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Interactions between Marine Bacteria and Dissolved-Phase and Beached Hydrocarbons after the *Exxon Valdez* Oil Spill

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Turnover times for toluene in Resurrection Bay after the *Exxon Valdez* grounding were determined to be decades, longer than expected considering that dissolved hydrocarbons were anticipated to drift with the current and stimulate development of additional hydrocarbon-utilizing capacity among the microflora in that downcurrent location. These turnover times were based on the recovery of $^{14}CO_2$ from added [^{14}C]toluene that was oxidized. The concentrations of toluene there, 0.1 to 0.2 μg/liter, were similar to prespill values. Oxidation rates appeared to be enhanced upstream near islands in the wake of the wind-blown slick, and even more within the slick itself. Specific affinities of the water column bacteria for toluene were computed with the help of biomass data, as measured by high-resolution flow cytometry. They were a very low 0.3 to 3 liters/g of cells · h^{-1}, indicating limited capacity to utilize this hydrocarbon. Since current-driven mixing rates exceeded those of oxidation, dissolved spill components such as toluene should enter the world-ocean pool of hydrocarbons rather than biooxidize in place. Some of the floating oil slick washed ashore and permeated a coarse gravel beach. A bacterial biomass of 2 to 14 mg/kg appeared in apparent response to the new carbon and energy source. This biomass was computed from that of the organisms and associated naphthalene oxidation activity washed from the gravel compared with the original suspension. These sediment organisms were very small at ≈0.06 μm^3 in volume, low in DNA at ≈5.5 g per cell, and unlike the aquatic bacteria obtained by enrichment culture but quite similar to the oligobacteria in the water column. Activity toward hydrocarbons was moderate, with specific affinities for naphthalene of 26 to 92 liters/g of cells/h (V_{max}, 0.36 μg/g of cells · h^{-1}; K_m, 1.4 μg/liter), giving residence times of only a few hours. A large population of carbon- and energy-starved, induced hydrocarbon oxidizers with metabolism limited by the physical and molecular recalcitrance of the heavier components is suggested. These are factors that should be addressed in bioremediation efforts. The effects of a surfactant that was widely applied were unremarkable on a test beach after 1.5 months. Unresolved components appearing in chromatograms from the remaining mixture were characteristic of partial oxidation products. Such compounds, known to accumulate when concentrations of smaller aqueous-phase hydrocarbons exceed the K_m, may form in sediments as well.

Hydrocarbons enter the ocean in large amounts (Table 1). Concerns include the fouling of birds and mammals, production of abnormal histology in fishes (27), and the esthetic quality of beaches. Most of these hydrocarbons are biodegraded by bacteria, yet the rates and the organisms responsible are poorly understood. On 24 March 1989, the oil tanker *Exxon Valdez* ran aground near Bligh Island (Fig. 1) and released a large quantity of Prudhoe Bay crude oil. Such spills coalesce into a lumpy emulsion or mousse within a few days due to evaporation, dissolution, wind mixing, and microbiological processing, and some of the hydrocarbons enter the water column (25). We had previously examined hydrocarbon oxidizer populations (9), oxidation rates (10), and concentrations in the region (see below) in a search for terpene hydrocarbons. These were located (8), as predicted from kinetics of toluene (12) and terpene (5) oxidation, and presumably arose from coastal conifers and moved into the region with the current. We therefore examined the microflora downstream from Bligh Island in anticipation of a similar response.

The toluene oxidation rate was again used as an indication of hydrocarbon-oxidizing organisms in the water column. At 0.6% (39) by volume, toluene is the most abundant hydrocarbon in Prudhoe Bay crude. It is quite soluble at 0.55 g/liter (4), is on the U.S. Environmental Protection Agency priority pollutant list, and is metabolized by well-studied pathways (42). The surface water of Resurrection Bay is normally about 10 days downstream from the spill along the Alaska Coastal Current (37), and we began measuring toluene oxidation there.

The wind-driven (26) oil slick moved southwest at about 20 km/day (17), became discontinuous when not replenished by fresh oil from beach flushings, formed walnut- and smaller-sized tar balls at and near the surface, and was visible 660 km southwest after 2 months. Some beaches were blackened, and the surface of those sprayed with emulsifiers appeared lighter in color. As a result, 120 km of beaches, according to U.S. Coast Guard public information, were sprayed with 230 tons of surfactants and 40 tons of a granular nitrogen-phosphate fertilizer. We examined several beaches, including one which was treated in an agency test program (29), and measured the populations of bacteria, their size distributions and DNA contents, concentrations of hydrocarbons remaining, and the degradation rates of added [^{14}C]naphthalene to characterize responses to the oil. The naphthalene was used to indicate hydrocarbon oxidation in the beaches because, having only 1% of the vapor pressure of toluene (41), some persisted with the oil, and with a solubility of 115 mg/liter (4), sufficient quantities enter the aqueous phase for kinetic studies.

The purpose of this communication is to report changes in

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TABLE 1. Recent oil spills

Event	Place	Date	10^3 tons of oil	Reference
Torrey Canyon	Cornwall, England, and France	March 1967	117	39
Ekofisk	North Sea	April 1977	25	39
Amoco *Cadiz*	Brittany, France	March 1978	223	39
IXTOC I well	Campeche Bay, Mexico	June 1979	350	39
Exxon Valdez	Gulf of Alaska	April 1989	33	17
Iraq/Kuwait	Persian Gulf	February 1991	1,000	21
All[a]	World ocean		15,000	39

[a] Annual average.

the characteristics of the indigenous bacteria and associated rates of hydrocarbon oxidation in response to an oil spill, improve understanding of hydrocarbon persistence in the environment, introduce novel methods for evaluating bioremediation procedures, and demonstrate the utility of high-resolution flow cytometry in environmental studies of processes involving heterotrophic bacteria.

