INFORMATION TO USERS

This manuscript has been reproduced from the microfilm master. UMI films the text directly from the original or copy submitted. Thus, some thesis and dissertation copies are in typewriter face, while others may be from any type of computer printer.

The quality of this reproduction is dependent upon the quality of the copy submitted. Broken or indistinct print, colored or poor quality illustrations and photographs, print bleedthrough, substandard margins, and improper alignment can adversely affect reproduction.

In the unlikely event that the author did not send UMI a complete manuscript and there are missing pages, these will be noted. Also, if unauthorized copyright material had to be removed, a note will indicate the deletion.

Oversize materials (e.g., maps, drawings, charts) are reproduced by sectioning the original, beginning at the upper left-hand corner and continuing from left to right in equal sections with small overlaps.

Photographs included in the original manuscript have been reproduced xerographically in this copy. Higher quality 6" x 9" black and white photographic prints are available for any photographs or illustrations appearing in this copy for an additional charge. Contact UMI directly to order.

ProQuest Information and Learning
300 North Zeeb Road, Ann Arbor, MI 48106-1346 USA
800-521-0600

NOTE TO USER

Page(s) not included in the original manuscript are unavailable from the author or university. The manuscript was microfilmed as received.

62-64, 84-86, 124,125,129-132,& 139

This is reproduction is the best copy available

UMI®

Effects of Multiple Bacterial Species and Nutrient Injection on Phenanthrene Transport and Bacterial Cell Elution

by

Brandolyn Maltese Patterson

Dissertation Submitted to the Faculty of the

DEPARTMENT OF SOIL, WATER, AND ENVIRONMENTAL SCIENCE

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA
2001

THE UNIVERSITY OF ARIZONA ®
GRADUATE COLLEGE

As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Brandolyn Maltese Patterson entitled The Effects of Multiple Bacterial Species and Nutrient Injection on Phenanthrene Degradation, Bacterial Cell Elution, and Species Distribution

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

Dr. Mark L. Brusseau	Date: 17 Apr. 01
Dr. Raina M. Maier	Date: 4/6/01
Dr. Robert Frye	Date
Dr. James Walworth	Date: 4/6/01
Dr. Ian Pepper	Date: 4/6/01

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copy of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

Dissertation Director
Dr. Mark L. Brusseau

Date: 17 Apr. 2001

STATEMENT BY AUTHOR

This dissertation has been submitted in partial fulfillment of requirements for an advance degree at The University of Arizona and is deposited in the University Library to be made available to borrowers under the rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Requests for permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department of the Dean of the Graduate College when in his or her judgement the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Brandon M. Patterson

ACKNOWLEDGMENTS

I wish to express my gratitude to Dr. Mark L. Brusseau for starting me on this project and to Dr. Raina M. Maier for all her help, patience, and direction. Sincere thanks to Dr. Robert Frye for his endless help with my statistics and his always supportive and positive attitude. My appreciation to Dr. James Walworth for taking the time to understand my project and offer helpful suggestions and to Dr. Ian Pepper for his insight.

I would also like to thank those in my lab that allowed me to bounce ideas off of them and who encouraged me until the end.

DEDICATION

to my family...

TABLE OF CONTENTS

TABLE OF CONTENTS

Page

TABLE OF CONTENTS

page

LIST OF ILLUSTRATIONS

Page

LIST OF ILLUSTRATIONS

Page

LIST OF TABLES

Page

ABSTRACT

The objectives of this study were to investigate the effects of multiple bacterial species and nutrient injection on phenanthrene degradation, cell elution, and cell distribution within a column system. Three indigenous, phenanthrene degrading species, *Actinobacter junii*, *Pseudamonas oleovorans*, and *Methylbacterium sp.*, were used. Characterization studies were conducted to determine the relative cell yield and cell hydrophobicity. Transport studies were conducted in a saturated column system. Results show single bacterial species produce relatively stable cell elution and phenanthrene biodegradation curves. In addition, it appears that cell density within the column may be dependent on cell hydrophobicity and possibly column carrying capacity. Conversely, phenanthrene degradation and cell elution was variable in the presence of multiple bacterial species. Results indicate that synergistic and antagonistic interactions occurred among the species. Injection of an alternative carbon source (R_2B) enhanced phenanthrene degradation only in the single species column containing *Actinobacter junii*. It caused increased cell elution in *Actinobacter junii* and *Pseudamonas oleovorans* single species columns. No increase in degradation was found in multi-species columns injected with R_2B. These studies illustrate that the dynamics of complex microbial communities should be considered when evaluating contaminant biodegradation and transport in subsurface systems. The findings presented here have important implications for representation of biodegradation in computer modeling and in the design of bioremediation strategies.

CHAPTER 1: INTRODUCTION

Explanation of Dissertation Format

This dissertation consists of 3 major parts: the literature review, investigations, and references.

There are 6 sections included in the literature review. In the first section, polycyclic aromatic hydrocarbons (PAHs) are defined and discussed with regard to their bioavailability. Biodegradation kinetics are then discussed with regard to their origin, development, and their impact on contaminant transport. The third section introduces the concept of community structure and the consequences that contamination may have with regard to system structure. In the fourth section, competition in microbial systems is explored. The fifth section reviews what is known to-date about the effects of multiple bacterial species on contaminant transport. The final section briefly discusses cell surface hydrophobicity.

Subsequent chapters are organized in the following manner: Chapter 3 deals with organism and substrate selection and characterization experiments conducted in batch type systems; Chapters 4-8 examine those biodegradation studies conducted in saturated column systems. Appendix A contains data from replicate column experiments and Appendix B contains cell distribution data from the column systems.

I was responsible for designing and conducting all of the aforementioned experiments and analyzing their results. There is one exception: the identification of phenanthrene

degraders obtained from a soil microcosm (Wang, 1999) which was conducted by Adria Bodour (Ph.D. student).

Research Problem

Contamination of soils and ground water has received increasing national attention over the past few decades. A variety of physical-chemical remediation technologies such as pump-and-treat and soil venting have traditionally been used. In an effort to increase efficiency and cost-effectiveness, *in situ* bioremediation technologies are currently being investigated. Although bioremediation shows great promise, lack of information regarding *in situ* biodegradation processes, indigenous microbial populations, and effects on contaminant transport is prohibiting an accurate assessment of contaminant transport and in many cases the application of bioremediation technology. To date, laboratory studies have investigated bioremediation parameters in the context of small contaminant input pulses and singular, well-identified, microbial species (Wang, 1999).

Models which address the effects of biodegradation on contaminant transport have also been constructed. Almost all models focus either on the impact of one microbial species or treat the microbial community as a homogenous composite (ie one growth rate, one half-saturation constant, etc). Microbial communities as they actually exist in soil complicate basic biodegradation models because biodegradation thresholds, toxic or inhibitory responses, and multiple-substrate responses cause deviations from ideal behavior (Rubin et al., 1982; Kanazawa and Filip, 1986; Scholtz-Muramatsu et al., 1988; Kuhn et al., 1985; Maier, 1989). These complications result in studies that provide an inaccurate assessment of the ultimate fate of the contaminant as well as projected clean-up costs and clean-up times. The major objectives of this study were to: investigate how

rates of contaminant biodegradation, by indigenous soil microbes, vary with increasing community complexity, examine changes in bacterial cell elution with increasing community complexity, assess changes in bacterial distrubution, and investigate the effect of nutrient addition on biodegradation and cell elution in a complex community.

CHAPTER 2: LITERATURE REVIEW

Polycyclic Aromatic Hydrocarbons

Definition

A polycyclic aromatic hydrocarbon (PAH) is a solid phase organic chemical that contains two or more fused benzene rings. Petroleum products, such as gasoline and jet fuel, are complex mixtures of hundreds of organic compounds including PAHs. Such compounds, on a total mass basis, are currently the most prevalent environmental pollutant (American Petroleum Institute, 1992; Welch and Yando, 1993). PAHs are persistent in the environment (Devlin and Barker, 1994; Wilson et al., 1993; Angley et al., 1992) due to their recalcitrant nature (Gudnlach et al., 1983; Vandermedlen, 1981).

PAHs persist in the environment for several reasons including: low water solubility, strong hydrophobic character, low mass-transfer rates in the soil, and a tendency to sorb to soil organic matter. These properties make remediation difficult (Volkering, 1996). Several studies have found selected PAHs to be toxic, carcinogenic, and mutagenic (Keith, 1979; Jacob et al., 1984; White, 1986). As such, the United States Environmental Protection Agency classifies several PAHs as priority pollutants (Keith and Telliard, 1979).

Phenanthrene ($C_{14}H_{10}$)is a component of petroleum hydrocarbons and is also a polycyclic aromatic hydrocarbon (Birman and Alexander, 1996). Its is composed of three benzene rings fused together.

Figure 1.Structural representation of phenanthrene

Similar to other PAHs, phenanthrene is characterized by low bioavailability (Alexander, 1973, 1975; Weissenfels et al., 1992) and low water solubility (1.28 mg/l) (Dzombak and Luthy, 1984).

Bioavailability

Investigations of PAH degradation on those PAHs that contain more than 3 rings have been conducted by Heitkamp et al. (1988) Mahaffey et al. (1988), Weissenfels et al. (1991), Kelley et al. (1991), and Walter et al. (1991). Overall, PAHs are not considered to be readily bioavailable due to their tendency to sorb to the soil, form separate solid phases, and their low water solubility. Thus, slow mass transfer rates into the aqueous phase limits biodegradation rates (Zhang et al., 1997). Despite these findings, soil microbes have been shown to degrade PAHs (Cerniglia, 1992; Davies and Evans, 1964; Evans et al., 1965). Microbial degradation of soil PAHs is believed to be one of the major processes that aids in the decontamination of soils (Sims et al., 1990).

Three general mechanisms of substrate uptake have been proposed: 1. the substrate is utilized from the dissolved state (Wodzinski and Bertolilni, 1972; Wodzinski and Coyle, 1974 in Köhler; 1994), 2. the production of bio-detergents or metabolites dissolves solid substrates into the aqueous media (pseudosolubilization) (Chakravarty et al., 1975; Cameotra et al., 1983 in Köhler, 1994), 3. direct contact occurs at the solid-material interface, whereby the substrate may transfer into the cell envelope through adsorption-desorption (interfacial adsorption) (Zilber et al., 1980; Goswami et al., 1983). It is believed that the first two mechanisms are the most probable (Maier et al., 2000).

Strictly speaking, bacterial degradation of a PAH would not be dependant on the amount of solid phase PAH present, but rather, would depend on the amount of dissolved solutes and the rate at which they move into the bulk solution (Woodzinski and Bertolini, 1972; Woodzinski and Coyle, 1974; Stucki and Alexander, 1987). Studies conducted by Wodzinski and Coyle (1974) do in fact show that phenanthrene up take is restricted by low solubilization rates.

Biodegradation Kinetics

Growth Kinetics

A great deal of interest exists in regard to bacterial growth kinetics and the appropriate manner in which to describe them. In general, the logarithmic or exponential growth phase of the microbial growth curve has been most widely studied. The exponential growth phase describes the phase in which bacterial numbers increase exponentially with time. This phase may occur when a small number of bacterial cells is introduced into an aqueous solution that contains a finite amount of nutrients and carbon source. The relationship between the increase in biomass over time (dB/dt) and the biomass concentration (B) may be mathematically described as follows:

$$\frac{dB}{dt}=\mu B \qquad [1]$$

This is a first order equation. The growth rate per unit of biomass, μ, is also termed the specific growth rate and carries units of time $^{-1}$. The specific growth rate is constant for a given carbon source, at a given concentration, and for a given species; however, it may vary if any of the aforementioned parameters, or other parameters such as temperature or pH, changes. A relationship between the rate of substrate utilization (dS/dt) and the rate of biomass growth (dB/dt) can be established by introducing the yield coefficient (Y). The yield coefficient is defined as the mass of cells produced per amount of substrate

consumed over a fixed period of time (Gaudy and Gaudy, 1980). Incorporating the yield coefficient allows the following mathematical relationship:

$$\frac{dB}{dt} = -Y\frac{dS}{dt} \qquad [2]$$

The negative sign indicates that the growth-limiting substrate concentration is declining over time. From this equation, the rate of substrate utilization can be expressed as a function of the biomass concentration (B), specific growth rate (μ), and yield coefficient (Y):

$$-\frac{dS}{dt} = \frac{\mu}{Y}B \qquad [3]$$

Monod Equation

Several biotransformation models describing biodegradation kinetics have been created. One of the most well-known and widely used models is that developed by Monod (1949). The Monod equation was developed for batch system use. It describes microbial growth kinetics in a well-controlled system where substrate, oxygen, and nutrients are not limiting. In addition, several assumptions are inherent to this model. The first assumption is that the system contains a single, pure culture. As well, the system must be well agitated, contain a water soluble, non-toxic carbon/energy source, and the metabolite being used should not be harmful to the degrading species. When these

assumptions are met, bacterial growth rate is proportional to substrate concentration. A decrease in substrate concentration produces a decrease in the bacterial growth rate, while increases in concentration produce an increase in the bacterial growth rate. Consequently, the bacterial growth rate is concentration specific and restricted by the concentration of the growth-limiting substrate. Monod developed an empirical mathematical expression to describe these relationships. This expression is written in the following manner:

$$\mu = \frac{\mu_{max} S}{K_s + S} \qquad [4]$$

where

μ = specific growth rate of biomass, [T^{-1}];

μ_{max} = maximum specific growth rate of biomass, [T^{-1}];

S= substrate concentration, [ML^{-3}];

K_s = the half-saturation constant, [ML^{-3}];

This equation [4], describes the biomass growth in response to the amount of substrate available. Mathematically, this expression is a hyperbolic function where increases in substrate (S) show μ asymptotically approaching μ_{max}. The Monod equation sets time "zero" at the end of the lag phase/beginning of the exponential phase (Kelly et al., 1996). As such, the Monod equation describes only the exponential growth phase. It does not describe the lag phase, in which bacteria are presumed to be acclimating but not

degrading, stationary phase, in which no net bacterial growth occurs, and the death phase, in which bacterial numbers decline.

A differential equation which describes substrate loss and microbial growth can be obtained by substituting for μ in equation [3]. The resulting equation:

$$-\frac{ds}{dt} = \frac{\mu_{max}}{Y}\frac{(S)}{(K_s + S)}B \qquad [5]$$

is valid for a liquid batch system that is maintained at a constant temperature, and contains a single limiting substrate and a pure culture microorganism. As the substrate concentration (S) increases, the rate of substrate utilization also increases. Eventually, the rate of substrate utilization will asymptotically approach a maximum value (μ_{max}/Y)B. As shown by this value, the fastest possible substrate utilization rate is a function of the maximum specific growth rate, the yield coefficient, and the biomass concentration. The relative values of S and K_s control the asymptotic approach.

Equation [5] is key, as it allows the dS/dt term to be substituted for the mass loss term in the advection-dispersion transport equation. This allows for the coupling of biodegradation and transport processes.

It is possible to rearrange the Monod equation a manner such that a plot of $1/\mu$ versus 1/S yields values for μ_{max} and K_s. This is done by taking the reciprocal of both sides of the Monod equation to give a linear formula:

$$\frac{1}{\mu}=\left(\frac{K_s}{\mu_{max}}\right)\frac{1}{S}+\frac{1}{\mu_{max}} \qquad [F]$$

When a plot of $1/\mu$ versus $1/S$ is performed, a straight line is created. The ordinate intercept ($1/\mu_{max}$) allows the calculation of μ_{max}. A regression line slope (K_s / μ_{max}) provides a value for K_s.

Half-Saturation Constant

The half-saturation constant, K_s, is one of several important parameters controlling biodegradation rates. The half-saturation constant, also known as the affinity constant, defines the substrate concentration, S, when μ is equal to $\mu_{max} / 2$ (Alexander, 1994). It carries the same units as substrate concentration [ML^{-3}]. In practical terms, the half-saturation constant may be used as an index in determining microbial population capacity for growth. A large K_s indicates a low biomass growth rate and a low substrate utilization rate (Brusseau et al., 1999). A lower value of K_s implies that the microorganism has a greater ability to grow in an environment with a low substrate concentration (Lynch and Poole, 1979). However, it must be noted that K_s values are species and substrate specific. For example, *B. ruminicola* is known to have a different K_s value for glucose, maltose, sucrose, and cellobiose. And *B. fibrisolvens* and *S. bovis* have different affinities for glucose (Russell and Baldwin, 1979). In addition, pure culture and multi-species microbial communities also exhibit different (van der Kooij and Hijenen, 1981; Larson, 1980), and in some cases concentration-dependent (Ishida et al., 1982), K_s values.

Modified Monod Equation

Two categories, with 6 variations (Alexander, 1994) of Monod kinetics have been proposed. All 6 variations (Monod-no-growth, zero-order, first-order, Monod-with-growth, logarithmic, and logistic) are based on a mass balance of carbon. That is, equations [1-5] assume that substrate disappearance is directly correlated to an increase in the number of cells; bacterial decay is ignored (Simkins and Alexander, 1984).

The Monod-no-growth model is used under conditions where the initial concentration of bacterial cells in the system is high. Thus, it is assumed that there is no growth in the system because there is a threshold or because the initial number of cells in the system is too large to permit growth on the existing substrate. In this situation, the biodegradation rate remains constant as there are no new cells available to influence the rate. The Monod-no-growth model, based on equation [5] can be expressed in the following manner:

$$\frac{-ds}{dt} = \frac{u\,max}{y}\left(\frac{S}{Ks+S}\right)Bo \qquad [7]$$

where

B_o = initial bacterial cell concentration , $S\text{-}S_o+B$

S_o = initial substrate (carbon) concentration

The Monod-with-growth model is the form of the Monod model that is most commonly used as growth within the system would yield a change in the biodegradation rate. It is valid for systems that initially contain a small biodegrading population relative to a large substrate concentration. That is to say, the amount of existing substrate is above any growth threshold that might exist. In this system, bacteria will continue to grow, but the growth rate will decrease accordingly as the substrate diminishes. The model can be rearranged to form a logistic kinetic ($S<<K_s$) model. To do this, equation [5] is reduced to the following first-order equation:

$$\frac{dS}{dt}=\frac{\mu_{max}}{Y}\frac{B}{K_s}S=-k_1S \qquad [8]$$

This equation is for biomass under steady-state conditions. Equation [7] can be integrated to form:

$$lnS=-k_1t+\ln S_o \qquad [9]$$

where k_1 is the first-order rate constant [T^{-1}]. The value for k_1 is the slope of the best fit line, on a plot of the natural log of the substrate concentration versus time. In the case where S is greater than K_s ($S>>K_s$), the following zero-order equation may be obtained from equation [5]:

$$\frac{dS}{dt}=-(\frac{\mu_{max}}{Y}\frac{B}{1})=-k_o \qquad [10]$$

Equation [10] can be integrated as well, to form

$$S=-k_o t+S_o \qquad [11]$$

where k_o is the zero-order rate constant with units [T^{-1}]. Here as well, k_o is the slope of the best fit line on a plot of substrate concentration versus time.

Limitations and Applications of Monod Kinetic Model

The Monod kinetic model is an empirical model, derived from experimental settings that were well controlled (i.e. no nutrient or electron acceptor limitations, pure culture, single substrate). Therefore, difficulty may be experienced when applying this model to more complex situations, such as those that contain multiple substrates, limiting nutrients or electron acceptors, and multiple species. Attempts to use the model to describe biodegradation in soil systems that contain sorbing substrates or low concentrations of substrates were not successful (Scow et al., 1986). Use of the model in situations where the substrate is toxic or insoluble or under varying experimental designs has also proven unsuccessful (Koch and Wang, 1982; Alexander, 1994). However, the successful application of this model to mixed cultures (Alexander and Scow, 1989), activated sludge reactors (Shamat and Maier, 1980), and continuous-flow bioreactor systems (Gaudy and Gaudy, 1980) has been demonstrated.

Due to some of the previously mentioned limitations, several modifications to the Monod model have been made and have produced successful results. Yoon et al. (1977)

and Papanastasiou and Maier (1982) successfully modified the model for use in competitive inhibition batch systems as well as for use in continuous flow, stirred tank reactor (CSTR) systems (Papanastasiou and Maier, 1983). Klečka and Maier (1988) included the effect of endogenous decay of cells in the Monod model.

Monod kinetics have been incorporated into solute transport models in an attempt to couple biodegradation processes with solute transport. Theoretically, this would allow models to better predict the mass loss of contaminant (substrate) that is due to biodegradation and thus better predict subsurface contaminant transport (Ardakani et al, 1973; Sykes et al., 1982; Corapcioglu and Haridas, 1985; Borden and Bedient, 1986; Molz et al, 1986; Bouwer and Cobb, 1987; Srinivasan and Mercer, 1988; Bosma et al., 1988; Kindred and Celia, 1989; MacQuarrie et al, 1990; Chen et al., 1992). However, limited success has been achieved matching modeling results with experimental data (Estrella et al., 1993; Maier et al., 1996; Hu and Brusseau, 1998). Through reviews of coupled-transport processes may be found in Brusseau et al. (1992) and Xie (1996). Systematic evaluations of the effects of μ_{max} and K_s on solute transport in soil columns may be found in Brusseau et al., (1999a; 1999b).

Effects on Contaminant Transport

Biodegradation is an extremely important process in the subsurface that must be considered if an accurate estimate of contaminant transport and fate is to be made (Budzinski et al., 1998; Cullen et al., 1994). Both McCary et al. (1981) and Borden and Bedient (1987) found that biodegradation a major, if not the most important, subsurface process taking place. As such, biodegradation has the potential to greatly influence contaminant transport (Dobbins et al., 1992). The effects of biodegradation on contaminant transport are noticeable for both steady state and non-steady state conditions. Brusseau et al. (1999a, 1999b) conducted a series of simulations to determine when it is appropriate to use first-order kinetics to describe biodegradation and when it is more appropriate to use zero- order Monod kinetics.

Zero-order biodegradation occurs in a system which contains sufficient substrate, electron acceptors, and nutrients. In this case, the nonlinear Monod equation may be used to describe the biodegradation process previously explained in this literature review. In zero-order systems, S>> Ks, therefore, microbial biomass is increasing as is the biodegradation rate (Alexander and Scow, 1989). In this case, the contaminant "break through curve" (BTC) exhibits a bell shaped curve, that may or may not be skewed, and C/C_o may eventually reach zero (Wang, 1999). Both Chen et al. (1992) and Estrella et al. (1993) have observed non-steady state behavior.

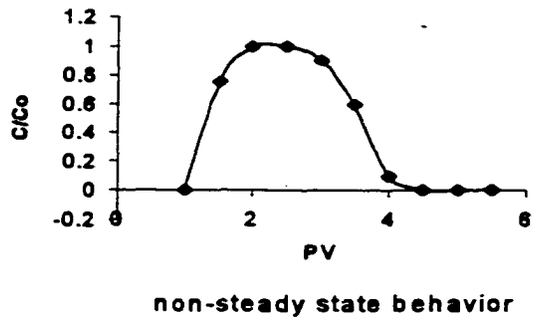

Figure 1.1. Non-steady state biodegradation. Co is influent concentration, C is the effluent concentration, PV (pore volume) is non-dimensional time.

Subsurface biodegradation usually takes place at the first-order kinetic level (Dobbins, 1990; Swindoll et al., 1988). Linear, first-order degradation occurs in a system when S << Ks (i.e. limited substrate concentration), or there is a lack of desired nutrients, or a limited electron acceptor concentration. In this case, there is steady-state degradation (Brusseau et al., 1999) and there is no net cell growth (Alexander and Scow, 1989). First-order systems exhibit steady state transport behavior, meaning that the effluent concentration plateaus at a concentration that is less than the influent concentration (C/C_o <1) (Brusseau et al., 1999; Wang, 1999). The kinetics of first-order biodegradation have been observed for various polycyclic aromatic hydrocarbons (PAHs) and environmental contaminants such as benzene, toluene, and xylene (Angely et al., 1992) and for 2,4-dicholorphenoxyacetic acid (2,4-D) (Hu and Brusseau, 1998).

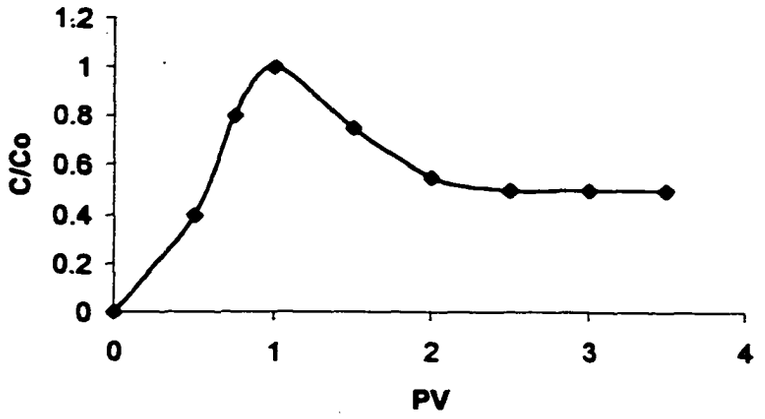

Figure 1.2. First-order (steady-state) biodegradation curve.

Community Structure

Long-term sustainability and system health are dependant on healthy soil biota function and community structure. Soil biota functions include recycling of nutrients, soil structure maintenance, biological control of pests, and degradation of chemicals (Parkinson and Coleman, 1991; Lee and Pankhurst, 1992). Microbial degradation processes play a key role in reducing the dissolved concentration of many chemical contaminants (Barker et al., 1987). Major factors influencing the magnitude and rate of biodegradation in a system, are the properties of the microbial population (Brusseau et al., 1999), and thus the structure of the microbial community. If microbial processes are to be harnessed for bioremediation, an increased understanding of microbial community structures, metabolic activities, and factors controlling microbial distrubution is critical (Fand and Barcelona, 1998). The following subsections will examine microbial community structure and its importance in a

contaminated system.

Biodiversity

Microbial biodiversity is a measure of the range of significantly different kinds of organisms found within the habitat or community of interest (Atlas, 1984). The degree of diversity found in a microbial community is related to the environment and the environment's heterogeneity and complexity (Dobbins et al., 1992). Bacteria, by nature, are not specialists. They consume combinations of substrates depending upon physico-chemical conditions (Brack and Madigan, 1988; Paul and Clark, 1989). Even so, many contaminants are not readily degradable by microbes. This may occur for several reasons including the lack of microbial enzymes necessary to degrade the compound (Dobbins et al., 1992). However, as illustrated by Wiggins et al. (1987) and Spain et al. (1980) it is possible for microbes to adapt, develop, or acquire the ability to degrade a specific contaminant. With this in mind, it is important for a microbial community to be biologically diverse. A biologically diverse community provides the potential for microbes to acquire degradation abilities via induction/repression of necessary enzymes, genetic transfer, genetic mutation, co-metabolism, and microbial competition (Heitkamp and Cerniglia, 1988; Madsen et al., 1992; Cerniglia, 1992). Likewise, Mary (1974), Tilman (1996), and Naeem and Li (1997) have proposed that increases in variability among species may actually lead to a stabilization of total community biomass or other total ecosystem functions and properties.

How is it possible for soil microbial biodiversity to be so high even though competition theory predicts that the number of limiting resources will dictate the number of coexisting species (Hardin, 1960; Phillips, 1973; Armstrong and McGehee, 1980; Grover, 1997)? This case is similar to that of the plankton that are found in the world's oceans. Plankton diversity is incredibly high (Hutchinson, 1961) despite a low number of limiting resources (nitrogen, phosphorus, silicon, iron, light, inorganic carbon and possibly vitamins or trace metals) (Hutsman and Welssings; 1999). Many have used the plankton system in resource competition models (Grover, 1997; Leon, 1975; Tilman, 1977; Hsu, 1981; Huisman and Weising, 1994). Hutsman and Welssings (1999) demonstrated that plankton population oscillations and associated chaotic behavior may be the result of species competition for three or more resources and that such oscillations in populations may be the reason that plankton biodiversity is so high. In addition, Hutsman and Welssings (1999) state that the processes examined in these models occur in other systems. As such, the idea of population oscillation and chaos may be applicable to the soil microbiological realm, where continual disturbance prevents attainment of equilibrium conditions and allows greater biodiversity than would otherwise be found (Armstrong, 1990; Sommer, 1985; Levins, 1979).

Functional versus Taxonomic Diversity

After knowledge regarding system biodiversity is obtained, one may begin to assess other aspects of a microbial population (Zak et al., 1994; Garland and Mills, 1994). The

most common community aspects examined are functional diversity and genetic (taxonomic) diversity. Functional and genetic diversity are both important factors in defining ecosystem processes (Ovreas and Torsvik, 1998).

Genetic diversity is a measure of DNA heterogeneity. It represents the total diversity of the soil microbial community including viable but non-culturable organisms. Such non-culturable organisms are thought to be K-selected organisms that are highly selected to grow on low concentrations of specific substrates (Atlas and Bartha, 1993).

Though genetic diversity provides a complete characterization of a system, functional (metabolic) diversity may be a better indicator of system change (Ovreas and Torsvik, 1998). Functional diversity is the range of significantly different functional activities found within a system (Walker, 1992). Diversity measured in this manner can provide insight into the significant changes in community structure and function that occur in a contaminated system (Thompson et al., 1999). The understanding of functional diversity is important in understanding the long-term stability of the ecosystem (Walker, 1992), and thus the continued ability of the system to perform biodegradation activities.

Soil Heterogeneity

Soil heterogeneity also plays a roll in determining community structure. Many microbial habitats exist, i.e. marine, terrestrial, aerial, etc.. Of these habitats, the terrestrial, soil environment is thought to be the most physically and chemically complex (Stotzky, 1972). This is due to the enormous number of micro-habitats that exist in a soil matrix.

Adjacent micro-habitats may vary greatly with respect to water content, oxygen availability, and pH. Micro-habitats allow for increased microbial diversity. In addition, the microbes existing in a particular habitat may change the habitat conditions over time (Dobbins et al., 1992). Single, localized soil sites may be quite heterogenous in nature with respect to physical/chemical characteristics as well as with respect to microbial activity. Dobbins (1990) found that single-site biodegradation rates may vary by as much as two orders of magnitude.

Subsurface soils were thought to be sterile or sparsely populated prior to the 1970's (Dockins et al., 1980; Dunlap and McNabb, 1973; Whitelaw and Edwards, 1980). Though recent studies have shown this to be untrue, it still appears that subsurface microbes generally have a lower activity level than do those in surface soils (Maier et al., 2000; Ward, 1985). Federle et al. (1986) showed that increasing soil depth leads to a decline in microbial activity and biomass; however, changes in microbial diversity were not examined. The study also showed that edaphic soil factors (chemical and physical) explained 88.6% of the microbial biomass variation, and 82.4% of the microbial activity variation. Based on these findings, Federle et al. (1986) believes that soil type may well determine the potential biodegradability of pollutants in the unsaturated zone.

In addition, soil porosity has been shown to influence both the establishment of microbial niches and contaminant flow patterns (du Plessis et al., 1998). Models which do not account for variations in soil porosity may not accurately represent contaminant migration, thus, inaccurately simulating microbial growth patterns (du Plessis et al., 1998).

Micropores may successfully protect microorganisms from high concentrations of toxic compounds and may increase microbial adhesion and biomass formation (du Plessis et al., 1998).

Effects of Contamination

Soil microbial communities are composed of many taxa, with ranging sensitivities to contaminant exposure (Welp and Brummer, 1997). Due to the important role that soil microbes play in nutrient recycling, soil health, and biodegradation processes, it is critical that microbe response to contaminant exposure be understood (Thompson et al., 1999). Both contaminated and uncontaminated sites have been screened for the presence of microbial populations. Results indicate that both types of sites contain sizable, actively metabolizing, microbial communities (Long and Aelion, 1999). Communities have shown the ability to adapt to repeated contaminant exposure (Atlas, 1981); therefore, microbes taken from a contaminated site would be expected to posses the ability to degrade contaminants found at that site (Budzinski et al., 1998). Indigenous microorganisms that are adapted to living in a polluted environment may have K_s values that are substantially lower than previously thought (e.g. $K_s < 1$mg/l) due to multi-substrate interactions or physiological adaptions (Rozkov et al., 1998).

There are several schools of thought in regard to changes that occur in a microbial community after contamination has occurred. Odum (1985) found that microbial communities are resilient; that is, they are able to rebound to their initial numbers and

community structure after a severe disturbance. Though microbial numbers appear to remain constant, it appears the overall community structure may change when exposed to a contaminant. For example, microbial growth and species richness were found to be reduced in systems contaminated with dichloroethene (Fries et al., 1997). And communities contaminated with oils have been shown to have significantly more gram-negative bacteria than oil- free samples (MacNaughton, 1999).

It appears that a single contaminant incident may cause very little change in community structure, while multiple contaminant incidents may cause significant changes in structure. A number of studies indicate that this is due to the diffusion and sequestration of the contaminant that occurs within the soil. In this scenario, the toxic effects of the first contaminant incident are ameliorated as the contaminant diffuses and sequesters into spatially-remote soil micro pores, causing the contaminant to be less bioavailable, and thus, less toxic. A subsequent incident would be increasingly toxic to the system as micro pores would already be filled, and the contaminant would be more bioavailable (Alexander, 1995; Hatzinger and Alexander, 1995).

Multiple applications of an organic pollutant have been shown to shift the microbial community away from opportunistic cells (ex. *Pseudamonas*) and move the community toward ***Bacillus*** cells (Thompson et al., 1999b) which are capable of handling more stressful environmental situations by producing resistant endospores (Sneath, 1986). In addition, Thompson et al. (1999b) showed that multiple exposure to contaminants causes a reduction in the metabolic potential of a community, possibly due to the loss of the

Pseudamonas sp. component. *Pseudamonas* appear to be one of the taxa that is sensitive to contaminant events (Thompson et al., 1999b).

Competition

Competition is the interaction of two organisms that are striving for the same thing (e.g. nutrients, light, space, etc.). In general, when two strains of bacteria are in competition for a resource, it appears that the strain that leaves the lowest residual substrate concentration will out compete the other strain a majority of the time (Duetz et al., 1994). An additional factor that also comes into play is which strain has the highest specific growth rate at low substrate concentrations (Harder et al., 1997). In addition, Steffensen and Alexander (1995) found competition for inorganic nutrients may slow degradation; however, addition of nutrients does not necessarily increase degradation. And the presence of additional bacterial species may also slow substrate degradation.

Exploitation vs. Interference

Two major types of competition have been identified: exploitation competition and interference competition. Exploitation competition is indirect competition through mutual exploitation of the same resource where the organisms do not necessarily come into contact (Atlas and Bartha, 1993). A prime example of exploitation competition among microbes can be found in work conducted by Steffensen and Alexander (1995). The

authors found that benzylamine was degraded by *P. putida* in the presence of phosphorous (100nM), but if *P. aeruginosa* was also present, degradation did not occur. The exception appeared to be if the concentration of phosphorous was increased. Based on these and other results, it was suggested that the competition for inorganic nutrients, phosphorous in this case, inhibited *P. putida* growth and therefore, biodegradation. While *P. aeruginosa* did not predate or directly harm *P. putida*, the presence of *P. aeruginosa* proved detrimental to the *P. putida* population or the population's ability to degrade benzylamine by exploiting the mutually desired phosphorous resource.

Interference competition is that in which one organism directly harms another organism through actions such as predation, secretion of toxic or inhibitory enzymes, etc. In many cases, interference competition replaces exploitation competition when resources become scarce (Atlas and Bartha, 1993). It is generally accepted that bacteria may secrete enzymes or other substances that are toxic or inhibitory to other microbes; however, evidence of bacterial predation has yet to be put forth (Maier et al., 2000). To date, very few studies have been conducted to investigate bacterial-bacterial interference. However, cases of protozoan-bacterial and fungal-bacterial interference have been documented. Many fungi produce anti-bacterial substances that slow or inhibit bacterial growth (Maier et al., 2000). Recently, Hahn and Höfle (1999) investigated protozoa-bacteria interference. The size and morphology of cells in a mixed bacterial community were found to be noticeably different after the community had undergone predation by *Ochromonas sp.* strain DS. *Ochromonas sp.* predation interferes with bacterial community structure by

reducing or eliminating bacteria of a specific size that compete with *Ochromonas sp.*. The bacteria of the intermediate size range, though able to compete in the absence of *Ochromonas sp.* strain DS, were unable to compete in the presence of the strain due to selective predation and interference competition. Field studies examining *in situ* microbial degradation of TCE by methanotrophs concluded that the oscillations in the soil methanotroph population were due to predation by protozoa, and also possibly by competition from other microbial populations (Travis and Rosenberg, 1997).

Competition has been shown to cause changes in bacterial diversity as well as in bacterial distribution and biomass density. Noguera et al. (1999) developed a mathematical model designed to simulate competition for substrate and space. Three conditions were tested: D. vulgaris grown alone, in association with M. formicium, and in the presence of M. formicium and sulfur. Results showed biomass densities of D. vulgaris to differ under each of the conditions imposed, indicating that type and degree of competition are important influences in bacterial distribution.

Recently, Lewandowski et al. (1998) showed that simple competition may not always be the only interaction between two bacterial species. The studies conducted in pure culture and with multiple species concluded that while a simple competition model is capable of predicting biomass growth, it may not be able to match experimental substrate utilization data indicating that additional interactions (such as inhibition, secondary metabolite utilization, and synergism) may take place in a multi-species system.

Lotka-Voltera Equations

Interspecific competition, that is competition between different species, can be described using the Lotka-Volterra equations (Lotka, 1932; Volterra, 1926). The Lotka-Volterra equations are actually extensions of the logistic equation [8]. Several terms are added to the logistic equation to account for competitive interactions. The following equations describe the population dynamics of a two-species interaction:

$$\frac{dN_1}{dt} = r_1 N_1 \frac{(K_1 - N_1 - \alpha N_2)}{K_1} \qquad [12]$$

$$\frac{dN_2}{dt} = r_2 N_2 \frac{K_2 - N_2 - \beta N_1}{K_2} \qquad [13]$$

where:

K= equilibrium level

N_1,N_2= number of species 1, 2 respectively

α=competition coefficient explaining the inhibitory effects of species 2 on species 1

β= competition coefficient explaining the inhibition of species 2 by species 1

Equation [12] describes how the growth of species one (N_1) is affected by interaction with species two (N_2). Equation [13] describes the growth of species two as affected by species one.

While the Lotka-Volterra equations are helpful in determining conditions under which coexistence may occur, they are not valid in all cases. Ayala (1970) conducted a series of laboratory experiments using *Drosophila pseudoobscura* and *D. serrata* (fruit flies). After applying the equations to the obtained experimental data, Ayala (1970) found a poor match. The final conclusion was that the detail of the Lotak-Volterra equations was not sufficient to describe the system studied. This lack of detail may be found in the competition coefficients (α and β) which remain constant. Constant competition coefficients create a model where competition is density independent. This assumption, as illustrated by Ayala (1970), may invalidate the use of the Lotka-Volterra model for many systems (Ricklefs, 1973).

Soil

Soil type is also believed to have an effect on competition, as the size of the soil granules and pore spaces may increase the number of micro niches. Micro niches may reduce predation of one species upon another, increase the availability of substrate and /or nutrients, or modify soil conditions to a point at which cellular physiology is also modified (van Elsas 1991).

Genetically Engineered Microbes

Of special interest in the field of bioremediation is the ability, or lack there of, of GEMs (genetically engineered microbes) to compete with indigenous soil populations. When placed in competition with indigenous microbes, GEMs generally show a decline in population numbers. The rate of decline may be a function of soil type (Zechman and Casida, 1982; van Elsas et al., 1986; Compeau et al., 1988; Dupler and Baker 1984). Several factors are believed to be involved in the competitive disadvantage of GEMs. The obvious genetic factors include, additional proteins required for degradation lead to energy deviation, disruption of cellular function by the introduction of additional proteins, or possibly that genes needed for survival are disrupted by the extra protein insertion (van Elsas et al., 1991). As well, there are a series of abiotic and biotic factors that may also affect GEM competition among soil populations. Biotic factors known to affect the survival of introduced organisms include antagonism, competition, and predation; abiotic factors include soil moisture levels (Dupler and Baker, 1984: Postma et al., 1989), soil temperature (Wessendof and Lingens, 1989) soil nutrients (van Elsas et al., 1991;Wessendof and Lingens, 1989), and soil texture (van Elsas et al., 1991;Wessendof and Lingens, 1989; van Elsas et al., 1989; van Elsas et al., 1990).

Multiple Species

Multiple species substrate use is an area that, until recently, has not received much attention. Previous studies, such as those performed by Lewandowski et al. (1998) use batch systems and focus on sewage treatment plant effluent. In an effort to better understand how a microbial community interacts with a substrate, Lewandowski et al. (1998) designed a model to be used with a 2-species community. Accompanying experiments using indigenous phenol degraders from a sewage treatment plant provided the data to be modeled. Though the authors were able to model changes in system biomass, they were unable to accurately model phenol degradation due to other factors (such as competition, inhibition, and toxicity) which were not represented in the model.

