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THE ROLE OF MICROORGANISMS IN THE BIOREMEDIATION OF THE OIL SPILL IN PRINCE WILLIAM SOUND, ALASKA

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The U.S. Environmental Protection Agency's Alaska Bioremediation Project was initiated in the aftermath of the March 24, 1989, EXXON VALDEZ oil spill. The objective of the project was to demonstrate an alternative cleanup method for oil-contaminated shorelines based on enhancing natural biodegradation of the oil through the addition of nitrogen and phosphorus nutrients. This enhancement process is a well-recognized and scientifically sound approach to bioremediation but had never been tested on a large scale in marine environments. The project was managed by EPA's Office of Research and Development with financial, scientific, and logistical support from the Exxon Company USA under the authority of the Federal Technology Transfer Act. The report describes the microbial aspects of the EPA field study in Prince William Sound, Alaska.

Two test sites, Snug Harbor and Passage Cove, were selected for the field demonstration project. Snug Harbor was selected to serve as a beach with oil contamination that approximated the degree of contamination remaining after a heavily oiled beach had been physically washed. Physical washing was the major cleanup available for testing early in the summer. In July, a second site was selected that had been physically washed by the Exxon operations. This site, Passage Cove, served as the main reference beach for the large-scale application of fertilizers and as a means to evaluate a sprayer system for fertilizer application.

Criteria for the selection of the test sites were based on six desired features:

- Typical shoreline of Prince William Sound, i.e., mixed sand and gravel and cobblestone beaches
- Sufficient area with fairly uniform distribution of sand, gravel, and cobble for the test plots

- Protected embayment with adequate staging areas and sufficient size to support several test and control plots
- Uniform oil contamination
- Minimal impact from freshwater inputs
- Shoreline with a gradual vertical rise

SNUG HARBOR PROJECT SITE

Snug Harbor is located on the southeastern side of Knight Island. The shoreline utilized for the demonstration is located on the western side of this harbor. The area is surrounded by mountains, reaching an elevation of approximately 2000 feet, with steep vertical ascents. Major sources of freshwater runoff are from precipitation and snowmelt, which is typical of islands in Prince William Sound. Although other shorelines in Snug Harbor were heavily contaminated with oil, it appeared that little oil was being released to the water, thus minimizing the prospect of reoiling on the beaches chosen for treatment and reference plots.

Table 1 identifies the beach types, dimensions, and treatment. Each plot was divided into 21 blocks, with 7 blocks in 3 tidal zones: high, intermediate, and low.

Table 1. Description of Fertilizer Treatment Demonstration Plots at Snug Harbor

Beach Name	Beach Type	Fertilizer Treatment	Length (m)	Depth (m)
Eagle	Sand, gravel	None-reference	21	12
Otter	Sand, gravel	Oleophilic fertilizer	21	12
Otter	Sand, gravel	Water-soluble fertilizer	35	12
Seal	Cobble	Water-soluble fertilizer	28	12
Seal	Cobble	Oleophilic fertilizer	28	12
Seal	Cobble	None-reference	21	8

Oil contamination in the test area represented a continuous band along the length of the beach. This band was approximately 15 to 20 meters wide and corresponded roughly with the average boundaries of the high and low tides observed in Snug Harbor. To determine the approximate distribution of oil on the beach, samples of beach material from one of the designated mixed sand and gravel plots were taken on May 25, 1989. The samples were extracted, and the oil weight and chemical composition were determined. It was clear from the oil residue weights and ratios of C17/pristane and C18/phythane at two different depths that some weathering of the oil had occurred. Oil concentrations varied considerably, ranging from a high of 67,000 mg/kg of beach material to a low of 37 found in the top 10 cm of the beach. Changes in the ratios, relative to fresh Prudhoe Bay crude, were also apparent in some samples, indicating biodegradation of the oil. Changes were quite variable, but biodegradation appeared to be occurring at the lower depths.

PASSAGE COVE PROJECT SITE

Passage Cove is located on the northwestern side of Knight Island. This site was originally heavily contaminated with oil and was physical washed by Exxon. Even after physical washing, considerable amounts of oil remained at this site, mostly spread uniformly over the surface of rocks and in the beach material below the rocks. Pools of oil and mousse-like material were minimal on the surface. Contamination was apparent to about 50 cm below the beach surface. All of the beach areas tested were cobblestone set on a mixed sand and gravel base. Table 2 lists Passage Cove beach designations and plot dimensions and fertilizer treatments.

