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Biostimulation for the treatment of an oil-contaminated coastal salt marsh

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Abstract

A field study was conducted on a coastal salt marsh in Nova Scotia, Canada, during the summer of 2000. The objective of the study was to assess the effectiveness of biostimulation in restoring an oil-contaminated coastal marsh dominated by *Spartina alterniflora* under north-temperate conditions. Three remediation treatments were tested with two additional unoiled treatments, with and without added nutrients, serving as controls. This research determined the effectiveness of nitrogen and phosphorus addition for accelerating oil disappearance, the role of nutrients in enhancing restoration in the absence of wetland plants, and the rate at which the stressed salt marsh recovered. Petroleum hydrocarbons were analyzed by gas chromatography/mass spectrometry (GC/MS). Statistically significant treatment differences were observed for alkanes but not aromatics in sediment samples. No differences were evident in above-ground vegetation samples. GC/MS-resolved alkanes and aromatics degraded substantially ($>90\%$ and $>80\%$, respectively) after 20 weeks with no loss of TPH. Biodegradation was determined to be the main oil removal mechanism rather than physical washout.

Introduction

Biodegradation appears to be the major process through which petroleum hydrocarbons are removed from sediments (Bragg et al. 1993). Wetland sediments, especially in the subsurface, are usually deficient in oxygen and/or nutrients, at least in concentrations needed to support optimal biodegradation of the contaminating hydrocarbons (Lee & Levy 1991; Mendelssohn & Lin 1995; Mitsch & Gosselink 1993). Biostimulation, the addition of nutrients to stimulate microorganisms to degrade the hydrocarbons, has been shown to enhance hydrocarbon biodegradation, especially in beach environments that are highly aerobic (Bragg et al. 1994; Pritchard & Costa 1991; Venosa et al. 1996) but also in low-energy sandy beaches (Lee et al. 1993; Lee & Trembley 1993). The redox condition of the sediment plays an important role in microbial degradation of toxic organics. Hydrocarbons degrade much more rapidly under aerobic conditions (Atlas 1981; Cerniglia 1992; Hambrick et al. 1980). Due to slow degradation in anaerobic sediments commonly found in marsh environments (Venosa et al. 2002), petroleum hydrocarbons may persist in wetlands for many years (Burns & Teal 1979; Macko et al. 1981). Artificially increasing the oxygen tension in marsh subsurface is difficult due to the slow diffusion of oxygen and the high oxygen demand in

saturated marsh sediment. Phytoremediation, a process mediated by the growth of plants to enhance rates of contaminant degradation in sediments and soil, has been shown effective for the removal of organic pollutants, including aromatics (Banks et al. 2000; Kruger et al. 1997; Liste & Alexander 2000), by direct plant uptake and indirectly by the stimulation of microbial populations within the rhizosphere. Also, the capacity of many wetland plant species to aerate the sediment rhizosphere has been hypothesized. However, a recent study involving *Scirpus pungens* concluded that oxygen introduced into the rhizosphere was insufficient to support biodegradation of the stressor (Venosa et al. 2002).

Treatments applied in this study included the presence of *Spartina alterniflora* with and without addition of fertilizer and addition of fertilizer with above ground vegetation cropped. The purpose of cropping was to determine to what extent remediation occurred through direct plant uptake vs. that caused solely by the microbial communities within the rhizosphere. The overall objective was to determine the effectiveness of fertilizer addition in accelerating the restoration of a highly stressed coastal salt marsh after an oil spill.

In a similar study conducted on the St. Lawrence River a year prior to this study (Venosa et al. 2002), we forced penetration of oil below the surface by raking the oil into the top few cm. This, indeed, resulted in anaerobic conditions, and biodegradation of oil was very slow. Despite this inhibition of oil disappearance in all treated plots, rapid and abundant vegetative recovery took place in all plots that received fertilizer compared to unfertilized plots. In the present salt marsh study, we did not force the oil into the subsurface. Rather, we allowed the oil to penetrate in whatever manner it would naturally occur, if at all, in order to see if the plants could induce aerobic conditions in the underlying rhizosphere through their own oxygen-transport mechanisms.

Materials and methods

Site description

The field study was conducted at a coastal salt marsh located at Conrod's Beach, near the mouth of Petpeswick Inlet (44°42′ N; 63°11′ W) on the Eastern Shore of Nova Scotia. Tides were semi-diurnal with a tidal range of about 2 m. The predominant wetland plant in the area was *Spartina alterniflora*.

Experimental Approach

Five different treatments were tested: (1) unoiled control, no nutrients added; (2) unoiled control, nutrients added; (3) oiled, vegetation intact, no nutrients added (natural attenuation); (4) oiled, vegetation intact, nutrients added; and (5) oiled, vegetation cropped to ground surface and removed from the plots to avoid detrital accumulation and artificially imposed surficial anaerobic conditions, nutrients added. The null hypothesis was that the amendments would not increase the rate or the extent of removal of oil components over that due to natural attenuation. A randomized complete block design was used to test this hypothesis. Each of the five treatments was replicated three times in separate blocks. The layout of the experiment consisted of 15 plots (5 treatments $\times$ 3 replicates) each measuring 3 m $\times$ 3 m in area. Treatments were distributed among the plots using a random number generation scheme. Pre-existing wetland plants were left intact in all test plots except the one in which the vegetation was cropped to ground level and maintained that way for the study duration.

The effectiveness of the tested amendments was determined by gas chromatography/mass spectrometry (GC/MS) analysis for the reduction of specific oil hydrocarbons (linear alkanes, C_{10} to C_{35} plus the isoprenoids pristane and phytane, and 2-, 3- and 4-ring aromatics and their alkylated homologs) and gravimetric measurements of Total Petroleum Hydrocarbons (TPH) for the gross assessment of crude oil removal. Analytes measured by GC/MS were normalized to the conservative biomarker C_{30}-17α-(H),21β(H)-hopane, naturally present in the test oil (Prince et al. 1994) to asses oil losses due to biodegradation. The degradation rates were derived from the hopane normalized oil degradation data according to the procedure outlined in the statistical analysis section.

Detailed description of the plots

The study was begun on June 5, 2000. Plots were laid out during the week prior to the beginning of

the field study. Each plot was surrounded by a skirt of sorbent pads hung vertically from a perimeter wire and anchored to the ground with rocks to safeguard the area from an accidental release of crude oil from the experimental plots into the surrounding environment.

