

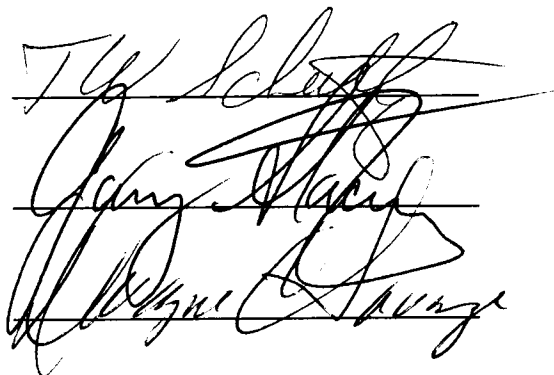
To the Graduate Council:

I am submitting herewith a dissertation written by Jeffrey Leblond entitled "Cometabolic Versatility of the NAH System of *Pseudomonas fluorescens*." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Microbiology.



Gary Sayler, Major Professor

We have read this dissertation
and recommend its acceptance:



Accepted for the Council:



Associate Vice Chancellor and
Dean of the Graduate School

COMETABOLIC VERSATILITY OF THE NAH SYSTEM
OF *PSEUDOMONAS FLUORESCENS*

A Dissertation
Presented for the
Doctor of Philosophy
Degree
The University of Tennessee, Knoxville

Jeffrey David Leblond
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Dedication

This dissertation is dedicated to my parents, Jeanne and Gerard Leblond, who have given me unwavering love and support for as long as I can remember.

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Abstract

Environmental sites contaminated by polycyclic aromatic hydrocarbons (PAHs) and heterocycles typically are characterized by the presence of approximately one-hundred different contaminants of varying ring structure and substitution patterns. The biodegradation of the majority of these contaminants by the NAH system for naphthalene degradation, which is currently the most well-studied enzyme system for the breakdown of PAHs, has not been examined in great detail. Using the bacterium *Pseudomonas fluorescens* 5R, and its mutant *P. fluorescens* 5RL, which contains a nonfunctional NAH system lower pathway, the metabolism of a suite of substituted naphthalenes and heterocyclic compounds by the NAH upper pathway was examined. Based on gas chromatography/mass spectrometry (GC/MS) analysis of metabolites, it was found that most naphthalenes substituted at the 1 and 2 position are transformed by the NAH upper pathway to their respective salicylate analogs. The heterocyclic compounds which were examined were characterized by an analogous cleavage of one aromatic ring. Substitution on both rings led to incomplete upper pathway metabolism and the formation of dead-end, hydroxylated parent compounds.

The ability of the cloned lower pathway genes for salicylate hydroxylase and catechol-2,3-dioxygenase of *P. fluorescens* 5R to cleave commercially available salicylate analogs and upper pathway metabolites was also examined. It was found that the lower pathway is limited in its ability to cleave many upper pathway products, except those with methyl- or chloro- substituents. The ability of these products to act as a surrogate for salicylate and induce NAH system transcription was examined using *P. fluorescens* 5RL. Many compounds were found to induce transcription. In general, with respect to the ability of the NAH system to facilitate growth on these substituted PAHs and heterocycles, only those compounds which undergo both the upper and lower pathway cleavage reactions and produce an inducer of transcription served as sole carbon and energy sources.

The capacity of the NAH system to preferentially degrade a mixture of PAHs and heterocycles was examined using *P. fluorescens* 5R and 5RL, an *E. coli* harboring the

gene for naphthalene dioxygenase (*nahA*), and a number of other bacterial isolates possessing the NAH system. It was found that naphthalene dioxygenase dictates the kinetics of mixed PAH degradation. In addition, preferential rates of degradation occurred for those compounds of least substitution which most resemble naphthalene structurally.

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PpG7

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Chapter I

Introduction

Over the last thirty years much information has been gained about the basic mechanistic framework of the degradation of naphthalene and a small number of other low-molecular weight polycyclic aromatic hydrocarbons (PAHs), such as anthracene and phenanthrene, by the NAH enzyme system of bacteria. However, given the almost ubiquitous presence of the NAH system in bacteria found at PAH-contaminated sites, it is relevant to examine the basic capacity for NAH-mediated transformation of other PAHs, such as methyl-substituted naphthalenes. This is especially relevant because microbial bioremediation of PAHs has been suggested as a treatment strategy for removing these low-molecular-weight polycyclic aromatic compounds [Mueller et al., 1989; Wilson and Jones, 1993; Luthy et al., 1994]. In addition, other types of substituted naphthalenes, such as halogenated naphthalenes, have been found at a number of contaminated sites. Therefore, it is of interest to determine the degradative potential of the NAH system for other structurally similar environmental contaminants.

Previous examinations of the degradative potential of the NAH system toward environmental contaminants have encompassed a thorough survey of the biotransformation products formed during parent compound catabolism. However, in spite of the wealth of information that such an experimentation provides, it does not give a complete understanding of other ancillary, yet vital, processes which occur simultaneously with parent compound degradation and biotransformation. For example, in order to induce the NAH enzyme system to maximum levels, the initiation of transcription of NAH system genes must be accomplished via an interaction between RNA polymerase, the NahR regulatory protein, and salicylate. The process of transcription induction is dependent upon the ability of the NAH system to transform a contaminant to salicylate or a salicylate analog, and the ability of this product to interact

favorably with the other components of the regulatory system. If either of these processes does not occur, then the operon is expressed at only basal levels and can only inefficiently utilize the compound as a carbon and energy source. If these two processes do occur successfully, then the organism must be able to further degrade the salicylate product and utilize it through Krebs cycle reactions. The success or failure of these reactions to occur defines the catabolic versatility of the NAH system with respect to various compounds.

An examination of the versatility of the NAH system is therefore not simply the examination of one of the above processes, but an effort to elucidate how all of the processes work together to determine the fate of a contaminant compound. Typically, such studies are performed using experiments to examine metabolism of individual compounds. However, such studies must be extended to elucidation of the degradation of mixed PAHs and other co-contaminants in the environment. For example, it is currently unclear which of the processes associated with the degradation of naphthalene are essential factors in the degradation of a contaminant mixture. This is important because PAH contaminants are rarely found alone in the environment. Rather, they exist in multicomponent mixtures of extremely diverse composition. Many physicochemical factors have been shown to contribute to the preferential rates at which PAHs in a mixture are degraded in the environment; however, the effect(s) of the many associated biological factors on preferential degradation is unknown. In addition, the effect(s) of the inherent structural diversity of PAHs and other similar contaminants on mixture degradation has not been examined.

The focus of this research was to develop a comprehensive understanding of the catabolic versatility of the NAH system toward a number of substituted naphthalenes, PAHs, and heterocycles, both singly and in diverse mixtures. This understanding is expected to contribute to better predictions on the fate of PAH contaminants in the environment, and to calculation of the environmental and ecological risk associated with

PAH contamination and remediation. Toward this end, the following specific objectives were examined:

- 1.) To identify the biotransformation products formed during the NAH system mediated degradation of co-contaminants.
- 2.) To determine the role of these biotransformation products as potential inducers of NAH system transcription.
- 3.) To elucidate which alternative and co-contaminant compounds serve as sole carbon and energy sources.
- 4.) To quantify the kinetics of mixed co-contaminant degradation mediated by the NAH system

Chapter II

Literature Review

The Presence of Polyaromatic Hydrocarbons (PAHs) in the Environment

During the early part of this century the United States relied heavily upon coal as a primary source of the natural gas used for heating and municipal lighting. As a result, large quantities of PAHs were produced as byproducts and released to the environment [English et al., 1992]. Years later these compounds continue to persist in contaminated soil as complex, multicomponent mixtures consisting of many PAHs of various structures and alkyl-substitutions. For example, in an analysis of supercritical fluid extracts of coal tar-contaminated soils, Yu et al. [1990] identified 120 different PAHs which range in molecular weight from 128 (naphthalene) to 302 (ex. dibenzo[*a,e*]pyrene). In general, PAHs with three or more rings were most prevalent. Among the lower molecular weight PAHs, naphthalene, mono-, di-, and trimethylnaphthalenes, fluorene, methylfluorenes, and methylbiphenyls were most predominant; however, they were present at much lower concentrations than the PAHs with three or more rings.

PAH-contaminated sites are also found globally in countries where industrial activity has led to uncontrolled waste disposal. For example, the study of an uncontrolled hazardous waste dump near the Nervion River in northern Spain by Villanueva et al. [1991] showed that the composition of PAH mixtures in sampled areas was dependent upon the presence of either industrial naphthalene mixtures, coal tars, and/or lubricating oils as source materials. Within those areas contaminated by industrial naphthalene mixtures, naphthalene mono- and dimethylnaphthalenes, fluorene, phenanthrene, and the heterocyclic compound dibenzofuran were the predominant contaminants. The contaminant profile of areas contaminated by coal tars resembled that observed by Yu et al. [1990] (see above). Halogenated PAHs, such as chlorinated naphthalenes, have been found to be present in municipal incinerator fly ash [Sovocool et al., 1988].

Hydroxylated asphaltine compounds have been found in coal-derived liquids [Cooper et al., 1986].

The occurrence of alkyl-substituted naphthalenes and other PAHs in the effluent of certain waste water treatment facilities of New York City has been shown by Tanacredi [1990]. The data presented illustrate the input of PAH contaminants into the Jamaica Bay estuarine system as a result of everyday municipal activities.

It should be noted that PAHs are also naturally introduced into the environment as a result of forest fires, volcanic eruptions and/or any other process which results in the incomplete combustion of organic material. The presence of PAHs has been detected in barley grains [Kirchmann and Tengsved, 1991]; it is thought that this has resulted either from the atmospheric deposition on plants of PAHs from fossil fuel combustion or root uptake of PAHs in the soil.

The Health Effects of PAHs and their Metabolites

PAHs and their metabolites display a variety of mechanisms of toxicity. Low molecular weight PAHs such as naphthalene and methyl-substituted naphthalenes are often individually soluble within a range of 1 to 30 mg/L, and are acutely toxic to higher eukaryotes through the mechanism of nonpolar narcosis; these hydrophobic compounds have been shown to partition into the lipid bilayers of both prokaryotic and eukaryotic cells to produce a reversible effect in which normal homeostatic processes, such as the maintenance of a membrane potential are interrupted until the compound(s) is removed [Sikkema et al., 1994 and 1995].

Higher molecular weight PAHs, such as benzo[a]pyrene (MW = 252), are much less water-soluble (less than 1 mg/L). They have been shown to be precursors to potent mutagens and carcinogens in humans. These compounds are typically oxidized in Phase I detoxification reactions by the cytochrome P-450 enzyme system prior to Phase II conjugation reactions and excretion. Phase II reactions protect against the possible

damaging effects of the products of Phase I oxidation [Prester et al., 1993]. The products from Phase I reactions, such as (+)-benzo[*a*]pyrene-7,8-diol-9,10-epoxide from the oxidation of benzo[*a*]pyrene, are reactive electrophiles which form DNA adducts and result in genotoxic effects [Williams and Weisburger, 1993]. For example, human red blood cells have been found to conduct Phase I reactions on benzo[*a*]pyrene [Lo Jacono et al., 1992]. An examination of the toxicity of a solvent extract from a coal-contaminated sediment has found that high-molecular-weight PAHs, such as benzo[*a*]pyrene, benzo[*ghi*]perylene, and indeno[1,2,3-*cd*]pyrene, were primarily responsible for mutagenic activity in an Ames *Salmonella typhimurium* assay [Marvin et al., 1995].

Known metabolites of bacterial degradation of naphthalene have been shown to be acutely toxic at physiological concentrations through a variety of mechanisms. Salicylate has been shown to be a mitochondrial respiratory uncoupler [Gutknecht, 1992]. Catechol, a metabolite in the bacterial degradation of naphthalene, and hydroquinone, a eukaryotic metabolite of benzene degradation, were found to act synergistically to disrupt the mitotic spindle in cultured human lymphocytes and interfere with chromosome segregation [Robertson et al., 1991].

A number of pure culture studies have revealed the accumulation of metabolites of unknown toxicity during the biodegradation of several PAHs by microorganisms isolated from contaminated sites. In each case the hydrophobic parent compound is transformed to a polar, more water-soluble product. Grifoll et al. [1995a] have shown that fluorene is utilized as a growth substrate by *Pseudomonas cepacia* F297 with the subsequent accumulation of 1-indanone. In addition, when this strain was grown in a mixture of creosote PAHs it was shown to accumulate the following dead-end metabolites: 4,5-dimethylsalicylate from 2,3-dimethylnaphthalene, 1-hydroxy-2-napthoic acid from phenanthrene, 2-hydroxy-3-napthoic acid from anthracene, 3-hydroxy-2-formylbenzothiophene from dibenzothiophene, and acenaphthenone and acenaphthoquinone from acenaphthene and acenaphthalene [Grifoll et al., 1995b]. A recent paper by Schneider et al. [1996] showed the accumulation of *cis*-7,8-dihydrodiol-

benzo[a]pyrene from the metabolism of benzo[a]pyrene by *Mycobacterium* sp. strain RJGII-135. The accumulation in the environment of metabolites from the biodegradation of PAHs is unclear at this time. In a study by Heitkamp et al. [1987], it was demonstrated that low amounts of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene, 1-naphthol, salicylate, and catechol could be detected in estuary sediment exposed to naphthalene. However, little can be said at this point about whether they may be ecotoxicologically deleterious compounds.

Instances have been observed in which the biodegradation of other types of aromatic contaminants has not led to a significant reduction in the toxicity of site material. For example, the biodegradation of pentachlorophenol (PCP) by *Pseudomonas* sp. SR3 resulted in an increase in teratogenicity and embryotoxicity to the inland silverside fish *Menidia beryllina* [Middaugh et al., 1993]. A study by Gersberg et al. [1995] demonstrated that bioremediation of a gasoline-contaminated aquifer in San Diego, California, removed virtually all contaminants but did not completely eliminate the toxicity to the test organism *Ceriodaphnia dubia*. In both instances, the failure of parent contaminant biodegradation to reduce toxicity was believed to be due to the production of toxic intermediate metabolites. However, in each study direct analytical evidence proving an association between metabolite accumulation and toxicity was inconclusive and warrants further investigation.

Catabolic Enzymes of the NAH System

The degradative enzymes of the NAH system are organized into an upper and a lower pathway, which are encoded by the *nah* (*nahABFCED*) and *sal* (*nahGHINLJK*) operons, respectively [Eaton and Chapman, 1992; Yen and Gunsalus, 1982; Harayama et al., 1987] (Transcriptional regulation is discussed in the next section) (figures 1 and 2). The upper pathway mediates the degradation of naphthalene to salicylate through an initial dioxygenase attack and subsequent *meta* cleavage of one of the aromatic rings. Salicylate

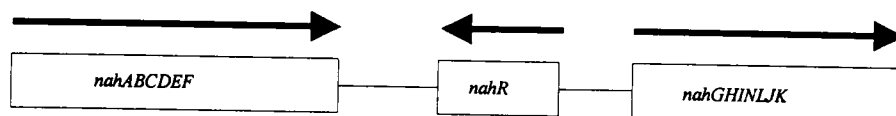
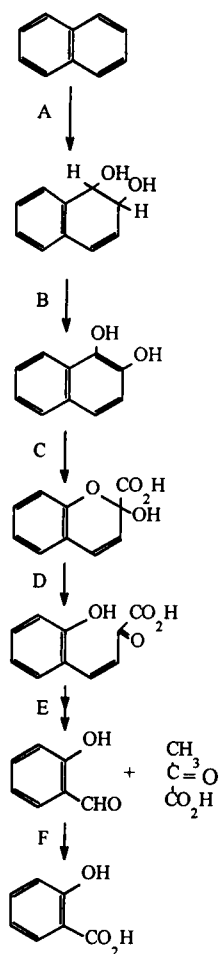


Figure 1. Genetic Organization of the NAH System. Arrows represent the direction of transcription.

Naphthalene Oxidation



Salicylate Oxidation

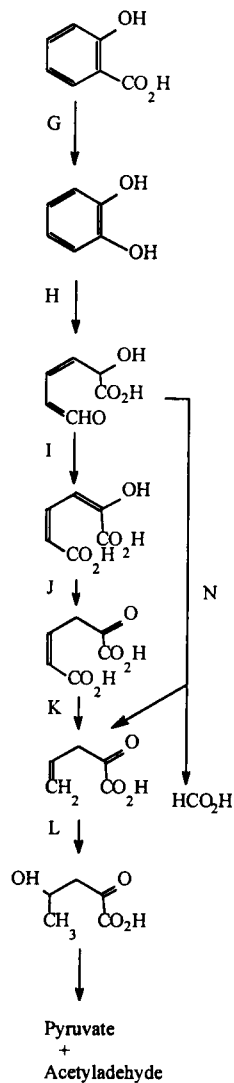


Figure 2. NAH Pathway for the Degradation of Naphthalene by Bacteria. Enzymes: A - naphthalene dioxygenase, B - *cis*-naphthalene dihydrodiol dehydrogenase, C - 1,2-dihydroxynaphthalene dioxygenase, D - 2-hydroxychromene-2-carboxylate isomerase, E - 2-hydroxybenzalpyruvate aldolase, F - salicylaldehyde dehydrogenase, G - salicylate hydroxylase, H - catechol dioxygenase, I - 2-hydroxymuconic semialdehyde dehydrogenase, J - 2-hydroxymuconate tautomerase, K - 4-oxalocrotonate decarboxylase, L - 2-oxo-4-pentenoate hydratase, N - 2-hydroxymuconic semialdehyde hydrolase [Adapted from Yen and Gunsalus, 1982; Yen and Serdar, 1988; Eaton and Chapman, 1992].

is degraded by the lower pathway through a second *meta* cleavage reaction to form pyruvate and acetaldehyde. In most cases, NAH system genes are plasmid-borne. The initial studies that first elucidated this fact were reported by Dunn and Gunsalus [1973] on plasmid pNAH7 using the environmental isolate *Pseudomonas putida* PpG7. A more recent study by Tsuda and Iino [1990] has indicated that the NAH system genes of plasmid pNAH7 reside within a defective transposable element. Since then a number of additional strains and their associated NAH plasmids have been studied in great detail. One of the most notable has been *Pseudomonas putida* NCIB 9816 [Serdar and Gibson, 1989], which contains the 83 kb plasmid pDTG1.

The initial incorporation of both atoms of the diatomic oxygen molecule into the structure of naphthalene to form *cis*-(1*R*,2*S*)-dihydroxy-1,2-dihydronaphthalene is accomplished by multicomponent enzyme naphthalene dioxygenase (*nahA*). Naphthalene dioxygenase is comprised of three components, an iron-sulfur flavoprotein reductase (*nahAa*), a ferredoxin reductase (*nahAb*), and an iron-sulfur protein (*nahAcAd*) (These specific gene designations and associated sequences have been arrived at by Simon et al [1993]). The steps in the transformation of naphthalene by the multicomponent enzyme naphthalene dioxygenase are as follows. The iron-containing flavoprotein reductase transfers two electrons from NADH to the ferredoxin component. The ferredoxin component then transfers the electrons to the iron-sulfur protein component. The iron-sulfur component binds naphthalene, accepts the two electrons from the ferredoxin, and incorporates the two atoms of molecular oxygen into the naphthalene molecule.

The iron-sulfur component has been shown to be composed of two 55,000 MW (α) and two 20,000 MW (β) subunits [Ensley and Gibson, 1983]. Further examination by Suen and Gibson [1993] has shown that each of the two α subunits of the iron-sulfur component of naphthalene dioxygenase possesses one Rieske-type [2Fe-2S] center. Suen and Gibson postulate also that the two Rieske-type centers are involved in the transfer of electrons from the ferredoxin component to the active site of the enzyme within the iron-sulfur component. It is also thought that each α subunit possesses an iron-binding site

which is involved in the activation of oxygen via a poorly understood mechanism [Mason and Cammack, 1992]. To date the function of the β subunit is not known. The ferredoxin component has been found to have a molecular weight of 13,600, and is also believed to have a Rieske-type [2 Fe-2 S] center similar to that found in the iron-sulfur component of the naphthalene dioxygenase component [Haigler and Gibson, 1990] (See Mason and Cammack [1992] and Butler and Mason [1997] for a review of Rieske-type proteins.).

1,2-Dihydroxynaphthalene dioxygenase, encoded by the *nahC* gene, initiates the cleavage of the 1,2-dihydroxynaphthalene produced by the sequential action of naphthalene dioxygenase and *cis*-naphthalene dihydrodiol dehydrogenase (NahB). NahC has a molecular weight of approximately 34 kDa [Harayama and Rekik, 1989]; the enzyme utilizes ferrous iron as a cofactor.

Aside from naphthalene dioxygenase, the two other most well-studied enzymes of the NAH system are salicylate hydroxylase and catechol-2,3-dioxygenase. As mentioned earlier, the combined action of these two enzymes cleaves the upper pathway product salicylate and sets in motion the processes which make it available for Krebs cycle processing. This is a crucial point in the pathway because the inability of these enzymes to cleave an upper pathway product leads to dead-end product accumulation and the inability of the cells to utilize it as a carbon and energy source.

Salicylate hydroxylase (NahG) has an approximate molecular weight of 45,000 [You et al., 1991]; it transforms salicylate to catechol. It is a monooxygenase enzyme which removes the carboxy group from salicylate and replaces it with one atom of molecular oxygen [Katagiri et al., 1965]. The transformation of salicylate to catechol has been shown to be dependent upon the presence of flavin adenine dinucleotide (FAD) as a cofactor [Katagiri et al., 1962]. During the reaction, NADH is oxidized as an electron donor in order to reduce FAD. FADH is then subsequently oxidized during the activation of oxygen prior to the formation of catechol [Katagiri et al., 1966].

