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Evaluation of Respirometric Data: Identification of Features That Preclude Data Fitting with Existing Kinetic Expressions

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The use of respirometric data for the evaluation of intrinsic biodegradation kinetic parameters for single organic compounds is discussed. Emphasis is placed on the preliminary assessment of the data set to determine whether it is suitable for kinetic parameter estimation. Careful preliminary examination of the data avoids attempting parameter estimation with unacceptable data. Furthermore, the use of unbiased respirometric data helps ensure that the estimated parameters truly reflect the intrinsic kinetics for biodegradation of a single substrate by the culture tested. Both experimental and theoretical oxygen uptake curves are used to illustrate how various conditions can limit the utility of a given data set. The effect of substrate inhibition, dual or multiple substrate limited growth, inaccuracies in the initial conditions assumed for curve fitting, and the use of poorly acclimated cultures are discussed. Techniques are presented which allow identification of whether a data set is unsuitable and should not be used for parameter estimation. In addition, experimental procedures which can help avoid the collection of aberrant data are discussed.

INTRODUCTION

Increasingly stringent environmental regulations require that maximal effluent concentrations be met for many synthetic organic chemicals discharged from point-sources. To predict effluent concentrations from waste treatment unit operations, adequate mass balances and rate terms for biotic and abiotic transformations must be developed and calibrated. For biodegradable organic compounds, biotic transformation rates are often the most important and two pieces of information are necessary to calibrate them. First, an estimate of the fraction of biomass active in the degradation of a given chemical is required. Second, estimates must be available for the specific parameters describing biotransformation of the chemical. The Monod (1949) and Andrews (1968) kinetic expressions are most commonly used to describe biotransformation rates. The Monod expression contains two parameters (μ_{max} and K_s), whereas the Andrews expression contains the same two, plus a third (K_i). Thus, characterization of biodegradation kinetics involves quantification of these parameters, as well as the true growth yield, Y. Several continuous and batch assays have been developed for parameter estimation (Grady, 1985). Since the introduction of automated respirometers in the 1980s, the use of respirometric techniques to study biodegradation capacity and biodegradation kinetics has experienced ever-growing popularity. This can be attributed to the fact that these techniques allow the collection of high-quality data with a relatively small input of experimental and analytical effort. In previous papers it has been demonstrated that respirometric data from batch growth experiments using acclimated biomass may be used to estimate the values of intrinsic kinetic parameters characterizing the biodegradation of individual organic compounds (Dang *et al.,* 1989; Brown *et al.,* 1990; Aichinger *et al.,* 1992; Naziruddin *et al.,* 1995). The application of nonlinear curve-fitting techniques to such data is necessary to achieve the most exact parameter estimates (Dang *et al.,* 1989; Brown *et al.,* 1990). However, the data must be critically inspected before they are subjected to any curve fitting routine to ensure that they meet the criteria assumed in the Monod or Andrews equations. The purpose of this paper is to illustrate how such an inspection can be accomplished from a preliminary evaluation of the data. It will also describe how initial estimates may be obtained for two of the parameters (Y and μ_{max}). The same concepts can also be applied to the evaluation of data on biomass growth or substrate depletion.

MATHEMATICAL DEVELOPMENT

Respirometric Equations

One of the key factors that allows the use of respirometric data for estimation of substrate biodegradation kinetics is the fact that substrate consumption, biomass growth, and oxygen consumption are stoichiometrically linked according to the following COD (chemical oxygen demand) balanced equation for aerobic chemoheterotrophic microbial growth:

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$$S - (1 - Y - Yp)O_2 = YX + YpP \quad (1)$$

where S is the substrate concentration, X is the biomass concentration, P is the soluble microbial product concentration (all measured in COD units), Y is the biomass yield coefficient (mg biomass COD formed/mg substrate COD used), and Yp is the product yield coefficient (mg product COD formed/mg substrate COD used). Therefore, the rate of oxygen consumption, r_{O_2}, is linked to the rate of biomass growth, r_X, the rate of substrate consumption, r_S, and the rate of product formation, r_P, according to the following equation:

$$\frac{-r_{O_2}}{1 - Y - Y_p} = \frac{r_X}{Y} = \frac{r_p}{Y_p} = -r_S. \quad (2)$$

This means that oxygen consumption data provide exactly the same information as biomass accumulation or substrate consumption data. If growth occurs in a batch reactor with a sealed aerobic environment, and all concentrations are expressed in COD units, the following equation describes the cumulative oxygen uptake at any time, O_{u_t}:

$$O_{u_t} = S_o - S_t + X_o - X_t + P_o - P_t. \quad (3)$$

This states that in the sealed environment of a batch bioreactor all of the substrate COD removed is either converted into biomass and microbial products or is oxidized to CO_2. Use is made of this concept to allow computation of theoretical oxygen consumption for the comparison with the actual measured oxygen consumption during nonlinear parameter estimation.

Typically, parameter estimation using batch respirometric data involves solution of the differential equations describing growth (Eq. (4)), substrate consumption (Eq. (5)), and product formation (Eq. (6)) (Dang *et al.*, 1989) thereby providing values of S_t, X_t, and P_t for substitution in Eq. (3). In these equations, μ_t is the microbial specific growth rate (hr^{-1}) and b_t is the specific decay rate (hr^{-1}).

$$\left(\frac{dX}{dt}\right)_t = \mu_t X_t - b_t X_t \quad (4)$$

$$\left(\frac{dS}{dt}\right)_t = -\frac{1}{Y}\mu_t X_t \quad (5)$$

$$\left(\frac{dP}{dt}\right)_t = -Y_p\left(\frac{dS}{dt}\right)_t. \quad (6)$$

Before the equation can be solved, the dependency of the specific growth rate on the substrate concentration must be expressed by either the Monod (Eq. (7)) or Andrews (Eq. (8)) expression.

$$\text{Monod: } \mu_t = \mu_{max}\frac{S_t}{K_s + S_t} \quad (7)$$

$$\text{Andrews: } \mu_t = \mu_{max}\frac{S_t}{K_s + S_t + \frac{S_t^2}{K_i}}. \quad (8)$$

Parameter estimation is an iterative process. Using initial estimates of the parameters Y, Y_p, μ_{max}, K_s, K_i, and b time-dependent values for S, X, and P are obtained by solving Eqs. (4)–(6) simultaneously. These values are then substituted in Eq. (3) and the resulting predicted O_u profile is compared with the actual profile. Deviations between the actual and predicted O_u profiles are quantified as the residual sum of squares error (RSSE). Using some form of logic, new estimates are made of the parameters and the process is repeated giving a new value of RSSE. Typically independent estimates may be obtained for Y, Y_p, and b, meaning that the nonlinear parameter estimation routine is only required for μ_{max}, K_s, and K_i. Parameter searching terminates when adequate agreement between the actual and predicted O_u profiles is obtained (i.e., when RSSE is sufficiently small). These parameters are called the best-fit parameters.

