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Using Respiration Rates and Stable Carbon Isotopes to Monitor the Biodegradation of Orimulsion by Marine Benthic Bacteria

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Carbon dioxide evolution rates and the $\delta^{13}C$ of produced CO_2 were measured in slurry experiments to determine the potential for biodegradation of Orimulsion, a new fuel consisting of 70% Cerro Negro bitumen, 30% water, and 0.1% emulsifying surfactant. Control slurries contained marine sediment as a bacterial inoculum and carbon source, while experimental slurries contained sediment amended with either Orimulsion or bitumen alone, surfactant-free. The $\delta^{13}C$ values of the substrates, marine organic matter and Orimulsion/bitumen were significantly different, −23‰ and −27‰. Respiration rates were 40−80% greater in hydrocarbon-amended slurries relative to controls. The $\delta^{13}C$ of the respired CO_2 in the Orimulsion-amended slurries was 2−3‰ depleted in ^{13}C relative to CO_2 produced in controls. Mass balance calculations showed that the $\delta^{13}C$ values of respired CO_2 due to marine organic matter utilization ranged from −20 to −23‰, while the $\delta^{13}C$ of excess CO_2 produced due to hydrocarbon degradation was generally more ^{13}C depleted (−21 to −27‰), evidence of bacterial degradation of Orimulsion and bitumen. Although Orimulsion biodegradation rates were considerably less than degradation rates of oil-based fuels, bioremediation could be enhanced and should be part of a coordinated effort for cleaning up Orimulsion.

Introduction

Orimulsion is a new heavy-end petroleum product that consists of 70% Orinoco Cerro Negro bitumen, 30% water, and approximately 0.1% surfactant (*1*). Bitumen is found in many marine deposits, especially in Venezuela, and is processed into the patented product Orimulsion by the addition of water and an emulsifying surfactant, Intan-100. Orimulsion is used as a fuel in Canada, South Korea, Japan, England, and Lithuania. The fuel is transported by tanker to the recipient nation and to the power plant via a pipeline. Should a spill occur at sea, Orimulsion would disperse into the water column and eventually settle into the sediments. Little is know about the biodegradability of Orimulsion in the native environment. Chromatographic profiles of Orimulsion appear similar to those of extremely weathered and degraded crudes (*2*), suggesting that bitumen is already relatively degraded. On the other hand, early tests of Orimulsion product storage indicated that marine bacteria could grow in Orimulsion storage vats, causing souring of the product (*15*). This observation suggested that Orimusion might be biodegradable. This study applied two techniques to determine the degradation potential of Orimulsion by benthic bacteria found in typical estuarine sediment.

The first technique measured microbial respiration rates in control sediment slurries containing only marine organic carbon and similar slurries amended with hydrocarbon. Orimulsion or bitumen degradation would be indicated by an increase in respiration as measured by CO_2 evolution in amended slurries relative to control flasks. However, the hydrocarbon additions increase the surface area within the slurries, which could alone enhance microbial respiration rates. Therefore, a second technique was used to directly demonstrate hydrocarbon degradation by measuring the isotopic ratio of the respired CO_2 in both control and amended slurries. This technique relied upon the two bacterial substrates, marine organic matter and hydrocarbon, having different isotopic ratios.

Studies have shown that stable carbon isotopes are useful in identifying the substrates supporting microbial activity. For example, Coffin et al. (*3*) used ^{13}C ratios to identify the diverse substrates supporting the growth of estuarine bacteria. Measurements of stable carbon isotopes of respired CO_2 were used to directly demonstrate the microbial degradation of hydrocarbon contaminants found in the subsurface (*4, 5*) and in salt marsh soils (*6*). Isotopic measurements of respired CO_2 were also used to monitor biodegradation of polycyclic aromatic hydrocarbons (*7*). Isotopic techniques appear to be valuable tools for the identification of contaminant origin (*8, 9*). In our system, the monitoring of biodegradation of contaminant hydrocarbons by stable carbon isotope analysis was possible due to the distinct difference between the isotopic composition of marine-derived organic carbon (∼ −20 to −23‰; *10*) and the hydrocarbons (∼ −25 to −30‰; *4, 9*).

