APPLIED AND ENVIRONMENTAL MICROBIOLOGY, June 1979, p. 1211-1216
0099-2240/79/06-1211/06$02.00/0
Vol. 37, No. 6

Effect of Concentration of Organic Chemicals on Their Biodegradation by Natural Microbial Communities

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Received for publication 4 October 1978

The effect of concentration on the biodegradation of synthetic organic chemicals by natural microbial communities was investigated by adding individual ^{14}C-labeled organic compounds to stream water at various initial concentrations and measuring the formation of $^{14}CO_2$. The rate of degradation of *p*-chlorobenzoate and chloroacetate at initial concentrations of 47 pg/ml to 47 μg/ml fell markedly with lower initial concentrations, although half or more of the compound was converted to CO_2 in 8 days or less. On the other hand, little mineralization of 2,4-dichlorophenoxyacetate and 1-naphthyl-*N*-methylcarbamate, or the naphthol formed from the latter, occurred when these compounds were present at initial concentrations of 2 to 3 ng/ml or less, although 60% or more of the chemical initially present at higher concentrations was converted to CO_2 in 6 days. It is concluded that laboratory tests of biodegradation involving chemical concentrations greater than those in nature may not correctly assess the rate of biodegradation in natural ecosystems and that low substrate concentration may be important in limiting biodegradation in natural waters.

The susceptibility of an organic chemical to microbial destruction in nature may be attributable to environmental factors or to the structure of the chemical itself. The influence of chemical structure on biodegradability is well documented (1), but the significance of only a few of the environmental factors has been established. Concentration of the compound may be a significant factor affecting its susceptibility to microbial attack, and organic chemicals may persist in some environments as a result of low prevailing concentration or low solubility in water. For example, evidence exists that the rates of microbial liberation of nitrogen from dialkylmethylenediureas (8) and of bacterial growth on a series of polycyclic aromatic compounds (15) are limited by solubility. In addition, Jannasch (9) has demonstrated that normally biodegradable substrates may not be metabolized by marine bacteria at significant rates when the compounds are present at low concentrations. Unfortunately, these studies have not led to useful generalizations regarding the role of substrate concentration in determining the biodegradability of organic pollutants in nature.

The present investigation was designed to determine the effect of concentration of synthetic organic chemicals on their biodegradation in aquatic microcosms. The experimental system used natural microbial communities from stream water and was designed to reflect the complex interactions among members of these communities rather than the behavior of an individual species.

MATERIALS AND METHODS

Stream water. Water samples were obtained from Fall Creek, a tributary of Cayuga Lake in central New York. The stream receives treated sewage from a village and runoff from adjacent agricultural areas upstream from the sampling location. The water was analyzed by standard methods (3), the analysis showing that pH, total alkalinity, total suspended solids, and turbidity ranges during the study period were 7.5 to 8.6, 80 to 140 μg of $CaCO_3$ equivalents per ml, <130 μg/ml, and <90 nephelometric turbidity units, respectively, with typical values being 8.0, 110, <40, and <30, respectively. The bacterial count ranged from 8×10^3 to 8×10^5 per ml, with the typical count being approximately 5×10^4/ml. Water samples were collected from April 1977 to April 1978, and the highest values for suspended solids, turbidity, and bacterial count were obtained in the spring and during periods of heavy rainfall.

Experimental system. Processing of the stream samples, the experimental system, methodology, means for determining bacterial counts, and inorganic nutrient supplements have been described (R. S. Boethling and M. Alexander, manuscript submitted). Microbial activity was assayed by measurement of $^{14}CO_2$ produced from ^{14}C-labeled organic chemicals added to stream water. Incubation was carried out at 29°C in 500-ml filtering flasks with specially designed closures. Respired $^{14}CO_2$ was collected quantitatively

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after acidification of the medium, and the radioactivity was counted by liquid scintillation in a Beckman LS-100C liquid scintillation spectrometer (Beckman Instruments, Inc., Fullerton, Calif.). The data were corrected for quench by the external standard-channels ratio method and expressed as disintegrations per minute. Control experiments showed that none of the ^{14}C-labeled compounds used was degraded to $^{14}CO_2$ in 5 days in 100-ml samples of stream water autoclaved for 30 min.

