

## AN ABSTRACT OF THE THESIS OF

David C. Phillips for the degree of Master of Science in Microbiology presented on September 5, 1996. Title: Growth Characteristics of 2,4-dichlorophenoxyacetic Acid Degrading Bacteria Recovered from an Oregon Soil.

*Redacted for Privacy*

Abstract approved: \_\_\_\_\_

Peter J. Bottomley

Man-made pesticides have been used extensively in agriculture for about 50 years, and some of them have been detected in ground water used for human consumption. We have limited information on the types of pesticide degrading bacteria present in the subsurface soil environment. The initial objective of my thesis research was to enumerate and isolate bacteria with the ability to grow on the herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), from different depths in the profile of a Willamette silt loam located at the North Willamette Research and Extension Center, Aurora, Oregon. Six 2,4-D degrading isolates (T1, D1, D2, T2, T3, T4) were recovered. I discovered that the buffering capacity (2.9mM phosphate) of the traditional mineral salts medium used in our laboratory was inadequate for the isolates to achieve maximal growth on 2,4-D as sole source of carbon. Indeed, as much as 90% of the initial 2,4-D remained in the growth medium after growth ceased and the medium pH had dropped to <4. Three isolates (T1, D1, and 712) required at least 22.9mM phosphate to achieve maximal growth yield, whereas another two isolates (D2 and T3) required at least 12.9mM phosphate. Although the slower growing isolates (T2 and T4) required 22.9mM phosphate to achieve adequate growth, their lag periods were extended by 24-30h when the phosphate concentration was >22.9mM. Although all of the isolates grew

with 2,4-D and ammonium nitrate as sole sources of carbon and nitrogen, respectively, evidence was obtained for 2,4-D being a poor quality substrate. The lag phase preceding growth was shortened (12-24h) by the inclusion of small amounts (50mg/liter) of yeast extract or casamino acids in the growth medium. The exponential growth rates and final cell yields achieved by isolates on 2,4-D were always slower and lower, respectively, than those achieved on glucose or succinate as sole carbon sources. Less than 20% of 2,4-D-carbon was assimilated by the isolates, whereas they assimilated about 40-50% of glucose-carbon. Data were obtained which indicated that the 2,4-D to cell biomass conversion value obtained for isolates in liquid culture might be extended to growth in soil only when 2,4-D is applied at  $>1\mu\text{g/g}$ . By recognizing the importance of the buffering capacity of the medium, and the low efficiency of conversion of 2,4-D-carbon to cell carbon, it is hoped that better success will be achieved at isolating 2,4-D degraders from soil samples in the future.

© Copyright by David C. Phillips  
September 5, 1996  
All Rights Reserved

Growth Characteristics of 2,4-dichlorophenoxyacetic Acid Degrading Bacteria  
Recovered from an Oregon Soil.

by

David C. Phillips

A THESIS

submitted to

Oregon State University

in partial fulfillment of  
the requirements for the  
degree of

Master of Science

Completed September 5, 1996  
Commencement June 1997

Master of Science thesis of David C. Phillips presented on September 5, 1996

APPROVED:

*Redacted for Privacy*

Major Professor, representing Microbiology

*Redacted for Privacy*

Chair of Department of Microbiology

*Redacted for Privacy*

Dean of Graduate School

I understand that my thesis will become part of the permanent collection of Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

*Redacted for Privacy*

David C. Phillips, Author

## ACKNOWLEDGMENTS

To my major professor, Peter Bottomley, thanks for being such a swell guy. It was a privilege to 'do some science' with you. Thank you also to Tom Sawyer, as the principle researcher on this project, for his hard work and for being a great source of help and information. I would also like to acknowledge Rockie Yarwood for contributing to the project in such a positive way, both in work and in many discussions.

I would like to acknowledge Dan Arp, Lyle Brown, and Michael Borman for being on my committee and contributing in such a positive manner.

Many thanks and affection goes to my lab mates during this period of study. Lesley, Ieda, Rockie, Tom, and Khrys, thanks for being understanding and for your kindness. I am glad that I could be the token red-neck (meant in a good way) of the group. You all contributed positively to the social and scientific education of this 'bubba' from Washington. The wheat-fields will never be the same again! The gift I leave to you is a radio seemingly permanently stuck on Country Music stations. It was a privilege to work with ya'll.

This work would not have been possible without the support of many friends. My profound thanks to Wendy Franz for proofreading countless documents in a professional manner, and to Scott Franz for many racquetball and swimming sessions. Above all I must thank my wife Janelle who married me despite this thesis project. Thank you for your energy, support and love. Finally I recognize my grandmother, Ada Phillips, who was dedicated to the education of her grandchildren.

This work was supported in part by the N. L. Tarter Fellowship from the Department of Microbiology, Oregon State University.

## TABLE OF CONTENTS

INTRODUCTION .....	1
MATERIALS AND METHODS .....	8
2,4-dichlorophenoxyacetic acid stock solution .....	8
Bacterial isolates.....	8
BIOLOG™ substrate utilization by 2,4-dichlorophenoxyacetic acid degrading isolates .....	9
Isolation of 2,4-dichlorophenoxyacetic acid degrading bacteria.....	12
Growth characteristics of isolates .....	14
Enumeration of 2,4-dichlorophenoxyacetic acid degrading populations from soil by a Most Probable Number procedure .....	15
Determination of the population densities of 2,4-dichlorophenoxyacetic acid degrading bacteria in Willamette silt loam soil after incubation with three concentrations of 2,4-dichlorophenoxyacetic acid .....	16
Analysis of 2,4-dichlorophenoxyacetic acid in culture filtrates by High Pressure Liquid Chromatography.....	16
RESULTS .....	18
Buffering studies.....	18
Organic supplement studies.....	25
Growth efficiency studies.....	25
Enumeration of 2,4-dichlorophenoxyacetic acid degrading soil microbial populations.....	32

## TABLE OF CONTENTS, CONTINUED

DISCUSSION.....	37
Buffering of medium .....	37
Growth yields .....	39
Isolation of 2,4-dichlorophenoxyacetic acid degrading bacteria from soil .....	40
LITERATURE CITED .....	41



## LIST OF FIGURES

<u>Figure</u>	<u>Page</u>
1. Growth characteristics of isolate T1 in minimal medium containing 1g/l (4.5mM) of 2,4-D with and without 1g/l (5.1mM) MES buffer.....	19
2. Growth characteristics of isolate D1 in minimal medium containing 1g/l (4.5mM) of 2,4-D with and without 1g/l (5.1mM) MES buffer.....	20
3. Growth characteristics of isolates T1, D1, and 712 in minimal medium containing 1g/l (4.5mM) of 2,4-D and various concentrations of phosphate.....	22
4. Growth characteristics of isolates D2 and T3 in minimal medium containing 1g/l (4.5mM) of 2,4-D and various concentrations of phosphate.....	23
5. Growth characteristics of isolates T2 and T4 in minimal medium containing 1g/l (4.5mM) of 2,4-D and various concentrations of phosphate.....	24
6. Growth characteristics of isolates 1173 and D1 in minimal medium containing 52.9mM phosphate, 1g/l (4.5mM) of 2,4-D +/- yeast extract or casamino acids at 50mg/l.....	26
7. Growth characteristics of isolates T2 and T4 in minimal medium containing 52.9mM phosphate, 1g/l (4.5mM) of 2,4-D +/- yeast extract or casamino acids at 50mg/l.....	27
8. Growth characteristics of isolates T1, D1, and 712 in minimal medium containing 52.9mM phosphate, and 27mM carbon supplied from various sources.....	29
9a. Growth characteristics of isolate D1 in minimal medium containing 52.9mM phosphate and different concentrations of 2,4-D. ....	30
9b. Maximal growth of D1 grown in minimal medium containing various concentrations of 2,4-D.....	30

## LIST OF TABLES

<u>Table</u>	<u>Page</u>
1. List of isolates used in this study. ....	11
2. Cell yield (mg dry weight/ml) and efficiency of growth (percent of carbon assimilated) of isolates T1, D1, 712, T2, and T4 grown on either glucose, succinate, or 2,4-D as sole carbon and energy source. ....	31
3. Comparison of (a) growth efficiency expressed in terms of substrate carbon processed per cell produced, and (b) final cell densities of isolates D1, T1, and 712 grown in minimal medium buffered with 52.9mM phosphate and either glucose, succinate or 2,4-D as the sole carbon and energy source. ....	33
4. Influence of incubation time and a yeast extract supplement on Most Probable Number (MPN) estimates of the population size of 2,4-D degraders in soil at different depths in the profile of a Willamette silt loam. ....	34
5. Comparison of the growth efficiency (2,4-D carbon used per cell produced) shown by an indigenous Willamette soil population of 2,4-D degraders exposed to three different concentrations of 2,4-D.....	36

# **GROWTH CHARACTERISTICS OF 2,4-DICHLOROPHENOXYACETIC ACID DEGRADING BACTERIA RECOVERED FROM AN OREGON SOIL.**

## **INTRODUCTION**

In recent years concern has been shown about the impact of agricultural practices on environmental quality. One particular area of concern centers around the fate of pesticides applied to agricultural lands. The seriousness of this concern is illustrated by the detection of pesticides in a high percentage of surface and groundwater throughout the USA (Flury, 1996; Thurman et al., 1992).

The fate of a field-applied pesticide is influenced by a variety of factors including its biodegradability, volatility, water solubility, and its sorptive properties to soil surfaces and organic matter. The physical and chemical properties of soils, and the types of cropping practices conducted on them can potentially affect the fate of pesticides. For example, sandy soils with high water infiltration rates are especially vulnerable to pesticide leaching (Boesten and van der Linden, 1991). Reduced tillage operations which reduce soil erosion also promote the activity of soil macrofauna and the persistence of root channels. Unfortunately these biologically created macropores can provide conduits for transmission of pesticides into the subsurface environment (Edwards et al., 1992). Crop residues, left on the soil surface to prevent soil erosion, have been shown to increase the infiltration of pesticides through soil (Green et al, 1995). The plant material prevents the herbicide from reaching the soil surface and adsorbing to soil particles.

Biodegradability of a field-applied pesticide is influenced by the presence of microorganisms with the appropriate enzymes to attack the chemical. The population density and the phenotypic attributes of these microbes will influence the speed and extent to which biodegradation of the herbicide occurs. The detection of a potentially degradable pesticide in groundwater indicates that the chemical has literally "escaped" microbial degradation. Several phenotypic properties of microorganisms could account for their

fallibility to degrade a pesticide. These include: inability to extract energy from the metabolism of the pesticide, inability to sequester low concentrations of the pesticide, intolerance of potentially toxic, high concentrations of the pesticide, and catabolite repression of pesticide degradation by the presence of other nutrient sources. Detection of pesticides in groundwater indicates they have escaped biodegradation by the surface soil microorganisms, and that conditions in the subsurface soil and groundwater environment are not conducive for biodegradation to occur efficiently (Flury, 1996). In this regard, few studies have been conducted to compare biodegradation of pesticides in surface and subsurface soils (Moorman, 1990).

Over the last 50 years chlorinated aromatic compounds have been used extensively as herbicides and insecticides in agriculture. An example of such a compound is the broad-leaf herbicide, 2,4-dichlorophenoxyacetic acid (2,4-D), which has been used extensively for the past 50 years and has a half-life of less than seven days in surface soils under field conditions (Ou, 1984). Unlike some chlorinated compounds, 2,4-D is readily degraded and mineralized in soil by microorganisms (Atlas and Bartha, 1993), and its metabolism has been studied extensively (Chaudhry and Chapalamadugu, 1991).

Numerous strains of bacteria have been isolated with the ability to degrade 2,4-D from different genera including; *Acinetobacter*, *Alcaligenes*, *Arthrobacter*, *Burkholderia*, *Corynebacterium*, *Flavobacterium*, and *Pseudomonas* (Chaudhry and Chapalamadugu, 1991). Studies in the 1950's and 1960's focused on phenotypic behavior of the organisms and on elucidating the biochemical pathway of degradation (Bollag and Alexander, 1968; Steenson and Walker, 1957; Tiedje et al., 1969; Walker and Newman, 1956). The complete enzymatic pathway of 2,4-D degradation has been described in *Arthrobacter* (sp) (Ghosal et al., 1985). In some bacteria, the 2,4-D degradative enzymatic pathway is encoded on transmissible plasmids (Don and Pemberton, 1981; Neilson et al, 1994; Pemberton and Fisher, 1977). Two plasmids have been studied extensively: pJP4 and RC101 (Chaudhry and Chapalamadugu, 1991; Ka et al. 1994a). *Alcaligenes eutrophus*

contains a native plasmid pJP4 which has been physically mapped (Weightman et al., 1984). Recent studies have shown that some 2,4-D degrading bacteria contain chromosomally encoded 2,4-D catabolic genes (Ka et al., 1994a).

