PAGE

Form Approved
OMB No. 0704-0188

1. AGENCY

3. REPORT TYPE AND DATES COVERED
FINAL 01 Dec 88 TO 30 Nov 91

4. TITLE AND SUBTITLE
MOLECULAR ECOLOGY OF BACTERIAL POPULATIONS IN INVIRONMENTAL HAZARDOUS CHEMICAL CONTROL

5. FUNDING NUMBERS
C F49620-89- C-0023
PE 61102F
PR 2312
TA AS

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10. SPONSORING/MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION/AVAILABILITY STATEMENT
Approved for public release; distribution unlimited.

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 words)

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14. SUBJECT TERMS

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
(U)	(U)	(U)	(U)

Molecular Ecology of Bacterial Populations in Environmental Hazardous Chemical Control

Draft Final Report
Contract #: F49620-89-C-0023

United States Air Force
Office of Scientific Research

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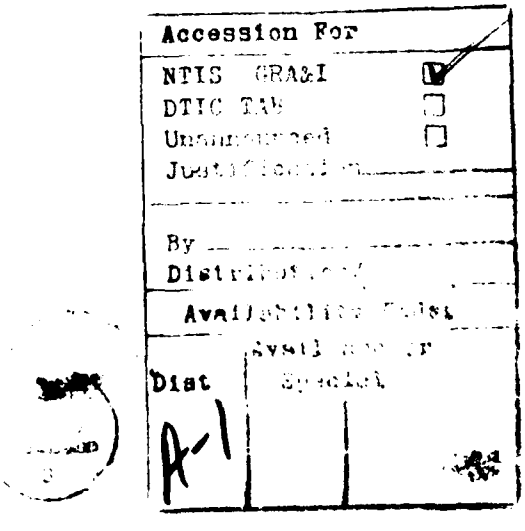

Abstract

Basic research was conducted to develop and explore the use of modern molecular biology techniques in understanding the dynamics of microbial populations engaged in biodegradation of environmental pollutants. The research focused on 1) the use of environmental DNA extraction and gene probing techniques to quantify the presence and distribution of degradative genes in the environment, 2) characterizing new degradative organisms and plasmids for eventual development of new catabolic gene probes for environmental use, and 3) construction of novel bioluminescent reporter bacteria to act as biosensors of catabolic activity in the environment.

The development of these methods contributes to the hypothesis that new quantitative molecular information is needed to evaluate the capacity of microbial communities to promote biodegradation and to develop specific environmental control and process analysis strategies for optimizing mixed culture biodegradation of hazardous waste.

The results of this investigation demonstrate the capacity for direct quantitative analysis of bacterial gene frequencies in the environment to describe the structure of biodegradative microbial communities. Bacterial plasmids from the environment have also been circumscribed. These plasmids have been shown to promote chlorobiphenyl and chrolobenzoate degradation in the environment. Initial characterization of the naphthalene pathway genetics in the chlorobenzene degrading Air Force strains, JS1, JS150, and JS6 has demonstrated a unique gentisate branch of naphthalene oxidation. Finally novel naphthalene (*nah*) - bioluminescent (*lux*) gene fusions were obtained that resulted in the development of bioluminescent reporter bacteria. These bacteria have been shown to act as light producing sensors to detect the presence and bioavailability of chemical pollutants in the environment. The organisms have been shown to be dynamic monitors of degradative activity in environmental or treatment reactor simulations.

Collectively, these results demonstrate the potential to develop further system analytical methods for biodegradative process optimization in complex environments and hazardous waste treatment systems.

Introduction

The world wide magnitude of hazardous waste pollution and environmental contamination problems is well recognized. A report by the Congressional Office of Technology Assessment (OTA) estimates that greater than 10,000 CERCLA/Superfunds alone may be identified by the year 2000 (19). In the U.S. alone, cost estimates for clean-up of environmental contamination and reduction of pollution loads range to $1.5 Trillion (25). These environmental health/protection costs are further inflated by regional disasters, such as the Exxon Valdez oil spill, deliberate release of petroleum to the Persian Gulf, and wide-spread discharge of carbonized petroleum from well-head fires of the Kuwait oil fields. These problems are further confounded by localized spills and discharges of solvents, gasoline, diesel, jet fuels, etc. to surface and subsurface soils, and groundwater. The contamination of subsurface soils and groundwater with toxic and recalcitrant organic and chlorinated organic compounds poses serious health threats. For example, polychlorinated biphenyls (PCBs), major environmental contaminants, are toxic, mutagenic and teratogenic with proven bioaccumulation and bioconcentration abilities (13). Removal of organic and chlorinated organic compounds from contaminated sites can be difficult and expensive especially if they are present at low concentrations or if they are sorbed onto the groundwater aquifer matrix.

Recently, the application of microbial biodegradation processes to "bioremediation" of chemically contaminated environments and waste mixtures has received considerably heightened attention as a possible cost-effective treatment technology. Bioremediation offers an attractive alternative for environmental cleanup because many strains of bacteria and microbial populations have biodegradative capabilities for one or more types of organic contaminants. Two approaches

can be used for *in situ* or in reactor bioremediation of environmental contamination: 1) the stimulation of degradative activity of indigenous bacterial populations, and 2) the introduction of microorganisms with known biodegradative potential. The first approach assumes that the microbial community contains the necessary genetic information for the biodegradative pathways. The second approach assumes that microorganism carrying the necessary genetic information can be added and maintained in the contaminated environment. Therefore, the success of a bioremediation effort ultimately depends on the genetic capacity for biodegradation. However, the gene abundance activity (hence biodegradation) is strongly influenced by environmental and ecological factors. Technical uncertainties are brought about by our limited understanding of 1) the distributions and dynamics of microbial populations capable of pollutant degradation in the environment, 2) environmental and ecological factors affecting microbial activity and biodegradation, 3) the interplay of physical-chemical processes such as sorption-desorption in biodegradation kinetics, and 4) the microbial capacity for sequential or simultaneous degradation of mixtures of pollutants in contaminated environments. These technical uncertainties at the microbiological level lead to suboptimal predictability and performance during engineering implementation and an increased regulatory requirement for bioprocess "credibility."

With these technical uncertainties and their ramifications in mind, this original investigation, USAF Contract #F49620-89-C-0023, was planned to incorporate the development of modern molecular methods into a paradigm for the analysis of biodegradative microbial community structure and activity. The broad goal for the research is to develop practical applications of available biotechnology, through ecological systems analysis, for optimization of natural or genetically engineered microbial biodegradation processes in the environmental

destruction of hazardous chemicals.

Although there are numerous reports of biodegradative organisms and biodegradative activity the rate, extent, and scope of biodegradation remains unpredictable. In order to predict these aspects of biodegradation one needs to determine the environmental, ecological, and genetic parameters that contribute to the long-term maintenance of biodegradative bacteria and their genes. Catabolic (biodegradative) genes in bacteria may either be plasmid encoded or chromosomally-encoded. Some catabolic genes, such as those encoding naphthalene degradation, tend to be located on plasmids whereas other catabolic genes, such as those encoding biphenyl degradation are often located on the chromosome. The location of the catabolic genes, i.e. plasmid versus chromosomal, has important implications for the manipulation of these genes in the laboratory and the maintenance and stability of these genes in the environment (28).

The study of biodegradative organisms in environmental matrices and the development of models to predict biodegradative capabilities requires methods that can determine the genetic potential of an environmental sample and also provide a direct measure of gene activity. With appropriate gene probes the structure and the function of biodegradative microbial communities can be determined.

As a measure of catabolic activity this research has developed bioluminescent reporter strains for *in situ* application in biodegradation process analysis and control (2,14). As a biotechnical approach for remediating waste contamination problem, a variety of technical, regulatory and societal limitations have been identified that impede progress toward a more universal application of the technology. Bioluminescence is a natural phenomenon associated with the microorganisms *Photobacterium* and *Vibrio*. The expression and regulation of the

bacterial luciferase (*lux*) genes has been studied (15) and the lux structural genes have been cloned and introduced into a variety of hosts (16). These new bioluminescent reporter strains can act as biosensors to measure the exposure of bacteria to chemicals and the induction of activity of degradative genes. The advantage of these bioluminescent assays compared to conventional activity assays are that they are non-invasive, non-destructive, rapid and population specific.

Goals and Objectives

The overall goal of the proposed research is to develop practical applications of available biotechnology, through ecological systems analysis, for optimization of natural or genetically engineered microbial biodegradation processes in the environmental destruction of hazardous chemicals. The specific objectives of the proposed research are outlined as follows:

1. Development and application of molecular microbial ecology techniques for measurement of catabolic gene expressions and regulation.

   a. Demonstrate use of bioluminescence gene cassettes for measurement of gene expression, as a surrogate reporter for catabolic genes.

   b. Application for catabolic DNA probe technology for detection as a measure of catabolic gene frequency.

2. Correlate the relationship between quantitative measures of biodegradation microbial community structure and function in relationship to degradation of hazardous chemicals.

3. Develop a ecological system analysis approach for determining control parameters that can be used to optimize in situ or in reactor microbial degradation process.

Experimental Methods

Chemicals and Reagents

Standard chemicals and reagents were purchased from Sigma Chemical Co., St. Louis, Missouri or from Baxter Chemical Co. Naphthalene (scintillation grade, 99% pure) was obtained from Aldrich Chemical Co., Milwaukee, WI. 4-chlorobiphenyl was purchased from Analab, Inc. and 4-chlorobenzoate was obtained from Pfaltz and Bauer, Inc. Most restriction endonucleases, T4 DNA Ligase and DNA molecular size markers were obtained from Bethesda Research Labs, Gaithersburg, MD.

[1-^{14}C] naphthalene with a specific activity of 8.0 mCi/mmol and a purity in excess of 98% was obtained from Sigma. [^{32}P] dCTP was obtained from New England Nuclear Corp., Boston MA.

Other specialty chemicals will be noted at appropriate places under material and methods.

Bacterial Strains

Environmental strains isolated from natural populations in contaminated substrates and genetically constructed strains were used in these studies. A list of relevant environmental strains and their phenotypes are presented in Table 1.

Table 1. Relevant environmental strains used in this study.

Strain	Catabolic Phenotype	Plasmid	Source
P. putida PB2440		none	M. Bagdasarian
P. putida PpG7	Nah	Nah7	I. Gunsalus
5R	Nah	pKA1	H. King
HK9	$Nah^{-}Sal^{+}$	multiple	H. King
A5	4-CB	pSS50	M. Shields
IC1	4-CB, 4-CBA	pSS60	C. Pettigrew
ALP83	4-CB, 4-CBA	pSS70	M. Shields

Abbreviations: Nah = naphthalene degrader, Sal = salicylate degrader
4CB = metabolism of 4-chlorobiphenyl to 4-chlorobenzoate
4CBA = metabolism of 4-chlorobenzoate to 4-hydroxybenzoate

Initially all environmental strains were grown in YEPG medium consisting of (in gL^{-1}) yeast extract, 0.2; polypeptone, 2.0; NH_4NO_3 0.2; and D-glucose, 1.0; at 28°C. Better selection for naphthalene degrading strains was obtained by growing these strains in Yeast extract-peptone-salicylate-succinate (YEPSS) medium consisting of (in gL^{-1}) yeast extract,0.2; polypeptone,2.0, NH_4NO_3 0.2; sodium salicylate 0.5; and sodium succinate, 2.7. For some *Pseudomonas* strains, faster growth was obtained in a L broth consisting of *5g* yeast extract, 10g tryptone, and *5g* NaCl. In all recipes agar plates were made by adding 1.5% bacto-agar.

Two different minimal salts media were used. Basal salts medium (pH 7.2) contains in gL^{-1}: KH_2PO_4,1.5; Na_2HPO_4, 0.5; $MgSO_4.7H_20$, 0.2; $NaNO_3$, 0.4, $FeCl_2$, 0.005; and CaC_{12}, 0.01. HK minimal salts consisted of in $gL^{-1}KH_2PO_4$, 0.68; K_2HPO_4, 1.73; $MgSO_4$. $7H_20$, 0.1; NH_4NO_3,1.0; and 0.1 ml mineral salts supplement (18).

Spontaneous rifampicin mutants of several environmental isolates were selected by spread plating cells on YEPG agar amended with 50 μg/ml rifampicin.

A list of *E. coli* strains, plasmids and genetically constructed strains are presented in Table 2. *E. coli* strains were typically grown in Luria-Bertani (LB) medium at 37°C (15). Plasmids were maintained by appropriate antibiotic selection.

Table 2. *E. coli* strains and genetically constructed strains used in these studies.

Strain (plasmid)	**Phenotype**	**Source**
E. coli DH5α		Bethesda Research Lab
E. coli (pUC18)	Cloning vector	Bethesda Research Lab
HB101 (pUCD623)	Lux transposon Tn4331	C. Kado
HB101 (pUCD615)	Lux promoter probe vector	C. Kado
E. coli (pE317)	Nah A clone	B. Ensley
HK53	PB2440 (Nah7)	H. King
DH5 (pUTK9)	Pu-lux	R. Burlage
RB1351	HK53 (pUTK9)	R. Burlage
5RL (pUTK21)	PKA1::Tn4331	H. King
HK44	HK9 (pUTK21)	H. King
DH5(pUTK2)	pSS50::Tn4331	R. Burlage
DH5(B-3)	PUC18: 5kb BamH1 fragment from pSS60	A. Layton

Molecular Techniques

Transposon mutagenesis

The lux transposon Tn4431 (31) was transferred to several environmental strains by filter matings with *E. coli* HB101 carrying the lux transposon on the plasmid pUCD623. For filter matings environmental isolates were grown overnight and *E. coli* cells were grown to log phase (OD 600 of 0.6 to 0.8). One ml aliquots of the donor and recipient cells were pelleted in an eppendorf microfuge (14,000 rpm) and the pellets were resuspended 1 ml 10 mM NaCl. One

ml mixtures of 1:10 or 1:5 donor to recipient cells were collected on sterile 0.45um filters with a vacuum. The filters were transferred to YEPG plates and incubated overnight at 28°C. Filters were resuspended in 10 ml YEPG and the cells were plated on YEPG agar plates containing an antibiotic for the selection of the recipient cells and tetracycline for the selection of the transposon.

Plasmid DNA Isolation and Molecular Analysis

Small-scale plasmid DNA minipreps were used to rapidly screen large numbers of bacterial strains for the presence of plasmids, whereas large scale plasmid DNA preps were used for molecular analysis of plasmids from individual cultures. The method of Anderson and McKay (1) was generally used for plasmid DNA minipreps from environmental isolates. A modified method by Promega was also used (23). Its through 0.6 to 0.7% agarose gels in 1X TBE buffer (16). For the examination of recombinant plasmids from *E. coli* cells plasmid DNA minipreps were done either by the boiling lysis method (11) or by the alkaline lysis method (16).

Large scale DNA plasmid preps from environmental isolates were performed by alkaline lysis following the method of Anderson and McKay (1) or Promega (23). Naphthalene type plasmids were further purified by a dye-buoyant density ultracentrifugation in cesium chloride and ethidium bromide (16). Smaller plasmids such as pSS50 were further purified by centrifugation through pZ523 spin columns (5',3',Inc., Paoli, PA). Large-scale DNA plasmid extractions were performed by a standard alkaline lysis procedure (16) or the Promega procedure (23). Plasmid purification was accomplished either by cesium chloride-ethidium bromide density gradient centrifugation or by pZ523 spin columns.

Molecular analysis of plasmid DNA's was accomplished by restriction endonuclease

digestion and separation of DNA fragments through agarose gels by electrophoresis following standard protocols (16). Restriction enzyme maps of plasmids were generated by analysis of restriction enzyme digest, digestion of subclones, and hybridization analysis.

Small plasmids were transformed into *E. coli* cells by the method of Hanahan (10) or into *E. coli* cells already made competent purchased from Bethesda Research Laboratories (Gaithersburg, MD). In some experiments, plasmid DNA was introduced into *P. putida* strains by electroporation. Electroporation was done with a BTX transfector 100 with a 400 microFarad capacitor coupled with an Optimizer (BTX, San Diego, CA). Approximately 10^8 cells in 10% glycerol with 10ng-1μg DNA were layered between electrodes. A field strength of 14 KV/cm and a pulse length of 5 msec with an exponential decay were used for each pulse. After electroporation the cells were resuspended in LB broth plus 20 mM glucose and incubated for 1 hour at 25°C. Selection for transformants was made on LB agar with appropriate antibiotics.

Direct Extraction of DNA from Sediments

DNA from soils and sediments was isolated by a modification of the direct extraction method from Ogram (20,21). 100 g samples of soils or sediments were resuspended in 125 ml of 0.12 M Na_2HPO_4, pH 8.0 and 25 ml 5% SDS and incubated for 1 hour at 70°C. 50 g of 0.1 mm bead-beats were added to the sample in a bead-beater chamber and the sample was beat for 5 minutes on ice. The samples were centrifuged in a JA14 Beckman rotor at 6,000 rpm at 10°C for 25 minutes. The supernatant was saved. The pellet was extracted 2 more times with 150 ml of 0.12 M NaP, pH 8.0 to recover the majority of the DNA. All three supernatants were combined and precipitated overnight at 4°C with 5M NaCl to yield a final concentration of 0.5M NaCl and 0.5 vol of 50% PEG. The samples were centrifuged at 6000 rpm at 4°C for

10 minutes. The pellets were resuspended in 40 ml of 10 mM Tris-HCl, 5 mM EDTA, pH 8.0. This solution was extracted 4 to 5 times with an equal volume of phenol saturated with Tris pH 8.0 and 1 time with an equal volume of chloroform/isoamyl alcohol (24:1). The aqueous phase was precipitated with 0.1 volume of 2 M sodium acetate and 2 volumes of 100% ethanol overnight at -20°C. The precipitates were centrifuged at 10,000 rpm at 4°C for 30 minutes. The pellets were dried and resuspended in 7 ml 0.12 M Na_2HPO_4, pH 6.8 in a warm waterbath (42°C-65°C). The sample was precipitated with 1 ml of 4 M potassium acetate and incubated on ice for 2 hours. The samples were centrifuged at 6,000 rpm at 4°C for 30 min and the pellets were resuspended in 10 mM Tris-HCl, 5 mM EDTA, pH 8.0. The DNA recovered at this point could be used for slot blot of southern blot analysis.

