Dissolved Hydrocarbons and Related Microflora in a Fjordal Seaport: Sources, Sinks, Concentrations, and Kinetics

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The continuous addition of toluene as a solute of treated ballast water from oil tankers into a well-defined estuary facilitated the study of the dynamics of dissolved hydrocarbon metabolism in seawater. Most rates of toluene oxidation were in the range of 1 to 30 pg/liter per h at 0.5 μ g of toluene per liter. Near the ballast water injection point, a layer of warm ballast water, rich in bacteria, that was trapped below the less-dense fresh surface water was located. Toluene residence times were approximately 2 weeks in this layer, 2 years elsewhere in Port Valdez, and 2 decades in the surface water of a more oceanic receiving estuary adjacent. Mixing was adequate for a steady-state treatment which showed that 98% of the toluene was flushed from Port Valdez before metabolism and gave a steady-state concentration of 0.18 μ g/liter. Total bacterial biomass from direct counts and organism size data was usually near 0.1 mg/liter, but ranged up to 0.8 mg/liter in the bacteria-rich layer. The origin of bacteria in this layer was traced to growth in oil tanker ballast during shipments. The biomass of toluene oxidizers in water samples was estimated from the average affinity of pure-culture isolates for toluene (28 liters per g of cells per h) and observed toluene oxidation kinetics. Values ranged from nearly all of the total bacterial biomass within the bacteria-rich layer down to 0.2% at points far removed. Because the population of toluene oxidizers was large with respect to the amount of toluene consumed and because water from a nearby nonpolluted estuary was equally active in facilitating toluene metabolism, we searched for an additional hydrocarbon source. It was found that terpenes could be washed from spruce trees by simulated rainfall, which suggested that riparian conifers provide an additional and significant hydrocarbon source to seawater.

Natural water systems are usually subsaturated in nonpolar, low-molecular-weight hydrocarbons so that rates of dissolution can exceed those of metabolism. The importance of such dissolved hydrocarbons to microorganisms was demonstrated by Yoshida et al. (37). Although petroleum biodegradation studies abound (2), only a few of these investigations (8, 19, 28) use hydrocarbons that are in true solution. Because usual working concentrations of hydrocarbons often exceed aqueous solubility (1), metabolism rates saturate at concentrations which are very small, and uptake rates are sufficiently slow that special procedures must be used to avoid artifactual results from long incubation times and isotope contaminants (10), kinetic studies are somewhat inconvenient. However, the development of sensitive techniques (10) has allowed direct measurement of hydrocarbon oxidation rates in seawater. Here, we utilize these methods in an attempt to quantify the dynamics of a dissolved hydrocarbon in seawater as well as the microflora that carries out its metabolism.

The rates of dissolved petroleum metabolism that occur in Port Valdez, Alaska, are considered because treated ballast water from oil tankers is pumped into the estuary in large amounts (2×10^6 liters per h) (20). It contains 11 mg of organic material per liter, about half of which is comprised of aromatic hydrocarbons. Toluene was selected for study here because it is a chief component of the soluble fraction. It enters the 20-km³ estuary at a rate of 2.4 metric tons per month. The receiving estuary is fjordal, shaped like a bathtub, and fairly well mixed (23). Processes can therefore be likened to those in the reactor of a laboratory continuous culture. We applied a similar theory (7, 22) to calculate concentrations. Toluene concentrations were estimated from rates of toluene input, utilization rates by microorganisms, estuary flushing rates, and the steady-state approximation. Values obtained were in agreement with recent analytical measurements (I. Lysyj and D. K. Button, manuscript in preparation). Toluene oxidizer populations were estimated from toluene oxidation

rates and concentration-dependent kinetics in much the same way that Jordan and Likens used sulfur uptake rates to estimate biomass (17). Required affinities were obtained both from mixed cultures subjected to dissolved hydrocarbons for long periods and from selected pure culture isolates. This method gave hydrocarbon oxidizer populations significantly higher than were previously thought to occur (1, 3, 15). The residence time of toluene and microbial growth rates from its utilization follow directly from toluene oxidation rates and attendant formulations presented.

With *in situ* analytical capabilities aboard ship, we were able to locate and define a layer of seawater that was rich in bacteria and trace the production of these organisms to the holds of oil tankers. However, since toluene utilization rates outside this bacteria-rich layer near the injection point of treated ballast in Port Valdez appeared not to be unusually high for seawater, we searched for an alternative hydrocarbon source. Reports of large quantities of airborne hydrocarbons from forests (27, 35) led to the hypothesis that, in cool northern climates, significant quantities of terpenes would remain on evergreen foliage to be washed by rain into streams and then to flow to estuaries. The hydrocarbons contained in stimulated rainwater from fresh spruce boughs were therefore examined.

MATERIALS AND METHODS

Sampling and incubations. Shallow samples of seawater were pumped with a hand-operated piston pump through weighted Tygon tubing into fired (550°C) Pyrex carboys aboard a rowed skiff. Those below 5 m were drained from a 30-liter Niskin sample bottle that was closed and returned from depth on the hydrowire of R/V *Alpha Helix*.

Biomass. Bacterial populations were enumerated from direct microscopic counts of acridine orange-treated organisms on filters (14); total biomass was determined from the dimensions of the fluorescent bodies (5) and verified when possible with Coulter Counter volume (model Z_{BI} and Channelyzer). Toluene oxidizer biomass was computed from toluene oxidation rates and specific affinity data.

