized analyte concentration inside of the plots at any time can be solved as:

$$\left(\frac{A_{treat}}{H}\right) = \left(\frac{A_o}{H_o}\right)e^{(k\ -k_T-k_{treat})t}\left[\frac{(k_{treat}-k_{con})+(k_T-k_B)e^{(k_T+k_{treat}-k_{con}-k_B)t}}{k_T+k_{treat}-k_{con}-k_B}\right] \tag{3.3}$$

Where k_T and k_H are the first-order loss coefficients for the nonbiodegradable tracer and hopane, respectively.

Representative results from this model are shown in Figure 3.3 using parameters obtained from Delaware field trial (Venosa *et al.*, 1996). It can be seen that the effect of exchange of oiled sand between the inside of treated plots and untreated beach is to reduce the observed degradation rate relative to the true rate. Sand exchange has no effect on the observed biodegradation rate in the control plots, because biodegradation is assumed to occur at the same rates inside and outside of those plots. The apparent rate of remediation in the treated plots, however, will decrease while relatively large amounts of oil remain even when bioremediation would be capable of achieving a complete cleanup if the entire contaminated shoreline were treated. This could lead to the

incorrect conclusion that, whereas bioremediation can stimulate the initial cleanup rate, it cannot restore the contaminated shorelines to acceptable conditions. The analysis shows that, if behavior of this type is observed, it is probably an artifact of treating a small fraction of the total contaminated area, and more complete remediation would be expected when a larger area is treated. Nevertheless, there are other possible explanations for incomplete remediation. These include the inability to maintain sufficient nutrients in the bioremediation zone and, especially on high-energy beaches, a high loss rate of bacteria from the oiled surfaces (e.g., due to scouring by waves). Therefore, a thorough monitoring program is a very important component of this research, because data on nutrient concentrations and microbial activity is required to properly interpret the results if the cleanup goals are not achieved during the field study.

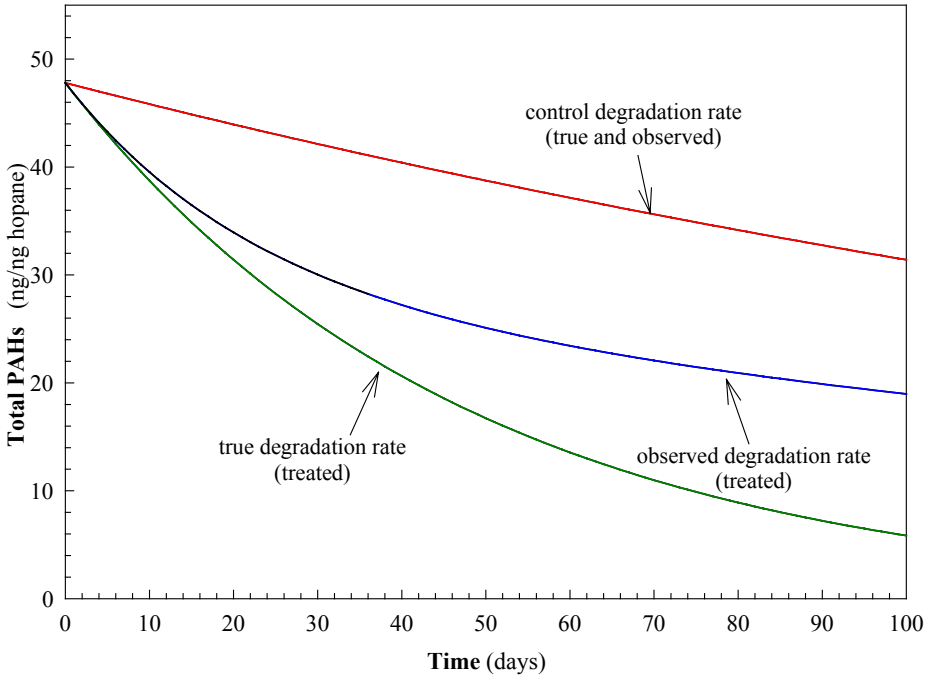


Figure 3.3 Reduction in the observed oil biodegradation rate due to exchange of oiled sand between the inside of treated plots and untreated surrounding beach

3.3 Sampling in the Field

Because oil contaminated sites are highly heterogeneous, representative sampling is difficult but also extremely important for proper evaluation of bioremediation. Field sampling procedures must be designed to achieve statistically valid sampling and to minimize contamination or changes in the samples. Variables that affect the representativeness of samples and their methods of collection include characteristics of media, concentration distribution of analytes, and bias introduced during collection, preparation, and transportation (Lee, 2000). General principles to achieving statistically valid sampling in soil environments and solid waste have been well-documented (Tan, 1996; USEPA, 1992). However, little information is available regarding sampling protocols for monitoring oil bioremediation in marine shorelines and freshwater

wetlands. Major considerations to achieve representative sampling are summarized as follows based on the soil-science literature and field experiences from recent oil bioremediation projects.

3.3.1 Sampling strategies

Types of sampling

Sampling methods can be classified into simple random sampling, systematic sampling, stratified sampling, and compositing (Tan, 1996). The Simple random sampling or the grab method involves collecting samples at random in a sampling area. This method depends completely on the luck of the draw without considering the variation of analytes in the sampling field. Purandare (1999) found that results of oil analysis in a wetland sediment were highly variable and unreliable using this sampling approach during the early stage of his study. Therefore, this approach may only be suitable for use in relatively homogeneous systems. Systematic sampling involves taking samples based on certain patterns, such as collecting samples in a grid pattern. This method will ensure that the entire sampling area is represented in the sample. Stratified sampling involves dividing the sampling field into a number of sectors or quadrants and taking independent samples in each sector according to the rule of proportionality (e.g., taking more samples in more heavily oiled sites). These approaches can often provide more accurate results than simple random sampling, because with this method the samples are distributed more evenly over the population. Compositing involves the mixing of sampling units to form a single sample, which has the advantage of increased accuracy through the use of large numbers of sampling units per sample. This approach in combination of stratified sampling has been frequently used in recent field studies on oil bioremediation (Venosa et al., 1996; Garcia-Blanco et al., 2001b).

Depth of sampling

Sampling depth in oil spill sites mainly depends on the distribution of the analytes of interest, especially the depth of oil penetration. Crude oil rarely penetrates coastal sediments to depths of greater than one foot (Gundlach, 1987). Penetration of oil in wetland environments will be even less deep than in most marine sediments. Purandare (1999) found that oil only penetrated to 2.5 cm in a wetland sediment in 16 weeks. The top 2 cm layers of the sample cores were then used for oil analysis. Therefore, a survey of oil penetration in the contaminated site is critical in determination of sampling procedures for a bioremediation application.

Size of sampling

The size of the sample required depends on the available resources, the required degree of confidence, and the objectives of the analysis (Rupp and Jones, 1993). Generally, the more heterogeneous the system, the more intense must be the sampling efforts to reach a given accuracy. However, economic considerations often restrict both the quantity and the number of samples taken, and a balance should be obtained between the size of samples to be taken for required confidence and economic factors. Following expressions are some examples of statistical approaches, which can be used to calculate the required number of samples with respect to an acceptable error (Peterson and Calvin, 1986; Tan, 1996):

$$n = 4\sigma/E^2 \tag{3.4}$$

or

$$n = t^2S^2/E^2 \tag{3.5}$$

where n is number of samples, t is t-test value, E is acceptable error, S^2 is sum of squares of sample deviation, and σ is standard deviation.

Sample handling and storage
Sample handling procedures must be designed to minimize cross-contamination or chemical and biological changes in the samples. For example, different sets of sampling tools should be used for different treatments to avoid cross contamination. Clean and proper containers, such as PVC bags and tin cans, should be used for sample storage. Samples, if not being analyzed in the field, should be frozen using blue ice or dry ice until they reach their destination. And once they arrive the destination, samples should be stored in a freezer at -18 to - 20 ^{o}C until needed.

3.3.2 Field sampling experiences

Two examples of well-designed sampling protocols used in definitive bioremediation field studies are shown below (Venosa *et al.*, 1996; Garcia-Blanco *et al*., 2001b).

Sampling protocols used in the Delaware field bioremediation study (Sandy Beach)
Each plot was divided into 4 quadrants for sampling purposes, and the sand samples were collected at the nodes of a 0.5 m by 0.5 m grid in each quadrant (Figure 3.4). As Figure 3.4 shows, all sample nodes were at least 0.5 m from the plot boundary on all sides. This buffer zone was designed to minimize the impact of edge effects on the observed extent of biodegradation. Since the plots were at least 9.5 m long, this provided a minimum of 28 sample nodes for each quadrant.

Samples were composited from 2 randomly selected nodes in each quadrant at 1 or 2 week intervals for a period of 14 weeks, but they were never collected from the same node twice. The sampling frequency was higher near the beginning of treatment and followed the order of Weeks 0 (i.e., just before treatment began), 1, 2, 3, 4, 6, 8, 10, 12, and 14. With 10 sample events in this study, 20 sample nodes were required per quadrant. Sand samples were collected with hand augers to a depth of 15 cm, which was determined based on preliminary oil penetration study.

Sampling protocols used in St. Lawrence River field study (Freshwater Wetland)
A randomized sampling plan was designed to eliminate sampling bias. Each plot was divided into six sectors, each measuring 1.5 m x 1.0 m, with the 1.5 m dimension parallel to the shoreline. Each sector was subdivided into 10 subsampling zones, corresponding to the predetermined sampling events. Each subsampling zone had dimensions 50 cm x 30 cm.

At each sampling event, a 9-cm core sample was collected using a tulip bulb planter from pre-assigned random subsampling zones from each of the six sectors within each plot. These samples were combined into 2 composites (3 predetermined samples per composite). Both composite samples were placed into quart size paint cans, frozen, and shipped to the University of Cincinnati (OH) for oil analysis. Samples were kept in the freezer (-18 to -20 ^{o}C) until they were extracted. Composite 1 served as the sample to be analyzed by GC/MS. Composite 2 was analyzed for three of the sampling events to check within-plot variability. The remaining composite 2 samples were frozen and archived. The first sampling event (week 0) was carried

out at low tide the day after the application of oil and nutrients. Subsequent sampling events were at weeks 1, 2, 4, 6, 8, 12, 16, 21, 48, and 65.

The sampling designs mentioned above are important because they incorporate replicate plots with random placement on the experimental plane. These are essential for permitting proper statistical analysis of treatment effects.

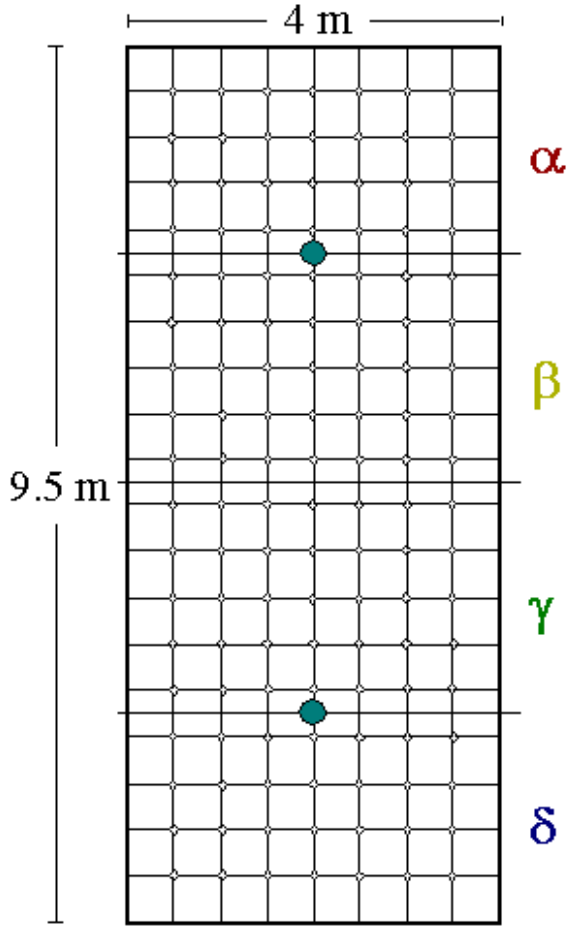


Figure 3.4 Example of an experimental plot in the Delaware field study showing sampling quadrants and nodes

3.4 Monitoring General Site Background Conditions

Monitoring general site background conditions is very important for properly evaluating effects of oil bioremediation. Major background conditions include dissolved oxygen, pore water pH, temperature, and salinity.

3.4.1 Oxygen

Oxygen availability is crucial for rapid bioremediation because hydrocarbon biodegradation is primarily an aerobic process. Therefore, the dissolved oxygen (DO) of pore water should be monitored on a regular basis. Water samples from the oil-contaminated region of the subsurface can be collected through the multi-port sample wells and sealed in DO bottles. Conventional methods for DO measurement include iodometric procedures and membrane electrode method (Eaton *et al*., 1995).

The iodometric technique is the most precise and reliable titrimetric procedure for DO analysis. Detailed procedure of this method is described in Standard Methods No. 4500-O B (Eaton *et al*., 1995). Various iodometric modifications have been developed. One procedure that is suitable for use in the field involves using Hach® high range dissolved oxygen ampoules (Hach Company, Loveland, CO). Once the Hach® ampoules are filled and capped and the reaction of the reagents with DO in the water sample is complete, the color is stable indefinitely. Therefore, the capped ampoules can be transported back to the laboratory trailer where the DO concentration in the water samples can be determined with a Hach® colorimeter. (Note: the caps often leak gaseous O_2 into the samples; so, the samples aren't really stable indefinitely, even though the color is stable as long as additional O_2 can be excluded.)

The membrane electrode method is more suitable for regular field monitoring and in situ DO determination. It is also recommended for DO analysis in highly polluted waters and colored waters. The general procedure for this method is described in Standard Methods No. 4500-OG (Eaton *et al*., 1995). Detailed analytical procedures may vary depending on the manufacturers of the DO probes.

3.4.2 pH

Biodegradation of oil is affected by the background pH (Atlas and Bartha, 1992). Oil biodegradation can also be severely inhibited by dramatic reductions in pH when ammonia is provided as the nitrogen source (Wrenn *et al*., 1994). The latter is true for closed environments such a laboratory flasks where no dilution is possible from continuously changing aqueous conditions such as tides. Nonetheless, monitoring pH in the field is of particular importance in evaluating the effect of oil biodegradation.

The pH values of pore-water samples are normally measured using a portable pH meter with a combination electrode. The pH can be measured in the field either immediately after the samples are collected or by putting pH electrodes directly into water in sampling wells. Sediment pH can also be measured in the field by mixing the soil samples with reagent water according to EPA method 9045c (US EPA, 1992).

3.4.3 Temperature

As discussed in Chapter 2, temperature affects both the properties of spilled oil and the biodegradation processes. All the other measurements are also temperature-dependent. Temperature profiles in air, water, and sediment should be monitored regularly using appropriate

thermometers. Many instruments for other analyses, such as DO or pH, have built-in thermometers, and the temperature should be recorded along with the other measurements. Any temperature measurement devices should be calibrated with a National Institute of Standards and Technology (NIST)-certified thermometer before field use (Eaton *et al*., 1995).

3.4.4 Salinity

Salinity of the environment may be an important factor in oil bioremediation, particularly in estuarine environments or in marine shorelines where regional seaward groundwater flow exists (Boufadel, *et al*., 1999b; Zobell, 1973).

Salinity of pore water can be measured by either a conductivity method or density methods (e.g., Standard Methods No. 2520B or 2520 C; Eaton *et al*., 1995). The conductivity method is the most commonly used method for salinity analysis due to its high sensitivity and ease of measurement. Conductivity meters can be installed in the field to monitor salinity profiles in marine shorelines. The density method involves using a precise vibrating flow densitometer. Water salinity can also be determined rapidly in the field using this method.

3.5 Monitoring of Biological Impacts

The public has responded favourably to bioremediation since its implicit goal is that of reducing toxic effects by converting organic molecules to cell biomass and other benign materials such as carbon dioxide and water (Atlas and Cerniglia, 1995). However, concerns about the net benefit of bioremediation strategies remain. This is attributed to lingering questions regarding the potential production of toxic metabolic by-products, possible toxic components in the formulation of bioremediation agents, and the ineffective degradation of the most toxic components of residual oils (Hoff, 1991; Office of Technology Assessment, 1991).

To date, a single ideal biological method - both sensitive and efficient - for the assessment of contaminant impacts to all sediment biota has not been identified. Two separate, yet complimentary, approaches have evolved: bioassessment and bioassays. Bioassessments are field-based analyses typically characterized by assessing the impacts of the contamination and treatment activity on environmental populations such as benthic communities, intertidal flora and fauna, etc. They are characterized as having limited experimental controls (Herricks and Schaeffer, 1984). Bioassays are laboratory-based tests that incorporate rigorous experimental protocols and controls. Both toxicity tests and bioaccumulation studies are bioassays (Chapman, 1989).

3.5.1 Bioassessment

Changes in benthic community structure can be used as a means of assessing ecosystem response to contaminated sediments in aquatic ecosystems. Since most contaminants such as crude oil within the aquatic ecosystem eventually bind to sediment particles, emphasis on benthic organisms (bottom dwelling vertebrates and invertebrates) as a primary means of assessing ecosystem response is warranted. Of particular importance are the macrobenthic invertebrates (organisms retained on screens of mesh size >0.2 mm) because of their basic longevity,

sedentary lifestyles, proximity to sediments, influence on sedimentary processes, and trophic importance. Microinvertebrates such as rotifers and nematodes are of particular ecological interest; however, their taxonomy is less well known. Hence, they have not been routinely monitored in environmental assessments.

While there is a vast bioassessment database on the effects of oil spills, the effects of clean up techniques have until recently been seldom addressed. Clearly, a database on the effects of clean-up operations would have obvious potential for guidance. For example, in a follow up of the *Exxon Valdez* oil spill clean up, Driskell *et al*. (1996) noted that total abundance, species richness, species diversity, and abundance of several major taxa (polychaetes, bivalves, and gastropods) were significantly lower in hot-water-washed beaches than in unoiled beaches. Infauna at oiled sites that were not hot-water washed rebounded quickly following the disturbance. Three years after the spill, recovery of infauna at sites that were cleaned still lagged significantly behind the oiled sites. Principal component analysis (PCA), a multivariate ordination technique, was used to track site recovery trends. Negative effects were indicated by reduction in size or biomass, mortality, and reduced or failed reproductive success. Conversely, the possibility of positive impacts was also identified (e.g., when oil tolerant species bloom during the period of reduced competition-predation). Changes in epifauna and infauna were also used to assess the rates of natural recovery and the impacts of intertidal clean-up activities on the coast of Saudi Arabia following the 1991 Gulf oil spill (Watt *et al*., 1993).

Macroinvertebrate bioassessment has been limited in field trials evaluating the efficacy of bioremediation strategies due to the amount of unrestricted surface area required for sample collection. The use of bioassessments in this context will expand with the development of bioassay protocols based on bioanalytical techniques (enyzmatic measurements, as well as immunoassay and biosensor techniques) aimed at the subcellular or multicellular level of biological organization. Application of these kinds of tests should be tailored for both the field and laboratory (Lee *et al*., 1998).

Bioassessment can readily include potential impacts on vegetation. Field surveys demonstrated that the 1991 Gulf War oil spill severely damaged intertidal vegetation along the Saudi Arabian Gulf coast (Boer, 1994). Along a 45 km stretch of intertidal mangroves and salt-marshes, *Salicornia europaea* was almost extinct. Dwarf mangrove (*Avicennia marina*) and salt-marshes dominated by *Arthrocnemum macrostachyum* and *Halocnemum strobilaceum* were severely damaged. *Halopeplis perfoliata* and *Limonium axillare* salt-marshes were relatively unaffected. It was noted that natural re-establishment of the vegetation would be protracted unless active measures were taken to aid recovery.

A bioassessment of vegetative growth was recently used to document the efficacy of bioremediation strategies to enhance the rate of habitat recovery within a tidal freshwater marsh located along the St. Lawrence River, Canada (Lee *et al*., 2001). *Scirpus pungens,* the dominant plant species at the study site was found to be tolerant to the oil, and its growth was significantly enhanced above that of the unoiled control by the addition of nutrients (Figure 3.5).

The aim of oil spill remediation is to restore a site to its pre-spill condition. In this context, monitoring the recolonization of impacted areas should be a primary goal in bioassessments.

Colonization and succession describe changes in the numbers and kinds of organisms making up the community over time. They provide an integrated measure of a toxicant's effect on immigration, emigration, competition and predation. Colonization is somewhat analogous to reproduction in a single species: it reflects the ability of the community to replicate and organize itself. Fleeger *et al*. (1996) showed that unweathered *Exxon Valdez* crude oil delayed, but did not preclude, colonization by meiofauna (harpacticoids) into azoic sediment of Prince William Sound.

To date, emphasis has been placed on the characterization of impacts on the macroinvertebrate community and vegetation. Nevertheless, sediment-associated contaminants enter the non-benthic environment and community through natural processes including resuspension, desorption, ingestion of benthic organisms, ingestion of sediment, and adsorption to or uptake through membranes during sediment contact. Due to mobility and sampling issues, it is inherently much more difficult to work on pelagic organisms such as fish. Nevertheless, given the holistic nature of toxicant perturbations on aquatic ecosystems and the multifaceted interactions between the water and sediment compartments, consideration should also be given to the bioassessment of fish and other nonbenthic community organisms (e. g., bacteria, phytoplankton, cladocera, and amphibians).

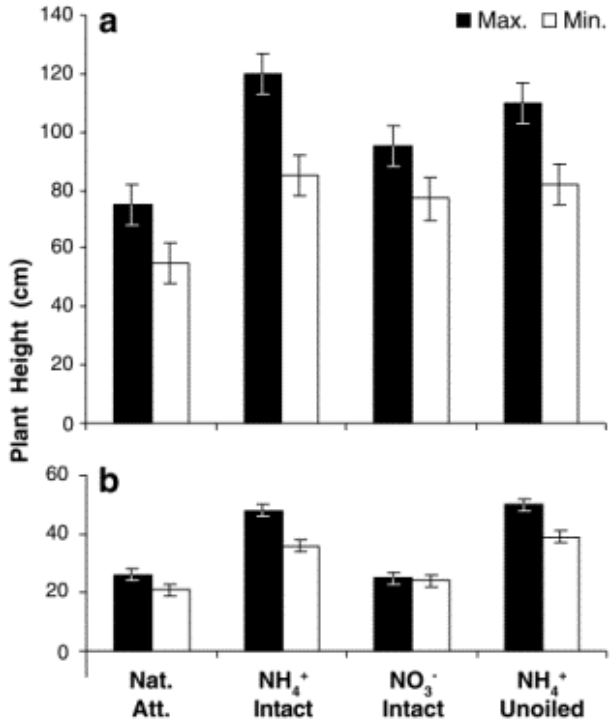


Figure 3.5 Minimum and maximum height of the (a) dominant (*Scirpus pungens)* and (b) secondary (*Eleocharis palustris)* plant species at Week15. Treatment of the oiled plots included natural attenuation (Nat. Att.); nutrient amendment with granular ammonium nitrate and super triple phosphate (NH_4^+ Intact); nutrient amendment with sodium nitrate instead of ammonium nitrate (NO_3^-). Unoiled plots were amended with granular ammonium nitrate and super triple phosphate (NH_4^+ Unoiled).

3.5.2 Bioassays

Bioassays provide a more accurate picture of ecosystem health at a contaminated site than chemical analyses because their result is an integration of the interaction that occurs between the contaminant and environmental variables. Bioassay endpoints are quantitative measures of toxicity. They compliment biological surveys, which describe communities of organisms in the field, and chemical analyses, which provide information on the nature of contaminants at a site, the magnitude of the remediation problem, and potential methods of treatment. Resource managers frequently use bioassays to identify the most toxic areas, thereby helping to prioritize sites for more thorough evaluation, including the selection of methods for chemical analyses.

Sediment toxicity tests are generally classified as "acute" or "chronic". They are usually performed on whole sediment (e.g., solid-phase), suspended sediment, sediment liquid phases (pore water, interstitial water), or sediment extracts (elutriates, solvent extracts). In general, assays using whole sediment samples are more sensitive than assays using elutriate or pore water samples. The American Society of Testing and Materials (ASTM, 1991) currently defines an acute toxicity test as a comparative study in which the organisms that are subject to different treatments are observed for a short period, usually not constituting a substantial portion of their life span. A chronic test is defined as a comparative study in which organisms that are subjected to different treatments are observed for a long period or a substantial portion of their life span. Acute tests often utilize mortality as the only measure of effect, while chronic tests usually include measures of growth, morphology, reproduction, behavioral effects, or other sublethal endpoints.