Rittmann and Manem have recently (1990) developed a multi species biofilm model that allows multiple species to coexist in the face of competition. This is due to competition for more than one resource, in this case substrate and space. The two most probable scenarios consist of the faster growing species occupying the top portion of the biofilm, close to the solution surface, while the second species, which is able to grow on a lower concentration of substrate, is found deeper in the biofilm. This same distrubution may also occur if the affinity for the substrates is the same, but one species grows faster than the other. Here, Rittmann and Manem suggest that one species would only be found deep in the biofilm as it would not be able to out compete the faster growing species unless the substrate supply was limiting. Even though the two species are in competition,

the presence of the faster growing species may be beneficial for the slower growing species as the faster growing species may help to protect the slower growing species.

This model is supported by Sturman et al. (1994). Laboratory experiments used *Pseudomonas aeruginosa* and *Klebsiella pneumoniae* as model organisms in a colonization, competition investigation. *P. aeruginosa* is characterized as a motile species, and obligate aerobe with a umax of 0.4/h, while *K. pneumoniae* is a non-motile species, a facultative anaerobe, and has a growth rate 5 times greater (umax = 2.0/h) than *P. aeruginosa*. *K. pneumoniae*, when introduced into a system that was already inhabited by *P. aeruginosa* was able to out compete the *P. aeruginosa*. An increase in *P. aeruginosa* numbers was prohibited, and a majority of the colonies were found in the center of the small spherical beads used as the solid growth stratum. However, *P. aeruginosa* was unable to out compete a previously established *K. pneumoniae* population. It was concluded the *K. pneumoniae* was able to out compete *P. aeruginosa* due to its faster growth rate and increased affinity for the substrate.

Recently, a long-term study conducted by Wang (1999) investigated multiple indigenous microbial species effects on phenanthrene degradation in a soil column system. Where a single microbial species generally produces a mono-modal substrate utilization curve, cyclic patterns of phenanthrene degradation were observed by Wang (1999). The patterns were attributed to shifts in the "dominant" phenanthrene degraders in the column. This was not unexpected as multiple microbial populations exhibit a variety of behaviors such as competition, antagonism, synergism, utilization of secondary metabolites, or

inhibition (Lewandowski et al., 1998). Such interactions create a dynamic community where population dominance, growth rates, and substrate utilization rates may change over time (Wang, 1999). As such, it would not be unexpected for a microbial population to produce a bi- or even tri- modal substrate utilization curve. However, such phenomena are generally not accounted for in mathematical biodegradation models as degradation by multiple microbial species is not well understood.

As demonstrated by recent studies, the effects of multiple microbial species and microbial succession on contaminant transport, bacterial cell elution, and species distribution are a field that merits further investigation.

Cell Surface Hydrophobicity

How bacteria are transported through the subsurface and their ultimate fate is a topic of interest to a variety of researchers in diverse industries including: oil recovery (Jang et al, 1989), bioremediation (Harms and Zehnder, 1994; Wilson et al., 1986; Reynolds et al., 1989), and public health and safety (Gerba, 1985). Microbial transport is influenced by many factors that, unfortunately, are not well understood (Yates and Yates; 1988; Harvey, 1989). Recently, cell adhesion has been shown to influence transport behavior (van Schie and Fletcher, 1999). But what influences cell adhesion?

Cell adhesion is predominantly influenced by cell surface hydrophobicity (van Loosdrecht et al., 1987a; Absolom et al., 1983; Busscher et al., 1984). However, conclusions regarding cell surface hydrophobicity research are varied (Albinger et al.,

1994; Baygents et al., 1998; Martin et al., 1996; Simoni et al., 1998). In addition, it has been suggested that the type of substrate consumed by the organism may also influence cell surface hydrophobicity (Lawrence and Hendry, 1996; McEldowney and Fletcher, 1986).

CHAPTER 3: MATERIALS AND METHODS

This chapter is divided into 5 sections: 1. Organism and Substrate Selection, 2. Determination of Yield in a Batch System, 3. Substrate Utilization in a Batch System, 4. Determination of Yield by Plate Count, and 5. Bath Assay Study.

Organism and Substrate Selection

The purpose of this study was to select: 1. a model substrate or pair of substrates, 2. three bacterial species, to be used in further investigations.

Five possible substrates were investigated. A series of paired substrate combinations were examined. Each substrate pair contained a substrate likely to sorb to a porous matrix, and a "minimally" sorbing substrate. The minimally sorbing substrate was also found in the biodegradation pathway of the sorbing substrate.

Previous investigations (Wang, 1999) isolated and identified approximately 120 indigenous microorganisms from a 20/80 mix of Mount Lemmon/Mexican soil, including 24 phenanthrene degrading species (150 isolates, 24 species). Three organisms, from the Wang (1999) study, were selected based on the following criteria, all three organisms: must be of different color when plated so as to be distinguished from one another, must have been present during different time periods as designated in Wang (1999) (3-day colony, 14-day colony, column breakdown colony), have different observed growth rates, and must be capable of growing on a common substrate.

Organism selection

Each of the 120 previously mentioned isolates was reviewed. Bacillus and other spore forming species were eliminated as the formation of spores would complicate the enumeration process. Three groups of organisms were then selected. Group A (Table 3.0) organisms are white, were observed to be fast growing, have been found throughout the effluent and column in previous experiments, and have been characterized as 3-day colonies or breakdown colonies (Wang, 1999). Group D (Table 3.0) organisms were found during the middle time period, were characterized as 14-day colonies, and are unmistakably yellow in color. Finally, Group 5 (Table 3.0) was selected as these organisms are pink in color and were found after 8,000 pore volumes in the previously mentioned experiment, and were characterized as a 14-day colony. Organisms from these three groups where then screened on several media types, as described below, and one isolate from each group was selected to be used in further investigations, based on the previously mentioned criteria.

Substrate selection

Growth on each substrate was determined in the following manner. Two hundred-fifty ml Erlenmeyer flasks were sterilized via autoclave (25 minutes) and filled with 25 ml of solution containing the substrate being investigated. Flasks were inoculated using colonies obtained from R_2A plates and placed on a rotary shaker at 200 rpm. After 3 days, bacterial samples were taken from each flask, serially diluted, and plated on media

corresponding to the substrate found in the flask as well as on R_2A plates (ie, bacterial samples from a flask that contains salicylate would be plated on salicylate plates as well as on R_2A plates). Plates were incubated and examined after 3, 7, 10, and 14 days. A bacterial species were considered to grow on a specific substrate if bacterial colonies were present after 14 days of incubation.

Results and Discussion

A total of 3 bacterial species were selected for further study. From Group A, #3, *Acinetobacter junii*, a white bacteria was chosen; Group D, *#M11, Pseudomonas oleovorans*, a yellow bacteria, and from Group 5, # 35, *Methylobacterium sp.*, a dark pink bacteria. These 3 species fulfilled the previously mentioned criteria in that they were capable of phenanthrene degradation, were morphologically different on R_2A and phenanthrene plates, had different observed dates of appearance on phenanthrene plates, and were present during different time periods in experiments conducted by Wang (2000). In addition, all three bacterial species are gram negative and rod shaped. The definitive size of each species was found to be similar (0.2 um to 2 um in diameter, 2 to 8 um in length). In addition, each species occurs in a semi-starvation state when phenanthrene is provided as the sole carbon source. This semi-starved state causes the cells to become rounded and facilitates their transport through porous media. As the porous media to be used in future experiments is a large grained silica sand there should be no transport restrictions due to size. Phenanthrene degradation is commonly plasmid borne as opposed

to chromosomally borne (Maier et al., 2000). The literature is replete with examples of phenanthrene degradation by *Pseudomonas sp.*. However, evidence of *Methylobacterium sp.* and *Actinobacter junii* degradation was not found.

Substrate Selection

Attempts to find sorbing/non-sorbing substrate pairs did not succeed. Growth on all substrates tested, with the exception of phenanthrene, was found to be negligible or non-existent (Table 3.1). Therefore, phenanthrene will be used in all further studies.

Table 3.0 Classification of isolates, Wang (1999).

Group A	Group A	Group B	Group C	Group D	Group E	Group F	Group G	Group H	Group I	Group J	Group K
M1	26	70	M22	M11	78	M8	M20	M17	72	M18	M19
M14	27		M23	M12	79	M9	M21	64	73	16	40
1	28		M24	M13	80	M15		65	74	17	77a
2	29					50		66	75	91	92
3	30					51		37			97
4	31					52a		68			98
5	32					52b		69			99
6	33							77b			
7	34							82			
8	37							86			
9	39							85			
10	41							86			
11	42							87			
12											
13	Group L	Group M	Group N	Group O	Group P	Group Q	Group 1	Group 2	Group 3	Group 4	Group 5
14	53	90	71a	M4a	95	M16	M3	M2	62	M10	35
15	54	84	71b	M4b	102	94	36	M5	63		
18	55				103	96	38	M6			
19	56				104	M25		M7			
20	57					M26					
21	58					M27					
22	59					M28					
23	60					M28					
24	61										
25	79										
	81										

Table 3.1 Growth on selected substrates. Growth is defined as the visible presence of CFU on petri plates.

Sorbing Substrate		Minimally Sorbing Substrate	
Naphthanene	No growth	Salicylate	No growth
		Benzoate	No growth
Phenanthrene	Growth	Catechol	No growth

Determination of Yield by Weight in a Batch System

Yield, denoted "y", is the amount (grams) of cell mass produced per amount (grams) of substrate consumed. The purpose of the following experiments was to determine yield for each of the 3 bacterial species being used.

Small flask studies:

The solubility of phenanthrene, 1.28 mg/L, does not provide sufficient energy to produce a measurable increase in cell mass. Therefore, the following experiments were conducted with greater than 1.28 mg/L total phenanthrene.

Sterilized 250 ml Erlenmeyer flasks were coated with a phenanthrene-chloroform mixture. The chloroform was then allowed to evaporate. The mixture contained sufficient phenanthrene to produce equivalent concentrations of 250, 100, 75, and 10 mg/l, when 25 ml of mineral salts broth (MSB) (KH_2PO_4, 1.0g/l; Na_2HPO_4, 1.0g/l; NH_4NO_3, 0.5g/l; $(NH_4)_2SO_4$, 0.5g/l; $MgSO_4$ $7H_2O$, 0.2 g/l; $CaCl_2$ $2H_2O$, 0.02g/l; $FeCl_3$, 0.002 g/l) was added; assuming all of the phenanthrene to be bioavailable, which it was not. Flasks were then placed on a rotary shaker at 200 rpm for 48 hours.

After 48 hours, flasks were inoculated with a pre-grown culture to produce a bacterial concentration of approximately 7 X 10^7 CFU/ml. Inoculum samples were plated and weighted to determine initial numbers and an aliquot oven dried to determine the weight of the initial inoculum.

Flasks shook for an additional 48 hours (a repeat of this experiment was conducted, where the flasks shook for 14 days) after which the contents of each flask were centrifuged (10,000 rpm, 10minutes) and the resulting cell pellet dried and weighed. Samples were also taken to determine final cell density via plate counts.

Large flask studies:

A procedure similar to that of the small flask investigations was used. Three large, 2,800 ml flasks were employed. Each flask contained 1,000ml of MSB with an equivalent phenanthrene concentration of 10 ug/l. At the time of centrifugation, all 3 flasks were combined to produce a single cell pellet.

Results and Discussion

The low solubility of phenanthrene combined with the possibility of a toxicity threshold in the organism (inferred from the decrease in cell numbers observed) made determination of yield problematic. In all treatments no increase in cell mass was found. In addition, treatments yielded no change or a decrease in cell mass.

The selected organisms have been observed to grow on petri plates that contain phenanthrene (0.25 mg) as the sole carbon source. Therefore, it is known that these organisms are capable of phenanthrene degradation and increasing their cell mass using solely phenanthrene. However, it is quite possible that the cells are not able to grow in a liquid broth medium. Growth may be prohibited by degradation intermediates or toxic

metabolic by-products of phenanthrene biodegradation. In addition, it is possible that the chosen organisms prefer to grow attached to a solid surface, attached to the phenanthrene itself, or that a larger amount of substrate is needed to produce measurable increases in cell numbers. These possibilities will be investigated.

Substrate Utilization in a Batch System

The purpose of the following investigations was to determine a substrate utilization rate as well as the lowest concentration to which each bacterial species can degrade phenanthrene.

Erlenmeyer flasks

Two thousand eight hundred ml squat Erlenmeyer flasks, each containing 1 liter of phenanthrene solution (at 1mg/l), were inoculated with approximately 10^7 CFU/ml bacteria. The flasks were placed on a rotary shaker at 200 rpm. Using a sterile, glass pipet, periodic samples were taken and analyzed for phenanthrene using a fluorescence spectrophotometer. The experimental duration was determined by the individual organism. Each experiment was terminated when 3 days of consecutive sampling revealed no further decrease in phenanthrene concentration. Data were plotted concentration vs. time and a regression line used to find the best fit line with the slope being the rate of substrate utilization. All experiments were performed in triplicate and repeated.

Radio-labeled experiments

Methods used for this experiment were similar to those discussed previously. Specially altered 250 Erlenmeyer flasks were sterilized via autoclave. Then the bottom of each flask coated with a phenanthrene/chloroform solution, sufficient to produce 1mg/l phenanthrene

solution when 25 ml of MSB were added. Flasks were allowed to shake on a rotary shaker for 24 hours, sufficient time for the phenanthrene coating the flask bottom to dissolve into the MSB and create a 1mg/l phenanthrene solution. Radio-labeled phenanthrene-9-^{14}C (Sigma) was then added. Each flask contained approximately 46,000 dpm. Flasks were then inoculated with *Acinetobacter junii* (3), *Peudomonas oleovorans* (M11), and/or *Methylobacterium sp.* (35). Trials were done in pure culture and in mixtures of 2 and 3 species. Inoculations resulted in a microbial density of at least 1 x 10^7 CFU/ml. All experiments were conducted in triplicate.

Flasks were sampled via a flushing tree designed to trap radio-labeled CO_2. Samples were collected periodically over a period of 80 hours. Disintegrations per minute were read on a scintillation counter.

Results and Discussion

Substrate utilization plots generated for species 3, *Acinetobacter junii*, show a slope of -0.007 (R^2 0.96) and a degradation limit of 0.72 mg/l (Figure 3.0). Substrate utilization, as described above, was not achieved for *Pseudomonas oleovorans* (M11) (Figure 3.0), nor *Methylobacterium sp.* (35) (Figure 3.0). It appears that these organisms do not have a very strong affinity for phenanthrene under the growth conditions and concentrations imposed.

Radio-labeled experiments

Data were plotted C/Co (cumulative dpms evolved/initial dpms) vs time (hours) in order to obtain the total dpms evolved from the flask as compared to dpms initially in the flask. In general, flasks containing pure cultures degraded more phenanthrene than was degraded by the mixed cultures. *Acinetobacter junii* degraded the most phenanthrene by far (13%) followed by *Pseudomonas oleovorans* (2%) and *Methylobacterium sp.* (0.3%) (Table 3.2). Data were also plotted log C/Co vs time. Regression analysis was then performed (Table 3.2).

Results once again indicate that phenanthrene degradation is minimal. It is possible that phenanthrene degradation by these three species is truly minimal, despite substantial growth on petri plates where phenanthrene is the sole carbon source. Conversely, it is possible that the growth conditions imposed are not favorable. This is supported by the results presented later in this chapter that show that cell growth on phenanthrene in a liquid medium is possible under very specific conditions.

Table 3.2. Summary of radio-labeled batch experiments. *Acinetobacter junii* (3), *Peudomonas oleovorans* (M11), *Methylobacterium sp.* (35). A regression was calculated for the plot C/Co versus time (hours).

Organism	Total dpms evolved/ Initial dpms in flask	Slope of Regression	R^2 Value
3	0.13	0.0306	0.3364
M11	0.02	0.0457	0.4817
35	0.003	0.3042	0.5708
35 & 3	0.0036	0.3173	1
3 & M11	0.006	0.0397	0.6762
M11 & 35	0.006	0.0397	0.6762
all species	0.0105	0.0481	0.713

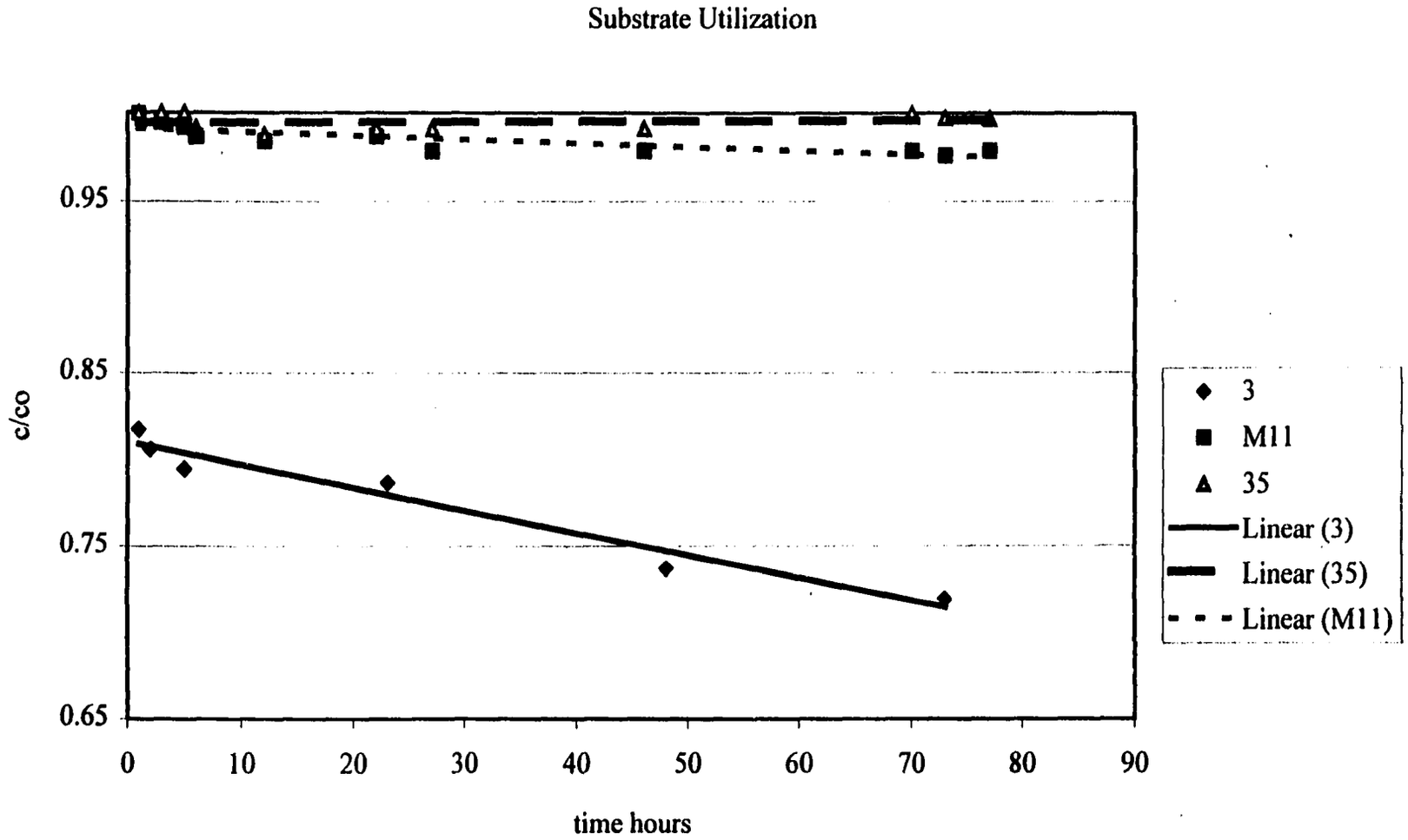

Figure 3.0. *Actinobacter junii* (3), *Pseudomonas oleavorans* (M11), and *Methylobaterium sp*. (35) substrate utilization over time.

the measurement of phenanthrene by conventional methods (i.e. fluorescence spectrophotometer of HPLC). In addition, the use of radio-labeled phenanthrene would also be problematic. First, the radio-labeled phenanthrene resides in the solution within the flask and would be degraded first. This would leave nothing left to "measure" phenanthrene degradation as more phenanthrene solubilizes. Second, cell growth is measured when cells are inoculated at $5x10^5$ CFU/ml and grow to $1x10^7$ CFU/ml. Detectable or noticeable degradation is not thought to occur until $1x10^7$ CFU/ml. Therefore, degradation would not be able to be detected in this system.

Bath Assay Study

This study used three species of indigenous microorganisms *(Acinetobacter junii, Pseudamonas oleovorans, Methylobacterium sp.)* to investigate variations in cell surface hydrophobicity. Specifically, the effects of substrate variation and buffer solution variation on cell surface hydrophobicity will be examined.

Materials and Methods

Bacteria: The three indigenous species of bacteria (*Acinetobacter junii, Pseudamonas oleovorans, Methylobacterium sp.*) used in this study were previously isolated from a soil mixture and identified as described in Wang (1999).

Substrates and Solutions: Bacteria were grown in petri plates containing sterile media. Two different types of media were used: R_2A and phenanthrene. R_2A media was purchased from Difco Laboratories (Detroit, MI) (yeast extract 0.5g/l; protease peptone no.3 0.5 g/l; casamino acids 0.5 g/l; dextrose 0.5 g/l; soluble starch 0.5 g/l; sodium pyruvate 0.3 g/l; dipotassium phosphate 0.3 g/l; magnesium sulfate 0.05 g/l; agar 15.0 g/l). Phenanthrene medium is a mineral salts medium (MSM) (KH_2PO_4, 1.0g/l; Na_2HPO_4, 1.0g/l; NH_4NO_3, 0.5g/l; $(NH_4)_2SO_4$, 0.5g/l; $MgSO_4$ $7H_2O$, 0.2 g/l; $CaCl_2$ $2H_2O$, 0.02g/l; $FeCl_3$, 0.002 g/l; purified noble agar, 15 g/l) with the addition of 0.25 mg phenanthrene

per plate. Organisms were pre-cultured, serially diluted, plated, and finally incubated at room temperature for 3 days in the case of R_2A, or 14 days in the case of phenanthrene.

Hexadecane 99% pure (Aldrich, Milwaukee, WI) was used as the hydrocarbon solution. Three different buffer solutions were used: MSB (mineral salts broth), phenanthrene, and phenanthrene/R_2B. The MSB buffer solution contained the same compounds as did MSM with the exception of the noble agar. The phenanthrene buffer solution was composed of MSB solution with the addition of phenanthrene to create a 1mg/l solution. And the phenanthrene/R_2B buffer solution was the above described phenanthrene solution with the addition of sufficient R_2B media to create a solution that derived 66% of its carbon from phenanthrene and 33% from R_2B. R_2B is identical to R_2A media with the omission of agar.

Bath Assay: A BATH assay (Bacterial Adherence to Hydrocarbon) was performed as described in Rosenberg (1984). For each species, six assays were performed: organisms grown on phenanthrene media were placed in buffer solutions of MSB, phenanthrene, phenanthrene/R_2B; likewise, organisms grown on R_2A media were placed in buffer solutions of MSB, phenanthrene, phenanthrene/R_2B. Hexadecane was used as the hydrocarbon solution in all treatments. Optical density (O.D.) was the parameter monitored. Analysis was performed on a Hitachi UV spectrophotometer at a wavelength of 600nm. All assays were performed in triplicate and all experiments were repeated.

Statistical Analysis: Percentage decrease in absorbency ((initial O.D. - final O.D.)/ initial O.D.)*100) was used for all comparisons. ANOVA was used to determine differences between and among treatments.

Results

As determined by ANOVA, differences between experiments were not significant ($p = 0.183$ or greater); thus, replicate experiments yielded comparable results. In all cases, organisms grown on R_2A were more hydrophobic (larger % decrease) than those grown on phenanthrene (Table 3.3). A significant substrate interaction was found for all three species ($p = 1.9 \times 10^{-11}$, *Acinetobacter junii;* $p = 1.0 \times 10^{-6}$, *Pseudamonas oleovorans;* 2.1×10^{-11} *Methylobacterium sp.*). Therefore, the substrate on which the aforementioned species were grown significantly affects cell hydrophobicity. Significant buffer solution interactions were found for *Acinetobacter junii* ($p = 2.3 \times 10^{-11}$) and *Methylobacterium sp.* ($p = 8.5 \times 10^{-7}$), however, not for *Pseudamonas oleovorans* ($p = 0.302$).

As shown in Table 1.1, *Acinetobacter junii* tends to be the most hydrophobic species (largest % decrease) followed by *Methylobacterium sp.* and *Pseudamonas oleovorans.*

Discussion

The transport of bacterial cells is influenced by cell hydrophobicity as well as other factors. Differences in cell hydrophobicity may lead to differences in cell elution behavior. Therefore, this experiment was conducted to determine if the selected species differ in their cell surface hydrophobicity, and if so, by how much. As demonstrated, the nutrient

media on which the aforementioned species are grown significantly influenced cell hydrophobicity. R_2A media, the more nutrient rich substrate, yielded a larger % decrease (increased hydrophobicity) over the phenanthrene media. The difference in "nutritional status" of the bacteria is one possible explanation for this behavior. While the bacteria in this study were not under strict starvation conditions, growth on phenanthrene plates is more limited (cells are observed to become round when examined under a microscope) than that on R_2A plates (cells are observed to be rods under a microscope). These results concur with those of Lawrence and Hendry (1996) who also found that a major factor influencing bacterial transport is the type of substrate that the bacteria consume (Lawrence and Hendry, 1996). In addition, Wrangstadh et al. (1986) observed a decrease in cell hydrophobicity when under starvation conditions.

Buffer solution significantly affected *Actinobacter junii* (3) and *Methylobacterium sp.* (35), yet not *Pseudamonas oleavorans* (M11) ($p=0.302$). The mechanism for this interaction is not known and would be an interesting set of investigations. However, it is not the mechanism that is of interest in this study, but the organisms response to different conditions that is of interest. The information obtained here will be referenced in subsequent chapters and used to explain observed behaviors.

Table 3.3. Percentage decrease in O.D. Mineral salts broth (MSB), Phenanthrene (Phen). A larger % decrease indicates increased hydrophobicity. Numbers are given in percentage; standard error is indicated.

		Buffer solution		
	Substrate	MSB	Phen	Phen/R_2B
Acinetobacter junii	R_2A	46.77 ± 0.89	41.06 ± 1.91	50.27 ± 0.98
	Phen	38.24 ± 0.90	19.35 ± 0.89	37.76± 1.85
Pseudomonas oleavorans	R_2A	4.63 ± 0.93	4.05 ± 0.83	6.09 ± 0.89
	Phen	-0.18 ± 0.89	1.61 ± 0.31	0.86 ± 0.76
Methylobacterium sp.	R_2A	13.85 ± 0.18	22.99 ± 0.69	26.45± 3.27
	Phen	1.33 ± 0.12	2.74 ± 1.99	12.75 ± 1.91

CHAPTER4: BIODEGRADATION IN A COLUMN SYSTEM

In order to investigate the effects of multiple species on contaminant degradation, it is first necessary to characterize the degradation behavior of the individual species. System complexity may then be increased by employing multiple-species communities that contain two and three bacterial species. The following column experiments were performed to meet such an objective.

Materials and Methods

System setup: All experiments were conducted using a stainless steel column (Aldrich), 15 cm in length, homogeneously packed with 40x50 Acusand (silica sand approximately 297-420 um in diameter, minimal organic matter content). A SSI piston pump was used and a flow rate of approximately 9 cm/hr was induced. All experiments were conducted under saturated conditions.

Bacteria: Bacteria were pre-cultured in a solution of R_2B. A colony, taken from an R_2A plate, was used as the initial inoculum for a flask containing 25 ml R_2B. After 48 hours on a rotary shaker, 1 ml of pre-culture was transferred to a flask containing 25 ml of fresh R_2B and returned to a rotary shaker, 200rpm. Cultures were incubated an additional 24 hours before harvesting. At harvest, the contents of each flask were transferred to sterile centrifuge tubes, and centrifuged at 10,000 rpm for 10 minutes. The supernatant was

decanted and the resulting pellet resuspended in 25 ml saline solution and centrifuged. Once again, the supernatant was decanted and the pellet resuspended in 10 ml mineral salts broth. The resulting mixture contained approximately 10^8 cfu/ml, as determined by plate counts. All bacteria in the following studies were enumerated by plate counts. Therefore, all bacterial data reflect culturable numbers of bacteria in the system. Exact inoculum densities and distribution within the column may be found in Appendix B.

Experimental Design: Column porous media was sterilized via autoclave (1 hour), inoculated with a bacterial cell culture, and then homogeneously packed into a sterile, stainless steel column. Porous media samples were sequentially taken 3 times during the packing process and plated in order to determine the total number of bacteria initially present and initial distribution of bacteria in the column. Once connected to a pre-sterilized pump (sterilization via 30 minutes bleach, 5 minutes sodium thiosulphate, 30 minutes sterilized deionized water), the column was saturated with mineral salts broth for a minimum of 18 hours to achieve complete saturation. A continuous flow of phenanthrene broth (MSB containing approximately 1 mg/l phenanthrene) was then begun. Input of phenanthrene broth continued for 3 weeks (approximately 300 pore volumes). Phenanthrene effluent concentration and dissolved oxygen concentration (mV), as well as bacterial elution (CFU/ml- determined by plate counts) were monitored throughout the course of the experiment. At the end of the experiment, the column was dissected using sterile technique and porous media samples plated to determine final bacterial counts and

distribution. Replicate columns were prepared in the same manner.

Results and Discussion

Single-species experiments

Phenanthrene in the single-species *Acinetobacter junii* column never reached C/Co (effluent concentration/influent concentration) of 1. Stable degradation, with a plateau C/Co of 0.70, was reached after 89 pore volumes. Degradation decreased with a concomitant increase in C/Co to 0.76 at 158 pore volumes, and remained stable for 45 pore volumes at which time degradation increased slightly to C/Co of 0.73 (Figure 4.0). No significant correlation between phenanthrene concentration and dissolved oxygen was found (df=1, F-ratio=1.987, p=0.178). No growth, calculated as the number of cells eluted plus the final number of cells present in the column minus the initial number of cells inoculated into the column, occurred in the column. The unaccounted for cells may be attributed to cell death during the study or may be due to a loss in culturability of the cells. In either case, the number of culturable and thus actively metabolizing cells was found to decrease (Table 4.0). Replicate data are shown in Appendix A.

Phenanthrene in the *Pseudomonas oleavorans* column reached a C/Co (effluent concentration/influent concentration) of 1 almost immediately. Phenanthrene effluent concentrations remained stable at C/Co of 1 for approximately 100 pore volumes. After 100 pore volumes, effluent concentrations decreased to 0.8 and remained between 0.8 and 0.85 until the 250th pore volume. At 250 pore volumes, degradation decreased and

effluent concentrations of phenanthrene returned to 0.9 (Figure 4.1). No significant correlation between dissolved oxygen and phenanthrene concentration was found (df=1, F-ratio=0.001, p=0.974).Growth calculations showed that $4.97x10^{10}$ to $5.00x10^{10}$ additional CFU were produced during the experiment (Table 4.0).

Phenanthrene in the *Methylobacterium sp.* column reached a C/Co (effluent concentration/influent concentration) of 1 almost immediately. Phenanthrene effluent concentrations remained stable at C/Co of 1 for approximately 115 pore volumes. After 115 pore volumes, effluent concentrations decreased to 0.8 and remained between 0.8 and 0.85 until the 230th pore volume. At 230 pore volumes, degradation decreased and effluent concentrations of phenanthrene returned to 0.95 (Figure 4.2). No significant correlation between phenanthrene and dissolved oxygen concentration was found (df=1, F-ratio=1.155, p=0.304). A cell mass balance shows a net loss of cells (i.e. cell death) during the experiment (Table 4.0).

Several aspects of this series of experiments proved to be interesting. Each of the three bacterial species produced a unique phenanthrene biodegradation breakthrough curve (Figure 4.3). *Acinetobacter junii* (3), observed to grow rapidly on plates, does not exhibit any degradation lag and phenanthrene C/Co therefore never reaches 1. This species also exhibits the greatest affinity for phenanthrene (i.e. degrades to the lowest C/Co). *Pseudomonas oleavorans* (M11) and *Methylobacterium sp* (35), exhibit a biodegradation lag time of roughly 90 pore volumes, with the lag for *Methylobacterium sp.* appearing to be slightly less than that of *Pseudomonas oleavorans*. This is an apparent

effect caused by a smaller pore volume in the *Pseudomonas oleavorans* column. Lag time (approximately 96 hours) is the same for both species. This concurs with observations of growth on plates in which colonies of both species begin to appear after 12 days. *Pseudamonas oleavorans* and *Methylobacterium sp.* affinity for phenanthrene appears to be similar.

Cell growth calculations showed that *Pseudomonas oleavorans* produced an increase in culturable cells (growth) over the experimental time course while *Actinobacter junii* and *Methylobacterium sp.* did not. The lack of growth for *Actinobacter junii* and *Methylobacterium sp.* was most likely due to "environmental conditions" rather than lack of substrates. As explained in Chapter 3, these organisms require a very specific set of conditions to be able to degrade phenanthrene and to increase in cell numbers. It seems likely that the flow-through nature of the column provided conditions which were not suitable for *Actinobacter junii* or *Methylobacterium sp.* growth. Perhaps these two species need to be in a system where extracellular metabolites are allowed to build-up, as in a batch system, rather than in the current system where they are washed away. As will be shown below, both *Actinobacter junii* and *Methylobacterium sp.* are capable of growth in systems where there is an additional bacterial species present, which may supply the metabolite(s) needed for growth. In addition, death of *Actinobacter junii* and *Methylobacterium sp.* decreases in columns injected with R_2B (Chapter 5), once again supporting the hypothesis that an additional metabolite may be required for cell growth.

In summary, the bacterial species appear to have different affinities for phenanthrene. *Acinetobacter junii* has the strongest affinity for phenanthrene, capable of substrate degradation to C/Co of 0.70, while *Methylobacterium sp.* and *Pseudomonas oleavorans* exhibited similar affinities (C/Co= 0.8). Such differences in phenanthrene affinity would be expected for members of a diverse soil bacterial community. This was an important aspect of this project as these organisms are meant to represent, on a simple level, the diversity that is found in a soil system. This raises interesting questions as to how degradation behavior may change when two or more species are inoculated into a soil column. Such questions will be addressed in the discussion of the two species column results that follows.

Acinetobacter junii and Methylobacterium sp.

Phenanthrene effluent concentrations for the *Acinetobacter junii (3) / Methylobacterium sp.(35)* experiment oscillated between C/Co of 1 and 0.8. Maximal degradation was achieved around 120 pore volumes and then again at 247 pore volumes, after which degradation decreased rapidly (Figure 4.4). *Methylobacterium sp.* appears to have a dampening effect on degradation in the *Methylobacterium sp./ Acinetobacter junni* column (Figure 4.5). At no time during the experiment did the extent of degradation reach that exhibited in the single-species *Acinetobacter junni* column. However, it is possible that the decrease in degradation is due to an order of magnitude fewer *Actinobacter junii* cells in the two-species column as compared to the single species

Actinobacter junii column, rather than inhibition. Lag time did appear to decrease in the *Acinetobacter junii/Methylobacterium sp.* species column.

Over all, the column experienced a net increase in cell numbers (ie. population growth) (Table 4.1). A cell mass balance, showed that *Methylobacterium sp.* experienced a net increase in cell numbers with $6.37x10^7$ to $1.1x10^9$ additional CFU produced, while *Actinobacter junii* showed a net increase in CFU with $7.40x10^7$ to $3.45x10^8$ additional CFU produced (Table 4.1).

Acinetobacter junii/Pseudomonas oleavorans

For the *Acinetobacter junii* (3)/ *Pseudomonas oleavorans (M11)* experiment, the phenanthrene concentration in the effluent did not reach C/Co of 1. Throughout the course of the experiment, degradation reached a high of C/Co 0.75 at 224 pore volumes. Degradation continued at this level for approximately 75 pore volumes and then decreased to C/Co 0.87 for the remainder of the experiment (Figure 4.6). In addition, lag was reduced in the *Pseudomonas oleavorans/ Acinetobacter junni* experiment as compared to the single-species *Pseudomonas oleavorans* experiment (Figure 4.7).

Pseudomonas oleavorans had a slight inhibitory effect in the two-species experiment, however, by 200 pore volumes concentrations were equivalent to those found at 200 pore volumes in the single species *Acinetobacter junni* column. After 200 pore volumes, concentrations increased, though were still less than those found in the single-species *Pseudomonas oleavorans* column. Over all, the column experienced a net increase in cell

numbers (Table 4.1). *Pseudomonas oleavorans* produced an additional 8.16×10^8 to 8.3×10^8 CFU during the experiment (Table 4.1). *Acinetobacter junii* showed the greater production of additional cells at 8.49×10^9 to 8.5×10^9 CFU.

Pseudomonas oleavorans and Methylobacterium sp.

For the *Pseudomonas oleavorans* (M11)/ *Methylobacterium sp.* (35) experiement, phenanthrene reached a C/Co of 1 within the first several pore volumes after which degradation began and C/Co of 0.70 was reached by 40 pore volumes. This level of degradation was sustained until 140 pore volumes. At 140 pore volumes, degradation ceased and C/Co returned to 1 (Figure 4.8). Minimal degradation occurred until just after 320 pore volumes at which time degradation increased again to C/Co of 0.9. Total cell growth (1.16×10^{10} to 1.18×10^{10} additional CFU produced) was achieved during the experiment (Table 4.2). *Pseudomonas oleavorans* (M11) showed the greater production of new, culturable cells (1.16×10^{10} CFU) than did *Methylobacterium sp.* (Table 4.1). The *Methylobacterium sp./ Pseudomonas oleavorans* column showed decreased lag and increased degradation during the first 150 pore volumes, versus the single-species experiments (Figure 4.9) and as for the single-species experiements, degradation decreased dramatically before increasing once again.

Three-Species Experiments

Phenanthrene effluent concentration in the three-species experiment oscillated between C/Co of 1 and 0.9. Maximal degradation was achieved during the first 170 pore volumes. Degradation appeared to cease between 220 and 270 pore volumes, increasing slightly there afterwards (Figure 4.10). Overall, the column experienced a net growth in cell numbers. *Acinetobacter junii* (3) showed the greatest production of new cells (6.28×10^9 to 1.69×10^9 CFU) followed by *Pseudomonas oleavorans* (3.28×10^8 to 4.25×10^7 CFU). Final culturable cell counts for *Methylobacterium sp.* were not obtained as *Methylobacterium sp.* was present at 2 or more orders of magnitude less than the other 2 species.

While lag is decreased, somewhat, by the presence of *Acinetobacter junii*, the combination of the three-species does not increase biodegradation of phenanthrene. Any synergism that might have been present in the *Methylobacterium sp./Pseudomonas oleavorans* system did not appear in the three-species system. On the contrary, dampening or averaging effects, such as those seen in the *Acinetobacter junii/Pseudomonas oleavorans* and *Acinetobacter junii/Methylobacterium sp.* systems appear to dominate.

Both *Acinetobacter junii* and *Pseudomonas oleavorans* show a net increase in culturable cells during the 3 species experiment (Table 4.3), suggesting once again a favorable or mutually beneficial relationship between the two species. *Methylobacterium sp.*, on the other hand, was present in such small numbers at the end of the experiment as to not be

possible to enumerate. This suggests that *Methylobacterium sp.* was not able to compete for sufficient phenanthrene to survive, and that it was not able to establish a "relationship" with *Acinetobacter junii* as it had in the two-species system, and/or that it did do both of these things on a more limited basis and therefore, existed at lower numbers.

Conclusions

As shown above, three-species and two-species systems produced biodegradation patterns different from those exhibited by the single-species systems, depending upon which species were present (Figure 4.11). In addition, substantial differences in cell growth were found in each system. The combination of *Methylobacterium sp./Pseudomonas oleavorans* resulted in a synergistic effect. First, lag time was substantially reduced as compared to the single- species systems (Figures 4.5, 4.7, 4.9). It is possible that each of these species requires the productions of a specific enzyme or other factor to degrade phenanthrene and the production of this factor requires time. *Pseudomonas oleavorans* and *Methylobacterium sp.* may each produce a factor required by the other. This would explain the decrease in lag time shown in the two-species system. In addition, phenanthrene degradation in this system was enhanced with the presence of these two species as compared to the single- species system. Perhaps the exchange of an abundant supply of required factors allowed more energy to be spent on degradation, rather than on factor production.

Degradation synergism to this degree was not present in the *Actinobacter junii/Pseudomonas oleavorans* nor *Methylobacterium sp./Actinobacter junii* system. However, a slight reduction in lag time for these systems as compared to the *Pseudomonas oleavorans* and *Methylobacterium sp.* single-species systems was found. This reduction in lag time would be due to the presence of *Actinobacter junii,* which shows no lag time when present as a single species. Furthermore, it appears that *Actinobacter junii* does not produce the "complementary factors" necessary for *Pseudomonas oleavorans* or *Methylobacterium sp.* to reduce their lag time as an incrase in degradation does not also occur.

