Variability in microbiological degradation experiments - analysis and case study

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Preface

This thesis is a result of a collaboration between IMM (Institute of Mathematical Modelling) and IMT (Department of Environmental Science and Engineering) at the Technical University of Denmark (DTU). The Groundwater Research Centre also at DTU has financed this Ph.D. project. The centre was established in 1988 with the main purpose of procuring basic technical knowledge for a successful and cost-effective protection, reclamation and utilization of soil and groundwater resources. The research program in the Groundwater Research Centre is divided into several sub-projects. The present study was carried out under project no. 3.3 : Biodegradation of aromatic compounds.

My work in context with this thesis has been divided into two parts. Part one, the carrying out of the microbiological degradations experiments in the laboratory (this period lasted a year) and part two, the modelling and statistical analysis of the data. In gaining a better understanding of the subject, it has been an advantage both to carry out the experiments and to implement the modelling and the statistical analysis as well. The advantage has been in designing the experiments and analyzing the samples from a statistically point of view, and in understanding the results of the measurements when modelling and testing the data.

It has been difficult writing this thesis since it treats subjects from two quite

different specialized areas (statistics and microbiology). The thesis is mainly written to microbiologists, interested in kinetics and who have some knowledge of the most elementary statistics. In case of no statistical knowledge, the reader can skip chapters and sections outlined in the Introduction. The treatment of biostatistics in context with microbiological degradation experiments is by no means exhaustive, but it is intended to show the aspects and possibilities of statistical modelling and testing applied to microbiological degradation experiments. The amount of mathematical/statistical formulae has been cut down to the most essential in order to make the reading more easy.

The thesis is not distinctly divided into theory and case studies. The necessary statistical and microbiological theories are mostly described along with good advice and illustrations from the experiments carried out in this study. Depending of the interest of the reader some chapters may be omitted as outlined in section ??. One type of reader may focus on nonlinear parameter estimation techniques and thus use especially chapter 4 as a reference "book", another reader may only be interested in the biological results and tend to omit the more statistically minded chapters. However, it is strongly recommended to read the whole thesis in order to achieve a better understanding of the problems in microbiological degradation experiments.

By

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Acknowledgements

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Summary

The variability of parameter estimates in microbiological degradation models has not received much attention in the literature. This in spite of the fact that the parameters are used in models for predicting and controlling microbiological processes of commercial interest. Furthermore, the accuracy of the parameter estimates are depending of the choice of estimation method, this fact has not either received much attention, all though an unsuitable estimation method can lead to estimates which are quite different from the "true" values.

The present thesis describes various nonlinear estimation techniques and describes analysis techniques for testing the reproducibility of a given experiment. The parameter estimation method employed for the experiments in this study is based on an iterative maximum likelihood method and the test statistic is an approximated likelihood ratio test. The estimations were carried out by the nonlinear estimation program Dekimo (developed at IMM by Bilbo and Sommer), available on request. The program successfully fitted all experiments. A few estimations were also carried out by the Lineweaver-Burk linearization, but the estimated parameters fitted the data poorly due to the inappropriate estimation method.

The examination of reproducibility/variability were carried out for two kinds of experiments: A single substrate experiment with toluene and a dual

substrate experiment with toluene and benzene. A pure culture, isolated from soil, grew with benzene and/or toluene as the only carbon and energy source. The substrates were degraded in batches under aerobic conditions. The Monod model was employed to describe the biological processes in the single substrate system, and Bailey & Ollis' model was employed to describe the processes in the dual substrate system. In the single substrate system 9 identical experiments were performed on three different days, and in the dual substrate system 12 identical experiments were performed on four different days. The data are available on the www address: http://www.imm.dtu.dk/documents/ftp/phdliste/phd31.abstract.html

Experimental observations indicate that these microbiological degradation experiments have a limited reproducibility, i.e. that a common set of parameter estimates could not be employed to describe all experiments in each of the two substrate systems. However, experiments carried out on the same days (within runs) were more uniform than experiment carried out on different days (between runs). In the single substrate system a common sets of parameter estimates for experiments within runs fitted the data very well, whereas common sets of parameter estimates for experiments between runs fitted the data poorly and were moreover strongly rejected to be identical by the likelihood ratio test. In the dual substrate system a common set of parameter estimates could not be accepted neither within the runs nor between the runs. Never the less, experiments within the runs were more uniform compared to experiments carried out on different days (between the runs). The lag phases within runs were thus exactly the same, but were quite different from experiments from different runs.

The limited reproducibility is probably caused by variability in the precultures, more precisely, variations in the activity level of the precultures just before used as inoculum. Facing the fact that these microbiological degradation experiments have a limited reproducibility one must in general expect large variability on the parameter estimates.

Resumé (in Danish)

Variabiliteten af parameterestimater i mikrobiologiske nedbrydningsforsøg har der i litteraturen ikke været fokuseret meget på. Det på trods af, at parametrene bliver anvendt som værende karakteristiske for de biologiske processer og bliver anvendt i modeller til prædiktion og overvågning af mikrobiologiske processer i kommercielle sammenhæng. Desuden har der i litteraturen været meget lidt opmærksomhed på de metoder og teknikker, som parametrene bliver estimeret ved hjælp af, selvom en uegnet estimationsmetode kan føre til estimater, som er en del forskellige fra de “sande” værdier.

Nærværende afhandling beskriver kort adskillige ikke-lineære estimationsteknikker og analyseteknikker til brug ved test af modelreduktion og reproducerbarhed. Den i dette studie anvendte parameterestimationsmetode er baseret på en iterativ maksimum likelihood metode, og det statistiske test er et likelihood kvotient test. Estimationerne blev udført v.h.a. det ikke-lineære estimationsprogram Dekimo (udviklet på IMM af Bilbo og Sommer), og programmet modellerede alle eksperimenter tilfredsstillende. Et par estimationer blev desuden udført v.h.a. Lineweaver-Burk metoden, men de estimerede parametre beskrev data dårligt p.g.a. den uegnede estimationsmetode.

Der blev i dette studie undersøgt reproducerbarhed/variabilitet af to forskel-

lige typer forsøg: Et enkelt-substrat-forsøg med toluen og et dobbelt-substrat-forsøg med toluen og benzen. En renkultur, isoleret fra jordbakterier, groede på toluen og benzen som de eneste karbon og energi kilder. Substraterne blev nedbrudt af bakterierne i batches under aerobe forhold. Monod modellen blev anvendt til beskrivelse af de biologiske processer i enkelt-substrat-forsøgene, og Bailey & Ollis' model blev anvendt til beskrivelse af processerne i dobbelt-substrat-forsøgene. I enkelt-substrat-systemet blev der udført 9 identiske forsøg på tre forskellige dage, og i dobbelt-substrat-systemet blev der udført 12 identiske forsøg på fire forskellige dage. (Alle rådata er tilgængelige på www adressen:
http://www.imm.dtu.dk/documents/ftp/phdliste/phd31.abstract.html,
og programmet Dekimo kan rekvireres på forespørgelse.)

Eksperimentelle observationer tyder på, at ovennævnte forsøg har en begrænset reproducerbarhed, d.v.s. at et fælles sæt parameterestimat ikke kunne anvendes til at beskrive alle de ens udførte eksperimenter i hver af de to substrat systemer. Forsøg, som var udført på samme dag (indenfor runderne), var imidlertid mere ensartede end forsøg, som var udført på forskellige dage (mellem runderne). For enkelt-substrat-forsøgene kunne man med held modellere forsøgene indenfor runderne med et fælles sæt parameterestimater, hvorimod et fælles sæt parameterestimater til modellering af forsøg, udført på forskellige dage, ikke var egnede til beskrivelse af data og var desuden forkastet af kvotient testet. For dobbelt-substrat-forsøgene kunne et fælles sæt parameterestimater ikke accepteres, hverken for forsøg udført på samme dag (indenfor runderne) eller for forsøg, udført på forskellige dage (mellem runderne). Ikke desto mindre var forsøgene udført på samme dag mere ensartede i forhold til forsøgene udført på forskellige dage. Lagfaserne indenfor runderne havde således den samme længde men var en del forskellige fra forsøg fra forskellige runder.

Den begrænsede reproducerbarhed skyldes sandsynligvis variabilitet i forkulturen, mere præcist, variation i aktivitets niveauet i forkulturen umiddelbart før denne blev anvendt som inoculum. Da disse mikrobiologiske forsøg har en begrænset reproducerbarhed må man således forvente en stor variabilitet på parameterestimaterne.

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Chapter 1

Introduction

1.1 Background

Obtaining reliable estimates of parameters in microbiological systems are prerequisites for predicting and controlling processes in wastewater treatments plants, production of useful intermediates of commercial interest, determination of the fate of toxic compounds in the groundwater etc. Biological treatment of contaminations in the aqueous environment receives increasing attention due to the widespread problem of groundwater contamination for example with oil products. Assessing the biodegradation kinetics of these compounds is essential e.g. in predicting the extent to which contamination will spread or in predicting the duration of *in situ* biodegradation cleanup operations. Many of the monoaromatic compounds found in gasoline (especially under aerobic conditions) can be degraded by microorganisms found in soil and in groundwater.

Four factors that play an important role in obtaining reliable estimates are

- the precision of the measurements,
- the number of samples,
- the estimation technique, and
- the reproducibility of the experiment.

Precision of measurements. Holmberg & Ranta (1982) carried out computer simulations with the Monod model and added random noise to the data to correspond to a measurement error. They reported that a noise on the measurement of up to 20% resulted in parameter estimates that varied up to a 100% ! This also emphasizes the importance of stating the parameter values not just as point estimates, but with the corresponding variances as well. The estimation was sensitive to measurement noise and this can explain the great variations which are often reported in parameter estimates obtained from different experiments performed under similar conditions.

Number of samples. The simulation studies by Vialas et al. (1978) indicated - not surprisingly - that the identification of the parameter K_s, was significantly easier in the case of 60 regular data points than in the case of 15 regular data points. If there are too few samples, many different models are likely to fit the data material.

Estimation technique. Even when the measurement errors are small and the chosen model is correct, major errors in the parameter estimates can be introduced by an unreliable estimation technique. The different estimation techniques are based on different assumptions about the measurement errors. If the assumptions are inappropriate for the actual data, the results of the parameter estimation will not be fully valid. Sàez & Rittmann (1992) emphasize the importance of using an estimation technique in microbiological experiments that matches the structure of the measurement errors, especially when the variance is strongly non-constant. 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 can thus be inaccurate and some times very time-consuming 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 for some of the parameters combined with linearization or with parameter estimates from the literature (Kong et al., 1996; Chang et al., 1993; Nakhla & Al-Hazazin, 1993; Thatipamala et al., 1992). These methods represent steps in the right direction, but when using linearization or parameter values from the literature, there is still a risk of serious validation problems. The best estimation result is obtained by use of nonlinear estimation for all the parameters.

"The intrinsic nature of environmental systems as multi-input multi-output systems in which input often can only be measured but not controlled, make the use of advanced system and parameter estimation techniques for the model building process important." (Reichert, 1994)

Reproducibility. It is obvious that any reported experiment ought to be reproducible, and if experimental results are claimed to be reproducible, it should be possible to obtain the previous results within some variations when the experiment is replicated. If the reproducibility of an experiment is confirmed, the next point of interest is to determine the variability, that is the variance of the experimental parameter values between replicated experiments. The choice of model, the measurement method, and the estimation technique are all merely tools. A poor choice of tool may cause irreproducibility. A few studies concerning variability of the parameter values have been reported (Arcangeli & Arvin, 1993), but reproducibility of the experiments was not examined explicitly. Blok & Booy (1984) reported a rather poor reproducibility in an inter laboratory test. In the test several laboratories participated using different chemicals and 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 & Booy carried out simulations performed on the basis

of the Monod model and explained the variability by the varying quality of the inoculum (mixed culture). By quality they meant the quantity of specific bacteria which are able to degrade the particular compound. In other words, they explained the variability of the results by the varying start concentrations of the specific bacteria in the different experiments. Pavlostathis & Giraldo-Gomez (1991) also suggest, that in order to obtain more reliable estimates the measurement of kinetic rates should be based on the viable microbial population density, as opposed to the total microorganism concentration. However, this is as they comment a very difficult task, especially in systems dealing with particulate organic substrates. Tanner, Souki & D'Ambrosi (1981) discovered large variability of parameter values estimated from identical experiments. They believed that this variability was caused by large measurement errors. We believe it is not the only explanation but the fact that it is different experiments (difference in inoculum) and that there may be considerable variance on the parameter estimates due to the estimation method (linearization). Considering the many microbiologic 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.

1.2 Scope of the thesis

The objective of the present study was to examine the reproducibility of two simple microbiological degradation experiments. In the present thesis the term "reproducibility" is used as a general term for describing variation between experiments that are repeated under more or less the same conditions in contrast to the strict definition given in the ISO 5725 standard. By a reproducible experiment we mean an experiment

- which is repeated a number of times and for which a common set of parameter estimates can be accepted when employing the likelihood ratio test.
- which replicates have been carried out on different days, using different precultures (inoculum) grown from the same biomass.

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 methods,
- which are carried out under the same conditions,
- which ideally should be governed by the same model parameters

As mentioned above there are several factors that play an important role in obtaining reliable parameter estimates. 1) the measurement errors were attempted minimized by not taking too small samples, and were examined by taking triplicates mainly in the beginning and at the end of the experiments. 2) the number of samples also have a great influence on the estimates, which is the reason why as many samples as possible were taken. 3) an appropriate estimation method is especially important for obtaining good parameter estimates and therefore a great deal of the present thesis concerns the estimation technique. The choice of an appropriate estimation technique has not been given much attention in articles concerning microbiological degradation experiments. Traditionally the chemical analyses used in the experiments are described thoroughly whereas the estimation and the assumptions made in that context are given little attention. This seems rather out of proportions.

Examination of reproducibility of degradation experiments is especially important when estimating parameter values. Special emphasis is laid upon repeating the experiment exactly the same way from one time to another. Focus has also been on the applicability and the development of a computer program used for estimation of kinetics parameters (part of the program already existed). With regard to the nonlinear estimation technique and the test statistic for common sets of parameter estimates, other experiments following a Monod-like model could have been used.

Two kinds of experiments were performed in the present thesis. One was a single substrate system with toluene and the other was a dual substrate

system with toluene and benzene. Both substrates are monoaromates and served as the only carbon and energy sources in the systems. The substrates were degraded by a pure culture isolated from soil bacteria. The experiments were kept relatively simple (a pure culture and one, respectively two substrates). Both kinds of experiments were repeated several times (see chapter 2). The Monod model and Bailey & Ollis' model were chosen to describe the single and the dual substrate experiments. No other competing models were fitted to the data material in search of the most suitable models. However, model reduction of Monod and Bailey & Ollis was examined. Since Bailey & Ollis' model contains 9 parameters there were many combinations of parameters with which the model could be reduced. In the present work only the most obvious parameters were tested.

1.3 Methods used in the present work

All methods used in the present work are thoroughly described in the different chapters. In this section the various methods are summarized without detailed descriptions. For the description of the biological processes in the single and the dual substrate systems, respectively, the Monod model and the Bailey & Ollis' model were chosen. The biomass concentrations were determined from protein measurements using a slightly modified version of the Lowry method and the substrates were measured on a gas chromatograph from samples taken from the liquid phase.

The parameter values were estimated using a nonlinear estimation method based on an iterative maximum likelihood routine. The variance of the measurement errors was assumed to depend on the response level - increasing with increasing response level. Moreover, the standard deviation on the errors for the biomass was estimated to be three times the standard deviation on the errors on the substrate(s). The log likelihood value was optimized by the quasi-Newton routine in order to obtain the parameter values. In the quasi-Newton expression the second derivative (the Hessian matrix) is used.

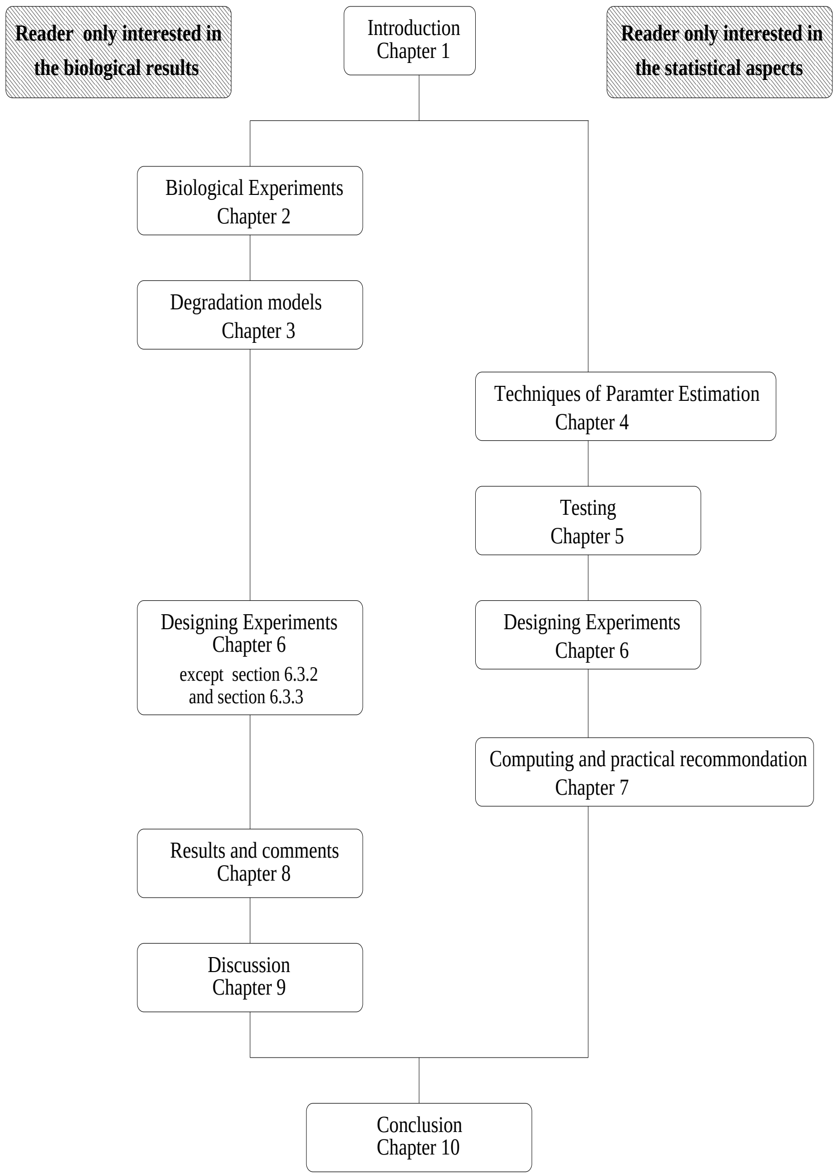
The Hessian matrix - or the inverse Hessian - was estimated or rather updated for every iteration by the BFGS (Broyden-Fletcher-Goldfarb-Shanno) algorithm. The test of reproducibility is based on an approximate likelihood ratio test and a significance level of 5% ($\alpha = 5\%$) was chosen.

1.4 Outline and reading guide

The thesis is not distinctly divided into theory and case studies. The necessary statistical models and microbiological methods are described along with good advice and illustrations of the experiments carried out. The thesis can be read and used on different levels, depending on the interest of the reader. One reader who is interested in learning more about the possibilities and advantages of using statistical methods for designing, estimating, and testing, may use the thesis more as a reference book. Another reader who is only interested in the biological results may tend to omit the more statistical minded chapters. However, it is strongly recommended to read these chapters, since they are written especially for non-statisticians and since one of the main objectives with the present thesis is to propagate a larger knowledge and understanding of statistical methods among microbiologists. At the end of this section a schematic reading guide is given.

The thesis is organized in the following chapters. Chapter **??** is the present chapter with the background and the scope of the project. Chapter 2 describes the biological experiments, the analysis methods, the setup, and gives an outline of all the experiments carried out in the present study. Chapter 3 emphasizes different aspects which are important when choosing an appropriate model to describe the biological processes. The models chosen (Monod and Bailey & Ollis) for the experiments performed in this study are described likewise. The 4^{th} chapter is an extensive chapter concerning general aspects of parameter estimation, not just the ones employed in this work. It starts by giving a mathematical formulation of the degradation models, defining the nonlinearity, and discusses different error structures.

The main focus of the chapter is on various often employed estimation techniques. The techniques are shortly introduced to the reader. Finally, techniques for determining the precision of the parameter estimates and for checking the assumed error structure are given. Chapter 5 presents the likelihood ratio test used in this study to test for model reduction and reproducibility. Chapter 6 focuses on the design of experiments in degradation models. The problem of choosing a specific sampling procedure is addressed and examples of optimal designs are given. Identification problems for the parameters in the Monod model are shown by use of sensitivity analysis. In chapter 7 general optimization problems are discussed and good advice is given on the use of the estimation program Dekimo or similar nonlinear estimation programs. Chapter 8 presents the main part of the experimental results for the single substrate and the dual substrate system - parameter estimates and variances, as well as results of tests for model reduction and reproducibility. Chapter 9 discusses the limited reproducibility of microbiological degradation experiments. Chapter 10 summarizes the results of the research carried out in the present study.


Reader only interested in the biological results
Introduction Chapter 1
Reader only interested in the statistical aspects
Biological Experiments Chapter 2
Degradation models Chapter 3
Techniques of Paramter Estimation Chapter 4
Testing Chapter 5
Designing Experiments Chapter 6 except section 6.3.2 and section 6.3.3
Designing Experiments Chapter 6
Computing and practical recommondation Chapter 7
Results and comments Chapter 8
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Chapter 2

Biological experiments

The main objective of this Ph.D. study has been to examine the variability/reproducibility of repeated microbiological degradation experiments carried out under conditions which were as identical as possible. In case of distrustful measurement results or large variability among the estimation results it is of great importance to be able to go back in the experimental procedure and seek for possible explanations. A careful description of the experiments is thus necessary for understanding, explaining and comparing the results of the experiments. In this chapter different aspects and conditions of the microbiological experiments are described.

2.1 Experimental design

Two types of degradation systems were examined 1) single substrate experiments with toluene as substrate and 2) dual substrate experiments with toluene and benzene as substrates. All experiments were carried out as

batch experiments. For the single substrate system three identical runs were carried out (I, II, and III) and for the dual substrate system four identical runs were carried out (IV, V, VI, and VII). Each run consists of three batch experiments (A, B, and C) plus a blank test (without biomass). Thus, for the single substrate system nine batch experiments plus three blank tests were carried out all together; and for the dual substrate system 12 batch experiments plus four blank tests were performed. For each run three batch experiments (A, B, and C) plus a blank test were carried out simultaneously and the biomass in these batches originate from the same precultur. In Fig. 2.1 a schematic outline of the experimental design is given for the single substrate system. A schematic outline of the dual substrate system would look the same except that there would be four runs instead of three.

Figure 2.1: Schematic overlook of the single substrate system

The reason for this design was that it was suspected that the biomass might cause the largest variability. Therefore the experimental procedure was designed using the blocking technique where each run represents a block (see chapter 6). Within each of the runs the biomass is assumed to be exactly the same, and between the runs the biomass may differ somewhat (activity level, mutation etc.) When examining reproducibility it is important that the experiments are carried out the same way in each replication and that factors like temperature, which influence the degradation rate, are kept constant for all the experiments. It is also important that the experiments are performed within the range of the models validity. The range was not known beforehand but learned along the way (sequentially) (for Run VIII and IX the Monod model was not valid, the initial toluene concentrations were higher than 10 mg/l - see section 2.4 for more details). For Run I - VII the toluene concentrations varied from 4-10 mg/l (Table 2.1).

The following procedure was used to obtain a biomass that varied as little as possible between all of the experiments. A few colonies of a pure culture (adapted to toluene over a 12 month period) were frozen in a glycerol medium (appendix A) at $-80°C$ in 2 ml tubes to obtain no activity in the biomass under the hole period of experiments (six month). Arvin et al. (1989) also froze the biomass in order to maintain as little activity as possible. Machado & Grady (1989) kept the biomass on agar slants during the period of experiments, they did not, however, state for how long time.

For each run (I, II, $\cdots$ X) a small amount of the frozen biomass was grown on an agar plate (Casein-peptone yeast agar) at 25°C in a dark cabinet in order to assure that the bacteria appeared "normal" (like the previous). 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), as well as growth medium, and chemicals. After about one day the toluene was completely degraded and the biomass was ready to be used for inoculum in the run of current interest. The preculture was shaken violently to ensure total mixing before 130 ml was removed and added to each batch (A, B, and C). The whole procedure was carried out under sterile conditions.

A pure culture instead of a mixed culture was chosen in order to ensure minimum variation of biomass in the repeated experiments. This is of great importance when comparing the experiments on the assumption that they are identical. In a mixed culture one or several groups of bacteria can be selected either during the thawing, in the preculture, or during the experiment (Yoon, Klinzing & Blanch, 1977). This effect would be likely to obscure or disturb the effect of the substrate degradation from experiment to experiment if a mixed culture was used.

The blank tests carried out served several purposes 1) to test whether the batches were gas tight, 2) to test sterile conditions (no contamination of other bacteria), and 3) to adjust the concentration of the substrate measurements if there had been any changes in external factors that would effect the measurements. (See appendix D for an example of adjusting). The blank tests were identical with the "real" experiments (A, B, and C) except that no biomass was added. The first two blank tests (from Run I and II) were carried out to ensure that the batches were gas tight. Concentrated acid (H_2SO_4) was added to the blank tests until pH reached 1-1.5 which would ensure no growth of any possible contaminating bacteria and thereby no degradation of toluene. The batches were found to be gas tight. The remaining 6 blank tests were mainly carried out to verify sterile conditions. For this purpose only the biomass was not added the blank tests, otherwise they were similar to the "real" experiments. These blank tests showed no growth of biomass and it could therefore be concluded that the experiments were carried out under sterile conditions.

The blank tests were also used to detect changes in experimental conditions concerning the substrate measurements. As an example, any changes in the pentane mixture or change to a new pentane mixture, with not exactly the same concentration as the former, were revealed by a jump in the constant concentration level in the blank tests. This knowledge was used for adjusting the substrate measurements in the "real" batch experiments.

Apart from the above mentioned experiments, preliminary experiments were carried out mainly for control and in order to achieve knowledge of the experimental conditions (such as constant temperature, the number of samples which could be taken before all substrate was degraded etc.). The preliminary experiments and their purpose are described in section 2.4. Table 2.1 summarizes all the experiments performed.

Number	Initial Concentration (mg/l)				Remarks
	Batch A	Batch B	Batch C	Blank test	
Single substrate					
Run I	4.6	4.0	6.1	6.6	
Run II	7.0	8.0	7.7	8.6	
Run III	6.2	7.4	8.2	6.6	
Dual substrate					
Run IV	4.0 & 6.7	4.7 & 5.1	4.8 & 5.7	7.8 & 6.8	
Run V	5.0 & 8.7	5.4 & 6.7	5.7 & 6.8	7.2 & 6.8	
Run VI	4.1 & 8.6	3.4 & 6.1	3.9 & 6.1	3.5 & 7.2	
Run VII	5.0 & 9.8	3.8 & 9.2	5.0 & 10.1	4.3 & 11.2	
Run VIII	14.2	15.9	15.9	15.8	inhibition
Run IX	9.6	-	12.1	11.6	inhibition
Run X	4.2 & 8.9	6.3 & 8.4	6.4 & 8.0	5.1 & 9.0	missing data
Preliminary					
Exp. i)	6.5				equilibrium
Exp. ii)	7.9	6.6	-	4.9	preculture
Exp. iii)	6.3	6.7	6.5	-	test exp.

Table 2.1: Summary of the batch experiments with their initial concentrations of substrate. Experiments above the line are used for parameter estimation and the three runs below the line are only used for verifying the parameter estimates obtained from the other experiments. The preliminary experiments are used to obtain information on the assumptions and the experimental procedure. Experiments with two initial concentrations contain benzene and toluene, respectively.

2.2 Experimental setup

The experiments were carried out in 5-litre batches under sterile aerobic conditions. Due to the large number of samples (50-60, each of 10 ml) taken from each batch, it was necessary to carry out the experiments in such relative large batches. The medium was stirred continuously to ensure total mixing and aerating. The aqueous medium consisted of benzene and/or toluene, bacteria, growth medium and four litres of distilled water Fig. 2.2. The biomass was a pure culture, identified as a Pseudomonas *cepacia* and originated from a groundwater sample from a former gaswork site at Fredensborg, Denmark (sampled by J. Flyvbjerg (1993)). H. M. Jensen (1994) adapted the bacteria to toluene in a chemostat over a period of 12 months and isolated the Pseudomonas *cepacia*.

Figure 2.2: Experimental batch. Completely mixed conditions were provided by a magnetic stirring bar. The experiments were conducted under a slight positive pressure to enable sampling.

Benzene and/or toluene served as the sole carbon source and the growth medium supplied the biomass with nitrogen, phosphorus, and other minerals necessary for the bacterial growth. The growth medium consisted of nutritive, trace metals, and a phosphate mixture. The composition of the growth medium is given in appendix A. The phosphate mixture also served as a buffer to ensure a stable pH value near 7.

As mentioned earlier three batch experiments plus a blank test were carried out simultaneously and repeated again 3-4 times separated by one or two weeks. In Fig. 2.3 the setup is illustrated. All batches were placed in a large plastic box insulated with Styrofoam on the sides and small plastic balls filled with air floating on the water surface. A combined pump and heating device was used to ensure a constant temperature of 28°C in the water surrounding the batches.

Figure 2.3: Experimental setup for single and dual substrate system

The sampling procedure was as followed: Substrate and biomass samples of 10 ml were taken from the bottom of each batch every 30 minutes by adding sterile filtered air in order to let the solution flow out as a result of positive pressure. Double or triple measurements were taken when changing to a new pentane mixture. The samples were collected in glass wares and treated immediately. Pentane mixture was added to the substrate samples which then were violently shaken, and trichloroacetic acid (TCA) was added to the biomass samples. The purpose of these steps was to halt the degradation process so that samples would accurately reflect the concentrations

in the batches at the time of sampling. The samples were stored at 5°C until analyzed (substrate samples the day after and biomass samples 3-5 days later). The reason for not sampling the substrate concentration from the headspace as some researchers have done (Alvarez-Cohen & McCarty, 1991), (Chang, Voice & Criddle, 1993) was 1) that the method is less precise (unpublished data) due to no use of internal standard (see the section below), 2) it is not possible to go back one or more days later to check the samples, and 3) that in this study it was impossible to sample and measure at the same time due to a tight time schedule.

A description of the preparation for the experiments (auto claving, making standard solutions, setting up, etc.) is given in appendix B. All materials, except from some tubes made of Teflon, were made of glass to prevent volatilization and minimize adsorption.

2.3 Chemical analysis

The toluene and benzene samples were analyzed in random order on a Shimadzu GC-9A gas chromatograph and later on a Carlo Erba, MEGA gas chromatograph both connected with 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 were extracted with a pentane mixture (1 ml, double distilled) and the peaks were quantified by internal standardization with heptane as a standard. Data acquisition and integration were achieved on a MAXIMA Chromatography Workstation. Standard curves for toluene and benzene were made for low (0-20 mg/l) and high (40-65 mg/l) concentrations (appendix C) in order to convert from peak area to concentration. It was obvious that one linear regression (Least Square method, LS) could not fit the data from low to high concentrations satisfactory. The linear regression curves for high concentrations were used when calculating the concentrations of the standard solutions of toluene and benzene. The regression curves for low concentration were used when calculating concentrations of the samples from the batch experiments.

The biomass samples were measured by the Lowry method (Lowry, Rosebrough, Farr & Randall, 1951) and (Peterson, 1979) - see appendix B for the procedure. The analysis of the protein content in the biomass measurements was carried out one week after the run was performed. The measurements were performed in random order in cuvettes on a Perkin Elmer, UV/VIS Spectrometer Lampda 2. Standard curves for the protein were made each time of measuring (for each run) in order to convert the response of absorption to a concentration (mg protein/l). All standard curves should ideally be exactly alike. However, the standard curves made for Run I and II were different from the other standard curves. This was due to lack of precision in the procedure (the protein standard solution needed for making the standard curve was measured and not weighted as in the later). The procedure was changed after this discovery. The two standard curves for Run I and II were recalculated using the remaining standard curves and could then be used to determine the protein concentration in these runs with the same accuracy as in the rest of the runs.