Table 2. Description of Fertilizer Treatment Demonstration Plots at Passage Cove

Beach Name	Beach Type	Nutrient Applications	Length (m)	Depth (m)
Raven	Cobble over mixed sand and gravel	None-reference	28	21
Tern	Cobble and mixed sand and gravel granules	Oleophilic and slow-release	35	21
Kittiwake	Cobble over mixed sand and gravel	Nutrient solution sprinkler system	28	21
Guillemot	Mixed sand and gravel with patchy cobble	Oleophilic and granules slow-release	21	7

MICROBIOLOGY OF SNUG HARBOR

Test beaches at Snug Harbor were moderately contaminated. Visually, the cobble plots had a thin coating of dry, sticky, black oil covering rock surfaces and gravel areas under the cobble. Oil did not penetrate more than a few centimeters below the gravel surface. In mixed sand and gravel plots, oil was well distributed over exposed surface areas and commonly was found at 20 to 30 cm below the surface. In many areas of the test plots, small patches of thick oil and mousse could be found. This material was very viscous and mixed with extensive amounts of debris.

Approximately 8 to 10 days following oleophilic fertilizer application to the cobble beach plot, reductions in the amount of oil on rock surfaces were visually apparent. The reduction was particularly evident from the air. The plot was a clean rectangle on the beach surface surrounded by oiled areas. The contrast also was impressive at ground level; there was a precise demarkation between fertilizer-treated and untreated areas.

Close examination of this treated cobble plot showed that much of the oil on the surface of the rocks was gone. Considerable amounts of oil still remained under rocks and in the mixed gravel below these rocks. The remaining oil was not dry and dull as was the case with oil in other areas of the beach, but appeared softened and more liquid. It also was very sticky, with no tendency to come off the rocks. At the time of these observations, no oil slicks or oily materials were observed leaving the beach during tidal flushing.

The mixed sand and gravel beach treated with oleophilic fertilizer also appeared to have reduced amounts of oil in an 8 to 10 day period. However, differences between treated and untreated plots were not as dramatic as on the similarly treated cobble beach. Loss of subsurface oil in treated areas was also visually apparent. Reduction of oil contamination was particularly evident at sampling times, as noticeably less oil remained on sampling equipment used on this beach plot.

At this time, all other plots looked as oiled as they did at the beginning of the field study. There were essentially no visual indications of oil removal on plots treated with slow-release fertilizer briquettes.

Over the next 2 to 3 weeks, the cleaned rectangle on the cobble beach remained clearly visible. Oil below the rocks remained but was less and less apparent and untreated reference plots appeared relatively unchanged. The oleophilic-treated mixed sand and gravel plot actually showed a greater loss of oil, appearing increasingly cleaner.

Six to eight weeks after fertilizer application, the contrast between the treated and untreated areas on the cobble beach narrowed. This was due to reoiling from subsurface material concurrent with the slow removal of oil on the beach material surrounding the plot. However, it was evident that the total amount of oil on the treated plots had decreased substantially relative to reference plots. The corresponding mixed sand and other plots still had observable oil contamination but generally less than that seen at the beginning of the study.

Toward the end of the summer, the area used for the bioremediation study became steadily cleaner, as did most of the areas surrounding the test plots. This was attributed to several storms and more frequent rainfall. A heavily contaminated area to the south that had never been treated, remained heavily contaminated.

Determinations of numbers of oil-degrading bacteria present in beach materials were made at each sampling of Snug Harbor, using all 21 sediment samples taken from each test plot to evaluate sediment chemistry. Numbers of degraders were assessed by serially diluting each sample in a minimal salts medium containing ammonium and phosphate, adding a small quantity of oil to each dilution, and incubating the dilutions for 21 days. The highest dilution series showing degradation was then scored, and a calculation was made based on dilution to extinction of the total oil degraders in the undiluted sample. Although similar in design to a single tube MPN procedure, the dilution to extinction procedure should not be mistaken for such.

