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Examination of reproducibility in microbiological degradation experiments

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Abstract

Experimental data indicate that certain microbiological degradation experiments have a limited reproducibility. Nine identical batch experiments were carried out on 3 different days to examine reproducibility. A pure culture, isolated from soil, grew with toluene as the only carbon and energy source. Toluene was degraded under aerobic conditions at a constant temperature of 28 °C. The experiments were modelled by a Monod model – extended to meet the air/liquid system, and the parameter values were estimated using a statistical nonlinear estimation procedure. Model reduction analysis resulted in a simpler model without the biomass decay term. In order to test for model reduction and reproducibility of parameter estimates, a likelihood ratio test was employed. The limited reproducibility for these experiments implied that all 9 batch experiments could not be described by the same set of parameter values. However, experiments carried out the same day (within the same run) were more uniform than experiments carried out on different days (between runs), and a common set of parameter estimates could be accepted for experiments within runs, but not for experiments from different runs. The limited reproducibility may be caused by variability in the preculture, or more precisely, variations in the physiological state of the bacteria in the precultures just before used as inoculum.

Introduction

The variability of parameter estimates in microbiological degradation models has received limited attention in the literature (an exception is Grady et al. 1996), although such parameters, for example, are used in models for predicting and controlling microbiological processes of commercial interest. Furthermore, the accuracy of the parameter estimates also depends on the estimation method used, and an unsuitable method can lead to poor estimates that are systematically different from the 'true' values.

If an experiment is claimed to be reproducible, almost identical results should be found when the experiment is replicated. Thus, it is of interest to determine the variability, that is the variance of the experimental parameter estimates when replicated experiments are considered. In the present paper, the term 'reproducibility' is used as a general term for describing lack of variation between experiments that are repeated under certain conditions. Our definition of reproducibility is not identical to the definition of reproducibility given by the ISO 5725 standard (which also takes variation between laboratories into account). If an experiment is repeated in the same way a number of times, and a common set of parameter estimates can be identified when employing the likelihood ratio test, we shall say that the experiment is reproducible. In order to incorporate natural variation from experimental conditions the replicates were carried out on different days using different precultures (inocula) grown from the same stock culture and within the same laboratory. Thus, the repeated identical experiments (replicates) are here defined as experiments:

- which examine degradation of the same compound(s),
- which use the same type of biomass,
- which use the same chemical analysis method,
- which are carried out under the same environmental conditions,
- which are carried out in the same laboratory, and
- which ideally should be governed by the same model parameters.

A few studies concerning variability of the parameter estimates have been reported (Grady et al. 1996; Arcangeli & Arvin 1994; Templeton & Grady 1988), but they did not examine measures of reproducibility of the experiments explicitly. Blok & Booy (1984) reported on a poor reproducibility in an interlaboratory test. In the test, several laboratories participated using different methods to test the so-called 'readily biodegradability' (positive if degraded more than 70% after 4 weeks and negative if 30% or less degraded) of several compounds. Large variability was found between the laboratories and between the different methods. Blok and Booy carried out simulations performed on the basis of the Monod model and explained the variability by the variable quality of the inoculum (mixed culture). By quality, they meant the quantity of specific bacteria that were able to degrade the particular compound. In other words, they explained the variability of the results by the different initial concentrations of the specific bacteria in the different experiments. Pavlostathis & Giraldo-Gomez (1991) also suggested that, in order to obtain more reliable parameter estimates, the measurement of kinetic rates should be based on the viable microbial population density, as opposed to the total microorganism concentration. However, especially in systems dealing with particulate organic substrates, this is a very difficult procedure, which was also noted by Pavlostathis & Giraldo-Gomez (1991).

Tanner, Souki & D'Ambrosi (1981) experienced large variability of parameter values estimated from identical experiments. They suggested that this variability was caused by large measurement errors. This explanation need not be the only possible, because, when repeated experiments are conducted, there may occur differences in inoculum (variations in the environment), and when a poor estimation method is employed (e.g. linearization), considerable errors in the parameter estimates may be introduced.

When examining reproducibility, one should (beside repeating the experiments as identically as possible) also be aware of the following points that play an important role for the results. Consider two identical experiments where the biological processes are governed by the same unknown model but with small differences between the parameter values. The choice of the model, the measurement method, and the estimation technique are all only tools. An inappropriate choice of tool can give rise to irreproducibility. The choice of biological degradation model can be difficult, but when considering experiments that are not too complicated, good suggestions can often be found in the literature. The importance of employing a sound estimation technique has been given little attention in microbiological degradation experiments. Sáez and Rittmann (1992) emphasized the importance of using an estimation technique that matches the structure of the measurement errors, especially when the variance is strongly non-constant, as in the experiments in this study.

Parameters in microbiological degradation models are often estimated by linearization or by heuristic methods (Criddle 1993; Alvarez et al. 1991; Folsom et al. 1990; Machado & Grady 1989; Vecht et al. 1988). Such methods do not account for the error structure and, thus, can be inaccurate or even lead to inconsistent results. Parameter estimates found by these methods can only be rough estimates. In recent years, however, more researchers have used nonlinear estimation methods. Often the method is used for *some* of the parameters in combination with linearization or with parameter values taken from the literature (Kong et al. 1996; Chang et al. 1993; Nakhla & Al-Hazazin 1993; Thatipamala et al. 1992). Such methods can represent improvements, but the estimates obtained still can be far from optimal with respect to uncertainty or skewness. If the model is nonlinear, the best estimation result is usually obtained by using nonlinear estimation of all the parameters.

Considering the many microbiological degradation experiments carried out, it is surprising that relatively few authors have found it worthwhile to test in a formalized way whether the experiments are reproducible or not. Therefore, it is the purpose of the present paper to outline a formalized procedure for examining reproducibility and to apply the procedure to a simple microbiological degradation experiment. Three identical runs (I, II, and III) were carried out, each consisting of three identical batch experiments A, B, and C (Figure 1). The parameter values were estimated using a nonlinear estimation procedure based on an iterative maximum likelihood routine. This method takes the measurement variance structure into account, but its

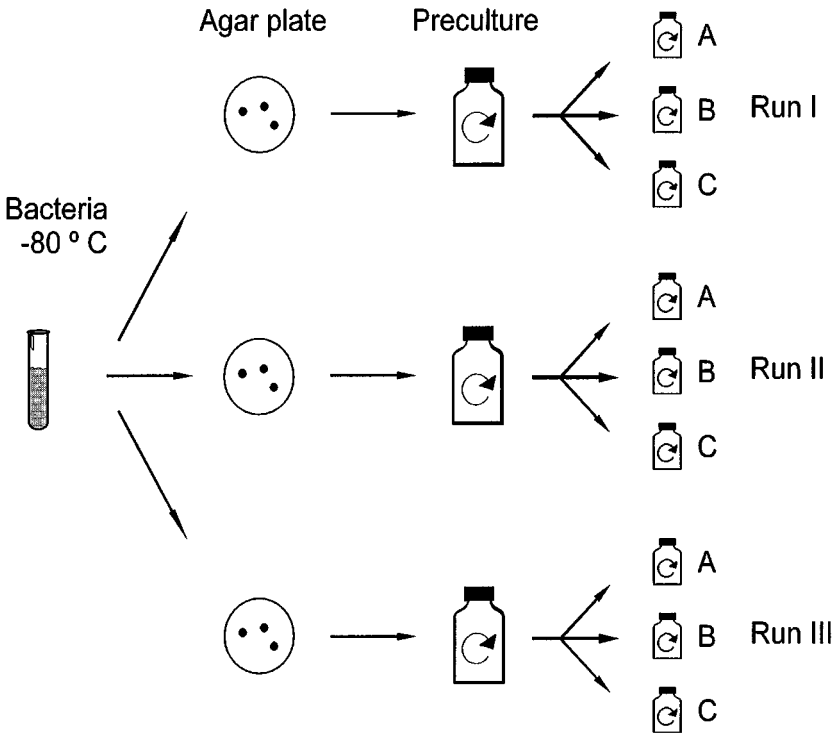

Figure 1. Biomass flow system.

main advantage over ordinary weighted least squares is that it leads to inference based on likelihood theory in a natural way. The test of reproducibility were based on an approximate likelihood ratio method. Tests for model reduction were performed by using 95% confidence intervals for the parameter estimates and by using an approximate likelihood ratio test.