Test plots were set up as follows (Figure 1): a buffer zone around most of the perimeter of each plot was reserved as a no-sample zone to minimize edge effects. Each plot had a half-meter wide walking path in the center; four benchmarks, one in each of the four corners; and a wooden signboard with the respective plot ID. Each plot was divided into four equal sectors, each measuring 1.0 m × 1.0 m. Each sector was subdivided into 9 sub-sampling zones, corresponding to the 8 sampling events. The ninth was used for ecological monitoring assays. Each sub-sampling zone measured 33 cm × 33 cm. They were identified as row (A to F) and column (1 to 6) coordinates, as shown in Figure 1.

Test oil application

The selected oil was Mesa, a Canadian light crude oil from Petro Canada. It had an API gravity of 29.7° and a flash point of 4 °C. About 200 l of this oil was artificially weathered by forcing air through it from an air compressor to evaporate the light fraction. The weathered oil was uniformly applied in two equal increments at a total load of 8 l/plot. The first oil application was carried out on June 6 at low tide. The rest of the oil (4 l/plot) was added on June 8. The oil was applied manually by two four-membered teams via a pump and spray apparatus similar to that used in the St. Lawrence River study (Venosa et al. 2002) and the Delaware experiment (Venosa et al. 1996). This application was conducted to simulate an oil slick that enters a salt marsh at high tide and contaminates the vegetation and underlying sediment as the tide recedes. A total of 72 l of oil was released onto the 9 oiled plots at an oil loading of 0.89 l/m^2 (equivalent to 0.043 l oil/kg dry sediment, assuming a dry sediment density of 1.03 kg/l and an oil penetration depth of 2 cm, and at 35 g oil/kg dry sediment for an oil specific gravity of 0.82).

Nutrient and oil application

A prilled granular form of NH_4NO_3 (ammonium nitrate) was used in specific treatments as a source of nitrogen. $Ca(H_2PO_4)_2{\cdot}H_2O$ was the selected phosphorus source. On June 9, after the first sampling event, nutrients were applied at low tide onto the appropriate plots. Granular nitrogen and phosphorus were initially added to each of the nutrient-amended plots (one control and two of

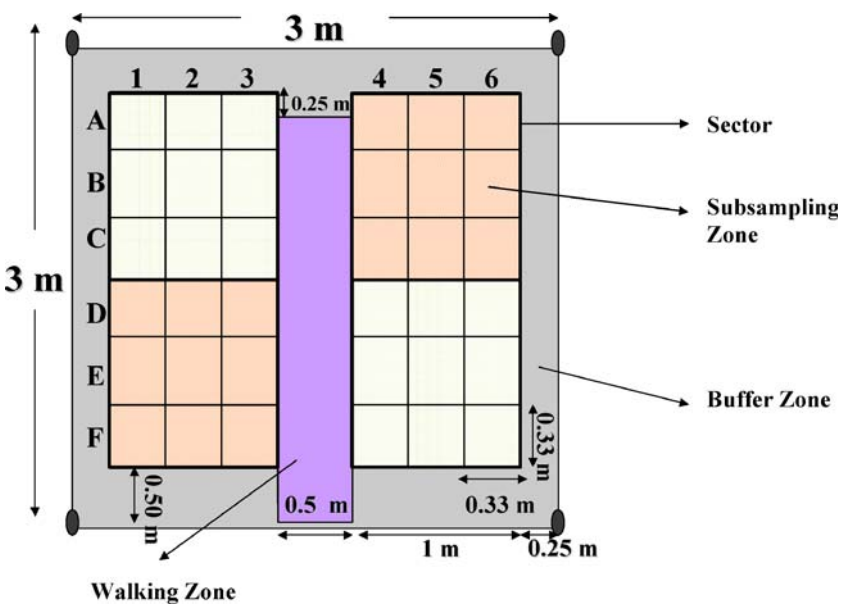

Figure 1. Schematic diagram of a test plot showing its divisions into sectors and sub-sampling zones.

the treatments) at a dosage of 450 g-N and 135 g-P per plot. This dosage represented a mass of 1284 g of NH_4NO_3, and 549 g of $Ca(H_2PO_4)_2{\cdot}H_2O$. Although background ammonium-N concentrations in the pore water were significantly higher than the minimum concentrations of 1.0–2.5 mg/l as N needed to support maximum growth rate of alkane degraders (Venosa et al. 1996), we decided to test the effect of supplemental addition of nitrogen and phosphorus for the following reasons. First, we wanted to ensure that sufficient nutrients would still be available in the pore water after commencement of bioremediation. Second, background phosphorus levels were sufficiently low to warrant its supplementation. Third, since the water temperature in the experimental area was low, we were not sure that the rate of formation (i.e., recycling) of nutrients by vegetative decay could keep up with the nutrient demand during the biodegradation of hydrocarbons. Finally, analysis of nutrients in tidal water indicated sub-0.1 mg/l levels, raising the question of nutrient persistence in the pore water as a result of tidal action. Nutrients were added at time 0 at N and P doses of 2 and 0.6% of the chemical oxygen demand of the oil, respectively. Subsequently, intermittent additions of N and P were added at the same dose on days 50 and 82 of the study. Nutrients were broadcast onto the plots as evenly as possible using a whirlybird sprayer. Vegetation was cropped from the cropped plots and removed to avoid the formation of a layer of detritus on the sediment surface, which could exacerbate anoxic conditions in the subsurface.

Sampling design, schedule, and handling

The first sampling time (week 0) was conducted at low tide on June 9, the day after the second incremental oiling. Subsequent sampling intervals took place on weeks 2, 4, 7, 9, 12, 16, and 20.

Samples were collected from the randomly assigned sampling locations in the sampling plan. Visual observation after oil application indicated that a considerable amount of oil was covering the vegetation within the oiled plots. Samples of the vegetation were collected from the sub-sampling areas by cutting the plants about 2-3 cm above the surface to quantify the amount of oil covering the plant surfaces. These samples were weighed to monitor plant growth and extracted and analyzed for petroleum hydrocarbons following the same procedure as the one used for the sediment samples. For a given sampling event, one sediment sample and one vegetative sample were taken from each of the sectors within the plot at the designated sub-sampling locations. This resulted in a total of 4 samples per plot per sampling event. These four samples were thoroughly mixed and composited into two composite samples. Each composite was composed of two samples. Samples were composited in diagonal directions on the plots (Figure 1). The sample from the sector on the top left hand side was mixed with the sample from the sector on the bottom right hand side to make Composite 1. The sample from the sector on the top right hand side was mixed with the sample from the sector on the bottom left hand side to make Composite 2, which was archived. Composite 1 was split into six sub-samples, which were distributed to different labs to be analyzed for oil chemistry. Each of the 4 sectors was subdivided into 9 equal subsampling zones, as shown in Figure 1. At each sampling event, a subsampling zone (example, A3) was sampled and composited with a sample taken from a subsampling zone of the diagonally opposite sector (example, D6). This procedure was adopted to randomize sampling and avoid resampling the same subsampling zone. The sequence that the subsections were sampled was determined by consulting a random number table.

