MICROBIAL DEGRADATION OF CRUDE OIL IN MARINE ENVIRONMENTS TESTED IN A FLASK EXPERIMENT

SALVADOR ALDRETT, JAMES S. BONNER*, MARC A. MILLS, ROBIN L. AUTENRIETH® and FRANK L. STEPHENS
Texas A&M University, Department of Civil Engineering, College Station, TX 77843-3136, U.S.A.

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Abstract—Thirteen different bioremediation products were evaluated for their effectiveness in biodegrading petroleum hydrocarbons. All 13 products tested in this experiment were listed on the NCP product schedule. Of these 13 products, 12 were bioaugmentation agents and one was a biostimulation agent. All the products were tested for toxicity levels initially, using standardized protocols. The products were sampled and analyzed three times over a 28-day period for most-probable number (MPN) of hydrocarbon degraders and total petroleum hydrocarbon as separate fractions. A subsample was analyzed for MPN, and the rest of the sample was extracted and fractionated in total saturated petroleum hydrocarbons (TsPH) and total aromatic petroleum hydrocarbons (TarPH). This experiment revealed that the petroleum hydrocarbons were biodegraded to an extent significantly greater than that achieved by the naturally occurring microorganisms. After 28 days, some products reduced the TsPH fraction to 60% of its initial weight and the TarPH fraction to 65%. Three of the 13 products tested enhanced microbial degradation of the petroleum to a degree significantly better than the nutrient control treatments. Of these three products, only one showed a toxicity level below that of the control treatment. © 1997 Elsevier Science Ltd

Key words—biodegradation, saturated, aromatics, total petroleum hydrocarbons, most probable number, microbial population

INTRODUCTION

Oil spills pose a great hazard for terrestrial and marine ecosystems. The total influx of oil into the sea is estimated to be between 1.7 and 8.8 million tonnes, the majority of which is derived from anthropogenic sources (Leahey and Colwell, 1990). From this estimate, approximately 8% is derived from natural sources (Farrington, 1985). The world’s oceans have received inputs of petroleum for a long time, probably for at least 100,000 years (Farrington, 1985); a few believe that oil seepage represents a significant source of petroleum pollution in oceans. MacDonald et al. (1993) reported that petroleum from oil seepage contributes an approximate 120,000 barrels per year to marine pollution in the Gulf of Mexico alone. Microbial degradation or biodegradation of crude oil appears to be the natural process by which the bulk of the polluting oil is eliminated and may be the reason that the oceans are not entirely covered with oil today (Zobell, 1964). Biodegradation is a microbial process whereby the petroleum is used as an organic carbon source, resulting in the breakdown of petroleum components to compounds of lower molecular weight or the transformation of petroleum components to more polar compounds of a carbon number equal to the parent compound.

Crude oil is composed of a wide range of hydrocarbons, including saturated compounds and polynuclear aromatic hydrocarbons, some of which are suspected carcinogens. Microbial utilization of these compounds as sole carbon sources is highly dependent on the chemical nature of the compounds within the petroleum mixture and on the environmental determinants (Atlas, 1981). Different components of crude oil are degraded at different rates; n-paraffins are oxidized more rapidly than either aromatics or naphthenes (Atlas and Bartha, 1973).

Other physical and chemical processes have been used to remove spilled oil from the environment; however, the use of these technologies has not always been successful. The use of commercially available products as bioremediation agents has gained importance in the United States during the past few years. Different companies have focused on the commercialization of these technologies. These products can be classified as either bioaugmentation or biostimulation agents, depending on their formulation. Alexander (1994) defines biostimulation as the modification of the environment to stimulate indigenous microbial populations to degrade the
materials of interest. In the same way, bioaugmentation is the addition of exogenous microbes or enzymes to the affected environment. Petroleum degradation enhancement has been attempted in some cases by adding highly adapted cultures (Dott et al., 1989). Many companies in the United States have focused on the commercialization of specific bioremediation agents.

