APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Feb. 1976, p. 189-197

Vol. 31, No. 2
Printed in U.S.A.

Measuring the Potential Activity of Hydrocarbon-Degrading Bacteria

J. D. WALKER[1] AND R. R. COLWELL*
Department of Microbiology, University of Maryland, College Park, Maryland 20742

Received for publication 30 January 1975

[^{14}C]hydrocarbons were utilized as a means of estimating the hydrocarbon-degrading potential of bacteria in estuarine and marine environments. Evaporation of the hydrocarbons must be considered in estimates of oxidation. Amount of mineralization of [^{14}C]hexadecane can be equated with the total number of petroleum-degrading bacteria and the percentage of the total heterotrophic population, which they represent. Mineralization activity was found to be related to the activity of the bacterial populations during in situ incubation. Rates of mineralization were observed, as follows, for [^{14}C]hexadecane > [^{14}C]naphthalene > [^{14}C]toluene > [^{14}C]cyclohexane. Increased rates of uptake and mineralization were observed for bacteria in samples collected from an oil-polluted harbor compared with samples from a relatively unpolluted, shellfish-harvesting area, e.g., turnover times of 15 and 60 min for these areas, respectively, using [^{14}C]hexadecane.

Ecological studies of microorganisms often are limited by difficulties in relating numbers of viable organisms present in natural waters at a given site with their activity, either actual or potential. In earlier studies carried out in our laboratory, we compared methods for enumerating petroleum-degrading microorganisms (25) and measuring degradation of petroleum (23). The paucity of information on the conversion of [^{14}C]hydrocarbons to CO_2 (14-16) and cellular material (15, 16) by marine bacteria, compared with the relatively large volume of information on the uptake and mineralization of ^{14}C-labeled carbohydrates and amino acids (1, 4, 7-13, 18, 19, 28-35), prompted us to examine this aspect of marine microbial ecology. In the study reported here, ^{14}C-labeled hydrocarbons were used to determine the activity of hydrocarbon-utilizing bacteria during laboratory and in situ incubation.

MATERIALS AND METHODS

Chemicals. ^{14}C-labeled hydrocarbons of at least 98% purity were purchased from Amersham-Searle (Arlington Heights, Ill.). [^{14}C]glucose and Omnifluor were purchased from New England Nuclear Corp. (Boston, Mass.). Scintanalyzed toluene was purchased from Fisher Scientific Co. (Fair Lawn, N.J.). Preblend cocktail (3a70) was purchased from Research Products International (Elk Grove Village, Ill.).

[1] Present address: Environmental Technology Center, Martin Marietta Corp., 1450 South Rolling Rd., Baltimore, Md. 21227.

Sampling. Water samples were collected aseptically using a Niskin sterile-bag sampler (General Oceanics, Miami, Fla.).

Radioactivity systems. Except where stated otherwise, 30-ml serum bottles were filled with 10 ml of an oil salts solution (22), after which they were covered with aluminum foil and autoclaved. Rubber caps for the vials were fitted with a plastic cup (Kontes Co., Vineland, N.J., no. K882320), which served as a center well. These were autoclaved in a beaker of distilled water. Immediately after autoclaving, the rubber caps were used to seal the serum bottles. Prior to inoculation, a 20- by 60-mm piece of accordian-folded Whatman no. 1 filter paper was inserted into the center well. Substrates (1 μl) were added via a 10-μl syringe prior to inoculation. The amounts of substrate added varied: 1 μl of [U-^{14}C]glucose solution (specific activity, 1 mCi/mM) per ml of oil salts yielded 0.0076 μg/ml; 1 μl of [1-^{14}C]hexadecane (specific activity, 54.4 mCi/mM) per ml of oil salts yielded 77.5 μg/ml; 1 μl of [U-^{14}C]cyclohexane (specific activity, 14.0 mCi/mM) per ml of oil salts yielded 77.9 μg/ml; 1 μl of [*methyl*-^{14}C]toluene (specific activity, 26.4 mCi/mM) per ml of oil salts yielded 86.7 μg/ml; and 1 μl of [1-^{14}C]naphthalene (specific activity, 5.1 mCi/mM) per ml of oil salts yielded 114.5 μg/ml. Naphthalene was dissolved in hexane and was added to each bottle in solution. The hexane was removed by placing the bottle in an autoclave for approximately 3 min. This resulted in an approximate loss of 20% of the naphthalene radioactivity. If all the hexane did not evaporate, 99% of the radioactivity appeared as "CO_2" (evaporated naphthalene) adsorbed to the filter paper in the uninoculated controls incubated at 15 C within 5 to 10 min. Those bottles incubated at $\leq$15 C required addition of hydrocarbon substrate prior to

incubation. Incubation at ≤15 C, followed by addition of certain hydrocarbons (e.g., hexadecane), resulted in solidification of the hydrocarbon in the barrel of the syringe when the barrel touched the wall of the bottle at the time of addition of the hydrocarbon. The bottles were incubated quiescently in the dark at 5, 15, 25, and 35 C for up to 144 h. Two controls were used, uninoculated and inoculated bottles containing 100 μg of $HgCl_2$ per ml. Water from Chesapeake Bay (0.1 ml) or the Atlantic Ocean, north of Puerto Rico (1.0 ml), served as the inoculum. Experiments were run in duplicate, and separate bottles were used for measuring microbial growth (plate counts 22) and uptake of radioactivity.