The Lowry method measures the protein in the biomass, and in order to convert to mg biomass/l, the measured protein was multiplied by 2 on the assumption that 50% of the biomass consists of protein. This assumption was checked by measuring the net weight from 9 dried samples (see appendix C). The net weight was found to be twice as high (conversion factor = 1.99), but with quite large variations. For batch A, B, and C the results were: 2.1 ± 0.8, 2.4 ± 1.0, 1.5 ± 0.2 (estimated mean values $\pm$ twice the standard deviation, which is approximatively 95% confidence interval). All the protein in the biomass, from living and dead biomass, is measured by the Lowry method. Since it was assumed that all the measured biomass was active, it was important to ensure a fully active biomass especially in the beginning of the experiment where the inert part composed a large part of the total measured biomass. Thus one of the reasons for a preculture was to activate the biomass so that only a small part of the biomass was

inert/inactive. Another reason for carrying out a preculture was to ensure adaption of the biomass to the batch environment described above so that an unnecessary lag phase could be avoided. (A lag phase is the time it takes the bacteria to undergo a change of chemical composition before they are capable of initiating growth).

2.4 Gaining information

Besides Run I, $\cdots$, VII three more runs were carried out (VIII, IX, X)(See Table 2.1). Two of these were similar to the single substrate system, but could not be used for estimating the kinetic parameter values. The initial toluene concentrations were higher in these runs (more than 10 mg/l) which resulted in some inhibition of the degradation. The reason for this may be a product inhibition, where an intermediate product toxic to the degradation accumulates. The spectra from the gas chromatograph measurements were examined in order to try to find any possible accumulation of intermediate products. Since examination of causes to inhibition was not part of the present study, no further investigations were performed.

The third run (Run X) was a dual substrate experiment carried out similar to Run IV,$\cdots$, VII but because of missing substrate data at the end of the experiment it could not be used for parameter estimation. All three runs (VIII, IX, X) were, however, used in verifying the parameter estimates found from the corresponding runs.

The three preliminary experiments were the first experiments to be conducted. The purpose of these was to gain information on the degradation system. Experiment i) served the purpose of examining the time it took for the toluene/benzene equilibrium between headspace and medium to adjust. The result was *less than 5 minutes* for a concentration of 20.5 mg/l to reach equilibrium of 16.4 mg/l. The rate is thus 0.82 mg/l/minute, which is much more than the greatest degradation rate (= 0.12 mg /l/minute) and therefore it seems reasonable to assume that the system is constantly in equilibrium.

Experiment ii) was carried out in order to gain information on the preculture with respect to the time it took before 10 mg/l toluene was completely degraded and how much biomass was produced from a few colonies from the agar plate. The biomass density was measured and an appropriate amount of the preculture to be added the runs was calculated. (Density = 10-12 mg dry weight biomass/l and amount to be added = 130 ml preculture).

The last preliminary experiment iii) was performed similar to the single substrate experiments. It was performed in order to gain information on the procedure - how many samples was it possible to take per hour, was it possible to maintain a constant temperature in the hole tank (see Fig. 2.3) throughout the experiment (answer: yes), how long time did it take before the substrate was degraded, were the initial substrate/biomass concentration appropriate etc. Experiment iii) confirmed the calculation that plenty of oxygen for the substrate degradation was available. Samples were taken at the end of the experiment and analysed for oxygen contents.

Chapter 3

Degradation models

It is desirable for most microbial ecologists and biologists in general, that a description of the processes in an experiment is supplemented with a quantitative approach also. The quantitative approach involves estimation of constants and/or parameters in the model chosen to represent the process under study, such as substrate degradation and biomass growth. In many situations, the functions that best represent biological behaviour in degradation processes are nonlinear with respect to their parameters and usually consist of two or more nonlinear coupled differential equations.

The kinetics of biodegradation have been described by a variety of mathematical models, increasing in complexity as they attempt to accommodate the numerous variables which can effect the rate of biological removal of the compound. These models are usually unstructured models, meaning that there is no description of the intra cellular components (Nielsen et al. 1991) and no description of the physiological state of the bacteria population - all cells in the whole population were assumed to be identical. In other words the models are more or less empirical. Description of natural systems by

mathematical models are drastically simplified and can only cover certain aspects of real systems. This is important to remember when modelling degradation experiments. One should not try to obtain a perfect fit of the data by extending the model, without having additional information of the system. Otherwise the parameters will be even more difficult to identify - the system may become overparametrized. Hence, the choice of a particular model should be based not only on how well experimental data fit the model, but also on the statistical reliability of the parameter estimates. Moreover, the structure of the model should be related as directly as possible to the causal mechanisms acting in the system under investigation. In other words modelling must be a compromise between making the model extensive enough to be realistic and reducing the number of parameters to a level where they can be estimated from available data. Before choosing a more complex model it is important to remember, that if the model doesn't fit the data well, several explanations may be possible: 1) large measurement errors, 2) too few data, 3) inaccurate estimation of the parameter values, and 4) important variables missing in the model. Only in the last mentioned case a new and more elaborate model should be employed. Among the several methods that exist for discriminating between competing models, Beck & Arnold (1977) recommend an F-test. Discrimination among competing models should not be performed as shown in the article of Luong (1987) with only 6 observations and little differences between the models, or as performed in Han & Levenspiel (1988) with only 4 observations! It is too few data to discriminate between models which are not tremendously different from each other.

The large majority of microbiological models are formulated as deterministic models. However, other models such as stochastic models exist. Deterministic models assume the future behaviour of a system to be completely determined by the knowledge of its present state and values of variables which describe the influence of the environment on the modelled system. Stochastic models also take in to account the random influences of the temporal evolution of the system itself. The main reason for the little (hardly any) use of stochastic models in microbiologic experiments may be a lack of data for the characterization of random variables and high requirement of computational resources for solving the stochastic equations. Bjørneboe (1990) and Steffensen (1991) examined applications of stochastic differential equations for the Monod model, and Wang (1994) and Spliid (1994) examined a microbiologic growth model. In chapter 4 more is written about stochastic models.

In this study competing models were not examined to fit the data sets. The purpose was not to find some optimal model for the data set, but to choose an appropriate, simple, and commonly used model for the biological system. Model reduction was, however examined. By use of the likelihood ratio test certain parameter values were tested for being equal to zero. When examining variability of replicated experiments it is advisable to start with a simple experiment and use a relative simple model, in order to achieve less correlation between parameters and between the variables. Afterwards the investigations can be extended to cover a more complicated experiment where a more elaborate model is needed. In this study a single substrate system with an easy degradable compound as toluene was chosen as the simple system. A dual substrate system with toluene and benzene as substrates was chosen as the more complicated system. The chosen models for these two systems are discussed in the following sections. As mentioned earlier, the biological system could for that matter be any other system, not necessary a single substrate or a dual substrate system with competing substrates, or with toluene and benzene as substrates - it is mainly the used estimation and testing technique that is in focus.

3.1 Single substrate degradation model

The choice of an appropriate, simple, and common used model for the single substrate system (Run I, II, and III) was fairly simple. Generally only the Monod model is employed for these single substrate systems where there

are no signs of self-inhibition (substrate inhibition) or product inhibition. Substrate inhibition occurs when high concentrations of substrate inhibit the growth of the biomass. Product inhibition occurs when a toxic product from the degradation pathway accumulates in the medium to such an extent that the metabolic activity is suppressed. Mulchandani & Luong (1989) have given a review on the many different models developed to describe substrate and product inhibition.

Other models than the Monod model for describing non-inhibition processes have been suggested (Contois, 1959: Grau et al., 1975; Chen & Hashimoto, 1978) but have by far not reached the same extension as the Monod model. The Monod model consists of two nonlinear first-order differential equations. The substrate concentration is denoted S, the biomass X, and the time t.

$$\frac{dS}{dt} = -k\frac{SX}{K_s+S} \tag{3.1}$$

$$\frac{dX}{dt} = Yk\frac{SX}{K_s+S} - bX \tag{3.2}$$

where the parameters are:
- k the maximum degradation rate for toluene,
- K_s the half-saturation coefficient,
- Y the yield coefficient, and
- b the decay coefficient.

Equation (3.1) was modified to correct for the continuous supply of target compound from the headspace as suggested by Broholm et al. (1992) . The right hand side of Eq. (3.1) is thus multiplied by the headspace factor h:

$$h = \frac{V_L}{V_L + H_C V_A}, \qquad H_C = exp\,(B_0 + B_1/T) \tag{3.3}$$

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 constants (Lamarch 1989), and

T is the absolute temperature.

The continuous stirring in the batches ensures equilibrium between liquid and air for toluene/benzene. The change in V_L due to the sampling was about 12.5%. This resulted in a changing of h of only 5%, we therefore ignored the fact that h was not a constant.

3.2 Dual substrate degradation model

The modelling of a biomass growing simultaneously on two substrates is complicated by the need to describe the rate at which each individual substrate is degraded. Several models have been proposed to qualitatively define different types of interactions between two substrates. Three common metabolic phenomena in connection with dual substrate experiments are: cometabolism, competitive inhibition, and simultaneously utilization. Substrate and product inhibition can also occur, but when looking at the data in this study there is no reason to believe that a substrate or product inhibition is present in the dual substrate experiments.

Cometabolism is a process where degradation of one substrate depends on the presence of another substrate, or as expressed by Alvarez-Cohen et al. (1991) "Cometabolism is the transformation of a compound by organisms that do not obtain energy or carbon for cell growth from the transformation and hence require an alternative source of carbon and energy". Different models have been suggested in order to model the cometabolic phenomena, Criddle (1993) summarize some of the models. Cometabolism can be excluded as describing the experiments with toluene and benzene degraded under aerobic conditions. Firstly, similar experiments have been carried

out showing that both toluene and benzene can be degraded independently without additionally substrates to induce the degradation process (Jensen 1992). Secondly, there is no indication in this study, that the degradation of benzene stops or slows down after toluene has been degraded.

When simultaneously utilization occurs the substrates are degraded simultaneously with no inhibition of any of the degradations rates. Competitive inhibition occurs when one or both of the substrates inhibit the other substrate's degradation. Many models can describe both simultaneously utilization and competitive inhibition, depending on the value of certain parameters. Since we did not know beforehand if the substrates (toluene and benzene) inhibited each others degradations, a model which could describe both simultaneous utilization and competitive inhibition was chosen.

The chosen model is referred to as Bailey & Ollis' model (Bailey & Ollis, 1977, pp.114). However, the model used here includes the modification suggested by Machado & Grady (1989) and Yoon et al. (1977). As for the single substrate system the chosen model is extended to meet the air/liquid system. Eq. (3.4) and Eq. (3.5) are multiplied by the headspace factors h_t and h_b for toluene and benzene respectively.

$$\frac{dS_t}{dt} = -h_t \cdot k\frac{S_t X}{K_{st} + S_t + z_b \cdot S_b} \tag{3.4}$$

$$\frac{dS_b}{dt} = -h_b \cdot k\frac{S_b X}{K_{sb} + S_b + z_t \cdot S_t} \tag{3.5}$$

$$\frac{dX}{dt} = -Y_t \cdot \frac{dS_t}{dt} - Y_b \cdot \frac{dS_b}{dt} - bX \tag{3.6}$$

where the parameters are:

k_t the maximum degradation rate for toluene,
K_{st} the half-saturation coefficient for toluene,
Y_t the yield coefficient for toluene,

z_t toluene inhibition coefficient
k_b the maximum degradation rate for benzene,
K_{sb} the half-saturation coefficient for benzene,
Y_b the yield coefficient for benzene,
z_b benzene inhibition coefficient, and
b the decay coefficient

The modification of Bailey & Ollis' model consisted of replacing K_{st}/K_{sb} and K_{sb}/K_{st} with the two independent parameters z_b and z_t. The reason for this replacement is that K_{sb} and K_{st} are difficulty to identify, thus the standard deviations of the estimates of these parameters will be quite large, which will result in even worse determined inhibition coefficients. Furthermore, the inhibition coefficient of toluene and benzene are independent of each other with this replacement. The drawback is that there are two more parameters to estimate. The inhibition parameters z_b and z_t describe how much one substrate inhibits the degradation of the other substrate. If both inhibition coefficients are zero, there is no inhibition of any of the degradations. And since both substrates give growth to the biomass, the substrates will be degraded faster than if the substrates were degraded alone. This process are referred to as simultaneously utilization. In the following the modified Bailey & Ollis' model with the extension to met the air/liquid system is referred to as the Bailey & Ollis' model.

The reason for choosing the Bailey & Ollis' model was that it satisfied the wish for a model that was appropriate for describing the data, that it was relative simple compared to other models for dual substrate systems, and that it was a commonly used model. In earlier experiments (Jensen 1992) which also used toluene and benzene as substrates, the Bailey & Ollis' model was found appropriate to describe the biological processes. Moreover the Bailey & Ollis' model is one of the simpler models for dual substrate degradation. For more complex models the parameter identification becomes more difficulty (Bates & Watts 1988). Bailey & Ollis' model is also commonly used (Folsom, Chapman & Pritchard, 1990; Strand, Bjelland & Stensel, 1990; and Chang, Voice & Criddle, 1993). Sambanis, Pavlou

& Fredrickson (1986) expressed the interactions between two substrates described by Tilman (1980) in mathematical terms using unstructured models all based on Bailey & Ollis' model but with no biomass growth.

Chapter 4

Techniques of parameter estimation

Parameter estimation consists of determining the optimal values of the parameters of a given model, which describes the measured data. It is important which estimation technique is chosen. The various estimation techniques rely on different assumptions of the measurement errors. The more realistic the assumptions about the errors are, the more correct is the result. The assumptions about the measurement error structure thus plays an important role, therefore the subject is discussed in the present chapter. The error structure is described by the measurement error variance, by the mutual dependences between errors, and by the statistical distribution.

Before estimating the parameters it may in some cases be advantageous to perform a transformation. The various transformation possibilities serve different purposes: 1) to obtain a nicer measurement error distribution (e.g. normal distribution or univariate), 2) to obtain an easier model to estimate in (e.g. a linear model), or 3) to reduce correlations between parameter

estimates. Such transformations are shortly described in section 4.4. No transformation was employed on the data in this study; linear transformation is, however, often employed by environmental researchers - often without full understanding of the effect it has on the results. Due to the widespread use of transformation the subject is included in this chapter.

Estimation of the parameter values can be performed by 1) linear transformation of the nonlinear model, followed by use of linear regression analysis, 2) nonlinear estimation methods (also referred to as nonlinear regression analysis), or 3) combining nonlinear estimation for some of the parameters, with either linearization or with parameter values found in the literature (for "similar" experiments) for other parameters.

In the present chapter a linearization technique is illustrated on one of the experiments from this study, and a short description of several common nonlinear estimation techniques is given. All the experiments in this study were modelled using a nonlinear estimation program Dekimo (developed at the Institute of Mathematical Modelling, DTU, part of the program is described in Bilbo, 1992). A few experiments were also modelled using linearization (Lineweaver-Burk) and AquaSim (a commercialized software program packages, Reichert, 1994) in order to compare the different parameter estimates obtained from the three methods. In chapter 8 the results are given. All parameters in a given model are written as a vector and estimated simultaneously. The values for the parameter vector can be estimated from one or more experiments. When estimating a set of parameter values based on several experiments, we call the estimates a *common set of parameter estimates*. The estimation technique is, however, the same as when estimating parameter values based on a single experiment. The technique of calculating the precision of the parameter estimates is given in section 4.6. Finally the assumptions of the measurement errors were checked in section 4.7 by use of residual analysis. Generally, the present chapter gives some basic definitions, review on different often employed techniques and examples and calculations using the data from this study.

4.1 General mathematical formulation

The models that best represent the microbiological degradation behaviour are usually nonlinear with respect to their parameters (see next section). The two models used in this study (Monod and Bailey & Ollis, see chapter 3) consist of two respectively three coupled differential equations. This implies that the depending variable (the responses) cannot be expressed explicit but must be approximated implicit. Several numerical approximation methods can be employed to solve the problem. The degradation models under consideration can be rewritten in two different types of mathematical formulation depending on the assumption of the measurement errors (also just referred to as errors). All small letters in bold represent a vectors and capitals in bold are matrices, if nothing else is written.

$$\boldsymbol{y} = \boldsymbol{f}(t, \boldsymbol{\theta}) + \boldsymbol{\varepsilon} \tag{4.1}$$

or

$$\frac{d\boldsymbol{y}}{dt} = \boldsymbol{g}(t, \boldsymbol{\theta}) + \boldsymbol{\varepsilon} \tag{4.2}$$

for the Monod model:

$$\boldsymbol{y} = \begin{bmatrix} S \\ X \end{bmatrix} \qquad \boldsymbol{f} = \begin{bmatrix} f_1 \\ f_2 \end{bmatrix} = \begin{bmatrix} S^{pred} \\ X^{pred} \end{bmatrix} \qquad \boldsymbol{\varepsilon} \in N(\boldsymbol{0}, \sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon)$$

$$\boldsymbol{g} = \begin{bmatrix} -k\frac{SX}{S+K_s} \\ Y \cdot k\frac{SX}{S+K_s} - bX \end{bmatrix} \qquad \boldsymbol{\theta} = \begin{bmatrix} k \\ K_s \\ Y \\ b \end{bmatrix}$$

and for Bailey & Ollis' model:

$$\boldsymbol{y} = \begin{bmatrix} S_b \\ S_t \\ X \end{bmatrix} \qquad \boldsymbol{f} = \begin{bmatrix} f_1 \\ f_2 \\ f_3 \end{bmatrix} = \begin{bmatrix} S_b^{pred} \\ S_t^{pred} \\ X^{pred} \end{bmatrix} \qquad \boldsymbol{\varepsilon} \in N(\boldsymbol{0}, \sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon)$$

$$\boldsymbol{g} = \begin{bmatrix} -k_b\frac{S_bX}{S_b+K_{sb}+z_tS_t} \\ -k_t\frac{S_tX}{S_t+K_{st}+z_bS_b} \\ Y_b \cdot k_b\frac{S_bX}{S_b+K_{sb}+z_tS_t} + Y_t \cdot k_t\frac{S_tX}{S_t+K_{st}+z_bS_b} - bX \end{bmatrix} \qquad \boldsymbol{\theta} = \begin{bmatrix} k_b \\ K_{sb} \\ Y_b \\ z_b \\ k_t \\ K_{st} \\ Y_t \\ z_t \\ b \end{bmatrix}$$

$\boldsymbol{y}$ is the dependent variable (response), t is the independent variable (time), $\boldsymbol{f}$ the predicted response (or expectation function), $\boldsymbol{\theta}$ the model parameter vector, and $\boldsymbol{\varepsilon}$ the error vector, assumed distributed after a normal distribution N with a mean of zero and a variance of $\sigma_\varepsilon^2\boldsymbol{\Sigma}_\varepsilon$. When $\boldsymbol{y}, \boldsymbol{\theta}, \boldsymbol{\varepsilon}, \boldsymbol{\sigma}, \boldsymbol{\Sigma}_\varepsilon$ are illustrated with a ^ (hat) it is the estimates of these.

The first mathematical formulation Eq.(4.1) expresses errors on the measured responses (substrate and biomass) and the second Eq.(4.2) expresses errors on the degradation/growth rates. There is no unambiguous answer which model formulation is the most correct. However, for the experiments in this study we found that Eq.(4.1) was the most adequate formulation, since it was the responses which were measured and not the rates. The nonlinearity of the model results from the functional form of the expectation function Eq.(4.1). If there is no correlation between errors, the model can also be written by:

$$y_{ij} = f_j(t_i, \boldsymbol{\theta}) + \varepsilon_{ij} \qquad \varepsilon_{ij} \in N(0, \sigma^2_{\varepsilon,ij}) \qquad \begin{cases} i = 1, \cdots, n \\ j = 1, \cdots, m \end{cases} \tag{4.3}$$

where i is the index for an observation and n is the total number of observations. j is the index for the type of response (substrate/biomass) and m is the total number of responses. One observation consists of measurements of two or three responses depending of the system (single or dual substrate). Systems with more than one type of response are also referred to as multiresponse systems.

4.2 Definition of nonlinearity

A model that is nonlinear in its parameters can be defined as one whose sensitivity equations depend on one or more of the model parameters (Beck & Arnold, 1977; Draper & Smith, 1981). A sensitivity equation mathematically describes how sensitive a model is, in terms of changes in the dependent variables, toward changes in parameters of the model. A sensitivity equation is defined as the first derivative of the expectation function (predicted response variable) with respect to a parameter p of the chosen model

$$\boldsymbol{f}'_\theta = \frac{\partial \boldsymbol{f}(\boldsymbol{t},\boldsymbol{\theta})}{\partial \theta_p} \qquad p = 1, \cdots, P \tag{4.4}$$

These equations cannot not be calculated explicitly, since explicit expressions of $\boldsymbol{f}(t,\boldsymbol{\theta})$ cannot be given. Two different methods are here given for treating the problem. 1) by approximating the functions $\boldsymbol{f}(t,\boldsymbol{\theta})$ numerically and then take the firsts derivation of the function with respect to a parameter or 2) by using the method shown in Holmberg & Ranta (1982), where the sensitive functions are obtained by solving a system of differential equations.

Here the sensitivity functions are estimated by using the first method mentioned above. The numerical approximation used was a 4'th order Runge-Kutta algorithm, given by

$$\hat{\boldsymbol{y}}_{i+1} = \hat{\boldsymbol{y}}_i + \frac{\boldsymbol{l}_1}{6} + \frac{\boldsymbol{l}_2}{3} + \frac{\boldsymbol{l}_3}{3} + \frac{\boldsymbol{l}_4}{6} \tag{4.5}$$

where $\boldsymbol{l}_1, \cdots, \boldsymbol{l}_4$ are defined by

$$\begin{aligned}
\boldsymbol{l}_1 &= h \cdot \boldsymbol{f}'_t(\hat{\boldsymbol{y}}_i, \boldsymbol{\theta}, t_i) \\
\boldsymbol{l}_2 &= h \cdot \boldsymbol{f}'_t(\hat{\boldsymbol{y}}_i + \boldsymbol{l}_1, \boldsymbol{\theta}, t_i + h/2) \\
\boldsymbol{l}_3 &= h \cdot \boldsymbol{f}'_t(\hat{\boldsymbol{y}}_i + \boldsymbol{l}_2, \boldsymbol{\theta}, t_i + h/2) \\
\boldsymbol{l}_4 &= h \cdot \boldsymbol{f}'_t(\hat{\boldsymbol{y}}_i + \boldsymbol{l}_3, \boldsymbol{\theta}, t_i + h)
\end{aligned}$$

and where $\boldsymbol{f}'_t$ is the derivative with respect to the independent variable and h is a time step.

$$\frac{d\hat{\boldsymbol{y}}}{dt} = \boldsymbol{f}'_t(\boldsymbol{y}_i, \boldsymbol{\theta}, t_i) \tag{4.6}$$

For a given set of parameter values $\boldsymbol{\theta}$, the value of a predicted response $\hat{\boldsymbol{y}}_{i+1}$ to a given time $t_i + h$ is obtained by use of the response value $\hat{\boldsymbol{y}}_i$ at time t_i plus the derivate function values (Eq.4.5). To demonstrate the idea, a few calculations are shown for the Monod model. Approximation of the substrate response by use of the Runge-Kutta method yields:

$$S_{i+1} = S_i - \frac{A}{6} - \frac{h}{3} \cdot \frac{kBX_i}{B + K_s} - \frac{h}{3} \cdot \frac{k(S_{i+\frac{1}{2}} - h\frac{kBX_i}{B+Ks})X_i}{Ks + (S_{i+\frac{1}{2}} - h\frac{kBX_i}{B+K_s})} - \frac{h}{6} \cdot \frac{k(\cdots)X_i}{K_s + (\cdots)}$$

where $A = h \cdot kS_iX_i/(S_i + K_s)$ and $B = S_{i+1/2} - A$. Now an approximation of the sensitivity equation for the parameter k can be indicated.

$$\frac{dS_{i+1}}{dk} = \frac{1}{6} \cdot \frac{A}{k} - \frac{h}{3} \cdot$$

$$\frac{(X_iS_{i+\frac{1}{2}} - 2AX_i) \cdot (S_{i+\frac{1}{2}} - A + K_s) - (kX_iS_{i+\frac{1}{2}} - kAX_i) \cdot (-A/k)}{(S_{i+\frac{1}{2}} - A + K_s)^2}$$

$$-\frac{h}{3}\cdots$$

$$\Updownarrow$$

$$\frac{dS_{i+1}}{dk} = \frac{h}{6} \cdot \frac{S_iX_i}{S_i + K_s} -$$

$$\frac{h}{3} \cdot \frac{k^2 \cdot \frac{h^2S^2X^3}{(S_i+K_s)^2} - 2k \cdot \frac{hS_iX_i^2(S_{i+\frac{1}{2}}+K_s)}{S_i+K_s} + X_iS_{i+\frac{1}{2}}(S_{i+\frac{1}{2}} + K_s)}{(S_i - k \cdot \frac{h}{2}\frac{X_iS_i}{S_i+K_s} + K_s)^2}$$

$$-\frac{h}{3}\cdots$$

From the above calculations of the approximative sensitivity equations it is easy to evaluate the dependence of the parameters under consideration. Only one of the sensitive equations need to depend on a parameter in order to declare the model nonlinear. For the Monod model no less than six of the eight sensitivity equations are depending on the parameters. For Bailey & Ollis' model 12 out of 18 sensitivity equations depend on one or more parameters. Thus both of these models have a high degree of nonlinearity. Several suggestions have been made to meet the need for a measure of "the amount of nonlinearity" in nonlinear models. Such a measure helps in deciding when linearized results provide acceptable approximations used in parameter estimation techniques. Bates & Watts (1988) present material concerning measurement of how severe nonlinear a particular model-data situation is. They developed relative curvature measures for the nonlinearity of an estimation problem using the first and second derivative of the expectation function.

$$\boldsymbol{f}'_\theta = \frac{\partial \boldsymbol{f}(t,\boldsymbol{\theta})}{\partial \theta_p} \quad \text{and} \quad \boldsymbol{f}''_\theta = \frac{\partial^2 \boldsymbol{f}(t,\boldsymbol{\theta})}{\partial \theta_p \partial \theta_q} \tag{4.7}$$

Models can, however, also be nonlinear with respect to the independent variable. To avoid confusion, the terms "linear" and "nonlinear" will be reserved for how a model behaves with respect to its parameters and not the independent variable.

4.3 Error structure

The assumptions made on the distribution and especially the mean and the variance structure of the errors, $\boldsymbol{\varepsilon}$, in a given model, is of great importance for the results of the analysis(the parameter estimates and the test statistic for reproducibility). Depending on the assumptions for the errors the most appropriate estimation technique (objective function) should be chosen. In the beginning of this section the assumptions made on the error structure

in the present work is given, and later it is shown, based on the data how they were estimated. At the end of the section a different error structure, depending on the time (stochastic different equations), is described shortly. This technique is, however, not used in the present study, but could be a technique for the future.

4.3.1 Assumptions

The mathematical models for Monod/Bailey & Ollis (Eq. 4.1) were in this study based on the assumption that the errors were normally distributed, uncorrelated between the different responses, and independent of time.

$$\boldsymbol{\varepsilon} \in N(\boldsymbol{0}, \sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon) \tag{4.8}$$

and for a given time t_i the diagonal covariance matrix can be written by

$$Cov(\varepsilon_{i,j1}\varepsilon_{i,j2}) = \sigma_\varepsilon^2 \boldsymbol{\Sigma}_{\varepsilon(i)} = \sigma_\varepsilon^2 \begin{pmatrix} w_{i1} & . & 0 \\ 0 & . & 0 \\ 0 & . & w_{im} \end{pmatrix} \tag{4.9}$$

where $i = 1, \cdots, n$ is the index for observations and j is the index for the type of responses (substrate/biomass) $j = 1, \cdots, m$ ($m = 2$ in the Monod model and $m = 3$ in the Bailey & Ollis' model). $\boldsymbol{\Sigma}_{\varepsilon(i)}$ are elements in the matrix $\boldsymbol{\Sigma}_\varepsilon$ given in Eq.(4.10). The errors on the two different responses taken at time t_i were assumed to be uncorrelated which is illustrated by the zeroes in the matrix Eq.(4.9). Moreover, errors were assumed independent on time, meaning that the errors on one type of response, measured at two different times were uncorrelated. This is illustrated by the zeroes outside the diagonal in Eq.(4.10). The structure of the covariance matrix $\boldsymbol{V}(\boldsymbol{\varepsilon}) = \sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon$ for two responses is given by

$$\boldsymbol{V}(\boldsymbol{\varepsilon}) = \sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon \quad = \quad \sigma_\varepsilon^2 \begin{pmatrix} \boldsymbol{\Sigma}_{\varepsilon(1)} & 0 & \cdots & 0 \\ 0 & \boldsymbol{\Sigma}_{\varepsilon(2)} & \cdots & 0 \\ . & & . & . \\ 0 & 0 & \cdots & \boldsymbol{\Sigma}_{\varepsilon(n)} \end{pmatrix} \tag{4.10}$$

$$= \sigma_\varepsilon^2 \begin{pmatrix} w_{11} & 0 & \cdots & & & & 0 \\ 0 & w_{12} & & & & & . \\ . & & w_{21} & & & & \\ & & & w_{22} & & & \\ & & & & . & & \\ & & & & & w_{n1} & \\ 0 & \cdots & & & & & w_{n2} \end{pmatrix} \quad (4.11)$$

The elements in the diagonal are different in magnitude reflecting that errors may not have the same magnitude for the different responses and for measurements taken at various stages (at different concentration levels). This situation is illustrated in Fig. 4.1.

Figure 4.1: Error structure in the experiments

The measured responses covered a large interval over which the magnitude of the errors changed. The concentration of the substrate was large initially and in the end, completely degraded. For the biomass it was more or less the opposite. It is thus natural to assume that the errors on the measured responses change during the experiment. This error structure is in the literature often referred to as heteroscedastic.

The different magnitudes for the errors on the various responses are often explained by the methods used when performing the chemical analysis (see chapter 2). But the natural variability of the processes considered will generally also vary with concentration. The concentrations of the substrates were determined with a higher precision than the biomass concentrations.

When estimating the parameter values the above described error structure should be accounted for. In order to assure that all responses are weighted appropriately, one has to transform the data initially or implement a weighting procedure in the estimation method. A weighting procedure in the estimation method was implemented in this study. Since the structure of the variance matrix $\boldsymbol{V(\varepsilon)}$ for the errors is a diagonal matrix, the distribution for the errors can be given as

$$\varepsilon_{ij} \quad \epsilon \quad N(0, \sigma_\varepsilon^2 w_{ij}) \qquad i = 1, 2, \cdots, n \qquad j = 1, \cdots, m \quad (4.12)$$

where i and j are index for observations and responses, respectively, and w_{ij} is the weight function. Among several possibilities we have for the single substrate system chosen (see next section) to describe the weight function by:

$$w_{i1} = f_1(t_i, \boldsymbol{\theta}) \qquad w_{i2} = 9 \cdot \sqrt{f_2(t_i, \boldsymbol{\theta})} \quad (4.13)$$

and for the dual substrate system:

$$w_{i1} = f_1(t_i, \boldsymbol{\theta}) \qquad w_{i2} = 9 \cdot f_2(t_i, \boldsymbol{\theta}) \qquad w_{i3} = f_3(t_i, \boldsymbol{\theta}) \quad (4.14)$$

where $f_j(t_i, \boldsymbol{\theta})$ are predicted values given by the model under consideration (Monod/Bailey & Ollis') for substrate ($j = 1$ and 3) and biomass ($j = 2$) and $\boldsymbol{\theta}$ is the parameter vector. These weights apply for the units used in this study. As mentioned earlier the biomass measurements were determined less precise than the substrate measurements. For the Bailey & Ollis' model the variance of the biomass was estimated to be about 9 times larger than the variance of the substrates; or the standard deviation of the biomass to be 3 times larger than the standard deviation of the substrates. The reason for a different structure in the dual substrate system was that the precision on the biomass measurements for the low concentrations were improved (see chapter 2). If the elements in the variance matrix $\boldsymbol{V}(\varepsilon)$ were all equal to one, there would be no weighting. When or if the predicted value f_j reached a certain value near zero the weighting procedure was turned of. This was done in order to prevent that a measurement with a very small concentration was weighted unreasonable high and thus more or less would dominate the whole estimation.

Figure 4.2: Trend in decay phase

4.3.2 Estimation of the error structure

The best way of examining and determine the error structure is by use of more than one measurements for all observations. At each observation the variances for the various responses can then be calculated. On basis of these estimated variance values the overall structure can be assessed. However, it is rather difficult and time consuming to carry out duplicate measurements in these degradation experiments, so only few were made. Instead one can use other measurements that can serve the same purpose. Measurements from the blank test, the lag phase, and the decay phase (if $b = 0$, also referred to as starvation phase) are all alternatives since the response levels are constant in these phases. Thus, they only dependent on the estimated mean values. Problems arise if there is a trend in the phases, which is

not accounted for. For example, if the biomass concentration is constant for some of the chosen period and then slowly dies away. In Fig. 4.2 the situation is illustrated. When using the lag and decay phase caution should also be taken on determining the beginning and the end of the phases. And when using a blank test one must ensure that the batches are gas tight, and that no degradation of the substrate occurs as in the experiments in this study.

