This is to certify that the

dissertation entitled

Diversity of Mono Hydrocarbon Degrading Bacteria and Their TCE Co-oxidation Potential

presented by

Marcos Rubens Fries

has been accepted towards fulfillment
of the requirements for

PhD degree in Crop and Soil Sciences

Major professor

Date 6-12-95

MICHIGAN STATE UNIVERSITY

RECORD OF DISSERTATION AND ORAL EXAMINATION REQUIREMENTS FOR DOCTORAL DEGREE CANDIDATE

Department of CROP & SOIL SCIENCES

Student's Name MARCOS RUBENS FRIES Student Number A11209766

1. Dissertation Title: DIVERSITY OF MONO AROMATIC HYDROCARBON DEGRADING BACTERIA AND THEIR TCE CO-OXIDATION POTENTIAL

2. Dissertation has been: X Accepted ____ Rejected ____ Accepted subject to revisions (beyond minor editorial changes) required by the Committee.

3. Oral examination in defense of the dissertation was conducted on 6-8-95 (Date)

The student X Passed ____ Failed: Reason ____

4. Dissenting opinions and signatures of dissenting examiners, if any:

5. Subject to the satisfactory completion of other requirements, this student is recommended for the degree Doctor of:

____ Philosophy ____ Education ____ Musical Arts

Signature of Examiners:	Printed Names	
1. (Chairperson of Committee)	1. DR JAMES M. TIEDJE	6-9-95 (Date)
2.	2. DR MICHAEL KLUG	6-8-95 (Date)
3.	3. DR STEPHEN BOYD	6-8-95 (Date)
4.	4. DR LEE JACOBS	6/8/95 (Date)
5.	5.	(Date)
6. (Outside Examiner (if applicable))	6.	(Date)

6. Major revisions required: ____

7. Revisions, if any, approved: (Chairperson of Guidance Committee) 6-8-95 (Date)

Approved: Department Chairperson

Associate/Assistant Dean ____

DISTRIBUTION:
Canary — Dean
Pink — Department
Blue — Guidance Committee
Goldenrod — Student

Forms supplied by Office of Registrar
10/87

MSU IS AN AFFIRMATIVE ACTION/EQUAL OPPORTUNITY INSTITUTION

Di ersi

Diversity of Mono Aromatic Hydrocarbon Degrading Bacteria and Their TCE Co-oxidation Potential.

By

Marcos Rubens Fries

A DISSERTATION

Submitted to
Michigan State University
in partial fulfillment of requirements
for the Degree of

DOCTOR OF PHILOSOPHY

Department of Crop and Soil Sciences

1995

ABSTRACT

Diversity of Mono Aromatic Hydrocarbon Degrading Bacteria and Their TCE Co-oxidation Potential.

BY

Marcos Rubens Fries

Trichloroethene (TCE) is the most frequently observed volatile organic contaminant in ground water. Bacteria with certain aromatic mono- or dioxygenases have the potential to co-oxidize TCE. A shallow anoxic aquifer was amended with phenol + O_2 and then toluene + O_2 to determine the effectiveness of these treatments in removing TCE. We isolated the phenol and toluene-degrading populations after both treatments from the terminal positive MPN dilution tubes using phenol and toluene as substrates. A high percentage of the isolates could grow on both phenol and toluene regardless of the substrate on which they were isolated . Half of the isolates were also denitrifiers, which may have been a result of high nitrate and low oxygen in this aquifer. When restricted genomic DNA from the isolates was probed in Southern blots with DNA probes encoding genes for the first steps of the five different catabolic pathways for toluene metabolism, we found that the toluene ortho-hydroxylase pathway was dominant among the isolates . Other organisms with this pathway are known to be effective TCE co-oxidizers. ARDRA analysis suggested similar community structures when phenol and toluene were the primary carbon sources, consistent with the interpretation from analysis of the

dominant

DNA wer

did isolat

were not

the popul

were dras

rebound t

dominant isolates. The majority of bands observed in ARDRA from community DNA were found in ARDRA for the most common isolates, suggesting that we did isolate the major populations from the site and that unculturable populations were not important in this study. When 1,1-DCE was injected into the aquifer, the population of heterotrophs and monoaromatic degraders as well as diversity were drastically reduced. Once 1,1-DCE was removed the microbial populations rebound to the original density and composition.

In memory of my grandfather

MILTON

who's love and support made my career possible

The a

Dr. J

course of this

Dr. M

committee

Lead

patience an

My

Fel

Inez Toro-S

Tamara Ts

Greg Thom

Frank Loff

Kirsti for t

M

laboratory

To

Dr. Larry R

and Dr. W

T

The a

providing

Departmen

ACKNOWLEDGMENTS

The author expresses his sincere thanks to:

Dr. James Tiedje for his patience, guidance, and encouragement throughout the course of this study and preparation of this dissertation. I could not have a better role model.

Dr. Michael Klug, Dr. Stephen Boyd and Dr. Lee Jacobs members of my guidance committee.

Leadir, my wife and friend, Milton, Kalynka and Henrique, my beloved children, for their patience and abnegation expressed during my training program.

My grandparents and family, for the loving support they have given to me.

Fellow lab colleagues, Joanne Chee-Sanford, Robert Sanford, Jorge Santo Domingo, Inez Toro-Suarez, Mary An Bruns, Klaus Nuesslein, Jim Champine, Suzane Thiem, Rick Ye, Tamara Tsoi, Jim Cole, Jizhong Zhou, Serguei Bezborodnikov, Roberta Fulthorpe, Al Rhodes, Greg Thom, Cindy Nakatsu, Cathy McGowan, Jong-ok Ka, Yuichi Suwa, Arturo Massol-Deya, Frank Loffler, Alenka Princic, Nanci Tonso, Yuichi Suwa, Amy Cascarelli, Grace Calabrese, Kirsti for the friendliness they have given to me.

Mrs. Joyce Widenthal, David Harris, Keith Nelson and Hellen Corlew-Neuman for laboratory help and friendship.

To the CME staff Ann, Lisa, Betty, Annette for their help and assistance.

Dr. Larry Forney, Dr. Perry McCarty and Dr. Gary Hopkins for participating in this exciting project and Dr. Wynne Lewis and Dr. Bill Holben for the Gene probe workshop.

To NSF for supporting CME.

The author is indebted to CME, CAPES and Departamento de Solos-UFSM, Brazil, for providing the opportunity and financial aid during the course of his training program and to the Department of Crop and Soil Sciences-MSU for accepting him into its Graduate Program.

TABLE OF CONTENTS

Table

LIST OF TABLES

Table **Page**

Chapter I

Chapter II

Chapter III

Chapter IV

Chapter V

Figure

LIST OF FIGURES

Figure **Page**

Chapter I

Chapter II

Chapter III

Figure 3.2.
chromos
for patte
indicate

Figure 3.3
levels of
isolated

Figure 3.4. D
XXX wer

Figure 3.5. A
aquifer.
(B). and
stained w
(AQ. GB)
water (Fil
beads fro

Figure 4.1. Di
products. (
homology

Figure 4.2. Clu
together wi
($r_{x,y}$=0.88)...

Figure 1. REP-P
using chromo
on the left....

Chapter IV

Chapter V

Figure 2. Ph
Isolate T
3 and Td

Figure 3. Sou
digested
heme nitr
(D). Hybr
conditions

Figure 4. Phy
constructe
and rooted
confidenc
sequences

Thr

Hazardous

Microbial E

Research

Environmen

program wa

enhance a

groundwater

cepacia G4

constitutively

effective in T

This

objectives aim

phenotypic pa

community and

the TCE degra

Chapter I

Introduction

Overview

Through the combined efforts of researchers within the Western Region Hazardous Substance Research Center at Stanford University, the Center for Microbial Ecology and the Great Lakes Mid Atlantic Hazardous Substance Research Center at Michigan State University, and the EPA Gulf Breeze Environmental Research Laboratory and associated collaborators a research program was initiated in 1993, for the field scale study of bioaugmentation to enhance and improve the in-situ bioremediation of TCE contaminated groundwater. The organism proposed for bioaugmentation is *Pseudomonas cepacia* G4 PR1, the nonrecombinant derivative of *P. cepacia* G4 that constitutively expresses toluene ortho-monooxygenase (TOM), and is highly effective in TCE degradation (Shields and Reagin, 1992).

This research was part of the main project and describes research objectives aimed at answering fundamental questions regarding genotypic and phenotypic patterns of natural biodegrading populations as well as to evaluate community and population changes when the primary carbon source stimulating the TCE degradation is switched from phenol to toluene. The general goal of

the isola

importan

recognize

communit

dominated

high level

determinin

were addr

group and

O

Fundamental

i. What portio

ii. How effecti

TCE oxidizing

cepacia G-4?

iii. What is th

judged by REP

iv. What is the

Ecology of the

i. What is the b

to the total hete

ii. How does th

carbon source fr

iii. Is dominanc

over time?

iv. What is the

confer a competit

the isolate portion of the study was to understand which populations are the important competitors of G4, what activity they have on TCE, and to learn how to recognize, and differentiate them from G4 in the field. The goal of the community analysis portion of the study was to determine which populations dominated the competition in the field study. I also investigated if the natural high levels of nitrate present in the low O_2 aquifer water played a role in determining which populations of denitrifiers were selected. These objectives were addressed in laboratory studies using samples collected by the Stanford group and sent to MSU.

Overall this study was designed to answer the following questions:

Fundamental properties:

i. What portion of the C-selected phenol degraders also degrade toluene and vise-versa?

ii. How effective are the selected indigenous populations at TCE degradation and how does their TCE oxidizing capacity compare to that of the well-studied strains, specially *Pseudomonas cepacia* G-4?

iii. What is the extent of diversity among the selected toluene and phenol degrading strains as judged by REP, FAME, and biochemical methods?

iv. What is the effect of anoxic conditions on TCE co-oxidation?

Ecology of the site:

i. What is the biological limit (population size) of phenol and toluene degraders when compared to the total heterotrophic population?

ii. How does the community structure respond to a perturbation, i.e., the change in the primary carbon source from phenol to toluene?

iii. Is dominance maintained during the field experiment, i.e., do the same populations repeat over time?

iv. What is the role that denitrification plays in the ecology of this site? Does denitrification confer a competitive advantage to certain microbial populations?

Sources o

A

enter oxic

rpimary and

have been

structural pl

against inva

compounds.

including hy

hydroxybenz

complex phe

(Harborne, 1

from non a

hydroxybenz

microorganis

Stachybotrys

Haider, 196

growing in glu

or both, and h

in 72% sulfuri

acids because

Background

Sources of aromatic compounds and biodegraders in the environment.

Aromatic compounds from natural and anthropogenic sources readily enter oxic or anoxic environments. Aromatic structures are found in many rpimary and secondary plant metabolites and several thousand plant phenols have been described (Harborne, 1980). They are an integral part of the structural plant matrix, serve as flower pigments, act as constitutive protection against invading organisms, function as signal molecules, act as allelopathic compounds, and affect cell and plant growth. Plants produce simple phenols, including hydroxyquinone, gallic acid, salicylic acid, protocatechuic acid, and p-hydroxybenzoic acid; more complex phenols, such as the flavonoids; and complex phenolic polymers, including lignins, catechol, melanins, and flavolans (Harborne, 1980). Phenolic units may also originate through microbial synthesis from non aromatic carbon and energy sources. Numerous phenolic and hydroxybenzoic acid compounds are synthesized by fungi and other microorganisms. Cultures of *Hendersonula toruloidea* (Martin et al, 1972) *and Stachybotrys atra*, *Stachybotrys chartarum* and *Epicoccum nigrum* (Martin and Haider, 1969) synthesize dark colored, humic acid-type substances when growing in glucose medium. They are formed in the culture media, in the cells, or both, and have been referred to as lignin-like because they resist degradation in 72% sulfuric acid, as melanins because they are dark colored, or as humic acids because they are soluble in alkali, precipitated by acid and have high

exchange

Compoun

included

Although

the origin

various so

such dep

toluene, e

anthracen

consumed

1.5 million

unprotecte

potential f

petroleum

modern env

Due t

is not surpri

these compo

of the carbo

communities

degradation

exchange capacities (Martin and Haider, 1969 and Martin et al, 1972). Compounds which could be separated from glucose -based culture medium included phenolic acids and hydroxylated toluenes (Martin and Haider, 1969).

Aromatic structures are also present in large amounts in fossil fuels. Although the detailed composition of crude petroleum deposits depends upon the origin and location of the petroleum, considerable similarities exist among various sources. Aromatic compounds comprise more than 50% by volume of such deposits and include homocyclic aromatics, both nuclear (benzene, toluene, ethylbenzene, xylenes) and polynuclear (naphthalene, phenanthrene, anthracene) (Cole, 1994). About 65% of the petroleum used as a fuel is consumed as gasoline, which is stored primarily underground in an estimated 1.5 million storage tanks. Almost all of the tanks installed prior to 1988 were unprotected steel underground storage tanks that have leaked or have the potential for leaking gasoline into the environment (Cole, 1994). Thus, petroleum products have become an increasingly common substrate in the modern environment.

Due to the ubiquitous nature of aromatic hydrocarbons in the environment, is not surprising that microorganisms have evolved with the ability to degrade these compounds. This activity can be considered as part of the normal process of the carbon cycle. Bacteria and fungi are dominant members of most soil communities and representatives of both groups are capable of hydrocarbon degradation (Bossert and Bartha, 1984). The ability to degrade and/or utilize

hydrocarb

Compilatio

presented

genera fou

hydrocarbo

and 31 gen

Based

the most fr

soil enviro

Bacillus, *P*

Among the

spp. are th

are the m

frequently

versus fu

extensivel

environme

bacteria a

was attrib

1986).

The

is estimate

hydrocarbon substrates is exhibited by several bacterial and fungal genera. Compilation of reported genera for hydrocarbon degrading bacteria and fungi is presented in Table 1.1. Hydrocarbon-degrading bacteria are represented in 25 genera found in the marine environment and 29 genera found in soil. Similarly hydrocarbon-degrading fungi are found in 25 genera from marine environment and 31 genera found in soil.

Based on the number of times that each genera was reported in this survey, the most frequently isolated hydrocarbon degrading bacteria in both marine and soil environments are *Achromobacter, Acinetobacter, Alcaligenes, Arthrobacter, Bacillus, Flavobacterium, Nocardia, Pseudomonas spp.* and the coyneforms. Among the fungi, *Aureobasidium, Candida, Rhodotorula, and Sporobolomyces* spp. are the most common marine isolates and *Tricoderma and Mortierella* spp. are the most common soil isolates. *Aspergillus and Penicillium* spp. have been frequently isolated from both environments. The relative contribution of bacteria versus fungi in the biodegradation of hydrocarbons in nature has not been extensively studied and may be a function of the ecosystem and local environmental conditions. In a comparative study of hydrocarbon degradation by bacteria and fungi in a sandy loam soil, 82% of the n-hexadecane mineralization was attributed to bacteria and only 13% was attributed to fungi (Song et al., 1986).

The fraction of monoaromatic (phenol and toluene) biodegraders in nature is estimated to be in the order of 1 per every 100 total heterotrophic bacteria

Table 1.1. Major genera of hydrocarbon degrading microorganisms[a].

Bacteria			Fungi		
Genera	Soil[b]	Marine and Brackish water environment[b]	Genera	Soil[b]	Marine and Brackish water environment[b]
Achromobacter	4	8	*Acremonium*	2	
Acinetobacter	3	4	*Allescheria*		1
Actinomyces		2	*Aspergillus*	3	4
Aeromonas		2	*Aureobasidium*	2	3
Alcaligenes	6	3	*Beauveria*	1	
Arthrobacter	7	5	*Botrytis*	1	2
Bacillus	2	5	*Candida*	2	5
Beneckea		1	*Cephalosporium*		1
Brevibacterium	1	1	*Chrysosporium*	1	
Chromobacterium	1		*Cladosporium*	1	2
Corynebacterium	5		*Cochliobolus*	1	
Coryneforms		7	*Cunninghamella*		1
Cytophaga	2		*Cylindrocarpon*	1	
Erwinia	1	1	*Debaromyces*	1	2
Flavobacterium	5	6	*Fusarium*	1	1
Klebsiella		1	*Geotrichum*	1	
Lactobacillus		1	*Gliocladium*	1	
Leucothrix		1	*Gonytrichum*		1
Micrococcus	3		*Graphium*	2	
Moraxella		1	*Hansenula*		1
Mycobacterium	3		*Helminthosporium*		1
Nocardia	3	6	*Humicola*	1	
Peptococcus		1	*Monilia*	1	
Proteus	1		*Mortierella*	3	
Pseudomonas	12	8	*Mucor*		1
Sarcina	2	1	*Oidiodendrum*		1
Serratia	1		*Paecilomyces*	1	1
Spherotilus		1	*Penicillium*	4	4
Spirillum	1	1	*Phialophora*		2
Streptomyces	1	1	*Phoma*	1	
Vibrio	1	7	*Rhodosporidium*		2
Xanthomonas	2	1	*Rhodotorula*	1	4
			Saccharomyces	2	2
			Saccharomycopsis		1
			Scolecobasidium	2	
			Scopulariopsis		1
			Spicaria	1	
			Sporobolomyces	1	4
			Sprotrichum	1	
			Tolypocladium	1	
			Torulopsis	1	1
			Trichoderma	6	1
			Trichosporon		2
			Verticillium	2	

[a] Data compiled by Bossert, I., and Bartha, R., (1984) for soils and by Floodgate, G. D., (1984) for marine and brackish water environment.

[b] Number of citations for each genera in different publications. There were 19 publications for marine and brackish water and 20 publications for soil data.

(Figure 1.1

compound

microbial g

Figure 1
applicati

(Figure 1.1.). Therefore, the focus for remediation technologies for this class of compounds should be on insuring that the environmental conditions do not limit microbial growth (Tiedje, 1993).

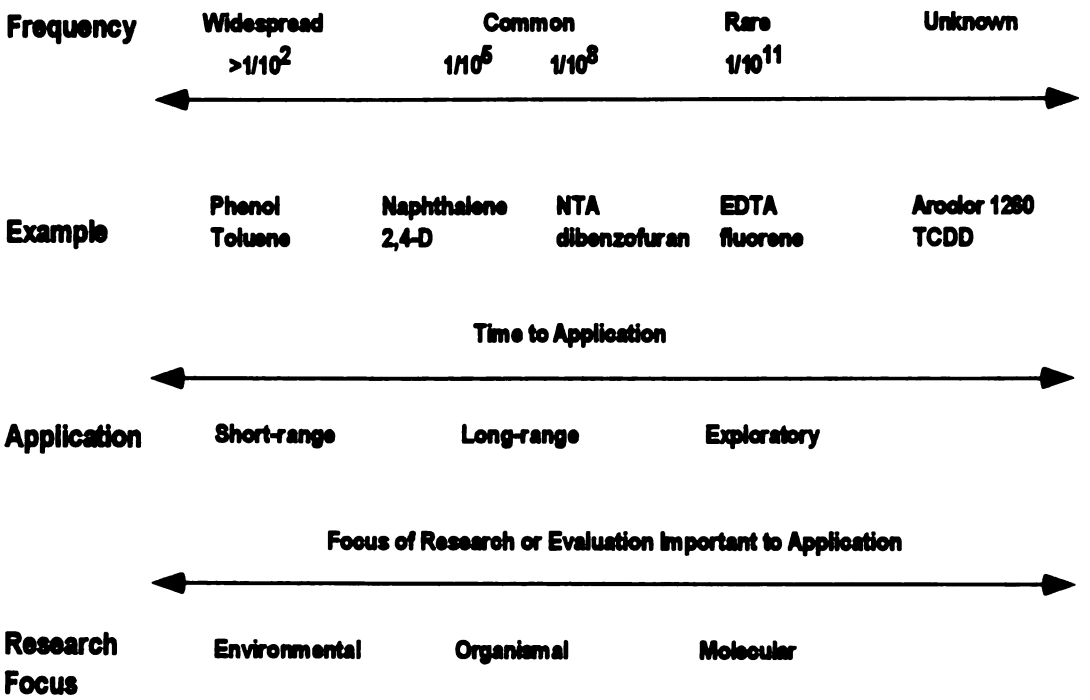


Figure 1.1. Relationship of frequency of biodegraders in the community to application of bioremediation (Tiedje, 1993).

Biochemi

Th

aromatic hy

in general

1994). Dur

benzene rin

attack or ri

hydroxyl gr

produce a

dihydroxyb

illustrated f

The fo

important

structure

(Dagley, 1

catechol ri

carbon-car

addition of

two differe

(*ortho*) dio

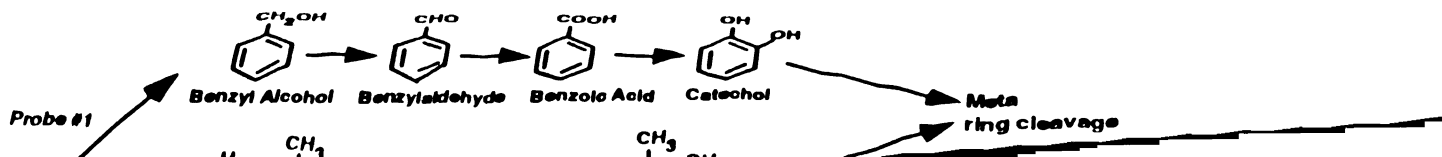
dioxygenas

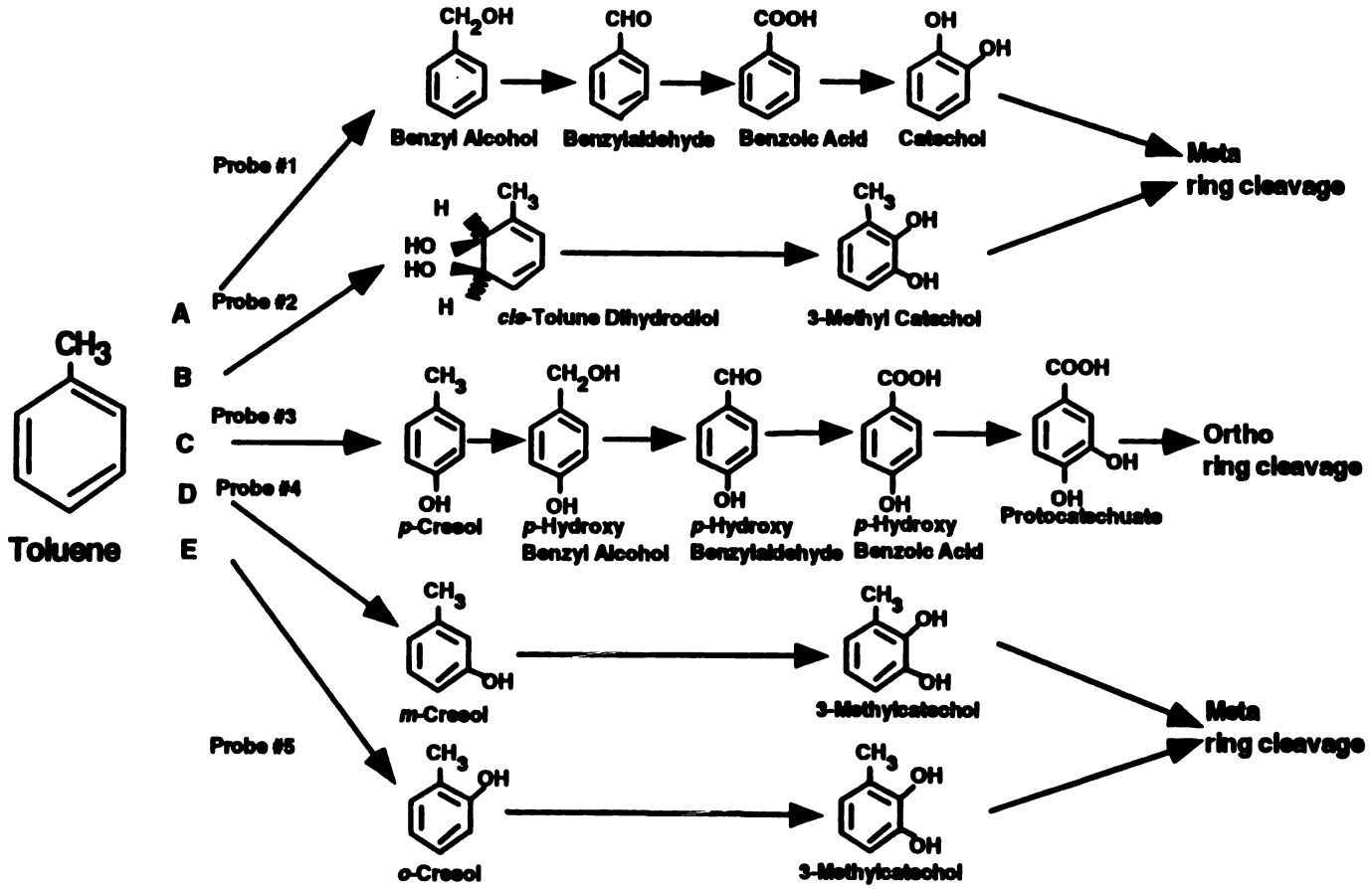
substitution

Biochemical pathways for aromatic hydrocarbon degradation

The aerobic biochemical mechanisms by which microorganisms utilize aromatic hydrocarbons have been studied in great detail in the last decades and in general can be sub-divided in three parts (reviewed by Williams and Sayers, 1994). During the first set of reactions, substituent groups are introduced on the benzene ring making possible alternative modes of biodegradation by side chain attack or ring attack. For almost all aromatic substrates, the introduction of hydroxyl groups onto the substrate is accomplished by mono or dioxygenases to produce a dihydroxyaromatic metabolite, most commonly catechol (1,2-dihydroxybenzene) or protocatechuate. The versatility of these reactions is best illustrated for toluene metabolism (Figure 1.2.).

The formation of compounds carrying two hydroxyl groups seems to be an important biochemical strategy to destabilize the chemically stable resonant structure of the aromatic ring in order to facilitate its subsequent opening (Dagley, 1986). The second set of reactions involves the opening of the catechol ring and is accomplished by dioxygenases which break one of the carbon-carbon bonds of the ring adjacent to the hydroxyl substitution by the addition of molecular oxygen, producing an unsaturated aliphatic acid. One of two different cleavage enzymes can be responsible for ring attack. An intradiol (*ortho*) dioxygenase which produces *cis-cis* muconate or an extradiol (*meta*) dioxygenase that yields 2-hydroxymuconate. Depending on the initial ring substitutions, analogous products can be observed at this stage. After ring


Benzyl Alcohol
Benzylaldehyde
Benzoic Acid
Catechol
Meta
ring cleavage
Probe #1



A- *Pseudomonas putida* mt-2 (PaW1) Probe # 1 toluene methyl-monooxygenase (Xyl MA genes)

B- *Pseudomonas putida* F1 Probe # 2 toluene dioxygenase (*tod* C1C2BA genes)

C- *Pseudomonas mendocina* KR Probe # 3 toluene para-hydroxylase (*tmo* genes)

D- *Burkholderia picckettii* PKO1 Probe # 4 toluene meta-hydroxylase (*tbu* genes)

E- *Burkholderia cepacia* G-4 Probe # 5 toluene ortho-hydroxylase (*tom* genes)
(also, *Pseudomonas* sp. JS 150)

FIGURE 1.2. Pathways for the aerobic metabolism of toluene and available DNA probes for the first step.

cleavage.

compounds

Of majo

Toluene, th

attack and

degradation

to tricarbo

reactions a

A for the c

collectively

pathway e

(plasmid p

oxidations

benzoate

followed by

enzymes c

(Worsey ar

Pathwa

Gibson ar

multicompo

incorporates

produce *cis*

cleavage, a third set of enzymes converts the products to small aliphatic compounds which can directly enter central metabolic routes.

Of major interest to this project, is the degradation of toluene and phenol. Toluene, the simplest of these substituted benzenes, is biodegraded by both ring attack and methyl group hydroxylation. There are five elucidated pathways for degradation of toluene and they share similar reaction sequences from catechol to tricarboxylic acid cycle intermediates, but each has a unique series of reactions and distinctive intermediates, prior to catechols (Figure 1.2.). Pathway A for the degradation of toluene is almost always encoded by large plasmids collectively called the TOL plasmids (Assinder and Williams, 1990). The pathway encoded on the TOL plasmid of *Pseudomonas putida* strain mt-2 (plasmid pWWO) involves the conversion of toluene to catechol by sequential oxidations of the methyl group, through benzyl alcohol and benzaldehyde to benzoate which is then converted to catechol in two steps, a dihydroxylation followed by a dehydrogenation / decarboxylation. Catechol is then degraded by enzymes of a meta cleavage pathway to CO_2, acetaldehyde and pyruvate (Worsey and Williams, 1975; Kunz and Chapman, 1981).

Pathway B, in Figure 1.2., characterized in *Pseudomonas putida* F1 by Gibson and co-workers, is chromosomally encoded and involves a multicomponent enzyme system, designated toluene dioxygenase, that incorporates two atoms of molecular oxygen directly into the aromatic nucleus to produce *cis*-toluene dihydrodiol (Zylstra and Gibson, 1989). The further

metabolis

form 3-me

6-oxo-2,4-

dienoate

metabolite

Gibson, 1

Three pat

groups by

of *Pseudo*

hydroxylat

p-hydroxy

complete

Pathway D

Pseudomo

al, 1994).

position o

of meta cr

is complete

1991). Pa

strain G4

monooxyge

metabolism of *cis*-toluene dihydrodiol involves a dehydrogenation reaction to form 3-methylcatechol and meta cleavage at the 2,3 position yields 2-hydroxo-6-oxo-2,4-heptadienoate , which is further metabolized to 2-hydroxypenta-2,4-dienoate and acetate. Further reactions in the pathway, eventually yielding metabolites that enter the TCA cycle, have not been investigated (Rogers and Gibson, 1977).

Three pathways have been elucidated involving the incorporation of hydroxyl groups by ring attack. Pathway C in Figure 1.2., was found to be characteristic of *Pseudomonas mendocina* strain KR. In this pathway, toluene is initially hydroxylated to ρ-cresol, followed by sequential oxidation of the methyl group to *p*-hydroxybenzoate, which is hydroxylated to form protocatechuate and then complete dissimilated by an ortho cleavage pathway (Whited and Gibson,1991). Pathway D in Figure 1.2., was detected in *Burkholderia pickettii* PKO1 (formerly *Pseudomonas pickettii*) and also has a ring hydroxylation mechanism (Olsen et al, 1994). However, in this case hydroxylation of toluene occurs at a different position on the aromatic ring to produce *m*-cresol. A subsequent hydroxylation of meta cresol by a separately inducible enzyme yields 3-methyl catechol which is completely mineralized by the meta ring cleavage pathway (Kukor and Olsen, 1991). Pathway E in Figure 1.2., was first demonstrated in *Burkholderia cepacia* strain G4 (formerly *Pseudomonas cepacia*). In this strain , a nonspecific monooxygenase hydroxylates toluene by two successive monooxygenations,

forming first ortho-cresol and then 3-methyl catechol, which is further degraded by enzymes of a meta cleavage pathway (Shields et al., 1989).

Trichloroethylene biodegradation

For many years, it was held that chlorinated aliphatic hydrocarbons (CAH's) could only be degraded anaerobically. In 1985, however, Wilson and Wilson presented some of the first evidence of aerobic degradation of TCE in soil enriched with natural gas (77% methane). After their work, aerobic degradation of CAH's, such as trichloroethylene, has been widely demonstrated, initially using methanotrophic bacteria. Unlike anaerobic degradation which can produce toxic by-products such as vinyl chloride (Vogel and McCarty, 1985), the products produced aerobically are thought to be relatively harmless for other organisms (Little et al., 1988; Oldenhuis et al., 1989; Tsien et al., 1989). Many studies have demonstrated that TCE can not be used as sole energy source by microbial consortia or pure cultures leading to the suggestion that aerobic degradation of TCE is a "co-metabolic" process. A definition proposed for cometabolism by Dalton and Stirling (1982), states " the transformation of a non-growth substrate in the obligate presence of a growth substrate or another transformable compound ". This definition however implies the concomitant presence of a growth substrate and does not contemplate transformations that occur after depletion of the primary growth and/or energy substrate.

Methanotrophs are bacteria that can oxidize methane for energy and growth. In the conversion of methane to methanol, the initial oxidation is catalyzed by the methane monooxygenase (mmo) enzyme system. Several investigators have examined the degradation of TCE by this group of microorganisms. A methanotrophic mixed culture transformed ^{14}C-labeled TCE to CO_2, cell biomass and nonvolatile or nonchlorinated compounds. Acetylene, a known inhibitor of mmo activity, inhibited degradation indicating that methane oxidizing bacteria probably initiated TCE oxidation (Fogel et al., 1986).

Working with a pure culture, strain 46-1, Little et al. (1988) demonstrated degradation of TCE only when growth was in the presence of methane. These studies were conducted using liquid cultures in inverted serum bottles. Degradation stopped when methane was depleted and continued if additional methane was added. These researchers concluded that TCE degradation is a cometabolic process that provides little or no benefit to methanotrophs because strain 46-1 initiated the degradation of TCE but was unable to metabolize the intermediates. Preliminary evidence indicated that glyoxylic acid and dichloroacetic acid were the breakdown products. They also proposed a mechanism of degradation, which is presented in Figure 1.3(a) and hypothesized that TCE is first converted to its epoxide, which breaks down spontaneously, yielding dichloroacetic acid, glyoxylic acid, formate and carbon monoxide. The end products are completely metabolized to CO_2 by heterotrophic bacteria in mixed cultures.

sys

mo

was

Nev

trich

mon

hydr

trich

Little

and

Pseu

reduc

substr

oxidize

substra

will st

transfo

Cohen

a conce

culture i

Henschler et al. (1979) evaluated TCE-epoxide reactivity in aqueous systems and found that the epoxide decomposes to form formate, carbon monoxide, glyoxylic acid and dichloroacetic acid. The amount of each product was pH dependent and at lower pH's, fewer one carbon products were observed. Newman and Wackett (1991) proposed a new mechanism for *Methylosinus trichosporium* OB3b. Four different methanotrophs expressing soluble methane monooxygenase produced 2,2,2-trichloroacetaldehyde (chloral hydrate). Chloral hydrate was shown to be biologically transformed to trichloroethanol and trichloroacetic acid. Figure 1.3. shows a combination of pathways proposed by Little et al. (1988) and Newman and Wackett (1991) for methanotrophic bacteria and by Li and Wackett (1992) for the toluene dioxygenase system of *Pseudomonas putida* F1.

The methane monooxygenase enzyme system requires a source of reducing power to carry out the transformations of TCE. When a growth substrate, such as methane or any one of the metabolites of methane is oxidized, reducing power is regenerated. When mmo oxidizes a non-growth substrate, such as TCE, reducing power is not regenerated, and the oxidation will stop. The effect of an alternative energy source addition on the transformation capacity of a mixed metanotrophic culture was tested by Alvarez-Cohen and McCarty (1991b). With the addition of 20 mM of formate and TCE at a concentration of approximately 20 mg/L, the transformation capacity of a culture increased from 0.036 to 0.073 mg TCE/mg of cells. However, significant

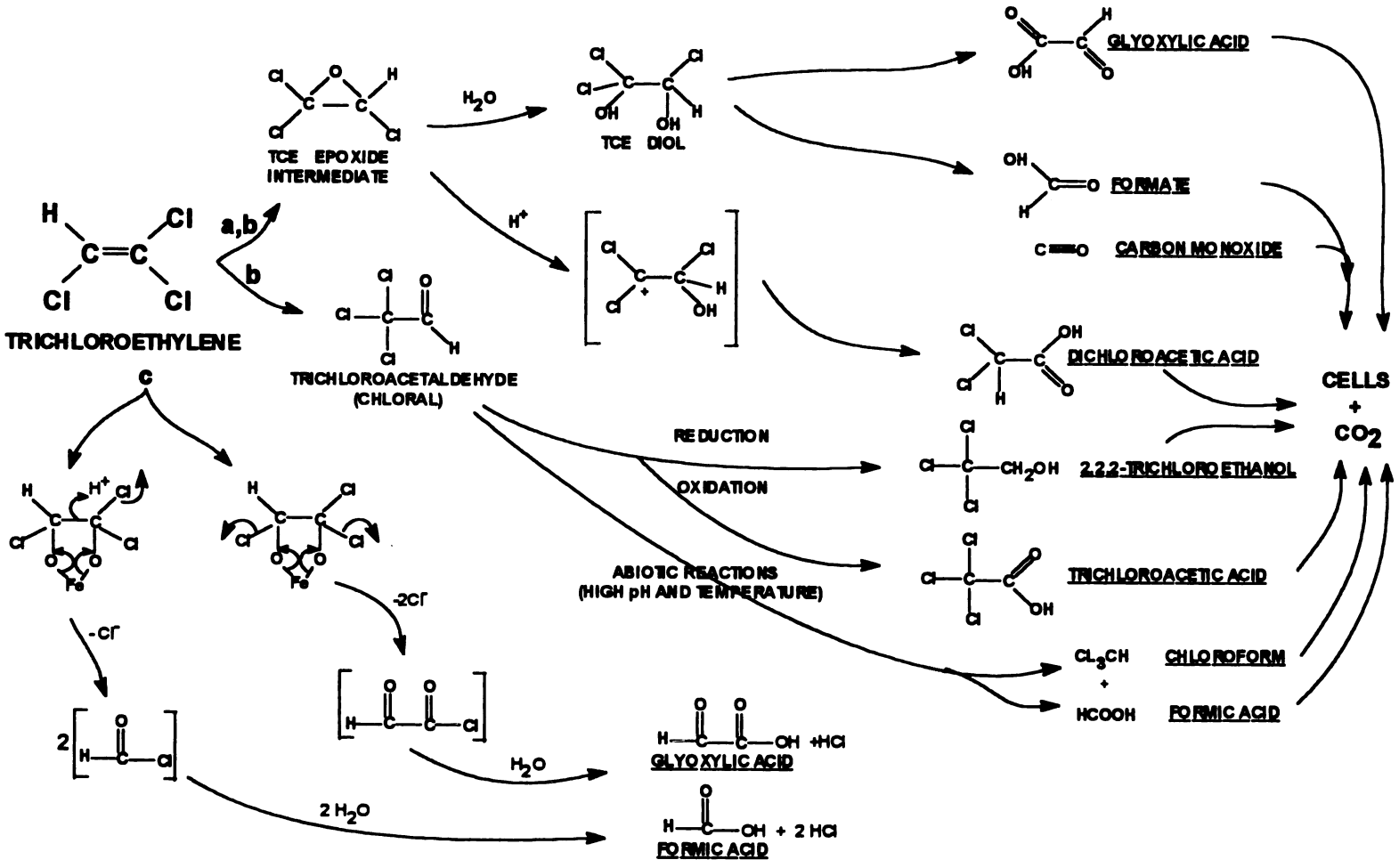


Figure 1.3. Proposed aerobic pathways for TCE co-metabolic biotransformation. (a)Methanotrophic bacteria (Little et al., 1988); (b) *M. trichosporium* OB3b (Newman and Wackett, 1990); (c) *Pseudomonas putida* F1, toluene dioxygenase system (Li and Wackett, 1992).

declines in methane conversion rates following exposure to TCE were observed for formate fed cells, suggesting toxic effects caused by TCE or its transformation products.

Even when methane is provided, reducing power can still be depleted. Using a pure culture, Henry and Grbic-Galic (1991b) showed that carbon monoxide competitively inhibited methane oxidation until formate was added as an exogenous electron donor. She also showed that formate addition to the mixed culture MM1 did not enhance degradation rates at low TCE concentrations and assumed this was because MM1 methanotrophs possess lipid storage granules which can serve as an alternate source of electrons.

Henry and Grbic-Galic (1991a) also tested the effect of formate addition on the rates of TCE transformation during methane starvation. At a TCE concentration of 30-60 μg/L, formate addition did not increase rates for the mixed culture MM1. However, the rates were enhanced for the first ten hours of methane starvation when the pure culture MM2 was incubated with 2 mM formate. When the culture was incubated without formate and the formate was added simultaneously with TCE, transformation rates remained significantly enhanced throughout 62 hours of testing.

Among the groups of bacteria that produce oxygenases and have so far been demonstrated to be capable of transforming TCE and other CAHs by co-metabolism include not only the methane oxidizers(Alvarez-Cohen and McCarty, 1991a; Alvarez-Cohen and McCarty, 1991b; Alvarez-Cohen et al., 1992; Fogel

et al., 1986; Henry and Grbic-Galic, 1990; Henry and Grbic-Galic, 1991a; Little et al,1988; Oldenhuis et al.,1989; Tsien et al., 1989); but also propane oxidizers (Wackett et al., 1989; Malachowski et al., 1994); propylene oxidizers (Ensingn et al.,1992); ammonia oxidizers (Arciero et al., 1989; Rasche et al., 1991);isoprene oxidizers(Ewers, et al.,1990); isopropylbenzene oxidizers (Dabrock et al., 1992); and monoaromatic hydrocarbon (toluene, phenol, or cresols) degrading organisms (Folsom et al., 1990; Harker and Kim, 1990; Nelson et al., 1987, Nelson et al, 1986, Nelson et al, 1988; Wackett and Gibson, 1988).

The first report of a pure culture capable of TCE degradation under non methanotrophic conditions was presented by Nelson et al. (1986). Studies with ^{14}C-labelled TCE resulted in 60% of the total ^{14}C-TCE converted to CO_2 and 35% remained as an unidentifed nonvolatile product. They also showed that an unidentified compound present in the water, from which the strain was isolated, and oxygen were required for TCE degradation. Later research using the isolated strain resulted in the identification of the required component present in the water as being phenol (Nelson et al., 1987). This was the first report involving an aromatic degradation pathway in the cometabolism of TCE. Later toluene, *o*-cresol and *m*-cresol were shown to stimulate TCE degradation.

