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Microbial Populations and Hydrocarbon Biodegradation Potentials in Fertilized Shoreline Sediments Affected by the T/V *Exxon Valdez* Oil Spill

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The effort to clean up the T/V *Exxon Valdez* oil spill in Prince William Sound, Alaska, included the use of fertilizers to accelerate natural microbial degradation of stranded oil. A program to monitor various environmental parameters associated with this technique took place during the summer of 1990. Microbiological assays for numbers of heterotrophic and oil-degrading microbes and their hydrocarbon mineralization potentials were performed in support of this program. Fertilizer addition resulted in higher hexadecane and phenanthrene mineralization potentials on treated plots than on untreated reference plots. Microbial numbers in treated and reference surface sediments were not significantly different immediately after the first nutrient application in May 1990. However, subsurface sediments from treated plots had higher numbers of hydrocarbon degraders than did reference sediments shortly after treatment. The second application of fertilizer, later in summer, resulted in surface and subsurface increases in numbers of hydrocarbon degraders with respect to reference sediments at two of the three study sites. Elevated mineralization potentials, coupled with increased numbers of hydrocarbon degraders, indicated that natural hydrocarbon biodegradation was enhanced. However, these microbiological measurements alone are not sufficient to determine in situ rates of crude oil biodegradation.

After the grounding of the T/V *Exxon Valdez* in Prince William Sound, Alaska, the U.S. Environmental Protection Agency and Exxon Corp. cooperated in a study during the summer of 1989 to determine the feasibility of bioremediation (i.e., enhancing natural biodegradation of the stranded oil by adding nutrients to oiled shorelines). In the spring of 1990, the state of Alaska was asked to concur with the U.S. Coast Guard, Exxon, and the National Oceanic and Atmospheric Administration for approval of nutrient addition as a main tool for 1990 oil cleanup activities. Bioremediation of beached crude oil in the summer of 1990 would involve the addition of oleophilic and/or slow-release, water-soluble nitrogen-phosphorus fertilizers to enhance biodegradation of the oil by indigenous microbes. Conditional approval for the widespread use of bioremediation was contingent upon favorable results of a detailed monitoring program. The results were to provide evidence within 6 weeks that the technique enhanced biodegradation of the oil without posing a significant toxicological threat to shoreline and nearshore biota. Hence, a bioremediation monitoring program was undertaken as a joint project by the State of Alaska Department of Environmental Conservation, the University of Alaska, Exxon, and the U.S. Environmental Protection Agency in the summer of 1990.

The monitoring program was designed to quickly examine a variety of parameters of concern to the state. These studies required the collection and processing of a large number of samples and the generation of results within a short period of time. The sampling program was initiated before fertilizer application and continued for up to 5 months after the study began. Concentrations of fertilizer nutrients and oxygen in shoreline interstitial water, sediment chemistry, microbiology, and ecological and toxicological parameters were measured in support of the monitoring program. Chemical analyses focused on changes in the amount and composition of oil in the beach sediments, the presence of petroleum hydrocarbons in the water column adjacent to treated shorelines, and the rate of disappearance of 2-butoxyethanol, a toxic component of the oleophilic fertilizer Inipol EAP22. Toxicological monitoring was concerned primarily with assessing the toxicity to aquatic biota of ammonia generated on treated shorelines after application of fertilizer. Additional monitoring was performed to assess the likelihood of algal blooms in response to increased nutrient loading in nearshore waters after fertilization.

Several studies have shown that suboptimal growth conditions can limit rates of biodegradation of hydrocarbons in the environment (for a review, see reference 5). While nutrient additions have often been used to stimulate biodegradation of terrestrial hydrocarbon spills, only rarely have such treatments been monitored for effectiveness in marine systems (1, 7). Microbiological analyses can be useful for monitoring stimulation of hydrocarbon biodegradation.

Microbiological analyses to assess relative differences among environmental samples necessitate the use of both microbial activity and biomass measures (3). Three main microbiological parameters were measured in this project: (i) numbers of marine heterotrophs, (ii) numbers of hydrocarbon-degrading microbes, and (iii) respirometric potential to degrade hydrocarbons. This report presents findings of the microbiological assays performed in support of the monitor-

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TABLE 1. Median (n = 5 to 9 samples) marine heterotroph MPN microbial counts per g (dry weight) of sediment for treated (T) and reference (R) plots[a]

Beach site	Day sampled[b]	Median MPN cells (10^4) per g of surface sediment		Different?[c]	Median MPN cells (10^4) per g of subsurface sediment		Different?
		T	R		T	R	
KN-135B	0	15.8	33.0	No	2.2	4.6	No
	2	23.9	45.3	No	24.7	4.7	No
	4	275.0	459.0	No	24.9	47.0	No
	8	240.0	98.5	No	251.0	25.1	Yes
	15	98.6	46.1	No	11.7	2.5	No
	32	453.0	241.0	No	254.0	25.6	No
	52	52.4	51.5	No	44.0	25.4	No
	56	2,510.0	84.3	Yes	652.0	25.0	Yes
	70	136.0	99.9	No	128.0	14.3	Yes
KN-211E	0*	15.6	45.6	Yes	24.6	46.4	No
	2*	24.1	46.1	Yes	46.2	98.7	No
	4	44.4	83.7	No	78.9	46.0	No
	16	59.7	96.1	No	46.1	24.4	No
	31	2.4	2.4	No	2.2	1.0	No
	45	22.9	172.0	No	27.4	24.1	No
KN-132B	0	44.6	24.0	No			
	2	96.4	44.7	No			
	4	244.0	99.1	No			
	8	459.0	45.6	Yes			
	16	47.3	4.1	Yes			
	29	46.1	77.5	No			
	43	277.0	58.2	Yes			
	60	177.0	29.7	Yes			

[a] All values obtained for each day were analyzed with a Mann-Whitney two-sample U test to determine whether the numbers were different at the 95% confidence level. KN-135B was initially treated after day 0 and was refertilized after day 52. KN-211E was initially treated after day 0 and was refertilized on day 42. KN-132B was fertilized after day 0 and again on day 40.