The assumption of uncorrelated errors between responses, Eq.(4.9), was examined by use of repeated measurements and by use of data from blank tests and lag phases. In Fig. 4.3 a strong correlation between the errors on the substrate responses (toluene and benzene) are significant. This correlation was probably due to the sampling method (one sample for both measurements). The correlation between the substrate responses was, however, ignored in the analysis due to the more complicated estimation method to be used when the variance matrix $\sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon$ is not diagonal. In chapter 9 it is discussed whether the ignoring of the correlation between the substrates has any serious influence on the parameter estimation and on the likelihood test.

Figure 4.3: Correlation between substrates

There are not enough replicated measurements of the substrate and the biomass taken at the same time in order to examine the correlation between these two kind of responses. However, the assumption of no correlation between substrate and biomass errors seemed reasonable since the substrate sample and the biomass sample were two physically different samples.

We do account for the influence on the errors from the magnitude of the response level and from the response type. Replicated measurements were used together with measurements from the lag and the decay phases (if $b = 0$) and from the blanc tests in predicting the variance structure (the diagonal in the variance matrix, Eq.(4.11)). The variance of each measurement was determined by

$$\sigma^2_{\varepsilon,ij} = \sigma^2_{\varepsilon} \cdot w_{ij} \tag{4.15}$$

where σ_ε was estimated by the program Dekimo and the weight functions were defined by the user. This means that the user determines the ratio between substrate and biomass errors, and the ratio on errors from low and high concentration levels. In order to compare the identical experiments, <u>one</u> common structure was chosen for all the single substrate experiments and <u>one</u> for all the dual substrate experiments (Eq. 4.13 and 4.14). In Fig. 4.4 and 4.5 the error structure for the single substrate system is illustrated, and in Fig. 4.6 and 4.7 the error structure for the dual substrate system is shown.

As seen in Fig. 4.8 and Fig. 4.9 the variance of the measurement errors on the biomass was about 9 times as large as the variance on the substrate measurements. The errors were as expected heteroscedastic, such that the variance is inversely proportional to the response. Cornish-Bowden & Endrenyi (1981) had found a weight function of $1/Y^2_{pred}$ (corresponding to $w = f(t, \boldsymbol{\theta})^2$ in our study) to be reasonable for an enzyme experiment.

Figure 4.4: Variance structure for substrate measurement errors in the single system

Figure 4.5: Variance structure for biomass measurement errors in the single substrate system

Sàez & Rittmann (1992) found a weighting of $1/Y_{pred}$ (corresponding to $w = f(t, \boldsymbol{\theta})$ in our study) to describe the variance structure of the measurement errors for their experiment. The experiment Sàez & Rittmann

Figure 4.6: Variance structure for substrate measurement errors in the dual substrate system

Figure 4.7: Variance structure for biomass measurement errors in the dual substrate system

carried out was a batch experiment with phenol. Depending on the data an inappropriate assumption of the error structure may result in unreliable parameter estimates and an incorrect result of the likelihood ratio test.

Figure 4.8: The estimated variance structure for the responses in the single substrate system

4.3.3 Stochastic differential equations

In most degradation experiments the errors are assumed to be independent of time (uncorrelated with time), mainly of the following three reasons. 1) the estimation method for time depending error structure is very little known to environmental researchers, 2) calculation of stochastic models requires some knowledge of the method in order to interpret the results, and 3) using stochastic difference equations in degradation models requires many observations (Steffensen, 1991 and Bjørneboe, 1990) in order to identify the stochastic variables. For the last mentioned reason stochastic differential equations were not used in this study. In future works more data may be obtained which could open up for the possibility of using stochastic differential equations in nonlinear degradation models. In this section a short introduction to the method is given. For a review on the stochastic differential equations, consult with Petersen (1991), Kloeden & Platen (1989), and Kloeden, Platen, & Schurz (1991).

The dependence of time of the errors can be more or less random with a

Figure 4.9: The estimated variance structure for the responses in the dual substrate system

stochastic part being a function W of time, response, and/or independent variable. $\boldsymbol{\varepsilon}$ is referred to as process error.

$$\boldsymbol{\varepsilon} \in \pounds(\mathbf{0}, W(t, Y, X)) \tag{4.16}$$

The dependence of time can be modelled by an approximative formulation that does not describe all aspects of importance for the system, because
1) the underlying relations are not exactly known,
2) the relations in the system are too complex to be handled, or
3) simply because some factors are stochastic by nature.

The modelling of degradation kinetics by stochastic differential equations is based on estimation of the model parameters and estimation of two error components, a process error component and a measurement error component. The model can be written as

$$\frac{d\boldsymbol{Y}(t)}{dt} = \boldsymbol{g}(\boldsymbol{Y}(t), \boldsymbol{\theta}) + \boldsymbol{W}(\boldsymbol{Y}(t), t) \cdot U(t) \tag{4.17}$$

where $\boldsymbol{g}(\boldsymbol{Y}(t),\ \boldsymbol{\theta})$ is a function of characteristic parameters, e.g. kinetic parameters. The function $U(t)$ is an appropriate process error, distributed

as a zero mean Gaussian noise, $U(t) \in N(0, Q(t))$. Generally, $\boldsymbol{W}(\boldsymbol{Y}(t), t)$ is a nonlinear matrix function of $\boldsymbol{Y}(t)$. $\boldsymbol{Y}(t)$ is a stochastic response vector with measurement error, $\boldsymbol{e}$

$$\boldsymbol{Y} = \boldsymbol{y} + \boldsymbol{e}, \qquad \boldsymbol{e} \in N(0, \boldsymbol{R}) \tag{4.18}$$

The measurement error is considered to be constant in time. The process error and the measurement error can be more or less confounded and to avoid problems in estimating the errors it is necessary that the variance of the measurement error do not exceed a certain level. As a rule-of-thumb the following expression can be used (Steffensen, 1991)

$$\frac{1}{3} < \frac{\sigma^2_{meas}}{\sigma^2_{proc}} < 3 \tag{4.19}$$

In obtaining satisfying identification of the two errors it is moreover necessary that the time interval between the samplings are not constant throughout the experiment (Bilbo, 1992).

Generally, the estimation problem cannot be handled within the traditional framework of mean square calculus, since the right hand side of Eq.(4.17) is not integrable in the mean square sense (Bilbo, 1992). Estimation of parameters in nonlinear stochastic differential equations can be expressed in terms of filtering techniques, e.g. the Kalman filter. The idea is to calculate the conditional means response, $\hat{\boldsymbol{y}}|(t-1)$ and the conditional variance, $V(\boldsymbol{y}|\boldsymbol{y}(t-1), \theta)$ and updating these estimates at each time step. Bjørneboe (1990), Steffensen (1991), Wang (1994), and Spliid (1994) have studied stochastic differential equations and the application in microbiological degradation systems as discussed in this thesis. In Table 4.1 an overview is given of the stochastic models they examined referring to the structure of the model in Eq. (4.17).

Steffensen applied a process error $\boldsymbol{W}(\boldsymbol{Y}(t), t) =$ constant and Bjørneboe applied a more complex process error function $\boldsymbol{W}(\boldsymbol{Y}(t), t) = c \cdot \boldsymbol{g}(X_t, S_t)$. Both Bjørneboe and Steffensen concluded that the estimation of the parameters (model parameters plus process and measurement error parameters) for the Monod model was connected with difficulties in case of "small" sample size (less than 200 observations of each response). However, Bjørneboe found that estimation of model parameters and the error components were possible with **simulated** data. He concluded that the large uncertainty of the parameter estimates was due to an incomplete model specification, i.e. an incomplete description of the error structure. Since the measurement errors in microbiological degradation experiments often are fairly large and since the deterministic part of the nonlinear models present an identification problem in itself, Bilbo does not recommend the use of stochastic differential equations in cases with small sample size, large variability in the data and with nonlinear models of the Monod-type.

$\frac{d\boldsymbol{Y}(t)}{dt}$	Deterministic part $\boldsymbol{g}(\boldsymbol{Y},\boldsymbol{\theta})$	Stochastic part $\boldsymbol{W}(\boldsymbol{Y},\boldsymbol{\theta})U(t)$	Study on real/ simulated data	Reference
$\frac{dS_t}{dt}$ $\frac{dX_t}{dt}$	$-k\frac{X_tS_t}{S_t+K_s}$ $Yk\frac{X_tS_t}{S_t+K_s} - bX_t$	$c_1 \cdot U(t)$	sim. data	[1]
		$c_2 \cdot U(t)$	sim. + real data	[2]
		$c_1 S_t \cdot U(t)$	sim. data	[1]
		$c_2 X_t \cdot U(t)$	sim. data	[2]
		$c_1 k\frac{S_tX_t}{S_t+K_s} \cdot U(t)$	sim. + real data	[1]
		$c_2 Y k\frac{S_tX_t}{S_tX_t} \cdot U(t)$	sim. data	[2]
$\frac{dX_t}{dt}$	$\theta \cdot X_t$	$\gamma \cdot X_t^{\lambda} \cdot U(t)$	sim. + real data	[3], [4]

Table 4.1: Stochastic models for microbiological degradation experiments. [1] Bjørneboe (1990), [2] Steffensen (1991), [3] Spliid (1994), and [4] Wang (1994).

Spliid (1994) and Wang (1994) examined the application of stochastic differential equation to a biomass growth model described by a first order growth

process $(\theta \cdot X_t)$. They suggested a more general process error than used by Bjørneboe and Steffensen, namely $W(X_t) = \gamma \cdot X_t^{\lambda}$. Wang estimated an appropriated set of γ and λ for a specific experiment.

4.4 Transformations

A common practice in estimating the parameters in degradation experiments is to transform the nonlinear model into a linearized form and then fit the transformed data to the linearized form by simple least squares. Hereby the estimates of the parameters can be obtained by using linear regression. The linearization technique (linear regression) is useful because of its simplicity, the estimates can be obtained by direct calculations, whereas nonlinear estimation procedures require complicated iterative schemes. The linearization technique can, however, have severe statistical faults. When transforming the data, the error structure is transformed as well, which can introduce heteroscedastic error structure and give rise to incorrect estimation results. The reason for this is that when using linear regression the error structure is assumed to be homoscedastic, which is often wrong. Different linearized forms of the same nonlinear degradation model typically yield dissimilar estimates of the same parameters, because each linearization transforms the error structure differently (Dowd & Riggs, 1965). In the present section transformation technique is described shortly in general followed by a previously very common transformation in nonlinear degradation models (Lineweaver-Burk), which is somewhat different from the general transformation technique. At the end of this section the Lineweaver-Burk technique is used in estimating parameter values for one of the experiments in this study.

Linearization of a nonlinear model can be obtained by one of several transformation techniques or a combination of these depending on the model under consideration.

- transformation of the parameters, θ,
- transformation of the independent variable, x,
- transformation of the dependent variable, y.

Transformation of the parameters and transformation of the independent variable do not change the error structure if the model is written as $y = f(x, \theta) + \varepsilon$. However, transformation of the response (the dependent variable) also involves a transformation of the measurement errors as well, which affects the assumption on it. In the following, simple examples are given to illustrate these transformations.

Transformation of parameters. Let

$$y = \frac{1}{\theta_1} + \frac{\theta_2}{\theta_1}x + \varepsilon \tag{4.20}$$

be our model equation, with θ_1 and θ_2 as parameters. Letting $\beta_1 = 1/\theta_1$ and $\beta_2 = \theta_2/\theta_1$, we have the linear model

$$y = \beta_1 + \beta_2 x + \varepsilon \tag{4.21}$$

The error structure is unaffected by this transformation. The linear regression analysis yields estimates of the new parameters β_1 and β_2 and the parameter covariance matrix $V(\beta) = \sigma_\varepsilon^2 \mathbf{\Sigma}_\beta$. From these estimates the original parameter values $\boldsymbol{\theta}$ and their covariance matrix $\boldsymbol{V(\theta)}$ can be calculated. Consult Bard (1974) for calculations of these values.

Transformation of the independent variables. Let

$$y = \theta_0 + \theta_1 \frac{1}{x_1} + \theta_2 \ln x_2 + \theta_3 x_3^{1/2} + \varepsilon \tag{4.22}$$

be our model equation, with θ_0, θ_1, θ_2, and θ_3 as parameters. Letting $z_1 = 1/x_1$, $z_2 = \ln x_2$, and $z_3 = x_3^{1/2}$, we have the linear model

$$y = \theta_0 + \theta_1 z_1 + \theta_2 z_2 + \theta_3 z_3 + \varepsilon \tag{4.23}$$

As we can see the error ε is unaffected of the transformation and, thus, has the same distribution as before transforming the independent variables. If

the independent variables were subject to error, there would be problems transforming them. There are many possible transformations of the independent variables. One useful type of transformation worth trying in many problems is the power transformation.

$$z_i = \begin{cases} x_i^{\alpha_i} & , \quad \text{for } \alpha_i \neq 0 \\ \ln x_i & , \quad \text{for } \alpha_i = 0 \end{cases} \tag{4.24}$$

for $i = 1, 2, \cdots, k$ where α_i are the transformation parameters to be estimated. The best way of estimating the α_i is to estimate them at the same time as the model parameters via nonlinear estimation methods (Draper & Smith, 1981), but then some of the idea of linearizating the nonlinear model is lost. Alternative, an iterative procedure can be used, as described by Box & Tidwell (1962) and Box & Draper (1987).

Transformation of the depending variable. Let

$$y = 1/(\theta_0 + \theta_1 x_1 + \theta_2 x_2) + \varepsilon \tag{4.25}$$

be our model equation, with θ_0, θ_1, and θ_2 as parameters. Letting $z = y^{-1}$, we have the linear model

$$z = \theta_0 + \theta_1 x_1 + \theta_2 x_2 + \varepsilon' \tag{4.26}$$

If $\varepsilon \in N(0, \sigma_\varepsilon^2 I)$ in the original model Eq. (4.25) the new error $\varepsilon' = -\varepsilon \cdot \frac{x'^2}{1+\varepsilon x'}$ will have the following mean and variance. $(x' = \theta_0 + \theta_1 x_1 + \theta_2 x_2)$

$$\varepsilon' = -\varepsilon \cdot \frac{x'^2}{1 + \varepsilon x'} = g(\varepsilon) \tag{4.27}$$

$$V(\varepsilon') = \left(\frac{\partial g(\varepsilon)}{\partial \varepsilon} \right)^2 V(\varepsilon) = \frac{x'^4}{(1 + \varepsilon x')^4} \cdot \sigma_\varepsilon^2 \tag{4.28}$$

Transformation of the response (dependent variable) to obtain a linear model also involves transformation of the error structure. Different transformations will have more or less effect on the distribution of the errors and thereby of the parameter estimates.

Stabilizing variance

The purpose of the above described transformations was to obtain a linear model so that linear regression analysis could be employed. The purpose of the here described transformation is however to stabilize the variance. If the structure of the errors is not normal, transformation of the response can be used to obtain "nicer" structure of the errors (closer to normally distribution). However, this may not result in a linearization of the nonlinear model and if the original model is linear it generally results in making the model nonlinear. Some well known transformations are shown in Draper & Smith (1981).

$$z = \begin{cases} (y^{\lambda} - 1)/\lambda & , \quad \text{for } \lambda \neq 0 \\ \ln y & , \quad \text{for } \lambda = 0 \end{cases} \tag{4.29}$$

Estimation of a λ that will stabilize the errors can be done by a maximum likelihood method using nonlinear estimation or by the method suggested by Box & Cox (1964).

Another way of dealing with error structure that is not normal distributed is by using a parameter estimation method that accounts for the "special" structure of the errors (for example weighted least squares or maximum likelihood, described in later sections).

Lineweaver-Burk transformation

Since most microbiological degradation models consist of two or more coupled nonlinear differential equations it is generally so that they cannot be solved analytically as $\boldsymbol{y} = \boldsymbol{f}(x, \boldsymbol{\theta})$, where $\boldsymbol{y}$ is the response vector. The Lineweaver-Burk expression was earlier very often used for transformation of degradation models. Bates & Watts (1988) call this kind of transformation "transformable linear". One of the problems with this linearization is that the "independent" variable (here S_i) occurs on both sides of the equal-sign and it is almost impossible to examine the variance structure of the measurement errors. For the Monod model the transformation becomes:

$$\frac{dS_i}{dt} = -k \cdot \frac{S_i X_i}{S_i + K_s} \tag{4.30}$$

$$\Updownarrow$$

$$\frac{X_i}{dS_i/dt} = \frac{K_s}{k} \cdot \frac{1}{S_i} + \frac{1}{k} = \beta_1 \frac{1}{S_i} + \beta_2 \quad (+\varepsilon_i') \tag{4.31}$$

where $\beta_1 = \frac{K_s}{k}$ and $\beta_2 = \frac{1}{k}$. X_i is usually calculated from $X_i = X_0 + Y(S_0 - S_i)$ under the assumption that $b \simeq 0$, and Y is calculated from $Y = (X_{t'} - X_0)/(S_{t'} - S_0)$ where t' is the time at which the substrate has been degraded. From a linear plot the parameters K_s and k can be calculated by: slope $= a = 1/K_s$ and cut off line $= b = k/K_s$ (Fig. 4.10).

Figure 4.10: Illustration of the Lineweaver-Burk method

In chapter 8 parameter estimates obtained by the Lineweaver-Burk linearization are compared with estimates obtained by use of the nonlinear programs AquaSim and Dekimo.

When using the ordinary (unweighted) least squares method in estimating the parameters β_1 and β_2 in the linearized model Eq.(4.31), we indirectly assume that the errors ε_i' are normally distributed with a constant variance, which is not the case. The wrong assumptions about the errors can give rise to seriously incorrect parameter estimates. For example, if the true variance structure followed the dashed line in Fig. 4.10, the "true" regression line could be quite different from the regression line shown in the figure and thus the "correct" parameter estimates would be quite different as well.

Linearized forms of nonlinear equations require that more data points are needed for parameter estimation than if nonlinear estimation techniques are used (Garfinkel et al.). Besides the Lineweaver-Burk linearization other methods can be used for linearizing the nonlinear Monod model, for example Eadie-Hofstee and all the linearized versions (Cornish-Bowden, 1979) of the integrated equation. However, they all violate the assumptions on the error structure and thus may give rise to more or less incorrect estimates.

4.5 Nonlinear estimation

Nonlinear estimation is not often used to estimate microbial kinetic parameters (Robinson, 1985) though lately more researchers have used nonlinear estimation for some of the parameter values. Part of the explanation for the limited use of nonlinear estimation techniques may be that an appropriate use requires some knowledge of the method in order to interpret the output and understand its limitations. In the nonlinear estimation procedure there are three phenomena to be considered.

- the error structure,
- the objective function or sum of squares function (e.g. ordinary least square),
- the minimization method (the method used to minimize the objective function).

First of all assumptions are made on the error structure and depending on these assumptions an appropriate objective function should be chosen. After the estimation of the parameter values, the assumptions on the error structure should be checked by use of residual analysis. If the residual analysis reveals an error structure significantly different from the one assumed earlier, new assumptions should be made and the estimation repeated.

The nonlinear minimization method is an iterative technique in which initial parameter estimates are sequentially improved until the "best" estimates

(i.e. those that minimize differences between the observed and predicted responses) are calculated. This involves many arithmetic operations which require the aid of a computer. Most nonlinear estimation methods require initial guesses for the parameters and if these are far away from the optimal values the estimation process may not succeed. Linearization methods can be used to obtain qualified initial guesses. In this section various objective functions (least squares estimates and maximum likelihood) are discussed, followed by a short description of various common methods of minimizing the chosen objective function in order to obtain the parameter estimates.

4.5.1 Objective function

In order to obtain parameter estimates an objective function must be chosen for minimization. In the statistical literature the objective function is also referred to as an expectation surface. There are several objective functions to choose among. Most of them involve a minimization of the residual sum of squares in some sense. The simple residual sum of squares is given by

$$SSE = \sum (Y_{obs} - Y_{pred})^2 \tag{4.32}$$

or written in mathematical terms

$$SSE(\boldsymbol{\theta}) = ||\boldsymbol{y} - \boldsymbol{f}(\boldsymbol{\theta})||^2 = \sum_{i=1}^{n} [\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta})]^\top [\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta})] \tag{4.33}$$

where $\boldsymbol{y}_i$ is the observed response vector at time t_i, $i = 1, \cdots, n$. $\boldsymbol{f}(t_i, \boldsymbol{\theta})$ is the corresponding vector of predicted values, and $\boldsymbol{\theta}$ is the unknown parameter vector. The parameter vector can be extended to include boundary conditions, such as initial concentrations of biomass and substrate, X_0 and S_0. These parameters are also referred to as system parameters and can, together with the model parameters, be estimated simultaneously using the same estimation technique. In this study X_0 has been included in the models.

In the following, the general least squares method with submodels (weighted least squares and ordinary least squares methods) and the maximum likelihood method are described.

General least squares (GLS) analysis
When applying general least squares assumptions are made that the measurement errors are normally distributed with a zero mean and a variance structure described by $\sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon$.

$$\boldsymbol{\varepsilon} \in N(\mathbf{0}, \sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon) \tag{4.34}$$

In this model the matrix $\boldsymbol{\Sigma}_\varepsilon$ does not need to be a diagonal matrix, it may be a general symmetric positive semidefinite matrix, which implies that the measurements (errors) can be correlated and of different magnitudes. $\boldsymbol{\Sigma}_\varepsilon$ contains previously discussed weights (section 4.3) and must be chosen by the user prior of the parameter estimation. The least squares estimator $\hat{\boldsymbol{\theta}}$ is obtained by minimization of $S(\boldsymbol{\theta})$ over the parameter space.

$$S(\boldsymbol{\theta}) = \sum_{i=1}^{n} [\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta})]^\top \boldsymbol{\Sigma}_\varepsilon^{-1} [\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta})] \tag{4.35}$$

The variance factor σ_ε^2 can be estimated from

$$\hat{\sigma}_\varepsilon^2 = \frac{1}{n-p} \sum_{i=1}^{n} [\boldsymbol{y}_i - \boldsymbol{f}(t_i, \hat{\boldsymbol{\theta}})]^\top \boldsymbol{\Sigma}_\varepsilon^{-1} [\boldsymbol{y}_i - \boldsymbol{f}(t_i, \hat{\boldsymbol{\theta}})] \tag{4.36}$$

The optimum is found for $\hat{\boldsymbol{\theta}}$ when $(\partial/\partial\boldsymbol{\theta})S(\hat{\boldsymbol{\theta}}) = 0$. $S(\boldsymbol{\theta})$ is once continuously differentiable on an open set Ω_0 with $\boldsymbol{\theta} \in \Omega$, when $\hat{\boldsymbol{\theta}}$ satisfies

$$\boldsymbol{f}'_\theta(\boldsymbol{t}, \hat{\boldsymbol{\theta}})^\top (\boldsymbol{y} - \boldsymbol{f}(\boldsymbol{t}, \hat{\boldsymbol{\theta}})) = 0 \quad \text{where} \quad \boldsymbol{f}'_\theta(\boldsymbol{t}, \hat{\boldsymbol{\theta}}) = \frac{\partial}{\partial \boldsymbol{\theta}^\top} \boldsymbol{f}(\boldsymbol{t}, \hat{\boldsymbol{\theta}}) \tag{4.37}$$

(Gallant 1987).

When using general least squares (GLS) for parameter estimation, knowledge of the error correlations is necessary. In order to obtain reliable estimates, a large number of observations is necessary. For this reason, among others, GLS is rarely used in microbiological degradation models.

Weighted least squares (WLS) analysis
The weighted least squares method is a sub-model of the general least squares method, brought about by an assumption of a simpler error structure. The errors are still assumed to be normally distributed with zero mean but with no correlations between the errors (no correlation between the responses and no time dependent correlation). The error structure can be written as

$$\boldsymbol{\varepsilon} \in N(\mathbf{0}, \sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon) \tag{4.38}$$

where $\boldsymbol{\Sigma}_\varepsilon$ is a diagonal matrix that may have different values in the diagonal - according to the different variances of the errors. Usually the exact error structure of $\boldsymbol{\Sigma}_\varepsilon$ is not known, but must be estimated. The matrix must be specified by the user prior of the parameter estimation. In section 4.6 it is shown how they can be estimated. The weighted least squares estimator of $\hat{\boldsymbol{\theta}}$ is obtained by minimization of $S(\boldsymbol{\theta})$

$$S(\boldsymbol{\theta}) = \sum_{i=1}^{n} [(\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta}))^\top \boldsymbol{\Sigma}_\varepsilon^{-1} (\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta}))] \tag{4.39}$$

If the diagonal elements of Σ_ε are w_{ij}, $S(\boldsymbol{\theta})$ can be written:

$$S(\boldsymbol{\theta}) = \sum_{j=1}^{m} \sum_{i=1}^{n} [(y_{ij} - f_j(t_i, \boldsymbol{\theta}))^2 w_{ij}^{-1}] \tag{4.40}$$

where w_{ij} act as weights. For this reason the method is often referred to as weighted least squares. The variance factor σ_ε^2 is estimated from

$$\hat{\sigma}_\varepsilon^2 = \frac{1}{n-p} \sum_{i=1}^{n} [(\boldsymbol{y}_i - \boldsymbol{f}(t_i, \hat{\boldsymbol{\theta}}))^\top \boldsymbol{\Sigma}_\varepsilon^{-1} (\boldsymbol{y}_i - \boldsymbol{f}(t_i, \hat{\boldsymbol{\theta}}))] \tag{4.41}$$

or

$$\hat{\sigma}_\varepsilon^2 = \frac{1}{n-p} \sum_{j=1}^{m} \sum_{i=1}^{n} (y_{ij} - f(t_i, \boldsymbol{\theta}))^2 w_{ij}^{-1} \tag{4.42}$$

where m is the number of responses, n is the number of observations, and p is the number of parameters. The nonlinear estimation program AquaSim uses the WLS method, and in chapter 7 an estimation of one of the experiments carried out in this study is performed using AquaSim.

Ordinary Least Squares Estimation (OLS) Analysis

The most commonly used method of the least squares methods in degradation experiments is the ordinary least squares method. OLS is a sub-model of the weighted least squares. In contrast to the weighted least squares the errors must be univariate, distributed with a constant variance over the measurement range.

$$\boldsymbol{\varepsilon} \in N(\mathbf{0}, \sigma_\varepsilon^2 \boldsymbol{I}) \tag{4.43}$$

where $\boldsymbol{I}$ is a identity matrix with 1's in the diagonal. The least squares estimator of $\hat{\boldsymbol{\theta}}$ is obtained by minimization of $S(\boldsymbol{\theta})$.

$$S(\boldsymbol{\theta}) = \sum_{i=1}^{n} [(\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta}))^\top (\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta}))] \tag{4.44}$$

or

$$S(\boldsymbol{\theta}) = \sum_{j=1}^{m} \sum_{i=1}^{n} (y_{ij} - f_j(t_i, \boldsymbol{\theta}))^2 \tag{4.45}$$

An estimate of the variance of the errors corresponding to the least squares estimator $\hat{\boldsymbol{\theta}}$ is

$$\hat{\sigma}_\varepsilon^2 = \frac{1}{n-p} \sum_{i=1}^{n} [(\boldsymbol{y}_i - \boldsymbol{f}(t_i, \hat{\boldsymbol{\theta}}))^\top (\boldsymbol{y}_i - \boldsymbol{f}(t_i, \hat{\boldsymbol{\theta}}))] \tag{4.46}$$

or

$$\hat{\sigma}_\varepsilon^2 = \frac{1}{n-p} \sum_{j=1}^{m} \sum_{i=1}^{n} (y_{ij} - f_j(t_i, \boldsymbol{\theta}))^2 \tag{4.47}$$

where n is the number of observations and p is the number of parameters in the nonlinear model.

OLS is used when nothing is known about the measurement errors, but if the measurement errors are known to be almost constant, then OLS is not

a poor method. If the standard deviation of the measurement errors varies by more than tenfold over the range in which it is measured, then WLS is usually superior to OLS (Beck & Arnolds, 1977). The measurements with the largest errors will dominate the estimation too much in the OLS analysis and result in incorrect estimates.

Maximum likelihood (ML) estimation

The maximum likelihood estimation method can, like the general least squares (GLS) method, account for correlations between the errors. Moreover, the errors do not necessarily have to be normally distributed. Any appropriate distribution can be employed

$$\boldsymbol{\varepsilon} \in \pounds(\mathbf{0}, \sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon) \tag{4.48}$$

where $\pounds$ can be any defined distribution describing the behaviour of the measurements errors and $\boldsymbol{\Sigma}_\varepsilon$ is not necessarily a diagonal matrix. For a normal distribution the log-likelihood function for the parameters $\boldsymbol{\theta}$ is

$$\begin{aligned} S(\boldsymbol{\theta}) &= L(\boldsymbol{\theta}) = \\ c & -\frac{N}{2} \log |\sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon| - \frac{1}{2} \sum_{i=1}^{n} [(\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta}))^\top \boldsymbol{\Sigma}_\varepsilon^{-1} (\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta}))] \end{aligned} \tag{4.49}$$

(Gallant, 1987).

The maximum likelihood estimators $\hat{\boldsymbol{\theta}}$ and $\hat{\sigma}_\varepsilon^2$ are obtained by minimization of the negative log-likelihood function. The variance of the errors is estimated by

$$\hat{\sigma}_\varepsilon^2 = \frac{1}{n-p} \sum_{i=1}^{n} [(\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta}))^\top \boldsymbol{\Sigma}_\varepsilon^{-1} (\boldsymbol{y}_i - \boldsymbol{f}(t_i, \boldsymbol{\theta}))] \tag{4.50}$$

The estimation program Dekimo uses the ML method, but does not account for correlations between errors - the correlation matrix $\boldsymbol{\Sigma}_\varepsilon$ is a diagonal matrix (only with elements different from zero in the diagonal). The measurement errors are assumed to be normally distributed. With the assumption that the errors are uncorrelated and normally distributed the weighted least squares method might as well have been applied for parameter estimation.

However, by use of ML the log-likelihood value is obtained which is of great value when estimating the parameter correlation matrix $\mathbf{\Sigma}_\theta$ (section 4.6 of parameter estimates) and a necessity when using the likelihood ratio test in testing identity between sets of parameters across several experiment.

In section 4.6 assumptions on the structure of the error matrix $\mathbf{\Sigma}_\varepsilon$ for the experiments in this study, are given.

4.5.2 Optimization methods

In contrast to linear models, explicit functions giving the best parameter estimates do not exist for nonlinear models (Draper & Smith, 1991). To overcome these difficulties iterative methods are used to determine values for parameters that minimize the chosen objective function (GLS, WLS, OLS, ML, or similar). In other words, an initial set of parameter estimates $\boldsymbol{\theta}^0$ is determined either by use of a linearized form of the chosen nonlinear model or by guessing. An initial value of the objective function $S(\boldsymbol{\theta}^0)$ is calculated and a new set of parameter values $\boldsymbol{\theta}^1$ is estimated to the corresponding objective function. The new value of the objective function $S(\boldsymbol{\theta}^1)$ is compared with the objective function for the initial parameter estimates $S(\boldsymbol{\theta}^0)$, and if the former is less than the value of the objective function for the initial estimates, then the second set of parameter estimates replaces the first.

$$\begin{aligned}\boldsymbol{\theta}^0 \rightarrow S(\boldsymbol{\theta}^0) \rightarrow \boldsymbol{\theta}^1 \rightarrow S(\boldsymbol{\theta}^1) \quad \Rightarrow \quad & \text{if} \quad S(\boldsymbol{\theta}^0) > S(\boldsymbol{\theta}^1) \quad \Rightarrow \boldsymbol{\theta}^2 \rightarrow S(\boldsymbol{\theta}^2) \quad \Rightarrow \\ & \text{if} \quad S(\boldsymbol{\theta}^1) > S(\boldsymbol{\theta}^2) \quad \cdots \end{aligned} \tag{4.51}$$

This process continues until the objective function reaches a minimum, at which point the best parameter values have been located. There is no universal method for determining the path to be taken from the initial parameter estimates to the values that minimize the objective function (Robinson, 1985; Bard, 1974). In this section a few common methods will be mentioned.

Trial and error technique
A simple but not very efficient method of minimizing the chosen objective function is by trial and error. Parameter values are chosen by guessing more or less randomly, and the set of parameter values that result in the smallest value of the objective function are chosen to be the final estimates. It is impossible to know if one has reached the global minimum, or even a local minimum (Fig. 4.11). Another set of parameter estimates which fits the data best (global optimum) may exist. The trial and error technique is some times used in connection with a spreadsheet where the predicted values of the responses are calculated by using small time steps and the objective function is usually ordinary least squares (Jensen, 1992 and Jensen, 1994). This method only requires a PC with a spreadsheet and patience for estimating. Others (Koeppe & Hanmann, 1980) have written a program to generate parameter guesses and to select the set that results in the lowest value of the objective function. The trial and error method becomes very difficult to use for models with say four or more parameters.