Five pathways differing in the first step have been elucidated for the degradation of toluene. These first step enzyme systems vary from oxidation of the methyl group to mono- or dioxygenations of the ring and seem to be the enzymes implicated in the cometabolic transformations of TCE. P*seudomonas*

putida F1 is the strain studied that contains the toluene dioxygenase enzyme system. A mutant strain defective in the *todC* gene, which encodes the oxygenase component of the toluene dioxygenase, failed to degrade TCE; but a mutant strain defective in *todE* encoding the 3-methyl catechol 2,3-dioxygenase, an enzyme dowstream in the pathway for toluene degradation, oxidized TCE as rapidly as the wild type (Wackett and Gibson, 1988). A spontaneous revertant selected from a *todC* culture regained simultaneously the ability to oxidize toluene and to degrade TCE. Evidence for the involvement of the first step enzyme on TCE degradation is also provided by the fact that in *Pseudomonas mendocina* KR, the toluene monooxygenase system (TMO), inserts a single atom of oxygen at the para-position of toluene to form p-cresol, however, p-cresol was not an inducer of TCE degradation (Winter et al., 1989).

In methanotrophic bacteria at least two classes of methane monooxygenase can be differentiated on the basis of their intracellular localization: a particulate form associated with the cell membrane (pmmo) and a soluble form (smmo) (Dalton et al., 1984). Soluble methane monooxygenase has a broader substrate range. Lower copper levels cause derepression of its synthesis increasing TCE oxidation (Oldenhius et al, 1989; Tsien et al., 1989; Alvarez-Cohen et al, 1992). Since TCE seems to be more efficiently degraded by the smmo form, practical applications based on this group of bacteria will have to be based on selective conditions for expression of this type of enzyme.

Little is known about the efficiency of TCE co-oxidation by the different monoaromatic hydrocarbon degrading pathways or if the presence of a specific enzyme system would be favorable for TCE degradation. Also, when one considers biodegradation of TCE by a pure culture containing catabolic oxygenases more than one oxygenase is probably induced for the degradation of the primary substrate. Independent monooxygenases involved in the TCE degradation were observed in an *Alcaligenes* strain degrading 2,4-dichlorophenoxyacetic acid (Harker and Kim, 1990). The toluene dioxygenase pathway of *Pseudomonas putida* F1 is one of the best studied enzyme systems for toluene degradation and was shown to be the only enzyme system required for TCE degradation (Wackett and Gibson, 1988). Working with toluene induced cells of *Pseudomonas putida* F1, Wackett and Gibson (1988) showed that the initial rate of TCE oxidation was linear with respect to substrate concentration over the range of 8 to 80 *u*M of TCE. Initially, over the first 20 minutes, a fairly rapid rate of 1.8 nmol/min per mg of protein was obtained using an initial TCE concentration of 80 uM, but the rate decreased rapidly over the next six hours of the experiment. No TCE oxidation was observed at an initial TCE concentration of 320 *u*M.

This rapid decrease in activity was attributed to toxic effects of TCE or TCE metabolites. The same toxic effects were attributed to the decrease in growth rates from 0.463 hr^{-1} to 0.139 hr^{-1} observed for *Pseudomonas putida* F1 growing in the presence of TCE (Wackett and Householder, 1989). Recombinant studies

using the toluene dioxygenase genes and *E. coli* JM109 as the host, showed a slower initial biodegradation but a prolonged linear rate of TCE disappearance when compared to the toluene dioxygenase genes expressed in the original donor *Pseudomonas putida* F1. The observation that TCE oxidation rates are more sustained in *E. coli* could be an indication that different hosts respond in different ways to cytotoxic effects from TCE or metabolites (Zylstra et al., 1989).

Folsom et al. (1990), evaluated the kinetic parameters for phenol and TCE degradation by *Burkholderia cepacia* G4, a strain that carries the toluene ortho-hydroxylase pathway. Whole cell studies showed that this enzyme has an apparent $K_m$and V_{max} for phenol oxidation of 8.5 *u*M and 466 nmol/min per mg of protein, respectively. The $K_m$and V_{max} for TCE were 3*u*M and 8 nmol/min per mg of protein. As both substrates are oxidized by the same enzyme system, competitive inhibition was likely to occur since the K_m values for both substrates are similar. Folsom et al. (1990) also showed where in experiments with equal concentrations of phenol and TCE, a decrease of about 50% in the rate of phenol degradation was observed.

The third type of toluene monooxygenase (toluene *para*-monooxygenase) studied for the kinetic parameters of TCE biodegradation is present in *Pseudomonas mendocina* strain KR which oxidizes TCE when grown in the presence of toluene (Winter et al., 1989). Recombinant *E. coli* strains containing the genes encoding this enzyme oxidized TCE at a rate of 1-2 nmol/min per mg of protein, which also confirmed the role of this enzyme system in the oxidation

of TCE. For the fourth type of toluene degrading enzyme system, the toluene meta-monooxygenase, identified in *Burkoldheria pickettii* (Olsen et al., 1994), no kinetic parameter data is avaliable for TCE oxidation. The fifth type of enzyme system involved in the biodegradation of toluene is present in *Pseudomonas putida* PaW1 and involves the hydroxylation of the methyl group of toluene to form benzoate(Worsey and Williams, 1975). This toluene degrader did not oxidize TCE (Nelson et al., 1988) and suggests that ring oxygenases are the enzyme systems efficient for the biodegradation of TCE.

The pathway for TCE degradation by enzymes involved in the biodegradation of aromatic compounds seems to be similar to the pathway proposed for methanotrophs, except for the dioxygenase system where TCE epoxide, the proposed universal intermediate for most monooxygenase enzyme systems, does not appear to be present. Metabolic studies and chloride release experiments during the degradation of TCE by *Pseudomonas mendocina* KR, resulted in non chlorinated water soluble products putatively identified as formic acid and glyoxylic acid, carbon dioxide and cellular constituents (Winter et al., 1989). Li and Wackett (1992), working with purified toluene dioxygenase obtained from *E. coli* recombinant strains and ^{14}C-TCE, found formic acid and glyoxylic acid as the major oxidation products for this enzyme system. None of the other intermediates identified for the methane monooxygenase system (Figure 1.3.) were detected indicating major differences in the mechanism of TCE oxygenation from those previously proposed. This also eliminates the

possibility that the dioxygenase system functions as a monooxygenase adding oxygen across the double bound. In one possible mechanism proposed by the authors, an iron bound dioxygenated intermediate might rearrange on the enzyme surface to yield formate from both carbon atoms as proposed in Figure 1. 3.

Determining the distribution of aromatic degrading organisms is important to the overall understanding of diversity and ecology of biodegrading communities. For the methane monooxygenase systems, where differences in TCE cometabolic transformation is well established, aromatic pathways may differ in their efficiency for cometabolic transformation. When this is the case, selection and introduction (i.e. bioaugmentation) of robust strains may increase the degradation rates of the pollutant in the field. I investigated the co-metabolic efficiency of different pathways involved in the degradation of toluene present in well-studied laboratory strains and compared those with isolates from a field site where successful TCE degradation occurs. To further investigate the relationship between pathway and strains, I used gene probes to determine the distribution of pathways in the new isolates.

Degradation of mono aromatic hydrocarbons under denitrifying conditions.

The availability of oxygen, due to its low solubility in water and inefficient transport through saturated porous matrices such as soil and sediments, is

usually the rate limiting parameter for BTEX removal from contaminated sites. It has only been in recent years that anaerobic degradation of these compounds has been conclusively established. Of the BTEX class of compounds, toluene seems to be the most easily degraded under anaerobic conditions . The degradation of toluene has been reported under denitrifying conditions (Chee-Sanford et al., 1992; Dolfing et al., 1990; Evans et al., 1991; Evans et al., 1992; Kukor and Olsen, 1990; Schocher et al., 1991), methanogenic conditions (Grbic-Galic and Vogel, 1987; Wilson et al., 1986), sulfate reducing conditions (Edwards et al., 1993; Rabus et al., 1993) and ferric iron reducing conditions (Lovley and Lonergan., 1990; Lovley et al., 1989). Very little was known about the organisms responsible for toluene degradation under denitrifying conditions and how widely they are distributed in nature. Hence one of my goals was to isolate such organisms and describe their physiological features.

List of References

Alvarez-Cohen, L. and P. L. McCarty. 1991a. Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene by methanotrophic resting cells. Appl. Environ. Microbiol. **57**:1031-1037.

Alvarez-Cohen, L. and P. L. McCarty. 1991b. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformations by a mixed methanotrophic culture. Appl. Environ. Microbiol. **57**:228-235.

Alvarez-Cohen, L. , P. L. McCarty, E. Boulygina, R. S. Hanson, G. A. Brusseau, and H. C. Tsien. 1992. Characterization of a methane urilizing bacterium from a bacterial consortium that rapidly degrades trichloroethylene and chloroform. Appl. Environ. Microbiol. **58**:1886-1893.

Arciero, D. T. T. Vanelli, M.Logan, and A. B. Hooper. 1989. Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Biochem. Biophys. Res. Commun. **159**:640-643.

Assinder, S.J. and P.A. Williams. 1990. The Tol plasmids: determinants of the catabolism of toluene and the xylenes. Advan. in Microb. Physiol. **31**: 1-69.

Bossert, I., and R. Bartha. 1984. The fate of petroleum in soil ecosystems, p. 434-476. *In* R. M. Atlas (ed.), Petroleum microbiology. Macmillan publishing Co., New York.

Chee-Sanford, J., M. R. Fries, and J. M. Tiedje. 1992. Anaerobic degradation of toluene under denitrifying conditions in bacterial isolate Tol-4, abst. Q-233, p.374. Abstr. 92th Annu. Meet. Am. Soc. Microbiol. 1992.

Cole, G. M. 1994. Assessment and Remediation of Petroleum Contaminated Sites. Lewis Publishers, Boca Raton, Florida.

Dabrock, B., J. Riedel, J. Bertram, and G. Gottschalk. 1992. Isopropylbenzene (cumene) a new substrate for the isolation of trichloroethylene-degrading bacteria. Arch. Microbiol. **158**:9-13.

Dagley, S. 1986. Biochemistry of aromatic hydrocarbon degradation in Pseudomonas, p.527-555. *In* J.R.Sokatch and L.N.Ornstron(eds.), The Bacteria. Academic Press, New York.

Dalton, H., S. D. Prior, D. J. Leak, and S. H. Stanley. 1984. Regulation and control of methane monooxygenase, p.75-82. *In* L. R. Crowford and R. S. Hanson (ed.), Microbial growth on C1 compounds. American Society for Microbiology, Washington, D.C.

Dalton, H., and D. I. Stirling. 1982. Co-metabolism. Phil. Trans. Soc. Lond. B. **297**:481-496.

Dolfing, J., J. Zeyer, P. Binder-Eicher, and R. P. Schwarzenbach. 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. **154**:336-341.

Edwards, E. A., L. E. Wills, D. Grbic-Galic, and M. Reinhard. 1993. Anaerobic degradation of toluene and xylene under sulfate-reducing conditions. Appl. Environ. Microbiol. **58**:794-800.

Ensingn, S. A., M. R. Hyman, and D. J. Harp. 1992. Cometabolic degradation of chlorinated alkenes by alkene monooxygenase in a propylene-grown Xantobacter strain. Appl. Environ. Microbiol. **58**:3038-3046.

Evans, P. J.,W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young. 1992. Metabolites formed during the anaerobic transformation of toluene and o-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. **58**:496-501.

Evans, P. J., D. T. Mang, K. S. Kim, and L. Y. Young. 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. **57**:1139- 1145.

Ewers, J., D. Freier-Schroder, and H. J. Knackmuss. 1990. Selection of trichloroethene(TCE) degrading bacteria that resist inactivation by TCE. Arch. Microbiol. **154**:410-413.

Floodgate, G. D. 1984. The fate of petroleum in marine ecosystems, p. 355-397. *In* R. M. Atlas (ed.), Petroleum microbiology. Macmillan publishing Co., New York.

Fogel, M. M., A. Taddeo, and S. Fogel. 1986.Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl. Environ. Microbiol. **51**:720-724.

Folsom, B. R., P. J. Chapman and P. H. Pritchard. 1990. Phenol and trichloroethylene degradation by Pseudomonas cepacia G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. **56**:1279-1285.

Grbic-Galic, D. and T. M. Vogel. 1987. Transformation of toluene and benzene by mixed methnogenic cultures. Appl. Environ. Microbiol. **53**:254-260.

Harborne, J. B. 1980. Plant phenolis, p.329-402. *In* E. A. Bell and B. V. Charlwood (ed.), Secondary plant products. Springer-Verlag, New York.

Harker, A. R. and Y. Kim. 1990. Trichloroethylene degradation by two independent aromatic independent pathways in *Alcaligenes eutrophus* JMP134. Appl. Environ. Microbiol. **56**:1179-1181.

Henry, S. H. and D. Grbic-Galic. 1990. Effect of mineral media on trichloroethylene oxidation by aquifer metanotrophs. Microb. Ecol. **20**: 151-169.

Henry, S. H. and D. Grbic-Galic. 1991a. Influence of endogenous and exogenous electron donors and trichloroethylene oxidation toxicity on trichloroethylene oxidation by methanotrophic cultures from a groundwater aquifer. Appl. Environ. Microbiol. **57**:236-244.

Henry, S. H. and D. Grbic-Galic. 1991b. Inhibition of trichloroethylene oxidation by the transformation intermediate carbon monoxide. Appl. Environ. Microbiol. **57**:1770-1776.

Henschler, D,. W. R. Hoos, H. Fentz, E. Dallmeir and M. Meltzler. 1979. Reactions of trichloroethylene epoxide in aqueous systems. Biochemical pharmacology. **28**:543-548.

Kukor, J. J., and R. H. Olsen. 1990. Diversity of toluene degradation following long term exposure to BTEX in situ, p. 405-421. *In* D. Kamely, A. Chakrabarty and G. S. Omenn, (ed.), Biotechnology and Biodegradation, Gulf Publishing Co., Houston, Texas.

Kukor, J. J., and R. H. Olsen. 1991. Genetic organization and regulation of a meta cleavage pathway for catechols produced from catabolism of toluene, benzene, phenol, and cresols *by Pseudomonas pickettii* PKO1. J. Bacteriol. **173**:4587-4594.

Kunz, D. A., and P. J. Chapman. 1981. Catabolism of pseudocumene and 3-ethyltoluene by Pseudomonas putida (arvila) mt-2: evidence for new functions of the Tol (pWWO) plasmid. J. Bacteriol. **146**:179-191.

Li, S. and Pl. L. Wackett. 1992. Trichloroethylene oxidation by toluene dioxygenase. Biochem. Biophys. Res. Comm: **185**:443-451.

Little, C. D., A. V. Palumbo, S. E. Herbes, M. E. Lidstrom, R.L. Tyndall, and P. J. Gilmer. 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl. Environ. Microbiol. **54**:951-956.

Lovley, D. R., M. J. Baedecker, D. J. Lonergan, I. M. Cozzarelli, E. J. P. Phillips, and D. I. Segal. 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature **339**:297-300.

Lovley,. R., D. J. Lonergan. 1990. Anaerobic oxidation of toluene, phenol, and p-cresol by the dissimilatory iron reducing bacterium GS-15. Appl. Environ. Microbiol. **56**:1858-1864.

Malachowsky, K. J., T. J. Phelps, A. B. Teboli, D. E. Minnikin and D. C. White. 1994. Aerobic mineralization of trichloroethylene, vinyl chloride, and aromatic compounds by *Rhodococcus* species. Appl. Environ. Microbiol. **60**:542-548.

Martin, J. P., and K. Haider. 1969. Phenolic polymers of *Stachybotrys atra*, *Stachybotrys chartarum* and *Epicoccum nigrum* in relation to humic acid formation. Soil Sci. 107:260-270.

Martin J. P., K. Haider, and D. Wolf . 1972. Synthesis of phenols and phenolic polymers by Hendersonula toruloidea in relation to humic acid formation. Soil Sci. Amer. Proc. 36:311-315.

Nelson, M. J. K.,S. O. Montgomery, E. J. O'Neil and P. H. Pritchard. 1986. Aerobic metabolism of trichloroethylene by a bacterial isolate. Appl. Environ. Microbiol. **52**:383-384.

Nelson, M. J. K.,S. O. Montgomery, E. J. O'Neil and P. H. Pritchard. 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53**:949-954.

Nelson, M. J. K.,S. O. Montgomery, and P. H. Pritchard. 1988. Trichloroethyleene metabolism by microorganisms that degrade aromatic compounds. Appl. Environ. Microbiol. **54**:604-606.

Newman, D., and L. P. Wackett. 1991. Fate of 2,2,2-trichloroacetaldehyde (chloral hydrate) produced during thrichloroethylene oxidation by methanotrophs. Appl. Environ. Microbiol. **57**:2399-2402.

Oldenhuis, R., R. L. J. M. Vink, D. B. Jansen, and B. Witholt. 1989. Degradation of chlorinated aliphatic hydrocarbons by Methylosynus trichosporium OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. **55**:2819-2826.

Olsen R. H., J. J. Kukor , and B. Kaphammer. 1994. A novel toluene-3-monooxygenase from Pseudomonas pickettii PKO1. J. Bacteriol. **176**:3749-3756.

Rabus, R., R. Nordhaus, W. Ludwig, and F. Widdel. 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. **59**:1444-1451.

Rasche, M. E., M. R. Hayman, and D. J. Harper. 1991. Factors limiting aliphatic chlorocarbon degradation by nitrossomonas europaea: cometabolic inactivation of ammonia monooxygenase and substrate specificity. Appl. Environ. Microbiol. **57**:2986-2994.

Rogers, J. E. and D.T. Gibson 1977. Purification and properties of *cis*-toluene dihydrodiol dehydrogenase *from Pseudomonas putida*. J. Bacteriol. **130**:1117-1124.

Schocher, R. J., B. Seyfried, F. Vazquez and J. Zeyer. 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. **157**:7-12.

Shields, M. S., S. O. Montgomery, P. J. Chapman, S. M. Cuskey, and P. H. Pritchard. 1989. Novel pathway of toluene catabolism in the trichloroethylene-degrading bacterium G4. Appl. Environ. Microbiol. **55**:1624-1629.

Shields, M.S. and M. J. Reagin. 1992. Selection of a *Pseudomonas cepacia* strain constitutive for the degradation of trichloroethylene. Appl. Environ. Microbiol. **58**:3977-3983.]

Song, H. G., T.A. Pedersen, and R. Bartha. 1986. Hydrocarbon mineralization in soil: relative bacterial and fungal contribution. Soil Biol. Biochem. **18**:109-111.

Tiedje, J. M. 1993. Bioremediation form an Ecological Perspective, p110-120. In In Situ Bioremediation . When does it work? National Academy Press. Washington , D.C.

Tsien, H. C., G. A. Brusseau, R. S. Hanson and L. P. Wackett. 1989. Biodegradation of trichloroethylene by methylosinus OB3b. Appl. Environ. Microbiol. **55**:3155-3161.

Vogel, T. M. and P. McCarty. 1985. Biodegradation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride , and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. **49**:1080-1083.

Wackett, L.P., G. A. Brusseau, S. R. Householder and R. S. Hanson. 1989. Survey of microbial oxygenases: trichloroethylene degradation by propane-oxidizing bacteria. Appl. Environ. Microbiol.**55**:2960-2964.

Wackett, L.P., and D. T. Gibson. 1988. Degradation of trichloroethylene by toluene dioxygenase in whole cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol.**54**:1703-1708.

Wackett, L.P., and S. R. Householder. 1989. Toxicity of trichloroethylene to *Pseudomonas putida* F1 mediated by toluene dioxygenase. Appl. Environ. Microbiol.**55**:2723-2725.

Whited, G. M., and D.T. Gibson. 1991. Toluene-4-monooxygenase, a three-componentenzyme system that catalizes the oxidation of toluene to p-cresol in *Pseudomonas mendocina* KR. J. Bacteriol. **173**:3010-3016.

Williams, P. A. and R. R. Sayers. 1994. The evolution of pathways for aromatic hydrocarbon oxidation in Pseudomonas. Biodegradation **5**:195-217.

Wilson, B. H., G. B. Smith, and J. F. Rees. 1986. Biotransformation of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: a microcosm study. Environ. Sci. Technol. **20**:997-1002.

Wilson, J. T. and B. H. Wilson. 1985. Biotransformations of trichloroethylene in soil. Appl. Environ. Microbiol. **37**:242-243.

Winter, R. B., K. Yen and B. D. Ensley. 1989. Efficient degradation of trichloroethylene by a recombinant Escherichia coli. Bio/Technology **7**:282-285.

Worsey, M.J. and P.A. Williams. 1975. Metabolismof toluene and Xylenes by *Pseudomonas putida(arvilla)* mt-2: evidence for a new function of the Tol plasmid. J. Bacteriol. **124**:7-13.

Zylstra, G. J. and D. T. Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1: Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli.* J. Biol. Chem. **264**:14940-14946.

Zylstra, G. J., L. P. Wackett and D. T. Gibson. 1989. Trichloroethylene degradation by Escherichia coli containing the cloned Pseudomonas putida F1 toluene dioxygenase genes. Appl. Environ. Microbiol. **55**:3162-3166.

Chapter II

Microbial populations of phenol and toluene degraders in an aquifer where successful TCE co-oxidation occurs.

Introduction

Contaminated water is a problem facing many communities throughout the world. Trichloroethylene (TCE), an Environmental Protection Agency priority pollutant, is widespread in the environment. TCE is an efficient industrial solvent and degreaser that can be transported into the environment by inadequate disposal techniques, accidental spillage, leaking storage tanks, and landfill leachates. TCE is relatively resistant to biodegradation in soil and the subsurface since microorganisms can not use this compound as a sole source of carbon or energy.

In 1985, Wilson and Wilson presented some of the first evidence of aerobic degradation of TCE in soil enriched with natural gas (77% methane). After their work, aerobic cometabolic degradation of TCE has been widely demonstrated, initially using methanotrophic bacteria. Unlike anaerobic degradation which can produce toxic by-products such as vinyl chloride (Vogel and McCarty, 1985), the products produced aerobically by methane oxidizers are thought to be relatively harmless for other organisms (Little et al., 1988; Oldenhuis et al., 1989; Tsien et al., 1989). The first report of a pure culture capable of TCE degradation under non-methanotrophic conditions was presented by Nelson et al. (1986). Among

the groups of bacteria that produce oxygenases and have so far been demonstrated to be capable of transforming TCE and other CAHs (chlorinated aliphatic hydrocarbons) by co-metabolism include not only the methane oxidizers (Alvarez-Cohen and McCarty, 1991a; Alvarez-Cohen and McCarty, 1991b; Alvarez-Cohen et al., 1992; Fogel et al., 1986; Henry and Grbic-Galic, 1990; Henry and Grbic-Galic, 1991a; Little et al., 1988; Oldenhuis et al., 1989; Tsien et al., 1989); but also propane oxidizers (Wackett and Householder, 1989; Malachowski et al., 1994); propylene oxidizers (Ensingn et al., 1992); ammonia oxidizers (Arciero et al., 1989; Rasche et al., 1991); isoprene oxidizers (Ewers et al., 1990); isopropylbenzene oxidizers (Dabrock et al., 1992); and monoaromatic hydrocarbon (toluene, phenol, or cresols) degrading organisms (Folsom et al., 1990; Harker and Kim, 1990; Nelson et al., 1987, Nelson et al., 1986, Nelson et al., 1988; Wackett and Gibson, 1988).

Initial attempts to stimulate TCE co-oxidation in the field were done by injecting methane and an oxygen source, but recently the injection of phenol and toluene along with oxygen have been investigated since they appear to result in more rapid TCE removal and are easier to engineer. The aerobic metabolism of toluene is known to be initiated by a variety of oxygenases that result in five different pathways leading to toluene mineralization. Phenol metabolism also involves an oxygenase attack, and its pathway of metabolism converges with some pathways of cresol metabolism that result from monooxygenase attack on toluene. Hence, toluene and phenol additions may be stimulating very similar if

not the same monooxygenases active in TCE co-oxidation. How similar the populations are that are stimulated by these two substrates or whether they are equivalent in their cooxidizing ability is unknown. The best studied strains with the five different toluene degrading pathways are very different in the TCE cooxidizing ability, varying from completely inactive to the most active known organism. Hence the success of phenol or toluene stimulated TCE cooxidation treatments would appear to depend on which type of population (and oxygenase) is stimulated at a contaminated site.

In this study we characterized the phenol and toluene degrading populations that grew in response to toluene, phenol and TCE additions to the Moffett Field aquifer. The isolates were taken from two sources: glass beads added to the aquifer in the flow path of the injected substrates so that only those organisms that grew *in situ* were obtained, and from filtered water pumped from the same hole so that any unattached organisms could also be obtained. Our objectives were: (*i*) to evaluate the sensitivity of the populations to toluene and phenol concentrations, (*ii*) to determine which populations were more frequently isolated, (*iii*) to elucidate major characteristics of the isolate collection such as gram reaction, frequency of use of both phenol and toluene, TCE cooxidation ability and hybridization to the toluene pathway probes , and (*iv*) to compare the rates of toluene use and TCE cooxidation among the more commonly selected aquifer strains and with the previously well-characterized toluene degraders.

Material and Methods

Field sample site description. The experimental site is located at the Moffett Federal Airfield (formerly the Moffett Naval Air Station), Mountain View, California, a few miles from Stanford University. It is located on the lower part of the Stevens Creek alluvial fan, approximately 3 km south of the southwest extremity of San Francisco Bay. The surface elevation at the site is 8.5 m above mean sea level. The test zone is a shallow, semiconfined aquifer, consisting of fine to coarse-grained sands and gravels. The alluvial sediments contained in the aquifer were deposited in the last 5000 years. This aquifer is about 4.5m below ground surface, and is about 1.4 m thick. Above the aquifer is approximately 4 m of silt and clay of a brownish-black to olive gray color, and a layer of dark greenish-gray silty clay about 7 m thick underlines the aquifer. The aquifer is spatially heterogeneous, with the composition varying appreciably over short distances. The test zone appears to have the structure of a buried stream channel containing sand and gravel in some areas and only sand in others (Roberts et al., 1990).

Pumping test results suggest that this is a leaky confined aquifer system, with a hydraulic conductivity of 100 m/day. Groundwater velocity in the test zone is relatively high, 1 m/day, based upon both bromide tracer tests and estimates from the measured hydraulic gradient across the field of 0.0032 and an estimated porosity of 0.33. The natural gradient moves in a south to north

direction, that is from the injection well towards the extraction well (Roberts et al., 1990).

The organic matter content of bulk aquifer samples is 0.11 percent. Sorption of TCE to aquifer material is rather high, as indicated by measured retardation factors with respect to groundwater movement of 8 to 12, which is consistent with sorptive properties measured in the laboratory. The groundwater is anoxic, of low salinity and near neutral pH. It is somewhat contaminated from other sources with chlorinated solvents, although the one of primary interest in this study, TCE, is not present. The only chlorinated aliphatic hydrocarbon(CAH) of significance is 1,1,1-trichlorethane, which is at a concentration of about 30 μg/L. These contaminants have posed no difficulties in the experiments conducted at the site, as they are either too low in concentration to be of significance, or else are not reactive biologically under the experimental conditions. Chemical analysis of water from the sampling site revealed the following concentrations: phosphate less than 0.5 mg/L, nitrate, 25 mg/L, sulfate, 700 mg/L (Hopkins et al., 1993).

For the phenol injection experiments conducted during 1991-1992, a new experimental leg was installed adjacent to the original one installed in 1986 for other studies. The injection and extraction wells consist of standard 2 inch PVC pipe, installed using a hollow stem auger. These wells contain 5-foot slotted screens that fully penetrate the aquifer zone. The sampling wells (Figure 2.1.) are located at 1(SSE1), 1.6(SSEGB), 2.2(SSE2), and 4 m(SSE3) from the

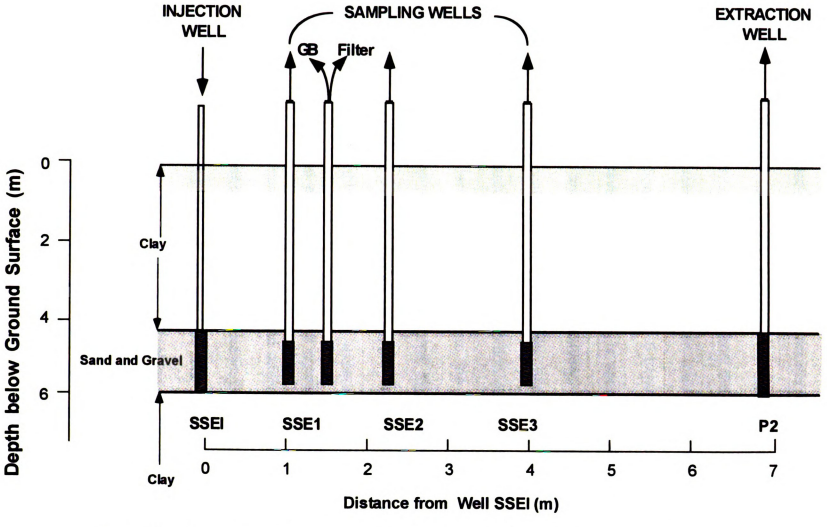


Figure 2.1. Cross section view of Moffett Field test site (modified from Roberts et al., 1990).

injection well, and consist of 1.25-inch stainless steel wire wound sand points with 2-foot screens, installed in the middle of the aquifer test zone. An additional sand point well with a 4.5-foot screen was placed 7m (extraction well-P2) from the injection well. Normally water was extracted from the aquifer at a flow rate of 10 L/min, creating a localized flow of groundwater towards the extraction well . Water was injected at a flow rate typically of 1.5 L/min. From bromide tracer tests, the mean time of travel of injected water is approximately 4,12, and 30 hours to the SSE1, SSE2, and SSE3 wells respectively.

Extracted water was treated by air stripping and filtration to remove volatile and suspended contaminants, respectively. A portion was used for the injection water , and the remainder was discharged into the Moffett Field drainage system. The test zone has many favorable features: high permeability, suitable groundwater chemistry, shallow depth, confined system providing two-dimensional flow, desirable size for field experiments, well controlled groundwater flow, and the ability to withdraw all chemicals or other materials added to the system. Details of the on-line field analytical system have been provided elsewhere (Roberts et al., 1990). An automated data acquisition and control system (DAC) was devised at the test site to implement the field experiments. Water samples for analysis were obtained from the monitoring well locations and from injected and extracted water by automated pumping to the analytical system. The automated analytical system permited the continuous measurement of the principal chemical constituents for a given experiment,

namely phenol, toluene and TCE, 1,1-DCE, c-DCE and t-DCE. The instruments, of interest for the field part of this project, operated by the DAC system are: a reverse phase high performance liquid chromatograph (HPLC) for phenol and toluene analysis, a gas chromatograph equipped with an electron capture detector (GC-ECD) and a Hall conductivity detector (GC-Hall) for CAC analysis. TCE was analyzed by GC using a purge and trap system described by Roberts et al., 1990. The gas chromatograph was equipped with a J&W Scientific (Folsom, CA) 30 meter, DB-624, megabore capillary column, and a Tremetrics (Houston, TX) Hall conductivity detector. Calibrations were made using an external standard. Phenol and toluene were analyzed by reverse phase HPLC, with separation on a Spherisorb ODS-2 column (Altech, Dearfield, IL) with 50% methanol in water as eluent, and detection using a Linear Model 200 UV detector. Chemicals were introduced into the injected water in an automatic programmed manner. The extracted water used for injection was treated before chemicals were added by filtration through a nominal 1μm filter, and UV disinfection. Chemicals introduced in the past were oxygen, phenol, bromide, TCE, cis and trans-1,2-dichloroethylene. Concentrations of the CAHs have generally been in the 40 to 150 μg/l range and, TCE concentrations up to 1000 μg/l were injected during the year of 1992. CAHs were added to injection water by continuous pumping of water solutions saturated with the particular CAH of interest. The injection water and sampled waters all pass through stainless-steel

tubing, which prevents passage of gases and CAHs through tubing walls. Thus, excellent mass balances of all chemicals can be maintained.

Chemical introduction into the aquifer - 1993 experiments. The field experiment was conducted by Gary Hopkins of Stanford University. For the experiments described in this work corresponding to the year of 1993, a summary of the injection concentrations for the chemicals of interest over this study period is given in Table 2.1. Before chemical augmentation, the groundwater was filtered with a nominal 1 um cotton filter, and UV disinfected. It was then passed through a gas absorption column where molecular oxygen was used to purge the column, producing a dissolved oxygen (DO) concentration in the groundwater of about 32 mg/L. Phenol and toluene were added in pulses during their respective test periods, once every 8 hours as controlled by a timing clock. For phenol, a solution was prepared, and an aliquot yielding 9 g phenol was added during each 10 min or less pulse, thus producing a 12.5 mg/L time-averaged concentration. Toluene was added in a similar fashion as phenol, but as pure solute: 6.5 g was added over a 30 min period to yield a time-averaged concentration of 9 mg/L for the eight hour cycle. A pair of static mixers with 24 elements was used to mix the toluene into the injection flow stream. Although the toluene was not perfectly dissolved within the contact time of less than 1s, it was nevertheless very finely dispersed upon exiting the static mixer, with an approximate pulse concentration of 200 mg/L (toluene solubility is approximately 600 mg/L). As in previous years, there was a period of 3 days in which the

Table 2.1. Time course of the four (*I-IV*) field experiments showing the concentrations of chemicals added to injected water and sampling schedule of glass beads [a].

Time period (h)	Concentration added to injected water						
	Phenol [b] (mg/L)	Toluene [b] (mg/L)	DO (mg/L)	TCE (μg/L)	1,1-DCE (μg /L)	c-DCE (μg /L)	t-DCE (μg /L)
I. -5760-0	0	0	0	0	0	0	0
-604 GB [c] Sample 1 taken							
II. 0[d]-200	12.5	0	32	230	0	0	0
200-500	12.5	0	32	230	134	0	0
500-680	12.5	0	32	230	65	0	0
504 GB Sample 2 taken							
III. 680-980	12.5	0	32	230	0	0	0
960 GB Sample 3 taken							
IV. 980-1316	0	9	32	230	0	0	0
1316-1422	0	9	32	230	0	119	0
1422-1465	0	9	32	230	0	0	0
1465-1656	0	9	32	230	0	0	81
1600 GB Sample 4 taken							

[a] Modified from Hopkins and McCarty,1995.
[b] Time-averaged concentrations
[c] GB=Glass beads taken from the GB sampling well.
[d] Time 0 estimated at the begining of experiments for the 1993 year.

microbial population was prestimulated with phenol (6.5 mg/L time-averaged) and oxygen (32 mg/L) addition. This was done to prevent potential biofouling of the extraction well and to provide time to correct the usual startup problems of the injection and analytical systems. At time zero, the chemical augmentation of TCE and bromide began along with the increase in the phenol concentration to a time-averaged 12.5 mg/L (Table 2.1). After TCE removal approached steady-state, the 1,1-DCE augmentation and evaluation began. After the termination of the 1,1-DCE addition and the quasi-steady state conditions were again achieved, the primary substrate was changed from phenol to toluene. Approximately one week later, augmentation with c-DCE began, followed by the augmentation with t-DCE.

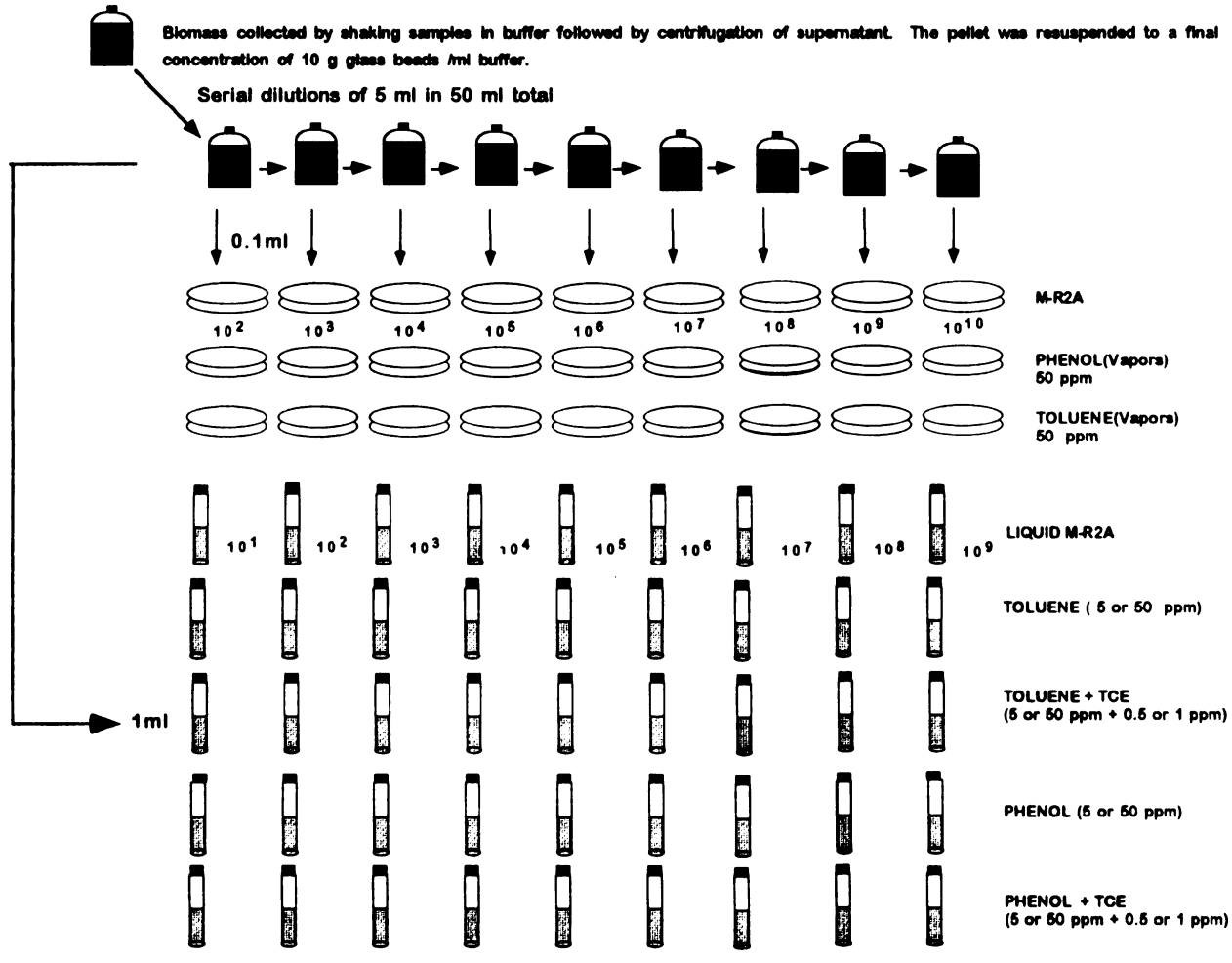
Samples for microbiological analysis. The 1993 study followed 8 months of inoperation of the field site. At about -604 hours before the beginning of the chemical augmentations for the 1993 season, three types of samples were sent to MSU for microbiological analysis of the phenol and toluene-degrading populations initially present on the field (sample 1). The samples consisted of microbial biofilms on glass beads (present in the field for 8 months) extracted from the SSEGB well (Figure 2.1.), and two filtered water samples. The first water sample resulted from filtering 500 ml of water through a 0.2 μm filter after extracting approximately 60% of the glass beads from the well (this sample is termed 0.22 μm filter sample in this work). A cotton filter (Micro-wynd II, J.N. Fauver Company, Flint, MI) was used to process all water extracted during the

removal of the glass beads from the well, approximately 150 L, and is termed here as the cotton filter sample. Samples 2, 3 and 4 consisted of glass beads deposited in a screen-fabric bag (approximately 100 g glass beads/bag) and hung in the fully penetrating well (SSEGB, Figure 2.1.). At indicated times (Table 2.1.), the bags of glass beads were removed from the well and sent on ice by Federal Express to MSU for microbiological analysis. New bags were deposited in the wells. For samples 2, 3 and 4, the period of microbial colonization of the glass bead bags in the field was 20-26 days.

Microbial biomass. To collect the biomass from the glass beads or filters, these samples were shaken with sterile saline solution, pH 7, in sterile 250 ml Nalgene plastic bottles. The supernatant was transferred to a clean, sterile bottle, followed by centrifugation. The pellet was retained and the supernatant discarded. At least eight extractions were performed in this manner from each sample. The final pellet was resuspended in saline to yield a biomass concentration correspondent to 10 g glass beads/ml of saline. The filter samples were resuspended to a simmilar visual turbidity as the resultant glass beads samples. This resuspended turbid solution was used for MPN determinations, direct isolation of microbial populations and total microbial community DNA extraction.