Materials and Methods

Five sediment slurry experiments were conducted. Sediments were collected from Bayboro Harbor, Tampa Bay, FL. In three of the experiments, sediment was amended with Orimulsion, while in two experiments sediment was amended with bitumen. Incubations were conducted in 160-mL glass serum bottles. Control slurries contained 20 mL of seawater and 1 g wet weight of sediment. Hydrocarbon-amended slurries contained 20 mL of seawater, 1 g wet weight of sediment, and 0.1 g wet weight of Orimulsion or bitumen. Bitumen was extracted by repeatedly freezing and thawing Orimulsion, which separated the water and surfactant from the bitumen, and decanting the aqueous fraction. The pH varied from 7.0 to 7.5 in the experiments and did not vary over time or between controls and amended slurries.

Slurry bottles were sealed with butyl rubber stoppers and aluminum crimp caps. Bottles were placed on a shaker at 3 rpm at room temperature (25−30 °C). The experiments were run for 21−60 days. On designated days, duplicate control and hydrocarbon-amended slurry bottles were sacrificed; they were collected and frozen for subsequent analysis of CO_2 concentration and $^{13}C/^{12}C$ ratio.

Carbon dioxide concentrations were determined by gas chromatography (GC). A 10-mL aliquot of nitrogen gas was injected into each bottle and mixed thoroughly. An 5-mL aliquot from the 140 mL of headspace was withdrawn by a 10-mL syringe and injected into a Shimadzu GC-8A equipped with a 90 cm × 3 mm size Haysep Q column, flame ionization detector (FID), and a methanizer.

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10.1021/es981158a CCC: $18.00 © 1999 American Chemical Society
Published on Web 05/06/1999

Samples were analyzed for stable carbon isotopes on a Finnigan Mat Delta S gas chromatography isotope ratio mass spectrometer (GCIRMS) (*11*). Carbon isotope ratios ($^{13}C/^{12}C$) are expressed in the conventional δ notation with units of per thousand or per mil, ‰:

$$\delta^{13}\text{C (‰)} = \left(\frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}}\right) \times 1000 \qquad (1)$$

where $R = {}^{13}C/{}^{12}C$ for both the sample and the standard. The standard for carbon is *Belemnitella americana* from the Cretaceous Peedee formation (PDB). The analytical precision (repeated determinations of the same sample) was 0.3‰. Precision between replicate slurries was 0.5‰.

To ensure aerobic conditions in slurry bottles, oxygen measurements were made on a Shimadzu GC-8A equipped with a 3 m × 3 mm size column, packed with 80/100 mesh molecular sieve, and a thermal conductivity detector (TCD). A 0.1-mL aliquot from the 140 mL of headspace gas was withdrawn by a 1-mL plastic syringe and injected into the GC−TCD. Outdoor air (20.8% O_2) was used as a standard for calibration, and replicate analyses of samples and standards were conducted.

Solid-phase samples of marine sediment (following acid treatment to remove carbonates), Orimulsion, bitumen, and Intan-100 surfactant were analyzed for ^{13}C isotopic composition at Isotope Services, Inc of Los Alamos, NM. Samples were weighed into small tin capsules and combusted in an N/A 1500 Carlo-Erba elemental analyzer. The combustion products are processed by a gas chromatic separation, and the carrier stream is sampled by a VG Isomass mass spectrometer, tuned for carbon, which yields the carbon isotope ratios.

Results

The $\delta^{13}C$ value of Tampa Bay sedimentary organic matter was −23.8 ± 0.6‰, Orimulsion's value was −27.5 ± 0.1‰, bitumen's value was −27.3 ± 0.1‰, and the surfactant's value was −27.9 ± 0.1‰. Due to the distinct $\delta^{13}C$ values of the marine organic matter relative to the hydrocarbon substrates, we could employ stable carbon isotope analysis of resultant CO_2 to evaluate hydrocarbon degradation.

Aerobic conditions were maintained in the headspace of all incubations (data not shown). In the three experiments with Orimulsion (exp 1, 2, and 4), the concentration of CO_2 increased linearly in both control and Orimulsion-amended slurries over 21−60 days (Figure 1). For example, in control slurries, CO_2 concentrations increased at a rate of 314 ppm/day while CO_2 in Orimulsion-amended slurries increased at a rate of 567 ppm/day, or an 80% increase in respiration rate in Orimulsion-amended slurries (Figure 1). Beginning on day 4, CO_2 produced within the Orimulsion-amended slurries was depleted in ^{13}C by 3‰ relative to controls (Figure 2). Similar results were obtained in two replicate experiments (Tables 1−3). To compare the $\delta^{13}C$ of CO_2 produced in control and amended slurry incubations, we averaged the $\delta^{13}C$ values from the final three time points for each experiment. The mean $\delta^{13}C$ values of the final three time points showed significant ^{13}C depletion in the Orimulsion incubations relative to the control incubations (Table 2), consistent with the degradation of the hydrocarbon. The hydrocarbon was ^{13}C depleted relative to the marine sedimentary organic matter (see above).