It must be realized that the implicit assumption in any parameter estimation algorithm is that the mathematical model employed is an accurate description of the physical observations. Because the parameter estimation routine is unable to verify this assumption, it is the researcher's responsibility to evaluate the experimental data prior to parameter estimation to determine whether they are adequately described by the mathematical model employed. The following sections illustrate how this can be done.

Data Transformation to Facilitate Preliminary Evaluation

It is very difficult to examine an oxygen consumption curve by eye and ascertain whether the data are consistent with the assumptions implicit in the models depicted by Eqs. (4) through (8). One important assumption is that the bacteria are fully acclimated to the substrate of interest and able to consume it immediately. Therefore, any lag or acclimation period is minimal and all bacteria are assumed to be active. In addition, Monod or Andrews kinetics assume that there is only one factor limiting the growth rate, the substrate whose biodegradation kinetics are being evaluated. Luckily, these assumptions are much easier to evaluate if the oxygen consumption data are transformed in two alternative forms.

It is also difficult to ascertain whether the Monod (Eq. (7)) or Andrews (Eq. (8)) expression is the most accurate depiction of the effect of substrate concentration on μ. Theoretically, one should be able to employ only the Andrews

equation during parameter estimation because it simplifies to the Monod equation when K_i is very large. In other words, if the Monod equation were more appropriate, then the curve fitting routine would simply return a large value for K_i. In practice, however, a two-parameter fit (Monod) is much easier to accomplish than a three-parameter fit (Andrews). Consequently, it is beneficial to be able to choose the appropriate model prior to beginning the parameter estimation routine. This choice can be facilitated by the same type of data transformation.

The rationale for the data transformation can be seen in the following. Taking the time derivative of all terms in Eq. (3) yields:

$$\left(\frac{dO_u}{dt}\right)_t = -\left(\frac{dS}{dt}\right)_t - \left(\frac{dX}{dt}\right)_t - \left(\frac{dP}{dt}\right)_t. \tag{9}$$

Based on Eq. (2), the following substitution can be made:

$$\left(\frac{dP}{dt}\right)_t = -Y_p\left(\frac{dS}{dt}\right)_t. \tag{10}$$

In batch growth experiments cell decay is minimal during the growth phase and becomes significant only when substrate is almost completely removed. Therefore, decay has successfully been modeled with an inverse Monod function during parameter estimation (Dang *et al.*, 1989). However, when a batch growth experiment is terminated immediately after substrate respiration, changes in biomass concentration are predominantly determined by substrate consumption and cell decay may be ignored. Thus, for purposes of data transformation, the following simplification may be made:

$$\left(\frac{dS}{dt}\right)_t = -\frac{1}{Y}\left(\frac{dX}{dt}\right)_t. \tag{11}$$

Equations (10) and (11) may be substituted in Eq. (9) to relate oxygen uptake rate to the rate of bacterial growth:

$$\begin{aligned}\left(\frac{dO_u}{dt}\right)_t &= \frac{1 - Y_p}{Y}\left(\frac{dX}{dt}\right)_t - \left(\frac{dX}{dt}\right)_t \\ &= \frac{1 - Y_p - Y}{Y}\left(\frac{dX}{dt}\right)_t.\end{aligned} \tag{12}$$

Equation (12) can be solved for dX/dt to yield:

$$\left(\frac{dX}{dt}\right)_t = \frac{Y\left(\frac{dO_u}{dt}\right)_t}{(1 - Y_p - Y)}. \tag{13}$$

Thus, it can be seen that the rate of bacterial growth is directly related to the rate of oxygen consumption in the absence of decay.

Recognizing that:

$$Y = \frac{X_t - X_o}{S_o - S_t} \text{ and } Y_p = \frac{P_t - P_o}{S_o - S_t}, \tag{14}$$

Eq. (3) can be rewritten as

$$O_{u_t} = \frac{(1 - Y_p - Y)}{Y}(X_t - X_o), \tag{15}$$

which can be solved for X_t:

$$X_t = X_o + \frac{Y}{(1 - Y_p - Y)} O_{u_t}. \tag{16}$$

Equation (16) states that the amount of biomass grown is directly related to the amount of oxygen consumed. Two useful data transformations result directly form Eqs. (13) and (16) and the fact that the bacterial specific growth rate is defined as:

$$\mu_t = \frac{1}{X}\left(\frac{dX}{dt}\right)_t. \tag{17}$$

The first comes from substitution of Eqs. (13) and (16) in Eq. (17):

$$\mu_t = \frac{\left(\frac{dO_u}{dt}\right)_t}{\frac{(1 - Y_p - Y)}{Y} X_o + O_{u_t}}. \tag{18}$$

Equation (18) reveals that μ may be determined solely from data on the rate of oxygen consumption and the total amount of oxygen consumed. Furthermore, since the residual substrate concentration is related to the amount of oxygen consumed (see Eq. (3)), Eq. (18) allows one to see how the substrate concentration affects the specific growth rate. The second useful transformation comes from rearranging Eq. (18) to yield:

$$\left(\frac{dO_u}{dt}\right)_t = \frac{(1 - Y_p - Y)}{Y}\mu_t X_o + \mu_t O_{u_t}. \tag{19}$$

As will be demonstrated later the shape of the plot of $(dO_u/dt)_t$ versus O_{u_t} is useful in identifying limitations during a batch growth experiment.

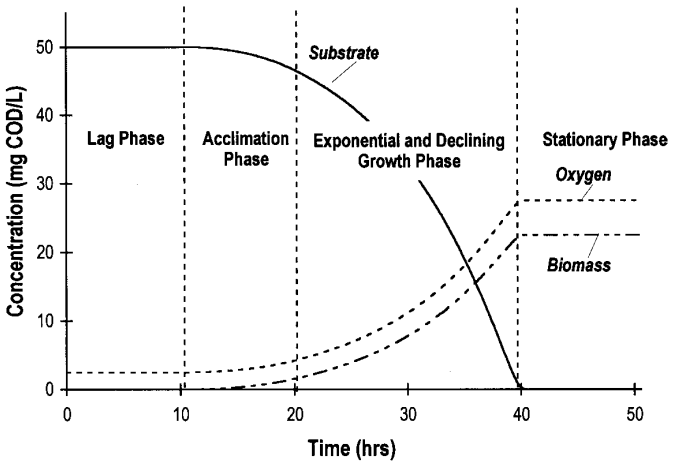

FIG. 1. Different phases that may be observed during microbial batch growth on a single limiting nutrient. Biomass concentration, substrate concentration, and oxygen consumption profiles are illustrated.

