DEVELOPMENT AND APPLICATION OF IN SITU PCR TECHNIQUES TO INVESTIGATE MICROBIAL COMMUNITY FUNCTIONAL AND PHYLOGENETIC DIVERSITY AT THE INDIVIDUAL CELL LEVEL

by

WENDY A. DUSTMAN

(Under the Direction of Robert E. Hodson)

ABSTRACT

We developed a number of *in situ* PCR techniques using model gene systems in laboratory-grown strains of bacteria, and then modified these techniques for use with natural bacterial communities. Depending on the particular technique used, we could target DNA, mRNA, or rRNA to examine either functional genes or gene activity or as a means to examine phylogenetic relationships amongst bacterial communities. We also applied these newly developed technologies to a pilot study in which we examined the response of both low-impact and high-impact microcosm communities to toluene. We found no influence of toluene on changes in bacterial cell number, nor on the numbers of BTEX-degraders as measured by *in situ* PCR. However, in high-impact communities, we observed a marked increase in total bacterial cell number as well as the number of BTEX degraders within the communities.

INDEX WORDS: in situ PCR dioxygenase BTEX benzene toluene ethylbenzene xylene microcosm degradation bioremediation

DEVELOPMENT AND APPLICATION OF IN SITU PCR TECHNIQUES TO INVESTIGATE MICROBIAL COMMUNITY FUNCTIONAL AND PHYLOGENETIC DIVERSITY AT THE INDIVIDUAL CELL LEVEL

by

WENDY A. DUSTMAN

B.A., Indiana University, 1991

A Dissertation Submitted to the Graduate Faculty of The University of Georgia in Partial Fulfillment of the Requirements for the Degree

DOCTOR OF PHILOSOPHY

ATHENS, GEORGIA

2002

DEVELOPMENT AND APPLICATION OF IN SITU PCR TECHNIQUES TO INVESTIGATE MICROBIAL COMMUNITY FUNCTIONAL AND PHYLOGENETIC DIVERSITY AT THE INDIVIDUAL CELL LEVEL

by

WENDY A. DUSTMAN

Major Professor: Robert E. Hodson

Committee: Mary Ann Moran
Brian Binder
William B. Whitman
Lawrence Shimkets

Electronic Version Approved:

Maureen Grasso
Dean of the Graduate School
The University of Georgia
December 2002

DEDICATION

I dedicate this tome to my husband Andy. His loving support through the many years of this endeavor kept me moving forward, always striving to do the best I could possibly do.

ACKNOWLEDGEMENTS

The completion of this dissertation would not have been possible without the support and assistance of a number of people. I would like to xtend my deepest gratitude to my major professor, Dr. Robert E. Hodson, for his generous support and friendship over the years. In addition, I would like to thank Dr. Mary Ann Moran for the tremendous amount of support and guidance that she provided every time I entered her door. I would also like to acknowledge the support and assistance of my committee, Dr. Brian Binder, Dr. William Whitman, and Dr. Lawrence Shimkets, who provided me with valuable comments and alternate insights over the years.

I also owe tremendous gratitude for the friendship of Kathryn Dudeck, Ed Sheppard, and Justin Hartmann; whether it be a chocolate and diet Coke® break, an impromptu EtOH committee meeting, or the many volunteered hours of free labor as my able assistants, you three have done more for me than words can express.

Finally, I'd like to thank my family for their love and support as I followed my dream.

TABLE OF CONTENTS

Page

LIST OF TABLES

Page

LIST OF FIGURES

Page

CHAPTER 1

INTRODUCTION AND LITERATURE REVIEW

In the past, studies of aquatic microbial ecology have been limited due to the lack of culturability of most microorganisms on existing laboratory media. While the examination of environmental isolates provides valuable information, these organisms represent less than 1% of the total bacterioplankton present in most communities and may not be representative of the bacteria that are active *in situ*. While there are more than a million bacteria in every cubic centimeter of seawater, less than a thousand can be grown on enriched nutrient agar plates for further study in the lab. Although much work has been done to demonstrate that bacteria are the organisms responsible for nutrient cycling in salt marsh and estuarine ecosystems by interacting as a "microbial loop" (33), almost nothing was known about the diversity of species present or the ways in which they interacted with one another. Much of the complexity regarding the pathways of carbon cycling, for example, might be better explained if we knew the identities of the bacteria involved and their spatial relationships to other microbes, higher organisms, and detrital material.

With the advent of recent molecular protocols, information regarding total microbial community compositions has become attainable. For example, various researchers have collected and extracted community DNA from a number of marine and freshwater habitats, and using a variety of molecular techniques, have investigated phylogenetic diversity of the microorganisms contained within (e.g. 13 and 26). Though these analyses look at the entire community and not just the strains cultivable in the

laboratory, they lack the ability to connect a particular genetic sequence with an individual bacterial cell. More recently, fluorescent *in situ* hybridization (FISH) targeting 16S rRNA has been used to visually examine the distributions of specific phylogenetic signatures at the individual cell level in many different natural milieus, including marine and freshwater environments (1-4, 10-12, 17, 22-25, 30, 36, and 18). Due to insufficient copy number of most functional genes, however, FISH has not been highly successful in the examination of nucleic acid sequences other than 16S rRNA.

We have attacked the problem of the non-culturability of aquatic bacteria and low level of target sequences by devising a novel visual way of studying the individual organisms and their interactions in the community without the need to culture and isolate them. We accomplished this goal by pioneering the process of *in situ* PCR, a method that enables the microbial ecologist to study both phylogenetic and functional information contained within the genomes of individual cells, as well as to investigate the activity of specific genes by targeting messenger RNA sequences inside whole cells. In short, the *in situ* PCR methodologies described herein allow us to conduct basic ecological and biogeochemical studies on individual organisms that possess a few copies, or even a single copy, of a gene or mRNA transcript.

Briefly, *in situ* PCR methodologies we have developed involve permeabilizing bacterial cell walls to allow the PCR reagents inside while maintaining cellular integrity, and then targeting and amplifying specific nucleic acid sequences within the cells by PCR. We have investigated, with success, many variations for detecting PCR amplicons inside intact bacteria including the direct incorporation of fluorescently-labeled nucleotides into the PCR product, and amplification using unlabeled nucleotides coupled

with *in situ* hybridization of fluorescently-labeled probe to an internal region of the PCR amplicon. In each case, using epifluorescence microscopy, we have been able to visually detect the cells that possess the gene or mRNA of interest within the microbial community, and distinguish them from the remainder of the microorganisms present.

As with other microscope-based methods used in microbial ecology, it is necessary to moderately concentrate the microbial community before analysis. Since natural aquatic bacterial communities have low population densities, without concentration, a microscope field of view will contain on average only a few cells. Our initial study employed centrifugation to concentrate bacteria, but this method is time consuming and labor intensive. In later studies, we employed membrane filtration, the same technique used for other microscopic characterizations of natural bacterial communities (i.e. direct microscope counting), to concentrate marine bacterial assemblages in preparation for *in situ* gene or RNA amplifications. Once filtered, the communities were then successfully subjected to *in situ* PCR methodologies and visually assessed using epifluorescence microscopy.

We used our *in situ* PCR methodology to target and detect genes involved in biodegradation of toluene and related aromatic hydrocarbons and to quantify the proportion of the microbial community engaged in this process in Georgia coastal waters. We were able to visually examine the microorganisms involved in toluene degradation and elucidate their spatial relationships to other bacterial cells. Significant differences in the responses to toluene were detected when bacterial communities from pristine and polluted coastal and estuarine seawater were compared. Using these newly developed molecular techniques, we can more clearly define the distribution and activity of

microbes involved in biodegradation processes, and ultimately develop predictive models of bacterial responses to chronic, and potentially even acute, contamination of our environment with a range of chemical compounds.

Contamination by petroleum compounds is a significant environmental problem, particularly in coastal areas that serve not only as recreational areas but also are home to fishing and aquaculture industries. Petroleum compounds are highly toxic to many organisms: plants, animals and microbes. Of particular threat are low molecular weight aromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene (or BTEX). Approximately 45% of the petroleum pollution in U.S. waterways from 1983 to 1984 was attributed to diesel fuel, a common energy source for marine engines (44); diesel fuel contains approximately 10% BTEX hydrocarbons (by weight) (8). BTEX compounds are also found as major components of gasoline (18% by weight) (34) and widely used as industrial solvents (37). These compounds are highly soluble in water compared to other petroleum components, and are a great environmental and public health concern due to their toxic and carcinogenic properties. Long-term exposure to benzene has been linked as causative agents of aplastic anemia and leukemia while similar exposure to toluene can result in brain damage (7); short-term exposure to toluene and xylene can result in headaches, nausea, and an irritation of the mucous membranes.

While many organisms are adversely affected by the presence of BTEX compounds, a number of bacterial species have been found to actively degrade these chemicals. While toluene degradation has been thoroughly investigated in *Pseudomonas* sp., a number of other genera, including Gram-negative bacteria as well as Gram-positive bacterial species (Table 1.1), have been shown metabolize BTEX compounds. However,

the majority of BTEX-degraders isolated thus far are Gram-negative rods. Bacteria capable of utilizing hydrocarbons as carbon and energy sources have been isolated from a number of contaminated environments including sites impacted by: fuel oil (5, 28), gasoline (16, 20, 38), diesel fuel (27), and jet fuel (40, 41). Physicochemical and biological factors affecting the microbial degradation of petroleum hydrocarbons in the environment have been well characterized (see 3 and 29 for reviews).

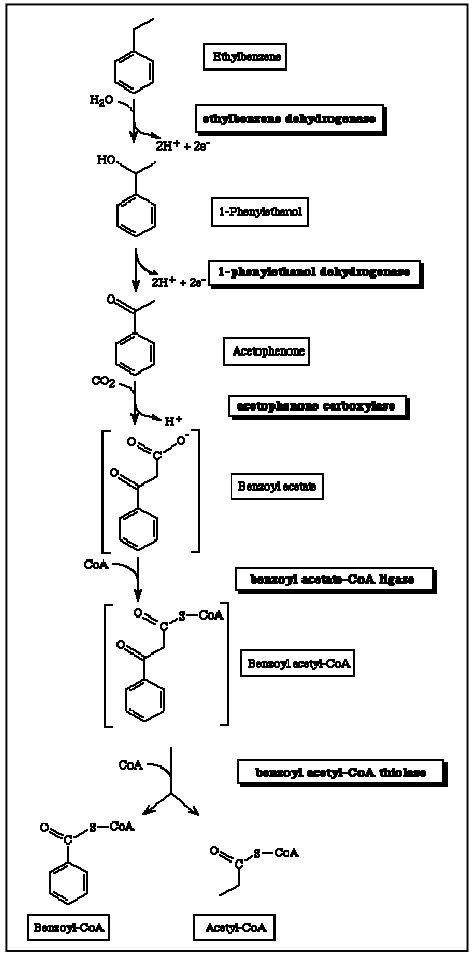
Metabolic pathways for the degradation of BTEX compounds are diverse, and can proceed either aerobically or anaerobically. Figures 1.1, 1.2, and 1.3 illustrate the known catabolic pathways for the degradation of benzene and ethylbenzene, toluene, and xylene, respectively. For this study, we focused on only the aerobic degradation mechanisms. Specifically, we used *in situ* PCR techniques to investigate the presence and distribution of a family of key enzymes called dioxygenases that are involved in BTEX-degradation. While dioxygenases are not involved in every pathway (i.e. toluene degradation has many monooxygenase pathways), there is some indication that the dioxygenase pathways dominate the biodegradation processes (43). Additionally, the gene sequences of BTEX dioxygenases have been well characterized and sequenced, including: benzene dioxygenase (*bdeD*) (15), ethylbenzene dioxygenase (*edoA*) (9), toluene dioxygenase (*todC1*) (21) and toluate dioxygenase (*xylX*, part of the xylene degradation pathways) (46).

TABLE 1.1. Bacteria capable of degrading aromatic hydrocarbons.

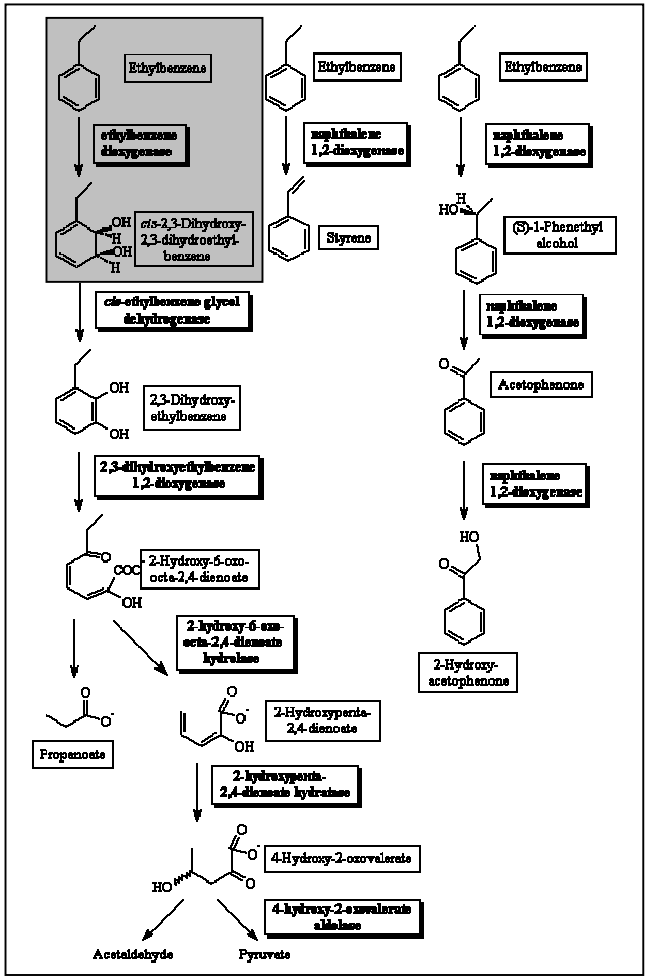
Genus	**Gram type**	**Reference(s)**
Pseudomonas sp.	-	32, 39, 42,47, 48, 49
Marinobacter sp.	-	18
Cycloclasticus sp.	-	13
Thauera aromatica	-	6
Desulfobacula sp.	-	35
Vibrio sp.	-	19
Rhodococcus sp.	+	31

Figure 1.1. Biochemical pathways for the microbial degradation of benzene and ethylbenzene. Adapted from The University of Michigan Biocatalysis/Biodegradation Database (http://umbbd.ahc.umn.edu).

Ethyl benzene
Anaerobic Pathway



Ethyl benzene
Aerobic Pathway



Benzene Pathway

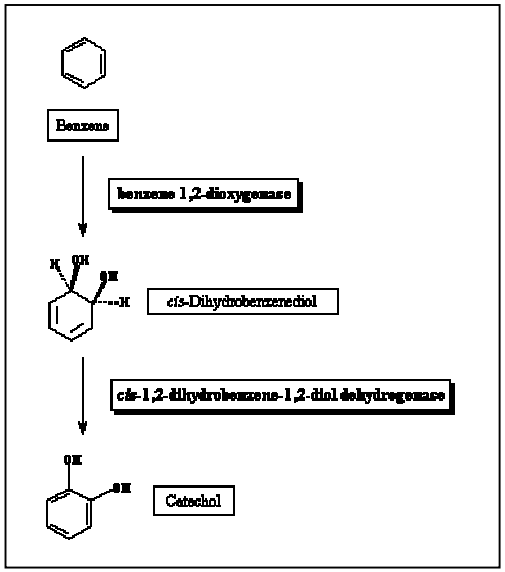


Figure 1.2. Biochemical pathways for the microbial degradation of toluene. Adapted from The University of Michigan Biocatalysis/Biodegradation Database (http://umbbd.ahc.umn.edu)

Aerobic

Anaerobic

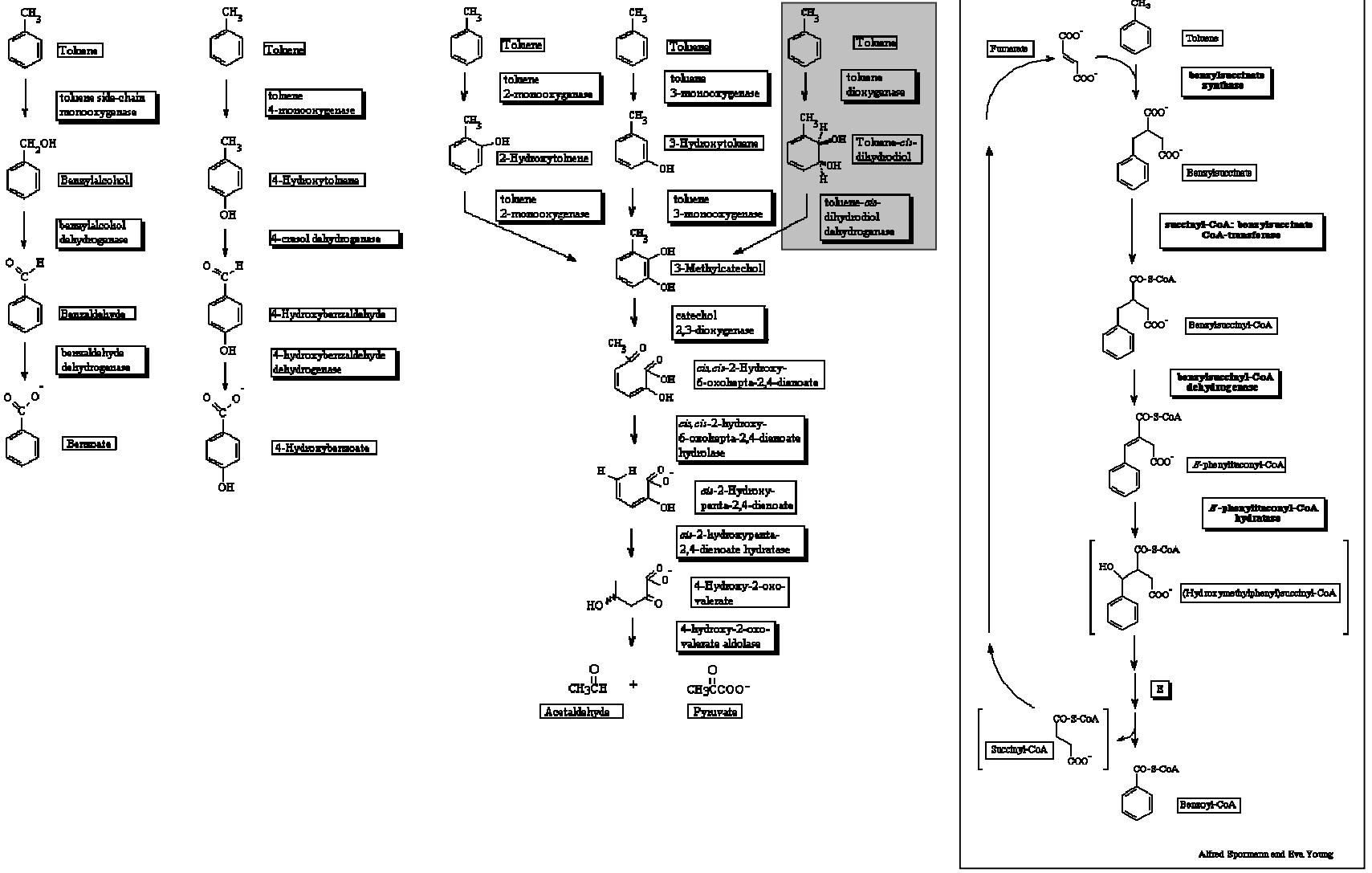
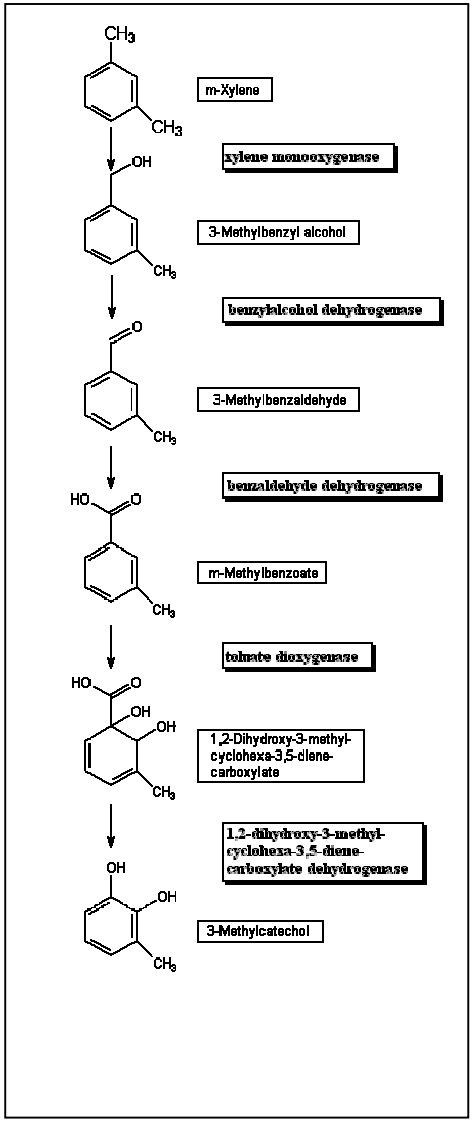
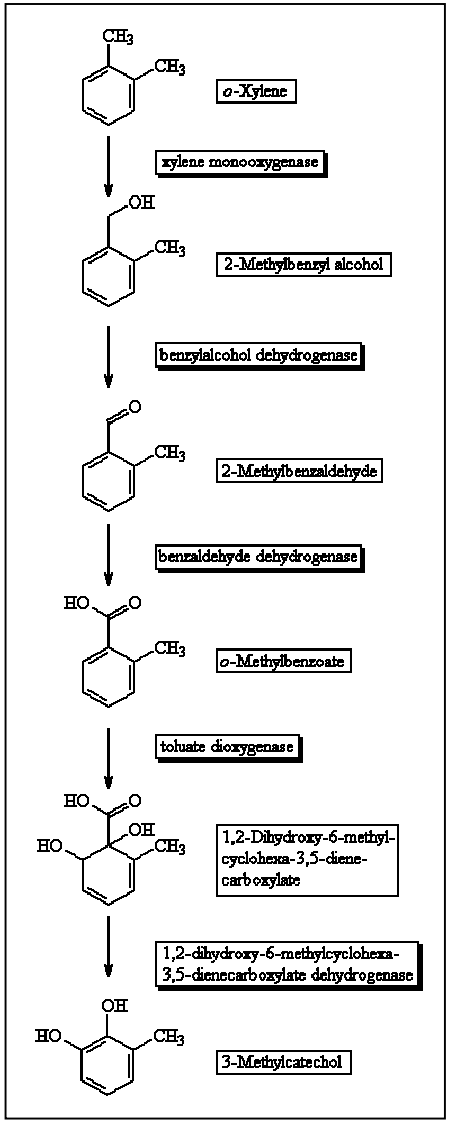
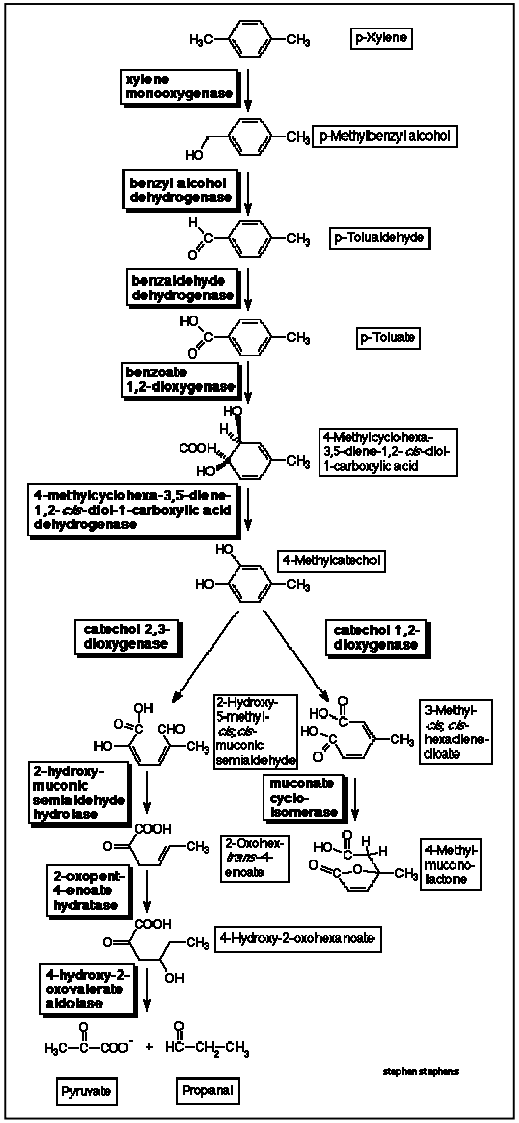


Figure 1.3. Biochemical pathways for the microbial degradation of xylene. Adapted from The University of Michigan Biocatalysis/Biodegradation Database (http://umbbd.ahc.umn.edu).


m-Xylene
xylene monooxygenase
3-Methylbenzyl alcohol
benzylalcohol dehydrogenase
3-Methylbenzaldehyde
benzaldehyde dehydrogenase
m-Methylbenzoate
toluate dioxygenase
1,2-Dihydroxy-3-methyl-cyclohexa-3,5-diene-carboxylate
1,2-dihydroxy-3-methyl-cyclohexa-3,5-diene-carboxylate dehydrogenase
3-Methylcatechol


o-Xylene
xylene monooxygenase
2-Methylbenzyl alcohol
benzylalcohol dehydrogenase
2-Methylbenzaldehyde
benzaldehyde dehydrogenase
o-Methylbenzoate
toluate dioxygenase
1,2-Dihydroxy-6-methyl-cyclohexa-3,5-diene-carboxylate
1,2-dihydroxy-6-methylcyclohexa-3,5-dienecarboxylate dehydrogenase
3-Methylcatechol


p-Xylene
xylene monooxygenase
p-Methylbenzyl alcohol
benzyl alcohol dehydrogenase
p-Tolualdehyde
benzaldehyde dehydrogenase
p-Toluate
benzoate 1,2-dioxygenase
4-Methylcyclohexa-3,5-diene-1,2-cis-diol-1-carboxylic acid
4-methylcyclohexa-3,5-diene-1,2-cis-diol-1-carboxylic acid dehydrogenase
4-Methylcatechol
catechol 2,3-dioxygenase
catechol 1,2-dioxygenase
2-Hydroxy-5-methyl-cis,cis-muconic semialdehyde
3-Methyl-cis, cis-hexadiene-dioate
2-hydroxy-muconic semialdehyde hydrolase
muconate cyclo-isomerase
2-Oxohex-trans-4-enoate
4-Methyl-muconolactone
2-oxopent-4-enoate hydratase
4-Hydroxy-2-oxohexanoate
4-hydroxy-2-oxovalerate aldolase
Pyruvate
Propanal
stephen stephens

REFERENCES

1. **Alfrieder, A. J. Pernthaler, R. Aman, B. Sattler, F. Glockner, A. Wille and R. Psenner.** 1996. Community analysis of bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by *in situ* hybridization. *Appl. Environ. Micriobiol.* **62**: 2138-2144.

2. **Amann, R.I., W. Ludwig, and K.-H. Schleifer. 1995.** Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* 59: 143-169.

3. **Atlas, R.M.** 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiol. Rev.* **45**: 180-209.

4. **Bano, N., and J.T. Hollibaugh**. 2000. Diversity and distribution of DNA sequences with affinity to ammonia-oxidizing bacteria of the β-Subdivision of the Class Proteobacteria in the Arctic Ocean. *Applied and Environmental Microbiology* **66**: 1960-1969.

5. **Bennaser, A., C. Guasp, and J. Lalucat.** 1998. Molecular methods for the detection and identification of Pseudomonas stutzeri in pure culture and environmental samples. *Microb. Ecol*. **35**: 22-33.

6. **Biegert, T., G. Fuchs, and J. Heider.** 1996. Evidence that anaerobic oxidtion of toluene in the denitrifying bacterium *Thauera aromatica* is initiated by the formation of benzylsuccinate from toluene and fumarate. *Eur. J. Biochem.* **238**: 661-668.

7. **Carlsen, A.** 1995. Toksikologiskie kvalite tskriterier for jord og drikkevand. Miljoe- & Energiministeriet.

8. **Chevron Phillips Chemical Company.** 1993. Material Safety Data Sheet #217110: 10% aromatic diesel fuel. Chevron Phillips Chemical Co., Houston, TX.

9. **Corkery, D.M. and A.D. Dobson.** 1998. Reverse transcription-PCR analysis of the regulation of ethylbenzene dioxygenase gene expression in Pseudomonas fluorescens CA-4. *FEMS Microbiol. Let.* **166**: 171-176.

10. **DeLong, E. F., D. G. Franks and A. L. Alldredge.** 1993. Phylogenetic diversity of aggregate-attached versus free-living marine bacterial assemblages. *Limnol. Oceanog.* **38**: 924-934.

11. **DeLong, E.F., G.S. Wickham, and N.R. Pace.** 1989. Phylogenetic stains: ribosomal RNA based probes for the identification of single cells. *Science* **243**: 1360-1362.

12. **DeLong, E.F., G.S. Wickham, and N.R. Pace.** 1989. Phylogenetic stains: ribosomal RNA based probes for the identification of single cells. *Science* **243**: 1360-1362.

13. **DeLong, E. F., K. Y. Wu, B. B. Prezelin and R. V. M. Jovine.** 1994. Archaea in Antarctic marine environments. *Nature* **371**: 695-697.

14. **Dykesterhouse, S.E., J.P. Gray, R.P. Herwig, J.C. Lara, and J.T. Staley.** 1995. Cycloclasticus pugettii gen. nov., sp., an aromatic hydrocarbon-degrading bacterium from marine sediments. *Int. J. Syst. Bacteriol.* **45**: 116-123.

15. **Fong, K.P., C.B. Goh, and H.M. Tan.** 1996. Characterization and expression of the plasmid-borne *bedD* gene from *Pseudomonas putida* ML2, which codes for a NAD+-dependent *cis*-benzene dihydrodiol dehydrogenase. *J. Bacteriol*. **178**: 5592-5601.

16. **Fredrickson, J.K., D.M. Balkwill, J.M. Zachara, S.W. Li, F.J. Brockman, and M.A. Simmons.** 1991. Physiological diversity and distributions of heterotrophic bacteria in deep Cretacious sediments in the Atlantic coastal plain. *Appl. Environ. Microbiol.* **57**: 402-411.

17. **Fuhrman, J. A., K. McCallum and A. A. Davis.** 1992. Novel marine archaebacterial group from marine plankton. *Nature* **356**: 148-149.

18. **Gauthier, M.J., B. Lafay, R.Christen, L. Fernandez, M. Acquaviva, P. Bonin, and J.C. Bertrand.** 1992. *Marinobacter hydrocarbonoclasticus* gen. nov., sp. nov., a new, extremely halotolerant, hydrocarbon-degrading marine bacterium. *Int. J. Syst. Bacteriol.* **43**: 568-576.

19. **Geiselbrecht, A.D., R.P. Herwig, J.W. Deming, and J.T. Staley.** 1996. Enumeration and phylogenetic analysis of polycyclic aromatic hydrocarbon-degrading marine bacteria from Puget Sound sediments. *Appl. Environ. Microbiol*. **62**: 3344-3349.

20. **Ghiorse, W.C., and D.L. Balkwill.** 1983. Enumeration and morphological characterization of bacteria indigenous to subsurface environments. *Dev. Inc. Microbiol.* **24**: 213-227.

21. **Gibson, D.T., G.E. Cardini, F.C. Maseles, and R.E. Kallio.** 1970. Incorporation of oxygen-18 into benzene by *Pseudomonas putida*. *Biochemistry* **9**: 1631-1635.

22. **Giovannoni, S. J., E.F. DeLong, G.J. Olsen, and N.R. Pace.** 1988. Phylogenetic group-specific probes for identification of single microbial cells. *J. Bacteriol.* **170**: 720-726.

23. **Giovannoni, S. J., T. B. Britschgi, C. L. Moyer, and K. G. Field.** 1990. Genetic diversity in Sargasso Sea bacterioplankton. *Nature* **345**: 60-63.

24. **Glockner, F.O., R. Amann, A. Alfrieder, J. Pernthaler, R. Psenner, K. Tresbisius, and K.-H. Schleifer.** 1996. An *in situ* hybridization protocol for detection and identification of planktonic bacteria. *System. Appl. Microbiol.* **19**: 403-406.

25. **Hicks, R.E., R.I. Amann, and D.A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4'6'-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. *Appl. Environ. Microbiol* **58**: 2158-2163.

