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Consideration of errors in estimating kinetic parameters based on Michaelis-Menten formalism in microbial ecology

Abstract—When microbial heterotrophic activity appears to conform to Michaelis-Menten kinetics, it is usual to estimate parameters of the model from linearly transformed, but unweighted, data. Theoretical error analysis indicates that if the uncertainties associated with untransformed data are homogeneous within a given experiment, it is inappropriate to fit transformed data in the usual manner. In such cases, an alternative method is proposed whereby untransformed and unweighted data are directly fit to an equivalent nonlinear equation. Results from experiments in the eastern Canadian arctic illustrate this alternative method of estimating kinetic parameters. A comparison of the two methods based on goodness-of-fit and precision of parameter estimates shows the alternative method to be more satisfactory.

The relationship between the concentration of available dissolved organic substrate and the rate of uptake of that substrate by heterotrophic microbial communities has long been described by a scheme analogous in form to that proposed by Michaelis and Menten (1913) to model the reaction kinetics of a system consisting of a single enzyme, a single substrate, and a single product (Parsons and Strickland 1962; Wright and Hobbie 1966). In spite of numerous features inherent in the complex biological processes carried out by heterogeneous microbial communities that invalidate the original scheme (Williams 1973; Krambeck 1979), many instances have been recorded (Hoppe 1978) for which the relationship of uptake rate to substrate concentration appears to conform, empirically, to the Michaelis-Menten equation:

$$V = \frac{V_m S}{K + S} \tag{1}$$

where V is the rate of uptake or metabolism ($\mu g \cdot liter^{-1} \cdot h^{-1}$) of a substance present at a concentration of S ($\mu g \cdot liter^{-1}$). The parameter V_m ($\mu g \cdot liter^{-1} \cdot h^{-1}$) is the asymptote toward which V tends at very large values of S. The parameter K ($\mu g \cdot liter^{-1}$) is the value of S for which V is half of V_m (half-saturation constant).

In many cases, the ambient concentration of the substrate, S_n ($\mu g \cdot liter^{-1}$) is unknown. Kinetic studies are performed by adding radiotracer to a concentration of A ($\mu g \cdot liter^{-1}$). Thus, the effective concentration S is the sum of S_n and A. Equation 1 then becomes

$$V = \frac{V_m(S_n + A)}{(K + S_n) + A}. \tag{2}$$

If an amount q of the radiotracer is taken up during an incubation of duration t in which an amount Q of the radiotracer is available in the medium, the following is true if isotopic discrimination is neglected:

$$V = \frac{q(S_n + A)}{Qt}. \tag{3}$$

Eliminating V from Eq. 2 and 3 gives

$$\frac{q}{Qt} = \frac{V_m}{K' + A} \tag{4}$$

where $$K' = K + S_n. \tag{5}$$

Notwithstanding the admonitions of Wilkinson (1961), Dowd and Riggs (1965), and Smith and Horner (1981) concerning

proper weighting of data in applications of the Michaelis-Menten equation, it is standard practice (Wright and Burnison 1979) to estimate V_m and K' by inverting Eq. 4 and performing unweighted linear regression of $(Qt)\cdot q^{-1}$ on A:

$$\frac{Qt}{q} = \frac{K'}{V_m} + \frac{A}{V_m}. \qquad (6)$$

The reciprocal of the slope yields V_m while the negative of the x-intercept yields K'.

Given a set of experimental data, the values of parameters estimated from Eq. 4 and 6 should in theory be identical. In practice, however, since experimental errors are inevitable, the accuracy of estimates will differ according to the form of the equation used. Since Eq. 4 and 6 are equivalent, the decision to use Eq. 4 must be based on the same biological considerations that are normally taken into account when Eq. 6 is used. For example, kinetic diversity of natural bacterial assemblages gives rise to different values of V_m and K' depending on the range of A considered (Williams 1973; Azam and Hodson 1981). In such a case, the use of Eq. 6 (and therefore also Eq. 4) is restricted to a narrow range of A, a description of the entire assemblage being given by specifying values of V_m and K' for successive ranges of A (Azam and Hodson 1981). My aim here is not to consider whether classical Michaelis-Menten formalism is realistic in describing substrate uptake by natural bacterial assemblages (it often appears to be so within narrow ranges of A, cf. Wright and Burnison 1979), but to discuss the merits of estimating parameters from Eq. 4 instead of 6. The following discussion applies to those experimental data that can in fact be described by either Eq. 4 or 6 within acceptable limits of statistical confidence.