Field and laboratory studies have been conducted to evaluate the efficacy of these bioremediation products (Venosa and Haines, 1991). In spite of the studies performed on these products, there is still some skepticism about their effectiveness. Often, naturally occurring microorganisms at a contaminated site are already biodegrading the pollutant. Addition of the microorganisms in these products neither harms nor enhances contaminant removal (Atlas, 1995).

To respond to oil and hazardous materials spills or catastrophes, the United States Government has developed a National Contingency Plan (NCP) to establish a set of procedures to deal with these situations. As a part of the NCP, a list (product schedule) was issued, including different biological remediation agents intended for the degradation of petroleum hydrocarbons. The present study tested 13 hydrocarbon bioremediation agents to determine their effectiveness in degrading petroleum hydrocarbons. The company names of the products tested are listed in alphabetical order: Acorn Biotechnical, AG-Technology, Alpha Environmental, BioEnviroTech, BioGEE, BioNutraTech, B&S Research, Enviro-Zyme, Medina Agricultural, Oil Spill Eater, Petrol Rem, Sybron Chemicals, and Waste Microbes. Twelve of these products are bioaugmentation agents and one is a biostimulation agent. This study evaluated the toxicity of every product at the concentrations indicated by the producer. The efficacy of these agents was measured in terms of the extent of total saturated petroleum hydrocarbons (TsPH), and total aromatic petroleum hydrocarbons (TarPH) removal; all treatments were compared against a nutrient and a non-nutrient control. The microorganisms in these products neither harms nor enhances contaminant removal (Atlas, 1995).

The objective of this research was to evaluate the bioremediation products in a laboratory using standardized protocols reproducing a marine environment. External transport of the contaminant into the microorganisms has been left aside, as has kinetics and mechanisms of reaction. In spite of this, rates of reaction are presented to provide an additional criteria for further comparison of the products.

**MATERIALS AND METHODS**

This research assessed the extent of crude oil biodegradation in emulated marine conditions. Seawater was collected from a clean, natural source (Baffin Bay, located on the Laguna Madre between Corpus Christi and Brownsville, Texas). The seawater was used within 48 h of collection; no microbial inoculum was added prior to testing. An standardized oil Alaska North Slope (ANS 521) was used in the study. The crude oil used was artificially weathered and purchased from NETAC (Pittsburgh, PA). The petroleum was weathered following standardized methods by heating to 521°F to remove the light-end hydrocarbons prior to the experiments; the weathering was done under a nitrogen environment to prevent oxidation. The main purpose of weathering the oil was to produce a standard or stable oil; this would give consistency to the results by alleviating the problem of losses due to volatilization. Besides, in an oil spill the light fraction of petroleum is lost faster than it can be biodegraded.

The experimental design included nutrient and non-nutrient controls and product treatments, each in triplicate, representing the sets of samples. Erlenmeyer (250 ml) flasks were used as incubation reactors. Being a blind study, every flask was coded to ensure anonymity and prevent any biasing of the data. The sterile flasks were filled with 100 ml of seawater, 0.5 g of ANS 521 crude oil, and the appropriate amount of product with or without nutrients (as indicated by the vendor). Flasks were shaken on a gyrorotatory shaker table at 200 rpm and 20°C. After 0, 7, and 28 days of incubation, a set of flasks was sampled.

The reactor was analyzed for microbial densities using a most-probable-number method (MPN). The sample was also analyzed for total petroleum hydrocarbons (TPH); TPH were analyzed as separate fractions. Products were tested for acute toxicity at day 0 as a separate analysis. To analyze for petroleum chemistry, the sample was extracted from the aqueous phase with methylene chloride, and the extracted sample was concentrated to 10 ml. To remove the polar materials from the sample, a 1-ml subsample was taken from the 10 ml; this subsample was concentrated and fractionated using liquid chromatography in a silica-gel/alumina column. The chromatography column was loaded with the sample and 100 ml of n-pentane and eluted; this first fraction or saturated fraction was collected in a boiling flask. The aromatics were extracted using the same procedure, but the solvent used was a mixture of 1:1 methylene-chloride:n-pentane. Both fractions were concentrated to 1 ml and quantified gravimetrically for saturated and aromatic compounds. The complete analytical procedures used in this research are described in detail by Mills (1994). All the reagents and solvents used in this research are of suitable GC-MS grade and were purchased from certified laboratories and suppliers.

