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GUIDELINES FOR THE BIOREMEDIATION OF OIL-CONTAMINATED SALT MARSHES

by

[1]Xueqing Zhu, [2]Albert D. Venosa, [1]Makram T. Suidan, and [3]Kenneth Lee

[1]University of Cincinnati
Cincinnati, OH 45221

[2]U.S. Environmental Protection Agency
National Risk Management Research Laboratory
Cincinnati, OH 45268

[3]Department of Fisheries and Oceans-Canada
Bedford Institute of Oceanography
Dartmouth, Nova Scotia B2Y 4A2

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Task Order Manager
Albert D. Venosa
Land Remediation and Pollution Control Division
National Risk Management Research Laboratory
Cincinnati, OH 45268

National Risk Management Research Laboratory
Office of Research and Development
U.S. Environmental Protection Agency
Cincinnati, OH 45268

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Lawrence W. Reiter, Acting Director
National Risk Management Research Laboratory

EXECUTIVE SUMMARY

Salt marshes are among the most sensitive ecosystems and, therefore, the most difficult to clean. Applications of some traditional oil spill cleanup techniques in wetland habitats have caused more damage than the oil itself. The objective of this document is to present a detailed technical guideline for use by spill responders for the cleanup of coastal wetlands contaminated with oil and oil products by using one of the least intrusive approaches – bioremediation technology. This manual is a supplement of the previously published “Guidelines for the Bioremediation of Marine Shorelines and Freshwater Wetlands” (Zhu *et al*., 2001), which has focused on the bioremediation of sandy marine shorelines and freshwater wetlands. This guidance document includes a thorough review and critique of the literature and theories pertinent to oil biodegradation and nutrient dynamics and provides examples of bioremediation options and case studies of oil bioremediation in coastal wetland environments. It also evaluates current practices and state-of-the-art research results pertaining to the bioremediation of hydrocarbon contamination, and presents a procedure for the design and evaluation of bioremediation processes applicable to the cleanup of oil contaminated coastal wetlands. Special attention is given to oil bioremediation of salt marshes since they are the most prevalent type of coastal wetland and have been the subject of the most extensive studies.

The document consists of two major parts. Part I presents the background and overview of bioremediation options, which include the characteristics of coastal wetlands, oil spill threats and countermeasures in salt marshes, and relevant state-of-the-art research. Part II provides guidelines for design and planning of oil bioremediation in salt marshes, which includes site characterization and evaluation, the selection of appropriate bioremediation technologies, and the design of sampling and monitoring programs.

The overall conclusions reached by the guidance manual are as follows. Unlike sandy beaches, oil biodegradation on marine wetlands is often limited by oxygen, not nutrient availability. Natural attenuation is increasingly becoming the preferred strategy for the restoration of oil-contaminated wetlands. However, field studies also show that on some coastal wetlands, nutrients might still be a limiting factor for oil biodegradation, particularly if the oil does not penetrate deeply into the anoxic zone of the wetland sediment. When biostimulation is selected, it is recommended that nitrogen concentrations of at least 2 to as much as 10 mg N/L should be maintained in the pore water to achieve optimal oil biodegradation, with the decision on higher concentrations to be based on a broader analysis of cost, environmental impact, and practicality. Furthermore, if ecosystem restoration is the primary goal rather than oil cleanup, at least one 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 resulting from the addition of extra nutrients. Therefore, the decision to bioremediate a site should depend on cleanup, restoration, and habitat protection objectives and other pertinent factors that may have an impact on success.

No effort was made to determine the quality of secondary data reviewed in the literature and the conclusions made from these data.

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1 INTRODUCTION AND OVERVIEW OF BIOREMEDIATION OPTIONS

1.1 Coastal Wetlands in the U.S.

Coastal wetlands are subjected to the influence of tidal action. They provide natural barriers to shoreline erosion, habitats for a wide range of wildlife including endangered species, and key sources of organic materials and nutrients for marine communities (Boorman, 1999, Mitsch and Gosselink, 2000). Coastal wetlands can be classified into tidal salt marshes, tidal fresh water marshes, and mangrove swamps (Mitsch and Gosselink, 2000).

- **Tidal Salt Marshes --** Salt marshes are those halophytic grasslands found in the middle and high latitudes along protected coastlines. They are subjected to tidal action as well as high salinities. In the United States, they are often dominated by the grass *Spartina alterniflora* in the low intertidal zone, and *Spartina patens* with the rush *Juncus* in the upper intertidal zone. Most of these wetlands are distributed along the Gulf of Mexico and the Atlantic coast.

- **Tidal Freshwater Marshes --** These wetlands are found inland from the salt marshes but still close enough to the coast to experience freshwater tidal effects. Since these wetlands lack the salinity stress of salt marshes, they are often very productive ecosystems and dominated by a variety of grasses and by perennial and annual broad-leafed aquatic plants.

- **Mangrove Swamps --** Mangroves are subtropical and tropical coastal wetlands dominated by halophytic trees and shrubs. In subtropical and tropical regions of the world, tidal salt marshes give way to mangrove swamps. In the United States, they are mostly distributed along the southern coast of Florida and generally dominated by the red mangrove (*Rhizophora*) and the black mangrove tree (*Avicennia*).

It the early 1990s, it was estimated that the total area of coastal wetlands in the United States was approximately 3.2 million ha (32,000 km^2), with about 1.9 million ha or 60 percent of the total coastal wetlands as salt marshes and 0.5 million ha as mangrove swamps (Mitsch and Gosselink, 2000). Coastal wetlands are no longer viewed as intertidal wastelands, and their ecological and economic values have been increasingly recognized. Major benefits and functions of coastal wetlands include:

- **Shoreline Protection** – Coastal wetlands provide a buffer between land and sea, protecting marine shorelines from the ravages of storms and erosion by wave action. Salt marshes, which sustain little damage from ocean storms, can shelter inland developed areas and reduce potential storm damage to coastal buildings and structures.

- **Support of Coastal Fisheries** – Tidal marshes provide spawning site and nursery areas for many fish and shellfish species. Due to their high productivity, coastal wetlands produce great volumes of detrital organic materials and nutrients, on which many small invertebrates and fish feed. It is estimated that over 95 percent of the commercial fish and shellfish species in the United States are wetland dependent (Feierabend and Zelazny, 1987).

- **Wildlife Habitat** – Coastal wetlands are the primary habitat for many plant and animal species and provide food, water, and shelter to indigenous and migratory species. More importantly, wetland habitats are essential for the survival of a large percentage of endangered species. For example, of the 209 animal species listed as endangered by the U.S. Department of Interior and the U.S. Fish and Wildlife Service in 1986, about 50 percent depend on wetlands for their survival (Mitsch and Gosselink, 1993).

- **Water Quality Management** – Coastal wetlands maintain and improve water quality by acting as sediment and chemical sinks (Baker *et al*., 1989). Under favorable conditions, wetland sediments, plants, and their associated microorganisms are able to contain, take up, and degrade various environmental contaminants, such as excess fertilizers, pesticides, and heavy metals.

Wetlands have suffered dramatic losses as a result of human activities, such as drainage for agricultural use. Overall, more than 50 percent of the wetlands in the continental U.S. were lost from the 1780s to the 1980s (Mitsch and Gosselink, 2000), and at a rate 7,300 ha/year from 1950s to 1970s (Tiner, 1984). Such losses have greatly diminished the nation's wetlands and their benefits. The rates of loss have been declining since the mid 1970s with the enactment of wetland protection laws and increased public appreciation. However, threats to coastal wetlands remain, including conversion for agricultural, industrial, and residential development, mean sea level rise, and chemical contamination from excessive nutrient inputs, chemical accumulations, and oil spills.

The threat of crude oil contamination to coastal wetlands is particularly high in certain parts of the U.S., such as the Gulf of Mexico, where oil exploration, production, transportation, and refineries are extensive (Lin and Mendelssohn, 1998). Oil and gas extraction activities in coastal marshes along the Gulf of Mexico have been one of the leading causes of wetland loss in the 1970s (Mitsch and Gosselink, 2000). Despite more stringent environmental regulations, the risk of an oil spill affecting these ecosystems is still high because of extensive coastal oil production, refining, and transportation.

1.2 Oil Spills in Salt Marshes: Threats and Countermeasures

1.2.1 Threats of oil spills

Marine wetlands are especially vulnerable to oil spills because the inherently low wave energy of a wetland does not physically remove oil effectively. They are flooded at high tide and their complex surface can trap large amounts of oil. Impacts of oil spills to coastal wetland ecosystems have been described and reviewed extensively (Baker *et al*., 1989; Fingas, 2001; NAS, 1985; Pezeshki *et al*., 2000). A brief summary on these impacts is provided in the following text.

1.2.1.1 Impact to wetland plants

Oil spills have been known to cause acute and long-term damage to salt marshes and mangroves (Baker *et al*., 1989; Burns *et al*., 1993; Lin and Mendelssohn, 1996; Pezeshki *et al*., 2000). These impacts include reduction in population and growth rate or abnormal growth and regrowth after initial impact. Mangroves are generally more vulnerable to oil spills than salt marshes because oil on the partially submerged roots of mangroves interferes with respiratory activity (Duke *et al*., 1997;

Evans, 1985).

The degree of oil impact also depends on various factors, such as the type and amount of oil, the extent of oil coverage, the plant species, the season of the spill, the soil composition, and the flushing rate. For example, No.2 fuel oil has been found to cause much higher mortality and damage to *Spartina alterniflora*, a dominant salt marsh grass along the Atlantic coast and Gulf of Mexico, than Arabian crude oil, Libyan crude oil, and No.6 fuel oil (Alexander and Webb, 1983 & 1985). Growth of *Spartina alterniflora* was not significantly affected by oil contamination at low to moderate concentrations (less than 5 mg crude oil/g sediment, Alexander and Webb, 1987; less than 50 mg crude oil/g sediment, DeLaune *et al.*, 1979) and sometimes was even stimulated (Li *et al.*, 1990). However, heavy contamination by light oil can lead to widespread mortality, and plants may require a decade or more to recover. Different wetland plants also respond differently to oil spills. Lin and Mendelssohn (1996) examined the effects of south Louisiana crude oil on three different types of coastal marshes and found that the sensitivity of these marshes to the crude oil increased in the order of *S. lancifolia* (freshwater marsh plant), *S. alterniflora* (salt marsh), and *S. patens* (brackish marsh). Plants are more sensitive to oiling during the growing season than other periods (Pezeshki *et al*., 2000). The sediment type also plays an important role. In general, oil remains longer in soils with higher organic matter and, therefore, has greater impact on resident plants. Some wetland sediment can act as a reservoir absorbing oil and leaching it out into adjacent coastal habitats, causing chronic impacts on biota (Levings *et al*., 1994).

1.2.1.2 Impact to wildlife and ecosystems

Oil spills on coastal wetlands not only damage plants but also have serious consequences for the wildlife and other organisms that rely on the wetlands as habitats and nursery grounds. These impacts include obvious immediate consequences, such as widespread animal mortality due to smothering and toxic effects, and more subtle long-term effects. Oil can affect the fish population by both direct toxicity and by a reduction in the benthic species on which they feed (NAS, 1985). Seabirds that congregate on the salt marshes suffer from the destruction of their feeding grounds. Oil can also change an animal’s feeding and reproductive behaviors. A light oiling of some birds can inhibit egg laying (Fingas, 2001). Furthermore, heavy mortality of seabirds is often observed because oiling effectively diminishes the natural water-repellant and insulation value of feathers.

The extent of the impacts also depends on many factors, such as the life cycle and the life habit of organisms, the time and season of oil spills, the type and amount of oil, and the duration of oil exposure (NAS, 1985). Sediment feeders could be more vulnerable to oil than epibenthic filter feeders. Larval fish are more vulnerable to oil than juveniles and adults. Avian mortality would be exacerbated by a spill occurring during their feeding and nesting season.

Considering the different sensitivity of wetland species and populations to oil, spills can significantly affect the overall balance of wetland ecosystems, especially if damage occurs to a dominant species. On the other hand, some long-term studies have suggested that many oiled marine wetlands could recover naturally after a long time (Baker, 1999; Hester & Mendelssohn, 2000; Sell *et al*., 1995). Recovery times vary from a few years for some salt marshes to over a decade for mangroves (NAS, 1985; Sell *et al*., 1995). In a few extreme cases, salt marsh ecosystems have not fully recovered decades after the initial oil spills (Baker *et al*., 1993; Teal *et al.*, 1992). The effects of oil on wetland ecosystems and recovery still require further investigation.

1.2.2 Response to oil spills in salt marshes

Since oil spills can cause serious damage to marine wetland ecosystems, effective countermeasures are essential to minimize these ecological impacts. Major oil spill response options in marine shorelines and freshwater environments have been briefly reviewed in Chapter 1 of the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001). However, salt marshes are among the most sensitive ecosystems and, therefore, the most difficult to clean. Applications of some traditional oil spill cleanup techniques in wetland habitats have caused more damage than the oil itself (Baker 1999; Owens and Foget, 1982, Sell *et al.*, 1995). Considering the characteristics of wetland ecosystems, a number of cleanup and treatment techniques have been proposed and tested to deal with oil contamination in coastal wetlands. The feasibility of these methods also depends on various factors, such as the type and amount of spilled oil, season of the year, and environmental conditions of the spill site.

1.2.2.1 Physical Methods

Many oil spill countermeasures based on physical clean up procedures, such as mechanical oil removal, high pressure or hot water flushing, and sediment relocation, have been reported to do more harm than good to wetland habitats. All physical methods that remain as options for use on marine wetland environments require some caution during deployment to minimize environmental damage.

- **Booming and sorbents** – Use of booms to contain and control the movement of floating oil at the edge of the wetland and removal of the oil by adsorption onto oleophilic materials placed in the intertidal zone. This method can be an effective strategy to prevent floating oil from reaching sensitive habitats with minimal physical disturbance if traffic of the cleanup crew is strictly controlled.

- **Low pressure flushing** – Oil is flushed with ambient-seawater at pressures less than 200 kpa or 50 psi to the water edge for removal (NOAA, 1992). This technique can be used selectively for quick removal of localized heavy oiling with minimal damage to wetland vegetation. However, the potential for oil release into the sediments and adjacent water bodies should be considered including appropriate containment measures.

- **Cutting vegetation**-- Cutting vegetation may be a useful cleanup technique to remove oils that form a thick coating on the vegetation and to prevent oiling of sensitive wildlife (Baker, 1989, NOAA 1992). However, the feasibility of this method depends strongly on the season in which the spill occurs. In general, winter cutting of dead standing vegetation has little effect on subsequence growth, but summer cutting could cause great damage to the regrowth of wetland plants and result in shoreline erosion. The use of cutting should also be avoided immediately prior to an anticipated rise in water levels because cutting followed by flooding could cut off necessary oxygen to plant roots (Pezeshki *et al*., 2000). Efforts should also be made to minimize the inevitable damage due to traffic.

- **Stripping** – Stripping of surface sediments can cause severe environmental impacts and may only be considered in the case of extremely oiled wetlands where the oil in the sediments is likely to kill the vegetation and prevent plant regrowth. To minimize erosion and habitat loss, it is critical to follow the stripping by the restoration of sediment elevation and replanting of the wetland species (Krebs & Tanner, 1981).

1.2.2.2 Chemical methods

Chemical methods have not been widely used in the United States mainly due to the concerns over their toxicity and long-term environmental impacts. However, with the development of less toxic chemical agents, the potential for their application will increase.

- **Dispersants** – Dispersants are chemicals that promote the dispersion of floating oil from the water surface into the water column. Fields studies have shown that application of dispersants in near shore waters can significantly reduce the retention of oil within the intertidal zone and, therefore, the impacts to wetland plants (Duke et al., 2000; Getter & Ballou, 1985). However, the use of dispersants in near shore water could have short-term toxic effects on adjacent coastal habitats, such as subtidal animal communities. Direct spraying or contact of dispersants with wetland plants may also have harmful effects on vegetation (Wardrop et al., 1987).

- **Cleaners** – Cleaners are chemicals that help wash oil from contaminated surfaces. These formulations have been used with low-pressure flushing operations to facilitate oil removal from wetland vegetation. Studies have shown that the application of cleaners can prevent mortality of salt marshes and mangroves (Pezeshki *et al*., 1995; Teas *et al*., 1993). However, their use has been limited because of the paucity of data available with respect to their long-term effects on wetland habitats. Also, concern has been expressed over the transfer of oil to the nearshore waters.

1.2.2.3 *In-situ* burning

In-situ burning involves controlled burning of the oil and oiled vegetation at the contaminated site. This technique is capable of rapidly removing large amounts of oil with limited equipment and personnel. However, the technique may result in severe damage to wetland habitats, temporary air pollution, and possibly toxic combustion residues. The degree of impact to salt marshes is seasonally dependent. Like cutting, the likelihood of damage is greatest during the summer and least during the period of dormancy in late fall and winter (Baker, 1989). In fact, fall burning of marshes has been a commonly used management strategy for controlling wetland overgrowth in many areas. The temporary air pollution caused by the airborne emissions are generally not considered a serious health threat or environmental concern, especially at distances greater than a few kilometers from the fire (Fingas, 2001). Limited data and applications have indicated that *in-situ* burning can be a viable option for removing a large volume of pooled oil at the right season when sediments are saturated (Mendelssohn *et al*., 1995; Pahl *et al*., 1997).