A quadrat made of tubular PVC with dimensions 25 cm by 25 cm was used to delineate the sampling area. Vegetation from the sub-sampling zone was cut using gardening shears and collected into a pre-labeled half-gallon aluminum can. Most of the sediment surface inside the quadrat was sampled by cutting sods (slabs of sediment about 2-cm in depth) with a serrated knife and placing the sample into a pre-labeled can. Care was taken to avoid cross-contamination between sub-sampling zones and plots.

For nutrient analyses, core sediment samples about 2 cm deep were collected every other day from the buffer zone within the plots of one block, to monitor nitrate + nitrite-N, ammonium-N, and orthophosphate concentrations. A different block of plots was sampled each time. Six cores were collected per plot using PVC cores about 5 cm in diameter, to give a mass of sediment of approximately 300 g. Samples were frozen and sent to Fisheries and Oceans Canada's

Maurice-Lamontagne Institute at Mont-Joli (Quebec) for analysis of the pore water.

Nutrient analysis

The pore water from the sediment was separated by centrifugation of the composited samples and directly analyzed for nutrients. Ammonium-N was measured colorimetrically by the Berthelot reaction (Fiore & O'Brien 1962), nitrite + nitrate-N by the cadmium reduction method (Armstrong et al. 1967), and ortho-phosphate colorimetrically after formation of phosphomolybdenum blue complex (Murphy & Riley 1962).

Oil chemistry

Marsh sediment samples were extracted in dichloromethane (DCM) using a Soxhlet apparatus. The detailed procedure has been described elsewhere (Venosa et al. 1996). Changes in the concentration and composition of residual crude oil in the sediments were determined by GC/MS with a Hewlett-Packard 5890 series II gas chromatograph coupled with a Hewlett-Packard 5971A mass selective detector (MSD) and a Hewlett-Packard 7673 autosampler. The MSD was operating in the selected ion monitoring (SIM) mode for quantitative analysis of resolved hydrocarbons: the targeted linear alkanes, C_{10} to C_{35}, plus the branched pristane and phytane and the predominant 2-, 3- and 4-ring aromatics and their alkylated homologues. The GC capillary column was MDN-5S by Supelco (30 m long, 0.25 mm i.d., and 0.25 μm film thickness). All analytes were normalized to the conservative biomarker C_{30}-17α-(H),21β(H)-hopane, naturally contained in the test oil. The raw weathered Mesa oil was analyzed with every set of field samples to ensure consistency in instrument performance.

Moisture was determined by gravimetric analysis of 10 g of sediment sample (or 5 g of vegetation sample) by drying for 24 h at 105 °C. TPHs were defined as Extractable Organic Material (EOM). They were calculated gravimetrically by evaporating a 1-ml aliquot of the concentrated solvent extract. Total hydrocarbon loss was determined by the difference in TPH concentrations normalized to dry sediment or vegetation weight at different times.

Statistical Analysis. Repeated measures analysis of variance (RMANOVA) (Freud et al. 1986) was used to analyze the response variables (oil analyte concentrations) to test the null hypothesis. When significant differences ($p < 0.05$) were revealed by RMANOVA, univariate ANOVAs were run at each time point. If significant differences were indicated at a specific time point ($p < 0.05$), treatment differences were evaluated by using the protected least significant difference (LSD) mean separations test. First order rate constants of oil biodegradation for all treatments were estimated by non-linear regression analysis. An *F*-test was performed to compare the slope coefficients statistically among the various treatments. For these calculations, the variance was estimated from the sum-of-squared residuals from the fit of the first-order model to the average concentrations (over all replicates) for each treatment at each time point.

Results

Visual observations

Walking around the plot area by the field crew had a negative effect on the condition of the marsh sediment and vegetation. Even one year after the end of the study, the marsh at Conrod's beach had not recovered its original appearance in areas where trampling and sampling had occurred. Very little *Spartina* vegetation was observed growing in the sampled areas of the oiled plots. However, this failure of plants to recover was attributed to the way the plots were sampled, not to the lack of stimulation by fertilizer addition. Whole pieces of sod were cut out of the sampling zones when the plots were sampled. If no plant material remained after sampling, it stands to reason that new plant growth would not occur. In fact, in areas of the plots where sod was not removed, evidence existed that the *Spartina* vegetation did indeed recover well. So, the observations above were a result of the sampling procedures. Most of the new growth in paths worn by personnel surrounding the experimental plots belonged to pioneer species other than *Spartina*. The growth of *Spartina* in the unsampled areas of the control plots was similar to that in the surrounding marsh, while the unsampled areas of the nutrient control plots had greener

and more abundant growth of *Spartina* than that surrounding the marsh.

Nutrient Concentrations and Persistence. Concentrations of ammonium-N, nitrite + nitrate-N, and orthophosphate were measured in the interstitial pore water over the 20 weeks of the study. Detailed data for the nitrogen forms are plotted in Figure 2 and phosphorus in Figure 3. The spikes in the figures correspond to the times when nutrients were re-applied to the test plots.

Average background nutrient concentrations in the pore water at Conrod's beach were 9.4 mg/l of NH_4^+–N, 0.1 mg/l of NO_3–N and, 1.2 mg/l of PO_4^{-3}–P. The addition of oil onto the natural attenuation plots did not deplete the existing nitrogen in the marsh (compare natural attenuation with the first control in Figure 2). Although nutrients were supplemented for the reasons stated earlier, *a posteriori* evidence indicates that the justification for nutrient addition was not valid, especially in regards to nitrogen, which persisted at levels exceeding the 0.5–2.5 mg/l needed for maximal growth rates (Venosa et al. 1996). Addition of phosphorus, however, led to increased levels of this nutrient that appear to have a significant impact on alkane biodegradation, as will be shown later. None of the plots appeared to be nutrient limited at any time during the 20 weeks of the study, as will be shown in later sections. The persistence of nutrients was similar in all the amended plots, although some differences did exist between oiled and unoiled treatments. The concentration of ammonium-N in the pore water was consistently higher than that of nitrite + nitrate-N. Since the nitrogen source in the fertilizer was ammonium nitrate, the mass of ammonium-N and nitrite + nitrate-N due to nutrient addition should be approximately equal. A possible explanation for this may be the fact that wetland soils may better retain ammonium than nitrate through ion exchange mechanisms (Mitsch & Gosselink 1993). Regarding phosphorus concentrations,