![Table 1. Definition of petroleum fractions](image-url)
Microbiological analysis

To determine bacterial growth, a modified MPN procedure was used (Wrenn and Venosa, 1996). The MPN method is a statistically based procedure that involves serial dilutions. This method is designed to detect saturated and aromatic hydrocarbon degraders. The method was performed using 96-well microtiter plates. Saturated and aromatic degraders are microorganisms able to support microbial metabolism having saturated or aromatic compounds, respectively, as the sole source of organic carbon. Each dilution was in replicates of eight and was incubated in minimal-salts Bushnell-Haas broth (Difco, Detroit, MI) with a separate carbon source. The aromatic carbon source mixture contained phenanthrene, anthracene, fluorene, and dibenzothiophene. Plates were read after an incubation time of 3 weeks. Positive wells turned yellow to greenish-brown from the oxidation of fluorene and dibenzothiophene. Plates were recounted 1 week after the initial count to verify that additional wells had not turned positive. Using statistical tables based on a Poison distribution, the bacterial count of aromatic degraders in each sample was estimated, based on the number of "positives" and "negatives" for each dilution. The MPN method was performed in the same way to detect saturated degraders using hexadecane as the carbon source. These plates were incubated for 2 weeks. Filter-sterilized p-iodonitrotetrazolium violet (INT) dye (5 g/liter) was added to each dilution replicate (25% v/v) and allowed to react overnight. A pink color denoted a "positive", while no color indicated "negative". Standard microbiological practices were followed to avoid and detect contamination of media or plates.

Toxicity analysis

Toxicity testing was performed using standardized Microbics Corporation (Carlsbad, CA) protocols. Toxicity tests were used to determine acute toxicity associated with the seawater, nutrient solutions, ANS oil, and/or bioremediation products. The objective of this part of the experiment was to evaluate whether the different commercial products increased the environmental toxicity more than the presence of oil on bioassay organisms.

The toxicity test was performed prior to each evaluation. Products were tested at the concentrations specified by the vendors for use in the product testing. Products were tested in seawater with and without the presence of oil. At day 0, two Erlenmeyer flasks were loaded with seawater. One flask contained oil and the product was added to. Samples were run in triplicate and set in a shaker table for 20 min at 200 rpm. After this period of time, samples were removed from the gyratory shaker table. A 10-ml aliquot was removed from each flask and centrifuged for 20 min to settle all the particles present in the sample. Samples were analyzed in a Microtox™ instrument using the "basic test". The "basic test" is outlined in the Microtox™ A Toxity Testing Handbook (Microbics Co., 1992). Toxicity values were reported as EC50, which is the concentration of sample required to reduce the luminescence of the bioassay organisms by 50%. Treatments were compared against a control to determine the extent of toxicity induced by the system with the presence of the product.

Statistical analysis

The separate fractions (TsPH and TarPH) from the different treatments were compared against the nutrient and non-nutrient control using a statistical hypothesis (Montgomery, 1984). In this hypothesis, μ1, μ2 represents the average of the two treatments to be compared—H0: μ1 = μ2 and H1: μ1 ≠ μ2 (where H0 is the null hypothesis and H1 is the alternative hypothesis).

The value t0 was compared against the t distribution with n1 + n2 - 2 degrees of freedom, and a 95% confidence interval. If |t0| > tα/2, n1+n2, H0 is rejected. Values for t0 = tα/2, n1+n2 are reported in statistical tables. The treatments were compared against the nutrient control according to these criteria.

RESULTS AND DISCUSSION

The efficacy of each product was measured by TPH analysis. Three of the 13 treatments were effective when compared statistically against the nutrient control. Figure 1 shows the saturated and aromatic microbial degradation as a fraction of the initial concentration for the 13 treatments. Analyzing these data, it was found that most of the treatments biodegraded the oil to a certain extent. However, for some treatments the degradation extent was not statistically significant relative to the nutrient control.