Figure 4.11: Local / global minimum

Simplex technique
In contrast to trial and error searches, most nonlinear estimation methods specify the direction and the magnitude of changes to be made to the para-

meter estimates during the recursive process. The simplex method (Nelder & Mead, 1965) requires only function evaluation, not derivatives of the objective function. It is not very efficient - it converges slowly - but the method is very robust especially for estimating parameters in coupled non-linear differential equations which appear in most microbiology degradation models. A simplex is a convex geometrical figure in P dimensions, defined by $P+1$ points (P is here the total number of parameters). From the initial parameter array specified by the user, the start simplex is generated. For a function of only two parameters, the simplex is a triangle. In three dimensions it is a tetrahedron. A simplex design is a design where $P+1$ values of p factors are given by the corners of the P dimensional simplex. The simplex method takes a series of steps, most steps just moving the point of the simplex where the objective function is largest through the opposite face of the simplex to a lower point. This is illustrated for a design for three parameters in Fig. 4.12.

Figure 4.12: Simplex method

The following expression is used to locate and replace a new point in the simplex.

$$S(\boldsymbol{\theta}^*) = \frac{1+\alpha}{P} \cdot [\sum_{\nu=1}^{P+1} S(\boldsymbol{\theta}_\nu) - S(\boldsymbol{\theta}_j)] - \alpha \cdot S(\boldsymbol{\theta}_j) \tag{4.52}$$

α controls the step size, and $S(\boldsymbol{\theta}^*)$ is the objective function in the new corner of the simplex and $S(\boldsymbol{\theta}_j)$ is the old corner, which is being replaced. In "Numerical Recipes" (Press et al., 1989) a recipe for a program "amoeba" using the simplex method is given. The simplex technique is implemented in the estimation program AquaSim.

Secant technique

The secant method is a more efficient algorithm for minimizing the chosen objective function compared with the simplex method. The simplex method slowly moves down the "gradient" of the objective function, whereas the secant method rapidly jumps to the position of a suggested solution of the problem found by parabolic extrapolation. The secant method is a derivative-free method which simply uses numerical approximations to derivatives. It is based on using a secant plane approximation to the objective function rather than a tangent plane approximation. The method uses earlier iterations to calculate an approximation to the secant and is therefore also called a two step method. It is recalled that the goal is to minimize the objective function by finding the solution θ^* to $S(\theta)/\partial\theta = 0$.

To illustrate the method the principles are shown in Fig. 4.13 for a one dimensional function $h(\theta) = \partial S(\theta)/\partial\theta$ and the secant $\phi(\theta)$ is expressed by

$$\phi(\theta) = \frac{h(\theta_k) - h(\theta_{k-1})}{\theta_k - \theta_{k-1}}(\theta - \theta_k) + h(\theta_k) \tag{4.53}$$

An estimate of θ^* is given by

$$\hat{\theta}_{k+1} = \frac{h(\hat{\theta}_k)\hat{\theta}_{k-1} - h(\hat{\theta}_{k-1})\hat{\theta}_k}{h(\hat{\theta}_k) - h(\hat{\theta}_{k-1})} \tag{4.54}$$

For each iteration the estimate of $S(\theta^*)$ decreases until the chosen convergence criterion are reached. For a multi dimensional function with more than one parameter, the secant expression becomes

$$\boldsymbol{\phi}(\boldsymbol{\theta}) = \frac{\sum_{j=1}^{m}(\boldsymbol{h}(\boldsymbol{\theta}_k) - \boldsymbol{h}(\boldsymbol{\theta}_{k-1}))}{\sum_{j=1}^{m}(\boldsymbol{\theta}_k - \boldsymbol{\theta}_{k-1})}(\boldsymbol{\theta} - \boldsymbol{\theta}_k) + \boldsymbol{h}(\boldsymbol{\theta}_k) \tag{4.55}$$

Figure 4.13: Secant method. Extrapolation or interpolation lines (dashed) are drawn through the two most recently evaluated points, whether or not they bracket the function. The points are numbered in the order that they are used.

The secant method is an option in the estimation program packet, AquaSim. The selected algorithm is an extension of the secant method DUD (Doesn't Use Derivatives) (Ralston & Jennrich, 1978). The "creators" of AquaSim recommend to start the parameter estimation with the secant algorithm and only switch to the more robust simplex method in case of numerical problems. Having roughly localized the solution with the simplex method, go back to the secant method to accelerate final convergence and obtain estimates of the standard deviations for the parameter estimates, which cannot be obtained by the simplex method.

Gaussian technique

The mathematical elements of the Gaussian method (also called the Gauss-Newton, Newton-Gauss, or linearization method) are derived through the application of a first order Taylor series expansion (Burden et al., 1978). This expansion essentially linearizes the nonlinear objective function, $S(\boldsymbol{\theta})$ (e.g. GLS, WLS, OLS, ML, or similar functions) in the neighbourhood of

the best parameter estimates. The best parameter estimates are obtained by iteratively improving the parameter values until there is no change. The Gaussian method is an attractive method because it is relatively simple and because it specifies direction and size of the corrections on the parameter vector, which is used for finding the new guess on parameter values. The Gaussian method is effective in seeking minima which are reasonably well-defined provided that the initial estimates are in the general region of the minimum. For strongly nonlinear models (like most microbiological degradation models) modifications must be made to ensure convergence. Linearization of the objective function is performed by the Taylor series about the best parameter estimate $\boldsymbol{\theta}^0$ given by

$$S(t_i,\boldsymbol{\theta}) = S(t_i,\boldsymbol{\theta}^0) + J_{i1}(\theta_1-\theta_1^0) + J_{i2}(\theta_2-\theta_2^0) + \cdots J_{iP}(\theta_P-\theta_P^0) \tag{4.56}$$

where $\boldsymbol{J}$ is the Jacobian matrix defined by

$$J_{ip} = \left.\frac{\partial S(t_i,\boldsymbol{\theta})}{\partial\theta_p}\right|_{\theta^0} \qquad p = 1,2,\ldots,P \tag{4.57}$$

and p is the index for the parameters. The Taylor series written in matrix notation is

$$S(t,\boldsymbol{\theta}) = S(t,\boldsymbol{\theta}^0) + \boldsymbol{J}(\boldsymbol{\theta}-\boldsymbol{\theta}^0) \tag{4.58}$$

By rearranging this expression it becomes clear that the equation has a linear form with $\boldsymbol{J}$ as the independent variable, $\boldsymbol{\beta} = \boldsymbol{\theta}-\boldsymbol{\theta}^0$ as parameter vector and $S(t,\boldsymbol{\theta}) - S(t,\boldsymbol{\theta}^0)$ as the dependent variable. To simplify the notation $S(t,\boldsymbol{\theta}) = S$ and $S(t,\boldsymbol{\theta}^0) = S^0$.

$$S - S^0 = \boldsymbol{\beta}\boldsymbol{J} + \boldsymbol{\varepsilon} \tag{4.59}$$

The estimate of $\boldsymbol{\beta}$ can now be obtained by use of the normal equations (method used in linear regression analysis).

$$\hat{\boldsymbol{\beta}} = (\boldsymbol{J}^\top\boldsymbol{J})^{-1}\boldsymbol{J}^\top(S-S^0) \tag{4.60}$$

The vector $\hat{\boldsymbol{\beta}}$ will thus minimize the sum of squares

$$SSE(\boldsymbol{\theta}) = \sum_{i=1}^{n}[S_i - S^0 - \boldsymbol{\beta}J_i]^2 \tag{4.61}$$

where $\boldsymbol{\beta} = \boldsymbol{\theta} - \boldsymbol{\theta}^0$. The values of $\boldsymbol{\theta}^0$ can be replaced by those of $\boldsymbol{\theta}^1$ and the same procedure is applied as described above by Eq.(4.56) except that all zero subscripts are replaced by ones. This will lead to another set of revised estimates $\boldsymbol{\theta}^2$, and so on. We can then write

$$\boldsymbol{\theta}^{u+1} = \boldsymbol{\theta}^u + \boldsymbol{\beta}^u \tag{4.62}$$

Since the Jacobian matrix, $\boldsymbol{J}$

$$\boldsymbol{J} = \partial S/\partial\boldsymbol{\theta} = -2\partial\boldsymbol{f}(t,\boldsymbol{\theta})/\partial\boldsymbol{\theta}\cdot(\boldsymbol{y}-\boldsymbol{f}(t,\boldsymbol{\theta})) = -2\boldsymbol{V}(\boldsymbol{y}-\boldsymbol{f}(t,\boldsymbol{\theta})) \tag{4.63}$$

(Bard, 1974) Eq.(4.62) can be rewritten by use of Eq. (4.60)

$$\boldsymbol{\theta}^{u+1} = \boldsymbol{\theta}^u + (\boldsymbol{V}^\top\boldsymbol{V})^{-1}\boldsymbol{V}^\top(\boldsymbol{y}-\boldsymbol{f}(t,\boldsymbol{\theta}^u)) \tag{4.64}$$

where $\boldsymbol{y}$ is the observation vector and $\boldsymbol{f}$ is the expectation function defined in section 4.1. The procedure of this optimization method is described by Robinson (1985), Draper & Smith (1981) and Bard (1974) among others. The Gauss procedure has a tendency to "overshoot", that is, to go beyond points $\boldsymbol{\theta}$ where smaller $S(\boldsymbol{\theta})$ values exist, to points where larger $S(\boldsymbol{\theta})$ values occur. To avoid this problem, a line search technique can be employed. Line search methods are particularly useful for models that exhibit a high degree of nonlinearity, since the objective functions of these models may be poorly approximated by the truncated Taylor series in the neighbourhood of the best parameter estimates (Bard, 1974). Which of the many methods is best in "helping" to find new parameter estimates, partly depends on the nonlinear model of interest (Robinson, 1985).

The Levenberg-Marquardt modification is one of many modification methods (Beck & Arnold, 1977). The method alters both the step size and direction taken by the Gaussian technique, attempting to ensure that the objective function $S(\boldsymbol{\theta})$ is sequentially reduced. Another modification of the Gaussian method is the Box-Kanemasu modification. It ensures that changes taken in the parameter search do not oscillate widely or diverge away from the parameter values defining the minimum of the objective function (Beck & Arnorld, 1977).

Newton-Raphson or quasi-Newton Technique

The quasi-Newton method, also called the variable metric method, requires calculation of the first and the second derivatives of the objective function.

$$S(t,\boldsymbol{\theta}) = S(t,\boldsymbol{\theta}^u) + \boldsymbol{J}^{u\top}(\boldsymbol{\theta}-\boldsymbol{\theta}^u) + 1/2(\boldsymbol{\theta}-\boldsymbol{\theta}^u)^\top\boldsymbol{H}^u(\boldsymbol{\theta}-\boldsymbol{\theta}^u) \tag{4.65}$$

where $\boldsymbol{J}$ is the Jacobian matrix defined in Eq.(4.57) and $\boldsymbol{H}$ is the Hessian matrix

$$\boldsymbol{H}^u = \left.\frac{\partial^2 S(t,\boldsymbol{\theta})}{\partial\boldsymbol{\theta}\partial\boldsymbol{\theta}^\top}\right|_{\theta=\theta^u} \tag{4.66}$$

In search of the optimal set of parameter estimates the objective function should be minimized by finding the solution to

$$\partial S^u/\partial\boldsymbol{\theta} = \boldsymbol{J}^u + \boldsymbol{H}^u(\boldsymbol{\theta}-\boldsymbol{\theta}^u) = \boldsymbol{0} \tag{4.67}$$

which, if $\boldsymbol{H}^u$ is nonsingular, has the solution

$$\boldsymbol{\theta}^{u+1} = \boldsymbol{\theta}^u - (\boldsymbol{H}^u)^{-1}\boldsymbol{J}^u \tag{4.68}$$

Eq.(4.68) defines the u^{th} iteration of the Newton-Raphson (also known as Newton) method. Since the Hessian matrix $\boldsymbol{H}$ is equal

$$\boldsymbol{H} = 2\boldsymbol{V}^\top\boldsymbol{V} - 2\frac{\partial\boldsymbol{V}^\top}{\partial\boldsymbol{\theta}^\top}(\boldsymbol{y}-\boldsymbol{f}(t,\boldsymbol{\theta})) \tag{4.69}$$

(Bates & Watts, 1988) Eq.(4.68) can be rewritten as

$$\boldsymbol{\theta}^{u+1} = \boldsymbol{\theta}^u - [\boldsymbol{V}^\top\boldsymbol{V} - \frac{\partial\boldsymbol{V}^\top}{\partial\boldsymbol{\theta}^\top}(\boldsymbol{y}-\boldsymbol{f}(t,\boldsymbol{\theta}^u))]^{-1}\cdot\boldsymbol{V}(\boldsymbol{y}-\boldsymbol{f}(t,\boldsymbol{\theta})) \tag{4.70}$$

where $\boldsymbol{y}$ is the observation vector and $\boldsymbol{f}$ is the expectation function.

When estimating a new set of parameter values the problem comes down to calculating the first and second derivatives of the expectation function, $\boldsymbol{f}$. However, the expectation function $\boldsymbol{f}$ in most microbiological degradation equations cannot be calculated analytically, but must be approximated by numerical algorithm (in this study by a 4$^{\text{th}}$ order Runge-Kutta approximation). This results in an unnecessary inaccuracy. Instead it is better to implement the formula given in Eq.(4.68) for the iterative parameter estimation. The Jacobian matrix $\boldsymbol{J}$ is not calculated from Eq.(4.63) but estimated by a central difference approximation

$$J_p \simeq \frac{S(t, \boldsymbol{\theta} + h\boldsymbol{e}_p) - S(\boldsymbol{\theta} - h_p \boldsymbol{e}_p)}{2h_p} \quad , \qquad p = 1, \cdots, P \tag{4.71}$$

where $\boldsymbol{e}_p$ is the p^{th} basis vector and h_p is the step-size for the parameter p. According to Dennis & Schnabel (1983) the optimal choice of step-size for the central difference approximation is

$$h_p = \kappa^{1/3} \theta_p \tag{4.72}$$

where κ is a constant larger than the machine precision.

And the Hessian matrix, needed in Eq.(4.68), is not calculated using a the direct numerical evaluation of the second derivative of the objective function $S(\boldsymbol{\theta})$ in Eq.(4.69), since these calculations generally result in very poor approximations. Instead an updating formula for the Hessian (or the inverse Hessian) was implemented in Dekimo. Using a secant approximation to the Hessian yields a very robust optimization procedure (Bilbo, 1992). The secant method is also classified as the quasi-Newton method, because basically the procedure is a modified Newton-Raphson method. The most successful quasi-Newton method seems to be the BFGS method for iterative Hessian approximation combined with so called soft line search (Dennis & Schnabel, 1983). The soft line search secures that the procedure always takes a step that decreases $S(\boldsymbol{\theta})$. Furthermore the soft line search ensures that the Hessian matrix ($\boldsymbol{H}$) has a positive definite solution for the next updating of the Hessian matrix (Dennis & Schnabel, 1983), which is a precondition for the functionality of the optimization routine. (See Madsen & Melgaard, 1991 for more information).

The estimation program, Dekimo, uses the quasi-Newton method as described above in optimizing of the maximum likelihood objective function.

4.6 Standard deviation of parameter estimates

Point estimates of microbial parameters are of little information by themselves. A parameter is never known with 100% precision, thus a knowledge of the uncertainty of the estimate is valuable. The most commonly used measure of the uncertainty of the parameter estimate is the standard deviation (SD or σ). If the parameters are estimated using a linearized form of the model, it is general difficult to assess the precision with which nonlinear parameters are determined. For example, when using the Lineweaver-Burk linearization together with linear regression analysis, estimates of the parameters $\boldsymbol{\beta}$ plus the corresponding standard deviations can be obtained. But $\boldsymbol{\beta}$ is not the parameter of interest since $\beta_1 = \frac{K_s}{k}$ and $\beta_2 = \frac{1}{k}$ (example from the Monod model). Estimates of σ for K_s and k are not easily obtained from β_1 and β_2 but it can be done by use of the equations given by Bard (1974).

Neither use of the trial and error method nor the simplex method in minimizing the chosen objective function provide estimates of the standard deviations. However, it is possible by use of likelihood inference results in Bates & Watts (1988) (p.6 or marginal confidence intervals p.22) to obtain approximate standard deviations. The method requires many additional function evaluations. (see also Nelder & Mead, 1965).

Standard deviations of the parameter estimates can be estimated from a single experiment or from more experiments. In this study a standard deviation estimated from a single experiment, will be call a "standard deviation within experiment" (σ_{we}). A standard deviation estimated from three experiments within the same run, is called "standard deviation within run" (σ_{wr}), and finally a standard deviation estimated from all the experiments is termed "standard deviation between runs" (σ_{br}). In the following the estimation of σ_{we} will be discussed. There are basically two methods of estimating σ_{we}. One way is by approximating the nonlinear model $\boldsymbol{f}(t_i, \boldsymbol{\theta})$ to a linear function in the area of the optimal parameter estimates, and then estimate the standard deviations of the parameter estimates like in a normal linear case. The nonlinear model can be approximated by a first order Taylor series.

$$\boldsymbol{f}(t_i,\boldsymbol{\theta}) = \boldsymbol{f}(t_i,\boldsymbol{\theta}^0) + \left.\frac{\partial \boldsymbol{f}(t_i,\boldsymbol{\theta})}{\partial \boldsymbol{\theta}^\top}\right|_{\theta=\theta^0} \cdot (\boldsymbol{\theta} - \boldsymbol{\theta}^0) \tag{4.73}$$

If we consider this as a linear model with $\boldsymbol{\theta}$ as the parameters, $\boldsymbol{\theta}^0$ and $\boldsymbol{f}(t_i,\boldsymbol{\theta}^0)$ as constants, and $\frac{\partial \boldsymbol{f}}{\partial \boldsymbol{\theta}^\top}|_{\theta=\theta^0} = \boldsymbol{X}$ as the variables, then the standard deviation for the parameter estimate can be calculated as (Robinson, 1985)

$$\begin{aligned}\hat{\sigma}_{we} &= \sqrt{V(\hat{\boldsymbol{\theta}})} = \sqrt{\hat{\sigma}_\varepsilon^2 \cdot (\boldsymbol{X}^\top \boldsymbol{X})^{-1}} \\ &\simeq \hat{\sigma}_\varepsilon \cdot \sqrt{\left(\left[\frac{\partial \boldsymbol{f}(t_i,\boldsymbol{\theta})}{\partial \boldsymbol{\theta}^\top} \right]^\top \left[\frac{\partial \boldsymbol{f}(t_i,\boldsymbol{\theta})}{\partial \boldsymbol{\theta}^\top} \right] \right)^{-1}} \end{aligned} \tag{4.74}$$

where σ_ε^2 is the variance connected with the measurement error.

The calculation of σ_{we} using Eq.(4.74) is statistically optimistic. The inaccuracy in using Eq.(4.74) arises because a nonlinear model is treated as a linear model in the neighbourhood of the best parameter estimates. The degree of optimism, and hence the extend to which σ_{we} is underestimated, depends on how close the linear Taylor series expansion approximates the model near the minimum. Especially when models have a high degree of nonlinearity the method of finding the standard deviation by linear approximation can be rather unrealistic. A more correct way of estimating the standard deviation of the parameter estimates for nonlinear models is by use of the Hessian matrix. The Hessian matrix is a measurement of the curvature of the likelihood objective function. By use of the Cramer-Rao's equation an estimate of the standard deviation for parameter estimates within the experiment is given by Cox & Hinkley (1974)

$$\hat{\sigma}_{we} \simeq \hat{\sigma}_\varepsilon \cdot \sqrt{1/\left[\frac{\partial^2 \log L(\boldsymbol{\theta})}{\partial \boldsymbol{\theta}^2} \right]} \tag{4.75}$$

The standard deviations for parameter estimates "within the runs" σ_{wr} and "between the runs" σ_{br} were estimated differently. Ordinary analysis of variance was employed as illustrated in the following.

$$\hat{\sigma}_{wr} = \sqrt{\frac{\sum_{j=1}^r \sum_{i=1}^s (x_{ij} - x_{.j})^2}{r\ (s-1)}} \tag{4.76}$$

$$\hat{\sigma}_{br} = \sqrt{\frac{\sum_{j=1}^r (x_{.j} - x_{..})^2}{r-1}} \tag{4.77}$$

where i and j here are indices for experiments within a run and for runs, respectively. s is the number of experiments within a run, and r is the number of runs. x_{ij} are parameter estimates, $x_{.j}$ are average values of parameter estimates belonging to the same run, and $x_{..}$ is the total average value for all estimates of the parameter under considerations.

The total standard deviation σ_{total} for each parameter estimate can be calculated from the following equation. The total variance can be split up into variance within runs and variance between runs.

$$\hat{\sigma}_{total}^2 = \frac{r(s-1)\ \hat{\sigma}_{wr}^2 +\ (r-1)\ \hat{\sigma}_{br}^2}{rs-1} \tag{4.78}$$

When parameter estimates are highly correlated, standard deviations are not always enough to characterize the uncertainty of the parameter estimates. It is also necessary to know the degree of correlation between the estimates. Thus, the parameter correlation matrix should be calculated. This can be done by use of the normalized inverse Hessian matrix (Bard, 1974).

$$Corr(\hat{\boldsymbol{\theta}}) = \begin{pmatrix} 1 & \rho_{12} & \rho_{13} & \cdot & \rho_{1P} \\ \rho_{21} & 1 & & & \\ \cdot & & 1 & & \\ \cdot & & & 1 & \\ \rho_{P1} & & & & 1 \end{pmatrix} \simeq \boldsymbol{H}^{-1} \tag{4.79}$$

The program Dekimo estimates the parameter correlation matrix by this method. The correlation matrix is symmetrically squared with dimensions set by the number of parameters of the nonlinear model. The elements range from -1 to +1 and the elements along the main diagonal are all ones, since any parameter is perfectly self-correlates. Elements outside the main diagonal constitute correlations for all pairwise combinations of the parameters. In this context, a high correlation (either positive or negative) is undesirable. The correlation depends on 1) the values of the independent variable (here t_i) chosen at which to measure the dependent variable (the responses y_{ij}) and 2) the nature of the nonlinear model itself. An optimal experiment design can minimize the correlation due to the chosen values of the independent variable (e.g. when to take a sample). In chapter 6 optimal designs are discussed. However, little can be done with the correlation due to the nature of the model. The Monod model or similar models are of such a nature that the parameter estimates have a high degree of correlation.

4.7 Residual analysis

Residual analysis is used to verify the appropriateness of assumptions made about 1) the model, and 2) the measurement errors. If the assumptions appear to be strongly violated, then the fitted model must be modified and the analysis (the fitting of a new model, estimation of parameters, analysis of residuals) continues until a satisfactory result is obtained. A new model could either be one with a new error structure or a new "biological" model ($f(t, \boldsymbol{\theta})$). Several techniques for examining residuals exist (Draper & Schnabel, 1981). Residuals are estimated measurement errors, $\hat{\boldsymbol{\varepsilon}} = \boldsymbol{r}$

$$\begin{aligned} \boldsymbol{y} &= \boldsymbol{f}(t, \boldsymbol{\theta}) + \boldsymbol{\varepsilon} \\ \boldsymbol{r} &= \boldsymbol{f}(t, \hat{\boldsymbol{\theta}}) - \boldsymbol{y} \end{aligned} \tag{4.80}$$

where $\boldsymbol{y}$ is the observed response vector and $\boldsymbol{f}(t, \boldsymbol{\theta})$ is the predicted response vector also referred to as the expectation function. Since the predicted values and thereby the residuals are calculated from the model under consideration, the residuals are depending on the precision of all the parameter estimates and on the correctness of the choice of model.

When a data set includes repeated measurements, it is possible to perform tests for lack of fit of the chosen degradation model. Such analyses are based on an analysis of variance in which the value of the objective function, $S(\hat{\boldsymbol{\theta}})$ is decomposed into the "replication", S_r (contribution to objective function of deviations of the replicated observations about their averages), and the "lack of fit", $S_l = S(\hat{\boldsymbol{\theta}}) - (S_r)$. In Fig. 4.14 this idea is illustrated. The average value of the repeated measurements $\bar{S}_{obs.}$ is compared to the predicted value given by the model $\boldsymbol{f}(x, \boldsymbol{\theta})$. To obtain a measurement of how well or ill the data fit the model, the following test can be carried out. The ratio of the lack of fit over the replication divided by their respective degrees of freedom is compared to an F-distribution.

$$\frac{S_l/f_l}{S_r/f_r} \sim F(f_l, f_r; \alpha) \tag{4.81}$$

where α is the significance level, and f_l and f_r are degrees of freedom, f_l is calculated as $f_l = n - P - f_r$, where n is the total number of observations and P is the total number of parameters. If α is less than 5% the lack of fit is considered significant, thus the chosen model is not appropriate for describing the given set of data (consult Bates & Watts (1988) for more information).

The data set rarely includes enough repeated measurements to carry out the lack of fit test. Instead the residuals $\boldsymbol{r}$ can be plotted against the dependent variable, $\boldsymbol{y}$, and against the independent variable, $\boldsymbol{x}$ (in this case time), which also provides a great deal of information. In Fig. 4.15 the most frequently occurring plots of residuals against the depending variable are illustrated.

Figure 4.15 a) shows independent equally distributed residuals with a common mean of zero. It is a homogeneous error structure. For this error structure an ordinary least squares function could be employed as objec-

Figure 4.14: Lack of fit

tive function. Figure 4.15 b) shows a relation between the residuals and the dependent variable (the response). The mean is constant zero, but the variance increases with the depending variable $\boldsymbol{y}_{pred} = f(\boldsymbol{x}, \hat{\boldsymbol{\theta}})$. This structure is often seen for degradation experiments, where the responses cover a wide range. The variance structure for the errors can then be described by $V(\boldsymbol{\varepsilon}) = \sigma_\varepsilon^2 \boldsymbol{\Sigma}_\varepsilon$, where $\boldsymbol{\Sigma}_\varepsilon$ is a diagonal matrix with increasing elements corresponding to the structure of the variance. Weighted least squares (WLS), maximum likelihood (ML), or similar functions should be employed when such a residual structure appears.

If correlation between the residuals show up as a trend in the residual plot, (Fig. 4.15 c)) it can be due to errors in the analysis (e.g. if the regression is forced on false conditions through zero). It could also result from the fact that the residuals are not completely independent of each other. This situation is best shown for a linear model $f(x_i, \boldsymbol{\theta}) = x_i \boldsymbol{\theta}$

$$y_i = x_i \boldsymbol{\theta} + \varepsilon_i \tag{4.82}$$

Assume that $\boldsymbol{\varepsilon} \in N(\boldsymbol{0}, \sigma^2 \boldsymbol{I})$ thus the least squares estimator is

$$\hat{\boldsymbol{\theta}} = (\boldsymbol{x}^\top \boldsymbol{x})^{-1} \boldsymbol{x}^\top \boldsymbol{y} \tag{4.83}$$

Figure 4.15: Frequently occuring residual plots

The residual vector is

$$\boldsymbol{r} = \boldsymbol{y} - \boldsymbol{x}\hat{\boldsymbol{\theta}} \tag{4.84}$$

$$\Updownarrow$$

$$\boldsymbol{r} = [\boldsymbol{I} - \boldsymbol{x}(\ \boldsymbol{x}^\top \boldsymbol{x})^{-1} \boldsymbol{x}^\top] \boldsymbol{y} \tag{4.85}$$

The covariance matrix for the residuals, also called the dispersion matrix is

$$Cov(r_i, r_j) = D(r) = \sigma_\varepsilon^2\ [\boldsymbol{I} - \boldsymbol{x}(\boldsymbol{x}^\top \boldsymbol{x})^{-1} \boldsymbol{x}] \tag{4.86}$$

From this matrix the correlation matrix can be calculated, which can show that the residuals are not always uncorrelated.

In Fig. 4.15 d) the correlation between the residuals is systematic, which can result from fitting a wrong model to the data. The chosen model may not be able to describe the process of the experiment. Care should be taken in drawing such conclusions since the systematic errors also may be a result of incorrect assumptions about the variance structure. A large number of repeated measurements is necessary in order to distinguish between the two possible causes.

In the following, residual plots for the experiments in this study are shown together with a 95% confidence interval calculated from the assumed variance structure given in section 4.3. The residual plots (Fig. 4.16, 4.17, 4.18, 4.19, and 4.20) correspond to the plot in Fig. 4.15 b). The variance increases with increasing response level, as expected. The columns of data seen in most of the plots are due to a lag phase or a starvation phase where the concentration levels were constant.

Figure 4.16: Residual plot for the toluene measurements in the single substrate system.

Recalling the symbols and indices given in section 4.1 the j^{th} error at the time instant t_i is called $\varepsilon_{i,j}$. Its variance is denoted $\sigma^2_{\varepsilon,i,j}$. The vector of parameters of the model $\boldsymbol{f}(t_i, \boldsymbol{\theta})$. Recalling the assumed variance structure for the responses (given in section 4.3.1), the structure for the substrates was given by $\sigma^2_{\varepsilon,i,j} = \sigma^2_\varepsilon \cdot \boldsymbol{f}(t_i, \boldsymbol{\theta})$ and for the biomass in the single substrate

Figure 4.17: Residual plot for the biomass measurements in the single substrate system.

Figure 4.18: Residual plot for the benzene measurements in the dual substrate system.

system by $\sigma^2_{\varepsilon,i,j} = \sigma^2_\varepsilon \cdot 9\sqrt{\boldsymbol{f}(t_i, \boldsymbol{\theta})}$ and in the dual substrate system by $\sigma^2_{\varepsilon,i,j} = \sigma^2_\varepsilon \cdot 9\boldsymbol{f}(t_i, \boldsymbol{\theta})$. In order to estimates the 95% confidence intervals the values of σ_ε were needed. The values (one for the single and one for

Figure 4.19: Residual plot for the toluene measurements in the dual substrate system.

Figure 4.20: Residual plot for the biomass measurements in the dual substrate system.

the dual substrate system) were obtained by taking the average values of σ_ε estimated by the program Dekimo (single sub. sys.: $\bar{\sigma}_\varepsilon = 0.132$ and dual sub. sys.: $\bar{\sigma}_\varepsilon = 0.083$). The 95% confidence interval for the residuals with

a mean of zero are thus given by:

$$\text{generally} \quad 0 \quad \pm\, \sigma_{\varepsilon,i,j} \cdot t(n-p)_{1-5\%/2} \tag{4.87}$$

$$\text{substrates :} \quad 0 \quad \pm\, \sigma_\varepsilon \cdot \boldsymbol{f}(t,\boldsymbol{\theta}) \cdot t(n-p)_{1-5\%/2} \tag{4.88}$$

$$\text{biomass, single sub. :} \quad 0 \quad \pm\, \sigma_\varepsilon \cdot 9\sqrt{\boldsymbol{f}(t,\boldsymbol{\theta})} \cdot t(n-p)_{1-5\%/2} \tag{4.89}$$

$$\text{biomass, dual sub. :} \quad 0 \quad \pm\, \sigma_\varepsilon \cdot 9\boldsymbol{f}(t,\boldsymbol{\theta}) \cdot t(n-p)_{1-5\%/2} \tag{4.90}$$

where n is the total number of observations in the system (single/dual) and p is the number of parameters in the model under consideration.

Outside the 95% confidence intervals 5% of the data should ideally be found. When calculating the percentage of data outside the intervals for the five plots the following results are obtained.

Single sub. sys.	residuals for toluene	4.3% outside
	residuals for biomass	8.3% outside
Dual sub. sys.	residuals for benzene	3.2% outside
	residuals for toluene	6.0 % outside
	residuals for biomass	6.7 % outside

The residuals seem to be reasonably equally distributed above and under the abscissa. This fact together with the acceptable percentage of data outside the 95% confidence interval, indicate that the models (Monod and Bailey & Ollis + the assumption on the error structure) were appropriate for fitting the data.

Chapter 5

Testing

After fitting a model to the experimental data sets, there is a need for summarizing the inferential results. Can some of the parameters for example have a certain value? Can the model under consideration be reduced? Can some or all of the parameter values, estimated from different data sets, take the same values? It could e.g. be of interest to test whether:

- some of the parameters can take values obtained from similar experiments reported in the literature,
- some of the parameters in a model can be said to have the same value e.g. for the yield constants for benzene and toluene,
- the model under consideration can be reduced by setting a parameter value equal to zero and thereby obtaining a simplified model,
- different sets of parameter values estimated from identical experiments can be said to have a common set of parameter values.

Concerning the experiments in this study, special interest was on testing common sets of parameter estimates in order to examine reproducibility.