The biological system consisted of a pure culture (*Pseudomonas cepacia*) with toluene as the only carbon and energy source. The experiment was kept very simple (one substrate and a pure culture) so that a standard Monod model was reasonable to apply. If more than one substrate is present, kinetic interactions very often occur, such as competitive inhibition or cometabolism, and for each of these phenomena, several models are suggested in the literature. For a single substrate system, however, generally the Monod model can be employed. The reason for choosing a pure culture instead of a mixed culture was to ensure as identical a biomass as possible in the repeated experiments and thereby avoid the phenomena Blok & Booy (1984) described concerning variable initial concentrations of specific degradation bacteria in different experiments.

Materials and methods

Experimental setup

The experiments were carried out in continuously stirred, 5-litre batch reactors under sterile aerobic conditions at 28 °C. The aqueous medium in the batches consisted of toluene (5–9 mg/l), bacteria, growth medium, and 4 litres of distilled water. The pure bacterial culture, identified as *Pseudomonas cepacia*, was isolated from soil and subsequently grown on toluene over one year. Besides the carbon/energy source (toluene), a mineral medium was added to provide the culture with required nutrients. The growth medium contained nitrogen sources, trace metals, and a phosphate mixture (see Sommer (1997) for a more detailed specification). The phosphate mixture also served as a buffer to ensure a stable pH value near 7. Samples of the liquid were taken every 15 minutes while air was injected to ensure overpressure (see Figure 2). The sampling frequency was decreased after the toluene was degraded. In Run I, only a few samples were taken in the decay phase, which implied that the biomass decay coefficient b was determined primarily by the last two biomass measurements in an

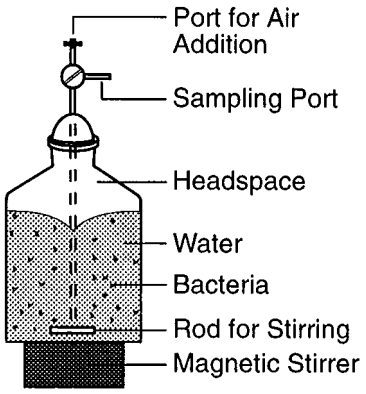

Figure 2. Illustration of a batch setup, which were used in all the microbiological degradation experiments.

experiment. In order to improve the estimation of b in the following experiments, more measurements of the biomass were taken in the decay phase.

Experimental design

When examining reproducibility, it is important that the experiments are carried out exactly in the same way in each replication and that environmental factors like temperature (which influence the degradation rate) are kept constant during the experiments. In order to limit the variation from the biomass inoculum as much as possible for all the experiments, the following procedure was used (see Figure 1). A few colonies of a pure culture (adapted to toluene) were frozen in a glycerol medium at −80 °C (Gibson & Khoury 1986). For each of the three runs (I, II, and III), a small amount of the frozen biomass was grown on an agar plate at 28 °C. A preculture of about 600 ml was then made from two or three colonies on the agar plate. Toluene stock solution was added to the preculture (about 10 mg/L) and mineral medium. After about one day, the toluene was completely degraded, and the biomass was ready to be used as inoculum for the actual experiment. A 130 - ml aliquot of the preculture was added to each batch.

The three identical runs (I, II, and III) each consisted of 3 batch experiments (A, B, and C) plus a blank test (without bacteria); all together, 9 batch experiments and 3 blank tests were carried out. Three batch experiments (A, B, and C) plus a blank test were carried out simultaneously, and the biomass in these 3 batches all came from the same preculture. The reason for this design was that we suspected that biomass characteristics might cause the largest variability. Within each of the three runs, the biomass was assumed to be exactly the same, but between the runs the biomass might differ somewhat due to small differences in the culture history (the type of environmental conditions imposed and the duration of their imposition (Grady et al. 1996)). The blank tests were used for checking that the batches were gastight and sterile (no contamination by other bacteria).

Chemical analysis

The toluene samples were analyzed in random order in a Shimadzu GC-9A gas chromatograph and later in a Carlo Erba MEGA gas chromatograph, both connected to a computing integrator. (Tests were made to ensure that the two gas chromatographs (Shimadzu and MEGA) gave the same results when analyzing the same samples). The samples (10 ml) were extracted with a pentane mixture (1 ml, double distilled pentane + internal standard), and the peaks were quantified by internal standardization with heptane as the standard. Data acquisition and integration were achieved on a MAXIMA Chromatography Workstation.

The biomass samples (also 10 ml) were measured as protein by the Lowry method (Lowry et al. 1951; Peterson 1979). The measurements were performed in random order in cuvettes on a Perkin Elmer UV/VIS Spectrometer Lambda2. This method measures the protein in the biomass, and, in order to convert to mg biomass/l, the protein weight was multiplied by 2 based on the assumption that the protein weight is 50% of the total dry weight of biomass. This assumption was checked by measuring 9 dried samples. The net dry weight was found to be twice as high as the protein weight (conversion factor = 1.99), although with quite large variations. For batches A, B, and C, the estimated ratios were: 2.1 ± 0.8, 2.4 ± 1.0, and 1.5 ± 0.2 ($\pm$ approximate 95% confidence intervals). Since the protein assay does not differentiate between active and inactive biomass, it was important to assure that the biomass was as active as possible, especially at the beginning of the experiment. Thus, one of the reasons for using a preculture was to activate the biomass, so that only a small fraction of the viable part was inert/inactive (Pavlostathis & Giraldo-Gomez 1991). Another reason for the preculture was to adapt the biomass to the batch environment, eliminating a lag phase.

Simple single-substrate Monod kinetics presumes that the reactions are not oxygen limited. In the present study, oxygen demand for degradation and respiration was calculated and compared to the available oxygen

in the system. The medium was treated 30 minutes with air to ensure a high oxygen content at the start of each experiment. Headspace was about 2 litres, containing approximately 600 mg oxygen. The medium contained approximately 9 mg O_2/l, i.e., about 40 mg oxygen in the liquid phase. Thus, the batch contained about 640 mg oxygen in total. The continuous stirring ensured the accessibility of the oxygen from the gas phase. During the experiments more air was added due to the sampling procedure (see section Experimental setup). The calculated demand for toluene oxidation is 3.2 mg O_2 per mg toluene (Verschueren 1996); thus the total oxygen demand for mineralisations was below 110 mg O_2 in all cases. In one experiment, an oxygen measurement was made. It showed ample oxygen in the medium at the end of the degradation.

Modelling and theoretical aspects

The biological model

The Monod model was used to describe the degradation of the substrate and the growth of the biomass for the single-substrate batch experiments in question. The model consists of two nonlinear first-order differential equations:

$$\frac{dS}{dt} = -k\frac{SX}{K_s + S} \tag{1}$$

$$\frac{dX}{dt} = Yk\frac{SX}{K_s + S} - bX \tag{2}$$

The concentration in the liquid of the substrate is denoted S, the biomass concentration is X, and t is the time. The substrate and the biomass concentrations were measured in the liquid phase. The model parameters are: k [mg/mg/hour] the maximum specific degradation rate for toluene, K_s [mg/l] the half-saturation concentration, Y [mg/mg] the true yield coefficient, and b [1/hour] the decay coefficient. Equation 1 was modified to correct for the exchange of target compound with the headspace, as suggested by Broholm et al. (1992). The right hand side of Equation 2 was thus multiplied by the headspace factor h:

$$\begin{aligned} h &= \tfrac{V_L}{V_L + H_C V_A}, \\ H_C &= exp(B_0 + B_1/T) \end{aligned} \tag{3}$$

where V_L and V_A are the volumes of the liquid and the air in the batch, respectively; H_C is the Henry's law constant, which is the equilibrium distribution coefficient between air and liquid for toluene (Atkins 1986); B_0 and B_1 are Henry's law constants (Lamarche & Droste 1989); and T is the absolute temperature. The continuous stirring in the batches ensured equilibrium between liquid and air for toluene (this was checked in a preliminary experiment). The change in V_L due to the sampling was about 12.5%. This resulted in a change of h of only 5%, and therefore it was decided to treat h as a constant.

