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Modification of the ^{14}C Most-Probable-Number Method for Use with Nonpolar and Volatile Substrates

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A method was developed to allow the use of volatile and nonpolar substrates in ^{14}C most-probable-number tests. Naphthalene or hexadecane was sorbed to filter paper disks and submerged in minimal medium. The procedure reduced the volatilization of the substrates while allowing them to remain available for microbial degradation.

The enumeration of microorganisms involved in the degradation of xenobiotic compounds is a logical step in understanding the fate of the compounds. The fraction of the microbial community with the ability to degrade hydrocarbons fluctuates widely from place to place and can be correlated with previous exposure to hydrocarbon pollution (1, 4, 9, 15, 18). Thus, the enumeration of specific degrader populations should be valuable in predicting the fate of hydrocarbons and may also prove important in assessing the effect of previous exposures.

A number of methods have been used to quantitate hydrocarbonoclastic organisms. Addition of petroleum hydrocarbons to agar-based media and hydrocarbon vapors in conjunction with solid media have been used extensively (3, 4, 7, 8, 14, 18). Seki (12) and Walker and Colwell (16) used silica gel as a solidifying agent to reduce the concentration of free organic compounds in the medium. Unfortunately, not all of the isolates from the hydrocarbon media can be confirmed as degraders (2).

Most-probable-number (MPN) methods eliminate the need for solidifying agents and permit greater chemical definition of the medium (6). Lehmicke et al. (10) developed a streamlined ^{14}C MPN method in which substrate concentrations are held to microgram-per-liter levels and evolution of the metabolic end product, $^{14}CO_2$, indicates mineralization of the substrate of interest. The method is particularly useful with water-soluble, nonvolatile compounds. MPN methods with hydrocarbon substrates are more difficult to apply because of the limited solubility of the test compounds. Even when appropriate concentrations can be attained, the substrate is often lost by volatilization. Carrier solvents can be used to introduce minute quantities of substrate to individual culture tubes, but care must be taken to account for the effect of the carrier on the inoculum. Gibson et al. (5) introduced benzene into a culture medium by mixing it with molten wax. After the wax solidified, it was aseptically placed into the appropriate medium. This method, however, is most applicable to high substrate concentrations and is impractical when a large number of replicate culture tubes are required. Wyndham and Costerton (17) added radiolabeled compounds to a maltene silica gel substrate for use in MPN estimation but noted problems with volatilization and recovery. Because the maltene fraction includes a complex mixture of hydrocarbons, it cannot be used for the enumeration of organisms degrading specific hydrocarbons as the sole carbon source. Similar limitations apply to the addition of radiolabeled hydrocarbons to crude oil as described by Atlas and co-workers (1, 11).

We developed a procedure by which hexadecane, naphthalene, and similar nonpolar compounds can be used as sole substrates in a ^{14}C MPN test such as that described by Lehmicke et al. (10).

The enumeration medium was a minimal salts broth (13) adjusted to pH 7.6. Salinity was adjusted by the addition of NaCl. Samples (1 ml) of the basal medium were dispensed into 4-ml plastic vials (Omnivials; Wheaton Industries, Millville, N.J.). The vials were capped, sterilized by autoclaving, and stored at 5°C until used. *N*-[^{14}C]hexadecane (53 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) was diluted in hexane to 8.25 mg/liter, and 5.0-μl samples were transferred to sterile filter paper disks (Sensi-Discs; BBL Microbiology Systems, Cockeysville, Md.). The hexane was allowed to evaporate for 10 min; then the disks were placed in separate vials of minimal salts broth. This procedure resulted in a nominal substrate concentration of 41 μg/liter and 2×10^4 dpm per vial.

[ring-U-^{14}C]naphthalene (4.7 mCi/mmol; kindly provided by D. T. Gibson, University of Texas) was diluted to 26 mg/liter in acetone; 7 μl of the solution was transferred to each filter paper disk, and the disks were immediately placed in vials containing minimal salts broth. The final substrate concentration was 144 μg/liter and 1.2×10^4 dpm per vial.

Vials were inoculated with 0.1 ml of an appropriately diluted environmental sample and incubated without a cap in a tightly capped scintillation vial that contained 1 ml of 1 N NaOH. Vials used for the naphthalene MPN tests were first inoculated with the samples, and the substrate was added just before they were placed in the scintillation vials. After incubation for 2 weeks, the small inner vials were removed, Carbon-14 scintillation cocktail (R. J. Harvey Instrument Co.) was added to the NaOH, and the radioactivity was measured by liquid scintillation counting. Any vial that exceeded the background by 1% of the total available counts was scored positive. Five uninoculated control vials were included with each experiment. MPN values were taken from tables in reference 9a.