Plant and animal communities are diverse; their members differ in their sensitivity to toxicants. A single species bioassay cannot represent the range of sensitivity of all biota within an ecosystem. To improve ecological relevance, a test battery approach with species from different trophic levels is required. Accountability for the influence of natural environmental factors in sediment bioassays is assisted by the testing of reference and control samples. Reference sediment may be defined as sediment collected from the vicinity of a study site, possessing similar characteristics to the test sediment, but without anthropogenic contaminants. Sediment characteristics, such as particle size distribution and percent organic carbon of the reference sediment should simulate, as closely as possible, that of the test sediment. In some cases the reference sediment might also show toxicity due to naturally occurring chemical, physical, or biological properties. This factor can be addressed by determining the toxicity of control sediments (natural or artificially prepared sediments known to be nontoxic) and the use of positive controls (a sediment of known toxicity to the test organism under the conditions of the test).

Bioassays have been developed and used extensively since the 1960s for the screening of chemicals and regulatory compliance monitoring. Sediment bioassays have been used extensively to diagnose the effects of oil spills (Teal, *et al*., 1992; Gilfillan *et al*., 1995; Neff and Stubblefield, 1995; Randolph *et al*., 1998). Their application has now been extended to include the documentation of effects and success of oil spill countermeasures like bioremediation (Lee *et al*., 1995b; Mearns *et al*., 1995). While any living organisms can be used in theory, toxicity tests with fish and macroinvertebrates have been standardized by environmental agencies to assess the

hazards of industrial wastes to aquatic systems (Blaise *et al*., 1988). Rapid advances are now being made in the development of cost-effective high-performance micro-scale procedures involving bacterial, protozoan, microalgal, and microinvertebrate indicators (Wells *et al*., 1998). Furthermore, the high demand for simple, rapid, and practical toxicological procedures has resulted in the creation of commercial bioanalytical products such as the Microtox® Test (AZUR Environmental Inc., USA).

Major criteria to consider in the selection of species for sediment toxicity testing include: (1) their behavior in sediment (habitat, feeding habits, etc.), (2) their sensitivity to test material, (3) their ecological and/or economic relevance, (4) their geographical distribution, (5) their taxonomic relation to indigenous animals, (6) their acceptability for use in toxicity measurement (standardized test method), (7) their availability, and (8) their tolerance to natural sediment characteristics such as grain size.

The response of the test organisms to the toxicant or test sediment is often affected by its life stage. Larval or juvenile life stages are generally more sensitive than adults.

3.5.2.1 Benthic invertebrates

In terms of benthic invertebrates, amphipods are among the most sensitive of benthic species. They are among the first to disappear from benthic communities in sediments impacted by pollution (Swartz *et al*., 1982; Mearns and Word, 1982). They have been used successfully to characterize shoreline impacts following oil spill incidents (Teal *et al*. 1992; Gilfillan *et al*., 1995; Wolfe *et al*., 1996).

Gilfillan *et al*. (1995) collected mussels from several locations for tissue hydrocarbon analysis to estimate bioavailable hydrocarbon concentrations in epifaunal species. In a newer approach, studies of sediment contamination and verification of laboratory bioassays involved controlled *in situ* exposures (caged animals) to expand the level of ecological relevance. In this case, oysters were used during a shoreline bioremediation experiment in Delaware Bay to document the loss of oil from the study area and to determine how the overall oiling may have impacted offshore resources (Mearns *et al*., 1997).

3.5.2.2 Microtox

Simple, sensitive, rapid, cost-effective, reproducible, and practical methods are needed for the screening of toxic impacts during oil spill response operations. The Microtox® Test, a commercial bioassay accepted by regulatory agencies, is based on the measurement of changes in light emission by a nonpathogenic, bioluminescent marine bacterium (*Vibrio fisheri*) upon exposure to test samples. The test has been used extensively worldwide over the last 18 years for toxicity screening of chemicals, effluents, water and sediment, and for contamination surveys and environmental risk assessment. Variations of this test have been applied to time-series monitoring of sediment and water toxicity. Ho and Quinn (1993) identified strong rank correlations between the Microtox response and polycyclic aromatic fractions of organic extracts of sediments. Its application for monitoring the efficacy of oil spill remediation methods has

been proven (Lee *et al.*, 1995b; 1997b). Mueller *et al*. (1999) quantified the effectiveness of intrinsic recovery within an oiled wetland by monitoring the rate of acute toxicity reduction using the Microtox 100% Test on a water extractable phase. The observed decrease in toxicity followed a pattern similar to the decrease seen in petroleum concentrations by GC/MS total target analyte measurements.

3.5.2.3 Fish

Due to their economic, recreational, and aesthetic value, fish have been historically selected as a primary bioassay organism. Difficulties in using fish as biomonitors of sediment contamination arise from their preference for particular sediments or habitats and their residence time in or over contaminated areas. Furthermore, their absence in a water body may more directly reflect water quality.

Biochemical and physiological alterations, if severe enough or protracted, can lead to structural alterations in organelles, cells, tissue, and organs. Detection of specific alterations using anatomical and cytological endpoints may indicate both prior and current exposure to chemical contaminants, so histopathology has been instrumental in assessing the toxicological impact of contaminated sediments. The documentation of neoplasms in fish and other aquatic organisms was perhaps the first use of histopathological indices in ecotoxicology.

Biomarkers (as distinguished from the oil biomarkers such as hopane discussed earlier) are used by resource managers as a means to identify a toxicological response from fish populations. Biomarkers can be defined as biochemical, physiological, or pathological responses measured in individual organisms on exposure to environmental contaminants, and which also provide information concerning sublethal effects arising from such exposures. The family of enzymes referred to as cytochrome P-450s (or P450) act on the functional groups of lipophilic substrates in a process referred to as mixed function oxidase (MFO) or monooxygenase reactions (Ortiz de Montellano, 1986). MFO reactions are induced by polycyclic aromatic hydrocarbons (PAHs) and a variety of halogenated hydrocarbons (notably certain chlorinated biphenyls, dibenzofurans, and dibenzodioxins). The enzyme system is sensitive to these contaminants at levels encountered in the environment. In fish, the most widely employed and readily performed techniques are measurements of enzyme activities, particularly aryl hydrocarbon hydroxylase (AAH) and ethoxyresorufin O-deethylase (EROD), that are highly associated with the P450 1A proteins.

Hodson *et al*. (2001) has monitored changes in the bioavailability and toxicity of oil-derived PAH to early life stages of fish in a field trial to evaluate the effectiveness of wetland bioremediation and phytoremediation strategies. For over 1.5 years, sediments from experimental plots were tested by bioassays of MFO (CYP1A) enzyme activity in livers of trout as an index of PAH exposure. Oil alone, oil mixed with sediments in the lab, and oiled sediments from the experimental plots all caused CYP1A induction relative to unoiled controls, indicating the presence and bioavailability of PAH. Induction did not vary markedly among treatments, but declined slowly with time. Concomitant chemical analysis suggested that PAHs were depleted primarily by weathering or sediment dispersion rather than by bioremediation

treatments. Sediments were also chronically toxic to developing stages of trout and medaka (*Oryzias latipes*), causing increased rates of deformities and mortality.

3.5.3 Application of bioassays to assess bioremediation in marine environments

Bioassays were used to document the effectiveness of shoreline bioremediation in accelerating toxicity reduction of a sandy shoreline at Fowler Beach, Delaware, USA, that had been oiled with weathered Nigerian Bonny light crude (Mearns *et al*., 1995). The bioassay suite included two solid phase (Amphipod Survival, Microtox Solid-Phase) and three pore water tests (Grass Shrimp Embryo Survival/Growth, Microtox, Sea Urchin Fertilization). Treatment with nutrients (sodium nitrate and sodium tripolyphosphate) or nutrients and oil degrading bacteria (isolated from the study site) did not accelerate toxicity reduction. However, results of the high-frequency test based on the hatching success of grass shrimp embryo suggested there may have been a substantial delay in pore water toxicity reduction due to the addition of the nutrients themselves during the first few weeks. The Sea Urchin Fertilization Test was least sensitive. The most sensitive tests were the 10-day amphipod and grass shrimp embryo bioassays.

Bioremediation by nutrient enrichment was investigated as a method of treating a mixture of Forties Crude Oil and Heavy Crude Oil stranded on Bullwell Bay, Milford Haven, UK, after the grounding of the Sea Empress in 1996 (Swannell *et al*., 1999b). Experimental results showed that the oil was significantly more biodegraded after two months as a result of application of the fertilizer. Based on the results of a bioassay that involved monitoring the development of oyster embryos, and the results of the Microtox Organic Solvent Basic Test, there was no evidence of detrimental effects associated with the bioremediation treatments.

To date, detrimental effects from nutrient enrichment have not been observed following actual field operations (Prince, 1993; Mearns *et al*., 1997) although the possibility of a future incident still exists. As an example, oxygen depletion and production of ammonia from excessive applications of a fish-bone meal fertilizer during one field experiment caused detrimental effects that included toxicity and the suppression of oil degradation rates (Lee *et al*., 1995b). Furthermore, in a subsequent bioremediation field trial it was reported that a commercial bioremediation product suppressed the rates of toxicity reduction as it increased the retention of residual oil within the sediments (Lee *et al*., 1997b). For safety assurance, future operational guidelines must include ecotoxicological monitoring protocols.

Chapter 4 TYPES OF AMENDMENTS AND CONSIDERATIONS IN THEIR APPLICATION

The success of oil spill bioremediation depends on our ability to optimize various physical, chemical and biological conditions in the contaminated environment. Existing amendments for enhancing oil biodegradation in marine shorelines and freshwater wetlands include addition of nutrients, addition of microbial cultures or enzymes, phytoremediation, and oxygen enhancement.

4.1 Nutrient Amendment

As reviewed in Chapter 2, nutrient addition has been proven to be an effective strategy to enhance oil biodegradation in various marine shorelines. Theoretically, approximately 150 mg of nitrogen and 30 mg phosphorus are consumed in the conversion of 1 g of hydrocarbon to cell material (Rosenberg and Ron, 1996). Therefore, a commonly used strategy has been to add nutrients at concentrations that approaches a stoichiometric ratio of C:N:P of 100:5:1. Recently, the potential application of resource-ratio theory in hydrocarbon biodegradation was discussed (Head and Swannell, 1999; Smith *et al*., 1998). This theory suggests that manipulating the N:P ratio may result in the enrichment of different microbial populations, and the optimal N:P ratio can be different for degradation of different compounds (such as hydrocarbons mixed in with other biogenic compounds in soil). However, the practical use of these ratio-based theories remains a challenge. Particularly, in marine shorelines, maintaining a certain nutrient ratio is impossible because of the dynamic washout of nutrients resulting from the action of tides and waves. A more practical approach is to maintain the concentrations of the limiting nutrient or nutrients within the pore water at an optimal range (Bragg *et al*., 1994; Venosa *et al*., 1996). Commonly used nutrients include water-soluble nutrients, solid slow-release nutrients, and oleophilic fertilizers. Each type of nutrient has its advantages and limitations. General characteristics of these nutrients and important factors affecting their persistence in the field, such as waves and tides, and physical intrusion effects, will be discussed in this section and summarized in Table 4.1. More practical issues such as nutrient application strategies will be discussed in Chapter 5.

4.1.1 Water-soluble nutrients

Commonly used water-soluble nutrient products include mineral nutrient salts (e.g. KNO_3, $NaNO_3$, NH_3NO_3, K_2HPO_4, $MgNH_4PO_4$), and many commercial inorganic fertilizers (e.g. the 23:2 N:P garden fertilizer used in *Exxon Valdez* case). They are usually applied in the field through the spraying of nutrient solutions or spreading of dry granules. This approach has been effective in enhancing oil biodegradation in many field trials (Swannell *et al*., 1996; Venosa *et al*., 1996). Compared to other types of nutrients, water-soluble nutrients are more readily available and easier to manipulate to maintain target nutrient concentrations in interstitial pore water. Another advantage of this type of nutrient over organic fertilizers is that the use of inorganic nutrients eliminates the possible competition of carbon sources. The field study by Lee *et al.* (1995a) indicated that although organic fertilizers had a greater effect on total heterotrophic microbial growth and activity, the inorganic nutrients were much more effective in stimulating crude oil degradation.

However, water-soluble nutrients also have several potential disadvantages. First, they are more likely to be washed away by the actions of tides and waves because of their water-solubility. The field study in Maine demonstrated that water-soluble nutrients can be washed out within a single tidal cycle in high-energy beaches (Wrenn, 2000, see section 2.6.2). Second, inorganic nutrients, ammonia in particular, should be added carefully to avoid reaching toxic levels. Existing field trials, however, have not observed acute toxicity to sensitive species resulting from the addition of excess water-soluble nutrients (Mearns *et al*., 1997; *Prince et al*., 1994). Third, water-soluble nutrients may have to be added more frequently than slow release nutrients or organic nutrients, resulting in more labor-intensive, costly, and physical intrusive applications.

4.1.2 Granular nutrients (slow-release)

Many attempts have been made to design nutrient delivery systems that overcome the washout problems characteristic of intertidal environments (Prince, 1993). Use of slow release fertilizers is one of the approaches used to provide continuous sources of nutrients to oil contaminated areas. Slow release fertilizers are normally in solid forms that consist of inorganic nutrients coated with hydrophobic materials like paraffin or vegetable oils. This approach may also cost less than adding water-soluble nutrients due to less frequent applications. Slow release fertilizers have shown some promises from oil bioremediation studies and applications. For example, Olivieri *et al*.(1976) found that the biodegradation of a crude oil was considerably enhanced by addition of a paraffin coated $MgNH_4PO_4$. Another slow-release fertilizer, Customblen (vegetable oil coated calcium phosphate, ammonium phosphate, and ammonium nitrate), performed well on some of the shorelines of Prince William Sound, particularly in combination with an oleophilic fertilizer (Atlas, 1995a; Pritchard *et al*., 1992; Swannell *et al*., 1996). Lee *et al*. (1993) also showed that oil biodegradation rates increased with the use of a slow release fertilizer (sulfur-coated urea) compared to water-soluble fertilizers.

However, the major challenge for this technology is control of the release rates so that optimal nutrient concentrations can be maintained in the pore water over long time periods. For example, if the nutrients are released too quickly, they will be subject to rapid washout and will not act as a long-term source. On the other hand, if they are released too slowly, the concentration will never build up to a level that is sufficient to support rapid biodegradation rates, and the resulting stimulation will be less effective than it could be. The field trials on of the shorelines of Prince William Sound showed that on certain beaches, Customblen granules were apparently washed away before any significant enhancement of bioremediation was recorded (*Swannell et al*., 1996). Several recent studies have shown that a slow release nutrient (Max Bac, a product similar to Customblen) failed to demonstrate enhancement of oil degradation because the nutrient release rate was too slow to affect oil biodegradation (Croft *et al*., 1995; Sveum and Ramstad, 1995).

4.1.3 Oleophilic nutrients

Another approach to overcome the problem of water-soluble nutrients being rapidly washed out was to utilize oleophilic organic nutrients (Atlas and Bartha, 1973; Ladousse and Tramier, 1991). The rationale for this strategy is that oil biodegradation mainly occurred at the oil-water

interface; since oleophilic fertilizers are able to adhere to oil and provide nutrients at the oil-water interface, enhanced biodegradation should result without the need to increase nutrient concentrations in the bulk pore water. A well-known oleophilic fertilizer is Inipol EAP 22, a microemulsion containing urea as a nitrogen source, lauryl phosphate (the phosphorus source), 2-butoxy-1-ethanol as a surfactant, and oleic acid to give the material its hydrophobicity. This fertilizer has been subjected to extensive studies under various shoreline conditions and was successfully used in oil bioremediation on of the shorelines of Prince William Sound. Other oleophilic organic fertilizers include polymerized urea and formaldehyde, and some organic fertilizers derived from natural products such as fishmeal (Lee *et al.,* 1995a; Rosenberg *et al*., 1992; Sveum and Ramstad, 1995).

Table 4.1 Major nutrient types used in oil bioremediation

Type of nutrients	Advantages	Disadvantages	Applications in the field or field trials
Water soluble	Readily available Easy to manipulate for target nutrient concentrations No complicated effect of organic matter	Rapidly washed out by wave and tide Labor-intensive, and physical intrusive applications Potential toxic effect	Alaska (Pritchard *et al.*, 1992) Delaware (Venosa *et al.*, 1996)
Slow release	Provide continuous sources of nutrients and may be more cost effective than other types of nutrients	Maintaining optimal nutrient release rates could be a challenge	Alaska (Pritchard *et al.*, 1992) Nova Scotia (Lee *et al*., 1993)
Oleophilic	Able to adhere to oil and provide nutrients at the oil-water interface	Expensive Effectiveness is variable Containing organic carbon, which may compete with oil degradation and result in undesirable anoxic conditions	Alaska (Pritchard *et al.*, 1992) Nova Scotia (Lee *et al*., 1987, 1989,1995a &b)

The effectiveness of oleophilic fertilizers also depends on the characteristics of the contaminated environment such as action of wave and tide, and sediment types. Based on several earlier studies, Sveum *et al.* (1994) indicated that oleophilic fertilizers proved to be more effective than water-soluble fertilizers when the spilled oil resided in the intertidal zone. But they have no advantages in enhancing oil biodegradation in the supralittoral zone where water transport is limited. Inipol EAP 22 was found to be more effective in coarse sediments than in fine sediments due to the difficulty in penetration for the oleophilic fertilizer in fine sediments (Sveum and

Ladousse,1989). Variable results have also been produced regarding the persistence of oleophilic fertilizers. Some studies showed that Inipol EAP 22 can persist in a sandy beach for a long time under simulated tide and wave actions (Santas and Santas, 2000; Swannell *et al*. 1995). Others found that Inipol EAP22 was rapidly washed out before becoming available to hydrocarbon-degrading bacteria (Lee and Levy, 1987; Safferman, 1991). Another disadvantage with oleophilic fertilizers is that they contain organic carbon which may be biodegraded by microorganisms in preference to petroleum hydrocarbons (Lee *et al*., 1995a; Swannell *et al*., 1996), and may also result in undesirable anoxic conditions (Lee *et al*., 1995b; Sveum and Ramstad, 1995).

In summary, the effectiveness of these various types of nutrients will depend on the characteristics of the contaminated environment. Slow-release fertilizers may be ideal nutrient sources if the nutrient release rates can be well controlled. Water-soluble fertilizers are likely more cost-effective in low-energy and fine-grained shorelines where water transport is limited. And oleophilic fertilizers may be more suitable for use in high-energy and coarse-grained beaches. However, successful application of bioremediation products will always require appropriate testing and evaluation based on the specific conditions of each contaminated site.

4.2 Microbial Amendments

Addition of oil-degrading microorganisms or bioaugmentation has been proposed as a bioremediation strategy. The rationale for this approach includes the contention that indigenous microbial populations may not be capable of degrading the wide range of substrates that are present in complex mixtures such as petroleum and that seeding may reduce the lag period before bioremediation begins (Forsyth *et al*., 1995; Leahy and Colwell, 1990). For this approach to be successful in the field, the seed microorganisms must be able to degrade most petroleum components, maintain genetic stability and viability during storage, survive in foreign and hostile environments, effectively compete with indigenous microorganisms, and move through the pores of the sediment to the contaminants (Atlas, 1977; Goldstein *et al*., 1985).

There are many vendors of bioremediation products, who claim their product (most of them are microbial agents) aids the oil biodegradation process. The U.S. EPA has compiled a list of bioremediation agents (USEPA, 2000) as part of the National Oil and Hazardous Substances Pollution Contingency Plan (NCP) Product Schedule, which is required by the Clean Water Act, the Oil Pollution Act of 1990, and the National Contingency Plan. A current list of bioremediation agents in NCP schedule is shown in Table 4.2. A product can be listed only when its safety and effectiveness have been demonstrated under the conditions of a test protocol developed by EPA (NETAC, 1993). However, listing does not mean that the product is recommended or certified for use on an oil spill (USEPA, 2000). The efficacy test protocol uses laboratory shake flasks to compare the degradation of artificially-weathered crude oil in natural seawater with and without a bioremediation product. Similar test protocols for freshwater conditions were recently proposed (Haines *et al*., 1999).

Table 4.2 Bioremediation agents in NCP product schedule (Adapted from USEPA, 2000)

Type	Name or Trademark	Manufacture
Biological additives (Microbial Culture or Enzyme additives)	BET BIOPETRO	BioEnviro Tech, Tomball, TX
	BIOGEE HC	RMC Bioremediation, Shreveport, LA
	BR (formerly ENVIRO-ZYME BR)	Enviro-Zyme, Inc., Stormville, NY
	ENZYT (LIQUID/CRYSTA)	Acorn Biotechnical Corporation Houston, TX
	MICRO-BLAZE	Verde Environmental, Inc., Houston, TX
	OPPENHEIMER FORMULA	Oppenheimer Biotechnology, Inc. Austin, TX
	PRISTINE SEA II	Marine Systems, Baton Rouge, LA
	PRP (Petroleum Remediation Product)	Petrol Rem, Inc., Pittsburgh, PA
	STEP ONE	B & S Research, Inc. Embarrass, MN
	SYSTEM E.T. 20	Quantum Environmental Technologies, Inc. (QET), La Jolla, CA
	WMI-2000	Waste Microbes, Inc., Houston, TX
Nutrient additives	INIPOL EAP 22 (oleophilic)	Societe, CECA S.A. France
	LAND AND SEA RESTORATION	Land and Sea Restoration LLC, San Antonio, TX
	OIL SPILL EATER II	Oil Spill Eater International, Corporation Dallas, TX
	VB591^{TM}WATER, VB997^{TM}SOIL, AND BINUTRIX (partially encapsulated & oleophilic)	BioNutraTech, Inc., Houston, TX

As reviewed in Chapter 2, however, even though the addition of microorganisms may be able to enhance oil biodegradation in the laboratory, its effectiveness has not been convincingly demonstrated in the field. Actually, most field studies indicated that bioaugmentation is not effective in enhancing oil biodegradation in marine shorelines, and nutrient addition or biostimulation alone had a greater effect on oil biodegradation than the microbial seeding (Jobson *et al*., 1974; Lee and Levy, 1987; Lee *et al*., 1997b, Venosa *et al*., 1996). The failure of bioaugmentation in the field may be attributed to the fact that the carrying capacity of most environments is likely determined by factors that are not affected by an exogenous source of microorganisms (such as predation by protozoans, the oil surface area, or scouring of attached biomass by wave activity), and that added bacteria seem to compete poorly with the indigenous population (Tagger *et al*., 1983; Lee and Levy, 1989; Venosa *et al*., 1992). Therefore, it is unlikely that externally added microorganisms will persist in a contaminated beach even when they are added in high numbers. In short, those criteria mentioned above for a successful colonization are very difficult to be met in the field.

Fortunately, oil-degrading microorganisms are ubiquitous in the environment, and they can increase by many orders of magnitude after being exposed to crude oil (Atlas, 1981; Lee and Levy, 1987, Pritchard and Costa, 1991). Therefore, in most environments, there is usually no need to add hydrocarbon degraders. In certain circumstances that have not been well defined, when the indigenous bacteria are incapable of degrading one or more important contaminants, addition of microbial inocula may be considered. Genetically engineered organisms are not likely to be used in the near or even distant future.

4.3 Plant Amendments (phytoremediation)

Phytoremediation has been defined as the use of green plants and their associated microorganisms to degrade, contain, or render harmless environmental contaminants (Cunningham *et al.*, 1996). This technique is emerging as a potentially cost-effective option for clean-up of soils contaminated with petroleum hydrocarbons (Frick *et al.*, 1999). As summarized by Macek *et al*. (2000), the main advantages of phytoremediation include less disruption to the environment, potential to treat a diverse range of contaminants, and high probability of public acceptance. Major concerns regarding this technology include dissolution and migration of contaminants, limitation by the toxicity of the contaminated environments, and it being a relatively slow process. Phytoremediation has been studied in a freshwater environment in Quebec, Canada (Garcia-Blanco *et al.,* 2000; Venosa *et al.,* 2002 (submitted)). These researchers found that addition of nutrients did not result in enhancement of biodegradation of crude oil contaminating the plots, whether or not plants were left intact or removed. It appeared that in a wetland environment, oxygen became limiting at depths within a few mm from the surface.

