DEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID IN EXPANDED BED REACTORS BY ALCALIGENES EUTROPHUS AE0106 WITH PLASMIDS pRO101 AND pRO103

By

CHING-PING YANG

Bachelor of Science in Engineering

Tamkang University

Taipei, Taiwan, R.O.C.

1983

Submitted to the Faculty of the
Graduate College of the
Oklahoma State University
in partial fulfillment of
the requirements for
the Degree of
MASTER OF SCIENCE
July, 1990

DEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID IN EXPANDED BED REACTORS BY ALCALIGENES EUTROPHUS AEO106 WITH PLASMIDS pRO101 AND pRO103

Thesis Approved:

Thesis Advisor

Dean of the Graduate College

ACKNOWLEDGEMENTS

Sincere appreciation is expressed to numerous acquaintances whose assistance has been so meaningful to my graduate study. First of all, I wish to thank my major advisor, Dr. William W. Clarkson, for his training of me to be a independent researcher which is beneficial to my career, and for advising and directing the writting of dissertation. Furthermore, the guidance of graduate studies and financial assistance provided by Dr. Clarkson is also greatly appreciated.

Appreciation is extended to my professorial colleagues for their support and friendship, and especially to Dr. Alan R. Harker, Dr. John N. Veenstra and Dr. William F. McTernan for their contributions.

A special thanks goes to my collaborator, John K. Delphon, for his cooperation in this research project.

Additional appreciation is extended to the family of David L. Fisher, for their encouragement of my language improvement.

Finally, my family has been the invisible source of strength for me to persevere. Having ventured so far away from home, I experience different cultures and have met some remarkable people along the way.

I dedicate this dissertation to my mother, Li-Shen Yang.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Development of This Research

Xenobiotic compounds such as herbicides and other pesticides have been introduced to the environment in increasing amounts in the last several decades. The sources of these chemicals are mainly run-off from agricultural lands or waste disposal sites. Both the chemical stability of these compounds and the ability of microbial biota to metabolize them would determine their environmental persistence. Oklahoma and surrounding states rely heavily on groundwater which recently has become increasingly contaminated by these compounds. The fate of natural and xenobiotic compounds presents challenges to microbiologists and environmental engineers.

While some xenobiotic compounds are nonbiodegradable, some may be transformed to less toxic structures by biological or chemical action after entering the environment. Microbial degradation of xenobiotic compounds has become increasingly feasible to control or eliminate such chemicals through enhanced microbial activity of soil bacteria, anaerobic in situ clean-up of aquifers, or treatment of

wastewater or drinking water prior to discharge or distribution. Biofilm reactor systems have been applied to remove xenobiotics in both research and industry. Ghosal, et al. (1985) suggested that a logical procedure to improve microbial strains to enhance the mineralization of persistent compounds is to understand the biochemical and genetic approach of microbial dissimilation of biodegradable halogenated compounds. However, most current biofilm systems employ uncharacterized mixed cultures as reactor inoculum, which are collected from contaminated areas where the microbial population has adapted to those chemicals.

2,4-Dichlorophenoxyacetic acid (2,4-D), one of the most widely used herbicides, is readily biodegradable by soil bacteria and is considered a less persistent compound. In addition, the bacterial decomposition of 2,4-D is now well known, and the nutritional requirements to maintain sufficient yields of consistently active cells to degrade 2,4-D have been developed.

Objectives and Goals

There are numerous well characterized strains of microorganisms which are capable of degrading xenobiotic compounds. The advantages of maintaining monoculture biofilms of these degradative strains over mixed culture biofilms may be as follows: (a) maintenance of specific pure culture biofilms would allow a treatment facility to

respond to sporadic contamination by chemicals at low concentration for which a mixed culture might not be adapted or induced; (b) because monocultures are well characterized genetically or physically, they could be recombined or manipulated *in vitro* to produce novel pathways or to alter the expression or regulation of current degradative pathways.

In this experiment, *Alcaligenes eutrophus* AE0106 which are previously engineered and characterized to express a 2,4-D degradative pathway have been used to establish biofilms in expanded bed reactors. The research addressed necessary requirements such as flow rates, aeration, support media, etc. to maintain viable cultures within the reactors. Furthermore, removal efficiencies of 2,4-D by inducible and constitutive strains of the bacteria have been compared.

CHAPTER II

BACKGROUND THEORY AND LITERATURE REVIEW

Biochemistry and Microbiology

Xenobiotic compounds have no counterpart in nature and are usually toxic and recalcitrant. Scientists and engineers have attempted to develop processes to control these environmental contaminants. Aly and Faust (1965) found that conventional chemical treatment processes such as coagulation and filtration cannot achieve effective removal efficiency of some synthetic compounds, including 2,4-D. Edwards and Schubert (1971) investigated the removal of 2,4-D sodium salt with initial concentration of 50 mg/L in batch studies with cellulose acetate reverse osmosis membranes. Removal efficiencies were highly variable, ranging from 1 to 65 %. Table I shows adsorption capacities of activated carbon at different equilibrium concentrations (Ware, 1988).

Microorganisms seem to have certain genetic plasticity which enables them to adapt to new substrate. Furthermore, new pathways might develop through long term exposure to non-lethal concentrations of xenobiotic compounds. Recently, many microorganisms have been collected

which have evolved certain ranges of enzymes and pathways in order to metabolize different compounds. Fisher and Pemberton (1977) found that bacterial degradation of xenobiotic compounds involved plasmids which play a significant role in the evolution of the microbial population. Plasmids, the extrachromosomal genetic elements, are naturally occurring circular duplex DNA molecules. They carry genes for the resistance of antibiotics, the production of toxins, the breakdown of natural xenobiotics. Plasmids can perform gene duplication, produce mutational change, and transport genetic information between microbial cells.

TABLE I

ADSORPTION CAPACITY OF ACTIVATED CARBON

Conc. of 2,4-D	10 μg/L	100 μg/L	1000 μ g/L
Adsorption Capacity (gm of 2,4-D/ gm of A.C.)	0.009	0.032	0.118

Pseudomonas, a group of soil bacteria, were known to possess plasmids to encode part or all of the degradation pathway in the breakdown and recycling of naturally

occurring aromatic and aliphatic compounds (Chakrabarty, 1976; Gunsalus and Marshall, 1971). Recent studies have shown that many synthetic chlorinated compounds are degraded by Alcaligenes (Fisher, et al., 1978).

In Alcaligenes eutrophus JMP134, the genes which encode the degradation pathway of the herbicide 2,4-D are plasmid-borne. pJP4 is an 80-kilobase, broad host range plasmid, belongs to the Inc P1 incompatibility group, encodes resistance to mercury, and carries essential genes to permit Alcaligenes eutrophus JMP134 to utilize 2,4-dichlorophenoxyacetic acid, 3-chlorobenzoate and 2-methyl-4-chlorophenoxyacetic acid as sole carbon and energy sources (Don and Pemberton, 1981 and 1985; Don, et al., 1985). The five gene products tfdA, tfdB, tfdC, tfdD and tfdE are the 2,4-D monooxygenase, 2,4-dichlorophenol hydroxylase, dichlorocatechol 1,2-dioxygenase, chloromuconate cycloisomerase and chlorodienelactone hydrolase, respectively, (Don et al., 1985 ; Streber, et al., 1987). These five enzymes are plasmid encoded and were described in concordance with the second, third, fourth, and fifth steps in the 2,4-D degradative pathway which was proposed by Evans, et al. in 1971 (Figure 1). Furthermore, the common functions which express the same degradation pathway between 2,4-D and 3-chlorobenzoate have been mapped by transposon insertion mutagenesis (Don,et al., 1985). Plasmid pRO101, derived by insertion of Tn1721 into plasmid PJP4, would express the

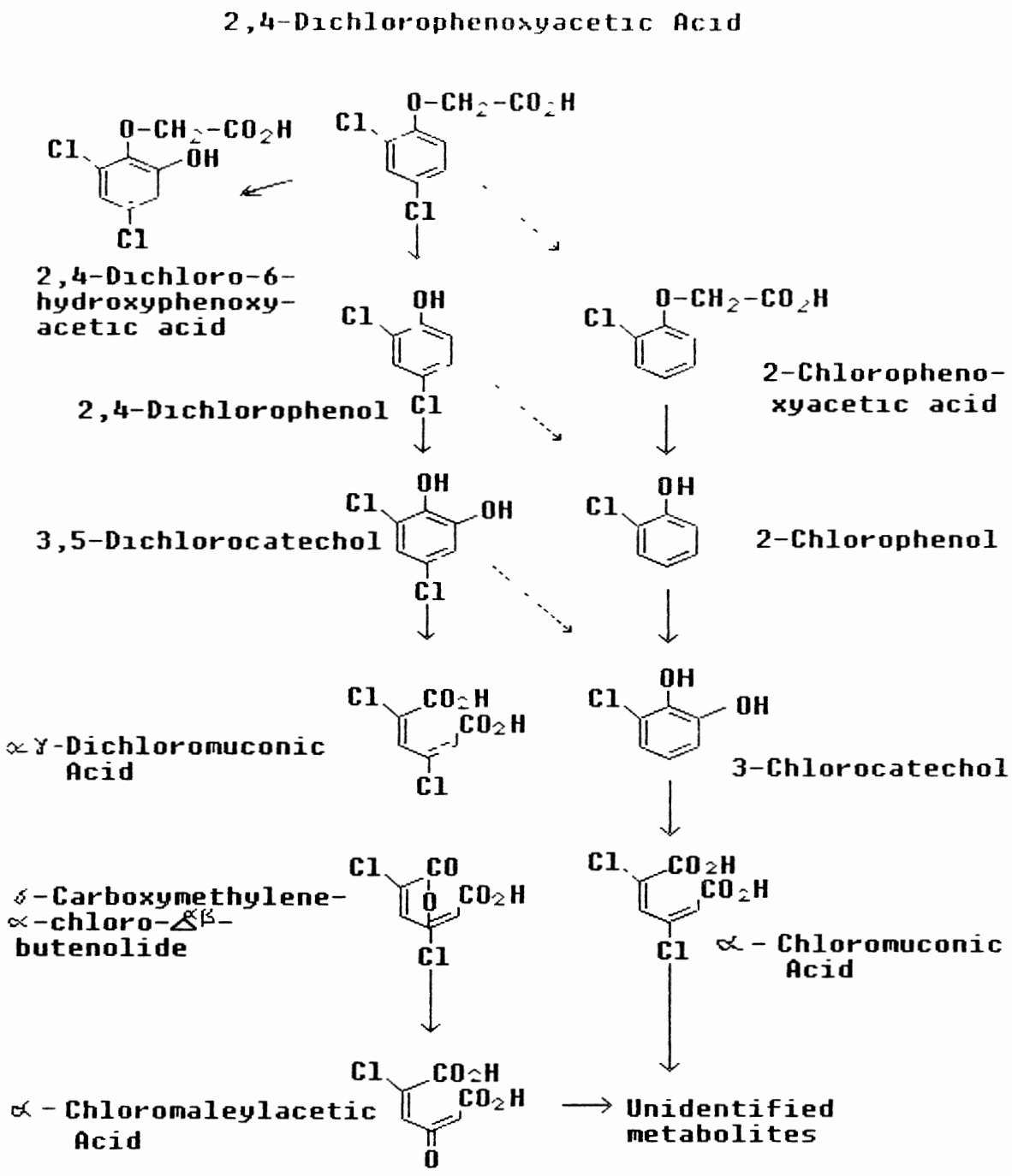

Figure 1. Degradation Pathway of 2,4-D (Evans, et al., 1971).

2,4-D pathway in the presence of inducer, such as 2,4-D or 3-chlorobenzoate, because the regulatory gene tfdR controls expression of the 2,4-D pathway. Plasmid pRO103, obtained by deletion of regulatory gene tfdR from pRO101, expresses constitutively the 2,4-D pathway, can completely metabolize 2,4-D and 3-chlorobenzoate and partially degrade phenoxyacetic acid (PAA) in the absence of inducer. Therefore, recombinant DNA technology could offer potential strains, such as Pseudomonas putida PPO300, to completely mineralize PAA or grow on PAA as sole carbon source (Harker, et al., 1989).