Uptake and sorption experiments. In uptake experiments, cells and suspended solids in stream water were collected on 0.45-μm membrane filters after addition of ^{14}C-labeled organic chemicals to the water. The filters were then dissolved in 9.0 ml of scintillation fluid (5) and counted. In some sorption experiments, 1.0 ml of stream water was removed before and after filtration, and the water samples, filtrate and filter were counted separately in 9.0 ml of scintillation fluid. Ultracentrifugation was carried out at 110,000 × *g* for 3 h. Apparent sorption of either 1-naphthyl-*N*-methylcarbamate (Sevin) or 1-naphthol or both to the incubation vessels was measured by counting 1.0-ml samples withdrawn at intervals from stream water autoclaved for 30 min and supplemented with ^{14}C-labeled Sevin. The difference between initial ^{14}C and the ^{14}C obtained in this way represents apparent sorption to the incubation vessel.

Analytical methods. Determinations of 2,4-dichlorophenoxyacetate (2,4-D), Sevin, and 1-naphthol were performed by measuring ultraviolet absorbancy at 283, 280, and 321 nm, respectively. In experiments in which initial concentrations of 22 μg/ml of 2,4-D and 30 μg/ml of Sevin were used, spectrophotometric analysis indicated that the actual initial concentrations were ±5% of the stated values. Measurements of dissolved O_2 were made by the azide modification of the Winkler method (3).

Radiochemicals and reagents. Unlabeled 2,4-D, chloroacetic acid, and *p*-chlorobenzoic acid were obtained from Aldrich Chemical Co., Milwaukee, Wis. Unlabeled Sevin (analytical reagent grade) was obtained from Union Carbide Corp., Salinas, Calif. The purity of these chemicals was confirmed by melting point analyses. The sources and specific activities of the radiochemicals used were as follows: 2,4-dichlorophenoxy[2-^{14}C]acetic acid (Amersham Corp., Arlington Heights, Ill.), 32 mCi/mmol; chloro[2-^{14}C]acetic acid (Amersham), 7.45 mCi/mmol; *p*-chloro[ring-*U*-^{14}C]benzoic acid (California Bionuclear Corp., Sun Valley, Calif.), 10.5 mCi/mmol; and 1-[1-^{14}C]naphthyl-*N*-methylcarbamate (California Bionuclear), 21 mCi/mmol.

RESULTS

The formation of $^{14}CO_2$ in the biodegradation of *p*-chloro[ring-*U*-^{14}C]benzoate added to stream water at four concentrations is shown in Table 1. The data are expressed as the percent of the initial ^{14}C-labeled compound recovered as $^{14}CO_2$ in the left portion of the table and, in the right portion, as the actual quantities of CO_2 formed. At the two lowest initial concentrations of *p*-chlorobenzoate, 47 pg/ml and 4.7 ng/ml, a substantial percentage of the initial material was degraded within 4 days. In contrast, a much smaller fraction was degraded within the time of study at the highest initial concentration, 47 μg/ml. When the data are expressed as absolute amounts of CO_2 formed, the yields of CO_2 were 1 to 2 orders of magnitude lower with each successively lower initial concentration, although residual substrate was still available.

Similar results were obtained with the herbicide chloroacetate (Table 2). Large percentages were degraded within 4 days at initial levels of 47 pg/ml and 4.7 ng/ml, but at least 8 days elapsed before a significant fraction of chloroacetate was degraded at 47 μg/ml. However, the actual rates of degradation, when expressed as nanomoles of CO_2 formed per liter, again were

TABLE 1. *$^{14}CO_2$ produced from p-chloro[ring-U-^{14}C]benzoate added to stream water*

Days	% of ^{14}C recovered as $^{14}CO_2$				nmol of CO_2 formed/liter			
	47 μg/ml[a]	470 ng/ml	4.7 ng/ml	47 pg/ml	47 μg/ml[a]	470 ng/ml	4.7 ng/ml	47 pg/ml
4	1.2	2.0	17.1	47.6	21,600	360	30.8	0.857
6	1.4	3.6	33.0	51.3	25,200	648	59.4	0.923
8			48.0	60.3			86.4	1.09
12	5.9	79.0			106,000	14,200		

[a] Initial *p*-chlorobenzoate concentration.