The in situ incubations were run by adding Chesapeake Bay water samples to a dark, sterile serum bottle containing labeled substrate. The bottle was resealed with a rubber cap fitted with a plastic cup containing filter paper. This experimental unit was lowered below the surface of the water at the site of study for given periods of time.

Application of Michaelis-Menton kinetics to the in situ studies required that the experiment include five concentrations of [^{14}C]hexadecane, i.e., 0.04, 0.08, 0.4, 0.8, and 4.0 μM. The procedures used in this study were those applied by Hobbie and Crawford (9) in their measurements of heterotrophic activity, originally proposed by Smith (17).

Radioisotope analysis. At appropriate intervals, control and experimental bottles were removed from incubation. A 23-gauge needle, attached to a 5-ml glass syringe containing 1 ml of concentrated H_2SO_4, was used to puncture the rubber cap and to add 1 drop of H_2SO_4 to the medium. The pH was thereby lowered from 7.0 to 2.5. After approximately 2 h, 0.5 ml of phenethylamine was added to the center well by a syringe, after which the phenethylamine was adsorbed by the filter paper. After 24 h, the filter was removed and placed in a scintillation vial containing 10 ml of an Omnifluor solution (4 g of Omnifluor per liter of scintanalyzed toluene). The hydrocarbon vapors arising from evaporation, as well as the $^{14}CO_2$ derived from mineralization of the substrate, were adsorbed onto the filter. The amount of label arising from the hydrocarbon vapors was calculated from the control, and the number obtained was subtracted from that for $^{14}CO_2$ resulting from mineralization. Cyclohexane and toluene (99%) evaporated within minutes at 15 C. Therefore, cold traps were used to trap vapors quantitatively prior to measurement of CO_2 for cyclohexane and toluene (2). Naphthalene and hexadecane volatized at much slower rates (4 and 6% after 24 h, respectively) and could be used in our system. Cells were removed by filtering through 0.4-μm Nucleopore membranes. The cells were washed with 3 ml of *n*-hexane (3 ml of water for non-hydrocarbon substrates). The cell-free medium and 3 ml of hexane wash were added to the original serum bottle (except in experiments designed to measure activity washed from cells, in which case the hexane was counted separately), and the pH was adjusted to 9.1 by addition of 2 drops of 50% NaOH from a pasteur pipette. NaOH was added after cells were removed, particularly in the case of the inoculated, poisoned controls, which formed a $Hg(OH)_2$ precipitate that adhered to the filter used to collect the cells. The alkaline solution was transferred to a test tube (16 by 150 mm) and gently vortexed. The hexane layer was removed and added to 10 ml of Omnifluor solution, and the process was repeated twice with 3-ml aliquots of hexane. This procedure efficiently removed hydrocarbons from the medium (Table 1). To recover extractable acidic products in the medium, the pH was reduced to approximately 2.0 by the addition of a drop of concentrated H_2SO_4, and the acidified medium was extracted three times with 3-ml aliquots of hexane. A typical fatty acid (palmitic) was extracted with 90% efficiency. Finally, 1 ml of the extracted medium was added to 10 ml of 3a70 preblended liquid scintillation cocktail.

The phenethylamine-saturated filter paper, cells, extracts, and spent medium from inoculated cultures, poisoned cultures, and uninoculated controls were counted on an Intertechnique model SL-40 liquid scintillation spectrophotometer, using channels ratio for the filters and external standard ratio for the spent media for quench correction. The radioactivity present in the different fractions of the poisoned cultures was used to determine the extent of abiotic substrate alteration (e.g., adsorption of substrate to cells in the inoculum); the uninoculated controls were used to quantitate the loss of hydrocarbon from the medium by evaporation and/or adsorption to the glass or to the rubber stoppers. These values were used to correct results obtained for the inoculated cultures. Results were calculated as disintegrations per minute and expressed as percent (average of duplicate samples which did not differ by more than 7%) because of differences in total

TABLE 1. *Efficiency of extraction for recovering ^{14}C-labeled hydrocarbons*

Extraction	Hydrocarbon							
	Hexadecane		Cyclohexane		Toluene		Naphthalene	
	dpm	%	dpm	%	dpm	%	dpm	%
First	89,219	96.4	140,007	83.9	26,555	90.6	718,366	85.6
Second	2,816	3.2	2,168	13.0	1,324	4.5	32,818	3.9
Third	202	<0.1	107	<0.1	478	1.6	11,811	1.4
Extracted medium	334	<0.1	408	2.4	960	3.3	76,311	9.1
Total								
Extracted	92,237	99.6	16,282	97.0	28,357	96.7	761,995	90.9
Recovered	92,571	99.6	16,690	99.4	29,317	100.0	839,306	100.0

radioactivity added to each bottle for each hydrocarbon.