5.1 Likelihood ratio test

When testing these hypothesis the likelihood ratio test is employed. Consider the problem of testing one of the hypothesis mentioned above (referred to as the null hypothesis H_0) against the alternative H_1

$$H_0 : \boldsymbol{\theta}_0 \in \Omega_0 \subset \Omega \quad \text{against} \quad H_1 : \boldsymbol{\theta}_1 \in \Omega \tag{5.1}$$

where Ω is the parameter space in the unrestricted case and Ω_0 is the parameter space in the restricted case. If the maximum of the likelihood function under the hypothesis H_0 is denoted by $L(\boldsymbol{\theta}_0)$ and the maximum of the likelihood function in the unrestricted case (H_1) denoted by $L(\boldsymbol{\theta}_1)$, then the ratio between the two likelihoods is:

$$\lambda = \frac{L(\boldsymbol{\theta}_0)}{L(\boldsymbol{\theta}_1)} \tag{5.2}$$

The distribution of $-2\log\lambda$ can be used for setting up a test statistic. Under the hypothesis H_0, the following holds asymptotically for large sample sizes. The multivariate case requires larger sample sizes than in the univariate case, before results can be trusted (Gallant, 1987) .

$$-2\log\lambda \in \chi^2(r)_{1-\alpha} \tag{5.3}$$

The number of degree of freedom in the $\chi^2(r)$ distribution is equal to the number of restrictions, r, imposed on the parameters under the null hypothesis compared to the number of parameters under the H_1 hypothesis. In other words, r is equal to the number of reduced parameters. α is the level of significance and $\chi^2(r)_{1-\alpha}$ denotes the $1-\alpha$ quantile of the χ^2-distribution. The greater the $-2\log\lambda$, the less we are inclined to accept the null hypothesis. The decision rule is; reject H_0 if $-2\log\lambda > \chi^2(r)_{1-0.05}$.

The distribution of $-2\log\lambda$ is only approximatively χ^2- distributed. In a few cases it is, however, possible to derive the exact distribution of the test quantity. For example, if the hypothesis $H_0 : \beta = 0$ is tested against $H_1 : \beta \neq 0$ in a general linear model $Y = X\beta + \varepsilon$, where the errors ε are independent, identically normally distributed (univariate), the likelihood ratio test can be rewritten to an exact F-test, and the following test is employed.

$$F = \frac{(n-p)(S(\theta_0) - S(\theta_1))}{r \cdot S(\theta_1)} > F(\alpha) \tag{5.4}$$

where S is the sum of squares under the H_0 and the H_1 hypothesis, respectively. n is the number of observations, p is the number of parameters, and r is the reduced number of parameters. In the multiresponse case with m responses, Gallant (1987) suggests that the number of degrees of freedom in the denominator should be $(nm - p)$. A conservative choice of $n - p$ may be more appropriate, as mentioned in Bilbo (1992) . The test (Eq. (5.4)) however, is not used in this study. The approximative likelihood ratio test is the nonlinear analogue to the F test (Beck & Arnold, 1977).

The log likelihood value itself, does not provide any information on how well the model fits the data. Only when compared to an alternative log likelihood value obtained from an alternative fit to the same data set, the magnitude of the log likelihood values has a meaning. The log likelihood value and the ratio test value depend on the choice of error structure. In some cases a test hypothesis is rejected with one error structure but accepted with another. Thus, it is important to determine the "true" error structure.

5.2 Test statistic in practice

In the following, it is shown how the likelihood ratio test is used in this study. First a couple of examples are given where one or more of the parameter values are fixed. Then an example of model reduction and finally the test method for reproducibility is illustrated.

5.2.1 Fixed parameter value

The following example is given to illustrate the test for, whether some of the parameter values can take specific values e.g. obtained from similar (or nearly similar) experiments reported in the literature. Two estimations were performed. One where the yield constant Y was fixed equal to 0.5 mg biomass/mg substrate, and another where all the parameters could assume any values. The likelihood values of the estimates are then compared. Consider an estimation in the Monod model for Run I, batch A, described in chapter 2.

$$H_0 : \boldsymbol{\theta}_0 = \begin{pmatrix} k \\ K_s \\ 0.5 \\ b \end{pmatrix} \qquad H_1 : \boldsymbol{\theta}_1 = \begin{pmatrix} k \\ K_s \\ Y \\ b \end{pmatrix} \tag{5.5}$$

The likelihood ratio test yields

$$\begin{aligned} -2 \log L(\boldsymbol{\theta}_0)/L(\boldsymbol{\theta}_1) &= -2\,(\log L(\boldsymbol{\theta}_0) - \log L(\boldsymbol{\theta}_1)) = \\ &-2\,(9.37 - 6.49) = 5.76 \;\; > \;\; \chi^2(1)_{1-0.05} \end{aligned} \tag{5.6}$$

The H_0 hypothesis cannot be accepted at a 5% significance level, since the test value = 5.76 were larger than the χ^2-value = 3.84. If two parameters were fixed the degree of freedom in the χ^2-distribution would equal 2. In the case of three fixed parameters, r would equal 3, etc.

An experiment from the present study (Run VI, batch A) is used in illustrating the test for whether some of the parameters in a model can be assumed to have identical values. In the example, a test is carried out to examine whether the value of the yield coefficient for benzene, Y_b could be assumed to equal the yield coefficient for toluene, Y_t.

$$H_0 : \boldsymbol{\theta}_0 = \begin{pmatrix} k_b \\ K_{sb} \\ Y \\ z_b \\ k_t \\ K_{st} \\ Y \\ z_t \\ b \end{pmatrix} \qquad H_1 : \boldsymbol{\theta}_1 = \begin{pmatrix} k_b \\ K_{sb} \\ Y_b \\ z_b \\ k_t \\ K_{st} \\ Y_t \\ z_t \\ b \end{pmatrix} \tag{5.7}$$

The likelihood ratio test yields

$$\begin{aligned} &-2\,(\log L(\boldsymbol{\theta}_0) - \log L(\boldsymbol{\theta}_1)) = \\ &\qquad -2\,(0.01 - 5.34) = 10.66 \;\; > \;\; \chi^2(1)_{1-0.05} = 3.84 \end{aligned} \tag{5.8}$$

The test hypothesis is rejected. The degree of freedom in the χ^2-distribution is 1, since there was one parameter less to estimate under the null hypothesis (Y instead of Y_b and Y_t), compared to the full model under the alternative hypothesis H_1. Thus the yield constant for benzene and toluene cannot be considered identical in this study.

5.2.2 Model reduction

In the test procedure for model reduction the parameter which is excluded from the model, is set to zero under the null hypothesis. The analysis is set up and calculated exactly in the same manner as the examination of fixed values for some of the parameters (Eq. (5.5) and Eq. (5.6)). An example is given here with the Monod model (Run II, batch A). The model was reduced by setting the decay coefficient b equal to zero.

$$H_0 : \boldsymbol{\theta}_0 = \begin{pmatrix} k \\ K_s \\ Y \\ 0 \end{pmatrix} \qquad H_1 : \boldsymbol{\theta}_1 = \begin{pmatrix} k \\ K_s \\ Y \\ b \end{pmatrix} \tag{5.9}$$

The likelihood value for the full model is denoted by $L(\boldsymbol{\theta}_0)$ and for the reduced model by $L(\boldsymbol{\theta}_1)$. The likelihood ratio test yields

$$\begin{aligned} &-2\,(\log L(\boldsymbol{\theta}_0) - \log L(\boldsymbol{\theta}_1)) \;=\; \\ &\qquad -2\,(-44.49 + 44.11) \;=\; 0.76 \;\; < \;\; \chi^2(1)_{1-0.05} = 3.84 \end{aligned} \tag{5.10}$$

In the classic analysis of variance the result of model reduction is often given in a table as shown in Table 5.1.

Model	$S(\boldsymbol{\theta})$	Test value	$\chi^2(1)$	Accept hypothesis ?
Full model	-44.11	0.76	3.84	Yes
Reduced model	-44.49			

Table 5.1: Scheme for test of model reduction.

Since the reduced model is not "an orthogonal submodel" of the full model, the parameter values (k, K_s, Y) changed when b was removed from the full model. New parameter values were therefor estimated.

By use of the 95% confidence interval for the parameter estimates, the investigator gets an idea of which parameters could be excluded from the model. A rough estimate of the 95% confidence interval was obtained by $\pm 2\cdot$ standard deviation. If the confidence interval includes zero, the parameter under consideration may be excluded. In nonlinear models it is not sufficient to look only at the parameter confidence intervals, since these are more or less skewed in distribution (Bates & Watts, 1988). A comparison of the log likelihood values using the likelihood ratio test is a more correct way of examining a model reduction.

In some cases the order in which the parameters are tested (for being equal to zero) has an influence on the result. If e.g. neither of the two parameter estimates are highly significant equal zero and if they are mutually correlated, the order in which they are tested may have an influence on the result. Usually the first parameter estimate which is tested has a larger chance of being accepted as equal to zero, than if this parameter was tested after that the other was accepted as being equal to zero. (Consult Montgomery, 1991 for more information on the subject).

Another well known test used for the examination of significant parameter values, is the t-test. The hypothesis $H_0 : \theta_p = 0$ against $H_1 : \theta_p \neq 0$ can be tested by using the statistic

$$T = \frac{\theta_p - 0}{\sqrt{\hat{\sigma}^2_{\theta_p}}} \tag{5.11}$$

which is compared to a t-distribution with $n - P$ degrees of freedom. The variances of the parameter estimates, $\hat{\sigma}^2_{\theta_p}$, $p = 1, \cdots, P$, are obtained from the estimation procedure by the program, Dekimo (see chapter 4). The test is also performed in Dekimo. It is, however, not a very reliable test of the H_0-hypothesis (Carstensen, 1994). Bates & Watts (1988) recommend that the likelihood ratio test be used in nonlinear cases, since it is less affected by the nonlinearity than the t test.

5.2.3 Reproducibility

Examination of reproducibility involves a test where log likelihood values from several experiments are compared. In principle the test statistic is the same as the test used in the above given examples, where only one experiment was considered at a time. The testing falls in two parts. 1) test for common sets of parameter estimates for experiments within the same run (within a block), and 2) test for common parameter estimates for all experiments (also between the blocks). If both 1) and 2) is accepted we will claim the experiment to be reproducible.

In the single substrate system 9 experiments were used in examining reproducibility, and in the dual substrate system 12 experiments were used.

Single substrate system		Dual substrate system	
Run I	Batch A Batch B Batch C	Run IV	Batch A Batch B Batch C
Run II	Batch A Batch B Batch C	Run V	Batch A Batch B Batch C
Run III	Batch A Batch B Batch C	Run VI	Batch A Batch B Batch C
		Run VII	Batch A Batch B Batch C

Table 5.2: Experiments carried out in the single substrate system.

In the classical analysis of variance, the setup shown in Table 5.2 could be characterized as a random effect model since there are no systematic effects. If the biomasses in the various runs were taken from different cultures of bacteria, the model could have been considered a deterministic (systematic) model. Our model is, however, somewhat different from the classical analysis of variance model since batch A, B, and C are complete experiments in themselves and not just single measurement as in the classical analysis of variance. The methods of the classical analysis of variance can therefore not be fully implemented, only the general idea. The test procedure always begins with the full model. In this case it means a null hypothesis that only restricts parameter values within the runs. The estimated parameter vectors for batch A, B, and C are assumed to be the same under the null hypothesis $H_0 : \boldsymbol{\theta}_A = \boldsymbol{\theta}_B = \boldsymbol{\theta}_C$. The hypothesis can be written as:

$$H_0 : \boldsymbol{\theta}_0 = \begin{pmatrix} \boldsymbol{\theta}_A \\ \\ \boldsymbol{\theta}_B \\ \\ \boldsymbol{\theta}_C \end{pmatrix} = \begin{pmatrix} k \\ K_s \\ Y \\ k \\ K_s \\ Y \\ k \\ K_s \\ Y \end{pmatrix} \qquad H_1 : \boldsymbol{\theta}_1 = \begin{pmatrix} k_A \\ K_{sA} \\ Y_A \\ k_B \\ K_{sB} \\ Y_B \\ k_C \\ K_{sC} \\ Y_C \end{pmatrix} \tag{5.12}$$

where A, B, and C are three batches within the same run. The $\log L(\boldsymbol{\theta}_0)$ value is given by summarizing the log likelihood values estimated for common sets of parameter values for each run. The $\log L(\boldsymbol{\theta}_1)$ value is given by summarizing the log likelihood values estimated for the individual estimations for all batch experiments. Twelve estimations using the program Dekimo were carried out in the single substrate system, and fifteen in the dual substrate system. The likelihood ratio test for common sets of parameter values within the runs is given by

$$-2\,(\sum_{j=1}^{r} \log L_{0,j}(\boldsymbol{\theta})_{(within)} - \sum_{i=1}^{rs} \log L_{1,i}(\boldsymbol{\theta})_{(individual)}\,) \tag{5.13}$$
$$\text{compared to} \quad \chi^2\,(p\,(rs-r))_{1-0.05}$$

where i and j here indicate experiments and runs, respectively. s is the number of experiments within a run, r is the number of runs, and p is the number of parameters in the model. If the hypothesis is accepted, this will be our new model consisting of three/four common estimations from the three/four runs. We will now test if these common sets of parameter estimates can be said to have the same values for all the runs. For the single

substrate system the hypothesis for a common set of parameter values between runs is given by

$$H_0 : \boldsymbol{\theta}_0 = \begin{pmatrix} \boldsymbol{\theta}_I \\ \\ \boldsymbol{\theta}_{II} \\ \\ \boldsymbol{\theta}_{III} \end{pmatrix} = \begin{pmatrix} k \\ K_s \\ Y \\ k \\ K_s \\ Y \\ k \\ K_s \\ Y \end{pmatrix} \qquad H_1 : \boldsymbol{\theta}_1 = \begin{pmatrix} k_I \\ K_{sI} \\ Y_I \\ k_{II} \\ K_{sII} \\ Y_{II} \\ k_{III} \\ K_{sIII} \\ Y_{III} \end{pmatrix} \tag{5.14}$$

The $\log L(\boldsymbol{\theta}_0)$ value is obtained from the estimation procedure for a common set of parameter values for all experiments within the system. The $\log L(\boldsymbol{\theta}_1)$ value is obtained by summarizing the log likelihood values estimated for common sets of parameter values for each run. The likelihood ratio test for a common set of parameter values for all experiments within the single or the dual substrate system is given by:

$$-2(\log L_0(\boldsymbol{\theta})_{(total)} - \sum_{j=1}^{r} \log L_{1,j}(\boldsymbol{\theta})_{(within)}\,) \tag{5.15}$$
$$\text{compared to} \quad \chi^2\,(p\,(r-1))_{1-0.05}$$

Due to program limitations in Dekimo, the latter test cannot be performed directly since it is not possible at present to estimate a common set of parameter values for more than 3 experiments. Instead of estimating a common set of parameter values for all experiments ($\log L(\boldsymbol{\theta})_{(total)}$), the test procedure has been split into three/four common estimations for experiments from three different runs ($\log L(\boldsymbol{\theta})_{(between)}$).

<u>For the single substrate system</u>
a) batch A Run I, batch A Run II, and batch A Run III
b) batch B Run I, batch B Run II, and batch B Run III
c) batch C Run I, batch C Run II, and batch C Run III

<u>For the dual substrate system</u>
a) batch A Run IV, batch A Run V, batch A Run VI
b) batch A Run VII, batch B Run IV, batch B Run V
c) batch B Run VI, batch B Run VII, batch C Run IV
d) batch C Run V, batch C Run VI, batch C Run VII

There is no special reason for comparing the batches in the order shown above. One batch experiment from each run is in principle chosen randomly. The likelihood ratio test for the new situation becomes

$$-2\left(\sum_{u=1}^{k} \log L_{0,u}(\boldsymbol{\theta})_{(between)} - \sum_{i=1}^{rs} \log L_{1,i}(\boldsymbol{\theta})_{(individual)}\right) \tag{5.16}$$
$$\text{compared to} \quad \chi^2\left(p\left(rs-k\right)\right)_{1-0.05}$$

where u is the index for test setup shown above (a), b), c), ..) and $\log L_{0,u}(\boldsymbol{\theta})_{(between)}$ is the log likelihood values for common estimations between runs. These test procedures are used in chapter 8.

Chapter 6

Designing experiments

Investigators should seek to design experiments which maximize the quality of information that can be extracted from data. Obtaining the best possible experiments involve several considerations before performing the experiments. In any experiment, the results and conclusions that can be drawn depend to a large extent on the manner in which the data were collected. Optimally designed experiments are therefore desirable since they provide the highest quality of information for a given expenditure of resources. In this chapter a discussion is given on designing experiments along with descriptions and explanations of what is done in connection with the experiments performed in this study. When and where to sample in the Monod model is also examined by use of D-optimal sampling and by use of sensitivity equations.

6.1 Objective of an experiment

The definition of an optimally designed experiment depends on the investigator's goal. Depending on the purpose different designs are employed, however, some of the purposes can be obtained in the same design. The goal of the investigator could be to

- determine the lack of fit of a certain model
- discriminate between competing models
- obtain good parameter estimates with minimum variance and minimum correlation (small and uniform confidence region)
- to test different treatments or factor effects on the experiment
- to test the variability of the experiment, etc.

Lack of fit
In determining the lack of fit for the chosen model, the data should include as many repeated measurements as possible. The analysis for testing the lack of fit is given in chapter 4, section 4.7.

Competing models
When considering different appropriate models to describe the biological system under consideration, a good idea is usually to look in the literature for earlier results and experiments on the subject. If there are several appropriate models to chose between, one should always start out with the simplest model with the fewest number of parameters, least correlated, and with the fewest number of non-physical parameters. When setting up the experiment to be conducted, the experimental conditions that maximize the differences among the competing models should be chosen. For example when examine competitive inhibition versus simultaneous utilization, the experiment should be performed with the combination of initial substrate concentration that gives rise to the largest different between the degradation models (Fig. 6.1). Fedorov (1972) and Beck & Arnold (1977) describes criteria for model discrimination.

Figure 6.1: Revealing differences between two models. a) competitive model with inhibition and b) simultaneously utilization model without inhibition.

Good parameter estimates

If the main objective of the experiment is to obtain good estimates of the parameters in a certain model, the researcher will be concerned about 1) which chemical analysis method to use in order to obtain good measurements, 2) how many samples to take and when to take them, 3) any replicates of the measurements, and 4) which estimation method to employ.

The first question is dealing with laboratory and chemical technique and the best method depends on what is measured. The second is related to choosing optimal design points. Box & Lucas (1959) have described an optimal design criterion for nonlinear models. In section 6.4 a more detailed description and specific examination on the optimal sampling points for the models in this study is found. Item 3) is of great important when examining the variance structure. No matter which method is used to estimate the parameter values, they all rely on some assumption of the variance structure. An incorrect assumption on the variance structure can have great influence on the modelling and thereby on the estimated parameter values (see chapter 4).

The precision and accuracy of the parameter estimates depend on the estimation method (item 4). Parameters in microbiologic degradation models are mostly estimated by linearization and by using parameter values from similar experiments found in the literature (Vecht et al., 1988; Folsom et al., 1990; Strand et al, 1990; Machado & Grady, 1988; Alvarez et al., 1991). If nonlinear estimation methods are employed it is usually only for a few parameters. The linearization method can give rise to incorrect parameter estimates, and when employing parameter values from the literature, one shall be aware of, that the parameter values are depending on the system in which they are estimated. Nonlinear estimation methods should always be employed when dealing with nonlinear microbiological degradation models, as described in more detail in chapter 4.

Blocking

When testing different factors effect on the experiment or when non-homogeneous conditions appear, it is necessary to use a blocking technique. Blocking is a technique used to increase the precision of an experiment. A block is a portion of the experiment which is considered to be more homogeneous than the entire set of material/experiments. The blocking technique is often used when many experiments are carried out. There may also be limits on for example how many experiments it is possible to carry out on one day, how many experiments can be carried out using a given chemical mixture, etc. These restrictions also give rise to blocks. More information can usually be obtained from an experiment when using the blocking technique and the corresponding analysis. (Consult with Hicks (1982) or Box, Hunter & Hunter (1978) for more information on using the blocking technique). With the blocking technique it is possible to more precisely reveal factors that have an effect on the experiments.

Variability

In examining the variability of an experiment, replicated experiments are carried out to be as identical as possible. If any suspicions on inhomogeneous conditions exist, the blocking technique described above should be used.

Our study
The purpose of the experiments in this study was mainly to examine the variability/reproducibility of microbiological degradation experiments. In this context, replicates of the experiments were conducted and parameter estimates were compared. Because changes in the biomass from preculture to preculture can occur, it was necessary to use the blocking technique. This resulted in the design shown in Fig. 6.2. Experiments with biomass originating from the same preculture constituted a block (referred to as a run) and were carried out simultaneously.

Figure 6.2: Experimental design for the single substrate system

Also, there were restrictions on the number of batch experiments carried out simultaneously, since it was only possible to place 4 batches in the water tank (see the experimental set up in chapter 2). Thus, the 4 batches (A, B, C, plus a blank) constituted a block. The runs and the precultures were thus confounded.

6.2 Controllable/uncontrollable noise

The process in which an experiment is performed can be regarded as a combination of machines/instruments, methods, people, and other resources that have or may have influence on the results. Some of the process variables are controllable, whereas other variables are uncontrollable. As examples of controllable variables for the microbiological degradation experiments carried out in this study, the choice of the Lowry method for biomass measurements, Pseudomonas *cepacia* (biomass), and a constant temperature of 28°C can be mentioned. The uncontrollable variables can be known or unknown. For the uncontrollable known variables it is important to measure their variations during the experiment and verify afterwards if any effect on the results can be identified (e.g. duration times of the precultures). For the unknown variables the problem is larger - if experiments are to be compared they should be carried out in the same way such that the unknown variables are kept constant and thereby have the same magnitude of effect on the results of the experiments. This is the reason why the preparation of the biomass and the experiments themselves were carried out the same way every time. If the unknown and uncontrollable variable is related to the order in which the samples are measured, it is important to randomize the measuring in order to “average out” the effect of extraneous factors, that may be present. Statistical methods require that the observations (or measurement errors) are independently distributed random variables. To avoid too many unknown factors that have an influence on the results of the experiments, we have chosen to carry out relatively simple experiments.

6.3 Sequential design

After the investigator has defined which objective the experiments have, how many experiments to perform, which model to employ, and so on, it still leaves many practical questions, which can only be answered by performing experiments. For this reason screening experiments are carried out and often they are smaller than the "real" experiments. However, not everything can be predicted. As more information is gained when the experiment is carried out, this new information should be utilized in the next experiment and by that means improve the experimental results. Such a design is referred to as a sequential design. However, when examining the natural variability of the parameter estimates, the investigator is interested in obtaining as identical experiments as possible in order to compare these. Thus sequential designs should be used with caution when examining reproducibility. Details about sequential designs are given in Juusola, Bacon & Downie (1972) and in Ford, Kitsos & Titterington (1989).

As examples of sequential learning in the present study, we can mention 1) the achieving of more knowledge of the procedure for biomass measuring, which improved the precision (see chapter 2), and 2) the discovering of inhibition of the degradation and the biomass growth when the initial toluene concentration exceeded 10 mg/l (Run VIII and IX). The initial toluene concentration in the later experiments was thus carefully kept under 10 mg/l.

6.4 Optimal design and sampling

There are three important considerations when designing the optimal sampling procedure. 1) in what range should the initial substrate and biomass concentrations be chosen, 2) how many samples should be taken and 3) where/when shall the samples be taken. These questions are important for the parameters identification and for the precision of the parameter estimates.

6.4.1 What range

The range for the initial biomass concentration should be chosen not too small and not too large. If chosen too small, it can e.g. be difficult to distinguish between a lag phase and a small growth rate, and if chosen too large the total biomass concentration would not change considerably which would make the estimation of the yield coefficient Y difficult. Moreover, the biomass will flocculate if the density becomes too large, and thus the biomass cannot be considered totally homogeneous.

The range for the initial substrate concentration also is limited. If chosen too small, the degradation of the substrate will occur too fast to obtain enough samples, and if chosen too large the degradation may be inhibited (as seen in Run VIII and IX). If the model is of the Monod type, it is moreover a prerequisite that $S >> K_s$ for identification and estimation of k and K_s. It is important that the degradation sequence runs through 0. and 1. order (Eq.(6.1), (6.2), (6.3), (6.4)) in order to identify both k and K_s. In the 0. order sequence k is identified and in the 1. order sequence, it is the relationship between k and K_s that is determined. If the experiment only is performed in the 1. order sequence, K_s and k would be very strongly correlated and almost impossible to separate. And if there are only measurements in the 0. order sequence and non in the 1. order sequence, estimation becomes very difficult or impossible. In Run X the last part of the benzene degradation is missing due to analysis problems, which destroyed the samples. Thus the estimation of all parameters becomes impossible. It was necessary to fix some of the parameter values (see chapter 8)

The Monod model
For $S >> K_s$ 0. order rate will be

$$\frac{dS}{dt} = k\frac{SX}{S+K_s} \approx kX \tag{6.1}$$

$$\frac{dX}{dt} = Yk\frac{SX}{S+K_s} - bX \approx YkX - bX \tag{6.2}$$

For $S << K_s$ 1. order rate will be

$$\frac{dS}{dt} = \frac{SX}{S+K_s} \approx \frac{k}{K_s}SX \tag{6.3}$$
$$\frac{dX}{dt} = Yk\frac{SX}{S+K_s} - bX \approx Y\frac{k}{K_s}SX - bX \tag{6.4}$$

where
S is the substrate concentration,
X is the biomass concentration, and
t is the time.
The parameters are:
k the maximum degradation rate for toluene,
K_s the half-saturation coefficient,
Y the yield coefficient, and
b the decay coefficient.

As for the single substrate system correlations between the parameter estimates in the dual substrate system cannot totally be avoid, only reduced. With many more parameters and two (in stead of one) degradation equation, it becomes less obvious how the correlations between the parameter estimates can be reduced. However, the ratio between S and K_s should as in the single substrate system be as large as possible. Moreover, the two substrate initial concentrations should be of such magnitudes that they are not totally degraded at the same time, otherwise the correlation between the two yield coefficients for toluene and benzene, respectively (Y_t and Y_b) will be very strong and impossible to identify separately.

If the investigator is interested in revealing e.g. the first substrate inhibition on the second or vice versa, the ratio between the two substrates should vary as much as possible. In the present work this was not done for two reasons 1) in order to examine reproducibility it was important to carry out experiments as identical as possible and 2) if initial toluene concentration was over 10 mg/l, inhibition would occur, and if much less than actually used the degradation would occur to fast to obtain enough observations.

6.4.2 How many observations

The necessary number of samples to be taken in order to insure a certain precision of the parameter estimates is of interest. A technique to obtain this goal is illustrated in the following. Consider a model:

$$\boldsymbol{y}_i = \boldsymbol{f}(x_i, \boldsymbol{\theta}) + \varepsilon_i \qquad \begin{aligned} \varepsilon_i &= N(0, \sigma_\varepsilon^2) \\ i &= 1, ..., n \end{aligned} \tag{6.5}$$

where $\boldsymbol{y}_i$ is the dependent variable vector, x_i is the independent variable, $\boldsymbol{f}$ is a linear or nonlinear function of the parameters, $\boldsymbol{\theta}$ is the vector of the parameters, ε_i is the measurement error associated in the i'th observation, and n is the number of observations. In degradation models x_i is replaced with t_i which is the time of sampling, $\boldsymbol{f}$ consists of two (or three) mathematical expressions - one for the biomass and one (or more) for the substrate(s), and $\boldsymbol{y}_i$ is the measurement of the biomass or substrate(s).

For a linear model it is possible to determine the necessary number of samples. The optimum only depends on a priori information on the standard deviation and the decided significance level, α. The marginal confidence region for a parameter θ_p is given by:

$$\theta_p \pm t(n-P)_{\alpha/2} \cdot \sqrt{V(\theta_p)} \tag{6.6}$$

where P is the total number of parameters, $V(\theta_p)$ is the variance of the parameter p, and t is the t distribution with $(n-P)$ degrees of freedom. For a required minimum significance level the number of samples, n can be calculated for each parameter and the largest number n is picked in the design.

For a nonlinear model, Bates & Watts have given a marginal confidence region:

$$\theta_p \pm t(n-P)_{\alpha/2} \cdot \hat{\sigma}_\varepsilon / \sqrt{\tilde{S}(\hat{\boldsymbol{\theta}}_{-p}) - S(\hat{\boldsymbol{\theta}})} \tag{6.7}$$

where $\tilde{S}(\hat{\boldsymbol{\theta}}_{-p})$ is the sum of squares function for the parameter vector $\hat{\boldsymbol{\theta}}_{-p} = (\hat{\theta}_1, ..., \hat{\theta}_{p-1}, \hat{\theta}_{p+1}, ..., \hat{\theta}_P)$ and $S(\hat{\boldsymbol{\theta}})$ is the sum of squares for the full model with all P parameters $\hat{\boldsymbol{\theta}}$.

However, for nonlinear models that are formulated as differential equations, i.e. implicit equations, it is very difficult to determine sample sizes to assure a minimum of uncertainty on the parameter estimates (Bilbo 1992). Generally, it is not possible to express an exact rule defining an optimal number of sampling points in a degradation kinetic model based on nonlinear differential equations. As pointed out by Box & Lucas (1959), the variance of the parameter estimates depend on the design matrix, i.e. the matrix of values of the independent variables, and on the parameter values themselves. As a result, one has to resort to the experience gained during previous experiments. It seems obvious that the more samples the better identification of the parameters. This is in good agreement with experiments in this study. The substrate measurements in Run V were half the number (25) as for the similar dual substrate experiments in Run IV, VI, and VII, and the identification of the parameter values were corresponding more difficult for the small experiment.

6.4.3 When/where to sample

Selecting an appropriate sampling procedure is critical to successful analysis of any experiment. The procedure for microbiologic experiments is concerning at which point of time during the experiments the samples should be taken. For this purpose different methods can be employed. 1) optimal design criteria and 2) sensitivity equations.

Optimal design criteria
Consider the following. When the variance structure of the measurement errors are normal distributed, the covariance matrix of the parameter estimates $\hat{\boldsymbol{\theta}}$ is described by

$$V(\hat{\boldsymbol{\theta}}) = (\boldsymbol{f'}_\theta^\top \boldsymbol{f'}_\theta)^{-1} \hat{\sigma}_\varepsilon^2 \tag{6.8}$$

where

$$\boldsymbol{f'}_\theta = \boldsymbol{f'}_\theta(\boldsymbol{x}, \boldsymbol{\theta}) = \begin{pmatrix} \frac{\partial f_1(x,\boldsymbol{\theta})}{\partial \theta_1} & \cdots & \frac{\partial f_1(x,\boldsymbol{\theta})}{\partial \theta_P} \\ \vdots & & \\ \frac{\partial f_k(x,\boldsymbol{\theta})}{\partial \theta_1} & \cdots & \frac{\partial f_k(x,\boldsymbol{\theta})}{\partial \theta_P} \end{pmatrix} \tag{6.9}$$

$\boldsymbol{f'}_\theta$ is also called the sensitivity matrix or the Jacobian matrix. In a linear regression model the optimal design points only depend on the design variables such as when the samples are taken, at what temperature, at what pressure, etc. The sensitivity matrix $\boldsymbol{f'}_\theta$ becomes equal to $\boldsymbol{X}$ (matrix of the independent variables, x) and is thereby independent on the parameter values. A good experimental design will be one that makes the matrix $(\boldsymbol{f'}_\theta^\top \boldsymbol{f'}_\theta)^{-1}$ small in some sense. Several functionals have been suggested in the literature as measurements of "smallness". To mention a few :

1. D-optimal designs - This design method minimizes the determinant of the matrix $(\boldsymbol{f'}_\theta^\top \boldsymbol{f'}_\theta)^{-1}$ meaning that the volume of the confidence ellipsoid is minimized (Fig. 6.3). Both the variance and the correlation between the parameter estimates are effected by this optimization design.

2. A-optimal designs - This design method minimizes the trace of the matrix, $\mathrm{tr}(\boldsymbol{f'}_\theta^\top \boldsymbol{f'}_\theta)^{-1}$, meaning that the variances of the parameter estimates are minimized.

3. E-optimal designs - this design method minimizes the maximal eigenvalue of $(\boldsymbol{f'}_\theta^\top \boldsymbol{f'}_\theta)^{-1}$, which makes the correlation between the parameter estimates less correlated. The ellipsoid in Fig. 6.3 will be closer to a circle.

Box & Draper (1987) describes more throughly these optimal criteria along with others.

Figure 6.3: Parameter confidence interval

Special design problems arise for nonlinear models. The properties of a design generally depend on the unknown parameter values, since $\boldsymbol{f'}_\theta$ is depending on the parameter vector $\boldsymbol{\theta}$. This leaves the investigator with the paradoxical position of having to know at the design stage the very same quantities one is conducting the experiment to estimate! A way of solving this problem is by sequential design. The first experiments (preliminary experiments) are used for checking the methods, the range in which the experiment is carried out, etc. The preliminary experiments can also be used for determining approximate values of the model parameters, which can be used in choosing good design points. The number of preliminary experiments depends on the amount of work, expenses, and time involved for the preparation of the experiments, compared with the number of samples taken in the "actual" experiment.

