Biomass Yield Dependence
on
Inhibitory Substrate Concentration

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Abstract

Variations of growth yield coefficient with substrate concentration of a mixed microbial population was studied. Substrates used for growth were 2,4-dichlorophenoxyacetic acid (2,4-D) and *para*-chloro-*ortho*-cresol (PCOC). The synthetic medium used was designed so that substrate was the limiting-nutrient. The microbial culture was obtained from an activated sludge system treating effluent containing 2,4-D and PCOC. This was acclimated to the particular substrate metabolised in the investigations. Growth was conducted in batch and chemostat configurations.

Experimental data obtained indicated variations in growth yield did occur and were dependent on substrate concentration. Growth yield and specific growth rate biokinetics were directly calculated from the data obtained. Analysis of specific growth rate help in understanding culture-substrate systems. Specific growth rate increased to a maximum then decreased with increasing substrate concentration in batch configuration. Decrease in growth rate began above 500 mg/l for 2,4-D and above 60 mg/l for PCOC. This is an indication of substrate inhibition. The design of the chemostat maintained a constant specific growth rate.

Growth yield decreased with increasing substrate concentration with growth on either 2,4-D and PCOC in batch and chemostat configurations. A review of the literature indicated maintenance coefficient is a key parameter in explaining variations in observed growth yield. Data analysis for determination of the biokinetic constants of maintenance coefficient, m_e, and half-saturation constant, K_s, was performed. Analysis techniques for these constants are traditionally derived from Monod kinetics. Monod kinetics adequately explains growth on innocuous substrates. However analysis of specific growth rate had indicated the substrates used were inhibitory. Determination of maintenance coefficient and half-saturation constant by Monod derived techniques was unsatisfactory.

The effect of maintenance coefficient on growth yield was considered. The literature indicated maintenance coefficient is constant for growth on innocuous substrates. The substrates used in the investigations have phenolic structures. Phenolic compounds are recognised to be destructive to cell membranes. It was proposed that maintenance coefficient increased with increasing inhibitory substrate concentration as a result of increasing cell damage. An explanation for the decreasing growth yield with substrate concentration is adequately given by considering the

variable maintenance coefficient. Substrate used for maintenance is substrate that is not available for growth.

The relationship between growth yield and substrate concentration is essentially linear. Linear regression of yield on substrate was performed for growth on 2,4-D and PCOC in batch configuration and PCOC in chemostat configuration. Fitting of the linear functional form was considered appropriate. Analysis of the linear models for the three biosystems indicated yield variations with substrate concentration are significant. The models for the three biosystems have been given:

For growth on 2,4-D in batch configuration

$$Y(s) = 0.334 - (2.8\times10^{-4})s$$

For growth on PCOC in batch configuration

$$Y(s) = 1.03 - (5.6\times10^{-3})s$$

For growth on PCOC in chemostat configuration

$$Y(s) = 0.799 - (6.0\times10^{-3})s$$

Growth yield models may be incorporated into an overall growth model when similar biosystem configuration and substrates are studied. It is considered that this will give a growth model with greater accuracy in design and operation of biological treatment plants.

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Chapter 1

Background and Introduction

Hazardous waste management is an evolving practice throughout the world. It involves continually developing policies and regulatory approaches to the problems posed by hazardous waste production and disposal. Many countries have developed a wide variety of technologies for dealing with hazardous waste problems. Significant research and development efforts have been sponsored in this field.

Hazardous wastes are defined as hazardous substances that have no further safe and/or economic use. They may be chemically reactive, explosive, flammable, corrosive, toxic, disease-causing, persistent, or may accumulate in the environment. Because of these characteristics, they pose a present or potential treat to the public or environmental health. Unwanted pesticides represent an example of a hazardous waste.

In New Zealand pesticide use has been practised for over forty years (Harris *et al.*, 1992). *Pesticides* also refer to herbicides and other biocides. Disposal options have been limited and in some instances non-existent. Inappropriate disposal methods have lead to wide spread public concern in recent years for public health and the environment. As a result policies and action plans have and are being developed to provide for the safe use and disposal of pesticides.

Many treatment processes have been applied to cleaning up hazardous wastes. Treatment processes may be categorised as physical (eg centrifugation, evaporation), chemical (eg neutralisation, ozonation), or biological (eg activated sludge, anaerobic digestion) (Biosystems Technology Development Program, 1990). The key factors considered in assessing the applicability of a particular technology are listed below:

Function	- the purpose and applicability
Description	- theoretical operating principles and design features.
Performance	- examples of demonstrated clean-up performance.
Limitations	- physical/chemical characteristics that limit applicability.
Economics	- capital, operating and maintenance costs.
Status	- current development status, availability, and research plans.

Some of the most promising of the new technologies for handling hazardous wastes are biological treatments. These appear to provide solutions where other technologies are expensive, inappropriate for the site, or ineffective.

Biological treatment uses microorganisms, such as bacteria or fungi, to transform harmful chemicals into less toxic or non-toxic compounds. Pollutants serve as an energy source for the microorganisms as they are broken down. These organisms have a wide range of abilities to metabolize different chemicals. Organisms that can break down a particular pollutant can be selected for use in a treatment system. Often technologies are developed utilizing the native microorganisms demonstrated to be actively metabolising pollutants at a contaminated site. The biosystems developed often allow for the addition of nutrients or other amendments promoting activity of the microorganisms. The processes are carefully monitored to reduce the possibility of a product of the process being more toxic than the original pollutant.

A biosystem for clean-up of a contaminated site may be based on a number of media or process types:

Liquid bioreactors
Ground-water treatment
Soil/sediment treatment

The process type is chosen to match the site's environment (Biosystems Technology Development Program, 1990). Liquid reactors have been particularly successful in bringing hazardous pollutants into contact with microorganisms for accelerated degradation. Landfill leachates are particularly amenable to liquid reactor treatment. Other treatments have been used with varying degrees of success.

Biodegradation is an attractive option because it is natural, and the products from the processes are usually harmlessly utilized in the biosphere.

Background

The disposal of significant amounts of toxic wastes to unsecured landfills has been practiced in many countries. With time leachate from these sites may migrate and pollute their surrounding

environment. In New Zealand there exits a small number of unsecured industrial hazardous waste landfill sites.

One such site containing industrial waste herbicides was brought to the publics attention in December 1982. Chemical odours were noticed on a popular foreshore. The dumpsite was located nearby. A study in 1984 determined that migration of hazardous components from the site was not significant (Collier and Oldham, 1986). However the nature of the unsecured site could not guarantee that migration of hazardous components would not occur further than the boundary of the landfill area. Containment of the existing site was not considered feasible. It was decided to construct a new secure landfill to contain the contents of the old site (Collier and Oldham, 1986).

The secure landfill was constructed following the guidelines of the Resource Conservation and Recovery Act (USA). Containment was ensured by a double liner and secondary leakage detection system. Water injection and primary leachate collection systems were installed. The contents at the old site were transferred to the new landfill. Leachate is collected from the landfill for biological treatment.

An analysis of the leachate showed it to contain significant quantities of phenoxyacetic acids, associated chlorophenols, and a number of alcohols. The significant specific compounds identified in these categories were: (phenoxies) 2,4-dichlorophenoxyacetic acid (2,4-D), 2-methyl-4-chlorophenoxyacetic acid (MCPA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T); (chlorophenols) 2,4-dichlorophenol (2,4-DCP), *para*-chloro-*ortho*-cresol (PCOC) and a trace quantity of 2,4,5-trichlorophenol (2,4,5-TCP); (alcohols) methanol, butan-1-ol and butan-2-ol.

In 1990 a study was completed at Massey University (McAlister, 1990) for describing a suitable process for the treatment of the leachate. A mathematical model was developed to describe the rate of biological growth. Preliminary experiments justified microbial growth to be described according to each of the three substrate categories: phenoxies, chlorophenols, or alcohols. As a result an interactive three-substrate model was developed. Mathematical models are useful for design and operation of biological treatment facilities. Applicability of the models is often dependent on the accuracy of the biokinetic growth constants used.

Microbial growth on inhibitory (tending to mild biotoxicity) carbon sources offers interesting challenges. Difficulties and uncertainties result from the nature of the substrate and from the

heterogeneity of the microbial populations. Consequently difficulties occur in obtaining and analyzing experimental data for the purpose of determining biokinetic constants. As a result there are reports of deficiencies in a representative data base for a range of numerical values for these constants (D'Adamo *et al.*, 1983). Investigators dependent upon these constants have often had to make assumptions and accept the resulting inaccuracies. A common assumption, and one used by McAlister (1990), involved the invariability of the growth yield coefficient. This was assumed for each of the three substrate categories. Growth yield is one of the biokinetic constants.

The growth yield coefficient is defined as the amount of biomass produced from a given amount of substrate metabolised. This parameter has significant importance in the design of biological treatment facilities. Growth yield is one of the parameters employed in kinetic models and in mass and energy balance equations. These are used to describe and predict the operational, and design characteristics of the treatment process. It also represents a large portion of the *sludge* which must be disposed of as a byproduct of the process.

Studies have indicated a variability of growth yield with changes in substrate concentrations and specific growth rates (Stouthamer, 1976). Experimental data suggests that continuous bioreactors can exhibit periodic oscillations in cell-substrate concentrations (Curds, 1971; Tsuchiya *et al.*, 1972). It was shown numerically that when the yield term is allowed to depend on the substrate concentration in the bioreactor, cell-substrate concentration oscillations can exist (Crooke and Tanner, 1982). No experimentally determined quantitative values describing growth yield variability have been cited in the literature.

Investigation

An investigation was conducted into the growth yield coefficient of an activated sludge biosystem defined and modelled by McAlister (1990). The investigation was performed under similar conditions used by McAlister (1990) to give compatibility of kinetic parameters.

Experiments performed used the pure substrates 2,4-D, a phenoxy, and PCOC, a chlorophenol. These were chosen to represent two of the three substrate categories present in the leachate media used by McAlister (1990). Leachate could not be used in the current investigation because of the pure substrate requirement. A synthetic medium with similar composition to the

leachate, without the assortment of carbon substrates, was developed. The pure substrates were added to the synthetic medium.

The activated sludge system developed by McAlister (1990) was maintained throughout the following investigations. This provided a compatible culture to work with. For the experimental purposes this culture was acclimated to the pure substrate media. Batch and chemostat operating configurations were used. It was considered analysis of growth under the different configurations would give results greater scope for application. The chemostat configuration is compatible with the original activated sludge system.

This thesis completes a one and a half year experimental study on aspects of the growth yield coefficient. Growth yield variability with changing environment, particularly substrate concentrations, were investigated. Values are given for the yield coefficient at a variety of substrate concentrations. A mathematical equation has been given describing the relationship of yield and substrate for the substrates used.

Chapter 2

Literature Review

2.1 Introduction

The literature has reported many inconsistencies in the kinetics of biodegradation. Not the least so, and of significant importance, has been the growth yield coefficient. Wide variations in the values of growth yield have been reported between authors even when similar or the same cultures have been grown under similar conditions (Gaudy and Ramanathan, 1971).

It is important to have an understanding of the biodegradation of the substrates to be studied in appreciating possible yield variations. The steps involved, the products formed and the extent of biodegradation have a marked effect on final yield. The microbial culture and environmental conditions also play a significant role. These factors and relevant information influencing growth yield have been investigated in the literature.

2.2 Biodegradation Pathways

Biodegradation of 2,4-dichlorophenoxyacetic acid (2,4-D) was first reported in 1945 by workers attempting to produce herbicides with significantly longer lasting toxicity characteristics (Nutman *et al.*, 1945). Since then studies have determined the biodegradability of chlorophenols and phenoxies by numerous microorganisms (Table 2.1). In studying the aerobic biodegradability of substituted phenoxy acids it is first useful to give a brief description of phenol degradation in microorganisms. There are key similarities between the two biochemical pathways.

2.2.1 Biodegradation of Phenoxies and Chlorophenols

2.2.1.1 Phenol Biodegradation

The pathways of biodegradation of phenol were elucidated studying almost exclusively

Pseudomonas species (Stanier and Ornston, 1973). Pathway variations have not been found using other species. Two pathways are responsible for the biodegradation of phenol in microorganisms:

(i) The β-ketoadipate pathway (also known as the ortho cleavage pathway). The phenol is firstly oxidised to catechol. Ring fission in the 1,2 position and oxidation via β-ketoadipic acid gives succinic acid.

(ii) The meta pathway. Again phenol is oxidised to catechol. Ring fission occurs at the 2,3 position to give muconic semialdehyde. Further degradation gives formate, acetaldehyde and pyruvate.

These pathways are outlined in Figures 2.1 and 2.2 respectively. Reasons for pathway selection during degradation is beyond the scope of this work.

2.2.1.2 2,4-Dichloro-Phenoxyacetic Acid (2,4-D) Degradation

The pathway for 2,4-D degradation was determined in the late 1960's by a group led by Alexander (Tiedje and Alexander, 1969; Bollag *et al.*, 1968a and b; Tiedje *et al.*, 1969; and Duxbury *et al.*, 1970) using an *Arthrobacter* species. The pathway has been widely accepted.

The pathway for 2,4-D degradation involves cleavage of the ether bond of 2,4-D to form 2,4-dichlorophenol (2,4-DCP) and glyoxylate. The latter is further metabolised to produce alanine (Tiedje and Alexander, 1969). 2,4-DCP is hydroxylated by molecular O_2 and NADPH to form 3,5-dichlorocatechol (Bollag *et al.*, 1968a). Chlorocatechol is then ortho cleaved and degraded to chloromaleylacetic acid (Tiedje *et al.*, 1969). This is further broken down to acetyl-CoA and succinate, releasing free chloride ions (Duxbury *et al.*, 1970). This pathway is outlined by Figure 2.3.

Evans confirmed the pathway using a pseudomonad (Evans *et al.*, 1971). Subsequent work with a variety of organisms have not significantly altered the pathway. Variations between organisms has predominantly involved variations in the timing of dechlorination reactions. For example chlorine removal has been observed prior to ring cleavage in some *Pseudomonas* (Evans *et al.*, 1971), *Nocardia* and *Arthrobacter* species (Sinton *et al.*, 1986), but this does not affect the general structures of the intermediates, or of the final products.

Table 2.1 Summary of Organisms Degrading Phenoxy Herbicides.

Organism	2,4-D	MCPA	2,4,5-T	Reference
Mycoplana sp.	*	*	*	Audus (1962)
Rhizobium meliloti	*	*		Audus (1962)
Corynebacterium sp.		*		Rogoff and Reid (1956)
Achromobacter	*		*	Audus (1962); Bell (1957)
Flavobacterium	*			Audus (1962)
Pseudomonas sp.	*		*	Tyler and Finn (1974); Pierce *et al.*(1982); Evans *et al.*(1971); Gaunt and Evans (1971a and b); Gamar and Gaunt (1971); Kim and Maier (1986)
Pseudomonas cepacia			*	Kilbane *et al.* (1982); Kilbane *et al.* (1983); Chatterjee *et al.*(1982); Karns *et al.* (1983a and b)
Pseudomonas fluorescens			*	Rosenberg and Alexander (1980a)
Alcaligenes sp.	*	*		Don and Pemberton (1981); Don *et al.* (1985)
Arthrobacter		*		Duxbury *et al.*(1970); Bollag *et al.*(1968a and b); Loos *et al.* (1967a and b) Tieje *et al.* (1969); Tieje and Alexander (1969); Sharpee *et al.* (1973)
Brevibacterium			*	Horvath (1971a)
Streptomyces viridochromogenes		*	*	Kearney and Kaufman (1975)
Phanerochaete chrysosporium			*	Ryan and Bumpus (1989)
Nocardia	*			Sinto *et al.* (1986)
Xanthanobacter	*	*		Ditzelmuller *et al.* (1989)

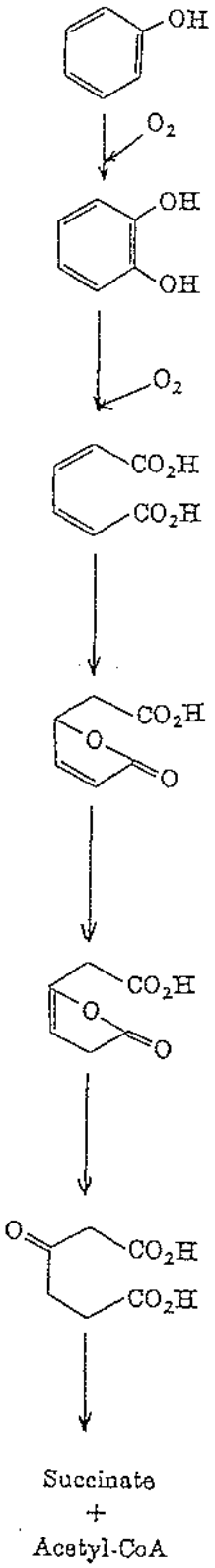

Figure 2.1 : Pathway for *ortho* Cleavage of Phenol (Dagley, 1971).

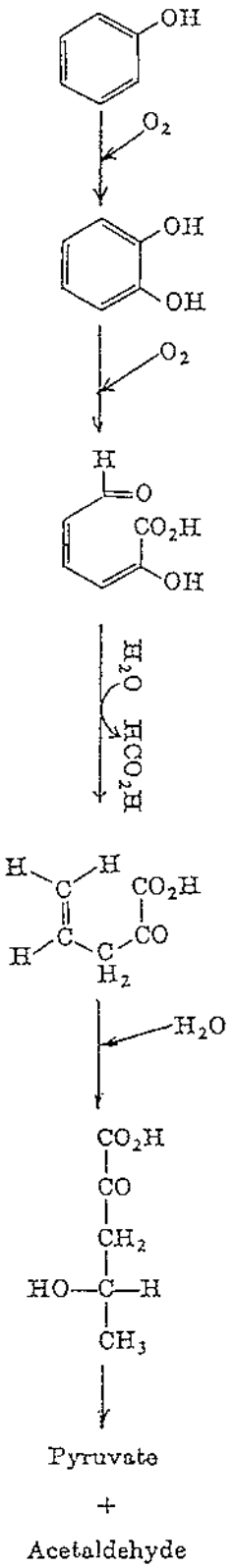

Figure 2.2 : Pathway for *meta* Cleavage of Phenol (Dagley, 1971).

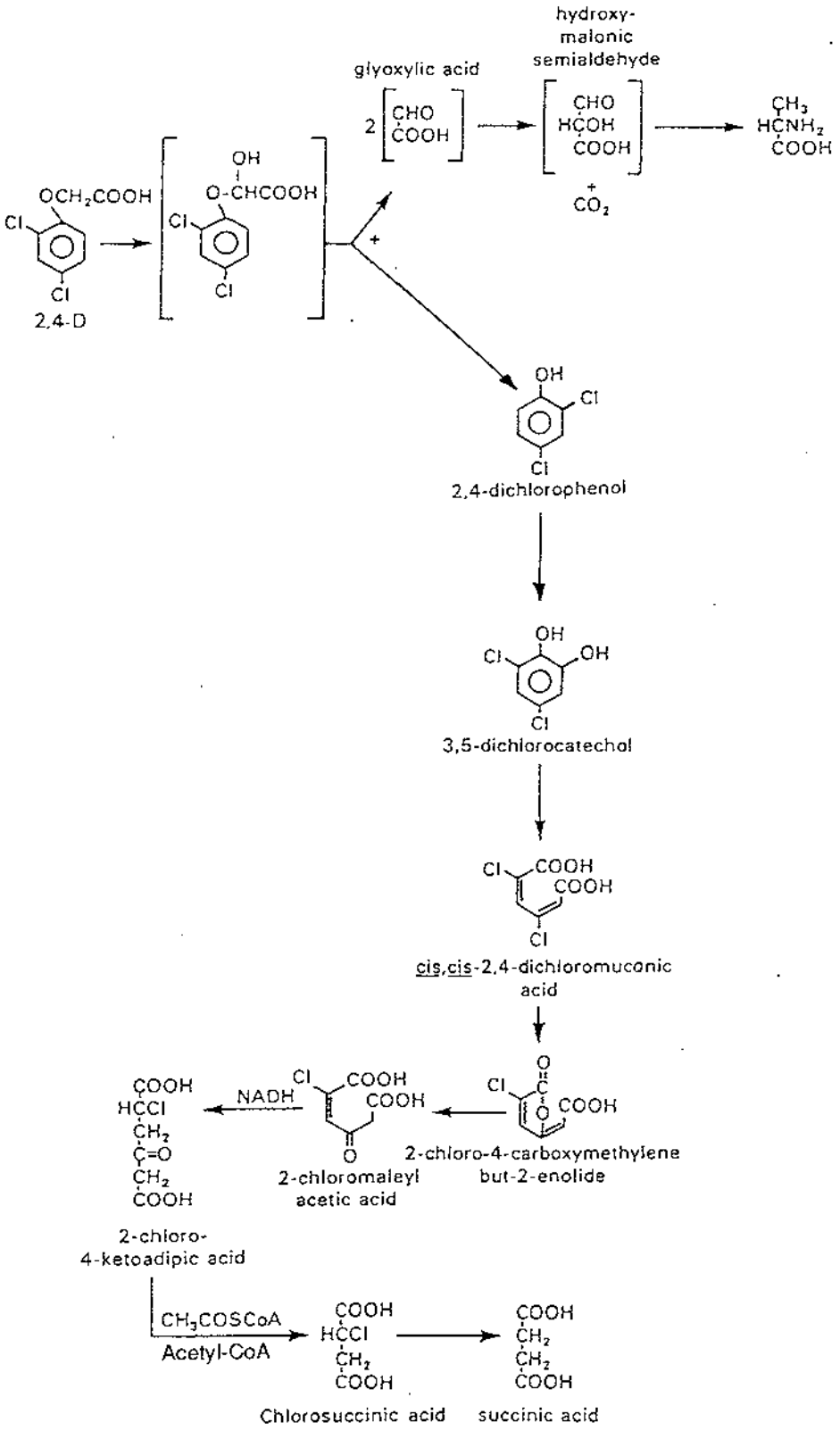

Figure 2.3 : Degradation Pathway of 2,4-D by *Arthrobacter*.
(Rochkind *et al.*, 1986).

2.2.1.3 *Para*-Chloro-*Ortho*-Cresol (PCOC) Degradation

PCOC is a chlorophenol. Chlorophenols are the first intermediate in the biodegradation of phenoxyacetic acids as given in the outline for 2,4-D degradation above. PCOC and other chlorophenols follow these catabolic pathways. Studies in their degradation have been considered by Loos *et al.* (1967a), Steiert *et al.* (1987), and Karns *et al.* (1983b).

2.2.2 Pathway Induction

Karns *et al.* (1983b) have postulated that an inducer is required for the metabolism of phenoxyacetic acids and chlorophenols. Work was performed using *Pseudomonas cepacia* AC1100. The organism lacked the ability to metabolise chlorophenols when grown on succinate. However 2,4-D and pentachlorophenol (PCP) were able to be metabolised by 2,4,5-trichlorophenoxyacetic acid (2,4,5-T) grown cells. It was postulated the inducer in this instance was 2,4,5-trichlorophenol (2,4,5-TCP) or a subsequent metabolite thereof.

The effects of other substrates on 2,4-D metabolism are varied. Lackmann *et al.* (1980) found that glucose or lactose added to a culture actively degrading 2,4-D were not metabolised until depletion of the 2,4-D. However if glucose was added when biodegradation commenced the overall rate of 2,4-D metabolism increased. This was attributed to increased active biomass concentration.

2.2.3 Side Reactions to the Catabolic Pathways

Side reactions have the potential to form products just as toxic as the initial products themselves. The formation of these products can have an important impact on the effectiveness of a biological process. They represent incomplete degradation and have an inhibitory effect on the bacteria involved. Some of the more inhibitory products are discussed below.

2.2.3.1 Chloroanisole Formation

Chloroanisoles can be formed during the biodegradation of phenoxyacetic acids as noted by Loos *et al.* (1967b), Smith (1985), and McCall *et al.*, (1981). They tend to bioaccumulate and are considered to be as toxic as chlorophenols (Neilson *et al.*, 1984). Allard *et al.* (1987), Loos *et al.* (1967b), and Smith, (1985), have shown detectable quantities of 2,4-dichloroanisole may be formed, as great as 10 percent of the substrate carbon applied, during the biodegradation of 2,4-D. Also 2,4-D degraders were found to have been unable to degrade 2,4-dichloroanisole.

2.2.3.2 Hydroxylated Phenoxyacetates

6-hydroxyphenoxyacetate was observed to have been formed during degradation of 2,4-D by *Pseudomonas* species (Evans *et al.*, 1971). Gaunt and Evans (1971a) found a phenoxyacetate degrading organism could not metabolise an hydroxylated phenoxyacetate and postulated that it was a product formed by a side chain reaction.

2.2.3.3 Partially Dechlorinated Chlorophenols

2-Chlorophenol and 2-muconic acid have been produced by organisms growing on 2,4-D (Evans *et al.*, 1971). This is evidence of dechlorination of the 4-chloro group prior to ring cleavage.

2.2.4 The Inhibitory Mechanisms of Toxic Metabolites

Toxic metabolites can be categorised as chlorocatechols (Knackmuss, 1984; Horvath, 1971a) or chlorophenols (Karns *et al.*, 1983a).

Knackmuss (1984) and Horvath (1971a) describe chlorocatechols as toxic however present no evidence indicating this. Klecka and Gibson (1981) have shown chlorocatechols to inhibit the catechol-2,3-dioxygenase activity in *Pseudomonas putida* by chelating iron.

High concentrations of chlorophenols have been shown to be inhibitory to degradation and toxic to cells (Karns *et al.*, 1983a; Tyler and Finn, 1974). This effect is thought to be due to a

decoupling mechanism separating electron transport from oxidative phosphorylation (Weinback and Garbus, 1965). This prevents aerobic energy release. Work with *Saccharomyces cerevisae* has indicated 2,4,5-T and 2,4,6-trichlorophenol (2,4,6-TCP) attack deoxyribonucleic acid (DNA) in the cell nucleus (Kleist-Welch Guerra and Lochmann, 1988).

2.2.5 Biodegradation Summary

Biodegradation of chlorophenols follows the ortho cleavage as opposed to the meta cleavage route. Knankmuss (1984) proposed that *Pseudomonas* initially exhibit meta cleavage during adaptation of the culture to chlorophenols. As the chlorocatechols produced by this process are not able to be broken down meta cleavage is suppressed by suicide inactivation. Ortho cleavage is induced and the accumulation of toxic chlorocatecols no longer occurs.

Dorn and Knackmuss (1978), using the same culture as above described the two enzymes responsible for the cleavage of catecols:

Pyrocatechase I, capable of cleaving only catechol and

Pyrocatechase II, able to cleave both catechol and chlorocatechols with a greater affinity for the latter. This includes the previously mentioned 3-methyl-5-chlorocatechol (Kilpi *et al.*, 1980).

Specific enzymes for chlorocatechol breakdown derivatives have not been described. McAlister (1990) assumed subsequent enzymes in the degradation pathway have a loose specificity regardless of whether the products are chlorinated. This implies the same inducers and control mechanisms apply to chlorophenol metabolism as apply to the phenol pathway beyond the catechol breakdown.

2.3 Microbiology and Genetics of Degradation

A large variety of microorganisms are potentially able to degrade phenoxy herbicides. Table 2.1 gives a number of these organisms found in the literature. The metabolic pathways described above were elucidated using predominantly *Pseudomonas* (Evans *et al.*, 1971) and *Arthrobacter* (Loos *et al.*, 1967a; Tiedje *et al.*, 1969) for 2,4-D and 2-methly-4-chlorophenoxyacetic acid (MCPA). *Pseudomonas fluorescens* (Rosenberg and Alexander, 1980) was predominantly used

for 2,4,5-T. The organisms most commonly associated with the degradation of phenoxies in the literature were from the family Pseudomonadaceae, and frequently *Pseudomonas* species.

Schmidt *et al.* (1983) used a defined mixed culture to study the dissimilation of chlorocatechols. These are recognised as the key step in the degradation of chlorophenols. The culture consisted of a chlorocatechol degrading *Pseudomonas* species, a phenol degrading *Alcaligenes* species, and a methanol degrading *Pseudomonad.* The mixed culture was capable of completely degrading a mixture of phenol, acetone, alcohols, and isomeric chlorophenols. A transconjugant strain of *Acaligenes* was isolated from the acclimated culture capable of total chlorophenol degradation, a property that none of the parent strains possessed (Schmidt *et al.*, 1983). The experiment implied that the genes for the degradation of chlorocatechols were transmissible and probably plasmid borne.

2.3.1 Plasmid Involvement in Biodegradation

Plasmid involvement in biodegradation of chlorophenols and phenoxy herbicides has been studied by several workers (Pemberton and Ficher, 1977; Don and Pemberton, 1981; Chatterjee *et al.*, 1981; Furukawa and Chakrabarty, 1982; Schwien and Schmidt, 1982; Don *et al.*, 1985; Chiura *et al.*, 1990).

Schwein and Schmidt (1982) demonstrated the transfer of chlorocatechol degrading genes from a *Pseudomonas* to an *Alcaligenes* species.

Chiura *et al.*, (1990) extracted plasmids from a phenoxy-herbicide degrading mixed culture and obtained several *Escherichia coli* transformants. Some of these transformants were capable of 2,4-D degradation in liquid culture. In this instance the rate of growth and 2,4-D degradation were lower for the transformant than with the mixed culture.

Don and Pemberton (1981) isolated two plasmids from an *Alcaligenes* phenoxy degrading species. These were designated pJP2 and pJP4. pJP2 was found capable of imparting 2,4-D and phenoxyacetic acid degrading characteristics, and pJP4 3-chlorobenzoic acid, MCPA, 2,4-D and merbromin and mercuric chloride resistance. Don *et al.* (1985) studied plasmid pJP4 and found five genes for enzymes involved in the catabolic metabolism of 2,4-D and 3-chlorobenzoate.

Four of the genes were found to code for 2,4-DCP hydroxylase, dichlorocatechol-1,2-dioxygenase,chloromuconate cycloisomerase and chlorodienelactone hydroxylase. Inactivation of the last three genes resulted in the inability to degrade both 2,4-D and 3-chlorobenzoate. This indicated a common pathway for chlorocatechol degradation and the reliance on plasmid encoded genes.

A culture consisting of *Pseudomonas cepacia* AC1100 capable of degrading 2,4,5-T was found to contain at least two plasmids (Ghosal *et al.*, 1985). Evidence indicated these plasmids were involved in 2,4,5-T degradation. Ghosal *et al.* (1985) demonstrated considerable homology between the fragment on plasmid pJP4 responsible for chlorocatecol degradation and a fragment of DNA on a plasmid isolated from *Pseudomonas cepacia* AC1100.

It has been demonstrated that plasmids can contain the complete set of genes capable of degrading phenoxy-herbicides. These plasmids can impart phenoxy degrading characteristics on their transformants whose strains were previously incapable of such degradation.

2.4 Microbial Population Kinetics

Qualitative observations of growth may provide interesting and useful descriptions of the current state of a microbial culture. However a quantitative approach consisting of a number of measurable parameters provides further information in describing the current state of a culture and for predicting its future characteristics with some accuracy. Mathematical relationships can be constructed between the various parameters. Experimental verification of these parameters and relationships can prove invaluable in describing and designing large scale microbial based processes.

The growth parameters are defined with reference to the growth of a simple homogenous batch culture. Such a system theoretically consists of a well-mixed batch of inoculated medium. It is assumed that mixing is sufficient to eliminate concentration gradients of biomass, nutrients and other medium constituents. A heterogeneous culture such as a colony growing on a surface clearly does not fit this description and requires a more complex description. There are a number of growth parameters frequently used to describe microbiological growth; growth yield, specific growth rate, growth lag, metabolic quotients for substrate utilization and product formation and substrate affinity. These parameters are briefly described below. Pirt (1975)

gives further useful descriptions of these.

2.4.1 Defining Kinetic Parameters

2.4.1.1 Growth Yield

An actively growing culture will experience an increase in biomass Δx and a decrease of substrate Δs in a defined time interval. Growth yield, Y, is described as $\Delta x/\Delta s$ where $\Delta s \rightarrow 0$. That is:

$$Y = -dx/ds \qquad (2\text{-}1)$$

The negative sign has been introduced as x and s vary in opposite consequence.

If x_o and s_o are the initial biomass and substrate concentrations respectively and x and s are the respective concentrations at an arbitrary time during the growth of the culture in batch mode then

$$x - x_o = Y(s_o - s) \qquad (2\text{-}2)$$

Where the substrate is growth-limiting and the biomass has reached its maximum, the substrate concentration will approximately be zero. Therefore the following may be written:

$$x_m - x_o = Ys_o \qquad (2\text{-}3)$$

,where x_m is the maximum biomass concentration.

Traditionally it has been considered that for constant growth conditions growth yield is a constant (Monod, 1942). Assuming constant yield given a growth-limiting substrate a plot of x_m against s_o should give a straight line of slope Y. There are various conditions where this has been shown to be otherwise. These will be described later in the review.

2.4.1.2 Specific Growth Rate

Where culture growth occurs then during a small time interval dt it is expected the increase in biomass, dx, will be proportional to the biomass initially present. That is

$$dx/dt = \mu x \qquad (2\text{-}4)$$

The parameter dx/dt expresses the growth rate. The growth rate per unit biomass, dx/(dt.x) gives the proportionality constant μ, known as the specific growth rate. This parameter has dimensions of reciprocal time, hr^{-1}, where time is in hours.
Assuming growth rate over a period is constant integration of eqn (2-4) gives

$$\ln x = \ln x_o + \mu t \qquad (2\text{-}5)$$

Often when initiating growth in a culture there is a lag period before a constant positive specific growth rate is obtained. This may be represented by

$$\ln x = \ln x_o + \mu(t - L) \qquad (2\text{-}6)$$

by rearranging eqn (2-5)

$$\ln (x/x_o) = \mu t \qquad (2\text{-}7)$$

and it follows

$$x = x_o e^{\mu t} \qquad (2\text{-}8)$$

Growth obeying this expression is constant exponential or logarithmic growth. Thus μ is a useful parameter for describing growth rate.

2.4.1.3 Metabolic Quotient

The rate of substrate utilization in a time increment, dt, in a growing culture is proportional to the biomass present;

$$ds/dt = qx \qquad (2\text{-}9)$$

ds is the substrate or nutrient used in time interval dt. q is the metabolic quotient or specific metabolic rate for the nutrient studied. The substrate carbon source and O_2 are common nutrients studied. It is important to ensure, or specify the period of, constant substrate utilisation rate when defining the metabolic quotient.

Metabolic quotient q is related to μ by the relationship

$$q = \mu/Y \qquad (2\text{-}10)$$

Metabolic quotients may also be used to express rates of product formation. The quotient q_p is similarly defined;

$$dp/dt = q_p x \qquad (2\text{-}11)$$

Again constant conditions should be ensured.

2.4.1.4 Substrate Affinity

The affinity of an organism for a substrate is inversely related to the saturation constant K_s. The occurrence of exponential growth over a wide range of substrate concentrations indicates growth exhibits zero order kinetics. However as the limiting substrate concentration is reduced to relatively low levels the specific growth rate will reduce, moving away from exponential growth. The substrate concentration giving half the maximum specific growth rate (1/2 μ_m) is the constant K_s (Figure 2.4).

It is often found that growth at low limiting substrate concentrations, affecting μ, closely follows Michaelis-Menton enzyme kinetics. Monod (1942) first demonstrated empirically this relationship relating bacterial growth to limiting substrate concentration;

$$\mu = \mu_m s/(s + K_s) \qquad (2\text{-}12)$$

This is often termed the Monod relation for describing microbiological growth. For estimating

the kinetic parameters, μ_m and K_s, accurately it generally better to rearrange eqn (2-12) to give a linear plot. The following options can be derived for data plotting and graphical parameter evaluation (Bailey and Ollis, 1977):

$$1/\mu = 1/\mu_m + K_s/\mu_m.s \qquad (2\text{-}13)$$

$$s/\mu = K_s/\mu_m + s/\mu_m \qquad (2\text{-}14)$$

$$\mu = \mu_m - K_s.\mu/s \qquad (2\text{-}15)$$

Figure 2.5 gives the various forms of the Monod equation. Bailey and Ollis, 1977, describes each of these equations:

When plotting eqn (2-12), μ vs. s, (Figure 2.5a) it is difficult to estimate μ_m accurately. Plotting eqn (2-13) as $1/\mu$ vs. 1/s (known as the Lineweaver-Burk plot) cleanly separates dependent and independent variables (Figure 2.5b). As μ values close to μ_m are more accurately measured these will tend to be clustered close to the origin. The lower rate values, least accurately measured, will be far from the origin and will most strongly determine the slope K_s/μ_m. Eqn (2-14) (Figure 2.5c) tends to spread out the data points for higher values of μ enabling accurate determination of the slope $1/\mu_m$. As the intercept often occurs close to the origin, determination of K_s is subject to large errors. Eqn (2-15) plots μ vs. μ/s (known as the Eadie-Hofstee plot, Figure 2.5d). The disadvantage here is that both variables contain the measured variable μ which is subject to errors.

2.4.2 Kinetic Parameter Values for Phenoxies and Chlorophenols

There has been very little work reported in the literature regarding the kinetic parameters for phenoxies and chlorophenols prior to the 1970's. D'Adamo *et al.* (1983) acknowledged an urgent need to develop a representative data base or a range of numerical values for kinetic constants employed in relationships between growth and substrate utilisation rates.

Few determinations of K_s values have been made because the values are often extremely low. These values are often at or below the sensitivity limits of the chemical assay methods. Also sampling and the assay are required to be instantaneous as the substrate level may fall

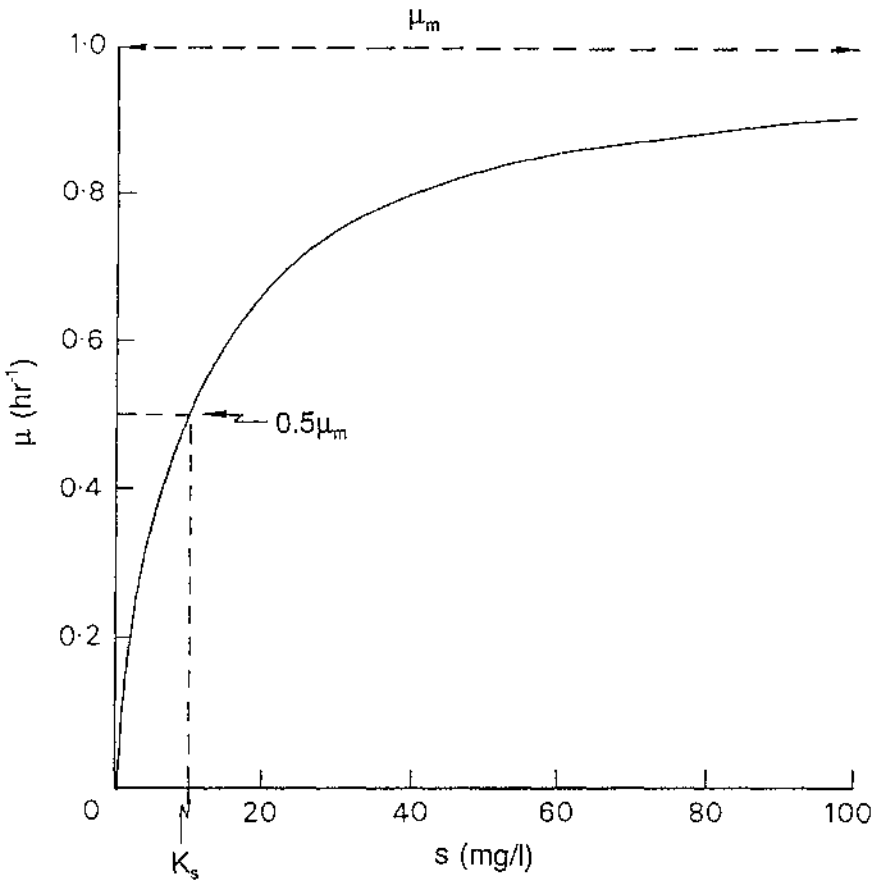

Figure 2.4 : Specific Growth Rate (μ) plotted as a function of Substrate Concentration (s) according to the Monod equation $\mu = \mu_m.s/(s + K_s)$ where $\mu_m = 1.0$ hr^{-1} and $K_s = 10$ mg/l. Note, when $s = K_s$, $\mu = 0.5\mu_m$.

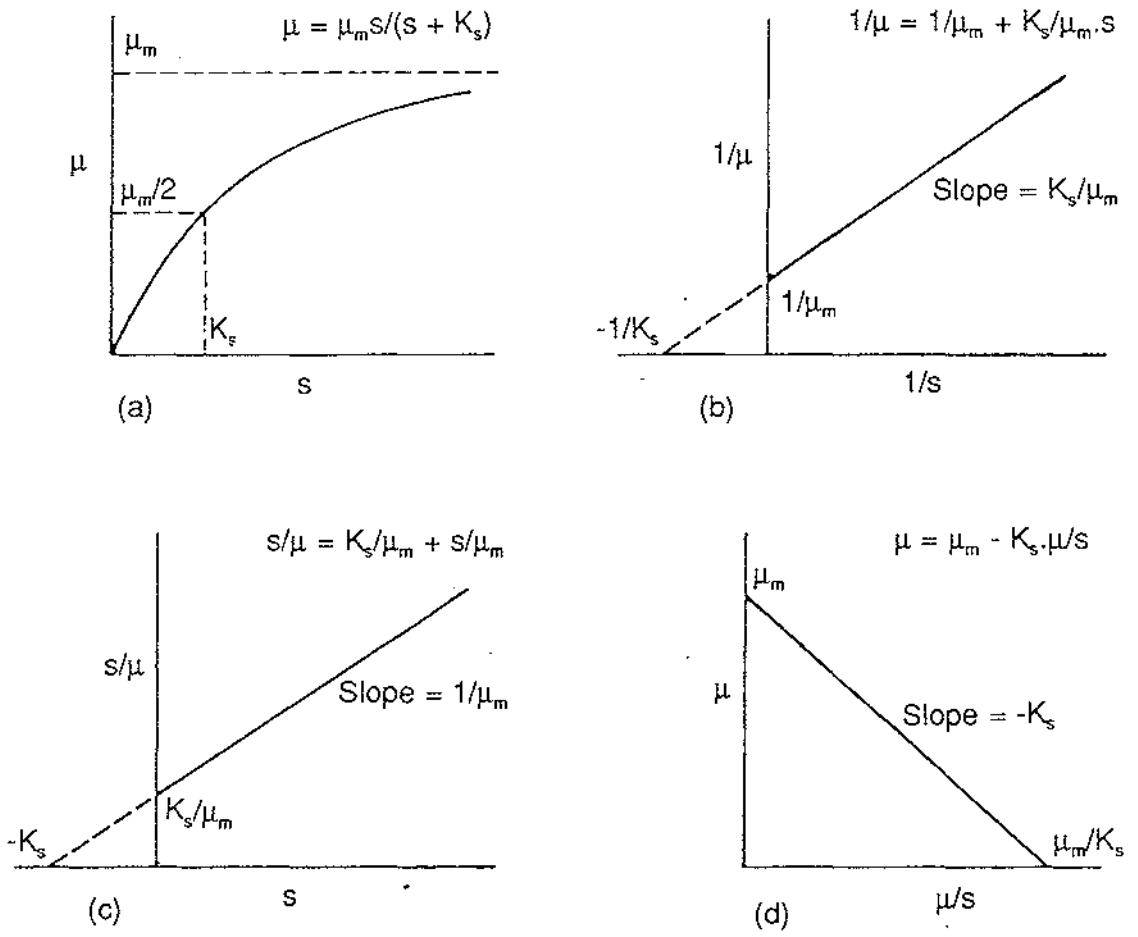

Figure 2.5 : Various forms of the Monod equation (rectangular hyperbola).

Table 2.2 Summary of kinetic parameters from the Literature.

Compound	μ_m (hr^{-1})	K_s (mg/l)	Y (g/g)	Ref	Comments
2,4-D	0.096	2.7	0.14	Shamat and Maier (1980)	20 C, 2,4-D not inhibitory up to 200 mg/l
2,4-D	0.09	0.6	0.14	Shaler and Klecka (1986)	25 C, 2,4-D not inhibitory up to 200 mg/l
2,4-D	0.14	ND	ND	Tyler and Finn (1974)	25 C, 2,4-D not inhibitory up to 2 g/l
2,4-DCP	0.12	5.1	ND	Tyler and Finn (1974)	25 C,2,4-DCP inhibitory at > 25 mg/l no growth at 100 mg/l
2,4-D	0.15	40	0.14	Papanastasiou and Maier (1982)	20 C, 2,4-D inhibitory at > 95 mg/l
Penta-chlorophenol	0.074	0.06	0.14	Klecka and Maier (1985)	20 C, PCP inhibitory at >0.4 mg/l

substantially with time. A number of the kinetic values found in the literature are given in Table 2.2. Yield coefficient for growth on 2,4-D and PCP is reported at 0.14.

The kinetic values reported for 2,4-D are reasonably consistent except for K_s of Papanastasiou and Maier (1982). This may well have been due to difficulty of analyzing at sufficiently low substrate concentrations as much as natural culture variations between workers.

