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Deterministic Three-Half-Order Kinetic Model for Microbial Degradation of Added Carbon Substrates in Soil

WALTER BRUNNER AND DENNIS D. FOCHT*

Department of Soil and Environmental Sciences, University of California, Riverside, California 92521

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The kinetics of mineralization of carbonaceous substrates has been explained by a deterministic model which is applicable to either growth or nongrowth conditions in soil. The mixed-order nature of the model does not require a priori decisions about reaction order, discontinuity period of lag or stationary phase, or correction for endogenous mineralization rates. The integrated equation is simpler than the integrated form of the Monod equation because of the following: (i) only two, rather than four, interdependent constants have to be determined by nonlinear regression analysis, (ii) substrate or product formation can be expressed explicitly as a function of time, (iii) biomass concentration does not have to be known, and (iv) the required initial estimate for the nonlinear regression analysis can be easily obtained from a linearized form rather than from an interval estimate of a differential equation. $^{14}CO_2$ evolution data from soil have been fitted to the model equation. All data except those from irradiated soil gave better fits by residual sum of squares (RSS) by assuming growth in soil was linear (RSS = 0.71) as opposed to exponential (RSS = 2.87). The underlying reasons for growth (exponential versus linear), no growth, and relative degradation rates of substrates are consistent with the basic mechanisms from which the model is derived.

The lack of formal kinetic theory to explain the nonlinear degradation rates of carbon substrates added to soil has resulted in a descriptive (see reference 20) rather than a quantitative approach. The basic nonlinear nature of progress curves is due mainly to changes in substrate and biomass (enzyme) concentration, in which the former decreases while the latter might increase. Consequently, progress curves of substrate disappearance usually follow two patterns that are either negative exponential or sigmoidal. The first case is generally observed with labile substrates (e.g., glucose or benzoate) and a high initial cell density that does not increase appreciably during the period of analysis. In this instance, the use of the half-life is a useful and valid kinetic term if the reaction follows first-order kinetics. The second case, however, is more complex and is generally observed with more refractile substrates that are also of more immediate concern in terms of environmental quality.

Negative sigmoidal (substrate disappearance) or positive sigmoidal (product formation) progress curves result from a low initial biomass concentration which increases as substrate concentration decreases with time (17). The Vehulst-Pearl (or logistic) equation has been used for fitting sigmoidal exponential growth curves where the population reaches a limit. The logistics equation has several drawbacks. First, it is unsuitable for substrate metabolism where growth does not occur. Second, it is a mere mathematical convenience rather than being deterministic: thus, the rate constants have no intrinsic meaning. Third, the biomass concentrations have to be measured during the experimental analysis.

Monod kinetics, though deterministic, are more suitable to continuous than to batch culture systems (including soil) because the $\Delta S/\Delta t$ linear approximation of the differential equation becomes highly inaccurate and impractical as the time intervals between measurements increase. Although the integrated equation is mathematically sound for long-term incubation studies, this equation is quite complicated and neither product formation nor substrate concentration can be expressed as an explicit function of time (15). Furthermore, the rate constants may have no intrinsic meaning in a nonaxenic system. K_s values are meaningful only when the substrates are soluble and their availability to the cells is not diffusion limited. More problematic is the inapplicability of yield coefficients which are generally orders of magnitude lower in soil than in vitro (2, 8).

The dependency of a rate process upon two functions, such as substrate concentration and biomass or substrate concentration and time, has been referred to as "second-order kinetics" (9, 13). Larson (9) considered biomass to be directly proportional to time and thus arrived at a second-order differential equation, $dS/dt = kSt$. He integrated the equation to obtain S as a discrete function of time. The model equation produces a symmetric sigmoidal curve and does not allow for metabolism without growth or for an "extended" (unsymmetrical) lag period a priori. Instead, the lag period must be arbitrarily assigned to make the data fit the model.