Many of the studies on the physiology, biochemistry, and genetics of 2,4-D breakdown have been conducted on *A. eutrophus* JMP134, in which almost all of the genes are encoded on plasmid pJP4 (van der Meer et al., 1992). Studies of the "classical" 2,4-D degrading pathway show that the genes are clustered into 3 operons consisting of *tfdA*, *tfdCDEF*, and *tfdB*. The first operon contains *tfdA* which encodes for 2,4-D dioxygenase. Genes of the modified ortho-fission pathway (*tfdCDEF*) make up the second operon, and *tfdB*, which encodes 2,4-dichlorophenol hydroxylase, constitutes the third operon (Kaphammer and Olsen, 1990). Kaphammer et al. (1990) showed that a regulatory gene, *tfdR*, encodes for a regulatory element of both *tfdA* and *tfdCDEF*. Another regulatory gene, *tfdS*, acts as a regulatory element for *tfdB* (Kaphammer and Olsen, 1990). Transcriptional activation of *tfdA* and *tfdCDEF* is promoted by the binding of *tfdR* to either 2,4-D, dichlorophenol, or 4-chlorocatechol. In the case of the regulation of *tfdB* by *tfdS*, it is thought that either 2-cis-chlorodienelactone (product of *tfdD*) or chloromaleylacetic acid (product of *tfdE*) is the effector of *tfdS*. *In vivo*, it is unclear whether the regulation of the *tfdA* and *tfdCDEF* operons is brought about primarily by the substrate (2,4-D), by the products of 2,4-D degradation, or by a combination thereof. It is well known that the end products of aromatic degrading pathways can act as the primary inducers of the genes in those pathways (Chaudhry, 1994). Whether or not substrates or products are the primary inducing molecules could be significant in field situations where herbicides are often "moving targets" that flow in soil water past surface-attached microorganisms. Rapid induction is probably important if a soil-applied herbicide is to be prevented from "escaping" the surface soil environment.

In soils, microbial populations are invariably exposed to complex mixtures of substrates present in variable concentrations. Under such situations catabolite repression

might occur and 2,4-D would not be degraded. In this context, Harker et al. (1989) showed that 2,4-D catabolic genes were not expressed in *Pseudomonas* in the presence of glucose, lactate, or succinate. However, these genes were expressed in the presence of 0.3% casamino acids and 0.05% 2,4-D. Dougherty and Karel (1994) showed that the rate of 2,4-D degradation by *Burkholderia cepacia* was affected by the presence of succinate. Degradation was repressed at low ratios of 2,4-D to succinate, but concurrent degradation of both substrates occurred with higher ratios of 2,4-D to succinate. Chen and Alexander, (1989) documented that 2,4-D breakdown by a bacterial isolate was suppressed in the presence of glucose.

Pesticides have found widespread application because of their biocidal effects (Alexander, 1994). Although certain bacteria can biodegrade these chemicals, often there is a fine line between concentrations suitable for supporting growth versus those that damage cells, or even cause death. This point is exemplified by a recent publication on anaerobic toluene degrading bacteria. Fries et al. (1994) reported that a decade of research on anaerobic toluene degrading bacteria had produced few isolates. However, by using much lower initial concentrations of toluene (5µg/g vs 250µg/g) in enrichments, 10 additional isolates were isolated and characterized. Circumstantial evidence exists for toxic effects of 2,4-D on soil microbial communities (Fournier, 1980). Miwa and Kuwatsuka (1990, 1991) showed there was a smaller population increase in soil treated with 100µg/g 2,4-D than in soil treated with 10µg/g 2,4-D. Studies on the kinetics of 2,4-D biodegradation in soil have shown that high concentrations of 2,4-D (20-500µg/g) can be toxic to degrading bacteria in the soil environment (Bellinck et al., 1979; Stott et al., 1983; Parker and Doxtader, 1982).

In most instances, pesticides are detected in soil and water at low concentrations (Flury, 1996; Veeh et al., 1996; Wilson and Cheng, 1976). As a result, questions have been raised about the ability of microorganisms to degrade pesticides such as 2,4-D at low concentrations. It has been suggested that different members of the soil microbial

community are responsible for degrading low and high 2,4-D concentrations (Fournier, 1980; Fournier et al., 1981; Parker and Doxtader, 1982; Soulas, 1993). Greer and Shelton (1992) determined that a 2,4-D degrading bacterium (designated strain 155 from either *Alcaligenes*, or *Pseudomonas* genera) with a  $K_s$  value of 34 mg/l had difficulty accessing 2,4-D applied to soil at 1  $\mu\text{g/g}$ . Boethling and Alexander (1979) found that <10% 2,4-D was degraded when it was applied to water microcosms at concentrations of 2 to 3 ng/ml.

Recently, direct evidence has been obtained for soil populations being composed of different types of 2,4-D degrading bacteria. Ka et al. (1994a) isolated 47 2,4-D degrading bacteria from soil treated with four different concentrations of 2,4-D (0, 1, 10, 100 $\mu\text{g/g}$ ). The bacterial strains were subdivided into four groups, based on the patterns of DNA fragments generated by repetitive extragenic palindromic polymerase chain reaction (REP-PCR), fatty acid methyl ester (FAME) analysis, and hybridization tendencies to several gene probes representing 2,4-D catabolic genes. Strains belonging to one bacterial genus (*Sphingomonas* sp.) were more frequently recovered from soil that had received the highest amounts of 2,4-D, and were rarely recovered from the plots receiving none or low applications of 2,4-D. Two other groups of 2,4-D degrading bacteria dominated the control plots and were not isolated from soil receiving the largest amounts of 2,4-D. In this context it is of interest to note that a wide, seemingly random, range of 2,4-D concentrations (50-900 $\mu\text{g/g}$ ) has been used to isolate 2,4-D degrading bacteria from natural environments (Diztelmuller et al., 1989; Fournier, 1980; Holben et al., 1992; Ka et al., 1994b; Miwa and Kuwatsuka, 1991; Ou, 1984; Sandmann et al., 1988; Xia et al., 1995). Another interesting fact from the Ka et al. (1994a) study was the observation that many of the organisms recovered showed no homology to the *tfd* gene probes from pJP4, indicating that the 2,4-D degrading genes of these isolates were different from the "model" strain, *A. eutrophus* JMP134. This lack of homology of 2,4-D degrading isolates to 2,4-D genes from plasmid pJP4 was confirmed by Xia et al. (1995) in bacteria isolated from soils in Washington State.

Both Ka et al. (1994a) and Xia et al. (1995) made an attempt to recover the most numerically dominant 2,4-D degrading bacteria from soil populations by enriching from the highest dilution of a Most Probable Number (MPN) assay that showed evidence of 2,4-D degradation. In both cases, researchers exposed their samples to relatively high concentrations of 2,4-D both in the MPN assay and the subsequent enrichments (500 and 900 µg/g respectively). Several questions arise from these findings which pertain to the properties of 2,4-D breakdown in soil. The data of Ka et al. (1994a) indicate that applications of 2,4-D can result in selective enrichment of specific members of the soil population. It is not clear if the competitive success of *Sphingomonas* in dominating the high concentration enrichments is related specifically to some unique 2,4-D degrading properties, or to other competitive attributes such as an inherently faster growth rate, or antibiotic production. It is possible, however, that there was no selective response from application of 2,4-D to the soil *per se*, and that enrichment conditions during and after the MPN analysis favored the recovery of *Sphingomonas* sp. It is unclear, at this time, to what extent different phenotypes present in a 2,4-D degrading soil population might influence the *in situ* degradation properties. In this context, data from our lab (Sawyer and Bottomley, unpublished observations) have shown that the kinetics of 2,4-D degradation differ according to season and soil depth.

At the time this thesis work was initiated, little work had been published on the composition of native populations of 2,4-D degrading bacteria in the soil profile. Furthermore, most isolates have been obtained by direct enrichment of surface soil (0-20cm) with relatively high concentrations (ranging from 250-500 µg/g) of 2,4-D (Ka et al., 1994b; Sandmann et al., 1988). The objective of my thesis was to attempt to isolate representatives of the most numerically dominant 2,4-D degrading bacteria existing at different depths in the profile of a Willamette silt loam soil, and to examine the 2,4-D degrading characteristics of these isolates. Isolation of 2,4-D degrading bacteria proved to be a difficult, unpredictable task. Indeed, recently Fulthorpe et al. (1996) showed they



could only recover five 2,4-D degrading strains from 600 soil samples taken throughout the world. This led me to address the topic of defining growth conditions for 2,4-D degrading bacteria so that a better isolation protocol could be developed.

## MATERIALS AND METHODS

### **2,4-dichlorophenoxyacetic acid stock solutions.**

Stock solutions of 2,4-D for Most Probable Number (MPN) experiments were prepared using 2,4-D (99% purity) purchased from Eastman Kodak Co., Rochester, N.Y. The 2,4-D was dissolved in 100% methanol and stored in glass stoppered volumetric flasks at room temperature.

$^{14}\text{C}$ -ring labeled 2,4-D (18.2 mCi/mmol) was purchased from Sigma Chemical Co., St. Louis, MO. A precious stock of radiolabeled 2,4-D was made by dissolving the original solid material in 1ml of 100% methanol. Working solutions were prepared by diluting aliquots of the methanolic stock 100-fold to achieve 1 $\mu\text{Ci/ml}$ .

### **Bacterial isolates.**

The studies in this thesis were carried out with a variety of bacterial isolates that were obtained from soil taken from Oregon State University's North Willamette Research and Extension Center, Aurora, Oregon. The soil at the site is described as a Willamette silt loam (fine-silty, mixed, mesic pachic Ultic Haploxeroll). Soils were sampled as follows: About 30 cores of soil were taken on 1m spacings from a 6 x 4m grid. Soil cores were recovered from the 0-20, 20-40, 40-60, 60-80, 80-100cm depth intervals using an extendible tube auger of 2cm diameter. Some simple precautions were taken to reduce the chances of contaminating the lower layers of soil with overlying material. (1) All samples were removed from a specific depth before moving to the next depth. (2) The inside surfaces of the bore holes were carefully reamed with the probe before sampling the next depth increment. (3) Loose soil on the top of the lower cores and extraneous soil on the auger were removed before bagging the samples. After thoroughly mixing the soil from a specific depth, a portion was sieved (<2mm) prior to experimentation. Although six

isolates (T1, D1, D2, T2, T3, T4) were obtained from this soil, the majority of studies were conducted with four isolates (T1, D1, T2, T4) and with isolates 712 (obtained from Dr. J. Tiedje, Michigan State University). Occasionally, an experiment was conducted with isolates D2, T3, and with isolate 1173 (also obtained from J. Tiedje). The isolates fell into two groups based upon their growth rates on 2,4-D. One group (T1, T3, D1, D2, 712, 1173) produced exponential generation times ranging between 4-10h, the other group (T2, T4) produced much longer exponential generation times ranging between 15-23h. An attempt was made to biotype the Oregon isolates (Table 1) with BIOLOG™ plates (Hayward, California). Two distinctly different types of response to the BIOLOG™ medium were obtained. Isolates T1 and D1 (putatively typed as *Burkholderia* [sp.]) used 65-85% of the substrates after 24h of incubation. The slower growing isolates T2, T3 and T4 (putatively typed as *Methylobacterium* [sp.]) and D2 (closest relative was *Pseudomonas cichorii*) used only 15-30% of substrates even after 65h of incubation.