Nucleic Acid Hybridizations

Both radioactive and nonradioactive DNA probes were used in these studies. [^{32}P] DNA probe was prepared with a nick-translation kit (Bethesda Research Laboratory, Gaithersburg, MD) using [^{32}P] dCTP as the sole labeling nucleotide and following the manufacturers protocol. The labelled probe was purified by using a Sephadex G-50 spun-column procedure (16) and the activity was quantified by liquid scintillation counting. Specific activity of the labelled DNA probe was approximately 10^7-10^8 dpm/μg of DNA. Conditions for prehybridization, hybridization, washing and autoradiography have been previously described (27). A low-salts (10mM NaCl) was used during the washing procedure to impose a 95% stringency on the hybridization of homologous DNA sequences.

Nonradioactive DNA probes were made by random primed incorporation of digoxigenin labelled dUTP (Boehinger Manaheim, Indianapolis, IN). The conditions used for

prehybridization and hybridization were those described by the manufacturer. Target: probe hybrids were detected by alkaline phosphatase linked antibodies made to digoxigenein following the manufacture's procedure.

The methods for DNA sample preparation were the same for either radioactive or nonradioactive detection methods. For DNA:DNA colony hybridization, colonies were transferred to Biotrans nylon filters (ICN, Irvine, CA) by overlaying the filters on the agar surface and lifting-off the colonies. The DNA was denatured on the filter by alkaline lysis following the manufacturers procedure. For southern hybridizations, DNA was separated through agarose by gel electrophoresis. DNA fragments were denatured and transferred to Biotrans membrane according to the manufacture's protocol.

Analytical Techniques

Mineralization Assays

Naphthalene mineralization assays were performed by placing each sample in 5 ml of minimal salts medium in a 25 ml glass EPA vial with a teflon-lined silicone septum cap (Pierce, Rockford, IL). A 7.5 ml glass vial containing 0.5 ml of 0.4 M NaOH to trap CO_2 was added to each EPA vial. Each sample was amended with cold naphthalene (100 mgL^{-1} final concentration) and 100,000 dpm of ^{14}C-naphthalene dissolved in 10 μl of acetone. Duplicate vials were prepared for each sample and the vials were shaken at 100 rpm in the dark at 20°C. The assay was terminated by the injection of 0.5 ml of 2N H_2SO_4 into the outer sample vial. The vials were allowed to sit for 30 minutes before the NaOH solution from the inner vial was removed and added to 10 ml of Beckman Ready-Protein scintillation cocktail (aqueous samples). $^{14}CO_2$ was quantified using liquid scintillation counting (LSC) on a Beckman LS3801

scintillation counter. The H# method was used for automatic quench compensation and dpm conversion of cpm based on standard quench curves. The sample was then extracted twice with 5 ml of 4:1 hexane:isopropanol. ^{14}C- naphthalene remaining in the vial was determined by the addition of 0.5 ml aliquots of the hexane phase extracts to Econofluor (DuPont, NEN Products, Boston, MA) followed by LSC. ^{14}C-labeled polar metabolites were estimated by performing a third extraction with 5 ml of distilled water followed by LSC.

Chloride Measurement

Chloride release was measured from cells incubated in chloride free minimal salts media plus 100 to 200 ppm 4-chlorobenzoate with an Orion expandable ion analyzer (model EA-920) equipped with an Orion chloride-selective electrode (model 94718) and a double junction reference electrode. Chloride concentrations were determined by comparison to chloride standards made with NaCl (22). Samples were incubated in 25 ml pierce vials with teflon seals containing 5 ml of media. Each vial was inoculated with 10^7 to 10^8 cells from an overnight YEPG culture, washed once with minimal salts. At least 3 sample vials and 2 killed control vials were sampled at each time point. Cells were killed at each time point by immersion into boiling H_2O for 45 seconds. The chloride concentration was measured for each vial at the end of the experiment. The initial cell concentration for each sample was determined by dilution spread plating on YEPG agar plates.

Substrate Monitoring by HPLC

Several different HPLC protocols were used to monitor different substrates and metabolites.

1. For mixed inoculum in continuous culture. Three ml of sample was extracted

with a equal volume of Hexane-Isopropanol (4:1). The organic phase was analyzed by a Perkin-Elmer-Series 410 HPLC with a C18 column using UV detection. Separation was accomplished with a mobile phase of methanol: water (85:15) at 1.5 ml/min.

2. Detection of naphthalene and metabolites. C^{14} labelled aqueous metabolites from naphthalene mineralization assays were analyzed by high-pressure liquid chromatography in conjunction with proportional counting. Metabolites in a 200-ul sample volume were separated on a Supelcosil LC-18 column (flow rate,2 ml min^{-1}) by elution for 5 minutes with water (pH 2.5) followed by a linear gradient to 60% acetonitrile: 40% water (5% min^{-1}). Detection was by a photodiode array detector at a wavelength of 255 nm. Eluant from the photodiode array detector was mixed on-line with scintillation cocktail (6 ml min^{-1}) and passed through a Flo-One Beta Radioactive Flow Detector Model IC/CR.

3. Detection of 4-chlorobenzoate and 4-hydroxybenzoate. Aqueous metabolites in a 50 μl sample volume from chloride release assay were separated on a Supelcosil LC-18 column (flow rate, 2ml min^{-1} by elution on a linear gradient rom 100% water (pH 2.5) to 60% acetonitrile: 40% water and held for 4 minutes. Detection was by a photodiode array detector at a wavelength of 255 nm. 4 - chlorobenzoate had an elution time of 13.5 minutes and 4-hydroxybenzoate had an elution time of 8.5 minutes. The concentration of each metabolite was calculated by comparison of the peak areas with known standards.

Bioluminescent Pollutant Bioavailability Assays

An overnight culture grown in YEPG medium was prepared from a frozen stock of P. fluorescens HK44. Of this culture, 5 ml were transferred to 100 ml of fresh medium and the optical density at 546 nm was monitored at 30 min intervals. At a predefined OD 546 of 0.35, aliquots of the exponentially growing culture were transferred to the test vials. For the starvation experiment 30 ml of the exponentially growing culture was harvested at the same optical density as described above and centrifuged at 7741 x g for 10 min at 27°C. The pellet was resuspended in 30 ml mineral salts medium and left at 27°C on a shaker for 3 hours. Two ml of either exponentially growing or starved culture were added to 2 ml of test solution in sterile 25 ml mineralization vials (pierce, Rockford, Illinois, USA) with teflon lids. Salicylate solutions were prepared in buffered mineral salts medium. The light output of the culture was then measured in 15 min intervals. The light readouts were obtained as an amperometric signal and are presented in nano ampere. The experiments were conducted in two independent sets using triplicate samples for each set. Data are presented as mean values with standard deviations. For the standard curves all values have been corrected for the light output of the control without inducing substrate. The corresponding standard deviations were calculated taking the error progation into account.

Light was measured with an Oriel (Stratford, CT) digital display model 7070 with a photomultiplier tube (model 77340) connected to a flexible liquid-light pipe and collimating beam probe. Light detection is based on a photoelectric effect and is measured as current. Detection by this system is sensitive to 10-12 amps of current. The photomultiplier was connected to an IBM PS/2 computer through a RS232 port. This allowed continuous sampling of the photomultiplier output over long periods of time. The software was written by Mr. Rod Bunn,

University of Tennessee. For experiments the photomultiplier was placed inside a growth chamber in constant dark at 25°C.

HPLC procedures for bioluminescent bioavailability assay: Samples were filtered through a 0.2 um teflon membrane filters prior to HPLC analysis. The HPLC system consisted of a model 5560 liquid chromatograph (Varian, Palo Alto, CA), a 25 cm VYDAC 201TP5 column (Separations Group, Hesparia, CA), a LS-4 fluorescence spectrophotometer (Perkin Elmer, Norwalk, CN). The fluorometer was equipped with a 3 μl flow sample cell. The gradient conditions used involved a continuous gradient form 0 to 50% aqueous acetonitrile between minute 1 and 2 and a second continuous gradient from 50 to 100% acetonitrile between 10 and 19 mins. At the end the program, the column was equilibrated for 3.5 min with water. A flow rate of 1.5 ml/min was used with sample injection occurring at 1 minute in the gradient program. An excitation wavelength of 290 nm and an emission wavelength of 360 nm were used to monitor the HPLC effluent for the first 5 minutes of the chromatogram for analysis of salicylate.

Soil experiments: For the soil experiments a loamy sand with a high organic content of 1.15% was used. The water content of the soil was 7.5% +/- 0.3%.

Preparation of soil extract: Before adding contaminants to the soils, 10 g portions of soil were autoclaved at 121°C for 20 min in 25 ml Corex glass centrifuge tubes (Corning Inc., Corning, N.Y., USA) with teflon sealed screw caps. The contaminant, 0.069 mg salicylate in 125 μl mineral salts medium were added to the soil and allowed to incubate for 1 hour. Following the 1 hour incubation 9.875 ml of buffered mineral salts medium was added. The resulting slurries were incubated at 27°C with mild shaking for 1 hour. The soil extracts were centrifuged at 7741

X g for 10 min at 25°C. Two ml aliquots form the clear supernatant were transferred to 25 ml mineralization vials with teflon sealed screw caps. The soil controls were processed exactly as described above but without the addition of the contaminants.

Preparations of soil slurries: Two grams of soil in 25 ml mineralization vials were autoclaved at 121°C for 20 min. The contaminant, salicylate, 0.0138 mg in 25 μl mineral salts medium was added to the soil. To the salicylate contaminated soil 1.975 ml of buffered mineral salts medium was added. The slurries were incubated, with mild shaking at 27°C for 1 hour. Control slurries were prepared identically without salicylate and naphthalene. Light measurements were conducted in 15 min intervals. The final concentration for salicylate was 3.45 mg/l this concentration represent the theoretical maximum aqueous concentrations of target compounds for the soil extract and soil slurry experiments. Light measurements were conducted in 15 min intervals for each the mineral salts medium, soil extract and soil slurry. After 1 hour the optical densities of the mineral salts and soil extract were compared after correction of the soil extract at 546 nm.

Reactor Conditions

Continuous Culture

Two different chemostats were used in these studies. One was a New Brunswick Scientific BioFLO Model C30 with a teflon headplate and teflon tubing. Media was supplied using a FMI peristaltic pump with Teflon Head. The reactor liquid volume consisted of 350 ml of buffered minimal salts with saturating concentrations of test compounds. The medium was agitated at 100 rpm at 25°C and sterilely aerated at 0.1L/min^{-1}.

A second chemostat system was used in combination with a bioluminescent detection system (Fig 1.).

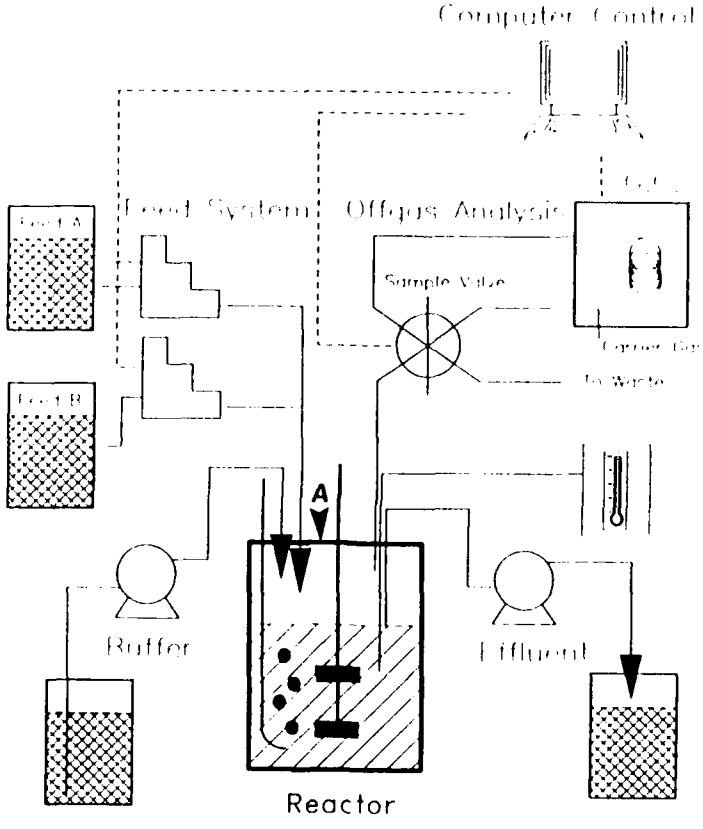

Figure 1. A schematic drawing of the bioreactor system for on-line studies of naphthalene degradation and light production by bioluminescent reporter strains. The port for liquid light pipe detection of bioluminescence is shown in A.

This system contained a photomultiplier as described in the bioluminescent monitoring section. The reactor system consisted of a L. H. Fermentation Series 500 continuous flow bioreactor with a 1 L glass reaction vessel, direct drive agitator, dissolved oxygen measurement, temperature control and air flow control modules. A dual feed system provided the opportunity to change from a naphthalene feed stock to another nonnaphthalene feed stock. Feed consisted of minimal salts medium containing sodium succinate (100 mg L^{-1}), yeast extract (100 mg L^{-1}), and tetracycline (14 mg L^{-1}). Naphthalene was added to one of the two feed supplies at 30 mgL^{-1}. Naphthalene was measured by off-gas analysis. Reactor offgas was routed to a computer-controlled Valco sampling valve with a 7.7 mL sample loop. The lines, valve, and sample loop were maintained at 140°C by means of heating tape and a solid state temperature control (Omega). The offgas sampling frequency, feed flow rate, and feed concentration were all controlled by an IBM PC personal computer. This chemostat was operated at 25°C, a dilution rate of 0.4 $hour^{-1}$ and a mean cell residence time of 2.5 hours.

Packed Bed Differential Volume Reactor

The packed bed reactor contains a soil wafer or differential volume of soil. The soil wafer or differential volume of soil is a thin layer of soil (0.8 com to 5.0 cm deep). Bacteria are maintained on the soil wafer. An HPLC pump is used to deliver liquid through the soil wafer. This reactor design eliminates the need for long soil columns with high residence times. A thin layer of soil has a short residence time for liquid flow which allows rapid chemical analysis feasible. The biodegradation of several PAHs (i.e. naphthalene, phenanthrene, acenaphathene, alkylated naphthalenes, and benzenes) in a 1.5 cm deep soil wafer has been demonstrated in this reactor.

This system will use the *Pseudomonas fluorescens* bioluminescence reporting strain, HK44. Strain HK44 contains a *nah-lux* gene fusion encoding for simultaneous naphthalene degradation and bioluminescence. Fiber optic bundles are inserted through ports in the rector walls to measure bioluminescence. A HPLC/fluorescence detection system measures the effluent concentrations.

Results

Direct Extraction of Nucleic Acids from Sediments

Previous results have demonstrated that nucleic acid can be extracted directly from soil and sediment samples without the need to culture the microorganisms from these samples. This DNA is suitable target DNA for nucleic acid hybridizations (20,21). Recent work has focused on using direct DNA extraction from samples with nucleic acid hybridizations to quantitate the abundance of catabolic genes for aromatic hydrocarbons to past or existing aromatic hydrocarbon contamination in environmental samples. A high yield of 100μg/100g of high quality DNA can be extracted with the methods used in this lab (Figure 2). This DNA approaches 20 kb in size and can be digested with restriction endonucleases. In previous studies environmental DNA was difficult to digest (20). This DNA was also an appropriate target for nucleic acid hybridization to detect community structural or functional genes. The gene probes used for hybridization were the nitrogen fixation genes from *Klebsiella* (*nif* K,D H) (4), photosystem reaction center from Rhodobacter (*puf*) (40), 23S rRNA from fluorescent pseudomonads (9), and naphthalene catabolism (*nah*A, courtesy of B. Ensley) and the plasmid pPG7 from *P. putida*. The sensitivity of detection for some of these gene probes was in the pg range (Figure 3). DNA extracted from several PAH contaminated soils was probed with the *nah*A gene from NAH7 (7). From almost

all of these soils the naphthalene genotype could be detected. Current experiments are directed at quantitating the number of genes present in these samples.

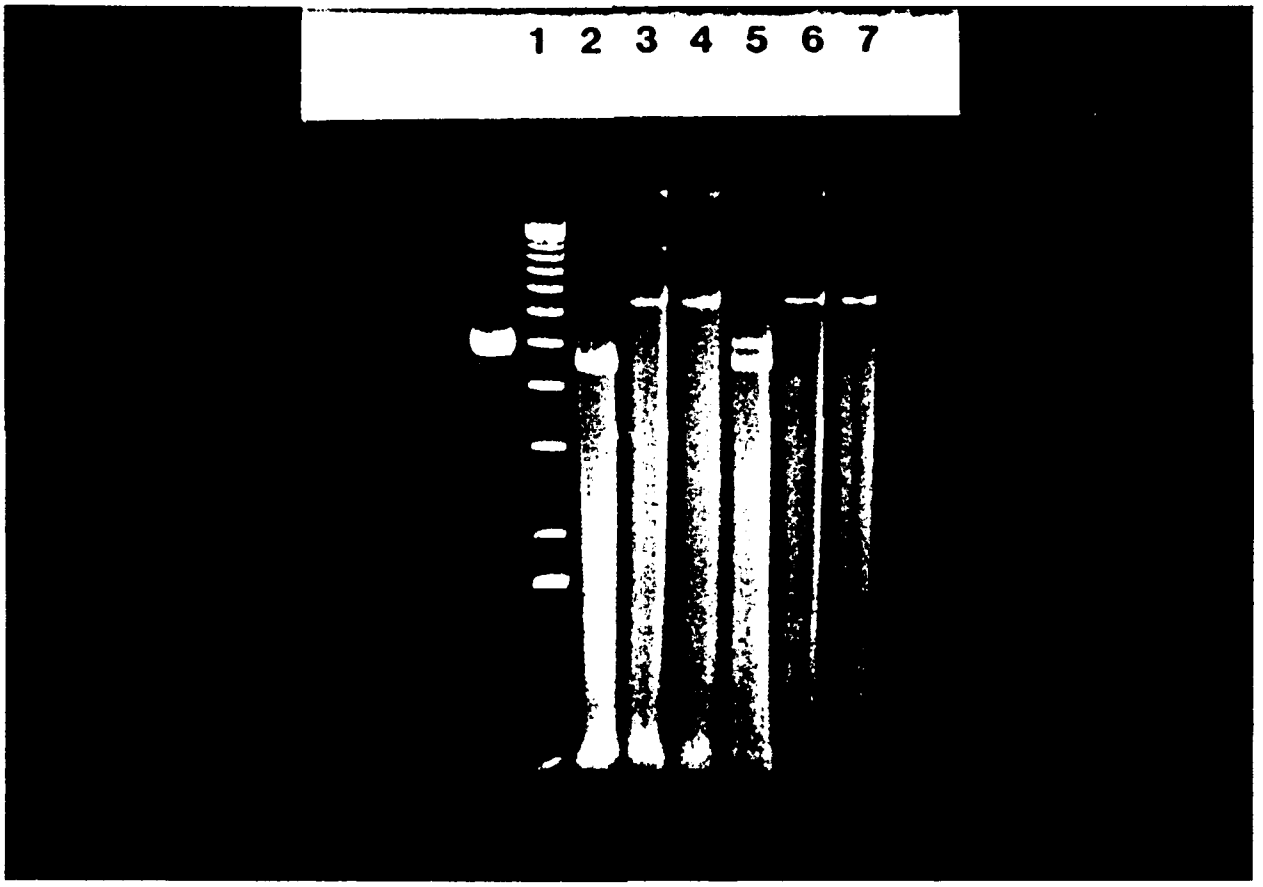

Figure 2. Comparative recovery and restriction digestion of DNA extracted from freshwater sediments. 2μg of an indicator plasmid, pUC8-ISP was added to 15μg of sediment DNA for each digestion. Lanes: 1. 1kb ladder; 2. Bam HI; 3. Sau3 AI; 4. not digested; 5. Bam HI; 6. Sau3 AI; 7. not digested.