The bitumen experiments (exp 3 and 5) show the same trends as the Orimulsion experiments. In control slurries, CO_2 increased at a rate of 109 ppm/day over 60 days, and the bitumen-amended slurries increased at a rate of 157 ppm/day over the same period (Figure 3, Table 1), resulting in an increase in respiration rate of 40% in bitumen slurries relative to controls. The isotopic composition of respired CO_2 in bitumen bottles was depleted in ^{13}C by 1−2‰ relative to the controls over the first 21 days of the experiment. However, at the 60-day time point, the isotopic values reversed. Therefore, the mean of the $\delta^{13}C$ values of the final three time points in the bitumen-containing slurries was not significantly different from control values due to the reversal at 60 days in experiment 5 and due to considerable scatter in the data of experiment 3 (Figure 4, Table 2).

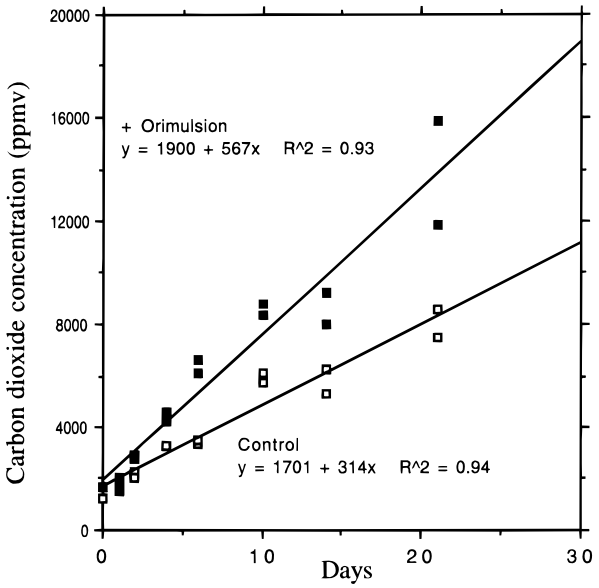

FIGURE 1. Experiment 2, CO_2 production rates in control sediment slurries (open symbols) and slurries amended with 0.1 g of Orimulsion (filled symbols). Each symbol represents an individual flask sacrificed for concentration and $\delta^{13}C$ analysis.

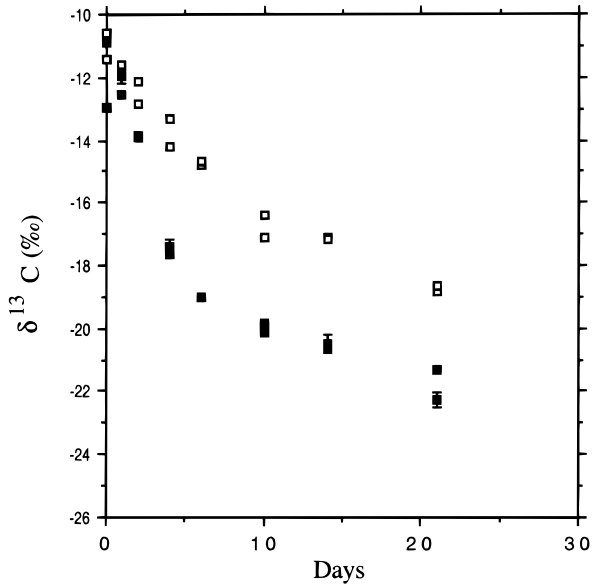

FIGURE 2. Experiment 2, $\delta^{13}C$ ‰ of CO_2 produced over the course of the incubation shown in Figure 1. Open symbols represent control incubations while filled symbols represent those amended with Orimulsion.

Discussion

In this study, we have presented two different lines of evidence that natural marine sediment bacteria can degrade Orimulsion. The results of the CO_2 production measurements indicate that, under aerobic conditions, both Orimulsion and the parent hydrocarbon, bitumen, were degraded. Experi-

TABLE 1. Respiration Rates (ppmv/day) As Measured by CO_2 Production for All Experiments[a]

exp	controls	hydrocarbon addition	% difference[b]
		Orimulsion	
1	312	456	46
2	314	567	81
4	106	147	39
		Bitumen	
3	68	110	61
5	109	157	44

[a] Controls consist of seawater slurries containing Tampa Bay sediment. Hydrocarbon amendments were made by the addition of either Orimulsion (bitumen + surfactant) or bitumen alone to seawater−sediment slurries. [b] % difference is a measure of the enhancement of the respiration by the hydrocarbon addition. (experimental rates − control rates)/(control rates).