As shown in Fig. 1, the aromatic fraction was less readily degraded than the saturated fraction. The reported values were obtained by dividing the fraction's weight at day 0 by the fraction's weight at day 28. Product 8 has a significantly greater biodegradation extent than the rest of the treatments, with 39% of the saturated fraction and 66% of the aromatic fraction remaining after 28 days. All the products showing a statistical difference were further analyzed and couples with the microbiological data. It was found that high microbial counts were not indicative of higher degradation extents. Most of the products showed microbial growth; however, in some cases the biodegradation extent was almost negligible (Figs 1 and 2). A positive correlation could not be found between the percent of oil degraded and the microbiological population size (Figs 3 and 4).

The separate fractions were quantified and the values were compared relative to the values of the nutrient controls. A 95% confidence interval was used in this comparison and the results are summarized in Tables 2 and 3. Four products have a t-test value lower than t0, indicating a significant statistical difference relative to the nutrient control. These products enhanced the microbial oxidation of saturated and aromatic petroleum hydrocarbons to an extent greater than that achieved by the nutrient controls.

Toxicity results are presented in Table 4. The EC50 index is the concentration at which 50% of the population no longer exhibits any luminescence. An EC50 of 11 means that it requires an 11% dilution of the original sample to reduce the number of bioassay organisms by 50%. Therefore, the lower the EC50, the higher the level of toxicity. Treatments showing a not-detected value were not toxic according to Microtox™ protocols at the concentrations tested. As presented in Table 4, products 2, 6, 8, 10, 12, and 13 showed a toxicity value higher than that of the control treatment (crude oil and seawater).

Total petroleum hydrocarbons analysis

The experiment assessed the efficacy of the different products based exclusively on the percentage of TPH
remaining in the sample, and, thus, we did not monitor other variables that could influence TPH biodegradation, such as nutrients, pH, or dissolved oxygen, among others, were not monitored. It was found that saturated were degraded 61% in the best case (Fig. 5), and, in the same way, aromatics were reduced to 40% of the original composition by the same product. In general, biodegradation rates for the aromatic compounds were lower. In some cases the aromatic fraction remained virtually undegraded. When the whole extract was compared before fractionation, most of the bioremediation products enhanced petroleum degradation to a greater extent relative to the nutrient control; however, the number was reduced to three products when the separate fractions were compared.
Figure 3 shows a correlation between the TsPH and the microbial population size and the fraction degraded at day 28. The figure indicates that product 11 is reaching a decay or death phase by day 28; however, the biodegradation by this product occurred mostly during this period. It is thought that during the first 7 days the microbial population is growing at the expense of an alternative source of organic carbon. After 7 days, the more readily degraded source is being depleted and the microorganisms are switching to petroleum hydrocarbons as the source of organic carbon. Following this period, the microbial culture starts...
utilizing the petroleum hydrocarbons as an organic carbon source; however, not all the organisms show this trait, and, thus, the microbial population is reduced. These findings are supported by the results presented in Fig. 5 for product 8. This product showed the best performance at degrading the petroleum hydrocarbons, although the microbial population size is smaller than for other products. At day 28, when product 8 shows microbial counts in the order of 10^5 counts/ml, product 11 is also reaching this number; however, product 11 seems to have reached the decay phase.

Aromatic petroleum hydrocarbons are being more readily degraded by product 10 during the first 7 days; after day 7 the degradation becomes almost negligible, with a final value of approximately 79% (Fig. 7). From the 21% degraded, almost 12% was achieved during the first 7 days. After this period, the population size is smaller than for other products. At day 28, when product 8 shows microbial counts in the order of 10^5 counts/ml, product 11 is also reaching this number; however, product 11 seems to have reached the decay phase.

Saturated-to-aromatic ratios decreased from 1.26 initially to an average of 0.5 at day 28 for the three successful treatments. Saturated compounds were more readily degraded in every treatment, and therefore decreased more rapidly through time, as observed in Figs 4, 5, and 7.