A great deal of knowledge in particular surrounds the properties of catechol-2,3-dioxygenase (NahH). This stems in large part to the high similarity of the NAH system

catechol-2,3-dioxygenase to the isoenzymes of other degradative systems, such as XylE of the well studied TOL system of plasmid pWW0 for the degradation of toluene. Many studies on the degradation of catechol have been performed since the pioneering work of Dagley et al. [1960] because catechol is a common intermediate in the degradation of a number of small aromatic compounds [Smith, 1990]. The catechol dioxygenase of the NAH system has been found to be approximately 35 kDa, and is thought to function as a 70 kDa dimer [Ghosal et al., 1987]. It contains ferrous iron as a sole cofactor. In a study on the nucleotide similarities of *nahH* and *xylE*, it was found that the two share sequence homologies of 81% at the nucleotide level and 85% at the amino acid level [Ghosal et al., 1987] (Similar results have been reported by Harayama et al. [1987]). A more recent comparison of the nucleotide and amino acid sequences of *nahH* and *xylE* of plasmid pDK1 of *Pseudomonas putida* HS1 showed homologies of 78% and 83%, respectively [Benjamin et al., 1991]. It is interesting to note that this study also identified the greatest predicted amino acid sequence homology (97%) between the respective catechol-2,3-dioxygenases of plasmids pNAH7 and pWW0 as being localized to the region containing nucleotides 1 to 213, the N-terminus. Conversely, the region of lowest homology amino acid sequence homology (55%) was localized to nucleotides 214 to 312. The C-terminus region, nucleotides 313 to 450, had a predicted amino acid homology of 94%. The region of low homology in the mid-section of the two proteins may play a role in the slight differences in catalytic capabilities between the two proteins (this is revisited later in the discussion section).

It should be noted that similarity between the NAH and TOL systems is not limited to their respective catechol-2,3-dioxygenases. Assinder and Williams [1988] have shown that the *nah* genes downstream of *nahH* in the lower pathway share a high level of homology with the respective TOL system *xyl* genes. The genes are also organized in the same order in both systems. Assinder and Williams [1988] and Harayama et al. [1987] speculate that the two operons are derived from a common ancestor and that the lower pathway has been joined with the upper pathway and the *nahR* region to form the three

functional units of the NAH system. The mechanism by which this organization occurred to ultimately form the defective transposon Tn4655 [Tsuda and Iino, 1990] is uncertain.

Biotransformation of PAHs, Heterocycles, and Other Substituted Naphthalenes by NAH System Organisms

Using the environmental isolate *P. fluorescens* 5R (*nah*⁺ *sal*⁺) and the mutant *P. fluorescens* 5RL (*nah*⁺ *Sal*⁻) it was shown that anthracene and phenanthrene are transformed to the salicylate-like compounds 2-hydroxy-3-naphthoic acid and 1-hydroxy-2-naphthoic acid, respectively [Menn et al., 1993] (All reactions discussed in this section are represented in figure 3.). Menn et al. [1993, 1994] have also shown that *P. fluorescens* 5RL transforms fluorene to 9H-fluoren-9-one, 9-fluorenol, and 2,3-dihydro-1H-inden-1-one (1-indanone).

With respect to methyl-substituted naphthalenes in particular, Williams et al. [1975] have shown that *Pseudomonas* P_G (NCIB 9816) degrades 2-methylnaphthalene through 4-methylsalicylate to its *meta*-cleavage product 2-hydroxy-5-methylmuconic semialdehyde. Selifonov et al. [1996] have recently reported an alternative mechanism of transformation of dimethylnaphthalenes by the cloned naphthalene dioxygenase of plasmid NAH7. This mechanism, an apparent monooxygenation of a methyl substituent of 1,5-, 1,8-, 2,6-, and 2,7-DMN, resulted in the formation of the corresponding methanolmethylnaphthalenes. No direct dioxygenase attack on an aromatic ring of these dimethylnaphthalenes was reported by Selifonov et al [1996].

Other studies have shown that the NAH system is able to transform a broad range of compounds via a number of mechanisms. An elegant study by Resnick and Gibson [1996b] has shown that the naphthalene dioxygenase of *Pseudomonas* sp. Strain NCIB 9816-4 catalyzes the oxidation of fluorene, dibenzofuran, and dibenzothiophene to form *cis*-dihydrodiols as major products. Fluorene was transformed primarily to (+)-(3*S*,4*R*)-*cis*-3,4-dihydroxy-3,4-dihydro-9*H*-fluorene (80-90% yield); 9-Fluorenol was formed as a

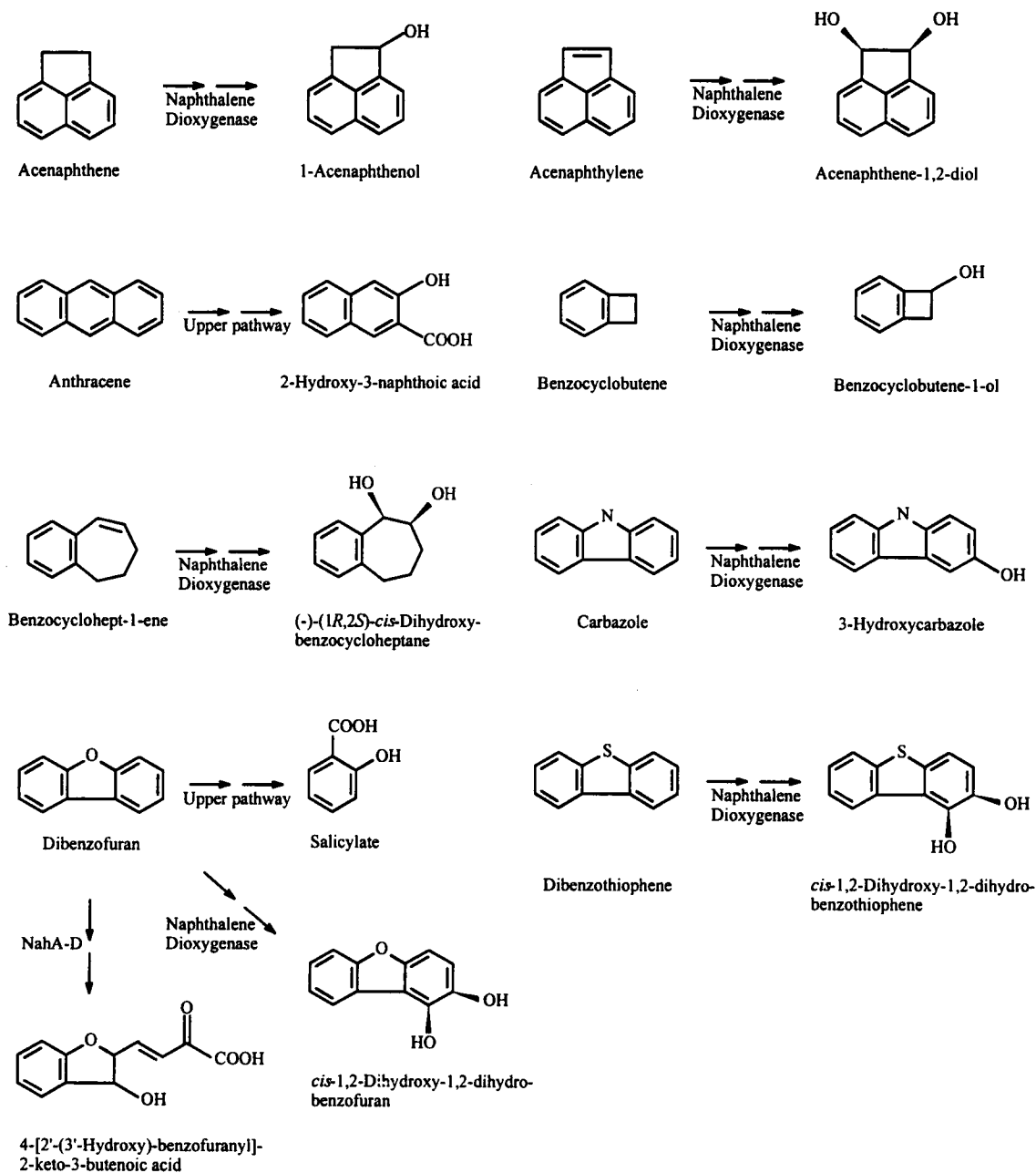


Figure 3. Selected Transformations of the NAH System. Note: the contribution of abiotic processes in certain transformations is discussed in the text. Postulated intermediates are not shown in this figure.

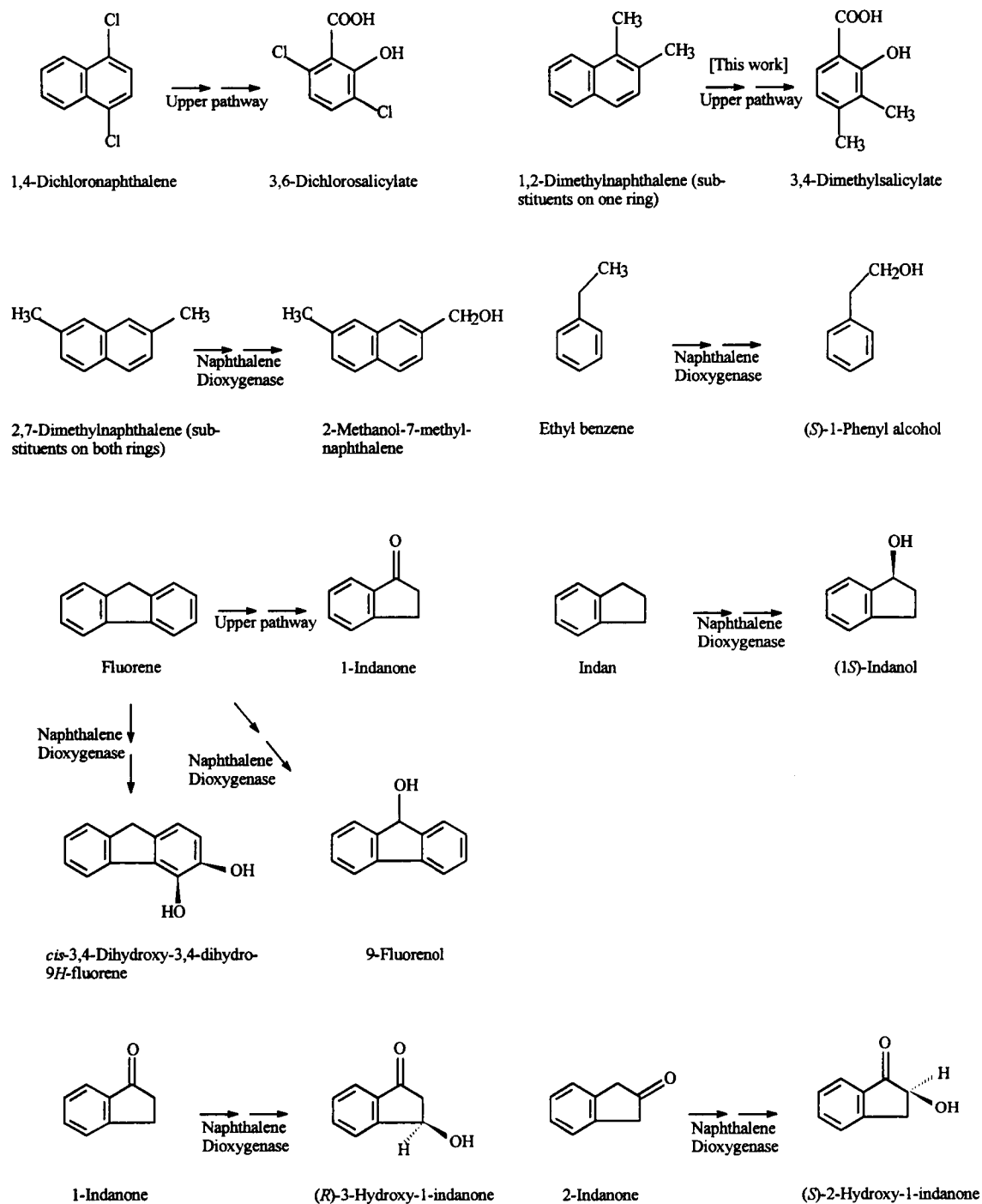


Figure 3 cont.

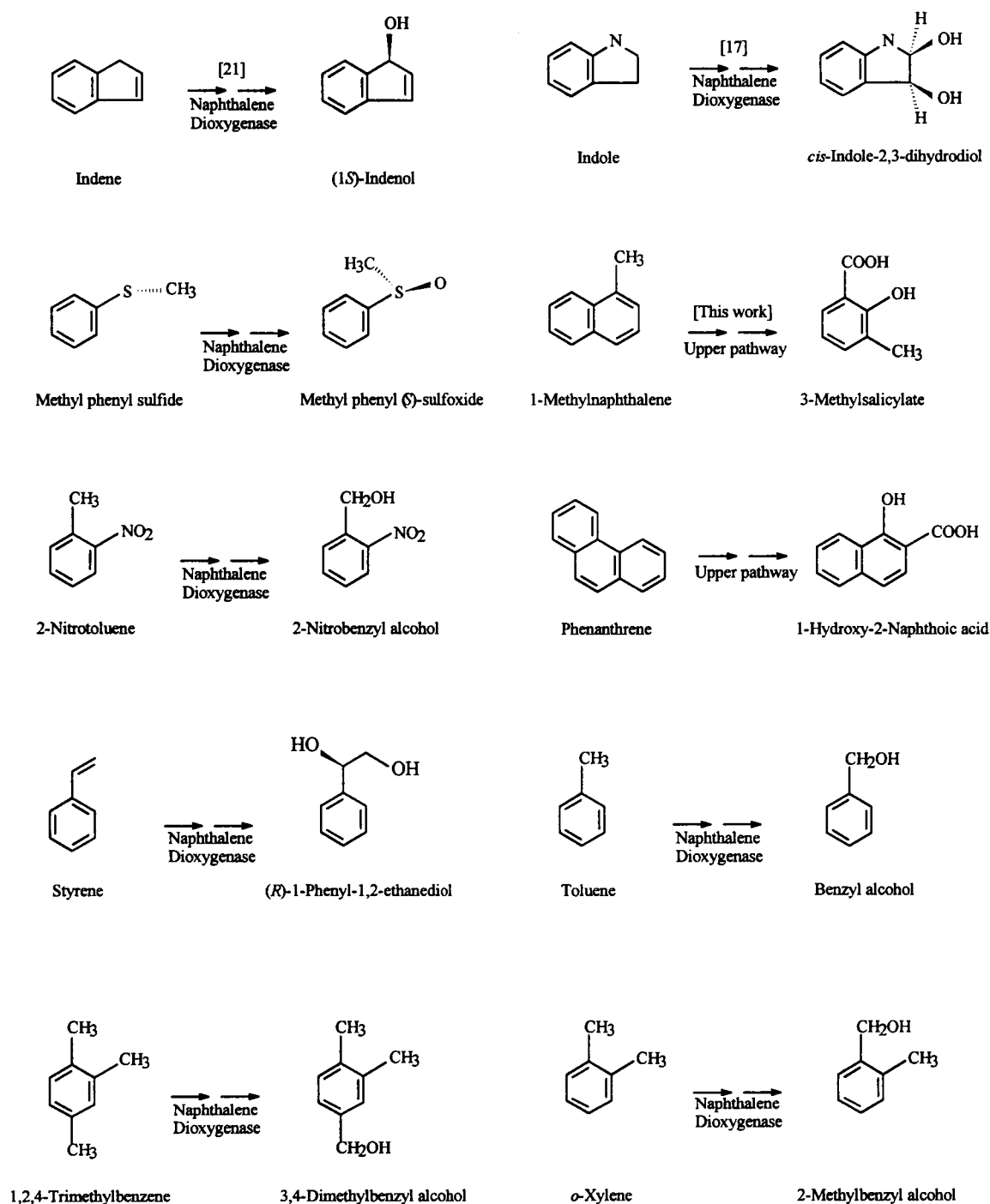


Figure 3 cont.

minor product (9-18% yield). Dibenzofuran was transformed to (1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzofuran and (3*S*,4*R*)-*cis*-3,4-dihydroxy-3,4-dihydrodibenzofuran with relative yields of 60-70 and 30-40%, respectively.

Dibenzothiophene was transformed by naphthalene dioxygenase of strain NCIB 9816-4 to (+)-(1*R*,2*S*)-*cis*-1,2-dihydroxy-1,2-dihydrodibenzothiophene (84-87% yield) and dibenzothiophene sulfoxide (9-14% yield). Resnick et al. [1993] have also shown that this strain oxidizes carbazole to 3-hydroxycarbazole. The authors were unsure whether this compound is formed by the dehydration of an unstable *cis*-carbazole-3,4-dihydrodiol or by a direct monooxygenation at the 3 position by naphthalene dioxygenase.

An earlier study by Selifonov et al. [1990] reported that the NAH system of *Pseudomonas putida* PpG7 transformed dibenzofuran to 4-[2'-(3'-hydroxy)-benzofuranyl]-2-keto-3-butenic acid, but was unable to cleave pyruvate from this molecule to form salicylate. Menn [personal communication] has shown that strain 5RL is capable of transforming dibenzofuran to salicylate.

Other examples of the ability of naphthalene dioxygenase to attack the aromatic ring structure of PAHs and heterocycles include the study by Selifonov et al. [1996] that demonstrated the formation of acenaphtho-1,2-quinone via the action of naphthalene dioxygenase from *P. putida* PpG7 on acenaphthene. Other oxygenated metabolites of acenaphthene, which represent the steps leading to the formation of this compound, were also identified. An earlier study by Schocken and Gibson [1984] had identified these metabolites in the degradation of acenaphthene by a *Beijerinckia* species. Ensley et al. [1983] have reported the transformation of the heterocyclic compound indole by the naphthalene dioxygenase of strain PpG7 to form the intermediate *cis*-indole-2,3-dihydrodiol. Resnick and Gibson [1996a] have shown that the naphthalene dioxygenase of *Pseudomonas* sp. strain 9816/11 transforms benzocyclohept-1-ene to (-)-*cis*(1*R*,2*S*)-benzocycloheptenediol. A study by Swanson [1992] has reported that a mutant of the naphthalene-degrading *Pseudomonas fluorescens* 127, which is apparently has a disrupted *nahB* gene, transforms the compound benzocyclobutene to benzocyclobutene-

1-ol. It is uncertain whether this product is that of a naphthalene dioxygenase-mediated monooxygenase reaction or a dioxygenation and subsequent loss of one hydroxyl group.

In certain cases, naphthalene dioxygenase has been shown unequivocally to catalyze monooxygenase reactions. For example, Resnick et al. [1994] have shown that strain 9816/11 transforms 1-indanone to (*R*)-3-hydroxy-1-indanone and (*R*)-2-hydroxy-1-indanone, and 2-indanone to (*S*)-2-hydroxy-1-indanone by strain 9816-11. Both indan and indene have been shown to be monooxygenated by the naphthalene dioxygenase of strain 9816/11 to form (1*S*)-indanol and (1*S*)-indenol, respectively [Gibson et al., 1995]. This study also demonstrated that naphthalene dioxygenase acts as a desaturase on indan to form indene, which is also further acted upon by naphthalene dioxygenase to form *cis*-(1*R*,2*S*)-indandiol. Torok et al. [1995] have also reported upon the ability of naphthalene dioxygenase to act as a desaturase. Lee and Gibson [1996a] have shown that naphthalene dioxygenase of strain 9816-4 transforms the monoaromatic compounds toluene, ethylbenzene, xylene isomers, and nitrotoluenes to benzyl alcohol, (*S*)-1-phenethyl alcohol, and the corresponding methylbenzyl alcohols, and nitrobenzyl alcohols, respectively, via monooxygenation of one of the substituents.

In a somewhat unusual and unexpected reaction, the naphthalene dioxygenase of strain 9816-4 was shown to transform styrene via dihydroxylation of the vinyl substituent to form (*R*)-1-phenyl-1,2-ethanediol [Lee and Gibson, 1996b]. No oxidation of the aromatic ring was detected. Lee et al. [1995] have also shown that naphthalene dioxygenase preferentially oxidized the sulfur atoms in the substituent groups of aryl alkyl sulfides; oxidation of the aromatic rings was not reported.

Few studies have examined the metabolism of halogenated compounds by NAH system enzymes. A study by Durham and Stewart [1987] has shown that *Pseudomonas putida* GX1005, which like strain 5RL has an inactive lower pathway due to a transposon insertion in *nahG*, is able to transform 1,4-dichloronaphthalene to 3,6-dichlorosalicylate. To date there are no reports of the ability of the NAH system to transform any PAHs with more than three rings.

Regulation of NAH System Transcription

Transcription of both the *nah* and *sal* operons is positively regulated by the NahR regulatory protein in the presence of salicylate, the product of the upper pathway-mediated degradation of naphthalene. In the absence of salicylate, a basal level of transcription of both the *nah* and *sal* operons occurs. This ultimately permits the low-level degradation of naphthalene and hence the formation of enough salicylate to lead to higher levels of expression. Once formed, salicylate interacts with NahR to facilitate transcription through the formation of fully operational RNA polymerase complexes on the promoters of the *nah* and *sal* operons [Huang and Schell, 1991; Schell and Poser, 1989; Schell et al., 1990].

Early insight which identified positive regulation as the mechanism of control for both operons was obtained from the work of Yen and Gunsalus [1985]. These researchers found that a Tn5 insertion into a region a few hundred bases upstream of the *nahG* gene for salicylate hydroxylase of plasmid NAH7 caused a loss of induction of enzymes in both operons. When *nahR* was supplied in *trans* the mutation was complemented. The early work of Schell [1985] also indicated that *nahR* is integral in the positive regulation of the *nah* and *sal* operons. The experiments of You et al. [1988] and Schell and Wender [1986] led to the determination that NahR is an approximately 35 kDa DNA-binding protein. Using subcloned portions of the *nahR* gene from plasmid NAH7 as target sequences in gel electrophoresis DNA-binding assays, these researchers localized the DNA-binding activity of NahR to the COOH-terminal domain. At the time of publication, it was also found that this region showed DNA sequence homology to the binding regions of the lambda repressor and the lambda Cro protein, which are two known α -helix-turn- α -helix DNA-binding proteins. More recent experimentation by Schell et al. [1990] has more accurately identified the following regions which are essential for the binding of NahR to the *nah* and *sal* promoters. The first spans residues 239 to 291 of the COOH-terminal domain, and the second spans residues 23-45 of the NH₂-terminal domain. NahR proteins that possess mutations in either of these regions

are defective in both their ability to bind and to activate transcription from the *sal* promoter. Schell et al. [1990] also elucidated that a third region, spanning residues 126-206, is essential for activation of transcription but not for binding to the promoters.