Substrate. [*ring*-1,2,3,4,5,6-¹⁴C]toluene (50 mCi/mmol; New England Nuclear Corp., Boston, Mass.) was purified by alkaline hydrolysis in concentrated NaOH and transferred from this basic solution at atmospheric pressure onto a cold finger. Success of the procedure was reestablished by finding small time-course intercepts and zero-order kinetics at high toluene concentrations (>20 µg/liter) when using an alternate lot of [¹⁴C]toluene as previously described (10).

Utilization rate measurement. Methodology for measuring utilization rate has been discussed previously (10). Briefly, ¹⁴C-hydrocarbon was added to samples within 30 to 120 min after collection. Incubation was at near surface seawater temperature (6 to 11°C)

in bottles or carboys that were shielded from light. Periodic (30- to 120-min) subsamples of 25 to 2,000 ml were acidified with 0.5 to 1 ml of 6 N H₂SO₄ and stripped with a nitrogen flow of 0.1- to 0.8-liter per min; smaller subsamples and reagent quantities correspond to samples with high biological activity. The ¹⁴CO₂-containing nitrogen was dried in concentrated H₂SO₄ and then by passing through a cold trap containing 4-mm glass beads at -70°C. Volatilized hydrocarbons were removed by a column (0.5 by 10 cm) of 60-80 mesh Tenax GC (Laboratory Data Control/ASD, Fairfield, Calif.) at -70°C, and the ¹⁴CO₂ was collected in 20 ml of a phenethylamine-based scintillation cocktail contained in a bubble tube (Midiget Impinger; Kontes, Vineland, N.J.), and counted.

Nomenclature. The following designations are used throughout this paper: *A_a*, concentration (grams per liter) of added toluene in incubations; *A_o*, influent toluene concentration (grams per liter) in treated ballast water; *A_{out}*, extracellular concentration (grams per liter); *A_n*, natural or background concentration (grams per liter); *F*, flushing rate of the estuary, estuary volumes exchanged per hour per estuary volume; *I*, influent flow rate (liters per hour) of treated ballast water; *K_A*, Michaelis constant (grams per liter) of *A*; *K_i*, Michaelis concentration (grams per liter) of component *i*; $\Sigma K_i/n$, weighted average Michaelis constant (grams per liter) *n*, microbial species for the *i*th substrate; *K_z*, vertical component of eddy diffusivity (square centimeters per second); *P*, CO₂ product of enzymatic activity (grams per liter) on substrate *A*; *Q*, intracellular organic product (grams per liter); *R_{Em}*, ratio of rate-limiting enzyme mass to cell membrane mass; *R_{mX}*, ratio of cell membrane mass to microbial biomass; *w*, quantity of *A* moving through a two-dimensional plane (grams per square centimeter per second); *X_T*, total microbial biomass (grams [wet weight] per liter); *X_A*, biomass of organisms that metabolize hydrocarbon *A* (grams [wet weight] per liter); *V*, volume (liters); *V_i*, maximal velocity (grams per liter per hour) of hydrocarbon metabolism by the *i*th species; ΣV_i , maximal velocity (grams per liter per hour) in the whole mixed culture; *Y_{QA}*, yield of organic product carbon produced from hydrocarbon carbon utilized (dimensionless); *Y_{XA}*, yield of cell carbon produced from hydrocarbon carbon utilized (dimensionless); *Z*, depth (centimeters); *a_A*, specific affinity (liters per gram of cells per hour) or second-order rate constant relating uptake to microorganisms *X* and substrate *A_{out}*; *k*, rate constant; *t*, time; *v*, rate (grams per liter per hour) of hydrocarbon utilization by a microflora; *v_a*, apparent (uncorrected for *A_n*) rate (grams per liter per hour); *v_i*, rate due to the *i*th species alone (grams per liter per hour); *μ_A*, specific growth rate (per hour) of the toluene oxidizers considering only that tissue laid down from substrate *A*; *μ_T*, specific growth rate (per hour) of the total biomass; *ξ*, partition coefficient of hydrocarbon between water and membrane lipid (dimensionless).

Formulations. The rate of consumption of hydrocarbon *A* (grams of hydrocarbon per liter per hour) from solution by organisms of biomass *X_A* is given at small (*A_{out}* < *K_A*) concentrations by

$$-dA_{out}/dt = a_A X_A A_{out} \quad (1)$$

where a_A is the affinity (9, 30) of organisms for hydrocarbon. The specific affinity is therefore given by the initial slope of the specific (per gram of cells) hydrocarbon utilization rate versus hydrocarbon concentration plot. By way of comparison with the rate constant-based definition for polar substrates (9), it can also be expressed as $a_A = kR_{mX}R_{Em}\xi$ where R_{mX} is the mass of lipid membrane per unit cell mass, R_{Em} is the portion of this cell membrane devoted to rate-limiting enzyme, ξ is the water-membrane partition coefficient for substrate, and k is the second-order rate constant for a substrate-enzyme rate-limiting step in the cell membrane. Alternatively the rate of hydrocarbon consumption v (grams of hydrocarbon per liter per hour) can be simply expressed as pseudo first order in hydrocarbon concentration

$$v = -dA_{out}/dt = k_1A_{out} \quad (2)$$

where $k_1 = a_AX_A$.