USE OF TRANSFORMED DATA PLOTS TO EVALUATE ACCLIMATION AND IDENTIFY SUBSTRATE INHIBITION

Impact of Acclimation

Figure 1 illustrates profiles of biomass concentration, substrate concentration, and cumulative oxygen consumption that could be observed during the batch growth of a microbial culture on a limiting carbon source. It is evident from Fig. 1 that growth may not commence immediately. Rather, a lag phase may be observed wherein cells adjust to the new growth environment and growth is absent, followed by an acclimation phase during which an increasing number of cells commence to grow and remove the substrate. Once all cells are growing maximally, the exponential growth phase can be observed until the substrate becomes limiting and declining growth ensues. Once all substrate is consumed, the cells enter the stationary phase. Recognition of the possible occurrence of the lag and acclimation phases is necessary because neither the Monod nor Andrews equation is able to model growth during those phases. If such phases are present in a data set, they must be eliminated before the remainder of the data set is used for parameter estimation. Because substrate utilization and biomass growth occur during the acclimation phase as illustrated in Fig. 1, elimination of that phase from the data set requires adjustment of the substrate and biomass concentrations present at the start of the exponential phase. The method for doing so is discussed below.

In order to illustrate the use to which the transformed plots can be put, a number of computer simulations were performed using the model depicted by Eqs. (3) through (8), providing theoretical data of oxygen consumption versus time. In all of those simulations, the following kinetic and stoichiometric parameters and initial conditions were used unless reported otherwise:

$$\mu_{max} = 0.1\ hr^{-1}$$
$$K_s = 1\ mg/liter\ as\ COD$$
$$K_i = 5, 10, 20, 50, 100, \infty\ mg/liter\ as\ COD$$
$$Y = 0.5\ mg\ biomass\ COD/mg\ substrate\ COD$$
$$Y_p = 0.05\ mg\ product\ COD/mg\ substrate\ COD$$
$$X_o = 2.5\ mg/liter\ as\ COD$$
$$S_o = 50\ mg/liter\ as\ COD.$$

In order to illustrate the impact of acclimation on the oxygen consumption curve for a batch bioreactor, use was made of the work of Kono (1968). Although the exact mechanism of adaptation has not yet been elucidated, Kono (1968) provides an empirical equation which mimics the effect of having more and more cells growing at their maximal rate as time progresses. His expression is

$$\left(\frac{dX}{dt}\right)_t = \mu_{max}[X_t - (1 - \alpha t)X_o], \qquad (20)$$

where α is an acclimation rate constant which account for the rate of increase of growth activity of the cells over time (Kono, 1968). To simulate acclimation, Eq. (20) was used in place of the Monod equation during acclimation, after which the Monod equation was applied until substrate was exhausted. Acclimation rate constants between 0.01 and 0.1 hr^{-1} were used. In addition, a simulation was performed in the absence of acclimation, in which case exponential growth began immediately.

Figure 2A illustrates the respirometric response of a batch culture in the presence (solid curve) and absence (dashed curve) of acclimation. Examination of Fig. 2A indicates that all of the curves are qualitatively similar. Consequently, it would be almost impossible to tell from examination of the oxygen consumption curve whether acclimation had been occurring during a test. Transformation of the data set according to Eq. (18) makes it immediately apparent that acclimation was occurring as presented in Fig. 2B. In the absence of acclimation, the specific growth rate starts immediately at a high value and declines as oxygen is consumed (i.e., as substrate is consumed). However, in the presence of acclimation, the specific growth rate rises initially, with the amount of oxygen and substrate consumed depending on the rate of acclimation.

Ideally, researchers should avoid kinetic parameter estimation with data from a growth experiment within which significant acclimation had occurred. Thus, kinetic experiments should be commenced with a fully acclimated inocu-

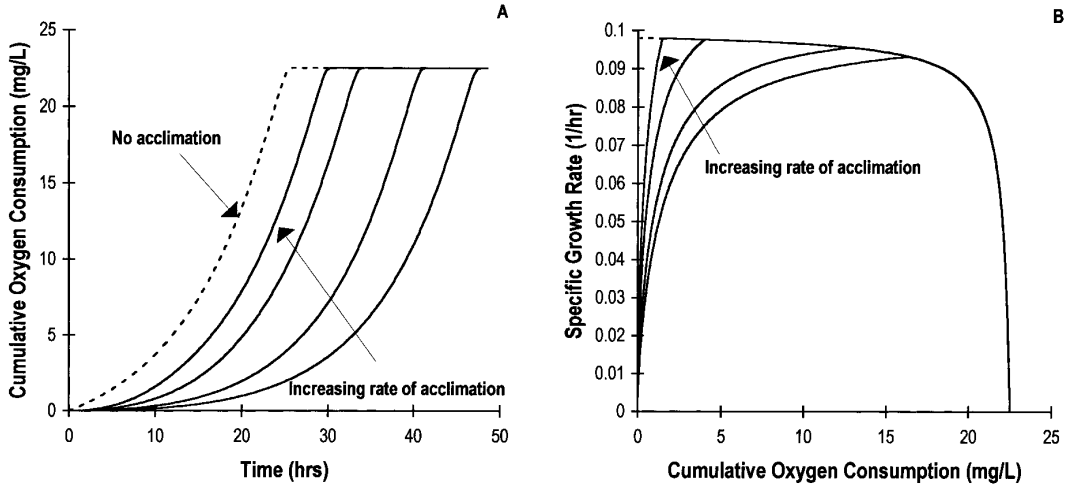

FIG. 2. The impact of an acclimation phase on oxygen consumption during batch growth and substrate mineralization. (A) Cumulative oxygen consumption profiles. (B) Specific growth rate profiles calculated with Eq. (18). The dashed lines represent the case without acclimation.

lum, which can easily be obtained by performing a preliminary respirometric test and harvesting the culture when it is in the exponential growth phase.

Experiments with a significant acclimation phase can, however, be used for kinetic parameter estimation provided the data set is truncated to exclude the acclimation phase. The end of the acclimation phase can be discerned most easily with the dO_u/dt vs O_u plot (Fig. 2B). When data are truncated in this way, the original conditions, S_o and X_o, must be altered to reflect the conditions at the end of the acclimation phase because some substrate utilization and biomass growth occurs during the acclimation phase, as discussed previously. The oxygen consumed during the acclimation phase allows estimation of the substrate removed during this phase. Again, this value is derived most easily from the dO_u/dt vs O_u plot (Fig. 2B). With knowledge of the yield coefficients (see below), the biomass which was formed during the acclimation phase can be computed with Eq. (15). A similar equation can be derived that allows calculation of the substrate consumed:

$$O_{u_t} = \frac{(1 - Y_p - Y)}{Y}(X_t - X_o) = \frac{(1 - Y_p - Y)}{Y}\Delta X \qquad (15'')$$

$$O_{u_t} = (1 - Y_p - Y)(S_t - S_o) = (1 - Y_p - Y)\Delta S. \qquad (21)$$

The values for biomass formed (ΔX) and substrate consumed (ΔS) thus calculated must be added to and subtracted from the initial biomass and substrate concentrations, respectively, to obtain the X_o and S_o values at the onset of the exponential growth.