Engineering Reactor Design

The mineralization of unwanted chemicals is of concern because many synthetic compounds are related to mutagens, carcinogens or toxicants. The decomposition of pollutant compounds in soils and water is primarily a consequence of microbial activity. When microorganisms degrade pollutant chemicals to intermediate or end products, they make use of some carbon to form cell constituents. Meanwhile, energy is released for biosynthesis. The biodegradation of chemical compounds has been enhanced through immobilized biofilm formation, either in natural systems or engineered treatment processes. Biofilm reactors, such as trickling filters and fluidized bed reactors, can accumulate a large quantity of microbial cells to maintain high solids residence times and promote system stability

and efficiency. Jewell and MacKenzie (1972) indicated that organic removal capacity in an attached biofilm reactor could be twice that in a suspended microbial reactor under aerobic conditions.

Fluidized bed reactors, combining some attributes of activated sludge and trickling filters, overcome the defects encountered in packed tower systems and minimize hydraulic head loss by upward expansion of smaller biofilm support materials. Jeris and Owens (1975) and Jeris, et al. (1977) have demonstrated high efficiencies in denitrification, nitrification and aerobic removal of organic compounds by fluidized bed reactors in wastewater treatment. Chen, et al. (1988) evaluated COD removal and methane production for industrial wastewater treatment by an anaerobic fluidized bed reactor. The system was efficient and achieved biomass concentration greater than 15 g/L. In addition, a predictive model of biomass concentration and particle terminal settling velocity was developed (Mulcahy and Shieh, 1987). Bull, et al. (1983) confirmed process stability and performance of an anaerobic fluidized bed reactor treating a synthetic dairy waste over a range of temperature and loading rate.

The attached biofilm expanded bed reactor was developed for aerobic and anaerobic waste treatment by Jewell and coworkers beginning about in 1970. Biomass concentrations in aerobic expanded bed reactors can reach greater

than 20 g/L and also are expected to be about 100 g/L for anaerobic beds with further process development (Jewell, 1981 and 1985). The lightweight support medium and low upflow velocity for 10-20 % bed expansion are the major differences between expanded and fluidized bed reactors. Thus, the input energy to the expanded bed could be reduced to a minimum. In addition, low hydraulic detention times also promote system economy. Furthermore, the expanded bed can trap and hydrolyze insoluble material with minimum clogging. Schraa and Jewell (1984) reported that high removal efficiency can be achieved for high COD loading rates in the anaerobic attached biofilm expanded bed system. Switzenbaum and Jewell (1980) verified the ability to treat dilute soluble wastewater. Rittmann and Brunner (1983) have shown nonsteady-state biofilms can perform with good removal efficiency on trace substrate below minimum growth-limiting substrate concentration for one year without reinoculation. Bouwer and McCarty (1982) investigated the removal of chlorinated organic compounds and assured that biofilm growth on adsorptive material such as granular activated carbon could contribute to higher stability and reliability of the treatment process. No literature was found to evaluate the application of aerobic expanded bed reactors to the type of waste considered here. Jewell and MacKenzie (1972) and Jewell (1981) described the occurrence of clogging and substrate transfer limitations if pure oxygen

was employed with aerobic expanded bed reactors, but the yields were significantly less than those obtained in suspended growth reactors.

Diatomaceous earth, unlike more commonly used support materials for fluidized bed reactors, has a high absorptive capacity of water because of its high specific surface area (5780 m^2/ m^3) and low bulk density (0.35 g/ cm^3). Comparing the capacity of diatomaceous earth and charcoal to adsorb different dissolved substances, the former shows significantly less removal capacity (Calvert, 1930). Diatomaceous earth also is chemically inert, has relatively low cost, and possesses unique particulate structure which could form voids to trap suspended solids. Furthermore, the low thermal conductance of diatomaceous earth gives this material a softening point of about 1430 ^oC (Anonymous, 1987), making it unaffected during analysis of biofilm volatile organic content.

CHAPTER III

MATERIALS AND METHODOLOGY

Bacterial Strains and Plasmids

Both plasmid pRO101 and pRO103, which carry certain genes to encode tetracycline resistance, were obtained from the microbiology laboratory of Professor Alan Harker of Oklahoma State University. Pseudomonas putida containing plasmids pRO101 and pRO103 separately were employed in start-up phase. The bacterial strain was changed to Alcaligenes eutrophus AE0106 for experimental phase. Genetically engineered bacterial strains were inoculated in 2,4-D solution (0.05%) with minimal salts medium in a model No. 3526 incubator-shaker (Lab-Line Instruments, Inc.) at 30 ^{o}C for 5 days. Then, each 10 mL inoculum was injected into reactors which were previously sterilized thoroughly.

Substrate and cultural medium

Because solubility of 2,4-D at neutral pH and room temperature is about 540 mg/L, concentrated 2,4-dichlorophenoxyacetic acid solution (0.05%) was prepared by titrating with 10 N sodium hydroxide solution to raise pH. 2,4-D was then totally dissolved at temperature of about 50-60 ^{o}C,

then poured into sterilized feed bottles to make up concentrations of 800, 1600, and 3200 mg/L. Dilution bottles containing autoclaved water with buffer solution in the same ratio as that in the feed bottles were used to dilute influent 2,4-D concentration to about 200, 400, and 800 mg/L. 2,4-D and solutions A, B, and C were prepared with pure water distilled from an Autostill 5 distiller (Wheaton Co.). Distilled water in both feed bottles and dilution bottles was obtained from a reverse osmosis system. The composition of minimum mineral medium which consists of buffer solution and elementary trace materials for bacterial growth is presented in Table II.

Apparatus

Two bench scale expanded bed reactors were constructed from identical glass chromatography columns fitted with fluoropolymer endplate, teflon seal, and stopcock. The schematic configuration of the reactors is presented in Figure 2. Silicone and tygon tubing were used for solution transport and in pumping heads, respectively. Tubing length was minimized to reduce any possible adsorption or attachment of bacterial growth on the surface. Silicone tubing was changed about every two months as soon as bacterial attachment become visible. Tygon tubing was replaced every other week to prevent any contamination caused by leaking. Diatomaceous earth was previously sifted through a No. 20

TABLE II

COMPOSITION OF MINIMUM MINERAL MEDIUM

Distilled water	930 mL
Solution A	40 mL
Solution B	20 mL
Solution C	10 mL

Solution A: 1 M Na/K Phosphate Buffer

Sodium Phosphate Dibasic (1 M)	220 mL
Potassium Phosphate Monobasic (1M)	190 mL

Solution B:

Nitrilotriacetate	10.0 g
Magnesium Sulfate	14.45 g
Calcium Chloride (Dihydrate)	3.34 g
Ammonium Molybdate	9.25 mg
Ferrous Sulfate (Heptahydrate)	0.1 g
Metal "44"	50 mL
Potassium Hydroxide	7.4 g

Dissolve above chemicals in distilled water to make up 1000 mL solution.

Solution C: 10 % Ammonium Sulfate

Distilled water	100 mL
Ammonium Sulfate	10 g

Metal "44"

Distilled water	100 mL
EDTA	0.25 g
Ferrous Sulfate (Heptahydrate)	0.5 g
Manganous Sulfate (Monohydrate)	0.154 g
Cupric Sulfate (Pentahydrate)	0.039 g
Cobalt Nitrate	0.025 g
Zinc Sulfate	0.11 mg
Sodium Tetraborate	0.018 mg

A few drops of sulfuric acid should be added to retard precipitation

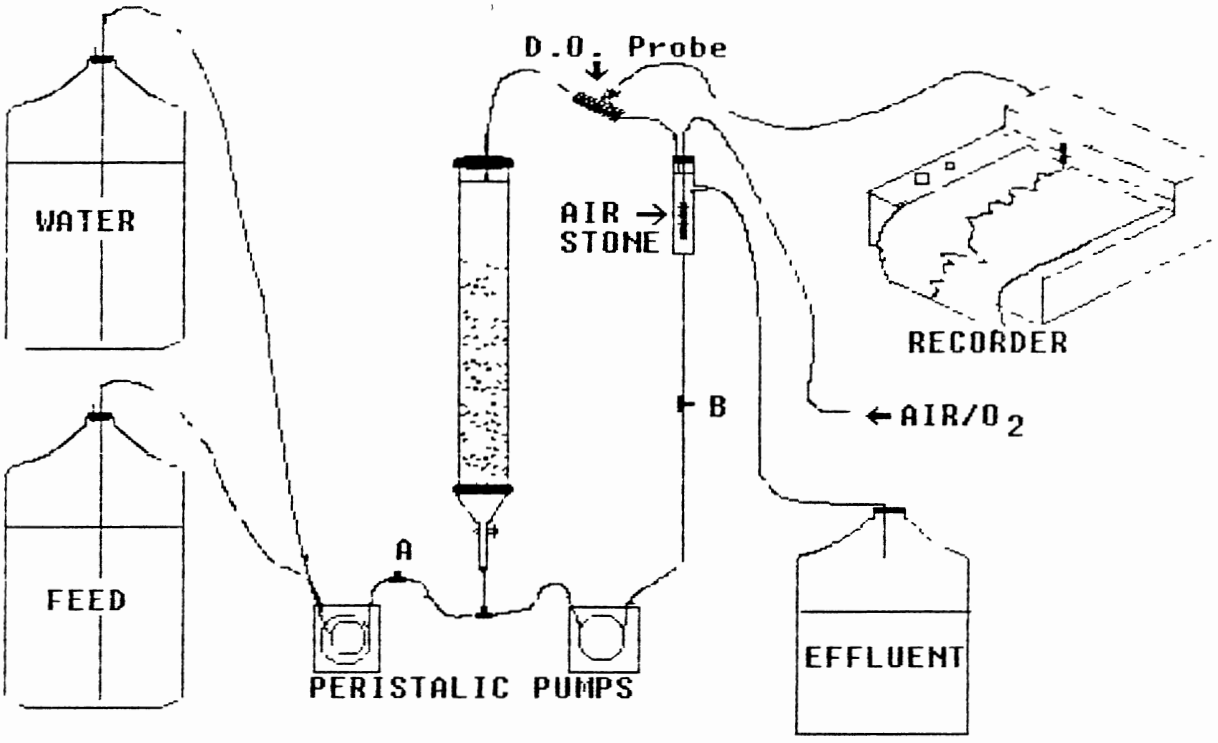

Figure 2. Schematic Configuration of The Reactor Systems.

mesh sieve (W.S. Tyler Co.) to ensure that particle size was restricted to less than 600 μm. The diatomaceous earth was ignited at 550 ±50 °C for one hour before being poured in the reactor column in order to eliminate volatile contaminants. Other utensils such as diffusing stones, cotton screens, air vents, and air filters (pore size 0.2 μm) were autoclaved prior to installation to block any bacterial invasion and balance system pressure. Whenever undesirable growth of organisms was observed, tetracycline was added to maintain the system as a monoculture.