[b] Days marked with an asterisk indicate that an unfertilized plot had significantly higher numbers than found in a fertilized plot.

[c] Statistically significant differences are reported for surface and subsurface sediments.

ing program. All of the data generated during the monitoring program are available in a public report to the Federal On-Scene Coordinator for the T/V *Exxon Valdez* oil spill (8).

MATERIALS AND METHODS

Fertilizers. Two fertilizer formulations, one water soluble and the other oleophilic, were used in the bioremediation effort in Prince William Sound. Customblen 28–8–0 (Sierra Chemicals, Milpitas, Calif.) is a slow-release formulation of soluble nutrients encased in a polymerized vegetable oil; it contains ammonium nitrate, calcium phosphate, and ammonium phosphates with a nitrogen-to-phosphorus ratio of 10.6:1. Customblen was applied to all bioremediation sites, with the application rate reduced when it was applied in conjunction with Inipol EAP22. Inipol EAP22 (CECA S.A., Paris La Defense, France) is an oleophilic fertilizer designed to adhere to oil. It is a microemulsion of a saturated solution of urea in oleic acid, containing tri(laureth-4)-phosphate and butoxy-ethanol. It was applied only where there was surface-exposed oil.

Shoreline treatment. The full monitoring program was designed to quantify the effects of fertilizer addition on stranded oil at three beaches in Prince William Sound. The beaches were chosen to represent a range of energies, substrate types, and depth of oiling within the beach substrate. The shoreline oiling condition, whether exposed on the shoreline surface or buried within the beach (not exposed at the surface), dictated the type of fertilizer (oleophilic and/or slow-release and water soluble) to be used. One site (KN-132B) had only surface oil and received both oleophilic and slow-release fertilizers. Another site (KN-135B) had both surface and subsurface oil and also received both fertilizers. The third site (KN-211E) had only subsurface oil and hence received only slow-release fertilizer.

Each beach site was split into two study plots. Each plot was periodically saturated with water through tidal action. Both portions of each site received similar manual treatment to remove oiled debris and tarmats before fertilizer application. One plot was fertilized (treatment) and one was not (reference) (8). Sampling began before fertilizer application and continued for several months after nutrient addition. KN-132B was treated on 2 June 1990 with 34 g of Customblen per m^2 and 302 g of Inipol EAP22 per m^2. A second application, consisting of 17 g of Customblen per m^2 and 302

TABLE 2. Median (n = 5 to 9 samples) hydrocarbon-degrader MPN microbial counts per g (dry weight) of sediment for treated (T) and reference (R) plots[a]

Beach site	Day sampled	Median MPN cells (10^4) per g of surface sediment		Different?[b]	Median MPN cells (10^4) per g of subsurface sediment		Different?
		T	R		T	R	
KN-135B	0	2.62	4.24	No	1.66	1.63	No
	2	4.79	1.58	No	1.02	0.47	No
	4	15.50	4.20	No	10.30	1.00	Yes
	8	1.56	15.60	No	10.10	2.27	No
	15	15.60	9.75	No	16.20	2.34	No
	52	13.70	23.40	No	75.40	36.00	No
	56	139.00	17.90	Yes	582.00	9.78	Yes
	70	149.00	25.20	Yes	126.00	13.00	Yes
	78	185.00	122.00	No	170.00	117.00	No
KN-211E	0	0.96	4.63	No	4.60	1.63	No
	2	77.00	127.00	No	193.00	2.23	Yes
	4	9.55	48.00	No	81.93	80.35	No
	16	45.40	44.44	No	46.22	97.49	No
	31	23.94	98.78	No	99.80	24.95	No
	45	30.83	53.23	No	33.18	25.32	No
	102	18.10	8.51	No	3.72	0.96	No
	112	3.19	11.70	No	8.51	1.28	Yes
KN-132B	0	24.90	23.00	No			
	2	155.00	21.70	No			
	4	77.70	16.00	No			
	8	160.00	37.10	No			
	16	97.30	15.70	Yes			
	29	28.00	16.00	No			
	43	135.00	0.59	Yes			
	60	84.10	1.78	Yes			
	95	117.00	53.20	Yes			

[a] All values obtained for each day were subjected to a Mann-Whitney two-sample U test to determine whether the sampled populations were different at the 95% confidence level. KN-135B was initially treated after day 0 and was refertilized after day 52. KN-211E was initially treated after day 0 and refertilized on day 42. KN-132B was fertilized after day 0 and again on day 40.

[b] Statistically significant differences are reported for surface and subsurface sediments.