Kinetics describing PCOC degradation could not be found. As the inhibitory effect of these chemicals appears to be correlated to the degree of substitution on the phenol ring the effects of PCOC would be expected to be similar to 2,4-D. The chlorophenols 2,4-DCP and Pentachlorophenol reported similar kinetic values to 2,4-D.

It is recognised that the kinetic values given for the respective chemicals above are for conditions where they are the sole carbon and energy source. The effect of a second carbon source on kinetics, for example the addition of glucose, may mask the true kinetics of the individual carbon source.

2.5 The Microbiology of Activated Sludge

Activated sludge is an aerobic process often employed for wastewater treatment. Here the liquid to be treated is mixed and aerated in a tank. As waste liquids are diverse so to are the cultures used to degrade them. Complex microbial interactions occur. Slime-forming bacteria grow and form flocs. These flocs form the substratum to which protozoa and other animals attach. Occasionally, filamentous bacteria and fungi are also present. Generally the more homogenous the waste to be treated the less diverse will be the microorganisms constituting the culture. The significant groups of microorganisms of an activated sludge are; bacteria, fungi, algae, protozoa and rotifers (Tchobanoglous, 1979). Crustaceans and viruses may be significant in some instances.

2.5.1 Bacteria

Bacteria are single-cell protists. They may be metabolically classified as autotrophic or heterotrophic. Important bacteria involved in degrading waste are heterotrophic because of their requirement of organic compounds for cell carbon. Common autotrophs are chemosynthetic, however a few are able to perform photosynthesis. Bacteria may be aerobic, anaerobic or facultative.

Bacteria vary widely in size. They may be spherical 0.5 to 1.0 μ, cylindrical 0.5 to 1.0 μ in width by 1.5 to 3.0 μ in length, or helical 0.5 to 5.0 μ in width by 6 to 15 μ in length (Tchobanoglous, 1979). Wastewater processes rely on flocculation to concentrate the sludge for recycle. This and the presence of predators inherently select for flocculating bacteria (Pike, 1975). It has been shown that flocculation results from the production of a sticky polysaccharide slime layer to which organisms adhere (Eckenfelder, 1989).

Bacteria are generally found to consist of about 80 percent water and 20 percent dry material. The dry material is 90 percent organic and 10 percent inorganic. The organic fraction may approximately be given by the formula $C_{60}H_{87}O_{23}N_{12}P$ (McCarty, 1970). The inorganic portion includes P_2O_5 (50 percent), SO_3 (15 percent), Na_2O (11 percent), CaO (9 percent), MgO (8 percent), K_2O (6 percent), and Fe_2O_3 (1 percent) (Tchobanoglous, 1979). These inorganic compounds must be available in the environment if they are not to be limiting.

Dissolved oxygen concentrations, temperature, pH and the presence of toxic compounds also have a profound effect on the organisms present (Pike, 1975). It has been observed that the rate of microorganism growth approximately doubles with every 10°C rise in temperature until some limiting temperature is reached (Kimball, 1966). Most organisms tolerate pH levels between 4.0 and 9.5. For optimal growth the pH lies between 6.5 and 7.5.

Filamentous bacteria (such as *Sphaerotilus*) can contribute to the bulking problem commonly caused by fungi (Pike, 1975).

2.5.2 Fungi

This group is often defined as those eukaryotic microorganisms that have rigid cell walls and lack chlorophyll. However the general agreement among microbiologists is not firmly established (Brock *et al.*, 1984). In sanitary engineering fungi are considered to be multicellular, non-photosynthetic, heterotrophic protists (Tchobanoglous, 1979).

Moulds or *true fungi* produce mycelium - filamentous masses. The terms mould and fungi are often used interchangeably. Yeasts are fungi that cannot form mycelium and are therefore unicellular.

The presence of large numbers of filamentous fungi results in bulking. This affects the performance of the activated sludge process (Eckenfelder, 1989). The sludge is unable to achieve rapid settling and concentration as wastewater treatment processes often require. Bulking may be caused by a number of factors such as low pH, underloading or overloading, or shock loads of toxic compounds (Tomlinson and Williams, 1975). It may be controlled by prolonged periods of anaerobiosis. Filamentous organisms are generally aerobic whereas most bacteria are facultative. Chlorine or hydrogen peroxide may be added or in more sever cases the majority of the filaments may be extracted by the addition of cationic polyelectrolytes (Eckenfelder, 1989).

Fungi can often tolerate an environment with a low pH. The optimum pH for most species is 5.6; the range is 2 to 9. They also have a low nitrogen requirement, often half that of bacteria.

2.5.3 Algae

Algae are unicellular or multicellular, autotrophic, photosynthetic protists. The group does not include the prokaryotic blue-green algae known as cyanobacteria. Some algae are motile and appear to be related to protozoa. The definition is not always clear, (Brock *et al.*, 1984).

Algae may provide an important source of dissolved oxygen in systems where mechanical or diffused aeration systems are insufficient. Diurnal fluctuations in dissolved oxygen concentrations will occur. However for a well aerated activated sludge system algae growths pose potential problems. Operating conditions designed to control algae growth may not allow proper sludge settling or result in the release of the odorous gases of decomposition (Eckenfelder, 1989).

Like other microorganisms, algae require inorganic compounds for growth. The principle nutrients other than carbon dioxide are nitrogen and phosphorous. Of lesser importance are iron, copper and molybdenum (Tchobanoglous, 1979). In natural waters prevention of excessive algal growth has centred around nutrient removal, particularly nitrogen and/or phosphorous.

2.5.4 Protozoa

Protozoa are motile uni- or multi-cellular microscopic protists. The majority are aerobic heterotrophs, although a few are anaerobic. They are generally an order of magnitude larger than bacteria and often consume bacteria as an energy source. As non-flocculating bacteria and particulate organic matter are most accessible to consumption the protozoa in effect act as polishers of effluents from biological wastewater treatment processes (Greenfield, 1987). There presence is considered a good indicator of a healthy activated sludge system.

Protozoa are sensitive to low dissolved oxygen, pH variations and excessive carbon dioxide (Curds, 1975).

2.5.5 Rotifers

The rotifer is an aerobic, heterotrophic, and multicellular animal (Tchobanoglous, 1979). They usually possess a foot for attachment and have two sets of rotating cilia on their head which are used for motility and capturing food. Rotifers consume dispersed and flocculated bacteria and particulate organic matter. By breaking up flocs, they provide nuclei for further floc formation as well as clearing the effluent of free swimming organisms (Greenfield, 1987). Their presence in an effluent indicates a highly efficient aerobic biological purification process.

2.6 General Nutrition

For a biological system to function properly it must have adequate amounts of nutrients. Nutrients required for growth and not used as an energy source may be classified into the following groups (Pirt, 1975): (i) principle nutrients C, H, O, N, and P; (ii) minor elements K, S, Mg; (iii) vitamins and hormones; (iv) trace elements. Growth factors are also sometimes included. These refer to essential organic nutrients, such as amino acids, which are incorporated into the cell whole.

Microbes were first cultivated in natural media. Examples are grape juice, milk, corn steep liquor, peptone, and serum. These are convenient as they contain all four groups of nutrients. However they are disadvantaged in that their composition is not clearly defined. Effects of particular nutrients cannot be accurately accessed. To determine this a *synthetic medium* must be used (Pirt, 1975). Raulin (1869), a student of Pasteur, developed the first fully defined synthetic medium. His work was significant in that he also determined the quantitative requirements for each nutrient.

A medium can be further defined as a minimal or a rich medium (Pirt, 1975). A minimal medium contains only those nutrients essential for growth. A rich medium usually supplements the essential nutrients with alternative sources of the nutrients such as in the form of amino acids or vitamins.

There are many examples of defined media for biological systems growing on inhibitory substrates reported in the literature. These can be used to estimate the concentration of nutrients required for maintenance and growth. Synthetic medium composition is reported for growth on

2,4-D (Bell, 1957), 2,4-DCP (Beltrame *et al.*, 1982), pentachlorophenol (Edgehill and Finn, 1983), and phenol (Kim *et al.*, 1981).

Based on the average composition of cell tissue the principle nutrients in a medium exist in the portions of $C_5H_7NO_2$. The phosphorous requirement is assumed to be about one fifth that of nitrogen. These portions may be varied according to the age of the culture and environmental conditions (Tchobanoglous, 1979).

Carbon, hydrogen and oxygen are the basic building blocks of organic material constituting a cell. Nitrogen is used mostly in protein, nucleic acids and cell wall polymers. Phosphate is mostly incorporated into the nucleic acids, phospholipids and cell wall polymers. Occasionally it may be stored in the cell as polymetaphosphate. Very little phosphate appears in the form of diffusible organic phosphates such as adenosine triphosphate (ATP).

The minor elements have been attributed to various functions in microorganisms, indicating their requirement (Pirt, 1975). Potassium appears to be bound up with ribonucleic acid (RNA) (Tempest, 1969). An increase in growth rate increases the potassium requirement due to the increase in the RNA content of the biomass. The ubiquitous nature of sodium ions means its requirement in microbial growth has rarely been demonstrated. A threshold concentration for magnesium is found to exist for stable growth in chemostats (Kurowski *et al.*, 1973). In algae, magnesium is required in the chlorophyll. Sulphur is often provided in the form of sulphate. Sulphur is required in sulphur containing amino acids and in some coenzymes.

Vitamins generally act as growth factors (Pirt, 1975). Their requirements for microbial cultures have rarely been defined quantitatively in terms of growth yield so their effect on biomass is unknown.

Studies on the role of hormones and any cell constituents indicate they may be anticipated to act or occur as growth factors (Pirt, 1975).

It is difficult to demonstrate the trace element requirements of a culture as these elements are often present in the medium as contaminants. Also the effects of different elements may not be *mono-specific*, allowing different ions to behave qualitatively in the same way (Dixon and Webb, 1967). Their requirements are often only known qualitatively and are therefore added to media in arbitrary amounts (Pirt, 1975). Rough estimates for some of the more important trace

elements are (given in g element/100 g dry biomass): Ca 0.10, Fe 0.015, Mn 0.005, Zn 0.005, Cu 0.001, Mo 0.001 (Pirt, 1975). The trace elements found essential for growth are given in Table 2.3. Pirt (1975) describes specific effects of trace elements.

In batch cultures a trace element deficiency is probably more apparent as a limitation of growth rate rather than a limitation of biomass concentrations. This effect was found with iron deficient growth of mammalian cells (Birch and Pirt, 1970). In a chemostat a trace element deficiency may be indicated by a decrease in the steady-state biomass with an increase in growth rate (Pirt, 1975).

Table 2.3 Trace elements which may be required in microbe and cell culture.
(Hutner, 1972; Tempest, 1969)

A	Elements which are frequently essential for growth	
		Ca, Mn, Fe, Co, Cu, Zn
B	Elements which are, rarely, essential for growth	
		B, Na, Al, Si, Cl, V, Cr, Ni, As, Se, Mo, Sn, I
C	Elements which may be, rarely, essential for growth	
		Be, F, Sc, Ti, Ga, Ge, Br, Zr, W

Hutner (1972) suggested the requirement for a trace element may increase seven fold when the culture is subjected to stress. This may be important when considering inhibitory substrates. Although little is known of the toxic concentrations of trace elements it is generally considered to be of the order 10^{-4} M.

Trace elements may be inadvertently removed from solution resulting in their deficiency. A common cause is precipitation as hydroxides, phosphates, or carbonates, and as ferrocyanides (Choudhary and Pirt, 1966). Other methods are extraction between polar/non-polar liquid phases (Donald, 1952), adsorption (Ratledge and chaudhry, 1971) and by metal chelating resins (Noguchi and Johnson, 1961).

Metal chelating agents can be used and are sometimes required to prevent the precipitation of metal ions, other than alkali metal ions, in culture medium. Many medium constituents and culture products such as amino acids and hydroxy acids act as metal ion complexants. Adding a chelating agent prevents this and controls the ions concentrations. Metal chelating agents are polybasic acids and act as metal ion buffers, for example ethylenediamine tetra-acetic acid (EDTA).

The quantitative and qualitative nutrient requirements of a culture vary with the conditions of a culture (Pirt, 1975). The temperature, pH and osmolality can be expected to affect the requirements. Also the total amount of nutrients will depend on the net mass of organisms produced (Tchobanoglous, 1979). This implies that nutrient quantities required will reduce for processes with long residence times.

In choosing a defined medium for growth experiments of substrate limitation it is important to ensure concentrations of essential nutrients are in excess. The importance of strictly defined culture conditions is also apparent for comparable, repeatable results.

2.7 Microbial Culture Process Configuration

Microbial cultures can be classified as closed or open systems. In a closed system the materials that constitute the system can neither enter or leave it. An open system allows materials to enter or leave in some manner (Pirt, 1975).

A batch culture is an example of a closed system. The reactor contains a limited amount of nutrients upon which the biomass grows. The growth rate of the biomass ultimately tends towards zero, as either the limiting nutrients are metabolised or products of growth concentrate to an inhibitory level. Growth in batch culture is inherently in a transient state.

Continuous-flow cultures are open systems. Examples of this are plug-flow and chemostat cultures. In plug-flow culture ideally the culture travels along a tube or channel without mixing. A chemostat culture consists of a perfectly mixed medium into which nutrients are constantly added and the mixed medium is constantly wasted at the same rate.

Studies of microbial growth have commonly used either batch cultures or single-stage chemostats

(Gaudy *et al.*, 1986). Each has their particular advantages and problems especially when dealing with inhibitory substrates (Hobson and Millis, 1990). Batch cultures have been used for growth studies on inhibitory substrates (Mizobuchi *et al.*, 1980; Rozich *et al.*, 1983; Luong, 1987). Problems have been encountered in obtaining data when the concentration is below the inhibitory level. This is because the concentration is often very low and biomass becomes difficult to measure. A chemostat on the other hand cannot be run at inhibitory concentrations of substrate. When the dilution rate exceeds the peak growth rate washout occurs. That is the chemostat cannot be run at substrate concentrations exceeding that which supports the peak growth rate. In order to obtain a complete study, data from both batch and chemostat processes are combined (Hill and Robinson, 1975; Rozich *et al.*, 1983; Yang and Humphery, 1975).

Some researchers have successfully used two-stage chemostats in overcoming substrate inhibition difficulties in obtaining a wide range of data (Jones *et al.*, 1973, Hobon and Millis, 1990). However Colvin and Rozich (1986) found it inapplicable for study of growth kinetics of a heterogenous population growing on an inhibitory substrate.

The research to be performed involved an essentially heterogenous population growing on inhibitory substrates. It was decided to obtain data using batch and single-stage chemostat configurations.

2.7.1 Batch Culture

2.7.1.1 Process Description

A batch culture is in a continual state of change as a limiting nutrient is exhausted or an inhibitory product is accumulated. The culture is well mixed, therefore homogenous, and always subject to the same physical conditions at any time. This results in definite phases able to be depicted as the culture grows then declines (Figure 2.6). After a lag period the culture experiences a rapid increase in growth rate to its maximum rate. This rate finally declines to zero as a nutrient becomes limiting or a product becomes inhibitory. At some stage after this, the amount of biomass declines due to maintenance metabolism or autolysis.

Although the growth curve shown in Figure 2.6 is typical of most simple batch cultures it is an

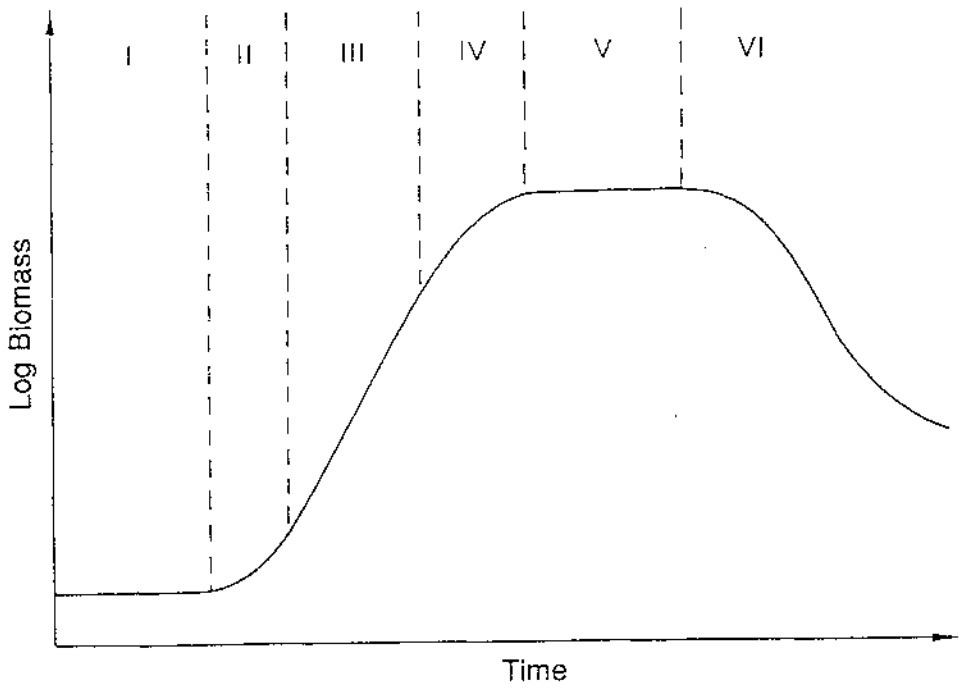

Figure 2.6 : Batch growth curve with six phases: I, lag; II, accelerating growth; III, exponential growth; IV, decelerating growth; V, stationary; VI, decline (Pirt, 1985).

idealised form. The duration of any of the phases may vary widely between cultures and experiments. For this reason single point estimates of growth cannot usually be compared, between experiments, at a chosen arbitrary time. Variations in biomass with time may reflect differences in lag periods, maximum specific growth rates, maximum population densities, concentrations of inhibitory products or biomass decline rates. Information obtained from growth curves is one of the foundations of cell biology. Further description of growth phases is given by Pirt (1975).

2.7.1.2 Growth Kinetics

The growth parameters described in section 2.4 (Microbial Population Kinetics) aptly apply for defining growth yield and growth rates. Rearranging eqn (2-2) for growth yield, Y, gives

$$Y = x\text{-}x_o/(s_o\text{-}s) \qquad (2\text{-}16)$$

The specific growth rate, μ, is defined by eqn (2-4)

$$dx/dt = \mu x \qquad (2\text{-}4)$$

When the substrate is the limiting factor to growth the Monod equation is often substituted for μ. However this requires the substrate to be non-inhibitory or to be at non-inhibitory concentrations. Hobson and Millis (1990) indicated batch culture studies have the advantage over other configurations of being able to be operated with inhibitory substrate influences. The Monod equation may not adequately describe growth on inhibitory substrates (McAlister, 1990). A number of equations have been proposed to more accurately describe this growth. Predominant amongst these is the Haldane equation. This equation and others are described in section 2.8 (Inhibited Microbial Growth Models).

2.7.2 Chemostat Culture

2.7.2.1 Process Description

A chemostat culture is a completely mixed system in which fresh medium is fed at a constant

rate and the mixed suspension is harvested at the same rate (Figure 2.7). The system is based on some fundamental concepts of microbiology indicating the importance of the development of theory before experiment. The method is applicable to all types of cells able to be grown in a homogenous submerged culture.

The mixing should ideally be perfect so that the fresh medium added is instantaneously dispersed throughout the culture. In practice this means the feed dispersion time should be small compared with the residence time, or the inverse of the dilution rate. Dilution rate is given by V/F, where V is culture volume and F is the medium feed flow rate. The feed contains nutrients, with no biomass, at concentrations where a chosen nutrient, usually the carbon source, is the limiting factor in the chemostat.

The chemostat permits control of both the population density and the growth rate of the culture. These are controlled by the concentration of the limiting nutrient in the feed, and the feed flow rate.

Effects of varying dilution rate are given in Figure 2.8 (Brock *et al.*, 1984). It is apparent from this figure that dilution rate affects growth rate, given by doubling time, over a wide range. However at very low dilution rates and at very high dilution rates the steady states breakdown. At high dilution rates the organisms growth rate cannot match the dilution and biomass washout occurs. At very low dilution rates the organisms begin to starve. It is generally accepted that there is a minimum amount of energy necessary to maintain cell structure and integrity (Brock *et al.*, 1984). When the limiting nutrient is not being added fast enough to permit maintenance of cell metabolism the population will slowly wash out. Small increases in steady-state substrate concentration with dilution rate are also observed before the point where washout is approached. Mathematical relationships for chemostats, expressing the parameters of Figure 2.8, are given in the following section.

The cell density in the chemostat is controlled by the level of the limiting nutrient supplied. If the concentration of the limiting nutrient in the feed is increased and the dilution rate remains constant the cell density will increase and the growth rate will be unchanged. The steady state limiting nutrient concentration will remain low (Brock *et al.*, 1984). However at very low concentrations of the essential nutrient, the growth rate will be proportional to the nutrient concentration (Figure 2.9). At these low nutrient concentrations the nutrient is quickly used by cell growth. Although the nutrient is continually supplied it will be quickly assimilated to

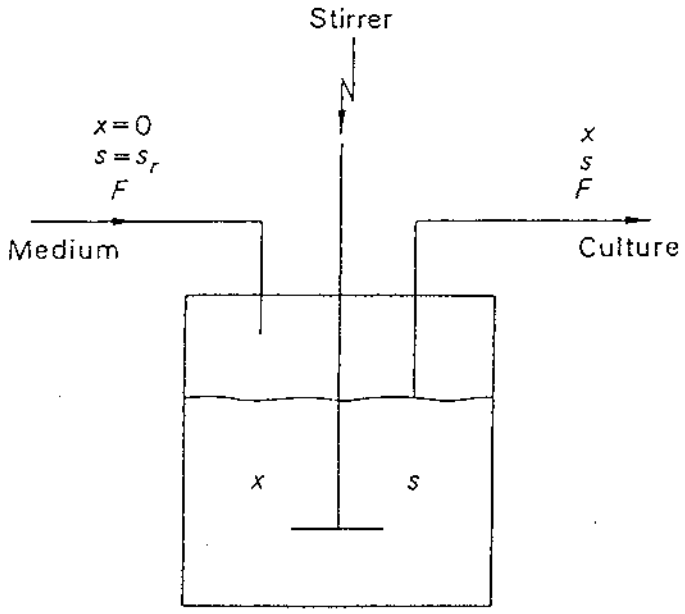

Figure 2.7 : The Chemostat (diagrammatic). The biomass and growth-limiting substrate concentrations at different points are represented by x and s respectively; F = flowrate; V = culture volume.

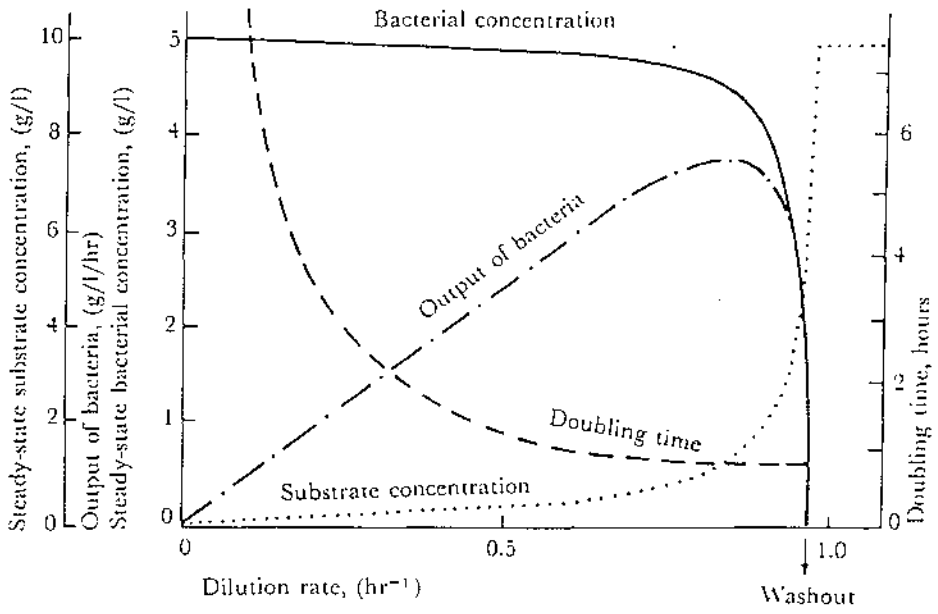

Figure 2.8 : Steady-state relationships in the chemostat. Note that at high dilution rates, growth cannot balance dilution, the population washes out, and the substrate concentration rises to a maximum (since there is no bacteria to use the incoming substrate). However throughout most of the range of the dilution rates shown, the population density remains constant and the substrate concentration remains at a very low value (Brock *et al.*, 1984).

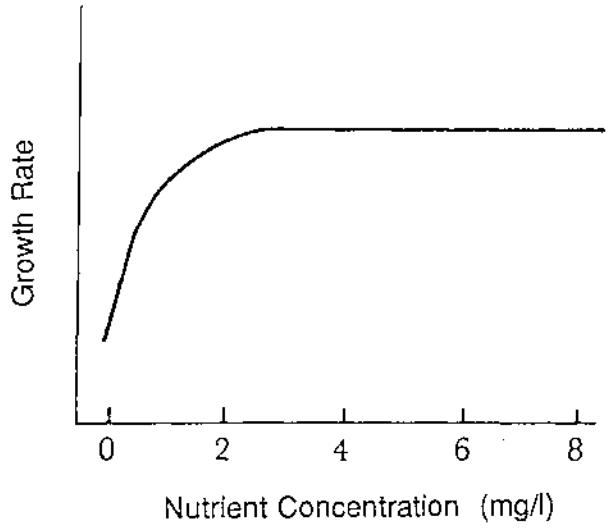

Figure 2.9 : Effect of limiting nutrient concentration on growth rate (Brock *et al.*, 1984).

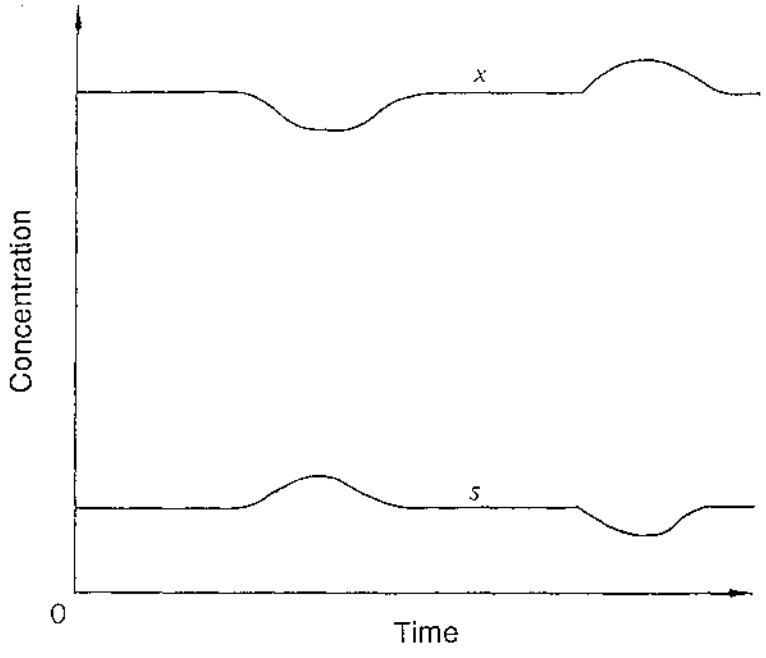

Figure 2.10 : Effect of temporary disturbances of steady-state conditions in a chemostat when the specific growth rate of the biomass is less than the maximum rate; x = biomass concentration; s = substrate concentration (Pirt, 1985).

maintain the biomass, giving almost zero nutrient concentration in the chemostat.

The steady-state in a chemostat is a self regulating one. The effects of temporary disturbances are given in Figure 2.10 (Pirt, 1975). For a given dilution rate a fall in biomass is accompanied by a rise in substrate concentration. This stimulates biomass production to restore steady-state. For an increase in biomass the converse is true.

Chemostats may be very useful for growth yield studies. The dilution rate and substrate level for a chemostat system may be controlled so that essentially all of the substrate is consumed (Brock *et al.*, 1984). The growth rate will then be controlled by the limiting substrate feed concentration. The growth yield becomes directly proportional to the concentration of substrate in the incoming medium. Calculation of growth yield is then very simple (Brock *et al.*, 1984). In practice however increasing levels of feed substrate concentration will result in low but significant substrate concentrations in the effluent stream.

2.7.2.2 Growth Kinetics and Mass Balance

In predicting the quantitative values of biomass and substrate concentration for various conditions the microbial parameters given in Figure 2.7 are used. The parameter D, dilution rate, is also of significance (D = F/V). Other growth parameters used are those previously mentioned in section 2.4 (Microbial Population Kinetics).

Specific growth rate and dilution rate

A biomass balance for the system is

net increase in biomass = growth - output

In a given small interval of time, dt, the balance may be written in terms of its mathematical parameters:

$$V.dx = V.\mu x.dt - Fx.dt \qquad (2\text{-}17)$$

Dividing eqn (2-17) through by v.dt gives

$$dx/dt = (\mu - D)x \qquad (2\text{-}18)$$

When the chemostat is at steady state dx/dt = 0. This is significant in chemostat theory, and gives

$$\mu = D \qquad (2\text{-}19)$$

Growth yield on growth-limiting substrate

The mass balance for growth-limiting substrate is

net increase = input - output - substrate used for growth

In a given small interval of time, dt, substituting mathematical parameters gives

$$V.ds = F.s_r.dt - Fs - V.\mu x.dt/Y$$

rearranging to make ds/dt the subject

$$ds/dt = D(s_r - s) - \mu x/Y \qquad (2\text{-}20)$$

At steady state ds/dt = 0. If the steady state values for s and x are shown by $\tilde{s}$ and $\tilde{x}$ respectively, rearranging eqn (2-20) gives

$$D(s_r - \tilde{s}) - \mu\tilde{x}/Y = 0 \qquad (2\text{-}21)$$

Substituting $\mu = D$ and rearranging for yield gives

$$Y = \tilde{x}/(s_r - \tilde{s}) \qquad (2\text{-}22)$$

Biomass and growth-limiting substrate concentrations

Rearranging eqn (2-22) making steady state biomass the subject gives

$$\bar{x} = Y(s_r - \bar{s}) \qquad (2\text{-}23)$$

Where an equation relating growth to substrate can be ascertained this can be substituted for $\bar{s}$. If the growth-limiting substrate is non-inhibitory, or at non-inhibitory concentrations, the Monod equation is often considered to adequately describe growth (Pirt, 1975).

$$\mu = \mu_m s/(s + K_s) \qquad (2\text{-}24)$$

Substituting μ in eqn (2-21) by the Monod equation and rearranging gives the steady state biomass concentration

$$\bar{x} = Y\{s_r - K_s D/(\mu_m - D)\} = Y(s_r - \bar{s}) \qquad (2\text{-}25)$$

The steady state substrate concentration may be obtained from eqn (2-24) by substituting $\mu = D$, and rearranging for $\bar{s}$ giving

$$\bar{s} = K_s D/(\mu_m - D) \qquad (2\text{-}26)$$

Critical dilution rate

The critical dilution rate, D_c, is the maximum dilution rate in a chemostat when the rate of growth of biomass can only just keep up to the outflow of biomass. Equivalently it is the minimum dilution rate at which growth and output of biomass are just imbalanced favouring the latter. At this value the steady state value of biomass will tend to and finally equal 0. When this exits $\bar{s} = s_r$. Inserting this in eqn (2-24) gives

$$\mu = D_c = \mu_m s_r/(s_r + K_s) \qquad (2\text{-}27)$$

When $s_r >> K_s$ it follows that $D_c = \mu_m$. Pirt, (1975), gives further theoretical description of this.

2.7.2.3 Chemostat Operation

Although the operation of a chemostat is relatively simple a number of things can significantly upset operation from fundamental principles (Veldkamp, 1976). The forms of the curves of biomass and growth-limiting substrate against dilution rate constitute important tests of the validity of the theory. Often excellent agreement can be obtained (Herbert, 1958). Where the chemostat is properly operated deviations from simple theory may be accounted for by important peculiar reactions of the biomass for its environment. Some recommendations for chemostat operation are given below.

(i) Good mixing. This implies that the time taken for added material to become homogenously dispersed throughout the culture is small in comparison to the mean residence time (1/D). Generally in laboratory-scale apparatus this is not difficult. However where mixing is not perfect pockets will develop in the culture in which the dilution rates are either less or greater than the medium dilution rate. The consequences of this are particularly apparent at the critical dilution rate (D_c). There will be some points in the vessel where $D < D_c$ and steady states can be obtained when $D > D_c$. This deviation has been termed an *apparatus effect* (Herbert, 1958). Further effects of incomplete mixing are discussed by Herbert *et al.* (1956), Hansford and Humphrey (1966), and Solomons (1972).

(ii) Avoidance of wall-growth. When wall growth occurs a steady state cannot be obtained (Solomons, 1972; Wilkinson and Hamer, 1974). Many organisms can adhere to glass and metal surfaces (Topiwala and Hamer, 1971). In long term chemostat cultures massive growths on vessel surfaces may occur. Topiwala and Hamer (1971) have modelled the effects of wall-growth in a chemostat. Vigorous agitation can prevent wall-growth, but splashes of the culture can cause accretion of biomass above the liquid level (Pirt, 1975). A temporary solution can be obtained by siliconing the surface of the vessel. Teflon provides a permanent non-stick surface but is unsatisfactory for applying to the surface of vessels. It's use is restricted to Teflon tubes and probes sheathed with these tubes.

(iii) Biomass clumping. Clumping is a source of inhomogeneity. Growth conditions within an aggregation are very different from those outside the clump. In difficult cases the organism may just not be suitable.

(iv) Verify the growth-limiting substrate. A shot of a concentrated solution of the

component injected into the culture should be followed by an increase in cell concentration. If a gas is limiting, cell concentration should continue to increase with a brief termination in the liquid feed.

(v) The gas blown through the culture should be equilibrated with water of the same temperature. This will considerably reduce evaporation. Evaporation effects can result in errors in measuring kinetic parameters (King *et al.*, 1972).

(vi) Inoculum size when starting the culture. Theoretically inoculum size is not important but in practice it often is. An example of an inoculum size effect in mixed culture studies is described by Meers and Tempest (1968).

(vii) The volume of liquid in the chemostat is not synonymous with the culture volume. This is particularly important when calculating dilution rates. Rapidly stirred cultures may hold up as much as 25 percent of air in their volume (Veldkamp, 1976).

Adequate agitation provides solutions for a number of the problems mentioned in the recommendations. Research has been done on the effects of shear by agitation contributing to organism damage. It has been claimed that the shear of stirring blades may damage organisms (Ugolini *et al.*, 1959). Obvious damage has been observed with filamentous organisms on some occasions (Pirt, 1975). However mammalian cells, regarded as very delicate, have been grown in laboratory fermenters at very high rates of stirring (Klein *et al.*, 1971). It may be that shear is not the prime cause of injury but it may enhance the effects of an adverse growth medium (Pirt, 1975). Consideration should be given to this.

2.7.2.4 Effect of an Inhibitory Substrate on an Activated Sludge System

Feed concentration of an inhibitory substrate can exhibit a marked effect on an activated sludge system. Increasing this substrate concentration has an increasingly inhibitory effect on the biomass. This effect continues until a critical maximum substrate concentration in the reactor is reached. At this point there is sudden system failure with the rapid washout of the biomass. This is accompanied by a step increase in reactor substrate concentration to the inflow concentration. The critical concentration is dependent on the growth rate and the inhibition characteristics of the biomass and substrate.

A similar effect is observed with increasing dilution rate with a constant inhibitory substrate feed concentration. Effluent substrate concentration will increase. System failure will occur at a critical dilution rate. This phenomenon is in distinct contrast to a non-inhibited system where a small change in dilution rate always results in a relatively small change in the effluent concentration (Gaudy and Rozich, 1982; Rozich and Gaudy, 1984). The difference is depicted in Figure 2.11, generated from model data, for the degradation of phenol (Gaudy and Rozich, 1982). The Monod model considers the substrate as non-inhibitory and the Haldane model considers the substrate as inhibitory.

2.8 Inhibited Microbial Growth Models

It can be appreciated from Figure 2.11 that modelling the effects of an inhibitory substrate may be difficult and/or complicated. Some researchers prefer to use an uninhibited model, for example Monods equation, for inhibitory substrates and restrict dilution rates to the range where inhibition does not occur (Gaudy and Rozich, 1982). However subsequent users of the model must always be aware of these constraints as they are not obvious in the model. For this reason the generation of more robust models has been desirable.

The most common expression used for modelling the degradation of inhibitory compounds is the Haldane model. It has been used to describe the degradation of phenol (Hill and Robinson, 1975; Pawlowsky and Howell, 1973a and b; Gaudy and Rozich, 1982), PCP (Klecka and Mailer, 1985), 2,4-DCP (Tyler and Finn, 1974) and 2,4-D (Papanastasiou and Maier, 1982). The model is

$$\mu = \mu_m s/(K_s + s + s^2/K_I) \qquad (2\text{-}28)$$

where K_I = inhibitor constant, the concentration where the growth rate is reduced to half of the maximum by the substrate (mg/l).

Pawlowsky and Howell (1973a) tested five different models from the literature for describing phenol degradation. They found no significant difference between them. They continued to use the Haldane model for subsequent work (Pawlowsky and Howell, 1973b).

The Haldane model suffers from the disadvantage that it will never predict total inhibition.

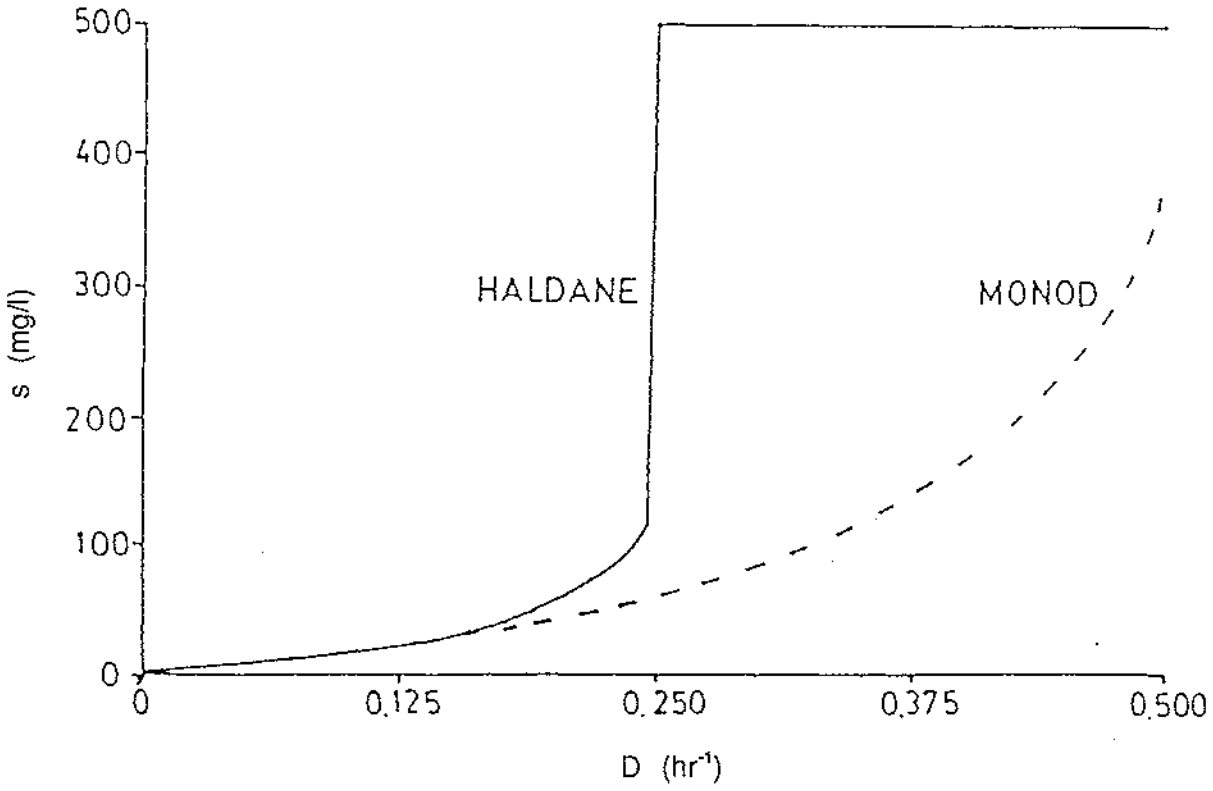

Figure 2.11 : Dilute out curves calculated for Monod and Haldane Models for Phenol (from Gaudy *et al.*, 1988). (μ_m = 0.6 hr^{-1}, K_s = 75 mg/l, K_I = 150 mg/l, Y = 0.5 mg/mg)

Linear inhibition models can be used to describe a linear reduction in growth with increasing substrate concentration to the point where there is no growth. This has been used to describe growth on 2,4-DCP (Tyler and Finn, 1974), and methanol and n-butanol (Wayman and Tseng, 1976).

Exponential and Teissier models have been used to describe substrate inhibition. Both are unable to predict total inhibition (Luong, 1987).

Luong (1987) proposed a generalised model for substrate inhibition. This adapts work done by Levenspiel (1980) on product inhibition. The model can predict Haldane equation type curves as well as linear inhibition curves.

Watkin and Eckenfelder (1989) proposed a generalised model based on work with 2,4-DCP. It can predict a variety of inhibition curves but cannot predict total inhibition.

These models are convenient in describing single substrate systems. Once systems move from one substrate to multiple substrates, multiple substrate models must be developed (Cloonan, 1984). McAlister (1990) developed a multi-substrate model to describe growth on inhibitory substrates.

2.9 Yield Studies in Microorganisms

Biological growth is dependent on the relationship between substrate utilisation, ATP generation and formation of new cell material. Growth yield studies on microorganisms can be better understood by considering energy generation and utilisation in cells. The energy carrying molecule in cells is adenosine triphosphate (ATP). Growth may be examined by comparison of cell yield to the amount of ATP generated during catabolism (Stouthamer, 1976).

ATP is generated in heterotrophs during respiration. Organic compounds are oxidised in the catabolic process. The metabolic pathways may be influenced by environmental conditions. An example is given by de Vries *et al.* (1970), in *Lactobacillus casei.* When grown in a glucose limited chemostat, the fermentation pattern of the organism is a function of growth rate. At low growth rates 3 moles of ATP are formed per mole of glucose fermented, and at high growth rates only 2 moles are formed. This accounts for the higher molar growth yields for glucose in

slow growing organisms.

The ATP formed in the dissimilatory processes is used for a number of processes as well as for biosynthetic purposes. ATP is needed for transport and for turnover of RNA. These and other energy processes not directly related to growth are considered *maintenance energy* (Veldkamp, 1976). Besides energy use for biosynthesis and maintenance the efficiency of ATP utilization for growth is dependent on the degree of coupling between catabolism and anabolism.

The maintenance energy of an organism can be observed in an organism grown in a chemostat under energy limitation. At low dilution rates the portion of ATP required for maintenance becomes relatively high. This results in a low growth yield at low growth rates. That is the yield is dependent on the growth rate as given in Figure 2.12 (Veldkamp, 1976). A mathematical expression relating yield, growth, and maintenance was given by Pirt (1965):

$$1/Y_o = m_e.1/\mu + 1/Y_g \qquad (2\text{-}29)$$

where Y_o is the observed molar growth yield (g dry biomass/mole substrate), m_e is the maintenance coefficient (moles substrate/g dry biomass/h), μ is specific growth rate (hr^{-1}), and Y_g is the molar growth yield corrected for the maintenance requirement.

Eqn (2-29) is not valid when ATP generation is a function of growth rate. In this instance the following equation (de Vries *et al.*, 1970) which considers the utilisation only of ATP should be used:

$$1/Y_{ATP} = m_e.1/\mu + 1/Y'_{ATP} \qquad (2\text{-}30)$$

where Y_{ATP} is g dry biomass produced/mole ATP used, and Y'_{ATP} is growth yield/ mole ATP corrected for the maintenance requirement.

It has been shown that there is sometimes relatively large discrepancies between Y_{ATP} and Y_{ATP}^{max} (Payne, 1970; Forrest and Walker, 1971) that cannot be accounted for by the relatively small maintenance energy requirement. This means not all the ATP is used for either growth or maintenance. It is considered that some ATP is used to give the required coupling between catabolism and anabolism (Belaich *et al.*, 1972; Lazdunski and Belaich, 1972). The following equation accounts for this:

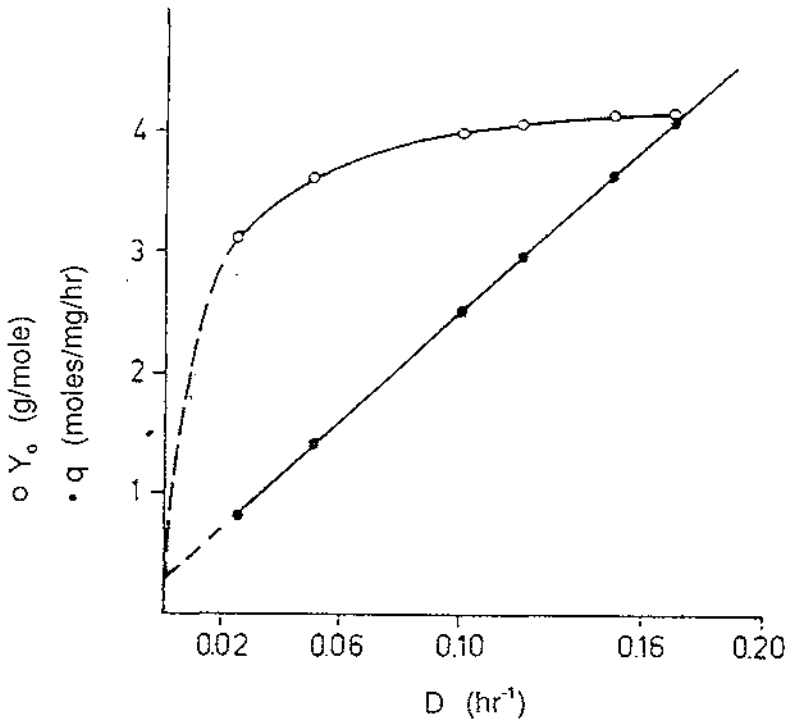

Figure 2.12: Molar growth yield (Y_o) and specific oxalate uptake rate (q) as a function of dilution rate (D). Theoretically dilution rate equals growth rate in the chemostat. Steady-state values were obtained at various dilution rates in oxalate-limited *Pseudomonas oxaiaticus*. (Harder, 1974).

$$Y'_{ATP} = K.Y_{ATP}^{max} \qquad (2\text{-}31)$$

Where K gives the degree of coupling.