In a degradation model one more problem arises - the function $\boldsymbol{f}$ cannot be given explicitly. This means that the predicted value of the i'th response (biomass and substrate), $\boldsymbol{y_i}$, has to be determined by numerical methods (we have used 4'th order Runge-Kutta iteration). As a result, we are not able to obtain analytical solutions for the - in some sense - optimal de-

sign points, $\boldsymbol{t}$. Box & Lucas (1959) outlined a procedure for determining the D-optimal design points for nonlinear models. The application of the Box-Lucas criterion for parameter estimation has received great attention by pharmacokineticists (Cobelli et al, 1983; Mori & DiStefano, 1979; DiStefano, 1980, 1981; Cobelli & DiStefano, 1980). Bilbo (1992) showed by using this procedure the estimation of the design points for a Monod model. Difficulties in optimization of the criteria $(\boldsymbol{f'}_\theta^\top \boldsymbol{f'}_\theta)^{-1}$ resulted in several local optima in the case of the smallest possible number of design points $n = P$, where P is the total number of parameters in the model. In the four-point design, Bilbo showed two sets of optimal design points and he was unable to select one of the design points as more preferable than the other (Fig. 6.4 and Fig. 6.5). The parameter values were determined from an earlier experiment. The first two design point in a set (4 measurements, two substrate + two biomass) can be interpreted as determining the degradation rate, k, and the yield coefficient, Y. Since the degradation and growth curves are approximately linear initially, two points will be "enough" to identify the steepness of the curves at this part, where the degradation takes on 0. order rate.

Figure 6.4: Illustration of an estimated optimal four-point design. • indicate the design-points.

Figure 6.5: Illustration of an estimated optimal four-point design. • indicate the design-points.

The last two design points (out of the four) determine the biomass decay, b. However, it is less obvious how K_s is determined, but it has been reported by Vialas, Cheruy & Gentil (1978) and Holmberg & Ranta (1982) that the sensitivity function reaches a peak just before the substrate is completely degraded (where degradation takes on 1. order rate). This is in agreement with our experience from the sensitivity functions (see later in this chapter). In the highest sensitive zone for a parameter, the influence of this parameter is at the greatest and that is where an observation should be taken. More information on optimal design points in a Monod model is described in Bilbo (1992). A four-point design has though little value in practice, since four observations general are too few in order to obtain reliable parameter estimates. The designs carried out by Bilbo are only included in order to give an idea of the technique. For a more elaborated six-point design, Bilbo reported that the optimization proved to be considerably more difficult due to singularities in the numerical estimation of $(\boldsymbol{f'}_\theta^\top \boldsymbol{f'}_\theta)$. An optimal design of e.g. 12 points would most likely be impossible to obtain.

The purpose of the D-optimal design was to minimize the number of sam-

ples and to minimize correlation and variance of the parameter estimates. However, for the Monod model and similar models even an optimal experimental design cannot quite eliminate the high degree of correlation exhibited by estimates of the parameters (Box & Lucas, 1959).

The application of the optimal sampling number and optimal design points technique for the experiment in this study has been less than hoped for. This has more than one reason 1) when there are relatively large uncertainties on the parameter estimates, on the variance estimates, and on the estimation of the D-matrix, optimization techniques becomes must less powerful, 2) the biological processes can easily get displaced which also will displace the optimal sampling points, and 3) large difficulties in estimating the design points (if more than four design points) makes the technique less attractive.

Sensitivity Equations

Sensitivity equation analysis treats the problem of the uniqueness of the determination of the parameter values. It can be used in order to give the investigator more information on how the model behaves and where/when it is optimal to sample. A sensitivity equation describes how sensitive a model is in terms of changes in the dependent variable caused by a given change of a parameter. Depending on whether absolute or relative measures of the variable and of the parameter are used, the following four sensitivity functions can be defined:

$$\boldsymbol{f'}_\theta^{a,a} = \frac{\partial \boldsymbol{f}}{\partial \theta_p} \tag{6.10}$$

$$\boldsymbol{f'}_\theta^{r,a} = \frac{1}{\boldsymbol{f}}\frac{\partial \boldsymbol{f}}{\partial \theta_p} \tag{6.11}$$

$$\boldsymbol{f'}_\theta^{a,r} = \theta_p \frac{\partial \boldsymbol{f}}{\partial \theta_p} \tag{6.12}$$

$$\boldsymbol{f'}_\theta^{r,r} = \frac{\theta_p}{\boldsymbol{f}}\frac{\partial \boldsymbol{f}}{\partial \theta_p} \tag{6.13}$$

In chapter 4 Eq.(6.10) was used in defining nonlinearity of a model. The two most often used sensitivity functions are Eq.(6.12) and Eq.(6.13), because the units of these functions do not depend on the units of the parameter.

This makes the comparison of the sensitivity of a variable to different parameters possible. Unique estimates of the parameters in a nonlinear model cannot be obtained if the sensitivity equations are multiples of one another. The more different the patterns of the sensitive functions are, the better can the parameters be identified (less correlated with other parameter estimates). In the following the sensitive functions for k, K_s, Y, and b in the Monod model are examined using Eq. (6.12). The sensitivity equations were estimated by use of the estimation program AquaSim. On basis of an experiment (batch A, Run I) a set of sensitivity equations was obtained. The derivatives required are calculated in AquaSim by the finite central difference approximation, which is more easy than using a derivation of a numerical approximation to $\boldsymbol{f}$.

$$\frac{\partial \boldsymbol{f}}{\partial \theta_p} \simeq \frac{\boldsymbol{f}(\boldsymbol{\theta} + \triangle\boldsymbol{\theta}) - \boldsymbol{f}(\boldsymbol{\theta} - \triangle\boldsymbol{\theta})}{2\triangle\boldsymbol{\theta}} \tag{6.14}$$

The central different approximation is more accurate than forward different approximation but it requires more evaluations of the function $\boldsymbol{f}$. In Fig. 6.6, 6.7, and 6.8 the sensitivity equations for the Monod model are shown.

Figure 6.6: Sensitivity curves, substrate

Figure 6.7: Sensitivity curves, biomass

Figure 6.8: Sensitivity curves, biomass

The influence of the maximum degradation rate (k) and the yield coefficient (Y) on the substrate curve Fig. 6.6 are very much the same, their curves are almost parallel. They can, however, be separated if measuring the biomass right after the substrate has been totally degraded, Fig. 6.7. In this region only Y has influence on the biomass curve and thus is easy to identify.

Since the estimate of b was very small, it's influence on any of the response curves shown in Fig 6.6 and 6.7 is hard to see. Therefore we have included Fig. 6.8, which shows the influence of the absolute values of the parameter estimates. Here it can be seen that the best place to measure the biomass decay coefficient is at the very end of the experiment. The half-saturation coefficient K_s is more difficult to identify - it is quite correlated with both k and Y and it's sensitivity to any of the two response curves is not very high. However, the best place to measure K_s is when it reaches it's peak just before the substrate is totally degraded.

These results are in good agreement with the estimated covariance matrix for Run I, batch A (Table 6.1), which show a strong correlation between k and K_s, and a strong negative correlation between k and Y.

	k	K_s	Y	b
k	1	0.70	-0.62	-0.36
K_s	0.7	1	0.1	0.08
Y	-0.6	0.1	1	0.70
b	-0.36	0.08	0.70	1

Table 6.1: Parameter correlation matrix for Run I, batch A.

When the sensitivity equations are nearly proportional, which is the case for many models of interest, the parameter estimates are highly correlated. The question arises, can the parameter be uniquely determined at all ?. Holmberg (1982) carried out a test (developed by Pohjanpalo 1978) with the Monod model and found that if the responses X and S were completely known all parameters could be uniquely determined. This implies that the model is theoretically globally identifiable. The practical identifiability with incomplete measurements and relative large measurements errors, cannot, however, be studied with this test (Pohjanpalo 1978). In order to illustrate the practical problems of parameter identification, an example from Run

I, batch A is used. In Fig. 6.9 it is shown how curve fitting for two quite different sets of parameter estimates suits the data almost equally well (the log likelihood values were -13.46 for the full line and -12.36 for the dashed line). (The estimation was carried out with weighting function equal to 1). The differences are especially large on the $k - K_s$ pair. The two Y-values were almost the same, which is in good agreement with the sensitivity function Fig. 6.7, where Y could be identified very well on the last part of the biomass curve.

Figure 6.9: Two different optima. □ biomass obs. ◇ toluene obs.

Because of these properties with quite different sets of parameter estimates which fit the data almost equally well, Holmberg & Ranta (1982b) suggest that the Monod model should be regarded rather as a black-box than as a physical model.

Chapter 7

Computing and practical recommendation

This chapter is of more practical character and focuses on giving some good advice on performing the experiments and analyzing the data. It also describes which kind of problems arises when using the nonlinear estimation program Dekimo or similar programs. What causes the problems and what can possible be done to illuminate/minimize them?

7.1 Advice on performing and analyzing data

One should always have a critical sense when sampling data. Detect possible error sources and concentrate on the large ones. If possible average out systematic errors by randomizing. Some examples on what was considered when performing the experiments in this study are given in the following.

- how large should the samples be in order to ensure measurements with reasonable small errors - about 10 ml was suitable,
- does the order in which the samples are measured on the gas chromatograph have any influence - no, unless a sample with very high concentration has been running just previously,
- how long time can the samples stay in the refrigerator without change in the concentrations - at least a couple of days, if stored properly,
- how large a flask should be used for the pentane mixture; if a large flask is used, too much of the internal standard will evaporate and leave the mixture with a different ratio, which has great influence on the calculation of the substrate concentration; if a smaller flask is used, it does not last throughout the run and a new pentane mixture much be made - the change of pentane mixture during the experiment does have a small influence on the concentration level, which was the reason why replicates were taken just before and after the changing. (These changes were as far as possible adjusted by the blank test carried out simultaneously).

When analyzing the data one will almost always run into problems concerning outliers or possible outliers. In this context it is of great value if one has made a thoroughly journal on the performing of the experiments. It makes it easier to identify "true" outliers. One is often in a conflict when classifying measurements as outliers - on one hand you take away a measurement from an already not too large sample size, on the other hand an outlier can violate the result of the estimation.

7.2 Optimization problems

Finding the best parameter estimates for nonlinear models are no trivial matter. For linear models, the surface described by the objective function or sum of squares function is parabolic and only one set of parameter estimates corresponds to a minimal value of the objective function. For nonlinear models, however, there may be several points along the surface where "the slope" equals zero. Some of the sets of parameter estimates may have meaningless values. These unrealistic values can be avoided by transforming the parameters into constrained parameters, which as a side profit often will improve the estimation time. The estimation routine will then be limited only to search in a certain parameter area. General techniques for estimating in models whose parameters are constrained can be found e.g. in Bard (1974) or Gill et al. (1981).

The estimation routine may end in a global optimum (truly the highest function value) or in a local optimum (the highest in a finite neighbourhood). When estimating in nonlinear models complete certainty cannot readily be given that the global optimum of the objective function has been found. As a general recommendation for minimizing functions, it is always advantageous to restart the algorithm with different initial parameter values to confirm the minimum found in the first trial. For some data sets it is best to restart the routine near the optimum and for others it is some times better to restart further away from the optimum. In the present study all model fittings were selected from the best (if not the same) of three or more estimations with different initial parameter values. In the single substrate system almost all estimations for each experiment ended in the same optimum. In the dual substrate system, however, often one of the estimations ended in a different optimum. Even when the estimations reached the same optimum the variance structure of the parameter estimates could vary. Most of the variations are due to the updating of the Hessian matrix. The Hessian matrix starts out as an identity matrix, and if there are too few iterations the matrix will not be properly updated. On the other hand if too many, very small inaccuracies in the approximation of the object function and in the updating of the Hessian can through the large number of iterations result in an incorrect estimation of the covariance matrix (this is often seen by a large conditional number given in the output file).

Difficult convergence of finding the optimum of the objective function is

among others related to ill-conditioned models (nearly proportional sensitivity equations), but not to the accuracy of the measurements (Beck & Arnold, 1977). When the parameters are strongly correlated the sensitivity equations are nearly proportional and many different sets of parameter values near the optimum may fit the data set almost equally well. In Fig. 7.1 a response surface for k and Y is shown. Run II, batch A were used in generating the surface where only k and Y varied. The relatively strong correlation between the parameters is reflected in the long ridge. In Fig. 7.2 the corresponding contour is shown.

Figure 7.1: Response surface for two parameters

In contrast to the accuracy of the measurements, the sample size does have an influence on the success of optimization. The smaller the sample size is, the more flat is the surface of the objective function near the optimum and a situation with many local minima as shown in Fig. 7.3 can occur (Steffensen, 1991). The larger the sample size is, the more well-defined is the surface of the objective function. This situation was also seen in the dual substrate system where the estimation of Run V was more difficult than the estimation of Run IV, VI, and VII due to less observations.

Figure 7.2: Contour for two parameters

Other problems of convergence can be due to numerical problems. The objective function must for example be twice continuous differentiable when using the maximum likelihood optimization method. The Hessian matrix (second derivative of the objective function) must be positive definite and non-singular. Roundoff errors can occur after many iterations and can cause the Hessian matrix to become nearly singular or non-positive definite. This may cause that the search direction does not lead downhill (closer to a minimum) and convergence becomes difficult to obtain. In trying to avoid this problem a Cholesky decomposition of the Hessian matrix was employed. However, should this situation occur anyway, it is recommendable to restart the algorithm with parameter values at the claimed minimum point, and see if it changes. Simple and easy, but not very elegant. For some nonlinear models, the initial values must be close to the best parameter estimates and linearized forms can then be used.

Figure 7.3: Ill-conditioned response surface

Of other numerical problems causing difficulties in convergence we can mention an inappropriate choice of step size control, convergence criteria and scaling. These problems are already thought of when building the estimation program. In Dekimo there are (should be) no convergence problems caused by the above mentioned cases.

7.3 Estimation of non-model parameters

Treating the initial value of the biomass, X_0, as a parameter to be estimated (a system parameter) besides the model parameters, is more realistic than assuming that this concentration is known. The value of X_0 has a relative large influence on the model fitting. The same could not be said about the initial substrate concentration(s), thus these values were not estimated by the program Dekimo. Changes in the substrate values only affects the degradation curve a little. In the estimation program Dekimo and in AquaSim the initial value of the biomass was estimated simultaneously with the model parameters. When using the Lineweaver-Burk linearization 4 different initial values for the biomass were examined and the best was chosen.

Another non-model parameter that needed to be estimated was the lag phase . The lag phase only occured in the dual substrate systems and was not estimated automatically by any of the estimation programs. It was estimated "by hand". In Dekimo the user support the program with information on the length of the lag phase . It is recommendable to test which of 3-4 different lengths of the lag phase, that result in the best fitting of the data (greatest log-likelihood value). The estimation program AquaSim cannot handle a lag phase and therefore the observations in the begining of the experiment must be removed from the data material. Since the estimated parameter values were not very sensitive to changes in the lag phase, it is for the time being good enough to estimate these parameters roughly by hand. In future work the lag phase may be incorporated in the parameter vector and estimated by the program Dekimo. However, estimation of one more parameter slows down the optimization routine and may give rise to convergence problems as a result of overparametrization.

Chapter 8

Results and comments

This chapter is mainly concerning results of the parameter estimations, tests for model reduction, and for reproducibility for the single and the dual substrate system. At the end of the chapter parameters are estimated using three different estimation techniques and the results are compared. The estimations were carried out by the computer program Dekimo (Bilbo 1992). Two types of estimation were performed.

- Individual estimation, where one experiment was estimated at a time.
- Common estimation, where three batches (within or between runs) were estimated commonly.

With the indices "1, 2, or 3" the above mentioned types of estimation indicate how they were estimated. "1" indicates that a full model was employed, "2" indicates that a reduced model was employed and that the examined experiments were carried out the same day (within the same run), and finally "3" indicates that a reduced model was employed and that the examined experiments were carried out on different days (between runs).

The examination of possible model reduction of respectively the Monod and Bailey & Ollis' model were treated as an entire block, meaning that only if reduction of the model under consideration was possible for all experiments in the single or the dual system, the model was reduced.

In order to simplify the models (Monod and Bailey & Ollis) they are examined for possible model reduction by comparing individual estimations of the full model to individual estimations of a reduced model. After these examinations, the reproducibility of the experiments are examined by comparing common estimations to individual estimations. Depending on the results of the model reduction, a full model or a reduced model was used. The procedure for model reduction and for reproducibility is described in chapter 5. In this study model reduction is examined first and then the reproducibility, it could also have been carried out in the opposite order.

8.1 Single substrate system

The observed responses and the estimated models for Run I, II, and III are shown in Fig. 8.1, 8.2, and 8.3. In the figures three kinds of curves are drawn. In the section above the different kinds of estimation methods are described. The model fitting for "Individual 1" for Run I and III seem to describe the data very well. For Run II the fitting is not quite as good. The estimation in this run should maybe have been carried out with a lag phase of e.g. 2 hours. Comments on the model fitting for the individual estimations "Individual 2" are given in section 8.1.1, and comments on the common estimations "Common 2" are given in section 8.1.2. The model fittings of "Individual 3" and of "Common 3" are shown in section 8.1.2. The estimates of the parameters corresponding to the shown curves in Fig.. 8.1, 8.2, and 8.3 are displayed in Table 8.1.

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

(SD) on the parameter estimates were calculated as averages of the variances within the batch experiments ($\hat{\sigma}^2_{we}$) from the same run. $\hat{\sigma}_{we}$ is automatically calculated by Dekimo.

$$\mathrm{SD} = \bar{\sigma}_{we} = \sqrt{\frac{\sigma^2_{we(A)} + \sigma^2_{we(B)} + \sigma^2_{we(C)}}{3}} \tag{8.1}$$

For all three runs K_s is the parameter which is determined with the largest standard deviation.

Before carrying out tests for common sets of parameter values within the runs, possible model reductions are examined.

	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	**
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	**
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	**
L	-10.7	-37.6	-32.4	-	-10.7	-38.4	-32.4	-	-130

Table 8.1: Estimated parameter values for batch A, B, and C in the single substrate system. "Individual 1" uses the full Monod model, "Individual 2" uses the reduced model A, and "Common 2" also uses the reduced model A. SD is the standard deviation, and L is the log likelihood value. * = fixed value, ** = the same X_0 as given for "Individual 2", and + = new estimates are given in Table 8.3 - see section 8.1.2 for explanation. 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).

Figure 8.1: Estimation within Run I a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs.

Figure 8.2: Estimation within Run II a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs.

Figure 8.3: Estimation within Run III a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs.

8.1.1 Model reductions

The most obviously parameter to reduce the Monod model with is the decay constant b. From Table 8.1 "Individual 1" it can be seen that all the estimates of b are near zero. In particular, a 95% confidence interval (roughly twice the standard deviation) around the b estimates would include zero. And when performing the likelihood ratio tests for every experiment, all χ^2-values were below the critical value confirming that the Monod model can be reduced to a more simple model without the biomass decay.

Reduced model A

$$\frac{dS}{dt} = -h \cdot k \frac{SX}{K_s + S} \tag{8.2}$$

$$\frac{dX}{dt} = Y \cdot k \frac{SX}{K_s + S} \tag{8.3}$$

The properties associated with the estimated parameters in the reduced model are improved (smaller standard deviations). All experiments were re-fitted and new estimates are given in Table 8.1, "Individual 2". The model fitting of the experiments look very much the same as the graphs for "Individual 1" (Fig. 8.1, 8.2, and 8.3), except from the fact that the last part of the biomass curves now are horizontal ($b = 0$). In Run I the biomass decay coefficient b were almost only determined by the last two biomass measurements in the experiments. Thus, in the following experiments many measurements of the biomass in the decay phase were taken.

Since the estimates of the half-saturation constant, K_s from the 9 experiments seem relatively small compared to the substrate initial values, it is relevant to examine if the Monod model (Eq.(8.2) and Eq.(8.3)) can be further reduced to a zero order degradation kinetic model, also called a logarithmic model (Simkins & Alexander 1984). This model also has the very attractive character of being linear.

Reduced model B
$$\frac{dS}{dt} = -h \cdot k \cdot X \quad (8.4)$$
$$\frac{dX}{dt} = Y \cdot k \cdot X \quad (8.5)$$

For Run I (Table 8.1, "Individual 1 and 2") 95% confidence intervals around K_s estimates do not include zero. This indicate that a reduction may not be possible. For Run II and III however, 95% confidence intervals around K_s would include zero. The results of the likelihood ratio tests are given in Table 8.2.

	Run I			Run II			Run III		
	A	B	C	A	B	C	A	B	C
Model A	5.2	5.4	-6.2	-44.5	-52.1	-66.0	-10.7	38.9	-32.4
Model B	-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 8.2: "Reduced model A" is the model given by Eq.(8.2) and Eq.(8.3), and "Reduced model B" is given by Eq.(8.4) and Eq.(8.5). The values are the estimated log likelihood values. The "Test values" were calculated from the equations given in chapter 5.

Not all of the experiments in this study can be described by the reduced model B (Eq.(8.4) and Eq.(8.5)). For the overall description we will therefore use the reduced model A given by Eq.(8.2) and Eq.(8.3). The conclusion is that the K_s estimates are small, but not always zero and have a relatively large variation.

8.1.2 Test for reproducibility

Within the runs

The common estimations for batches within the runs are shown in Fig. 8.1, 8.2, and 8.3. The fitted substrate curves for the individual and the common estimations are practically identically. The largest difference between the two estimations (Individual 2 and Common 2) is seen for the biomass curves, especially in the decay phases . The reason for this is the fact that the biomass measurements is weighted less than the substrate measurement in the estimation routine, due to the larger variance on the biomass measurements compared to the substrate measurements.

A test for the hypothesis that the parameter values for the three batches A, B, and C belonging to the same run, were the same, is carried out. The log likelihood values were used in forming the test statistic, which was compared to a quantile in the χ^2-distribution:

$$-2\,\left(\sum_{j=1}^{r} \log L_0(\boldsymbol{\theta})_{(within)} - \sum_{i=1}^{rs} \log L_1(\boldsymbol{\theta})_{(individual)}\right) \quad \text{compared to} \quad \chi^2(p\,(rs-r))_{1-0.05} \quad (8.6)$$

The joint test:

$$-2\,(-298.2 - (-240.2)) = 116.0 \;>\; \chi^2(18) = 28.9$$

Using α=5% the hypothesis of common sets of parameter estimates cannot be accepted. However, if the test is split up in the three runs to find which sub hypothesis cannot be accepted, the following result is obtained.

$$-2\,\left(\log L_0(\boldsymbol{\theta})_{(within)} - \sum_{i=1}^{s} \log L_1(\boldsymbol{\theta})_{(individual)}\right) \quad \text{compared to} \quad \chi^2(p\,(s-1))_{1-0.05} \quad (8.7)$$

Test in Run:

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

The hypothesis for Run I and II is accepted, but for Run III the hypothesis of a common set of parameter estimates is rejected. It seems to be batch A that is causing the trouble. The yield coefficient Y is much larger for batch A than for the other two batches, and it is the only experiment out of all 9, where the concentration of the biomass exceed the start concentration for the substrate. If only batch B and C in Run III are compared, the estimates are much more alike. In Table 8.3 the parameter values are recalculated. The values of the initial concentrations X_0 for batch B and C are changed a little due to the new common estimation consisting only of batch B and C. The new initial concentrations for batch B and C resulted in small changes in the estimated parameter values.

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
L	-38.64	-33.72	-	-72.00

Table 8.3: New estimates for batch B and C Run III. L is the log likelihood. 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).

After having removed batch A from the data in Run III the hypothesis of a common set of parameter values can easily 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:

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

It is difficult to guess upon the reason why batch A in Run III behaves so differently compared to batch B and C. A more detailed microbiological information on the biomass in the three batches might have given an explanation. We are aware of the "danger" in singling out one experiment when assuming that the experiments are truly representative of a larger population of possible experiments. The three experiments should ideally be considered as random realizations of a larger population of experiments.

Between the runs

The hypothesis of common sets of parameter values for experiments between the runs (days) is examined in this section. According to the procedure described in chapter 5, three parallel tests for common set of parameter values from different runs were carried out :

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

Any other combination of batches from three different runs could have been chosen in the three parallel tests. The examination of common sets of parameter estimates were treated the same way as when examining common sets of parameter values within the runs. This means that 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. In Fig. 8.4, 8.5, and 8.6 the common estimations between the runs are shown together with the individual estimations (Individual 3) and together with the individual estimation (Individual 2) given in Table 8.1. Common estimation for Run I is fitted worse than for Run II and III, this is due to less observations in Run I compared to Run II and III, and not because Run I is pronounced more different from the other two.

The results of the likelihood ratio tests were the following:
The joint test:

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

Test in:

$$\begin{aligned}
&\text{a)} \; -2 \cdot (-103.6 - (-9.1 - 45.9 - 13.8)) = 69.6 > \chi^2(6)_{95\%} = 12.6 \\
&\text{b)} \; -2 \cdot (-107.2 - (-4.3 - 54.6 - 40.9)) = 14.8 > \chi^2(6)_{95\%} = 12.6 \\
&\text{c)} \; -2 \cdot (-139.1 - (-19.3 - 70.6 - 39.6)) = 19.2 > \chi^2(6)_{95\%} = 12.6
\end{aligned}$$

Using $\alpha = 5\%$ the hypothesis of a common set of parameter estimates for experiments from different runs is rejected for all three tests. When looking at the common estimations and the individual estimations (Individual 3) in Fig. 8.4, 8.5, and 8.6, the differences do not seem very large. However, when comparing the common estimations or Individual 3 with Individual 2 the differences become much larger, and it is clear to see that the common estimation does not fit the data as well. This indicates that the new initial concentrations were not appropriate, and together with the rejection of the common sets of parameter estimates between the runs, we will conclude that the single substrate experiment has a limited reproducibility with respect to variation between runs.

8.1.3 Experiments with inhibition

In Run VIII and IX, which consist of experiments also carried out as single substrate experiments, product or substrate inhibition possibly occured. The biological processes in these experiments probably followed some unknown product inhibition process. It seems as though the intermediate product was not a "problem" for the degradation until it accumulated to a

Figure 8.4: Estimation between runs a) Run I batch A, b) Run II batch A, and c) Run III batch A. □ biomass obs. ◇ toluene obs.

Figure 8.5: Estimation between runs a) Run I batch B, b) Run II batch B, and c) Run III batch B. □ biomass obs. ◇ toluene obs.

Figure 8.6: Estimation between runs a) Run I batch C, b) Run II batch C, and c) Run III batch C. □ biomass obs. ◇ toluene obs.

certain concentration and then inhibited the degradation of the substrate and the growth of the biomass. It was, however, not possible to find any accumulation of intermediate products when examining the spectra from the gas chromatograph measurements. This does not exclude the possibility of an accumulated intermediate. The intermediate may for example not have been extracted from the sample by pentane (if the intermediate was polar it would not have been extracted since pentane is non-polar) and could therefore not be seen on the spectra. The inhibition only occured when the initial toluene concentration was greater than 10 mg/l.

Alvarez et al. (1991) found no substrate inhibition (or self-inhibition) for toluene concentrations < 30 mg/l for a mixed culture from sandy aqueous material. Run VIII and IX were not modelled with any product inhibition model, they were only used in comparison with the non-inhibited experiments from the single substrate system. That some kind of inhibition occured when S_0 was greater than 10 mg/l is most clearly seen in Run VIII (Fig. 8.7). The biomass growth stopped before the toluene was totally degraded.

Outliers from the biomass measurements shown in Fig. 8.7 have not been removed since these experiments were not modelled. The outliers from the biomass measurements are usually much less than the measurements in the same area. This is due to the analysis method. After the samples were centrifuged the liquid was sucked up and by that process some or all of the biomass at the bottom of the test tube was easily sucked up as well.

In Run IX, batch A was not inhibited since the initial substrate concentration for toluene was less than 10 mg/l. Batch B was cut out of the experiment due to an accident with the glass top of the batch-bottle. Batch C was inhibited. If we use the parameter estimates obtained from batch A to model the data in batch C (Fig. 8.8), it becomes very clear that some inhibition of the degradation and the growth processes have occured, and that the Monod model is insufficient for describing the processes.

Figure 8.7: Run VIII a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs.

Figure 8.8: Run IX a) batch A and b) batch C. □ biomass obs. ◇ toluene obs.

8.2 Dual substrate system

The dual substrate system consisted of four identical runs. The observed responses and the estimated models for Run IV, V, VI, and VII are shown in Fig. 8.9, 8.10, 8.11, and 8.12. "Individual 1" are estimations, where the full Bailey & Ollis' model was employed, "Common 1" are estimations for batch A, B, and C within the same run, where the full model was employed, and "Individual 2" are estimates, where the reduced model B was employed.

The model fitting for "Individual 1" for all the runs in the dual substrate system seem to describe the data very well. Comments on the “Common 1” estimation is given in section 8.2.1. The model fittings of “Individual 3” and of “Common 3” are shown in section 8.2.2. The model fittings of “Individual 2” are not shown.

The estimates of the parameters are displayed in Table 8.4 and 8.5. The values of the estimated initial concentrations, X_0 for batch A, B, and C, were determined from the common estimation. The lag phases were estimated "by hand", meaning that for each run 3 or 4 different lag phases were tested and the one that resulted in the largest log likelihood value was chosen. The standard deviations (SD) on the parameter estimates were calculated as averages of the variances within the batch experiments ($\hat{\sigma}^2_{we}$) from the same run. $\hat{\sigma}_{we}$ is automatic calculated by Dekimo.

$$\mathrm{SD} = \bar{\sigma}_{we} = \sqrt{\frac{\sigma^2_{we(A)} + \sigma^2_{we(B)} + \sigma^2_{we(C)}}{3}} \tag{8.8}$$

The inhibition coefficient z_t (which inhibit the benzene degradation) is the most inaccurate determined parameter in the model in contrast to the z_b inhibition coefficient which take the value of zero for all 12 experiments except from one ($z_b = 0.006$). The half-saturation coefficients K_{sb} and K_{st} have as in the single substrate system a relative large SD-value. The SD-value for the yield coefficient Y_b is larger compared to Y_t. A reason for the better determination of Y_t-estimate could be due to the larger initial value of S_0 for toluene compared to the S_0-value for benzene. The effect of the yield coefficient is larger the larger the substrate concentration. In general, the standard deviation values for the estimates in the dual substrate system are larger than in the single substrate system, which very likely is due to the larger number of parameters in the Bailey & Ollis model.

	Individual 1				Common 1	Individual 2			
Run IV	A	B	C	SD	A, B, C	A	B	C	SD
k_b	0.19	0.25	0.33	0.06	0.28	0.12	0.15	0.15	0.01
K_{sb}	0.11	0.56	0.87	0.18	0.59	0.01	0.04	0.07	0.04
Y_b	0.79	0.48	0.45	0.17	0.44	1.03	0.57	0.75	0.29
z_b	0.00	0.00	0.00	0.00	0.00	0*	0*	0*	-
k_t	0.44	0.45	0.41	0.02	0.43	0.44	0.44	0.39	0.02
K_{st}	0.68	1.30	0.67	0.10	0.87	0.65	1.18	0.59	0.14
Y_t	0.93	1.17	1.12	0.09	1.13	0.81	1.12	1.03	0.16
z_t	0.67	1.08	1.88	0.34	1.44	0*	0*	0*	-
b	0.04	0.04	0.04	6^{-3}	0.03	0.04	0.04	0.05	0.01
σ_ε	0.08	0.07	0.06	0.01	0.08	0.10	0.08	0.09	0.01
X_0	0.71	0.58	0.78	0.03	**	0.71	0.58	0.78	0.03
lag	2	2	2	-	2	2	2	2	-
L	6.2	25.6	36.1	-	37.5	-14.3	13.6	1.0	-
Run V									
k_b	0.19	0.33	0.25	0.02	0.46	0.13	0.21	0.17	0.01
K_{sb}	0.16	0.36	0.51	0.10	2.01	0.01	0.53	0.07	0.01
Y_b	0.12	0.24	0.78	0.10	0.55	0.64	0.27	0.37	0.03
z_b	0.00	0.00	0.01	0.01	0.00	0*	0*	0*	-
k_t	0.42	0.44	0.42	0.01	0.44	0.42	0.43	0.38	0.01
K_{st}	0.15	0.01	0.02	0.07	0.01	0.09	0.01	0.01	0.01
Y_t	0.85	0.66	0.62	0.06	0.67	0.67	0.67	0.82	0.03
z_t	0.95	0.87	0.61	0.20	2.53	0*	0*	0*	-
b	0.01	2^{-3}	0.02	7^{-3}	0.02	0.02	2^{-3}	0.01	5^{-3}
σ_ε	0.07	0.10	0.09	0.01	0.10	0.09	0.11	0.10	0.01
X_0	0.41	0.43	0.48	0.02	**	0.41	0.43	0.48	0.02
lag	7	7	7	-	7	7	7	7	-
L	6.7	-20.5	-8.2	-	-58.7	-14.2	-25.5	-13.5	-

Table 8.4: Estimated parameter values in the dual substrate system for Run IV and V. "Individual 1" and "Common 1" use the full Bailey & Ollis' model, and "Individual 2" uses the reduced model B. SD is the standard deviation, L is the log likelihood value, and lag is the lag phase. * = fixed value and ** = the same X_0 values as given for "Individual 1". The units of the parameters are given by the following: k_b (mg substrate/mg biomass/hour), K_{sb} (mg substrate/l), Y_b (mg biomass/mg substrate), z_b (non), k_t (mg substrate/mg

biomass/hour), K_{st} (mg substrate/l), Y_t (mg biomass/mg substrate), z_t (non), b (/hour), X_0 (mg biomass/l), and lag phase (hour).