When the level of complexity in the column system is increased, by the addition of all three species to the system, the degradation synergism and decrease in degradation lag time found in the *Pseudomonas oleavorans/Methylobacterium sp.* column disappears. This suggests that the complementary factors produced in this system are either not produced or they do not reach the target organism.

Calculations to determine the number of culturable bacteria produced by each column system support the synergistic behavior found in the *Pseudomonas oleavorans/Methylobacterium sp.* column. The number of culturable bacteria produced by *Pseudomonas oleavorans* in the *Pseudomonas oleavorans/Methylobacterium sp.* column is similar to that produced in the single species *Pseudomonas oleavorans* column (Table 4.4). In addition, the number of culturable bacteria produced by *Methylobacterium sp.* is significantly greater when a second bacterial species is also present in the system (Table

4.4), suggesting once again that the interaction among bacterial species involves an exchange of factors or compounds that effect bacterial growth and thus biodegradation.

The differences in biodegradation and culturable cell production among those bacteria in a single species column system versus a two or three species system are significant. These results show that doing individual "pure-culture" experiments to measure biodegradation would not give accurate results for a consortium. Future work must be conducted to better understand bacterial interactions and how such interactions will effect contaminant transport and biodegradation.

From the investigations conducted it is clear that the species used in these investigations interact in a variety of ways (ie. synergism and inhibition). Such interactions produce dramatic changes in cell growth and biodegradation. Growth rates of each species are different in single-species systems versus those in the multiple-species systems. Phenanthrene degradation is also different with each different species combination. Such variations in behavior indicate mutualistic and inhibitory interactions are occurring and that the nature of the interactions is dependent on the species present. These findings support the idea that the community acts as a consortium in degradation and nutrient cycling. They also support the idea that each species within the consortium has a specific function or niche, without which proper functioning of the system does not occur.

Table 4.0. Single species column cell elution and growth. Values given are a range (minimum possible to maximum possible) as calculated using 95% confidence intervals.

	Initial number of cells	Number of cells eluted	Final number of cells remaining in the column	Number of cells eluted/ Initial number of cells	Final number of cells remaining in the column/ Initial number inoculated	Growth (number of cells eluted + final number of cells in the column- initial number of cells inoculated into the column)
Acinetobacter junii	2.06×10^{9}	1.43×10^{8}	1.58×10^{8} to 1.53×10^{8}	0.069	-0.340 to 0.490	-1.61×10^{9} to -1.63×10^{9}
Pseudomonas oleavorans	7.46×10^{9}	5.47×10^{10}	7.45×10^{6} to 7.18×10^{6}	7.38	0.000 to 0.004	4.97×10^{10} to 5.00×10^{10}
Methylobacterium sp.	4.57×10^{10}	1.38×10^{9}	2.84×10^{3}	0.003	0.000 to 0.000	-4.11×10^{10} to -4.26×10^{10}

	initial number of cells inoculated	remaining in the column/ initial number inoculated	
Actinobacter junii/Methylobacterium sp. total cell numbers	3.57 to 3.57	-0.455 to 0.665	9.32×10^9 to 9.39×10^9
Actinobacter junii in *Actinobacter junii/ Methylobacterium sp.* column	1.31 to 1.31	-0.185 to 0.601	7.49×10^7 to 3.45×10^8
Methylobacterium sp. in *Actinobacter junii/Methylobacterium sp.* column	1.150 to 1.150	-0.027 to 0.140	6.37×10^7 to 1.11×10^9
Actinobacter junii/ P. oleavorans total cell numbers	3.57 to 3.57	-0.455 to 0.665	9.32×10^9 to 9.39×10^9
Actinobacter junii in *Actinobacter junii/P. oleavorans* column	3.59 to 3.59	-0.094 to 1.12	8.49×10^9 to 8.54×10^9
P. oleavorans in *Actinobacter junii/P. oleavorans* column	3.37 to 3.37	-0.066 to 0.264	8.16×10^8 to 8.30×10^8
P. oleavorans/Methylobacterium sp. total cell numbers	0.000 to 0.000	0.053 to 0.076	1.16×10^{10} to 1.16×10^{10}
P. oleavorans in *P. oleavorans/Methylobacterium sp.* column	7.03 to 7.03	-0.177 to 0.271	1.16×10^{10} to 1.16×10^{10}
Methylobacterium sp. in *P. oleavorans/Methylobacterium* column	1.02 to 1.02	0.000(8) to 0.000(3)	-2.08×10^7 to 1.34×10^8

Table 4.3. Three species column cell elution and growth. Values given are a range (minimum possible to maximum possible) as calculated using 95% confidence intervals.

	cells eluted/ initial number of cells inoculated	final number of cells remaining in the column/ initial number inoculated	growth
Species total	2.766 to 2.770	-0.241 to 0.689	1.5×10^{10} to 1.5×10^{10}
Actinobacter junii	2.67 to 2.67	-0.233 to 0.874	6.28×10^{9} to 1.69×10^{9}
Pseudomonas oleavorans	5.64 to 5.64	-0.027 to 0.270	3.25×10^{8} to 4.25×10^{7}
Methylobacterium sp.	2.40 to 2.40	n/a	n/a

* *Methylobacterium sp.* was present at 2 or more orders of magnitude less than *Actinobacter junni and Pseudomonas oleavorans* at the end of the experiment and thus was not able to be enumerated via plate counts.

Table 4.4. Final cell density within each column. *Pseudamonas oleavorans* (M11), *Actinobacter junii* (3), *Methylobacterium sp.* (35). Final density is independent of growth or inoculation density for *Actinobacter junii* and *Pseudomonas oleavorans*, but not for *Methylobacterium sp.*. Data shown are a range as determined by 95% confidence interval.

	Inoculation Density (avg.)	Growth	Final Density
M11 grown alone	5.73×10^7 CFU/g sand	4.97×10^{10} to 5.0×10^{10}	2×10^5 to 7×10^5
M11 grown with 3	3.5×10^6 CFU/g sand	8.16×10^8 to 8.3×10^8	8×10^4 to 1×10^6
M11 grown with 35	2.14×10^7 CFU/g sand	1.16×10^{10} to 1.16×10^{10}	1×10^6 to 1×10^6
3 grown alone	2.18×10^7 CFU/g sand	-1.6×10^9 to -1.63×10^9	5×10^5 to 1×10^7
3 grown with M11	2.99×10^7 CFU/g sand	8.49×10^9 to 8.54×10^9	5×10^6 to 5×10^7
3 grown with 35	3.8×10^6 CFU/g sand	7.49×10^7 to 3.45×10^8	5×10^5 to 2×10^6
35 grown alone	7.55×10^8 CFU/g sand	-4.11×10^{10} to -4.26×10^{10}	1x102 to 1×10^2
35 grown with 3	1.25×10^7 CFU/g sand	6.37×10^7 to 1.11×10^9	5x105 to 1×10^6
35 grown with M11	3.95x107 CFU/g sand	-2.08×10^7 to 1.34×10^8	5x104 to 2×10^4

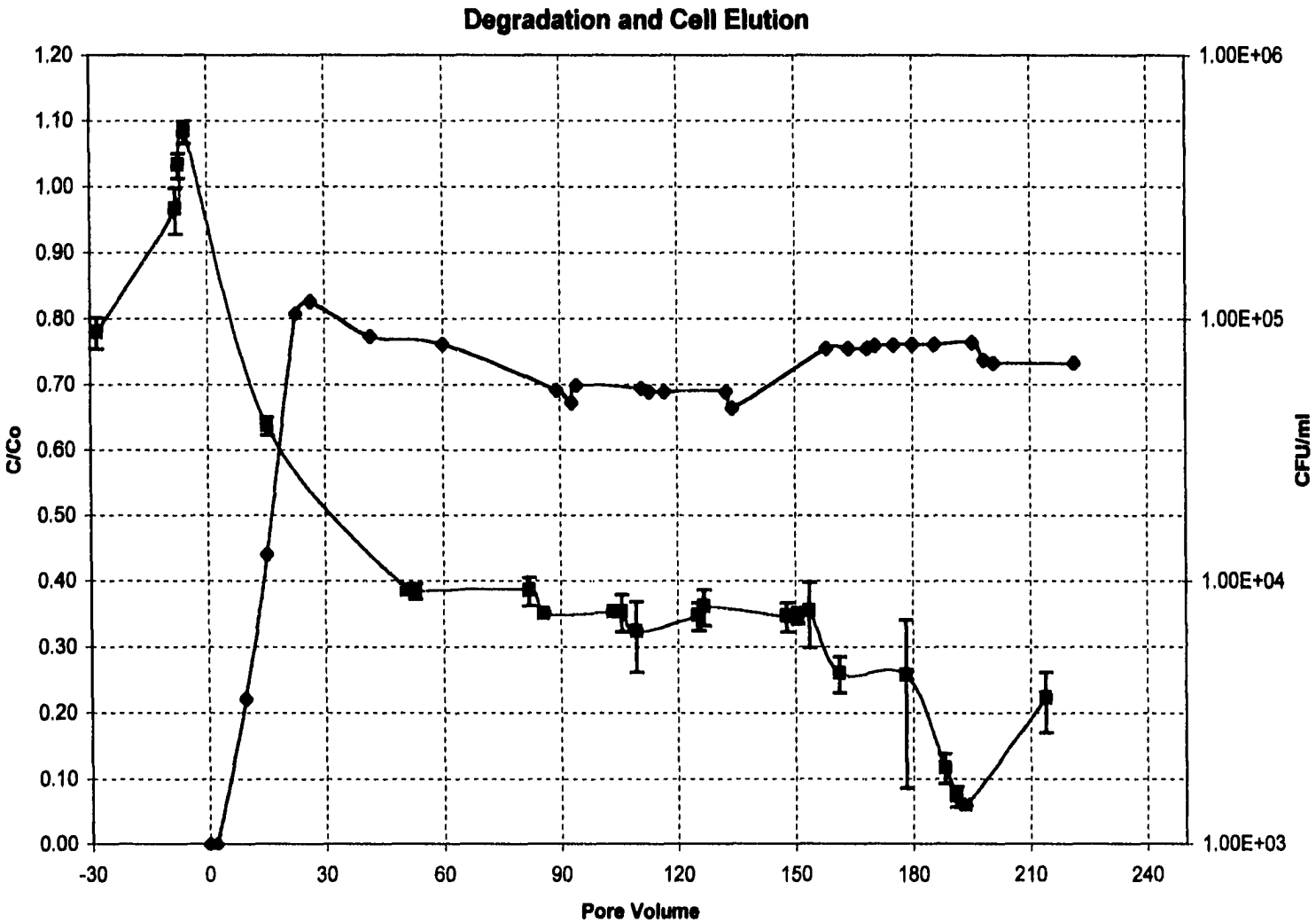

Figure 4.0 *Actinobacter junii (3)* biodegradation and cell elution in a single species column.

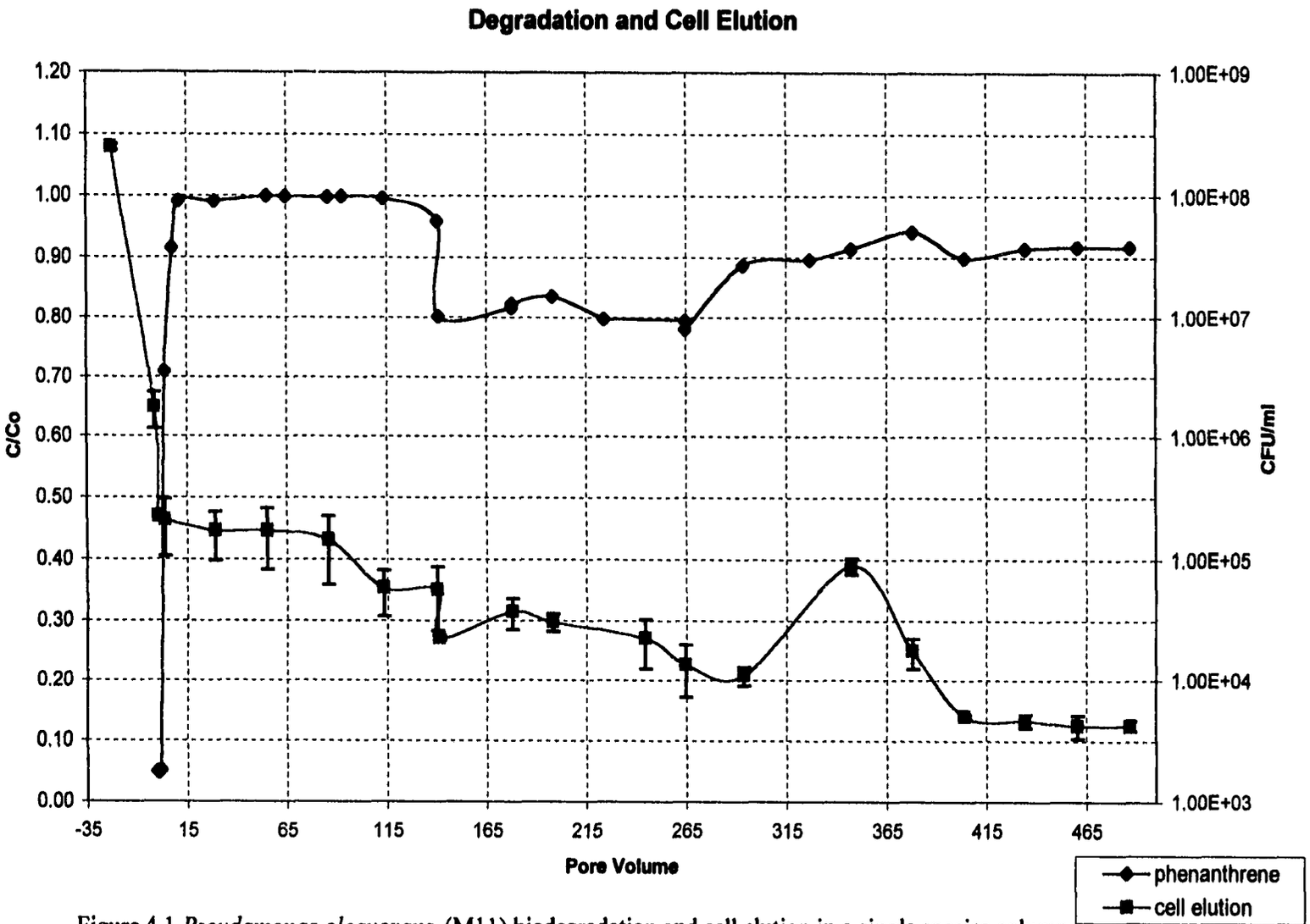

Figure 4.1 *Pseudamonas oleavorans* (M11) biodegradation and cell elution in a single species column.

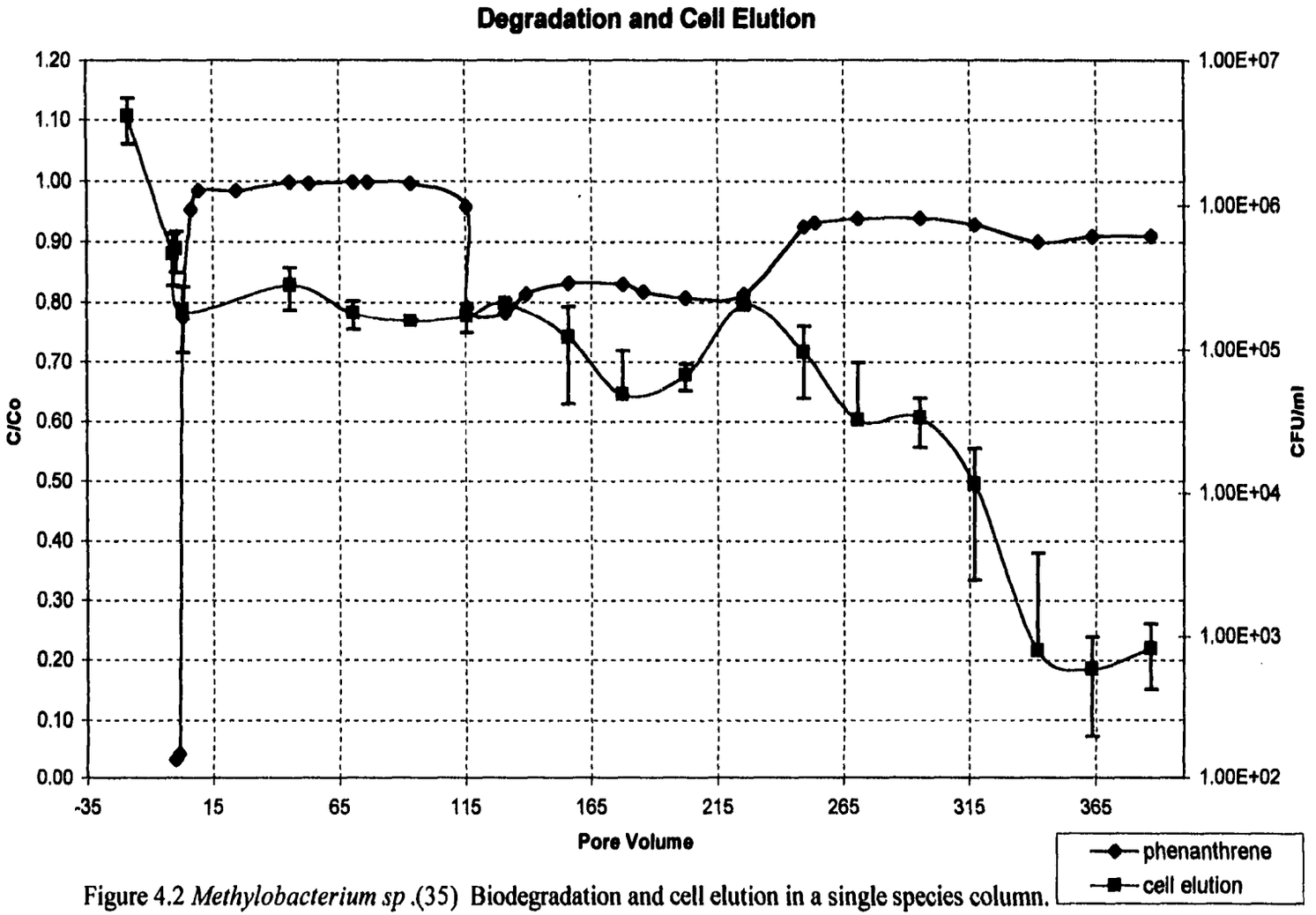

Figure 4.2 *Methylobacterium sp* .(35) Biodegradation and cell elution in a single species column.

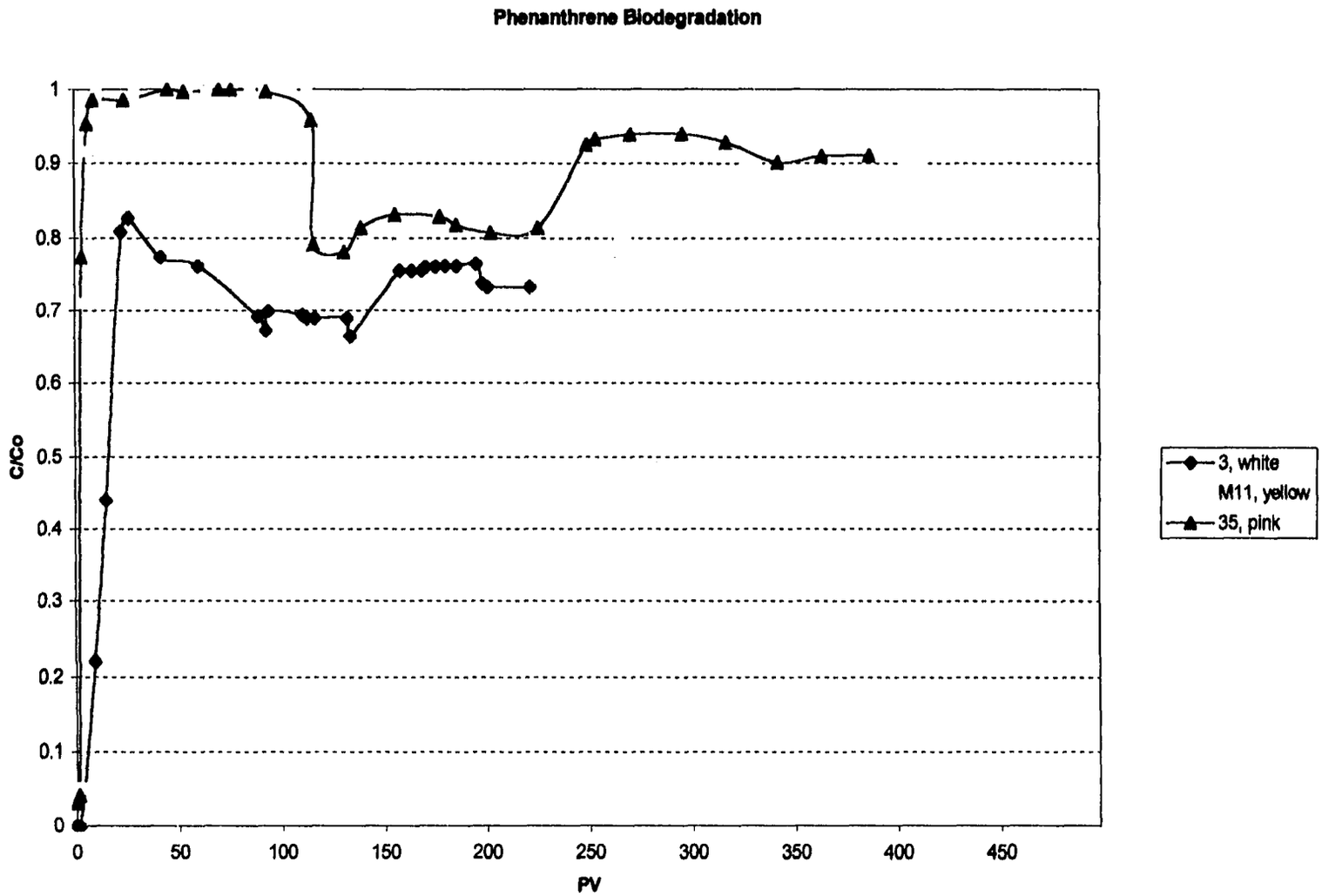

Figure 4.3. Comparison of single species biodegradation columns. *Actinobacter junii (3), Pseudamonas oleavorans (M11), Methylobacterium sp. (35).*

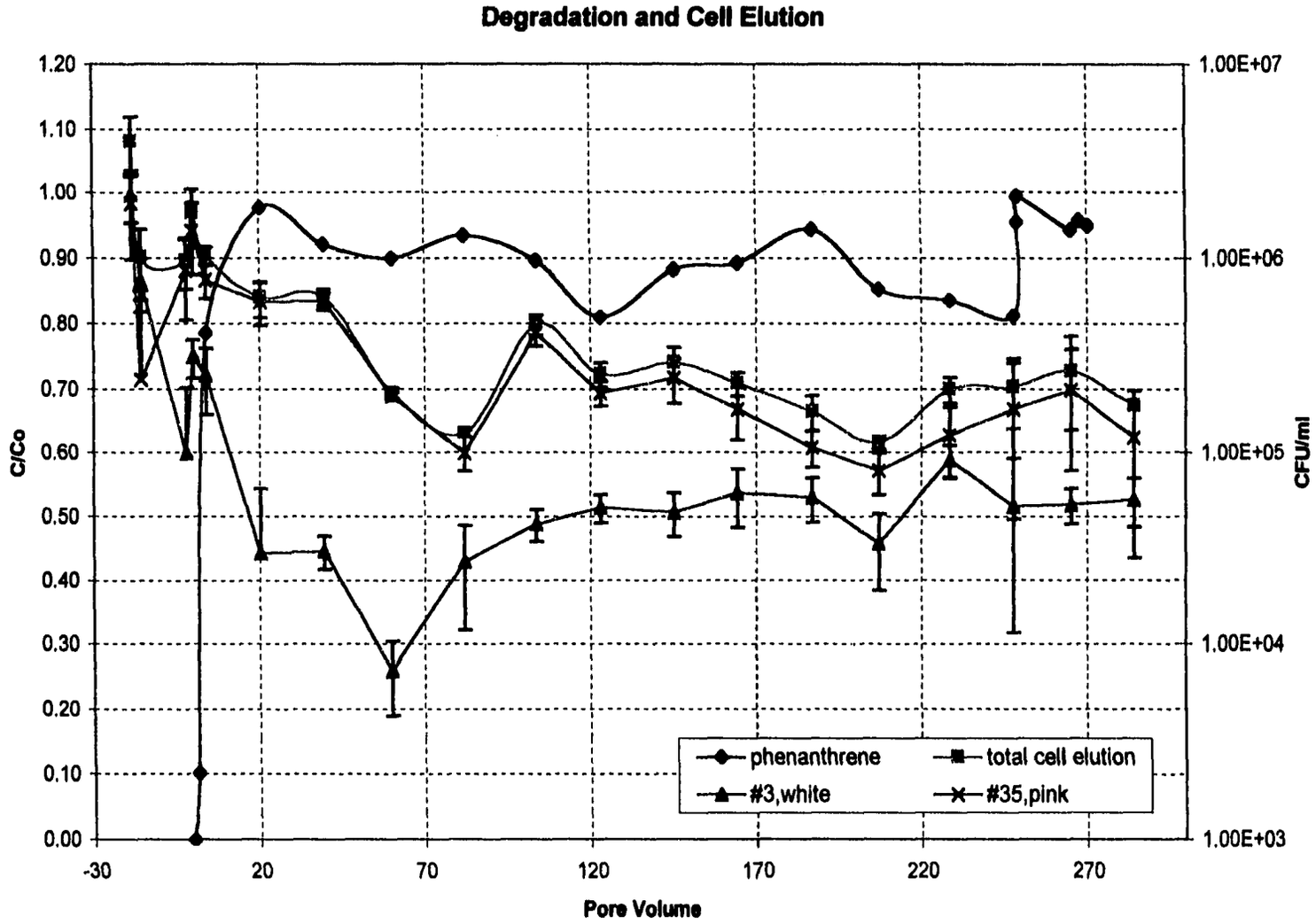

Figure 4.4. *Actinobacter junii (3) & Methylobacterium sp(35)* . Biodegradation and cell elution in a two column system.

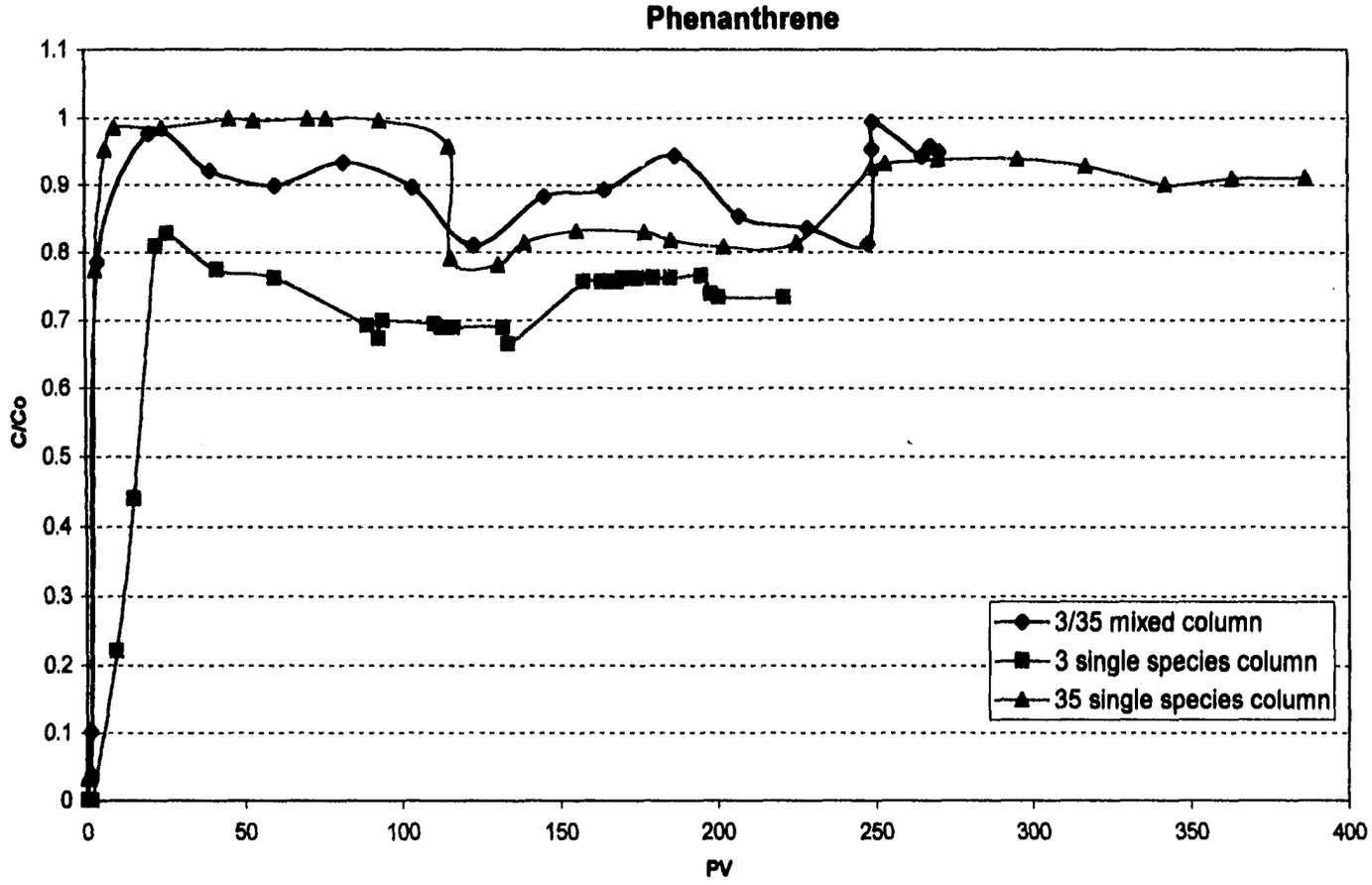

Figure 4.5 *Actinobacter junii(3) & Methylobacterium sp.(35)* biodegradation in a two species system in comparison with single species systems.

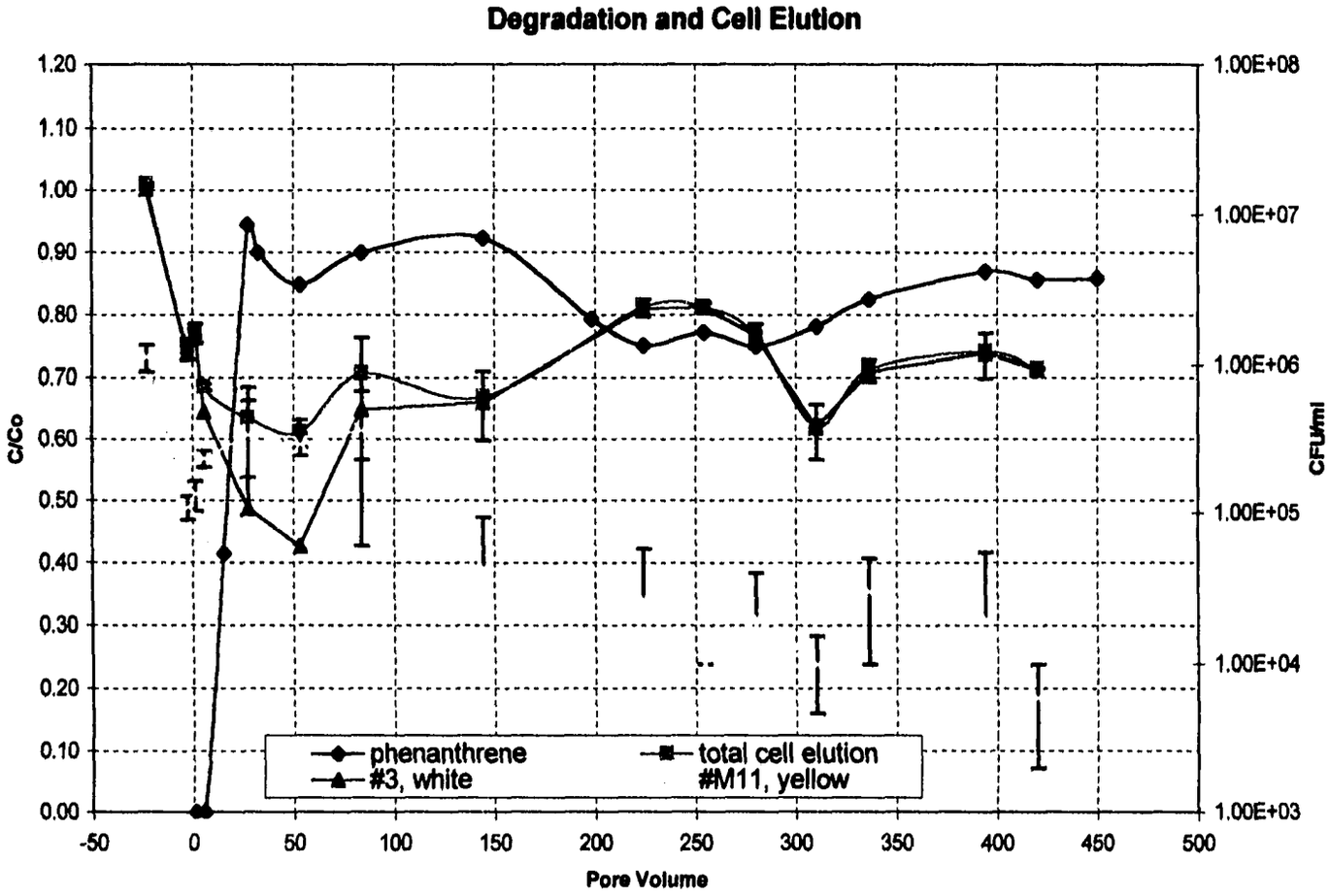

Figure 4.6 *Actinobacter junii (3) & Pseudamonas oleavorans (M11)* biodegradation and cell elution in a two species column system.

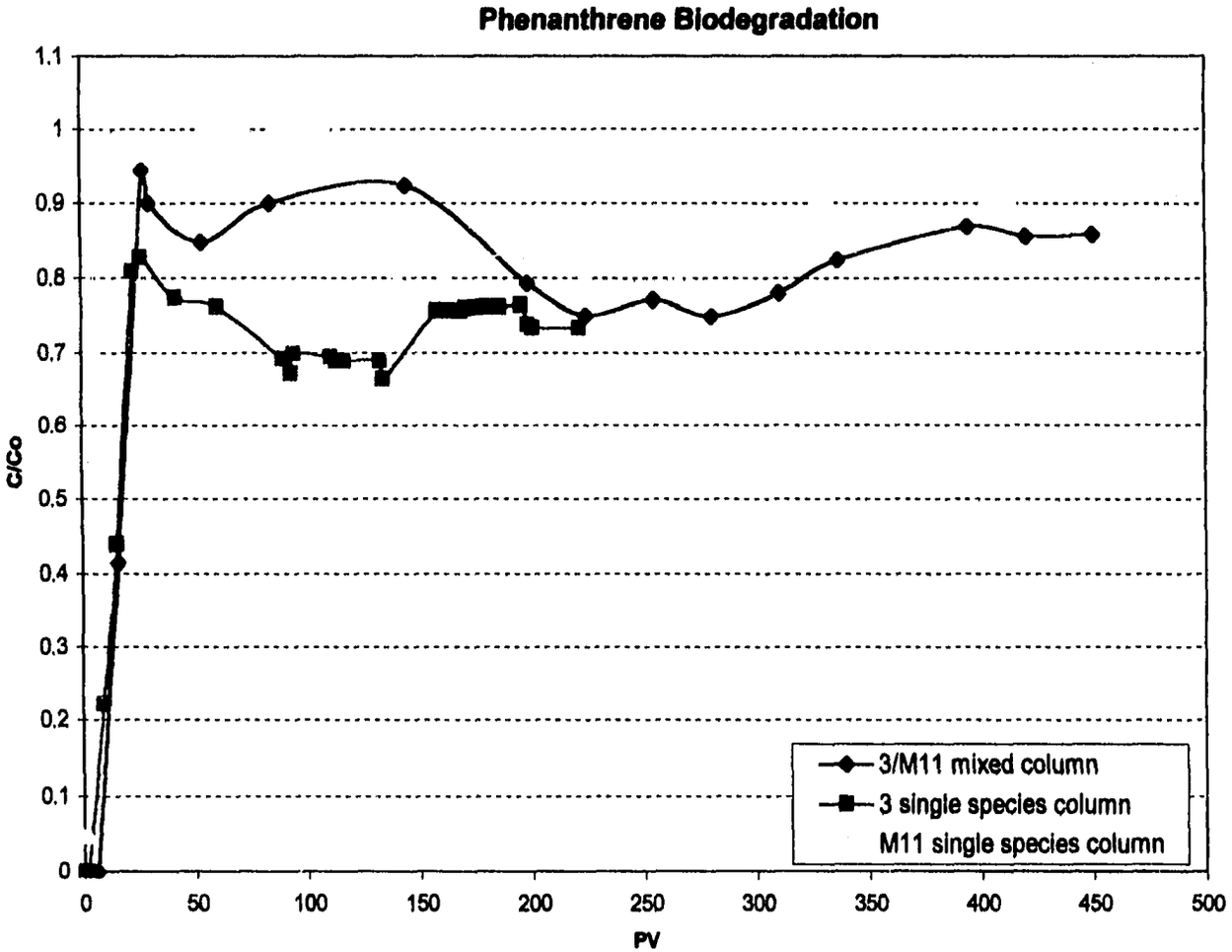

Figure 4.7 *Actinobacter junii(3) & Pseudamonas oleavorans(M11)* biodegradation and cell elution in a two species system in comparison with single species sytems.

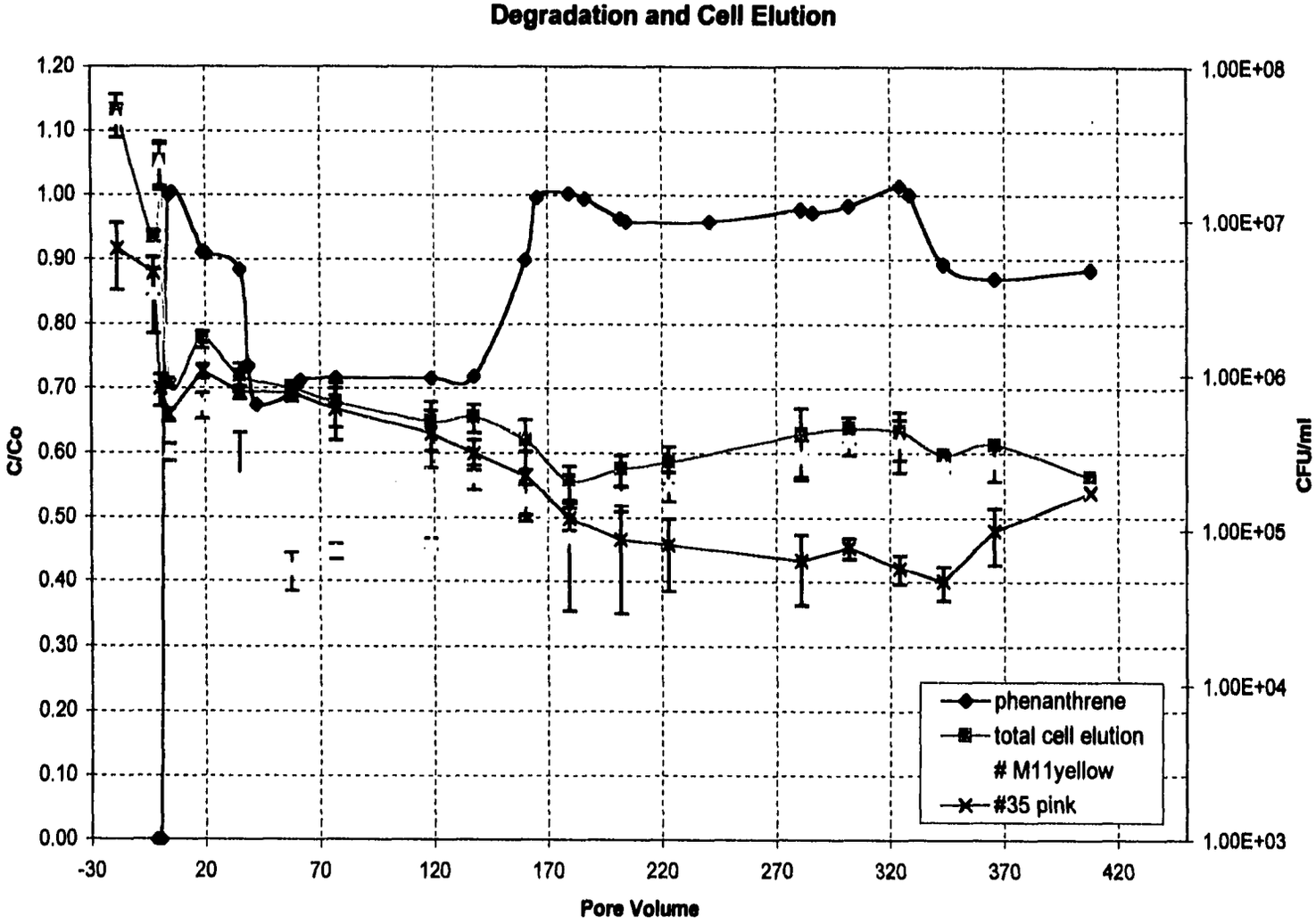

Figure 4.8. *Pseudamonas oleavorans(M11) & Methylobacterium sp* .(35) Biodegradation and cell elution in a two species column.