The rate of hydrocarbon consumption exceeds the rate of CO_2 production (dP_{out}/dt) by the amount of cell material and other products formed from the hydrocarbon consumed. Expressing cell production as the yield Y_{XA} of cells formed from hydrocarbon consumed on a carbon basis

$$v = -dA_{out}/dt = (dP_{out}/dt)[(1/1 - Y_{XA} - Y_{QA})] \quad (3)$$

In the environment Y_{XA} and Y_{QA} are unknown. If they are taken to be small and neglected, the hydrocarbon consumption rate is given by the approximation

$$v = -dA_{out}/dt \approx dP_{out}/dt \quad (4)$$

If added ^{14}C -hydrocarbon A_a is supplemented by naturally occurring hydrocarbon A_n , the oxidation rate v exceeds the apparent rate v_a observed from radioactive product formation. Where the Michaelis constant for uptake K_A exceeds total hydrocarbon A_{out} ($= A_a + A_n$) so that saturation is insignificant

$$v = v_aA_{out}/A_a \quad (5)$$

Fortuitously, k_1 is unaffected because ^{14}C -labeled P and A_a are equally diluted by A_n . Thus, k_1 is given by v_a data directly without knowledge of the population in the water sample used.

The population of organisms (wet weight) that can oxidize the particular hydrocarbon used X_A is given, from the above, by the rate of hydrocarbon uptake together with the affinity (grams of hydrocarbon oxidizers per liter)

$$X_A = k_1/a_A = v/a_AA_{out} \quad (6)$$

where K_A exceeds A_{out} so that again saturation is insignificant. The uptake rate of each species i (or homogeneous metabolism unit where an organism has multiple types of systems with similar function [16, 21]) of a mixed culture is

$$v_i = V_iA_{out}/(K_i + A_{out}) \quad (7)$$

and for the whole mixed culture of n species the sum is

$$v = \Sigma V_iA_{out}/[(\Sigma K_i/n) + A_{out}] \quad (8)$$

where V_i is the maximal velocity for each species and $\Sigma K_i/n$ is the weighted average Michaelis constant for uptake. Equation 7 is more sharply curved in the region of K_i than equation 8 and is slightly less accurate. For consideration of the lightly saturated (in hydrocarbon) systems here either equation 7 or 8 is adequate. The residence time of A in the system from either equation 4 or 8 is v/A_{out} . Michaelis constants are used here mainly to reflect the ambient hydrocarbon concentration where consumption increases at a decelerating rate. Affinities are normalized to both hydrocarbon concentration and biomass and reported as an index of the ability of the microflora to oxidize hydrocarbons.

The specific rate of growth μ_A (grams of cells from A per gram of cells using A per hour) of the hydrocarbon oxidizers X_A from hydrocarbon used is given by

$$\mu_A = (dX_A/dt)(1/X_A) = vY_{XA}/X_A \quad (9)$$

All substrates considered, the growth rate μ_{AT} of the hydrocarbon oxidizers becomes $\mu_{AT} = \mu_A + \mu_B + \mu_C \dots$ where $A, B, C \dots$ indicate the contributions of each of the substrates used (amino acids, carbohydrates, and the like) to the growth rate. The growth rate μ_T (grams of cells formed per gram of cells present per hour) of the total bacterial biomass X_T in the water column that is due to the hydrocarbon alone is

$$\mu_T = vY_{XA}/X_T \quad (10)$$

Turning to the average hydrocarbon concentration in an estuary, the material balance of a well mixed system is: change = input - output. A steady-state treatment gives

$$V(-dA_{out}/dt) = IA_o - k_1A_{out}V - FA_{out}V \quad (11)$$

where V is the volume of the system, A_o is the concentration of hydrocarbon in the influent flow I , and F is the flushing rate of the system. Dividing through by V gives the hydrocarbon concentration (grams per liter) at steady state

$$A_n = (I/V)[A_o/(k_1 + F)] \quad (12)$$

RESULTS

In situ activity. Sampling locations in Port Valdez, together with a more oceanic station in Prince William Sound, are shown in Fig. 1. Microbial populations at these locations (determined by fluorescence microscopy) and the rate at which they utilize added toluene are given in Table 1. When seawater samples are returned to the laboratory for careful kinetic measurement, the rate of toluene utilization in them consistently exhibits saturation kinetics. Half-maximal rates in these mixed populations are in the very small concentration range of $\Sigma K_i/n = 0.5$ to $1.5 \mu g/liter$. Kinetics for another hydrocarbon, biphenyl, are similar (28). Whereas absolute rates in fresh samples are much slower than rates in the same seawater after it has been returned to the laboratory, saturation appears to occur at

about the same concentration. The slope of a Hofstee plot (v versus v/A_{out}) of the April 24, 1980 data of Table 1 gives an average Michaelis constant $\Sigma K_i/n$ of 2.8 $\mu\text{g/liter}$. We selected 0.5 $\mu\text{g/liter}$ as the optimal concentration of toluene to make measurements of microbial activity.

Typical data from which the toluene utilization rates were determined are shown in Fig. 2. Values were calculated from scintillation spectrometry data collected in the laboratory by using $^{14}\text{CO}_2$ samples from experiments conducted at sea. Data fit a straight line with an

FIG. 1. Location map showing station numbers.

