BIODEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4-DICHLOROPHENOL

by

GREGORY L. SINTON

B. S., Colorado State University, 1984

A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

College of Engineering
Department of Chemical Engineering

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1987

Approved by:

Larry E. Erickson, Major Professor

LD
2668
.T4
CHE
1987
S56
c. 2

ACKNOWLEDGMENTS

A11207 309969

I wish to express my gratitude to Dr. Larry E. Erickson and Dr. L. T. Fan for their valuable guidance throughout the course of this work.

I would also like to thank Dr. Alexander P. Mathews for reviewing this manuscript and for serving on my committee and Dr. S. S. Yang for his assistance with statistics.

I also appreciate the financial support I recieved through the Chemical Engineering Department and the Dow fellowship.

This work was conducted under the sponsorship of the Engineering Experiment Station (Office of Hazardous Waste Research) of Kansas State University.

TABLE OF CONTENTS

page

ACKNOWLEDGEMENTS.......................................i

TABLE OF CONTENTS......................................ii

CHAPTER I. INTRODUCTION................................ 1.1

OVERVIEW OF CHAPTERS.................................. 1.3

REFERENCES.. 1.5

CHAPTER II. BACKGROUND AND LITERATURE REVIEW.......... 2.1

ORGANISMS CAPABLE OF DEGRADING 2,4-D................. 2.2

METABOLIC PATHWAYS.................................... 2.3

ENVIRONMENTAL FACTORS AFFECTING BIODEGRADATION...... 2.5

BIODEGRADATION MODELS................................. 2.13

REFERENCES.. 2.21

FIGURE.. 2.27

TABLE... 2.28

CHAPTER III. BIODEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4-DICHLOROPHENOL IN SHAKE FLASKS........... 3.1

MATERIALS AND METHODS................................. 3.1

RESULTS AND DISCUSSION................................ 3.4

CONCLUSIONS... 3.8

REFERENCES.. 3.9

FIGURES... 3.10

TABLES.. 3.24

CHAPTER IV. EFFECT OF pH ON 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4-DICHLOROPHENOL BIODEGRADATION.................. 4.1

THEORY.. 4.1

MATERIALS AND METHODS............................... 4.5

RESULTS AND DISCUSSION.............................. 4.9

CONCLUSIONS... 4.21

REFERENCES.. 4.23

NOMENCLATURE.. 4.24

FIGURES... 4.26

TABLES.. 4.54

CHAPTER V. RECOMMENDATIONS FOR FUTURE WORK............ 5.1

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY.............. 5.1

BIOMASS MEASUREMENT................................. 5.2

STERILIZATION....................................... 5.4

CULTURE MAINTENANCE................................. 5.5

REFERENCES.. 5.8

APPENDIX A. STANDARD CURVE DATA.......................A.1

APPENDIX B. PROGRAMS..................................B.1

CHAPTER I

INTRODUCTION

2,4-Dichlorophenoxyacetic acid (2,4-D) and other phenoxyalkanoic herbicides have been used as aquatic and terrestrial herbicides since the late 1940s. They are highly effective against selected broadleaf plants and have found applications in a wide variety of situations, from weed control in lawns, gardens, cereal crops and pastures to defoliants in forestry and warfare. In the early 1980s U.S. production of 2,4-D alone was almost 13 million pounds per year (1). The fact that large quantities of 2,4-D and related phenoxyalkanoic herbicides are manufactured and applied each year indicates the importance of having effective means of treating production wastes and a thorough understanding of the fate of these chemicals in the environment.

The study of the biodegradation of phenoxyalkanoic herbicides and their metabolites is essential not only because of their own ubiquity, but also because of the structural similarity between these and numerous other toxic compounds currently of interest, such as other halogenated aromatic and phenolic compounds (2). Many of these compounds have been found to be highly recalcitrant under

certain conditions, and some, e.g., dioxins, possess extreme toxicity. In addition, 2,4-D is among 38 compounds that the U.S. Environmental Protection Agency (EPA) has proposed to add to a list of chemicals used for identifying wastes as hazardous and appropriate for management under the Resource Conservation and Recovery Act (3).

The increasing amount of hazardous and toxic waste legislation emphasizes the need for quantifiable hazard assessments that can be used to set and enforce environmental standards. Environmental persistence is a fundamental feature to consider in assessing the potential hazard of a given compound. Since biodegradation is often a major factor determining persistence, appropriate models for estimating environmental biodegradation rates must be developed. As part of the hazard assessment, the EPA has been directed to identify the most dangerous existing toxic waste sites and target them for highest priority clean-up efforts. The EPA has estimated that some 2500 sites may be identified as priority sites (4). Since many of these sites are the results of traditional waste treatment methods, it is clear that new waste treatment alternatives are needed both for cleaning old sites and phasing out unacceptable methods. Acceptable treatment methods will also have to be developed to treat newly regulated compounds.

The primary goal of this research is to examine the effect of pH on the rate of 2,4-D and 2,4-DCP biodegradation. The forms of 2,4-D biodegradation models and the values of their kinetic parameters need to be clarified to understand the mechanism of 2,4-D biodegradation under a variety of conditions. An understanding of the effects of pH is essential for the design and evaluation of biological treatment options to eliminate production wastes and to manage biodegradation in field applications. The pH should also be considered in assessing environmental persistence of 2,4-D and in determining if undesirable metabolic products are produced as a result of 2,4-D biodegradation.

OVERVIEW OF CHAPTERS

Chapter 2 provides background information on the biodegradation of 2,4-D and related compounds. The literature is reviewed to present models for the biodegradation process and to identify the significant environmental factors influencing the biodegradation of 2,4-D.

A preliminary study of 2,4-D biodegradation by Pseudomonas sp. NCIB 9340 in 250 mℓ shake flasks is presented in Chapter 3. The effects of different 2,4-D

concentrations on 2,4-D biodegradation are reported. The techniques used for the analysis of substrate, products, and biomass concentrations throughout all of the experimental work are also described in Chapter 3.

The biodegradation of 2,4-D and 2,4-DCP in one and two liter fermenters is discussed in Chapter 4. The effects of pH on biodegradation rate and product formation are examined. Estimates for the maximum specific growth rate and biomass and available electron yield are also presented.

Chapter 5 discusses some of the problems encountered in the present research and identifies possible improvements in the experimental procedure. The four topics that are examined are high performance liquid chromatography, biomass measurement, sterilization, and culture maintenance.

REFERENECES

1. United States Department of Agriculture, Agricultural Statistics, United States Printing Office, Washington D.C., p 430 (1983).

2. Peyton, T. O., Enzyme Microb. Technol., 6, 146-154 (1984).

3. Rich, L. A., Chemical Week, 139, 26-27 (July 2, 1986).

4. Boraiko, A. A., National Geographic, 167, 318-351 (1985).

CHAPTER II

BACKGROUND AND LITERATURE REVIEW

In this chapter the literature is reviewed to provide a background for research on the biodegradation of 2,4-D and related compounds. Basic chemical and physical data for 2,4-D are not included but can be found elsewhere (1,2). The initial topics covered are the organisms capable of degrading 2,4-D and the most commonly observed pathways of 2,4-D metabolism. Environmental factors affecting 2,4-D biodegradation rates including pH, temperature, aeration, supplemental nutrient supplies, culture enrichment, acclimation, and substrate concentration are discussed. A major portion of the chapter is directed toward the various models proposed to describe the biodegradation of 2,4-D and similar compounds. These models include traditional growth associated models such as the Monod and Haldane expressions as well as other approaches including physicochemical parameter correlations and the consideration of cometabolic degradation of substrates. Finally, several studies involving cell immobilization are briefly discussed.

ORGANISMS CAPABLE OF DEGRADING 2,4-D

A large variety of microorganisms have been isolated that are capable of degrading 2,4-D and related compounds (3-42). The most commonly cited 2,4-D degrading genera are Pseudomonas, Alcaligenes, and Arthrobacter. Organisms found to degrade one phenoxyacetic acid can often degrade related compounds such as 2-methyl-4-chloro phenoxyacetic acid (MCPA) and 2,4,5-trichlorophenoxyacetic acid (2,4,5-T).

The capacity of some organisms to degrade 2,4-D has been linked to the presence of certain plasmids, many of which have now been well characterized (9,11,43). Don and Pemberton (10) have found that these plasmids can be transferred by conjugation between various species of bacteria; however, among the organisms studied the 2,4-D degrading capability is only expressed in Alcaligenes eutrophus, A. paradoxus and P. putida. A recent review by Ghosal et al. (43) describes the current understanding of the genetic mechanisms involved in the biodegradation of 2,4-D and related halogenated compounds.

Past studies of 2,4-D biodegradation often employed mixed cultures. Most of these cultures were obtained from river water, pond water, soil, or domestic or industrial wastewater sludge. Whenever dominant genera have been mentioned for mixed cultures they have been among the genera known to degrade 2,4-D in pure culture (44,45).

METABOLIC PATHWAYS

The majority of the works on metabolic pathways for 2,4-D biodegradation have been carried out with aerobic cultures of Arthrobacter and Pseudomonas species (30,46). Primarily through the works of the groups led by Alexander (12-20) and Evans (33-37,47-50) the pathways for these organisms, known to be among the dominant species involved in 2,4-D degradation, have been well established. These basic pathways are presented in Fig. 2.1.

In general, all the aerobic pathways observed involve the removal of the acetic acid side chain followed by ortho hydroxylation of the phenol to produce a catechol. The catechols then undergo ortho cleavage of the aromatic ring to yield a muconic acid. The end product of the pathway shown in Fig. 2.1 is succinic acid, which can readily be used in the citric acid cycle.

The main variations reported in these aerobic pathways involve chlorine removal. As indicated by Fig. 2.1, the chlorine in position 4 can be removed from either 2,4-D, 2,4-dichlorphenol (2,4-DCP), or 3,5-dichlorocatechol. Chlorine removal before ring cleavage has been observed in Arthrobacter (51), Pseudomonas (33), and Nocardia species (52), as well as in the fungus Aspergillus niger (23). Early studies with A. niger indicated that the fungus could

hydroxylate 2,4-D, but further degradation was not observed (24-26). In a more recent study, A. niger has been found to degrade 2,4-D completely and use it as its sole source of carbon and energy (23).

Recent studies have indicated that 2,4-D and related halogenated aromatics can also be degraded under anaerobic conditions. Suflita et al. (53) have detected anaerobic degradation of 2,4,5-T to yield 2,5-dichlorophenoxyacetic acid; no further degradation was observed. Gibson and Suflita (54) observed complete degradation of 2,4-D and 2,4,5-T in three different anaerobic environments; pond sediment, sewage sludge, and a methanogenic aquifer. 2,4-D and other halogenated aromatics tested in this study were not significantly degraded under sulfate-reducing conditions. Using 2,4,5-T as a test substrate it was determined that the lack of degradation was at least partially due to inhibitory effects of sulfate rather than a lack of metabolic potential. Based on observations of initial product formation Gibson and Suflita (54) have proposed that methanogenic degradation of phenoxyacetates, e.g. 2,4-D, involves removal of the side chain followed by reductive dehalogenation to yield phenol before eventual degradation to methane and carbon dioxide.

ENVIRONMENTAL FACTORS AFFECTING BIODEGRADATION

Various environmental parameters affect biodegradation rates. Significant among them are pH, temperature, aeration, substrate form and concentration, supplemental nutrients and culture adaptation or acclimation to the substrate. While the effects of these parameters are organism specific, some generalizations can be made. When pH and temperature can be controlled readily, these parameters can be maintained at levels determined to be optimal for the particular organisms involved. In many cases these conditions may vary from substrate to substrate, being different from those determined using 'standard' substrates, e.g., glucose. This situation can arise due to the effects of pH and temperature on properties such as ionization state, solubility, and vapor pressure, which can affect the observed rate of substrate disappearance. In aqueous cultures, temperatures in the lower mesophilic range have been found to be the most favorable for the biodegradation of 2,4-D and related compounds. In experiments with lake water DeMarco _et al_. (55) found 2,4-D degradation to be faster in the 22-26 C range than at cooler temperatures. Tyler and Finn (40) determined the optimum temperature to be 25 C for the degradation of 2,4-D and 2,4-DCP by _Pseudomonas_ sp. NCIB 9340 but not for other substrates such as glucose and succinate. The results of

Nesbit and Watson (56), who have suggested an optimum of 21 to 25 C based on their river water studies, provide further support for this optimum temperature range.

Tyler and Finn (40) also studied the effect of pH on growth rate. They have found that the optimum pH range for 2,4-DCP degradation (7.1-7.8) is significantly higher than the range for 2,4-D (6.2-6.9). They have hypothesized that this shift can be at least partially explained by the higher dissociation constant of 2,4-D, which results in a lower concentration of the conjugate acid form of 2,4-D than that of 2,4-DCP at a given pH. The conjugate acid forms are believed to be more readily transported across the cell membrane and thus are the primary source of both metabolic and inhibitory activities. In aqueous mixed cultures from peatlands, Williams and Crawford (57) have found a lower pH optimum around 5.5 for 2,4-D degradation.

Since the biodegradation of 2,4-D and related compounds is often observed to be an aerobic process, oxygen supply can be a significant factor in determining biodegradation rates. A number of studies, such as those of DeMarco et al.(55) and Williams and Crawford (57) have shown that increasing oxygen supplies can lead to an increase in the rate of 2,4-D biodegradation. These studies do not appear to show the full extent or limits of this effect. Shaler and Klecka (58) have found 2,4-D biodegradation to be a

hyperbolic function of dissolved oxygen concentration. They estimated the oxygen half saturation constant to be 1.2 mg/L. While they observed little increase in the 2,4-D biodegradation rate as the dissolved oxygen level was increased above 2.0 mg/L, a significant reduction in biodegradation rate occurred at dissolved oxygen concentrations less than 1.0 mg/L.

Numerous researchers have investigated the effects of supplemental nutrients on the biodegradation of xenobiotic compounds such as 2,4-D. Nutrient supplementation could be especially useful for situations where the target substrate is present in concentrations too low to develop sufficient biomass for rapid degradation. In an early study by Schwartz (59), the addition of a nutrient broth to mixed cultures growing on minimal salts and 2,4-D was found to have no effect on the degradation rate. In contrast, most other studies have indicated that the degradation can be stimulated by a variety of supplemental nutrients under both natural and artificial conditions. Nesbitt and Watson (56) found that their river water samples were deficient in both nitrogen and phosphorus. In order for maximum degradation rates to be achieved, additional sources of both of these elements were needed. Results similar to those of Nesbitt and Watson (56) were observed by Williams and Crawford (57) for peatland cultures.

In several experiments with supplemental nutrients 2,4-D has been observed to be degraded concurrently with nutrient supplements. Kim and Maier (60) observed concurrent degradation of nutrient broth and 2,4-D. They found that with initial 2,4-D concentrations of 100 or 10 mg/L the time for 2,4-D degradation was significantly reduced by the addition of nutrient broth. At lower initial 2,4-D concentrations this effect was less evident. At the lowest initial 2,4-D concentration tested, 0.14 mg/L, the addition of nutrient broth appeared to retard degradation. The rate of nutrient broth consumption was slightly reduced by the presence of 2,4-D. Papanastasiou and Maier (44,45) found that glucose and 2,4-D are mutually inhibitory. Although glucose slows the cellular rate of 2,4-D metabolism, the effect can be overcome resulting in an increase in the 2,4-D degradation rate due to rapidly increasing biomass concentrations caused by the utilization of glucose.

In addition to the basic nutrient requirements, such as sources of carbon, nitrogen and phosphorous, trace requirements are essential. Careful consideration of these nutrients may be especially important in anaerobic systems where relatively little is known about trace requirements. Speece (61) has suggested that inadequate supplies of trace elements, such as iron, cobalt, nickel and sulphide, might

have given rise to negative results in numerous anaerobic treatability studies.

It has been demonstrated that biodegradation rates of 2,4-D and other xenobiotic compounds can be greatly increased if enrichment cultures are formed by repeated exposure of the organisms to these compounds (30,32,62, 60). Exposure of cultures to substrates like 2,4-D for the first time results in a lag phase of limited utilization followed by relatively rapid degradation. Such a lag phase essentially disappears and biodegradation rates are enhanced in subsequent exposures. Hemmett and Faust (62) observed a maximum degree of acclimation occurred after 60 days of repeated exposure.

Two mechanisms generally discussed in connection with adaptation during enrichment are induction and mutation. Two observations have led Loos (30) to suggest that induction is probably the more significant of these two mechanisms. First, for a given sample of soil, independent adaptation experiments resulted in the same dominant organisms in the final cultures. Second the length of the lag period was observed to be approximately constant; if mutation were responsible, a widely varying lag period corresponding to a stochastic genetic event would be expected.

Among other factors that may be important in determining lag times are initial biomass concentration,

plasmid availability and transfer rates, and substrate concentration (54,60). If the initial biomass concentration is small, part of the observed lag could be the time required to sufficiently increase the microbial population to a level where significant degradation is possible. This factor could be one reason for the observation that 2,4-D and other phenoxyalkanoic acids are generally degraded more rapidly in soils than in aqueous environments (59,62). The previously mentioned role of plasmids in xenobiotic biodegradation suggests that they could also be a factor in the lag phase duration. If the transfer of plasmids between organisms occurs at a relatively constant rate, a consistent lag phase duration could be observed. Several researchers have noted that the duration of the lag period is dependant on the initial concentration of the xenobiotic substrate (40,63). Parker and Doxtader (63) studied 2,4-D biodegradation in soil and determined that as the initial substrate concentration was increased the duration of the lag phase of slow degradation increased linearly.

When acclimatized cultures were used to seed new cultures, relatively short lag periods were observed (40, 62). This lag appears to be at least partially due to factors such as osmotic shock, but the xenobiotic concentration used in acclimation also appears to be a factor. Tyler and Finn (40) observed that the transfer of

inocula from chemostats with higher xenobiotic concentrations reduces subsequent lag times.

The range of substrate concentrations to be examined is an important consideration when attempting to model biodegradation. The entire mechanism of biodegradation can change for different concentration ranges. Significant errors manifest themselves when attempts are made to extrapolate results to concentration ranges outside those used to develop the models. In mixed cultures this can be caused by the dominance of different organisms at different concentrations, as briefly discussed below (64).

At very low concentration levels, a threshold may be reached below which very little substrate is utilized. This could occur, for example, if insufficient substrate is present to induce the proper enzyme systems or if the supply is inadequate for organism maintenance. At slightly higher concentrations, e.g., less than about 1 μg carbon per milliliter, oligotrophic organisms with high affinity for the substrate may dominate. At still higher concentrations, oligotrophic organisms may be killed or inhibited by the substrate and eutrophic organisms may dominate. Eventually, as xenobiotic concentrations are increased further, degradation by eutrophic organisms may become inhibited.

In pure culture the situation is simplified to two basic concerns: the levels of substrate and nutrients necessary to be above the threshold level and the level at which inhibition occurs. The threshold levels are generally very low, sometimes below detectable levels. Often, therefore, thresholds do not prevent compounds from being degraded to acceptably low concentrations. Inhibition is generally a more prevalent concern since the xenobiotics involved are often very toxic.

BIODEGRADATION MODELS

A number of different studies have been conducted in attempts to develop kinetic models for the biodegradation of 2,4-D and related compounds. The results of these studies have given rise to various biodegradation models, some of which appear to yield conflicting results. Part of the diversity among the proposed models can be attributed to variability of conditions, e.g., pH , as previously discussed.

Many of the proposed models do not account for any inhibition effects (40,62,65-68,69). Most of these efforts can be described based on the Monod model:

$$-d[S]/dt = \mu_m[S][X]/\{Y_s(K_s + [S])\} \qquad (1)$$

Some of the values that have been reported for the parameters in equation (1) are given in Table 2.1. When experiments are performed over relatively short time intervals, the biomass concentration is sometimes considered constant. This assumption leads to the so-called pseudo-zero and pseudo-first order forms for large and small substrate concentrations respectively. Several researchers have resorted to a zero-order model to describe xenobiotic degradation (62,66). This form has generally not provided

an accurate description of observed degradation rates. The zero order model is a simplification of the Monod model that applies only at higher substrate concentrations. One reason for poor results may be inhibition effects.

The first order dependence on substrate concentration appears to be a useful form of the Monod model (65,68,70). Though limited to applications at low concentrations, this model provides valid descriptions of biodegradation rates for a variety of xenobiotic compounds. Paris et al. (65) studied the biodegradation of the butoxyethyl ester of 2,4-D in natural waters at concentrations from 0.1 to 1.0 mg/L. The culture time was very short (5 hours) and thus the biomass concentration was again considered constant and pseudo-first order rate constants, K[X], were determined.

The complete Monod model given by eq. (1) sometimes fails to provide an accurate description of the biodegradation of 2,4-D and related compounds (40,66,67), presumably because of inhibition effects. Tyler and Finn (40) have reported that this model accurately describes growth on 2,4-D up to 2000 mg/L, but fails to describe growth on 2,4-DCP above 25 mg/L.

Considerable uncertainty exists as to where and if 2,4-D itself is actually inhibitory. Some researchers have reported inhibitory effects for 2,4-D at levels such as

35mg/L and 40 μg/g-soil (44,45,63). Others have successfully employed versions of the Monod model that completely neglect inhibition effects. The uncertainty about 2,4-D inhibition may be caused in part by the inhibitory effects of one or more metabolic products, e.g., 2,4-DCP. Most of the inhibition models reviewed are based on inhibition caused by the original substrate. Consideration of the inhibitory effects of the degradation products including 2,4-DCP may improve resultant models. This could involve product inhibition or substrate inhibition in steps other than the original one. In any case, possible interaction of the various substrates and products should be taken into account.

As noted earlier, 2,4-DCP is generally accepted as the first product in the 2,4-D biodegradation pathway. The notion that 2,4-DCP may be responsible for the inhibition associated with 2,4-D biodegradation is supported by the data of Tyler and Finn (40), indicating that 2,4-DCP induces inhibitory effects at concentrations as low as 25 mg/L. Other researchers have also found 2,4-DCP to be inhibitory at low concentrations. Beltrame et al. (71) reported that microbial phenol utilization is reduced by 50 % by the presence of 47.6 mg/L 2,4-DCP, and Liu et al. (72) observed a 50 % reduction in bacterial dehydrogenase activity at 2,4-DCP levels of 75 mg/L.

The Haldane model is expressed as

$$-d[S]/dt = [X][S]k_1/(Y_s(k_2 + [S] + k_3[S]^2)) \qquad (2)$$

This model appears to be the most promising model for the description of inhibitory substrate degradation. The results of Papanastasiou and Maier (45), indicate a good fit for growth on 2,4-D using $k_1 = 0.15$, $k_2 = 40$, and $k_3 = 1/31$. In contrast, the Haldane model does not appear to provide an accurate description of 2,4-DCP or 2,4-D biodegradation for the experimental results of Tyler and Finn (40); however, numerous researchers besides Papanastasiou and Maier have recommended the Haldane model based on studies with inhibitory substrates other than 2,4-D; these substrates include phenols and benzoate (73-75). Edwards (74) compared the Haldane model to four other models. By studying the inhibition data of eight different substrates, Edwards has concluded that the Haldane model provides the best overall fit. Pawlowsky and Howell (75) examined the same five models using phenol as the substrate. They have determined that the fit is satisfactory with all five models and that the differences among them are not statistically significant. Thus, they have concluded that the Haldane

model is preferable on the basis of its relative simplicity. According to Sokol and Howell (73), the Haldane model still provides an accurate description of the degradation even when it is simplified to the following form;

$$-d[S]/dt = [X][S]k_4/(Y_s(k_5 + [S]^2)) \qquad (3)$$

In mixed cultures different organisms may exhibit the dominant mode of degradation in distinct substrate concentration ranges. This may have been the case in the work of Parker and Doxtader (63) with a mixed culture in soil. Their data indicate two separate peaks of biodegradation activity. This led them to propose that one organism or enzyme system, system 1, is active only at concentrations less than 10 μg 2,4-D/g-soil, while another, system 2, is active over the entire range of concentrations. To model the cumulative effect, separate models were developed for each system and then added together. A model equivalent to the Haldane model was used to describe system 2.

The models discussed so far generally relate the substrate concentration to the growth rate. Difficulties materialize when the substrates are degraded by cometabolism (64,68,76-82) Microorganisms cometabolising substrates

convert them to organic products without obtaining a significant amount of carbon or energy from the degradation. Since no growth appears to be associated with cometabolic substrate utilization, models relating substrate concentration to growth rate are not applicable. Schmidt et al. (77) have modeled the biodegradation of organic compounds not supporting growth. They found that the kinetics of mineralization at concentrations too low to support growth are best described by a first-order model or by kinetic expressions of the metabolising population based on other growth supporting substrates. Venkataramani and Ahlert (83) have proposed a biodegradation model incorporating cometabolism coupled with cellular maintenance based on the Haldane expression to account for substrate inhibition. Hsieh and Wang (84) have developed a kinetic model for microbial cooxidation based on the Monod model with the addition of a maintenance term.

Physicochemical parameter models can be applied to either growth associated or cometabolic substrate degradation. Banerjee et al. (68) have developed a fairly successful model correlating the octanol-water partition coefficient to biodegradation rates. Other physicochemical parameters that have been used to predict biodegradation rates include the van der Waal's radii, Taff's steric parameter, hydrophobic parameters, Hammetts substituent

constant, the Swain and Lupton field factor, molecular connectivities, and atomic charge difference (85,86). Though the various physicochemical parameter models do provide correlations enabling rates to be estimated, the validity of any proposed mechanisms is difficult to assess because the mathematical forms of the models are often the common first and second order relations that arise from many different mechanisms.

Due to the relatively slow growth rate of microorganisms on most xenobiotic substrates, the biomass concentration in continuous free-cell systems is limited to low levels. To increase the overall biodegradation rates, methods for increasing the biomass concentrations need to be developed. Two such methods are immobilization of cells in polymer beads such as alginate, polyacrylamide hydrazide and polyurethane (87,88), and adsorption of microorganisms to support particles such as activated carbon and polyurethane (88,89).

The immobilization of cells can have additional advantages besides increasing biomass concentrations. Rehm et al (87,89) have found that while free-cell cultures can not tolerate phenol concentrations above 1.5 g/L, polymer entrapped organisms are sheltered and can degrade phenol at bulk concentrations up to 3 g/L. Anselmo _et al_. (88) observed degradation of phenol by polyurethane entrapped

cells of <u>Fusarium</u> flocciferum at concentrations up to 4 g/L. Organisms adsorbed to activated carbon can survive temporary concentrations up to 15 g/L. Thus, these forms of immobilization appear to provide protection against substrate toxicity as well as providing a means for increasing biomass concentration. As more complex treatment methods, such as cell immobilization, are developed, kinetic models for free-cell systems will need to be extended to account for additional factors such as diffusion through polymer beads and biofilms and different reactor configurations.

REFERENCES

1. Hee, Shane S. Que and Sutherland, Ronald G., The Phenoxyalkanoic Herbicides Volume I: Chemistry, Analysis, and Environmental Pollution, CRC Press, Boca Raton, Florida (1981).

2. Buchel, K. H. (ed.), Chemistry of Pesticides, John Wiley and Sons, New York (1983).

3. Sinton, G. L., Fan, L. T., Erickson, L. E., and Lee, S. M., Enzyme Microb. Technol., 8, 395-403 (1986).

4. Steenson, T. I. and Walker, N., J. Gen. Microbiol., 18, 692-697 (1958).

5. Steenson, T. I. and Walker, N., J. Plant Soil, 8, 17-32 (1956).

6. Steenson, T. I. and Walker, N., J. Gen. Microbiol., 16, 146-155 (1957).

7. Bell, G. R., Can. J. Microbiol., 3, 821-840 (1957).

8. Bell, G. R., Can. J. Microbiol., 6, 325-337 (1960).

9. Friedrich, B., Meyer, M. and Schlegal, H. G., Arch. Microbiol., 134, 92-97 (1983).

10. Don, R. H. and Pemberton, J. M., J. Bacteriol., 145, 681-686 (1981).

11. Fisher, P. R., Appleton, J. and Pemberton, J. M., J. Bacteriol., 135, 798-804 (1978).

12. Duxbury, J. M., Tiedje, J. M., Alexander, M. and Dawson, J. E., J. Agr. Food Chem., 18, 199-201 (1970).

13. Tiedje, J. M., Duxbury, J. M., Alexander, M. and Dawson, J. E., J. Agr. Food Chem., 17, 1021-1026 (1969).

14. Bollag, J. M., Helling, C. S. and Alexander, M., J. Agr. Food Chem., 16, 826-828 (1968).

15. Bollag, J. M., Briggs, G. G., Dawson, J. E. and Alexander, M., J. Agr. Food Chem., 16, 829-833 (1968).

16. Helling, C. S., Bollag, J. M., and Dawson, J. E., J. Agr. Food Chem., 16, 538-539 (1968).

17. Loos, M. A., Roberts, R. N. and Alexander, M., Can. J. Microbiol., 13, 679-689 (1967).

18. Loos, M. A., Roberts, R. N. and Alexander, M., Can. J. Microbiol., 13, 691-698 (1967).

19. Loos, M. A., Bollag, J. M. and Alexander, M., J. Agr. Food Chem., 15, 858-860 (1967).

20. Bollag, J. M., Helling, C. S. and Alexander, M., Appl. Microbiol., 15, 1393-1398 (1967).

21. Tiedje, J. M. and Alexander, M., J. Agr. Food Chem., 17, 1080-1084 (1969).

22. Audus, L. J., Nature, 166, 356 (1950).

23. Shailubhai, K., Sahasrabudhe, S. R., Vora, K. A. and Modi, V. V., FEMS Microbiol. Lett., 18, 279-282 (1983).

24. Faulkner, J. K. and Woodcock, D., J. Chem. Soc., 1187-1191 (1961).

25. Faulkner, J. K. and Woodcock, D., J. Chem. Soc., 5397-5400 (1965).

26. Clifford, D. R. and Woodcock, D., Nature, no. 4946, 763 (1964).

27. Rogoff, M. H. and Reid, J. J., J. Bacteriol., 71, 303-307 (1956).

28. Jensen, H. L., Petersen, H. I., Nature, 170, 39-40 (1952).

29. Jensen, H. L., Petersen, H. I., Acta Agr. Scand., 2, 215-231 (1952).

30. Loos, M. A. in Herbicides: Chemistry, Degradation, and Mode of Action (Keaney, P. C. and Kaufman, D. D. eds.), Vol. 1, 2nd ed., Marcel-Dekker, Inc., New York, 1-56 (1975).