Most probable number (MPN) population estimates. For the MPN determinations the basic experimental protocol presented in Figure 2.2. was followed. The estimated population density and the 95% confidence intervals

EXPERIMENTAL DESIGN FOR MPN DETERMINATION AND ISOLATIONS



MOST DILUTE POSITIVE TUBES WERE PLATED AS FOLLOWS (NEXT PAGE):

1 ml from end point positive tube / 9 ml saline

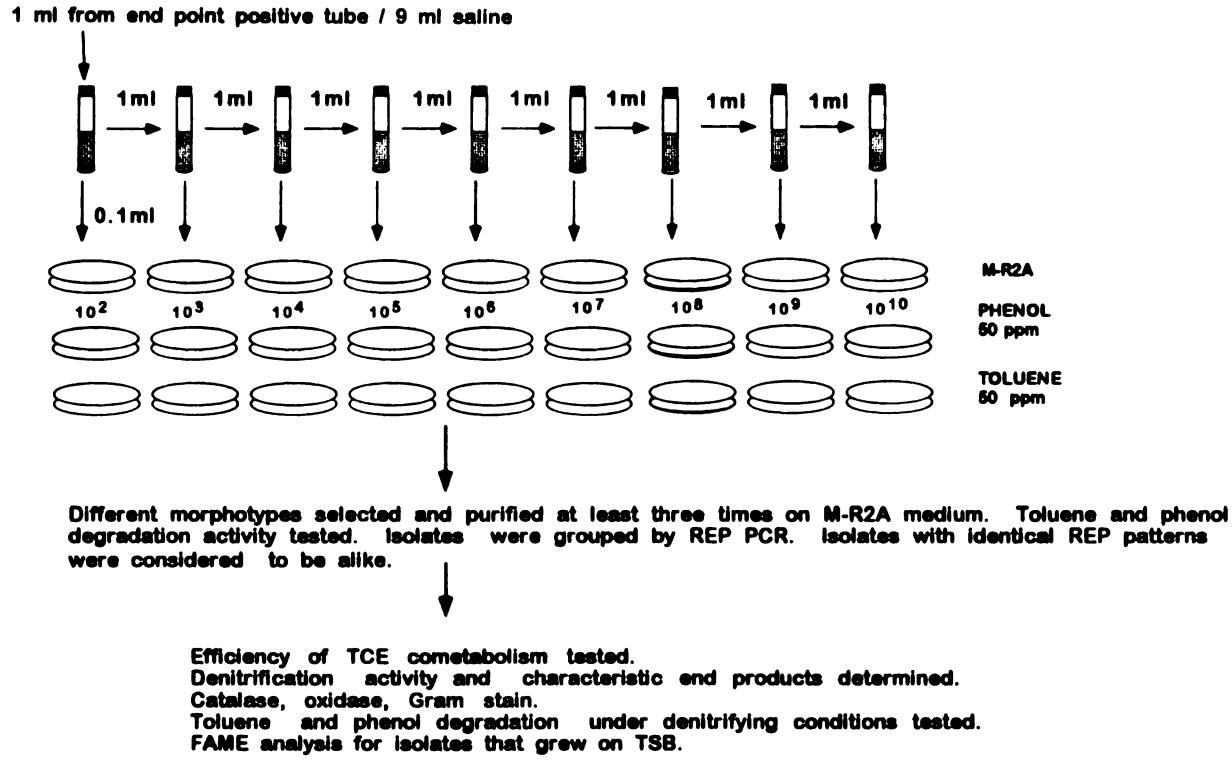


Figure 2.2. Experimental protocol followed for the determination of the Most Probable Number of toluene and phenol degraders, TCE co-oxidizers, total viable heterotrophs and for isolation of the most dominant populations from the aquifer.

were calculated by standard methods (Alexander,1982). Resuspended material collected from glass beads and filters were serially diluted and 1 ml of each dilution was transferred to sterile 20 ml vials. Five tubes were inoculated from each serial dilution. The samples were incubated without shaking after addition of 9 ml of basal salts (BS) medium (Owens and Keddie, 1969) amended with 5 mM KNO_3 and sealed with Teflon-lined stoppers. For sample 1, basal salts medium containing toluene or phenol at 5 or 50 ppm concentrations without or with TCE at 0.5 or 1 ppm, respectively, was added. For samples 2, 3 and 4, basal salts medium containing toluene or phenol at 25 ppm without or with TCE at 0.5 ppm was added. A vial was assumed to be positive for biodegradation of the primary substrate and TCE co-oxidation when more than 50 and 95 % of the TCE and toluene or phenol, respectively, had disappeared.

For the determination of total heterotroph population the vials were inoculated with liquid M-R2A medium and analyzed for visible turbidity. Modified R2A (M-R2A), is based on the original carbon composition provided by Difco (Detroit, MI), combined with a low phosphate plus trace salts mixture. M-R2A had the following composition per liter: salt mixture (SM) consisted of KH_2PO_4, 0.25g; K_2HPO_4, 0.4g; KNO_3, 0.505g; $CaCl_2.2H_2O$, 0.015g; $MgCl_2.6H_2O$, 0.02g; $FeSO_4.7H_2O$, 0.007g; Na_2SO_4, 0.005g; NH_4Cl, 0.8g; $MnCl_2.4H_2O$, 5mg; H_3BO_3, 0.5mg; $ZnCl_2$, 0.5mg; $CoCl_2.6H_2O$, 0.5mg; $NiSO_4.6H_2O$, 0.5mg; $CuCl_2.2H_2O$, 0.3mg; $NaMoO_2.2H_2O$, 0.01mg. For solid medium 15 g of Bacto agar (Difco, Detroit, MI) was added. The pH was adjusted to 7.0 before

autoclaving. The carbon sources were per liter: yeast extract, 0.5g; Proteose peptone, 0.5g; casamino acids, 0.5g; Dextrose, 0.5g; soluble starch, 0.5g; sodium pyruvate, 0.5g. The incubation temperature throughout this work was 30°C.

Isolation of dominant populations. To isolate most of the culturable populations present on the aquifer we followed the protocol presented in Figure 2.2. We used the most dilute MPN tubes showing disappearance of phenol and toluene with or without TCE from all samples, to isolate the most dominant phenol and toluene degraders. For samples 2, 3, and 4, we also used the original dilution tubes with samples used for MPN and direct plated these samples for microbial isolations because in sample 1 the number of toluene and phenol degraders were below the expected levels in relation to the total heterotrophic population and we attempted to verify if the most dominant heterotrophs were capable of toluene or phenol degradation and TCE co-oxidation. Samples from MPN tubes were serially diluted and plated on three different solid media: 1) BS + 5 mM NO_3^- + toluene vapors, 2) BS + 5 mM NO_3^- + phenol and 3) Solid M- R2A. Glass Petri dishes were used for phenol and toluene-vapor based growth. Toluene was added in a small vial inside an incubation jar. The amount of toluene added was calculated based on the total volume of agar in the incubation jar to provide a final concentration of 50 ppm. After one week of incubation sufficient toluene to provide 50 ppm was again added. The jar used in the phenol and toluene vapor experiments was sealed

with a Teflon-lined aluminum sheet (Cole-Palmer, Chicago, Il) to prevent phenol or toluene absorption by the rubber sealer of the jar. The plates were incubated under aerobic conditions. The selection strategy employed for isolation was based on bacterial colonies obtained on the different media (toluene and phenol vapors and M-R2A) and selecting all colonies that had differences in colony morphology, size and pigmentation. For purification of isolates, single colonies from different media and from all dilutions showing positive-distinct-colony-growth were selected and purified at least three times on M-R2A. For very small colonies at least three individual colonies were transferred to new plates to assure the maintenance of these morphotypes because our experience has shown that many isolates can be lost at this stage.

Characterization of isolates. Gram stain, catalase and oxidase tests were performed on pure cultures of isolates by standard methods (Smibert and Krieg, 1981). Confirmation of phenol and toluene degradation was done by transferring a heavy inoculum of each isolate to sterile 20 ml vials after which 5 ml of aerobic BS + NO_3^- + 25 ppm phenol or toluene medium was added. The vials were sealed with sterile butyl rubber Teflon-lined septa and incubated for at least 2 weeks before phenol or toluene disappearance was evaluated. Controls from the same batch of medium without cells were incubated at the same time to determine the non-biological phenol or toluene loss. Positive degradation activity was defined as at least 95% loss of toluene in the headspace as

measured by GC analysis and at least 95% loss of phenol in the supernatant of the growth media as measured by HPLC analysis.

Standard strains. Laboratory standard strains of *Pseudomonas putida* F1, *Pseudomonas putida* PaW1, *Pseudomonas mendocina* KR, *Burkholderia cepacia* G-4, *Burkholderia pickettii* PKO1 and *Pseudomonas sp.* JS-150 were kindly provided by Dr. Ronald Olsen, University of Michigan.

Trichloroethylene co-oxidation. To test for the isolates' capabilities to co-oxidize TCE during degradation of toluene or phenol, inocula were grown on liquid M-R2A under aerobic conditions. Inocula (1/2 ml) were transferred to 20 ml sterile auto sampler vials and 4.5 ml of BS + NO_3^- + 25 ppm of the aromatic substrate and 0.5 ppm TCE was added to the vials. The vials were sealed with Teflon-lined stoppers. Positive degradation activity was determined by TCE disappearance in the headspace after 15 days of incubation as measured by GC analysis, and after comparison to non-inoculated controls. For the TCE co-oxidation rate studies, the cultures were grown on BS + NO_3^- + 50 ppm toluene. Inocula (10 %) was transferred to 160 ml serum bottles containing 130 ml of BS + NO_3^- + 50 ppm of toluene and 1 ppm TCE. This culture was subdivided in 5 ml portions and transferred to 20 ml sterile auto sampler vials and duplicate Hungate tubes and sealed with Teflon-lined stoppers. Three vials were sacrificed by adding 0.1 ml of 10 N HCl and the vials were frozen at - 20°C for later analysis of initial toluene and TCE concentrations. Growth in Hungate tubes was followed by optical density at 600 nm; at different time intervals vials

were sacrificed and preserved as before, for later analysis of remaining toluene and TCE.

FAME analysis. The isolates analyzed for cellular fatty acids (FAME) were precultured on M-R2A and inoculated into Erlenmeyer flasks containing 0.3% (w/v) tryptic soy broth (TSB), (Krieg, 1981). Cells were harvested by centrifugation. The cell pellet was placed into a screw-capped test tube and stored at -20°C until prepared for analysis. Saponification, methylation and extraction were performed using the procedure described in the MIDI manual (Sasser, 1990). Gas chromatography data was compared to a fatty acid identification library version 3.8 (Microbial Identification Systems, Inc., Newark, Del.) for possible isolate identification.

Molecular methods. Repetitive extragenic palindromic REP- PCR patterns were obtained from cells using Rep-1 and Rep-2 primers and the polymerase chain reaction (de Bruijn, 1992). Amplification was performed using a Model 9600 Perkin-Elmer Cetus Thermocycler. Products (10 µl) were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide. Amplification was primarily done using individual colonies grown on M-R2A, but for isolates with poor or no amplification, DNA extracted from cells was used as the template for PCR amplification. Half of the amplified REP products were run in a prelliminar gel that was scanned for band patterns using the Ambis System. Band pattern cluster analysis of sets of isolates that could include up to 130 strains revealed which isolates had possible close REP patterns. The remaining

REP amplified product were re-run side-by-side as suggested by the computer cluster analysis to eliminate siblings from further characterizations. For isolates with very close (not identical) REP patterns, the same stock of primers and reagents were used in the analysis to confirm differences.

The gene probes used in this study are listed in Table 2.2. *E. coli* cultures carrying the plasmids with probes were grown for plasmid amplification in the presence of the appropriate antibiotic. Plasmids were extracted by standardy protocol (Maniatis et al., 1982). The probes were isolated as restriction fragments from their respective vectors in 1% low melting point agarose, purified with the Gene clean kit (Bio 101, Inc. La Jolla, CA) and labelled with alpha-[^{32}P] dCTP (3,000 Ci/mM; Dupont, NEN Research Products, Wilmington, DE) using a random hexamer priming kit from Boehringer Mannheim Biochemicals. Labelled probes were separated from unincorporated nucleotides prior to use with a spun column (Maniatis et al., 1982). The probes were used at approximately 10^6 cpm/ml of hybridization fluid.

Genomic DNA was obtained by standard methods (Elmerich et al., 1982) from pure cultures of isolates and selected strains grown on M-R2A broth under aerobic conditions. Restriction endonuclease digestion of DNA was performed according to manufacturer specifications. Digested DNA was size fractioned by electrophoresis in 0.7% agarose gels and transferred to nitrocellulose (polyester-supported BAS 68380; Schleicher & Schuell, Keene, N.H.) as previously described (Maniatis et al., 1982) using 20 X SSPE (1 XSSPE is

Table 2.2. List of DNA probes used in this study.

Organism (probe source)	Gene encoded and size of the probe	Plasmid	Lab. source
Pseudomonas putida PaW1	Methyl monooxygenase (hydroxylase and NADH-ferredoxin reductase), 2.35 Kb (*Sal I/Hind* III).	pG5H2836	S. Harayama
Pseudomonas putida F1	Toluene dioxygenase(large and small subunits of the oxygenase, ferredoxin and part of the reductase), 3.5 Kb (*Eco* RI/Bgl II)	pDTG601	D. Gibson
Pseudomonas mendocina KR	Toluene parahydroxylase (monooxygenase and ferredoxin), 3.6 Kb (*Eco* RI/*Eco* RI)	pMY421	M. DeFlaun
Burkholderia pickettii PKO1	Toluene metahydroxylase (α subunit of monooxygenase), 0.68 Kb (*Apa I/Ava* I).	pAB14ΔAva I	R. Olsen
Pseudomonas sp JS-150	Toluene orthohydroxylase, 2.2Kb (*Eco* RV/*Hind* III).	pRO20116	R. Olsen
Burkholderia cepacia G-4	Toluene orthohydroxylase, 2.7Kb (*EcoRI/Hind*III).	pMS80	M. Shields

[a] Laboratories from which the clones were obtained from.
[b] This strain was originally provided by J.C.Spain.

0.18 M NaCl, 10 mM NaH_2PO_4, pH 7.04, and 1 mM EDTA). The DNA on the filters was cross-linked by U.V. light (Stratagene, La Jolla, CA). The solutions used for DNA hybridization analysis have been described elsewhere (Holben et al., 1988). The membranes were prehybridized for at least 24 h in heat sealed bags containing 100 µl of prehybridization fluid per cm^2 of filter. Prehybridization fluid contained 5 X Denhardt solution, 5 X SSPE, 50% formamide, and 200 µg of sonicated and denatured salmon sperm DNA per ml. The hybridization solution was the same as for prehybridization but included 10% (w/v) of dextran sulfate, and was added at 50 µl per cm^2 of filter. Membranes were incubated for at least 24 h. Two hybridization temperatures were used: low stringency at 30°C and high stringency at 42°C. After hybridization, the filters were washed once for 15 min with agitation at 30°C with 2 X SSC (1 X SSC is 0.15 M NaCl plus 0.015 M sodium citrate), plus 0.1% SDS. For low stringency hybridization a second wash of 30 min with 0.5 X SSC plus 0.1% SDS was performed. For high stringency hybridization a second wash of 15 min with 0.5 X SSC plus 0.1% SDS followed by a third wash with 0.1 X SSC plus 0.1% SDS was performed. This was followed by a final wash at 55°C for 30 min with the latter solution. After washes, hybridization signals were visualized using the Betascope radioactive blot analyzer (Betagen Corp., Waltham, MA) or by autoradiography using Kodak X-Omat AR film (Kodak, Rochester, NY) exposed at -70°C with a Quanta III (Sigma, St. Louis, MO) intensifying screen. Exposure times were 1 to 3 days depending on the intensity of the radioactive

signal. For reuse of the same blot for another probe, the blots were stripped by washing for 10 min in boiling water and 0.1% SDS at least two times, depending on the signal left on the blot after evaluation with the Betascope. A final wash with 2 X SSPE + 0.1% SDS for 10 min completed the stripping protocol.

Analytical methods. Toluene and TCE were measured by gas chromatography equipped with a flame ionization detector(GC/FID), a DB-624 capillary column (J&W Scientific, Folson, CA.) and a headspace sampler. The autosampler vials were equilibrated at 30°C, the column at 90°C and injector and detector at 200°C. He was the carrier gas. Phenol was analyzed by reverse-phase HPLC with a Hibar RT C_{18} column (E. Merck), with a flow rate of 1.5 ml/min of 66:33:0.1 H_2O-CH_3CN-H_3PO_4 and a UV detector set to 218 nm.

Results

Population densities. The effect of toluene and phenol concentrations on population densities of toluene and phenol degraders and TCE co-oxidizers were evaluated. The mean initial population density of microorganisms able to degrade toluene was $7.2x10^2$ cells/g of glass beads from sample 1 when the concentration of toluene in the medium was 50 ppm and $4.9x10^4$ cells/g of glass beads when the concentration of toluene in the mediium was 5 ppm (Figure 2.3a). This trend of a greater than one log unit increase in populations of toluene degraders detected at 5 ppm versus 50 ppm was also noted for the water populations obtained from the two filters ($2.4x10^5$ and $3.5x10^5$ cells/ml of

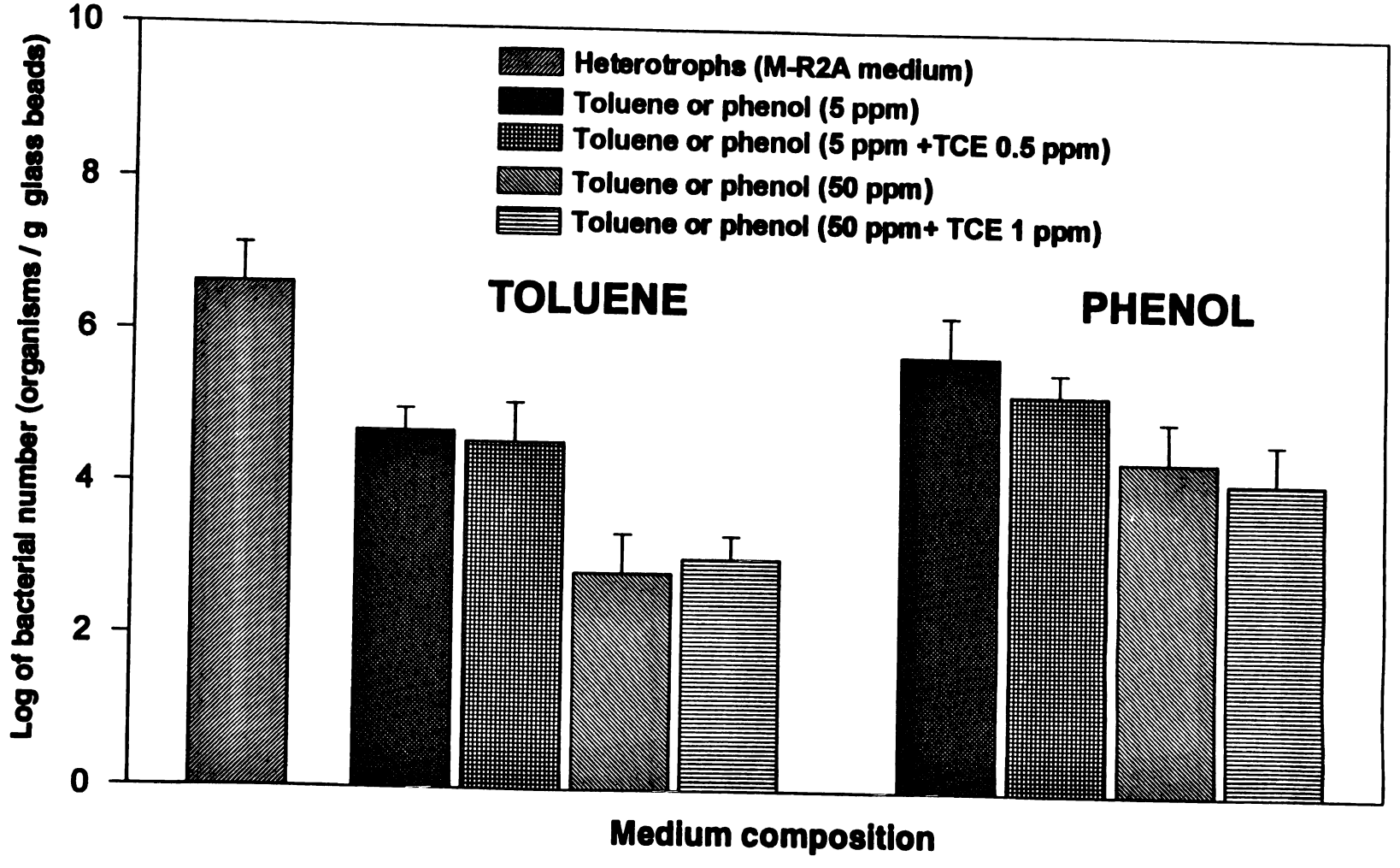


Figure 2.3a. Effects of toluene and phenol concentration on total number of toluene and phenol degraders detected by MPN on glass beads from aquifer well. The total number of heterotrophs present in the same sample was also determined by MPN.

slurry at 5 ppm versus $3.3x10^3$ and $3.3x10^4$ cells/ml of slurry at 50 ppm for the 0.22 *um* and cotton filters respectively, Appendix A. The presence of TCE at 0.5 ppm or 1 ppm together with 5 ppm or 50 ppm toluene, respectively, had no effect on MPN estimates of toluene degraders for the glass beads sample (Figure 2.3a) or for the the two filtered water samples (Appendix A).

Phenol concentrations had less of an impact on MPN numbers of phenol degraders than toluene (Figure 2.3a) but there still was a significant increase in numbers, nearly one log when the phenol concentration was decreased from 50 to 5 ppm (Figure 2.3a). Based on the sensitivity of the aquifer population to higher toluene and phenol concentrations, isolates from samples 2, 3, and 4 where enriched in MPN tubes at 25 ppm, the lower concentration feasible for evaluation of TCE cooxidizers.

The number of toluene and phenol degraders on the glass beads and filters was compared to the number that could cooxidize 1 ppm of TCE in 2 weeks. A vial was assumed to be positive for co-oxidation when more than 50% of TCE had disappeared. When toluene was the primary carbon source $7.0x10^3$, $7.9x10^3$ and $2.4x10^4$ cells of TCE cooxidizers/ml of slurry was observed for the glass beads, 0.22*um* filter and cotton filter samples, respectively (Figure 2.3b). For the same samples, when phenol was the primary carbon source $7.9x10^4$,$5.4x10^5$ and $3.5x10^5$ cells/ml of slurry were obtained, indicating a one log greater number of TCE co-oxidizers growing on phenol than on toluene. At 50 ppm of toluene or phenol, the population density of TCE degraders was

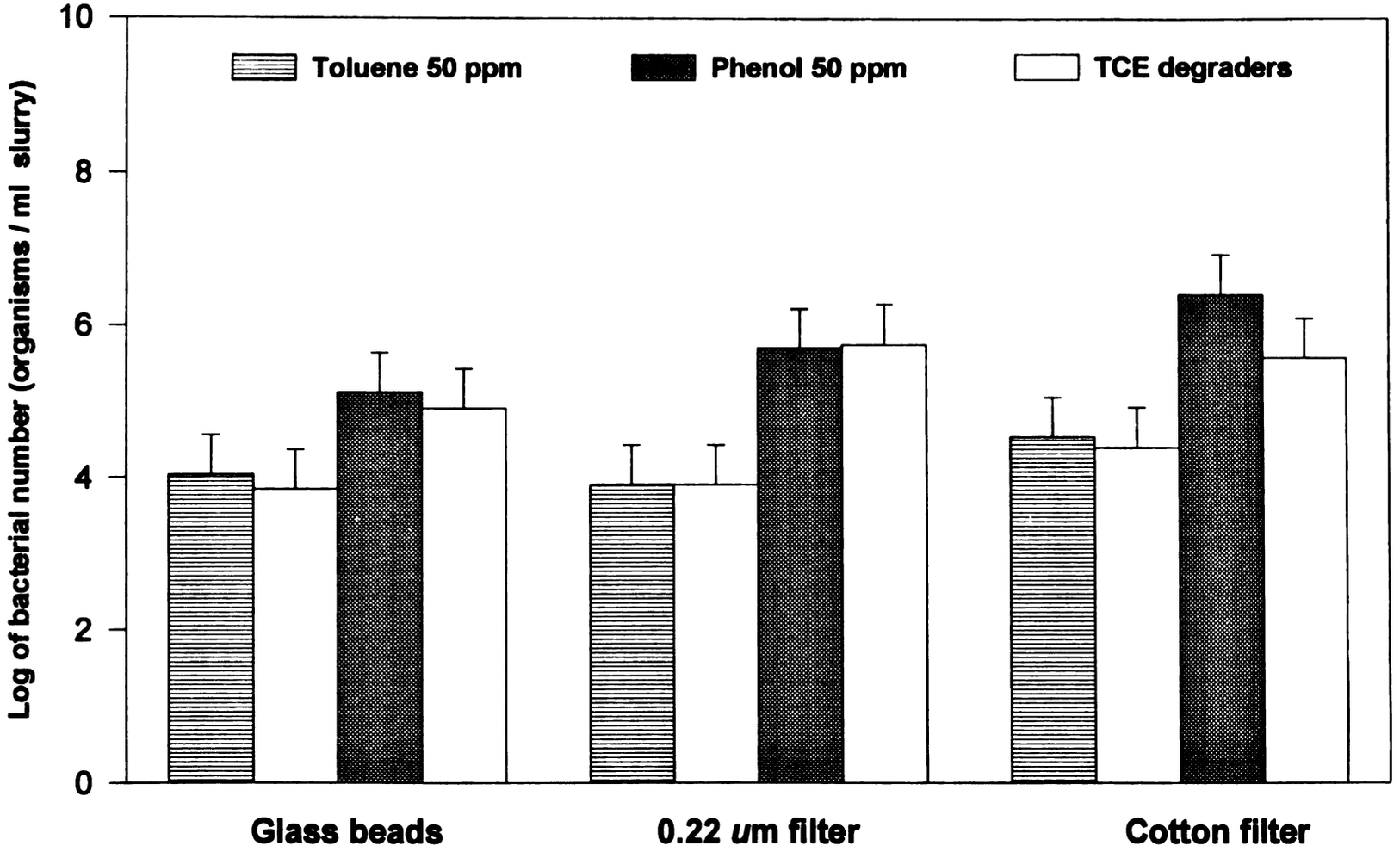


Figure 2.3b. Number of TCE co-oxidizers and primary substrate degraders estimated from vials containing toluene or phenol as primary carbon sources. Data are from three different samples taken from the aquifer at the first sampling period.

identical to that of toluene degraders and phenol degraders, suggesting that most organisms able to degrade toluene or phenol produced the necessary enzyme to degrade at least half of the TCE present. The same experiment, but done at 5 ppm of the primary carbon source, did not yield consistent data. The low concentration of the carbon substrate may not have resulted in enough enzymes for TCE co-oxidation (Appendix A) or may indicate that TCE at a 10% concentration may be toxic for some populations.

The total numbers of heterotrophs detected for the three samples were $4.3x10^7$, $2.4x10^8$ and $2.4x10^8$ cells/ml of slurry for the glass beads, 0.22um filter and cotton filter samples, respectively (Figure 2.3a and Appendix A). Using these values, toluene degraders represent 1.8%, 0.1% and 0.15% of the total heterotrophs, and phenol degraders represent 12.6%, 2.2% and 13.8% of the total heterotrophic population present on the the glass beads, 0.22 um filter and cotton filter samples, respectively. The populations of toluene or phenol degraders is likely higher than this since some isolates selected from direct plating on M-R2A of the most dilute tube were positive for toluene and/or phenol degradation. This results further indicates, that the conventional MPN method can understimate the population of phenol or toluene degraders. We attribute this to toxicity of these compounds, even at concentrations as low as 5 ppm. Lower concentrations of substrates could be used, but the detection method, especially for toluene would be compromised, when working with the number of samples required for MPN estimations.

Isolation of populations. To examine a broader spectrum of culturable populations capable of degradation of the primary substrates and in doing so gain a greater understanding of the entire community, we attempted to go beyond enriching for the best competitors in the laboratory. We used the most dilute MPN tubes showing disappearance of phenol and toluene with or without TCE from all field treatments, to isolate the most dominant phenol and toluene degraders. We also plated the original dilution series samples 2, 3, and 4 on M-R2A and selected isolates from these plates. For sample 2, isolates were obtained only on M-R2A medium since no MPN tube was positive. The isolate obtained from sample 3 on M-R2A as the most dominant heterotroph, MF-H-3-3, was not retrieved from the MPN tubes when phenol or toluene were the carbon sources. (For strain nomenclature, MF= Moffett Field; H, if present indicates heterotrophic isolate from M-R2A, the next digit indicates the sample number (1, 2, 3, or 4), and the last digit is the strain number in the sample series). One isolate, MF-H-3-5 obtained by direct plating on M-R2A was also isolated from MPN tubes (e.g. MF-301). As expected, some isolates obtained as heterotrophs from M-R2A plates were incapable of toluene or phenol degradation. For sample 4, some degraders obtained from MPN tubes, namely isolates MF-416, MF-421 and MF-441 were also isolated as heterotrophs: MF-H-4-2, 4, and 6, respectively. Interestingly, the most dominant isolate from this sample, MF-H-4-5, was also not isolated from dilution tubes and does not grow on toluene as the

primary carbon source, but it does grow on phenol. These results suggest that toluene and phenol were toxic at the concentrations used in the very dilute MPN tubes (low cell number/unit of substrate) and that in the most dilute MPN positive tubes, these strains were unable to compete for the carbon subtrates with the other populations present in the MPN tube and hence were not isolated.

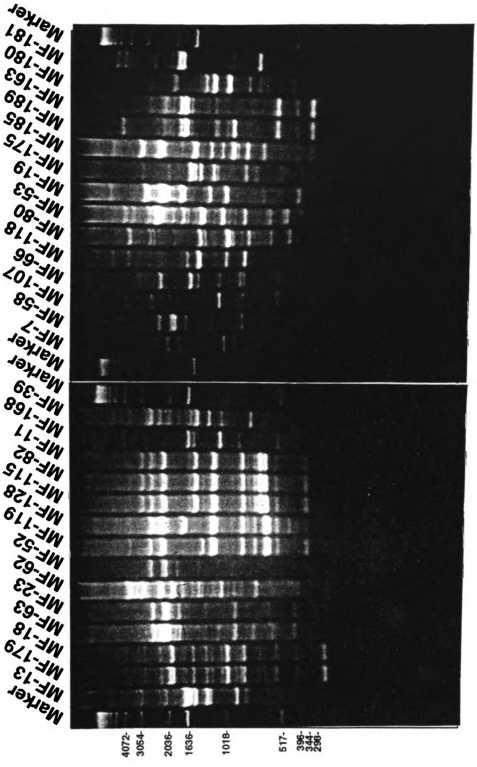
Discrimination of isolates by REP-PCR analysis. We obtained 348 numerically dominant isolates from the Moffett Field experimental site from July 1993 through November 1993. These represent bacterial populations present after an untreated 8 month period before the start of the 1993 field experiments (sample 1) or after treatment with phenol or toluene at different times in 1993 (samples 2, 3,4). These isolates were further screened for their ability to degrade phenol or toluene; 273 isolates were shown to be positive for degradation of at least one of the carbon substrates. Degraders of toluene or phenol were first grouped by similar colony morphology as much as possible. To focus the further characterization, we eliminated very closely related isolates or siblings using REP-PCR patterns. Visual comparison of patterns on several gel pictures is a difficult task so we used the Ambis system to scan and cluster isolates with similar patterns. New gels were then run with closely related strains side-by-side. This confirmed differences or similarities in REP patterns. Isolates with identical REP-PCR patterns were considered the same and only one representative of that pattern was analyzed further. The REP-PCR analysis

of the isolates revealed that the 273 degraders produced 63 different REP fingerprint patterns (Figure 2.4). Isolate MF-88, produced two different unstable (reverting spontaneously to the original appearance) colony morphologies on M-R2A medium, but the REP pattern remained the same (Figure 2.4).

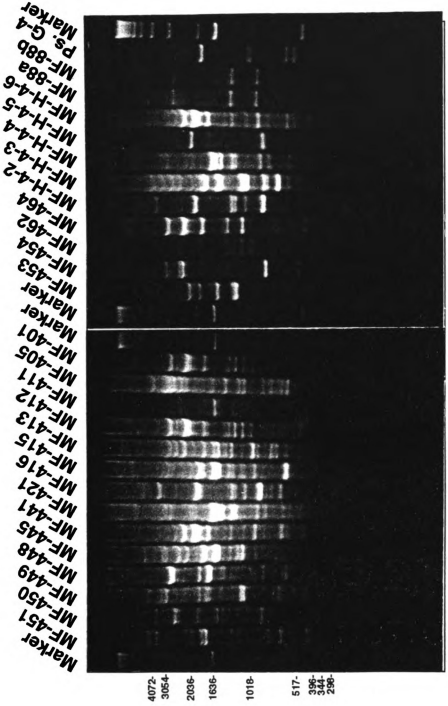
Isolate distribution. One dominant REP group (MF-19) contained 15% of the isolates (41 strains). Analysis of isolates obtained from glass beads, representing the attached population and isolates obtained from the filtered water, representing the planktonic community (Figure 2.5.) shows that five of the 6 types most frequently isolated, MF-19, MF-63, MF-11 and MF-175 were retrieved from both sources. Isolates MF-163, MF-180, MF-415 and MF-419 were isolated only from glass beads and isolates MF-185 and MF-168 only from filtered water, but these types were less frequently recovered.

FAME/taxonomic and degradative diversity analysis. The isolates obtained during the course of this experiment were analyzed for their cellular fatty acid composition. Species identification based on total fatty acids was poor for most of the isolates since the analysis gave in many cases a low similarity index by using the MIDI database (Table 2.3). From the 63 bacterial isolates with unique REP-PCR patterns, 16 isolates were assigned to 11 different genera and 15 different species with profile similarity index of $\geq$ 0.5 (range, 0 to 1.0). The taxonomic designations suggested were: *Acinetobacter johnsonii* (strain MF-24),

Figure 2.4. REP-PCR fingerprint patterns of Moffett Field isolates generated by using genomic DNA or whole cells. Outside lanes show DNA size markers indicated (in base pairs) on the left. Strains are ordered by similarity in band patterns according to the Ambis System clustering program.


Marker
MF-13
MF-179
MF-18
MF-63
MF-23
MF-62
MF-52
MF-119
MF-128
MF-115
MF-82
MF-11
MF-168
MF-39
Marker
Marker
MF-7
MF-58
MF-107
MF-66
MF-118
MF-80
MF-53
MF-19
MF-175
MF-185
MF-189
MF-163
MF-180
MF-181
Marker
4072-
3054-
2036-
1636-
1018-
517-
396-
344-
298-

Marker
MF-121
MF-182
MF-92
MF-108
MF-88
MF-89
MF-147
MF-24
MF-5
MF-84
MF-84W
MF-15
MF-54
MF-97
Marker
MF-183
MF-H-2-1
MF-H-2-4
MF-H-2-9
MF-H-2-12
MF-H-2-13
MF-301
MF-304
MF-340
MF-342
MF-H-3-3
MF-H-3-5
Marker
4072-
3054-
2036-
1636-
1018-
517-
396-
344-
298-


Marker
MF-451
MF-450
MF-449
MF-448
MF-445
MF-441
MF-421
MF-416
MF-415
MF-413
MF-412
MF-411
MF-405
MF-401
Marker
MF-453
MF-454
MF-462
MF-464
MF-H-4-2
MF-H-4-3
MF-H-4-4
MF-H-4-5
MF-H-4-6
MF-88a
MF-88b
Ps. G-4
Marker
4072-
3054-
2036-
1636-
1018-
517-
396-
344-
298-

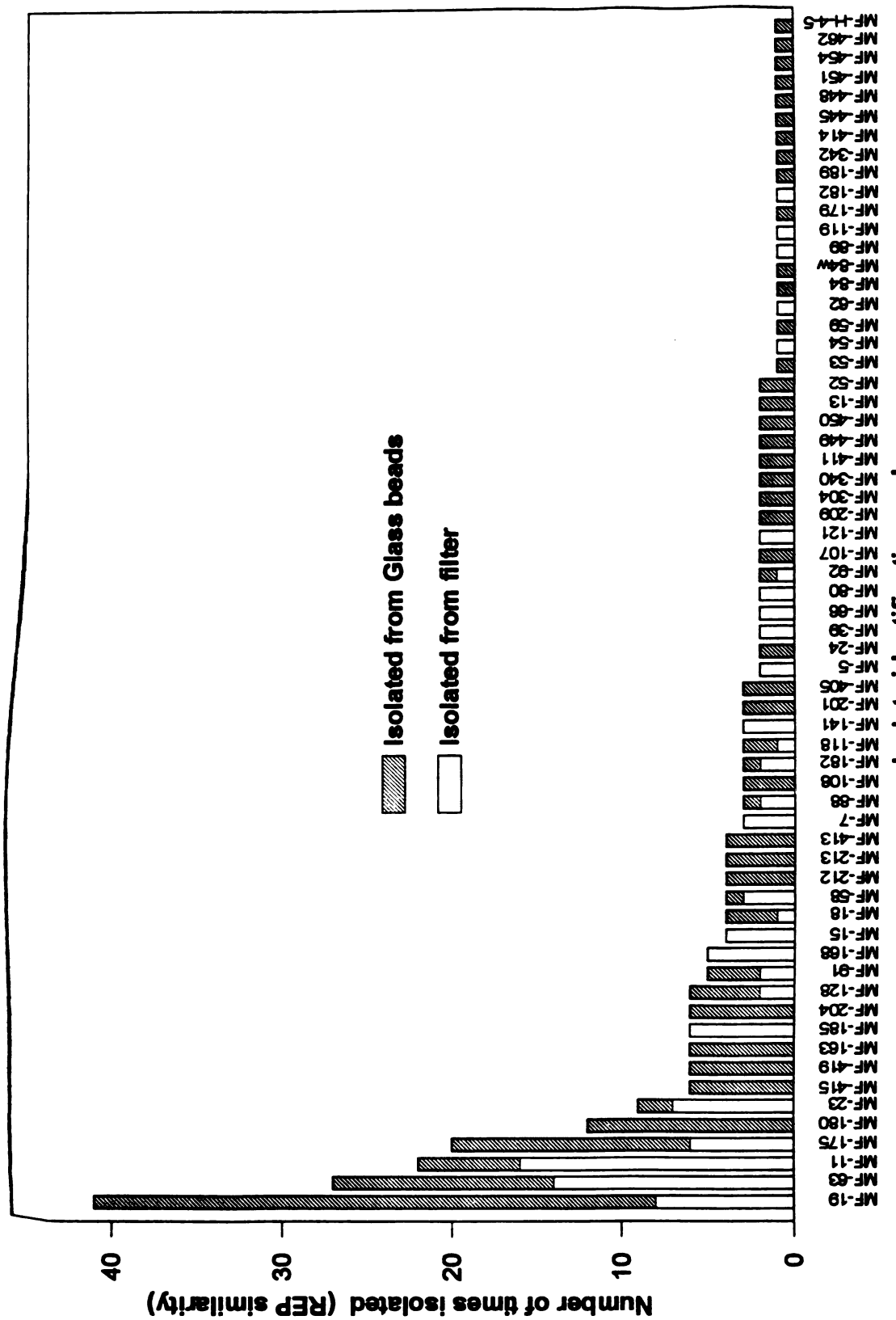


Figure 2.5. Frequency distribution of isolates according to REP analysis.

Table 2.3. Cellular, physiologic and genotypic properties of all isolates with different REP patterns obtained from the Moffett Field aquifer, from the four sampling times.