#### **BIOLOG™ substrate utilization by 2,4-dichlorophenoxyacetic acid degrading isolates.**

Isolates were grown overnight in one-half strength tryptic soy broth (Difco Laboratory, Detroit, MI) (TSB) containing in g/l: bactotryptone, 8.5; bactosoytone, 1.5; bactodextrose, 1.25; NaCl, 2.5; K<sub>2</sub>HPO<sub>4</sub>, 1.25g. Cells were harvested by centrifugation and washed twice in mineral salts (M.S.), composed of the following (grams per liter): NH<sub>4</sub>NO<sub>3</sub>, 0.5g; K<sub>2</sub>HPO<sub>4</sub>•3H<sub>2</sub>O, 0.655g [0.5g anhydrous]; MgSO<sub>4</sub>, 0.2g; CaCl<sub>2</sub>, 0.05g. pH was adjusted to 6.5 with concentrated HCl). Cells were then resuspended in 50ml of sterile M.S. solution to produce an O.D. 0.1 (660 nm). An 8-channel pipetter was used to inoculate 150µl portions of cells into each well of the BIOLOG™ plates. Plates were incubated for 24-48h at 27°C in a partially closed zip-lock bag containing a moistened paper towel. Plates were examined for substrate responses at 12, 18, 24 and 48h.

Results of the BIOLOG™ substrate analysis were analyzed with the BIOLOG™ database to determine how closely the isolates resembled other microorganisms.

**Table 1:** List of isolates used in this study.

isolate #	soil depth (cm)	Date of soil sampling	isolation protocol	Closest relative in the BIOLOG™ Database Identification System	SIMM <sup>1</sup>
T1	0-20		I	<i>Burkholderia caryophyllii</i>	0.58
T2	80-100		II	<i>Methylobacterium radiotolerans</i>	0.53
T3	0-20	8.13.93	III	<i>Methylobacterium rhodinum</i>	0.46
T4	80-100	8.13.93	III	<i>Methylobacterium mesophilicum</i>	0.35
D1	80-100	4.20.94	IV	<i>Burkholderia cepacia</i>	0.67
D2	80-100	4.20.94	IV	<i>Pseudomonas cichorii</i>	0.20
D35	80-100	7.13.95	V	N.D. <sup>2</sup>	N.D.
1173				<i>Alcaligenes eutrophus</i> <sup>3</sup>	N.D.
712				<i>Burkholderia picketti</i> <sup>3</sup>	N.D.

<sup>1</sup>Similarity index as reported by BIOLOG™ database identification system.

<sup>2</sup>Not determined.

<sup>3</sup>Obtained from Dr. J. Tiedje, Michigan State University.

### **Isolation of 2,4-dichlorophenoxyacetic acid degrading bacteria.**

Over the course of these studies the protocol for isolating 2,4-D degrading bacteria from soil was modified because success rates were poor. The isolation protocols used to recover the bacteria described in this thesis are presented below.

- I. Isolate T1 was obtained from a sample of Willamette silt loam collected in April, 1993 from a depth of 0-20cm. The soil was incubated with 100µg/g of 2,4-D at 27°C for 10d. Then, a 10 gram portion of this soil was diluted through a 10-fold series to 10<sup>-8</sup> dilution in sterile M.S. solution. Aliquots (10ml) of the dilution series were incubated for two weeks in 60ml serum bottles containing “cold” 10µM 2,4-D and 0.05µCi of <sup>14</sup>C-2,4-D. <sup>14</sup>CO<sub>2</sub> was trapped on NaOH-saturated wicks placed in Kontes buckets inserted through rubber stoppers into the vials. Portions (30µl) of the highest soil dilutions (10<sup>-6</sup>, 10<sup>-7</sup>, and 10<sup>-8</sup>) showing 2,4-D mineralizing ability were spread onto plates containing 1g/l 2,4-D in M.S. with Noble agar as the solidifying agent (1.5% w/v) and incubated for 7d. A 2,4-D degrading isolate (T1) was recovered from the 10<sup>-6</sup> dilution.
- II. Isolate T2 was recovered from a sample of Willamette silt loam collected in April, 1993 from 80-100cm depth. The soil was incubated with 100µg/g of 2,4-D at 27°C for 10d. Then, a 10g portion of this soil was diluted and incubated with <sup>14</sup>C-2,4-D as described above to determine the highest soil dilution with 2,4-D mineralizing activity. Aliquots of the (10<sup>-6</sup>, 10<sup>-7</sup>, 10<sup>-8</sup>) dilutions were streaked onto plates containing 1g/l 2,4-D in M.S. with Noble agar as the solidifying agent (1.5% w/v) and incubated. An isolate (T2) was obtained from the 10<sup>-8</sup> dilution.
- III. Isolates T3 and T4 were obtained from a sample of Willamette silt loam collected in August 1993, from 0-20cm and 80-100cm depths, respectively. The soil was

incubated with 100 $\mu$ g/g of 2,4-D at 27°C for 20d, diluted, and incubated with  $^{14}$ C-2,4-D as described above. Portions (10ml) of the  $10^{-5}$ ,  $10^{-6}$ ,  $10^{-7}$ ,  $10^{-8}$  dilutions were added to 100ml of M.S. containing 0.1g/l 2,4-D and incubated at 27°C for 10d. Portions (10 $\mu$ l) of these cultures were streaked to plates containing 1g/l 2,4-D in M.S. solidified with Noble agar (1.5% w/v) and incubated for 7d. Isolate T3 was obtained from the  $10^{-8}$  dilution of 0-20cm soil, and T4 was obtained from the  $10^{-6}$  dilution of the 80-100cm soil.

- IV. Isolates D1 and D2 were recovered from a sample of Willamette silt loam collected in April, 1994 from 80-100cm depth. A 25g sample of soil was treated with a low concentration (0.6 $\mu$ g/g) of 2,4-D and the rate of  $^{14}$ CO<sub>2</sub> evolution was monitored until its rate had dropped to  $\leq 1\%$  of the initial label released per day. Another sample of soil was incubated in a moist state (30% w/w water content) without a 2,4-D supplement. Samples of each soil were diluted 10-fold to  $10^{-6}$  in sterile M.S., and a portion of each dilution was incubated with  $^{14}$ C-2,4-D. Aliquots of the highest soil dilutions that scored positive for 2,4-D mineralization ( $10^{-3}$  and  $10^{-4}$ ) were transferred either to liquid M.S. medium containing 0.1g/l 2,4-D or plated directly onto plates containing 1g/l 2,4-D in M.S. with Noble agar as the solidifying agent (1.5% w/v). No visible growth was detected in liquid enrichments but colonies developed on the Noble agar plates with 2,4-D as sole carbon source. Individual colonies were re-streaked onto plates containing 1g/l 2,4-D, streaked twice on 1/2 strength TSB plates, and finally streaked onto 1g/l 2,4-D plates. Isolate D1 was recovered from the  $10^{-3}$  dilution of 2,4-D treated soil, and isolate D2 was obtained from the  $10^{-4}$  dilution of untreated soil.

The 2,4-D degrading ability of isolates was confirmed by following the disappearance of 2,4-D from liquid culture (0.1g/l in M.S.) by high pressure liquid chromatography (HPLC), and by following the release of  $^{14}\text{CO}_2$  from ring labeled  $^{14}\text{C}$ -2,4-D.

### **Growth characteristics of isolates.**

Cultures of each isolate were grown for 8-16h at 27°C in one-half strength tryptic soy broth. Portions (25ml) of these cultures were centrifuged for 15 minutes at 12,000 x g, resuspended in M.S. medium, centrifuged, and resuspended in 1-2ml of M.S. Portions of this suspension were added dropwise to 50ml of M.S. until an optical density of approximately 0.1 was achieved. Portions (100 $\mu$ l) of this suspension were added to 100ml samples of growth medium composed of the following per liter of deionized water:  $\text{NH}_4\text{NO}_3$ , 0.5g;  $\text{K}_2\text{HPO}_4 \cdot 3\text{H}_2\text{O}$ , 0.655g (0.5g anhydrous);  $\text{MgSO}_4$ , 0.2g;  $\text{CaCl}_2$ , 0.05g; trace elements, 10ml of a stock solution [containing per liter]:  $\text{H}_3\text{BO}_3$ , 143mg;  $\text{MnSO}_4 \cdot 4\text{H}_2\text{O}$ , 102mg;  $\text{ZnSO}_4 \cdot 2\text{H}_2\text{O}$ , 22mg;  $\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$ , 8mg;  $\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$ , 5mg.  $\text{Na}_2\text{EDTA}$ , 3.3ml (stock solution 3mg/ml);  $\text{FeCl}_3$ , 1ml (stock solution 4mg/ml in 0.1M HCl); 0.5ml of a filter-sterilized vitamins stock solution was added after autoclaving. The stock contained per liter: p-aminobenzoate, 30mg; pyridoxine, 60mg; riboflavin, 100mg; choline, 10mg; myo-inositol, 100mg; Ca-pantothenate, 250mg; thiamine, 125mg; nicotinic acid, 20mg. Medium pH was adjusted to 6.5 with concentrated HCl before autoclaving. To this basal medium (minimal mineral salts, M.M.S. medium) varying quantities of 2,4-D, phosphate, yeast extract and other carbon sources were added to meet specific experimental objectives. 2,4-D was dissolved in M.M.S. with stirring at room temperature for 4-18h. After the 2,4-D dissolved, the pH was adjusted to 6.5 with 1M KOH. Media containing various phosphate ( $\text{K}_2\text{HPO}_4$ ) concentrations were prepared by adding the appropriate amounts of phosphate to M.M.S. Stock solutions (10g/l) of yeast extract and



casamino acids were prepared in deionized water, autoclaved and added to growth media prior to final autoclaving.

Cultures were grown at  $27^{\circ}\pm 2^{\circ}\text{C}$  in a New Brunswick Scientific Controlled Environment Incubator Model G-25 shaker at 300 rpm. Growth was monitored routinely by aseptically sampling the cultures and measuring turbidity at 660nm in a 1cm path length cuvette in a Beckman Model 34 Spectrophotometer. pH readings were taken with a Corning Scientific Instruments Model 10 pH Meter.

Growth efficiency measurements were conducted on isolates grown on limiting quantities of three carbon sources; 2,4-D, succinate (disodium salt), and D-glucose. These carbon sources were supplied in equivalent amounts (27mM C). Growth of the isolates was monitored until stationary phase was reached. HPLC analysis was carried out to confirm that all 2,4-D was utilized. Cells were filtered and dried at  $65^{\circ}\text{C}$  for 24h. Final cell densities of the cultures were determined by the plate count technique. Cultures were taken through a 10-fold dilution series in M.S. and 100 $\mu\text{l}$  of the dilutions were plated in duplicate onto one-half strength tryptic soy agar plates. Plates were incubated 14 or 96h (fast and slow isolates, respectively) at  $27^{\circ}\text{C}$  and colonies were counted.

#### **Enumeration of 2,4-dichlorophenoxyacetic acid degrading soil microbial populations by a Most Probable Number procedure.**

Ten gram portions of soil were suspended in 95 ml portions of M.S. solution. After shaking vigorously for five minutes, the soil suspensions were diluted through either a 10 or five-fold dilution series depending upon the depth from which the soil had been recovered. Portions (100 $\mu\text{l}$ ) of 1mM cold 2,4-D (10 $\mu\text{M}$  final concentration) in methanol along with  $\approx 0.05\mu\text{Ci}$  of  $\text{UL-}^{14}\text{C}$  ring-labeled 2,4-D were added to warm, sterile 60ml serum bottles. After the methanol had evaporated, 10 ml portions of the appropriate soil dilution were added to each of four replicate vials. Plastic buckets (Kontes of California) containing filter paper wicks, were inserted into red rubber septa and  $\approx 100\mu\text{l}$  portions of 1M NaOH were soaked onto the wicks. Incubations were conducted at room temperature

( $\approx 21^{\circ}\text{C}$ ) for varying periods of time ranging between 7 and 40d. After incubation for the appropriate time interval, wicks were removed and placed into 20ml scintillation vials containing 6ml of NaOH, and 10ml of scintillation fluid, Scinti-Verse II (Fisher Scientific, N. J.). Radioactivity trapped in the filter wick was counted in a scintillation counter using a quench curve standardized with known amounts of  $\text{Na}_2^{14}\text{CO}_3$  in NaOH and Scinti-Verse II. Tubes were considered positive for 2,4-D degradation if  $\geq 10\%$  of the initial radiolabel was found in the NaOH-saturated wicks.