Estimation

Estimation of Monod parameters from batch data is not a simple matter, because the model involves two coupled, non-linear differential equations, and the parameter estimates can be strongly correlated (Box & Lucas 1959). In this study a nonlinear estimation method was employed. All parameters were estimated simultaneously, and, furthermore, the different variance structures for the concentration S and the biomass X were taken into account. Accounting for different variance structures is important when estimating the parameters in order to avoid unreliable and/or skewed estimates. A computer program called Dekimo (Bilbo 1992; Sommer 1997) was developed for this purpose.

We assumed that the measurements for the substrate (S_i) and the biomass (X_i) at a given time t_i were normally distributed. The mean values of the two responses (the predicted values) given by the Monod model are denoted by $f(.)$ and $g(.)$, respectively. The model can be written as:

$$\begin{aligned} &S_i \epsilon N(f(t_i, \underline{\theta}), \sigma_\varepsilon^2 \cdot w_i) \\ &X_i \epsilon N(g(t_i, \underline{\theta}), \sigma_\varepsilon^2 \cdot v_i) \qquad i = 0, 1, ..., n \end{aligned} \tag{4}$$

where $N(\mu, \sigma^2)$ denotes the normal distribution with mean μ and variance σ^2. The parameters in the model are collected in the vector $\underline{\theta} = (k, K_s, Y, b)$. The variance parameters w_i and v_i are weight functions, and σ_ε^2 is a common coefficient of variance. Finally, n is the total number of observations in a given experiment. One observation consists of a measurement of S_i and/or X_i taken at time t_i.

The substrate and the biomass responses (S_i and X_i) were not determined with the same precision. This difference can be reflected through the two weight functions (w_i and v_i) and the constant c, introduced

in Equation 5. The variances of S_i and X_i increase with the predicted responses. Therefore, increasing functions of f and g were chosen for w_i and v_i. The biomass measurements were often less precise than the substrate measurements. Based on these considerations, the following functions were chosen:

$$w_i = f(t_i, \underline{\theta}), \qquad v_i = c \cdot \sqrt{g(t_i, \underline{\theta})} \tag{5}$$

where c is a constant. These functions were compared with other possibilities (by a visual judgment), but among the alternatives considered, they performed best. Especially it can be noted that the relative increase of the variance was higher for the substrate than it was for the biomass.

Two main problems arise when determining a proper structure for the weight functions. First, a number of independently replicated measurements of the responses at high and low concentrations in each experiment should be available. This requirement is often difficult to meet. Second, the chosen structure should be able to describe a common variance structure for *all* the identical experiments. The reason for this is that it is very advantageous when comparing log-likelihood values if a common variance structure can be assumed. Although the data revealed some variation in this respect, it seemed appropriate to choose such a common structure. For the present experiments, the variance of the biomass measurements were found to be around 9 times greater than the variance of the substrate measurement, and $c = 9$ was chosen. In other words, the standard deviation of the biomass measurements was about 3 times the standard deviation of the substrate measurements (after taking f and $\sqrt{g}$, respectively, into consideration). Other values for c were tried ($c = 2, 6, 16, 20$), but the ratio between the residual variances for S and X from the estimation was close to one for $c = 9$. A more correct way of estimating the variance ratio could be to consider replicates of the response measurements, especially in the beginning and in the end of the experiment. However, it is impractical to carry out so many independent repeated measurements in the experiments considered here.

These assumptions about the distribution of the measurements were used in the nonlinear estimation method. Estimation of the parameters in the Monod model Equation 1 and Equation 2 was based on an iterative maximum likelihood method. The optimization of the log-likelihood function in Equation 6 was performed using a quasi-Newton method (Melgaard & Madsen 1991), and a fourth-order Runge-Kutta iterative algorithm was used to obtain values for the predicted responses $f(t_i, \underline{\theta})$ and $g(t_i, \underline{\theta})$. By optimization of the log-likelihood function in Equation 6, estimates of the model parameters and of the common variance coefficient σ_ε^2 were obtained. A more general statistical description is given by Bates & Watts (1988) and Gallant (1987).

$$\begin{aligned} &\log L(\underline{\theta}, \sigma_\varepsilon^2 | y) = \\ &-1/2 \textstyle\sum_{i=1}^{n} [\log(2\pi\sigma_\varepsilon^2 \cdot w_i) + \frac{(S_i - f(t_i, \underline{\theta}))^2}{\sigma_\varepsilon^2 \cdot w_i}] \\ &-1/2 \textstyle\sum_{i=1}^{n} [\log(2\pi\sigma_\varepsilon^2 \cdot v_i) + \frac{(X_i - g(t_i, \underline{\theta}))^2}{\sigma_\varepsilon^2 \cdot v_i}] \end{aligned} \tag{6}$$

The results of the estimation were influenced by the value of the initial biomass concentration at time t_0, denoted X_0. For this reason, X_0 was estimated simultaneously with the model parameters. The model fitting was, however, not very dependent on the initial substrate concentration, S_0. Thus, S_0 was not estimated; instead the measured values were used. In order to improve the precision on the parameter estimates, possible model reductions were examined and discussed in the Results section.

Tests

Two kinds of tests were carried out in order to examine model reduction and reproducibility. Both tests used the same test statistic, namely the approximative likelihood ratio, which uses the log-likelihood values given by the nonlinear estimation program (Dekimo). The likelihood ratio decision rule is

$$-2 \cdot (logL_0 - logL_1) > \chi^2_{1-\alpha}(df) \tag{7}$$

where α is the level of significance and $\chi^2_{1-\alpha}(df)$ denotes the $1 - \alpha$ quantile of the χ^2-distribution with df degrees of freedom. In order to test the hypothesis that parameters in a certain model are zero or equal, for example, the quantity $\log L_1$ is computed as the log-likelihood for the full model, while $\log L_0$ is the log-likelihood computed for the reduced or restricted model. If the left hand quantity exceeds the χ^2-quantity, the hypothesis in question is rejected.

Model reduction

When examining model reduction in nonlinear models, it can be misleading only to use conventional confidence interval procedures, since the distributions of the estimates can be very skewed (Bates & Watts

1988). A comparison of the log-likelihood values using the likelihood-ratio test, Equation 7, is a more correct way of testing whether a model reduction is justified or not. The model was reduced only if the hypothesis was acceptable for all the experiments involved.

Reproducibility

Examination of reproducibility was based on tests for which the log-likelihood values from several experiments were used. In principle, the test statistic was computed as described above, but the log-likelihood values were computed as sums of the log-likelihoods over all experiments involved. The procedure contains two parts: 1) testing whether a common set of parameters could be applied for experiments carried out the same day (within the same run), and 2) testing whether a common set of parameters could be applied for all experiments carried out on different days (between different runs). If both 1) and 2) lead to acceptance, the experiment was claimed to be reproducible.

1) Variation within runs. For each batch within each run, individual parameter estimates and corresponding log-likelihood values were calculated (all together $3 \cdot 3 = 9$ estimations). The sum of these 9 log-likelihood values is $\log L_1$. Next, a set of common parameters for the batches within each run was estimated together with the corresponding log-likelihood values (all together 3 estimations). The sum of these 3 log-likelihood values is $\log L_0$. The model had p parameters. df was calculated as the total number of parameters for the individual estimation ($9 \cdot p$) minus the total number of parameters for the common estimation ($3 \cdot p$). The resulting χ^2-quantity will then have $df = 9 \cdot p - 3 \cdot p = 6 \cdot p$ degrees of freedom.

2) Variation between the runs. Ideally, the parameter variation between runs should be tested by comparing the log-likelihood value, $\log L_1$, obtained corresponding to the common estimates for each run, with the log-likelihood value, $\log L_0$, obtained for one common set of parameters covering all batches in all runs.