1.2.2.4 Restoration

In cases of coastal wetlands being catastrophically damaged, plant and animal species have been re-introduced as restoration strategies. (Bergen *et al.*, 2000; Frink and Gauvry, 1995; Teas *et al.*, 1989). *S. alterniflora* was successfully replanted to restore the salt marshes in New Jersey after the 1990 *Arthur Kill* oil spill (Bergen *et al.*, 2000). Three years after the replanting, over 70% of the plant coverage was restored as compared to only 5% by natural recolonization at the unplanted reference sites. Mangroves were also successfully replanted to restore oil-killed mangrove forest in Panama after the 1986 *Refineria Panama* oil spill (Teas *et al.*, 1989). However, this approach may also upset the ecological balance or natural succession processes if it is not carried out appropriately (Fingas, 2001).

1.3 Bioremediation of Oil Spills in Salt Marshes

Bioremediation is an emerging technology that involves the addition of materials (e.g. nutrients or other growth-limiting cosubstrates) to contaminated environments to accelerate the natural biodegradation processes (OAT, 1991). This technology has been recognized as one of the least intrusive methods and has been shown to be an effective tool for the treatment of oil spills in medium and low-energy marine shorelines (Lee *et al*, 1997; Swannell *et al*., 1996; Venosa *et al*., 1996, Zhu *et al*., 2001). However, until a few years ago, only limited information was available on the effectiveness and impacts of the bioremediation of oil spills in coastal wetlands (Lee *et al*., 1991; Wood *et al*., 1997; Wright *et al*., 1997). Recently, several long-term field studies on oil bioremediation in coastal wetlands have been carried out. These studies provide better understanding of the potential of oil remediation in such environments (Burns *et al*., 2000; Garcia-Blanco and Suidan, 2001; Jackson and Pardue, 1999; Shin *et al*., 1999). In this section, an in-depth review of current practices and research on oil bioremediation in coastal wetland environments is presented with emphasis on the findings of these field trials.

1.3.1 Environmental factors affecting oil biodegradation in salt marshes

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. Environmental factors affecting oil biodegradation include temperature, nutrients, oxygen, pH, and salinity. These factors have been discussed in general in Chapter 2 of the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001) and in Section 5.5 of the document with respect to freshwater wetlands. The limiting conditions for oil biodegradation in salt marshes can be significantly different from other marine shorelines and even freshwater wetlands. A brief summary of these conditions in salt marshes is given here.

In terms of nutrient supply, coastal marshes are considered high-nutrient wetlands (Mitsch and Gosselink, 2000), but most of the nutrients, and nitrogen in particular, are present in the form of organic matter and not readily available for microbial or plant uptake (Cartaxana *et al*., 1999). Figure 1.1 illustrates the nitrogen cycling that occurs in a wetland environment. The amount of inorganic nitrogen or available nitrogen for oil biodegradation will depend on many processes, such as nitrogen fixation, nutrient mineralization, plant uptake and release, denitrification, and wetland hydrodynamics. Studies also show that the concentration of inorganic nitrogen (mostly ammonium) in salt marsh sediments exhibits a seasonal pattern with a concentration peak during the summer months probably due to higher mineralization rates associated with elevated temperatures (Cartaxana *et al*., 1999). A similar trend was also reported for available phosphorus (Nixon *et al*., 1980). Therefore, when a major oil spill occurs in salt marshes, it is still likely that nutrient availability becomes a limiting factor for oil degradation, depending on the type of sediment, the season, and quantity of oil spilled. Hence, nutrient addition may be an important remedy to contemplate when considering any or all of these factors.

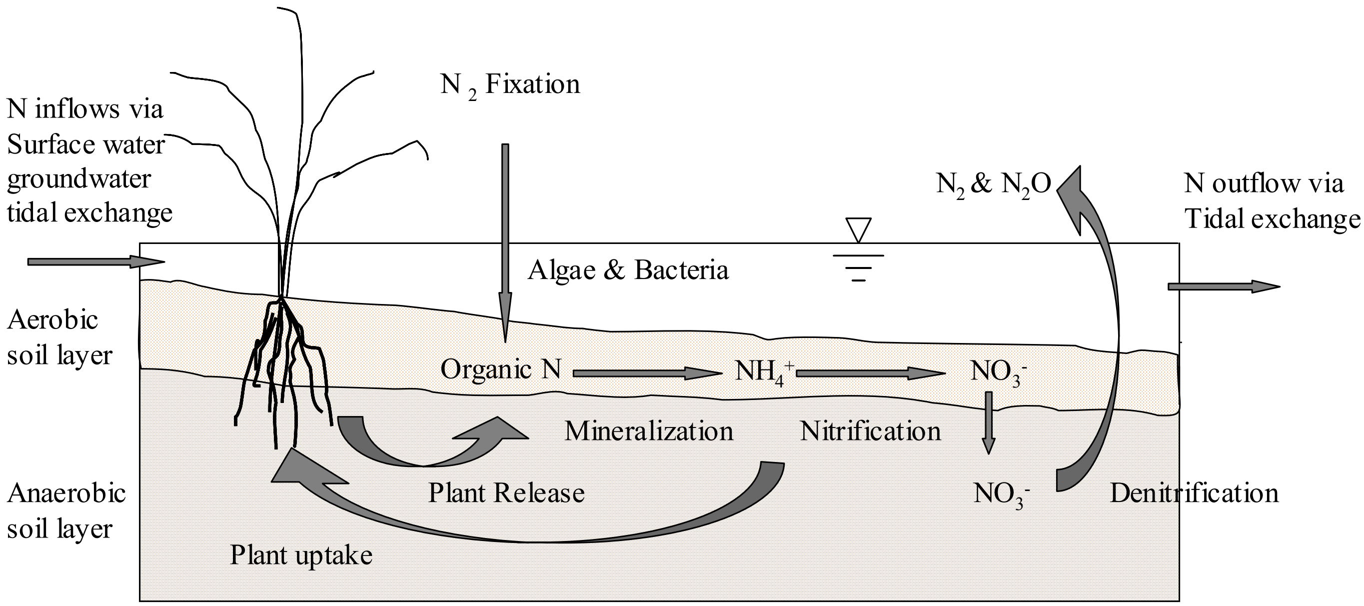

Figure 1.1 Major processes involved in nitrogen cycles in a coastal wetland

Unlike other marine shorelines, the substrates of coastal wetlands are saturated or flooded with water, and oxygen diffusion rates through these hydric soils are very slow. As a result, available oxygen in the soils and in the interstitial water is quickly depleted through metabolism by aerobic organisms and chemical oxygen demand due to reduced chemical species. Typically, there is a thin layer of oxidized soil (a few millimeters) at the surface, below which the environment becomes anaerobic (Gambrell and Patrick, 1978). The thickness of this oxidized layer depends on the population of oxygen utilizers, the rate of photosynthetic oxygen production by algae and plants, and the rate of oxygen transport through the sediments, which is related to wind, tide and wave action. A study of oxygen demand in an oil contaminated salt marsh sediment indicated that oxygen availability could be a limiting factor for oil degradation (Shin *et al*., 2000). These authors reported that significant biodegradation occurred only when the tidal cycle exposed the surface of the salt marsh to the atmosphere. The dominant electron acceptor in the anaerobic soil layer in salt marshes is also different from most freshwater wetlands. In freshwater wetlands, methanogenesis is often the dominant process for the oxidation of organic carbon in the reduced soil layer, while in marine wetlands, sulfate reduction is usually the most important process when oxygen is limited since seawater contains abundant sulfate (Mitsch and Gosselink, 2000). Studies have shown that in some marine sediment, 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 this process in the biodegradation of oil in salt marsh environments still requires further demonstration, especially in the field. The inherent ability of many wetland plant species to transfer oxygen to the rhizosphere may also play a role in reducing the effect of oxygen limitation. However, little research has been conducted on the capacity of this mechanism in enhancing oil biodegradation.

Other important environmental factors affecting biodegradation of petroleum hydrocarbons include pH and salinity. The optimal pH for oil biodegradation is between 6 and 9 (Atlas and Bartha, 1992). The pH of wetland sediments and the overlying water depend on both soil type and hydraulic condition. Sediments in salt marshes and mangroves are mostly organic and often acidic. In addition, the anoxic condition and high sulfur content in coastal wetlands often lead to the production of hydrogen sulfide. When exposed to air, sulfide can be reoxidized to sulfate and result in a further drop in sediment pH. However, in areas with frequent tidal inundation, the pH of wetland sediments and pore water is determined by seawater and is near neutral or slightly alkaline. The salinity of pore water in coastal wetlands may also vary dramatically and depends on the frequency of tidal inundation, rainfall, coverage of vegetation, groundwater and freshwater inflow, and soil type (Mitsch and Gosselink, 2000). In areas adjacent to coastlines or receiving frequent tidal inundation, the salinity of wetland water is close to that of seawater. Elevated salinity levels are frequently reported at higher elevations or areas with little rainfall and other freshwater supply. Brackish marshes are frequently found in estuarine areas and other sites that extend away from the open ocean. Salinity can significantly affect the rates of oil biodegradation. Most marine microorganisms 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). Studies have also shown the rates of hydrocarbon degradation to decrease with increased salinity above that of seawater (Rhykerd *et al.*, 1995; Ward and Brock, 1978).

Although many factors can affect oil biodegradation, not many environmental factors can be easily manipulated to enhance this natural process. For example, it is not practical to alter wetland salinity, and nothing can be done to change the climate. There are two main approaches to oil spill bioremediation. (1) *bioaugmentation,* in which oil-degrading microorganisms are added to supplement or augment 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 co-substrates. Extensive studies have been carried out recently on the bioremediation of oil contaminated coastal wetlands both on a laboratory scale and in the field (see next section).

1.3.2 Laboratory studies

Most of the laboratory studies have focused on the potential of using nutrient amendments to enhance oil biodegradation in salt marsh environments. This is because studies conducted in other shoreline environments have demonstrated that the microbial population is rarely a limiting factor, and nutrient addition alone had a greater effect on oil biodegradation than did the addition of microbial products (Lee *et al*, 1997; Venosa *et al*., 1996).

Jackson and Pardue (1999) conducted microcosm and mesocosm studies to investigate the effect of different nutrient types on enhancing biodegradation of a Louisiana crude oil in Louisiana salt marsh sediment. The microcosms contained a 60:1 (water/soil) slurry produced from a salt marsh sediment at an oil concentration of 0.7 g oil/g soil and were operated in a completely mixed and aerated mode, where oxygen limitation was non-existent. Nutrient species examined included phosphate, ammonium, nitrate, and phosphate plus ammonium. The results showed that oil degradation was limited by nitrogen but not phosphorus under these conditions. Optimal nitrogen concentrations in pore water were in the range of 100-670 mg N/L. Among the nitrogen species, ammonium was found to be generally more effective in stimulating oil degradation than nitrate. Thus, ammonium might be advantageous in the enhancement of the degradation of certain oil components because ammonium is less likely to be lost from the system by washout due to its higher adsorptive capacity to organic matter. Ammonium is also the more toxic species of nitrogen in the environment.

In a follow-on mesocosm study in the same paper (Jackson and Pardue, 1999), large intact cores (900 cm^2) of salt marsh sediments were contaminated with crude oil and treated with various concentrations of ammonium salts. The results showed that ammonium amendments had limited success in enhancing oil biodegradation. Even at the highest ammonium loading (10 mg/m^2), the nutrient amendment was only able to increase the degradation of lower chain length alkanes ($<C_{20}$) by10-40% and no significant enhancements in the degradation of PAHs and longer chain alkanes were observed.

Wright *et al*. (1996 and 1997) also conducted a mesocosm study to investigate the influence of nitrogen and phosphorus using 7.5-L buckets containing salt marsh sediments with and without transplanted *Spartina alterniflora* under total flooded conditions. The effect of season was also examined by conducting this study during winter and summer months. Weathered Arabian crude oil was applied to the mesocosms at a rate of 0.46 g oil/m^2. Nutrient treatments included urea, ammonium, nitrate, a slow release fertilizer (Max Bac), and an oleophilic fertilizer (Inipol EAP-22). Each of them was also evaluated with and without supplemental phosphate. The study showed that the effectiveness of bioremediation depended on both the types of nutrients and the season of the spill. During the 80 days of the winter experiment, phosphorus was the limiting nutrient for oil degradation in this salt marsh sediment. Addition of urea, ammonium, and nitrate without P did not enhance hydrocarbon degradation, while phosphorus addition led to an increase in hydrocarbon degradation from 43% for the no-nutrient control to 53 -75% for various N and P combinations. MaxBac and Inipol EAP-22, which contain both N and P, performed slightly better than inorganic nitrogen supplemented with phosphorus. However, during the 40 days of the summer experiment, although the overall oil degradation rates were higher than in winter, nutrient addition did not significantly stimulate oil degradation beyond the 70% reported for all the treatments. This was attributed to the higher mineralization rates of organic nutrients in the sediments during the summer, which is consistent with the findings by Cartaxana *et al*. (1999) and Nixon *et al*. (1980). The study also indicated that the existence of *Spartina alterniflora* did not significantly affect oil degradation as compared to treatments without the wetland plant.

Garcia-Blanco *et al.* (2001a) conducted a microcosm study to further simulate the bioremediation of oil-contaminated salt marsh under tidal conditions. The study was carried out in glass columns filled to a depth of 10 cm with sediment collected from a salt marsh in Nova Scotia, Canada. Each microcosm was operated on a 24 hr tidal cycle with a 12-hour submergence period. The entire sediment was mixed with weathered No.2 fuel oil to a concentration of 20 g/kg of wet sediment. Three inorganic nitrogen sources were tested: (1) a slow-release granular fertilizer (prilled ammonium nitrate); (2) sodium nitrate; (3) ammonium chloride. Each nitrogen fertilizer was supplemented with sodium tripolyphosphate and was added to microcosms at a N:P ratio of 5:1. The study showed that the addition of nutrients did not enhance the rate of degradation over the natural attenuation rate. The extent of microbial degradation of No. 2 fuel oil in all the microcosms averaged only 20% for the total aliphatic hydrocarbons and 12% for the total PAHs. Degradation was greater in all cases in the top layers than in the bottom layers of the columns, suggesting that oil degradation may have been limited by oxygen availability under the conditions of this study.

Because these laboratory studies seem to suggest that adding nutrients may be effective under non-oxygen limiting conditions, or during certain seasons, further field experiments are necessary to determine the potential of oil bioremediation in coastal wetlands.

1.3.3 Full-scale demonstrations

From north temperate salt marshes to tropical mangroves, several field studies on the performance of oil bioremediation have been carried out in recent years. These have provided more convincing demonstrations of the effectiveness of oil bioremediation since laboratory studies may not consider many real world conditions such as spatial heterogeneity, biological interactions, and mass transfer limitations.

1.3.3.1 Nova Scotia, Canada, 1989

Lee and Levy (1991) conducted one of the first field trials on oil bioremediation in a salt marsh environment. The study involved periodic addition of water-soluble fertilizer granules (ammonium nitrate and triple super phosphate) to enhance biodegradation of waxy crude oil in a salt marsh dominated by *Spartina alterniflora* and located in Nova Scotia, Canada (Lee and Levy, 1991). Two levels of oil concentrations were used (0.3 and 3.0% v oil/v sediment) and two concentrations of the NH_4NO_3 were tested (0.34 and 1.36 g/L sediment). In this study, pristane was used as a biomarker for evaluation of biodegradation of crude oils. Results showed that the effectiveness of nutrient addition was related to oil concentration. Enhancement by fertilizer was significant at the 0.3% contamination level, but no enhancement occurred at 3%, which was attributed to the penetration of the oil at higher concentrations into the reduced soil layers where little degradation is expected. This study indicated that bioremediation might have a role in the cleanup of coastal wetlands lightly contaminated with oil.

1.3.3.2 San Jacinto Wetland Research Facility (SJWRF), Texas, 1994-1997

To evaluate the effectiveness of various bioremediation options, a series of field trials were carried out in a Texas coastal wetland by a research group from Texas A&M University (Mills *et al*., 1997; Mills *et al*., 2003; Simon *et al*., 1999; Townsend *et al*., 1999; Mills *et al*., 2003). This brackish wetland was set aside for a long-term research program after an oil spill from ruptured pipelines in 1994. The 21-plot site, named San Jacinto Wetland Research Facility (SJWRF), has been used for a series of studies on oil spill countermeasures. Studies on oil bioremediation included three phases. Phase I of the research evaluated the intrinsic bioremediation or natural attenuation process after the initial oil spill. The effect of biostimulation was investigated in phase II by evaluating the use of diammonium phosphate and diammonium phosphate plus nitrate. Phase III involved the evaluation of two commercial bioaugmentation products and a repeated diammonium phosphate treatment. The

21 5 x 5 m plots were arranged in a randomized complete block experimental design, and Arabian light and medium crude oils were used in phases II and III, respectively. Oil constituents were determined using gas chromatography/mass spectroscopy (GC/MS) and were normalized to 17α(H), 21β(H)-hopane to reduce the effects of sample heterogeneity and physical losses. The results of phase II showed that the diammonium phosphate treatment significantly enhanced the biodegradation rates of both total resolved saturates and total resolved PAHs, and the addition of diammonium phosphate plus nitrate only enhanced the biodegradation of total resolved saturates (Mills *et al*., 1997). The field trial on oil bioaugmentation in phase III showed as with other shoreline types (Lee *et al*, 1997a; Venosa *et al*., 1996), that the addition of microbial products does not significantly enhance oil biodegradation rates (Simon *et al*., 1999). However, the performance of the nutrient treatment in this phase also failed to demonstrate the enhancement observed in Phase II.

