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Kinetic Analysis of Enhanced Biodegradation of Carbofuran

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Mineralization of 0.01, 0.1, 5.0, and 50 mg of carbonyl-^{14}C-labeled 2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate (carbofuran) per kilogram of soil was measured in soil that had not been exposed to the pesticide and in soil that had been previously treated with the same concentrations of carbofuran. The stimulation in mineralization rate as a result of previous treatment of the soil with carbofuran was not the result of a substantial increase in the size of the microbial population able to use the compound, as indicated by most probable number counts. Of the Monod (single-substrate) and dual-substrate models of biodegradation kinetics, model I of the dual-substrate models provided the best fit to all curves of mineralization of carbonyl-labeled carbofuran. The fact that model I fit the data supports the hypothesis that the microorganisms mineralizing the carbonyl-labeled molecule do not grow at the expense of the methylcarbamate moiety. This study demonstrates the usefulness of kinetic models for characterizing microbial processes in soil.

The rate of biodegradation of organic compounds in soil is often faster following the second than the first addition of the chemical. Several hypotheses have been proposed to explain this phenomenon of enhanced degradation: growth of the population, induction of enzymes, and selection of new metabolic capabilities produced by genetic change (Spain et al., 1980). Multiplication of the microorganisms carrying out the transformation was responsible for an increase in the rate of degradation of a second application of pentachlorophenol in soil (Watanabe, 1978), 2,4-dichlorophenoxyacetic acid (2,4-D) in soil (Fournier et al., 1981), and *p*-nitrophenol in sediment (Spain et al., 1980). Increased rates of degradation without an increase in the number of organisms able to degrade the test compound were found in soils exposed to 2,4-D (Torstensson et al., 1975) and *S*-ethyl dipropylthiocarbamate (EPTC) (Moorman, 1988). Increased rates of degradation were attributed to the appearance of a new organism, apparently following a mutation, in a mixed microbial population exposed to 2,2-dichloropropionic acid (Senior et al., 1976) and in river water containing aniline (Wyndham, 1986).

Many agricultural soils show enhanced rates of degradation of carbofuran (2,3-dihydro-2,2-dimethylbenzofuran-7-yl methylcarbamate) after repeated applications of the insecticide (Felsot et al., 1981; Hendry and Richardson, 1988), and considerable work has been performed to determine the factors associated with the stimulation. Because of the large number of variables potentially involved in enhanced biodegradation, it is difficult to determine the contribution of a change in an individual factor, such as an increase in the growth rate, to the enhancement.

Kinetics models permit a quantitative determination of the degree of dependence of the rate of biodegradation on each of the parameters controlling the rate; thus, models may provide new insight into the phenomenon of enhanced degradation. A number of theoretical models have been developed to describe the kinetics of biodegradation of organic compounds (Alexander and Scow, 1989). In the Monod family of kinetics models, it is assumed that the rate of biodegradation is controlled only by the substrate concentration and the population density of the microbial population (Simkins and Alex-

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ander, 1984). A similar assumption underlies the dual-substrate models, but it is further assumed that biodegradation is controlled by growth on a substance other than the compound of interest (Schmidt et al., 1985). Having been formulated for pure cultures of microorganisms in homogeneous media, the Monod and dual-substrate models are relatively simple, but certain of these models still have been applied successfully in the analysis of the kinetics of biodegradation in soil (Scow et al., 1989; Focht and Shelton, 1987).

The purpose of this study was to measure the kinetics of enhanced biodegradation of carbofuran and 2,4-D in soil and, with use of kinetics models, to test several hypotheses to explain the enhancement. For purposes of comparison, kinetic analyses were also performed on data describing enhanced biodegradation of 2,4-D, which has been attributed to microbial growth on the pesticide (Fournier et al., 1981).