However, due to program limitations in Dekimo, this last test could not be performed directly, since it was not possible to estimate one common set of parameters for all 9 experiments. Only 3 experiments could be treated simultaneously. Therefore, a heuristic test procedure was used. Each run was represented by only one batch as follows in three test cases

Case a) Run I (batch A), Run II (batch A), Run III (batch A)
Case b) Run I (batch B), Run II (batch B), Run III (batch B)
Case c) Run I (batch C), Run II (batch C), Run III (batch C)

The labelling of the batches within the runs was random, such that the above choice of batches to represent the runs in the three test cases also was random; any other random allocation could have been used. For each case individual estimates were computed for the runs, and the sum of the corresponding log-likelihood values was $\log L_1$. Next, common parameter estimates for the runs within each case were computed and the sum of the corresponding log-likelihood values was $\log L_0$. It is noted that this method did not result in a single common set of parameter estimates, but in 3 sets corresponding to each of the three cases. The degrees of freedom of the χ^2-quantity in one case was $(3p - p)$, and for the summed test quantity, based on 3 cases, it was $3(3p - p) = 6p$.

Results

Estimated parameters

As described above, in principle, two types of estimation were performed.

- *Individual estimation*, where one experiment was estimated at a time.
- *Common estimation*, where a common set of parameters for three batches (from the same run or from different runs) were estimated.

In the figures '1', '2', or '3' are used to indicate the kind of model employed. '1' indicates a full model, '2' indicates a reduced model for batches from the same run, and '3' indicates a reduced model for batches from different runs. Thus, for example, 'Individual 2' is a reduced model estimated for one batch, and 'Common 2' is a reduced model estimated commonly for 3 batches from the same run, while 'Common 3' is a reduced model estimated commonly for 3 batches from 3 different runs.

The observed responses and the estimated models (types '1' and '2') for Run I, II, and III are shown in Figures 3, 4, and 5, respectively. The 'Individual 1' models for Run I and III seem to describe the data very well. For Run II the fitting is not quite as good, but possibly it could have been improved by incorporation of a lag phase of about 2 hours. Comments on the 'Individual 2' and 'Common 2' models are given below.

Table 1. Estimated parameter values for batch A, B, and C. 'Individual 1' uses the full Monod model, 'Individual 2' uses the reduced model A, and 'Common 2' also uses the reduced model A

	Individual 1				Individual 2				Common 2
Run I	A	B	C	SD	A	B	C	SD	A, B, C
k	0.85	0.72	0.82	0.03	0.82	0.71	0.79	0.04	0.78
K_s	0.36	0.36	0.49	0.09	0.38	0.36	0.50	0.10	0.39
Y	0.69	0.89	0.75	0.04	0.75	0.89	0.80	0.03	0.81
b	−0.01	0.00	-9^{-3}	0.01	0*	0*	0*	–	0*
σ_ε	0.09	0.10	0.12	0.01	0.10	0.10	0.12	0.01	0.11
X_0	0.09	0.10	0.09	0.01	0.09	0.10	0.09	0.01	**
log L	6.49	5.38	−4.52	–	5.24	5.37	−6.24	–	−1.84
Run II									
k	0.43	0.43	0.44	0.02	0.44	0.44	0.46	0.01	0.44
K_s	0.19	0.01	0.08	0.07	0.01	0.01	0.07	0.03	0.01
Y	0.77	0.73	0.74	0.04	0.70	0.71	0.68	0.03	0.70
b	0.01	2^{-3}	9^{-3}	0.01	0*	0*	0*	–	0*
σ_ε	0.16	0.17	0.21	0.01	0.17	0.17	0.21	0.01	0.19
X_0	0.15	0.16	0.15	0.02	0.15	0.16	0.15	0.02	**
log L	−44.1	−52.0	−65.0	–	−44.5	−52.1	−66.0	–	−166
Run III									
k	0.50	0.59	0.58	0.02	0.49	0.60	0.58	0.02	0.56
K_s	0.01	0.01	0.04	0.06	0.01	0.02	0.01	0.06	0.01
Y	0.89	0.72	0.72	0.03	0.92	0.71	0.72	0.03	0.76
b	-1^{-3}	2^{-3}	0.00	3^{-3}	0*	0*	0*	–	0*
σ_ε	0.10	0.15	0.13	0.02	0.10	0.15	0.13	0.02	0.16
X_0	0.12	0.15	0.18	0.01	0.12	0.15	0.18	0.01	**
log L	−10.7	−37.6	−32.4	–	−10.7	−38.4	−32.4	–	−130

SD is the standard deviation, and log L is the log-likelihood value. * = fixed value, ** = the same X_0 as given for 'Individual 2', and + = new estimates are given in Table 3 (explained in sections below). The values with raised index are read as $\cdot\, 10^{-3}$. The units of the parameters are given by the following: k (mg substrate/mg biomass/hour), K_s (mg substrate/l), Y (mg biomass/mg substrate), b (/hour), and X_0 (mg biomass/l)

The estimates of the parameters corresponding to the shown curves are displayed in Table 1. For all three runs K_s is the parameter which was determined with the largest standard deviation. Average standard deviations (SD) for the various parameter estimate were calculated from the average of the variances of the parameter estimates for the individual experiments from the same run:

$$SD = \hat{\bar{\sigma}}_{we} = \sqrt{\frac{\hat{\sigma}^2_{we(A)} + \hat{\sigma}^2_{we(B)} + \hat{\sigma}^2_{we(C)}}{3}} \qquad (8)$$

where $\hat{\sigma}^2_{we(A)}$ denotes the variance of the estimate of a parameter estimated from batch A. This variance was calculated by the program Dekimo using the methods given by Bates & Watts (1988) p. 133 ff.

The values of the estimated initial concentrations, X_0 for batches A, B, and C, were determined from the common estimation.

Model reduction

When estimating the decay constant b in Run I, it turned out that the estimate was essentially determined solely by the last two biomass measurements in the experiments. To improve this estimation, more measurements were taken during the anticipated decay phase. However, as shown in Table 1, all estimates of b are close to zero. In particular, all 95% confidence intervals (roughly $\hat{b} \pm$ twice its standard deviation) for the individual or common b estimates include zero. And when carrying out the likelihood ratio tests for

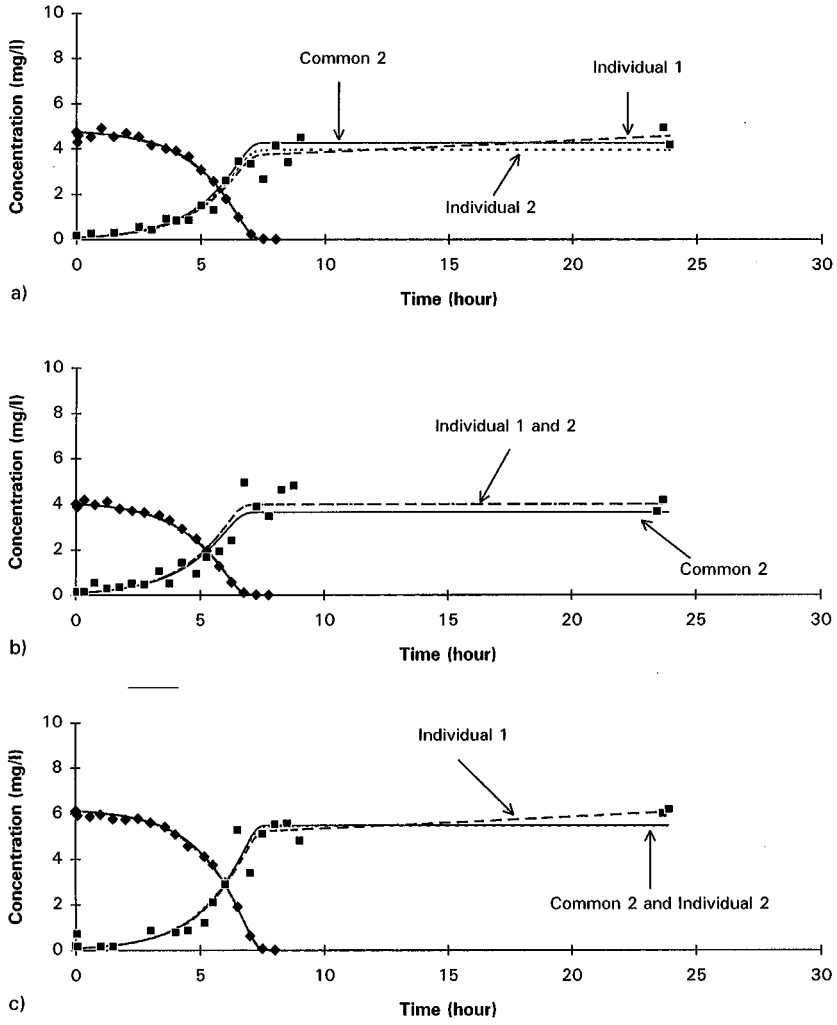

Figure 3. Estimation within Run I a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs. '1' indicates that a full model was employed and '2' indicates that a reduced model was employed (see Table 1).

all experiments, all χ^2 values were below the critical value, which confirmed that the Monod model could be reduced to a more simple model without biomass decay.