Percentage of petroleum-degrading bacteria was calculated as the number of colonies utilizing petroleum in liquid culture/total number of colonies on silica gel oil plates (25).

RESULTS AND DISCUSSION

Reaction termination. Acidifying, poisoning, and filtering are used to terminate reactions at the end of an incubation period. These methods were compared for hexadecane and naphthalene (Table 2). The addition of H_2SO_4 to the reaction mixture terminates activity but increases the temperature from 15 to 15.5 C which, in turn, causes increased volatilization of hydrocarbon, which then is recorded with the labeled CO_2 in the uninoculated and poisoned samples. Acidification of the sample is necessary to release all of the CO_2 from the solution. In the case of naphthalene, less radioactivity was associated with cells that were acidified, suggesting that naphthalene or an assimilated product of naphthalene is released into the medium (Table 2). After this work was completed, a report by Griffiths et al. (6) provided similar results for glutamic acid uptake. That is, cells recovered after washing at pH 7 contained 6×10^3 dpm of glutamate, whereas cells at pH 2 contained $<10^3$ dpm of glutamate. Since a relatively small amount of hexadecane was incorporated into the cells after 24 h, the effects of acidification on release of hexadecane or assimilated products could not be assessed.

Terminating the reaction by poisoning with $HgCl_2$ stopped further activity and did not cause the release of material by the cells, but did not release all of the CO_2 from solution.

Terminating the reaction by filtering yielded the highest counts of radioactivity associated with cells. Therefore, it is suggested that separate samples, with appropriate controls, be used for terminating the reaction by filtration to measure radioactivity associated with cells and for terminating the reaction by acidification to measure mineralization. This procedure was used for the remainder of the experiments reported in this paper as well as for those reported elsewhere (15, 16).

In addition to comparing methods for terminating activity, two other parameters were also examined. Cells not washed and cells washed with hexane to remove extracellular hydrocarbon were compared. Cells incubated in the presence of hexadecane and naphthalene, but not washed with hexane, yielded a higher percentage of radioactivity than those washed with hexane. Hexane, when used as a wash in this procedure, removes only extracellular hydrocarbon, as determined previously by Walker and Cooney (27). The hexane extract was separated into equal portions for examination of extracellular hydrocarbons, extracted alkaline medium, and extracted re-acidified medium of cells cultured on hexadecane. Results showed that 0.2% of the total radioactivity was associated with extracellular hydrocarbon, 2.1% with acidic products, and the remainder with unaltered hydrocarbon. The percentage of radioactivity associated with these fractions corresponded to the difference between unwashed and washed cells (extracellular hexadecane)

TABLE 2. *[^{14}C]n-hexadecane and [^{14}C]naphthalene activity associated with uninoculated and inoculated samples*

Sample	Treatment	% [^{14}C]n-hexadecane associated with:				%[^{14}C]naphthalene associated with:			
		CO_2	Cells	Medium	Extract	CO_2	Cells	Medium	Extract
Uninoculated	None	6.0	<0.1	<0.1	93.9	4.1	0.1	6.0	89.6
	Acidified	10.9	<0.1	<0.1	89.0	8.0	0.1	7.2	84.4
	Filtered	11.4	0.1	<0.1	88.2	6.2	0.1	8.1	85.5
	Filtered, acidified	25.6	0.1	<0.1	74.1	7.3	0.1	8.3	84.1
Inoculated and poisoned	None	6.0	0.1	<0.1	93.8	4.5	0.3	7.0	88.0
	Acidified	11.7	<0.1	<0.1	88.2	8.1	0.1	7.7	84.1
	Filtered	13.1	0.2	<0.1	86.6	5.8	0.3	7.9	85.9
	Filtered, acidified	26.4	0.2	<0.1	73.3	7.3	0.3	9.1	83.3
Inoculated	None[b]	9.5	0.1	2.0	88.0	8.3	0.4	8.0	83.0
	Acidified	16.7	0.1	2.1	80.9	12.1	<0.1	8.8	79.1
	Acidified[b]	17.0	0.3	<0.1	82.6	13.0	0.6	8.5	77.6
	Filtered	16.4	0.6	2.1	70.8	6.0	0.6	9.1	83.9
	Filtered, acidified	26.0	0.6	2.0	71.1	6.8	0.6	8.1	84.5

[a] Poisoned with 100 μg of HgCl per ml after 24 h of incubation at 15 C.

[b] Cells were not washed with hexane and no NaOH was added to the medium prior to extraction.

and between alkaline and acidified medium (acid products) (Table 2).