Truncation of the acclimation phase will provide sufficient data for kinetic parameter estimation provided that the acclimation phase is not too long. With increasing acclimation phase lengths, more of the substrate consumption occurs during acclimation, providing only a very short period of subsequent exponential growth (Fig. 2B). Under such conditions, truncated data will not allow good estimation of μ_{max}.

Identification of Substrate Inhibition

Another problem facing the experimenter during data analysis is the selection of the appropriate model for use in parameter estimation. Figure 3A illustrates oxygen consumption curves (in the absence of acclimation) associated with Monod kinetics ($K_i = \infty$) and with Andrews kinetics with varying degrees of substrate inhibition (low K_i values represent strong inhibition). As with Fig. 2A, it is evident from Fig. 3A that it would be very difficult to identify substrate inhibition from examination of just an oxygen consumption curve. Luckily, the type of transformation indicated by Eq. (19), i.e., a plot of $(dO_u/dt)_t$ vs O_{u_t} makes it much easier to discern substrate inhibition.

Figure 3B illustrates the dO_u/dt vs O_u profiles for the different degrees of inhibition kinetics depicted in Fig. 3A. All of the profiles are monotonically increasing functions of O_u until the substrate is nearly depleted. At that point the substrate concentration approaches K_s causing dO_u/dt to rapidly fall off and approach zero. In actual experiments, dO_u/dt will not decrease to zero. Rather, it will precipitously decrease to a fairly constant but very low dO_u/dt indicative of endogenous oxygen consumption. The monotonic increase in the profile is because during unrestricted growth (when $S > K_s$) the growth rate is a first order function of the biomass concentration. Because the oxygen consumption rate is coupled to the growth rate (Eq. (2)) and the biomass concentration is linked to the total oxygen consumed (Eq.

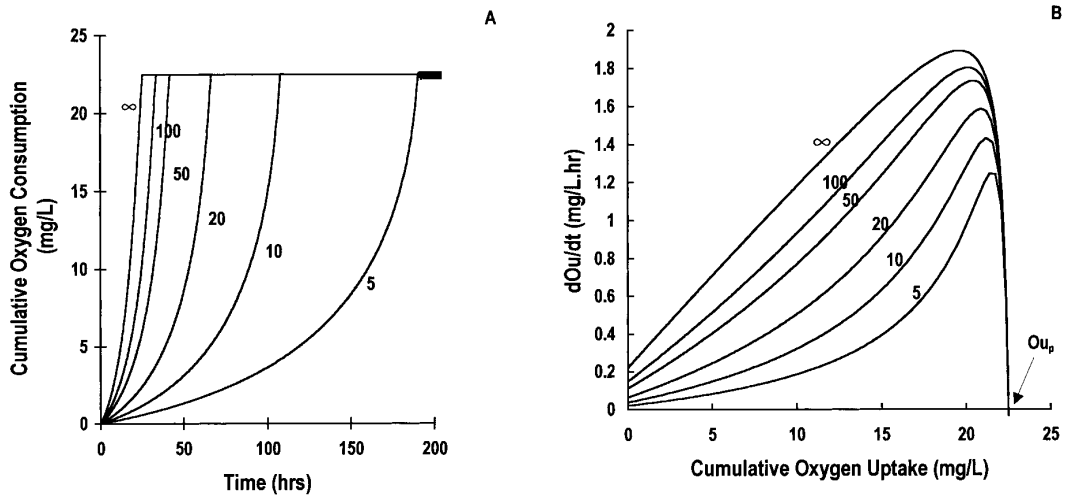

FIG. 3. The impact of the degree of substrate inhibition on the oxygen consumption rate during batch growth substrate mineralization. K_i = 5, 10, 20, 50, 100, or ∞ mg/liter. (A) Cumulative oxygen consumption profiles. (B) Oxygen consumption rate profiles.

(16)), the oxygen consumption rate is also an increasing function of the cumulative oxygen consumption. With increasing substrate inhibition (i.e., at decreasing values of K_i) dO_u/dt is still an increasing function of O_u, although the dependence is less than first order making the curve concave upward.

The dO_u/dt vs O_u curves, therefore, allow identification of the presence of substrate inhibition. In the absence of inhibition, the initial part of the plot will be linear or concave downward. The presence of substrate inhibition is reflected by upward concavity. This simple data transformation, therefore, makes clear the need to subject the data to a fitting routine that incorporates substrate inhibition kinetics.

When experimental data are transformed, attention must be paid to the number of data points used to calculate each value of dO_u/dt. Because of the error associated with the experimental data, it is recommended that the oxygen consumption rate not be calculated using sequential data points. Rather, the use of three to five points is suggested. By shifting one point at a time, a smoothing of the slope can be obtained that makes it much easier to identify the trends in the data. The actual number used will depend on the quantity of data points collected during the experiment, with more smoothing possible with larger data sets.

IDENTIFICATION OF FACTORS PRECLUDING PARAMETER ESTIMATION

In the preceding section it was illustrated how transformed data sets can be used to determine when acclimation has been achieved and to identify the appropriate kinetic model describing the effect of substrate concentration on biodegradation. Figure 3B indicated that for a culture growing with only the carbon and energy source as the growth-limiting substrate, a plot of dO_u/dt versus the cumulative oxygen consumption increases monotonically until the substrate becomes limiting, at which time the plot drops precipitously. This is a very important characteristic of the plot and in this section it will be demonstrated that deviations from that characteristic can be used to identify data sets which are not consistent with the assumptions inherent to the Monod and Andrews models. Such data sets cannot be used to obtain kinetic parameter values.

Oxygen Supply Limitation

Figure 3B demonstrated that the rate of oxygen consumption continually increases throughout a batch experiment until the substrate becomes limiting, at which time the rate drops rapidly. However, if the maximum rate at which the respirometer can deliver oxygen is less than the maximum rate at which the bacteria need to use it, an artificial limit is imposed upon the system which results in a constant growth phase rather than an exponentially increasing one. Data collected under such a condition are not representative of the effect of substrate concentration on the specific growth rate and thus cannot be used for parameter estimation.