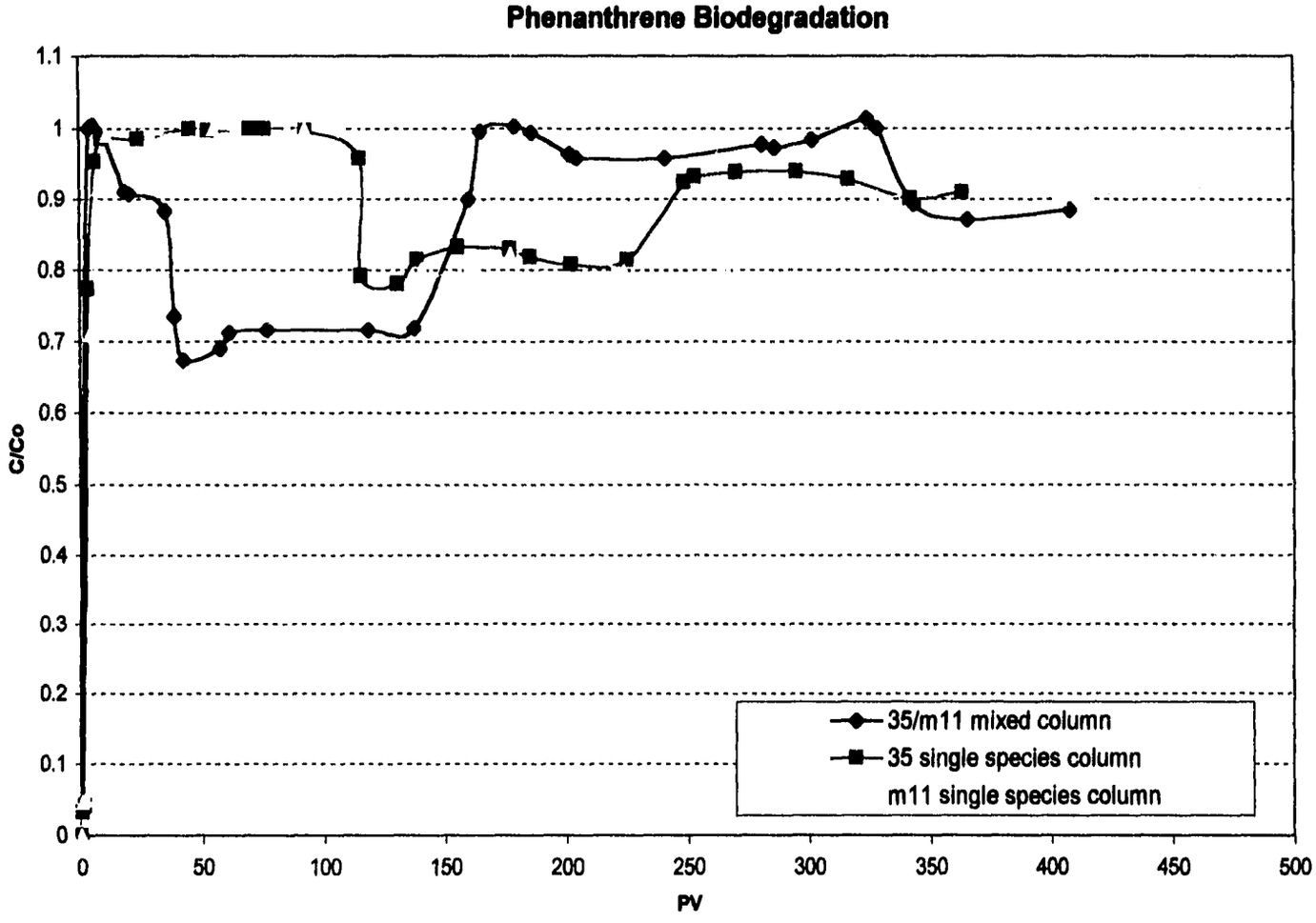

Figure 4.9 *Pseudamonas oleavorans & Methylobacterium sp*. biodegradation in a two species column in comparison with degradation in single species columns.

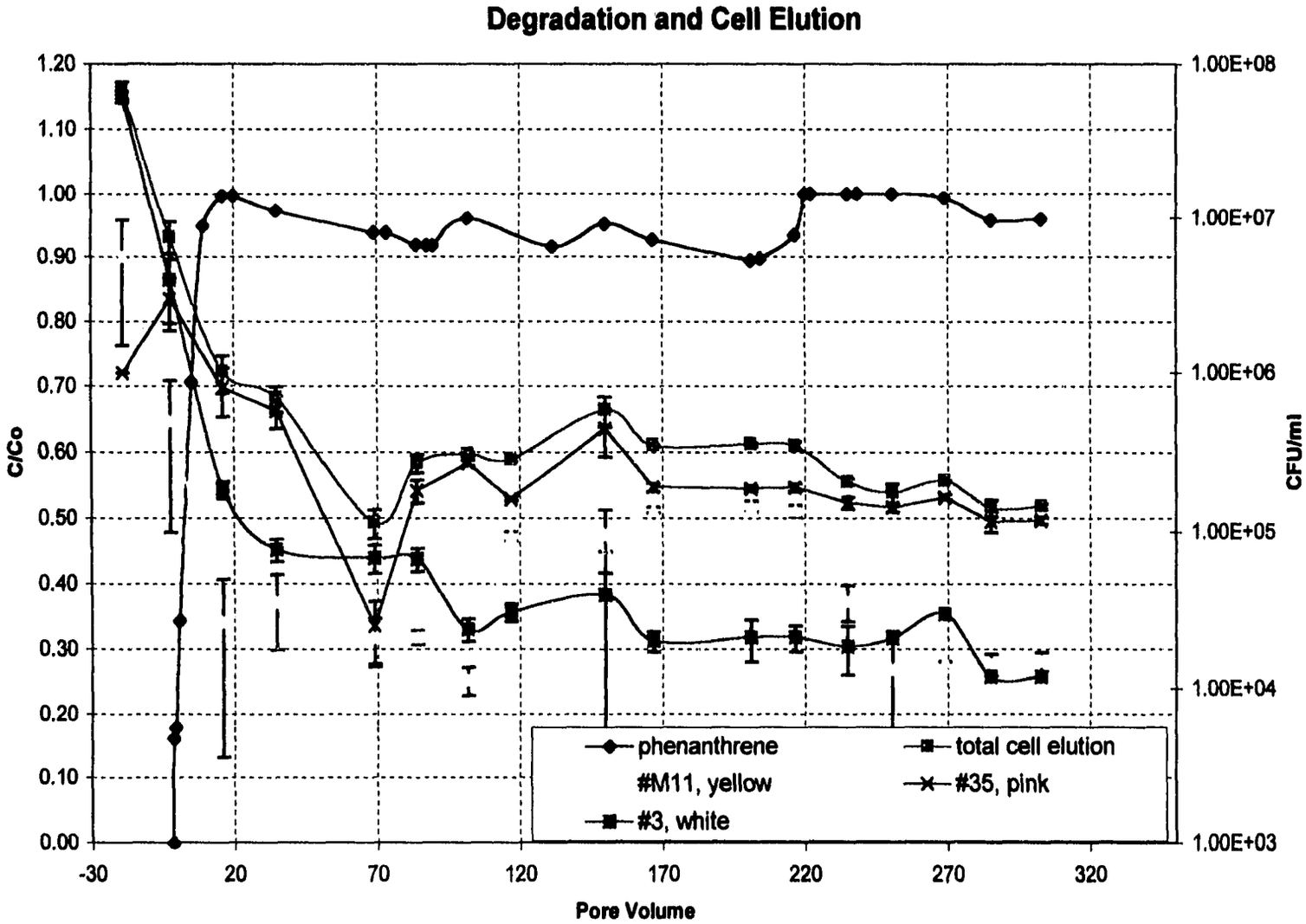

Figure 4.10 Biodegradation and cell elution in a three-species column system. *Actinobacter junii (3), Pseudamonas oleavoreans (M11), Methylobacterium sp. (35).*

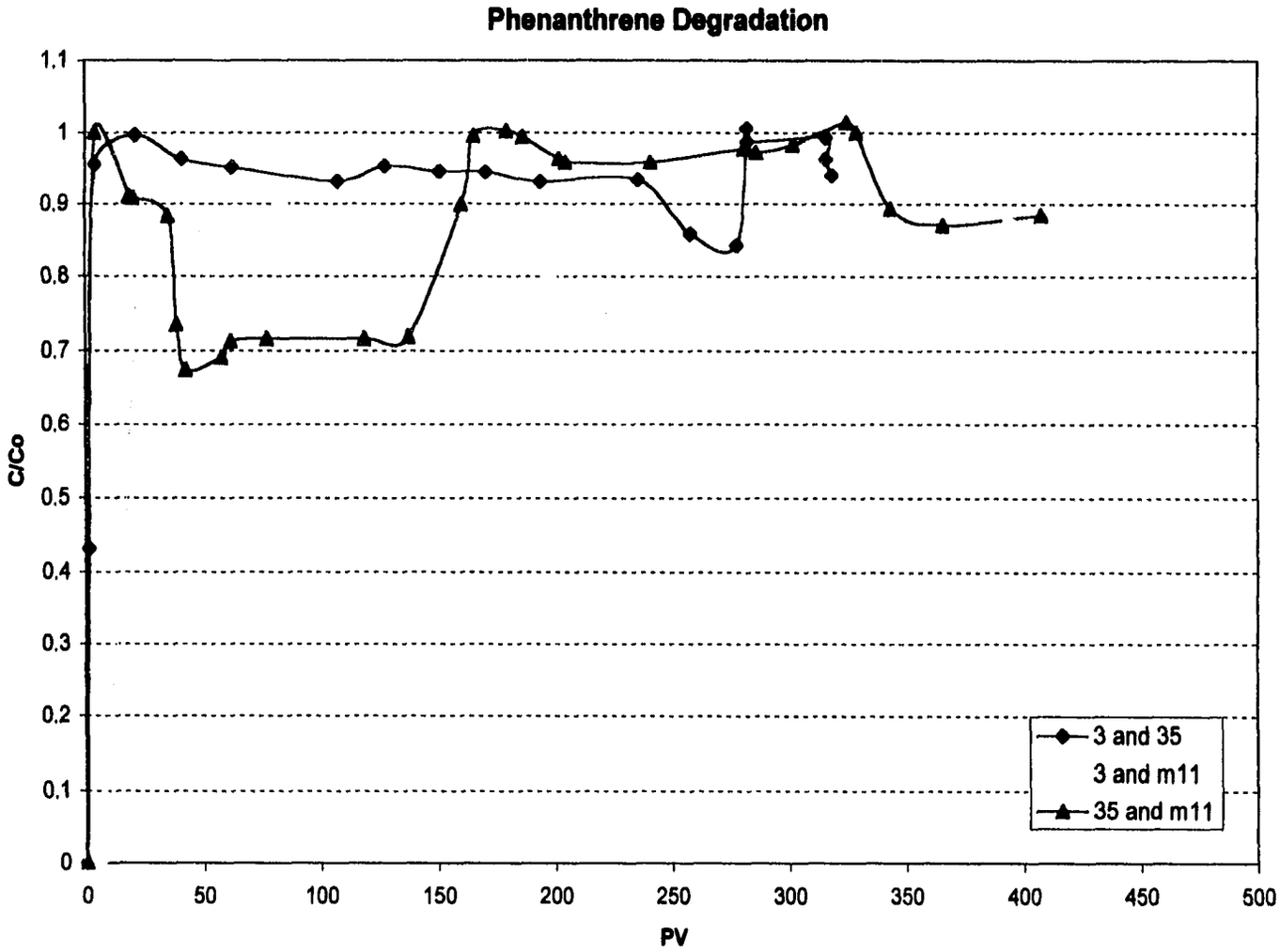

Figure 4.11 Comparisons between two species column systems. *Actinobacter junii (3), Pseudamonas oleavorans (M11), Methylobacterium sp. (35).*

CHAPTER 5: IMPACT OF NUTRIENT INJECTION ON BIODEGRADATION

Introduction

Nutrient (carbon) addition is a method commonly employed in bioremediation to aid in increasing cell numbers and biodegradation. However, the effects, other than possible increased degradation are not well defined. Therefore, this study is an introductory look at the effects of nutrient (carbon) addition to a system.

Materials and Methods

System setup: All experiments were conducted using a stainless steel column (Aldrich), 15 cm in length, homogeneously packed with 40x50 Acusand (silica sand approximately 297-420 um in diameter, minimal organic matter content). A SSI piston pump was used and a flow rate of approximately 9 cm/hr was induced. All experiments were conducted under saturated conditions. The following solutions were used throughout the experiment: Mineral Salts Broth (MSB) (KH_2PO_4, 1.0g/l; Na_2HPO_4, 1.0g/l; NH_4NO_3, 0.5g/l; $(NH_4)_2SO_4$, 0.5g/l; $MgSO_4$ $7H_2O$, 0.2 g/l; $CaCl_2$ $2H_2O$, 0.02g/l; $FeCl_3$, 0.002 g/l), phenanthrene (MSB and phenanthrene at approximately 1 mg/l), and phenanthrene/R_2B (10 ml R_2B for every 990 ml MSB, and phenanthrene at approximately 1 mg/l; R_2B contains yeast extract 0.5g/l; protease peptone no.3 0.5 g/l; casamino acids 0.5 g/l; dextrose 0.5 g/l; soluble starch 0.5 g/l; sodium pyruvate 0.3 g/l; dipotassium phosphate 0.3 g/l; magnesium sulfate 0.05 g/l)

Bacteria: Bacteria were pre-cultured in a solution of R_2B. A colony, taken from an R_2A plate, was used as the initial inoculum for a flask containing 25 ml R_2B. After 48 hours on a rotary shaker, 1 ml of pre-culture was transferred to a flask containing 25 ml of fresh R_2B and returned to a rotary shaker, 200rpm. Cultures were incubated an additional 24 hours before harvesting. At harvest, the contents of each flask were transferred to sterile centrifuge tubes, and centrifuged at 10,000 rpm for 10 minutes. The supernatant was decanted and the resulting pellet resuspended in 25 ml saline solution and centrifuged. Once again, the supernatant was decanted and the pellet resuspended in 10 ml mineral salts broth. The resulting mixture contained approximately 10^8 cfu/ml, as determined by plate counts. Exact inoculum densities and distribution within the column may be found in Appendix B.

Experimental Design: Column porous media was sterilized via autoclave (1 hour), inoculated with a bacterial cell culture, and then homogeneously packed into a sterile, stainless steel column. Porous media samples were sequentially taken 3 times during the packing process and plated in order to determine the total number of bacteria initially present and initial distribution of bacteria in the column. Once connected to a pre-sterilized pump (sterilization via 30 minutes bleach, 5 minutes sodium thiosulphate, 30 minutes sterilized deionized water), the column was saturated with mineral salts broth for a minimum of 18 hours to achieve complete saturation. A continuous flow of phenanthrene broth (MSB containing approximately 1 mg/l phenanthrene) was then begun. After

several days, the phenanthrene solution was replaced with the phenanthrene/R_2B solution. The phenanthrene/ R_2B solution pulse lasted for at least 24 hours but not more than 48 hours after which the solution was returned to phenanthrene only. Experiments were conducted in pure culture with a single species and in a mixed culture where all three species were inoculated. Inoculation densities were as follows: single species *Actinobacter junii* $1.99x10^7$ CFU/g sand, *Pseudomonas oleavorans* $1.18x10^8$ CFU/g sand, *Methylobacterium sp.* $4.88x10^7$ CFU/g sand; mixed species *Actinobacter junii* $4.51x10^7$ CFU/g sand, *Pseudomonas oleavorans* $7.44x10^6$ CFU/g sand, *Methylobacterium sp.* $6.40x10^6$ CFU/g sand. Phenanthrene effluent concentration and dissolved oxygen concentration (mV), as well as bacterial elution (CFU/ml- determined by plate counts) were monitored throughout the course of the experiment. At the end of the experiment, the column was dissected using sterile technique and porous media samples plated to determine final bacterial count and distribution. Replicate columns were prepared in the same manner.

Result and Discussion

Acinetobacter junii initially degraded phenanthrene to a C/Co of 0.65. Degradation increased, resulting in a decrease in C/Co to 0.24, upon addition of R_2B. Degradation returned almost to pre-R_2B levels (C/Co= 0.72) after the pulse was terminated (Figure 5.0). A dramatic increase in cell elution occured during the R_2B pulse. Cell elution was found to be 1.14 times greater per pore volume during the pulse than during non-pulse

time (Figure 5.0). It was determined that a net increase in culturable cells did not occur over the time course of the experiment (Table 5.0).

Pseudamonas oleovorans initially degraded phenanthrene to a C/Co of 0.92 (Figure 5.1). Degradation of phenanthrene increased slightly upon R_2B addition, after which degradation then decreased. Cell elution during the R_2B pulse increased dramatically (Figure 5.1), accounting for 2.6 times more cell elution per pore volume than occurred during non-pulse time (Table 5.0).

Methylobacterium sp. degraded phenanthrene to a C/Co of 0.86 prior to the R_2B pulse (Figure 5.2). However, during the R_2B pulse, phenanthrene concentration increased to almost C/Co = 1. Phenanthrene degradation after the R_2B pulse stabilized at a C/Co of 0.86 for 66 pore volumes, suddenly increasing to C/Co of 1 thereafter. *Methylobacterium sp.* did not exhibit a significant increase in cell elution during the R_2B pulse (Figure 5.2). The experiment yielded neither a net increase nor decrease in cell numbers (Table 5.0).

Addition of nutrients to the system did not affect all three bacterial species in the same manner. The increased degradation coinciding with the R_2B pulse in the *Actinobacter junii* column is quite interesting. Cells did not experience net growth in the column and the inoculated cell density was $1x10^7$ CFU/g soil, which is sufficient to degrade phenanthrene at measurable levels. Thus, it is reasonable to say that the increase in degradation was a non-numeric (i.e. no change in cell growth/numbers) response. This suggests that either R_2B provides an additional nutrient needed for degradation that is in

limiting supply or that phenanthrene degradation by *Actinobacter junii* is somewhat co-metabolic.

Previous investigations (Chapter 4) showed that *Actinobacter junii* and *Methylobacterium sp.* single-species systems that received only phenanthrene did not produce additional culturable bacterial cells. However, pure-culture investigations with *Pseudomonas oleavorans* did produce an increase in culturable bacteria. What is also interesting to note is that in this series of experiments the R_2B pulse decreased the amount of cell death in the *Actinobacter junii* and *Methylobacterium sp.* columns, as compared to that which occurred in the those single-species experiments with no R_2B addition (Table 5.1). Conversely, the addition of a second carbon source was detrimental to the *Pseudomonas oleavorans* population (Table 7.1). The decreased death in the *Actinobacter junii* and *Methylobacterium sp.* columns was most likely due to the additional nutrients provided by the R_2B solution. The addition of nutrients to the *Pseudomonas oleavorans* system was not necessary. In fact, it decreased *Pseudomonas oleavorans'* ability to produce new, culturable bacteria possibly due to a toxic build-up of by-products or some other form of system stress. Similar behavior has been see for other substrates, such as salicylate (Sandrin et al., 2001) Where large concentrations of salicylate become toxic and inhibit growth.

Cell elution during the R_2B pulse increased dramatically in *Actinobacter junii* and *Pseudamonas oleavorans* columns. The instantaneous response of the cells to the R_2B pulse suggests that the increase in cell elution is a chemical response in which a cell

"adhesion" changes upon addition of R_2B. This is not uncommon and the literature contains several examples where the introduction of a particular substrate results in an increase in cell elution (Sandrin et al., 2001; Yolcubal et al., in review). Based on this evidence, it is reasonable to conclude that the increase in cell elution during the R_2B/phenanthrene pulse is due to a chemical response.

During the pulse of R_2B in the three species column, a significant increase in C/Co phenanthrene was observed. This indicates a decrease in phenanthrene degradation occurred upon addition of R_2B (Figure 5.3). In addition, during the R_2B/phenanthrene pulse an increased cell elution response was elicited from *Actinobacter junii* and *Pseudamonas oleavorans*, but not detected for *Methylobacterium sp.*.

Conclusions

R_2B, a second carbon source, impacts the three-species system by causing decreased phenanthrene degradation despite the system containing *Actinobacter junii*, which, when alone, degrades more phenanthrene in the presence of R_2B. Thus, the addition of an additional carbon source is not always "beneficial" in terms of increasing degradation. The reduction in phenanthrene degradation with the addition of R_2B to the three species column has several possible explanations. The first is that the density of *Actinobacter junii* in the system was not sufficient to produce noticeable degradation even with the addition of the R_2B. Final plate counts revealed approximately $5x10^6$ CFU/g sand *Actinobacter junii* in the column. This amount of bacteria should be sufficient to produce

detectable phenanthrene degradation.

Another possible explanation for the lack of phenanthrene degradation during the pulse is that either *Pseudamonas oleavorans* or *Methylobacterium sp.* (or both) were able to out-compete *Actinobacter junii* for the R_2B. As shown in the previous chapter, a single-species *Actinobacter junii* column showed increased phenanthrene degradation during the R_2B pulse. With competition occurring in a multiple species column, R_2B may have been preferentially consumed by *Pseudomonas oleavorans* and *Methylobacterium sp.*, and would therefore not have been available to aide *Actinobacter junii* in phenanthrene degradation. This is supported by the results of a mixed-culture batch experiment which shows that *Pseudomonas oleavorans* out competes the other two species for R_2B when all three species are present in an R_2B solution. Specifically, as shown in Figure 5.4, *Pseudomonas oleavorans* exhibits the largest increase in culturable cells over time. Therefore, it is reasonable to conclude that an increase in phenanthrene degradation, as was observed for the *Actinobacter junii* single-species system, did not occur for the three-species system because *Pseudomonas oleavorans* was able to out- compete *Actinobacter junii* for the R_2B. The second carbon source impacts the system by causing decreased phenanthrene degradation despite the potential of the system to increase in degradation due to the presence of *Actinobacter junii*. Thus, the addition of an additional carbon source may not always be "beneficial" in terms of increasing degradation, depending on specific interactions within the community.

Table 5.0. Single species cell elution and growth in an R_2B pulsed column. Values given are a range (minimum possible to maximum possible) as calculated using 95% confidence intervals.

	cells eluted/ initial number of cells inoculated	final number of cells remaining in the column/ initial number inoculated	cell lost /pv in R_2B pulse vs cell lost /pv w/o pulse	growth
Actinobacter junii	0.153 to 0.153	-0.174 to 0.286	1.14 to 1.14	-1.26×10^9 to 1.23×10^9
Pseudomonas oleavorans	0.594 to 0.594	-0.071 to 0.138	2.61 to 2.61	-3.84×10^9 to -3.98×10^9
Methylobacterium sp.	0.619 to 0.619	0.021 to 0.209	0	-1.14×10^9 to 9.74×10^8

Table 5.1. Compares growth in a single species column to that of a single species column that received an R_2B pulse. Values given are in CFU/g sand, and indicate a 95% confidence interval.

	Cell growth in a pulsed column	Cell growth in a non-pulsed column
Actinobacter junii	-1.26×10^9 to 1.23×10^9	-1.61×10^9 to -1.63×10^9
Pseudomonas oleavorans	-3.84×10^9 to -3.98×10^9	4.97×10^{10} to 5.00×10^{10}
Methylobacterium sp.	-1.14×10^9 to 9.74×10^8	-4.11×10^{10} to -4.26×10^{10}

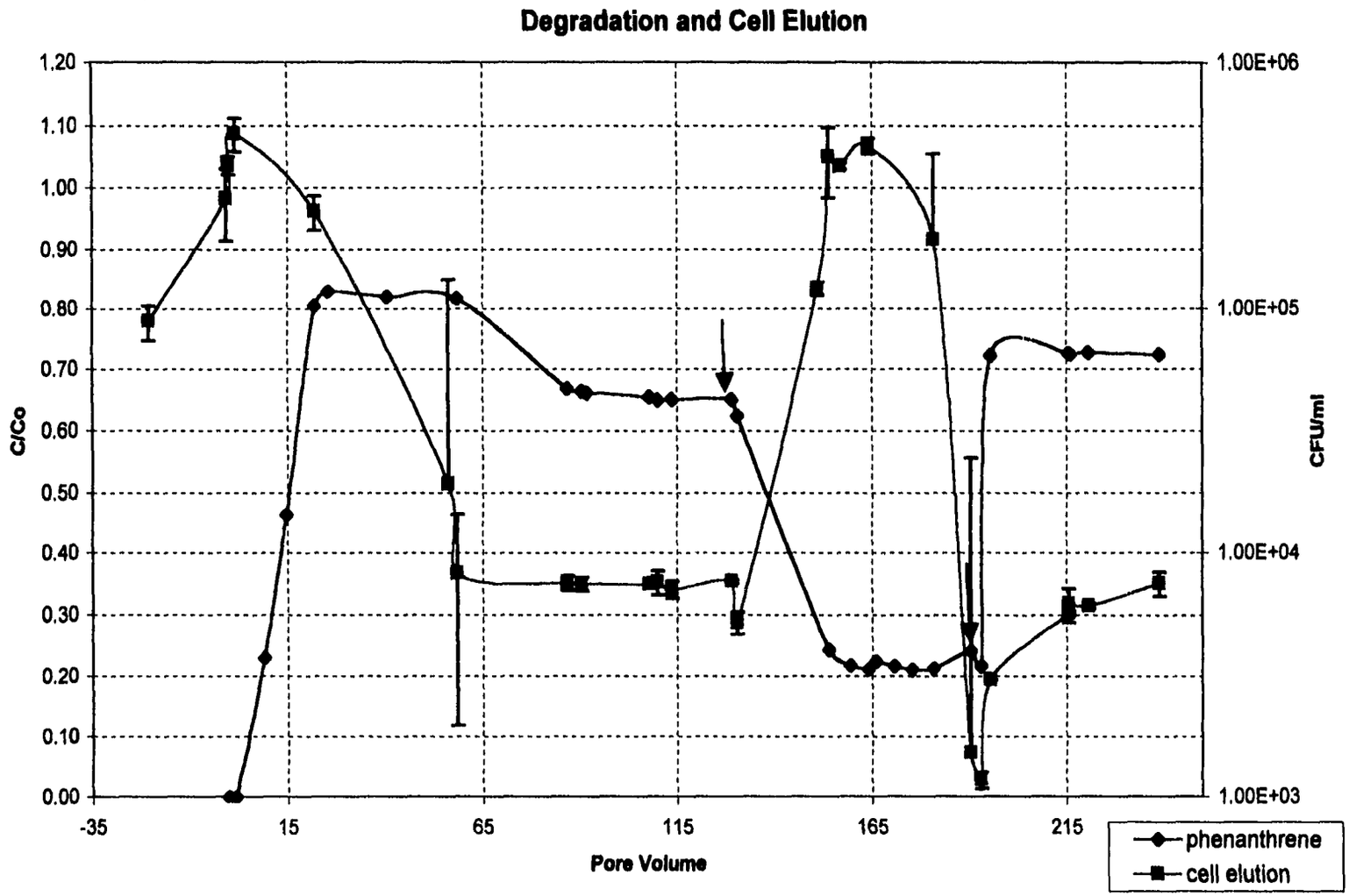

Figure 5.0 *Actinobacter junii* (3) biodegradation and cell elution in a single species system with an R_2B pulse. Arrows indicate the start/stop of the pulse

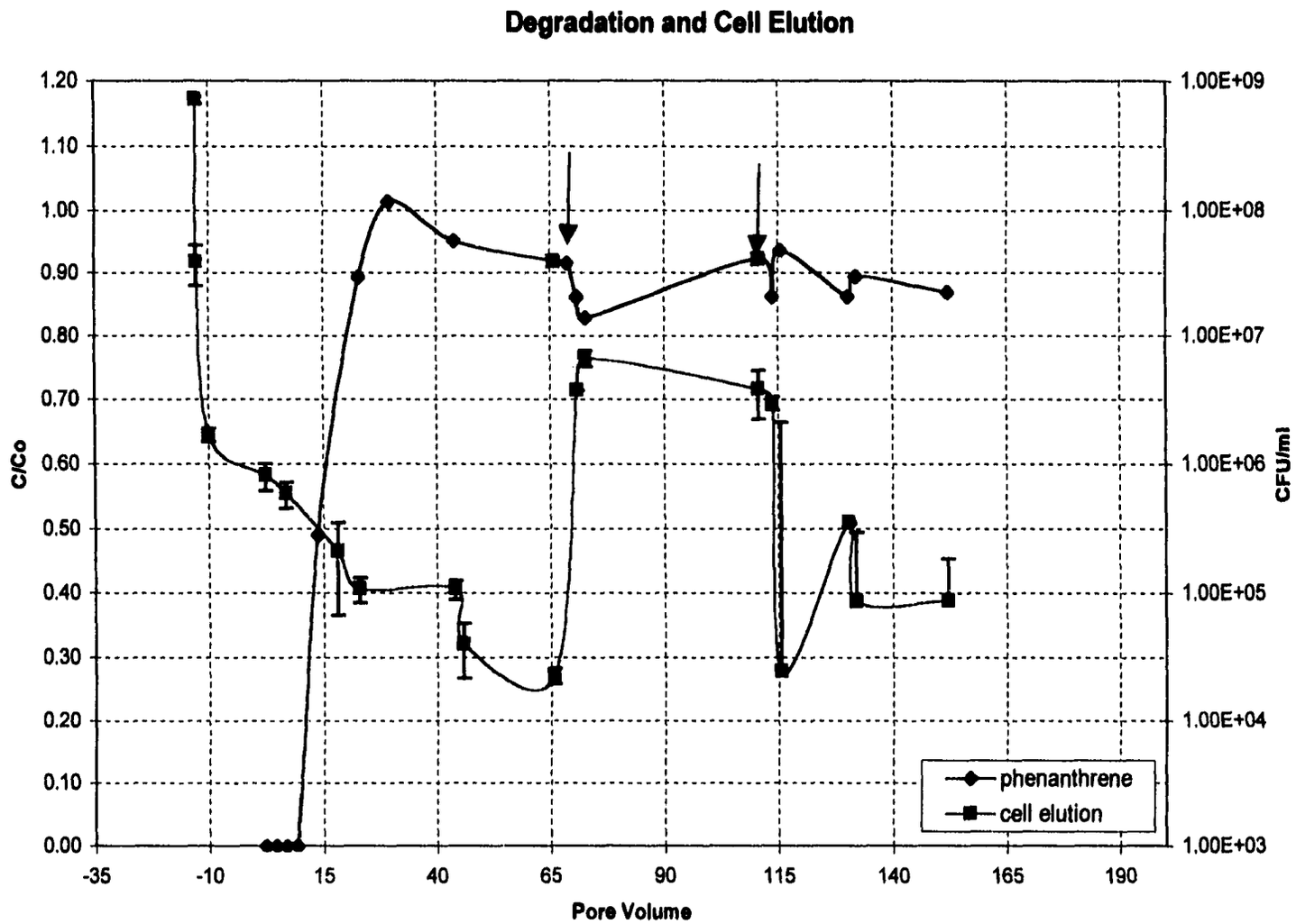

Figure 5.1 *Pseudamonas oleavorans* (M11) biodegradation and cell elution in a single species system with an R_2B pulse. Arrows indicate the start/stop of the pulse.

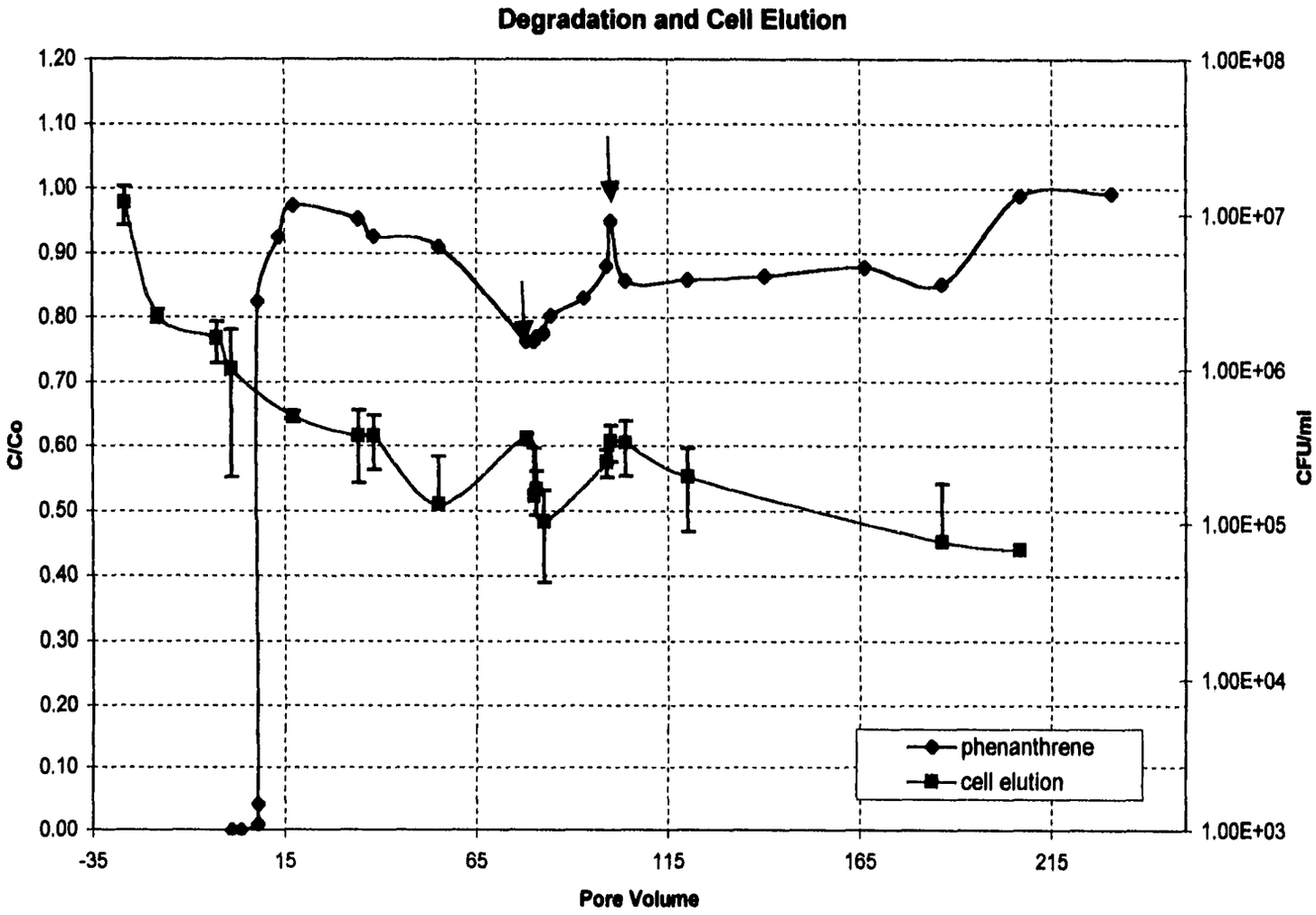

Figure 5.2 *Methylobacterium sp.* (35) biodegradation and cell elution in a single species system with an R_2B pulse. Arrows indicate the start/stop of the pulse.

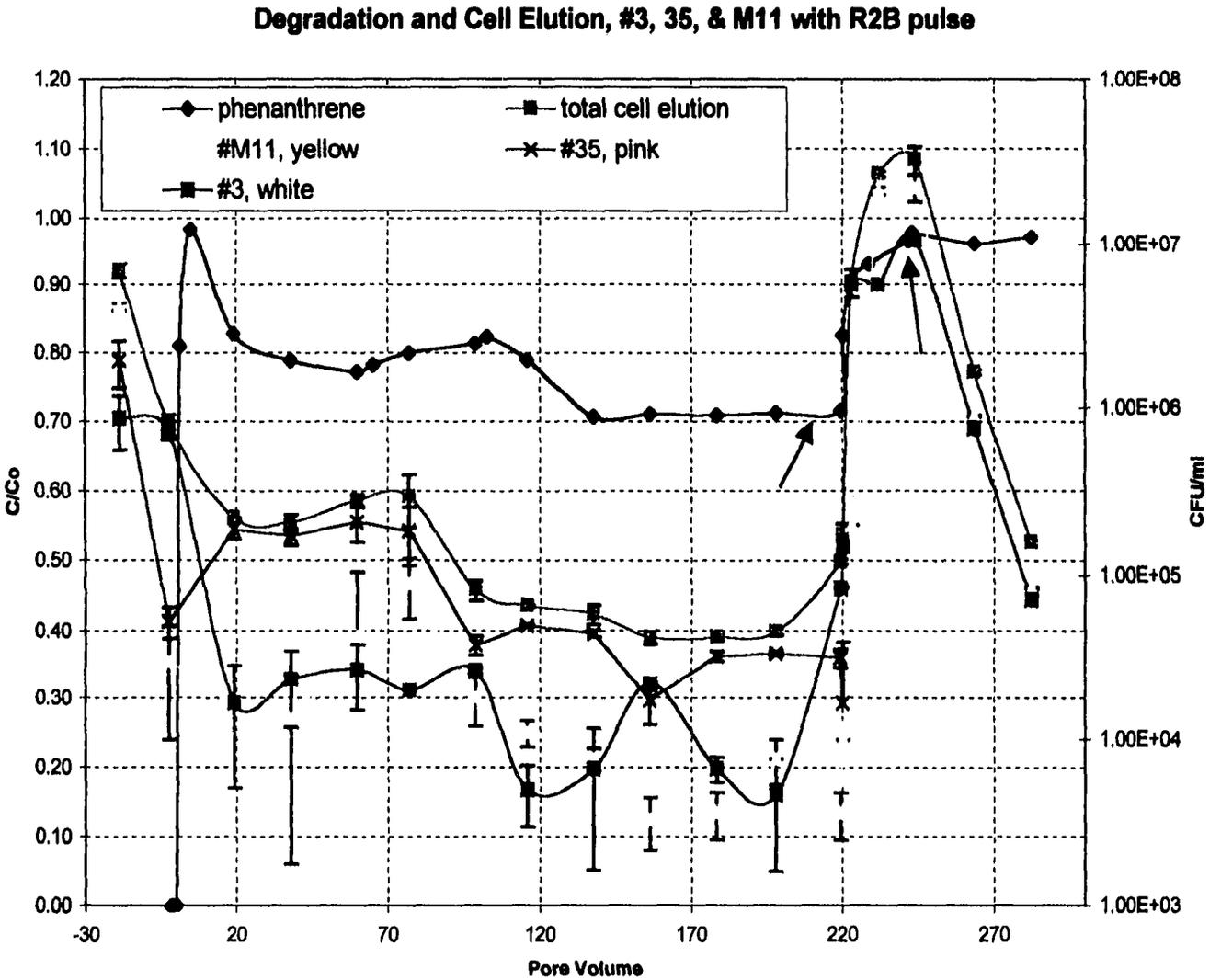

Figure 5.3. Biodegradation and cell elution in a multi-species system with an R_2B pulse. *Actinobacter junii (3), Pseudamonas oleavorans (M11), Methylobacterium sp. (35)*. Arrows indicate start/stop of the pulse.