Combining eqns (2-30) and (2-31) gives

$$1/Y_{ATP} = m_e.1/\mu + 1/K.Y_{ATP}^{max} \qquad (2\text{-}32)$$

Eqn (2-32) was used by Harder and van Dijken (1976) to calculate yields for organisms growing on methane. m_e and K vary with environmental conditions, however they are independent of growth rate and growth-limiting substrate concentration (Veldkamp, 1976).

Stouthamer (1976) showed that the lower molar growth yields observed at low growth rates, due to maintenance requirement, are more apparent with C-limited cultures than with a limitation of any other nutrient.

Experimental determination of growth yields, with respect to ATP (energy) requirement, are generally only easily done with fermentative organisms (Stouthamer, 1976). This is because the amount of ATP produced per molecule of substrate catabolized can be calculated exactly from knowledge of the metabolic pathway of the organism. Factors influencing Y_{ATP} will affect the overall growth yield, Y, whether the organisms mode of catabolism is aerobic or anaerobic. The difference in growth yields between various organisms and the influence of the medium composition on yield are attributed to the following factors (Stouthamer, 1976):

(1) The maintenance coefficient of the organism under the conditions of the experiment.
(2) The specific growth rate, which together with the maintenance coefficient determine the relative amount of the energy source which is used as maintenance.
(3) The complexity of the medium, which determines the monomers required to be synthesised by the organism.
(4) The nature of the carbon source. This strongly influences the amount of ATP needed for monomer synthesis.
(5) The nature of the nitrogen source.
(6) The macromolecular composition of the microbial cells. Differences between

species are clearly reflected in differences in Y_{ATP}^{max}.

(7) Energy-requiring processes other than formation of new cell material which are related to growth rate in energy limited cultures.

Growth yields may vary for other reasons where the culture is a mixed microbial population and also by considering microbial viability. These are discussed in the following sections.

2.9.1 Mixed Microbial Populations Grown on a Single Growth-Limiting Substrate

The various species of a mixed microbial population will compete with each other for a growth-limiting substrate. Where the concentration of nutrients is in excess, such as in a batch system, selection of the organism with the maximum specific growth rate would be favoured (Veldkamp, 1976). It has however been shown that heterotrophic organisms exist which show a high substrate affinity (K_s) for various nutrients, but which show a low μ_{max} (Veldkamp and Jannasch, 1972; Veldkamp and Kuenen, 1973; Jannasch and Matels, 1974). Organisms of this type never come to the fore in batch culture. However in a chemostat operated at low dilution rates such an organism is likely to be selected. This indicates that the organisms present in culture collections, isolated and maintained at high substrate concentrations, are not those responsible for mineralization processes in nutritionally poor environments.

Selection of organisms of a mixed microbial population have also been shown to be influenced by temperature (Harder and Veldkamp, 1971), and light (van Gemerden, 1974).

In the above selection processes the organism favoured will with time dominate and completely take over the system providing the favouring factors remain effective. The selective power of the chemostat can be used to illustrate this. For complete selection however competition between organisms must be for a single growth-limiting substrate. The presence of preditor-prey relations in a mixed microbial population will upset this condition. The existence of such relations means substrate is provided not just by the carbon containing compounds added to the medium but also by the bacteria themselves becoming a source of substrate to bacterial consuming protozoa and rotifers. Most natural mixed microbial populations will be subject to predator-prey relations. The favoured organism may therefore not eliminate less favoured organisms. Such effects may be avoided by filtering the inoculum through a 2 µm membrane filter, removing these larger predators.

Jost *et al.* (1973a) illustrated the effect of a predator-prey relationship by growing *Azotobacter vinelandii* and *Escherichia coli* in a chemostat under glucose limitation. It was discovered that independent of dilution rate *Azotobacter* was selectively eliminated. However when the predatory ciliate *Tetrahymena pyriformis* was introduced to the same system both bacterial cultures were found to co-exist.

Valuable contributions to these complex systems are given by Canale (1969, 1970), Curds (1971), and Jost *et al.* (1973a). A classical theoretical basis of predator-prey relations was given by Lotka (1925). He developed a model for a system in which cell/substrate oscillations are inherent. However, Tsuchiya *et al.* (1972), and Jost *et al.* (1973a), have indicated that oscillations in a single stage chemostat are not inherent to predator prey relations as such. They are dependent on other environmental factors such as dilution rate and growth-limiting substrate concentration. With some combinations of these factors no oscillations occur (cf. Jost *et al.*, 1973b).

It has been demonstrated that the major role of protozoa in the activated sludge process is to remove the dispersed non-flocculated bacteria (Curds and Cockburn, 1968). The predatory activities of ciliated protozoa alone could account for the removal of suspended effluent bacteria (Curds and Cockburn, 1968).

2.9.2 Viability as a Function of Growth Rate

Bacterial cells which become incapable of growth in an environment which is normally suitable for growth, must either be dead or dormant cells (Pirt, 1975). Cells may die as a result of an adverse physical factor such as high temperature, toxic chemicals, starvation or *mistakes* in autosynthesis. In a growing culture dormant and dead cells behave similarly in that they make no contribution to the growth of the population.

It has generally been observed that deceasing the dilution rate of a chemostat results in a decrease in viability of the microbial population (Postgate and Hunter, 1962; Tempest *et al.*, 1967; Sinclair and Topiwala, 1970; Postgate , 1973). Such studies have usually been carried out with non-inhibitory substrates.

At growth rates below $0.25\mu_{max}$ C-limited cells show a marked decrease in viability with

decreasing growth rate (Stouthamer, 1976). It is proposed that this is due to a lack of energy for maintenance purposes when the carbon (and energy) supply is restricted.

In contrast several workers (Hobson and Millis, 1990; Mink *et al.*, 1982; Green, 1978; and Ng, 1982) have shown increased sensitivity of cells grown at fast growth rates when subjected to a variety of stress causing agents. In the chemostat a decreasing viability was observed at the faster dilution rates with inhibitory substrates. Millis and Hobson (1990) indicated that given the apparent greater susceptibility of fast-growing cells to stress-causing agents, it seems reasonable that the viability of the population may fall. The maintenance requirement was shown to be dependent on whether growth conditions were inhibitory or non-inhibitory. At high steady-state inhibitory substrate concentrations the maintenance requirements became significant. It has been suggested (Tempest and Neijssel, 1984) that most of the maintenance energy used by a cell is used for the maintenance of the ionic gradient. In that case, the presence of high concentrations of known membrane-dissociating compounds like phenolics could result in a significant increase in the maintenance requirement of a culture.

2.10 Activated Sludge Respirometric Measurements for Determining Microorganism Activity

Respirometric measurements of the oxygen consumptions of activated sludge have been recognised as one of the existing possibilities to determine microorganism activity (Suschka and Ferreira, 1986).

Microorganism activity and viability are related. Traditional methods used to determine culture viability allow the individual organisms to multiply and produce colonies which can be counted. The dilution count method and the membrane filter method are common examples (Meynell and Meynell, 1965). These methods however depend on bacterial cells being individually dispersed throughout the growth medium. They are therefore unsuitable for flocculated cells.

The respirometric method is independent of cell dispersion. Two common techniques used to employ this method are: (i) manometric and (ii) direct oxygen uptake measurement using an oxygen probe (Suschka and Ferreira, 1986).

The manometric technique was developed by Sierp (1928). The technique, however, is only

convenient for non-growing or *resting* cells (Pirt, 1975). The development of the oxygen probe applied in respiratory is more convenient.

A galvanic cell oxygen probe inserted in a BOD bottle, containing the culture, can be used to measure the rate of oxygen uptake. By measuring the biomass in the bottle the specific oxygen uptake rate (SOUR) can be calculated. A comparison of these rates between different samples, of the same culture, enables a comparison of relative microorganism viabilities.

2.11 Yield Variation in a Model for a Continuous Reactor

Section 2.9.1 describing growth of mixed microbial populations indicated that under certain environmental conditions, in a chemostat, oscillations in cell concentration and substrate concentration may be observed. Crooke and Tanner (1982) noted reports of this phenomenon and investigated the mathematical intricacies of the Monod model in an attempt to account for this behaviour. The simplicity of the monod model meant mathematical analysis could easily be adopted by the experimentalist.

The mathematical model was developed for a single species grown in a continuously stirred homogenous reactor, continuously fed by a limiting nutrient and the cells continuously drawn off. It was shown numerically that when the substrate yield was allowed to depend on the substrate concentration in the reactor, then under certain conditions for the system parameters (kinetic and physical parameters) it is possible to have periodic orbitals in cell-substrate concentrations.

The model developed by Crooke and Tanner (1982) assumed the growth yield, Y(s), to be a linear function of substrate

$$Y(s) = A + Bs \qquad (2\text{-}33)$$

where A and B are positive constants.

2.12 Summary and Conclusions

The literature has indicated the following:

(1) Growth and yield characteristics of a microbial culture may be influenced by a wide range of environmental factors.

(2) The microbial degradation of phenoxies and PCOC have been well documented and are considered to be inhibitory substrates.

(3) The chemostat can be used to control growth rate.

(4) There is experimental evidence indicating growth yield is not constant even when the same cultures are grown under similar conditions.

(5) Growth-limiting substrate concentration has been shown to be a significant parameter in influencing the growth characteristics of a culture.

(6) It has been suggested that a model incorporating a variable yield term could give a more representative indication of growth of a microbial culture.

(7) An equation for a variable yield term has been presented. This was based on qualitative experimental observations and not quantitative measurements.

In conclusion the literature has indicated a model describing microbial growth incorporating a variable yield term could give a reliable representation of the growth. Development of a variable yield term dependent on a growth-limiting substrate, from experimental measurements, would be valuable for use in such a model. This thesis will describe a study examining the degradation of two inhibitory substrates. It will attempt to measure yield variations with substrate concentration and describe this by a variable yield function.

Chapter 3

Materials and Methods

3.1 Introduction

This chapter describes the materials and methods common to subsequent chapters. Methods were for: the temperature maintenance and mixing of cultures; biomass determinations; substrate concentration determination; feed and effluent chemostat flow operations; specific oxygen uptake rate determination; and culture acclimation.

Materials and chemicals mentioned are given with details and suppliers where relevant.

3.2 Temperature Maintenance and Mixing of Cultures

Batch and Chemostat cultures were maintained at a constant temperature of 25°C and mixed at approximately 150 rev/min.

Constant temperature was achieved by placing the glass bioreactors in a flat bottomed plastic container. This container was sufficient in size to hold four 2 litre flask reactors or two 20 litre glass reactors without difficulty. This allowed multiple runs when performing experiments.

With the reactors in place the container was filled with approximately 10 centimetres of water or to a lesser level if the reactors tended to float. A water heating pump (MR 2002, Watson Victor, NZ) was clamped to the side of the container with intake and outlet ports under the water's surface. The heater setting was adjusted to 25°C.

Constant mixing was achieved by means of magnetic stirrers and assisted by air sparging. For each reactor placed in the plastic container a magnetic stirrer (Heidolph MH2002, Watson Victor, NZ) was centred directly under the container where the reactor stood. Depending on the number of reactors and runs performed up to four magnetic stirrers were used at one time. Each reactor had a 2.5 centimetre magnetic flea added and the stirrers adjusted to 150 rev/min.

Air was sparged through the medium at approximately 300 ml/l.min. This ensured sufficient oxygenation of the medium and assisted with mixing. Air was obtained from an external compressed air source and used after passing through an oil trap (F39-100, Norgran, NZ).

3.3 Biomass Determinations

Biomass was determined gravimetrically as mixed liquor suspended solids (MLSS). As the media used did not contain any significant quantity of suspended material MLSS was taken as being equal to the biomass in the system.

One of two techniques were used. Where the biomass seed was determined for the initiation of a batch run the first (i) of the following techniques was used. All subsequent determinations during the course of the batch run and those of chemostat runs were determined using the second (ii) technique described.

(i) The biomass of a substrate acclimated culture grown in a total working volume of 2 litres was allowed to settle in a 2 litre measuring cylinder for 30 minutes. The supernatant was poured off leaving approximately 500 mls of concentrated biomass. This was centrifuged (RC5C Sorvall centrifuge, Watson Victor, Wellington, NZ., using a GS3 head) at 5000 rev/min for 15 minutes at 4°C. The centrate was carefully poured off the resultant biomass pellet leaving about 50 mls in the centrifuge tube. This remaining liquid was used to slurry up the biomass pellet from where it was transferred to a vacuum filter using a 0.45 µm membrane filter (Whatman, cellulose nitrate, plain white, 47 mm diameter).

When filtering was complete (within 30 minutes) the filter apparatus was dismantled and the biomass *paste* was scraped onto a glass evaporating dish. The paste was divided into portions of either 0.4 g or 4 g weights depending on whether the paste was being transferred as seed to a 1 l or 10 l working volume reactor respectively. All paste portions were accurately weighed (Mettler AE160 digital balance, Watson Victor, Wellington, NZ) then added to the appropriate bioreactor but one. This one was accurately weighed then placed in a 105°C oven (Watvic Oven, Cat No. 01124, Watson Victor, Wellington, NZ) on a glass evaporating dish covered with tin foil for 14 hours. The dish was then allowed to cool in a desiccator in the presence of silica gel (BDH, Poole, England) for 30 minutes after which the dried biomass weight was determined.

As one of the paste portions had been weighed as a paste then dried, the dry biomass weights for other portions separated could be reasonably estimated. As the paste was commonly found to be 10 percent dry weight after this procedure the resulting biomass seeding concentrations were generally 40 mg/l.

(ii) A sample volume was withdrawn during the time course of a batch or chemostat run. For all batch runs this first sample volume was 1 litre (In the instance of the preliminary 2,4-D batches this was also the final sample and consisted of the entire working volume). The second was 0.75 litres and subsequent samples were 0.5 litres. In a few instances the final sample volumes were reduced to as low as 0.2 litres. The decreasing volumes taken approximately corresponded to the increasing biomass with time, during the progression of batch runs. This meant the biomass filtered was approximately constant.

Samples were placed in 0.5 litre centrifuge bottles and centrifuged (RC5C Sorvall centrifuge, Watson Victor, Wellington, NZ., using a GS3 head) at 5000 rev/min for 15 minutes at 4°C. The centrate was carefully poured off. A pre-dried (2 hours at 105°C and cooled in a desiccator for 30 minutes) then weighed 0.45 µm membrane filter (Whatman, cellulose nitrate, plain white, 47 mm diameter) was placed in a vacuum filter apparatus. The biomass pellet was slurried using distilled water and quantitatively transferred to the filter. On completion of filtering the filter was placed on a glass evaporating dish and covered with tin foil. This was placed in a 105°C oven for 14 hours. The dish was then transferred to a desiccator for 30 minutes. After this the filter and dried biomass was weighed.

It is noted that the membrane filter was originally dried for 2 hours and not 14 hours as the weight differences between these times was found to be insignificant. In all cases at least 10 mg (dry weight) were present on the filter to minimise weighing errors.

3.4 Determination of Substrate Concentrations

Substrates used were 2,4-dichlorophenoxyacetic acid (2,4-D) or *para*-chloro-*ortho*-cresol (PCOC) (supplied by DowElanco, NZ, Ltd.). Their detection and quantification were determined using a spectrophotometer (Spectro PU 8625 UV/VIS, Phillips, NZ). Wavelengths were adjusted to 283 nm for 2,4-D and 281 nm for PCOC. Matched sets of quartz cells were used throughout.

Two standard curves were constructed. One for 2,4-D and another for PCOC. Standard solutions for each compound were made to concentrations between 0 and 150 mg/l and adjusted to pH 6.65 measured using an Orion Research 701A/Digital Ionalyser (Watson Victor, Wellington, NZ). Standard curves are given by Figures 3.1 and 3.2.

Samples of 5 mls were taken from batch or chemostat culture using a 5 ml automatic pipette (Gilson k-82-14282, France). These were transferred to 5 ml plastic centrifuge tubes and centrifuged (using a Clandon T52.1 centrifuge, Clandon Scientific, England) at 5000 rev/min for 10 minutes at room temperature. The centrate was carefully pipetted off and 3 mls added to the quartz cell for spectrophotometric analysis.

3.5 Chemostat Operation

Chemostats were run in two litre Erlenmeyer flasks. These were set up as described in Section 3.2 describing the temperature maintenance and mixing.

Each chemostat was fed from a 20 litre glass reservoir (Pyrex, England) containing the synthetic medium and appropriate substrate. The feed solution was dispensed using a Masterflex 7014 pump head (with C-flex tubing) mounted on a pump drive (Cole-Parmer 7546-10, peristaltic pump, Salmond Smith Biolab, Palmerston North, NZ).

The chemostat working volume was maintained by adjusting the effluent intake tube to the surface of the medium. Effluent was pumped to a 20 litre holding reservoir using a Masterflex 7016 pumphead (with C-flex tubing) also mounted on the feed pump drive. This ensured the effluent flowrate always matched the feed flowrate.

3.6 Specific Oxygen Uptake Rate

Samples of 300 mls were directly withdrawn from the chemostat to a 1 litre measuring cylinder. The sample was aerated at approximately 50 ml/min for 1 minute using an oil trapped (F39-100, Norgan, NZ) external compressed air source. During this 1 minute 2 mls of 4000 mg/l PCOC solution was added to the sample. This ensured sufficient substrate for the following oxygen uptake determination procedure. The sample was then transferred to a 300 ml BOD bottle. A

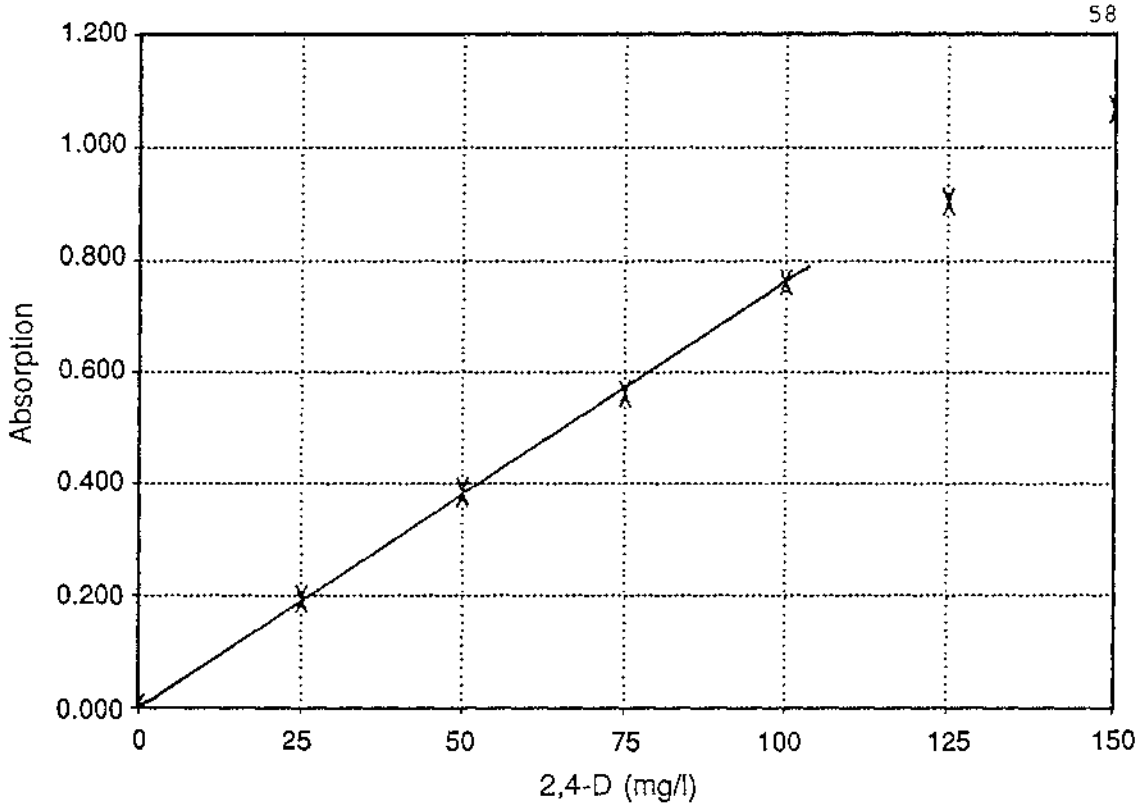

Figure 3.1: Standard Absorption Curve (2,4-D).
283 nm, pH 6.65.

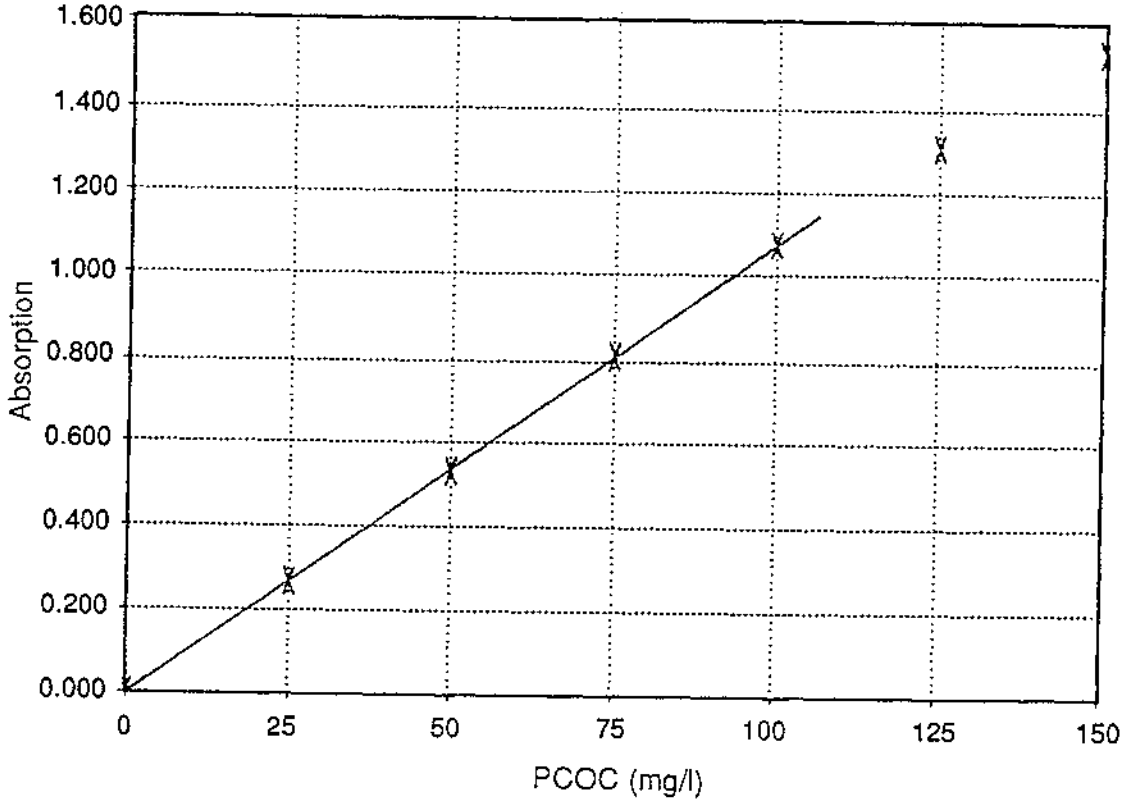

Figure 3.2: Standard Absorption Curve (PCOC).
281 nm, pH 6.65.

1.5 cm magnetic flea was added.

Dissolved oxygen was determined using a YSI M57 dissolved oxygen meter (Watson Victor, Wellington, N.Z.), fitted with a YSI 5739 probe equipped with a 0.001" standard membrane. This was standardised according to the instruments instructions. The probe was inserted into the neck of the BOD bottle. The sample was well mixed using a magnetic stirrer (Heidolph MH2002, Watson Victor, NZ) at 150 rev/min.

Dissolved oxygen concentration was recorded at 1 to 3 minute intervals until a constant concentration was reached for more than 10 minutes. This gave the oxygen uptake rate (OUR). The biomass of the sample was then determined using the procedure described by Section 3.3 (ii) above. From this specific oxygen uptake rate (SOUR) could be determined.

3.7 Synthetic Medium

The following synthetic medium described was used for all experiments. Four concentrated stock solutions were made up and used according to the desired concentrations in the synthetic medium. Stock solutions consisted of the salt solution, nutrient solution, 2,4-D concentrate and PCOC concentrate.

The salts and concentrations used in the salt stock solution are given by Table 3.1 (all Analar grade, BDH, Poole). The salt stock was made to 50 times the required concentration of synthetic medium. A total volume of 2 litres was made up at a time. To effectively dissolve all the salts the pH was adjusted to below 4 using 1 M HCl (Pronalys Grade, May and Baker, Australia).

The nutrient stock was produced using the nutrients and quantities given by Table 3.2 (all Analar grade, BDH, Poole). The stock was made to 100 times the required concentration of synthetic medium. A total volume of 1 litre was made up at a time. The nutrients were found to easily dissolve.

2,4-D and PCOC concentrate stocks were made to 4000 mg/l in 2 litre volumes. 2,4-D and PCOC was supplied in pure form by (DowElanco, NZ, Ltd). A weight of 8 g of either substance was used for making a stock. Dissolving the compound required the pH to be raised

above 10, using 10 M NaOH (Analar grade, BDH, Poole). The solution was vigorously stirred for up to 1 hour using a magnetic stirrer (Heidolph MH2002, Watson Victor, NZ).

Table 3.1 Synthetic Medium Salt Stock (Concentrate 50×).

Compound	Concentration (mg/l)
NaCl	1271
K_2SO_4	1114
$CaCl_2.2H_2O$	3668
$MgCl_2.6H_2O$	3345
$FeSO_4.7H_2O$	1245
$MnCl_2.4H_2O$	720
$CoCl_2.6H_2O$	101
$CuSO_4.5H_2O$	20
$ZnCl_2$	63
$Na_2MoO_4.2H_2O$	63

Table 3.2 Synthetic Medium Nutrient Stock (Concentrate 100×).

Compound	Concentration (g/l)
$(NH_4)H_2PO_4$	35.6
$(NH_4)_2HPO_4$	21.0
$(NH_4)_2SO_4$	24.4

The synthetic medium was made by adding 20 mls/l salt stock, 10 mls/l nutrient stock and a volume/l for the desired concentration of substrate stock to distilled water. The pH was adjusted to 6.65 by the addition of HCL or NaOH as required and measured using an Orion Research 701A/Digital Ionalyser (Watson Victor, Wellington, NZ). The meter was calibrated prior to measurements at pH 4.0 and pH 7.0 using colour key buffer solutions (Laboratory reagent grade, BDH, Poole, England).

3.8 Culture Acclimation

Culture used was obtained from a *parent bioreactor* established by McAlister (1990). The operation and culture characteristics are described by McAlister (1990). The composition of the leachate is given in Table 3.3. The bioreactor feed consisted of 10 percent leachate (McAlister, 1990).

Table 3.3 Composition of Leachate.

Component	Concentration (mg/l)
methanol	260
butan-1-ol	390
butan-2-ol	330
2,4-dichlorophenoxyacetic acid (2,4-D)	1420
4-chloro-2-methylphenoxyacetic acid (MCPA)	2020
para-chloro-*ortho*-cresol (PCOC)	520
2,4-dichlorophenol (2,4-DCP)	50
2,4,5-trichlorophenoxyacetic acid (2,4,5-T)	390
2,4,5-trichlorophenol (2,4,5-TCP)	0.5
4-chloro-2-methylphenoxybutyric acid (MCPB)	4
4,5-dichloro-2-methylphenoxyacetic acid (MDCPA)	30

An effluent volume of 0.5 litres from the *parent bioreactor* was placed in a 2 litre Erlenmeyer flask. A volume of 0.5 litres of synthetic medium was made up with the substrate and to the concentration of the future desired pure substrate batch run. For PCOC chemostat culture acclimation a PCOC concentration of 50 mg/l was generally used. The synthetic medium was added to the 2 l flask. This was run as a reactor in batch mode according to the conditions given by Section 3.2 above. Degradation was determined by spectrophotometric analysis (Section 3.4). When this was about 80 percent complete the working volume was transferred to a measuring cylinder and allowed to settle for 30 minutes. The supernatant was poured off leaving 50 mls of biomass slurry.

A 1 litre working volume batch of synthetic medium, substrate concentration determined as above, was prepared. The 50 ml biomass slurry was added to this in a 2 litre Erlenmeyer flask. This was again treated to batch culture conditions.

Sub-culturing was repeated on the biomass for two batches of 1 litre total synthetic volume batches. Seeding of the experimental runs was carried out as given by Section 3.3 (i) for batch culture or directly added as the 50 ml biomass slurry to chemostat cultures.

Chapter 4

Growth Yield and Specific Growth Rate in Batch Cultures.

4.1 Introduction

Determination of growth parameters by batch process configuration has the advantage that it is able to be operated with inhibitory substrate influences (Hobson and Millis, 1990). The experiments conducted used either 2,4-D or PCOC as a growth-limiting substrate. Substrate concentrations were taken up to the threshold of possible growth.

It has been indicated that growth yield variations of a culture may be attributed to a number of factors (Section 2.9). The following batch experiments were designed to meet a consistent set of conditions while controlling and allowing variations in the growth-limiting substrate concentration. While substrate concentration in itself can affect yield, it can also affect specific growth rate which has been shown to influence yield (Stouthamer, 1976).

This chapter describes the degradation of a growth-limiting substrate, 2,4-D or PCOC, in batch configuration. Analysis of biodegradation is carried out to relate growth yield, specific growth rate and substrate concentration.

4.2 Initial 2,4-D Batch Experiment

Preliminary batch runs were operated using 2,4-D as the growth-limiting substrate. Growth yield was determined. Operating conditions and results would allow better design of subsequent experiments.

4.2.1 Experimental Procedure

Multiple batches were designed to run at various concentrations of 2,4-D: duplicates at 50 mg/l and triplicates at 100, 200, 300, 400, 600, and 800 mg/l, in synthetic medium (Section 3.7). All

batch runs were carried out at 25°C, using 1 litre working volumes, in 2 litre Erlenmeyer flask reactors. Operating conditions were the same as described in Section 3.2. Biomass was determined gravimetrically and 2,4-D concentration by spectrophotometry (Sections 3.3 and 3.4). Biomass was determined at the beginning and end of each batch run.

4.2.2 Results

Growth yield against initial 2,4-D concentration is given by Figure 4.1. Yield significantly decreases with increasing substrate concentration up to 400 mg/l. Yield remains at a constant low after this concentration. Statistical data for the results are given by Table A1.1 (Appendix 1).

4.2.3 Discussion

A decrease in growth yield with substrate concentration was observed in Figure 4.1. This decrease was significant as given by relative small standard errors in the growth yields (Table A1.1).

The experiment was operated using an inhibitory substrate thereby placing the system under stress. Stouthamer (1976) gave environmental stress as a factor increasing cell maintenance requirement. Substrate (energy source) used for maintenance is substrate that is not available for productive increase in biomass. As the substrate concentration was increased, increasing stress on the culture would result in an increased maintenance requirement. The measured yield would correspondingly fall as observed.

The constant low yield at high substrate concentrations, but below the threshold concentration, is more difficult to interpret. However as the experiment was run as an initial trial it is possible experimental weighing errors may have contributed to this.

4.3 2,4-D Batch Experiment

A set of batch runs were designed to operate at various 2,4-D concentrations. Measurements

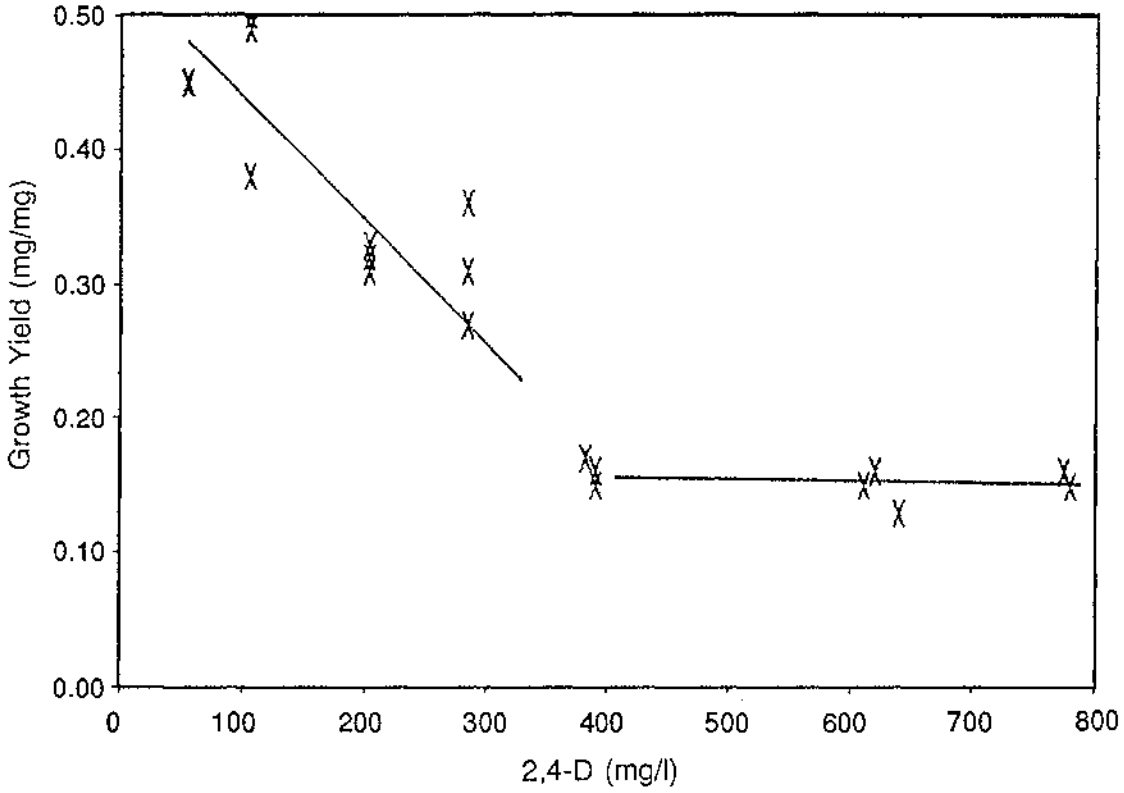

Figure 4.1: Growth Yield versus Initial 2,4-D.
Initial Batch Experiment.

were taken to enable growth yields and specific growth rates to be determined.

4.3.1 Experimental Procedure

Batch runs were operated with initial substrate-limiting concentrations of 2,4-D between 100 and 600 mg/l: duplicates at 100, 300, and 600 mg/l, and single batches at 200 and 400 mg/l. Batch runs were carried out in 20 litre glass reactors using 10 litre working volumes. Operating conditions were the same as described in Section 3.2.

Biomass was determined gravimetrically and 2,4-D concentration by spectrophotometry (Sections 3.3 and 3.4). Biomass measurements were taken at approximately five even time intervals from the beginning to completion of degradation in each batch.

4.3.2 Results

Growth yield and specific growth rate against 2,4-D concentration are given by Figures 4.2 and 4.3 respectively. Growth yield decreases with increasing substrate concentration. Specific growth rate rises constantly to a maximum then begins to decline when initial substrate concentrations are greater than 500 mg/l.

Figure 4.4 gives reciprocal yield against reciprocal specific growth rate. The slope of this plot can generally be used to indicate the maintenance coefficient, m_e. A positive slope is not apparent so no attempt to determine maintenance coefficient has been made.

An Eadie-Hofstee plot is given by Figure 4.5. The absolute of a negative slope of this line will give the saturation constant, K_s, for the culture-substrate system. A K_s value of 100 mg/l has been estimated.

Raw data for these experiments is given in Appendix 1.

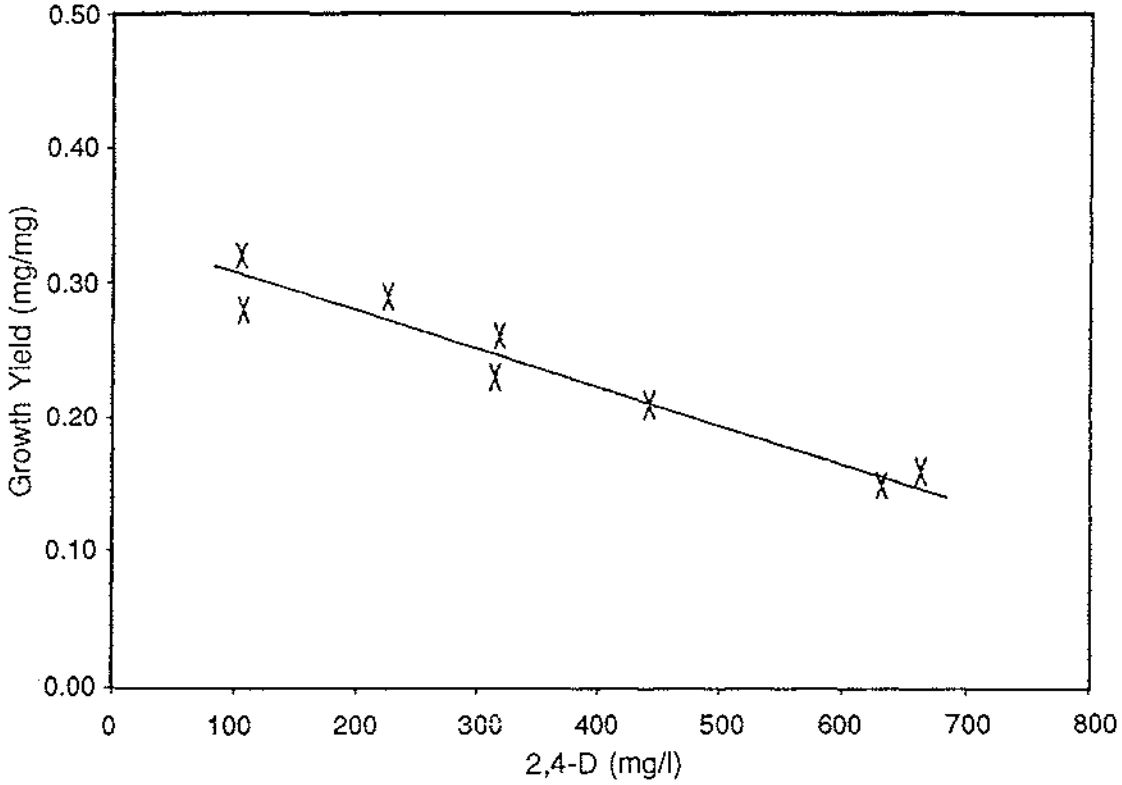

Figure 4.2: Growth Yield versus Initial 2,4-D.
Final Batch Experiment.

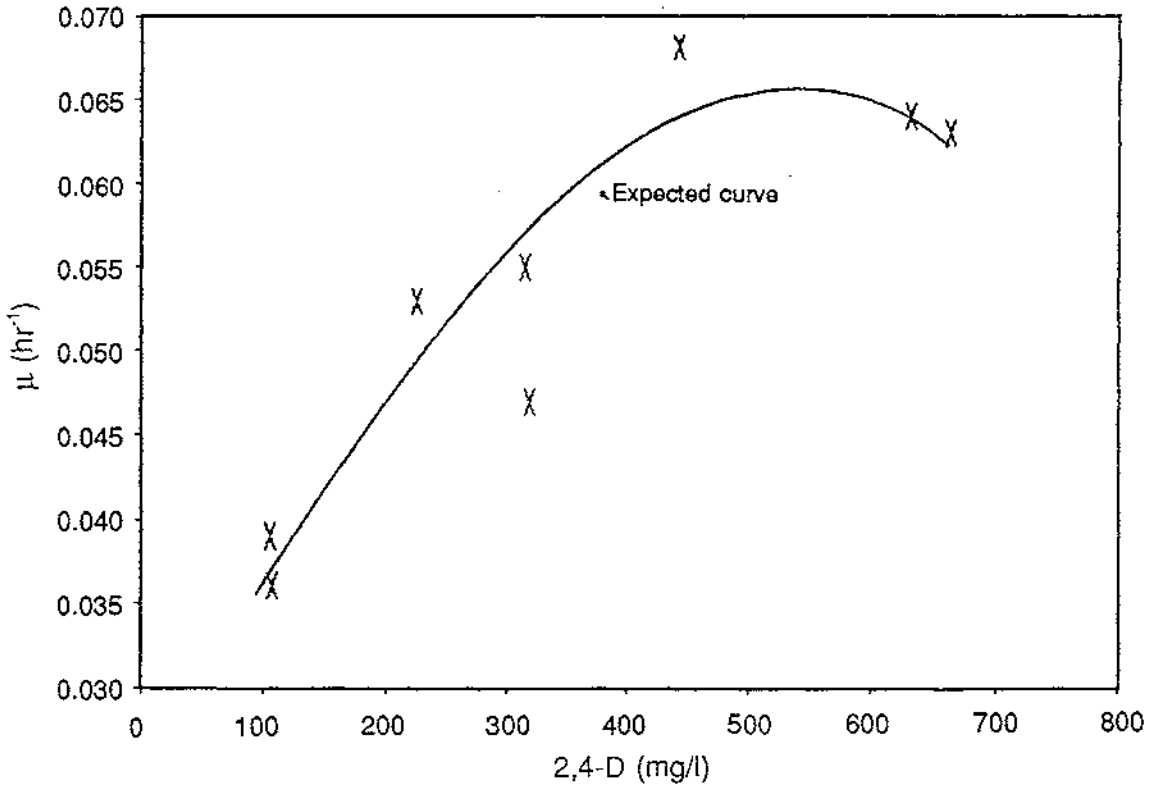

Figure 4.3: Specific Growth Rate versus Initial 2,4-D.
Final Batch Experiment.

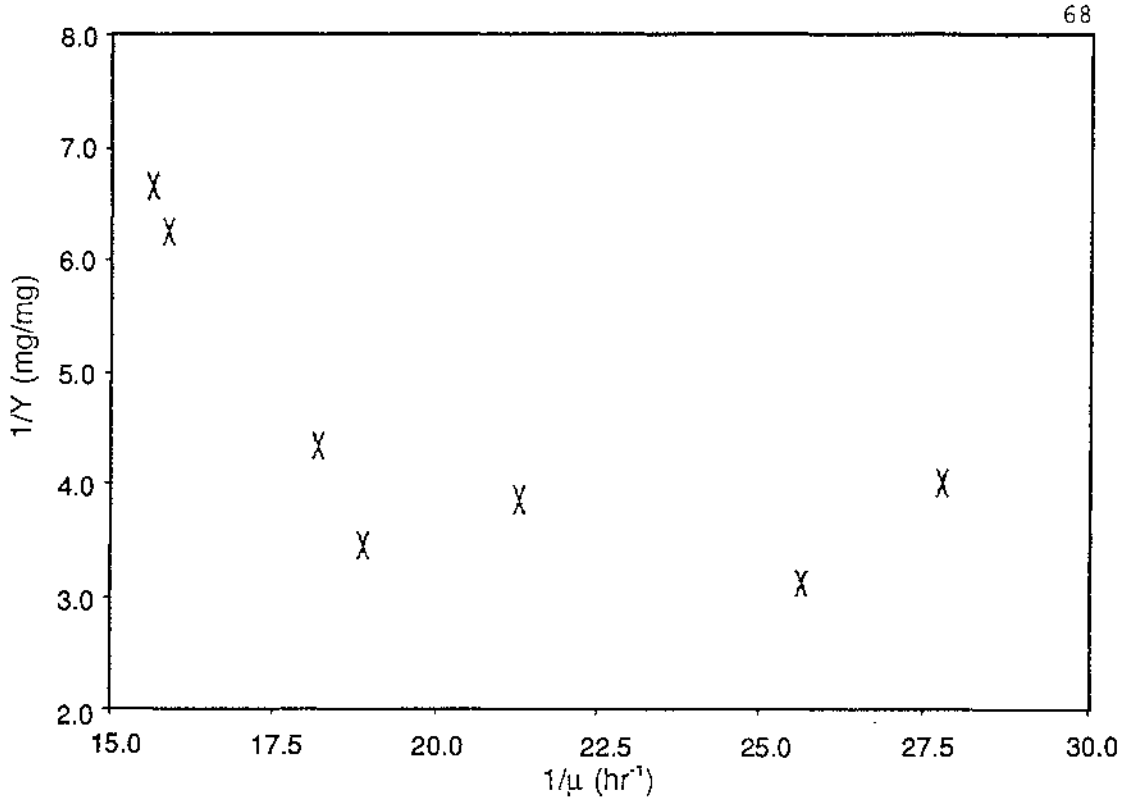

Figure 4.4: Reciprocal Yield versus Reciprocal Specific Growth Rate (2,4-D). Final Batch Experiment.