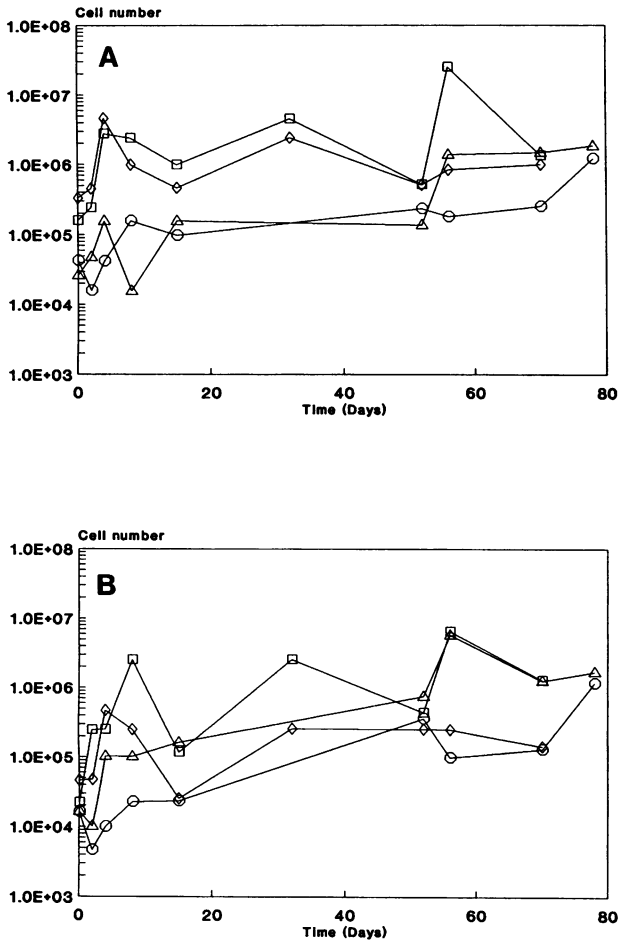

FIG. 1. Site KN-135B. Median (n = 5 to 9) cell numbers of marine heterotrophs for treated (□) and reference (◇) sediments and of oil degraders for treated (△) and reference (○) sediments. (A) Surface sediment samples; (B) subsurface sediment samples. Statistical treatment of data is found in Tables 1 and 2.

g of Inipol EAP22 per m^2, was made on 12 July (day 40). KN-135B was initially treated on 21 May 1990, receiving 103 g of Customblen per m^2 and 361 g of Inipol EAP22 per m^2. This site received a second application of 17 g of Customblen per m^2 and 303 g of Inipol EAP22 per m^2 on 13 July (day 53). The entire site at KN-135B (both treated and reference plots) was fertilized with Customblen at a rate of 91 g/m^2 on 1 August (day 70). A final application of Inipol at 361 g/m^2 and Customblen at 17 g/m^2 was made to the entire segment again on 5 September 1990. KN-211E was initially treated on 30 May with Customblen at a rate of 95 g/m^2. A second application to the treatment plot, at the same rate, was made on 13 July 1990 (day 42). Finally, the entire segment was treated, on an experimental basis, with Inipol EAP22 at 361 g/m^2 on 8 September 1990 (day 99).

The fertilizer applications were intended to follow the application guidelines used for the 1990 cleanup program, but in fact the initial application of Customblen on KN-132B was double the recommended amount, while that on KN-135B was sixfold higher than the recommended amount. In terms of nitrogenous nutrients, KN-132B received approximately 115% of the recommended amount and KN-135B received 200%. All other applications conformed to the application guidelines.

Sampling. Eighteen sediment samples from each treated plot and 18 sediment samples from each reference plot were collected from sites KN-135B and KN-211E before fertilizer application and at various times after fertilizer application. Half of the samples were taken from the surface layer of the sediment, and the other half were taken from a depth of 20 to 30 cm. Only surface sediment samples were collected at KN-132B, with sample collection following essentially the same schedule as that of the other two sites. Sediment samples were placed in sterile plastic bags, placed in coolers with ice, and shipped via floatplane and commercial airline to our laboratory at University of Alaska within 12 h of collection in the field.

Microbiological analyses. Upon receipt in the laboratory, a fraction of each sediment sample was mixed 1:10 (wt/vol) in a sterile 500-ml glass bottle containing filtered and autoclaved seawater from Prince William Sound. To measure the hydrocarbon mineralization potential of the microorganisms in each sediment sample, the University of Alaska Fairbanks (UAF) protocol, essentially as described by Brown et al. (3), was followed. After shaking for 30 min on a wrist-action shaker, 10-ml aliquots were pipetted into sterile, 40-ml, precleaned glass incubation vials fitted with Teflon-lined

TABLE 3. Median (n = 8 to 18 samples) hexadecane mineralization activity measurements from treated (T) and reference (R) sediments expressed as percentage of added hexadecane mineralized[a]

Beach site	Day sampled	% Mineralized in surface sediments		Different?[b]	% Mineralized in subsurface sediments		Different?
		T	R		T	R	
KN-135B	0	6.25	6.35	No	5.10	4.37	No
	2	15.00	8.19	Yes	12.61	10.41	No
	4	12.91	7.80	No	16.82	5.64	Yes
	8	11.34	7.05	Yes	13.14	4.41	Yes
	15	13.24	4.39	Yes	4.54	1.09	Yes
	32	7.53	1.92	Yes	10.82	1.01	Yes
	52	12.55	2.11	Yes	10.02	1.17	Yes
	56	15.55	3.02	Yes	14.01	2.01	Yes
	70	22.21	1.42	Yes	6.71	0.35	Yes
	78	7.73	1.19	Yes	5.03	0.97	Yes
KN-211E	0	2.79	4.69	No	4.82	5.13	No
	2	7.91	12.88	No	6.79	3.40	Yes
	4	5.57	6.69	No	6.74	3.79	Yes
	16	7.81	3.66	Yes	5.99	1.31	Yes
	31	6.59	6.60	No	4.87	1.56	Yes
	45	11.18	1.67	Yes	5.78	0.64	Yes
	102	12.67	3.60	Yes	12.61	1.11	Yes
	112	13.04	3.31	Yes	3.33	1.38	Yes
KN-132B	0	3.40	2.50	No			
	2	12.83	5.97	Yes			
	4	4.69	1.53	Yes			
	8	7.35	2.18	Yes			
	16	10.17	2.89	Yes			
	29	14.73	3.67	Yes			
	43	6.02	0.90	Yes			
	60	3.57	1.99	Yes			

[a] All treated and reference values obtained for each day were subjected to a Mann-Whitney two-sample U test to determine whether the two groups of values were different at the 95% confidence level.

[b] Statistically significant differences are reported for surface and subsurface sediments.