	Individual 1				Common 1	Individual 2			
Run VI	A	B	C	SD	A, B, C	A	B	C	SD
k_b	0.14	0.21	0.27	0.04	0.52	0.12	0.15	0.16	0.01
K_{sb}	0.01	0.28	0.32	0.13	1.78	0.01	0.08	0.01	0.02
Y_b	0.01	0.41	0.12	0.20	0.81	0.20	0.43	0.09	0.13
z_b	0.00	0.00	0.00	0.00	0.00	0*	0*	0*	-
k_t	0.75	0.56	0.53	0.02	0.73	0.75	0.54	0.52	0.02
K_{st}	2.07	0.74	0.65	0.09	1.21	1.87	0.71	0.61	0.09
Y_t	0.75	0.73	0.84	0.09	0.51	0.68	0.73	0.87	0.07
z_t	0.34	0.37	0.83	0.24	2.05	0*	0*	0*	-
b	0.00	0.00	-1^{-3}	4^{-3}	5^{-3}	1^{-3}	0.00	-1^{-3}	5^{-3}
σ_ε	0.09	0.08	0.08	0.01	0.14	0.11	0.08	0.09	0.01
X_0	0.25	0.31	0.28	0.03	**	0.25	0.31	0.28	0.03
lag	0	0	0	-	0	0	0	0	-
L	5.3	28.3	16.4	-	-93.8	-10.9	23.6	5.7	-
Run VII									
k_b	0.30	0.16	0.28	0.03	0.28	0.13	0.09	0.11	0.01
K_{sb}	0.61	0.06	1.28	0.12	0.89	0.01	0.00	0.00	0.01
Y_b	0.62	0.50	0.00	0.21	0.28	1.14	0.22	0.03	0.04
z_b	0.00	0.00	0.00	0.00	0.00	0*	0*	0*	-
k_t	0.48	0.63	0.66	0.02	0.57	0.46	0.59	0.65	0.02
K_{st}	0.58	1.51	2.07	0.14	1.13	0.49	1.44	1.98	0.13
Y_t	0.80	0.74	0.80	0.06	0.77	0.64	0.83	0.80	0.02
z_t	1.48	0.78	1.69	0.29	1.47	0*	0*	0*	-
b	4^{-3}	4^{-3}	0.01	5^{-3}	-6^{-3}	0.01	1^{-3}	-0.01	3^{-3}
σ_ε	0.09	0.08	0.11	0.01	0.13	0.12	0.11	0.13	0.01
X_0	0.41	0.88	0.56	0.04	**	0.41	0.88	0.56	0.04
lag	5	5	5	-	5	5	5	5	-
L	-0.9	10.2	-18.3	-	-131	-30.2	-20.5	-35.1	-

Table 8.5: Estimated parameter values in the dual substrate system for Run VI and VII. "Individual 1" and "Common 1" use the full Bailey & Ollis' model, and "Individual 2" uses the reduced model B. SD is the standard deviation, L is the log likelihood value, lag is the lag phase. * = fixed value

and ** = the same X_0 values as given for "Individual 1". The units of the parameters are given by the following: k_b (mg substrate/mg biomass/hour), K_{sb} (mg substrate/l), Y_b (mg biomass/mg substrate), z_b (non), k_t (mg substrate/mg biomass/hour), K_{st} (mg substrate/l), Y_t (mg biomass/mg substrate), z_t (non), b (/hour), X_0 (mg biomass/l), and lag phase (hour).

8.2.1 Model reductions

The Bailey & Ollis' model, which was used for modelling the processes in the dual substrate system, consists of 9 parameters. With 9 parameters there are many combinations of parameters that could be examined for being eliminated from the model. In the present study the obviously ones and those of special interest were tested. The benzene inhibition constant is number one. As can be seen from Table 8.4 and 8.5 all estimates of z_b are zero, except for one ($z_b = 0.006$) but here the 95% confidence interval includes zero. Also the likelihood ratio test showed that z_b could be accepted as being equal to zero. Thus, the Bailey & Ollis' model could be reduced to a more simple model without the inhibition constant z_b.

Reduced model A

$$\frac{dS_t}{dt} = -h_1 \cdot k \frac{S_t X}{K_{st} + S_t} \tag{8.9}$$

$$\frac{dS_b}{dt} = -h_2 \cdot k \frac{S_b X}{K_{sb} + S_b + z_t \cdot S_t} \tag{8.10}$$

$$\frac{dX}{dt} = -Y_t \cdot \frac{dS_t}{dt} - Y_b \cdot \frac{dS_b}{dt} - bX \tag{8.11}$$

It is also interesting to see if the toluene inhibition constant z_t could be eliminated from the model - in such a case the model would turn into a simultaneously utilization model (reduced model B). Reducing the model with z_t does, however, not seem likely, since far from all 95% confidence intervals around z_t include zero.

Figure 8.9: Estimation within Run IV a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs. △ benzene obs.

Figure 8.10: Estimation within Run V a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs. △ benzene obs.

Figure 8.11: Estimation within Run VI a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs. △ benzene obs.

Figure 8.12: Estimation within Run VII a) batch A, b) batch B, and c) batch C. □ biomass obs. ◇ toluene obs. △ benzene obs.

Reduced model B
$$\frac{dS_t}{dt} = -h_1 \cdot k \frac{S_t X}{K_{st} + S_t} \tag{8.12}$$
$$\frac{dS_b}{dt} = -h_2 \cdot k \frac{S_b X}{K_{sb} + S_b} \tag{8.13}$$
$$\frac{dX}{dt} = -Y_t \cdot \frac{dS_t}{dt} - Y_b \cdot \frac{dS_b}{dt} - bX \tag{8.14}$$

By carrying out new estimations with the reduced model B the following likelihood ratio tests were obtained.

	Run IV			Run V		
	A	B	C	A	B	C
Reduced model A	6.2	25.6	36.1	6.7	-20.8	-7.4
Reduced model B	-14.3	13.5	1.0	-14.2	-25.5	-13.5
Test value	41.0	24.2	70.2	41.8	9.4	12.2
$\chi^2(1)_{95\%}$	3.8	3.8	3.8	3.8	3.8	3.8
Further reduction	No	No	No	No	No	No

	Run VI			Run VII		
	A	B	C	A	B	C
Reduced model A	5.3	28.3	16.4	-0.9	10.2	-18.3
Reduced model B	-10.9	23.6	5.7	-30.2	-20.5	-35.1
Test value	32.4	9.4	21.4	58.6	61.4	33.6
$\chi^2(1)_{95\%}$	3.8	3.8	3.8	3.8	3.8	3.8
Further reduction	No	No	No	No	No	No

Table 8.6: "Reduced model A" is the model given by Eq.(8.9), Eq.(8.10), and Eq.(8.11), and "Reduced model B" is given by Eq.(8.12), Eq.(8.13), and Eq.(8.14), and the values are the estimated log likelihood values. The "Test values" were calculated from the likelihood ratio test, given in chapter 5.

From the test results it was not possible to reduce the Bailey & Ollis' model to a simultaneously utilization model (reduced model B). The reduced model B also fitted the data in the 12 experiments very poorly (graphs not shown). When z_t takes a value greater than zero, it means that the toluene inhibits the benzene degradation. And when all the toluene has gone the benzene degradation rate will accelerate. This phenomena can only be seen in a small part of the degradation curve, namely between the time where toluene has been totally degraded or nearly, and until benzene is totally degraded. Only this part separate the two models. In Fig. 8.9, 8.10, 8.11, and 8.12, the situation is more or less seen depending on the time interval and on the parameter values.

Other parameters of interest to reduce the model by were the maximum degradation coefficient, the half-saturation coefficient, and the yield coefficient for toluene (k_t, K_{st}, Y_t). These parameter values were fixed equal the mean values obtained from the single substrate system. This new model is denoted reduced model C. Estimations were carried out with these fixed values and compared with the values from the reduced model A. The reduced model C was not compared with reduced model B, since model B was not accepted. The likelihood ratio tests gave the results, displayed in Table 8.7.

From the results of the test it was not possible to use the parameter estimates for toluene obtained in the single substrate system.

The biomass decay constant was not examined to be equal zero as in the single substrate system, since the 95% confidence interval did not include zero in most of the cases.

	Run IV			Run V		
	A	B	C	A	B	C
Reduced model A	6.2	25.6	36.1	6.7	-20.5	-8.2
Reduced model C	-14.3	13.5	1.0	-14.2	-25.5	-13.5
Test value	41.0	24.1	70.1	41.9	10.0	10.8
$\chi^2(1)_{95\%}$	3.8	3.8	3.8	3.8	3.8	3.8
Further reduction	No	No	No	No	No	No
	Run VI			Run VII		
	A	B	C	A	B	C
Reduced model A	5.3	28.3	16.4	-0.9	10.2	-18.3
Reduced model C	-10.9	23.6	5.7	-30.2	-20.5	-35.1
Test value	32.4	9.3	21.5	58.5	61.5	33.7
$\chi^2(1)_{95\%}$	3.8	3.8	3.8	3.8	3.8	3.8
Further reduction	No	No	No	No	No	No

Table 8.7: "Reduced model A" is the model given by Eq.(8.9), Eq.(8.10), and Eq. (8.11), and "Reduced model C" is also given by Eq.(8.9), Eq. (8.10), and Eq. (8.11), but k_t, K_{st}, Y_t have fixed values. The values given in the table are the estimated log likelihood values, and the "Test values" were calculated from the likelihood ratio test given in chapter 5.

8.2.2 Test for reproducibility

Within runs

A test for the hypothesis that the parameter values for three batches A, B, and C belonging to the same run could be said to have the same values, is carried out. The log likelihood values from the three batches were used in forming the test statistic (given in chapter 5) which is compared to a quantile in the χ^2-distribution:

The joint test:

$$-2 \cdot (-246.37 - (-87)) = 666.8 > \chi^2(64)_{95\%} = 90$$

Test in Run:

$$
\begin{array}{rlcl}
\text{IV:} & -2 \cdot (37.5 - (6.2 + 25.6 + 36.1)) = 60.8 & > & \chi^2(16)_{95\%} = 26.3 \\
\text{V:} & -2 \cdot (-58.7 - (6.7 - 20.5 - 8.2)) = 73.0 & > & \chi^2(16)_{95\%} = 26.3 \\
\text{VI:} & -2 \cdot (-93.8 - (5.3 + 28.3 + 16.4)) = 287.6 & > & \chi^2(16)_{95\%} = 26.3 \\
\text{VII:} & -2 \cdot (-131.4 - (-0.9 + 10.2 - 18.3)) = 244.8 & > & \chi^2(16)_{95\%} = 26.3
\end{array}
$$

All the above shown tests reject the hypothesis of common sets of parameter estimates within the runs. For Run IV the rejection may not seem reasonable, since the common estimation is practically identical with the individual estimation as seen in Fig. 8.9. For Run V, VI, and VII the differences between common and individual estimation is larger, see Fig.8.10, 8.11, and 8.12. These observations reflect the magnitude of the test values. The test value for Run V is the smallest, whereas the test values for Run VI and VII are considerable larger. The test value for Run V is relatively large compared to the small number of observations in contradistinction to the number of observations in Run IV, VI, and VII.

Between runs

A test for the hypothesis that the parameter values for three batches belonging to different runs could be said to have the same values, was carried out. According to the procedure described in chapter 5, four parallel tests for common set of parameter values for experiments from different runs were carried out.

a) Run IV batch A, Run V batch A, Run VI batch A

b) Run VII batch A, Run IV batch B, Run V batch B

c) Run VI batch B, Run VII batch B, Run IV batch C

d) Run V batch C, Run VI batch C, Run VII batch C

Any other combination of batches from three different runs could have been chosen in the four parallel tests. The examination of common sets of parameter estimates were treated the same way as when examining common sets of parameter values within the runs. This means that new initial concentrations for the biomass were estimated for the new common estimation (Common 3), and new individual estimates (Individual 3) with the same new initial concentration were carried out as well. In Fig. 8.13, 8.14, 8.15 and 8.16 the common estimations (Common 3) between the runs are shown together with the individual estimations (Individual 3).

The results of the tests were the following.

The joint test:

$$-2 \cdot (-343.7 - (-62.2)) = 811.8 > \chi^2(64)_{95\%} = 90$$

Test in:

$$
\begin{array}{rlcl}
\text{a)} & -2 \cdot (-126.3 - (6.2 + 0.6 - 3.8)) = 258.3 & > & \chi^2(16)_{95\%} = 26.3 \\
\text{b)} & -2 \cdot (-63.4 - (-10.0 + 34.3 - 20.4)) = 134.6 & > & \chi^2(16)_{95\%} = 26.3 \\
\text{c)} & -2 \cdot (-24.6 - (27.1 + 16.7 + 31.9)) = 200.6 & > & \chi^2(16)_{95\%} = 26.3 \\
\text{d)} & -2 \cdot (-129.5 - (-10.1 + 6.8 - 17.7)) = 217.0 & > & \chi^2(16)_{95\%} = 26.3
\end{array}
$$

Using $\alpha = 5\%$ the hypothesis of a common set of parameter estimates for experiments from different runs is rejected for all four tests. For a few graphs the rejection of a common set of parameter estimates seem less obviously. As a whole we must conclude that common sets of parameter estimates do not describe the observed measurements very well. Thus, one common set of parameter estimates for all 12 experiments would fit the graphs even worse. The conclusion is then that the dual substrate system has a limited reproducibility as the single substrate system.

Figure 8.13: Estimation between runs a) Run IV batch A, b) Run V batch A, and c) Run VI batch A. □ biomass obs. ◇ toluene obs. △ benzene obs.

Figure 8.14: Estimation between runs a) Run VII batch A, b) Run IV batch B, and c) Run V batch B. □ biomass obs. ◇ toluene obs. △ benzene obs.

Figure 8.15: Estimation between runs a) Run VI batch B, b) Run VII batch B, and c) Run IV batch C. □ biomass obs. ◇ toluene obs. △ benzene obs.

Figure 8.16: Estimation between runs a) Run V batch C, b) Run VI batch C, and c) Run VII batch C. □ biomass obs. ◇ toluene obs. △ benzene obs.

8.2.3 Experiment with missing data

Due to an accident in the chemical analysis of the substrate samples the last measurements of the benzene degradation in all three experiments in Run X were lost. As a result, the simultaneous estimation of all parameters was impossible. However, when fixing minimum two of the parameters at a time, estimation of the rest of the parameters was possible. The first estimation was performed with fixed inhibition constants z_b and z_t. The values were set to 0 and 0.96 respectively, which were equal the average values from the dual substrate experiments. The results of individually and commonly estimations are shown in Fig. 8.17 and the parameter estimates are given in Table 8.8.

	Individual				Common
Parameters	Batch A	Batch B	Batch C	SD	Batch A, B, and C
k_b	0.219	0.191	0.191	0.045	0.236
K_{sb}	3.066	0.075	0.000	1.911	2.551
Y_b	0.000	0.334	0.462	0.100	0.466
z_b	0*	0*	0*	-	0*
k_t	0.447	0.403	0.449	0.020	0.449
K_{st}	1.282	0.671	1.113	0.177	1.121
Y_t	0.928	0.856	0.782	0.048	0.789
z_t	0.96*	0.96*	0.96*	-	0.96*
b	0.004	0.000	0.003	0.001	0.003
σ_ε	0.092	0.082	0.080	0.005	0.093
X_0	0.871	0.794	0.979	0.037	0.871, 0.794, 0.979
lag phase	2.5	2.5	2.5	-	2.5
L	-23.71	-12.61	-15.41	-	-77.42

Table 8.8: Estimated parameter values for Run X, batch A, B, and C, individually and commonly. SD is the standard deviation, L is the log likelihood value, and values with * were fixed during the estimation. The units of the parameters are given by the following: k_b (mg substrate/mg biomass/hour), K_{sb} (mg substrate/l), Y_b (mg biomass/mg substrate), z_b (non), k_t (mg substrate/mg biomass/hour), K_{st} (mg substrate/l), Y_t (mg biomass/mg substrate), z_t (non), b (/hour), X_0 (mg biomass/l), and lag phase (hour).

Figure 8.17: Estimation within Run X, z_b and z_t were fixed (0 and 0.96) a) batch A, b) batch B), and c) batch C. □ biomass obs. ◇ toluene obs. △ benzene obs.

Concerning the test for common set of parameter estimates within the run, the test result is not different from the previously tests in the dual substrate system - the test rejects the hypothesis of a common set of estimates within the run, even though the common estimation seems to describe the data reasonable well. However, the more observations (as in the dual substrate system) the better can possible differences between the two sets of estimates be revealed.
Test in Run X:

$$-2 \cdot (-77.4 - (-23.7 - 12.6 - 15.4)) = 51.4 > \chi^2(14) = 23.7$$

The second estimation for Run X was performed with fixed inhibition and half-saturation coefficients for benzene z_b and K_{sb}. The values were set to average values, respectively equal to 0 and 0.413. The results are shown in Fig. 8.18 and the parameter estimates are given in Table 8.9. As for the previous estimation a common set of estimates could not be accepted.

	Individual				Common
Parameters	Batch A	Batch B	Batch C	SD	Batch A, B, and C
k_b	0.116	0.213	0.274	0.053	0.240
K_{sb}	0.425*	0.425*	0.425*	-	0.425*
Y_b	0.000	0.344	0.465	0.120	0.448
z_b	0*	0*	0*	-	0
k_t	0.441	0.395	0.446	0.024	0.451
K_{st}	1.243	0.608	1.083	0.201	1.099
Y_t	0.926	0.848	0.772	0.060	0.772
z_t	0.425	1.171	1.982	0.590	1.640
b	0.004	0.000	0.003	0.002	0.003
σ_ε	0.092	0.080	0.080	0.006	0.093
X_0	0.893	0.821	1.006	0.037	0.893, 0.821, 1.006
lag phase	2.5	2.5	2.5	-	2.5
L	-24.34	-11.31	-15.35	-	-77.42

Table 8.9: Estimated parameter values for Run X, batch A, B, and C, individually and commonly. SD is the standard deviations, and L is the log likelihood value, and values with * were fixed during the estimation.

Figure 8.18: Estimation within Run X, z_b and $K_s b$ were fixed (0 and 0.425) a) batch A, b) batch B), and c) batch C. □ biomass obs. ◇ toluene obs. △ benzene obs.

Many of the parameter estimates changed surprisingly little between the two estimations ($z_b = 0$, $z_t = 0.96$ and $z_b = 0$, $K_{sb} = 0.425$), even though the fixed values were quite different. With the relatively strong parameter correlation one would have expected the opposite. The reason for the small changes in the estimates could, however, be due to the large number of parameters in the model.

8.3 The structures of the correlation matrices

The structure of the correlation matrices varied from experiment to experiment, but the main structures were the same. The examination of the correlation matrices is divided into two parts; 1) for the single substrate system and 2) for the dual substrate system.

Single substrate system

A strong positive correlation between k and K_s, a negative correlation between k and Y, and a small positive correlation between K_s and Y were in common for all the structures in the single substrate system. An average correlation matrix for the single substrate system based on the correlation matrices, is given in Table 8.10. A few covariance matrices could not be used, due to a very large conditional number - see chapter 7.

Avoiding correlations between the parameter estimates are impossible due to the structure of the model (Box & Lucas, 1959), however the correlations can be minimized. See chapter 6 for more on the subject.

	k	K_s	Y
k	1	0.7	-0.6
K_s	0.7	1	0.1
Y	-0.6	0.1	1

Table 8.10: Average correlations matrix for the single substrate system.

Dual substrate system

An average correlation matrix for the dual substrate system based on the correlation matrices from the 12 experiments, is given in Table 8.11. A few covariance matrices could not be used, due to very large conditional number - see chapter 7.

	k_b	K_{sb}	Y_b	k_t	K_{st}	Y_t	z_t	b
k_b	1	**0.8**	0.2	0.3	0.0	-0.2	**0.9**	0.0
K_{sb}	**0.8**	1	0.1	0.1	0.1	-0.1	**0.8**	0.1
Y_b	0.2	0.1	1	**0.6**	0.1	**-0.6**	0.0	0.5
k_t	0.3	0.1	**0.6**	1	0.4	**-0.8**	0.1	0.0
K_{st}	0.0	0.1	0.1	0.4	1	0.0	0.2	0.0
Y_t	-0.2	-0.1	**-0.6**	**-0.8**	0.0	1	0.0	0.0
z_t	**0.9**	**0.8**	0.0	0.1	0.2	0.0	1	0.0
b	0.0	0.1	0.5	0.0	0.0	0.0	0.0	1

Table 8.11: Average correlations matrix for the dual substrate system.

A strong positive correlation between k_b and K_{sb}, between k_b and z_t, and between z_t and K_{sb} were in common for all the structures in the dual substrate system. And likewise a strong negative correlation between k_t and Y_t, and between Y_b and Y_t, and a positive correlation between k_t and Y_b. For minimizing the correlations between the parameter estimates see chapter 6.

When comparing the results from Table 8.11 with the single substrate system (Table 8.10), one will notice that the strong negative correlation between k_t and Y_t is also found in the dual substrate system, but that the strong correlation between k_t and K_{st} is not found in the dual substrate system. This indicates that parameter estimates are more or less depending on the system in which they are conducted. It is not so that k_t and K_{st} are not correlated, they are just less correlated compared to the other parameter correlations.

8.4 Overlook of the estimates from single and dual substrate systems

The Monod model in the single substrate system was reduced to a Monod model with no biomass decay; and the Bailey & Ollis' model in the dual substrate system was reduced to a competitive model where benzene did not inhibit the toluene degradation but toluene inhibited the benzene degradation. The reduction of the Bailey & Ollis' model was very convincing since all estimates of z_b (except from one) were already equal to zero when using the full model.

In spite of the fact that the experiments have a limited reproducibility it is still of interest to know the magnitude of the parameter estimates and of the variances that one could expect to obtain in a future experiment like these. For this reason averages and corresponding standard deviations for the parameter estimates are calculated (using the equations given in chapter 4) and the results are displayed in Table 8.12.

In the single substrate system it was as we recall possible to describe experiments within the same run with a common set of parameter estimates, but not for experiments from different runs. This situation is reflected in the standard deviations given in Table 8.12, where the standard deviations within the runs are smaller than the standard deviations between the runs. The standard deviations within the experiments are in general smaller than any of the other standard deviations - this holds for both the single and the dual substrate system. In the dual substrate system it was as mentioned earlier not possible to describe the experiments with a common set a parameter estimates neither for experiments within the runs nor for experiments from different runs. This situation is also reflected in the standard deviation given in Table 8.12, where $\hat{\sigma}_{wr}$ for some parameter estimates are smaller than $\hat{\sigma}_{br}$ and for other larger, in other words, there is no difference in the magnitude of the standard deviations within and between the runs.

Experiment	Parameter	Average value	$\hat{\sigma}_{we}$	$\hat{\sigma}_{wr}$	$\hat{\sigma}_{br}$	$\hat{\sigma}_t$
Single sub.	k_t (mg/mg/hour)	0.60	0.02	0.03	0.17	0.09
	K_{st} (mg/mg/hour)	0.16	0.06	0.05	0.23	0.13
	Y_t (mg/mg)	0.75	0.03	0.04	0.06	0.05
Dual sub.	k_b (mg/mg/hour)	0.21	0.03	0.07	0.05	0.06
	K_{sb} (mg/mg/hour)	0.43	0.13	0.33	0.20	0.30
	Y_b (mg/mg)	0.38	0.17	0.28	0.16	0.25
	k_t (mg/mg/hour)	0.52	0.19	0.08	0.10	0.08
	K_{st} (mg/mg/hour)	0.72	0.10	0.70	0.60	0.68
	Y_t (mg/mg)	0.83	0.07	0.10	0.17	0.12
	z_t	0.96	0.27	0.42	0.37	0.41
	b (/hour)	0.01	0.01	0.01	0.02	0.01

Table 8.12: The average values were calculated from all batch experiments belonging to the single or the dual substrate system. $\hat{\sigma}_{we}$ is the standard deviation for parameter estimates within the experiment and is given 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 $\hat{\sigma}_t$ is the total standard deviation.

A general result in the dual substrate system is that the estimates of the maximum degradation coefficient for toluene, k_t, were larger than for benzene, k_b, and that the estimates of the yield coefficient for toluene, Y_t, were larger than for benzene, Y_b.

The parameter values given in Table 8.12 are not independent of each other. One should be aware of that some of the parameter estimates are strongly correlated (see the section above).

8.5 Three different estimation techniques

Different optimization methods used for estimation in the same nonlinear model describing the same experiment can yield different parameter estimates. In the following parameter estimates obtained from Lineweaver-Burk linearization, AquaSim, and from Dekimo are compared. The experiment used for illustrating all three estimation methods was Run I, batch A. Run VII, batch B (a dual substrate experiment) was moreover used in comparing AquaSim and Dekimo. For matter of simplicity the measurement errors were assumed univariate, i.e. the weight function w_{ij} in Dekimo was set to 1. Linear regression analysis was carried out for the Lineweaver-Burk linearization (Fig. 8.19).

Figure 8.19: Linear regression used in the Lineweaver-Burk linearization

The regression line is mostly controlled by only the two last points, which are the two substrate measurements with the lowest concentrations. From the cut off and the slope of the line, k and K_s were determined (see chapter 4 for calculations of the parameter values). In Table 8.13 the parameter estimates from the Lineweaver-Burk are given together with the estimates from AquaSim and Dekimo for Run I, batch A. The model fitting based on parameter estimates from Lineweaver-Burk is shown in Fig. 8.20, and the

model fitting based on parameter estimates from AquaSim and Dekimo, respectively is shown in Fig. 8.21. In order to compare the three different fits the log likelihood values were calculated for all of them by fixing all parameter values and letting Dekimo estimate the likelihood values. Parameter estimates obtained from Dekimo fit the data best. The estimation with AquaSim was almost as good, however, standard deviation and parameter correlation matrix was not estimated by the program.

Parameter	Lineweaver-Burk	AquaSim	Dekimo	SD
k_t (mg/mg/hour)	0.73	1.00	1.28	0.33
K_{st} (mg/mg/hour)	0.41	0.86	2.39	1.28
Y_t (mg/mg)	0.85	0.85	0.86	0.03
b (/hour)	0	0	0	-
X_0 (mg/l)	0.08	0.04	0.04	0.03
log likelihood	-25.68	-13.46	-12.36	-

Table 8.13: Parameter estimates for Run I, batch A, estimated by Lineweaver-Burk, AquaSim, and Dekimo. SD ($\hat{\sigma}_{we}$) is the standard deviation estimated by Dekimo.

The same picture is seen with the estimations in the dual substrate system. The log likelihood values for the two kinds of estimations (Dekimo and AquaSim) were practically the same, but AquaSim did not calculate the standard deviations on the parameter estimates. The model fittings are shown in Fig. 8.5 and 8.23. With different initial values for the parameters (Table 8.14) three quite different sets of estimates were obtained (Table 8.15) in AquaSim in spite of that the model fits were almost identical. With the same three initial values, three different sets of estimates were obtained in Dekimo. The estimates obtained by Dekimo were, however, much more alike as seen in Table 8.14. Dekimo seems better in finding the global optimum, since the three sets of parameter estimates were must more alike compared to the optimum found by AquaSim. However, since the parameters are highly correlated, all 6 optima (global or not) fit the data very well.

Figure 8.20: Run I, batch A estimated by the Lineweaver-Burk method. □ biomass obs. ◇ toluene obs.

Parameter	Initial values		
k_b (mg/mg/hour)	0.170	0.377	0.493
K_{sb} (mg/mg/hour)	0.244	1.525	1.946
Y_b (mg/mg)	0.451	0.241	0.321
z_b	3.694	0.489	0.431
k_t (mg/mg/hour)	2.100	0.747	0.729
K_{st} (mg/mg/hour)	0.041	1.117	1.159
Y_t (mg/mg)	0.852	0.952	0.914
z_t	0.085	1.192	1.654
b (/hour)	0.001	0.003	0.003
X_0 (mg/l)	0.090	0.143	0.200

Table 8.14. Three different sets of initial values used for estimating in AquaSim and Dekimo.

Figure 8.21: Run I, batch A estimated by AquaSim and Dekimo. □ biomass obs. ◇ toluene obs.

Parameter	AquaSim			Dekimo			SD
k_b (mg/mg/hour)	0.170	0.494	0.602	0.147	0.152	0.160	0.03
K_{sb} (mg/mg/hour)	0.153	1.795	2.354	0.001	0.006	0.031	0.17
Y_b (mg/mg)	0.439	0.241	0.308	0.315	0.360	0.341	0.16
z_b	3.877	0.295	0.318	0.520	0.379	0.201	0.18
k_t (mg/mg/hour)	1.971	0.702	0.660	0.746	0.644	0.563	0.07
K_{st} (mg/mg/hour)	0.086	1.475	1.093	0.339	0.000	0.000	0.05
Y_t (mg/mg)	0.844	0.948	0.915	0.794	0.767	0.773	0.08
z_t	0.157	1.914	2.789	0.003	0.040	0.103	0.14
b (/hour)	0.000	0.003	0.003	0.001	-0.001	-0.001	0.002
X_0 (mg/l)	0.133	0.206	0.230	0.200	0.222	0.248	0.05
log likelihood	-40.68	-45.46	-42.36	-39.57	-39.56	-39.27	-

Table 8.15. Parameter estimates for Run VII, batch B, estimated by AquaSim and Dekimo. SD ($\hat{\sigma}_{we}$) is the standard deviation estimated by Dekimo. With three different initial values of the parameters, different estimates were obtained.

Figure 8.22: Run VI, batch B estimated by AquaSim for three different initial values. □ biomass obs. ◇ toluene obs. △ benzene obs.

Figure 8.23: Run VI, batch B estimated by Dekimo for three different initial values. □ biomass obs. ◇ toluene obs. △ benzene obs.

Chapter 9

Discussion

Reproducibility

For both the single and the dual substrate system the experiments which were carried out the same days (within the runs) resembled each other more than experiments which were carried out on different days (between the runs). In the single substrate system, the likelihood ratio test was in agreement with these observations, since the test could accept common sets of parameter estimates within the runs but not between the runs. In the dual substrate system the likelihood ratio test rejected all hypothesis of common sets of parameter estimates. However, the fact that the lag phases between the runs varied considerably but were exactly the same within the runs, indicates that the experiments were more uniform within the runs than between the runs.

Since the experiments were more uniform within the runs than between, it seemed reasonable to suspect the biomass of causing the variability. The biomass in experiments carried out the same day came from the same preculture, whereas the biomass in experiments carried out on different days

came from physical different precultures, which though originated from the same frozen stock culture (see Fig. 2.1, chapter 2). This fact is the only known variation between the runs, all other factors should be as identical as within the runs. A possible reason for the variation in the preculture may be due to variations on the agar plate in the early growth phases of the bacteria. The bacteria may e.g. have mutated and when starting the "real" experiments the biomass could therefore be genetically different from the biomasses in other runs. Even though the biomass was grown on agar plates after each experiment and showed the same morphology as previously. Another explanation of the greater variation between the runs involves the conversion of mg protein/l into mg biomass/l. As mentioned in chapter 2 the conversion with a factor 2 was not exact, but showed some variation. If the protein measurements from one or more batch experiments should have been converted by e.g. 1.8 instead of 2, the estimated parameter values would have been different, so would the log likelihood values and the tests likewise (see appendix E). A third explanation could be the variation of duration time of the precultures (the time between starting the preculture by adding bacteria from the agar plates until the preculture was used as inoculum in the “real experiments”). The biomass is likely to be more active just after the exponential phase than later on. Several hours later, part of the biomass may have died or would be in a starvation phase. In Table 9.1 a review of the duration time of the precultures together with the estimated lag phases is shown.

	Single substrate			Dual substrate			
Run	I	II	III	IV	V	VI	VII
Duration time	51	$46\frac{1}{2}$	47	$44\frac{1}{2}$	46	$42\frac{1}{2}$	45
Lag phase	0	0(2)	0	2	7	0	5

Table 9.1.: Review of the duration time (in hours) of the precultures and of the lag phases in the runs. The (2) refers to experiments where the lag phase is uncertain and maybe should be two.