TABLE 2. *$^{14}CO_2$ produced from chloro[2-^{14}C]acetate added to stream water*

Days	% of ^{14}C recovered as $^{14}CO_2$				nmol of CO_2 formed/liter			
	47 μg/ml[a]	470 ng/ml	4.7 ng/ml	47 pg/ml	47 μg/ml[a]	470 ng/ml	4.7 ng/ml	47 pg/ml
4	0.5	24.3	52.6	44.8	2,500	1,200	26.3	0.224
6	3.9	64.6	69.6	51.6	19,500	3,230	34.8	0.258
8	5.4	73.5			27,000	3,680		
10	72.6	75.9			363,000	3,800		

[a] Initial chloroacetate concentration.

proportional to the initial substrate concentration.

The apparent microbial assimilation of ^{14}C from labeled substrates was measured by collecting and counting the cells and suspended solids from 100-ml samples of stream water incubated for 7 or 14 days with two initial concentrations of each compound. The formation of $^{14}CO_2$ was also measured, but different flasks were used for measurements of apparent ^{14}C assimilation and $^{14}CO_2$ formation. This procedure avoided difficulties in interpretation of data owing to the loss of ^{14}C-labeled solutes from cells after acidification of the water (13). The data in Table 3 show that only a small fraction of the initial ^{14}C-labeled chemical was assimilated, regardless of the initial concentration.

No loss of ^{14}C from solution was observed when autoclaved stream water was incubated with 4.7 ng/ml of *p*-chlorobenzoate or chloroacetate at 29°C for 2 days, and the water samples were centrifuged as described. Thus, the ^{14}C-labeled material recovered by filtration represented microbial uptake rather than sorption of these chemicals to suspended solids in the stream water.

The effect of concentration on the rate of biodegradation was strikingly different for 2,4-D from that observed for *p*-chlorobenzoate and chloroacetate. At initial 2,4-D concentrations of 22 μg/ml and 220 ng/ml, more than two-thirds of the C-2 carbon of the acetate moiety was converted to CO_2 within 6 days, but less than 10% was converted to CO_2 in 8 days at initial levels of 2.2 ng/ml and 22 pg/ml (Fig. 1). This difference was even more pronounced if the actual amounts of CO_2 generated at the four initial 2,4-D concentrations are compared. The rate of

TABLE 3. *$^{14}CO_2$ produced and apparent assimilation of ^{14}C from organic compounds added to stream water*[a]

Compound	Initial concn (ng/ml)	% of ^{14}C recovered as $^{14}CO_2$	% of ^{14}C recovered on filter
2,4-Dichlorophenoxy[2-^{14}C]-acetate (2,4-D)	22,000	73.2	4.2
	2.2	6.2	1.2
1-[1-^{14}C]naphthyl-*N*-methylcarbamate	30,000	72.3	
	3.0	13.4	8.8
Chloro[2-^{14}C]acetate	47,000	6.3	3.5
	4.7	73.7	9.6
p-Chloro[ring-*U*-^{14}C]benzoate	47,000	1.6	0.2
	4.7	24.5	4.9

[a] Average of two determinations. The incubation period was 14 days for 2,4-D and 7 days for the other compounds.

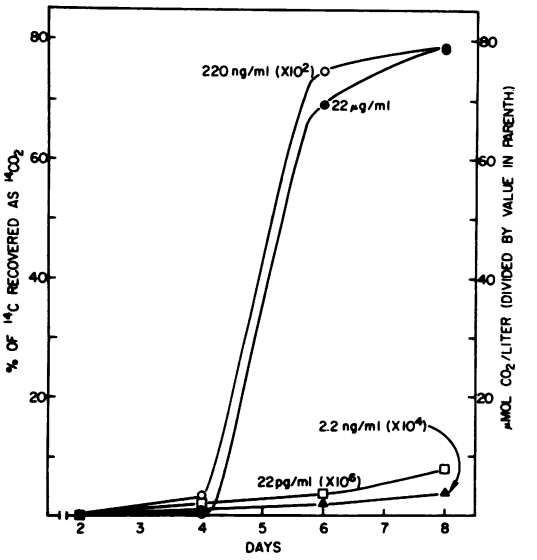

FIG. 1. *Formation of CO_2 from 2,4-D added to stream water at four initial concentrations.*

CO_2 formation at an initial 2,4-D concentration of 2.2 ng/ml was more than an order of magnitude lower than expected based on the assumption that the degradation rates are directly proportional to substrate concentration and by using the data from studies at levels of 22 μg/ml and 220 ng/ml. The C-2 carbon of 2,4-D was eventually degraded to CO_2, even at the lowest initial concentrations. Thus, in a separate experiment, more than 40% was converted to CO_2 after 18 days of incubation of stream water with an initial level of 2.2 ng/ml of 2,4-D, and more than 80% was converted to CO_2 in 12 days or less at 220 ng/ml (Table 4).