Start-up Phase

Each column was filled with 625 mL of support materials composed of 75 mL glass beads (3 mm) for distribution of flow and 550 mL diatomaceous earth for bacterial attachment. Total reactor volume was 1070 mL which consisted of 900 mL in glass chromatography column, 160 mL in a separate aeration vessel and 10 mL in tubing. A photograph of the system is shown in Figure 3. Influent concentrations of casamino acid were 3000 mg/L for the flow rate 1.75 L/d which had a hydraulic detention time of 14.6 hours, and 630 mg/L for the flow rates 8.5 and 18.3 L/d which had hydraulic detention times of 3 and 1.56 hours, respectively. The use of glass beads (diameter 3 mm) as bacterial attachment material was also examined in this experiment because of their minimal adsorption properties and regular geometry

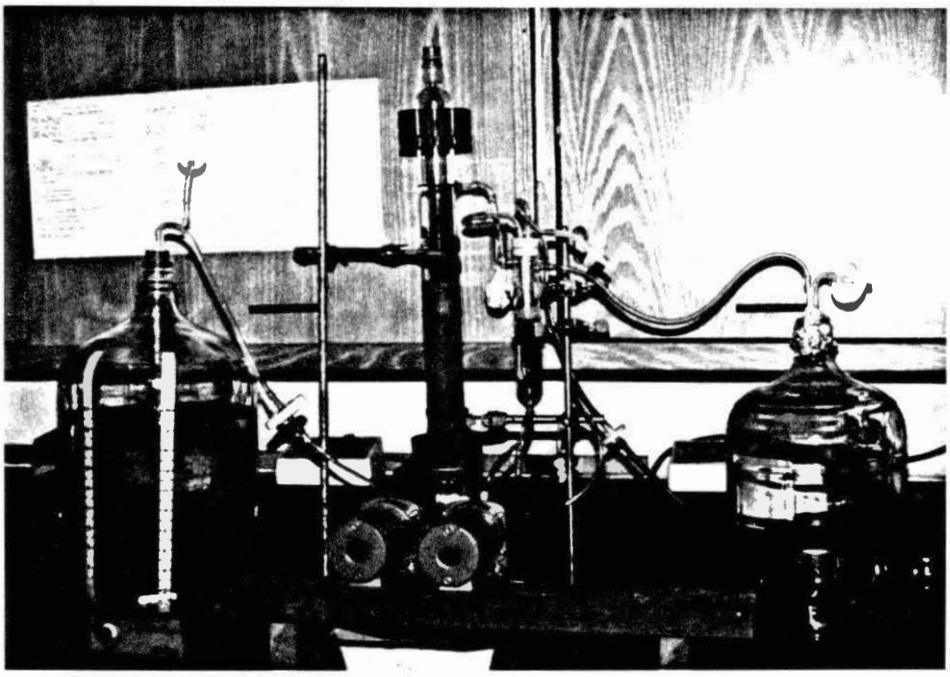

Figure 3. The Reactor Set-up of Start-up Phase.

However, our recycle pump could not expand 50 mL of glass beads due to their high specific gravity, and diatomaceous earth was chosen instead.

Experimental Phase

Each expanded bed reactor in this phase had a total volume of 185 mL, including 160 mL in the glass chromatography column, 15 mL in the aeration vessel and 10 mL in tubing. Bed volume was 90 mL of diatomaceous earth. Photograph of one of the two reactors is given in Figure 4. Five holes (one-quarter inch diameter) were uniformly drilled in the endplate of these reactors. This was used instead of glass beads in order to improve the regulation of upward flow to make the reactor mix completely. The variables studied are outlined in Table III. Recycle pumping was adjusted periodically to keep a constant bed expansion of about 20 %.

Engineered bacterial strains were inoculated into reactors which were filled with media containing 200 mg/L of 2,4-D. In the beginning of inoculation, influent was fed periodically to balance vaporization loss from the aeration vessel to submerge the diffusing stone for the dissolution of oxygen into solution. Continuous 2,4-D feed was not started until significant degradation could be measured, and biofilm build-up could be observed. Loading rate of 2,4-D was doubled either by increasing

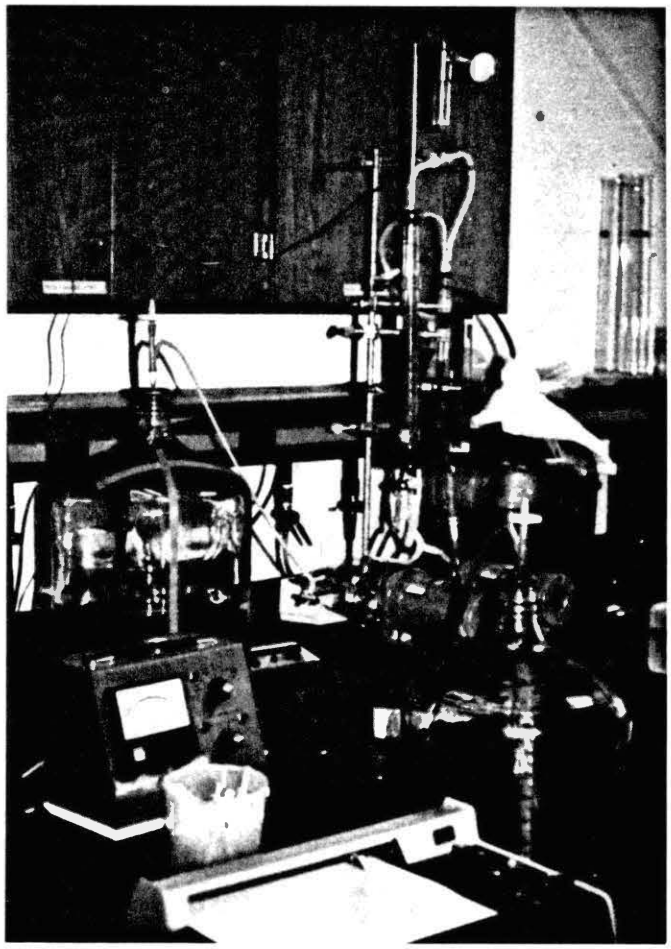

Figure 4. The Reactor Set-up of Experimental Phase.

TABLE III

SCOPE OF STUDY

Variable	Value	
Influent 2,4-D Concentration	200 - 800	mg/L
Hydraulic Detention Time	2 - 0.5	hr
2,4-D Loading Rate	4 - 64	kg/m^3*d
Temperature	20 $\pm$ 2	^oC

influent concentration or feed flow rate in stages, as shown in Table IV. In stage 3, pure oxygen feed was initiated. A YSI 4004 Clark oxygen probe connected to a YSI model 53 biological oxygen monitor with a chart recorder was installed to detect the dissolved oxygen residual of effluent. The dissolved oxygen in the effluent was adjusted to remain above 20 % of saturation to prevent oxygen limitation.

TABLE IV

LOADING TESTS

Stage	2,4-D Concentration (mg/L)	Flow Rate L/d (mL/min)	2,4-D Loading Rate kg/m^3*d
1	200	2.16 (1.5)	4
2	200	4.32 (3)	8
3	200	8.64 (6)	16
4	400	8.64 (6)	32
5	800	8.64 (6)	64

Methods of Analysis

2,4-D Concentration

All spectrophotometric tests were performed on a Shimadzu model UV-160A visible recording spectrophotometer. Minimal medium salts in the same concentration as in 2,4-D solution was used as absorbance standard. The absorbances of a series of 2,4-D concentrations (50, 100, 150, 200, 250 mg/L) were measured to construct a standard curve. 2,4-D was identified by scanning spectra between 240 and 320 nm. Maximum absorbance was exhibited at around 283 nm. After influent 2,4-D concentration was increased to higher than 250 mg/L, samples would be diluted to below 250 mg/L. Each 1 mL sample of influent and effluent was removed every day by sterile syringe from point A or B (see Figure 2) and centrifuged in an Eppendorf 5414 centrifuge for 3 minutes to separate cells and supernatant.

COD Tests

Total COD data were determined every two or three days for influent and effluent by the colorimetric culture tube method 508 C of Standard Methods (APHA *et al.*, 1985). All tests were performed on the model UV-160A visible recording spectrophotometer. Potassium hydrogen phthalate was used to construct the standard COD curve. The coefficient of correlation for all standard curves was greater than 0.99.

Biomass Concentration

Biomass concentrations were determined in duplicate by taking representive samples (10 mL sample with 5 mL diatomaceous earth and 5 mL liquid) at a point 5 cm below the top of the expanded bed. Liquid supernatant was transferred to an ashed, pre-weighed crucible. Diatomaceous earth was agitated vigorously by distilled water, and supernatant was transferred to the above crucible until a clear supernatant remained over the diatomaceous earth. The purpose of this was to dislodge and separate any entrapped solids from the biofilm attached to diatomaceous earth. Then, both diatomaceous earth and supernatant were dried at 103 ^{o}C and ashed at 550 $\pm$ 50 ^{o}C. These samples represent the attached and suspended biomass concentrations (Clarkson, 1986; APHA, 1985).

Viable Count Number

Viable count was executed in triplicate by a coworker in the microbiology laboratory. Diatomaceous earth (0.5 mL) was removed at the same point as described above. Samples were rinsed gently in 1 mL buffer solution until there were no obvious entrapped materials in the solution. Samples were vortexed with 2 mL buffer solution to detach the biofilm. Then, 3 more mL buffer solution was added to make up a 10^{-1} dilution. Serial dilutions (10^{-3}, 10^{-4}, 10^{-5}, 10^{-6}) were

made and 1 mL of each spread on a TNA plate. Viable count number could be obtained a few days later by incubating those plates at 37 ^{o}C.

CHAPTER IV

RESULTS AND DISCUSSION

Start-up Phase

Theoretically, casamino acid alone does not induce any degradative activities encoded by plasmid pRO101 (Harker, 1989). It was predicted that the inducible strain should have better biofilm formation from casamino acid solution because energy requirements for the inducible strain to grow and sustain themselves are less than that for the constitutive strain due to the secretion of 2,4-D degradative enzymes in the constitutive strain. Furthermore, when bacterial growth substrate was shifted from casamino acid to 2,4-D, the acclimation of bacterial strains in the absence of inducer (casamino acid) to the presence of inducer (2,4-D) can be compared. In the initial start-up period, 3000 mg/L of casamino acid in minimal salts medium was fed to reactors at 1.2 mL/min producing a hydraulic detention time of about 14.5 hours. A large amount of cell material was observed at the top of the columns independent of the diatomaceous earth matrix. This phenomenon indicated the hydraulic detention time was too long to wash out excessive suspended cells. Feed medium as described above

was diluted to about 630 mg/L and feed flow rate was increased to 5.8 mL/min and 11.8 mL/min corresponding to hydraulic detention times of 3 and 1.56 hours. In the former condition, attached biomass concentrations for AE0106 pRO101 and pRO103 were measured as 26.1 and 12.5 g/L, respectively. Furthermore, both reactors were contaminated by undesired organisms which could not be eliminated effectively even after the addition of tetracycline. Diatomaceous earth could not be mixed completely in the reactors due to the imperfect mixing. In the latter condition, attached and suspended biomass concentrations of both reactors were measured and tabulated in Table V.

TABLE V

ATTACHED AND SUSPENDED BIOMASS CONCENTRATIONS OF EXPERIMENT I IN WHICH HDT = 1.56 HR

	Pseudomonas putida pRO101	Pseudomonas putida pRO103
Attached Biomass Concentration (g/L)	8.2	4.3
Suspended Biomass Concentration (g/L)	5.3	6.8

Data in Table V show that suspended biomass made up a high percentage of the total population in both reactors. The reactors were not established as completely attached biofilm systems. Bacterial growth rate in high nutrient substrates such as casamino acids, which provide ready-made amino acids for bacterial utilization, should be faster than that in refractory carbon sources in which bacteria have to synthesize their necessary amino acids. Thus, a hydraulic detention time shorter than bacterial doubling time was recommended to increase washout of excessive suspended growth of cells. Bacterial doubling times of Pseudomonas and Alcaligenes in substrate casamino acid at room temperature were estimated to be about 45 minutes (Harker, 1989).

Experimental Phase

2,4-Dichlorophenoxyacetic acid was used as sole carbon source instead of casamino acid for bacterial growth. Concentrated 2,4-D solution in feed bottles (see Figure 2) were made up to 800, 1600, and 3200 mg/L and applied to the reactors over 5 stages. They were diluted with distilled water with the same concentrations of minimal salts medium as that in feed bottles in order to feed the reactors at desired 2,4-D concentrations. Dilution ratios over the experimental period (113 days) of both reactors were randomly observed on the 48th, 52th, and 96th day and tabulated in Table VI. Average dilution ratio was assumed to be

constant over the entire experiment period. The prepared influent 2,4-D concentrations were defined as concentrations in feed bottles divided by dilution ratio. Prepared influent 2,4-D concentrations of both reactors over 5 stages were calculated and listed in Table VII.