In the single substrate system, a slow start or perhaps a lag phase in Run II, cannot be explained by a longer duration time of the preculture compared to Run I and III. However, this does not exclude the explanation that the biomass in the preculture for Run II had suffered the longest period of starvation or death before it was used as inoculum in the "real experiments". The toluene in this preculture may have degraded faster due to less toluene and more bacteria initially than in the precultures for Run I and II.

In the dual substrate system it seems more likely that there may be a connection between the duration time of the precultures and the lag phases. The lag phases cannot be explained by the presence of benzene, since there was no lag phase in Run VI.

Likelihood ratio test
In the discussion of the likelihood ratio tests appropriateness for these microbiological degradation experiments, several aspects are considered. The appropriateness of the test is evaluated by looking at the figures and the standard deviations of the estimates. In the single substrate system the likelihood ratio test accepts common sets of estimates within the runs and rejects between the runs. This result is reflected in the model fittings. The common estimations within the runs fit the data very well compared to the individual estimation. Between the runs the fittings by common estimations are less good. These observations are also reflected in the average standard deviation (SD) for the parameter estimates. SD within the runs are smaller than SD between the runs (see chapter 8, section 8.4).

In the dual substrate system the likelihood ratio test rejects all hypothesis of common estimates, both within and between the runs. When looking at the average standard deviations there are no difference between the SD for parameter estimates within the runs compared to the SD for estimates between the runs. When looking at the model fittings the rejection of common sets of parameter estimates may seems reasonable except for one run (Run IV). The common estimation for Run IV is practically identical to the individual estimations. The exact reason why the likelihood ratio test rejected

the common set of parameter estimates is not known. One reason might be that the strong correlation between the substrate responses (toluene and benzene), which is not accounted for in the likelihood expression, has some influence on the test. In order to examine this, it would require a large number of simulations with known parameter values and known structure of the measurement errors.

Statistics
The program (Dekimo) used in this study to estimate the parameters of the microbiological degradation models, are based on different assumptions. One of these assumptions was, however, violated, namely the assumption of no correlations between the responses. It turned out that the substrate measurements (toluene and benzene) were strongly correlated. In spite of this, the analysis showed that a simple model based on no correlation between the responses proved to be effective. The strong correlation could give rise to singularity in the design matrix and cause difficulties in convergence. The strong correlation between the responses may as mentioned earlier also have an influence on the likelihood ratio test. One of two solutions to this problem may be considered 1) instead of one sample to determine benzene and toluene two samples could be taken from the batches or 2) include the correlation in the model building.

A more numerically related aspect is the time step size, which has relative large influence on the value of the estimates. The step sizes in Dekimo are variable, large when little change in the rate of differential equations and small when a great change in the rate was detected. Experience from estimating in a spreadsheet, where the integration of the differential equations were performed by step-wise linearization, showed however that the size of the steps have a large influence on the modelling and thereby on the parameter estimates. The step sizes in Dekimo should be adequately small in order to ensure correct parameter estimates - the step sizes are determined by the BDF method. However, if the change in rate is too large e.g. when the curve breaks off convergence problems may occur due to difficulties in determining an appropriate time step size. This situation may occur if e.g.

the Monod model is modelled with a very small K_s value (less than 0.001).

The model employed to describe the microbiological degradation experiments in this study consists of a biological model, which is deterministic and a "statistic" part (measurement error), which is stochastic. Other models may also have been used in describing the processes under consideration e.g. the stochastic differential models. Stochastic differential equations introduce the possibility of formulating more elaborate dynamical models for degradation kinetics. Parameterization of the stochastic nature of the measurement error component makes it possible to account for the occurrence of highly correlated responses with time (not to be mistaken with the correlation between different responses). However, statistical models of degradation kinetics based on nonlinear, stochastic differential equations require more accurate measurements of both the substrate compounds and the biomass compound, compared to currently available methods.

Chapter 10

Conclusions

Nonlinear parameter estimation methods and examination of reproducibility in microbiological degradation experiments are two very important but overlooked phenomena. This is quite disturbing since it is current practice to use the parameters as characteristics of the biological processes.

Incorrect parameter estimates are easily obtained if an inappropriate estimation method is used, e.g. linearization, including parameter values from other experiments, or an incorrect error structure in a nonlinear estimation method. The parameter estimates vary from one experiment to another even though they are conducted under almost exactly the same experimental conditions. Therefore the variability and the reproducibility should also be examined.

In the present study an iterative maximum likelihood estimation routine was implemented for parameter estimation, and the measurement error structure was mainly determined from repeated measurements. The estimation program (Dekimo) successfully found the global optimum, for the two non-

linear models, Monod and Bailey & Ollis. Parameter estimation using two other methods than the one employed in the study by Dekimo showed some interesting results. The linearization method Lineweaver-Burk resulted in parameter estimates which did not provide a good fit of the data. The nonlinear program AquaSim gave good model fits but had trouble finding the global optimum especially for Bailey & Ollis' model. Moreover, the program did not estimate standard deviations for the parameter estimates due to a poor functioning secant routine. Instead it was necessary to use the more simple Simplex routine, which did not provide the user with standard deviations of the parameter estimates.

In the present study of microbiological degradation experiments it was not possible to reproduce the experiment completely, that is it was not possible to describe all 9 and 12 experiments in the single and the dual substrate systems, respectively, with one common set of parameter values for each system. However, for the single substrate system it was possible to estimate common sets of parameter values within the runs (experiments carried out on the same day). Looking at the fitted models for the dual substrate system common sets of parameter estimates within the runs seemed possible, but the likelihood ratio test rejected this hypothesis. The fact that the hypothesis was rejected might be due to the strong correlation between the substrate responses, which were not accounted for in the likelihood ratio test. For both the single and the dual substrate system, common sets of parameter estimates between the runs did not seem likely, because the differences were too large. It is believed that it was the variability of the biomass in the preculture that caused the limited reproducibility. It is disturbing that some of the least complicated experiments on microbiological degradation seems to have limited reproducibility.

In spite of the limited reproducibility, the experiments in the single and the dual systems, had several features in common. All experiments in the single substrate system could be estimated without the biomass decay ($b = 0$). All experiments in the dual substrate system were estimated without inhibition of the toluene degradation ($z_b = 0$). All the estimations showed that the yield coefficients Y for toluene were larger than for benzene, and that the maximum degradation coefficients k for toluene were larger than for benzene. Furthermore, the correlation matrices were very much alike within the single and the dual substrate systems, which also show some similarity even though the experiments are said to have limited reproducibility.

It is often seen that several sets of parameter estimates with quite different values fit a given data set almost equally well (present thesis and Holmberg & Ranta, 1982). This can be due to
1) strong correlations between the parameter estimates,
2) relatively large measurements errors,
3) nonlinearity of the model (Box & Lucas, 1959).

The large variations which are often reported in parameter estimates obtained from different experiments performed under similar conditions (Holmberg, Sievänen & Carlberg, 1980) can thus be explained. The reproducibility of experiments is often uncertain because it can be difficult to obtain exactly the same environmental conditions and prevent changes in the internal state of the organism. In this study we have concentrated on performing experiments under the same environmental conditions for all the runs. The procedure of the precultures should though maybe have been even more identically performed, however, preventing changes in the internal state of the organism was not possible.

The parameters should not be used as biological characteristics, which is current practice. They depend on the system in which they were estimated. This was also seen in the present study when the average parameter values for toluene obtained in the single substrate system were used in the dual substrate system - the modelling fitted the data very poorly. The idea of comparing the parameters and using them as biological characteristics, should therefore be critically reconsidered.

Appendix A

Chemical recipes

Glycerol medium

Tryptonesoybroth (TSB)	30 g
Glucose	5 g
Skim-milk powder	20 g
Glycerol 87%	40 g
Distilled water	1 litre

Stock solution of toluene and benzene

1 litre of distilled water was autoclaved. When cooled off, concentrated toluene (96%) or benzene was added using a sterile pipette. The stock solution was stirred for one day to ensure total dissolution. Stock solution used in the experiments were taken from the bottom of the "stock solution batch".

Growth medium - concentrations i batch experiments

Stock solution	Concentration in experiments (mg/l)
Nutrient	
KNO_3	14.25
$Mg(NO_3)_2$, $6H_2O$	58.15
$Ca(NO_3)_2$, $4H_2O$	65.00
$Fe(NO_3)_2$, $9H_2O$	3.98
NH_4NO_3	63.1
Tracer metals	
$MnSO_4$, H_2O	0.093
$Co(NO_3)_2$, $6H_2O$	0.160
$Na_2B_4O_7$, $10H_2O$	0.052
$Zn(NO_3)_2$, $6H_2O$	0.164
Na_2MoO_4, $2H_2O$	0.133
$NiSO_4$, $7H_2O$	0.077
KJ	0.020
EDTA (di-Na)	1.100
$CuSO_4$	0.138
Phosphate buffer	
Na_2HPO_4, 12 H_2O	2865
KH_2PO_4	626

Concentrated solutions of the nutrient and the trace metals were made up as stock solutions, stored at room temperature in a dark closet, and diluted appropriately for use. The phosphate buffer was added as powder.

Appendix B

Procedures

PROCEDURE FOR PREPARING AND PERFORMING EACH RUN

Agar plate
The agar media was made from Casein-peptone glucose yeast. Five plates were made (one for use as inoculum, one for use after the preculture experiment, and one for use after the "real" experiments). The plates were put in a dark cabinet at 30° C for a day in order to ensure that the plates were sterile (if contaminated bacteria would grow on the plates).

Grafting
In order to prevent that the biomass would defrost during the transportation from the basement to the laboratory, a small ice/water bath were made. With a sterile grafting knife a small amount of biomass was put on a plate and placed in a dark cabinet at 30° C for a day.

Autoclave
The autoclaving lasted 4 hours.

- Four 5 litre bottles (for batch A, B, C, and blank test) each containing 4 litre of distilled water + nutrient (2 ml of each standard solutions, see appendix A) + a magnet.
- One 1 litre bottle (for the preculture) containing 0.6 litre of distilled water + nutrient + magnet.
- Two 1 litre bottles (for production of standard solutions of benzene and toluene) containing 1 litre of distilled water.
- Four large and three smaller glass tops for the bottles.
- Three measuring cups with phosphor mixture, various small bottles, measuring cups, and flasks

Preculture
In order to measure the amount of toluene, which was added to the preculture, the following procedure was used. 50 ml pentane mixture was produced (345 mg heptane + 50 ml double distilled pentane). 1 ml toluene standard solution were dissolved in a 10 ml flask and after that measured on a gas chromatograph by extracting toluene into the pentane mixture. The amount of toluene was then calculated and added to the preculture together with the phosphor mixture and 3 colonies of bacteria from the agar plate. When carrying out the grafting one should be aware of not to use a too hot grafting knife, otherwise the heat would kill the bacteria. The inner side of the bottleneck was smeared with silicone (in order to make the bottle gas tight) before the glass top was put on. The bottle was then put on a magnet stirrer and a sample was taken. The next day more samples were taken to see if the toluene was degraded. If and when the toluene was degraded the preculture was ready to be use as inoculum and the last preparations of the "real" experiments could begin.

Preparing for the batches experiments
Two flasks of pentane mixture were prepared (each consisting of 100 ml pentane mixture) and the amount of toluene/benzene were calculated and

poured into sterile flasks (this should not be done to much time ahead of the start of the experiment, otherwise the substrate(s) will evaporate from the flasks). The phosphate mixture was added the 4 autoclaved 5-litre bottles each containing 4 l of distilled water, grow medium, and a magnet. Before starting the experiments the bottles were heated up to 28° C. The measured amount of substrate and about 130 ml biomass from the preculture for each bottle were then poured into the 5-litre bottles under sterile conditions. Each batch was shaken violently and stirred for 15 minutes before sampling. This was done in order to ensure homogeneously condition in the medium.

The experiment

Each 15 minutes two samples were taken from one of the four batches by injecting air through a sterile filter to insure overpressure. One sample for the biomass determination and the other for the substrate(s) determination. TCA was added to the biomass sample, a lid was put on and the tube was shaken violently and then placed in a refrigerator. To the substrate sample 1 ml pentane mixture was added, a lid was put on and the tube was shaken violently and then placed in a refrigerator upside down. After the experiment was done samples were taken, diluted and spread on an ager plate to ensure that the bacteria look the same as before the preculture was started.

LOWRY METHOD FOR PROTEIN DETERMINATION

Reactions

Formation of the protein-copper complex. Reduction of the phosphomolybdate-phosphotungstate reagent (Folin-Ciocalteu phenol reagent) by tyrosine and tryptophan residues.

Reagents

Production of reagent 1:
A: 2 g Na_2CO_3 dissolved in 100 ml 0,1 N NaOH
B: 2 g Na-K-tartrate (Sodium potassium tartrate) in 100 ml distilled water
C: 1 g $CuSO_4$, $5H_2O$ in 100 ml distilled water.

Reagent 1: Mix 100 ml A + 1 ml B + 1 ml C

For each sample 2.5 ml reagent 1 is used. Solution A is durable for 2 weeks in a refrigerator. The durability of solution B and C are months. Reagent 1 must be fresh daily.

Reagent 2
Folin-Ciocalteus phenol reagent. Durable until change of colour to green.

Protein 1
300.0 mg Bovin Serium Albumin
100.0 ml distilled water

The solution can be frozen at −20° C in batches of 1.5 ml. Durability: about a year in a freezer.

Protein 2
Dilute Protein 1 10 times by 1) weighting a small flask, 2) adding 1.0 ml Protein 1, and 3) adding distilled water corresponding to 9 x the weight of the protein. Protein 2 is made fresh daily

3 M Trichlor acetic acid (TCA)
Dissolve 122.6 g TCA in 250 ml distilled water. Use gloves ! the solution is corrosive and suspected of being carcinogenic.

0.66 N NaOH
Dissolve 13.2 g NaOH in 500 ml distilled water.

1.32 N NaOH
Dissolve 10.6 g NaOH in 200 ml distilled water.

Sampling

Immediately after sampling from a batch, TCA is added and the tube is shaken. The concentration of TCA in the tube should be about 0.5 M, e.g., to a sample of 10 ml, 2 ml is added. The tubes are closed with a lid and placed in the refrigerator until analysed. The tubes used when sampling should be the same as used when centrifuging.

Preparation of the samples
The samples are centrifuged at 4500 rotations/min. for 20 minutes. When done, the samples are decanted and as much water as possible is removed e.g. by sucking up the water with a pipette. Be careful - it is better to leave a drop of water instead of risking to suck up some of the biomass at the bottom of the tube. 0.5 ml 0.66 N NaOH is added to each sample and shaken on a Whirley mixer and the lids are put back on. The samples are then incubated for 48 hours at 37° C.

Analyzing the samples and the standard curve

- Prepare Reagent 1
- Construct a standard curve as the shown in Table 1 with replicates. Be (as always) very accurate when adding the preparations. Protein 2 should be added as the last.

Standard No.	Total protein μg	Distilled water μl	1.32 N NaOH μl	Protein 2 μl
1	0	250	250	0
2	3	240	250	10
3	6	230	250	20
4	15	200	250	50
5	30	150	250	100
6	45	100	250	150
7	60	50	250	200
8	75	0	250	250

Table 1. Scheme for making standard curve.

- Prepare the spectrophotometer for use (see the manual in the laboratory).
- Add 2.5 ml Reagent 1 to standards/samples. Incubate standards/ samples precise 10 min. (use stop watch, the timing is critical). Prepare e.g. 8-10 samples at a time with an interval of e.g. 20 second apart.
- Add 250 μ l Reagent 2 at the same interval as above and shake with Whirley mixer.
- Incubate at precise 30 min. before measuring.

Calculation of biomass
Concentration of protein = The measured amount of protein divided by ml sample which was taken from the batch. The result is given in μ g protein/ml or in mg protein/l. If converted into mg dry weight biomass/l, it is necessary to know the percentage concentration of protein in the biomass (usually about 50%).

Appendix C

Calculations

STANDARD CURVES FOR TOLUENE

The regression curve for all 16 standards seems to describe the data very well (Fig. C.1). But when looking only at the low concentrations the regression curve does not describe the data so well (Fig. C.2). The regression curve for the full data set was:

$$\text{Conc.} = 0.8289 + 6.3109 \cdot \text{Area}$$

Thus two regression curves were calculated. For the low concentrations the regression curve was:

$$\text{Conc.} = 0 + 7.1476 \cdot \text{Area}$$

The intercept was not significantly different from zero and was therefor set to zero (Fig. C.3). For the high concentrations the regression was (Fig. C.4):

$$\text{Conc.} = 4.1364 + 6.0139 \cdot \text{Area}$$

Figure C.1: Regression curve for the full data set for toluene standards

Figure C.2: Regression curve for the full data set for toluene standards, low concentrations

Figure C.3: Regression curve for the low concentrations of the toluene standards

Figure C.4: Regression curve for the high concentrations of the toluene standards

STANDARD CURVES FOR BENZENE

There were made two regression curves for benzene of the same reason as for the toluene standard curve. For the low concentrations the intercept was set to zero (not significantly different from zero) and the regression was thus found to be:

$$\text{Conc.} = 0 + 6.1624 \cdot \text{Area}$$

For the high concentrations the regression was:

$$\text{Conc.} = 3.5293 + 5.3097 \cdot \text{Area}$$

In Fig. C.5 and C.6 the regression curves are seen for low and high concentrations respectively.

Figure C.5: Regression curve for the low concentrations of the benzene standards

Figure C.6: Regression curve for the high concentrations of the benzene standards

DETERMINATION OF THE DRY WEIGHT OF THE BIOMASS

IMT's laboratory procedure was followed. From each batches (A, B, and C) three samples were taken - some of 200 ml and some of 400 ml. The measured mg dry weight biomass/l were compared to the measured average values of mg protein/l found by the Lowry method. The conversion factors for the three batches are given in the following table.

Batch	Conversion factors			Average values	Standard deviations
A	1.7	2.0	2.5	2.1	0.4
B	1.7	2.5	3.1	2.4	0.5
C	1.4	1.5	1.5	1.5	0.1

Appendix D

Adjustment with the blank test

The blank tests were in some situations used for adjusting the substrate concentrations. If an unusually pattern were found in the three batch experiments A, B, and C and the same pattern were recognized in the blank test, when the blank test was used for adjusting. The unusually pattern could occur when using the last of an extractions solvent bottle (pentane + heptane) and changing to a new bottle. When using the last 20% of the solvent of the bottle the pentane/heptane concentration changed dramatic. After the two first runs more that 80% of the pentane mixture was never used (this is also referred to as learning sequential). Not all the experiments were adjusted by the blank tests. In this appendix an example from Run II is given. All three batches A, B, and C were adjusted but only batch C is shown here. In Fig D.1 the original measurements are shown and in Fig. D.2 after adjustments. The same characteristic drop in concentration level were found in the blank test (Fig. D.3). In this case the drop in concentration level is probably due to the equilibrium (of substrate between

headspace and media), which was not quite in balance at the start of the experiment The adjustment was carried out by determine a regression of the 7 measurements seen in Fig. D.3 of the blank test, calculating it back to a horizontal line, and use this recalculation on the measurements from the batch experiment.

Figure D.1: Original data set, Run II, batch C

Figure D.2: Adjusted data set, Run II, batch C

Figure D.3: Regression line in the blank test, Run II

Appendix E

Protein conversion factor

The conversion factor (from mg protein/l to mg biomass/l) used in this study was equal 2. This factor may though vary from experiment to experiment. The conversion factor has influence on the parameter estimates. An example is here given with a factor equal 3.5. In practice less variation of the estimated conversion factor is though expected.

Parameter	Conversion factor=2	Conversion factor=3
k_b (mg/mg/hour)	1.572	1.208
K_{sb} (mg/mg/hour)	0.622	0.296
Y_b (mg/mg)	0.476	0.412
b (/hour)	-0.010	-0.007
X_0 (mg/l)	0.030	0.055

Figure E.1: □ biomass obs. ◇ toluene obs.

References

ALVAREZ, P. J. J. and VOGEL, T. M., 1991, Substrate interactions of benzene, toluene, and para-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries, *Appl. Environ. Microbiol.*, **57**, 2981–2985.

ALVAREZ-COHEN, L. and MCCARTY, P. L., 1991, A cometabolic biotransformation model for halogenated aliphatic compounds exhibiting product toxicity, *Environ. Sci. Technol.*, **25**, 1381–1387.

ARVIN, E., JENSEN, B. K., and GUNDERSEN, A. T., 1989, Substrate interactions during aerobic biodegradation of benzene, *Appl. Environ. Microbiol.*, **55**, 3221–3225.

ATKINS, P. W., 1986, *Physical Chemistry*, Oxford University Press, third edition edition.

BAILEY, J. E. and OLLIS, D. F., 1977, *Biochemical Engineering Fundamentals*, McGraw-Hill, New York, international student edition.

BARD, Y., 1974, *Nonlinear Parameter Estimation*, Academic Press, New York.

BATES, D. M. and WATTS, D. G., 1988, *Non-linear Regression Analysis and it's applications*, John Wiley and Sons, New York.

BECK, J. V. and ARNOLD, K. J., 1977, *Parameter Estimation in Engineering and Science*, Wiley, New York.

BILBO, C. M., 1992, *Statistical Analysis of Multivariate Degradation Models*, PhD thesis, Technical University of Denmark, DTU, Institute of Mathematical Modelling, Buil.321, Lyngby.

BJØRNEBOE, K., 1990, Stochastic differential equations and biological systems., Master's thesis, Technical University of Denmark, DTU, Institute of Mathematical Modelling, Build. 321, Lyngby.

BOX, G. E. P. and COX, D. R., 1964, An analysis of transformation, *J. Roy. Statiat. Soc.*, **B-26**, 211–243, discussion 244–252.

BOX, G. E. P. and DRAPER, N. R., 1987, *Empirical Model-Building and Response Surfaces*, Probability and Mathematical Statistics. Wiley, New York.

BOX, G. E. P. and LUCAS, H. L., 1959, Design of experiments in nonlinear situations, *Biometrica*, **46**, 77–90.

BOX, G. E. P. and TIDWELL, P. W., 1962, Transformation of the independent variables, *Technometrics*, **4**, 531–550.

BROHOLM, K., CHRISTENSEN, T. H., and JENSEN, B. K., 1992, Modelling tce degradation by a mixed culture of methane-oxidizing bacteria, *Wat. Res.*, **26**, 1170–1185.

BURDEN, R. L., FAIRES, J. D., and REYNOLDS, A. C., 1978, *Numerical Analysis*, Prindle, Weber and Schmidt, Boston.

CARSTENSEN, J., 1994, *Identification of Wastewater processes*, PhD thesis, Technical University of Denmark, IMM, Build.321, 73.

CHANG, M. K., VOICE, T. C., and CRIDDLE, C. S., 1993, Kinetics of competitive inhibition and cometabolism in the biodegradation of benzene, toluene, and p-xylene by two pseudomonas isolates, *Biotechnol. and Bioeng.*, **41**, 1057–1065.

Chen, Y. R. and Hashimoto, A. G., 1978, Kinetics of methane fermentation, *Biotechnol. Bioeng. Symp.*, **22**, 2081–2095.

Cobelli, C. and DiStefano III, J. J., 1980, Parameter and structurel concepts and ambiguities: A critical review and analysis, *Am. J. Physiol*, **239**, R7–R24.

Cobelli, C., DiStefano III, J. J., and Ruggeri, A., 1983, Minimal sampling schedules for identification of dynamic models of metabolic systems of clinical interest: Case studies for two liver function tests, *Math. Biosci.*, **63**, 173–186.

Contois, D. E., 1959, Kinetics of bacterial growth. relationship between population density and specific growth rate of continuous cultures, *J. Gen. Microbiol.*, **21**, 40–50.

Cornish-Bowden, A. and Endrennyi, L., 1981, Fitting of enzyme kinetic data without prior knowledge of weights, *Biochem J.*, **193**, 1005–1008.

Cox, D. R. and Hinkley, D. V., 1974, *Theoretical Statistics.*, Chapman and Hall, London.

Criddle, C. S., 1993, The kinetics of cometabolism, *Biotechn. and Bioeng.*, **41**, 1048–1056.

Dennis, J. E. J. and Schnabel, R. B., 1983, *Numerical Methods for Unconstrained Optimation and Nonlinear Equations.*, Computational Mathematics. Prentice-Hall, New Jersey.

DiStefano III, J. J., 1980, Design and optimization of tracer experiments in physiology and medicine, *Fed. Proc.*, **39**, 84–90.

DiStefano III, J. J., 1981, Optimized blood sampling protocols and sequential design of kinetic experiments, *Am. J. Physiol.*, **240**, R259–R265.

Dowd, J. E. and Riggs, D. S., 1965, A comparison of estimates of michaelis-menten kinetic constants from various linear transformations, *J. Biol. Chem.*, **240**, 864–869.

Draper, N. R. and Smith, H., 1981, *Applied Regression Analysis*, Wiley, New York.

Fedorov, V. V., 1972, *Theory of Optimal Experiments*, Adademic Press, New York.

Flyvbjerg, J., 1992, *Anaerobic degradation of creosote-related aromatic compounds under nitrate-reducing conditions*, PhD thesis, Technical University of Denmark, DTU, Department of Environmental Engineering, Build. 115, Lyngby.

Folsom, B. R., Chapman, P. J., and Pritchard, P. H., 1990, Phenol and trichloroethylene degradation by pseudomonas cepacia g4: Kinetics and interactions between substrates, *Appl. Environ. Microbiol.*, **56**, 1279–1285.

Ford, I., Kitsos, C. P., and Titterington, D. M., 1989, Recent advances in nonlinear experimental design., *Technometrics*, **31**, 49–60.

Gallant, A. R., 1987, *Nonlinear statistical Models.*, Probability and Mathematical Statistics. Wiley, New York.

Garfinkel, L., Kohn, M. C., and Garfinkel, D., 1977, System analysis in enzyme kinetics, *CRC Crit. Rev. Bioeng.*, **2**, 329–361.

Gill, P. E., Murray, W., and Wright, M. H., 1981, *Practical Optimization*, Academic Press, New York.

Grau, P., Dohanyos, M., and Chudoba, J., 1975, Kinetics of multicomponent substrate removal by activated sludge, *Wat. Res.*, **9**, 637–642.

Han, K. and Levenspiel, O., 1988, Extended monod kinetics for substrate, product, and cell inhibition, *Biotech. and Bioengng.*, **32**, 430–437.

HICKS, C. R., 1982, *Fundamental Concept in the Design of Experiments*, Rinehart and Winston, third edition.

HOLMBERG, A., 1982, On the practical identifiability of microbial growth models incorporating michaelis-menten type nonlinearities, *Math. Biosci.*, **62**, 23–43.

HOLMBERG, A. and RANTA, J., 1982, Procedures for parameter and state estimation of microbial growth proces models, *Automatica*, **18**, 181–193.

HOLMBERG, A., SIEVÄNEN, R., and CARLBERG, G., 1980, Fermentation of Bacillus thuringiensis for exotoxin production - process analysis study, *Biotech. Bioengng*, **22**, 1707.

JENSEN, H. M., 1992, Nedbrydningskinetik for toluen og benzen, Master's thesis, Technical University of Denmark, DTU, Lyngby, 15/92.

JENSEN, H. M., 1994, *Cometabolic degradation of chlorinated aliphatic hydrocarbons*, PhD thesis, Technical University of Denmark, DTU, Institute of Environmental Science and Engineering, Buil. 115, Lyngby.

JUUSOLA, J. A., BACON, D. W., and DOWNIE, J., 1972, Sequential statistical design strategy in an experimental kinetic study., *Canadian J. Chem. Eng.*, **50**, 796–801.

KLOEDEN, P. E. and PLATEN, E., 1989, A survey of numerical methods for stochastic differential equations, *Stoch. Hydrology and Hydraulics*, **3**, 155–178.

KLOEDEN, P. E., PLATEN, E., and SCHURZ, H., 1991, The numerical solution of nonlinear stochastic dynamical systems: A brief introduction, *Interna. Jour. of Bifurcation and Chaos*, **1**, 277–286.

KOEPPE, P. and HAMANN, C., 1980, A program for nonlinear regression analysis to be used on desk-top computers, *Comp. Programs Biomed.*, **12**, 121–128.

LAMARCH, P. and DROSTE, R. L., 1989, Air-stripping mass transfer correlations for volatile organics, *Jour. AWWA*, p. 78.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDALL, R. J., 1951, Protein measurement with the folin phenol reagent, *J. Biol. Chem.*, **193**, 265–275.

LUONG, J. H. T., 1987, Generalization of monod kinetics for analysis of growth data with substrate inhibition, *Biotech. and Bioengng.*, **29**, 242–248.

MACHADO, R. J. and GRADY-JR., C. P. L., 1989, Environmental systems engineering, *Biotechnol. and Bioeng.*, **33**, 327–337.

MADSEN, H. and MELGAARD, H., 1991, The mathematical and numerical methods used in ctlsm, Technical Report 7/1991, IMM, Lyngby.

MONTGOMERY, D. C., 1991, *Design and Analysis of Experiments*, John Wiley and Sons, third edition.

MORI, F. and DISTEFANO III, J. J., 1979, imal nonuniform sampling interval and test input design for identification of physiological systems from very limited data, *IEEE, Trans. Automat. Control*, **AC-26**, 893–900.

MULCHANDANI, A. and LUONG, J. H. T., 1989, Microbial inhibition kenitics revisited, *Enzyme Microb. Technol.*, **11**, 66–73.

NELDER, J. A. and MEAD, R., 1965, A simplex method for function minimization, *Computing Journal*, **7**, 308–313.

NIELSEN, J., PEDERSEN, A. G., STRUDSHOLM, K., and VILLADSEN, J., 1991, Modeling fermentations with recombinant microorganisms: feromulation of a structured model, *Biotechn. Bioeng.*, **37**, 802–808.

PETERSEN, J. H., 1991, Stochastic differential equations, Master's thesis, Institute of Mathematical Modelling, DTU, 2800 Lyngby, Denmark.

PETERSON, G. L., 1979, Rewiew of the phenol protein quantization method of lowry, rosebrough, farr and randall, *Anal. Chem.*, **100**, 201–220.

Press, W. H., Flannery, B. P., Teukolsky, S. A., and Vetterling, W. T., 1989, *Numerical Recipes - The Art of Scientific Computing*, Cambridge University Press, Cambridge.

Ralston, M. L. and Jennrich, R. I., 1978, Dud, a derivative-free algorithm for nonlinear least squares, *Technometrics*, **20(1)**, 7–14.

Robinson, J. A., 1985, *Determining microbial kinetic parameters using nonlinear regression analysis.*, volume 8, chapter In: Advances in Microbial Ecology, pp. 61–114, Plenum Press, London.

Sáez, P. B. and Rittmann, B. E., 1992, Model-parameter estimation using least squares, *Wat.Res.*, **26**, 789–796.

Sambanis, A., Pavlou, S., and Fredrickson, A. G., 1986, Analysis of the dynamics of ciliate-bacterial interactions in cstr, *Chem. Eng. Sci.*, **41**, 1455–1469.

Simkins, S. and Alexander, J., 1984, Models for mineralization kinetics with the variables of substrate concentration and population density, *Appl. Environ. Microbiol.*, **47**, 1229–1306.

Spliid, H. and Tørsløv, J., 1994, Statistical analysis of joint toxicity in biological growth experiments, *Ecotox. and Environ. Safety*, **28**, 181–192.

Steffensen, P., 1991, Estimation af stokastiske differentialligninger, Master's thesis, Technical University of Denmark, DTU, Institute of Mathmatical Modelling, Build. 321, Lyngby.

Strand, S. E., Bjelland, M. D., and Stensel, H. D., 1990, Kinetics of chlorinated hydrocarbon degradation by suspended cultures of methane-oxidizing bacteria, *Research J. WPCF*, **62**, 124–129.

Tilman, D., 1980, Resources: a graphical-mechnistic approch to competition and predation, *Am. Nat.*, **116**, 362–393.

Vecht, S. E., Platt, M. W., Er-El, Z., and Goldberg, I., 1988, The growth of pseudomonas putida on m-toluic acid and on toluene in batch and in chemostat cultures, *Appl. Micro. Biotech.*, **27**, 587–592.

Vialas, C., Cheruy, A., and Gentil, S., 1978, An experimental approch to improve the monod model identification, In *IFAC Modelling and Control of Biotechnological Processes*, pp. 175–179.

Wang, C., 1994, *Stochastic differential equations and a biological system*, PhD thesis, Technical University of Denmark, DTU, IMM, Build. 321, 2800 Lyngby, Denmark.

Yoon, H., Klinzing, G., and Blanch, H. W., 1977, Competition for mixed substrates by microbial populations, *Biotechnol. and Bioeng.*, **19**, 1193–1210.

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