TABLE 4. Median (n = 8 to 18 samples) phenanthrene mineralization activity measurements from treated (T) and reference (R) sediments expressed as percentage of added phenanthrene mineralized[a]

Beach site	Day sampled	% Mineralized in surface sediments		Different?[b]	% Mineralized in subsurface sediments		Different?
		T	R		T	R	
KN-135B	0	0.87	1.19	No	1.16	1.58	No
	2	4.50	8.09	No	15.49	16.45	No
	4	7.08	11.26	No	14.76	10.42	No
	8	3.18	7.69	No	5.95	2.84	Yes
	15	19.79	5.12	Yes	3.27	2.39	No
	32	13.83	4.84	Yes	14.71	2.14	Yes
	52	3.42	0.77	Yes	2.07	0.29	Yes
	56	2.98	1.04	Yes	2.61	0.42	Yes
	70	1.05	0.48	Yes	1.20	0.45	Yes
	78	2.19	1.76	No	1.97	0.88	Yes
KN-211E	0	4.70	7.92	No	12.05	16.83	No
	2	1.11	5.25	No	6.62	9.76	No
	4	1.62	1.72	No	7.09	4.61	No
	16	5.02	4.02	No	6.02	3.95	No
	31	4.68	2.65	Yes	5.19	7.00	No
	45	7.80	0.53	Yes	2.85	0.83	Yes
	102	17.11	5.50	Yes	12.89	5.72	Yes
	112	8.33	2.92	Yes	4.74	4.43	No
KN-132B	0	2.25	2.66	No			
	2	2.00	1.87	No			
	4	2.28	0.79	Yes			
	8	4.05	1.52	Yes			
	16	7.34	1.94	Yes			
	29	3.72	1.84	No			
	43	0.92	0.48	No			
	60	0.54	0.46	No			

[a] All treated and reference values obtained for each day were subjected to a Mann-Whitney two-sample U test to determine whether the two groups of values were different at the 95% confidence level.

[b] Statistically significant differences are reported for surface and subsurface sediments.

septa (I-Chem Research, Hayward, Calif.) for hydrocarbon mineralization potential assessment. Either a linear alkane (n-hexadecane) or a polynuclear aromatic (phenanthrene) was used as the hydrocarbon substrate to assay mineralization potential. The incubation times were 48 h for hexadecane and 72 h for phenanthrene.

Replicate vials of a 10-ml slurry from each of the 18 sediment samples were injected with 50 μl of a 2-g/liter solution (in acetone) of radiolabelled hexadecane (1-^{14}C labelled) or phenanthrene (9-^{14}C labelled). The resulting initial concentration of added hydrocarbon was then 100 μg per vial (100 μg/g of wet sediment; 10 μg/ml of slurry; approximately 50,000 dpm). By adding 100 μg of hydrocarbon substrate to each vial, the hydrocarbon mineralization potential of the microorganisms was independent of the degree of oil contamination in the sediment tested. Immediately after injecting the vials with radiolabelled hydrocarbon, 1 ml of 10 N NaOH was injected into a series of vials at time zero to stop microbial activity and trap CO_2. The remaining vials were incubated for 48 h (hexadecane) or 72 h (phenanthrene) before the addition of NaOH.

To recover the $^{14}CO_2$ in each killed sample, the sample was first acidified with 1.5 ml of 12 N HCl. The acidified samples were then purged for 15 min with N_2 gas (30 ml/min) through a Harvey trap (Harvey Biological Supplies, Hillsdale, N.J.) containing 15 ml of acidified toluene. The trap effectively scavenged unoxidized or partially oxidized volatile hydrocarbon purged from the sample along with the CO_2. After passing through the Harvey trap, the gaseous stream was bubbled through a standard liquid scintillation vial containing 10 ml of CO_2-sorbing phenethylamine cocktail. The radioactivity in the vial was counted in a model LSC 1800 liquid scintillation counter (Beckman Instruments, Irvine, Calif.) with automatic quench correction.

Because of the large number of time-zero controls processed and the numerous sources of variability (ranging from sediment heterogeneity to liquid scintillation counting), all of the negative control data for each hydrocarbon were averaged. The mean (in units of quench-corrected counts per minute) was subtracted from all of the mineralization potential samples independent of source to yield a corrected disintegrations-per-minute value. This corrected disintegrations-per-minute value was divided by the added disintegrations per minute to obtain the percent mineralization of added hydrocarbon substrate. The percent hydrocarbon mineralized, in units of percent CO_2 evolved divided by time of incubation, is referred to as the mineralization potential of the sample.

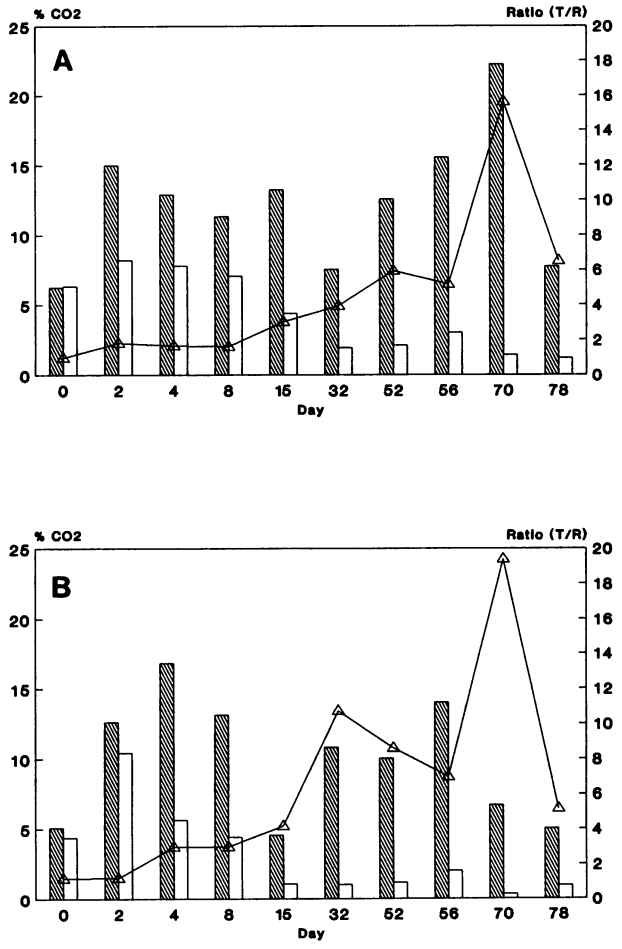

FIG. 2. Site KN-135B. Median (n = 8 to 18) hexadecane mineralization potentials for treated (▨) and reference (□) sediments, and ratio of treated to reference median potentials (△). (A) Surface sediments; (B) subsurface sediments. Statistical treatment of data is found in Tables 3 and 4.