TABLE 1. Microbial populations and oxidation rates in Port Valdez

Station no.	Depth (m)	No. of small organisms per ml ($\times 10^6$)	Filament 10- μm segments per ml ($\times 10^4$)	Mass of organisms ^a ($\mu\text{g/liter}$)			Toluene utilization rate, v_a (ng/liter/h)
				Small organisms	Filaments	Total X_T	
1 ^b	5	1.4	<0.1	68		68	0.0025
2 ^b	5	1.4	1.18	68	52	120	
2 ^b	5	1.5	2.60	73	114	188	0.0008
3 ^b	5	1.5	1.18	73	52	125	0.0046
4 ^b	5	1.5	0.65	73	28	102	0.009
5 ^b	5	1.5	2.60	73	114	188	0.015
5 ^b	0	1.4	3.61	68	159	227	0.026
5 ^b	5	2.0	3.67	97	162	259	0.020
5 ^b	35	0.37	3.24	18	143	161	0.60
5 ^b	50	0.37	8.42	18	371	389	8.77
5 ^b	50 ^c	0.31	20.70	15	914	929	>7.07
5 ^b	60	0.34	5.18	16	228	245	2.41
5 ^b	75	0.34	5.23	16	230	247	0.027
5 ^b	200	0.32	0.26	15	11	27	0.027
5 ^d	5	6.5				34	0.01
5 ^e	5	6.5				34	0.05

^a The wet weight of small organisms was estimated from the value of 0.047 μm^3 per organism (5); the mass of filaments was estimated by using the average diameter of 0.7 μm .

^b Samples were collected 27 through 29 August 1980 and incubated with 0.33 to 0.42 μg of [^{14}C]toluene per liter at 13°C.

^c We returned to the same location after 12 h and collected a second sample.

^d Samples were collected 24 April 1980 and incubated at 6°C. Population is total count from 1 m with 10% consisting of 0.5- by 2.0- μm rods.

^e Same as *d*, except that added toluene was increased from 0.38 to 3.9 $\mu\text{g/liter}$.

FIG. 2. Typical $^{14}\text{CO}_2$ evolution data from which toluene utilization rates were calculated. Seawater samples were collected from a depth of 5 m at the stations shown in Fig. 1.

average correlation coefficient of $r = 0.94$. Preliminary counts on shipboard were nearly as accurate. Very high activity was noticed in 50-m samples at station 5 from preliminary counts which caused us to return later that day for more extensive sampling. Activity was sufficient that subsample volumes could be reduced to 25 ml and incubation times could be reduced to 4 h, which allowed processing of additional samples. The average correlation coefficient for these data was $r = 0.68$. Considering that the replicate rate data at 5 and 50 meters were collected on different days using different sample sizes, reproducibility was quite good. A depth profile of utilization rates is shown in Fig. 3. These data were computed directly from $^{14}\text{CO}_2$ recovery (equation 4); values corrected for background toluene (equation 5) are given in Table 2.

One striking feature of the depth profile is the peak in the rate of toluene utilization at 50 m. The outlet of treated ballast wastewater from Alyeska Pipeline Service Co.'s operation lies 500 m to the southeast (Fig. 1). According to dye studies (Colonell, personal communication), this warm (7 to 17°C) effluent floats upward to become trapped below the less-dense surface water which is enriched 10 to 20% by freshwater from stream runoff. Although added hydrocarbons from the effluent would tend to decrease observed oxidation rates if $A_n > K_A$, the rate maximum of Table 1 showed strong correlation with the plume location from dye studies and from direct hydrocarbon concentration measurements (20).

FIG. 3. Depth profile of toluene oxidation rates v_a at station 5 showing the active layer. The right-hand plot is an expanded view. Both the injection depth of treated ballast water and the trap depth (below less saline but cooler fresh water) are shown.

Origin of biomass. The data of Table 1 also show a peak in total microbial biomass which corresponds with the peak rate of toluene utilization at 50 m. We questioned the origin of these organisms because computation showed that growth rates were too slow to generate this layer before turbulence would disperse it (*vide infra*). Toluene utilization rates were only approximately $0.01 \mu\text{g/liter per h}$ in the high-activity layer which is small when compared to the 0.4 to 0.9 mg of microbial biomass per liter present. Treated ballast water contains just four major components which are potential growth-supporting heterotrophic substrates: benzene, toluene, xylene, and ethylbenzene (20). Even if 10 other petroleum hydrocarbons were included and endogenous metabolism was neglected, the resulting growth rate from this input would be on the order of 2 per year. This computed growth rate is quite insufficient to form the observed layer because dispersal rates are at least 4 per week (*vide infra*). We therefore searched for other sources of bacteria upstream from the layer of bacteria-rich water formed.

Toluene utilization rates and microbial populations at various points of processing within the ballast water treatment facility that feeds Port Valdez are shown in Table 3. Stripping of these samples with air removed background volatile hydrocarbons. This served to reduce competition for added ^{14}C toluene (equation 5) and therefore increased the apparent utilization rates shown to more nearly correct values. However, oil droplets still remained which would also reduce apparent utilization rates by absorbing ^{14}C toluene. This was probably not a serious problem because affinity values for ^{14}C toluene in the whole mixed culture (averaging 49.1 liters per g of cells per h) were near the maximum

TABLE 2. *Distribution and activity of toluene-oxidizing microorganisms in Port Valdez^a*

Station no.	Sampling depth (m)	Total toluene $A_a + A_n$ ($\mu\text{g/liter}$)	Toluene oxidation rate v (ng/liter/h)	Toluene residence time k_1^{-1} (years)	Toluene oxidizers X_A ($\mu\text{g/liter}$)	Ratio X_A/X_T (%)	Growth rate μ_T (per century)
1	5	0.68	0.0034	22.8	0.07	0.25	0.048
2	5	0.45	0.0012	42.8	0.09	0.06	0.009
5 ^b	5	0.56	0.014	4.6	0.09	0.26	0.020
5	5	0.57	0.027	2.4	1.7	1.3	0.125
5	50	0.56	12.3	>0.005	784	118	16.5
5	200	0.63	0.037	1.9	2.1	7.7	1.2

^a Values are computed from equations 2, 4, 5, 6, and 10 with v_a and X_T from Table 1 and an average affinity a_A of 28 liters per g of cells per h from the pure culture data of Table 4.