In consideration of all these prior conceptual and mathematical difficulties, we propose a single deterministic model, which (i) requires no a priori assignment of a lag phase, (ii) contains only two interdependent constants as opposed to four for the Monod equation, and (iii) is suitable for substrate metabolism with (pseudo-second order) or without (pseudo-first order) growth. The applicability of the model to the last point is why we designate the reaction as being "3/2 order" or mixed order. We show in this study how the model equation can be fitted to CO_2 evolution data from soil incubations by nonlinear least-squares regression analysis.

MATERIALS AND METHODS

Theory and methods. The rate of substrate change in a substrate-limited system may be described as

$$\frac{dS}{dt} = -KS \quad (1)$$

where K represents a first-order proportionality constant. The substrate disappearance depends only on the concentra-

* Corresponding author.

tion, and equation 1 does not account for any change in the biomass. If growth occurs during degradation, such that the level of cell or enzyme concentration E increases, we define

$$K = k_1 + aE \tag{2}$$

where k_1 is a proportionality constant per unit time and a is a proportionality constant per unit of biomass concentration per unit time. Thus

$$dS/dt = -k_1S - aES \tag{3}$$

To solve the above equation, E must be described as a function of t. Two such functions are as follows:

$$aE = k_2t \tag{4}$$

$$aE = E_0e^{\mu t} \tag{5}$$

Equation 4 assumes linear growth, where k_2 is in units of reciprocal time squared, whereas equation 5 assumes the classical exponential growth, in which $E_{0/a}$ is the starting cell concentration and μ is the growth rate constant.

Substitution of equations 4 and 5 into equation 3 gives the two respective differential equations

$$\frac{dS}{dt} = -k_1S - k_2St \tag{6}$$

$$\frac{dS}{dt} = -k_1S - SE_0e^{\mu t} \tag{7}$$

Integrating both equations over time yields

$$S = S_0e^{-k_1t - (k_2t^2)/2} \tag{8}$$

$$S = S_0e^{-k_1t - \frac{E_0}{\mu}(e^{\mu t} - 1)} \tag{9}$$

where S_0 is the substrate concentration at zero time.

We are primarily interested in final product formation (CO_2) since it is easier to measure and represents complete mineralization of substrate. The rate of product formation P can be expressed as

$$dP/dt = -dS/dt + k_0 \tag{10}$$

where k_0 is a zero-order rate constant that represents the rate of indigenous mineralization of soil organic matter or of residual ^{14}C that has become synthesized into new humus. It is assumed that the amount of carbon in the form of transient intermediates or biomass is relatively small. Further discussion and verification of this point will be considered later. The advantage to using CO_2 product formation as opposed to substrate concentration is that the latter is both tedious and impractical to determine in a soil incubation since it disturbs the system.

Integration of equation 10 gives

$$P = S_0 - S + k_0t \tag{11}$$

so that substitution of equations 8 and 9, respectively, into the above equation gives

$$P = S_0\,(1 - e^{-k_1t - (k_2t^2)/2}) + k_0t \tag{12}$$

$$P = S_0\left[1 - e^{-k_1t - \frac{E_0}{\mu}(e^{\mu t} - 1)}\right] + k_0t \tag{13}$$

It should be noted that although S_0 can represent the amount of substrate added at zero time, it can also be redefined to represent only the amount of substrate that is converted into the product CO_2. This has the added convenience of normalizing S_0 to exclude the amount of carbon which goes into biomass or humus. Depending on the nature of the added substrate, a varying fraction of the added carbon is stabilized in soil humic material (17).

We assumed linear growth in the development of equation 4. The apparent reaction rate is not a simple first-order rate even though in equation 6 the rate is dependent on substrate to the first power. When we do not have to account for growth, k_2 disappears (as E in equation 2 would be constant), and equation 12 is simplified to a first-order equation (plus a linear term). To distinguish between a first- and apparent "second"-order reaction, we refer to equation 11 as being mixed or three-half order. Equations 12 and 13 contain four and five unknown parameters, respectively. Both equations are intrinsically nonlinear, as determined by analysis of the sensitivity coefficients, which are the partial derivatives of P for each parameter (1). It is therefore not possible to transform them into a linear form, so we must use a nonlinear regression method (1, 3) that allows for the estimation of these parameters. As we will see, equation 13 represents a special and rather rare case for soil incubation situations. The following mathematical discussion will therefore be restricted to equation 12. The mathematical requirements and restrictions of nonlinear regression analysis have recently been discussed thoroughly by Robinson and Tiedje (15) and will not be repeated here.