TABLE 5. Ratio of treated to reference (T:R) median hexadecane and phenanthrene mineralization potentials for surface and subsurface sediments

Beach site	Day sampled	T:R ratio of mineralization potentials			
		Surface		Subsurface	
		Hexadecane	Phenanthrene	Hexadecane	Phenanthrene
KN-135B	0	0.98	0.74	1.17	0.74
	2	1.83	0.56	1.21	0.94
	4	1.65	0.63	2.98	1.42
	8	1.61	0.41	2.98	2.10
	15	3.02	3.87	4.16	1.37
	32	3.93	2.86	10.74	6.88
	52	5.94	4.45	8.60	7.19
	56	5.16	2.86	6.98	6.23
	70	15.64	2.20	19.40	2.70
	78	6.52	1.25	5.18	2.24
KN-211E	0	0.59	0.59	0.94	0.72
	2	0.61	0.21	2.00	0.68
	4	0.83	0.95	1.78	1.54
	16	2.13	1.25	4.56	1.52
	31	1.00	1.76	3.11	0.74
	45	6.70	14.84	9.08	3.44
	102	3.52	3.11	11.32	2.25
	112	3.94	2.86	2.41	1.07
KN-132B	0	1.36	0.85		
	2	2.15	1.07		
	4	3.08	2.89		
	8	3.37	2.66		
	16	3.52	3.79		
	29	4.02	2.02		
	43	6.70	1.90		
	60	1.80	1.17		

Positive controls showed that the purging system would recover greater than 99% of $^{14}CO_2$ from processed radiolabelled bicarbonate as if it were a sediment sample. The potential for carry-over between samples was monitored by periodically running blank controls through the purging line. Blank controls run in this manner always fell within the range for time-zero control samples.

Another portion of the suspension was assayed for numbers of hydrocarbon-degrading microorganisms and marine heterotrophs by use of a miniaturized most-probable-number (MPN) method. The Sheen Screen method (2), with sterilized Prudhoe Bay crude oil as the carbon source and growth indicator, was used to enumerate hydrocarbon degraders. Marine heterotrophs were enumerated in a similar manner, except that the growth medium was marine broth (Difco, Detroit, Mich.) and growth was indicated by turbidity. All MPN arrays were incubated at 16 ± 2°C. The heterotroph MPN arrays were incubated for 1 week after inoculation, and the Sh en Screen MPN arrays were incubated for 3 weeks after inoculation.

RESULTS

Given the variety of factors potentially affecting the mineralization values in our assay, as well as the underlying heterogeneity of the environment sampled, we did not expect or obtain normally distributed data from the sets of nine surface or nine subsurface samples collected each sampling time. Thus, the data are presented as median values or in relative frequency polygons (9). We used nonparametric statistical analyses to test for differences in distributed data between treatment and reference plots. To determine whether biomass and mineralization potential were significantly different on treatment versus reference plots, a simple Mann-Whitney U test was performed on the MPN and respirometry data for each day (9).

The median marine heterotroph MPN values for each sampling day are presented in Table 1. The results of the Mann-Whitney test to determine if the populations were significantly different (at the 95% confidence level) on any given sampling day are also presented in this table. The same data for the oil-degrader MPNs are presented in Table 2. The microbial population data for beach site KN-135B are presented graphically in Fig. 1.

The median hexadecane mineralization potentials for treated and reference plots at the three study sites are presented in Table 3. The results of the Mann-Whitney test for differences at the 95% confidence level are also given in Table 3. Similar data, including the results of the statistical analyses, for phenanthrene mineralization potentials are

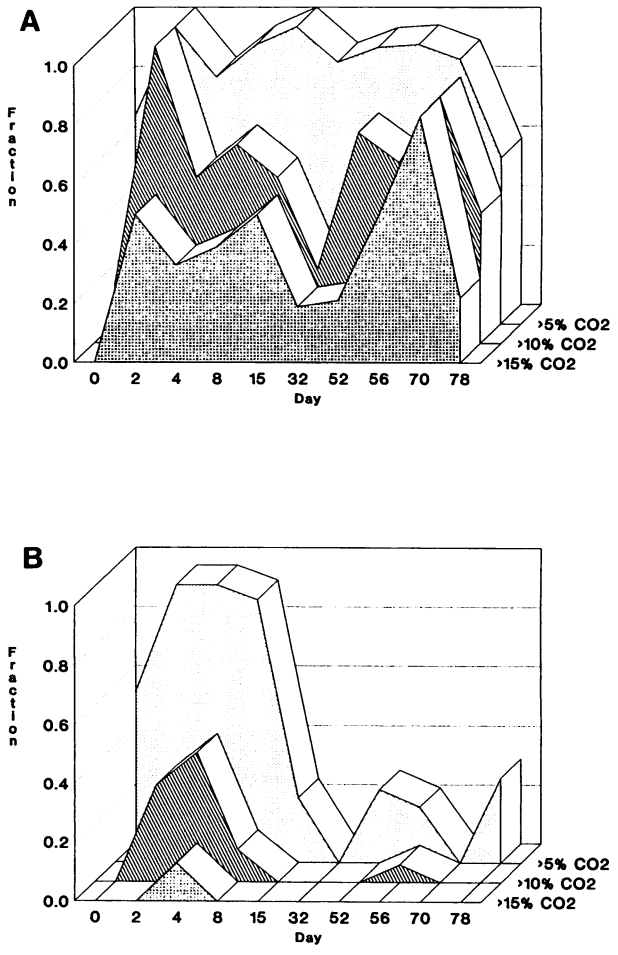

FIG. 3. Hexadecane mineralization frequency polygon for surface sediments, site KN-135B. (A) Treated sediments; (B) reference sediments. The area under the curve is proportional to the fraction of all samples collected which exhibited at least 5, 10, or 15% mineralization of added radiolabelled substrate.