The filter paper disks sink when placed in an aqueous medium; therefore, the distribution of the substrate is determined by its partition coefficient. Hexadecane is insoluble in

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TABLE 1. Enumeration of hexadecane degraders in water from Escambia River near Pensacola, Florida[a]

Dilution	Radioactivity recovered as $^{14}CO_2$		Scoring[b]
	dpm	%	
10^{-3}	2,613	13	+
	2,628	13	+
	3,077	15	+
	3,886	20	+
	3,150	16	+
10^{-4}	2,776	14	+
	2,754	14	+
	3,493	18	+
	3,653	18	+
	4,013	20	+
10^{-5}	88	<1	−
	2,362	12	+
	78	<1	−
	2,629	13	+
	79	<1	−
10^{-6}	134	<1	−
	115	<1	−
	206	<1	−
	103	<1	−
	112	<1	−

[a] Initial hexadecane concentration was 39 μg/liter (1.9×10^4 dpm/vial). Background radioactivity in NaOH from uninoculated controls averaged 109 ± 10 dpm. MPN for this example was 5.0×10^4 hexadecane degraders per ml.

[b] +, Radioactivity exceeded the background by at least 1% of the total available counts; −, radioactivity did not exceed the background by at least 1% of the total available counts.

water and remains almost entirely associated with the disk. After 50 h of incubation, over 98% of the radioactivity initially added to the uninoculated control vials could be recovered from the disk; less than 2% was detected in the overlying water. Over 90% of the radioactive hexadecane could be recovered from uninoculated vials after the 2-week incubation period. Mineralization of [^{14}C]hexadecane in inoculated vials indicated that the substrate was available for microbial degradation (Table 1). Because of the relatively

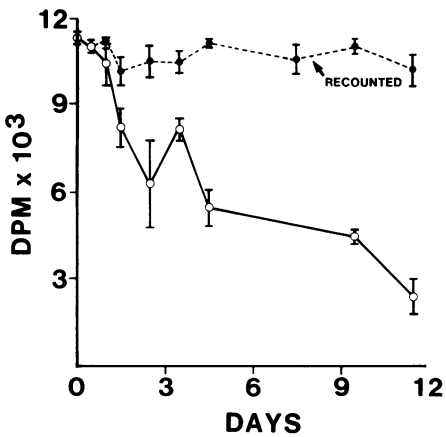

FIG. 1. [^{14}C]naphthalene remaining in plastic vials. Radioactivity was measured immediately after the addition of scintillation cocktail (○) and again after standing for 12 h (●). Points are means of triplicate samples, and error bars represent ± one standard deviation.

TABLE 2. Enumeration of naphthalene degraders in water from Santa Rosa Sound near Pensacola, Florida[a]

Dilution	Radioactivity recovered as $^{14}CO_2$		Scoring[b]
	dpm	%	
10^{-1}	1,204	10	+
	1,004	8	+
	1,144	9	+
	1,285	10	+
	1,125	9	+
10^{-2}	1,489	12	+
	1,411	11	+
	1,613	13	+
	972	8	+
	672	5	+
10^{-3}	52	<1	−
	124	<1	−
	54	<1	−
	685	5	+
	56	<1	−

[a] Initial naphthalene concentration was 144 μg/liter (1.2×10^4 dpm per vial). Background radioactivity in NaOH from uninoculated controls averaged 50 ± 2 dpm. MPN for this example was 3.5×10^2 naphthalene degraders per ml.

[b] See Table 1, footnote *b*.

brief incubation period, only 12 to 20% of the added radioactivity was recovered as $^{14}CO_2$.

Naphthalene is much more volatile than hexadecane. It evaporated within 2 min from paper disks exposed to air. When disks were submerged in sterile minimal salts broth in the mineralization test system, the naphthalene gradually disappeared from the paper disks and from the aqueous medium (Fig. 1). Attempts to recover the radioactive naphthalene from the atmosphere of the larger scintillation vial and from the NaOH were unsuccessful. When the plastic inner vials were filled with scintillation cocktail and allowed to stand overnight, the radioactivity increased to the initial level. Either the naphthalene sorbed reversibly to the plastic vials and was extracted by solvents in the scintillation cocktail, or the scintillation cocktail penetrated the walls of the vial and came in contact with the sorbed naphthalene.

Naphthalene was available for mineralization by microorganisms in the plastic vials during the 2-week incubation period (Table 2). The fraction of the radioactivity converted to $^{14}CO_2$ was low but easily distinguishable from background. When small glass vials were substituted for the plastic vials, the naphthalene volatilized from the test systems and could not be recovered. Wyndham and Costerton (17) noted similar problems with volatilization even when the naphthalene was sorbed to silica gel. Mineralization was extensive when large inocula were included, but the glass vials would not be suitable in situations in which metabolism of the substrate was delayed.

The ^{14}C MPN method for the enumeration of hydrocarbon degraders is unique in that it provides direct, unambiguous evidence for the mineralization of a specific compound within a culture medium. Lehmicke et al. (10) refined the basic technique to determine the number of degrader organisms active at natural substrate concentrations. Our modification allows the use of compounds that are not readily incorporated into liquid media. In addition, the method eliminates the need for a carrier solvent or inclusion in a complex hydrocarbon mixture.

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