26. **Hollibaugh, J.T., P.S. Wong and M.C. Murrell.** 2000. Similarity of particle-associated and free-living bacterial communities in northern San Francisco Bay, California. *Aquatic Microbial Ecology* **21**: 103-114.

27. **Hosien, S.G., D. Millette, B.J. Butler, and C.W. Greer.** 1997. Catabolic gene probe analysis of an aquifer related microbial community degrading creosote-related polycyclic aromatic and heterocyclic hydrocarbons. *Microb. Ecol*. **34**: 81-89.

28. **Laurie, A.D., and G.Lloyd-Jones.** 1999. The *phn* genes of *Burholderia* sp. strain RP007. *J. Bacteriol*. **181**: 531-540.

29. **Leahy, J.G., and R.R. Colwell.** 1990. Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev*. **54**: 305-315.

30. **Lemke, M.J., C.J. McNamara, and L.G. Leff.** 1997. Comparison of methods for the concentration of bacterioplankton for *in situ* hybridization. *J. Microbiol. Methods* **29**: 23-29.

31. **Malachowsky, K.J., J.T. Phelps, A.B. Teboli, D.E. Minnikin, and D.C. White.** !994. Anaerobic mineralization of trichloroethylene vinyl dioxide and aromatic compounds by *Rhodococcus* sp. *Appl. Environ. Microbiol.* **60**: 542-548.

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

33. **Pomeroy, L.R.** 1974. The ocean's food web, a changing paradigm. *BioScience* **24**: 499-504.

34. **Potter, T.L.** 1992. Fingerprinting petroleum products: unleaded gasolines. p. 83-92. *In* P.T. Kostecki and E.J. Calabrese (eds.), Petroleum contaminated soils, Lewis Publishers, Chelsea MI.

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

36. **Ramsing, N.B., M. Kühl, and B.B. Jørgensen.** 1993. Distribution of sulfate-reducing bacteria, O_2, and H_2S in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. *Appl. Environ. Microbiol.* **59**: 3840-3849.

37. **Reisch, M.S.** 1992. Top 50 chemicals production stagnated last year. Chem. Eng. News 70: 16-22.

38. **Sanseverino, J., C. Werner, J. Flemming, B. Applegate, J.M.H. King, and G. S. Sayler.** 1993. Molecular diagnostics of polycyclic aromatic hydrocarbon degradation in manufactured gas plant soils. *Biodegradation* **4**: 303-321.

39. **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.

40. **Stahl, D.A. and R. Raman.** 1991. Development and application of nucleic acid probes. *In* Stackebrandt, E. and M. Goodfellow (eds.) Nucleic acid techniques in bacterial systematics. John Wiley and Sons, Inc. Chichester England

41. **Stahl, D.A., B. Fleischer, H.R. Mansfield and L. Montgomery.** 1988. Use of phylogenetically based hybridization probes for studies of ruminal ecology. *Appl. Environ. Microbiol.* **54**: 1079-1084.

42. **Sobecky, P.A., M.A. Schell, M.A. Moran, and R.E. Hodson.** 1992. Adaptation of model genetically engineered microorganisms to lake water: growth rate enhancements and plasmid loss. *Appl. Environ. Microbiol.* **58**: 3630-3637.

43. **Tsao, C.-W., H.-G. Song, and R. Bartha.** 1998. Metabolism of benzene, toluene, and xylene hydrocarbons in the soil. *Appl. Environ. Microbiol.* **64**: 4924-4929.

44. **United States Coast Guard**. 1994. Polluting incidents in and around U.S. waters, calendar year 1983 and 1984. United States Coast Guard Service, COMDTINST M16450.26. NTIS 87-186821.

45. **Wagner, M., G. Rath, R. Amann, H.-P. Koops, and K.-H. Schleifer.** 1995 *In situ* identification of ammonia-oxidizing bacteria. *System. Appl. Microbiol.* **18**: 251-264.

46. **Wessler, H.G., D.R. Hares, M.D. Poulter, J.A. Voss, H.S. Khedairy, R.F. Baker, E.E. Azadpour, X. Luo, and R.C. Benjamin.** (In press) Sequence of the lower (meta-cleavage) xyl operon of the *Pseudomonas putida* TOL plasmid pDK1.

47. Whited, G.M. and D.T. Gibson. 1991. Toluene-4-monooxygenase, a tree component enzyme system that catalyzes the oxidation of toluene to *p*-cresol in *Pseudomonas mendocina* KR1. *J. Bacteriol.* 173: 3010-3016.

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

49. Zylstra, G.J., W.R. McCombie, D.T. Gibson, and B.A. Finnette. 1988. Toluene degradation by *Pseudomonas putida* F1: genetic organization of the tod operon. *Appl. Environ. Microbiol*. **54**: 1498-1503.

CHAPTER 2

PROKARYOTIC *IN SITU* PCR: VISUALIZATION OF MICROSCALE DISTRIBUTION OF SPECIFIC GENES IN PROKARYOTIC COMMUNITIES[1]

[1] R.E. Hodson, W.A.Dustman, R.P. Garg, and M.A. Moran, and R.E. Hodson. 1995. *Applied and Environmental Microbiology*. 61:4074-4082. Exerpts reprinted here with permission of publisher.

ABSTRACT

Obtaining information on genetic capabilities and phylogenetic affinities of individual prokaryotic cells within natural communities is a high priority in the fields of microbial ecology, microbial biogeochemistry, and applied microbiology, among others. A method is presented here for prokaryotic *in situ* PCR (PI-PCR), a technique which will allow single cells within complex mixtures to be identified and characterized genetically. The method involves amplification of specific nucleic acid sequences inside intact prokaryotic cells, followed by color or fluorescence detection of the localized PCR product via brightfield or epifluorescence microscopy. Prokaryotic DNA was used successfully as a target for PI-PCR. We demonstrate the use of PI-PCR to identify *nahA* positive cells in pure and mixed cultures of *Pseudomonas* sp.

INTRODUCTION

Techniques currently exist to measure the distribution of various microbial processes at macroscale levels in natural systems. For example, methods for characterizing the spatial variation in such parameters as rates of degradation of individual compounds (3, 28), cycling rates of inorganic nutrients (7), or average rates of bacterial growth and respiration (8, 13) have been available for a number of years. However, at the microscale, genetic composition of bacterial communities is virtually unknown. With few exceptions, it has been impossible to determine which individual cells in a natural bacterial assemblage possess the genetic capability to carry out a specific process or how the organisms which mediate a process are spatially associated with each other, with other organisms, or with non-living particles in water, sediments, or soils.

Even with microscale sampling, techniques based on viable counts offer little promise for providing insight into bacterial community structure, since cultivation efficiency of bacteria from natural communities is ordinarily very low. Likewise, light or electron microscopic techniques generally identify only those cells possessing unusual morphologies, though microscopy often reveals the extraordinary complexity of the physical structure and heterogeneity of microbial communities (24). The presence of specific genes in extracts of bulk DNA from natural bacterial communities can be determined semi-quantitatively by using dot blot hybridizations (23), but as with older techniques, such methods fail to provide information at the microscale or individual cell levels.

Recently, development of *in situ* (or "whole cell") hybridization methods for ribosomal RNA-based oligonucleotide probes has made taxonomic identification of single cells within natural bacterial communities possible for the first time (1, 2, 5, 11, 15, 27, 29). However, these methods rely on the presence of naturally-occurring multiple targets within the bacterial cell to provide a detectable signal, and thus are limited in utility by the low number of rRNA molecules in many slow-growing or dormant bacteria from environmental samples (10, 11). Detection of individual genes present in single or low copy numbers in intact bacterial cells with *in situ* hybridization methods is not possible.

One potential approach to characterizing the microscale genetic and taxonomic properties of natural bacterial communities would be via *in situ* PCR, a unique modification of PCR in which amplification and detection of specific target nucleic acid sequences is carried out inside individual cells rather than on bulk extracted nucleic acid (18). Individual genes, mRNA, and rRNA are all candidate targets for *in situ* PCR, so

that genetic capabilities, expression of those capabilities, and taxonomic information are all potentially accessible on the individual cell level. This approach has been used successfully only in eukaryotic cells, primarily in biomedical applications (9, 14, 16, 19). Heretofore, no complementary approaches have been developed either for prokaryotic cells or for environmental or clinical samples containing prokaryotes. We report here the development of a method for prokaryotic *in situ* PCR (PI-PCR), and provide examples of its use in single-cell detection of a specific gene and specific gene transcripts in mixtures of bacterial isolates and in simple model communities of marine bacteria.

MATERIALS AND METHODS

Bacterial samples and cell fixation. Strains of *Pseudomonas aeruginosa* and *Pseudomonas putida* used in this study are listed in Table 2.1. The NAH7 plasmid was mobilized to *P. aeruginosa* (ATCC 19712) by triparental conjugation with pRK2013 as the helper plasmid (4). All strains used in PI-PCR were grown in Luria-Bertani (LB) broth which contained per liter 10 g tryptone, 5 g yeast extract, and 10 g NaCl (17). Model marine bacterial communities were assembled from eight strains of bacteria. Five of the strains were random isolates from coastal Georgia sediments which had been cultured on half-strength YTSS agar (4 g yeast extract, 2.5 g tryptone, 200 ml 2.5X sea salts solution per liter) at room temperature. The other three strains were *P. putida* AC10R-7, *P. aeruginosa* 19712 and *Escherichia coli* HB101.

Single colonies of each strain were taken from fresh plates, inoculated into 25 ml broth, and grown at 30°C and 250 rpm for 6-12 hours or until the cultures reached 1.0 OD. Cells were harvested by centrifugation at 2500 x g for 10 min and washed twice with phosphate buffered saline (PBS; 120 mM NaCl, 2.7 mM KCl in 10 mM phosphate buffer, pH 7.6). Cells were resuspended in 4% fresh paraformaldehyde in PBS for 4-16 hr, after

TABLE 2.1. Bacterial strains used in PI-PCR procedures

Strain	Species (plasmid)	Reference or source
AC10R	*P. putida*	Sobecky et al. (1992)
AC10R-7	*P. putida* (NAH7)	Sobecky et al. (1992)
19712	*P. aeruginosa*	ATCC[a]
19712-7	*P. aeruginosa* (NAH7)	This work
HB101	*E.coli*	ATCC

[a] ATCC, American Type Culture Collection

which cells were twice washed with PBS and then resuspended in 5 ml of 50% ethanol in PBS. Fixed cells were aliquoted into 100 µl portions and stored at -20°C.

Oligonucleotides. The 20-mer oligonucleotide primers which were used for the amplification of the *nahA* gene on the NAH7 plasmid (25) were as follows: NAH A (5'-terminal, positions 653-674) TAC AAG CAT CAA GTT GAG CG and NAH B (3'-terminal, positions 1758-1779) GGA ATC AGG CTG TCA TGA GT. A third 19-mer oligonucleotide primer, NAH I, which was used for primer extension, was complementary to a region internal to the first two primers (positions 825- 843) and had the sequence CAG TCA GCA AGA CCT CTA C. All primers were synthesized on an Oligo 1000 DNA Synthesizer (Beckman Instruments Inc., Fullerton, CA). The primers were cleaved and deprotected using an Ultrafast Cleavage and Deprotection Kit (Beckman Instruments Inc.), vacuum-dried using a Speed Vac Concentrator (Savant Instruments Inc., Farmingdale, NY), and resuspended in 500 µl ultrapure water. Oligonucleotide concentrations were determined spectrophotometrically and diluted to 100 pmol μl^{-1} before storage at -20°C.

Cell wall permeabilization. Fixed cells were washed twice in PBS buffer and resuspended in 200 µl lysozyme buffer (100 mM Tris, 50 mM EDTA, pH 8.0). Cell wall permeabilization was achieved by treating with lysozyme at a final concentration of 0.5 mg ml^{-1} for 30 min at room temperature. Lysozyme was removed from the cells by three consecutive washes with PBS. Permeabilization was furthered by treatment with either proteinase K at a final concentration of 0.10 µg ml^{-1} or trypsinogen at a final concentration of 0.50 µg ml^{-1} for 10 min at room temperature. Protease was removed by four consecutive washes with PBS and cells were re-suspended in 200 µl of PBS. If the amplification target was DNA, RNA was removed from the cells during the cell permeabilization steps by adding DNase-free RNase at a final concentration of 0.5 mg

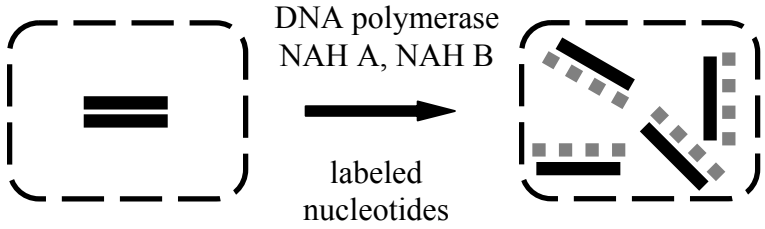
ml^{-1} along with the lysozyme. However, if the amplification target was RNA, permeabilized cells were treated with RNase-free DNase (10 U μl^{-1} final concentration) at room temperature for 2-3 hours to degrade DNA.

One stage PI-PCR. A 2-primer PCR protocol was developed for *in situ* amplification inside prokaryotic cells (Fig. 2.1). Amplification of the *nahA* gene inside permeabilized cells was performed using Thermalase Tbr (AMRESCO Inc., Solon, OH) and the NAH A and NAH B oligonucleotides as primers. The reaction mixture was prepared according to manufacturer's instructions with the exception that $MgCl_2$ concentration was raised to 2 mM and digoxygenin- or fluorescein-labeled nucleotides were included in the nucleotide mix. For the DIG-dUTP mix, nucleotide concentrations were 1 mM dATP, dCTP and dGTP; 0.65 mM dTTP; and 0.35 mM DIG-11-dUTP (DIG-DNA labeling mixture; Boehringer Mannheim, Indianapolis, IN). For the FLOUS-dUTP mix, nucleotide concentrations were 1 mM dATP, dCTP and dGTP; 0.65 mM dTTP; and 0.35 mM fluorescein-12-dUTP. Typically, 10 µl of permeabilized cell suspension (approximately 10^6 - 10^7 cells) were used per 50 µl reaction mixture in Oil-Free Tubes (Barnstead Thermolyne Corp., Dubuque, IA). Using a DNA Thermal Cycler 480 (Perkin-Elmer Corp., Norwalk, CT), a hot start technique (10 min at 82°C) was employed prior to the addition of the polymerase, and was followed by initial denaturation at 94°C for 3 min. Amplification of the target DNA occurred during 45 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min and extension at 72°C for 1 min. Cells were harvested and washed with PBS, resuspended in 10 µl PBS. The resuspended cells were spotted onto wells of printed microscope slides (Cel-Line Associates, Inc., Newfield, NJ) in 2-5 µl aliquots and allowed to air dry.

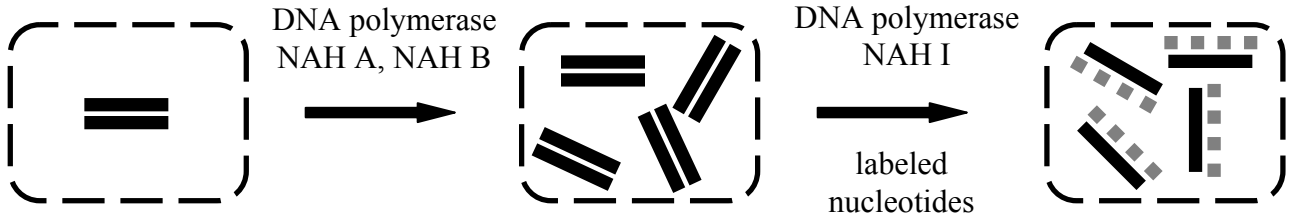
Two stage PI-PCR. A two-stage prokaryotic *in situ* PCR protocol was developed based on the primed *in situ* DNA amplification (PIDA) method for eukaryotic cells as described

Figure 2.1. Schematic of methods for *in situ* detection of specific nucleic acid sequences in prokaryotic cell.

One-Stage PI-PCR



Two-Stage PI-PCR



by Moss and Kaliner (16). In Stage 1 (amplification stage), cell preparation and conditions for PCR amplification were the same as described above, except that nucleotides (10 mM final concentration for each) were unlabeled. Following target sequence amplification, cells were harvested and washed twice with 0.5X SSC (750 mM NaCl, 75 mM trisodium citrate, pH 7.0), and either resuspended in 10 µl PBS (for immediate use) or stored at 4°C in 4% paraformaldehyde overnight. In Stage 2 (primer extension stage), the reaction mixture was the same as for amplification, with the exception that a labeled nucleotide mix (either DIG-dUTP mix or FLOUS-dUTP mix) was used. Forty µl of reaction mixture was added directly to the 10 µl of resuspended cells from Stage 1. NAH I at 0.2 µM concentration was added as the primer for an internal region of the sequence amplified in Stage 1. The purpose of Stage 2 was to increase the specificity of detection of the amplified target sequence; amplified products of mispriming during Stage 1 will not bind the NAH I primer in Stage 2. The hot start technique was again employed to denature DNA duplexes. Primer extension was carried out using 5 cycles of denaturation at 94°C for 1 min, annealing at 42°C for 1 min, and extension at 72°C for 1 min. Cells were harvested, washed with PBS and resuspended in 10 µl PBS. Cells were spotted onto wells of printed glass slides in 2-5 µl aliquots and allowed to air dry.

PI-PCR on microscope slides. *In situ* PCR was also carried out on microscope slides placed directly on top of the block of the thermal cycler. Permeabilized cell suspensions were spotted into a 10 mm diameter well of a printed glass slide (Cel-Line Assoc., Inc.) and allowed to dry. Slides were passed through an ethanol dehydration series (50-80-98%, 3 min each) to fix the cells to the slide surface. A plastic "Gene Cone" chamber (15 mm diameter; Gene Tech, Durham, NC) was mounted around the periphery of the well to serve as a reservoir for the PCR reaction mixture, and two-stage PI- PCR was carried out as described above with the following exceptions: the concentration of NAH I was

increased to 2 μM, the number of primer extension cycles was raised to 10, and a step for degradation of residual primers was added following stage 1. Primer degradation was performed by adding 50 μl of a nuclease S1 solution at a final concentration of 100 U ml^{-1} in nuclease S1 buffer (200 mM NaCl; 50 μM sodium acetate, pH 4.5; 1 mM $ZnSO_4$; 0.5% glycerol) for 30 min at 37°C; nuclease S1 was inactivated by chelation in nuclease stop buffer (1 mM EDTA, pH 8.0; 10 mM sodium phosphate) followed by three consecutive washes with PBS for 10 min each at room temperature. After Stage 1 amplification and before the washing and primer degradation steps, the Gene Cone ring was removed to facilitate effective treatment of the cells. A second ring was then mounted in the same place on the slide, the cover was put in place, and the primer extension step (Stage 2) with labeled nucleotide mix was carried out as described above.

Another variant of *in situ* amplification and detection was carried out with cells immobilized on glass slides immersed in reaction mixture inside PCR tubes. Permeabilized cells were spotted onto the lower third of thin glass slides (approximately 2 mm x 20 mm) which had been cut from standard glass cover slips (26). The slides were inserted into standard PCR tubes so that the reaction mixture covered the cell spot. One-stage or two-stage PI-PCR was then carried out as described above, using an oil overlay to prevent evaporation during thermal cycling. Following PCR, slides were briefly washed with 98% ethanol to remove the oil and placed on top of standard microscope slides for detection procedures.

Detection of amplified genes. Following completion of the PCR procedures and spotting of cells onto printed slides, cells were dehydrated in ethanol (50-80-98%; each for 3 min), dried at 50°C, and washed twice in 0.5X SSC at 45°C for 15 min to remove excess primer and unincorporated nucleotides. For PI-PCR on microscope slides, a second nuclease S1 digestion step as described above was included in addition to the 0.5X SSC washes.

For PCR procedures using FLOUS-dUTP nucleotide mix, dehydrated and washed cell spots were viewed directly via epifluorescence microscopy. For PCR procedures using the DIG-dUTP nucleotide mix, slides were placed in blocking solution [50 mM Tris, 150 mM NaCl, 0.1% SDS, 3% Radfree blocking powder (Schleicher & Schuell, Keene, NH), pH 7.5] for 1 hr at 50°C and then transferred to Genius Buffer 1 (Boehringer Mannheim) containing 1:100 anti-DIG Fab fragments conjugated to alkaline phosphatase (for color detection) or to fluorescein or rhodamine (for fluorescence detection) (Boehringer Mannheim) for 1 hour at room temperature. Nonspecifically bound antibody was removed by rinsing twice in wash solution (5 mM Tris, 15 mM NaCl, 0.1% SDS, pH 7.5) for 15 min at room temperature followed by washing twice with Genius Buffer 1 for 15 min each at room temperature. Cells detected with alkaline phosphatase conjugate were briefly washed in Genius Buffer 3 (Boehringer Mannheim) prior to color development using the alkaline phosphatase reaction in color developing solution [10 ml Genius Buffer 3 plus 35 µl BCIP and 45 µl NBT substrates (Boehringer Mannheim)]. This reaction was allowed to proceed for 5 min to 1 hr at 37°C before rinsing with distilled water to stop the reaction. After air drying, the slides were observed using brightfield microscopy for the presence of a blue precipitate and phase contrast microscopy for the presence of phase-bright cells. Photographs were taken using an Olympus OM-2 camera (Olympus America Inc., Lake Success, NY) with automatic exposure settings and Kodak Ektachrome Elite 400 slide film or Kodak Enhanced Multi-Purpose 400 speed print film. Cells detected using fluorescein- or rhodamine-labeled antibody were dried for immediate examination via epifluorescence microscopy using an Olympus NIB filter cube. Photography was carried out as described above.

Dot blot hybridizations were carried out to confirm the presence of the correct PCR product by immobilizing target (PCR reaction mixture or intact cells) to Nytran nitrocellulose membranes (Schleicher & Schuell) using a dot-blot apparatus (Schleicher & Schuell) and UV cross-linking (Fisher Scientific, Pittsburgh PA). DNA was denatured with NaOH after lysis with 10% SDS (21). The oligonucleotide probe (NAH I) was labeled with $^{32}P[\alpha]$ dCTP using terminal transferase (Boehringer Mannheim) as per the manufacturer's recommendations. Hybridization was carried out in 50% formamide at 39°C for 16 hrs after which membranes were washed under stringent conditions (21).

RESULTS

Cell wall permeabilization. Successful prokaryotic *in situ* PCR depends on a methodology which will permeabilize the bacterial cell membranes to allow entry of reagents for amplification and detection, yet retard the diffusion of PCR product away from the cells while not destroying the morphology of the cells or the microscale structure of the microbial community. To optimize permeabilization procedures for *P. putida* and *P. aeruginosa* prior to *in situ* PCR, final lysozyme concentrations were varied between 0.03 and 0.5 mg ml^{-1}, and time of exposure of the cells to lysozyme between 0.5 and 3 hr. The effectiveness of cell permeabilization was evaluated by exposing treated cells to RNase and DNase and then staining cells with acridine orange (12) or DAPI (20) to determine whether or not nucleic acid had been effectively degraded, thus indicating optimal entry of enzymes into the cell. The lysozyme treatment that permitted free entry of enzymes into the cells of the two strains of *Pseudomonas* while maintaining the morphological integrity of the cell was 0.5 mg ml^{-1} (final concentration) at room temperature for 30 minutes.

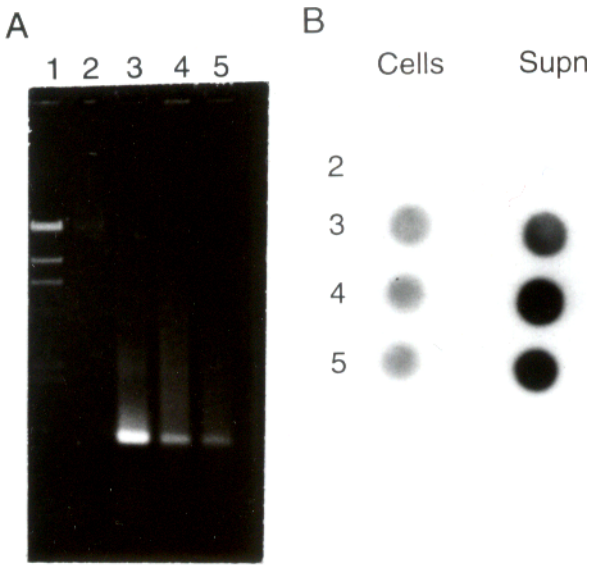
Following optimization of lysozyme treatment, protease concentrations were likewise varied to achieve optimum entry of *in situ* amplification reagents. Trypsinogen

was used initially, but we ultimately switched to proteinase K because it had greater stability to freezing and thawing as well as a broader range of action. Cell lysis occurred at high proteinase K concentrations, but we found that cells retained integrity when treated with a concentration of 0.10 μg ml^{-1} for 10 minutes at room temperature. The action of the protease in effecting *in situ* PCR may be either that of freeing nucleic acids from crosslinkages with proteins established during paraformaldehyde fixation, or of further degrading the cell membrane to facilitate entry of enzymes and reagents (18), or both.

One stage PI-PCR. The one stage PI-PCR approach was successful in amplifying target genes inside intact cells, as determined by the presence of strong color or fluorescence signals, although interfering levels of labeled non-specific PCR products were produced simultaneously when this protocol was used. Amplification of specific target DNA inside *Pseudomonas* cells was confirmed by dot blot hybridizations of cell pellets (recovered from PCR reaction mixtures) with the NAH I probe (Fig. 2). Amplification of target sequences also occurred outside the cells in the reaction mixture, presumably with DNA released from lysed cells serving as the template. The presence of specific products outside the cells was confirmed by analysis of the cell-free reaction mixture in agarose gels and by dot blot hybridization of this mixture with the NAH I probe (Fig. 2). $MgCl_2$ concentrations in the range of 1 to 5 mM were required for successful amplification inside cells, and no signal was detected either inside or outside cells when $MgCl_2$ was omitted from the reaction mixture. The optimum concentration of $MgCl_2$ was found to be affected by a number of variables, including density of cells in the reaction mixture as well as the batch of polymerase. For most one-stage PI-PCR procedures, we found 2 mM $MgCl_2$ and 10^6-10^7 cells to be a workable combination.

Non-specific products of PI-PCR were recognized by two methods. First, reaction mixtures from *in situ* PCR amplifications analyzed on agarose gels were found to contain a range of products of varying size along with the desired 1.1 kb product of specific

Figure 2.2. Two-Stage PI-PCR amplification of the *nahA* sequence in *Pseudomonas putida* (NAH7) AC10R-7 carried out in solution. (A) Agarose gel electrophoresis of supernatants and (B) dot blot hybridizations of cell pellets and supernatants. Lanes: 1, ladder (0.12 - 23.1 kbp λ-DNA cleaved by *Hind*III); 2, negative control (obmission of both NAH A and NAH B primers); 3, 1.1 kb *nahA* fragment amplified in the presence of 1 mM $MgCl_2$; 4, 1.1 kb *nahA* fragment amplified in the presence of 4 mM $MgCl_2$; 5, 1.1 kb *nahA* fragment amplified in the presence of 5 mM $MgCl_2$. Dot blot numbers correspond to lane numbers.

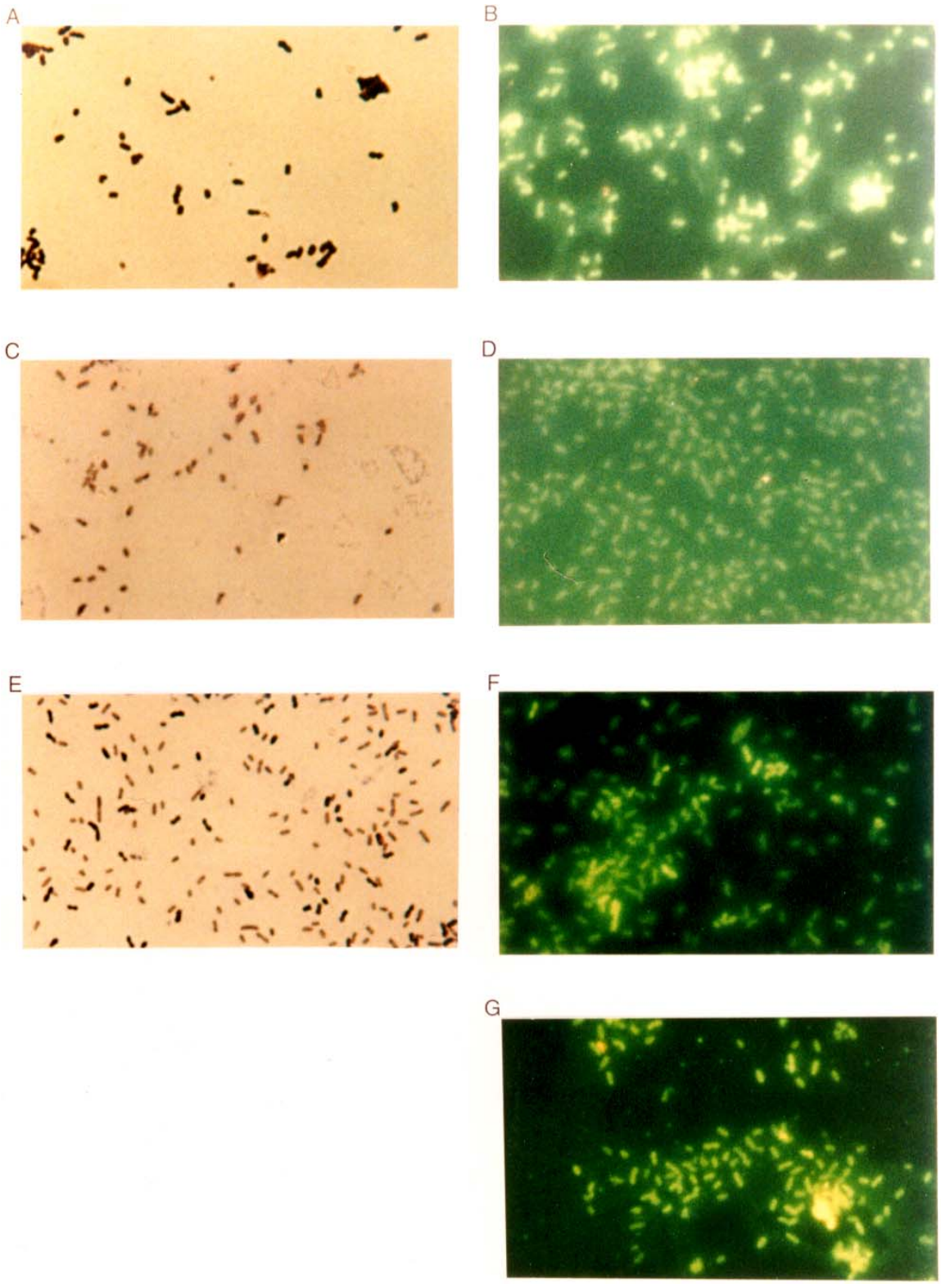

A
1 2 3 4 5
B
Cells
Supn
2
3
4
5

amplification. Second, amplification products were detected inside cells in the absence of added primers (although never in the absence of DNA polymerase), suggesting that small pieces of DNA native to the cell were acting as PCR primers (21) or that preexisting replication forks were being extended. Attempts to optimize reaction conditions when using the single-stage variant of PI-PCR with direct incorporation of labeled nucleotides failed to achieve specific labeling of target in PI-PCR.

Two stage PI-PCR. To increase the specificity of PI-PCR, three approaches were used: 1) increasing the stringency of amplification conditions such that only the target DNA was amplified; 2) increasing the specificity of detection after amplification such that only the correct PCR product was detected; and 3) using cDNA copies of RNA as the amplification target (Fig. 2.2). Using the first option, we varied a number of amplification parameters including $MgCl_2$ concentration, number of PCR cycles, primer concentration, and annealing temperature. However, when conditions were sufficient for product detection inside prokaryotic cells, non-specific amplification was always found to occur simultaneously. Modifying the other amplification parameters likewise did not sufficiently increase the specificity of labeled product formation inside the cells to allow us to visually distinguish between NAH7-containing cells and NAH7-free cells (though target amplification was achieved).