4.3.1 Mechanisms of phytoremediation

Phytoremediation of petroleum hydrocarbons generally involves three major mechanisms: (1) degradation, (2) containment and (3) the transfer of contaminants from soil to the atmosphere (Cunningham *et al.*, 1996; Frick *et al.*, 1999).

Degradation can be accomplished by both plants and their associated microorganisms. One of the most important processes involved in the degradation is the interaction between plants and microorganisms in the rhizosphere (root zone). Plants can stimulate the growth and metabolism of soil microorganisms by providing root exudates of carbon, enzymes, nutrients, and oxygen, which can result in more than 100-fold increase in microbial counts (Macek *et al*., 2000). This process is also mutual beneficial. The microbes can reduce the phytotoxicity of contaminants so that plants can grow in adverse soil conditions. Cometabolism may also play an important role in phytodegradation. Ferro *et al.* (1997) suggested that plant exudates might have served as co-metabolites in enhancing the biodegradation of pyrene in the rhizosphere.

Other major mechanisms of phytoremediation include containment of petroleum hydrocarbons and their transfer from the soil to the atmosphere. Containment involves the accumulation of contaminants within the plants, adsorption of contaminants onto roots, and binding of contaminants in the rhizosphere through enzymatic activities (Cunningham *et al.*, 1996; Frick *et al.*, 1999). Plants can also transport volatile petroleum hydrocarbons to the atmosphere through leaves and stems. However, these effects are less important than the degradation mechanism in phytoremediation of petroleum hydrocarbons (Ferro *et al.* 1997).

4.3.2 Considerations in application of oil phytoremediation

While phytoremediation had been used successfully within the terrestrial environment to decontaminate soils (Banks and Schwab, 1993; Schnoor *et al.*, 1995), the technique has not been employed as an operational oil spill countermeasure. Until recently, only limited research had been carried out on the effectiveness of phytoremediation in freshwater wetlands (Lin and Mendelssohn, 1998). Most of the studies were greenhouse experiments rather than field studies. Like bioaugmentation, studies on phytoremediation of petroleum hydrocarbons have produced mixed results. The effectiveness of phytoremediation is site-specific, which can be affected by factors as oil properties, types of plants, and environmental conditions.

Oil concentrations

Plants can tolerate oils with certain concentration ranges. When oil concentrations are too high, toxic effects will lead to growth inhibition or death of plants. When oil concentrations are too low, phytoremediation will not be effective either due to poor bioavailability. Longpre *et al.* (1999) investigated the impact of oil concentrations on a freshwater wetland plant (*Scirpus pungens)* along the shore of the St. Lawrence River. The results showed that the plant growth was stimulated in the presence of crude oil at a concentration less than 4.56g/Kg sediment when compared to the growth of the control plants. At higher oil concentrations, up to 27.4g/kg sediment, growth inhibition or no growth increase was observed. When the oil concentration was above 36.4 g/kg sediment, plant growth was significantly reduced. The study concluded that the plants were likely to survive and grow in sediments contaminated with crude oil in a range of concentrations comparable to oil spill incidents.

Plant species

Another important factor in considering phytoremediation is establishment of appropriate plants. Lin and Mendelssohn (1996) studied the effect of oil spills on four freshwater marsh plant species. Two of them (*C. ordoratus*, and *A. teres*) failed to survive in any of the oiled sods and *E.*

quadrangulata could only persist at oil levels up to 8 L/m^2. In contrast, the growth of *Sagittaria lancifolia* was enhanced in response of oil addition up to 24 L/m^2. Generally, legume and grass species have been the choices for their potential use in phytoremediation of petroleum hydrocarbons (Frick *et al.*, 1999). Legumes are nitrogen-fixing plants, which may have advantages in competing with non-legume species in oil-contaminated sediments. Native plants should normally be selected since they have better chance to survive or out-compete non-indigenous inocula (Cunningham et al., 1996).

Environmental factors

Similar to the environmental factors affecting microbial biodegradation discussed in chapter 2, major environmental factors affecting phytoremediation of petroleum hydrocarbons include soil types, nutrients, oxygen and temperature. Detailed description can be seen in Cunningham *et al.*, 1996 and Frick *et al.*, 1999.

4.3.3 Applications in marine shoreline and freshwater wetlands

Current applications of phytoremediation in marine shorelines and freshwater wetlands have been limited to accelerate recovery and restoration of oiled wetland. For example, mangroves were successfully replanted to restore oil-killed mangrove forest in Panama after the 1986 *Refineria Panama* oil spill (Teas *et al*., 1989). Only a few field studies have been carried out on the effectiveness of phytoremediation in enhancing oil degradation in marine shorelines and freshwater wetlands.

Lin and Mendelssohn (1998) investigated the effects of biostimulation and phytoremediation in enhancing habitat restoration and oil degradation in a coastal wetland environment (greenhouse study). They found that application of fertilizer in conjunction with the presence of transplants led to much higher oil degradation rates than phytoremediation alone. The results were attributed to a higher microbial number and activity induced by the fertilizer. However, it was still not clear whether this effect was due to biostimulation of soil microorganisms or due to phytoremediation via fertilizer-increased plant biomass. These confounding effects perhaps could have been distinguished by adding one more treatment (biostimulation with absence of transplants).

In 1999 and 2000, a major research study was conducted on the shoreline of the St. Lawrence River (Garcia-Blanco *et al.*, 2000; Venosa *et al.*, 2002 (submitted)). The experimental design was similar to the one used on the marine shoreline in Delaware Bay (Venosa *et al.*, 1996). There were 5 treatments: a no oil control and four oiled treatments. The oiled treatments included a natural attenuation control plot with no amendments, a plot receiving ammonium nitrate and orthophosphate nutrients but with the wetland plants continually cut back to ground surface to suppress photosynthetic activity and growth, a plot receiving the same nutrients as Treatment B but with the plants left intact, and a plot similar to Treatment C but with only nitrate (no ammonium) serving as the nitrogen source. The no-oil control also received the same nutrients as the oiled treatments receiving nutrients. Findings are summarized as follows: (1) alkane degraders increased only marginally in all treatments while the PAH degraders were stimulated to increase by 3.5 orders of magnitude in response to exposure to crude oil; (2) nitrogen in the form of ammonium was partly adsorbed to negatively charged soil particles, partly taken up by the root system of the wetland plants, and partly leached into the pore water. Nitrogen in the

form of nitrate leached into the pore water, and some was taken up in the root system (Lee *et al*., 2002 (submitted); (3) the primary mechanism of oil mass loss from all the plots, regardless of treatment, was physical rather than biological; (4) with respect to biodegradation of total alkanes and PAHs during the first 21 weeks of the investigation as measured by GC/MS analysis, only about 35% biodegradation occurred in all treatments on average, and no significant differences among any of the treatments were observed ($p > 0.05$); (5) a substantial increase in plant biomass was observed due to fertilizer addition; (6) better biodegradation occurred in surface samples in plots where the plants had been removed than in any of the core samples because of the oxic nature of the surface and the lack of competition for nutrients by the plant species. Enhanced oxygen transfer to the rhizosphere by the plants through their roots did not appear to take place, at least at the level needed by hydrocarbon degraders to metabolize the oil rapidly.

The major reason for the lack of biodegradation beyond only about 35% was ascribed to the fact that the oil had been raked into the top 2-3 cm of sediment to make sure that penetration had occurred. When such oil penetration occurs, little oxygen is available to allow significant biodegradation to take place throughout the oiled zone. If oil contaminates only the surface where more aerobic conditions exist, and if it does not penetrate deeply into the subsurface, better biodegradation should take place, at least theoretically. The major conclusion reached from this study was that bioremediation of an oil-contaminated freshwater wetland *where significant penetration of oil has taken place into the sediment* has limited potential for enhanced cleanup of the contamination.

In summary, the effectiveness of phytoremediation in enhancing oil degradation in freshwater wetlands is highly site-specific and does not promise to be an effective oil cleanup technique. However, it does show promise in accelerating the recovery and restoration of wetland environments contaminated with oil and oil products.

4.4. Oxygen Amendment

Oxygen usually is not a limiting factor on many sandy beaches. However, oxygen limitation may occur in wetlands and fine-grained shorelines as indicated by some field studies (Garcia-Blanco *et al.*, 2001b, Lee and Levy, 1991, Purandare, 1999). Under such circumstances, oxygen amendment may be considered as a bioremediation strategy. Although oxygen supply has been widely used for bioremediation of oil contaminated soils and groundwater, such as at many subsurface fuel contaminated sites, this strategy has not been applied to enhance oil degradation in marine shoreline and freshwater wetlands. This is because oxygen amendment usually involves expensive and environmentally intrusive operations. For bioremediation of a large-scale oil spill, use of this approach is probably not practical even when oxygen is a limiting factor. However, under certain circumstance that involves high oil contamination in smaller scale and in less sensitive habitats, oxygen amendment can still be considered as an alternative for oil bioremediation. Commonly used oxygen supply techniques include tilling, forced aeration, and chemical methods (Atlas, 1991; Brown and Crosbie, 1994; Riser-Roberts, 1998). These methods are summarized below. They are mostly based on studies and practices in soil environments. Special attention will be given to their potentials of application in marine shorelines and freshwater wetlands.

4.4.1 Tilling

Tilling has been a conventional physical method to accelerate natural oil removal by exposing oiled sediments to a higher level of physical abrasion and biochemical degradation (Owens, 1998). This technique is also an effective means of aeration for surface layer of sediments. It has been successfully used to accelerate biodegradation in landfarming (Atlas, 1991; Jerger *et al*., 1994). Traditional tilling machines, such as disk harrows and rototillers, can aerate surface soils to a depth of 6 to 24 inches. Sediments deeper than about 2 ft (60 cm) can be aerated by using construction equipment, such as a backhoe (Riser-Roberts, 1998).

Currently, tilling has been recommended as a physical method to accelerate natural weathering processes of oil in sandy or coarse-sediment beaches (Owens, 1998). The main purpose of this practice is to increase physical abrasion of oils rather than to enhance aeration since oxygen is usually not a limiting factor in these environments. However, based on existing experiences in landfarming, this technique may have some potential in enhancing oil biodegradation in some fine-sediment beaches where oxygen is limited. Tilling is also considered a low-cost technology among the available aeration methods (Jerger *et al*., 1994).

Major concerns regarding this technique include disturbance of both the natural shape of shorelines and local habitats and the potential of releasing of oil and oiled sediment into adjacent locations. The experience from the St. Lawrence River field trial also suggests that the tilling of surface soil may cause oil penetration deep into the shoreline sediments and may reduce the overall oil biodegradation rates if the oil penetrates into anaerobic sediments (Garcia-Blanco *et al*., 2001; Venosa *et al.*, 2002 (submitted); see Section 5.5.2).

4.4.2 Forced aeration

Forced aeration techniques, including injection of aerated water, air and pure oxygen, are expensive methods and commonly used for enhancing bioremediation in subsurface sediments and groundwater contaminated with petroleum hydrocarbons (Brown and Crosbie, 1994; Riser-Roberts, 1998). Oil contamination of coastal and wetland environments, however, usually occurs near the surface, especially when the contamination is the result of an offshore spill. Furthermore, crude oil rarely penetrates coastal sediments to depths of greater than one foot (Gundlach, 1987). Therefore, these techniques of subsurface aeration are probably not appropriate for use in bioremediation of oil spill in marine shorelines and freshwater wetlands.

4.4.3 Chemical methods

Chemical methods involve addition of alternative oxygen sources such as hydrogen peroxide (H_2O_2), or alternative electron acceptors such as nitrate. Hydrogen peroxide can provide oxygen at a rate up to two orders of magnitude faster than the forced aeration methods (Brown and Norris, 1994). It also requires less equipment and capital cost. However, problems including too rapid decomposition, gas blockage, and inefficient use were encountered in some sites when using H_2O_2 (Brown and Norris, 1994). The chemical also can be toxic to microorganisms at high concentrations (Riser-Roberts, 1998).

Nitrate has received most attention as an alternative electron acceptor because it is relatively inexpensive, very soluble in water, and does not decompose. Since nitrate is also commonly used nutrient source for oil biostimulation, addition of nitrate may be a promising option for oil bioremediation under oxygen limiting conditions. A potential disadvantage of this method is that nitrate may be effective for degradation of fewer classes of compounds than oxygen. It has been reported that nitrate-utilizing bacteria can degrade many aromatics but do not degrade aliphatic compounds and benzene under denitrifying conditions (Brown *et al.*, 1993). However, others recently found that degradation of alkanes could take place under denitrifying conditions (Hess *et al*., 1996). Sulfate is another potentially useful electron acceptor especially in certain marine environments, such as salt marshes, where sulfate reduction is one of most important natural processes (Mitsch and Gosselink, 2000). Some laboratory studies have shown that PAHs and alkanes can be degraded under sulfate-reducing conditions at similar rates to those under aerobic conditions in some marine sediment (Caldwell *et al.*, 1998; Coates *et al*., 1997). However, these high oil degradation rates under sulfate-reducing conditions have not been reported or demonstrated in the field.

In summary, the potential of using oxygen amendment for enhancing oil biodegradation in marine shorelines and freshwater wetlands is limited. Tilling may be considered as an aeration strategy for enhancing oil biodegradation in the upper layer of sediments in less sensitive habitats. Nitrate could be a potential alternative electron acceptor for use in a wide range of environments, but its use as an effective enhancer of biodegradation is questionable. More studies, particularly field trials, are still required to evaluate the effectiveness of these strategies.

Chapter 5 GUIDELINES FOR BIOREMEDIATION OF MARINE SHORELINES AND FRESHWATER WETLANDS: DECISION-MAKING AND PLANNING

Existing research and applications have demonstrated that bioremediation is an effective technology that can be used to treat certain oil-contaminated environments. Typically, it is used as a polishing step after conventional mechanical cleanup options have been applied, although it could also be used as a primary response strategy if the spilled oil does not exist as free product and if the contaminated area is remote enough not to require immediate cleanup or not accessible by mechanical tools. However, one of the major challenges in the application of oil bioremediation is lack of guidelines regarding the selection and use of this technology. Although extensive research has been conducted on oil bioremediation in the last decade, most existing studies have been concentrated on either evaluating the feasibility of bioremediation for dealing with oil contamination or testing favored products and methods (Mearns, 1997). Only a few limited operational guidelines for bioremediation in marine shorelines have been proposed (Lee, 1995; Lee and Merlin, 1999; Swannell *et al*., 1996). The following two chapters will present a more detailed and workable guidance for use by spill responders for the bioremediation of marine shorelines and freshwater wetlands based on recent field studies and current understandings on bioremediation processes.

As a result of recent field studies (Lee *et al*, 1997b; Venosa *et al*., 1996), we now know that there is usually little need to add hydrocarbon degrading microorganisms because this approach has been shown not to enhance oil degradation more than simple nutrient addition. Therefore, this document will only present guidelines for oil bioremediation using biostimulation strategies, mainly nutrient addition.

A general procedure or plan for the selection and application of bioremediation technology is illustrated in Figure 5.1. The major steps in a bioremediation selection and response plan include:

1. Pre-treatment assessment – This step involves the evaluation of whether bioremediation is a viable option based on the type of oil that has been spilled, its concentration, the presence of hydrocarbon-degrading microorganisms, concentrations of background nutrients, the type of shoreline that has been impacted, and other environmental factors (pH, temperature, presence of oxygen, remoteness of the site, accessibility of the site and logistics, etc.).

2. Design of treatment and monitoring plan – After the decision is made to use bioremediation, further assessments and planning are needed prior to the application. This involves selection of the rate-limiting treatment agents (e.g., nutrients), determination of application strategies for the rate-limiting agents, and design of sampling and monitoring plans.

3. Assessment and termination of treatment – After the treatment is implemented according to the plan, assessment of treatment efficacy and determination of appropriate treatment endpoints are performed based on chemical, toxicological, and ecological analysis.

This chapter covers operational guidelines for decision-making in the use of bioremediation and describes the planning process for bioremediating marine shorelines and freshwater wetlands.

The next chapter (Chapter 6) will present guidelines for assessment of field results and establishment of appropriate treatment endpoints.

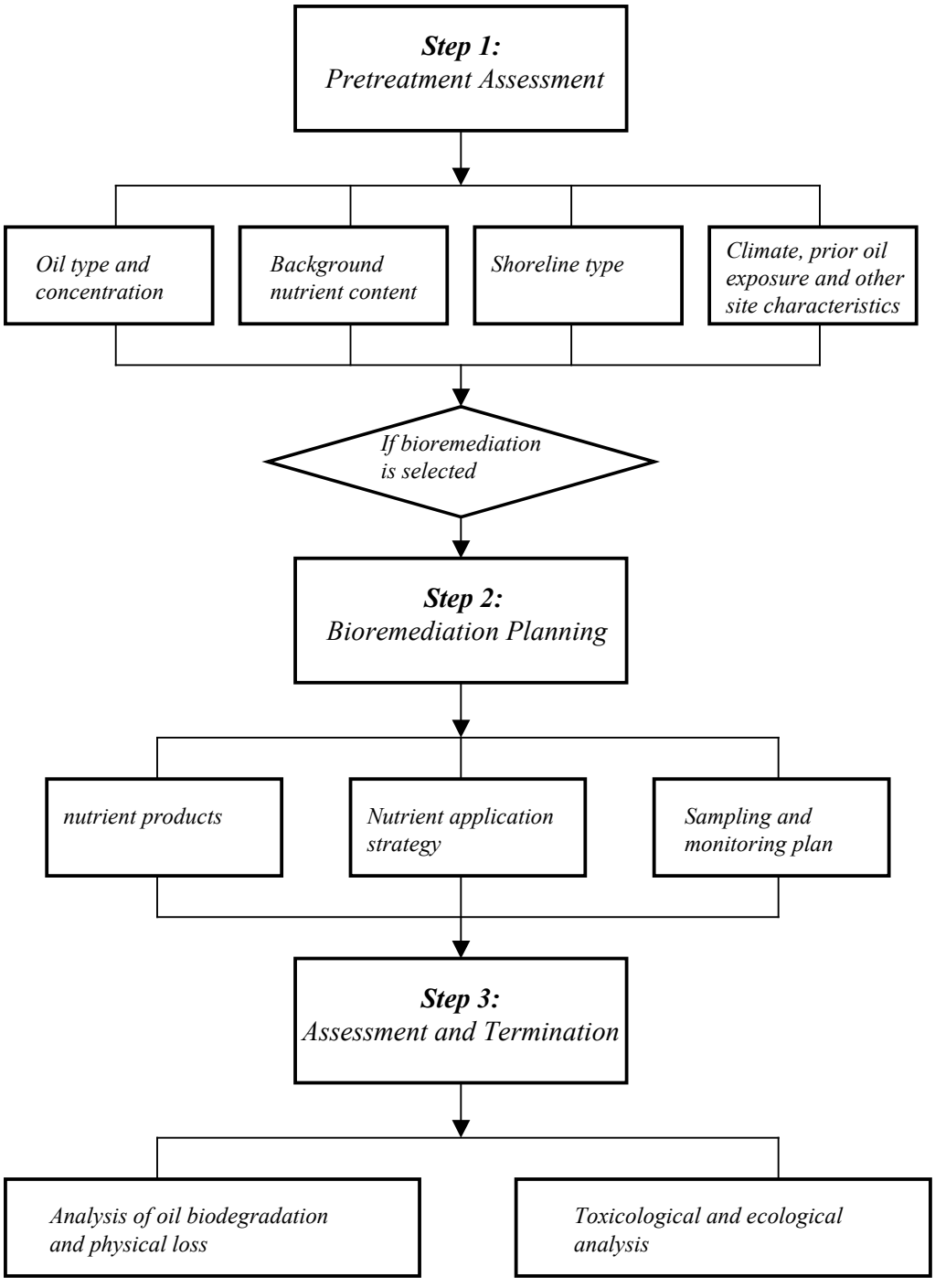


Figure 5.1 Procedures for the selection and application of oil spill bioremediation

5.1 Pre-treatment Assessment

Pretreatment assessment involves some preliminary investigations to assess whether bioremediation is a viable option and determination of the rate determining process, which include the evaluation of (1) oil types and concentrations, (2) background nutrient content, (3) shoreline types, and (4) other environmental factors such as the prevalent climate and prior oil exposures.

5.1.1 Oil type and concentration

Oil Type

As reviewed in Chapter 2, the biodegradability of different types of oils and petroleum products varies greatly depending on the distribution of oil components. In general, the susceptibility to microbial degradation for petroleum hydrocarbons is in the order of n-alkanes > branched alkanes > low-molecular-weight aromatics > cyclic alkanes > high molecular-weight aromatics, although this pattern is not universal (Perry, 1984). The degradation rate for the same oil components may also vary significantly for different oils. It has been found that the rate and extent of biodegradation of biodegradable components (e.g. n-alkanes) decreases with the increase of non-biodegradable fractions (e.g., resins and asphaltenes) (Uraizee *et al*., 1998; Westlake *et al*., 1974). Therefore, the heavier crude oils are less likely biodegradable than lighter crude oils. McMillen *et al.* (1995) investigated the biodegradability of 17 crude oils with API gravity ranging from 14° to 45°. They concluded that crude oil with greater than 30° API gravity can be considered readily biodegradable, and those with less than 20° API gravity (heavier oils) are slow to biodegrade. Similar results were obtained by other researchers (Hoff *et al*., 1995; Sugiura, *et al*., 1997). Wang and Bartha (1990) also investigated the effects of bioremediation on residues of fuel spills in soils. The results showed that the treatability by bioremediation for the fuel residues are in the order of jet fuel > heating oil > diesel oil. However, more work is still required to classify crude oils and refined products with respect to their theoretical amenability to cleanup by bioremediation.

The biodegradation potential of oils also depends on the weathering processes that alter oil compositions and properties. For example, evaporation leads to removal of the more toxic, lower molecular weight components from the spilled oil. Therefore, there is less need to bioremediate a spill of light petroleum products such as gasoline since it would evaporate rapidly. The formation of water-in-oil emulsion may increase mass transfer limitation for oxygen and nutrients and decrease the oil biodegradation rate. Interactions between oil and various types of shorelines also play important roles in oil degradation, which will be discussed later. Field experience also suggested that oils that have been subjected to substantial biodegradation might not be amenable to bioremediation due to the accumulation of polar components in the oils (Bragg *et al*., 1994; Oudet *et al*., 1998).

Oil concentrations

The concentration of oil is another important consideration in determining whether bioremediation is a viable option. Very low concentrations of hydrocarbons in the environment may be inefficiently attacked by microbes (Foght and Westlake, 1987). For sites contaminated with oils at low concentrations, biodegradation is also less likely to be limited by nutrients or

oxygen. Therefore, bioremediation may not be effective in enhancing biodegradation in these cases. Natural attenuation may be a more viable option.

High concentrations of hydrocarbons may cause inhibition of biodegradation due to toxic effects, although the inhibitory concentration varies with oil composition. Therefore, there should be an optimum oil concentration range for bioremediation applications, below which degradation is not easily stimulated, and above which inhibition occurs. However, this concentration range, particularly the maximum concentration of oil amenable to bioremediation, has not been well quantified. Field experiences in Prince William Sound, Alaska showed that less than 15 g oil/kg sediments could be treated using bioremediation (Swannell *et al*., 1996). Xu *et al*. (2001) recently investigated the effect of oil concentration in a microcosm study using weathered Alaska North Slope crude oil. The results showed that crude oil concentrations as high as 80 g oil/kg dry sand were still amenable to biodegradation. Favorable oil concentrations for bioremediation are also related to background conditions, such as shoreline types, which will be discussed later in this chapter.

5.1.2 Background nutrient content

Since nutrient addition has been chosen as the primary strategy to enhance oil biodegradation, assessment of background nutrient concentrations becomes critical in determining whether bioremediation is a viable option, whether natural attenuation should be considered, and/or which nutrient (nitrogen or phosphorus) should be added for oil bioremediation.