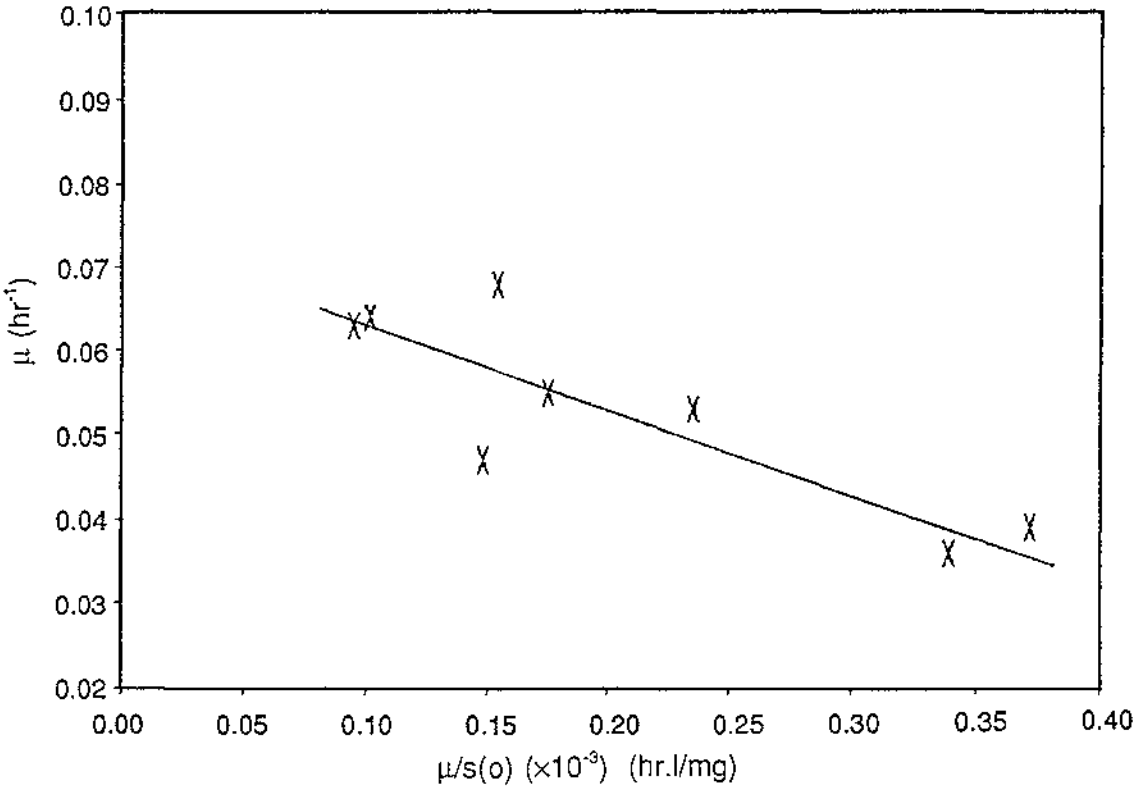

Figure 4.5: Eadie-Hofstee Plot (2,4-D). Final Batch Experiment.

4.3.3 Discussion

Large working volumes of 10 litres were used in the batch runs. This was as a result of the large samples required for biomass determinations (Section 3.3). Sampling should not reduce the working volume more than 30 percent. It was considered that a reduction greater than this would significantly alter system geometry and possibly lead to biosystem variations due to geometrical effects.

A decrease in growth yield was observed with increasing limiting-substrate concentration. This was similar to, though not as pronounced, as the decreasing yield observed in the initial 2,4-D batch experiment (Section 4.2). Hobson and Millis (1990) indicated that phenolic compounds may damage cell membranes increasingly as phenolic concentration increases. It is expected that the increasing maintenance requirement with damaging inhibitory substrate results in this decreasing yield.

Stouthamer (1976) indicated specific growth rate together with the maintenance coefficient determine the relative amount of the substrate (energy source) which is used as maintenance. In assuming this the maintenance coefficient is considered to be constant. This was used to explain low growth yields at low growth rates. However such observations were made for growth on innocuous carbon sources. It is reasonable to expect an increase in maintenance with growth on an inhibitory substrate as described above.

A cell culture may become more susceptible to the adverse effects of an inhibitory substrate with increasing growth rate (Hobson and Millis, 1990; Mink *et al.*, 1982; Green, 1978; Ng, 1982). High growth rates would therefore lead to increased cell damage increasing the maintenance coefficient.

Specific growth rates have been determined by regressing the natural logarithm of the biomass against time. These plots for batch runs performed are given by Figures A1.9 to A1.16, Appendix 1.

Figure 4.3 indicates an increasing growth rate with substrate concentration to about 500 mg/l. After this concentration the growth rate falls slightly. The effect of growth rate increasing maintenance (Hobson and Millis, 1990) reinforces the effect of decreasing yield with substrate concentration in Figure 4.2. Above 500 mg/l specific growth rate may decrease due to excessive

cell damage caused by the inhibitory substrate. The maintenance coefficient would remain high and the growth yield low.

Analysis of the results has indicated that maintenance coefficient most probably increases with increasing substrate concentration and specific growth rate. Pirt (1965) gave a mathematical equation, eqn (2-29), enabling the maintenance coefficient to be determined from growth yield and specific growth rate. The maintenance coefficient is determined as the slope of a plot of reciprocal yield against reciprocal specific growth rate. This is given by Figure 4.4.

The maintenance coefficient is expected to be positive. Between the specific growth rates 1/28 (0.036) to 1/18 (0.056) hr^{-1}, maintenance is essentially zero. These growth rates correspond to 2,4-D substrate concentrations between 100 and 400 mg/l. Above the specific growth rate 1/18 hr^{-1} (closer to the origin on Figure 4.4) the maintenance coefficient is negative.

Pirt (1965) gave equation (2-29) to describe variations in observed yield with growth rate for growth on innocuous substrates. The effects of inhibitory substrates were not considered. The current analysis for the inhibitory substrate system is considered inappropriate.

Figure 4.5 gives an Eadie-Hofstee plot for the batch runs. Bailey and Ollis (1977) describe the plot (Section 2.4.1.4). This may be used to determine the affinity the culture has for the substrate. The affinity is measured by the saturation coefficient, K_s. This is given by the absolute value of the slope of the Eadie-Hofstee plot. Figure 4.5 indicates a K_s value of 100 mg/l. This value is relatively large when compared to those in the literature (Table 2.2).

A saturation constant evaluated by this method is considered to be vulnerable to the errors contained in the measured variable μ. Also the inhibitory nature of the substrate will effect the analysis. The Eadie-Hofstee plot is derived from Monods equation, based on growth on innocuous substrates. The inhibitory nature of the substrate used increases with increasing concentration. Greater accuracy in applying the Eadie-Hofstee plot would therefore be attained by designing an experiment to give more points at lower substrate concentrations.

4.4 PCOC Batch Experiment

A set of batch runs were designed to operate at various PCOC concentrations. Measurements were taken to enable growth yields and specific growth rates to be determined.

4.4.1 Experimental Procedure

Batch runs were operated with initial substrate-limiting concentrations of PCOC between 25 and 100 mg/l: duplicates at 25 mg/l, triplicates at 50 and 75 mg/l, and a single batch at 100 mg/l. Batch runs were carried out in 20 litre glass reactors using 10 litre working volumes. Operating conditions were the same as described in Section 3.2.

Biomass was determined gravimetrically and PCOC concentration by spectrophotometry (Sections 3.3 and 3.4). Biomass measurements were taken between four to seven even time intervals for each batch during substrate degradation.

4.4.2 Results

Growth yield and specific growth rate against initial PCOC concentration are given by Figures 4.6 and 4.7 respectively. Growth yield decreases with increasing substrate concentration. Specific growth rate rises to a maximum, at initial substrate concentration 55 mg/l, then constantly decreases as substrate concentration continues to increase.

Figure 4.8 gives reciprocal yield against reciprocal specific growth rate. The slope of the linear relation given by this plot is usually used to determine the maintenance coefficient, m_e. The given plot is inadequate for this determination.

An Eadie-Hofstee plot is given by Figure 4.9. A negative relationship is not apparent to enable an estimate of the saturation constant, K_s, to be determined from the plot.

Raw data for these experiments is given in Appendix 2.

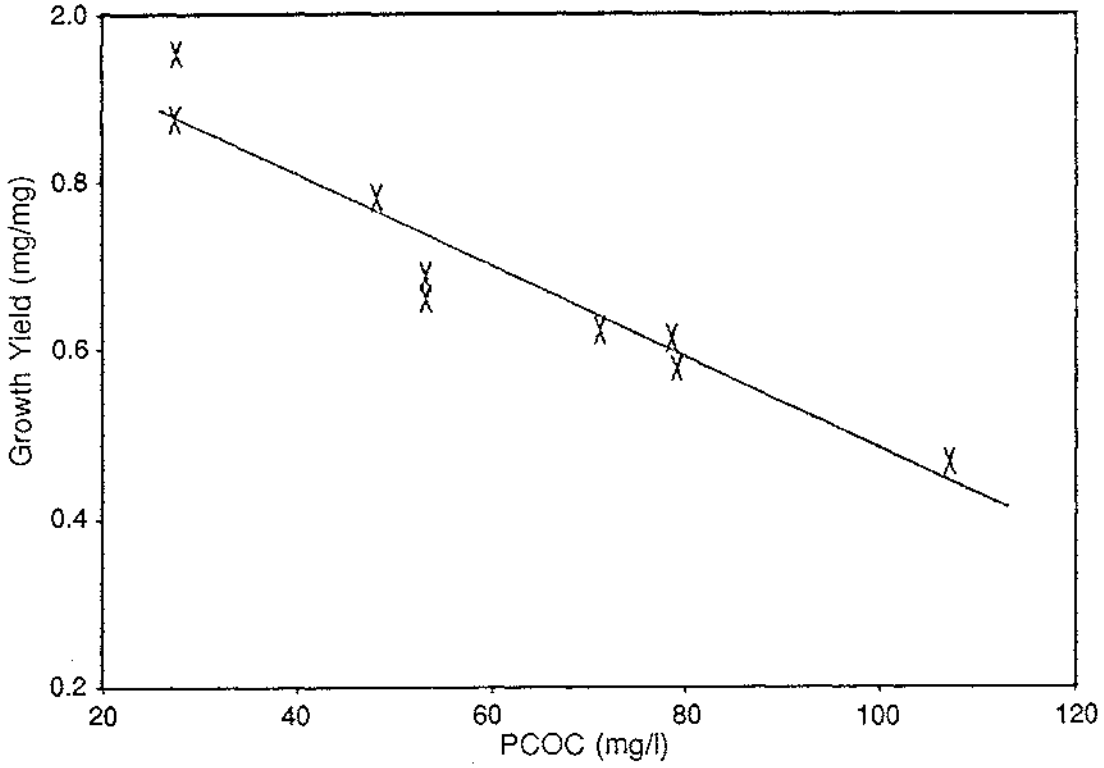

Figure 4.6: Growth Yield versus Initial PCOC.
Final Batch Experiment.

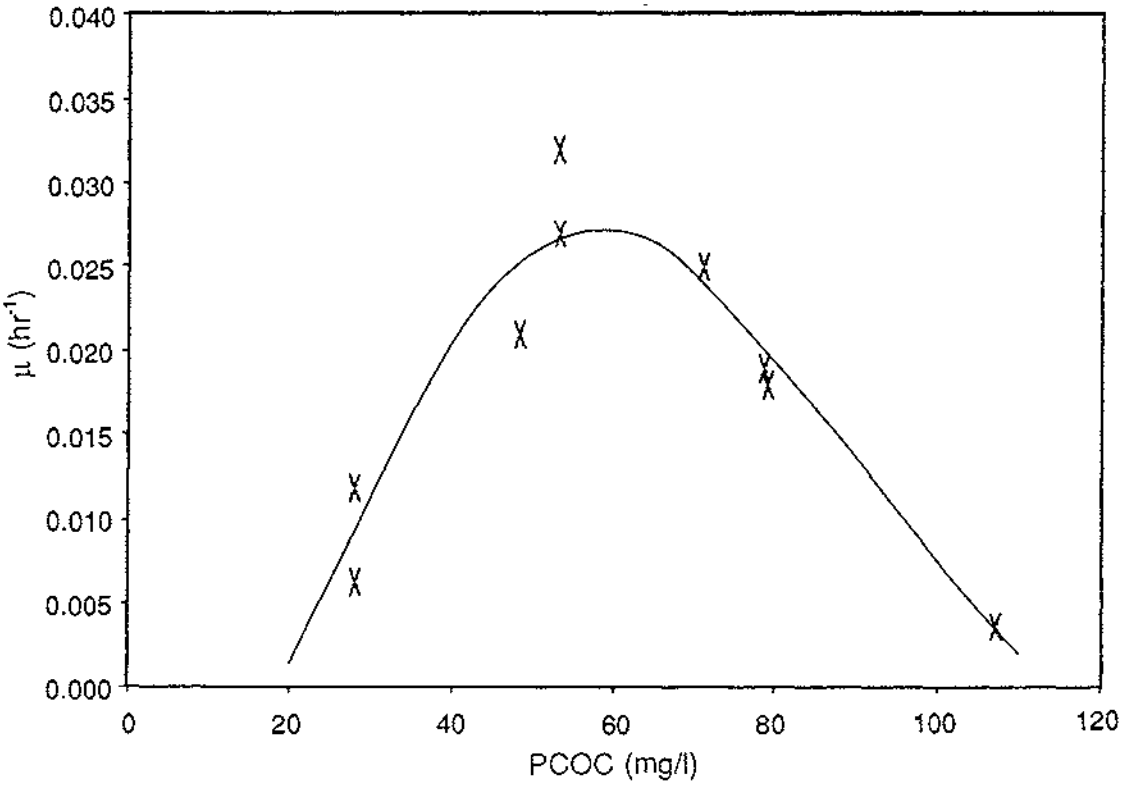

Figure 4.7: Specific Growth Rate versus Initial PCOC.
Final Batch Experiment.

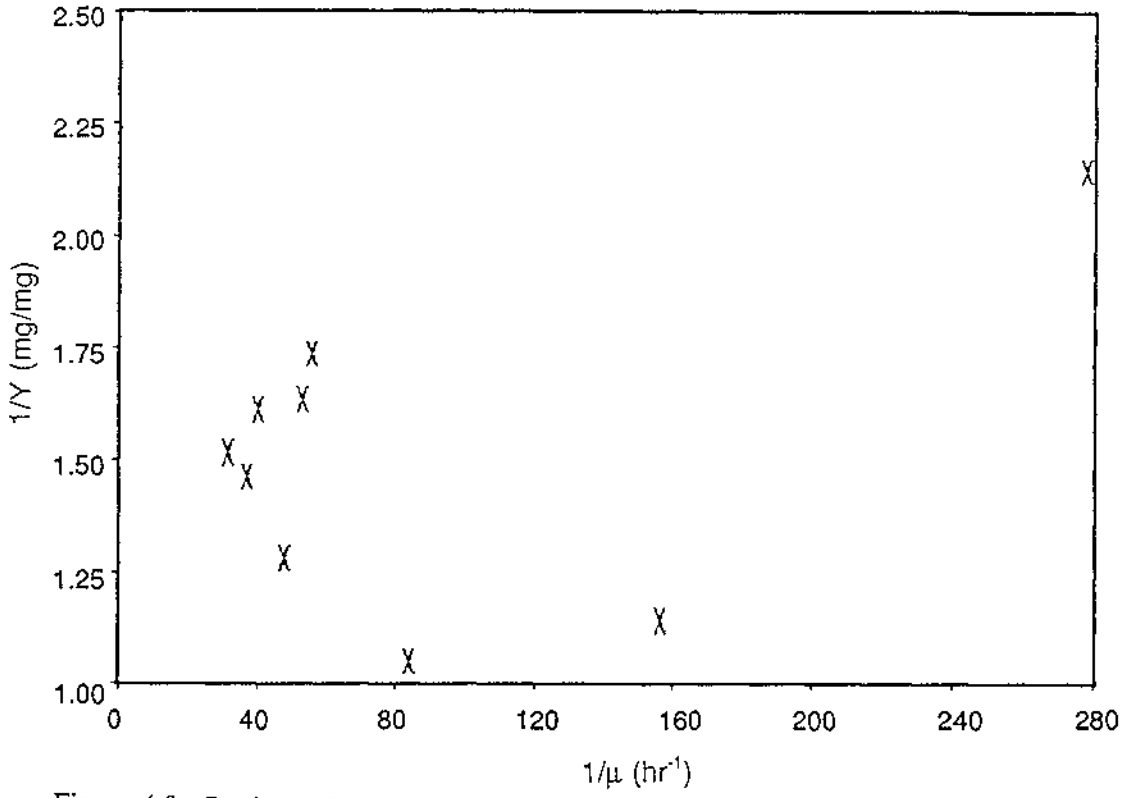

Figure 4.8: Reciprocal Yield versus Reciprocal Specific Growth Rate (PCOC).
Final Batch Experiment.

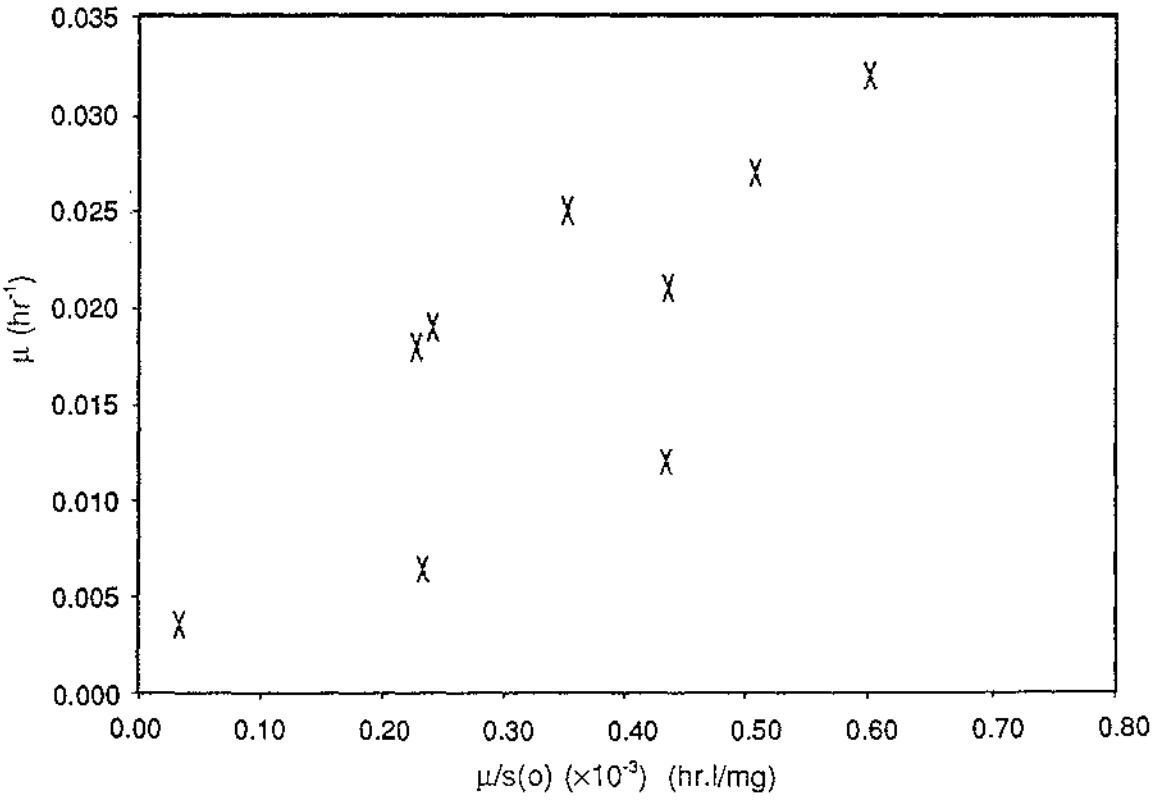

Figure 4.9: Eadie-Hofstee Plot (PCOC).
Final Batch Experiment.

4.4.3 Discussion

Large working volumes of 10 litres were again used in the batch runs. This allowed a number of large samples to be taken for biomass determinations (Section 3.3). A 30 percent drop in working volume was not exceeded for any of the batch runs, thereby minimising the effects of a change in geometry on the system.

Growth yield decreased with increasing initial growth-limiting substrate concentration. This can be explained by an increasing maintenance requirement of the culture as cell damage increases with the increasing phenolic concentrations (Hobson and Millis, 1990; Mink *et al.*, 1982; Green, 1978; Ng, 1982).

Cells become more sensitive to environmental stress agents as growth rate increases (Hobson and Millis, 1990). Specific growth rate variations between batch runs were studied. An increase in specific growth rate would result in increased cell damage therefore increasing the expected maintenance coefficient.

Specific growth rates have been determined by regressing the natural logarithm of the biomass against time (Appendix 2).

Figure 4.7 gives specific growth rate against initial substrate concentration. Growth rate increases with substrate concentration to 55 mg/l. Figure 4.6 shows the growth yield decreasing over this concentration range. This can be explained by an increasing maintenance coefficient (Hobson and Millis, 1990). From substrate concentrations 55 to 110 mg/l (Figure 4.7) the specific growth rate constantly decreases. This would be expected at a high concentration of an inhibitory substrate where the cell damage cannot be adequately repaired and growth is inhibited. The maintenance requirement is expected to remain high over this period. This is indicated by the continuing low growth yields at the higher substrate concentrations in Figure 4.6.

An estimation of maintenance coefficient was attempted by use of a mathematical equation given by Pirt (1965), eqn (2-29). This indicates the slope of a plot of reciprocal yield against reciprocal specific growth rate will give the maintenance coefficient. For the current investigation this is given by Figure 4.8. The data used to generate this is in Table A2.1. The maintenance, and therefore slope of the plot, is always expected to be a positive value. It is difficult to put any line or curve through the points with any assurance.

Eqn (2-29), given by Pirt (1965), was designed for use with data derived from innocuous carbon sources. The data derived from biosystems using inhibitory substrate is inappropriate for analysis using the equation. Pirt (1975) indicated that lower growth rates would give decreased observed growth yields. Figures 4.6 and 4.7 show that this variation has not occurred. At low growth rates growth yield has been shown to be at its highest. This is consistent with the explanation of effects of stress agents given by Hobson and Millis (1990).

An Eadie-Hofstee plot is given by Figure 4.9 for the batch runs. Bailey and Ollis (1977) describe the plot (Section 2.4.1.4). This has been found useful for determining the saturation constant, K_s, of a cell-substrate system. K_s is given by the absolute of a negative slope of the plot. Figure 4.9 gives no indication of a negative slope. The data used to generate this figure is given in Table A2.1.

To understand this anomaly the origins of the Eadie-Hofstee plot need to be considered. The plot is derived from the Monod equation (Monod, 1942) given in Section 2.4.1.4 by eqn (2-12). This equation was designed to explain cell growth at relatively low substrate concentrations (avoiding inhibitory effects) on innocuous carbon sources. As the substrate concentration is increased Monods equation indicates the specific growth rate is expected to increase towards its maximum. Figure 4.7 indicates this up to a substrate concentration of 55 mg/l. However the increase is so great when compared to the increase in substrate concentration that a positive relation is formed between the axis of the Eadie-Hofstee plot. The cause of this sensitivity of growth rate to substrate concentration is uncertain. At higher substrate concentrations specific growth rate decreased. This is consistent with inhibitory growth (Hobson and Millis, 1990) and is not explained by Monods equation.

4.5 Summary and Conclusions

Batch runs were performed using 2,4-D or PCOC as growth-limiting substrate. Similarities in variations of measured parameters for growth on each of the substrates were observed:

(i) Growth yield decreased constantly with increasing initial substrate concentration. An increasing maintenance requirement accounts for this.

(ii) Specific growth rate increased constantly to a maximum then decreased with increasing

initial substrate concentration. This is considered a result of growth inhibition.

(iii) Determination of a maintenance coefficient for the 2,4-D and PCOC systems was given. Results obtained were inconclusive. This was explained by the method of analysis used, based on Monods equation, being unable to take account of inhibitory substrate influences.

(iv) An Eadie-Hofstee plot was used to obtain saturation constants, K_s. The 2,4-D system gave a constant of 100 mg/l. This is high when compared to values in the literature. A K_s value could not be determined for the PCOC system. As the plot is derived from Monods equation and the substrate concentrations used were relatively high and inhibitory, determination of K_s is considered inaccurate or inadequate.

Growth yield and specific growth rate variations were similar for growth on both the growth-limiting substrates 2,4-D and PCOC. An explanation using maintenance requirements and inhibition effects adequately explains this.

Chapter 5

Growth Studies in PCOC fed Chemostats.

5.1 Introduction

The chemostat permits control of both the population density and the growth rate of a culture. These are controlled by the concentration of the limiting nutrient in the feed and the flow rate respectively. The chemostat suffers from the disadvantage that it cannot be operated in inhibitory growth conditions (Hobson and Millis, 1990).

When a culture is grown under conditions of stress its maintenance requirement is expected to increase (Stouthamer, 1976). Stress may be caused by an increased concentration of an inhibitory nutrient. The culture may also become more susceptible to the effects of a stress causing agent with increasing growth rate (Hobson and Millis, 1990; Mink *et al.*, 1982; Green, 1978; Ng, 1982). The following experiments have been designed to operate at a constant growth rate on the growth-limiting substrate PCOC. Variations in the growth yield will reflect changes in the maintenance requirement independent of growth rate. Brock *et al.* (1984) have indicated that the chemostat configuration is convenient for estimating growth yields.

Respirometric measurements may be used to indicate cell activity (Suschka and Ferreira, 1986). Cell activity is a measure of culture viability and gives useful information in understanding the behaviour of a culture under varying conditions.

This chapter studies growth yield of a culture growing on PCOC as the growth-limiting substrate in a chemostat configuration. Analysis of growth relates growth yield, cell activity and substrate concentration.

5.2 Initial PCOC Chemostats

A preliminary experiment was carried out using PCOC as growth-limiting substrate. This was to give an indication of the stability of the biomass-substrate system. Also, effluent biomass and

substrate concentration data would allow better design of subsequent experiments.

5.2.1 Experimental Procedure

Four chemostats were designed to run at PCOC feed concentrations of 50, 100, 150 and 200 mg/l in synthetic medium (Section 3.7). Chemostats were run at a constant dilution rate of 0.14 hr^{-1}. The experiments were carried out at 25°C, using approximately 1.4 litre working volumes, in 2 litre Erlenmeyer flask reactors. All conditions were the same as those described in Sections 3.2 and 3.5.

Samples were taken for biomass and PCOC analysis approximately once every 24 hours. Biomass was determined gravimetrically and PCOC concentration by spectrophotometry (Sections 3.3 and 3.4).

5.2.2 Results

Biomass and substrate concentrations are given in Table 5.1. Biomass is represented by MLSS. At the higher substrate (PCOC) feed concentrations (s_r) of 144 and 189 mg/l, MLSS could not be accurately estimated. At lower PCOC feed concentrations, 58 and 103 mg/l, MLSS concentrations are given with estimated errors. A significant increase in MLSS concentration from 41 to 71 mg/l is apparent. Corresponding yield estimates are given. Although there is a decrease in yield, from 0.77 to 0.74, the estimated errors indicate that this is not significant.

An increase in effluent substrate concentration (s) is apparent with increasing feed substrate concentration (s_r), given by Figure 5.1.

Appendix 3 gives raw data for the chemostat runs.

5.2.3 Discussion

Preliminary investigations with the chemostat system indicated a dilution rate of 0.14 hr^{-1} was high. Washout had frequently occurred at higher rates indicating the critical dilution rate to be

Table 5.1 Summary of initial PCOC Chemostat Experiment.

Chemostat (Designed s_r mg/l)	s (mg/l)	Std. Deviation	s_r (mg/l)	Std. Deviation	s_r-s (mg/l)	Std. Deviation
50	4.8	0.5	58	0.3	53	0.8
100	5.8	1.0	103	1.5	96	2.5
150	7.0	0.8	144	1.7	138	2.5
200	9.4	1.0	189	2.7	180	3.7

Chemostat (Designed s_r mg/l)	MLSS (mg/l)	Std. Deviation	Growth Yield	Estimated Error (±)
50	41	1	0.77	0.03
100	71	6	0.74	0.07
150	(Chemostat not at Steady-State)			
200	(Chemostat not at Steady-State)			

close to this value.

Under true chemostat conditions dilution rate is equal to specific growth rate of the culture (Pirt, 1975). An increase in growth rate can give a culture greater susceptibility to cell damaging agents (Mink *et al.*, 1982; Ng, 1982). Phenolic compounds, for example PCOC, have been shown to attack cell membranes. From such evidence it is expected that the maintenance requirement of a culture would increase with increasing growth rate even with a constant concentration of a stress causing agent such as PCOC.

The experiment described was designed to analyze the effects of variations in feed substrate concentrations, independent of other factors, on growth parameters. Dilution rate was maintained

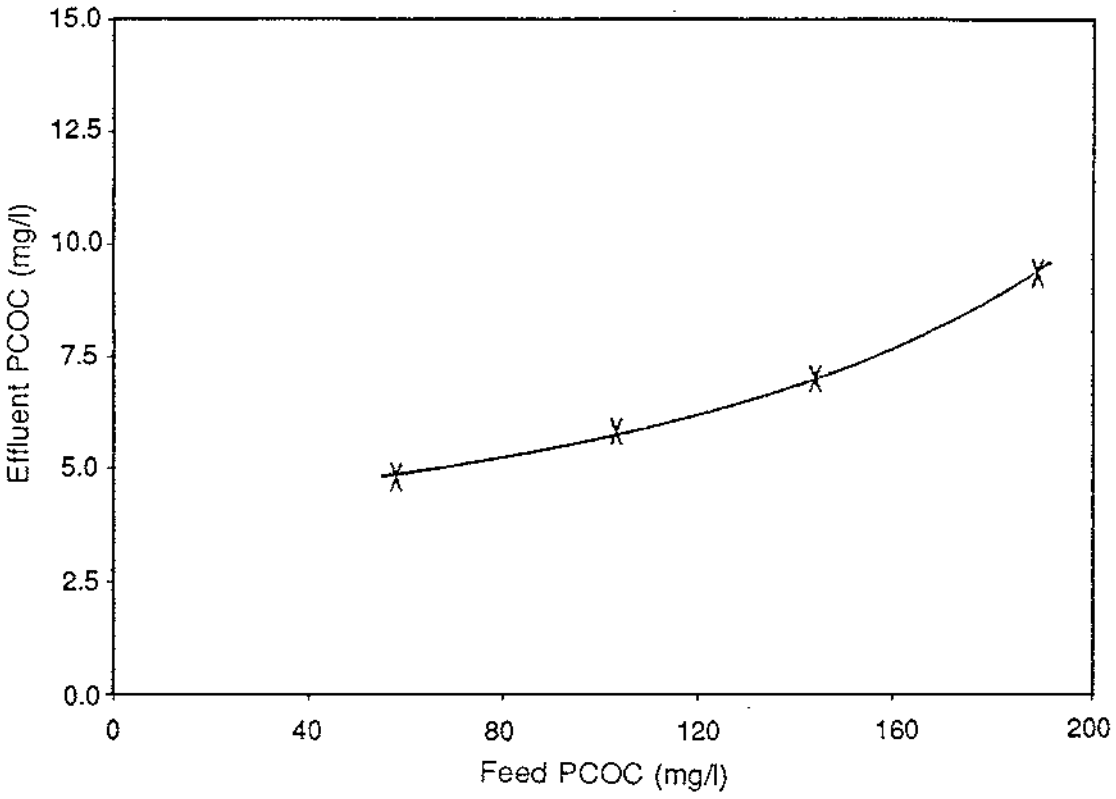

Figure 5.1: Effluent PCOC versus Feed PCOC.
Initial Chemostat Experiment.

at a constant thereby avoiding possible effects caused by variations in growth rate. A high dilution rate was used as this was expected to maximise cell viability (Postgate and Hunter, 1962; Tempest *et al.*, 1967).

Measurements of biomass, substrate, and growth yield are useful for analyzing the biomass system:

Biomass may be described by MLSS. This is considered to be a good estimate for the given synthetic medium as all components other than cell mass are dissolved. Table 5.1 shows that MLSS increases from 41 to 71 mg/l as substrate concentration increases from 58 to 103 mg/l. This variation is consistent with conventional chemostat theory, eqn (2-25), indicating increased biomass with feed substrate concentration for non-inhibitory substrates (Pirt, 1975).

MLSS for feed concentrations 144 and 189 mg/l have a large degree of uncertainty. This is indicated by Figures A3.6 and A3.8 respectively (Appendix 3). The oscillations show the chemostats have not attained steady-state. Oscillations in chemostat parameters are considered later in this section.

Effluent substrate concentration against feed substrate concentration is given by Figure 5.1. A gradual increase in effluent substrate concentration with feed substrate is apparent. Preliminary investigations with the chemostat system showed for the given dilution rate a maximum feed concentration of 200 mg/l was possible without incurring wash out. Chemostat theory indicates that effluent substrate concentration should remain constant, and usually very low, for a given dilution rate regardless of the limiting substrate feed concentration (Brock *et al.*, 1984; Pirt, 1975). This is indicated by eqn (2-26). Estimated errors given for effluent substrate (Table 5.1) indicate the increase in concentration to be significant. This may be explained by the inhibitory effect of the substrate. Increased feed substrate concentrations stress the cells in culture up to the point where washout occurs (s_r = 200 mg/l). With increasing stress the efficiency of substrate metabolism decreases resulting in increased substrate residue in the medium.

Growth yield variations with feed substrate for the experiment are difficult to estimate due to the uncertainties of MLSS at higher substrate concentrations. An initial yield drop from 0.77 to 0.73 is given for increasing feed concentrations from 58 to 103 mg/l. However estimated errors indicate this is not significant.

The uncertainties in MLSS determinations for chemostats with substrate feed rates 144 and 189 mg/l are due to wide MLSS parameter oscillations. For all chemostats no significant oscillations in feed or effluent substrate concentrations were apparent. MLSS oscillations may be a result of insufficient time allowed for the chemostat to reach steady state. Subsequent experiments would overcome this by allowing greater operating time. The frequency of sampling may also affect stability. 100 to 200 ml samples were withdrawn directly from the chemostat approximately once every 24 hours. This represents 7 - 14 percent of the working volume. Alternatively oscillatory behaviour may be inherent in the chemostat system at higher feed substrate concentrations (Curds, 1971; Crooke and Tanner, 1982). Again extended operation time would determine this.

5.3 PCOC Chemostats with Cell Activity Determination

Chemostats were operated using PCOC as growth-limiting substrate. Conditions were carefully controlled so as to obtain steady state biomass and substrate concentrations. Biomass, substrate and cell activities were determined to enable a better understanding of the biosystems studied.

5.3.1 Experimental Procedure

Six chemostats were designed to operate at PCOC feed concentrations of 50, 75, 100, 125, 150 and 175 mg/l in synthetic medium (Section 3.7). Chemostats were operated at a constant dilution rate of 0.13 hr^{-1} at 25°C. Working volumes used were approximately 1.4 litres in 2 litre Erlenmeyer flask reactors (Section 3.2). Other operating conditions were the same as those described in Section 3.5.

Samples were taken for combined biomass, PCOC concentration and oxygen uptake rate determinations at intervals of no less than 48 hours. Cell activities are indicated by oxygen uptake rates. Biomass was determined gravimetrically, PCOC by spectrophotometry and oxygen uptake rates by direct oxygen probe measurements (Section 3.6).

5.3.2 Results

Direct parameter measurements of biomass (MLSS) and substrate concentrations are given in Appendix 4 by Table A4.1. Figures A4.1 to A4.20 give dissolved oxygen concentrations for samples taken. These were used, with biomass, to give specific oxygen uptake rates (SOUR) given in Table A4.1.

Figure 5.2 gives MLSS against substrate used (s_r-s). An essentially constant increase in MLSS with metabolised substrate is apparent.

Effluent substrate against feed substrate concentrations are given by Figure 5.3. The effluent substrate is observed to increase with feed substrate. Standard errors for effluent substrate concentrations are given by Table 5.2. These indicate increases in effluent substrate observed are significant.

Growth yield is plotted against effluent substrate concentration in Figure 5.4. Yield appears to be slightly decreasing with substrate concentration between 5 and 20 mg/l. However there is substantial scatter in the data. Standard errors are given in Table 5.2.

SOUR against effluent substrate concentration is given by Figure 5.5. SOUR initially increases and peaks when the effluent substrate concentration is 15 mg/l. This begins to decline as the effluent concentration continues to rise. Standard errors are given in Table 5.2.

SOUR against MLSS is given by Figure 5.6. SOUR initially increases to a maximum, then decreases with MLSS. Standard errors are given in Table 5.2.

5.3.3 Discussion

Preliminary investigations with the chemostat system had determined the critical dilution rate to be approximately 0.15 hr^{-1}. A high dilution rate traditionally is thought to give maximum culture viability (Postgate and Hunter, 1962; Tempest *et al.*, 1967). While this is desirable a dilution rate too close to the critical rate may result in washout with minor operating fluctuations. By considering this a dilution rate of 0.13 hr^{-1} was chosen.

Table 5.2 Summary of PCOC Chemostat and Cell Activity Experiment.

Chemostat (Designed s_r mg/l)	s (mg/l)	Std. Deviation	s_r (mg/l)	Std. Deviation	MLSS (mg/l)	Std. Deviation
50	6.2	1.7	58.5	2.2	40.0	1.9
75	10.6	0.5	88.1	1.6	53.3	3.8
100	10.9	2.1	107.1	0.8	72.7	5.1
125	15.0	0.9	131.8	0.6	82.2	4.9
150	15.7	1.3	152.2	0.9	94.9	3.0
175	18.9	2.4	171.5	2.3	109.4	4.7

Chemostat (Designed s_r mg/l)	SOUR ($\times 10^{-3}$) (mg/l)	Std. Deviation ($\times 10^{-3}$)	Growth Yield	Std. Deviation
50	2.2	0.05	0.76	0.050
75	2.2	0.14	0.69	0.068
100	2.9	0.21	0.75	0.049
125	3.2	0.00	0.70	0.039
150	3.2	0.26	0.70	0.024
175	3.0	0.09	0.71	0.029

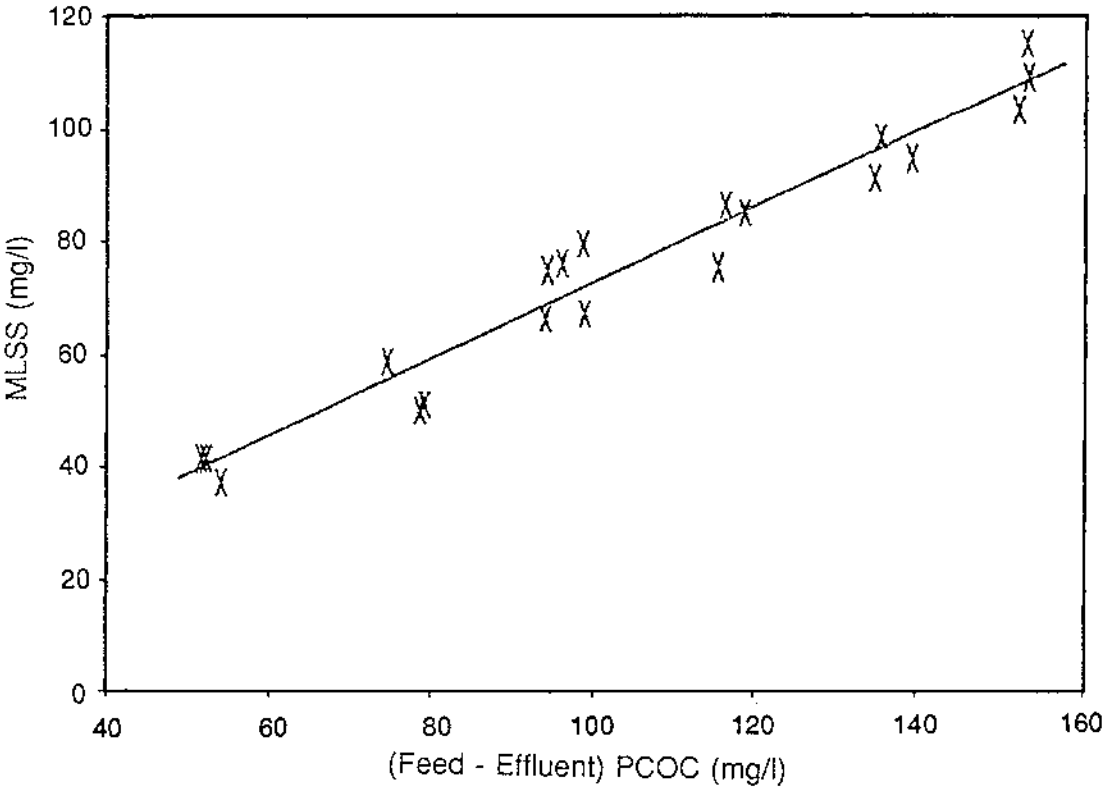

Figure 5.2: MLSS versus PCOC utilised.

Final Chemostat Experiment.

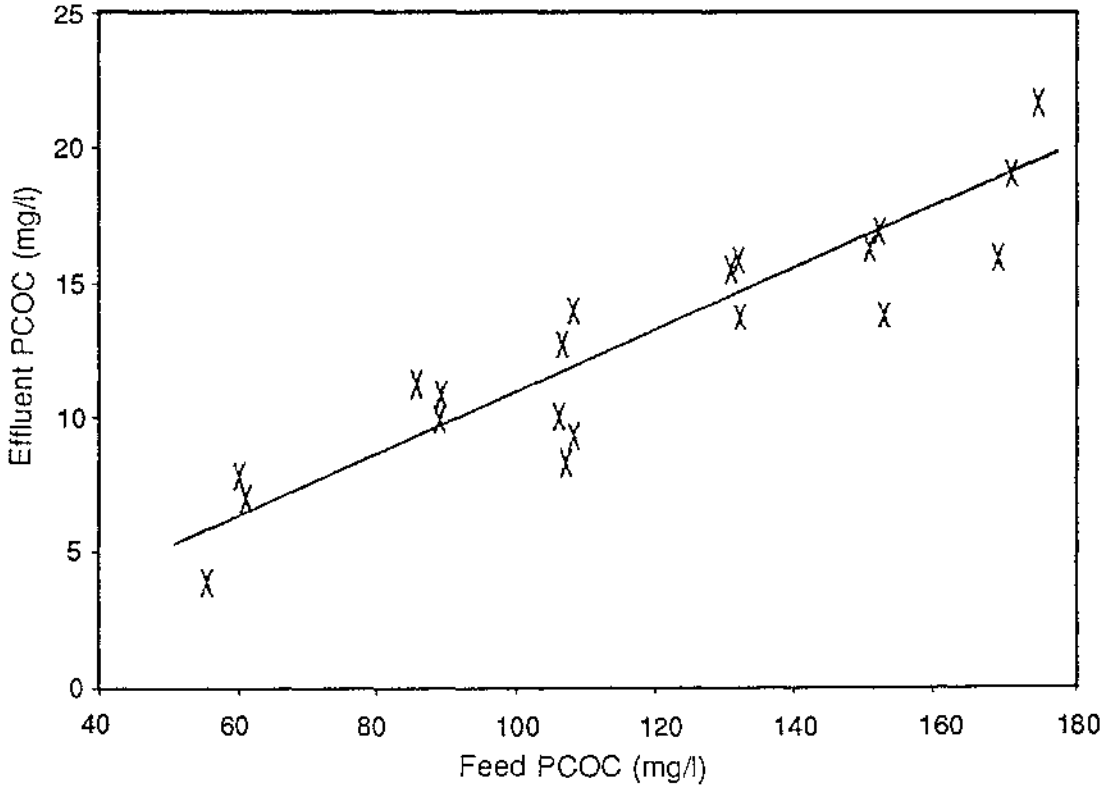

Figure 5.3: Effluent PCOC versus Feed PCOC.

Final Chemostat Experiment.