^b Samples were collected on 24 April 1980; other samples were collected on 27 through 29 August 1980.

TABLE 3. *Population characteristics of organisms in the ballast water-treatment facility*

Location ^a	Rate v (ng/liter/h) ^b		Population ^c X_T		Affinity ^d a_A (liters/g of cells/h)
	Before aeration	After aeration	Organisms/liter	mg (wet)/liter	
Fresh ballast	1.9	24.5	6.9×10^9	3.5	35.1
pH basin	0.2	22.4	3.5×10^9	1.8	81.4
Lagoon (top)	1.8	28.9	4.9×10^9	2.5	53.6
Lagoon (bottom)	2.9	19.3	7.4×10^9	3.7	26.5

^a Fresh ballast water, from the tanker *Mobile Arctic* in this case, is stored briefly, skimmed, aerated, skimmed again, stored in a pH basin potentially for chemical adjustment, pooled in a lagoon, and then, after a half-day residence time in the whole system, discharged from near the bottom of the lagoon into Port Valdez at a depth of 70 m.

^b The rate of toluene utilization calculated from the $^{14}\text{CO}_2$ recovered from 0.23 to 0.35 μg of added [^{14}C]toluene per liter according to equation 4. Aerated cultures were sparged overnight to remove volatile hydrocarbons before toluene addition.

^c From direct microscopic counts. Some organisms were hidden from view because of penetration into or attachment to oil droplets.

^d Calculated from direct count and organism size data and toluene utilization rate data together with equations 1 and 4.

obtained for known toluene oxidizers isolated (Table 4). Utilization kinetics in these toluene experiments were not confused by an oil phase because the higher solubility of toluene allows true solution.

With a flow rate of 2×10^6 liters per h for treated ballast water containing 4 mg of hydrocarbon utilizing organisms per liter, about 1 metric ton of biomass from the bottom of the holding lagoon (Table 3) per week is injected, along with dissolved and entrained hydrocarbons, into Port Valdez. Photomicrographs of these organisms from ballast water within a ship, from the treatment plant, from the bacteria-rich layer in Port Valdez, and from a typical lightly contaminated area are shown in Fig. 4.

Toluene concentration. The residence times of toluene (Table 2) as $1/k_1$ are given by toluene consumption rates (v) and the concentration of toluene (equation 2) so long as enzymatic processes are not saturated. Comparison of populations in the bacteria-rich layer (Table 1) with those in the holding lagoon (Table 3) where the toluene concentration is known (1.9 mg/liter [20]) gives an estimate of the concen-

tration to which toluene in the 50-m sample is diluted based on the assumptions that both bacteria and toluene are equally diluted by fresh seawater and that little growth occurs within the layer itself (Table 2). This computed concentration of A_n (610 $\mu\text{g/liter}$) is sufficient to saturate metabolism ($\Sigma K_i/n \approx 2.8 \mu\text{g/liter}$; see below) so that the toluene utilization rate is, from equation 8, $v = 1.3 \mu\text{g/liter per h}$. The residence time of toluene in the bacteria-rich layer, using this toluene concentration estimate and disregarding dispersal, is 19 days.

Horizontal currents of approximately 4 km/day (23) with associated vertical components should disperse the hydrocarbons in the bacteria-rich layer before appreciable metabolism can occur (vide infra) and justify a steady-state treatment. With a toluene input rate of $A_d I = 2.4$ metric tons per month a rate of flushing of the estuary of $F = 9$ per year, and a toluene metabolism rate constant for the whole 5- by 22-km fjord of $k_1 = 2.5 \times 10^{-5}$ per h from average toluene utilization rate data (station 5 at 200 m and station 2 at 5 m) and equation 2, the steady-state concentration of toluene is 0.18 $\mu\text{g/liter}$.

TABLE 4. Specific affinities^a of selected isolates and of mixed populations for hydrocarbons

Culture	Substrate	Growth conditions	Affinity (liter/g of cells/h)
Seawater isolates			
<i>Pseudomonas</i> T-1	Toluene	Toluene alone, 10°C	9.3
<i>Pseudomonas</i> T-2	Toluene	Toluene alone, 10°C	9.2
<i>Pseudomonas</i> T-2	Toluene	Amino acids then toluene, 10°C	87 ± 8 (n = 3)
<i>Pseudomonas</i> T-3	Toluene	Toluene alone, 10°C	18.5
<i>Pseudomonas</i> T-3	Toluene	Amino acids then toluene, 10°C	15 ± 1 (n = 3)
<i>Pseudomonas</i> T-4	Toluene	Toluene alone, 10°C	5.7
<i>Pseudomonas</i> B ^b	Toluene	Various, 10°C	0.0 to 13
Five dodecane isolates	Toluene	Toluene alone	0.0
<i>Corynebacterium</i> 198	Dodecane	Continuous culture, 25°C (8)	14.0
Oil tanker ballast direct	Toluene	Aerated, 25°C (Table 3) ^c	53 ± 27 (n = 4)
Oil tanker ballast flora, toluene enrichment culture	Toluene	Aerated, 10°C ^c	87.7
Seawater; Seward, Alaska	Toluene	Stored 30 days at 10°C (28)	47
Seawater; Seward, Alaska	Biphenyl	Stored 30 days at 10°C (28)	116
Port Valdez, station 1, 5 m	Toluene	In situ, 13°C ^d	0.054
Port Valdez, station 5, 50 m	Toluene	In situ, 13°C ^d	36

^a The specific affinities of the isolates were calculated from equations 1 and 4; hydrocarbon concentrations were always less than 2 µg/liter and were determined from radioactivities; pure culture biomass was determined from integrated Coulter Counter size distributions, and the biomass of mixed cultures was determined by direct microscopic counts and organism size data. Standard error is shown where multiple determinations were made.