In the second approach, we added an additional stage to the *in situ* PCR protocol such that amplification and detection (by incorporation of labeled nucleotides) were temporally separated (Fig. 2.1). With two-stage PI-PCR, the non-specific products of the first amplification remain undetected because they are not complementary to the internal primer used in the second stage (and thus do not get labeled). Two-stage PI-PCR was successful in differentiating between cells with and without the target gene. With this approach, *P. putida* cells containing the NAH7 plasmid gave strong signals with either color or fluorescence detection (Fig. 2.3 A, B), while *P. aeruginosa* cells without NAH7

Figure 2.3. Two-Stage PI-PCR amplification of *nahA* sequence in fixed cells of *Pseudomonas putida* (NAH7) AC10R-7, *P. aeruginosa* 19712, and *P. aeruginosa* (NAH7) 19712-7. Brightfield (left) and epifluorescence (right) photomicrographs are shown. Exposure times were held constant to allow direct comparison of staining intensity. The *nahA* sequence was amplified using DIG-dUTP nucleotides in Stage II and detected by alkaline phosphatase-labeled anti-DIG antibodies (A, C, E) or FLOUS-labeled anti-DIG antibodies (B, D, F, G). (A, B) Strain AC10R-7; (C, D) Strain 19712; (E, F) Mixture of strains AC10R-7 and 19712; (G) Strain 19712-7.


A
B
C
D
E
F
G

were only weakly visible (Fig. 2.3 C, D). Cells of the two types could be readily distinguished in mixtures (Fig. 2.3 E, F). To demonstrate that possible differences between the two *Pseudomonas* species (in cell wall structure, for example, or other phenotypic characteristics) were not responsible for the variations in signal intensity observed, two- stage PI-PCR was also carried out with *P. aeruginosa* 19712-7 (derived by inserting the NAH7 plasmid into *P. aeruginosa* 19712). These cells also gave a strong positive signal (Fig. 2.3 G).

To optimize conditions for the second stage of two-stage PI-PCR, $MgCl_2$ concentrations were varied from 1 to 5 mM; primer concentrations were varied 1.0 to 2.0 μM; and DNA polymerase concentrations were varied from 0.1 to 1.0 μl per 100 μl of reaction mixture. Optimum concentrations for stringent extension of the NAH I internal primer in suspended cells were found to be 2 mM $MgCl_2$, 0.2 μM primer, and 0.2 to 0.5 μl DNA polymerase. Optimum reagent concentrations for stringent extension of the NAH internal primer for cells fixed to slide surfaces were found to be 2mM $MgCl_2$, 2.0 μM primer, and 1.0 μl DNA polymerase.

DISCUSSION

To date, it has not been possible to determine with any reliability which individual cells within a complex natural bacterial community possess a specific genetic trait and whether or not that trait is being expressed. Unless information on actual numbers and microscale patchiness and physical associations (e.g., free-living cells vs. attachment to particulate matter or other organisms, etc.) of bacterial cells is available, their functional niche cannot be described. The development of an *in situ* PCR protocol for prokaryotic cells makes such information available to the researcher and holds significant promise for addressing many of the deficiencies currently limiting our understanding of natural

bacterial community structure and function. This approach has the potential to provide information on specific genes and gene products at the scale of the individual cell.

Permeabilization of the cell envelope was determined to be a critical step in *in situ* PCR, since it is necessary to modify the membrane such that enzymes and reagents freely pass into and out of the cell, while the amplified PCR products remain inside and the cell retains its integrity for microscopy-based detection. We used a combination of treatment with lysozyme and a mild protease to accomplish the cell permeabilization, optimizing conditions to balance product labeling and retention in positive cells with effective washing of non-incorporated labeled nucleotides from negative cells. Amplification and detection reagents used in the *in situ* PCR procedures included several large molecules, such as DNA polymerase (95,000 daltons) and anti-DIG Fab fragments conjugated to alkaline phosphatase (>244,000 daltons). Thus, even relatively large molecules were able to freely pass the bacterial cell membrane following permeabilization. Similarly, Zarda et al. (29) report the penetration of alkaline-phosphatase-labeled anti-DIG Fab fragments into lysozyme-treated gram negative cells.

Amplification of non-target DNA was the most significant problem encountered during the development of the *in situ* PCR protocol. We found that a two-stage *in situ* PCR procedure involving two sequential rounds of thermal cycling greatly reduces the problem of formation of labeled non-specific products. The first phase of two-stage PI-PCR is a conventional exponential amplification using two primers and non-labeled nucleotides; the second phase is an arithmetic amplification of only the correct PCR products by extension (with labeled nucleotides) of a third primer complementary to a region of the product of Stage 1. An alternative method for increasing specificity of *in situ* detection of target DNA suggested by Nuovo (18), but not used in the present study, involves hybridization of a labeled oligonucleotide probe to products formed in a standard one-stage PCR.

Each of the detection methods used for PI-PCR was found to have advantages and disadvantages. Direct incorporation of fluorescent nucleotides into the PCR product was the most simple approach, and it required entry of the smallest-sized suite of reagents into the cells. However, with this approach we sometimes observed higher levels of non-specific staining. Likewise, incorporation of DIG-labeled nucleotides followed by detection with fluorescently-labeled Fab fragments sometimes yielded less distinct differences between positive and negative cells. With both fluorescence detection approaches, fluorescence from the labeled products tended to fade over time with light excitation. Ultimately, differences in fluorescence signal strength between positive and negative cells may be more easily detected by replacing direct microscopic examination with image analysis approaches (e.g., 27) or flow cytometric procedures (e.g., 1).

In most cases, detection with alkaline phosphatase-labeled Fab fragments (the largest of the detection reagents) gave good subjective visual differences between positive and negative *Pseudomonas* cells, and the color precipitate was permanent. Color-based detection, however, may be less well suited for adapting PI-PCR for use with natural bacterial communities. In preliminary experiments, we found that the small size of many natural cells made distinguishing positive from negative cells difficult. Likewise, distinguishing darkly stained cells from detrital or sediment material was more problematic. In these experiments, best results were obtained by simultaneously using DAPI (4', 6-diamidino-2-phenylindole) as a general fluorescent stain for total counts (excitation wavelength: 330 nm; emission wavelength: 450 nm) and rhodamine-labeled anti-FAB fragments (excitation wavelength: 560; emission wavelength: 610) to detect PCR products in positive cells.

PI-PCR was successfully carried out both with cells suspended in a reaction mixture and cells immobilized on glass slides. With suspension PI-PCR, a significant percentage of the cells sometimes were lost during the procedure, presumably due to inefficient centrifugation during intermediate washing steps. However, this approach is the simplest technically; washing steps are rapid, and the resulting intensity of signal from positive cells is uniform. When cells were immobilized on glass, washes sometimes were not as effective, as evidenced by fairly substantial formation of background signal in the negative cells. The addition of a nuclease S1 digestion step after amplification was successful in reducing the background in the negative cells while not affecting the intensity of the positive cells. When PI-PCR was carried out on microscope slides, we initially had a high failure rate of the chamber adhesive seal (about 50%) due to the high-temperature denaturation step (94°C). However, this problem was minimized by using printed microscope slides for better adherence of the chambers and by blocking the top of the chamber with melted wax. The benefits of conducting PI-PCR on microscope slides are that almost all of the bacterial cells remain at the end of the procedure and the microscale distribution of cells (e.g., association in microcolonies or attachment to detrital particles) can be preserved.

PI-PCR carried out on strips of slide cover slips suspended in reaction mixture was problematic at the wash steps due to difficulties in manipulating the small, fragile slides and interference by the mineral oil used as an overlay for thermal cycling. We are currently developing a PI-PCR method which involves collecting bacteria on filters prior to amplification (6). This approach may ultimately prove to be the most versatile approach for characterizing natural prokaryotic communities, since it will allow efficient

concentration of cells yet maintain physical associations of bacteria with each other and with particulate matter (11).

Optimization of *in situ* PCR conditions is clearly a more straightforward undertaking for a defined system, such as the *Pseudomonas* species used here, than for a complex bacterial community in an environmental sample. Several challenges remain for the continued development of PI-PCR techniques for use with natural communities or other heterogeneous samples such as clinical tissue preparations or bacterial-eukaryotic symbioses. These include: 1) identifying permeabilization and amplification conditions appropriate for a specific organism in a community (for example, when targeting expression of one gene in a single bacterial species) or appropriate for all prokaryotic species in the community, including those which are not presently culturable (for example, when targeting one gene harbored by a number of phylogenetically distinct species); and 2) ensuring that the PI-PCR protocols are applicable for non-growing or slowly-growing cells typical of many natural communities. The successful development of the PI-PCR methodology, however, promises to provide the first opportunity to link distribution and identity of prokaryotic cells in natural environments with their genetic potential and *in situ* activities.

ACKNOWLEDGMENTS

We thank Dr. Gerard Nuovo for valuable advice offered in early phases of this work and Dr. John Hobbie for comments on an early draft of this manuscript. Kirsten Jordan and Kim Watkinson provided excellent laboratory assistance in the development of this technique. We thank Dr. Patty Sobecky, Elaine Hardwick, Lucy Feingold, and Laura Rutherford for suggestions and help.

This research was funded by a grant from the Office of Naval Research, Marine Environmental Quality Program (N00014-93-0850) to R.E.H. and M.A.M. Support for collection of field samples for isolation of marine bacterial strains was provided by the Georgia Rivers LMER (NSF DEB-9412089).

REFERENCES

1. **Amann, R. I., B. J. Binder, R. J. Olson, S. W. Chisolm, R. Devereux, and D. A. Stahl.** 1990. Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. Appl. Environ. Microbiol. **56:**1919-1925.

2. **Amann, R. I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and in situ detection of individual microbial cells without cultivation. Microbiol. Rev. **59:**143-169.

3. **Azam, F., and R. E. Hodson.** 1977. Dissolved ATP in the sea and its utilization by marine bacteria. Nature **267:**696-698.

4. **Ditta, G., S. Stanfield, D. Corbin, and D. R. Helinski.** 1980. Broad host range DNA cloning system for gram-negative bacteria: construction of a gene bank of *Rhizobium meliloti*. Proc. Nat. Acad. Sci. USA **77**:7347-7351.

5. **De Long, E. F., G. S. Wickham, and N. R. Pace.** 1989. Phylogenetic stains: ribosomal RNA-based probes for the identification of single cells. Science **243**:1360-1362.

6. **Dustman, W. A and R. E. Hodson.** Unpublished data.

7. **Fenchel, T. and T. H. Blackburn.** 1979. Bacteria and mineral cycling. Academic Press, London.

8. **Fuhrman, J. A., and F. Azam**. 1980. Bacterioplankton secondary production estimates for coastal waters of British Columbia, Antarctica, and California. Appl. Environ. Microbiol. **39**:1085-1095.

9. **Gressens, P., and J. R. Martin.** 1994. *In situ* polymerase chain reaction: localization of HSV-2 DNA sequences in infections of the nervous system. J. Virol. Meth. **46:**61-83.

10. **Hahn, D., R. I. Amann, W. Ludwig, A. D. L. Akkermans, and K. H. Schleifer.** 1992. Detection of micro-organisms in soil after *in situ* hybridization with rRNA-targeted, fluorescently labelled oligonucleotides. J. Gen. Microbiol. **138:**879-887.

11. **Hicks, R. E., R. I. Amann, and D. A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4',6-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. Appl. Environ. Microbiol. **58:**2158-2163.

12. **Hobbie, J. E., R. J. Daley, and S. Jasper.** 1977. Use of Nucleopore filters for counting bacteria by fluorescence microscopy. Appl. Environ. Microbiol. **33**:1225-1228.

13. **Kirchman, D. L., E. K'nees, and R. Hodson.** 1985. Leucine incorporation and its potential as a measure of protein synthesis by bacteria in natural aquatic systems. Appl. Environ. Microbiol. **49**:599-607.

14. **Komminoth, P., and A. A. Long.** 1993. *In-situ* polymerase chain reaction: an overview of methods, applications and limitations of a new molecular technique. Virchows Archiv B **64:**67-73.

15. **Manz, W., U. Szewzyk, P. Ericsson, R. Amann, K.-H. Schleifer, and T.-A. Stenström.** 1993. *In situ* identification of bacteria in drinking water and adjoining biofilms by hybridization with 16S and 23S rRNA-directed fluorescent oligonucleotide probes. Appl. Environ. Microbiol. **59:**2293-2298.

16. **Moss, R. B., and M. A. Kaliner.** 1994. Primed in situ DNA amplification (PIDA). J. Clin. Lab. Anal. **8:**120-122.

17. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory. Cold Spring Harbor, N.Y.

18. **Nuovo, G. J.** 1992. PCR *in situ* hybridization. Protocols and applications. Raven Press, NY.

19. **Nuovo, G. J., F. Galley, and P. MacConnell.** 1992. Detection of amplified HPV6 and 11 DNA in vulvar lesions by hot start PCR *in situ* hybridization. Mod. Pathol. **5**:444-448.

20. **Porter, K. G. and Y. S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. Limnol. Oceanogr. **25**:943-948

21. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

22. **Sällström, J. F., I. Zehbe, M. Alemi, and E. Wilander.** 1993. Pitfalls of *in situ* polymerase chain reaction (PCR) using direct incorporation of labelled nucleotides. Anticancer Res. **13**:1153-1154.

23. **Sayler, G. S., and A. C. Layton.** 1990. Environmental application of nucleic acid hybridization. Ann. Rev. Microbiol. **44**:625-648.

24. **Sieburth, J. M.** 1975. Microbial seascapes: a pictoral essay on marine microorganisms and their environment. University Park Press, Baltimore.

25. **Sobecky, P. A., M. A. Schell, M. A. Moran, and R. E. Hodson.** 1992. Adaptation of model genetically engineered microorganisms to lake water: growth rate enhancements and plasmid loss. Appl. Environ. Microbiol. **58:**3630-3637.

26. **Terkelsen, C., J. Koch, S. Kølvraa, J. Hindkjaer, S. Pedersen, and L. Bolund.** 1993. Repeated primed *in situ* labeling of specific DNA sequences ("PCR-PRINS") in metaphase chromosomal spreads. BM Biochemica **10**.

27. **Trebesius, K., R. Amann, W. Lugwig, K. Mühlegger, and K.-H. Schleifer.** 1994. Identification of whole fixed bacterial cells with nonradioactive 23S rRNA-targeted polynucleotide probes. Appl. Environ. Microbiol. **60:**3228-3235.

28. **Wright, R. T., and J. E. Hobbie.** 1966. Use of glucose and acetate by bacteria and algae in aquatic ecosystems. Ecology **47:**447-464.

29. **Zarda, B., R. Amann, G. Wallner, and K.-H. Schleifer.** 1991. Identification of single bacterial cells using digoxigenin-labelled, rRNA-targeted oligonucleotides. J. Gen. Microbiol. **137:**2823-2830.

CHAPTER 3

FILTER CONCENTRATION OF NATURAL SEAWATER BACTERIAL COMMUNITIES FOR ANALYSIS OF FUNCTIONAL DIVERSITY BY *IN SITU* AMPLIFICATION TECHNIQUES[2]

[2] Dustman, W.A., M.A. Moran, F. Chen and R.E. Hodson. To be submitted to *Limnology and Oceanography*.

ABSTRACT

In situ nucleic acid sequence amplification techniques were modified for detecting phylogenetic information (rRNA), functional gene information (DNA), and gene expression (mRNA) in natural aquatic bacterial populations collected on standard membrane filters. The filter-concentrated bacteria were subjected to various recently developed *in situ* amplification techniques. We successfully amplified target sequences using each *in situ* amplification protocol and either 16SrDNA primers or a functional gene primer set designed for detection of aromatic hydrocarbon ring-cleaving dioxygenases involved in benzene, toluene, ethyl-benzene and xylene (BTEX) degradation. The recovery efficiency of filter-adhered cells post-amplification by all *in situ* techniques examined was found to exceed 85% in all but three samples. Using positive and negative control strains of *Pseudomonas putida*, we demonstrate that nearly 100% of the cells that adhered to filters can be detected by *in situ* amplification methods. False positive results were not obtained in control strain studies. Thus, filter concentration of natural water samples prior to application of *in situ* amplification techniques provides a good protocol for bacterial community concentration for the characterization of genetic diversity and functional gene activity in natural assemblages.

INTRODUCTION

Given that only 0.1 to 1.0% of all bacteria in natural assemblages are cultivable in the laboratory (8, 39), information regarding the taxonomic diversity and functional capabilities of natural bacterial populations has been quite limited. However, recently developed culture-independent molecular tools have significantly advanced our understanding of microbial communities, especially in aquatic environments. *In situ* hybridization (ISH) of RNA within intact cells, with radioactively or fluorescently labeled probes, has provided a wealth of new knowledge regarding the taxonomic

composition of environmental communities which had previously been uncharacterizable by microscopic methods due to the lack of complex cellular morphology (1, 6, 11). While ISH techniques have been used to characterize the functional roles played by bacteria in various environments (e.g. sulfate reduction [36], ammonia oxidation [44]), based on conserved regions of 16S rRNA of the groups of bacteria involved in those processes, such methods are not amenable to demonstrating the single or low-copy number genes that may be present in a given population due to the requirement for sufficient numbers of target molecules (10-20 copies minimum) within the cells for detection by ISH (25, 31, 32). ISH is effective in taxonomic investigations because 16S rRNA is present at a level of several thousand copies per cell.

More recently, *in situ* PCR was developed to investigate the functional roles of bacteria and their activity in the environment based on the presence of either specific catabolic genes or their gene products (mRNA) (16). Amplification of the genetic material occurs within the bounds of the cell membrane, increasing the number of targets to an easily detectable level. Many variants of *in situ* PCR have sprung forth (Table 3.1), providing a number of different ways in which the genetic characteristics of intact cells can be elucidated. A number of genes have been successfully subjected to *in situ* amplification variants: 1) *nahA* in *Pseudomonas putida* (16), 2) *nifH* in *Azotobacter vinelandii* (16) 3) *todC*1 in *Pseudomonas putida* (3), 3) *xylE* in *Escherichia coli* and *Pseudomonas putida* (35), 4) *amoA* in *Nitrococcus oceanii* (7) and *Nitrosomonas europea* (19), 5) phenol hydroxylase (41), 6) *lacZ* (21) and 7) a larger fragment of the *lac* operon (43). Additionally, expression products (mRNA) of the *todC*1 (3, 4, 5,), *dnaK* (27), *lac* (42), *groEL* (18) and *tsf* (18) genes were successfully detected by *in situ* RT-PCR and/or

TABLE 3.1. Comparison of recently published *in situ* amplification methods.

***In situ* technique**	**Target(s)**	**Brief Description**	**Reference(s)**
One stage *in situ* PCR	DNA	Exponential amplification of target sequence using labeled (i.e. FITC, CY3, biotin or DIG) nucleotides which are directly incorporated into product	Hodson *et al.* 1995 Hoshino *et al.* 2001 Porter *et al.* 1995
Two stage *in situ* PCR	DNA	Exponential amplification of target sequence using unlabeled nucleotides Secondary exponential amplification of region internal to that amplified in first stage and using labeled (i.e. FITC, CY3, biotin or DIG) nucleotides which are directly incorporated into product	Hodson *et al.* 1995
In situ RT-PCR	mRNA	Reverse transcription of mRNA to CDNA Exponential amplification of cDNA target sequence using labeled (i.e. FITC, CY3, biotin or DIG) nucleotides which are directly incorporated into product	Hodson *et al.* 1995, Tolker-Neilsen *et al.* 1997 Lange *et al*. 2000
RNA probe extension (RPE)	mRNA	Linear amplification of target sequences using a single primer and labeled (i.e. FITC, CY3, biotin or DIG) nucleotides which are directly incorporated into product	Hodson *et al.* 1995
In situ reverse transcription (ISRT)	rRNA, mRNA	Reverse transcription of mRNA to CDNA using labeled (i.e. FITC, CY3, biotin or DIG) nucleotides which are directly incorporated into product	Chen *et al.* 1999 a, b
In situ PCR with hybridization (*in situ* PCR/H)	DNA	Exponential amplification of target sequence using unlabeled nucleotides Hybridization of labeled (i.e. FITC, CY3, biotin or DIG) probe internal to the region amplied	This study Tani *et al.* 2002
In situ RT-PCR with hybridization (*in situ* RT-PCR/H)	mRNA	Reverse transcription of mRNA to CDNA Exponential amplification of target sequence using unlabeled nucleotides Hybridization of labeled (i.e. FITC, CY3, biotin or DIG) probe internal to the region amplied	Chen *et al.* 1999 a

ISRT methodologies in laboratory-grown cultures. The successful application of *in situ* PCR methodologies to natural aquatic bacterial assemblages has also been established. The distribution of the *amoA* gene in estuarine communities (7) has been compared to the distribution of a functional group of nitrifying bacteria, and the distribution of *amoA* in biofilms of a nitrifying reactor (19) has been characterized. Microbes involved in bioaugmentation of TCE degradation in a contaminated aquifer were also monitored by *in situ* PCR (41). Expression products of the *todC*1 gene have been successfully detected by *in situ* RT-PCR in seawater microcosms after exposure to 2% toluene vapors (3, 4, 5).

Not only do ISH and *in situ* PCR methodologies allow the investigation of all cells present in a given sample, but they also provide an opportunity for examining the spatial relationships of cells within natural assemblages. Recently, ISH techniques have also been coupled with direct microscopic counting techniques (15, 34) to provide a quantitative measurement of the phylogenetic distribution of bacteria in the aquatic environment (9, 12, 13, 29). Aquatic samples filtered through polycarbonate membranes were transferred to gelatin coated slides before ISH (13, 29) or ISH was performed directly on the filters (9, 12, 29) and cells enumerated accordingly. Transfer efficiency rates from filter to slide were variable (18.3 - 79.2 % (29), and 48- 69% (13)), while ISH performed directly on filters showed that greater than 90% of the cells were retained on the filter after processing (9, 12). Since membrane filtration techniques are routinely used to quantify levels of bacteria, especially in aquatic environments, and the techniques have been successfully combined with ISH, membrane filtration could also serve to concentrate aquatic microbial communities so that a quantitative estimate of functional gene presence and/or gene activity can be made using *in situ* PCR techniques as well.

One obstacle of *in situ* PCR techniques, however, is that cells must be adequately fixed to retain cell morphology as in ISH, but must also be permeabilized to the extent that large molecules like DNA polymerase may easily enter the cell. Too much permeabilzation can result in a loss of amplified product from the cell into the surrounding PCR cocktail (16), lessening signal inside cells significantly or even creating a false negative result or completely destroy cell morphology. Therefore, great care must be taken to find a combination of fixation and permeabilization in which cell morphology is unchanged, successful amplification of the target is achieved, and the loss of amplified material from the cell is minimized. Much of the previous work has been done on laboratory batch cultures grown on rich medium, or samples enriched in microcosms. It is expected that natural assemblages, because of their more oligotrophic growth conditions, may be more sensitive to permeabilization procedures than their robust batch-cultured relatives.

The goals of this study were 1) to optimize cell fixation and permeabilization conditions for use with natural assemblages, 2) to determine whether or not *in situ* amplification techniques were compatible with available filter substrates and if amplification and detection is hindered by the filter material, 3) to optimize PCR conditions for successful amplification on the filter substrate, and 4) to obtain a quantitative estimate of the recovery efficiency of filter-concentrated cells from various samples after *in situ* reverse transcription (ISRT), *in situ* PCR/hybridization (*in situ* PCR/H) and *in situ* RT-PCR/hybridization (*in situ* RT-PCR/H) were applied. Additionally, we report the optimized conditions for use of a new primer set for

amplifying a suite of dioxygenase genes involved in the initial biodegradation of aromatic hydrocarbons including benzene, toluene, ethyl-benzene and xylene (BTEX).

MATERIALS AND METHODS

Cultures. *Pseudomonas putida* F1 was used as a positive control strain for BTEX dioygenase genes and was grown in Luria-Bertani broth (LB) (30) or a basal salt medium (BSM) supplemented with toluene vapors as the sole carbon source, as described by Chen et al. (3). *Pseudomonas putida* AC10-7 was used as a negative control strain for BTEX dioxygenase genes and was grown in either LB or in BSM supplemented with salicylate (37). Strain AC10-7, however, harbored NAH7 plasmids that encode naphthalene dioxygenase, and while functionally related, is not a target of the BTEX primer/probe set. Cultures were grown overnight at 30°C and 200 rpm to produce dense cultures in mid-logarithmic growth phase. For ISRT studies wherein rRNA was the target, cells grown in LB were used. For ISRT studies in which the target was mRNA, and for *in situ* PCR/H and *in situ* RT-PCR/H studies, cells grown at the expense of toluene in BSM were used.

Natural aquatic samples. Sample types included size-fractionated natural estuarine or coastal water samples as well as subsamples taken from experimental microcosms (Table 3.2). For each estuarine or coastal sample, approximately 500 ml was collected from the surface in a sterile Nalgene bottle and transported on ice back to the laboratory. Estuarine samples were then passed through a 3.0 μm pore size polycarbonate membrane (47 mm, Nucleopore) under gentle vacuum (300 mbar) to remove large particles and

TABLE 3.2. Sampling sites used in this study.

Estuarine sample	**Location**
Hudson Creek[a]	Meridian GA
St. Andrew Sound [b]	Jekyll Island GA
Duplin River[c]	Sapelo Island GA
Sapelo River[c]	Eulonia GA
Dean Creek[c]	Sapelo Island GA
Lazaretto Creek[c]	Tybee Island GA
Savannah River[c]	Savannah GA
Wilmington River A[c]	Thunderbolt GA
Wilmington River B[c]	Wassau Island GA

[a]Estuarine water sample and microcosm

[b]Estuarine water sample only

[c]Microcosm sample only

plankton, then stored at 4°C. For microcosms, estuarine water samples were also size-fractionated in the same manner and then diluted 100-fold in an artificial seawater/basal salt medium (AS/BSM) (BSM [3] using sea salts [Sigma Chemical Co.] at a final concentration of 20 g liter^{-1} to simulate estuarine salinities). Microcosms were either supplemented with toluene vapors (3) or left unamended.

Cell fixation. When *P. putida* F1 or *P. putida* AC10R-7 were used, an initial 100-fold dilution of mid-log phase culture in phosphate-buffed saline (PBS) [pH 7.6] (Sigma Chemical Co.) was made before fixation to achieve cell densities similar to what was expected in environmental samples (approximately 10^6 cells ml^{-1}) (22). Freshly prepared 10% paraformaldehyde (in PBS) was then added to diluted cultures or natural samples (1 volume fixative: 3 volumes sample) (13) and incubated at 4°C for 2 hrs.

Concentration by membrane filtration. Once cell fixation was complete, a 25 mm diameter glass filter tower (Millipore, Bedford, MA), was employed to concentrate samples onto membrane filters. Membranes from various manufacturers (Table 3.3) (Nucleopore, Poretics, Millipore, and Whatman) were used initially to determine compatibility with *in situ* amplification techniques. Filters were placed upon a cellulose nitrite support filter (25 mm diameter, 0.2 μm pore size, Millipore) previously moistened with 0.2 μm-filtered distilled water. Sample volume differed by location. Concentration of the samples to yield a target cell density of between 30 and 300 cells per microscope field was achieved by applying a gentle vacuum (300 mbar). Fixative was removed by two consecutive washes with 10.0 ml of 0.2 μm-filtered PBS. Filters were then removed

TABLE 3.3. Filter types examined, their autofluorescence under CY3 excitation, and compatibility with *in situ* amplification techniques.

Product/ Manufacturer	Material	Pore Size (µm)	Autofluorescence Intensity[a]	CY3 Signal
Nucelopore	Polycarbonate, black, hydrophilic	0.2	++	Yes
Nucelopore	Polycarbonate, white, hydrophilic	0.2	+	Yes
Poretics	Polycarbonate, black, PVP free, hydrophilic	0.2	++	Yes
Poretics	Polycarbonate, white, PVP free, hydrophilic	0.2	+	Yes
Isopore GTTP, Millipore	Polycarbonate, black, hydrophilic	0.2	++	Yes
Isopore GTTP, Millipore	Polycarbonate, white, hydrophilic	0.2	+	Yes
Durapore GVHP, Millipore	Polyvinylidene difluoride, hydrophilic	0.2	++	Yes[b]
Fluoropore FGLP, Millipore	Polytetrafluoroethylene with a polyethylene backing, hydrophobic	0.2	+	nd[c]
Anodisc 25, Whatman	Alumina matrix with a polypropylene support	0.2	+ to +++[d]	Yes

[a] As determined under CY3 excitation conditions: +, faintly autofluorescent; ++ moderately autofluorescent; +++, strongly autofluorescent

[b] Although *in situ* amplification signals were achieved, cell loss from the filters was extensive (>75%, data not shown)

[c] nd, not determined; Polyethylene backing on filters made them difficult to work with and samples routinely dried out during ISRT procedures

[d] We observed a high degree of variability in the autofluorescence of this filter type and eliminated them from further use in the study.

to sterile tissue culture dishes (Corning), and allowed to completely air-dry, then stored at -20°C until *in situ* PCR techniques were performed.

Oligonucleotide primers and probes. The sequences of the primers used in this study are listed in Table 3.4. All primers and probes were synthesized by IDT DNA Inc. Primer 1492R was used for rRNA detection protocols, while primers BTEX1 and BTEX2 served to investigate functional gene presence (DNA) as well as gene activity (mRNA). Probe BTEX4 was labeled with CY3-dye as a fluorescent marker. The BTEX primers and probe were designed to detect a sequence common to a group of related dioxygenase genes involved in the degradation of aromatic compounds including benzene, toluene, ethyl-benzene and xylene (BTEX) (Dr. Feng Chen, personal communication). BTEX primers and probe were designed based on the sequence alignments of 12 BTEX dioxygenase genes for characterized enzymes including: toluene dioxygenase; biphenyl dioxygenase; benzene dioxygenase; chlorobiphenyl dioxygenase; 2,3-dihyrdoxybiphenyl 1,2-dioxygenase, and isopropylbenzene 2,3-dioxygenase.

Cell wall permeabilization. Each replicate filter was cut into quarters before further analysis. One quarter of each filter was placed back into a tissue culture dish and stored at -20°C for a later assessment of cell recovery efficiency rates. This filter piece represented the original number of cells adhered to the filter prior to any *in situ* amplification steps that might have resulted in a loss of cells from the filter (see below). Another quarter section of each replicate was overlaid with 1 ml of freshly prepared lysozyme (Sigma Chemical Co.) at a final concentration of 0.5 mg ml^{-1} in lysozyme

TABLE 3.4. Sequences and targets of oligonucleotide primers and probes used for *in situ* amplification in this study.

Primer/ Probe	**Primer Sequence (5' - 3')**	**Target**	**Reference**
1492R	GGTTACCTTGTTACGACTT	Eubacteria, 16S RNA	Lane 1991
BTEX1	GTVTTCCTVAACRTGYCG	BTEX dioxygenases	This study
BTEX2	CCVCGVGCMGCYTCNTCG	BTEX dioxygenases	Chen *et al.* 1999 b
BTEX4	[a]AGYTAYCAYGGSTGGGCYTACGA	BTEX dioxygenases	This study

[a] Labeled at the 5' end with CY3 fluorescent marker.

buffer (100 mM tris, 50 mM EDTA [pH 8.0]) for 10 min at 25°C to permeabilze the cell walls, thus allowing entry of polymerases into the cells. Lysozyme was removed by transferring the filter section to a new tissue culture dish containing 0.2 μm-filtered PBS and gently moving the filter through the liquid for 30 seconds. After this wash, filters were placed on Whatman 3M filter paper and dried in an oven at 55°C to completely deactivate any lysozyme remaining in the cells. In cases where rRNA or mRNA analyses were intended, the lysozyme buffer and PBS solutions were treated with DEPC to minimize RNase contamination (3, 4).

Nuclease Pre-treatment. Prior to ISRT and ISRT-PCR/H analyses, permeabilized cells were treated with RNase-free DNase (Boehringer Mannheim) at a final concentration of 0.1 U μl^{-1} at room temperature for 2 hrs, then rinsed with 0.2 μm-filtered PBS to remove the enzyme. DNased filters were then dried once again on Whatman 3M filter paper in an oven at 55°C. When the target was DNA, permeabilized cells were treated with DNase-free RNase I (Boehringer Mannheim) at a final concentration of 0.01 U μl^{-1} at room temperature for 2 hrs, then rinsed and dried as described above.

PCR optimization for BTEX primers. DNA from *P. putida* F1 and *P. putida* AC10R was used to optimize the conditions necessary for successful amplification of the dioxygenase genes targeted by the BTEX primers. Bacterial DNA was purified from the positive and negative control strains grown in Luria-Bertani broth using a QIAmp Blood and Tissue Kit (QIAGEN Inc.) following manufacturer's instructions.