31. Walker, R. L., Newman, A. S., Appl. Microbiol., 4, 201-206 (1956).

32. Audus, L. J. in The Physiology and Biochemistry of Herbicides, Academic Press, New York, 1963-189 (1965).

33. Evans, W. C., Smith, B. S. W., Fernley, H. N. and Davies, J. I., Biochem. J., 122, 543-551 (1971).

34. Gaunt, J. K. and Evans, W. C., Biochem. J., 122, 519-526 (1971).

35. Gaunt, J. K. and Gamar, Y., Biochem. J., 122, 527-531 (1961).

36. Gaunt, J. K. and Evans, W. C., Biochem. J., 122, 533-542 (1971).

37. Gaunt, J. K. and Evans, W. C., Biochem. J., 79, 25P-26P (1961).

38. Kilpi, S., Backstrom, V. and Korhola, M., FEMS Microbiol. Lett., 18, 1-5 (1983).

39. Kilpi, S., Backstrom, V. and Korhola, M., FEMS Microbiol. Lett., 8, 177-182 (1980).

40. Tyler, J. E. and Finn, R. K., Appl. Microbiol., 28, 181-184 (1974).

41. Kilbane, J. J., Chatterjee, D. K., Karns, J. S., Kellogg, S. T. and Chakrabarty, A. M., Appl. Environ. Microbiol., 44, 72-78 (1982).

42. Kilpi, S., Microbio. Ecol., 6, 261-270.

43. Ghosal, D., You, I. S., Chatterjee, D. K. and Chakrabarty, A. M., Science, 228, 135-142 (1985).

44. Papanastasiou, A. C. and Maier, W. J., Biotechnol. Bioeng., 25, 2337-2346 (1983).

45. Papanastasiou, A. C. and Maier, W. J., Biotechnol. Bioeng., 24, 2001-2011 (1982).

46. Cripps, R. E. and Roberts, T. R., in Pesticide Microbiology (Hill, I. R. and Wright, S. L. J. eds) Academic Press, New York, 670-677 (1978).

47. Evans, W. C. and Smith, B. S. W., Biochem. J., 57, XXX (1954).

48. Evans, W. C. and Moss, P., Biochem. J., 65, 8P (1957).

49. Evans, W. C., Smith, B. S. W., Moss, P. and Fernley, H. N., Biochem. J., 122, 509 (1971).

50. Evans, W. C. and Fernley, H. N., Biochem. J., 73, 22P (1959).

51. Ruisinger, S., Klages, U., and Lingens, F., Arch. Microbiol., 110, 253-256 (1959).

52. Klages, U. and Lingens, F., FEMS Microbiol. Lett., 6, 201-203 (1979).

53. Suflita, J. M., Stout, J. and Tiedje, J. M., J. Agric. Food Chem., 32, 218-221 (1984).

54. Gibson, S. A. and Suflita, J. M. Appl. Environ. Microbiol., 52, 681-688 (1986).

55. DeMarco, J., Symons, J. M. and Robeck, G. G., Am. Water Works Assoc., 59, 965-976 (1967).

56. Nesbitt, H . J. and Watson, J. R., Water Res., 14, 1689-1694 (1980).

57. Williams, R. T. and Crawford, R. L., Can. J. Microbiol., 29, 1430-1437 (1983).

58. Shaler, T. A. and Klecka, G. M., Appl. Environ. Microbiol., 51, 950-955 (1986).

59. Schwartz, H. G., J. Water Pollut. Control. Fed., 39, 1701-1714 (1967).

60. Kim, C. J., Maier, W. J., J. Water Pollut. Control Fed., 58, 157-164 (1986).

61. Speece, R. E., Environ. Sci. Technol., 17, 416A-427A (1983).

62. Hemmett, R. and Faust, S., Res. Review, 29, 191-206 (1969).

63. Parker, L. W. and Doxtader, K. G., J. Environ. Qual., 11, 679-684 (1982).

64. Alexander, M., Environ. Sci. Technol., 18, 106-111 (1985).

65. Paris, D. F., Steen, W. C., Baughman, G. L. and Barnett, J. T. Jr., Appl. Environ. Microbiol., 41, 603-609 (1981).

66. Salerno, S., McMullen, N., Lewandowski, G., "Biodegradation of Toxic Substances with Commercial Preparations", Summer National Meeting of AIChE, Philadelphia, Aug. 19-22 (1984).

67. Lewandowski, G., Baltzis, B. and Varuntanya, C., "Kinetics of Biodegradation of Toxic Organic Compound", Summer National Meeting of AIChE, Philadelphia, Aug. 19-22 (1984).

68. Banerjee, S., Howard, P. H., Rosenberg, A. M., Dombrowski, A. E., Sikka, H. and Tullis, D. L., Environ. Sci. Technol., 18, 416-422 (1984).

69. Shamat, N. A., Maier, W. J., J. Water Pollut. Control Fed., 52, 2158-2166 (1980).

70. Paris, D. F., Lewis, D. L., Barnett, J. T., Jr., and Baughman, G., "Microbial Degradation and Accumulation of Pesticides in Aquatic Systems"; U.S. Environmental Protection Agency, Washington D.C. EPA-660/3-75007 (1975).

71. Beltrame, P., Beltrame, P. L. and Carniti, P., Chemosphere, 13, 3-9 (1984).

72. Liu, D., Thomson, K. and Kaiser, K.L.E., Bull. Environm. Contam. Toxicl., 29, 130-136 (1982).

73. Sokol, W. and Howell, J. A., Biotechnol. Bioeng., 23, 2039-2049 (1981).

74. Edwards, V. H., Biotechnol. Bioeng., 12, 679-712 (1970).

75. Pawlowsky, U., and Howell, J. A., Biotechnol. Bioeng., 15, 889-896 (1973).

76. Wang, Y. S., Subba-Rao, R. V. and Alexander, M., Appl. Environ. Microbiol., 47, 1195-1200 (1984).

77. Schmidt, S. K., Simkins, S. and Alexander, M., Appl. Environ. Microbiol., 50, 323-331 (1985).

78. Alexander, M. in Microbial Degradation of Pollutants in Marine Environments, U.S. EPA, Washington D.C., EPA-600/9-79-012 (1979).

79. Dalton, H. and Sterling, D. I., Phil. Trans. R. Soc. London, Ser. B, 297, 481-496 (1982).

80. Hovarth, R. S., Bacteriol. Rev., 36, 146-155 (1972).

81. Hulbert, M. H. and Krawiec, S., J. Theor. Biol., 69, 287-291 (1977).

82. Perry, J. J., Microbial Rev., 43, 59-72 (1979).

83. Venkataramani, E. S. and Ahlert, R. C., Biotechnol. Bioeng., 27, 1306-1311 (1985).

84. Hsieh, J. H. and Wang, S. S., Enzyme Microb. Tech., 2, 299-304 (1980).

85. Paris, D. F., Wolfe, N. L. and Steen, W. C., Appl. Environ. Microbiol., 44, 153-158 (1982).

86. Dearden, J. C., Nicholson, R. M., Pestic. Sci., 17, 305-310 (1986).

87. Bettmann, H. and Rehm, H. J., Appl. Microbiol. Biotechnol., 20, 285-290 (1984).

88. Anselmo, A. M., Mateus, M., Cabral, J. M. S., and Novais, J. M., Biotechnol. Lett., 7, 889-894 (1985).

89. Ehrhardt, H. M. and Rehm, H. J., Appl. Microbiol. Biotechnol., 21, 32-36 (1985).

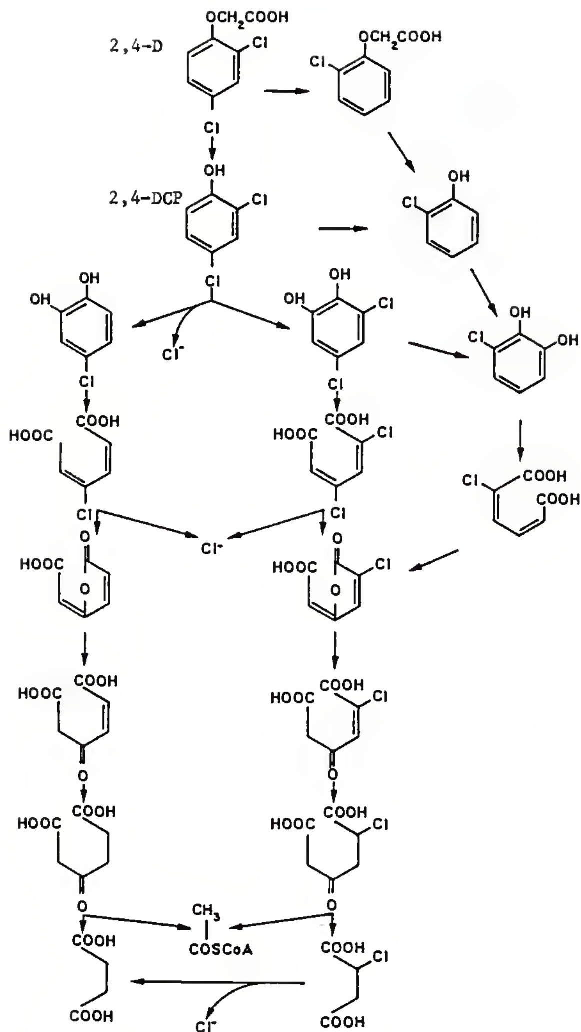

Figure 2.1. Aerobic pathways for 2,4-D biodegradation (11, 30, 46).

Table 2.1. Reported values of Monod model parameters for 2,4-D biodegradation.

μ_m (1/hr)	K_s (mg/L)	Y_s (g/g)	Reference	Culture Type
0.096	5.4	0.14	Shamat & Maier (69)	Batch
0.092	2.7	-	Shamat & Maier (69)	Continuous
0.14	5.1	-	Tyler & Finn (40)	Batch & Cont.
0.09	0.6	0.14	Shaler & Klecka (58)	Batch
-	-	0.14	Papanastasiou & Maier (45)	Cont.

μ_m = maximum specific growth rate; K_s = Monod half-saturation constant; Y_s = biomass yield (g biomass produced per g substrate consumed).

CHAPTER III

BIODEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4-DICHLOROPHENOL IN SHAKE FLASKS

This chapter represents a preliminary study of 2,4-D biodegradation by *Pseudomonas sp.* NCIB 9340. Shake flasks were used as a simple means of growing the organism to examine the effects of different 2,4-D concentrations on 2,4-D biodegradation. The primary goal was to roughly identify what, if any, 2,4-D concentration is inhibitory or toxic to the organism and if there is a minimum threshold level of 2,4-D that is required for biodegradation to occur in the concentration range being examined. These experiments also provided a simple test of the analytical methods to be used in one and two liter batch fermentations and initial estimates of some of the parameters describing 2,4-D biodegradation.

MATERIALS AND METHODS

The organism used in this work was *Pseudomonas sp.* NCIB 9340 obtained from the National Collections of Industrial and Marine Bacteria Ltd., Aberdeen, Scotland. The growth media for this organism contained the following: 1.5 grams/liter (g/L) of K_2HPO_4; 0.2 g/L of $MgSO_4 \cdot 7H_2O$; 0.05 g/L

of $CaSO_4 \cdot 2H_2O$; 0.5 g/L of NH_4NO_3; 0.0005 g/L of $FeSO_4 \cdot 7H_2O$; de-ionized water; and 2,4-D as the sole source of carbon. Inocula for the experiments were obtained from cultures that were continuously maintained by regular subculturing in shake flasks with 2,4-D provided as the carbon source. The maintenance cultures were kept at room temperature (21-25 C) at pH 6.4 to 7.0.

Substrate and product concentrations were assayed using high performance liquid chromatography (HPLC). The chromatography system consisted of a Varian Model 5000 liquid chromatograph equipped with a Varian Model 9176 strip chart recorder, an ISCO ISIS auto-sampler and auto-injector, and a Varian Vari-Chrom UV-Vis detector. Separation was accomplished by reverse-phase HPLC utilizing a Varian MCH-10 column. The column is packed with octadecylsilane ($-C_{18}H_{37}$ covalently bound to silica). In reverse-phase chromatography the separation is governed by the hydrophobic character of the solute compounds; the more hydrophobic the compound, the longer it is retained in the column. HPLC grade acetonitrile and 0.015N H_2SO_4 prepared with HPLC grade water served as solvents in a linear gradient elution with the acetonitrile concentration increasing from 30% to 80%. The HPLC program and instrument settings are described in Tables 3.1 through 3.4. Detection of 2,4-D, 2,4-DCP and

related compounds was accomplished by UV absorption at 283 nanometers. Figure 3.1 demonstrates the separation achieved using 2,4-D, 2,4-DCP and several possible degradation products. Concentrations are recorded in integrator units (I.U.) by the 9176 recorder. The standard curves for converting the integrator units into 2,4-D and 2,4-DCP concentrations are given in Figures 3.2 and 3.3. The data for these figures and conversion equations are given in Appendix A.

The HPLC procedure described here made it possible to analyze aqueous samples directly without any extraction or concentration. The detection limits for 2,4-D and 2,4-DCP were 1.0 and 0.1 mg/L, respectively. The only sample preparation required was filtration through a 0.45 micrometer nitrocellulose filter to remove the biomass and other particulates.

Biomass concentration was determined by monitoring absorbance with a Bausch and Lomb Spectronic 20 at 545 nm. Absorbance values were converted to biomass concentration by using a standard curve based on biomass dry-weight measurements. Biomass dry-weight was determined by filtering samples through 0.45 micrometer nitrocellulose filters. The filters were then placed in an oven at 105 C for approximately 24 hours of drying before weighing. the standard curve produced is shown in Fig. 3.4 with the data

and conversion equation given in Appendix A.

The experiments were conducted in 250 mℓ shake flasks at 25 C and 180 rpm. A solution of the nutrient media containing approximately 400 mg/L of 2,4-D was diluted with varying amounts of media with no 2,4-D to provide flasks with initial concentrations of 10.5, 25.3, 50.4, 94.5, 198 and 370 mg/L. Each flask was then inoculated with 10 mℓ of the Pseudomonas maintenance culture to make the total initial volume in each flask 100 mℓ. Table 3.5 indicates the contents and initial conditions of each flask. No control was provided during the experiments except for temperature. The pH was monitored with an Orion Research model 701A digital ionalyzer. Biomass concentration, substrate concentration and product concentration were also measured at intervals throughout the experiment.

RESULTS AND DISCUSSION

The shake flask experiments give no indication of substrate inhibition or toxicity to the organism in the concentration range from 0.0 to 370 mg/L. Estimates of the maximum specific growth rate have been obtained from the slopes of logarithmic plots of biomass concentration against time during the exponential growth phase. Figures 3.5 through 3.8 show these plots for the four flasks with the highest initial 2,4-D concentrations. The data in these

figures are accurately described by straight lines through the experimental region from the outset of the experiments until the end of the exponential growth phase; there is no decrease in slope at the beginning of the experiments that would be indicative of substrate inhibition. The growth phase was so short in the two flasks with the lowest initial 2,4-D concentrations that only two points have been used to estimate the maximum specific growth rate. In addition, the initial 2,4-D concentrations in these two experiments are close enough to the K_s value that even these two point initial rate estimates are probably significantly below the actual maximum specific growth rate. The estimates for the maximum specific growth rates for individual flasks are given in Table 3.6. The average value is 0.081 h^{-1} with a standard deviation of 0.012. Figures 3.9 through 3.14 illustrate the concentration profiles of biomass, 2,4-D, and 2,4-DCP in each flask as well as biomass and substrate concentrations predicted by the Monod model. Tables 3.7 through 3.12 list the data recorded for each experiment. Though there is not enough data at low concentrations to render precise parameter estimates possible, the Monod model with half saturation constant values, K_s, approximately in the range between 1.0 and 5.1 mg/L seems to provide an adequate description of the data. This is in agreement with

the results obtained by Tyler and Finn (2) based on the Monod model with a K_s value of 5.1 mg/L for 2,4-D biodegradation. The organism appears to grow well at all the initial concentrations tested indicating that the minimum threshold concentration required to stimulate growth, if there is a threshold, is below 10.5 mg/L.

In the shake flasks with initial 2,4-D concentrations of 50.4, 94.5, 198, and 370 mg/L, accumulation of 2,4-DCP was observed to reach levels of 0.1, 0.7, 13.2, and 16.9 mg/L respectively. Accumulation of 2,4-DCP could lead to inhibition of 2,4-D biodegradation. As previously mentioned in chapter 2, 2,4-DCP has been shown to cause inhibition in other experiments (1-3). The 2,4-DCP apparently did not reach sufficiently high concentrations to cause inhibition in these experiments as indicated by the logarithmic plots of biomass against time; these plots are essentially linear even after accumulation of 2,4-DCP.

Even though the pH was not controlled, it was monitored throughout the experiments as indicated in Tables 3.7 through 3.12. At the highest initial 2,4-D concentration the pH decreased from 6.83 to 6.48 over the course of the experiment. The flasks with the highest initial 2,4-D concentrations exhibited the greatest decrease in pH during biodegradation. No significant decrease in pH was observed in the two flasks with the lowest initial 2,4-D

concentrations. Similar decreases in pH concurrent with the degradation of chlorinated compounds have been reported elsewhere (4,5). Shamat and Maier (5) have observed that the biodegradation of certain chlorinated compounds results in the stoichiometric release of chloride and hydrogen ions that can reduce the buffering capacity of the media which may lead to a decrease in pH.

Biomass yields, Y_s, have been estimated using the initial biomass and substrate concentrations and biomass and substrate concentrations corresponding to the last recorded non-zero substrate concentrations. These estimates are presented in Table 3.6. The mean value of the biomass yield is 0.257 with a standard deviation of 0.043. There is some indication that the biomass yield increases as the substrate concentration decreases. At the highest initial 2,4-D concentration of 370 mg/L, the biomass yield is 0.175, while at the lowest initial concentration, 10.5 mg/L, the biomass yield is 0.291. In addition, Figs. 3.13 and 3.14 indicate that the yield estimates for the flasks with initial 2,4-D concentrations of 25.3 and 10.5 mg/L are too low to accurately describe the data. A biomass yield of about 0.35 is more consistent with the results of these two low concentration experiments.

CONCLUSIONS

The shake flask experiments give no indication of inhibition of growth by 2,4-D in the concentration range from 0.0 to 370 mg/L. Growth occurred at all of the initial concentrations tested; this indicates that if there is a minimum threshold concentration required to stimulate growth it is below 10.5 mg/L. The maximum specific growth rate observed in these experiments is 0.095 h^{-1}. The average biomass yield is 0.257. The Monod model with K_s around 1.0 to 5.1 mg/L adequately describes 2,4-D biodegradation. Accumulation of 2,4-DCP was observed, but it did not appear to reach inhibitory levels. All 2,4-D and 2,4-DCP was eventually biodegraded to below detectable levels.

REFERENCES

1. Papanastasiou, A. C., and Maier, W. J., Biotechnol. Bioeng., 24, 2001-2011, (1982).

2. Tyler, J. E., and Finn, R. K., Appl. Microbiol., 28, 181-184, (1974).

3. Parker, L. W., and Doxtader, K. G., J. Environ. Qual., 11, 679-684, (1982).

4. Kim, C. J., and Maier, W. J., J. Water Pollut. Control Fed., 58, 157-164, (1986).

5. Shamat, N. A., and Maier, W. J., J. Water Pollut. Control Fed., 52, 2158-2166, (1980).

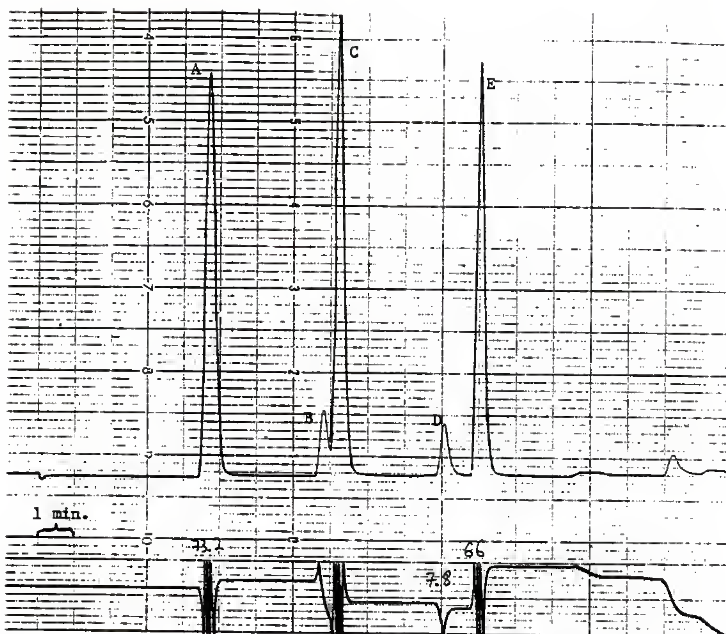

Figure 3.1. HPLC chromatogram for 2,4-D and related compounds including possible biodegradation metabolites.

peak	compound	retention time (min.)	detection limit(mg/L)*
A	phenol	8.3	not determined
B	2-chlorophenoxyacetic acid	11.4	not determined
C	2-chlorophenol	11.8	not determined
D	2,4-dichlorophenoxyacetic acid	14.6	1.0
E	2,4-dichlorophenol	15.6	0.1

Column: Varian MCH-10 (monomeric octadecasilane bonded to silica)
Detection: UV absorbance at 283 nm.

* using aqueous samples directly without any extraction or concentration.

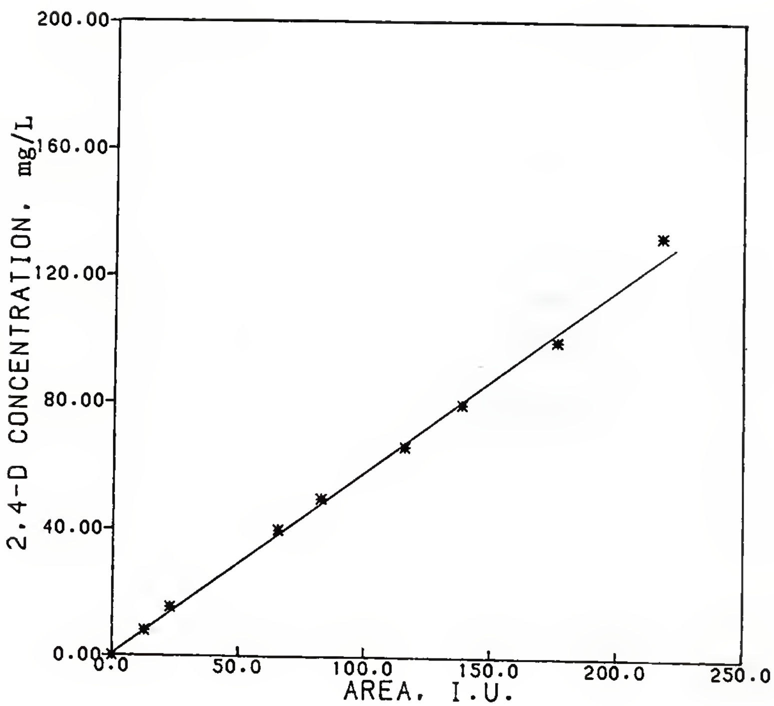

Figure 3.2. Standard curve for determining 2,4-D concentration from HPLC analysis.

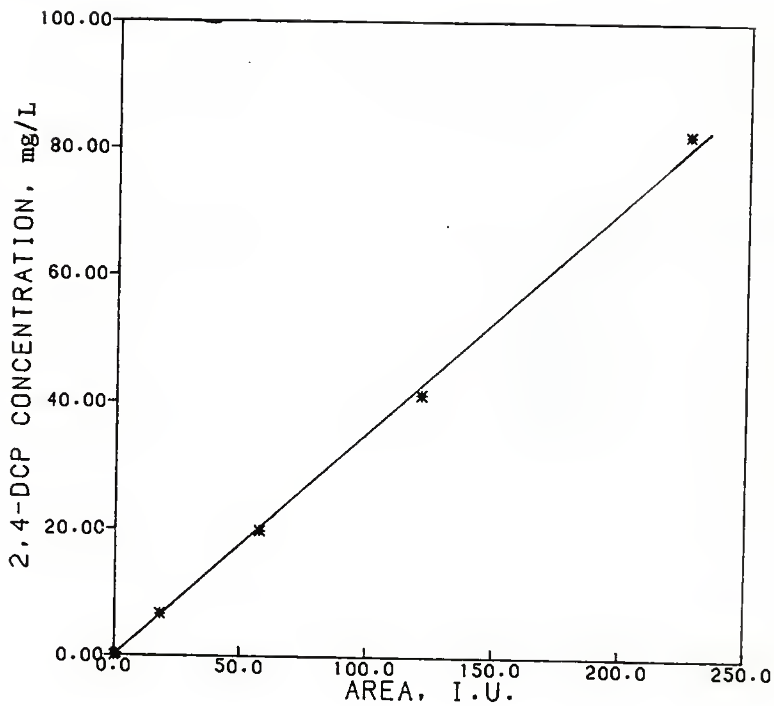

Figure 3.3. Standard curve for determining 2,4-DCP concentration from HPLC analysis.

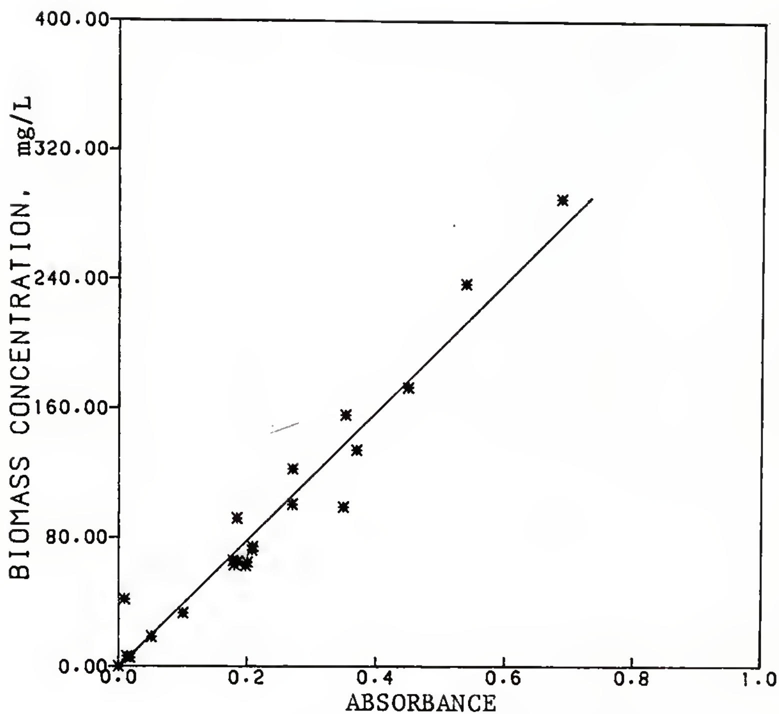

Figure 3.4. Standard curve for determining biomass concentration from absorbance.

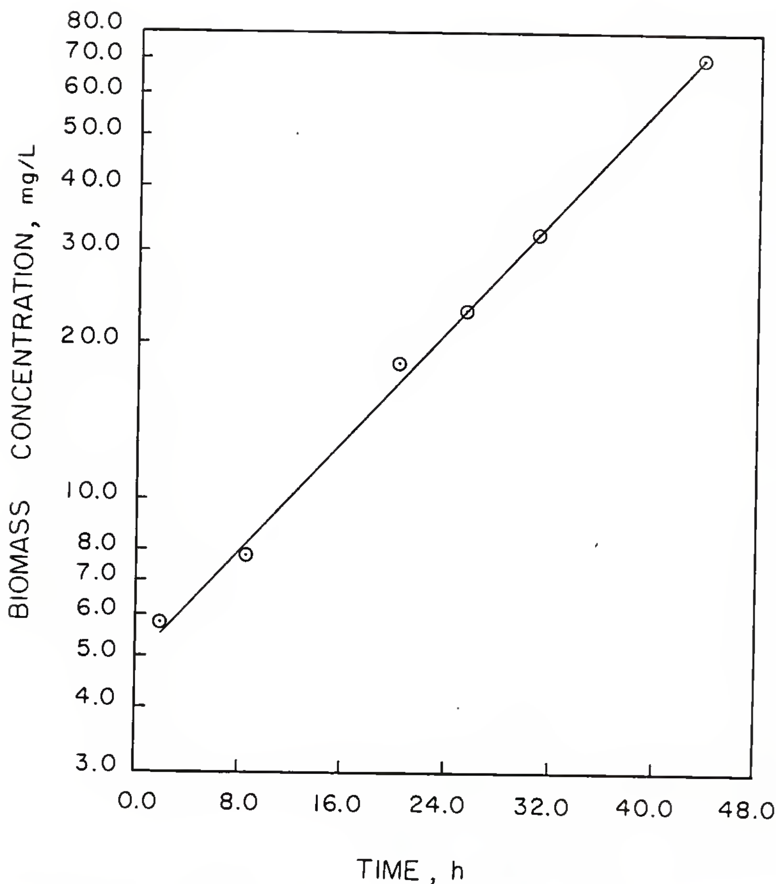

Figure 3.5. Logarithmic plot of biomass concentration against time for shake flask experiment with initial 2,4-D concentration of 370 mg/L; initial pH 6.8; temperature 25°C; ——, predicted biomass concentration over the exponential growth period with $\mu = 0.061\ h^{-1}$. Data from Table 3.7.