Reduced model A:

$$\frac{dS}{dt} = -h \cdot k \frac{SX}{K_s + S} \tag{9}$$

$$\frac{dX}{dt} = Y \cdot k \frac{SX}{K_s + S} \tag{10}$$

The properties associated with the estimated parameters in the reduced model were in most cases improved (smaller standard deviations) compared to the full model. All experiments were refitted, and the new estimates are given in Table 1, 'Individual 2'. These models look very much like the 'Individual 1' models (Figures 3, 4, and 5), except that the last part of the biomass curves now are horizontal ($b = 0$).

Since the estimates of the half-saturation constant, K_s from the 9 experiments seem relatively small compared to the substrate initial values, it was relevant to examine if the reduced Monod model, Equation 9 and Equation 10, could be further reduced to a zero order degradation kinetic model, also called a logarithmic

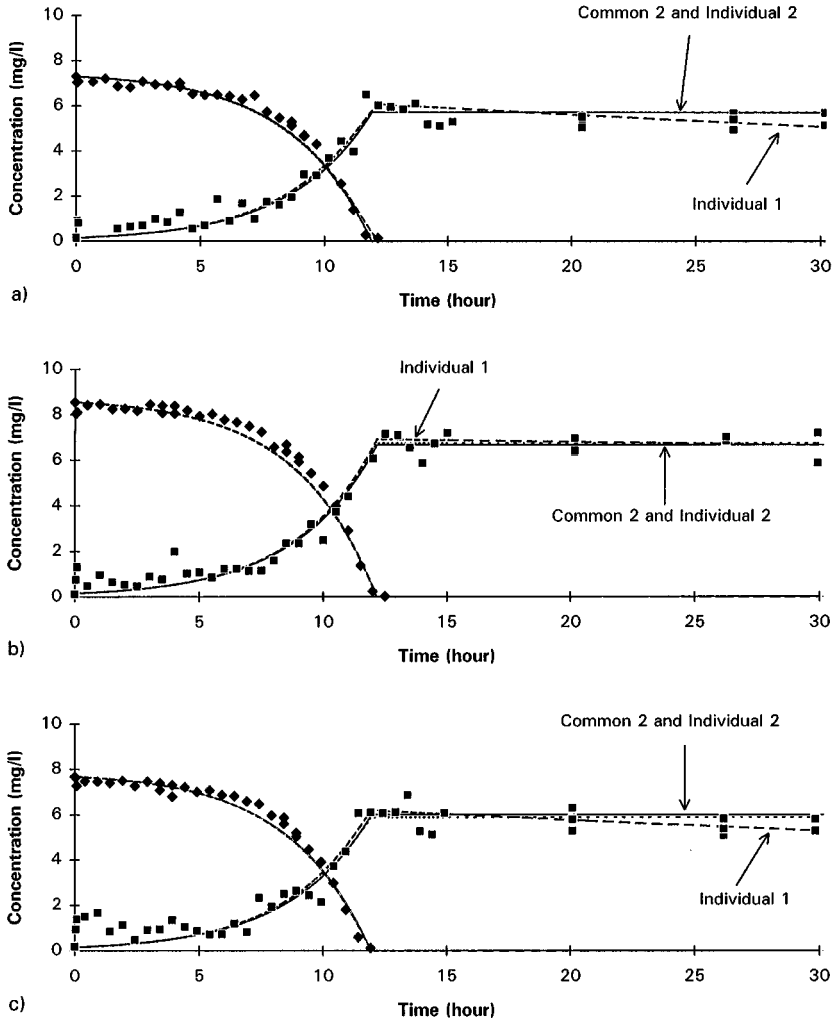

Figure 4. Estimation within Run II a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs. '1' indicates that a full model was employed and '2' indicates that a reduced model was employed (see Table 1).

model (Simkins & Alexander, 1984). This model also has the very attractive character of being linear.

Reduced model B:

$$\frac{dS}{dt} = -h \cdot k \cdot X \tag{11}$$

$$\frac{dX}{dt} = Y \cdot k \cdot X \tag{12}$$

For all batches in Run I (Table 1, 'Individual 1 and 2') 95% confidence intervals around the K_s estimates do not include zero. For Run III, however, 95% confidence intervals about K_s all include zero. For Run II some intervals include zero and some do not. The results of the corresponding likelihood ratio tests are given in the Table 2.

Thus, not all of the experiments in this study could be described by the reduced model B (Equation 11) and Equation 12). For the overall description, the model A given by Equation 9 and Equation 10 will therefore be used. The conclusion is that K_s estimates are small, but not always zero, and they have a relatively large variation.

Reproducibility tests

Within runs

The common estimations for batches within the runs are shown in Figures 3, 4, and 5. The fitted substrate curves for the individual and the common estimations are practically identical. The largest difference between the two estimations ('Individual 2 and Common

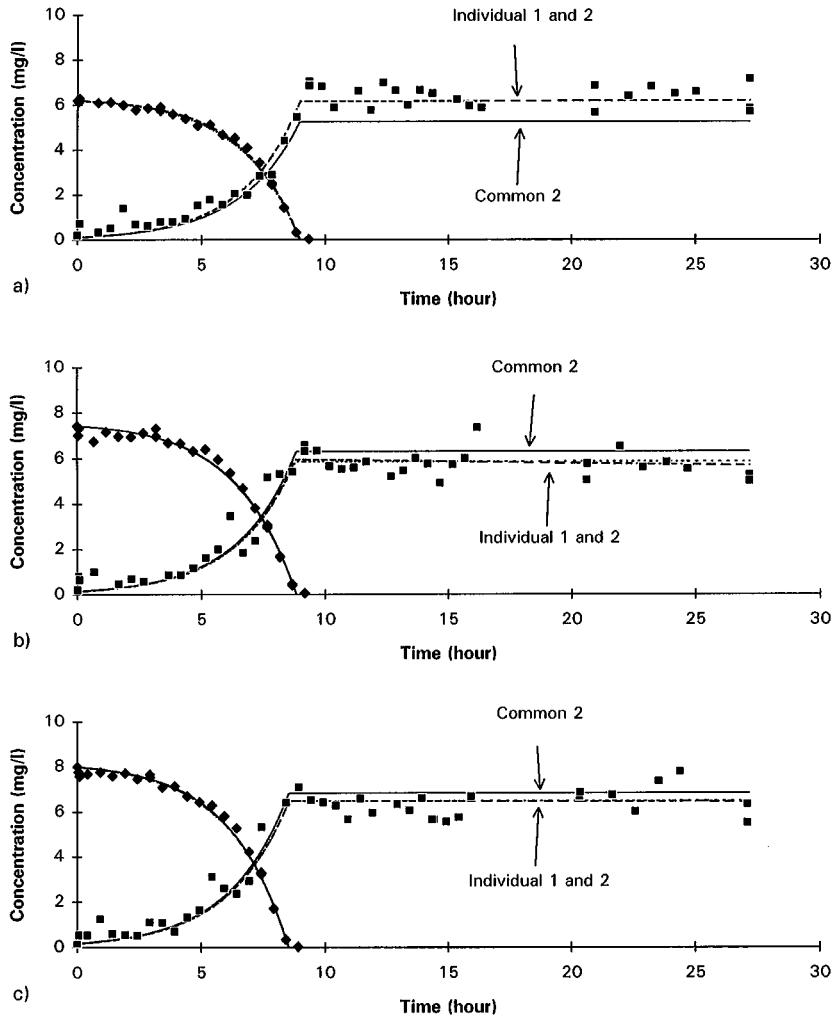

Figure 5. Estimation within Run III a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs. '1' indicates that a full model was employed and '2' indicates that a reduced model was employed (see Table 1).