^b This isolate may have been selected for its ability to oxidize an ethyl acetate contaminant of unpurified [¹⁴C]toluene (10).

^c Background volatile hydrocarbons were stripped out with air before [¹⁴C]toluene addition.

^d Oxidation rate data were collected from incubations begun immediately after sample collection.

Hydrocarbon oxidizers. Outside the bacteria-rich layer, populations of bacteria are in the order of 100 µg/liter (Table 1) or 2,000 metric tons in the estuary. The population of toluene oxidizers can be estimated from toluene oxidation rates (Table 1) and affinities (Tables 3 and 4) together with equation 6. Using an average toluene utilization rate of 14 pg/liter per h (above) at 0.37 µg of toluene per liter and an average affinity of 28 liters per mg of cells per h from the pure culture data of Table 4 gives a population of toluene oxidizers of 1.3 µg/liter or 26 metric tons in all of Port Valdez. If large numbers of toluene oxidizers in seawater are more able to metabolize it than those selected, then the population of toluene oxidizers in Port Valdez will be proportionately higher. Notice, however, that the affinities of the various toluene oxidizing isolates varied by less than 1 order of magnitude from the mixed cultures that were enriched for toluene utilization (Tables 3 and 4). We were unable to demonstrate toluene metabolism at all with several parallel isolates from dodecane enrichment media as shown.

By comparing metabolism rates (k_1 above) with flushing rates ($F = 1 \times 10^{-3}$ liters per h), it

can be seen that only 2.5% of the 90-kg/day toluene input is consumed within the estuary. Thus the 81-kg/month toluene consumption rate is supplying the toluene diet of at least 26,000 kg of bacteria, but contributes only a maximum of 6.9/century to their growth rate. The rate of tissue accumulation for the total population of hydrocarbon oxidizers and others that can occur from the consumption of toluene is even less (Table 2).

Hydrocarbon sources. The large factor by which hydrocarbon oxidizer biomass exceeds hydrocarbon consumption quantities, together with the fact that, outside the bacteria-rich layer, toluene consumption rates in water from Port Valdez were no higher than that in water from Resurrection Bay (near Seward, Alaska; data not shown), suggests that hydrocarbon sources in addition to ballast exist. Coastal Alaska is bordered by a 10⁶-ha conifer forest (O. K. Hutchison, 1964 Alaska's forest resource [p. 9], U.S. Department of Agriculture, 1967). If hydrocarbons are washed from these evergreens in significant quantities by rain and these resulting crown flow hydrocarbons are preserved during runoff by cool temperatures and swift cur-

FIG. 4. Photomicrographs of microflora collected on filters and used to calculate bacterial biomass. After treatment with acridine orange, 5-ml samples were filtered and photographed with ASA 400 film (bar, 5 μ m). A, Typical 5-m sample of seawater (station 2). B, Bacteria-rich layer of station 5 at 50 m; the large object at left is a particle of glacial flour. C, Untreated ballast water from the Mobile Arctic; the central clump consists of organisms attached to a particle of oil. D, Water from the holding lagoon (near surface water) which is injected as shown in Fig. 3.

rents, riparian conifers could represent the missing hydrocarbon source. Indeed, we found that monoterpene hydrocarbons are easily washed from white spruce trees (*Picea glauca*) by sprinkling water over them (Fig. 5). Quantitative analysis of water in which fresh spruce boughs were briefly dipped (data not shown) provided an estimate of total production. Some of the hydrocarbons were particulate (not filterable), and some components were liberated in larger quantities when subjected to simulated wind damage. Total production was estimated to be roughly 10,000 metric tons per rain for coastal Alaska and 30 metric tons in the forested 10-square-mile (ca. 26-km²) watershed of Port Valdez. Structures of these hydrocarbons, e.g., limonene (Fig. 5), are closely related to the water-soluble constituents of petroleum such as toluene. Although the substrate specificity of hydrocarbon oxidizers is not universal (Table 4), it is fairly broad (29), so that regular terpene input

would probably enhance the capacity of an estuary to metabolize toluene.

DISCUSSION

Kinetics. The rates of toluene oxidation were obtained at concentrations selected to be near the Michaelis constant for sustained uptake. This concentration avoids appreciable rate-concentration disproportionality due to saturation and thus provides for near maximum sensitivity. Yet, because of the small quantities involved, minimal chemical perturbation to the system is caused and isotope impurity problems are minimized. The value of the Michaelis constant (2.8 μ g/liter) from the April data of Table 1 is in agreement with laboratory data from raw seawater flora where $\Sigma K_i/n$ from equation 8 is 0.5 to 3 μ g/liter (not shown). Use of a Hofstee plot to obtain the Michaelis constant is compatible with equation 7 only. The refined version, equation 8, for hydrocarbon-limited multiple micro-