**Determination of the population densities of 2,4-dichlorophenoxyacetic acid degrading bacteria in Willamette silt loam soil after incubation with three concentrations of 2,4-dichlorophenoxyacetic acid.**

Twenty-gram portions of soil were added to deionized water to provide a final water content of 30g per 100g oven dry soil and sufficient 2,4-D to provide either 6, 0.6, or  $0.06\text{ }\mu\text{g}$  2,4-D/g of soil. The 2,4-D solution was supplemented with approximately  $0.1\text{ }\mu\text{Ci}$  of ring-labeled 2,4-D. Samples of sterile soil amended with radiolabeled 2,4-D, and of radiolabel without soil were included in each study to serve as controls.  $^{14}\text{C}$ -labeled  $\text{CO}_2$  was collected by passing the effluent air through vials containing 6ml portions of 1M NaOH. The vials were changed at daily intervals until the amount of radioactivity evolved daily was  $<1\%$  of the initial amount applied. The soil samples were processed and incubated as described earlier to determine the population densities with the MPN procedure. A duplicate series of soil dilutions was supplemented with  $50\mu\text{g/ml}$  YE.

**Analysis of 2,4-dichlorophenoxyacetic acid in culture filtrates by High Pressure Liquid Chromatography.**

Samples (2ml) of cultures were filtered through  $0.45\mu\text{m}$  pore size nitrocellulose membrane filters. Filtrates were then diluted 4:1 with membrane filtered M.S. ( $0.45\mu\text{m}$  pore size) to achieve a suitable maximum peak height on the strip chart recorder. Samples (2ml) were injected into a HPLC (Waters Associate Inc. Milford, MA: model M-6000A

pump, Model 440 detector fitted with a C18 column). The eluent consisted of: methanol:  
dH<sub>2</sub>O: glacial acetic acid, 65:35:0.1, (v/v/v) prefiltered through a 0.2µm nylon filter.

## RESULTS

### Buffering studies.

Although Bottomley's laboratory has many years of experience in growing different bacterial species, my results indicated that the traditional laboratory mineral salts medium, buffered at pH 6.5 with 0.5g/liter (2.9mM) potassium phosphate, was inadequately buffered for isolate T1 to achieve optimal growth on 2,4-D as sole source of carbon. With 2,4-D at 1g/liter (4.5mM), T1 reached stationary phase at an optical density (O.D. 660nm) of 0.1. Analysis of the culture medium by HPLC showed that 90% of the 2,4-D remained and the pH had dropped to 4.0 (Figure 1). Initially, I was reluctant to enhance the buffering capacity of the medium by simply increasing its phosphate concentration. Autoclaving causes high phosphate concentrations to precipitate in the medium which would interfere with turbidimetric growth measurements. When the buffering capacity of the medium was augmented with 1g/liter (5.1mM) of the non-metabolizable buffer, 2-N-morpholino-ethanesulfonic acid (MES), neither the length of the time interval required to detect turbidity nor the growth rate (5h) of T1 were changed (Figure 1). However, the growth yield of T1 doubled and only 25% of the 2,4-D remained in the spent growth medium. Nonetheless, the buffering capacity was still inadequate to prevent the pH from dropping to 5.0.

The poor growth of T1 on 2,4-D in the traditional mineral salts medium was not unique. Isolate D1 grew poorly (doubling time of 10h), reaching a final O.D. of only 0.05 (Figure 2). Ninety percent of the 2,4-D remained in the medium at stationary phase and the final medium pH was 3.8. Again, improved cell growth (O.D. of 0.2) was achieved when the mineral salts medium was supplemented with 1g/liter of MES (Figure 2). In this case, even though the pH of the medium dropped to 3.5, virtually all (90-95%) of the 2,4-D was consumed.

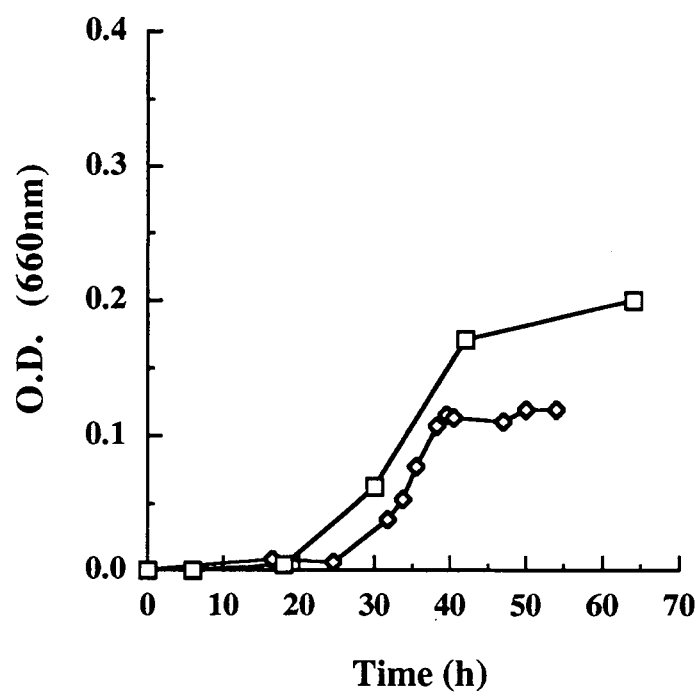


Figure 1: Growth characteristics of isolate T1 in minimal medium containing 1g/l (4.5mM) of 2,4-D with (—□—) and without (—◇—) 1g/l (5.1mM) MES buffer.

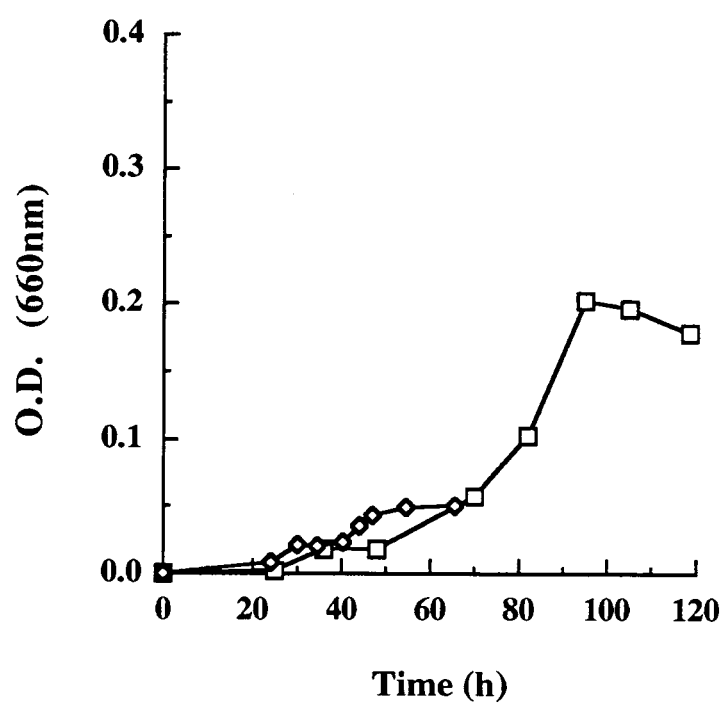


Figure 2: Growth characteristics of isolate D1 in minimal medium containing 1g/l (4.5mM) of 2,4-D with (—□—) and without (—◇—) 1 g/l (5.1mM) MES buffer

I excluded MES as a permanent solution to buffering the growth medium because of its cost. Furthermore, preliminary data were obtained which showed that the time interval required to detect turbidity was lengthened when the concentration of MES was increased above 5mM (data not shown). Therefore, an experiment was conducted to determine the optimal phosphate concentration required for growth of various 2,4-D degrading bacteria on 2,4-D. Growth of the isolates on 4.5mM 2,4-D was improved significantly by increasing the concentration of phosphate in the medium. Three types of growth responses were observed among the isolates (Figures 3-5). Although the maximum growth rates were not influenced by different phosphate concentrations, final culture turbidity was affected. Three isolates (T1, D1, and 712) grew with exponential phase generation times of 4h, 4h, and 7h respectively. They required >12.9mM phosphate to achieve maximum culture turbidity during growth on 4.5mM 2,4-D (Figure 3). In 12.9mM phosphate-buffered medium, isolates T1, D1, and 712 left 20, 25, and 35%, respectively, of the 2,4-D in the medium after stationary phase was reached and the medium pH had dropped to 3.6, 3.6, and 4.8, respectively. Two additional isolates (D2, and T3) also grew with exponential phase generation times of 7h, and 10h respectively on 4.5mM 2,4-D (Figure 4). In contrast with the three previous isolates (see Figure 3), isolates D2 and T3 reached final growth turbidities in 12.9mM phosphate-buffered medium that were no different from those reached in media with concentrations of phosphate >12.9mM (Figure 4). In contrast to T1, D1, and 712, isolates D2 and T3 consumed all of the 2,4-D in the 12.9mM phosphate treatment despite the final medium pH being lowered to 3.7.

Isolates T2 and T4 showed distinctly different growth responses to 2,4-D in the medium buffered with different phosphate concentrations (Figure 5). The maximum exponential phase generation times (15h) of isolates T2, and T4 on 4.5mM 2,4-D were markedly longer than the other five isolates. In addition, the length of time interval required to detect turbidity was less in medium supplemented with 12.9 and 22.9mM

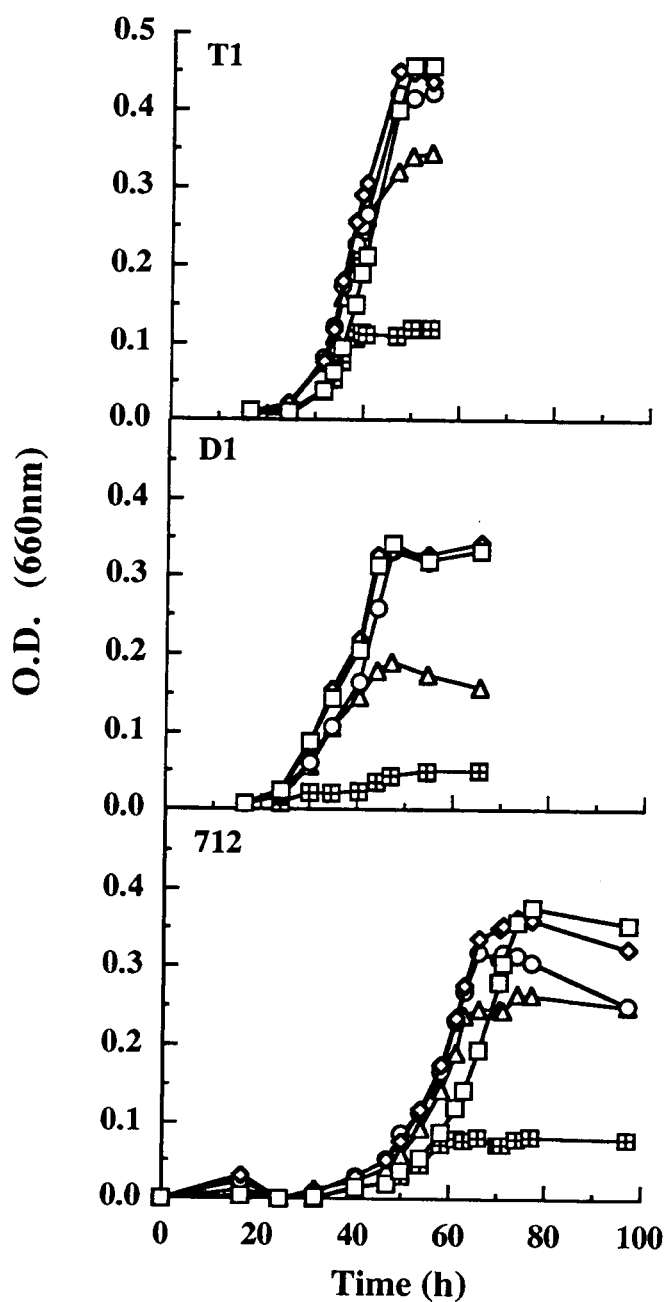


Figure 3: Growth characteristics of isolates T1, D1, and 712 in minimal medium containing 1g/l (4.5mM) of 2,4-D and various concentrations of phosphate. —□— 52.9mM; —◇— 42.9mM; —○— 22.9mM; —△— 12.9mM; —▣— 2.9mM.