MATERIALS AND METHODS

Measurements of the mineralization of carbofuran were made on Kendaia clay loam, a member of the fine-loamy, mixed, nonacid, mesic family of Aeric Haplaquepts. Carbofuran (FMC Corp., Princeton, NJ) was radiolabeled with ^{14}C either in the carbonyl (533 MBq/mmol) or on the ring (uniformly labeled, 466 MBq/mmol). Mixtures of labeled and unlabeled carbofuran were incubated in 50 g of soil (dry weight) in biometer flasks at 21 ± 2 °C (carbofuran at 0.1 and 5.0 mg/g) or 29 ± 1 °C (0.01 and 50 mg/g) by the method of Scow et al. (1986). The $^{14}CO_2$ evolved was trapped in 0.5 N NaOH, and the base was periodically removed and counted in a liquid scintillation counter (Model LS7500; Beckman Instruments Inc., Irvine, CA). Full details of the procedures will be published separately. Mineralization of 0.01, 0.10, 5.0, or 50 mg of carbofuran/kg of soil was determined in soil samples not previously amended with the insecticide and in samples to which those concentrations were previously added either once or twice (or three times at 0.10 and 5.0 mg/kg). The subsequent additions were made when the radioactivity in the CO_2 evolved from the previous addition fell to less than 100 dpm/day. A ^{14}C most probable number (MPN) method adapted from Moorman (1988) was used to quantify microorganisms able to degrade carbonyl- and ring-labeled carbofuran. The population sizes were determined from MPN tables given by Alexander (1982), and the significance of the differences between two populations was tested according to Cochran (1950).

Nonlinear regression analyses were performed with the data from all tests of carbofuran mineralization. Two families of kinetics models describing biodegradation were used to analyze the data. The Monod family of models, which are described by Simkins and Alexander (1984), is applicable to the degradation of a single substrate and includes the first-order, Michaelis–Menten, logistic, logarithmic, Monod, and zero-order equations. For situations in which the presence of other substrates or factors influences the degradation of the test substrate, the dual-substrate models, developed by Schmidt et al. (1985), were used to analyze the data. Model I of the dual-substrate models was derived for situations in which the kinetics of growth of the microorganisms responsible for the mineralization observed are controlled by a substrate other than the one being measured. Thus, the overall kinetics of mineralization result from the combined effects of logistic growth on a second substrate and the simultaneous pseudo-first-order degradation of the test chemical by the growing population. The integral form of model I is

$$S = S_0[\phi(e^{\mu_{max}t} - 1) + 1]^{-k_{lu}/\mu_{max}}$$

where S is substrate concentration, S_0 is the initial substrate concentration, μ_{max} is the maximum specific growth rate, ϕ equals B_0/B_{max} (B_0 and B_{max} are the initial and maximum population sizes of the active organisms, respectively), and k_{lu} equals $V_{max}B_{max}/K_m$, where V_{max} is the maximum specific reaction rate and K_m is the half-saturation constant.

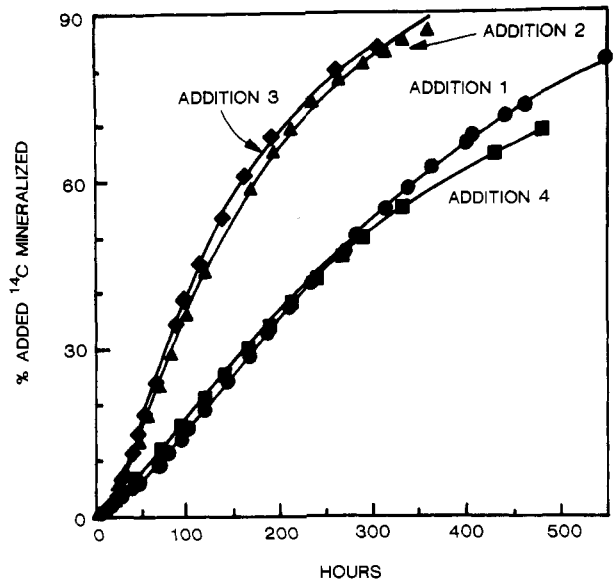

Figure 1. Mineralization of 0.1 mg of carbonyl-^{14}C-labeled carbofuran/kg in soil that was (additions 2–4) and was not (addition 1) previously amended with carbofuran. The curves were fit by model I of the dual-substrate models.

The TOMLIN computer program (Scow et al., 1989), which is an adaptation of the MARQFIT program (Simkins and Alexander, 1984) designed to include the dual-substrate models of Schmidt et al. (1985), was used to fit the data. This program fits data by minimizing the least squares of the differences between the data and the model curve with the Marquardt method (Bard, 1974). The mineralization data were converted from total disintegrations per minute to a percentage of the initial substrate ^{14}C mineralized per unit time. Curves for the percentage of the labeled substrate converted to $^{14}CO_2$, after correction for background radioactivity, were adapted for use with those models formulated for substrate disappearance. Sensitivity analyses were conducted for several concentrations of carbofuran with respect to each set of initial parameter estimates to ensure that selection of poor initial estimates did not lead to convergence of the models with an incorrect set of parameter estimates.

