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Enhanced biodegradation of diesel fuel through the addition of particulate organic carbon and inorganic nutrients in coastal marine waters

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Abstract

Diesel fuel pollution in coastal waters, resulting from recreational boating and commercial shipping operations, is common and can adversely affect marine biota. The purpose of this study was to examine the effect of additions of particulate organic carbon (POC) in the form of naturally-occurring marsh grass (*Spartina alterniflora*), inorganic nutrients (nitrogen and phosphorus), inert particles, and dissolved organic carbon (DOC) on diesel fuel biodegradation and to attempt to formulate an effective bioremedial treatment for small diesel fuel spills in marine waters. Various combinations of treatments were added to water samples from a coastal marina to stimulate diesel fuel biodegradation. Diesel fuel was added in concentrations approximating those found in a spill and biodegradation of straight chain aliphatic constituents was estimated by measuring mineralization of ^{14}C hexadecane added to diesel fuel. All treatments that included POC showed stimulation of biodegradation. However, the addition of inert particles (glass fiber filters and nylon screening) caused no stimulation of biodegradation. The addition of nitrogen and phosphorus alone did not result in stimulation of biodegradation, but nitrogen and *Spartina* (although not phosphorus and *Spartina*) did result in stimulation above that of *Spartina* alone. Maximum biodegradation rates were obtained by the addition of the *Spartina* POC, ammonium, and phosphate. The addition of mannitol, a labile DOC source with POC and phosphate resulted in a decrease in diesel fuel biodegradation as compared to POC and phosphate alone. The seasonal pattern of diesel fuel biodegradation showed a maximum in the summer and a minimum in the winter. Therefore, of the treatments tested, the most effective for bioremediation of diesel fuel in marine waters is the addition of POC, nitrogen, and phosphorus.

Introduction

Petroleum hydrocarbons are the most common family of anthropogenic pollutants in the marine environment. Distribution of petroleum hydrocarbon pollution in the marine environment is uneven, with a majority occurring in bays, harbors, and estuaries (Levy et al. 1981). One common petroleum hydrocarbon pollutant in the marinas and coastal waters of most of the United States is diesel fuel. Enclosed areas such as marinas frequently are exposed to small spills of fuels such as diesel as a result of over-filling of gas tanks and other standard boating operations. There is a need for a bioremedial technique to deal with these small spills, which as a group may constitute a significant portion of marine petroleum pollution.

Many methods designed to clean up large-scale spills are neither effective nor efficient in dealing with environmentally-relevant small to medium sized spills of a few hundred to a thousand gallons, because they often include the use of large and expensive mechanical methods. Equipment such as booms and hot water sprayers are costly to get to sites and operate, and often cause considerable habitat destruction as a result of their use (Foster, et al. 1990). The design of remedial techniques for these smaller spills must take into account the ecological complexity of the bays, harbors, and estuaries while weighing the costs and benefits of any remedial actions proposed.

The fate of crude and refined petroleum products in the marine environment is influenced by many factors, including type and amount of pollution, temperature, hydrodynamics of the environment, rates of volatilization and solubilization, and various transformation processes that may occur at the site (National Research Council 1985). Bioremediation of both crude and refined petroleum in the marine environment has been described in many studies (Atlas 1981; Floodgate 1984; Pritchard & Costa 1991; Prince 1992). Diesel fuel has a relatively low viscosity which allows it to disperse fairly quickly, thus increasing the potential for microbial degradation, as microorganisms tend to degrade petroleum hydrocarbons at water and oil interfaces (Pritchard & Costa 1991). Microorganisms capable of utilizing petroleum hydrocarbons appear ubiquitous in the marine environment (Atlas 1981; Floodgate 1984; Prince 1992). If the indigenous microflora have the ability to degrade petroleum hydrocarbons and are not doing so at a reasonable rate, then environmental constraints at the site of the spill may be controlling the level of microbial degradation.