Mineralization potential at different temperatures. Preliminary experiments were conducted using bacteria indigenous to water from Colgate Creek of Baltimore Harbor in Chesapeake Bay as the inoculum. Colgate Creek is an area continuously exposed to oil and thus represents an active hydrocarbon-utilizing microbial population (20). Water collected from Colgate Creek during January 1974 was used to inoculate bottles incubated at 5, 15, 25, and 35 C containing 1 ml of [1-^{14}C]*n*-hexadecane. The rate of bacterial growth and cellular uptake of hexadecane increased with temperature, reaching a maximum at 25 C (Fig. 1). However, the percentage of hexadecane mineralized to CO_2 decreased as the temperature increased from 5 to 35 C (Fig. 1). The percentage of hexadecane found to be mineralized at 5 C substantiates previous results on the utilization of mixed hydrocarbon substrate at 0 C by Colgate Creek water bacteria collected in January 1973 (22). The amount of radioactivity associated with CO_2 (disintegrations per minute) remains about the same after the initial mineralization. However, the percentage of radioactivity associated with CO_2 decreases because of the increased activity associated with the cells (Fig. 1).

Correlation of activity with number or percentage of petroleum-degrading bacteria. Water collected from Colgate Creek in late November 1972 was used to compare log number and percentage of petroleum-degrading bacteria present in the water (25) with the amount of [^{14}C]hexadecane mineralized in Colgate Creek water incubated at 5, 15, 25, and 35 C for 12 h (Fig. 2). A stronger correlation was observed between percentage of petroleum-degrading bacteria and percentage of [^{14}C]hexadecane mineralized (r = 0.99) than between log number of petroleum-degrading bacteria and percentage of [^{14}C]hexadecane mineralized (r = 0.86) (Fig. 3). Presenting the data as percentages provides better correlation because a normalization for total numbers on each plate at each depth, station, etc. is achieved.

We used this approach in comparing percentage of petroleum-degrading bacteria with per-

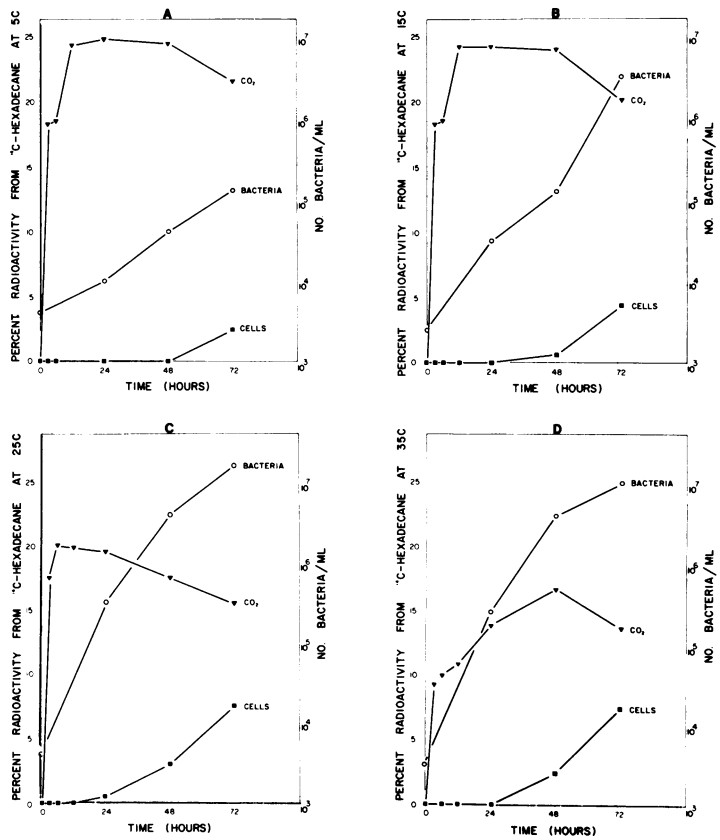

FIG. 1. *Growth of Colgate Creek bacteria (○) on [^{14}C]hexadecane, uptake of radioactivity by the cells (■), and mineralization to CO_2 (▼) at 5 C (A), 15 C (B), 25 C (C), and 35 C (D).*

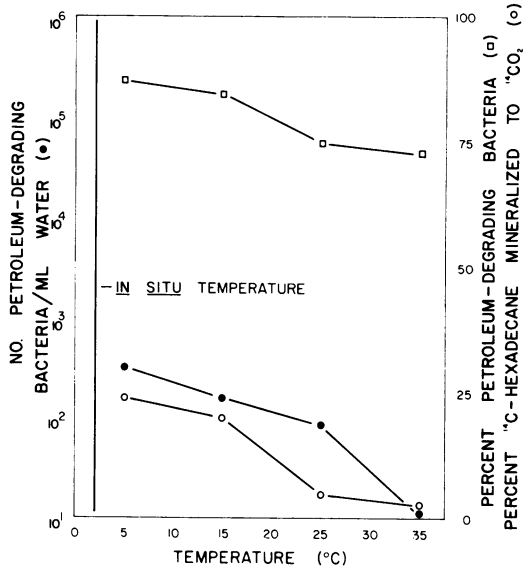

FIG. 2. *Comparison of number and percentage of petroleum-degrading bacteria with percentage of [^{14}C]hexadecane mineralized to $^{14}CO_2$.*