Model fits were compared by using an F-test (Snedecor and Cochran, 1967). The model giving the lowest residual sum of squares for a particular data set was deemed the model of best fit if the difference between it and models with fewer parameters was significant at the 95% confidence level or higher ($p < 0.05$) when a standard F-test was used (Robinson, 1985). Also considered when comparing model fits were how realistic were the parameter estimates and the standard error associated with each estimate.

RESULTS

The best fit of the Monod or dual-substrate models to the curves of mineralization of all concentrations of carbonyl-labeled carbofuran was provided by model I of the dual-substrate models ($p < 0.05$) of Schmidt et al. (1985). The curves depicting mineralization of 0.1 mg of carbofuran/kg of soil in previously unamended soil (designated addition 1) and in soil previously amended one, two, or three times with the insecticide (designated additions 2–4) are shown in Figure 1. Also shown are the fits to the data by model I. In the curves depicting mineralization of the second and third addition of carbofuran, the initial rate of mineralization was greater and the curves were more sigmoidal than in the degradation of the first addition. Approximately 100% of the ^{14}C was converted to CO_2 (data not shown). The parameters estimated by the model for fits to curves depicting mineralization of 0.01–50 mg of carbonyl-labeled carbofuran/kg of soil are presented in Table I. The rates of growth (μ_{max}) and degradation (k_{lu}) were greater during the bio-

Table I. Parameter Estimates[a] from Fits of Model I to Mineralization of Carbonyl-Labeled Carbofuran

carbofuran, mg/kg	no. of carbofuran addns	time of addn, days	μ_{max}	ϕ	k_{lu}
0.01	1	0	0.037 ± 0.002	0.44 ± 0.02	0.0196 ± 0.0005
	2	31	0.140 ± 0.010	0.20 ± 0.02	0.0306 ± 0.0004
	3	52	0.113 ± 0.021	0.23 ± 0.06	0.0283 ± 0.0001
0.10	1	0	0.017 ± 0.001	0.52 ± 0.03	0.0034 ± 0.0002
	2	28	0.066 ± 0.014	0.26 ± 0.10	0.0055 ± 0.0002
	3	70	0.061 ± 0.003	0.22 ± 0.02	0.0084 ± 0.0002
	4	350	0.024 ± 0.001	0.97 ± 0.03	0.0033 ± 0.0002
5.0	1	0	0.015 ± 0.001	0.54 ± 0.02	0.0021 ± 0.0001
	2	28	0.024 ± 0.007	0.69 ± 0.20	0.0042 ± 0.0002
	3	70	0.032 ± 0.004	0.36 ± 0.04	0.0053 ± 0.0018
	4	350	0.007 ± 0.003	0.42 ± 0.25	0.0060 ± 0.0034
50	1	0	0.007 ± 0.001	0.52 ± 0.03	0.0044 ± 0.0002
	2	64	0.008 ± 0.002	0.46 ± 0.07	0.0037 ± 0.0009
	3	104	0.005 ± 0.002	0.53 ± 0.18	0.0058 ± 0.0017

[a] Units for parameters: μ_{max} and k_{lu}, h^{-1}; ϕ, dimensionless.

degradation of the second addition of 0.01, 0.10, and 5 mg of carbofuran/kg of soil than during the first. Incubation of the soil for 350 days resulted in a fall in the value of the parameter μ_{max} for samples receiving 0.10 and 5.0 mg of carbofuran/kg and in the value of k_{lu} in the samples receiving 0.10 mg/kg. In the soil treated with 50 mg of carbofuran/kg of soil, the values of μ_{max} and k_{lu} did not vary greatly after the first and second additions of carbofuran (Table I).

In soil amended for the first time with the pesticide, the initial biomass ($X_0 = \phi S_0$) was proportional to the concentration of carbofuran added. The estimated biomass in the soil amended with 0.01 and 0.1 mg of carbofuran/kg of soil declined by a factor of 2 after the soil was amended once with carbofuran and then remained the same after the second addition. In soil amended with 5.0 and 50 mg/kg, the biomass remained at approximately the same level after the first and second amendments with the chemical, and it declined slightly after the second addition of 5.0 mg/kg (Table I).