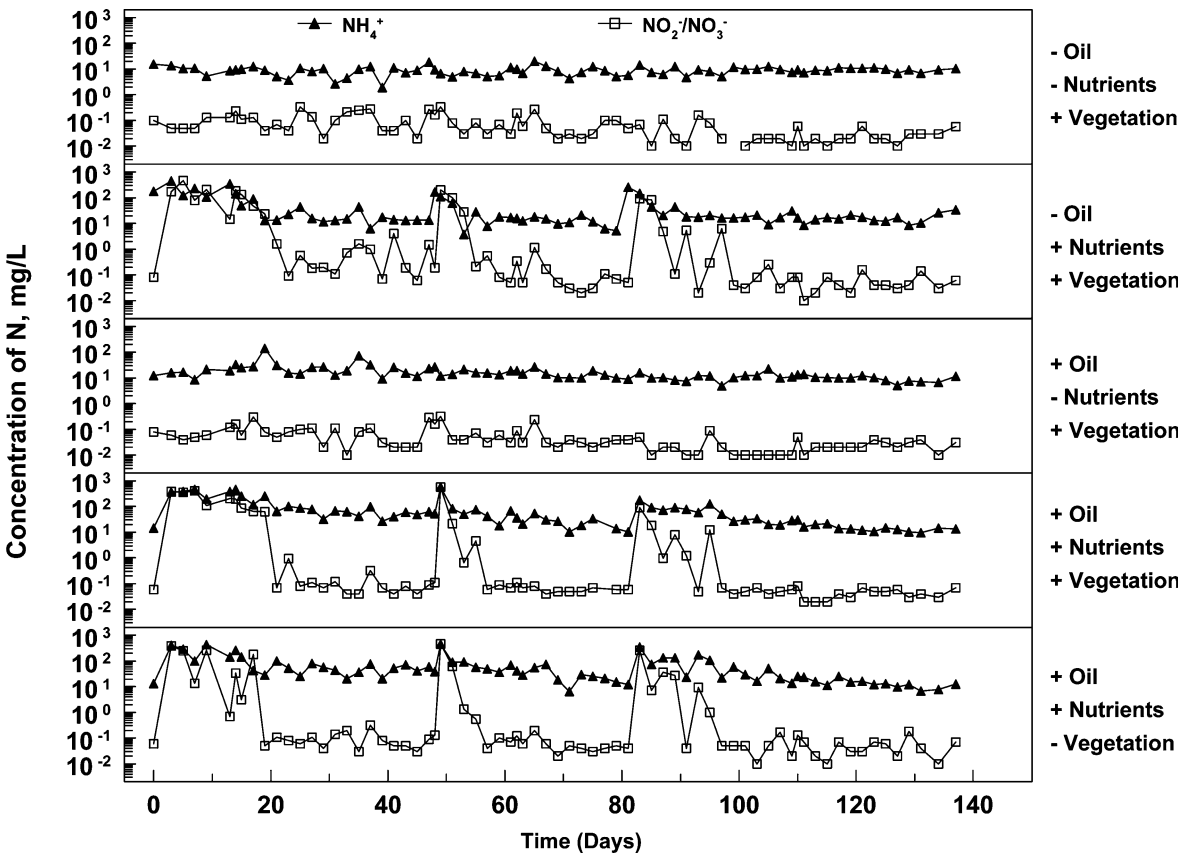

Figure 2. Nitrogen concentrations in the pore water for each of the different treatments.

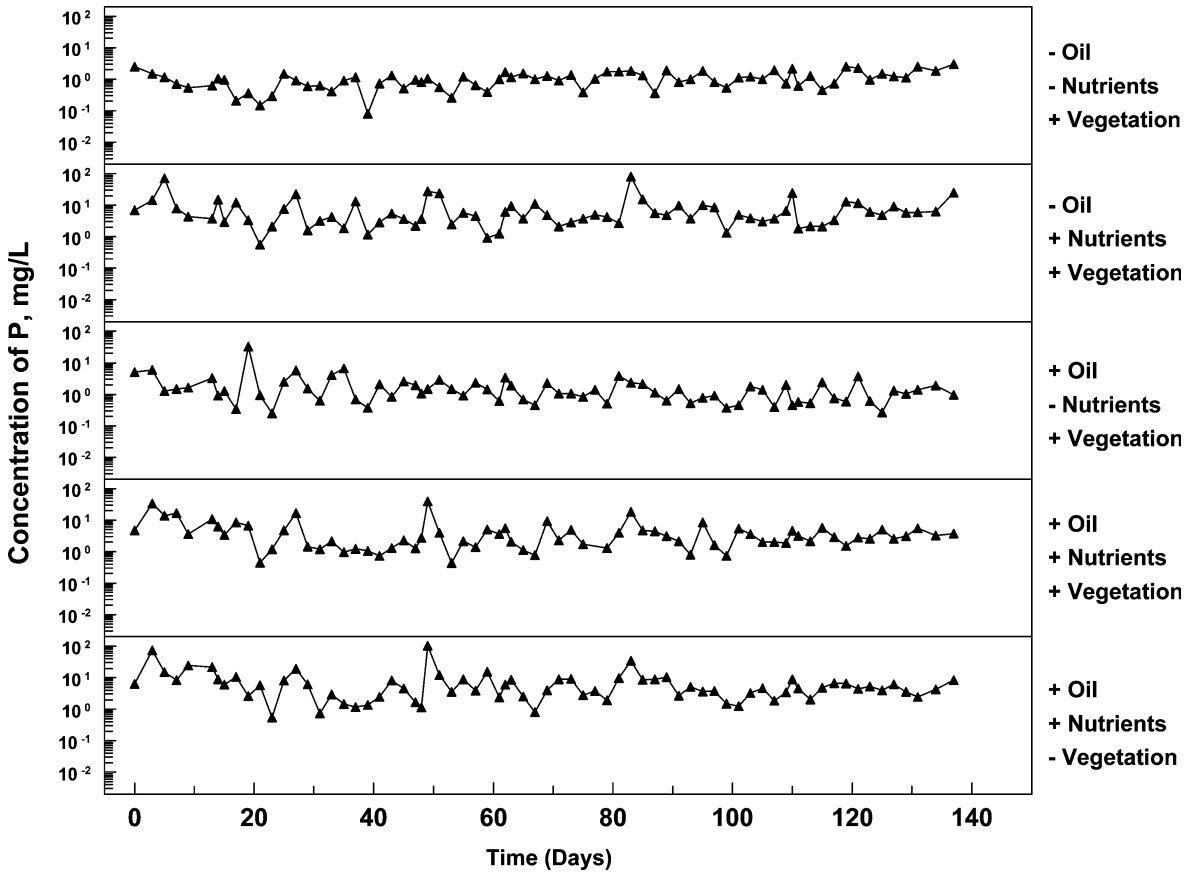

Figure 3. Phosphorus concentrations in the pore water for each of the different treatments.

nutrient amended plots exhibited average concentrations that were about one order of magnitude higher than the plots with no nutrients added.