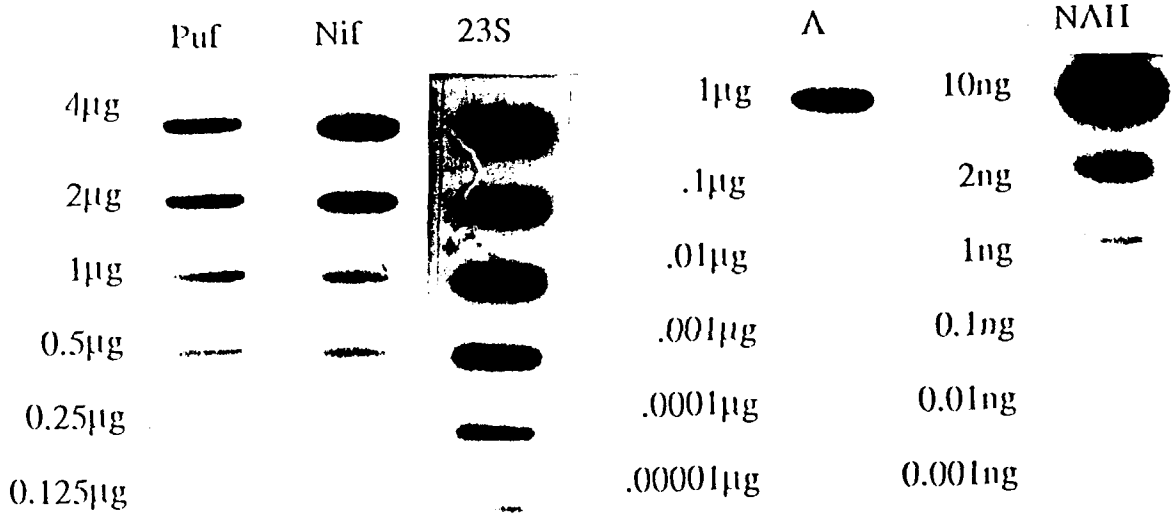

Figure 3. The use of DNA extracted from freshwater sediments as target DNA in slot blot hybridization with population and function specific gene probes.

Characterization of Plasmids from 4-chlorobiphenyl Degrading Bacteria

pSS50 belongs to incompatibility group P: The original *Alcaligenes* strain A5 was able to mineralize 4-chlorobiphenyl (4-CB) and contained a 75 kb plasmid (32). The plasmid has undergone a deletion(s) so that it is now 51 kb. The strain A5 no longer mineralizes 4-CB, but it still has the ability to metabolize 4-CB to 4-chlorobenzoate (4-CBA). The 51 kb plasmid is apparently cryptic, and has been mapped with restriction enzymes (12). pSS50 was mutagenized with Tn4331 (lux transposon) so that pSS50 would contain a selectable tetracycline resistance gene. One transposon mutant, pUTK2 was used to determine the incompatibility group of pSS50 because no deleterious results could be detected from the mutagenesis. The incompatibility group of pSS50 was determined using pUTK2 in the mini-gal plasmid incompatibility test described by Davey etal (6). pUTK2 was tested for compatibility with several E.coli incompatibility groups including: B, FIα, L/M, FII, I1, I2, N, P, Q, T, W, X, 9, F1β, K, U, Y, Z, FV, and Iα. The only tester plasmid that pUTK2 was incompatible with was the Inc P plasmid. Southern Blot hybridization of pSS50 with RK2 *oriV* sequence demonstrated homology in pSS50. This signal was weaker for pSS50 than RK2 but was comparable to R751 (Figure 4). On this basis pSS50 has been assigned to the IncPβ group.

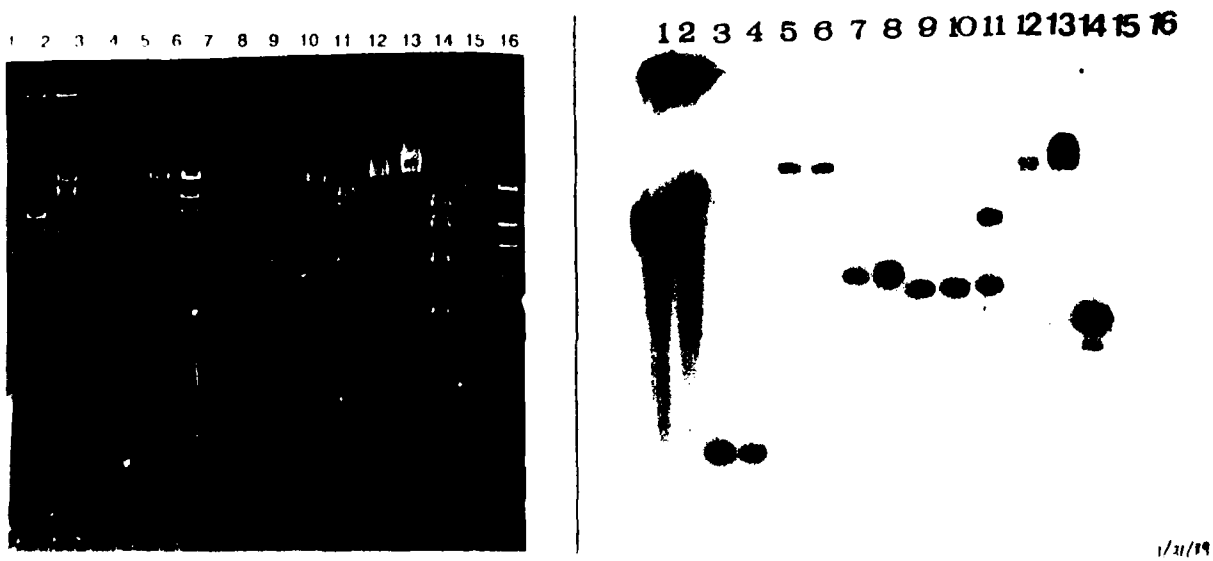

Figure 4. Hybridization analysis with probe 5 (RK2 *oriV*). (A) agarose gel and (B) autoradiograph of 5 IncP plasmids. Lanes (plasmid/restriction enzymes). 1. RK2/SphI, 2. RK2/SmaI, 3. R751/EcoRI, 4. R751/EcoRi, PstI, 5. pSS50/SmaI, 6. pSS60/SmaI, 7. pSS50/SphI, 8. pSS60/SphI, 9. pSS50/SstII, 10. pSS60/SstII, 11. pBR60/EcoRI, 12. pBR60/Bg1II, 13. pJP4/EcoRI, HinDIII, 14. pJP4/SphI, 15. NAH7/EcoRI 16. lambda phage/HinDIII.

A detailed comparison of the IncP backbone of pSS50, pSS60 (described in the next section) , R751, pJP4 (encodes 2,4D degrading genes) and BR60 (encodes 3-chlorobenzoate degrading genes) was made using gene probes from RK2 (3). A description of DNA sequences used as gene probes for this study is presented in Table 3. These probes were kindly provided by C. M. Thomas (33). The results from these probe experiments were uniform in their association of functions with specific fragments (Figure 5) and indicate that pSS50, pSS60, pJP4 and R751 are closely related. In degradative plasmids the backbone is interrupted only once between the *oriV* and *trfA* loci to accommodate a restriction-rich region of catabolic genes. Also of interest is the fact that all of these degradative plasmids have at least a remnant of a mercury resistance gene although the plasmids pSS50 and pSS60 do not confer mercury resistance.

Table 3. Source of gene probes used to locate function on several IncP plasmids.

Probe	**Plasmid/digest**	**Size**	**Relevant Markers**
1	RK2/HinDIII,SstII	4.5 kb	*pri*
2	pCAS50/SstII	14.4 kb	TRA 1, *oriT*
3	pCT16/EcoRI,SstII	2.3 kb	*kor*
4	pUB476/EcoRI	4.5 kb	*kil*
5	pCAS220/EcoRI,HinDIII	1.0 kb	*oriV*
6 & 7	pVI123.2/EcoRI,HinDIII	2.8 kb	*trfA*
8	R751/EcoRI,SstII	7.9 kb	TRA 2
9	pUB476/EcoRI	6.0 kb	*mer*
10	pUB476/EcoRI	2.2 kb	*mer*

See Table 1 for relevant host strains. kb = kilobasepair.

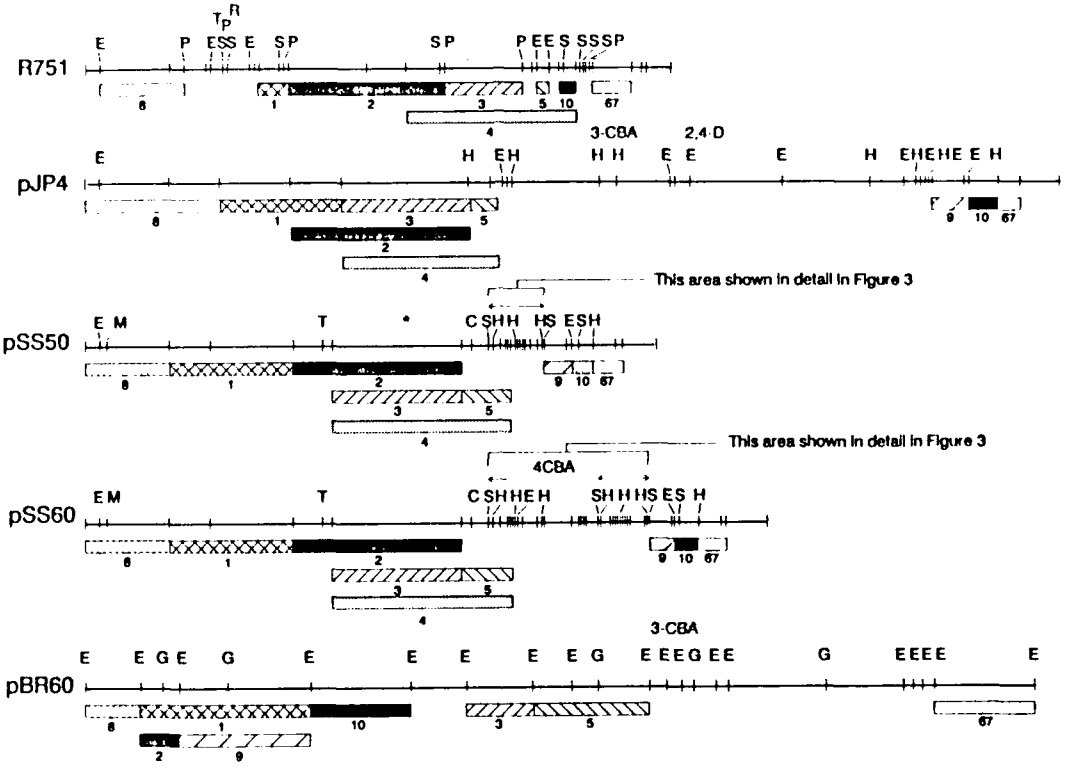

Figure 5. Restriction maps of IncP plasmids and results from hybridization with probes from RK2. All plasmids have been linearized at an SphI site except for pBR60, in which a EcoRI site was used. All restriction sites are listed except for 12 SphI sties in a 22 kb fragment of pJP4, which has been omitted. The map of R751 is from Thomas and Smith (36), pBR60 from Wyndham *et al.* (38), and pSS50 from Hooper *et al.* (12). Putative repeat sequences in pSS50 and pSS60 are denoted and oriented by arrows. Identity of probe-specific sequences are denoted by cross-hatching and appropriate probe numbers; see Table 3 for description of probes. Symbols: Tpr, trimethoprim resistance; 3-CBA, 3-chlorobenzoate degradation; 2,4-D, 2,4-dichlorophenoxyacetate degradation; 4-CBA, 4-chlorobenzoate dehalogenation; *, insertion point for Tn4431.

Restriction Maps of pSS60, and pSS70: Isolate ICI (pSS60) was obtained by its ability to clear 4-CB spray plates and to hybridize to the plasmid pSS50 from A5 (20,35). Initial digests of pSS60 with Sph I indicated that it was 10 kb larger than pSS50 (3,22). The restriction map of pSS60 is very similar to pSS50 excepting that it has a unique 7kb region which does not hybridize to pSS50 (Figure 6). The 5kb BamH1 fragment from pSS60 was cloned into pUC18 (B-3) and used as a gene probe to screen other bacteria isolated and frozen at the same time as A5. The strain ALP83 hybridized to the 5kb fragment from pSS60. This strain also had a pSS50-like plasmid designated pSS70. The backbone of pSS70 has the same restriction pattern as pSS50 and cross hybridizes with pSS50. It has a region of approximately 10 kb that is unique from pSS50 and cross hybridizes to the 7 kb region of pSS60 (Figure 6). The 6.5 kb and 7.0 kb BamHI fragments of pSS70 were subcloned into pUC18. These subclones were used to confirm the restriction map of pSS70 and determine the exact regions of homology with pSS50 and the 5kb BamHI fragment from pSS60. The 3.0 kb Bam HI -Cla I fragment from the 6.5 kb BamHI fragment also cross- hybridizes to a 4.5 kb Bgl II- Bam HI fragment in the adjacent 7.0 kb region suggesting that this region may be duplicated.

Also of interest in these restriction maps is the presence of inverted repeats found on all three plasmids. pSS50 contains two 2.2 kb inverted repeats (first noted by Hooper et al.(12)), pSS60 contains three inverted repeats, and pSS70 contains three inverted repeats plus an additional 1.5 kb region with homology to pSS50. The extra 3.0 kb region in pSS70 including both pSS50-like sequences and unique sequences may account for the apparent instability of the pSS70 plasmid (discussed in the next section). This instability could be due to: 1) a gene duplication found in the unique region of pSS60 and pSS70, or 2) a potential extension of the

2.2 kb repeat to a 3.4 kb BamHI-Cla I fragment as seen on the left hand border of pSS50, pSS60 and pSS70.

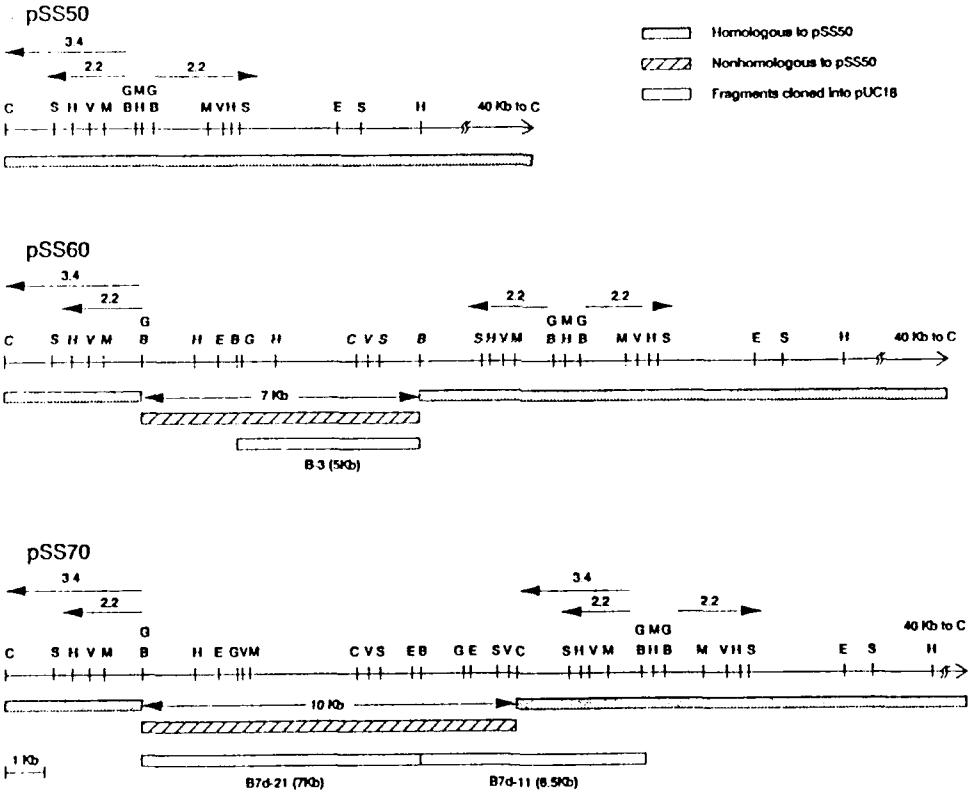

Figure 6. Restriction maps of the restriction rich region of pSS50, pSS60 and pSS70 and the unique region of pSS60 and pSS70. Abbreviations for the restriction enzymes- B= BamHI, C=ClaI, E=EcoRi, G=Bg1II, H=HindIII, M=SmaI, V=PvuII.