MATERIALS AND METHODS

Sampling. Most surfacewater samples were collected at the forward-moving end of a rowed boat or drifting float plane with a glass carafe and transferred into a 20-liter fired carboy. Subsurface samples were raised in a washed and exhaustively rinsed Niskin bottle and pooled in the same way. Locations were downcurrent from the spill (Resurrection Bay), in and adjacent to the wake of the moving slick, and within the slick itself (Fig. 1). Beach samples were collected at Snug Harbor from plots established by the Environmental Protection Agency (28). One test plot (12 by 21 m) had been treated with Inipol EAP 22, a mixture of oleic acid, tri(laureth-4)-phosphate, 2-butoxy-1-ethanol, and urea (27a), at a rate of 225 g/m^2 (two passes with a sprayer over 9 days) in conjunction with an ongoing study (29) 2.5 months following the spill. After 45 days, a 2-kg sample was pooled from material at 5-cm depth from beaches of rather well sorted fine gravel and coarse sand which appeared to be evenly coated with oil. Another was taken from a row of heavily oiled cobble at the high-tide line. Non-oil-coated controls were from Resurrection Bay and included a gravel beach like that sampled at Snug Harbor, but because activity was undetectable in the gravel washwater, fine sediment from a stable tide flat beach was also used.

Remote samples were returned by float plane, and those from the oil slick were returned by boat. Water samples were processed at our Marine Station in Seward within 4 h; the beach samples were maintained at beach temperature, flushed once with seawater to simulate the tide, and processed within 12 h.

Oxidation rates. Water column samples of 5 to 20 liters were amended with 0.9 ± 0.2 μg of [^{14}C]toluene per liter, incubated for 6 to 12 h at 10°C, and occasionally shaken by hand; 1-liter subsamples were collected, and the $^{14}CO_2$ was stripped, purified, and quantitated as described previously (13). Values for microbial activity toward hydrocarbons were based on total bacterial biomass present, as determined by flow cytometry.

For sediments, 400 g of beach material was amended with 0.2-μm-filtered seawater to 500 ml; the mixture was shaken and allowed to settle, and the supernatant was decanted. A portion of the aqueous fraction was preserved for flow cytometry. The remainder was amended with [^{14}C]naphtha-

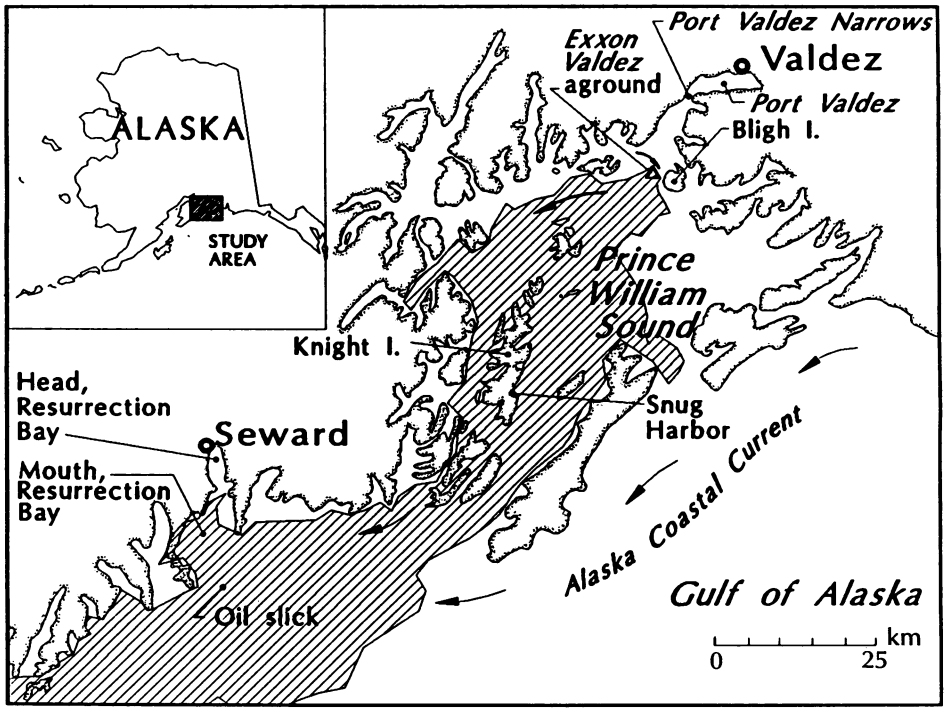

FIG. 1. Location of the *Exxon Valdez* grounding, area covered by oil slick after 13 days (hatched area), flow of the Alaska Coastal Current (curved arrows), and sampling points.

lene at 10 to 35 μg/liter and incubated at 10°C with gentle shaking, and $^{14}CO_2$ evolution from the subsamples was measured over time as above. Remaining gravel was amended with another 500 ml of seawater and with 30 μg of [^{14}C]naphthalene, mixed vigorously for 30 min, and incubated and subsampled as above.