As mentioned in Chapter 2, in marine environments, nutrients are generally limiting due to the naturally low nitrogen and phosphorus concentrations in seawater (Floodgate, 1984). Nutrient content is more variable in freshwater systems and is normally abundant in freshwater wetlands (Cooney, 1984; Mitsch and Gosselink, 1993). However, background nutrients also depend on other site characteristics such as local industrial and domestic effluents and agricultural runoff.

Recent field studies indicate that natural nutrient concentrations in some marine shorelines can be high enough to sustain rapid intrinsic rates of biodegradation without human intervention (Oudet *et al*., 1998; Venosa *et al.*, 1996). The field trial in Delaware showed that although biostimulation with inorganic mineral nutrients significantly accelerated the rate of hydrocarbon biodegradation, the increase in biodegradation rate over the intrinsic rate (i.e. slightly greater than twofold for the alkanes and 50% for the PAHs) would not be high enough to warrant a recommendation to actively initiate a major, perhaps costly, bioremediation action in the event of a large crude oil spill in that area (Venosa, *et al*., 1996). The high intrinsic biodegradation rate was attributed to the high background nutrient concentrations (0.8 mg N/L on average) because the Fowler Beach area of Delaware Bay was adjacent to farmland. The relatively high organic content of both the Delaware Bay seawater and the underlying geology of the site and the presence of a saltwater marsh several hundred meters landward from the beach could also account for the high nitrogen levels encountered. The study investigators observed that maintenance of a threshold nitrogen concentration of 3-6 mg N/L in the interstitial pore water was biostimulatory for hydrocarbon biodegradation.

A similar conclusion was also reached in a field trial to evaluate the influence of a slow-release fertilizer on the biodegradation rate of crude oil spilled on interstitial sediments of an estuarine environment in the bay of Brest, France (Oudet *et al*., 1998). Due to the high background levels of N and P at the study site, no significant difference in biodegradation rates was detected following nutrient addition. It was proposed that bioremediation by nutrient enrichment would be of limited use if background interstitial pore water levels of N exceed 1.4 mg/L, which is close to the finding from the Delaware study (Venosa *et al*., 1996).

Phosphorus is another essential nutrient related to microbial growth. Although no field study of critical phosphorus concentrations on marine shoreline and freshwater wetlands has been reported, it has been generally accepted that the optimal N:P ratio for microbial growth is in a range of 5:1 to 10:1. Therefore, the threshold phosphorus concentration for maintaining optimal hydrocarbon degradation can be derived based on this ratio and critical nitrogen concentrations obtained from existing field studies. However, further research is still required in determining the influence of phosphorus on oil bioremediation under various marine shoreline and freshwater wetland environments.

These results suggest that, in the event of a catastrophic oil spill impacting a shoreline, one of the first tasks in pretreatment assessment is to measure the natural nutrient concentrations within the interstitial water in that environment. If they are high enough, further investigation is required to determine whether such a nutrient loading is typical for that area and season (i.e., determine the impact of chronic runoff from nearby agricultural practice and local industrial and domestic effluents). The decision to use bioremediation by addition of nutrients should be based on how high the natural levels are relative to the optimal or threshold nutrient concentrations.

5.1.3 Type of shorelines

The characteristics or type of the contaminated shoreline also play an important role in the decision to use bioremediation. This preliminary investigation involves the assessment of the need for bioremediation based on wave and tidal energy, the sediment characteristics, and geomorphology of the shoreline.

Shoreline energy and hydrology

Oil can be removed rather rapidly under high wave and tide influence, typically in rocky shorelines. In high-energy environments, bioremediation products are also more difficult to apply successfully since they can be washed out rapidly. High wave energy will also scour microorganisms attached to the sediment particles, and diminish the net oil biodegradation rate that can be achieved. The Maine field study demonstrated that washout rate of nutrients from the bioremediation zone will be strongly affected by the wave activity of the contaminated beach. However, washout due to tidal activity alone is relatively slow, and nutrients will probably remain in contact with oiled beach material long enough to effectively stimulate oil biodegradation on low-energy beaches (Suidan and Wrenn, 2001; see Section 2.6.2).

However, many of the same characteristics that make low-energy beaches favorable for bioremediation cleanup from a nutrient persistence perspective might make other conditions unfavorable with respect to other important factors. For example, availability of oxygen is more

favorable on high-energy beaches than on low-energy beaches. Aeration mechanisms for near-surface coastal sediments involve exchange of oxygenated surface water with oxygen-depleted pore water by wave-induced pumping and tidal pumping. For low energy beaches, tidal pumping is the only likely aeration mechanism, and as a result, the surface sediments are more likely to be anoxic than are similar depths on high-energy beaches (Brown and McLachlan, 1990). The probability of moisture (or water activity) limitation is also higher on low-energy beaches, because wave runup provides water to supratidal sediments on high-energy beaches during neap tides (Suidan and Wrenn, 2001). Therefore, it is essential to thoroughly characterize the factors that are likely to be rate limiting on each contaminated site before deciding and designing a bioremediation response strategy.

Shoreline substrate

Although successful bioremediation application and field trials have been carried out on cobble, medium sand, fine sand, and some salt marsh shorelines (Bragg *et al*., 1994; Lee and Levy, 1991; Swannell *et al.*, 1999a; Venosa *et al.*, 1996), different shoreline substrates or sediment types will affect the feasibility and strategies of using bioremediation. In a 7-month field study, Lee and Levy (1991) compared the bioremediation of a waxy crude oil on a sandy beach and a salt marsh shoreline. Terra Nova crude oil was added at two concentrations, 3% (v/v) and 0.3% (v/v) to beach sand and salt marsh sediments retained in *in-situ* enclosures in a low energy environment. The results showed that at the lower oil concentrations (0.3%) within the sand beach, oil biodegradation proceeded rapidly in both the fertilized plot and the unfertilized control. The application of a bioremediation treatment provided no advantage. However, at the higher oil concentrations (3%) on the sandy beach, oil biodegradation rates appeared to be nutrient limited and were enhanced by nutrient addition. In contrast, the addition of nutrients to the salt marsh sediments containing the lower (0.3%) oil concentration resulted in enhanced rates of biodegradation. This additional need for nutrients at the lower oil concentrations is consistent with the notion that nutrient demands within a salt marsh environment are higher, due to the size of the microbial population within an organic-carbon rich environment. At the higher oil concentration (3%) within the salt marsh sediments, insignificant rates of oil degradation were reported following fertilization. The results clearly demonstrated that the success of bioremediation depends on the characteristics of the shoreline.

On the sandy beach with low concentrations of oil, neither nutrient nor oxygen was a limiting factor. Under these conditions, nutrient enrichment appears to provide little or no benefit, and monitored natural attenuation can be considered as an alternative. However, at higher oil levels, the microbial community within the sand beach may become nutrient-limited, and bioremediation treatment could effectively enhance the rate of oil removal. In the salt marsh environment, nutrient addition was only effective at low oil concentrations. Oxygen limitation was more likely at higher oil concentrations due to the finer particle size and higher organic content of the sediment in these environments. Similar results have been obtained in the field study conducted in a freshwater wetland (Garcia-Blanco *et al*., 2001b; Venosa *et al.*, 2002), which also indicated that oxygen availability was likely a major rate-limiting factor in the wetland environments. A field study sponsored by EPA and the Department of Fisheries and Oceans-Canada was recently conducted on the shoreline of Nova Scotia to further investigate the potential of using bioremediation in salt marshes. Guidelines for oil bioremediation in this type of shoreline will be available upon completion of the data analysis from this investigation.

5.1.4 Other factors

Prevalent climate

Prevalent climate, the ambient temperature in particular, is an important consideration when assessing the feasibility of using bioremediation. As discussed in Chapter 2, the ambient temperature of an environment affects both the properties of spilled oil and the activity or population of microorganisms. At low temperatures, the viscosity of the oil increases, delaying the onset of biodegradation (Atlas, 1981), and the volatility of toxic low-molecular-weight hydrocarbons is reduced. Although the rates of biodegradation generally decrease with decreasing temperature, bioremediation has been tested and applied successfully to enhance oil biodegradation in cold arctic, alpine, and Antarctic environments (Margesin and Schineer, 1999). This is probably because psychrophilic bacteria are plentiful and generally the dominant species in these marine environments (Karrick, 1978).

A more important consideration regarding the effect of climate or weather on the use of bioremediation perhaps is the seasonal factor. Significant seasonal differences in the size of hydrocarbon degrader populations have been observed. The numbers of hydrocarbon degraders may be much lower during winter than summer in some environments (Atlas, 1981). Oil biodegradation slows significantly and even ceases when the contaminated sediments are frozen. Therefore, oil bioremediation will be more effective during warmer seasons and probably should only be considered during the summer for cold environments such as arctic regions.

Prior exposure to oil

Prior exposure of a microbial community to hydrocarbons either from natural sources (e.g. chronic seeps and plant derived hydrocarbons) or as a result of pollution (e.g. spills and waste disposal) may affect the rate at which subsequent hydrocarbon input can be biodegraded (Leahy and Colwell, 1990). Those environments with a history of oil pollution or natural oil inputs have been found to have a much higher percentage of hydrocarbon degraders and a generally greater potential of hydrocarbon degradation than previously unpolluted areas (Atlas, 1981; Lee and Levy, 1987, Pritchard and Costa, 1991). Therefore, for oil bioremediation in environments with no prior oil exposure, there may be a lag and adaptation period before any significant oil biodegradation occurs. This usually is not a concern since bioremediation itself is a relative slow process and typically is used as a polishing step after conventional mechanical cleanup operations. In contrast, those environments with prior exposure to oil need a shorter lag period before initiation of biodegradation and thus will likely have a higher potential for oil biodegradation. Thus, this type of environment is generally considered a favorable condition for using bioremediation.

5.1.5 Summary of pretreatment assessment

In summary, the following pretreatment assessments should be conducted to determine whether bioremediation is a viable option in response to a spill incident:

- Determine whether the spilled oil is potentially biodegradable – Light petroleum products and light crude oils (API gravity > 30°) are relatively biodegradable; products rich in normal

alkanes are relatively biodegradable; heavy crude oils (API gravity < 20°) and residual fuel oils, which are high in polar compounds (asphaltenes and resins) are less biodegradable. High concentrations of oil may also inhibit biodegradation.

- Determine whether the nutrient content at the impacted area is likely to be an important limiting factor by measuring the background nutrient concentrations within the interstitial water in that environment – The decision to use bioremediation by addition of nutrients should be based on how high the natural levels are relative to the optimal or threshold nutrient concentrations (e.g., > 2 mgN/L on sandy marine shorelines). It should also be determined if the natural nutrient concentrations present are typical of the area or sporadic. If sporadic, biostimulation may still be appropriate when the nutrient levels fall to limiting values; if chronic, biostimulation may not be necessary.

- Determine whether the shoreline characteristics are favorable for using bioremediation – High-energy rocky beaches and some low energy shorelines such as some wetlands are considered not likely to be very amenable to nutrient addition.

- Determine whether climatic or seasonal conditions are favorable for using bioremediation – bioremediation will be more effective during warmer seasons, particularly in cold environments. Prior exposure to oil will also be a favorable but not a solely determinative condition for selecting bioremediation.

5.2 Selection of Nutrient Products

After bioremediation is determined to be a potentially effective cleanup option based on the preliminary investigations, further assessments and planning are needed before its application. The first task is to select appropriate nutrient products through both screening tests and assessments based on characteristics of the contaminated site.

5.2.1 Nutrient selection based on efficacy and toxicity

To assist response personnel in the selection and use of spill bioremediation agents, it is useful to have some simple, standard methods for screening the performance and toxicity of bioremediation products as they become available (Blenkinsopp, *et al*., 1995; Haines *et al*., 1999, Lepo and Cripe, 1998a). One of the most comprehensive examples of such protocols is the tiered approach developed by EPA, in cooperation with the National Environmental Technology Applications Center (NETAC, 1993; Thomas *et al*., 1995). Conducting treatability tests using micro- or mesocosms is another commonly used approach.

EPA/NETAC protocols

The NETAC/EPA protocols consist of five progressive tiers, which increase in environmental cost and complexity with each tier of testing (Table 5.1). The approach begins with a Base Tier in which basic information on the agent's toxicity is gathered based on a review of its formulation. During this tier, the presence of chemicals or biological components that are normally considered unacceptable (i.e. pathogens, carcinogens, or hazardous substances) would

be identified. Tier I provides the basis for a preliminary evaluation of whether an agent could be effective and safely applied, which includes a description of how the product will be used, and information on previous usage. Tier II provides empirical evidence through the use of laboratory shake flask treatability studies to estimate a product's effectiveness. This tier also provides information on the relative changes in aliphatic and aromatic oil constituent concentrations over time and the total hydrocarbon degrading microbial activity. Tier III proposes the use of flow-through microcosm systems to study biodegradation effectiveness. Tier IV is the use of field demonstrations to predict a product's potential effectiveness in the natural environment. Tiers III and IV are no longer considered viable options when evaluating a product for use in an oil spill due to overwhelming economic considerations.

It is clear that field studies can provide the most convincing demonstration of the effectiveness of oil bioremediation because laboratory studies simply cannot simulate real world conditions such as spatial heterogeneity, climate change, and mass transfer limitations. Since conducting a field study just to determine that a product might work is unrealistic and economically burdensome, the practical approach in selection of nutrient products for the bioremediation of an oil spill would be through laboratory tests, microcosm tests in particular, as well as evaluations based on existing field study results in similar environmental conditions.

Table 5.1 Bioremediation product test protocols developed by EPA and in cooperation with NETAC.

Test Levels	Description
Base Tier	Collection and analysis of basic information on product safety including formulation and acceptability of its chemical or biological components
Tier I	Feasibility assessment concerning production capabilities, potential effectiveness, and safety certification, including a description of how the product will be used, and information on previous usage
Tier II	Efficacy and safety evaluation using shake flasks to compare the degradation of artificially-weathered crude oil in natural seawater with and without a bioremediation agent
Tier III	Efficacy and safety evaluation using microcosm systems to simulate various environments (e.g. open water, beaches, and marshes)
Tier IV	Efficacy and safety evaluation through a field demonstration

Nutrient selection through microcosm tests

The laboratory treatability tests, especially well-designed microcosm tests, are most commonly used approaches to determine the type and level of amendments, such as the types of fertilizer

and the optimal nutrient concentrations. A good example of these microcosms is illustrated in Figure 5.2, which has been used for various screening and treatability tests (Ahn, 1999, Du *et al*., 1999, Xu *et al*., 2001). These microcosms have at least three advantages over the batch reactors that are often used in this type of study: (1) they are connected to respirometers, allowing non-destructive acquisition of kinetic data by continuously recording the oxygen consumption that accompanies oil biodegradation; (2) they are open systems that can simulate the nutrient washout that will occur in contaminated intertidal zones; and (3) they are designed to simulate tidal flushing by filling and draining on a 12-hour cycle, thus simulating the periodic anoxia that can occur due to tidal flooding. The potential for oxygen limitation in these reactors is a particularly important advantage over more conventional microcosms. Because nutrient concentrations do not limit the oil biodegradation rate when oxygen becomes sufficiently depleted, these systems will provide more realistic estimates of maximum biodegradation rates than well-aerated shake flasks will provide. Also, some fertilizers contain large amounts of readily biodegradable organic compounds that can accentuate dissolved oxygen depletion. Although they might be very effective in well-aerated microcosms, oxygen availability can limit their effectiveness in the field (Lee *et al*., 1995a).

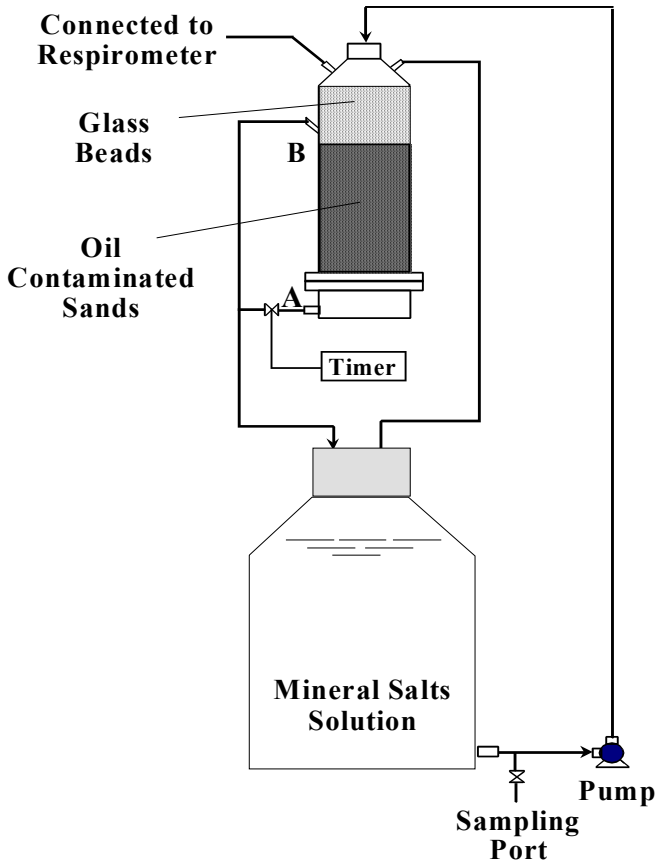


Figure 5.2 Schematic diagram of a beach microcosm for laboratory treatability testing of oil spill bioremediation treatment.

Using this microcosm system, Xu *et al*. (2001) investigated the effect of different nitrogen nutrients on the bioremediation of weathered Alaska North Slope crude oil under simulated tidal conditions. Three oil concentrations of 5, 20, and 80 g oil/kg dry sand were used. Two types of nitrogen nutrients (KNO_3 and NH_4Cl) were applied at a concentration of 100 mg-N/L. Oil biodegradation was evaluated by monitoring CO_2 production, oxygen uptake, nitrogen consumption, as well as oil constituent analysis. Results indicated that more biomass growth occurred in the submerged (sometimes anoxic) portion of the sand, and better oil degradation was observed in microcosms to which nitrate-nitrogen was applied. This result suggested that nitrate might also have enhanced oil bioremediation by serving as an electron acceptor when oxygen was limiting. However, the role of nitrate still requires further investigation. Ramstad and Sveum (1995) also found that nitrate had the most pronounced effect in stimulating oil degradation when comparing the effect of nitrate, ammonia, and an organic nitrogen product on biodegradation of topped Statfjord crude oil in a continuous-flow seawater column system.

Effect of nutrient type may also depend on the properties of shoreline substrates. Jackson and Pardue (1999) found that addition of ammonia as compared to nitrate appeared to more effectively simulate degradation of crude oil in salt marsh soils in a microcosm study. The ammonia requirement was only 20% of the concentration of nitrate to achieve the same increase of degradation. The authors concluded that ammonia was less likely to be lost from the microcosms by washout due to its higher adsorptive capacity to sediment organic matter. However, in a microcosm study using sandy sediments, Suidan and Wrenn (2000) found that there were no significant differences in the nutrient washout rates or the abilities of ammonium and nitrate to support oil biodegradation. These results suggest that although cation-exchange adsorption may be an important difference between ammonium and nitrate in sediments with high cation-exchange capacities (CECs), such as marsh sediments, it is unlikely to be significant in sediments with low CECs, such as sand.

Toxicity and other environmental impacts

In addition to demonstrating the efficacy of nutrient products in enhancing oil degradation, it is also necessary to demonstrate that bioremediation products have low toxicity and do not produce any undesired environmental and ecological effects. Various toxicity test protocols have been developed (NETAC, 1993; Lepo and Cripe, 1998a, See Section, 3.5). For example, the EPA Tier II safety evaluation consists of 7-day toxicity tests with the bioremediation product (without oil) in natural seawater using a crustacean (*Mysidopsis bahia*, mysid), and a fish (*Menidia beryllina*, the inland silverside). Additional Tier II toxicity tests evaluate the potential for interaction between the product and the water-soluble-fraction of a weathered crude oil. Indirect effects of nutrient products should also be evaluated, which include oxygen depletion through increase in organic carbon or eutrophication, and enhanced production of toxic oil degradation metabolites (Lepo and Cripe, 1998b).

So far, no detrimental effects from bioremediation by nutrient enrichment have been observed following actual field operations (Prince, 1993; Mearns *et al*., 1997). However, the possibility that harmful effects might occur remains. For example, oxygen depletion and production of ammonia from excessive applications of a fish-bone meal fertilizer during a field study caused detrimental effects, including a slowing in oil degradation rates and toxicity reduction rates

measured by Microtox® Solid-Phase Test (Lee *et al*., 1995b). For safety reasons, proper ecotoxicological assessment is always necessary in selecting nutrient products.

5.2.2 Environmental factors affecting nutrient selection

Nutrient selection also depends on environmental factors such as temperature, shoreline energy, and substrate. A field study conducted by Lee *et al*. (1993) indicated that the effectiveness of specific nutrient formulations might be influenced by temperature conditions. The study investigated the efficacy of water-soluble inorganic fertilizers (ammonium nitrate and triple superphosphate) and a slow release fertilizer (sulfur-coated urea) to enhance the biodegradation of a waxy crude oil in a low energy shoreline environment. The results showed that at temperate conditions above 15°C, the slow-release fertilizer appeared to be more effective in retaining elevated nutrient concentrations within the sediments and more effective in enhancing oil degradation than water-soluble fertilizers. However, lower temperatures were found to reduce the permeability of the coating on the slow-release fertilizer and suppressed nutrient release rates. Water-soluble fertilizers such as ammonium nitrate were then recommended under these temperature conditions.

The action of wave, tide, and sediment type will also affect the selection of nutrients. Some studies suggested that oleophilic fertilizers might be more suitable for use in high-energy and coarse-grained beaches (Sveum *et al*., 1994; Sveum and Ladousse, 1989; See Chapter 4), although stronger evidence is needed to confirm this suggestion. Therefore, for optimal effectiveness, the nutrient selection should always take into account the environmental conditions, the type of contaminated shoreline, and the methods of application, which will be discussed later in this chapter (Lee *et al*., 1993; Prince, 1993; Swannell *et al*., 1996).

5.3 Determination of the Optimal Nutrient Loading and Application Strategy

After the initial selection of nutrient products that meet the requirements of efficacy and safety, the next step is to determine the proper nutrient loading and the best nutrient application strategies. Major considerations in this task include the determination of optimal nutrient concentration, frequency of addition, and methods of addition. Finally, selection of appropriate nutrient products should also be conducted in conjunction with this process.

5.3.1 Concentration of nutrients needed for optimal biostimulation

Since oil biodegradation largely takes place at the interface between oil and water, the effectiveness of biostimulation depends on the nutrient concentration in the interstitial pore water of oily sediments (Bragg *et al*., 1994; Venosa *et al*., 1996). The nutrient concentration should be maintained at a high enough level to support maximum oil biodegradation based on the kinetics of nutrient consumption. Higher concentrations will not only provide no added benefit but also may lead to potentially detrimental ecological and toxicological impacts.

Studies on optimal nutrient concentrations have been conducted both in the laboratory and in the field. Boufadel *et al*., (1999a) investigated the optimal nitrate concentration for alkane biodegradation in continuous flow beach microcosms (Figure 5.2) using heptadecane as a model

alkane immobilized onto sand particles at a loading of 2g heptadecane/kg sand. They determined that a continuous supply of approximately 2.5 mg N/L supported maximum heptadecane biodegradation rates. Du *et al.* (1999) also investigated the optimal nitrogen concentration for oil biodegradation using weathered Alaska North Slope crude oil in the same microcosms with an oil loading of 5g/kg sand. The results showed that nitrate concentrations below approximately 10 mg N/L limited the rate of oil biodegradation. The higher nutrient requirement was attributed to the more complex substrate (crude oil) compared to the pure heptadecane of Boufadel *et al.* (1999a). The more complex substrate (crude oil) of Du *et al.* (1999) also likely selected a different population of degraders than those that grew on the pure heptadecane (Boufadel *et al.*, 1999a), which might have contributed to the different growth rate characteristics observed.