Isolation of Plasmid Deletions from pSS70

The unique fragment in pSS70 was easily lost from the strain ALP83. In YEPG media, the ALP83 culture typically persisted as a mixed culture with colonies of different sizes. By hybridization with the gene probes pSS50 and the 5 kb insert from pSS60 two types of colonies were detected; those with a pSS50 type plasmid with the insert and those with only the pSS50 plasmid. Restriction digests of a deletion plasmid obtained from pSS70 suggests that the deletions resulted in plasmids identical to the pSS50 plasmid. Growth of the ALP83 culture in MS plus 4-CB sifted the population to >90% with plasmid deletions. Single colonies inoculated in minimal salts with 4-CBA as a sole carbon source grew poorly so the plasmid insert could not be enriched by growing ALP83 in minimal salts with 4-CBA. A deletion mutant was not obtained from pSS60 even when grown in restrictive temperatures or in minimal salts with 4-CB.

Chloride Release from Strains Grown in 4-CBA

Chloride release from strains grown in MS with 200 ppm 4-CBA were initially assayed in 250 ml batch cultures in flasks. The strains TM1, ALP83, ICI and A5 were tested for the ability to dechlorinate 4-CBA and spent supernatants were analyzed by HPLC for intermediate production (Figure 7). ALP83 and the control strain TM1 dechlorinated 4-CBA in two days without the accumulation of any intermediates. ICI dechlorinated 4-CBA at a slower rate and accumulated the intermediate 4-hydroxybenzoate as determined by comparison with a 4-hydroxybenzoate standard and mass spectral analysis. A5 did not dechlorinate 4-CBA and did not accumulate 4-hydroxybenzoate.

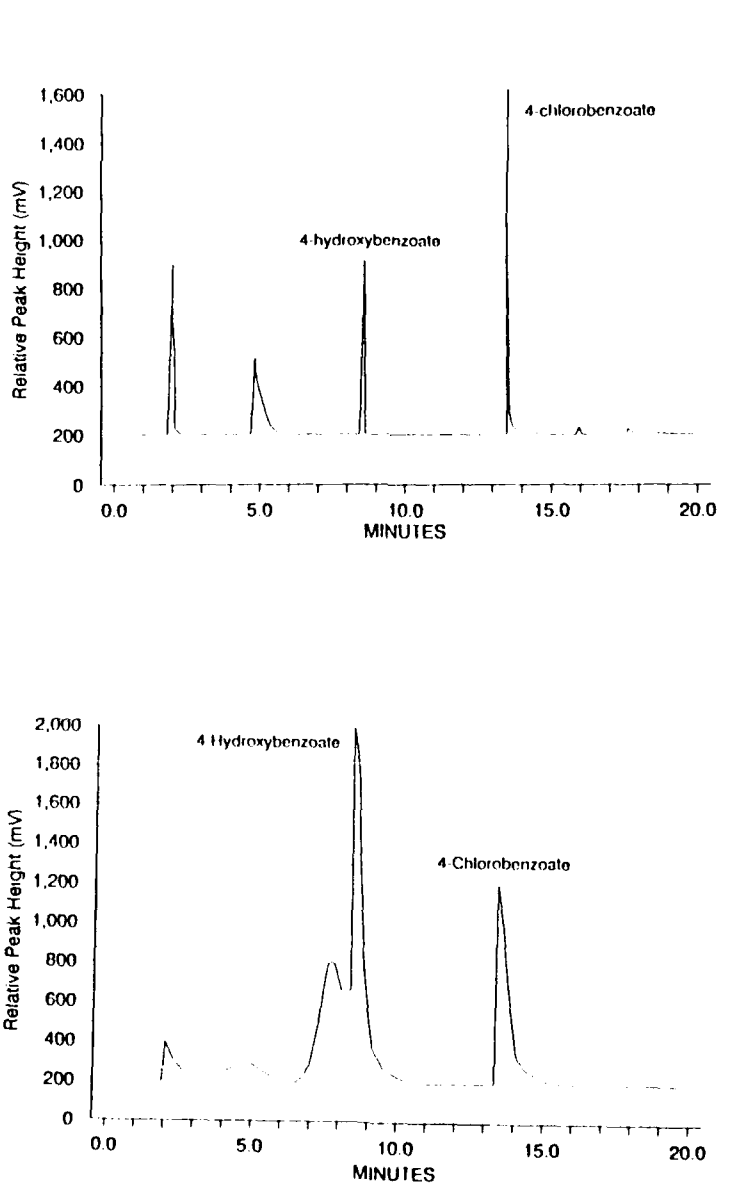

Figure 7. HPLC analysis of supernatants of strain ICI cells grown in 4-chlorobenzoate.

a. Elution of 4-hydroxybenzoate and 4-chlorobenzoate 40ppm standards at 8.5 and 13.4 minutes respectively.

b. Elution of 4-hydroxybenzoate and 4-chlorobenzaote from ICI supernatants.

Since A5 (pSS50) cannot dechlorinate 4-CBA, whereas ALP83 (pSS70) can, vial experiments were designed to determine whether dechlorination of 4-CBA was associated with this plasmid insert. In a two day dechlorination assay AL3007 containing the insert dechlorinated 4-CBA whereas the deletion mutant AL3019 and A5 (pSS50) did not (Table 4). However, in vials the ALP83 cultures accumulated the intermediate 4-hydroxybenzoate. The strain TM1 was used as a positive control for the dechlorination of 4-CBA and did not accumulate the intermediate 4-hydroxybenzoate.

Table 4. Chloride release from 4-chlorobenzoic acid and production of 4-hydroxybenzoic acid by 4-chlorobiphenyl degrading and 4-chlorobenzoate degrading strains.

Isolate	cell #/ml	% with 5kb Insert	Initial Conc. (mM)	Final Conc. (mM)		
			4-CBA	4-CBA	4-HBA	Cl^-
TM1	7×10^6	0	.65	.04	0	.7
A5	9×10^7	0	.67	.67	0	0
ALP83	2×10^7	38	.58	.28	.28	.34
AL3007	1×10^7	100	.68	.2	.68	.5
AL3019	4×10^7	<1	.73	.69	0	0

Expression of the cloned 7 kb fragment: In *E. coli* neither the 7.0 kb or the 6.5 kb BamHI fragments cloned into pUC18 were capable of dehalogenating 4-CBA under the control of their own promotor in 2 day vial experiments. The 7.0 kb fragment from pSS70 carried in pUC18 was cloned into the broad host range cloning vector pPZ101 and transferred into *P. areuginosa.* No dehalogenation of 4-CBA occurred with this construct.

Experiments design to get expression of the 7.0 kb BamH1 fragment are underway. A Alcaligenes host containing plasmid pSS70 has been shown to express dehalogenase activity suggesting the Alcaligenes strain may be a good host for expression of cloned fragments. The 7.0 kb BamH1 fragment has been cloned into the broad-host range vector pLAFR1 to facilitate mobilization and maintenance in an Alcaligenes host strain BR6020. The 7.0 kb BamH1 fragment has been moved into pLAFR1 in both orientations and these plasmid constructs transferred to the BR6020 Alcaligenes host. Experiments are presently underway to determine if this host expresses the dehalogenase activity.

Construction of Bioluminescent Naphthalene Degrading Strains

The Naphthalene Promotor Inserted in Front of the Lux Cassette

The promotor for the upper pathway of the naphthalene plasmid NAH7 (7,29) carried in pE317 (8) was cloned into pUC18 (Figure 8). A 2.3 kb *Xho* II fragment from this construction was subcloned into the promoterless *lux* plasmid vector pUCD615 (22). The resulting plasmid, pUTK9, forms a transcriptional fusion between the nah promotor and the lux genes (Figure 9). The strain RB1351 was constructed by transferring pUTK9 into HK53 (a *P. putida* strain carrying the NAH7 (Table 2)). The NAH7 plasmid is necessary to provide the nahR regulatory gene, and for the ability to grow on naphthalene.

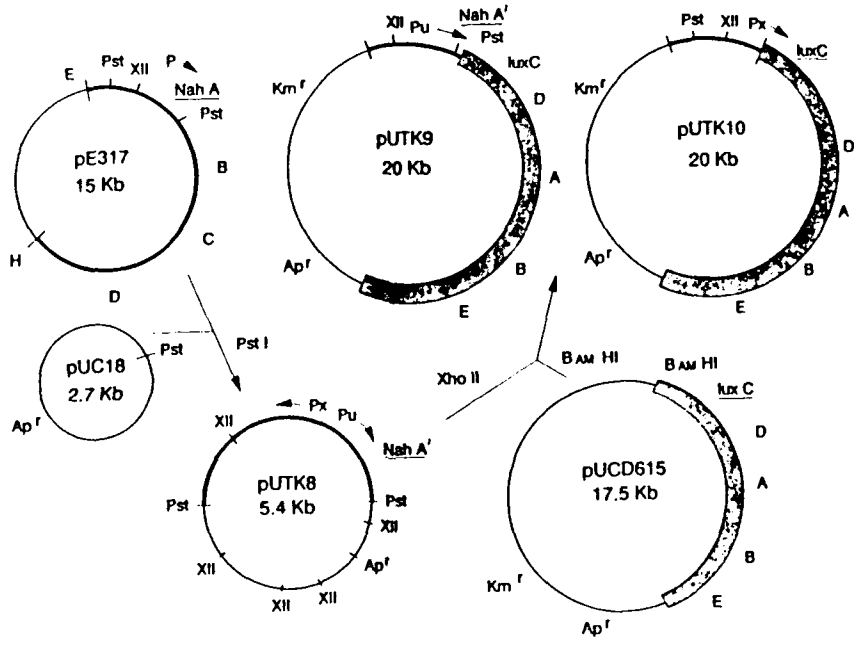

Figure 8. Subcloning of the *nah* promoter and construction of pUTK9 and pUTK10. A *Pst* I fragment of pE317 was cloned into the *Pst*I site of pUC18, creating pUTK8. An *Xho* II fragment of pUTK8 was cloned into the *lux* vector pUCD615 at the BamHI site to create both pUTK9 and pUTK10. Pu-*nah* upper pathway promoter, Px-putative divergent promoter, XII-*Xho*II, E-*Eco* RI; Apr- ampicillin resistance, Kmr-kanamycin resistance. Arrows show direction of transcription.

Transposon Mutagenesis of P. fluorescens Strain 5R

A second reporter strain was made by transposon mutagenesis of strain 5R with the promotorless lux transposon Tn4331 (Figure 9). Strain 5R is an environmental isolate from Manufactured Gas Plant (MGP) soil and contains a plasmid homologous to NAH7 designated pKA1. Tn4331 was introduced into 5R by conjugation with *E.coli* HB101 (pUCD623). The transconjugant 5RL containing the *lux* recombinant plasmid pUTK21 was selected for tetracycline resistance and the ability to grow on naphthalene. The transposon inserted into the *nah*G as demonstrated by gene probing and by the accumulation of salicylate when supplied with naphthalene. Salicylate hydroxylase is the product of the *nah*G gene, and an interruption in this gene prevents the further degradation of salicylate. The accumulation of salicylate, which is the inducer of both the upper and lower pathways (7), the recombinant plasmid pUTK21 was moved into the strain HK9 which is able to degrade salicylate but not naphthalene. The resulting strain, HK44, is able to mineralize naphthalene and has the same bioluminescent characteristics as strain 5RL.

Light emission in response to naphthalene induction: Colonies of RB1351 grown on agar medium and exposed to naphthalene vapor or in a liquid reactor had a large and rapid light response (Figure 10). Light induction was rapid (less than 10 minutes for the maximum response) and stable for many hours. The approximately 20-fold increase in light output compares favorably with the results of other investigators who have studied the expression of the upper pathway operon of NAH7. Light induction in response to naphthalene in the strain HK44 was comparable in both magnitude and response to RB1351. Control experiments with strains without the *nah*R regulatory gene or without the nah-lux fusion would not emit light

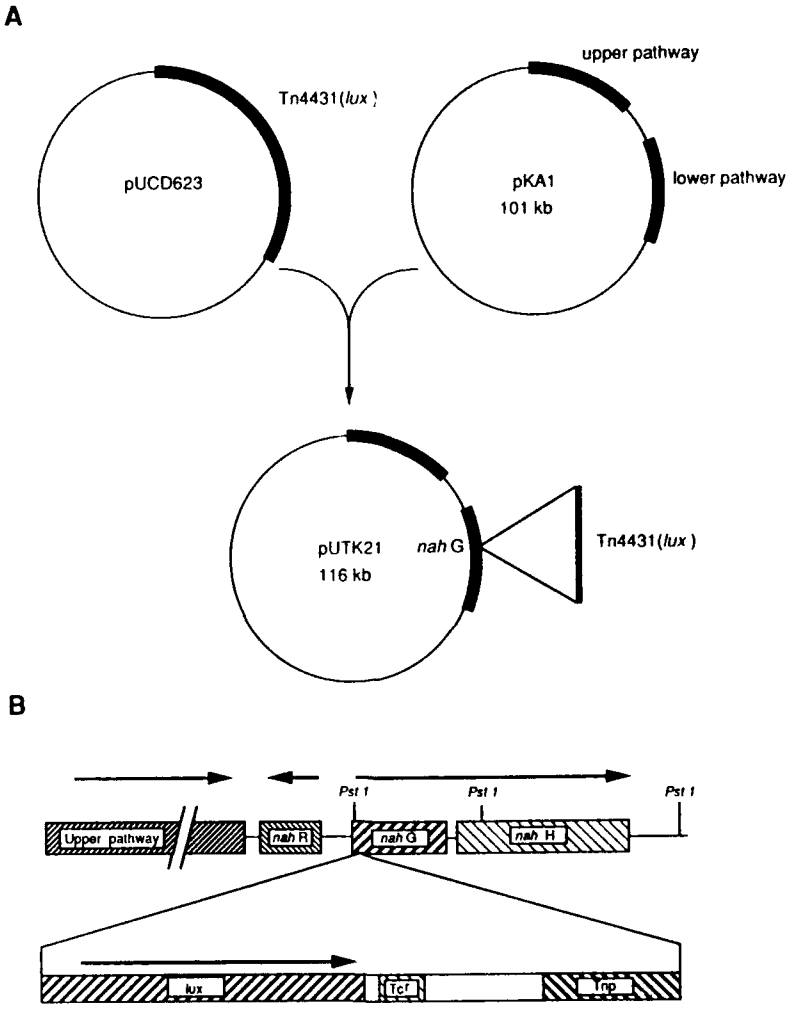

Figure 9. A. Construction of bioluminescent reporter plasmid pUTK21.

B. Proposed location of *lux* transposon in pUTK21.

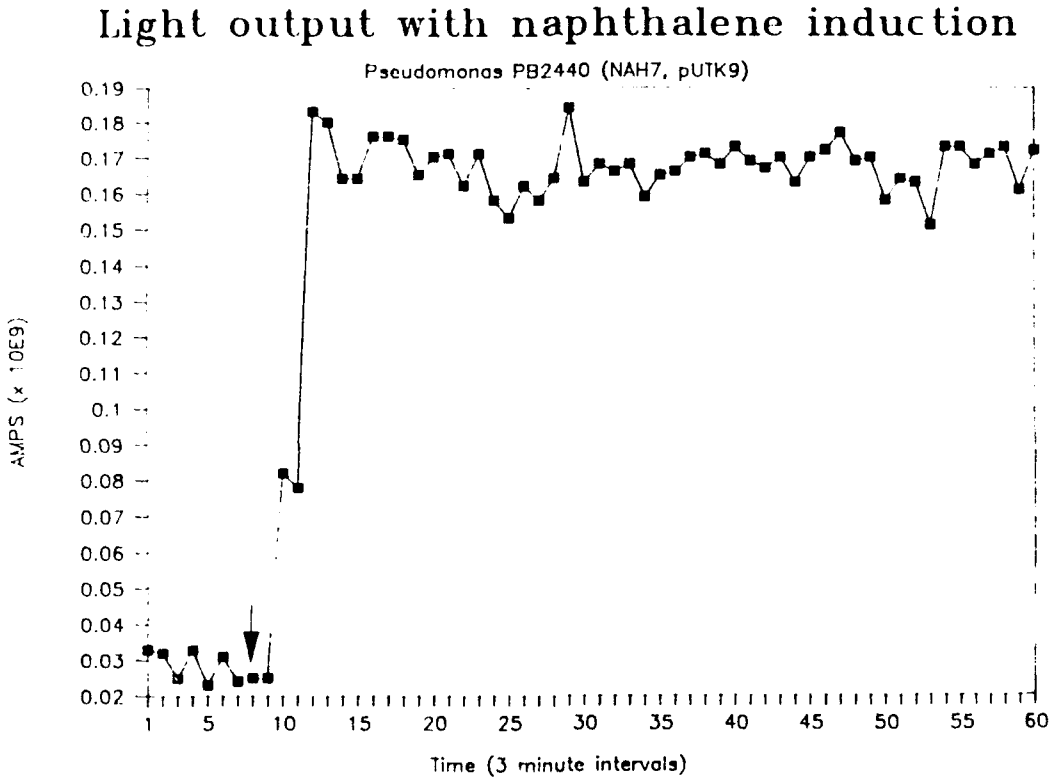

Figure 10. Response of RB1351 colonies to naphthalene. RB1351 was grown to mature colonies on LB agar medium. Naphthalene was supplied as vapor from crystals placed on the lid of an inverted plate at time point 8 (arrow).

under any conditions, demonstrating the stringent control of this system.

Correlation of naphthalene degradation with light production: In order to use these strains as reporter strains it is necessary to correlate the signal (light production) with the naphthalene degrading phenotype. In RB1351 light production was correlated with mineralization of C^{14}-labelled naphthalene (Figure 11). The growth rate of RB1351 in the minimal salts medium with naphthalene as the sole carbons source was very slow and the viable count was unchanged throughout the experiment. Both naphthalene degradation and light production occurred shortly after the addition of naphthalene to the culture medium, demonstrating that the promotors controlling light expression and naphthalene degradation are regulated in an identical manner.

The addition of naphthalene to a growing culture in liquid medium was expected to result in a rapid bioluminescent response. However, when naphthalene was added to a culture in an exponential growth rate light generation was delayed several hours until the culture had entered a slower rate of growth (Figure 12). Mineralization experiments demonstrated that significant naphthalene utilization did not occur during the exponential phase of growth and growth and increased during the slower rate of growth. This unexpected result demonstrates the potential for the *lux* system to describe genetic expression.