Isolate[a]	Rep[b]	Gram Stain	Catalase	Oxidase	TOM[c]	Fame identification[d]	Index[e]
MF-5	1	-V	+	++	+	*Pseudomonas fluorescens*	0.013
MF-7	2	-	+	-	-	*Hydrogenophaga pseudoflava*	0.321
MF-11	3	-	W	-	+	*No match*	
MF-13	4	-	+	w	+	*Actinobacillus lignieresii*	0.227
MF-15	5	-	++	++	+	*No match*	
MF-18	6	-	+	-	+	*Pseudomonas putida*	0.157
MF-19	7	-	+	-	+	*Comamonas acidovorans*	0.074
MF-23	8	-	-	-	+	*Hydrogenophaga pseudoflava*	0.386
MF-24	9	-	+	-	-	*Acinetobacter johnsonii*	0.595
MF-39	10	-	+	+	+	*Variovorax paradoxus*	0.634
MF-52	11	+	+	-	-	*Staphylococcus aureus*	0.010
MF-53	12	-	W	W	+	*Hydrogenophaga pseudoflava*	0.434
MF-54	13	-	+	+	-	*No match*	
MF-58	14	-	+	+	+	*Hydrogenophaga pseudoflava*	0.365
MF-59	15	-	+	+	-	*No growth*	
MF-62	16	-	++	W	+	*Hydrogenophaga pseudoflava*	0.502
MF-63	17	-	-	-	+	*Hydrogenophaga pseudoflava*	0.421
MF-66	18	-	W	W	+	*Hydrogenophaga pseudoflava*	0.191
MF-80	19	-	W	++	+	*Pseudomonas syringae*	0.114
MF-84	20	+V	+	W	-	*Bacillus sphaericus*	0.214
MF-84w	21	+V	+	W	-	*Bacillus sphaericus*	0.151
MF-88	22	-V	+	-	+	*Variovorax paradoxus*	0.382
MF-89	23	+	+	-	-	*Bacillus subtilis*	0.572
MF-91	24	-V	+	-	+	*no growth*	
MF-92	25	-	+	-	+	*Comamonas acidovorans*	0.572
MF-107	26	-	+	W	-	*Hydrogenophaga pseudoflava*	0.388
MF-108	27	+	+	-	-	*Nocardia asteroides*	0.516
MF-118	28	-	W	W	+	*Janthinobacterium lividum*	0.548
MF-119	29	-V	+	W	+	*No match*	

Table 2.3. (continued)

MF-121	30	+	+	-	-	*No match*	
MF-128	31	-	+	+	+	*Acidovorax avenae*	0.068
MF-141	32	-	+	W	+	*Corynebacterium diphteriae*	0.472
MF-163	33	+	+	-	-	*No match*	
MF-168	34	-	+	+	+	*No match*	
MF-175	35	-	+	+	+	*Burkholderia pickettii*	0.019
MF-179	36	-			+	*Pseudomonas putida*	0.193
MF-180	37	-			+	*Pseudomonas fluorescens*	0.012
MF-181	38	+			-	*Bacillus pumilus*	0.882
MF-182	39	-	+	+	+	*Pseudomonas putida*	0.333
MF-185	40	-	+	+	-	*No match*	
MF-189	41	+	+	-	-	*No match*	
MF-H-2-1	42	+	+	-	nd	*Micrococcus varians*	0.273
MF-H-2-4	43	+V	+	-	-	*Methylobacterium mesophilicum*	0.348
MF-H-2-9	44	-	+	+	-	*Acinetobacter radioresistens*	0.216
MF-H-2-12	45	+	+	-	-	*Staphylococcus haemolyticus*	0.776
MF-H-2-13	46	+	+	-	-	*No match*	
MF-301	(7)	-	+	-	+	*Comamonas acidovorans*	0.381
MF-304	47	-V	+	+	-	*Pseudomonas putida*	0.580
MF-340	48	-	+	+	-	*Enterococcus faecium*	0.020
MF-342	49	+	+	+	-	*Bacillus amyloliquefaciens*	0.741
MF-H-3-3	(33)	+	+	-	-	*no match*	
MF-H-3-5	(7)	-	+	-	nd	*Variovorax paradoxus*	0.339
MF-401	(7)	-V	+	++	+	*Comamonas acidovorans*	0.302
MF-405	50	-	+	-	-	*no match*	
MF-411	51	+	+	-	nd	*Corynebacterium aquaticum*	0.327
MF-413	52	-	W	+	+	*Acidovorax delafieldii*	0.893
MF-414	53	-	W	+	nd	*Acidovorax facilis*	0.969
MF-415	54	-V	W	+	+	*Acidovorax delafieldii*	0.813
MF-416	(24)	+	+	+	nd	*Bacillus macerans*	0.485
MF-419	55	-	+	+	nd	*no match*	
MF-421	(35)	-	+	+	nd	*Alcaligenes eutrophus*	0.682
MF-441	(17)	-	-	-	nd	*Hydrogenophaga pseudoflava*	0.303
MF-445	56	-	+	-	nd	*Hydrogenophaga pseudoflava*	0.062

Table 2.3. (continued)

MF-448	57	-	+	++	nd	*no match*	
MF-449	58	+	+	-	nd	*Gordona bronchialis*	0.098
MF-450	59	+	+	-	nd	*Nocardia restricta*	0.553
MF-451	60	+	+	-	-	*no match*	
MF-453	(28)	+	+	-	-	*Nocardia asteroides*	0.525
MF-454	61	-	+	-	nd	*Acinetobacter radioresistens*	0.336
MF-462	62	+	W	-	nd	*no match*	
MF-464	(7)	-	+	W	-	*Variovorax paradoxus*	0.552
MF-H-4-5	63	-	+	+	-	*Pseudomonas putida*	0.418
MF-H-4-2	(24)	+	+	-	-	*Bacillus amyloliquefaciens*	0.774
MF-H-4-4	(35)	-	+	+	nd	*Burkholderia pickettii*	0.743
MF-H-4-6	(17)	-	-	-	nd	*Hydrogenophaga pseudoflava*	0.264

[a] Isolates numbered as 001-199 (sample 1) were obtained before the treatments with phenol and toluene were applied to the field for the year of 1993.
Isolates numbered as 201-299 (sample 2) were obtained after the phenol treatment and 1,1 DCE + TCE.
Isolates numbered as 301-399 (sample 3)were obtained after the phenol treatment + TCE.
Isolates numbered as 401-499 (sample 4)were obtained after the toluene treatment + TCE.
Isolates termed MF-H-..., were purified from the positive terminal dilution tube of the original sample and plated on M-R2A medium and were originally isolated as dominant heterotrophs.

[b] REP-PCR pattern group. Isolates in parentheses represent REP groups identified as identical to a previously observed REP profile.

[c] Results from Southern blot hybridization using the toluene ortho monooxygenase probe from Pseudomonas JS-150.

[d] FAME analysis (Sasser,M. 1990)

[e] Index of similarity to the database for FAME in the MIDI System (version 3.8).

Hydrogenophaga pseudoflava (strain MF-62), *Bacillus subtilis* (strain MF-89), *Nocardia asteroides* (strain MF-108), *Comamonas acidovorans* (strain MF-92), *Janthinobacterium lividum* (strain MF-118), *Bacillus pumilus* (strain MF-181), *Staphylococcus haemolyticus* (strain MF-212), *Pseudomonas putida* (strain MF-304), *Bacillus amyloliquefaciens* (strain MF-342), *Acidovorax delafieldii* (strain MF-413 and MF-415), *Acidovorax facilis* (strain MF-414), *Nocardia restricta* (strain MF-450), *Burkholderia pickettii* (strain MF-H-4-4) and *Variovorax paradoxus* (strains MF-39) (Table 2.1). Of the remaining 47 isolates, 30 isolates presented identification profiles with a similarity index below 0.5, 15 isolates had no match at all to the MIDI database and two isolates did not grow on TSB medium, the medium recommended for comparison to the database. MIDI specifies similarity indices of > 0.5 as having a high probability of being correct and those between 0.6 and 1.0 as being excellent matches. The low similarity indices observed for most of these isolates of environmental origin may be a reflection of the fact that the MIDI-FAME data base is primarily composed of clinical isolates. Gram stain results were in agreement with the identification provided by FAME (Table 2.3). Overall, 30% of the isolates were gram positive and 70% gram negatives (Table 2.4).

Analysis of the collection with different REP patterns revealed that 65% of the isolates can degrade toluene, 90% can degrade phenol and 57% can degrade both carbon substrates. Only 8% of all isolates could degrade toluene exclusively and 35% could degrade phenol exclusively (Table 2.4). Gram

Table 2.4. Distribution of main characteristics observed for the 63 isolates with different REP patterns from Moffett Field.

	% of Total	Growth substrates		
		Toluene only	Phenol only	Phenol and Toluene
% of Total		8	35	57
% Gram positive	30	0	53	47
% Gram negative	70	12	27	61
% TOM positive	55	4	18	78
% TCE degraders	60	5	45	50

	Gram positives		Gram negatives		Total
	Toluene[a]	Phenol[a]	Toluene[a]	Phenol[a]	
% TCE degraders	22	42	56	70	60
% TOM positive	0	0	72	70	55
% Toluene degraders	100	47	100	70	65
% Phenol degraders	100	100	88	100	90
% Phenol and toluene degraders	100	47	84	70	57

[a]Toluene or phenol used as carbon source. Strains that use both carbon sources are repeated under both columns.

positives isolates were more frequently exclusive-phenol degraders (53%) compared to gram negatives (27%). No gram positive isolate was an exclusive toluene degrader, whereas 12% of gram negatives could only degrade toluene. Of the gram positive isolates, 100% are phenol degraders and 47% are toluene degraders. For gram negative isolates that can degrade phenol, 70% can also degrade toluene and for gram negatives that can degrade toluene, 88% can also degrade phenol. Analysis of the cometabolic TCE transformation potential showed that 60% of the isolates were TCE co-oxidizers. Gram positives (42%) and gram negatives (70%) were more effective TCE co-oxidizers when phenol was the primary carbon source, where only 22% and 56%, respectively, were effective TCE cometabolizers (Table 2.4).

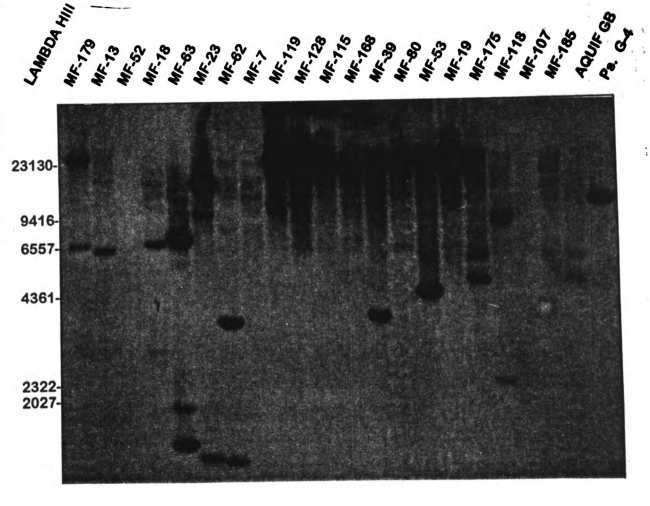
Diversity of toluene degradation genes. Chromosomal DNA isolated from isolates and five well studied aerobic toluene degraders was hybridized to various toluene pathway probes to determine which strains carried similar sequences. Probes for the first steps in all five aerobic toluene degrading pathways were used. In a preliminary search, slot blots were used to analyze for the presence of homology between DNA from each isolate and the five different probes. Under low stringency conditions the results were inconsistent because many isolates hybridized to all probes, probably as a result of the low specificity inherent to the hybridization conditions. Under high stringency conditions only the probe for the *ortho*-hydroxylase gene showed significant signals. We then

used Southern blot analysis for a more specific evaluation of hybridization. Only the probes for the ortho-hydroxylase from *Pseudomonas* sp. JS-150 genes showed hybridization under low and high stringency conditions (Figure 2.6) corroborating the results from slot blots. On the basis of the hybridization signal intensity under high-stringency conditions, different levels of sequence homology were observed for the different isolates (Figure 2.6). Fifty-five percent of the tested isolates showed homology to the gene encoding the toluene *ortho*-hydroxylase probe (Table 2.4). Only 18% and 4% of the exclusive phenol or toluene degraders, respectively, showed homology to the TOM probe, whereas 78% of toluene and phenol degraders were positive for the presence of this gene sequence (Table 2.4). Hence, these results demonstrate that DNA highly homologous to the genes encoding toluene *ortho*-hydroxylase is present in the majority of the bacterial strains tested that catabolize both phenol and toluene. Interestingly, no gram positive isolate tested showed homology to this gene.

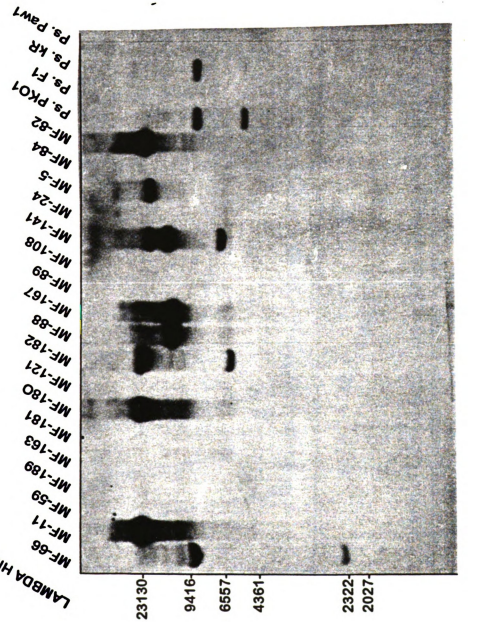
TCE co-oxidation rates. The rate of TCE degradation by five different well known aerobic toluene degraders that harbor different first step mechanisms for toluene attack is shown in Figure 2.7. TCE was metabolized at a high initial rate that decreased over time for strains F1, KR, PKO1 and G-4. Toluene was degraded at a very fast rate by strain PaW1, but no co-oxidation of TCE was observed for this organism. For strains F1, KR, PKO1 and G-4, the onset of TCE degradation was delayed until a substantial fraction of toluene had been

Figure 2.6. RFLP profiles of genomic DNA from Moffett Field isolates digested with *EcoR1* and hybridized with the gene probe encoding for the toluene ortho-hydroxylase of *Pseudomonas sp.* JS-150.

A


LAMBDA HIII
MF-179
MF-13
MF-52
MF-18
MF-63
MF-23
MF-62
MF-7
MF-119
MF-128
MF-115
MF-168
MF-39
MF-80
MF-53
MF-19
MF-175
MF-118
MF-107
MF-185
AQUIF GB
Pa. G-4
23130
9416
6557
4361
2322
2027

B


LAMBDA HIII
MF-66
MF-11
MF-59
MF-189
MF-183
MF-181
MF-180
MF-121
MF-182
MF-88
MF-167
MF-89
MF-108
MF-141
MF-24
MF-5
MF-84
MF-82
Ps. PKO1
Ps. F1
Ps. KR
Ps. Paw1
23130
9416
6557
4361
2322
2027

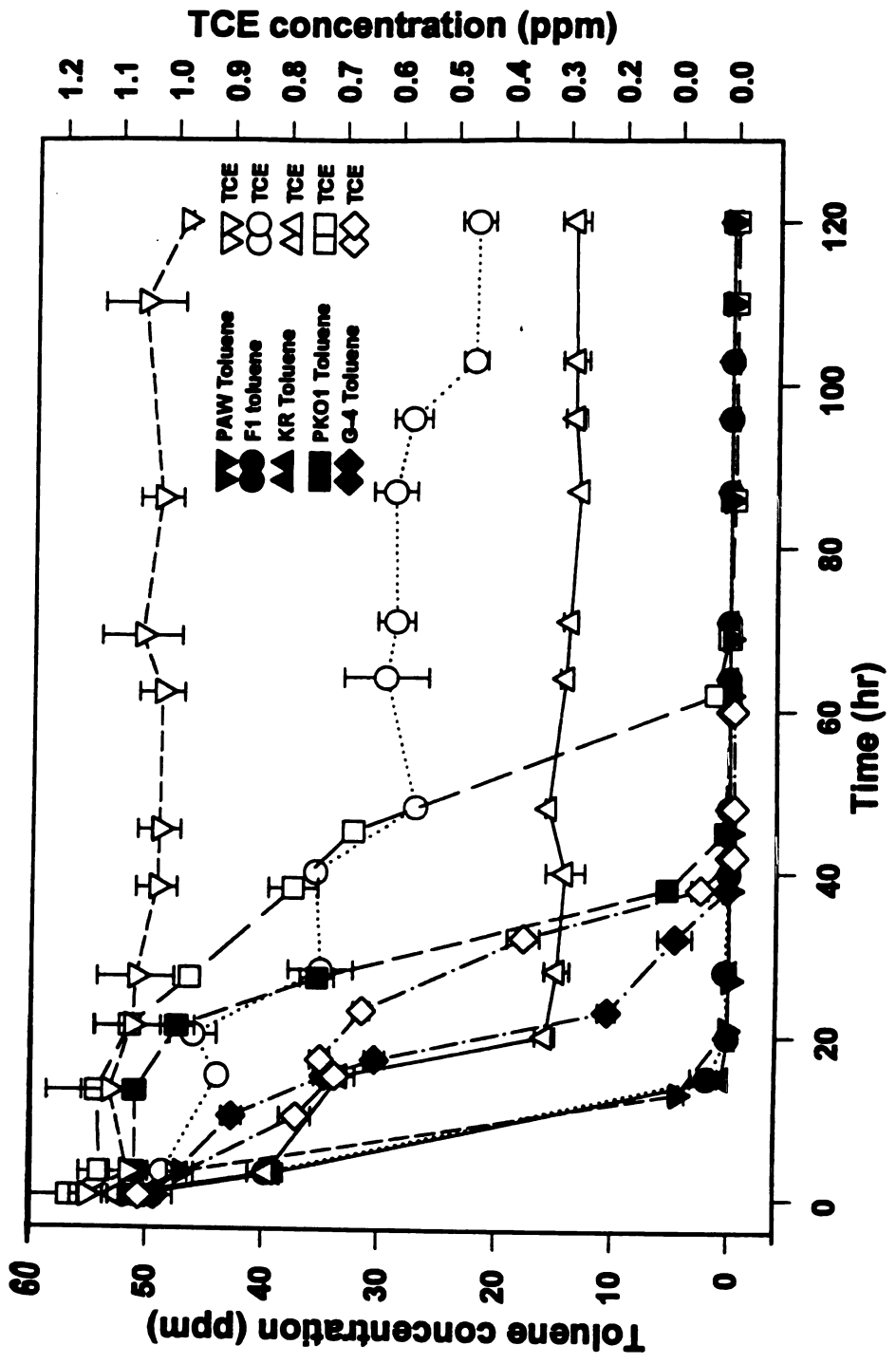


Figure 2.7. Pattern of toluene consumption (filled symbols) and TCE removal (open symbols) by different strains representing the five known aerobic toluene degrading pathways. Toluene was the primary carbon source.

metabolized, notably for strain F1. At 45 h from the beginning of the experiment toluene was consumed by all strains, but G-4 was the only strain to show extensive cometabolism of TCE (to below detection).

The isolates from Moffett Field showed variable rates of TCE co-oxidation, and for many strains TCE was cometabolized at a high initial rate that decreased over time (Figure 2.8). The extent of TCE degradation was strain dependent. At 120 h, toluene was totally consumed by all strains but less than 50% of TCE was co-oxidized by strains MF-7, MF-163 and MF-H-3-3, whereas more than 50% was co-oxidized by strains MF-23, MF-15, MF-168, MF-182, MF-183, MF-179 and MF-80. *Burkholderia cepacia* strain G-4, used as a control in this experiment, showed complete removal of TCE and had the fastest co-oxidation rate of any strain in this group. Some strains degraded toluene at rates that are not significantly different from those of *Burkholderia cepacia* G-4 (Figure 2.9.). However, extensive degradation (to below detection) of TCE was only observed with strain G-4.

The relative change in TCE concentration between inoculated and uninoculated controls measured at 15 days of incubation was used to evaluate the relative TCE transformation capacity of the entire collection. Isolates varied markedly in their ability to co-oxidize TCE. The rank ordered cometabolic transformation capacity profiles for isolates grown on phenol and toluene are illustrated in Figure 2.10. Isolates with homology to the toluene *ortho*-hydroxylase gene (TOM) were distinctive in both the proportion of isolates with

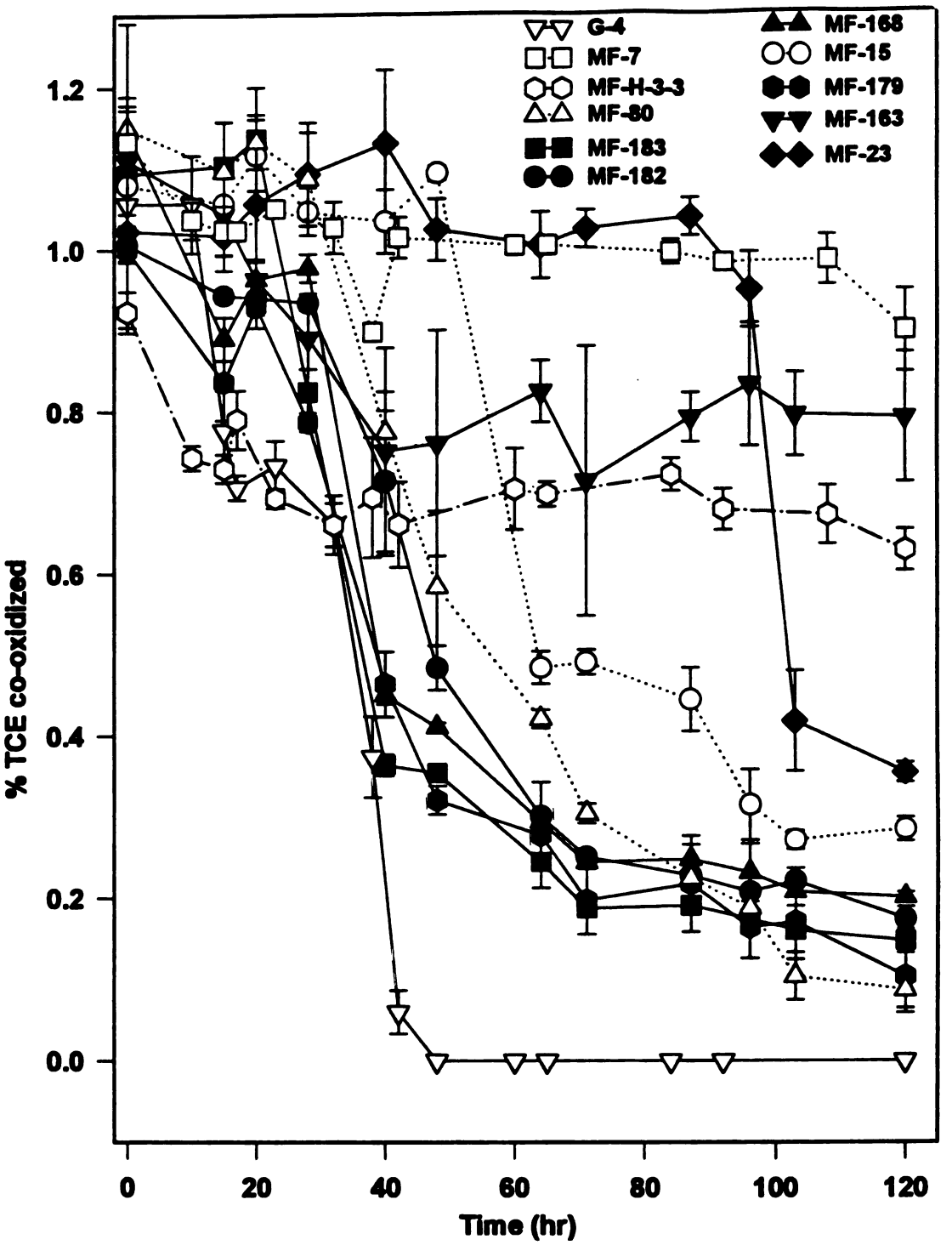


Figure 2.8. TCE co-oxidation patterns of selected isolates. Toluene was the primary carbon source and was totally consumed by all strains at 120 hours.

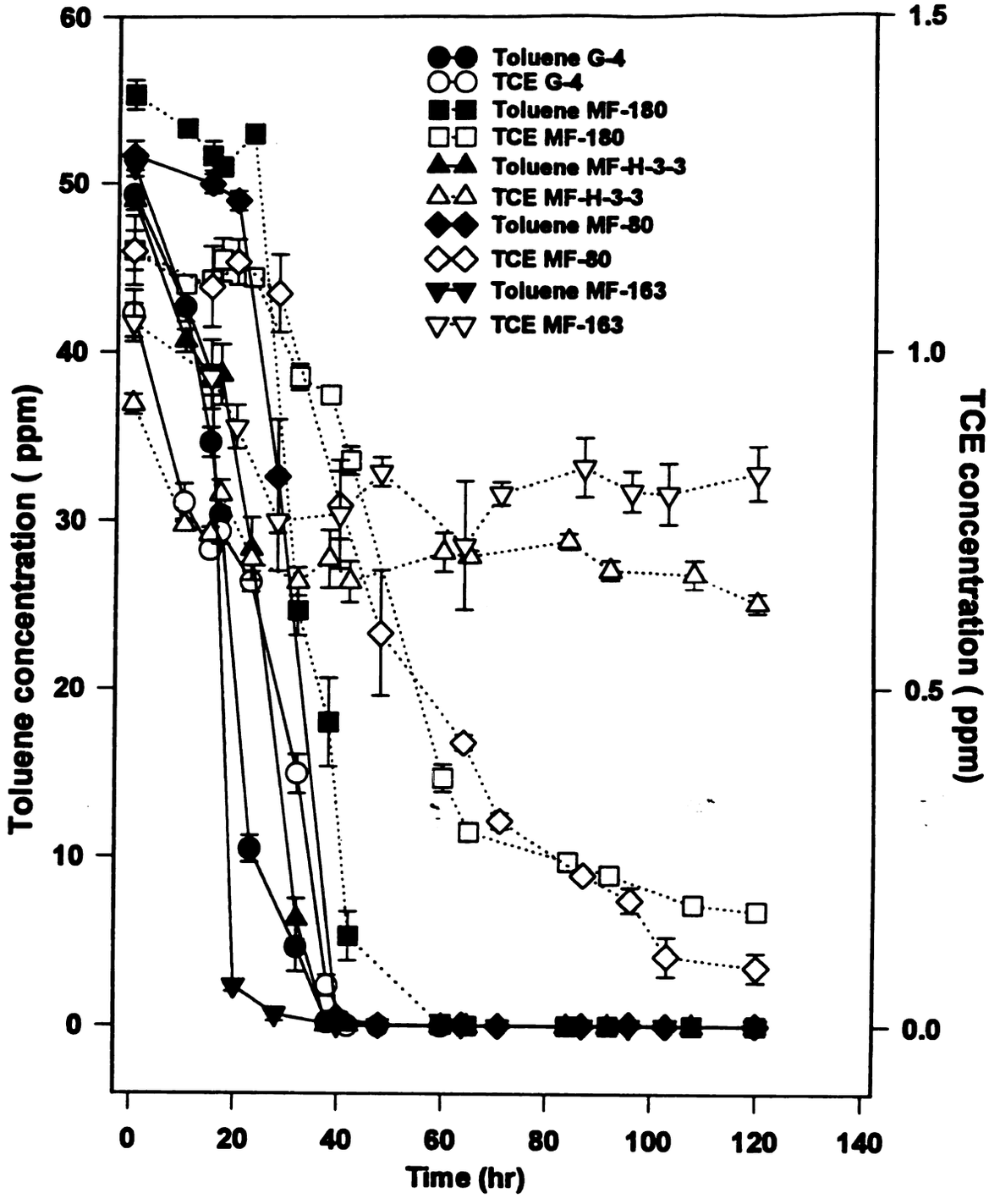


Figure 2.9. TCE co-oxidation profile of selected isolates that show fast toluene degradation rates

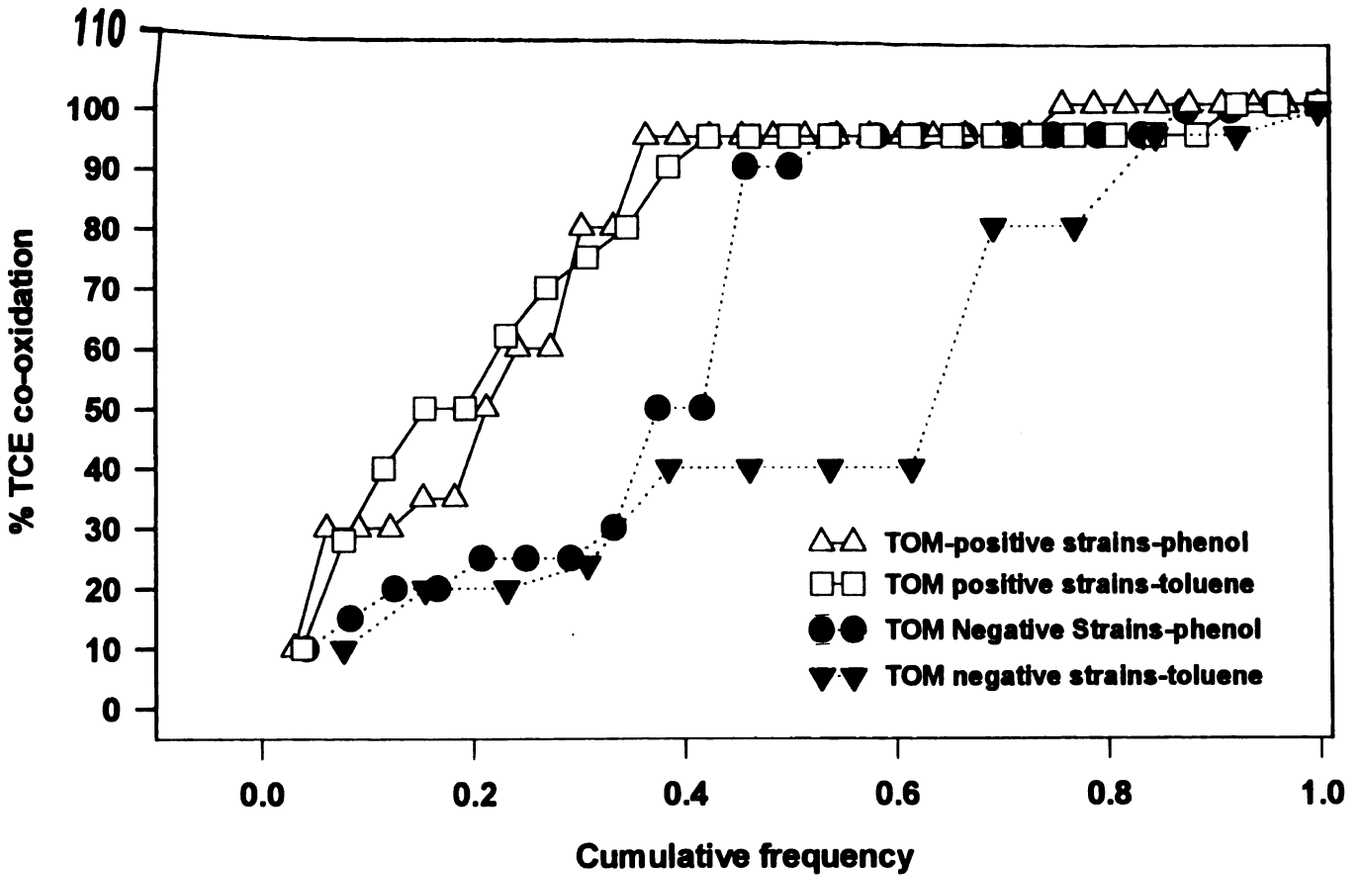


Figure 2.10. Distribution of TCE co-oxidation ability by Moffett Field isolates with or without homology to the *ortho*-hydroxylase gene (TOM) carried by *Burkholderia cepacia* strain G-4. Evaluation at 15 days of incubation.

high co-oxidation capacities (more than 60% with transformation capacities > than 90%) and the overall number of isolates obtained (55% TOM positives). There was no effect of the primary carbon source on the transformation capacity profile for TOM positive strains, but differences can be seen when TOM negative strains are analyzed. More than 50% of the TOM negative strains grown on toluene showed less than 50% co-oxidation capacity when toluene was the primary carbon source, whereas more than 50% of TOM negative strains co-oxidized TCE when grown on phenol.

Discussion

We analyzed population levels and the diversity of dominant phenol and toluene degrading bacteria isolated at different times from an aquifer that has been treated with different CAH's, phenol and toluene by using molecular and conventional microbiological techniques. Microbial population density is an important determinat of biodegradation rates (Simkins and Alexander, 1984). We used standard MPN techniques (Alexander, 1982) to quantify numbers of specific phenol and toluene degraders and respective TCE co-oxidizers. For the evaluation of the number of toluene and phenol degraders, the concentration of substrate used was critical. Our results showed that populations were probably underestimated by the previous methods used. High concentration of substrates may also select resistant populations that have little significant ecological importance since this condition is usually found in contaminated sites where

biodegradation is occurring. We have previously shown that toluene concentration was also critical for enrichments and isolation of pure cultures of toluene-degrading denitrifiers (Fries et al., 1994). Phenol and toluene degraders were found to be 9.5% and 0.7%, respectively, of total heterotrophic bacteria. Phenol has been the substrate used at this site for two previous feeding regimens which may have enriched phenol degraders in the site.

The majority of the isolates can grow on both phenol and toluene as the primary carbon source although phenol degradation was a trait found in 90% of the isolates. A larger percentage of phenol grown strains were more effective in cooxidizing TCE than toluene grown strains. This suggests that phenol may be better than toluene for field application.

Most of the dominant strains isolated from the glass beads were also isolated from the water samples suggesting that they are good surface colonizers. Very few strains were isolated from water samples exclusively. Species identification by FAME analysis recognized only 25% of the isolates at an index above 0.5, and these isolates were placed in 11 different genera. Forty-eight percent of the isolates could not be identified by FAME analysis. This is similar to what is found with other groups of environmental isolates, namely that the database inadequately reflects environmental strains.

The most surprising feature of this study was the dominance of the toluene ortho-monoxygenase gene in these aromatic degraders. Hybridization signals from Southern blots showed that the toluene ortho-hydroxylase is the dominant

toluene degrading pathway. The variation in RFLP patterns obtained, signal intensity and restriction size of the bands suggests divergence of sequences for this gene in the populations, although it may have had a common ancestral origin. The lack of hybridization of many strains to any of the five probes tested suggests unknown pathways or enzymes. Indeed, a new gene sequence for toluene degradation that is biochemically similar to the dioxygenase pathway of *Pseudomonas putida* F1 has been isolated (Gerben J. Zylstra, personal communication). Following toluene injection into the aquifer, ortho-cresol was detected as an intermediate (Hopkins and McCarty, 1995) providing additional evidence for dominance of the ortho-hydroxylase pathway among the organisms present and effective in this field site.

Although separate information on TCE degradation rates for the five well known toluene degraders that harbor different genes is available in the literature (Winter et al., 1989; Wackett and Gibson, 1988, Folsom et al, 1990), direct comparison among organisms is difficult because of significant differences among methods used. This is the first study that compared these strains under the same conditions. The efficiency of TCE cooxidation was related to the gene sequence present on the organisms. The same observations has been made for methane monooxygenase where the soluble form of that enzyme is more effective in TCE degradation (Oldenhius et al, 1989; Tsien et al., 1989; Alvarez-Cohen et al, 1992). We found some cross hybridization between the clones for the toluene ortho-hydroxylase gene and the toluene meta-hydroxylase, and the

strains harboring those sequences, *Burkholderia cepacia* G-4 and *Burkholderia pickettii* PKO1, were the most efficient TCE cooxidizers. TCE transformation capacity varied widely between the Moffett Field strains and ranged from approximately 1 to 100%. The diversity in the transformation capacity profile also indicates that a single population was not dominant at this site. A possible explanation for this fact is that populations may respond in different ways to the interactions between the natural substrate and other chemicals serving as substrates for the oxygenases systems. This was also shown by Folsom et al. (1990) in experiments with equal concentrations of phenol and TCE. A decrease of about 50% in the rate of phenol degradation was observed, suggesting a competitive mechanism, and possibly toxic effects, between the natural substrate and the fortuitous substrate, TCE.

In the present study we have shown the diversity of multiple phenol and toluene degrading strains from glass bead biofilms and planktonic communities. The majority of these isolates harbor pathways similar to the toluene ortho-hydroxylase gene, but the lack of signal to all known genes for more than half of the isolates suggests that more diversity exist at the gene sequence level of toluene oxidation than what is currently known.

Acknowledgments

We thank Helen Corlew-Neuman for conducting the FAME analysis. This work was supported by EPA , Grant N° CR 822029-01-0 and the NSF Center for Microbial Ecology, Grant N° BIR 9120006.

List of References

Alexander, M. 1982. Most probable number method for microbial populations, p. 815-820. *In* A. L. Page (ed.), Methods of soil analysis. Part 2. Chemical and microbiological properties. American Society of agronomy, Madison, Wi, USA.

Alvarez-Cohen, L. , P. L. McCarty, E. Boulygina, R. S. Hanson, G. A. Brusseau, and H. C. Tsien. 1992. Characterization of a methane urilizing bacterium from a bacterial consortium that rapidly degrades trichloroethylene and chloroform. Appl. Environ. Microbiol. **58**:1886-1893.

Alvarez-Cohen, L. and P. L. McCarty. 1991a. Product toxicity and cometabolic competitive inhibition modeling of chloroform and trichloroethylene by methanotrophic resting cells. Appl. Environ. Microbiol. **57**:1031-1037.

Alvarez-Cohen, L. and P. L. McCarty. 1991b. Effects of toxicity, aeration, and reductant supply on trichloroethylene transformations by a mixed methanotrophic culture. Appl. Environ. Microbiol. **57**:228-235.

Arciero, D. T. T. Vanelli, M.Logan, and A. B. Hooper. 1989. Degradation of trichloroethylene by the ammonia-oxidizing bacterium *Nitrosomonas europaea*. Biochem. Biophys. Res. Commun. **159**:640-643.

Dabrock, B., J. Riedel, J. Bertram, and G. Gottschalk. 1992. Isopropylbenzene (cumene) a new substrate for the isolation of trichloroethylene-degrading bacteria. Arch. Microbiol. **158**:9-13.

de Bruijn, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bateria. Appl. Environ. Microbiol. **58**:2180-2187.

Elmerich, C. ,B. L. Dreyfus, and J. P. Aubert. 1982. Genetic analyses of nitrogen fixation in a tropical fast-growing *Rhizobium.* EMBO J. 1:499-503.

Ensingn, S. A., M. R. Hyman, and D. J. Harp. 1992. Cometabolic degradation of chlorinated alkenes by alkene monooxygenase in a propylene-grown Xantobacter strain. Appl. Environ. Microbiol. 58:3038-3046.

Ewers, J., D. Freier-Schroder, and H. J. Knackmuss. 1990. Selection of trichloroethene(TCE) degrading bacteria that resist inactivation by TCE. Arch. Microbiol. 154:410-413.

Fogel, M. M., A. Taddeo, and S. Fogel. 1986.Biodegradation of chlorinated ethenes by a methane-utilizing mixed culture. Appl. Environ. Microbiol. 51:720-724.

Folsom, B. R., P. J. Chapman and P. H. Pritchard. 1990. Phenol and trichloroethylene degradation by Pseudomonas cepacia G4: kinetics and interactions between substrates. Appl. Environ. Microbiol. 56:1279-1285.

Fries, M. R., J. Zhou, J. Chee-Sanford, and J. M. Tiedje. 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. 60:2802-2810.

Harayama, S., M. Rekik, M. Wubbolts, K. Rose, R. A. Leppik, and K. N. Timmis. Characterization of five genes in the upper-pathway operon of tol plasmid pWWO from *Pseudomonas putida* and identification of the gene products. J. Bacteriol. 171:5048-5055.

Harker, A. R. and Y. Kim. 1990. Trichloroethylene degradation by two independent aromatic independent pathways in *Alcaligenes eutrophus* JMP134. Appl. Environ. Microbiol. 56:1179-1181.

Henry, S. H. and D. Grbic-Galic. 1991. Influence of endogenous and exogenous electron donors and trichloroethylene oxidation toxicity on trichloroethylene oxidation by methanotrophic cultures from a groundwater aquifer. Appl. Environ. Microbiol. 57:236-244.

Henry, S. H. and D. Grbic-Galic. 1990. Effect of mineral media on trichloroethylene oxidation by aquifer metanotrophs. Microb. Ecol. 20: 151-169.

Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. 54:703-711.

Hopkins, G. D., L. Semprini, and P. L. McCarty. 1993. Microcosm and *In situ* field studies of enhanced biotransformation of trichloroethylene by phenol-using microorganisms. Appl. Environ. Microbiol. **59**:2277-2285.

Hopkins, G. D., and P. L. McCarty. 1995. Field evaluation of In-Situ aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates. Environ. Sci. Technol. (submitted for publication)

Johnson, G. R., and R. H. Olsen. 1993. Characterization of clones from *Pseudomonas sp.* JS150 encoding substituted phenol and benzene metabolic pathways analogous to *Pseudomonas* strains PKO1 and G4, abstr. K-82, p.274. Abstr. 93th Annu. Meet. Am. Soc. Microbiol. 1993.

Krieg, N. R. 1981. Enrichment and isolation, p. 112-142. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips. (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.

Little, C. D., A. V. Palumbo, S. E. Herbes, M. E. Lidstrom, R.L. Tyndall, and P. J. Gilmer. 1988. Trichloroethylene biodegradation by a methane-oxidizing bacterium. Appl. Environ. Microbiol. **54**:951-956.

Malaichowsky, K. J., T. J. Phelps, A. B. Teboli, D. E. Minnikin and D. C. White. 1994. Aerobic mineralization of trichloroethylene, vinyl chloride, and aromatic compounds by *Rhodococcus* species. Appl. Environ. Microbiol. **60**:542-548.

Maniatis, T., E. F. Fritsch, and J. Sambrook. 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

Nelson, M. J. K.,S. O. Montgomery, and P. H. Pritchard. 1988. Trichloroethyleene metabolism by microorganisms that degrade aromatic compounds. Appl. Environ. Microbiol. **54**:604-606.

Nelson, M. J. K.,S. O. Montgomery, E. J. O'Neil and P. H. Pritchard. 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53**:949-954.

Nelson, M. J. K.,S. O. Montgomery, E. J. O'Neil and P. H. Pritchard. 1986. Aerobic metabolism of trichloroethylene by a bacterial isolate. Appl. Environ. Microbiol. **52**:383-384.

Oldenhuis, R., R. L. J. M. Vink, D. B. Jansen, and B. Witholt. 1989. Degradation of chlorinated aliphatic hydrocarbons by Methylosynus

trichosporium OB3b expressing soluble methane monooxygenase. Appl. Environ. Microbiol. 55:2819-2826.

Olsen R. H., J. J. Kukor , and B. Kaphammer. 1994. A novel toluene-3-monooxygenase from Pseudomonas pickettii PKO1. J. Bacteriol. 176:3749-3756.

Owens, J. D., and R. M. Keddie. 1969. The nitrogen nutrition of soil and herbage coryneform bacteria. J. Appl. Bacteriol. 32:388-347.

Rasche, M. E., M. R. Hayman, and D. J. Harper. 1991. Factors limiting aliphatic chlorocarbon degradation by nitrossomonas europaea: cometabolic inactivation of ammonia monooxygenase and substrate specificity. Appl. Environ. Microbiol. 57:2986-2994.

Roberts, P. V., G. D. Hopkins, D. M. Mackay and L. Semprini. 1990. A field evaluation of in-situ biodegradation of chlorinated ethenes: Part 1, Methodology and field site characterization. Ground water 28:591-604.

Sasser, M. 1990. Technical Note#102: Tracking a strain using the Microbial Identification System. MIS Inc., North Newark, Del.