Ahn (1999) further studied the effect of nitrate concentrations under tidal flow conditions instead of continuous flow. He used the same beach microcosms as Du *et al.* (1999) filled with sand loaded with weathered Alaska North Slope crude oil at 5g/kg sand. A nutrient solution with nitrate concentrations ranging from 6.25 to 400 mg N/L was supplied semi-diurnally to simulate tidal flow. The results indicated that the optimum nitrate concentration for maximum oil biodegradation rate was over 25 mg N/L. Some laboratory studies have reported that greater than 100 mg N/L was required to stimulate maximum biodegradation rates (Atlas and Bartha, 1992; Reisfeld *et al.*, 1972), but this observation probably reflects a stoichiometric rather than a kinetic requirement, since these experiments were conducted in closed batch reactors.

Compared to the results from laboratory studies, nutrient concentrations that supported high oil biodegradation rates were found to be lower in field studies. For example, the field tests that were conducted after the *Exxon Valdez* oil spill in Prince William Sound, Alaska showed that the rate of oil biodegradation was accelerated by average interstitial nitrogen concentrations of about 1.5 mg N/L (Bragg *et al*., 1994). A similar result was obtained from the study conducted in the Bay of Brest, France, in which nitrogen was not a limiting factor when the interstitial pore water concentrations exceeded 1.4 mg N/L (Oudet *et al*., 1998). The Delaware field trial also showed that the background nitrate concentration (0.8 mg N/L) was sufficient to support fairly rapid natural (but not maximal) rates of alkane and PAH biodegradation (Venosa *et al*., 1996). Increasing the average nitrate concentration in the bioremediation zone of the experimental plots to between 3 and 6 mg N/L resulted in a moderate increase in the oil biodegradation rate.

Observations from the referenced field studies suggest that concentrations of approximately 1 to 2 mg/L of available nitrogen in the interstitial pore water is sufficient to meet the minimum nutrient requirement of the oil degrading microorganisms for the approximately 6-hour exposure time to the contaminated substrate during a tidal cycle. However, laboratory microcosm results as well as the Delaware field study suggest that higher concentrations of nitrogen can lead to accelerated hydrocarbon biodegradation rates. Since the minimum nitrogen concentration needed to satisfy the nitrogen demand in a tidal cycle is 1 to 2 mg N/L, and since concentrations of nitrogen in pore water that lead closer to maximum rates of biodegradation can be several-fold to as much as an order of magnitude higher, it is recommended that biostimulation of oil impacted beaches should occur when nitrogen concentrations of at least 2 to as much as to 5-10 mg N/L are maintained in the pore water with the decision on higher concentrations to be based on a broader analysis of cost, environmental impact, and practicality. In practice, a safety factor should be used to achieve target concentrations, which will depend on anticipated nutrient

washout rates, selected nutrient types, and application methods. For example, in the Delaware study, since nitrate in the interstitial pore water was quickly diluted to background levels whenever the incoming tide completely submerged the plots, water-soluble nutrients were applied every day using a sprinkler system. A 100-fold safety factor to account for dilution was used to achieve the 3-6 mg/L average interstitial pore water concentrations experienced at Delaware. A lower safety factor may be needed when using slow release nutrients.

5.3.2 Nutrient application strategies

Once the optimal nutrient concentrations have been determined, the next task is to design nutrient application strategies, which include nutrient application frequency and delivery methods.

Frequency of nutrient addition

The frequency of nutrient addition to maintain the optimal concentration in the interstitial pore water mainly depends on shoreline types or nutrient washout rates. On marine shorelines, contamination of coastal areas by oil from offshore spills usually occurs in the intertidal zone where the washout of dissolved nutrients can be extremely rapid. Oleophilic and slow-release formulations have been developed to maintain nutrients in contact with the oil, but most of these rely on dissolution of the nutrients into the aqueous phase before they can be used by hydrocarbon degraders (Safferman, 1991). Therefore, understanding the transport of dissolved compounds in intertidal environments is critical in designing nutrient addition strategies, no matter what type of fertilizer is used.

The Maine field study on nutrient hydrodynamics (See Section 2.6) has demonstrated that during spring tide, nutrients can be completely removed from a high-energy beach within a single tidal cycle. But it may take more than two weeks to achieve the same degree of washout from a low-energy beach. Washout during the neap tide can be much slower because the bioremediation zone will be only partially covered by water in this period. Since nutrients may be completely washed out from high-energy beaches within a few days, and remain in low energy beaches for several weeks, the optimal frequency of nutrient application should be based on observations of the prevalent tidal and wave conditions in the bioremediation zone. For example, a daily nutrient application may be needed for a high-energy beach during spring tide. But weekly or monthly additions may be sufficient for low-energy beaches when the nutrients are applied during neap tide. Nutrient sampling, particularly in beach pore water, must also be coordinated with nutrient application to ensure that the nutrients become distributed throughout the contaminated area and that target concentrations are being achieved. The frequency of nutrient addition should be adjusted based on the nutrient monitoring results.

Methods of nutrient addition

Nutrient application methods should be determined based on the characteristics of the contaminated environment, physical nature of the selected nutrients, and the cost of the application.

Shoreline energy and geometry are important factors in determination of nutrient application methods. The study in Maine suggested that surface application of nutrients may be ineffective

on high-energy beaches because wave action in the upper intertidal zone may cause nutrients from the surface layers of the beach to be diluted directly into the water column, resulting in their immediate loss from the bioremediation zone. Daily application of water-soluble nutrients onto the beach surface at low tide could be a feasible approach (Venosa *et al*., 1996), although this method is highly labor-intensive. Nutrients that are released from slow-release or oleophilic formulations will probably behave similarly to water-soluble nutrients with respect to nutrient washout. Formulations with good long-term release characteristics probably will never achieve optimal nutrient concentrations in environments with high washout rates. Therefore, they will not be effective on high-energy beaches unless the release rate is designed to be high enough to achieve adequate nutrient concentrations while the tide is out.

Another potentially effective strategy is the subsurface application of nutrients onto high-energy beaches. Wise *et al.* (1994) found that application of nutrients through a trench or subsurface drain placed above the high-tide level, rather than directly on the beach by sprinklers, would result in significantly longer retention times. However, since nutrients move downward and seaward during transport through the intertidal zone of sandy beaches, nutrient application strategies that rely on subsurface introduction must provide some mechanism for insuring that the nutrients reach the oil-contaminated area near the surface. The approach that was proposed by Wise *et al*. (1994) assumes that nutrients dissolved in the freshwater plume will be brought into contact with the oiled beach material periodically by the rising tide because the freshwater plume should float on top of the saltwater. However, the finding of freshwater trapped between two saltwater wedges by Boufadel *et al*. (1999) indicates that subsurface injection of nutrients above the high tide level would likely not be an effective method for providing nutrients to the bioremediation zone.

Compared to high-energy shorelines, application of nutrients on low-energy beaches is much less problematic. Since washout due to tidal activity alone is relatively slow (Suidan and Wrenn, 2001), surface application of nutrients is an effective and economical bioremediation strategy on low-energy beaches.

The physical forms of fertilizers are also critical in determination of appropriate nutrient application methods. Generally, available fertilizers can be classified into four types in terms of their physical forms: (1) slow release fertilizer briquettes, (2) dry granular fertilizers, (3) liquid oleophilic fertilizers, and (4) water-soluble inorganic nutrient solutions (Glaser *et al*., 1991; Swannell *et al*., 1996). The application of the briquette forms is problematic in regards to buoyancy of the briquettes and redistribution by tide and wave action (Glaser *et al.,* 1991). The method used during the *Exxon Valdez* spill involved packing the briquettes in mesh bags tethered to steel bars driven into the beach subsurface. The poor distribution problem occurs by channeling of nutrients vertically down the beach rather than lateral spreading.

Dry granular fertilizers can be slow-release (e.g., Customblen in Alaska) or water-soluble, solid granules (e.g., prilled ammonium nitrate). Granular fertilizers are easier and more flexible to apply using commercially available whirlybird-type hand spreaders. Although this type of fertilizer is also subject to washout by wave and tidal action, dry granular fertilizers are probably the most cost-effective way to control nutrient concentrations. Liquid oleophilic nutrients, such as Inipol EAP 22, are also relatively easy to apply by using hand-held or backpack sprayers. One

of the problems when using Inipol EAP 22 during the *Exxon Valdez* spill cleanup was its low pour point (11°C), which led to clogging of spraying nozzles and poor uniformity of application (Glaser *et al.,* 1991). The problem was later resolved by warming the product. This type of fertilizer is significantly more expensive than granular fertilizers. Water-soluble nutrient solutions are normally delivered to the beach by a sprinkler system after dissolving nutrient salts in a local water source. Although this type of nutrient may be easier to manipulate to maintain target concentrations in interstitial pore water, its application may require more complicated equipment such as large mixing tanks, pumps, and sprinklers.

Based on current experiences and understandings, application of dry granular fertilizer to the impact zone at low tide is probably the most cost-effective way to control nutrient concentrations.

5.4 Sampling and Monitoring Plan

To properly evaluate the progress of bioremediation, a comprehensive and statistically valid sampling and monitoring plan should be developed before the application of bioremediation. The sampling and monitoring plan should include important efficiency and toxicity variables, environmental conditions, and sampling strategies.

5.4.1 Important variables

Important variables to be monitored in an oil bioremediation project include limiting factors for oil biodegradation (e.g., interstitial nutrient and oxygen concentrations), evidence of oil biodegradation (e.g., concentrations of oil and its components), microbial activity (e.g., bacterial numbers), environmental effects (e.g., ecotoxicity levels) and other environmental conditions (e.g., temperature and pH). A comprehensive monitoring plan proposed for a bioremediation field study is listed in Table 5.2. A monitoring plan for a full-scale bioremediation application should be similar to this and at least should include those critical measurements.

Since oil biodegradation in the field is usually limited by availability of nutrients, nutrient analysis, particularly the nutrient concentrations in the pore water, is one of the most important measurements in developing proper nutrient addition strategies and assessing the effect of oil bioremediation. The frequency of nutrient sampling must be coordinated with nutrient application, making certain that the treatment is reaching and penetrating the impact zone, target concentrations of nutrients are being achieved, and toxic nutrient levels are not being reached. Otherwise, nutrient application strategies should be adjusted accordingly. The location from which nutrient samples are collected is also important. Recent research on solute transport in the intertidal zone has shown that nutrients can remain in the beach subsurface for much longer time periods than in the bioremediation zone (Wrenn *et al*., 1997a). Nutrient concentration profiles along the depth of the oil-contaminated region can be monitored by using multi-port sample wells or sand samples collected from the oil-contaminated region.

The sampling depth should be determined based on the preliminary survey of oil distribution. It can be established by determining the maximum depth of oil penetration, then adding a safety factor, which will be chosen based on the observed variation in oiled depth, to ensure that the

samples will encompass the entire oiled depth throughout the project. The safety factor will be modified if observations during the bioremediation application suggest that the depth of oil penetration has changed.

The success of oil bioremediation will be judged by its ability to reduce the concentration and environmental impact of oil in the field. As discussed in Chapter 3, to effectively monitor biodegradation under highly heterogeneous conditions, it is necessary that concentrations of specific analytes (i.e., target alkanes and PAHs) within the oil be measured using chromatographic techniques (e.g., GC/MS) and are reported relative to a conservative biomarker such as hopane. On the other hand, from an operational perspective, more rapid and less costly analytical procedures are also needed to satisfy regulators and responders on a more real time, continual basis. Existing TPH technologies are generally not reliable and have little biological significance (See Chapter 3). TLC-FID seems to be a promising screening tool for monitoring oil biodegradation (Stephens *et al*., 1999).

Table 5.2 Monitoring plan for a bioremediation field study

Analysis	Matrix	Recommended Methods
*dissolved nitrogen	Sediment (interstitial pore water)	extract in acidified 0.1% NaCl 4500-N_{org} D (persulfate oxidation to NO_3^-) 4500-NO_3^- F (automated Cd-reduction)
dissolved phosphorus	Sediment (interstitial pore water)	extract in acidified 0.1% NaCl 4500-P B.5 (persulfate oxidation) 4500-P E (ascorbic acid method)
*residual oil	Sediment	extract into methylene chloride analyze components by GC/MS-SIM
dissolved oxygen	Aqueous	Hach® high range assay
pore-water pH	Aqueous	potentiometric with combination electrode
microbial populations	Sediment	MPN for alkane and PAH degraders
metabolic activity	Sediment	CO_2 production from sand slurries
*toxicity of residual oil	Sediment	Microtox® Solid-Phase Test
toxicity of residual oil	Sediment	10-day amphipod survival bioassay
toxicity of pore-water	Aqueous	Microtox® Acute Test
toxicity of pore-water	Aqueous	sea-urchin fertilization bioassay
bioaccumulation potential	SPMDs**	2-week adsorption into SPMDs
beach profile		surveying using fixed markers (e.g., wells, plot boundary markers) in intertidal zone
beach profile		surveying relative to fixed benchmarks in the supratidal zone

* Critical measurements

** Semi-permeable membrane devices

In addition to monitoring the treatment efficacy for oil degradation, the bioremediation monitoring plan should also incorporate reliable ecotoxicological endpoints to document treatment effectiveness for toxicity reduction. Commonly used ecotoxicity monitoring techniques, such as the Microtox® assay and an invertebrate survival bioassay, have been summarized in chapter 3. These microscale bioassays may provide an operational endpoint indicator for bioremediation activities on the basis of toxicity reduction (Lee *et al*., 1995b). Considerations for selecting an appropriate bioremediation endpoint based on both oil degradation and toxicity reduction will be presented in detail in the next chapter.

Other important variables in a comprehensive monitoring plan include site background conditions (e.g., oxygen, pH and temperature) and shoreline profiles. Oxygen availability is crucial for rapid bioremediation, because hydrocarbon biodegradation is primarily an aerobic process. Therefore, dissolved oxygen (DO) in the pore water should be monitored on a regular basis. The frequency of DO sampling should also be coordinated with nutrient application, particularly when organic nutrients are used (Lee *et al*., 1995b; Sveum and Ramstad, 1995; See Section 4.1.3), to insure that anoxic conditions do not result. When oxygen does become limited, the nutrient dosage and application frequency should be adjusted accordingly. Alternatively, oxygen amendment strategies, such as tilling or addition of chemical oxygen sources, may be considered, although these approaches are likely to be expensive and potentially environmentally hazardous (see Chapter 4).

Measurement of pH in the pore water is also important in monitoring oil bioremediation. Biodegradation of oil in marine environments is optimal at a pH of about 8 (Atlas and Bartha, 1992). The pH of seawater is usually around 8.5, which is adequate to support rapid oil biodegradation.

5.4.2 Statistical considerations in the sampling plan

To ensure that monitored results reflect the reality in a highly heterogeneous environment, it is important that a bioremediation sampling plan be designed according to valid statistical principles such as randomization, replication, and proper control.

To minimize bias, a random sampling plan should be used to evaluate treatment effects and their variance within the bioremediation zone. For samples with a high degree of spatial heterogeneity, which will be the case for most oil spill sites, stratified sampling strategies should be used. For example, the sampling field on a marine shoreline can be divided into a number of sectors or quadrants based on the homogeneity of geomorphology within each sector (e.g., upper and lower intertidal zones), and independent samples should be taken in each sector according to the rule of proportionality (e.g., taking more samples in more heavily oiled sites).

Although economic factors may be restrictive, efforts should be made to ensure that an adequate number of samples are taken to reach a given accuracy and confidence. Power analysis should be used to assist in the determination of sample replications required in a monitoring plan. A statistical power test was performed in the Delaware field study to determine the number of replicates that would be needed in future studies to detect significant treatment differences under

similar conditions (Venosa *et al*., 1996). The study indicated that the required replicates to detect treatment effects depend on expected variance and expected treatment differences. For example, if oil distribution and shoreline characteristics are highly heterogeneous, variance will be high, thus requiring more replicates to detect significant treatment effects. If background nutrients are high, treatment differences will be low, and more replicates will also be required. By comparing three shoreline assessment designs used for the *Exxon Valdez* oil spill, Gilfillan *et al*. (1999) also proposed several strategies to increase power (i.e., the probability that significant differences between two or more treatments are detected when indeed they exist). One of the approaches to increase power is to select sampling sites from only the most heavily oiled locations. This strategy may not be feasible for assessing the oil degradation within the whole bioremediation zone, although it may be useful for evaluating the effect of bioremediation on ecological recovery since the ecological injury most likely occurs at the heavily oiled locations.

A control area normally refers to a set-aside untreated site, which has similar physical and biological conditions as the treated site. Although on-scene coordinators prefer not to leave oiled sites uncleaned, it is difficult to assess the true impact of a treatment without control or set-aside areas (Hoff and Shigenaka, 1999). When selecting control areas, one must consider not only the similarity of the conditions but also the effect of sand and nutrient exchanges between the treated and untreated areas (See Section 6.1.2).

5.5 Considerations for Freshwater Wetland Bioremediation

Guidelines proposed in earlier sections are mostly derived from studies and practices on marine shorelines. However, freshwater conditions or habitats may differ sufficiently from marine situations so that a simple transfer of response strategies may not be necessarily the most appropriate. Special considerations for oil bioremediation in freshwater wetlands are summarized here based on current understandings, particularly the findings of the St. Lawrence River field study (Garcia-Blanco *et al*., 2001b; Venosa *et al.,* 2002; Lee *et al*., 2001a).

5.5.1 Characteristics of freshwater wetlands

Wetlands occupy the interface between terrestrial and aquatic systems. They have been defined by EPA and the U.S. Army Corps of Engineers as: "*Those areas that are inundated or saturated by surface or groundwater at a frequency or duration to support, and that under normal conditions do support, a prevalence of vegetation typically adapted for life in saturated soil conditions. Wetlands generally include swamps, marshes, bogs, and similar areas*." (EPA, 40 CFR 230.3 and Corps, 33 CFR 328.3). Wetlands normally should have the following three characteristics or diagnostic parameters: (1) at least periodically, the land supports predominantly hydrophytes (i.e., plants adapted to the flooded conditions), (2) the substrate is predominantly undrained hydric soil (i.e., a soil with unique physical and chemical characteristics, such as highly reduced conditions, due to repeated and prolonged saturation), and (3) the substrate is saturated with water or covered by shallow water for a significant part of the growing season each year (Greene, 2000; Mitsch and Gosselink, 1993).

Wetland ecosystems have enormous ecological and environmental value, contributing to aquifer recharge, water quality improvement, flood mitigation, and shoreline erosion protection. They

also provide unique and extensive habitats for a wide variety of flora and fauna. Furthermore, wetlands play an important role in the global cycles of nitrogen, sulfur, methane, and carbon dioxide (Mitsch and Gosselink, 1993).

The lower 48 states contained an estimated 100 million acres (400,000 square kilometers) of wetlands in the mid-1980s, an area about the size of California, among which freshwater wetlands are estimated to make up 80 to 95 percent (Mitsch and Gosselink, 1993, Greene, 2000). Freshwater wetlands also cover extended areas of Alaska and Canada. Their proximity to areas of human activity makes them susceptible to contamination by petroleum hydrocarbons, via leakage, runoff or spill. Necessary measures need to be taken to protect these ecosystems since they are among the environments most sensitive to oil and clean-up activities (Hayes *et al*., 1995, 1997). Furthermore, there are reports that the application of traditional oil spill cleanup techniques in wetland habitats caused more damage than the oil itself (Baker *et al.*, 1993). When looking for both inexpensive and environmentally friendly technologies for wetland cleanup and restoration, bioremediation and phytoremediation have potential for being the most suitable options (Atlas, 1995; Cunningham et al, 1996).

The limiting conditions for oil biodegradation in freshwater wetlands may be significantly different from most marine environments. In terms of nutrient supply, freshwater wetlands can be divided into eutrophic wetlands (e.g., tidal marsh) and oligotrophic wetlands (e.g., cypress domes (Mitsch and Gosselink, 1993). Many freshwater wetlands also exhibit a seasonal pattern of uptake and release of nutrients. During the growing season (i.e., late spring and summer), there is a high rate of nutrient uptake by vegetation from both the water and sediments. And when higher plants die in the fall, a substantial portion of nutrients will be released to the water and sediments. The amount of inorganic nutrients or nutrients available for oil biodegradation also depends on many other processes, such as nutrient mineralization, denitrification, anaerobic release of phosphorus, and wetland hydrodynamics (Mitsch and Gosselink, 2000).

When wetland soils are inundated with water, oxygen diffusion rates through the soil are drastically reduced. Available oxygen in the soil and in the interstitial water is quickly depleted through metabolism by aerobic organisms. Below a few centimeters—and sometimes only a few millimeters—of the soil surface, the environment becomes anaerobic (Gambrell and Patrick, 1978). When the metabolic demand for oxygen by soil organisms exceeds that of supply, the redox potential in the soil drops and other ions (nitrate, manganese, iron, sulfate, and carbon dioxide) are used as electron acceptors (Mitsch and Gosselink, 1993). Therefore, in freshwater wetland environments, petroleum biodegradation is likely to be limited by oxygen availability.

Because wetland sediments are generally more fine-grained and, more importantly, saturated with water, the extent of oil penetration is expected to be much lower in freshwater wetlands than on a porous sandy marine beach. In a microcosm study, Purandare (1999) investigated the penetration of weathered light Arabian crude oil in freshwater wetland sediment under two different water levels. For the case of low water level, where the sediment was saturated but not covered with water, the oil was found to penetrate only about 2.5 cm in 16 weeks. For the case of high water level, where the water level was 10 cm above the sediment surface, most of the oil was floating on the surface of the water and the penetration depth in the sediment for some settled and dissolved oil was also about 2.5cm during this study. The depth of oil penetration in

the case of the St. Lawrence River study was higher (about 9 cm), due to the initial raking of the wetland sediments after they were oiled. This depth is still much lower than the depth of oil penetration that occurs in marine sandy beaches (up to 30 cm, Gundlach, 1987) and cobble beaches (up to 1 m, Wolfe *et al.*, 1994).

Another important feature of wetlands is that at least periodically, the land supports predominantly hydrophytes, or plants "growing in water, soil, or on a substrate that is at least periodically deficient of oxygen as a result of excessive water content." (Greene, 2000). These wetland plants may play important roles in oil bioremediation and wetland restoration. On the one hand, they may be involved in degradation, containment, and transfer of petroleum hydrocarbons from the soil to the atmosphere (Frick, *et al*, 1999). On the other hand, these wetland plants may also compete with hydrocarbon-degrading microorganisms for nutrients.

5.5.2. Bioremediation strategies in freshwater wetlands

Although the same decision-making and planning principles that were described earlier in this chapter for bioremediation of marine shorelines should also apply to freshwater wetland environments, the feasible bioremediation strategies are likely to be different due to the distinct characteristics of wetlands. The potential effectiveness of different amendments is discussed as follows mainly based on the findings of St. Lawrence River field study (Garcia-Blanco *et al*., 2001b; Venosa *et al.* 2002; Lee *et al*., 2001a).

Nutrient amendment

Since nutrients could be limited in wetland sediments during the growing season in particular, addition of nutrients would seem to have some potential for enhancing oil biodegradation in such an environment. However, the results from the St. Lawrence River field study (See Section 2.5) showed that no significant enhancement was observed in terms of the oil biodegradation following biostimulation through addition of nutrients (either ammonium or nitrate). After 21 weeks, reduction of target parent and alkyl-substituted PAHs averaged 32% in all treatments. Reduction of target alkanes was of similar magnitude. The removal of PAHs in nutrient-amended plots was only slightly better than natural attenuation after 64 weeks of treatment. Oil analysis from the top 2 cm sediment samples showed that the plots amended with ammonium nitrate and with *Scirpus pungens* plants cut back demonstrated a significant enhancement in target hydrocarbon reduction over natural attenuation as well as all other treatments. This suggests that biostimulation may be effective only in the top layer of the soil, where aerobic conditions are greater, and when hydrocarbon-degrading microorganisms do not have to compete for nutrients with the growing wetland plants.

Another potential problem with respect to the use of biostimulation in wetlands is that some plant communities may be sensitive to nutrient additions. Repeated and excessive nutrient additions may alter the nature of the wetland ecosystem as indicated by the effects of chronic nutrient additions to the Everglades in Florida (Davis, 1994).

Oxygen amendment

Since oxygen has been found the most likely limiting factor in oil biodegradation in freshwater environments, oxygen amendment may be considered. However, an appropriate technology for

increasing the oxygen concentration in such environments, other than reliance on the wetland plants themselves to pump oxygen down to the rhizosphere through the root system, has yet to be developed. Existing oxygen amendment technologies developed in terrestrial environments, such as tilling, forced aeration, and chemical methods (See Section 4.4), are not likely to be cost-effective for bioremediation of freshwater wetlands since they often involve expensive and environmentally intrusive practices.