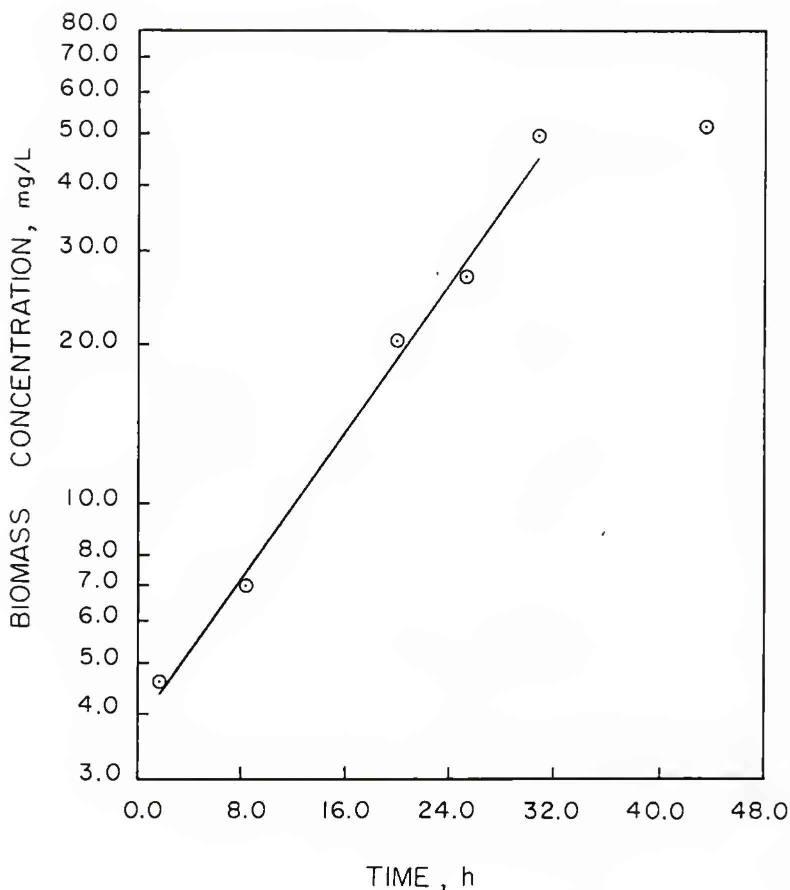

Figure 3.6. Logarithmic plot of biomass concentration against time for shake flask experiment with initial 2,4-D concentration of 198 mg/L; initial pH 6.8; temperature 25 °C; ——, predicted biomass concentration over the exponential growth period with μ = 0.081 h^{-1}. Data from Table 3.8.

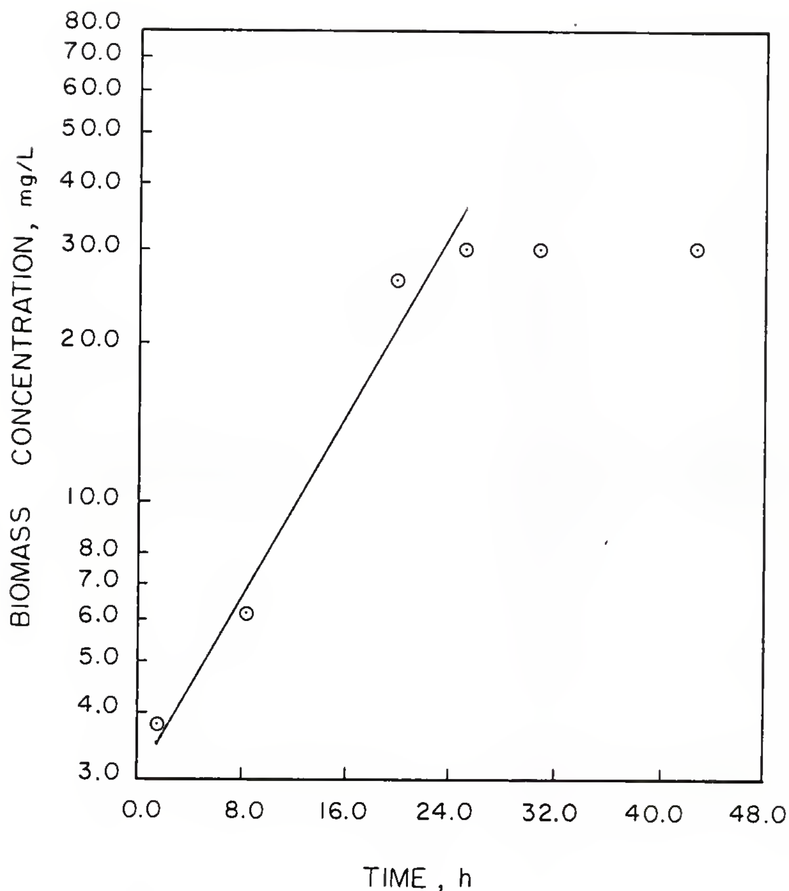

Figure 3.7. Logarithmic plot of biomass concentration against time for shake flask experiment with initial 2,4-D concentration of 94.5 mg/L; initial pH 6.8; temperature 25°C; ——, predicted biomass concentration over the exponential growth period with $\mu = 0.095\ h^{-1}$. Data from Table 3.9.

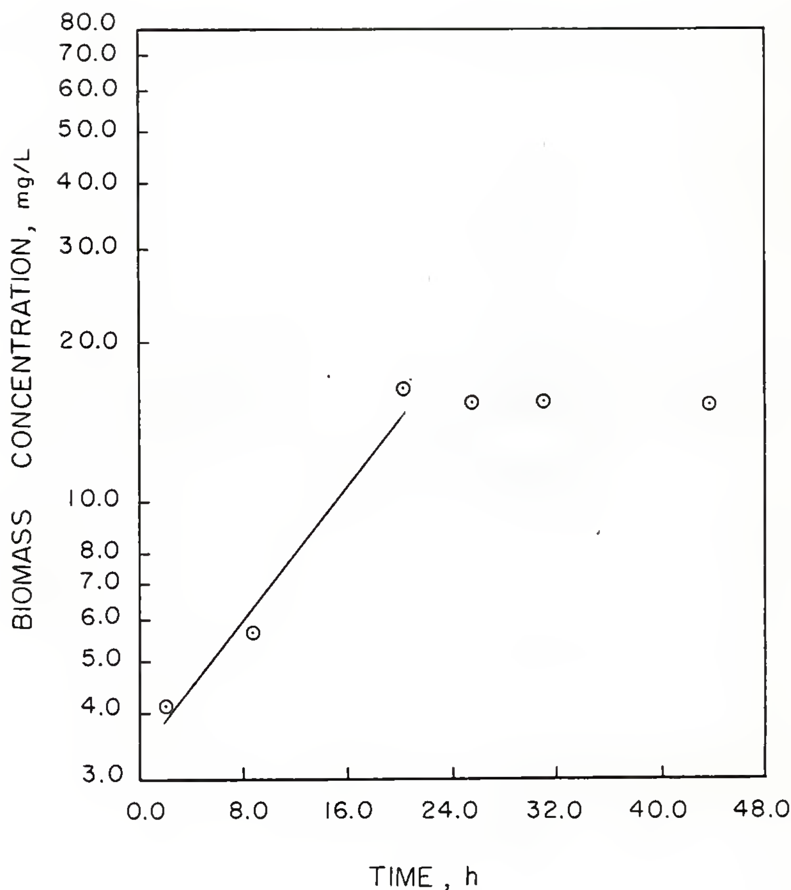

Figure 3.8. Logarithmic plot of biomass concentration against time for shake flask experiment with initial 2,4-D concentration of 50.4 mg/L; initial pH 6.8; temperature 25°C; ——, predicted biomass concentration over the exponential growth period with μ = 0.081. Data from Table 3.10.

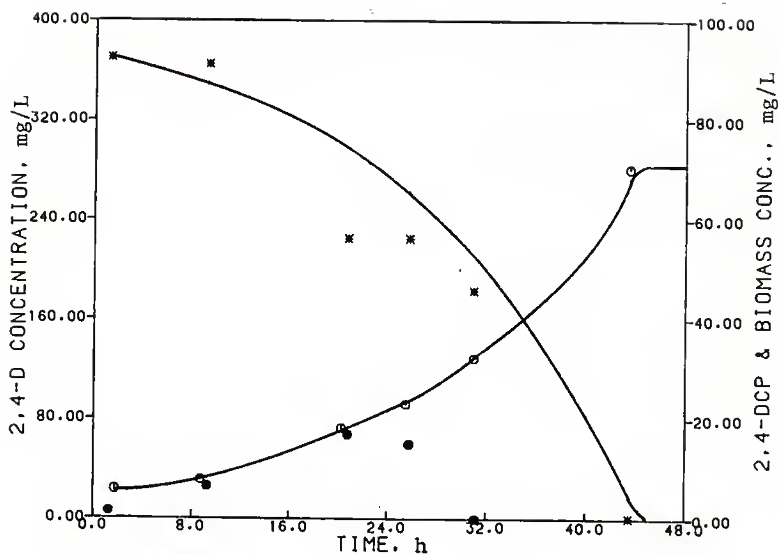

Figure 3.9. Substrate, product, and biomass concentration profiles for shake flask experiment with initial 2,4-D concentration of 370 mg/L; temperature 25°C; initial pH 6.8; * , 2,4-D concentration; o , biomass concentration; ● , 2,4-DCP concentration; ——, biomass and substrate concentrations predicted by the Monod model with μ_m = 0.061 h^{-1}, Y_s = 0.175, K_s = 5.1 mg/L. Data from Table 3.7.

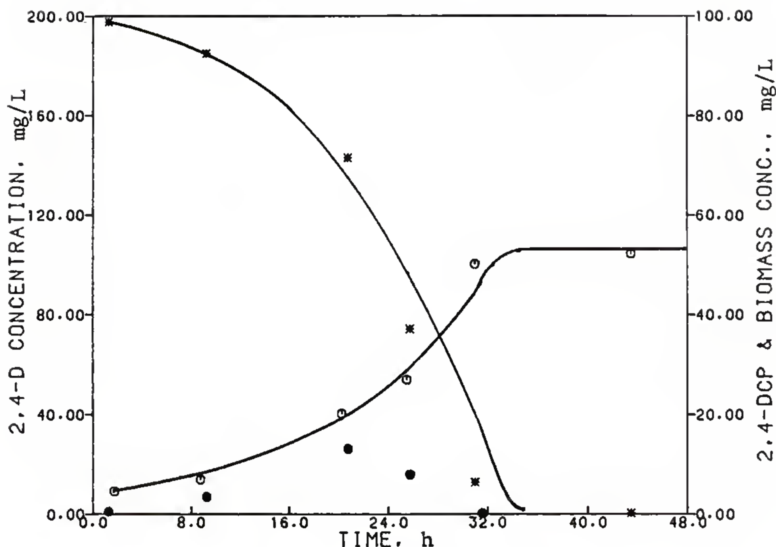

Figure 3.10. Substrate, product, and biomass concentration profiles for shake flask experiment with initial 2,4-D concentration of 198 mg/L; temperature 25°C; initial pH 6.8; * , 2,4-D concentration; o , biomass concentration; ● , 2,4-DCP concentration; ———, biomass and substrate concentrations predicted by the Monod model with μ_m = 0.081 h^{-1}, Y_s = 0.246, K_s = 5.1 mg/L. Data from Table 3.8.

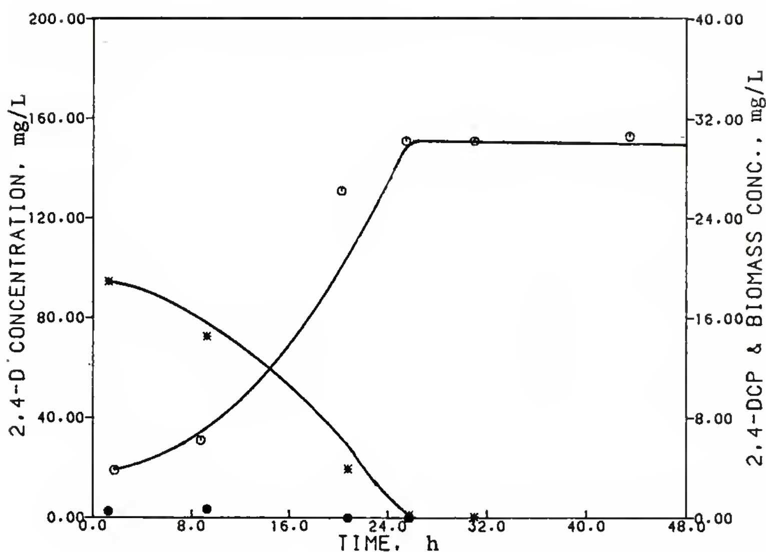

Figure 3.11. Substrate, product, and biomass concentration profiles for shake flask experiment with initial 2,4-D concentration of 94.5 mg/L; temperature 25°C; initial pH 6.8; * , 2,4-D concentration; o , biomass concentration; • , 2,4-DCP concentration; ——, biomass and substrate concentrations predicted by the Monod model with μ_m = 0.10 h^{-1}, Y_s = 0.281, K_s = 5.1 mg/L. Data from Table 3.9.

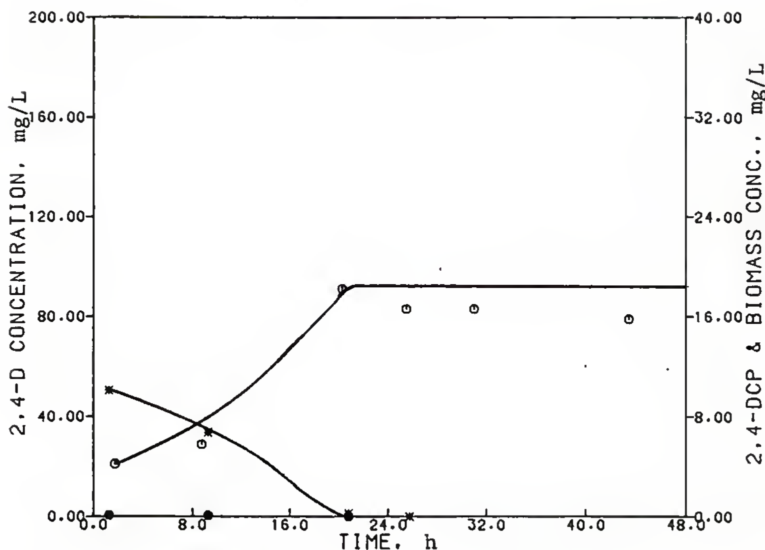

Figure 3.12. Substrate, product, and biomass concentration profiles for shake flask experiment with initial 2,4-D concentration of 50.4 mg/L; temperature 25°C; initial pH 6.8; * , 2,4-D concentration; o , biomass concentration; ● , 2,4-DCP concentration; —— , biomass and substrate concentrations predicted by the Monod model with μ_m = 0.10 h^{-1}, Y_s = 0.285, K_s = 5.1 mg/L. Data from Table 3.10.

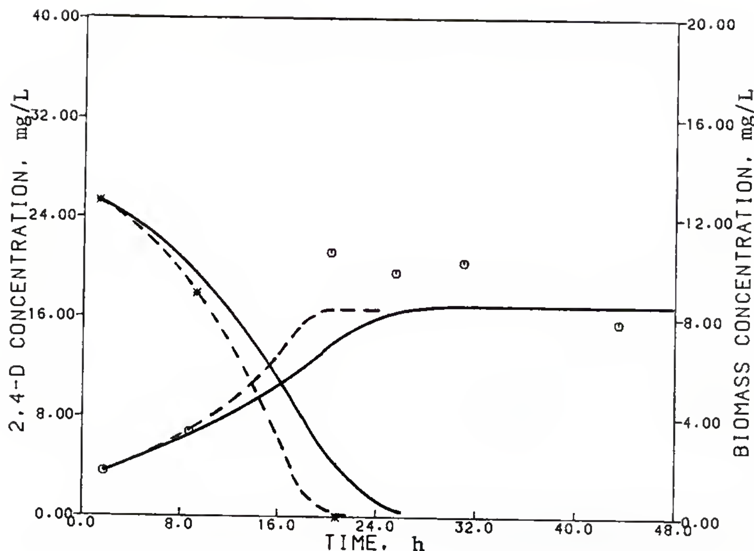

Figure 3.13. Substrate and biomass concentration profiles for shake flask experiment with initial 2,4-D concentration of 25.3 mg/L; temperature 25°C; initial pH 6.8; * , 2,4-D concentration; o , biomass concentration; ——, biomass and substrate concentrations predicted by the Monod model with $\mu_m = 0.10\ h^{-1}$, $Y_s = 0.261$, $K_s = 5.1$ mg/L; ----, biomass and substrate concentrations predicted by the Monod model with $K_s = 1.0$ mg/L. Data from Table 3.11.

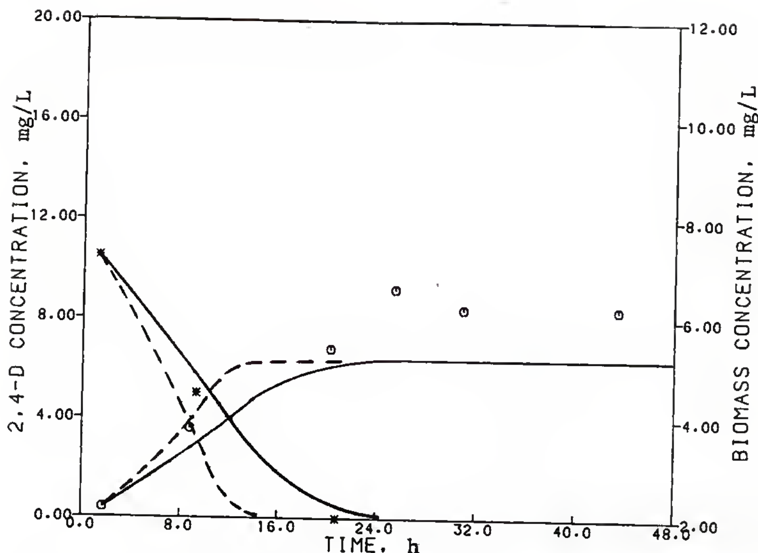

Figure 3.14. Substrate and biomass concentration profiles for shake flask experiment with initial 2,4-D concentration of 10.5 mg/L; temperature 25°C; initial pH 6.8; * , 2,4-D concentration; o , biomass concentration; ———, biomass and substrate concentrations predicted by the Monod model with μ_m = 0.10 h^{-1}, Y_s = 0.291, K_s = 5.1 mg/L; - - -, biomass and substrate concentrations predicted by the Monod model with K_s = 1.0 mg/L. Data from Table 3.12.

Table 3.1. Varian model 5000 chromatograph settings and conditions for HPLC analysis.

Parameter	Value
column	Varian MCH-10
solvent A	0.015N H_2SO_4 in HPLC water
solvent B	HPLC acetonitrile
Pmax	120 atm
Pmin	0 atm
temperature	30 B
reservoir	AB
external events	0
analog out	%

Table 3.2. HPLC programs.

	Time (min)	Code	Value
Program 1:*			
	0.0	FLOW	0.1
	0.0	%	30.0
	0.0	RSVR	AB
	0.0	EVNT	0
	5.0	FLOW	1.0
	7.0	FLOW	1.0
	7.0	PROG	2
Program 2:			
	0.0	FLOW	1.0
	0.0	%	30
	0.0	RSVR	AB
	0.0	EVNT	0
	20.0	%	80
	22.0	%	30
	38.0	%	30
	38.0	PROG	2

*Program 1 is used only to bring the column to the initial conditions when starting up the HPLC.

Table 3.3. ISCO ISIS auto-injector settings for HPLC analysis.

Parameter	Value
repeat size	1/4
transfer pump*	auto
wash	auto
loop loading time	11 sec
injections per sample	1 (usually)
analysis time	38 minutes
remote inject	off

*The transfer pump itself is set to FWD.

Table 3.4. Varian UV-Vis detector and model 9176 strip chart settings for HPLC analysis.

Parameter	Value
UV-Vis detector:	
wavelength	283 nm
time constant	normal
bandwidth control	8
absorbance range	0.05 (usually)
sample cell position	front
Chart Recorder:	
chart speed	1 cm/min
span	x 1
span mV/FS	1

Table 3.5. Contents and initial conditions for experiments in 250 ml shake flasks.

C_o	CM	NSM	Initial pH	Acid Added (ml) *	Adjusted pH
360	90	0	7.07	0.03	6.83
200	50	40	7.37	0.10	6.82
100	25	65	7.55	0.10	6.76
50	12.5	77.5	7.64	0.125	6.84
24	6	84	7.7	0.15	6.83
12	3	87	7.65	0.15	6.80

C_o, calculated initial 2,4-D concentration (mg/L); CM , amount of media containing nutrient salts and 400 mg/L 2,4-D (ml); NSM , amount of media containing only nutrient salts.

* 0.5 N H_2SO_4 was used to adjust the pH.

Note: 10 ml of inoculum was added to each flask making the initial volume 100 ml.

Table 3.6. Yield and specific growth rate estimates for shake flask experiments.

Initial 2,4-D Concentration (mg/L)	Y_s	μ_m	r	Time Span for Estimating μ_m (hr)
370	0.175	0.061	0.998	1.75 to 43.5
198	0.246	0.081	0.996	1.75 to 25.5
94.5	0.281	0.095	0.987	1.75 to 25.5
50.4	0.285	0.081	0.985	1.75 to 20.25
25.3	0.261	0.090	1.0 *	1.75 to 8.75
10.5	0.291	0.078	1.0 *	1.75 to 8.75

Y_s, biomass yield (g biomass produced per g substrate consumed); μ_m, maximum specific growth rate (h^{-1}); r , correlation coefficient for the μ_m estimate.

* these μ_m estimates are based on only two data points.

Table 3.7. Shake flask experiment with initial 2,4-D concentration of 370 mg/L.

Time (h)	pH	Biomass Concentration (mg/L)	2,4-D Concentration (mg/L)	2.4-DCP Concentration (mg/L)
0.0	-	-	-	-
0.5	6.83	-	-	-
1.25	-	-	370	1.4
1.75	-	5.8	-	-
8.25	6.87	-	-	-
8.75	-	7.8	-	-
9.25	-	-	365	6.5
20.0	6.82	-	-	-
20.25	-	18.2	-	-
20.75	-	-	225	16.9
25.0	6.85	-	-	-
25.5	-	23.0	-	-
25.75	-	-	225	15.0
30.75	6.76	-	-	-
31.0	-	32.2	183	0.0
43.0	6.48	-	-	-
43.5	-	70.2	0.9	0.0

T = 25 C; initial pH = 6.8.

Table 3.8. Shake flask experiment with initial 2,4-D concentration of 198 mg/L.

Time (h)	pH	Biomass Concentration (mg/L)	2,4-D Concentration (mg/L)	2.4-DCP Concentration (mg/L)
0.0	-	-	-	-
0.5	6.82	-	-	-
1.25	-	-	198	0.5
1.75	-	4.6	-	-
8.25	6.84	-	-	-
8.75	-	7.0	-	-
9.25	-	-	185	3.5
20.0	6.80	-	-	-
20.25	-	20.2	-	-
20.75	-	-	143	13.2
25.0	6.77	-	-	-
25.5	-	27.0	-	-
25.75	-	-	74.3	8.0
30.75	6.66	-	-	-
31.0	-	50.2	12.9	0.0
43.0	6.65	-	-	-
43.5	-	52.2	0.5	0.0

T = 25 C; initial pH = 6.8.

Table 3.9. Shake flask experiment with initial 2,4-D concentration of 94.5 mg/L.

Time (h)	pH	Biomass Concentration (mg/L)	2,4-D Concentration (mg/L)	2.4-DCP Concentration (mg/L)
0.0	-	-	-	-
0.5	6.76	-	-	-
1.25	-	-	94.5	0.5
1.75	-	3.8	-	-
8.25	6.79	-	-	-
8.75	-	6.2	-	-
9.25	-	-	72.6	0.7
20.0	6.70	-	-	-
20.25	-	26.2	-	-
20.75	-	-	19.6	0.0
25.0	6.70	-	-	-
25.5	-	30.2	-	-
25.75	-	-	1.2	0.0
30.75	6.70	-	-	-
31.0	-	30.2	0.5	0.0
43.0	6.69	-	-	-
43.5	-	30.6	0.0	0.0

T = 25 C; initial pH = 6.8.

Table 3.10. Shake flask experiment with initial 2,4-D concentration of 50.4 mg/L.

Time (h)	pH	Biomass Concentration (mg/L)	2,4-D Concentration (mg/L)	2.4-DCP Concentration (mg/L)
0.0	-	-	-	-
0.5	6.84	-	-	-
1.25	-	-	50.4	0.1
1.75	-	4.2	-	-
8.25	6.85	-	-	-
8.75	-	5.8	-	-
9.25	-	-	33.6	0.1
20.0	6.81	-	-	-
20.25	-	18.2	-	-
20.75	-	-	1.3	0.0
25.0	6.84	-	-	-
25.5	-	16.6	-	-
25.75	-	-	0.0	0.0
30.75	6.83	-	-	-
31.0	-	16.6	0.0	0.0
43.0	6.81	-	-	-
43.5	-	15.8	-	-

T = 25 C; initial pH = 6.8.

Table 3.11. Shake flask experiment with initial 2,4-D concentration of 25.3 mg/L.

Time (h)	pH	Biomass Concentration (mg/L)	2,4-D Concentration (mg/L)	2.4-DCP Concentration (mg/L)
0.0	-	-	-	-
0.5	6.83	-	-	-
1.25	-	-	25.3	0.0
1.75	-	1.8	-	-
8.25	6.85	-	-	-
8.75	-	3.4	-	-
9.25	-	-	17.9	0.0
20.0	6.85	-	-	-
20.25	-	10.6	-	-
20.75	-	-	0.0	0.0
25.0	6.86	-	-	-
25.5	-	9.8	-	-
30.75	6.86	-	-	-
31.0	-	10.2	-	-
43.0	6.84	-	-	-
43.5	-	7.8	-	-

T = 25 C; initial pH = 6.8.

Table 3.12. Shake flask experiment with initial 2,4-D concentration of 10.5 mg/L.

Time (h)	pH	Biomass Concentration (mg/L)	2,4-D Concentration (mg/L)	2.4-DCP Concentration (mg/L)
0.0	-	-	-	-
0.5	6.80	-	-	-
1.25	-	-	10.5	0.0
1.75	-	2.2	-	-
8.25	6.82	-	-	-
8.75	-	3.8	-	-
9.25	-	-	5.0	0.0
20.0	6.83	-	-	-
20.25	-	5.4	-	-
20.75	-	-	0.0	0.0
25.0	6.84	-	-	-
25.5	-	6.6	-	-
30.75	6.83	-	-	-
31.0	-	6.2	-	-
43.0	6.81	-	-	-
43.5	-	6.2	-	-

T = 25 C; initial pH = 6.8.

CHAPTER IV

EFFECT OF pH ON 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4-DICHLOROPHENOL BIODEGRADATION

In this chapter the biodegradation of 2,4-D and 2,4-DCP in one and two liter fermenters is examined. The primary goal is to study the effects of pH on biodegradation. An understanding of the effects of pH is essential for the design and evaluation of biological treatment options to eliminate production wastes and to manage biodegradation in field applications. The pH should also be considered in assessing environmental persistence of 2,4-D and in determining if undesirable metabolic products are produced as a result of biodegradation.

THEORY

Biomass yield and specific growth rate can be assumed to be constant during the exponential growth phase. Thus, the specific growth rate can be determined directly from biomass concentration data using

$$\mu = \frac{1}{X}\frac{dX}{dt} \qquad (1)$$

During exponential growth, the specific growth rate determined by this relation is the maximum specific growth rate. Substrate and biomass data can be used to estimate the biomass concentration based on substrate from

$$Z = X_o + Y_s(S_o - S) \qquad (2)$$

Thus, a second estimate of the specific growth rate can be obtained from

$$\mu = \frac{1}{Z}\frac{dZ}{dt} \qquad (3)$$

Integration of equations (1) and (3) gives, respectively,

$$\ln(X) - \ln(X_o) = \mu(t - t_o) \qquad (4)$$

$$\ln(Z) - \ln(Z_o) = \mu(t - t_o) \qquad (5)$$

Letting $\ln X - \ln X_o = Y_1$, $\ln Z - \ln Z_o = Y_2$, and $t - t_o = \theta$, the data can be described by the following models;

$$Y_1 = \mu\theta + \epsilon_1 \qquad (6)$$

$$Y_2 = \mu\theta + \epsilon_2 \qquad (7)$$

Estimates of μ can be obtained from equations (6) and (7) by determining the values that minimize the sum of the squares of the error terms, ϵ_i. In addition, all the data can be utilized simultaneously by determining the least squares fit for the regression model by regarding the average as the dependent variable as follows:

$$\overline{Y} = (Y_1 + Y_2)/2 = \mu\theta + (\epsilon_1 + \epsilon_2)/2 \quad (8)$$

Another estimate that uses all the data can be made by employing the covariate adjustment method (1-4). This method also considers the average value, $\overline{Y}$, as the dependent variable of the regression model, but also includes additional information gained by taking the difference between equations (6) and (7) to obtain

$$C = (Y_1 - Y_2)/2 = (\epsilon_1 - \epsilon_2)/2 \quad (9)$$

This term appears as a covariate in the following multiple regression model.

$$\overline{Y} = \mu\theta + \beta C + \epsilon_3 \quad (10)$$

The interval of exponential growth can be determined by

identifying the linear section of the logarithmic plot of biomass against time. If qualitative analysis is inadequate for determining the linear region, more quantitative statistical methods can be employed (3-5). The statistical method still requires that an initial interval be selected in the exponential region. This region is then extended one set of points at a time using statistics such as the root mean square error, residual, and p value to determine if the newly added points should be included as part of the exponential region. This analysis resorts to two different regression equations:

$$\ln(X) = \beta_o + \beta_1 t \qquad (11)$$

$$\ln(X) = \beta_o + \beta_1 t + \beta_2 t^2 \qquad (12)$$

The root mean square error, MSE, and the residual, R, are computed from the least squares fit of equation (11) to the data. A small MSE indicates that the linear model provides a good fit. The difference in MSE before and after addition of the new data point is also an important consideration. The residual is the value of the deviation from the expected value predicted by equation (11) for the added point. The p value is the level of significance of the test statistic

testing if the quadratic term in equation (12) is needed. The smaller the p value the greater the evidence for rejecting the null hypothesis that $\beta_2 = 0$. A p value > 0.1 indicating that the second order term is not significant might be selected as an acceptance level for including an additional point.