The impact of limiting oxygen delivery rates on oxygen consumption profiles is illustrated in Fig. 4 using experimental data from the batch mineralization of phenol. Figure 4A illustrates the cumulative oxygen consumption data from two flasks with different maximal oxygen delivery rates. (Note that the initial substrate concentrations in the two flasks were different, giving a different total oxygen consumption.) From Fig. 4A it is not immediately apparent that flask 1 had an oxygen transfer limitation, and in fact, both experimental curves could be fitted with a Monod equation describing

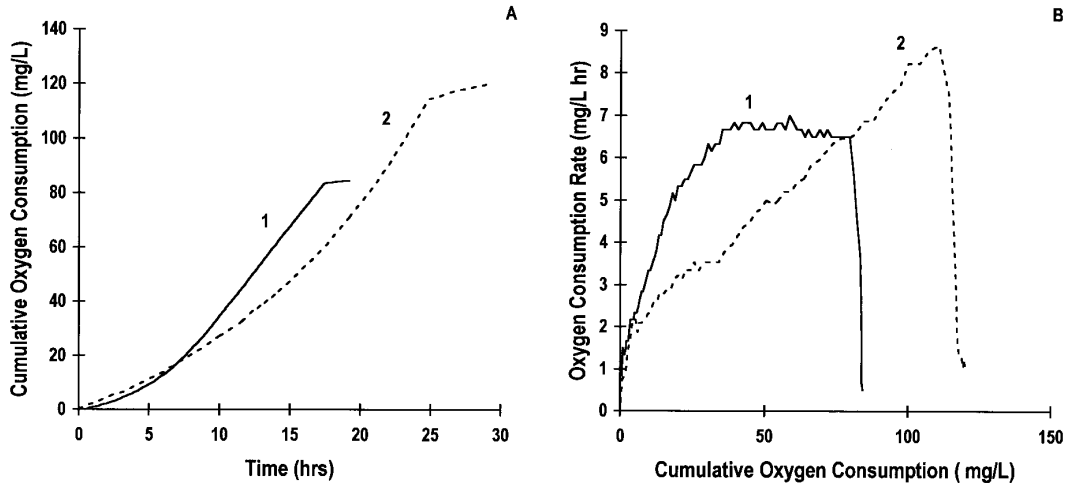

FIG. 4. The impact of an oxygen delivery limitation on oxygen consumption rate during batch growth substrate mineralization experiments. Flask 1, solid line; flask 2, dashed line. (A) Cumulative oxygen consumption profiles. (B) Oxygen consumption rate profiles. Note that the two flasks received different initial substrate concentrations.

growth. Detailed inspection, however, revealed that oxygen transfer limited the growth rate in flask 1. This can clearly be seen much more easily in Fig. 4B which illustrates the change in oxygen consumption rate as a function of the cumulative oxygen consumption for both flasks. Flask 2 followed the expected profile illustrated in Fig. 3B. Flask 1, however, had a period of constant oxygen consumption rate indicative of an oxygen transfer limitation. Parameters obtained by fitting curve 1 to the Monod equation are not valid and should be discarded because the assumption of a single substrate limitation inherent to the Monod equation was violated.

In order to ensure that all experimental data are valid and can be used for parameter estimation, the oxygen delivery system in the respirometer should be adjusted so that it is capable of delivering oxygen at a rate in excess of the maximum rate that the culture will experience. An estimate of that maximum rate can be made using likely values for the kinetic parameters. Recognizing that at any time the biomass concentration $X_t = X_o + Y(S_o - S_t)$, Eqs. (11) and (17) can be combined to yield:

$$\left(\frac{dS}{dt}\right)_t = -\frac{1}{Y}\mu_t(X_o + Y(S_o - S_t)). \tag{22}$$

The μ_t term is subsequently replaced by the appropriate Monod or Andrews rate term (Eqs. (7) and (8)) to yield an expression for dS/dt with S as the sole dependent variable. By taking the second derivative of S with respect to time, and solving the equation for the condition $d^2S/dt^2 = 0$, values for S^* can be obtained. These values represent the substrate concentrations during the growth experiment at which the substrate transformation rates, and hence the oxygen consumption rates, are maximal. The following expressions for S^* were derived for Monod and Andrews kinetics, respectively:

$$S^*_{\text{Monod}} = -K_s + \sqrt{K_s^2 + K_s\left(S_o + \frac{X_o}{Y}\right)} \tag{23}$$

$$S^*_{\text{Andrews}} = \frac{-K_s + \sqrt{K_s^2 + K_s\left(S_o + \frac{X_o}{Y}\right)\left[\left(S_o + \frac{X_o}{Y}\right)\frac{1}{K_i} + 1\right]}}{\left[\left(S_o + \frac{X_o}{Y}\right)\frac{1}{K_i} + 1\right]}. \tag{24}$$

Using preliminary estimates of K_s, K_i, and Y, values for S^* can be calculated and used to find the maximum substrate uptake rate, using Eqs. (22) and (7) or (8). The corresponding oxygen consumption rate can then be calculated with Eq. (2).

The oxygen delivery rate of the apparatus should be set at a safe multiple of the calculated maximum oxygen consumption rate to avoid oxygen limitations. Care should be taken, however, to not set the oxygen delivery rate too high because this will result in a respirogram with less resolution. The researcher can also easily evaluate the maximum O_2 transfer rate achievable by a given experimental system, by performing a test with excess biological activity. The excess biological activity ensures that O_2 transfer will be limiting, and its value can be observed from the dO_u/dt vs O_u plot.

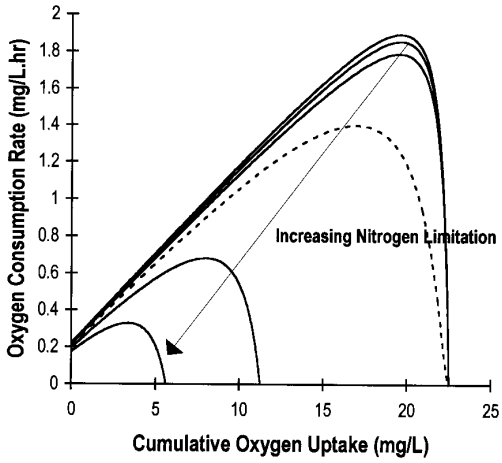

FIG. 5. The impact of $NH_4^+ - N$ limitation on the oxygen consumption rate during batch growth on a single substrate as calculated by modeling. $NH_4^+ - N$ was assumed to be present in excess, or at 4, 2, 1 (dashed line), 0.5, or 0.25 times the stoichiometrically required dose.