Shields, M. S. (1994) Personal communication.

Simkins, S., and M. Alexander. 1984. Models for mineralization kinetics with the variables of substarte concentration and population density. Appl. Environ. Microbiol. 47:1299-1306.

Smibert, R. M., and N. R. Krieg. 1981. General characterization, p.409-443. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips. (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.

Tsien, H. C., G. A. Brusseau, R. S. Hanson and L. P. Wackett. 1989. Biodegradation of trichloroethylene by methylosinus OB3b. Appl. Environ. Microbiol. 55:3155-3161.

Vogel, T. M. and P. McCarty. 1985. Biodegradation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride , and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. 49:1080-1083.

Wackett, L.P., and S. R. Householder. 1989. Toxicity of trichloroethylene to *Pseudomonas putida* F1 mediated by toluene dioxygenase. Appl. Environ. Microbiol.55:2723-2725.

Wackett, L.P., D. T. Gibson. 1988. Degradation of trichloroethylene by toluene dioxygenase in whole cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol.**54**:1703-1708.

Wilson, J. T. and B. H. Wilson. 1985. Biotransformations of trichloroethylene in soil. Appl. Environ. Microbiol. **37**:242-243.

Winter, R. B., K. Yen and B. D. Ensley. 1989. Efficient degradation of trichloroethylene by a recombinant Escherichia coli. Bio/Technology **7**:282-285.

Yen, K.-M, M. R. Karl, L. M. Blatt, M. J. Simon, R. B. Winter, P. R. Fausset, H. S. Lu, A. A. Harcourt, and K. K. Chen. 1991. Cloning and characterization of a *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. J. Bacteriol. **173**:5315-5327.

Zylstra, G. J. 1995. Personal communication

Zylstra, G. J. and D. T. Gibson. 1989. Toluene degradation by *Pseudomonas putida* F1: Nucleotide sequence of the *todC1C2BADE* genes and their expression in *Escherichia coli.* J. Biol. Chem. **264**:14940-14946.

Appendix A

Table A.1. Total number of heterotrophs, toluene, phenol and TCE degraders recovered using different concentrations of substrates, and present on three different samples from the Moffett Field aquifer.

Sample	Concentration (μg/ml)				MPN (cells/ml of slurry)			
					Primary substrate		TCE co-oxidizers	
	Tol.	Phe.	TCE	M-R2A	cell n°	95% CI[a]	cell n°	95% CI
Glass beads	50	-	-	-	$7.0x10^3$	$2.1x10^3$, $2.3x10^4$		
	50	-	1	-	$1.1X10^4$	$3.3X10^3$, $3.6X10^4$	$7.0x10^3$	$2.1x10^3$, $2.3x10^4$
	5	-	-	-	$4.9x10^5$	$1.5x10^5$, $1.6x10^6$		
	5	-	0.5	-	$3.5x10^5$	$1.1x10^6$, $1.2x10^6$		
	-	-	-	3000	$4.3x10^7$	$1.3x10^7$, $1.4x10^8$		
	-	50	-	-	$2.4x10^5$	$7.3x10^4$, $7.9x10^5$		
	-	50	1	-	$1.3x10^5$	$3.9x10^4$, $4.3x10^5$	$7.9x10^4$	$2.4x10^4$, $2.6x10^5$
	-	5	-	-	$5.4x10^6$	$1.6x10^6$, $1.8x10^7$		
	-	5	0.5	-	$7.9x10^5$	$2.4x10^5$, $2.6x10^6$		
0.22μm filter	50	-	-	-	$3.3x10^3$	$1.0x10^3$, $1.1x10^4$		
	50	-	1	-	$7.9x10^3$	$2.4x10^3$, $2.6x10^4$	$7.9x10^3$	$2.4x10^3$, $2.6x10^4$
	5	-	-	-	$2.4x10^5$	$7.3x10^4$, $7.9x10^5$		
	5	-	0.5	-	$2.4x10^5$	$7.3x10^4$, $7.9x10^5$		
	-	-	-	3000	$2.4x10^8$	$7.3x10^7$, $7.9x10^8$		
	-	50	-	-	$3.5x10^5$	$1.1x10^5$, $1.2x10^6$		
	-	50	1	-	$4.9x10^5$	$1.5x10^5$, $1.6x10^6$	$5.4x10^5$	$1.6x10^5$, $1.8x10^6$
	-	5	-	-	$5.4x10^6$	$1.6x10^6$, $1.8x10^7$		
	-	5	0.5	-	$3.5x10^6$	$1.1x10^6$, $1.2x10^7$		
Cotton filter	50	-	-	-	$3.3x10^4$	$1.0x10^4$, $1.1x10^5$		
	50	-	1	-	$3.3x10^4$	$1.0x10^4$, $1.1x10^5$	$2.4x10^4$	$7.3x10^3$, $7.9x10^4$
	5	-	-	-	$3.5x10^5$	$1.1x10^5$, $1.2x10^6$		
	5	-	0.5	-	$1.3x10^6$	$3.9x10^5$, $4.3x10^6$		
	-	-	-	3000	$2.4x10^8$	$7.3x10^7$, $7.9x10^8$		
	-	50	-	-	$3.3x10^7$	$1.0x10^7$, $1.1x10^8$		
	-	50	1	-	$2.4x10^6$	$7.3x10^5$, $7.9x10^6$	$3.5x10^5$	$1.1x10^5$, $1.2x10^6$
	-	5	-	-	$2.4x10^6$	$7.3x10^5$, $7.9x10^6$		
	-	5	0.5	-	$2.4x10^6$	$7.3x10^5$, $7.9x10^6$		

Table A.1. (continued)

S-2	25		-		0.0	0.0	0.0	0.0
	25		0.5		0.0	0.0	0.0	0.0
		25	-		0.0	0.0	0.0	0.0
		25	0.5		0.0	0.0	0.0	0.0
				3000	$1.3 10^{3}$	$3.9x10^{2}$, $4.3x10^{4}$		
S-3	25		-		$2.7x10^{3}$	$0.8x10^{3}$, $8.9x10^{3}$		
	25		0.5		$1.1x10^{4}$	$0.3x10^{4}$, $3.6x10^{4}$	$4.0x10^{2}$	$1.1x10^{2}$, $1.3x10^{3}$
		25	-		$4.9x10^{5}$	$1.5x10^{5}$, $1.6x10^{6}$		
		25	0.5		$4.9x10^{5}$	$1.5x10^{5}$, $1.6x10^{6}$	$1.1x10^{4}$	$0.3x10^{4}$, $3.6x10^{4}$
				3000	$2.4x10^{8}$	$7.3x10^{7}$, $7.9x10^{8}$		
S-4	25		-		$7.0x10^{3}$	$2.1x10^{3}$, $2.3x10^{4}$		
	25		0.5		$3.5x10^{4}$	$1.1x10^{4}$, $1.2x10^{5}$	$2.9x10^{3}$	$0.9x10^{3}$, $9.6x10^{3}$
		25	-		$3.5x10^{4}$	$1.1x10^{4}$, $1.2x10^{5}$		
		25	0.5		$3.5x10^{4}$	$1.1x10^{4}$, $1.2x10^{5}$	$3.5x10^{4}$	$1.1x10^{4}$, $1.2x10^{5}$
				3000	$2.4x10^{8}$	$7.3x10^{7}$, $7.9x10^{8}$		

[a] CI, confidence interval

Appendix B

Table B.1. Original data on all isolates from Moffett Field. Isolates are arranged according to common REP-PCR groups, source of isolate, and use of toluene and phenol are also shown.

Isolate[a]	REP[b]	Source of isolate[a]	Toluene[c]	Phenol[c]
MF-5	1	E-F50T 10-4 a(10-5)	100	100
			100	100
169		E-C50T 10-4a(10-5)	100	100
MF-7	2	E-F50T 10-4b(10-5)	100	0.0
			100	0.0
12		E-C50T 10-4a(10-5)	100	0.0
9		E-C50T 10-4a(10-5)	100	0.0
MF-11	3	E-F50T 10-4a(10-6)	0.0	100
			100*	100
2		E-GB50T 10-5a(10-5)	100	100
3		E-GB50T 10-5a(10-6)	100	100
4		E-GB50T 10-5a(10-5)	100	100
MF-13	4	E-C50T 10-4a(10-6)	100	100
			100	100
MF-15	5	E-C50T 10-4c(10-7)	100	100
			100	100
20		E-C50T 10-4a(10-6)	100	100
21		E-C50T 10-4a(10-8)	100	100
6		E-F50T 10-4a(10-7)	100	100
MF-18	6	E-GB50T 10-5a(10-6)	80	100
			99	100
178		E-GB50TT 10-5a(10-5)	80	100
103		E-C50T 10-4a(10-6)	70	100
55		E-GB50TT 10-5a(10-6)	80	100
MF-19	7	E-F50T 10-4c(10-6)	100	100
			100	100
14		E-C50T 10-4a(10-6)	100	100
10		E-F50T 10-4a(10-6)	100	100
176		E-C50T 10-4a(10-6)	100	100
177		E-GB50TT 10-5a(10-6)	100	100
1		E-GB50TT 10-5a(10-6)	100	100
25		E-F50T 10-4a(10-6)	100	100
184		E-F50T 10-4a(10-6)	100	100
45		E-GB50TT 10-5a(10-6)	100	100
49		E-C50T 10-4a(10-6)	100	100
50		E-GB50TT 10-5a(10-6)	100	100
172		E-C50T 10-4a(10-6)	100	100

Table B.1. (continued)

MF-23	8	E-GB50P 10-5a(10-6)	0.0	100
			0.0	100
27		E-GB50P 10-5a(10-7)	0.0	100
28		E-F50P 10-5a(10-6)	0.0	100
29		E-F50P 10-5a(10-6)	0.0	100
30		E-F50P 10-5a(10-6)	0.0	100
31		E-F50P 10-5a(10-6)	0.0	100
32		E-C50P 10-7a(10-6)	0.0	100
33		E-C50P 10-7a(10-6)	0.0	100
34		E-C50P 10-7a(10-7)MD	0.0	100
MF-24	9	E-GB50P 10-5a(10-4)	0.0	0.0
			0.0	100 *
38		E-GB50P 10-5b(10-5)	0.0	100
MF-39	10	E-F50PT 10-6b(10-6)	50	100
			40	100
41		E-F50PT 10-6b(10-6)	30	100
MF-52	11	E-GB50T 10-5a(10-4)	0.0	100
			40	100
MF-53	12	E-GB50T 10-5a(10-4)	100	100
			100	100
MF-54	13	E-F50T 10-4a(10-5)	100	100
			100	100
MF-58	14	E-GB50P 10-5b(10-7)	100	100
			100	100
60		E-C50P 10-7a(10-6)	100	100
61		E-C50P 10-7a(10-6)	100	100
123		E-C50TT 10-5a(10-6)	100	100
MF-59	15	E-GB50P 10-5a(10-5)	100	100
			100	100
MF-62	16	E-C50P 10-7a(10-5)	100	100
			100	100
MF-63	17	E-GB50PT 10-5b(10-5)	100	100
			100	100
64		E-GB50PT 10-5b(10-4)	100	100
65		E-GB50PT 10-5b(10-5)	100	100
37		E-GB50PT 10-5b(10-6)	100	100
42		E-C50PT 10-6a(10-6) MD	100	100
43		E-C50PT 10-6a(10-6)MD	100	100
44		E-F50PT 10-6b(10-6)	100	100
35		E-GB50PT 10-5b(10-5)	100	100
36		E-GB50PT 10-5b(10-5)	100	100
69		E-F50PT 10-6b(10-6)	100	100
70		E-C50PT 10-6a(10-6)	100	100
71		E-C50PT 10-6a(10-6)	100	100
72		E-C50PT 10-6a(10-6)	100	100
73		E-C50PT 10-6a(10-6)	100	100
74		E-C50PT 10-6a(10-6)	100	100
75		E-C50PT 10-6a(10-6)	100	100
76		E-C50PT 10-6a(10-6)	100	100
77		E-F50PT 10-6b(10-6)	100	100
78		E-F50PT 10-6b(10-6)	100	100
79		E-F50PT 10-6b(10-6)	100	100

Table B.1. (continued)

MF-66	18	E-F50PT 10-6b(10-6)	40	100
			10	100
40		E-F50PT 10-6b(10-6)	20	100
MF-80	19	E-F50T 10-4a(10-6)	100	100
			100	100
101		E-F50PT 10-6a(10-6)	100	100
MF-82	(3)	E-C50T 10-4a(10-5)	100	100
			0.0	100
85		E-F50T 10-4a(10-6)	80	100
MF-84	20	E-GB50T 10-5a(10-5)	100	100
			100	100
MF-84w	21	E-GB50T 10-5a(10-5)	100	100
			100	100
MF-88	22	E-F50T 10-4a(10-6)	100	100
			100	100
167		E-F50T 10-4a(10-5)	100	100
MF-89	23	E-F50T 10-4a(10-5)	0.0	100
			0.0	100
MF-91	24	E-GB50TT 10-5a(10-5)	0.0	100
			0.0	100
117		E-F50TT 10-4a(10-4)	0.0	100
130		E-F50P 10-6a(10-5)	0.0	100
MF-92	25	E-GB50T 10-5a(10-5)	100	100
			100	100
90		E-C50P 10-7a(10-5)	100	100
MF-107	26	E-GB50TT 10-5a(10-5)	100	100
			100	100
112		E-GB50TT 10-5a(10-6)	100	100
MF-108	27	E-GB50TT 10-5a(10-5)	0.0	100
			0.0	100
109		E-GB50T10-5a(10-4)	0.0	100
110		E-GB50TT 10-5a(10-4)	0.0	100
MF-115	(3)	E-GB50TT 10-6a(10-5)	0.0	100
			0.0	100
116		E-GB50TT 10-5a(10-6)	0.0	100
105		E-C50T 10-4a(10-6)	0.0	100
140		E-C50PT 10-6a(10-6)	0.0	100
51		E-GB50T 10-5a(10-6)	0.0	100
150		E-C50PT 10-6a(10-6)	0.0	100
151		E-C50PT 10-6a(10-6)	0.0	100
152		E-C50PT 10-6a(10-6)	0.0	100
153		E-C50PT 10-6a(10-6)	0.0	100
154		E-C50PT 10-6a(10-6)	0.0	100
155		E-C50PT 10-6a(10-6)	0.0	100
156		E-C50PT 10-6a(10-6)	0.0	100
157		E-C50PT 10-6a(10-6)	0.0	100
158		E-C50PT 10-6a(10-6)	0.0	100
159		E-C50PT 10-6a(10-6)	0.0	100
MF-118	28	E-F50TT 10-4a(10-5)	100	65
			100	30
MF-119	29	E-F50TT 10-4a(10-5)	0.0	100
			0.0	100

Table B.1. (continued)

MF-121	30	E-C50TT 10-5b(10-6)	100	100
			100	100
120		E-C50TT 10-5b(10-6)	100	100
MF-128	31	E-GB50P 10-5a(10-6)	100	100
			100	100
126		E-GB50P 10-5a(10-6)	100	100
122		E-GB50P 10-5a(10-5)	100	100
124		E-GB50P 10-5a(10-5)	100	100
125		E-GB50P 10-5a(10-6)	100	100
MF-141	32	E-C50PT 10-6a(10-7)	100	100
			100	100
137		E-C50PT 10-6a(10-6)	100	100
139		E-C50PT 10-6a(10-5)	100	100
MF-163	33	E-GB50T 10-5a(10-5)	100	100
			100	100
160		E-GB50T 10-5a(10-4)	100	100
MF-168	34	E-F50T 10-4a(10-5)	100	100
			100	100
170		E-C50T 10-4a(10-6)	100	100
171		E-C50T 10-4a(10-5)	100	100
173		E-C50T 10-4a(10-5)	100	100
174		E-C50T 10-4a(10-6)	100	100
MF-175r	35	E-C50T 10-4a(10-7)	100	100
			100	100
127		E-GB50P 10-5a(10-6)	100	100
129		E-GB50P 10-5a(10-6)	100	100
131		E-F50P 10-6a(10-5)	100	100
132		E-F50P 10-6a(10-6)	100	100
133		E-F50P 10-6a(10-4)	100	100
134		E-F50PT 10-6a(10-6)	100	100
138		E-F50PT 10-6a(10-6)	100	100
96		E-GB50PT 10-5a(10-5)	100	100
97		E-GB50PT 10-5a(10-5)	100	100
102		E-F50PT 10-6a(10-6)	100	100
56		E-GB50PT 10-5a(10-5)	100	100
47		E-GB50PT 10-5a(10-5)	100	100
142		E-GB50PT 10-5a(10-6)	100	100
143		E-GB50PT 10-5a(10-7)	100	100
144		E-GB50PT 10-5a(10-5)	100	100
145		E-GB50PT 10-5a(10-6)	100	100
146		E-GB50PT 10-5a(10-4)	100	100
147		E-GB50PT 10-5a(10-7)	100	100
148		E-GB50PT 10-5a(10-5)	100	100
149		E-GB50PT 10-5a(10-6)	100	100
MF-179	36	E-GB50TT 10-5a(10-6)	100	100
			100	100
MF-180	37	E-GB50TT 10-5a(10-6)	100	100
			100	100
MF-181	38	E-GB50TT 10-5a(10-6)	20	100
			10	100
MF-182	39	E-GB50TT 10-5a(10-6)	100	100
			100	100
94		E-F50PT 10-6a(10-6)	100	100
95		E-F50P 10-6a(10-5)	100	100

Table B.1. (continued)

MF-183	(37)	E-GB50TT 10-5a(10-6)	100	100
			100	100
MF-185	40	E-C50TT 10-5a(10-7)	0.0	100
			0.0	100
188		E-C50TT 10-5a(10-6)	0.0	100
190		E-C50TT 10-5a(10-6)	0.0	100
191		E-C50TT 10-5a(10-7)	0.0	100
165		E-F50P 10-6a(10-5)	0.0	100
166		E-F50P 10-6a(10-6)	0.0	100
MF-189	41	E-GB50T 10-5a(10-4)	100	100
			100	100
No growth on toluene or phenol				
16,17,161,93,162		E-GB50T 10-5a	0.0	0.0
111,114,113		E-GB50TT 10-5a	0.0	0.0
57,26,		E-GB50P 10-5a	0.0	0.0
67,98,100		E-GB50PT 10-5a	0.0	0.0
8,87,86,164		E-F50T 10-4a	0.0	0.0
46		E-F50TT 10-4a	0.0	0.0
68,99		E-F50PT 10-6a	0.0	0.0
22,81,83,104,106		E-C50T 10-4a	0.0	0.0
48		E-C50TT 10-5a	0.0	0.0
186,187		E-C50P 10-7a	0.0	0.0
136,135,192		E-C50PT 10-6a	0.0	0.0
MF-H-2-1	42	D-10-2c	0.0	100
			0.0	100
2		D-10-2c	0.0	100
3		D-10-2a	0.0	100
MF-H-2-4	43	D-10-2c	0.0	100*
			0.0	100
6		D-10-2a	0.0	100
7		D-10-2a	0.0	100
8		D-10-2c	0.0	100
10		D-10-2a	0.0	100
11		D-10-2c	0.0	100
MF-H-2-9	44	D-10-2c	0.0	100
			0.0	100
24		D-10-2c	0.0	100
MF-H-2-12	45	D-10-2a	0.0	100
			0.0	100
18		D-10-2a	0.0	100
19		D-10-2a	0.0	100
20		D-10-2a	0.0	100
MF-H-2-13	46	D-10-2a	0.0	100
			0.0	100
16		D-10-2a	0.0	100
17		D-10-2a	0.0	100
26		D-10-2a	0.0	100
No growth on toluene or phenol				
14,15,21		D-10-2a	0.0	0.0
22,23,25,5		D-10-3a	0.0	0.0

Table B.1. (continued)

MF-301	(7)	E-T-10-4b (10-6)MD	100	100
			100	100
302		E-T-10-4b (10-6)	100	100
303		E-T-10-4b (10-6)	100	100
305		E-TT-10-5b (10-6)	100	100
306		E-TT-10-5b (10-6)	100	100
316		E-P-10-5b (10-5)	100	100
318		E-PT-10-5a (10-5)	100	100
321		E-PT-10-5a (10-5)	100	100
322		E-PT-10-5a (10-5)	100	100
323		E-PT-10-5a (10-5)	100	100
324		E-PT-10-5a (10-4)	100	100
332		E-PT-10-5a (10-5)	100	100
333		E-PT-10-5a (10-5)	100	100
338		E-PT-10-5a (10-5)	100	100
339		E-PT-10-5c (10-5)	100	100
343		E-PT-10-5c (10-5)	100	100
MF-304	47	E-TT-10-5a(10-5)	0.0	0.0
			0.0	100
317		E-TT-10-5a(10-4)	0.0	100
MF-340	48	E-P-10-5a(10-5)	100	0.0
			100	0.0
341		E-P-10-5a(10-5)	100	0.0
MF-342	49	E-P-10-5a(10-6)	0.0	0.0
			100*	100*
No growth on toluene or phenol				
307,308,327		E-T-10-5a	0.0	
309,310,311,319,320,334		E-TT-10-5a	0.0	
312,313,314,315,325,326		E-P-10-5a	0.0	
328,329,330,331,334,335,336,337		E-PT-10-5a	0.0	
MF-H-3-3 (7,8,9)	(33)	D-10-7a MD	100	100
			100	100
Mf-H-3-5	(7)	D-10-5a	100	100
			100	100
No growth on toluene or phenol				
MF-H-3-1		D-10-6a		
MF-H-3-2		D-10-5a		
MF-H-3-4		D-10-6a		
MF-H-3-6		D-10-5a		
MF-401	(7)	E-T-10-5a(10-6) MD	100	100
			100	100
402		E-T-10-5a(10-6) MD	100	100
403		E-P-10-5a(10-5)	100	100
404		E-P-10-5a(10-5)	100	100
406		E-T-10-5a(10-5)	100	100
407		E-T-10-5a(10-5)	100	100
408		E-TT-10-5a(10-5)	100	100
409		E-TT-10-5a(10-5)	100	100
423		E-TT-10-5b(10-5)	100	100
424		E-TT-10-5b(10-5)	100	100
425		E-TT-10-5b(10-5)	100	100

Table B.1. (continued)

MF-405	50	E-P-10-5a(10-5)	0.0	100
			0.0	100
443		E-P-10-5b(10-5)	0.0	100
447		E-P-10-5b(10-5)	0.0	100
MF-411	51	E-T-10-5a(10-5)	0.0	97
			0.0	100
410		E-T-10-5a(10-5)	0.0	100
MF-412	(7)	E-T-10-5a(10-6)	100	100
			100	100
426		E-T-10-5b(10-5)	100	100
MF-413	52	E-TT-10-5a(10-7)MD	100	100
			100	100
427		E-TT-10-5a(10-7)	100	100
428		E-TT-10-5a(10-7)	100	100
429		E-TT-10-5a(10-7)	100	100
MF-414	53	E-TT-10-5(10-5)	100	100
			100	100
MF-415	54	E-TT-10-5a(10-6) MD	40	100
			100	100
430		E-T-10-5a(10-6)	100	100
431		E-T-10-5a(10-6)	100	100
432		E-PT-10-5a(10-6)	100	100
435		E-PT-10-5a(10-6)	100	100
446		E-P-10-5a(10-5)	100	100
MF-416	(24)	E-P-10-5a(10-5)	0.0	0.0
			0.0	100
455		E-P-10-5a(10-5)	0.0	100
MF-419	55	E-P-10-5a(10-5)	0.0	0.0
			100	0.0
417		E-T-10-5a(10-4)	100	0.0
436		E-TT-10-5a(10-5)	100	0.0
437		E-PT-10-5a(10-4)	100	0.0
438		E-P-10-5a(10-5)	100	0.0
456		E-P-10-5a(10-4)	100	0.0
MF-421	(35)	E-T-10-5a(10-5)	100	100
			100	100
418		E-PT-10-5a(10-4)	100	100
422		E-PT-10-5a(10-5)	100	100
433		E-TT-10-5a(10-5)	100	100
419		E-T-10-5a(10-6)	100	100
420		E-T-10-5a(10-5)	100	100
440		E-P-10-5a(10-4)	100	100
434		E-P-10-5a(10-6)	100	100
461		E-TT-10-5a(10-6)	100	100
MF-441	(17)	E-PT-10-5a(10-6)	100	100
			100	100
442		E-P-10-5a(10-5)	100	100
457		E-P-10-5a(10-4)	100	100
458		E-PT-10-5a(10-5)	100	100
459		E-T-10-5a(10-6)	100	100
444		E-T-10-5a(10-4)	100	100
MF-445	56	E-TT-10-5a(10-6)	100	0.0
			100	0.0

Table B.1. (continued)

MF-448	57	E-PT-10-5a(10-5)	0.0	100
			100	100
MF-449	58	E-T-10-5b(10-4)	100	100
			100	100
460		E-T-10-5b(10-4)	100	100
MF-450	59	E-T-10-5b(10-5)	0.0	100
			100	100
452		E-T-10-5b(10-5)	100	100
MF-451	60	E-T-10-5b(10-5)	100	100
			100	100
MF-453	(28)	E-T-10-5a(10-5)	0.0	100
			0.0	100
463		E-T-10-5a(10-5)	0.0	100
MF-454	61	E-T-10-5b(10-5)	0.0	100
			0.0	100
MF-462	62	E-P-10-5c(10-6)	0.0	100
			0.0	100
MF-464	(7)	E-PT-10-5a(10-6)	100	100
			100	100
No growth on Toluene or Phenol				
439,465		E-T-10-5a		
446,447,455		E-TT-10-5a		
456		E-P-10-5a		
466,467,468		E-PT-10-5a		
MF-H-4-1	(63)	D-10-8a MD	0.0	100
MF-H-4-5	63	D-10-7a MD	0.0	100
			0.0	100
MF-H-4-2	(24)	D-10-7a MD	0.0	100
			0.0	100
MF-H-4-4	(35)	D-10-6a MD	100	100
			100	100
MF-H-4-6	(17)	D-10-6a	100	100
			100	100
No growth on toluene or phenol				
MF-H-4-3	64	D-10-7a	0.0	30
			0.0	0.0

[a]Isolates were obtained after dilution series of original samples (D) or from MPN Positive tube at extiction (E) from the same samples amended with phenol (P) or toluene (T) at 50 ppm, with (PT or TT) or without TCE. Original dilutions and MPN positive tubes at extinction were diluted and plated on: M-R2A (a), toluene vapors (b), phenol vapors. The first number indicates the dilution at which the isolate was obtained from the natural sample and the second indicates the level of the isolate in the enriched MPN tube (e.g. 10-7=10^7). Isolates were purified by three tranfers on M-R2A medium, tested for their ability to degrade Toluene or phenol. Sources of inoculum for the first sample from the aquifer: GB=Glass beads, F=filter (water from the aquifer filtered through a 0.2 μm filter), C= cotton filter (bacteria collected in a cotton filter exposed to the flux of water from the aquifer). Samples 2, 3, and 4 consisted of glass beads only. Samples 2, 3, and 4 MPN tubes had 25 ppm phenol or toluene with or without TCE.

Isolates numbered as 001-199 (sample 1) were obtained before the treatments with phenol and toluene were applied to the field for the year of 1993.
Isolates numbered as 201-299 (sample 2) were obtained after the phenol treatment and 1,1-DCE + TCE.
Isolates numbered as 301-399 (sample 3) were obtained after the phenol treatment + TCE.
Isolates numbered as 401-499 (sample 4) were obtained after the toluene treatment + TCE.

[b] REP-PCR grouping of isolates. Positive degraders were grouped by REP PCR analysis (plates were grouped by similar colony morphologies). Isolates in parentheses represent REP groups identified as identical to a previous observed pattern.

[c] % of toluene removed from the headspace of a vial or phenol disapearance from the medium when compared to a noninoculated control and measured by GC/HPLC analysis, respectively, at 15 days of incubation; or (*) second evaluation at 30 days.

Chapter III

Microbial succession during a field evaluation of phenol and toluene as the primary substrates for TCE co-oxidation.

Introduction

Trichloroethylene (TCE) is one of the most common pollutants of groundwater throughout the United States. Anaerobic degradation of TCE and tetrachloroethylene (PCE) in the laboratory has been reported (Bower and McCarty, 1983; Vogel et al, 1987; Vogel and McCarty, 1985), but these transformations are often incomplete and can result in the accumulation of equally harmful lesser chlorinated metabolites, c-DCE, t-DCE, 1,1-DCE and vinyl chloride (Vogel et al., 1987). In 1985, Wilson and Wilson demonstrated aerobic degradation of TCE in soil enriched with natural gas. Later, several aromatic compounds were also shown to induce TCE degradation (Nelson et al., 1987; Wackett asnd Gibson, 1988; Winter et al., 1989). These aromatic oxygenases are responsible for the cometabolic transformation of this compounds.

Bioremediation remains potentially the most cost effective cleanup technology for chlorinated aliphatic hydrocarbons (CAH's). Field experiments have been carried out at the Moffett Federal Airfield, Mountain View, California, to test the effectiveness of several primary carbon and energy sources for stimulating the bioremediation of CAH's. Methane was an effective substrate for

the transformation of t-DCE and vinyl chloride, but not for TCE and c-DCE (Roberts et al., 1990; Semprini et al., 1990; Semprini and McCarty, 1991; Semprini et al 1991). Phenol was subsequently found to be effective for TCE and c-DCE removal (Hopkins et al., 1993a; Hopkins et al., 1993b). The successful field demonstration of phenol stimulating TCE removal prompted further investigations to optimize its use and to compare its effectivenes for the removal of TCE and its lesser chlorinated products, c-DCE, t-DCE, 1,1-DCE, and vinyl chloride (Hopkins and McCarty, 1995).

Many studies have been carried out in the laboratory on selected culturable isolates responsible for biodegradation of aromatic compounds and their TCE co-metabolic potential, but few studies have focused on a more comprehensive understanding of the community selected by such cometabolic treatments and the dynamics of that community in response to treatments.

In this work we studied microbial population succession as a result of injection into the aquifer of different CAH's. In addition, we evaluated the influence of a shift in carbon source from phenol to toluene on aquifer microbial community structure. We evaluated community composition by both classical growth dependent microbiological procedures, as well as by analysis of 16S rRNA patterns derived from DNA extracted from the entire community. The dominant populations selected were rather stable over treatments and quite resilient following introduction of a toxic CAH.

Material and Methods

Field sample site. The experimental site is located at the Moffett Federal Air Field (formerly the Moffett Naval Air Station) Mountain View, California. A complete description of the site is provided in chapter 2.

Field treatments and sampling. The field experiment was conducted by Gary Hopkins of Stanford University. The experiments analyzed in this chapter are the same as described for 1993 in chapter 2 but the emphasis here is on the sucession of populations responding to the four treatments. A summary of the sequence of chemicals injected into the aquifer is given in Table 2.1. of chapter 2. Briefly, sample 1 represented the microbial population present in the aquifer following phenol and TCE injections the previous field seasons and after 8 months of inoperation of the field test site. Sample 2, was exposed to phenol, TCE and 1,1-DCE, sample 3 was exposed to phenol and TCE and sample 4 was exposed to toluene and TCE. For samples 2, 3 and 4, new sterilized glass bead bag were placed in the sampling well on the same day new substrate addition began and remained in the field for 20-26 days before removal for analysis. Since new, uncolonized samples were provided before each treatment the results reflect only colonization (growth) due to treatments and not survival by indigenous flora which would have been the case if aquifer solids were sampled.

Analysis of microbial biomass and extraction of total microbial community DNA. To collect the biomass from the glass beads or filters, these samples were shaken with sterile saline solution, pH 7, in sterile 250 ml Nalgene plastic

bottles. The supernatant was transferred to a clean, sterile bottle, followed by centrifugation. The pellet was retained and the supernatant discarded. At least eight extractions were performed in this manner from each sample. The final pellet was resuspended in saline to yield a biomass concentration that corresponded to 10 g glass beads/ml of saline. This turbid resuspended solution was used for MPN determinations, direct isolation of microbial populations and total microbial community DNA extraction (chapter 2). DNA was extracted according to the direct lysis protocol developed by Holben et al. (1988).

For the MPN determinations and isolation of dominant populations the basic experimental protocol presented in Figure 2.2. (chapter 2) was followed. The estimated population density and the 95% confidence intervals were calculated by standard methods (Alexander,1982). The dilution level from which each isolate was obtained was recorded to determine which strains repeated overtime, and their approximate population levels.

Species diversity index. Species richness (d) was calculated using the expression of Pielou (1975): d= S-1 / log N, in which S=number of species and N= number of individuals. We used identical REP patterns as a surrogate for bacterial "species". While this does not reflect true species, it does reflect patterns of diversity in the community.

FAME - The isolates analyzed for cellular fatty acids (FAME) were precultured on M-R2A and inoculated into Erlenmeyer flasks containing 0.3% (w/v) tryptic

soy broth (TSB) (Krieg, 1981). Cells were harvested by centrifugation. The cell pellet was placed into a screw-capped test tube and stored at -20°C until analysis. Saponification, methylation and extraction were performed using the procedure described in the MIDI manual (Sasser, 1990). Gas chromatography data was compared to a fatty acid identification library (Microbial Identification Systems, Inc., Newark, Del.) for possible isolate identification. Cluster analysis and dendograms of fatty acid profiles were generated using NTSYS software (Applied Biostatistics, Inc., Setauket, N.Y.). An Euclidian similarity matrix was computed from non-standardized data and distances between strains were calculated using unpaired group mean averages(UPGMA). Goodness of fit between the Euclidian matrix and resulting tree was compared using the cophonetic correlation coefficient (Romesburg, 1984). For the construction of the dendogram, we determined the fatty acid composition of the following standard strains grown on the same media as the isolates: *Hydrogenophaga pseudoflava* (MIDI database version 3.8), *Arthrobacter* sp. strain 53 (Haack et al., 1994), *Pseudomonas putida* F1, *Pseudomonas putida* PaW1, *Pseudomonas mendocina* KR, *Burkholderia cepacia* G-4 and *Burkholderia pickettii* PKO1 (ref. Zhou). The FAME profiles of the *Azoarcus* strains were determined in this study.

Molecular methods. Repetitive extragenic palindromic REP- PCR patterns were obtained from cells using Rep-1 and Rep-2 primers and the polymerase chain reaction (de Bruijn, 1992). Amplification was performed using a Model 9600 Perkin-Elmer Cetus Thermocycler. Products (10 µl) were separated by

electrophoresis in 1.5% agarose gels and stained with ethidium bromide. Amplification was primarily done using individual colonies grown on M-R2A, but for isolates with poor or no amplification, DNA was extracted from cells and used as the template for PCR amplification. For isolates with very close patterns, the same stock of primers and reagents were used in the analysis.

The biomass extracted and resuspended from glass beads and filters from samples 1, 2, 3 and 4 was used for the community DNA extraction. The pellet was resuspended in phosphate buffer and DNA was extracted according to the direct lysis protocol developed by Holben et al. (1988). Extracted DNA was subjected to amplification of the ribosomal RNA genetic loci by the polymerase chain reaction (PCR). Universal primers were used to amplify an approximately 1,500 base pairs fragment using a forward primer which corresponds to nucleotide positions 8-27 (5'-AGAGTTTGATCCTGGCTCAG-3'; primer A) of *Escherichia coli* 16S rRNA and a reverse primer corresponding to the complement of positions1541 to 1518 (5'-AAGGAGGTGATCCAGCCGCA-3'; primer H) (Ulrike et al., 1989). All primers were synthesized with an Applied Biosystem DNA synthesizer at the Macromolecular Structure and Sequencing facility at Michigan State University. In general, amplification was done in 100 µl total reaction volume containing 100 ng of total community extracted DNA or DNA from pure cultures of different isolates as template, 1 µM of each primer, 250 µM of each deoxynucleoside triphosphate, 10 µl of 10X *Taq* buffer, and 2.5 Units of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, CT). PCR was

performed in an automated thermal cycler(9600 Perkin-Elmer Cetus, Norwalk, CT) with an initial denaturation [95°C,130 s], followed by 30 cycles of denaturation [92°C, 70 s], annealing [55°C, 30 s], and extension [72°C, 130 s], and a single final extension [72°C, 6 min]. An aliquot of 10 µl was run in 0.7% agarose gel to evaluate the quality of the amplified fragment. In general amplification yielded greater than 5 ug of PCR product. Amplified DNA from community (25µl) or bacterial isolates (15 µl) was digested according to manufacturers specifications for at least 3 h with restriction endonucleases (*Hae*III, *Hpa*II and *Sau*3A; Boehringer Mannheim, Indianapolis, IN). The 16S rDNA restriction fragments were then concentrated to a lesser volume by lyophilization for 10 min and then electrophoresed on 4% (w/v) NuSieve 3:1 agarose gel (FMC, Indianapolis, IN) in 0.5X TAE [Tris acetate-EDTA] at 7 volts/cm, and stained in a 0.5 µg/ml ethidium bromide solution. The resulting fragmentation patterns were then used as the fingerprint for the identification of bacterial or community genomes.

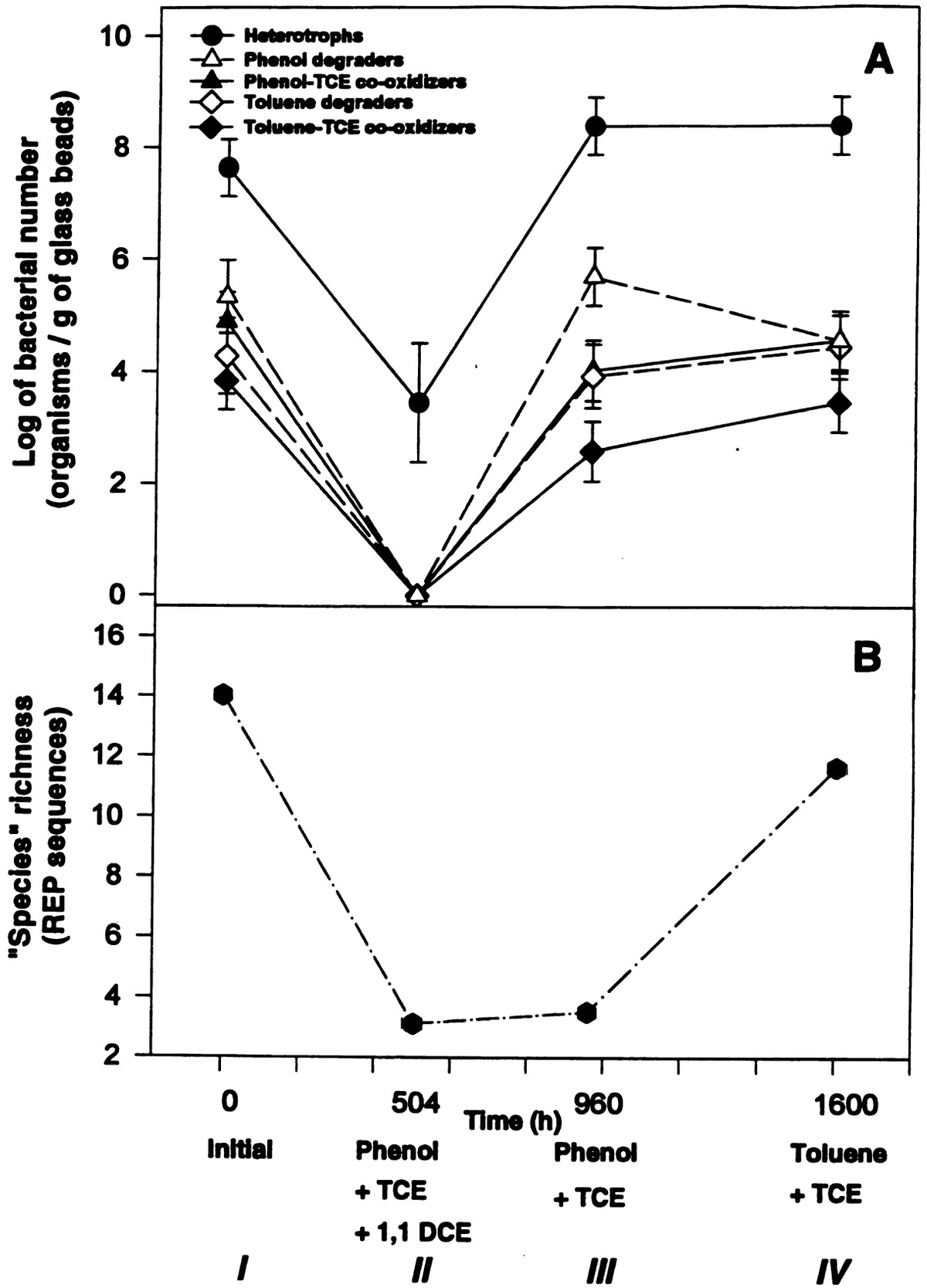
Analytical methods - Toluene and TCE were measured by gas chromatography equipped with a flame ionization detector(GC/FID), a DB-624 capillary column (J&W Scientific, Folson, CA.) and a headspace sampler. The autosampler vials were equilibrated at 30°C, the column at 90°C and injector and detector at 200°C. He was the carrier gas. Phenol was analyzed by reverse-phase HPLC with a Hibar RT C_{18} column (E. Merck), with a flow rate of 1.5 ml/min of 66:33:0.1 H_2O-CH_3CN-H_3PO_4 and a UV detector set to 218 nm.