Nonlinear regression analysis for any equation that is nonlinear in its parameters requires initial estimates. As we have explained in the evaluation of the initial differential equation, product formation becomes zero order (and therefore linear) after the added "labile" substrate is metabolized. S_0 and k_0 can therefore be determined rather accurately from the data at higher values of t. Equation 12 can be rearranged (4) to yield

$$Y = -k_1 - k_2t/2 \tag{14}$$

with Y being

$$\frac{1}{t}\,[\ln(S_0 - P + k_0t)/S_0] \tag{15}$$

Values of Y can be determined and plotted against t. This yields a straight line, and initial estimates for k_1 and k_2 can be determined from intercept and slope.

Nonlinear regression analysis was performed with the "NLIN" program by SAS (16) on the Prime 750 computer at the Academic Computing Center at the University of California, Riverside. The program uses the Marquardt algorithm for stepwise interation, which yields rapid convergence even when the initial estimates are considerably wrong.

RESULTS AND DISCUSSION

Evaluation of regression analysis procedure. Nonlinear regression analysis does not proceed by an analytical solution. Any parameter estimate therefore contains two error components, which are introduced by the random errors in

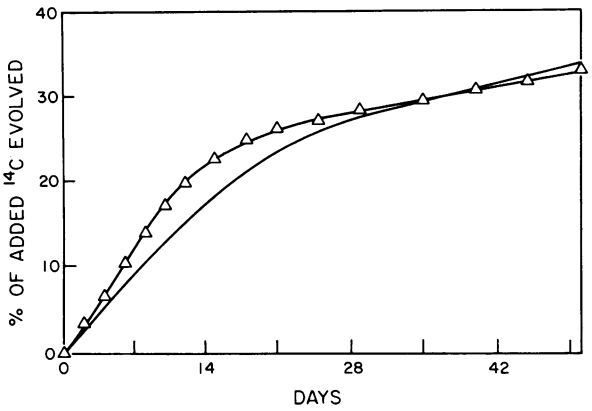

FIG. 1. Comparison of theoretical curves calculated by using initial versus final (best) estimates of parameters S_0, k_0, k_1, and k_2 with simulated data points (containing a random error with sigma = 0.05). (Parameter estimates used are the same as in row 3, Table 1.)

measurement (considered to be additive and uncorrelated) and by an added uncertainty from the regression analysis (1, 3). It becomes important to test the reliability of the regression algorithm or program for the equation in question. This is achieved by fitting simulated data with known variability to the model equation. Large differences between the estimated parameters and the true values under certain conditions indicate limitations of the usefulness.

Several data sets were produced by calculating $P_{(t)}$ for different values of S_0, k_0, k_1, and k_2, and random errors with a constant standard deviation of 5% were added. Initial estimates of S_0 and k_0 were obtained by fitting data from the linear part of the curve at high values of time. These values were then used in equation 14, and starting estimates for the regression analysis were calculated. The same procedure to obtain starting values for the parameters was used with actual data. The values obtained by linear approximation were quite accurate (within 10% of the true values). Equation 14 can therefore be used as a substitute for nonlinear regression analysis when no computer programs are available. The regression with the Marquardt algorithm proceeded quickly to reduce the residual sum of squares drastically (Fig. 1; Table 1). Introducing initial estimates for k_1 and k_2 that were off by more than one order of magnitude did not impair rapid convergence of the iteration process. The quick convergence confirms the conclusion, from the analysis of the sensitivity coefficients, that the two exponential parameters k_1 and k_2 are not correlated, and unique estimates are possible (1). The regression process is highly efficient and yields good results even when only 8, instead of 15, data points were used (see Table 1). The assymptotic standard error and the deviation from the "true" theoretical values did not change significantly. The regression analysis for the proposed model can distinguish between the cases for first or mixed-reaction order. The algorithm used in the program sets k_2 to zero for the final calculation if it drops below a very small value during the iteration process. The distinction between exponential or linear growth as the underlying mechanism, however, has to be made by comparing the residual sum of squares after fitting the data to both equations 12 and 13.

