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Project Summary

Interim Protocol for Measuring Microbial Transformation Rate Constants for Suspended Bacterial Populations in Aquatic Systems

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An interim protocol for performing research to measure microbial degradation rates of organic chemicals in freshwaters is presented. Microbial degradation is a major transformation pathway influencing the environmental fate of chemicals. The interim protocol presented provides a basis for measurement of microbial degradation rates such that reliable, comparable and consistent data can be obtained by different laboratories and research investigations. As additional research and information is gathered on the environmental factors affecting microbial degradation by suspended bacterial populations in freshwater, the outlined protocol will be modified.

This Project Summary was developed by EPA's Environmental Research Laboratory, Athens, GA, to announce key findings of the research project that is fully documented in a separate report of the same title (see Project Report ordering information at back).

Introduction

Under the Toxic Substances Control Act of 1976 (PL 94-469), EPA's Office of Toxic Substances is required to review the potential risk to human health and the environment posed by new chemicals before manufacture and use are permitted. For many chemicals, microbial degradation is a major transformation pathway that influences their environmental fate. Therefore, in assessing risk, it is necessary to have some estimate of the microbial transformation rate of each chemical.

The Office of Toxic Substances estimates microbial transformation rates of chemicals proposed for manufacture by comparing each new chemical's organic structure (or other known properties) with those of chemicals whose microbial transformation rates have been established. Chemicals with similar structures/properties are expected to have similar microbial transformation rates. Unfortunately, microbial transformation rates and rate constants have been measured for only a few chemicals. Investigations are being expanded, however, as a better understanding of the many environmental factors that influence microbial degradation is achieved. Much remains to be learned about ways in which population density and diversity, accessibility of chemical substrate within microcosms, and other factors influence transformation rates.

While these investigations continue, it is necessary to provide measured microbial transformation rates for chemicals based on current knowledge. These microbial rates must be measured in a manner that is reproducible and that assures the results of measurement of one chemical can be compared to those

of another with confidence, even though the influence of some environmental factors is not fully understood.

This report represents a sampling and measurement protocol that has been applied over several years at the Environmental Research Laboratory, Athens, GA. The methodology has been found to provide reproducible second-order rate constants using suspended natural populations in aerobic aquatic systems. Transformation rate is based on the rate of disappearance of the test chemical.

The interim protocol provides a stepwise description of methods for establishing aerobic biodegradation investigations. In a typical biodegradation study, natural aquatic sites within the vicinity of the investigator's laboratory are sampled. Water temperature is recorded, and the samples are transported to the laboratory. After receipt at the laboratory, samples are filtered to remove coarse debris.

Population densities should be measured by standard plate counts (heterotrophic plate counts). This characterization serves to establish the baseline population density of the ambient water and to determine the need for a concentration procedure for increasing population densities in order to observe measurable rates in the transformation of test chemicals.

The time lapse between sampling and initiation of the rate constant measurement phases should be no more than 12 hours. Once the microbial samples have been returned to the laboratory, handling prior to experimental rate constant measurement can take two courses.

In one procedure, the sample is used at the natural population density when sampled. The test chemical is added and the chemical's transformation rate is measured.

In the alternative procedure, the natural microbial population is enhanced through a concentration step. This 10:1 concentration step requires larger volumes of source water.

Bacterial populations in water samples are concentrated 10-fold by filtering 10 liters through a membrane filter (0.2 μm pore diameter, Nucleopore or equivalent) that has been prewashed with distilled water. After filtration, filters are placed in a 2-liter, wide-mouth, cotton-plugged Erlenmeyer flask containing 1 liter of the original source sample.

Sterile, aqueous, concentrated stock solutions of nutrients are prepared to yield concentrations (g/L) of each of the following: NH_4Cl (0.5), $(NH_4)_2SO_4$ (0.5), Na_2HPO_4 (0.5), KH_2PO_4 (0.5), $MgSO_4$ (0.001), and $FeCl_3$ (0.001). No more than 1 mL of each nutrient is added to the concentrated bacterial population. These bacterial suspensions are then incubated for 48 hours at 22°C in a temperature-controlled shaker (150 to 200 rpm) before each experiment is initiated. This procedure enhances the bacterial population 10- to 100-fold.