When a spill occurs, a surfeit of carbon is available to the microbial community. The rate at which this carbon can be utilized is limited by the lability of the carbon, the organisms present, the availability of other nutrients, and characteristics of the physical environment. Generally, oxygen, nitrogen, and phosphorus are the chemical constituents that may limit or control the biodegradation processes (Leahy & Colwell 1990). Petroleum spills often introduce more carbon to an environment than the indigenous microflora can degrade. To increase the microbial population to a size which is capable of assimilating the hydrocarbons at a reasonable rate, one of two basic actions must be taken. Either organisms must be added, or the environment must be modified to increase the activity and growth of indigenous population (Prince 1992).

Concentrations of inorganic nutrients (nitrogen and phosphorus) often limit the biodegradation of petroleum hydrocarbons in the marine environment. Atlas & Bartha (1972) found that microbial degradation and mineralization were not increased by the addition of nitrate or phosphate alone but were increased dramatically when nitrate and phosphate were added together. Pfaender et al. (1985) found that the concentration of inorganic nutrients in estuaries, rivers, and lakes correlated with the potential for biodegradation. Simple experiments designed to determine the limiting nutrients at a specific site can and should be performed prior to any bioremedial effort (Prince 1992). The addition of readily-degradable carbon substrate has been utilized as a biostimulatory technique to attempt to increase microbial degradation of organics (Schmidt & Alexander 1985; Shimp & Pfaender 1985; Ladousse & Tramier 1991; Pfaender 1992; Swindoll et al. 1988). POC additions have also been shown to enhance biodegradation of xenobiotics by reducing the toxicity of the polluted area (Apajalahti & Salkinoja-Salonen 1984).

This work examined the effectiveness of additions of inorganic nutrients, POC, inert particles, and DOC in various combinations at increasing diesel fuel degradation and attempted to formulate a treatment which significantly enhanced diesel fuel biodegradation in marine waters.

Experimental materials and methods

Water samples

Water samples were obtained from the Morehead City Yacht Basin, which is on a full salinity channel adjacent to the Morehead City Port, NC, USA. The yacht basin is just off Bogue Sound on the fringe of the Newport River estuary. The geological composition of the bottom is a mixture of sand, silt, and clay (Kirby-Smith & Costlow 1989). The natural substrate in the water column is largely detritus from adjacent salt marsh vegetation. The dominant aspect of the hydrology of the basin is the semi-diurnal tidal pattern, with an approximately 1 meter difference between high and low tides (Kirby-Smith & Costlow 1989). Nutrient concentrations in the study area are generally low (0.13–3.0 μM ammonium and 0.2–1.2 μM phosphate) and show a diel variation in concentration with a night time maximum and near zero levels of ammonium through most of the daylight hours (Litaker et al., 1993). Nitrogen is known to be growth limiting in this system (Kirby-Smith & Costlow 1989; Litaker et al. 1993). Because of boat traffic, docking operations, and the presence of docked boats in the water throughout the year, the basin is chronically exposed to petroleum hydrocarbon pollution.

Five liter water samples were acquired and divided to assess diesel fuel degradation. For all assays, 30 ml of sample water was placed in a 60 ml serum vial along with varied additions. All samples were incubated on an air powered outdoor shaker table (APOST), either outdoors or in a greenhouse. When the shaker table was outside it was shaded with neutral density screen

Table 1. All treatments included 30 ml water sample and 0.3 ml diesel fuel (^{14}C hexadecane spiked). All *Spartina* additions were made at 0.05 g/vial. Nitrogen and phosphorus were added to supply 1 mM ammonium and 50 μM phosphate, respectively, unless otherwise noted.

Acronym	Treatment description
C	control-water sample only
NP	nitrogen (1 mM ammonium) and phosphorus (50 μM phosphate)
S	*Spartina* (0.05 g)
SN	*Spartina* and nitrogen
S2N	*Spartina* and nitrogen (2 mM ammonium)
S3N	*Spartina* and nitrogen (3 mM ammonium)
SP	*Spartina* and phosphorus
SNP	*Spartina*, nitrogen, and phosphorus
S2NP	*Spartina*, nitrogen (2 mM ammonium), and phosphorus (50 μM phosphate)
S2N2P	*Spartina*, nitrogen (2 mM ammonium), and phosphorus (100 μM phosphate)
S3NP	*Spartina*, nitrogen (3 mM ammonium), and phosphorus (50 μM phosphate)
S3N3P	*Spartina*, nitrogen (3 mM ammonium), and phosphorus (300 μM phosphate)
ScP	nylon screen particles (0.05 g) and phosphorus
GP	glass filter particles (0.05 g) and phosphorus
SPM	*Spartina*, phosphorus and mannitol (5 mM)

during the day to prevent excessive irradiance and overheating of the samples. Dissolved oxygen (DO) was measured on several occasions and was low (1.6 ppm) late in incubations, but never zero. The range of DO values measured was from 1.6–6.0 ppm.