Experiments were performed in eastern Canadian arctic waters on board CSS *Hudson* (cruise 80-027) during July and August 1980. Uptake of [^{3}H]glucose by natural microbial communities exposed to different concentrations of glucose was measured. Details of sampling, experimental methods, and discussion of other aspects of the data will by published elsewhere. I thank T. Platt for help in error analysis and critical review of the manuscript, C. L. Gallegos for programing the computer, and D. Rudderham for running the program.

Of the four measurements made, t, A, Q, and q, the first three are controlled by the investigator and can usually be made with high precision. On the other hand, the measured biological response q is subject to both systematic and random errors. In Eq. 4, q appears as measured and has an uncertainty σ_q. The turnover rate (R) is designated as

$$R = \frac{q}{Qt}. \qquad (7)$$

Q and t are assumed to be error-free so that the uncertainty associated with R, σ_R, is

$$\sigma_R = \frac{\sigma_q}{Qt}. \qquad (8)$$

On the other hand, q appears as the transformed variable q^{-1} in Eq. 6. The turnover time (T) is designated as

$$T = R^{-1} = \frac{Qt}{q}. \qquad (9)$$

The uncertainty of any function $f(R)$ is given in terms of the uncertainty in R as (Bevington 1969)

$$\sigma_{f(R)} = \frac{df(R)}{dR}\cdot\sigma_R. \qquad (10)$$

Therefore, the uncertainty associated with T, σ_T, is

$$\sigma_T = \frac{-\sigma_R}{R^2} = \frac{-\sigma_q Qt}{q^2}. \qquad (11)$$

When an equation is fitted to unweighted data, it is assumed implicitly that the uncertainties are equal for all data in the given set. In the present instance, this means that if we fit

$$R = \frac{V_m}{K' + A}, \qquad (12)$$

we are assuming σ_R to be statistically indistinguishable for all A. On the other

Table 1. Two-way ANOVA of $\hat{\sigma}_R$ based on five experiments conducted in eastern Canadian arctic waters.

Source of variation	df	SS (h^{-2})	F_s
A	8	3.92×10^{-7}	1.24 ($P>0.25$)
Experiments	4	7.82×10^{-7}	4.96 ($P<0.005$)
Error	32	1.26×10^{-6}	

hand, if, according to standard practice, we fit

$$T = \frac{K'}{V_m} + \frac{A}{V_m}, \qquad (13)$$

we are assuming σ_T to be statistically indistinguishable for all A.

The above considerations can be illustrated with results from the experiments previously mentioned. To test the hypothesis that σ_R was homogeneous within each experiment under the conditions tested, ANOVA was performed on data from five separate experiments in which triplicate measurements of q were made at each of the same nine levels of A. R was calculated according to Eq. 7. For each experiment, at each level of A, the triplicate values of R allowed calculation of $\hat{\sigma}_R$, where $\hat{\sigma}_R$ symbolizes the sample statistic used to estimate the population statistic σ_R. A two-way, mixed model ANOVA (Sokal and Rohlf 1969, p. 322) was performed on $\hat{\sigma}_R$ with A being the fixed treatment effect and the particular experiments being the random effects. There was no significant effect of A on $\hat{\sigma}_R$ (Table 1). However, $\hat{\sigma}_R$ was not the same for all experiments (Table 1); this is not entirely surprising since the data were derived from samples taken at different locations, depths, times of day, incubated at different temperatures, and for different lengths of time and included different size fractions of the communities.