Bacteria. Bacterial characteristics were determined by high-resolution flow cytometry. Samples were analyzed as described previously (32) except that they were preserved with formaldehyde at 0.5%, stored cold, permeabilized with Triton X-100 at 0.1%, and stained with 4'6-diamidino-2-phenylindole (DAPI) at 0.5 μg/ml and 10°C for 1 h before analysis. Size was determined by forward light scatter (35). DNA content was measured by DAPI-DNA fluorescence by comparison with *Escherichia coli* (34) treated with rifampin (40) to produce integral numbers of genome replicates. Resulting standard curves were linear (correlation coefficient of 0.999) for between two and eight genome copies (34). Population values were based on an internal standard of latex spheres independently quantitated by Coulter counter. Biomass was computed from population and mean cell volume obtained from forward-scatter histograms, assuming a cell density of 1.04. Biomass in the sediment was estimated from that in the washwater and the relative rates of naphthalene oxidation in both fractions, assuming that the distribution of organism types was similar in both and that the rate was saturated and maximal at V_{max}.

Hydrocarbons. Water column hydrocarbons were measured by the strip-and-purge method in 10-liter volumes as reported previously (22), with deuterotoluene and 6-methylhept-5-en-2-one as internal standards. The sediment hydrocarbon standard was 8-[^{2}H]naphthalene. Total hydrocarbons from sediments were determined from integrated areas of gas chromatographic peaks derived from methylene chloride-methanol Soxhlet extracts of beach material. Recoveries of 50% were obtained from weighed samples and used to estimate levels present. Naphthalene values were from gas chromatography-mass spectrometry data for methylene chloride-methanol extracts, with an internal standard of [^{2}H]naphthalene. Samples were processed in a well-ventilated room at seaside and returned to F. Jüttner's laboratory or to Fairbanks for analysis.

RESULTS

Water column activity. Toluene oxidation in fresh seawater was linear over 6 to 10 h, as shown in Fig. 2. The subsequent increase in rate is normal. It can be ascribed to bottle effect (40%/day) (9) and enzyme induction, known to occur after a few hours at added concentrations (33), and indicates the value of rapid sample processing. The rate at 10°C was 2.1 times faster than that at 3°C, the prevailing surface water temperature. This is a normal increase in rate with temperature, as indicated by the corresponding Arrhenius activation energy of 16.3 kcal/mol. Other measurements were made at 10°C to normalize bacterial activity measurements to temperature.

Bacterial populations, according to flow cytometry, are indicated by the bivariate histograms of Fig. 3. Water column organisms are indicated in Fig. 3A and C, and a typical hydrocarbon enrichment marine isolate, *Pseudomonas* sp. strain T2 (33), is shown in Fig. 3B for comparison. Due to its much larger size, 0.2 to 1.5 μm^3, it remains mostly off scale even on this logarithmic plot, emphasizing the small size of typical hydrocarbon oxidizers compared with the bacteria usually studied. DNA content is small as well;

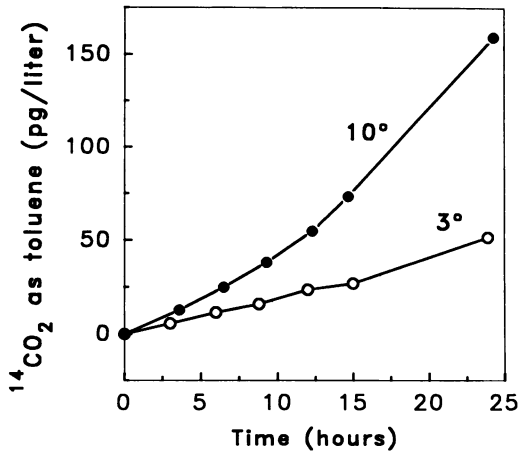

FIG. 2. Rate curves for toluene oxidation. Sample was taken from the 3°C water at the head of Resurrection Bay, and portions were incubated at either 3 or 10°C. Incubation time was extended to show the increase in rate after 6 h at 10°C.

growing *E. coli* and strain T2 normally have DNA contents of about 8 fg per cell, compared with the ≈5 fg per cell shown in Table 4. The characteristics of the bacteria in hydrocarbon-impacted sediments are shown in Fig. 3D. The large subpopulation of small, weakly fluorescent particles that appears in the sediment sample is mostly below the cutoff values of 0.03 μm^3 and 2 fg of DNA per cell usually indicating bacteria. The location of the bacterial peak has been confirmed in other samples by epifluorescence counts, which agree with flow cytometry signals from the assigned region, with sorting by flow cytometry and subsequent microscopic examination, and by sorting samples previously exposed to radiolabeled amino acids and determining radioactivity, where the smallest fraction was found to be particularly active (unpublished data). Other experiments showed that, unlike phytoplankton, natural and cultured bacteria could be preserved with little change in cell size, DNA content, or population (34).

Rates of toluene oxidation to CO_2 in surface water at the head of Resurrection Bay between 10 and 20 days following the spill are shown in Table 2. Values did not increase significantly with time (Spearman rank-order correlation coefficient: $r = 0.6$, $P = 0.3$) and amount to an average residence time of 50 years (Table 3), similar to the residence times measured previously. Bacterial biomass also remained constant over the period, ranged from 28 to 36 μg/liter, and correlated with rate ($r = 0.9$, $P = 0.09$). Data are from single analyses of the pooled sample; for triplicate subsamples from a single cast, the standard deviation was ±4.5% for the population, ±15% for cell volume, ±3.8% for DNA content (see below), and ±12% for total biomass.