Vegetation samples vs. sediment samples

Oil concentrations in the removed vegetation samples were about 10-fold higher than in the sediment samples when expressed on a dry weight basis. In vegetation samples, TPH values were in the range of 200–600 mg TPH/g dry vegetation, while in the sediment samples these values were between 20 and 60 mg TPH/g dry sediment. The data from the last sampling event suggest that most of the applied oil did not reach the sediment and remained on the *Spartina*. Since vegetation samples were less homogeneous than sediment samples, most of the data shown in the data figures are from sediment samples. No statistical analysis of data was conducted for the vegetative samples.

Biodegradation of GC/MS-resolved alkanes and aromatics

Analysis of the unoiled control plots showed that aliphatic hydrocarbons but not hopane were present in the area that did not appear to have come from the test oil (chromatographic fingerprints were quite different). Therefore, hopane-normalized total alkanes and aromatics were corrected for values of alkanes and aromatics in the background unoiled control plots of the same block at every sampling event. The assumption behind this correction was that these background hydrocarbons were generated as a result of previous and/or ongoing exposure to a different oil source. Figures 4a (alkanes) and 4b (aromatics) show changes in the normalized concentrations of resolved hydrocarbons versus time. The degradation rates were derived from the hopane-normalized oil concentration data by non-linear regression analysis

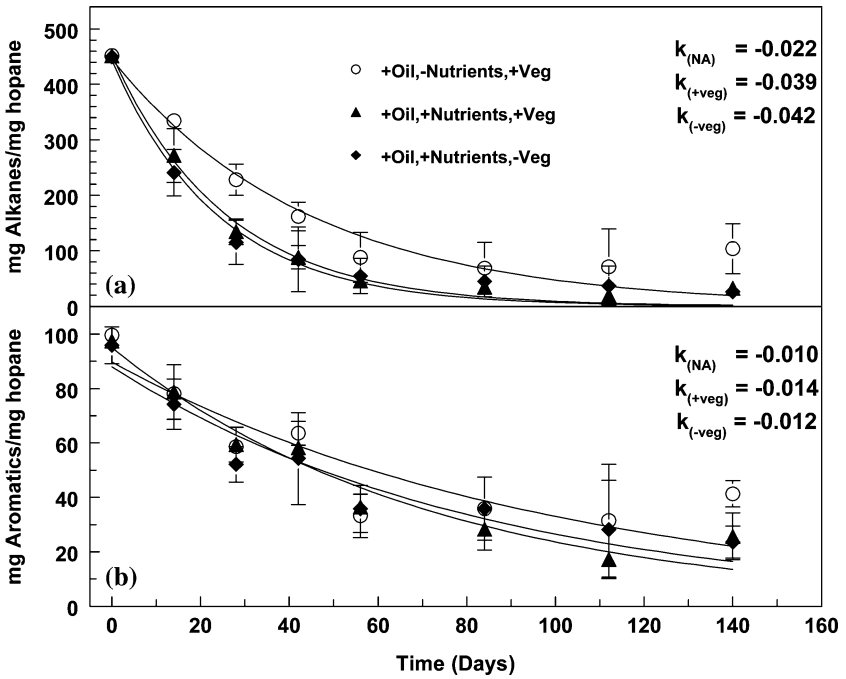

Figure 4. Temporal decrease in the (a) hopane-normalized total alkanes and (b) hopane-normalized total aromatics for each treatment after correcting for background hydrocarbons in the unoiled plots. For all plots, points represent average values of three replicates. Bars represent one standard deviation unit for the 3 replicates. Non-linear regression lines represent first-order fits of the data for each treatment, and the rate coefficients are shown.

using a first-order model to fit the data. The rate coefficients are shown on the figure. For all treatments, biodegradation rate constants were greater for total alkanes than for total aromatics.

Significant differences ($p < 0.05$) were detected in the total resolved alkane rate coefficients for the amended treatments compared to natural attenuation, using the LSD Mean Separation test. The extent of degradation 20 weeks after oil application (Table 1) was also statistically significant ($p < 0.05$), although differences were slight and standard deviations nearly overlapped. At that time, 76.0% of the resolved alkanes in the marsh sediment had been degraded in the natural attenuation plots vs. an average of 93.2% for the plots to which nutrients had been added. For the vegetation samples, the average extent of degradation in all the oiled treatments was higher (about 97%) than the sediment samples. This was expected since the surfaces of the *Spartina* plants were exposed to aerobic conditions for longer periods of time than the wetland sediment. Other factors may have also contributed to the observed rate differences. On the positive side, the increased surface area of the vegetative growth and exposure to UV light may have led to greater disappearance of the hydrocarbons, whereas on the negative side nutrient limitations on the vegetative growth may have contributed to decreased rates. The observations are the sum total of all these factors.

Table 2 presents the first-order biodegradation rate constants calculated for the individual resolved alkanes in the sediment. The pattern of disappearance of saturated hydrocarbons was characteristic of biodegradation: the lower molecular weight alkanes (C_{10} through C_{19}) declined at a greater rate than the higher carbon-number alkanes (C_{20} through C_{35}). Moreover, rate coefficients were lower for the branched alkanes pristane and phytane than the normal alkanes. Except for the alkanes with 10, 11, and 12 carbon atoms, differences in the resolved alkane rate coefficients between the nutrient-amended treatments and natural attenuation were statistically significant ($p < 0.0001$). The presence of above ground vegetation did not have any effect on the rate or extent of degradation of total or individual resolved alkanes.

Table 1. Extent of degradation (%) for total alkanes and total aromatics in sediment samples 20 weeks after oil application*

Analyte	Substrate	Column c: + Oil − Nutrients + Vegetation	Column d: + Oil + Nutrients + Vegetation	Column e: + Oil + Nutrients − Vegetation
Total alkanes	Sediment	76.0 (10.0) de	92.4 (3.0) c	93.9 (1.4) c
	Vegetation	94.7 (3.4)	98.2 (0.7)	99.1 (0.4)
Total aromatics	Sediment	58.4 (4.8) de	73.3 (9.0) c	75.4 (6.4) c
	Vegetation	85.7 (5.2)	90.2 (1.4)	88.5 (1.1)

Values in parentheses represent one standard deviation.
*Letters of treatments means that are significantly different from the current column are displayed.