MATERIALS AND METHODS

The basic measurement techniques, organism, and media employed for the work described in this chapter were the same as those described in the materials and methods section of chapter 3. Biomass concentration was measured by absorbance at 545 nm, and 2,4-D and 2,4-DCP concentrations were monitored using HPLC with UV detection at 283 nm. Inocula for the experiments were provided from cultures growing in shake flasks with either 2,4-D or 2,4-DCP as the carbon source depending on which compound was to be used as the substrate in the given experiment.

The experiments are named according to the type of experiment (B = Batch, FB = Fed-Batch) followed by the date the experiment was started, the vessel (e.g. V1 = Vessel 1) and a letter if the experiment is a continuation of a previous experiment.

Batch experiments were conducted in one or two liter L H Fermentation 500 series fermentation systems. The pH

was controlled with 505D controllers with Ingold type 465 electrodes using 0.1M NaOH and 0.1M H_2SO_4. Temperature control was provided by model 503 temperature control modules. The air flow rate was set by rotameters. Mixing was accomplished using model 502 direct drive units with two impellers in vessels with four baffles. The vessels were equipped with outlet gas condensers.

In the early experiments, strict aseptic conditions were not maintained because it was believed that the toxic nature of the substrates would prevent contamination. After contamination of experiments by protozoa was observed a sterilization procedure was developed. The vessels, complete with pH probe, temperature sensor, heater, and cooling finger, were steam sterilized in an autoclave for 20 minutes at 15 psig. Nutrient salts media was then added after filtration through 0.45 μm nitro-cellulose filters with autoclaved filtration equipment. The growth substrate, either 2,4-D or 2,4-DCP, was then supplied directly to the vessels.

For most of the experiments the exponential growth region was clearly evident from examination of the logarithmic plots of biomass against time. In the less obvious cases the statistical methods based on equations (11) and (12) were employed to identify the exponential region.

The biomass yield was determined using the biomass data included in the exponential interval. Biomass concentrations at each end of the interval were estimated by using linear regression with equation (11) to determine expected values at the endpoint times based on all the biomass data in the exponential region. The substrate concentrations used for the yield estimates were those directly measured at the start and end times of the exponential growth period. Thus, the biomass yield was estimated as

$$Y_s = (\hat{X}_2 - \hat{X}_1)/(S_1 - S_2) \qquad (13)$$

where subscript 1 refers to the starting time and subscript 2 refers to the end time of the exponential growth region. Assuming that chlorine has a valence of -1, the available electron yield coefficient, η, can be determined by multiplying the biomass yield by 1.253. This factor is determined as follows:

$$\eta = (\sigma_b\gamma_b/\sigma_s\gamma_s)Y_s = 1.253Y_s \qquad (14)$$

where $\sigma_b = 0.462$ and $\gamma_b = 4.291$ (6-9).

Whenever possible, the maximum specific growth rate was

estimated by using biomass data (equation (6)), substrate data (equation (7)), and the covariate adjustment method (equation (10)). Two sets of growth rate estimates were made using equations (6) and (10). One set of estimates are based on the Y_s values determined for the individual experiment, with the second set based on the average biomass yield determined from all of the experiments. Since the biomass yield is actually a constant, the best estimate of the maximum specific growth rate is taken to be the one with the smallest 95% confidence interval obtained from equations (6), (7), and (10) using the average Y_s value.

The regression analysis has been performed primarily with the general linear model procedures contained in the SAS statistical package (10). The programs used for the exponential interval selection, specific growth rate estimates and first order rate constant estimates are given in appendix B.

RESULTS AND DISCUSSION

The results of batch fermentation experiments with initial 2,4-D concentrations of approximately 200 mg/L indicate that pH is a significant factor in determining growth rates. The experiments were conducted over a pH range from 5.1 to 9.4. The data for these experiments are given in Tables 4.1 through 4.11. Figures 4.1 through 4.9 illustrate the concentration profiles of biomass, 2,4-D and 2,4-DCP for the pH 5.5 through 8.9 experiments as well as biomass and substrate concentrations predicted by the Monod model. Figures 4.10 through 4.18 contain the logarithmic plots of biomass against time, from which the exponential region for these experiments have been determined. Statistical analysis was performed to aid in the selection of the exponential interval for the pH 6.0, 7.0, 7.9, and 8.9 experiments. The results of this analysis are given in Table 4.12. Table 4.13 gives the point and 95% confidence interval estimates for the maximum specific growth rates for the experiments in the range of pH from 5.5 to 8.9. The results of the pH 5.1 to 9.4 experiments are summarized in Table 4.14, with Fig. 4.19 demonstrating the relation between the pH and maximum specific growth rate. The highest growth rates were observed in the pH range from 6.5 to 7.9. This is slightly higher than the 6.2 to 6.9 optimum pH range observed by Tyler and Finn (11). The average

growth rate observed in the 6.5 to 7.9 pH range was 0.14 h^{-1}, which is the same growth rate that Tyler and Finn observed in the 6.2 to 6.9 pH range. The highest growth rate, 0.15 h^{-1}, was observed at pH 7.9. The growth rate decreased as the pH was increased or decreased from the 6.5 to 7.9 range with no growth occurring at pH 5.1 or 9.4. Cultures exhibiting no growth for seven days at pH 5.1 could be revived to resume normal growth and substrate consumption by increasing the pH to 6.0.

The biomass yield estimates for the pH 5.1 to 8.9 experiments are given in Table 4.14. There does not appear to be any relation between pH and biomass yield. The average biomass yield was 0.247 with a standard deviation of 0.059. The relatively low value for the biomass yield is at least in part due to the two chlorines in the substrate that account for a considerable amount of mass that is not incorporated into biomass. The average available electron yield is 0.309. This represents the fraction of available electrons in the organic substrate that are transferred to biomass. This value is consistent with the lower range of values reported for the energetic yield in hydrocarbon fermentations (12).

Appreciable accumulation of 2,4-DCP was observed in the experiments conducted at pH 5.5 and 5.7. These were the two

lowest pH levels where growth was observed. In both cases the accumulation of 2,4-DCP appears to have completely stopped the biodegradation of 2,4-D. At pH 5.7, the 2,4-DCP accumulation reached 49.5 mg/L with 2,4-D biodegradation ceasing at 38 mg/L as indicated by Fig. 4.2. No further growth or degradation was observed even after the 2,4-DCP concentration was decreased to below 20 mg/L by non-biodegradation mechanisms and additional 2,4-D substrate was supplied. At pH 5.5, 2,4-DCP accumulated to 44.5 mg/L with 2,4-D degradation ceasing at 87 mg/L of 2,4-D as shown in Fig. 4.1. The specific growth rates given for the pH 5.5 and 5.7 experiments are based on growth before significant amounts of 2,4-DCP accumulation. Figures 4.1 and 4.2 show that accumulation of 2,4-DCP was accompanied by a reduction in biodegradation rate, presumably due to inhibitory effects of 2,4-DCP.

The Monod model with a half saturation constant of 5.1 mg/L as suggested by the work of Tyler and Finn (11) provides an adequate description of the biodegradation of 2,4-D in the pH range from 6.0 to 8.1 as indicated in Figs. 4.3 through 4.8. In the low pH experiments shown in Figs. 4.1 and 4.2, the Monod model is inadequate due to the inhibitory effects of 2,4-DCP accumulation. The pH 7.9 experiment shown in Fig. 4.7 gives an indication of inhibitory effects after about 30 hours. This may be caused

by the accumulation of some metabolic product other than 2,4-DCP. An unrealistically rapid increase in biomass concentration in relation to substrate consumption is indicated by the results shown in Fig. 4.9 for the pH 8.9 experiment. The biomass concentration also appears to level off prematurely. Assuming that the biomass data is in error and utilizing the average biomass yield value of 0.247 a satisfactory description of the substrate data can be obtained from the Monod model with a K_s value of 5.1 mg/L.

Biodegradation is probably not a major factor in the decrease in the 2,4-DCP concentration shown in Figs. 4.1 and 4.2 for the experiments conducted at pH 5.5 and 5.7. Tables 4.15 through 4.19 show the results of five experiments conducted to examine the changes in 2,4-D and 2,4-DCP concentrations in the absence of biological activity. Table 4.20 summarizes these results and values obtained for the experiments conducted at pH 5.5 and 5.7. The decreases in the 2,4-DCP concentration observed in these experiments were adequately described by first order rate expressions. Figure 4.20 demonstrates the fit of a first order model to the data from experiment B9/12V1. Even though these results yield the lowest correlation coefficient of all four 9/12 experiments, they still indicate a good fit. Though the first order models provide a good fit for the individual experiments the value of the rate constant varies

significantly ranging from 0.0018 to 0.0143 h^{-1}. The four experiments started on 9/12 are all described by similar rate constants ranging from 0.00500 to 0.00676 h^{-1} in spite of different condenser water temperatures and vessel sizes; these factors do not significantly affect the rate of disappearance. Experiments started on different dates produced what appear to be unrelated rate constant values. The dependence of the first order rate constants on the dates of the experiments seems to indicate that some unmeasured factor or factors such as photolysis may be responsible for the observed decreases in the 2,4-DCP concentrations. The pH may also be a factor in determining rate constant magnitudes. 2,4-D photolysis rates have been shown to increase with increasing pH (13). The opposite trend seems to be indicated for 2,4-DCP by the results in Table 4.20. Microbial degradation, adsorption or absorption may also be important. This could explain the relatively high rate constants observed in the pH 5.5 and 5.7 experiments. In the four experiments started on 9/12 and experiment B6/17V1 microbial activity was precluded by the absence of nutrients other than the carbon source.

The vapor pressure of 2,4-DCP at 25 C has been estimated to be 0.0002 atm by fitting an Antoine type equation to the available vapor pressure data at various temperatures (14). Assuming equilibrium, ideal liquid and

ideal gas conditions the 2,4-DCP vapor loss was estimated to be 0.0006 mg/h using the following conditions: air flow rate, 650 ml/min.; total pressure, 1 atm; temperature, 25 C; volume, 1 liter; 2,4-DCP concentration, 100 mg/L. This as well as the experimental results at different condenser temperatures indicates that 2,4-DCP vapor loss is insignificant.

Table 4.20 indicates that changes in the 2,4-D concentration in the absence of microbial activity were too small to significantly affect experimental results. 2,4-D vapor loss was estimated based on the same assumptions and conditions as in the 2,4-DCP estimate with a 2,4-D concentration of 200 mg/L and a vapor pressure of 1.38×10^{-5} atm (15). The estimated rate of 2,4-D loss is 8×10^{-5} mg/h. Again, though this estimate is very approximate, 2,4-D vapor loss does not appear to be a significant cause of concentration changes. Reductions in volume due to water loss account for much of the observed changes in 2,4-D concentration. Assuming 30 % relative humidity for the inlet air at 25 C and saturated exit gas, the vapor loss for outlet condenser temperatures of 22, 19, and 9 C have been estimated to be 11.9, 8.9, and 1.3 ml/day, respectively. The water loss measured in experiment B6/17V1 with condenser water at 22 C was 7.8 ml/day indicating that the loss may be somewhat lower than predicted by the calculated values.

There may be a small non-biodegradation loss of 2,4-D. In experiment B9/12V3 where the condenser water temperature of 9 C should have kept the volume constant, a decrease of approximately 0.03 mg/L-h was observed. As mentioned previously, photolysis could be a factor in this loss (13).

Results from the experiments initiated on 5/12 are listed in Tables 4.21 through 4.24. In these experiments the biomass concentration fluctuated with little or no net increase in vessels two and four where the initial 2,4-D concentrations were 64.5 and 62.7 respectively. In vessels one and three with initial 2,4-D concentrations of around 220 mg/L a net increase was observed in the biomass concentration, however, it also fluctuated with the maximum observed biomass concentrations indicating biomass yields of only 0.056 to 0.087. The overall rate of 2,4-D biodegradation was slow in all of these experiments with vessel three being the slowest having 3.0 mg/L of 2,4-D left after 222.5 hours.

Microscopic examination of samples from vessels one and three revealed that the cultures were contaminated with protozoa. Presumably, predation by these protozoa was the cause of the fluctuating biomass concentrations, low apparent biomass yields, and low biodegradation rates. No protozoa were observed in vessels two or four. The cultures were not examined until the day after the last experiment

was completed, so protozoa that might have been present in vessels two and four might have died. All the vessels were inoculated from the same maintenance culture which may have been contaminated by protozoa. Experiments with sewage organisms were conducted in flasks adjacent to the maintenance cultures at that time. The maintenance culture started with the residual inoculum from the 5/12 experiments failed to degrade 2,4-D. No predatory protozoa were observed in this culture, but there was considerable delay before it was tested.

Fifteen experiments were conducted in 2-liter fermenters with 2,4-DCP as the only source of carbon. The data for these experiments are given in Tables 4.25 through 4.39. Based on the results of the non-biodegradation experiments (B6/17V1 and the four 9/12 experiments), loss of 2,4-DCP as described by first order rate constants ranging from 0.002 to 0.008 h^{-1} can be considered to be normal loss by non-biodegradation mechanisms.

Only six of the 2,4-DCP experiments yielded clear indications of biodegradation. The specific growth rates were roughly estimated for these experiments by using the substrate data to calculate z values for use in regression equation (7). Because the experimental results with 2,4-DCP were not adequate to provide a direct estimate of the biomass yield a value was calculated by assuming that the

available electron yield is the same for 2,4-D and 2,4-DCP. The available electron yield for 2,4-DCP can be calculated using equation (14) to obtain $\eta = 1.13Y_s$. This leads to an estimate of $Y_s = 0.27$ for 2,4-DCP assuming Y_s for 2,4-D is 0.247. The data used to calculate the z values are corrected for substrate loss due to non-biodegradation mechanisms by subtraction of substrate loss predicted by first order models. The biomass data for the 2,4-DCP experiments was not used for direct growth rate estimates because of large errors associated with biomass measurements at the low concentrations observed.

Table 4.40 shows the six specific growth rate estimates for 2,4-DCP biodegradation. The average growth rate is 0.078 h^{-1} with a standard deviation of 0.053. Due to the inhibitory nature of 2,4-DCP, the method employed in this research to estimate growth rates probably does not provide an accurate estimate of the maximum specific growth rate. Tyler and Finn (11) extrapolated results of 2,4-DCP growth experiments to estimate a maximum specific growth rate of 0.14 h^{-1} for 2,4-DCP. The six growth rate estimates cover a wide range of values even though the pH was similar in all of these experiments. The non-biodegradation first order rate constants for the three B11/13 experiments were determined from substrate concentration data at the

beginning of each experiment when the 2,4-DCP concentration was high enough to preclude significant biodegradation activity. The non-biodegradation rate constant for the FB11/30 experiments was determined from experiment B11/23V3 which was conducted at the same pH but at a high 2,4-DCP concentration where there was no evidence of biodegradation activity. Figures 4.21 through 4.26 compare the uncorrected 2,4-DCP concentrations to the values predicted by the non-biodegradation first order models. There is no clear indication of biodegradation occurring above approximately 35 mg/L of 2,4-DCP. Degradation more rapid than the predicted non-biodegradation rate was first observed in the range from 30 to 35 mg/L in the 11/13 and 11/23 experiments. Rapid degradation started immediately in the 11/30 experiments where the initial 2,4-DCP concentrations were 26.6 and 34.5 mg/L.

Biomass yield estimates for growth on 2,4-DCP based on the biomass and substrate data for the six experiments where growth was most evident ranged from 0.056 to 0.13. The average yield was 0.093 with a standard deviation of 0.03.

Table 4.41 lists first order rate constants as determined from least squares fits of the data of all fifteen 2,4-DCP experiments, the pH 5.5 and 5.7 2,4-D experiments, and the non-biodegradation loss experiments. No growth was observed in any of the four experiments with

initial 2,4-DCP concentrations above 50 mg/L. The loss of 2,4-DCP in these experiments can be fairly well described by first order models with rate constants consistent with normal non-biodegradation loss ranging from .0032 h^{-1} to .0052 h^{-1}. The data in experiment B12/7V2 can also be described by a first order model, but with a rate constant higher than expected for non-biodegradation.

The results from experiments B12/7V1 and B12/10V2 are especially unusual. In these experiments the large estimates of first order rate constants provide a poor description of the data as indicated by Figs. 4.27 and 4.28. It appears as if there may be significant biodegradation occurring early in these experiments where rapid decreases in substrate concentration are observed; however, after the rapid initial disappearance, the 2,4-DCP concentration stabilizes and even appears to increase slightly. A similar pattern was observed in experiment B12/10V1, but with a smaller initial decrease. The observed increases in 2,4-DCP concentrations are completely unexpected because degradation or removal by some mechanism is assumed to occur even in the absence of biodegradation.

The large inocula used may be a factor in the unusual results obtained in the 12/7 and 12/10 experiments. The inoculum size was increased in these later experiments because of what appeared to be extended lag

periods and total lack of growth in many of the earlier experiments. High biomass concentrations present at the start of the experiments could cause the initial rapid decrease in the 2,4-DCP concentration through absorption or adsorption uptake mechanisms. The subsequent stabilization of the 2,4-DCP concentration could represent the point where equilibrium is reached between the bulk liquid 2,4-DCP concentration and that associated with the biomass. The observed increases in the bulk 2,4-DCP concentration could be due to cell lysis, experimental error, or water loss. The lack of clear evidence of extensive growth or biodegradation in any of the experiments started on 12/7 or 12/10 even though the initial 2,4-DCP concentrations were relatively low may indicate that these experiments were inoculated with mostly non-viable cultures.

No relation between the first order rate constants or growth rates and pH is evident from the 2,4-DCP experiments. This is probably due to the large degree of error in the various estimates and uncertainty concerning what factors are influencing 2,4-DCP disappearance. To accurately examine 2,4-DCP biodegradation the non-biodegradation removal mechanisms need to be clarified. Three factors that may be involved in the non-biodegradation disappearance that need to be investigated further are pH, photolysis, and biomass adsorption/absorption phenomena.

CONCLUSIONS

The experiments with 2,4-D indicate that pH is an important factor in determining growth rates. The highest growth rates on 2,4-D occur between pH 6.5 and 7.9. The average growth rate observed in this region is 0.14 h^{-1}. The method of statistical analysis employed in this research can be useful in selecting the exponential growth regions for growth rate estimates. Growth on 2,4-D is observed over a pH range from 5.5 to 8.9. Cultures exhibiting no growth at pH 5.1 can resume normal growth when the pH is increased to 6.0. The average biomass yield with 2,4-D is 0.25 with no apparent relation between pH and biomass yield. Accumulation of 2,4-DCP during 2,4-D biodegradation is also dependent on pH; accumulation occurred at pH 5.5 and 5.7, the lowest pH levels where growth was observed. In these two cases, the accumulation of 2,4-DCP appears to have stopped the biodegradation of 2,4-D, possibly killing the microbial population. The Monod model with a half saturation constant of 5.1 mg/L provides a satisfactory description of the 2,4-D biodegradation process in the pH range from 6.0 to 8.1, but is inadequate for the low pH range where significant 2,4-DCP accumulation occurs. Protozoa can tolerate 2,4-D in the concentration range examined and, presumably by predation, cause considerable reduction in the biodegradation rate.

Experiments with 2,4-DCP as the carbon source indicate that it is strongly inhibitory at concentrations above 30 to 35 mg/L. No growth was observed in any experiments with initial 2,4-DCP concentrations above 50 mg/L. Significant reductions in 2,4-DCP concentrations occur even in the absence of microbial activity. These losses can be described by first order rate models. The growth rate and biomass yield observed with 2,4-DCP are lower than those observed for growth on 2,4-D. In order to accurately examine 2,4-DCP biodegradation the influence of factors such as photolysis and absorption or adsorption of 2,4-DCP by biomass need to be clarified. No relation between first order rate constants or growth rates and pH is evident from these 2,4-DCP experiments.

REFERENCES

1. Yang, S. S., Solomon, B. O., Oner, M. D., and Erickson, L. E., Technometrics, 26, 355-361,(1984).

2. Solomon, B. O., Erickson, L. E., and Yang, S. S., Biotechnol. Bioeng., 25, 2683-2705, (1983).

3. Buono, M., Masters Thesis, Kansas State University (1985).

4. Oner, M. D., Ph. D. Dissertation, Kansas State University, (1985).

5. Oner, M. D., Erickson, L. E., and Yang, S. S., Biotechnol. Bioeng., 28, 895-901, (1986).

6. Minkevich, I. G., and Eroshin, V. K., Folia Microbiol., 18, 376-385, (1973).

7. Erickson, L. E., Minkevich, I. G., and Eroshin, V. K., Biotechnol. Bioeng., 20, 1595-1621, (1978).

8. Erickson, L. E., Minkevich, I. G., and Eroshin, V. K., Biotechnol. Bioeng., 21, 575-591, (1979).

9. Erickson, L. E., Biotechnol. Bioeng., 22, 451-456, (1980).

10. SAS User's Guide, SAS Institute, 1982, Cary, North Carolina.

11. Tyler, J. E., and Finn, R. K., Appl. Microbiol., 28, 181-184, (1974).

12. Ferrer, A., and Erickson, L. E., Biotechnol. Bioeng., 1979, 21, 2203-2233.

13. Maki, A. W., Dickson, K. L., Cairns, J. Jr., Biotransformation and Fate of Chemicals in the Aquatic Environment, American Society for Microbiology, Washington, D. C., 1980.

14. Weast, R. C. (ed.), CRC Handbook of Chemistry and Physics, 59th edition, CRC Press, Boca Raton, Florida, (1979).

15. Hee, S. S. Q., and Sutherland, R. G., The Phenoxyalkanoic Herbicides Volume I: Chemistry, Analysis, and Environmental Pollution, CRC Press, Boca Raton, Florida, (1981).

NOMENCLATURE

C	covariate defined by eq. (9)
MSE	root mean square error
R	residual
S	substrate concentration (mg/L)
S_o	initial substrate concentration (mg/L)
t	time (h)
t_o	initial time (h)
X	biomass concentration (mg/L)
X_o	initial biomass concentration (mg/L)
$\hat{X}_i$	expected value of biomass concentration determined from the least squares fit of eq. (11)
$\overline{Y}$	average of biomass and substrate based dependent variables; defined by eqts. (8) and (10)
Y_1	biomass based dependent variable for eq. (6); $Y_1 = \ln X - \ln X_o$
Y_2	substrate based dependent variable for eq. (7); $Y_2 = \ln Z - \ln Z_o$
Y_s	biomass yield (g biomass formed /g substrate consumed)
Z	equivalent biomass concentration calculated from substrate consumption with eq. (2)
β_i	parameters in regression models
γ_b	reductance degree of biomass; equivalents of available electrons/g mol carbon. γ_b=4.291
γ_s	reductance degree of organic substrate; equivalents of available electrons/ g mol carbon

ϵ_i error terms in regression models

η available electron yield coefficient; fraction of available electrons in organic substrate that is converted to biomass

θ time minus initial time (h)

μ specific growth rate (h^{-1})

σ_b weight fraction carbon in biomass; $\sigma_b = 0.462$ (dimensionless)

σ_s weight fraction carbon in organic substrate (dimensionless)

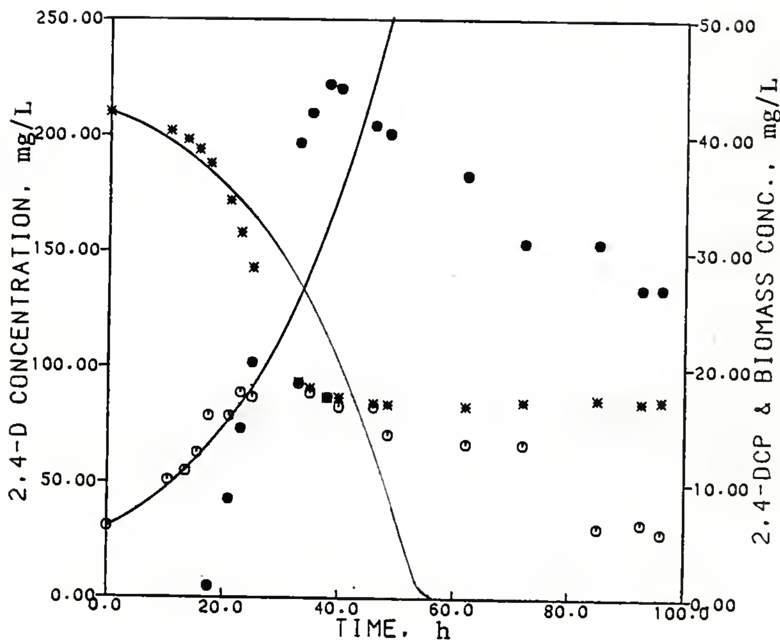

Figure 4.1. Substrate, product, and biomass concentration profiles for batch fermentation experiment B7/9V1 with 2,4-D at pH 5.5; * , 2,4-D concentration; o , biomass concentration; ● , 2,4-DCP concentration; ——— , biomass and substrate concentrations predicted by the Monod model with $\mu_m = 0.045\ h^{-1}$, $Y_s = 0.270$, $K_s = 5.1$ mg/L. Data from Table 4.2.

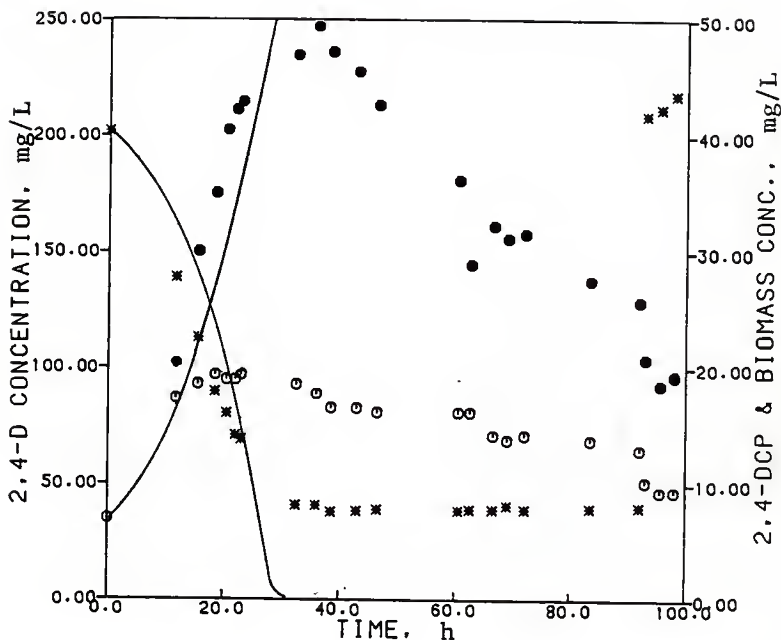

Figure 4.2. Substrate, product, and biomass concentration profiles for batch fermentation experiment B8/28V2 with 2,4-D at pH 5.7; * , 2,4-D concentration; ⊙ , biomass concentration; ● , 2,4-DCP concentration; ——, biomass and substrate concentrations predicted by the Monod model with $\mu_m = 0.078\ h^{-1}$, $Y_s = 0.244$, $K_s = 5.1$ mg/L. Data from Table 4.3.

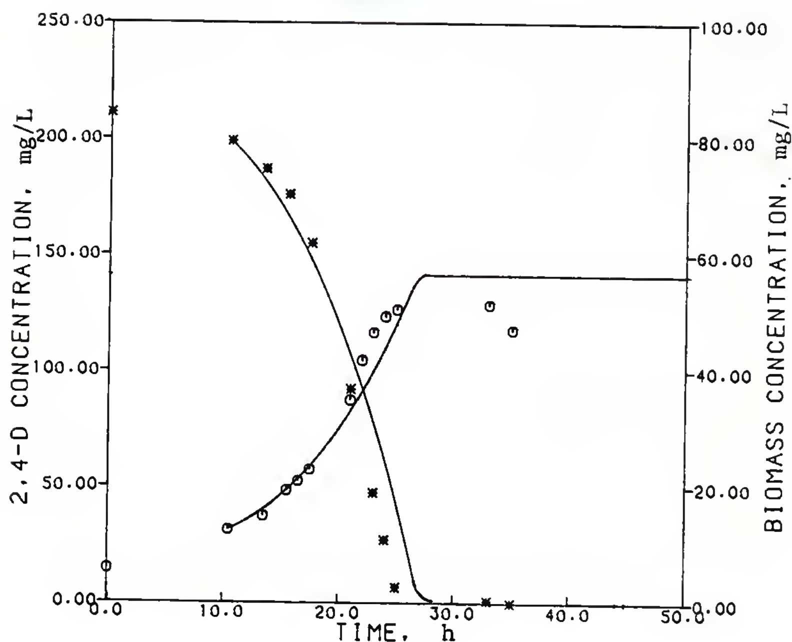

Figure 4.3. Substrate and biomass concentration profiles for batch fermentation experiment B7/9V2 with 2,4-D at pH 6.0; * , 2,4-D concentration; ⊖ , biomass concentration; ——— , biomass and substrate concentrations predicted by the Monod model with $\mu_m = 0.098\ h^{-1}$, $Y_s = 0.220$, $K_s = 5.1$ mg/L. Data from Table 4.4.