Nutrient Limitation

A second common growth limitation that can occur during respirometric experiments is due to deficiencies in the mineral composition of the medium used. The ability of the medium to allow unrestricted growth should be verified by the researcher based on preliminary knowledge of the yield and stoichiometry of the bacterial cell. In order to illustrate the impact of an inorganic nutrient limitation, simulations were performed using a kinetic model in which both the organic substrate and the nitrogen source ($NH_4^+ - N$) were growth limiting. Instead of the single substrate limitation (Eq. (7)), a dual substrate limitation model was used to compute the specific growth rate (Droop, 1973):

$$\mu_t = \mu_{\max} \frac{S_{1t}}{K_{s_1} + S_{1t}} \frac{S_{2t}}{K_{s_2} + S_{2t}} \qquad (25)$$

wherein S_1 and K_{s_1} refer to the concentration of and the affinity for the organic substrate and S_2 and K_{s_2} refer to the concentration of and affinity for $NH_4^+ - N$, respectively. A typical K_{s_2} values of 0.5 mg/liter as $NH_4^+ - N$ was assumed. Based on the empirical formula $C_5H_7O_2N$ for bacterial mass (Hoover and Porges, 1952), a constant ratio of 0.15 g N consumed per gram of substrate COD consumed was calculated for balanced microbial growth and no substrate storage was assumed to occur during N limitation.

The impact of an increasing $NH_4^+ - N$ limitation resulting from these simulations is illustrated in Fig. 5. At the onset of growth, $NH_4^+ - N$ was either provided in great excess (using the single substrate limitation model) or was provided at 4, 2, 1, 0.5, or 0.25 times the stoichiometrically required dose. Figure 5 illustrates three key points. First, even when the N dose exceeds the stoichiometrically required amount by as much as fourfold, oxygen consumption rates are impacted. This means that any kinetic parameters obtained by fitting with a single substrate model would be incorrect. Second, when the N dose is less than the nonlimiting amount, the dO_u/dt profiles qualitatively change. Oxygen consumption rates level off too soon and gradually decrease to zero. This means that any kinetic parameters obtained by fitting with a single substrate model would be incorrect. Third, when the N dose is less than the stoichiometrically required amount, the total oxygen consumed is less than expected. Consequently, atypically high yield values are calculated (see Eq. (25) below). Such high yields provide an easy means for identifying severe nutrient limitation. The degree of impact of a second nutrient limitation on the substrate and oxygen consumption rates is also dependent on the affinity coefficient for the second nutrient. At higher affinities, the secondary limitation impacts the oxygen consumption less. However, to be conservative, it is recommended that all nutrients be provided in at least a 10-fold stoichiometric excess to avoid the impact of a second limitation on the biodegradation and resulting oxygen consumption profiles.

The impact of a nutrient limitation on experimental oxygen consumption profiles is illustrated in Fig. 6. The data were collected from the batch growth mineralization of toluene (initial concentration of 100 mg/liter as COD) by an enrichment culture. The inoculum size to all flasks was identical, while different amounts of a mineral salts solution were added to each flask. The full-strength mineral salts solution provides stoichiometrically sufficient micro- and macronutrients to allow mineralization of 5000 mg/liter as COD of an organic substrate (Daigger, 1979). The mineral salts solution in the three examined flasks was provided at 50-, 5-, or 0.5-fold the stoichiometrically required dose for mineralization of 100 mg/liter as COD of toluene. Figure 6A clearly indicates the nutrient limiting effect when only 0.5-fold the stoichiometrically required dose was added. The observed oxygen consumption rates during mineralization were significantly lowered, as was the total cumulative oxygen consumption. This very low oxygen consumption, or conversely very high apparent yield (see Eq. (25) below), is indicative of incomplete substrate respiration. The difference in the oxygen consumption profiles for the cases in which the nutrient excess was 50- or 5-fold is less apparent. Both curves plateaued at approximately the same total cumulative oxygen uptake indicating complete substrate respiration. Some indication of lower growth rates, however, was apparent in the 5*X* curve. This is more evident in Fig. 6B illustrating again the utility of the transformed data plot. Thus, even when nutrients were provided in 5-fold excess, growth rates were reduced, with the result that Monod kinetic parameters obtained under these experimental conditions would not truly reflect the kinetics of biodegradation under a single

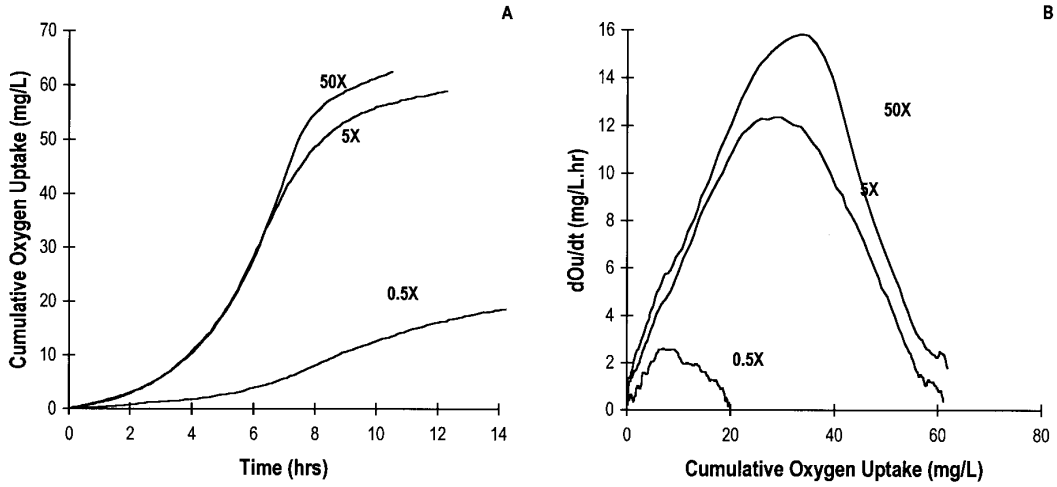

FIG. 6. The impact of a nutrient limitation on the oxygen consumption rate as observed during batch growth experiments on a single substrate. Nutrients were provided at 0.5, 5, or 50 times the stoichiometric amount. (A) Cumulative oxygen consumption profiles. (B) Oxygen consumption rate profiles.

substrate limitation. Better estimates for the single substrate growth parameters on toluene were derived from the experiment wherein nutrients were at 50-fold excess. This experimental curve allowed fitting with the Monod equation in a model which accounts for the partitioning of toluene between the gaseous and the aqueous phase (Naziruddin *et al.*, 1995).

Whenever a second limitation is observed due to oxygen transfer or nutrient limitation, the proposed μ/S relationships embodied in the Monod or Andrews equations are no longer valid. Further data fitting should be abandoned because neither the Monod or Andrews equation are adequate models to describe these dual or multiple limitation phases. Rather, after identification of the cause of the limitation, the experiment should be revised to remove the additional limitation and repeated.