Treatments

Additions to samples assayed for diesel fuel degradation included *Spartina alterniflora* particles, glass filter particles, nylon screen particles, inorganic nitrogen (NH_4Cl), inorganic phosphorus (KH_2PO_4), and 5 mM mannitol ($C_6H_{14}O_6$). Acronyms and descriptions of all treatments used are contained in Table 1.

The *Spartina* particles were obtained from standing dead *Spartina* collected from nearby marshes. The dead leaves and stems were washed with filtered seawater, dried in an oven at 50° C, sorted to remove extraneous material, autoclaved, and ground up in a coffee grinder. In samples with *Spartina* additions, 0.05 g of dry *Spartina* was added to a 30 ml water sample. This quantity was chosen by examining the effect of the addition of increased amounts of *Spartina* on dissolved oxygen levels and the quantity of plant particles used to enhance nitrogen fixation in another study (Bebout & Paerl, 1988). The sources for the glass filter particles and the nylon screen particles were Whatman 934-AH glass microfibre filters and neutral density nylon screen, respectively. These sources materials were autoclaved and chopped in the same manner as the *Spartina* and yielded similar particle sizes. They were also added at 0.05 g per 30 ml water sample. The size of all particle types was approximately 1000 microns.

Degradation experiments

Microbial respiration of petroleum hydrocarbons was measured using the radiolabel method of Atlas (1979). Diesel fuel (Amoco Oil Company marine diesel fuel), spiked (0.2μCi/ml diesel fuel) with ^{14}C hexadecane (terminal carbon labeled, Amersham Inc.), was added to all the sample vials at 0.3 ml per 30ml water sample. Hexadecane was selected for the ^{14}C-labeled spike compound because a sixteen carbon aliphatic chain represents the 'average' constituent of diesel fuel straight chain aliphatics. Samples were incubated in 60 ml serum vials sealed with 20 mm rubber-sleeved stoppers fitted with hanging center-well collectors containing 2 N KOH on a piece of folded filter paper. The strong base collected the $^{14}CO_2$ generated by degradation of the labeled hexadecane. After incubation (usually 1, 2, and 4 weeks), the filter paper was removed and placed in 7 ml scintillation vials with 5 ml Cytoscint (ICN Inc.) cocktail. Counts per minute of ^{14}C were determined by counting for 10 minutes on a Beckman LS5000TD scintillation counter.