Counts of the number of microorganisms able to mineralize the insecticide showed that there was little or no increase in the population of organisms able to use carbonyl-labeled carbofuran after amendment of soil with 0.1 or 50 mg of carbofuran/kg. Thus, 5.3×10^5 cells were present/g of soil that were able to mineralize 0.1 mg of carbofuran/kg of soil before soil amendment, and 2.0×10^6 cells were found in soil previously treated with the same concentration of the pesticide. The 95% confidence intervals for these two population estimates were 2.3×10^5 to 1.2×10^6 and 8.5×10^5 to 6.5×10^6, respectively. There was no increase in the population as a result of the addition of 50 mg/kg to previously unamended soil. These observations support the results of model I that also showed that the addition of carbofuran resulted in no increase in the value of ϕ, which reflects the size of the population able to use the insecticide.

The best fit to the curves of mineralization of 0.01, 0.1, and 50 mg of ring-labeled carbofuran/kg of soil was provided by the first-order model, which is a single-substrate model. The mineralization of 0.1 mg of ring-labeled carbofuran, which is shown in Figure 2, was more rapid in the soil previously treated with the insecticide than in soil not so pretreated, but the shapes of the curves were similar. The parameter estimates determined by the fits of this model are shown in Table II. The degradation rate, k_1, increased in the soil receiving 0.01 and 50 mg/kg as a result of preincubation with the pesticide. The percent of C incorporated into either cell material

Table II. Estimates of Rate Constants[a] from Fits of the First-Order Model to Mineralization of Ring-Labeled Carbofuran

carbofuran, mg/kg	no. of carbofuran addns	time of addn, days	k_1 ($\times 10^{-3}$)	ζ
0.01	1	0	8.5 ± 0.1	0.69 ± 0.01
	2	31	11.8 ± 0.4	0.66 ± 0.01
	3	52	13.9 ± 0.7	0.57 ± 0.01
0.1	1	0	4.4 ± 0.1	0.71 ± 0.01
	2	52	4.6 ± 0.1	0.60 ± 0.01
50	1	0	0.8 ± 0.1	0.75 ± 0.01
	2	64	2.4 ± 0.1	0.82 ± 0.01
	3	104	1.8 ± 0.1	0.82 ± 0.01

[a] Units for parameters: k_1, h^{-1}; ζ, dimensionless.

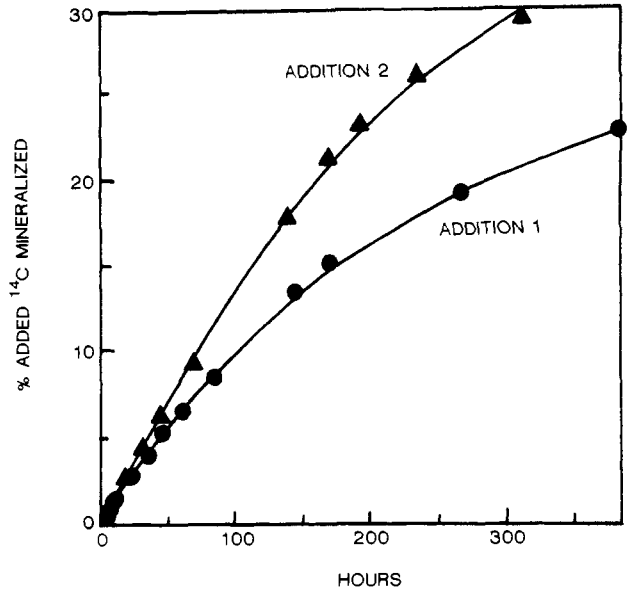

Figure 2. Mineralization of 0.1 mg of ring-^{14}C-labeled carbofuran/kg in unamended soil and soil previously amended with carbofuran. The curves were fit by the first-order model.

or soil organic matter, ζ, decreased after the addition of 0.01 and 0.1 mg of carbofuran/kg; however, ζ increased after the addition of 50 mg/kg.

In the first-order equation, the derived parameter k_1 is equal to $\mu_{max}X_0/K_s$ (Simkins and Alexander, 1984). Therefore, if the stimulation in rate is attributed entirely to an increase in X_0, the biomass can be assumed to have increased only by 1.4 and 1.6 times the initial density after one or two additions, respectively, of 0.01 mg of carbofuran/kg. Similarly, after treatment once with 50 mg/kg, the increase in rate constant can be attributed to the biomass increasing 3-fold compared to the initial density.