Results from these studies are shown in Table 3. The values reported are the $\log_{10}$ mean and standard deviation of 18 to 21 dilution series for each mixed sand and gravel plot (no cobble beach material was analyzed). When a plot was sampled on two separate days, the results represented eight to ten dilution series per day. Table 3 has been keyed to indicate the number of determinations within a plot in which every dilution in the series was positive for oil degradation. The greater the number of positive dilutions, the greater the underestimation of the relative oil-degrading population.

Results suggested that an increase in oil-degrading microorganisms occurred within the oleophilic fertilizer-treated plots between the time zero and 9 days after application. The results from the water-soluble treatment showed the same trend, but the differences in both cases were not statistically supportable. For unexplained reasons, oil degraders increased more than 100- to 200-fold on day 31 in control and water-soluble fertilizer-treated plots.

It was concluded from the available data that an increase in oil-degrading microorganisms may have occurred as a result of fertilizer application, but it could not be statistically verified. The apparent increase in organism populations in the fertilized plots at day 9 corresponds to the high level of nutrients seen immediately following the application of nutrients. In these tests, the presence of high numbers of oil-degrading bacteria in the control beaches made it difficult to detect subtle differences in the numbers of degrading organisms between treatments.

MICROBIOLOGY OF PASSAGE COVE

Original oil contamination in Passage Cove was heavy. Following complete physical washing, oil was well distributed over most of the surface of all cobble and all gravel under the cobble. The oil was black, dry, and dull with considerable stickiness. It was spread as a thin layer over the beach material. Relatively few patches of pooled oil or mousse were present but where they were present the oil was thick and viscous. Oil also was found at depth in the beach, generally 30 to 40 cm below the surface. It was well distributed within the beach material.

Table 3. Relative Concentrations (Log_{10} of the Cell Numbers/g of Beach Material) of Oil-Degrading Microorganisms in Snug Harbor Mixed Sand and Gravel Test Plots[a]

Sampling Date[b]			Fertilizer	
Before Application	Days	Control	Water Soluble	Oleophilic
6/8/89	0	6.58 ±1.00		5.95 +/-1.29
6/11/89	1	6.16 ±0.89	5.80 ±0.91	
After Application				
6/17/89	9	>6.24*	>6.62*	>6.91**
6/24/89	16	5.96 ±0.83	5.86 ±1.15	5.96 +/-1.10
7/8/89	30	6.61 ±1.34		5.86 +/-0.67
7/9/89	31	>8.47*	>9.39**	

[a]No. of dilution series positive in all dilution tubes (0-25%); *(25-50); **(50-75).

[b]Samples on 6/8/89 and 6/11/89 are preapplication of the fertilizer.

Within approximately 2 weeks following application of oleophilic fertilizer and slow release granular fertilizer, it became apparent that the treated beach was considerably cleaner relative to the reference plots. In contrast to the observations at Snug Harbor, not only did the rock surfaces look cleaner but the oil under the rocks and on the gravel below was also disappearing. In another 2 weeks, oil could be found only in isolated patches and at 10 cm and below in the subsurface. At no time were oil slicks or oily material seen leaving the beach area. During this time, no loss of oil from the rock surfaces was apparent in the reference plot.

The beach treated with fertilizer solution from the sprinkler system behaved in a very similar manner to the oleophilic-granule-treated plot; that is, it became clean. The only difference was that it lagged behind the oleophilic/granular-treated beach by about 10 to 14 days. By the end of August, both beaches--the oleophilic and fertilizer solution treated beaches looked equally clean. In contract, the reference plot appeared very much as it did in the beginning of the field study. Oil in the subsurface still remained in all plots. In the fertilizer-treated plots, however, oil was apparent visually only below a depth of 20 to 30 cm.

The number of oil-degrading bacteria present on beach materials also was determined for Passage Cove. Samples of beach material were taken from grids 1, 3, 5, 7, 8, 10, 12, 14, 15, 17, 19, and 21. Numbers of degraders were assessed by a modification of the dilution to extinction method used for Snug Harbor. Five replicate dilution series were prepared from the initial 1:10 dilution. The relative numbers of bacteria in each sample was an average of the five replicate dilution series.