Extracted DNA (20 ng) was added to 50.0 µl of PCR cocktail containing a final concentration of 1X Expand High Fidelity PCR Buffer (Boehringer Mannheim), 2.5 mM $MgCl_2$, 200.0 µM dNTP mix, 1.0µM each of BTEX1 and BTEX2, and 0.5 U of Expand High Fidelity Enzyme (Boehringer Mannheim). Additionally, a negative control tube containing all reagents except DNA template was included in each PCR trial. PCR was conducted using a PTC-200 DNA Engine (MJ Research) under the following parameters: 1) initial denaturation at 94ºC for 3 min, 2) 35 cycles (1 min each) of: denaturation at 94ºC, annealing at 64ºC, and extension at 72ºC, and 3) 5 min at 72ºC. For RT-PCR, an initial reverse transcription step of 30 min at 64ºC was included prior to the above cycling protocol. Products were visualized via electrophoresis in a 1.5% agarose gel. Based on sequence alignments, amplification of the dioxygenase genes was expected to produce a product approximately 1.2 kb in size.

The optimized DNA amplification parameters were determined to be: 1) initial denaturation at 94ºC for 3 min, 2) 35 cycles (1 min each) of: denaturation at 94ºC, annealing at 64ºC, and extension at 72ºC, and 3) 5 min at 72ºC. These parameters were used for the remainder of the study.

Optimization of hybridization conditions for BTEX4 following *in situ* PCR. Permeabilized cells of *P. putida* F1 and *P. putida* AC10R-7 concentrated onto polycarbonate membrane filters were used as positive and negative control strains, respectively, to optimize in situ hybridization conditions for the BTEX4 probe. After *in situ* amplification of BTEX gene targets, 55 µl of hybridization solution (900 mM NaCl, 20 mM Tris-HCl, 0.01% sodium dodecyl sulfate; pH 7.2) containing 5 ng/µl of CY3-labeled BTEX4 probe were sealed inside a FrameSeal chamber with a quarter section of membrane filter containing one control strain or the other. Slides were placed in a block assembly specific for slide PCR in a PTC-200 DNA Engine and initially denatured for 3 min at 94°C, followed by one cycle (2 hrs) of hybridization ranging from 55°C to 68°C.

Post hybridization, excess probe was removed by a brief wash with 0.2 µm-filtered 0.5X SSC which had been pre-warmed to the temperature of hybridization followed by a brief dip in with 0.2 µm-filtered PBS. Wet filters were then subjected to dual staining (see below), mounted in oil and viewed under epifluorescence to assess the specificity of probe hybridization. The optimal hybridization temperature was determined to be 63°C.

***In Situ* Reverse Transcription (ISRT).** ISRT procedures, specifically the CY3-dUTP incorporation method, were followed from Chen et al. (5) with the following modifications to our original methods: 1) fixed cells were concentrated onto polycarbonate filters; 2) the ethanol dehydration series used to affix cells to the surface of glass slides was omitted; 3) RNase inhibitor (0.8 U μl^{-1}, Boehringer Mannheim) was added in the primer hybridization solution; 4) only AMV reverse transcriptase

(Boehringer Mannheim) was used for reverse transcription; and 5) hybridization solution and ISRT cocktails were prevented from drying out during incubation by using FrameSeal Chambers (MJ Research). Studies subsequent to our initial ISRT publication (5) revealed that the omitted steps were unnecessary, at least under these culture conditions.

Briefly, the ISRT protocol was as follows. After permeabilization, filter quarters were placed in a FrameSeal chamber (65 µl capacity) with 55 µl of ISRT solution containing primers and RNase inhibitor. Slides were placed in a PTC-200 DNA Engine thermal cycler (MJ Research) block designed for slides. Primers were allowed to hybridize to the target rRNA or mRNA for 3 hr at 45°C, then unbound primers were removed by washing with pre-warmed (45°C) 0.5X SSC that had been treated with DEPC and filtered through a 0.2 µm filter. Filter quarters were then placed in new FrameSeal chambers and along with 55 µl of ISRT cocktail containing CY3-labeled UTP. Reverse transcription was allowed to proceed for 3 hr at 45°C, during which CY3-labeled dUTP was incorporated into the product. Excess CY3 label was removed from the filter-adhered cells by gentle washing with 0.2 µm-filtered 0.5X SSC. Cells were then counterstained with a general nucleic acid stain to visualize all cells present in the sample (see below).

***In Situ* PCR/Hybridization (*in situ* PCR/H).** Briefly, a quartered section of membrane filter containing permeabilized cells was placed in a FrameSeal chamber along with 55 µl of PCR cocktail and optimized parameters for in situ BTEX dioxygenase gene amplification were applied (described above). After PCR cycling was complete, filter

sections were rinsed briefly in 0.2 μm-filtered PBS, and allowed to dry on Whatman 3M filter paper in a 55°C oven for 5 min. Filter sections were then placed in new FrameSeal chambers with 55 μl of hybridization solution containing the CY3-labeled BTEX4 probe (final concentration 5 ng μl^{-1}), placed back into the slide block and subjected to optimized hybridization conditions: 1) initial denaturation at 94°C for 3 min, 2) hybridzation at 63°C for 2 hrs and 3) washing briefly with pre-warmed 0.2 μm-filtered 0.5X SSC to remove excess probe. After air-drying on Whatman 3M filter paper, filters were subjected to the dual staining procedures described below and viewed with epifluorescence microscopy for further enumeration of total cell number, as well as number of amplification-positive cells.

***In Situ* RT-PCR/Hybridization (*in situ* RT-PCR/H).** Generally, *in situ* RT-PCR/H procedures were followed exactly as for *in situ* PCR/H described above with the following exceptions: 1) RNase inhibitor (0.8 U μl^{-1}, Boehringer Mannheim) was added into RT-PCR cocktail; 2) a Titan One Tube RT-PCR System kit (Boehringer Mannheim) was used in place of an Expand High Fidelity PCR System (Perkin Elmer); 3) filters were initially held at the optimized annealing temperature (64°C) for 30 min to allow for reverse transcription of mRNA into cDNA before amplification; and 4) distilled water and wash solutions were treated with DEPC (3) to prevent contamination of samples with RNases.

Dual Staining. After *in situ* amplification (or amplification/hybridization), YO-PRO (Molecular Probes Inc.) was used at a 0.1 μM final concentration in PBS for 2 min as a counterstain to the amplification (CY3) signal. Excess stain was removed by a gentle

rinsing in 0.1% Nonidet P40 (Sigma Chemical Co.) in 0.2 μm-filtered PBS, followed by a brief rinse in 0.2 μm-filtered distilled water to remove any salts. Filter sections were allowed to air dry, and were then mounted in FF immersion oil (R.P. Cargille Laboratories Inc.).

Digital Image Analysis. Slides were examined under an epifluorescence microscope (Olympus BX 40) with a high-resolution 100X U Plan Oil objective lens (numerical aperture, 1.35 to 0.50) (C-squared Corp.). Single band excitation filters (Chroma Technology, set 82000) for CY3 (555 ± 12.5 nm) and FITC (484 ± 7.5 nm) channels were used in conjunction with a triple emission filter (458 ± 9.5 nm; 518 ± 15.5 nm; and 602 ± 21 nm). BTEX-positive target cells labeled with CY3 were viewed under green light (CY3 excitation filter) while cells stained with YO-PRO (all cells containing DNA or RNA) were viewed under blue light (FITC excitation filter). Since the samples were counterstained, samples were first exposed to the CY3 excitation conditions to visualize in situ amplification (ISRT or in situ PCR/H or in situ RT-PCR/H) positive cells. Next, the same field was exposed to YOPRO excitation wavelengths to visualize all cells adhered to the filter.

Images were acquired with a cooled charge-coupled device (CCD) Sensys 1400 camera (1317 by 1035 image array, 6.8 by 6.8 μm pixel, 12 bit) (Photometrics) and processed with the Oncor Image (Oncor Inc.) software package ver. 2.02 on a Power Macintosh 9500 (Apple Computer Inc., Cupertino CA). For image capture, exposure times were controlled by the software package and were 1.5 sec for CY3 images and 1.0 sec for YOPRO images.

Bacterial Enumeration and Data Analysis. As described above, one half of each replicate filter was left untreated and stored at -20°C. so that the total number of cells originally present on each filter could be determined after staining with YOPRO. For untreated filters, as well as filters subjected to *in situ* amplification protocols, the cell densities for each replicate sample were calculated from the mean cell count of 10 microscopic fields multiplied by the filter-to-microscope conversion factor. Care was taken to exclude fields directly adjacent to the cut edge of the filter pieces in order to account for bias caused by excessive filter manipulation, otherwise fields were chosen randomly within the interior area of the filter section. Recovery efficiency rates for each replicate were calculated by dividing the mean number of cells ml^{-1} present on each filter piece after in situ amplification by the mean number of cells ml^{-1} present on the matching untreated filter piece.

RESULTS

Compatibility of membrane filter materials. Membrane filters are available from several manufacturers in an array of materials (Table 3), though the most widely used for microbial counts are made of polycarbonate. The presence of some filter materials, including polycarbonate, has been observed to produce detrimental effects on in vitro PCR amplification of DNA and the effects ranged from complete inhibition to a drastic reduction in amplification efficiency (2). Thus, the compatibility and sensitivity of various filter types from different manufacturers were assessed for use with bacterial *in situ* amplification including ISRT, *in situ* PCR/H and *in situ* RT-PCR/H protocols.

In preliminary experiments using polycarbonate filter-adhered cells, we observed CY3-labled dUTP incorporation via ISRT techniques to yield visible but somewhat high background signals (3). Polycarbonate filters pre-stained with Irgalan Black yielded a much higher autofluorescence under CY3 excitation conditions that nearly eclipsed the observation of ISRT signals within the cells. Glockner et al. (9) also noted that black polycarbonate filters had increased background fluorescence compared to white filters during ISH experiments using CY3-labeled probes. We also observed that during the thermocycling steps of *in situ* PCR and *in situ* RT-PCR, Irgalan Black diffused from the membranes into the PCR cocktail, essentially rendering the polycarbonate membranes unstained. Thus, black polycarbonate filters were omitted from future use. White polycarbonate filters, independent of manufacturer, were routinely found to produce the least background fluorescence when used under CY3 excitation conditions (Table 4). However, using 4',6'-diamidino-2-phenylindole (DAPI) as a total DNA counterstain on white polycarbonate filters (34) was problematic due to high levels of filter material autofluorescence under UV excitation. Consequently, YOPRO (14) was used in place of a DAPI as a counterstain for enumeration of total cells present on a given filter section. Cells adhered to white polycarbonate membranes were successfully subjected to ISRT, *in situ* PCR/H, and *in situ* RT-PCR/H protocols using our control bacterial strains.

Alumina matrix filters (i.e. Anodisc 25 [Whatman]) had been reported previously to have low levels of autofluorescence when used for ISH with Texas Red-labeled probes in filter-concentrated stream bacterial communities (29). While ISRT signals were discernible from cells collected on alumina filters, we observed significant variations in the amount of autofluorescence between the filters themselves under CY3 excitation

conditions. Further, these filters proved to be too fragile to survive the extensive manipulations involved in *in situ* amplification protocols. We investigated the use of Durapore and Fluoropore (Millipore) filter substrates as well, and although autofluorescence of the filter material under CY3 excitation conditions was absent overall, neither substrate was as compatible with our techniques as the standard white polycarbonate filters. Although we achieved successful ISRT of *P. putida* F1 cells concentrated on Duropore GVHP filters, the numbers of cells lost during the process routinely exceeded 75%. Because the backing of Fluoropore FGLP filters made them difficult to section without destroying the rest of the filter, they were completely eliminated from either ISRT or other *in situ* amplification trials.

Cell fixation and permeabilization. One of the main objectives of fixation is to maintain cellular integrity during extensive periods of thermocycling. Fixation also prevents change in bacterial numbers due to growth and the breakdown of organic materials within cells. Originally, paraformaldeyde fixation of samples for *in situ* amplification was modeled after those used in *in situ* hybridization protocols (6, 11). However, the overnight fixation times (≈16 hr) used in standard ISH protocols coupled with the time-intensive *in situ* PCR techniques made the techniques somewhat impractical for large numbers of samples. Additionally, extended fixation time has been shown to be detrimental to successful amplification of targets in *in situ* PCR with eukaryotes (31) and may have similar effects on prokaryotic in situ amplification.

During paraformaldehyde fixation, proteins are crossed-linked to one another (23) creating structural barriers that can impede entry of PCR enzymes and critical reagents

into the cell and potentially limit their access to the nucleic acids targets. As a result, the bacterial cells must be subjected to a permeabilization treatment sufficient to allow penetration of chemicals into the cells, yet mild enough to prevent significant loss of amplified DNA from the cells.

P. putida F1 cells grown in LB and fixed in paraformaldehyde and concentrated onto polycarbonate membranes were used initially. Fixation times of 1, 2, and 4 hr were tested. Fixed and filter-adhered cells from each fixation treatment group were then subjected to permeabilization by lysozyme at either 0.5 mg ml^{-1} or 1.0 mg ml^{-1} for 10 min at 25°C in order to determine the best combination of fixation and permeabilization treatments. Lysozyme-treated cells were then subjected to thermocycling procedures in PCR cocktail, washed, and then incubated in hybridization buffer under optimized conditions. Finally, the treated, filter-adhered cells were given a final wash after hybridization was complete, then stained with YOPRO. Cells were viewed under the epifluorescence microscope to ascertain that cellular integrity had been preserved, and that DNA had remained inside the cells post-treatment. As a comparative control, fixed cells that had been subjected to lysozyme treatment but no PCR or hybridaization were used.

In cells fixed for 1 hr, the relative fluorescence of permeabilized cells was low compared to the untreated cells, indicating a significant loss of DNA post-treatment. Additionally, the morphology of the treated cells appeared deformed; cells appeared shrunken and shriveled. Lysozyme-treated cells that had been fixed for a minimum of 2 hr remained brightly stained after thermocycling, indicating that DNA loss from the cells was minimal. However, the morphology of *P. putida* F1 cells treated with 1.0 mg ml^{-1}

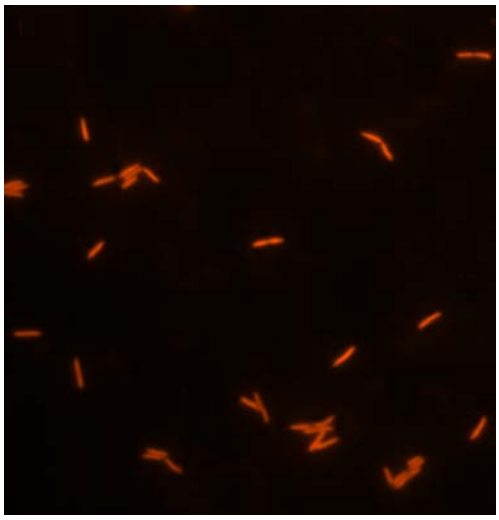
lysozyme after 2 hr of fixation was somewhat compromised; a small halo of fluorescence was observable outside the cell wall indicating that DNA loss from the cells was beginning to occur at this permeabilization level. When cells were fixed for 2 hrs and subsequently permeabilized with 0.5 mg ml^{-1} lysozyme, the halo was absent. When fixation time was lengthened to 4 hr, we observed results highly similar to those observed in the 2 hr fixation period. Thus, the fixation time was reduced from an initial 16 hr in our previous studies to 2 hr, and the lysozyme concentration was reduced to 0.5 mg ml^{-1}. To ensure that these conditions were also compatible with cells from natural aquatic environments, filter-adhered cells from size-fractionated (< 3.0 μm) estuarine samples were examined for morphological quality after treatment with these optimized conditions. As observed with the control strain, a 2 hr fixation period combined with permeabilization for 10 min at 25°C by 0.5 mg ml^{-1} lysozyme resulted in brightly stained cells. To ensure that fixed and permeabilized cells were still amenable to *in situ* amplification procedures under these new conditions, we subjected the treated, filter-adhered cells of our control strains to ISRT, *in situ* PCR/H and *in situ* RT-PCR/H with success.

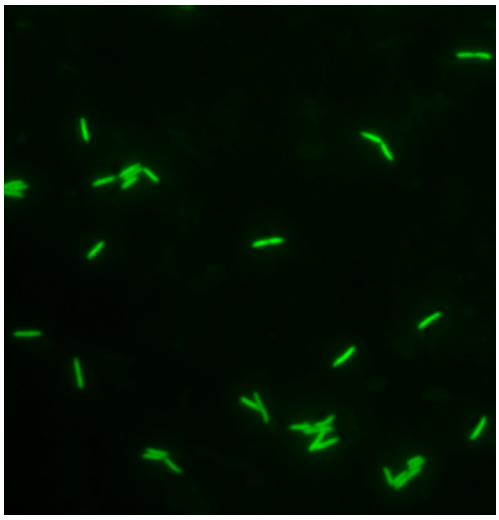
Sensitivity and specificity of detection. Initially, *P. putida* F1 was used to determine the sensitivity and specificity of ISRT on filter-concentrated samples. A single primer (1492R) was used in conjunction with CY3-labeled nucleotides and reverse transcriptase to produce CY3-labeled cDNA from rRNA sequence targets within the filter-adhered cells. ISRT amplification signal (CY3) was successfully achieved in all replicate samples, indicating that our newly optimized conditions for fixation and permeabilization

were suitable for use with in situ amplification techniques. Figure 3.1 A shows cells that were visible under CY3 excitation and represents the number of amplification-positive cells collected onto the filter. Figure 3.1 B shows the same field of cells, except that it shows all cells adhered to the filter. As expected given the high level of target that 16S rRNA provides within the cell, we were able to detect 97.7% (Table 3.5) of *P. putida* F1 cells present on the filter using 16S rRNA as a target. In negative controls where enzyme was omitted from the ISRT cocktail, no amplification signal was detectable within the P. putida F1 cells under CY3 excitation conditions (Figure 3.1C), yet nucleic acids remained present inside intact cells as revealed by YOPRO staining (Figure 3.1D).

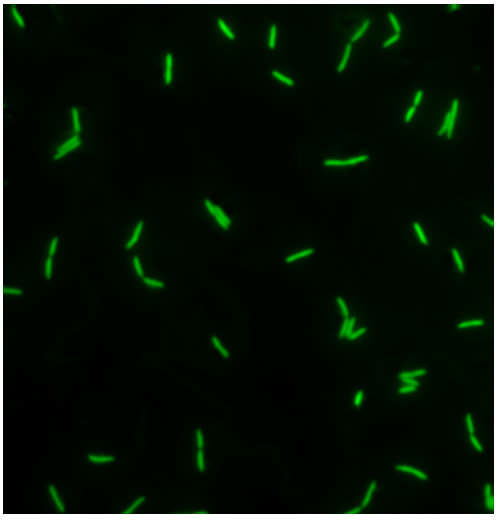
P. putida F1 and *P. putida* AC10R-7, served as positive and negative control strains, respectively, for *in situ* PCR/H and *in situ* RT-PCR/H using BTEX primers to detect either the presence of functional genes involved in toluene degradation or their gene products. Strain F1 carries a single (nominally) chromosomal copy of the gene encoding toluene dioxygenase (*todC*1) (10). Strain AC10R-7 lacks the above gene, but carries a single copy of NAH7 plasmid which possesses genes specific for production of a dioxygenase involved in naphthalene degradation (34), but which is not a target of the BTEX primer set. Cultures were grown in BSM medium supplemented with either toluene or salicylate as the sole carbon source to induce the transcription of BTEX and naphthalene dioxygenases, respectively. Since either toluene or naphthalene was the sole carbon source, we assumed that all cells present in the culture must possess the appropriate dioxygenase in order to reproduce. Thus, it was expected that amplification positive cells would approach 100% of the total cells present on the filter if they had the target gene when *in situ* PCR/H was applied. Similarly, because cells growth occurred

FIGURE 3.1. Detection of ISRT in filter-concentrated bacterial strains. (A) Viewed under CY3 excitation conditions, target cells of *P. putida* F1 show the red fluorescence of CY3 after CY3-labeled nucleotides were directly incorporated into the amplification product using a eubacterial primer and ISRT. (B) The same field of cells as in (A), except viewed under YOPRO excitation conditions so that all cells can be visualized. (C) When enzyme was omitted, *P. putida* F1 cells showed no red fluorescence under CY3 excitation conditions, indicating no amplification had occurred. (D) The same field of cells as in (C), except viewed under YOPRO excitation conditions so that all cells can be visualized.

A 

B 

C 

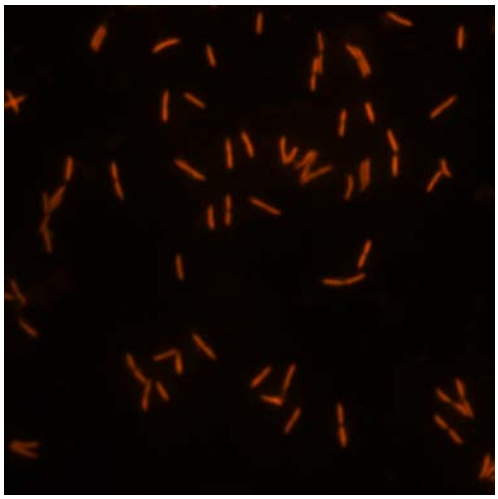
D 

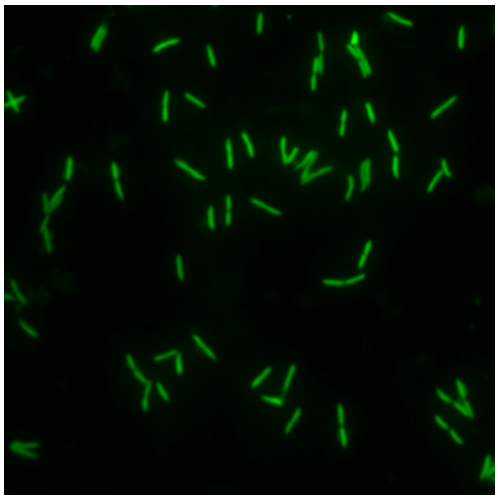
under conditions inducing transcription of the dioxygenases, we assumed that nearly 100% of the filter-adhered cells possessing the target mRNA would also be detected by *in situ* RT-PCR/H signal.

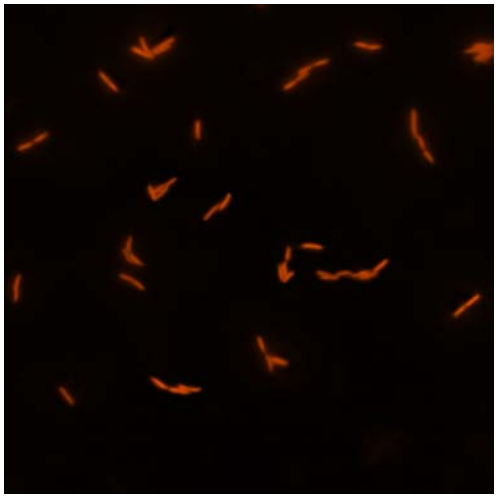
Figure 3.2 shows typical results achieved in *P.putida* F1 after *in situ* PCR/H (A and B) and after *in situ* RT-PCR/H (C and D). Overall, we were able to detect 99.2 % and 98.8% of *P. putida* F1 cells on filters after *in situ* PCR/H and *in situ* RT-PCR/H, respectively (Table 3.5). Cells of strain AC10R-7 (negative controls) consistently did not yield an observable signal under CY3 conditions after hybridization with probe BTEX4 during *in situ* PCR/H and *in situ* RT-PCR/H (figure not shown), indicating that our primers, probe, and optimized amplification/hybridization conditions were well-suited for the specific detection of BTEX dioxygenases.

Once successful amplification was established in pure laboratory control strains, the techniques were applied to natural aquatic bacterial samples. Estuarine and coastal water samples were fixed, filter-concentrated, permeabilized and subjected to ISRT (eubacterial primer, 16S rRNA target) or *in situ* RT-PCR/H (BTEX primers and probe, mRNA target). With the 16S rRNA primers, we found that 23.3% and 44.8%, depending on sample site, of all putative bacterial cells present on the filter were also observed under the CY3 excitation conditions after ISRT (Table 3.5). Figure 3.3 shows a typical example of ISRT results from natural estuarine samples where less than 50% of the bacterial cells detected by nucleic acid stain (B) are also detected by ISRT (CY3-labeled nucleotide incorporation) (A). Since the cellular 16S rRNA levels were unknown in these natural samples, and may be significantly lower in inactive cells (28), this result

FIGURE 3.2. Detection of ISPCR/H and ISRT-PCR/H in filter-concentrated bacterial strains. (A) *P. putida* F1, grown at the expense of toluene, was subjected to ISPCR/H using a set of BTEX primers. Subsequently, amplified product was detected using a CY3-labeled probe under stringent hybridization conditions. ISPCR/H positive cells were viewed under CY3 excitation conditions. (B) The same field of cells as in (A), except viewed under YOPRO excitation conditions so that all cells can be visualized. (C) *P. putida* F1, grown at the expense of toluene, was subjected to ISRT- PCR/H using a set of BTEX primers. Subsequently, amplified product was detected using a CY3-labeled probe under stringent hybridization conditions. ISRT-PCR/H positive cells were viewed under CY3 excitation conditions. (D) The same field of cells as in (C), except viewed under YOPRO excitation conditions so that all cells can be visualized.

A 

B 

C 

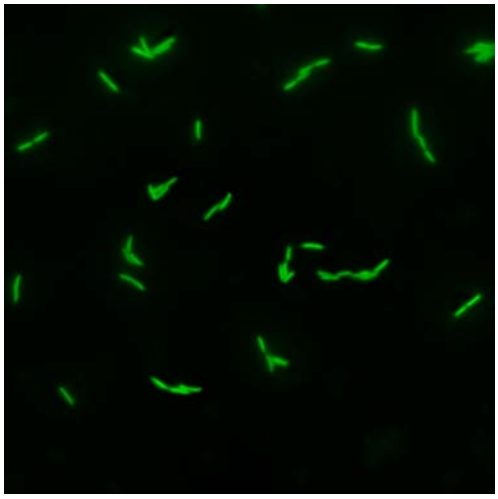
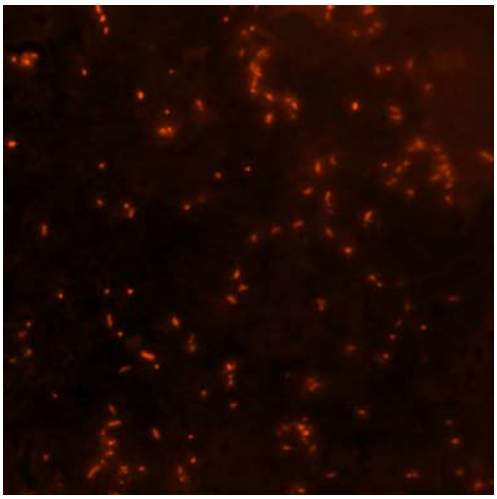
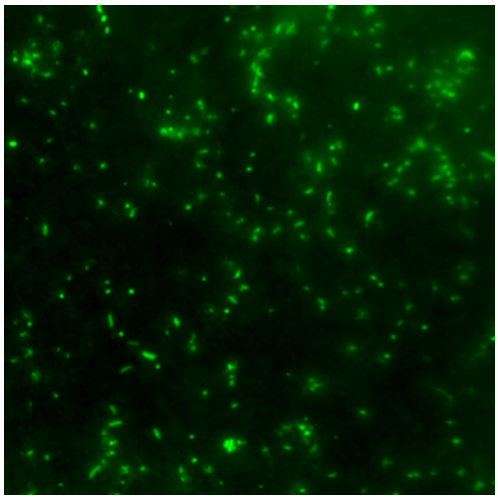
D 

TABLE 3.5. Sensitivity of *in situ* amplification techniques applied to filter-concentrated bacterial strains and assemblages.

Sample Type	**Number of Replicates**	In Situ *Technique*	**Primer(s)/ Probe Used**	**Percentage CY3+ Cells Detected**[a]	**Mean Percentage CY3+ Cells Detected**
P. putida F1, Luria broth	6	ISRT	1492R	96.8 – 98.5	97.7
St. Andrew Sound (July 1999)	3	ISRT	1492R	20.8 – 23.9	23.3
Hudson Creek (May 1999)	3	ISRT	1492R	44.1 – 45.3	44.8
P. putida F1, BSM + toluene vapor	3	ISRT-PCR/H	BTEX1, BTEX2	98.1 – 100.3	98.8
St. Andrew Sound (July 1999)	3	ISRT-PCR/H	BTEX1, BTEX2	0.0 – 1.4	1.2
Hudson Creek (May 1999)	3	ISRT-PCR/H	BTEX1, BTEX2	0.9 – 1.2	1.0
P. putida F1, BSM + toluene vapor	5	ISPCR/H	BTEX1, BTEX2/ BTEX4	97.4 – 99.8	99.2

[a] Defined as: $\frac{\text{mean \# cells/ field giving CY3 signal on filter after amplification}}{\text{mean \# cells/field stained with YOPRO on filter after amplification}} \times 100$

FIGURE 3.3. Detection of ISRT in a filter-concentrated bacterial assemblage. A natural assemblage of aquatic bacteria from Hudson Creek was subjected to ISRT using a eubacterial primer. CY3-labeled nucleotides were incorporated directly into the amplification product and viewed under CY3 excitations to visualize all ISRT positive cells (A) and under YOPRO excitation conditions to visualize all cells present on the filter (B).

A  B 

was not unexpected. Also, the permeabilization protocols are optimized for Gram negative bacteria only. When ISRT-PCR/H was applied to estuarine samples using the BTEX primer set, only 1.0 to 1.2% of the community adhered to the filters was observed to yield a CY3 signal (Table 3.5) indicating a low level of activity for BTEX degradation at the time of sampling.

Recovery efficiency. Total bacteria counts were compared between two sections of the same polycarbonate filter, one untreated and the other subjected to *in situ* techniques, in order to determine the recovery efficiency of cells after in situ amplification procedures were applied. Recovery efficiency, or the percentage of cells remaining attached to the filter, was observed to be consistent within each sample; a range of recovery efficiencies for replicate sample is given in Table 3.6. Mean recovery efficiencies for all replicate samples of each control strain, estuarine samples and estuarine microcosm samples were calculated. Mean recovery efficiency varied from 75.7% to slightly greater than 100% in a few circumstances (Table 6). Overall, only 3 samples out of 88 resulted in less than an 85% recovery efficiency.

DISCUSSION

Marine bacterial assemblage densities range from 10^4 cells ml^{-1} to 10^8 cells ml^{-1} (22), often making it necessary to concentrate the natural populations before microscopic analyses can be performed. Standard filtration protocols provide a rapid and acceptable means of concentrating samples with bacteria from low cell densities, like those found in aquatic environments. While centrifugation could also serve to increase cell densities in

TABLE 3.6. Recovery efficiency ranges and means of samples concentrated onto 0.2 μm polycarbonate filters and subjected to *in situ* amplification protocols. Part I.

Sample/Growth medium	**Replicates**	In Situ *Technique*	**Primer(s)/ Probe Used**	**Range of Recovery Efficiencies (%)**[a]	**Mean Recovery Efficiency (%)**[b]
P. putida F1, LB	6	ISRT	1492R	94.3 – 95.4	94.8
Hudson Creek (May 1998)	4	ISRT	1492R	80.3 – 82.8	81.5
St. Andrew Sound (July 1999)	3	ISRT	1492R	86.3 – 91.8	89.1
Hudson Creek (May 1999)	3	ISRT	1492R	87.6 – 92.3	89.2
PpF1, BSM + toluene vapor	3	ISRT-PCR/H	BTEX1, BTEX2	96.6 – 98.9	97.5
St. Andrew Sound (July 1999)	3	ISRT-PCR/H	BTEX1, BTEX2	89.5 – 92.3	91.5
Hudson Creek (May 1999)	3	ISRT-PCR/H	BTEX1, BTEX2	87.1 – 92.3	90.4
P. putida F1, BSM + toluene vapor	5	ISPCR/H	BTEX1, BTEX2/ BTEX4	98.9 – 100.3	99.2
Hudson Creek (July 2001), AS/BSM microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND[b]	t = 0: 87.1 t = 12: 85.3 t = 24: 94.2 t= 72: 96.2 t= 120: 98.9
Hudson Creek (July 2001), AS/BSM + toluene vapor microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 76.2 t = 12: 89.5 t = 24: 99.3 t= 72: 96.2 t= 120: 100.0

TABLE 3.6. Recovery efficiency ranges and means of samples concentrated onto 0.2 μm polycarbonate filters and subjected to *in situ* amplification protocols. Part II.