Regression analysis of actual data. To evaluate whether the proposed model describing CO_2 evolution from soil amended with a carbon substrate was really applicable, we fitted data obtained from the literature to the model equation. For the regression analysis, we chose data from Malik and Haider (11). Following the procedure for simulated data, S_0 and k_0 were first approximated by linear regression. Early values were then linearized and initial estimates of k_1 and k_2 were calculated. The results of the nonlinear regression analysis are presented in Fig. 2. The regression analysis for all four amendments were carried out with equation 12, assuming three-half-order kinetics. For cytoplasm, the parameter estimation resulted in k_2 being determined as zero, indicating that $^{14}CO_2$ evolution from this "labile" amendment of amino acids, peptides, and carbohydrates proceeded as a first-order reaction. The mean value for k_0 for all 12 incubations was 0.27 ± 0.04, confirming the assumption that the linear term k_0 is the inherent soil mineralization rate and is therefore independent of added substrate. S_0 for the four different fractions varied. The values for cytoplasm and mycelium were 47.1 ± 2.7 and 38.6 ± 1.9, respectively. This means that roughly the same proportion from each of the three fungal cytoplasm and from mycelium was metabolized to CO_2. The values for the cell wall fractions and the melanins were 31.5 ± 7.7 and 20 ± 2.6, respectively, indicating greater diversity in the amount of mineralized portions of these substrates. The k_1 and k_2 values varied considerably even within a given substrate, indicating differences of degradability between the different fungi. To determine whether exponential growth could also be assumed for the degradation of these compounds, melanin data were fitted to equation 13. Figure 3 shows a comparison of the results from nonlinear regression analysis for *Alternaria alternata*. When taking the differences in the residual sum of squares (2.87 versus 0.708), it becomes clear that the goodness of fit for linear growth (equation 12) is superior to that for exponential growth (equation 13). This was the case for all CO_2 evolution curves obtained from reference 11.

TABLE 1. Comparison of results from nonlinear regression analysis for simulated data[a] with different number of data points and different initial estimates

No. of data points used	Initial estimates				Final estimates[b]				RSS_i/RSS_f[c]
	S_0	k_0	k_1	k_2	S_0	k_0	k_1	k_2	
15	21	0.25	0.1	0.005	22.22	0.207	0.0524	0.012	168
8	21	0.25	0.1	0.005	22.12	0.209	0.0497	0.0127	1,403
8	21	0.25	0.04	0.005	22.12	0.209	0.0497	0.0127	12,109
25	38	0.31	0.005	0.06	37.76	0.31	0.0083	0.0999	1,592
11	38	0.31	0.04	0.04	37.74	0.31	0.005	0.101	7,378
8	29	0.25	0.4	0.005	28.54	0.216	0.448	0	120

[a] Data contain errors having a constant standard deviation (0.05).

[b] True parameter values were: $S_0 = 22.5$, $k_0 = 0.2$, $k_1 = 0.05$, and $k_2 = 0.0.12$ for rows 1 to 3; $S_0 = 37.3$, $k_0 = 0.316$, $k_1 = 0.0054$, and $k_2 = 0.099$ for rows 4 and 5; and $S_0 = 28.6$, $k_0 = 0.21$, $k_1 = 0.43$, and $k_2 = 0$ for row 6.

[c] Ratio of residual sum of squares for initial parameter estimates (RSS_i) to that of final parameter estimates (RSS_f).