1.3.3.3 Terrebonne Parish, Louisiana, 1998

Due to the mixed results from the earlier trials, field studies were conducted to verify the feasibility of oil bioremediation and to determine the limiting factors in oil biodegradation in coastal wetland environments. Shin *et al*. (1999 & 2000) investigated the effect of nutrient amendment on the biodegradation of a Louisiana "sweet" crude oil and oxygen dynamics in a Louisiana salt marsh, which has a tidal range of 20 cm and is vegetated by *Spartina alterniflora*. Four treatments (unoiled control, oiled control, oil plus ammonium nitrate, and oil plus a slow release fertilizer) were examined in forty field plots arranged in a randomized complete block design. Oil components were measured by GC/MS, and hopane was used as a biomarker. Oxygen dynamics were investigated by monitoring sediment oxygen demand (SOD), and the importance of sulfate reduction was determined using a $^{35}SO_4^{2-}$ radiotracer technique. Overall, the nutrient amendments did not significantly stimulate oil biodegradation, which might have been related to the high background nutrient concentrations at this site. Throughout this study, the background nitrogen concentrations in the interstitial pore water were higher than the threshold nitrogen concentration of 1 - 2 mg N/L required for maximum hydrocarbon biodegradation as found by Venosa *et al*. (1996) in an unrelated field trial on a sandy beach. The addition of oil and fertilizers did increase the SOD and sulfate reduction rates in marsh soils. About 2/3 of the oxygen demand was due to aerobic respiration with the majority of this demand exerted by hydrocarbon degrading organisms, indicating aerobic biodegradation of the crude oil was the main mechanism. The remaining 1/3 of the oxygen demand was attributed to sulfide oxidation. Data also showed that significant biodegradation of crude oil in the salt marshes occurred only when the tidal cycle exposed the surface of the marsh to air (Shin *et al*., 2000). This study indicated that oxygen availability appears to control the oil biodegradation process in salt marshes.

1.3.3.4 Gladstone, Australia, 1997-1998

A field study on the performance of oil bioremediation in both mangrove and salt marsh ecosystems was carried out recently in a tropical marine wetland located at Gladstone, Australia (Burns *et al*., 2000; Duke *et al*., 2000; Ramsay *et al*., 2000). This study evaluated the influence of a bioremediation protocol on the degradation rate of a medium range crude oil (Gippsland) and a Bunker C oil stranded in a tropical *Rhizophora* sp. mangrove environment and in *Haloscarcia* sp. salt marshes behind the mangroves. The bioremediation strategy used in this study involved pumping air beneath sediment that was supplemented with a slow release fertilizer (Osmocote™

Tropical fertilizer) for the mangrove sites, and nutrient addition alone for the salt marsh sites. No aeration was tested in the salt marsh experiments because the sediment of the salt marshes was much less anoxic than that of the mangroves based on preliminary investigations. Four oiled treatments (two types of oils with and without the bioremediation treatments) and two unoiled controls (enclosure and ambient controls) were tested. Each treatment was studied with replicates of three plots for the mangrove sites and replicates of four plots for the salt marsh sites. The oils were added to mangrove plots and salt marsh plots at target loadings of 5 L/m^2 and 2 L/m^2, respectively. The fertilizer was added at a loading of 0.15 kg/m^2 40 hours after oiling for both mangrove and salt marsh plots and then again after three months in mangrove plots only. Aeration in mangrove plots started 40 hr after oiling and lasted for about four months. In this study, other than total hydrocarbons (THCs), only individual alkanes were analyzed using GC-FID, and phytane was used as a biomarker. Oil analysis for the mangrove sediments over 13 months showed that no significant change in oil composition due to biodegradation was observed until two months after oiling, and by that time 90% of the THCs were removed from the sediments through evaporation and dissolution. The remediation strategy did not significantly enhance the degradation of either the remaining Gippsland oil or the Bunker C oil. A similar lag phase before the start of oil biodegradation was observed in the 9-month salt marsh experiment. The addition of the fertilizer to the salt marshes did show a stimulation of the degradation of the lighter Gippsland oil and resulted in about 20% more oil loss as compared with the untreated plots. However, the nutrient amendment did not significantly impact the rate of loss of Bunker C oil in the salt marsh plots. Microbial analysis for the mangrove sediments showed that the bioremediation treatment had a significant effect on alkane degraders and increased the population size by one to three orders of magnitude, as compared to the oil only plots. However, the population of aromatic degraders only increased slightly (one order of magnitude). Due to the limitation of the experimental design, the study could not distinguish whether nutrient addition or aeration stimulated the microbial growth.

In the same study, Duke *et al*. (2000) investigated the ecological effects of the bioremediation strategy in the mangroves and compared the results to a previous field trial involving use of a dispersant at the same site. Although the authors suggested that the dispersant (but not bioremediation) significantly reduced the mortality of mangrove trees, the data appeared to show that both treatments had some positive effects on the wetland habitats. The increase in the tree mortality in bioremediation plots occurred only months after aeration and nutrient addition was stopped. Even though some aspects of the design are questionable (e.g., lack of independent tests of the effect of nutrients and aeration in mangrove environments, different oiling and treatment conditions for the salt marsh and mangrove experiments), the Gladstone field trial did provided useful insights on the potential of oil bioremediation, particularly in tropical marine wetland environments.

1.3.3.5 Nova Scotia, Canada, 2000-2001

A comprehensive field trial conducted on oil bioremediation in a salt marsh environment was carried out recently by the U.S. Environmental Protection Agency, University of Cincinnati, and Fisheries and Oceans Canada in a coastal salt marsh site situated on the Eastern Shore of Nova Scotia, Canada (manuscript not published at the time of this writing). This study explored various options for restoring salt marshes heavily contaminated with petroleum hydrocarbons under north-temperate conditions. Treatment options included natural attenuation, phytoremediation, and/or bioremediation by nutrient amendment and disking (gentle tilling). Like most North American salt marshes, this tidal salt marsh was dominated by *Spartina alterniflora*. Tides were semi-diurnal with a range of about 2 m. The commencement of the experiment coincided with the spring tide. Test plots for the study were set up throughout the wetland in a way that all of them were exposed to the same tidal inundation.

A randomized block design was used in the study. Eighteen 3 m x 3 m plots were set up in three replicate blocks. Each block contained six treatments randomly distributed: (a) unoiled, no-nutrient control; (b) unoiled with nutrient amendments $\{NH_4NO_3 + Ca(H_2PO_4)_2.H_2O\}$; (c) oiled with no nutrient amendments and plants intact (*natural attenuation*); d) oiled with nutrient amendments and plants intact; (e) oiled with nutrient amendments and vegetation continually cut back to the ground surface and removed to suppress the influence of plants and anaerobiosis associated with the accumulation of plant detritus; (f) oiled with nutrient amendments and disked daily to introduce oxygen into the rhizosphere.

A weathered Mesa crude oil was applied to the plots at a rate of 35 mg oil/g dry sediment during the first two days of the study. Granular nitrogen and phosphorus nutrients were initially added to each of the treated plots at a dosage of 450 g-N and 135 g-P per plot. Subsequent applications took place on an as-needed basis as determined by residual nutrient analysis in the interstitial pore spaces. When the nitrogen levels fell below a specified concentration range of 5-10 mg N/L in the pore water, another application of the same magnitude was made. The effectiveness of various treatments was determined by monitoring the reduction of oil constituents in both soil and grass samples using GC-MS techniques. Hopane was used as a biomarker to reduce the effects of sample heterogeneity and to distinguish bioremediation removal from physical losses. In addition to these detailed chemical analyses, this project also used biological endpoints such as evidence of wetland plant recovery and reduction of toxic responses to verify the success of the bioremediation/phytoremediation treatments (discussed in Section 2.2.4).

The study showed that the biodegradation of targeted aliphatic hydrocarbons and PAHs took place to a very high extent at this north-temperate salt marsh. After 20 weeks, the extent of degradation of target n-alkanes within the experimental plots averaged 87% and 97 %, respectively, for the oil in sediment and the oil associated with emergent vegetative growth. Reduction of parent and alkyl-substituted PAHs was about 69% in the soil samples and 88% in the plant samples. However, targeted alkanes and PAHs only represent a small fraction (less than 10%) of the total petroleum hydrocarbons (TPHs). Biodegradation of TPH averaged only 35% in the soil samples and 42% in the grass samples (very little of this TPH was comprised of high molecular weight plant waxes). More than half of the applied oil remained in the marsh 20 weeks after oil application. Based on the extent of oil washout (measured as mg hopane per kg dry soil) and the total oil loss (g TPH per kg dry soil), the main mechanism for oil disappearance was attributed to biodegradation. These results contrast with those reported in the St. Lawrence River freshwater wetland bioremediation study (Venosa *et al*., 2002) and the study on a tropical marine wetland at Gladstone, Australia, in which most of the TPH was removed through physical mechanisms (Burns *et al*., 2000).

In this study, as in other reported field studies, no significant differences were observed among treatments, either in the degradation of alkanes or PAHs. No significant enhancement of biodegradation through the addition of nutrients or the use of disking was observed. The average nutrient concentrations in the interstitial pore water for various treatments during this study are summarized in Table 1.1. Data indicated that the background nitrogen (mostly ammonium nitrogen) and phosphorus concentrations in the interstitial pore water were always far in excess of 2-5 mg N/L and 1 mg P/L, suggesting that nutrients may not be a limiting factor for oil biodegradation in this salt marsh. Enhanced oxygen transfer to the rhizosphere by the plants through their roots or by disking did not appear to take place either, at least to the level needed by hydrocarbon degraders to metabolize the oil rapidly. From the extent of degradation of the targeted aliphatic and aromatic hydrocarbons, it can be inferred that there was no oxygen or nutrient limitation in this particular salt marsh site.

Table 1.1 Average nutrient concentrations in pore water for different treatments during Nova Scotia study

	Treatment A *Background*	Treatment B *Nutrient Control*	Treatment C *Natural Attenuation*	Treatment D *Phyto-remediation*	Treatment E *Nutrient Amendment*	Treatment F *Disking*
NH_4-N mg N/L	9.43	60.63	18.08	92.49	80.83	104.6
NO_3-N mg N/L	0.09	28.57	0.07	37.11	30.56	28.89
PO_4-P mg P/L	1.25	12.42	2.17	5.66	9.42	14.75

However, nutrient addition did stimulate microbial growth, as in the Gladstone study (Ramsay *et al*., 2000). Alkane degraders in this wetland seemed to be nutrient limited, since the addition of nutrients without oil led to an increase in number of about two orders of magnitude relative to background levels. However, when oil was added to the plots without any nutrient amendment (natural attenuation plots), the increase in alkane degraders was also on the same order, suggesting the existence of different populations that can degrade alkanes under different conditions. However, PAH degraders were clearly limited by their carbon sources. Only oiled plots showed an increase in the number of PAH degraders. These populations did not seem to be limited by nutrients since the addition of nitrogen and phosphorus did not have an effect on either the number of microorganisms or on the rate of PAH degradation.

In summary, these field trials suggest that nutrient amendments may be less effective in stimulating oil biodegradation rates in coastal wetlands than sandy beaches. Oil biodegradation on marine wetlands is often limited more by oxygen than by nutrient availability. A large fraction of the total nutrients in wetland sediments is bound in organic matter (i.e., plants and detritus) that is not readily available for microbial uptake. In such cases, natural ongoing mineralization processes may provide an effective means to overcome this restraint. However, field experience also suggests that some coastal wetlands may be nutrient limited, and in these cases, biostimulation with nutrient enrichment may still be an appropriate countermeasure treatment if the oil does not penetrate deeply into the anaerobic layer of the wetland sediments (Lee and Levy, 1991; Venosa *et al*., 2002; Mills *et al*, 1997).

1.3.4 Kinetics of oil biodegradation

Knowledge of the kinetics of oil biodegradation is important for assessing the potential fate of targeted compounds, evaluating the efficacy of bioremediation, and determining appropriate strategies to enhance oil biodegradation. The rates of biodegradation vary greatly among the various components of crude oils and petroleum products and depend on many environmental factors, such as temperature, nutrient concentration, and oxygen content. The heterogeneity of oil distribution on shorelines or wetland sediments makes kinetics studies even more difficult. To reduce the variability associated with heterogeneous oil distribution, Venosa *et al.* (1996) utilized hopane normalization in studying the kinetics of oil biodegradation and developed first-order biodegradation rate constants for resolvable alkanes and important two- and three-ring PAH groups present in a light crude oil on a sandy beach in the Delaware field study. The first order relationship was expressed as:

$$\left(\frac{A}{H}\right) = \left(\frac{A}{H}\right)_0 e^{-kt} \tag{1.1}$$

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 analyte, A.

Table1.2 Summary of first order biodegradation rate constants from field studies

Field Study Location	Shoreline Type	Oil Type	Treatment	First order biodegradation rate day^{-1}		Reference
				Alkanes	PAHs	
Delaware	Sandy beach	Bonny light crude oil	Control Nutrient Inoculum	0.026 0.056 0.045	0.021 0.031 0.026	Venosa *et al.*, 1996
Quebec Canada	Tidal freshwater wetland	Mesa light crude oil	Control Nutrient	0.0028 0.0023-0.0034	0.0028 0.0016-0.0041	Venosa *et al.*, 2002
Texas	Brackish wetland	Phase II: Arabian light crude oil Phase III: Arabian medium Crude oil	Control Nutrient Control Nutrient Inoculum	0.019 0.042-0.061 0.020 0.024 0.019-0.030	0.017 0.018-0.027 0.015 0.013 0.016-0.017	Simon *et al.*, 1999
Louisiana	Salt marsh	South Louisiana crude oil	Control Nutrient	0.005 0.005	N/A	Shin *et al.*, 1999
Nova Scotia Canada	Salt marsh	Mesa light crude oil	Control Nutrient	0.020 0.026-0.039	0.010 0.011-0.013	Unpublished data

Since the Delaware study, several field trials conducted in other types of environments, including salt marshes and a freshwater wetland, have reported first-order oil biodegradation rate constants obtained using the same approach (Shin *et al.*, 1999; Simon *et al.*, 1999; Venosa *et al.*, 2002). The results of these kinetic studies in the field are summarized in Table 1.2. It can be seen that except for

Phase II of the San Jacinto field study (Mills *et al*., 1997; Simon *et al*., 1999), nutrient amendments did not show significant biodegradation rate enhancements in most of these wetland environments. Nonetheless, it is encouraging to see that crude oils can be biodegraded intrinsically on marine wetlands at similar rates as on sandy beaches (i.e., the Delaware site). The higher intrinsic oil biodegradation rates reported for salt marshes when compared to freshwater wetlands may be attributed to the generally greater oxygen limitation in freshwater wetland environments (Mitsch and Gosselink, 2000). It should be noted here that in the St. Lawrence River freshwater wetland study (Venosa *et al*., 2002), the oil was manually raked into the sediment, causing penetration of oil into the anaerobic zone. It is not known whether higher biodegradation rates would have ensued had this raking not been done.

1.3.5 Monitoring biological responses to quantify the efficacy of remediation treatment

In addition to the demonstration that remedial treatments reduce the concentration of residual oil, it is necessary to demonstrate that they do not produce any undesired environmental and ecological effects. As discussed in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001), two complimentary approaches are available: (1) bioassessments, which typically monitor changes in populations and communities of flora and fauna (Herricks and Schaeffer, 1984); and (2) bioassays, which include toxicity tests and bioaccumulation studies (Chapman, 1989).

1.3.5.1 Bioassessment

The monitoring of alterations in benthic community structure is frequently used to assess the potential impacts of residual oil within sediments. For example, in a follow up of the Exxon Valdez oil spill clean up, Driskell *et al*. (1996) noted negative effects including reductions in size, biomass, fecundity, and increased mortality as a result of hot water washing. 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 Persian Gulf oil spill (Watt *et al*., 1993). The possibility of adverse ecological effects such as algal blooms and invertebrate mortality from excessive nutrient amendments associated with bioremediation treatments is also a concern (Lee, 2000a; Lee *et al*., 2001a, 2001b).

To date, sediment bioassessments have been largely based on the tracking of changes in macroinvertebrate community structure. For a holistic approach, it is recommended that consideration should also be given to the bioassessment of fish and other non-benthic community organisms (e.g. bacteria, phytoplankton, cladocera, and amphibians). Furthermore, with recent advances in biotechnology, micro-scale bioassays are now available to monitor alterations at the subcellular or multicellular level of biological organization (Lee *et al*., 1998; Wells *et al*., 1998). In wetland environments, quantification of potential impacts on vegetative growth can be used to document the efficacy of bioremediation strategies. For example, in a tidal freshwater wetland experiment, the predominant plant species (*Scirpus pungens*) was reported to be tolerant to the oil, and its growth was significantly enhanced above that of the unoiled control by the addition of nutrients (Lee *et al*., 2001a). Monitoring of recolonization within impacted areas should be considered as an endpoint in bioassessments, as it provides integrated information on the impact of contaminants on processes such as immigration, emigration, competition, and predation.