Inappropriate S_o Value

How an oxygen transfer limitation can be identified from a region of constant oxygen uptake rate in a plot of dO_u/dt was illustrated earlier. Other factors, however, can also affect the shape of the dO_u/dt curve and thus care must be exercised while interpreting such plots. One of those factors is the affinity of the microorganisms for the substrate as reflected in the value of the half-saturation coefficient, K_s, and more particularly the initial substrate concentration used in an experiment relative to that half-saturation coefficient. To illustrate this point, simulations were performed using a model incorporating Monod kinetics and Fig. 7 illustrates the different oxygen consumption rate profiles obtained when K_s was varied while holding S_o constant. From this figure it can be seen that as K_s becomes larger relative to S_o, it becomes increasingly difficult to identify a secondary limitation. Perhaps more importantly, however, is the fact that as the ratio of S_o to K_s becomes smaller, it becomes more difficult to properly quantify the individual Monod parameters by nonlinear curve fitting to experimental data (Wang, 1988). Therefore, the presence of a constant or quasi-constant phase in the dO_u/dt vs O_u curve is undesirable and should be avoided. In such cases, the experimental design need to be redone so that a growth experiment with a higher initial substrate concentration is performed. For optimum parameter identifiability, the S_o/K_s value should be at least 1.0 (Wang, 1988).

OTHER USES OF TRANSFORMED DATA PLOTS

Although the focus has, to a large degree, been on how transformed data plots can be used to identify conditions

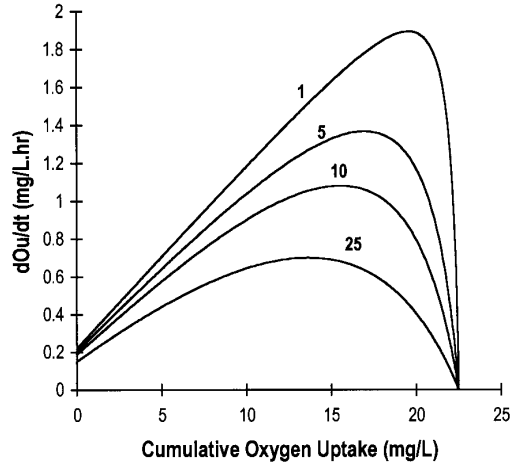

FIG. 7. The theoretical impact of the degree of substrate affinity on the oxygen consumption rate during batch growth on a single substrate. K_s was 1, 5, 10, or 25 mg/liter as COD while S_o was constant at 25 mg/liter as COD.

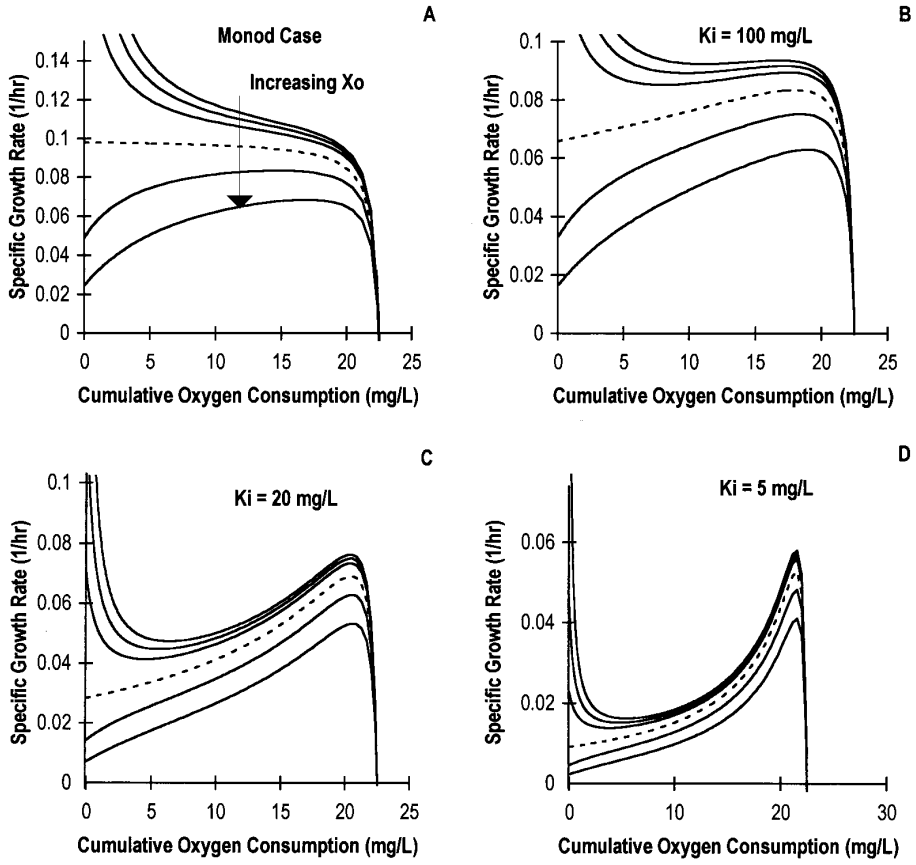

FIG. 8. The impact of the degree of substrate inhibition and the estimate of the initial biomass concentration on the calculated specific growth rate profiles. (A) $K_i = \infty$, (B) $K_i = 100$, (C) $K_i = 20$, (D) $K_i = 5$. In all panels, profiles from bottom to top were calculated with X_o estimates of 10, 5, 2.5 (dashed line), 1, 0.5, and 0.1 mg/liter as COD. The exact initial biomass concentration was 2.5 mg/liter.

that preclude use of a data set for parameter estimation, there are other more positive uses of them as well. Among them are obtaining initial estimates for μ_{max} and Y, and verification that the initial biomass concentration, X_o, used during curve fitting is indeed correct.

Initial Estimation of μ_{max} and Y

Examination of Eq. (19) reveals that the slope of the dO_u/dt vs O_u curve is μ_t. For Monod kinetics, $\mu_t = \mu_{max}$ when $S \gg K_s$. Therefore, the initial slope of the dO_u/dt vs O_u profile provides a good estimate of μ_{max} for Monod kinetics. However, when significant substrate inhibition occurs (i.e., when Andrews kinetics apply), accurate estimates of μ_{max} cannot be readily obtained because the specific growth rate is depressed at higher substrate concentrations. As a rough approximation, the construction of a line that goes through the origin and is tangential to the dO_u/dt vs O_u curve near its highest value is suggested. Its slope can be used as a first estimate of μ_{max}. This estimate is fair at low substrate inhibition, but may underestimate the true μ_{max} value by as much as 50% (Fig. 3B) at increasing levels of inhibition. Nevertheless, it should still provide an adequate initial μ_{max} estimate for subsequent curve fitting.