Results from these studies are shown in Table 4. The values reported are the log_{10} normal mean and standard deviation of 11-12 dilution series for each mixed sand and gravel sample. Results suggested that no consistent increase in oil-degrading microorganisms occurred as a result of fertilizer application. This means that, even in the plot treated with nutrient solutions from a sprinkler system where nutrient exposure to the bacteria should be optimized, no increase in oil-degrading microorganisms occurred. This could be the result of a relatively constant sloughing of microbial biomass from the surfaces of the beach material, perhaps as caused by tidal flushing action. Grazing by protozoans also could keep the microbial numbers at a specific density. The presence of high numbers of oil-degrading bacteria in the reference beaches made it difficult to detect subtle differences in the numbers of degrading organisms between treatments.

Table 4. Relative Concentration (Log_{10} of the cell number/g of beach material) of Oil-Degrading Microorganisms in Passage Cove.

Sampling Date		Plots Fertilizer-Treated	
Before Application	Reference	Water Soluble	Oleophilic & Water soluble
07/22/89	6.44 ±1.44	6.31 ±1.36	6.44 ±1.33
After Application			
08/06/89	5.32 ±1.12	5.78 ±1.45	5.71 ±0.67
08/19/89	6.60 ±1.83	5.47 ±1.34	5.66 ±0.35

MICROBIOLOGY OF UNCONTAMINATED BEACHES

In early August, several beaches that had not been affected by the oil spill were sampled to determine relative levels of oil-degrading microorganisms. Samples were collected from the high, mid, and low tide areas at each beach. The bacterial densities are shown in Table 5. The range in concentration of oil-degrading organisms was much greater than that observed for oil-impacted beaches. It is clear that the number of oil degraders in uncontaminated areas were 1000-100,000 times lower than in contaminated areas. Thus, the presence of oil causes a significant enrichment of oil degrading microorganisms.

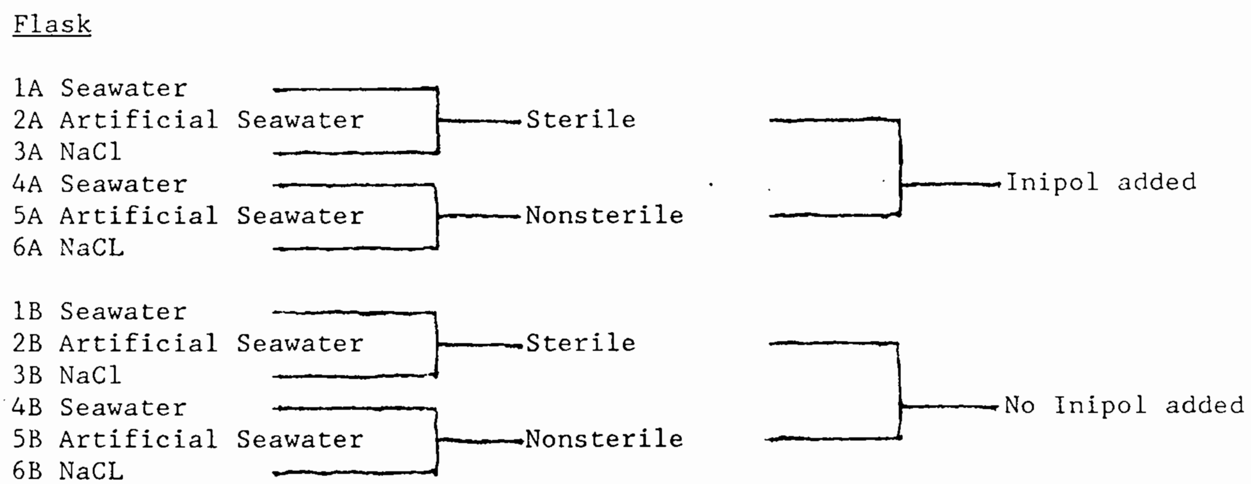
Table 5. Relative Concentration (Log_{10} of the cell numbers/g of beach material and standard deviation) of Oil-Degrading Microorganisms in Samples from Beaches That Were Not Impacted by Oil.