TABLE VI

DILUTION RATIO

Reactor	Flow Rate of Distilled Water /Flow Rate of 2,4-D feed			Average Dilution Ratio
Day	48	52	96	
pRO101	3.55	3.29	3.45	3.45
pRO103	3.2	3.5	3.5	3.4

TABLE VII

PREPARED INFLUENT 2,4-D CONCENTRATIONS OVER 5 STAGES

Stage	Prepared Influent Concentration (mg/L)			
	Reactor pRO101		Reactor pRO103	
1	180	(6-24th day)	182	(6-24th day)
2	180	(25-38th day)	182	(25-38th day)
p 3	180	(39-81th day)	182	(39-80th day)
4	360	(82-98th day)	364	(81-98th day)
5	719	(99-113th day)	729	(99-113th day)

" p " Pure oxygen was added to reactor pRO101 and pRO103 on the 77th and 54th day, respectively.

Because concentrated 2,4-D solution and dilution water might not mix perfectly enough in point A (see Figure 2) for instantaneous grab samples to be representive, three point rolling average data of influent 2,4-D and COD concentrations were calculated (Tables VIII, IX and X). Thus, the data are smoothed by this technique and should represent a true picture of substrate feed to the reactor. The first and last composite 2,4-D concentrations of each sample population were the average value of the first and last two grab samples, respectively. Sample population are those data of Column A in Table VIII, IX and X. Each of the other 2,4-D concentrations is an average of three grab sample concentrations. For example, the first value, 122 mg/L, in Column B of Table VIII is the average of the first two actual grab samples, 137 and 106 mg/L, which were listed in Column A. The second value, 138 mg/L, of Column B of Table VIII is the average of 138, 106 and 172 mg/L of Column A in the same Table, and so on through all the data. Degradation efficiency and loading rate were calculated based on both prepared influent 2,4-D concentrations (Table VII) and composite influent 2,4-D concentrations (Table VIII, IX).

Influent composite 2,4-D and COD concentrations over 5 stages of both reactors are presented in Figures 5, 6, 7 and 8. Effluent 2,4-D and COD concentrations also shown in Figures 5, 6, 7 and 8 should not be subject to

the same potential sampling errors because samples from any point of a completely mixed reactor have the same characteristics. Viable count numbers of both reactors over the experimental period are plotted in Figure 9.

Stage 1

Five days after inoculation of bacterial strains to reactors, viable count numbers of reactors pRO101 and pRO103 were measured as 34 $*10^6$ and 8.6 $*10^6$ CFU/mL, respectively. Then 2,4-D solution was fed to the systems continuously. In the beginning of this stage, effluent residual 2,4-D showed more significant degradation of 2,4-D in reactor pRO101 (Table XI), and viable counts also consistently indicated more cell formation in strain pRO101 (Table XII and Figure 9). Eventually, complete 2,4-D degradation was achieved (Figure 5, 6 and Table XI) and effluent COD was about 50 mg/L for both reactors (Figure 7, 8 and Table XI). Although more bacterial activity and 2,4-D degradation was observed in strain pRO101, data in Table XII showed higher biofilm concentration of strain pRO103 at the 20th day. This might indicate the attachment of strain pRO103 is stronger. The anomalous viable count number (< 1) at the 24th day was not plotted in Figure 9.

Stage 2

Once influent flow rates were increased to 3 mL/min,

TABLE VIII

COMPOSITE INFLUENT 2,4-D CONCENTRATIONS
OF REACTOR pRO101 OVER 5 STAGES

Day	A	B	Day	A	B
Stage 1 through Stage 3:			(Continued)		
6th	137	122	62th	237	209
7th	106	138	63th	180	165
11th	172	139	64th	178	189
13th	139	156	67th	209	197
15th	158	171	68th	203	197
18th	216	183	70th	178	193
20th	174	208	72th	198	204
22th	230	208	74th	237	239
			75th	281	258
			78th	257	228
25th	221	224	79th	156	226
28th	221	230	80th	264	211
29th	250	232	81th	212	238
30th	225	222			
32th	192	219	Stage 4:		
33th	242	213	82th	464	489
38th	206	200	83th	514	493
			85th	502	468
			86th	387	468
39th	153	158	88th	514	426
40th	114	152	91th	377	410
42th	188	180	94th	339	376
43th	237	213	96th	413	400
44th	214	222	97th	447	430
45th	216	209			
46th	196	201	Stage 5:		
47th	270	199	99th	656	714
48th	211	199	100th	771	664
49th	197	214	102th	565	659
50th	234	233	103th	640	632
51th	269	215	105th	690	675
55th	143	193	106th	694	691
56th	166	172	107th	689	671
57th	207	188	109th	631	682
58th	190	213	110th	727	647
59th	242	228	111th	582	709
60th	253	235	112th	818	702
61th	211	234	113th	707	763

Column A - grab samples taken from influent (mg/L).
Column B - composite samples calculated from column A (mg/L), as three point rolling average.

TABLE IX

COMPOSITE INFLUENT 2,4-D CONCENTRATIONS
OF REACTOR pRO103 OVER 5 STAGES

Day	A	B	Day	A	B
Stage 1 through Stage 3:			(Continued)		
6th	190	165	64th	117	138
7th	200	183	67th	124	128
11th	159	187	68th	142	166
13th	202	186	70th	232	188
15th	196	199	72th	189	208
18th	200	189	75th	202	195
20th	171	190	78th	195	194
22th	198	186	79th	185	175
			80th	144	165
25th	189	197	Stage 4:		
28th	209	216	81th	272	343
29th	250	220	82th	414	363
30th	201	217	83th	404	354
32th	200	197	84th	243	386
33th	190	198	85th	510	370
38th	205	190	88th	358	455
			89th	496	390
			91th	315	358
39th	175	178	92th	262	270
40th	154	154	94th	234	345
42th	134	154	97th	539	387
43th	174	138			
44th	106	140	Stage 5:		
45th	141	142	99th	400	568
46th	179	162	101th	735	683
48th	167	166	102th	913	804
50th	153	150	103th	765	878
51th	131	129	104th	956	826
52th	104	120	105th	757	854
53th	126	135	106th	849	758
54th	174	171	107th	669	686
55th	214	180	110th	539	556
57th	153	176	111th	459	599
58th	161	164	112th	799	702
59th	179	165	113th	848	819
60th	154	169			
61th	173	148			

Column A - grab samples taken from influent (mg/L).
Column B - composite samples calculated from column A (mg/L), as three point rolling average.

TABLE X

COMPOSITE INFLUENT COD CONCENTRATIONS OF REACTOR pRO101 AND pRO103 OVER 5 STAGES

Reactor pRO101				Reactor pRO103			
Day	A	B		Day	A	B	
Stage 1 through Stage 3:				Stage 1 through Stage 3:			
12th	241	240		12th	195	213	
16th	238	224		16th	231	202	
			(232)				(216)
26th	192	216		21th	181	234	
28th	219	206		26th	291	239	
32th	207	217		28th	244	254	
34th	224	199		32th	228	227	
36th	167	200		34th	210	220	
38th	209	184		36th	222	217	
			(204)				(217)
39th	175	201		38th	219	207	
44th	219	201		39th	181	198	
46th	210	218		42th	195	188	
48th	225	227		46th	189	188	
50th	247	211		48th	181	181	
55th	161	212		50th	172	164	
57th	227	224		53th	138	180	
60th	284	235		55th	229	179	
64th	193	236		57th	171	191	
67th	231	206		60th	174	160	
70th	195	249		67th	134	190	
75th	322	230		70th	261	200	
79th	172	247		75th	206	221	
			(223)	79th	195	201	
Stage 4:							(188)
82th	501	423		Stage 4:			
86th	344	417		82th	374	445	
91th	407	377		89th	516	393	
94th	379	415		91th	290	439	
97th	458	419		97th	510	400	
			(410)				(419)
Stage 5:				Stage 5:			
100th	885	828		103th	875	903	
103th	770	804		106th	930	890	
106th	757	750		112th	865	898	
109th	724	745					(897)
113th	753	739	(773)				

Column A, Column B as previously defined.
Parenthesis means the average composite influent COD value of the stage.