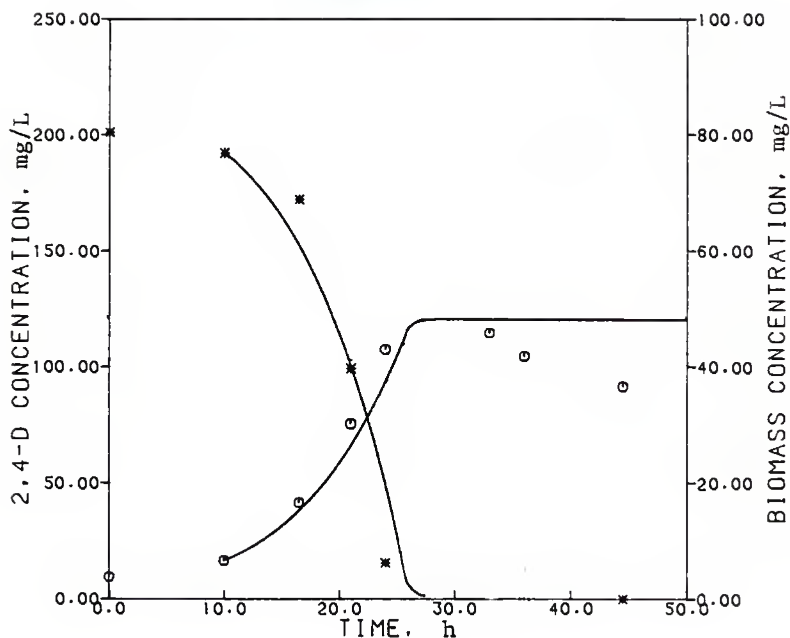

Figure 4.4. Substrate and biomass concentration profiles for batch fermentation experiment B6/12V1 with 2,4-D at pH 6.5; * , 2,4-D concentration; ʘ , biomass concentration; ——, biomass and substrate concentrations predicted by the Monod model with μ_m = 0.133 h^{-1}, Y_s = 0.218, K_s = 5.1 mg/L. Data from Table 4.5.

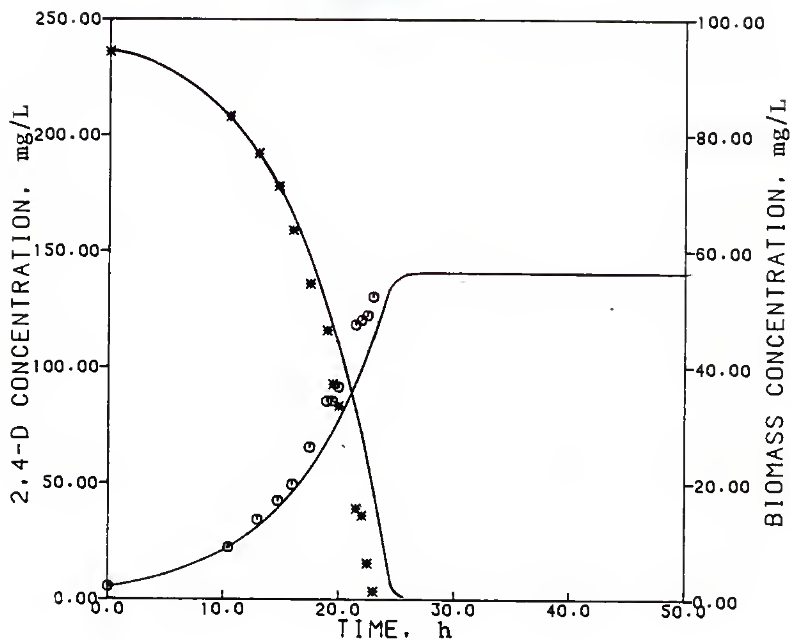

Figure 4.5. Substrate and biomass concentration profiles for batch fermentation experiment B11/30 with 2,4-D at pH 7.0; * , 2,4-D concentration; ⊙ , biomass concentration; —— , biomass and substrate concentrations predicted by the Monod model with μ_m = 0.140 h^{-1}, Y_s = 0.229, K_s = 5.1 mg/L. Data from Table 4.6.

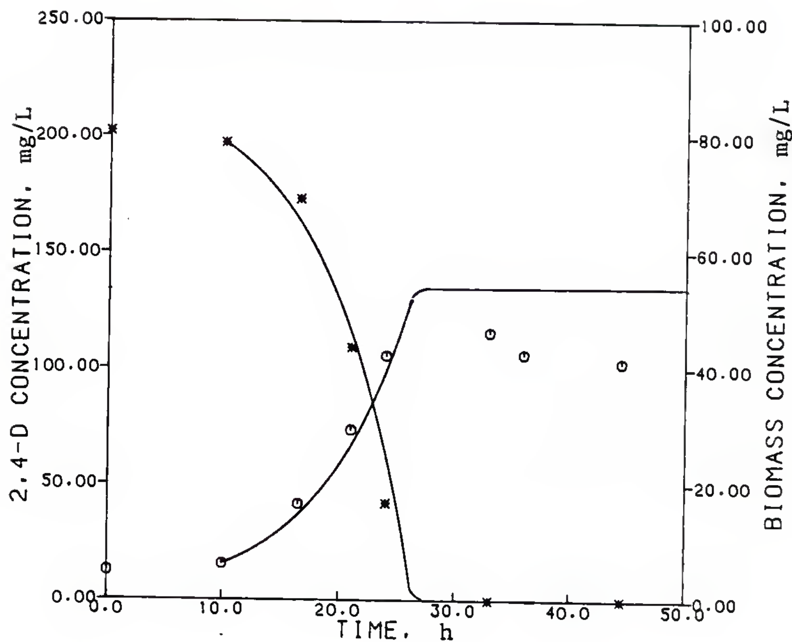

Figure 4.6. Substrate and biomass concentration profiles for batch fermentation experiment B6/12V2 with 2,4-D at pH 7.3; * , 2,4-D concentration; ⊙ , biomass concentration; —— , biomass and substrate concentrations predicted by the Monod model with μ_m = 0.139 h^{-1}, Y_s = 0.241, K_s = 5.1 mg/L. Data from Table 4.7.

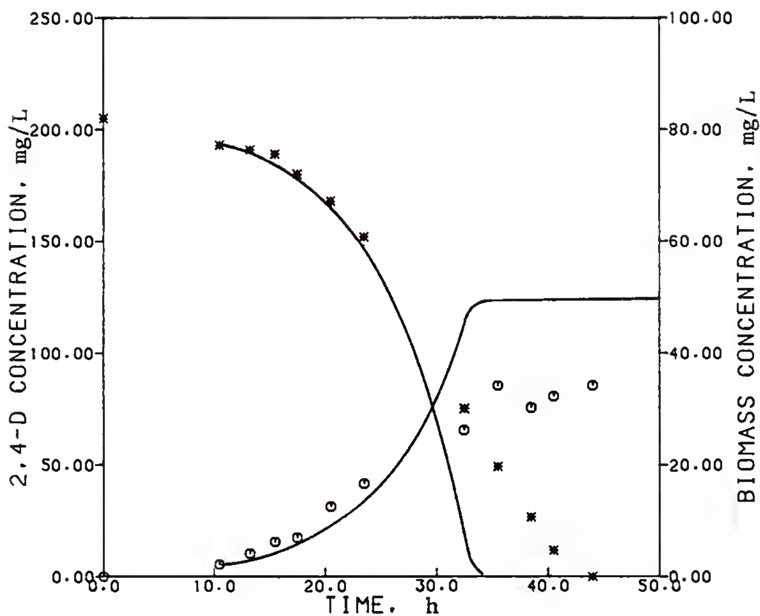

Figure 4.7. Substrate and biomass concentration profiles for batch fermentation experiment B6/16V2 with 2,4-D at pH 7.9; * , 2,4-D concentration; ⊙ , biomass concentration; ——, biomass and substate concentrations predicted by the Monod model with $\mu_m = 0.147\ h^{-1}$, $Y_s = 0.247$, $K_s = 5.1$ mg/L. Data from Table 4.8.

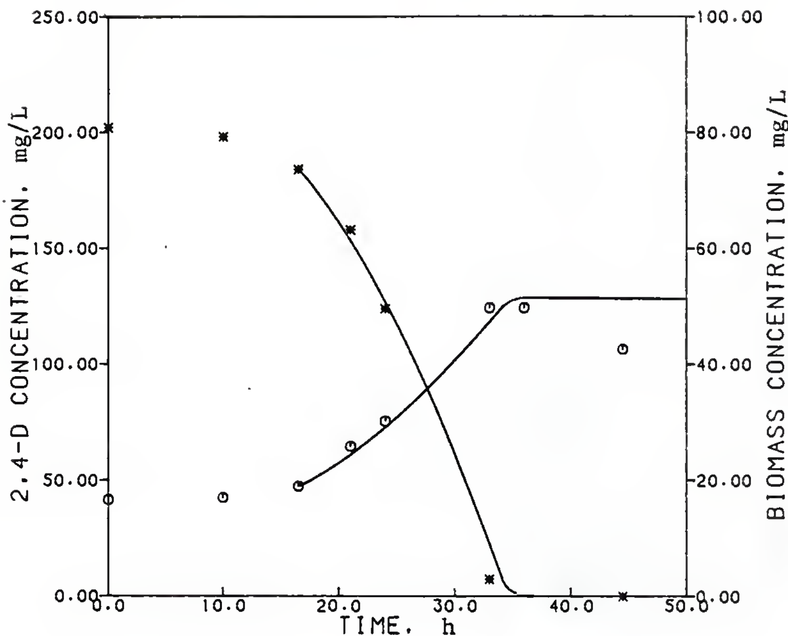

Figure 4.8. Substrate and biomass concentration profiles for batch fermentation experiment B6/12V3 with 2,4-D at pH 8.1; * , 2,4-D concentration, ⊙ , biomass concentration; ——, biomass and substrate concentrations predicted by the Monod model with $\mu_m = 0.059\ h^{-1}$, $Y_s = 0.176$, $K_s = 5.1$ mg/L. Data from Table 4.9.

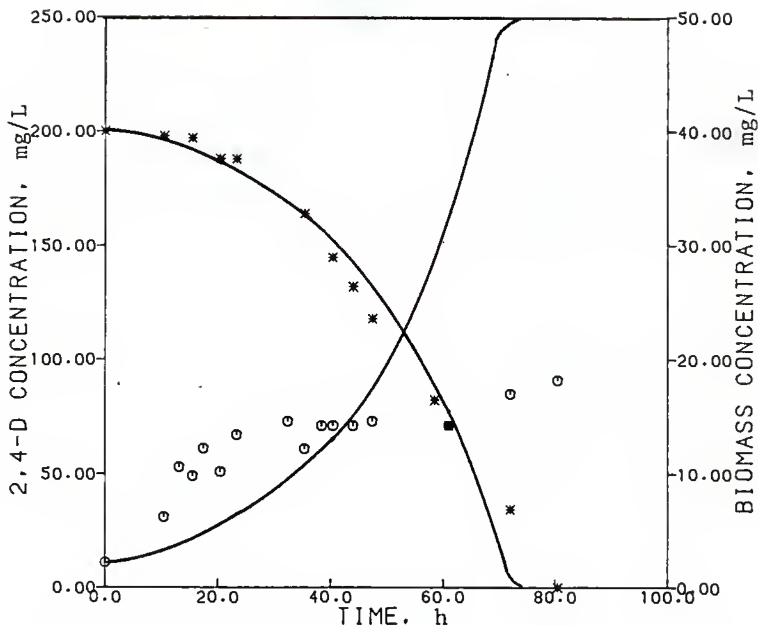

Figure 4.9. Substrate and biomass concentration profiles for batch fermentation experiment B6/16V3 with 2,4-D at pH 8.9; * , 2,4-D concentration; o , biomass concentration; ——, biomass and substrate concentrations predicted by the Monod model with μ_m = 0.046 h^{-1}, Y_s = 0.247, K_s = 5.1 mg/L. Data from Table 4.10.

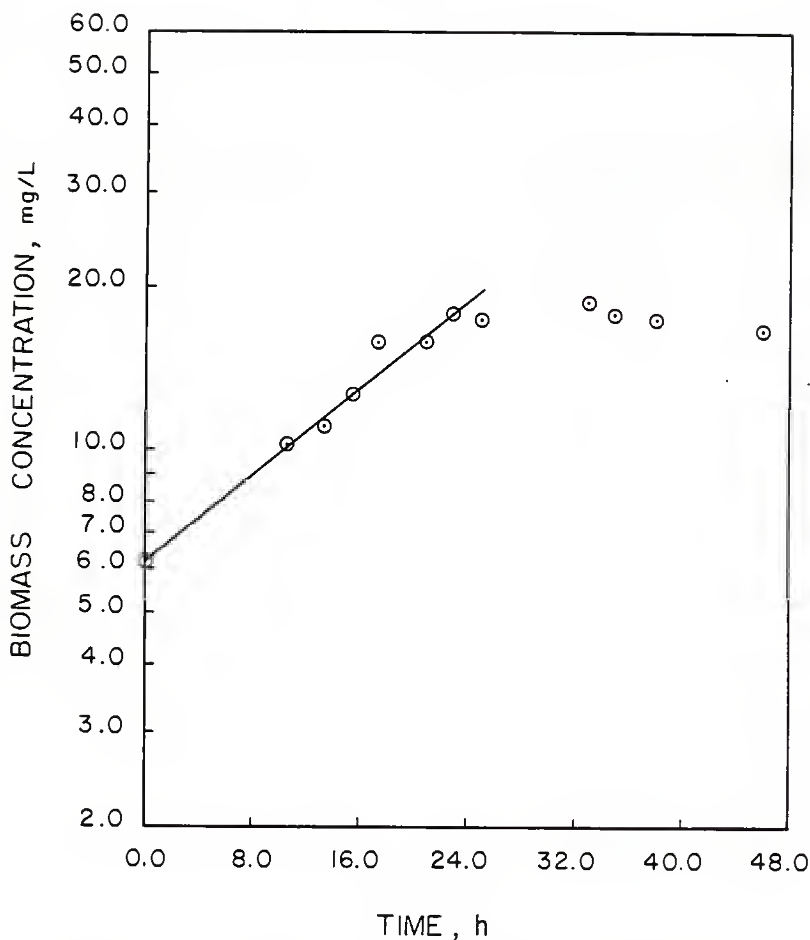

Figure 4.10. Logarithmic plot of biomass concentration against time for batch fermentation experiment B7/9V1 with 2,4-D at pH 5.5; ——, predicted biomass concentration over the exponential growth region with $\mu = 0.0452\ h^{-1}$. Data from Table 4.2.

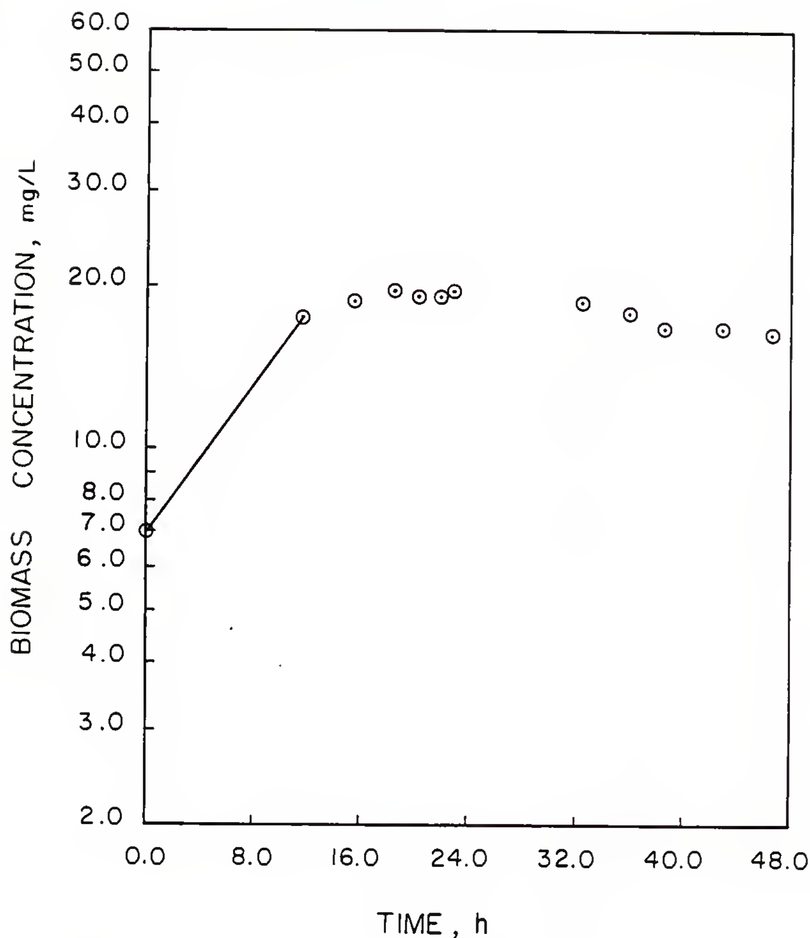

Figure 4.11. Logarithmic plot of biomass concentration against time for batch fermentation experiment B8/28V2 with 2,4-D at pH 5.7; ——, predicted biomass concentration over the exponential growth region with $\mu = 0.0775\ h^{-1}$. Data from Table 4.3.

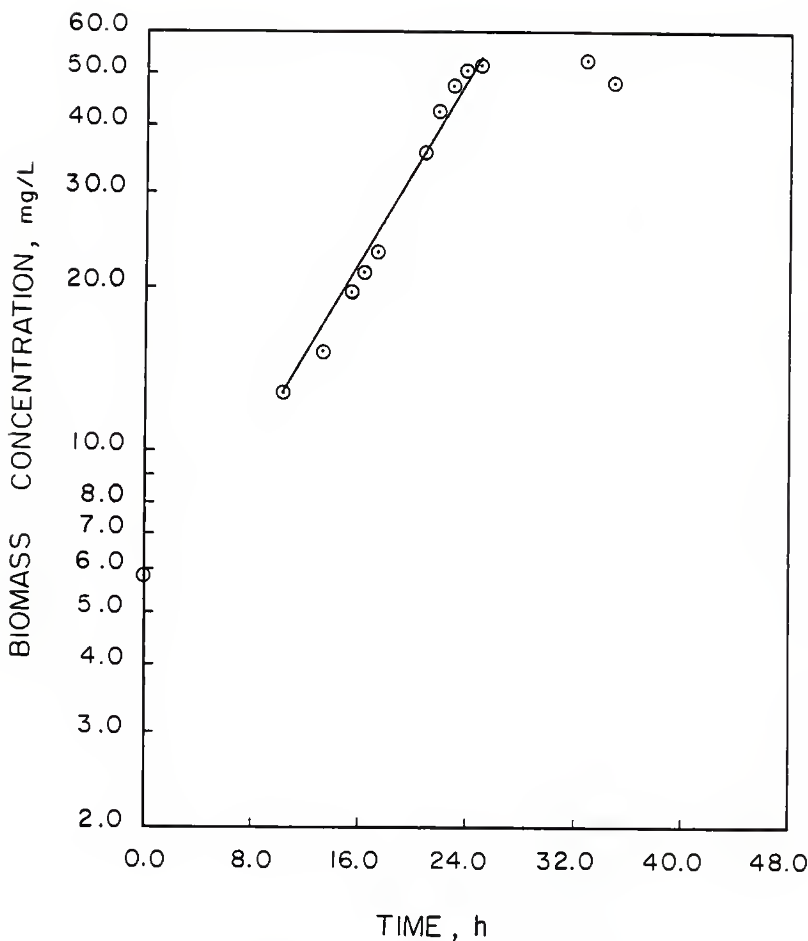

Figure 4.12. Logarithmic plot of biomass concentration against time for batch fermentation experiment B7/9V2 with 2,4-D at pH 6.0; ——, predicted biomass concentration over the exponential growth region with $\mu = 0.0978\ h^{-1}$. Data from Table 4.4.

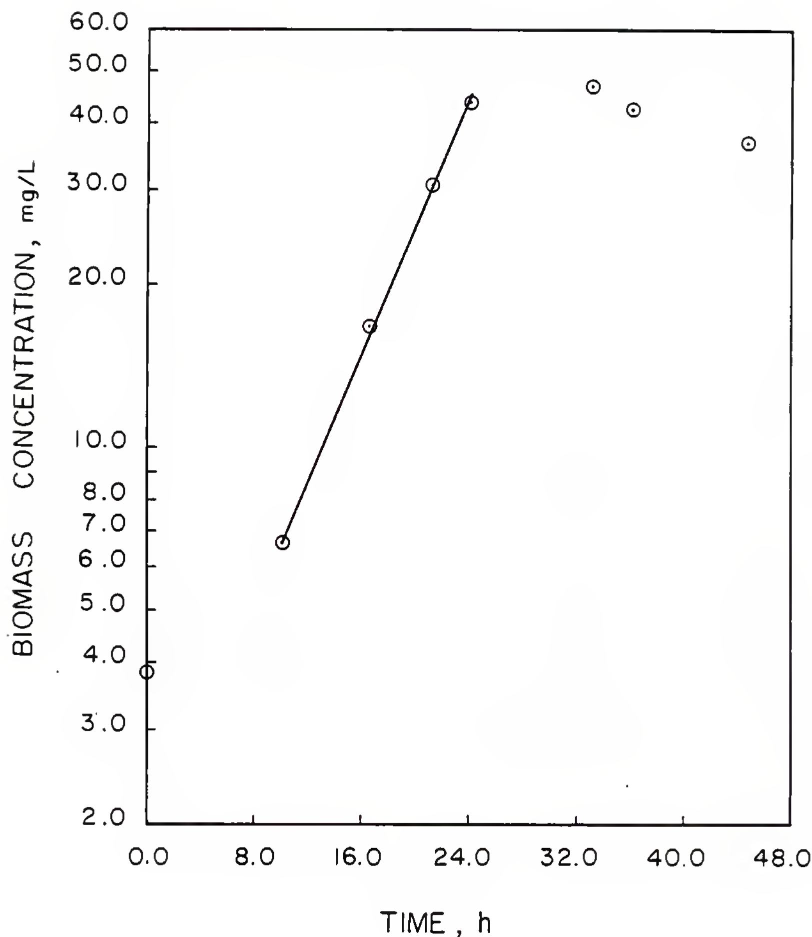

Figure 4.13. Logarithmic plot of biomass concentration against time for batch fermentation experiment B6/12V1 with 2,4-D at pH 6.5; ——, predicted biomass concentration over the exponential growth region with $\mu = 0.1336\ h^{-1}$. Data from Table 4.5.

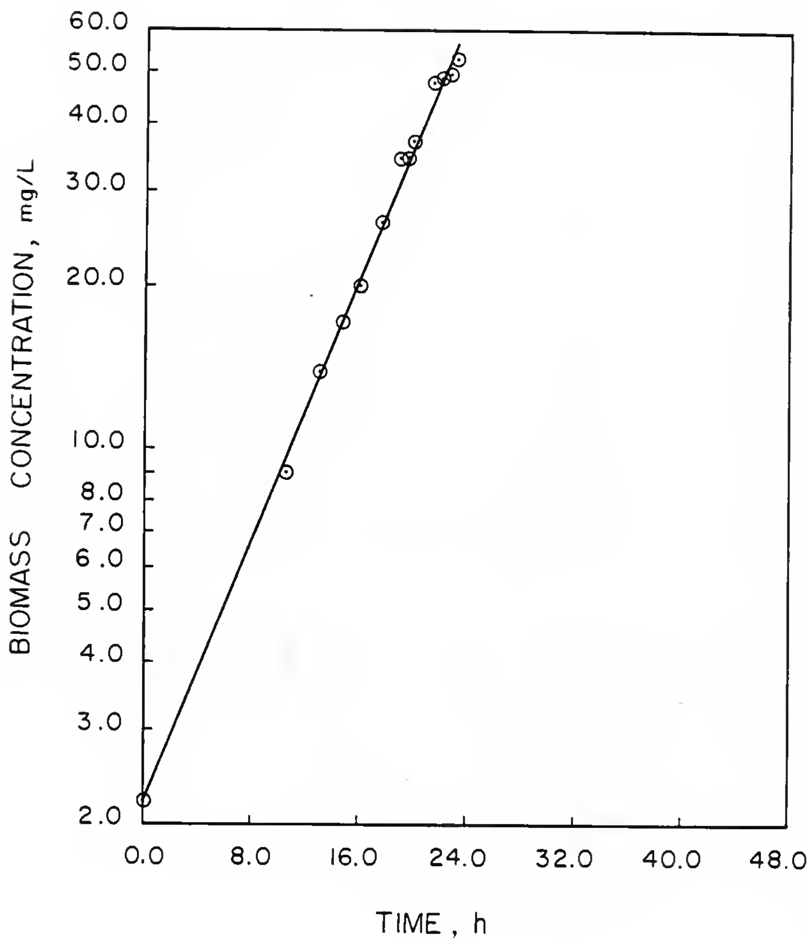

Figure 4.14. Logarithmic plot of biomass concentration against time for batch fermentation experiment B11/30 with 2,4-D at pH 7.0; ——, predicted biomass concentration over the exponential growth region with $\mu = 0.1401\ h^{-1}$. Data from Table 4.6.

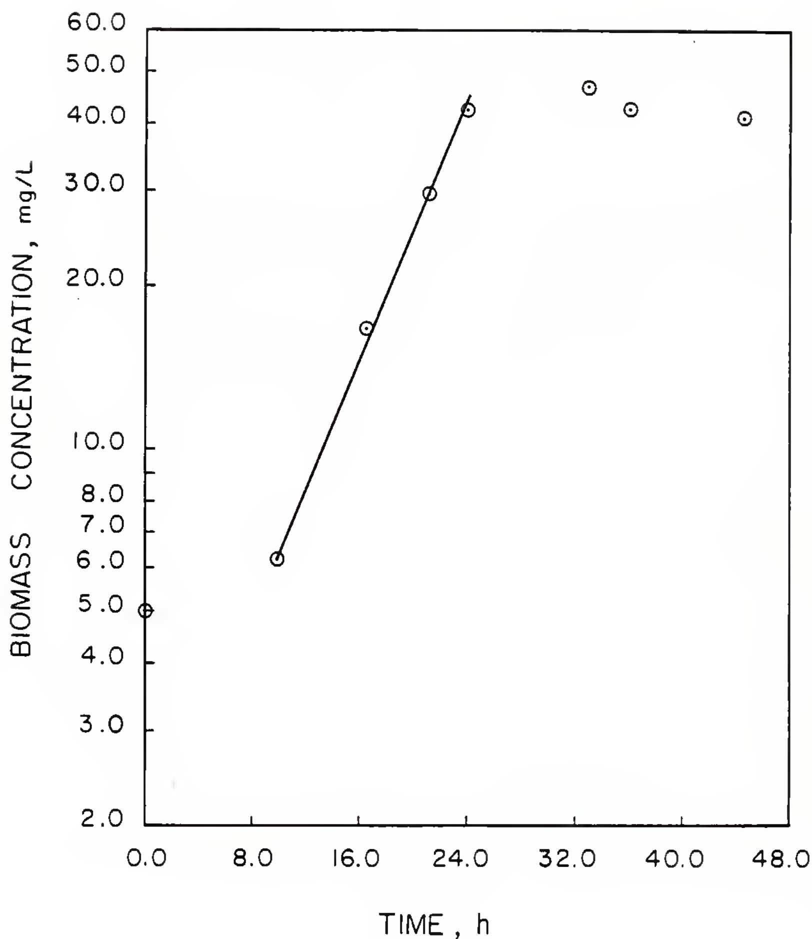

Figure 4.15. Logarithmic plot of biomass concentration against time for batch fermentation experiemnt B6/12V2 with 2,4-D at pH 7.3; ——, predicted biomass concentration over the exponential growth region with $\mu = 0.1402\ h^{-1}$. Data from Table 4.7.

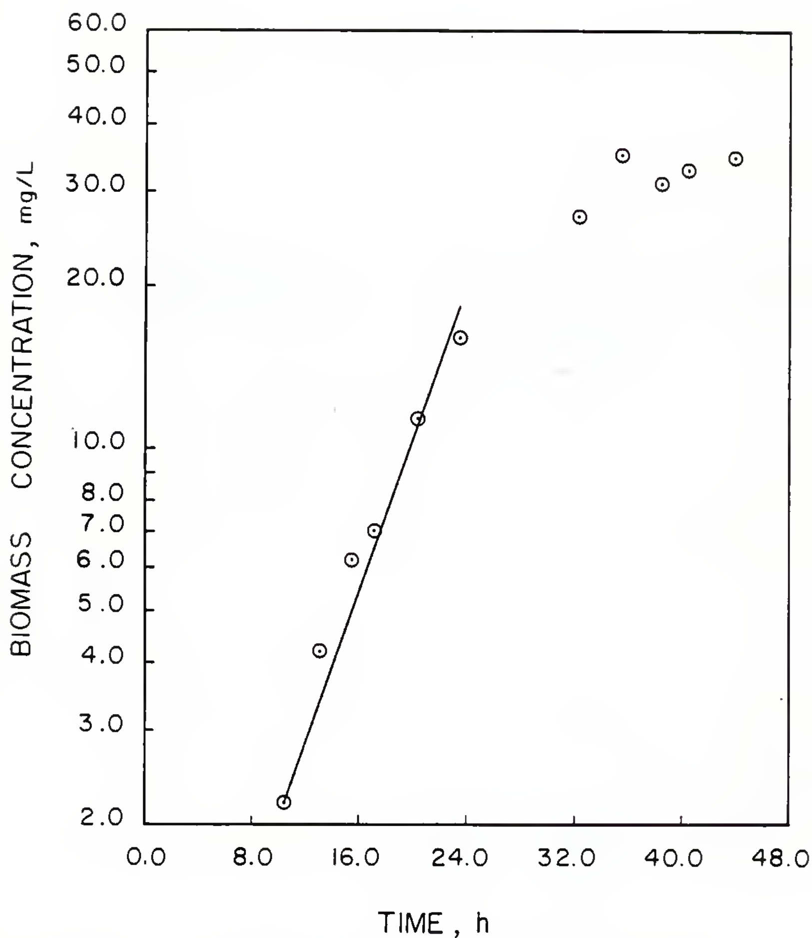

Figure 4.16. Logarithmic plot of biomass concentration against time for batch fermentation experiment B6/16V2 with 2,4-D at pH 7.9; ——, predicted biomass concentration over the exponential growth region with $\mu = 0.1677\ h^{-1}$. Data from Table 4.8.