Site	High Tide	Mid Tide	Low Tide
Tatitlek	2.41 ± .58	4.31 ±1.14	6.11 ±2.05
Fish Bag	<1.51	<1.31	<2.71
Snug Corner Cove	2.31 ± .54	2.51 ± .55	<1.11
Hell's Hole	<2.11	2.51 ± .89	< .91
Commander Cover	4.51 ±1.14	1.31	3.11 ± .45

MECHANISM OF ACTION OF INIPOL-ENHANCED OIL DEGRADATION

A laboratory study was conducted to investigate the mechanism by which the Inipol fertilizer enhanced oil degradation. Numbers of oil-degrading microorganisms and oleic acid-degrading microorganisms were specifically examined along with changes in oil composition. The study was performed in a manner that would, to some extent, simulate environmental conditions, i.e., no shaking and daily water change to simulate tidal flushing. Results are currently available for oil-degrading and oleic acid-degrading microbial populations.

The experimental design is shown in Figure 1. Studies were conducted in chemically clean (I-Chem) jars, each containing approximately 200 g of oiled rocks and either seawater, defined nutrient medium, or sodium chloride solution (20%). The defined nutrient medium (DNM) used in these tests contained (per liter of distilled water): NaCL (24g) $MgSO_4 \cdot 7H_2O$ (1.0 g) KCL (0.7 g) KH_2PO_4 (2.0 g). Na_2HPO_4 (3.0 g), and NH_4NO_3 (1.0 g). The pH of the medium was adjusted to 7.4 with 1.0 N NaOH following autoclaving. For sterile systems, the oil-contaminated rocks were autoclaved in I-Chem jars. This removed the water from the oil, but did not remove the oil from the rocks. Inipol application consisted of dripping 3 ml of Inipol (sterile) over the rock surface and allowing the treated rocks to incubate for 3 hours before filling the jars with the appropriate aqueous phase (about 100 ml). Except for the jar containing unautoclaved seawater, sterile medium (seawater, defined nutrient medium, or NaCl solution) was used in each microcosm. Subsamples of 1.0 ml for bacterial enumeration were collected from all jars at 24-hour intervals. Oleic acid- degrading bacteria were enumerated on oleic acid-containing agar plates supplemented with nitrogen and phosphorous. Oil-degrading bacteria were enumerated by the dilution to extinction technique. After collection of bacterial enumeration samples, the aqueous phase from one set of jars was decanted into a sterile I-Chem jar and replaced with fresh sterile medium (fresh seawater was added to the nonsterile seawater jar). The decanted solution was frozen for analysis of residual oil components.



Note: Two parallel series were either incubated, as originally planned, or the aqueous phase was replaced each day.

Figure 1. Experimental Design for Laboratory Microcosm Study

The results from these studies indicated that the addition of Inipol led to a substantial increase in the number of organisms capable of growth on oleic acid-agar plates (Figure 2). High background concentrations of oleic acid-degrading bacteria were observed in the water even before Inipol treatment.

Because the aqueous phase at each water change was sterilized, the number of oleic acid degraders possibly reflected those that sloughed off the oiled rocks during a 24-hour period. However, no obvious differences were observed for the different aqueous phases. Similar results were observed in systems that did not have daily water changes.