2') is seen for the biomass curves, especially in the decay phases. The reason for this is that the biomass measurements were given smaller weights compared to the weights for the substrate measurements in the estimation routine, because the variance of the biomass measurements were larger than the variance of the substrate measurements.

The hypothesis that the parameter values for the three batches A, B, and C (from the same run) could be considered identical was tested. The result of the joint likelihood ratio test covering all three runs is:

$$-2(-298.1-(-240.2)) = 115.8 > \chi^2(18)_{95\%} = 28.9$$

Using α=5%, the hypothesis of common sets of parameter estimates could not be accepted. However, if the test was split up in the three runs to find which sub hypothesis could not be accepted, the following results were obtained:

$$\begin{aligned} \textit{Run I}: \quad & -2\cdot(-1.8-(5.2+5.4-6.2)) = \\ & 12.4 < \chi^2(6)_{95\%} = 12.6 \\ \textit{Run II}: \quad & -2\cdot(-166.1-(-44.5-52.1-66.0)) = \\ & 7.0 < \chi^2(6)_{95\%} = 12.6 \\ \textit{Run III}: \quad & -2\cdot(-130.2-(-10.7-38.9-32.4)) = \\ & 96.4 > \chi^2(6)_{95\%} = 12.6 \end{aligned}$$

The hypothesis for Run I and II was accepted, but for Run III the hypothesis of a common set of parameters was rejected. The trouble seems to be caused by batch A. The yield coefficient Y is much larger for batch A in Run III than for the other two batches in Run III, and it is the only experiment out of all 9 experiments

Table 2. 'Reduced model A' is the model given by Equation 9 and Equation 10, and 'Reduced model B' is given by Equation 11 and Equation 12. The values are the estimated log-likelihood values. The 'Test values' were calculated from equations Equation 7

	Run I			Run II			Run III		
	A	B	C	A	B	C	A	B	C
Model A ($\log L_1$)	5.2	5.4	−6.2	−44.5	−52.1	−66.0	−10.7	38.9	−32.4
Model B ($\log L_0$)	−4.5	2.3	−11.6	−46.0	−52.5	−74.5	−11.0	−39.1	−34.1
Test value	19.4	6.2	10.8	3.0	0.8	17.0	0.6	0.4	3.4
$\chi^2(1)_{95\%}$	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8	3.8
Further reduc.	No	No	No	Yes	Yes	No	Yes	Yes	Yes

Table 3. New estimates for batch B and C in Run III. log L is the log likelihood value

Run III	Individual 2			Common 2
Parameter	Batch B	Batch C	SD	B and C
k	0.560	0.540	0.002	0.552
K_s	0.003	0.003	0.023	0.005
Y	0.703	0.737	0.016	0.716
σ_ε	0.144	0.130	0.013	0.142
X_0	0.183	0.223	0.011	0.183, 0.223
log L	−38.64	−33.72	–	−72.00

The units of the parameters are given by the following: k (mg substrate/mg biomass/hour), K_s (mg substrate/l), Y (mg biomass/mg substrate), and X_0 (mg biomass/l).

where the highest concentration of the biomass during the experiment exceeds the initial concentration for the substrate. If only batches B and C in Run III were compared, the estimates were much more alike. In Table 3, the recalculated parameter values are displayed. The values of the initial concentrations X_0 for batches B and C were changed a little due to the new common estimation based only on batch B and C. The new initial concentrations for batch B and C resulted in small changes in the estimated parameter values. After having removed batch A from the data in Run III, the hypothesis of a common set of parameter values could be accepted:

Test in Run III:

$$-2 \cdot (-72.0 - (-38.6 - 33.7)) = 0.6 \ < \ \chi^2(3)_{95\%} = 7.8$$

The joint test:

$$-2 \cdot (-240.0 - (-230.6)) = 18.8 \ < \ \chi^2(15)_{95\%} = 25.0$$

Without more detailed microbiological data on the individual batches, it is difficult to explain why batch A in Run III behaved so differently compared to batch B and C. On the other hand one must be aware of the 'danger' in singling out one experiment among a number of experiments that are designed to be essentially identical, especially when no real cause can be identified.

Between runs

The hypothesis of a common set of parameter values for experiments from different runs (days) was examined. The same procedure as used when examining common sets of parameter values within the runs was used. Hence, three parallel tests (referred to as case a), b), and c), see Modelling and theoretical aspects) for common set of parameter values for three batches from different runs were carried out.

New initial concentrations for the biomass were estimated from the new common estimation, and new individual estimates ('Individual 3') with the new initial concentrations were computed as well (Table 4). In Figure 6, 7, and 8 the common estimations (considering 3 batches from 3 different runs ('Common 3')) are shown together with the individual estimations ('Individual 3') and the individual estimations ('Individual 2') given in Table 1. The 'Common 3' fit for Run I seems poorer than the fit for Run II and for III. The reason for this is primarily that there are fewer measurements in Run I compared to Run II and III (and not because the variability in Run I is larger that the variability in Run II and III).

The results of the likelihood ratio tests were as follows. The joint test, whether the parameters for all 9 batches could be considered identical:

$$-2 \cdot (-349.9 - (-298.1)) = 103.6 > \chi^2(18)_{95\%} = 28.9$$

Table 4. Estimated parameter values for batch A, B, and C. Case a), b), and c) refer to the allocation of experiments in connection with examination of common sets of parameter estimates between the runs. 'Individual 3' uses the reduced model A, and 'Common 3' also uses the reduced model A

	Individual 3				Common 3
Case a)	Run I, A	Run II, A	Run III, A	SD	Run I,II, III, A
k	0.51	0.52	0.44	0.01	0.48
K_s	1^{-3}	2^{-3}	3^{-3}	0.01	3^{-3}
Y	0.71	0.71	0.91	0.02	0.80
σ_ε	0.14	0.17	0.10	0.01	0.17
X_0	0.29	0.07	0.18	0.03	**
log L	−9.1	−45.9	−13.8	–	−103.6
Case b)	Run I, B	Run II, B	Run III, B	SD	Run I, II, III, B
k	0.47	0.52	0.53	0.02	0.52
K_s	0.03	0.03	0.01	0.08	0.00
Y	0.85	0.71	0.70	0.03	0.71
σ_ε	0.13	0.19	0.15	0.02	0.16
X_0	0.27	0.08	0.23	0.02	**
log L	−4.3	−54.6	−40.8	–	−107.2
Case c)	Run I, C	Run II, C	Run III, C	SD	Run I, II, III, C
k	0.48	0.53	0.50	0.02	0.50
K_s	0.03	0.24	3^{-3}	0.08	2^{-3}
Y	0.76	0.69	0.71	0.03	0.71
σ_ε	0.16	0.23	0.15	0.02	0.20
X_0	0.36	0.10	0.32	0.01	**
log L	−19.3	−70.6	−39.6	–	−139.1

SD is the standard deviation, and log L is the log-likelihood value. ** = the same X_0 as given for 'Individual 3'. The values with raised index are read as $\cdot 10^{-3}$. The units of the parameters are given by the following: k (mg substrate/mg biomass/hour), K_s (mg substrate/l), Y (mg biomass/mg substrate), and X_0 (mg biomass/l).

Test situations a), b) and c) for identical parameters in different runs:

$a)$ $-2 \cdot (-103.6 - (-9.1 - 45.9 - 13.8)) = 69.6 > \chi^2(6)_{95\%} = 12.6$

$b)$ $-2 \cdot (-107.2 - (-4.3 - 54.6 - 40.9)) = 14.8 > \chi^2(6)_{95\%} = 12.6$

$c)$ $-2 \cdot (-139.1 - (-19.3 - 70.6 - 39.6)) = 19.2 > \chi^2(6)_{95\%} = 12.6$

The log-likelihood values used in the above tests are shown in Table 4. Using $\alpha = 5\%$, the hypothesis of a common set of parameter values from different runs was rejected for all three tests. When looking at the common estimations ('Common 3') and the individual estimations ('Individual 3') in Figure 6, 7, and 8, the differences do not seem very large. However, when comparing 'Common 3' estimations with the 'Individual 2' the differences becomes larger, and it is clear, that the common estimation does not fit the data very well.