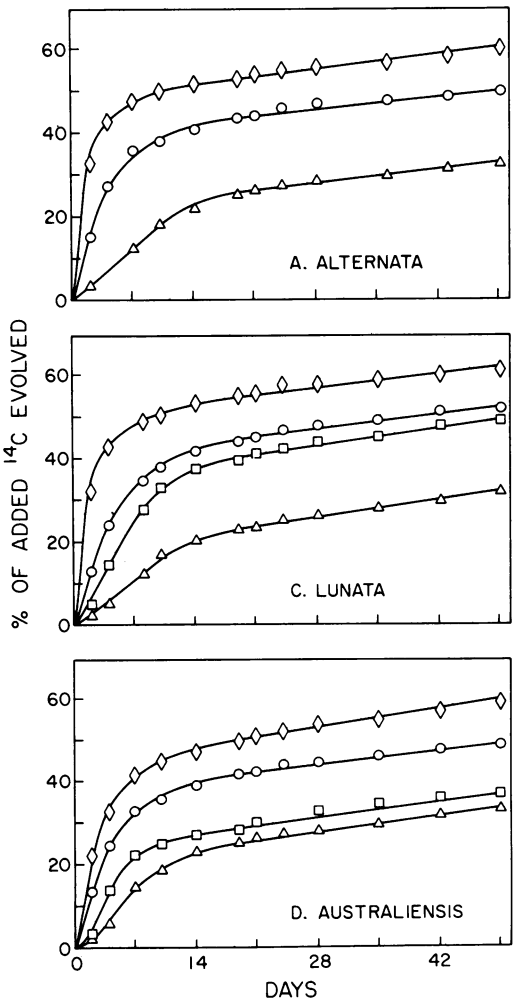

FIG. 2. Comparison of theoretical curves with measured percent $^{14}CO_2$ for incubation of three fungi, *A. alternata*, *Drechsleria australiensis*, and *Curvularia lunata*: mycelium (○), cytoplasm (◇), cell wall (□), and melanin (△). Experimental data are from Malik and Haider (11).

The better fit of CO_2 evolution to linear, rather than exponential, growth (except in irradiated soil) appears to be surprising since exponential growth is one of the basic paradigms in microbiology. Nonexponential growth is treated as a deviation from the norm (14) since it is rarely encountered in liquid cultures. The situation in soil, however, is quite different from liquid culture. Growth of microorganisms in soil is limited by diffusion of substrate and nutrients because of the soil matrix. Although this matrix in itself prevents diffusion of nutrients, the more significant aspect probably relates to the predominant distribution of bacteria on soil surfaces (6, 12, 21). When the cell density exceeds the surface that can be physically occupied, such that more than one layer of cells is formed, diffusion of substrate to the inner cells is restricted. Conversely, diffusion of essential nutrients associated with soil (e.g., cations, phosphate) is diffusion limited to the outermost cells. Conceptually, both gradients would establish a different nutrient-limited environment depending on the position of each cell in the "floc." These restrictions would result in the apparent linear growth.

When population densities are very low as in the case of gamma-irradiated soil (10), the surface area is occupied by only a very small fraction of microorganisms, and nutrient diffusion does not become a problem until after development of a monolayer. Although no quantitative data regarding bacterial numbers were given by Lichtenstein et al. (10), we can calculate that the total surface area available for cell attachment in a silty clay loam would be about 2×10^7 μm^2 per cm^3 of bulk soil, assuming an average particle size of 50 μm. Since an average *Bacillus* cell of 2 by 1 μm would take up a "one-sided" surface area of 1.57 μm^2, the soil would support a monolayer of about 10^7 bacteria per cm^3. Lichtenstein et al. reported that "no bacteria" (i.e., $<10^3$) were found on petri plates after irradiation. Thus, an increase in numbers by at least three, and possibly four, orders of magnitude was possible before incurring diffusion-related problems. It is not surprising, therefore, that in nonirradiated soil the $^{14}CO_2$ evolution data from glucose addition were best fitted to a first-order reaction that is indicative of no growth. The $^{14}CO_2$ data from the irradiated soil, however, could only be fitted to equation 13, assuming exponential growth. The parameters obtained were as follows: $k_1 = 0.01$ day^{-1}; growth rate, $\mu = 0.42$ day^{-1} and $E_0 = 0.001$ day^{-1}. The growth rate μ is still much slower than the rate which would be observed in vitro considering the substrate is glucose. The data could only be fitted to linear growth by assuming a 5-day lag after addition of the substrate (during