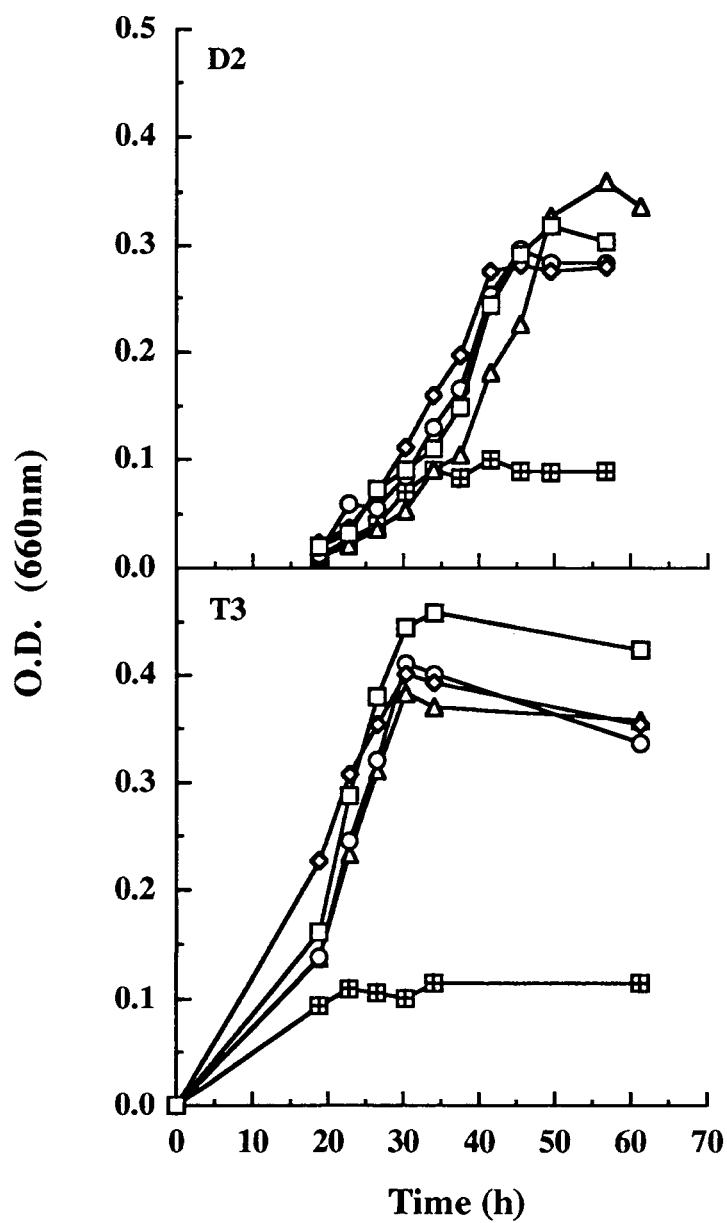


Figure 4: Growth characteristics of isolates D2 and T3 in minimal medium containing 1g/l (4.5mM) of 2,4-D and various concentrations of phosphate.

—□— 52.9mM; —◇— 42.9mM; —○— 22.9mM;  
 —△— 12.9mM; —▣— 2.9mM.

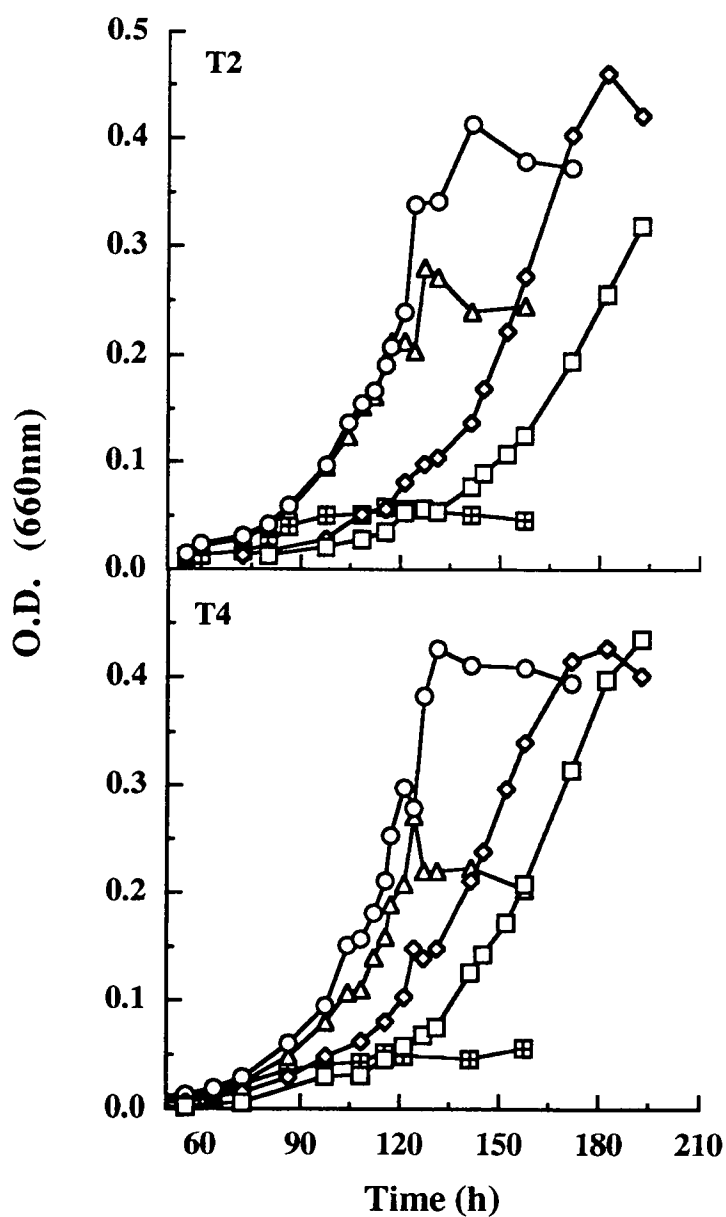


Figure 5: Growth characteristics of isolates T2 and T4 in minimal medium containing 1g/l (4.5mM) of 2,4-D and various concentrations of phosphate. —□— 52.9mM; —◇— 42.9mM; —○— 22.9mM; —△— 12.9mM; —■— 2.9mM.

phosphate (about 80-100h) compared to medium containing 42.9 and 52.9mM phosphate (about 110-140h). Furthermore, the exponential phase generation times at the two higher phosphate concentrations were longer (20-23h). Although the final O.D. of T2 and T4 cultures were suboptimal at 12.9mM phosphate, all 2,4-D was consumed by both isolates regardless of phosphate concentration and the pH only declined to 4.5. It is unclear if this phenomenon is specifically related to high phosphate, or high potassium, or if it is a general osmotic effect.

### **Organic supplement studies.**

My initial attempts at isolating 2,4-D degrading bacteria were based upon the assumption that isolates could be recovered that would grow on 2,4-D and ammonium nitrate ( $\text{NH}_4\text{NO}_3$ ) as the sole sources of carbon and nitrogen, respectively. Although all of the isolates mentioned so far grew under the aforementioned conditions, the time intervals required to detect visible turbidity were shortened considerably when cells were inoculated into growth medium containing 2,4-D and a small amount (50mg/l) of yeast extract (YE) or casamino acids (CA). However, exponential phase generation times were approximately the same (4h) regardless of the organic supplement. Growth of cultures supplemented only with YE or CA showed that the isolates could grow on either YE or CA as carbon sources (Figure 6). No discernible diauxie was shown by the fast isolates in the YE supplemented medium. YE and CA reduced the time it took for slow growing isolates T2, and T4 to reach O.D. 0.1 by about 40h (Figure 7). Once visible turbidity was reached, the growth rates of these slower isolates were unaffected by the organic supplements (20h).

### **Growth efficiency studies.**

Since all isolates grew in buffered minimal medium on 2,4-D as sole carbon source, stimulation of growth on 2,4-D by small amounts of yeast extract or casamino acids was

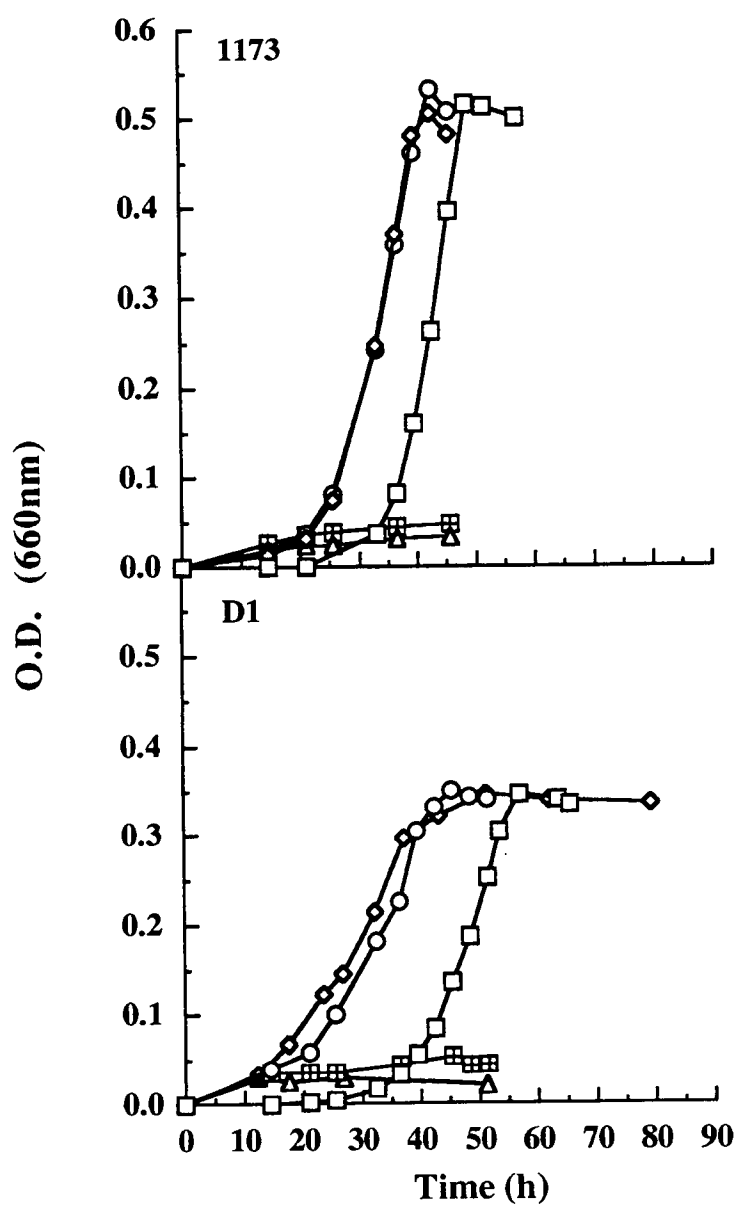


Figure 6: Growth characteristics of isolates 1173 and D1 in minimal medium containing 52.9mM phosphate, 1g/l (4.5mM) of 2,4-D +/- yeast extract (YE) or casamino acids (CA) at 50mg/l.

—□— 2,4-D; —◇— 2,4-D + YE; —○— 2,4-D + CA;  
 —△— YE; —▣— CA.