The invariance of $\hat{\sigma}_R$ within individual experiments having now been established, it is appropriate to fit Eq. 12 using unweighted values of R. At the same time, this means that σ_T varies with A in the following manner (by eliminating R from Eq. 11 and 12):

$$\sigma_T = \frac{-\sigma_R(K' + A)^2}{V_m^2}. \qquad (14)$$

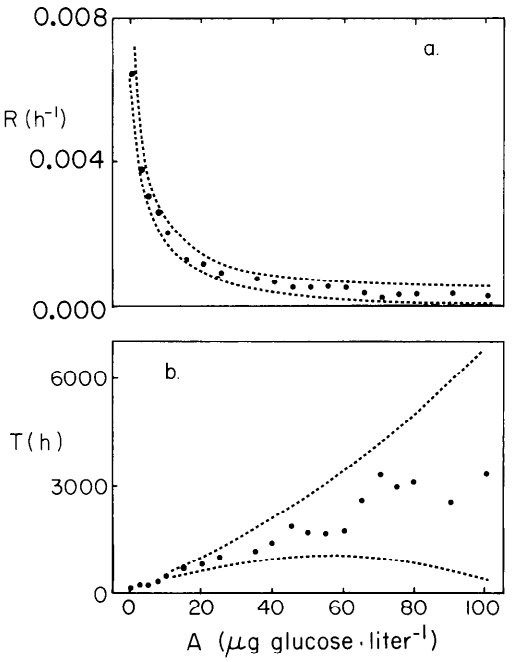

Fig. 1. [^{3}H] glucose uptake by microbes collected on 14 August 1980 at 74°28.7'N, 92°2.3'W from a depth of 29 m. a. Turnover rate (R) as a function of the concentration of added glucose (A). Curves represent $R = V_m \cdot (K' + A)^{-1} \pm \hat{\sigma}_R$ where V_m = 0.0285 μg glucose · liter^{-1} · h^{-1}, K' = 4.28 μg glucose · liter^{-1}, and $\hat{\sigma}_R$ = 0.00024 · h^{-1}. b. Linear transform of above data. Curves represent $T = (K' + A) \cdot V_m^{-1} \pm \hat{\sigma}_R \cdot (K' + A)^2 \cdot V_m^{-2}$ where V_m, K', and $\hat{\sigma}_R$ are as above.

It is evident from this that in an experiment where the values of V_m, K', and σ_R are constant, σ_T will vary as a function of A. Thus it would be inappropriate to fit Eq. 13 using unweighted values of T.

Figure 1 illustrates measurements of [^{3}H]glucose uptake by microbes collected on 14 August 1980 from a depth of 29 m. The data were fit to Eq. 12 directly by nonlinear parameter estimation according to the Gauss-Newton method (Bard 1974) using a computer. The parameters thus obtained, together with their standard deviations, were V_m = 28.5 ± 1.0 ng glucose · liter^{-1} · h^{-1} and K' = 4.28 ± 0.19 μg glucose · liter^{-1}. The dashed curves in Fig. 1a represent positive and negative deviations of $\hat{\sigma}_R$ from a curve with values of V_m and K' given above. Since $R(A)$ was not replicated in this particular experiment, I used a typical value of $\hat{\sigma}_R$ =

$0.00024 \cdot h^{-1}$, merely for illustrative purpose. This typical value is the mean from the five separate experiments for which $\hat{\sigma}_R$ was available (Table 1).

In contrast, Fig. 1b shows the plot of the same data transformed by multiplicative inversion. The dashed curves represent positive and negative deviations ($\hat{\sigma}_T$) from Eq. 13 according to 14 with values of V_m, K', and $\hat{\sigma}_R$ as for Fig. 1a. This shows clearly that less weight should be attached to values of T at large A when Eq. 13 is used.

The merit of using Eq. 12 rather than 13 for unweighted data can be shown by considering the following questions. First, for which equation do the data more closely agree with the fitted function? Second, for which equation do we have a more precise estimate of the widely used parameter—natural turnover time ($T_n = K' \cdot V_m^{-1}$)—i.e. turnover time of the dissolved substrate at the in situ concentration S_n? Related to this is a third consideration: for which equation is the estimated value of T_n closer to a value of T measured experimentally by using a very small amount of high specific activity tracer such that $A \ll S_n$ (Azam and Holm-Hansen 1973)?