NahR, which is a member of the LysR family of regulatory proteins [Schell and Sukordhaman, 1989], is encoded by the *nahR* gene and is divergently transcribed from a locus immediately upstream of the transcriptional start site of the *sal* operon [Schell, 1990; Schell and Poser, 1989; You et al., 1991]. Using S1 nuclease mapping and sequence analysis, Schell [1986] elucidated the location and sequence of the *nahR* gene promoter. It was found that the start of the *nahR* promoter overlaps with the -35 region of the adjacent, divergently transcribed *sal* promoter [Schell and Wender, 1986]. Because of this overlap, transcription of the *nahR* gene, which does not require the NahR protein, is negatively autoregulated because it cannot be transcribed when NahR and RNA polymerase are bound to the *sal* promoter. Analysis of the sequences of the *nah* and *sal* promoters has also produced insight as to why these two operons are coordinately regulated in the presence of salicylate (see below). The two promoters share three highly homologous regions (given as percentages): 1.) -80 to -60 (81%), 2.) -40 to -28 (75%), and 3.) -1 to +15 (70%) [Schell, 1986].

Insight into the mechanism of transcriptional activation by the NahR protein was brought forth by Schell and Poser [1989] in a series of DNA-binding experiments involving a 390 bp fragment which contained the *nahA* promoter and a portion of the *nahA* gene. Their studies found that NahR binds equally well to the *nah* operon promoter in both the presence and the absence of salicylate. In particular, they found that through footprinting assays that the -82 to -47 regions of both the *sal* and *nah* promoters, respectively, are bound by NahR under both conditions. Bound NahR, in the form of a complex with salicylate, promotes conformational changes in the DNA of the promoter regions initiation of transcription by RNA polymerase [Huang and Schell, 1991]. These conformational changes are believed to be the driving force behind the activation of transcription by NahR and salicylate.

Environmental Predominance of NAH System Bacteria at PAH-Contaminated Sites and the Degradation of PAHs by Other Genetic Systems

A vast number of bacteria have been isolated from a number of environments for their ability to grow on naphthalene and/or other PAHs. In the presence of either naphthalene or salicylate, many strains show inducible enzyme activities which correspond to NAH system enzymes, such as naphthalene dioxygenase, salicylate hydroxylase, and catechol 2,3-dioxygenase [Garcia-Valdes et al., 1988; Tagger et al., 1990]. However, the exact genetic composition of their degradative system(s) is often undetermined.

Because of the demonstrated ability of NAH system organisms to degrade certain PAHs, the *nahA* gene of the upper pathway has traditionally been used as a determinant in the quantification of indigenous PAH-degrading bacteria via gene-probing techniques. Organisms containing *nahA* gene sequences are broadly distributed in the environment and are positively enriched in manufactured gas plant (MGP) and creosote-contaminated soils [Herrick et al., 1993; Sanseverino et al., 1993b] (Herrick et al. [1997] have recently presented evidence which links the ubiquitous nature, in a global sense, of *nahA* to natural horizontal transfer of plasmid-borne genes responsible for naphthalene degradation). In such soils population densities of NAH system organisms can approach 10^{10} organisms per gram of soil based on DNA extraction and detection with *nahA* gene probes [Sanseverino et al., 1993b]. In general, the population densities arrived at via direct DNA extraction and probing were one to two orders of magnitude higher than those densities achieved through colony hybridization techniques. These discrepancies may have implications concerning the interpretation of data from other similar studies that do not utilize direct DNA extraction in their estimates of the population densities of PAH degraders. In addition, it has been possible to demonstrate that the genes are functionally expressed in contaminated soils by measuring quantitatively the mRNA abundances of the genes and correlating the mRNA with mineralization rates of naphthalene [Fleming et al., 1993; Sanseverino et al., 1993b].

Bacteria that possess the NAH system have also been observed in environments not traditionally considered to be as highly enriched for PAH degrading bacteria at MGP or creosote-contaminated sites. For example, using ^{32}P -labelled DNA from plasmid DTG113 (a NAH system plasmid) as a probe in colony hybridization procedures, Blackburn et al. [1987] have determined the presence and phenotypic activity of NAH system organisms which were amended to activated industrial sludge. These researchers were also able to correlate the *nah* genotype in a bioreactor containing the sludge with the removal and eventual mineralization of ^{14}C -naphthalene from a stream of industrial wastewater. MacGillivray and Shiaris [1994] have also presented evidence for the presence of organisms with a NAH system genotype in PAH-contaminated freshwater and marine sediments. Colony hybridization experiments which utilized a *nahABCD* probe (derived from plasmid NAH7) with naphthalene-degrading bacteria isolated aerobically from estuarine, freshwater, and soil environments revealed a number of key insights. On the average, *nahABCD*⁺ organisms comprised 48.5% of all naphthalene degraders isolated from all three environments. Interestingly, the estuarine and freshwater environments were characterized by the presence of only 28.8 and 22.6% *nahABCD*⁺ naphthalene degraders, respectively. Conversely, 87.2% of the naphthalene degraders in the soil environment were *nahABCD*⁺. Garcia-Valdes et al. [1994] have also presented evidence for the presence of the *nahGH* genes in a naphthalene degrading *Pseudomonas stutzeri* strain isolated from a marine environment.

To date, the degradation of unsubstituted PAHs by other genetic systems has in most cases revealed very little genetic and mechanistic divergence from the framework of the NAH system. Denome et al. [1993] and Kiyahara et al. [1994a, 1994b] have cloned systems which possess a high degree of sequence similarity to NAH system genes, and which carry out identical reactions. However, this does not preclude the involvement of other apparently unrelated systems in the degradation of PAHs. Goyal and Zylstra [1996] have recently reported the cloning from three *Comamonas testosteroni* strains of genes which are responsible for the degradation of naphthalene and phenanthrene, yet which show no apparent homology to the NAH system. Fredrickson et al. [1991] have also

reported the isolation of an organism which mineralizes ^{14}C -naphthalene but does not hybridize to DNA probes derived from plasmid pNAH7. Foght and Westlake [1991] have also reported that using probes derived from NAH system genes may miss a number of non-NAH PAH-degrading bacteria.

The vast majority of studies of PAH-degrading bacteria, whether they be pure culture studies or examinations of the environmental distribution of PAH-degrading bacteria, have been conducted under mesophilic conditions. Whyte et al. [1997] have isolated psychrotrophic strains from Baffin Island, NWT, Canada, which utilize naphthalene and possess an archetypal naphthalene dioxygenase gene. Sediment cores taken from the ocean floor of the Guaymas Basin in the Pacific Ocean were examined over a range of incubation temperatures for the ability of autochthonous organisms to mineralize ^{14}C -labelled naphthalene. Evidence was found for mineralization of naphthalene at temperatures up to 55°C ; no mineralization occurred at 80°C [Bazylnski et al., 1989]. It would be an interesting scientific endeavor to further examine the biodegradative abilities of organisms from these less studied environments.

The Biodegradation of PAH Mixtures

Compared to the study of the biotransformations of individual PAHs and related compounds by PAH-degrading bacteria, relatively little is known of the biodegradation of PAH mixtures. Specifically, there is a general paucity of information concerning the mechanistic and kinetic aspects of the degradation of complex contaminant mixtures by bacterial species. This is true when examined either on the scale of biodegradation in the environment by a consortium of microorganisms, or by a single species in the laboratory with a defined enzyme system for PAH metabolism. In spite of this deficiency, the gathering of such information is a vital conceptual leap from the study of the degradation of individual PAHs since it is well established that PAHs exist in the form of diverse mixtures at contaminated sites. The ability to better understand the process of PAH

mixture degradation may lead to more effective treatment regimes. However, it is in fact the diverse nature of these contaminant sites, and the experimental difficulties surrounding their study, that has hindered the study of mixture degradation. There is often little homogeneity in the composition of the contaminant mixture throughout an actual contaminated site [Harkey et al., 1995]. In addition, the original composition and concentrations of contaminants is often unknown. Therefore, it is difficult to ascertain what has been degraded over an unknown period of time at an actual contaminated site prior to scientific examination.

Although contaminated sites may be characterized by the predominance of bacteria with the NAH, or a related, degradative system, these organisms will most likely be in a wide variety of nutritional states and metabolic activity due to a variety of physicochemical conditions associated with their immediate environment. For example, contaminated sites are characterized by variations in temperature, moisture content, available nutrients, pH, and oxygen tension. The physicochemical properties of PAHs themselves is also an important factor to consider. A number of studies have shown that PAHs, particularly those of high molecular weight, adsorb very strongly to the surface of soil particles and organic matter and are hence not readily available for biodegradation [Weissenfels et al., 1992; Fu et al., 1994]. Fu et al. [1994] observed that 30 to 50% of naphthalene, the most soluble PAH, resisted desorption from an Oklahoma soil consisting of 92% sand. Guerin and Boyd [1992] have shown that the ability to mineralize naphthalene sorbed to different types of soil is variable for different naphthalene-degrading bacterial strains. They concluded that organism-specific properties, such as possible surfactant production, also affect the amount of naphthalene available for degradation. In addition, the dissolution rate of crystalline PAHs not attached to soil particles has been shown to be a limiting factor in their bioavailability to bacteria and hence their slows their biodegradation [Thomas et al., 1986; Stucki and Alexander, 1987; Volkerling et al., 1992].

The few studies that have been performed on the degradation of PAH mixtures have typically been conducted in two different manners. The first has essentially been

centered upon the degradation of PAHs in a mixture by a well-characterized bacterial isolate(s) under ideal laboratory conditions (i.e. none of the physical condition variation found at contaminated sites) using an artificial, defined contaminant mixture. This approach is generally made in an attempt to avoid the experimental difficulties described above. The second manner of experimentation has been based more upon the recreation of what occurs in the environment using microcosm-type experimental setups. Typically, PAH-contaminated soil, which is usually artificially contaminated, is incubated under certain conditions and the disappearance of PAHs is traced analytically. Both methodologies have their benefits and deficiencies. The first manner of experimentation involves much fewer variables, and thus provides much clearer insight as to the role of specific variables in the degradation of a mixture. Conversely, it may not simulate the environmental factors and processes as well as the second manner and thus ignores an important variable found only under actual environmental conditions. The second manner is intended to be a better simulation of what may happen in the environment. However, the experimental difficulties associated with adsorption of PAHs to soil cannot be avoided. More studies have been conducted in the second manner. A number of relevant ones will be discussed here.

A study by Trzesicka-Mlynarz and Ward [1995] on the degradation of a simple mixture of PAHs (acenaphthene, fluorene, phenanthrene, fluoranthene, anthracene, and pyrene) by individual and mixed cultures of fluoranthene-degrading bacteria has shown that a mixture of four different species was more effective in degrading these PAHs than any single organism. The reason for this is unknown; however, it is speculated that certain strains may act to remove intermediates produced by other members of the consortium and hence facilitate total PAH removal. Degradation kinetics were not extensively examined in this study.

In a similar study by Mueller et al. [1991], the degradation of a 42-component mixture (intended to be representative of the compounds found at a creosote-contaminated site) by an uncharacterized inoculum of creosote-degrading organisms showed a pattern of preferential degradation. Many of the cresol-type compounds were most rapidly

degraded by the consortium; low molecular weight PAHs, such as naphthalene, biphenyl, and methyl-substituted naphthalenes were also degraded rapidly. Anthracene and 2-methylanthracene were among the most recalcitrant compounds. This study is also one of the few that have attempted to link the biodegradation of a contaminant mixture with a decrease in toxicity. For unknown reasons, the toxicity and teratogenicity of this mixture were not significantly decreased after biodegradation.

In an examination of the degradation of binary mixtures of naphthalene and anthracene, and naphthalene and phenanthrene, by an uncharacterized consortium of organisms in sediment taken from a salt marsh on Long Island, NY, it was found that naphthalene was always the more rapidly degraded component [Bauer and Capone, 1988]. The authors of this paper did not interpret their results as evidence for the preferential degradation of one PAH over another. However, their data clearly show that under all of their experimental conditions naphthalene was more readily degraded than either anthracene or phenanthrene.

In a study of the ability of a native consortium of microorganisms from the beach material of Prince William Sound, Alaska, to degrade a mixture of aliphatic and aromatic hydrocarbons representative of those found in the non-volatile portion of Alaskan North Slope crude oil, it was found that a preferential degradation of certain PAHs and heterocycles did occur [Elmendorf et al., 1994]. A generalized trend in which the ring structure of the compounds dictated the nature of preferential degradation showed that the rates of degradation were naphthalenes>fluorenes>dibenzothiophenes>phenanthrenes>chrysenes. The authors also observed that an increase in the methyl substitution of each type of ring slowed the rates of degradation. In a separate study by Heitkamp and Cerniglia [1987], a general trend of PAH degradation was seen in freshwater and marine microcosms. They observed that the preferential degradation of a mixture occurred in the order of naphthalene>phenanthrene>2-methylnaphthalene>pyrene>3-methylcholanthrene>benzo[α]pyrene. Grosser et al. [1995] have also presented a study on the comparative mineralization of a number of higher-molecular-weight PAHs. They also observed that an increase in ring number slowed the rates of mineralization.

A recent paper by Stringfellow and Aitken [1995] has examined the biodegradation of binary PAH mixtures by two soil isolates, *Pseudomonas stutzeri* P-16 and *Pseudomonas saccharophila* P-15, able to grow on naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, and phenanthrene. These experiments, conducted under ideal laboratory conditions, demonstrated that the presence of either naphthalene, 1-methylnaphthalene, 2-methylnaphthalene, or fluorene in a binary mixture with phenanthrene, acted to competitively inhibit the uptake and degradation of phenanthrene. Although this paper presents valuable information on the effect of the presence of one PAH on the degradation rate of another, the analytical processes in the experiments involved the use of a spectrophotometer for tracing phenanthrene disappearance and were not sufficient enough to determine the rates of disappearance for the other component of the mixture. This methodology is also limiting in that it does not provide the potential to examine the fates of compounds in mixtures of greater complexity.

Structure-Activity Relationships (SARs)

Investigations of the type of chemical structure of a substance which will interact with a molecular site of action and produce a well-defined endpoint are commonly referred to as structure-activity studies. The objective of structure-activity analyses is to define as accurately as possible the limits of variation in the structure of a chemical moiety that are consistent with the production of a specific effect and the ways in which alterations in the environment of this moiety and overall properties of the molecule influence activity.

The structure of a chemical substance is typically given as a two-dimensional representation of the molecule. However, its biodegradability is a manifestation of the actual three-dimensional structure. Associated with the three-dimensional structure are molecular properties (descriptors). In addition to molecular properties, there are also associated physicochemical properties, chemical properties, and binding properties. Molecular properties include electron distribution, spatial disposition (conformation,

geometry, and shape), and molecular volume. Physicochemical properties include hydrophobicity and solubility, whereas chemical properties include electrophilicity. Binding properties are important in receptor-mediated activities such as biodegradation. As discussed later, the interaction between a compound and naphthalene dioxygenase is a crucial step in determining its rate of biodegradation. The SAR approach is based on two assumptions. The first is that the structure of a molecule (i.e., its geometric and electronic properties) must contain the features responsible for its physical, chemical, and biological properties. The second assumption is that chemical(s) must be able to be represented by one or more numerical molecular descriptors.

The biodegradability of a compound is governed by its various molecular, chemical, and physicochemical properties, which in turn are determined by its chemical structure. Therefore, there is an interrelationship of the structure, properties, and biodegradability. To be biodegraded two events must take place. First the compound must move from the environment into the biota (e.g., bacterium). Secondly the compound must interact with an intracellular target site (i.e., an enzyme). The first of these eventualities is the toxicokinetics phase, which includes compound uptake and compound movement within the organism itself. The second eventuality is the toxicodynamic phase. It includes interaction at the enzyme as well as the resulting biochemical and cellular effects which result from the interaction. While progress is being made in modeling biodegradation using SAR methodology, these efforts typically require information about binding or enzyme structure and often defy quantitative techniques.

From a physicochemical standpoint, the biodegradation of a chemical is controlled by three major classes of properties- hydrophobic, electronic, and steric. The uptake of a compound by a biological system, such as a bacterial cell, is governed largely by its partitioning between lipid and aqueous phases. Partitioning and dissolution vary with hydrophobicity and water solubility, respectively. The latter is influenced by pH. Transport of most xenobiotics to biological systems is via passive diffusion. This is a process of transfer from an outer aqueous phase to lipid membrane phase to an intracellular aqueous phase. The rate of transport depends on properties such as

hydrophobicity, polarity, degree of ionization, molecular shape, and size. It is assumed that this partition of a chemical between a biophase and water is related to the 1-octanol-water partition coefficient (K_{ow}). Biodegradation or metabolism itself is controlled by electronic and steric factors governing interaction with the corresponding metabolizing enzymes and the energetics of covalent bond cleavage. Steric factors (size and shape) can also shield a bond from metabolic attack.

Chapter III

Materials and Methods

Compounds

Acenaphthene, 2-aminonaphthalene, 4-aminosalicylate, 5-aminosalicylate, 1-bromonaphthalene, 2-bromonaphthalene, carbazole, 1-carboxynaphthalene, 2-carboxynaphthalene, 1-chloronaphthalene, 3-chlorosalicylate, 4-chlorosalicylate, dibenzofuran, dibenzothiophene, 1,2-dimethylnaphthalene, 1,3-dimethylnaphthalene, 1,4-dimethylnaphthalene, 1,5-dimethylnaphthalene, 1,6-dimethylnaphthalene, 1,7-dimethylnaphthalene, 1,8-dimethylnaphthalene, 2,3-dimethylnaphthalene, 2,6-dimethylnaphthalene, 2,7-dimethylnaphthalene, 1-ethylnaphthalene, 2-ethylnaphthalene, 1-fluoronaphthalene, 2-hydroxynaphthalene, 3-hydroxysalicylate, 4-hydroxysalicylate, 5-hydroxysalicylate, 1-iodonaphthalene, 1-methanolnaphthalene, 2-methanolnaphthalene, 1-methoxynaphthalene, 2-methoxynaphthalene, 3-methoxysalicylate, 4-methoxysalicylate, 5-methoxysalicylate, 2-naphthaldehyde, naphthalene, methylsalicylate, 1-methylnaphthalene, 2-methylnaphthalene, 3-methylsalicylate, 4-methylsalicylate, 5-methylsalicylate, 1-nitronaphthalene, phenanthrene, thiosalicylate, 2-thionaphthalene were purchased from Aldrich Chemical (Milwaukee, WI). 5-Chlorosalicylate, fluorene, and salicylate were purchased from Sigma Chemical (St. Louis, MO). 2-Chloronaphthalene was purchased from Acros Organics (Pittsburgh, PA). 1-Hydroxynaphthalene was purchased from Fisher (Fair Lawn, NJ). 3-Nitrosalicylate was purchased from TCI America (Portland, OR). Naphthalene-2-carbonitrile was purchased from Lancaster Synthesis (Windham, NH). Carbaryl was purchased from ChemService (West Chester, PA). All chemicals were of the highest purity available, generally greater than 95% as listed by the manufacturers. Analysis of all chemicals by gas chromatography/mass spectrometry (described later) did not reveal any

contamination that would require further compound repurification. The numbering schemes for substituted naphthalenes and salicylates are shown in figure 4.

Organisms

Pseudomonas fluorescens 5R and 5RL have been described previously [King et al., 1991]. Briefly, *P. fluorescens* 5R contains the 100 kb archetypal NAH plasmid, pKA1 (table 1). *P. fluorescens* 5RL (*nah*⁺ *Sal*⁻ *lux*⁺) contains the *lux* reporter plasmid pUTK21 [King et al., 1990], which is a product of the insertion of the *lux* transposon Tn4431 [Shaw et al., 1988] approximately 300 base pairs downstream from the start of the *nahG* gene [Menn et al., 1993] (figure 5). In addition to the *luxCDABE* genes, Tn4431 contains a tetracycline resistance marker. *Pseudomonas* sp. DFC49 and DFC50 have been described by Sanseverino et al. [1993a]. *Pseudomonas putida* PpG7 has been described in a number of references, including Dunn and Gunsalus [1973].

Detailed descriptions of strain construction are given by Applegate [1997]. Construction of the *nahA* clone was initiated by cloning the 11 kb *nahABFCE* *Eco*RI *Hind*III restriction fragment [Eaton and Chapman, 1992; Yen and Serdar, 1988] from plasmid pNAH7 into pBluescript II SK(+) (Stratagene, LaJolla, CA.). Colonies were screened for conversion of indole to indigo on LB agar [Sambrook et al., 1989] plates containing 50 mg/L ampicillin. The recombinant plasmid containing *nahABFCE* was designated pUTK201. The final *nahA* construct was completed by partial digestion of pUTK201 with *Xho*I and subsequent religation to form plasmid pUTK202. All transformants were verified by miniprep plasmid isolation [Holmes and Quigley, 1981] followed by restriction digestion with the appropriate enzymes.

The *nahGH* clone was generated by amplification of a 3.1 kb fragment encompassing the *nahGH* genes from plasmid pKA1 of *P. fluorescens* 5R [Sanseverino et al, 1993a]. The nucleotide sequences of the upstream and downstream primers were GCGCAATATTCATGTTGATGA and CGTCCTAGGTCATAACGGTCATG,

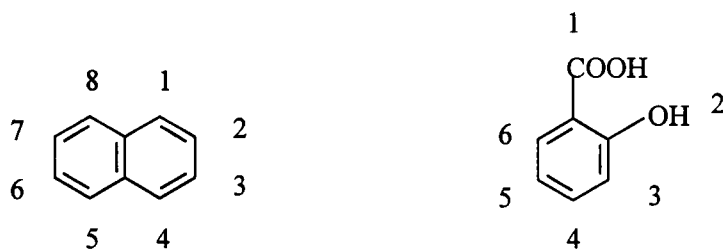


Figure 4. Numbering Schemes of Naphthalene and Salicylate.

Table 1. Bacterial Strains and Plasmids Used in this Study.