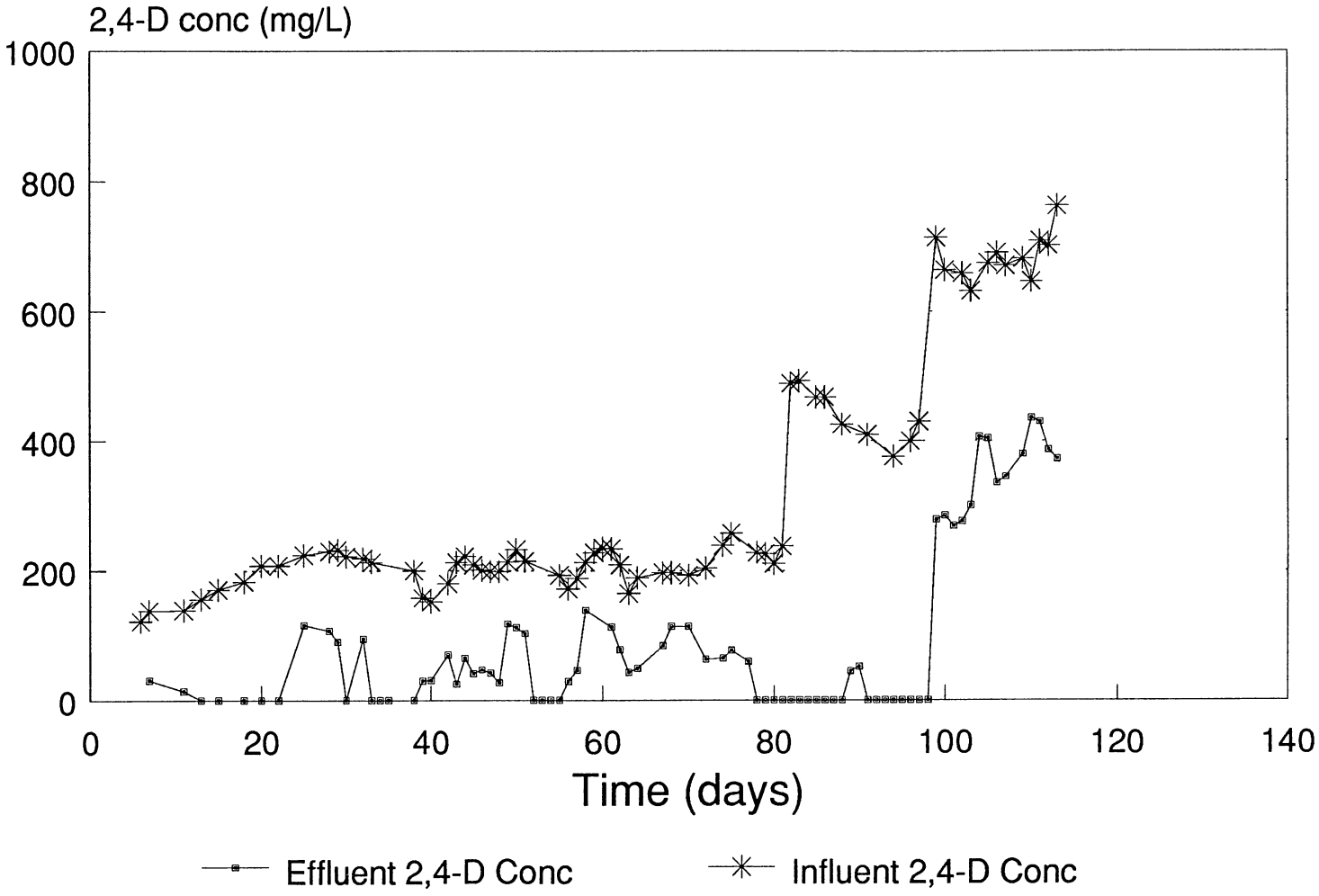

Figure 5 2,4-D Concentrations of Reactor pRO101

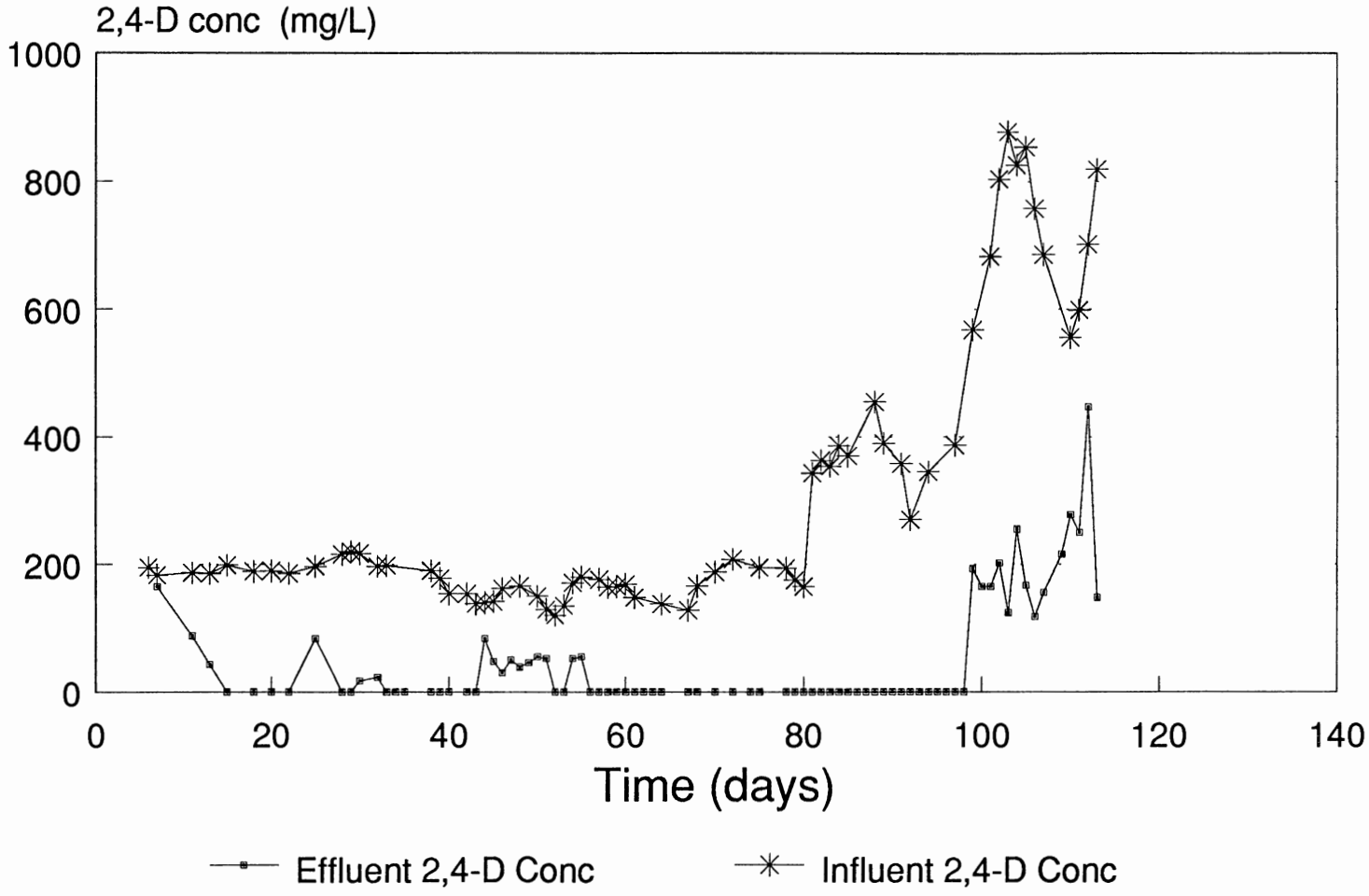

Figure 6 2,4-D Concentrations of Reactor pRO103

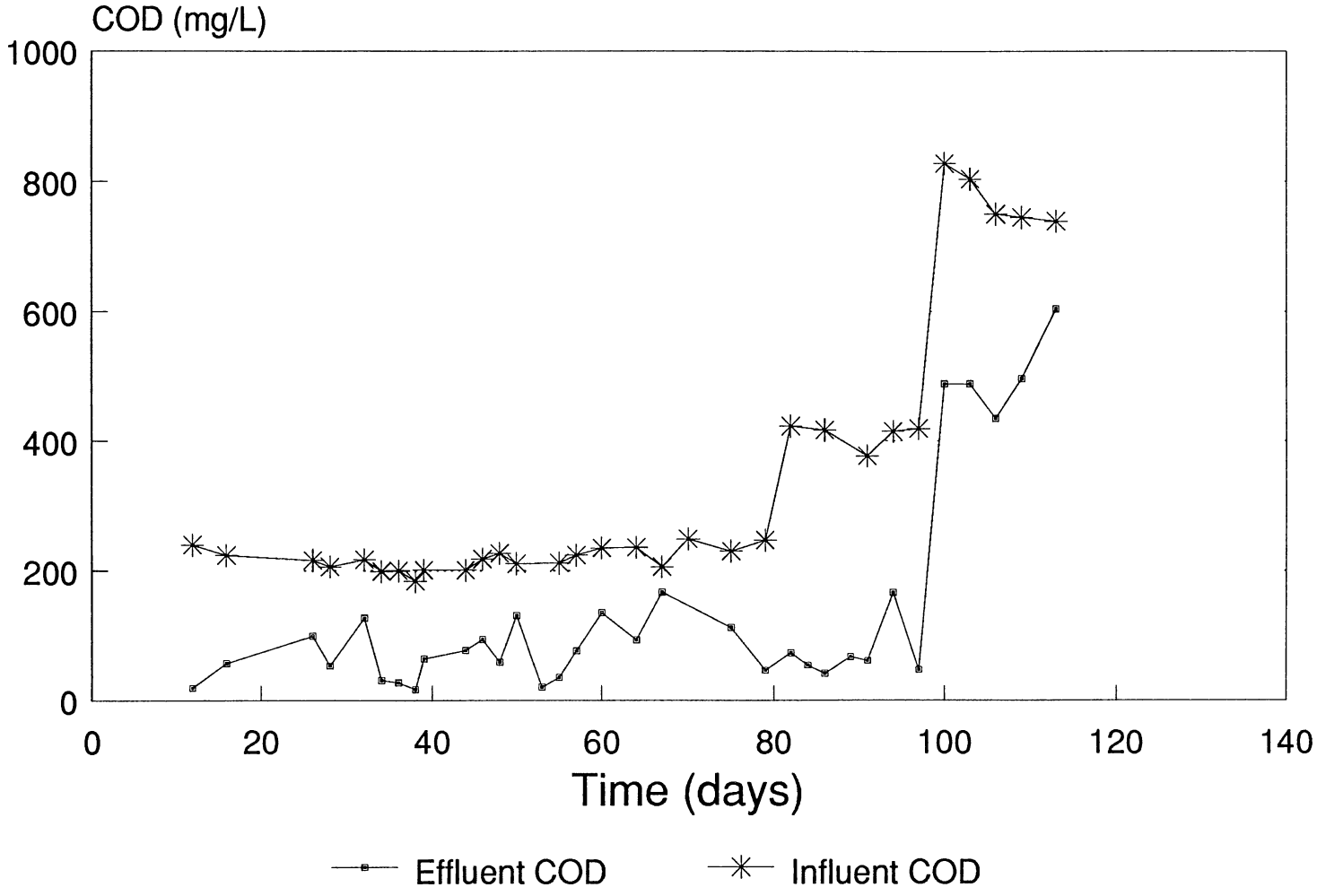

Figure 7 COD Concentrations of Reactor pRO101

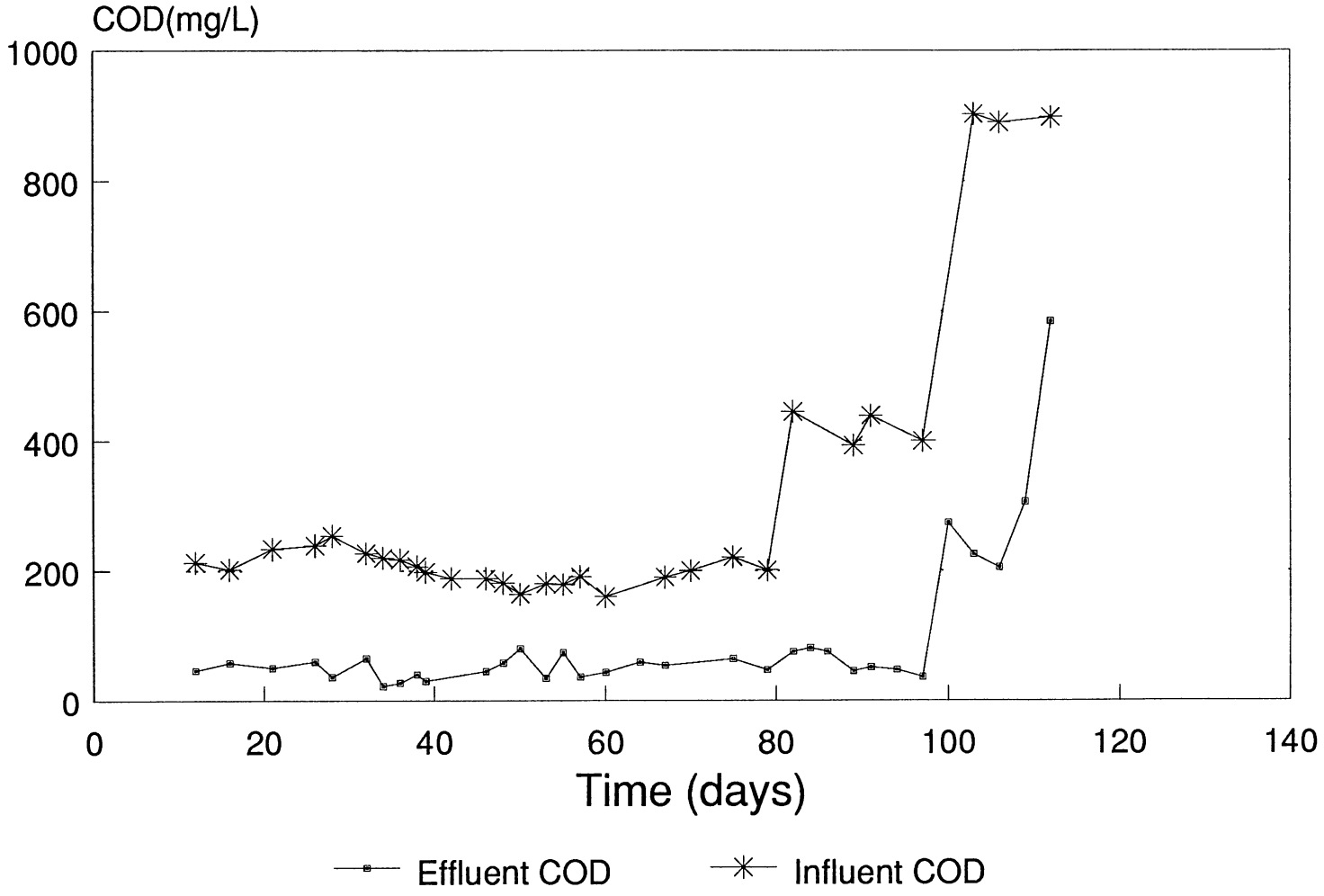

Figure 8 COD Concentrations of Reactor pRO103

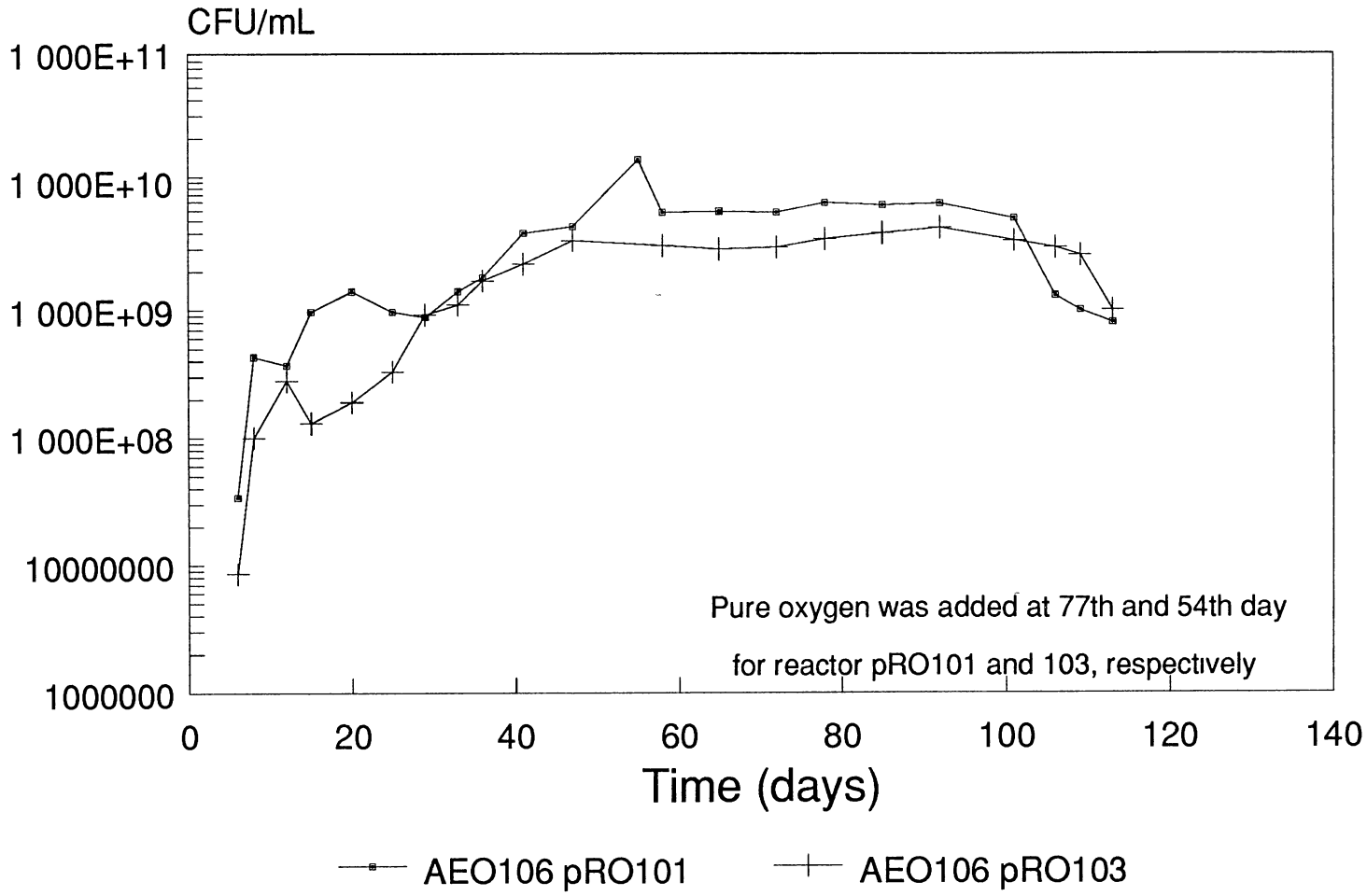

Figure 9 Viable Count Number of AEO106 pRO101 and pRO103

TABLE XI

EFFLUENT 2,4-D AND COD CONCENTRATIONS OF STAGE 1

Day	Effluent 2,4-D Concentration (mg/L)		Effluent COD Concentration (mg/L)	
	pRO101	pRO103	PRO101	pRO103
7th	31	165		
11th	14	88		
12th			19	46
13th	b	43		
15th	b	b		
16th			57	58
18th	b	b		
20th	b	b		50
22th	b	b		

" b " 2,4-D Concentration below detectable.

TABLE XII

BIOFILM CONCENTRATIONS AND VIABLE COUNT NUMBERS OF STAGE 1

Day	Biofilm Concentration (g/L)		Viable Count Number CFU 10^6/mL	
	pRO101	pRO103	pRO101	pRO103
7th	2.1	1.7		
8th			430	100
12th			370	280
15th			970	130
20th	4.24	8.35	1400	190
24th			< 1	

Viable count number 970, 1400, 130 and 190 CFU 10^6 /mL were used to calculate the substrate removal rates for strain pRO101 and pRO103, respectively.

the still-developing biofilm reactors experienced a rise in effluent 2,4-D and COD concentrations (Figure 5, 6 and Table XIII). However, attached biofilm was still being established (Table XIV and Figure 9) and about a week later, complete degradation of 2,4-D was achieved again. Effluent COD was reduced to about 30 mg/L in both reactors (Table XIII and Figures 7, 8). Attached biomass concentrations were not sampled at this stage due to concern over the removal of 10 mL samples of diatomaceous earth. It was felt that this relatively large sample size would significantly influence the reactors during their development. Although complete 2,4-D removal was achieved by both strains, viable count numbers (Table XIII) still showed unstable biofilm populations.

Stage 3

2,4-D residual concentrations in both reactors dropped below detection limit and then rose again (Table XV). This phenomenon indicates that before the installation of pure oxygen, effluent quality tended to fluctuate in both reactors. The reasons might be substrate transfer limitation, oxygen transfer limitation, or insufficient supply of dissolved oxygen. Viable count numbers indicate that virtually steady-state biofilm populations were reached in this period (Table XVI and Figure 9), particularly after pure oxygen was supplied to reactor pRO103. Reactor pRO101

TABLE XIII

EFFLUENT 2,4-D AND COD CONCENTRATIONS OF STAGE 2

Day	Effluent 2,4-D Concentration (mg/L)		Effluent COD Concentration (mg/L)	
	pRO101	pRO103	pRO101	pRO103
25th	116	84		
26th			99	60
28th	107	b	53	36
29th	90	b		
30th	b	17		
32th	95	23	127	65
33th	b	b		
34th	b	b	31	22
35th	b	b		
36th			27	27
38th	b	b	17	40

" b " Below detection limit.

TABLE XIV

VIABLE COUNT NUMBERS OF STAGE 2

Day	Viable Count Number CFU 10^6/mL	
	pRO101	pRO103
25th	970	330
28th	880	920
32th	1400	
35th		1100
36th	1800	1700

2,4-D residue below detectable level achieved after the 33th day. Viable count number 1800, 1100 and 1700 CFU 10^6/mL were used to calculate the substrate removal rate for strain pRO101 and pRO103, respectively.

TABLE XV

EFFLUENT 2,4-D AND COD CONCENTRATIONS OF STAGE 3

Day	Effluent 2,4-D Concentration (mg/L)		Effluent COD Concentration (mg/L)	
	pRO101	pRO103	pRO101	pRO103
39th	30	b	64	30
40th	31	b		
43th	25	b		
44th	65	84	77	92