Results

Succession determined from studies of isolates. No significant differences in the relative abundance of total heterotrophs, phenol degraders, toluene degraders, phenol catalyzed TCE co-oxidizers and toluene catalyzed TCE co-oxidizers were observed when TCE, c-DCE or t-DCE were injected into the aquifer along with phenol or with toluene (Figure 3.1.a.). In contrast, when 1,1-DCE was injected along with phenol and TCE (sample 2), a drastic reduction in all populations was observed (Figure 3.1.a.). Once 1,1-DCE was removed (sample 3) populations levels increased and reached densities similar to those at the beginning of the experiment. While 1,1-DCE had an effect on population densities, the primary carbon source did not since the addition of phenol (sample 3) or toluene (sample 4) produced the same population densities. It is noteworthy that the populations of toluene degraders rose markedly after feeding with phenol (sample 3) and that phenol degraders were at equal density to toluene degraders after feeding with toluene (sample 4).

Species diversity indices were used to express changes in relative diversity among successional stages. Due to technical difficulties in speciating a large number of bacterial isolates we used REP band patterns to reflect the effects of CAH's and primary substrates on species richness. Species richness was greatly reduced by 1,1-DCE and remained low during the subsequent phenol feeding (sample 3) but increased to original levels following the toluene feeding (Figure 3.1.b.). A taxonomic evaluation of the isolates showed that

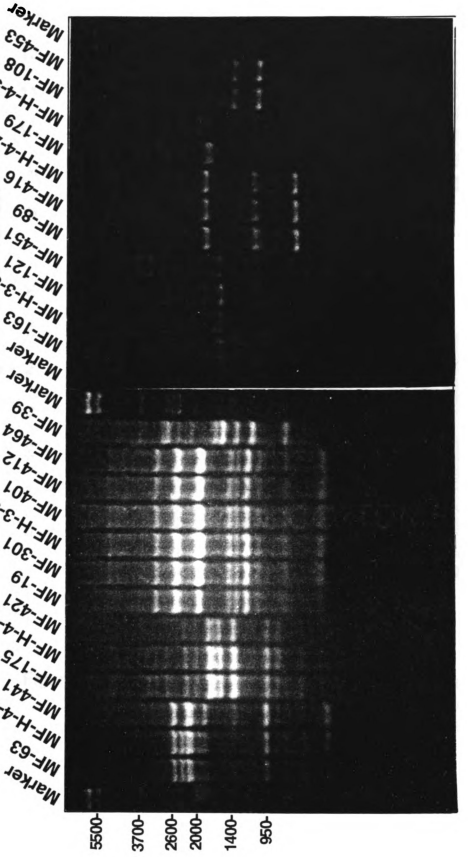
Figure 3.1. (A) Populations of total heterotrophs, phenol and toluene degraders and TCE co-oxidizers obtained from glass beads sampled following each of the four indicated treatments, *I, II, III, IV.* For the initial sample, the populations of toluene and phenol degraders were obtained with 50 ppm carbon substrate. Populations for samples *II, III,* and *IV* were obtained with 25 ppm of carbon substrate. (B) Species richness index calculated as suggested by Pielou (1975) considering isolates from glass beads with different REP sequences as different "species".


Heterotrophs
Phenol degraders
Phenol-TCE co-oxidizers
Toluene degraders
Toluene-TCE co-oxidizers
A
Log of bacterial number
(organisms / g of glass beads)
10
8
6
4
2
0
B
"Species" richness
(REP sequences)
16
14
12
10
8
6
4
2
0
504
960
1600
Time (h)
Initial
Phenol
+ TCE
+ 1,1 DCE
Phenol
+ TCE
Toluene
+ TCE
I
II
III
IV

disturbance by 1,1-DCE led to the survival of relatively few populations compared with unperturbed communities, and that these surviving populations were predominantly gram positives (Figure 3.4, and chapter 2).

We also investigated the effect of carbon sources and CAH's on the composition of the aquifer bacterial communities by tracking changes in specific populations using REP-PCR. Isolates from successive sampling that had similar REP sequences were identified and run side-by-side on agarose gels to confirm their similarities (Figure 3.2). Strains that were found at more than one sampling time and from several samples or media were judged to be more dominant in the field. The distribution of the REP group members isolated was influenced by the field treatments. Isolates represented by REP groups MF-19, MF-63, MF-89, MF-108, MF-163 and MF-175 were among the most commonly isolated and dominant populations in the initial sample (Figure 3.3.). However, after the phenol and 1,1-DCE treatment very few isolates were obtained, and those found (sample 2) had REP sequences not seen before. This suggests that one of these compounds was detrimental to the initial bacterial populations dominating the aquifer. Once 1,1-DCE was removed, but phenol was still being applied, two isolates with REP patterns resembling groups MF-19 and MF-163 were again detected as the most commonly isolated populations (sample 3). When the phenol injection was switched to toluene, isolates representing REP groups MF-19, MF-63, MF-89, MF-108 and MF-175 (sample 4) were again isolated at a high frequency. These isolates accounted for almost all of the isolates recovered in

Figure 3.2. REP-PCR fingerprint patterns of Moffett Field isolates generated by using chromosomal DNA. Isolates that were observed to repeat overtime were runned side-by-side for pattern comparisons. Lanes 1, 15, 16 and 28 are size markers, with the base pairs indicated on the left.


Marker
MF-63
MF-H-4-6
MF-441
MF-175
MF-H-4-4
MF-421
MF-19
MF-301
MF-H-3-5
MF-401
MF-412
MF-464
MF-39
Marker
MF-163
MF-H-3-3
MF-121
MF-451
MF-89
MF-416
MF-H-4-2
MF-179
MF-H-4-5
MF-108
MF-453
Marker
5500-
3700-
2600-
2000-
1400-
950-

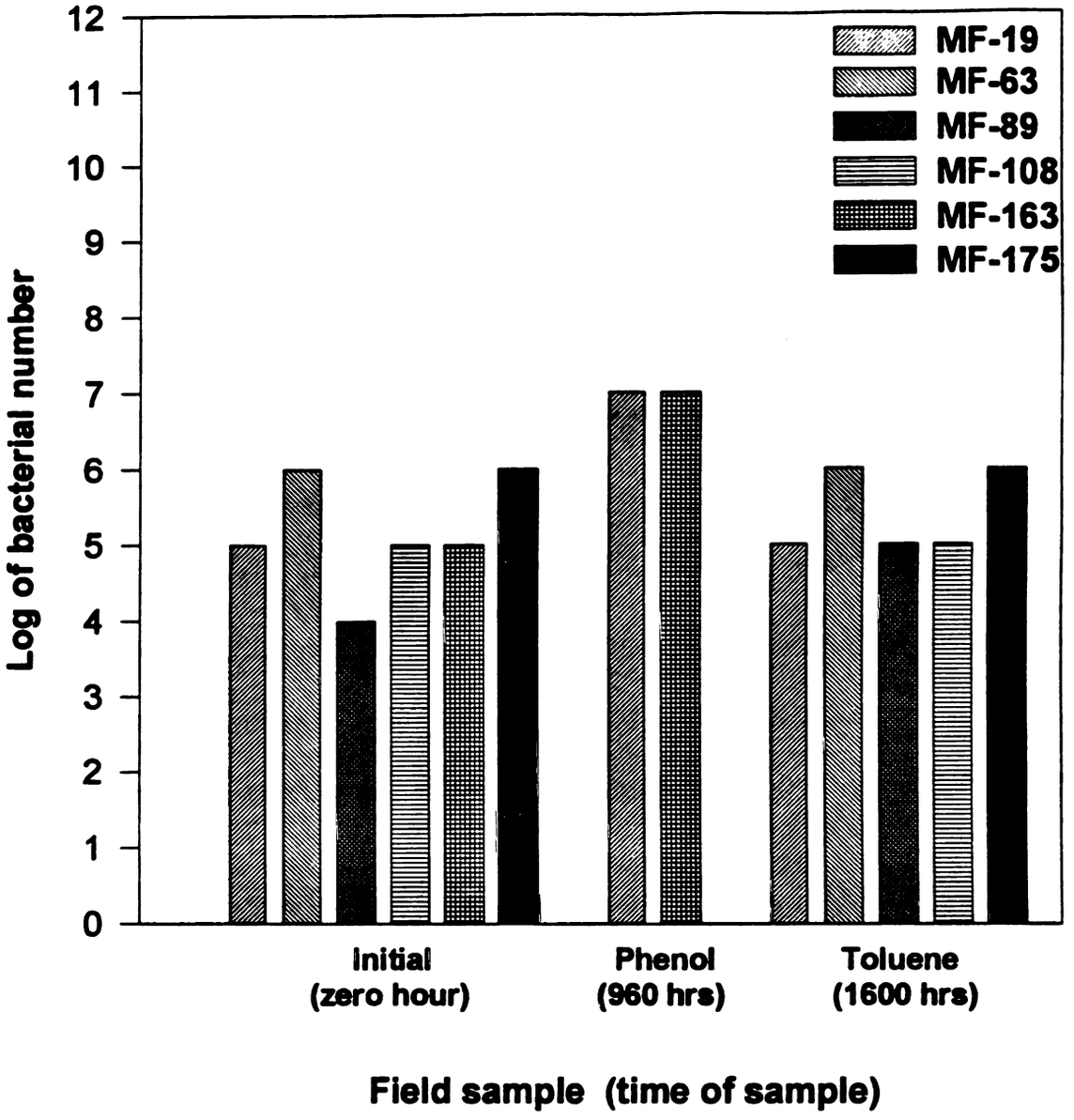


Figure 3.3. REP genotypes persistent during the course of the experiment. The population levels for each isolate is estimated using the most dilute tube from which the strain was isolated.

the initial sample indicating a succession back to the original microbial community in this aquifer.

Comparison of the microbial groups from different aquifer samples. FAME analysis was done on all isolates with different REP patterns from different samples. FAME was then used as an independent method to evaluate differences among isolates including some with the same REP pattern but isolated from different samples. Both FAME and REP data could then be used to evaluate which isolate types were more frequently isolated over the treatments. Initial populations present in the field were very diverse based on the number of isolates with different REP patterns (chapter 2) and on the number of fatty acid methyl ester (FAME) groups obtained. The FAME analysis grouped the isolates with different REP patterns from the four samples into 30 distinct groups of strains at Euclidian distances of less than 20 (Figure 3.4). MIDI literature suggests that the Euclidian distance scale permits a preliminary indication of the relatedness of entries at the genus, species, and subspecies levels, which are respectively, 25, 10, and 6 Euclidian distances (Sasser, 1990). In our cluster analysis we included fatty acid data of well characterized strains that are known to harbor five different pathways for toluene degradation. None of the isolates clustered (< 20) with these isolates: *Pseudomonas putida* F1, *Pseudomonas putida* PaW1, *Pseudomonas mendocina* KR or *Burkholderia cepacia* G-4, or with a common soil isolate *Arthrobacter* sp. One isolate (MF-13) clustered at an Euclidian distance of less than 10 with *Burkholderia pickettii*

Figure 3.4. Dendogram based on the fatty acid profiles of the isolates. FAME groups I through XXX were defined at a Euclidian distance of 20. ($r_{x,y}$= 0.86).

(Isolates from, ▭ sample *I*, ● *II* ★ *III*, and ⬭ *IV*).


I
II
III
IV
V
VI
VII
VIII
IX
X
XI
XII
XIII
XIV
XV
XVI
XVII
XVIII
XIX
XX
XXI
XXII
XXIII
XXIV
XXV
XXVI
XXVII
XXVIII
XXIX
XXX
MF-108
MF-453
MF-H-2-13
MF-121
MF-451
MF-163
MF-189
MF-H-3-3
MF-449
MF-450
MF-H-2-9
MF-24
MF-454
MF-462
MF-115
MF-128
MF-119
MF-415
Azoarcus tolulitycus Td-21
MF-53
MF-92
Azoarcus tolulitycus TOL-4
Azoarcus tolulitycus Td-17
Azoarcus tolulitycus Td-19
Azoarcus sp. S5b2
MF-441
MF-62
MF-H-4-6
MF-63
MF-23
MF-7
MF-66
MF-58
MF-118
Azoarcus tolulitycusTd-15
MF-107
Azoarcus tolulitycus Td-1
Azoarcus tolulitycus Td-2
Azoarcus tolulitycus Td-3
Hydrogenophaga pseudoflava
MF-304
MF-413
MF-414
MF-88
MF-421
MF-H-4-4
MF-175
MF-5
MF-90
MF-183
MF-185
MF-141
MF-445
MF-82
MF-11
MF-182
MF-179
MF-H-4-5
MF-18
Azoarcus indigens VB32T
MF-301
MF-464
MF-39
MF-401
MF-19
MF-412
MF-H-3-5
MF-13
Burkholderia pickettii PKO1
Pseudomonas putida F1
Pseudomonas putida PAW1
MF-419
Burkholderia cepacia G-4
Pseudomonas mendocina KR
MF-15
MF-168
MF-H-2-4
MF-H-4-3
MF-405
MF-190
MF-340
MF-448
MF-54
MF-H-2-1
MF-52
MF-89
MF-H-4-2
MF-416
MF-342
MF-411
MF-H-2-12
MF-84
MF-84W
MF-181
Arthrobacter sp. strain 53
70
60
50
40
30
20
10
0
Euclidian distance

PKO1. However, many isolates and a known control, *Azoarcus indigens* VB32T a nitrogen fixing organism (Reinhold-Hurek et al., 1993), also clustered in this group at an Euclidian distance of less than 20. We also included in this cluster analysis, the fatty acid composition of the recently identified new genus of toluene-degrading denitrifiers *Azoarcus tolulyticus*, since many of the isolates from Moffett Field resembled this genus based on FAME analysis, toluene degradation and denitrification properties (chapter 4). In our earlier work (Fries et al.,1994), we isolated several toluene-degrading denitrifiers from different geographical regions of the world. Those isolates when analyzed by FAME were reported as *Hydrogenophaga pseudoflava* (MIDI system Version 3.8). Cluster analysis of these isolates and the inclusion of the FAME profile for *Hydrogenophaga pseudoflava* and *Azoarcus* sp. S5b2 (another nitrogen fixing isolate), confirmed that the new isolates clustered with *H. pseudoflava* (Figure 3.4.). Gram positive isolates clustered in 12 different groups(I, II, III, IV, V, VI, XVII, XXIV, XXVI, XXVIII, XXIX, and XXX). Most of the gram positive isolates were retrieved in specific samples. However, group III gram positives was very common in the aquifer since isolates belonging to this group were obtained from samples 1, 3 , and 4 (Figure 3.3).

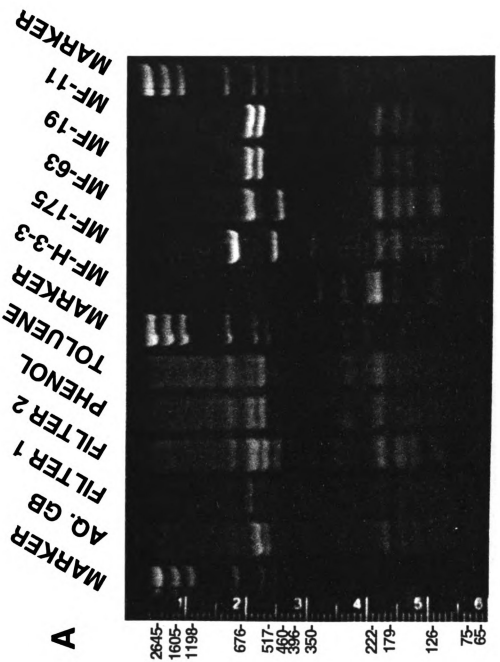
Community composition determined by ARDRA. We used ARDRA of community DNA to evaluate (i) how similar the glass bead biofilm community was to the planktonic community, (ii) whether the community selected by phenol

was different from the one selected by toluene and (iii) whether the dominant strains determined from the isolation studies also appeared to be the dominant organisms by DNA analysis. The relatively low number of bands generated for aquifer community DNA suggests that such communities are low in diversity or that the diversity resides in a number of closely related organisms with the same band patterns. The microbial community profile obtained from glass beads, representing the attached community was very similar to the profiles obtained for the microbial community profiles obtained for filtered water samples and representing the planktonic community (Figure 3.5. A,B and C).

The results obtained with the RFLP profile from the original community and subsequent communities obtained under different carbon sources strongly supports the hypothesis that the structure of the aquifer microbial community did not change under the treatments being compared (Figure 3.5). The number of unique bands per treatment out of the total restriction bands seen using three different restriction enzymes were 2/15 for the initial sample, 1/15 after phenol treatment and 1/15 after toluene treatment (Table 3.1). These numbers show considerable stability in the community over treatments.

All but one of the most dominant isolated strains showed a distinctive restriction fragment pattern for the amplified rDNA when digested with *Hpa*II (Figure 3.5.A). The exception was MF-19 and MF-11 which showed similar patterns. Digestion with *Hae* III (Fig. 3.5.B), and *Sau*3A (Fig. 3.5.C) distinguished between these isolates. The patterns observed for all dominant

Figure 3.5. ARDRA analyses of aquifer community DNA and of pure cultures dominant in the aquifer. The PCR amplified 16S rRNA gene product was digested with *Hpa*II (A), *Hae* III (B), and *Sau*3A (C), electrophoresed on a 0.5XTAE (W/V) NuSieve (FMC) agarose, and stained with ethidium bromide. The lanes show PCR products from aquifer glass beads (AQ. GB) of sample *I*, cells retained on the 0.22 μm filter (Filter 1) and cotton fiber filtered water (Filter 2) from sample *I*, glass beads from sample *III* (phenol amendment), glass beads from sample *IV* (toluene amendment) and the indicated pure cultures.


A
MARKER
AQ. GB
FILTER 1
FILTER 2
PHENOL
TOLUENE
MARKER
MF-H-3-3
MF-175
MF-63
MF-19
MF-11
MARKER
2645-
1605-
1198-
676-
517-
460-
396-
350-
222-
179-
126-
75-
65-
1
2
3
4
5
6

B
MARKER
MF-H-3-3
MF-175
MF-63
MF-19
MF-11
AQ. GB
FILTER 1
FILTER 2
PHENOL
TOLUENE
MARKER
2645-
1605-
1198-
676-
517-
460-
396-
350-
222-
179-
126-
75-
65-
1
2
3
4
5

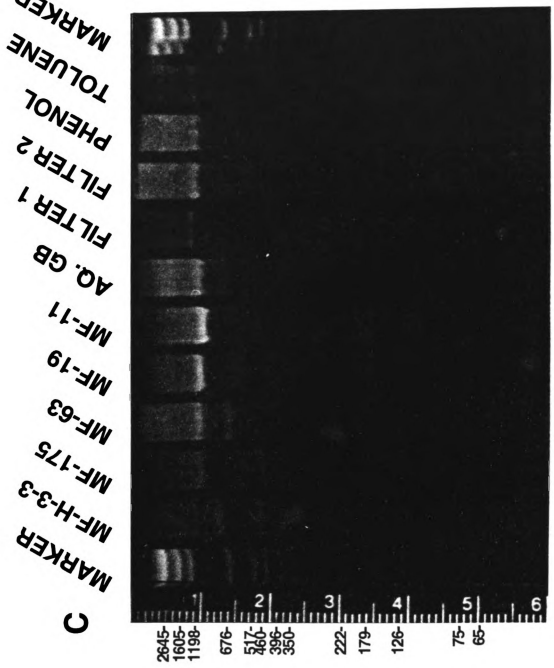

C
MARKER
MF-H-3-3
MF-175
MF-63
MF-19
MF-11
AQ. GB
FILTER 1
FILTER 2
PHENOL
TOLUENE
MARKER
2645-
1605-
1198-
676-
517-
460-
396-
350-
222-
179-
126-
75-
65-
1
2
3
4
5
6

Table 3.1. Number of unique ARDRA bands for the indicated sample relative to the total bands in that sample. The template DNA was extracted from glass beads sampled after three field treatments.

Enzyme	Initial sample[a]	Phenol+TCE[b]	Toluene+ TCE[c]
Hae III	2/9	1/9	1/9
Hpa II	0/4	0/4	0/4
Sau 3A	0/2	0/2	0/2
Total	2/15	1/15	1/15

[a]Beginning of the experiment (8 months of field inoperation)
[b]after phenol-TCE injection (20-26 days exposure)
[c]after toluene-TCE injection (20-26 days exposure).

aquifer isolates could also be discerned in the community DNA for the three samples under study (Table 3.2.). This finding indicates that the dominant community profiles can be accounted for by the dominant isolates suggesting that these isolates are dominant members of the community. Only one band, of approximately 460 to 517 base pairs (Figure 3.5.B) was present in all community samples but could not be accounted for by the bands from culturable isolates. The correspondence in dominance determined by isolation with dominance determined from extracted community DNA suggests that non-culturable forms are not significant in this study.

Discussion

We evaluated succession of microbial populations in an aquifer field site in order to understand how different CAH's and two primary carbon sources affected the dynamics of that natural community. Similar population densities for total heterotrophs, toluene and phenol degraders, as well as TCE co-oxidizers, were observed when the aquifer was injected with TCE, c-DCE or t-DCE and with phenol or toluene as the primary carbon sources. However, all population sizes were drastically reduced when 1,1-DCE was injected into the aquifer. TCE, which is known to have a toxic effect on cultures of TCE co-oxidizers (Wackett and Gibson, 1988; Wackett and Householder, 1989; Zylstra et al.,1989; Winter et al., 1989) did not cause a measured effect on populations detected. In this study, the presence of TCE did not affect field population levels

Table 3.2. Number of common ARDRA bands obtained from pure cultures thought to be the most dominant members of the community compared to the ARDRA bands obtained from DNA extracted from glass beads of samples *I*, *II*, *III* and *IV*. The number of bands seen in community DNA but not seen in isolates tested is shown in parentheses.

Enzyme	Glass beads[a]				
	MF-11	MF-19	MF-63	MF-175	MF-H-3-3
Hae III	6/9(4)	6/9(4)	7/9(2)	8/9(3)	5/9(3)
Hpa II	2/4(6)	2/4(5)	3/4(2)	2/4(2)	2/4(1)
Sau 3A	2/2(4)	2/2(4)	2/2(2)	2/2(0)	0/2(2)
Total	10/15(14)	10/15(1)	12/15(6)	12/15(5)	7/15(6)
Enzyme	Glass beads[b]				
	MF-11	MF-19	MF-63	MF-175	MF-H-3-3
Hae III	6/9(3)	6/9(3)	6/9(3)	5/9(3)	5/9(3)
Hpa II	2/5(3)	3/5(2)	3/5(0)	3/5(1)	3/5(2)
Sau 3A	2/2(0)	2/2(0)	2/2(0)	2/2(0)	0/2(2)
Total	10/16(6)	11/16(5)	11/16(3)	10/16(4)	8/16(7)
Enzyme	Glass beads[c]				
	MF-11	MF-19	MF-63	MF-175	MF-H-3-3
Hae III	6/9(3)	6/9(3)	6/9(3)	5/9(3)	5/9(3)
Hpa II	2/5(3)	3/5(2)	3/5(0)	3/5(1)	3/5(2)
Sau 3A	2/2(0)	2/2(0)	2/2(0)	2/2(0)	0/2(2)
Total	10/16(6)	11/16(5)	11/16(3)	10/16(4)	8/16(7)

[a]Beginning of the experiment (8 months of field inoperation)
[b]after phenol-TCE injection (20-26 days exposure)
[c]after toluene-TCE injection (20-26 days exposure).

but 1,1-DCE seems to have caused extreme toxicity effects for many microbial populations.

The first signs of environmental stress usually occur at the population level, affecting especially sensitive species. If there is sufficient redundancy, other species may fill the functional niche occupied by the sensitive species (Odum, 1985; 1990; Schindler, 1990). Indeed, we observed a drastic reduction in species diversity, as measured by patterns of REP richness during the injection of 1,1-DCE. The populations isolated after this field treatment were predominantly gram positive bacteria and were only able to degrade phenol. These survivors may have a unique resistance to 1,1-DCE. From a bioremediation point of view, this fact must be considered carefully, since these surviving populations may not be efficient for the cometabolic transformation of the desired CAH. None of the isolates obtained after injection of 1,1-DCE (sample 2) carried the toluene ortho-monooxygenase gene despite the fact that this was the most common gene observed in isolates from sample 1 (chapter 2). One explanation is that toxicity of 1,1-DCE was directly related to this enzyme system, since the 1,1-DCE injection prevented isolates with homology to this gene from colonizing the glass beads.

Some populations were observed to repeat overtime as well as following the injection of the toxic 1,1-DCE. Population levels in sample 3, returned to original levels in sample 1, but not in species richness. Toluene addition helped return the original richness to the community. The same populations found after

phenol addition occured after toluene addition. This observation can be explained by the fact that most phenol degraders isolated from the site are also toluene degraders (chapter 2). Therefore, we conclude that some populations are better competitors for the primary carbon sources, but at the same time, very sensitive to 1,1-DCE.

By cluster analysis of the cellular fatty acids isolates representing many different groups of degraders (30) were obtained. This indicates that a taxonomically very diverse community was selected in this aquifer. This includes twelve distinct gram positive groups and a group that, based on fatty acid methyl esters, resembles *Azoarcus sp.* (chapter 4). From all four characterized communities these results indicate that the initial (community 1) and the final (community 4) were very similar, but the community obtained from sample 2 (community 2) was very different from the others and of low diversity. These results corroborate the other evidence found for toxicity and selection of particular populations by 1,1-DCE.

ARDRA analysis provided an alternative method that does not rely on culturing to examine the structure of microbial communities by describing 16s rRNA fingerprint patterns of bacterial assemblages. In this study, ARDRA analysis suggested similar community structures when phenol and toluene were the primary carbon sources, consistent with the interpretation from analysis of the dominant isolates. The majority of bands observed in ARDRA from community DNA were found in ARDRA for the most common isolates,

suggesting that we did isolate the major populations from the site and that unculturable populations were not important in this study.

In this study we examined microbial structure of an aquifer communities by standard MPN techniques, isolation of the most dominant populations and differentiation of these populations by REP-PCR analysis, and restriction endonuclease analysis of amplified 16S rRNA gene fragments from pure cultures and total community DNA. A "Species" richness index showed a marked effect on population diversity when 1,1-DCE was injected into the aquifer. Both the structure and biochemical ability for primary carbon source oxidation of the aquifer community were altered due to this CAH. We also found a rebound of microbial populations to the original community structure once 1,1-DCE was removed. This study also showed that toluene selected the same population that had been selected on phenol suggesting that these two carbon sources were rather equivalent in their biochemical potential.

Acknowledgments

This work was supported by EPA , Grant N° CR 822029-01-0 and the NSF Center for Microbial Ecology, Grant N° BIR 9120006.

List of References

Alexander, M. 1982. Most probable number method for microbial populations, p. 815-820. *In* A. L. Page (ed.), Methods of soil analysis. Part 2. Chemical and microbiological properties. American Society of agronomy, Madison, Wi, USA.

Bower, E. J., and P. L. McCarty. 1983. Transformation of 1 and 2-carbon halogenated aliphatic compounds under methanogenic conditions. Appl. Environ. Microbiol. **45**:1286-1294.

de Bruijn, F. J. 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bateria. Appl. Environ. Microbiol. **58**:2180-2187.

Fries, M. R., J. Zhou, J. Chee-Sanford, and J. M. Tiedje. 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. **60**:2802-2810.

Haack, S. K., H. Garchow, D. A. Odelson, L. J. Forney, and M. J. Klug. 1994. Accuracy, reproducibility, and interpretation of fatty acid methyl ester profiles of model bacterial communities. Appl. Environ. Microbiol. **60**:2483-2493.

Holben, W. E., J. K. Jansson, B. k. Chelm, and J. M. Tiedje. 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**:703-711.

Hopkins, G. D., J. Munakata, L. Semprini, and P. L. McCarty. 1993a. Trichloroethylene concentration effects on pilot field-scale in-situ groudwater bioremediation by phenol-oxidizing microorganisms. Environ. Scie. and Technol. **27**:2542-2547.

Hopkins, G. D., L. Semprini, and P. L. McCarty. 1993b. Microcosm and *In situ* field studies of enhanced biotransformation of trichloroethylene by phenol-utilizing microrganisms. Appl. Environ. Microbiol. **59**:2277-2285.

Hopkins, G. D., and P. L. McCarty. 1995. Field evaluation of In-Situ aerobic cometabolism of trichloroethylene and three dichloroethylene isomers using phenol and toluene as the primary substrates. Environ. Scie. and Technol. (submitted for publication)

Krieg, N. R. 1981. Enrichment and isolation, p. 112-142. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B.

Phillips. (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.

Nelson, M. J. K.,S. O. Montgomery, E. J. O'Neil and P. H. Pritchard. 1987. Biodegradation of trichloroethylene and involvement of an aromatic biodegradative pathway. Appl. Environ. Microbiol. **53**:949-954.

Odum, E. P. 1985. Trends expected in stressed eco-system. Bioscience **35**:419-422.

Odum, E. P. 1990. Field experimental tests of eco-system-level hypotheses. Trends Ecol. Evol. **5**:204-205.

Pielou, E. C. 1975. Ecological diversity. John Wiley and Sons, New York.

Reinhold-Hurek, B., T. Hurek, M. Gillis, B. Hoste, M. Vancannet, K. Kersters, and J. Ley. 1993. *Azoarcus gen. nov.*, nitrogen-fixing proteobacteria associated with roots of Kallar grass (*Leptochloa fusca* (L.) Kunth), and description of two species, *Azoarcus indigens sp. nov.* and *Azoarcus communis sp. nov.* Intern. Journal of System. Bacteriol. **43**:574-584.

Roberts, P. V., G. D. Hopkins, D. M. Mackay and L. Semprini. 1990. A field evaluation of in-situ biodegradation of chlorinated ethenes: Part 1, Methodology and field site characterization. Ground water. **28**:591-604.

Romesburg, H. C. 1984. Cluster analysis for researchers. Lifetime Learning Publications, Belmont, Calif.

Sasser, M. 1990. Technical Note#102: Tracking a strain using the Microbial Identification System. MIS Inc., North Newark,Del.

Schindler, D. W. 1990. Experimental perturbations of whole lakes as tests of hypotheses concerning ecosystem structure and function. *Oikos* **57**:25-41.

Semprini, L., and P. L. McCarty. 1991. Comparison between model simulations and field results for in-situ biorestoration of chlorinated aliphatics. Part 1. Biostimulation of methanotrophic bacteria. Ground Water **29**:365-374.

Semprini, L. , G. D. Hopkins, P. V. Roberts, D. Grbic-Galic, and P. L. McCarty. 1991. A field evaluation of in-situ biodegradation of chlorinated ethenes. Part 3. Studies of competitive inhibition. Ground Water **29**:239-250.

Semprini, L. , P. V. Roberts, G. D. Hopkins, and P. L. McCarty. 1990. A field evaluation of in-situ biodegradation of chlorinated ethenes . Part 2. Results of biostimulation and biotransformation experiments. Ground Water **28**:715-727.

Ulrike, E., T. Rogall, H.Blocker, M. Emde, and E. C. Bottger. 1989. Isolation and direct complete nucleotide determination of entire genes. Characterization of a gene coding for 16*S* ribossomal RNA. Nucleic Acids Res. **17**:7843-7853.

Vogel, T. M. and P. McCarty. 1985. Biodegradation of tetrachloroethylene to trichloroethylene, dichloroethylene, vinyl chloride , and carbon dioxide under methanogenic conditions. Appl. Environ. Microbiol. **49**:1080-1083.

Vogel, T. M., C. S. Criddle, and P. McCarty. 1987. Transformation of halogenated aliphatic compounds. Environ. Sci. Technol. 21:722-736.

Wackett, L.P., and D. T. Gibson. 1988. Degradation of trichloroethylene by toluene dioxygenase in whole cell studies with *Pseudomonas putida* F1. Appl. Environ. Microbiol.**54**:1703-1708.

Wackett, L.P., and S. R. Householder. 1989. Toxicity of trichloroethylene to *Pseudomonas putida* F1 mediated by toluene dioxygenase. Appl. Environ. Microbiol.**55**:2723-2725.

Winter, R. B., K. Yen and B. D. Ensley. 1989. Efficient degradation of trichloroethylene by a recombinant Escherichia coli. Bio/Technology 7:282-285.

Wilson, J. T. and B. H. Wilson. 1985. Biotransformations of trichloroethylene in soil. Appl. Environ. Microbiol. **37**:242-243.

Zylstra, G. J., L. P. Wackett and D. T. Gibson. 1989. Trichloroethylene degradation by Escherichia coli containing the cloned Pseudomonas putida F1 toluene dioxygenase genes. Appl. Environ. Microbiol. **55**:3162-3166.

Chapter IV

Natural selection of denitrifiers at a field site amended with phenol and toluene for TCE co-oxidation.

Introduction

Denitrification is the dissimilatory reduction of nitrate or nitrite to nitrogen gases. A variety of bacteria occupying diverse habitats including soil, water, foods, marine environment, and digestive tract have been reported as denitrifiers (Zumft, 1991). Their numbers in relation to total culturable populations in soil and water habitats can vary from 0.01 to 9% and are in this range for all soil and water habitats studied (Tiedje, 1988).

Four enzymes are required to convert nitrate to nitrogen gas in respiratory denitrification. Most of the knowledge about the enzyme systems is from gram negative bacteria. The first enzyme, nitrate reductase is membrane bound with its active site facing the cytoplasm (Southamer, 1988). Nitrite reductase is located in the periplasmic space. There are two major types of dissimilatory nitrite reductases: those containing cytochrome cd_1 (cd_1-dNirs) and those containing copper (Cu-dNirs) in the active sites (Shapleigh and Payne, 1985; Hochstein and Tomlinson, 1988; Coyne et al., 1989). The major product of nitrite reduction by nitrite reductase is nitric oxide, which is subsequently reduced to nitrous oxide by nitric oxide reductase. Nitric oxide reductases

purified so far are membrane bound and contain a *b* and *c* cytochrome (Heiss et al., 1989; Carr and Ferguson, 1990; Demastia et al., 1991). In some bacteria like *P. aureofaciens* and *P. chlororaphis*, N_2O formation is the last step of denitrification (Firestone et al., 1979). In most denitrifiers, however, N_2O is reduced to dinitrogen gas by a Cu-containing nitrous oxide reductase located in the periplasmic space (Hochstein and Tomlinson, 1988; Stouthamer, 1988).

Biodegradation of aromatic compounds under aerobic conditions is well known; oxygen is utilized for ring activation and cleavage as well as serves as the electron acceptor for the complete oxidation of these compounds (Gibson and Subramanian, 1984). The availability of oxygen, due to its low solubility in water and inefficient transport through saturated porous matrices such as soil and sediments, is usually the rate limiting parameter for aromatic compound biodegradation. It's only been in recent years that anaerobic degradation of these compounds has been conclusively established. The degradation of toluene and phenol has been reported by pure cultures under denitrifying conditions (Bakker, 1977; Dolfing et al., 1990; Schocher et al., 1991; Evans et al., 1992; Chee-Sanford et al., 1992; Fries et al., 1994), methanogenic conditions (Wilson et al., 1986; Grbic-Galic and Vogel, 1987), sulfate reducing conditions (Edwards et al., 1993; Rabus et al., 1993) and ferric iron reducing conditions (Lovley et al., 1989; Lovley and Lonergam, 1990). We have developed techniques for the isolation of populations of anaerobic phenol and toluene-degrading denitrifers where low (e.g. 5 ppm) concentrations of the

aromatic compound was an important factor to success (Fries et al., 1994). Also, the stimulatory effects of nitrate addition on biorestoration of a fuel contaminated site has been reported (Hutchins, 1991; Hutchins et al., 1991a; Hutchins et al., 1991b). Therefore, the ability to degrade aromatic compounds under anaerobic conditions is now well documented.

An aquifer at the Moffett Federal Air Field, Mountain View, California, has been under test with phenol and toluene as the primary carbon and energy sources for stimulating the cometabolic remediation of several CAH's (Hopkins et al., 1993; chapter 2). The cometabolic transformation of CAH's is dependent on the expression of oxygenases by microbial populations that use the aromatic compound for growth. However, the limiting O_2 , below 0.2 mg/L (Roberts et al., 1990) and high nitrate (25 mg/L) in the ground water (Hopkins et al., 1993) may favor the development of denitrifers. In the present study, we investigated whether the high levels of nitrate and low O_2 in the groundwater favored the selection of denitrifiers following the injection of aromatic compounds. Furthermore, if aromatic compounds are injected for the purpose of inducing the production of oxygenases, it must be ensured that the levels of oxygen are maintained since a different set of enzymes are induced under denitrifying conditions for the degradation of these substrates (Altenschmidt and Fuchs, 1992; Evans et al., 1992; Seyfried et al., 1994; Chee-Sanford et al., 1995). We also confirmed that anoxic conditions resulted in no TCE co-oxidation by isolates able to degrade toluene under denitrifying conditions.

Material and Methods

Field sample site. The experimental site is located at the Moffett Federal Air Field (formerly the Moffett Naval Air Station) Mountain View, California. Chemical analysis of water from the sampling site revealed the following concentrations: phosphate less than 0.5 mg/L, nitrate, 25 mg/L, sulfate, 700 mg/L (Hopkins et al., 1993). A complete description of the site is provided elsewhere (chapter 2).

Microbial strains. Isolation procedures and some characteristics of the strains from the Moffett Field used in this work are provided in chapter 2 and chapter 3. Standard strains, *Azoarcus tolulyticus* Td-isolates have been described (Fries et. al., 1994), *Pseudomonas pickettii* PKO1 was kindly provided by Dr. Ron Olsen and *Azoarcus* sp.S5b2 and *Azoarcus indigens* VB32T was kindly provided by Dr. Barbara Reinhold-Hurek.

Most probable number of heterotrophs and denitrifiers. Two samples were used to compare population levels of denitrifiers and heterotrophs from the site. Sample 1 consisted of filtered water from the SSEGB well (Figure 2.1., chapter 2) using a 0.22 *um* filter, and sample 2 was a core sample from aquifer material collected during the construction of a new (third) experimental leg at the field site in 1994, on a location adjacent to the old wells but which had no previous exposure to the water flow from previous field experiments. To collect the biomass from the filter, this sample was shaken with sterile saline solution, pH

7, in sterile 250 ml Nalgene plastic bottle. To collect the biomass from the sand-gravel, 300 g of this sample was shaken with sterile saline solution, pH 7, in a sterile 250 ml Nalgene plastic bottle. The supernatant was collected and resuspended to a visual turbidity similar to the one obtained for the filter. The slurry obtained from the filter and the sand core sample were used for MPN determinations.

The estimated population density and the 95% confidence intervals were calculated by standard methods (Alexander,1982). Resuspended material collected from filters and subsurface material were serially diluted and 1 ml of each dilution was transferred to sterile 20 ml vials after which 9 ml of the specific medium was added. Two media were used for MPN determination of denitrifier population density: nutrient broth amended with 5 mM NO_3^- (Tiedje, 1982) and a denitrification medium. The denitrification medium consisted of the salt mixture (SM) (chapter 2) and the following carbon sources (final concentration per liter): Proteose peptone, 0.5 g; yeast extract, 0.5 g; casamino acids, 0.5 g; potassium acetate, 1 g; sodium succinate, 1 g. The tubes were sealed with Teflon-lined stoppers and incubated without shaking. Denitrification was evaluated by observing gas bubble formation in inverted Durham tubes (N_2), by measuring the presence of NO and N_2O in the dilution tube headspace by gas chromatography and by measuring the concentrations of nitrate and nitrite in the medium supernatant by HPLC analysis. A vial was assumed to be positive for denitrification when bubbles were detected in Durham tubes, or when we

detected NO in the vial headspace. The total heterotrophic population in both samples was determined by the MPN method also using two media: the standard TSB medium (Krieg, 1981) and liquid M-R2A (chapter 2) medium. The results were scored by analyzing visible turbidity. Five tubes were inoculated from each serial dilution. The incubation temperature throughout this work was 30°C.

FAME . Protocols for FAME analyzes and construction of a dendogram have been described in chapter two. For the construction of the dendogram, the fatty acid composition of *Hydrogenophaga pseudoflava* was obtained from MIDI database version 3.8.

Isolate characterization. The denitrification ability of individual isolates was evaluated by observing gas bubble formation in inverted Durham tubes, by detecting the presence of NO and/or N_2O in the vial headspace by gas chromatography and by measuring the concentration of nitrate and nitrite in the medium by HPLC analysis. A 10% inoculum grown aerobically on the denitrification media was used for the denitrification test. To test the capabilities of the isolates to grow on phenol or toluene under denitrifying conditions, a heavy inoculum from M-R2A plates, of each isolate was transferred to sterile 20 ml vials after which 5 ml of anaerobic BS + NO_3^- medium plus 25 ppm phenol or toluene was added (Fries et al., 1994). The media with the aromatic substrates were added in an anaerobic chamber filled with nitrogen gas and 3% hydrogen. The vials were sealed with sterile butyl rubber Teflon-lined septa and

incubated for at least 2 weeks before phenol or toluene disappearance was evaluated. Controls from the same batch of medium but without cells were incubated at the same time as a reference to determine the amount of phenol or toluene removed. Degradation activity was defined as at least 95% loss of substrate in the headspace as measured by GC analysis for toluene or in the culture's supernatant for phenol.

Trichloroethylene co-oxidation under aerobic versus anaerobic denitrifying conditions. Toluene-degrading denitrifiers were grown under anaerobic conditions in BS + NO_3^- + 50 ppm of toluene (chapter 2). Inocula (1/2 ml) were transferred to 20 ml sterile auto sampler vials and 4.5 ml of BS + NO_3^- + 50 ppm of toluene and 0.8 ppm TCE was added to the vials and the vials were sealed with Teflon-lined stoppers. For evaluating anaerobic TCE co-oxidation vials were prepared similarly except that the medium was added in the anaerobic chamber. Degradation was determined by substrate disappearance in the headspace after 15 days of incubation as measured by GC analysis, when compared to non-inoculated controls.