During the St. Lawrence River field trial (Garcia-Blanco *et al*., 2001b; Venosa *et al.*, 2002), after the first nutrient and oil applications, the top 1-2 cm surface soil in all plots was manually raked using cast iron rakes. This was done to minimize loss of oil from the plots due to tidal action and to uniformly incorporate the nutrients and the oil into the soil. However, the oil analysis results suggested that the tilling of surface soil might have slowed the overall oil biodegradation rates by enhancing oil penetration deep into the anaerobic sediments. Based on these observations, surface tilling will not be an effective strategy for increasing the oxygen concentration in freshwater wetlands (although this was not the intent of the raking). The slightly better but statistically insignificant performance in both alkane and PAH degradation with addition of nitrate compared to ammonium after 64 weeks of treatment implied that nitrate may have served as an alternative electron acceptor in enhancing oil biodegradation when oxygen was limiting. However, the limited increase in biodegradation rate over natural attenuation would not warrant a recommendation to use nitrate as an oxygen amendment agent in such an environment.

Phytoremediation

Since plants cover wetlands at least periodically, the use of phytoremediation becomes a natural option for wetland cleanup and restoration. Phytoremediation is emerging as a potentially viable technology for cleanup of soils contaminated with petroleum hydrocarbons (Frick *et al.*, 1999 See Section 4.3). However, this technique has not been used as a wetland oil spill countermeasure. Only limited studies have been carried out on the effectiveness of phytoremediation in enhancing oil degradation in marine shorelines and freshwater wetlands. Lin and Mendelssohn (1998) found in a greenhouse study that application of fertilizers in conjunction with the presence of transplants (*S. alterniflora* and *S. patens*) significantly enhanced oil degradation in a coastal wetland environment. In the case of freshwater wetlands, the St. Lawrence River study suggested that although application of fertilizers in conjunction with the presence of a wetland plant *(Scirpus pungens)* may not significantly enhance oil degradation, it could enhance habitat recovery through the stimulation of vigorous vegetative growth and reduction of sediment toxicity and oil bioavailability (Lee *et al*., 2001a). The effectiveness of oil phytoremediation in freshwater wetland environments still requires further study.

Natural attenuation

Natural attenuation has been defined as the reliance on natural processes to achieve site-specific remedial objectives (USEPA, 1999b). This approach has been increasingly recognized as a possible viable option for oil spill cleanup with more understanding gained over the past decade about the advantages and disadvantages of active treatment versus natural attenuation (Owens *et al*., 1999). As indicated by Sell *et al*. (1995), the decision-making should focus more on a preference for natural attenuation except when a large amount of viscous oil is present, where natural removal will be slow, or when non-ecological factors are of greater importance.

The St. Lawrence River Study demonstrated that the availability of oxygen, not nutrients, is likely to be the limiting factor for oil biodegradation in freshwater wetlands. However, no feasible technique is currently available for increasing oxygen concentration under such an environment. As a result of this study, natural attenuation is recommended as the most cost-effective strategy for oil spill cleanup in freshwater wetlands when the oil concentration is not high enough (e.g., less than 30 g/kg soil; Longpre *et al.,* 1999) to destroy wetland vegetation.

Chapter 6 GUIDELINES FOR ASSESSMENT OF FIELD RESULTS AND TERMINATION OF TREATMENT

After the treatment is implemented according to the bioremediation plan, the next or final step in an oil bioremediation project is to assess the treatment efficacy and terminate the bioremediation action at appropriate treatment endpoints. Key questions to be answered in this task include "what are the measurements of oil bioremediation success?" and "how clean is clean?" or "when should bioremediation efforts be terminated?". Actually, these issues should be dealt with during the bioremediation planning stage, when proper treatment objectives, strategies, and sampling protocols should be established. On the other hand, more definite answers to these questions can only be reached during the bioremediation actions and based on the findings of comprehensive monitoring programs. Cost-benefit analysis (e.g., net environmental benefit analysis, Baker, 1995&1999) and sometimes political considerations should also be taken into account in this process, however, which are beyond the scope of this document. From a technological point of view, the measurements of bioremediation success and establishment of operational endpoints should be based on both the efficacy of oil biodegradation and the evidence of ecotoxicity reduction and ecological recovery, each of which will be discussed in this chapter.

6.1 Assessment of Oil Biodegradation Efficacy

6.1.1 Verification of oil biodegradation

Evidence for the effectiveness of oil bioremediation in terms of oil biodegradation should include: (1) faster disappearance of oil in treated areas than in untreated areas, and (2) a demonstration that biodegradation was the main reason for the increased rate of oil disappearance. As described earlier in this document, assessing the effectiveness of oil bioremediation in oil spill sites is difficult due to the heterogeneous conditions of contaminated sites and lack of control over the oil distribution. Nevertheless, the success of bioremediation can be verified through well-designed monitoring programs and proper data interpretation.

Distinguishing biodegradation from abiotic loss

Oil constituents can be lost from a shoreline by physical washout, dissolution, volatilization, and biodegradation. To demonstrate the effectiveness of a bioremediation treatment, biodegradation should be identified as the main mechanism for the increased rate of oil disappearance. As described in Chapter 3, to effectively distinguish biodegradation from abiotic loss, specific oil components or analytes should be analyzed using GC/MS techniques and then these analytes should be normalized to a conserved biomarker, such as hopanes and chrysenes. This approach has been successfully used to distinguish between biodegradation and the physical or chemical loss of oil in recent bioremediation field studies (Bragg *et al.*, 1994; Venosa *et al*., 1996; *Lee et al*., 1997b). It should also be noted, however, that hopane normalization is most useful for reducing the variability associated with heterogeneous oil distribution. The use of hopane normalization to distinguish biodegradation from physical loss of oil is valid mostly when losses due to dissolution and volatilization are negligible. Biodegradation can also be verified as the main removal mechanism by examining the relationships between the degradation rates and the substrate structure such as the relative degradation rates for homologous series of alkylated PAHs. These relationships, when used in conjunction with other oil analysis data, can be very

useful in accurate assessment of the extent and progress of oil biodegradation (Venosa *et al.*, 1997a; Wang *et al*., 1998; See section 3.3).

In addition to the demonstration of oil biodegradation based on chemical analysis, the effectiveness of oil bioremediation can also be verified by monitoring the changes in growth and activity of oil degrading microorganisms. Growth of hydrocarbon degrading bacteria can be determined by Most Probable Number (MPN) techniques, particularly the procedure proposed by Wrenn and Venosa (1996), which can separately enumerate aliphatic and aromatic hydrocarbon-degrading bacteria and is simple enough for use in the field. Because many viable microorganisms are unculturable (Atlas & Bartha, 1987, Macnaughton *et al*., 1999), the emergent culture-independent molecular techniques, such as the PLFA-DGGE techniques (See section 3.1.1.2), are becoming important tools to identify the diversity and composition of uncultivated microbial communities and to enumerate bacteria in more precise ways. Other useful tools in monitoring biological activities include *in-situ* measurement of microbial CO_2 production by the use of respirometric or radiorespirometric methods (Sugai *et al*., 1997; Swannell *et al*., 1997).

Assessing treatment significance

To show that a treatment increases the rate of oil biodegradation, the concentrations of the target analytes (e.g., hopane normalized total resolvable alkanes or aromatics) should be significantly lower in treated than untreated areas within the time frame of bioremediation applications (e.g., several months to a year). Convincing demonstration of an increased rate of oil degradation requires taking a sufficient number of true replicate samples that are randomly interspersed throughout the sampling domain (see section 5.4). The field results should then be interpreted using proper statistical analysis (Venosa *et al*., 1996), including analyzing field data using standard statistical models and analysis of variance (ANOVA) techniques, which are usually done at each sampling event. In addition to the ANOVAs, the entire data set should be analyzed by non-linear regression analysis to estimate the rate of decline in biodegradable analytes for each treatment over the entire course of the bioremediation treatment. As demonstrated in the Delaware field trial (Venosa *et al*., 1996), the interpretation of treatment effectiveness will be affected by reaction kinetics, background nutrient concentration, the variance of analytical results, the number of replicates, and statistical significance required to demonstrate the differences between treated and control areas.

An example of a likely treatment effect under different kinetic conditions is shown in Figure 6.1. The performance of oil biodegradation expected on an oiled shoreline can be estimated based on the first-order kinetic models described in Section 2.5.3. In the Delaware field study, first-order biodegradation rate coefficients ranging between 0.026 and 0.056 day^{-1} for total resolvable alkanes and 0.021 to 0.031 day^{-1} for total resolvable aromatics were observed (Venosa *et al*., 1996). These first-order biodegradation rate coefficients are also a function of the nutrient concentration (See equation 2.4). The half-saturation concentration K_n for nitrate is approximately 0.5 mg N/L (Boufadel *et al.*, unpublished). In this example, the background nitrogen concentration is assumed to be 0.1 mg N/L. The assumption of low background nutrient concentrations is reasonable since this is one of the prerequisites that bioremediation actions should be selected. Two cases of oil biodegradation rates (i.e., 0.026 and 0.056 day^{-1}) in the treated areas are examined, which covers both the high (treated) and low (natural attenuation)

ends of what was observed in the Delaware study (Venosa *et al*., 1996). A theoretical control rate of 0.0093 day^{-1} was assumed based on a background pore water nitrogen concentration of 0.1 mg/L and a Kn of 0.5 mg/L. This is the natural attenuation rate that would have occurred had the background nitrogen concentration been 0.1 mg/L rather than the actual 0.8 mg/L.

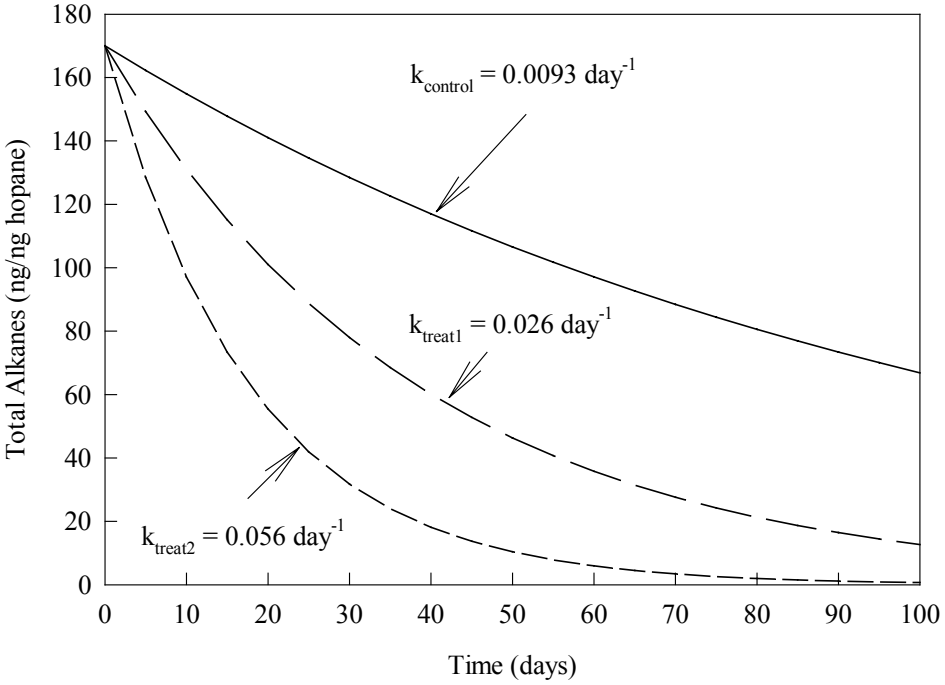


Figure 6.1 Influences of biodegradation rates a on detectability of treatment effect

It can be seen from Figure 6.1 that differences between treated and control plots are prominent very early in the project period when first order decay coefficient was 0.056 day^{-1}. Differences were also evident when the decay rate was only 0.026 day^{-1}, but they were not as prominent. Thus, had the background nitrogen concentration been closer to 0.1 mg/L, the overall conclusion from the study would have been to recommend bioremediation since a substantial enhancement would have been evident with nutrient addition. This would have been true even considering the aromatic fraction, which had a lower decay rate but still higher than the theoretical control.

To determine the effect of variance on the ability to detect differences between treatments, Figure 6.2 was developed. This figure was based on the power analysis conducted by Venosa *et al.* (1996). Using 5 replicates and at a statistical power of 80% (i.e., the probability that significant differences between two or more treatments are detected when they actually exist), the minimum detectible difference clearly increases linearly with variance. Thus, if the variance doubles, the ability to detect a difference between treatments lessens inversely. Figure 6.3 was developed to show how the minimum detectable difference varies at a constant variance but at an increasing number of replicate plots. Obviously, the fewer the number of replicate plots established, the more difficult it is to detect statistically significant treatment differences. These figures point out why it is so important to minimize variance by hopane normalization and increasing the number of replicate plots.

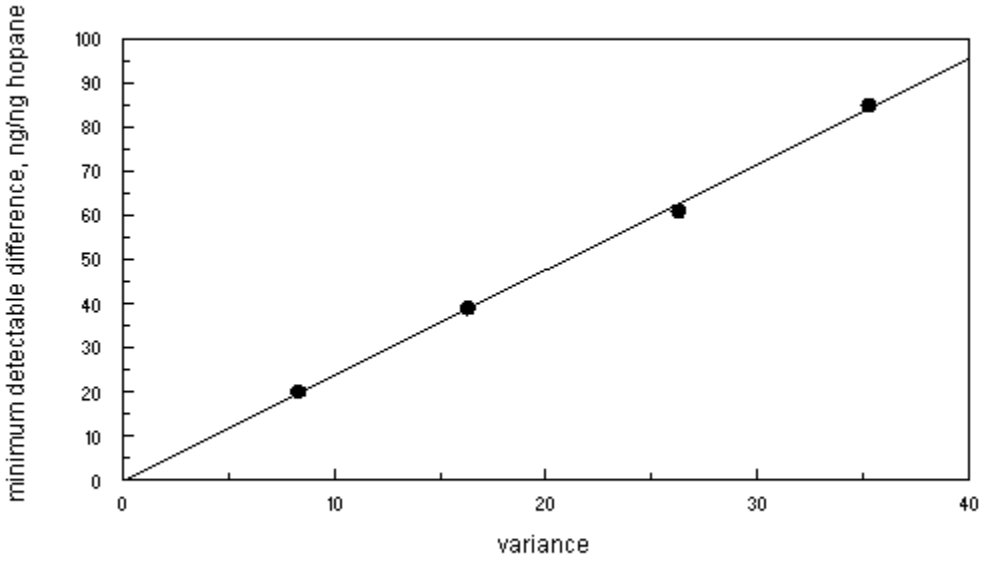


Figure 6.2 Minimum detectible treatment difference as a function of variance.

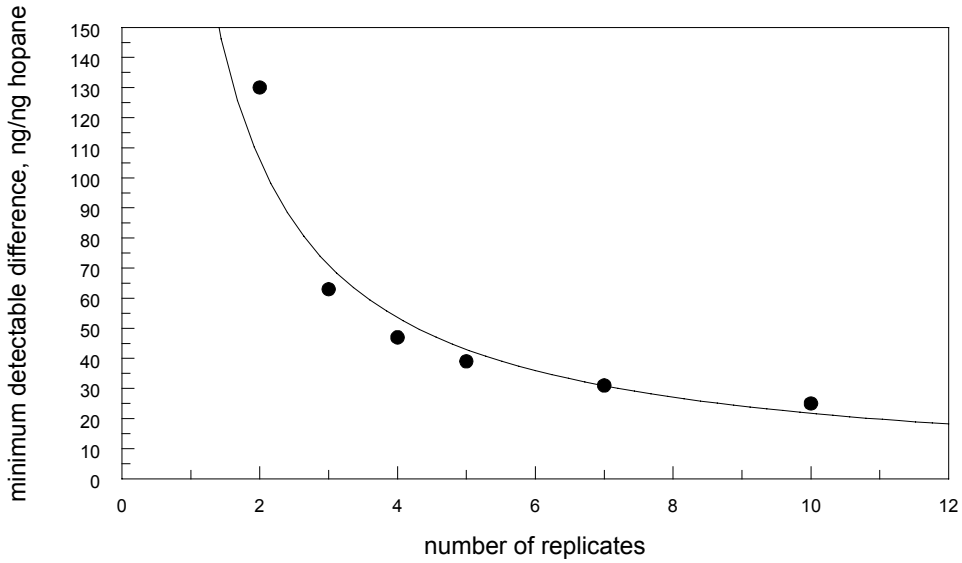


Figure 6.3. Minimum detectible treatment difference as a function of number of replicate plots.

6.1.2 Assessment of physical loss

To better evaluate oil bioremediation performance, one should be able to distinguish between physical loss and biodegradative loss. This requires a mass balance. However, little information is available in this regard for most oil bioremediation field studies and applications due to the lack of comprehensive monitoring programs, reliable measurement tools, and proper data interpretation.

So far, the most complete mass balance of any major oil spill was the *Exxon Valdez* incident. In the early 1990s, Wolfe et al. (1994) undertook a comprehensive monitoring program. They estimated that about 3 years after the spill, approximately 20% of the spilled oil had evaporated and undergone photolysis in the atmosphere; approximately 14% was recovered and disposed of; approximately 2% remained on intertidal shorelines and 13% in subtidal zones. Approximately 30% of the spilled oil was biodegraded in the water column, and nearly 20% was biodegraded on the shorelines. However, the paper did not provide a mass balance for the 113 km of shorelines in Prince William Sound where bioremediation applications (nutrient additions) were performed.

A methodology was proposed by Venosa *et al*. (1996) to conduct a mass balance and to distinguish biodegradation from physical loss of oil in the Delaware field study. As mentioned earlier, spilled oils can be lost from a shoreline by physical washout, dissolution, volatilization, and biodegradation. The method assumes that a nonbiodegradable component of oil (namely, hopane) can be used to estimate the first three loss rates, and that the actual biodegradation rate of an analyte can be estimated from the difference between its total loss rate and its physical loss rate. For this approach to be valid the physical washout rate of the oil must be dominant, and the oil loss due to dissolution and volatilization must be negligible. Volatilization and dissolution can cause some preferential loss of oil components particularly in the early stage of an oil spill (see Chapter 2). However, these factors are unlikely to be important during the bioremediation treatment since bioremediation is typically used as a polishing step after conventional mechanical cleanup options have been applied and is often initiated weeks to months after an oil spill. Wolfe *et al.* (1994) reported that evaporation was no longer an important loss mechanism three months after the *Exxon Valdez* spill.

Figure 6.4 shows the overall first order disappearance of hopane and total extractable organic matter (EOM) based on the results of the Delaware study. Over 90% of the spilled oil was removed from the shoreline through physical washout based on the rate of hopane loss. The EOM first-order rate coefficient was higher than the hopane disappearance rate. The difference in loss rates between hopane and EOM was attributed to biodegradation because EOM includes both biodegradable and nonbiodegradable components. However, because EOM and other total petroleum hydrocarbon (TPH) measurements are not sensitive enough, no differences between bioremediation treatments and control could be determined using this approach in the study. This observation suggests that losses due to bioremediation may not be detectable using TPH analysis. This again demonstrates that the success of bioremediation should be judged by analyzing variables of biological significance, such as the reduction of concentrations of oil components of ecological concern (e.g., PAHs), toxicity of the oil, and ultimately the ability to accelerate the recovery of the oil contaminated ecosystem.

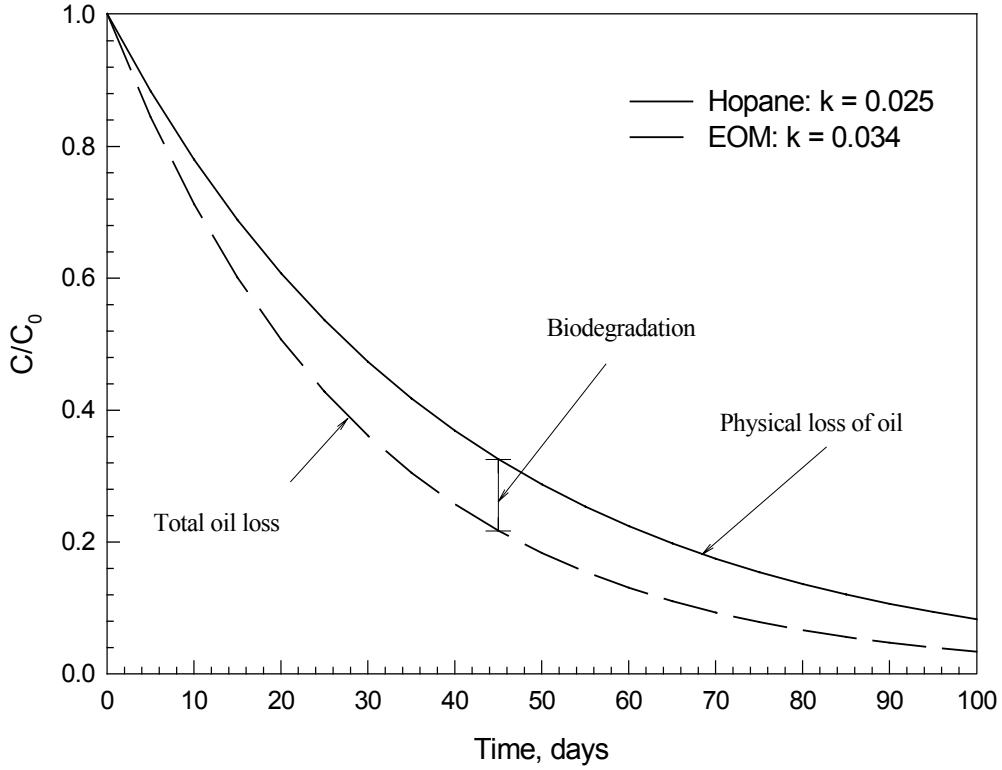


Figure 6.4 Fate of oil disappearance from shoreline during the Delaware study

Another important consideration in assessing physical loss of oil and bioremediation success in a marine shoreline is the effect of shoreline substrate transport. Suidan and Wrenn (2001) found that substantial sand transport occurs over short time scales on marine shorelines, particularly on high-energy beaches, although the net amount of shoreline sediment did not change significantly during the tracer study. Sediment transport will affect oil transport since oiled sand will move with the bulk sand. As discussed in Section 3.2.2, the physical redistribution of oil between the inside and outside of experimental plots can affect interpretation of results from bioremediation field studies that are conducted on shorelines contaminated by real oil spills. Studies of this type often involve treatment of oiled sand in small plots that are surrounded by large areas of untreated, contaminated beach. Because the treated sand will become mixed with untreated sand from surrounding areas of the beach by wave-induced sediment transport, the apparent effectiveness of the treatment will be reduced. Hopane normalization cannot correct for this underestimation of the treatment effect since the hopane concentration inside the plots will not be affected by sand transport. However, this effect will be less important in cases of an actual bioremediation application when the entire contaminated shoreline is treated. The potential effect of shoreline substrate transport on data interpretation in these cases, however, will be to overestimate the treatment effect due to the sand transport from large treated areas to small untreated control areas. Therefore, the control area set aside for assessing oil bioremediation should be either large enough to reduce this effect or relatively isolated from the treated area to

minimize the exchange of sand and nutrients between the two areas. The influence of shoreline substrate transport should always be taken into account in both the design of the sampling plan and the interpretation of data from the field, particularly for high-energy beaches.

6.1.3 Operational endpoints based on oil biodegradation

Bioremediation endpoints for many soil and groundwater sites contaminated with petroleum products are often selected based on predetermined remediation target concentrations, such as 10-10,000 mg/kg TPH and 0.1-500 mg/kg BTEX, which are adopted by various regulatory agencies (King, *et al*., 1998; Salanitro *et al.*, 1997). However, for reasons discussed previously in regard to the inadequacies of TPH analysis, such targets are not appropriate for protecting the environment. It is a good idea, however, for parties involved in a remediation project to have some measurable endpoints for management and regulatory purposes. Based on existing experiences, the following criteria are suggested in determining bioremediation endpoints with respect to oil biodegradation.