Based on the rejection of the hypothesis of common sets of parameters for batches from different runs, it is concluded that the experiment considered has a limited reproducibility with respect to variation between runs.

Despite the fact that the experiment has a limited reproducibility, it is still of interest to know the order of magnitude of the parameters and of the variances that one could expect to experience in future experiments like the ones considered here. For this reason, averages and approximate standard deviations for the parameter estimates were calculated and the results are displayed in Table 5. The calculation was based on the natural model for the total variance, σ^2_{total}, of a parameter:

$$\sigma^2_{total} = \sigma^2_{we} + \sigma^2_{wr} + \sigma^2_{br}$$

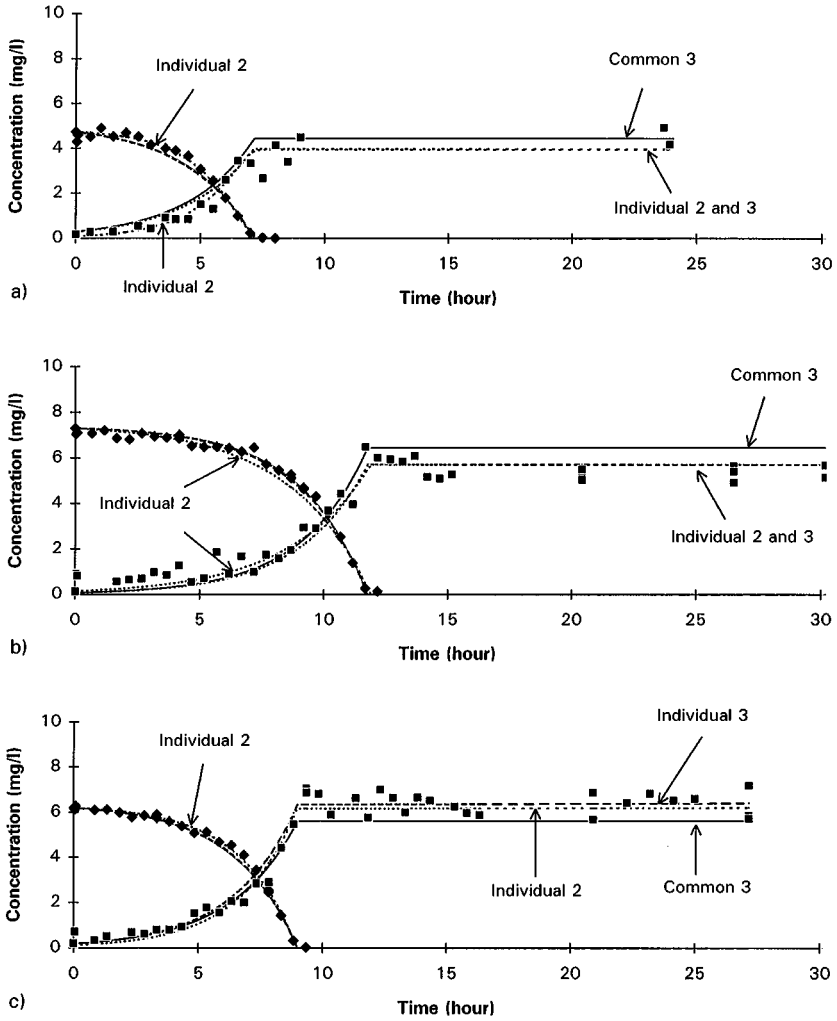

Figure 6. Estimation between runs a) Run I batch A, b) Run II batch A, and c) Run III batch A. □ biomass obs. ◇ toluene obs. '2' indicates a reduced model for batches from the same run and '3' indicates a reduced model for batches from different runs.

where σ^2_{we} is the estimation variance within one experiment, σ^2_{wr} is the variance contribution from different batches within one run, and σ^2_{br} is the variance contribution from batches from different runs. As shown in Table 5, the main part of the variation originates from variation between the runs (br), while the variation within runs (wr) and the variation within experiments (we) are smaller, which is in accordance with the results of the described statistical tests.

The parameter estimates, given in Table 5, are correlated. The correlation primarily originates from the model under investigation. The highest correlation is between k and K_s (0.7), which means that k and K_s are difficult to distinguish, and they are skewed in the same direction. The correlation between k and X_0 is likewise very high (-0.7), which underlines the importance of estimating the initial biomass concentration instead of using the X_0 measurement directly. A matrix with the approximately correlation values for the experiments carried out in this study is shown in Table 6.

The goodness of fit of the models can be judged by considering the figures and Table 1, 3, and 4. The precision of the parameter estimates is given by their standard deviations (SD). The residual standard deviation, σ_ε, indicates how well the model fits the observations.

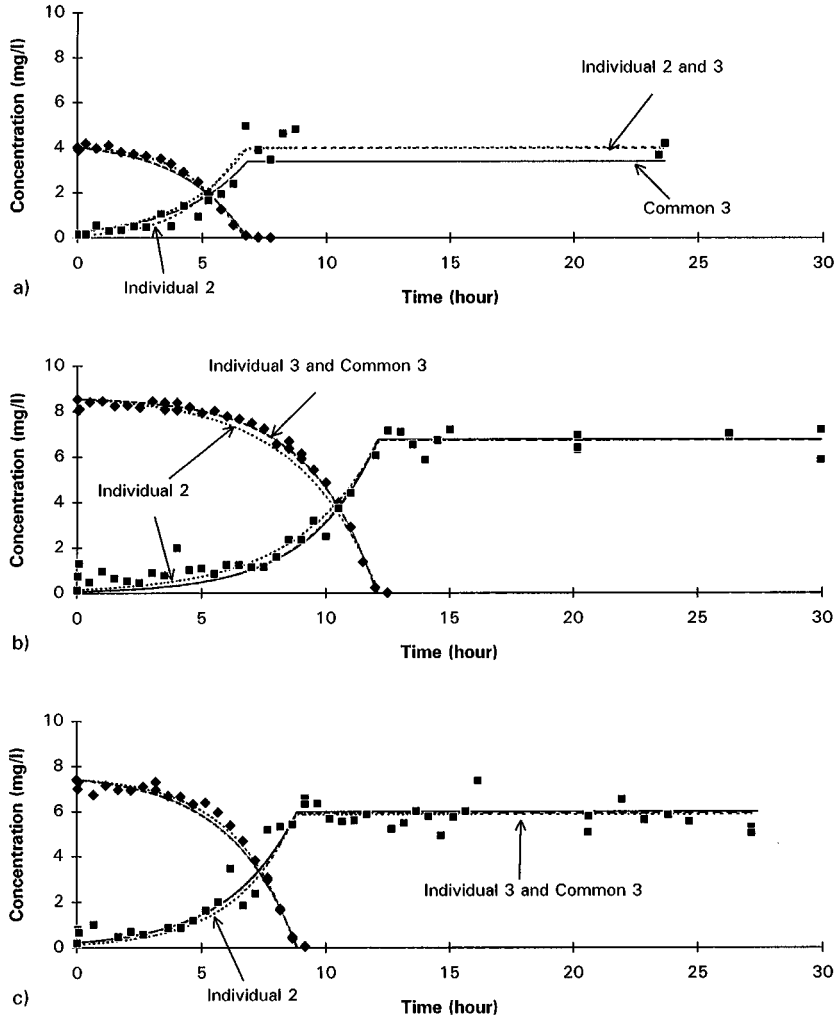

Figure 7. Estimation between runs a) Run I batch B, b) Run II batch B, and c) Run III batch B. □ biomass obs. ◇ toluene obs. '2' indicates a reduced model for batches from the same run and '3' indicates a reduced model for batches from different runs.