The concentrations of toluene (Table 4), one of numerous water column hydrocarbons (Fig. 4), were also no different than those measured earlier (Table 5). Thus, the anticipated response in Resurrection Bay to the spill was not reflected in either toluene concentrations or oxidation rates. Rates in surface water from Port Valdez were unchanged from pre-spill values as well. Port Valdez lies north of the usual current flow from Bligh Island (Fig. 1) and is the site of oil tanker loading. Ballast water-derived bacteria discharged from the tankers through a treatment plant are responsible for the excessive activity at 50 m prior to the spill (12).

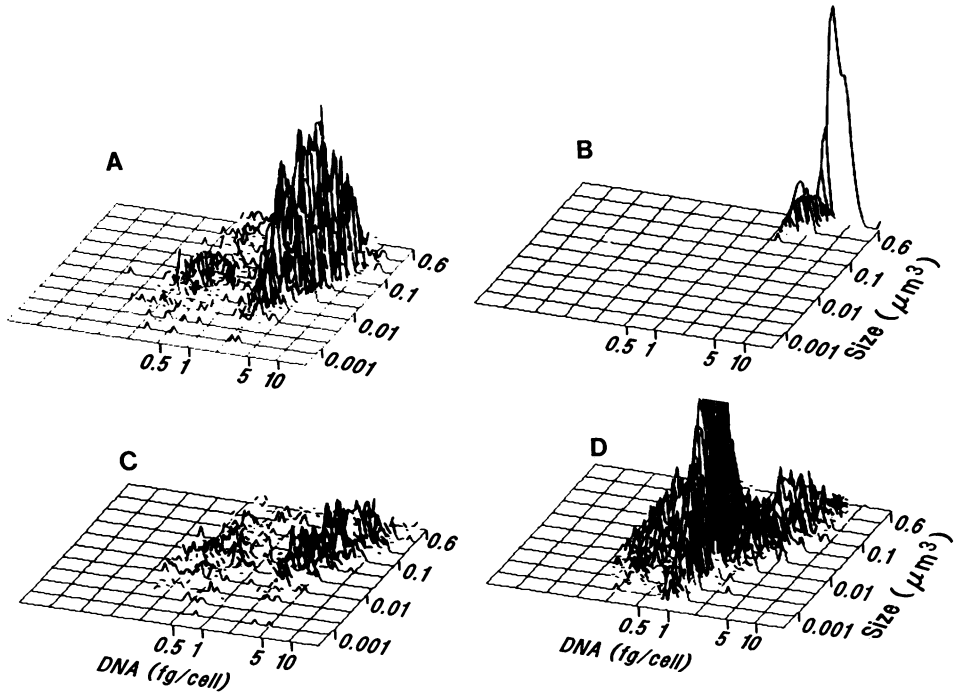

FIG. 3. Unedited bivariate histograms showing size distributions and DNA contents of seawater and sediment. (A) Surface water from the head of Resurrection Bay. (B) Culture of a typical marine bacterial isolate, *Pseudomonas* sp. strain T2, isolated by enrichment culture and having a mean cell size of 1.2 μm^3 (1,150 fg) and 12 fg of DNA; instrument settings were the same as used for panels A, C, and D, placing most cells off the scale. (C) Organisms from within the oil slick off Resurrection Bay. (D) Washwater of oiled gravel from Knight Island; signal below 0.02 μm^3 in size, shown here as a truncated peak and resolved more clearly in contour plots, was taken as mineral-associated nonbacterial background.

Toluene oxidation was also measured in relatively open water near Knight Island, where large amounts of floating oil were beached. Although values were similar to those in Resurrection Bay, they were at least sixfold higher than prespill values, rates which were below our 0.5 pg/liter · h^{-1} detection limits (10, 12). Twelve days following the spill, rates 5 km outside Resurrection Bay in open water of the Gulf of Alaska were also unusually large (Table 4); biomass and toluene concentrations were more like estuarine than open-water values and were probably enhanced by either current-driven dissolved hydrocarbons from the initial spill for which we were searching or by fringes of the wind-driven slick.

In the slick itself, organism size, DNA content, and total biomass remained normal, but rates of toluene oxidation were higher; like benzene in the subsurface IXTOC blowout (25), toluene appeared to remain in substantial quantities (Table 4), but some could have been extracted from microdroplets of oil. The concentrations measured exceeded K_m, the 0.5 to 2 μg/liter Michaelis constant for toluene oxidation by mixed microflora (10), and could have been sufficient for a reduction in the increase in rate with concentration due to enzymatic saturation.

Kinetics. K_m is often taken as an index of microbial affinity for a substrate, but specific affinity (a_A°) is the preferred term (18) because it is not normalized to maximal rate and specifies the value of the substrate flux. It is defined by the initial slope of a specific uptake rate-versus-concentration curve (11) and, according to janusian kinetics (7), defined somewhat by the amount of cytoplasmic and mostly by the amount of membrane-bound transporters. When substrate concentration (A) is below the K_m, saturation can be ne-

TABLE 2. Oxidation rate of added [^{14}C]toluene in Resurrection Bay, normalized to 0.9 μg of toluene per liter, between 10 and 20 days following the spill

Date in April 1989	Biomass (μg/liter)	Rate[a] (pg/liter · h^{-1}) ± SE
3	30.9	3.5 ± 0.9
4	28.2	1.2 ± 0.4
5	32.6	7.7 ± 0.4
9	31.5	4.5 ± 1.3
13	36.0	7.1 ± 0.4

[a] The standard error among subsamples is shown.