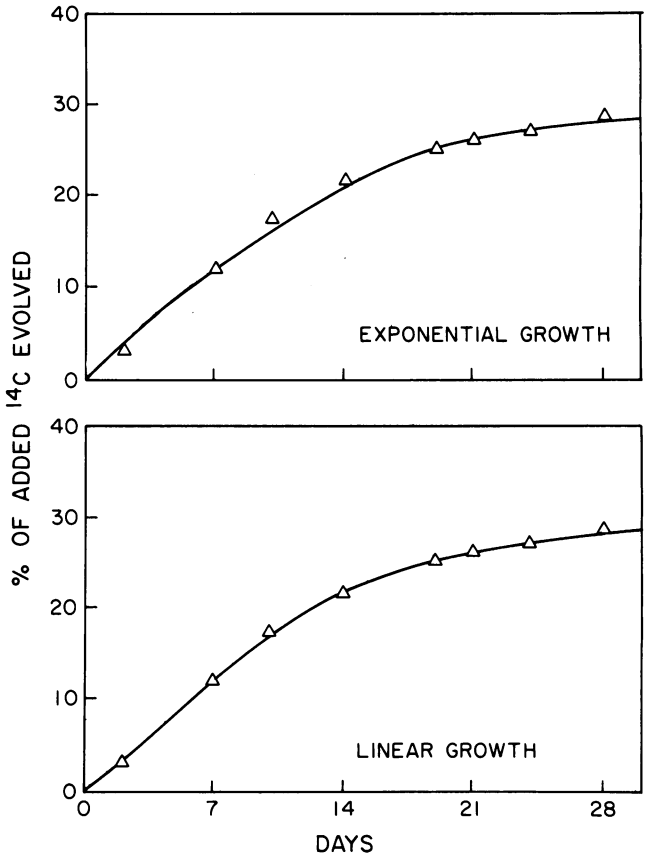

FIG. 3. Comparison of goodness of fit for theoretical curves versus measured percent $^{14}CO_2$ for *A. alternata* melanin fraction incubated in soil. Exponential growth model (top) and linear growth model (bottom). Parameter estimates used: $S_0 = 22.75$, $k_0 = 0.191$, $k_1 = 0.073$, $\mu = 0.202$, $E_0 = 0.007$ (exponential growth); $S_0 = 22.7$, $k_0 = 0.2$, $k_1 = 0.05$, $k_2 = 0.012$ (linear growth).

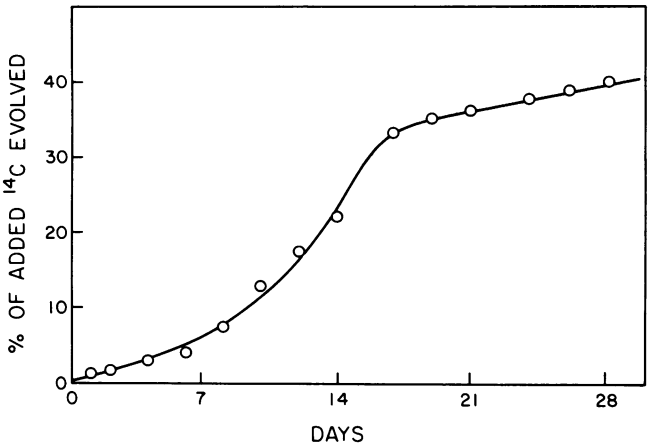

FIG. 4. Comparison of theoretical curve with measured percent $^{14}CO_2$ data from glucose added to gamma-irradiated soils. Parameter estimates used: $S_0 = 25.5$, $k_0 = 0.5$, $k_1 = 0.001$, $\mu = 0.42$, $E_0 = 0.001$. Experimental data are from Lichtenstein et al. (10).

which only the zero-order rate process produced some CO_2). With the exponential growth model, no a priori provisions had to be made for what appears to be a lag in Fig. 4.