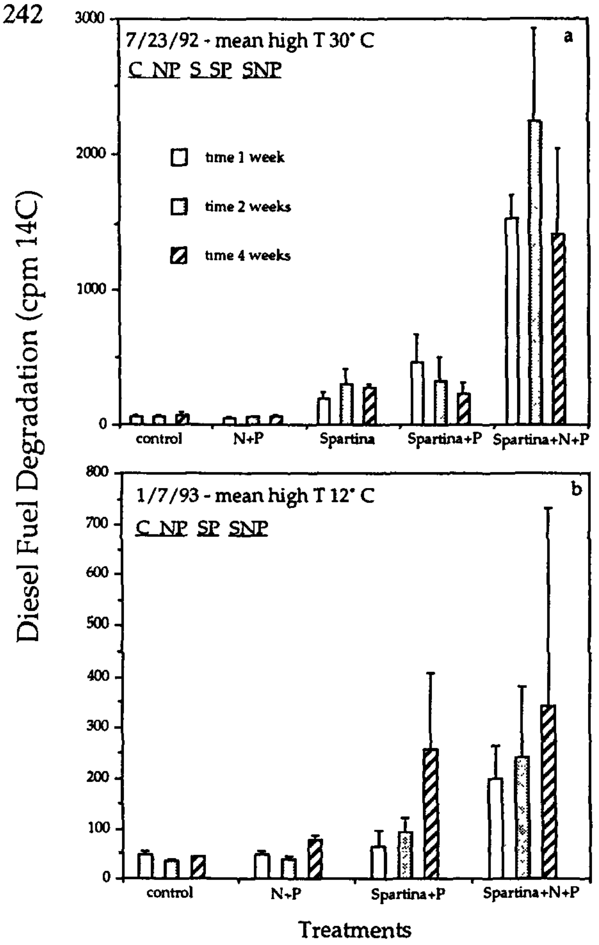

Figure 1. Comparison of the effectiveness of the addition of *Spartina* and inorganic nutrients at stimulating diesel fuel degradation in two experiments run in the summer and winter, respectively (*a* and *b*). Error bars are ± standard deviation.

Data analysis

Data were analyzed using a two-way ANOVA with treatment and time as the two factors. Data were checked for normality and $\log_{10}$ transformed where necessary to achieve normality. A-posteriori comparisons of means were performed using the Bonferroni multiple range test (Moore & McCabe 1993). The seasonal data were subjected to a Student-Newman-Keuls multiple range test due to the size of the data set. Linear regressions were run on the seasonal degradation data to test for relationships to temperature.

Results

An experiment beginning on 7/23/92 examined the impact of POC and inorganic nutrient additions on diesel fuel degradation (Figure 1a) after 1, 2, and 4 weeks. In this experiment there was a significant treatment effect ($p<0.01$). The treatments varied in effectiveness in increasing diesel fuel degradation. The hierarchy of degradation stimulation by the treatments was C = NP < S = SP < SNP. Similar treatments were evaluated for effectiveness in increasing diesel fuel degradation (Figure 1b) in an experiment beginning 1/7/93. Again, there was a significant treatment effect ($p<0.01$). The treatments ranked in effectiveness in increasing diesel fuel degradation in the following order: C = NP < SP < SNP. In each experiment, the most effective treatment was *Spartina* plus nitrogen and phosphorus. Results from these experiments were compared to test for differences between summer and

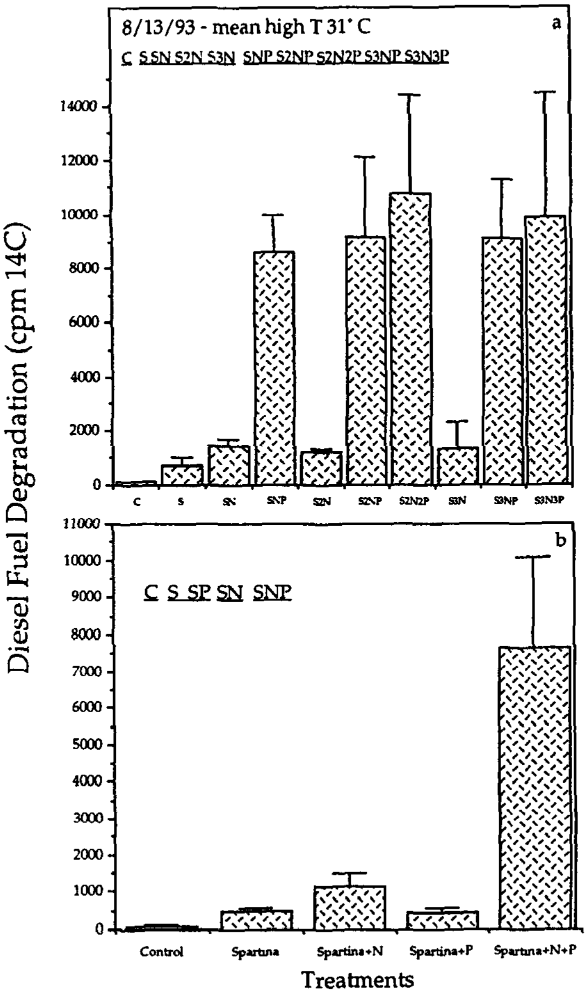

Figure 2. Comparison of the effectiveness of the addition of *Spartina* and inorganic nutrients in varying concentrations (*a*) and *Spartina* and inorganic nutrients in different combinations (*b*) at stimulating diesel fuel degradation in a one week incubation. Error bars are ± standard deviation.