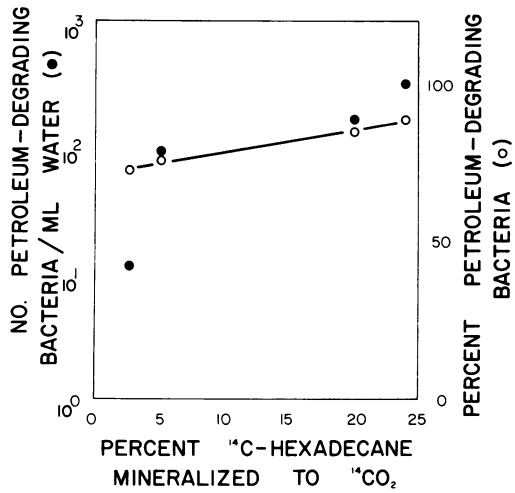

FIG. 3. *Correlation of percentage of petroleum-degrading bacteria and number of petroleum-degrading bacteria with percentage of [^{14}C]hexadecane mineralized to CO_2.*

centage of [^{14}C]hexadecane mineralized (r = 0.96) to evaluate the activity of bacterial populations in sea water. Water samples collected in the open ocean approximately 50 miles (ca. 80.5 km) north of Puerto Rico were used to inoculate bottles of oil salts incubated at 15 C for 12 h and to enumerate petroleum-degrading bacteria (Fig. 4). The activity of the petroleum-degrading population was found to be correlated with a lower percentage of petroleum degraders at the midlevel depth of 2,300 m (Fig. 4). It is suggested that hydrocarbon-utilizing activity be examined in conjunction with percentage of petroleum degraders present in the total heterotrophic population, as well as with amount of petroleum present in the environment under study (25). The amount of hydrocarbon used in our experiments (0.08 μg/g of medium) compares with the amount of hydrocarbon present in Eastern Bay and Colgate Creek water (0.05 to 0.5 μg/g of water).

Mineralization of different hydrocarbons. Using methods described above, the mineralization of four [^{14}C]hydrocarbons by bacteria in Colgate Creek water collected in May 1974 and incubated at 15 C in oil salts was compared (Fig. 5). Naphthalene was mineralized to the same extent as hexadecane, although the rate of hexadecane mineralization was higher. Toluene, the methylated analogue of benzene, is considered to be more toxic than naphthalene. Greater utilization of naphthalene than toluene was observed, which corroborates results obtained previously, using Colgate Creek water as inoculum, in studies of the degradation of pure hydrocarbons (3), mixed hydrocarbon substrate (21), motor oil (26), and Colgate Creek petroleum extracts (24). Cyclohexane is most likely mineralized by co-metabolism or commensalism (2).

Mineralization of glucose and hexadecane. Comparison of [^{14}C]glucose and [^{14}C]hexadecane utilization by microorganisms in Colgate Creek collected in July 1974 indicated that there was a lag in utilization of glucose, but not for hexadecane (Fig. 6). Similar results have been reported in laboratory studies (5). The

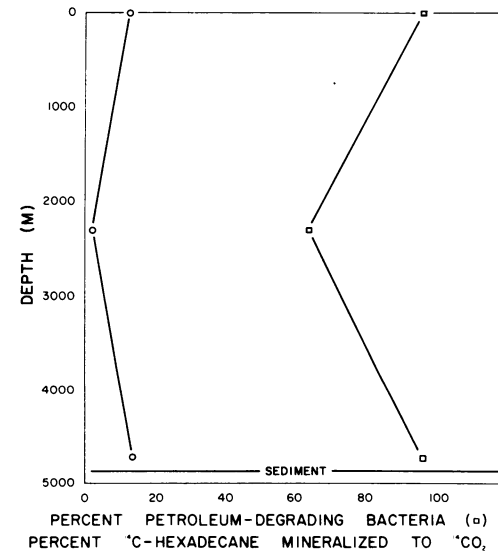

FIG. 4. *Comparison of percentage of petroleum-degrading bacteria with percentage of [^{14}C]hexadecane mineralized to $^{14}CO_2$.*

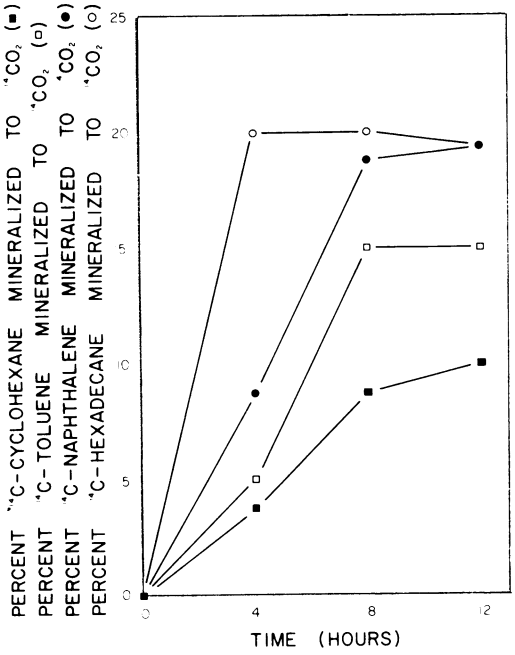

FIG. 5. *Comparison of mineralization of [^{14}C]hexadecane (○), [^{14}C]naphthalene (●), [^{14}C]toluene (□), and [^{14}C]cyclohexane (■) by Colgate Creek water bacteria.*