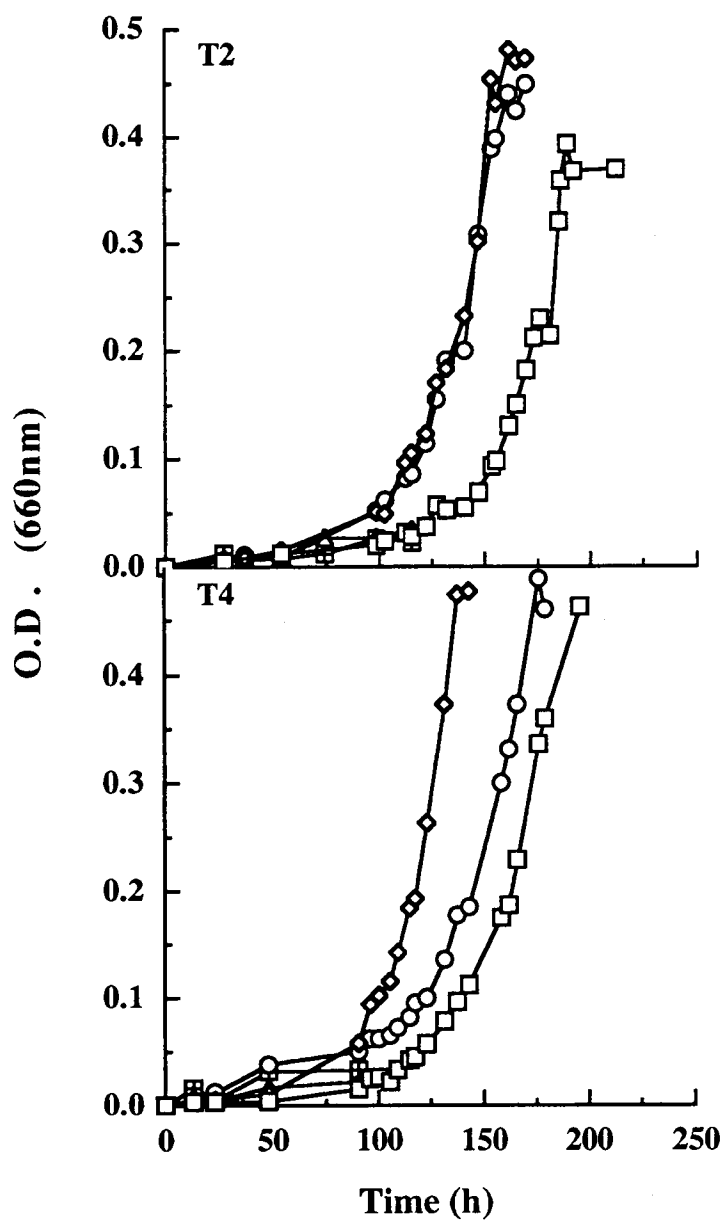


Figure 7: Growth characteristics of isolates T2 and T4 in minimal medium containing 52.9mM phosphate, 1g/l (4.5mM) of 2,4-D +/- yeast extract (YE) or casamino acids (CA) at 50mg/l.

—□— 2,4-D; —◇— 2,4-D + YE; —○— 2,4-D + CA;  
 —△— YE; —▣— CA.

probably a reflection faster growth on the latter than on 2,4-D. This observation led me to examine aspects of the growth efficiency of isolates on 2,4-D. Growth of isolates D1, T1, and 712 were compared on equivalent amounts (27mM carbon) of 2,4-D, succinate (disodium salt), and D-glucose (Figure 8). It can be seen that the isolates tested grew more rapidly on glucose and succinate (exponential phase generation times of 2-2.5h) than on 2,4-D (exponential phase generation times of 4h, 5h, and 7h for D1, T1, and 712 respectively). In addition these isolates grew to greater O.D. on glucose and succinate (0.9, and 0.5 respectively) than they did on 2,4-D (0.3-0.4) [Figure 8]. It is not known why isolate 712 exhibited such an extended delay in the response to glucose. The final O.D.s of the glucose and succinate grown cultures were approximately 300% and 25-50% higher, respectively, than the 2,4-D grown cultures.

The effect of 2,4-D concentration on overall growth of isolate D1 was examined in medium buffered with 52.9mM phosphate and supplemented with four concentrations of 2,4-D (0.1, 0.5, 0.75, and 1g/liter)[Figure 9a]. Isolate D1 exhibited a linear relationship between the amount of cell growth and 2,4-D concentration (Figure 9b). In the case of 0.1g/liter, a maximum O.D. of 0.05 was obtained which was barely visible to the eye.

Table 2 shows the final cell yield data obtained from cultures of T1, D1, 712, T2, and T4 grown on glucose, succinate, or 2,4-D. Averaging the three isolates, glucose-grown cells produced about 0.42mg dry weight/ml, whereas 2,4-D-grown cells produced about 0.13mg/ml. This dry weight ratio of the two substrates agrees favorably with the ratio of the final O.D.s observed in the cultures. In the case of succinate-grown isolates, cell dry weight yields averaged 0.18mg/ml, and were more similar to 2,4-D cell yields than to glucose yields. Again this yield difference agrees with the final O.Ds. which were about 25-50% higher on succinate than on 2,4-D-grown cells. If 0.13mg dry weight of cells are produced from 4.5 $\mu$ moles of 2,4-D, a cell yield value of 28.9g of cells per mole of 2,4-D can be calculated.

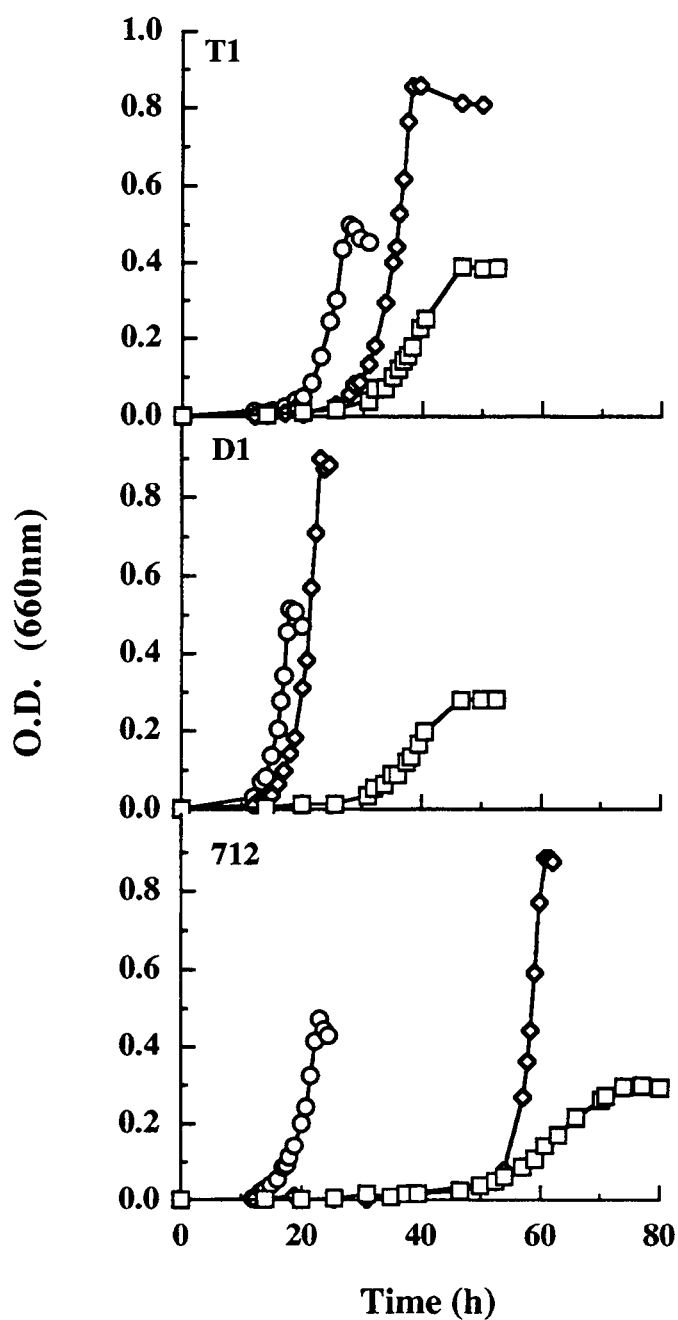


Figure 8: Growth characteristics of isolates T1, D1, and 712 in minimal medium containing 52.9mM phosphate, and 27mM carbon supplied from various sources. —□— 2,4-D; —◇— glucose; —○— succinate.

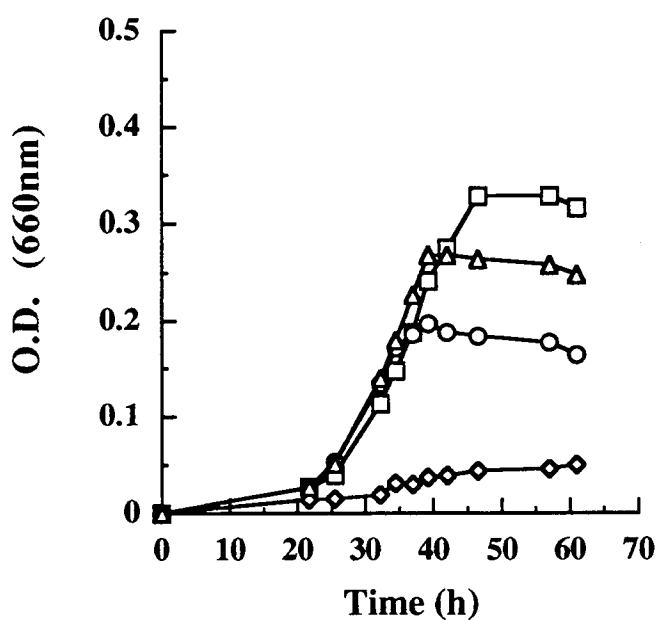


Figure 9a: Growth characteristics of isolate D1 in minimal medium containing 52.9mM phosphate and different concentrations of 2,4-D. —◆—, 0.1g/l; —○—, 0.5g/l; —△—, 0.75g/l; —□—, 1g/l.

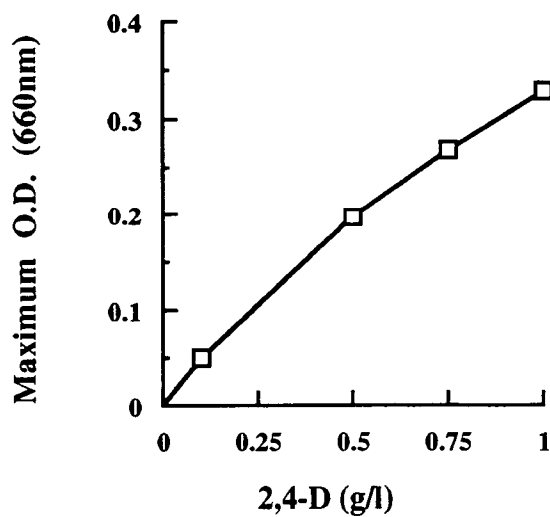


Figure 9b: Maximal growth of D1 grown in minimal medium containing various concentrations of 2,4-D.



**Table 2:** Cell yield (mg dry weight/ml) and efficiency of growth (percent of carbon assimilated) of isolates T1, D1, 712, T2, and T4 grown in minimal medium buffered with 52.9mM phosphate on either glucose, succinate, or 2,4-D as sole carbon and energy source.

Isolate	growth substrate					
	glucose		succinate		2,4-D	
	dry weight	% C assim <sup>2</sup>	dry weight	% C assim.	dry weight	% C assim.
T1	0.3	47	0.16	25	0.16	25
D1	0.25 (0.02) <sup>1</sup>	38.3 (2.5)	0.21 (0.03)	31.3 (4.2)	0.11 (0.01)	15.5 (1.0)
712	0.45	70	0.16	25	0.14	21
T2	0.26	40.5	0.18	27	0.12	18
T4	N.D. <sup>3</sup>	N.D.	N.D.	N.D.	0.13	20

<sup>1</sup>Numbers in parentheses refer to the standard deviation of the means from 2 to 4 separate experiments.

<sup>2</sup>Assumes that cell dry weight consists of 50% carbon. Assim. abbreviation for assimilated.

<sup>3</sup>Not determined.

The final cell density values for the isolates grown on the three carbon sources are presented in Table 3. In this case, two of the three values for the glucose-grown cultures were only about 50-70% greater than for the 2,4-D cultures despite the much greater difference in cell dry weights. The discrepancy could reflect the possibility that some of the glucose grown-cells were dead at the time that the plate counts were conducted, or perhaps, that glucose-grown cells are larger than the 2,4-D cells, or that glucose-grown cells produce a larger amount of capsular material. It can be calculated that on average it takes 0.017 and 0.03pmoles of glucose and 2,4-D-carbon, respectively, to produce a cell, which is equivalent to 0.54 and 0.83pg of glucose and 2,4-D, respectively, to produce a cell.

### **Enumeration of 2,4-dichlorophenoxyacetic acid degrading soil microbial populations.**

I used the information obtained about the growth requirements of 2,4-D degrading bacteria in several ways. First, the mineral salts medium buffered with 52.9mM phosphate, and either supplemented with or without yeast extract (50mg/l) was used in a Most Probable Number assay (MPN) to determine the population densities of 2,4-D degrading bacteria in soil recovered from different depth increments in a Willamette silt loam. Data in Table 4 show that the presence of yeast extract did not influence the MPN estimates and that an incubation period of 21d was adequate to obtain the maximum number of positive vials. Even though the population density of 2,4-D degrading bacteria was <100/g at depths of 60cm and below, all potentially positive vials had mineralized 2,4-D as CO<sub>2</sub> after 15d of incubation with 30-40% of the 2,4-D radiolabel released as carbon dioxide. By the final assay time (31d), 70 to 85% of the 2,4-D radiolabel had been evolved as carbon dioxide.