Table 2. First-order biodegradation rate coefficients (day^{-1}) for individual alkanes in sediment samples and significant differences among treatments as determined by the Protected LSD Mean Separation Test*

Analyte	Column b: + Oil− Nutrients + Vegetation		Column c: + Oil + Nutrients + Vegetation		Column d: + Oil + Nutrients − Vegetation	
nC−10	−13.1		−42.2		−42.3	
nC−11	−6.4		−6.9		−5.1	
nC-12	−3.5		−5.1		−4.0	
nC−13	−2.7	cd	−4.5	b	−3.7	b
nC-14	−2.1	cd	−3.8	b	−3.6	b
nC-15	−1.9	cd	−3.5	b	−3.7	b
nC−16	−1.7	cd	−2.8	b	−3.4	c
nC-17	−1.4	cd	−2.3	b	−2.4	b
Pristane	−0.4	c	−0.6	b	−0.6	c
nC-18	−1.4	cd	−2.3	c	−2.6	c
phytane	−0.4	cd	−0.6	c	−0.6	c
nC-19	−1.3	cd	−2.30	c	−2.5	c
nC−20	−1.2	cd	−2.1	c	−2.3	c
nC−21	−1.2	cd	−2.0	c	−2.4	c
nC-22	−1.2	cd	−2.0	c	−2.3	c
nC-23	−1.2	cd	−1.9	c	−2.3	c
nC-24	−1.1	cd	−1.9	c	−2.1	c
nC-25	−1.1	cd	−1.8	c	−2.2	c
nC-26	−0.9	cd	−1.6	c	−1.8	c
nC-27	−0.8	cd	−1.5	b	−1.9	c
nC-28	−0.9	cd	−1.9	c	−1.8	c
nC-29	−0.5	d	−1.0		−1.5	b
nC-30	−0.6	d	−2.6		−1.3	c
nC-31	−0.6	cd	−1.2	c	−1.4	c
nC-32	−0.6	cd	−1.6	c	−1.3	c
nC-33	−0.5	cd	−1.1	c	−1.1	c
nC-34	−0.4	cd	−1.2	b	−1.1	b
nC-35	−0.4	cd	−1.0	c	−0.9	c

*The letters of the treatment means that are significantly different from the current column mean, as indicated by the Protected *T*-tests, are displayed.

In contrast to the total resolved alkanes, rate differences were not statistically significant ($p = 0.104$) among treatments for the total resolved aromatics. However, the extent of degradation 20 weeks after oil application (Table 1) was significantly different ($p < 0.05$) between the natural attenuation plots and the other treatments. Degradation of resolved aromatics

averaged 74.4% in the nutrient amended plots versus 58.4 % in the natural attenuation plots. No such differences were observed in the vegetation samples, which yielded an average PAH degradation of 88.1% regardless of treatment.

Rate coefficients for the individual aromatics are summarized in Table 3. Similar to the alkanes, the pattern of disappearance of aromatics was characteristic of biodegradation in that rate coefficients for the lower molecular weight aromatics (2-ring species) were higher compared to the higher molecular weight aromatics (3- and 4-ring species). This was also true when comparing the rate coefficients for the higher alkyl-substituted aromatics to the lower alkyl-substituted homologs (Table 3). In regards to statistical treatment differences among individual aromatics, the most consistent differences appeared to be between natural attenuation and the treatment with intact vegetation, mostly for the 3-ring aromatics and their alkylated homologs.

Physical oil loss. To assess cleanup and recovery of an oil-contaminated coastal marsh ecosystem, it is desirable to quantify the fate and effect of the whole oil over time. Loss of hopane is the best indicator of physical washout, since this compound has been found to be relatively non-biodegradable (Prince 1993; Venosa et al. 1996). Figure 5 summarizes the hopane concentrations in the sediment normalized to the dry weight of

Table 3. First-order degradation rate constants (day^{-1}) for individual aromatics in sediment samples and significant differences among treatments as determined by the Protected LSD Mean Separation*

Analyte	Column b: + Oil – Nutrients + Vegetation		Column c: + Oil + Nutrients – Vegetation		Column d: + Oil + Nutrients – Vegetation	
Nap	−4.3		−4.1		−3.9	
C_1-Nap	−2.1		−2.2		−2.4	
C_2-Nap	−1.2		−1.4		−1.5	
C_3-Nap	−0.8		−1.0		−1.0	
C_4-Nap	−0.6		−0.8		−0.7	
Flu	−0.8		−1.0		−1.0	
C_1-Flu	−0.6	c	−0.8	b	−0.7	
C_2-Flu	−0.3	c	−0.5	c	−0.4	
C_3-Flu	−0.2	c	−0.4	bd	−0.2	c
Dbt	−0.6	c	−0.9	b	−0.8	
C_1-Dbt	−0.5	c	−0.7	b	−0.6	
C_2-Dbt	−0.2	c	−0.4	b	−0.3	
C_3-Dbt	−0.2		−0.3		−0.2	
Phe	−0.6	c	−0.9	b	−0.8	
C_1-Phe	−0.4	c	−0.7	b	−0.6	
C_2-Phe	−0.2	c	−0.4	b	−0.3	
C_3-Phe	−0.2	c	−0.3	b	−0.2	
C_4-Phe	−0.2		−0.2		−0.2	
Nbt	−0.2		−0.2		−0.2	
C_1-Nbt	−0.1	c	−0.2	bd	−0.1	c
C_2-Nbt	−0.1		−0.1		−0.1	
C_3-Nbt	−0.1		−0.1		−0.1	
Pyr	−0.2		−0.2		−0.2	
C_1-Pyr	−0.1		−0.2		−0.1	
C_2-Pyr	−0.2		−0.2		−0.2	
Cry	−0.1		−0.1		−0.3	
C_1-Cry	−0.1		−0.2		−0.1	
C_2-Cry	−0.1		−0.1		−0.1	

*Letters of the treatment means that are significantly different from the current column mean are displayed. Abbreviations: Nap = naphthalene; Flu = fluorine; Dbt = dibenzothiophene; Phe = phenanthrene; Nbt = napthobenzothiophene; Cry = Chrysene; $C_{1,2,3,4...}$ = no. of alkyl substituted groups.