1.3.5.2 Bioassays

As discussed in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001), acute and/or chronic bioassays can be performed on whole sediment (e.g., solid-phase), suspended sediment, sediment liquid phases (pore water, interstitial water), or sediment extracts (elutriates, solvent extracts). Since various forms of biota differ in their sensitivity to toxicants, it is highly recommended that a test battery approach with species from different trophic levels be utilized in environmental assessments to ensure ecological relevance.

Sediment bioassays have been used extensively to diagnose the effect of oil spills (Teal *et al*., 1992; Gilfillan *et al*., 1995; Neff and Stubblefield, 1995; Randolph *et al*., 1998) and their countermeasures such as bioremediation (Lee *et al*., 1995b; Mearns *et al*., 1995; Mueller *et al*., 1999). Criteria to consider for the selection of bioassays include: (1) sensitivity to test material, (2) ecological and/or economic relevance, and (3) the availability of regional expertise for the analysis and interpretation of results.

1.3.5.2.1 Numerous bioassays can be used to document the impact of oil spills in coastal environments. Benthic invertebrates such as amphipods and shellfish have been found to be highly sensitive to residual hydrocarbons following oil spill incidents (Teal et al. 1992; Gilfillan et al., 1995; Mueller *at al*., 1999; Wolfe et al., 1996). They have been used in both field and laboratory studies to monitor the impact and effectiveness of oil spill countermeasures such as bioremediation (Mearns et al., 1997, 1995; Lee et al., 2001a,b). In terms of quantifying a microbial response, the Microtox test is based on the measurement of changes in light emission by a nonpathogenic, bioluminescent marine bacterium (*Vibrio fisheri*) upon exposure to test samples. This commercial assay has been used by regulatory agencies for toxicity screening of chemicals, effluents, water and sediment, and for contamination surveys and environmental risk assessment, and its application for monitoring the efficacy of oil spill remediation methods has been proven (Lee et al., 1995b, 1997; Mueller *et al*., 1999).

1.3.5.2.2 Due to their economic, recreational, and aesthetic value, fish have been historically selected as a primary bioassay organism. Biochemical and physiological alterations induced by toxicant exposures can result in: 1) anatomical changes, 2) structural alterations in organelles, cells, tissue, and organs, and 3) alteration of metabolic processes. For example, the observation of neoplasms in fish was one of the first histopathological indices used in ecotoxicology. Biomarkers, defined as biochemical, physiological, or pathological responses measured in individual organisms on exposure to environmental contaminants, such as mixed function oxidase (MFO) reactions (Ortiz de Montellano, 1986) are also used. MFO reactions induced by PAHs and a variety of halogenated hydrocarbons are highly sensitive to contaminants. In the tidal freshwater wetland study with early life stages of fish, Hodson et al. (2001) noted that oil alone, oil mixed with sediments in the lab, and oiled sediments from the experimental plots all caused induction of MFO (CYP1A) enzyme activity 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.

To date, detrimental effects from nutrient enrichment have not been observed following full-scale field operations (Prince, 1993; Mearns *et al*., 1997). However, field experiments have suggested that the possibility of detrimental effects from bioremediation treatments cannot be fully discounted (Mueller *et al*., 1999). For 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*., 1995a). 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*., 1997). Bioassays used to document the effectiveness of bioremediation treatments in sandy intertidal shoreline sediments oiled with a weathered light crude oil showed an inhibitory effect on the hatching of grass shrimp due to the addition of nutrients (Mearns *et al*., 1995). Furthermore, most recently, in the tidal freshwater study described in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001), it was noted that amphipod toxicity levels became elevated during the study due to excessive nutrient enrichment (Lee *et al*., 2001a). It is recommended that future operational guidelines include ecotoxicological-monitoring protocols.

1.3.6 Bioremediation options on salt marshes

Major bioremediation options have been described in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001). This document provides a summary of the most current information on restorative techniques pertaining to salt marshes.

1.3.6.1 Nutrient Amendment

As stated in previous sections, biostimulation has been ineffective in accelerating the disappearance of oil on certain oil-contaminated salt marshes (Garcia-Blanco and Suidan, 2001; Shin *et al*., 1999) due to either the presence of high background nutrient concentrations or oxygen limitation. However, a few field studies did show enhanced oil biodegradation through nutrient addition (Lee and Levy, 1991; Mills *et al*., 1997); therefore, nutrient amendment may still be a viable option for removing hydrocarbons from an oil-contaminated wetland when nutrients are limiting. Nutrients used for biostimulation can be classified as water-soluble, slow-release, oleophilic, and organic.

- **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. 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. The main disadvantage is that they are more likely to be washed away by tidal and wave action. However, this washout effect is of lesser concern in salt marshes, since they generally represent low-energy environments that are subject to little turbulent mixing. A field study on nutrient hydrodynamics showed that water-soluble nutrients could remain in contact with oiled sediments for weeks on low energy shorelines before being washed out (Wrenn *et al*., 1997a; Harris *et al*., 1999).

- **Slow-release fertilizers** -- Slow release fertilizers are normally available in solid forms that consist of inorganic nutrients coated with hydrophobic materials like paraffin or vegetable oils or organic nutrients encapsulated by semi-permeable or controlled-rate degradable surface coatings. They are designed to overcome the washout problems and provide a continuous supply of nutrients to oil contaminated areas. This approach may also cost less than adding water-soluble nutrients due to less frequent applications (Lee et al., 1993). The Gladstone field trial has shown promise for the application of slow-release fertilizers in coastal wetlands (Burns *et al*., 2000). In this study, the degradation of a Gippsland crude oil in salt marsh plots was stimulated by the addition of Osmocote^{TM}, a slow release fertilizer consisting of a mixture of inorganic nutrients coated with an organic resin.

- **Oleophilic nutrients** -- Another approach to overcome the problem of water-soluble nutrient washout is to utilize oleophilic organic nutrients. The rationale for this strategy is that oil biodegradation mainly occurs at the oil-water interface, and 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. Results have been mixed. Some studies have suggested that oleophilic fertilizers might be more suitable for use in high-energy, coarse-grained environments due to poor penetration of fine sediments by oleophilic fertilizers (Sveum *et al*., 1994; Sveum and Ladousse, 1989). Bioremediation agents containing organic substrates such as meat and fish-bone meal and yeast extracts may have the capacity to provide essential micro-nutrients and organic growth substrates that may be limiting. However, the large amount of organic carbon within this type of amendment may also cause problems. For example, the organic carbon in the product may be biodegraded by microorganisms preferentially over petroleum hydrocarbons, thus contributing to oxygen depletion and resulting in undesirable anoxic conditions (Lee and Levy, 1987,1989; Lee *et al*., 1995a,b; Swannell *et al*., 1996). Considering their high cost and lack of demonstrated effectiveness, oleophilic fertilizers are unlikely to be the choice biostimulation agent for oil cleanup in coastal wetlands.

1.3.6.2 Microbial amendments

Addition of oil-degrading microorganisms (bioaugmentation) has been proposed as another type of 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 (Leahy and Colwell, 1990). Although many vendors of microbial agents claim that their product aids the oil biodegradation process based on laboratory tests, the effectiveness of microbial amendments has not been convincingly demonstrated in the field (Zhu *et al*., 2001). Actually, results from most field studies indicate that bioaugmentation is not effective in enhancing oil biodegradation on marine shores. Field studies conducted on sandy beaches have shown that nutrient addition or biostimulation alone had a greater effect on oil biodegradation than microbial seeding (Lee and Levy, 1987; Lee *et al*., 1997, Venosa *et al*., 1996). The San Jacinto study, the only reported field trial on oil bioaugmentation in a coastal wetland environment, also revealed that addition of microbial products did not significantly enhance oil biodegradation rates (Simon *et al*., 1999). This is because hydrocarbon-degrading microorganisms are ubiquitous in the environment, and their density can increase by many orders of magnitude after exposure to crude oil, as evidenced in recent studies of coastal wetlands (Garcia-Blanco and Suidan, 2001; Ramsay *et al*., 2000; Townsend *et al*., 1999). Also, added bacteria may not be able to compete with the indigenous, well-adapted population (Lee and Levy, 1989; Venosa *et al*., 1992). The mass of the hydrocarbon-degrading bacterial population on coastal wetlands is also limited 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 tidal activity. Therefore, it is unlikely that exogenous microorganisms would persist in contaminated wetlands even when they are added in large numbers. As a result, microbial amendments will not have any long-term or short term beneficial effects in shoreline cleanup operations.

1.3.6.3 Oxygen amendment

Because wetland soils are inundated with water, the diffusion rates of oxygen through the soils are very slow, and oxygen in the interstitial water is quickly depleted by aerobic metabolism of detritus that is abundant in wetlands. A few centimeters, and often only a few millimeters below the sediment surface, the wetland sediments are anaerobic. Therefore, oxygen is likely a limiting factor for oil biodegradation in marine wetlands. 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. Many of the oxygen amendment technologies developed in terrestrial environments (e.g. tilling, forced aeration, and the addition of chemical oxidants), are currently not considered viable options for use in coastal wetlands. There is concern that their deployment is expensive and environmentally intrusive. Furthermore, their effectiveness in enhancing oil biodegradation in wetland environments is unproven.

The Gladstone field trial showed that a forced aeration strategy was only able to increase the depth of the aerobic layer of the wetland sediments from 1 mm to 2 mm, and could not significantly stimulate oil biodegradation in the anaerobic mangrove environment (Burns *et al*., 2000). Strategies involving the mixing of surface sediments, such as tilling or disking, have also been proven ineffective in recent field studies (Garcia-Blanco and Suidan, 2001; Garcia-Blanco *et al*., 2001b; Venosa *et al*., 2002). Not only does this approach cause severe ecological damage to wetlands, it also enhances oil penetration deep into the anaerobic sediments, resulting in slower oil biodegradation. As for adding alternative electron acceptors, there is no strong evidence yet to suggest that the addition of nitrate as an electron acceptor can enhance oil biodegradation when oxygen is limiting (Garcia-Blanco and Suidan, 2001; Townsend *et al.*, 1999). The high oil degradation rates under sulfate-reducing conditions found in some laboratories (Caldwell *et al.*, 1998; Coates *et al*., 1997) have not been convincingly demonstrated in the field. Therefore, further research is still required to explore cost-effective oxygen amendment techniques for the bioremediation of coastal wetlands.

1.3.6.4 Plant amendment (phytoremediation)

Phytoremediation, the stimulation of contaminant degradation by the growth of plants and their associated microorganisms, is emerging as a potentially cost-effective option for cleanup of petroleum hydrocarbons in terrestrial environments (Banks *et al*., 2000; Frick *et al.*, 1999a). Mechanisms responsible for oil phytoremediation may include degradation, containment, and the transfer of contaminants from soil to the atmosphere (Cunningham *et al.*, 1996). Frick *et al*. (1999b) indicated that the primary loss mechanism for petroleum hydrocarbons is the degradation of these compounds by microorganisms in the rhizosphere of plants. Phytoremediation was hypothesized to be particularly effective when used together with nutrient enrichment because hydrocarbon contamination may result in nutrient deficiencies in contaminated soil. Added fertilizers could increase the rate of oil degradation by indigenous microorganisms in the rhizosphere and simultaneously stimulate plant biomass production, thereby increasing the effectiveness of phytoremediation and accelerating the recovery of the affected wetland plant ecosystem.

Extensive studies have been conducted on the phytoremediation of petroleum hydrocarbons in terrestrial environments (Frick *et al.* 1999a,b). Researchers at University of Saskatchewan, Canada, recently developed a catalogue of plants with the potential to phytoremediate hydrocarbon contaminated soils following a review of information in the literature and the conduct of field surveys (Godwin *et al*., 1999; and Frick *et al*., 1999c). Nevertheless, only limited studies have been carried out on the effectiveness of phytoremediation in enhancing oil degradation in coastal wetland environments. Lin and Mendelssohn (1998) found in a greenhouse study that application of fertilizers in conjunction with the presence of salt marsh and brackish marsh transplants significantly enhanced oil degradation. In another mesocosm study, Dowty *et al*. (2001) evaluated the effects of soil organic matter content, plant species, soil oxygen status and nutrient content on oil degradation and plant growth response in fresh marsh environments. The study found that the amount of oil remaining after 18 months was lowest in aerated and fertilized mesocosms containing either *P. hemitomon* or *S. lancifolia* and a substrate of low organic matter content. Field studies, however, have not demonstrated such significant effects as in the mesocosm studies. A recent Nova Scotia field trial showed that addition of nutrients did not result in significant enhancement of biodegradation of crude oil, whether or not plants were left intact or removed (Garcia-Blanco and Suidan, 2001). Similar results were also found in the St. Lawrence River freshwater wetland field study (Garcia-Blanco *et al.,* 2001b; Venosa *et al.,* 2002). On the other hand, the results of these field trials did suggest that although application of fertilizers in conjunction with the presence of wetland plants may not significantly enhance oil degradation, it could accelerate habitat recovery. There is evidence that nutrient amendments could stimulate vigorous vegetative growth, reduce sediment toxicity and oil bioavailability (Lee *et al*., 2001a).

In summary, on the basis of field trials conducted to date, the effectiveness of phytoremediation in enhancing oil degradation in coastal wetlands is highly site-specific and does not promise to be an effective oil cleanup technique *per se*. However, it does show promise in accelerating the recovery and restoration of wetland environments contaminated with oil and oil products, which is the ultimate goal of the treatment.

1.3.6.5 Monitored natural attenuation

Natural attenuation has been defined as the reliance on natural processes to achieve site-specific remedial objectives (USEPA, 1999). When used as a clean up method, a monitoring program is still required to assess the performance of natural attenuation. This approach is increasingly viewed as the most cost-effective, although the least cosmetically appealing, option for the cleanup of oil spills in coastal wetland environments since it causes the least adverse ecological impacts often associated with cleanup activities (Baker, 1999; Owens *et al*., 1999; Sell *et al*., 1995). Sell *et al*. (1995); Mills *et al*., 2003) compared the rates of recovery between treated and untreated wetlands based on 20 case studies of heavily oiled salt marshes. They concluded that most traditional cleanup methods did not promote significant long-term ecosystem recovery.

Recent field studies on oil bioremediation have demonstrated that the availability of oxygen, not nutrients, is often the limiting factor for oil biodegradation in coastal wetlands. However, as discussed earlier, no feasible technique is currently available for increasing the availability of oxygen in such an environment. Fortunately, these field studies also showed that the natural biodegradation of alkanes and PAHs could occur to a very high extent and at similar rates in coastal wetlands as in sandy beaches (See section 1.3.4). Therefore, in consideration of the potential impacts associated with physical clean-up procedures in wetlands (i.e. trampling), natural attenuation should be given more preference in decision making for oil spill cleanup in coastal wetlands when the oil concentration is not high enough to destroy the ecosystem.

2 RECOMMENDED APPROACHES TO BIOREMEDIATION IN SALT MARSHES

Existing studies have demonstrated that oil biodegradation on marine wetlands is often limited by oxygen, not nutrient availability. Natural attenuation is increasingly becoming a promising and even a preferred strategy for the restoration of oil-contaminated wetlands. However, field studies also showed that on some coastal wetlands, nutrients might still be a limiting factor for oil biodegradation, particularly if the oil does not penetrate deeply into the anoxic zone of the wetland sediment (Lee and Levy, 1991; Mills *et al*., 1997; Venosa *et al*., 2002). Therefore, biostimulation with nutrient amendment can still be an appropriate countermeasure treatment under some circumstances. General guidelines for the bioremediation of oil-contaminated marine shorelines, which are mostly derived from studies and practices on sandy beaches, have been presented in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001). Although the general principles for achieving successful oil bioremediation for all types of marine shorelines are the same, a simple transfer of response strategies may not be necessarily the most appropriate since salt marsh habitats are significantly different from other marine situations. Therefore, guidelines and special considerations for oil bioremediation in coastal wetland environments are presented here based on current understandings and field studies, particularly the findings of the Nova Scotia field study (Garcia-Blanco and Suidan, 2001).

Similar to the general protocol presented in the sandy shoreline and freshwater wetland guidance document (Zhu et al., 2001), a general procedure or plan for the selection and application of bioremediation technology in salt marshes is illustrated in Figure 2.1. 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 biodegradability of the spilled oil, the depth of oil penetration and oxygen availability, concentrations of background nutrients, the presence of hydrocarbon-degrading microorganisms, the type of shoreline substrate, and other logistic and environmental factors (pH, temperature, 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 step 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 document will focus on the operational guidelines for decision-making and planning of oil bioremediation in salt marshes. Guidelines with respect to the assessment of field results and establishment of appropriate treatment endpoints can be found in the Chapter 6 of the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001).