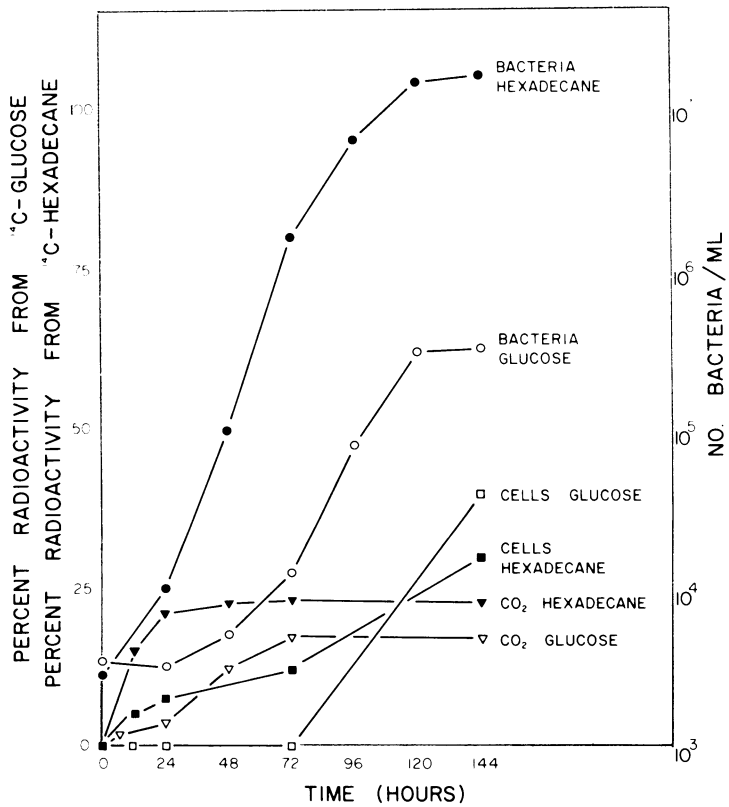

FIG. 6. *Growth of Colgate Creek bacteria on [^{14}C]glucose (○) and [^{14}C]hexadecane (●), cellular uptake of radioactivity from [^{14}C]glucose (□) and [^{14}C]hexadecane (■), and mineralization of [^{14}C]glucose (▽) and [^{14}C]hexadecane (▼) to CO_2.*

presence of hydrocarbons in bacterial cultures from oil-contaminated Colgate Creek may inhibit utilization of glucose until intracellular hydrocarbons have been assimilated.

Mineralization potential of Colgate Creek and Eastern Bay bacteria. Previous studies comparing Colgate Creek and Eastern Bay, a commercially productive shellfish area in Chesapeake Bay, demonstrated that there were greater numbers of petroleum-degrading microorganisms in Colgate Creek samples and an increased petroleum-degrading potential displayed by the microorganisms from Colgate Creek. Comparison of [^{14}C]hexadecane utilization, using water collected in March 1974, provided results confirming the observations of the earlier studies (Fig. 7).

As another means of comparing [^{14}C]-hexadecane mineralization by bacteria in Colgate Creek and Eastern Bay water, in situ incubations were conducted at each station during June 1974, when the water temperature was 24.5 C. Bottles containing substrate and inoculum were incubated in the water for 1 h. The turnover times (incubation time/fraction of added radioactivity mineralized = minutes) for [^{14}C]hexadecane were calculated to be 15 min and 1 h for heterotrophs in Colgate Creek and Eastern Bay water samples, respectively (Fig. 8). V_{max} was 1 μg of C/liter per h for Colgate Creek and 0.3 μg of C/liter per h for Eastern Bay (Fig. 8).

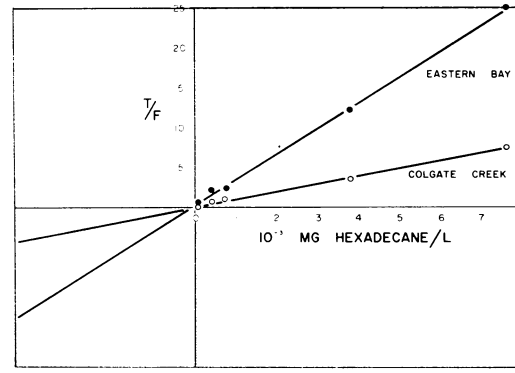

FIG. 8. *T (incubation time)/F (fraction of the added radioactivity mineralized) versus amount of substrate added to yield mineralization of [^{14}C]-hexadecane by bacteria from water of Colgate Creek and Eastern Bay, according to a modified Michaelis-Menten equation (9).*