TABLE 3. Prespill toluene oxidation rates, given as residence times assuming a 50% yield of carbon dioxide from toluene

Location	Date (mo/yr)	Depth (m)	Temp (°C)	Residence time (yr)	Reference
Resurrection Bay, Alaska	5/89	0	3	50	This study
	5/89	0	10	24	This study
	10/81	0	10	>200	10
Aransas Bay, Texas	5/81	5	17	0.33	10
Valdez, Alaska	5/80	5	10	22	13
	5/80	50	10	>0.003	13
Valdez Narrows, Alaska	5/80	5	13	>200	13

TABLE 4. Characteristics and activity of water and contained organisms toward toluene from various locations in the Gulf of Alaska[a]

Location	Rate (pg of toluene/liter · h^{-1})	Population (10^8/liter)	Cell size (μm^3/cell)	DNA (fg/cell)	Biomass (μg/liter)	Toluene (μg/liter)	Specific affinity (liters/g of cells · h^{-1})	Residence time (yr)
Head, Resurrection Bay	4.8 ± 2.6	6.5	0.040	4.5 ± 0.9	31	0.17	0.32 ± 0.14	13
Port Valdez	6.8 ± 0.09	8.6	0.037	4.9	33	ND[b]	2.6	9.3
Near Knight Island	3.1 ± 0.03	7.1	0.057	5.6	42	0.17	0.07	20
Gulf, off Resurrection Bay	2.1 ± 0.04	5.2	0.061	5.5	33	0.23	0.11	30
Gulf, in oil slick	6.7 ± 0.42	2.4	0.064	5.5	16	36	3.3	3.5
5 m under oil slick	1.6 ± 0.11	3.2	0.053	5.4	17	32	0.83	3.5

[a] Standard deviations from the mean of five independent determinations of rate, DNA, and specific affinity are shown for Resurrection Bay; standard errors for other rates show variance among subsamples.
[b] ND, not determined.

glected and a_A° can be computed from uptake rate (v), biomass (X), and the rate equation

$$v = a_A^\circ XA \quad (1)$$

The first four specific affinities of Table 4 were calculated from isotope distributions and equation 1. Ambient toluene was assumed to be insufficient to cause enzymatic saturation, and errors in A and v cancel (12). Saturation should have been negligible away from the slick but could reduce apparent specific affinities near it. For the last two values (slick associated), where saturation may have been significant, specific affinities were calculated from

$$V_{max} = a_A^\circ K_m \quad (2)$$

where V_{max} is the maximal rate of uptake. Assumptions were that all the toluene measured was available (not sequestered in oil particles), K_m was 1.5 μg/liter (10), and the observed flux was maximal (V_{max}) for the bacteria present. Values from within the slick but calculated from equation 1 were 0.9 and 0.2 liters/g of cells · h^{-1}, similar (i.e., within a factor of 3) to those calculated from equation 2 and shown in Table 4, so uncertainties about the true dissolved toluene concentration had a limited effect on computed microbial activity and hydrocarbon residence times. These oxidation rates were 5- to 100-fold above the detection level. Prespill oxidation rates that were undetectable in open water (Table 3), together with a general homogeneity in marine bacterial population densities, led to an expectation that, without an unusual hydrocarbon input, rates should have been undetectable. Data therefore suggest enhancement due to the spill of ≥100-fold.

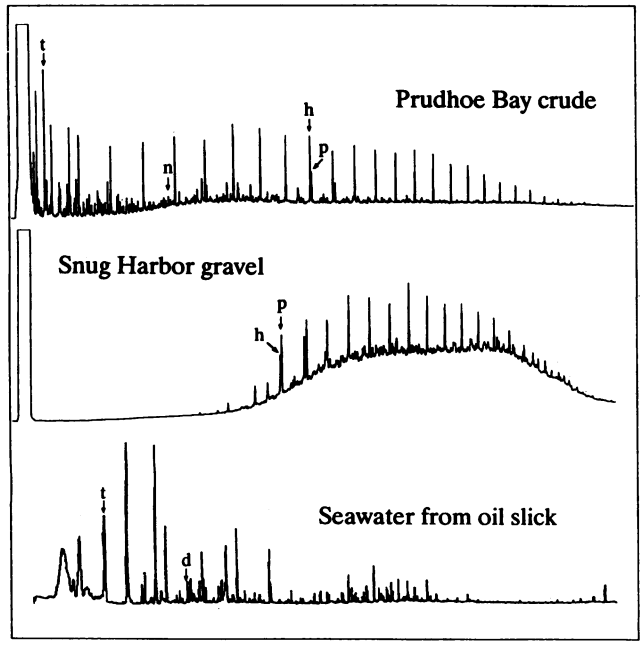

FIG. 4. Chromatograms of fresh Prudhoe Bay crude oil of the type carried in the *Exxon Valdez*, from oiled beach gravel on Knight Island at Snug Harbor, and from seawater in the oil-slick area 13 days following the spill. Symbols: t, toluene; n, naphthalene; h, heptadecane; d, *n*-decane; p, pristane.