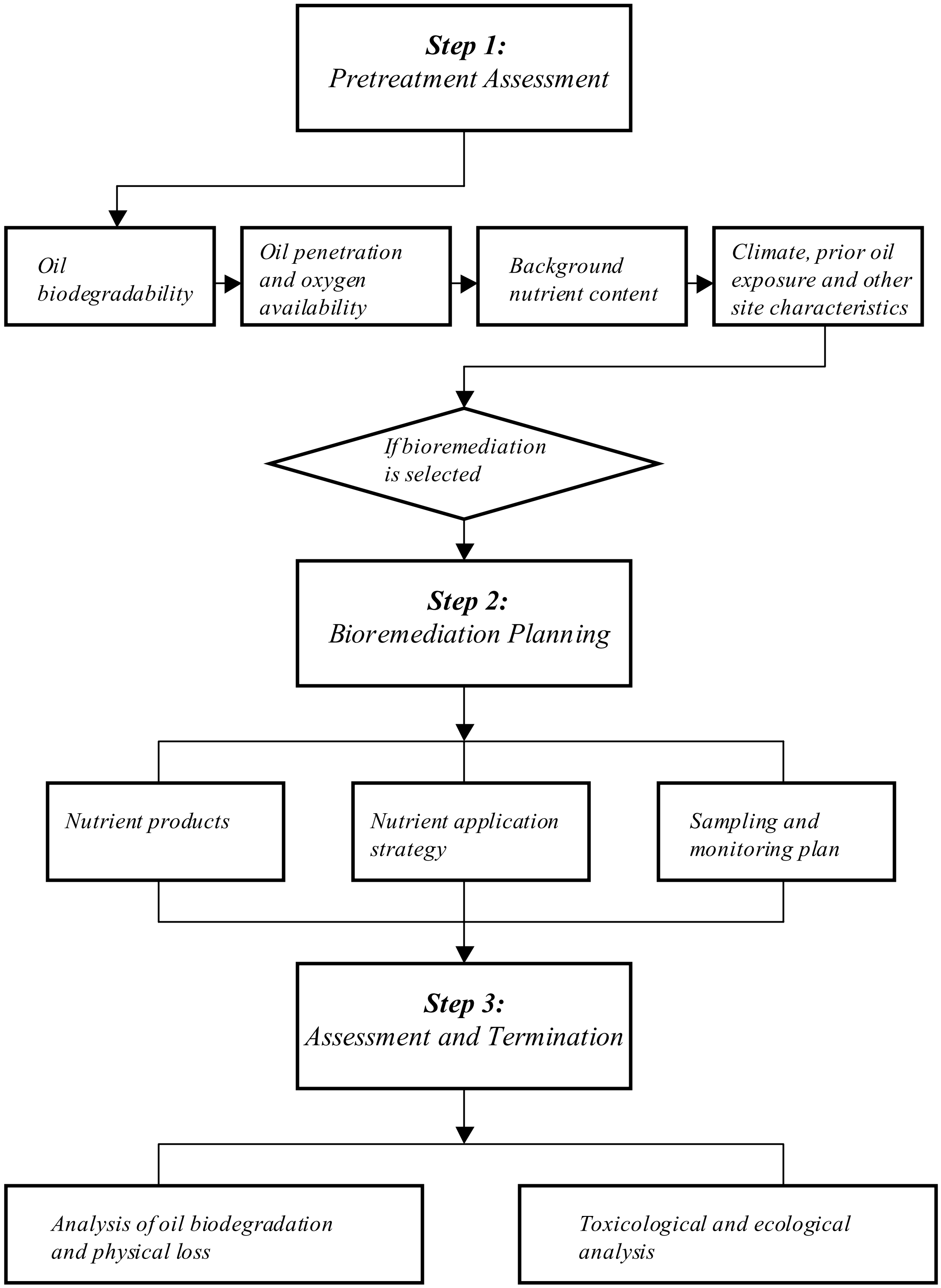

Figure 2.1 Procedures for the selection and application of oil spill bioremediation in salt marshes

2.1 Pre-treatment Assessment

Major considerations in the assessment of the need for biostimulation in salt marshes include the evaluation of 1) oil types and concentrations, 2) oil penetration and oxygen availability 3) background nutrient content, and 4) other environmental factors such as the prevalent climate and prior oil exposures. Among these factors, the assessments of oil penetration, oxygen availability and background nutrient content are of particular importance for bioremediation of salt marshes and will be discussed in following sections. Detailed discussion on the assessments of oil types and concentrations, oil biodegradability, climate and other environmental factors can be found in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001).

2.1.1 Oil penetration and oxygen availability

Unlike other types of marine shorelines (e.g. sandy beaches), the most important limitation for cleanup of an oil-contaminated marine wetland is oxygen availability. Wetland sediments become anoxic often below a few millimeters to centimeters of the soil surface. When substantial penetration of spilled oil into anoxic sediments has taken place, available evidence suggests that biostimulation with nutrient addition has limited potential for enhancing oil biodegradation, and it would likely be best simply to leave it alone and not risk further damage to the environment by trampling and the associated bioremediation activities. Therefore, the evaluation of oil penetration and oxygen availability is probably the most important pre-treatment assessment for determining whether bioremediation is a viable option.

The thickness of the oxidized layer within wetland sediments varies from a few millimeters to several centimeters, depending on the population of oxygen utilizers, the rate of photosynthetic oxygen production by algae, the soil chemical composition, and the rate of oxygen transport into the wetland sediments (Mitsch and Gosselink, 2000; Shin *et al*, 2000). For example, soil organic matter is a major oxygen sink in salt marshes and, therefore, oxygen deficiency is more likely to occur in wetland soil with high organic matter content. Oxygen limitation will be less severe in the area where the wetland surface is exposed to the atmosphere or is subjected to strong surface mixing by convection currents and wave action.

The depth of the aerobic layer can be identified through both visual observation and measurements of DO and redox potential. The wetland surface or the aerobic layer is often a brown or brownish-red color due to the presence of ferric ions. The anoxic zone in wetland sediments is either bluish gray due to the presence of ferrous ions or, more often in salt marshes, black along with a foul odor associated with the production of hydrogen sulfide under sulfate reducing conditions. Anoxic conditions can also be determined by measuring dissolved oxygen in pore water. Oxygen will become a limiting factor when DO concentration in pore water approaches zero. When using DO probe, a reading of 0.1 or 0.2 mg/L indicates the depletion of dissolved oxygen. Redox potential is a more sensitive measurement of the degree of reduction of wetland sediments. For example, denitrification occurs at a redox potential of approximately 250 mV and sulfates are reduced to sulfides at a redox potential between –100 and –200 mV (Mitsch and Gosselink, 2000).

The depth of oil penetration also depends on many factors, such as oil type, concentration and shoreline substrate. In general, fresh crude oils and heavy oils tend to adhere to the marsh surface sediment or pool on the sediment surface. Light oils and oil components can generally penetrate the top few centimeters of wetland sediment. However, penetration can be much deeper into burrows and cracks extending up to one meter (NOAA, 1992). A microcosm study on the penetration of weathered light Arabian crude oil in freshwater wetland sediments showed that the oil was able to penetrate about 2.5 cm in 16 weeks for both a flooded condition and a saturated but non-flooded condition (Purandare, 1999). However, the amount of the oil able to penetrate into the sediment was much less for flooded sediments, where most of the oil floated on the surface of the water. The depth of oil penetration also increases with the increase of oil concentration and therefore affects the potential of oil biodegradation. In the field trial reported by Lee and Levy (1991), the rates of oil degradation in the salt marsh were not stimulated by nutrient amendments at the higher test concentration (3.0% v oil/v sediment), where oil penetrated to anaerobic layers of sediment. However, bioremediation was effective at the lower test concentration (0.3 % v oil/v sediment), where the oil did not penetrate beyond the aerobic sediment surface layers. In the same field study, Lee and Levy (1991) also examined the effect of oil concentration on a sandy beach and found that oil biodegradation rates were enhanced by the nutrient amendment at the higher oil concentrations (3%), where oxygen was not a limiting factor. The result suggested that the favorable concentrations for using bioremediation would be much lower in salt mashes than on sandy beaches.

The type of shoreline substrate is another important factor affecting the oil penetration and the feasibility of using bioremediation. Shoreline substrate can affect oil penetration from the perspectives of both the sediment texture and the soil chemical composition. Generally, oil penetrates coarse sediments more readily than fine sediments. However, because the texture of all wetland sediments is normally very fine, the substrate chemical composition plays a more important role in oil penetration in salt marsh environments. Studies have shown that the rates of oil penetration and biodegradation are strongly related to the soil organic matter content (Dowty *et al*., 2001; Lin and Mendelssohn, 1996). Oil is more likely to penetrate into sediments with higher organic content since it associates more readily with organic matter than with mineral particles. In a greenhouse study, Lin and Mendelssohn (1996) investigated the performance of oil biodegradation in three types of coastal wetlands -- salt, brackish, and freshwater marsh. They found that the rates of oil degradation were highest in the salt marshes and lowest in the freshwater marshes. The difference in oil residue was mainly attributed to the difference in the soil organic content, which was lowest in the salt marsh sediments and highest in the freshwater marsh sediments. The study also measured the concentrations of the oil that penetrated the soils in digested (to remove the associated organic matter) and undigested marsh soil and found that the oil concentrations were 41-279 times higher in the undigested soil than the digested one. Similar results were observed by Dowty *et al*. (2001) in a mesocosm study conducted in fresh marsh environments. They found that the rates of oil degradation were significantly higher in the inorganic sediments than the organic ones under different oil concentrations and nutrient levels. These results are consistent with the notion that oxygen demand is higher and oil is more readily able to penetrate into organic sediments. Therefore, oil bioremediation seems more likely to be successful when applied in a wetland with lower organic matter content.

2.1.2 Background nutrient content

To determine whether nutrient amendment is a viable option, it is necessary to assess the background nutrient levels in the contaminated site, particularly the nutrient concentrations within the interstitial water in that environment. There is no need to add nutrients if natural nutrient concentrations are high enough to sustain rapid intrinsic rates of oil biodegradation. However, because oxygen is often the determining factor in oil degradation on coastal wetlands, the assessment of background nutrient concentration is important and needed only after the assessments of oil penetration and oxygen availability conclude that oxygen limitation is not a serious impediment. In other words, when substantial oil penetration into the anoxic zone of the wetland sediments occurs, nutrient amendment is not likely to be effective even if nutrient deficiency exists. As shown in the St. Lawrence River field trial (Venosa *et al*., 2002), the average pore water nitrogen concentration in natural attenuation plots was only about 0.74 mg N/L, well below the levels needed for maximum hydrocarbon biodegradation (Venosa *et al*., 1996). However, the dramatic increase in nutrient levels in the biostimulation plots did not enhance oil biodegradation above that achievable in the natural attenuation plots due to the oxygen limitation within that freshwater wetland sediment.

However, when oxygen availability is not a limiting factor, the decision to use nutrient amendments should be based on how high the natural levels are relative to the optimal or threshold nutrient concentrations. It has been recommended in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001) that the threshold concentration for optimal hydrocarbon biodegradation on marine shorelines is in the range of 2 to 10 mg N/L based on the field experiences on sandy beaches (Bragg *et al*., 1994; Venosa *et al*., 1996) as well as in an estuarine environment (Oudet *et al*., 1998). Although no such threshold concentration has been experimentally identified in salt marsh environments, recent field experiences did provide some insights. The Nova Scotia study found that the average background nitrogen concentration in pore water was about 10 mg N/L at the experimental site. Thus, nitrogen limitation was not an important factor (Garcia-Blanco and Suidan, 2001). The ineffectiveness of nutrient amendments in enhancing oil biodegradation under this high background nutrient level suggested that the nitrogen threshold concentration should be lower than 10 mg N/L. However, the San Jacinto River study suggested that the threshold nitrogen concentration may be higher than 2 mg N/L on coastal wetlands. During Phase II of the study, nutrient addition apparently enhanced oil degradation even when the background nitrogen concentration was about 5 mg N/L (Harris *et al*, 1999; Mills *et al*., 1997). This study, however, was inconclusive because the same enhancement was not observed when the treatment was repeated during the following year (Simon *et al.*, 1999). Although further research is still needed, it appears from existing evidence that the threshold nitrogen concentration for optimal oil biodegradation in salt marshes is likely similar to that obtained in other shoreline types (e.g. 2-10 mg N /L).

The investigation of background nutrients should also determine whether the present nutrient concentrations are typical of the area or sporadic (i.e., determine the impact of chronic runoff from nearby agricultural practices and local industrial and domestic effluents). As described in Part I, coastal marshes are generally considered high-nutrient wetlands. However, inorganic bioavailable nutrient concentrations in salt marsh sediments may exhibit a strong seasonal pattern with a concentration peak usually during the summer months probably due to a high mineralization rate at a higher temperature (Cartaxana *et al*., 1999; Nixon *et al*., 1980). The available nutrient levels can also be elevated as a result of runoff, fire and death of plants. If these events are sporadic, biostimulation may still be appropriate when the nutrient levels fall below threshold concentrations.

2.1.3 Summary of pretreatment assessment

Based on the current understandings discussed in the previous sections as well as in the sandy shoreline and freshwater wetland guidance document (Zhu et al., 2001), the following pretreatment assessments should be conducted to determine whether bioremediation is a viable option in response to a spill incident in salt marsh environments:

- 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 also 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 (of any weight) may also inhibit biodegradation. For details, see Zhu et al. (2001).

- Determine whether oxygen is a factor limiting oil biodegradation by measuring the depth of oxidized sediment layer and the extent of oil penetration – When a substantial portion of the spilled oil has penetrated into anoxic sediments, biostimulation with nutrient addition has limited potential for enhancing oil biodegradation. Oxygen limitation is less likely to occur in wetland sediments with lower organic matter and/or contaminated with oil at moderate concentrations.

- Determine whether the nutrient content at the impacted area is likely to be a limiting factor by measuring the background nutrient concentrations within the interstitial water in that environment – If oxygen is not the limiting factor, 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., > 5 mg N/L). 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 climatic or seasonal conditions are favorable for using bioremediation – Bioremediation may be more effective during warmer seasons, particularly in cold environments, since oil biodegradation rates are higher during these seasons. However, this does not necessarily mean that summer is the most favorable season. Because inorganic nutrient levels in salt marsh sediments often peak during the summer, biostimulation will not be effective if the nutrient content is no longer the limiting factor during warmer seasons. Prior exposure to oil will also be a favorable but not a solely determinative condition for selecting bioremediation.

2.2 Treatment Selection and Design

If biostimulation by nutrient addition is determined to be a potentially effective cleanup option based on the pretreatment assessments, further evaluation and planning are needed before its application. This step involves selection of the rate-limiting nutrients, determination of optimal nutrient concentrations and application strategies, and design of sampling and monitoring plans.

2.2.1 Nutrient selection

One of the first tasks during the stage of treatment selection and design would be to select appropriate nutrient products. The laboratory treatability tests, especially well-designed microcosm or mesocosms tests, are most commonly used approaches to determine the type and level of amendments. However, responders will likely not have time or resources to conduct a treatability study. This section, therefore, serves to support a reasonable approach to deciding which type of formulation to use.

Screening and treatability tests that have been reported in the literature involve the determination of rate limiting nutrients as well as optimal forms of nutrient species. Nitrogen, phosphorus, or both can limit oil degradation in salt marshes. In a microcosm study, Jackson and Pardue (1999) found that nitrogen but not phosphorus was the rate-limiting nutrient for oil degradation in sediment from a Louisiana salt marsh. Wright *et al.* (1996, 1997) reported the opposite result for a mesocosm study where oil degradation was mainly limited by the concentration of phosphorus in sediment from a Texas salt marsh.

The molecular form of nutrients is also important. For example, although both ammonium and nitrate are capable of enhancing oil degradation when nitrogen is a limiting factor, their effectiveness may differ depending on the type of oil and the properties of shoreline substrate. Jackson and Pardue (1999) found that addition of ammonium appeared to stimulate degradation of crude oil more effectively than nitrate in salt marsh soils in a microcosm study. The ammonium requirement was only 20% of the concentration of nitrate to achieve the same increase in degradation. The authors concluded that ammonium was less likely to be lost from the microcosms by washout due to its higher adsorptive capacity to sediment organic matter. A recently completed study at a salt marsh in Nova Scotia also showed that the ammonium spikes after nutrient addition were always substantially higher than the nitrate spikes, even though the only exogenous source of nitrogen was NH_4NO_3 (Table 1.1). The lower pore water nitrate concentrations can be attributed to the higher washout rate for nitrate and its loss through denitrification within the anoxic sediments. Under such circumstances, ammonium based nutrients may be superior to nitrate based nutrients because the nutrient dosage will be much lower when using ammonium than nitrate to achieve the same pore nitrogen concentration. However, this may not always be the case. Actually, the St. Lawrence River field study showed that the pore water nitrate concentrations were always higher than the ammonium concentrations after NH_4NO_3 was added (Venosa *et al*., 2002). This finding was attributed to the adsorption of NH_4^+ onto the negatively charged soil particles and its uptake by the root systems of the wetland plants. This result also suggests that the effects of nitrate washout and denitrification were less important in this fresh water marsh.

Nutrient selection might also be influenced by temperature conditions. In a field study, Lee *et al*. (1993) investigated the efficacy of water-soluble inorganic fertilizers (ammonium nitrate and triple super phosphate) 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 suppress nutrient release rates. Water-soluble fertilizers such as ammonium nitrate were then recommended under these temperature conditions. Based on the above discussion, it is recommended that, if temperature conditions allow use of slow-release fertilizer (i.e., temperatures in excess of 15°C), then that would be the preferred fertilizer to use in a salt marsh. If the temperature were lower, then ammonium nitrate would be appropriate. In either case, the amount of fertilizer to use should be based on maintenance of a minimal amount that would not limit biodegradation (i.e., something greater than about 5 mg N/L and 0.5 mg P/L).