Microbial growth curves were inconclusive in this study. For some treatments, the MPN values suggest a decay stage reached by day 28. This phenomenon was observed in a few treatments, but it was not constant, and the lack of information through time led to inconclusive results. It is believed that the accumulation of toxic metabolites, or more recalcitrant ones, along with the depletion of the more readily degradable fraction of the petroleum hydrocarbons, may have contributed to the decay of several microbial cultures present in some products.

High microbial counts were not indicative of high hydrocarbon degradation rates (Figs 3 and 4). Some biological additives are formulated as oleophilic compounds by adding chemicals that tend to stay at the oil-water interface. Venosa et al. (1991) found oleophilic fertilizers to be effective in enhancing oil degradation; however, some of these products are composed of paraffinic compounds or waxes that are more readily degradable, and could be degraded faster than the more resistant hydrocarbons. The results from this research suggested that some of the bioremediation agents introduced to the system a more readily degradable source of organic carbon (such as waxes), and this interfered with the hydrocarbon degradation process. Due to the design of this experiment and the proprietary nature of the products, it was not possible to determine the specific factors producing this effect.

Three of the 13 products showed a statistically significant difference in the extent of microbial degradation, but the rest of the products did not enhance the microbial degradation of the petroleum hydrocarbons as tested in this experiment. These results support the findings of Dott et al. (1989), who reported that fuel oil degradation did not depend on, nor was enhanced by, the application of highly adapted commercially available cultures. Because the set-up of this experiment did not emulate a natural environment, it is thought that the application of these products in the field should be tested before any decision is made. It is believed that this test provides a database for further evaluations.

### Table 2. Statistical summary—comparison of the different treatments against the nutrient control (total saturated petroleum hydrocarbons)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>t-test value</th>
<th>tO</th>
<th>Result</th>
</tr>
</thead>
<tbody>
<tr>
<td>Product 1</td>
<td>2.20</td>
<td>2.132</td>
<td>†</td>
</tr>
<tr>
<td>Product 2</td>
<td>-0.42</td>
<td>2.132</td>
<td></td>
</tr>
<tr>
<td>Product 3</td>
<td>1.08</td>
<td>2.132</td>
<td></td>
</tr>
<tr>
<td>Product 4</td>
<td>-4.44</td>
<td>2.132</td>
<td></td>
</tr>
<tr>
<td>Product 5</td>
<td>-2.54</td>
<td>2.132</td>
<td></td>
</tr>
<tr>
<td>Product 6</td>
<td>0.54</td>
<td>2.132</td>
<td></td>
</tr>
<tr>
<td>Product 7</td>
<td>-5.72</td>
<td>2.132</td>
<td></td>
</tr>
<tr>
<td>Product 8</td>
<td>3.29</td>
<td>2.132</td>
<td>†</td>
</tr>
<tr>
<td>Product 9</td>
<td>-4.92</td>
<td>2.132</td>
<td></td>
</tr>
<tr>
<td>Product 10</td>
<td>23.69</td>
<td>2.132</td>
<td>†</td>
</tr>
<tr>
<td>Product 11</td>
<td>11.09</td>
<td>2.132</td>
<td>†</td>
</tr>
<tr>
<td>Product 12</td>
<td>-5.19</td>
<td>2.132</td>
<td></td>
</tr>
<tr>
<td>Product 13</td>
<td>-5.71</td>
<td>2.132</td>
<td></td>
</tr>
</tbody>
</table>

† Product showing statistical significance relative to the nutrient control.

### Table 4. Toxicity values with and without the addition of oil (EC50 (% of dilution))

<table>
<thead>
<tr>
<th>Treatment</th>
<th>EC50 (no oil added)</th>
<th>EC50 (oil added)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>Nutrients</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>P1</td>
<td>20.34</td>
<td>N/D</td>
</tr>
<tr>
<td>P2</td>
<td>36.21</td>
<td>N/D</td>
</tr>
<tr>
<td>P3</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>P4</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>P5</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>P6</td>
<td>15.98</td>
<td>N/D</td>
</tr>
<tr>
<td>P7</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>P8</td>
<td>75.31</td>
<td>N/D</td>
</tr>
<tr>
<td>P9</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>P10</td>
<td>7.54</td>
<td>N/D</td>
</tr>
<tr>
<td>P11</td>
<td>N/D</td>
<td>N/D</td>
</tr>
<tr>
<td>P12</td>
<td>8.97</td>
<td>N/D</td>
</tr>
<tr>
<td>P13</td>
<td>58.6</td>
<td>N/D</td>
</tr>
</tbody>
</table>