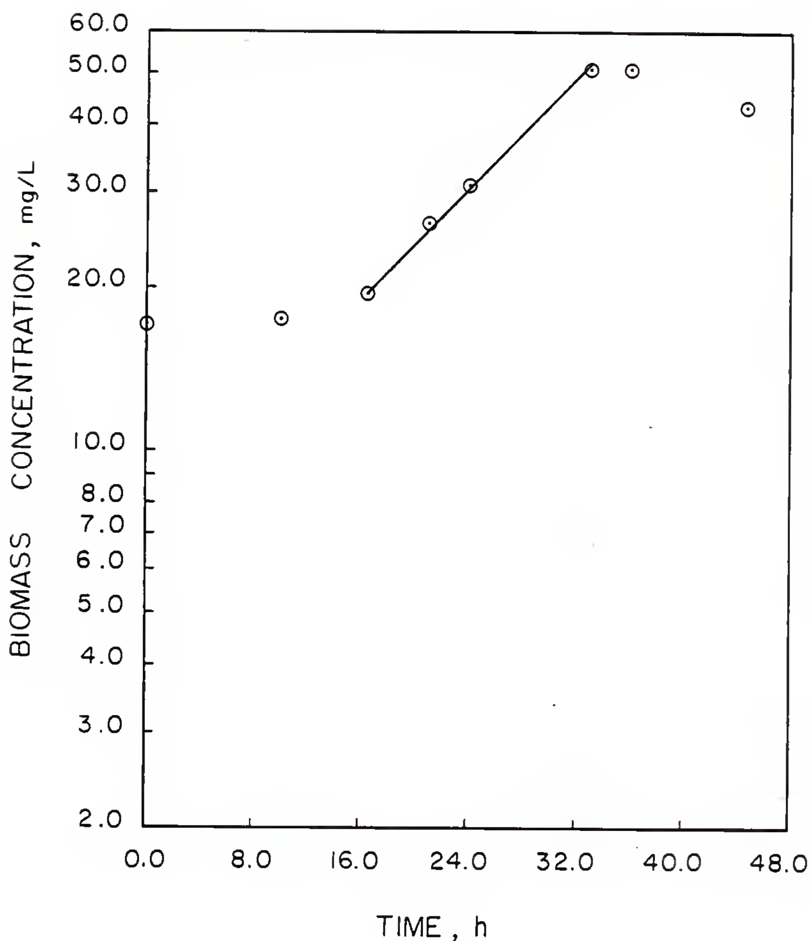

Figure 4.17. Logarithmic plot of biomass concentration against time for batch fermentation experiment B6/12V3 with 2,4-D at pH 8.1; ——, predicted biomass concentration over the exponential growth region with $\mu = 0.0595\ h^{-1}$. Data from Table 4.9.

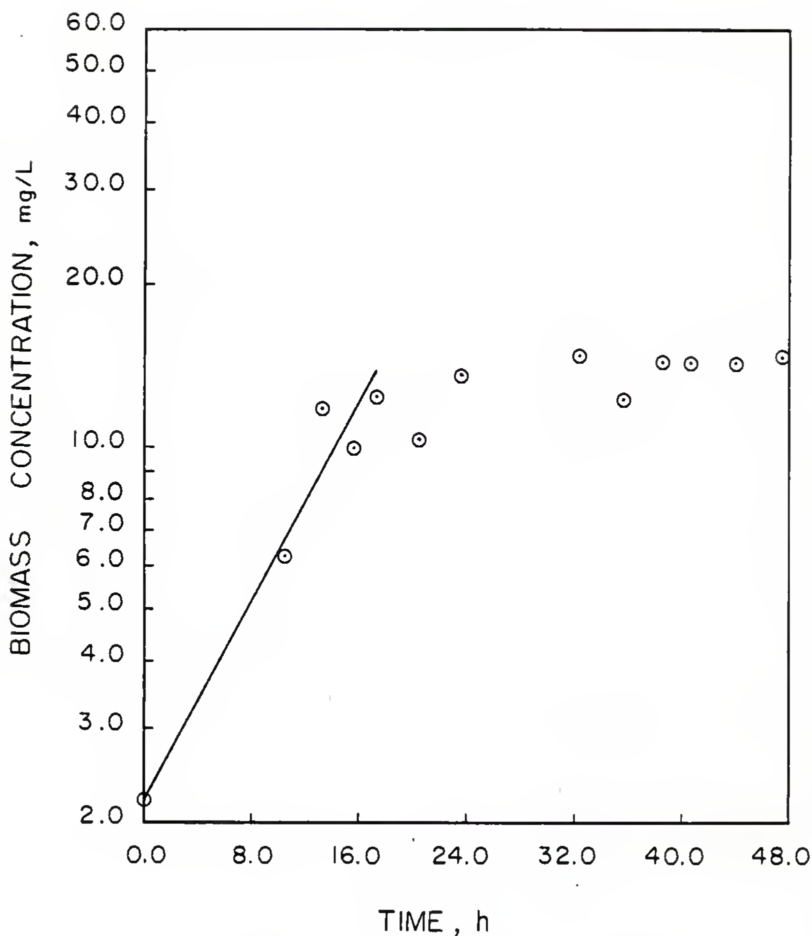

Figure 4.18. Logarithmic plot of biomass concentration against time for batch fermentation experiment B6/16V3 with 2,4-D at pH 8.9; ——, predicted biomass concentration over the exponential growth region with $\mu = 0.1022\ h^{-1}$. Data from Table 4.10.

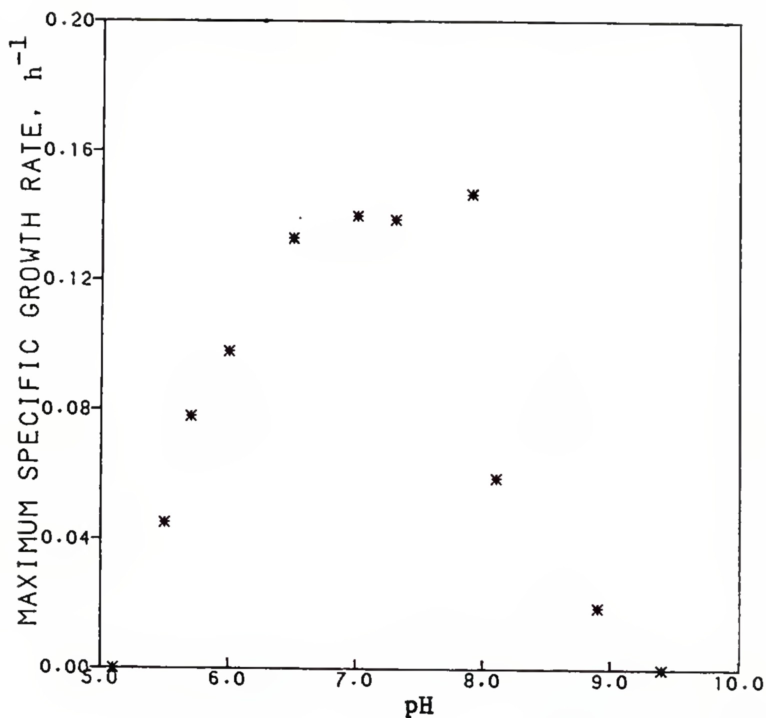

Figure 4.19. Effect of pH on the maximum specific growth rate on 2,4-D in 1-liter batch fermenters at 25 C.

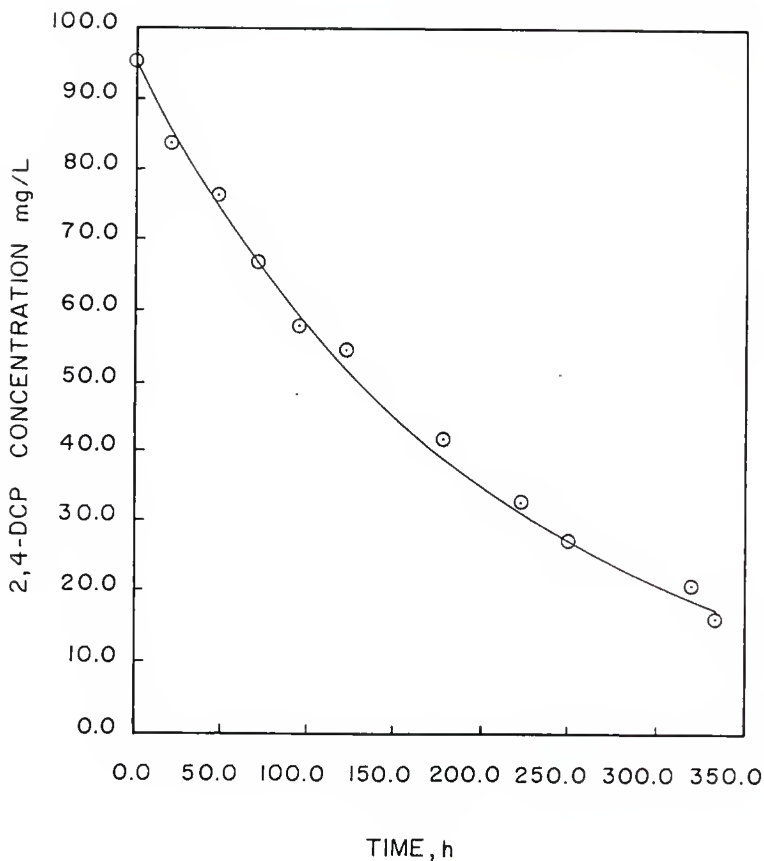

Figure 4.20 Change in 2,4-DCP concentration in the absence of microbial activity in experiment B9/12V1; o , observed 2,4-DCP concentration; —— , concentration profile predicted by a first order model with $k = 0.005\ h^{-1}$.

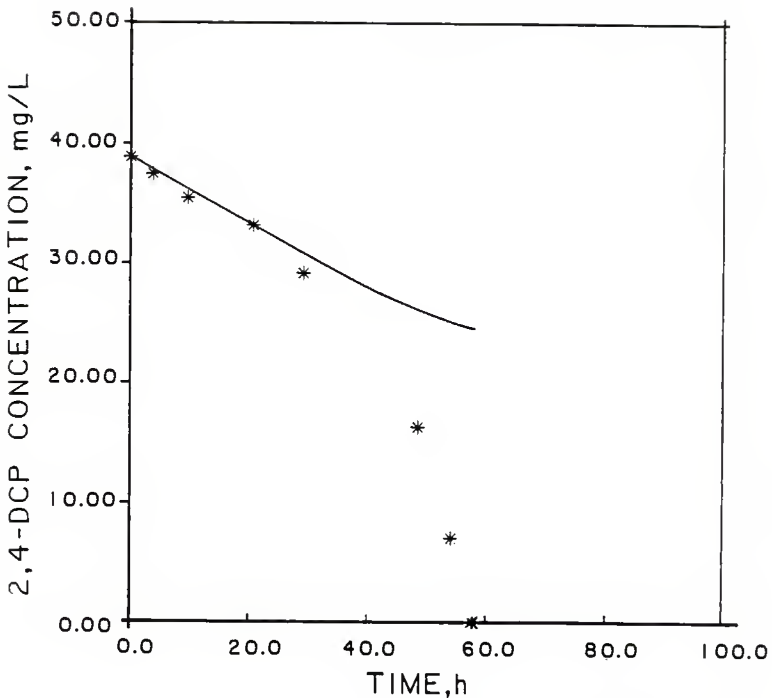

Figure 4.21. Comparison of observed 2,4-DCP concentrations and the concentration profile predicted by a non-biodegradation first order model for experiment B11/13V2B; * , observed 2,4-DCP concentration; ——, 2,4-DCP concentration predicted by a first order model with $k = 0.0079\ h^{-1}$. Data from Table 4.28.

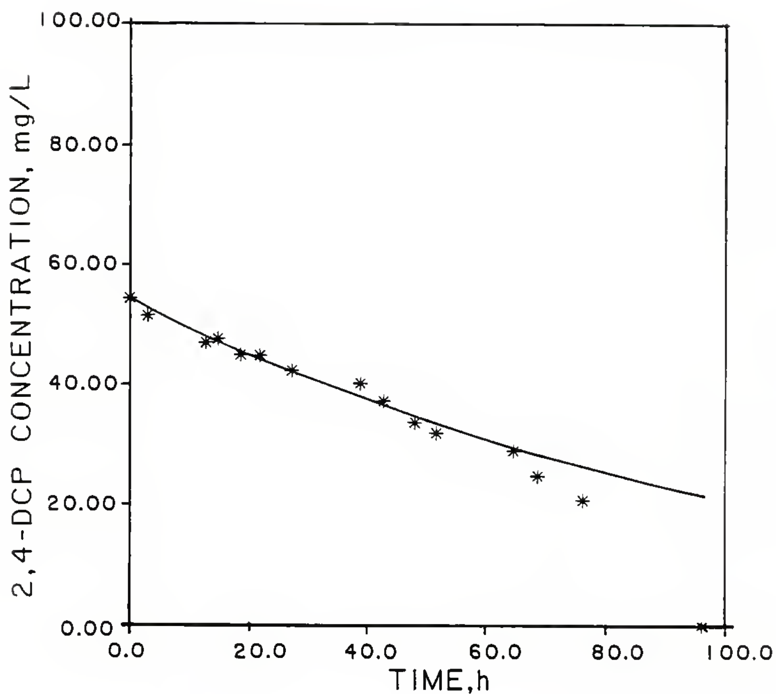

Figure 4.22. Comparison of observed 2,4-DCP concentrations and the concentration profile predicted by a non-biodegradation first order model for experiment B11/23V2; * , observed 2,4-DCP concentration; ——, 2,4-DCP concentration predicted by a first order model with k = 0.0095 h^{-1}. Data from Table 4.29.

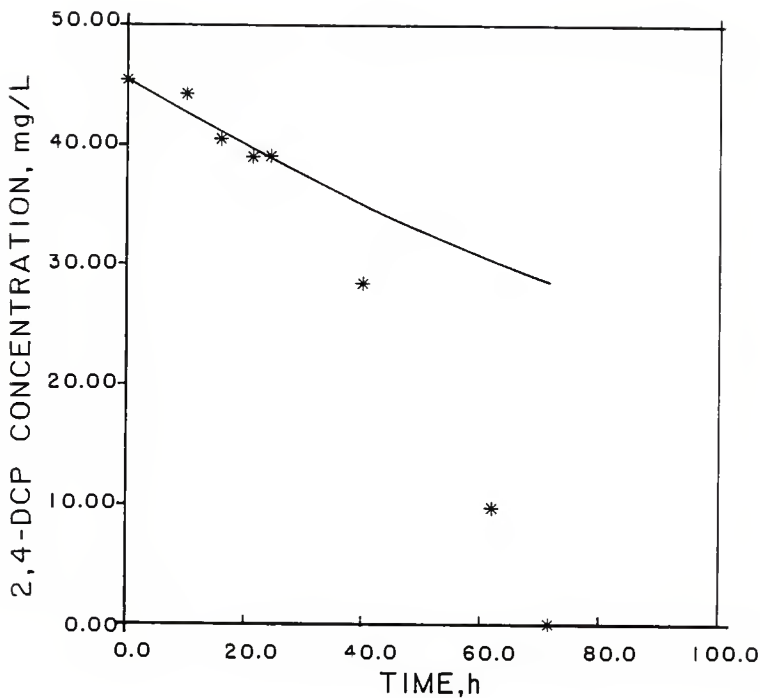

Figure 4.23. Comparison of observed 2,4-DCP concentrations and the concentration profile predicted by a non-biodegradation first order model for experiment B11/13V3; * , observed 2,4-DCP concentration;——, 2,4-DCP concentration predicted by a first order model with $k = 0.0065\ h^{-1}$. Data from Table 4.30.

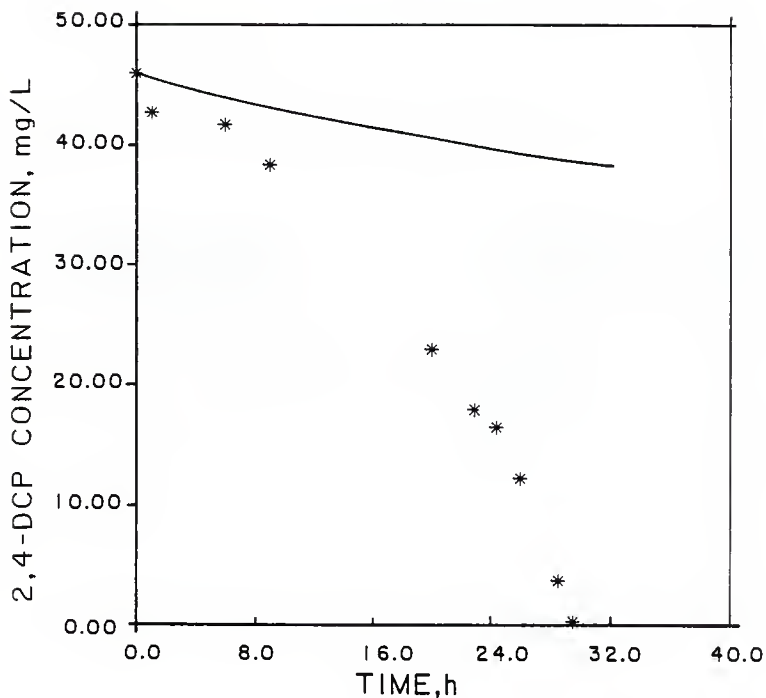

Figure 4.24. Comparison of observed 2,4-DCP concentrations and the concentration profile predicted by a non-biodegradation first order model for experiment B11/13V3B; * , observed 2,4-DCP concentration; ——, 2,4-DCP concentration predicted by a first order model with $k = 0.0065\ h^{-1}$. Data from Table 4.31.

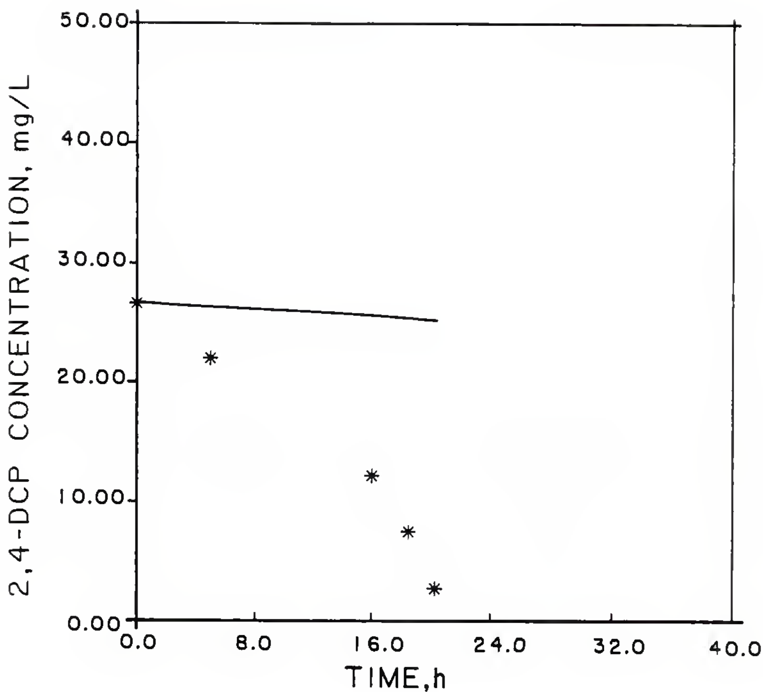

Figure 4.25. Comparison of observed 2,4-DCP concentrations and the concentration profile predicted by a non-biodegradation first order model for experiment FB11/30; * , observed 2,4-DCP concentration; ——, 2,4-DCP concentration predicted by a first order model with $k = 0.0032\ h^{-1}$. Data from Table 4.33.

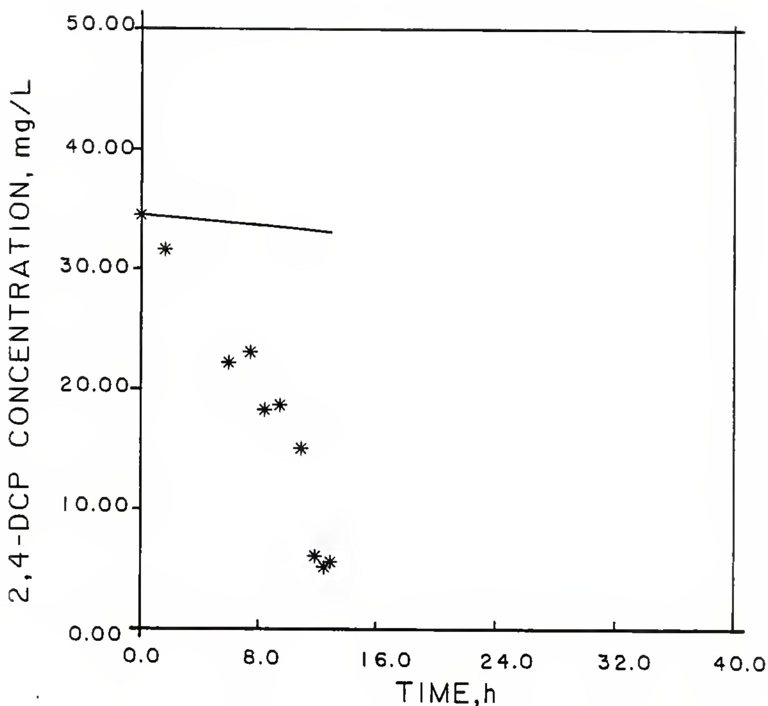

Figure 4.26. Comparison of observed 2,4-DCP concentrations and the concentration profile predicted by a non-biodegradation first order model for experiment FB11/30B; * , observed 2,4-DCP concentration; ——, 2,4-DCP concentration predicted by a first order model with $k = 0.0032\ h^{-1}$. Data from Table 4.34.

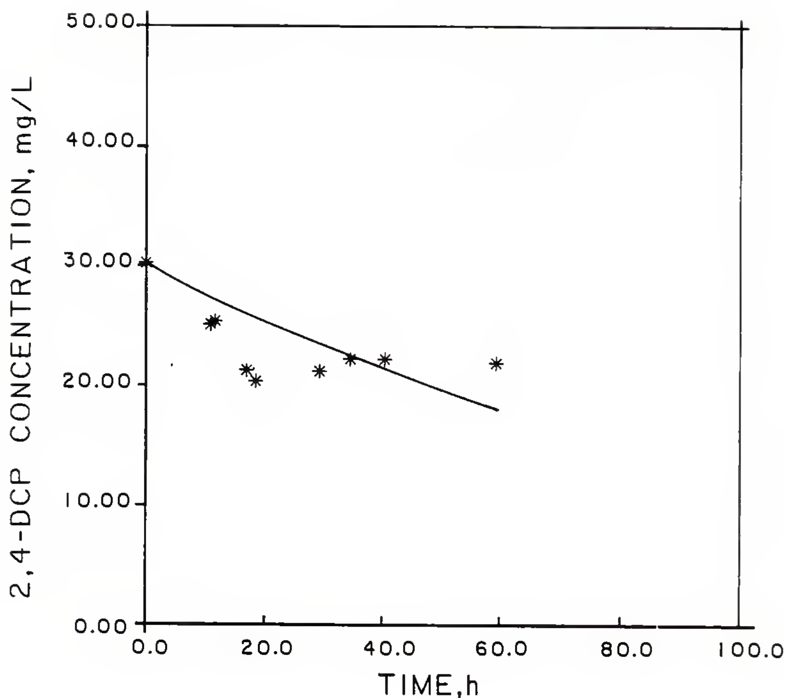

Figure 4.27. Comparison of observed 2,4-DCP concentrations and the least squares fit first order model for experiment B12/7V1; * , observed 2,4-DCP concentration; ——, 2,4-DCP concentration predicted by the best fit first order model ($k = 0.0085\ h^{-1}$). Data from Table 4.36.

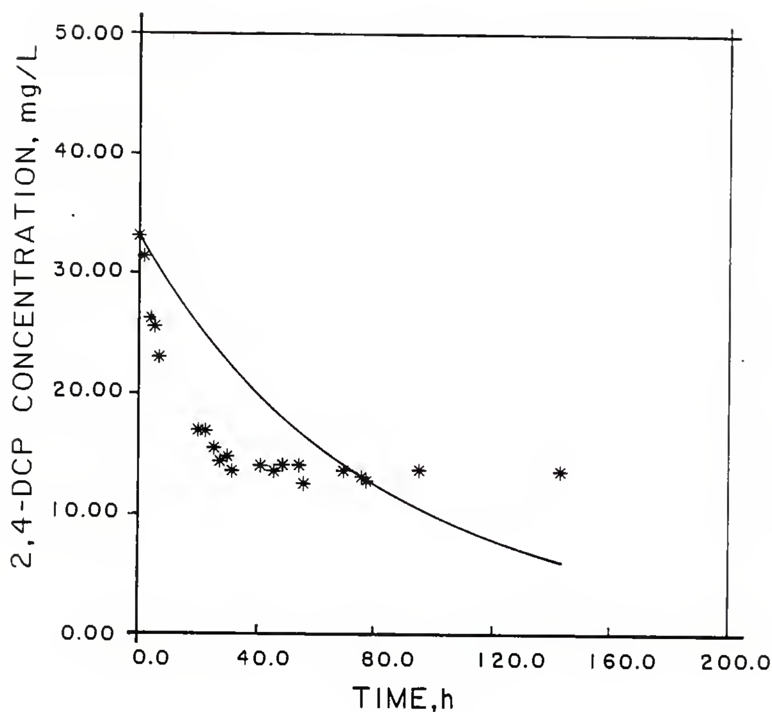

Figure 4.28. Comparison of observed 2,4-DCP concentrations and the least squares fit first order model for experiment B12/10V2; * , observed 2,4-DCP concentration; ——, 2,4-DCP concentration predicted by the best fit first order model ($k = 0.0122\ h^{-1}$). Data from Table 4.39.

Table 4.1. Batch fermentation experiment B8/28V1 with 2,4-D at pH 5.1.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] (mg/L)	pH	Sample Volume (ml)
0.0	9.0	2	200	5.13	8
11.75	5.8	2	205	5.10	50
15.5	5.8	2	202	5.12	8
18.5	5.4	2	205	5.11	40
22.0	5.4	2	206	5.11	40
32.5	5.8	2	206	5.16	30
36.0	3.4	2	-	5.18	8
38.5	3.4	2	-	5.21	8
46.5	5.0	2	-	5.26	8
61.0	3.4	2	207	5.22	24
66.5	2.6	2	208	5.24	8
72.0	3.0	2	-	5.24	8
83.5	5.8	2	209	5.28	8
98.0	2.2	2	211	5.22	8
111.0	1.8	2	211	5.24	8

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 200 mg/L; air flow rate = 650 ml/min.; inoculum volume = 125 ml; total volume at time 0, 1033 ml.

Table 4.2. Batch fermentation experiment B7/9V1 with 2,4-D at pH 5.5.

Time (hr)	Biomass Concentration (mg/L)	[2,4-D] (mg/L)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	6.2	210	0.0	5.58	8
10.5	10.2	202	0.0	5.47	8
13.5	11.0	198	0.0	5.49	8
15.5	12.6	194	0.0	5.52	40
17.5	15.8	188	1.0	5.47	8
21.0	15.8	172	8.6	5.51	30
23.0	17.8	158	14.7	5.53	20
25.0	17.4	143	20.4	5.56	8
33.0	18.6	93.4	39.4	5.65*	8
35.0	17.8	91.0	42.0	5.55	8
38.0	17.4	86.9	44.5	5.57	20
40.0	16.6	86.9	44.1	5.57	8
46.0	16.6	84.5	40.9	5.59	8
48.5	14.2	83.9	40.2	5.59	20
62.0	13.4	83.3	36.6	5.61	20
72.0	13.4	85.1	30.8	5.50	8
85.0	6.2	86.3	30.8	5.53	20
92.5	6.6	85.1	26.9	5.56	8
96.0	5.8	85.7	26.9	5.56	8

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 210 mg/L; air flow rate = 650 ml/min.; inoculum volume = 100 ml; total volume at time 0, 1007 ml.**

*Added 1 ml of 0.1M H_2SO_4 to reduce pH to 5.54.

1 ml of 0.1M NaOH was added at time 15.5 hours.

** 7 ml of 0.1M H_2SO_4 was added before time 0 making the total volume 1007 ml.

Table 4.3. Batch fermentation experiment B8/28V2 with 2,4-D at pH 5.7.

Time (hr)	Biomass Concentration (mg/L)	[2,4-D] (mg/L)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	7.0	202	0.0	5.62	8
11.5	-	-	-	6.42*	0
11.75	17.4	139	20.4	5.66	50
15.5	18.6	113	30.1	5.66	8
18.5	19.4	89.8	35.1	5.66	25
20.5	19.0	80.4	40.5	5.66	8
22.0	19.0	71.0	42.3	5.67	20
23.0	19.4	69.2	43.0	5.68	8
32.5	18.6	40.9	47.0	5.72	20
36.0	17.8	40.9	49.5	5.68	8
38.5	16.6	38.0	47.3	5.67	8
43.0	16.6	38.6	45.6	5.66	8
46.5	16.2	39.2	42.7	5.66	8
60.5	16.2	38.6	36.2	5.66	16
62.5	16.2	39.2	29.0	5.67	8
66.5	14.2	39.2	32.3	5.68	8
69.0	13.8	40.9	31.2	5.68	8
72.0	14.2	39.2	31.6	5.68	8
83.5	13.8	39.8	27.6	5.71	8
92.0	13.0	40.4	25.8	5.68	8
92.5	Added 200 ml more media with approx. 189 mg 2,4-D**				
93.0	10.2	209	20.8	5.65	8
95.5	9.4	212	18.6	5.65	8
98.0	9.4	218	19.3	5.64	8
107.5	12.2	218	17.6	5.66	8
111.0	9.4	221	17.6	5.65	8

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 202 mg/L; air flow rate = 650 ml/min.; inoculum volume = 125 ml; total volume at time 0, 1032 ml.