FIG. 5. Trace from a gas chromatographic-mass spectrometer used to identify hydrocarbons in the wash water from a small (2-m) white spruce tree. The tree was collected locally in January and sprinkled with distilled water. Drippings were funneled through heavy-gauge aluminum foil into Pyrex casseroles, and the product was collected through a Pyrex fiber filter; all material was cleaned by baking at 525°C. The wash water was extracted with methylene chloride and separated compounds were quantified from other chromatograms by use of peak area and internal standards.

bial species systems is not always distinguishable experimentally from equation 7. It is easily shown, however, that the Woolf transformation (A_{out}/v versus A_{out}) of equation 7 (36) yields abscissa intercepts that depend not only on A_n , the background level of toluene, but on several other factors as well. Examples are (i) concentration thresholds (30), (ii) competitive inhibitors, and (iii) the presence of multiple substrate accumulation systems having Michaelis constants with different values. Since computation of A_n from kinetic data was an objective of this work, equation 8 is introduced simply to demonstrate that manipulation of equation 7 is insufficient for evaluation of A_n . For this reason we developed the alternative method defined by equation 12.

Yields of cell material from toluene of $Y_{XA} = 0.32 \pm 0.12$ were obtained from laboratory studies of the mixed microflora of seawater (10). However, since endogenous metabolism rates in seawater may be significant at the low substrate concentrations which occur, the correct value of Y_{XA} for use in equation 3 may be much lower. It was therefore taken as zero in the rate computations. If the value of 0.3 is correct, it would have the effect of increasing toluene oxidation rates and reducing residence times by one-third. Specific rates of formation of organic products from toluene by mixed cultures are not specifically defined; however, preliminary indications

are that their effect on the value Y_{XA} is often less than that of CO_2 production.

Metabolic activity was an ubiquitous property of the water column. The rate of toluene oxidation in Prince William Sound may have been enhanced by phytoplankton (11), because light appeared to stimulate activity somewhat (data not shown). Rates in all samples collected were well above the limits of detection. A rate of 0.5 pg/liter per h at station 2 may best typify the integrated value for toluene (A_n) consumption in Port Valdez because that region is well mixed and this rate was obtained when the tidal outflow (current = 3 cm/s) was near the end of the cycle.

Mixing. The assumption of perfect mixing used to derive equation 12 and on which the calculation of steady-state toluene concentration depends might be questioned because of the variation in populations observed. Spectacular as the utilization rates in the bacteria-rich layer are, the influence of these high rates on the hydrocarbon concentration is minimal. A lower estimate for the vertical component of diffusivity near the thermocline of a fjordal estuary is $K_z = 0.05 \text{ cm}^2/\text{s}$ (25). Values for most estuarine systems are larger (12). The rate of material transport from this layer W is related to K_z by Fick's first law, $W = K_z dA_{out}/dZ$, where the concentration gradient is given by the differential. At a microbial concentration gradient of $dX_T/dZ = 0.41 \text{ } \mu\text{g}/\text{cm}^3$ over 10 m (Table 1), the population will diffuse downwards at a rate of $0.28 \text{ } \mu\text{g}/\text{cm}^2$ per h. Diminution of the $660\text{-}\mu\text{g}/\text{cm}^2$ population in the 1,000-cm lower half of the layer, due to downward diffusion, should be first order in cell population according to $dA_{out}/dt = k_d X_T$, giving a rate constant k_d of 0.12/h. Then the half-life of the layer due to downward diffusion alone is 5.8 h. This rate constant for vertical diffusive loss k_d compares with the much smaller rate constant for toluene metabolism of $k_1 \leq 1.5 \times 10^{-3}/\text{h}$ in the bacteria-rich layer. Because (i) metabolism rates are even slower elsewhere (Table 1), (ii) the horizontal transport greatly exceeds vertical mixing, and (iii) the vertical components may be significantly larger than those used (12), the assumption of steady state appears to be justified.

Steady-state toluene. The toluene concentration $A_n = 180 \text{ ng/liter}$ which arises from this steady-state treatment is quite compatible with several direct measurements in seawater: 10 to 60 ng of toluene per liter over Georges Bank (4), 10 ng of toluene per liter in Vineyard Sound off Massachusetts (33), and 60 to 500 ng of total hydrocarbons per liter in the Caribbean and Gulf of Mexico (32). More important, toluene concen-

trations of 300 ng/liter have been obtained in Port Valdez away from the contaminated area with appropriately much higher values obtained in the bacteria-rich layer (I. Lysyj, personal communication). Where concentrations of toluene exceed the Michaelis constant, uptake rates are affected by isotope dilution (equation 5) and saturation (equation 8) phenomena. These effects, if present, would increase the value of the toluene uptake rate in the bacteria-rich layer over that reported here. This is because the isotope is diluted by cold toluene which results in increased real rates and because saturation prevents proportionate increase in the observed rate of [^{14}C]toluene metabolism due to the dilution of isotope.

The dominance of flushing in the calculation of A_n for this system should be noted. Weather-related phenomena are thought to modify flushing on a regular basis. However, the value reported for steady-state toluene should remain within the correct order of magnitude.

Residence time. Toluene residence times based on metabolic rates ranged from years in Port Valdez to decades in the surface waters of the larger receiving estuary adjacent. These long residence times indicate that most hydrocarbons are flushed out of small estuaries and then diffuse downward to where the residence times, which vary inversely with microbial population, should be many centuries. The fact that metabolism rates are first order in concentration below the Michaelis constant (unpublished data show no threshold of the type observed for growth [18, 30]) has an equalizing effect on hydrocarbon concentrations in the world ocean because those hydrocarbons which accrue to higher concentrations are metabolized at higher rates.