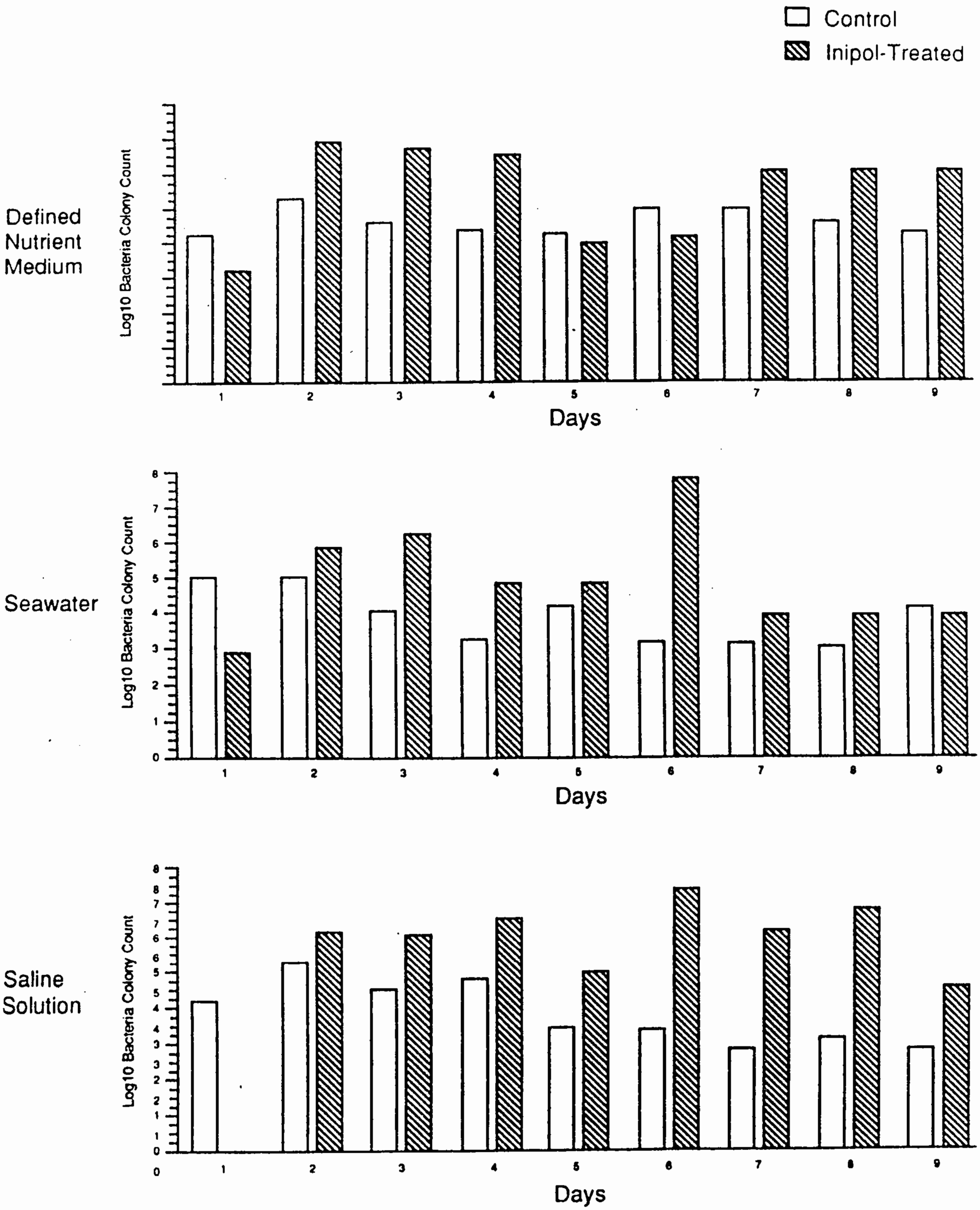
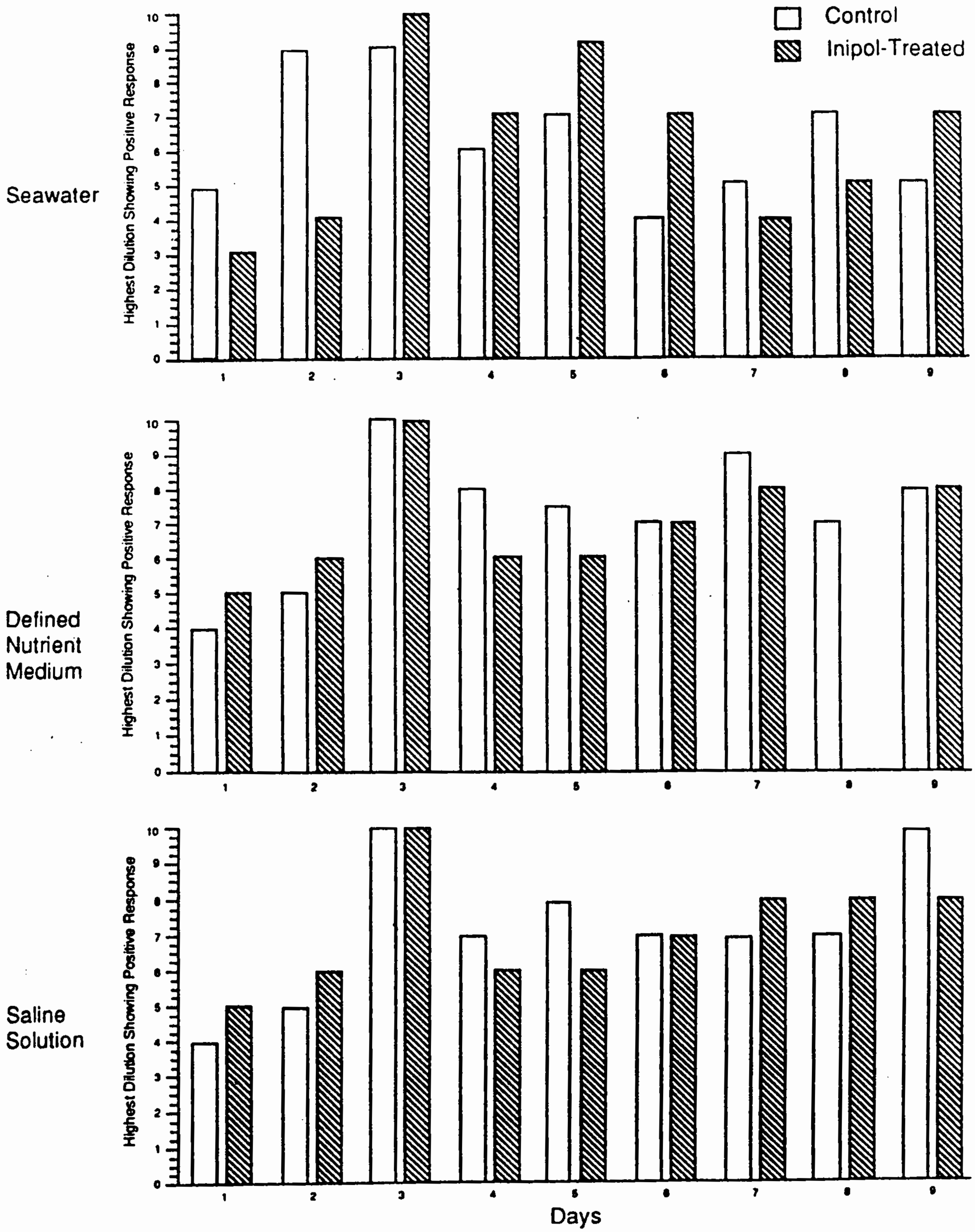