Several experiments were done to determine possible sorption of 2,4-D to sides of the incubation vessel or to suspended solids in stream water. The results of two experiments demonstrated the absence of detectable sorption of 2,4-D to the incubation vessel or to particulate matter retained by 0.45-μm membrane filters (Table 5). In addition, no loss of 2,4-D from solution was detected when stream water autoclaved for 30 min was incubated with 2.2 ng/ml of 2,4-D for 36 h and then centrifuged as described. The degradation of 2,4-D did not result in significant O_2 depletion in the experimental flasks even at the highest initial 2,4-D concentration (22 μg/ml); thus, in a 10-day period, the O_2 level was 97.4% of saturation without 2,4-D and 94.0% with the herbicide. During the same period, 82.7% of the ultraviolet absorbancy resulting from 2,4-D disappeared from the solu-

tion, indicating that the stream water remained aerobic even with such extensive cleavage of the aromatic nucleus of the 2,4-D molecule.

The effect of substrate concentration on the biodegradation of Sevin was similar to that observed with 2,4-D. Figure 2B shows the results obtained when Sevin was added to stream water at four initial concentrations. More than 60% of the starting material was degraded to CO_2 within 4 days at initial levels of 30 μg/ml and 300 ng/ml, but 10% or less was converted to CO_2 at 3.0 ng/ml and 30 pg/ml. At these two lower levels, CO_2 was generated at rates not exceeding 3% of the starting material per day. Again, the differences in the actual amounts of CO_2 generated were even more striking, the rates of CO_2 formation at initial Sevin concentrations of 3.0 ng/ml and 30 pg/ml being much lower than those predicted by Michaelis-Menten kinetics. The smaller proportions of Sevin degraded to CO_2 at these levels were not the result of more efficient assimilation by the responsible microflora as indicated by the results in Table 3, which show that less than 10% of the starting material was recovered on 0.45-μm membrane filters after 7 days of incubation of stream water with Sevin at an initial concentration of 3.0 ng/ml.

Sevin undergoes spontaneous hydrolysis to 1-naphthol under mildly alkaline conditions, as in the Fall Creek water used in these studies. At an initial concentration of 30 μg/ml, 60% was hydrolyzed to 1-naphthol in 2 days, and 90% was hydrolyzed in 4 days in autoclaved stream water. Therefore, in the present experiments, either Sevin, 1-naphthol, or both may have been subject to microbial degradation. Approximately 20% of either the Sevin or the 1-naphthol or both was apparently sorbed to the incubation vessel after 5 days of incubation of autoclaved stream water with 3.0 ng/ml of Sevin (Table 5). Of the 81% in solution or sorbed to cells and

TABLE 4. *$^{14}CO_2$ produced from 2,4-dichlorophenoxy[2-^{14}C]acetate (2,4-D) added to stream water*[a]

Days	% of ^{14}C recovered as $^{14}CO_2$		nmol of CO_2 formed/liter	
	220 ng/ml[b]	2.2 ng/ml	220 ng/ml[b]	2.2 ng/ml
6	1.7	1.6	17	0.16
12	81.0	8.9	810	0.89
18	85.3	43.4	853	4.34

[a] Average of two determinations.
[b] Initial 2,4-D concentration.