- Bioremediation treatment should be terminated when the extent of oil degradation tends to level off based on oil analysis results. Cost-benefit analysis should be used in establishing target bioremediation levels. It is unrealistic and uneconomical to remove all traces of oil hydrocarbons using bioremediation technologies.

- The concentrations of target oil analytes can also be used as endpoint indicators, particularly when the treatment is highly effective. Emphasis should be given to those chemicals of environmental concerns, such as PAHs. The target concentrations should be agreed upon in the treatment plan and can be determined based on existing standards used for other environments (e.g., oil contaminated surface and subsurface soils, Bell *et al.*, 1994).

- The change in oil composition may also help to establish the bioremediation endpoint. As oil becomes more biodegraded, the fraction of less biodegradable components (e.g., resins and asphaltenes) in the oil become enriched. Studies following the *Exxon Valdez* spill showed that oil biodegradation slowed substantially when the polar content of the North Slope crude oil reached 60-70% of the total mass (Bragg *et al*.,1994). Therefore, the polar fraction as a percent of the total oil mass remaining may potentially serve as an endpoint indicator although more research is still required to establish quantitative criteria. If rapid removal of the resin and asphaltene fractions of the oil is the desired endpoint, the only way to achieve this is by excavation and hauling to a contained or secure facility. That is because removal of these constituents in nature is known to occur only through dispersion and dilution. The rate of these processes can be very site specific and depend heavily on the type of substrate (i.e., cobble, sand, etc.) and wave energy.

6.2 Environmental Assessments

Bioremediation is among the least intrusive of the current operational physical and chemical oil spill countermeasure options available. Nevertheless, apprehension remains about the environmental impact of bioremediation agents released into the environment (Hoff, 1993; Holloway, 1991; Lee *et al.*, 1995a; Office of Technology Assessment, 1991). In addition to

potential effects on wildlife and humans, there is concern that the by-products of enhanced oil biodegradation may be more toxic than the parent compounds. For general acceptance of bioremediation as an oil spill countermeasure, we must demonstrate that it does not induce negative effects that suppress the rates of natural habitat recovery. Environmental assessment should be an integral part of guidelines governing the application of bioremediation treatments to ensure protection of the environment.

Both ecosystem structure and function must be considered in environmental assessments. Ecosystem structure is studied by examining species abundance, biomass, and diversity and other components at one point in time. Bioassessment field surveys (Chapter 3.5.1) provide this basic information on ecosystem structure. Ecosystem function describes the dynamics or changes in the system over time. Information on ecosystem function is provided by the quantification of rates of biological processes like production, respiration, mineralization, and nutrient regeneration. In addition, bioassays (Chapter 3.5.2) provide a means of quantifying the potential effects of toxicants on ecosystem function.

6.2.1 Operational guidance from environmental assessments for treatment application

Controlled studies suggest that during remedial operations, optimal rates of degradation in sediments can be achieved by sustaining elevated interstitial nutrient levels that do not elicit an adverse effect (Lee *et al*., 1993; Swannell *et al.*, 1996; Lee *et al*., 1997b; Venosa *et al*., 1996; Boufadel *et al*., 1999b). Bioassessment field surveys can be used to guard against detrimental effects such as the stimulation of toxic algal blooms associated with eutrophication.

Environmental assessments can also provide guidance for bioremediation by pinpointing the optimal time for the onset of treatment. It has become clear in numerous studies that treatments have limited or no success as long as the residual oil is retaining its most toxic compounds, which are typically low molecular weight compounds that are removed through natural weathering processes. In such a case, it is better to wait one to two weeks before treatment to allow for toxicity levels to decline, which can be delineated by time-series monitoring of sediment or water toxicity using bioassays.

The results of toxicity tests have been used to explain the mode of action and performance results of commercial bioremediation agents containing biostimulation (nutrients) and bioaugmentation (bacterial inocula) properties. Lee *et al.* (1997b) observed that increases in microbial activity following treatment are not necessarily correlated with toxicity reduction or habitat recovery. Natural attenuation was more effective because the bioremediation agent inhibited the physical loss of residual oil from the sediments.

Standard bioassay test protocols are now being developed by regulatory agencies for toxicity evaluation of oil spill bioremediation agents (Thomas *et al.*, 1995; Blenkinsopp *et al.*, 1995). Field application of bioremediation products should be limited to those that have passed regulatory screening procedures for performance and toxicity. For a conservative approach, it is recommended that feasibility studies be conducted prior to full-scale operations. This can be accomplished by conducting contaminant biodegradability studies in the laboratory and monitoring the effectiveness of the proposed treatment on several untreated but oiled shoreline

segments. If the desired treatment endpoint (reduction of residual oil concentration or enhanced rate of habitat recovery) has been identified, chemical analysis and bioassays can be used to quantify the efficacy of bioremediation treatments.

6.3 Case Study: Environmental Assessment of Bioremediation Treatments in a Tidal Freshwater Marsh

Since environmental assessment is a relatively new approach in evaluating the effectiveness of oil bioremediation treatments, a case study is presented in detail here to help spill responders better understand and conduct this type of assessment. To evaluate the efficacy of nutrient amendment and phytoremediation as bioremediation strategies, a controlled oil spill field trial was recently conducted in a tidal freshwater marsh on a shoreline of the St. Lawrence River, Canada to determine if nutrient enrichment would enhance the rates of residual oil loss and habitat recovery (Blanco-Garcia *et al.*, 2001b; Venosa *et al.*, 2002; Lee *et al.*, 2001a). The experimental design and bioremediation performance with respect to oil biodegradation has been reviewed in Chapter 2 (See Section 2.5.2). Environmental assessment of the extent of habitat recovery, which included a suite of bioassays for the identification of possible detrimental treatment effects (e.g. toxicity of the bioremediation agent or oil degradation by-products), is described as follows.

6.3.1 Alterations in ecosystem structure

Vegetative recovery of the predominant plant species (*Scirpus pungens*) was monitored by determining changes in species composition (predominant *S. pungens* and secondary *Eleocharis palustris*), total biomass, height, and percent cover. During the first growing season *S. pungens* tolerated the experimental oil concentrations, but suffered oil-induced growth inhibition (Figure 6.5), which is consistent with the results of recent greenhouse studies (Longpré *et al.*, 1999). The effects of growth inhibition by oil were still evident within natural attenuation plots at Week 65. The enhanced recovery of the vegetation in nutrient amended plots during the second field season could not be attributed to the concentration of nutrients in the porewater, as they had diminished to background levels before the previous winter. Analysis of nitrogen content within the roots of plants suggested that the abundant growth was attributable to the recycling of organic nitrogen stored by these plants over the first season.

With the reduction of residual oil concentrations (primarily by physical removal) and regrowth of the predominant vegetative species, extensive recolonization by indigenous invertebrate species such as the mystery snail (*V. georgianus*) was also observed during the second field season in all experimental plots (Week 45).

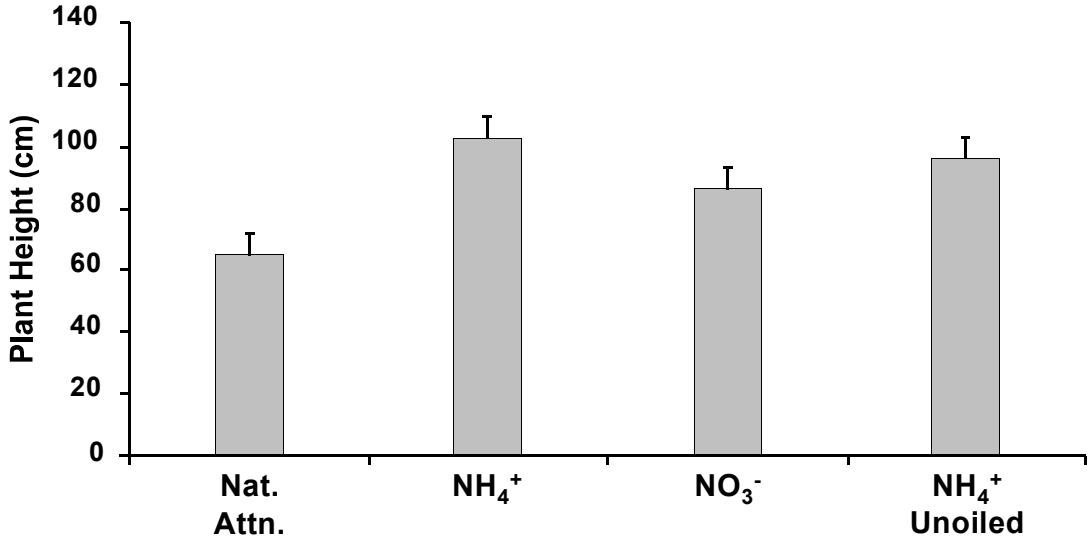


Figure 6.5 Average height of the predominant plant species (*Scirpus pungens*) at Week 15 in three oiled treatments (Nat. Attn., NH_4^+, NO_3^-) and the unoiled but fertilized (NH_4^+) treatment.

6.3.2 Alterations in ecosystem function

The environmental impact of contaminants in aquatic ecosystems cannot be assessed accurately with a single species bioassay, which cannot represent the range of sensitivity of all biota. To address this issue, a test battery with organisms from several trophic levels was used: (1) microbial response; (2) Microtox solid phase; (3) algal solid phase; (4) cladoceran survival; (5) amphipod survival; (6) gastropod survival/histopathology; and (7) acute and chronic effects in fish.

6.3.2.1 Microbial response

Concentrations of bacteria are up to seven orders of magnitude higher in the surface sediments than in the water column. This concentration of microbial activity makes sediments the most active site for transformations of organic carbon, nitrogen, phosphorus, magnesium, and sulfur. If the processes of decomposition, mineralization, and nutrient regeneration are disturbed by contaminants, the nature of the ecosystem will be changed. Despite the importance of these and other processes concentrated in the sediments, relatively few assessments have focused on functional changes attributable to sediment-associated contaminants.

Detailed studies on the microbial response to bioremediation treatments within experimental enclosures were conducted by Greer *et al.* (2000). The viable bacterial population density showed a slight increase during the first 4 weeks following oil and fertilizer application. The increase was clearly due to the fertilizer, as evident from the contrasting population densities in untreated areas which remained relatively unchanged throughout the monitoring period. Patterns of hydrocarbon mineralization activity and distribution of hydrocarbon-degrading microorganisms can be used as an indication of *in-situ* biodegradation of petroleum (Braddock *et al.*, 1996). The microbial populations demonstrated a rapid and sustained increase in naphthalene mineralization activity in the plots that were both oiled and fertilized. Activity was somewhat lower in unfertilized/oiled and fertilized-only plots. Hexadecane mineralization

activity increased in response to fertilizer application, especially ammonium nitrate, in comparison to sodium nitrate: activity in the unfertilized/oiled plots and unoiled reference control areas remained relatively low. Laboratory assays to monitor various pathways in the nitrogen cycle (nitrification, denitrification, nitrogen fixation) have been developed (Pritchard and Bourquin, 1985) and can be used to assess changes in function due to contaminant stress. Field and laboratory evaluation of nitrogen metabolism indicated significant denitrification activity in sediments following fertilizer application, which was not adversely affected by oiling. In contrast to the results of chemical analysis that showed no treatment effect on oil biodegradation rates, the results demonstrated that the application of fertilizers stimulated the activities of indigenous hydrocarbon degrading and denitrifying bacteria, and the presence of oil did not have a detrimental effect on these activities.

6.3.2.2 Microtox solid phase test

In the Microtox® Solid Phase Test (Microbics Corporation 1995), the bacterium, *Vibrio fisheri*, was exposed to oiled sediment. A significant decrease in bioluminescence relative to water-only controls was indicative of sediment toxicity. Toxicity levels were calculated as the concentration of sample that would result in a 50% reduction in luminescence ('effective concentration,' EC_{50}). To account for interferences from differences in sample grain size distribution, turbidity, and to a lesser extent, color of the sample dilutions, sample test results were compared with results from unoiled sediments from the immediate study area.

Oil toxicity was evident on comparison of oiled with unoiled plots (Figure 6.6). While the fertilized plots showed a trend towards a reduction in toxicity, there was no such evidence in the natural attenuation plots over 65 weeks. Major treatment effects were not observed in the first field season. However, in the second field season the relative toxicity levels in nutrient amended plots were similar to values of the unoiled controls.

6.3.2.3 Algal solid phase assay

An algal solid-phase assay (ASPA) was used to assess the toxicity of sediments recovered from the experimental test plots (Blaise and Ménard, 1998). The endpoint for this assay is based on the concentration of sediment causing 50% inhibition (IC_{50}) of esterase enzyme activity in *Selenastrum capricornutum* due to toxicants. This test is an excellent biomarker for environmental assessments, as esterases are a key group of ubiquitous enzymes found in both plants and animals (Dorsey *et al.* 1989 ; Gala and Giesy 1990).

Results of the algal solid-phase assay (Figure 6.7) showed that there was no toxicity in the unoiled reference plots throughout the course of the experiment (IC_{50} > 7%), and that nutrient additions to unoiled sediment appear to cause no detrimental effects. For the first 21 weeks, elevated levels of toxicity were observed in all the oiled plots. Significant reduction in sediment toxicity was not observed until the second field season. At this point, a marked decrease in toxicity was observed for the oiled plots amended with nutrients (either ammonium nitrate or sodium nitrate). Nutrient amended plots were deemed non toxic by Week 65. While nutrient treatments were terminated at the end of the first field season, treatments resulted in significant positive effects that were not observed until the second field season. This lag suggests that toxicity reduction may be correlated with enhanced vegetative growth associated with nutrient enrichment.

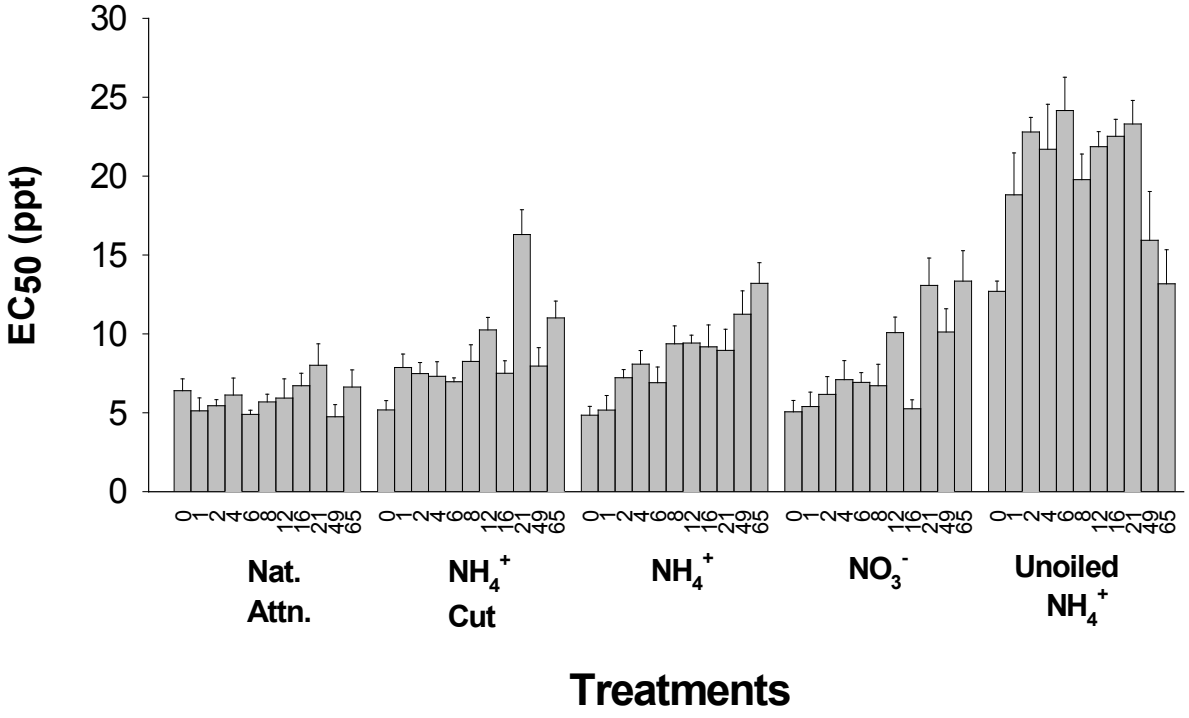


Figure 6.6. Time-series changes in sediment toxicity as quantified by the Microtox® Solid Phase Test. Error bars = 1 standard deviation

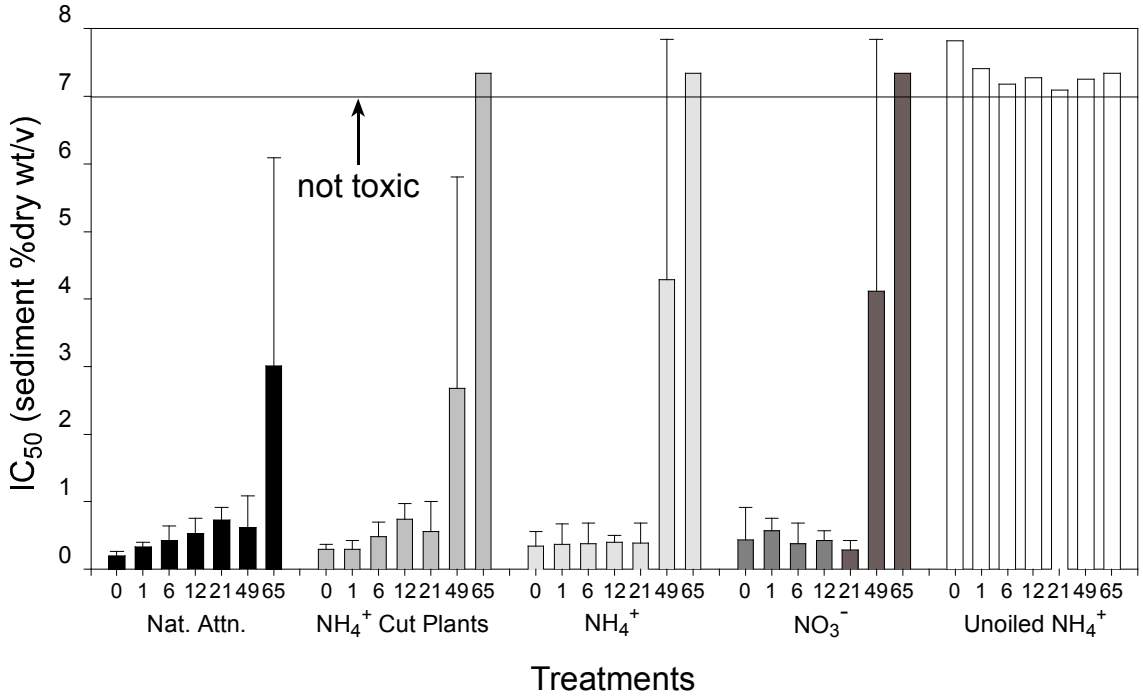


Figure 6.7. Algal solid phase assay (ASPA) as IC_{50} for sediment samples collected at Weeks 0, 1, 6, 12, 21, 49 and 65. Bars represent means ± standard deviation.

6.3.2.4 Cladoceran survival test

The cladoceran *Daphnia magna* ('water flea') was used to assess the toxicity of elutriates from sediment samples (Environment Canada, 1990). Treatment of unoiled plots with ammonium nitrate and super triple phosphate appeared to cause negligible impacts on the survival of *D. magna* (Figure 6.8a). In contrast, immediate, but limited, toxic responses were observed in all oiled plots. Ammonium nitrate and triple super phosphate additions appeared to reduce residual oil toxicity to background levels by Week 2. Amendments with sodium nitrate appeared less effective. Since the sensitivity of this assay appeared to be limited — the effect of residual oil was deemed negligible by Week 6 — this assay was excluded from the second field season.

6.3.2.5 Amphipod survival test

The Amphipod Test measured the effects of sediment samples on survival of sediment-dwelling *Hyalella azteca* neonates, 2-9 days old (Environment Canada, 1997). Both the mean percent survival and the mean weight of animals in each treatment were compared with mean percent survival and mean weight of amphipods in reference control sediments to determine if the treatments caused a significant decrease in organism survival or growth.

Hyalella azteca mortality (Figure 6.8b) was a more sensitive bioassay endpoint (i.e. higher response) than *Daphnia* mortality (Figure 6.8a). This may be due to species differences and the fact that the Amphipod Survival Test is a direct-contact sediment test. Oil derived toxicants within the sediment may not have been effectively transferred to the elutriate used in the cladoceran test. Furthermore, in a laboratory test, the uptake of sediment-associated anthracene by the freshwater amphipod *Hyalella azteca* was reported to occur at a rate much higher than predicted. It was concluded that selective feeding of *H. azteca* on smaller particles results in a diet of fines containing the highest organic matter concentration and, hence, contaminant levels (Landrum and Scavis, 1983). *H. azteca* mortality was consistently lower in the unoiled nutrient amended plot. High mortality was observed during the first week in all oiled plots. The presence or absence of plants appeared to have no significant effect.

At the latter part of the first field season there appeared to be a pronounced increase in sediment toxicity in the oiled plots amended with prilled ammonium nitrate. The sensitivity of *H. azteca* to ammonia (LC_{50} = 14.9 mg-N/L) was verified in the laboratory. Many samples, notably Weeks 12 and 21, had overlying water ammonia levels exceeding the established toxicity limits, with the highest values corresponding to the oiled plots amended with ammonium nitrate. The fertilized unoiled site had lower elevated ammonia levels. This and a possible synergistic effect between oil and elevated NH_4^+ concentrations may explain the observation of little or no toxic response observed in the fertilized unoiled plots (Figure 6.8b). In response to a reduction in residual oil concentrations, percentage mortality was near background levels by the second field season. The results of this bioassay suggested that the experimental treatments offered little advantage on an operational scale.

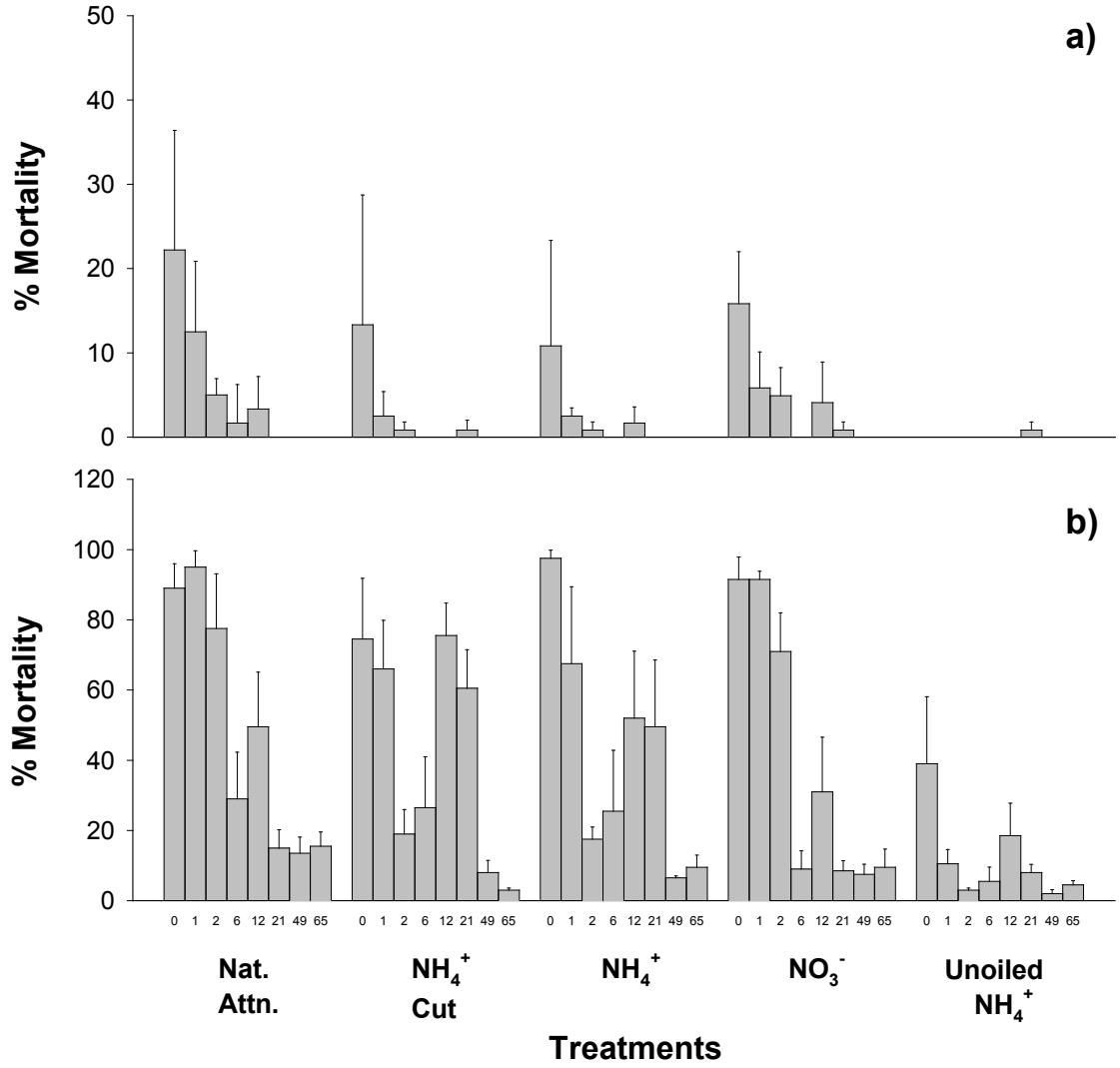


Figure 6.8 Time-series changes in sediment toxicity as quantified by: a) Cladoceran Survival Test; b) Amphipod Survival Test. Error bars = 1 standard deviation

6.3.2.6 Gastropod survival/histopathology

Although many organisms have been used as sentinels or biomonitors of environmental contaminants (LeBlanc and Bain, 1997), there is still a need to identify and exploit alternative species that are sensitive and amenable to ecotoxicological testing. Molluscs are abundant and widely distributed and their use as *in situ* biomonitors has been on the rise (Lagadic and Caquet, 1998). In this study (Lee *et al.*, 2001b), gastropod survival and histopathology assays were conducted with the mystery snail, *Viviparus georgianus*. They were specifically selected for use as an *in situ* biomonitor as they feed on sediment detritus, algae and decaying organic matter within the wetland. Snails (n = 50/treatment/sampling time) were caged within 20 × 20 × 22 cm open mesh polypropylene baskets moored to the sediment surface of experimental plots, and in designated 'untreated control' areas within the vicinity of the plots. Cages were recovered after 30, 60 and 90 days of exposure to evaluate effects on survival at the end of the second field

season (Week 65). Healthy snails were also exposed for a 30 day period to test sediments recovered from the plots, under laboratory conditions, to determine survival rates.