Molecular methods. The gene probes used in this study, *i.e.* the heme-type nitrite reductase, from *Pseudomonas stutzeri* JM 300, the toluene-ortho-monooxygenase from *Pseudomonas sp.* JS150 and *Pseudomonas cepacia* G-4 along with the protocols for hybridization are described in chapter 2 and 5. Genomic DNA was obtained by standard methods (Elmerich et al.,1982) from pure cultures of isolates grown on M-R2A broth under aerobic conditions.

Restriction endonuclease digestion of DNA was performed according to manufacturer's specifications. Digested DNA was size fractioned by electrophoresis in 0.7% agarose gels and transferred to nitrocellulose (polyester-supported BAS 68380; Schleicher & Schuell, Keene, N.H.) as previously described (Fries et al., 1994).

Analytical methods. Toluene and TCE were measured by gas chromatography equipped with a flame ionization detector (GC/FID), a DB-624 capillary column (J&W Scientific, Folson, CA.) and a headspace sampler. The autosampler vials were equilibrated at 30°C, the column at 90°C and injector and detector at 200°C. He was the carrier gas. NO_3^- and NO_2^- concentrations were determined in culture supernatant by HPLC analysis using a Partisil 10 SAX column (Whatman, Clifton, NJ.), UV detection at 210 nm, and 50 mM phosphate (pH 3.0) as eluant. N_2O and NO were measured by GC/ECD, using a Poropak Q column at 55°C, 300°C detector temperature and 95% argon-5% methane as carrier gas. Phenol was analyzed by reverse-phase HPLC with a Hibar RT C_{18} column (E. Merck), with a flow rate of 1.5 ml/min of 66:33:0.1 H_2O-CH_3CN-H_3PO_4 and a UV detector set to 218 nm.

Results

Heterotrophic and denitrifying population densities. The populations of cultivatable heterotrophs and denitrifiers were compared for two samples from the aquifer using two different media to estimate each group. Similar heterotrophic populations ($7.9x10^5$- $2.4x10^6$ cells/ml of slurry) were observed, irrespective of the medium, for the core sample from an untreated area from the site, and the filtered water sample from the aquifer that had received carbon additions for the two previous field seasons (Table 4.1). The proportion of the total heterotrophic population comprised of denitrifiers was also similar for the two habitas with 4.9% and 8.1% capable of denitrification in the water (treated) and sand (untreated) samples, respectively.

Denitrification products in MPN tubes. During the MPN evaluation of denitrifier populations some of the MPN tubes at the low and high dilutions showed no turbidity for the water sample yet some nitrate was consumed. This occured in both nutrient broth and denitrification medium. Headspace analysis of these tubes (40 % of the total positive MPN tubes) revealed the presence of NO (nitric oxide) (Table 4.2.). None of the tubes where NO was detected produced N_2 gas, but all accumulated some N_2O.

We obtained 348 numerically dominant isolates from the Moffett Field experimental site, of which 273 were positive for toluene and/or phenol degradation. REP-PCR analysis revealed that the 273 isolates procuced 63

Table 4.1. Number of denitrifiers and heterotrophs observed in a sample that is on the path of carbon flow (filter) compared with a sample from the aquifer (sand) that has not been subjected to treatments at the Moffett Field site.

Sample	Medium[a]	Cell number ($X 10^4$)	95% CI[b] ($X 10^4$)
Filter	DM	7.9	2.4, 26
	NB	2.4	0.7, 7.9
	TSB	79	24, 260
	M-R2A	130	39, 430
Sand	DM	13	3.9, 43
	NB	17	52, 56
	TSB	130	39, 430
	M-R2A	240	73, 790

[a] DM (denitrification media) and NB (nutrient broth) amended with 5 mM nitrate were used to evaluate total numbers of denitrifiers. TSB (Tryptic soy broth) and M-R2A (modified R2A) minus nitrate were used to evaluate the total number of heterotrophs.

[b] 95% confidence interval.

Table 4.2. An example of diversity of denitrification products in MPN tubes from a Moffett Field filtered water sample.

MPN tube	Turbidity	Denitrification products				
		NO_3^-	NO_2^-	NO	N_2O	N_2
10^{-1}	+	-	-	-	-	+
10^{-1}	+	-	-	-	-	+
10^{-1}	-	2.2	-	+	+	-
10^{-1}	+	-	1.8	-	-	+
10^{-1}	-	0.13	4.0	+	+	-
10^{-2}	+	-	-	-	-	+
10^{-2}	+	-	-	-	-	+
10^{-2}	-	2.3	1.4	+	+	-
10^{-2}	+	-	-	-	-	+
10^{-2}	+	-	-	-	-	+
10^{-3}	+	-	-	-	-	+
10^{-3}	+	-	-	-	-	+
10^{-3}	+	-	-	-	-	+
10^{-3}	+	-	-	-	-	+
10^{-3}	+	-	-	-	-	+
10^{-4}	+	-	4.5	+	+	-
10^{-4}	+	-	0.1	+	+	-
10^{-4}	+	-	1.7	+	+	-
10^{-4}	+	-	-	-	-	+
10^{-4}	+	-	-	-	-	+
10^{-5}	+	-	-	+	+	-
10^{-5}	+	-	4.0	+	+	-
10^{-5}	+	-	-	+	+	-
10^{-5}	-	4.3	-	-	-	-
10^{-5}	-	4.0	-	-	-	-
10^{-6}	-	3.9	-	-	-	-
10^{-6}	-	4.0	-	-	-	-
10^{-6}	-	4.0	-	-	-	-
10^{-6}	-	4.5	-	-	-	-
10^{-6}	-	4.4	-	-	-	-

different REP-groups (chapter 2). Forty-six percent of the isolates are denitrifiers with nitrogen gas as the end product, 5% produced nitrous oxide, one isolate produced nitric oxide, 25% reduced nitrate to nitrite. Twenty-two percent of the isolates did not reduced nitrate in denitrification media (Table 4.3. and Figure 4.1). Three of the most dominant and most commonly isolated strains from sample 1, MF-175, MF-19, MF-163 were reisolated again in samples 3, and 4, on heterotrophic (non-selective) medium, MF-H-4-4, MF-H-3-5, MF-H-3-3, respectively. All of these isolates are denitrifiers. When probed for the type of nitrite reductase gene , 41% of the denitrifier isolates (Table 4.3. and Figure 4.1) hybridized to the heme cd_1 nitrite reductase gene probe from *Pseudomonas stutzeri* JM300. This type of nitrite reductase was shown by Coyne et al. (1989) to be the most frequent in strains collections from nature.

Characteristics of an unusual NO-producing denitrifier, MF-18. The results obtained in the MPN tubes, were unusual in respect to the appearance of nitric oxide in some tubes. These results can be potentially explained by one of isolates, strain MF-18. This strain was independently isolated four times from glass beads and cotton filter samples in medium containing toluene and presence or absence of TCE. This strain produced large quantities of NO when given nitrate or nitrite under aerobic and anaerobic conditions (Table 4.4.). This NO concentration could be sufficient to prevent turbidity from developing in MPN tubes or pure culture. The isolate did not grow with nitrous oxide as an electron

Table 4.3. Characteristic denitrification products of Moffett Field isolates, hybridization to the heme-type nitrite reductase gene probe, and capability of toluene or phenol degradation under denitrifying conditions.

Isolate	Denitrification product[a]	Hybridization to cd_1-dNir[b]	Phenol degradation[c]	Toluene degradation[c]
MF-5	NO_2^-	-	-	-
MF-7	+	+	-	+
MF-11	+	+	-	-
MF-13	N_2O	+	-	-
MF-15	+	nd	-	-
MF-18	NO	-	-	-
MF-19	+	[illegible]	-	-
MF-23	+	+	-	-
MF-24	-	-	-	-
MF-39	NO_2^-	-	-	-
MF-52	-	-	-	-
MF-53	+	+	-	-
MF-54	+	nd	-	-
MF-58	+	nd	+	+
MF-59	+	-	-	-
MF-62	+	+	-	-
MF-63	+	+	-	-
MF-66	+	+	+	+
MF-80	+	-	-	-
MF-84	+	nd	-	-
MF-84w	+	nd	-	-
MF-88	-	-	-	-
MF-89	-	-	-	-
MF-91	+	nd	-	-
MF-92	NO_2^-	nd	-	-
MF-107	+	-	-	+
MF-108	+	-	-	-
MF-118	+	+	-	+
MF-119	+	+	-	-
MF-121	NO_2^-	-	-	-
MF-128	+	+	-	-
MF-141	+	+	-	-
MF-163	+	-	-	-
MF-168	+	-	-	-
MF-175	N_2O	+	-	-
MF-179	NO_2^-	-	-	-
MF-180	-	-	-	-
MF-181	-	-	-	-

Table 4.3. (continued)

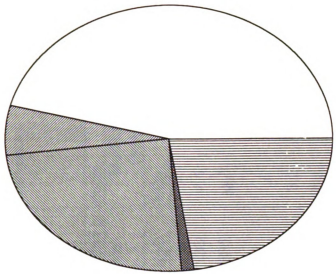
MF-182	NO_2^-	-	-	-
MF185	+	+	-	-
MF-189	NO_2^-	-	-	-
MF-H-2-1	NO_2^-	nd	nd	nd
MF-H-2--4	+	nd	nd	nd
MF-H-2-9	-	nd	nd	nd
MF-H-2-12	-	nd	nd	nd
MF-H-2-13	NO_2^-	nd	nd	nd
MF-304	+	nd	nd	nd
MF-340	NO_2^-	nd	nd	nd
MF-342	NO_2^-	nd	nd	nd
MF-405	-	nd	nd	nd
MF-411	-	nd	nd	nd
MF-413	+	nd	nd	nd
MF-414	NO_2^-	nd	nd	nd
MF-415	-	nd	nd	nd
MF-419	+	nd	nd	nd
MF-445	-	nd	nd	nd
MF-448	N_2O	nd	nd	nd
MF-449	NO_2^-	nd	nd	nd
MF-450	NO_2^-	nd	nd	nd
MF-451	NO_2^-	nd	nd	nd
MF-454	-	nd	nd	nd
MF-462	NO_2^-	nd	nd	nd
MF-H-4-5	-	nd	nd	nd
B. cepacia G-4	NO_2^-	-	-	-
P. stutzeri JM300	+	+	-	-
B. pickettii PKO1	+	-	-	-

[a] Observed denitrification products were N_2 (+) (as gas in Durham tubes), NO_2^- by HPLC, and NO and N_2O by GC/ECD detector.

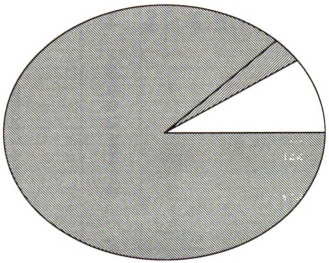
[b] Results from Southern blot hybridizations using the heme type nitrite reductase gene probe.

[c] Growth of the isolates under anaerobic denitrifying conditions using phenol or toluene as the only carbon and energy source.

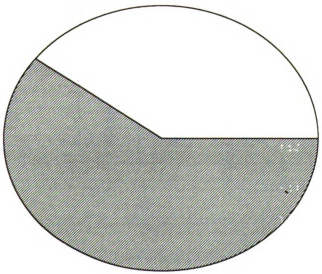
Figure 4.1. Distribution among the 63 REP-PCR groups of the (a) characteristic denitrification products, (b) capability to degrade phenol or toluene under denitrifying conditions and (c) homology to the heme type nitrite reductase gene probe.



46.5 % Nitrogen gas
5% Nitrous oxide
25% Nitrite
1.5% Nitric oxide
22 % Non-denitrifiers



8% Toluene-degrading denitrifiers
3% Phenol-degrading denitrifiers
89% Non-Phenol or -toluene-degrading denitrifiers



41% Homology to heme-type nitrite reductase
59% No homology to cd_1-dNir

Table 4.4. Characteristic denitrification products of isolate MF-18 when grown under different atmospheres.

Headspace	e^- acceptor[a]	Increase in turbidity	End products			
			Headspace (ppm)		Medium (mM)	
			NO	N_2O	NO_3^-	NO_2^-
Argon	none	-	-	-	-	-
	NO_3^-	-	84	136	4.1	1.7
	NO_2^-	-	188	468	Na[b]	6.6
Air	O_2	+	-	-	-	-
	NO_3^-	+	105	910	3.2	2.5
	NO_2^-	+	327	750	Na	5.6
N_2O	N_2O	-	Nd	Nd	Nd	Nd
Glove box[c]	NO_3^-	-	Nd	Nd	Nd	Nd
	NO_2^-	-	Nd	Nd	Nd	Nd

[a] Initial concentrations were 5.8 mM for NO_3^- and 7.2 mM for NO_2^-

[b] Not applicable

[c] Open tube in glove box (N_2 + H_2 headspace).

acceptor. The isolate did appear to begin growth as a denitrifier under argon since N-anions were consumed and N gases were produced, but turbidity did not developed. Growth could have been arrested early by the high concentration of NO. To test this hypothesis we tried to grow this isolate in a glove box (97% nitrogen and 3 % hydrogen) with nitrate or nitrite as an electron acceptor and a cotton plug on the tube so that any nitric oxide (a very insoluble gas) could be exchanged into the headspace of the glove box. No apparent turbidity was observed (Table 4.4.). Growth was observed in tubes without nitrite or nitrate in an aerobic headspace , and no NO was detected under this conditions.

Strains that degrade toluene and phenol under denitrifying conditions. We evaluated the ability of the isolates obtained from sample 1 (chapter 2) representing different REP-PCR groups to degrade toluene or phenol under anaerobic (denitrifying conditions). Five isolates (8%) were able to degrade toluene and two of those isolates also degraded phenol using nitrate as an electron acceptor (Table 4.3. and Figure 4.1.). All isolates were also aerobic toluene degraders and three showed a distinct band of homology in Southern blots when hybridized to the toluene *ortho*-hydroxylase of *Pseudomonas cepacia* G-4. The efficiencies for TCE removal under aerobic conditions varied from 30% to 95% among the isolates. Under anaerobic denitrifying conditions, no TCE was removed from the vial headspace of any of the isolates although all toluene was consumed (Table 4.5.). This result was expected since it is

Table 4.5. Degradation of toluene under aerobic and anaerobic-denitrifying conditions and percent removal of TCE by toluene-degrading denitrifier isolates from Moffett Field[a].

Isolate	TOM probe[b]	Aerobic[c]		Anaerobic[c]	
		Toluene	TCE	Toluene	TCE
MF-7	-	100	30	100	0
MF-58	+	100	95	100	0
MF-66	+	50	50	100	0
MF-107	-	100	40	100	0
MF-118	+	100	40	100	0

[a] The inoculum was grown under denitrifying conditions (50 ppm toluene) and, a 10 % inoculum was transferred to a new vial before addition of test medium.

[b] Southern blot hybridization to the toluene ortho monooxygenase gene (TOM) from *Pseudomonas sp.* JS 150 and from *Burkholderia cepacia* G-4..

[c] % Toluene and TCE removal from the headspace vials after two weeks of incubation (initial concentrations were 50 ppm toluene and 0.8 ppm TCE).

generally accepted that oxygenases are necessary for TCE co-oxidation; this is additional evidence for this fact.

The five new toluene-degrading denitrifier isolates appeared to be closely related to each other and to the members of the new species *Azoarcus tolulyticus*. The probable identification is based on the fact that these isolates share the following key features with the described *Azoarcus tolulyticus* strains (Fries et al., 1994; Zhou et al., 1995): characteristic growth and colony morphology on M-R2A medium, the same type and proportion of cellular fatty acids , the same gram stain, catalase reaction, and characteristic cell morphology when growing on M-R2A plates. We used the fatty acid data for the new isolates and from the previously isolated *Azoarcus tolulyticus* as well as some other *Azoarcus sp.*, in a cluster analysis. All isolates clustered at an Euclidian distance of less than 20 suggesting that they belong to the same genus (Figure 4.2.).

Discussion

We investigated if selection for denitrifying populations occurred in an aquifer that has high natural levels of nitrate and low levels of oxygen, by evaluating population numbers, characterizing denitrification products of isolates from the site and using molecular methods for identification of denitrifying genes. We used two different media for this comparison and found that 4.9-8.1% of the total viable population was composed of denitrifiers. This proportion is on the

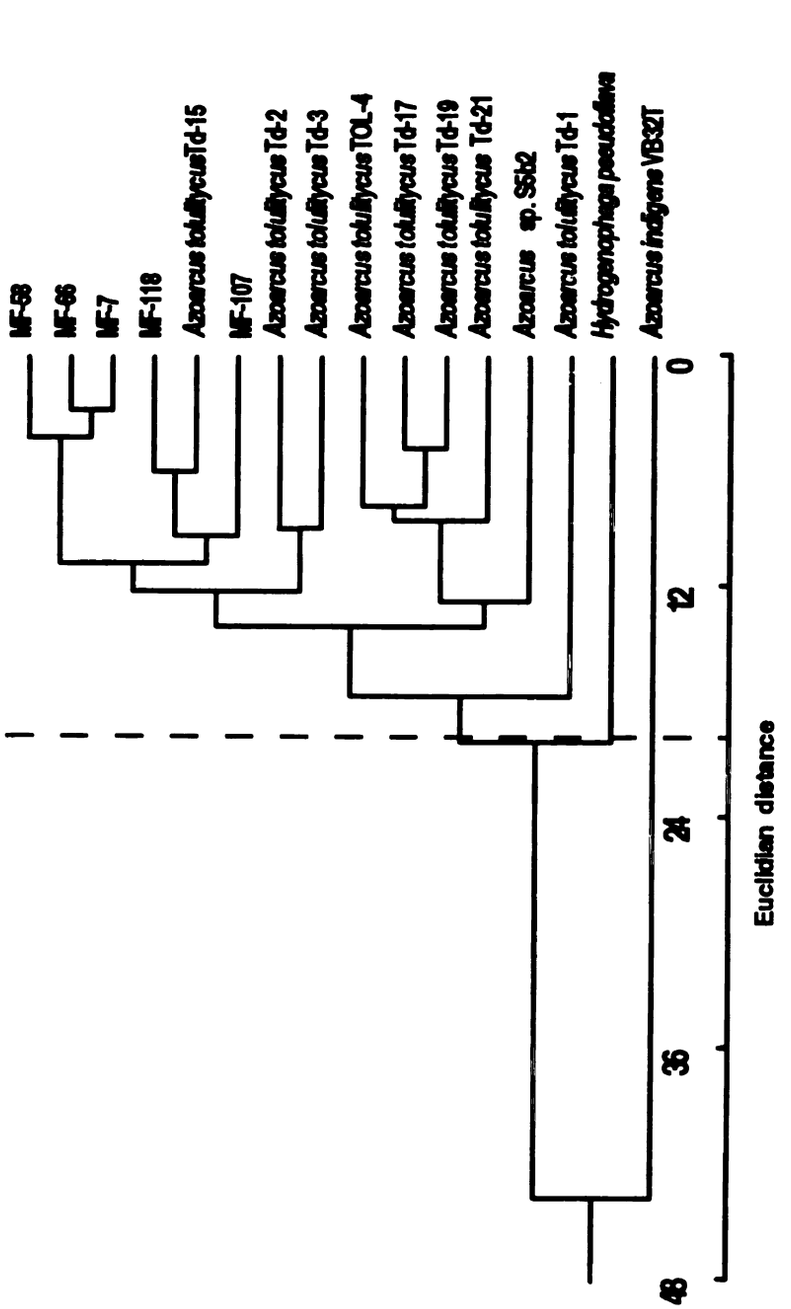


Figure 4.2 Cluster analysis of FAME data from toluene-degrading denitrifiers from Moffett Field together with well characterized *Azoarcus* strains from different geographic regions ($r_{x,y}$=0.88).

high end of the range for populations of denitrifiers present in common soils (Tiedje, 1988). The similarities in denitrifier numbers and their proportion of the total heterotrophic population for a sample collected outside the flow path of injected toluene or phenol and from a water sample in the flow path indicates that the injected carbon sources were not responsible for this selection. However, the high nitrate low oxygen condition may have been sufficient to select a high proportion of denitrifiers in both samples regardless of whether extra carbon was added.

More than half of the isolates obtained from the site (chapter 2) are true denitrifiers with either nitrogen gas or nitrous oxide as end products. The number of nitrite accumulators was half the number of denitrifiers. This is in contrast to results obtained by Gamble et al. (1977), when evaluating samples from world soils, who found a greater number for nitrite accumulators than denitrifiers. Only 22% of the isolates presented no reaction to nitrate when tested for this property. Three of the most commonly isolated and most dominant strains are also denitrifiers. Based on these several observations, we believe that the denitrification property helped enriched the denitrifying populations at this site. Murray et al. (1992) has shown that denitrification efficiency by an isolate was responsible for the competitive advantages for growth under denitrifying conditions when compared with an inefficient denitrifier.

One isolate, MF-18, produced NO when growing on medium supplemented with nitrate or nitrite and in tubes with an aerobic headspace. NO was also produced by this strain when inoculated into an anaerobic medium, but no increase in turbidity was observed. We suspected that the organism was producing enough NO to be toxic and prevent its further growth. Hence, we tried to grow this strain in a glove box in a non-sealed tube, thus allowing the toxic NO to diffuse from the tube. However, no growth was observed under this condition. We are not certain that the experimental protocol was appropriate for NO removal or that the production of NO by the cell still damaged the cell before it could escape. NO production by strains like MF-18 still offer the best explanation why some MPN tubes at low dilution had no turbidity, but high headspace NO and only partial NO_3^- and NO_2^- removal.

In natural communities we could postulate that other species may consume this compound and in this way the isolate would maintain its niche by preventing growth of other sensitive species. We consider this isolate to be an unusual denitrifier based on the lack of visible growth under denitrifying conditions. This, however, might be explained by NO toxicity. This is the first report of the isolation of a denitrifier that produces NO in high amounts apparently preventing its own growth. One explanation for the NO accumulation is that the nitrite reductase is much more rapid than the nitric oxide reductase. Another possibility is that other enzymatic mechanisms could produce NO in the absence of a removal enzyme. Nitric oxide reductase cytochrome P450*nor* is one novel

mechanism for nitrate reduction that has been found in fungi, e.g. *Fusarium oxysporum* (Shoun and Tanimoto, 1991).

We also found that 8% of the isolates harbor the unique property of anaerobic growth on monoaromatic hydrocarbons, using nitrate as their electron acceptor. This field site was amended with phenol for two field seasons prior to our sampling, and this may have enriched for anaerobic phenol (and toluene) degraders. Monoaromatic hydrocarbons are common contaminants of ground water, and also, biogenic sources of aromatic compounds have been described for bacteria and fungi. Hydroxylated toluene, toluene and benzene has been reported to be produced by aerobic and anaerobic populations in culture media, and by natural anoxic hypolimnia of stratified lakes and anoxic river sediments (Martin and Haider, 1969; Juttner and Henatsch, 1986; Juttner, 1988, 1990,1991; Fisher and Juttner, 1994). The fact that aromatic compounds are toxic even at concentrations of 5-10 ppm, (Fries et al., 1994; chapter two) and that many aromatic compounds are synthesized by microbial populations at aerobic and anaerobic conditions led us to speculate that the ability of populations to use these resources from an habitat, provides these organisms with greater versatility anaerobic micro-niches. A natural source for toluene in nature helps to explain the wide distribution and high population levels of anaerobic toluene degraders in nature. All these isolates resemble *Azoarcus tolulyticus* (Fries et al., 1994; Zhou et al., 1995) from tests done so far; further comparisons are under way to confirm their identity.

TCE is a solvent that is postulated to be co-oxidized by many oxygenase enzyme systems. We compared the aerobic versus denitrifying growth of toluene-degraders and the effects of TCE levels on the isolates capable of growth under these two oxic conditions. We demonstrated that anaerobic pathways are not effective on TCE co-metabolism. This provides additional evidence of the requirement for O_2 and oxygenases for TCE co-metabolism.

The presence on this site of aromatic-degrading denitrifiers is an important consideration for bioremediation technologies. Populations of TCE degraders may still be enriched under anaerobic conditions so that once any O_2 is added the TCE oxidation rate may proceed at a higher rate.

Acknowledgments

We thank Gary Hopkins for collecting the samples from the aquifer during the field experiment. This work was supported by EPA , Grant N° CR 822029-01-0 and the NSF Center for Microbial Ecology, Grant N° BIR 9120006.

List of References

Alexander, M. 1982. Most probable number method for microbial populations, p. 815-820. *In* A. L. Page (ed.), Methods of soil analysis. Part 2. Chemical and microbiological properties. American Society of agronomy, Madison, Wi, USA.

Altenschmidt, U. and G. Fuchs. 1992. Anaerobic toluene oxidation to benzylalcohol and benzaldehyde in a denitrifying Pseudomonas strain. J. Bacteriol. **174**:4860-4862

Bakker, G. 1977. Anaerobic degradation of aromatic compounds in the presence of nitrate. FEMS Letters 1:103-108.

Carr, G. J. and S. J. Ferguson. 1990. The nitric oxide reductase of *Paracoccus denitrificans*. Biochem. J. **269**:423-430.

Chee-Sanford, J., J. W. Frost, M. R. Fries, J. Zhou, and J. M. Tiedje. 1995. Characteristics of a denitrifying toluene-degrading bacterium, Azoarcus tolulitycus strain Tol-4, and evidence for its anaerobic toluene mineralization pathway involving acetyl-CoA and cinnamoyl-CoA. Submmited for publication.

Chee-Sanford, J., M. R. Fries, and J. M. Tiedje. 1992. Anaerobic degradation of toluene under denitrifying conditions in bacterial isolate Tol-4, abst. Q-233, p.374. Abstr. 92th Annu. Meet. Am. Soc. Microbiol. 1992.

Coyne, M. S. A. Arunakumari, B. A. B. A. Averil, and J. M.. Tiedje. 1989. Immunological identification and distribution of dissimilatory heme cd_1 and nonheme copper nitrite reductases in denitrifying bacteria. Appl. Environ. Microbiol. **55**:2924-2931.

Demastia, M., T. Turk, and T. C.. Hollocher. 1991. Nitric oxide reductase. Purification from *Paracoccus denitrificans* with use of a single collumn and some characteristics. J. Biol. Chem. **266**:10899-10905.

Dolfing, J., J. Zeyer, P. Binder-Eicher, and R. P. Schwarzenbach. 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. **154**:336-341.

Edwards, E. A., L. E. Wills, D. Grbic-Galic, and M. Reinhard. 1993. Anaerobic degradation of toluene and xylene under sulfate-reducing conditions. Appl. Environ. Microbiol. **58**:794-800.

Elmerich, C. ,B. L. Dreyfus, and J. P. Aubert. 1982. Genetic analyses of nitrogen fixation in a tropical fast-growing *Rhizobium*. EMBO J. **1**:499-503.

Evans, P. J., D. T. Mang, K. S. Kim, and L. Y. Young. 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. **57**:1139- 1145.

Evans, P. J.,W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young. 1992. Metabolites formed during the anaerobic transformation of toluene and o-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. **58**:496-501.

Firestone, M. K., R. B. Firestone and J. M. Tiedje. 1979. Nitric oxide as an intermediate in denitrification: evidence from nitrogen-13 isotope exchange. Biochem. Biophys. Res. Commun. **91**:10-16.

Fischer, C. and F. Juttner. 1994. Isolation of a new toluene producing Clostridium from anoxic sediments of a freshwater lake. Abstr. Annu. Meet. Swiss Soc. Microbiol. 1994.

Fries, M. R., J.-Z Zhou, J. Chee-Sanford, and J. M. Tiedje. 1994. Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats. Appl. Environ. Microbiol. **60**:2802-2810.

Gamble, T. N., M. R. Betlach, and J. M. Tiedje. 1977. Numerically dominant denitrifying bacteria from world soils. Appl. Environ. Microbiol. **33**:926-939.

Gibson, D. T., and V. Subramanian. 1984. Microbial degradation of aromatic hydrocarbons, p. 181-252. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.

Grbic-Galic, D., and T. M. Vogel. 1987. Transformation of toluene and benzene by mixed methnogenic cultures. Appl. Environ. Microbiol. **53**:254-260.

Heiss, B., K. Frunzke and W. Zumft. 1989. Formation of the N-N bond from nitric oxide by menbrane-bound cytochrome *bc* complex of nitrate-respiring (denitrifying) *Pseudomonas stutzeri.* J. Bacteriol. **171**:3288-3297.

Hochstein, L. I. and G. A. Tomlinson. 1988. The enzymes associated with denitrification. Annu. Rev. Microbiol. **42**:231-261.

Hopkins, G. D., L. Semprini, and P. L. McCarty. 1993. Microcosm and In situ field studies of enhanced biotransformation of trichloroethylene by phenol-using microorganisms. Appl. Environ. Microbiol. **59**:2277-2285.

Hutchins, S.R. 1991. Biodegradation of aromatic hydrocarbons by aquifer microorganisms using oxygen, nitrate, or nitrous oxide as the terminal electron aceptor. Appl. Environ. Microbiol. **57**:2403-2407.

Hutchins, S.R., W. C. Downs, J. T. Wilson, G.B. Smith, D.A. Kovacs, D. D. Fine, R. H. Douglass, and D.J. Hendrix. 1991. Effect of nitrate addition on

biorestoration of fuel-contaminated aquifer: Field demonstration. Groundwater 29:68-76.

Hutchins, S.R., G.W. Sewell, D.A. Kovacs, and G.A. Smith. 1991a. Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. Environ. Sci. Technol. 25:68-76.

Juttner, F. 1988. Benzene in the anoxic hypolimnion of a freshwater lake. Naturwissenschaften. 75:151-153.

Juttner, F. 1990. Distribution of toluene in tratified lakes and river dams of southwest Germany. Verh. Internat. Verein. Limnl. 24:279-281.

Juttner, F. 1991. Formation of toluene by microorganisms from anoxic freshwater sediments. J. Anal. Chem. 339:785-787.

Juttner, F., and J. J. Henatsch. 1986. Anoxic hypolimnion is a significant source of biogenic toluene. Nature. 223:797-798.

Krieg, N. R. 1981. Enrichment and isolation, p. 112-142. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips. (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.

Lovley, D. R., M. J. Baedecker, D. J. Lonergan, I. M. Cozzarelli, E. J. P. Phillips, and D. I. Segal. 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature 339:297-300.

Lovley,. R., and D. J. Lonergan. 1990. Anaerobic oxidation of toluene, phenol, and p-cresol by the dissimilatory iron reducing bacterium GS-15. Appl. Environ. Microbiol. 56:1858-1864.

Martin, J. P., and K. Haider. 1969. Phenolic polymers of *Stachybotrys atra*, *Stachybotrys chartarum* and *Epicoccum nigrum* in relation to humic acid formation. Soil Sci. 107:260-270.

Murray, R. E., L. L. Parsons, and M. S. Smith. 1992. Competition between two isolates of denitrifying bacteria added to soil. Appl. Environ. Microbiol. 58:3890-3895.

Rabus, R., R. Nordhaus, W. Ludwig, and F. Widdel. 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. 59:1444-1451.

Roberts, P. V., G. D. Hopkins, D. M. Mackay and L. Semprini. 1990. A field evaluation of in-situ biodegradation of chlorinated ethenes: Part 1, Methodology and field site characterization. Ground water. **28**:591-604.

Schocher, R. J., B. Seyfried, F. Vazquez and J. Zeyer. 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. **157**:7-12.

Seyfried, B., G. Glod, R. Schocher, A. Tschech, and J. Zeyer. 1994. Initial reactions in the anaerobic oxidation of toluene and m-xylene by denitrifying bacteria. Appl. Environ. Microbiol. **60**:4047-4052.

Shapleigh, J. P., and W. J. Payne. 1985. Differentiation of *c,d1* cytochrome and copper nitrite reductase production in denitrifiers. FEMS Microbiol. Lett. **26**:275-279.

Shoun, H., and T. Tanimoto. 1991. Denitrification by the fungus *Fusarium oxysporum* and involvement of cytochrome P-450 in the respiratory nitrite reduction. J. Biol. Chem. **266**:11078-11082.

Stouthamer, A. H. 1988. Dissimilatory reduction of oxidized nitrogen compounds. p. 245-303. ***In***: A. J. B. Zehnder (ed), Biology of anaerobic microorganisms. John Wiley & Sons, Inc., New York.

Tiedje, J. M. 1982. Denitrification, p. 1011-1026. ***In*** A. L. Page (ed.), Methods of soil analysis. Part 2. Chemical and microbiological properties. American Society of agronomy, Madison, Wi, USA.

Tiedje, J. M. 1988. Ecology of denitrification and dissimilatory nitrate reduction to ammonium, p. 179-244. ***In*** A. Zehnder (ed.), Biology of anaerobic microorganisms. Jonh Wiley& Sons, Inc., New York.

Wilson, B. H , G. B. Smith and J. F. Rees. 1986. Biotransformation of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: a microcosm study. Environ. Sci. Technol. **20**:997-1002.

Zhou, J.-Z., M. R. Fries, J. Chee-Sanford, and J. M. Tiedje. 1995. Phylogenetic analyses of a new group of denitrifiers capabla of anaerobic growth on toluene and description of Azoarcus tolulyticus sp. nov. Intern. Journal of System. Bacteriol. 45:.....-.....

Zumft, W. G. 1991. The denitrifying prokariotes. p.554-582, ***In*** A. Balows, et al., (ed.), The prokaryotes, second edition. Springer-Verlag, Ina., New York.

Chapter V

Isolation, characterization, and distribution of denitrifying toluene degraders from a variety of habitats

APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Aug. 1994, p. 2802–2810
0099-2240/94/$04.00+0

Vol. 60, No. 8

Isolation, Characterization, and Distribution of Denitrifying Toluene Degraders from a Variety of Habitats

MARCOS R. FRIES,[1] JIZHONG ZHOU,[1] JOANNE CHEE-SANFORD,[2] AND JAMES M. TIEDJE[1,2]*

Departments of Crop and Soil Sciences[1] and of Microbiology,[2] Center for Microbial Ecology, Michigan State University, East Lansing, Michigan 48824

Received 18 March 1994/Accepted 6 June 1994

Enrichments capable of toluene degradation under O_2-free denitrifying conditions were established with diverse inocula including agricultural soils, compost, aquifer material, and contaminated soil samples from different geographic regions of the world. Successful enrichment was strongly dependent on the initial use of relatively low toluene concentrations, typically 5 ppm. From the enrichments showing positive activity for toluene degradation, 10 bacterial isolates were obtained. Fingerprints generated by PCR-amplified DNA, with repetitive extragenic palindromic sequence primers, showed that eight of these isolates were different. Under aerobic conditions, all eight isolates degraded toluene, five degraded ethylbenzene, three consumed benzene, and one degraded chlorobenzene. *meta*-Xylene was the only other substrate used anaerobically and was used by only one isolate. All isolates were motile gram-negative rods, produced N_2 from denitrification, and did not hydrolyze starch. All strains but one fixed nitrogen as judged by ethylene production from acetylene, but only four strains hybridized to the *nifHDK* genes. All strains appeared to have heme nitrite reductase since their DNA hybridized to the heme (*nirS*) but not to the Cu (*nirU*) genes. Five strains hybridized to a toluene *ortho*-hydroxylase catabolic probe, and two of those also hybridized to a toluene *meta*-hydroxylase probe. Partial sequences of the 16S rRNA genes of all isolates showed substantial similarity to 16S rRNA sequences of *Azoarcus* sp. Physiological, morphological, fatty acid, and 16S rRNA analyses indicated that these strains were closely related to each other and that they belong to the genus *Azoarcus*. The activity and isolation of at least one toluene-degrading denitrifier from the majority of the habitat types studied suggest that microbes with the capacity to grow anaerobically on toluene are common in nature.

The monoaromatic hydrocarbons known as BTEX (benzene, toluene, ethylbenzene, and xylenes) are one of the major problems in environmental pollution. Their presence in groundwater is a widespread problem because of the leakage of underground petroleum storage tanks and spills at petroleum production wells, refineries, pipelines, and distribution terminals. Many governments have established cleanup standards for these chemicals in groundwater because of their carcinogenic potential (4, 14).

Biodegradation of BTEX under aerobic conditions is well-known; oxygen is utilized for ring activation and cleavage and serves as the electron acceptor for the complete oxidation of this compounds (15). The availability of oxygen, due to its low solubility in water and its low rate of transport through saturated porous matrices such as soil and sediments, is usually the rate-limiting parameter for BTEX removal from contaminated sites. Therefore, BTEX biodegradation in the absence of oxygen would be a very beneficial remediation process. It has only been in recent years that anaerobic degradation of these compounds has been conclusively established. Of the BTEX class of compounds, toluene seems to be the most easily degraded under anaerobic conditions. The degradation of toluene under denitrifying (3, 8, 11, 12, 21, 25, 36), methanogenic (16, 40), sulfate-reducing (9, 32), and ferric iron-reducing (27, 28) conditions has been reported. For bioremediation, the most attractive electron acceptor is nitrate since it is water soluble, not costly, and not seriously toxic and does not react with other inorganic species present, such as ferric iron. However, very little is known about the organisms responsible and how widely they are distributed in nature.

We report here on a new group of bacteria that grow on toluene under denitrifying conditions and show that they appear to be widely distributed in nature.

MATERIALS AND METHODS

Enrichments and isolations. Soils and sediments were collected independently from various locations and handled by procedures to prevent any cross-contamination. Samples (5 to 10 g) were incubated without shaking with 10 ml of basal salts (BS) medium (31) amended with 5 mM KNO_3 in sterile centrifuge tubes. After 3 days of incubation, the enrichments were centrifuged (1,300 × *g* for 10 min), the supernatant was removed, and fresh sterile medium was added to the samples. The samples were vortexed and reincubated. This protocol was repeated one more time to deplete easily oxidizable carbon from the samples that could potentially reduce the selection for anaerobic toluene degraders. After the third incubation period, the samples were centrifuged and the pellet was resuspended in fresh medium; the large soil particles were then allowed to settle. The tubes were transported to a Coy anaerobic chamber, and 5 ml of the supernatant was transferred with a sterile syringe to a serum bottle containing 45 ml of BS medium containing 5 mM KNO_3 and 5 ppm of toluene that had been prepared by strict anaerobic protocol. Toluene disappearance was evaluated by headspace analyses. Enrichments positive for toluene degradation were spiked again with toluene at a concentration of 5 ppm and then at 25 ppm. The bottles were inverted and incubated, and the production of bubbles on the surface of the Teflon-lined septa indicated denitrification activity. Once bubble production ceased, typi-

* Corresponding author. Mailing address: Center for Microbial Ecology, Plant and Soil Science Building, Michigan State University, East Lansing, MI 48824-1325. Fax: (517) 353-2917. Electronic mail address: 21394jmt@msu.edu.

cally after 3 days at this last stage of enrichment, three serial transfers of a 10% inoculum were made per sample into fresh medium containing 25 ppm of toluene. Anaerobic manipulations throughout this work were done in the anaerobic chamber and the resultant headspace of the bottles, and agar plates was nominally 10% H_2-90% N_2. The incubation temperature throughout this work was 30°C.

These enriched samples were serially diluted and plated on two different solid media: (i) BS medium plus 5 mM NO_3^- plus toluene vapors and (ii) modified R2A (M-R2A), based on the original composition provided by Difco (Detroit, Mich.). M-R2A had the following salt mixture composition per liter: KH_2PO_4, 0.25 g; K_2HPO_4, 0.4 g; KNO_3, 0.505 g; $CaCl_2 \cdot 2H_2O$, 0.015 g; $MgCl_2 \cdot 6H_2O$, 0.02 g; $FeSO_4 \cdot 7H_2O$, 0.007 g; Na_2SO_4, 0.005 g; NH_4Cl, 0.8 g; $MnCl_2 \cdot 4H_2O$, 5 mg; H_3BO_3, 0.5 mg; $ZnCl_2$, 0.5 mg; $CoCl_2 \cdot 6H_2O$, 0.5 mg; $NiSO_4 \cdot 6H_2O$, 0.5 mg; $CuCl_2 \cdot 2H_2O$, 0.3 mg; and $NaMoO_2 \cdot 2H_2O$, 0.01 mg. For solid medium, 15 g of Bacto agar (Difco) was added. The pH was adjusted to 7.0 before autoclaving. The carbon sources and their concentrations, per liter, were yeast extract, 0.5 g; peptone, 0.5 g; Casamino Acids, 0.5 g; dextrose, 0.5 g; soluble starch, 0.5 g; and sodium pyruvate, 0.5 g. Glass petri dishes were used for toluene vapor-based growth, and toluene was added to a small vial inside an incubation jar. The amount of toluene added to provide a final concentration of 25 ppm was calculated in the basis of the total volume of agar in the incubation jar. After 1 week of incubation, sufficient toluene to provide 25 ppm was again added. The jar used in the toluene vapor experiments was sealed with a Teflon-lined aluminum sheet (Cole-Palmer, Chicago, Ill.) to prevent toluene absorption by the rubber sealer of the jar. The plates were incubated under anaerobic and aerobic conditions.

For purification of isolates, single colonies from different plates and different dilutions were selected and purified at least three times on M-R2A before further evaluation. Confirmation of toluene degradation under denitrifying conditions was done by transferring a heavy inoculum of each isolate to sterile 20-ml vials, recapping the vials with foam plugs, and transporting the vials to an anaerobic chamber for headspace gas exchange (at least 6 h), after which 10 ml of anaerobic BS medium–NO_3^-–25 ppm of toluene was added. The vials were sealed with sterile butyl rubber Teflon-lined septa and incubated for at least 2 weeks before toluene disappearance was evaluated. As a control, medium without nitrate containing cells and toluene was incubated at the same time as the medium with nitrate and was used as a reference to determine nitrate-dependent toluene removal.