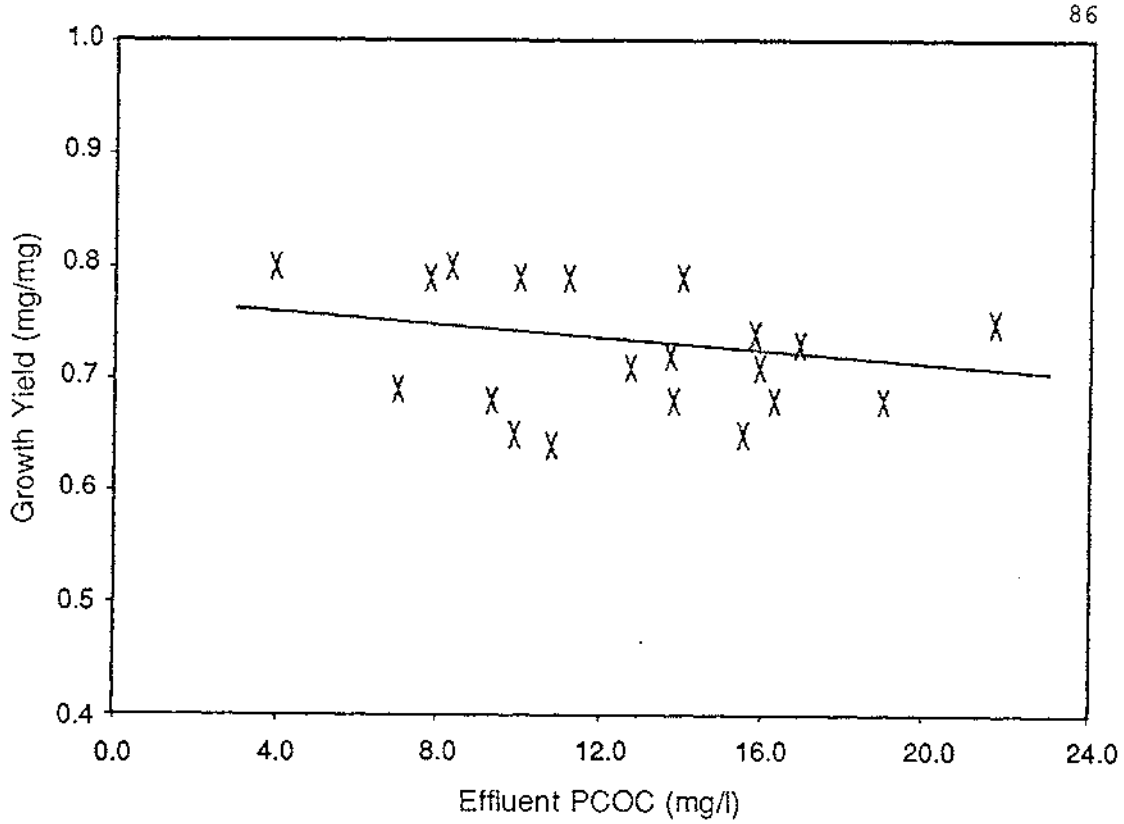

Figure 5.4: Growth Yield versus Effluent PCOC.
Final Chemostat Experiment.

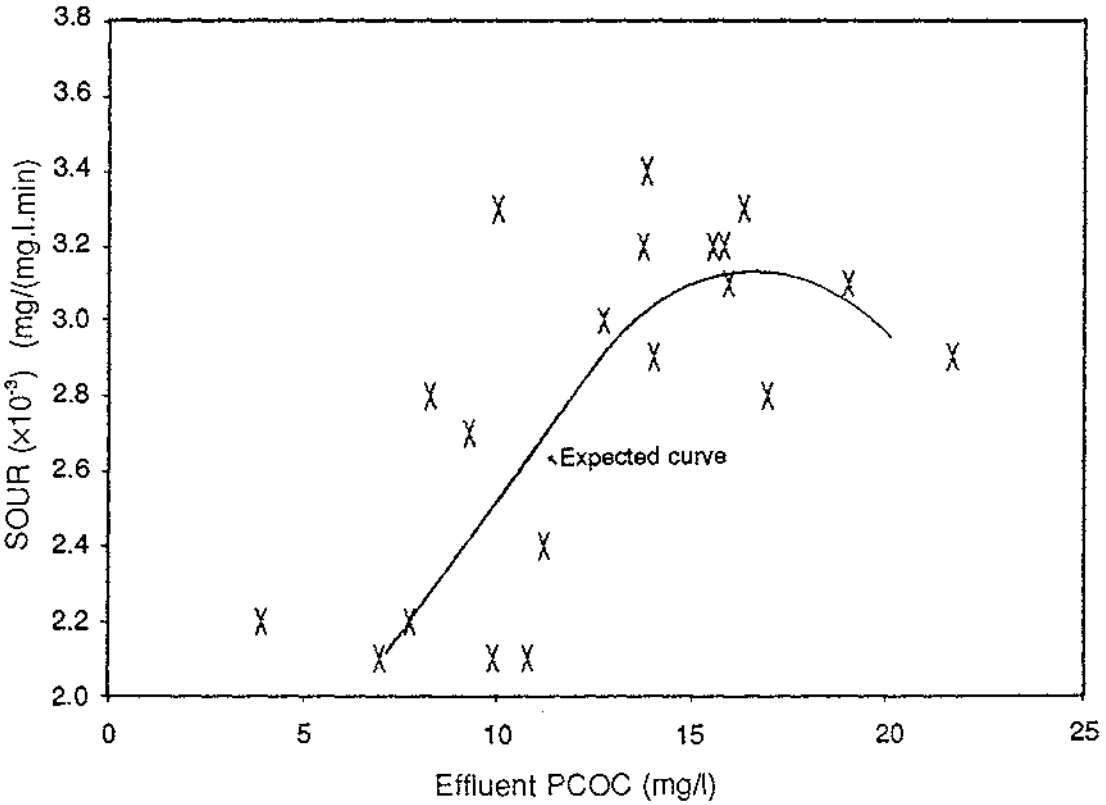

Figure 5.5: SOUR versus Effluent PCOC.
Final Chemostat Experiment.

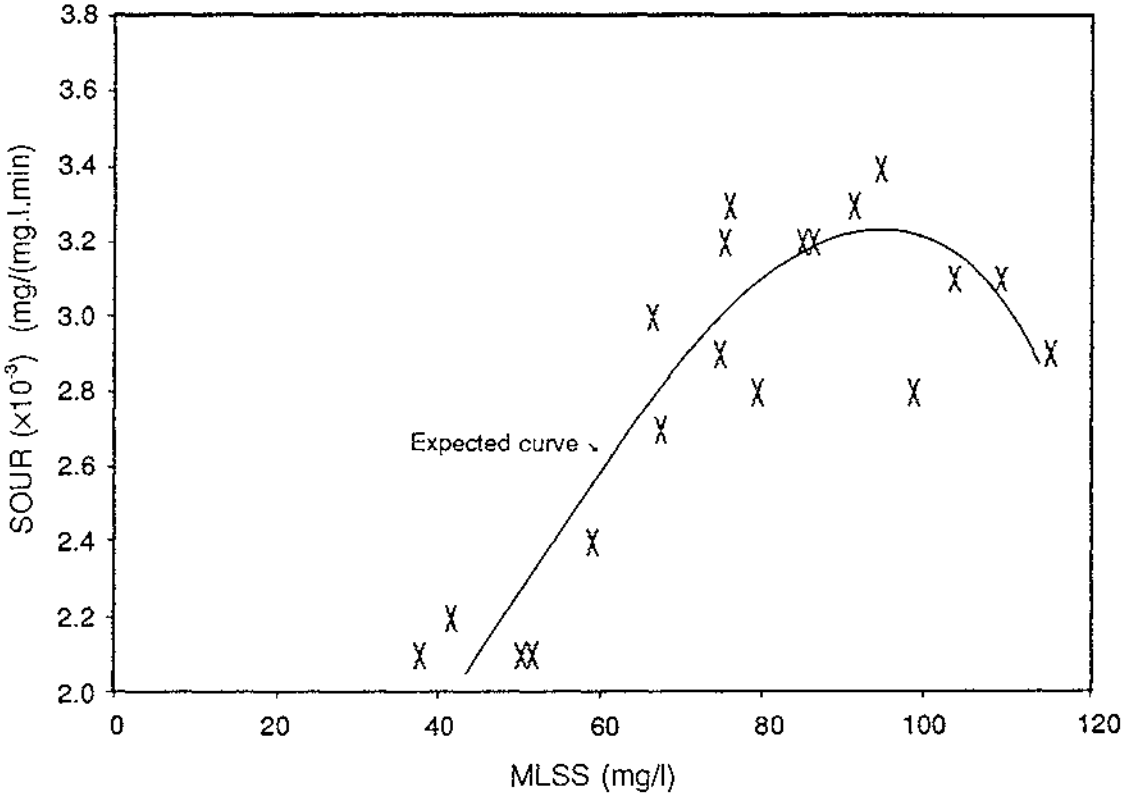

Figure 5.6: SOUR versus MLSS.

Final Chemostat Experiment.

The specific growth rate of a culture is approximately equal to dilution rate when grown in chemostat configuration (Pirt, 1975). An increase in specific growth rate has been shown to increase the maintenance requirement of a culture in the presence of a stress causing agent (Mink *et al.*, 1982; Green, 1978; Ng, 1982). PCOC is considered to be inhibitory to growth (McAlister, 1990) and therefore may act to stress cell growth. Increasing cell maintenance will decrease growth yield (Stouthamer, 1976). To minimise the effects of specific growth rate on the growth yield coefficient dilution rate was held constant for all chemostat runs.

By altering the feed substrate concentration (s_r), which is growth-limiting, and holding all other chemostat conditions constant, the effect of s_r on effluent substrate concentration, biomass and cell activity can be studied. The parameters measured also allow the calculation and analysis of the growth yield coefficient.

Figure 5.2 gives MLSS against substrate used. Biomass is measured by MLSS as the synthetic medium is well defined where all suspended solids are essentially cell mass. Growth yield is defined as biomass produced divided by substrate used (Section 2.7.2.2). The slope of Figure 5.2 gives growth yield. The relationship appears to be essentially linear. This is consistent with conventional chemostat theory (Pirt, 1975).

Brock *et al.* (1984) described chemostats as very useful and convenient for growth yield studies. It was suggested that a chemostat could be operated to produce negligible effluent substrate, when the substrate is growth-limiting. In this situation biomass becomes directly proportional to the feed substrate concentration.

In the current investigation the effluent substrate concentration was not negligible and therefore yields were estimated using differences of feed to effluent substrate concentrations.

Figure 5.3 gives effluent substrate against feed substrate concentrations. An increase in effluent substrate is apparent. Standard errors for the effluent substrate concentrations, given by Table 5.2, indicate this increase to be significant. Chemostat theory indicates that effluent substrate concentration should remain constant and low for a given dilution rate (Brock *et al.*, 1984; Pirt, 1975). The preliminary experiment (Section 5.1) detected an increase in effluent substrate concentration and it was proposed to be a result of the inhibitory nature of PCOC. Increasing feed substrate (PCOC) concentration stresses the culture up to the point where washout occurs. The increasing stress possibly decreases the effectiveness of substrate metabolism resulting in

increased substrate residue in the effluent. Effluent substrate may therefore be considered a measure of culture stress.

Figure 5.4 gives growth yield against effluent substrate concentration. Growth yield slightly decreases with increasing substrate concentration. Standard errors are given for yield in Table 5.2. Effluent substrate has been described as a measure of stress on the culture (above). Also the discussion (above) on the theoretical basis for altering feed substrate alone, describes how stress affects maintenance requirement which in turn affects growth yield. The decrease in growth yield as effluent substrate increases can be explained in terms of increasing stress and maintenance requirement.

Figure 5.5 describes an increasing then decreasing SOUR with effluent substrate concentration. SOUR is a measure of cell activity (Suschka and Ferreira, 1984), which may be extended to indicate cell viability. Studies of mixed cultures growing on phenolics have indicated that ignoring viability can lead to significant miscalculation of growth parameters (Hobson and Millis, 1990). Knowledge of viability can give a greater understanding of variations in biokinetic parameters of a chemostat system.

Measurement of SOUR as a relative measure of cell viability is a convenient technique for flocculating cells. The culture investigated was highly flocculent. The flocs were unable to be broken without damaging individual cells themselves. This disallowed the more conventional use of pour plate or streak plate techniques for actual viable cell counts.

Several workers have shown increased sensitivity of cells growing at fast growth rates to a variety of stress-causing agents (Mink *et al.*, 1982; Green, 1978; Ng, 1982). PCOC may become inhibitory (McAlister, 1990) and therefore a stress factor when considering growth. Effluent substrate has been described as an indicator of stress. The chemostats were operated at fast growth rates. The conventional assumption is that viability increases at fast growth rates (Postgate and Hunter, 1962; Tempest *et al.*, 1967). This assumption was based on experimental evidence using innocuous substrates. Hobson and Millis (1990) found that viability decreased with growth on inhibitory substrates even at high dilution rates.

Figure 5.5 shows SOUR, and therefore cell viability, increases up to an effluent substrate concentration of 15 mg/l. This is compatible with the conventional assumption of a high viability with a high dilution rate given sufficient substrate. When the effluent substrate

concentration exceeded 15 mg/l, SOUR decreased. This may be explained by the inhibitory effect of the substrate affecting growth above 15 mg/l. This indicates viability in the chemostat system decreases above this concentration.

Figure 5.6 gives SOUR against MLSS. The shape of Figure 5.6 is similar to that in Figure 5.5 for similar reasons. Chemostat theory indicates steady state biomass increases with feed substrate concentration, assuming yield and effluent substrate variations are comparatively small (Pirt, 1975). This is given by eqn (2-23). Figure 5.3 shows effluent substrate concentration increases relatively slightly with feed concentration. Therefore viability is expected to behave similarly when plotted against MLSS or effluent substrate concentration.

5.4 Summary and Conclusions

Chemostats were operated using PCOC as growth-limiting substrate. A high constant dilution rate (0.13 hr^{-1}) was used and PCOC feed concentrations were varied. Steady-state conditions were attained for all chemostats with extended operation time and sampling at intervals of no less than 48 hours. A number of parameter variations, some contrary to conventional microbial and chemostat theory, were measured:

(i) Effluent substrate concentrations remained relatively low with increasing feed substrate to the point of washout.

(ii) Effluent substrate concentrations increased a small but significant amount, from 5 to 20 mg/l, with increasing feed substrate.

(iii) Growth yield coefficient slightly decreased with increasing effluent substrate concentration.

(iv) SOUR, which is considered a measure of relative cell viability, increased with effluent substrate concentration to a maximum after which SOUR began to decline.

It is concluded that the growth-limiting substrate PCOC is a stress causing agent to cell growth and becomes inhibitory as its working concentration increases. At low concentrations, below 15 mg/l at a dilution rate of 0.13 hr^{-1}, PCOC behaves as an innocuous substrate and essentially

obeys the rules of Monod kinetics and conventional chemostat assumptions. At concentrations above 15 mg/l, with the same dilution rate, PCOC becomes inhibitory.

Inhibitory concentrations of PCOC are considered to increase damage to cell membranes. This decreases culture viability and the efficiency of substrate utilisation. Small increases in effluent substrate concentrations occur. The increase in cell maintenance requirement decreases the amount of available substrate for cell production. This results in decreased growth yields at the higher PCOC concentrations.

Chapter 6

Modelling of Yield Variation

6.1 Introduction

A linear model describing a variable yield term dependent on substrate concentration has been described by Crooke and Tanner (1982). The model is of the form

$$Y(s) = A + Bs \qquad (6.1)$$

where A and B are positive constants, s is the independent substrate concentration term, and Y(s) is the dependent growth yield variable.

In a theoretical investigation it was shown that when growth yield is allowed to depend on the underlying substrate concentration in a bioreactor then it is possible to have oscillations in cell-substrate concentrations (Crooke and Tanner, 1982). It was proven that a generally accepted model for biodegradation could not exhibit periodic solution if the growth yield term was constant. Such oscillations are supported by experimental data (Curds, 1971; Tsuchiya *et al.*, 1972). Studies have also indicated a variability of growth yield with changes in substrate concentrations and specific growth rates (Stouthamer, 1976; Hobson and Millis, 1990). However there has been a lack of experimental data to support suggested relationships.

The current work has investigated growth yield variability against the controlled variable of substrate concentration. The substrates biodegraded by acclimated culture were 2,4-dichlorophenoxyacetic acid (2,4-D) and *para*-chloro-*ortho*-cresol (PCOC). The data obtained is applicable to fitting and verifying the proposed model (Crooke and Tanner, 1982). An initial examination of yield and substrate concentration data has indicated a negative linear relationship. The negative relationship is considered to be a result of using inhibitory substrates for growth. The constraints on the proposed model are relaxed allowing the constant B to be a negative.

This chapter will first examine the principles and assumptions for model fitting and verification.

It will then analyze growth yield and substrate concentration data sets obtained in previous chapters. Data sets have been obtained using batch and chemostat configurations. It will fit the model of Crooke and Tanner (1982) to the data. A model will be verified for each set of data. For the verified models a significance test will be applied. This will establish the significance of the relationship between yield and substrate concentration. Confidence intervals for model constants will also be given.

6.2 Principles and Assumptions for Model Fitting and Verification

6.2.1 Model Fitting

A scatter diagram is often convenient for deciding whether a linear model is appropriate for sets of data. A scatter diagram plots the dependent variable against an independent variable. Where a straight line can be fitted to the data points a linear relationship is said to exist.

Where an exact functional relationship exists between two variables and measurement error is very small no statistical analysis of the fitting is really required. However this is seldom the case. A systematic method to determine a model and it's coefficients is given by Gunst and Mason (1980).

In the problem investigated, the growth yield coefficient is dependent, or expected to be dependent, on the substrate concentration. The substrate concentration was able to be controlled during experimental runs and is called the controlled, independent or regressor variable. The yield coefficient is called the dependent or response variable. The model proposed by Crooke and Tanner (1982) relating these two variables is of a linear form (eqn (6.1)).

Sometimes linear theory is used for a non-linear relationship. To achieve this the variables are transformed by some *pre-model function.* An example of this is the taking of logarithmic values of the variables to express an exponential relationship linearly. Variable transformation is not suggested by Crooke and Tanner (1982).

The problem therefore is to fit a line to the yield-substrate data. This line is called the regression of yield on substrate. In using eqn (6.1) the task is to find estimates for A and B. This is known as regression. The method chosen for doing this is the *method of least squares.*

The theory behind this method is explained by Gunst and Mason (1980). To summarise; least squares estimates of A and B are obtained by choosing the values that minimise the sum of squares of the deviations between the observed and expected (predicted) yields.

For least squares estimates to be *good* estimates of the unknown parameters of the regression equation the following conditions should apply:

(1) For a fixed value of the controlled variable (substrate) the dependent variable (yield) follows a normal distribution. The mean of this distribution should be the estimated yield for the given substrate concentration.

(2) The conditional variance of the distribution of yield for a fixed value of substrate is a constant. This conditional variance should not depend on the value (concentration) of the substrate.

The properties of normality and constant variance are important assumptions in many types of model. These assumptions are also necessary to establish the confidence intervals for the true values of A and B and of the mean values of the estimated, or predicted, yields.

6.2.2 Model Verification

Two assumptions have been made in the previous section describing linear regression. If any of the assumptions are false some or all of the preceding analysis may be invalidated. A good indication of applicability of the assumptions can be checked by looking at the residuals. The residuals are the deviations between the observed and predicted yields. An important procedure is the plotting of the residuals. The usual plots are the residuals versus the predicted values, and the residuals versus the controlled variable (Ryan *et al.*, 1982).

A number of common patterns may be apparent in the residual plots (Gunst and Mason, 1980):

(1) The worst possibility is the residuals are not random. For example alternating series of positive then negative residuals. This indicates an uncontrolled factor is systematically affecting the results. The possibility of non-randomness is overcome by randomization. Alternatively a technique called blocking can be used.

(2) The residuals are not normally distributed. The F-test and T-test are both robust to departures from the normality assumption. However occasionally data transformation may be required.

(3) The magnitude of the residuals may vary with the controlled variable values. This indicates changing conditional variance. If this is observed then the least squares procedure must be modified by giving more weight to those observations which have the smaller variance (Hald ,1952). Alternatively it may be possible to transform the data so that the error variance is constant.

Another important use of residuals is to detect *outliers* in a set of data (Gunst and Mason, 1980). These are data points which appear to be inconsistent with the rest of the data. A common procedure is to reject observations whose residual is more than four times the residual standard deviation. Rejection of observations must always be carefully considered as a large residual may indicate a fault in the functional form of the model. A rejected outlier can be completely removed or replaced with an average value.

6.3 Linear Regression of Biosystem Data

Sets of yield and substrate concentration data were obtained for each substrate, 2,4-D and PCOC, biodegraded in batch configuration. Additionally a data set was obtained for degradation of PCOC in chemostat configuration. These data sets are given in Appendix 5. Each data set is analyzed separately in the following sections.

Linear regression of yield on substrate has been performed. The *method of least squares* was chosen. A computer program was used to perform the analysis. Estimates of the coefficients A and B are obtained. R-squared values given by the regression indicates how well the equation fits the data. The fitted model is then verified by considering residuals (section 6.2.2). When the assumptions on which the model is based are accepted, a significance test is performed and confidence intervals for the model coefficients, A and B, are obtained.

6.3.1 2,4-D Substrate, Batch Configuration

Growth yield against initial substrate concentration is given by Figure 6.1. Initial inspection of the figure indicates a linear functional relationship may be appropriate.

The results of linear regression, by computer, of yield on substrate concentration are given by Appendix 5. The fitted linear equation is:

$$Y = 0.334 - (2.8\times10^{-4})s \qquad (6.2)$$

where Y is growth yield coefficient, s is initial substrate concentration, coefficient A = 0.334, B = -2.8×10^{-4}. The R-squared value of 93.04 percent indicates the equation gives a reasonable fit to the data points. Residuals versus predicted yields and substrate concentrations are given by Figures 6.2 and 6.3 respectively.

Conditions are considered for accepting model assumptions to verify the model (section 6.2.2):

(1) The residuals appear random.
(2) Insufficient data points are available to indicate non-normal residual distribution.
(3) Unequal residual variance with different substrate concentrations, given by Figure 6.3, is difficult to determine.

For detecting *outliers* the procedure of rejecting observations more than four times the residual standard deviation is used. The residual standard deviation calculated from residual data (Appendix 5) is ± 0.017. Four times this is ± 0.07. No residuals lie outside this range to consider rejecting.

Linear regression assumptions will be accepted for the data analysis performed. The robust T-test will be used for considering the model.

A key function of the investigation was to determine if growth yield is dependent on substrate concentration. The slope of eqn (6.2) is given by B coefficient. A yield dependence exists where B is significantly different from zero. Examination of regression results (Appendix 5) indicates B is more precisely -2.750×10^{-4}.

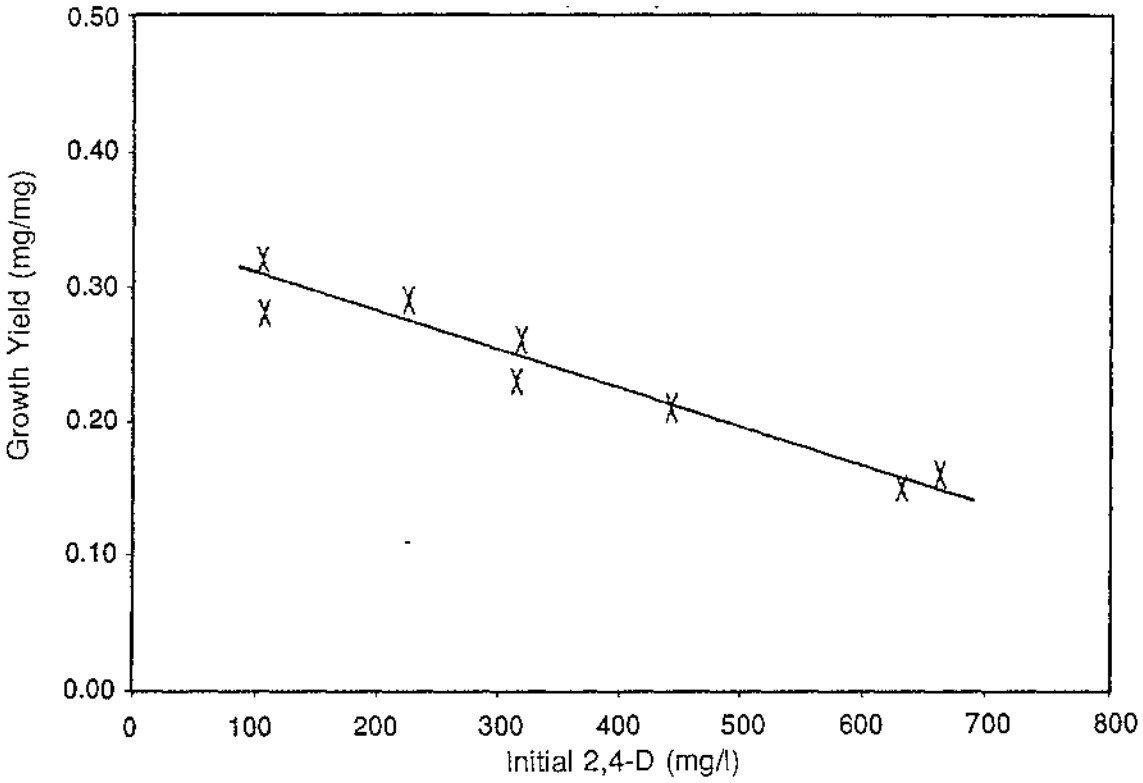

Figure 6.1: Growth Yield versus Initial 2,4-D.

Final Batch Experiment.

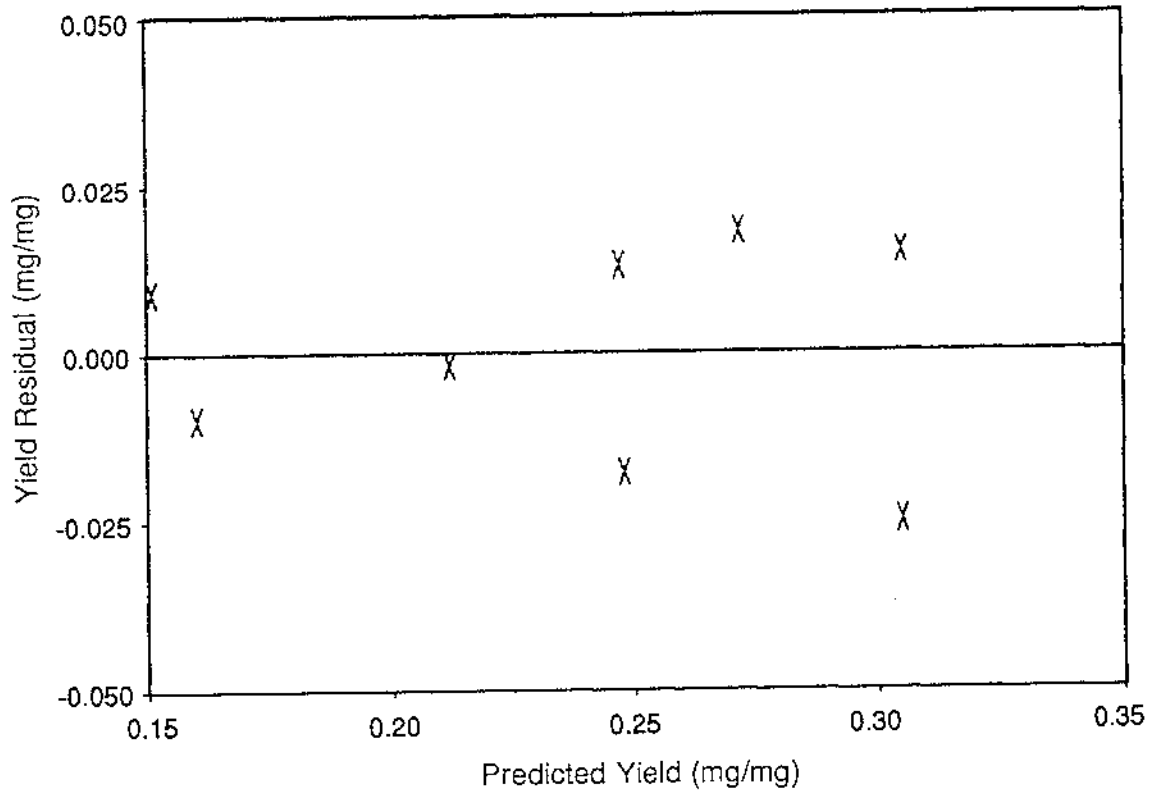

Figure 6.2: Growth Yield Residuals versus Predicted Yield (2,4-D).
Final Batch Experiment.

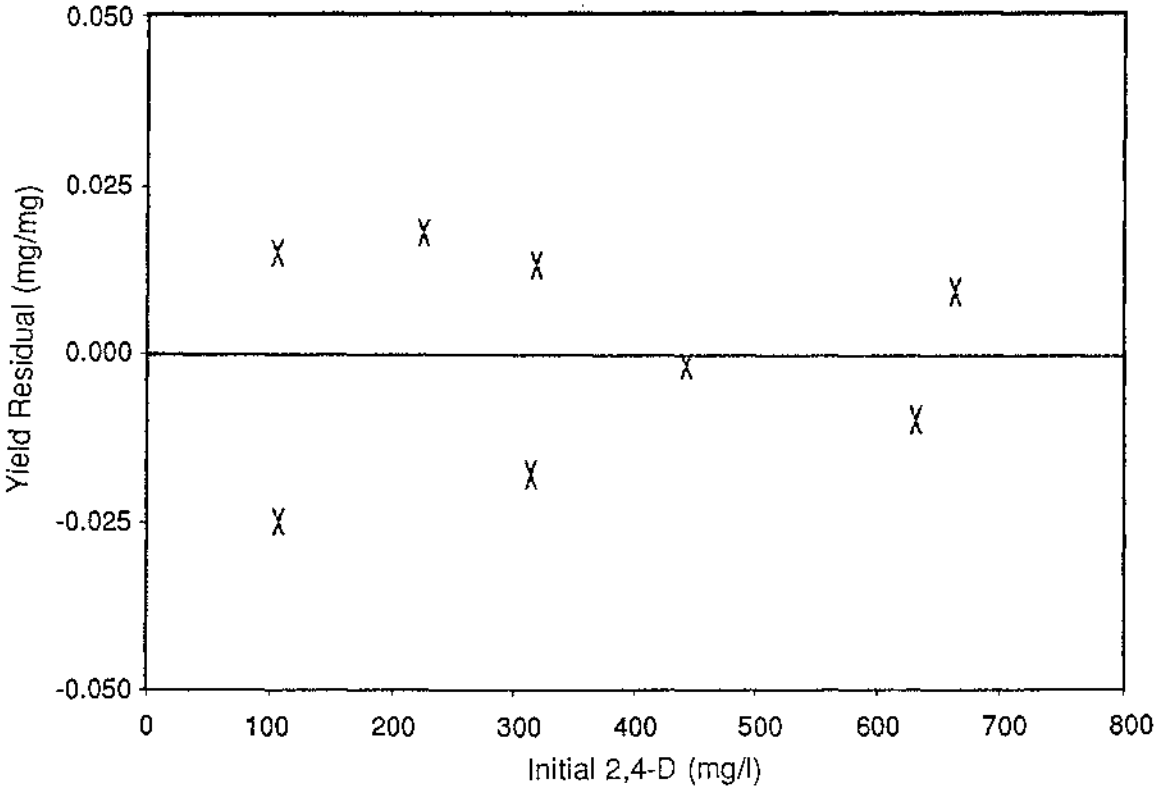

Figure 6.3: Growth Yield Residuals versus Initial 2,4-D.
Final Batch Experiment.

It is desired to test the hypothesis H_0:B = 0 against the alternative hypothesis H_1:B ≠ 0. The test statistic (t-value) is 8.95 (ignoring the sign, Appendix 5). Degrees of freedom is 6 (Appendix 5). Using Student's t distribution, $t_{0.05,6} = 2.45$. Thus the result is significant at the 5 per cent level. Therefore the slope of the regression line is significantly different from zero.

A 95 percent confidence interval for B is given by

$$B \pm t_{0.05,6} \times SD(coef)_B$$

where $SD(coef)_B$ is the standard deviation of B (Appendix 5). The confidence interval is:

$$-2.8\times10^{-4} \pm 0.8\times10^{-4}$$

A 95 percent confidence interval may be given for coefficient A by

$$A \pm t_{0.05,6} \times SD(coef)_A$$

where $SD(coef)_A$ is the standard deviation of A (Appendix 5). The confidence interval is:

$$0.334 \pm 0.029$$

6.3.2 PCOC Substrate, Batch Configuration

Growth yield against initial substrate concentration is given by Figure 6.4. Inspection of the figure indicates a linear functional relationship may be appropriate.

The results of linear regression of yield on substrate concentration are given by Appendix 5. The fitted equation is:

$$Y = 1.034 - (5.6\times10^{-3})s \qquad (6.3)$$

coefficient A = 1.034, B = 5.6×10^{-3}. An R-squared value of 91.48 percent indicates the equation gives a good fit to the data. Residuals versus predicted yields and substrate concentrations are given by Figures 6.5 and 6.6 respectively.

Conditions are considered for accepting model assumptions to verify the model (section 6.2.2):

(1) The residuals are essentially random. Residual sign variation indicates no pattern.
(2) Insufficient data points are available to indicate non-normal residual distribution.
(3) Unequal residual variance with different substrate concentrations (Figure 6.6) is inconclusive.

Observations more than four times the residual standard deviation may be considered outliers. The residual standard deviation calculated from residual data (Appendix 5) is ± 0.048. Four times this is ± 0.19. No residuals lie outside this range to consider rejecting.

The assumptions of linear regression will be accepted for the current analysis. The robust T-test will be used for considering the model.

The yield dependence on substrate concentration can now be considered. If the slope of eqn (6.3), B coefficient, is significantly different from zero a yield dependence exists. Regression results given by Appendix 5 indicate B is -5.629×10^{-3}.

The test hypothesis is H_0:B = 0 against the alternative hypothesis H_1:B ≠ 0. The test statistic (t-value) is given as 8.67 (ignoring the sign, Appendix 5). Degrees of freedom is 7 (Appendix 5). Using Student's t distribution, $t_{0.05,7} = 2.37$. Thus the result is significant at the 5 percent level. Therefore the slope of the regression line is significantly different from zero.

A 95 percent confidence interval for B is given by

$$B \pm t_{0.05,7} \times SD(coef)_B$$

where $SD(coef)_B$ is the standard deviation of B (Appendix 5). The confidence interval is:

$$-5.6\times10^{-3} \pm 1.5\times10^{-3}$$

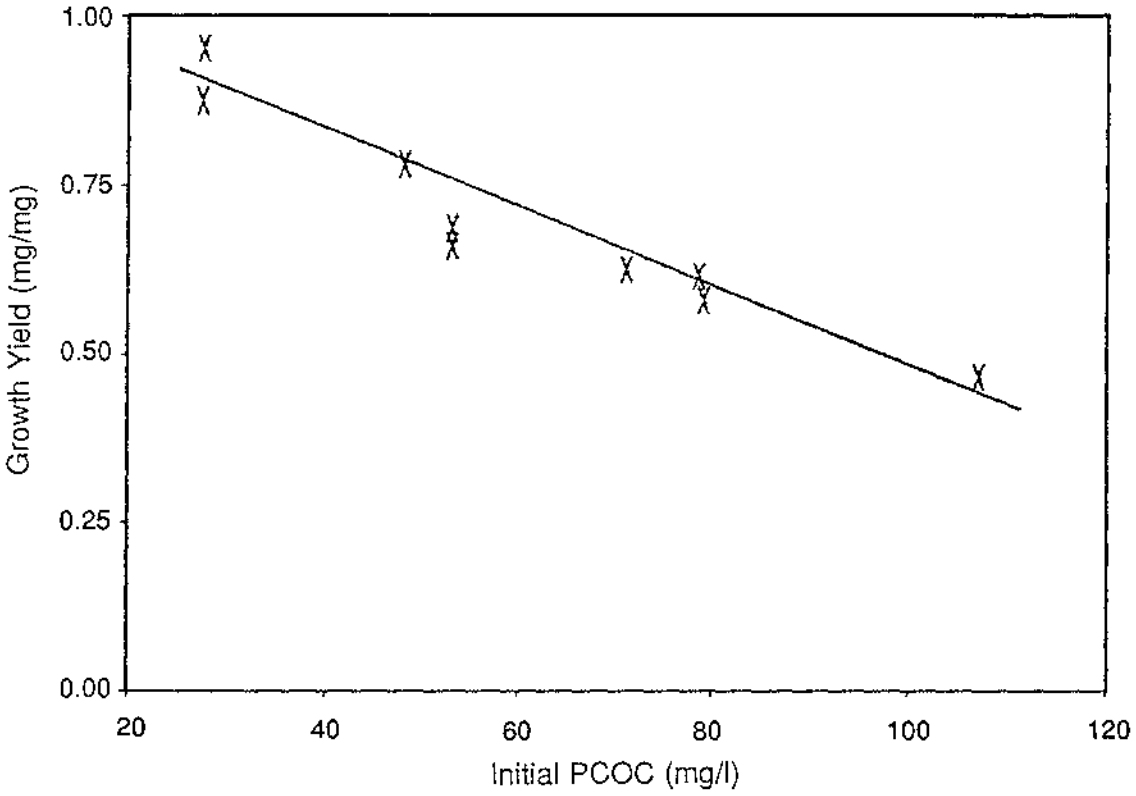

Figure 6.4: Growth Yield versus Initial PCOC.

Final Batch Experiment.

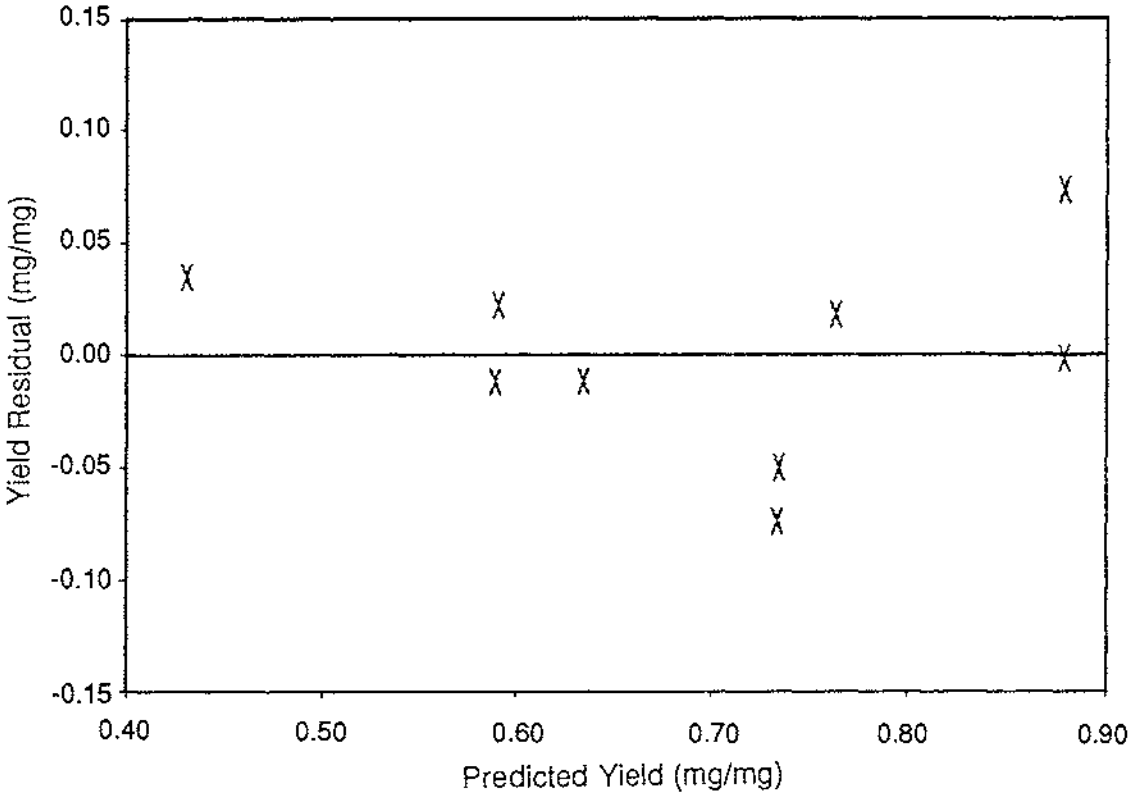

Figure 6.5: Growth Yield Residuals versus Predicted Yield (PCOC).
Final Batch Experiment.

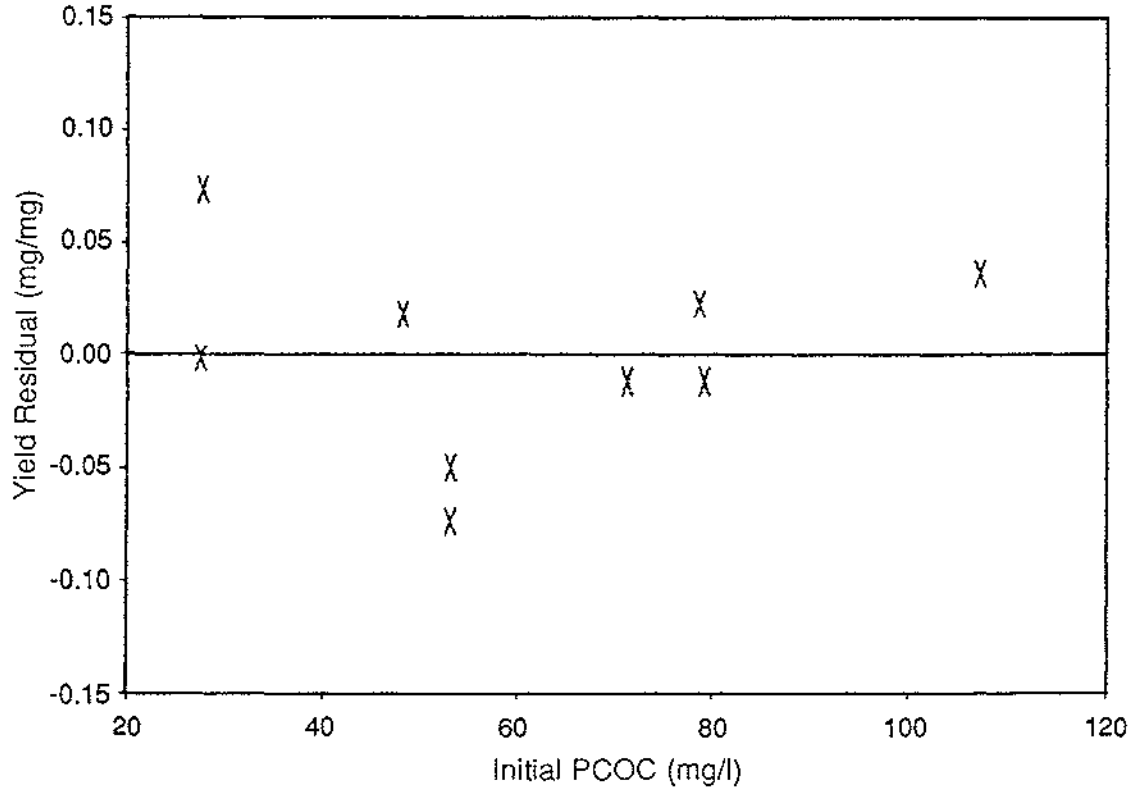

Figure 6.6: Growth Yield Residuals versus Initial PCOC.
Final Batch Experiment.

A 95 percent confidence interval may be given for coefficient A by

$$A \pm t_{0.05,7} \times SD(coef)_A$$

where $SD(coef)_A$ is the standard deviation of A (Appendix 5). The confidence interval is:

$$1.03 \pm 0.10$$

6.3.3 PCOC Substrate, Chemostat Configuration

Growth yield against initial substrate concentration is given by Figure 6.7. Inspection of the data indicates a linear functional relationship may be appropriate.

The results of linear regression of yield on substrate concentration are given by Appendix 5. The fitted linear equation is:

$$Y = 0.799 - (6.0\times10^{-3})s \qquad (6.4)$$

coefficient A = 0.799, B = -6.0×10^{-3}. Investigating the low R-squared value of 24.5 percent indicates this be a result of the large scatter of data points. Residuals versus predicted yields and substrate concentrations are given by Figures 6.8 and 6.9 respectively.

Conditions are considered for accepting model assumptions to verify the model (section 6.2.2):

(1) The residuals appear random.
(2) Insufficient data points are available to indicate non-normal residual distribution.
(3) Unequal residual variance with different substrate concentrations (Figure 6.9) is not apparent.

Observations more than four times the residual standard deviation may be considered outliers. The residual standard deviation calculated from residual data (Appendix 5) is ± 0.047. Four times this is ± 0.188. No residuals lie outside this range to consider rejecting.