Figure 2. Effect of Inipol on the relative numbers of oleic acid-degrading bacteria in jars containing oiled rocks and seawater, defined nutrient medium, or saline solution. Incubated with daily change of water.

Results from the enumeration of oil-degrading organisms (Figure 3) indicated that, in all cases, the populations increased to a high value by day 3 and then decreased to an intermediate but variable level for the following 6 days. Similar results were seen in those jars that did not have a daily water change. Although all the samples showed a peak after 3 days of incubation, jars containing only seawater appeared to have the fewest microorganisms in the 6 days following the 3-day peak. Chemical analysis of the water samples is being performed. Information on how effectively the enriched oleic acid degraders can degrade the oil also is forthcoming.

Inipol increased the number of oleic acid-degrading bacteria in flask studies designed to approximate field conditions. This situation would theoretically result in competition for available nutrients between oleic acid-degrading and oil-degrading bacteria. This competition could explain the decrease in oil-degrading bacteria following their initial rise after initiation of the experiment. Tests of oleic acid-degrading bacteria currently are being conducted to determine the percentage that are also hydrocarbon degraders. Supplying dissolved nutrients in addition to those nutrients in Inipol did not seem to affect the oleic acid- and oil-degrading bacterial populations.



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16. ABSTRACT

The U.S. Environmental Protection Agency's Alaskan Bioremediation Project was initiated in the aftermath of the March 24, 1989, EXXON VALDEZ oil spill. The objective of the project was to demonstrate an alternative cleanup method for oil-contaminated shorelines based on enhancing natural biodegradation of the oil through the addition of nitrogen and phosphorus nutrients. This enhancement process is a well-recognized and scientifically sound approach to bioremediation but had never been tested on a large scale in marine environments. The project was managed by EPA's Office of Research and Development with financial, scientific, and logistical support from the Exxon Company USA under the authority of the Federal Technology Transfer Act. This paper covers the microbial aspects of the EPA field study in Prince William Sound, Alaska.

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