The number of microorganisms able to use ring-labeled carbofuran increased from less than 10 (confidence interval not determinable) to 65 (95% confidence interval of 28–151) cells/g after two additions of 0.1 mg of carbofuran/kg. The population did not change from an initial size of <10/g after addition of 50 mg/kg.

Fournier et al. (1981) found that an increase in the rate of degradation of 2,4-D in soil resulted from growth of the population able to metabolize this herbicide. To permit a comparison of an enhancement in the rate of mineralization under conditions in which growth was not responsible for the increase in rate, such as found above in the case of carbofuran, to a situation in which an increase in the rate resulted from growth of the degrading population, some of the curves of Fournier et al. (1981) depicting mineralization of 2,4-D were analyzed by nonlinear

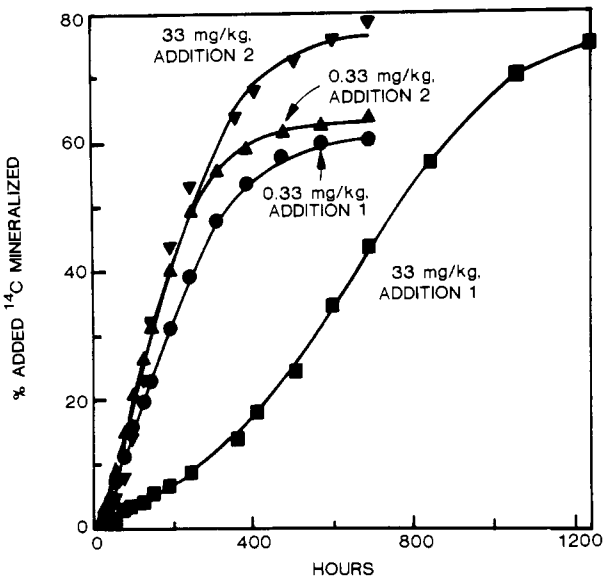

Figure 3. Mineralization of 0.33 and 33 mg of 2,4-D in unamended soil and soil previously amended with 2,4-D. The curves were fit by the logistic (0.33 mg/kg, additions 1 and 2), the Monod with growth (33 mg/kg, addition 1), and the Michaelis–Menten (33 mg/kg, addition 2) equations. Data are adapted from Fournier et al. (1981).

Table III. Parameter Estimates[a] from Fits of Models of the Monod Family to Mineralization of 2,4-D[b]

2,4-D, mg/kg	no. of 2,4-D addns	model	rate constant	K_s	X_0
0.33	1	logistic	$k_4 = (6.64 \pm 0.1) \times 10^{-5}$	NA[c]	32.1 ± 2.3
	2	logistic	$k_4 = (8.17 \pm 0.1) \times 10^{-5}$	NA[c]	34.6 ± 5.0
33	1	Monod	$\mu = (8.02 \pm 2.60) \times 10^{-3}$	125.9 ± 55.1	7.7 ± 1.3
	2	Michaelis–Menten	$k_1 = 0.51 \pm 0.12$	44.6 ± 26.8	64[d]

[a] Units for parameters: μ_{max} and k_1, h^{-1}; k_4, (percent of initial substrate concentration × h)$^{-1}$; K_s and X_0, percent of initial substrate concentration. [b] Data from Fournier et al. (1981). [c] NA, not applicable. [d] Estimated parameter from derivation of Michaelis–Menten equation where $X_0 = k_1/\mu$ (Simkins and Alexander, 1984).

regression analysis. The mineralization of 0.33 and 33 mg of 2,4-D/kg and the model fit to these data are shown in Figure 3. The models providing the best fits to the data were members of the Monod family that are applicable to situations in which the population grows on the added substrate. The parameters estimated by the model fits to the data are summarized in Table III. The logistic model provided the best fit to data depicting the degradation of 0.33 mg of 2,4-D/kg in soil previously amended or not pretreated with the compound. The Monod model provided the best fit ($p < 0.05$) to the degradation of 33 mg of 2,4-D/kg of soil not previously amended with the chemical, whereas the Michaelis–Menten model provided the best fit to the curve of the same concentration in soil that had been pretreated. The models indicate that no growth occurred following treatment with 0.33 mg of 2,4-D, whereas the population increased by a factor of 8 after treatment with 33 mg of 2,4-D/kg of soil (Table III). Similarly, Fournier et al. (1981) found no increase in the number of organisms (determined by the most probable number procedure) able to metabolize 2,4-D after exposure to 0.33 mg/kg and a significant increase in numbers after exposure to 33 mg/kg.