Sediments. Most of the beaches affected by floating oil are of gravel or cobble (≈3-cm stones), highly permeable, often moistened by rain, and subject to vigorous (4-m amplitude) tidal flushing. Knight Island (Fig. 1) had received a moderate coating of oil. We examined the beaches of Snug Harbor 5 months following the spill and 2.5 months following Inipol treatment. Cobble at the high-tide line was heavily covered with brown material with the consistency of Vaseline. The oil coating the gravel was thinner, black, and appeared to be rather uniformly distributed, perhaps due to successive recoating by tides. Coverage was less uniform on nearby rocky areas, where bands of pliable black material several centimeters long remained.

Recovered hydrocarbons were in the grams-per-kilogram range, and most were larger than C_{16} (Fig. 4, center, and Table 6). They reflect microbiological processing by a reduction of the ratio of C_{17}-*n*-hydrocarbon to the more recalcitrant pristane (1) in the original crude (Fig. 4, top), consistent with the data of Pritchard and Costa (29), by the emergence of an unresolved envelope (compare top and middle chromatograms) (19), and by reduction of the more volatile and soluble components of less than C_{16}. Components in this size range on the heavily oiled cobble were more abundant than they were on the other sediments.

The rate of $^{14}CO_2$ evolution from gravel washwater increased with added naphthalene concentration in a hyperbolic manner, giving a K_m of 1.4 μg/liter (specific affinity of 26 liters/g of cells/h and V_{max} of 0.36 μg/g of cells/h). Analytical values for naphthalene are given in Table 6. Since these values exceeded the K_m and some naphthalene gradually disappeared into the particulate phase (at a rate of 5% per 16 h, according to aqueous-phase total radioactivity), we

TABLE 5. Prespill hydrocarbon concentrations for Resurrection Bay for 1987

Depth (m)	Concn (ng/liter)				
	Toluene	*m*-Xylene	Mesitylene	Hemellitene	Naphthalene
0	129	55	3.8	4.6	4.8
5	126	41	3.2	3.9	5.2
10	67	35	4.0	6.6	5.1

TABLE 6. Characteristics of bacteria in beach material and their activity toward naphthalene

Material	Hydrocarbons recovered (mg/kg)	Naphthalene			Bacteria				Specific affinity (liters/g of cells · h^{-1})
		Ambient (μg/kg)	$^{14}CO_2$ produced[a]		Population (10^6/ml)	Vol (μm^3/cell)	DNA (fg/cell)	Biomass (μg/kg)	
			ng/liter/h	ng/kg of solids · h^{-1}					
Clean									
Gravel	0.4	0.10	0.2	1.0				>340	
Gravel washwater		0.89	<0.01		0.13	0.11	1.9	3.4	<2.1
Sediment	7.8	5.2	0.17	0.9				900	
Sediment washwater		0.92	0.01		2.5	0.063	4.1	164	0.04
Oil impregnated									
Cobble	2,080	10	94.6	236				2,300	
Cobble washwater		16	30.2		5.1	0.15	4.0	822	26
Gravel	720	6	723	1,810				14,000	
Gravel washwater		21	3.5		0.4	0.065	4.9	27	92
Oil impregnated and treated[b]									
Gravel	640	3	600	1,500				11,500	
Gravel washwater		18	1.3		0.1	0.10	3.2	10	75

[a] Reported as naphthalene
[b] Treated with oleophilic mixture.

assumed incomplete partitioning between the dissolved and oil phases, and computed naphthalene oxidation rates from the amount added. These rates are given in Table 6 together with characteristics of the responsible organisms in and on the sediments and of the organisms washed from them.

Organism size, usually <0.1 μm^3, was small, like that of the oligobacteria of the water column (7), and different from the 1-μm^3 organisms developing in culture media (Fig. 3). DNA content was low as well, about half that of the organisms usually grown and equivalent to only approximately a single genome copy in *E. coli* DH1 of 4.16 fg (20). The method used for DNA analysis, DAPI fluorescence of single cells by flow cytometry, has uncertainties. It assumes a 50% AT/GC ratio in the cells, and despite excellent linearity of the standard curve and good stability of marine bacterial fluorescence, there can be some drift in *E. coli* fluorescence over time.

The biomass shown for the washwater was computed directly from flow cytometry data and reflects those pelagic bacteria that are <1 μm^3 in size. That in the sediments reflects activity toward naphthalene measured in the sediments. Specific affinities shown were computed for bacteria in the washwater and quantify the ability of the organisms washed from the sediment to accumulate naphthalene. Values were computed from equation 2 and again with the assumptions that all bacteria washed from the sediment remained in the aqueous phase and that $v = V_{max}$.

DISCUSSION

Values for population, cell size, and DNA content of the bacteria, both in the water column and in beach sediment samples, were quite instructive. Measurements of populations by flow cytometry are more convenient than microscopic direct counts and less exclusive than plate count or most-probable-number techniques, allow computation of microbial biomass, and provide a base for direct evaluation of bacterial activity and nutrition. For example, it is generally observed that cell size decreases with the severity of carbon and energy limitation in mineral-sufficient systems (35), with decreasing phosphate concentration in carbon-sufficient systems (31), and with DNA content over a range of growth rates (15). Population measurement by flow cytometry is generally similar to that by epifluorescence microscopy if one excludes the very small signals which are thought to be viruses and counts attached groups as single cells. These measurements are reproducible to at least 5%. False signals from organism-contaminated reagents are also more easily located. Some sediments generate substantial background, as shown at the lower left of Fig. 3D, but it appears below the signals from marine bacteria (note the logarithmic scaling). Such background also results from brown lakewater but not from fine marine sediments or clear lake and seawater. Any overlap could increase apparent populations, reduce apparent cell size and DNA content, and affect specific affinities. Upper values as well as mean values indicate that the cells in the sediment are much smaller than cultured bacteria (Fig. 3), so background-generated errors are not responsible for the small sizes and DNA contents reported for the organisms in the sediment. Background problems are generally absent for water column samples from the ocean.