APPENDIX A: COLUMN REPLICATE DATA

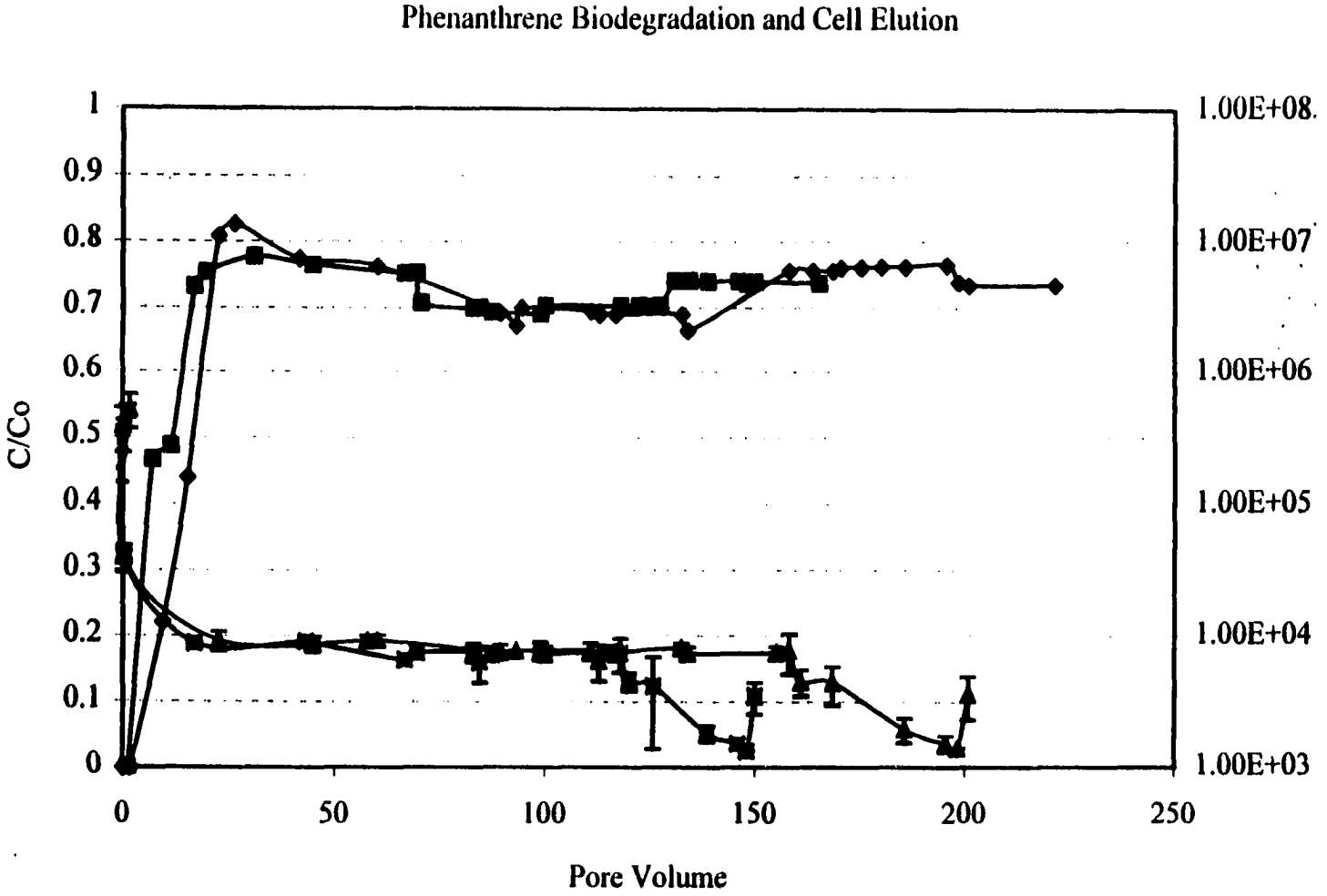

Figure A.1. Phenanthrene biodegradation. *Actinobacter junii* (3). Cell Elution. *Actinobacter junii* (3). Replicate experiments shown.Replicate experiments are shown.

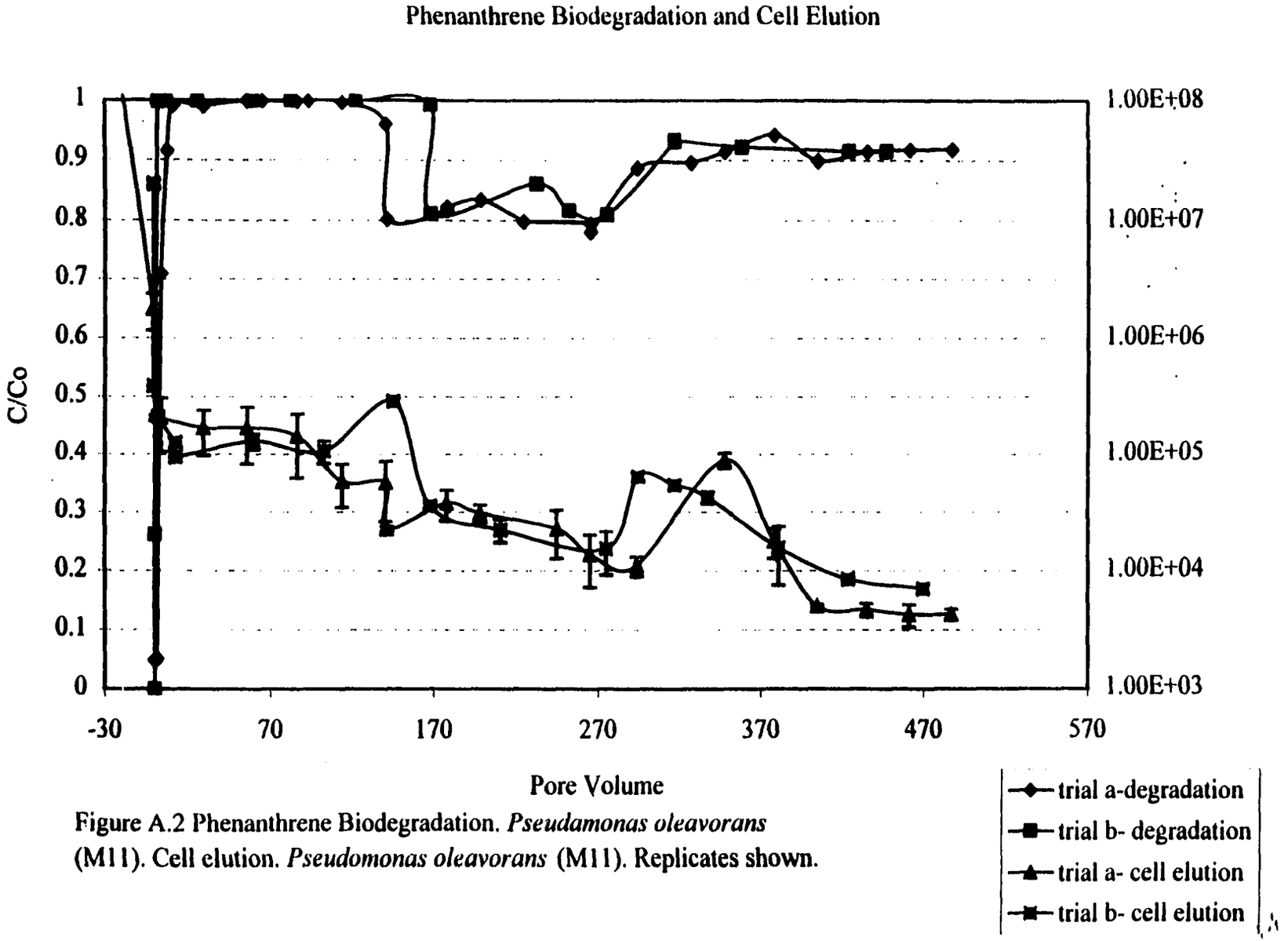

Figure A.2 Phenanthrene Biodegradation. *Pseudamonas oleavorans* (M11). Cell elution. *Pseudomonas oleavorans* (M11). Replicates shown.

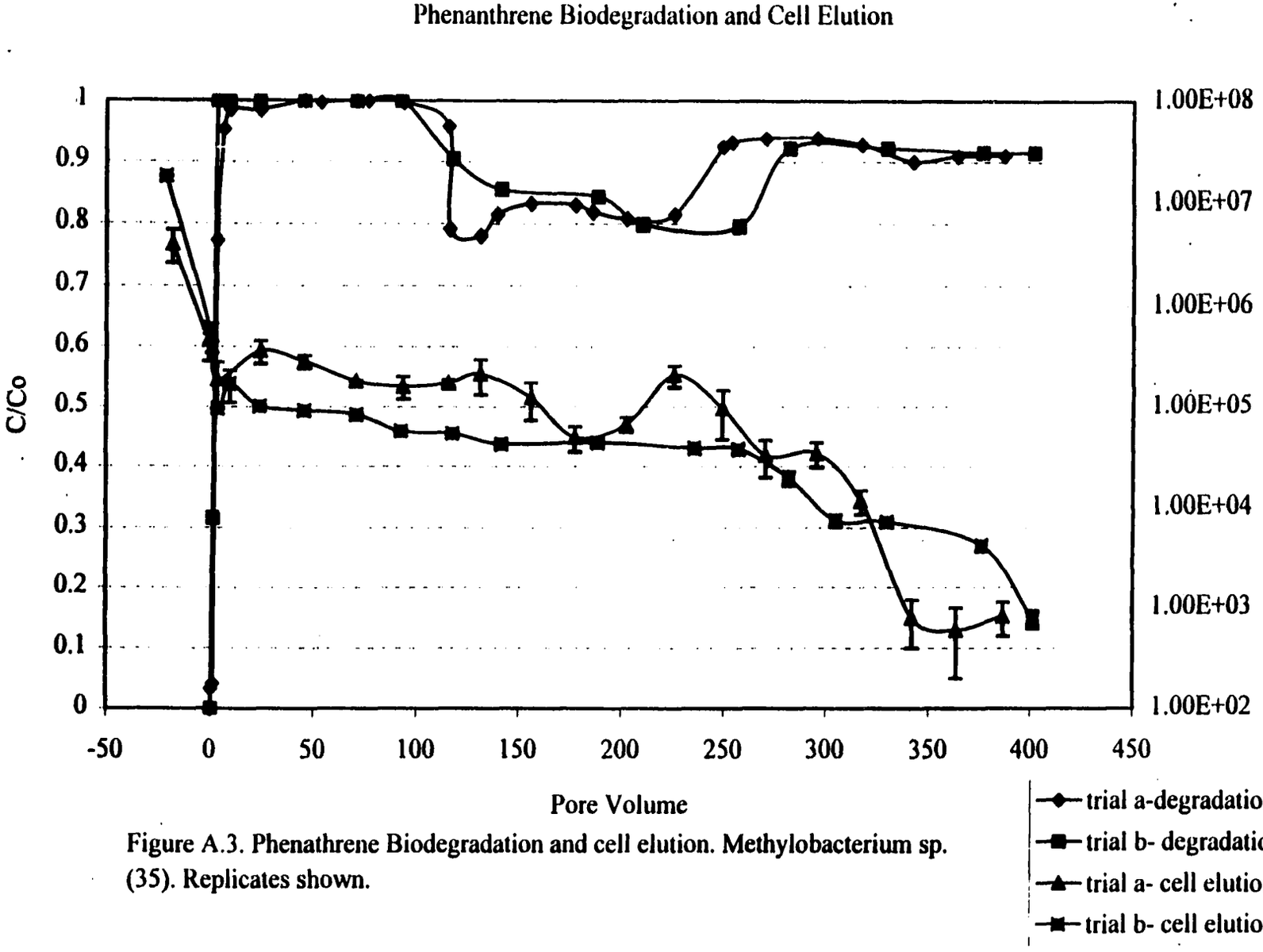

Figure A.3. Phenathrene Biodegradation and cell elution. Methylobacterium sp. (35). Replicates shown.

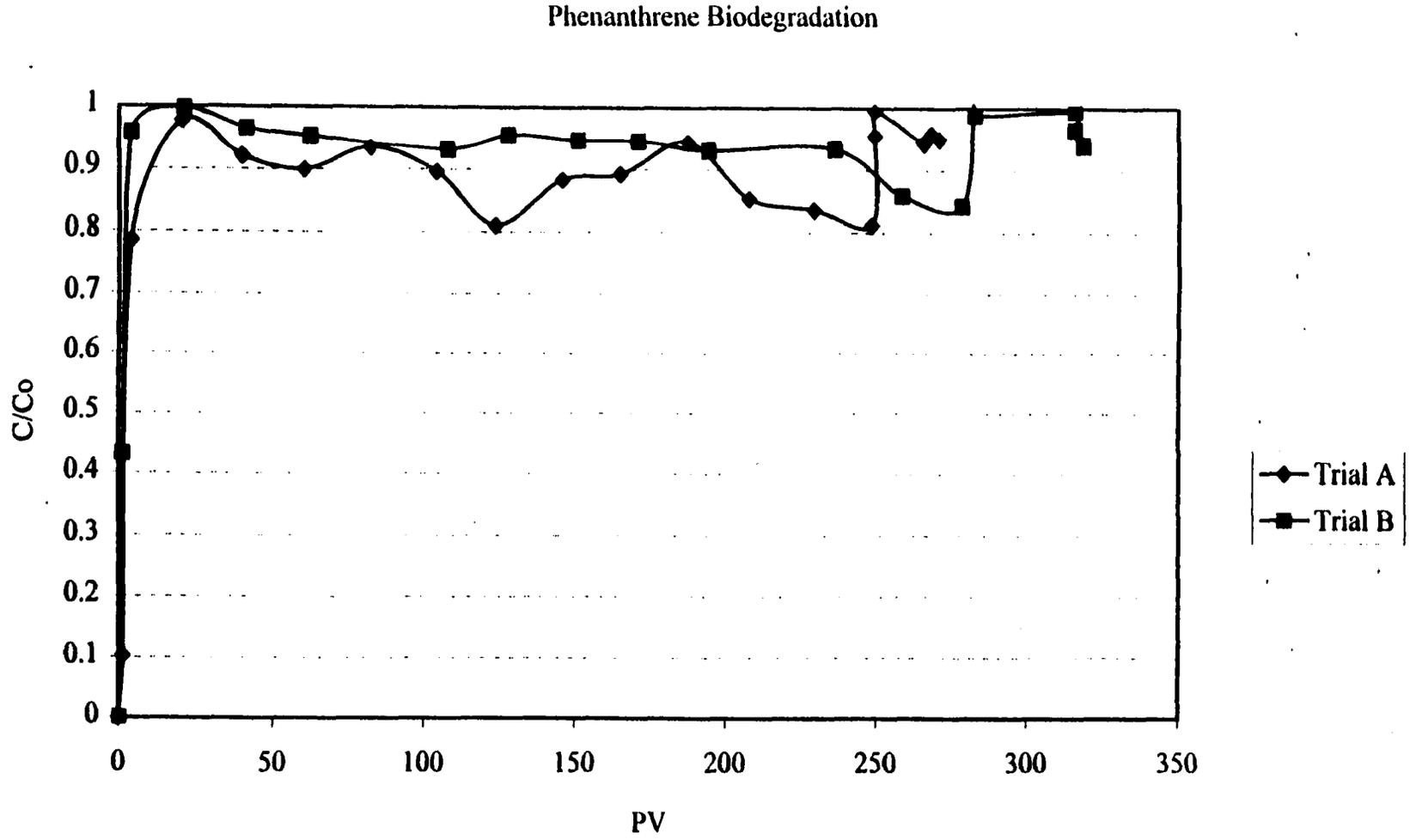

Figure A.4 Phenanthrene Biodegrdation. *Actinobacter junii* (3) and *Methylobacterium sp.* (35) mixed column. Replicates shown.

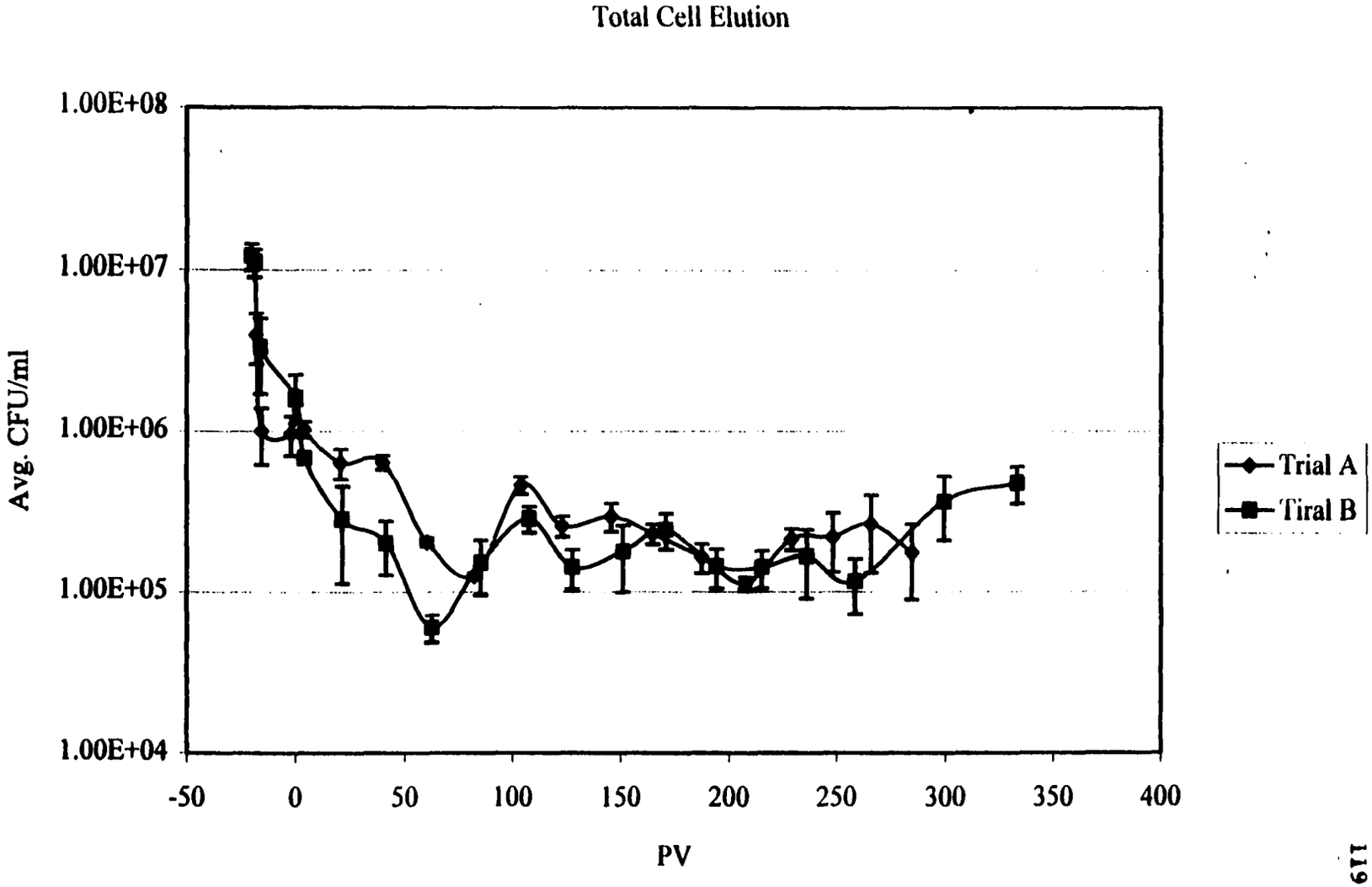

Figure A.5 Total cell elution from a mixed species column (*Actinobacter junii* (3) and *Methylobacterium sp* (35). Replicates shown.

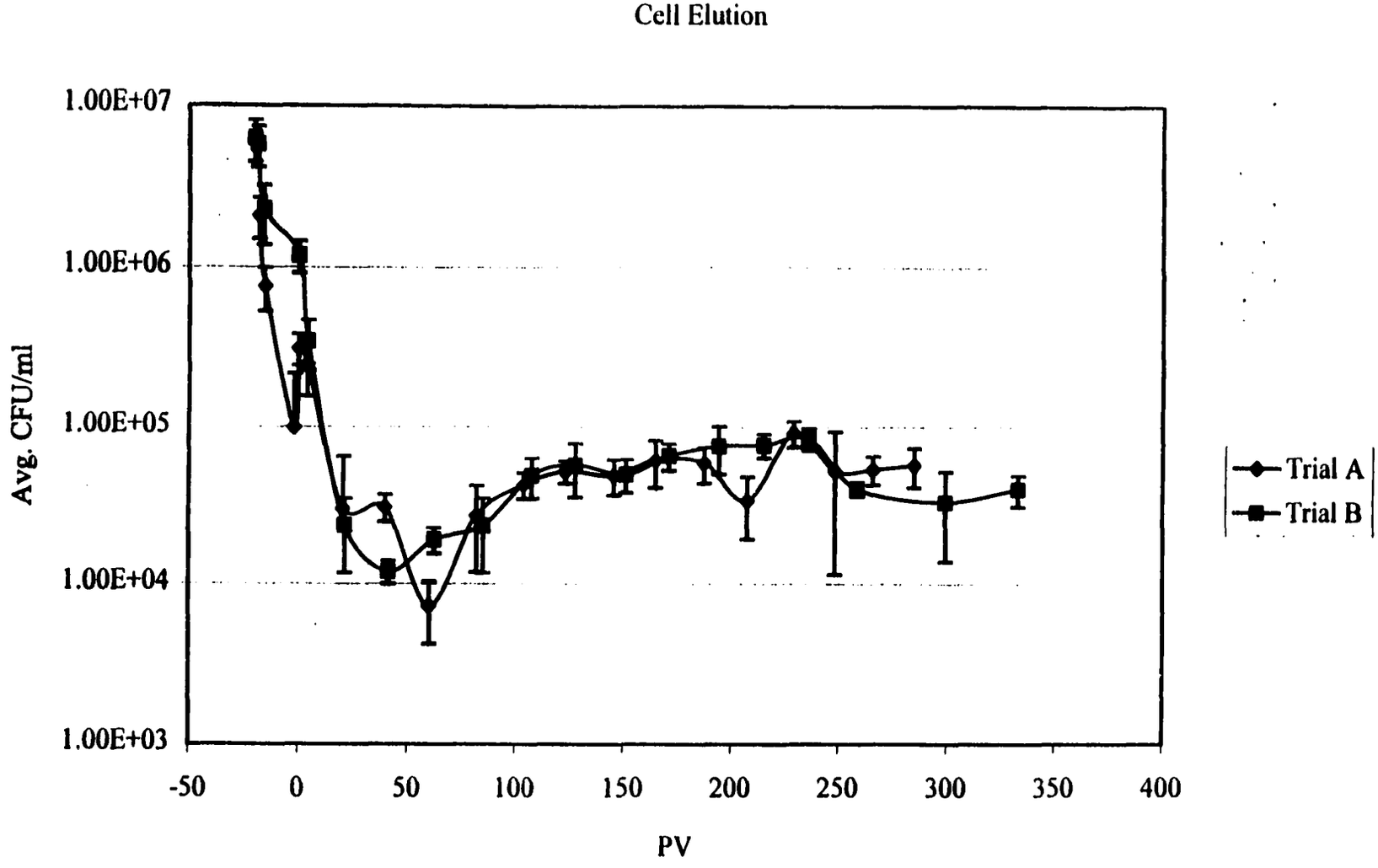

Figure A.6. *Actinobacter junii* (3) cell elution from a mixed species column (*Actinobacter junii* and *Methylobacterium sp.* (35). Replicates shown.

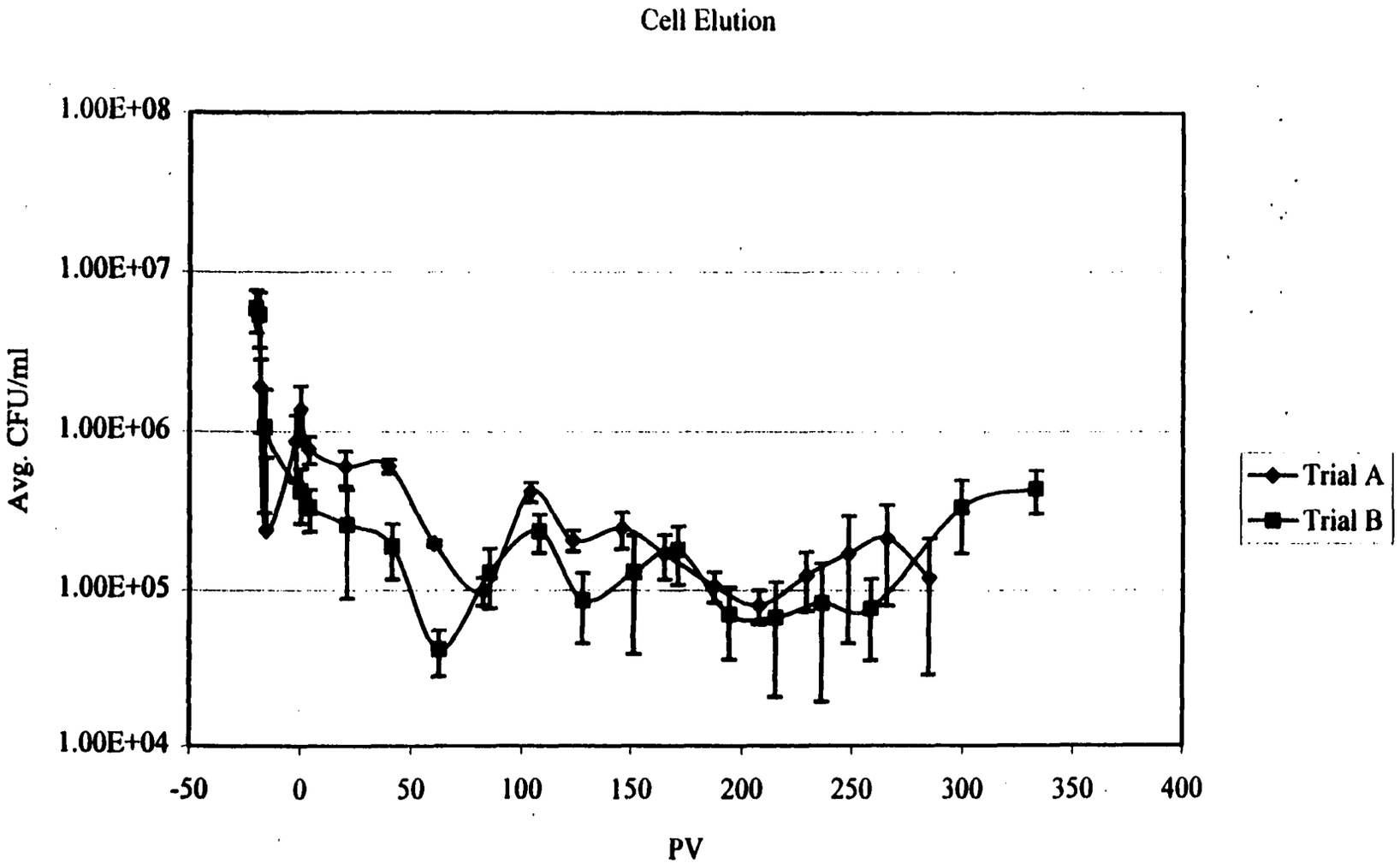

Figure A.7. *Methylobacterium sp*. (35) cell elution from a mixed species column (*Actinobacter junii* (3) and *Methylobacterium sp*. (35). Replicates shown.

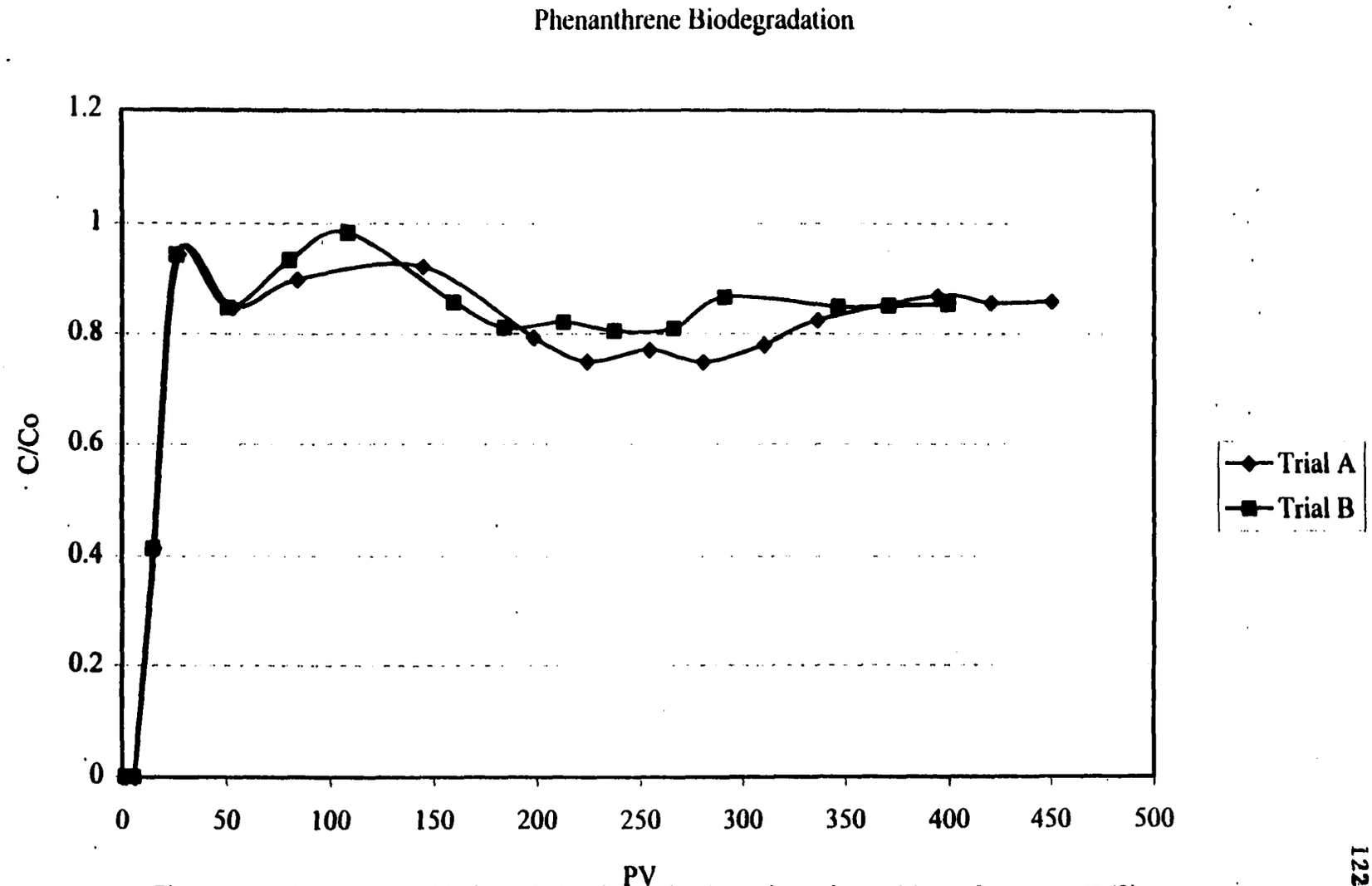

Figure A.8 Phenanthrene biodegradation in a mixed species column (*Actinobacter junii* (3) and *Pseudomonas oleavorans* (M11). Replicates shown.

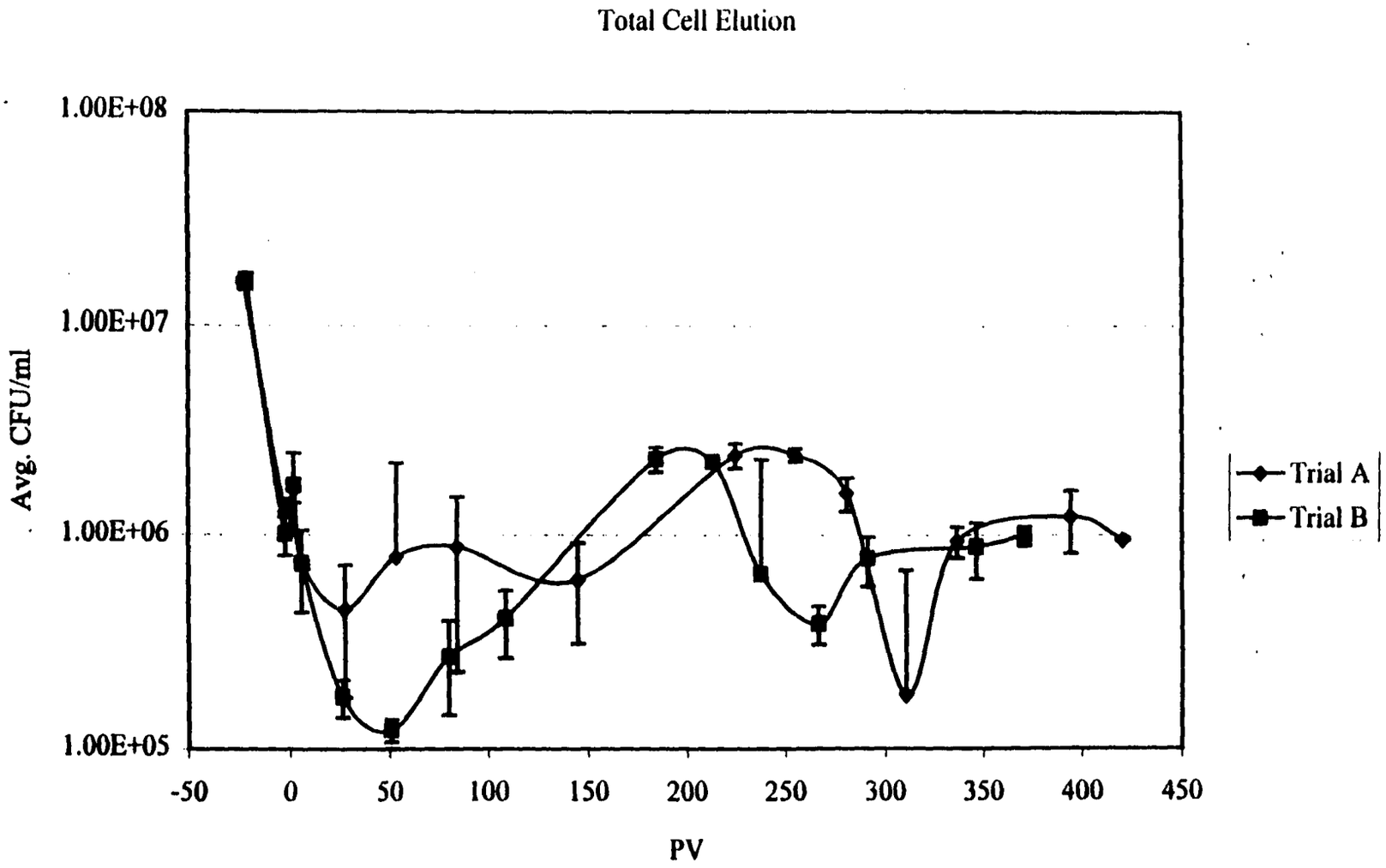

Figure A.9. Total cell elution from a mixed species column (*Actinobacter junii* (3) and *Pseudomonas oleavorans* (M11). Replicates shown.

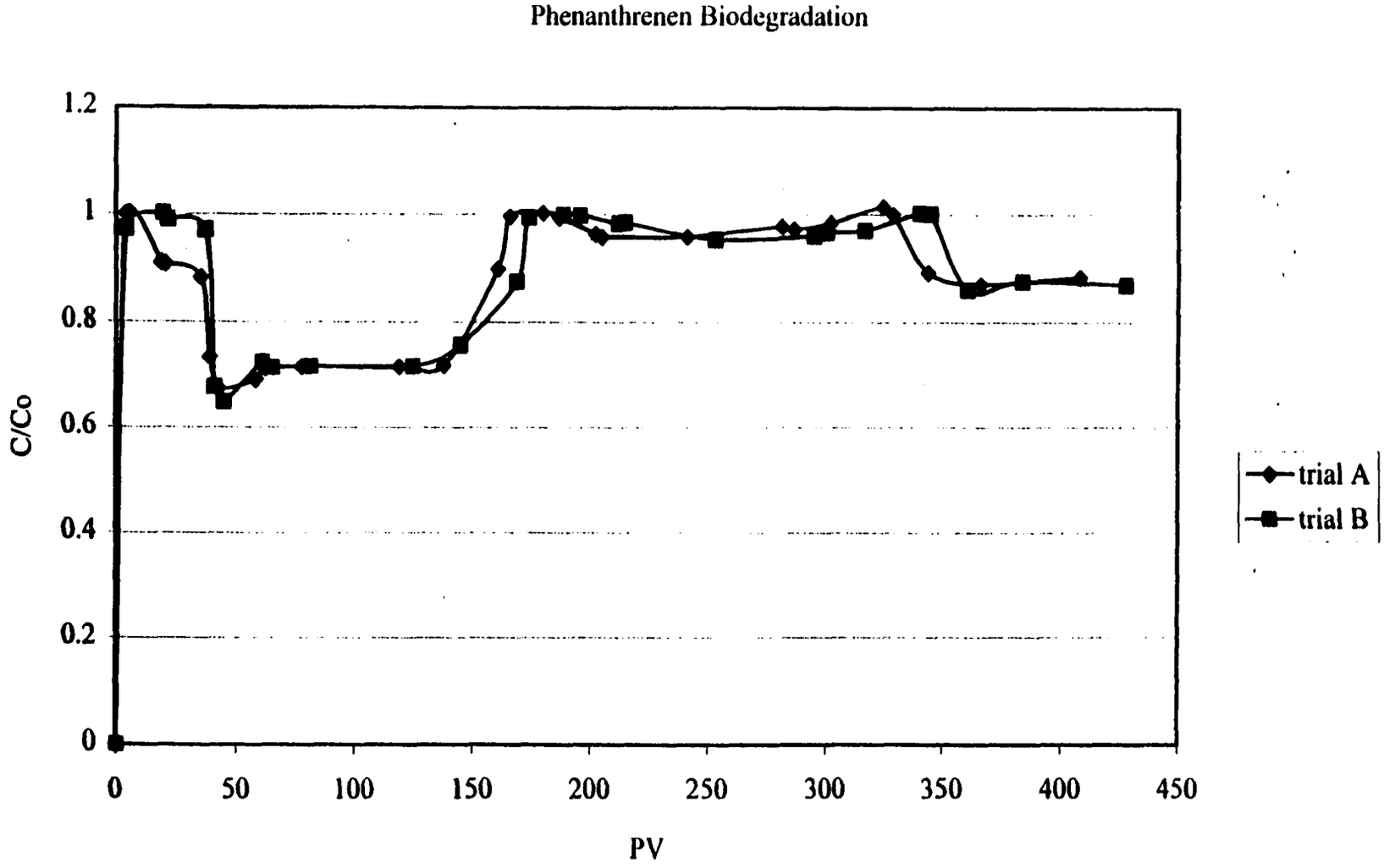

Figure A.12 Phenanthrene biodegradation in a mixed species column (*Methylobacterium sp*.(35) and *Pseudomonas oleavorans* (M11). Replicates shown.

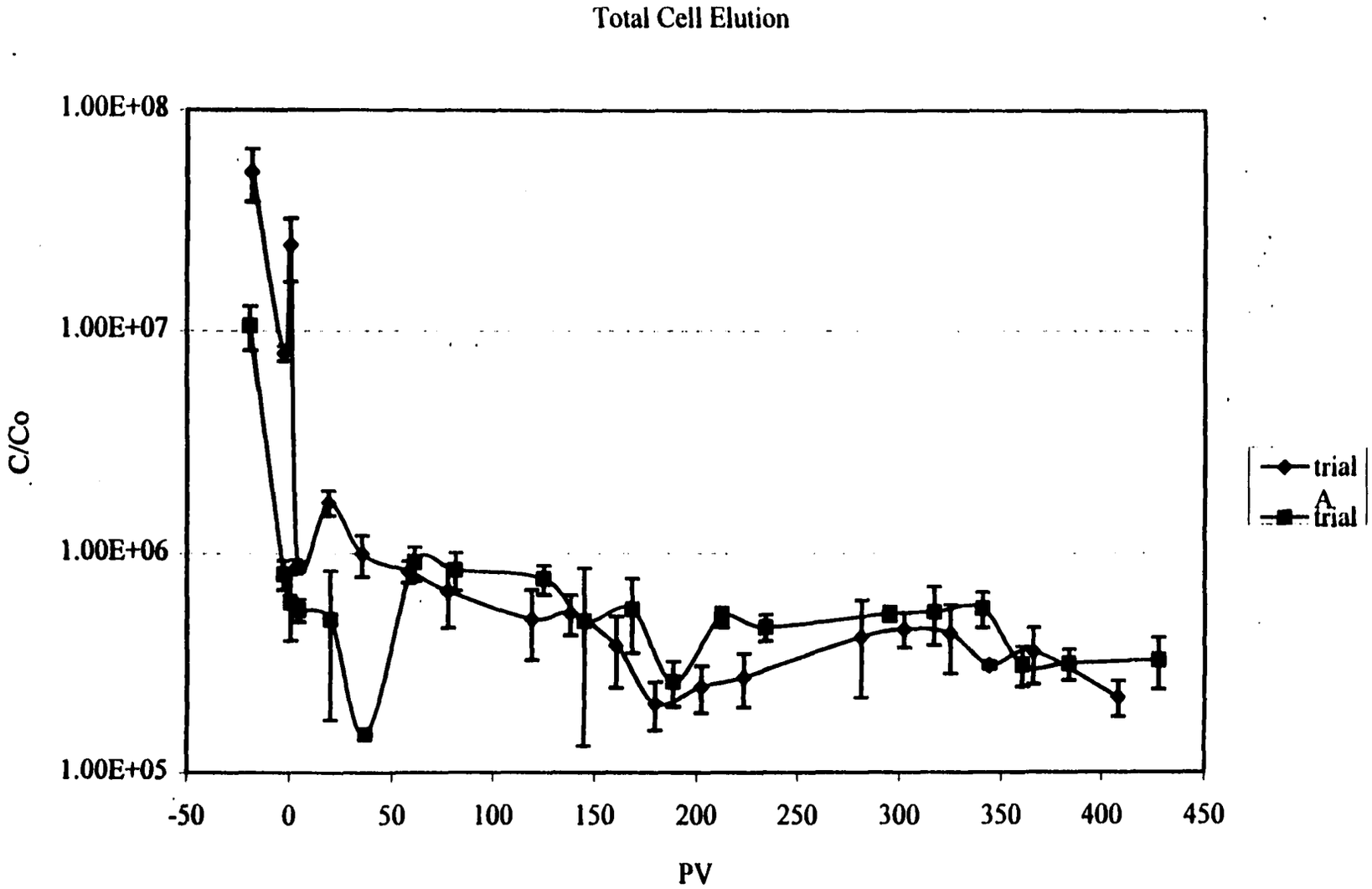

Figure A.13. Total cell elution from a mixed species column *(Methylobacterium sp.* (35) and *Pseudomonas oleavorans* (M11). Replicates shown.