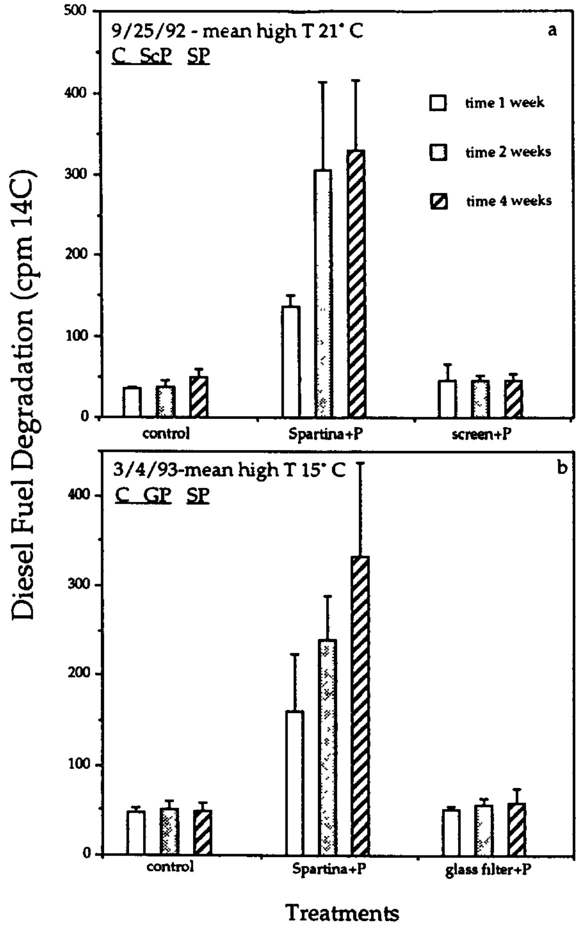

Figure 3. Comparison of the effectiveness of the addition of *Spartina* and nylon screen particles (*a*) and *Spartina* and glass fiber filter particles (*b*) at stimulating diesel fuel degradation. Error bars are ± standard deviation.

winter degradation rates. There was a significant difference (p<0.01) between treatment effectiveness in the 7/23/92 experiment and the 1/7/93 experiment, with greater degradation rates during the summer experiment.

Having established the positive impact of POC and inorganic nutrient (N and P) additions on diesel fuel degradation, further experiments were performed to evaluate different concentrations and combinations of the amendments. Figure 2a shows the effectiveness of several treatments in increasing diesel fuel degradation after a one week incubation beginning 8/13/93. Nitrogen and phosphorus concentrations were varied to test the optimal concentration of each to stimulate diesel fuel degradation. A significant treatment effect existed (p<0.01, one- way ANOVA). The treatments fell into three categories in terms of effectiveness in increasing diesel fuel degradation. The control was alone in the least effective group. The next most effective group encompassed those treatments that included *Spartina* and one or no inorganic nutrients (S, SN, S2N, S3N). The most effective treatments were those which included *Spartina*, nitrogen and phosphorus (at any concentration); these treatments were SNP, S2NP, S2N2P, S3NP, and S3N3P.

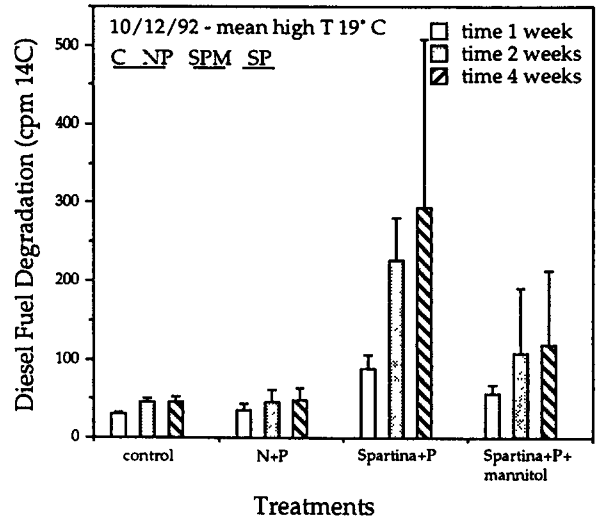

Figure 4. Comparison of the effectiveness of the addition of *Spartina* and inorganic nutrients and DOC at stimulating diesel fuel degradation. Error bars are ± standard deviation.