In addition to demonstrating the efficacy of nutrient products in enhancing oil degradation, it is also critical to demonstrate that bioremediation products have low toxicity and do not produce any undesired environmental and ecological effects, especially when applied to such sensitive ecosystems as salt marshes. Various toxicity test protocols have been discussed in part I as well as in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001). A case study on the assessment of bioremediation treatment through monitoring biological responses in an oil-contaminated salt marsh will also be presented later in this document.

2.2.2 Concentrations 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 provide no added benefit but may lead to potentially detrimental ecological and toxicological impacts.

Only a few studies have been reported on the optimal nutrient concentration in salt marsh environments. In a microcosm study using salt marsh sediment slurry, Jackson and Pardue (1999) found that oil degradation rates could be increased with increasing concentrations of ammonia in the range of 10 – 670 mg N/L, with most of the consistent rate increases occurring between 100 -670 mg N/L. They further proposed a critical nitrogen concentration range of 10-20 mg N/L. Harris *et al*. (1999) examined the nutrient dynamics during natural recovery of an oil-contaminated brackish marsh and found that there was an interdependency between the natural nutrient levels and the extent of oil degradation when the background nitrogen concentration in pore water declined from 40 mg N/L to 5 mg N/L. Evidence from bioremediation field studies also suggested that concentrations of approximately 5 to 10 mg/L of available nitrogen in the interstitial pore water is sufficient to meet the minimum nutrient requirement of the oil degrading microorganisms (Garcia-Blanco and Suidan, 2001; Mills *et al*., 1997; See Section 2.1.2). As mentioned earlier, the threshold concentration range for optimal hydrocarbon biodegradation on marine shorelines is around 2 to 10 mg N/L based on field experiences on sandy beaches (Bragg *et al*., 1994; Venosa *et al*., 1996) and in an estuarine environment (Oudet *et al*., 1998). The apparent higher threshold nitrogen concentrations in salt marshes are mainly due to the lack of information with respect to oil biodegradation under lower nitrogen concentrations, since all the existing field studies were conducted in salt marshes with background nitrogen concentrations of at least 5 mg N/L (Garcia-Blanco and Suidan, 2001; Harris *et al*., 1999; Mills *et al*., 1997; Shin *et al*., 1999). Therefore, it is reasonable to recommend, as for other types of shorelines, that biostimulation of oil impacted salt marshes 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. The safety factor used in salt marsh environments may generally be smaller than that used in higher energy beaches due to the reduced degree of nutrient washout expected in salt marshes. One needs always to keep in mind, however, that nutrient toxicity might exist if too much nutrient is applied to a coastal wetland (Mueller et al., 1999). The factors that lead to higher nutrient losses in wetland environments may also be important, such as sediment adsorption, plant uptake, and denitrification (if applicable).

2.2.3 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.

2.2.3.1 Frequency of nutrient addition

The frequency of nutrient addition to maintain the optimal nutrient concentration in the interstitial pore water mainly depends on shoreline nutrient loss rates. A tracer study conducted on a low-energy beach and a high-energy beach in Maine demonstrated the influence of shoreline types on nutrient washout rates (Wrenn *et al*., 1997a; Zhu *et al*., 2001)). The study shows 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 during this period. Salt marshes are low-energy systems and nutrient washout rates in such environments should be similar to the observations made on the low energy beach in the Maine study. In salt marshes, the washout rates may be further reduced when using ammonium-based nutrients due to their higher affinity to adsorb onto the sediment as compared to nitrate-based fertilizers (Jackson and Pardue, 1999). Therefore, weekly to monthly additions may be sufficient for biostimulation of salt marshes when the nutrients are applied during neap tide. It is even possible that only one nutrient dose is required for the bioremediation of some coastal marshes. A study on the nutrient dynamics in an oil contaminated brackish marsh showed that it took more than one year for nutrient concentrations to decrease to background levels after being naturally elevated by flooding and perturbations due to the spill (Harris *et al*., 1999). However, this may not be truly indicative of nutrient application dynamics, since exogenous nutrients were not added in this case. Nutrient sampling, particularly in sediment pore water, must 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.

2.2.3.2 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. In many intertidal environments, particular high-energy shorelines, the primary consideration in developing and selecting a nutrient application method has been how to overcome the washout problems. Many attempts have been made in this regard, including the use of slow release and oleophilic fertilizers

(Prince, 1993) and the subsurface application of nutrients (Wise *et al.*, 1994). However, since nutrient washout in coastal wetland environments is relatively slow, the more important considerations in such cases should be on the use of less expensive and less environmentally intrusive application methods. As discussed in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001), current experience indicates that surface application of dry granular fertilizer (either slow-release or water-soluble) to the impact zone at low tide is probably the most cost-effective and less environmentally intrusive way to control nutrient concentrations.

2.2.4 Sampling and Monitoring Plan for Bioremediation Operations

2.2.4.1 Important variables and recommended measurements

Important variables to be monitored in an oil bioremediation project include the environmental factors that limit oil biodegradation rates (e.g., temperature, interstitial nutrient and oxygen concentrations), evidence of oil biodegradation (e.g., concentrations of oil and its components), microbial activity (e.g., bacterial numbers and activity), and toxicological effects. Primary variables recommended for monitoring of bioremediation field programs in coastal wetland environments are listed in Table 2.1.

If pretreatment assessments determine that oil biodegradation in the field is likely to be limited by nutrient rather than oxygen availability, pore water nutrient analysis becomes 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. This is to make certain that (1) the treatment is reaching and penetrating the impact zone, (2) target concentrations of nutrients are being achieved, and (3) toxic nutrient levels are not being reached. The location from which nutrient samples are collected is also important. Recent research on solute transport in the intertidal zone has shown that nutrients may remain in the beach subsurface for much longer periods than in the bioremediation zone (Wrenn *et al*., 1997b). Nutrient concentration profiles along the depth of the oil-contaminated region may be monitored by using multi-port sample wells or by the extraction of sediment samples collected from the oil-contaminated region (Venosa *et al*., 2002; Lee *et al*., 2001a). The sampling depth should be established from the results of site surveys to determine the maximum depth of oil penetration. To counter the inherent heterogeneity observed in field studies, a positive “margin of error” should be added to ensure that the samples will encompass the entire oiled depth throughout the project. The sampling depth must 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 of the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001), 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 occasionally using chromatographic techniques (e.g., GC/MS) and are reported relative to a conservative biomarker such as hopane. However, 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 protocols for the measurement of TPH, especially those using infrared absorption of Freon-extracts, are generally not reliable and have limited biological significance. Using GC/FID and integrating the area under the chromatogram is better. TLC-FID appears to be a promising screening tool for monitoring oil biodegradation (Stephens *et al*., 1999), although not enough experience is available to make any firm recommendations on its use at this time.

As suggested in the foregoing paragraph, GC/MS operated in the selected ion monitoring mode (SIM) is the preferred method to use to assess the progress of biodegradation. One sampling per month of composited samples from the site analyzed by GC/MS should suffice to provide evidence that hydrocarbons are being biodegraded. To this end, normalization of biodegradable constituents in the oil to hopanes, steranes, and/or other potential biomarkers (e.g., highly substituted 4- or 5-ring PAHs like C_4-chrysene) is essential to ensure that the disappearance observed is due to the bioremediation action rather than physical washout. Samples are normally extracted with dichloromethane and cleaned up using column chromatography prior to conducting the GC/MS. However, due to the expense and expertise involved with GC/MS analysis, more frequent analysis of TPH is appropriate to follow the temporal progress of treatment. It is suggested that at least one TPH sampling event per week be conducted at the spill site. Either the gravimetric or GC/FID method of TPH analysis should be used. Interpretation of chromatographic methods may be confounded by the presence of plant lipids and other biogenic compounds present in the environment; thus, care should be exercised in interpreting results. For example, plant lipids normally give rise to peaks in the chromatograms at retention times that coincide with odd-numbered higher molecular weight alkanes in the range of C_{25}, C_{27}, C_{29}, C_{31}, and C_{33}. Thus, it is essential that the chromatogram of the spilled oil be known to compare to actual samples analyzed.

In addition to monitoring treatment efficacy, 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 also been summarized in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001). These micro-scale bioassays may provide an operational endpoint indicator for bioremediation activities on the basis of toxicity reduction (Lee *et al*., 1995b). A summary of criteria for selecting an appropriate bioremediation endpoint based on both oil degradation and toxicity reduction has been presented in the sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001). Examples for a salt-marsh study are presented in the following sections.

Table 2.1 Monitoring plan for an oil bioremediation project in a coastal wetland environment

Analysis	Matrix	Recommended Methods	References
*Dissolved nitrogen	Sediment (interstitial pore water)	Extract in acidified 0.1% NaCl. 4500-NH_3 H (Automated Phenate Method) and 4500-NO_3^- F (automated Cd-reduction)	Eaton *et al*., 1995 Page *et al*, 1986
Dissolved phosphorus	Sediment (interstitial pore water)	Extract in acidified 0.1% NaCl. 4500-P E (ascorbic acid method)	Eaton *et al*., 1995 Page *et al*, 1986
*Residual oil constituents	Sediment	Extract into dichloromethane (DCM). Analyze components by GC/MS-SIM	Venosa *et al*, 1996
*Total petroleum hydrocarbons	Sediments	Gravimetric analysis (dichloromethane extraction) or GC/FID analysis of DCM extracts.	NETAC, 1993
Redox and sulfide	Sediment (interstitial pore water)	Redox and sulfide electrodes	ATI Orion,1991a,b
*Dissolved oxygen of pore water	Aqueous	Hach® high range assay	Hach Company, Loveland, CO
pH of pore water	Aqueous	Potentiometric with combination electrode	Page *et al*, 1986
Microbial populations	Sediment	MPN for alkane and PAH degraders Genetic biomarkers	Wrenn and Venosa, 1996 Macnaughton *et al*., 1999
Microbial activity	Sediment	Uptake/respiration of radiolabelled substrates. *In-situ* respiration	Lee and Levy, 1989 Prince *et al*, 1999
*Toxicity of residual oil	Sediment, pore water	Biotests (e.g. Microtox Test, Amphipod survival test, MFO induction, etc.)	See Section 1.3.5 and 2.2.4.2
Shoreline profile	Contaminated site	Intertidal/supratidal zone surveys using fixed benchmarks at the study site.(e.g., wells, plot boundary markers).	Wrenn *et al*, 1997a,b

* Critical measurements

The sandy shoreline and freshwater wetland guidance document (Zhu *et al*., 2001) presents other important variables in a comprehensive monitoring plan, including site background conditions (e.g., oxygen, redox, pH, sediment grain size, and temperature) and shoreline profiles. Oxygen availability is crucial for rapid bioremediation since hydrocarbon biodegradation is primarily an aerobic process. Although the pretreatment assessments may have determined that oxygen availability might not be a serious concern for the on-going project, oxygen limitation is always a potential problem in a wetland environment. 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 available oxygen does become limiting, the nutrient dosage and application frequency should be adjusted accordingly. Monitoring oil penetration and analyzing redox potential and sulfide concentrations with depth of wetland sediments will assist in determining whether oil has penetrated into the anoxic zone during the process of bioremediation. This assessment can also be used as a criterion in determining treatment endpoints.

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. For accurate interpretation of field data, analysis of sediment grain size should be conducted to verify study site homogeneity.

2.2.5 Environmental assessment of an oil-contaminated salt marsh: a case study

Since environmental assessment is a relatively new approach in evaluating the effectiveness of oil bioremediation treatments, a case study outline is presented as a means to provide operational guidance to spill responders. This example is based on a controlled oil spill field trial recently conducted in a salt marsh at Conrod's Beach, Nova Scotia, Canada, to determine if bioremediation by nutrient enrichment or phytoremediation by enhanced plant growth would accelerate the rates of residual oil loss and habitat recovery. The experimental design and bioremediation performance with respect to oil biodegradation has been reviewed (Section 1.3.3). Standard bioassessment and biotest procedures (Section 1.3.5) were used to quantify the rates of habitat recovery and to identify detrimental treatment effects (e.g., toxicity of the bioremediation agent or oil degradation by-products). The overall success of the remedial operations was based on the integration of results from a suite of assays, which were chosen on the basis of ecological relevance to the site of concern, cost considerations, and the availability of technical expertise (Venosa and Lee, 2002).

2.2.5.1 Bioassessments.

2.2.5.1.1 Recovery of vegetation. Growth (biomass) of the predominant plant species (*Spartina alterniflora*) within the salt marsh was significantly suppressed by oiling, and recovery was not observed during the first growing season. While initial results showed some recovery in all oiled plots in the following spring, there was also evidence of changes in species composition within the treated plots. Another opportunistic plant species that was more tolerant to the altered site conditions increased its percentage of cover. By the end of the second growing season, the treated plots showed substantial evidence of recovery in the sections of the plots that were not removed by sampling.

2.2.5.1.2 Microbial responses.

- *Oil degradation potential*. Potential hydrocarbon degradation rates of representative alkane and PAH components within sediment samples were determined by quantifying the respiration rate of added ^{14}C-labelled hexadecane and ^{14}C-labelled phenanthrene as representatives of *n*-alkane and PAH class components within the test oil (Lee and Levy, 1989, Caparello and LaRock 1975, Walker and Colwell, 1976). Time-series changes (Week: 4, 7, 9, 12, 16, 20) in the turnover time of these specific tracers were calculated with the actual concentrations of residual hexadecane and phenanthrene in each sample determined by GC/MS to account for dilution by the unlabelled fraction of the specific substrates under study.

  Results of the added ^{14}C-labeled hexadecane studies clearly illustrated the stimulation of indigenous organisms with the potential to degrade alkanes within the first 10-weeks after the application of oil (Figure 2.2). The lower the turnover time, the greater is the stimulatory effect (lower turnover times mean higher biodegradation rates). A stimulatory effect on potential hexadecane degradation rates by the addition of nutrients to unoiled sediments was also observed. However, within the oiled sediments, remedial treatments based on nutrient additions did not appear to cause a stimulatory effect that could be adequately resolved by measurement of hexadecane respiration rates. Natural attenuation (Treatment C: oil without nutrients in the figure) appeared to be relatively effective. These radiotracer studies are in agreement with detailed chemical analysis that showed that 87% of the target *n*-alkanes were degraded in the test sediments within 20 weeks. Similar observations were made for the biodegradation of PAHs (represented by phenanthrene, a 3-ringed polycyclic aromatic hydrocarbon) with the exception that nutrient amendments to the unoiled control sediments had no stimulatory effect (Figure 2.3) as contrasted with the hexadecane results. These observations are in full agreement with the corresponding field studies on microbial growth by MPN analysis. It was noted that besides oil, the addition of nutrients to unoiled plots also resulted in an increase in the number of potential *n*-alkane degraders by two orders of magnitude with respect to background levels. Only oiled plots showed an increase in the number of PAH degraders.

- *Denitrification activity*. Denitrification is a primary process that regulates the nitrogen cycle in wetland sediments (Figure 1.1). Microbial denitrification activity was monitored on each sampling occasion by placing a gas chamber on each plot and taking headspace samples over a 30-minute period, which were subsequently analyzed for nitrous oxide, an intermediate in the denitrification of nitrate. The seasonal average denitrification activity, plotted against treatment type showed that in all cases where nutrients were applied (Treatments D = oil + nutrients, E = oil + nutrients + cut plants, F = oil + nutrients + disking), there was a net positive denitrification potential (Fig. 2.4). Natural attenuation (Treatment C: oil without nutrients) and Treatment A (unoiled control) showed a net negative denitrification potential. These results indicate that nutrient application resulted in increased denitrification activity in the sediments.

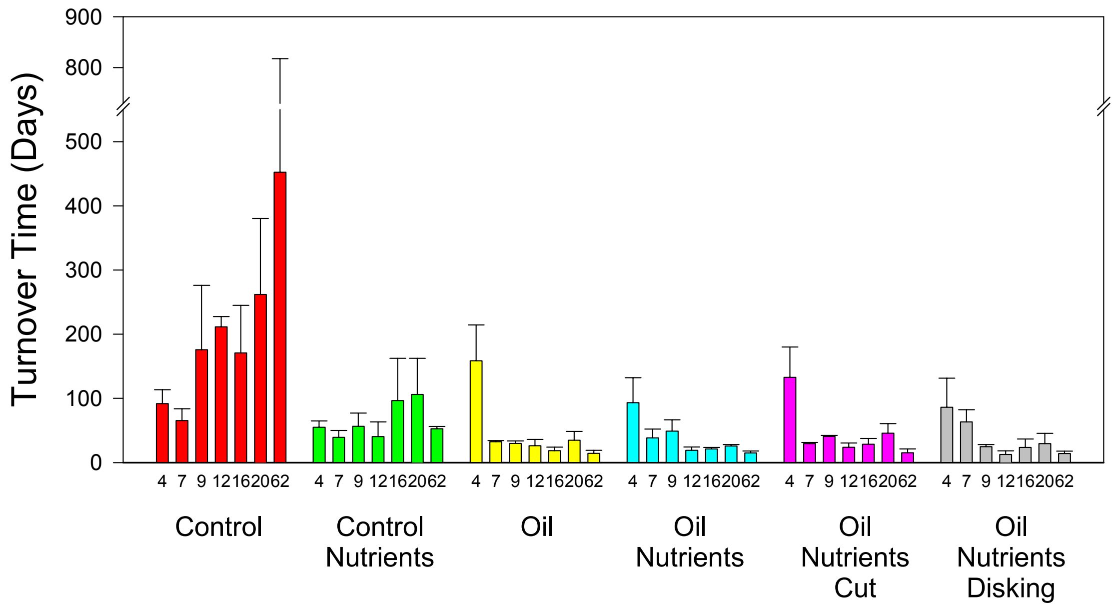

Figure 2.2. Average turnover time of hexadecane. Error bars = 1 standard error.