Dynamic Sensing of Naphthalene Degradation and Light Output

A continuous culture system was used to determine the dynamic response nature of the bioluminescent reporter strains to periodic naphthalene exposure. The response of strain HK44 to square wave perturbations in naphthalene exposure with a frequency of four hours is shown in Figure 13,14,15. A 15 minute lag was observed in the bioluminescent response to changes in naphthalene exposure. Bioluminescence increased linearly at a rate of 0.39 μamps Hr^{-1}

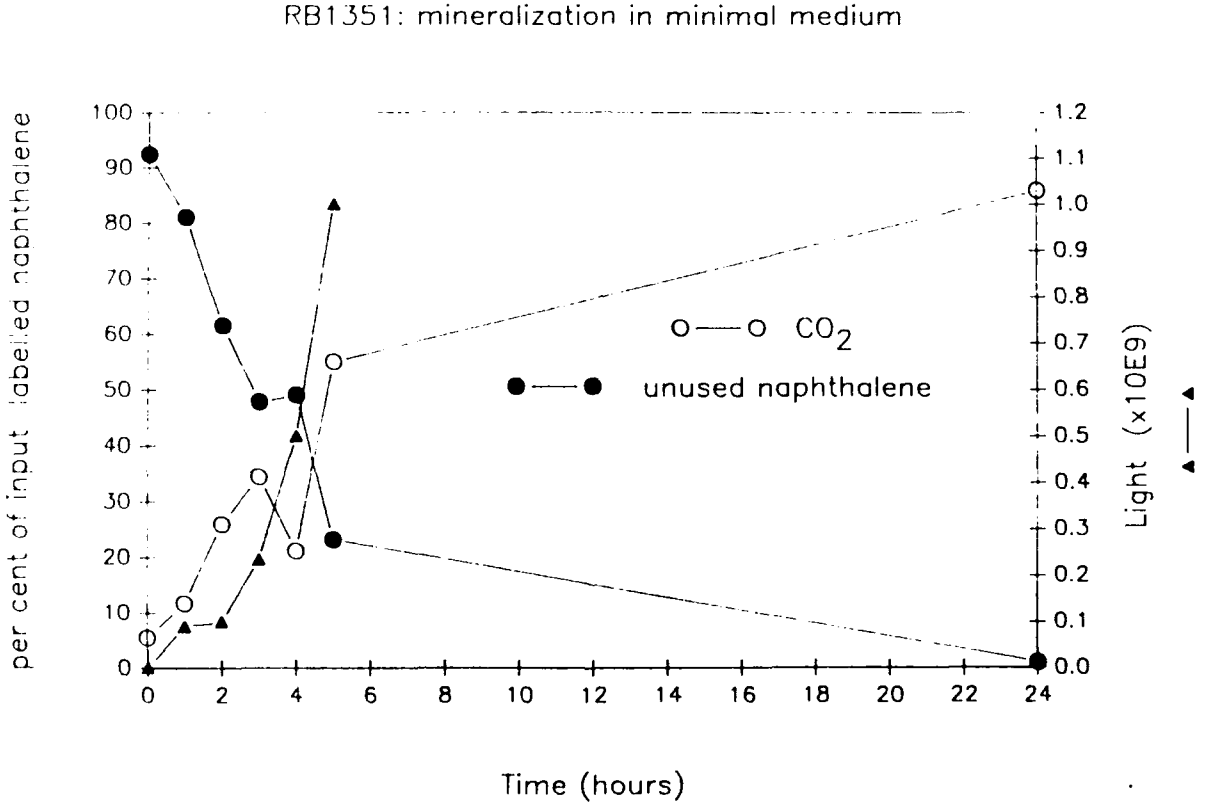

Figure 11. Mineralization assay with RB1351. Duplicate vials were incubated with ^{14}C-naphthalene in basal salts medium. Light output was measured immediately before fraction analysis. Cell concentration remained at 3 x 10^7 throughout the experiment.

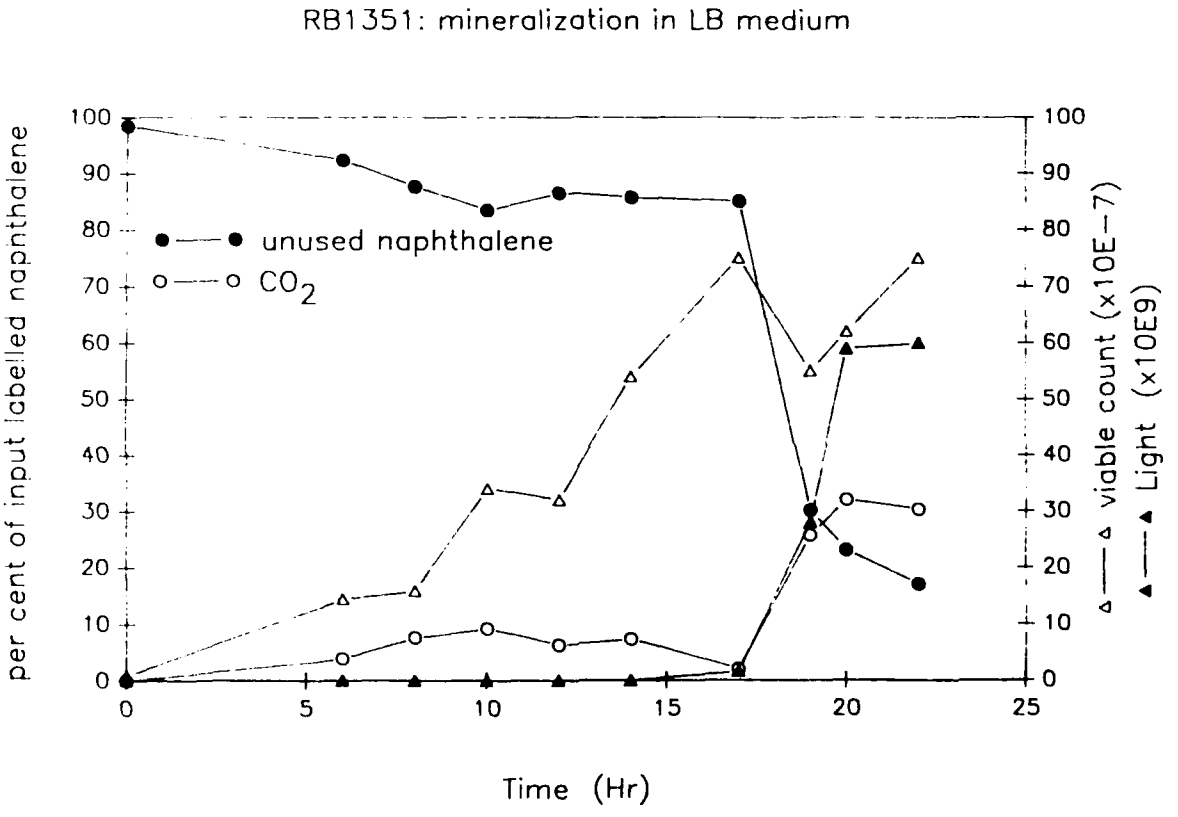

Figure 12. Mineralization assay of RB1351 in LB medium. Duplicate vials were incubated with ^{14}C-naphthalene. Light output was measured immediately before fraction analysis.

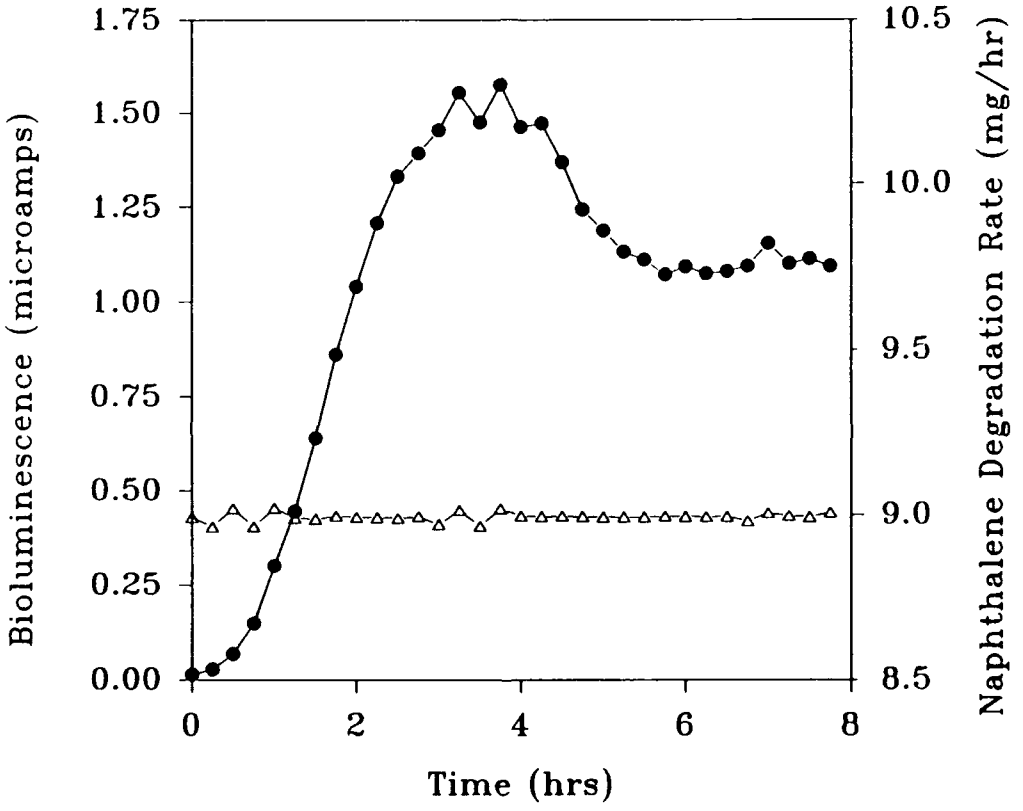

Figure 13. Induction of bioluminescence of strain HK44(pUTK21) in continuous culture: (●) bioluminescence and (▵) naphthalene degradation rate. Data represents single time-point determinations and three results are representative of three independent experiments.

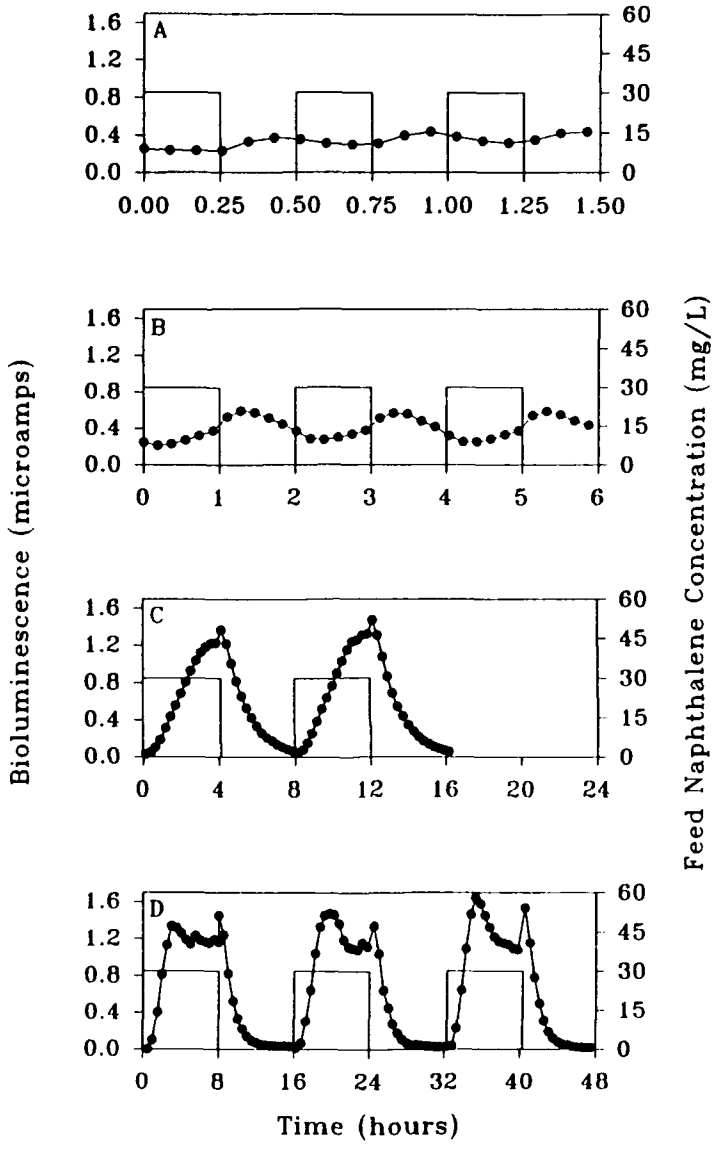

Figure 14. Bioluminescence response to square-wave perturbations in naphthalene feed concentration (rate): (A) 0.5-hour cycle; (B)2-hour cycle; (C) 8-hour cycle; and (D) 16-hour cycle. (●) Bioluminescence and (—) Naphthalene feed concentration. Perturbation cycles were repeated at each frequency to demonstrate the reproducibility of the bioluminescent response. The results are representative of two independent experiments.

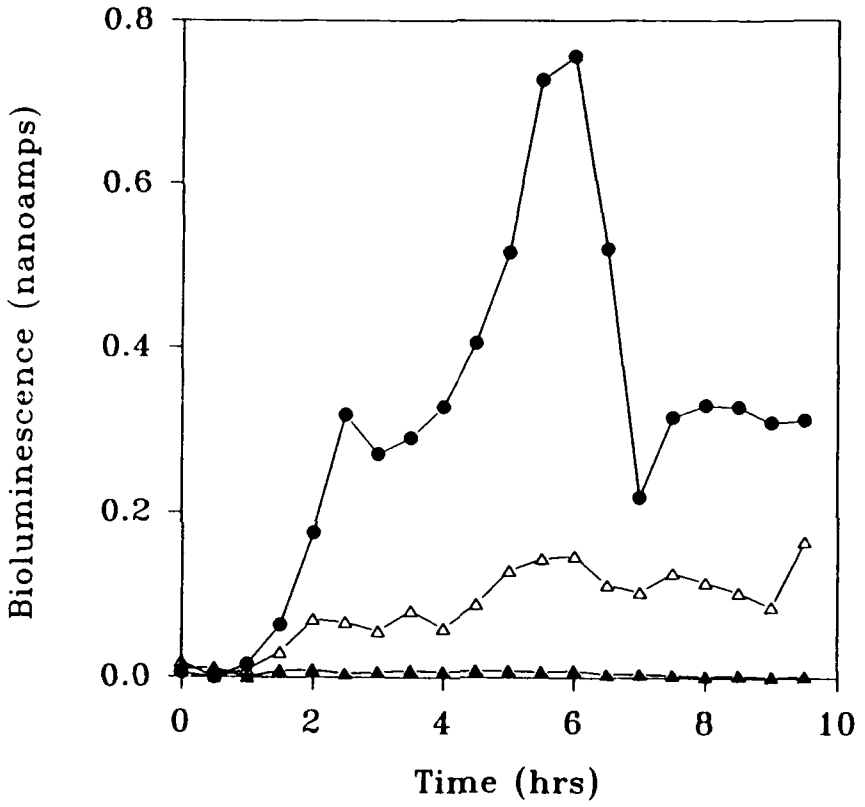

Figure 15. Detection of bioluminescence in soil slurries containing strain HK44. The test system consisted of 10 g of soil and 10 ml of uninduced HK44 cells (10^9 cells ml^{-1}) in phosphate buffered saline. The slurry was stirred to provide aeration and bioluminescence was monitored b y placing the liquid light pipe into the slurry.

(▲) Control soil; (△) Control soil amended with 10 mg naphthalene; and (●) MGP soil. The results are representative of two independent experiments.

during the two hour naphthalene exposure period followed by a near linear reduction in light output during the two hour period with no naphthalene. During the naphthalene exposure phase of the feed cycle the liquid naphthalene concentration in the chemostat increased to a steady-state concentration of between 0.4-0.5mg L^{-1} and the naphthalene degradation rate remained almost constant. When naphthalene addition to the reactor was ceased the reactor liquid naphthalene concentration decreased exponentially to 0.1 mg L^{-1}. Interestingly in the third perturbation cycle when the feed naphthalene concentration was reduced from 30 to 18 mg, both the rate increase in bioluminescence (0.26 μamps Hr^{-1}) and the peak bioluminescence attained were reduced (Figure 13). This suggests that the rate of increase in bioluminescence may be proportional to the naphthalene degradation rate. By using square wave perturbations ranging in periodicity from 0.5 to 16 hour bioluminescence was shown to be related to naphthalene exposure. During prolonged continuous exposure under steady-state conditions, the level of bioluminescence remained constant.

In Situ Simulations

Remote sensing of bioluminescence in complex matrices such as soils and sediments is more problematical than it is in liquid culture systems. Quenching of light by opaque matrix, particulate interference, and the development of biofilms might all be affect sensitivity and light monitoring. To demonstrate the utility of the bioluminescent reporter technology in complex matrices, experiments were done with stirred batch soli slurries inoculated with 10^{-9} uninduced cells of HK44. Light production was monitored by placing the liquid light pipe probe in the slurry. Two soil types were used, an uncontaminated Etowah soil and a soil from a MGP site contaminated with polycyclic aromatic hydrocarbons (Figure 14). As expected no

bioluminescence was detected in the uncontaminated soil. Whereas, bioluminescence indicative of naphthalene exposure and degradation was detected in the MGP soil and Etowah soil amended with naphthalene. The light production in the MGP soil was induced by endogenous naphthalene and illustrates the use of reporter strains as an assay for the bioavailability of contaminants in environmental matrices. Additional experiments are needed to correlate the bioluminescence levels reported in Figure 14 with biodegradative activity levels. However, the results are a qualitative indicator of activity. The rate of increase in light production was greater in the MGP soil than in the Etowah soil possibly indicating a higher initial biodegradative activity. Two factors which could explain the subsequent transient peak in activity are an exhaustion of bioavailable naphthalene or a decrease in the survival of the introduced reporter strain. It was hoped that the latter effect could be minimized by using strain HK44 which was originally isolated from an MGP soil. In the Etowah soil bioluminescence and hence naphthalene biodegradation activity remained constant for up to 16 hours.