N/D, not detected.
Fig. 5. Saturated and aromatic fraction removal and microbial growth compared with the nutrient control for product 8.

**Biodegradation rates**

The rate at which oil undergoes microbial oxidation is influenced by the abundance and kinds of microorganisms, the dispersion of hydrocarbons in the medium, oxygen supply, temperature, the chemical composition of the oil, and several other factors (Zobell, 1964). The present author estimated the biodegradation rates for the three successful products. The time and concentration data were fitted to a first-order model \((r = KC^n, \text{ where } n = 1)\) as suggested by different studies (Stewart et al., 1993; Venosa et al., 1996). When the rate constant was obtained by plotting \(\ln(C)\) versus time, the slope of the regression line gave us the value of the constant.
Fig. 7. Saturated and aromatic fraction removal and microbial growth compared with the nutrient control for product 10.

The data was fitted to a first-order and zero-order model, but it was found that the first-order model fit best according to the determination coefficients ($r^2$); these parameters are included in Tables 5 and 6, respectively. The predicted line was fitted using only three points in time. It was found that more data is needed to reduce the error associated with this calculation, although the predicted data gave an estimation of the biodegradation rates. The objective of the work was to measure the extent of petroleum hydrocarbon degradation as measured by total saturated and total aromatic hydrocarbons. Rate analyses only were provided to give a relative comparison of the rates of the bioremediation products to offer further comparison for product efficacy. The rate data should be interpreted in this context only. These rates are presented in Tables 5 and 6. In general, the rates for saturated degradation are faster than the rates of aromatics, and they increased by the addition of the bioremediation agents (Tables 4 and 5). These rates are calculated using the initial concentration. Making a materials balance on the reactor, and assuming a first-order rate, came up with the following expression for the rate of reaction:

$$\frac{\partial C}{\partial t} = -KC$$

From this equation, the half-life of the petroleum mixture can be obtained as

$$0.5 = \exp(-Kr)$$

According to the findings, the half-life for the saturated and aromatic petroleum fractions was approximately 31 and 66 days, respectively. These results support the findings of Stewart et al. (1993), who reported an average half-life from different experiments of approximately 2 months.

**SUMMARY AND CONCLUSIONS**

Hydrocarbon microbial degradation was enhanced effectively by 3 of the 13 treatments. A saturated
reduction of up to 61% of the initial amount was achieved by some products, and 40% was achieved for the aromatics. In general, saturated compounds were more readily degraded than aromatic compounds, and the ratio of aliphatic compounds to aromatics was reduced by more than 50% from day 0 to day 28. From the different treatments evaluated, only one presented a level of toxicity lower than that of the control treatment.

Biodegradation rates increased with the addition of some bioremediation agents, with an average half-life for saturated and aromatic hydrocarbons of 33 and 66 days, respectively. For some of the products tested, the majority of the biodegradation occurred during the first 7 days. After day 7 the change of concentration with respect to time was almost negligible. The microbial populations increased during the first 7 days, and by day 28 most of the cultures reached a decay stage, but this was not a constant trend. It is thought that this is due to the recalcitrant nature of the crude oil relative to that of specific bacterial culture, a nutrient depletion occurring after day 7, or the toxicity induced by degradation metabolites or intermediate products.

The different bioremediation agents proved to be significantly better than the control treatment. This experiment showed that the bioremediation agents positively enhanced the biodegradation process. In spite of these results, caution is a must due to the specific conditions involved in this test. The variety of conditions and factors present in a real spill suggests that additional research work should be conducted to test the bioremediation agents' performance under such specific conditions.

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