A one week experiment that attempted to identify the essential components of an effective treatment for stimulation of diesel fuel bioremediation began on 9/17/93 (Figure 2b). There was a treatment effect (p<0.01, one-way ANOVA). The treatments divided into the following groups in terms of increased diesel fuel degradation: C < S = SP < SN << SNP. Again, the most effective treatment included *Spartina* and both inorganic nutrients (SNP).

Two experiments were run which compared the effectiveness of POC and inert particles at stimulating diesel fuel biodegradation (Figures 3a and 3b). There was a significant treatment effect and a significant time effect observed (p<0.01, two-way ANOVA). The SP treatment was found to be more effective than the C or ScP treatments (Figure 3a), which were statistically similar in terms of degradation level. The SP treatment was also significantly more effective than the C and GP treatments which were equally effective (Figure 3b).

Four treatments were tested in the 10/12/92 experiment (Figure 4) that examined the effect of the addition of DOC in conjunction with POC and inorganic

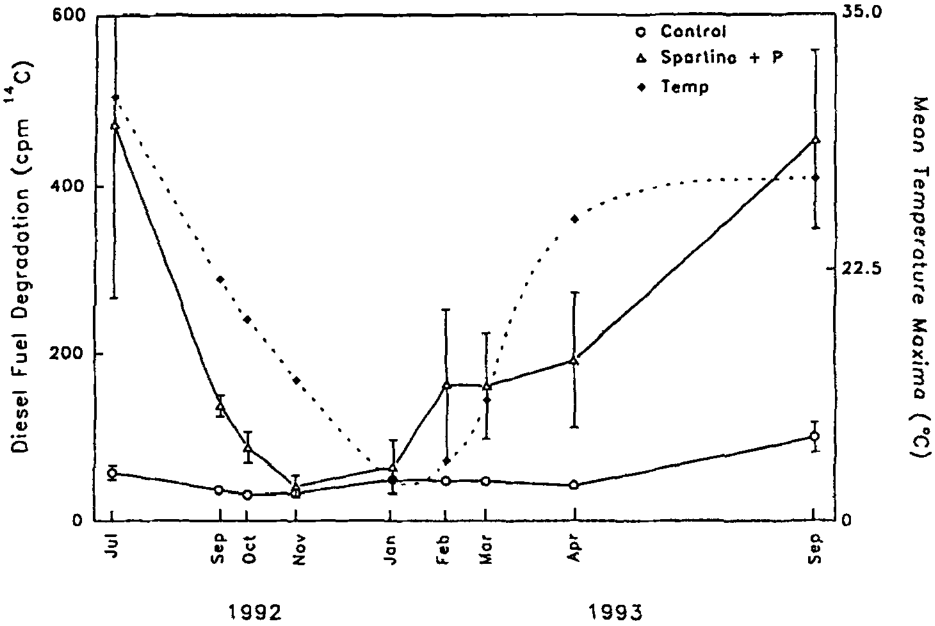

Figure 5. Seasonal pattern of diesel fuel degradation through a one week incubation for the period 7/23/92–9/17/93 plotted with mean high temperature for the experimental periods. Error bars are ± standard deviation.

nutrients. A two-way ANOVA found a time effect and a treatment effect ($p<0.01$). The control and the NP treatment exhibited the least degradation, followed by the SPM treatment, and the SP treatment which was most effective in stimulating diesel fuel degradation.

In order to examine seasonal trends, the control and SP treatments were followed throughout one full season (7/92–9/93). The degradation after one week and the mean high temperatures are shown in Figure 5. There were significant time and treatment effects ($p<0.01$). The SP treatment was found to be more effective than the control throughout the season. Linear regression reveled a significant relationship between degradation in the SP treatment and the mean high temperature through the incubation ($N=9$, $R^2=0.64$, $F=0.009$). No relationship was observed between mean high temperature and degradation in the control ($N=9$, $R^2=0.18$, $F=0.26$).