Bacterial Strain	Plasmid	Genotype	Reference
<i>P. fluorescens</i> 5R	pKA1	<i>nah⁺ sal⁺</i>	Sanseverino et al. [1993a]
<i>P. fluorescens</i> 5RL	pUTK21	<i>nah⁺ sal⁻ lux⁺</i>	Sanseverino et al. [1993a]
<i>Pseudomonas</i> sp. Strain DFC49	pKA2	<i>nah⁺ sal⁺</i>	Sanseverino et al. [1993a]
<i>Pseudomonas</i> sp. Strain DFC50	pKA3	<i>nah⁺ sal⁺</i>	Sanseverino et al. [1993a]
<i>Pseudomonas putida</i> PpG7	pNAH7	<i>nah⁺ sal⁺</i>	Dunn and Gunsalus [1973]
<i>E. coli</i> DH5 α	pUTK200	<i>nahGH</i>	Applegate [1997]
<i>E. coli</i> DH5 α	pUTK201	<i>nahABFCE</i>	Applegate [1997]
<i>E. coli</i> DH5 α	pUTK202	<i>nahA</i>	Applegate [1997]

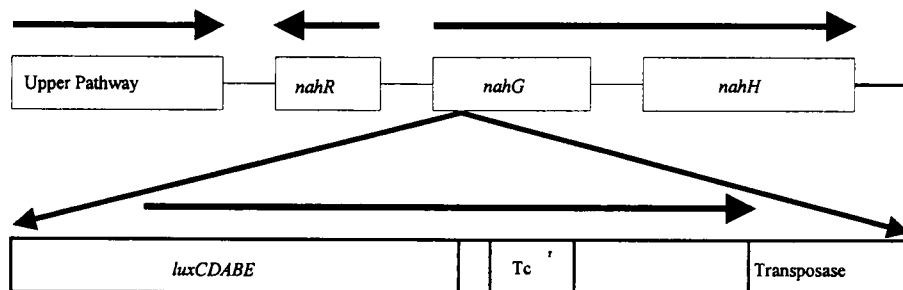


Figure 5. Schematic Representation of the *lux* Insertion into *nahG*. Arrows represent direction of transcription. *Tc^r* - tetracycline resistance. This schematic is not to scale.

respectively [Ghosal et al., 1987, You et al., 1991]. The reaction was performed using the manufacturers protocol (Gene^{amp}, Perkin-Elmer, Norwalk, CT.) with the following thermocycler conditions: 1 minute at 94°C (denaturation), 1 minute at 55°C (annealing) and 3 minutes at 72°C (extension). The resultant fragment was then directly cloned into the TA cloning vector pCRII (Invitrogen, San Diego, CA) using the manufacturer's protocol. Clones were screened for inserts by the addition of catechol to the plates. Colonies producing the characteristic yellow ring cleavage product were replated and exposed to salicylate. Colonies producing the yellow ring cleavage product were analyzed by miniprep plasmid isolation and restriction digestion analysis. One strain containing pCRII with the 3.1 kb *nahGH* fragment designated pUTK200 was chosen for further studies.

Biotransformation Assays

The transformation of methyl-substituted naphthalenes by *P. fluorescens* 5RL was performed according to a modified version of the procedure described by Menn et al [1993]. Strain 5RL was pregrown to late log phase at 28°C and 150 rpm in 100 ml of a yeast extract-peptone-glucose (YEPG) medium (34) amended with 14 mg/L of tetracycline. Ten ml of cells were then transferred to two 4.0-liter Erlenmeyer flasks, each of which contained 1 L of a yeast extract-peptone-salicylate-succinate medium (YEPSS) with the same concentration of tetracycline [Menn et al., 1993]. Cells were harvested in the exponential phase of growth by centrifugation for 10 min. at 22,095x *g* and 4°C. The cells were washed three times and resuspended in 200 ml of 50 mM sodium phosphate buffer (pH 7.0) [Breznak and Costilow, 1994] to an approximate concentration of 1.0 g of cells per 100 ml. The 200 ml were then divided in half into two 500 ml Erlenmeyer flasks. Into one flask was added 20 mg of substrate dissolved in 0.5 ml of *N,N*-dimethylformamide (DMF). The negative control consisted of adding DMF to 100 ml of cells. Abiotic controls were performed separately and consisted of adding

substrate in DMF to 100 ml of Na₃PO₄ buffer. All flasks were then incubated at 28°C and 150 rpm for approximately 18 hrs.

After centrifugation (described above), the cell-free suspensions were extracted with ethyl acetate (neutral extract; three 100-ml volumes). The aqueous layer was then acidified (pH < 2.0) with concentrated H₂SO₄ and extracted with ethyl acetate (acid extract) as described above. Both the neutral and acid ethyl acetate extracts were dried over anhydrous sodium sulfate, and the solvent was removed in vacuo at 30°C [Menn et al., 1993]. The residues were dissolved in 2.0 ml of acetone and then prefiltered through a 0.2 µm-pore-size PTFE filter (Gelman Sciences, Ann Arbor, MI) prior to analysis. Samples were not derivatized prior to analysis. Biotransformation experiments involving pUTK202 were performed identically with the exception that the strain was grown on minimal salts medium (composition listed under Growth Assays) amended with 50 mg/L of ampicillin and 100 mg/L glucose.

Analysis and Identification of Upper Pathway Metabolites

Two methods were used for metabolite separation and identification. In the first, which was utilized in analysis of the metabolites of only methyl-substituted naphthalenes, metabolite separation was performed using a Fisons Instruments AS8000 series gas chromatograph (Beverly, MA) with a Restex RT_x-5 capillary column (Bellefonte, PA) according to the method described by Selifonov et al. [1996]. Metabolite identification was performed using a Fisons Instruments VG Platform II quadrupole mass spectrometer. Analysis was carried out by using the electron impact mode at an ionization voltage of 70 eV. All identifications were based on comparisons to commercially available standards and/or reported mass spectra fragmentation values.

The second method was utilized in the analyses of metabolites of all other compounds. Metabolite separation was performed using a Hewlett-Packard 6890 series gas chromatograph (Palo Alto, CA) with an HP-5MS capillary column (30 m, 250 µm inner

diameter, 0.25 μm film thickness). Helium was used as a carrier gas at a flow rate of 1 ml/min. Injection was performed using a 1 μL injection with 1:10 split ratio at a flow rate of 10 ml/min. The injector temperature was set at 250°C. The oven temperature program began at 50°C for 1 min, and was then increased at a rate of 10°C/min until a final temperature of 290°C. The final temperature was held for a period of 2 min. Metabolite identification was performed using a Hewlett-Packard 5973 series quadrupole mass spectrometer in the electron impact mode at an ionization voltage of 70 eV. The transfer line was set at 300°C.

Bioluminescence Reporter Assays and mRNA Analysis

The *lux::nahG* gene fusion of *P. fluorescens* 5RL was used according to a modified version of the procedure developed by Heitzer et al. [1992] as a reporter to measure induction of NAH system transcription. Two ml of sodium phosphate buffer saturated with approximately 20 mg/L of naphthalene, 1-methylnaphthalene, or 2-methylnaphthalene were added to a 20 ml scintillation vial containing 2 ml of 5RL grown to an optical density at 600 nm (OD_{600}) of 0.35. The vials were then shaken at 100 rpm at room temperature in the dark for one hour. After this time interval, the light output of the cultures was measured in amperes using an Oriel photomultiplier (model 77340, Oriel, Stratford, CT) with an Oriel digital display (model 7070) and a liquid light cable. Light production was normalized to cell density by dividing the value for amperes by the OD_{600} of each vial after one hour.

The ability of the NAH system to utilize individual methyl-substituted salicylates as inducers of transcription was tested by initially preparing a stock solution of 20 mg/L and then diluting it appropriately with sodium phosphate buffer to achieve a range of final concentrations (in the presence of 5RL) from 0.1 to 10 mg/L. The assays were then carried out as described above. All experiments included negative controls which consisted of 5RL and sodium phosphate buffer.

Methyl-substituted salicylates and a number of other salicylate analogs were also examined in the following manner. In order to correct for the potential difficulty of any of the tested compounds to pass through a biological membrane, and hence enter the interior of cells of strain 5RL due to the chemical nature of their substituents, this experiment was conducted using molar concentrations of test compounds which were normalized to the predicted internal concentration of salicylate when salicylate is tested at 10 mg/L. No mechanism is known for the transport of salicylate or its analogs into cells which possess the NAH system. The uptake of these compounds into strain 5RL is believed to occur via diffusion through cellular membranes. This has been modeled by correlating the log of the octanol-water partition coefficient ($\log K_{ow}$) to the ability of a compound to pass through a membrane. Therefore, the test compound concentrations were normalized using the following equation:

$$\text{Test compound concentration} = (\log k_{ow}(\text{salicylate}) / \log k_{ow}(\text{test compound})) * (\text{molar concentration of salicylate at 10 mg/L}) * \text{MW of test compound}$$

Values for $\log K_{ow}$ of each chemical were secured as either a measured or computer estimated value from CLOGP for windows software (BIOBYTE Corp., Claremont, CA, USA). When available, the measured value was used in preference to a calculated one. Thus, for the more hydrophobic compounds, such as the chlorosalicylates and methylsalicylates, a lower concentration of compound was examined because of the greater ease of passage through the cell membrane. Compounds such as aminosalicylates, which are less hydrophobic than salicylate, involved the testing of much higher concentrations. Based on this equation, the concentration of 5-aminosalicylate to be tested was impracticably high. Therefore, it was tested at one-half of its normalized concentration. Also, the $\log K_{ow}$ of 3-nitrosalicylate was not available at the time of testing. It was tested at a concentration of 100 mg/L.

In order to verify that the increases in bioluminescence were due to increases in induction of the *sal* operon, the levels of *lux* mRNA were compared for cells incubated separately with salicylate, 4-methylsalicylate, or buffer alone. Immediately after the bioluminescence readings were taken, 2 ml of cells were transferred to an Eppendorf tube and centrifuged at 22,095x g for 10 min. The supernatant was decanted and the cell pellet was rinsed with 2 ml of STE buffer [Sambrook et al., 1989]. After centrifugation, the STE buffer was decanted and the cell pellets were stored at -80° C until RNA extraction. RNA extraction was performed using a RNeasy kit (Qiagen, Chatsworth, CA) according to the manufacturer's protocol. Equivalent amounts of RNA were then immobilized on a Zeta-probe nylon membrane (Biorad, Richmond, CA) according to the manufacturer's protocol. The comparative levels of *lux* mRNA were quantified using a single-stranded 350 bp anti-sense *luxB* ³²P-DNA probe [Johnston, 1996] generated from sequences published by Baldwin et al [1989]. RNA slot blots were exposed to x-ray film at -80°C. Developed film was scanned and analyzed using SigmaGel software (Jandel Scientific, San Rafael, CA).

Meta-Cleavage Assays

The ability of the salicylate hydroxylase (*nahG*) and catechol 2,3-dioxygenase (*nahH*) to facilitate aromatic ring cleavage of commercially available methyl-substituted salicylates was examined with *P. fluorescens* 5R and/or pUTK200 using a modification of the biotransformation assay used by Menn et al. [1993]. At one hour intervals, a 2 ml sample from a flask containing 5R and the test compound was centrifuged and scanned over ultraviolet and visible wavelengths (200 to 600 nm) using a Beckman DU-70 spectrophotometer (Palo Alto, CA). Control experiments were conducted in the same manner as described above for biotransformation experiments. Control experiments for pUTK200 included the use of the cloning vector without the *nahGH* insert.

Growth Assays

The ability of *P. fluorescens* 5R to utilize methyl-substituted naphthalenes as sole sources of carbon and energy was examined according to the following procedure. One hundred mg of test compound dissolved in 600 μ l of acetone was added in excess of solubility to 100 ml of a minimal salts medium in a 250 ml Erlenmeyer flask after autoclaving (done in triplicate). The medium is a modification of that used by Davis and Mingioli [1950], and is composed of 0.68 g KH_2PO_4 , 1.73 g K_2HPO_4 , 0.1 g $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$, and 1.0 g NH_4NO_3 per liter of water. After autoclaving, 0.1 ml of a filtered trace element solution [Meynell and Meynell, 1965] was also added. Three ml of strain 5R, grown overnight in YEPG medium, was added to each flask containing the test compound(s) and minimal medium (Due to the small amounts of 1,6- and 1,7-dimethylnaphthalene available, these two compounds were examined in test tube experiments at approximately 1:50 the scale.). Flasks were then shaken at 150 rpm at room temperature. Samples were taken for optical density (OD_{600}) readings using a Beckman DU-70 spectrophotometer. Experiments utilizing naphthalene were performed as a positive control. Negative controls were performed through the use of stain 5R and minimal salts medium without the test compound. No growth was observed on acetone.

The ability of strain 5R to utilize commercially available salicylate analogs as sole sources of carbon and energy was examined at a concentration of 225 mg/L. Positive control experiments were performed using salicylate at this concentration. Negative controls were performed as described above. In order to test the possibility that salicylate analogs may only serve as a carbon and energy source in the presence of the known inducer molecule salicylate experiments were also performed using all binary mixture combinations with salicylate. Each compound was present in the mixture at an individual concentration of 225 mg/L.

PAH Mixture Biodegradation

The PAH mixture under study was prepared fresh prior to each experiment by dissolving 0.05 g of each compound together in 5 ml of acetone. This concentrated solution was then added to 1 L of minimal salts medium (see above for composition), and allowed to sit in the dark for approximately 2 to 3 days in order to achieve full saturation. On the day of the experiment, 5 ml of this solution was filtered through a 0.2 μm PTFE syringe top filter and added to baked, sterilized 12 ml screw cap tubes with teflon-coated caps. The starting concentration (mol/L) of each component of the mixture at time 0 is represented in the graphs located in the appendix. In general, the starting concentrations did not vary much from experiment to experiment when the same mixture composition was used.

Bacterial strains utilized in PAH mixture biodegradation experiments were pregrown overnight in YEPSS in the same manner as in biotransformation experiments. The strain in use was centrifuged and washed three times, and then finally resuspended in 20 ml of minimal salts medium to achieve a 100-fold concentration of cells. Fifty μL of this concentrated solution of cells were added to tubes containing the PAH mixture to achieve a cell density of approximately 10^8 cells/ml. Serial dilutions and plating on YEPG were performed approximately every 24 hrs. On the average, the number of colony forming units (cfu) was maintained at 10^8 cells/ml through the 48 hr time point. From 48 hrs to 96 hrs, the cfu concentration dropped one order of magnitude. The tubes were then placed on a Glas-col rotary shaker at 75% of its maximum speed. Triplicate samples were taken every half-hour from time zero up to seven hours by adding 5 ml of hexane to the appropriate sacrificed tubes and then shaking horizontally for 2 hrs at 150 rpm. After this time, approximately 2 ml of the hexane phase was taken from the tube, crimp-sealed in an autosampler vial with a teflon-lined cap, and then stored at -20°C until analysis. Samples were also usually taken at 12, 24, 48, 72, and 96 hrs into the experiment. Negative controls, killed with concentrated sulfuric acid prior to time zero, were treated

identically. No appreciable loss of the mixture components was observed in killed controls for any experiment.

GC analysis was performed using a Shimadzu GC-9AM (Kyoto, Japan) with a J&W Scientific DB-5 column (30m, 0.25 mm inner diameter and film thickness; Folsom, CA), using either of two methods depending upon the mixture under study. The temperature profile of the first method consisted of the following steps: 2 min at 60°C, an increase from 60 to 230°C at 10°C per min, and then a hold at 230°C for 14 min. The temperature profile of the second method consisted of the following steps: 2 min at 80°C, an increase from 80 to 230°C at 5°C per min, and then a hold at 230°C for 5 min. Both methods utilized a N₂ as a carrier gas at 40 ml/min and an injection volume of 2.5 µL. Both the detector and injector temperatures were set at 300°C. Injection was performed in the splitless mode. Detection of peaks was performed using a flame ionization detector (FID).

Chapter IV

Results

Biotransformation of Methyl-Substituted Naphthalenes by *P. fluorescens* 5RL and pUTK202

The products of the biotransformation of methyl-substituted naphthalenes by the upper pathway of the NAH system of strain 5RL were characterized by GC/MS analysis and comparison to commercially available standards. Two apparently different modes of oxygenase attack occurred in the transformation of 1- and 2-methylnaphthalene. The first, a classical upper pathway-mediated dioxygenase attack at positions 7 and 8 of the unsubstituted ring of each compound, led to the transformation of each test compound to the respective salicylate analogs 3- and 4-methylsalicylate as major products (All metabolites for the biotransformation of methyl-substituted naphthalenes are shown schematically in figure 6). These products, each with a molecular weight of 152 (All data for this section is shown in table 2), were identified via comparison to standards prepared from commercially purchased 3- and 4-methylsalicylate.

The second mode of attack led to the incorporation of one hydroxyl substituent into each compound to form minor products with molecular weights of 158. Based on a comparison of the mass spectrum and the retention time to available standards, the product of this transformation for 1-methylnaphthalene was found to be 1-methanolnaphthalene. The transformation of 1-methylnaphthalene also yielded a small amount of 1-carboxynaphthalene (1-naphthoic acid) as a product. In the case of the transformation of 2-methylnaphthalene, the product of molecular weight 158 did not have a mass spectrum and retention time that matched authentic 2-methanolnaphthalene. Rather, the hydroxylated methylnaphthalene produced in this transformation had a base peak (m/z) of 158. A comparison to a library of mass spectra has shown that this is more

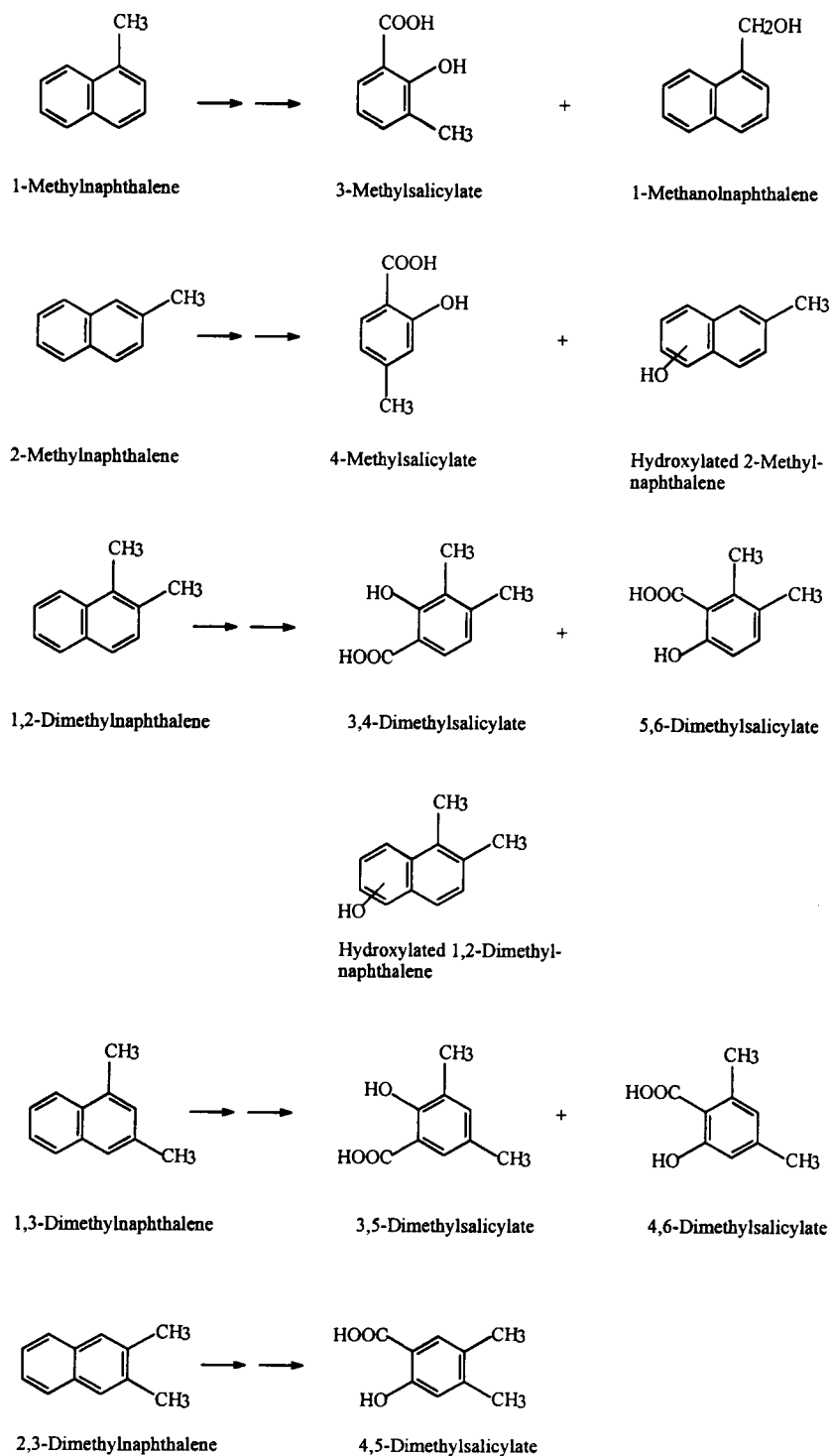


Figure 6. The Biotransformation of Methyl-Substituted Naphthalenes by *P. fluorescens* 5RL.