*23 ml of 0.1M NaOH was inadvertently added due to a siphoning problem. 5 ml of 0.1 H_2SO_4 was added to return the pH to 5.7.

**Also added 2 ml of 0.1M H_2SO_4 to make the pH 5.65.

Table 4.4. Batch fermentation experiment B7/9V2 with 2,4-D at pH 6.0.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] (mg/L)	pH	Sample Volume (ml)
0.0	5.8	0	211	6.07	8
10.5	12.6	0	199	5.96	8
13.5	15.0	2	187	5.96	8
15.5	19.4	4	176	6.00	40
16.5	21.0	4	-	5.98	8
17.5	23.0	4	155	6.00	8
21.0	35.0	9	92.2	5.99	20
22.0	41.8	9	-	5.98	8
23.0	46.6	11	47.4	6.02	15
24.0	49.4	12	27.4	6.02	15
25.0	50.6	12	6.8	6.02	8
33.0	51.4	12	1.0	6.02	8
35.0	47.0	12	0.0	6.02	8

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 211 mg/L; air flow rate = 650 ml/min.; inoculum volume = 100 ml; total volume at time 0, 1005 ml.

Table 4.5. Batch fermentation experiment B6/12V1 with 2,4-D at pH 6.5.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] mg/L	pH	Sample Volume (ml)
0.0	3.8	0	201	6.52	8
10.0	6.6	15	192	6.63	50
16.5	16.6	18	172	6.50	8
21.0	30.2	29	99.3	6.62	8
24.0	43.0	36	15.6	6.75*	15
33.0	45.8	50	0.0	6.92*	8
36.0	41.8	58	0.0	6.51	8
44.5	36.6	58	-	6.55	30

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 201 mg/L; air flow rate = 650 ml/min.; inoculum volume = 150 ml; total volume at time 0, 1042 ml.

*Added 0.1M H_2SO_4 to reduce pH to 6.5 (less than 2 ml added)

** Unusually large volume of base addition is due to siphoning.

Table 4.6. Batch fermentation experiment B11/30 with 2,4-D at pH 7.0.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] (mg/L)	pH	Sample Volume (ml)
0.0	2.2	2	236	7.02	7.5
10.5	9.0	2	208	7.02	7.5
13.0	13.8	8	192	7.05	7.5
14.75	17.0	8	178	7.03	7.5
16.0	19.8	8	159	7.00	7.5
17.5	26.2	14	136	7.03	7.5
19.0	34.2	22	116	7.05	7.5
19.5	34.2	22	92.8	7.04	7.5
20.0	36.6	22	83.3	7.03	7.5
21.5	47.4	28	39.2	7.05	7.5
22.0	48.2	28	36.2	7.04	7.5
22.5	49.0	28	15.6	7.02	7.5
23.0	52.2	28	3.8	7.01	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 236 mg/L; air flow rate = 650 ml/min.; inoculum volume = 200 ml; total volume at time 0, 2000 ml.

Table 4.7. Batch fermentation experiment B6/12V2 with 2,4-D at pH 7.3.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] (mg/L)	pH	Sample Volume (ml)
0.0	5.0	0	202	7.36	8
10.0	6.2	1	197	7.34	50
16.5	16.6	1	173	7.28	8
21.0	29.4	3	109	7.25	8
24.0	42.2	3	42.1	7.26	8
33.0	46.2	6	0.0	7.32	8
36.0	42.4	6	0.0	7.32	8
44.5	41.0	6	0.0	7.32	30

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 202 mg/L; air flow rate = 650 ml/min.; inoculum volume = 150 ml; total volume at time 0, 1042 ml.

Table 4.8. Batch fermentation experiment B6/16V2 with 2,4-D at pH 7.9.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] (mg/L)	pH	Sample Volume (ml)
0	-	0	205	7.88	50
10.5	2.2	8	193	7.95	50
13.25	4.2	8	191	7.92	8
15.5	6.2	10	189	7.92	8
17.5	7.0	11	180	7.94	8
20.5	12.6	11	168	7.92	40
23.5	16.6	12	152	7.92	8
32.5	26.2	17	75.1	7.91	8
35.5	34.2	23	49.2	8.05	35
38.5	30.2	27	26.8	8.03	8
40.5	32.2	27	11.8	8.01	8
44.0	34.2	27	0.0	8.07	30

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 205 mg/L; air flow rate = 650 ml/min.; inoculum volume = 150 ml; total volume at time 0, 1050 ml.

Table 4.9. Batch fermentation experiment B6/12V3 with 2,4-D at pH 8.1.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] (mg/L)	pH	Sample Volume (ml)
0.0	16.6	0	202	8.24	8
10.0	17.0	5	198	8.17	50
16.5	19.0	7	184	8.13	8
21.0	25.8	13	158	8.16	8
24.0	30.2	13	124	8.13	8
33.0	49.8	24	7.4	8.17	8
36.0	49.8	24	0.0	8.25	8
44.5	42.6	24	-	8.27	30

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 202 mg/L; air flow rate = 650 ml/min.; inoculum volume = 150 ml; total volume at time 0, 1042 ml.

Table 4.10. Batch fermentation experiment B6/16V3 with 2,4-D at pH 8.9.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] (mg/L)	pH	Sample Volume (ml)
0.0	2.2	0	200	8.92	50
10.5	6.2	41	198	8.94	50
13.25	10.6	49	-	8.96	8
15.5	9.8	67	197	8.96	46
17.5	12.2	74	-	8.96	8
20.5	10.2	79	188	8.94	40
23.5	13.4	82	188	8.93	8
32.5	14.6	91	-	8.95	8
35.5	12.2	94	164	8.93	35
38.5	14.2	98	-	8.93	8
40.5	14.2	102	145	8.97	8
44.0	14.2	106	132	8.96	40
47.5	14.6	108	118	8.96	8
58.5	8.2*	108	82.2	8.93	42
61.0	14.2*	110	71.0	8.93	8
72.0	17.0*	115	34.5	8.93	8
80.5	18.2*	115	0.0	8.94	8

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 200 mg/L; air flow rate = 650 ml/min.; inoculum volume = 150 ml; total volume at time 0, 1050 ml.

*Fuzzy clumps observed between the baffels and the vessel wall.

Table 4.11. Batch fermentation experiment B7/9V3 with 2,4-D at pH 9.4.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] (mg/L)	pH	Sample Volume (ml)
0.0	5.8	0	210	9.46	8
10.5	8.2	57	206	9.29	8
13.5	7.0	68	204	9.43	8
15.5	4.6	82	-	9.42	50
17.5	6.6	96	-	9.41	8
21.0	2.6	112	-	9.40	50
25.0	6.6	122	-	9.43	8
33.0	5.8	139	-	9.44	8
35.0	7.0	143	204	9.40	8
38.0	3.0	159	-	9.45	50
48.5	3.4	201	-	9.44	50
62.0	2.6	214	-	9.44	50
72.0	5.0	219	-	9.44	8
85.0	-	222	-	9.41	50
92.5	-	227	208	9.44	8

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 210 mg/L; air flow rate = 650 ml/min.; inoculum volume = 100 ml; total volume at time 0, 1000 ml.

Table 4.12. Results of statistical analysis used to select the interval of exponential growth for the pH 6.0, 7.0, 7.9, and 8.9 experiments with 2,4-D.

Experiment	pH	time interval	p value	MSE	R_i	R_f
B7/9V2*	6.0	10.5-25	0.3171	0.0598	0.0835	-0.0625
B7/9V2	6.0	0-25	0.0015	0.1018	0.1529	0.0054
B11/30	7.0	10.5-22	0.5863	0.0356	-0.0201	-0.0367
B11/30	7.0	0-22	0.172	0.0397	0.0342	-0.0186
B11/30	7.0	0-22.5	0.5892	0.0432	0.0237	-0.0611
B11/30	7.0	10.5-22,23	0.1437	0.0450	-0.0358	-0.0774
B11/30	7.0	10.5-23	0.0759	0.0470	-0.0429	-0.0639
B11/30*	7.0	0-23	0.9956	0.0454	0.0143	-0.0581
B6/16V2	7.9	13.25-23.5	0.7762	0.0859	-0.0224	-0.0409
B6/16V2*	7.9	10.5-23.5	0.1557	0.1268	-0.1427	-0.1019
B6/16V3*	8.9	0-17.5	0.5832	0.1363	-0.0255	-0.0672
B6/16V3	8.9	0-20.5	0.1012	0.2377	-0.1387	-0.3218
B6/16V3	8.9	0-23.5	0.0380	0.2430	-0.2142	-0.2077

* Interval selected

MSE, root mean square error; R_i, residual for the initial point in the time interval; R_f, residual for the final point in the time interval.

Table 4.13 Point and 95% confidence interval estimates for the maximum specific growth rate, μ_m, in batch fermentations growing on 2,4-D.

Culture	pH	Regression Equation	Point Estimate	95% Confidence Interval	
B7/9V1	5.5	6	0.0452	[0.0419	0.0485]
B7/9V1	5.5	7	0.0369*	[0.0328	0.0410]
B7/9V1	5.5	10	0.0417*	[0.0382	0.0452]
B7/9V1	5.5	7	0.0393	[0.0351	0.0435]
B7/9V1	5.5	10	0.0427	[0.0397	0.0457]
B8/28V2	5.7	6+	0.0775	-	
B8/28V2	5.7	7+	0.0781*	-	
B8/28V2	5.7	10+	0.0778*	-	
B7/9V2	6.0	6 (10 points)	0.0982	[0.0929	0.1035]
B7/9V2	6.0	6 (8 points)	0.0978	[0.0917	0.1039]
B7/9V2	6.0	7 (8 points)	0.1058*	[0.0980	0.1136]
B7/9V2	6.0	10 (8 points)	0.0963*	[0.0828	0.1098]
B7/9V2	6.0	7 (8 points)	0.0991	[0.0912	0.1070]
B7/9V2	6.0	10 (8 points)	0.0976	[0.0906	0.1046]
B6/12V1	6.5	6	0.1336	[0.1295	0.1377]
B6/12V1	6.5	7	0.1305*	[0.0963	0.1647]
B6/12V1	6.5	10	0.1333*	[0.1295	0.1371]
B6/12V1	6.5	7	0.1226	[0.0884	0.1568]
B6/12V1	6.5	10	0.1326	[0.1285	0.1367]
B11/30	7.0	6	0.1401	[0.1386	0.1416]
B11/30	7.0	7	0.1430*	[0.1412	0.1448]
B11/30	7.0	10	0.1412*	[0.1394	0.1430]
B11/30	7.0	7	0.1393	[0.1413	0.1373]
B11/30	7.0	10	0.1398	[0.1384	0.1412]
B6/12V2	7.3	6	0.1402	[0.1319	0.1485]
B6/12V2	7.3	7	0.1350*	[0.1135	0.1565]
B6/12V2	7.3	10	0.1388*	[0.1355	0.1421]
B6/12V2	7.3	7	0.1334	[0.1116	0.1552]
B6/12V2	7.3	10	0.1383	[0.1350	0.1416]

Table 4.13 continued

Culture	pH	Regression Equation	Point Estimate	95% Confidence Interval
B6/16V2	7.9	6	0.1677	[0.1479 0.1875]
B6/16V2	7.9	7	0.1269*	[0.1074 0.1464]
B6/16V2	7.9	10	0.1472*	[0.1367 0.1577]
B6/16V2	7.9	7	0.1601	[0.1404 0.1798]
B6/16V2	7.9	10	0.1639	[0.1552 0.1726]
B6/12V3	8.1	6	0.0595	[0.0550 0.0640]
B6/12V3	8.1	7	0.0726*	[0.0679 0.0773]
B6/12V3	8.1	10	0.0658*	[0.0509 0.0807]
B6/12V3	8.1	7	0.0581	[0.0535 0.0627]
B6/12V3	8.1	10	0.0588	[0.0566 0.0610]
B6/16V3	8.9	6	0.1022	[0.0908 0.1136]
B6/16V3	8.9	7	0.0189*	[0.0187 0.0191]
B6/16V3	8.9	10	0.0182*	[0.0157 0.0207]
B6/16V3	8.9	7	0.0180	[0.0177 0.0183]
B6/16V3	8.9	10	0.0181	[0.0145 0.0217]
B6/16V3	8.9	7	0.0459**	[0.0405 0.0513]

* The average biomass yield, 0.247, was used to make the maximum specific growth rate estimates marked by asterisks.

\+ The estimates for B8/28V2 are based on only two points.

** This estimate is based on the exponential region selected using substrate data instead of biomass data; the interval selected is time = 0 to 47.5 hours.

Table 4.14. Yield and maximum specific growth rate estimates for batch fermentations growing on 2,4-D at different pH.

Culture	pH	Y_s (g/g)	μ_m (1/h)	N	time span used for parameter estimates (h)
B8/28V1	5.1	-	0.0	-	-
B7/9V1	5.5	0.270	0.045	8	0.0 to 25.0
B8/28V2	5.7	0.244	0.078	2	0.0 to 11.75
B7/9V2	6.0	0.220	0.098	10	10.5 to 25.0
B6/12V1	6.5	0.218	0.133	4	10.0 to 24.0
B11/30	7.0	0.229	0.140	13	0.0 to 23.0
B6/12V2	7.3	0.241	0.139	4	10.0 to 24.0
B6/16V2	7.9	0.388	0.147	6	10.5 to 23.5
B6/12V3	8.1	0.176	0.059	4	16.5 to 33.0
B6/16V3	8.9	0.233	0.019	6	0.0 to 17.5
B7/9V3	9.4	-	0.0	-	-

μ_m, maximum specific growth rate as selected by comparing the estimates obtained from regression equations (6),(7), and (10) using $Y_s = Y_{Ave} = 0.247$ and selecting the estimate with the smallest 95% confidence interval; N, number of points used for Y_s and μ_m estimates; Y_s, biomass yield (g biomass produced per g substrate consumed).

Table 4.15. Experiment B6/17V1: Water, 2,4-D, and 2,4-DCP loss in the absence of microbial activity.

Time (hr)	Volume before sample (ml)	[2,4-D] (mg/L)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	1000	75.7	58.1	6.82	8
20.5	990	80.7	60.3	6.68	8
44.0	972	81.0	51.8	6.73	8
72.0	957	82.8	46.6	-	8
107.0	937	83.3	44.5	6.88	8
165.0	905	87.5	39.1	7.03	8
286.0	858	91.6	32.1	7.21	8
496.0	788	101.0	24.4	7.26	8

T = 25 C; impeller speed = 700 rpm; condenser water T = 22 C air flow rate = 650 ml/min.; total volume at time 0, 1000 ml

Table 4.16. Experiment B9/12V1: Changes in 2,4-D and 2,4-DCP concentration in the absence of microbial activity.

Time (hr)	2,4-D Concentration (mg/L)	2,4-DCP Concentration (mg/L)
0.0	207	95.8
23.25	213	83.6
48.0	216	76.1
72.0	219	66.7
96.25	219	57.8
122.75	221	54.5
177.25	217	41.6
223.25	215	32.6
249.25	222	27.2
318.25	220	20.8
332.75	-	16.1

T = 25 C; impeller speed = 700 rpm; condenser water T =22 C; air flow rate = 400 ml/min.; volume = 1000 ml.

Table 4.17. Experiment B9/12V2: Changes in 2,4-D and 2,4-DCP concentration in the absence of microbial activity.

Time (hr)	2,4-D Concentration (mg/L)	2,4-DCP Concentration (mg/L)
0.0	208	98.7
23.25	216	88.3
48.0	219	74.6
72.0	221	62.8
96.25	223	56.0
122.75	223	45.2
177.25	221	31.9
223.25	216	21.5
249.25	223	18.3
318.25	223	12.5
332.75	221	10.0

T = 25 C; impeller speed = 700 rpm; condenser water T =22 C; air flow rate = 400 ml/min.; initial volume = 1000 ml.

Table 4.18. Experiment B9/12V3: Changes in 2,4-D and 2,4-DCP concentration in the absence of microbial activity.

Time (hr)	2,4-D Concentration (mg/L)	2,4-DCP Concentration (mg/L)
0.0	213	106.0
23.25	221	96.2
48.0	220	81.8
72.0	221	71.8
96.25	218	60.6
122.75	214	54.5
177.25	213	39.1
223.25	219	29.0
249.25	208	25.1
318.25	210	17.9
332.75	207	13.6

T = 25 C; impeller speed = 700 rpm; condenser water T = 9 C; air flow rate = 400 ml/min; initial volume = 1000 ml.

Table 4.19. Experiment B9/12V4: Changes in 2,4-D and 2,4-DCP concentration in the absence of microbial activity.

Time (hr)	2,4-D Concentration (mg/L)	2,4-DCP Concentration (mg/L)
0.0	211	112
23.25	218	97.6
48.0	218	84.0
72.0	215	70.7
96.25	218	60.3
122.75	205	51.3
177.25	215	34.1
223.25	218	24.0
249.25	218	20.1
318.25	215	14.0
332.75	213	11.1

T = 25 C; impeller speed = 700 rpm; condenser water T = 9 C; air flow rate = 650 ml/min.; initial volume = 2000 ml.

Table 4.20. Summary of observed changes in 2,4-D and 2,4-DCP concentrations in the absence of microbial activity.*

Experiment	pH	V (ml)	T_c (C)	T_m (C)	$D_{2,4-D}$ (mg/L h)	k_{DCP} (1/h)	r_k^2
B6/17V1	7.0	1000	22	25	+0.046	0.00191	0.9765
B9/12V1	6-7	1000	22	25	+0.026	0.00500	0.9973
B9/12V2	6-7	1000	22	25	+0.021	0.00665	0.9986
B9/12V3	6-7	1000	9	25	-0.032	0.00581	0.9981
B9/12V4	6-7	2000	9	25	+0.0013	0.00676	0.9990
B8/28V2	5.7	1000	22	25	-	0.01427	0.9869
B7/9V1	5.5	1000	22	25	-	0.00889	0.9905

V, vessel size; T_c, temperature of outlet condenser water; T_m, temperature of fermentation media; $D_{2,4-D}$, linear regression estimate of the rate of change of the 2,4-D concentration; k_{DCP}, the first order rate constant for the disappearance of 2,4-DCP ($-d[DCP]/dt = k[DCP]$); r_k^2, the square of the correlation coefficient for the k_{DCP} estimate.

* The pH 5.5 and 5.7 experiments may include effects of microbial activity.

Table 4.21. Batch fermentation experiment B5/12V1 with 2,4-D at pH 7.0.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] (mg/L)	pH	Sample Volume (ml)
1.25	2.2	64	218	6.96	13
4.75	0.2	137	-	6.96	15.5
8.75	1.8	164	205	6.99	50
17.75	2.2	169	-	6.96	8
21.5	1.8	171	205	6.96	8
26.5	3.0	187	-	6.96	8
27.0	-	187	-	-	13
33.0	3.0	209	205	6.95	7
41.75	1.8	209	-	7.02	7
49.25	1.4	216	201	6.97	9
55.25	2.2	226	-	6.97	7
66.5	5.4	226	-	7.01	11
69.5	2.2	226	-	7.00	12
72.5	2.2	227	201*	6.98	10
76.25	4.2	228	-	6.97	8
79.75	2.2	228	-	6.99	8
90.5	7.0	228	195	6.98	7
114.5	10.6	232	175*	7.00	8
140.0	14.6	235	95.7*	7.01	8
144.5	12.6	235	88.7	7.01	8
152.0	11.8	235	66.9	6.99	7
163.5	16.6	235	36.8	6.95	9
168.5	18.6	240	20.9	6.96	7
175.25	19.0	245	0.0	6.99	9
187.0	17.4	245	0.0	6.99	8

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 218 mg/L; air flow rate = 1450 ml/min.; inoculum volume = 100 ml; total volume at time 0, 900 ml.

*2,4-DCP at concentrations of 1.0, 2.8, and 0.3 mg/L was detected at times 72.5, 114.5, and 140 hours, respectively.

Table 4.22. Batch fermentation experiment B5/12V2 with 2,4-D at pH 7.0.

Time (hr)	Biomass Concentration (mg/L)	[2,4-D] (mg/L)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
1.25	0.0	64.5	0.0	7.01	13
4.75	0.0	63.3	0.0	7.01	10
8.75	0.0	-	-	7.01	11
17.75	1.0	-	-	7.02	9
21.25	0.2	-	-	7.01	8
26.5	1.8	-	-	7.01	9
27.0	-	57.2	1.0	-	7
33.0	0.0	-	-	7.00	7
41.75	2.2	-	-	7.00	7
49.25	1.0	-	-	6.99	8
55.25	2.2	46.2	0.0	6.99	8
66.5	1.4	-	-	6.99	11
69.5	0.6	-	-	6.97	12
72.5	0.0	-	-	6.97	8
76.25	0.0	43.3	0.0	6.97	8
79.75	2.2	-	-	6.96	8
90.5	4.2	-	-	6.97	7
114.5	1.8	30.9	0.0	6.95	8
140.0	0.2	-	-	6.98	8
144.5	0.0	16.8	-	6.97	8
152.0	0.0	13.8	0.0	6.97	7
163.5	0.0	-	-	6.96	9
168.5	2.2	9.7	0.0	6.95	7
175.25	0.0	-	-	6.95	9
187.0	0.0	6.2	0.0	6.94	8
198.5	3.0	5.0	0.0	7.00	10
222.5	1.8	3.0	0.0	7.30*	10

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 64.5 mg/L; air flow rate = 1450 ml/min.; inoculum volume = 100 ml; total volume at time 0, 900 ml.

*25 ml of 0.1M NaOH siphoned in to cause this increase in pH

Table 4.23. Batch fermentation experiment B5/12V3 with 2,4-D at pH 6.9.

Time (hr)	Biomass Concentration (mg/L)	[2,4-D] (mg/L)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
1.25	0.2	222	0.0	7.1	14
4.75	1.4	222	1.4	6.7	14
8.75	0.0	-	-	6.96	10
17.75	1.8	-	-	6.96	9
21.25	0.0	-	-	-	9
26.5	0.0	-	-	-	8
27.0	-	-	-	6.94	7
33.0	0.0	-	-	-	7
41.75	2.2	220	0.0	6.93	7
49.25	1.8	-	-	-	8
55.25	3.0	-	-	6.90	8
66.5	6.2	-	-	-	11
69.5	6.2	-	-	6.85	12
72.5	6.2	-	-	-	8
76.25	3.8	207	0.0	6.85	8
79.75	2.6	-	-	-	8
90.5	2.2	205	0.0	-	7
114.5	1.8	199	0.0	6.80	8
140.0	9.8	95.7	0.0	-	8
144.5	10.2	39.2	0.0	6.56	8
152.0	8.2	2.1	0.0	-	7
163.5	12.6	-	-	-	9
168.5	11.0	0.0	0.0	-	7

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 222 mg/L; air flow rate = 1450 ml/min.; inoculum volume = 100 ml; total volume at time 0, 900 ml.

Table 4.24. Batch fermentation experiment B5/12V4 with 2,4-D at pH 6.7.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-D] (mg/L)	pH	Sample Volume (ml)
1.25	0.6	3	62.7	6.72	12
4.75	2.6	7	62.1*	6.72	13
8.75	0.0	10	-	6.72	10
17.75	0.0	15	-	6.72	9
21.5	0.0	16	-	6.72	10
26.5	0.0	17	-	6.73	8
27.0	-	17	62.1	-	7
33.0	0.0	47	-	6.71	7
41.75	0.0	47	-	6.72	7
49.25	0.0	48	-	6.71	9
55.25	0.2	49	58.0	6.70	8
66.5	0.0	51	-	6.75	12
69.5	0.0	51	-	6.75	12
72.5	0.0	52	-	6.75	8
76.25	0.0	53	53.3	6.75	8
79.75	0.0	53	-	6.77	8
90.5	0.6	53	43.3	6.77	7
114.5	1.8	53	36.2	6.76	8
140.0	1.8	53	25.0	6.73	8
144.5	0.0	53	-	6.72	8
152.0	0.0	53	18.6	6.73	7
163.5	0.0	53	10.9	6.72	9
168.5	0.0	53	6.8	6.71	7
175.25	0.0	53	2.4	6.73	9
187.0	0.0	53	0.0	6.73	8

T = 25 C; impeller speed = 700 rpm; initial 2,4-D concentration = 62.7 mg/L; air flow rate = 1450 ml/min.; inoculum volume = 100 ml; total volume at time 0, 900 ml.

*A 2,4-DCP concentration of 1.4 mg/L was detected at time 4.75.

Table 4.25. Batch fermentation experiment B11/13V1 with 2,4-DCP at pH 5.1.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	8.6	0	50.6	5.11	9.0
10.0	5.8	0	49.6	5.08	7.5
16.0	1.8	0	47.8	5.11	7.5
18.0	1.4	0	-	5.11	7.5
21.5	0.0	0	44.2	5.15	7.5
24.5	0.0	0	47.0	5.11	7.5
40.0	0.0	13	42.7	5.16	7.5
62.0	0.0	13	37.3	5.19	7.5
71.5	0.0	13	-	5.09	7.5
85.75	0.0	15	36.6	5.17	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 50.6 mg/L; air flow rate = 650 ml/min.; inoculum volume = 215 ml; total volume at time 0, 1715 ml. condenser water T = 19 C.

Table 4.26. Batch fermentation experiment B11/13V1B with 2,4-DCP at pH 5.1. (continuation of B11/13V1)*

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	0.0	0	40.9	5.08	7.5
4.0	0.0	0	41.7	5.06	7.5
10.0	0.0	0	33.7	5.13	7.5
21.0	0.0	2	36.6	5.17	7.5
29.5	0.0	5	38.1	5.13	7.5
48.5	0.0	7	33.0	5.09	7.5
58.0	0.0	7	32.3	5.12	7.5
69.5	0.0	7	29.1	5.16	7.5
92.0	0.0	7	29.8	5.18	7.5
105.0	0.0	7	26.2	5.22	7.5
131.5	0.0	7	21.5	5.05	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 40.9 mg/L; air flow rate = 650 ml/min.; new inoculum volume = 80 ml; condenser water T = 19 C.
* new inoculum and 2,4-DCP added to B11/13V1 at time 87 hr.

Table 4.27. Batch fermentation experiment B11/13V2 with 2,4-DCP at pH 6.1.

Time (hr)	Biomass Concentration (mg/L)	2,4-DCP Concentration (mg/L)	pH	Sample Volume (ml)
0.0	6.6	54.2	6.08	7.5
10.0	2.2	49.9	6.09	7.5
16.0	2.2	48.5	6.08	7.5
18.0	0.2	-	6.08	7.5
21.5	2.2	47.0	6.08	7.5
24.5	0.6	44.9	6.08	7.5
40.0	0.6	42.7	6.10	7.5
62.0	0.0	38.8	6.10	7.5
71.5	0.0	-	6.11	-
85.5	0.0	36.6	6.12	-

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 54.2 mg/L; air flow rate = 650 ml/min.; inoculum volume = 212 ml; total volume at time 0, 1712 ml. condenser water T = 19 C.

Table 4.28. Batch fermentation experiment B11/13V2B with 2,4-DCP at pH 6.1. (continuation of B11/13V2)*

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	0.0	0	38.8	6.09	7.5
4.0	0.0	0	37.7	6.08	7.5
10.0	0.0	0	35.5	6.08	7.5
21.0	0.0	0	33.0	6.07	7.5
29.5	0.0	0	29.1	6.06	7.5
48.5	1.4	0	16.5	6.00	7.5
54.0	1.8	3	7.1	6.02	7.5
58.0	4.6	8	0.0	6.05	7.5
69.5	3.0	8	0.0	6.05	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 38.8 mg/L; air flow rate = 650 ml/min.; new inoculum volume = 80 ml; condenser water T = 19 C.
* new inoculum and 2,4-DCP added to B11/13V2 at time 75 hr.

Table 4.29. Batch fermentation experiment B11/23V2 with 2,4-DCP at pH 6.1.*

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	3.0	0	48.5	6.09	8.0
6.0	1.4	0	54.6	6.08	7.5
9.0	1.4	0	51.4	6.07	7.5
19.0	2.2	0	47.4	6.07	7.5
21.0	0.6	0	47.5	6.06	7.5
25.5	0.2	0	45.2	6.07	7.5
28.0	0.0	0	44.9	6.06	7.5
33.5	0.2	0	42.4	6.05	7.5
45.0	0.0	0	39.9	6.04	7.5
49.0	1.8	0	37.3	–	7.5
54.5	2.2	0	33.7	6.03	7.5
58.0	2.2	0	31.9	6.02	7.5
71.0	0.0	2	29.1	6.08	7.5
75.0	1.4	2	24.8	6.07	7.5
82.5	1.0	2	20.8	6.04	7.5
102.5	5.4	6	0.0	6.05	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 48.5 mg/L; air flow rate = 650 ml/min.; condenser water T = 19 C.

*started experiment by adding 2,4-DCP to B11/13V2B

Table 4.30. Batch fermentation experiment B11/13V3 with 2,4-DCP at pH 7.0.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	9.8	0	45.6	7.02	7.5
10.0	1.4	0	44.2	7.03	7.5
16.0	2.6	0	40.6	7.02	7.5
18.0	0.6	0	-	7.02	7.5
21.5	1.4	0	39.1	7.02	7.5
24.5	0.0	0	39.1	7.02	7.5
40.0	1.8	0	28.4	7.03	7.5
62.0	2.2	5	9.7	7.01	7.5
71.5	2.6	5	0	6.98	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 45.6 mg/L; air flow rate = 650 ml/min.; inoculum volume = 210 ml; total volume at time 0, 1710 ml. condenser water T = 19 C.