45th	41	48		
46th	47	30	94	45
47th	42	50		
48th	27	39	59	58
49th	118	46		
50th	112	55	131	80
51th	103	52		
52th	b	b		
53th	b	b	20	34
54th	b	p 52		
55th	b	p 55	35	p 74
56th	29	p b		
57th	46	p b	76	p 36
58th	139	p b		
59th		p b		
60th		p b	135	p 43
61th	113	p b		
62th	78	p b		
63th	43	p b		
64th	49	p b	93	p 59
67th	84	p b	167	p 54
68th	114	p b		
72th	63	p b		
74th	65	p b		
75th	77	p b	112	p 64
77th	p 60	p b		
78th	p b	p b		
79th	p b	p b	p 46	p 47
80th	p b	p b		
81th	p b	---		---

" p " Pure oxygen placed on line.
" b " Below detection limit.

TABLE XVI

VIABLE COUNT NUMBERS OF STAGE 3

Day	Viable Count Number CFU 10^6/mL	
	pRO101	pRO103
40th	4000	2300
46th	4500	3500
54th	15000	----
57th	5800 p	3200
64th	5900 p	3000
71th	5800 p	3100
77th	p 6900 p	3600

" p " Pure oxygen.

was fed pure oxygen about two weeks later, and although viable counts remained higher in this reactor, biofilm growth was not as apparent.

Stage 4

Complete 2,4-D degradation was achieved (Figures 5, 6 and Table XVII) and effluent COD concentrations were reduced below 90 mg/L (Figures 7, 8 and Table XVII) in both reactors throughout this stage. Viable count data indicate that the addition of pure oxygen stabilized the attached biomass population (Figure 9 and Tables XVI, XVIII). Thus, it could be inferred that the instability of systems occurring in Stage 3 was caused by insufficient dissolved oxygen. Effluent 2,4-D concentrations of 45 and 52 mg/L noted on the

89th and 90th day in reactor pRO101 might be due to irregularities in adjusting the pure oxygen flow rate. Once flow rate of pure oxygen was regulated to keep residual dissolved oxygen above 20 % saturation, the residual 2,4-D dropped below detection limit.

Stage 5

After influent concentrations were doubled from 400 to 800 mg 2,4-D/L, both systems experienced a decline in efficiency. Average 2,4-D degradation efficiencies dropped to about 50 % and 70 % (average effluent 2,4-D concentrations of 350 mg/L and 205 mg/L) for reactor pRO101 and pRO103, respectively (Figure 6, 7 and Table XIX). The degeneration of biofilm activity in both reactors might suggest that this loading rate inhibited both bacterial strains. The enervation is particularly obvious in reactor pRO101, which seemed to display less acclimation ability to new loading rates after Stage 3. Furthermore, viable count data also show a decrease of biofilm population, especially in strain pRO101 (Figure 5 and Table XX).

At the end of the experiment, biofilm concentrations were measured as 22 g/L and 65 g/L for reactor pRO101 and pRO103, respectively (Table XX). The biofilm formation of strain pRO103 was visible from the end of Stage 3 through Stage 5. This also corroborated that stronger attachment of strain pRO103 occurred in Stage 1. Because the drop in

TABLE XVII

EFFLUENT 2,4-D AND COD CONCENTRATIONS OF STAGE 4

Day	Effluent 2,4-D Concentration (mg/L)		Effluent COD Concentration (mg/L)	
	pRO101	pRO103	pRO101	pRO103
81th	p b	p b		
82th	p b	p b	p 73	p 75
83th	p b	p b		
84th	p b	p b	p 54	p 81
85th	p b	p b		
86th	p b	p b	p 41	p 75
87th	p b	p b		
88th	p b	p b		
89th	p 45	p b	p 67	p 45
90th	p 52	p b		
91th	p b	p b	p 61	p 51
92th	p b	p b		
93th	p b	p b		
94th	p b	p b	p 166	p 47
95th	p b	p b		
96th	p b	p b		
97th	p b	p b	p 47	p 36
98th	p b	p b		

" p " Pure oxygen.
" b " Below detection limit.

TABLE XVIII

VIABLE COUNT NUMBERS OF STAGE 4

Day	Viable Count Number CFU 10^6/mL	
	pRO101	pRO103
84th	p 6600	p 4000
91th	p 6800	p 4400

" p " Pure Oxygen.