Table 5. The average values were calculated from all batch experiments performed in this study. $\hat{\sigma}_{we}$ is the standard deviation for parameter estimates within the experiment and is calculated by the program Dekimo, $\hat{\sigma}_{wr}$ is the standard deviation within the runs, $\hat{\sigma}_{br}$ is the standard deviation between the runs, and $\hat{\sigma}_{total}$ is the total standard deviation

Parameter	Average value	$\hat{\sigma}_{we}$	$\hat{\sigma}_{wr}$	$\hat{\sigma}_{br}$	$\hat{\sigma}_{total}$
k (mg/mg/hour)	0.60	0.02	0.02	0.17	0.17
K_s (mg/mg/hour)	0.16	0.06	0.00	0.22	0.23
Y (mg/mg)	0.75	0.03	0.03	0.04	0.06

Table 6. Average correlation matrix for parameter estimates

	k	K_s	Y	X
k	1	0.7	−0.6	−0.7
K_s	0.7	1	0.1	−0.6
Y	−0.6	0.1	1	0.2
X	−0.7	−0.6	0.2	1

Discussion on reproducibility

The experiments which were carried out the same days (within runs) resembled each other more than the experiments which were carried out on different days (between runs). These observations were in good agreement with the results of the likelihood ratio test, which accepted common sets of parameter estimates within the runs, but not between the runs, and in good

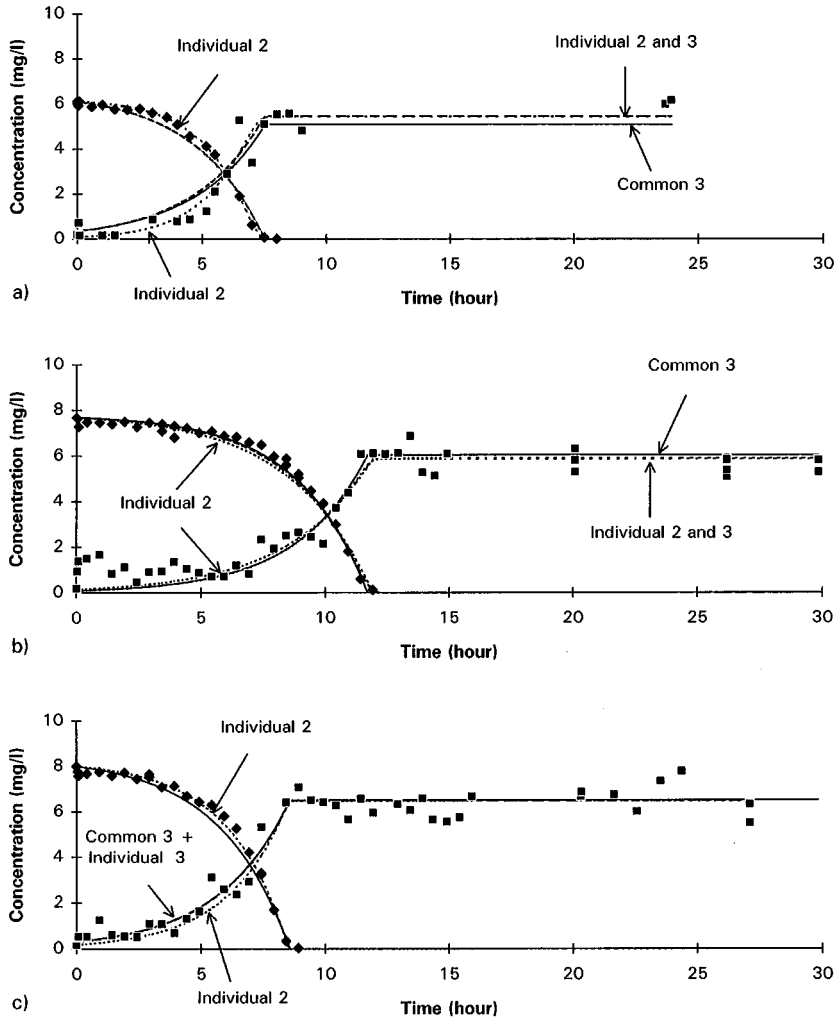

Figure 8. Estimation between runs a) Run I batch C, b) Run II batch C, and c) Run III batch C. □ biomass obs. ◇ toluene obs. '2' indicates a reduced model for batches from the same run and '3' indicates a reduced model for batches from different runs.

agreement with the estimated standard deviation for the whole system seen in Table 5, where the largest variation was the variation between the runs. Since the experiments were more uniform within the runs than between them, it seemed reasonable to suspect that biomass caused the variability. The biomass in the experiments carried out the same day came from the same preculture, whereas the biomass in experiments carried out on different days came from physically different precultures, which, however, all originated from the same frozen stock culture, see Figure 1. Differences in the culture history for the three runs may result in differences in the physiological state, which is the sum total of a cell's macromolecular composition. Changes in the macromolecular composition (consisting of cell-envelope, protein, RNA, and DNA) is known as physiological adaptation and the macromolecular composition at a given time is referred to as the organism's physiological state (Grady et al. 1996). Thus, differences in the physiological state of the bacteria in the precultures when they were introduced as inoculum in the 'real' experiments, is one possible explanation of the variation between runs.

The physiological state of the biomass just after the exponential phase was likely to be different from the physiological state of the bacteria, which had entered the dying phase. And since the duration times before the three precultures were used as inoculum were not the same, the physiological state of the bacteria

Table 7. S_0/X_0-values for the 9 experiments carried out in this study

	Bathes			Average
Run I	52.8	40.2	68.0	53.7
Run II	48.7	53.4	51.2	51.1
Run III	51.7	49.5	44.4	48.5
Overall Average				51.1

may have varied. (The toluene was in all cases totally degraded before the preculture was used as inoculum.)

This explanation to the observed variability of parameter estimates is in agreement with the statement given by Grady et al. (1996), who reported that culture history has been suggested as a major factor contributing to measured differences in kinetic parameters describing biodegradation. The way in which a culture has been grown often determines the nature of the enzyme systems that are expressed (how rapid it can synthesize enzymes, as well as how rapid those enzymes react).

Grady et al. (1996) point out that the outcome of the experiment may be influenced by the initial substrate to biomass ratio, S_0/X_0, because the ratio influences parameter identifiability. In this study S_0 and X_0 were chosen so that little identification problems occurred, and also the ratio was almost constant in all experiments, see Table 7.

Grady et al. also discuss how S_0/X_0 may influence the kinetic parameters through physiological adaptation, but since S_0/X_0 was almost constant in all experiments, this effect cannot explain why the variaton of the parameter estimates between runs was larger than within runs. Instead we believe that the effect relates to the physiological state in the preculture just before being used as inoculum.

Conclusions

In this study of microbiological degradation of toluene, it was not possible to reproduce the experiment completely, that is, it was not possible to describe all 9 performed batch experiments with one common set of parameter values. However, it was possible to estimate a common set of parameter values for experiments carried out simultaneously (within runs). It is believed that it was the variability of the biomass in the precultures that caused the limited reproducibility. Despite the limited reproducibility some similarities were found. For example, all experiments could be modelled without biomass decay ($b = 0$), and also the correlations between parameters estimates were almost the same in all experiments.

The reproducibility of biological experiments can be limited, because it is difficult to assure fixed environmental conditions and to prevent changes in the physiological state of the organism. In this study, major efforts were made to perform experiments under identical and fixed environmental conditions. However, variations in the duration time of the precultures still may have caused the larger variations between runs (based on different precultures). The biomass used in the three runs originating from three precultures was most likely in different physiological states when used as inoculum for the 'real experiments'.

Even a very simple experiment on microbiological degradation seems to have limits to its reproducibility. For more complex degradation systems, the number of sources of variation can be much larger. Many biological systems are more complex, with possible interactions between different compounds. Consequently there will be doubts in the choice of model, and the estimation procedure will become more cumbersome due to inherent correlations between the parameter estimates. The result will often be larger uncertainties for the estimated parameters.

Finally, it is emphasized that the estimation method plays an important role for the parameter estimates and for the tests of reproducibility. Unprecise or skewed parameter estimates may result if an inappropriate method based on linearization, for example, is employed, or if unrealistic assumptions concerning the variance of the measurements are used.

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