Table 4.31. Batch fermentation experiment B11/13V3B with 2,4-DCP at pH 7.0. (continuation of B11/13V3)*

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	2.6	0	29.4	7.02	7.5
1.0	--Added more 2,4-DCP-----------		46.0	-------------	
2.0	2.2	0	42.7	7.02	7.5
4.0	1.8	0	-	7.02	90.0
7.0	2.2	0	41.7	7.02	7.5
10.0	2.2	0	38.4	7.02	7.5
21.0	2.6	2	23.0	7.01	7.5
24.0	5.0	4	17.9	6.99	7.5
25.5	4.2	4	16.5	7.00	7.5
27.0	5.8	6	12.2	7.01	7.5
29.5	5.8	6	3.6	7.01	7.5
30.5	6.2	6	0.3	7.00	75.0

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 29.4 mg/L; air flow rate = 650 ml/min.; new inoculum volume = 80 ml; condenser water T = 19 C.
* new inoculum and 2,4-DCP added to B11/13V3 at time 87 hr.

Table 4.32. Batch fermentation experiment B11/23V3 with 2,4-DCP at pH 7.1.

Time (hr)	Biomass Concentration (mg/L)	2,4-DCP concentration (mg/L)	pH	Sample Volume (ml)
0.0	0.0	83.3	7.13	7.5
1.5	0.0	85.1	7.29	7.5
11.5	0.0	81.5	7.06	7.5
13.5	0.0	81.9	7.00	7.5
18.0	0.0	83.0	7.06	7.5
20.5	0.0	79.0	7.06	7.5
26.0	0.0	77.6	7.06	7.5
37.5	0.0	73.3	7.06	7.5
47.0	0.0	71.1	7.07	7.5
50.5	0.0	-	7.07	7.5
63.5	0.0	66.8	7.07	7.5
67.5	0.0	-	7.07	7.5
75.0	0.0	-	7.07	7.5
95.0	0.0	61.8	7.09	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 83.3 mg/L; air flow rate = 650 ml/min.; inoculum volume = 250 ml; total volume at time 0, 1750 ml. condenser water T = 19 C.

Table 4.33. Fed-batch fermentation experiment FB11/30 with 2,4-DCP at pH 7.1.

Time (hr)	Biomass Concentration (mg/L)	Cumulative Feed Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	0.0	0	26.6	7.25	7.5
5.0	0.0	0	21.9	7.07	7.5
16.0	1.8	0	12.2	7.07	7.5
18.5	1.8	0	7.5	7.06	7.5
20.25	2.2	0	2.8	7.04	7.5
23.0	3.0	0	-	7.04	7.5
24.5	3.0	138	0.0	7.09	7.5
25.0	2.6	180	-	7.10	0.0
25.5	3.0	219	-	7.11	0.0
26.75	3.4	325	-	7.14	0.0
27.25	4.6	367	-	7.15	0.0
27.75	3.8	410	0.0	7.16	7.5
28.0	5.4	433	-	7.15	0.0

Feed 2,4-DCP concentration = 58 mg/L; T = 25 C; condenser water T = 19 C; impeller speed = 400 rpm; initial 2,4-DCP concentration = 26.6 mg/L; inoculum volume = 100 ml; air flow rate = 650 ml/min.; initial volume = 1200 ml.

Table 4.34. Fed-batch fermentation experiment FB11/30B with 2,4-DCP at pH 7.1 (continuation of FB11/30).

Time (hr)	Biomass Concentration (mg/L)	Cumulative Feed Added from time = 28 h (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
28.75	4.2	21*	11.1	7.17	7.5
29.0	3.8	55	-	7.07	0.0
29.25	5.0	89	-	7.08	0.0
29.5	3.4	125	-	7.09	0.0
29.75	4.6	161	-	7.09	0.0
30.0	4.2	196	-	7.11	0.0
30.25	5.0	229	-	-	0.0
30.5	4.6	265	15.4	7.12	7.5
30.75	3.8	301	-	7.13	0.0
31.0	4.2	336	-	7.13	0.0
31.25	3.8	372	-	-	0.0
31.5	4.2	408	16.8	7.15	7.5
32.0	3.8	408	15.8	7.09	7.5
41.0	9.4	408	0.0	7.07	7.5
44.0	Added about 0.05 g 2,4-DCP				
45.0	7.0	408	34.5	7.06	7.5
46.75	6.6	408	31.6	7.05	7.5
51.0	7.4	408	22.2	7.03	7.5
52.5	9.0	408	23.0	7.01	7.5
53.5	7.8	408	18.3	7.01	7.5
54.5	7.4	408	18.6	7.01	7.5
55.5	7.4	408	-	7.00	7.5
56.0	7.4	408	15.1	7.00	7.5
57.0	8.2	408	6.1	7.00	7.5
57.5	7.8	408	5.4	7.00	7.5
58.0	8.6	408	5.7	6.99	7.5

Feed 2,4-DCP concentration = 97 mg/L; condenser water T= 19C T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 11.1 mg/L; air flow rate = 650 ml/min.;
* Added 0.02 g 2,4-DCP in addition to feed.

Table 4.35. Batch fermentation experiment B11/23V1 with 2,4-DCP at pH 8.0.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	0.2	15	54.2	8.07	7.5
4.0	3.0	45	60.3	8.01	7.5
6.5	3.0	72	57.1	8.03	7.5
16.5	5.4	105	57.5	8.03	7.5
18.5	3.8	105	53.9	7.97	7.5
23.5	5.4	120	58.2	8.02	7.5
25.5	4.2	136	57.5	7.97	7.5
31.0	5.8	136	52.4	7.91	7.5
42.5	5.4	156	49.9	8.00	7.5
46.0	5.8	170	47.8	8.02	7.5
55.0	5.0	176	50.3	8.02	7.5
66.0	5.4	192	49.5	8.02	7.5
70.0	6.2	197	–	8.04	7.5
77.5	3.8	205	–	8.00	7.5
85.5	5.0	224	49.5	8.04	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 54.2 mg/L; air flow rate = 650 ml/min.; inoculum volume = 200 ml; total volume at time 0, 1700 ml. condenser water T = 19 C.

Table 4.36. Batch fermentation experiment B12/7V1 with 2,4-DCP at pH 8.0.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	0.2	0	28.4	8.11	7.5
1.0	0.2	0	30.1	7.41	7.5
11.5	-	66	-	8.02	-
12.0	5.4	71	25.1	8.11	7.5
13.0	5.4	94	25.5	8.15	7.5
15.0	5.8	132	18.6*	7.96	7.5
18.0	6.2	161	21.2	7.96	7.5
19.5	6.2	164	20.4	7.78	7.5
21.0	5.8	189	-	7.95	7.5
30.5	5.4	198	21.2	7.99	7.5
35.5	4.2	200	22.2	7.98	7.5
41.5	3.0	202	22.2	7.99	7.5
60.5	2.6	208	21.9	8.03	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 28.4 mg/L; air flow rate = 650 ml/min.; inoculum volume = 250 ml; total volume at time 0, 1950 ml. condenser water T = 19 C.

*analysis of this sample was delayed.

Table 4.37. Batch fermentation experiment B12/10V1 with 2,4-DCP at pH 8.0.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	19.0	0	34.1	7.94	7.5
1.0	18.2	2	31.6	7.93	7.5
2.0	18.6	2	-	7.97	7.5
4.5	16.6	5	-	7.97	7.5
5.5	17.0	5	31.2	-	90.0
7.0	15.0	6	-	7.99	7.5
20.5	10.2	26	29.4	7.95	7.5
25.5	9.4	31	-	7.96	7.5
27.75	8.2	39	-	7.98	7.5
30.0	9.4	42	31.2	7.95	7.5
32.0	6.6	72	-	7.97	7.5
46.75	6.2	89	28.4	7.98	7.5
55.5	5.8	102	-	7.99	7.5
69.75	6.2	107	-	7.98	7.5
76.5	6.2	115	28.4	8.00	7.5
95.0	3.8	120	-	8.03	7.5
105.5	3.0	122	-	8.01	7.5
118.0	3.0	125	27.1	7.96	7.5
129.0	4.6	129	-	7.97	7.5
143.5	4.6	131	29.4	7.99	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 34.1 mg/L; air flow rate = 650 ml/min.; inoculum volume = 250 ml; total volume at time 0, 1550 ml. condenser water T = 19 C.

Table 4.38. Batch fermentation experiment B12/7V2 with 2,4-DCP at pH 8.8.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	1.0	0	29.1	8.75	7.5
1.0	2.2	0	30.5	8.78	7.5
11.5	-	26	-	8.77	7.5
12.0	5.4	26	24.8	8.75	7.5
13.0	6.2	31	22.6	8.75	7.5
15.0	5.4	39	22.2	8.78	7.5
18.0	6.6	46	21.9	8.75	7.5
19.5	7.0	46	20.4	8.73	7.5
21.0	6.6	53	21.5	8.78	7.5
30.5	9.8	61	17.9	8.77	7.5
32.5	10.2	61	17.2	8.74	7.5
35.5	10.2	61	17.6	8.89	7.5
41.5	11.8	61	20.1	8.75	7.5
60.5	12.2	62	-	8.79	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 29.1 mg/L; air flow rate = 650 ml/min.; inoculum volume = 250 ml; total volume at time 0, 1950 ml. condenser water T = 19 C.

Table 4.39. Batch fermentation experiment B12/10V2 with 2,4-DCP at pH 9.0.

Time (hr)	Biomass Concentration (mg/L)	Cumulative 0.1M NaOH Added (ml)	[2,4-DCP] (mg/L)	pH	Sample Volume (ml)
0.0	12.6	0	33.0	8.98	7.5
1.0	12.2	0	31.6	8.97	7.5
2.0	13.4	0	31.2	9.00	7.5
4.5	11.0	0	26.2	9.02	7.5
5.5	13.0	0	25.5	-	100
7.0	-	0	23.0	8.96	7.5
20.5	17.4	3	16.9	8.95	7.5
22.5	18.2	3	16.9	-	7.5
25.5	18.2	3	15.4	8.94	7.5
27.75	19.0	3	14.3	8.95	7.5
30.0	21.0	3	14.7	8.98	7.5
32.0	20.6	3	13.6	9.01	7.5
41.5	20.6	4	14.0	8.94	7.5
46.75	19.0	4	13.6	8.94	7.5
49.5	19.4	4	14.0	8.99	7.5
54.5	19.8	5	14.0	9.01	7.5
56.5	20.6	5	12.5	8.97	7.5
69.75	21.0	6	13.6	8.93	7.5
76.5	20.2	6	12.9	8.94	7.5
78.5	18.2	7	12.5	9.01	7.5
81.75	19.0	7	-	8.96	7.5
95.0	17.4	7	13.6	8.98	7.5
105.5	16.6	8	-	8.96	7.5
143.5	17.0	8	13.6	8.94	7.5

T = 25 C; impeller speed = 700 rpm; initial 2,4-DCP concentration = 33.0 mg/L; air flow rate = 650 ml/min.; inoculum volume = 250 ml; total volume at time 0, 1550 ml. condenser water T = 19 C.

Table 4.40. Maximum specific growth rate estimates for batch fermentations growing on 2,4-DCP.

Culture	pH	μ_m	r^2	N	T.S.	k
B11/13V2B	6.1	0.111	0.9998	4	29.5-58	0.0079
B11/13V3	7.0	0.012	0.9996	4	24.5-71.5	0.0065
B11/13V3B	7.0	0.057	0.9943	10	2-30.5	0.0065
B11/23V2	6.1	0.068	0.9999	3	75-102.5	0.0095
FB11/30	7.1	0.163	0.9999	3	16-20.25	0.0032
FB11/30B	7.1	0.054	0.9889	10	45-58	0.0032

μ_m, (h^{-1}), maximum specific growth rate obtained from regression equation 2 with Y_s = 0.25; r^2, square of the correlation coefficient for the μ_m estimate; N, number of points used for the μ_m estimate; T.S., (h), time span used to estimate μ_m.

Table 4.41. First order rate constants describing 2,4-DCP degradation.

Culture	pH	T_e	T_k	k	r^2	C_{sd}	C_i	C_f
B7/9V1	5.5	0-58*	0-58	.0089	.991	-	44.5	26.9
B8/28V2	5.7	0-75*	0-75	.0143	.987	26-21	49.5	17.6
B6/17V1+	7.0	0-496	0-496	.0016	.965	-	58.1	28.9
B6/17V1	7.0	0-496	0-496	.0019	.977	-	58.1	24.4
B9/12V1	-	0-333	0-333	.0050	.997	-	95.8	16.1
B9/12V2	-	0-333	0-333	.0066	.999	-	98.7	10.0
B9/12V3	-	0-333	0-333	.0058	.998	-	106	13.6
B9/12V4	-	0-333	0-333	.0068	.999	-	112	11.1
B11/13V1	5.1	0-86	0-86	.0042	.973	-	50.6	36.6
B11/13V1B	5.1	0-132	0-132	.0044	.958	-	40.9	21.5
B11/13V2	6.1	0-86	0-86	.0052	.974	-	54.2	36.6
B11/13V2B	6.1	0-70	0-54	.0218	.854	16-7	38.8	0.0
B11/13V2B	6.1	0-70	0-48.5	.0144	.921	29-16	38.8	0.0
B11/13V2B	6.1	0-70	0-29.5	.0090	.989	33-29	38.8	0.0
B11/13V2B	6.1	0-70	0-21	.0079	.996	33-29	38.8	0.0
B11/23V2	6.1	0-103	0-77	.0106	.982	29-25	48.5	0.0
B11/23V2	6.1	0-103	0-65	.0095	.994	34-32	48.5	0.0
B11/13V3	7.0	0-72	0-62	.0181	.838	28-10	45.6	0.0
B11/13V3	7.0	0-72	0-40	.0093	.920	39-28	45.6	0.0
B11/13V3	7.0	0-72	0-24.5	.0065	.975	39-28	45.6	0.0
B11/13V3B	7.0	1-30.5	1-30.5	.0795	.703	12-4	46.0	0.3
B11/13V3B	7.0	1-30.5	1-27	.0420	.967	16-12	46.0	0.3
B11/13V3B	7.0	1-30.5	1-25.5	.0382	.975	23-18	46.0	0.3
B11/13V3B	7.0	1-30.5	1-10	.0194	.929	38-23	46.0	0.3
B11/23V3	7.1	0-95	0-95	.0032	.972	-	83.3	61.8
FB11/30	7.1	0-20	0-20	.0798	.899	8-6	26.6	2.8
FB11/30B	7.1	45-58	45-58	.1103	.896	15-6	34.5	5.7

Table 4.41. Continued.

Culture	pH	T_e	T_k	k	r^2	C_{sd}	C_i	C_f
B11/23V1	8.0	4-85.5	4-85.5	.0034	.884	-	60.3	49.5
B12/7V1	8.0	0-61	0-59.5	.0085	.792	-	28.4	21.9
B12/10V1	8.0	0-144	0-144	.0018	.733	-	34.1	29.4
B12/7V2	8.8	0-61	0-41.5	.0139	.946	-	29.1	20.1
B12/10V2	9.0	0-144	0-144	.0122	.783	-	33.0	13.6
B12/10V2	9.0	0-144	0-79	.0166	.892	-	33.0	13.6
B12/10V2	9.0	0-144	0-30	.0301	.983	-	33.0	13.6
B12/10V2	9.0	0-144	0-20.5	.0359	.964	-	33.0	13.6

T_e, (h), time span of experiment; T_k, (h), time span used for k estimate; k, (h^{-1}), first order rate constant; r^2, square of correlation coefficient for k estimate; C_{sd}, (mg/L), 2,4-DCP concentration range where observed 2,4-DCP degradation becomes significantly more rapid than that predicted by the given first order model; C_i, (mg/L), 2,4-DCP concentration at the beginning of the time span used for estimating k; C_f, (mg/L), 2,4-DCP concentration at the end of the experiment.

*For this analysis the pH 5.5 and 5.7 experiments are considered to start at the point where the maximum 2,4-DCP accumulation occurred.

+corrected for water loss.

CHAPTER V

RECOMMENDATIONS FOR FUTURE WORK

In this chapter some of the problems encountered in the present research and possible improvements in the experimental procedures are identified. The changes in methods were not implemented in the current research so that consistency of methods could be maintained. The four topics discussed are HPLC, biomass measurement, sterilization, and culture maintenance. The suggestions for the HPLC procedure may be particularly useful in simplifying future efforts by significantly reducing the time required for sample analysis.

HIGH PERFORMANCE LIQUID CHROMATOGRAPHY

The HPLC procedure developed for this research, as described in chapter 3, uses a solvent gradient to maximize separation between 2,4-D and any possible degradation products. The main disadvantage of this method is that it is very time consuming, mainly due to the time required to regenerate the initial conditions. In addition, the gradient increases baseline drift and thus requires increased monitoring and causes some complication of data analysis. Only 2,4-D and 2,4-DCP were observed in actual

experiments; thus, it may not be necessary to use the complex and time consuming gradient elution on every sample. A simple isocratic program could be used for most samples, with occasional tests using the more sensitive gradient program to verify the absence of other products.

The optimal conditions for the isocratic program could easily be determined experimentally. All the variables except the solvent concentrations could remain the same as in the original gradient program described in chapter 3. Different solvent concentrations could then be tested to determine the optimal isocratic condition. Based on the results obtained with the gradient scheme, a constant setting of about 70% acetonitrile and 30% 0.015 N H_2SO_4 would probably yield good results. The results obtained with the isocratic method could be compared to the gradient elution program to determine the need for occasional monitoring with the gradient procedure. It may be possible to develop an isocratic program that would completely eliminate the need for the gradient elution.

BIOMASS MEASUREMENT

The measurement of biomass dry weight presented some problems with consistency of results as indicated by the variance in the absorbance-biomass dry weight standard curve

shown in chapter 3. Throughout the current research a number of possible means of improving biomass dry-weight measurements have become apparent. One improvement needed is a means of consistently removing detergent from the filters. This could be accomplished by soaking the filters and then passing a consistent volume of de-ionized water through each filter. The method of drying also needs improvement. Oven drying at 105 C seems to cause a reduction in filter weight other than that due to water loss. This makes careful timing and the use of control filters important. The 24 hour drying time used in these experiments is probably much longer than required. Shorter, precisely measured drying times would be desirable. Drying after pre-rinsing before taking the initial weights of the filters could probably be done in about an hour. Another possibility that might improve results would be to use a lower oven temperature or to use desiccaters at room temperature for drying. Finally, because the actual amount of biomass measured needs to be very small to make rapid filtration possible, the use of multiple samples at each concentration is very useful in getting a good value and estimating the sample variance.

STERILIZATION

The method used to sterilize the fermentation systems could be improved. Initially, aseptic techniques were not used in the fermentation systems because it was assumed that the toxic nature of the substrates would prevent contamination. However, after several experiments the fermenters became contaminated with predatory protozoa, thus indicating the need for sterilization. One method for controlling predatory protozoa that has been used is the addition of antibiotics (1), but in the laboratory where autoclaving is possible this is probably not a reasonable alternative.

The first method tested for sterilization of the fermentation systems was autoclaving the entire unit together including the media. This method resulted in the precipitation of some of the nutrient salts which could potentially cause problems with biomass absorbance readings and might also change the concentration of nutrients available to the organisms. These factors led to the use of the filtration method described in chapter 4.

There are two methods that could be used to improve the sterilization procedure. First, the salts that cause the precipitation could be identified experimentally and then separated into different solutions. Most of the media could then be sterilized in the intact fermenter, with the other

part of the media autoclaved separately and added afterward. This would make it possible to complete the sterilization procedure with one transfer of media without the need for sterilization of filtration equipment or the extensive environmental exposure resulting from the filtration method. The second alternative would be to use filtration sterilization with a regular pressure vessel system to force the media through a sterile filtration device as is often done for heat sensitive media for eukaryotic organisms.

CULTURE MAINTENANCE

The determination of an appropriate method of storage for Pseudomonas sp. NCIB 9340 to provide a consistent source of inocula was another difficulty encountered in the present research. It has been demonstrated that biodegradation rates of 2,4-D and other xenobiotic compounds can be greatly increased by allowing the organisms responsible for the degradation to become acclimated to the new substrates (2-4). Thus, it is desirable to have experiments inoculated with organisms that are equally acclimated to the test substrate. In an attempt to provide a supply of organisms with a uniform history of acclimation, the original freeze dried sample of Pseudomonas sp. NCIB 9340 was revived and grown on 2,4-D. It was then dispensed into a large number of test tubes containing the nutrient salts media described

in chapter 3 with varying amounts of 2,4-D and 10 weight percent glycerol. Subsequently, these samples were placed in a freezer at -10 C for long term storage. Unfortunately this method of storage caused the organism to loose its ability to degrade 2,4-D and 2,4-DCP. Storage on refrigerated agar slants was also attempted; however, revival of 2,4-D degrading organisms was generally not possible after more than about two weeks and was very inconsistent even over shorter intervals.

The genes required for the biodegradation of xenobiotic materials are often found on plasmids (5-8). Based on the assumption that the ability of Pseudomonas sp. NCIB 9340 to degrade 2,4-D is plasmid mediated, Leslie (9) at the National Collections of Industrial and Marine Bacteria Ltd. investigated several storage methods in response to inquiries concerning long term storage of the organism. The organism was well maintained by regular subculturing on 2,4-D. Storage in liquid nitrogen also maintains the plasmids, but at a somewhat lower level. Storage in 50% glycerol at -20 C is unsuitable for plasmid maintenance.

The regular subculturing method of culture maintenance was selected because of the failure of the glycerol and agar slant methods. It has been observed that after a certain period of acclimation organisms may not be greatly affected by further exposure to a given substrate (3), thus the

regular subculturing method should provide fairly consistent inocula.

Improvements in the maintenance procedure are desirable for three major reasons. The first reason is that regular subculturing is a time consuming tedious chore. Second, this method provides many opportunities for contamination of the cultures. Finally, continuously maintaining the organism in rapid growth conditions may lead to mutations that could change some of the characteristics that are being examined. While the tendency of the organism to mutate is an important factor to examine in a separate study, it needs to be avoided in order to obtain consistent values of various parameters describing the growth of the organism and to accurately determine the influence of different environments.

A method of long term storage that maintains the organism in a dormant or near dormant state should be developed for future work. Two methods that should be investigated based on the information presented here are storage in liquid nitrogen and re-freeze drying samples of the organisms. In both cases, organisms could be stored in a large number of samples with identical histories. If this was done then each time a new source of inocula was required one of the storage samples could be revived and exposed to some consistent level of acclimation.

REFERNECES

1) Wiggins, B. A. Chemical Week, 138, 29 (April 2, 1986).

2) Audus, L. J. in The Physiology and Biochemistry of Herbicides, Academic Press, New York, pp 163-189 (1964).

3) Hemmett, R. and Faust, S. Residue Rev., 29, 191-206 (1969).

4) Loos, M. A. in Herbicides: Chemistry, Degradation and Mode of Action, (Kearney, P. C. and Kaufman, D. D., eds) 2nd edn, Marcel Dekker Inc., New York, Vol. 1, pp 1-56 (1975).

5) Don, R. H. and Pemberton, J. M. J. Bacteriol., 145, 681-686 (1981).

6) Fisher, P. R., Appleton, J. and Pemberton, J. M. J. Bacteriol., 135, 798-804 (1978).

7) Friedrich, B., Meyer, M. and Schlegel, H. G. Arch. Microbiol., 134, 92-97 (1983).

8) Ghosal, D., You, I.-S., Chatterjee, D. K. and Chakrabarty, A. M. Science, 228, 135-142 (1985).

9) Leslie, C. E., private communication (December 4, 1986).

APPENDIX A. STANDARD CURVE DATA

Table A-1. Data for the 2,4-D Standard Curve for UV Detection at 283 nm with the Absorbance Range at 0.05. Integrater Units = I.U.

Area (I.U.)	Average Area (I.U.)	2,4-D Concentration (mg/L)
0.0	0.0	0.0
12.8	12.8	8.0
20.5, 23.3, 24.8	22.8	15.4
64.8, 66.4	65.6	39.9
82.6	82.6	49.9
116.8, 114.4	115.6	66.5
138.2	138.2	79.8
177.0, 169.0, 181.0	175.7	99.8
217.0	217.0	133.1

Linear regression of this data gives the following equation to convert I.U. to 2,4-D concentration:

2,4-D Concentration = 0.589(I.U.) + 0.3
correlation coefficient = 0.998

Table A-2. Data for the 2,4-DCP Standard Curve with UV Detecter Wavelength at 283 nm and Absorbance Range at 0.05. Integrater Units = I.U.

Area (I.U.)	2,4-DCP Concentration (mg/L)
0.0	0.0
0.8	0.33
18.1	6.6
57.6	19.8
121.5	41.3
227.5	82.5

Linear regression of this data gives the following equation to convert I.U. to 2,4-DCP concentration:

2,4-DCP Concentration = 0.359(I.U.) - 0.4
Correlation coefficient = 0.999

Table A-3. Data for the Biomass Concentration Versus 545 nm Absorbance Standard Curve.

Absorbance	Biomass Concentration (mg/L)
0.0	0.0
0.018	5.3
0.013	6.4
0.051	18.4
0.10	33.0
0.009	41.6
0.198	62.3
0.18	62.9
0.201	64.6
0.186	65.1
0.178	65.7
0.208	72.2
0.209	74.4
0.184	91.8
0.35	98.7
0.27	100.3
0.271	122.4
0.37	133.8
0.353	155.3
0.45	172.3
0.54	236.2
0.69	288.6
0.835	442.0

Linear regression of the data for biomass concentrations less than 300 mg/L gives the following equation to convert absorbance to biomass concentration:

Biomass Concentration = 400.2(Absorbance at 545 nm) - 1.8

Note: The relation should not be used if the absorbance reading is above 0.7, i.e., if the biomass concentration is above 300 mg/L.

APPENDIX B. PROGRAMS

EXPONENTIAL INTERVAL SELECTION

```
// EXEC SAS
/*REGION        1000K
//SYSIN DD *
DATA;
INPUT T X;
Y = LOG(X) - LOG(Xo);                 (Xo is input for each run)
TA = T - To;                          (To is input for each run)
CARDS;
(input T X data here)
PROC GLM;
MODEL Y = TA/P;                       (P is a SAS option)
PROC GLM;
MODEL Y = TA TA*TA/P;
PROC PRINT;
```

FIRST ORDER RATE CONSTANT DETERMINATION

```
// EXEC SAS
/*REGION
//SYSIN DD *
DATA;
INPUT T C;                    (T = time, C = concentration)
Y = -LOG(C/Co);               (co input for each run)
CARDS;
(input T, C data here)
PROC GLM;
MODEL Y = T/NOINT P;          (NOINT, P are SAS options)
PROC PRINT;
```

SPECIFIC GROWTH RATE ESTIMATES

```
// EXEC SAS
/*REGION     1000K
//SYSIN DD *
DATA;
INPUT T Z X;
Y1 = LOG(X) - LOG(Xo);          (Xo input for each run)
Y2 = LOG(Z) - LOG(Zo);          (Zo input for each run)
C = (Y2 - Y1)*0.5;
YAV = (Y1 + Y2)*0.5;
TA = T - To;                    (to input for each run)
CARDS;
(input T, Z, X data here)
PROC GLM;
MODEL Y1 = TA/NOINT;
PROC GLM;
MODEL Y2 = TA/NOINT;
PROC GLM;
MODEL YAV = TA C/NOINT;
PROC PRINT;
```

BIODEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID AND 2,4-DICHLOROPHENOL

by

GREGORY L. SINTON

B. S., Colorado State University, 1984

AN ABSTRACT OF A MASTER'S THESIS

submitted in partial fulfillment of the

requirements for the degree

MASTER OF SCIENCE

College of Engineering
Department of Chemical Engineering

KANSAS STATE UNIVERSITY
Manhattan, Kansas

1987

ABSTRACT

Considerable uncertainty exists as to if and how 2,4-dichlorophenoxyacetic acid (2,4-D) inhibits microbial growth. The uncertainty may be due to inhibitory effects of the metabolic product 2,4-dichlorophenol (2,4-DCP). Experiments with *Pseudomonas* sp. NCIB 9340 in one and two liter fermenters have shown that culture pH is an important factor in determining the growth rate of this organism and the extent of 2,4-DCP accumulation. Experiments with one liter batch fermenters over a pH range of 5.1 to 9.4 have shown that the highest growth rates occur between pH 6.5 and 7.9; the specific growth rate decreases as the pH is increased or decreased from this range until it reaches zero at 9.4 or 5.1 respectively. Cultures exhibiting no growth for seven days at pH 5.1 can be revived to resume normal growth by increasing the pH to 6.0. The average growth rate on 2,4-D between pH 6.5 and 7.9 is 0.14 h^{-1}. The average biomass yield is 0.25 g dry biomass/g 2,4-D.

Shake flask experiments with initial 2,4-D concentrations ranging from 10.5 to 370 mg/L give no indication of inhibition of growth by 2,4-D. In addition, there is no indication of a minimum threshold concentration of 2,4-D required to stimulate growth in this concentration range. Shake flask and one liter batch fermentation experiments both indicate that the Monod model with a half saturation constant approximately in the range from 1.0 to

5.1 mg/L provides an adequate description of 2,4-D biodegradation.

Extensive accumulation of 2,4-DCP occurred at a low pH; it was accompanied by a reduction in the biodegradation rate, presumably due to inhibitory effects of 2,4-DCP. 2,4-D biodegradation ceased completely when the concentration of 2,4-DCP reached about 44 mg/L. No growth was observed even after the 2,4-DCP concentration was reduced by non-biodegradation mechanisms and new 2,4-D substrate was added to the culture.

Two liter batch experiments with 2,4-DCP as the carbon source indicate that it is strongly inhibitory at concentrations above 30 to 35 mg/L. No growth was observed in any experiments with initial 2,4-DCP concentrations above 50 mg/L. Significant reductions in 2,4-DCP concentrations occur even in the absence of microbial activity. These losses can be described by first order rate models. The growth rate and biomass yields with 2,4-DCP are lower than those observed for growth on 2,4-D. In order to accurately examine 2,4-DCP biodegradation the influence of factors such as photolysis and absorption or adsorption uptake of 2,4-DCP by biomass need to be clarified.