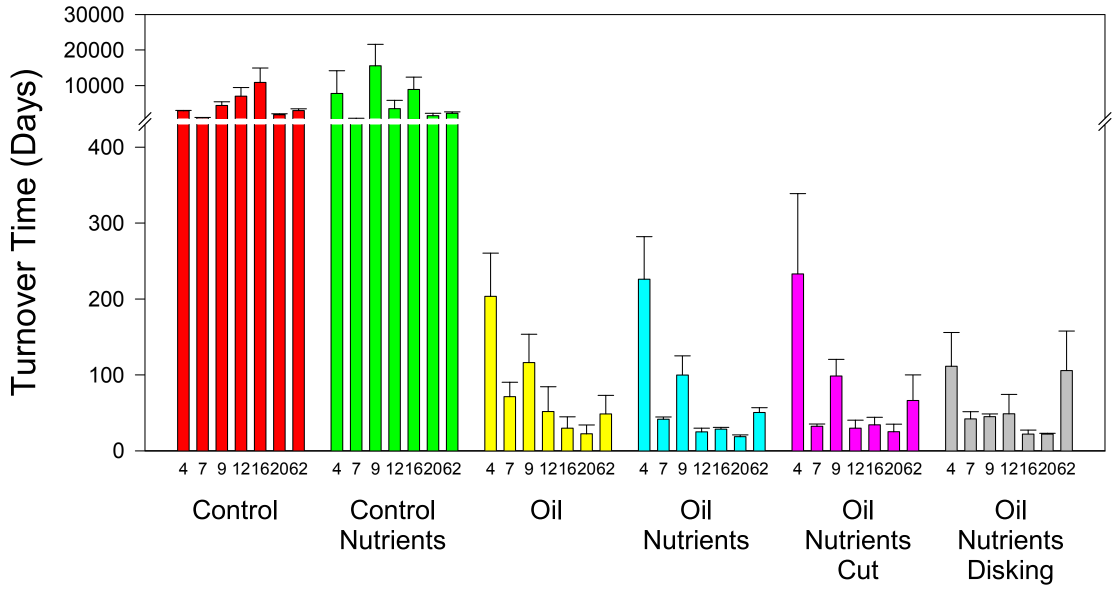

Figure 2.3. Average turnover time of phenanthrene. Error bars = 1 standard error.

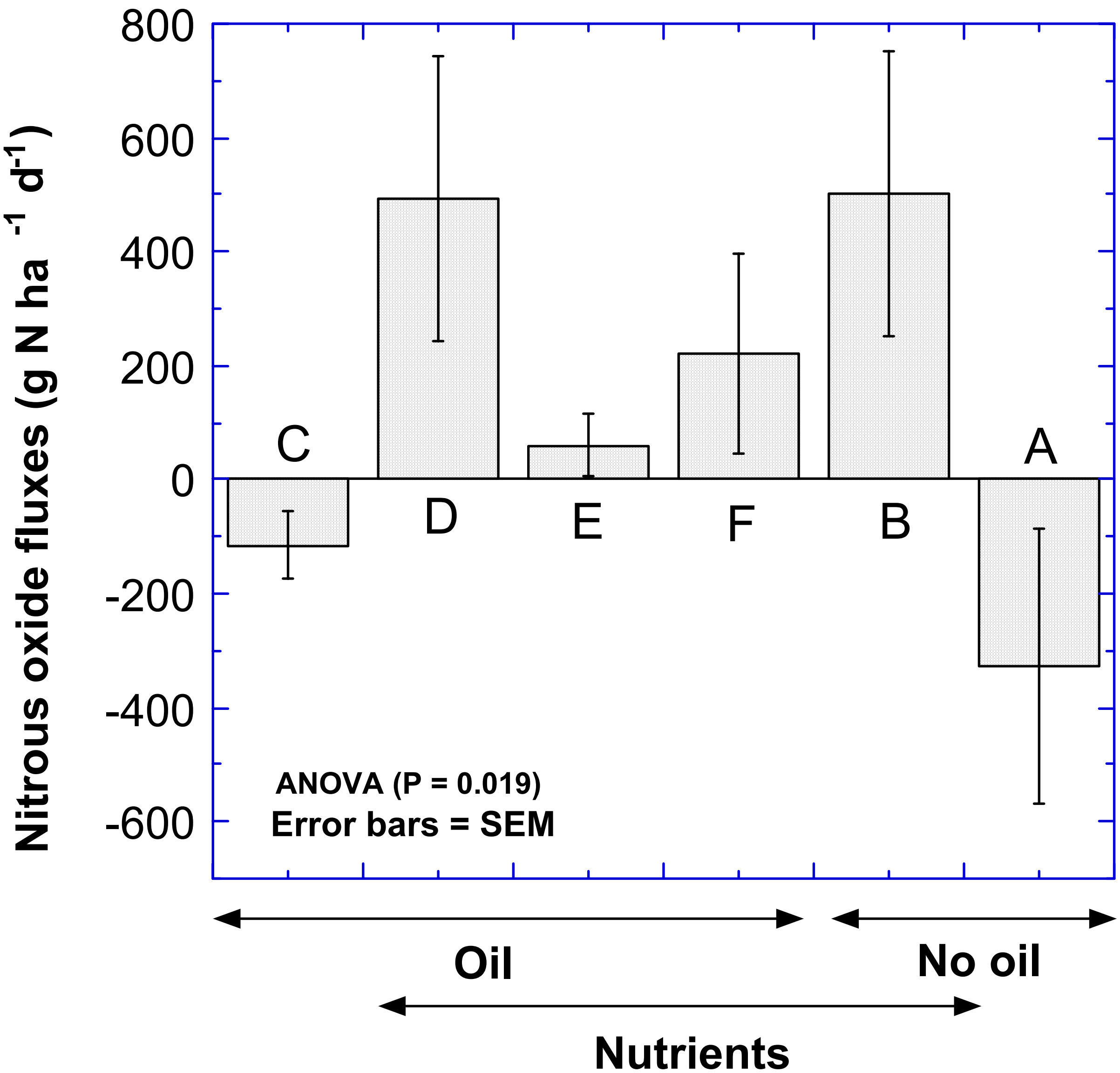

Figure 2.4. Seasonal average in denitrification activity with different treatments from Conrod's Beach sediments from June to November, 2000. Letter designations are the same as in Figure 2.2.

- *Structural deformity of Foraminifera*. For ecological relevance in the monitoring of potential effects in contaminated environments, it is preferred to use native (indigenous) species as indicators. In this study, Foraminifera (forams), single-celled microorganisms (protozoa) that construct a shell from available mineral particles or secrete one of calcium carbonate or of silica, were found to be a unique indicator species, due to their sensitivity to residual oil. This is attributed to the fact that the process of forming their shell has been reported to be highly susceptible to certain types of environmental pollution resulting in deformities. Foram skeletons are also resistant to decay, and many are found as fossils. Having these properties, foram tests and deformities can be used to monitor the ecological effects of oil spills and treatments.

The forams under observation at the study site were typically between 63-500 μm. They occurred in the sediment at an abundance of 400-4000 species per sample. Sediment samples (1 cm depth) were taken with a metal 10-cc core bi-weekly for the first two months and monthly for the last three months until the end of the study. The samples were sieved, processed, and analyzed under a stereomicroscope to determine the types of species, the number of living vs. dead, and normal vs. deformed populations. Preliminary results from the first study season suggest that the oil impacted at least one particular species of forams, *Miliamina fusca*, resulting in a high percentage of structural deformity in comparison to non-oiled specimens. Time-series studies can prove an estimate of the time required for natural attenuation or remedial treatments to reverse this biological effect.

2.2.5.1.3 Bioassays. Establishing the actual exposure level of biota to residual oil is difficult. While chemical measures of oil in sediments, water, and tissues are routine, there is no guarantee that all biological organisms accumulate oil or its components equally or in proportion to environmental concentrations. Further, many of the components of oil such as alkanes and PAHs are metabolized, so that chemical analyses of tissue may not represent the true dose or dose rate. The key to sediment assessment is bioavailability since elevated concentrations of toxic compounds may not necessarily result in adverse effects to the organisms living within the sediments. 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 to determine 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.

- *Microtox solid phase test*. In the Microtox® Solid Phase Test (AZUR Environmental, 1999; Lee *et al*., 1995b; Microbics Corporation, 1992), the bacterium, *Vibrio fisheri*, is exposed to test sediments. A significant decrease in bioluminescence relative to water-only controls is indicative of sediment toxicity. Toxicity levels are calculated as the concentration of sample that would result in a 50% reduction in luminescence (‘effective concentration,’ EC_{50}). To account for interference 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 2.5a). If one sets an arbitrary EC_{50} toxicity threshold at 1,000 mg/L [which Environment Canada uses in its regulations (Tay *et al*., 1997)], then even though there was a detrimental response observed in the control sediments treated with nutrients only, all unoiled sediment samples would be deemed non-toxic according to this guideline, while toxicity was identified following oil treatments (Fig. 2.5a). There is no implied suggestion that the 1,000 mg/L threshold is being or should be adopted by EPA. The threshold was reported as an example to demonstrate how one may utilize toxicity data in decision-making. On comparison of results, it appears that natural attenuation (the bars labeled Oil in the figure) could account for most recovery. By week 9, all treatments were non-toxic. The significance of natural attenuation was also illustrated by a comparison of the relative recovery of the plots using EC_{50}’s for each treatment and sampling time normalized to the unoiled control (Fig. 2.5b).

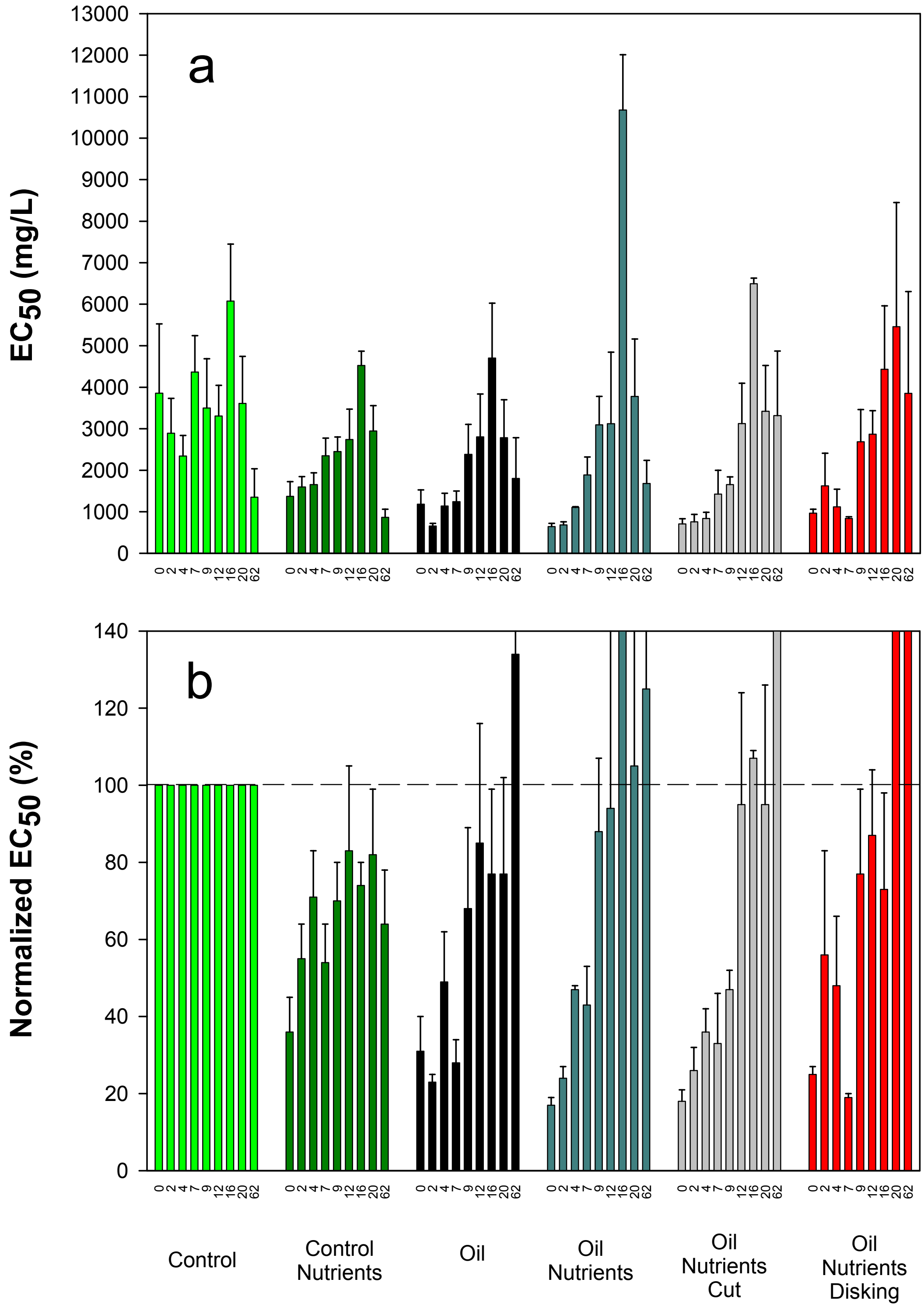

Figure 2.5. Sediment toxicity for sediment samples from Conrod's Beach, Nova Scotia, at weeks 0 to 62, as reflected by (a) EC_{50} for experimental treatments and (b) EC_{50} normalized to the mean control value at each sampling time. Error bars = 1 standard deviation.

- *Amphipod survival test*. The Amphipod Test measured the effects of sediment samples on survival of sediment-dwelling *Eohaustorius estuarius* (Environment Canada, 1992). 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. The results are reported as percent mortality (Figure 2.6). Mortality was high in all of the oiled treatments, but it began to decrease by Week 12 largely as the result of natural processes. Addition of nutrients accompanied by disking appeared to cause the most rapid rates of detoxification (recovery) within the oiled plots as measured by this test. However, the results of chemical analyses (GC/MS) indicated that this observation could also be attributed to the physical removal of oil (enhanced dispersion with tides) mediated by disking operations. By Week 62, the difference between the disked plots and the natural attenuation plots was highly significant (32% vs. 5% mortality).

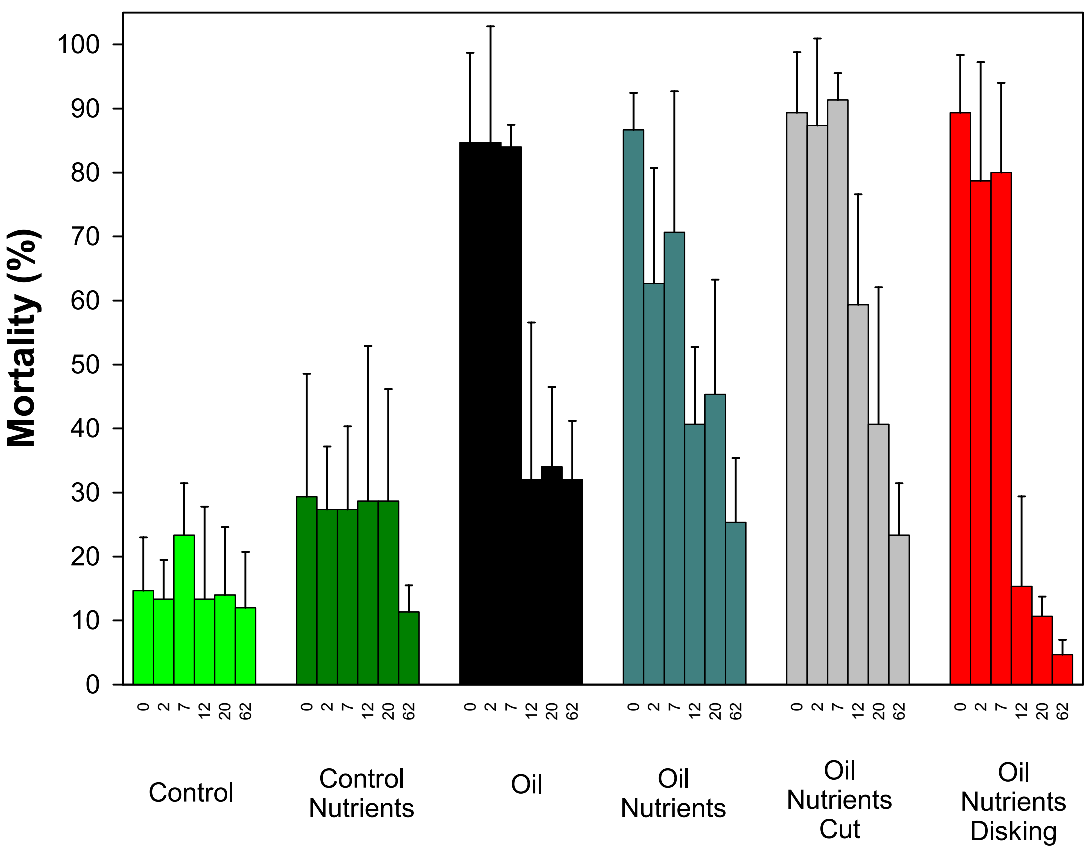

Figure 2.6. Changes in sediment toxicity from 0 to 62 weeks as quantified by the Amphipod Survival Test. Error bars = 1 standard deviation

- *Gastropod survival*. Although many organisms have been used as sentinels or bio-monitors 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. Mollusks are abundant and widely distributed, and their use as *in situ* bio-monitors has been on the rise (Lagadic and Caquet, 1998). Saltwater marshlands present unique restoration challenges following oil spills due to the sediment's high capacity for oil absorption, low oxygen content, fluctuating salinity, and tidal flow. The mud-snail, *Ilyanassa obsoleta*, an abundant detritovore inhabiting these marshlands, was evaluated for its suitability as a bio-monitor to assess the impacts or efficacy of the bioremediation treatments. It was selected for use as an *in situ* bio-monitor as it feeds on sediment detritus, algae and decaying organic matter within the wetland. Snails (n = 50/treatment/sampling time) were caged in 20 × 20 × 22 cm open mesh polypropylene baskets moored to the sediment surface of experimental plots. At the end of the second year, cages were recovered after being exposed to the experimental plots for 30, 60 and 90 days to evaluate effects on survival at the end of the second field season (Week 62). Healthy snails were also exposed for a 30-day period under laboratory conditions to test sediments recovered from the plots, and to determine survival rates. The mud snails did not survive long in captivity in the field, and laboratory exposures were erratic. Mortality in the field was likely due to environmental factors as mortality was high even in control cages. It is unlikely that anoxia due to crowding and/or eutrophic conditions was a factor since these snails are tolerant of anoxic conditions and can grow in dense aggregates. Mortality after 5 d exposure was generally higher for snails caged within the experimental plots amended with nutrients. This toxic response was attributed directly to the use of fertilizers.