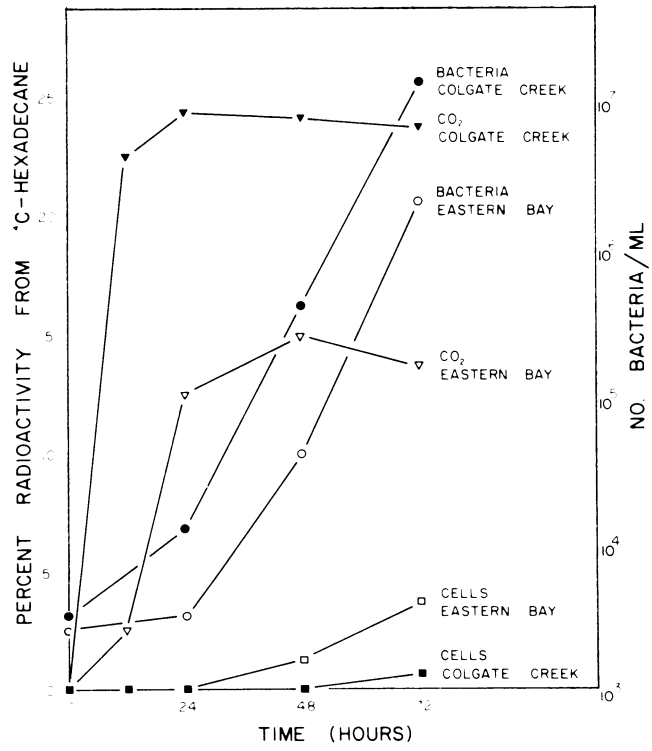

FIG. 7. *Growth of bacteria from Colgate Creek (●) and Eastern Bay (○) on [^{14}C]hexadecane, uptake of radioactivity from [^{14}C]hexadecane by cells from Colgate Creek (■) and Eastern Bay (□), and mineralization of [^{14}C]hexadecane to CO_2 by bacteria of Colgate Creek (▼) and Eastern Bay (▽) at 10 C.*

In summary, [^{14}C]hydrocarbons can be used to evaluate the hydrocarbon-utilizing activity of bacteria in the marine environment. However, proper controls are mandatory for correct interpretation of results, as has also been emphasized by Wright (35).

ACKNOWLEDGMENTS

The excellent technical assistance of H. F. Austin and B. F. Conrad is gratefully acknowledged.

This work was supported by contract no. N00014-67-A-0239-0027-02 between the Office of Naval Research and the University of Maryland.