The goodness-of-fits were evaluated by comparing the sums of squared residuals (SSR). As emphasized by Silvert (1979), residuals should be computed as the discrepancies between the actual data and the fitted curve, not between transformed data and the linearized curve. Consider the example in Fig. 1: SSR based on a direct nonlinear fit of R to Eq. 12 was $2.6 \times 10^{-7} \cdot h^{-2}$. On the other hand, linear regression of unweighted T on A (Eq. 13) gave values of $V_m = 29.6$ ng glucose$\cdot$liter$^{-1} \cdot h^{-1}$ and $K' = 5.81$ μg glucose$\cdot$liter^{-1}. When these values were substituted into Eq. 12, SSR was $10.0 \times 10^{-7} \cdot h^{-2}$. Therefore, there is a closer fit of the model when parameters estimated from unweighted values of R are used rather than those from unweighted values of T.

Besides its utility as an ecological concept (Azam and Holm-Hansen 1973), T_n serves a useful purpose in the present context of error analysis since it is the ratio of the two parameters estimated. We can calculate $\hat{\sigma}_{Tn}$ according to the formula for the standard deviation of the ratio of two variables (e.g. Bevington 1969, p. 61) using the values of V_m, K', and the variances and covariance estimated directly by nonlinear fitting of Eq. 12. For example in Fig. 1a, this calculation gives $T_n \pm \hat{\sigma}_{Tn} = 150 \pm 3$ h. On the other hand, Eq. 13 gives T_n directly as the y-intercept. As Sokal and Rohlf (1969) note, for linear regression of y on x, a biconcave confidence belt is obtained. The further away from the mean, the less reliable are the estimates of y because of the uncertainty about the true slope of the regression line. For the example in Fig. 1b, the y-intercept gives $T_n \pm \hat{\sigma}_{Tn} = 172 \pm 122$ h. The coefficient of variation ($\hat{\sigma}_{Tn}/T_n$) for T_n arising from the nonlinear method is 2% while that from linear regression is 71%.

Finally, we can compare values of T_n estimated by the two methods with an experimental value measured at a value of A presumed to be much less than S_n (cf. Gocke 1977; Barvenik and Malloy 1979; Litchfield 1980). For the example in Fig. 1, $T = 154$ h at the lowest level of A tested (0.18 μg glucose$\cdot$liter^{-1} = 1 nM). The percentage deviation of T_n estimated from the nonlinear method from this measured T was −3%. On the other hand, the percentage deviation of T_n estimated from transformed data from this measured T was 12%. Figure 2 shows frequency distributions of these percentage deviations for 102 experiments performed during the arctic cruise. It is clear from these histograms that close agreement between estimated T_n and T measured at $A \ll S_n$ occurred more often when the estimates were derived from a direct fit of Eq. 12 rather than a fit of transformed data to Eq. 13. If there is no deviation from the Michaelis-Menten model, T_n (i.e. T estimated at $A = 0$) should always be $< T$ measured at some finite value of A, no matter how small A is (Gocke 1977). When T_n was calculated by the nonlinear method, this expectation was fulfilled in 83% of the experiments (Fig. 2a). On the other hand, estimation of T_n by linear transformation resulted in an almost normal dis-