The MPN procedure was also used to determine the final population densities of 2,4-D degrading bacteria that had grown in soil samples recovered from 80-100cm depth and treated with three different concentrations of 2,4-D (0.06μg, 0.6μg, and 6μg/g of soil)

**Table 3:** Comparison of (a) growth efficiency expressed in terms of substrate carbon processed per cell produced, and (b) final cell densities of isolates D1, T1, and 712 grown in minimal medium buffered with 52.9mM phosphate and either glucose, succinate or 2,4-D as the sole carbon and energy source.

	Isolate		
	D1	T1	712
Growth substrate:	pmoles of C processed/cell produced		
glucose	0.01	0.02	0.02
succinate	0.02	0.07	0.03
2,4-D	0.02	0.04	0.03
	Final Cell Density ( $\times 10^{-9}$ )		
glucose	5.0 <sup>1</sup>	1.2	1.4
succinate	1.3	0.4	0.9
2,4-D	1.3	0.7	0.9

<sup>1</sup>Cell density values represent the average of duplicates from a single experiment.

**Table 4.** Influence of incubation time and a yeast extract supplement on Most Probable Number (MPN) estimates of the population size of 2,4-D degraders in soil at different depths in the profile of a Willamette silt loam.

		Population density, #/g ( $\times 10^{-3}$ ) estimated after:		
Soil Depth (cm)		15d	21d	28d
0-20	- <sup>1</sup>	1.8 -(7.0)-26.7 <sup>2</sup>	4.7-(17.8)-67.6	4.7-(17.8)-67.6
	+	3.4-(12.9)-48.9	10.8-(41.1)-156.4	10.8-(41.1)-156.4
20-40	-	0.3-(1.1)-4.2	1.1-(4.2)-16.1	1.1-(4.2)-16.1
	+	0.7-(2.7)-10.3	0.7-(2.7)-10.3	0.7-(2.7)-10.3
40-60	-	0.4-(1.4)-5.4	0.4-(1.4)-5.4	0.4-(1.4)-5.4
	+	0.2-(0.7)-2.8	0.2-(0.7)-2.8	0.4-(1.4)-5.4
60-80	-	0.7-(3.2)-14.8	0.7-(3.2)-14.8	0.7-(3.2)-14.8
	+	0.3-(1.4)-6.6	0.3-(1.4)-6.6	0.3-(1.4)-6.6
80-100	-	0.3-(1.5)-6.9	0.3-(1.5)-6.9	0.3-(1.5)-6.9
	+	0.3-(1.5)-6.9	0.3-(1.5)-6.9	0.3-(1.5)-6.9

<sup>1</sup> - indicates no yeast extract. + indicates 50mg/l yeast extract.

<sup>2</sup> Values outside of parentheses represent the lower and upper 95% confidence limits of the population size determined by the MPNES program.

[Table 5]. In this case, supplementation of the MPN vials with yeast extract produced a larger number of positive vials than the minus yeast extract series of vials after 7d of incubation. Nevertheless, after 14d of incubation, there were no differences in the number of positive vials between plus and minus yeast extract for the 0.06 and 0.6  $\mu\text{g/g}$  treatments. However, in the case of the 6  $\mu\text{g/g}$  treatment, a larger number of positive vials were detected in the yeast extract supplemented series throughout the incubation period (31d). The cell densities calculated by MPN were used to determine the cell yield values (assuming all of the 2,4-D applied to the soil had been metabolized). The highest MPN estimate of cell density for the 6 $\mu\text{g/g}$  application was equivalent to a yield of 0.09 pmole 2,4-D carbon/ cell. This value is comparable with those obtained for isolates grown in liquid culture (Table 3). However, as the concentration of 2,4-D decreased, the apparent efficiency of 2,4-D conversion into cells decreased to between 0.27 and 0.94 pmole C/cell.

**Table 5:** Comparison of values of 2,4-D carbon used per cell produced for an indigenous Willamette soil population of 2,4-D degraders that was exposed to three different concentrations of 2,4-D.

2,4-D added to soil ( $\mu\text{g/g}$ )	2,4-D carbon added (pmole/g)	MPN estimate ( $\# \times 10^{-4}/\text{g}$ ) <sup>1</sup>	2,4-D carbon used per cell produced (pmole/cell) <sup>5</sup>
0.06	2168	0.05-(.23)-1.1 <sup>2</sup>	0.94
0.6	21680	1.7-(8.0)-37.3 <sup>2</sup>	0.27
6.0	216800	8.5-(40.0)-186.9 <sup>3</sup>	0.54
6.0	216800	51.3-(240.0)-1121.8 <sup>4</sup>	0.09

<sup>1</sup> Values outside of parentheses represent the lower and upper 95% confidence limits of the population size determined by the MPNES program.

<sup>2</sup> MPN values were not influenced by the presence of a yeast extract supplement in the MPN assay.

<sup>3</sup> MPN value determined without a yeast extract supplement in the incubation vials.

<sup>4</sup> MPN value determined with a yeast extract supplement in the incubation vials.

<sup>5</sup> Values are based on the assumption that all 2,4-D was processed by the soil microorganism.

## DISCUSSION

Growth is central to the roles that microorganisms play on planet Earth and our ability to study them. In the specific case of biodegradation of xenobiotic chemicals, our ability to quantify populations of xenobiotics degrading bacteria in natural samples by the MPN technique requires conditions in which microorganisms can proliferate from a small number. Furthermore, isolation of xenobiotic degrading organisms from the environment depends upon establishing conditions for growth at the expense of the xenobiotic in question. The ability of a mathematical model to accurately describe the kinetics of degradation of a xenobiotic chemical, depends in part, upon whether or not growth occurs during degradation of the chemical (Alexander, 1994). From my studies, I have made some basic observations on the growth characteristics of 2,4-D degrading bacteria which could have ramifications to the properties of 2,4-D degradation in natural environments.

### **Buffering of medium.**

My data illustrate that serious generation of acidity occurs when bacteria degrade 2,4-D. The data show that the decline in pH of the medium was greater from the metabolism of 2,4-D than from equivalent amounts of glucose or succinate carbon. From the Henderson and Hasselbach equation it can be calculated that 2.9mM phosphate used in the traditional mineral salts medium buffered at pH6.5 would contain only about 1mM  $\text{HPO}_4^{2-}$ . Assuming that 2mM of HCl are generated per mM of 2,4-D metabolized, only 0.5mM of the 2,4-D (11%) can be degraded before the buffering capacity of the medium is compromised. This calculation agrees with the observation that about 90% of the 2,4-D remained after growth ceased in our original minimal medium. Other researchers have used a wide range of phosphate concentrations to buffer 2,4-D media (4.4-50mM) [Ka et al., 1994a; Amy et al., 1985]. Although Focht (1994) recommended a medium buffered with 13mM phosphate for studying breakdown of chlorinated aromatics, he suggested that the

concentration of phosphate might need to be increased if higher concentrations of substrates are used. My data from the faster growing isolates are in agreement with that statement, however my observations with the slower growing isolates indicate that caution should be taken before indiscriminately using high concentrations of phosphate to buffer growth media.

However, it was interesting to observe that isolates D2 and T3 were not so sensitive to inadequate buffering. Presumably, these bacteria are less sensitive to acid conditions than either T1, D1, or 712. Differential sensitivity of bacterial species, or strains of a species to pH has been well described in soil-borne bacteria such as *Rhizobium*, *Bradyrhizobium* and ammonia-oxidizing bacteria (Bottomley, 1992; Schmidt and Belser, 1994). Since shifts in the populations of 2,4-D degrading bacteria have been observed in soil treated repetitively with high concentrations ( $\geq 100\mu\text{g/g}$ ) of 2,4-D (Ka et al., 1994a), it would be interesting to know if acid sensitivity played a role in this population shift. It has been documented that 2,4-D breakdown occurs more slowly in acid soils (Sandmann et al., 1988; Stott et al., 1983). Another possibility is that different metabolic pathways exist which allow some organisms to metabolize 2,4-D under acidic conditions. It is also possible that 2,4-D itself might play a negative role in the cessation of growth as pH declines. It is well documented that the toxicity of 2,4-D to plants increases as pH decreases because 2,4-D uptake into plant cells increases as a greater proportion of the phenoxyacetic acid exists in the unassociated form (Devin et al., 1993). Furthermore, 2,4-D has been shown to disturb membrane functions in bacteria (de Peretti, 1992). Finally, it would be interesting to know if acid generation is a serious impediment to sustaining biodegradation of polychlorinated aromatic compounds such as polychlorinated biphenyls (P.C.B.s) and pentachlorophenol in poorly-buffered polluted environments.



## Growth yields.

The slower growth rates of the isolates on 2,4-D relative to the other carbon sources, in conjunction with the low efficiency of carbon assimilation, indicate that 2,4-D is a substrate of low energy content. One would predict therefore, that 2,4-D degrading bacteria, which generally belong to genera that show great nutritional versatility (e.g. *Burkholderia*, *Pseudomonas*, and *Alcaligenes*), would prefer to use carbon sources of greater energy content than 2,4-D if both occurred simultaneously. It would be interesting to know if biodegradation of herbicides containing energy-poor structures, such as the triazine ring, is influenced by the simultaneous presence of alternate substrates (Mandelbaum et al., 1995; Radosevich et al., 1995).

The 2,4-D growth yield value calculated from the cell yield data was similar for all of the isolates and similar to the value reported by Ka et al. (1995). However, the substrate-to-cell conversion value of 0.83pg/cell is considerably lower than the values of 1.3pg/cell and 3pg/cell calculated by Holben et al. (1992), and Chen and Alexander (1989), respectively. Perhaps this discrepancy reflects upon differences in cell size, or upon different efficiencies of 2,4-D metabolism by the isolates.

The cell yield value can be used to predict size of the population of 2,4-D degrading bacteria produced by adding a known amount of 2,4-D to a soil. In this context the population that grew in Willamette soil in response to the highest application of 2,4-D converted the latter to cells at an efficiency similar to what I had observed in pure liquid cultures. At lower concentrations, however the conversion efficiencies were much lower. There are several interesting possibilities that might account for this observation. (1) the MPN procedure might under-estimate the population size of 2,4-D degrading bacteria. (2) A certain proportion of the low concentrations of 2,4-D applied to soil is not bioavailable. (3) A certain percentage of the 2,4-D is metabolized by microorganisms that do not grow on the latter. Yadav and Reddy (1993) have shown that the fungus *Phanerochaete chrysosporum* can metabolize 2,4-D under co-metabolic conditions.

### **Isolation of 2,4-dichlorophenoxyacetic acid degrading bacteria from soil.**

One of the earlier goals of my thesis project was to isolate 2,4-D degrading bacteria from the surface and subsurface soil environment. One of my strategies involved using low concentrations (0.1g/l) of 2,4-D in liquid culture. My data show that isolates can barely grow to visible turbidity on 0.1g/liter of 2,4-D (Figure 9b). Therefore, even though there is evidence that high concentrations of 2,4-D may be toxic to some soil bacteria it will be difficult to follow enrichment of bacteria at 0.1g/liter by simply monitoring growth visually. All the isolates that I recovered and tested grew on 2,4-D in a minimal medium supplemented with vitamins. However, I observed that a yeast extract supplement permitted detection of 2,4-D degrading bacteria in higher dilutions of Willamette soil treated with 6 $\mu$ g/g 2,4-D. These data indicate that some degrading bacteria may need essential growth factors. Recently, preliminary evidence has been obtained for the existence of 2,4-D degrading bacteria in Willamette soil that require a yeast extract supplement to grow on 2,4-D (Rockie Yarwood, personal communication). Although only a few 2,4-D degrading bacteria were isolated from the Willamette soil, two of the isolates (T2 and T4) recovered from the subsurface were quite slow-growing relative to those recovered from surface soil. Whether or not such organisms influence the biodegradation potential of the subsurface soil environment will require further study.