The hypothesized increase in microbial activity with time in Resurrection Bay due to current-borne hydrocarbons from the oil spill was not detected. Light rainfall over Southeast Alaska during the spring of 1989 caused an unusual dissipation of the Alaska Coastal Current (37). Such a breakdown in normal current flow could explain our failure to observe a signal in microbial activity toward toluene. Enhancement of hydrocarbon oxidizer activity was probably significant in the region of the oil slick and perhaps near Knight Island, where toluene oxidation rates, particularly during a season of low coastal runoff, were anticipated to be undetectable. We previously measured an induction constant, concentration at half-maximal induction rate, of 1.9 ± 0.06 μg of toluene per liter in the area (9); based on toluene analyses, therefore, some increase in specific affinity for toluene would be expected. The small standard error indicated a lack of random variability among independent rate determinations.

Specific affinities for the water column bacteria were comparatively low for marine bacteria in general and hydrocarbon oxidizers in particular. Values of 1,000 liters/g of cells/h or more are common for various substrates, including hydrocarbons (6, 33). They specify the contribution of a particular substrate to the rate of growth, μ_A: $\mu_A = a_A^\circ AY$,

where Y is cell yield from substrate consumed. The data from Resurrection Bay show that toluene contributes 3×10^{-8}/h to specific growth rate, and assuming that $v = V_{max}$ in the oil, the contribution in the oil slick was 2×10^{-7}/h. The sum of all carbon sources, including other hydrocarbons, should give approximately 0.1/h (24), so toluene was only a minor substrate. Measurements in continuous cultures showed that disturbances can significantly reduce specific affinities (31), which emphasizes the need for well-developed procedures to obtain accurate as well as reproducible kinetic data.

With these low affinities, oxidation rates of water column toluene were slow. Circulation times are about a year in Prince Williams Sound, 60 years in the Gulf of Alaska, and 600 years through the deep ocean (17). Most of the residence times (A/v) measured here were decades or centuries (Table 3). Bacterial populations affect residence times (equation 1), so deepwater populations of only about 10^7/liter (3), together with small inducing concentrations of hydrocarbons, could increase residence times by 10^2 or more. Since these residence times greatly exceed oceanic mixing times, dissolved hydrocarbons have global influence.

Hydrocarbon metabolism rates in oiled gravel can greatly exceed those in the water column because of higher concentrations of both bacteria and hydrocarbons. Activity toward naphthalene due to the added oil (clean beach versus oiled gravel, Table 6) was enhanced more than 1,000-fold, and the increase in affinity was more than 50-fold. Biomass washed from oiled gravel and measured directly by flow cytometry was nearly 10 times that washed from the control, and additional organisms would be expected to remain with the oiled gravel. Even so, the increase in computed biomass on the basis of hydrocarbon oxidation rates approached 40-fold. That the change in activity over controls exceeded the change in biomass is consistent with either induction or selection of a population rich in hydrocarbon oxidizers. Compared with total beach material, this population amounted to 2 to 14 mg/kg of the untreated oiled gravel and was a relatively large 0.1 to 2% of the weight of the oil present. Enhancement estimates are lower limits because activity in the control beach gravel washwater was undetectable. These biomass measurements include assumptions that all the bacteria in the aqueous phase were pelagic and responsible for all the oxidation, that the activity of organisms in oil on the sediment was the same as that of those washed from it, and that hydrocarbon oxidizers within oil on the sediment were not shielded from access to the added naphthalene by the oil. Apparent specific affinities were high enough to classify all the organisms from oiled gravel as hydrocarbon oxidizers. These affinities could be inflated by exclusion of some oil phase bacteria in wash water by the flow cytometric measurements but not the naphthalene oxidation rate determinations. This biomass of approximately 10 mg/kg, less on cobble due to smaller surface area, is thought to be composed mostly of hydrocarbon-oxidizing bacteria.

There was substantial depletion of naphthalene in the sediments, both as measured and according to the hyperbolic kinetics together with the low K_m observed. That analytical naphthalene exceeded the K_m indicated that induction was probably complete, and the small value of K_m indicated that the organisms were operating at a near-maximal rate. However, hyperbolic kinetics in a homogeneous environment are inconsistent with ambient concentrations that exceed the K_m. There may have been incomplete partitioning of the ambient naphthalene into the wash water, where bacteria acted mostly on added [^{14}C]naphthalene, consistent with the fact that some analytical naphthalene remained in the sediments after 5 months. This phenomenon would cause an additional underestimate of naphthalene oxidizer biomass in the sediment. These concentrations were sufficient for the organisms present to metabolize it at near maximal rates, but only because the K_m was expectedly small, and even then concentrations were near the point of rate limitation. These methods are new and give first estimates of bacterial biomass in oiled sediments, as far as we know.