Quantifying bioluminescent response: For the concentration range between 0 and 3.45 mg/l salicylate a good linear relationship between the concentration and light response could be established (Figure 16). In Figure 17, a comparison of the time course of the bioluminescence response between a mineral salts sample (Figure 17a), a soil extract (Figure 17b) and a soil surry (Figure 17c) which are all expected to contain the same amount of salicylate per assay volume are presented together with the corresponding controls without salicylate. While the mineral salts medium and the soil extract exhibited a similar response, the bioluminescence in the soil slurry was reduced by a factor of approximately 15. However, the overall relative response pattern in the soil slurry was quite similar to the ones observed in the mineral salts

medium and the soil extract.

In situ bioluminescent response was simulated using a differential volume reactor: Figure 18 depicts the on-line measurement of bioluminescence and naphthalene effluent concentration data from the reactor. At time zero, feed begun flowing at 0-.5 ml/min into the reactor. The initial baseline bioluminescence measured 0.5 namps. The initial reactor pressure was 80 psig. As the experiment proceeded, naphthalene absorbed onto the biofilm in the reactor. After four hours, the naphthalene concentration in the feed reached an equilibrium on the biofilm. At this point, the effluent concentration remained constant until induction of the naphthalene pathway occurred. After eight hours. bioluminescence began to increase and naphthalene effluent concentration began to drop simultaneously. The bioluminescence continued to rise reaching a peak of 6.8 namps; meanwhile, the naphthalene concentration continued to drop until naphthalene could not be detected leaving the reactor. Naphthalene was not detected during the remainder of the experiment. The bioluminescence also decreased and leveled off at 2 namps. Plate counts estimated the number of viable cells to be 4.2 x 108 cells/gram dry sand. AODC estimated the total cells to be 4.68 x 10 8 cells/ gram of dry sand. The total quantity of sand in the reactor equaled 44.08 g.

Comparative Culture Maintenance in Reactor Systems

Mixed culture and mixed contaminant removal: The degradation of different classes of compounds, specifically naphthalene and 4-chlorobiphenyl, can be achieved simultaneously by different populations. The population maintenance of a naphthalene degrading strain and a 4-chlorobiphenyl degrading strain growing on naphthalene and 4-chlorobiphenyl respectively s shown in Figure 19. The data show that the naphthalene and 4-chlorobiphenyl degrading strains

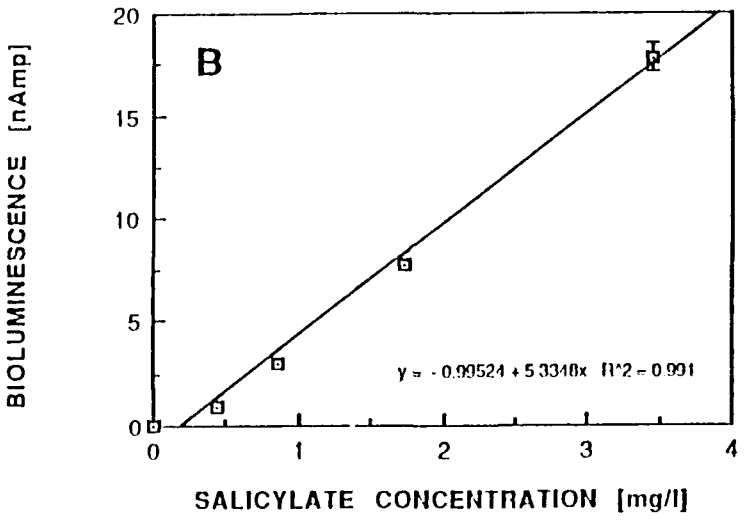

Figure 16. Relationship between salicylate concentration and bioluminescence. *nahG-lux* gene expression in exponentially growing *Pseudomonas fluorescens* HK44, 1 hour after incubation. Bioluminescence values are corrected for the response observed in the control (without salicylate), and show the linear range and regression fit.

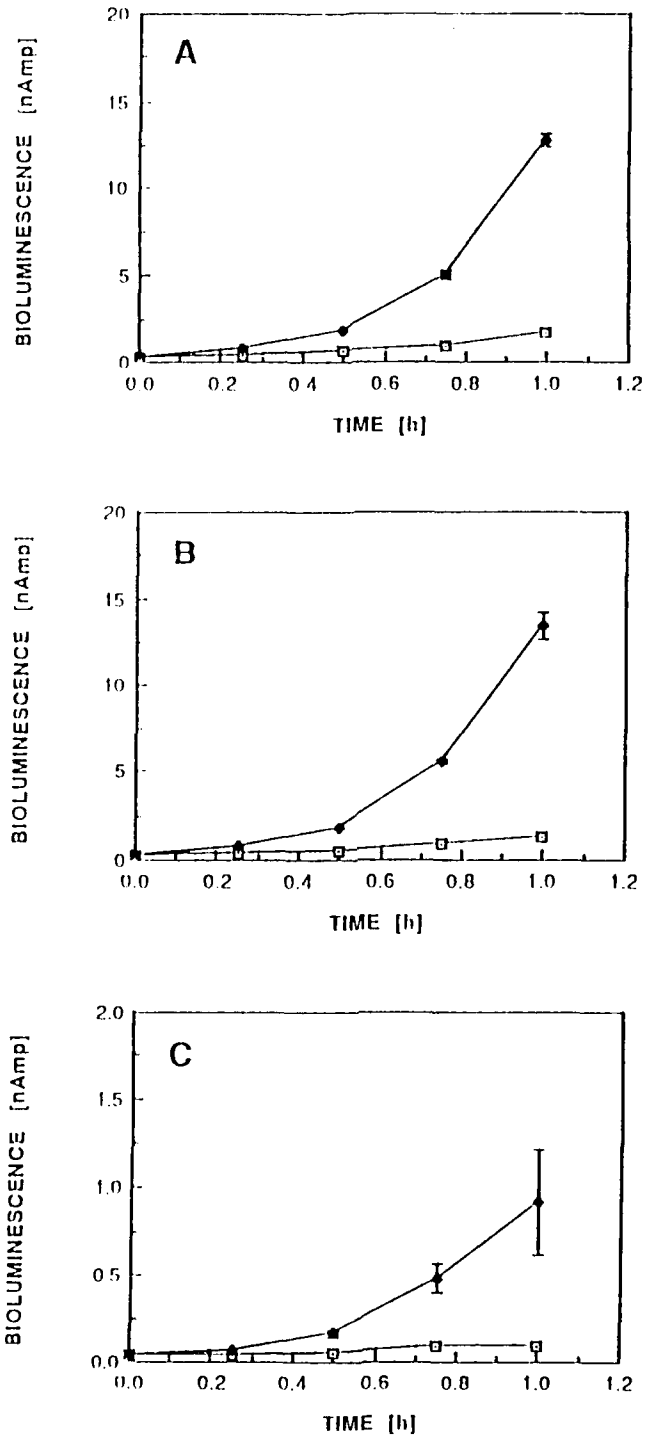

Figure 17. Time course of salicylate induced bioluminescence in growing *Pseudomonas fluorescens* HK44. In: A) mineral salts medium with 3.45 mg/l salicylate, B) soil extract and C) soil slurry where the soils received 6.9 mg/kg salicylate, resulting in a theoretical maximum aqueous concentration of 3.45 mg/l in the assay. (♦) salicylate samples, (⊡) control samples (without salicylate)

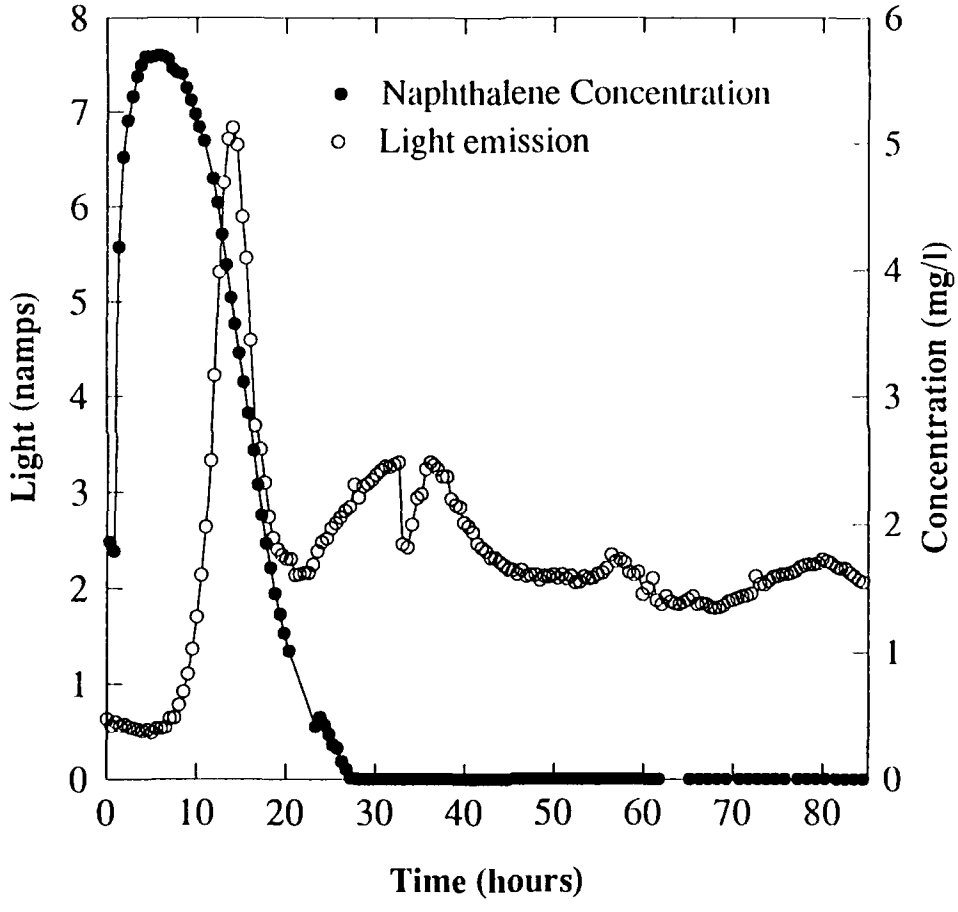

Figure 18. Light emission from strain HK44 and naphthalene effluent concentration from the differential volume reactor.

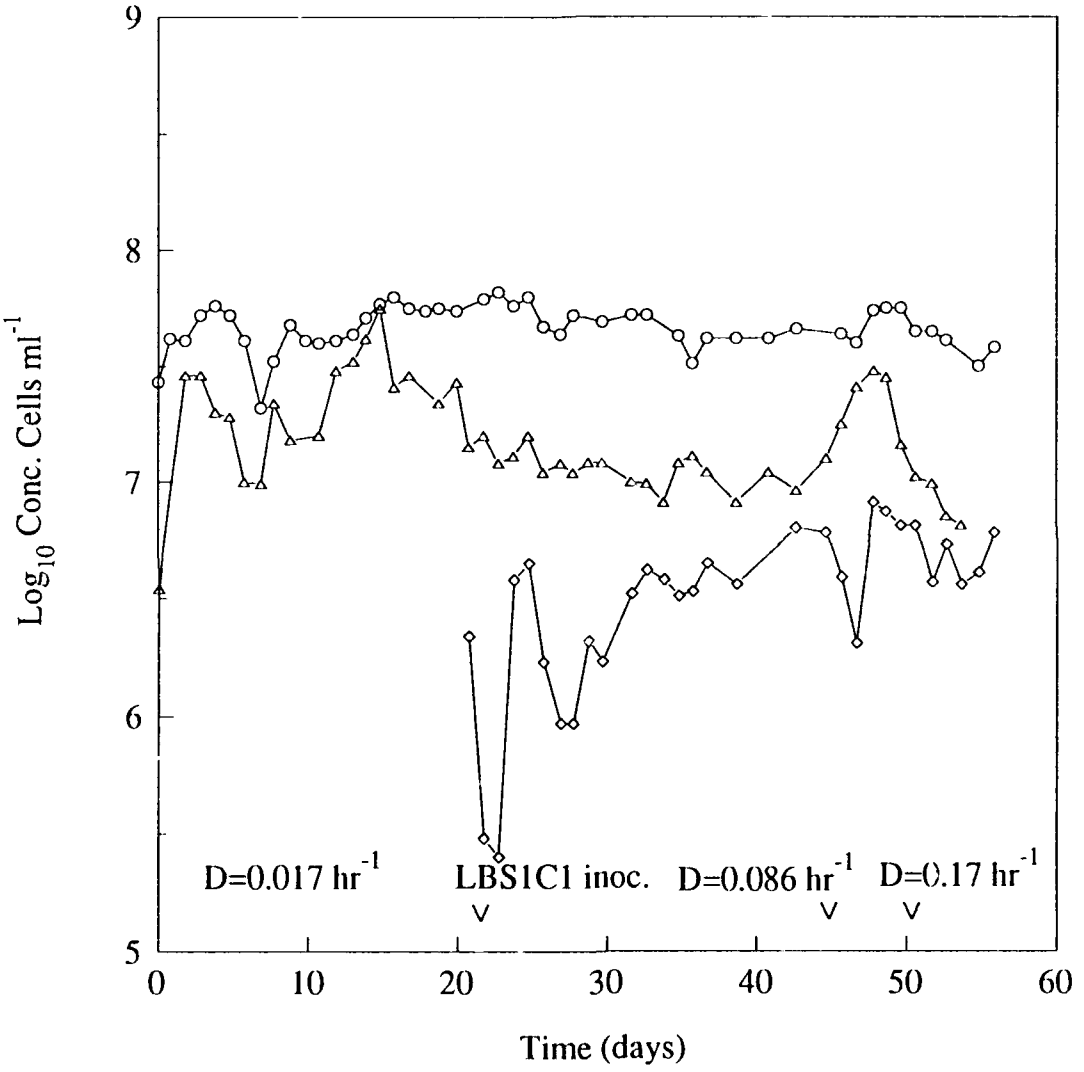

Figure 19. Population maintenance of a wild type PAH degrading strain (▵ *P. fluorescens* 5R) and a 4-chlorobiphenyl strain (◇ *Pseudomonas* sp. LBS1C1) growing on saturating concentrations of naphthalene and 4-chlorobiphenyl respectively in minimal media. Total cell numbers are shown (○).

were maintained at $\sim 10^7$ and $\sim 2 \times 10^6$ cfu/ml respectively for 27 generations at a dilution rate of 0.017 hr^{-1}. At a dilution rate of 0.086 hr^{-1}, the populations were maintained for an additional 14 generations. At a dilution rate of 0.17 hr^{-1}, the strains were maintained for an additional 60 generations. None of the parent compounds or metabolites were detected (data not shown).

Competitive maintenance of engineered and wild type strains: The mixed culture results indicate that factors other than competition for naphthalene are important since competitive exclusion was not observed. The population maintenance of the wild type degrading strain and two engineered reporter strains growing on naphthalene is shown in Figure 20. The data show that the wild type strain was maintained at a level 4-5 orders of magnitude higher than either engineered strain. The wild type strain *Ps. fluorescens* 5R, *Ps. fluorescens* 5RL, and *Ps. putida* RB1351 were maintained at $\sim 10^8$, $\sim 1.3 \times 10^3$ and $\sim 5 \times 10^4$ cfu/ml respectively at a dilution rate of 0.017 hr^{-1} for 74 generations. At a dilution rate of 0.17 hr^{-1}, the *Ps. fluorescens* 5R and *Ps. putida* RB1351 were maintained as before for an additional 99 generations but *Ps. fluorescens* 5RL was nearly washed out. The results do show that *Ps. putida* RB1351 was maintained at 10^4-10^5 cfu/ml even at higher dilution rates. *Ps. fluorescens* 5RL was nearly washed out at higher dilution rates possibly due to the inability to completely mineralize naphthalene. The sharp increase in total cell number and in the population level of *Ps. fluorescens* 5R corresponded with the decline in the population of *Ps. putida* RB1351 and the near washout of the *Ps. fluorescens* population. No naphthalene or metabolites were detected (data not shown). The fact that no salicylate was detected in the culture effluent indicates that *Ps. fluorescens* 5RL provided an additional substrate for growth to *Ps. fluorescens* 5R and *Ps. putida* RB1351 thus providing a

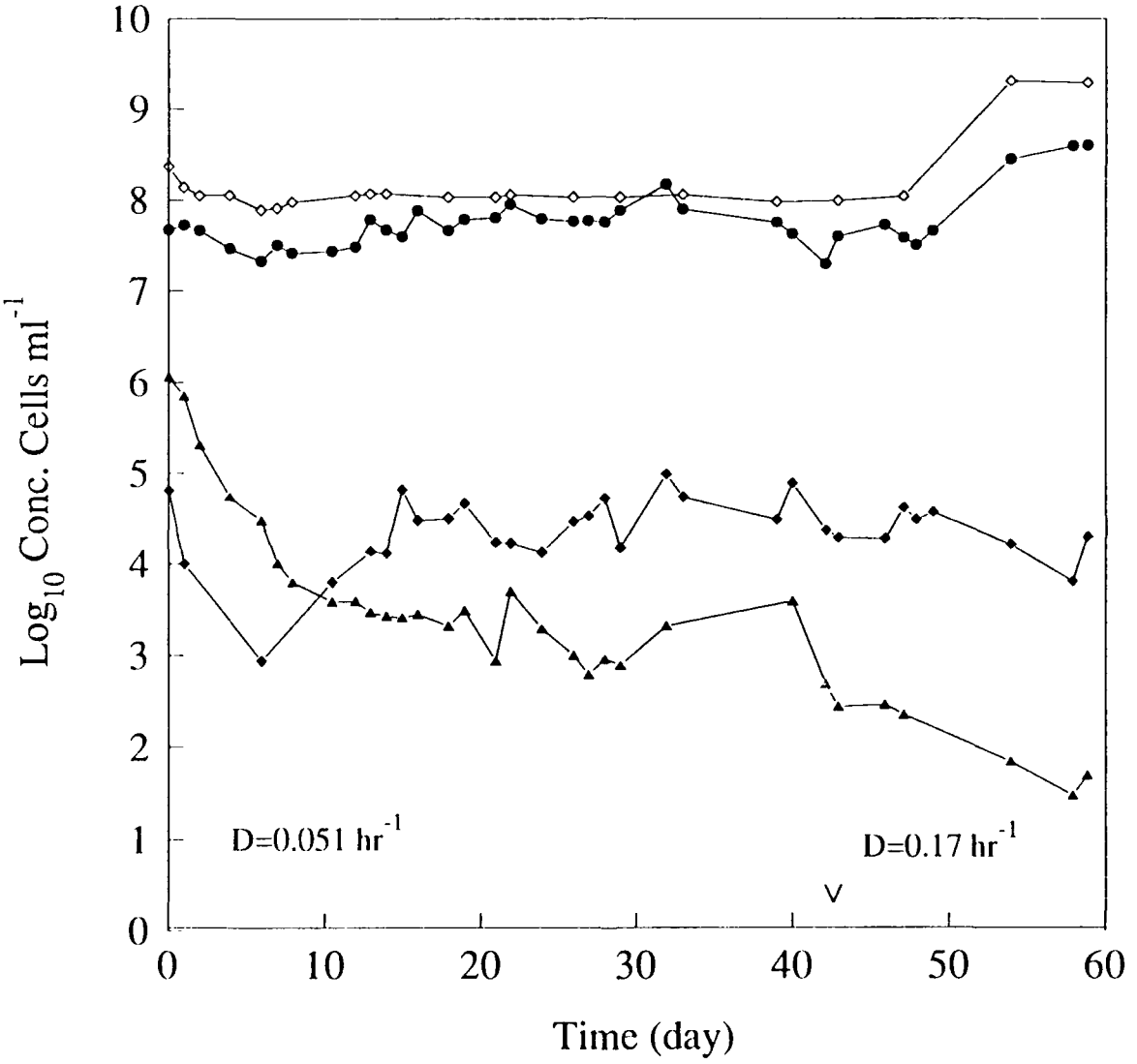

Figure 20. Population maintenance of a wild type PAH degrading strain (●, *P. fluorescens* 5R) and two engineered *lux* reporter strains (♦, *P. putida* RB1351 and ▲, *P. fluorescens* 5RL) growing on saturating levels of naphthalene in minimal media. Total cell numbers are shown (◇).

competitive advantage to those strains.