Sample/Growth medium	**Replicates**	In Situ *Technique*	**Primer(s)/ Probe Used**	**Range of Recovery Efficiencies (%)[a]**	**Mean Recovery Efficiency (%)[b]**
Duplin River (July 2001), AS/BSM microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 99.8 t = 12: 99.0 t = 24: 99.8 t= 72: 96.7 t= 120: 101.2
Duplin River (July 2001), AS/BSM + toluene vapor microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 95.6 t = 12: 99.1 t = 24: 97.4 t= 72: 98.8 t= 120: 98.3
Sapelo River (July 2001), AS/BSM microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 96.9 t = 12: 97.4 t = 24: 96.4 t= 72: 100.7 t= 120: 99.8
Sapelo River (July 2001), AS/BSM + toluene vapor microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 91.8 t = 12: 91.8 t = 24: 98.6 t= 72: 100.6 t= 120: 97.0
Dean Creek (July 2001), AS/BSM microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 90.4 t = 12: 99.1 t = 24: 95.5 t= 72: 100.7 t= 120: 99.8

TABLE 3.6. Recovery efficiency ranges and means of samples concentrated onto 0.2 μm polycarbonate filters and subjected to *in situ* amplification protocols. Part III.

Sample/Growth medium	**Replicates**	In Situ *Technique*	**Primer(s)/ Probe Used**	**Range of Recovery Efficiencies (%)[a]**	**Mean Recovery Efficiency (%)[b]**
Dean Creek (July 2001), AS/BSM + toluene vapor microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 94.1 t = 12: 91.3 t = 24: 85.6 t= 72: 96.6 t= 120: 98.9
Lazaretto Creek (August 2001), AS/BSM microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 95.6 t = 12: 106.7 t = 24: 90.2 t= 72 : 99.3 t= 120: 97.4
Lazaretto Creek (August 2001), AS/BSM + toluene vapor microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 98.5 t = 12: 96.8 t = 24: 90.4 t= 72: 99.4 t= 120: 98.3
Savannah River (August 2001), AS/BSM microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 95.4 t = 12: 93.1 t = 24: 93.4 t= 72: 99.3 t= 120: 100.2
Savannah River (August 2001), AS/BSM + toluene vapor microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 92.5 t = 12: 98.2 t = 24: 91.7 t= 72: 99.4 t= 120: 99.8

TABLE 3.6. Recovery efficiency ranges and means of samples concentrated onto 0.2 μm polycarbonate filters and subjected to *in situ* amplification protocols. Part IV.

Sample/Growth medium	**Replicates**	In Situ *Technique*	**Primer(s)/ Probe Used**	**Range of Recovery Efficiencies (%)[a]**	**Mean Recovery Efficiency (%)**
Wilmington River A (August 2001), AS/BSM microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 91.8 t = 12: 75.7 t = 24: 104.6 t= 72: 94.0 t= 120: 104.4
Wilmington River A (August 2001), AS/BSM + toluene vapor microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 89.6 t = 12: 86.3 t = 24: 98.5 t= 72: 99.5 t= 120: 99.2
Wilmington River B (August 2001), AS/BSM microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 107.1 t = 12: 99.2 t = 24: 88.9 t= 72: 96.8 t= 120: 99.9
Wilmington River B(August 2001), AS/BSM + toluene vapor microcosm	3 per timepoint	ISPCR/H	BTEX1, BTEX2/ BTEX4	ND	t = 0: 100.0 t = 12: 90.5 t = 24: 89.9 t= 72: 98.0 t= 120: 98.8

[a] Defined as: $\frac{\text{mean \# cells/field stained with YOPRO on filter piece after amplification}}{\text{mean \# cells/field stained with YOPRO on untreated filter piece}} \times 100$

[b] ND = not determined

natural samples, concentration of cells by these means is less amenable to *in situ* amplification procedures. Centrifugation has been shown to result in a significantly lower retention of cell numbers after concentration than filter-concentration when coupled with ISH techniques (29).

Filtration is compatible with all of our recent *in situ* amplification (ISRT, *in situ* PCR/H and *in situ* RT-PCR/H) protocols. Of the filter substrates examined, white polycarbonate filters routinely demonstrated low levels of background fluorescence using a CY3-label detection system and did not inhibit PCR reactions. ISRT, *in situ* PCR/H and *in situ* RT-PCR/H were all successfully demonstrated to amplify phylogenetically specific sequences (rRNA), a single-copy functional gene (DNA), and functional gene activity (mRNA), respectively, within intact cells of laboratory cultures and natural bacterioplankton assemblages that had been concentrated onto polycarbonate filters.

By using membrane filtration to concentrate naturally occurring microbial communities, we can not only investigate the genetic capabilities of each individual member of the community on a cellular basis and the spatial relationships of these individuals within the community, but we can begin to more accurately quantify the distribution of functional activities within the community. Previously, the assignment of specific capabilities (e.g. degradation of certain organic substrates) has been dependent upon the ability to first cultivate environmental organisms and then to demonstrate their growth on the specific substrate in the laboratory (e.g. isolation on selective substrate [40], and mineralization rates of radioactively labeled substrates [33]), or the ability to confirm gene and/or mRNA presence in bulk nucleic acids (i.e. dot blot hybridization [17]; extracted mRNA [20]). Unlike *in situ* amplification, these aforementioned

approaches, though providing substantial information, do not provide a picture of the entire community at the cellular level.

Although *in situ* amplification protocols require extensive manipulation (i.e. permeabilization, heating cycles, washes, detection protocols, etc.), the vast majority of cells remain adhered to the filter medium throughout the process. We observed that greater than 85% of the cells initially present on a filter were recovered at the completion of amplification and/or hybridization. Thus, filter-concentration represents a very convenient way to quantify the presence of genes within a community, or their expression.

We have successfully demonstrated ISRT, ISPCR/H and ISRT-PCR/H in laboratory cultures concentrated by membrane filtration, as well as in natural assemblages concentrated in the same manner. The permeabilzation step is critical in these processes, and needed to be optimized for use with natural samples. When performed under those conditions that were previously found acceptable for laboratory batch cultures (16), cells in natural samples were observed to lose a significant amount of DNA and their integrity to be compromised. A lower lysozyme concentration of 0.5 mg ml^{-1} proved sufficient to yield successful amplification in intact bacterial cells of natural estuarine microbial communities as well as laboratory strains, while maintaining cell integrity without substantial loss of amplified sequences. It should be noted, however, that differences in cell wall composition between Gram positive and Gram negative bacteria mean that the permeabilization protocols published herein have only been optimized for amplification of sequences in Gram negative organisms. Conditions optimal for permeabilizing Gram positive bacteria and Archea remain to be determined.

Although the cell wall structure of methanogens differs dramatically from that of Gram negative bacteria (24), successful *in situ* PCR has recently been reported for the *Methanosarcina mazei* using a protocol with low levels of lysozyme for an extended period of time (27).

The successful *in situ* amplification of sequences targeted by the set of BTEX primers as described here will facilitate further investigations of how individual cells within natural microbial communities respond to the presence of aromatic compounds. *In situ* PCR/hybridization may serve as a valuable tool for monitoring changes in the distribution of BTEX dioxygenases over time as a community responds to chronic contamination with BTEX compounds. *In situ* PCR/H used in conjunction with other techniques that ellucidate the phylogenetic relationships of the bacteria which possess the amplified sequences may also provide a direct, visual way to study the horizontal transmission of plasmids carrying bioremediative genes in the natural environment. Similarly, ISRT and *in situ* RT-PCR/H techniques can be applied to communities actively degrading aromatic contaminants to investigate how different cells in the assemblage contribute to the overall degradation process.

REFERENCES

1. **Amann, R.I., W. Ludwig, and K.-H. Schleifer.** 1995. Phylogenetic identification and *in situ* detection of individual microbial cells without cultivation. *Microbiol. Rev.* **59**: 143-169.

2. **Bej, A.K., M.H. Mahbubani, J.L. Dicesare, and R.M. Atlas.** 1991. Polymerase chain reaction-gene probe detection of microorganisms by using filter concentrated samples. *Appl. Environ. Microbiol.* **57**: 3529-3534.

3. **Chen, F, W.A. Dustman, and R.E. Hodson.** 1999 a. Microscopic detection of the toluene dioxygenase gene and its expression inside bacterial cells in seawater using prokaryotic *in situ* PCR. *Hydrobiologia* **401**: 131-138.

4. **Chen, F., W.A. Dustman, and R.E. Hodson.** 1999 b. Application of *in situ* reverse transcription to estuarine bacterial community analysis. *In* C.R. Bell, M. Brylinsky and P. Johnson-Green (ed.), Microbial Biosystems: New Frontiers (Proceedings of the 8th International Symposium on Microbial Ecology). Atlantic Canada Society for Microbial Ecology, Halifax Canada.

5. **Chen, F., J.M. Gonzalez, W.A. Dustman, M.A. Moran, and R.E. Hodson.** 1997. *In situ* reverse transcription, an approach to characterize genetic diversity and activities of prokaryotes. *Appl. Environ. Microbiol.* **63**: 4907-4913.

6. **DeLong, E.F., G.S. Wickham, and N.R. Pace.** 1989. Phylogenetic stains: ribosomal RNA based probes for the identification of single cells. *Science* **243**: 1360-1362.

7. **Dudeck, K.L.** 2001. M.S. thesis. University of Georgia, Athens GA.

8. **Ferguson, R.L., E.N. Buckley, and A.V. Palumbo.** 1984. Response of marine bacterioplankton to differential filtration and confinement. *Appl. Environ. Microbiol*. **47**: 49-55.

9. **Glockner, F.O., R. Amann, A. Alfreider, J. Pernthaler, R. Psenner, K. Tresbesius, and K.H. Schleifer.** 1996. An *in situ* hybridization protocol for detection and identification of planktonic bacteria. *System. Appl. Microbiol.* **19**: 403-406.

10. **Gibson, D.T., M. Hensley, H. Yoshioka, and T.J. Mabry.** 1970. Formation of (+)-*cis*-2,3,-dihydroxy-1-methlcyclohexa-4,6,-diene from toluene by *Pseudomonas putida*. *Biochem*. **9**: 1626-1630.

11. **Giovannoni, S. J., E.F. DeLong, G.J. Olsen, and N.R. Pace.** 1988. Phylogenetic group-specific probes for identification of single microbial cells. *J. Bacteriol.* **170**: 720-726.

12. **Heidelberg, J.F., K.R. O'Neill, D. Jacobs, and R.R. Colwell.** 1993. Enumeration of *Vibrio vulnificans* on membrane filters with a fluorescently labeled oligonucleotide probe specific for kingdom-level 16S rRNA sequences. *Appl. Environ. Microbiol*. **59**: 3474-3476.

13. **Hicks, R.E., R.I. Amann, and D.A. Stahl.** 1992. Dual staining of natural bacterioplankton with 4'6'-diamidino-2-phenylindole and fluorescent oligonucleotide probes targeting kingdom-level 16S rRNA sequences. *Appl. Environ. Microbiol* **58**: 2158-2163.

14. **Hirons, G.T., J.J. Fawcett, and H.A. Crissman.** 1994. TOTO and YOYO: New very bright fluorochromes for DNA content analyses by flow cytometry. *Cytometry* **15**: 129-140.

15. **Hobbie, J.E., R.J.Daley, and S. Jasper.** 1977. Use of Nucelopore filters for counting bacteria by fluorescence microsopy. *Appl. Environ. Microbiol*. **33**: 1225-1228.

16. **Hodson, R.E., W.A. Dustman, R.P. Garg, and M.A. Moran.** 1995. *In situ* PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. *Appl. Environ. Microbiol*. **61**: 4074-4082.

17. **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.

18. **Holmstrøm, K., T. Tolker-Nielsen, and S. Molin.** 1999. Physiological states of individual *Salmonella typhimurium* cells monitored by *in situ* reverse transcription-PCR. *J. Bacteriol*. **181**: 1733-1738.

19. **Hoshino, T., N. Noda, S. Tsuneda, A. Hirata, and Y. Inamori.** 2001. Direct detection by *in situ* PCR of the *amoA* gene in biolfilm resulting from a nitrogen removal process. *Appl. Environ. Microbiol.* **67**: 5261-5266.

20. **Hurt, R.A., X.Qiu, L. Wu, Y. Roh, A.V. Palumbo, J.M. Tiedje and J. Zhou.**

    2001. Simulatneous recovery of RNA and DNA from soils and sediments. *Appl.*

    Envrion. Microbiol. **67**: 4495-4503.

21. **Jacobs, D., M.L. Angles, A.E. Goodman, and B.A. Nielan.** Improved methods for *in situ* enzymatic amplification and detection of low copy number genes in bacteria. *FEMS Microbiol. Lett.* **152**: 65-73.

22. **Kennish, M.J. (ed)** Practical Handbook of Marine Science, 3rd edition. CRC Press LLC, Boca Raton FL.

23. **Kiernan, J.A.** 1990. Histological and histochemical methods: theory and practice, 2nd Edition. Pergamon Press, New York NY.

24. **Kreisl, P., and O. Kandler.** 1986. Chemical structure of the cell wall polymer of *Methanosarcina*. *Syst. Appl. Microbiol*. **7**: 293-299.

25. **Komminoth, P., and A.A. Long.** 1993. *In-situ* polymerase chain reaction: an overview of methods, applications and limitations of a new molecular technique. *Virchows Arch. B. Cell Pathol*. **64**: 67-73.

26. **Lane, D.J.** 1991. 16S/23S sequencing, p. 115-175. *In:* E. Statckenbrandt and M. Goodfellow (eds.), Nucleic Acid Techniques in Bacterial Systemetics. John Wiley and Sons, Ltd., London.

27. **Lange, M., T. Tolker-Nielsen, A. Molin and B.K. Ahring.** 2000. *In situ* reverse transcription-PCR for monitoring gene expression in individual *Methanosarcina mazei* S-6 cells. *Appl. Environ. Microbiol.* **66**: 1796-1800.

28. **Lee, S., and P.F. Kemp.** 1994. Single-cell RNA content of natural marine planktonic bacteria measured by hybridization with multiple 16S rRNA-targeted fluorescent probes. *Limnol. Oceanogr*. **39**: 869-879.

29. **Lemke, M.J., C.J. McNamara, and L.G. Leff.** 1997. Comparison of methods for the concentration of bacterioplankton for *in situ* hybridization. *J. Microbiol. Methods* **29**: 23-29.

30. **Miller, J.H.** 1972. Experiments in molecular genetics. Cold Spring harbor Laboratory, Cold Spring Harbor, N.Y.

31. **Nuovo, G.J.** 1992. PCR *in situ* hybridization: protocols and applications. Raven Press, New York NY.

32. **Nuovo, G.J., F. Gallery, R. Hom, P. MacConnell, and W. Block.** 1993. Importance of different variables for enhancing *in situ* detection on PCR-amplified DNA. *PCR Methods Appl.* **2**: 305-312.

33. **Piehler, M.F., J.G. Swistak, J.L. Pinckney, and H.W. Paerl .** 1999. Stimulation of diesel fuel biodegradation by indigenous nitrogen fixingn bacterial consortia. *Microbial Ecology* **38**: 69-78.

34. **Porter, K., and Y.S. Feig.** 1980. The use of DAPI for identifying and counting aquatic microflora. *Limnol. Oceanogr. 25*: 943-948.

35. **Porter, J., R. Pickup, and C. Edwards.** 1995. Flow cytometric detection of specific genes in genetically modified bacteria using *in situ* polymerase chain reaction. *FEMS Microbiol. Lett.* **134**: 51-56.

36. **Ramsing, N.B., M. Kühl, and B.B. Jørgensen.** 1993. Distribution of sulfate-reducing bacteria, O_2, and H_2S in photosynthetic biofilms determined by oligonucleotide probes and microelectrodes. *Appl. Environ. Microbiol.* **59**: 3840-3849.

37. **Schell, M.A.** 1985. Transcriptional control of the *nah* and *sal* hydrocarbon degradation operons by the *nahR* product. *Genes* **36**: 301-309.

38. **Sobecky, P.A., M.A. Schell, M.A. Moran, and R.E. Hodson.** 1992. Adaptaion of model genetically engineered microorganisms to lake water: growth rate enhancements and plasmid loss. *Appl. Environ. Microbiol.* **58**: 3630-3637.

39. **Staley, J.T., and A. Konopka.** 1985. Measurement of *in situ* activities of nonphotosynthetic microorganisms in aquatic and terrestrial habitats. *Ann. Rev. Microbiol.* **39**: 321-346.

40. **Stapleton, R.D., N.G. Bright and G.S. Sayler.** 2000. Catabolic and genetic diversity of degradative bacteria from fuel-hydrocarbon contaminated aquifers. *Microbial Ecology* **39**:211-221.

41. **Tani, K., M. Muneta, K. Nakamura, K. Shibuya, and M. Nasu.** 2002. Monitoring of *Ralstonia eutropha* KT1 in groundwater in an experimental bioaugmentation field by *in situ* PCR. *Appl. Environ. Microbiol.* **68**: 412-416.

42. **Tolker-Nielsen, T., K. Holmstrøm, and S. Molin.** 1997. Visualization of specific gene expression in individual *Salmonella typhimurium* cells by *in situ* PCR. *Appl. Environ. Microbiol.* **63**: 4196-4203.

43. **Tolker-Nielsen, T., K. Holmstrøm, L. Boe and S. Molin.** 1998. Non-genetic population heterogeneity studied by *in situ* polymerase chain reaction. *Mol. Microbiol*. **27**: 1099-1105.

44. **Wagner, M., G. Rath, R. Amann, H.-P. Koops, and K.-H. Schleifer.** 1995. *In situ* identification of ammonia-oxidizing bacteria. *System. Appl. Microbiol*. **18**: 251-264.

CHAPTER 4

USE OF *IN SITU* PCR/H TO VISUALIZE THE BTEX DEGRADATION RESPONSE OF INDIVIDUAL BACTERIAL CELLS WITHIN NATURAL ASSEMBLAGES[3]

[3] Dustman, W.A. and R.E. Hodson. To be submitted to *Applied and Environmental Microbiology*.

ABSTRACT

Seawater samples from salt marshes and estuaries along the Georgia coast were examined for the presence of bacteria possessing genes for the degradation of several aromatic hydrocarbons (i.e. BTEX: benzene, toluene, ethlybenzene and xylene). A new visual approach involving *in situ* PCR/hybridization and epifluorescence microscopy was employed. A range of sampling sites was chosen to represent conditions of little-to-no pollution by petroleum hydrocarbons (low-impact) as well as areas where hydrocarbon contamination was clearly evident (high-impact). Our aim was to examine the distribution of individual BTEX-degrading microorganisms *in situ* and, characterize the dynamics of bacterial communities' responses to increased hydrocarbon selection pressure. Only a small percentage of the organisms comprising the microbial communities at the sites contained BTEX dioxygenase genes, about 1 to 5% in the low- and high-impact sites, respectively. As selective pressure was applied, only the communities from high-impact sites exhibited significant shifts toward enrichment in BTEX degraders, nearing 50% of the total community. BTEX-positive cells were found as a combination of free-living cells, small cell aggregates of BTEX-degraders, and complex microcolonies associated with cells lacking BTEX dioxygenase genes. Our findings demonstrate that *in situ* PCR can be an effective, visual tool for examining complex inter-relationships between degrader microorganisms and the rest of the community, such as cooperative catabolism of complex substrates.

INTRODUCTION

Management of hazardous wastes in water is a worldwide concern. As coastal development intensifies, pollution of marine ecosystems increases proportionately, leading to long-term, low-level contamination of coastal marshes and estuaries, and aquatic organisms. Among the many pollutants, petroleum hydrocarbon contamination has been shown to be especially harmful to marine organisms (29, 38, 41) and because they are subject to bioaccumulation, can be transferred to humans via seafood consumption (29). Chronic petroleum contamination from normal ship operations, transfer spills, and leaky storage tanks occurs continuously in rivers, harbors, coastal waters, and marinas while disastrous tanker accidents, though rarer, also add significantly to waste loading.

Natural attenuation of these contaminants is mediated primarily by the degradation activities of indigenous bacteria. A large literature base describing degradation of petroleum and petroleum products by natural and introduced bacteria exists (see 5, 24, and 27 for reviews). Much has been learned about the enzymes catalyzing degradation of aliphatic and aromatic hydrocarbons and the distribution of these enzymes among various bacterial taxa (5). However, much less is known about the activities of the natural or introduced genetically enhanced degraders in the environment. Many studies showing that a particular bacterial species effectively degraded a particular petroleum component in the lab were followed by disappointing failures of those same organisms to enhance the removal of contaminants from the natural environment (e.g. 19, 23, and 39). It was assumed that added bacteria either did not survive or were otherwise inactive after release into freshwater or estuarine systems (6, 23). In some strains of

bacteria, genes for hydrocarbon degradation, though stable under laboratory conditions, were found to be lost under low nutrient, environmental conditions (40). Moreover, some indigenous bacterial populations respond to the presence of contaminants while others do not (3). In the notorious case of the Exxon Valdez tanker accident in Prince William Sound, Alaska, many different remediation approaches were attempted. After multiple failures, researchers concluded that the only reliable bacterial treatment they had available was addition of inorganic nitrogen and phosphorous to the water to enhance degradation activity by the indigenous microflora (7).

Recently, the interactions of mixed bacterial species, or cocultures, rather than a single species alone, have been investigated, and these cultures have shown enhanced abilities to degrade mixed hydrocarbon substrates (1, 4, 9). Currently, little is known regarding the community dynamics of such mixed cultures. For example, degraders may develop very specific physical associations with other bacteria, or perhaps higher organisms, in order to combine their metabolic capabilities to jointly break down recalcitrant compounds. Microscopic studies could help characterize these systems if they could reveal information about the degradative or genetic capabilities of individual bacteria.

Considering the complexity of factors controlling hydrocarbon degradation in soil and aquatic systems, and the frequent failure of additions of exogenous bacteria to significantly enhance bioremediation in the field, much research focused recently on the development of new methodologies for examining genetic biodegradative capabilities of individual bacterial species, even individual bacterial cells, *in situ* in the natural water and sediment matrices. Our lab responded to this need by developing a distinct, but

related, set of methodologies, collectively referred to as prokaryotic *in situ* PCR. The approach facilitates the detection, within individual bacterial cells, of specifically targeted DNA, mRNA or rRNA sequences to taxonomically identify the bacterium and/or even single-copy genes or their expression. Details of the approaches have been described in previous reports (11, 12, 13, 25).

This report describes the initial application of a bacterial *in situ* amplification protocol that targets the genes encoding enzymes involved in degradation of a group of petroleum hydrocarbons known as BTEX (benzene, toluene, ethyl-benzene and xylene). Diesel fuel, an example of a common petroleum contaminant which accounts for 45% of the total volume of petroleum pollution in U.S. waterways (43), is a mixture of various aromatic hydrocarbons and contains, on average: > 80% paraffinic hydrocarbons, <10% aromatic hydrocarbons, and <5% olefinic hydrocarbons (14). The aromatic hydrocarbon fraction includes, on average, 1.35 mg toluene g^{-1} and 1.43 mg of *m*- plus *p*-xylene g^{-1} (29) as well as other volatile organic compounds such as benzene, and ethyl-benzene (33). BTEX compounds represent a group of petroleum hydrocarbons that are relatively soluble in water and, consequently, very toxic. Thus, bioremediation of these chemicals has been the focus of much effort in bioremediation, whether by natural attenuation (e.g. 20 and 42) or through the use of introduced bioengineered microorganisms (e.g. 6).

As mentioned above, the use of cocultures dominated by one or several species is now showing great promise for field bioremediation. While biodegradation rates have been calculated in these studies, the organismal interactions within these populations remains to be elucidated. The ability to visually identify members of the communities that are actively involved in degradation would greatly increase understanding of the

complex inter-relationships within the microbial assemblages that catabolize toxic compounds like the BTEX group.

The aim of this study was to investigate the use of recently developed *in situ* PCR and hybridization (*in situ* PCR/H) techniques to examine the distribution of bacteria involved in natural attenuation of BTEX compounds in coastal marine waters and the spatial relationships of these microorganisms with respect to the rest of the bacterial community. The new procedure facilitates detection of the gene in individual cells by epifluorescence microscopy. Selective isolation by spread plate was used in tandem with molecular methods in order to assess the specificity of our newly developed *in situ* PCR/H techniques.

By using microcosms selectively enriched with toluene, we demonstrate the potential of *in situ* PCR/H techniques for the direct, visual enumeration of degrader populations in the environment, as well as the sensitivity of these methodologies. At the onset of our experiment, the proportion of BTEX degraders from low-impact sites was approximately 1% of the total population while BTEX degraders represented nearly 5% of the community from high-impact microcosms. Selective, cultivation-based enumeration of toluene degraders by plate count was, as anticipated, less sensitive. No toluene degraders were ever enumerated by plate count for the low-impact sites, regardless of the selective pressure applied during microcosm incubation. Enumeration of toluene degraders via this plate count was successful from high-impact sites. However, enumeration and isolation of the degrader microorganisms from these sites was limited to only those microcosms supplemented with toluene, and sampled at 24 h or beyond.

MATERIALS AND METHODS

Cultures. *Pseudomonas putida* F1 (40) from a –80 °C freezer stock culture was used as a positive control strain for BTEX dioxygenase genes and was grown in artificial seawater/basal salt medium (AS/BSM) (BSM plus sea salts [Sigma Chemical Co.] at a final concentration of 20 g liter^{-1} to simulate estuarine salinities) supplemented with toluene vapors as a primary carbon source (11). This preparation was free of significant other organic substrates, however, it has been reported that the sea salt reagents used here may contain varying low-level concentrations of nonvolatile dissolved organic carbon (DOC), equivalent to 1 mg marine DOC l^{-1} after standard re-hydration (personal communication, Dr. Mary Ann Moran). Cultures were grown overnight at 30°C and 200 rpm to yield dense populations in mid-logarithmic growth phase.

Oligonucleotide primers and probes. All primers and probes were synthesized by IDT DNA, Inc. Primers BTEX1 and BTEX2 (16) were used to amplify, via *in situ* PCR/H, a sequence common to a group of related dioxygenase genes involved in degradation of the aromatic compounds benzene, toluene, ethyl-benzene and xylene (BTEX). Probe BTEX4 (16), labeled with CY3-dye as a fluorescent marker, targeted an internal region of the DNA amplified during *in situ* PCR. BTEX primers and probe were designed (Dr. Feng Chen, personal communication) based on sequence alignments of 12 BTEX dioxygenase genes for characterized enzymes including: toluene dioxygenase; biphenyl dioxygenase; benzene dioxygenase; chlorobiphenyl dioxygenase; 2,3-dihyrdoxybiphenyl 1,2-dioxygenase, and isopropylbenzene 2,3-dioxygenase.

Sampling sites and sample collection. Eight sites along the Georgia coast were selected for this study (Table 4.1). Of the 8 stations, 4 (hereafter termed high-impact) were specifically chosen because visible signs of contamination with petroleum compounds were present at the air-water interface. These high-impact sites were the locations of continual small boat and/or shipping activity. Conversely, we selected the 4 other sites (low-impact) from areas where petroleum contamination was expected to be minimal and where petroleum contamination was neither evident nor had ever been reported. These low-impact sites were in areas devoid of significant shipping or marine activity. Two of these locations, Dean Creek and Marsh Landing, were within the bounds of the NOAA Sapelo Island Research Reserve and adjacent to the University of Georgia Marine Institute on Sapelo Island. All four low-impact sites are also NSF GA-LTER sites and in addition, Dean Creek serves as an NSF Microbial Observatory site.

At each estuarine or coastal sampling site, approximately 500 ml of seawater was collected from the surface in a sterile Nalgene bottle and transported to the laboratory on ice. Samples were then passed through a sterile 3.0 μm pore-size polycarbonate membrane (47 mm, Nucleopore) under gentle vacuum (300 mbar) to remove larger particles and plankton. Samples were then stored at 4°C for a maximum of 24 hr before further processing.

Microcosms. Two ml of size-fractionated (≤ 3.0 μm particle size) estuarine or coastal samples were diluted 100-fold by aseptically transferring into ashed 250-ml Erlenmeyer flask microcosms containing 198 ml of an artificial seawater medium containing basal salts (AS/BSM [11]) each. Microbial communities from all sites were suspended in this artificial seawater medium to eliminate the effects of the differing chemical

TABLE 4.1. Sampling sites used in this study. Microcosms amended with toluene are indicated with "TOL" as part of the microcosm name.

Impact Type	Sampling Site	Location	Microcosm ID
Low	Hudson Creek	Meridian GA	MD, MDTOL
	Duplin River	Sapelo Island GA	ML, MLTOL
	Sapelo River	Eulonia GA	EU, EUTOL
	Dean Creek	Sapelo Island GA	DC, DCTOL
High	Lazaretto Creek	Tybee Island GA	LZ, LZTOL
	Savannah River	Savannah GA	SV, SVTOL
	Wilmington River A	Thunderbolt GA	IAC, IACTOL
	Wilmington River B	Wassau Island GA	WR, WRTOL

compositions of the water from the various sites, then sealed with an autoclaved Nalgene stopper. Half the microcosms were supplemented with toluene and half were left unamended. Previous reports have shown that the addition of aromatic substances to culture medium has resulted in a significant toxicity to cultures unless supplied in low levels. Thus toluene vapors were supplied from a 2% aqueous solution (vol/vol) (21, 45). Specifically, 2% toluene solution (500 µl) was placed in an ashed scintillation vial suspended from the Nalgene stopper by heat-sterilized nickel chromium wire (Arcor Inc.), allowing the vapors to dissolve into the medium during incubation. Toluene-amended and control microcosms were incubated separately at 25°C and 25 rpm, and sampled at intervals over the course of 5 days. To ensure aerobic conditions were maintained throughout the study, and to account for the volatility of toluene, each toluene-amended microcosm was opened for a period of 45 sec daily while depleted toluene solution was removed and replaced with fresh solution. Control microcosms were treated similarly. Subsamples were aseptically removed from each microcosm for analysis at the following timepoints: 0, 12, 24 h, 3 d and 5 d.

The rationale for the microcosm selection studies of BTEX degraders used herein was that we had previously determined that BTEX degraders in Georgia coastal waters ranged from undetectable to 1.4% of total bacterioplankton in filtered (< 3.0 µm pore-size) when measured by *in situ* RT-PCR/H (16) or were undetectable using ISRT in samples in an estuarine environment on the Georgia coast unless the sample was pre-incubated with toluene (12). We wanted to both determine the sensitivity of the *in situ* PCR/H method and follow the dynamics of the community selection for BTEX degraders at the single cell level. The dilution and subsequent re-growth of the free-living bacterial

community over time ensured that we were looking at active cells from within the assemblages, not a high percentage of 'husks'. Parallel treatments (enrichment with toluene as the sole and essentially unlimited carbon substrate for growth vs. no additional DOC supplied) allowed us to determine the sensitivity of the *in situ* PCR method, as well as how the size of the population of BTEX-degraders changes with overall bacterial population size with and without selective pressure (i.e. in the presence of toluene vs. in the absence of added toluene). By using viable counts and standard and selective isolation techniques, viable bacteria were screened for the presence of BTEX dioxygenase genes as a control to ensure that the *in situ* PCR/H BTEX-positive results were not artifacts of the techniques.

Enumeration of total viable bacteria and viable toluene degraders by plate counting. At each timepoint except at 12 h, subsamples were diluted serially (10-fold) in phosphate buffered saline (PBS) [pH 7.6] (Sigma Chemical Co.) for viable count enumerations. Each dilution, as well as 1 ml of undiluted subsample, was then spread-plated onto one-half strength yeast extract tryptone sea salts agar (½ YTSS = g/L dH_20: 4 g tryptone, 2.5 g yeast extract, 20 g sea salts and 15 g agar-agar) for enumeration of total cultivable bacteria and onto AS/BSM agar (AS/BSM with 20 g $liter^{-1}$ molecular grade agarose [Sigma Chemical Co.] supplemented with vapors from a 2% aqueous toluene solution) for enumeration of total bacteria capable of growth on toluene as a sole carbon source. In addition, all dilutions were also spread-plated onto AS/BSM agar minus the toluene supplement as a check against the possibility that the growth on this medium might be at the expense of agarose rather than toluene.

All spread plates were prepared in duplicate and incubated as follows: YTSS plates at 25°C for 2 d, AS/BSM plates with and without toluene at 25°C for 5 d. Colony numbers on plates were monitored daily until no further increases occurred. AS/BSM plates were supplemented with toluene vapors by placing a sterile 25 mm diameter paper disc (Whatman), previously dipped in a 2% aqueous toluene solution, onto the lid of each culture dish. AS/BSM with toluene plates were sealed in plastic bags (Ziploc) to prevent loss of toluene by evaporation, and incubated inverted in a separate chamber from the unsupplemented plates. Aerobic conditions were maintained and toluene was replenished on a daily basis by removing the plates from the plastic bags, inserting a new paper disc with fresh toluene solution, then re-sealing the bags for continued incubation.