TABLE 2. Comparison of the Measured $\delta^{13}C$ ‰ of CO_2 within the Headspace of Slurry Incubations for All Experiments[a]

exp	controls $\delta^{13}C$ ‰	hydrocarbon addition $\delta^{13}C$ ‰	difference ‰
		Orimulsion	
1	-16.6 ± 2.2	-20.3 ± 1.0	3.7 ± 2.4
2	-17.6 ± 0.9	-20.8 ± 0.9	3.2 ± 1.3
4	-22.7 ± 0.3	-24.1 ± 0.4	1.4 ± 0.5
		Bitumen	
3	-21.0 ± 1.2	-21.5 ± 1.2	0.5 ± 1.7
5	-22.4 ± 1.0	-22.5 ± 0.8	0.1 ± 1.3

[a] Values are the means of the final three sampling time points for control slurry flasks and amended slurry flasks.

TABLE 3. $\delta^{13}C$ ‰ of Produced CO_2 over the Indicated Time Period Corrected for the Initial CO_2 Concentration (Eq 3, for Both Controls and Hydrocarbon Addition Flasks) and for the Respiration of Tampa Bay Sedimentary Organic Matter (Eq 4) for Hydrocarbon Addition Flasks[a]

exp, duration	sediment organic matter controls $\delta^{13}C$ ‰	hydrocarbon addition $\delta^{13}C$ ‰
	Orimulsion	
1, 21 days	−20.2	−27.0
2, 21 days	−20.1	−27.3
4, 22 days	−22.6	−27.2
4, 60 days	−23.0	−27.4
	Bitumen	
3, 21 days	−21.4	−23.4
5, 21 days	−21.5	−23.8
5, 60 days	−23.4	−21.0

[a] The second column is the $\delta^{13}C$ of CO_2 produced from sediment organic matter in controls, and the third column is the $\delta^{13}C$ of CO_2 in excess of that produced from respiration of sediment organic matter and represents the respiration of the added hydrocarbon.

ments 1 and 2 used marine sediment preincubated at room temperature for several days increasing the activity of the resident bacterial community. Therefore, these experiments show greater respiration rates than those observed in experiment 4 (Table 1). In all five experiments for both Orimulsion and bitumen, the data indicate respiration rates that are 40−80% greater in hydrocarbon-amended slurries relative to controls (Table 1).

The isotopic composition of CO_2 generated within the incubation flasks containing Orimulsion was always ^{13}C depleted relative to CO_2 generated within the control flasks (Figure 2 and Table 2). This trend was less pronounced in the flasks containing bitumen alone. Orimulsion is clearly

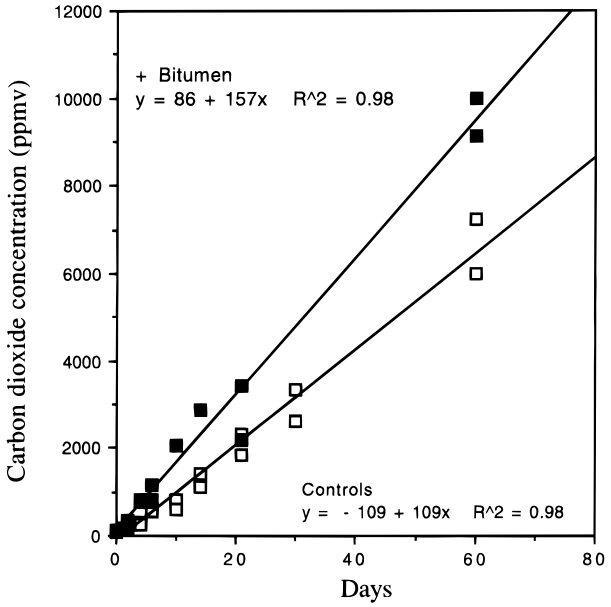

FIGURE 3. Experiment 5, CO_2 production rates in control sediment slurries (open symbols) and slurries amended with 0.1 g of bitumen (filled symbols). Each symbol represents an individual flask sacrificed for concentration and $\delta^{13}C$ analysis.