DISCUSSION

The finding that a dual-substrate model fits the curves depicting mineralization of carbonyl-labeled carbofuran better than the Monod models supports the hypothesis that the soil microflora does not use the methylcarbamate moiety of the insecticide as a C and energy source for growth. The pathway by which the methylcarbamate moiety is cleaved to yield carbofuran phenol, methylamine, and CO_2 occurs in bacteria that use methylamine as a source of N (Chaudhry and Ali, 1988; Karns et al., 1986) or C (Chaudhry and Ali, 1988).

It is unlikely that C in the carbofuran ring is supporting the growth of the population mineralizing the carbonyl-C because the population mineralizing the ring was 3–4 orders of magnitude smaller than that mineralizing the side chain. Chaudhry and Ali (1988) found that bacteria able to mineralize the carbonyl of carbofuran were different from those degrading the ring. The source of C for the growth of the carbonyl-mineralizing microflora in Kendaia clay loam may be constituents of soil organic matter. These C sources presumably influence the kinetics of biodegradation of carbofuran in a way similar to that in which dissolved organic C present in water alters the kinetics of the biodegradation of test chemicals in solution (Schmidt et al., 1985).

Hendry and Richardson (1988) reported that an enhancement in the mineralization of the carbonyl-C in Norfolk loamy sand was associated with growth of the microbial population able to degrade the insecticide. The population increased from 1.6×10^3 to 3.1×10^5 cells/g following two additions of 9.0 mg of carbofuran/kg. This final population size is quite similar to the predicted value (5×10^5) if the methylamine moiety is the sole C source for the bacteria, if it is assumed that 1 pg of C needs to be oxidized to form one cell. Thus, it is plausible to believe that, in soils containing only a small number of organisms able to degrade carbofuran, application of the insecticide may lead to increases in the number, and hence activity, of degrading organisms.

The patterns of mineralization of ring- and carbonyl-labeled carbofuran were different. A much lower percentage of the ring-labeled compound was mineralized. In addition, the model that provided the best fit to the data depicting mineralization of ring-labeled carbofuran was the first-order model, which describes situations in which the population presumably is metabolizing only the test compound. First-order kinetics assume that growth does not occur and that the substrate concentration is substantially below K_s. Given the relatively high concentrations of carbofuran added, the number of organisms able to degrade the ring-labeled compound might be expected to be more than the low numbers (<10 cells/g) found. The fact that first-order kinetics fit these data suggests that much of the aromatic moiety of carbofuran is not available to the microflora. Evidence exists that the carbofuran ring, but presumably not the carbonyl, is strongly sorbed to soil organic matter and is not freely available (Getzin, 1973). Furthermore, the results of the model estimates and direct measurements of biomass indicate that little or no growth of the microbial population able to mineralize ring-labeled carbofuran occurred when the soil was treated with 0.01, 0.10, or 50 mg of carbofuran/g.

The results of our kinetic analysis of data showing an enhancement in the rate of 2,4-D degradation indicate that the single-substrate models of the Monod family describe these data. The analysis also supports the findings of Fournier et al. (1981) that the number of 2,4-D-degrading microorganisms did not increase following the addition to soil of 0.33 mg of 2,4-D/kg but did increase upon the addition of 33 mg/kg because of growth on the herbicide.

In contrast with 2,4-D, enhancement of the rate of mineralization of the carbonyl moiety of carbofuran after addition of the insecticide to Kendaia clay loam does not result from a large increase in the size of the microbial population able to degrade the compound. Because model I provides the best fit to the curves depicting mineralization of the carbonyl moiety of carbofuran, the microflora probably mineralizes the side chain while growing to a slight extent with another source of C. The increases in the kinetic parameters μ_{max} and k_{lu} suggest that the enhancement of biodegradation upon repeated application of carbofuran results from an increase in the biodegradative activity per cell in the population rather than from a substantial increase in the population size of carbofuran-degrading microorganisms.

The results of this study illustrate the usefulness of kinetic models as tools for characterizing microbial processes in soil. Use of these models, when combined with information from independent measurements of model parameters, can help in formulating testable hypotheses concerning the microbial populations that are responsible for the biodegradation of synthetic chemicals.

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