Isolation of representative toluene degraders and screening for BTEX dioxygenase gene presence. After enumeration, colonies growing on either complex organic medium (½ YTSS) or solely on toluene (AS/BSM plus toluene) were examined for distinct colonial morphologies. Unique colonies were aseptically picked from plates and purified by successive streaking at least three times on the medium from which they were isolated. Isolates were then maintained on ½ YTSS agar. Isolates were designated by their site of origin, and the medium on which they were isolated (Table 2). Each pure culture isolate was grown in ½ YTSS broth in preparation for extraction of bacterial DNA using a QIAmp Blood and Tissue Kit (QIAGEN Inc.) according to manufacturer's instructions. Bacterial DNA from each isolate was screened for the presence of BTEX dioxygenase genes.

TABLE 4.2. Designations used for isolates in this study.

Isolate Code	Toluene Present	Isolation Medium
MD ML EU DC LZ SV WR IAC	Yes	½ YTSS
MDC MLC EUC DCC LZC SVC WRC IACC	No	½ YTSS
MDT MLT EUT DCT LZT SVT WRT IACT	Yes	AS/BSM + toluene

Briefly, 20 ng of extracted DNA from an isolate was added to 50.0 µl of PCR reaction mixture containing a final concentration of 1X Expand High Fidelity PCR Buffer (Boehringer Mannheim), 2.5 mM $MgCl_2$, 200.0 µM dNTP mix, 1.0 µ M each of the oligonucleotide primers BTEX1 and BTEX2, and 0.5 U of Expand High Fidelity Enzyme (DNA polymerase) (Boehringer Mannheim). *In vitro* PCR conditions were as follows: 1) initial denaturation at 94ºC for 3 min; 2) 35 cycles (1 min each) of: denaturation at 94ºC, annealing at 64ºC, and extension at 72ºC; and 3) 5 min at 72ºC.

Dot blot hybridizations were carried out to confirm the presence of the amplified dioxygenase sequences by immobilizing 25 µl of spent PCR reaction mixture to Nytran nitrocellulose membranes, pre-printed with a 96-well grid, (Schleicher & Schuell) using a dot-blot apparatus (Schleicher & Schuell) and UV cross-linking for 1 min at 120 mjoules (Stratagene). The double-stranded DNA was denatured to single-strand with NaOH buffer, then neutralized (35). The oligonucleotide probe used in these hybridizations (BTEX4) carried the FITC label at the 5'end (rather than the CY3 used for *in situ* PCR). Hybridization protocols described in the manual included with the Genius System for Filter Hybridization kit (Boehringer Mannheim) were followed with two exceptions: 1) the hybridization solution used was the same buffer solution used for the hybridization step of *in situ* PCR/H, and 2) hybridization time and temperature were identical to those used for *in situ* PCR/H: 2 hrs at 63°C. Post-hybridization, anti-FITC Fab fragments conjugated to alkaline phosphatase were employed using manufacturer's instructions (Genius System for Filter Hybridization kit, Boehringer Mannheim) for colorimetric detection of probe hybridization and the results compared to those of a positive control strain *Pseudomonas putida* F1 or a negative control strain *P. putida* AC10R-7.

Cell fixation and collection. For the microcosm studies, at each timepoint, freshly prepared, 0.2 μm-filtered 10% paraformaldehyde (in PBS) was added to aliquots of subsample from each microcosm (1 volume fixative: 3 volumes sample) and incubated at 4°C for 2 hrs before collection of bacteria onto white polycarbonate membrane filters (0.22 μm pore-size, 25 mm diameter, Poretics) in triplicate. A 25 mm diameter glass filter tower (Millipore, Bedford, MA), was employed to concentrate samples onto the membrane filters using a gentle vacuum (<300 mbar). Filters were placed atop cellulose nitrite support filters (25 mm diameter, 0.2 μm pore size, Millipore), previously moistened with 0.2 μm-filtered distilled water. The volume of each subsample used for collection of the bacteria from the microcosms varied with seawater collection site and incubation and timepoint between 0.5 and 1.0 ml per filter. However, achieving a target cell density of between 30 and 300 cells per microscope field (1200X magnification) was the goal in each case. Filters were washed twice with 10.0 ml each of 0.2 μm-filtered PBS to remove remaining fixative. Filters were then removed to sterile tissue culture dishes (Corning), and allowed to completely air-dry, then stored at -20°C until direct microscopic counts or *in situ* PCR/ISH was performed.

Enumeration of total bacteria by direct epifuorescence microscopic counting. Filters with collected, fixed bacterial cells were removed from –20°C storage and cut into quarters using a sterile razor blade. A one-quarter section of each membrane filter from each timepoint was chosen randomly to be used for direct microscopic counts, and was removed to a new sterile tissue culture dish for staining. Nucleic acid in the bacteria on the filters was stained with YO-PRO (Molecular Probes Inc.) at 0.1 μM final

concentration in PBS for 2 min. Excess stain was removed by freely floating the filter sections in 0.1% Nonidet P40 (Sigma Chemical Co.) in 0.2 μm-filtered PBS, and followed by a brief dip in 0.2 μm-filtered distilled water to remove any salts. Filter sections were allowed to air dry on Whatman 3M paper at room temperature, and then mounted onto plain glass microscope slides between two layers of FF immersion oil (R.P. Cargille Laboratories Inc.). Slides were then examined under epifluorescence excitation conditions optimal for YOPRO visualization (see below). Community (bacterial) cell densities (total cells ml^{-1}) for each replicate sample were calculated from the mean cell count of 10 microscopic fields (viewed under YOPRO) multiplied by the appropriate filter-to-microscope field conversion factor. Fields directly adjacent to the cut edges of the filter pieces were excluded in order to eliminate bias caused by excessive filter manipulation, otherwise fields were chosen randomly within the interior area of the filter section.

Each field used for direct microscopic counts was also viewed under CY3 excitation conditions (see below) to visualize any particles that could autofluorescence at these wavelengths. Particles that were excited under both the YOPRO and the CY3 filter sets were assumed to be DNA-containing bacterial cells with autofluorescent pigments. Cell densities (cells ml^{-1}) of the autofluorescing fraction of the community were calculated in the same manner as total community densities. However, we previously determined that approximately 15% of the cells originally collected on polycarbonate filters from this study were lost when subjected to the same *in situ* PCR/H procedure described below (16). Therefore, it was necessary to correct the data with regard to recovery efficiency for each sampling site and timepoint. Briefly, recovery efficiencies

(of filters undergoing *in situ* PCR/H) were calculated by enumerating counterstained cells under YOPRO excitation conditions to visualize all cells remaining on the filter. Cell numbers (total cells ml^{-1}) for the fraction of the community remaining after *in situ* PCR/H were calculated in the same manner as described above for total bacteria. Recovery efficiencies were calculated by the formula:

$$\left(\frac{\text{Mean cells ml}^{-1}{}_{\text{no } in\ situ \text{ PCR/H}} - \text{Mean cells ml}^{-1}{}_{in\ situ \text{ PCR/H}}}{\text{Mean cells ml}^{-1}{}_{\text{no } in\ situ \text{ PCR/H}}}\right) \text{ x } 100$$

and applied to correct the values of autofluorescent cell numbers remaining adhered to the filters after *in situ* PCR/H.

***In situ* PCR/H.** *In situ* PCR/H procedures were followed as previously described (16). Three replicate filter sections from each timepoint of each microcosm were used. Briefly, the procedure is as follows. Filter sections containing collected bacteria were subjected to lysozyme treatment (0.5 mg ml^{-1}at 25°C for 1 min) to permeabilize cell membranes. Filter quarters were allowed to float freely, face up, in 1.0 ml lysozyme solution in a sterile tissue culture dish (25 mm diameter, Corning), similarly washed in 0.2 μm-filtered PBS, then placed on Whatman 3M filter paper and dried in an oven at 55°C to deactivate any remaining lysozyme. The filters, containing the permeabilized cells, were then sealed in FrameSeal chambers (65 μl capacity, MJ Research) along with 55 μl of PCR reaction mixture containing a final concentration of 1X Expand High Fidelity PCR Buffer (Boehringer Mannheim), 2.5 mM $MgCl_2$, 200.0 μM dNTP mix, 1.0 μM each of BTEX1 and BTEX2, and 0.5 U of Expand High Fidelity Enzyme (Boehringer Mannheim). Slides were placed in a PTC-200 DNA Engine (MJ Research) thermal cycler block designed specifically to hold microscope slides and subjected to the

following amplification regime: 1) initial denaturation at 94ºC for 3 min, 2) 35 cycles (1 min each) of: denaturation at 94ºC, annealing at 64ºC, and extension at 72ºC, and 3) 5 min at 72ºC.

After the *in situ* PCR treatment, the rinsed and dried filters were placed in new FrameSeal chambers with 55 µl of hybridization solution containing the CY3-labeled BTEX4 probe (final concentration 5 ng μl^{-1}). After incubation at 94°C for 2 min to denature the double-stranded amplicons, the probe was allowed to hybridize for 2 hr at 63°C. Post hybridization, unbound probe was removed by gently washing the filter sections by immersion in pre-warmed 0.5X SSC in sterile culture dishes. The filters quarters were rinsed briefly in PBS, then subjected to dual staining with YOPRO (as described above) as a counterstain to visualize all bacteria, and mounted in FF oil as described above for epifluorescence microscopic analysis.

Enumeration of toluene degraders by direct epifuorescence microscopic counting. Slides, containing dual-stained sections of filters having undergone *in situ* PCR/H, were examined under epifluorescence excitation conditions optimal for visualization of CY3 fluorescence (see below). Densities (cells ml^{-1}) of CY3-excited cells for each replicate sample were calculated from the mean cell count of 10 microscopic fields (viewed under CY3) multiplied by the filter-to-microscope field conversion factor. Field selection for enumeration was random, except that care was taken to exclude fields directly adjacent to the cut edges of the filter pieces, thus eliminating bias caused by our sample preparation.

Because visual discrimination between naturally autofluorescent cells and BTEX-positive cells was not possible by these techniques, it was imperative to correct the values

to represent solely the BTEX-degrading fraction of the community. Thus, assuming 1) that the distribution of autofluorescent cells on each filter was even, 2) that auto-fluorescence was not removed as a result of *in situ* PCR/H, and 3) that autofluorescent cells were as equally prone to disassociation from the membrane filters as non-autofluorescent cells during the *in situ* PCR/H process; the mean autofluorescent cell density (corrected with respect to recovery efficiency) was subtracted from the CY3-excited cell density, thereby resulting in a calculation of all BTEX-positive cells.

Microscopy and digital image analysis. Slides were examined under an epifluorescence microscope (Olympus BX 40) with a high-resolution 100X U Plan Oil objective lens (numerical aperture, 1.35 to 0.50) (C-squared Corp.). Single band excitation filters (Chroma Technology, set 82000) for CY3 (555 ± 12.5 nm) and FITC (484 ± 7.5 nm) channels were used in conjunction with a triple emission filter (458 ± 9.5 nm; 518 ± 15.5 nm; and 602 ± 21 nm). BTEX-positive target cells labeled with CY3 (or autofluorescent cells) were viewed under green light (CY3 excitation filter) while cells stained with YO-PRO (all cells containing DNA) were viewed under blue light (FITC excitation filter). Since the samples were counterstained, samples were first examined to the CY3 excitation conditions to visualize BTEX-positive cells. Next, the same field was viewed under YOPRO-specific excitation wavelengths to visualize all cells adhered to the filter.

Images were acquired with a cooled charge-coupled device (CCD) Sensys 1400 camera (1317 by 1035 image array, 6.8 by 6.8 μm pixel, 12 bit) (Photometrics) and processed with the Oncor Image (Oncor Inc.) software package ver. 2.02 on a Power

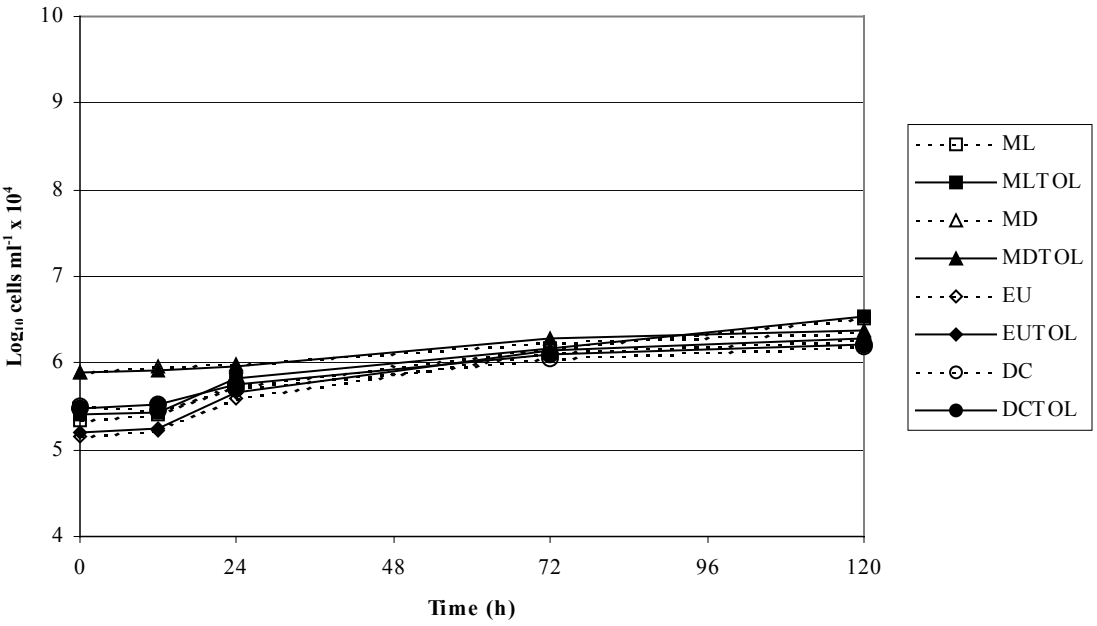
Macintosh 9500 (Apple Computer Inc., Cupertino CA). For image capture, exposure times were controlled by the software package and were 1.5 sec for CY3 images and 1.0 sec for YOPRO images.

RESULTS

Response of total and viable cells. Although the marine bacterial communities inoculated into each microcosm initially were diluted 100-fold from the *in situ* cell densities at the onset of the experiment, at least 10-fold re-growth of the diluted assemblages occurred in all microcosms including those without added toluene during incubation, as evidenced by direct microscopic counts (Figure 4.1 A and B). Initial bacterial number ranged from 1.02×10^5 cells ml^{-1} to 1.06×10^5 cells ml^{-1} in the amended microcosms and unamended microcosms, respectively (Figure 4.2 A and B). Numbers steadily increased in all microcosms over the course of the 120 h incubation. This result suggests there was DOC in the reconstituted artificial seawater medium that served as a growth substrate in absence of toluene. Interestingly, in the low-impact site microcosms, the time-dependent increase in the numbers of total bacteria in the toluene-amended treatments and the unamended treatments remained very similar throughout the 5-day incubation (Figures 4.2 and 4.3). For example, in the ML and MLTOL communities, bacterial numbers were 3.3×10^6 cells ml^{-1} and 3.4×10^6 cells ml^{-1}, respectively, at the termination of the experiment (Figure 4.2 A). In the high-impact site microcosms, however, there was a substantial increase in cell number of all microcosms provided with toluene compared with those that did not receive the carbon supplement (Figure 4.2B and

Figure 4.1. Increases (log_{10} cells ml^{-1}) in bacterial cell numbers over time in (A) low-impact and (B) high-impact microcosms over time measured by direct microscopic count.

A.


10
9
8
7
6
5
4
0
24
48
72
96
120
Time (h)
Log10 cells ml-1 x 10^4
ML
MLTOL
MD
MDTOL
EU
EUTOL
DC
DCTOL

B.

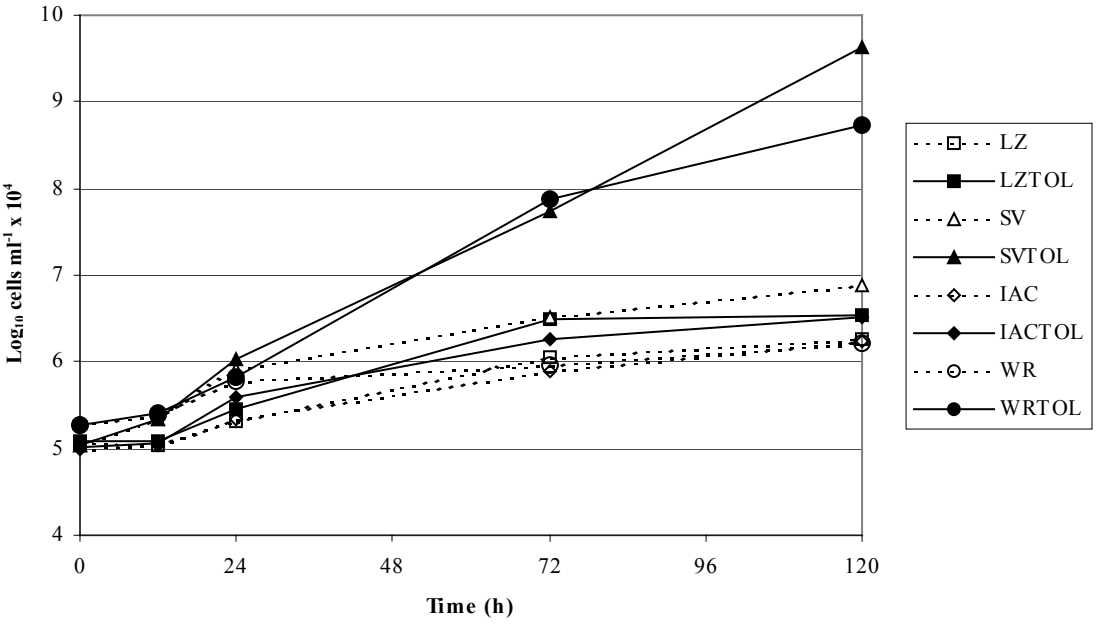
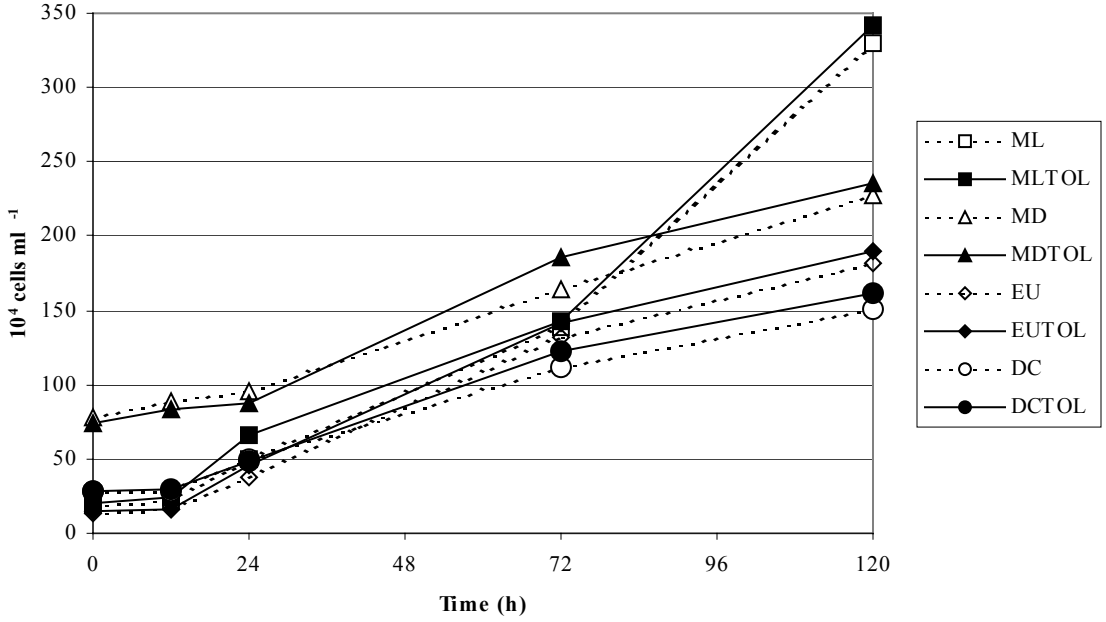

10
9
8
7
6
5
4
0
24
48
72
96
120
Time (h)
Log10 cells ml-1 x 10^4
LZ
LZTOL
SV
SVTOL
IAC
IACTOL
WR
WRTOL

Figure 4.2. Increases (untransformed) in bacterial cell numbers over time in (A) low-impact and (B) high-impact microcosms measured by direct microscopic count. Values for SV at 120 h (7.8×10^6 cells ml^{-1}), SVTOL at 72 and 120 h (5.5×10^7 cells ml^{-1} and 4.2×10^9 cells ml^{-1} respectively), and WRTOL at 72 and 120 h (7.6×10^7 cells ml^{-1} and 5.4×10^8 cells ml^{-1}, respectively) are not shown.

A.


350
300
250
200
150
100
50
0
10^4 cells ml $^{-1}$
0
24
48
72
96
120
Time (h)
ML
MLTOL
MD
MDTOL
EU
EUTOL
DC
DCTOL

B.

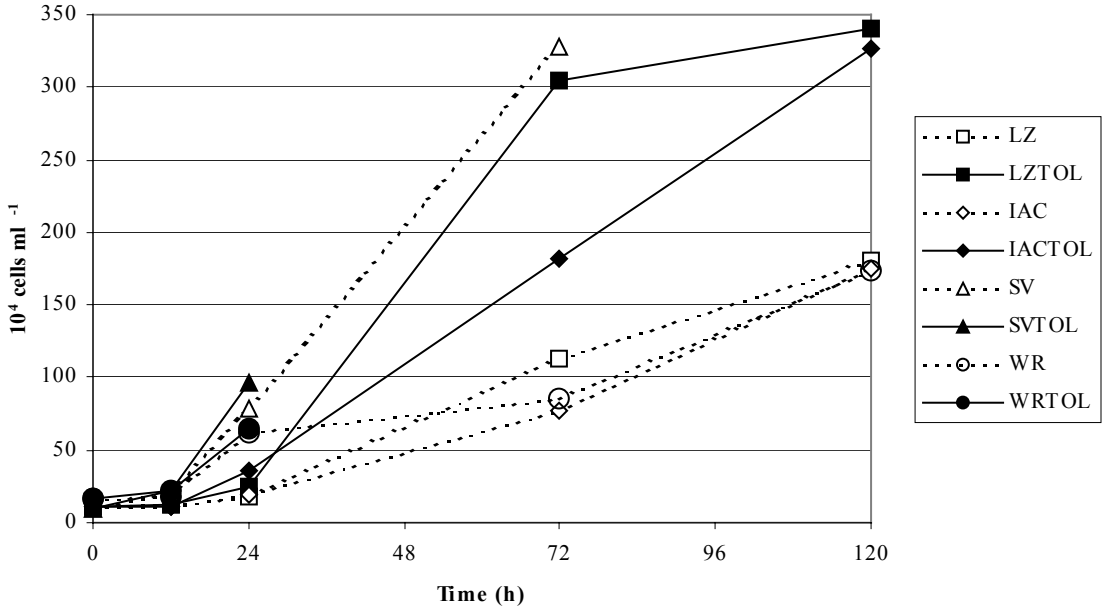
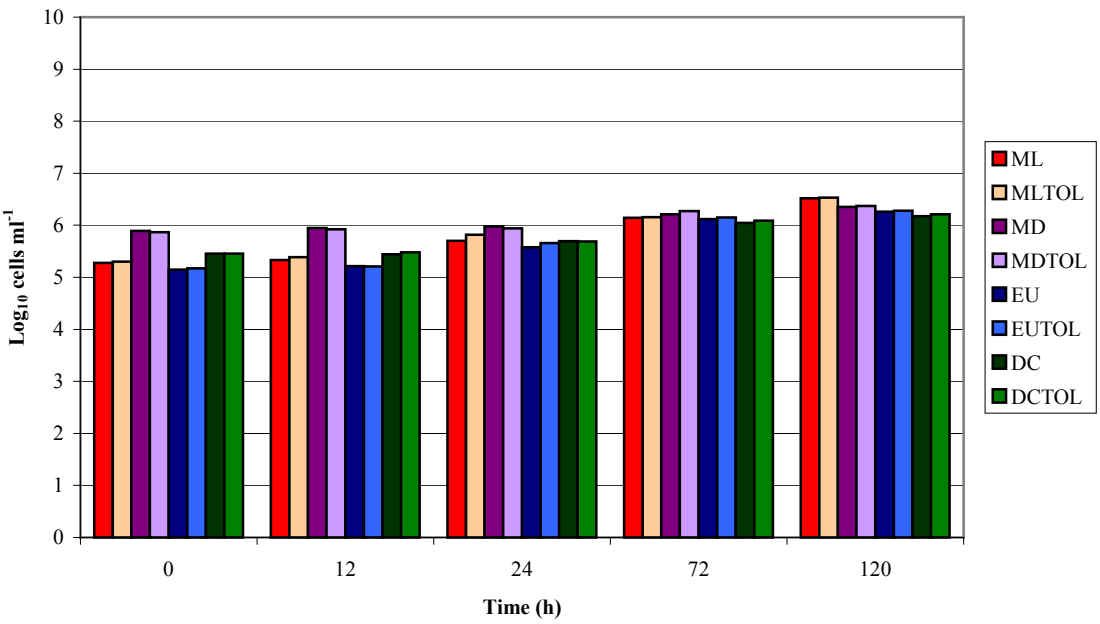
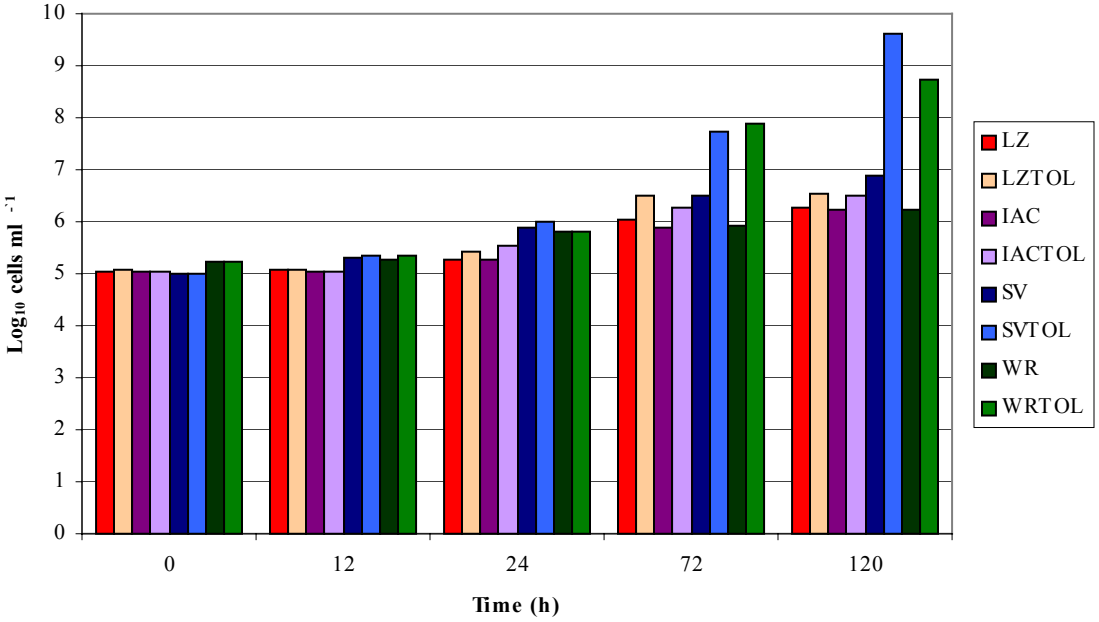

350
300
250
200
150
100
50
0
10^4 cells ml $^{-1}$
0
24
48
72
96
120
Time (h)
LZ
LZTOL
IAC
IACTOL
SV
SVTOL
WR
WRTOL

Figure 4.3. Change in bacterial numbers (log_{10} cells ml^{-1}) in (A) low-impact and (B) high-impact microcosms over time measured by direct microscopic count.

A.


10
9
8
7
6
5
4
3
2
1
0
Log10 cells ml-1
0
12
24
72
120
Time (h)
ML
MLTOL
MD
MDTOL
EU
EUTOL
DC
DCTOL

B.


10
9
8
7
6
5
4
3
2
1
0
Log10 cells ml -1
0
12
24
72
120
Time (h)
LZ
LZTOL
IAC
IACTOL
SV
SVTOL
WR
WRTOL

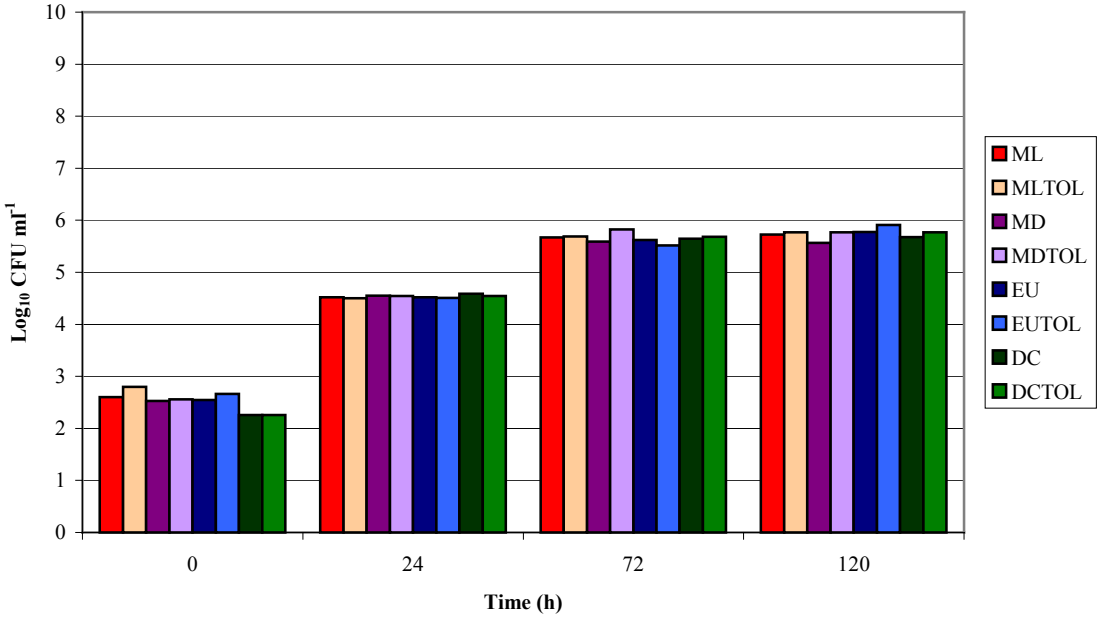
4.3B). More specifically, in two of these microcosms, SVTOL and WRTOL, re-growth of bacteria was sufficient to counter the initial 100-fold dilution of the ambient bacterial populations (Figure 4.1 B and 4.2 B). There was a rise from 1.03 x 10^5 cells ml^{-1} to 4.16 x 10^9 cells ml^{-1} in the SVTOL community, and from 1.63 x 10^5 cells ml^{-1} to 5.37 x 10^8 cells ml^{-1} in the WRTOL community (Figures 4.2 B and 4.3 B). These results indicate that bacterial growth, at least in part, was at the expense of toluene in the high-impact microcosm systems.

Viable counts on ½ YTSS agar also revealed steady increases in bacterial cell numbers over time in each of the microcosms comparable to those measured by direct microscopic counts (Figure 4.4A and B) although as expected, the numbers of culturable bacteria were much lower at all sampling times. Again, in the low-impact site microcosms, toluene enrichment had no positive or negative effect on bacterial growth (Figure 4.4 A). Initially, viable bacteria numbers ranged from as low as 180 CFU ml^{-1} to as high as 1.9 x 10^3 CFU ml^{-1}. When toluene was supplied to high-impact site bacterial communities, significant increases in viable cell numbers were observed, particularly in the SVTOL and WRTOL microcosms (Figure 4.4 B). By 120 h, viable cell numbers had reached a maximum of 9.4 x 10^8 CFU ml^{-1} and 1.03 x 10^7 CFU ml^{-1} in the SVTOL and WRTOL microcosms, respectively.

Table 3 shows the time-dependent changes in the percent of culturability of agar plate-cultivable bacteria compared with the percent culturability of the communities. Over time in low-impact microcosms, culturability on ½ YTSS agar ranged from 0.04% to 0.18% at 0 h and from 16.04% to 42.51% at 120 h (Table 4.3). Culturability in the high-impact microcosms was initially higher than in the low-impact microcosms at 0hr,

Figure 4.4. Increases (log_{10} CFU ml^{-1}) bacterial cell numbers over time in (A) low-impact and (B) high-impact microcosms over time measured by viable plate count on ½ YTSS agar.