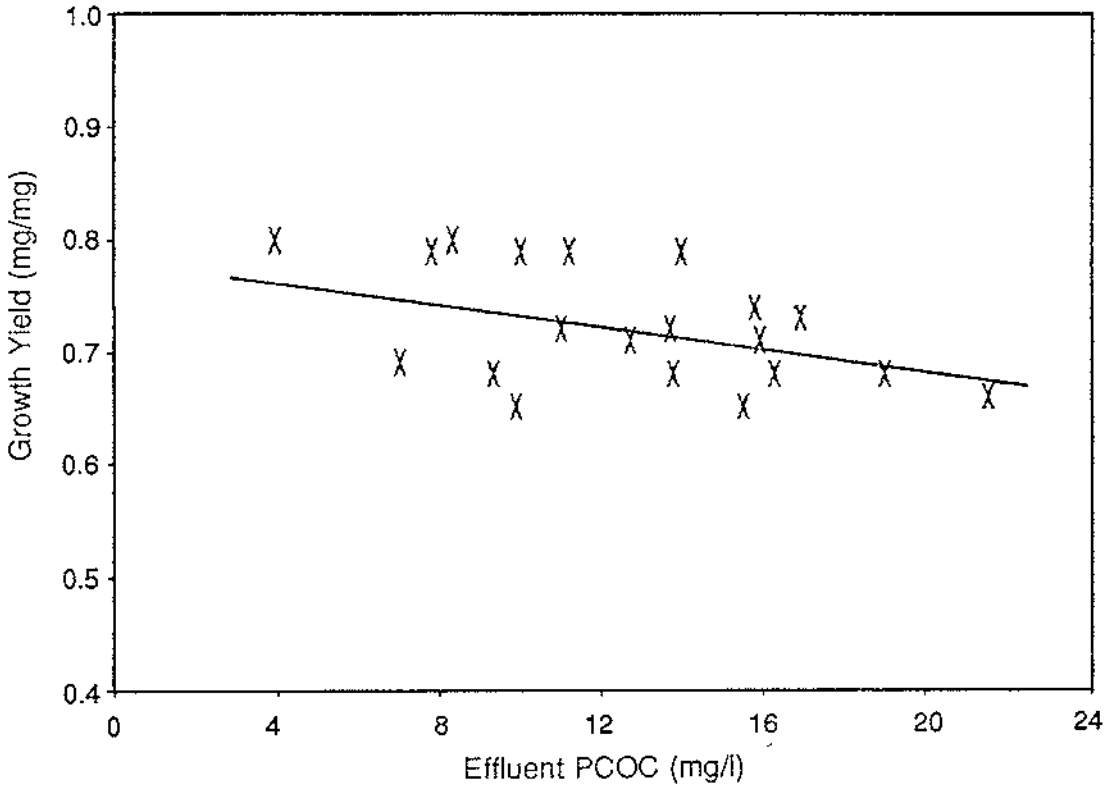

Figure 6.7: Growth Yield versus Effluent PCOC.
Final Chemostat Experiment.

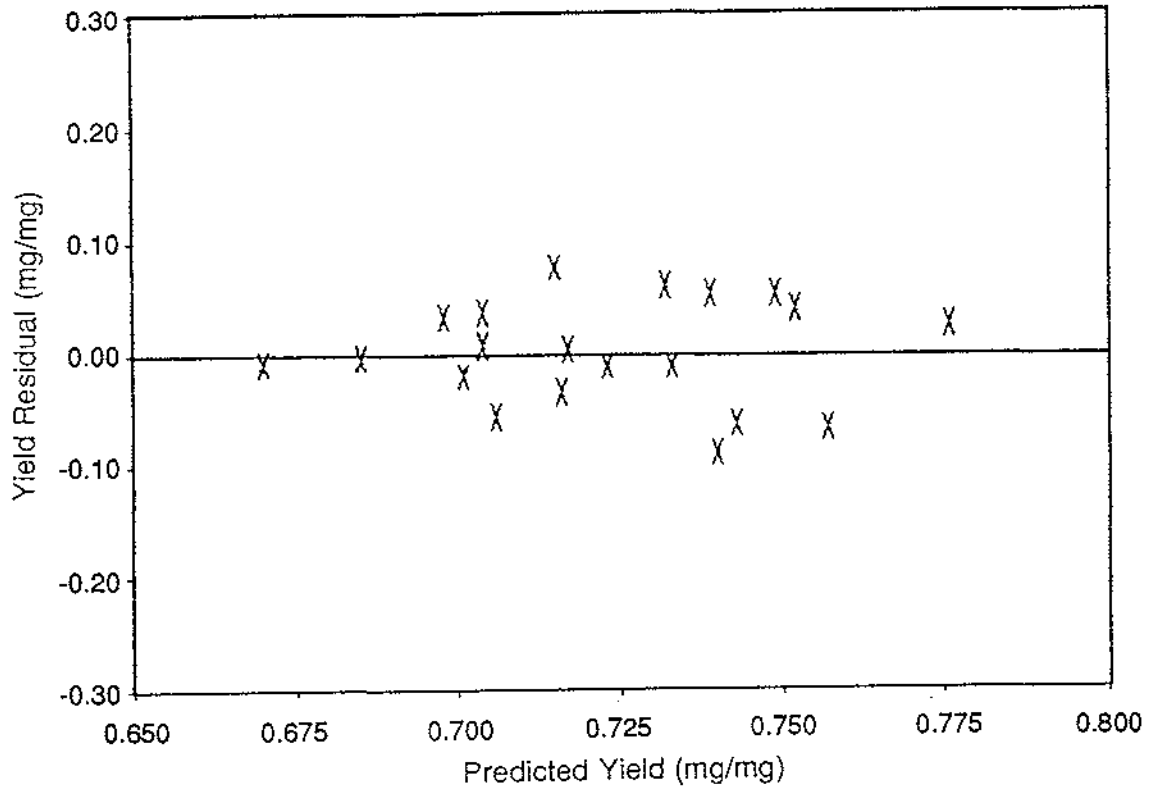

Figure 6.8: Growth Yield Residuals versus Predicted Yield (PCOC).
Final Chemostat Experiment.

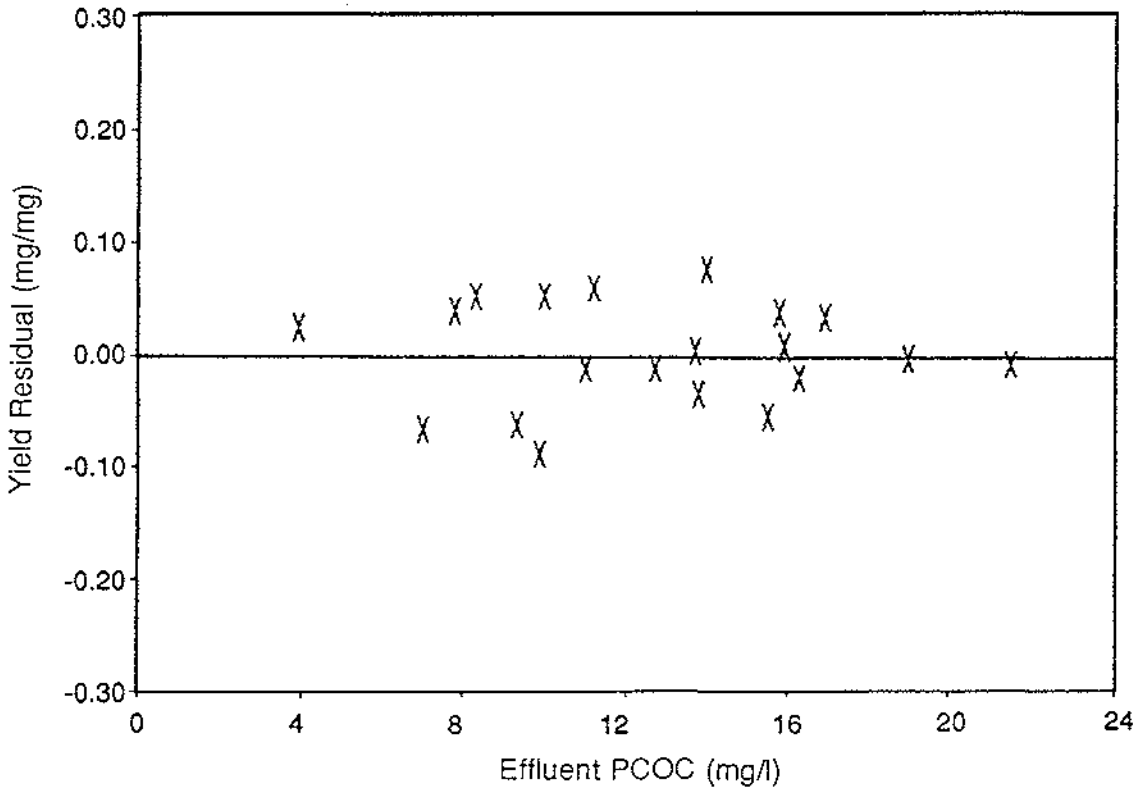

Figure 6.9: Growth Yield Residuals versus Effluent PCOC.
Final Chemostat Experiment.

Linear regression assumptions will be accepted for the data analysis performed. The robust T-test will be used for considering the model.

The yield dependence on substrate concentration can now be considered. If the slope of eqn (6.4), B coefficient, is significantly different from zero a yield dependence exists. Regression results given by Appendix 5 indicate B is -6.008×10^{-3}.

Again the test hypothesis is H_0:B = 0 against the alternative hypothesis H_1:B $\neq$ 0. The test statistic (t-value) is given as 2.42 (ignoring the sign, Appendix 5). Degrees of freedom is 18 (Appendix 5). Using Student's t distribution, $t_{0.05,18} = 2.10$. Thus the result is significant at the 5 percent level. Therefore the slope of the regression line is significantly different from zero.

A 95 percent confidence interval for B is given by

$$B \pm t_{0.05,18} \times SD(coef)_B$$

where $SD(coef)_B$ is the standard deviation of B (Appendix 5). The confidence interval is:

$$-6.0\times10^{-3} \pm 5.2\times10^{-3}$$

A 95 percent confidence interval may be given for coefficient A by

$$A \pm t_{0.05,18} \times SD(coef)_A$$

where $SD(coef)_A$ is the standard deviation of A (Appendix 5). The confidence interval is:

$$0.799 \pm 0.069$$

6.4 Summary and Conclusions

The linear model proposed by Crooke and Tanner (1982) has been applied to 2,4-D and PCOC batch and chemostat data. The model is of the form

$$Y(s) = A + Bs$$

where A and B are constants, s is substrate concentration and Y(s) is the substrate dependent growth yield. An original constraint on the constants, A and B, as positives was relaxed to allow negative B values.

For the data analyzed the linear model was considered an acceptable functional form. A regression line of yield on substrate concentration was fitted. The method of least squares was used. Estimates of A and B were obtained.

Liner regression makes a number of assumptions about the data. These assumptions on which the model is based were checked. The data sets were small but were considered to meet the requirements to verify the model. The estimates for A and B were considered to give the minimum least squares. The robust student's t distribution was applied.

The T-test was applied to determine if there is a growth yield dependence on substrate concentration:

For growth on 2,4-D and PCOC in batch configuration the dependence is significant at the 5 percent level.

For growth on PCOC in chemostat configuration the dependence again is significant at the 5 percent level.

The model was given for each data set. Confidence intervals on the constants A and B were also given:

- For growth on 2,4-D in batch configuration

$$Y(s) = 0.334 - (2.8\times10^{-4})s$$

The 95 percent confidence interval for B is

$$(-2.8\times10^{-4}) \pm (0.8\times10^{-4})$$

The 95 percent confidence interval for A is

$$0.334 \pm 0.029$$

- For growth on PCOC in batch configuration

$$Y(s) = 1.03 - (5.6\times10^{-3})s$$

The 95 percent confidence interval for B is

$$(-5.6\times10^{-3}) \pm (1.5\times10^{-3})$$

The 95 percent confidence interval for A is

$$1.03 \pm 0.10$$

- For growth on PCOC in chemostat configuration

$$Y(s) = 0.799 - (6.0\times10^{-3})s$$

The 95 percent confidence interval for B is

$$(-6.0\times10^{-3}) \pm (5.2\times10^{-3})$$

The 95 percent confidence interval for A is

$$0.799 \pm 0.069$$

Chapter 7

Summary

It has been recognised that biological treatment can be an effective means to transform harmful chemicals into non-toxic compounds. Biological treatment involves the metabolism of chemicals by microorganisms. The rate of microbial or biological growth in these systems may be quantified by a mathematical model incorporating the relevant biokinetics for the system. Such models are useful for design and operation of biological treatment facilities. Their applicability however is often highly dependent on the accuracy of the biokinetic growth constants used.

The investigations conducted have studied the biodegradation of 2,4-D, a phenoxy herbicide, and PCOC, a chlorophenol in batch and chemostat configurations. Acclimated cultures were used throughout.

The substrates used demonstrated inhibitory effects on growth as a result of their biotoxicity. Researchers have often experienced difficulties in obtaining growth data for inhibitory substrates. The biokinetic parameter of growth yield has been described for growth in various inhibitory systems. Growth yield was found to be a variable contrary to the common assumption that it is a constant.

Growth yield variations studied were particularly related to dependence on substrate concentration, other conditions being held constant. Specifically three biosystems were studied:

(i) 2,4-D degraded in batch configuration
(ii) PCOC degraded in batch configuration
(iii) PCOC degraded in chemostat configuration

Each of the three biosystems were found to exhibit inhibitory behaviour under some growth conditions. Difficulties with analyzing the data for determining biokenetic constants arose when inhibition caused a significant departure from uninhibited (Monod) kinetics. These departures cannot be explained by simple uninhibited growth models. Techniques for determining biokinetic constants have generally depended on these simple models. Similar departures from Monod kinetics were observed for growth in batch and chemostat configurations.

In batch configuration Monod kinetics indicate specific growth rate increases with increasing substrate concentration. For the low substrate concentrations investigated this was apparent. However as substrate concentrations were increased relatively high, specific growth rates began to fall sharply. This is due to substrate inhibition.

In chemostat configuration the investigations indicated similar variations of specific oxygen uptake rate (SOUR), as previously with growth rates, with an increasing underlying substrate concentration. SOUR is considered a measure of culture activity, or viability. SOUR was observed to increase at low underlying substrate concentrations. At relatively high concentrations SOUR decreased. Again this is due to substrate inhibition.

Data analysis for determinations of the saturation constant and maintenance coefficient for the biosystems presented difficulties. The techniques employed were derived from Monod kinetics and were found to be inappropriate.

Growth yield in all instances was found to be significantly dependent on substrate concentration. As yield could no longer be considered constant, mathematical models were developed for each biosystem for predicting yield from substrate concentrations. An appropriate functional form was found to be linear as described by Crooke and Tanner (1982):

$$Y(s) = A + Bs$$

where A and B are constants. An initial constraint requiring positive constants was relaxed for B. Y(s) is the dependent growth yield concentration and s is the controlled substrate concentration.

The constants A and B were estimated by linear least squares. The robust student's t distribution was applied and 95 percent confidence intervals were calculated. These are given corresponding to each of the above biosystems:

(i) Growth on 2,4-D in batch configuration

$A = 0.334 \quad \pm 0.029$

$B = (-2.8\times10^{-4}) \quad \pm (0.8\times10^{-4})$

(ii) Growth on PCOC in batch configuration

$A = 1.03 \quad \pm 0.10$

$B = (-5.6\times10^{-3}) \quad \pm (1.5\times10^{-3})$

(iii) Growth on PCOC in chemostat configuration

$A = 0.799 \quad \pm 0.069$

$B = (-6.0\times10^{-3}) \quad \pm (5.2\times10^{-3})$

The constant B is important for determining growth yield dependence on substrate concentration. The further from zero the greater the dependence. It is interesting to note the large 95 percent confidence interval for B for growth on PCOC in chemostat configuration. The interval comes close to zero. If the interval crossed zero, yield dependence on substrate concentration would be insignificant. The B constants for batch configurations were relatively further from zero when considering their confidence intervals. A description of yield by Stouthamer (1976) and explanations by Hobson and Millis (1990) clarify this observation:

Stouthamer (1976) indicated observed growth yield variations to be a result of the maintenance coefficient and varying specific growth rates. Maintenance was considered to be constant. This concept was proposed for an innocuous substrate. Hobson and Millis (1990) indicated that cell damage, and therefore maintenance coefficient, is likely to increase with increasing inhibitory substrate concentration. The result would be decreasing yield with increasing substrate. The negative B constants above for batch and chemostat configurations support this.

Hobson and Millis (1990); Mink *et al.* (1982); Green (1978); and Ng (1982) have shown that cells were more susceptible to damage at faster growth rates. In batch configuration cells grow at their maximum possible rate. In chemostat configuration growth rate is controlled at some constant rate less than the maximum. By considering this, cells grown under batch configuration would be expected to have a greater yield decrease with increasing substrate than those grown under chemostat configuration.

To summarize: significant variations in growth yield have been found to exist with growth on inhibitory substrates. It has been found appropriate to describe yield by negative linear regression against the controlled substrate variable. Models have been given describing growth yield for growth on 2,4-D and PCOC in batch configuration, and for PCOC in chemostat configuration. Yield has shown a greater dependence on substrate concentration for growth in

batch configuration. Explanations for this have been given.

Abbreviations and Nomenclature

2,4-D	2,4-dichlorophenoxyacetic acid
2,4-DCP	2,4-dichlorophenol
MCPA	2-methyl-4-chlorophenoxyacetic acid
PCOC	*Para*-chloro-*ortho*-cresol
PCP	Pentachlorophenol
2,4,5-T	2,4,5-trichlorophenoxyacetic acid
2,4,5-TCP	2,4,5-trichlorophenol
2,4,6-TCP	2,4,6-trichlorophenol

ATP	Adenosine triphosphate
$(coef)_A$	Coefficient A
$(coef)_B$	Coefficient B
D	Dilution Rate (hr^{-1})
D_c	Critical Dilution Rate (hr^{-1})
DNA	Deoxyribonucleic acid
e	Exponential Constant
eqn	Equation
F	Medium Flow Rate (l/hr)
K	Coupling Constant between catabolism and anabolism
K_I	Inhibition Constant (mg/l)
K_s	Saturation Constant (mg/l)
L	Lag Period (hr)
M	Molar (moles/l)
m_e	maintenance coefficient (moles/g/hr)
MLSS	Mean Liquor Suspended Solids (mg/l)
NADPH	Nicotinamide adenine dinucleotide phosphate (reduced form)
q	Metabolic Quotient, substrate utilisation (mg/mg/hr)
q_p	Metabolic Quotient, product formation (mg/mg/hr)
RNA	Ribonucleic acid
s	Substrate Concentration (mg/l)
$\tilde{s}$	Steady-State Substrate Concentration (mg/l)
s_o	Initial Substrate Concentration (mg/l)

s_r	Substrate Concentration in medium feed (mg/l)
SD	Standard Deviation
SOUR	Specific Oxygen Uptake Rate (mg/mg/minute)
t	Time (hr)
$t_{0.05,\varkappa}$	Student's t value at the 5 percent level of significance with $\varkappa$ degrees of freedom.
x	Biomass Concentration (mg/l)
$\bar{x}$	Steady-State Biomass Concentration (mg/l)
x_o	Initial biomass Concentration (mg/l)
x_m	Maximum Biomass Concentration (mg/l)
V	Culture Volume (l)
Y	Growth Yield Coefficient (mg/mg)
Y_g	Molar Growth Yield corrected for maintenance (g/mole)
Y_o	Observed Molar Growth Yield (g/mole)
Y_{ATP}	Overall ATP Yield (g/mole)
Y'_{ATP}	ATP Yield corrected for maintenance (g/mole)
Y_{ATP}^{max}	Maximum Overall ATP Yield (g/mole)
μ	Specific Growth Rate (hr^{-1})
μ_m	Maximum Specific Growth Rate (hr^{-1})

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Appendices

Appendix 1

2,4-D Batch Experiments

Table A1.1 2,4-D Initial Batch Experiment Result Summary

Initial s(o) Conc. (mg/l)	Final s(o) Conc. (mg/l)	Initial MLSS Conc. (mg/l)	Final MLSS Conc. (mg/l)	Growth Yield (Y)	Mean Growth Yield (Y)	Std. Dev.
53	19	37.6	52.8	0.45		
55	23	32.5	46.9	0.45	0.45	0
105.6	37.5	32.7	66	0.49		
105.6	27.5	34.6	73.3	0.5		
105.6	22.5	37.7	68.9	0.38	0.46	0.07
203	5	10.8	74.7	0.32		
203	5	11.8	77.2	0.33		
203	6	13.3	75.2	0.31	0.32	0.01
284	86	30	101.3	0.36		
284	5	39.6	115.5	0.27		
284	61	33.6	103.3	0.31	0.31	0.05
383	21.3	33.7	93.8	0.17		
390	24.4	43.5	99.4	0.15		
390	24.4	47.1	106.2	0.16	0.16	0.01
640	153	38.1	102.2	0.13		
620	151	39	115.9	0.16		
610	151	38.6	107.3	0.15	0.15	0.02
775	302	37.1	111.9	0.16		
780	305	49	121.5	0.15		
775	306	45	120.1	0.16	0.16	0.005

Table A1.2 2,4-D (Final) Batch Experiment
Result Summary

Batch Title No.	Initial s(o) Conc. mg/l	Growth Yield (Y)	1/Growth Yield (1/Y)	Specific Growth Rate (μ)	1/Specific Growth Rate (1/μ)	μ/So (*0.001)
1 100	105	0.32	3.125	0.039	25.64102	0.371428
2 100	106	0.28	3.571428	0.036	27.77777	0.339622
1 200	225	0.29	3.448275	0.053	18.86792	0.235555
1 300	314	0.23	4.347826	0.055	18.18181	0.175159
2 300	318	0.26	3.846153	0.047	21.27659	0.147798
1 400	442	0.21	4.761904	0.068	14.70588	0.153846
1 600	664	0.16	6.25	0.063	15.87301	0.094879
2 600	632	0.15	6.666666	0.064	15.625	0.101265

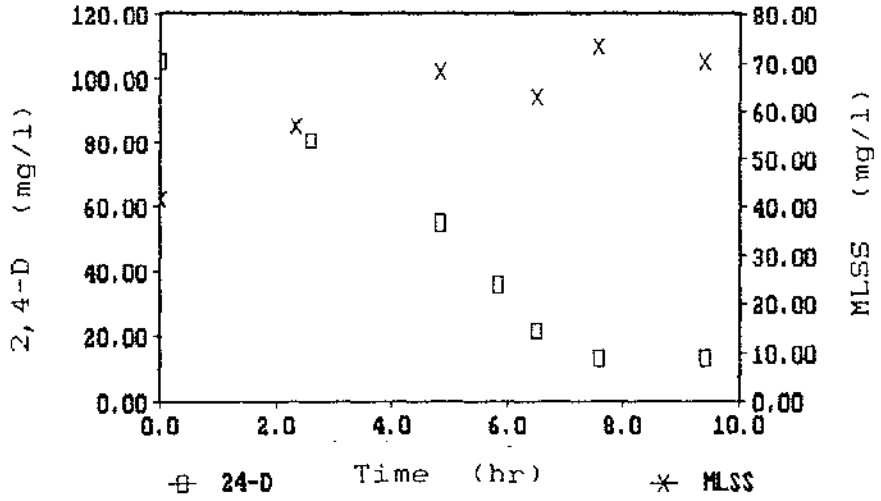


Figure A1.1: 2,4-D 100 mg/l, Batch 1. s, MLSS v t

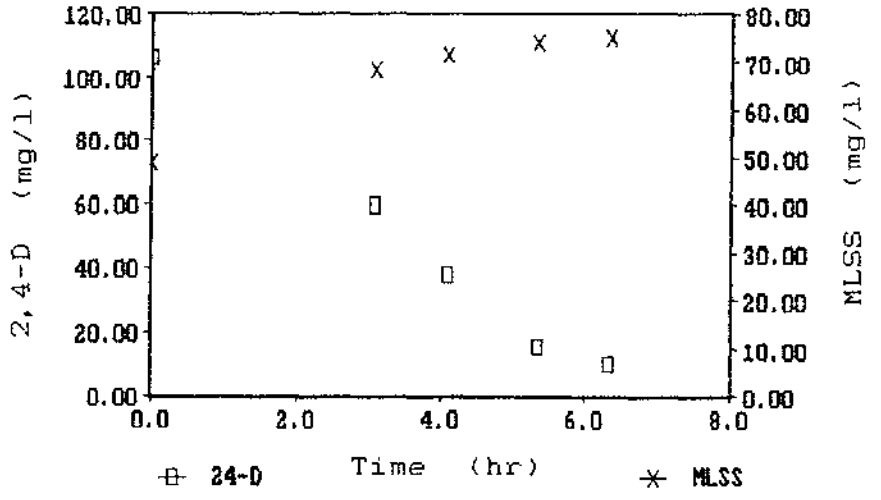


Figure A1.2: 2,4-D 100 mg/l, Batch 2. s, MLSS v t

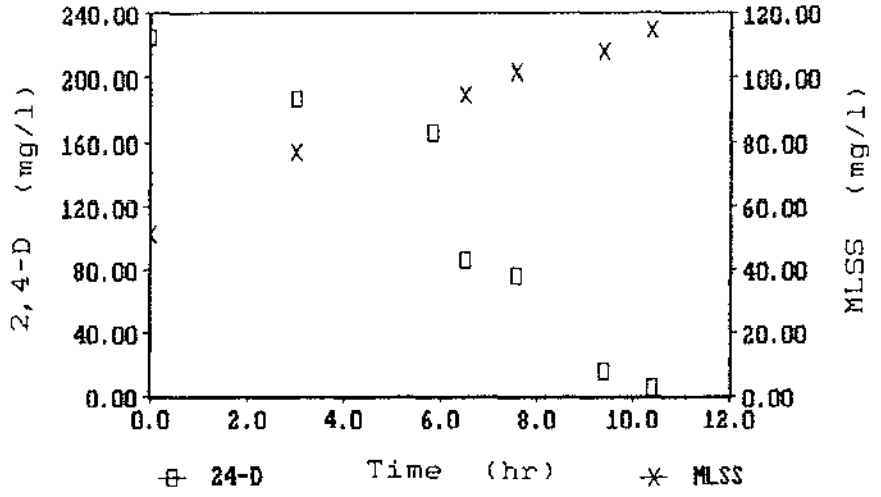
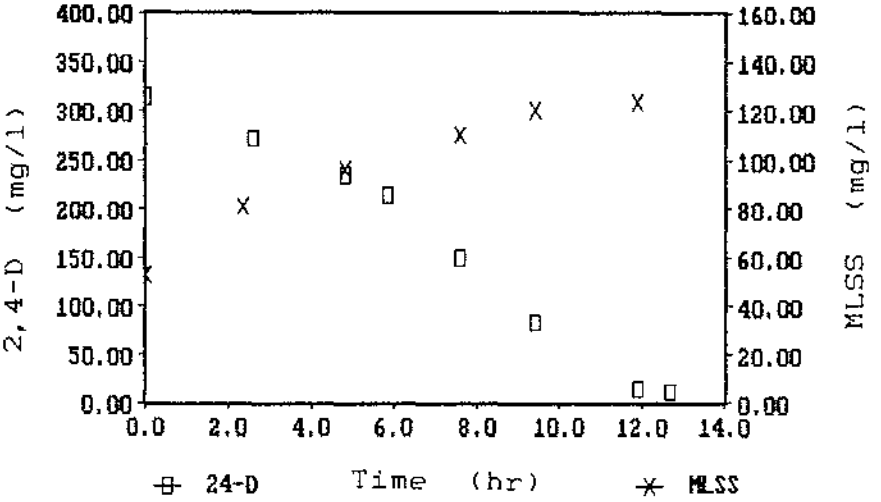


Figure A1.3: 2,4-D 200 mg/l, Batch 1. s, MLSS v t



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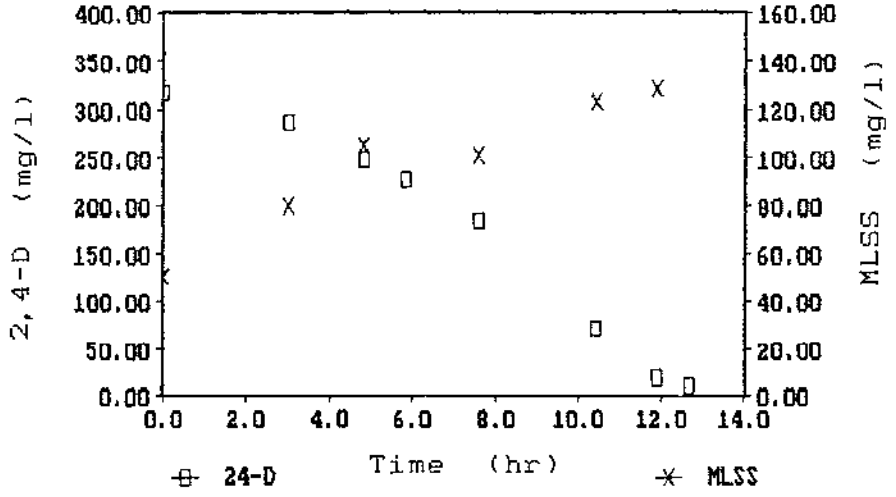


Figure A1.5: 2,4-D 300 mg/l, Batch 2. ε, MLSS v t

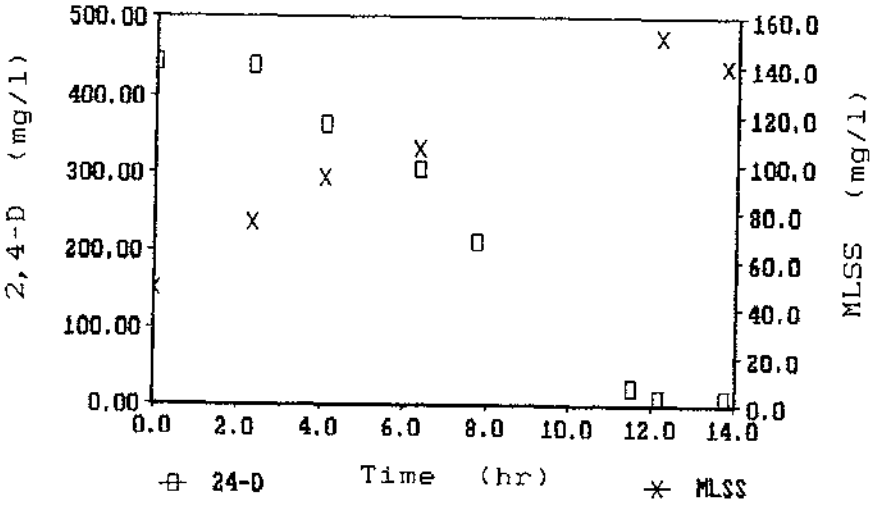


Figure A1.6: 2,4-D 400 mg/l, Batch 1. ε, MLSS v t

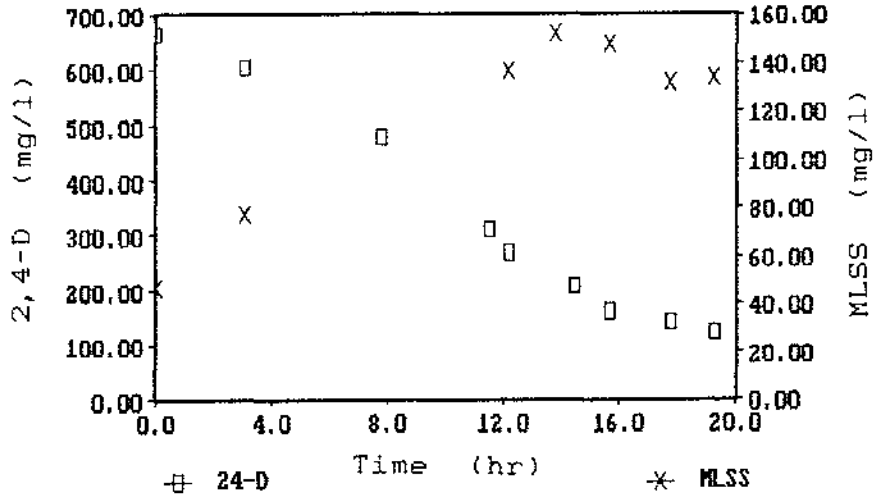


Figure A1.7: 2,4-D 600 mg/l, Batch 1. s, MLSS v t

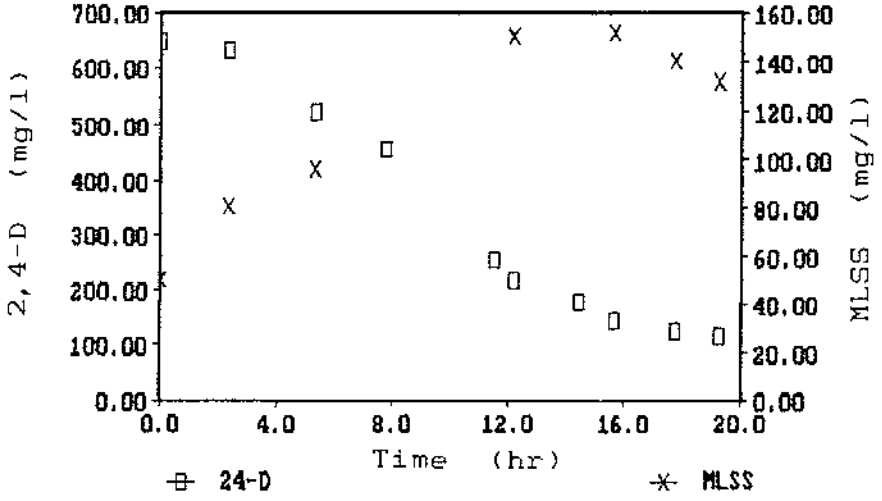


Figure A1.8: 2,4-D 600 mg/l, Batch 2. s, MLSS v t

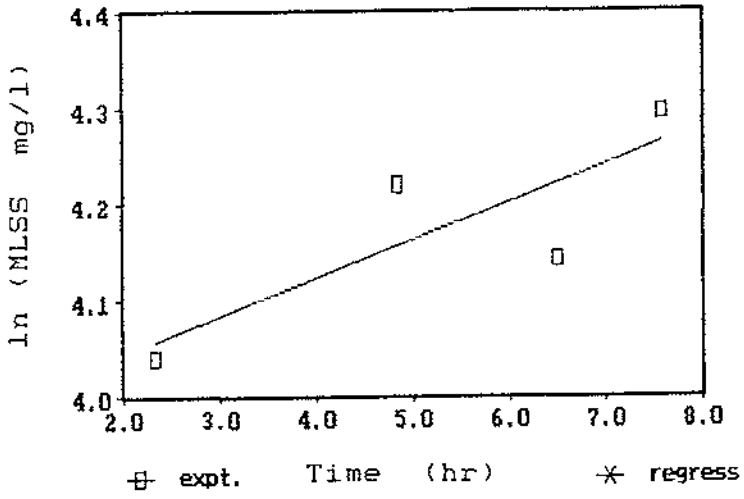


Figure A1.9: 2,4-D 100 mg/l, Batch 1.
ln (MLSS) v t. Slope (μ) = 0.039 hr⁻

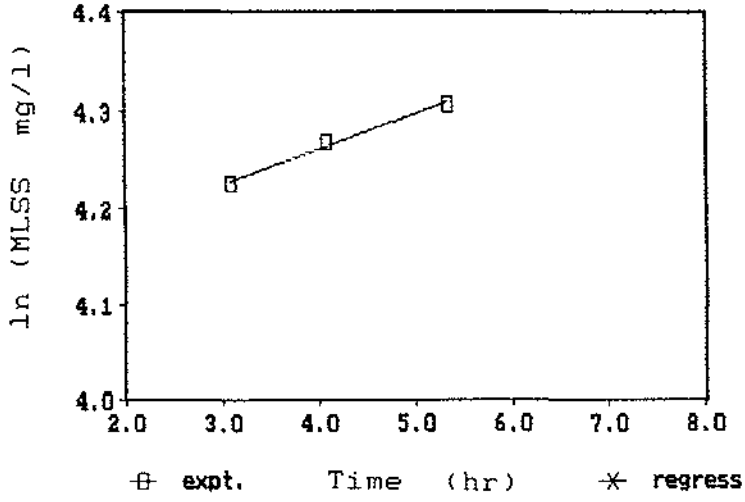


Figure A1.10: 2,4-D 100 mg/l, Batch 2.
ln (MLSS) v t. Slope (μ) = 0.036 hr⁻

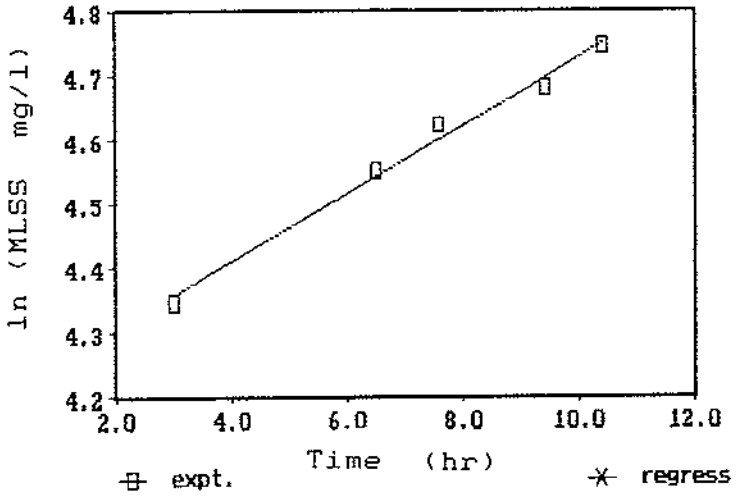


Figure A1.11: 2,4-D 200 mg/l, Batch 1.
ln (MLSS) v t. Slope (μ) = 0.053 hr^{-1}

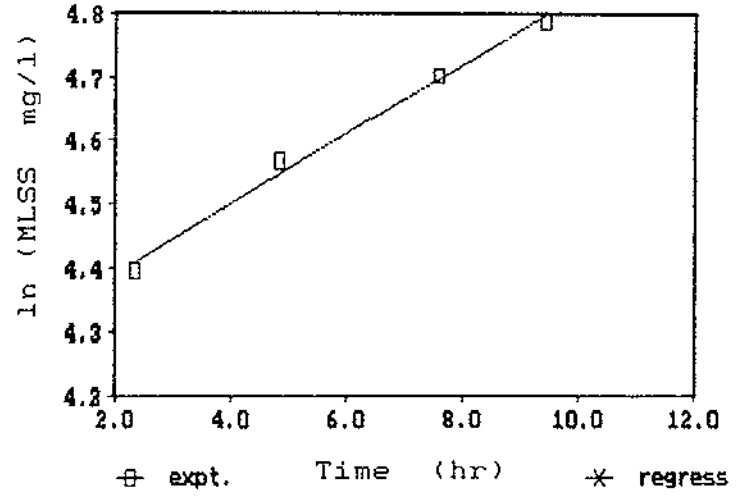


Figure A1.12: 2,4-D 300 mg/l, Batch 1.
ln (MLSS) v t. Slope (μ) = 0.055 hr^{-1}

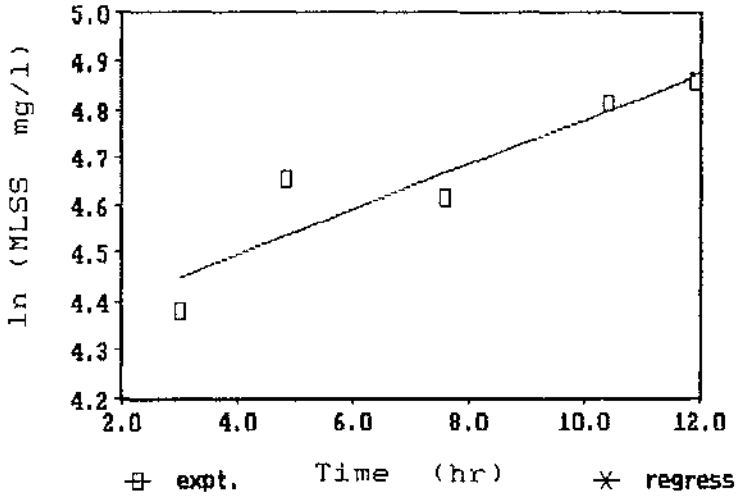


Figure A1.13: 2,4-D 300 mg/l, Batch 2.
ln (MLSS) v t. Slope (μ) = 0.047 hr^{-1}

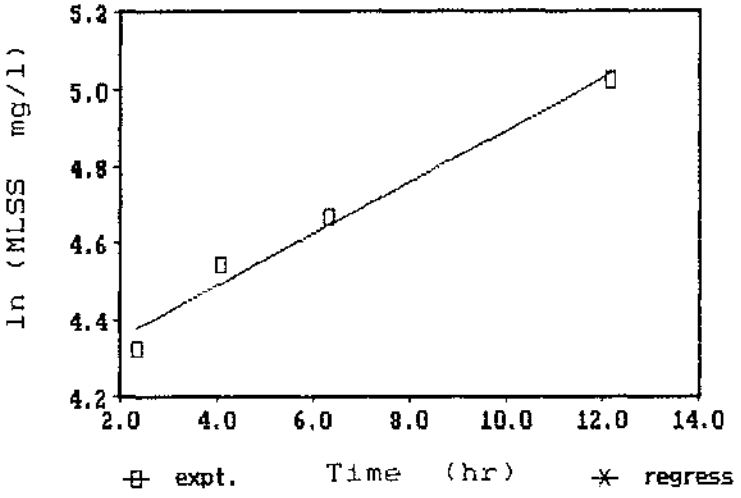


Figure A1.14: 2,4-D 400 mg/l, Batch 1.
ln (MLSS) v t. Slope (μ) = 0.068 hr^{-1}

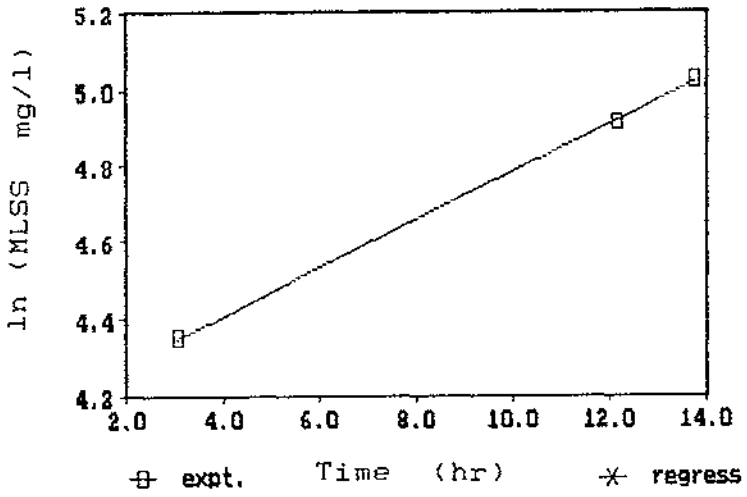


Figure A1.15: 2,4-D 600 mg/l, Batch 1.
ln (MLSS) v t. Slope (μ) = 0.063 hr^{-1}

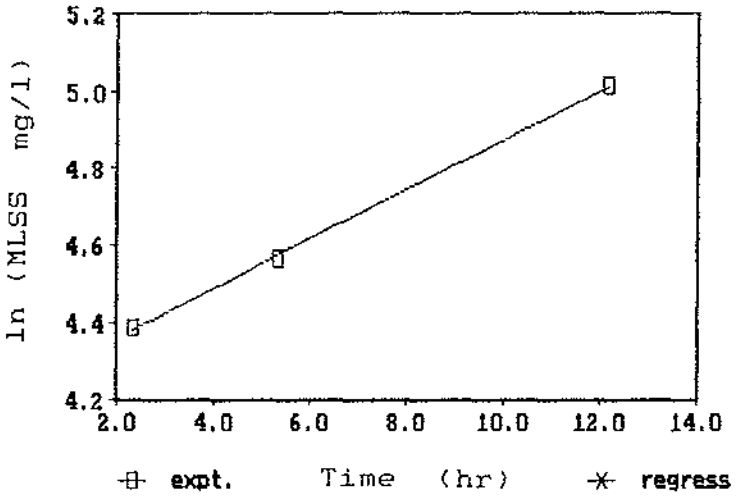


Figure A1.16: 2,4-D 600 mg/l, Batch 2.
ln (MLSS) v t. Slope (μ) = 0.064 hr^{-1}

Appendix 2

PCOC Batch Experiment

Table A2.1 PCOC Batch Experiment
Result Summary

Batch Title No.	Initial s(o) Conc. mg/l	Growth Yield (Y)	1/Growth Yield (Y)	Specific Growth Rate (μ) (*0.001)	1/Specific Growth Rate (1/μ)	μ/So (*0.001)
1 25	27.5	0.877	1.140250	6.4	156.25	0.232727
2 25	27.6	0.953	1.049317	12	83.33333	0.434782
1 50	53.1	0.685	1.459854	27	37.03703	0.508474
2 50	53.2	0.66	1.515151	32	31.25	0.601503
3 50	48.2	0.781	1.280409	21	47.61904	0.435684
1 75	79.1	0.577	1.733102	18	55.55555	0.227560
2 75	78.7	0.613	1.631321	19	52.63157	0.241423
3 75	71.1	0.622	1.607717	25	40	0.351617
1 100	107.1	0.466	2.145922	3.6	277.7777	0.033613