Bacterial concentrations are determined by pour plating techniques using Tryptone Glucose Extract Agar (TGE) from serial dilutions of each reaction flask. Each dilution is plated in duplicate or triplicate. Pour plates are then incubated for 48 hours at 22 ± 1°C in the temperature-controlled incubator/shaker. Following the 48-hour incubation, plates are removed and bacteria are counted (using a suitable counting instrument), tabulated, and arranged.

Following preparation of abiotic and biotic treatment flasks, test chemical disappearance is measured within each reaction flask by either gas chromatographic or high performance liquid chromatographic methods. Raw data on the test chemicals are obtained by measuring the concentration of chemical remaining in the reaction flask at specific time intervals. The measured concentration at time zero serves as the reference point for the remaining points. From these data, the first-order slope or rate constant (k, hr^{-1}) is determined either through standard laboratory computer programs or manually through graphical manipulations using semi-log paper.

Using the mean of bacterial concentration determined from plate counts and first-order slopes (k, hr^{-1}) for chemical disappearance, the second-order rate constant (k_b, L org^{-1} hr^{-1}) is calculated. The applicable form of the second-order rate expression used throughout is:

$$-ds/dt = k_b[B_T][S_T]$$

where:

- k_b = Second-order rate constant (L org^{-1} hr^{-1})
- $[B_T]$ = Measured bacterial concentration (CFU or org per liter)
- $[S_T]$ = Measured substrate (chemical) concentration (mg per liter)

The utility of this protocol has been demonstrated by comparative determinations of the second-order microbial rate constants of standard reference chemicals. Two standar reference chemicals, methyl ester (2,4-dichloro phenoxy acetic acid (2,4 DME) and propanil, were investigate using natural pond water over a period (1 year. Second-order rate constant ranged from 7.8 to 9.2 X 10^{-9} l org hr^{-1} for nine determinations with th methyl ester of 2,4-D. Propanil yielde rate constants ranging from 1.1 to 6.4 10^{-11} l org^{-1} hr^{-1} for six determir ations. Coefficients of variation were 3 to 72% for the methyl ester of 2,4- and 42 to 69% for propanil. No season effects were observed. Use of a standar reference chemical directly aids th investigator in establishing his confidenc in the protocol measurements and allow for interlaboratory comparativ investigations in application of th protocol.

Several basic assumptions serve a the foundation for measurements c microbial transformation rate constant under this protocol. Use of total viabl plate count as a measure of microbi population concentrations provides measurement that is assumed to b proportional to the number c microorganisms participating in th biodegradation process. For chemical for which the concept was developed an tested, many of the culturabl populations indigenous to aquati systems support the necessar constitutive enzymes for microbiall mediated hydrolysis and oxidatio reactions. Moreover, the transform ation/biodegradation process is pseudc first-order with respect to bacteri concentration and, therefore, i proportional to the density of total viabl bacteria in the system plated on TGE. is further assumed that the substrate (tes chemical) concentration is much les than the theoretical Ks half-saturatio concentration and that reaction kinetic are first-order with respect to substrat concentration. It also is assumed tha carbon and energy contributions from th chemical under investigation are nc sufficient to cause measurable growth c the constitutive populations. Th second-order mathematical descriptio of biodegradation serves as reproducible and reliable measurement c microbial transformation rate constant for organic chemicals in aquatic system

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The complete report, entitled "Interim Protocol for Measuring Microbial Transformation Rate Constants for Suspended Bacterial Populations in Aquatic Systems," (Order No. PB 88-165 709/AS; Cost: $12.95, subject to change) will be available only from:

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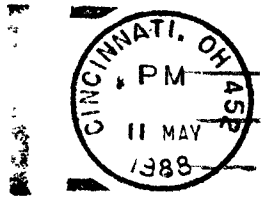

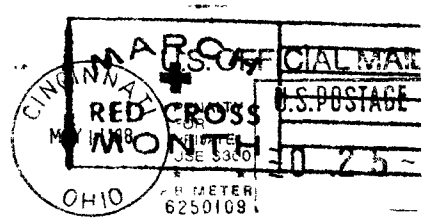

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