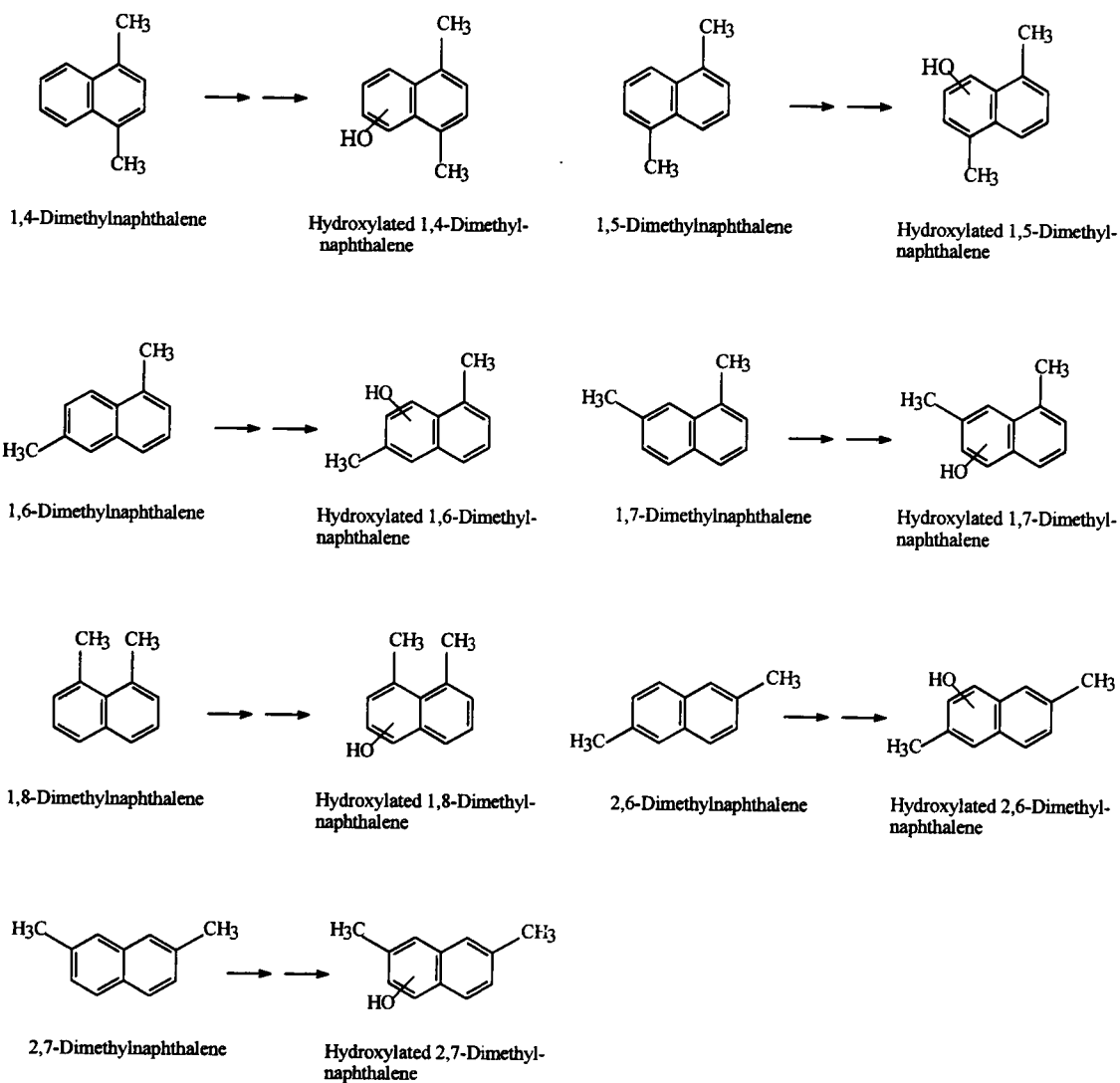


Figure 6 cont.

Table 2. GC/MS Data from the Biotransformation of Methyl-Substituted Naphthalenes by *P. fluorescens* 5RL.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
1-MN	A	15.0	49	152 (M ⁺ , 30), 135 (11), 134 (100), 106 (85), 105 (31), 78 (31), 77 (28)	3-Methylsalicylate ^a	Bestetti et al. [1994]
	N	18.4	85	158 (M ⁺ , 16), 141 (10), 130 (14), 129 (100), 128 (40), 127 (26), 115 (9), 77 (5)	1-Methanol-naphthalene ^a	
	A	21.0	3.5	172 (M ⁺ , 42), 155 (26), 128 (15), 127 (100), 126 (35), 115 (18), 77 (14)	1-Carboxynaphthalene ^b	
2-MN	A	16.6	53	152 (M ⁺ , 26), 135 (11), 134 (100), 106 (20), 105 (22), 78 (18), 77 (11)	4-Methylsalicylate ^a	Hydroxylated 2-methylnaphthalene ^c
	N	19.6	42	158 (M ⁺ , 100), 157 (20), 139 (12), 130 (32), 129 (84), 128 (69), 127 (31), 115 (46), 77 (8)		
	A	18.1	44	166 (M ⁺ , 19), 148 (62), 121 (12), 120 (100), 119 (32), 105 (14), 91 (31), 77 (11)	3,4-Dimethylsalicylate ^c	
1,2-DMN	A	18.8	11	166 (M ⁺ , 24), 149 (11), 148 (100), 121 (19), 120 (91), 119 (40), 107 (11), 105 (42), 91 (52), 77 (18)	5,6-Dimethylsalicylate ^c	Hydroxylated 1,2-dimethylnaphthalene ^c
	N	20.6	4.5	172 (M ⁺ , 35), 157 (11), 155 (11), 154 (36), 153 (19), 143 (52), 141 (31), 139 (21), 129 (89)		

Table 2 cont.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
1,3-DMN	A	17.9	35	128 (100), 127 (30), 115 (44), 89 (5), 77 (10), 76 (15)	3,5-Dimethylsalicylate* ^c	
				166 (M ⁺ , 18), 148 (50), 121 (11), 120 (100), 119 (28), 105 (5), 91 (39), 77 (7)		
	A	18.8	38	166 (M ⁺ , 28), 149 (11), 148 (100), 121 (12), 120 (83), 119 (46), 105 (13), 92 (16), 91 (66), 77 (12)	4,6-Dimethylsalicylate* ^c	
1,4-DMN	N	21.6	1.5	172 (M ⁺ , 45), 157 (21), 155 (11), 144 (13), 143 (57), 141 (38), 139 (20), 129 (94), 128 (100), 127 (30), 115 (58), 91 (11), 77 (9)	Hydroxylated 1,4-dimethylnaphthalene* ^c	
1,5-DMN	N	21.7	33	172 (M ⁺ , 32), 157 (12), 153 (12), 144 (12), 143 (100), 141 (39), 139 (24), 129 (70), 128 (97), 127 (25), 115 (37), 89 (3), 77 (4)	Hydroxylated 1,5-dimethylnaphthalene* ^c	Selifonov et al. [1996]
1,6-DMN	N	21.2	25	172 (M ⁺ , 33), 157 (12), 155 (11), 144 (12), 143 (100), 141 (36), 139 (21), 129 (55), 128 (88), 127 (21), 115 (32), 89 (3), 77 (3)	Hydroxylated 1,6-dimethylnaphthalene* ^c	
1,7-DMN	N	21.0	19	172 (M ⁺ , 36), 157 (11), 155 (11),	Hydroxylated 1,7-	

Table 2 cont.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
1,8-DMN	N	21.5	6.9	144 (13), 143 (100), 141 (33), 139 (21), 129 (60), 128 (95), 127 (25), 115 (37), 89 (3), 76 (4)	dimethylnaphthalene ^{*c}	Selifonov et al. [1996]
2,3-DMN	N	19.6	89	172 (M ⁺ , 34), 154 (54), 153 (93), 152 (22), 143 (25), 141 (37), 139 (29), 129 (41), 128 (100), 127 (33), 115 (51), 89 (6), 77 (11), 76 (20)	Hydroxylated 1,8-dimethylnaphthalene ^{*c}	Bestetti et al. [1994] Grifoll et al. [1995b] Mahajan et al. [1994]
2,6-DMN	N	21.1	29	172 (M ⁺ , 40), 157 (14), 155 (12), 144 (11), 143 (100), 142 (11), 141 (48), 139 (27), 129 (67), 128 (93), 127 (25), 115 (37), 89 (4), 85 (11), 77 (5)	4,5-Dimethylsalicylate ^b Hydroxylated 2,6-dimethylnaphthalene ^{*c}	Miyachi et al. [1993] Barnsley [1988] Grifoll et al. [1995b] Selifonov et al. [1996]
2,7-DMN	N	24.3	5.1	186 (M ⁺ , 69), 169 (16), 142 (16), 141 (100), 140 (12), 139 (39), 128 (10), 115 (44), 89 (6), 79 (4)	2-Carboxy-6-methylnaphthalene ^b	Miyachi et al. [1993] Barnsley [1988] Grifoll et al. [1995b]
2,7-DMN	N	21.3	66	172 (M ⁺ , 34), 157 (13), 155 (10), 144 (12), 143 (100), 142 (11), 141 (42), 139 (24), 129 (56), 128 (86), 127 (20), 115 (31)	Hydroxylated 2,7-dimethylnaphthalene ^{*c}	Selifonov et al. [1996]

Table 2 cont.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
	N	24.3	3.8	89 (3), 77 (3) 186 (M ⁺ , 88), 169 (17), 142 (15), 141 (100), 140 (12), 139 (36), 128 (11), 115 (59), 89 (5), 77 (3)	2-Carboxy-7-methylnaphthalene ^b	Miyachi et al. [1993]

^a Identification based on comparison to commercially available standard.

^b Identification based on comparison to references.

^c Suggested structure.

^d Abundance represents peak area percentage for the corresponding extract. In the case of the neutral fraction, it includes any peak area from untransformed parent compound.

*Isomer not identified.

Compound abbreviations: DMN - dimethylnaphthalene, MN - methylnaphthalene.

indicative of direct hydroxylation of the aromatic ring. The location of the hydroxyl substituent is uncertain.

Biotransformation assays using strain 5RL and a suite of dimethylnaphthalenes (DMN) exhibited both the classical upper pathway sequence of reactions and the incorporation of a single hydroxyl substituent. However, only in the instance of 1,2-DMN did both modes occur in the transformation of a single compound as they did in the transformation of 1- and 2-methylnaphthalene. The location of hydroxyl group incorporation into 1,2-DMN is uncertain. 1,2-DMN and 1,3-DMN were each transformed to two products of molecular weight 166. According to classical upper pathway reactions, the identification of these products is suggested to be 3,4- and 5,6-dimethylsalicylate for 1,2-DMN, and 3,5- and 4,6-dimethylsalicylate for 1,3-DMN. 2,3-DMN was transformed to a single product of molecular weight 166. Comparison of this data to the results of a number of other studies [Bestetti et al., 1994; Grifoll et al., 1995b; Mahajan et al., 1994] leads to the identification of this product as 4,5-dimethylsalicylate. No incorporation of a single hydroxyl group was observed in the transformation of 1,3-DMN and 2,3-DMN by strain 5RL.

Transformation of 1,4-, 1,5-, 1,6-, 1,7-, 1,8-, 2,6-, and 2,7-DMN by strain 5RL was characterized by the notable absence of a classical upper pathway attack. No salicylate analogs were formed during their biotransformation. Those compounds which are symmetrical, 1,4-, 1,5-, 1,8-, 2,6-, and 2,7-DMN, were each transformed to one product of molecular weight 172. This represents the incorporation of one hydroxyl group into the compound; however, the location of this substitution is unclear. 1,4- and 1,8-DMN were both characterized by a comparatively low amount of metabolite formation (1.5 and 6.9% abundances, respectively); this may represent an enzymatic difficulty in transforming these two compounds. The two non-symmetrical compounds 1,6-DMN and 1,7-DMN were also each transformed to one product of molecular weight 172. The location of the hydroxyl substituent is also unclear for these two products. The formation of carboxylated intermediates was also observed from the transformation of 2,6- and 2,7-DMN; each was transformed to a minor product of molecular weight 186. Comparison to

the results of Barnsley [1988], Miyachi et al. [1993], and Grifoll et al. [1995b] indicates that these compounds are 2-carboxy-6-methylnaphthalene and 2-carboxy-7-methylnaphthalene, respectively.

The products of the naphthalene dioxygenase-mediated transformation of 1- and 2-methylnaphthalene were reexamined using the *nahA* clone, pUTK202. 1-Methylnaphthalene and 2-methylnaphthalene were selected because 1- and 2-methanolnaphthalene are commercially available for use as standards; no methanolmethylnaphthalenes are currently available. No direct products of a dioxygenase reaction upon either 1- or 2-methylnaphthalene were detected in experiments utilizing this construct. It is currently unclear whether the absence of a dioxygenase attack, in this instance and in experiments which utilized pUTK202, is an artifact of the analytical conditions, or if the reactions actually did not occur. 1-Methanolnaphthalene was formed in the biotransformation of 1-methylnaphthalene by pUTK202. In the case of the transformation of 2-methylnaphthalene by this clone, the product had a base peak of m/z 158 and was similar to the product formed during the transformation of 2-methylnaphthalene by strain 5RL. The location of this hydroxyl substituent is again uncertain; however, this product has a different fragmentation pattern and retention time from 2-methanolnaphthalene. Oxidation of the aromatic ring appears to have occurred.

The formation of 1-carboxynaphthalene was also not observed in pUTK202. When 1- and 2-methanolnaphthalene were used as parent compounds in biotransformation assays with strain 5RL, there was no evidence to suggest that it initiates any further reactions, such as the formation of 3- or 4-methanolsalicylate, on either compound. Results of negative controls using strain 5RL and pUTK202 in the absence of test compound, and of abiotic controls support these findings of NAH system activity on 1- and 2-methylnaphthalene. In addition, experiments using pBluescript II SK, the precursor to pUTK202, with either 1- or 2-methylnaphthalene showed no transformation of parent compound.

Biotransformation of Other Substituted Naphthalenes by *P. fluorescens* 5RL

The biotransformation products of the following substituted naphthalenes by strain 5RL were also examined using GC/MS analysis: 1- and 2-bromonaphthalene, 1- and 2-chloronaphthalene, 1- and 2-ethylnaphthalene, 1-fluornaphthalene, 1- and 2-hydroxynaphthalene, 1-iodonaphthalene, 1- and 2-methoxynaphthalene, 1-naphthoic acid, 1-nitronaphthalene, and 2-thionaphthalene (All data and schematic representations concerning these experiments are located in table 3 and figure 7, respectively). In most cases, the classical upper pathway reactions leading to the formation of a substituted salicylate were most prominent. For example, 1- and 2-ethylnaphthalene, which are most structurally similar to the methyl-substituted naphthalenes discussed earlier, were transformed to 3- and 4-ethylsalicylate, respectively. 4-Ethylsalicylate, comprised 82.7% of the total ion chromatogram (TIC), whereas 3-ethylsalicylate comprised 53.2% of the TIC. No other intermediates were observed in the transformation of 2-ethylnaphthalene; however, the transformation of 1-ethylnaphthalene did yield two different hydroxylated 1-ethylnaphthalene products of molecular weight 172. No compounds of this nature are available commercially for comparison.

The transformation of 1- and 2-methoxynaphthalene yielded 3- and 4-methoxysalicylate as the primary products (17 and 91%, respectively). Only in the case of 1-methoxynaphthalene was the presence of a hydroxylated product detectable.

Surprisingly, 1- and 2-hydroxynaphthalene were each transformed to salicylate (50.7 and 25.9% TIC, respectively). This indicates that the NAH upper pathway of strain 5RL is able to incorporate one hydroxyl group to form 1,2-dihydroxynaphthalene as in the transformation of naphthalene to salicylate. 1- and 2-Hydroxynaphthalene were also each transformed to dihydroxynaphthalene products (3.0 and 8.4% TIC, respectively) which did not have mass spectra similar to that of 1,2-dihydroxynaphthalene. This spectrum of this minor product of 1-hydroxynaphthalene resembled elements of the spectra of 1,3-, 1,4-, 1,5-, 1,6-, 1,7-, and 1,8-dihydroxynaphthalene. Although an identification is difficult to discern, this product must have a hydroxyl substituent at the 1 position

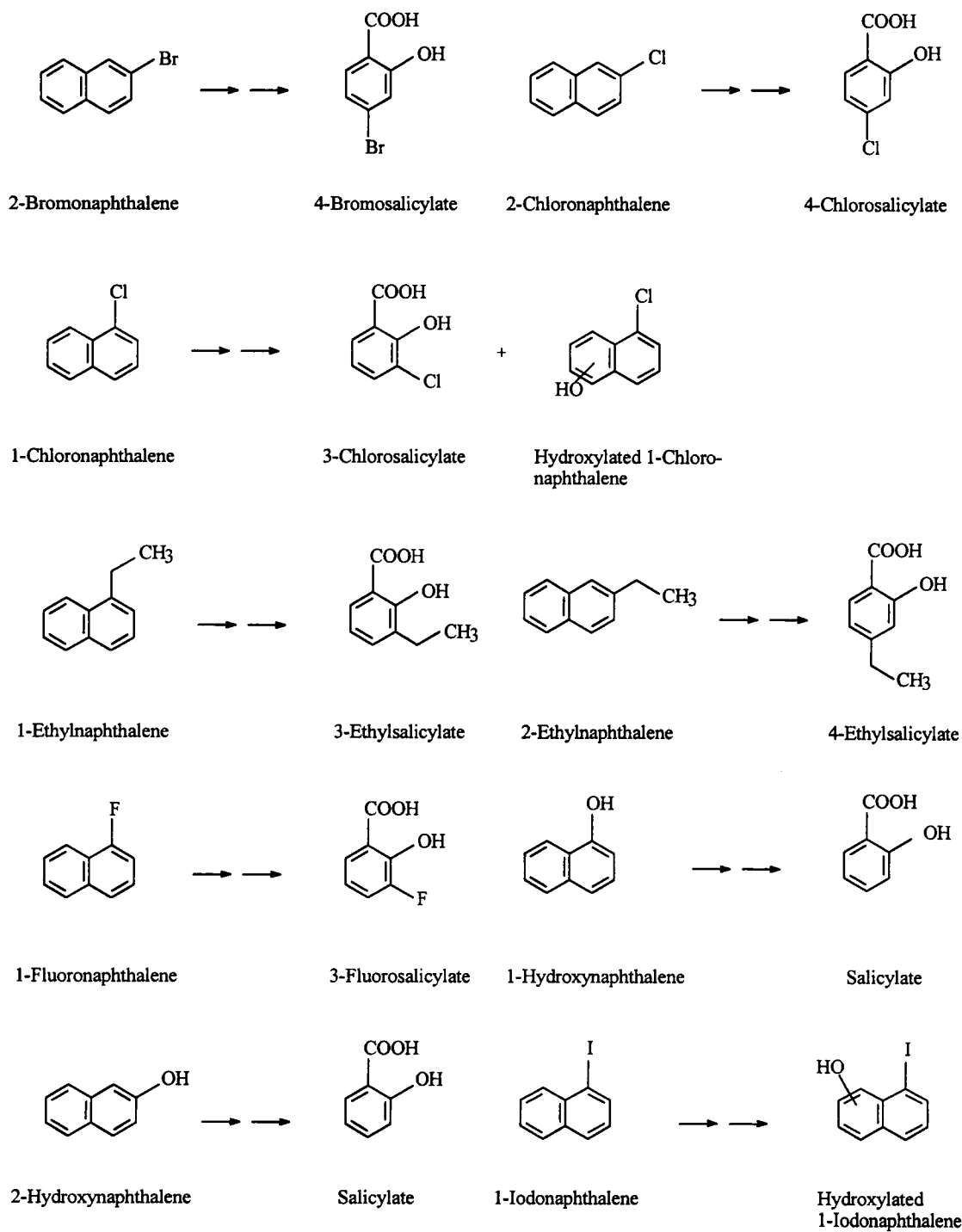


Figure 7. The Biotransformation of Other Substituted Naphthalenes by *P. fluorescens* 5RL.

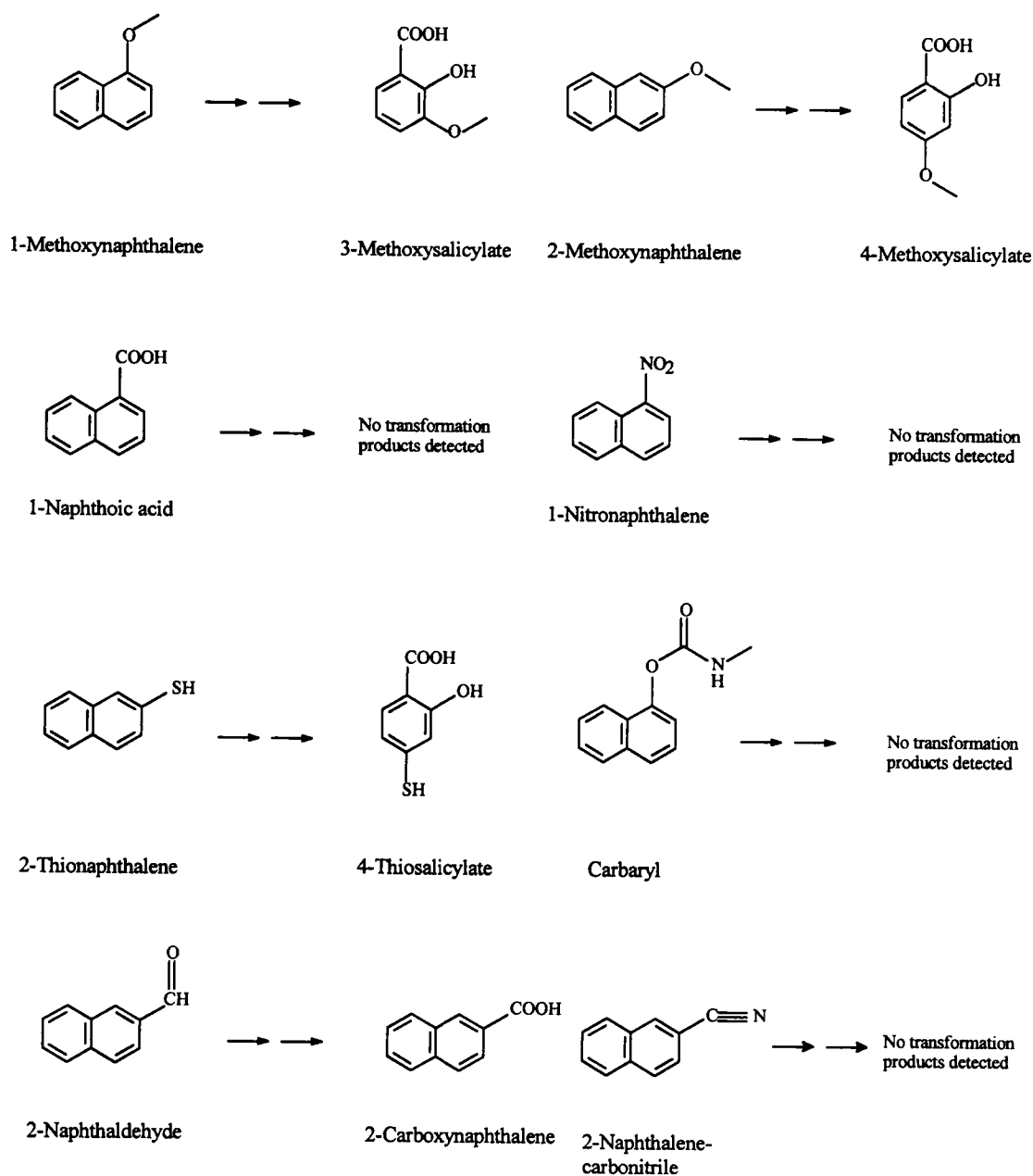


Figure 7 cont.