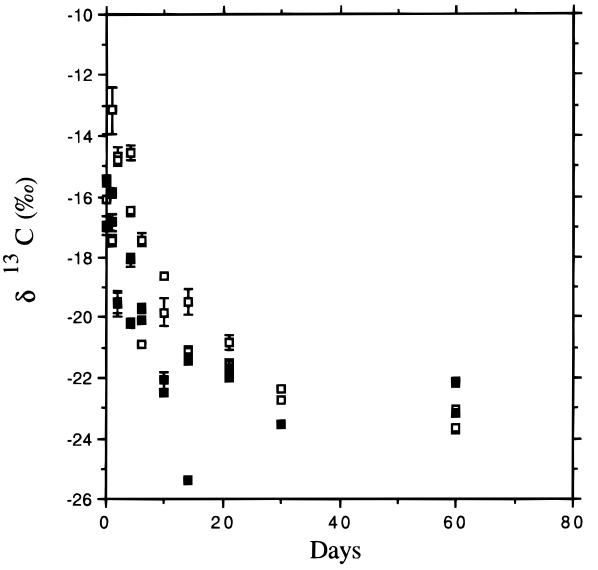

FIGURE 4. Experiment 5, $\delta^{13}C$ ‰ of CO_2 produced over the course of the incubation shown in Figure 3. Open symbols represent control incubations while filled symbols represent those amended with bitumen.

being degraded whereas the bitumen without the surfactant may be less bioavailable.

To determine whether the produced CO_2 was due to the degradation of marine organic matter or Orimulsion/bitumen, mass balance calculations were performed. The isotopic value of the CO_2 produced over the course of the incubation $(\delta^{13}C)_{prod}$ was calculated from

$$[CO_2]_0(\delta^{13}C)_0 + [CO_2]_{prod}(\delta^{13}C)_{prod} = [CO_2]_t(\delta^{13}C)_t \quad (2)$$

by rearrangement

$$(\delta^{13}C)_{prod} = \{[CO_2]_t(\delta^{13}C)_t - [CO_2]_0(\delta^{13}C)_0\}/[CO_2]_{prod} \quad (3)$$

where $[CO_2]$ is the concentration of CO_2 at an initial (0) and

final (t) time, $\delta^{13}C$ is the isotopic composition at an initial and final time, and $[CO_2]_{prod}$ and $(\delta^{13}C)_{prod}$ are the concentration and isotopic values of respired or produced CO_2, and $[CO_2]_{prod} = [CO_2]_t - [CO_2]_0$. In all experiments, the isotopic value of the respired or produced CO_2 due to marine organic matter was −20.3 to −24.1‰ (Table 3), bracketing the ^{13}C values we obtained for Tampa Bay marine sedimentary organic matter (23.8‰).

A similar mass balance equation was set up to determine the isotopic composition of the CO_2 produced in the hydrocarbon-containing flasks due to the respiration of the hydrocarbon alone ($(\delta^{13}C)_{hc}$). That is, the concentration and $\delta^{13}C$ of the total CO_2 ($[CO_2]_T(\delta^{13}C)_T$) produced in the hydrocarbon-containing flasks were corrected for the time 0 intitial CO_2 and the CO_2 produced from the respiration of the sedimentary organic matter as measured in the control flasks. After correction for the initial CO_2 present in the beginning of the experiment (using eq 3), the CO_2 produced in the hydrocarbon flasks was corrected for the CO_2 produced from the decomposition of marine organic matter as measured in the control flasks using

$$(\delta^{13}C)_{hc} = \{[CO_2]_T(\delta^{13}C)_T - [CO_2]_{con}(\delta^{13}C)_{con}\}/[CO_2]_{hc} \quad (4)$$