A.


10
9
8
7
6
5
4
3
2
1
0
Log10 CFU ml-1
0
24
72
120
Time (h)
ML
MLTOL
MD
MDTOL
EU
EUTOL
DC
DCTOL

B.

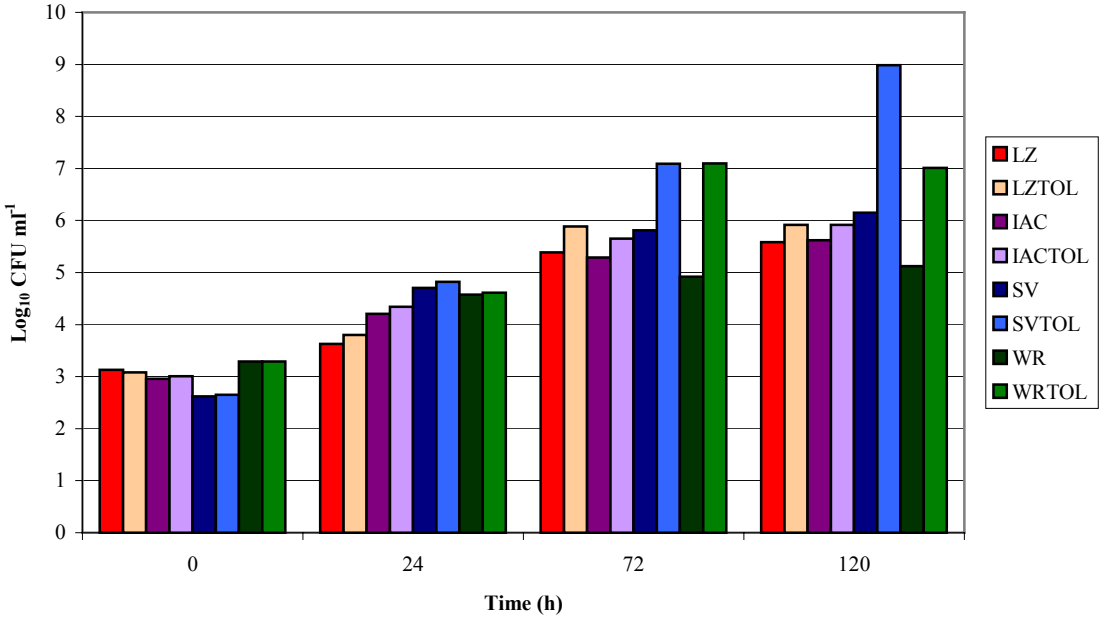

10
9
8
7
6
5
4
3
2
1
0
Log10 CFU ml-1
0
24
72
120
Time (h)
LZ
LZTOL
IAC
IACTOL
SV
SVTOL
WR
WRTOL

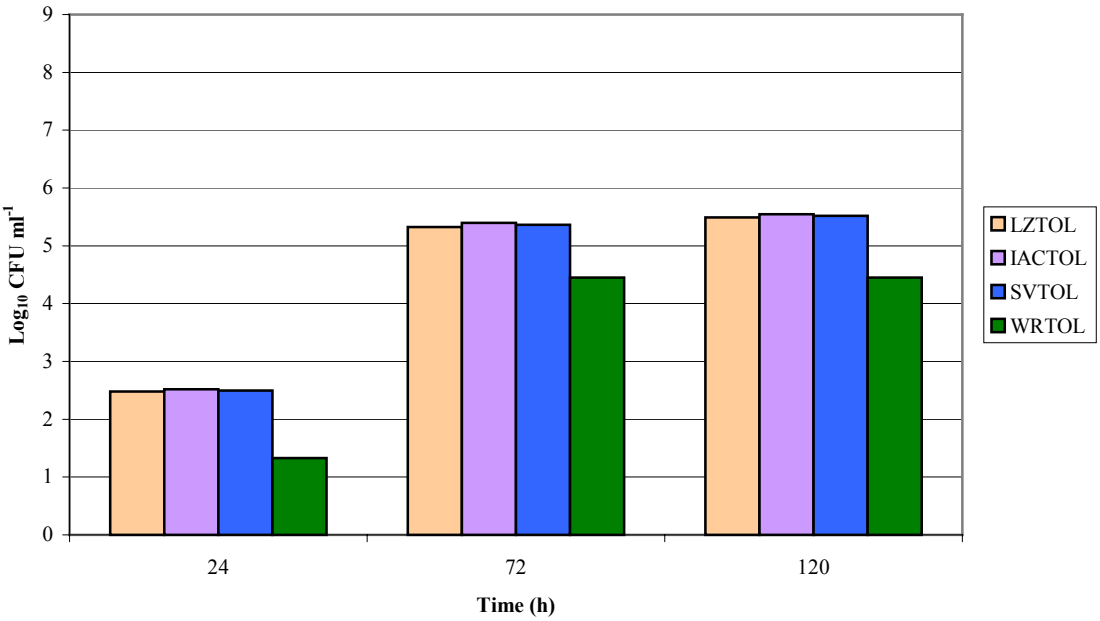
TABLE 4.3. Percent culturability ([CFU_{YTSS}/DMC_{YOPRO}) x 100]) changes over time in low- and high-impact microcosms. Microcosms amended with toluene are indicated with "TOL" as part of the microcosm name.

Microcosm		% Culturability			
		0 h	**24 h**	**72 h**	**120 h**
Low Impact	ML	0.18	6.22	32.54	16.04
	MLTOL	0.23	4.83	32.82	16.80
	MD	0.04	3.76	22.86	16.45
	MDTOL	0.05	3.90	35.77	24.27
	EU	0.24	8.47	31.98	32.68
	EUTOL	0.27	6.80	23.74	42.51
	DC	0.06	7.30	38.45	30.69
	DCTOL	0.06	6.08	36.17	34.57
High Impact	LZ	1.20	2.09	21.58	20.72
	LZTOL	1.05	2.30	24.53	24.28
	IAC	0.92	7.35	24.65	23.88
	IACTOL	0.97	6.03	25.07	25.74
	SV	0.39	6.01	19.65	18.05
	SVTOL	0.41	6.37	21.77	22.55
	WR	1.04	6.31	9.20	7.99
	WRTOL	1.04	5.95	16.30	18.57
Mean of All Low Impact		0.14	5.92	31.79	26.75
Mean of All High Impact		0.88	5.30	20.34	20.22
Mean Low Impact		0.13	6.44	31.46	23.97
Mean Low Impact + Toluene		0.15	5.40	32.12	29.53
Mean High Impact		0.88	5.44	18.77	17.66
Mean High Impact + Toluene		0.87	5.16	21.91	22.78

ranging from 0.39% to 1.20% (Table 4.3). Though culturability continued to increase during incubation in the high-impact microcosms, the overall increase was less than observed in their low impact counterparts: 8.0% to 25.7% (Table 4.3).

Isolation and enumeration of toluene degraders. We attempted to enumerate bacteria capable of growing on toluene as a sole carbon source. However, regardless of the dilution plated, no colonies grew on AS/BSM (plus toluene) plates that were inoculated with seawater from any of the low-impact bacterial communities (microcosms ML, MLTOL, MD, MDTOL, EU, EUTOL, DC and DCTOL) over the 5-day experiment, whether or not the microcosm had received a toluene amendment. Likewise, no toluene degraders could be detected from the high-impact community microcosms that were not amended with toluene (LZ, SV, IAC and WR). Only high-impact community microcosms amended with toluene (LZTOL, SVTOL, IACTOL, and WRTOL) yielded viable toluene degraders on AS/BSM (plus toluene) agar (Figure 4.5). Degraders were undetected in the initial diluted seawater communities of these microcosms but were observed at subsequent samplings made at 24, 72, and 120 h. Numbers of toluene degraders increased over the course of the incubation from undetectable to maximum values of 2.2×10^4 CFU ml^{-1}, 3.3×10^5 CFU ml^{-1}, 8.0×10^6 CFU ml^{-1}, and 8.8×10^7 CFU ml^{-1} in microcosms IACTOL, LZTOL, WRTOL and SVTOL, respectively, at 120 h. Overall, 21 morphologically distinct isolates capable of growth using toluene as a sole carbon source were obtained from the high –impact communities plated on AS/BSM (plus toluene). No isolates were ever obtained from AS/BSM plates without toluene supplement, indicating that growth of isolates obtained from this medium was at the

Figure 4.5. Increases (log_{10} CFU ml^{-1}) bacterial cell numbers over time in high-impact microcosms over time measured by selective viable plate count on AS/BSM agar plus toluene vapors.


9
8
7
6
5
4
3
2
1
0
Log10 CFU ml-1
24
72
120
Time (h)
LZTOL
IACTOL
SVTOL
WRTOL

expense of the toluene vapors supplied and not at the expense of agar digestion or trace organic substances.

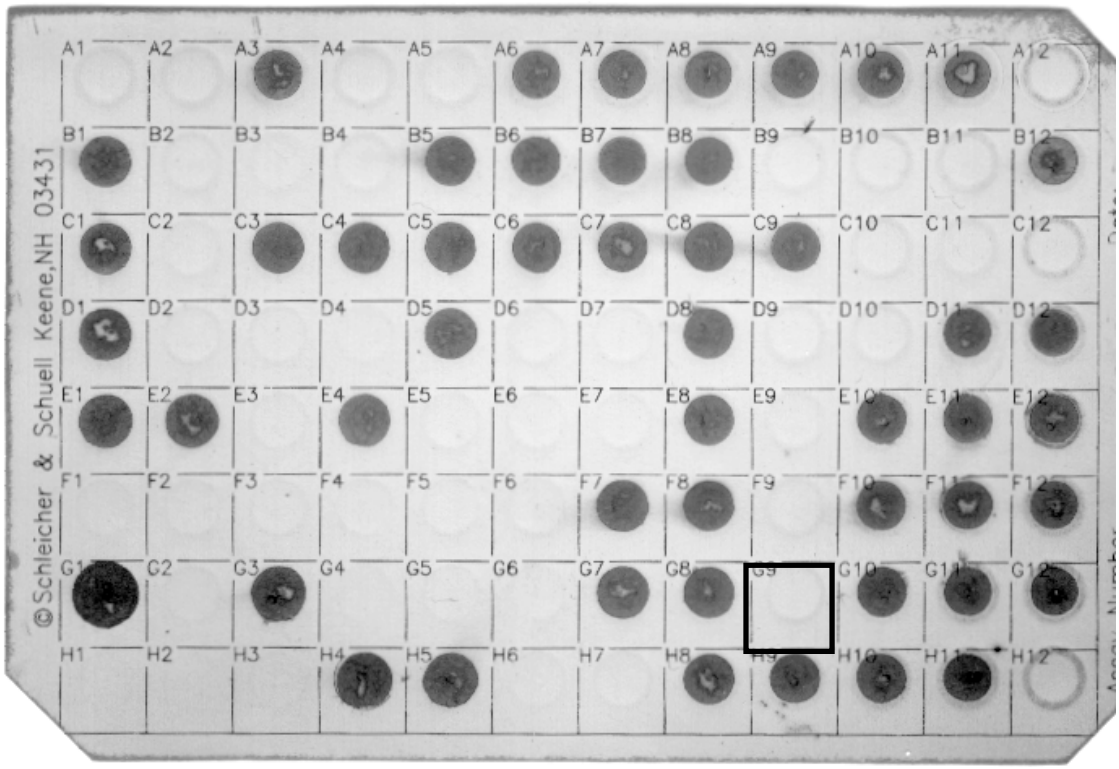
Morphologically distinct isolates were also selected from ½ YTSS agar plates over the course of the experiments at various timepoints. From the low-impact site microcosms, a total of 28 morphotypes (from both the toluene-amended and unamended treatments) were selected and purified. The high-impact microcosms yielded a greater diversity of colonial morphotypes than did the low-impact microcosms: a total of 66 unique isolates (from both the toluene-amended and unamended treatments) were purified.

Using the same *in vitro* BTEX PCR amplification and hybridization conditions as were used in the *in situ* PCR/H experiments, DNA from each of the isolates was screened for the presence of BTEX dioxygenase gene sequences. No strains from the low-impact microcosms (isolated on ½ YTSS agar) were positive for BTEX dioxygenase genes. Among 66 high-impact community isolates from ½ YTSS agar, 30 were determined to possess BTEX dioxygenase genes detectable by using *in vitro* amplification and dot blot hybridization conditions (Figure 4.6). Isolation on AS/BSM agar plus toluene vapors was only achieved from high-impact community microcosms that had been supplemented with toluene vapors during incubation. In total, 21 morphologically distinct strains were isolated from these communities, and 20 of these yielded positive hybridization signals with BTEX4 after amplification (Figure 4.6).

Correction for autofluorescence. Low levels of interfering autofluorescence under CY3 excitation conditions were observed on filter sections used for direct microscopic

Figure 4.6. (A) Results of dot blot hybridization for BTEX4 probe of PCR amplifications of dioxygenase genes using BTEX1 and BTEX2 primers in isolates from high-impact community microcosms. The open square around the negative dot blot area represents the one strain isolated by selective growth on toluene that did not hybridize to the BTEX4 probe. (B) A map of the isolates used in this dot blot hybridization.

A.



B.

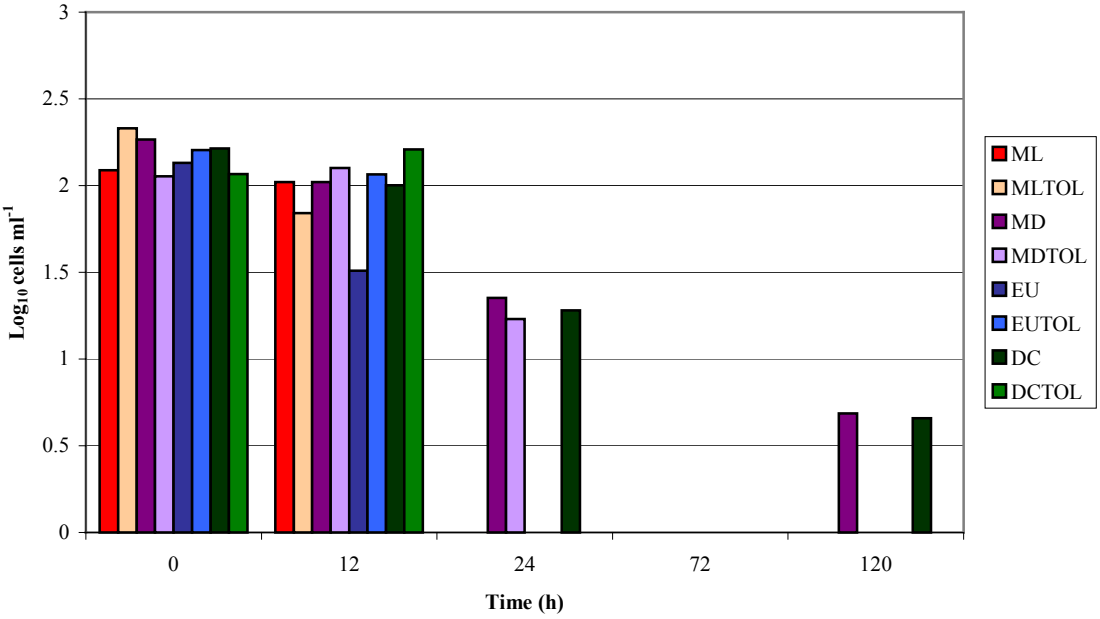
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**	**11**	**12**
A	WRC 1	WRC 2	WRC 3	WRC 4	WRC 5	WRC 6	WRC 7	WRT 1	WRT 2	WRT 3	WRT 4	
B	WR 1	WR 2	WR 3	WR 4	WR 5	WR 6	WR 7	WR 8				PPF1
C	IACC 1	IACC 2	IACC 3	IACC 4	IACC 5	IACT 1	IACT 2	IACT 3	IACT 4			
D	IAC 1	IAC 2	IAC 3	IAC 4	IAC 5	IAC 6	IAC 7	IAC 8	IAC 9		LZT 1	LZT 2
E	LZ 1	LZ 2	LZ 3	LZ 4	LZ 5	LZ 6	LZ 7	LZ 8	LZ 9	LZ 10	LZ 11	LZT 3
F	LZC 1	LZC 2	LZC 3	LZC 4	LZC 5	LZC 6	LZC 7	LZC 8		LZT 6	LZT 5	LZT 4
G	SV 1	SV 2	SV 3	SV 4	SV 5	SV 6	SV 7	SV 8	SVT 1	SVT 2	SVT 3	SVT 4
H	SVC 1	SVC 2	SVC 3	SVC 4	SVC 5	SVC 6	SVC 7	SVC 8	SVT 5	SVT 6	SVT 7	

counts. To account for this, appropriate corrections were made to the direct count estimates of BTEX-positive cell numbers, producing a conservative estimate of the abundance of BTEX dioxygenase gene-containing cells in natural assemblages from coastal Georgia waters. Generally, as population densities increased in the microcosms, the number of autofluorescing cells decreased (Figure 4.7 A and B). Initial autofluorescent cell numbers ranged from 2.4 x 10^3 cell ml^{-1} to 4.4 x 10^3 cell ml^{-1} in the low-impact microcosm communities and from 2.3 x 10^3 cell ml^{-1} to 1.68 x 10^4 cell ml^{-1} in the high-impact communities. After 72 of incubation, numbers of autofluorescent cells decreased, often to undetectable levels, within the community.

Enumeration of toluene degraders by *in situ* PCR/H. At timepoints 0, 12h and 24 h, the corrected *in situ* PCR/H (BTEX-positive) signals (calculated cell numbers) were numerically close to the numbers of autofluorescing cells in all microcosms (Figure 4.8 A and B). In a few cases at the 0 h timepoint, the number of autofluorescing cells exceeded the number of BTEX-positive cells (Figure 4.8 A [EU, MD, and MDTOL] and 4.8 B [LZ, SV]). Additionally, autofluorescent cell numbers were higher than BTEX-positive cell number in the MDTOL and DC microcosms at the 12 h sampling (Figure 4.8 A). In order to determine if there was an overall significant difference between BTEX-positive cell number and autofluorescent cell number within the first 24 h, BTEX-positive cell numbers for each timepoint were pooled into 4 groups (e.g. low impact, low impact plus toluene, high impact, high impact plus toluene), as were mean autofluorescence values. Nonparametric statistical tests (Mann-Whitney $\alpha = 0.05$) were then applied to the pooled data sets.

Figure 4.7. Change in numbers of autofluorescent cells (log_{10} cells ml^{-1}) in (A) low-impact and (B) high-impact microcosms over time measured by direct microscopic count of filters not subjected to *in situ* PCR/H.

A.


3
2.5
2
1.5
1
0.5
0
Log10 cells ml-1
0
12
24
72
120
Time (h)
ML
MLTOL
MD
MDTOL
EU
EUTOL
DC
DCTOL

B.

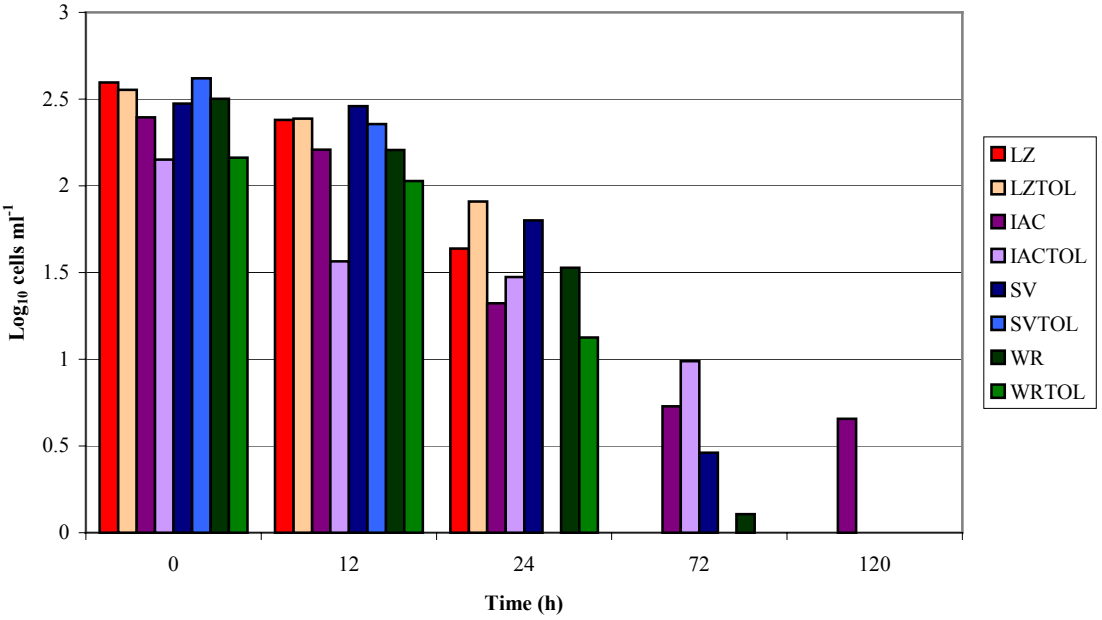
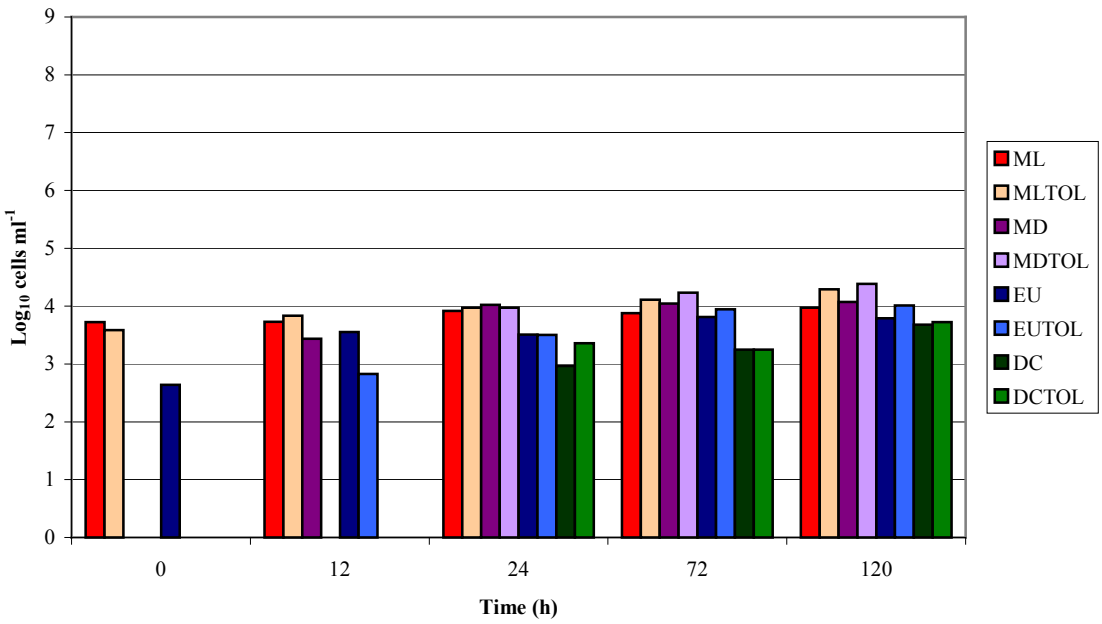
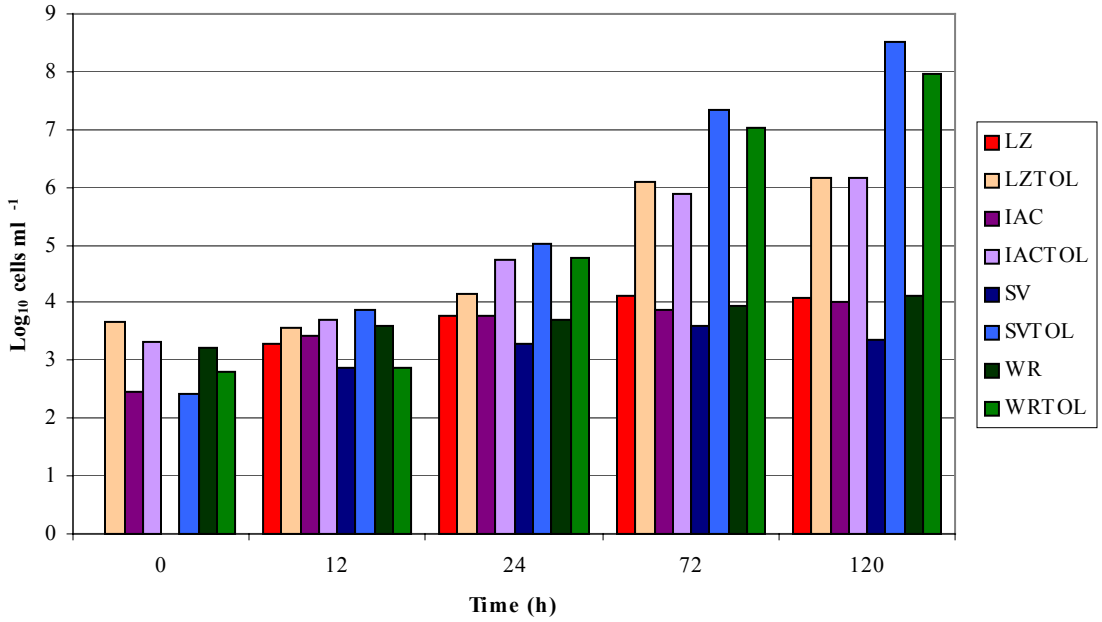

3
2.5
2
1.5
1
0.5
0
Log10 cells ml-1
0
12
24
72
120
Time (h)
LZ
LZTOL
IAC
IACTOL
SV
SVTOL
WR
WRTOL

Figure 4.8. Increases (log_{10} cells ml^{-1}) in BTEX-positve bacterial cell numbers over time in (A) low-impact and (B) high-impact microcosms measured by direct microscopic count after *in situ* PCR/H. Timepoints for which no bar appears for a particular microcosm indicates that the number of autofluorescent cells exceeded the number of BTEX-positive cells.

A.


9
8
7
6
5
4
3
2
1
0
Log10 cells ml-1
0
12
24
72
120
Time (h)
ML
MLTOL
MD
MDTOL
EU
EUTOL
DC
DCTOL

B.


9
8
7
6
5
4
3
2
1
0
Log10 cells ml -1
0
12
24
72
120
Time (h)
LZ
LZTOL
IAC
IACTOL
SV
SVTOL
WR
WRTOL

In the low-impact community microcosms as well as the low-impact toluene-amended microcosms, autofluorescing cell numbers of the 0 h and 12 h pooled subsamples were not found to be significantly different from the estimated numbers of BTEX-positive bacteria ($p > 0.05$) (Table 4.4). In the remaining toluene-amended and unamended low-impact microcosm communities, the pooled subsamples from timepoints 24 h, 72 h and 120 h were determined to be significantly different ($p < 0.005$) from one another (Table 4.4). In the case of the high-impact microcosm communities, as well as the high impact plus toluene microcosm communities, statistical analyses showed that populations of autofluorescent cells were significantly ($p < 0.005$) different from the BTEX-positive cells in all cases except the 0 h subsamples (Table 4.4).

Excluding samples in which autofluorescent cell numbers exceeded BTEX-positive cell numbers, a general trend regarding the number of BTEX degraders in the microcosms was apparent. Numbers of BTEX degraders were fairly constant in the low impact microcosms over the 120 h experiment, regardless of selective pressure of toluene addition or its absence (Figure 4.8). For example, at the completion of 120 h incubation, the numbers of BTEX-positive cells were 4.8×10^3 cells ml^{-1} and 5.3×10^3 cells ml^{-1} in microcosms DC and DCTOL, respectively (Figure 4.8). Similarly, in the unamended high-impact community microcosms, the number of BTEX degraders underwent slight increases as compared to those observed in the microcosms grown under the selective pressure of toluene enrichment. In microcosm WR, the initial number of BTEX-positive cells was 1.6×10^4 cells ml^{-1} and increased to a maximum of 1.3×10^5 cells ml^{-1}, while in microcosm WRTOL, initial numbers of BTEX-positive cells

TABLE 4.4. Results of Mann-Whitney statistical tests using pooled samples vs. autofluorescence ($\alpha = 0.05$).

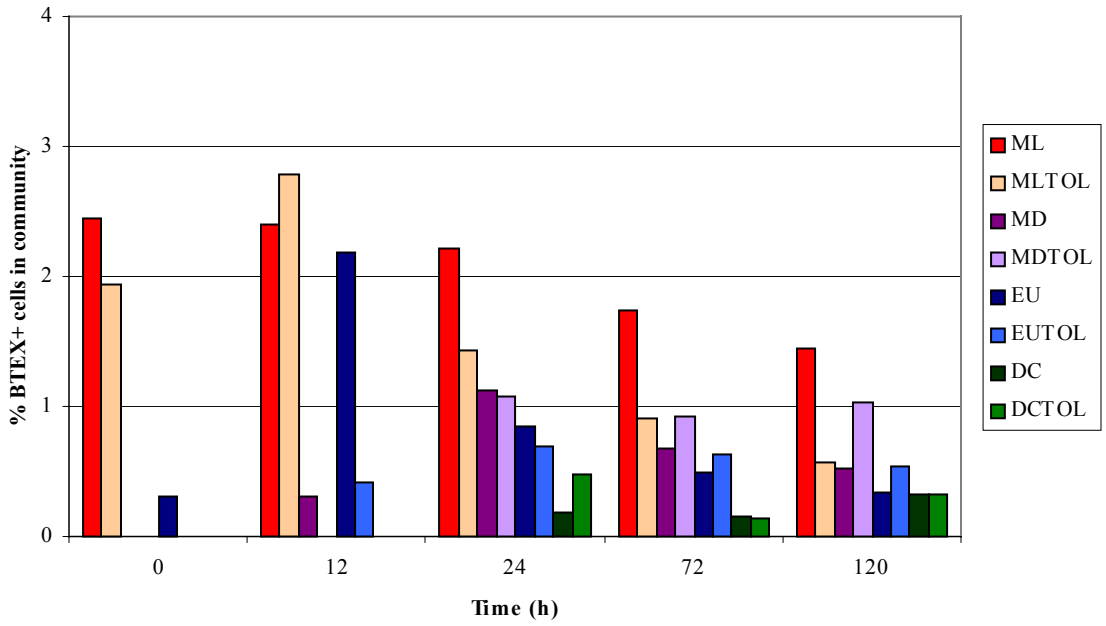
Time	**Low Impact**	**Low Impact with Toluene**	**High Impact**	**High Impact with Toluene**
0	p = 1.0000	p = 0.6912	p = 0.4743	p = 0.1077
12	p = 0.1330	p = 0.5063	p = 0.0018	p = 0.0169
24	p = 0.0004	p = 0.0002	p = 0.0001	p = 0.0002
72	p = 0.0001	p = 0.0001	p = 0.0002	p = 0.0002
120	p = 0.0001	p = 0.0001	p = 0.0002	p = 0.0002

increased dramatically from 6.3 x 10^2 cells ml^{-1} to 9.0 x 10^7 cells ml^{-1}. Moreover, BTEX degraders represented only a very slim portion of the entire community in the low impact microcosms, ranging from about 0.5% to near 2.8% of the total population (Figure 4.9 A). While BTEX degraders approached 50% of the community at 120 h in the high impact microcosms supplemented with toluene, the proportion of BTEX remained under 5% of the total cells present in the community when only background DOC was available as a growth substrate (Figure 4.9 B). Figure 10 A and B show a typical microscope field of BTEX-degraders from a high-impact microcosm under YOPRO and CY3 excitation wavelengths, respectively. Similarly, Figure 10 C and D represent a typical microscope field from a low-impact microcosm.

Relationship between selective growth on toluene and BTEX-positive signals. We found a strong positive correlation between numbers of toluene degraders isolated on selective media (AS/BSM plus toluene vapors) and the number of BTEX-positive cells after *in situ* PCR/H using primers and probe targeting dioxygenases such as the one encoded by *tod*C1. Analysis of communities from LZTOL and IACTOL produced similar correlation coefficients (r = 0.76 each), while the SVTOL and WRTOL communities showed the strongest correlations (r = 0.99 each). Moreover, nearly all (21 of 22) strains isolated by growth on toluene as a sole carbon source appear to possess the dioxygenase genes (targeted in the *in situ* PCR/H method) as determined by *in vitro* PCR and dot blot hybridization using our degenerate BTEX dioxygenase primers and probe (Figure 4.6).