Table 3. GC/MS Data from the Biotransformation of Other Substituted Naphthalenes by *P. fluorescens* 5RL.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
1-BN	N	14.7	16.7	272 (M ⁺ , 27), 256 (11), 216 (43), 213 (25), 198 (100), 170 (22), 154 (6), 142 (4), 126 (4), 119 (41), 92 (10), 91 (19), 63 (33)	yet to be identified	
				224 (M ⁺ , 89), 200 (9), 199 (9), 197 (9), 196 (100), 170 (6), 117 (42), 98 (7), 89 (53), 87 (8), 63 (41), 62 (23), 61 (8)		
				209 (M ⁺ , 100), 131 (34), 130 (21), 103 (11), 102 (18), 77 (15), 75 (10), 51 (17)		
				209 (M ⁺ , 100), 131 (33), 130 (12), 103 (15), 102 (15), 77 (21), 51 (17)		
	N	15.3	1.7	272 (M ⁺ , 100), 257 (38), 213 (92), 149 (9), 119 (14), 107 (8), 63 (12)	yet to be identified	
				272 (M ⁺ , 12), 257 (5), 216 (39), 213 (11), 198 (100), 170 (21), 119 (39), 91 (16), 63 (30)		
				266 (M ⁺ , 13), 251 (9), 209 (100), 198 (5), 144 (13), 130 (13), 115 (21), 102 (6), 89 (6), 63 (6)		
	A	12.5	16.9	216 (M ⁺ , 49), 198 (100), 170 (48), 148 (12), 144 (8), 142 (8), 120 (10), 91 (11), 63 (10), 62 (10), 53 (10)	4-Bromosalicylate ^c	
				272 (M ⁺ , 91), 257 (37), 213 (100), 198 (6), 170 (6), 149 (10), 134 (6),		
2-BN	N	13.97	56.8			
	N	14.88	6.6		yet to be identified	

Table 3 cont.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
CBY	No transformation products detected			119 (5), 89 (6), 63 (12)		
	N	15.29	3.9	272 (M ⁺ , 4), 226 (100), 198 (69), 184 (10), 172 (7), 156 (17), 147 (12), 119 (19), 91 (61), 78 (37), 63 (20)	yet to be identified	
	1-CN	12.85	4.8	172 (M ⁺ , 52), 155 (11), 154 (100), 126 (65), 99 (7), 98 (15), 92 (7), 73 (7), 63 (26)	3-Chlorosalicylate ^a	
	N	13.74	11.7	228 (M ⁺ , 10), 213 (6), 178 (100), 170 (14), 154 (47), 142 (5), 126 (16), 115 (25), 114 (63), 98 (5), 89 (10), 63 (18)	yet to be identified	
2-CN	N	15.82	14.4	178 (M ⁺ , 100), 149 (13), 115 (45), 114 (15), 113 (8), 89 (9), 63 (5)	Hydroxylated 1-chloronaphthalene ^{a,c}	
	A	13.69	45.9	228 (M ⁺ , 14), 213 (8), 172 (42), 154 (100), 126 (39), 119 (10), 98 (10), 91 (6), 73 (5), 63 (25)	yet to be identified	
	A	14.1	11.9	180 (M ⁺ , 70), 152 (100), 124 (8), 89 (41), 63 (21)	yet to be identified	
	N	12.84	42.8	172 (M ⁺ , 53), 154 (100), 126 (65), 98 (15), 73 (6), 63 (25)	4-Chlorosalicylate ^a	
	N	13.74	11.6	228 (M ⁺ , 61), 213 (39), 169 (100), 154 (5), 126 (6), 63 (9)	yet to be identified	
	N	13.97	2.0	228 (M ⁺ , 80), 213 (35), 169 (100),	yet to be identified	

Table 3 cont.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
1-EN	N	14.10	12.4	154 (19), 126 (14), 112 (45), 69 (19), 63 (12)	yet to be identified	
				182 (M ⁺ ,100), 154 (74), 147 (11), 140 (10), 125 (12), 119 (29), 112 (30), 91 (45), 89 (13), 77 (25), 63 (14), 51 (12)		
1-EN	N	12.79	53.1	166 (M ⁺ ,48), 148 (52), 133 (21), 121 (11), 120 (100), 102 (16), 91 (18), 77 (14)	3-Ethylsalicylate ^c	
				172 (M ⁺ ,77), 158 (12), 157 (100), 153 (8), 152 (14), 129 (24), 128 (22), 127 (15), 115 (10)		
				172 (M ⁺ ,50), 158 (14), 157 (100), 128 (17), 115 (8)		
2-EN	N	13.16	82.7	166 (M ⁺ ,42), 149 (12), 148 (100), 120 (7), 105 (48), 91 (49), 77 (10)	4-Ethylsalicylate ^c	
1-FN	A	10.17	2.3	156 (M ⁺ ,52), 138 (100), 110 (99), 82 (26), 81 (10), 63 (9), 57 (12)	6-fluorosalicylate ^c	
				156 (M ⁺ ,42), 138 (100), 110 (37), 83 (9), 82 (26), 63 (7), 57 (8)		
				212 (M ⁺ ,23), 197 (14), 165 (9), 164 (99), 153 (32), 138 (10), 136 (100), 108 (35), 107 (43), 81 (10), 57 (6)		
1-FN	A	11.27	40.2		3-Fluorosalicylate ^c	
1-FN	A	11.59	2.4		yet to be identified	

Table 3 cont.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
1-HN	N	16.72	3.0	160 (100), 132 (13), 131 (51), 105 (16), 104 (10), 103 (15), 77 (17)	Dihydroxynaphthalene* ^c	
	A	10.58	50.7	138 (M ⁺ , 57), 121 (12), 120 (100), 92 (74), 93 (8), 65 (10), 64 (18), 63 (13), 53 (6)	Salicylate ^a	
2-HN	N	10.56	25.9	138 (M ⁺ , 58), 121 (12), 120 (100), 93 (8), 92 (73), 65 (9), 64 (18), 63 (13), 53 (5)	Salicylate ^a	
	N	12.52	19.1	162 (M ⁺ , 100), 134 (16), 106 (25), 105 (9), 78 (37), 77 (14), 63 (5), 51 (10)	yet to be identified	
	N	13.67	30.9	166 (M ⁺ , 22), 147 (5), 135 (24), 120 (5), 108 (12), 107 (100), 79 (12), 77 (27)	yet to be identified	
	N	14.20	6.5	178 (M ⁺ , 6), 166 (29), 148 (67), 132 (13), 131 (14), 120 (100), 119 (25), 112 (24), 107 (43), 106 (9), 103 (12), 92 (19), 91 (50), 79 (10), 78 (25), 77 (34), 69 (10), 65 (11), 63 (9), 51 (11)	yet to be identified	
	N	16.92	8.4	160 (M ⁺ , 100), 131 (36), 103 (9), 77 (10), 51 (5)	Dihydroxynaphthalene* ^c	
1-IN	N	18.25	10.1	270 (M ⁺ , 100), 134 (6), 115 (51),	Hydroxylated	

Table 3 cont.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
1-MxN	N	18.31	14.8	114 (10), 89 (5), 63 (4)	iodonaphthalene* ^c	
				270 (M ⁺ ,100), 143 (11), 115 (41), 114 (8), 89 (5), 63 (4)	Hydroxylated iodonaphthalene* ^c	
	A	15.72	15.6	320 (M ⁺ ,100), 305 (24), 261 (67), 149 (13), 119 (10), 91 (5), 63 (5)	yet to be identified	
				174 (M ⁺ ,100), 159 (99), 131 (49), 115 (15), 103 (11), 102 (9), 77 (10), 63 (4)	Hydroxylated 1-methoxynaphthalene* ^c	
2-MxN	N	13.73	91.3	168 (M ⁺ ,64), 150 (79), 149 (15), 122 (100), 121 (47), 120 (36), 108 (9), 107 (45), 105 (9), 92 (26), 79 (16), 63 (8), 55 (8), 53 (12), 51 (16)	3-Methoxysalicylate ^a	
				168 (M ⁺ ,58), 151 (14), 150 (100), 124 (8), 122 (42), 108 (7), 107 (38), 94 (10), 79 (19), 63 (6), 53 (7), 51 (8)	4-Methoxysalicylate ^a	
1-NaN	No transformation products detected	14.83	3.8	178 (M ⁺ ,100), 177 (8), 161 (6), 150 (33), 149 (22), 137 (14), 136 (28), 121 (11), 108 (19), 91 (7), 79 (10), 78 (10), 77 (13), 65 (9)	yet to be identified	

Table 3 cont.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
1-NN	No transformation products detected					
2-TN	A	14.49	37.6	170 (M ⁺ , 69), 153 (12), 152 (100), 124 (22), 123 (11), 112 (15), 96 (38), 69 (12), 63 (6), 53 (7)	2-Thiosalicylate ^e	
	A	15.77	17.5	226 (M ⁺ , 88), 211 (20), 207 (12), 180 (10), 179 (9), 169 (9), 168 (15), 167 (100), 152 (30), 151 (12), 138 (14), 137 (13), 119 (9), 110 (14), 91 (14), 77 (7), 69 (8)	yet to be identified	
2-NPA	N	15.90	97.7	172 (M ⁺ , 100), 155 (55), 127 (59), 115 (6), 101 (5), 86 (3), 77 (6)	2-Carboxynaphthalene ^e	

^a Identification based on comparison to commercially available standard.

^b Identification based on comparison to references.

^c Suggested structure.

^d Abundance represents peak area percentage for the corresponding extract. In the case of the neutral fraction, it includes any peak area from untransformed parent compound.

^e Isomer not identified.

Compound abbreviations: CBY - carbaryl, CN- chloronaphthalene, EN - ethylnaphthalene, FN - fluoronaphthalene, HN - hydroxynaphthalene, IN - iodonaphthalene, MxN - methoxynaphthalene, MN - methylnaphthalene, NaN - naphthoic acid, NN - nitronaphthalene, TN - thionaphthalene, NPA - naphthaldehyde.

because of the configuration of the parent compound. Alternatively, the product of the transformation of 2-hydroxynaphthalene must have a hydroxyl substituent at the 2 position. The number of dihydroxynaphthalene compounds with a hydroxyl substituent at the 2 position are fewer. In this case, a reasonable library match was found with 2,7-dihydroxynaphthalene. The transformation of 2-hydroxynaphthalene also yielded two additional products of molecular weights 162 and 166 (19.1 and 30.9% TIC, respectively). The identifications of these two products are currently uncertain.

2-Bromonaphthalene, 1- and 2-chloronaphthalene, 1-fluoronaphthalene, and 2-thionaphthalene were all transformed to their respective salicylates by the upper pathway of strain 5RL. 4-Bromosalicylate was the primary product of the transformation of 2-bromonaphthalene. Two other minor brominated products of molecular weight 272 were also detected in the transformation of 2-bromonaphthalene; however, they have not been identified. The transformation of 1-bromonaphthalene was not characterized by the formation of 3-bromosalicylate. It was however also transformed to two products of molecular weight 272. A very minor product (1.7% TIC), which may represent hydroxylated 1-bromonaphthalene, was also detected. Mono-hydroxylated parent compounds were also detected in the transformation of 1-chloronaphthalene and 1-iodonaphthalene; none were detected in the transformation of 1-fluoronaphthalene. With respect to the 1-halonaphthalenes, only in the case of 1-fluoronaphthalene and 1-chloronaphthalene were the respective salicylates formed. 3-Fluorosaliclyate was the major product (40.2% TIC) in the transformation of 1-fluoronaphthalene, whereas 3-chlorosalicylate was a relatively minor product (4.8% TIC). This trend suggests that a halogen atom larger than fluorine at the 1 position impedes the formation of a halo-substituted salicylate.

A number of compounds were characterized by no evidence for transformation by the upper pathway of the NAH system. Among these were carbaryl (methyl carbamic acid 1-naphthyl ester), 1-naphthoic acid, and 1-nitronaphthalene. 1-Naphthoic acid has been previously observed to be degraded by a set of enzymatic steps similar to those of the upper pathway to form 3-carboxysalicylate [Phale et al., 1995]; however this was not

observed with strain 5RL. 2-Naphthaldehyde was transformed to 2-naphthoic acid and did not undergo classical upper pathway reactions.

Biotransformation of Other PAHs and Heterocycles by *P. fluorescens* 5RL

Acenaphthene, biphenyl, carbazole, and dibenzothiophene were all found to be transformed by strain 5RL (All data and schematic representations concerning these experiments are located in figure 8 and table 4, respectively). Acenaphthene was transformed to four products, all of which have been previously identified by Selifonov et al. [1996]. The most prominent of these was found to be 1-acenaphthenone (74.1% TIC). The remaining minor products were identified as acenaphthylene-1,2-diol, *cis*-acenaphthene-1,2-diol, and acenaphtho-1,2-quinone (0.6, 4.8, and 8.9% TIC, respectively).

Biphenyl was transformed to a primary product with a molecular weight of 160 (52.3% TIC). The suggested identity of this compound is 1-phenyl-1,4-butenedial. A minor product was also produced in this biotransformation with a molecular weight of 175. It is similar to the 5-oxo-5-phenylpentanoic acid observed in the transformation of biphenyl by a fluorene-degrading isolate [Grifoll et al., 1995b].

Carbazole was transformed by strain 5RL to three primary products. The formation of one of these, 3-hydroxycarbazole (13.0% TIC), by naphthalene dioxygenase has been observed previously by Resnick et al. [1993]. 1*H*-Indole-2,3-dione (4.8% TIC) was also identified as a product via a library match with a mass spectra database. The final product (9% TIC) is thought to be 2,3-dihydroxyindole. Comparison of its mass spectrum indicates that it shares a similar structure to that of 1*H*-Indole-2,3-dione.

Dibenzothiophene was transformed to two major products. The first (66.0% TIC) was identified as 3-hydroxy-2-formylbenzothiophene. The second product (24.1% TIC) has a molecular weight of 200 and is most likely dibenzothiophene sulfoxide or a hydroxylated dibenzothiophene. The formation of 3-hydroxy-2-formylbenzothiophene in the

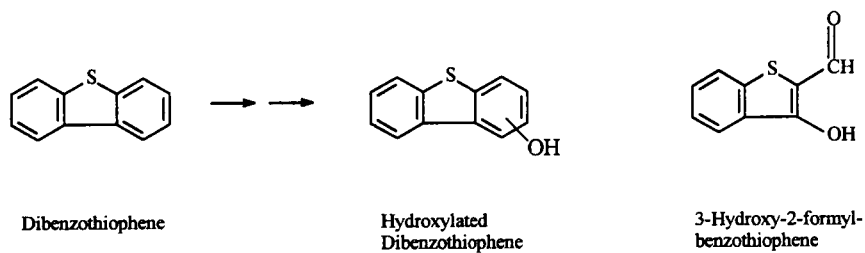
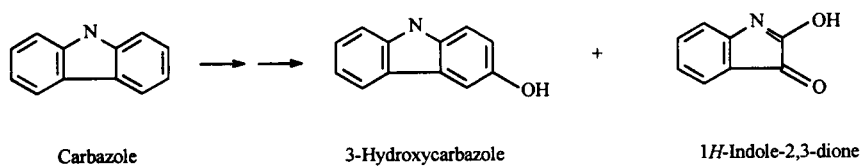
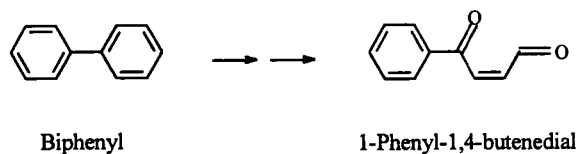
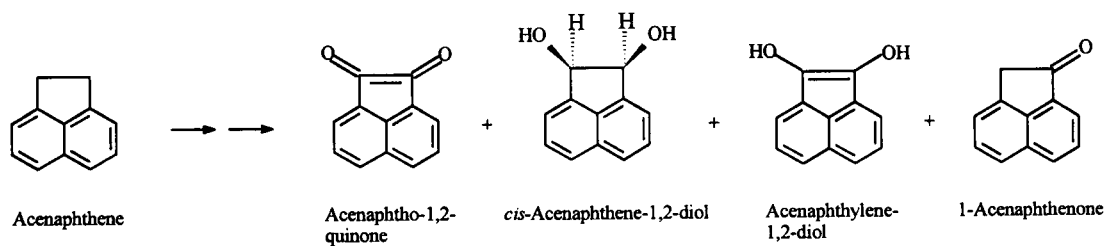


Figure 8. The Biotransformation of Other PAHs and Heterocycles by *P. fluorescens* 5RL.

Table 4. GC/MS Data from the Biotransformation of Other PAHs and Heterocycles by *P. fluorescens* 5RL.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
ACE	N	15.1	74.1	168 (M ⁺ , 100), 141 (14), 140 (95), 139 (82), 113 (9), 89 (5), 87 (5), 70 (9), 63 (6)	1-Acenaphthenone ^b	Selifonov et al. [1996]
				184 (M ⁺ , 96), 168 (24), 156 (52), 155 (83), 140 (19), 139 (21), 128 (100), 127 (79), 126 (26), 101 (10), 77 (12), 75 (9), 63 (10)		
	N	16.6	0.6	186 (M ⁺ , 68), 169 (16), 168 (100), 155 (19), 141 (22), 140 (99), 139 (56), 129 (31), 128 (39), 127 (33), 115 (6), 102 (4), 77 (6), 70 (8)	Acenaphthylene-1,2-diol ^b	Selifonov et al. [1996]
				182 (M ⁺ , 61), 155 (13), 154 (100), 127 (11), 126 (97), 76 (8), 74 (11), 63 (12)		
BPH	A	15.3	52.3	160 (M ⁺ , 5), 145 (1), 131 (2), 115 (4), 106 (9), 105 (100), 104 (6), 99 (4), 91 (4), 77 (22), 51 (6)	1-Phenyl-1,4-butenedial ^c	
				175 (M ⁺ , 22), 154 (5), 148 (9), 147 (19), 120 (8), 115 (3), 106 (9), 105 (100), 91 (4), 77 (38), 55 (10)		
	A	17.3	2.6		yet to be identified	
CBZ	N	14.41	9.0	149 (M ⁺ , 88), 121 (21), 93 (100), 92 (16), 77 (15), 66 (20), 65 (16), 51 (9)	yet to be identified	
	N	17.4	8.9		Acenaphtho-1,2-quinone ^b	Selifonov et al. [1996]

Table 4 cont.

Parent Compound	Extract	GC Retention Time (min.)	Abundance (%) ^d	m/z of Major Ion Peaks (abundance [%])	Metabolite Identification	Relevant References
	N	15.46	4.8	147 (M ⁺ , 61), 120 (9), 119 (100), 92 (60), 91 (14), 76 (6), 64 (19), 63 (15)	1H-Indole-2,3-dione ^b	library match
	N	20.13	13.0	183 (M ⁺ , 100), 182 (15), 154 (26), 127 (9), 91 (8), 77 (6)	3-Hydroxycarbazole ^b	Resnick et al. [1993]
DBT	N	13.97	66.0	178 (M ⁺ , 100), 177 (70), 160 (5), 150 (5), 121 (35), 77 (13)	3-Hydroxy-2-formylbenzothiophene ^c	
	N	19.78	24.1	200 (M ⁺ , 79), 185 (15), 184 (100), 172 (44), 171 (77), 152 (12), 139 (27), 127 (7), 92 (7), 79 (6), 63 (5)	Dibenzothiophene sulfoxide or a hydroxylated dibenzothiophene	Resnick and Gibson [1996b]

^a Identification based on comparison to commercially available standard.

^b Identification based on comparison to references.

^c Suggested structure.

^d Abundance represents peak area percentage for the corresponding extract. In the case of the neutral fraction, it includes any peak area from untransformed parent compound.

*Isomer not identified.

Compound abbreviations: ACE - acenaphthene, BPH - biphenyl, CBZ - carbazole, DBT - dibenzothiophene.

degradation of dibenzothiophene was originally observed by Kodama et al. [1970] in a study of dibenzothiophene-utilizing bacteria.