where $[CO_2]_T$ is the total concentration of CO_2 at the final time point in the hydrocarbon containing flasks, $[CO_2]_{con}$ is the total concentration in the control flasks at the same time point, $\delta^{13}C$ is the measured isotopic composition in the hydrocarbon-containing flasks (T) or control flasks (con) at the final time point, and $[CO_2]_{hc} = [CO_2]_T - [CO_2]_{con}$. The excess CO_2 produced from hydrocarbon respiration in the amended slurries was found to vary from −21 to −27‰ (Table 3). The $\delta^{13}C$ depleted values calculated from the slurries amended with Orimulsion are clearly consistent with hydrocarbon degradation, while the results of incubations amended with bitumen alone are more ambiguous. For experiments 4 and 5, which ran for 60 days, we calculated produced CO_2 values for 21−22 days in addition to the 60-day final time to allow better comparison with the other three experiments. In experiment 4, the $\delta^{13}C$ of the CO_2 produced was not dependent upon the incubation period, but for experiment 5, it did make a difference primarily due to the anomalous isotopic values at 60 days (Figure 4). In this experiment, the bitumen-amended slurries consistently produced ^{13}C depleted CO_2 relative to the controls except for this last time point. It may be that anaerobic conditions developed in the slurry of the bitumen flask incubated over this long period. Although oxygen was never depleted in the flask headspace, the existence of anoxic microzones is possible. Carbon dioxide enriched in ^{13}C could result from methane production, which fractionates isotopes extensively to produce ^{13}C-depleted CH_4 and ^{13}C-enriched CO_2 (*12*). If we favor the shorter time period incubation results in experiment 5, which are consistent with the majority of the data in that experiment and with the incubation times of the other experiments, we find consistency in the data. The incubations containing bitumen provide some evidence for the production of ^{13}C-depleted CO_2, suggesting bitumen degradation. This interpretation is also consistent with the results of Figures 1 and 3 and Table 1, where we observed higher CO_2 production in bitumen-containing flasks relative to controls.

To further evaluate bitumen as opposed to surfactant degradation, we calculated the total amount of the hydrocarbon respired in each experiment by summing the quantity of CO_2 in each slurry bottle at the end of the experiment. We assumed equilibrium between the headspace and the water and used standard equilibrium equations (*13*). There was 0.1 g of Orimulsion or bitumen present in each amended incubation which, based upon measurements of the carbon composition and water content of these mixtures, translates to 4666 or 6667 μmol of C, respectively. (The Orimulsion contained more water.) Final total CO_2 amounts ranged from 33 to 171 μmol in control flasks and from 55 to 289 μmol in the amended flasks. After correction for control values, we calculate that from 0.6 to 2.7% of the original hydrocarbon was degraded over the 21−60 days.

The results indicate that we measured degradation of the bitumen and not the surfactant alone. While earlier formulations of Orimulsion contained 0.2−0.5% surfactant (*2*, *15*) the more recent formulation (which we used) is closer to 0.1−0.15% surfactant by weight (*15*). Furthermore, most of the surfactant degrades to stable organic intermediates rather than CO_2 when subjected to bacterial oxidation on the time scale of these experiments, so the CO_2 production from the surfactant should only be 25% of the amount of surfactant present (*14*).

The degradation rate of Orimulsion/bitumen (1−3% over 21−60 days) is relatively slow as compared to crude oil degradation rates measured in a comparable experiment (*6*). Here roughly 10% of alkanes and 50% of PAHs were degraded over similar time scales in unfertilized experiments. However bitumen is considered to be a highly degraded hydrocarbon so that any microbial respiration of this compound at all is remarkable. Our results indicate that many fuels not considered biodegradable may in fact have bioavailable components. Furthermore, this suggests that native bacteria may continue to degrade hydrocarbons in the environment long after the initial labile components have been mineralized.

The $\delta^{13}C$ values of the Orimulsion, bitumen, and surfactant are the same, approximately −27‰. Therefore, isotopic values do not allow distinction between bitumen or surfactant degradation. However the calculation above is consistent with bitumen degradation. Overall, the evidence for bitumen degradation is greater in the presence of the surfactant, e.g., when the slurries were amended with Orimusion rather than bitumen alone. The surfactant could act like a co-metabolite, providing a readily available carbon and energy source to the bacteria and increasing bitumen degradation. Alternatively, the surfactant may merely increase bitumen reactivity by promoting its dissolution in water. Additional studies are currently underway in our laboratory to investigate the effects of co-metabolism on bitumen degradation and other techniques to enhance Orimulsion bioremediation.

Acknowledgments

This research was supported primarily by Florida Power and Light Corporation and by a cooperative grant from the U.S. Department of Energy to Florida A&M University and Florida State University (DEFG0297ER62449). We particularly thank Mr. Jerry Kirk. We thank Dr. Jennifer Cherrier and Ms. Susan Egolf for assistance in the laboratory and Drs. Paul Kostecki, Tom Potter, Larry Robinson, and Richard Hogg for helpful discussions and support over the course of the project.

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Received for review November 10, 1998. Revised manuscript received March 15, 1999. Accepted March 19, 1999.

ES981158A