Figure 4.9. Increases in the percentage BTEX-positive bacterial cell numbers of the total community cell numbers over time in (A) low-impact and (B) high-impact microcosms over time measured by direct microscopic count after *in situ* PCR/H. Timepoints for which no bar appears for a particular microcosm indicates that the number of autofluorescent cells exceeded the number of BTEX-positive cells.

A.


4
3
2
1
0
% BTEX+ cells in community
0
12
24
72
120
Time (h)
ML
MLTOL
MD
MDTOL
EU
EUTOL
DC
DCTOL

B.

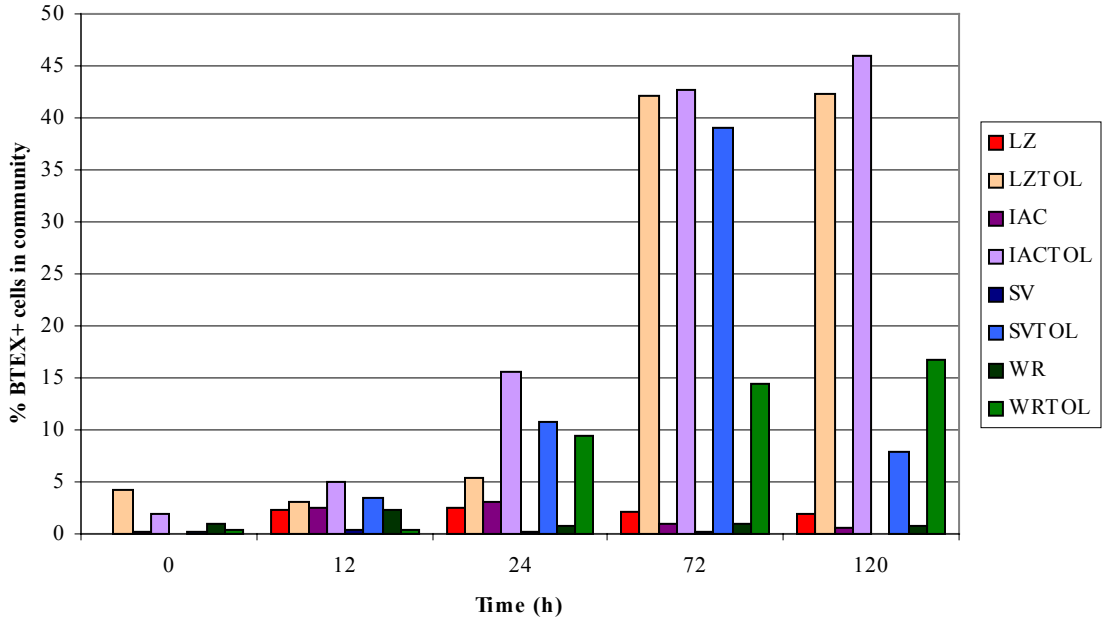
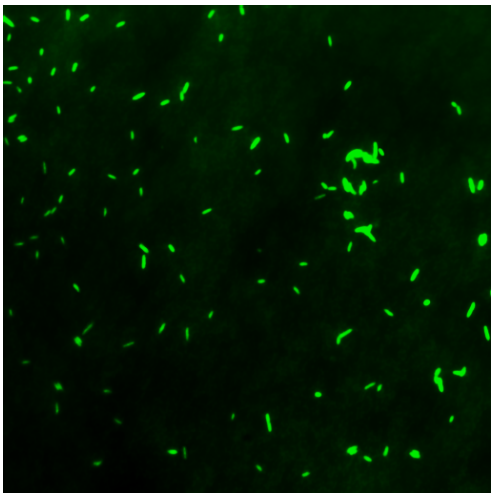
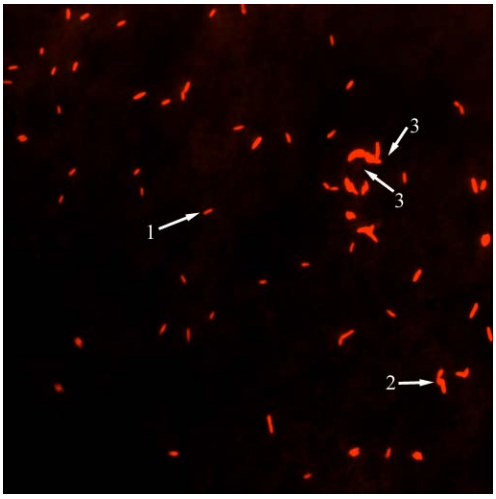

50
45
40
35
30
25
20
15
10
5
0
% BTEX+ cells in community
0
12
24
72
120
Time (h)
LZ
LZTOL
IAC
IACTOL
SV
SVTOL
WR
WRTOL

Figure 4.10. Detection of total bacterial numbers (green under YOPRO excitation) vs. BTEX-positive cell numbers (red under CY3 excitation) in filter-concentrated bacterial communities from microcosms: (A) and (B) a high-impact microcosm community, LZTOL at 72 h, under YOPRO and CY3 wavelengths, respectively; (C) and (D) a low-impact microcosm community, DCTOL at 72 h, under YOPRO and CY3 wavelengths, respectively. Note the spatial associations of the BTEX-positive cells with community bacteria denoted by arrows in (B).

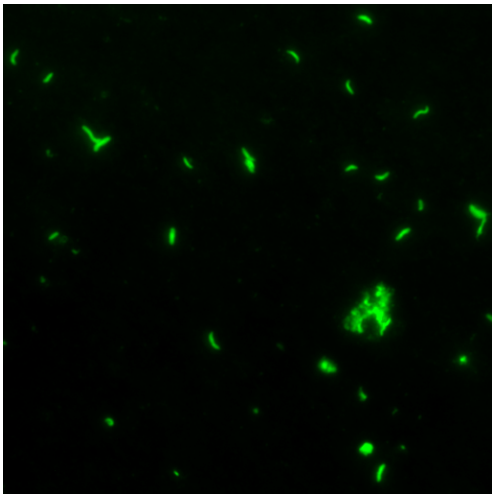
A



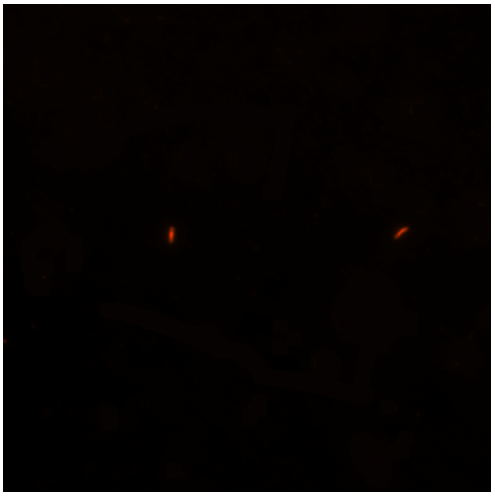
B



C



D



Spatial relationships of BTEX-positive cells with other organisms. Figure 10 A shows the total bacterial community present in the high-impact community microcosm LZTOL at 72 h under YOPRO excitation conditions, Figure 10 B, shows only the cells that are BTEX-positive after *in situ* PCR/H. Note, BTEX-positive organisms include free-living cells (arrow 1) as well as multi-cellular associations with either other BTEX-positive cells (arrow 2) or cells that do not possess BTEX dioxygenase genes (arrow 3) (Figure 12 B). It is possible, however, that these spatial associations are not naturally occurring, and are, instead, an artifact of the filter collection procedure that effectively reduces a three-dimensional matrix to a two-dimensional one. Some of the floc holding together the large aggregates can be seen under YOPRO illumination. A typical field of a low-impact community microcosm (EUTOL) at the 72 h timepoint is shown in Figure 10 C and D, under YOPRO and CY3 excitation conditions, respectively.

DISCUSSION

In this study, we have shown that *in situ* PCR/H can be a sensitive visual method to detect specific individual bacterial cells capable of performing a specific metabolic activity, specifically the degradation of BTEX compounds, within a mixed bacterial community. While other cultivation-independent methods (e.g. *in vitro* hybridizations using extracted community DNA) can provide similar estimates of of genes for specific metabolic activities, these methods are dependent upon the use of one to several calculations to convert experimental results (e.g. bulk signal strength) to a number representing the original bacterial population size present in the sample (i.e. amount of DNA examined/mean amount of DNA per cell). One of the advantages of *in situ* PCR/H

as reported here, is that each positive organism can be examined individually, rather than merely as a contribution to the bulk community nucleic acid signal. Because this technique can preserve the physical associations of bacteria and other particles, we have been able to begin examining the spatial relationships, and hence the complex inter-relationships, of bacteria capable of catabolizing BTEX hydrocarbons in marine environments. By increasing the complexity of the growth substrates in step-wise manner, and using *in situ* PCR/H with primer/probe sets for key specific enzymes of bioremediation pathways, we should be able to produce a direct visual link between disappearance of a particular pollutant and specific cells within an indigenous bacterial assemblage.

While toluene is a simple hydrocarbon and may be fully metabolized by a single organism (i.e. free living cells), some BTEX degraders may also work in concert with other microorganisms to metabolize substrates, especially more recalcitrant hydrocarbons. Recent studies have shown that as few as two different species of *Pseudomonas* completely metabolize a suite of BTEX compounds when grown as a co-culture, even when both of the individual strains cannot utilize any of the individual BTEX components as measured under selective growth conditions (8). It is interesting to note, since we amended our microcosm communities with toluene, that Attaway *et al.* (8) found toluene was the only BTEX compound individually degraded by both of the two *Pseudomonas* species.

Although we diluted the ambient bacterial community 100-fold below its original concentration at the onset of our experiment, we were still able to detect low levels of BTEX-positive organisms comprising less than 1% to about 5% of the diluted

assemblages in the first 24 h by *in situ* PCR/H. This data indicates that these techniques may be sensitive enough to detect a specific sequence present in less than one organism out of 1000, depending on the volume of seawater filtered. While the logistics of manually assessing several thousand cells per sample seem daunting, *in situ* PCR/H techniques are adaptable for use with more automated forms of analysis such as flow cytometry (10), allowing fast and efficient analysis of hundreds of thousands of organisms per sample. Moreover, the joint use of *in situ* PCR/H with flow cytometry would provide an additional advantage in that they can be further subdivided and subjected to further analyses (i.e. DNA sequencing) after they are assessed for *in situ* PCR/H signal.

Despite the fact that we specifically chose sampling sites where we were reasonably sure BTEX contamination was either negligible or absent (i.e. our low-impact community sites), we observed BTEX-positive cells at all locations, regardless of whether or not toluene was supplied as selective pressure. Conversely, in cultivation experiments, only the high-impact community microcosms supplemented with toluene yielded isolates when plated with toluene as a sole carbon source. While these results seem to contradict one another, we must recall that the *in situ* PCR/H and selective isolation techniques measure two different things. We used *in situ* PCR/H to demonstrate the presence of dioxygenase genes commonly part of an operon involved in BTEX degradation. Their presence does not necessarily confer the ability to catabolize the compounds and/or grow at their expense. More specifically, the degradation of BTEX compounds, specifically the toluene substrate used in these microcosms, may occur via several different metabolic pathways. The known pathways for the aerobic degradation

of toluene include: 1) toluene 2,3-dioxygenase: *todC1C2BADE* (47, 48, 49); 2) xylene monooxygenase: *xylCMAB* and *xylXYXLTEGFJQIH* (45); 3) toluene 4-monoxygenase: *tmoABCDE* (44); 4) toluene 2-monooxygenase: *tbmABCDEF* (26); 5) toluene-ortho-monooygenase: *tomAB* (37); 6) toluene 3-monooxygenase: *tbuABCDEFGKIHJ* (32); and 7) toluene-ortho-xylene-monooxygenase: *touABCDEFR* (2). Selective isolation as used here, however, requires that the organism use the toluene for growth and be able to grow on solid medium.

It is possible that a small percentage of bacteria in the low-impact communities retain incomplete operons for BTEX degradation, and hence, when supplied with toluene as a sole carbon substrate, are unable to utilize it for growth. They may, in contrast, have been growing at the expense of other trace DOC components and metabolites present in the artificial seawater medium of the microcosms. As stated above, we had not intended to introduce any carbon source other than the toluene supplement along with whatever DOC was present in the 2 ml of inoculum. It should be noted that newly available DOC could also be introduced from exudates of living cells, or as a result of cell death and lysis. We learned subsequently of the variability in DOC content of the sea salts used in our medium preparation and assume that these trace carbon sources were partially responsible for re-growth in the low-impact community microcosms (with and without the toluene supplement), as well as in the unsupplemented high-impact community microcosms. Re-growth of bacterial numbers was also likely due to the lack of protozoan grazers since the medium was sterile and the inoculum was filtered (pore-size 3.0 μm). During the course of the 5-day microcosm studies, the percent of the high-impact microcosms bacterial community that was BTEX dioxygenase-positive increased from

initial values averaging 1.3% to maximum values often exceeding 25% but never exceeding 46% in our studies (Table 4.3). Without the toluene supplement, numbers of BTEX-degrading bacteria from high-impact community assemblages remained below 3.3% (Table 4.3). The percentage of BTEX-positive cells in either of the low-impact microcosm treatments remained steady, never rising above 3.8% during the course of the experiment (Table 4.3).

The fact that the proportion of BTEX-positive cells did not increase in the low-impact community microcosms amended with toluene is consistent with the results of previous studies. Armstrong *et al*. (3) monitored mineralization of toluene in water samples from a groundwater aquifer that was chronically contaminated with toluene, and compared these rates with those supported by bacteria in a groundwater control site, adjacent to but not impacted by hydrocarbon contamination. While toluene mineralization was readily and repeatedly detected in water from the impacted site samples, no measurable mineralization was observed in the un-impacted site samples (3). Furthermore, the authors incubated control site water in the presence of toluene for up to one month in an attempt to select for toluene degraders before assaying for toluene mineralization. However, even after one month of pre-adaptation to toluene, no toluene mineralization could be detected in the groundwater microbial community. Since many genes for biodegradation are plasmid-bound, it is possible that conditions in these groundwater aquifer sites, as well as our own study's low-impact sampling sites, were sufficiently oligotrophic to cause bacterial cells present to reject plasmids. A similar phenomenon was observed by Sobecky *et al.* (40).

Although we estimated the fraction of the communities capable of degrading any BTEX compound, including toluene, the selective culturable methods we employed only estimated the number of bacteria within the community that are able to degrade toluene. Although many bacteria are capable of degrading multiple BTEX compounds, not necessarily all BTEX-degraders are capable of metabolizing toluene. Thus, the viable plate counts of (toluene) degraders may only represent a portion of the BTEX-degrading community present.

Yet another likely explanation for the discrepancy between *in situ* PCR/H and culturable results lies in the phenomenon of viable-but-not-culturable (VNBC) bacteria. VNBC bacterial cells from marine and freshwater habitats have been shown to remain intact, retaining metabolic activity, yet incapable of growing on conventional culture media (22, 31, 34, 46). While our *in situ* PCR/H results indicated a much higher number of BTEX-degrading bacteria than what we estimated by selective viable plate counts, it is likely that the latter was seriously underestimated due to the presence of VNBC bacterial cells within the communities.

Our results indicate that the strains comprising the bacterial communities from our low-impact coastal marine sites were less culturable (mean = 0.14%, Table 3) at the experiment onset than were the bacteria comprising the communities at sites impacted by marine and shipping activities (mean = 0.88%, Table 3). This difference may simply be an artifact of our sample number. On the other hand, were these differences to prove typical of more sites and sampling times, it might be indicative of direct or indirect pollutionally induced changes in bacterial community structure. Inoculation of these communities in microcosms for up to five days resulted in dramatic and consistent

increases in the percent culturability (up to a maximum of 42.5 %), and these changes were independent of the selective pressure exerted by toluene amendments (Table 3). Such profound changes in percent culturability have been noted previously and are related to "wall effects", selective outgrowth of high-nutrient adapted heterotrophs, and/or the absence of bacteriovores in the confined samples (17). Our results suggest follow-up studies in which changes in bacterial community structure in microcosms with or without toluene could be followed using denatured gradient gel electrophoresis (DGGE) (18) or terminal restriction fragment length polymorphism (T-RFLP) analysis (30), each of which can provide an index of community composition and diversity without the requirement of laboratory cultivation on rich media. Studies with DGGE or T-RFLP could, in turn, suggest which particular degrader organisms might warrant further examination with *in situ* PCR/H.

REFERENCES

1. **Alvarez, P.J.J., and T.M. Vogel.** 1991. Substrate interactions of benzene, toluene, and para-xylene during microbial degradation by pure cultures and mixed culture aquifer slurries. *Appl. Environ. Microbiol*. **57**: 2981-2985.

2. **Arenghi, F.L.G., M. Pinti, E. Galli, and P. Barbieri**. 1999. Identification of the *Pseudomonas stutzeri* OX1 toluene-*o*-xylene monooxygenase regulatory gene (*tou*R) and of its cognate promoter. *Appl. Environ. Microbiol.* **65**: 4057-4063.

3. **Armstrong, A. Q., R. E. Hodson, H. M. Hwang, and D. L. Lewis.** 1991. Environmental factors affecting toluene degradation in ground water at a hazardous waste site. *Environ. Tox. and Chem.* **10**: 147-158.

4. **Arvin, E., B.K. Jensen, and A.T. Gundersen.** 1989. Substrate interactions during aerobic biodegradation of benzene. *Appl. Environ. Microbiol*. **55**: 3221-3225.

5. **Atlas, R.M.** 1981. Microbial degradation of petroleum hydrocarbons: an environmental perspective. *Microbiol. Rev.* **45**: 180-209.

6. **Atlas, R.M**. 1992. Persistence and survival of genetically modified microorganisms released into the environment. *In* D.E.S. Stewart-Tull and M. Sussman (eds.), The release of genetically engineered microorganisms, Plenum Press, New York NY.

7. **Atlas, R.M.** 1995. Bioremediation of petroleum pollutants. *Intl. Biodeter. Biodeg.* **35**:317-327.

8. **Attaway, H.H., and M.G. Schmidt.** 2002. Tandem biodegradation of BTEX compounds by two *Pseudomonas* sp. *Curr. Microbiol*. **45**: 30-36.

9. **Chang, M-K., T.C. Voice, and C.S. Criddle.** 1993. Kinetics of competitive inhibition and cometabolism in the biodegradation of benzene, toluene and p-xylene by two *Pseudomonas* isolates. *Biotechnol. Bioeng*. **41**:1057-1065.

10. **Chen, F., B.J. Binder, and R.E. Hodson.** 2000. Flow cytometric detection of specific gene expression in prokaryotic cells using in situ RT-PCR. *FEMS Microbiology Letters* **184**: 291-295.

11. **Chen, F, W.A. Dustman, and R.E. Hodson.** 1999 a. Microscopic detection of the toluene dioxygenase gene and its expression inside bacterial cells in seawater using prokaryotic *in situ* PCR. *Hydrobiologia* **401**: 131-138.

12. **Chen, F., W.A. Dustman, and R.E. Hodson.** 1999 b. Application of *in situ* reverse transcription to estuarine bacterial community analysis. *In* C.R. Bell, M. Brylinsky and P. Johnson-Green (ed.), Microbial Biosystems: New Frontiers (Proceedings of the 8th International Symposium on Microbial Ecology). Atlantic Canada Society for Microbial Ecology, Halifax Canada.

13. **Chen, F., J.M. Gonzalez, W.A. Dustman, M.A. Moran, and R.E. Hodson.** 1997. *In situ* reverse transcription, an approach to characterize genetic diversity and activities of prokaryotes. *Appl. Environ. Microbiol.* **63**: 4907-4913.

14. **Chevron Phillips Chemical Company.** 1993. Material Safety Data Sheet #217110: 10% aromatic diesel fuel. Chevron Phillips Chemical Co., Houston, TX.

15. **Dudeck, K.L**. 2001. M.S. thesis. University of Georgia, Athens GA.

16. **Dustman, W.A., F. Chen, M.A. Moran and R.E. Hodson.** 2002. Filter concentration of natural seawater bacterial communities for nnalysis of genetic and functional diversity by *in situ* amplification techniques. (In preparation).

17. **Ferguson, R.L., E.N. Buckley, and A.V. Palumbo.** 1984. Response of marine bacterioplankton to differential filtration and confinement. *Appl. Environ. Microbiol*. **47**: 49-55.

18. **Ferrari, V.C. and J.T. Hollibaugh.** 1999. Distribution of microbial assemblages in the central Arctic Ocean basin studied by PCR/DGGE: Analysis of a large data set. *Hydrobiologia* **401**: 55-68.

19. **Fujita, M., M. Ike, and K. Uesugi.** 1994. Operation parameters affecting the survival of genetically engineered microorganisms in activated sludge processes. *Water Research* **28**: 1667-1672.

20. **Geiselbrecht, A.D., B.P. Hedlund, M.A. Titchi, and J.T. Staley.** 1998. Isolation of marine polycyclic aromatic hydrocarbon (PAH)-degrading *Cycloclasticus* strains from the Gulf of Mexico and comparison of their PAH degradation ability with that of Puget Sound *Cycloclasticus* strains. *Appl. Environ. Microbiol.* **64**: 4703-4710.

21. **Gibson, D.T., G.E. Cardini, F.C. Maseles, and R.E. Kallio.** 1970. Incorporation of oxygen-18 into benzene by *Pseudomonas putida*. *Biochemistry* **9**: 1631-1635.

22. **Grimes, D. J., R. W. Atwell, P. R. Brayton, L. M. Palmer, D. M. Rollins, D. B. Roszak, F. L. Singleton, M. L. Tamplin, and R. R. Colwell.** 1986. The fate of enteric pathogenic bacteria in estuarine and marine environments. *Microbiol. Sci.* **3**: 324-329.

23. **Goldstein, R.M, L.M. Mallory and M. Alexander.** 1985. Reasons for possible failure of inoculation to enhance biodegradation. *Appl. Environ. Microbiol.* **50**: 977-983.

24. **Harayama, S., and K.N. Timmis.** 1989. Catabolism of aromatic hydrocarbons by *Pseudomonas*, p. 99-156. *In* D.A. Hopwood and K.F. Chater (eds.), Genetics of bacterial diversity, Academic Press, San Diego CA.

25. **Hodson, R.E., W.A. Dustman, R.P. Garg, and M.A. Moran.** 1995. *In situ* PCR for visualization of microscale distribution of specific genes and gene products in prokaryotic communities. *Appl. Environ. Microbiol*. **61**: 4074-4082.

26. **Johnson, G.R., and R.H. Olsen**. 1995. Toluene/benzene-2-monoxygenase from *Pseudomonas* sp. strain JS150. *Appl. Environ. Microbiol.* **61**: 3336-3346.

27. **Leahy, J.G., and R.R. Colwell.** 1990. Microbial degradation of hydrocarbons in the environment. *Microbiol. Rev*. **54**: 305-315.

28. **Meador, J.P., J.E. Stein, W.L. Reichert, and U. Varanasi.** 1995. Bioaccumulation of polycyclic aromatic hydrocarbons by marine organisms. *Rev. Environ. Contam. Toxicol.* **143**: 79-165.

29. **Millner, G.C., A.C. Nye, and R.C. James.** 1992. Human health based soil cleanup guidelines for diesel fuel no.2., p. 165-216. *In* P.T. Kostecki and E.J. Calabrese (eds.), Contaminated soils: diesel fuel contamination, Lewis Publishers, Chelsea MI.

30. **Moeseneder, M.M., J. M. Arrieta, G. Muyzer, C. Winter, and G. J. Herndl.** 1999. Optimization of terminal-restriction fragment length polymorphism analysis for complex marine bacterioplankton communities and comparison with denaturing gradient gel electrophoresis. *Appl. Envir. Microbiol.* **65**: 3518-3525.

31. **Nilsson, L., J. D. Oliver, and S. Kjelleberg.** 1991. Resuscitation of *Vibrio cholerae* from the viable but nonculturable state. *J. Bacteriol.* **173**: 5054-5059.

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

33. **Potter, T.L.** 1992. Fingerprinting petroleum products: unleaded gasolines. p. 83-92. *In* P.T. Kostecki and E.J. Calabrese (eds.), Petroleum contaminated soils, Lewis Publishers, Chelsea MI.

34. **Rollins, D. M., and R. R. Colwell.** 1986. Viable but nonculturable stage of *Camylobacter jejuni* and its role in survival in the natural aquatic environment. *Appl. Environ. Microbiol.* **52**: 531-538.

35. **Sambrook, J., E.F. Fritsch, and T. Maniatis.** 1989. Molecular Cloning: A Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

36. **Shi, Y., M.D. Zwolinski, M.E. Schreiber, J.M. Bahr, G.W. Sewell and W.J. Hickey.** 1999. Molecular analysis of microbial community structures in pristine and contaminated aquifers: field and laboratory microcosm experiments. *Appl. Environ. Microbiol*. **65**: 2143-2150.

37. **Shields, M.S., M.J. Reagin, R.R. Gerger, R. Campbell, and C. Somerville.** 1995. TOM, a new aromatic degradative plasmid from *Burkerholderia (Pseudomonas)cepacia* G4. *Appl. Environ. Microbiol.* **61**: 1352-13-56.

38. **Sikkema, J., J.A.M. duBont, and B. Poolman.** 1995. Mechanisms of membrane toxicity of hydrocarbons. *Microbiol. Rev.* **59**:201-222.

39. **Sims, J.L., J.M. Sulfita, and H.H. Russell.** 1992. Groundwater Issue: In situ bioremediation of contaminated groundwater. EPA540/S-92/003, U.S. Environmental Protection Agency Office of Solid Waste and Emergency Response, Washington D.C.

40. **Sobecky, P.A., M.A. Schell, M.A. Moran, and R.E. Hodson.** 1992. Adaptaion of model genetically engineered microorganisms to lake water: growth rate enhancements and plasmid loss. *Appl. Environ. Microbiol.* **58**: 3630-3637.

41. **Stegeman, J.J.** 1981. Polynuclear aromatic hydrocarbons and their metabolism in the environement, p. 1-60. *In* H.V. Gelboin and P.O.P. Tsao (eds.), Polycyclic hydrocarbons and cancer, vol. 1. Academic Press, Inc., New York NY.

42. **Tsao, C-W., H-G. Song, and R. Bartha.** 1998. Metabolism of benzene, toluene, and xylene hydrocarbons in soil. *Appl. Environ. Microbiol.* **64**: 4924-4929.

43. **United States Coast Guard**. 1994. Polluting incidents in and around U.S. waters, calendar year 1983 and 1984. United States Coast Guard Service, COMDTINST M16450.26. NTIS 87-186821.

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

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

46. **Xu, H. S., N. Roberts, F. L. Singleton, R. W. Attwell, D. J. Grimes, and R. R. Colwell.** 1982. Survival and viability of nonculturable *E. coli* and *V. cholerae* in the estuarine and marine environment. *Microb. Ecol.* **8**: 313-323.

47. **Ziffer, H., D.N. Jerina, D.T. Gibson, and V.M. Kobal.** 1973. Absolute stereochemistry of the dextro *cis*-1,2-dihydroxy-3-methlycyclohexa-3,5-diene produced from toluene by *Pseudomonas putida*. *J. Am. Chem. Soc*. **95**: 4048-4049.

48. **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. Biochem*. **264**: 14940-14946.

49. **Zylstra, G.J., W.R. McCombie, D.T. Gibson, and B.A. Finnette.** 1988. Toluene degradation by *Pseudomonas putida* F1: genetic organization of the tod operon. *Appl. Environ. Microbiol*. **54**: 1498-1503.

CHAPTER 5

SUMMARY

SUMMARY

The intent of studies described herein was to develop a molecular-based protocol to enable the visual detection of specific functional genes or gene expression within individual bacterial cells using epifluorescence microscopy, and to apply the methodology to investigate the distribution of a specific genetic capability, hydrocarbon degradation, in bacterioplankton communities in coastal Georgia waters.

Strains of *Pseudomonas putida* and *P. aeruginosa* containing a single copy of the NAH7 plasmid, as well as an isogenic strains lacking the plasmid, was used to verify the new methodology's sensitivity and specificity. We successfully amplified *nahA* sequences within individual bacterial cells using direct incorporation of labeled-nucleotides in a one-stage *in situ* PCR protocol; detectable levels of nonspecific incorporation of the labeled-nucleotides were also seen (Chapter 2, Figure 2). To increase the specificity of our techniques, we modified the protocol to include two separate rounds of PCR. The first round of amplification was performed with unlabeled nucleotides and served to increase copy number of our target sequence while also repairing any nicked DNA. A second round of PCR involving primer extension using a single primer internal to the region amplified in the first round and labeled nucleotides was then employed. Nonspecific products of the first amplification round remain undetected because they are not complimentary to the internal primer used in the second stage, and thus, are not labeled. When both positive and negative control strains were mixed prior to *in situ* PCR using this variation, we were able to reliably and repeatedly differentiate cells possessing the *nahA* gene from those that did not (Chapter 2, Figure 3).

Once proven in laboratory strains, we adapted the new methodologies for use in natural aquatic samples. In the environment, bacterioplankton are much more dilute than when grown in laboratory medium. Thus, one obstacle to adapting these techniques for use with natural water samples was to somehow concentrate the communities. To this end, we employed membrane filtration, a protocol widely used in microbial ecology studies. We examined a variety of membrane filter materials (Chapter 3, Table 3), and determined which membranes were most compatible with our techniques in terms of low background fluorescence signal and recovery efficiency. Although *in situ* PCR techniques require extensive manipulations that can result in loss of cells adhered to the filter, we defined conditions under which we routinely obtained a greater than 85% recovery efficiency (Chapter 3, Table 6).

To verify the applicability of the filter collection of bacteria, we employed two other variations of *in situ* PCR developed in our lab: *in situ* reverse transcription (ISRT) and *in situ* PCR/hybridization (*in situ* PCR/H). Positive and negative control strains, *Pseudomonas putida* F1 (which carries the gene for toluene dioxygenase) and *P. putida* AC10R-7 (which carries a non-homologous naphthalene dioxygenase gene on a NAH7 plasmid), respectively, were used to examine the specificity and sensitivity of both techniques. ISRT using 16S rRNA primers successfully detected both strains, essentially 100% of the total cells present on the filters. Although both strains carried genes for dioxygenases, our primers were aimed specifically at those involved in benzene, toluene, ethylbenzene and xylene cleavage. When either ISRT or *in situ* PCR/H was performed with the BTEX primers/probe, only strain F1, which carried a target gene, was detectable while the protocols gave the expected negative response with strain AC10R-7, which

carried a functionally similar but non-homologous gene, but not one targeted by the BTEX primer/probe set (Chapter 3, Figures 2 and 3). Much like the sensitivity of ISRT, the sensitivity of the procedure for detecting BTEX dioxygenase genes in the positive control strain approximated 100%.

When these techniques were applied to size-fractionated (< 3.0 μm pore-size) seawater samples, we found that ISRT using 16S rRNA primers detected between 23 and 45 % of all bacterioplankton on the filters. While these values were significantly less than what we observed for positive control strains where100% of all cells were detected, these results are consistent with reports of low rRNA levels of cells in the natural bacterioplankton communities. Similarly, when we used *in situ* RT-PCR/H using the BTEX primer/probe set on the same samples, we detected approximately 1% of the bacterial fraction as BTEX degraders, indicating that Georgia coastal and estuarine waters had low activity for BTEX degradation at the time of sampling.

In the final study, seawater samples from eight salt marsh or estuarine sites along the Georgia coast were collected and examined for the distribution of BTEX-degrading bacterioplankton using our newly developed *in situ* PCR/H techniques. We chose a range of sampling sites representing conditions of little-to-no pollution by petroleum hydrocarbons (low-impact) as well as areas where hydrocarbon contamination was known, and was clearly evident (high-impact). BTEX dioxygenase genes were found in an average of about 1 and 5% of the cells comprising the bacterial communities in the low-and high-impact sites, respectively.

We then examined (in microcosms) the response of these communities to selective pressure from added hydrocarbon (toluene). When incubated under conditions

selectively enriching for growth of toluene degraders, only the communities from high-impact sites exhibited significant shifts, cells containing the target aromatic hydrocarbon dioxygenases eventually comprised almost 50% of the total community after 120 h. Due to the visual nature of detection, we demonstrated that were able to associate the specific degradative potential with individual cells and examine the cell-to-cell relationships in small cell aggregates which formed in the microcosm communities. Interestingly, the bacterial communities from the pristine coastal sites did not respond significantly to toluene selection (over 120 h) as measured either by total bacterial community numbers of cells capable of growing on toluene, or as percent of the community possessing BTEX dioxygenase genes (Chapter 4, Figure 10 C and D). These findings demonstrate that *in situ* PCR can be an effective, visual tool for examining complex inter-relationships (i.e. cooperative catabolism of complex substrates) of individual organisms with the community.