- *Acute and chronic effects on fish*. Fish biotests were performed with salt marsh sediments recovered from the experimental site at Conrod's Beach, Dartmouth, Nova Scotia using euryhaline rainbow trout. Bioavailability was assessed by quantifying the extent of CYP1A (MFO enzyme, see Section 1.3.5.2) induction (Guiney *et al*., 1997) in fingerling trout following 96 h exposures to test sediments. S-9 fractions from liver homogenates were prepared for the measurement of ethoxyresorufin-o-deethylase (EROD, CYP1A enzyme) activity (Hodson *et al*.,1996). Each bioassay included negative controls (water only), un-oiled sediment controls, and positive water controls (fish exposed to the compound ß-naphthoflavone, which is a model inducer).

  All data were analyzed after log transformation and the extent of induction calculated by normalizing to control activity, i.e. induction equaled activity of treated fish divided by activity of control fish, and hence had no units. The Mesa crude oil contained sufficient PAH to cause high levels of EROD induction in trout, as shown by a preliminary bioassay of clean sediments spiked with oil in the lab (10 mL oil/L of sediment). By diluting the spiked sediment with clean sediment, a clear exposure-dependent gradient of induction was found (Figure 2.7). The plateau of activity at the highest oil concentrations suggests acute toxicity (Hodson *et al*. 1996), likely due to the combined narcotic effects of all the components of oil. A similar effect was observed when oil was simply added to water (data not shown). The threshold concentration causing induction was about 0.1 mL oil/L of reference sediment (Figure 2.7).

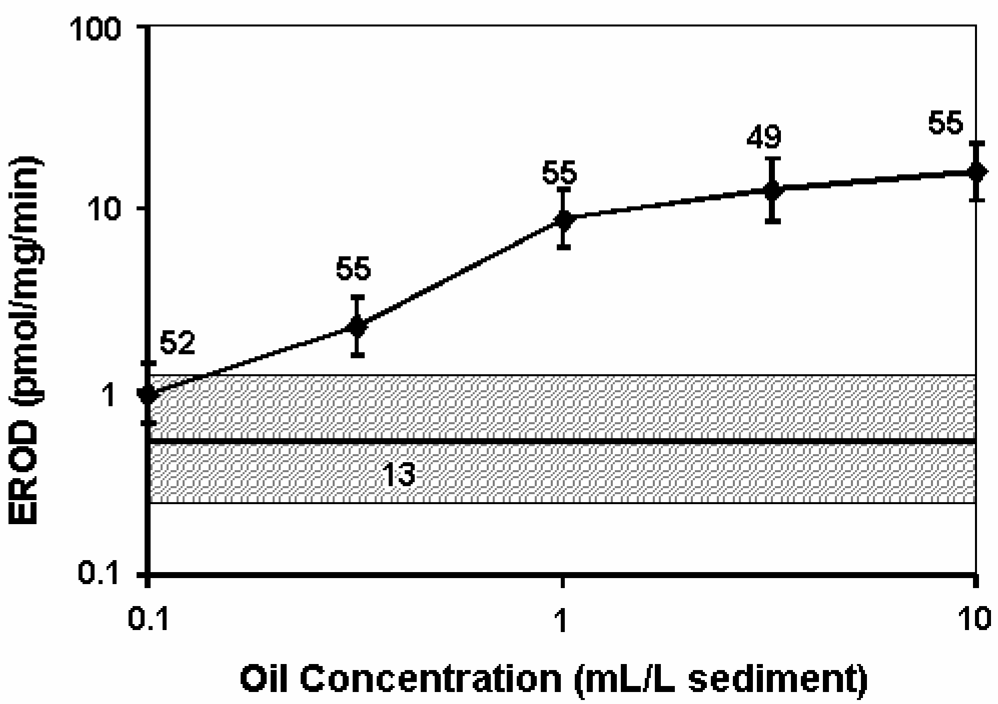

Figure 2.7. EROD activity of rainbow trout exposed to reference sediments spiked with oil. Error bars are 95% confidence limits while the shaded zone represents the 95% confidence limits of control activity. Numbers represent sample sizes.

Study results showed that PAHs were bioavailable from Conrod's Beach oiled sediments. While EROD induction was evident for fish exposed to sediments sampled 1 d after oiling, induction actually increased in July (50 d later) before decreasing somewhat in October (140 d; Figure 2.8). The initial low extent of induction may have been caused by oil toxicity. With higher oil concentrations, it is likely that EROD induction was inhibited, as was evident from the leveling-off of the exposure-response curve in the test of sediments spiked with oil in the lab (Figure 2.7). There did not appear to be a major effect of bioremediation treatments, with the possible exception of disking. Plots disked to aerate the sediment showed 45% lower EROD induction potency ($p<0.05$) than plots with or without nutrients (Figure 2.9). The plots with plants cut showed 44% lower induction potency, but the difference was just below the level of statistical significance. Disking may have reduced induction potency by facilitating the transport of oiled surface sediments into deeper underlying sediments, enhancing the dispersion of disturbed surface sediments by tides, and stimulating microbial activity by improving oxygen availability within the wetland sediments (Lee 2000b).

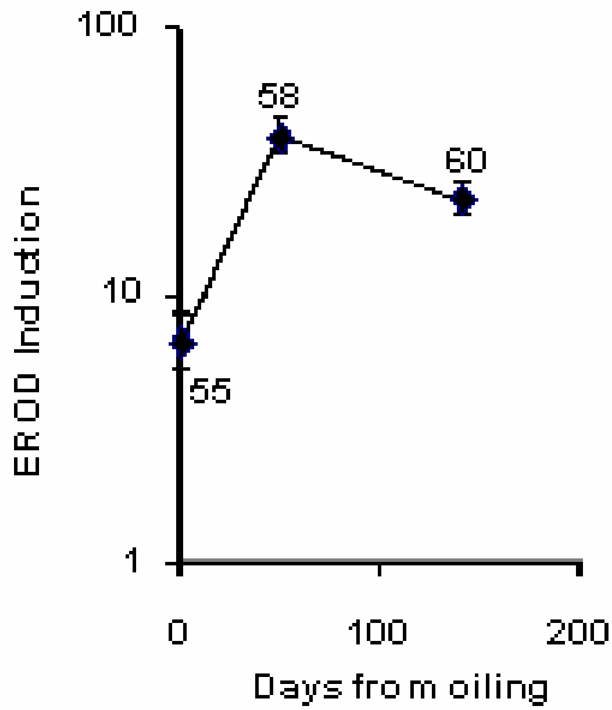

Figure 2.8. MFO induction in fish exposed to oiled sediments. Each point is the average of data pooled across all treatments. Error bars = 95% confidence limits. Numbers = sample size.

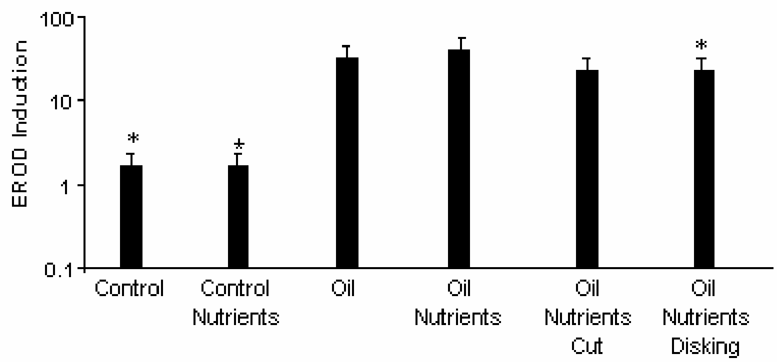

Figure 2.9. Effect of experimental treatments on EROD induction of trout exposed to oiled sediments. Asterisk indicates induction was significantly lower than the highest activity. Error bars = 95% confidence limits. N = 15/treatment.

In summary, because there was a strong link between concentrations of PAHs in beach sediments and the extent of induction in exposed trout, the induction bioassay successfully tracked the changes over time in the concentrations and bioavailability of PAH and of the crude oil itself. Over the long term, we would expect that the relationship between induction and uptake of non-PAH hydrocarbons would weaken due to the differential rates of degradation and weathering of different components of oil. However, within the time frame of this study, it appears that both the bioremediation and phytoremediation treatments did not markedly affect the rate of PAH degradation. While there appeared to be a significantly greater loss of PAH from aerated sediments, the overall enhancement was less than two-fold, which is at the limit of detection of the induction bioassay for the sample sizes tested.

Preliminary studies with eyed-eggs (about 15 d post fertilization) of trout also suggested that symptoms of Blue sac disease (BSD - characterized by yolk sac and pericardial edema, hemorrhaging, deformities, and induction of mixed function oxygenase enzymes) were more frequent in fish exposed to oiled than to un-oiled sediments (Zambon *et al*., 2000); indicating a risk to early life stages of species that spawn on tidal beaches.

These laboratory bioassays with fish represent a 'worst-case' scenario as the test organisms could not avoid exposure to sediments that have been mixed, thereby destroying surface layers that might be depleted of oil due to weathering. The ratio of water to sediment was also fixed, which is very different from the situation in well-flushed tidal beaches. Finally, the test species is a useful model, but is not a beach spawner. Nevertheless, the model fish do provide a useful surrogate for other species, such as smelt, capelin, and herring that spawn on both freshwater and marine beaches or in marine littoral zones. In many estuaries, contaminated water is often not well mixed, but moves back and forth with the tide, thereby causing prolonged exposure of fish entrained in the water mass (Elson et al. 1972). As illustrated by the *Exxon Valdez* spill, eggs deposited in beach sediments cannot move and are also subject to continuous exposure. The utility of freshwater species as a surrogate for marine species might also be questioned. However, it is worth noting that eggs of pink salmon, a species closely related to rainbow trout, were exposed to oil from the *Exxon Valdez* because they were spawned in river mouth shoals (Marty *et al*. 1997). The eggs were bathed alternately in fresh and salt water as the tide rose and fell, so that an exposure of trout to oiled sediments in saline water is not entirely unrealistic. To resolve the uncertainties associated with assessing exposure and effects, it is clear that the next step is to refine and adapt bioassays for application in situ, using species endemic to the test sites.

2.2.5.2 Risk assessment

In this case study, overall sediment quality was determined from the integration of results from analysis of sediment chemistry, community structure, alteration of primary metabolic processes, and sediment toxicity. The results of detailed chemical analysis, bioassessments, and bioassay tests suggested that in the Conrod Beach study, natural attenuation was the primary process that reduced residual oil concentrations and toxic effects. The biotest results showed that the remediation strategies under evaluation, stimulation of bioremediation and phytoremediation activity by nutrient amendments and physical mixing (disking), were not highly effective in regards to restoration. It was also evident in the results of some biotests (e.g. Amphipod Survival Test, Microtox Test) that possible detrimental effects may be linked to the addition of fertilizers.

It is imperative that one fully understands the various processes that may affect biotest endpoints. Failure of the Gastropod Survival Test to resolve differences in experimental treatments could be attributed to adverse environmental conditions that caused high levels of inherent variability within the test matrix. The Biotox Solid-Phase Flash Assay (Lappalainen et al, 1999) is currently being considered as a relevant adjunct (or alternative) test to the Microtox solid-phase assay, since it allows the evaluation of large numbers of environmental samples at a more reasonable cost using the same test organism. This assay was used with success to provide evidence of toxicity reduction by remedial activities in an oil-impacted freshwater environment (Blaise et al., 2002). However, in this case study, all sediments collected during the first 2 sampling events (Week 0, 2) show marked and similar levels of toxicity. It is hypothesized that the assay was unable to discriminate between toxicity of oiled and unoiled sediments, as it was responding to the presence of natural contaminants present in the anaerobic sediments (e.g., NH_3 and/or H_2S) and possibly also to their degree of oil contamination (in the case of the oiled sediments). Similarly, the Algal Toxicity Test with *Selenastrum capricornutum* (Blaise and Ménard, 1998) that readily identified the inhibitory effects of residual oil in sediments on esterase enzyme activity in the previous freshwater marsh case study was ineffective in this test case. This was attributed to interferences associated with benthic diatom growth. This has necessitated the development of a new algal toxicity test using the marine macro-alga *Champia parvula* to assess archived sediments from this study.

With further refinement, guidelines for selection of bioassessment and bioassay test suites will be provided to oil spill response managers that are tasked to implement and verify the success of countermeasure operations including the extent of habitat recovery. For this case study in a marine salt marsh environment, the results of the ecological risk assessment with all available quantitative chemical and biological data suggest that natural attenuation may be the most environmentally sound and cost-effective treatment option. Although there was some evidence of changes in microbial community structure and activity, no significant differences were observed among treatments in oil degradation rates or toxicity reduction. Active remedial treatment is not supported by cost-to-benefit analyses.

2.3 Summary and Recommendations

Most of the information presented in this guidance document was based on only a few field studies of oil bioremediation. Not many studies have been done in a definitive manner. The Conrod Beach experiment in Dartmouth, Nova Scotia, demonstrated that biodegradation of the alkane fraction and some of the PAH fraction was stimulated following the application of inorganic fertilizers directly to the plots. Disking (or tilling) caused substantial damage to the rhizosphere, and such drastic measures cannot be recommended as a means of increasing oxygen content in the root zone. Not much can be done in that regard. Thus, if significant penetration has taken place into the subsurface, then not much hope of acceleration in hydrocarbon disappearance is possible since anaerobic conditions rapidly set in at greater depths. If, however, penetration is limited to the top several mm, then sufficient oxygen might be available to permit biostimulation to accelerate greater hydrocarbon disappearance than via natural attenuation. So, of major importance in the event of an oil spill in a salt marsh (or any wetland oil spill) is to assess the degree to which penetration has taken place below the surface. If it is minor, then biostimulation could be considered as a viable strategy for cleanup. If it is more than a few mm penetration, then biostimulation will have diminished effectiveness due to the increased likelihood of limiting oxygen concentration in the oil impact zone.

Salt marshes are among the most sensitive ecosystems and, therefore, the most difficult to clean. Applications of some traditional oil spill cleanup techniques in wetland habitats have caused more damage than the oil itself. Several long-term field studies have been carried out in coastal wetlands to evaluate the potential of oil bioremediation, one of the least intrusive technologies. The studies have shown that oil biodegradation on coastal wetlands is often limited by oxygen, not nutrient availability. Natural attenuation is increasingly becoming the preferred strategy for the restoration of oil-contaminated wetlands. However, field studies also indicate that nutrient amendments may still be a viable option for removing hydrocarbons from an oil-contaminated wetland if the oil does not penetrate deeply into the anoxic zone of wetland sediments and when nutrients are limiting. When biostimulation is selected, it is recommended that nitrogen concentrations of at least 2 to as much as 10 mg N/L should be maintained in the pore water to achieved optimal oil biodegradation, with the decision on higher concentrations to be based on a broader analysis of cost, environmental impact, and practicality. The overall success of the remedial operations should be not only based on the efficiency of oil degradation but also the integration of results from a suite of assays, which are chosen on the basis of ecological relevance to the site of concern, cost considerations, and the availability of technical expertise.

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