The maintenance of the reporter plasmid pUTK9 from *Ps. putida* RB1351 is shown in Figure 21. This graph shows that the reporter plasmid pUTK9 was maintained in ~78% of the *Ps. putida* RB1351 population. Although the population of *Ps. putida* RB1351 not harboring pUTK9 fluctuates, there is no identifiable trend in plasmid loss.

A similar mixed culture was composed of *Ps. putida* RB1351, *Ps. fluorescens* 5Rrif, and *Ps. fluorescens* HK44. Since *Ps. fluorescens* HK44 has a complete salicylate pathway, it was hypothesized that this strain could compete and be maintained in continuous culture. The maintenance of the respective populations are shown in Figure 22. The wild type strain *Ps. fluorescens* 5Rrif and *Ps. putida* RB1351 were maintained at 1.2-8.2 x 10^6 cfu/ml for 4.3 generations at a dilution rate of 0.017 hr^{-1}. The concentration of *Ps. fluorescens* HK44 dropped from ~5 x 10^5 to ~4 x 10^5 cfu/ml. At a dilution rate of 0.034 hr^{-1}, *Ps. flourescens* 5Rrif and *Ps. putida* RB1351 increased to ~9 x 10^6 cfu/ml then gradually decreased to ~4 x 10^5 cfu/ml then again increased to ~5 x 10^6 cfu/ml over 8.2 generations. *Ps. fluorescens* HK44 gradually decreased until no CFUs could be detected. This eventual washout of *Ps. fluorescens* HK44 may have been due to the 10-fold lower inoculum of this strain even though there was an attempt to inoculate all strains at the same titer. This situation would provide a competitive advantage to the strains present in higher numbers. The bioluminescent response of the mixed culture was dynamic in character. Upon inoculation of the respective strains, bioluminescence increased to 0.2 x 10^{-9} amps and remained constant until the continuous addition of medium after 24 hr. At this time, the bioluminescence increased to 0.63 x 10^{-9} amps. The response fluctuated over the next 5 days. Upon increasing the dilution rate from 0.017 hr^{-1} to 0.034 hr^{-1}, the bioluminescence increased 2.5 fold, fluctuated, then decreased to ~0.6 x 10^{-9} amps. This data

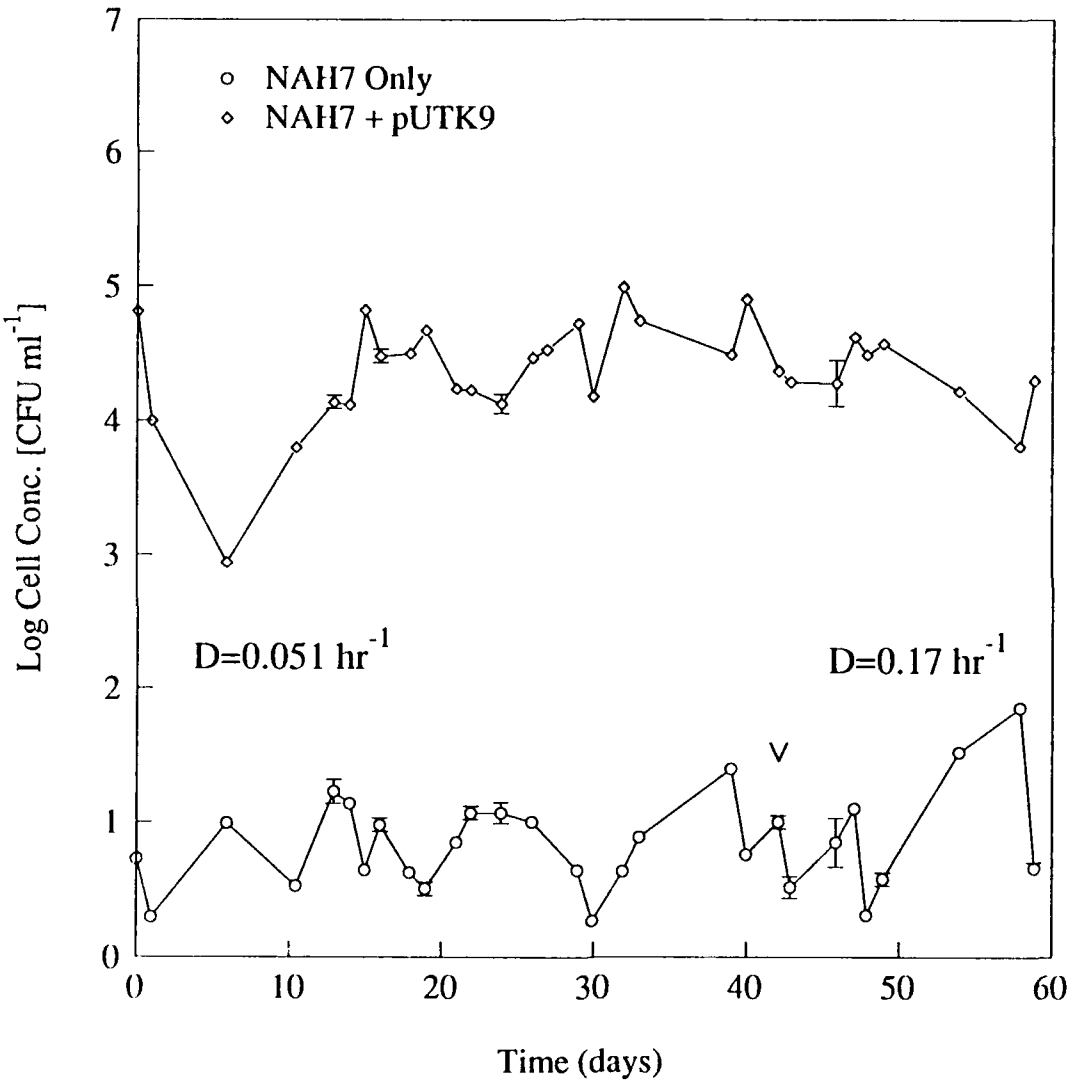

Figure 21. Maintenance of pUTK9 in a mixed chemostat culture containing *P. fluorescens* 5R, and two engineered *lux* reporter strains, *P. putida* RB1351 and *P. fluorescens* 5RL, growing on saturating levels of naphthalene in minimal media.

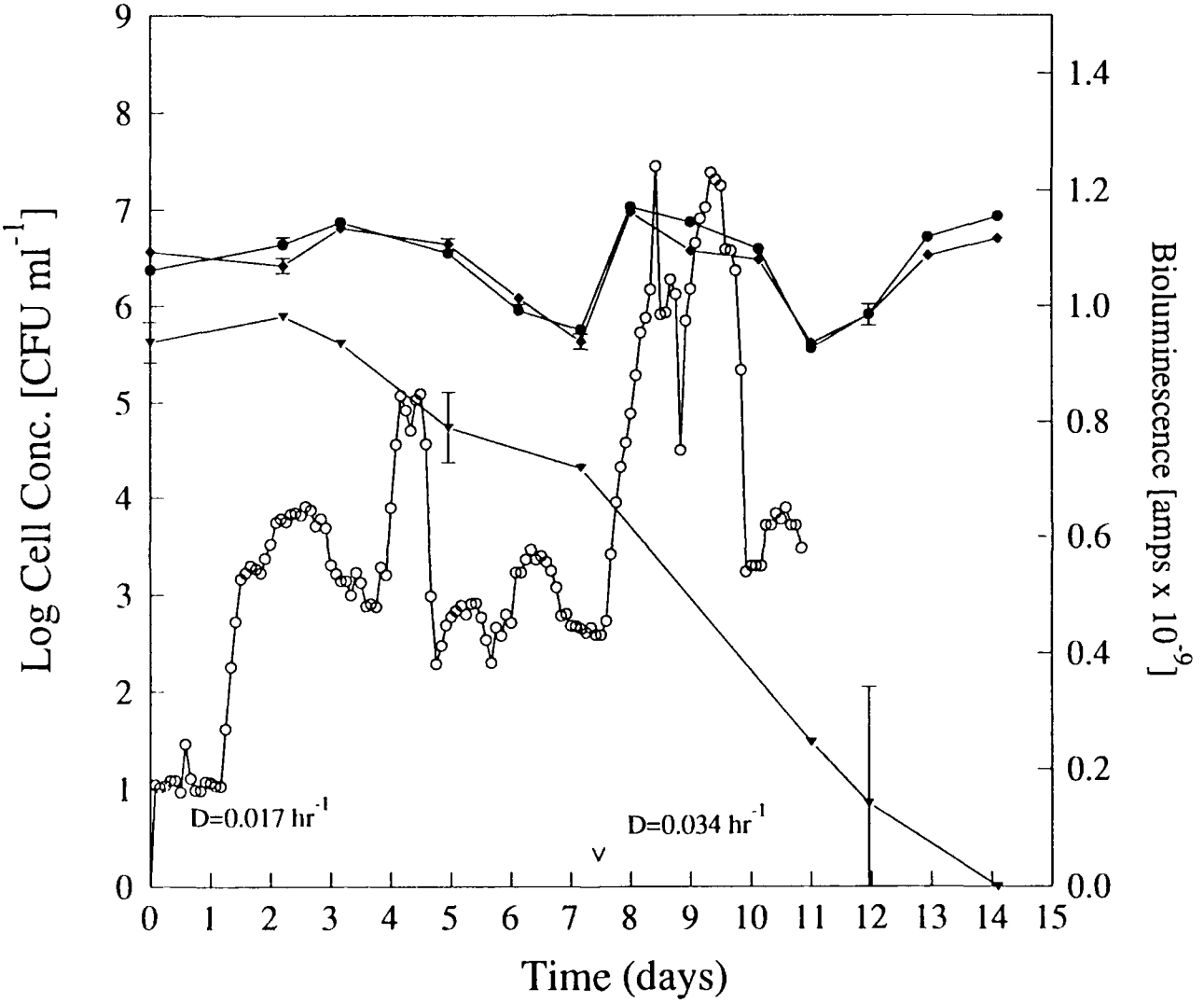

Figure 22. Population maintenance of a wild type PAH degrading strain (● , *P. fluorescens* 5Rrif) and two engineered *lux* reporter strains (◆ , *P. putida* RB1351 and ▼ , *P. fluorescens* HK44) growing on saturating levels of naphthalene in minimal media. Bioluminescence is indicated (○).

demonstrates that the bioluminescent response is sensitive to perturbations such as a change in dilution rate (growth rate). A steady-state was regained indicating that the bioluminescent response is probably not related to growth rate.

Since *Ps. fluorescens* HK44 was not maintained in the previous experiment, this strain was continuously cultured alone with naphthalene as a sole source of carbon. The experiment was set up to test the hypothesis that *Ps. fluorescens* HK44 was not maintained in mixed continuous culture due to the loss of pUTK21. Although naphthalene serves as selective pressure for pUTK21, it is conceivable that the plasmid is gradually lost over time and the host strain washes out of the culture. The culture was monitored using selective and non-selective media. If a large portion of the population were losing pUTK21, then large differences in plate counts on the respective media would be expected. There were no such differences beyond the inherent error in plate counts (data not shown). The population maintenance of *Ps. fluorescens* HK44 is shown in Figure 23. The data show that the population started at $\sim 1.2 \times 10^6$ cfu/ml. This level increased to 1×10^8 - 7×10^7 cfu/ml for 9 generations at a dilution rate of 0.026 hr^{-1}. The culture was maintained for another 17 generations at a dilution rate of 0.06 hr^{-1}. Upon addition of *Ps. fluorescens* 5Rrif, the population of *Ps. fluorescens* HK44 decreased to $\sim 5 \times 10^7$ cfu/ml but was maintained for another 21 generations at a dilution rate of 0.06 hr^{-1}.

The maintenance of pUTK21, as determined by probing colony blots taken from culture samples, with *lux*AB specific DNA is shown in Figure 22. The results agree with the viable count data shown in Figure 5. These data indicate that the reporter plasmid pUTK21 is not lost during continuous culture. The *lux* genotype was stably maintained for 9 generations at a dilution rate of 0.026 hr^{-1}. Upon increasing the dilution rate to 0.06 hr^{-1}, the *lux* genotype continued to be maintained for another 6.5 generations. When the wild type PAH degrading

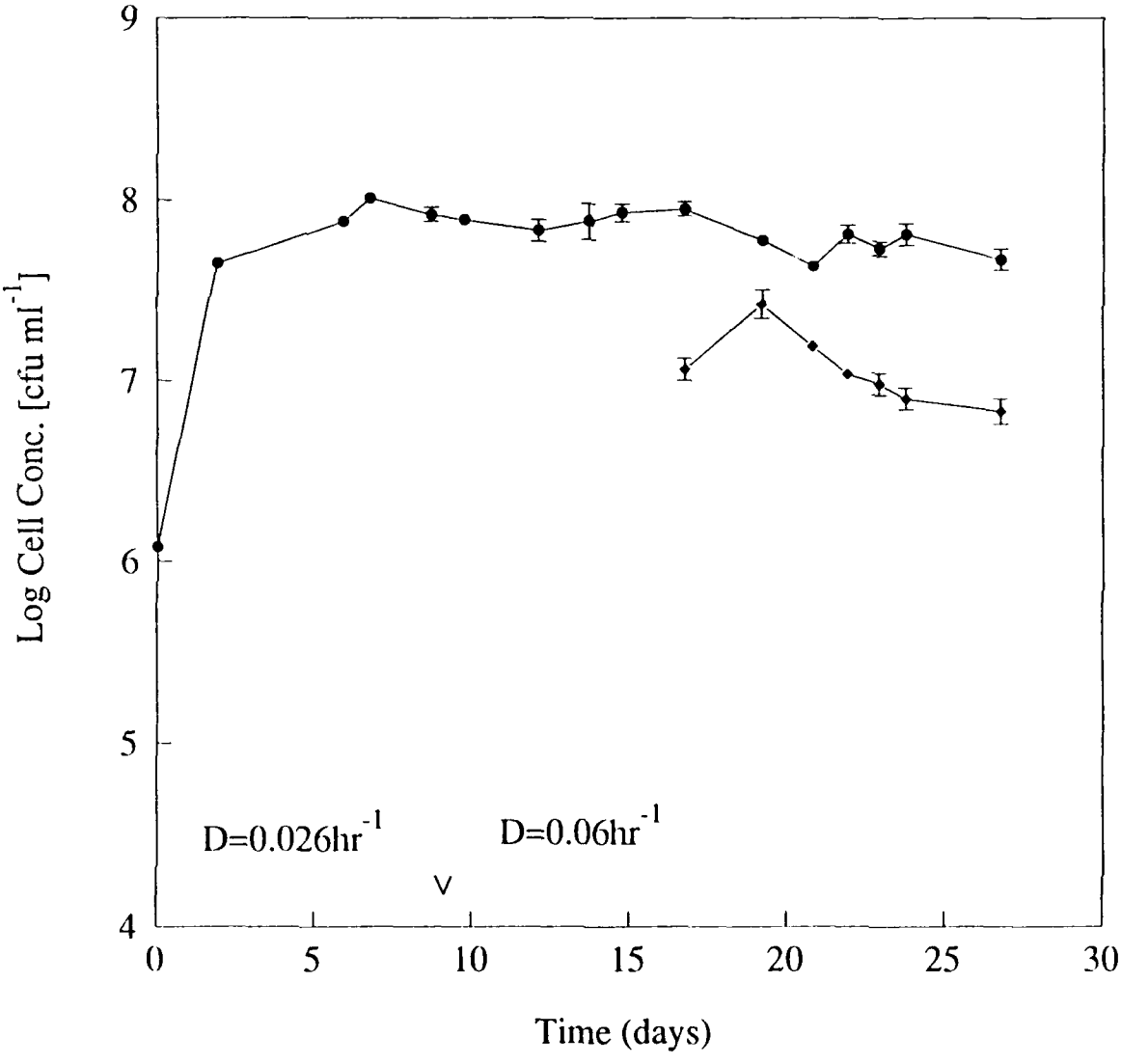

Figure 23. Population maintenance of *P. fluorescens* HK44 (●) growing on saturating levels of naphthalene in minimal media in continuous culture. The wild type PAH degrading strain, *P. fluorescens* 5Rrif (♦), was inoculated into the culture after 17 days of cultivation.

strain *Ps. fluorescens* 5Rrif was added to the culture, the *lux* genotype continued to be maintained for another 21 generations. Plasmid preparations derived from random culture isolates showed that the *lux* transposon in pUTK21 remained stable (data not shown). Although these blots were not probed for naphthalene degrading genes, there did not appear to be any deletions or rearrangements in any of the isolates. The bioluminescent response of the culture is also shown in Figure 24. Over the first 3 generations, the bioluminescence was dynamic but reached a steady-state at $\sim 0.27 \times 10^{-8}$ amps. These data agree with the viable counts shown in Figure 23 with respect to cell number and bioluminescent output. The bioluminescence becomes steady about the same time the viable count of *Ps. fluorescens* HK44 becomes steady. The bioluminescence remains steady for 9 generations at a dilution rate of 0.026 hr^{-1}. Upon increasing the dilution rate to 0.06 hr^{-1}, the bioluminescent response shows a brief fluctuation and then attains its former level for another 6.5 generations. This fluctuation was much smaller in magnitude than the one in Figure 22. The dilution rates were different, thus it is impossible to judge whether such a perturbation is more pronounced in a mixed culture than in a pure culture. Upon addition of *Ps. fluorescens* 5Rrif to the culture, the bioluminescence dropped 2 fold. The bioluminescence regained its former level after an additional 21 generations. It seems that the sudden burden of competition has a pronounced effect on the bioluminescent response in this situation. At the conclusion of the experiment, the culture was poisoned with the detergent Quatsyl 256. The bioluminescence dropped to 0×10^{-8} amps. This demonstrates that the response was indeed light from the culture and not due to light leakage or dark current.