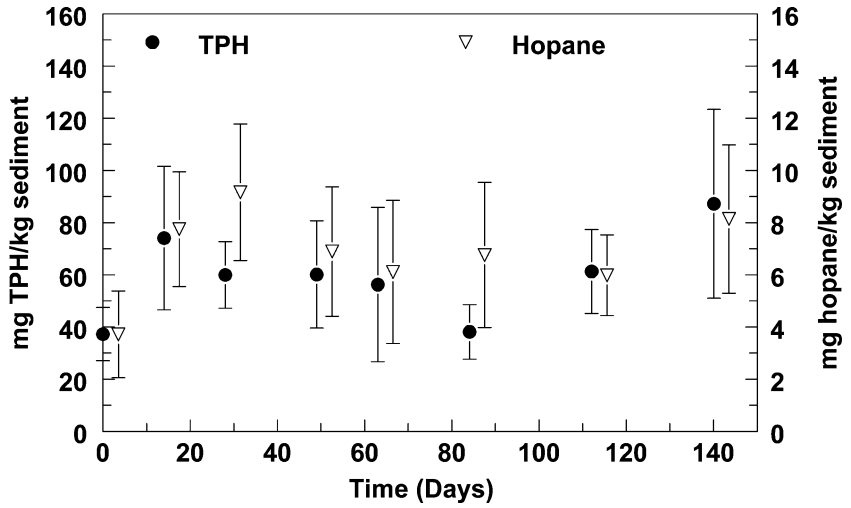

Figure 5. Concentrations of hopane and TPH as a function of time averaged over all treatments. Error bars are the standard deviations of the 3 replicates. The hopane and TPH data are offset slightly from each other to facilitate viewing.

sample versus time for each of the oiled treatments. Although variability was quite high, little physical loss occurred in any of the treatments during the 20 weeks of the study. This figure also shows little, if any, temporal change in TPH normalized to dry weight of sediment. Normalizing TPH to hopane confirms the lack of physical loss.

Total oil loss

Total loss is the sum of all loss mechanisms from the plots, including evaporation, biodegradation, and washout. Volatilization was assumed to be negligible since the crude oil was weathered before application to the plots. Temporal changes in concentrations of resolved alkanes and aromatics normalized to dry weight of sample represent the total loss of these compounds. Total resolved alkane concentrations decreased to levels close to those in unoiled plots after 20 weeks (data not shown). However, PAH concentrations were still significantly higher than background PAH concentrations. Despite the fact that percent degradation of resolved alkanes and aromatics was high, these resolved compounds constitute the most readily biodegradable and bioavailable fraction of the oil. A large fraction of oil in the form of TPH remained in the oiled test plots 20 weeks after the beginning of the experiment. In fact, TPH and hopane values shown in Figure 5 suggest very little, if any, biodegradation of the gross TPH since the ratio of TPH to hopane remained constant for the study duration.

Discussion

In this study, the null hypothesis in terms of rates was rejected for alkanes but not for aromatics. The oil was applied to the surface of the salt marsh, and deep penetration of oil did not take place as the wetland sediments were saturated with water. The oil either remained floating on top of the water or penetrated < 1 cm where conditions were still aerobic. Thus, biodegradation of resolved hydrocarbons was not oxygen-limited under these conditions. Average percent degradation values were similar for saturated hydrocarbons and aromatics in the sediment samples and for the same hydrocarbons coating the emergent marsh vegetation, where conditions were aerobic at all times. The literature states clearly that the rate of biodegradation of alkanes and, especially, of aromatics, in the absence of oxygen is much slower than under aerobic conditions (Caldwell et al. 1998; Coates et al. 1997; Hambrick et al. 1980).

First order biodegradation rate coefficients were greater for total alkanes than for total aromatics for all the tested treatments. These results differ from those reported by others. Venosa et al. (2002) in a freshwater wetland artificially contaminated with crude oil observed that rate

coefficients were about the same for total alkanes and aromatics. Jackson & Pardue (1999) found that total aromatics degraded faster than total alkanes when studying biodegradation potential of a salt marsh in Louisiana. These results indicate that either biodegradation of oil hydrocarbons is a site-specific process or that the limiting factors affecting biodegradation were different at each of the study areas due to the experimental conditions imposed by the study design. In another study, Jackson & Pardue (1997) found that the capacity of a Louisiana salt marsh to mineralize oil without nutrient addition was much lower than that of a Louisiana fresh marsh. Comparing rates and extent of degradation of the northern salt marsh in this study vs. the northern freshwater wetland in Venosa et al. (2002), we concluded that in the salt marsh case the inherent potential for biodegradation of oil hydrocarbons was higher because penetration of oil was not forced into the Conrod Beach sediment as was the case in the freshwater wetland, where the applied oil was raked into the subsurface.

Addition of nitrogen and phosphorus fertilizer to oiled plots significantly accelerated alkane degradation rate over the natural attenuation rate ($p < 0.05$). Biostimulation, however, did not affect aromatic degradation rate, which agreed with the Jackson & Pardue (1999) study.

These results are consistent with the fundamental research of Garcia-Blanco (2004) concerning the optimum nutrient ratios and concentrations for stimulating specific degrading communities in a mixed substrate environment. In 1982, Tilman (1982) developed the resource ratio theory, in which he postulated that different ratios of N:P may bring about significant changes in the dominant populations within a community. This theory was recently applied to the study of hydrocarbon biodegradation in laboratory experiments (Garcia-Blanco 2004). Different (low, medium, and high) absolute concentrations of N and P were varied at different constant ratios of N:P ranging from 1:5 to 100:1. Hydrocarbons studied included a simple mixture of octadecane and phenanthrene in one experiment, and a more complex mixture of 14 alkanes and 14 aromatics in another experiment. In our field study, alkane degradation coefficients were in all cases higher than aromatic degradation coefficients, where the N:P ratio was approximately 10:1 in all treatments with relatively high concentrations of phosphorus ($\sim$10.6 mg/l as P). This is consistent with the findings of Garcia-Blanco (2004) in which alkane degradation rates were higher than aromatic degradation rates when the N:P ratio was 10:1 or lower with relatively high phosphorus concentrations. That ratio was also found by Smith et al. (1998) to be optimal for biodegradation of a simple alkane. Garcia-Blanco (2004) observed that alkane degradation was much more sensitive to changes in phosphate concentrations than aromatic degradation. This could explain the statistical differences observed for alkane biodegradation coefficients in the biostimulation treatments (with or without cropped vegetation) compared to the natural attenuation treatment where the hydrocarbon degrading communities had to rely on background nutrients to support degradation of the oil. The average levels of phosphorus in the nutrient-amended treatments ($\sim$10.6 mg/l as P) were much higher than in the natural attenuation treatment (1.2 mg/L as P). These results appear to be consistent with the conclusions of Garcia-Blanco (2004), suggesting that, to stimulate greater PAH degradation in the presence of alkanes, little or no phosphorus should be added relative to nitrogen, which was not done in the present field study where the phosphorus concentrations were relatively high in the nutrient-amended treatments. More research is needed to confirm this conclusion, but it is an attractive speculation that might affect the way bioremediation is practiced in the future.