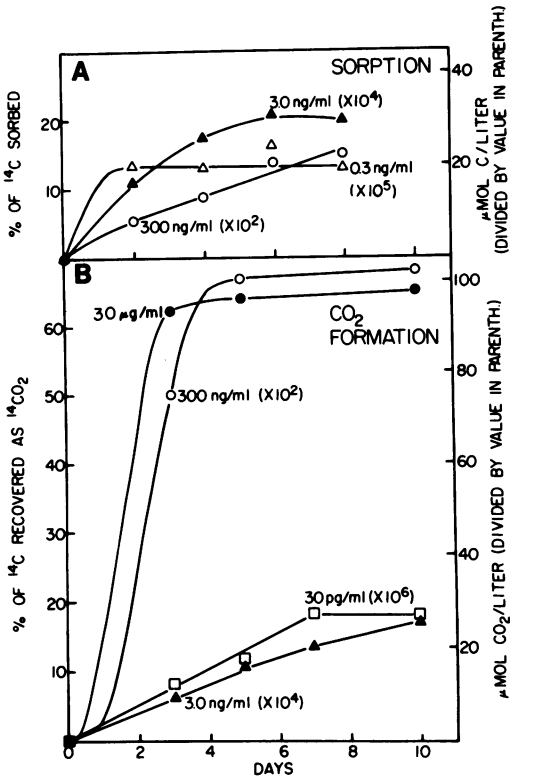

FIG. 2. *Formation of CO_2 and apparent sorption to the incubation vessel of either 1-naphthyl-N-methylcarbamate or 1-naphthol or both at four initial Sevin concentrations.*

TABLE 5. *Sorption of 2,4-dichlorophenoxyacetate (2,4-D) and 1-naphthyl-N-methylcarbamate (Sevin) added to stream water*[a]

Compound	Sterile[b]	Days	% of ^{14}C recovered from:		
			Water	Filtrate	Filter
2,4-D[c]	−	2	99.8 ± 0.7[d]	99.1 ± 1.2	<0.1
2,4-D[c]	+	5	100.6 ± 0.7	99.9 ± 0.4	<0.1
Sevin[e]	+	5	80.9 ± 1.5	68.9 ± 1.2	9.4 ± 0.6

[a] Three determinations.
[b] Either autoclaved (+) or nonsterile (−).
[c] Initial concentration, 2.2 ng/ml.
[d] Standard error of the mean.
[e] Initial concentration, 3.0 ng/ml.

suspended solids, nearly 10% was retained by 0.45-μm membrane filters.

The kinetics of sorption of either Sevin or 1-naphthol or both to the incubation vessels was studied at three initial Sevin concentrations. The results are shown in Fig. 2A. The kinetics of apparent loss of ^{14}C from solution and concomitant binding to the incubation vessel varied with the initial Sevin concentration, but significant proportions were sorbed at all levels. Nevertheless, the differences in the proportions of either Sevin or 1-naphthol or both sorbed at these levels were not sufficient to account for the observed differences in CO_2 formation.

DISCUSSION

These findings are significant for making extrapolations from laboratory assessments of biodegradation to predict the microbial destruction of organic compounds in natural waters. The laboratory test is almost invariably conducted with concentrations of test chemicals far higher than those found in rivers, lakes, and marine waters, and little attention is usually given to how the rate of degradation in the artificial microcosm approximates the rate under natural circumstances. It is evident from the present study that at least two possibilities exist. (i) The rate may be directly proportional to concentration. In this instance, a chemical at a 10- or 100-fold lower concentration in nature than is used in the biodegradation test would be mineralized at a 10- or 100-fold lower rate, but the percent of the compound converted to CO_2 will be independent of concentration. This relation between concentration and rate is predicted by Michaelis-Menten kinetics for enzyme reactions, but it has rarely been tested in microbial cultures or in aquatic or terrestrial microcosms. (ii) Little or no biodegradation may occur at low chemical concentrations, and a threshhold would exist below which no significant mineralization occurs. The usual tests involve relatively high chemical concentrations and thus would not predict this behavior, and the wrong conclusion would be reached from the laboratory trial. The existence of a threshhold is not predicted by Michaelis-Menten kinetics, but it is not surprising. One possible explanation is that energy is obtained too slowly from oxidation of the low substrate concentrations to meet the energy demands of the initially small population active on the compound, these organisms thus being unable to proliferate to reach cell densities sufficient to cause appreciable chemical loss.

Sorption of 2,4-D to the incubation vessel or to suspended solids in stream water was not detected. Moreover, the smaller percentage of 2,4-D and Sevin converted to CO_2 at the lowest concentrations of these chemicals was not the result of more efficient assimilation by the microbial community. Either Sevin or 1-naphthol or both did sorb to a significant extent to the glass incubation vessels at the concentrations of Sevin used, but there was no obvious relationship between the extent of binding and the proportion degraded to CO_2. The sorption data for both 2,4-D and Sevin are consistent with published reports; for example, Schwartz (14) and DeMarco et al. (6) did not find significant sorption of 2,4-D to suspended mineral solids in natural surface waters, and Aly and El-Dib (2) demonstrated that both Sevin and 1-naphthol sorb to clay in aqueous suspension.