## LITERATURE CITED

- Alexander, M. 1994. Biodegradation and Bioremediation. Academic Press, San Diego pp.8-40.
- Amy, P. S., J. W. Schulke, L. M. Frazier, and R. J. Seidler. 1985. Characterization of aquatic bacteria and cloning of genes specifying partial degradation of 2,4-dichlorophenoxyacetic acid. *Appl. Environ. Microbiol.* 49:1237-1245.
- Atlas, R. M. and R. Bartha. 1993. *Microbial Ecology: Fundamentals and Applications*. Benjamin Cummings Publishing Co. Inc. New York. pg 388.
- Bellinck, C., L. Batistic, J. Mayaudon. 1979. Breakdown of 2,4-dichlorophenoxyacetic acid in soils. *Rev. Ecol. Biol. Sol.* 16:161-168.
- Boethling, R. S., and M. Alexander. 1979. Effect of concentration of organic chemicals on their biodegradation by natural microbial communities. *Appl. Environ. Microbiol.* 37:1211-1216.
- Boesten, J. J. T. I., and A. M. A. van der Linden. 1991. Modeling the Influence of Sorption and Transformation on Pesticide Leaching and Persistence. *J. Environ. Qual.* 20:425-435.
- Bollage, J. M., C. S. Helling, and M. Alexander. 1968. 2,4-D metabolism: Enzymatic hydroxylation of chlorinated phenols. *J. Agric. Food Chem.* 16:826-828.
- Bottomley, P. J. 1992. Ecology of *Bradyrhizobium* and *Rhizobium*. pp. 293-348. *Biological Nitrogen Fixation*. G. Stacy, R. H. Burris, and H. J. Evans, editors. Chapman and Hall, N. Y.
- Chaudhry, G. R., and S. Chapalamadugu. 1991. Biodegradation of halogenated organic compounds. *Microbiol. Rev.* 55:59-79.
- Chaudhry, G. R. 1994. *Biological degradation and bioremediation of toxic chemicals*. Dioscorides Press Portland, Oregon.
- Chen, S., and M. Alexander. 1989. Reasons for the acclimation for 2,4-D biodegradation in lake water. *J. Environ. Qual.* 18:153-156.
- Daugherty, D. D., and S. F. Karel. 1994. Degradation of 2,4-dichlorophenoxyacetic acid by *Pseudomonas cepacia* DBO1 (pRO101) in a dual-substrate chemostat. *Appl. Environ. Microbiol.* 60:3261-3267.
- de Peretti, A. F., R. Duffard, and A. M. E. de Duffard. 1992. Effects of 2,4-dichlorophenoxyacetic acid on *Rhizobium sp.* membrane fluidity. *Arch. Environ. Contam. Toxicol.* 23:301-306.
- Devine, M. D., S. O. Duke, and C. Fedtke. 1993. *Physiology of herbicide action*. Prentice Hall, N. J. p. 441.

- Ditzelmuller, G., M. Loidl, and F. Streichsbier. 1989. Isolation and characterization of a 2,4-dichlorophenoxyacetic acid-degrading soil bacterium. *Appl. Microbiol. Biotech.* 31:93-96.
- Don, R. H., and J. M. Pemberton. 1981. Bacterial Plasmids of Agricultural and Environmental Importance. *Agric-Environ.* Amsterdam, Elsevier. pp. 22-32.
- Edwards, W. M., M. J. Shipitalo, W. A. Dick, and L. B. Owens. 1992. Rainfall Intensity Affects Transport of Water and Chemicals Through Macropores in No-Till Soil. *Soil Sci. Soc. Am. J.* 56:52-58.
- Flury, M. 1996. Experimental Evidence of Transport of Pesticides through Field Soils-A Review. *J. Environ. Qual.* 25:25-45.
- Focht, D. 1994. Microbiological Procedures for Biodegradation Research. pp. 409-410. In *Methods of Soil Analysis Part 2: Microbiological and Biochemical Properties*. R. W. Weaver, J. S. Angle, and P. J. Bottomley, editors. SSSA, Madison, WI.
- Fournier, J. C. 1980. Enumeration of the Soil Micro-Organisms able to Degrade 2,4-D by Metabolism or Co-Metabolism. *Chemosphere* 9:169-174.
- Fournier, J. C., P. Codaccioni, and G. Soulas. 1981. Soil adaptation to 2,4-D degradation in relation to the application rates and the metabolic behaviours of the degrading microflora. *Chemosphere* 10:977-984.
- Fries, M. R., J. Zhou, J. Chee-Sanford, and J. M. Tiedje. 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. *Appl. Environ. Microbiol.* 60:2802-2810.
- Fulthorpe, R. R., A. N. Rhodes, and J. M. Tiedje. 1996. Pristine soils mineralize 3-chlorobenzoate and 2,4-dichlorophenoxyacetate via different microbial populations. *Appl. Environ. Microbiol.* 62:1159-1166.
- Ghosal, D., I. S. You, D. K. Chatterjee, and A. M. Chakrabarty. 1985. Microbial degradation of halogenated compounds. *Science* 288:135-142.
- Green, J. D., R. Horton, and J. L. Baker. 1995. Crop residue effects on the leaching of surface-applied chemicals. *J. Environ. Qual.* 24:343-351.
- Greer, L. E., and D. R. Shelton. 1992. Effect of inoculant strain and organic matter content on kinetics of 2,4-dichlorophenoxyacetic acid degradation in soil. *Appl. Environ. Microbiol.* 58:1459-1465.
- Harker, A. R., R. H. Olsen, R. J. Seidler. 1989. Phenoxyacetic acid degradation by the 2,4-dichlorophenoxyacetic acid (TFD) pathway of plasmid pJP4: mapping and characterization of the TFD regulatory gene, *tfdR*. *J. Bacteriol.* 171:314-320.
- Holben, W. E., B. M. Schroeter, V. G. M. Calabrese, R. H. Olsen, J. K. Kukor, V. O. Biederbeck, A. E. Smith, and J. M. Tiedje. 1992. Gene probe analysis of soil microbial populations selected by amendment with 2,4-dichlorophenoxyacetic acid. *Appl. Environ. Microbiol.* 58:3941-3948.

- Ka, J. O., W. E. Holben, and J. M. Tiedje. 1994a. Genetic and phenotypic diversity of 2,4-dichlorophenoxyacetic acid (2,4-D) degrading bacteria isolated from 2,4-D-treated field soils. *Appl. Environ. Microbiol.* 60:1106-1115.
- Ka, J. O., W. E. Holben, and J. M. Tiedje. 1994b. Use of gene probes to aid in recovery and identification of functionally dominant 2,4-dichlorophenoxyacetic acid-degrading populations in soil. *Appl. Environ. Microbiol.* 60:1116-1120.
- Ka, J. O., P. Burauel, J. A. Bronson, W. E. Holben, and J. M. Tiedje. 1995. DNA probe analysis of microbial community selected in field soil by long-term 2,4-D application. *Soil Sci. Soc. Am. J.* 59:1581-1587.
- Kaphammer, B., J. J. Kukor, and R. H. Olsen. 1990. Regulation of *tfdCDEF* by *tfdR* of the 2,4-dichlorophenoxyacetic acid degradation plasmid pJP4. *J. Bacteriol.* 172:2280-2286.
- Kaphammer, B., and R. H. Olsen. 1990. Cloning and characterization of *tfdS*, the repressor-activator gene of *tfdB*, from the 2,4-dichlorophenoxyacetic acid catabolic plasmid pJP4. *J. Bacteriol.* 172:5856-5862.
- Mandelbaum, R. T., D. L. Allan, and L. P. Wackett. 1995. Isolation and characterization of a *Pseudomonas* sp. that mineralizes the s-triazine herbicide atrazine. *Appl. Environ. Microbiol.* 61:1451-1457.
- Miwa, N., and S. Kuwatsuka. 1990. Enrichment process of 2,4-D degraders in different soils under upland conditions. *Soil Sci. Pl. Nutr.* 36:261-266.
- Miwa, N., and S. Kuwatsuka. 1991. Characteristics of 2,4-dichlorophenoxyacetic acid (2,4-D)-degrading microorganisms isolated from different soils. *Soil Sci. Plant Nutr.* 37:575-581.
- Moorman, T. B. 1990. Adaptation of microorganisms in subsurface environments: Significance to pesticide degradation. pp. 167-180. In: *Enhanced biodegradation of pesticides in the environment*. K. D. Racke, and J. R. Coats, editors. Amer. Chem. Soc. Washington D. C.
- Neilson, J. W., K. L. Josephson, I. L. Pepper, R. B. Arnold, G. D. DiGiovanni, and N. A. Sinclair. 1994. frequency of horizontal gene transfer of a large catabolic plasmid (pJP4) in Soil. *Appl. Environ. Microbiol.* 60:4053-4058.
- Ou, Li-Tse. 1984. 2,4-D degradation and 2,4-D degrading microorganisms in soils. *Soil Science* 137:100-107.
- Parker, L. W., and K. G. Doxtader. 1982. Kinetics of microbial decomposition of 2,4-D in soil: effects of herbicide concentration. *J. Environ. Qual.* 11:679-684.
- Pemberton, J. M., and P. R. Fisher. 1977. 2,4-D Plasmids and persistence. *Nature* 268:732-733.
- Radosevich, M., S. J. Traina, Y-L. Hao, and O. H. Tuovinen. 1995. Degradation and mineralization of atrazine by a soil bacterial isolate. *Appl. Environ. Microbiol.* 61:297-302.

- Sandmann, E. R. I. C., M. A. Loos, and L. P. van Dyk. 1988. The microbial degradation of 2,4-dichlorophenoxyacetic acid in soil. *Rev. Environ. Contam. Toxic.* 101:1-53.
- Schmidt, E. L., and L. W. Belser. 1994. Autotrophic nitrifying bacteria. pp. 159-195. In *Methods of Soil analysis Part 2: Microbiological and Biochemical Properties*. R. W. Weaver, J. S. Angle, and P. J. Bottomley, editors. SSSA Madison, WI.
- Soulas, G. 1993. Evidence for the existence of different physiological groups in the microbial community responsible for 2,4-D mineralization in soil. *Soil Biol. Biochem.* 25:443-449.
- Stenson, T. I., and N. Walker. 1957. The pathway of breakdown of 2,4-dichloro- and 4-chloro-2-methyl-phenoxyacetic acid by bacteria. *J. Gen. Microbiol.* 16:146-155.
- Stott, E. E., J. P. Martin, D. D. Focht, and K. Haider. 1983. Biodegradation, stabilization in humus, and incorporation into soil biomass of 2,4-D and chlorocatechol carbons. *Soil. Sci. Soc. Am J.* 47:66-70.
- Tiedje, J. M., and M. Alexander. 1969. Enzymatic cleavage of the ether bond of 2,4-dichlorophenoxyacetate. *J. Agric. Food Chem.* 17:1080-1084.
- Thurman, E. M., D. A. Goolsby, M. T. Meyer, M. S. Mills, M. L. Pomes, and D. W. Kolpin. 1992. A reconnaissance study of herbicides and their metabolites in surface water of the midwestern United States using immunoassay and gas chromatography/mass spectrometry. *Environ. Sci. Technol.* 26:2440-2447.
- van der Meer, J. R., W. M. de Vos, S. Harayama, and A. J. B. Zehnder. 1992. Molecular mechanisms of genetic adaptation to xenobiotic compounds. *Microbiol. Rev.* 56:677-694.
- Veeh, R. H., W. P. Inskeep, and A. K. Camper. 1996. Soil depth and temperature effects on microbial degradation of 2,4-D. *J. Environ. Qual.* 25:5-12.
- Walker, R. L., and A. S. Newman. 1956. Microbial decomposition of 2,4-dichlorophenoxyacetic acid. *Applied Microbiol.* 4:201-206.
- Weightman, A. J., R. H. Don, P. R. Lehrbach, and K. N. Timmis. 1984. Genetic control of environmental pollutants. G. S. Omenn and A. Holsander, editors. Plenum, New York. p 47.
- Wilson, R. G., and H. H. Cheng. 1976. Breakdown and movement of 2,4-D in the soil under field conditions. *Weed Sci.* 24:461-466.
- Xia, X., J. Bollinger, and A. Ogram. 1995. Molecular genetic analysis of the response of three soil microbial communities to the application of 2,4-D. *Molec. Ecol.* 4:17-28.
- Yadav, J. S., and C. A. Reddy. 1993. Mineralization of 2,4-dichlorophenoxyacetic acid (2,4-D) and mixtures of 2,4-D and 2,4,5-trichlorophenoxyacetic acid by *Phanerochaete chrysosporium*. *Appl. Environ. Microbiol.* 59:2904-2908.