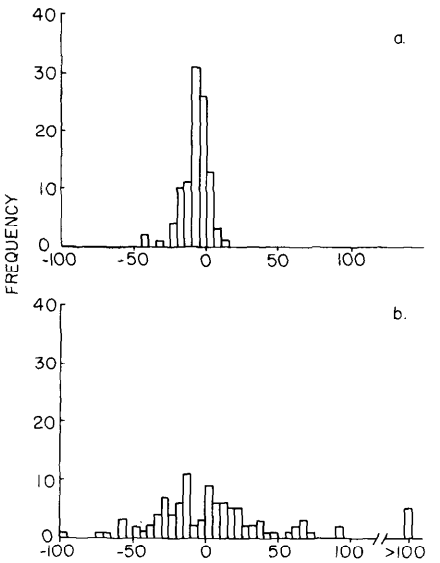

Fig. 2. Frequency distribution of percentage deviation of estimated natural turnover time (T_n) from turnover time measured at the lowest tested concentration of added glucose. a. T_n calculated as the ratio $K':V_m$ where K' and V_m were estimated by direct nonlinear fit of data to $R = V_m \cdot (K' + A)^{-1}$. b. T_n estimated as the y-intercept arising from linear regression of transformed data according to $T = (K' + A) \cdot V_m^{-1}$.

tribution of percentage deviation about the measured T (Fig. 2b). In this latter case, 47% of the experiments represented negative deviations. This indicates that at low levels of A, the agreement between measured and predicted values are poorer when data have been transformed but left unweighted before fitting. The undue emphasis given values of T at high A according to Eq. 13 results in poor estimates of T_n.

The importance of proper weighting of transformed data in applications of the classical Michaelis-Menten equation has already been emphasized (Wilkinson 1961; Dowd and Riggs 1965). Microbial ecologists use a slightly modified version of this equation (Eq. 12 or 13) because absolute rates cannot be calculated when S_n is not known. The results of my study emphasize the need for proper consideration of errors in the use of the modified equation. The usual practice of estimating parameters from linear regression of unweighted T on A is statistically less satisfactory than from a direct nonlinear fit of R to A. The former method is no longer defensible when nonlinear fitting by computer is accessible to the investigator.

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A method for determining exoproteolytic activity in natural waters

Abstract—A very sensitive method allowing determination of exoproteolytic activity in natural water is based on the use of aminoacyl-β-naphthylamide, which gives rise to a fluorescent product upon hydrolysis of its peptidelike bond by filtered or unfiltered water samples. Specificity of the method to exoproteases is demonstrated by a strong competitive inhibition effect of added proteins.

A good correlation is found between exoproteolytic activity and the rate of amino acid utilization by microheterotrophs in various aquatic environments.

Organic nitrogen supplied by phytoplanktonic production or decomposition in natural waters consists of about 85% proteins and peptides; only small amounts of free amino acids are directly produced (Billen 1982). Such high molecular weight material, either dissolved or particulate, cannot be directly taken up by bacteria but can only be absorbed after exoenzymatic hydrolysis (Rogers 1961).

Exoproteases can therefore be inferred to play an important role in the nitrogen cycle of aquatic ecosystems. Unfortunately, due to the lack of a convenient and sensitive method, little information is available concerning their occurrence and activity in natural waters. By the use of an insoluble synthetic protein-dye, releasing a soluble color upon enzymatic hydrolysis, some workers (Kim and ZoBell 1974; Little et al. 1979; Meyer-Riel 1981) have demonstrated free exoproteolytic activity in lake or seawater samples. This method, however, is not sensitive enough for rapid measurements and requires either very long incubation times (a few days) or preconcentration of the samples by dialysis or ultrafiltration. Here we report a very sensitive and reliable method of determining exoprotease activity in a few minutes, without any concentration of the sample even in oligotrophic waters.

The method is adapted from the procedure of Roth (1965) in clinical analysis. It is based on the use, as a substrate for proteolytic exoenzymes, of aminoacyl-β-naphthylamide, which gives rise to a fluorescent product after hydrolysis of the peptidelike bond (Fig. 1). The standard procedure is described below. Two milliliters of an unconcentrated water sample, either unfiltered of filtered through a cellulose acetate membrane, are transferred to a sterile quartz fluorimeter cell kept at about 20°C; 50μl of a sterile 40 mM solution of aminoacyl-β-naphthylamide solution is added, and the increase of fluorescence at 410 nm under 340 nm excitation is measured as a function of time over 10–100 min with a Perkin Elmer 2000 fluorimeter.