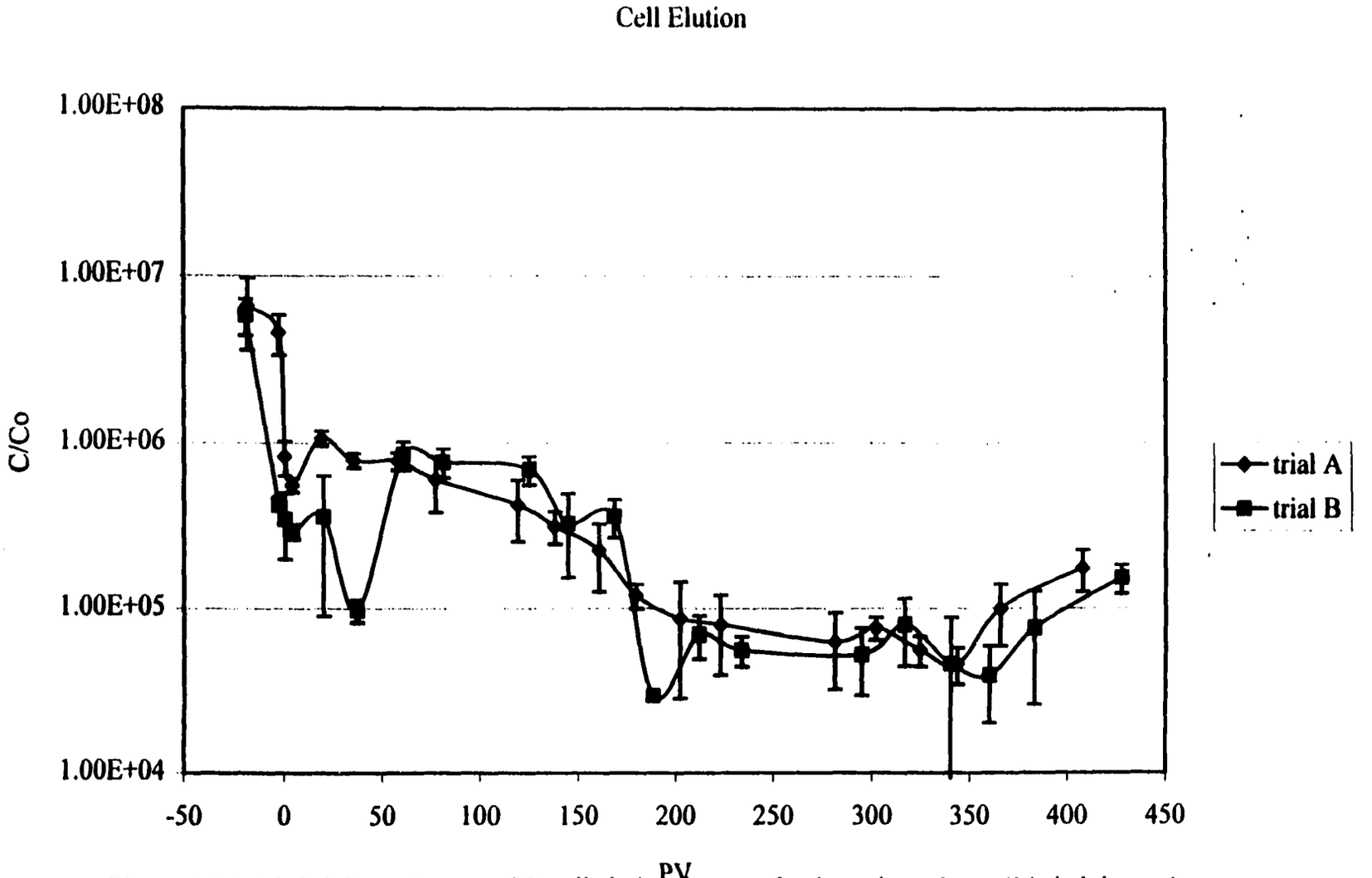

Figure A.14. *Methylobacterium sp.* (35) cell elution from a mixed species column (*Methylobacterium sp.* (35) and *Pseudomonas oleavorans* (M11). Replicates shown.

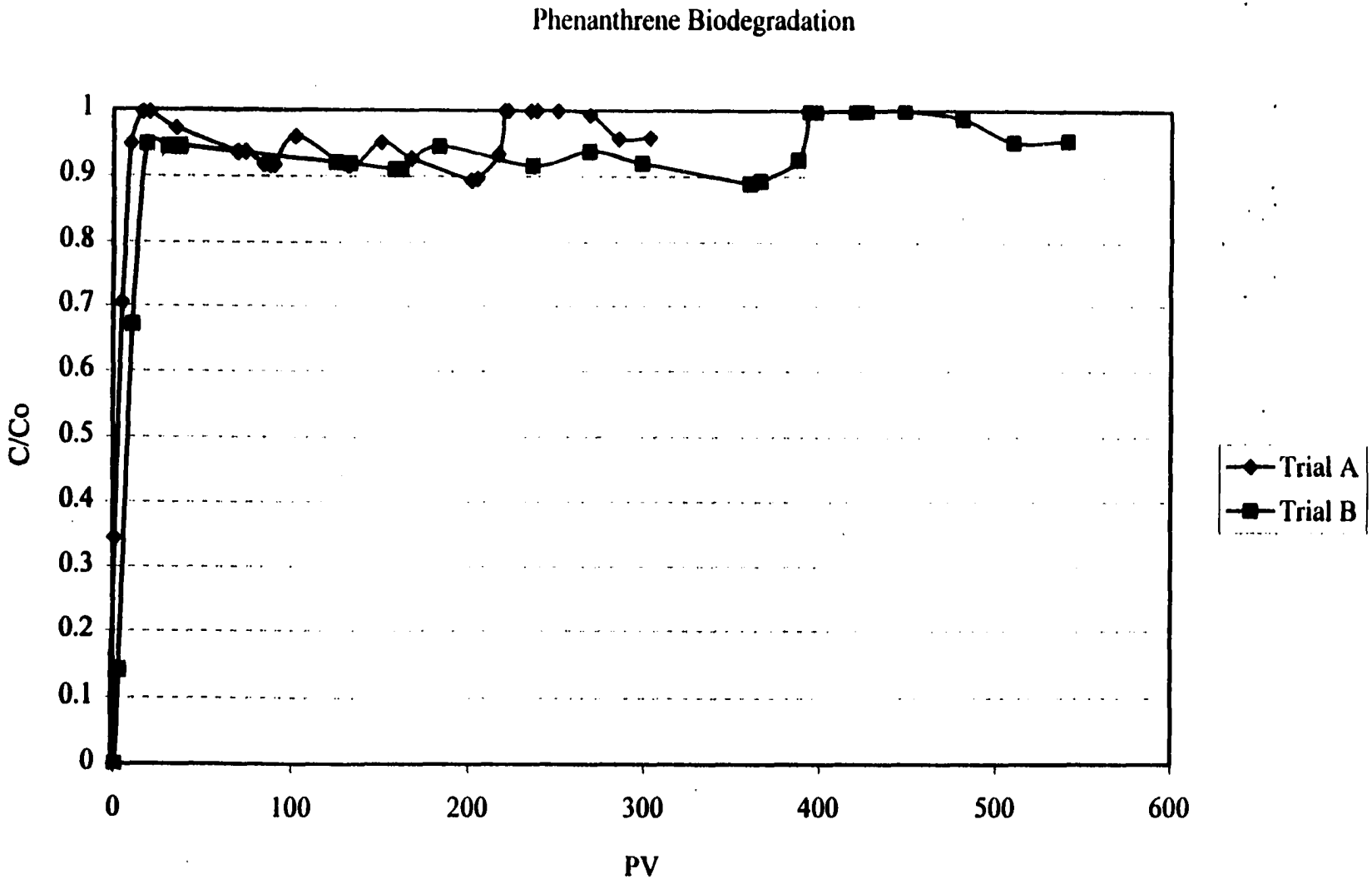

Figure A.16 Phenanthrene biodegradation in a mixed column (*Actinobacter junii* (3), *Methylobacterium sp*. (35), and *Pseudomonas oleavorans* (M11). Replicates shown.

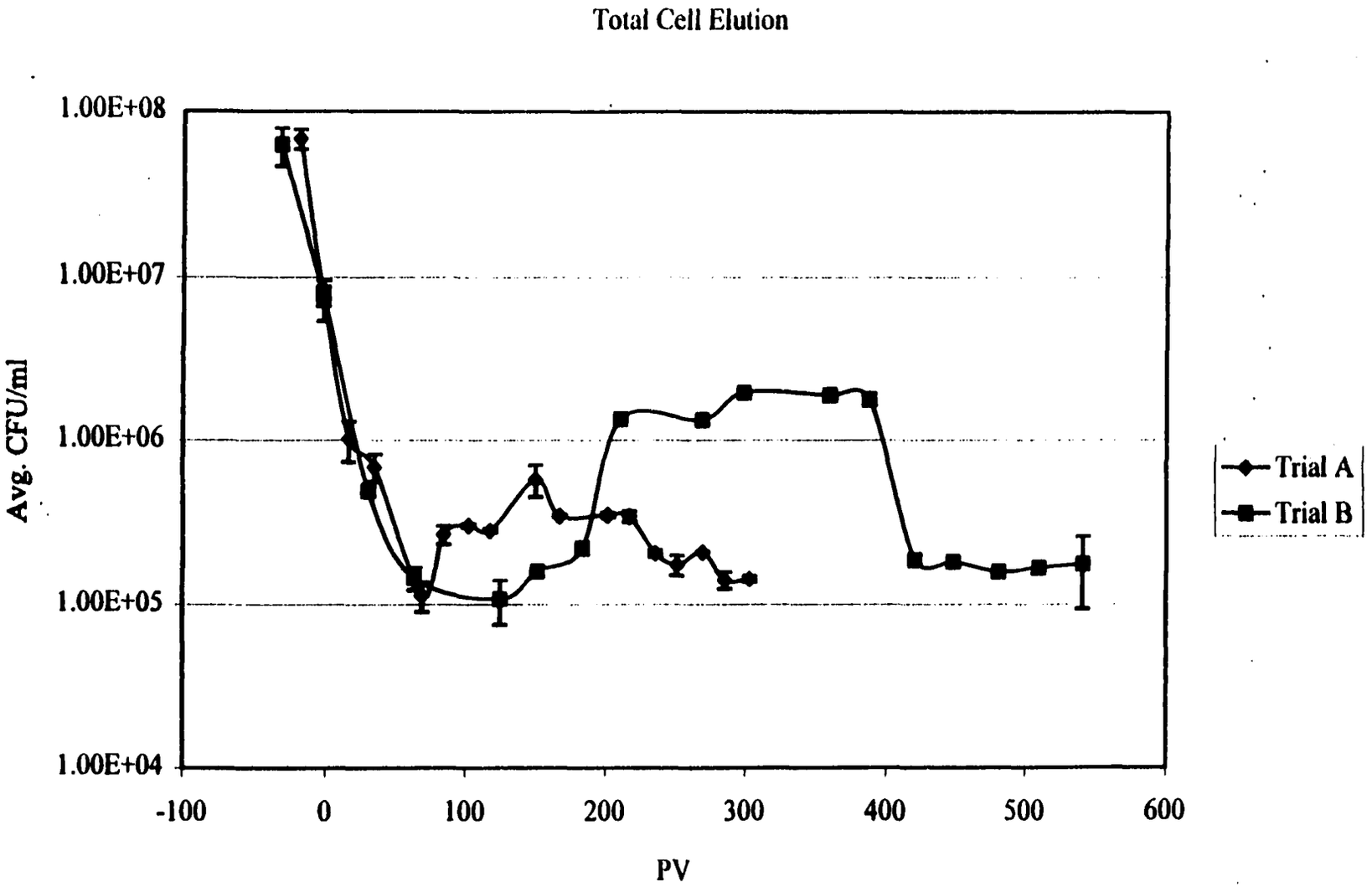

Figure A.17 Total cell elution in a mixed species column (*Actinobacter junii* (3), *Methylobacterium sp*. (35), and *Pseudomonas oleavorans* (M11). Replicates shown.

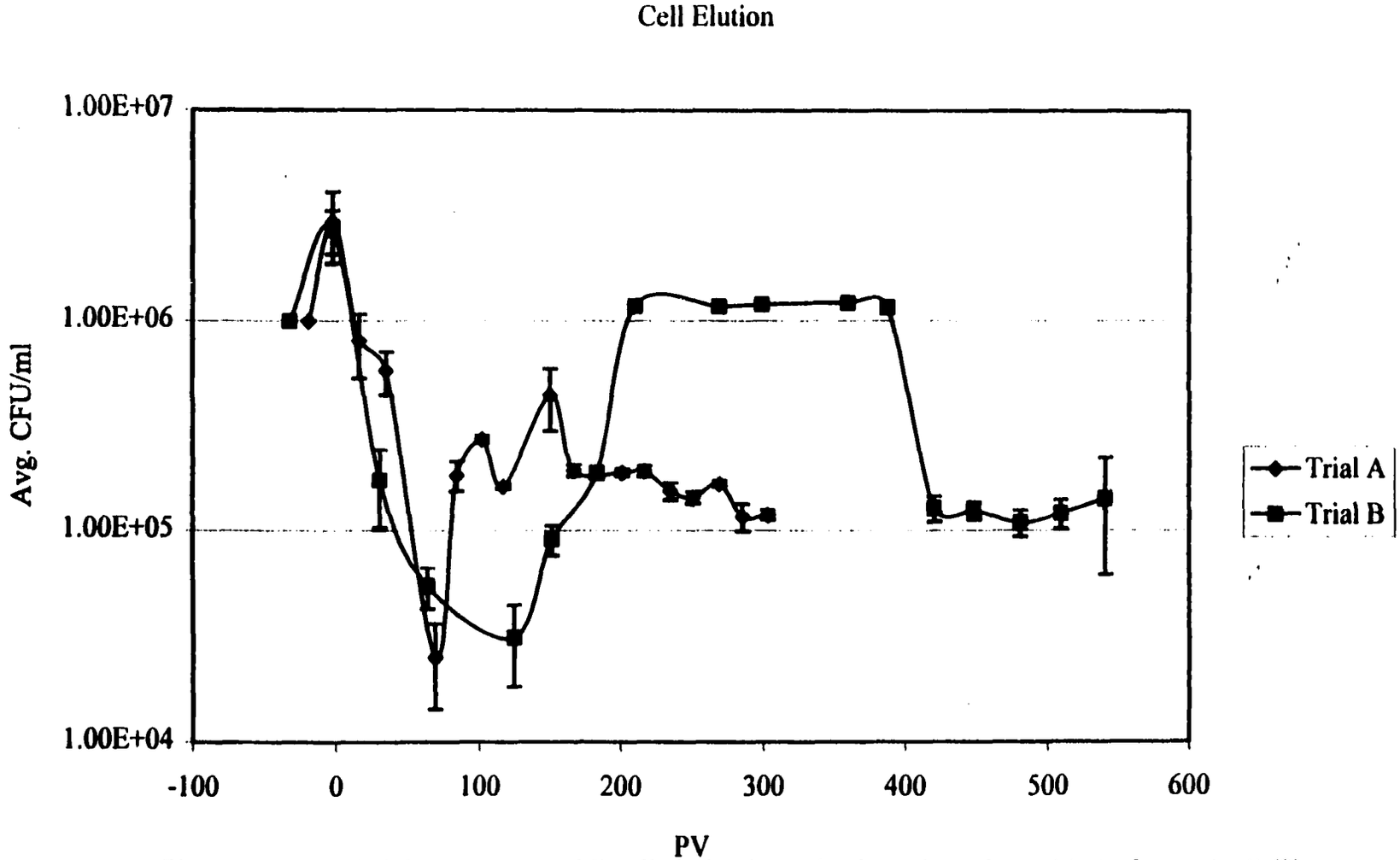

Figure A 19. *Methylobacterium sp.* (35) cell elution in a mixed species column (*Actinobacter junii* (3), *Methylobacterium sp*. (35), and *Pseudomonas oleavorans* (M11)). Replicates shown.

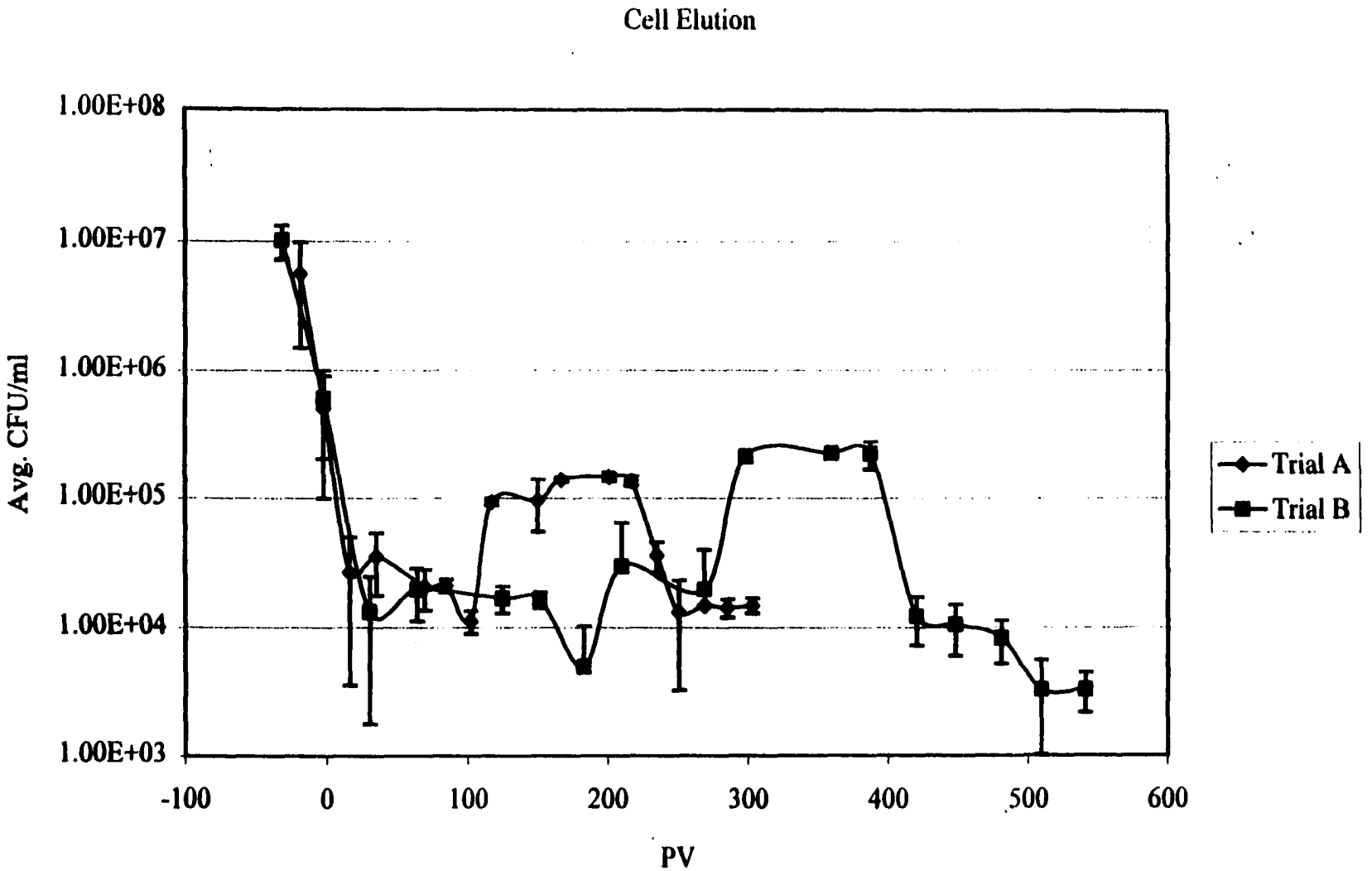

Figure A.20. *Pseudomonas oleavorans* (M11) cell elution in a mixed species column (*Actinobacter junii* (3), *Methylobacterium sp.* (35), and *Pseudomonas oleavorans* (M11)). Replicates shown.

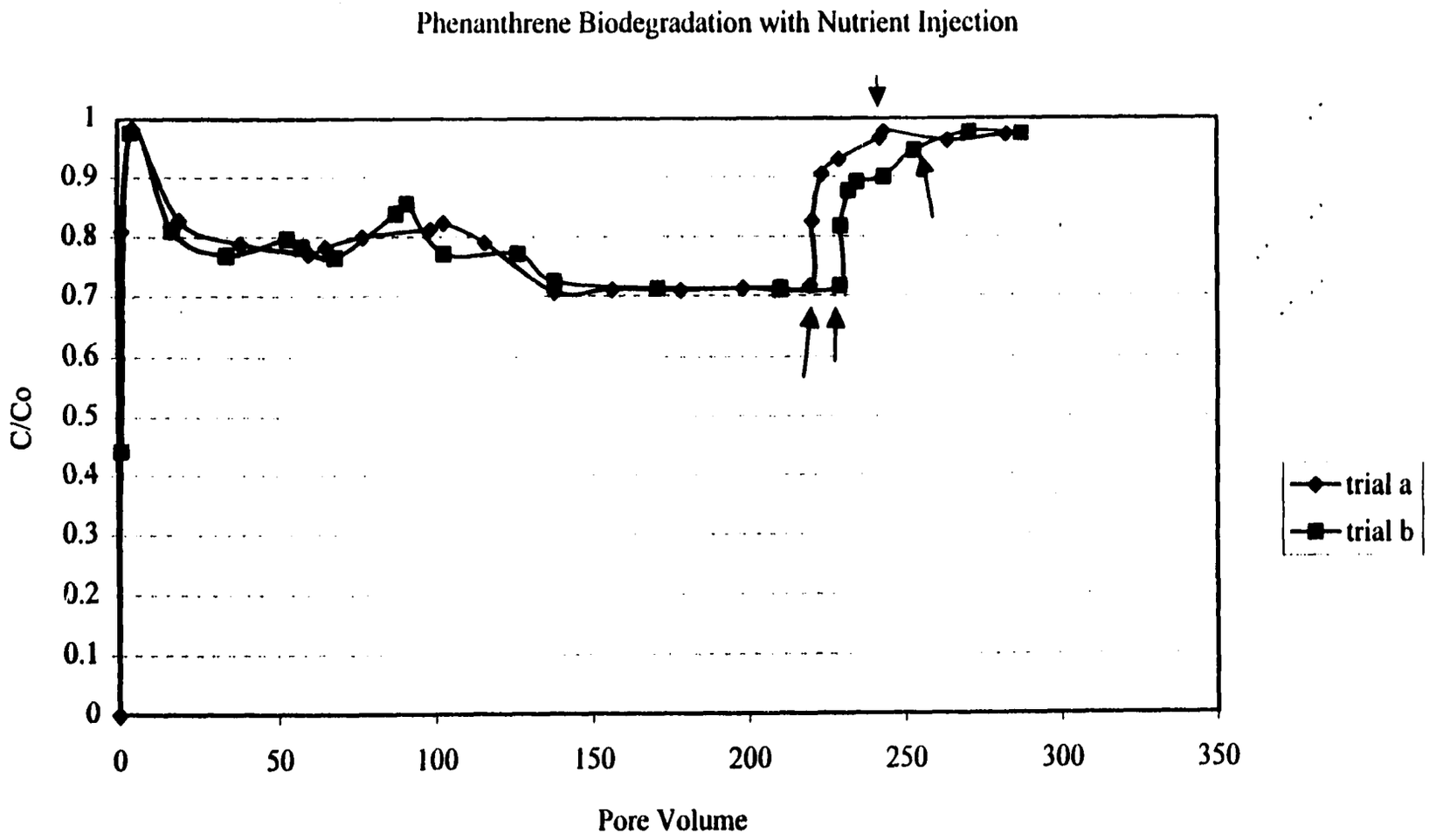

Figure A.21. Phenanthrene biodegradation in a three species mixed column system. Arrows indicate start/stop of the R_2B pulse. Replicates shown.

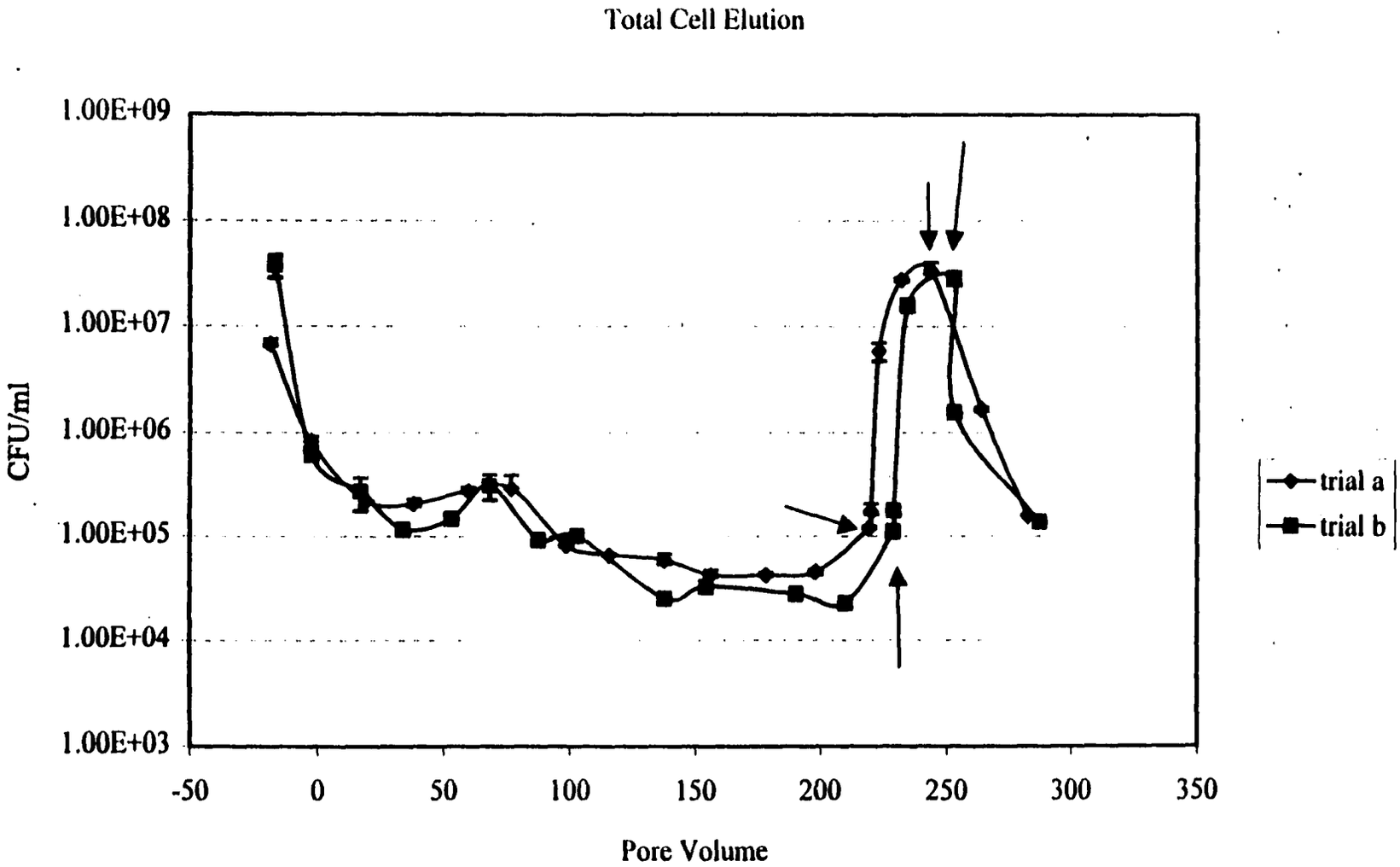

Figure A.22. Total cell elution in a three species mixed column. Arrows indicate start/stop of R_2B pulse. Replicates shown

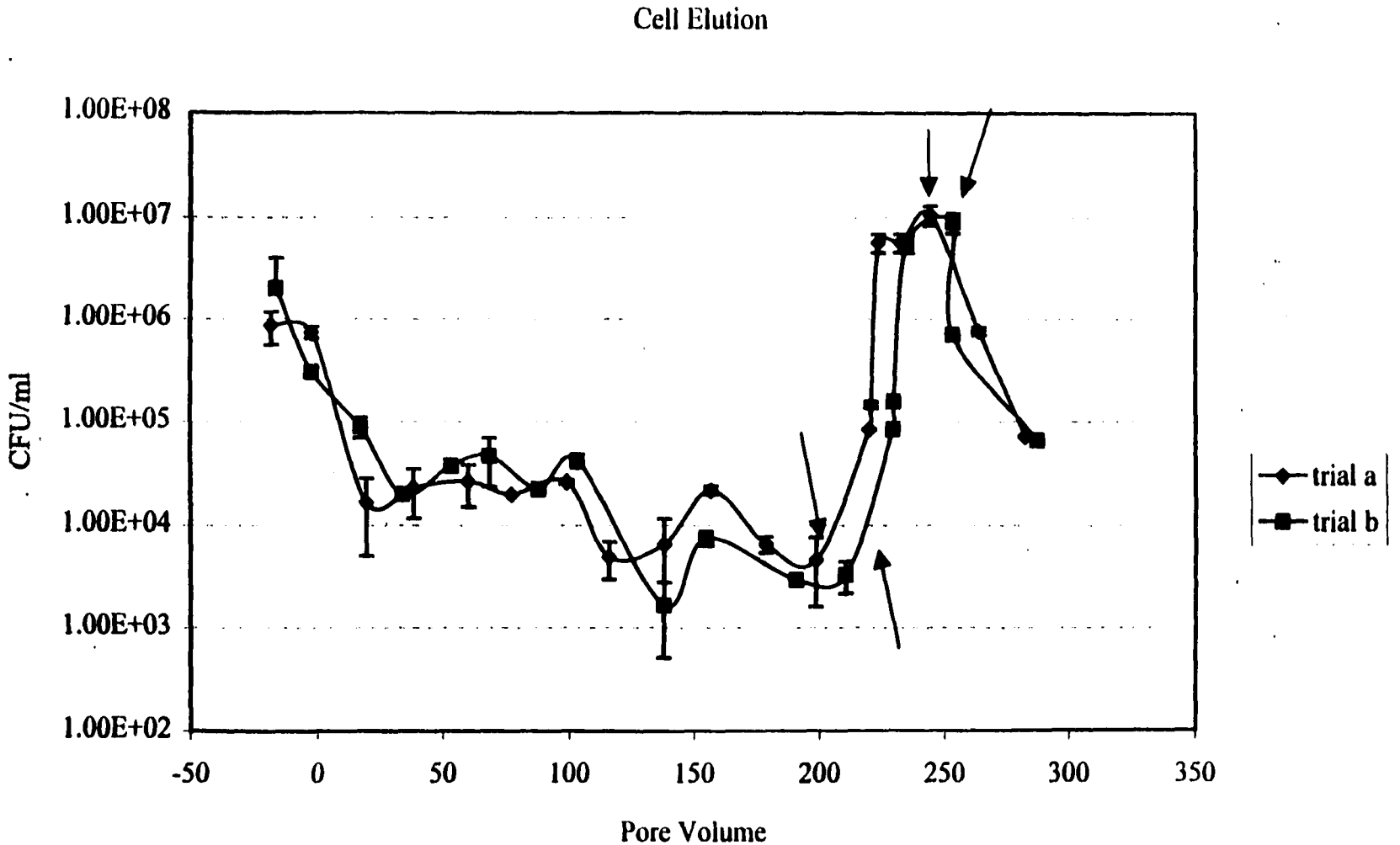

Figure A.23. *Actinobacter junii* (3) cell elution in a three species mixed column. Arrows indicate start/stop of the R_2B pulse. Replicates shown.

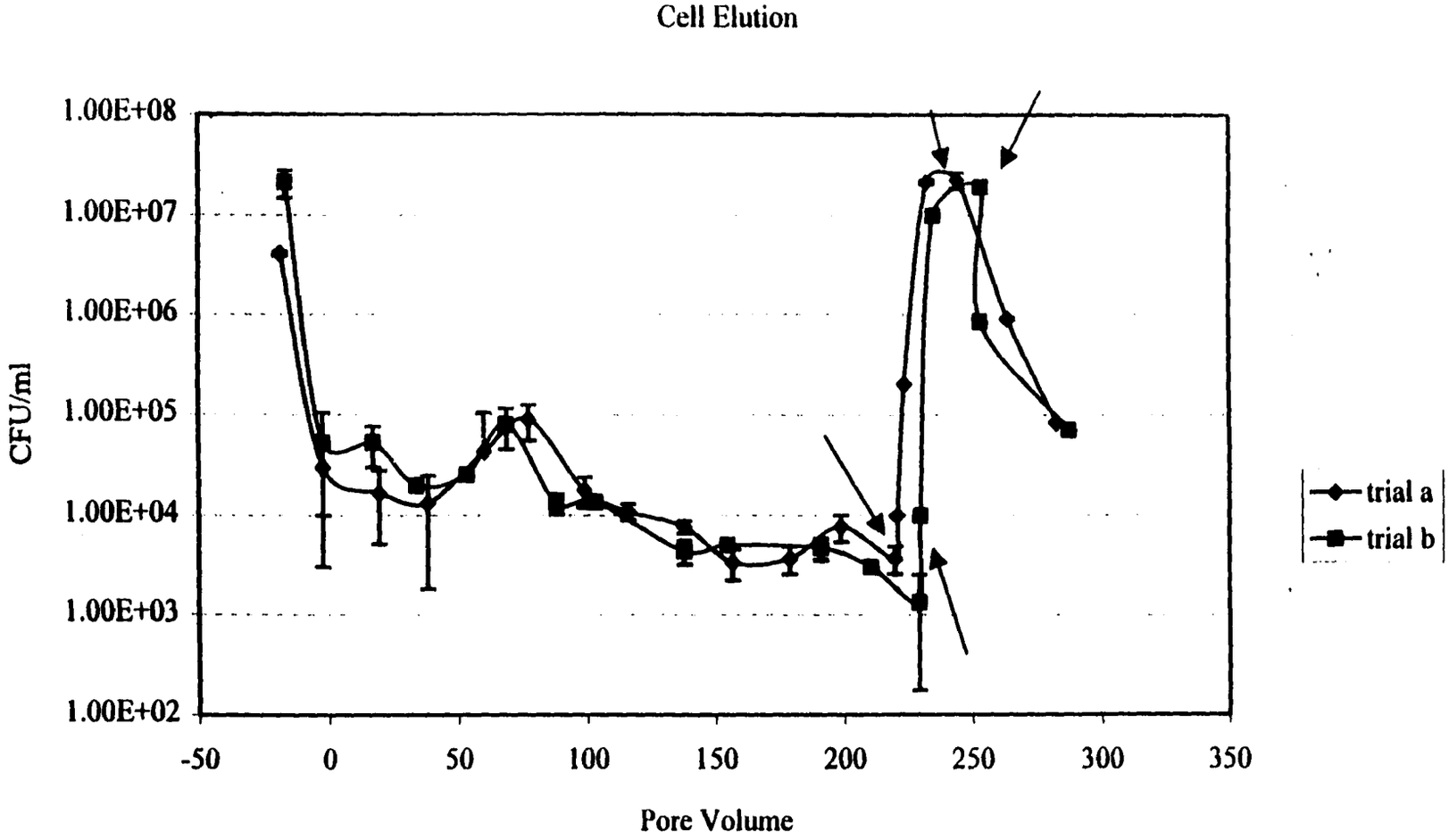

Figure A.24. *Pseudomonas oleavorans* (M11) cell elution in a three species mixed column. Arrows indicate start/stop of the R_2B pluse. Replicates shown.

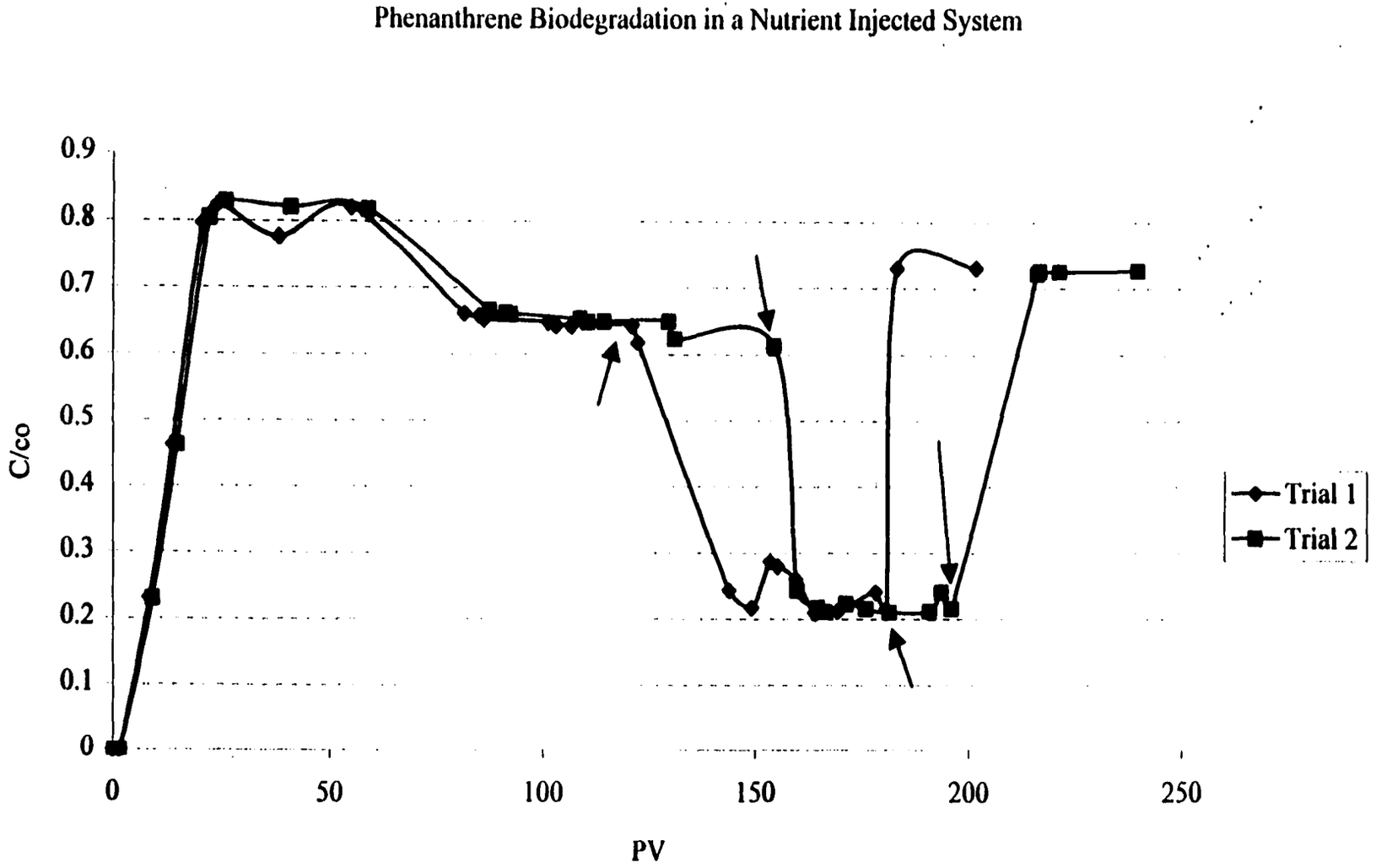

Figure A.26. *Actinobacter junii* (3) phenanthrene biodegradation. Arrows indicate start/stop of the R_2B pulse. Replicates shown.