The model assumes that addition of labile, readily degraded substrates such as carbohydrates, aromatic compounds, or amino acids is not followed by significant growth of microorganisms in soil. Experimental data (7) show that only a small amount of added labile carbon is incorporated into biomass. Likewise, Larson (9) found that <10% of ^{14}C from alkyl ethoxylate added to Ohio River water was detected in the particulate matter after incubation. Kinetic theories postulate that maintenance requirements at low growth rates become much higher and that the yields drop under these circumstances (14). This has been demonstrated in chemostat studies where cell yields at low dilution rates were markedly lower (19).

We would like to put forward another explanation that can account for the apparent absence of growth after substrate addition. The soil ecosystem in general has to be considered as a nutrient-limiting environment. Whereas the concentration of macronutrients such as nitrogen and phosphorus are seemingly high enough, their rapid availability for microorganisms might be low due to adsorption at exchange sites in soil (12). Amending the soil with a labile carbon source results in an excess of available growth substrate that is not balanced by other nutrients. Soil microorganisms with low k_m for the substrate uptake systems would have a selective advantage in general. In the case of an unusual high substrate concentration, however, this could prove to be disastrous for their internal metabolic balance. Tempest and Neijssel discussed the problem recently in an elegant review (19). As they pointed out, organisms have to react to an unbalanced nutrient situation to prevent intracellular accumulation of suicidal intermediates. There are several possible ways for the organism to react. One possibility is the excretion of partially oxidized metabolites. In a nonaxenic system, these intermediates, however, would not accumulate outside the cells. They would be taken up by other organisms with high affinities for these compounds, and they would subsequently be converted to CO_2. Oxidation and excretion of intermediates constitute a way for organisms under limiting conditions to bypass the threat posed by high growth substrate concentrations. However, it does leave them with a problem in their energy balance. A spilloff oxidation produces reducing equivalents, resulting in a high ATP concentration.

Limitation by nutrients other than carbon is typical for resting cell suspensions in which the respiratory system is uncoupled from ATP production (18) while oxygen consumption and CO_2 production increase. Nutrient limitation for soil microorganisms may closely resemble that of resting cells whereby the reducing power of substrate oxidation might be likewise "wasted" in a similar manner. This would explain how up to 90% of the added carbon is released as CO_2 while growth yields are very low. Substrates that are degraded only slowly (e.g., melanin) would allow for some growth since the rate of uptake of a limiting nutrient would be comparably similar to the rate of substrate catabolism. This is unlike labile substrate, which would be catabolized at a rate far in excess of the rate of limiting nutrient uptake.

It has been shown that some activity from ^{14}C-labeled substrate is found in biomass. For glucose this can amount to about 28% after 2 weeks and <20% after 12 weeks. For aromatic compounds the values after 12 weeks do not exceed 6.7% (18). However, these data do not provide information about net increases in biomass since ^{14}C-labeled biomass may simply reflect the turnover and exchange of labeled substrate without any increase in viable cells.

The proposed model describing CO_2 production in soil provides a tool for quantitative comparative studies of the fate of organic amendments to soil. It allows for indirect, nondisturbing analysis of growth and metabolism in soil. A collection of kinetic parameters (rate constants) obtained by incubating the same substrate in different soils should yield additional quantitative characteristics of the soils.

Determining rate constants from pesticides or other environmental chemicals from this model could be a useful step in offering a quantitative framework for the interpretation and evaluation of data proposed by Greaves et al. (5). Finally, the proposed model provides directions for the design of soil incubation experiments. Both the sampling interval and the time of the experiment can be determined from theoretical calculations based on preliminary data.

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