Treatment of beaches with the organophosphate-surfactant-urea mixture has been credited for an increase in culturable hydrocarbon oxidizers of 2 orders of magnitude (14), and significant cleanup of a beach treated both with this mixture and a slow-release nitrogen-phosphorous source after 5 to 10 months has been reported (28), but data were not shown. We observed no change in visible or analytical oil or microbial populations on the treated gravel compared with the oiled control. Undoubtedly some stimulation of biodegradation occurred, but effects from a single treatment were undramatic. Inipol is composed of 50% saturated hydrocarbon with a C/N/P ratio of 62:5:1 and was used in amounts sufficient to produce a hydrocarbon oxidizer biomass of approximately 1 g/dm^2 or 500 mg/kg of gravel in the absence of oil, compared with the 2 to 14 mg/kg already present. Other fertilizers used contain biodegradable hydrocarbons as well (28). Perhaps treatment-derived hydrocarbons supplement near-surface hydrocarbon oxidizer populations, which generate emulsifiers that enhance activity before being flushed away.

The naturally developed population should, at a specific growth rate of 0.2 h^{-1} and constant population, consume all the oil on the gravel in 5 days; however, substantial quantities remain after 2 years. Something other than petroleum carbon therefore limits growth. This is generally presumed to be either minerals or recalcitrant molecular structure (36). Nitrogen and phosphorus are therefore added as a main component during bioremediation by fertilization. However, they are difficult to retain on flushed or rocky beaches, and supply to organisms in the oil is thought to be reduced by low permeability of oil to the ions (23). In any case, kinetic studies indicate that bacteria, which have higher specific affinities for minerals than algae have (6), should not be N and P limited except where prevented from regular flushing by seawater.

Good candidates for biomass limitation include resistance of the oil to penetration by hydrophilic nutrients as well as metabolic recalcitrance of remaining hydrocarbons (2). That carbon limits growth is suggested by the very small cell size observed, because carbon is the chief component of bacterial biomass and bacteria are easily miniaturized by restricting it; limitation by minerals such as phosphate increases cell size, as mentioned above. Accordingly, the organisms were larger on the cobble, where a larger complement of light aliphatics remained. Inherent limitation by hydrocarbons as a poorly utilized class of nutrients is not suggested, however, since large, fast-growing organisms have been obtained from hydrocarbon media in numerous studies. Thus, the data here suggest that bioremediation mixtures for weathered oil should include a metabolically labile, mobile, water-soluble hydrocarbon that will partition into the remaining residue, decrease its viscosity, and invigorate those hydrocarbon oxidizers present. Monoterpenes are an example of such hydrocarbons and are already normally present from natural sources (8).

The 14-mg/kg biomass reported here corresponds to about 10^8 cells per g, a large number considering the absence of fine sediments in the gravel. Determination of hydrocarbon oxidizer populations usually depends on sufficient growth in hydrocarbon-amended media to disrupt oil slicks or liberate $^{14}CO_2$ from labeled substrate (30). Mean populations of $\leq 10^4$ per gram of mud have been reported for the area, and 10 to 1,000 hydrocarbon oxidizers per ml have been reported for the water (29). We recently enumerated organisms by diluting small volumes of raw seawater into sterile unsupplemented aliquots and found 2 to 100% viability for bacteria present in the water column, but that viability was reduced by nutrient addition (38). Whether these numerous oligobacteria can oxidize petroleum is not known, but many appear to be able to oxidize terpenes (5). Populations of this class of organisms could easily be overlooked, resulting in substantial underestimation of microbial populations in oiled sediments, particularly after weathering has eliminated most of the soluble components which typically cultured organisms tolerate in large quantity.

The microbial oxidation of hydrocarbons is not always complete. When toluene concentrations exceed the K_m, large amounts of partly oxidized products such as 2-hydroxy-6-oxohepta-2,4-dienoic acid can be released (33). This is thought to be due to a high ratio of initial-pathway enzymes in the bacterial membranes to cytoplasmic catabolic enzymes and results in high specific affinities and low apparent K_ms (7). According to the vectorial partitioning hypothesis (6), hydrocarbon transport avoids the metabolic costs of membrane transport by reliance on high solute concentrations partitioned into the lipids of the cell membrane, where initial oxidative enzymes are located. The polar products of hydrocarbon metabolism probably require conventional transport mechanisms, causing them to have high Michaelis constants and small specific affinities. We found a higher K_m for 3-methylcatechol (33) and for toluene dihydrodiol (0.14 to 1.7 mg/liter with *Pseudomonas* sp. strain T2; unpublished data) than the K_m for the parent toluene, which is in the range of 1 μg/liter for environmental samples (10). If high Michaelis constants reflect low partitioning into the cell membrane and a deficiency in membrane transport proteins, then product persistence is likely to exceed that of the parent hydrocarbons. Organic products were indicated here in Fig. 4C, which suggests that they accumulate in sediments, consistent with the idea that they are normally associated with unusually large concentrations of hydrocarbons. The environmental significance of these electrophilic alkylating agents is unknown.

While focus is often on the visible effects of spills and on culturable organisms, we draw attention to the long residence times of hydrocarbons in the water column, the even longer residence times of their end products in accord with smaller associated specific affinities, the potential biological reactivity of these electrophiles, the importance of small and often unculturable bacteria to biodegradation, and the large component of high-molecular-weight oleophilic compounds added in current bioremediation efforts. Data show a need for detailed understanding of the physical and molecular mechanisms that affect hydrocarbon metabolism rates for the design of effective bioremediation procedures.

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