TABLE XIX

EFFLUENT 2,4-D AND COD CONCENTRATIONS OF STAGE 5

Day	Effluent 2,4-D Concentration (mg/L)		Effluent COD Concentration (mg/L)	
	pRO101	pRO103	pRO101	pRO103
99th	p 279	p 193		
100th	p 285	p 165	p 488	p 274
101th	p 269	p 165		
102th	p 276	p 202		
103th	p 301	p 124	p 488	p 225
104th	p 407	p 255		
105th	p 404	p 167		
106th	p 336	p 118	p 435	p 205
107th	p 345	p 156		
109th	p 380	p 216	p 496	p 306
110th	p 436	p 278		
111th	p 430	p 250		
112th	p 387	p 447		
113th	p 373	p 148	p 604	p 585

" p " Pure oxygen.

TABLE XX

BIOFILM CONCENTRATIONS AND VIABLE COUNT NUMBER OF STAGE 5

Day	Biofilm Concentration (g/L)		Viable Count Number CFU 10^6/mL	
	pRO101	pRO103	pRO101	pRO103
100th			p 5200	p 3500
105th	----	p 62.7	p 1300	p 3100
109th	----	p 72.9	p 1000	p 2700
113th	p 21.8	----	p 800	p 1000

" p " Pure Oxygen.

viable count number of strain pRO101 was larger than that of strain pRO103, it is hard to determine the actual maximum biofilm concentration of strain pRO101 in this experiment. Furthermore, bacterial API tests developed by API Laboratory Products Ltd. were carried out, both before the inoculation of bacteria to reactors and at the end of the experiments, to ensure the engineered organisms were still the same as those inoculated in the beginning. The API tests combine 20 biochemical and assimilation tests to diagnose gram-negative, nonfermentative bacteria. All the tests were executed at 30 ^{o}C for 24 hours during the incubation period. Some of the tests are enzymatic tests in which metabolic endproducts would produce a color change after microorganisms metabolize the reagents. Others are assimilation tests in which microbial growth in the presence of single carbon source will be observed. Tabulating the results according to the coding principles (API tests manual), and comparison with API Codebook would identify the unknown microorganisms.

The use of a spectrophotometer to measure 2,4-D concentration is not as sensitive as high-pressure liquid chromatography (HPLC) or gas chromatography (GC) because the light might be absorbed by not only 2,4-D molecules but also water molecules or other impurities. Furthermore, some unavoidable properties of light such as deflection and scattering might also decrease the accuracy of measurement. Usually, an UV spectrophotometer is capable

of detecting 2,4-D at 1 mg/L. HPLC is 100 times more sensitive than the UV spectrophotometer and can detect 2,4-D at 5 μg/L (Shaler and Klecka, 1986). 2,4-D residuals which are below detection limits of the spectrophotometer may still be above EPA drinking water limits of 100 μg/L (maximum contaminant level, MCL). Thus, HPLC and GC analysis of 2,4-D residual concentration are recommended for further study.

2,4-D volumetric removal rates based on both prepared and composite influent concentrations over the experiment period are listed in Table XXI.

2,4-D volumetric loading rates were calculated as follows:

2,4-D volumetric loading rate (based on composite influent concentration) = Average of composite influent 2,4-D concentration (Table VIII and IX) * Flow rate divided by bed volume.

2,4-D volumetric loading rate (based on prepared influent concentration) = Prepared influent concentration (Table VII) * Flow rate divided by bed volume.

In the first four stages, because 2,4-D degradation was assumed to be achieved completely due to 2,4-D residual concentrations below detectable limit, 2,4-D volumetric removal rates are equal to volumetric loading rates.

In the 5th stage, 2,4-D removal efficiency decreased and was calculated as :

2,4-D removal efficiency = 100% - (Average of

residual concentration / Average influent concentration) * 100 %.

2,4-D volumetric removal rates in 5 stage must be multiplied by an efficiency factor since removal was incomplete.

Because 2,4-D was the only carbon source for bacterial growth, influent COD values were totally due to 2,4-D. Effluent total COD would include 2,4-D residue, degradative intermediate products and detached biomass. COD removal efficiencies were calculated as 100% - (total effluent COD divided by the average composite influent COD) * 100 %. The percentages of COD removed at steady-state over 5 stages presented in Table XXII are relatively high except in Stage 5, because of the breakdown of reactor stability. It is evident that maximum conversion rates were achieved in the pRO101 system and approached in the pRO103 system. However, the makeup of residual COD is unknown. For future research, soluble effluent COD is recommended to be measured. Furthermore, the analysis of effluent residual composition by HPLC and the detection of CO_2 production are also suggested to know how far the 2,4-D degradation pathway could be carried out.

TABLE XXI

OVERALL 2,4-D VOLUMETRIC REMOVAL RATES (BASED ON PREPARED AND COMPOSITE INFLUENT 2,4-D CONCENTRATIONS)

Stage	Bed Volume (mL)		2,4-D Volumetric Removal Rate (g/L*d)			
			Based on Prepared Influent Concentration		Based on Composite Influent Concentration	
	pRO101	pRO103	pRO101	pRO103	pRO101	pRO103
1	100	100	3.89	3.93	3.58	4.09
2	95	95	8.19	8.28	10.00	9.32
3	118	112	13.18	14.04	13.60	11.58
4	145	135	21.45	23.30	26.22	23.40
5	113	134	26.83	31.43	25.52	31.27

TABLE XXII

COD Removal Efficiencies

Stage	Total COD Removal Efficiency (%)	
	pRO101	pRO103
1	75	75
2	88	87
3	79	73
4	82	86
5	35	65

Discussion

Besides the advantages of biological degradation over other removal approaches to reduce 2,4-D concentration in wastewater, it is also beneficial to degrade 2,4-D in a continuous flow, completely mixed reactor. This can largely relieve the toxicity of intermediate products, such as 2,4-dichlorophenol, to the bacterial strains. However, as this research shows, it is only feasible to build up and maintain a monoculture biofilm in a continuous flow aerobic expanded bed reactor to degrade 2,4-D with engineered bacterial strain Alcaligenes eutrophus AE0106 with plasmids pRO101 and pRO103 in the laboratory level as a research exercise. In practical application, it should be difficult and not economically feasible to build up a monoculture reactor because most plasmids are transmissible from one bacterial cell to another by conjugation. From the engineering point of view, it would be acceptable if plasmids transfer to other species of bacteria and still encode those bacteria to degrade unwanted xenobiotics. Furthermore, the injection of this engineered bacteria to groundwater aquifer to clean up xenobiotic pollutants might be of concern to public health because of the bacterial resistance to antibiotics.

The substrate removal efficiency is a function of hydraulic detention time and organic loading rate. For

steady-state biofilm, the substrate removal efficiency would drop if hydraulic detention time were decreased, or organic loading rate were increased. In these experiments, the decrease of hydraulic detention time from 2 hours to 1 hour corresponding to Stage 1 and Stage 2 instead increased the COD removal efficiencies from 75 % to 88 % for both reactors. This increase may be due to more cell development in Stage 2 (Table XII, XIV). When influent 2,4-D concentrations were doubled from 200 to 400 mg/L between Stage 3 and Stage 4, COD removal efficiencies were in creased 4 to 12 % for reactor pRO101 and pRO103, respectively. Although there was no significant change in viable count numbers between the end of Stage 3 and Stage 4, the addition of pure oxygen might vitalize the biofilm activity.

COD volumetric loading rate and total COD removal rate of both strains were presented in Table XXIII. Substrate removal rate is dependent upon the mass of biofilm present. It is intuitive that the mass of biofilm will be large when the substrate volumetric loading rate is high because the bacteria will be able to capture chemical energy and convert it partially to cell materials at a fast rate. Conversely, when the substrate volumetric loading rate is low, the substrate removal rate must be low. At sufficiently low substrate volumetric loading rates, the rate of energy capture may be less than the rate of energy required to maintain the viability of the bacteria. From this point,

TABLE XXIII

COD VOLUMETRIC LOADING RATES AND SPECIFIC COD REMOVAL RATES OF STRAIN pRO101 AND pRO103

Strain	Stage	COD Volumetric Loading Rate (g/L*d)	Specific COD Removal Rate[c] (COD g/10^{13}CFU)
pRO101	1	5.01	1.465
	2	9.26	0.99
	3	16.32	0.255
	4	24.44	0.502
	5	59.12	0.832
pRO103	1	4.67	8.54
	2	10.33	1.41
	3	14.49	0.425
	4	26.83	0.858
	5	57.83	1.767

" c " Because biofilm concentrations were not measured for each stage of the experiment, colony forming units (CFU) were used as an alternative parameter to represent the biofilm population. Specific COD removal rate was defined as the rate of COD removed by per 10^{13}CFU. [Usually, substrate removal rate is expressed as COD removed per gram volatile solids (g COD/g VS)].

the specific COD removal rate in Stage 1 and 2 (see Table XXIII) evidently prove the instability of biofilm formation during these two stages. Figure 10 shows the relationship between COD removal rate and volumetric loading rate after biofilm development stabilized. Obviously, the substrate removal rate of pRO103 is higher than that of pRO101. Furthermore, COD removal rates before pure oxygen was placed on line in Stage 3 were 0.18 and 0.41 COD g/10^{13}CFU

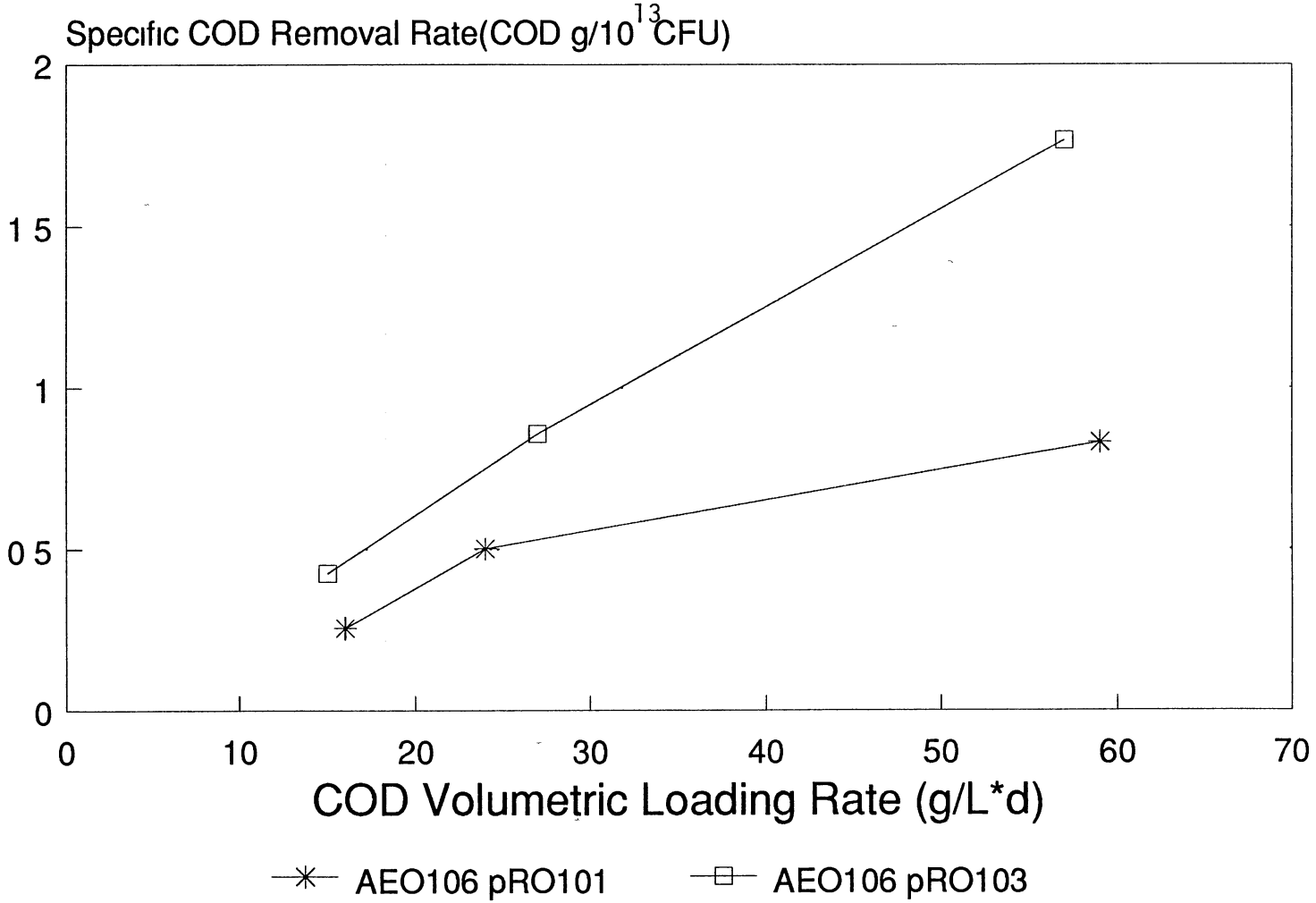

Figure 10 The Relationship Between COD Removal Rates and Volumetric Loading Rates

for strain pRO101 and pRO103, respectively. COD removal rates of Stage 3 in Table XXIII quantitatively demonstrate the influence of pure oxygen on biofilm activity.