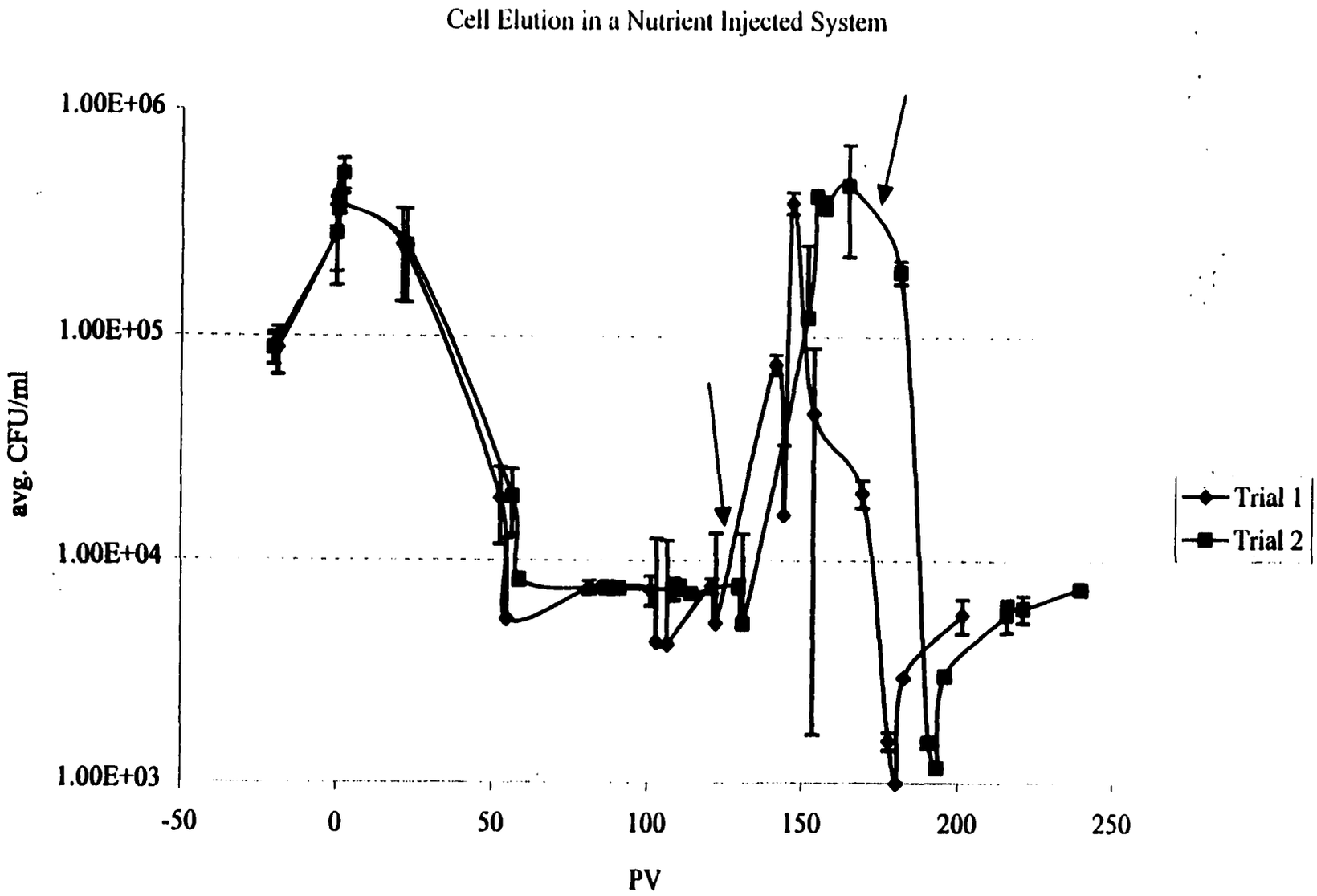

Figure A.27. *Actinobacter junii* (3) cell elution. Arrows indicate start/stop of the R_2B pulse. Replicates shown.

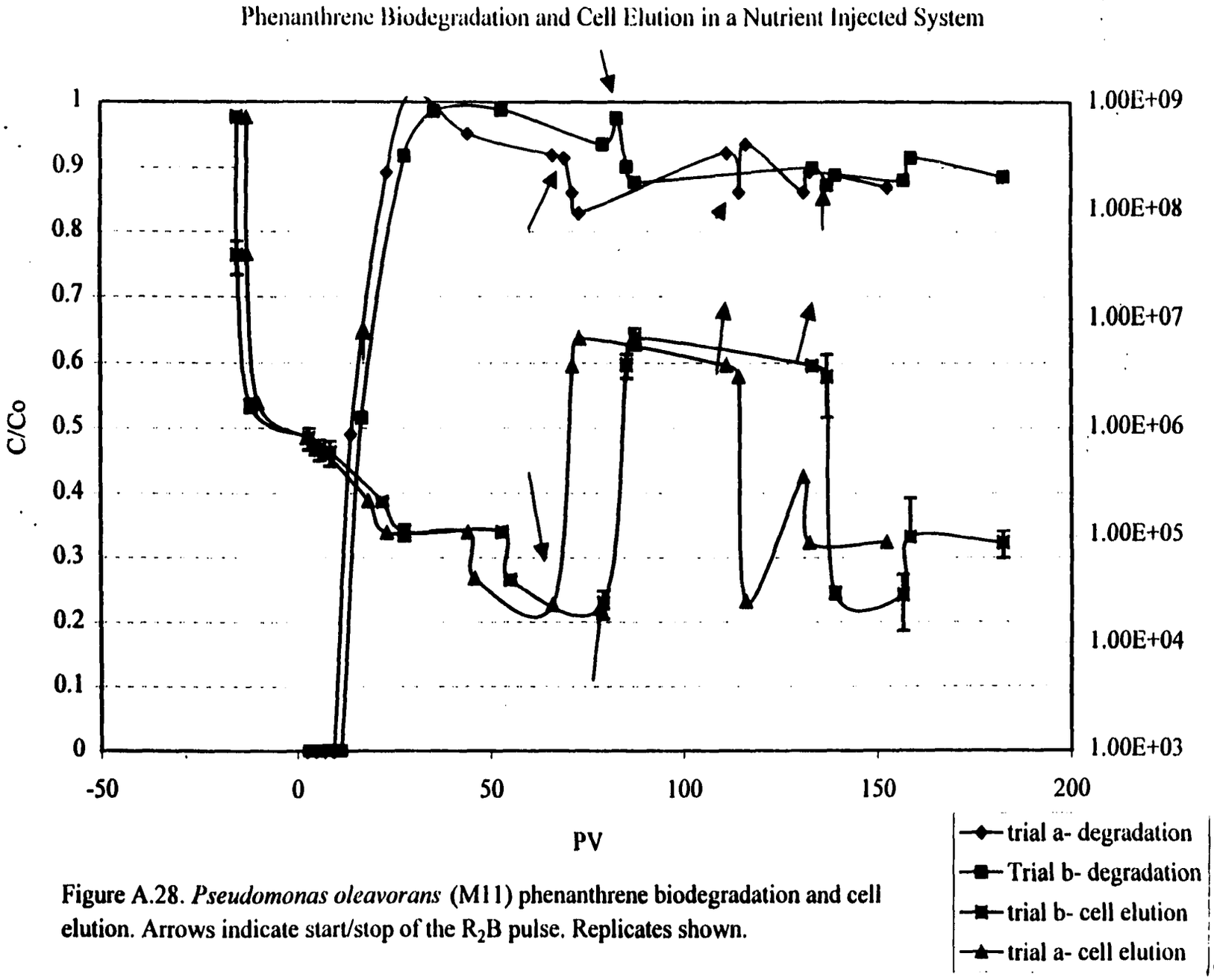

Figure A.28. *Pseudomonas oleavorans* (M11) phenanthrene biodegradation and cell elution. Arrows indicate start/stop of the R_2B pulse. Replicates shown.

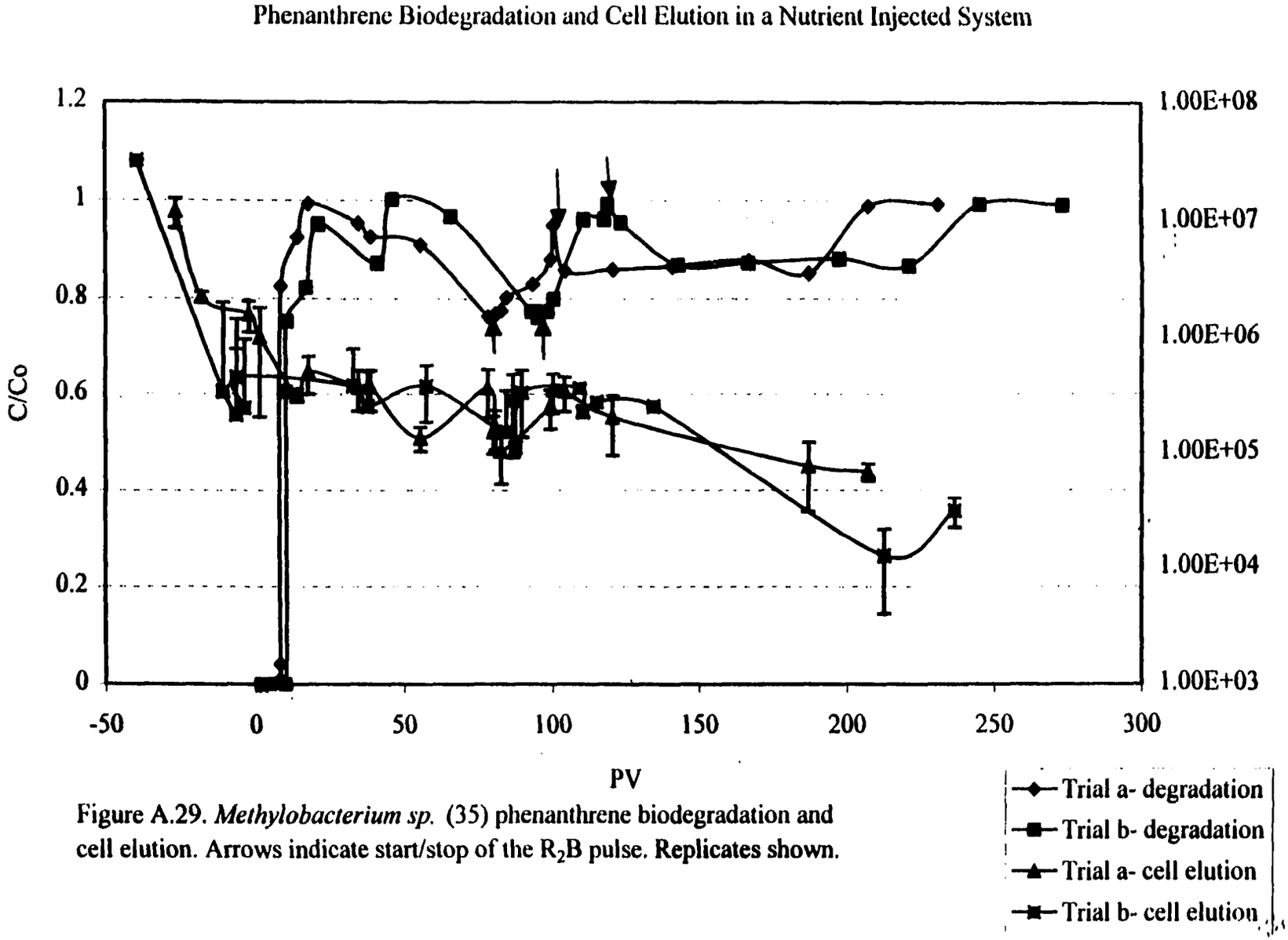

Figure A.29. *Methylobacterium sp.* (35) phenanthrene biodegradation and cell elution. Arrows indicate start/stop of the R_2B pulse. Replicates shown.

APPENDIX B: CELL DISTRIBUTION DATA

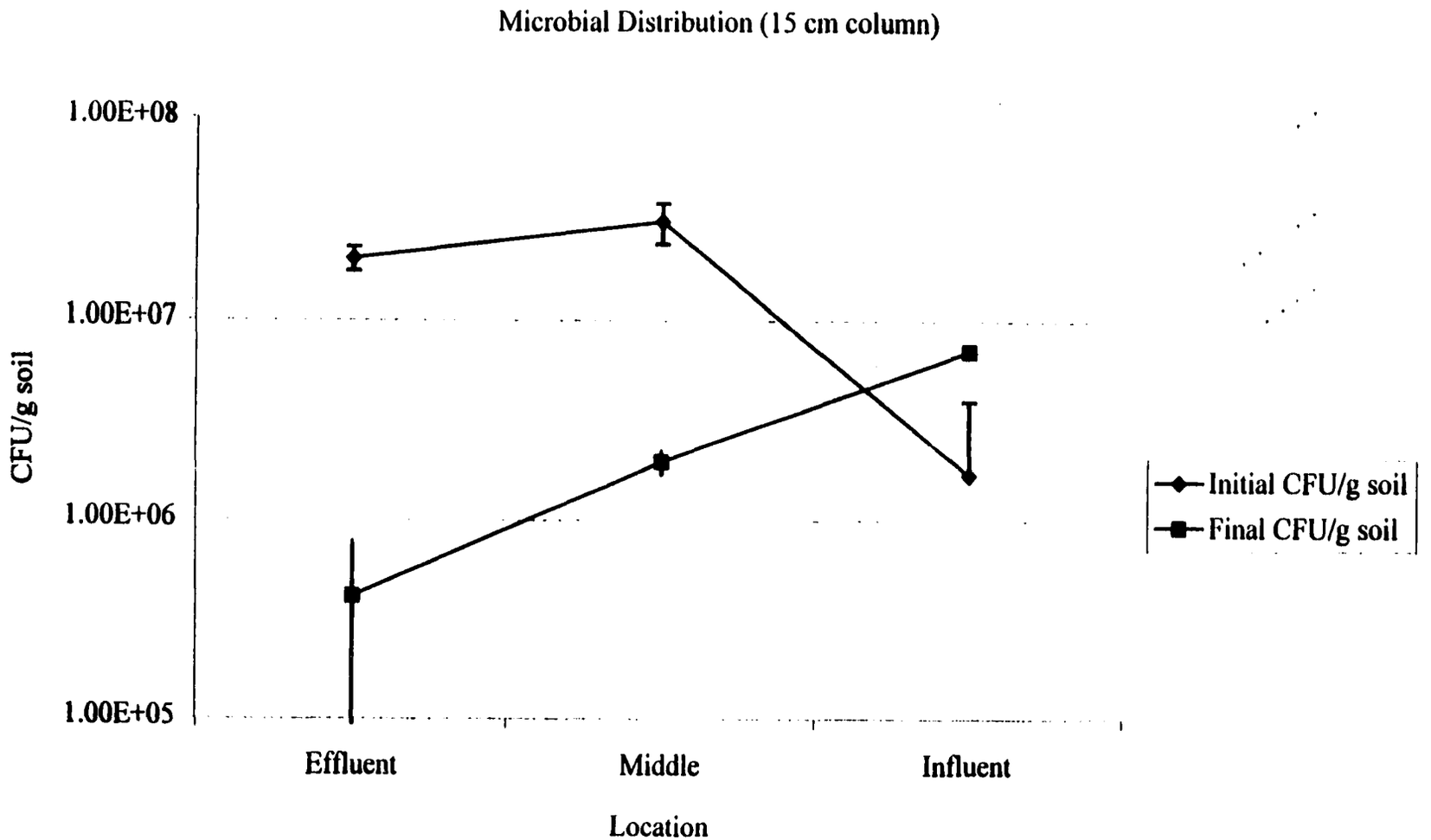

Figure B.1. *Actinobacter junii* (3) cell distribution.

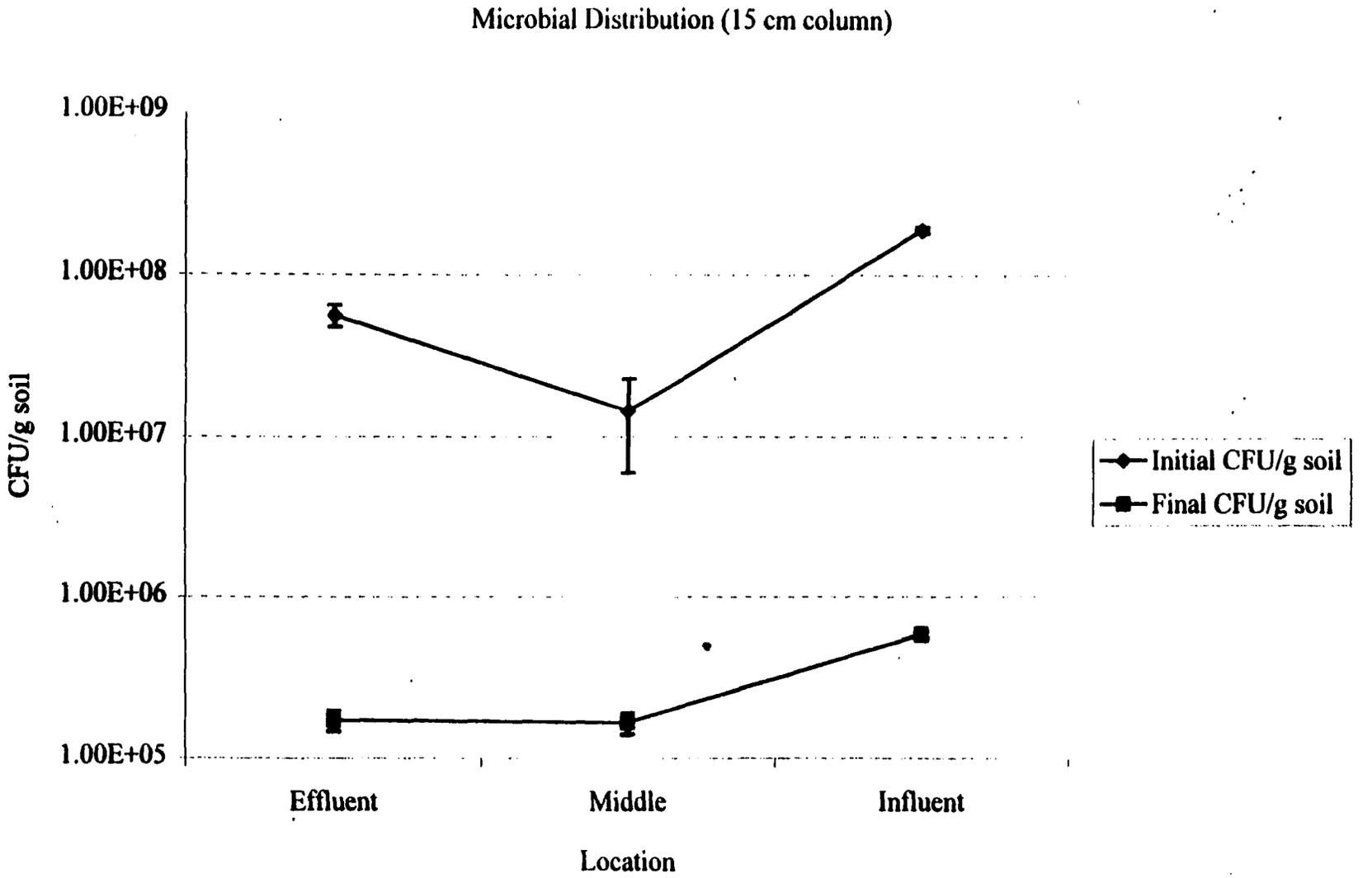

Figure B.2. *Pseudomonas oleavorans* (M11) cell distribution.

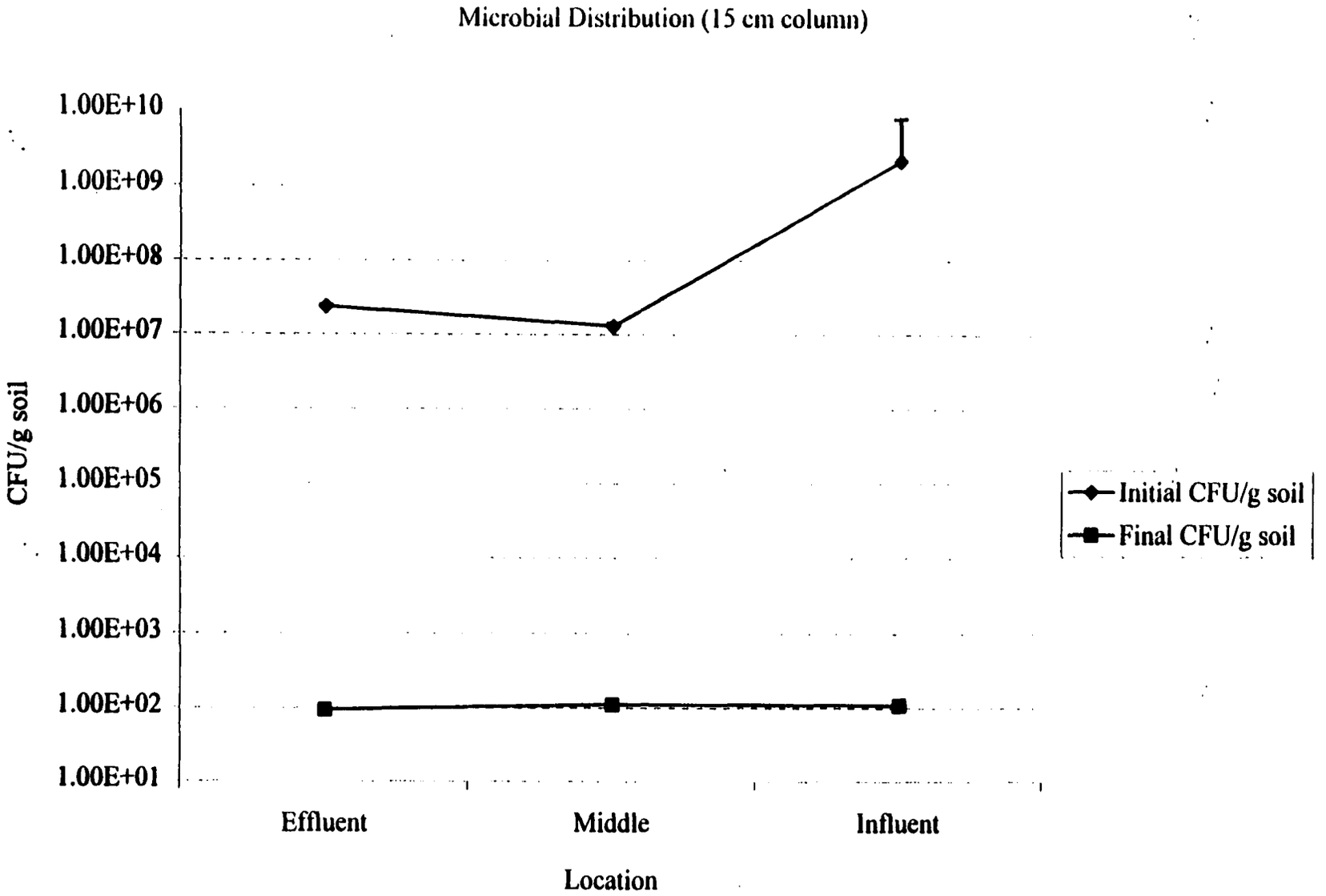

Figure B.3.. *Methylobacterium sp*. (35) cell distribution.

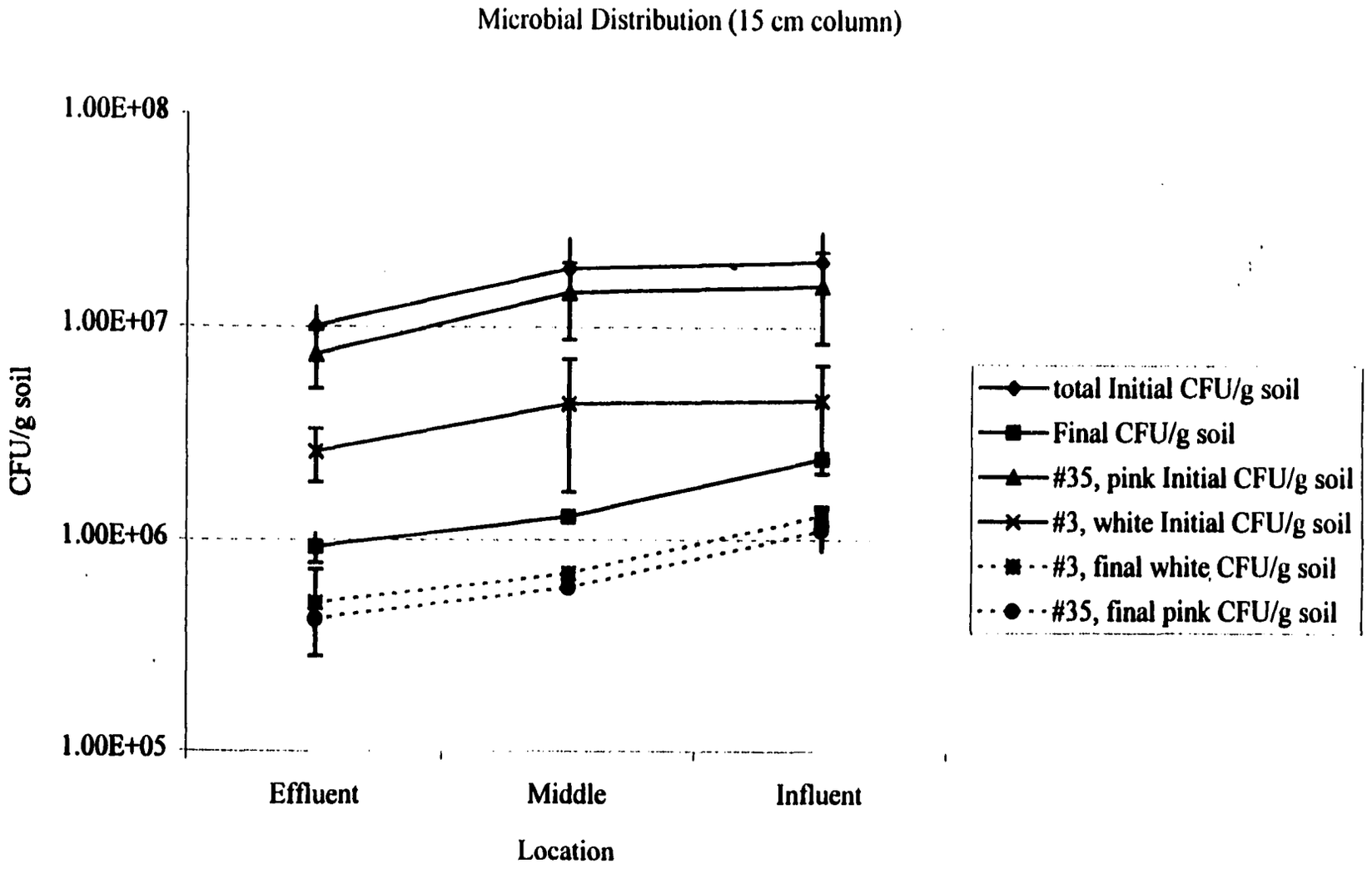

Figure B.4. *Actinobacter junii* (3) and *Methylobacterium sp.* (35) cell distribution.

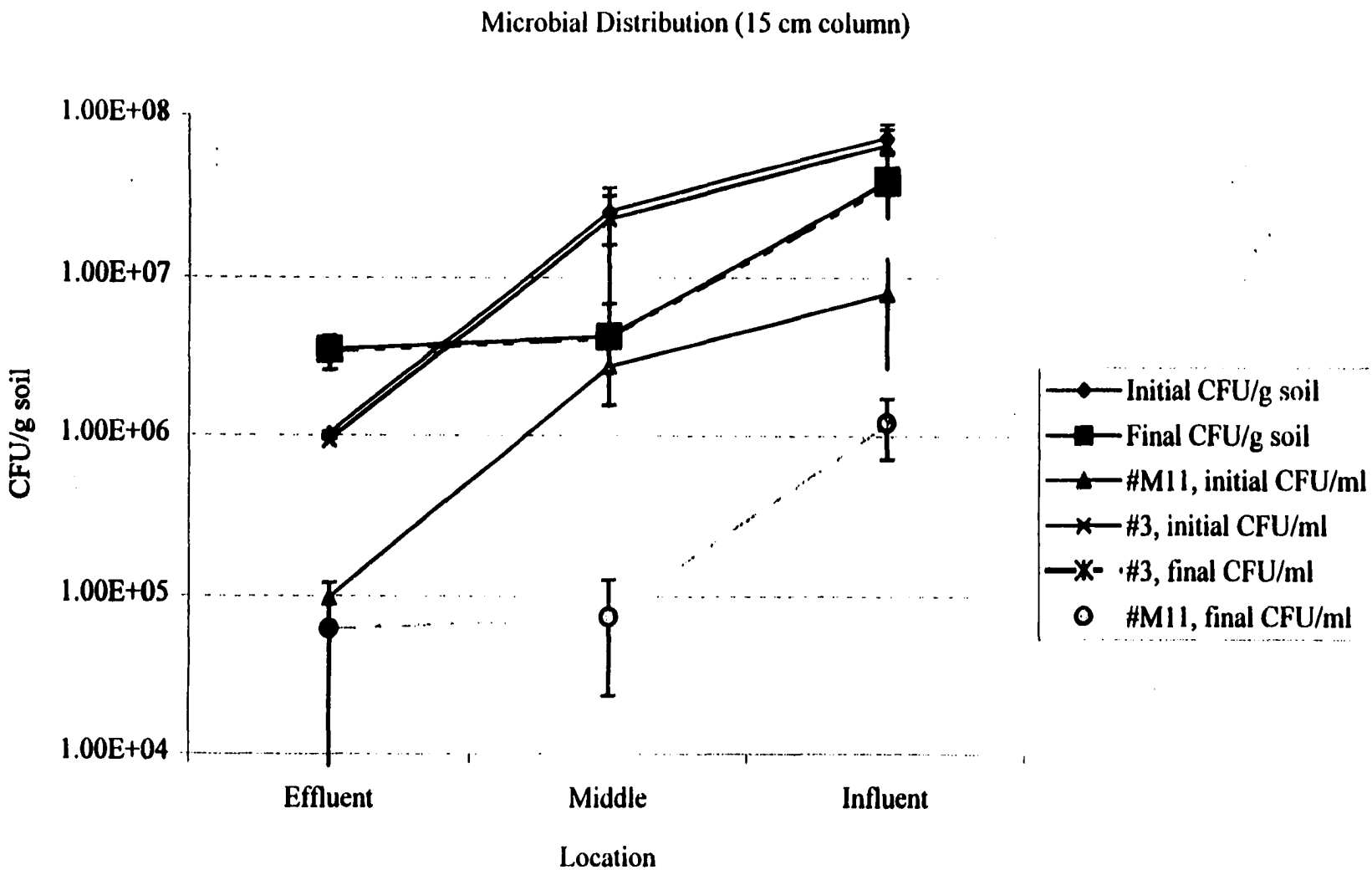

Figure B.5. *Actinobacter junii* (3) and *Pseudomonas oleavorans* (M11) cell distribution.

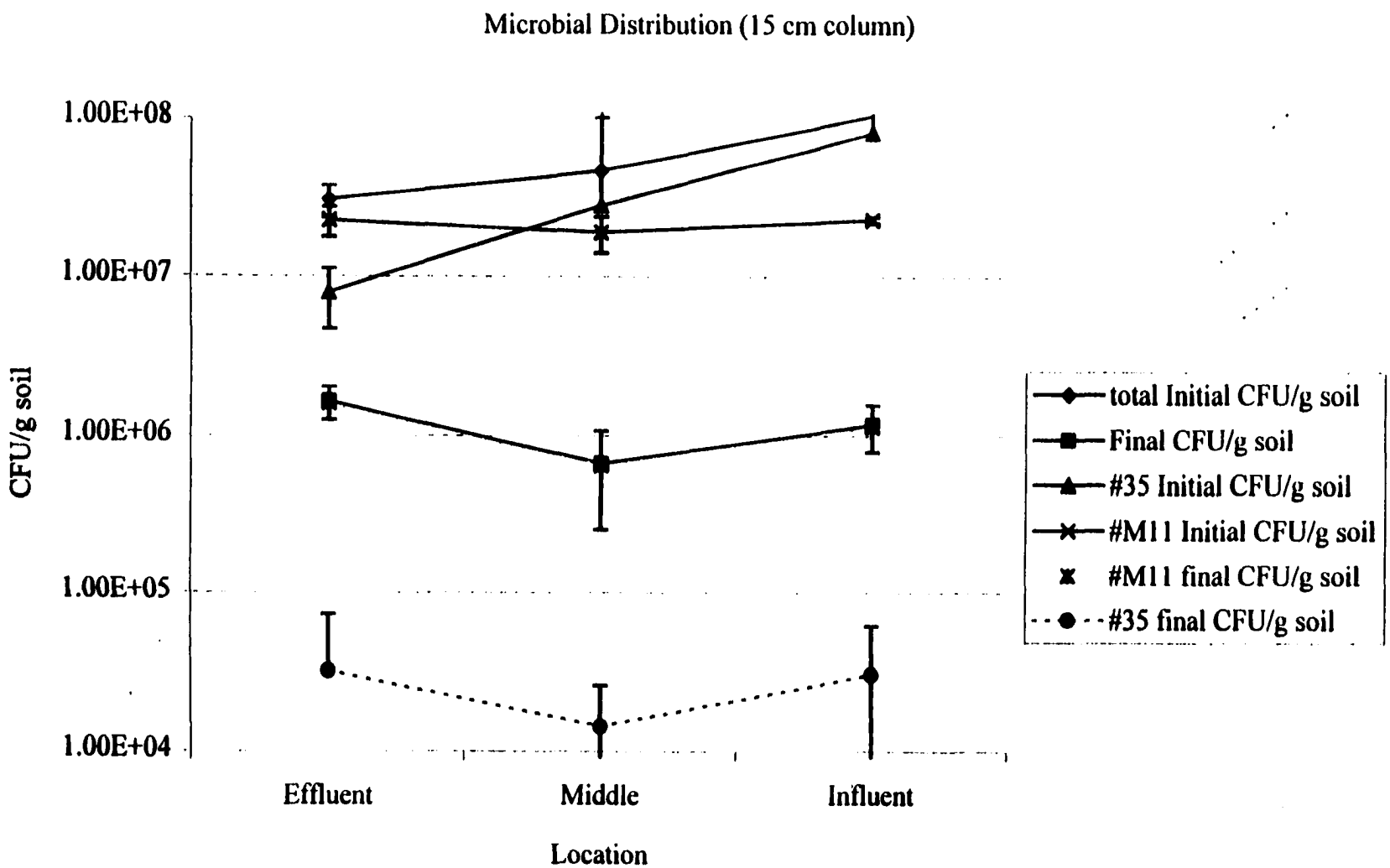

Figure B.6. *Methylobacterium sp* (35) and *Pseudomonas oleavorans* (M11) cell distribution.

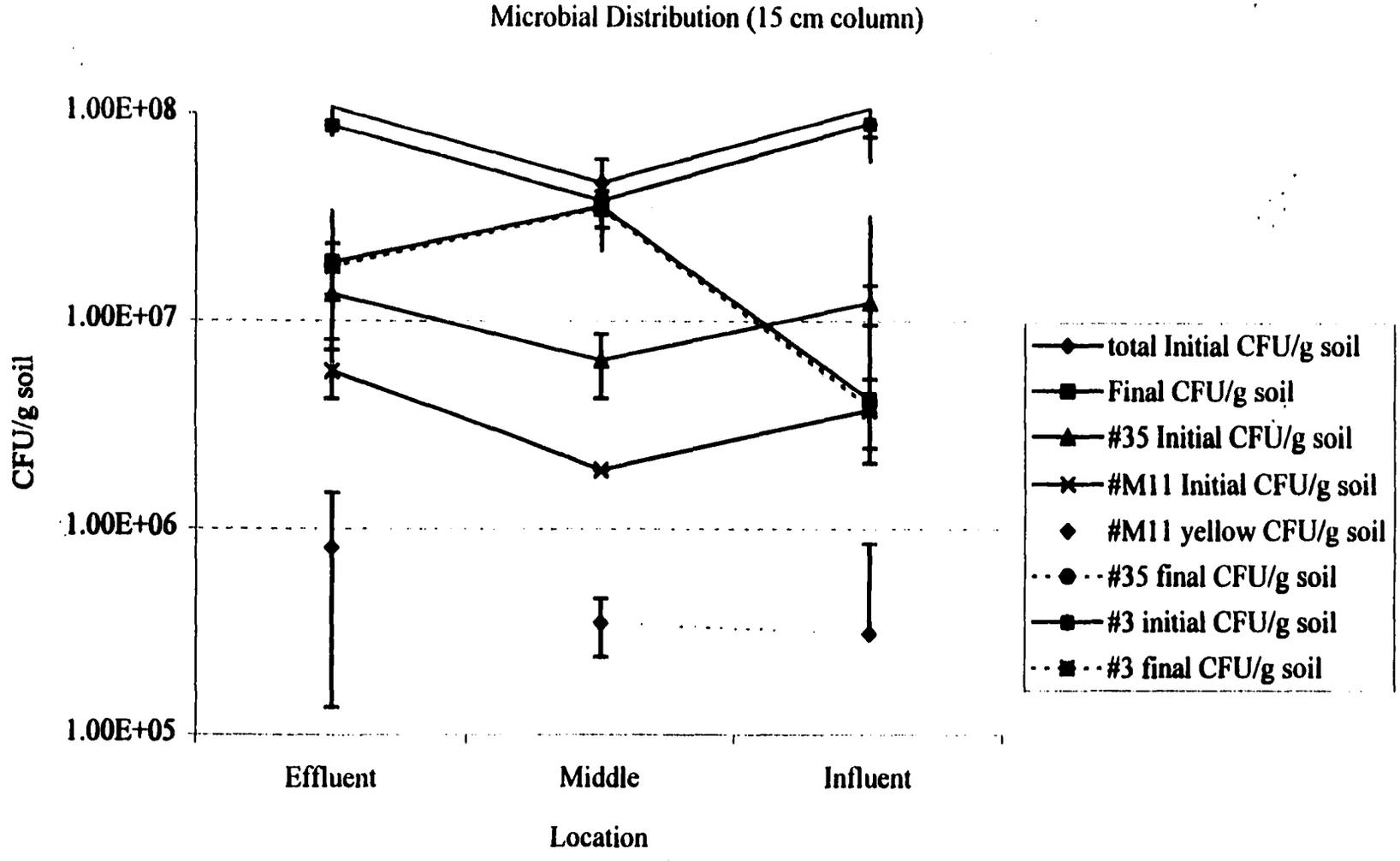

Figure B.7. *Actinobacter junii* (3), *Pseudomonas oleavorqns* (M11), and *Methylobacterium sp.* (35) cell distribution.