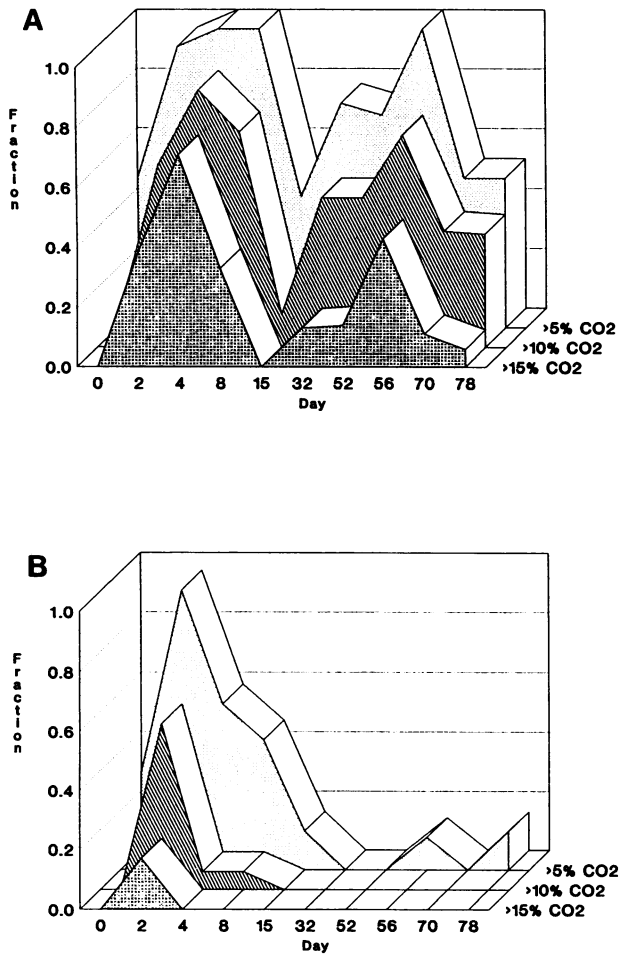

FIG. 4. Hexadecane mineralization frequency polygon for subsurface sediments, site KN-135B. (A) Treated sediments; (B) reference sediments. The area under the curve is proportional to the fraction of all samples collected which exhibited at least 5, 10, or 15% mineralization of added radiolabelled substrate.

shown in Table 4. The median hexadecane mineralization potentials for KN-135B are graphically presented in Fig. 2. The ratios of the median potentials for each day are also plotted in this figure to illustrate the relative enhancement of mineralization potential after fertilizer application. Table 5 shows the mineralization potential ratios for both hydrocarbon substrates for all sampling days and all beach sites.

Another way of visualizing the mineralization potentials is to plot the fractions of all samples exhibiting a certain level of activity. For example, the hexadecane mineralization potentials for the same beach shown in Fig. 1 and 2 (KN-135B) are presented as relative frequency polygons (9) in Fig. 3 (surface sediments) and Fig. 4 (subsurface sediments). A comparison of the treated and reference plots shows the relative abundance of low, moderate, and high mineralization potentials of microorganisms from samples on a temporal scale. The greater the area inside the polygon, the greater the proportion of collected samples which exhibited at least that level of mineralization potential. The fractions of all samples exhibiting various levels of mineralization potential for each substrate, treatment, and beach site are given in Tables 6 and 7.

DISCUSSION

Over the course of the study (mid-May through late August), in both treated and reference plots, the populations of marine heterotrophs and oil degraders generally increased one to two orders of magnitude. The measured biomass increases were typically greater and more sustained in the treated plots than in the reference plots. A statistical treatment of the data shows that, despite the overall biomass increases in both treated and reference plots, there were significantly more microbes in the fertilized areas on some of the sampling days (Tables 1 and 2). These differences became more pronounced later in summer.

Populations of both marine heterotrophs and hydrocarbon degraders (Tables 1 and 2) in surface sediments were not significantly different in treated and reference plots immediately after the initial application of fertilizer. However, at those sites where subsurface samples were collected, there were higher numbers of hydrocarbon degraders in treated subsurface sediment samples 2 (KN-211E) and 4 days (KN-135B) after fertilizer application. After the second application of fertilizer to the treated plot, the populations of both marine heterotrophs and hydrocarbon degraders at two of the beaches, KN-135B and KN-132B, were higher in surface- and subsurface-treated samples. Beach site KN-211E, which had no surface oiling and received only slow-release fertilizer, never had higher microbial numbers in treated surface sediments than the associated reference sediments.

Samples taken from fertilized sediments commonly exhibited higher hydrocarbon mineralization potential than those from the associated reference plots (Fig. 2). On all but a few days after initial fertilizer application at all three beach sites, there were statistically higher hexadecane mineralization potentials in samples collected from the treated plots than in the associated reference samples (Table 3). The phenanthrene mineralization potentials (Table 4) showed a generally similar trend, although there were more days when there was no difference between treated and reference potentials, especially at site KN-211E.

Measurements of the dissolved oxygen, salinity, and temperature of the interstitial water revealed no trend that could be correlated with the decrease in activity on the unfertilized portion of the beach (Fig. 2) (7). The dissolved-oxygen levels on the reference portion of the beach were always close to those found in the nearshore water. The salinity varied substantially, depending on freshwater input, but in a similar fashion throughout the beach, and the temperature was relatively constant between 10 and 17°C. The possibility remains that the decline in activity in the unfertilized portion of the beach is an artifact of the collection, transport, or laboratory treatment of the samples. In this regard, however, it is important to recognize that the conclusions on the efficacy of fertilizer addition are based on comparisons of samples that were collected, transported, and treated at the same time (Tables 1 to 7).