Significant growth ($p < 0.001$) of the snails was observed during the study, but no significant differences ($p > 0.05$) were observed in tissue weight, shell size or shell thickness between the experimental treatments. Under adverse conditions *V. georgianus* is known to retract into its shell and show no motility for prolonged periods, so animal vitality was assessed by the presence of an operculum. No surviving snails were found in any of the continuous exposure cages after three months (Figure 6.9). To factor out the influence of stress from the caging of animals for extended periods, data at each sampling event were normalized to unoiled control plots adjacent to the test blocks, which received no oil or nutrient amendments (4%, 47% and 100% mortality at 30, 60 and 90 days, respectively). The only oiled treatment that gave a higher percentage survival after 30 days exposure was the ammonium nitrate amended plots with intact plants. The other treatments were more toxic than the natural attenuation plots. Elevated nutrient concentrations for 60 days did not reduce the toxic effects of the oiled sediments to *V. georgianus*. Indeed, exposure to the treatment probably exacerbated stress.

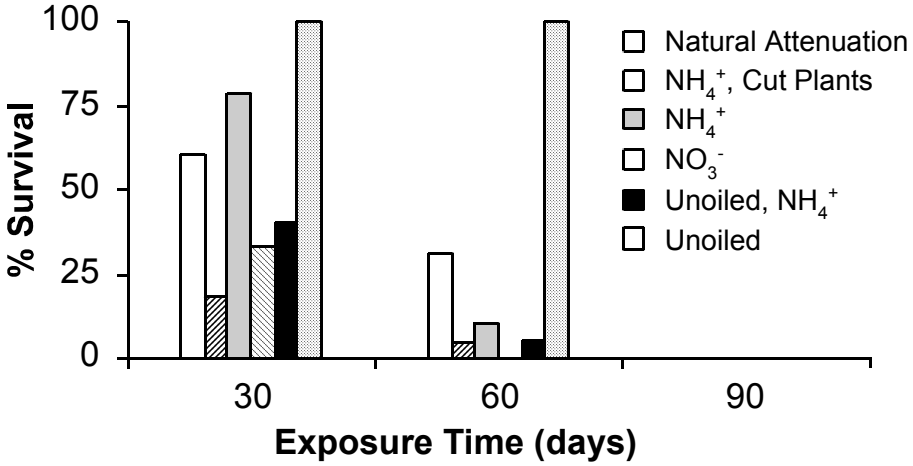


Figure 6.9 Percent survival of the mystery snail, *Viviparus georgianus*, following exposure to test treatments for 30, 60 and 90 days. Results normalized to survival in unoiled sediment without nutrient amendment.

Toxicity of nitrogenous compounds at high levels has been noted with various organisms. Among invertebrates, ammonia toxicity was assessed with a freshwater snail (Hickey and Vickers 1994). Acute values were derived for the snail to be 0.15 g/m ammonia compared to the EPA value of 0.52 for salmonids, and they found that the snail was more sensitive than the normally accepted sensitive species such as mayflies and stoneflies. After 30 days of continuous exposure, the highest mortality was reported in the fertilized plots with plants cut back. Direct observations suggested that these animals suffered from harsh conditions in the absence of natural ground cover. All treatments containing nutrients with or without oil caused higher mortalities than no oil or oil alone. Laboratory exposures using sediment recovered from the experimental plots at Week 65 (which had less oil, and of lower bioavailability) showed that the application of treatments in the first season had insignificant long-term effects (Figure 6.10).

Based on the results of the amphipod and snail bioassays, bioremediation using nutrients *in situ* will need to take the above into consideration.

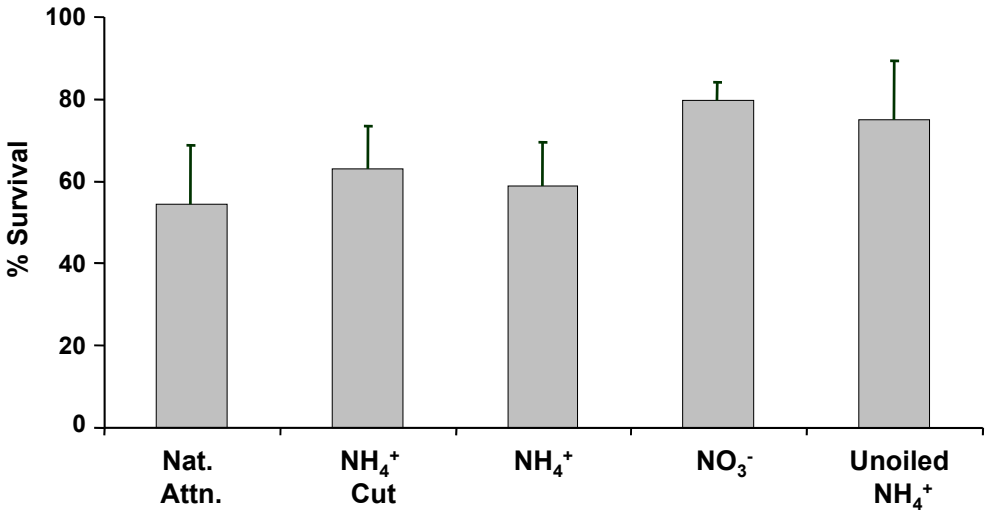


Figure 6.10 Percent survival of the mystery snail, *Viviparus georgianus* under laboratory conditions following 30 days of exposure to sediments sampled at Week 65. Error bars = 1 standard deviation

When organisms are exposed to xenobiotics, cellular changes have been observed to occur. Evaluation of histological changes can provide important information as to the stress of the organism and mode of action of pollutants. Specimens of live snails were preserved for histological analysis. All tissues showed degenerative changes over time in snails exposed to fertilizers. Pathological changes were most obvious in epithelial cells lining such tissues as gills, intestine and digestive gland, but some degenerative changes were also observed in other organs such as gonads. The most dramatic changes were observed in snails from plots treated with sodium nitrate (Figure 6.11). Effects of treatment on reproductive success of *V. georgianus* were noted. Gravid females contained on average 10 young (range 3 to 15) visible with the naked eye. At 1-week post oil exposure, gravid females showed some degenerating embryos in all but the control treatments. This was also true for some of the 1-month post exposure gravid females, but number of gravid females with macroscopic embryos decreased.

6.3.2.7 Acute and chronic effects on fish

Experience with the *Exxon Valdez* demonstrated that oil deposition in shoals where pink salmon (*Oncorhynchus gorbuscha*) and Pacific herring (*Clupea pallasi*) spawn, caused blue sac disease (BSD) of newly hatched larvae (Hose *et al*., 1996; Marty *et al.*, 1997). BSD is characterized by yolk sac and pericardial edema, hemorrhaging, deformities, and induction of mixed function oxygenase enzymes (Marty *et al.*, 1997; Billiard *et al.*, 1999; Fragoso *et al.*, 1998). As a consequence, there is considerable interest in the application of bioremediation strategies that accelerate the removal of oil from intertidal beaches, which provide the nursery habitat for many fish species. The success of these technologies should be judged not simply on how quickly oil disappears, but on a demonstrated reduction in risk, i.e. on how quickly toxicity (hazard) and exposure to oil are reduced.

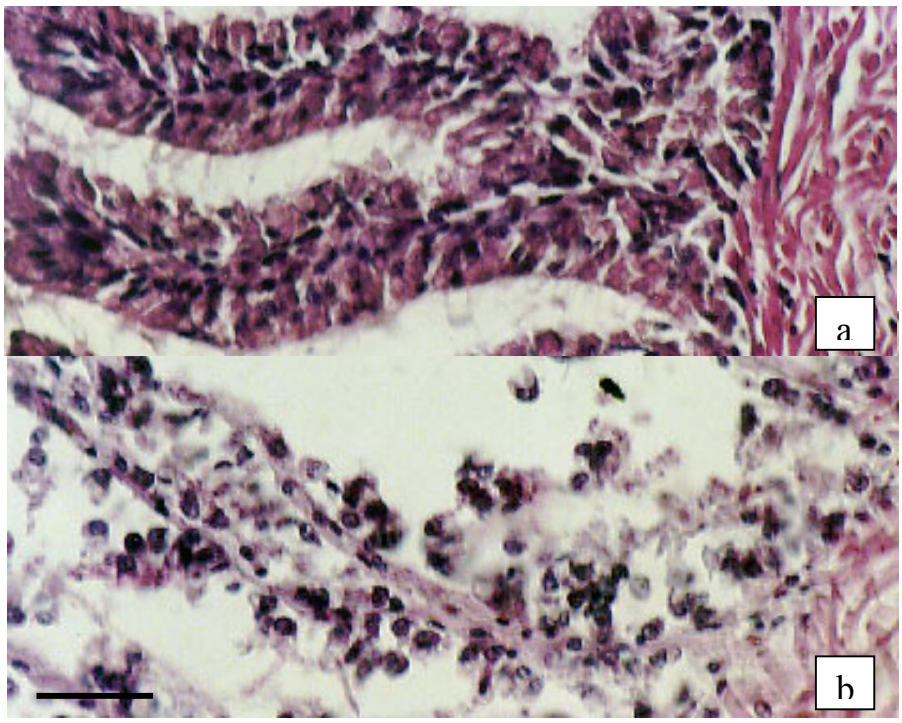


Figure 6.11. Representative sections through the intestine of Viviparus showing normal villi seen in control snails (a) and degenerated villus with hemocytic infiltration seen in snails from the sodium nitrate treatment after 1 month exposure (b). Bar = 75 µm.

Establishing the exposure of fish to oil is difficult. While chemical measures of oil in sediments, water and tissues are routine, there is no guarantee that fish accumulate oil or its components equally or in proportion to environmental concentrations. Further, many of the components of oil (e.g. alkanes, polynuclear aromatic hydrocarbons (PAH)), are metabolized by fish, so that chemical analyses of fish tissues may not represent the true dose or dose rate.

Assessing hazard is equally difficult. Oil is a complex mixture, and many of its components have different modes of toxicity. At acutely lethal concentrations, mortality is rapid and is likely due to narcosis caused by monoaromatics (benzenes, toluenes, xylenes, etc.) and alkanes. At lower concentrations (Billiard *et al.*, 1999), chronic toxicity (BSD) may be linked to the concentrations of alkyl PAH such as 7-isopropyl-1-methylphenanthrene (retene, a C-4 phenanthrene). This mechanism may involve metabolism of alkyl PAH to more toxic forms by CYP1A enzymes. Toxicity and rate of excretion of phenanthrene and retene can be modulated by inhibiting or inducing CYP1A activity (Hawkins *et al.*, 2000). Delayed responses, such as the long-term onset of cancer, may also follow brief exposures to pro-carcinogens such as benzo(a)pyrene (BaP). In this case, the mechanism involves oxygenation of BaP by CYP1A enzymes to carcinogenic diols and epoxides, which cause genotoxicity by forming adducts with bases of DNA (Varanasi *et al.*, 1989).

In this case study, bioassays of CYP1A induction in juvenile rainbow trout (*Oncorhynchus mykiss)*, BSD in embryo-larval stages (ELS) of trout, and reproductive developmental studies

with Japanese medaka (*Oryzias latipes*) were used to evaluate the presence, bioavailability, and toxicity of PAH (and parent mixture of oil) in contaminated beach sediments (Hodson *et al.*, 2001). Changes in bioavailability and toxicity with time were monitored by analysis of time-series samples.

The key to sediment assessment is bioavailability. Although sediments might contain relatively high concentrations of toxic compounds, this condition does not necessarily lead to adverse effects on organisms living in the sediments (Payne *et al.*, 1988). The only means of measuring bioavailability is by measuring or determining biological response. Such testing has often involved measures of bioaccumulation (the ability of an organism to accumulate contaminants in tissues. However, because bioaccumulation is a phenomenon, not an effect (and can be relatively expensive due to costly chemical analyses), emphasis has shifted towards indicative endpoints that are based on sediment toxicity tests, which are effects-based and relatively inexpensive.

Bioavailability was assessed by the extent of CYP1A induction in fingerling trout after a 4 d exposure to sediments. S-9 fractions were prepared from liver homogenates and activity of ethoxyresorufin-o-deethylase (EROD, CYP1A enzyme) was measured by a kinetic microplate fluorescence method following the protocols outlined by Hodson *et al.* (1996). Each bioassay included negative controls (water only), unoiled sediment controls, and positive water controls (fish exposed to ß-naphthoflavone, a model inducer). Results indicated that one day after oiling, EROD activity of fish exposed to oiled sediments was on average 25-fold higher than that in the control sediments (range = 13-35-fold). Over a 17 month period, there was about an 80% decline in induction potency by oiled sediments sampled from the beach. The decline in EROD activity paralleled declines in total hydrocarbons, total PAH, and total alkyl-PAH, measured by GC/MS with correlation coefficients between log of EROD activity and log of hydrocarbon concentrations of 0.96 or higher (Hodson *et al.*, 2001).

Blue sac disease of trout was assessed by exposing 50 eyed-eggs (about 15 d post fertilization) to sediments until they had hatched, resorbed the majority of their yolk sac, and begun to swim up (Zambon *et al.*, 2000). After 32 d of exposure, larvae were removed and the % survival and prevalence of the symptoms of BSD (edema, hemorrhaging, deformities) were measured (Guiney *et al.*, 1997). Results showed that trout embryos and larvae exposed to oiled sediments from Ste. Croix exhibited a low prevalence of symptoms of BSD, although rates were higher than observed for fish exposed to the reference sediments. All oil-exposed fish examined histologically demonstrated intense staining for CYP1A protein, indicating a significant exposure to CYP1A-inducing compounds such as PAH. These preliminary results suggested that symptoms of BSD were more frequent in fish exposed to oiled than to un-oiled sediments, indicating a risk to early life stages of species that spawn on tidal beaches.

Medaka larvae exposed to oiled sediments for 90 days (maturity) experienced a higher mortality rate (42.5± 4.2%) than those exposed to un-oiled sediments (9.1±7.4). The growth of surviving medaka in the oiled sediment treatments was impaired (Figure 6.12), as shown by their smaller size compared to controls (20.6±1.3 vs 16.4±3.3 mm). In addition, nearly all medaka from the oiled sediment treatments had deformed or missing fins. Preliminary histological examination of

fixed medaka indicated a degenerative liver lesion, *spongiosis hepatis*, in >95% of the fish from the oiled-sediments, but not in controls. There was also a low incidence of male medaka (<10%) with intersex gonads (testis-ova) in fish exposed to oiled sediments.

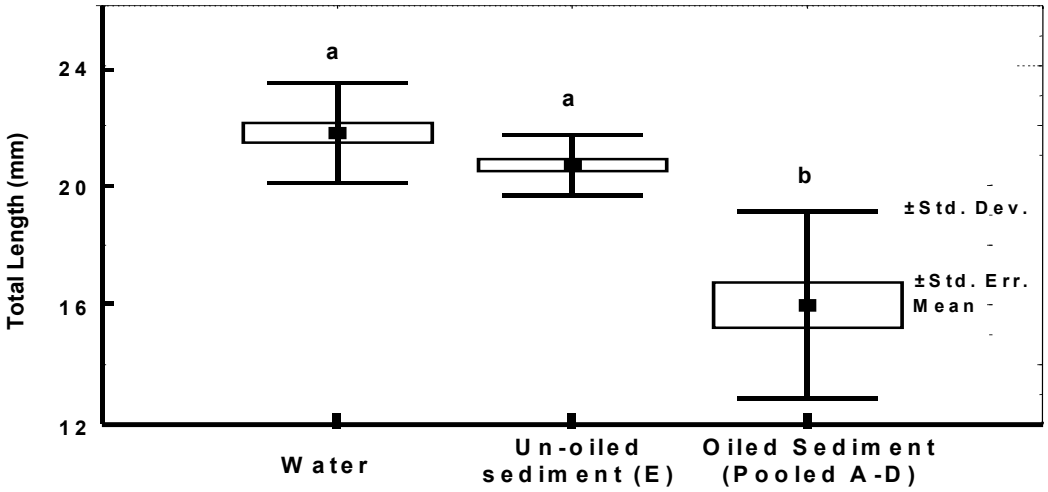


Figure 6.12. Effect of exposure to Ste. Croix oiled sediment (Oct. 1999) on growth of medaka. Treatments sharing the same letters are not statistically different.

The laboratory fish bioassay represents a 'worst-case' scenario as the fish could not avoid exposure. Furthermore, mixing of the sediments in the test chambers might have disrupted the top-most sediment layer (that might have been depleted of oil in the field by weathering) and exposed more oily layers of sediment. The ratio of water to sediment is also fixed, which is very different from the situation in well-flushed tidal beaches. Finally, the test organisms were not beach spawning species of fish such as smelt, capelin, and herring.

Semi-permeable membrane devices (SPMDs) were used to assess *in-situ* changes in the bioavailability and toxicity of residual oil components following treatment. These passive *in-situ* samplers, developed by Huckins *et al.* (1990), contain purified triolein, a substance that constitutes a major fraction of the neutral lipid of fish. When immersed in water, SPMDs absorb non-ionic, organic chemicals having a log K_{ow}>1, a size <1 Å, a molecular weight of about 600 or less, and possibly neutral organo-metal complexes. These characteristics correspond to that of known mixed-function oxygenase (MFO) inducing compounds, including PAHs. The operational advantage of SPMDs is that they can be deployed within the environment to provide an integrated sample over time. This characteristic makes the assay highly advantageous for field use. In terms of ecological relevance, the diffusion of dissolved neutral organic chemicals into the triolein through the pores of the polyethylene membrane within SPMDs simulates the diffusion of compounds across a live fish gill membrane. The lipid can be analyzed by traditional chemical techniques to provide a list of chemicals absorbed, their concentrations in

the SPMDs, and, by back calculation, their concentrations in water or sediment. In this study SPMDs were used as concentrating devices for biological testing with the Microtox® Assay.

SPMD units (12.5 cm) enclosed in protective cases were deployed at the water sediment interface at predetermined intervals and recovered for analysis after one week of exposure. SPMD concentrated samples were diluted with organic solvents for toxicological analysis by Microtox. The results showed that by 4 weeks, the toxicity of sediments within the oiled plots had declined to the levels of the unoiled but fertilized control as the result of a reduction in bioavailability (Johnson *et al.*, 2000).

6.4 Ecotoxicological Tests for Risk Assessment

The application of *in-situ* bioremediation operations is not expected to generate a large volume of waste materials like *ex-situ* operations do. If the program is effective, any residual hydrocarbons will elicit little or no biological effect due to physical, chemical, and biological processes that reduce their bioavailability. As illustrated in the case study, sediment quality can be assessed by a number of methods that tend to fit into one of five categories: sediment chemistry, sediment toxicity, community structure, tissue chemistry, and pathology. Ideally, all five components would be utilized to assess sediment quality. However, in reality, environmental managers are faced with limitations in both resources and time. They must optimize use of their resources by selecting the information that will have the greatest utility. Thus, monitoring programs should be focused on the measurement of variables that allow quantification of treatment success against a pre-defined endpoint. Project coordinators must strive to strike a balance between level of effort and type or quality of information needed to make effective decisions.

While detrimental effects have not been linked to the application of bioremediation strategies based on nutrient enrichment in actual spill response operations (Mearns *et al.*, 1997; Prince, 1993), the results of recent field trials clearly demonstrate that the possibility exists. In the case study presented (Section 6.3), improper application of bioremediation agents (the addition of excess fertilizer) was detrimental to the environment. For example, synergistic effects between ammonia and the test oil were observed in the Amphipod Survival Test. However, the nutrient additions had no effect on the unicellular algal species, *Selenastrum capricornutum*, and actually enhanced the growth and productivity of the dominant plant species within the oiled plots relative to those that received no treatment. Rapid recovery of vegetation is critical within an impacted wetland for erosion control. Depending on the desired endpoint of the remedial operation, a balance must be made among the many positive (e.g. enhanced recovery of vegetation) and negative effects of treatment (e.g. changes in productivity, species composition, and diversity among the remaining wetland plants).

With refinement, bioassays can be used in oil spill operations to provide real-time guidance to the treatment operations (e.g. determining the optimal nutrient concentration that does not elicit a detrimental effect), to verify the success of countermeasures and to quantify the extent of habitat recovery. The demonstration of species dependent responses in previous investigations and the current case study suggest that future environmental risk assessments be based on a multi-species, multi-trophic level test battery approach. The results of the ecotoxicological tests should

be used to build an ecological risk assessment — an estimate of the probability of harm to the aquatic environment derived from the synthesis of results of separate exposure and effects components in a scientific manner (Gentile *et al.*, 1989). It is often stated that the objective of oil spill countermeasures are to return an impacted site to its immediate pre-spill condition. This is an unrealistic goal as the environment is a dynamic rather than a static system. On an operational scale, our goal should be to return the structure and function of an ecosystem to within the limits of pre-defined, acceptable criteria.

6.5 Ecotoxicological Tests to Identify Operational Endpoints

The effectiveness of oil spill countermeasures ultimately must be judged by their ability to reduce injury to aquatic life. The worse case scenario would be the use of a response method that is not effective in reducing exposure and increases injury to aquatic life. In bioremediation operations the application of ecotoxicological monitoring protocols may be used to verify the efficacy for toxicity reduction over that of no treatment.

Bioremediation treatments should be terminated when it is deemed that: (1) treatments offer no operational advantage over natural recovery, (2) the contaminant concentrations and toxicity values are reduced to acceptable levels, or (3) detrimental effects from the treatment strategy are identified. Cost-benefit analysis should be considered in the decision of the acceptable level. It is futile to expect bioremediation techniques to remove all traces of residual hydrocarbons. In terms of ecological relevance, declaration of habitat recovery can be made when toxicity limits are within regulatory guidelines and there is evidence for the return of the original community structure (Lee *et al.*, 1995b; Mearns *et al.*, 1995).

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