LITERATURE CITED

1. **Banoub, M. W., and P. J. Le B. Williams.** 1972. Measurements of microbial activity and organic material in the Western Mediterranean Sea. Deep Sea Res. 19:433-443.
2. **Beam, H. W., and J. J. Perry.** 1974. Microbial degradation of cycloparaffinic hydrocarbons via co-metabolism and commensalism. J. Gen. Microbiol. 82:163-169.
3. **Colwell, R. R., J. D. Walker, and J. D. Nelson.** 1973. Microbial ecology and the problem of petroleum degradation in Chesapeake Bay, p. 185-197. *In* D. G. Ahearn and S. P. Meyers (ed.), The microbial degradation of oil pollutants. Louisiana State University Publ. no. LSU-SG-73-01, Baton Rouge.
4. **Crawford, C. C., J. E. Hobbie, and K. L. Webb.** 1973. Utilization of dissolved organic compounds in an estuary, p. 169-180. *In* L. H. Stevenson and R. R. Colwell (ed.), Estuarine microbial ecology. University of South Carolina Press, Columbia.
5. **Gill, C. O., and C. Ratledge.** 1973. Inhibition of glucose assimilation and transport by *n*-decane and other *n*-alkanes in *Candida* 107. J. Gen. Microbiol. 75:11-22.
6. **Griffiths, R. P., F. J. Hanus, and R. Y. Morita.** 1974. The effects of various water-sample treatments on the uptake of glutamic acid by natural marine microbial populations. Can. J. Microbiol. 20:1261-1266.
7. **Hamilton, R. D., and J. E. Preslan.** 1970. Observations on heterotrophic activity in the Eastern Tropical Pacific. Limnol. Oceanogr. 15:395-401.
8. **Herbland, A. M., and J. F. Bois.** 1974. Assimilation and mineralization of the dissolved organic matter in the sea: liquid scintillation counting method. Mar. Biol. 24:203-212.
9. **Hobbie, J. E., and C. C. Crawford.** 1969. Respiration corrections for bacterial uptake of dissolved organic compounds in natural waters. Limnol. Oceanogr. 14:528-532.
10. **Hobbie, J. E., C. C. Crawford, and K. L. Webb.** 1968. Amino acid flux in an estuary. Science 159:1463-1464.
11. **Hobbie, J. E., O. Holm-Hansen, T. T. Packard, L. R. Pomeroy, R. W. Sheldon, J. P. Thomas, and W. J. Wiebe.** 1973. A study of the distribution and activity of microorganisms in ocean water. Limnol. Oceanogr. 17:544-555.
12. **Jannasch, H. W., and C. O. Wirsen.** 1973. Deep-sea microorganisms: *in situ* response to nutrient enrichment. Science 180:641-643.
13. **Parsons, T. R., and J. D. H. Strickland.** 1962. On the production of particulate organic carbon by heterotrophic processes in sea water. Deep Sea Res. 8:211-222.
14. **Robertson, B., S. Arhelger, P. J. Kinney, and D. K. Button.** 1973. Hydrocarbon biodegradation in Alaskan waters, p. 171-184. *In* D. G. Ahearn and S. P. Meyers (ed.), The microbial degradation of oil pollutants. Louisiana State University Publ. no. LSU-SG-73-01, Baton Rouge.
15. **Schwarz, J. R., J. D. Walker, and R. R. Colwell.** 1974. Deep-sea bacteria: growth and utilization of hydrocarbons at ambient and in situ pressure. Appl. Microbiol. 28:982-986.
16. **Schwarz, J. R., J. D. Walker, and R. R. Colwell.** 1975. Deep-sea bacteria: growth and utilization of *n*-hexadecane at *in situ* temperature and pressure. Can. J. Microbiol. 21:682-687.
17. **Smith, D. E.** 1967. Location of estrogen effect on uterine glucose metabolism. Proc. Soc. Exp. Biol. Med. 124:747-749.
18. **Vaccaro, R. F., and H. W. Jannasch.** 1966. Studies on heterotrophic activity in seawater based on glucose assimilation. Limnol. Oceanogr. 11:596-607.
19. **Vaccaro, R. F., and H. W. Jannasch.** 1967. Variation in uptake kinetics for glucose by natural populations in seawater. Limnol. Oceanogr. 12:540-542.
20. **Walker, J. D., and R. R. Colwell.** 1973. Microbial ecology of petroleum utilization in Chesapeake Bay, p. 685-690. *In* API/EPA/USCG conference on prevention and control of oil spills. American Petroleum Institute, Washington, D.C.
21. **Walker, J. D., and R. R. Colwell.** 1975. Degradation of hydrocarbons and mixed hydrocarbon substrates by Chesapeake Bay microorganisms. Prog. Water Technol., vol. 7, in press.
22. **Walker, J. D., and R. R. Colwell.** 1974. Microbial degradation of model petroleum at low temperatures. Microb. Ecol. 1:63-95.
23. **Walker, J. D., and R. R. Colwell.** 1974. Microbial degradation of petroleum: use of mixed hydrocarbon substrates. Appl. Microbiol. 27:1053-1060.
24. **Walker, J. D., and R. R. Colwell.** 1975. Role of autochthonous bacteria in removal of spilled oil from sediment. Environ. Pollut., in press.
25. **Walker, J. D., and R. R. Colwell.** 1976. Enumeration of petroleum-degrading microorganisms. Appl. Environ. Microbiol. 31:198-207.
26. **Walker, J. D., R. R. Colwell, and L. Petrakis.** 1975. Bacterial degradation of motor oil. J. Water Pollut. Control Fed. 47:2058-2066.
27. **Walker, J. D., and J. J. Cooney.** 1973. Aliphatic hydrocarbons of *Cladosporium resinae* cultured on glucose, glutamic acid, and hydrocarbons. Appl. Microbiol. 26:705-708.
28. **Williams, P. J. Le B.** 1970. Heterotrophic utilization of dissolved organic compounds in the sea. I. Size distribution of population and relationship between respiration and incorporation of growth substrates. J. Mar. Biol. Assoc. U.K. 50:859-870.
29. **Williams, P. J. Le B., and P. Andrews.** 1971. Heterotrophic utilization of dissolved organic compounds in the sea. III. Measurement of the oxidation rates and concentrations of glucose and amino acids in seawater. J. Mar. Biol. Assoc. U.K. 51:111-125.
30. **Williams, P. J. Le B., and C. Askew.** 1968. A method of measuring the mineralization by microorganisms of organic compounds in seawater. Deep Sea Res. 15:365-375.
31. **Williams, P. J. Le B., and R. W. Gray.** 1970. Heterotrophic utilization of dissolved organic compounds in the sea. II. Observations on the response of heterotrophic marine populations to abrupt increases in amino acid concentration. J. Mar. Biol. Assoc. U.K. 50:871-881.
32. **Wirsen, C. O., and H. W. Jannasch.** 1974. Microbial transformations of some ^{14}C-labeled substrates in coastal water and sediment. Microb. Ecol. 1:25-37.
33. **Wood, L. W.** 1973. Monosaccharide and disaccharide interactions on uptake and catabolism of carbohydrates by mixed microbial communities, p. 181-197.

In L. H. Stevenson and R. R. Colwell (ed.), Estuarine microbial ecology. University of South Carolina Press, Columbia.

34. **Wright, R. T.** 1970. Glycollic acid uptake by planktonic bacteria, p. 521-536. *In* D. W. Hood (ed.), Organic matter in natural waters. Institute of Marine Science, University of Alaska, Fairbanks.
35. **Wright, R. T.** 1973. Some difficulties in using ^{14}C-organic solutes to measure heterotrophic bacterial activity, p. 199-217. *In* L. H. Stevenson and R. R. Colwell (ed.), Estuarine microbial ecology. University of South Carolina Press, Columbia.