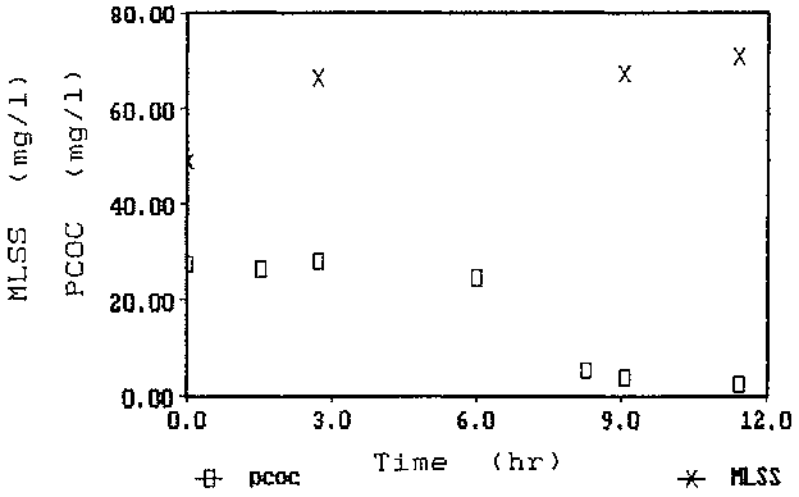


Figure A2.1: PCOC 25 mg/l, Batch 1. s, MLSS v t

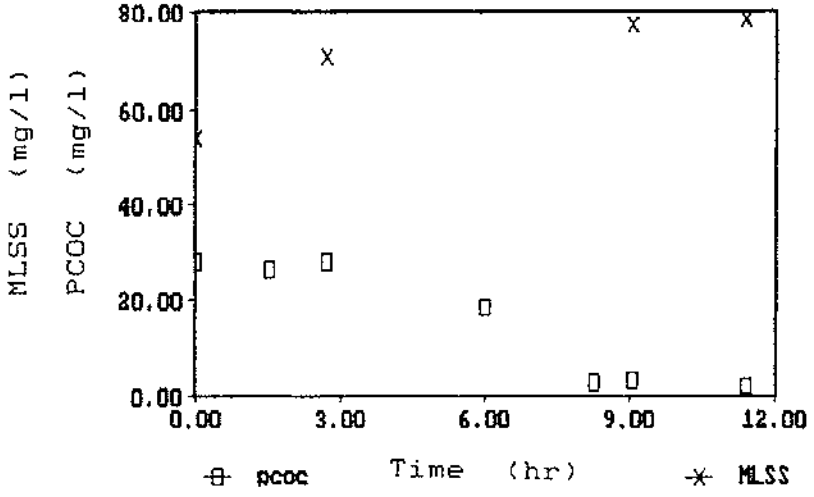


Figure A2.2: PCOC 25 mg/l, Batch 2. s, MLSS v t

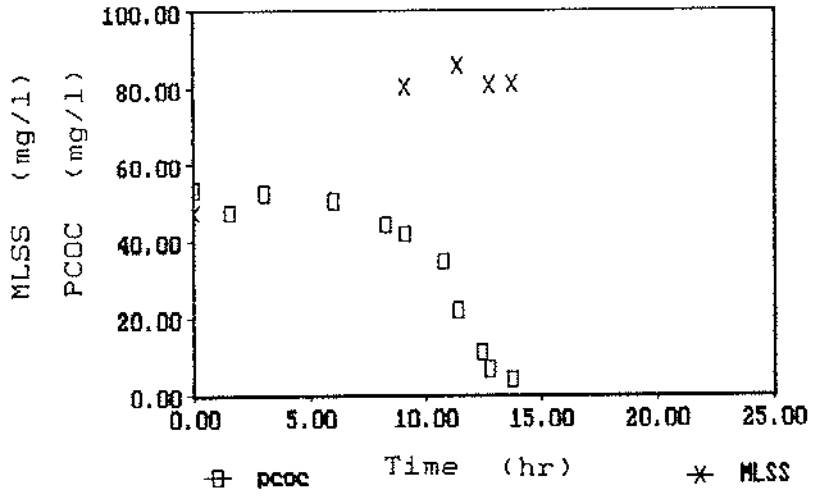


Figure A2.3: PCOC 50 mg/l, Batch 1. s, MLSS v t

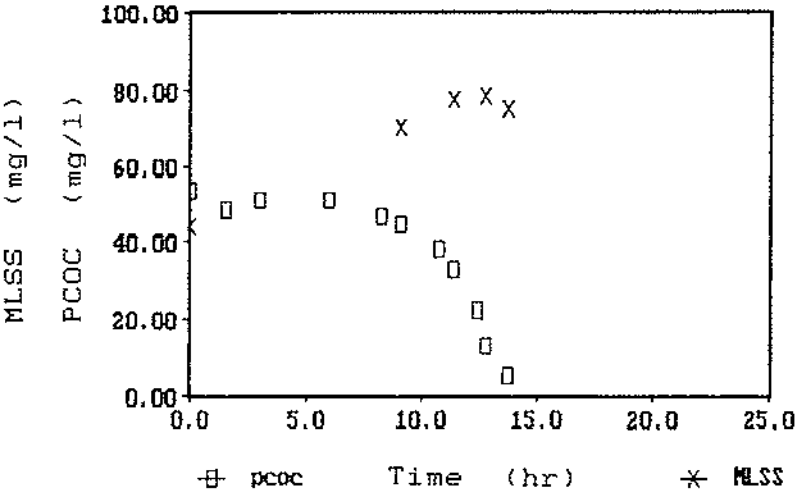


Figure A2.4: PCOC 50 mg/l, Batch 2. s, MLSS v t

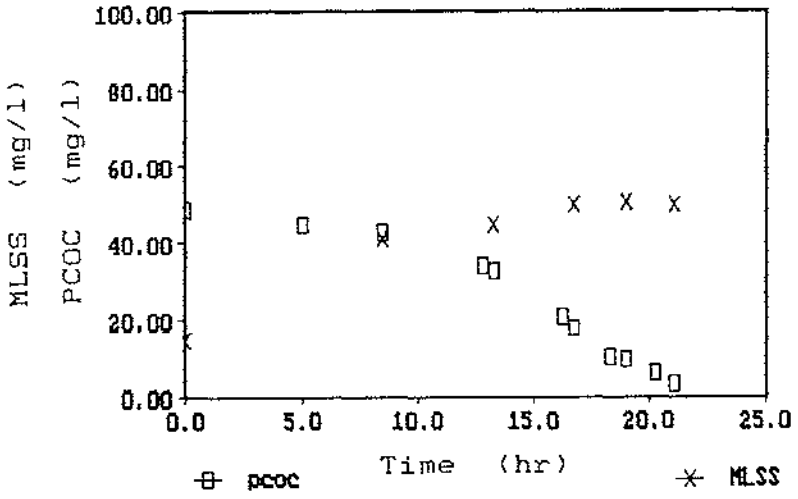


Figure A2.5: PCOC 50 mg/l, Batch 3. s, MLSS v t

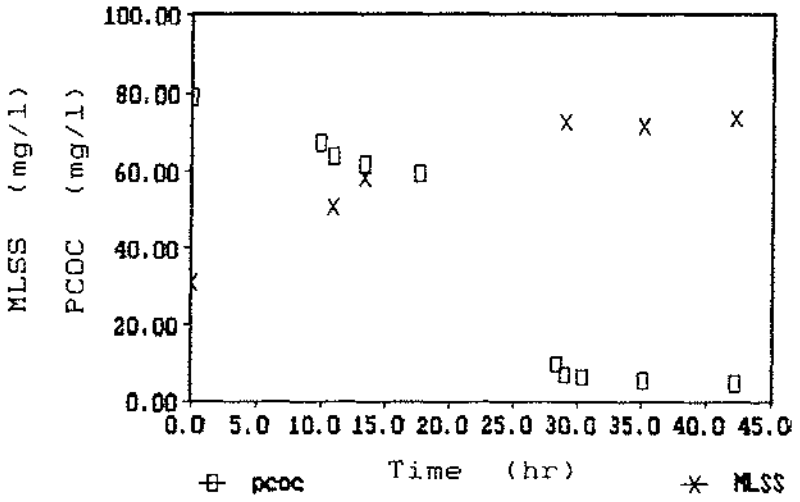


Figure A2.6: PCOC 75 mg/l, Batch 1. s, MLSS v t

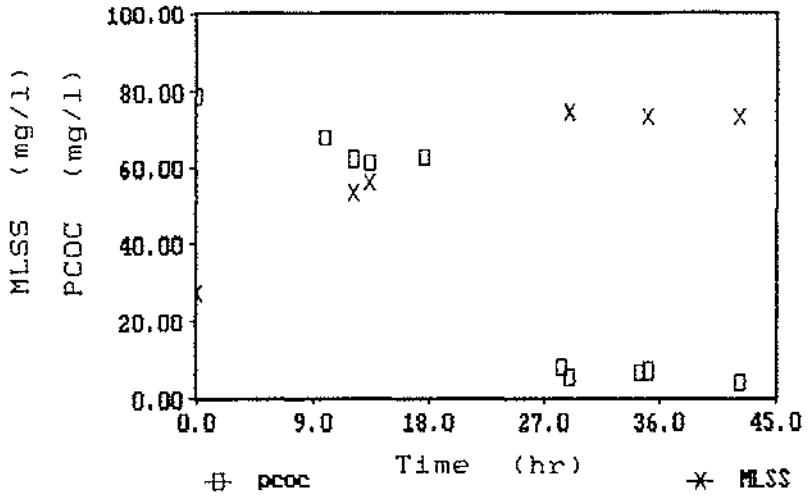


Figure A2.7: PCOC 75 mg/l, Batch 2. s, MLSS v t

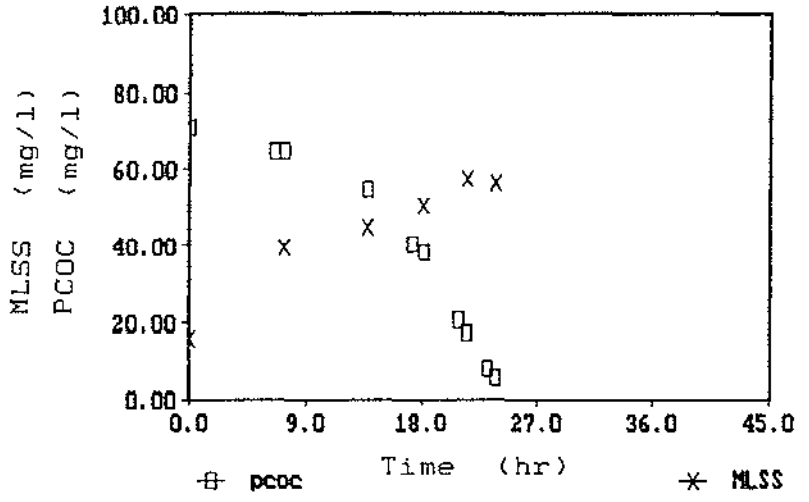


Figure A2.8: PCOC 75 mg/l, Batch 3. s, MLSS v t

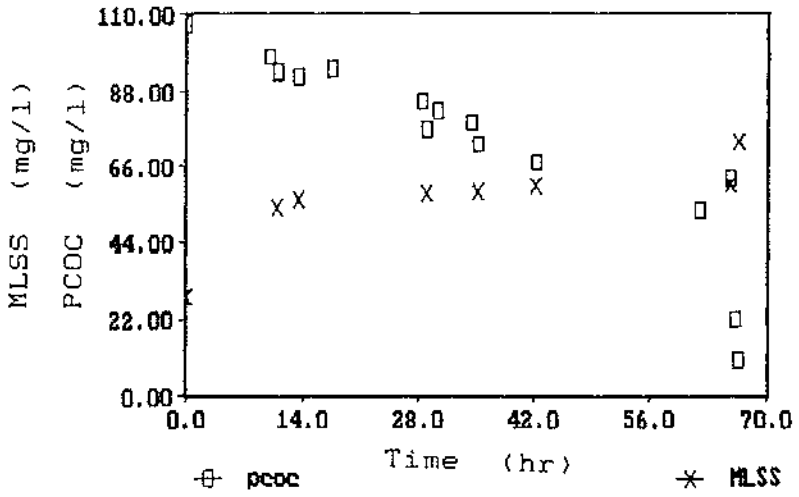


Figure A2.9: PCOC 100 mg/l, Batch 1. s, MLSS v t

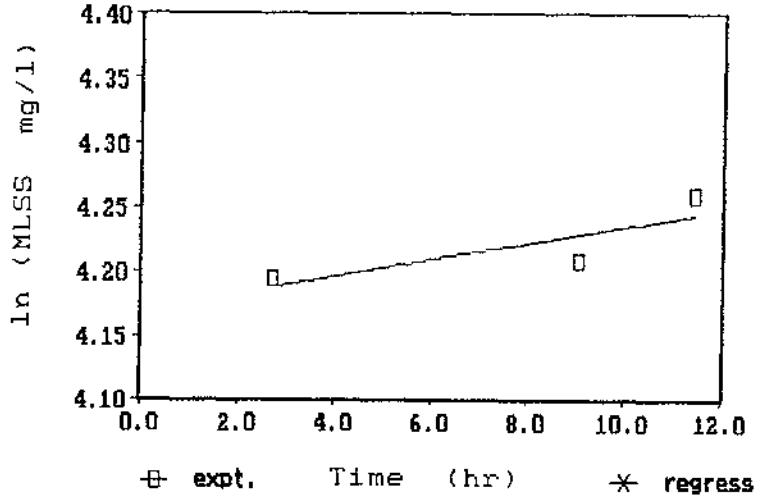


Figure A2.10: PCOC 25 mg/l, Batch 1.
ln (MLSS) v t. Slope (μ) = 0.0064 hr^{-1}

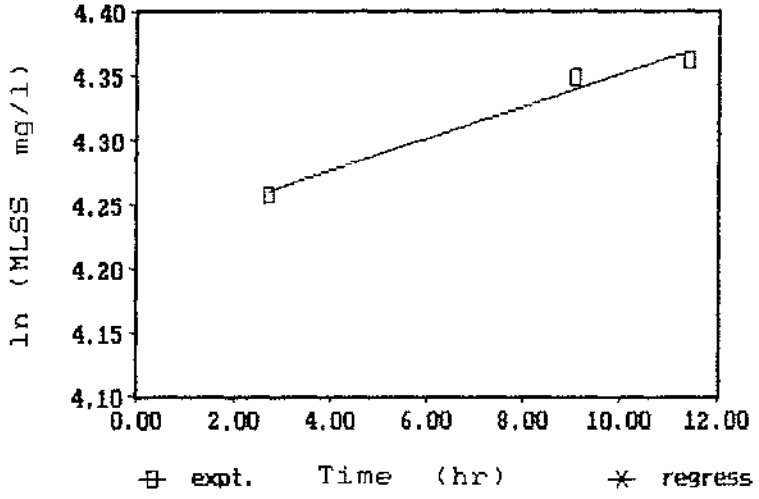


Figure A2.11: PCOC 25 mg/l, Batch 2.
ln (MLSS) v t. Slope (μ) = 0.012 hr^{-1}

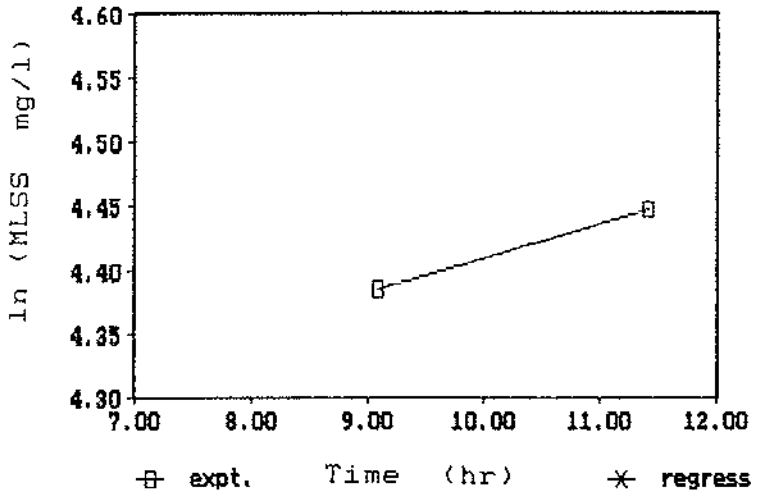


Figure A2.12: PCOC 50 mg/l, Batch 1.
ln (MLSS) v t. Slope (μ) = 0.027 hr^{-1}

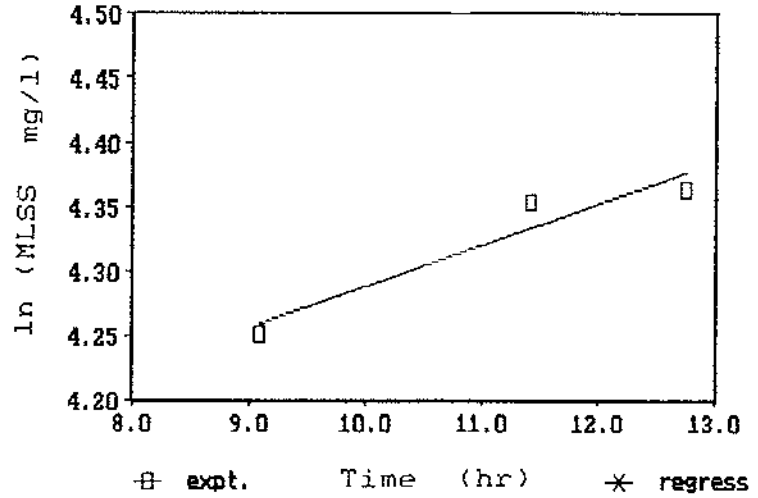


Figure A2.13: PCOC 50 mg/l, Batch 2.
ln (MLSS) v t. Slope (μ) = 0.032 hr^{-1}

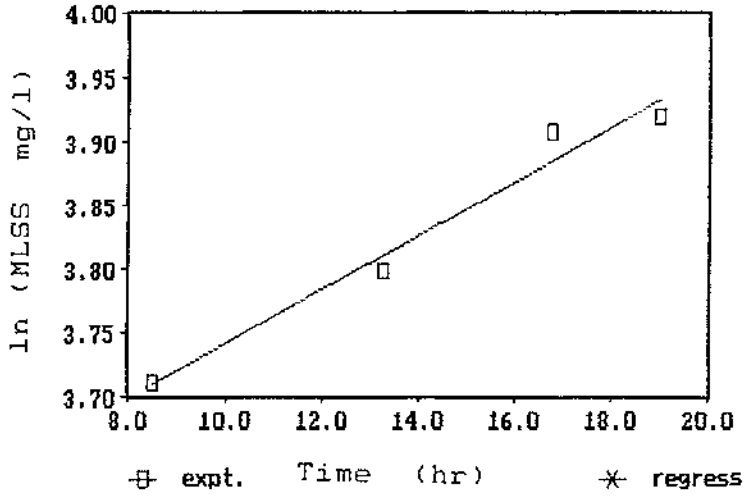


Figure A2.14: PCOC 50 mg/l, Batch 3.
ln (MLSS) v t. Slope (μ) = 0.021 hr^{-1}

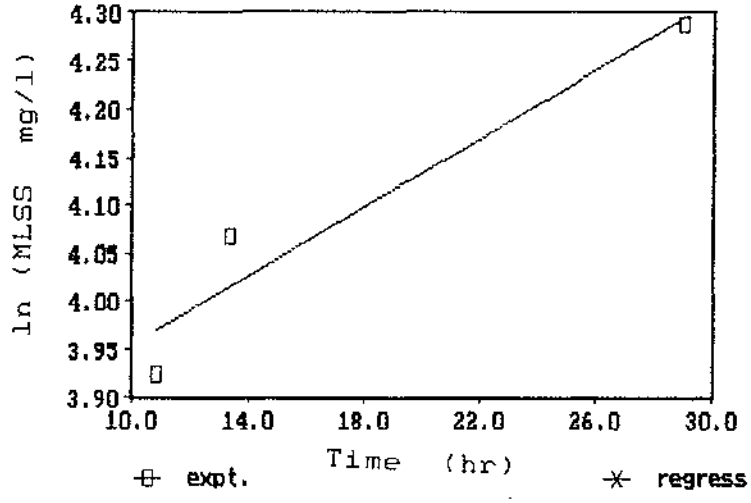


Figure A2.15: PCOC 75 mg/l, Batch 1.
ln (MLSS) v t. Slope (μ) = 0.018 hr^{-1}

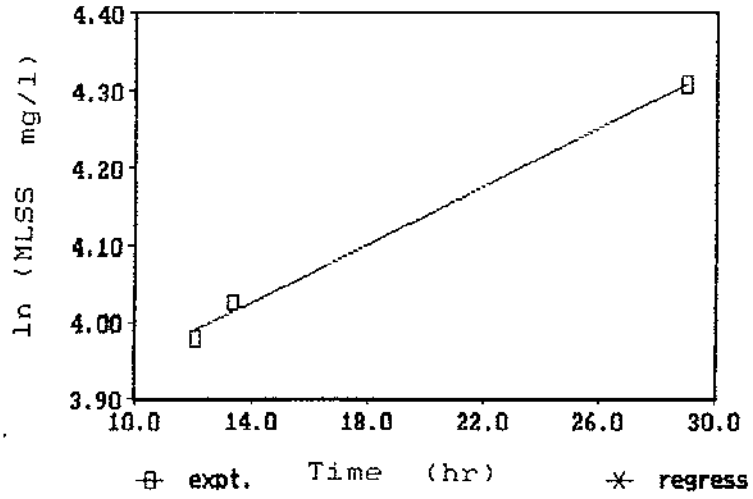


Figure A2.16: PCOC 75 mg/l, Batch 2.
ln (MLSS) v t. Slope (μ) = 0.019 hr^{-1}

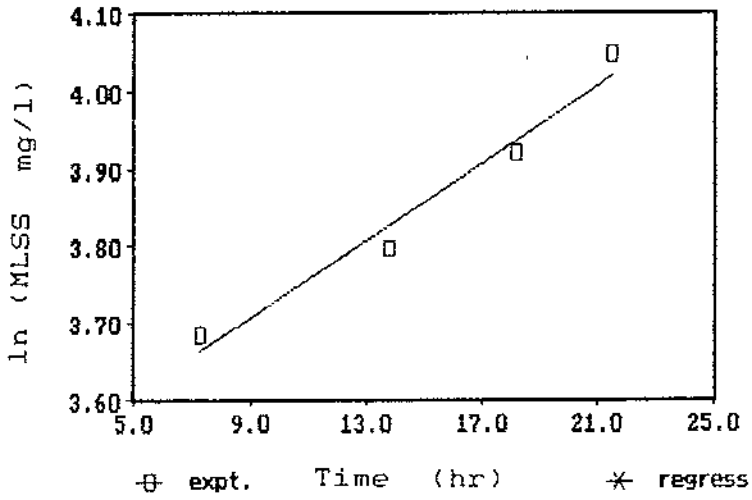


Figure A2.17: PCOC 75 mg/l, Batch 3.
ln (MLSS) v t. Slope (μ) = 0.025 hr^{-1}

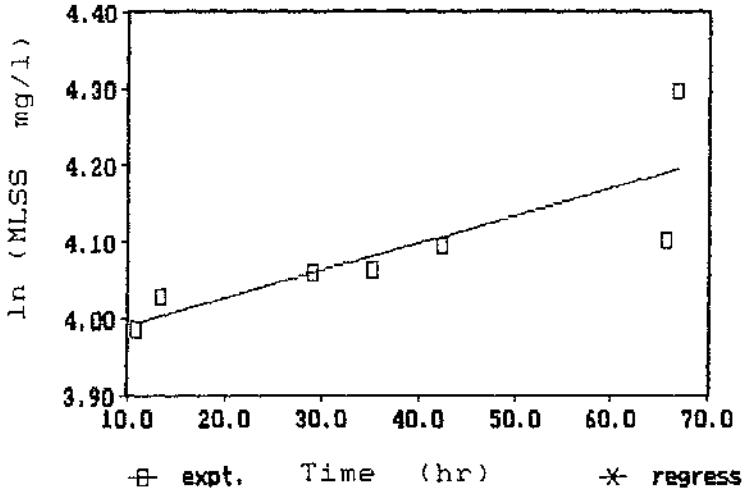


Figure A2.18: PCOC 100 mg/l, Batch 1.
ln (MLSS) v t. Slope (μ) = 0.036 hr^{-1}

Appendix 3

Initial PCOC Chemostats

Table A3.1 PCOC CSTR (50 mg/l)

SPECIFICATIONS

Feed
Conc. 50 mg/l

Reactor (final)
Volume 1.5 Litres

Dilution
Rate 0.14 1/hr

Day	Month	Time (24 hr) (hrs)	Time Absolute	Feed Abs.	Analysis Dil.	Feed Conc. (mg/l)	Effl.Abs.	Analysis Dil.	Effl Conc. (mg/l)
28	6	12	0	0.621	1	57.753	0.056	1	5.208
29	6	17.5	29.5	0.615	1	57.195	0.061	1	5.673
30	6	17	53	0.619	1	57.567	0.052	1	4.836
1	7	16	76	0.629	1	58.497	0.055	1	5.115
2	7	19.5	103.5	0.626	1	58.218	0.055	1	5.115
				Chemostat	Cleaned				
5	7	13.5	169.5	0.628	1	58.404	0.045	1	4.185
6	7	12.33	192.33	0.624	1	58.032	0.044	1	4.092
7	7	13.5	217.5	0.629	1	58.497	0.045	1	4.185

Chemostat Volume (ml)	Flowrate (ml/min)	Dilution Rate (1/hr)	Memb. wt.(mg)	Memb.+dry cells (mg)	Dry cell wt.(mg)	Sample Vol.(L)	MLSS (mg/L)
1450	3.4	0.140689	78.3	82.4	4.1	0.1	41
1450	3.4	0.140689	79.4	87.6	8.2	0.2	41
	3.45		77.1	85.5	8.4	0.2	42
	3.4		80.1	88.5	8.4	0.2	42
1450	3.4	0.140689	78.2	86.4	8.200000	0.2	41
	3.3		78.3	85.5	7.2	0.2	36
1450	3.4	0.140689	78	85.8	7.8	0.2	39
			78	85.9	7.9	0.2	39.5

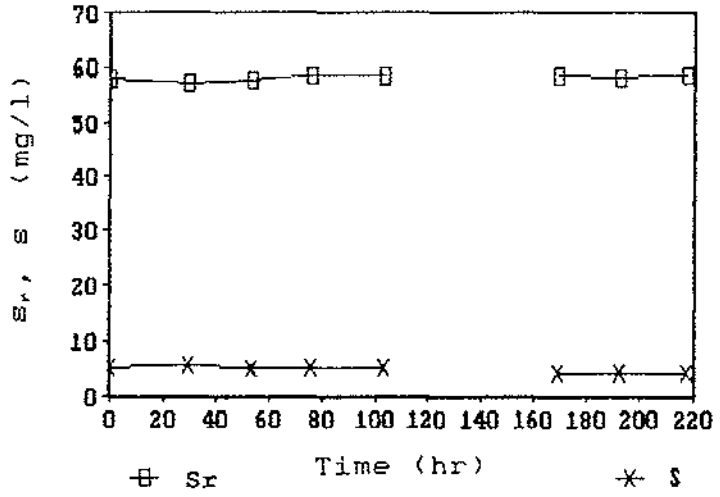


Figure A3.1: PCOC Chemostat, 50 mg/l
S_r, S v t

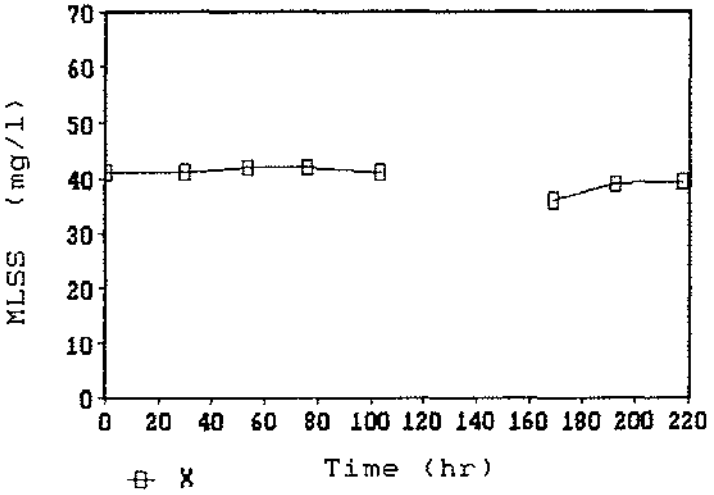


Figure A3.2: PCOC Chemostat, 50 mg/l
MLSS v t

Table A3.2 PCDC CSTR (100 mg/l)

SPECIFICATIONS

Feed
Conc. 100 mg/l

Reactor (final)
Volume 1.5 Litres

Dilution
Rate 0.14 1/hr

Day	Month	Time (24 hr) (hrs)	Time Absolute (hrs)	Feed Abs.	Analysis Dil.	Feed Conc. (mg/l)	Effl.Abs.	Analysis Dil.	Effl Conc. (mg/l)
14	6	17	0	0.564	2	104.304	0.07	1	6.51
15	6	16	23	0.558	2	103.788	0.047	1	4.371
17	6	10	65	0.548	2	101.928	0.053	1	4.929
18	6	14.5	93.5	0.556	2	103.416	0.055	1	5.115
19	6	12	115	0.567	2	105.462	0.049	1	4.557
20	6	10.5	137.5	0.56	2	104.16	0.051	1	4.743
21	6	10	161	0.565	2	105.09	0.067	1	6.231
23	6	18	217	0.55	2	102.3	0.089	1	8.277
24	6	14	237						
25	6	12	259	0.561	2	104.346	0.057	1	5.301
26	6	17	288	0.549	2	102.114	0.068	1	6.324
27	6	14	309	0.559	2	103.974	0.066	1	6.138
28	6	22.5	341.5	0.539	2	100.254	0.072	1	6.696

Chemostat Volume (ml)	Flowrate (ml/min)	Dilution Rate (1/hr)	Memb.wt. (mg)	Memb+dry cells(mg)	Dry cell wt.(mg)	Sample Vol.(L)	MLSS (mg/L)
1450	3.4	0.140689	74.8	83.1	8.299999	0.1	82.99999
			74.7	81.2	6.5	0.1	65
	3.4		74.7	81.2	6.5	0.1	65
1475	3.4	0.138305	75.3	82.1	6.799999	0.1	67.99999
	3.4		76.5	83.8	7.3	0.1	73.00000
	3.3		74.7	82.2	7.5	0.1	75
	3.35		76.1	83.1	7	0.1	70
	3.3		76.7	84.5	7.8	0.1	78
1450	3.4	0.140689					
1450	3.4	0.140689	76.7	83.5	6.8	0.1	68
	3.4		76.7	83	6.3	0.1	63
	3.4		76.6	83.6	7	0.1	70
	3.4		78.3	85.2	6.899999	0.1	68.99999

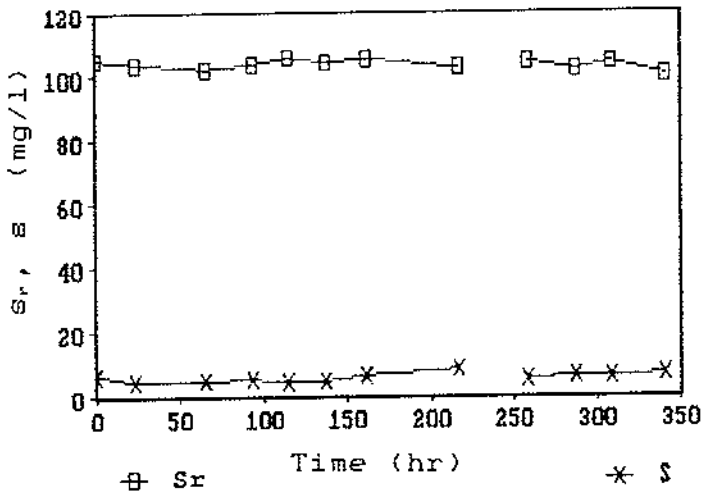


Figure A3.3: PCOC Chemostat, 100 mg/l
S_r, S v t

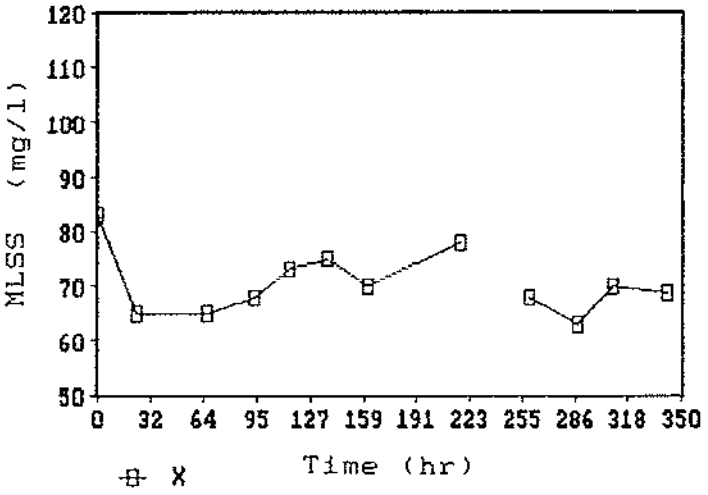


Figure A3.4: PCOC Chemostat, 100 mg/l
MLSS v t

Table A3.3 PCBC CSTR (150 mg/l)

SPECIFICATIONS

Feed
Conc. 150 mg/l

Reactor (final)
Volume 1.5 Litres

Dilution
Rate 0.14 1/hr

Day	Month	Time (24 hr) (hrs)	Time Absolute	Feed Abs.	Analysis Dil.	Feed Conc. (mg/l)	Effl.Abs.	Analysis Dil.	Effl Conc. (mg/l)
6	6	17.5	0	0.777	2	144.522	0.078	1	7.254
7	6	14	20.5	0.784	2	145.824	0.079	1	7.347
7	6	15	21.5						
8	6	22.5	53	0.766	2	142.476	0.189	1	17.577
9	6	15.5	70	0.782	2	145.452	0.183	1	17.019
10	6	14	92.5	0.773	2	143.778	0.095	1	8.835
11	6	17	119.5						
12	6	15	141.5						
12	6	21.5	148	0.782	2	145.452	0.08	1	7.44
13	6	21	171.5	0.784	2	145.824	0.082	1	7.626
14	6	17	191.5	0.771	2	143.406	0.07	1	6.51
15	6	16	214.5	0.772	2	143.592	0.064	1	5.952
17	6	10	256.5	0.775	2	144.15	0.078	1	7.254
18	6	14.5	285	0.765	2	142.29	0.069	1	6.417
19	6	12	306.5	0.774	2	143.964	0.073	1	6.789
20	6	10.5	329	0.772	2	143.592	0.065	1	6.045

Chemostat Volume (ml)	Flowrate (ml/min)	Dilution Rate (1/hr)	Memb wt. (mg)	Memb+dry cells(mg)	Dry cell wt.(mg)	Sample Vol.(L)	MLSS (mg/L)
1440							
1440	2.4	0.1					
	2.2						
	2.3						
	3.3		74.9	87.5	12.6	0.1	126
1475							
1475	3.1	0.126101	74.8	86.8	12	0.1	120
1330	3.1	0.139849	74.9	85.6	10.7	0.1	107
1325	3.1	0.140377	74.9	85	10.1	0.1	101
			74.9	85.4	10.5	0.1	105
	3.2		75.6	87.2	11.6	0.1	116
1350	3.1	0.137777	75.9	87.8	11.9	0.1	119
	3.2		76.6	86.2	9.6	0.1	96
	3.2		76.3	86.6	10.3	0.1	103

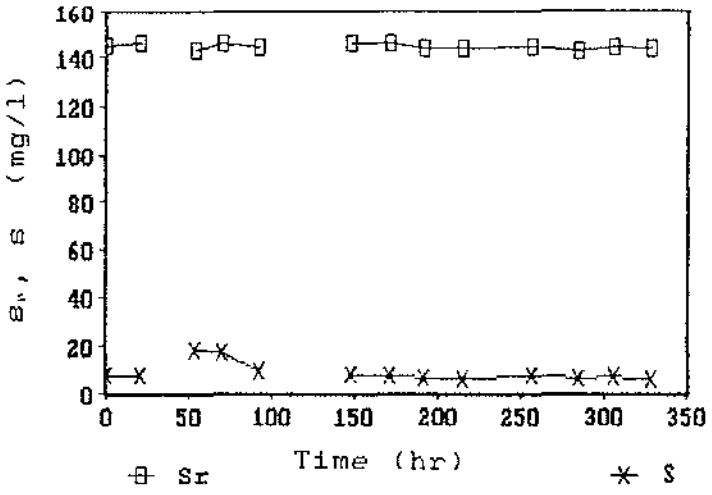


Figure A3.5: PCOC Chemostat, 150 mg/l
s_r, s v t

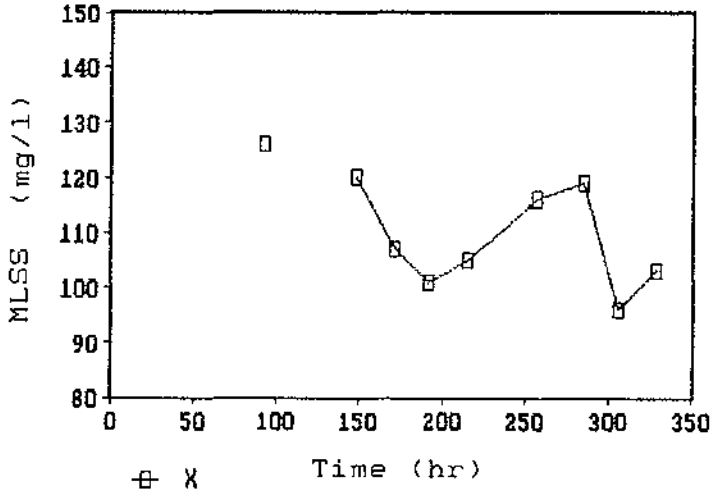


Figure A3.6: PCOC Chemostat, 150 mg/l
MLSS v t

Table A3.4 PCDC CSTR (200 mg/l)

SPECIFICATIONS

Feed
Conc. 200 mg/l

Reactor (final)
Volume 1.5 Litres

Dilution
Rate 0.14 l/hr

Day	Month	Time (24 hr) (hrs)	Time Absolute (hrs)	Feed Abs.	Analysis Dil.	Feed Conc. (mg/l)	Effl.Abs.	Analysis Dil.	Effl Conc. (mg/l)
20	6	22	0	0.493	4	183.396			
21	6	10	12	0.5	4	186	0.098	1	9.114
23	6	18	68	0.508	4	188.976	0.073	1	6.789
24	6	14	88						
25	6	12	110	0.505	4	187.86	0.09	1	8.37
26	6	17	139	0.517	4	192.324	0.102	1	9.486
27	6	14	160	0.511	4	190.092	0.097	1	9.021
28	6	22.5	192.5	0.511	4	190.092	0.104	1	9.672
29	6	17.5	211.5	0.514	4	191.208	0.118	1	10.974
30	6	17	235	0.508	4	188.976	0.095	1	8.835
1	7	16	258	0.515	4	191.58	0.114	1	10.602
2	7	19.5	285.5	0.518	4	192.696	0.119	1	11.067
6	7	12.33	374.33	0.507	4	188.604			

Chemostat cleaned
1/7/91

Chemostat Volume (ml)	Flowrate (ml/min)	Dilution Rate (1/hr)	Memb.wt. (mg)	Memb+dry cells(mg)	Dry cell wt.(mg)	Sample Vol.(L)	MLSS (mg/L)
	3.1						
	3.05		76.5	88.8	12.3	0.1	123
	3.1		77.1	91.2	14.1	0.1	141
1400	3.2	0.137142					
1450	3.4	0.140689	77.2	95.6	18.4	0.1	184
	3.4		76.3	94.3	18	0.1	180
	3.4		76.7	95.3	18.6	0.1	186
	3.4		77.8	92	14.2	0.1	142
1450	3.4	0.140689	80.3	96	15.7	0.1	157
	3.35		78	93.1	15.1	0.1	151
	3.2		78.5	84.5	6	0.1	60
1375	3.2	0.139636	82.2	81	-1.2	0.1	
	3.1		77.9	92.8	14.9	0.1	149

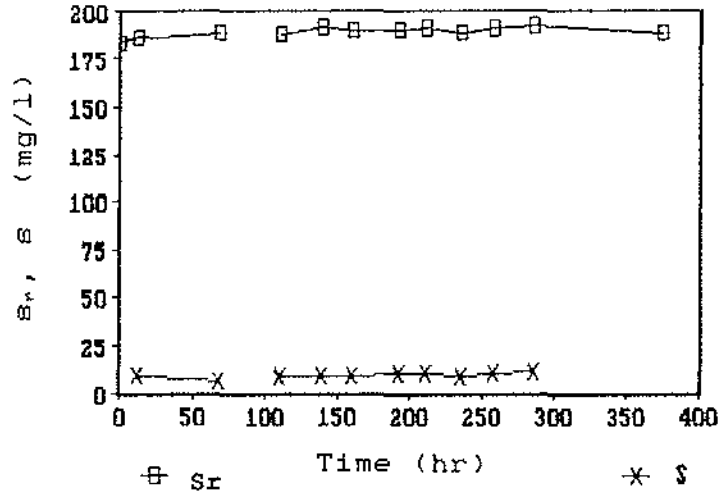


Figure A3.7: PCOC Chemostat, 200 mg/l S_r, S v t

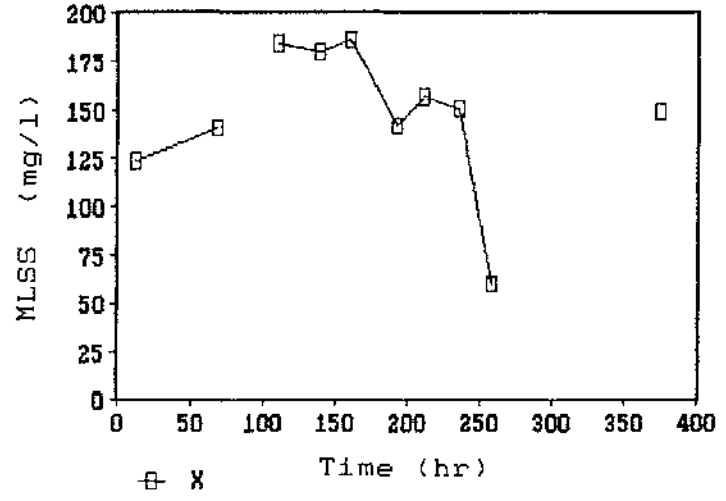


Figure A3.8: PCOC Chemostat, 200 mg/l MLSS v t

Appendix 4

PCOC Chemostats with Cell Activity Determination

Table A4.1 PCOC OXYGEN UPTAKE RATE EXPERIMENT

Results from Chemostats of Vol. 1500ml (approx)
and Dilution rate 0.13 +/- 0.005

SUMMARY

Chemostat (Expt.no. +Feed conc.)	s (mg/l)	sr (mg/l)	sr-s (mg/l)	MLSS (X) (mg/l)	OUR (mg/l(O2) /min)	SOUR (mg/l(O2) /(X).min	Growth Yield (Y)
1,50mg/l	3.9	55.4	51.5	41.3	0.09	0.0022	0.8
2,50mg/l	7.8	60.1	52.3	41.3	0.092	0.0022	0.79
3,50mg/l	7	61.1	54.1	37.3	0.079	0.0021	0.69
1,75mg/l	11.2	85.8	74.6	58.7	0.14	0.0024	0.79
2,75mg/l	9.9	89.1	79.2	51.3	0.11	0.0021	0.65
3,75mg/l	10.8	89.3	78.5	50	0.105	0.0021	0.64
1,100mg/l	9.3	108.1	98.8	67.3	0.18	0.0027	0.68
2,100mg/l	10	106	96	76	0.25	0.0033	0.79
3,100mg/l	8.3	106.9	98.6	79.3	0.22	0.0028	0.8
4,100mg/l	14	108.1	94.1	74.7	0.22	0.0029	0.79
5,100mg/l	12.7	106.6	93.9	66.3	0.2	0.003	0.71
1,125mg/l	15.5	130.9	115.4	75.3	0.24	0.0032	0.65
2,125mg/l	15.8	132.1	116.3	86.3	0.28	0.0032	0.74
3,125mg/l	13.7	132.5	118.8	85	0.27	0.0032	0.72
1,150mg/l	16.9	152.4	135.5	98.7	0.28	0.0028	0.73
2,150mg/l	16.3	151.1	134.8	91.3	0.3	0.0033	0.68
3,150mg/l	13.8	153.2	139.4	94.7	0.32	0.0034	0.68
1,175mg/l	19	171	152	103.7	0.33	0.0031	0.68
2,175mg/l	15.9	169	153.1	109.3	0.34	0.0031	0.71
3,175mg/l	21.7	174.6	152.9	115.3	0.34	0.0029	0.75

Table A4.2 PCOC Chemostat 1, 50 mg/l

Date 6/9/91 50 mg/l Feed

Sample Vol (mls)	300	Feed Abs	0.565
Dry Memb. wt. (mg)	77.4	Feed conc mg/l	55.37
Dry Memb +cells(mg)	89.8	Effl Abs	0.04
		Effl conc mg/l	3.92
Dry Cells (mg)	12.4	Growth Yield	0.803368
MLSS (mg/L)	41.33333		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	8.9	60	3.3
1	8.7	62	3.1
2	8.5	64	2.9
3	8.4	66	2.7
4	8.3	68	2.5
5	8.2	70	2.3
6	8.1	72	2.2
8	7.9	74	2.1
10	7.7	76	1.9
12	7.5	78	1.7
14	7.3	80	1.6
16	7.1	82	1.6
18	6.9	84	1.6
20	6.8		
22	6.6		
24	6.4		
26	6.2		
28	6.1		
30	5.9		
32	5.7		
34	5.6		
36	5.4		
38	5.2		
40	5		
42	4.8		
44	4.6		
46	4.4		
48	4.3		
50	4.1		
52	3.9		
54	3.8		
56	3.6		
58	3.4		

Table A4.3 PCOC Chemostat 2, 50 mg/l

Date 27/9/91 50 mg/l Feed

Sample Vol (mls)	300	Feed Abs	0.613
Dry Memb. wt. (mg)	77.4	Feed conc mg/l	60.074
Dry Memb +cells(mg)	89.8	Effl Abs	0.08
Dry Cells (mg)	12.4	Effl conc mg/l	7.84
MLSS (mg/L)	41.33333	Growth Yield	0.791310

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	8.9	50	5.2
2	8.8	52	5
6	8.7	54	4.8
8	8.6	56	4.6
9	8.5	58	4.4
11	8.4	60	4.2
12	8.3	62	4
14	8.2	64	3.8
16	8.1	66	3.7
17	8	68	3.5
18	7.9	70	3.3
19	7.8	72	3.2
21	7.7	74	3.1
22	7.6	76	3
24	7.5	78	3
26	7.3	80	3
28	7.1	82	2.9
30	6.9		
32	6.8		
34	6.6		
36	6.4		
38	6.3		
40	6.1		
42	5.9		
44	5.8		
46	5.6		
48	5.4		

Table A4.4 PCOC Chemostat 3, 50 mg/l

Date 29/9/91 50 mg/l Feed

Sample Vol (mls)	300	Feed Abs	0.623
Dry Memb. wt. (mg)	80.4	Feed conc mg/l	61.054
Dry Memb +cells(mg)	91.6	Effl Abs	0.071
		Effl conc mg/l	6.958
Dry Cells (mg)	11.2	Growth Yield	0.690131
MLSS (mg/L)	37.33333		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	9.5	50	6
2	9.4	52	5.9
4	9.3	54	5.7
6	9.2	56	5.5
8	9.1	58	5.3
10	8.9	60	5.2
12	8.8	62	5
14	8.7	64	4.8
16	8.5	66	4.7
18	8.4	68	4.5
20	8.3	70	4.3
22	8.1	72	4.1
24	8	74	3.9
26	7.8	76	3.8
28	7.7	78	3.6
30	7.6	80	3.4
32	7.4	82	3.3
34	7.3	84	3.2
36	7.1	86	3
38	6.9	88	2.9
40	6.8	90	2.8
42	6.7	92	2.8
44	6.5		
46	6.3		
48	6.2		

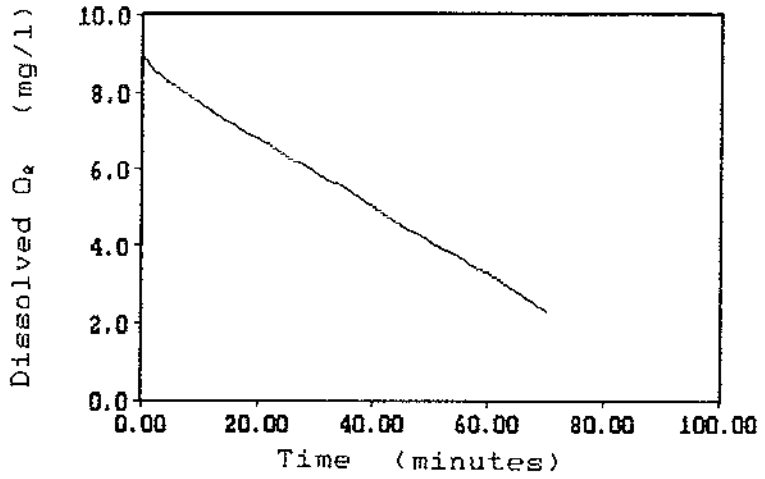


Figure A4.1: Chemostat 1, 50 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.09 mg/l/min.