Growth Assays and *Meta*-Cleavage Product Assays

P. fluorescens 5R did not display the ability to utilize 1-methylnaphthalene or any dimethylnaphthalene as a sole carbon and energy source. Conversely, it was capable of growing solely on 2-methylnaphthalene (data not shown). It was not unexpected that those dimethylnaphthalenes which are hydroxylated to dead-end products would not permit growth since no ring cleavage occurred. Experiments were thus performed to determine whether the ability to grow on 2-methylnaphthalene and naphthalene (determined previously), and the inability to grow on 1-MN, 1,2-DMN, 1,3-DMN, and 2,3-DMN, corresponds to the capacity of strain 5R to utilize the salicylates as sole carbon and energy sources. Because 3-, 4-, and 5-methylsalicylate are the only methyl-substituted salicylates which are commercially available, they were chosen as the model upper pathway products for this group of four compounds

3-Methylsalicylate did not serve as a sole carbon and energy source. Cells in the presence of 3-methylsalicylate did not achieve an OD₆₀₀ greater than that of negative controls. 4-Methylsalicylate and 5-methylsalicylate, however, replaced the role of the native substrate salicylate and were able to serve as sole growth substrates (figure 9; data not shown for 5-methylsalicylate). Cells exposed to 4-methylsalicylate (1.48 mM) achieved an OD₆₀₀ of 0.10; this result was very similar to the OD₆₀₀ of cells which grew in a salicylate solution of approximately the same concentration (1.63 mM). In order to determine whether 3-methylsalicylate could serve as an additional carbon source in the presence of either salicylate or 4-methylsalicylate, growth of 5R on binary mixtures of these substrates was examined. It was found that the addition of 3-methylsalicylate to medium containing either salicylate or 4-methylsalicylate did indeed lead to an increase in OD₆₀₀ over those levels attained by exposure to either salicylate or 4-methylsalicylate

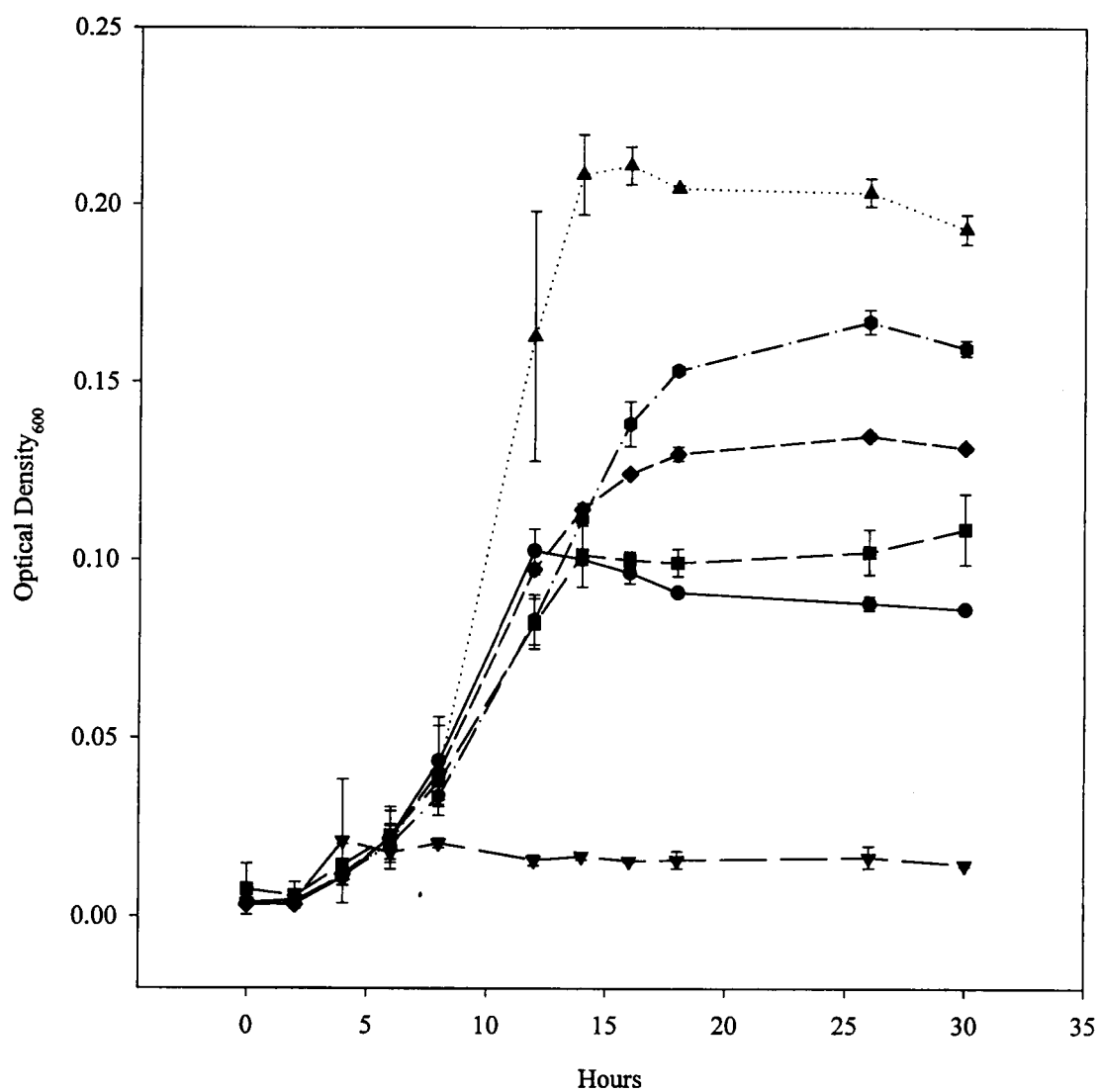


Figure 9. Growth of *Pseudomonas fluorescens* 5R on Salicylate, 3-Methylsalicylate, and 4-Methylsalicylate. Legend: —●— Salicylate, —▼— 3-Methylsalicylate, —■— 4-Methylsalicylate, —◆— Salicylate and 3-Methylsalicylate, ...▲... Salicylate and 4-Methylsalicylate, and —●— 3-Methylsalicylate and 4-Methylsalicylate.

alone. Strain 5R in the presence of a combination of salicylate and 3-methylsalicylate achieved a maximum OD₆₀₀ of 0.13, whereas the combination of 3-methylsalicylate and 4-methylsalicylate led to a maximum OD₆₀₀ of 0.17. The combination of salicylate and 4-methylsalicylate produced a maximum OD₆₀₀ of 0.21.

In separate experiments it was found that 4-methoxysalicylate, the product of 2-methoxynaphthalene metabolism, appeared to allow a minimal amount of growth (see figure 10; discussed below). 3-Hydroxysalicylate also led to an increase in OD₆₀₀. These results, together with the results of *meta*-cleavage assays, are discussed below. No other salicylate analogs were found to serve as carbon and energy sources either individually or in the presence of salicylate.

2-Methoxynaphthalene and 2-chloronaphthalene also appeared to serve as a sole carbon and energy source for strain 5R (data not shown). However, in spite of an increase in the measured OD₆₀₀ over time, no visible turbidity was produced by cells inoculated into a solution of either 2-methoxynaphthalene or 2-chloronaphthalene. This increase in OD₆₀₀ is attributed to the accumulation of colored products which cannot be degraded further (discussed later). Of the other PAHs and heterocycles examined, dibenzofuran and dibenzothiophene both permitted a slight amount of growth by strain 5R. 1-Chloronaphthalene, 1- and 2-hydroxynaphthalene, 1-methoxynaphthalene, and 2-thionaphthalene did not permit the growth of strain 5R. The inability of 1- and 2-hydroxynaphthalene, and the weak ability of dibenzofuran, to permit growth was not expected since all three compounds are transformed to salicylate. It is possible that under these conditions, which are quite different from those in which the biotransformation experiments were performed, the upper pathway reactions do not occur at a fast enough rate to support growth. Selifonov et al. [1990] have previously shown that *Pseudomonas putida* PpG7 is unable to grow on dibenzofuran.

The ability of the NAH lower pathway to cleave 3-methylsalicylate and initiate the reactions necessary for its use as a carbon and energy source is supported by *meta*-cleavage assays using strain 5R and pUTK200. On the basis of spectrophotometric measurements of culture supernatant, salicylate, 3-methylsalicylate, 4-methylsalicylate,

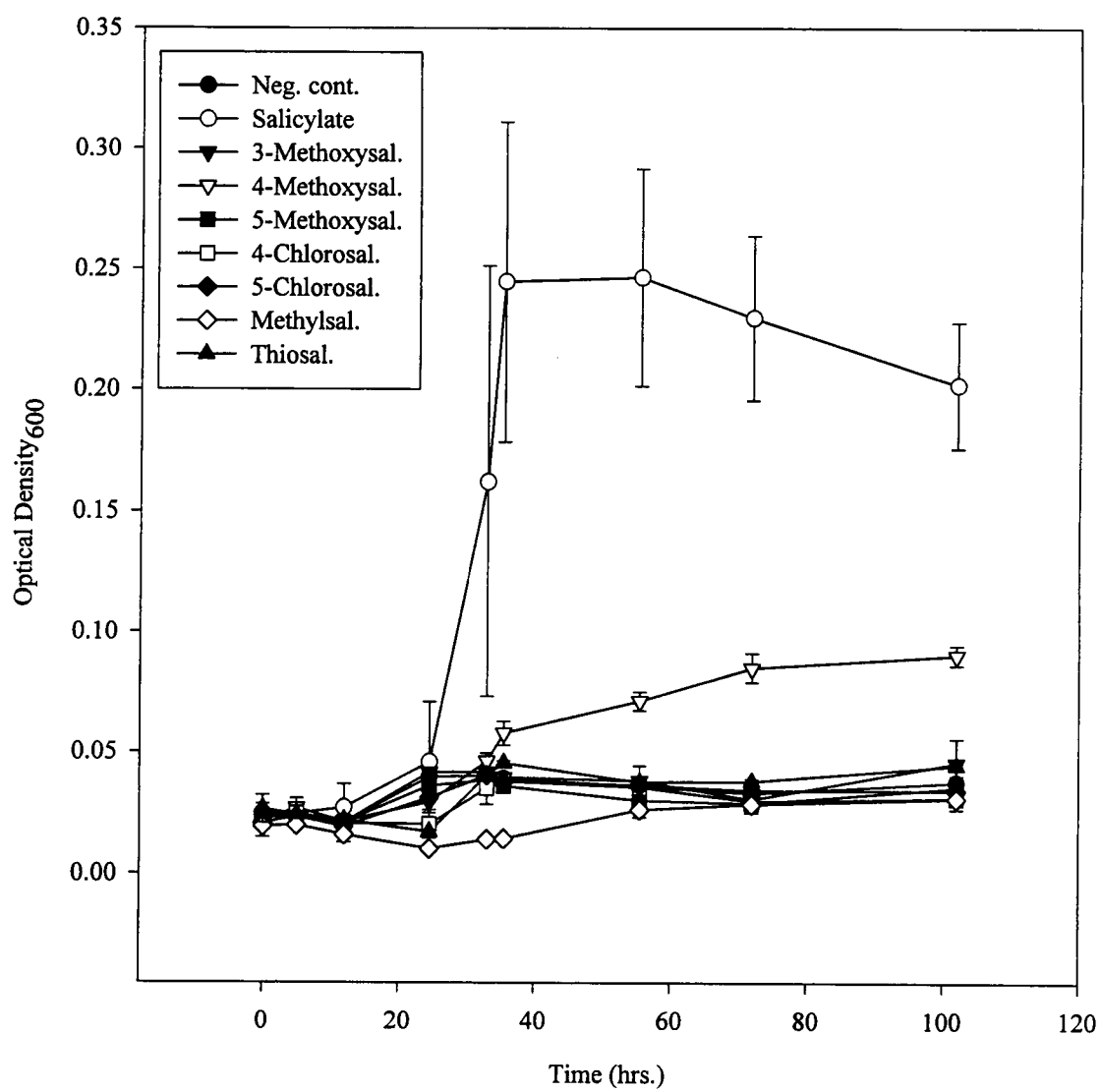


Figure 10. Growth of *P. fluorescens* 5R on Various Salicylate Analogs.

and 5-methylsalicylate were all found to be degraded via *meta*-cleavage reactions to products with absorption maxima of 375, 381, 380, and 380 nm, respectively. These results were verified through an examination of the *meta*-cleavage capabilities of the cloned *nahGH* genes in plasmid pUTK200. *Meta*-cleavage products of methylcatechols, which are produced from the action of salicylate hydroxylase on methylsalicylates as demonstrated here, have previously been shown to produce strong absorbance peaks in the vicinity of 375-400 nm near neutral pH [Bayly et al., 1966]. These products of the *meta*-cleavage of 3- and 4-methylcatechol have been identified as 4-hydroxy-2-oxovalerate and 4-hydroxy-2-oxohexanoate as the respective products [Bayly et al., 1966]. Bayly et al. also showed that 4-hydroxy-2-oxovalerate is further degraded to acetaldehyde and pyruvate, whereas 4-hydroxy-2-oxohexanoate is further degraded to propionaldehyde and pyruvate. Pyruvate is an end product of the *meta*-cleavage of catechol and serves as a growth substrate in the degradation of naphthalene.

Meta-cleavage product assays involving pUTK200 and a number of other commercially available substituted salicylates revealed that only 4- and 5-chlorosalicylate were cleaved by the action of salicylate hydroxylase and catechol dioxygenase (table 5). These two compounds were both transformed to cleavage products with absorbance maximum of 380 nm. The following compounds were not cleaved by the combined action of these two enzymes: 4- and 5-aminosalicylate, 3-, 4-, and 5-hydroxysalicylate, 3-, 4-, and 5-methoxysalicylate, methylsalicylate, 3-nitrosalicylate, and thiosalicylate. The inability of the lower pathway to cleave these compounds is an impediment to their utilization as carbon and energy sources. Of this suite of commercially available salicylates, the data concerning 3- and 4-chlorosalicylate, and 3- and 4-methoxysalicylate are most noteworthy because these compounds represent actual upper pathway products in the transformation of the respective chloro- and methoxynaphthalenes.

Table 5. *Meta*-Cleavage Product Formation by Salicylate Hydroxylase and Catechol 2,3-Dioxygenase of pUTK200.

Test Compound	Absorbance Maximum (nm) of Product
<u>Electrically neutral substituents:</u>	
3-Chlorosalicylate	ND
4-Chlorosalicylate	380
5-Chlorosalicylate	380
3-Methoxysalicylate	ND
4-Methoxysalicylate	ND
5-Methoxysalicylate	ND
3-Methylsalicylate	385
4-Methylsalicylate	380
5-Methylsalicylate	380
Salicylate	375
<u>Electron-releasing substituents:</u>	
4-Aminosalicylate	ND
5-Aminosalicylate	ND
3-Hydroxysalicylate	ND
4-Hydroxysalicylate	ND
5-Hydroxysalicylate	ND
<u>Electron-withdrawing substituents:</u>	
3-Nitrosalicylate	ND
<u>Variation in the 2 position:</u>	
Methylsalicylate	ND
Salicylate	375
Thiosalicylate	ND
Negative Control	ND

ND - none detected

Bioluminescence Reporter Assays and mRNA Analysis

The roles of 3-, 4-, and 5-methylsalicylate in the regulation of NAH system transcription was examined using *P. fluorescens* 5RL according to the procedure outlined by Heitzer et al. [1992]. It was found that 4-methylsalicylate produced a bioluminescent response that was greater than that produced by salicylate as the test compound concentrations were increased from 0.1 to 200 mg/L. This difference was not pronounced at 0.10 mg/L (table 6). At this concentration (the actual levels are 0.073 mM salicylate and 0.066 mM 4-methylsalicylate), 4-methylsalicylate induced an average bioluminescent response of $2.4 \times 10^0 \pm 0.2 \times 10^0$ nanoamps ($\pm$ denotes standard deviation). Cells exposed to salicylate produced an average response of $2.2 \times 10^0 \pm 0.2 \times 10^0$ nanoamps. Negative controls without any test compound produced a bioluminescent response of $2.2 \times 10^0 \pm 0.6 \times 10^0$ nanoamps (data not shown). In separate experiments involving 3-methylsalicylate and 5-methylsalicylate it was found that 5-methylsalicylate at 100 and 200 mg/L induced a light response slightly greater than that induced by 4-methylsalicylate at the same concentration ($1.2 \times 10^4 \pm 2.0 \times 10^2$ and $1.2 \times 10^4 \pm 3.5 \times 10^2$, respectively, for 5-methylsalicylate vs. $8.2 \times 10^3 \pm 1.7 \times 10^2$ and $9.8 \times 10^3 \pm 1.0 \times 10^2$, respectively, for 4-methylsalicylate). However, 5-methylsalicylate produced a much lower light response than 4-methylsalicylate at 10 mg/L. 3-Methylsalicylate was shown to be a relatively poor inducer at concentrations at or below 10 mg/L. At concentrations of 100 and 200 mg/L, 3-methylsalicylate ($1.8 \times 10^3 \pm 2.8 \times 10^1$ and $1.1 \times 10^3 \pm 6.2 \times 10^1$, respectively) induced much lower light responses than 4-methylsalicylate, 5-methylsalicylate, and salicylate ($6.9 \times 10^3 \pm 3.9 \times 10^2$ and $7.5 \times 10^3 \pm 2.7 \times 10^2$, respectively).

In an analysis of the respective parent compounds at one-half of their aqueous solubility, it was found that 2-methylnaphthalene produced a bioluminescent response of $1.4 \times 10^3 \pm 3.2 \times 10^1$ nanoamps/OD₆₀₀. Naphthalene produced a bioluminescent response of $3.4 \times 10^2 \pm 3.5 \times 10^1$ nanoamps/OD₆₀₀, whereas 1-methylnaphthalene produced a bioluminescent response of $1.4 \times 10^2 \pm 2.0 \times 10^0$ nanoamps/OD₆₀₀. These results mirror the trends seen in experiments performed with the respective salicylate analogs. The

Table 6. Bioluminescent Response of *P. fluorescens* 5RL Exposed to Methyl-Substituted Salicylates over a Range of Concentrations.

Test Compound Concentration (mg/L)	Salicylate	Specific Bioluminescence (nanamps/OD ₆₀₀)		
		3-Methylsalicylate	4-Methylsalicylate	5-Methylsalicylate
10	$3.1 \times 10^3 \pm 8.4 \times 10^1$	$2.7 \times 10^1 \pm 0.5 \times 10^0$	$8.0 \times 10^3 \pm 2.1 \times 10^2$	$2.7 \times 10^2 \pm 2.4 \times 10^1$
7.5	$2.9 \times 10^3 \pm 6.1 \times 10^1$	$2.0 \times 10^1 \pm 0.7 \times 10^0$	$7.9 \times 10^3 \pm 1.3 \times 10^2$	$1.5 \times 10^2 \pm 6.5 \times 10^0$
5.0	$2.5 \times 10^3 \pm 4.3 \times 10^0$	$1.6 \times 10^1 \pm 0.6 \times 10^0$	$8.6 \times 10^3 \pm 9.5 \times 10^1$	$6.2 \times 10^1 \pm 8.9 \times 10^0$
2.5	$1.2 \times 10^3 \pm 6.7 \times 10^1$	$1.4 \times 10^1 \pm 0.1 \times 10^0$	$7.7 \times 10^3 \pm 2.0 \times 10^2$	$2.1 \times 10^1 \pm 1.2 \times 10^0$
1.0	$1.5 \times 10^2 \pm 7.1 \times 10^0$	$1.4 \times 10^1 \pm 0.4 \times 10^0$	$3.7 \times 10^3 \pm 1.5 \times 10^2$	$1.5 \times 10^1 \pm 0.4 \times 10^0$
0.75	$9.7 \times 10^0 \pm 0.7 \times 10^0$	NT	$7.9 \times 10^2 \pm 5.2 \times 10^1$	NT
0.5	$3.8 \times 10^0 \pm 0.1 \times 10^0$	NT	$1.7 \times 10^2 \pm 2.0 \times 10^1$	NT
0.25	$2.4 \times 10^0 \pm 0.1 \times 10^0$	NT	$9.1 \times 10^0 \pm 0.4 \times 10^0$	NT
0.1	$2.2 \times 10^0 \pm 0.2 \times 10^0$	NT	$2.4 \times 10^0 \pm 0.2 \times 10^0$	NT

NT = not tested.

± = standard deviation

Negative control = $2.2 \times 10^0 \pm 0.6 \times 10^0$

negative control in this experiment produced a bioluminescent response of $3.2 \times 10^1 \pm 2.1 \times 10^0$ nanoamps/OD₆₀₀. In a separate experiment, 1,2-, 1,3-, and 2,3-DMN produced bioluminescent responses of $3.5 \times 10^1 \pm 0.6 \times 10^0$, $3.7 \times 10^1 \pm 6.3 \times 10^0$, and $2.2 \times 10^1 \pm 0.3 \times 10^0$ nanoamps/OD₆₀₀. The negative control in this experiment had a light response of $1.5 \times 10^1 \pm 0.4 \times 10^0$ nanoamps/OD₆₀₀.

A number of other commercially-available upper pathway products and their structural analogs were tested for their ability to induce NAH system transcription. It was found that compounds with a substituent at the 4 position were most successful at producing a light response (table 7). For example, 4-aminosalicylate, 4-chlorosalicylate, 4-methoxysalicylate, and 4-methylsalicylate, at their normalized concentrations, produced light responses of $8.4 \times 10^2 \pm 5.3 \times 10^1$, $2.4 \times 10^1 \pm 5.3 \times 10^0$, $2.2 \times 10^2 \pm 2.3 \times 10^1$, $1.1 \times 10^3 \pm 2.1 \times 10^2$ nanoamps/OD₆₀₀, respectively. With the exception of 5-aminosalicylate (which was tested at ½ its normalized concentration due to solubility limitations), no other substituted salicylate produced a light response significantly greater than that of the negative control.

Quantification of *lux* mRNA in cells incubated with salicylate and 4-methylsalicylate was performed in order to verify that the production of light by cells exposed to 4-methylsalicylate corresponded to actual induction of NAH system transcription. It was found that cells exposed to 4-methylsalicylate did show levels of *lux* mRNA that increased as the concentration of 4-methylsalicylate was increased from 0.1 to 1.0 mg/L. As with the bioluminescent responses, the difference between *lux* mRNA produced by cells exposed to salicylate and 4-methylsalicylate was more pronounced at a higher test compound concentration. In this case, the level of *lux* mRNA at 1.0 mg/L 4-methylsalicylate was 0.09 µg/ml with a standard deviation of ± 0.01 . Exposure to salicylate at 1.0 mg/L produced a *lux* mRNA concentration of 0.04 µg/ml with a standard deviation of ± 0.01 . At test compound concentrations of 0.10 mg/L there was no measurable difference in the amount of *lux* mRNA produced, and it was approximately the same concentration as that of the negative controls.

Table 7. Bioluminescent Response *P. fluorescens* 5RL Exposed to Various Salicylate Analogs.