To assess whether the *Spartina* additions themselves may have been an innoculum of microorganisms an experiment with sterile (filtered and autoclaved) seawater was run. There was no increase in biodegradation in treatments that included Spartina and sterile seawater compared to sterile seawater alone ($p<0.01$). To obtain an estimate of the amount of hexadecane sorbed to the *Spartina* particles samples were incubated with sterilized seawater, *Spartina* and diesel fuel spiked with ^{14}C hexadecane. Particles were separated from the aqueous phase and the amount of radioactivity sorbed to the particles was found to be approximately 10% of the total added.

Discussion

In the event of an oil spill the addition of the limiting nutrients (usually nitrogen and phosphorus) would be expected to increase microbial degradation of the petroleum hydrocarbons over levels with no additions (Prince 1992; Atlas & Bartha 1972). Nitrogen and phosphorus were found by Atlas and Bartha (1972) to co-limit marine microbial degradation. Contrary to these expectations the NP treatment was never more effective than the control in stimulating microbial diesel degradation (Figures 1 and 4). A possible explanation for the lack of degradation associated with the NP treatment is the presence of an additional limiting factor.

Addition of POC in the form of *Spartina alterniflora* particles stimulated diesel fuel degradation in every experiment. The POC was not a source of additional microorganisms so stimulation is probably attributable to several other factors. Although not a source of microorganisms, the *Spartina* may have provided a good substratum for attached microbial growth. *Spartina* is a naturally- occurring substrate in the water column and is colonized by the indigenous microflora (Newell et al. 1983). Organisms which attach to the particles are likely to be larger and metabolically more active and the particles often support a higher proportion of the active microbial community than the water column (Paerl 1980; Jannasch & Pritchard 1972). In addition, the *Spartina* may have increased the availability of the diesel fuel to the attached microorganisms. A portion of the *Spartina* remained suspended in the bottles which could have increased the potential for microbial diesel fuel degradation by allowing the attached microorganisms to remain in contact with the separate phase diesel fuel. Also, degradation may have been augmented due to the sorption of dissolved hydrophobic diesel fuel components to colonized *Spartina* particles (van Loosdrecht et al. 1990). Alternatively, *Spartina* addition may also have decreased the toxicity in the bottles by sorbing the higher solubility components of the diesel fuel. Decreased toxicity in the bottles may have led to increased biodegradation of diesel fuel components. A similar scenario was described with bark chips and pentachlorophenol biodegradation by Apajalahti and Salkinoja-Salonen (1984).

Several experiments were conducted to determine whether POC was essential for stimulation of biodegradation or whether the addition of inert particles (nylon screen particles and glass filter particles) would serve the same purpose. Figure 3 shows the effect of addition of an inert particle in place of *Spartina*. The addition of these inert particles with phosphorus did not enhance diesel fuel degradation. Therefore, it was not just the presence of the solid particle surfaces that stimulated the biodegradation. Rather, the stimulating effect is dependent on some property of the POC. Some possible reasons for the inability of the inert particles to substitute for *Spartina* are that the physical or chemical properties of the surface of the inert particles might have made microbial attachment difficult (Fletcher & McEldowney 1984) or that other necessary substances (nutrients, diesel fuel) did not associate with these particles.

Because of the high level of microbial activity and the potential for attraction of pollutants, particles and other surfaces may be important sites for biodegradation (Kirchman 1993). We observed that approximately 10% of the total ^{14}C hexadecane added in the diesel fuel mixture was sorbed to *Spartina* particles. Hydrophobic compounds would be expected to sorb to *Spartina* because they have a tendency to avoid contact with water and associate with a non- polar non-aqueous environment such as an organic particle (Stumm & Morgan 1996). The inorganic nutrients utilized in these experiments were positively charged and would be likely to associate with the negatively charged *Spartina* particles and glass fibers, but less likely to do so with the inert nylon screen (Stumm & Morgan 1996). Microbial attachment to surfaces is not fully understood, but it is likely that microorganisms would attach preferentially to the organic carbon containing *Spartina* particles (Paerl 1985).