If persistence is sometimes a consequence of the inability of microorganisms to metabolize biodegradable molecules at low concentrations, this lack of activity may account in part for the presence of trace levels of many synthetic organic chemicals in natural waters. For example, Faust and Aly (7) found that 2,4-D and 2,4-dichlorophenol in wastewater from a chemical manufacturing plant contaminated ground waters and persisted for years in this environment at low concentrations. Moreover, numerous studies have demonstrated the presence at low concentrations of an enormous variety of synthetic organic chemicals in surface waters (10, 11), and the existence of trace amounts of soluble organic matter in oceans may have a similar explanation.

Organic compounds that persist in natural waters, even in trace quantities, could present environmental problems in several ways. Such trace substances could lead to serious problems if they are susceptible to biomagnification and are subsequently toxic to species at higher trophic levels in food chains. In addition, organic chemicals can impart objectionable tastes and odors to water at levels of nanograms per milliliter (7). Finally, some organic chemicals are toxic to aquatic microorganisms at nanograms-per-milliliter levels and lower (4, 12). Further inquiry is necessary, therefore, to define more precisely the influence of concentration on the rates of microbial degradation of organic molecules in aquatic ecosystems.

ACKNOWLEDGMENT

This research was supported in part by Public Health Service Training Grant ES00098 from the Division of Environmental Health Sciences and by National Science Foundation Grant ENV75-19797.

LITERATURE CITED

1. **Alexander, M.** 1965. Biodegradation: problems of molecular recalcitrance and microbial fallibility. Adv. Appl. Microbiol. 7:35–80.
2. **Aly, O. M., and M. A. El-Dib.** 1972. Studies of the persistence of some carbamate insecticides in the

aquatic environment, p. 210-243. *In* S. D. Faust (ed.), Fate of organic pesticides in the aquatic environment. American Chemical Society, Washington, D.C.

3. **American Public Health Association.** 1976. Standard methods for the examination of water and wastewater, 14th ed. American Public Health Association, Washington, D.C.
4. **Batterton, J., K. Winters, and C. Van Baalen.** 1978. Anilines: selective toxicity to blue-green algae. Science **199**:1068-1070.
5. **Bray, G. A.** 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. **1**:279-285.
6. **DeMarco, J., J. M. Symons, and G. G. Robeck.** 1967. Behavior of synthetic organics in stratified impoundments. J. Am. Water Works Assoc. **59**:965-976.
7. **Faust, S. D., and O. M. Aly.** 1964. Water pollution by organic pesticides. J. Am. Water Works Assoc. **56**:267-279.
8. **Hays, J. T., and W. W. Haden.** 1969. Effect of structure on nitrification of urea derivatives. J. Agric. Food Chem. **17**:1077-1079.
9. **Jannasch. H. W.** 1967. Growth of marine bacteria at limiting concentrations of organic carbon in seawater. Limnol. Oceanogr. **12**:264-271.
10. **Junqclaus, G. A., V. Lopez-Avila, and R. A. Hites.** 1978. Organic compounds in an industrial wastewater: a case study of their environmental impact. Environ. Sci. Technol. **12**:88-96.
11. **Meijers, A. P., and R. C. Van der Leer.** 1976. The occurrence of organic micropollutants in the river Rhine and the river Maas in 1974. Water Res. **10**:597-604.
12. **Powers, C. D., R. G. Rowland, H. B. O'Connors, Jr., and C. F. Wurster.** 1977. Response to polychlorinated biphenyls of marine phytoplankton isolates cultured under natural conditions. Appl. Environ. Microbiol. **34**: 760-764.
13. **Ramsay, A. J.** 1976. The effect of acidification on measurements of microbial uptake of radioactive glucose in freshwater. Limnol. Oceanogr. **21**:922-926.
14. **Schwartz, H. G., Jr.** 1967. Adsorption of selected pesticides on activated carbon and mineral surfaces. Environ. Sci. Technol. **1**:332-337.
15. **Wodzinski, R. S., and M. J. Johnson.** 1968. Yields of bacterial cells from hydrocarbons. Appl. Microbiol. **16**: 1886-1891.