Persistence of engineered reporter strain in mixed culture degrading mixed contaminants: The *lux* reporter strain *Ps. putida* RB1351 could successfully compete with a wild type undefined assemblage. Population maintenance of *Ps. putida* RB1351 in a mixed culture containing a

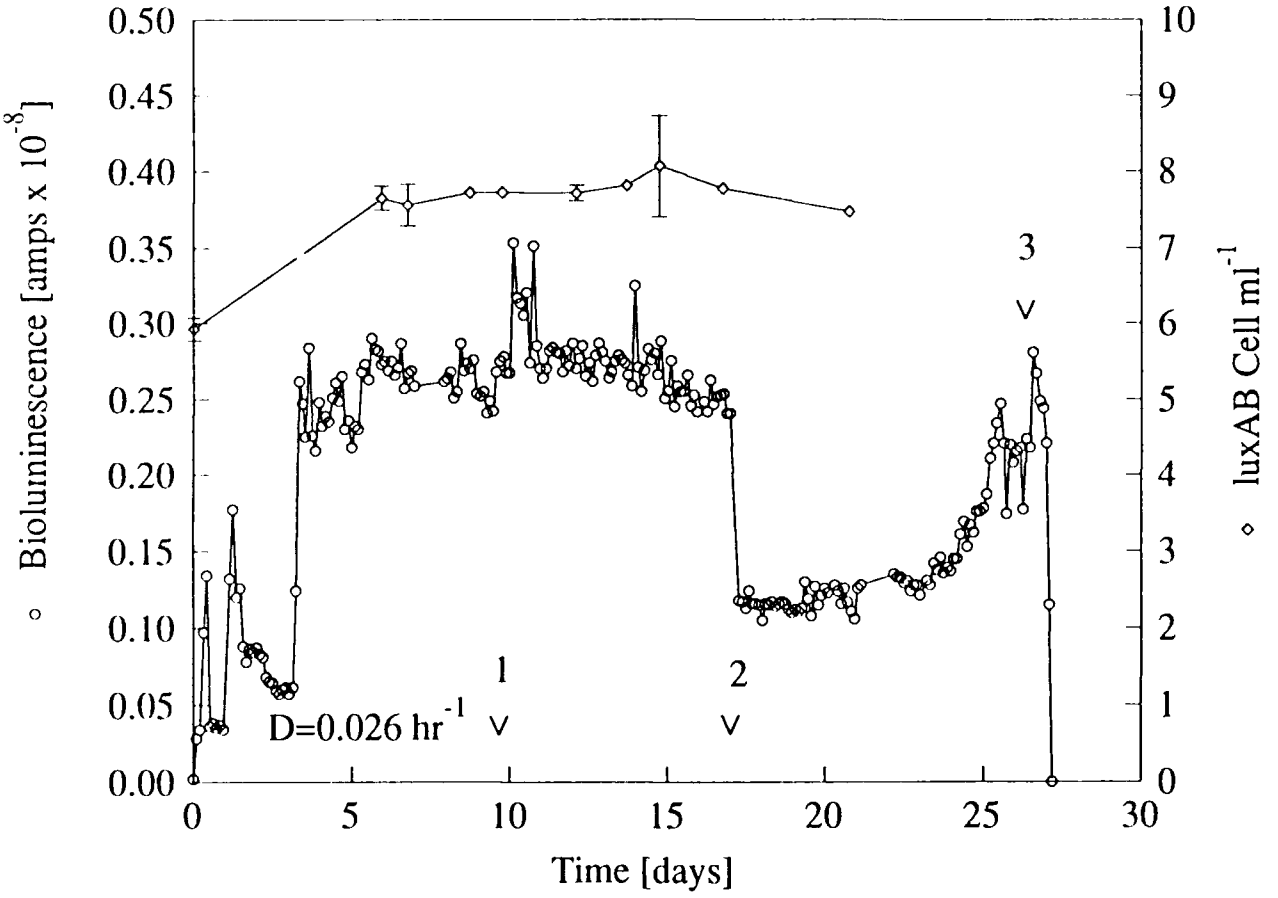

Figure 24. Maintenance of pUTK21 (◇) in *P. fluorescens* HK44 growing in continuous culture on saturating levels of naphthalene in minimal media. The bioluminescent response is indicated (○). The system was perturbated in the following ways: (1) The dilution rated was increased to 0.06 hr^{-1}; (2) The strain *P. fluorescens* 5Rrif was inoculated into the system; (3) The culture was poisoned with the detergent Quatsyl 256.

community derived from a PAH contaminated soil is shown in Figure 25. The data show that after 11.5 generations at a dilution rate of 0.17 hr^{-1}, the *lux* reporter strain was maintained at a level of 2.4-2.6 x 10^6 cfu/ml. The wild type assemblage was maintained at 2.3 x 10^6-8.5 x 10^6 cfu/ml. No naphthalene was detected after day one, and phenanthrene could not be detected after day six. The abrupt rise in the level of the wild type assemblage correlates with the utilization of phenanthrene. *Ps. putida* RB1351 is unable to utilize phenanthrene thus the wild type assemblage may have gained a competitive advantage from an additional carbon source. The *lux* strain *Ps. putida* 1351 was capable of maintaining a detectable output of bioluminescent light for long periods of time. Bioluminescence from this strain in a mixed culture (same as Figure 25) is shown in Figure 26. The results indicate that bioluminescence was maintained at 1.4-2.3 x 10^{-8} amps. The gap in the data was due to a computer malfunction and subsequent loss of data.

Characterizing JS150

Characterization of the naphthalene utilization in JS150 was initiated during this project investigation. DNA hybridization experiments with Pseudomonas strain JS150 demonstrate the presence of the nah A gene (naphthalene dioxygenase) similar to the nah A gene found on the NAH7 plasmid. DNA hybridization experiments with the nah G gene was not detectable in strain JS150. These results suggest strain JS150 may contain an upper pathway for naphthalene degradation that is similar to the pathway found in NAH7 but the degradation of salicylate to TCA cycle intermediates may be different from the NAH7 plasmid encoded pathway.

Salicylate degradation by strain JS150 was examined by mineralization studies. Strain JS150 was able to convert 60 % of salicylate to CO2 in 120 hours. Preliminary HPLC data

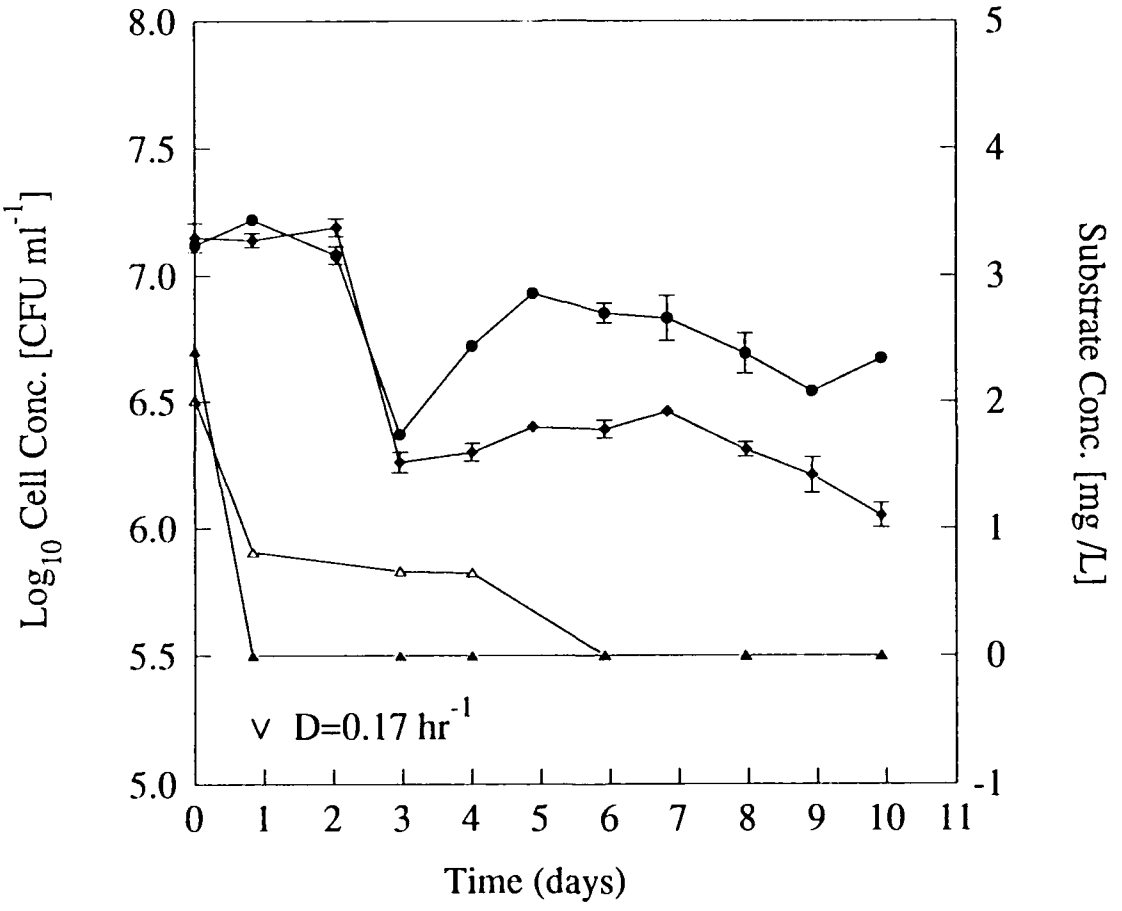

Figure 25. Maintenance of an engineered *lux* reporter strain (◆), *P. putida* RB1351 in a mixed culture containing a community derived from a PAH contaminated soil. The wild type community (●), naphthalene (▲), and phenanthrene (▵) were monitored. Naphthalene and phenanthrene were supplied at saturating concentrations in minimal media supplemented with a soil extract (DOC=10 mg/L).

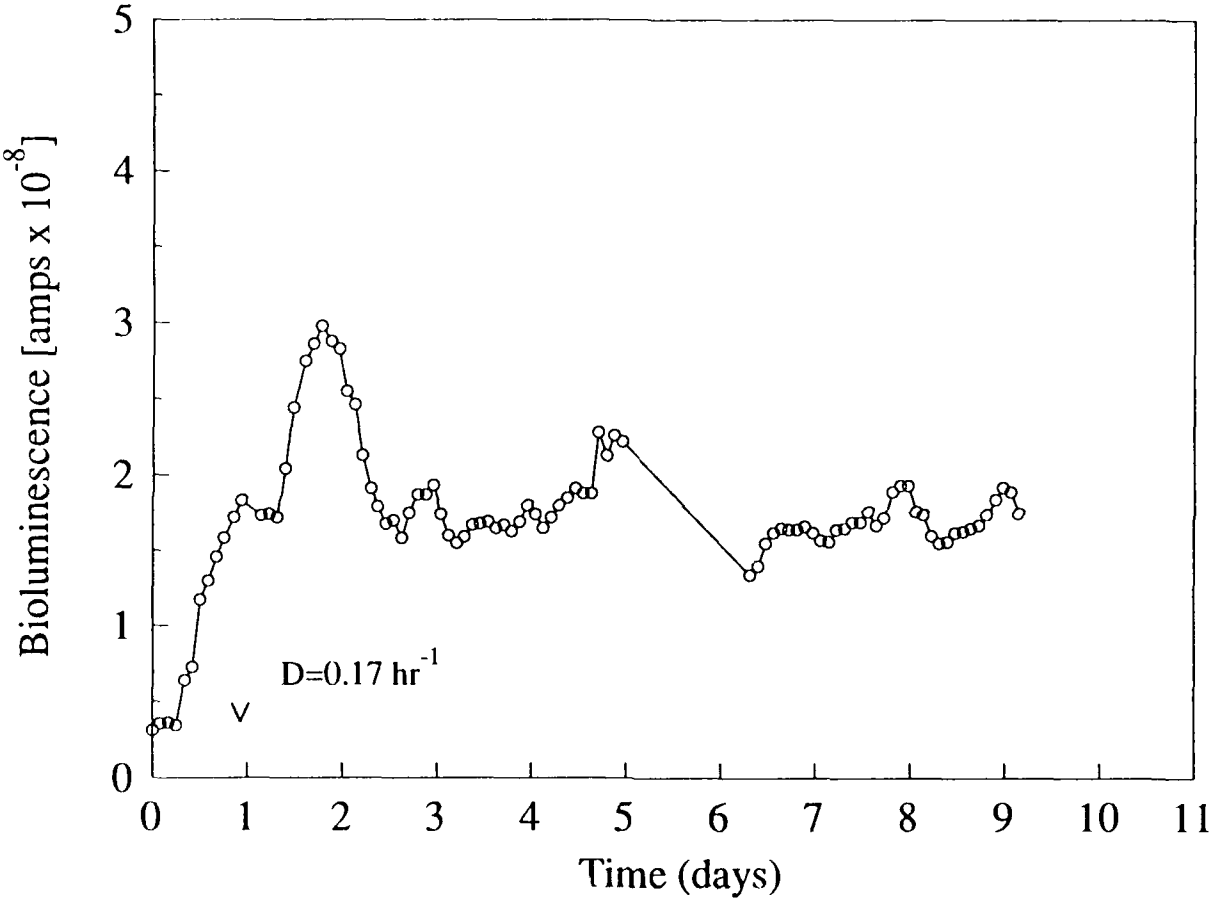

Figure 26. Bioluminescence from *lux* reporter strain *P. putida* RB1351 in a mixed culture containing a community derived from PAH contaminated soil. Media conditions were the same as in Fig. 25.

identifying gentisate in culture supernatants from strain JS150 grown on 5 mM salicylate possibly suggest JS150 metabolizes salicylate via the intermediate gentisate. Further experiments with radiolabbeled salicylate should provide the necessary information to elucidate this hypothesis.

Summary Research Progress and Findings

Significant research progress was accomplished across the three major research objectives. The research focused on 1) the molecular construction of *lux* transcriptional fusions (Bioluminescent) with naphthalene catabolic genes of the NAH7 naphthalene catabolic plasmid, 2) application and analysis of *nah-lux* fusions as a bioluminescent reporter for naphthalene exposure and biodegradation, 3) the dynamic response of bioluminescent sensing of naphthalene degradation in waste treatment reactor simulations, 4) a comparative gene probe analysis of naphthalene degradative gene frequency and dynamics in soils and waste treatment simulations, and 5) molecular analysis of a chlorobiphenyl degradative plasmid in preparation for the construction of bioluminescent reporter systems for PCB biodegradation.

Publications

Eight total publications were derived from this USAF supported research effort:

Hooper, S.W., C.A. Pettigrew, and G.S. Sayler. 1990. Ecological fate, effects, and prospects for the elimination of environmental polychlorinated biphenyls (PCBs). Environmental Toxicology and Chemistry. Vol.9 No.5. pp.655-667.

Burlage, R.S., G.S. Sayler, F. Larimer. 1990. Bioluminescent Monitoring of Naphthalene Catabolism using *nah-lux* Transcriptional Fusions. J. Bacteriol. Vol. 172, No. 9, p. 4749-4757.

Packard, J., A. Breen, G.S. Sayler, A.V. Palumbo. 1989. Monitoring Populations of 4-Chlorobiphenyl-degrading Bacteria in Soil and Lake Water Microcosms Using Colony Hybridization. Proc. 2nd Natl. Conf. Hazardous Materials Control Research Institute. Washington, D.C. pp. 119-125.

King, J.M.H., P.M. DiGrazia, B. Applegate, R. Burlage, J. Sanseverino, P. Dunbar, F. Larimer, and G.S. Sayler. 1990. Rapid and Sensitive Bioluminescent Reporter Technology for Naphthalene Exposure and Biodegradation. Science. Vol. 249, p. 778-781.

Sayler, G.S. and J.W. Blackburn. 1990. Analysis and Control of Biodegradative Processes and Biological Waste Treatment. In: (J.E. Glass and G. Swift, eds.) Agricultural and Synthetic Polymers: Biodegradability and Utilization. ACS Symposium Series No. 433. pp. 13-32.

Sayler, G.S., S.W. Hooper, A. Layton, and J.M.H. King. 1990. Catabolic plasmids for environmental applications: A review. Microbial Ecology. Vol. 19, No. 1. pp. 1-20.

Burlage, R.S., L.A. Bemis, A.C. Layton, G.S. Sayler, and F. Larimer. 1990. Comparative Genetic Organization of Incompatibility Group P Degradative Plasmids. J. Bacteriol. Vol. 172, No. 12, p. 6818-6825.

Layton, A.C. and G.S. Sayler. 1990. Environmental Application of Nucleic Acid Hybridization. Annual Review of Microbiology. Vol. 44, 625-648.

Layton, A.C., J. Sanseverino, W. Wallace, C. Corcoran, and G.S. Sayler. 1992. Evidence for 4-Chlorobenzoic Acid Dehalogenation Mediated by Plasmids Related to pSS50. Appl. Environ. Microbiol. Vol.58, No. 1, p.399-402.

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