Characterization of isolates. Cell size and shape were observed by phase-contrast microscopy for cells grown anaerobically on toluene in liquid medium and for cells grown on M-R2A agar. The latter were resuspended in water, and 5 μl was added to a microscopic slide containing 5 μl of molten 0.4% agarose solution, mixed gently, and covered with a coverslip. The medium for the nitrogen fixation assay consisted of BS free of nitrogen sources supplemented with filter-sterilized solutions of (final concentration per liter) biotin (0.1 mg), $Na_2MoO_4 \cdot 2H_2O$ (0.002 g), malic acid (5 g), and KOH (4.5 g), adjusted to pH 7. Agarose (0.07%) was added to provide for establishment of microaerobic conditions (7). Inocula were grown on liquid M-R2A, and cells washed in saline solution were inoculated into 20-ml serum vials containing 5 ml of medium. The vials were incubated for 48 h, and nitrogenase activity was evaluated by the acetylene reduction assay (1) after 6 and 24 h of incubation with acetylene. *Azospirillum brasiliense* sp7 was used as a positive control.

Denitrification was evaluated by observing gas bubble formation in inverted tubes and by measuring the presence of N_2O in the vial headspace by gas chromatography (GC). The denitrification medium consisted of the salt mixture and the following carbon sources (final concentration per liter): Proteose peptone (0.5 g), yeast extract (0.5 g), Casamino Acids (0.5 g), potassium acetate (1 g), and sodium succinate (1 g). Argon was the headspace for the samples analyzed by GC.

Starch hydrolysis was performed by the standard method (37) on M-R2A plates supplemented with 0.2% starch.

The isolates analyzed for cellular fatty acids were precultured on M-R2A and streaked onto plates containing 0.3% (wt/vol) tryptic soy broth solidified with 15 g of tryptic soy agar (TSA) per liter (24). At least three plates of each isolate were incubated for 72 to 96 h in order to obtain enough biomass for the analysis. Cells were harvested from the plates by scraping with a sterile loop. Saponification, methylation, and extraction were performed by the procedure described previously (35). Cluster analysis was carried out by using an in-house cluster program and the MIDI software.

Detectable plasmids were screened for in isolates cultured anaerobically on toluene-BS-NO_3^- medium. Cells were lysed, and plasmids were screened as described by Kado and Liu (23).

The anaerobic growth rate of the isolates in BS (containing one-fifth of the original EDTA concentration)–5 mM NO_3^-–50 ppm of toluene was determined by measuring the optical density at 600 nm. The inoculum was grown under the same conditions. Growth in TSA was evaluated every 12 h, and the colony size was compared with that of M-R2A-grown cells.

To test for the isolates' capabilities to degrade benzene; ethylbenzene; *o*-, *m*-, and *p*-xylenes; and chlorobenzene, inocula were grown on BS-NO_3^--toluene medium under both denitrifying and aerobic conditions. Inocula (0.5 ml) were transferred to 20-ml of sterile auto sampler vials. For aerobic growth, 4.5 ml of BS–NO_3^-–25 ppm of the aromatic substrate was added to the vials, and the vials were sealed with Teflon-lined stoppers. For anaerobic growth, the vials were prepared similarly except that the media with the aromatic substrates were added in the anaerobic chamber. Positive degradation activity was defined as at least 80% loss of substrate in the headspace as measured by GC analysis, compared with that for noninoculated controls.

Molecular methods. Repetitive extragenic palindromic (REP)-PCR patterns were obtained from cells with Rep-1 and Rep-2 primers by PCR (5). Amplification was performed with a model 9600 Perkin-Elmer Cetus thermocycler. Products (10 μl) were separated by electrophoresis in 1.5% agarose gels and stained with ethidium bromide. Amplification was primarily done by using individual colonies grown on M-R2A, but for isolates with poor or no amplification, DNA extracted from cells was used as the template for PCR amplification. For isolates with very similar patterns, the same stock of primers and reagents were used in the analysis.

The gene probes used are described in Table 1. *Escherichia coli* cultures carrying the plasmids with probes were grown for plasmid amplification in the presence of the appropriate antibiotic. Plasmids were extracted by a standard protocol (29). The probes were isolated as restriction fragments from their respective vectors in 1% low-melting-point agarose, purified with the Gene Clean kit (Bio 101, Inc., La Jolla, Calif.), and labelled with [α-^{32}P]dCTP (3,000 Ci/mM; Dupont, NEN Research Products, Wilmington, Del.) by using a random hexamer priming kit from Boehringer Mannheim Biochemicals. Labelled probes were separated from unincorporated nucleotides prior to use with a spun column (29). The probes were used at approximately 10^6 cpm/ml of hybridization fluid.

Genomic DNA was obtained by standard methods (10) from

TABLE 1. List of DNA probes used in this study

Organism (probe source)	Gene encoded and probe size	Plasmid	Laboratory source and reference[a]
Pseudomonas putida PaW1	Methyl monooxygenase (hydroxylase and NADH-ferredoxin reductase), 2.35 kb (*SalI-Hin*dIII)	pG5H2836	S. Harayama (18)
Pseudomonas putida F1	Toluene dioxygenase (large and small subunits of oxygenase, ferrodoxin, and part of reductase), 3.5 kb (*Eco*RI-*Bgl*II)	pDTG601	D. Gibson (44)
Pseudomonas mendocina KR	Toluene *para*-hydroxylase (monooxygenase and ferredoxin), 3.6 kb (*Eco*RI-*Eco*RI)	pMY421	M. DeFlaun (42)
Pseudomonas pickettii PKO1	Toluene *meta*-hydroxylase (α subunit of monooxygenase), 0.68 kb (*Apa*I-*Ava*I)	pAB14ΔAva I	R. Olsen (2, 30)
Pseudomonas sp. strain JS-150	Toluene *ortho*-hydroxylase, 2.2 kb (*Eco*RV-*Hin*dIII)	pRO20116	R. Olsen (22)[b]
Pseudomonas stutzeri JM300	Heme containing nitrite reductase, 0.7 kb(*Dde*I-*Dde*I)	pBsGTh 2.4	J. Tiedje (38)
Pseudomonas sp. strain 179	Copper containing nitrite reductase, 1.9 kb (*Eco*RI-*Bam*HI)	pRTc1.9	J. Tiedje (41)
Pseudomonas stutzeri ZoBell	Nitrous oxide reductase, 1.2 kb (*Pst*I-*Pst*I)		W. Zumft (39)
Micrococcus luteus	23S rRNA, 0.47 kb (*Eco*RI-*Hin*dIII)	pAR17	K. Schleifer (33)
Rhizobium sp.	Nitrogenase (*nifHDK* genes), 3.6 kb (*Bgl*II-*Xho*I)	pRS2	F. de Bruijn (10)

[a] Laboratories from which the clones were obtained from.
[b] This strain was provided by J. C. Spain (17). The probe has strong hybridization to *P. cepacia* G-4 (22) (Fig. 3C) and likely reflects G-4-like sequences as well.

pure cultures of isolates and selected strains grown on M-R2A broth under aerobic conditions. Restriction endonuclease digestion of DNA was performed according to the manufacturer's specifications. Digested DNA was size fractioned by electrophoresis in 0.7% agarose gels and transferred to nitrocellulose (polyester-supported BAS 68380; Schleicher & Schuell, Keene, N.H.) as previously described (29) with 20× SSPE (1× SSPE is 0.18 M NaCl, 10 mM NaH_2PO_4 [pH 7.04], and 1 mM EDTA). The DNA on the filters was cross-linked by UV light (Stratagene, La Jolla, Calif.). The solutions used for DNA hybridization analysis have been described elsewhere (19). The membranes were prehybridized for at least 24 h in heat-sealed bags containing 100 μl of prehybridization fluid per cm^2 of filter. Prehybridization fluid contained 5× Denhardt solution, 5× SSPE, 50% formamide, and 200 μg of sonicated and denatured salmon sperm DNA per ml. The hybridization solution was the same as that for prehybridization but included 10% (wt/vol) dextran sulfate and was added at 50 $\mu l/cm^2$ of filter. The membranes were incubated for at least 24 h. Two hybridization temperatures, 30°C (low stringency) and 42°C (high stringency), were used. After hybridization, the filters were washed once for 15 min with agitation at 30°C with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–0.1% sodium dodecyl sulfate (SDS). For low-stringency hybridization, a second wash for 30 min with 0.5× SSC–0.1% SDS was performed. For high-stringency hybridization, a second wash for 15 min with 0.5× SSC–0.1% SDS followed by a third wash with 0.1× SSC–0.1% SDS was performed. This was followed by a final wash at 55°C for 30 min with the last solution. After the washes, hybridization signals were visualized by using the Betascope radioactive blot analyzer (Betagen Corp., Waltham, Mass.) or by autoradiography with X-Omat AR film (Kodak, Rochester, N.Y.) exposed at −70°C with a Quanta III (Sigma, St. Louis, Mo.) intensifying screen. Exposure times were 1 to 3 days, depending on the intensity of the radioactive signal. For reuse of the same blot for another probe, the blots were stripped by washing for 10 min in boiling water–0.1% SDS at least two times, depending on the signal left on the blot after evaluation with the Betascope. A final wash with 2× SSPE–0.1% SDS for 10 min completed the stripping protocol.

The 16S rRNA gene was amplified from genomic DNA by PCR and cloned into a plasmid vector as described previously (43). The 16S rRNA gene was sequenced from both directions with an automated fluorescence sequencer (model 373A; Applied Biosystems, Foster City, Calif.) with the forward and reverse primers, which span *E. coli* 16S rRNA gene positions 785 to 805 and 1115 to 1100, respectively. The DNA sequences were compared with those in the Ribosomal Database Project (26) and analyzed by using the programs in the Genetics Computer Group software package (6) and in PHYLIP phylogeny inference package (13).

Analytical methods. Toluene; ethylbenzene; *o*-, *m*-, and *p*-xylenes; benzene; and chlorobenzene concentrations were measured with a GC equipped with an flame ionization detector, a DB-624 capillary column (J&W Scientific, Folson, Calif.), and a headspace sampler. The vials and bottles were equilibrated at 30°C, the column was equilibrated at 90°C, and the injector and detector were equilibrated at 200°C. He was the carrier gas. Acetylene and ethylene were assayed by a GC/-flame ionization detection by using a DB-23 megabore column (J&W Scientific) at 75°C, an injector at 160°C, and a detector at 320°C with N_2 as the gas carrier. NO_3^- and NO_2^- concentrations in culture supernatant were determined by high-performance liquid chromatography (HPLC) analysis using a Partisil 10 SAX column (Whatman, Clifton, N.J.), UV detection at 210 nm, and 50 mM phosphate (pH 3.0) as the eluant. N_2O was measured by GC-electron capture detection with a Poropak Q column at 55°C, 300°C detector temperature, and 95% argon–5% methane as the carrier gas.

Nucleotide sequence accession numbers. The partial 16S rRNA sequences of all new isolates have been placed in Genbank under accession no. L 33687 to L33694.

RESULTS

Enrichments. Successful enrichments for denitrifying toluene degraders were obtained from about half of the samples from both chemically contaminated and noncontaminated sites (Table 2). Enrichments positive for toluene degradation were obtained from widely separated and dissimilar habitats. Ten isolates that were confirmed as pure cultures and were able to grow on toluene in the presence of nitrate and the complete absence of oxygen were obtained (Table 2). The isolates also came from a wide range of environments. Additional clones that appeared to have toluene-degrading denitrifying activity were obtained, but they were not studied further because it was too difficult to confirm purity, growth was too variable, or they appeared to be identical or closely related to isolates already in pure culture by REP-PCR analysis.

TABLE 2. Source of inoculum, number of enrichments with positive activity for toluene degradation under denitrifying conditions, and number of isolates obtained with this activity from each sample

Source of inoculum and description of sample	Enrichments (no. positive/ total no. tested)	Isolates obtained[a] (strain no.)
Noncontaminated		
Cameroon, rainforest soil	1/2	IU
Michigan, muck soil	2/2	2 (Td-20, Td-21)
Michigan, compost pile	1/1	1 (Td-15)
Michigan, agricultural soil	1/2	1 (Td-16)
Hawaii, Big Island forest soil	2/3	INA
Hawaii, Kauai sugar cane soil	0/1	
Siberia, Kolyma Valley permafrost soil	0/2	
Total	7/13	4
Contaminated with organic pollutants		
Sao Paulo, Brazil; industrial waste	2/3	2 (Td-17, Td-19)
Rio Grande do Sul, Brazil; industrial sludge	2/3	INA
Ontario, Canada; pulp mill lagoon sediment	1/4	IU
Bear Lake, Michigan; aquifer, sand, petroleum (24 to 26-m deep)	1/1	1 (GR-3)
Wexford, Michigan; aquifer, sand, petroleum	0/1	
Huntington Beach, California; marine, petroleum	1/1	1 (Td-3)
Washington State	2/2	2 (Td-1, Td-2)
Six undescribed chemically contaminated soils	1/6	IU
Total	10/21	6

[a] IU, isolation unsuccessful; INA, isolation not attempted.

Successful enrichment and isolation were strongly dependent on the use of relatively low toluene concentrations, typically 5 ppm. This strategy was used because our early isolation and enrichment attempts were unsuccessful after extensive effort with 250 ppm and because we had noted a considerable increase in the most probable number estimate of aerobic toluene degraders when the toluene concentration was decreased from 250 to 50 ppm and again when reduced to 5 ppm. Thus, we reasoned that toluene toxicity could also be an important factor for successful enrichment and isolation of anaerobic toluene degraders. Once isolated, the cultures were routinely cultured in 25 ppm of toluene and, once growing, could be fed 50 ppm to obtain higher cell yields.

All isolates were obtained from plates of the M-R2A medium incubated aerobically. Some isolates were obtained from the anaerobic toluene vapors plus nitrate or M-R2A plus nitrate medium, but by REP-PCR they were identical to the ones isolated from the same source on the aerobic medium. In all cases, the denitrifying toluene degraders were pinpoint colonies. Larger colonies were also picked, but these isolates were either denitrifiers or toluene degraders. Some of them were capable of both functions but did not carry out both under the same conditions. In some cases, it was very difficult to separate contaminating cells from the denitrifying toluene degrader. Purity was based primarily on uniform and repeated colony morphology after at least 2 weeks of incubation.

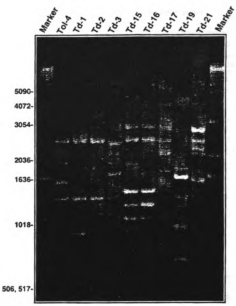


FIG. 1. REP-PCR fingerprint patterns of toluene-degrader denitrifier isolates generated by using chromosomal DNA. Lanes 1 and 11 show size markers, with the base pairs indicated on the left.

Consistent REP-PCR patterns and consistent cell morphology were used as confirmatory methods for purity.

We also carried out parallel enrichments for denitrifying benzene degraders, using the same enrichment conditions and environmental samples as those used for the toluene enrichments. No activity could be confirmed by measuring benzene removal from any of the enrichments.

We used REP-PCR to screen for sufficiently different strains for further study. There were eight distinct profiles from the 10 confirmed denitrifying toluene degraders (Fig. 1). One of the isolates, GR-3, had a REP-PCR pattern identical to that of our previous isolate, Tol-4 (3), and was not studied further. One set of isolates with identical patterns came from the same site (Michigan muck soil) and may be siblings; therefore, only isolate Td-21 was studied further. The other set of isolates with identical patterns came from Michigan agricultural soil and a compost pile; only the compost isolate, Td-15, was studied further since it came from a very different environment.

Characteristics. The ability of the seven new isolates to degrade related aromatic compounds under aerobic or anaerobic conditions is very limited, except that all isolates can also degrade toluene aerobically (Table 3). Several of the cultures were initially negative for aerobic toluene use; but after repeated experiments and optimizing conditions, all were shown to be capable of aerobic toluene consumption, albeit some showed weak ability. Five strains could use the alkylated analog, ethylbenzene, aerobically, but none could use it anaerobically. Three isolates used benzene, and one used chlorobenzene aerobically. *meta*-Xylene was the only substrate used anaerobically other than toluene, and it was used by only one isolate. The anaerobic pathway seems very specific for toluene, and the aerobic substrate range is much more limited than it is for the well-studied aerobic toluene degraders (Table 3).

TABLE 3. Removal of different substrates by toluene-degrading isolates and well-known aerobic strains in BS medium under aerobic and anaerobic (denitrifying) conditions after 2 weeks of incubation[a]

Isolate	Benzene		Toluene		Ethyl benzene		*o*-Xylene		*m*-Xylene		*p*-Xylene		Chlorobenzene	
	Ae[b]	Ana[c]	Ae	Ana	Ae	Ana	Ae	Ana	Ae	Ana	Ae	Ana	Ae	Ana
Tol-4	−	−	+	+	+	−	−	−	−	−	−	−	−	−
Td-1	−	−	+	+	+	−	−	−	−	−	−	−	−	−
Td-2	−	−	±	+	−	−	−	−	−	−	−	−	−	−
Td-3	±	−	±	+	−	−	−	−	−	−	−	−	−	−
Td-15	−	−	+	+	+	−	−	−	−	+	−	−	−	−
Td-17	+	−	+	−	+	−	−	−	−	−	−	−	−	−
Td-19	−	−	±	−	−	−	−	−	−	−	−	−	+	−
Td-21	−	−	+	+	+	−	−	−	−	−	−	−	−	−
Pseudomonas cepacia G4	+	−	+	−	+	−	+	−	−	−	+	−	+	−
Pseudomonas mendocina KR	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Pseudomonas picketti PKO1	+	−	+	−	+	−	+	−	+	−	+	−	+	−
Pseudomonas putida F1	+	−	+	−	+	−	+	−	+	−	+	−	−	−
Pseudomonas putida PaW1	+	−	+	−	+	−	+	−	−	−	+	−	−	−

[a] Denotes more than 80% removal from headspace vial: −, negative activity: ±, activity often delayed. All substrates at 25 ppm concentration.
[b] Ae, aerobic conditions.
[c] Ana, anaerobic (denitrifying) conditions.

All seven isolates had similar major features but also showed minor differences which confirmed that they were not identical strains (Table 4). All were gram-negative rods and, motile, produced N_2 from denitrification, and did not hydrolyze starch. They did not grow well aerobically on complex media such as TSA. Growth could begin to be seen on 1/10 strength TSA as very sparse tiny colonies only after 48 h of incubation. M-R2A is the best medium that we have found for growth on plates. Colonies of 1 to 4 mm in diameter can be obtained after 36 to 72 h of incubation. N_2 fixation was shown by subsurface pellicle formation in semisolid medium free of combined nitrogen, by ethylene production from acetylene (all but isolate Td-15), and by DNA from four of seven strains hybridized to the *nifHDK* genes (Table 4).

The major fatty acids for all strains studied and their concentration ranges were cis:9 16:0 (42.3 to 61.9%), 16:0 (21.5 to 38.6%), 12:0 (4.4 to 12.9%), 3-OH-10:0 (1.7 to 7.7%), 14:0 (0.9 to 1.55%), cyclo 17:0 (0 to 4.11%), and 18:0 (less than 1%). Species identification based on total fatty acids and cluster analysis was not possible since the analysis gave a similarity index of less than 0.4 by using the MIDI database.

The morphology of all strains grown anaerobically on toluene was small rods, typically 1.4 to 2.1 μm in length (Fig. 2A). When grown on M-R2A agar, however, all formed longer cells, 2.1 to 2.8 μm (Fig. 2B), and some isolates (i.e., Td-3, Td-15, Td-17, and Td-19) had a tendency to form chains (Fig. 2C).

DNA isolated from the seven new isolates and five well-studied aerobic toluene degraders was digested and hybridized on Southern blots to various probes to determine which strains carried similar sequences. Hybridization with a universal 23S rRNA probe confirmed that sufficient DNA was in all lanes and that all strains were different (Fig. 3A). The probe for the denitrifying Cu-nitrite reductase gene (*nirU*) showed no hybridization (data not shown), but the heme-nitrite reductase probe (*nirS*) hybridized to all strains, suggesting that they are all denitrifiers with the heme-type enzyme (Fig. 3B). The nitrous oxide reductase probe (*nosZ*) hybridized to all strains and in different positions (data not shown). Probes for the first steps in all five aerobic toluene-degrading pathways were used, but only the probe for the *ortho*-hydroxylase (*Pseudomonas* sp. strain JS150) (Fig. 3C) and the *meta*-hydroxylase (*P. pickettii* PKO1) (Fig. 3D) genes showed hybridization. Five strains (all

TABLE 4. Summary of characteristics of the different denitrifying toluene degraders[a]

Isolate	Growth on M-R2A for 48 h	Growth on 1/10 TSA		Growth (h) rate[b]	Denitrification			Nitrogen fixation			Starch hydrolysis	Detectable plasmids
		48 h	96 h		N_2[c]	N_2O	NO_2^-	Pellicle[d]	Acetylene[e]	Probe[f]		
Tol-4	−	±	+	8–13	+	+	+	+	+	+	−	−
Td-1	+	±	−	6–7	−	+	±	+	+	+	−	−
Td-2	+	±	−	5–7	+	+	+	+	+	+	−	−
Td-3	+	−	−	7–8	+	+	+	+	+[g]	−	−	−
Td-15	+	−	+	6–7	+	+	+	−	−	−	−	−
Td-17	+	−	+	5–7	+	+	−	+	+	−	−	−
Td-19	+	±	+	5–7	−	+	+	+	+[g]	−	−	+
Td-21	+	−	+	5–7	+	+	+	+	+	+	−	−

[a] +, positive activity; −, negative activity; ±, variable activity.
[b] Cells grown on BS–50 ppm of toluene under denitrifying conditions. Doubling time is expressed in hours.
[c] Observation of bubbles on the surface of the Teflon-lined septum.
[d] Formation of growth pellicle on nitrogen-free medium.
[e] Detection of ethylene from acetylene after at least 24 h of incubation.
[f] Presence of distinct band on Southern blot hybridized with *nifHDK* probe.
[g] Ethylene production detectable only after 24 h of incubation with acetylene.

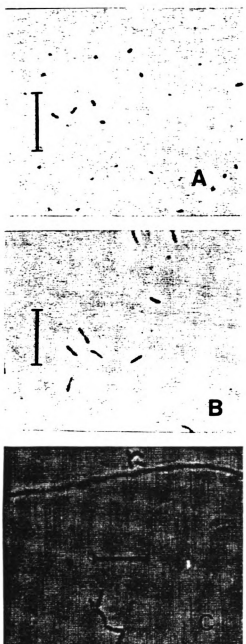


FIG. 2. Phase-contrast photomicrographs of isolates Td-2 (A), Td-3 (B), and Td-17 (C). Isolate Td-2 was grown on BS-toluene liquid medium to late exponential phase. Isolates Td-3 and Td-17 were grown on M-R2A solid medium for 48 h. Bars, 14 μm.

but isolates Td-3 and Td-15) showed strong signals to the *ortho*-hydroxylase probe. The *meta*-hydroxylase probe hybridized to DNA from two strains that also showed hybridization to the *ortho*-hydroxylase probes, but the hybridizing bands were in different positions for the two probes.

The sequence of approximately 280 nucleotide bases, corresponding to the *E. coli* 16S rRNA gene sequence from nucleotide 810 to 1090, was obtained for the seven isolates. The percent similarities among these isolates ranged from 97.8 to 100%. The partial sequences of these isolates showed strong similarity to 16S rRNA gene sequences of the nitrogen-fixing genus *Azoarcus* (20). The similarities are *Azoarcus* sp. strain Sbs2 (90.7 to 91.5%), Azoarcus sp. strain BH72 (92.6 to 94.1%), and *Azoarcus indigens* (93.4 to 94.9%). The phylogenetic tree constructed by the maximum parsimony method showed that all the toluene-denitrifying isolates form a phylogenetically coherent unit clustered with *A. indigens* and *Azoarcus* sp. strain BH72 (Fig. 4). Very similar tree topologies were also obtained by distance matrix and maximum likehood methods (data not shown).

DISCUSSION

Pure cultures of anaerobic toluene degraders have not been easy to isolate. After a decade of effort by many capable laboratories, seven isolates that use nitrate as an electron acceptor (3, 8, 12, 36), one that uses Fe(III) (28), and one that uses sulfate have been reported (32). This study yielded 10 new isolates and additional active enrichments. We believe that the most important reason for the improved success rate of enrichment and isolation was the strategy of avoiding toxicity by never exposing the culture to more than 5 ppm of toluene during the initial enrichment process. Also important were exhausting the residual available carbon before adding toluene, isolating cells by aerobic growth on M-R2A, and being sure to select the pinpoint colonies. This procedure, however, may have selected for only a certain group of denitrifying toluene degraders and may explain why we were unsuccessful in obtaining isolates from some of the active enrichments.

The seven new isolates appear to be closely related to each other and to be members of the genus *Azoarcus*. The identification is based on the fact that these isolates share the following key features with the described *Azoarcus* strains (20, 34): all 16S rRNA sequences fall within the cluster for this genus, they fix nitrogen, they have the same type and proportion of cellular fatty acids as do the described strains, and they have similar morphology when grown on complex medium. They do have some phenotypic and ecologic differences from the described *Azoarcus* strains. The new strains all denitrify and grow poorly on TSA, which are not characteristics of the previously described strains (34). Also, 11 of 12 previously described strains were isolated from the roots of tropical grasses and are considered to be rhizosphere-associative nitrogen fixers. None of our isolates came from plant rhizospheres. Three came from environments in which plants had recently grown (Michigan muck and agricultural soil), but four came from soils contaminated with chemical wastes in industrial areas, one from an aquifer 24 to 26 m underground and one from a compost pile. Thus, the ecological niche of the new isolates may be very different from that described for the previous isolates.

All our isolates are able to use toluene aerobically as well as anaerobically, which would not be expected if these are independent traits. Perhaps the pathways share some common steps, reflect a common phylogenetic heritage, or were a result of concurrent selection resulting from continued use of the

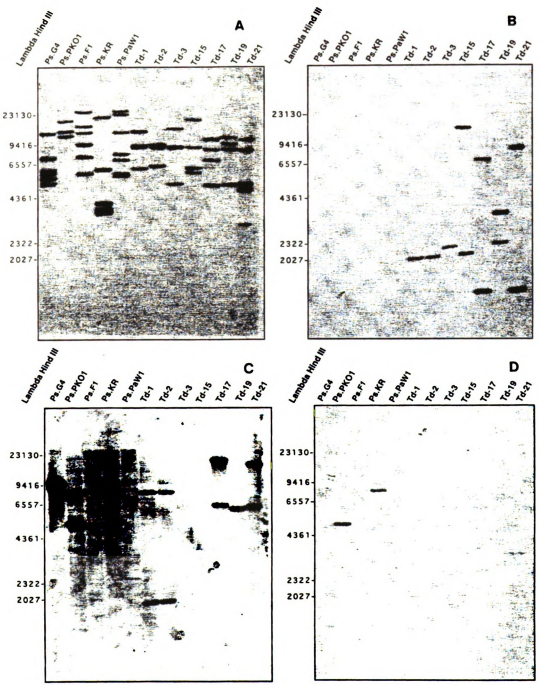


FIG. 3. Southern hybridization of genomic DNA from pure cultures of toluene degraders digested with *Eco*RI and hybridized with the following gene probe: universal 23S rRNA (A), heme nitrite reductase (B), toluene *ortho*-hydroxylase (C), and toluene *meta*-hydroxylase (D). Hybridizations were done under high (A and B) and low (C and D) stringency conditions. Size markers (in base pairs) are indicated on the left.

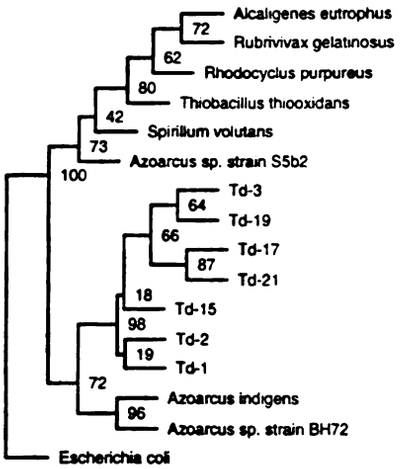


FIG. 4. Phylogenetic position of the toluene-degrading denitrifier (Td) isolates. This tree was constructed by using the programs SEQBOOT, DNAPARS, and CONSENSE in PHYLIP 3.5 and rooted by reference to *E. coli*. The numbers under the nodes are the bootstrap confidence estimates on the branches in 100 replicates. All other 16S rRNA gene sequences were obtained from the Ribosomal Database Project (26).

same substrate under both aerobic and anaerobic conditions. The phylogenetic heritage of the aerobic pathway at least is not necessarily expected because the aerobic toluene pathway has often been found on transmissible plasmids. The aerobic substrate range, however, is far more limited than that found for the well-studied aerobes, suggesting that the *Azoarcus* aerobic pathway(s) has unique features.

Hybridization at high stringency of the subunit probe for the toluene *ortho*-hydroxylase pathway to DNA from five of the *Azoarcus* isolates suggests the presence of this gene in these strains and hence that the *ortho*-hydroxylase pathway may be responsible for aerobic toluene metabolism. Two of the strains may also have the *meta*-hydroxylase pathway since they also hybridized to this probe at different positions on the Southern blot. Having three different toluene pathways, two aerobic and at least one anaerobic, in one strain is perhaps unexpected. Two of the strains had detectable plasmids, but the aerobic toluene pathway probes did not hybridize to the plasmids.

This study indicates that denitrifying toluene degraders are widely distributed in nature; and thus if nitrate were present or added, toluene should be removed. This conclusion is based on finding anaerobic toluene removal in such diverse and widely distributed environments as a pristine rain forest in Cameroon, industrial sites in two states of Brazil, a forest preserve in the young geographically isolated island of Hawaii, a marine beach in California, a deep sandy aquifer in Michigan, a wood pulp treatment lagoon in Ontario, and a compost pile in Michigan. The only different environment that did not yield an enrichment was permafrost soils collected from a region adjacent to the Arctic Ocean in eastern Siberia. Many samples did not yield enrichments, however. This may be due to the difficulty of successfully enriching these organisms or to the fact that such organisms may not be present in every gram of nature. The poor enrichment record for the undescribed contaminated samples is likely due to general toxicity from the chemical contaminants since chemical odors were apparent in these samples.

ACKNOWLEDGMENTS

We thank Helen Garchow for performing the FAME analysis and Frans de Bruijn for the use of facilities for the nitrogen fixation assay.

This project was supported by NIEHS Superfund Research Program, grant no. ES04911, and the facilities were provided to the Center for Microbial Ecology by NSF grant no. BIR9120006. M.R.F. acknowledges Fellowship support from CAPES, Brazil.

REFERENCES

1. **Bergersen, F. J.** 1980. Measurement of nitrogen fixation by direct means. p. 65–110. *In* F. J. Bergersen (ed.), Methods for evaluating biological nitrogen fixation. John Wiley & Sons, Chichester, United Kingdom.
2. **Byrne, A. M., J. J. Kukor, and R. H. Olsen.** 1993. Nucleotide sequence analysis of the *tbu* operon encoding the toluene-3-monooxygenase from *Pseudomonas pickettii* PKO1, abstr. K-81, p. 274. Abstr. 93th Gen. Meet. Am. Soc. Microbiol. 1993. American Society for Microbiology, Washington, D.C.
3. **Chee-Sanford, J., M. R. Fries, and J. M. Tiedje.** 1992. Anaerobic degradation of toluene under denitrifying conditions in bacterial isolate Tol-4, abstr. Q-233, p. 374. Abstr. 92th Gen. Meet. Am. Soc. Microbiol. 1992. American Society for Microbiology, Washington, D.C.
4. **Dean, B. J.** 1985. Recents findings on the genetic toxicology of benzene, toluene, xylenes and phenols. Mutat. Res. **154**:153–181.
5. **de Bruijn, F. J.** 1992. Use of repetitive (repetitive extragenic palindromic and enterobacterial repetitive intergeneric consensus) sequences and the polymerase chain reaction to fingerprint the genomes of *Rhizobium meliloti* isolates and other soil bacteria. Appl. Environ. Microbiol. **58**:2180–2187.
6. **Devereaux, J., P. Haeberli, and O. Smithies.** 1984. A comprehensive set of sequence analysis programs for the VAX. Nucleic Acids Res. **12**:387–395.
7. **Dobereiner, J., and J. M. Day.** 1976. Associative symbiosis in tropical grasses: characterization of microorganisms and dinotrogen fixing sites, p. 518–538. *In* E. W. Newton, and C. J. Nyman (ed.), Proceedings of the 1st International Symposium on Nitrogen Fixation. Washington State University Press, Pullman, Wash.
8. **Dolfing, J., J. Zeyer, P. Binder-Eicher, and R. P. Schwarzenbach.** 1990. Isolation and characterization of a bacterium that mineralizes toluene in the absence of molecular oxygen. Arch. Microbiol. **154**:336–341.
9. **Edwards, E. A., L. E. Wills, M. Reinhard, and D. Grbić-Galić.** 1992. Anaerobic degradation of toluene and xylene by aquifer microorganisms under sulfate-reducing conditions. Appl. Environ. Microbiol. **58**:794–800.
10. **Elmerich, C., B. L. Dreyfus, and J. P. Aubert.** 1982. Genetic analyses of nitrogen fixation in a tropical fast-growing *Rhizobium*. EMBO J. **1**:499–503.
11. **Evans, P. J., W. Ling, B. Goldschmidt, E. R. Ritter, and L. Y. Young.** 1992. Metabolites formed during the anaerobic transformation of toluene and *o*-xylene and their proposed relationship to the initial steps of toluene mineralization. Appl. Environ. Microbiol. **58**:496–501.
12. **Evans, P. J., D. T. Mang, K. S. Kim, and L. Y. Young.** 1991. Anaerobic degradation of toluene by a denitrifying bacterium. Appl. Environ. Microbiol. **57**:1139–1145.
13. **Felsenstein, J.** 1989. PHYLIP—phylogeny inference package (version 3.2). Cladistics **5**:164–166.
14. **Fishbein, L.** 1985. An overview of environmental and toxicological aspects of aromatic hydrocarbons. II. Toluene. Sci. Total Environ. **42**:267–288.
15. **Gibson, D. T., and V. Subramanian.** 1984. Microbial degradation of aromatic hydrocarbons, p. 181–252. *In* D. T. Gibson (ed.), Microbial degradation of organic compounds. Marcel Dekker, Inc., New York.
16. **Grbić-Galić, D., and T. M. Vogel.** 1987. Transformation of toluene and benzene by mixed methanogenic cultures. Appl. Environ. Microbiol. **53**:254–260.

17. **Haigler, B. E., C. A. Pettigrew, and J. C. Spain.** 1992. Biodegradation of mixtures of substituted benzenes by *Pseudomonas* sp. strain JS150. Appl. Environ. Microbiol. **58**:2237–2244.
18. **Harayama, S., M. Rekik, M. Wubbolts, K. Rose, R. A. Leppik, and K. N. Timmis.** Characterization of five genes in the upper-pathway operon of TOL plasmid pWWO from *Pseudomonas putida* and identification of the gene products. J. Bacteriol. **171**:5048–5055.
19. **Holben, W. E., J. K. Jansson, B. K. Chelm, and J. M. Tiedje.** 1988. DNA probe method for the detection of specific microorganisms in the soil bacterial community. Appl. Environ. Microbiol. **54**:703–711.
20. **Hurek, T., S. Burggraf, C. R. Woese, and B. Reinhold-Hurek.** 1993. 16S rRNA-targeted polymerase chain reaction and oligonucleotide hybridization to screen for *Azoarcus* spp., grass-associated diazotrophs. Appl. Environ. Microbiol. **59**:3816–3824.
21. **Hutchins, S. R., G. W. Sewell, D. A. Kovacs, and G. A. Smith.** 1991. Biodegradation of aromatic hydrocarbons by aquifer microorganisms under denitrifying conditions. Environ. Sci. Technol. **25**:68–76.
22. **Johnson, G. R., and R. H. Olsen.** 1993. Characterization of clones from *Pseudomonas* sp. JS150 encoding substituted phenol and benzene metabolic pathways analogous to *Pseudomonas* strains PKO1 and G4. abstr. K-82. p. 274. Abstr. 93th Gen. Meet. Am. Soc. Microbiol. 1993. American Society for Microbiology, Washington, D.C.
23. **Kado, C. I., and S.-T. Liu.** 1981. Rapid procedure for detection and isolation of large and small plasmids. J. Bacteriol. **145**:1365–1373.
24. **Krieg, N. R.** 1981. Enrichment and isolation, p. 112–142. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
25. **Kukor, J. J., and R. H. Olsen.** 1990. Diversity of toluene degradation following long term exposure to BTEX in situ, p. 405–421. *In* D. Kamely, A. Chakrabarty, and G. S. Omenn (ed.), Biotechnology and biodegradation. Gulf Publishing Co., Houston, Tex.
26. **Larsen, N., G. J. Olsen, B. L. Maidak, M. J. McCaughey, R. Overbeek, T. J. Macke, T. L. Marsh, and C. R. Woese.** 1993. The Ribosomal Database Project. Nucleic Acids Res. **21**(Suppl.):3021–3023.
27. **Lovley, D. R., M. J. Baedecker, D. J. Lonergan, I. M. Cozzarelli, E. J. P. Phillips, and D. I. Segal.** 1989. Oxidation of aromatic contaminants coupled to microbial iron reduction. Nature (London) **339**:297–300.
28. **Lovley, D. R., and D. J. Lonergan.** 1990. Anaerobic oxidation of toluene, phenol, and *p*-cresol by the dissimilatory iron-reducing organism, GS-15. Appl. Environ. Microbiol. **56**:1858–1864.
29. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
30. **Olsen, R. H., J. J. Kukor, and B. J. Kaphammer.** 1994. A novel toluene-3-monooxygenase pathway cloned from *Pseudomonas pickettii* PK01. J. Bacteriol. **176**:3749–3756.
31. **Owens, J. D., and R. M. Keddie.** 1969. The nitrogen nutrition of soil and herbage coryneform bacteria. J. Appl. Bacteriol. **32**:338–347.
32. **Rabus, R., R. Nordhaus, W. Ludwig, and F. Widdel.** 1993. Complete oxidation of toluene under strictly anoxic conditions by a new sulfate-reducing bacterium. Appl. Environ. Microbiol. **59**:1444–1451.
33. **Regensburger, A., W. Ludwig, and K.-H. Schleifer.** 1988. DNA probes with different specificities from a cloned 23S rRNA gene of *Micrococus luteus*. J. Gen. Microbiol. **134**:1197–1204.
34. **Reinhold-Hurek, B., T. Hurek, M. Gillis, B. Hoste, M. Vancanneyt, K. Kersters, and J. De Ley.** 1993. *Azoarcus* gen. nov., nitrogen-fixing *Proteobacteria* associated with roots of Kallar grass (*Leptochloa fusca* (L.) *Kunth*), and description of two species, *Azoarcus indigens* sp. nov. and *Azoarcus communis* sp. nov. Int. J. Syst. Bacteriol. **43**:574–584.
35. **Sasser, M.** 1990. Technical note 101: identification of bacteria by gas chromatography of cellular fatty acids. MIDI, Inc. North Newark, Del.
36. **Schocher, R. J., B. Seyfried, F. Vazquez, and J. Zeyer.** 1991. Anaerobic degradation of toluene by pure cultures of denitrifying bacteria. Arch. Microbiol. **157**:7–12.
37. **Smibert, R. M., and N. R. Krieg.** 1981. General characterization, p. 409–443. *In* P. Gerhardt, R. G. E. Murray, R. N. Costilow, E. W. Nester, W. A. Wood, N. R. Krieg, and G. B. Phillips (ed.), Manual of methods for general bacteriology. American Society for Microbiology, Washington, D.C.
38. **Smith, G. B., and J. M. Tiedje.** 1992. Isolation and characterization of nitrite reductase gene and its use as a probe for denitrifying bacteria. Appl. Environ. Microbiol. **58**:376–384.
39. **Viebrock, A., and W. G. Zumft.** 1988. Molecular cloning, heterologous expression, and primary structure of the structural gene for the copper enzyme nitrous oxide reductase from denitrifying *Pseudomonas stutzeri*. J. Bacteriol. **170**:4658–4668.
40. **Wilson, B. H., G. B. Smith, and J. F. Rees.** 1986. Biotransformation of selected alkylbenzenes and halogenated aliphatic hydrocarbons in methanogenic aquifer material: a microcosm study. Environ. Sci. Technol. **20**:997–1002.
41. **Ye, R. W., M. R. Fries, S. G. Bezborodnikov, B. A. Averill, and J. M. Tiedje.** 1993. Characterization of the structural gene for a copper containing nitrite reductase and its homology to other denitrifiers. Appl. Environ. Microbiol. **59**:250–254.
42. **Yen, K.-M., M. R. Karl, L. M. Blatt, M. J. Simon, R. B. Winter, P. R. Fausset, H. S. Lu, A. A. Harcourt, and K. K. Chen.** 1991. Cloning and characterization of a *Pseudomonas mendocina* KR1 gene cluster encoding toluene-4-monooxygenase. J. Bacteriol. **173**:5315–5327.
43. **Zhou, J.-Z., M. R. Fries, J. C. Chee-Sanford, and J. M. Tiedje.** Unpublished data.
44. **Zylstra, G. J., and D. T. Gibson.** 1989. Toluene degradation by *Pseudomonas putida* F1: nucleotide sequence of the *todC1C2 BADE* genes and their expression in *Escherichia coli*. J. Biol. Chem. **264**:14940–14946.