In addition to its potential effects on substrate availability and toxicity, it is also possible that the *Spartina* was providing some limiting nutrients or growth factor. Analysis of the carbon, hydrogen, and nitrogen content of *Spartina alterniflora* from the same location found that leaves and stems contained approximately 1% nitrogen by weight (Coston-Clements & Ferguson 1985). Clearly the nitrogen in the *Spartina*, even if accessible to the microbial community, is not sufficient to support high levels of diesel fuel degradation. The SN treatment was significantly more effective than the S treatment (except in Figure 2a), indicating that once the limitation relieved by *Spartina* was removed, nitrogen availability was limiting hydrocarbon biodegradation in this experiment. The addition of phosphorus with *Spartina* did not significantly enhance degradation over the level obtained with the S treatment in either experiment in which they were both tested, indicating that phosphorus availability alone was not restricting diesel fuel biodegradation.

The highest observed degradation levels were recorded when POC, nitrogen, and phosphorus were all added together. The large difference in effectiveness between the SNP and all other treatments suggests that the experimental system requires POC to influence some key limiting factor and is subsequently limited first by the availability of N and then by P. The POC could be necessary as: a substratum for microbial attachment that may in turn influence substrate availability, it may be mitigating the toxicity of the diesel fuel, or it could be providing some other undetermined growth factor. The addition of all three amendments in the SNP treatment optimized conditions for diesel fuel degradation in this system. Increased amounts of inorganic nutrients added above the standard additions (1mM N, 50 μM P) did not enhance degradation. The SNP treatment was as effective at increasing diesel fuel degradation as the S2N2P and the S3N3P treatments.

The addition of readily-degradable carbon has been shown to stimulate microbial degradation of xenobiotics (Shimp & Pfaender 1985). However, in this study, the addition of mannitol decreased the amount of degradation of diesel fuel. Rather than stimulating the microbial community and in turn increasing the metabolism of diesel fuel, mannitol was probably a preferable carbon and energy source. Many microorganisms may have metabolized mannitol rather than the less energetically efficient ß-oxidation of diesel fuel. The addition of a second carbon substrate can have varied impacts on biodegradation of synthetic organic compounds at low concentrations. The effect of the addition of a second substrate can enhance or inhibit degradation, depending on the relative recalcitrance of the substrates (Schmidt & Alexander 1985) and the mechanism of transformation.

Throughout the year, the diesel fuel degradation was significantly enhanced by the addition of *Spartina* and phosphorus. The highest values were obtained in the summer and the lowest in the winter with a gradual decrease through fall and increase through spring. This pattern fits the generally accepted model of seasonal microbial carbon mineralization in salt marshes (Howes, et al. 1985). The variation in diesel fuel degradation potentials was probably attributable to several factors, including, temperature, nutrient concentrations in the marshes, microbial abundance and metabolic activity level, and the number of petroleum hydrocarbon degraders present. There was a relationship observed between degradation in the SP treatment and mean high temperature, but not between temperature and degradation in the control. Temperature was also a factor contributing to observed differences between two similar experiments run in the summer and winter (Figure 1).

Based on these data, the most effective treatment for cleaning up a diesel fuel spill is the SNP treatment. It appears necessary to include *Spartina* in any treatment to enhance degradation. Following the addition of POC, biodegradation of diesel fuel in this study seems to be limited by available nitrogen and then by available phosphorus. Thus, any bioremedial technique would have to include POC and potentially varying amounts of inorganic nutrients. It is also possible that it may be feasible to rely on a contribution of N from increased particle associated microbial N_2 fixation and, in turn, be able to reduce the amount of nitrogen added. This question is currently being evaluated in our laboratory.

Based on the results of this study, in a small scale spill situation the SNP treatment may be an effective tool to increase diesel degradation by the indigenous marine microflora. In addition there are several potential refinements for improving the SNP treatment utilized in this work. Incorporating the addition of POC with oleophilic inorganic nutrients (nitrogen and phosphorus) may produce a hybrid fertilizer that would maximize microbial attachment and the efficiency of nutrient delivery and decrease toxicity in the solution. If a fertilization technique such as this is to become widely used, there is also a need to find an appropriate substitute for *Spartina* particles. Post harvest cornslash is an agricultural waste product and has been found to stimulate N_2 fixation and facilitate microbial attachment in marine environments (Bebout & Paerl 1988). Determining its effectiveness at stimulating petroleum hydrocarbon degradation is currently being assessed. The incorporation of the improvements mentioned above could lead to the development of an easily applied and relatively inexpensive bioremedial technique for small-scale oil spills.

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