Previous reports of hydrocarbon residence times (19, 34) have not considered the multiple methodological problems of impurities in the substrate, long incubation times, saturation phenomena, and the presence of an oil phase, all of which affect the rates of metabolism of dissolved hydrocarbons (10). It is reported that low water temperatures and low nutrient concentrations seriously constrain hydrocarbon metabolism rates (34). However, the data in Table 1 show appreciable rates at 6°C, and metabolism appears to occur normally at -1.8°C (1). It is also reported that clays absorb significant quantities of hydrocarbons (19) and alter metabolic rates. However, we have not observed this to be the case for either dodecane (8) or glucose (6). Rates and kinetic constants observed here are quite similar to those for biphenyl (Table 4), with monochlorination causing only slightly slower rates (28). Neither is dissolved dodecane, a com-

mon straight-chain hydrocarbon, metabolized significantly faster than toluene (Table 4), so that rates reported here can be regarded as somewhat typical for dissolved hydrocarbons. Although affinities reported here are lower for hydrocarbons than they are for most polar substrates (9), low Michaelis constants also appear to restrict rates in some experimental systems because the rates are saturated at such small concentrations.

Populations. Error in the estimate of the biomass of toluene oxidizers depends strongly on the integrity of the average specific affinity value used. That affinity values were quite consistent among the various isolates has a precedent in the finding that the affinities of various organisms for various polar substrates are also quite consistent (9). The large difference between affinities for polar substrates and nonpolar substrates is thought to result from comparing aqueous external concentrations of polar substrates with membrane concentrations of hydrocarbon after they have partitioned from the aqueous phase into lipid (vide supra). Direct count data and computed biomass from kinetic data are used over population estimates according to plate counts or most-probable-number techniques because few of the organisms in seawater will respond to these latter techniques. A toluene oxidizer biomass of 0.1 $\mu\text{g/liter}$ amounts to about 5×10^6 organisms per liter; however, only about 10^4 organisms per liter can be cultured with mixed hydrocarbon enrichments (1, 31). Moreover, the large variation in organism size, as well as clumping characteristics (Fig. 4), would suggest that organism biomass is a preferred indicator of metabolic activity, particularly for comparing diverse systems. Consistency of the data of Table 4, together with the fact that ample opportunity for selection of the best toluene oxidizers available probably occurred in oil tankers, suggests that the specific affinity values reported are near an upper limit. However, some organisms, including those whose hydrocarbon metabolism pathways remain repressed, may have lower affinities; isolate *Pseudomonas* B is one example. If the average affinity of the ballast water (Table 3) is used rather than the average pure culture value (Table 4), then the computed toluene oxidizer biomass estimate is reduced from 118% of the total to 71%. Since most of the biomass in this layer was probably derived from hydrocarbons rich in toluene, either affinity value gives a believable estimate of toluene oxidizer biomass which lies within the standard deviation of the affinity values collected.

The population responsible for most of the

toluene oxidation appears, from microscopic observations, to be bacteria (blue-green algae [cyanobacteria] and actinomycetes included). These transthreptic (heterotrophic and non-phagocytotic) microorganisms comprise at least 2,000 metric tons of biomass in Port Valdez. This biomass compares with an annual average phytoplanktonic productivity of about 150 metric tons per day (13), so the bacteria biomass considered is quite significant indeed.

Growth rate. The growth rate estimates μ_A considered the formation rate of only that part of the microbial tissue that is derived from the carbon of hydrocarbon utilized. This method gave generation times of only 0.03 to 100 centuries from toluene in Port Valdez, which indicated that submicrogram concentrations of a single hydrocarbon are a minor food source. Small Michaelis constants indicate that larger concentrations ($>5 \mu\text{g/liter}$) provide for little additional growth. Yet various mixed cultures will grow in the laboratory on toluene vapors supplied. In other studies we have found that mixed polar organic substrates enhanced growth capabilities at low concentrations (18), and laboratory air is far from organic carbon free. Because of the low Michaelis constants involved, the energy provided by multiple hydrocarbons utilized by separate initial pathways would be additive at moderate ($>\sim 1 \mu\text{g/liter}$) as well as small concentrations. These interactions are under current investigation. However, it is quite possible that the numerous hydrocarbons of seawater in concert can provide for faster growth of these organisms than does toluene alone.

Population source. Given the substantial populations in the bacteria-rich layer, the corresponding populations in the treated ballast water added, and the slow growth rates above, it is clear that the biomass of this layer is derived directly from organisms within the treated ballast water. Since the residence time of tanker effluent in the ballast water treatment facility is short (Table 3) and the microbial populations in the effluent are equivalent both before and after treatment, it is also clear that the organisms in the bacteria-rich layer are grown, for the most part, during transit time between refineries (often approximately 10 days from the west coast of the United States) and Port Valdez.

Hydrocarbon sources. The finding of substantial terpene hydrocarbons in the crown flow from conifers suggested an explanation for the ubiquity of hydrocarbon-oxidizing microorganisms in seawater. Slow turnover of the hydrocarbons in the water column indicated that (i) terrestrial hydrocarbons (27, 35) can reach the ocean, particularly when they are contained in

the swift cool waters of spring runoff, and (ii) hydrocarbons from terrestrial sources become integrated into the ocean as a whole and provide a significant part of the dissolved organic chemicals in seawater. Probable catabolic similarities between terpenes and petroleum hydrocarbons (24, 26) suggest that terrestrial plant hydrocarbons support a seed-stock microflora which is available to propagate at the expense of the frequent accidental oil spills.

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