The COD/2,4-D ratio of complete mineralization was calculated as follows:

Reaction equation of complete mineralization:

$$C_8H_6O_3Cl_2 + 15/2\ O_2 \text{ ----> } 8\ CO_2 + 2\ H_2O + 2\ HCl$$

Theoretical COD value of each mole 2,4-D :

$$32 * 15/2 = 240 \text{ g/mole}$$

The molecular weight of each mole 2,4-D is 221 g.

The COD/2,4-D ratio :

$$COD/2,4\text{-}D = 240/221 = 1.086$$

Comparing the effluent 2,4-D and COD concentrations from the 99th to 109th day, there was 120 and 60 mg/L in the effluent of reactor pRO101 and pRO103 which might consist of degradative intermediate substances and detached biomass. The analysis of effluent composition is recommended to know whether concentrations of any intermediate products in this stage were high enough to inactivate the biofilm activity. Data of strain pRO103 in Table XX consistently indicated that the decrease of viable count number was due to the loss of biomass activity instead of surface biofilm concentration.

Maximum 2,4-D complete removal rates of 8 and 21 g/L*d for air and pure oxygen, respectively, were achieved in this study. These results are significantly higher than

the degradation rate (600 mg/L*d) achieved by Kelly *et al.* (1989) in a mixed culture study.

CHAPTER V

CONCLUDING REMARKS

During the experimental period, imperfect mixing such as channeling and stagnant regions occurred in both of the reactors. Both reactors were contaminated by undesired organisms. By the end of the experiment the contaminant organisms seemed to be increasingly predominant. The mechanism of interaction between engineered and contaminant bacteria, if any, was not addressed in these experiments. Thus, there is no conclusive evidence to confirm if the inducible or constitutive engineered strain is more efficient. However, the substrate removal rates show the strain pRO103 reactor was more vigorous. Further observations may be presented as follows:

1. For the use of Pseudomonas putida and Alcaligenes AEO106 to build up attached biofilm, it is recommended that hydraulic detention time should be shorter than bacterial doubling time, or less readily degradable compounds might be used as an alternative carbon source. The purpose of either the decrease of hydraulic detention time or the use of an alternative carbon source is to make bacterial doubling time longer than hydraulic detention time in the reactor. Thus, most of the bacteria in the system will

be washed out before their duplication. Then only bacterial cells with stronger ability to attach could survive on the surface of particles and construct the biofilm.

2. Strain pRO103 was more potent in attachment to particlate surfaces. Micrometric measurement of biofilm thickness is suggested for further research.

3. From Stage 3 to 5, it was observed that inducible strain pRO101 seemed slower to acclimate to twofold increases in loading rate.

4. When using air to aerate reactor systems, 100 percent 2,4-D removal was achieved at loading rates below 8 g/L*d. Effluent COD remained below 60 mg/L for both strains, and both reactors were stable.

5. When loading rates were over 13 g/L*d, pure oxygen was required for 100 percent 2,4-D removal and maintenance of system stability. With pure oxygen, complete removal of 2,4-D was achieved at a loading rate 21 g/L*d.

6. Loading rates over 45 g/L*d resulted in a drop of viable count numbers and reduced the removal efficiencies of both reactors.

7. Removal efficiencies of COD over 5 stages ranged between 88 and 35 percent for pRO101 and 87 and 65 percent in the pRO103 reactor.

8. Biomass concentration greater than 20 g/L is achievable for both strains, with much higher concentration (70 g/L) occurring in the pRO103 biofilm.

REFERENCES

Aly, O.M. and S.D. Faust. 1965. Removal of 2,4-D derivatives from natural water. J. Am. Waterworks Assoc. 57:221-230.

Am. Pub. Health Assn., Am. Water Works Assn., Water Poll. Control Fed. Standard Methods for the examination of water and wastewater. 1985 (16th Ed.)., New York, N.Y.

Anonymous. 1987. Diatomite-no skeletons in the cupboard. Industrial Minerals. May 1987. pp. 22-39.

Bouwer, E.J. and P.L. McCarty. 1982. Removal of trace chlorinated organic compounds by activated carbon and fixed-film bacteria. Environ. Sci. Technol. 16:836-843.

Bull, M.A., R.M. Sterritt, and J.N. Lester. 1983. Response of the anaerobic fluidized bed reactor to transient changes in process parameters. Water Research. 17: 1563-1568.

Calvert, R. 1930. Diatomaceous earth. American Chemical Society Monograph Series. No. 52.

Chakrabarty, A.M. 1976. Plasmids in Pseudomonas. Annu. Rev. Genet. 10: 7-30.

Chen, S.S., C.T. Li, and W.K. Shieh. 1988. Anaerobic fluidized bed treatment of an industrial wastewater. J. Water Poll. Control Fed. 60:1826-1832.

Clarkson, W.W. 1986. Fermentation of particulate organic matter to methane in a thermophilic anaerobic attached film expanded bed reactor. Ph.D. Thesis, Cornell Univ., Ithaca, N.Y.

Don, R.H. and J.M. Pemberton. 1981. Properties of six pesticide degradation plasmids isolated in Alcaligenes paradoxus and Alcaligenes eutrophus. J. Bacteriol. 145:681-686.

Don, R.H. and J.M. Pemberton. 1985. Genetic and physical

map of the 2,4-dichlorophenoxyacetic acid degradative plasmid pJP4. J. Bacteriol. 161:466-468.

Don, R.H., A.J. Weightman, H.-J. Knackmuss, and K.N. Timmis. 1985. Transposon mutagenesis and cloning analysis of the pathway for degradation of 2,4-dichlorophenoxyacetic acid and 3-chlorobenoate in Alcaligenes eutrophus JMP134 (pJP4). J. Bacteriol. 161:85-90.

Edwards V.H. and P.F. Schubert. 1971. Removal of 2,4-D and other persistent organic molecules from water supplies by reverse osmosis. J. Am. Waterworks Assoc. 13:610-616.

Evans, W.C., B.S.W. Smith, H.N. Zenk, and J.I. Davids. 1971. Bacterial metabolism of 2,4-dichlorophenoxyacetate. Biochem. J. 122:543-551.

Fisher, P.R., J. Appleton, and J.M. Pemberton. 1978. Isolation and characterization of the pesticide-degrading plasmid pJP1 from Alcaligenes paradoxus. J. Bacteriol. 135: 798-804.

Fisher, P.R. and J.M. Pemberton. 1977. 2,4-D plasmids and persistence. Nature (London) 268:732-733.

Ghosal, D., I.-S. You, D.K. Chatterjee, and A.M. Chakrabarty. 1985. Microbial degradation of halogenated compounds. Science 228:135-142.

Gunsalus, I.C., and V.P. Marshall. 1971. Monoterpene dissimilation: chemical and genetic models. Crit. Rev. Microbiol. 1: 291-310.

Harker, A.R. 1989. personal communication.

Harker, A.R., R.H. Olsen, and R.J. Seidler. 1989. Phenoxyacetic acid degradation by the 2,4-D pathway of the plasmid pJP4: Mapping and characterization of the 2,4-D regulatory gene tfdR. J. Bacteriol. 171:314-320.

Jeris, J.S. and R.W. Owens. 1975. Pilot-scale, high-rate biological denitrification. J. Water Poll. Control Fed. 47:2043-2057.

Jeris, J.S., R.W. Owens, and R. Hickey. 1977. Biological fluidized-bed treatment for BOD and nitrogen removal. J. Water Poll. Control Fed. 49: 816-831.

Jewell, W.J. 1981. Development of the attached microbial film expanded-bed process for aerobic and anaerobic waste treatment. in "Biological fluidized bed treatment of water and wastewater." edited by P.F. Cooper and B. Atkinson. Ellis Horwood Limited, England.

Jewell, W.J. 1985. The development of anaerobic wastewater treatment. presented at National Science Foundation Sponsored Seminar on Anaerobic Treatment of Sewage at the University of Massachusetts, Amherst, Massachusetts.

Jewell, W.J. and S.E. MacKenzie. 1972. Microbial yield dependence on dissolved oxygen in suspended and attached systems. presented at 6th Water Resources Symposium, University of Texas, Austin, Nov. 13, 1972.

Kelly, M.P., K.B. Hallberg, and O.H. Tuovinen. 1989. Kinetics and metabolic fate of 2,4-dichlorophenoxyacetic acid in mixed culture. Abstracts of The Annual Meeting of American Society for Microbiology. 1989. P335.

Mulcahy, L.T. and W.K. Shieh. 1987. Fluidization and reactor biomass characteristics of the denitrification fluidized bed biofilm reactor. Water Research. 21:451-458.

Rittmann, B.E. and C.W. Brunner. 1983. The nonsteady-state-biofilm process for advanced organic removal: Concept, model, and experiment evaluation. presented at the 4th Annual Conference of the Illinois Water Pollution Control Association, Naperville, IL, May 17-19, 1983. Also presented at the Research Symposia of the Annual Conference of the Water Pollution Control Federation, Atlanta, GA, 3 October, 1983.

Schraa, G. and W.J. Jewell. 1984. High rate conversions of soluble organics with a thermophilic anaerobic attached film expanded bed. J. Water Poll. Control Fed. 56:226-232.

Shaler, T.A. and G.M. Klecka. 1986. Effects of dissolved oxygen concentration on biodegradation of 2,4-dichlorophenoxyacetic acid. Applied and Environ. Microbiol. 51:950-955.

Streber, W.R., K.N. Timmis, and M.H. Zenk. 1987. Analysis, cloning, and high level expression of the 2,4-dichlorophenoxyacetate monooxygenase gene tfdA of Alcaligenes eutrophus JMP134. J. Bacteriol. 169:2950-2955.

Switzenbaum, M.S. and W.J. Jewell. 1980. Anaerobic attached-film expanded-bed reactor treatment. J. Water Poll. Control Fed. 52:1953-1965.

Ware, G.W. 1988. Reviews of environmental contamination and toxicology. Volume 104. Springer-Verlag Inc., New York.

VITA

Ching-Ping Yang

Candidate for the Degree of

Master of Science

Thesis: DEGRADATION OF 2,4-DICHLOROPHENOXYACETIC ACID IN EXPANDED BED REACTORS BY ALCALIGENES EUTROPHUS AE0106 WITH PLASMIDS pRO101 AND pRO103

Major Field: Environmental Engineering

Biographical:

Personal Data: Born in Taoyuan, Taiwan, Republic of China, October 7, 1960, the son of Yin-Jiunn Yang and Shen Lee.

Education: Graduated from The High School of National Taiwan Normal University, Taipei, Taiwan, June 1979; received Bachelor of Science Degree in Civil Engineering from Tamkang University at Taipei, Taiwan in June, 1983; complete requirements for the Master of Science degree at Oklahoma State University in July, 1990.

Professional Experience: Teaching Assistant, Department of Civil Engineering, Oklahoma State University, January, 1988, to May, 1988. Research Assistant, Department of Civil Engineering, Oklahoma State University, August, 1988, to December, 1989.