Treatment differences were absent in the aboveground vegetation samples as evidenced by overlapping standard deviations among treatment means. Most of the oil applied to the plots coated the surface of the *Spartina* plants. Coastal marshes are typically deficient in nutrients, especially nitrogen (Mitsch & Gosselink 1993). However, in this study, background nutrient concentrations were high enough to support similar rates of degradation as the fertilized plots. In addition, the application of oil onto the non-fertilized plots did not deplete the existing nitrogen in those plots.

The average concentration of ammonium-N in the unoiled control plots was lower than in the oiled plots even though those treatments received the same amount of fertilizer. This could be explained by a higher ammonium-N uptake by the healthier unoiled wetland plants than by the plants

recently stressed by exposure to crude oil. Ammonium-N concentrations in the pore water were consistently higher than nitrate-N concentrations. These differences cannot be attributed to nitrate utilization for oil biodegradation but more likely to greater washout losses and/or the anoxic conditions in the marsh substrate that promoted reduction of oxidized forms of nitrogen to ammonium or nitrogen gas. Lee et al. (2002) showed that when *Scirpus pungens* vegetation in the St. Lawrence River study were left intact in the plots, ammonium-N uptake was substantially higher than in the plots where the vegetation was cropped. These differences did not exist in the case of *Spartina alterniflora* at Conrod's Beach. Measured nitrogen concentrations in the pore water were approximately the same for oiled plots with or without intact vegetation. One reason for the higher biodegradation rate in the present study compared to the St. Lawrence River study was that the lower nutrient uptake rate at Conrod Beach might reflect a slower growth rate, which might be due to physiological differences between *S. alterniflora* and *S. pungens*. Another reason was that the oil had not been manually raked deeply into the anaerobic or anoxic sediment as it had in the latter study. Deep penetration into the sediment did not occur, so aerobic conditions predominated in this study.

The presence of above ground vegetation did not enhance the rate of oil degradation compared to the cropped-vegetation treatment, suggesting that direct uptake by plants had no additional influence on hydrocarbon losses in the rhizosphere. These results agreed with mesocosm experiments conducted by Wright et al. (1997). Tilling was attempted in the present study but had no positive effect on hydrocarbon degradation (data not shown). It was, in fact, highly destructive to the marsh environment, causing the loss of the marsh vegetation, destruction of the root system, and erosion of marsh sediment.

The temporal changes in hopane (and TPH) in the present study were insignificant. The hopane data taken alone suggest that physical loss of the oil during the study was negligible. This is not surprising considering the fact that all oiled plots were skirted with sorbent sheets for the duration of the study to minimize this occurrence. The consistency of the ratio of TPH to hopane suggests very little biodegradation of TPH. This is not inconsistent with the study findings that proclaim a high degree of biodegradation of the monitored analytes, since these analytes represent less than about 5–7% of the total oil mass. This conclusion differed from the findings in the St. Lawrence River study, where physical loss was a very important mechanism of oil removal (Venosa et al. 2002).

Bioremediation activities in this study, both from trampling and from sampling, strongly affected the physical state of the wetland environment. Unlike the previous study in the St. Lawrence River shoreline (Venosa et al. 2002) in which the vegetation completely recovered after a year, the oil and the effect of trampling and sampling were detrimental to *Spartina alterniflora*. However, this does not mean that biostimulation should not be a recommended response action for a salt marsh. Indeed as illustrated by remaining sectors in the plots that were not sampled, the addition of fertilizer had a beneficial effect. Thus, if rapid restoration is the goal (e.g., to avoid erosion of wetland), biostimulation should be considered an effective and even appropriate response action to accelerate habitat recovery. Natural attenuation might also be an effective strategy for oil removal, since the rate of biodegradation was almost as rapid as the nutrient-amended plots. This is especially true for coastal salt marshes in or near estuaries because such environments are likely to be rich in nutrients. Careful monitoring of background concentrations of nutrients at a spill site will clearly influence decisions to proceed with biostimulation. Although background nutrient concentrations at Conrod Beach were sufficiently high to support rapid biodegradation of hydrocarbons, simply measuring high nutrient concentrations might not be sufficient to suggest that biostimulation is not necessary. What is important is not necessarily the absolute nutrient concentrations in a marsh but rather if continued supplementation of nutrients due to vegetative decay or transport from the sea or other sources is high enough to support continued rapid biodegradation of the oil. In this study, the data suggest that nutrient addition did not significantly enhance the biodegradation rates of the aromatic analytes of interest. This finding could not have been predicted *a priori* by simply measuring background nutrient levels. For such a prediction to be made, an extensive nutrient dynamics study should be conducted for the environment of interest. This

may not be feasible in the case where a rapid response to a spill is needed.

Persistence and recycling of nutrients are key factors and perhaps even more important than the instantaneous concentration of N and P observed at any given time. This is explainable with a brief theoretical discussion of C:N. The amount of oil applied to each plot in this study was 8 l, and each plot consisted of 9 m^2 of wetland surface. If we conservatively assume that oil penetrates to a depth of 10 cm into the wetland subsurface (it penetrated much less than that), then the volume of wetland substrate that receives the oil is 0.9 m^3 or 900 l. Assuming that the porosity of the wetland substrate is 50% (much less than that in reality), the water volume exposed to oil is 450 liters. If the ammonia-N concentration in the pore water is assumed to be 10 mg/l, then, neglecting the mass of nutrient sorbed onto the sediment substrate, this gives a total mass of ammonia-N of only 4.5 g. If only 10% of the oil is biodegraded and considering the density of oil to be 0.82 kg/l, then the mass of oil that can biodegrade is 656 g, which is equivalent to almost 2300 g of chemical oxygen demand (COD). Consequently the COD:N ratio is 510, which should not be a sustainable ratio for continued biodegradation without human intervention (nutrient supplementation). Any less conservative assumptions than above will result in an even greater COD:N ratio. Notwithstanding the foregoing theoretical argument, rapid biodegradation did take place, so replenishment of nutrients due to recycling occurred rapidly enough to satisfy the COD demand of the oil in the marsh system.

Due to destructive impacts on vegetative growth, by no means should tilling, even in the mildest form, be considered as a means of accelerating aerobic biodegradation of salt marshes and wetlands.

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