Relative median mineralization potentials (expressed as the ratio of treated to reference median potentials) usually increased within 2 weeks of initial fertilization (Fig. 2). After a second application of fertilizer to the treated plots, further enhancement of relative mineralization potentials was observed. At the end of the 1990 shoreline treatment season, when fertilizer was applied to both treated and reference plots at KN-135B and KN-211E, the relative mineralization

TABLE 6. Fraction of all samples exhibiting at least 5, 10, or 15% mineralization of added hexadecane spike

Beach site	Day sampled	Fraction of samples exhibiting mineralization in:											
		Surface sediments						Subsurface sediments					
		>5%[a]		>10%		>15%		>5%		>10%		15%	
		T[b]	R[b]	T	R	T	R	T	R	T	R	T	R
KN-135B	0	0.70	0.58	0.20	0.00	0.00	0.00	0.50	0.33	0.10	0.00	0.00	0.00
	2	1.00	0.94	1.00	0.33	0.50	0.00	0.94	0.94	0.62	0.56	0.39	0.17
	4	0.83	0.94	0.56	0.44	0.33	0.13	1.00	0.56	0.86	0.06	0.71	0.00
	8	0.94	0.89	0.67	0.11	0.39	0.00	1.00	0.44	0.72	0.06	0.33	0.00
	15	1.00	0.22	0.56	0.00	0.50	0.00	0.44	0.13	0.11	0.00	0.00	0.00
	32	0.88	0.00	0.25	0.00	0.19	0.00	0.75	0.00	0.50	0.00	0.13	0.00
	52	0.93	0.25	0.71	0.00	0.21	0.00	0.71	0.00	0.50	0.00	0.14	0.00
	56	0.94	0.19	0.61	0.06	0.50	0.00	1.00	0.11	0.71	0.00	0.43	0.00
	70	0.89	0.00	0.83	0.00	0.83	0.00	0.50	0.00	0.39	0.00	0.11	0.00
	78	0.56	0.29	0.44	0.00	0.22	0.00	0.50	0.13	0.38	0.00	0.06	0.00
	2–78	0.92	0.42	0.65	0.11	0.43	0.01	0.75	0.26	0.52	0.08	0.25	0.02
KN-211E	0	0.19	0.50	0.00	0.17	0.00	0.00	0.50	0.50	0.00	0.11	0.00	0.00
	2	0.61	0.89	0.39	0.78	0.17	0.28	0.69	0.11	0.19	0.00	0.06	0.00
	4	0.56	0.56	0.22	0.06	0.00	0.00	0.94	0.33	0.17	0.00	0.06	0.00
	16	0.72	0.44	0.33	0.19	0.11	0.06	0.69	0.11	0.31	0.00	0.25	0.00
	31	0.61	0.89	0.39	0.28	0.33	0.06	0.44	0.00	0.11	0.00	0.00	0.00
	45	0.88	0.13	0.56	0.00	0.19	0.00	0.63	0.00	0.13	0.00	0.06	0.00
	102	1.00	0.35	0.86	0.15	0.29	0.05	0.75	0.00	0.63	0.00	0.31	0.00
	112	0.89	0.25	0.67	0.13	0.39	0.00	0.28	0.00	0.11	0.00	0.11	0.00
	2–112	0.74	0.53	0.48	0.24	0.21	0.07	0.63	0.08	0.23	0.00	0.12	0.00
KN-132B	0	0.06	0.11	0.00	0.00	0.00	0.00						
	2	0.72	0.67	0.70	0.11	0.39	0.00						
	4	0.44	0.00	0.00	0.00	0.00	0.00						
	8	0.61	0.00	0.44	0.00	0.28	0.00						
	16	0.78	0.06	0.67	0.00	0.61	0.00						
	29	0.67	0.44	0.44	0.00	0.22	0.00						
	43	0.50	0.00	0.38	0.00	0.31	0.00						
	60	0.25	0.00	0.17	0.00	0.00	0.00						
	2–60	0.58	0.21	0.40	0.02	0.27	0.00						

[a] Percent mineralization exhibited.
[b] T, treated; R, reference.

potential enhancement (i.e., ratio of potentials) diminished after fertilizer application to both plots.

The frequency polygons provide an illustration of the relative abundance of active populations in sediments taken from treated and reference plots at KN-135B (Fig. 3 and 4). Mineralization potentials in the treated plots were fairly high, in general, through the first 2 weeks after fertilization, but showed a decrease by the week 4. The frequency polygons for the reference plot also showed a fairly high abundance of low to moderately active samples but a lower abundance of highly active samples than the treated plot for the same days. After a second fertilizer application (day 53 at KN-135B), the treated plot again exhibited high mineralization potentials while the reference plot continued to show a low abundance of active samples. The effect of a third fertilizer application (day 70 at KN-135B), this time to both treated and reference plots, can be seen in both treated and reference polygons as a maintenance of or increase in the relative abundance of active samples.

The similarity in frequency polygons for treated and reference plots through the first 2 weeks of the study may be due to the fact that both plots received manual treatment (e.g., soil disturbance through tilling, raking, tarmat removal, etc.) which may have stimulated mineralization activity in both plots by enhancing bioavailability of the substrates of interest. When fertilizer was applied to the treated plot a second time (this time with no manual treatment to disturb the soil), the relative abundance of active samples increased in the treated plot while there was no similar increase in the reference samples.

An alternative explanation of the polygon shapes may involve the time of the season when treatment occurred. Early spring in Prince William Sound is characterized by warmer weather and the melting of snowpack in the uplands adjacent to the studied shorelines. The snow runoff at the time the study began may have carried enough nutrients to stimulate hydrocarbon mineralization activities in the untreated plot without added fertilizer. Later in the season, when the second treatment occurred, natural nutrient inputs usually decrease, as a result of either decreased nutrient runoff, increased competition for available nutrients, or both. Studies of the Port Valdez area have shown that marine nutrient levels fluctuate seasonally, reaching a minimum during the summer (4). Note that despite the apparent similarity in frequency polygons (for low and moderately active samples) for the early weeks of the study (Fig. 3 and 4), the potentials for the treated plots are still statistically higher than for the reference samples on all but a few days after initial treatments (Table 3).