Y can be estimated from the total oxygen consumption at the onset of the plateau in oxygen consumption O_{u_p}, after which the endogenous phase occurs. First, however, Y_p must be estimated from the soluble COD fraction remaining in the respirometric at the plateau. That COD is comprised of soluble microbial products. Therefore, Y_p is calculated as the ratio of the soluble COD at the onset of the endogenous phase to the initial substrate concentration, S_o. By knowing O_{u_p} and Y_p, the biomass yield can be estimated as:

$$Y = 1 - \frac{O_{u_p}}{S_o} - Y_p. \tag{26}$$

O_{u_p} can be obtained from the O_u vs t curve as the oxygen consumption at the plateau, but is typically difficult to discern. It is easier to estimate O_{u_p} from the dO_u/dt vs O_u curve,

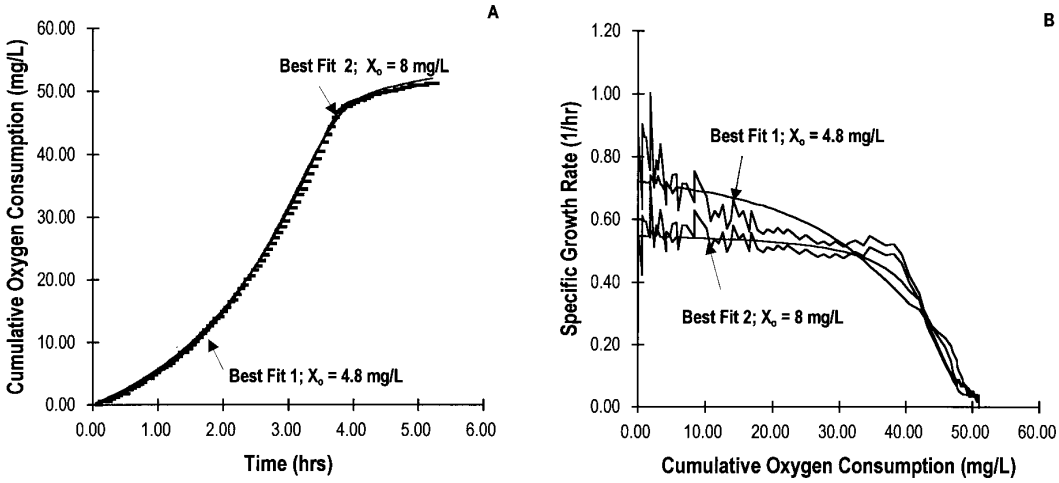

FIG. 9. The identification of a wrong X_o estimate and its impact on the best fit parameters estimates for a batch growth substrate mineralization experiment. (A) Cumulative oxygen consumption profiles and best fit curves. (B) Calculated specific growth rate profiles. The smooth continuous lines are the predicted curves from the model fits.

as the O_{u_p} value obtained by extending the rapidly falling region of the curve to the abscissa as presented in Fig. 3B.

Verification of the Initial Biomass Concentration, X_o

The other useful transformation of the experimental data allows evaluation of the accuracy of the X_o value to be used during parameter estimation. To calculate μ according to Eq. (18), values for Y, Y_p, and X_o must be known. Y and Y_p are estimated independently as described above, while X_o, or a best estimate thereof, is typically measured at the beginning of the experiment. Because Eq. (18) does not depend upon μ_{max}, K_s, and K_i, it may be used to check the accuracy of the estimate of X_o by examining the shape of the resulting μ vs O_u curve.

The dashed curve in Fig. 8 illustrate the shape of the μ profiles associated with different degrees of substrate inhibition when the value of X_o is exact. The solid curves illustrate how the calculated μ profiles behave for incorrect X_o values. The impact of an incorrect X_o estimate is the most apparent early in the profile because the term containing X_o in the denominator of Eq. (18) becomes less significant with increasing oxygen consumption. If the value of X_o is larger than the true value, the curves of μ vs O_u will lie below the correct μ profile and rise toward it. Conversely, if the assumed X_o value is smaller than the true value, then the curves will lie above the correct profile and fall toward it. The impact of incorrect X_o values is easily discernible when substrate mineralization obeys Monod kinetics (Fig. 8A), or when substrate inhibition is small (Fig. 8B). When substrate inhibition is large, however, incorrect X_o values are harder to discern, especially if they are larger than the true value (Fig. 8D).

The impact of an incorrect X_o value on the subsequent estimation of the best fit kinetic parameters is illustrated in Fig. 9. The oxygen consumption data in Fig. 9A were obtained from the mineralization of 100 mg/liter as COD of phenol by an enrichment culture. The initial inoculum concentration was measured experimentally as 4.8 mg/liter and Y and Y_p were measured at the end of the experiment as described before. Nonlinear parameter estimation techniques (Brown *et al.*, 1990) were applied with those values to estimate μ_{max} and K_s for the Monod equation. The resulting parameter values were $\mu_{max} = 0.855\ hr^{-1}$, $K_s = 18.26$ mg COD/liter which resulted in a RSSE value of 35.36. The theoretical oxygen consumption plot corresponding to those parameters is presented as best fit 1 in Fig. 9A. It appears to track the data set very well. However, when the transformation of Eq. (18) is made for both the experimental data and the fitted curve, it becomes apparent that the X_o value was in error. This can be seen in Fig. 9B in which the smooth curve corresponds to the fitted curve and the jagged curve corresponds to the data. The fact that the data curve falls initially indicates that X_o was too small. Adjustments were made to X_o until the experimental μ vs O_u curve was relatively flat, yielding and X_o value of 8.0. The nonlinear curve fit was then repeated giving a μ_{max} value of $0.571\ hr^{-1}$ and a K_s value of 4.08 mg COD/liter, which resulted in a much lower RSSE value of 6.48. Examination of Fig. 9A reveals that it is very difficult to discern a difference between best fit 1 and best fit 2 when plots of O_u versus time are examined by eye. However, examination of Fig. 9B illustrates that after the transformation of Eq. (18) is applied, the superiority of best fit 2 is clearly evident. This illustrates two important points. First, use of an incorrect X_o value during curve fitting will result in incorrect estimates of μ_{max} and K_s. Second, the goodness of fit associated with a given set of parameter

estimates is often more easily discerned in transformed plots than in plots of oxygen consumption versus time, even though parameter estimates were obtained by fitting the latter.

CONCLUSION

This paper addressed the preliminary evaluation of respirometric data obtained during biodegradation of organic compounds in batch reactors. Based on theoretically exact respirometric profiles, how those profiles are impacted by substrate inhibition, nutrient limitation, oxygen delivery limitation, the presence of an acclimation phase, and incorrect estimates of the active inoculum size was examined. The impact of these complicating factors is frequently difficult to discern in the untransformed data set, but can be detected with greater ease when the data are transformed and the oxygen consumption rate profile or the specific growth rate profile is inspected. Such evaluation can be carried out fairly simply using a spreadsheet program and should be executed prior to subjecting the data set to nonlinear curve fitting. This preliminary evaluation allows the investigator to examine whether the conditions implicit to the two commonly used expressions to describe substrate limited microbial growth, the Monod and Andrews equation, are met. Such examination ensures that the reported kinetic parameters truly reflect the kinetics of degradation under single substrate limited conditions by an acclimated culture.

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