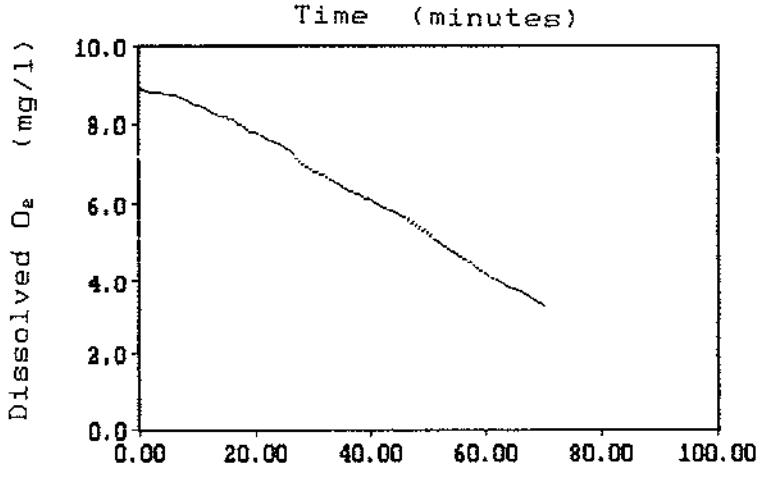


Figure A4.2: Chemostat 2, 50 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.092 mg/l/min.

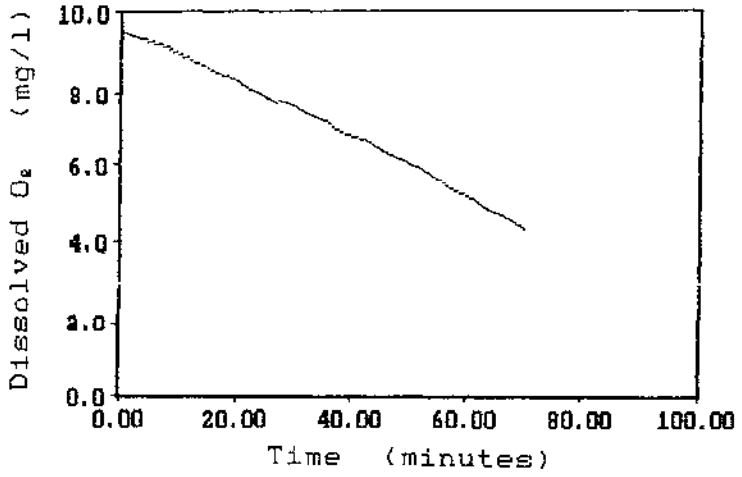


Figure A4.3: Chemostat 3, 50 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.079 mg/l/min.

Table A4.5 PCOC Chemostat 1, 75 mg/l

Date 2/10/91 75 mg/l Feed

Sample Vol (mls)	300	Feed Abs	0.876
Dry Memb. wt. (mg)	77.4	Feed conc mg/l	85.848
Dry Memb +cells(mg)	95	Effl Abs	0.114
		Effl conc mg/l	11.172
Dry Cells (mg)	17.6	Growth Yield	0.785616
MLSS (mg/L)	58.66666		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	9.5	40	3.3
1	9.3	42	3
2	9.1	44	2.8
3	8.7	46	2.7
4	8.4	48	2.6
6	8.2	50	2.4
8	7.8	52	2.3
10	7.5		
12	7.3		
14	7		
16	6.7		
18	6.4		
20	6.1		
22	5.8		
24	5.5		
26	5.3		
28	5		
30	4.7		
32	4.4		
34	4.1		
36	3.8		
38	3.6		

Table A4.6 PCOC Chemostat 2, 75 mg/l

Date 4/10/91 75 mg/l Feed

Sample Vol (mls)	300	Feed Abs	0.909
Dry Memb. wt. (mg)	89.7	Feed conc mg/l	89.082
Dry Memb +cells(mg)	105.1	Effl Abs	0.101
		Effl conc mg/l	9.898
Dry Cells (mg)	15.4	Growth Yield	0.648279
MLSS (mg/L)	51.33333		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	9.3	40	4.3
1	9.1	42	4.1
2	8.8	44	3.9
3	8.6	46	3.7
4	8.4	48	3.5
5	8.3	50	3.3
6	8.1	52	3.2
8	7.8	54	3.1
10	7.6	56	2.9
12	7.4	58	2.8
14	7.2	60	2.7
16	7	62	2.6
18	6.7		
20	6.4		
22	6.2		
24	6		
26	5.8		
28	5.6		
30	5.3		
32	5.1		
34	4.9		
36	4.7		
38	4.5		

Table A4.7 PCOC Chemostat 3, 75 mg/l

Date 6/10/91 75 mg/l Feed

Sample Vol (mls)	300	Feed Abs	0.316
Dry Memb. wt. (mg)	78.2	Feed conc mg/l	89.768
Dry Memb +cells(mg)	95.2	Effl Abs	0.112
		Effl conc mg/l	10.976
Dry Cells (mg)	17	Growth Yield	0.719193
MLSS (mg/L)	56.66666		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	8.8	30	4.8
1	8.4	32	4.6
2	8.1	34	4.4
3	7.9	36	4.2
4	7.7	38	4
6	7.4	40	3.8
8	7.2	42	3.6
10	7	44	3.4
12	6.8	46	3.3
14	6.6	48	3.1
16	6.4	50	2.9
18	6.2	52	2.8
20	6		
22	5.8		
24	5.5		
26	5.3		
28	5.1		

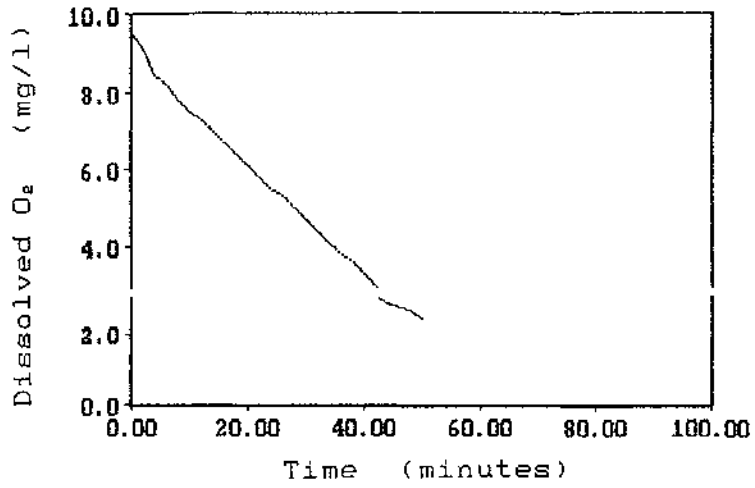


Figure A4.4: Chemostat 1, 75 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.14 mg/l/min.

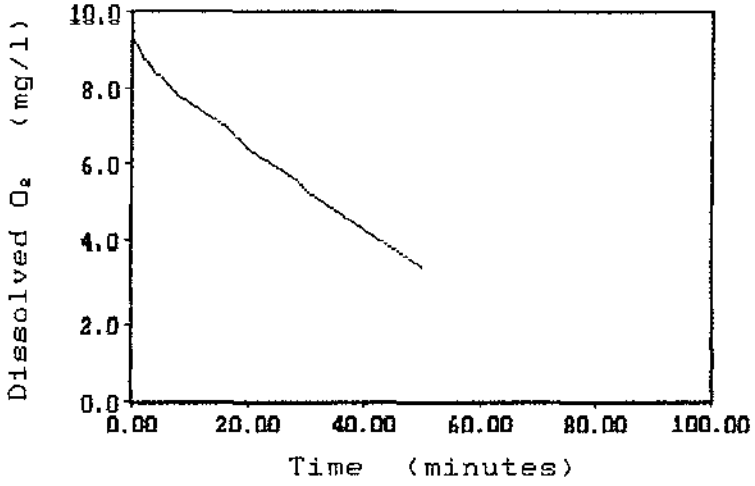


Figure A4.5: Chemostat 2, 75 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.11 mg/l/min.

Figure A4.6: Chemostat 3, 75 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.12 mg/l/min.

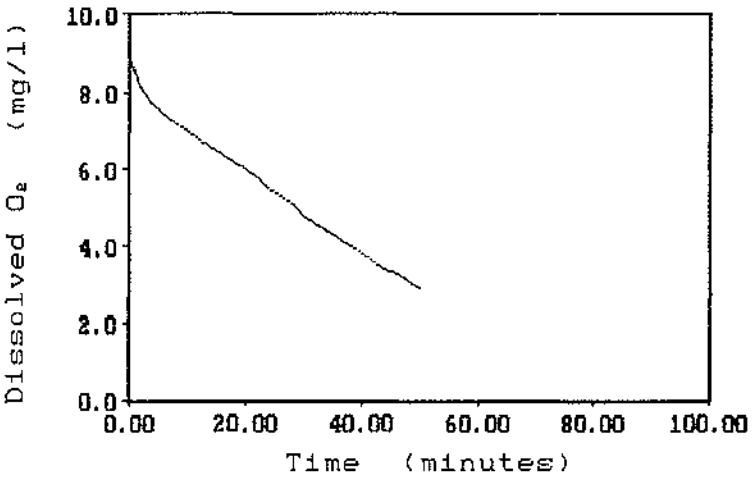


Table A4.8 PCOC Chemostat 1, 100 mg/l

Date 5/9/91 100 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.103
Dry Memb. wt. (mg)	75.9	Feed conc mg/l	108.094
Dry Memb +cells(mg)	96.1	Effl Abs	0.095
		Effl conc mg/l	9.31
Dry Cells (mg)	20.2	Growth Yield	0.681621
MLSS (mg/L)	67.33333		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	9.2	21	5.1
1	8.8	22	4.9
2	8.6	24	4.6
3	8.4	26	4.3
4	8.3	28	4
5	8.1	30	3.6
6	7.9	32	3.2
7	7.7	34	2.8
8	7.5	36	2.4
9	7.3	38	2.1
10	7.2	40	1.8
11	7	42	1.6
12	6.8	44	1.3
13	6.7	46	1.3
14	6.5	48	1.2
15	6.3		
16	6.1		
17	5.9		
18	5.7		
19	5.5		
20	5.3		

Table A4.9 PCOC Chemostat 2, 100 mg/l

Date 7/9/91 100 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.082
Dry Memb. wt. (mg)	76.4	Feed conc mg/l	106.036
Dry Memb +cells(mg)	99.2	Effl Abs	0.102
		Effl conc mg/l	9.996
Dry Cells (mg)	22.8	Growth Yield	0.791336
MLSS (mg/L)	76		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	9.3	21	3.8
1	8.9	22	3.6
2	8.7	23	3.4
3	8.3	24	3.3
4	8.1	25	3.2
5	7.8	26	3.1
6	7.5	28	2.8
7	7.2	30	2.6
8	6.9	32	2.4
9	6.6	34	2.2
10	6.3	36	1.7
11	6.1	38	1.4
12	5.8	40	1.3
13	5.6	42	1.2
14	5.4	44	1.2
15	5.2	46	1.1
16	4.9	48	1.1
17	4.7		
18	4.4		
19	4.2		
20	4		

Table A4.10 PCOC Chemostat 3, 100 mg/l

Date 9/9/91 100 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.091
Dry Memb. wt. (mg)	77.6	Feed conc mg/l	106.918
Dry Memb +cells(mg)	101.4	Effl Abs	0.085
		Effl conc mg/l	8.33
Dry Cells (mg)	23.8	Growth Yield	0.804695
MLSS (mg/L)	79.33333		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	8.3	22	3.2
1	8.1	24	2.8
2	7.8	26	2.3
3	7.6	28	2
4	7.3	30	1.8
5	7	32	1.7
6	6.8	34	1.6
7	6.6	36	1.6
8	6.4	40	1.6
9	6.2		
10	5.9		
12	5.6		
14	5.2		
16	4.7		
18	4.2		
20	3.7		

Table A4.11 PCOC Chemostat 4, 100 mg/l

Date 25/9/91 100 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.103
Dry Memb. wt. (mg)	77.1	Feed conc mg/l	108.094
Dry Memb +cells(mg)	99.5	Effl Abs	0.143
		Effl conc mg/l	14.014
Dry Cells (mg)	22.4	Growth Yield	0.793650
MLSS (mg/L)	74.66666		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	8.8	22	4
1	8.4	24	3.7
2	8.1	26	3.5
3	7.7	28	3.3
4	7.4	30	3.2
5	7.2	32	3
6	6.9	34	2.9
7	6.8	36	2.8
8	6.6	38	2.7
9	6.4	40	2.6
10	6.2	42	2.6
11	6.1		
12	5.9		
13	5.7		
14	5.4		
15	5.2		
16	5		
17	4.8		
18	4.6		
19	4.4		
20	4.3		

Table A4.12 PCOC Chemostat 5, 100 mg/l

Date 27/9/91 100 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.088
Dry Memb. wt. (mg)	78.6	Feed conc mg/l	106.624
Dry Memb +cells(mg)	98.5	Effl Abs	0.13
		Effl conc mg/l	12.74
Dry Cells (mg)	19.9	Growth Yield	0.706545
MLSS (mg/L)	66.33333		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	9.1	22	4.8
1	9	24	4.5
2	8.8	26	4.2
3	8.6	28	3.9
4	8.4	30	3.7
5	8.2	32	3.5
6	7.9	34	3.3
7	7.7	36	3.2
8	7.5	38	3
9	7.3	40	2.8
10	7.1		
11	6.8		
12	6.6		
13	6.4		
14	6.2		
16	5.8		
18	5.4		
20	5.1		

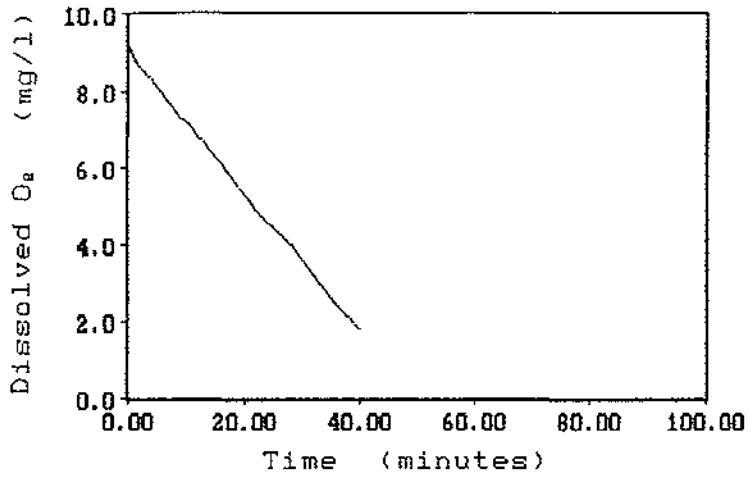


Figure A4.7: Chemostat 1, 100 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.18 mg/l/min.

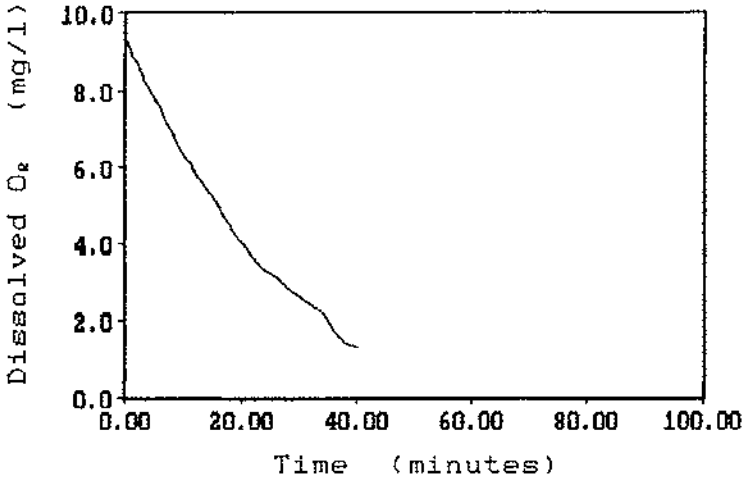
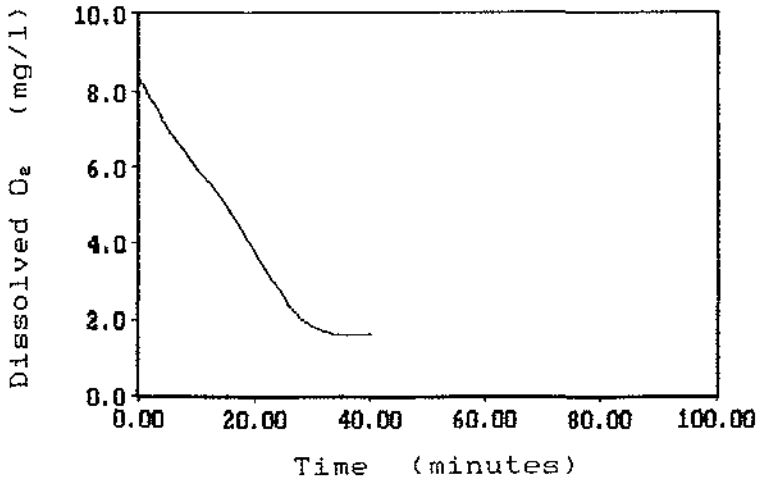


Figure A4.8: Chemostat 2, 100 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.25 mg/l/min.

Figure A4.9: Chemostat 3, 100 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.22 mg/l/min.



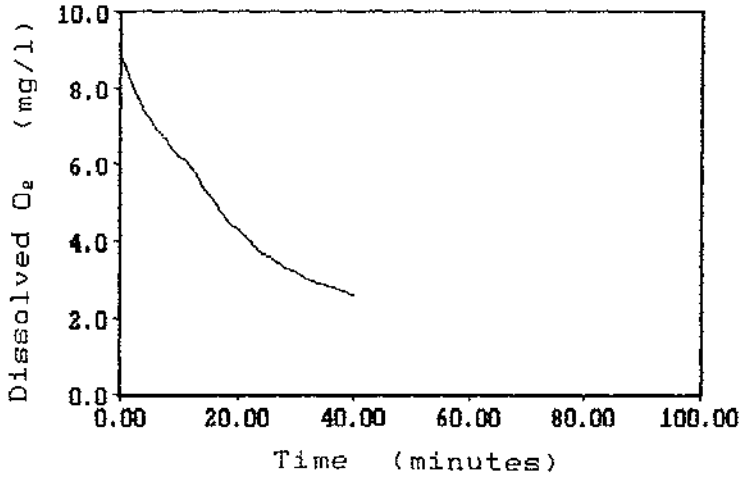


Figure A4.10: Chemostat 4, 100 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.22 mg/l/min.

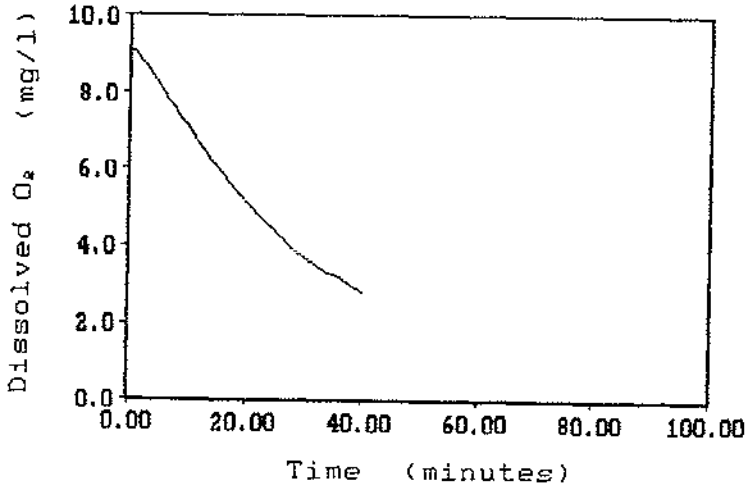


Figure A4.11: Chemostat 5, 100 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.20 mg/l/min.

Table A4.13 PCOC Chemostat 1, 125 mg/l

Date 2/10/91 125 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.336
Dry Memb. wt. (mg)	79.4	Feed conc mg/l	130.928
Dry Memb +cells(mg)	102	Effl Abs	0.158
		Effl conc mg/l	15.484
Dry Cells (mg)	22.6	Growth Yield	0.652553
MLSS (mg/L)	75.33333		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	9.3	21	4.6
1	9.1	22	4.4
2	8.8	24	4.1
3	8.5	26	3.8
4	8.3	28	3.7
5	8.1	30	3.5
6	7.8	32	3.3
7	7.6	34	3.2
8	7.4	36	3
9	7.2	38	2.9
10	6.9		
11	6.7		
12	6.4		
13	6.2		
14	5.9		
15	5.7		
16	5.5		
17	5.3		
18	5.1		
19	4.9		
20	4.7		

Table A4.14 PCOC Chemostat 2, 125 mg/l

Date 4/10/91 125 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.348
Dry Memb. wt. (mg)	80.2	Feed conc mg/l	132.104
Dry Memb +cells(mg)	106.1	Effl Abs	0.161
Dry Cells (mg)	25.9	Effl conc mg/l	15.778
MLSS (mg/L)	86.33333	Growth Yield	0.742167

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	9.4	22	3.4
1	9.1	24	3.2
2	8.7	26	3
3	8.3	28	2.8
4	8	30	2.6
5	7.7	32	2.5
6	7.4		
7	7.1		
8	6.8		
9	6.5		
10	6.3		
11	6		
12	5.8		
13	5.6		
14	5.3		
15	5		
16	4.7		
17	4.5		
18	4.2		
19	3.9		
20	3.7		

Table A4.15 PCOC Chemostat 3, 125 mg/l

Date 8/10/91 125 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.352
Dry Memb. wt. (mg)	80.5	Feed conc mg/l	132.496
Dry Memb +cells(mg)	106	Effl Abs	0.14
Dry Cells (mg)	25.5	Effl conc mg/l	13.72
MLSS (mg/L)	85	Growth Yield	0.715632

Time (min)	Diss Oxy
0	8.4
1	8
2	7.7
3	7.4
4	7.1
5	6.8
6	6.6
7	6.3
8	6
9	5.8
10	5.5
11	5.2
12	4.9
13	4.7
14	4.4
15	4.2
16	3.9
17	3.7
18	3.4
19	3.2
20	3
22	2.6
24	2.3
26	2.1
28	2
30	2

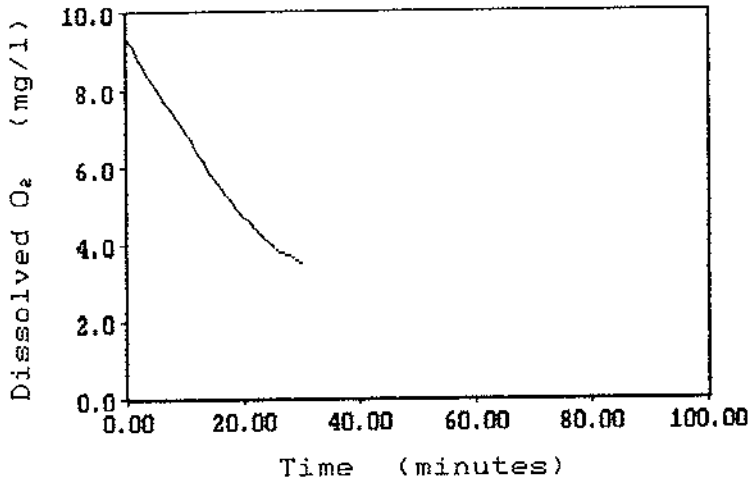


Figure A4.12: Chemostat 1, 125 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.24 mg/l/min.

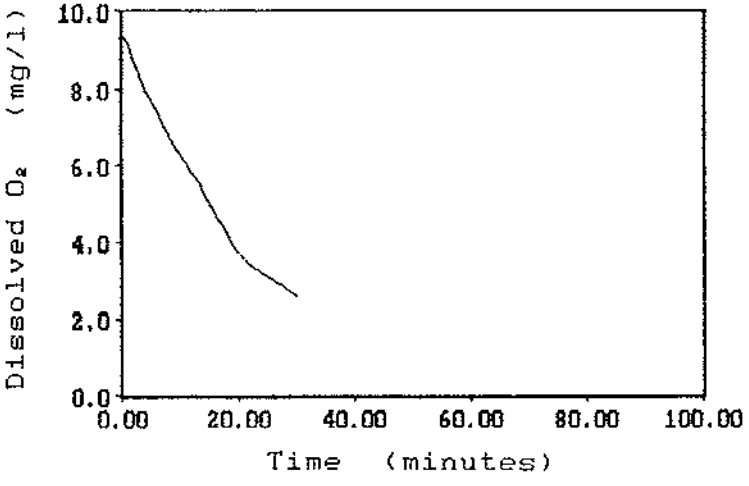


Figure A4.13: Chemostat 2, 125 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.28 mg/l/min.

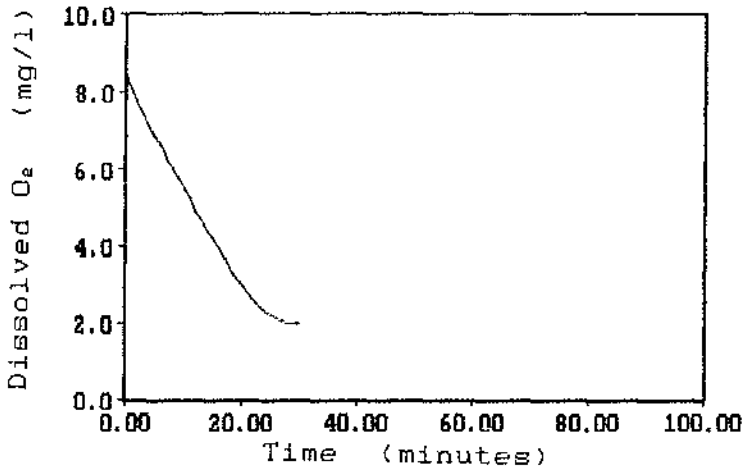


Figure A4.14: Chemostat 3, 125 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.27 mg/l/min.

Table A4.16 PCOC Chemostat 1, 150 mg/l

Date 4/9/91 150 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.555
Dry Memb. wt. (mg)	77.4	Feed conc mg/l	152.39
Dry Memb +cells(mg)	107	Effl Abs	0.172
		Effl conc mg/l	16.856
Dry Cells (mg)	29.6	Growth Yield	0.727984
MLSS (mg/L)	98.66666		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	9.4	22	3.3
1	8.9	24	2.9
2	8.6	26	2.6
3	8.3	28	2.2
4	8.2	30	2
5	7.9	32	1.8
6	7.7	34	1.6
7	7.3	37	1.4
8	7	40	1.4
9	6.7	43	1.4
10	6.4		
11	6.1		
12	5.9		
13	5.6		
14	5.3		
16	4.7		
18	4.4		
20	3.7		

Table A4.17 PCOC Chemostat 2, 150 mg/l

Date 27/9/91 150 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.542
Dry Memb. wt. (mg)	82.1	Feed conc mg/l	151.116
Dry Memb +cells(mg)	109.5	Effl Abs	0.166
		Effl conc mg/l	16.268
Dry Cells (mg)	27.4	Growth Yield	0.677305
MLSS (mg/L)	91.33333		

Time (min)	Diss Oxy	Time (min)	Diss Oxy
0	8.8	21	3.5
1	8.6	22	3.3
2	8.3	23	3.2
3	8.1	25	3
4	7.7	27	2.8
5	7.4	29	2.7
6	7.1	31	2.6
7	6.8	33	2.5
8	6.5	35	2.5
9	6.2		
10	5.9		
11	5.6		
12	5.3		
13	5.1		
14	4.8		
15	4.6		
16	4.3		
17	4.2		
18	4		
19	3.8		
20	3.7		

Table A4.18 PCOC Chemostat 3, 150 mg/l

Date 4/10/91 150 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.563
Dry Memb. wt. (mg)	76.9	Feed conc mg/l	153.174
Dry Memb +cells(mg)	105.3	Effl Abs	0.141
		Effl conc mg/l	13.818
Dry Cells (mg)	28.4	Growth Yield	0.679315
MLSS (mg/L)	94.66666		

Time (min)	Diss Oxy
0	9.5
1	9.2
2	8.9
3	8.6
4	8.2
5	7.8
6	7.5
7	7.2
8	6.8
9	6.4
10	6.1
11	5.8
12	5.5
13	5.2
14	5
15	4.7
16	4.5
17	4.3
18	4.2
19	4
20	3.8
22	3.5
24	3.2
26	3
28	2.8
30	2.7
32	2.6
34	2.5
36	2.4

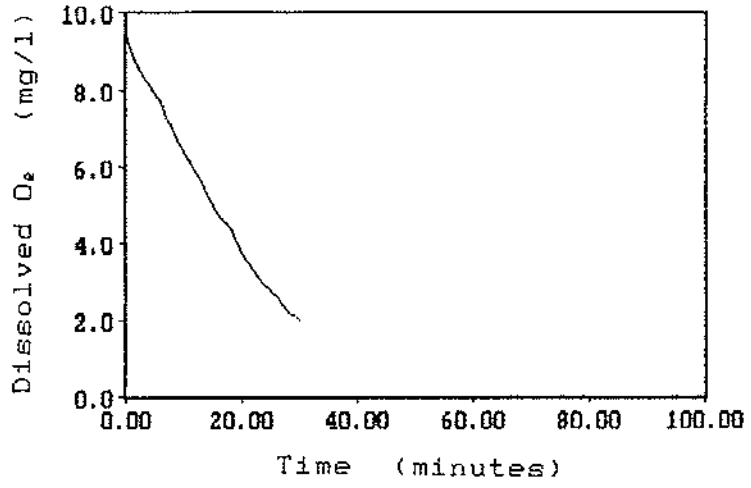


Figure A4.15: Chemostat 1, 150 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.28 mg/l/min.

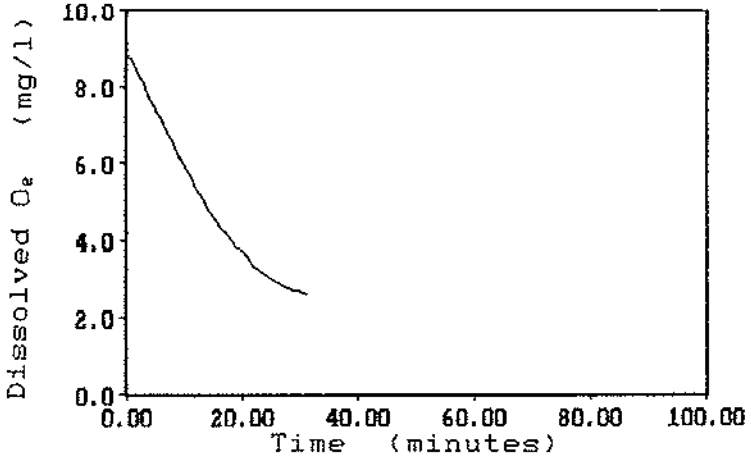


Figure A4.16: Chemostat 2, 150 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.30 mg/l/min.

Figure A4.17: Chemostat 3, 150 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.32 mg/l/min.

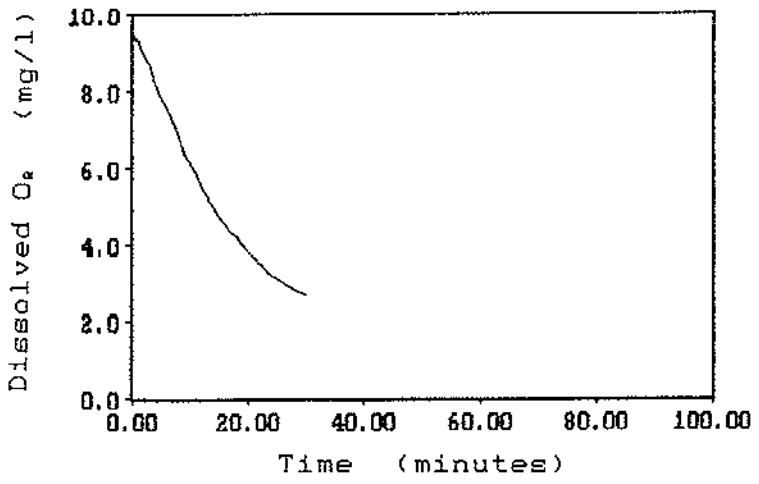


Table A4.19 PCOC Chemostat 1, 175 mg/l

Date 7/10/91 175 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.745
Dry Memb. wt. (mg)	78.6	Feed conc mg/l	171.01
Dry Memb +cells(mg)	109.7	Effl Abs	0.194
Dry Cells (mg)	31.1	Effl conc mg/l	19.012
MLSS (mg/L)	103.6666	Growth Yield	0.682026

Time (min)	Diss Oxy
0	8.7
1	8.3
2	8
3	7.8
4	7.4
5	7
6	6.6
7	6.3
8	5.9
9	5.6
10	5.4
11	5
12	4.7
13	4.5
14	4.3
15	4.1
16	3.8
17	3.7
18	3.5
19	3.3
20	3.2
22	2.9
24	2.7
26	2.6
28	2.5

Table A4.20 PCOC Chemostat 2, 175 mg/l

Date 9/10/91 175 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.724
Dry Memb. wt. (mg)	77.3	Feed conc mg/l	168.952
Dry Memb +cells(mg)	110.1	Effl Abs	0.162
		Effl conc mg/l	15.876
Dry Cells (mg)	32.8	Growth Yield	0.714242
MLSS (mg/L)	109.3333		

Time (min)	Diss Oxy
0	9.3
1	8.9
2	8.5
3	8.2
4	7.8
5	7.5
6	7.1
7	6.8
8	6.5
9	6.1
10	5.7
11	5.4
12	5.1
13	4.8
14	4.5
15	4.3
16	4
17	3.8
18	3.6
19	3.5
20	3.3
22	3.1
24	2.9
26	2.8

Table A4.21 PCOC Chemostat 3, 175 mg/l

Date 11/10/91 175 mg/l Feed

Sample Vol (mls)	300	Feed Abs	1.76
Dry Memb. wt. (mg)	76.8	Feed conc mg/l	172.48
Dry Memb +cells(mg)	106.5	Effl Abs	0.219
Dry Cells (mg)	29.7	Effl conc mg/l	21.462
MLSS (mg/L)	99	Growth Yield	0.655550

Time (min)	Diss Oxy
0	8.8
1	8.4
2	8.1
3	7.7
4	7.3
5	7
6	6.7
7	6.3
8	6
9	5.6
10	5.3
11	4.9
12	4.7
13	4.4
14	4.2
15	3.9
16	3.7
17	3.4
18	3.2
19	3
20	2.8
22	2.7
24	2.5
26	2.4

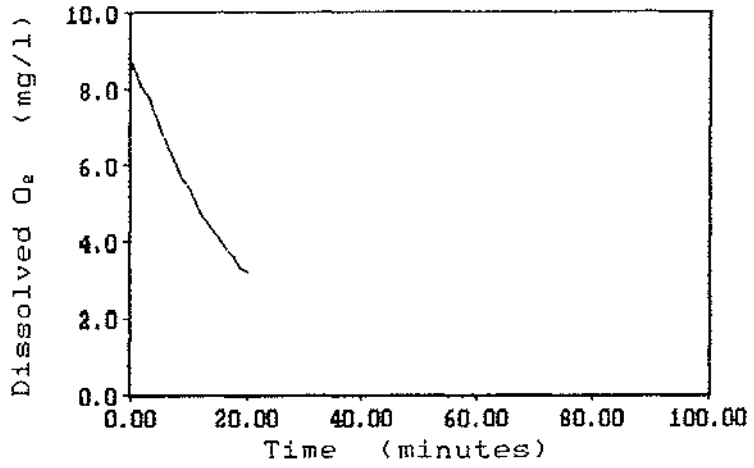


Figure A4.18: Chemostat 1, 175 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.33 mg/l/min.

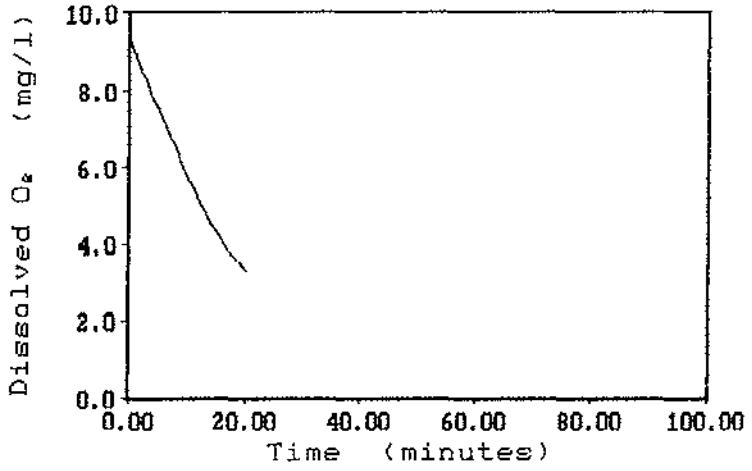
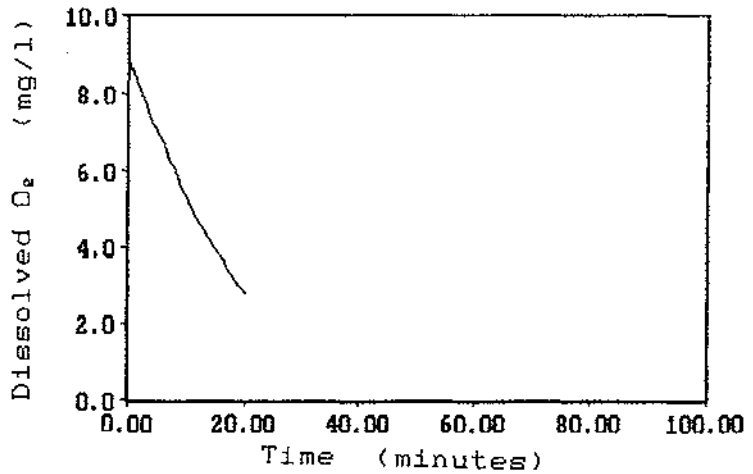


Figure A4.19: Chemostat 2, 175 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.34 mg/l/min.

Figure A4.20: Chemostat 3, 175 mg/l, Dissolved O_2 v Time -Slope (OUR) = 0.29 mg/l/min.



Appendix 5

'Method of Least Squares' Linear Regression

Table A5.1 2,4-D Batch Experiment. Yield v Substrate Regression Data.

PRINT C1 C2 C3 C4

Column Count Row	Substrate (mg/l)	Observed Yield	Yield Residuals	Predicted Yield
1	105	0.320	0.015	0.305
2	106	0.280	-0.025	0.305
3	225	0.290	0.018	0.272
4	314	0.230	-0.019	0.248
5	318	0.260	0.013	0.247
6	442	0.210	-0.002	0.212
7	664	0.160	0.009	0.151
8	632	0.150	-0.010	0.160

REGRESS 'Y' 1 'S'

The fitted equation is :
Y = 0.334 - 0.000*S

Column	Coeff	SD(Coeff)	t-value
constant	0.334	0.012	26.89
S	-2.750E-04	3.072E-05	-8.95

the residual standard deviation about the model is S = 0.017

with 8 - 2 = 6 degrees of freedom

R-squared = 93.04%

Analysis of Variance

Due to	SS	DF	MS=SS/DF
Regression	0.025	2	0.012
Residual	1.835E-03	6	3.058E-04
Total	0.026	8	

Table A5.2 PCOC Batch Experiment. Yield v Substrate Regression Data.

Column Count Row	Substrate (mg/l)	Observed Yield	Yield Residuals	Predicted Yield
1	27.500	0.877	-0.002	0.879
2	27.600	0.953	0.074	0.879
3	53.100	0.685	-0.050	0.735
4	53.200	0.660	-0.074	0.734
5	48.200	0.781	0.018	0.763
6	79.100	0.577	-0.012	0.589
7	78.700	0.613	0.022	0.591
8	71.100	0.622	-0.012	0.634
9	107.100	0.466	0.035	0.431

REGRESS 'Y' 1 'S'

The fitted equation is :
Y = 1.034 - 0.006*S

Column	Coeff	SD(Coeff)	t-value
constant	1.034	0.042	24.36
S	-5.629E-03	6.493E-04	-8.67

the residual standard deviation about the model is S = 0.048

with 9 - 2 = 7 degrees of freedom

R-squared = 91.48%

Analysis of Variance

Due to	SS	DF	MS=SS/DF
Regression	0.171	2	0.085
Residual	0.016	7	2.273E-03
Total	0.187	9	

Table A5.3 PCOC Chemostat Experiment. Yield v Substrate Regression Dat:

	Substrate (mg/l)	Observed Yield	Yield Residuals	Predicted Yield
1	3.900	0.800	0.024	0.776
2	7.800	0.790	0.038	0.752
3	7.000	0.690	-0.067	0.757
4	11.200	0.790	0.058	0.732
5	9.900	0.650	-0.090	0.740
6	11.000	0.720	-0.013	0.733
7	9.300	0.680	-0.063	0.743
8	10.000	0.790	0.051	0.739
9	8.300	0.800	0.051	0.749
10	14.000	0.790	0.075	0.715
11	12.700	0.710	-0.013	0.723
12	15.500	0.650	-0.056	0.706
13	15.800	0.740	0.036	0.704
14	13.700	0.720	0.003	0.717
15	16.900	0.730	0.032	0.698
16	16.300	0.680	-0.021	0.701
17	13.800	0.680	-0.036	0.716
18	19.000	0.680	-0.005	0.685
19	15.900	0.710	0.006	0.704
20	21.500	0.660	-0.010	0.670

REGRESS 'Y' 1 'S'

The fitted equation is :
Y = 0.799 - 0.006*S

Column	Coeff	SD(Coeff)	t-value
constant	0.799	0.033	24.06
S	-6.008E-03	2.484E-03	-2.42

the residual standard deviation about the model is S = 0.047

with 20 - 2 = 18 degrees of freedom

R-squared = 24.53%

Analysis of Variance

Due to	SS	DF	MS=SS/DF
Regression	0.013	2	6.577E-03
Residual	0.040	18	2.240E-03
Total	0.054	20	