Measured by the UAF protocol (3), the increase in mineralization potentials after fertilizer addition can be the result of an increase in biomass of hydrocarbon degraders,

TABLE 7. Fraction of all samples exhibiting at least 2, 5, or 10% mineralization of added phenanthrene spike

Beach site	Day	Fraction of samples exhibiting mineralization in:											
		Surface sediments						Subsurface sediments					
		<5%[a]		>10%		>15%		>5%		>10%		15%	
		T[b]	R[b]	T	R	T	R	T	R	T	R	T	R
KN-135B	0	0.00	0.33	0.00	0.08	0.00	0.00	0.14	0.39	0.07	0.22	0.00	0.00
	2	0.81	1.00	0.44	0.57	0.31	0.50	1.00	0.94	0.89	0.94	0.89	0.72
	4	0.94	1.00	0.56	0.88	0.44	0.69	1.00	0.88	0.89	0.81	0.61	0.50
	8	0.78	0.83	0.33	0.56	0.28	0.22	1.00	0.79	0.67	0.43	0.33	0.21
	15	0.89	0.94	0.89	0.50	0.72	0.38	0.69	0.56	0.31	0.28	0.13	0.00
	32	1.00	0.94	0.94	0.44	0.81	0.00	0.94	0.56	0.78	0.11	0.56	0.00
	52	0.72	0.00	0.22	0.00	0.11	0.00	0.50	0.00	0.00	0.00	0.00	0.00
	56	0.56	0.06	0.44	0.00	0.13	0.00	0.71	0.11	0.21	0.00	0.07	0.00
	70	0.39	0.00	0.06	0.00	0.00	0.00	0.19	0.00	0.00	0.00	0.00	0.00
	78	0.56	0.39	0.22	0.06	0.06	0.00	0.44	0.13	0.00	0.06	0.00	0.00
	2–78	0.73	0.57	0.45	0.33	0.31	0.19	0.74	0.46	0.45	0.31	0.32	0.17
KN-211E	0	0.78	1.00	0.44	0.72	0.22	0.44	0.89	1.00	0.83	0.89	0.61	0.89
	2	0.33	1.00	0.22	0.61	0.22	0.17	1.00	1.00	0.69	0.94	0.19	0.50
	4	0.39	0.44	0.11	0.22	0.00	0.00	0.83	0.89	0.56	0.39	0.11	0.06
	16	0.83	0.89	0.50	0.44	0.22	0.11	0.75	0.78	0.63	0.44	0.19	0.00
	31	0.78	0.67	0.50	0.11	0.11	0.00	0.89	1.00	0.61	0.83	0.22	0.06
	45	0.81	0.00	0.63	0.00	0.31	0.00	0.78	0.14	0.11	0.00	0.00	0.00
	102	1.00	0.89	1.00	0.56	0.75	0.17	1.00	1.00	1.00	0.78	0.67	0.00
	112	1.00	0.69	0.72	0.31	0.39	0.13	0.94	0.89	0.39	0.28	0.17	0.00
	2–112	0.73	0.65	0.52	0.32	0.28	0.08	0.89	0.84	0.57	0.54	0.22	0.09
KN-132B	0	0.69	0.67	0.00	0.11	0.00	0.00						
	2	0.50	0.36	0.28	0.00	0.00	0.00						
	4	0.64	0.14	0.07	0.00	0.00	0.00						
	8	0.75	0.39	0.42	0.00	0.25	0.00						
	16	1.00	0.50	0.78	0.06	0.33	0.00						
	29	0.56	0.33	0.33	0.00	0.00	0.00						
	43	0.25	0.06	0.00	0.00	0.00	0.00						
	60	0.25	0.08	0.00	0.00	0.00	0.00						
	2–60	0.57	0.28	0.29	0.01	0.08	0.00						

[a] Percent mineralization exhibited.
[b] T, treated; R, reference.

an increase in specific mineralization activity, or both. However, because of sample heterogeneity, the naturally active microbial biomass, and the precision of the MPN technique, this study could not address the mechanism. Nevertheless, any increase in hydrocarbon mineralization activity in situ, whether due to increased biomass or specific activity elevation, bodes well for enhancing hydrocarbon biodegradation rates. The radiorespirometry assay is an in vitro assay and thus will not address the in situ case. The evidence of higher cell numbers by using a measurement of the in situ population (e.g., Sheen Screen), however, supports the observed increased activity potentials in fertilized sediments.

The addition of fertilizer to oiled shoreline sediments resulted in increases in hexadecane and phenanthrene mineralization potentials of microorganisms from these sediments compared with those of the reference sediments. The differences in mineralization potential between treated and reference plots appear to have been more pronounced later in the study (i.e., after about 4 weeks) than at its beginning. Fertilizer addition also resulted in increased numbers of hydrocarbon degraders.

Many complex factors affect rates of hydrocarbon biodegradation in nature, and determining an actual in situ crude oil biodegradation rate in Prince William Sound is difficult, if not impossible. This is because crude oil represents a complex mix of hydrocarbon substrates, all diminishing at different rates. In addition, hydrocarbon mass balances are very difficult to obtain in such open and heterogeneous environments where a variety of abiotic factors may compete with biodegradation for removal of the substrate. Proof of in situ biodegradation requires demonstration of a decrease in the mass of contaminant due to microbiological activity (6). Thus, direct proof of statistically significant hydrocarbon biodegradation enhancement in the environment remains elusive. However, logically linked indirect evidence, such as increases in numbers of hydrocarbon degraders, in vitro assays showing increased hydrocarbon mineralization potentials, and decreases in dissolved oxygen, when taken together can substantiate claims regarding in situ biodegradation (6). In our study, increases in in situ numbers of hydrocarbon degraders, along with the increased mineralization potential of microorganisms from shoreline sediments, support the assertion that biodegradation of hydrocarbons was enhanced by the addition of fertilizers.

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