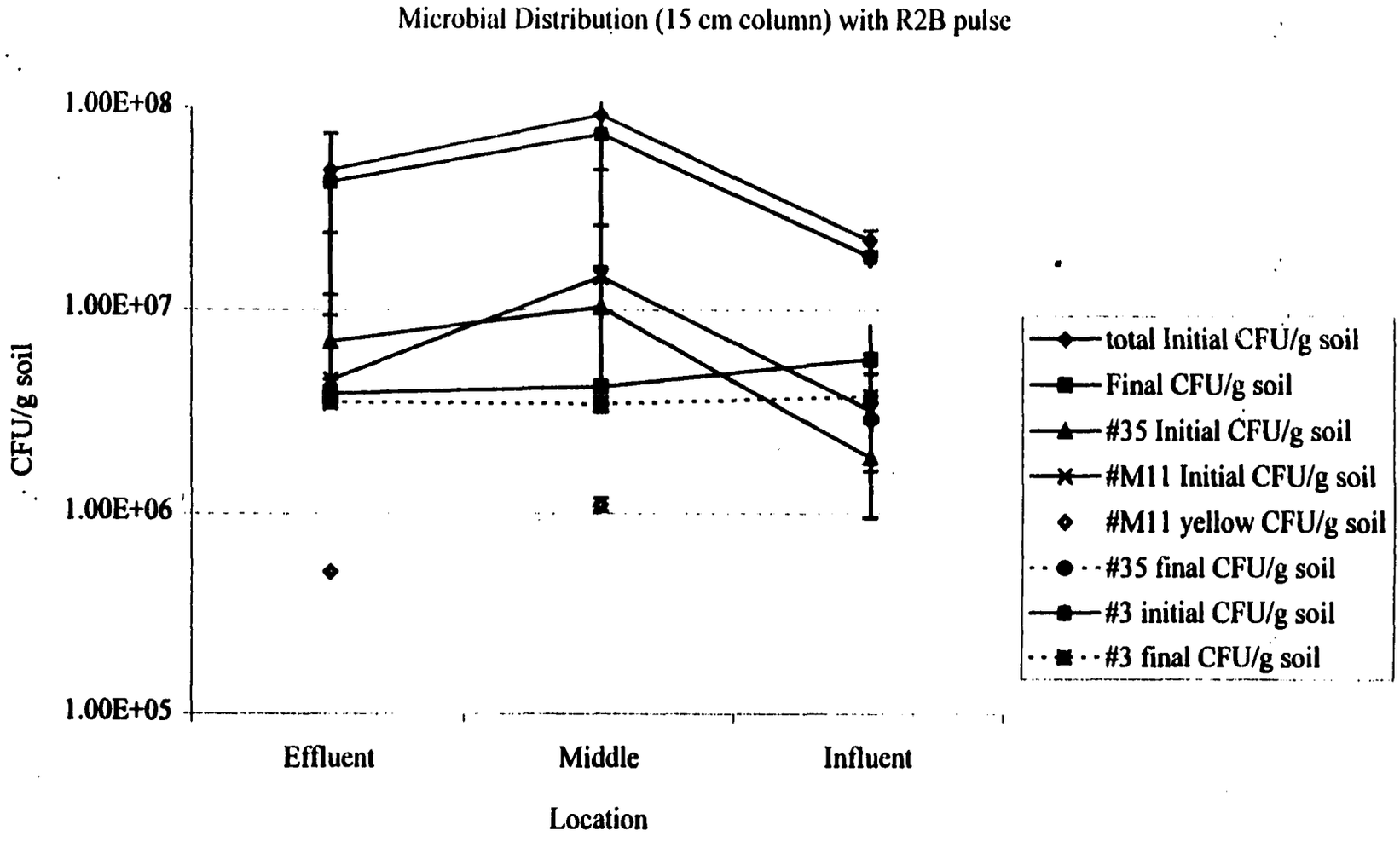

Figure B.8. ***Actinobacter junii*** **(3),** ***Pseudomonas oleavorans*** **(M11), and** ***Methylobacterium sp.*** **(35) cell distribution in a pulsed column.**

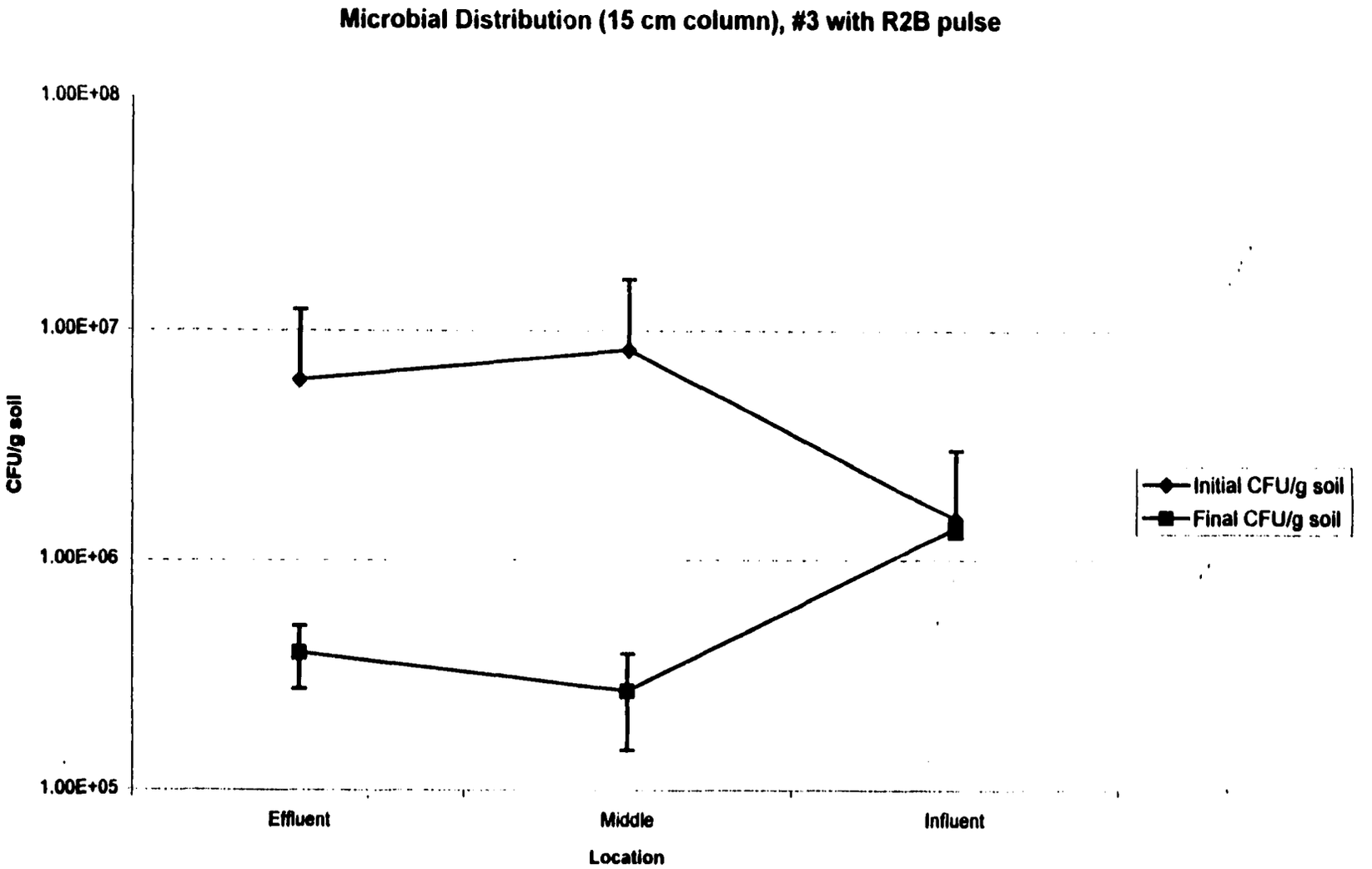

Figure B.9. *Actinobacter junii* (3) cell distribution in s pulsed column.

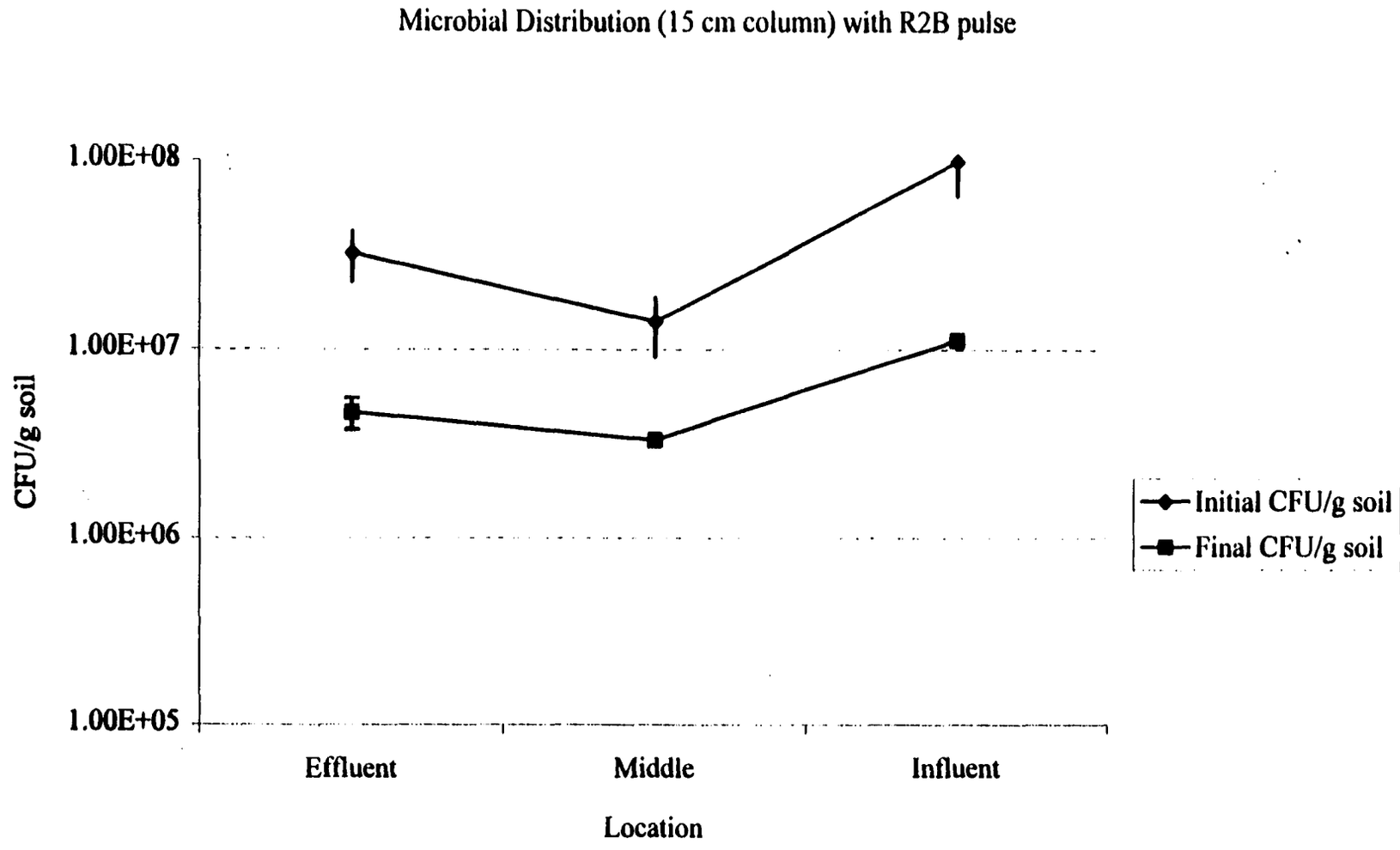

Figure B.10. *Methylobacterium sp.* (35) cell distribution in a pulsed column.

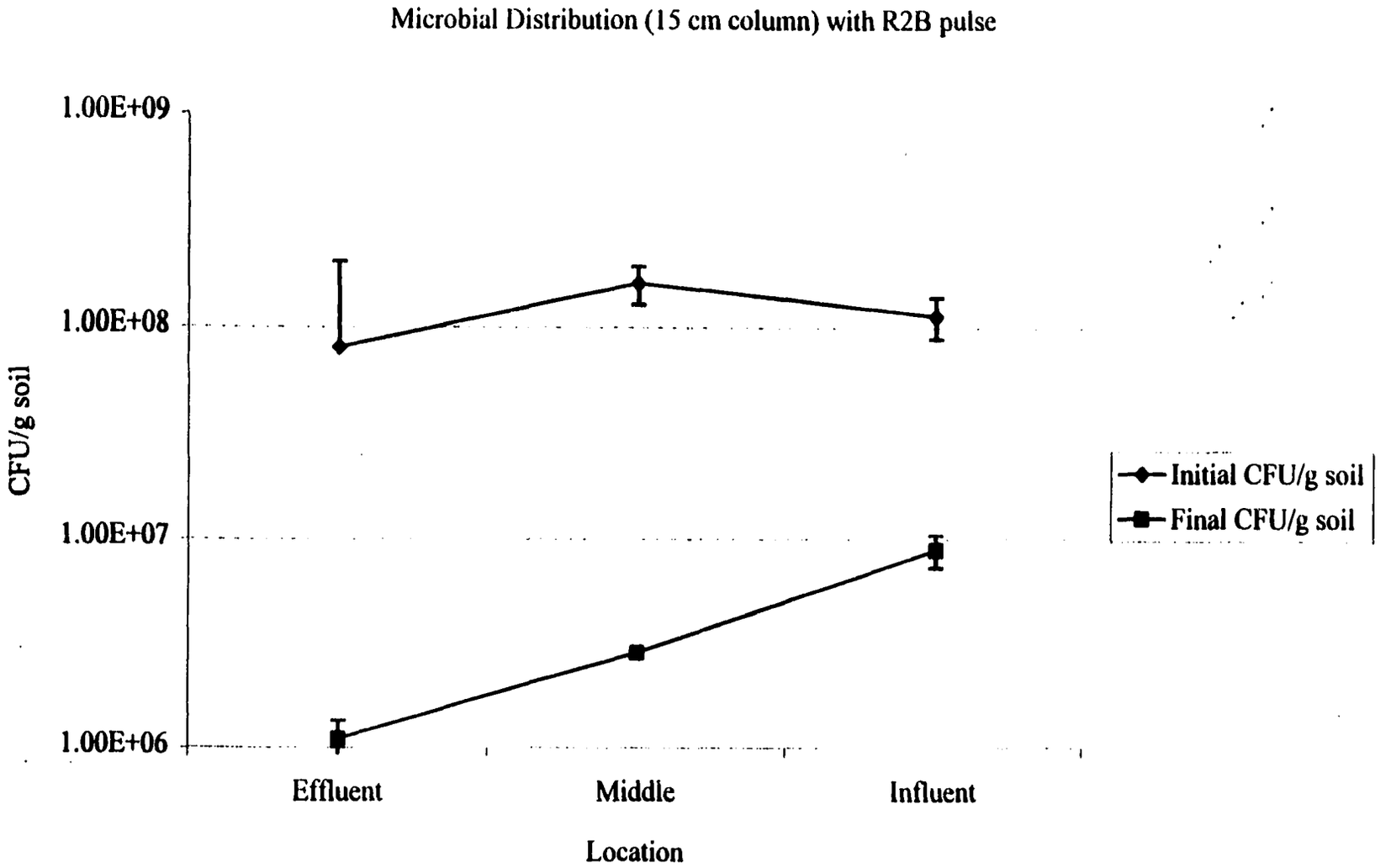

Figure B.11. *Pseudomonas oleavorans* (M11) cell distribution in a pulsed column.

APPENDIX C: ADDITIONAL RESULTS

Additional results pertaining to cell elution and cell distribution may be obtained from Dr. Mark L. Brusseau in the Department of Soil, Water and Environmental Science.

REFERENCES

Absolom, D.R., F.V. Lamberti, Z. Policova, W. Zingg, C.J. van Oss, and A.W. Neumann. 1983. Surfact thermodynamics of bacterial adhesion. Appl. Environ. Microbiol. 46: 90-97.

Albinger, O., B.K. Biesemeyer, R.G. Arnold, and B.E. Logan. 1994. Effect of bacterial heterogeneity on adhesion to uniform collectors by monoclonal populations. FEMS microbiol Letters 124: 321-326.

Alexander, M. 1965. Biodegradation; problems of molecular recalcitrance and microbial fallibility. Adv. Appl. Microbiol. 7: 35.

_____. 1973. Nonbiodegradable and other recalcitrant molecules. Biotechnol Bioeng 15:611-647

_____.1975. Environmental and microbiological problems arising from recalcitrant molecules. Microbio Ecol 2: 17-27

_____. 1981. Biodegradation of chemicals of environmental concern. Science. 211: 132.

_____. 1994. Biodegradation and bioremediation. Academic, San Diego, CA, USA

_____. 1995. How toxic are toxic chemicals in soil? Environmental Science and Technology 29: 2713-2717.

Alexander, M. and K.M. Scow. 1989. Kinetics of Biodegradation in Soil. P243-269. *In* B.L. Sawhney and K.W. Brown (ed.) Reactions and Movement of Organic Chemicals in Soils. Soil Science Society of America: American Society of Agronomy, Inc., Madison, WI.

American Petroleum Institute. 1992. Basic petroleum data book: petroleum industrial statistics. 8(3):. API, Washington, D.C..

Angley, J.T., M.L. Brusseau, W.L. Miller, and J.J. Delfino. 1992. Nonequilibrium Sorption and Aerobic Biodegradation of Dissolved Alkylbenzenes during Transport in Aquifer Material: Column Experiments and Evaluation of a Coupled-Process Model. Environ. Sci. Technol. V 26(7). P1404-1410.

Armstrong, R.A., and R. McGehee. 1980. Competitive exclusion. Am Nat. 115: 151-170.

Atlas, R.M. 1991. Bioremediation of fossil fuel contaminated sites (p 14-32). *In* R.E. Hinchee an R.F. Olfenbuttel, eds. In situe bioreclamation: applicatons and investigations for hydrocarbon and contaminated site remediaton. Battelle, Columbus, OH.

_____. 1993. Bioaugmentation to enhance microbial bioremediation. (P 19-37). *In* M. Gealt and M. Levin (eds). Biotreatment of industiral and hazardous waste. McGraw-Hill, NY.

Atlas, R.M. 1984. Diversity of microbial communities. Adv. Microb. Ecol. 7: 1-47.

Atlas, R.M. 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. Microbiological Reviews 45: 180-209.

Ayala, F.J. 1970. Competition, coexistence, and evolution. In: Essays in evolution and genetics. Eds. M.K. Hecht and W.C. Steere, pp. 121-158. New York: Appleton-Centuary-Crofts.

Barker, J.F., G.C. Patrick, and P.D. major. 1987. Natural attenuation of aromatic hydrocarbons in a shallow sand aquifer. Ground Water Monitor Rev 7: 64-71.

Baygents, J.C., J.R. Glynn, O. Albinger, B.K. Biesemeyer, K.L. Ogden, and R.G. Arnold. 1998. Variation of surface charge density in monoclonal bacterial populations: implications for transport through porous media. Environ Sci Technol 32: 1596-1603.

Birman, I., and M. Alexander. 1996. Optimizing biodegradation of phenanthrene dissolved in nonaqueous-phase liquids. Appl Microbiol Biotechnol 45:267-272

Borden, R.D. and T.B. Bedient. 1986. Transport of dissolved hydrocarbons influenced by oxygen-limited biodegradation: 1. Theoretical development. Water Resour. Res. 22: 1973-1982.

Borden, R.D. and T.B. Bedient. 1987. In situ measurement of adsorption and biotransformation at a hazardous waste site. Water Resour. Bull. 23: 629.

Brusseau, M.L. and P.S.C. Rao. 1989. Sorption Nonideality during Organic Contaminant Transport in Porous Media. CRC Crit. Rev. Environ. Control. 19(1). P33-99.

Brusseau, M.L., P.S.C. Rao, and C.A. Bellin. 1992. Modeling Coupled Processes in Porous Media: Sorption, Transformation, and Transport of Organic Solutes. *In* R.J. Wagenet, P. Baveye, and B.A. Stewart (ed.) Interacting Processes in Soil Science,

Advances in Soil Science. Lewis, Ann Arbor, MI. p147-184.

Brusseau, M.L., L.H. Xie, and L. Li. 1999. Biodegradation during Contaminant Transport in Porous Media: 1. Mathematical Analysis of Controlling Factors. Journal of Contaminant Hydrology. v 37. P269-293.

Brusseau, M.L., M.Q. Hu, J.M. Wang, and R.M. Maier. 1999. Biodegradation during Contaminant Transport in Porous Media: 2. Influence of Physicochemical Factors. Environ. Sci. Technol. v33(1). p96-103.

Budzinski, H., M. Raymond, T. Nadalig, M. Gilewicz, P. Farrigues, J.C. Bertrand, and P. Caumette. 1998. Aerobic biodegradation of alkylated aromatic hydrocarbons by a bacterial community. Org. Geochem. 28(5): 337-348.

Busscher, H.J., A.H. Werrkamp, H.C. van der Mei. A.W.J. van Pelt, H.P. de Jong, and J. Arends. 1984. Measurement of the surface free energy of bacterial cell surfaces and its relevance for adhesion. Appl. Environ. Microbiol 48: 980-983.

Cerniglia, E.C.. 1984. Microbial transformation of aromatic hydrocarbons. *In* Petroleum Microbiology,. Atlas, Rm. (ed.) Chapter 3. Macmillan: NY.

Cerniglia, E.C.. 1984. Microbial metabolism of polycyclic aromatic hydrocarbons. Adv Applied Microbiology 30: 31-71.

Cerniglia, C.E. 1992. Biodegradation of polycyclic aromatic hydrocarbons. Biodegradation, 3: 351-368.

Cerniglia, C.E.. 1993. Biodegradation of polycyclic aromatic hydrocarbons. Current Opinions in biotechnology 4: 331-338.

Cerniglia, C.E., J.B. Sutherland, and S.A. Crow. 1992. Fungal metabolism of aromatic hydrocarbons (p 193-217) *In* G. Winkelmann, (ed). Microbial degradation of natural products. VCH Verlagsgesellschaft, Weinheim, Germany.

Chen, Y.M., L.M. Abriola, P.J.J. Alvarez, P.J. Anid, and T.M. Vogel. 1992. Modeling Transport and Biodegradation of Benzene and Toluene in Sandy Aquifer Material: Comparisons with Experimental Measurements. Water Resources Research. V28(7). P1833-1847.

Compeau, G., A. Jadoun, E. Platsouka, and S.B. Levy. 1988. Survival of rifampin-resistant mutants of *Pseudomonas fluorescens* and *Pseudomonas putida* in soil systems. Appl. Environ. Microbiol. 54:2432-2438.

Corapcioglu, M.Y. and A. Haridas. 1984. Transport and fate of microorganisms in porous media: a theoretical investigagion. J. Hydrol 72: 149-169.

Cullen, W.R., S.F. Li, and K.J. Reimer. 1994. Degradation of phenanthrene and pyrene by microorganisims isolated from marine sediment and seawater. Science of Total Environment 156: 27-37.

Davies, J.M. and J.M. Addy, R.A. Blackman, J.R. Blanchard, JE. Ferbrache, D.C. Moore, H.J. Somerville, A. Whitehead, and T. Wilkinson. 1984. Environmental effects of the use of oil-based drilling muds in the North Sea. Mar. Pollut. Bull. 15:363-70.

Devlin, J.F. and J.F. Barker. 1994. A semipassive nutrient injection scheme for enhanced in situ bioremediation. Groundwater. 32(3): 374-80.

Dobbins, D.C.. 1990. Rates of organic substrate biodegradation in samples of aquifer solids, Ph.D. thesis, University of North Carolina at Chapel Hill.

Dobbins, D.C., C.M. Aelion, and F. Pfaender. 1992. Subsurface, terrestrial microbial ecology and biodegradation of organic chemicals: a review. Critical Reviews in Environmental Control 22 (½): 67-136.

Dockins, W.S., G.J. Olson, G.A. McFeters, and S.C. Turbak. 1980. Dissimilatory bacterial sulfate reduction in Montana ground waters. Geomicrobiol. J. 2: 53.

du Plessis, C.A., E. Senior, and J.C. Hughes. 1998. Growth kinetics of microbial colonisation of porous media. South African Journal of Science 94: 33-38.

Duetz, W.A., C. De Jong, P.A. Williams, and J.G. Van Andel. 1994a. Competition in chemostat culture beween *Pseudomonas* strains that use different pathways for the degradation of toluene. Appl Environ Microbiol 60: 2858-2863.

Dunlap, W.J., and J.F. McNabb. 1973. Subsurface biological activity in relation to foundwater pollution. EPA-6601/273-014. National Environmental Research Center. Office of Research Monitoring. U.S. EPA, Corvallis, OR, 1973.

Dupler, M. and R. Baker. 1984. Survival of *Pseudomanas putida*, a biological control agent, in soil. Phytopthology 74: 195-200.

Dzombak, D.A., and R.G. Luthy. 1984. Estimating adsorption of pylycyclic aromatic hydrocarbons on soils. Soil Sci 137: 122-307

Estrella, M.R., M.L. Brusseau, R.S. Maier, I.L. Pepper, P.J. Wierenga, and R.M. Miller. 1993. Biodegradation, Sorption, and Transport of 2,4-Dichlorophenoxyacetic Acid in Saturated and Unsaturated Soils. Appl. Environ. Microbiol. V59(12). P4266-4273.

Evans, C.G.T., D. Hervert, and D.W. Tempest. 1970. The continuous cultivation of microoganisims. 2. Construction of a chemostat. *In*: Norris J.R., and D.W. Ribbons (eds.), Methods of microbiology, vol2. Academic Press, London, pp 277-327.

Fang, J., and J.J. Barcelona. 1998. Biogeochemical evidence for microbial community change in a jet fuel hydrocarbons-contaminated aquifer. Org. Geochem. 29 (4): 899-907.

Fries, M.R., G.D. Hopkins, P.L. McCarty, L.J. Forney, and J.M. Tiedje. 1997. Microbial succession during a field evaluaton of phenol and toluene as the primary substrates for trichloroethene cometabolism. Applied and environmental microbiology. Apr. 1515-1522.

Garland, J.L. and A.L. Mills. 1994. A community-level physiological approach for studying microbial communities. In: Ritz, K. Dighton, J. Giller KE (ed) Beyond the biomass. John Wiley & Sons, Chichester, UK

Gerba, C.P., 1985. Microbial contamination of the subsurface. p 53 -67. In C.H. Ward. W. Ginger, and P.L. McCarty (ed.) Ground water quality. John Wiley & Sons, Inc, New York, N.Y.

Grover, J.P. 1997. Resource Competition (Chapman and Hall, London.)

Gundlach, E.R., P.D. Boehm, M. Marchand, R.M. Atlas, D.M. Ward, and D.A. Wolfe. 1983. The fate of Amoco Cadiz. Oil Sci 221: 122-129.

Harder, W. J.G. Kuenen, A. Matin. 1977. Microbial selection in continuous culture. J Appl Bacteriol 43: 1-24.

Hardin, G. 1960. The competitive exclusion principle. Science 131: 1292-1298.

Harms, H., and A.J.B. Zehnder. 1994. Influence of substrate diffusion on degradation of dibenzofuran and 3-chlorodibezofuran by attached and suspended bacteria. Appl Environ Microbiol 60: 2736-2745.

Harvey, R.W., L.H. George. R.L. Smith. And D.R. LeBlanc. 1989. Transport of microshperes and indigenous bacteria through a sandy aquifer: results of natural- and forced-gradient tracer experiments. Environ. Sci. Technol. 23: 51.

Hatzinger, P.B. and M. Alexander. 1995. Effect of aging of chemicals in soil on their

biodegradability and extractability. Envoronmental Science and Technology 29: 537-545.

Heitkamp, M.A. and C.E. Cerniglia. 1988. Mineralization of polycyclic aromatic hydrocarbons by a bacterium isolated from sediment below an oil field. Appl. Environ. Microbiol. 54: 1612-1641.

Hsu, S.B., K.S. Cheng, S.P. Hubel. 1981. Exploitative competition of micro-organisims for two complementary nutrients in continuous cultures. SIAM. J. Appl. Math 41: 422-444.

Hu, M.A. and M.L. Brusseau. 1998. Coupled effects of nonlinear, rate-limited sorption biodegradation on transport of 2,4-dicholrophenoxyacetic acid in soil. Environmental Toxicology and Chemistry. 17(9). p1673-1680.

Huisman, J. and F.J. Weisings. 1994. Light-limited growth and competition for light in well-mixed aquatic environments: an elementary model. Ecology 75: 507-520.

Hutchinson, G.E. 1961. The paradox of the plankton. Am. Nat. 95: 137-145

Jacob, J., W. Karcher, and PlJ. Wagstaffe. 1984. Polycyclic aromatic compounds of environmental and occupational importance. Their occurrence, toxicity and the development of high purity certified reference materials. Part I. Fresnis Z. Analytical Chemistry 317: 101-114.

Jang, I.K., T.F. Yen, G.V. Chiliagarian, and E.C. Donaldson. 1989. Bacterial migration through nutrient-enriched sand-pack columns for in-situ recovery of oil. p. 151-164. In. E.C. Donaldson, G.V. Chilingarian, and T.F. Yen (ed). Microbial enhanced oil recovery. Elsevier, New York, N.Y.

Kanazawa, S., and Z. Filip. 1986. Effects of trichloroethylene, tretrachloroethylene and dichloromethane on enzymatic activities in soil. Appl. Microbiol. Biotechnol., 25: 76.

Keith, L.H., and W.A. Telliard. 1979. Priority pollutants 1: A perspective view. Environ. Sci Technol 13: 416-423.

Kelley, I., J.P. Freeman, F.E. Evans, and C.E. Cernigllia. 1991. Identification of a carboxylic acid metabolite from the catabolism of fluoranthrene by a Mycybacterium sp. Appl Environ Microbiol 57: 636-641.

Koch, A.K., O Kappeli, A. Fiechter, and A. Reiser. 1991. Hydrocarbon assimilation and

biosurfactant production in *Pseudomonas aeruginosa* mutants. J. Bacteriol, 173: 4212-4219.

Kuhn, E.P., P.J. Colberg, J.I. Schnoor, O.Wanner, A.J.B. Zennder, and R.P. Schwarzenbach. 1985. Microbial transformations fo substituted benzenes during infiltration of river water to groundwater: laboratory column studies. Environ. Sci. Technol. 19: 961.

Larsen, P.F. D.F. gadbois, a.C. Johnson, L.F. Dogget. 1983. Distribution of polycyclic aromatic hydrocarbons in the surficial sediments of Casco Bay, Maine. Bull. Environ. Contam. Toxicol. 30:530-5.

Lawrence, J.R. and M.J. Hendry. 1996. Tramsport of bacteria thourhg geologic media. Can. J. Microbiol 42 (4): 410-422.

Lee, K.E. and Pankhurst, C.E. 1992. Soil organisims and sustainable productivity. Aust. J. Soil Res. 30: 855-92.

Leon, J.A. and D.B. Tumpson. 1975. Competition between two species for two complementary or substitutable resources. J. Theor. Biol 50, 185-201.

Lewandowski, G., B. Baltizis, and C. P. Varuntanya. 1998?. The use of pure culutres as a means of understanding the performance of mixed cultures n biodegradation of phenolics. Biotechnology for degradation of toxic chemicals.

Long, S.C. and C.M. Aelion. 1999. Metabolite formation and toxicity measurements in evaluating bioremediation of a jet-fuel-contaminated aquifer. Applied Biodhemistry and Biotechnology 76: 79-95.

Lotka, A.J. 1932. The growth of mixed populations: two species competing for a common food supply. Journal of the Washington Academy of Sciences, 22: 461-469.

MacQuarrie, K.T.B., and E.A. Sudicky. 1990. Simulation of biodegradable organic contaminants in grounwater: 2. Plume behavior in uniform and random flow fields. Water Resour. Res. 26:223-239.

Madsen, E.L., A. Winding, K. Malachowsky, C.T. Thomas, W.C. Ghiorse. 1992. Contrasts between subsurface microbiol communities and their metabolic adaption to polycyclic aromatic hydrocarbons at a forested and an urban coal-tar disposal site. Microb Ecol 24: 199-213.

Mahaffey, W.R., D.T. Gibson, and C.E. Cerniglia. 1988. Bacterial oxidation of chemical

carcinogens: formation of polycyclic aromatic acids from benz(a)anthracene. Appl Environ Microbiol 54: 2415-2423

Maier, W.J., 1989. Kinetics-dynamics of biodegradation of potentially toxic organic chemicals. In 43rd Purdue Industrial Waste Conf. Proc. Lewis Publishers, Chelsea, MI.

May, R.M. 1974. Stability and Complexity in model ecosystems (Princeton Univ. Press, Princeton).

McCarty, P.L., M. Reinhard, and B.E. Rittman. 1981. Trace organics in groundwater. Environ. Sci. Technol. 15: 40.

McEldowney, S. and M. Fletcher. 1986. Effect of growth conditions and surface characteristics of aquatic bacteria on their attachment to solid surfaces. Journal of General Microbiology. 132:513-523.

Moltz, F.J. , M.A. Widdowson, and L.D. Benefield. 1990. Simulation of microbial growth dynamics coupled to nutrient and oxygen transport in porous media. Water Resour. Res 22(8): 1270-1216.

Monod, J. 1949. The growht of bacterial cultures. Ann. Rev. Microbiol. 3. p371-394.

Naeem, S., and S. Li. 1997. Biodiversity enhances ecosystem reliability. Nature 390: 507-509.

Noguera, D.R., G. Pizarro, D.A. Stahl, and B.E. Rittmann. 1999. Simulation of multispecies biofilm development in three dimenstions. Water Sci. Tech. 39 (7): 123-130.

Odum, E.P. 1985. Trends expected in stressed eco-systems. BioScience 35:419-422.

Øvreås, L., and V. Trosvik. 1998. Microbial diversity and community structure in two different agricultural soil communities. Microb. Ecol. 36: 303-315.

Reynolds, P.J., P. Sharma, G.E. Jenneman, and M.J. McInerney. 1989. Mechanisms of microbial movement in subsurface materials. Appl Environ Microbiol 55: 2280-2286.

Ricklefs, R.E. 1973. Ecology. Chiron Press, Inc. Great Britain.
Rittman, B.E. and J.A. Manem, 1992. Development and experimental evaluation of steady-state, multispecies biofilm model. Biotechnology and Bioengineering. 39: 914-922.

Rozkov, A., A. Käärd, and R. Vilu. 1998. Biodegradation of dissolved jet fuel in chemostat by a mixed bacterial culture isolated from a heavily polluted site.

Biodegradation 8: 363-369.

Rubin, H.E., R.V. Subba-Rao, and M. Alexander. Rates of mineralization of trace concentrations of aromatic compounds in lake water and sewage samples.Appl. Environ. Microbiol. 43: 1133.

Sandrin, S.K., and..... in review

Scholtz-Muramatus, H., V. Schneider, S. Gaiser, and D. Vardtke. 1988a. Biological elimination of dichloromethane from contaminated groundwater - interference by components of the goundwater. Water Sci. Technol., 20: 393.

Scow, K.M, and M. Alexander. 1992. Effect of difustion on the kinetics of biodegration: experimental results with synthetic aggregates. Soil Sci. Soc. Am. J.. 51: 128-134.

Sims, J.L., Sims, R.C. and J.E. Matthews. 1990. Approach to bioremediaton of contaminated soil. Hazardous Waste and Hazardous Material. 7: 117-149.

Sneeth, P.H.A. 1986. Endosport-forming Gram-positive rods and cocci. In: Sneath. P.H.A. (Ed.) Bergey's Manual of Sytematic Bacteriology. Vol. 2. The Williams and Wilkins Company, Baltimore. pp.. 1104-1207.

Snyder, S.K., F.L. Jordan, R.M. Maier, and M.L. Brusseau. 1999. The impact of microbial lag and bacterial cell growth on the biodegradation and transport of organic compounds. Environ. Sci. Technol. (In Press).

Sommer. U. 1985. Comparison between steady state and non-steady state competition: experiments with natural phytoplankton. Limnol. Oceanogr. 30: 335-346.

Spain, J.C., P.H. Pritchard, and A.W. Bourquin. 1980. Effects of adaption on biodegradation rates in sediment/water cores from estuarine and freshwater environments. Appl. Envirn. Microbiol. 40: 726.

Steffensen, W.S., and M. Alexander. 1995. Role of competition of inorganic nutrients in the biodegradation of mixtures of substrates. Applied and Environmental Microbiology, 61(8): 2859-2862.

Stotzky, G. 1972. Activity, ecology, and population dynamics of microorganisims in soil. Crit. Rev. Microbiol. 2: 59.

Stucki, G., and M. Alexander. 1987. Role of dissolution rate and solubility in biodegradation of aromatic compounds. Appl Environ Microbiol 53: 292-297

Sturman, P.J., W.L. Jones, and W.G. Characklis. 1994. Interspecies competition in colonized porous pellets. Wat. Res. 28(4): 831-839.

Swindoll, C.M., C.M. Aelion, and F.K. Pfaender. 1988. Influence of inorganic and organic nutrients on aerobic biodegratioin and on the adaption response of subsurface microbial communities. Appl. Environ. Microbiol., 54: 211-217.

Sykes, J.F., S. Soyupak, and G.J. Farquhar. 1982. Modeling of leachate organic migration and attenuation in groundwaters below sanitary landfills. Water Resour. Res. 18(1): 135-145.

Thompson, I.P., M.J. Bailey, R.J. Ellis, N. Maguire, and A.A. Meharg. 1999b. Response of soil microbial communities to single and multiple doses of an organic pollutant. Soil Biol. Biochem 31:95-105.

Thompson, I.P., M.J. Bailey, E.M. Boyd, N. Maguire, A.A. Meharg, and R.J. Ellis. 1999. Concentration effects of 1,2-dichlorobenzene on soil microbiology. Environmental Toxicology and Chemistry 19 (9): 1891-1898.

Tilman, D. 1977. Resource competition between planktonic algae; an experimental and theoretical approach. Ecology 58: 338-348.

Travis, B.J., and N. D. Rosenberg. 1997. Modeling *in situ* bioremediation of TCE at Savannah River: effects of product toxicity and microbial interactions on TCE degradation. Environmental Science and Tech. 31(11): 3093.

van Elsas, J.D., A.F., Dijkstra, J.M. Govaert, and J.A. Van Veen. 1986. Survival of *Pseudomonas fluorescens* and *Bacillus subtills* introduced into two soils of different texture in field microplots. FEMS Microbiol. Ecol. 38:151-160.

van Elsas, J.D., L.S. van Overbeek, A.M. Feldmann, A.M. Dullemans, and O. de Leeuw. 1991. Survival of genetically engineered *Pseudomonas fluorescens* in soil in competition with the parent strain. FEMS Microbiology Ecology, 85: 53-64.

van Loosdrecht, M.C.M., J. Lyklema, W. Norde, G. Schraa, and A.J.B. Zehnder. 1987. The role of bacterial cell wall hydrophobicity in adhesion. Appl. Environ. Microbiol 53: 1893-1897.

van Loosdrecht, M.C.M., J. Lyklema, W. Norde, G. Schraa, and A.J.B. Zehnder. 1987. Electrophoretic mobility and hydrophobicity as a measure to predict the initial steps of bacterial adhesion. Appl Environ Microbiol 53: 1898-1901.

Van Schie, P.M., and M. Fletcher. Adhesion of biodegradative anaerobic bacteria to solid surfaces. Applied and Environmental Microbiology, 65: 5082-5088.

Volkering, F. 1996. Bioavailabiltiy and biodegradation of polycyclic aromatic hydrocarbons. Ph.D. thesis. Wageningen Agricultural University, Wageningen, The Netherlands.

Volterra, V. 1926. Variations and fluctuations of the numbers of individuals in animal species living together. (Reprinted in 1931. In: R.N. Chapman, Animal Ecology. McGraw Hill, New York.

Walker, B.H. 1992. Biodiversity and ecological redundancy. Conserv. Biol. 6: 18-23.

Walter, U., M. Beyer, J. Klein, and H.J. Rehm. 1991. Degradation of pyrene by Rhodococcus sp. UW1. Appl Microbiol Biotechnol 34: 671-676.

Wang, J-M., A. Bodour, R.M. Maier, and M.L. Brusseau. 1999. The influence of bacterial population heterogeneity on biodegradation and transport o f phenanthrene in soil. Environmental Science and Technology (in press).

Ward, T.E.. 1985. Characteriaing the aerobic and anaerobic microbial activities in surfac and subsurface soil. Environ. Toxicol. Chem. 4: 727.

Weissenfels, W.D., H.J. Klewer, and J. Langhoff. 1992. Adsorption of polycyclic aromatic hydrocarbons (PAHs) by soil particles: Influence on biodegradability and biotoxicity. Appl Microbiol Biotechnol 36: 671-676

Wessendorf, J. and F. Lingens. 1989. Effect of culture and soil conditions on survival of *Pseudomonas fluorescens* R1 in soil. Appl. Microbiol. Biotechnol 31: 97-102.

White, K.L. 1986. An overview of immunotoxicology and carcinogenic polycyclic aromatic hydrocarbons. Journal of Environmental Sciences and Health, Environmental Carcinogenic Reviews C4(2): 163-202.

Whitelaw, K., and R.A. Edwards. 1980. Carbohydrates in the unsaturatedzone of the Chalk, England,Chem. Geol. 29:281.

Wiggins, B.A., S.H. Jones, and M. Alexander. 1987. Explanations for the acclimation period preceding the mineralization of organic chemicals in aquatic environments. Appl. Environ. Microbiol. 53: 791.

Wilson, M.S., and E.L. Madsen. 1996. Field extraction of a transient intermediary metabolite indicative of real time in situ naphthalene biodegradation. Environ. Sci. Technol. 30:2099-2103.

Wilson, J.L., S.H. Conrad, W.R. Mason, W.Peplinski, and E.Hagan. 1990. Laboratory investigation of residual liquid organics from spills, leaks, and the disposal of hazardous wastes in groundwater. U.S. Environmental Protection Agency publication no. EPA/600/6-90/004. U.S. Environmental Protection Agency, Washington, D.C.

Wilson, J.T., D.K. Kampbell, S.R. Hutchins, and D.F. Pope. 1993. Subsurface bioremediation. Proceedings, EPA Seminar on Bioremediation of Hazardous Waste Sites: Practical Approaches to Implementation, 10-11 June, Chicago, IL.

Woodzinski, R.S., and D. Bertolini. 1972. Physical state in which naphthzalene and bibenzyl are utilized by bacteria. Appl Microbiol 23: 1077-1081

Woodzinski, R.S., and J.E. Coyle. 1974. Appl Microbiol 27:1080-1084.

Wodzinski, R.S., and D. Larocca. 1977. Bacterial growth kindetics on diphenylmethane and naphthalene-heptamethylononane mixtures. Appl Environ Microbiol 33: 660-665

Yates, M.V., and S.R. Yates. 1988. Modeling microbial fate in the subsurface environmnet CRC Crit Rev Environ Control 17: 307-344.

Yolcubal, I., and ..... in review

Zak, J.C., M.R. Willig, D.L. Moorhead, and H.G. Wildman. 1994. Functional diversity of microbial communities: a quantitive approach. Soil Biol Biochem 26:1101-1108.

Zang, T.C., and P.L. Bishop. 1994. Density, porosity and pore structure of biofilms. Wat. Res. 28: 2267-2277.

Zang, T.C., Y.C. Fu, and P.L. Bishop. 1993. Competition in biofilms. Proc. Second Int. Specialized Conf. On Biofilm Reactors, Paris, pp 705-712.

Zhang, Y., W.J. Maier, and R.M. Miller. 1997. Effect of rhamnolipids on the dissolution, bioavailability, and biodegradation of phenanthrene. Environ. Sci. Technol. 31:2211-2217.