Test Compound	Log K _{ow} ^a	Concentration ^b	Specific Bioluminescence (Nanoamps/OD ₆₀₀)
<u>Electrically neutral substituents:</u>			
3-Chlorosalicylate	3.04	1.9	NSD
4-Chlorosalicylate	3.04	1.9	2.4x10 ¹ ±5.3x10 ⁰
5-Chlorosalicylate	3.09	1.9	NSD
3-Methoxysalicylate	1.94	25.5	NSD
4-Methoxysalicylate	2.23	25.5	2.2x10 ² ±2.3x10 ¹
5-Methoxysalicylate	1.94	25.5	NSD
3-Methylsalicylate	2.86	2.8	NSD
4-Methylsalicylate	2.99	2.1	1.1x10 ³ ±2.1x10 ²
5-Methylsalicylate	2.78	2.1	NSD
Salicylate	2.26	NP	1.1x10 ³ ±6.7x10 ¹
<u>Electron-releasing substituents:</u>			
4-Aminosalicylate	0.89	260.0	8.4x10 ² ±5.3x10 ¹
5-Aminosalicylate	-0.16	1600.0	7.3x10 ¹ ±0.8x10 ⁰
3-Hydroxysalicylate	1.20	128.0	NSD
4-Hydroxysalicylate	1.63	48.8	NSD
5-Hydroxysalicylate	1.74	37.0	NSD
<u>Electron-withdrawing substituents:</u>			
3-Nitrosalicylate	-0.73	100.0	NSD
<u>Variation in the 2 position:</u>			
Methylsalicylate	2.55	5.7	NSD
Salicylate	2.26	NP	1.1x10 ³ ±6.7x10 ¹
Thiosalicylate	2.39	8.3	NSD

^aDerivation of log K_{ow} values discussed in text.

^bTest compound concentrations (mg/L) were normalized to the same internal concentration of salicylate using the following equation:

$$(\log k_{ow(sal)} / \log k_{ow(test\ compound)}) * (\text{molar conc. of salicylate at } 10 \text{ mg/L}) * \text{MW of test compound}$$

NP = Not applicable

NSD = Not statistically different from negative control

± = standard deviation

Negative control = 8.6x10⁰±1.7x10⁰

Elucidation of Biological Factors Contributing to the Preferential Degradation of PAHs in Complex Mixtures

It was of interest to determine whether the NAH system, in the absence of potential degradation-limiting physical surroundings such as a soil matrix, mediates the preferential degradation of certain PAHs when present in a mixture. As described earlier, previous researchers have shown that PAH mixture degradation in a soil matrix with an undefined bacterial consortium occurs in a preferential manner. Based on a number of experiments which examined the physicochemical properties of PAHs in soil, it can be assumed that this preferential degradation is due at least in part to the adsorption of higher molecular weight PAHs to soil matrices. In spite of these valuable findings, the role of bacteria in preferential degradation has not been elucidated. No data have been published concerning the existence of specific biological factors, such as differing enzyme specificities for certain compounds, that may also contribute to preferential PAH degradation. The experiments described below were intended to examine the biological basis for preferential relative rates of degradation of mixed PAHs by the NAH system. The compositions of the mixtures were chosen to include low-molecular-weight compounds known to be degraded by the NAH system.

The first experiment performed utilized *P. fluorescens* 5R pregrown under inducing conditions in YEPSS medium. Based on GC analysis of hexane extracts, naphthalene and 2-methylnaphthalene disappeared first within 2 hrs. 1-Methylnaphthalene, dibenzothiophene, acenaphthene, and phenanthrene disappeared slightly afterward; 1-methylnaphthalene disappeared within 10 hrs. Dibenzothiophene also disappeared within 10 hrs, although this was not entirely clear due to an outlier of high concentration at the 10 hr timepoint. Acenaphthene and phenanthrene disappeared within 22 hrs. The first-order rate constants of these two compounds are very similar, $-0.206/\text{h}$ and $-0.045/\text{h}$, respectively. Biphenyl, dibenzofuran, and fluorene disappeared within 48 hrs. 2,6-Dimethylnaphthalene was the most slowly degraded compound. It did not disappear during the duration of the experiment and had a first-order rate constant of $-0.0031/\text{h}$.

No appreciable parent compound disappearance was observed in killed controls; this was also true for every strain and mixture combination examined in subsequent experiments. A series of sample chromatograms is shown in figure 11; the rapid disappearance of naphthalene and 2-methylnaphthalene compared to the other components of the mixture is quite evident in this figure. Rate constant values are shown in figure 12; disappearance curves for all experiments discussed from this point are included in the appendix. This disappearance curves for this experiment are represented in figure 24 in the appendix.

Similar experiments were also performed with a number of other well-characterized NAH system strains (all grown on YEPSS) in order to determine whether they respond similarly when presented with the same mixture. This is an important point of experimentation because it verifies that unknown, species-specific factors other than the activity of the NAH system are not an issue in the degradation of a PAH mixture. It was found with *Pseudomonas* sp. strain DFC50 that the same general trends are observed as for strain 5R. Naphthalene, 2-methylnaphthalene, and 1-methylnaphthalene, respectively, were again the first three compounds to disappear. In this case, naphthalene and 2-methylnaphthalene both disappeared within 2 hrs and had first-order rate constants of $-6.852/\text{h}$ and $-1.938/\text{h}$, respectively. 1-Methylnaphthalene disappeared within 3 hrs and had a first-order rate constant of $-1.094/\text{h}$. Dibenzothiophene also disappeared within 3 hrs and had a first-order rate constant of $-0.346/\text{h}$. Acenaphthene, dibenzofuran, and phenanthrene disappeared within 12 hrs with respective first-order rate constants of $-0.350/\text{h}$, $-0.420/\text{h}$, and $-0.177/\text{h}$, whereas fluorene was degraded within 48 hrs. Biphenyl appeared to be a less favorable substrate than for strain 5R; it, along with 2,6-dimethylnaphthalene, was not removed within the course of the experiment (Refer to figure 13 for disappearance rate constants and to figure 25 in the appendix for disappearance curves).

Experiments which utilized strains DFC49 and PpG7 general also showed similar patterns of preferential degradation. In the case of strain DFC49, naphthalene and 2-methylnaphthalene were completely disappeared within 1-2 hrs. The majority of the 1-

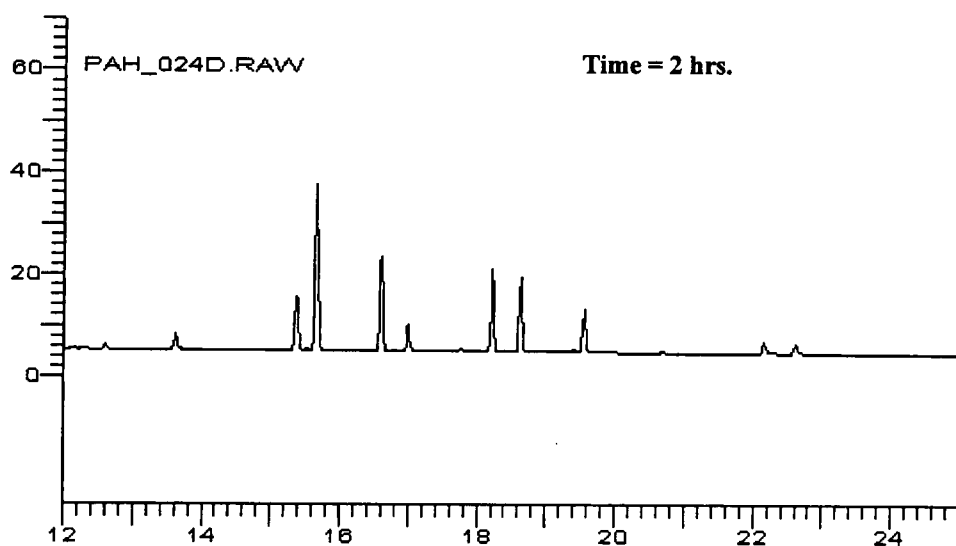
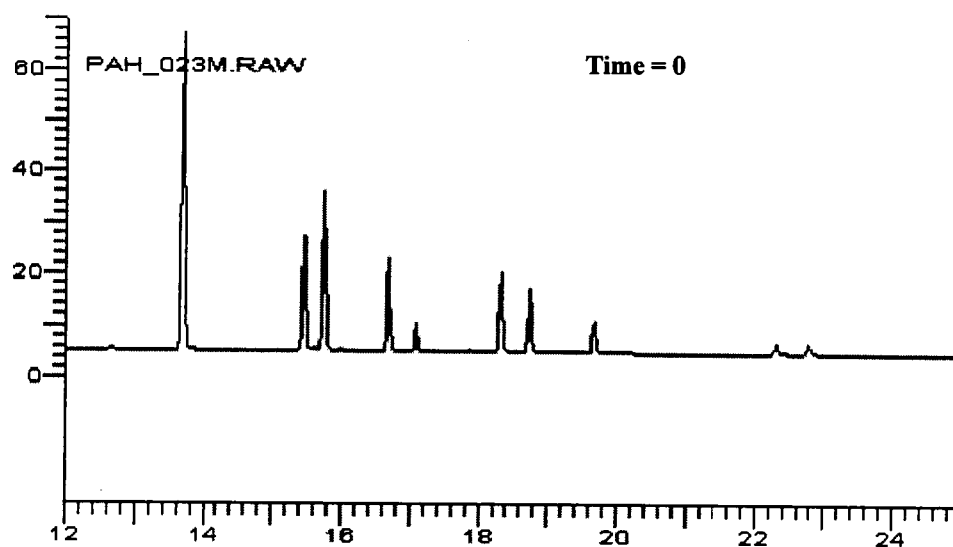


Figure 11. Sample Chromatograms from Different Time Points in the Biodegradation of a PAH and Heterocycle Mixture by *P. fluorescens* 5R. In each chromatogram the left axis represents the FID signal in mV and the bottom axis is the retention time in minutes. Peak identifications are the following: naphthalene (13.7 min.), 2-methylnaphthalene (15.5 min.), 1-methylnaphthalene (15.7 min.), biphenyl (16.7 min.), 2,6-dimethylnaphthalene (17.1 min.), dibenzofuran (18.3 min.), acenaphthene (18.7 min.), fluorene (19.7 min.), dibenzothiophene (22.3 min.), and phenanthrene (22.8 min.).

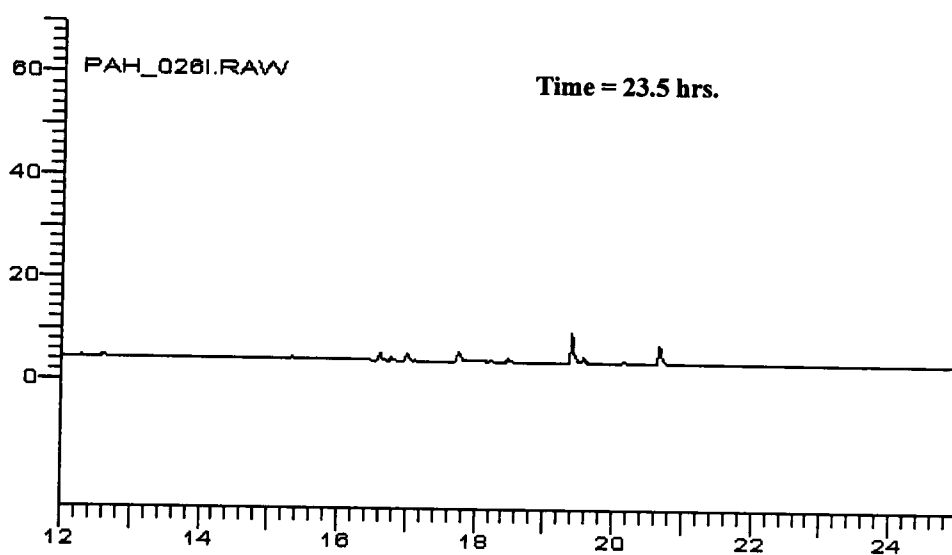
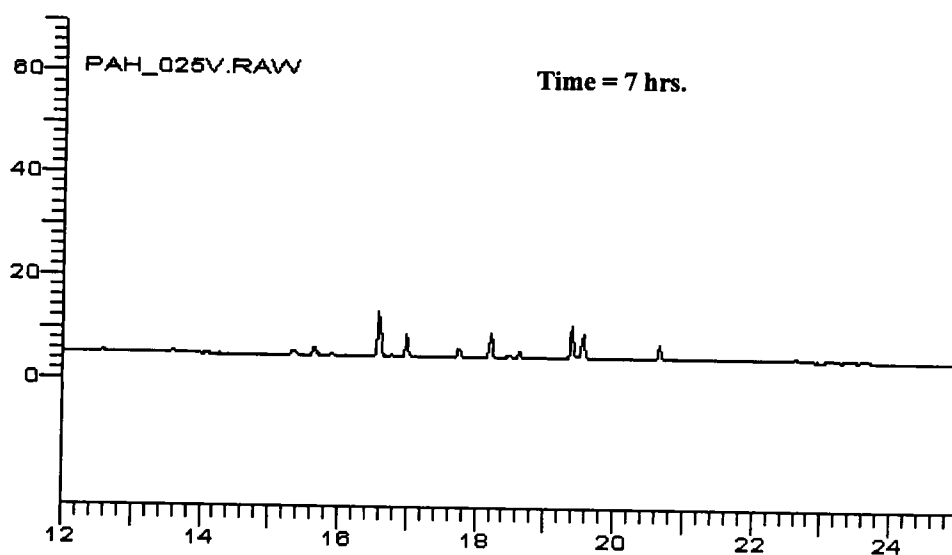


Figure 11 cont.

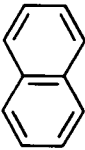
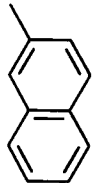
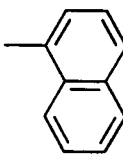
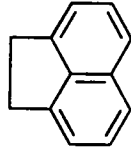
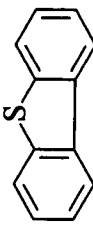
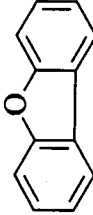
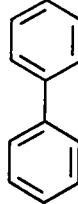
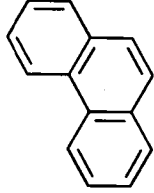
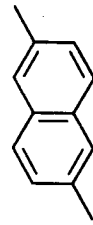
	Naphthalene $k_1 = -4.960/\text{h}$ $r^2 = 0.694$		2-Methylnaphthalene $k_1 = -2.571/\text{h}$ $r^2 = 0.818$		1-Methylnaphthalene $k_1 = -0.252/\text{h}$ $r^2 = 0.584$		Acenaphthene $k_1 = -0.206/\text{h}$ $r^2 = 0.637$		Dibenzothiophene $k_1 = -0.104/\text{h}$ $r^2 = 0.610$
	Dibenzofuran $k_1 = -0.071/\text{h}$ $r^2 = 0.515$		Biphenyl $k_1 = -0.051/\text{h}$ $r^2 = 0.484$		Phenanthrene $k_1 = -0.045/\text{h}$ $r^2 = 0.312$		Fluorene $k_1 = -0.025/\text{h}$ $r^2 = 0.140$		2,6-Dimethylnaphthalene $k_1 = -0.0031/\text{h}$ $r^2 = 0.0019$

Figure 12. Rate Constants for the Biodegradation of a PAH and Heterocycle Mixture by *P. fluorescens* 5R.

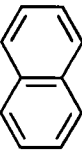
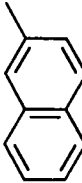
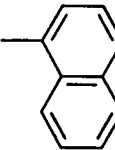
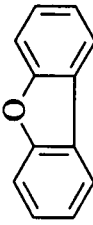
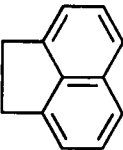
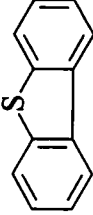
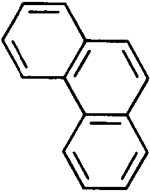
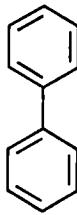
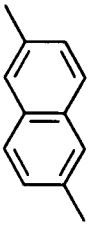
	Naphthalene $k_1 = -6.852/h$ $r^2 = 1.000$		2-Methylnaphthalene $k_1 = -1.938/h$ $r^2 = 0.962$		1-Methylnaphthalene $k_1 = -1.094/h$ $r^2 = 0.917$		Dibenzofuran $k_1 = -0.420/h$ $r^2 = 0.951$		Acenaphthene $k_1 = -0.350/h$ $r^2 = 0.937$
	Dibenzothiophene $k_1 = -0.346/h$ $r^2 = 0.837$		Phenanthrene $k_1 = -0.177/h$ $r^2 = 0.623$		Fluorene $k_1 = -0.096/h$ $r^2 = 0.868$		Biphenyl $k_1 = -0.025/h$ $r^2 = 0.949$		2,6-Dimethylnaphthalene $k_1 = -0.013/h$ $r^2 = 0.922$

Figure 13. Rate Constants for the Biodegradation of a PAH and Heterocycle Mixture by *Pseudomonas* sp. DFC50.

methylnaphthalene disappeared within 10 hrs; it completely disappeared within 24 hrs. Acenaphthene, dibenzofuran, dibenzothiophene, and phenanthrene disappeared after naphthalene, 2-methylnaphthalene, and 1-methylnaphthalene. Dibenzothiophene was the most rapidly removed of these four compounds within an interval of 48 hrs.

Acenaphthene and dibenzofuran showed very similar patterns of disappearance and first-order rate constants. The majority of each was removed within 48 hrs; their respective rate constants were $-0.016/\text{h}$ and $-0.017/\text{h}$. The degradation of phenanthrene was erratic with a large amount of variation within each of the latter time points. As in the case of strain DFC50, fluorene, biphenyl, and 2,6-dimethylnaphthalene were the most slowly removed compounds. None achieved complete disappearance. Their respective rate constants were $-0.006/\text{h}$, $-0.004/\text{h}$, and $-0.002/\text{h}$ (refer to figure 14 in text and to figure 26 in the appendix).

Strain PpG7 also removed naphthalene and 2-methylnaphthalene most rapidly; both completely disappeared within 2 hrs. Acenaphthene was transformed more rapidly, with respect to the relative disappearance of the other components of the mixture, by PpG7 than by any other strain. It completely disappeared along with 1-methylnaphthalene within 12 hrs. The first-order rate constants for acenaphthene and 1-methylnaphthalene were $-0.302/\text{h}$ and $-0.239/\text{h}$, respectively. As with strains 5R, DFC50, and DFC49, dibenzothiophene was rapidly removed within 6 hrs; it had a first-order rate constant of $-0.174/\text{h}$. Phenanthrene completely disappeared within 12 hrs with a rate constant of $-0.088/\text{h}$. However, strain PpG7, as in the manner of strain 5R, removed biphenyl more rapidly with respect to the other components of the mixture than did strains DFC49 and 50. It was degraded within 48 hrs with a rate constant of $-0.100/\text{h}$. Dibenzofuran, fluorene, and phenanthrene both completely disappeared within 48 hrs with similar rate constants. The trend of 2,6-dimethylnaphthalene being the most slowly removed compound was repeated in this experiment. It did not completely disappear over the course of the experiment and had a first-order rate constant for disappearance of $-0.020/\text{h}$ (refer to figure 15 in the text and to figure 27 in the appendix).

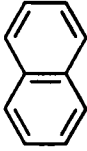
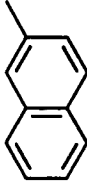
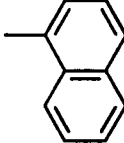
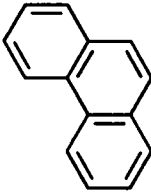
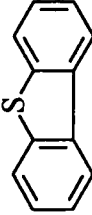
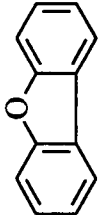
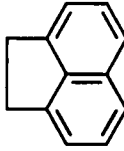
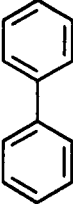
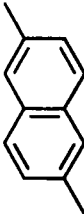
	Naphthalene	$k_1 = -7.279/\text{h}$ $r^2 = 0.981$		2-Methylnaphthalene	$k_1 = -1.390/\text{h}$ $r^2 = 0.915$		1-Methylnaphthalene	$k_1 = -0.322/\text{h}$ $r^2 = 0.795$		Phenanthrene	$k_1 = -0.059/\text{h}$ $r^2 = 0.585$		Dibenzothiophene	$k_1 = -0.047/\text{h}$ $r^2 = 0.616$
	Dibenzofuran	$k_1 = -0.017/\text{h}$ $r^2 = 0.687$		Acenaphthene	$k_1 = -0.016/\text{h}$ $r^2 = 0.753$		Fluorene	$k_1 = -0.006/\text{h}$ $r^2 = 0.677$		Biphenyl	$k_1 = -0.004/\text{h}$ $r^2 = 0.732$		2,6-Dimethylnaphthalene	$k_1 = -0.002/\text{h}$ $r^2 = 0.445$

Figure 14. Rate Constants for the Biodegradation of a PAH and Heterocycle Mixture by *Pseudomonas* sp. DFC49.

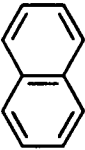
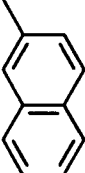
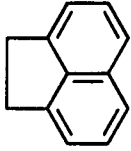
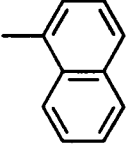
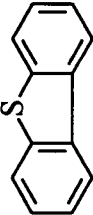
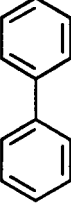
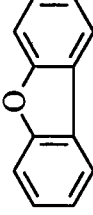
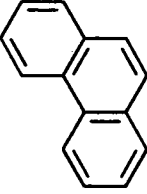
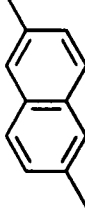
	Naphthalene	$k_1 = -3.489/\text{h}$ $r^2 = 0.933$		2-Methylnaphthalene	$k_1 = -2.163/\text{h}$ $r^2 = 0.900$		Acenaphthene	$k_1 = -0.302/\text{h}$ $r^2 = 0.912$		1-Methylnaphthalene	$k_1 = -0.239/\text{h}$ $r^2 = 0.888$		Dibenzothiophene	$k_1 = -0.174/\text{h}$ $r^2 = 0.725$
	Biphenyl	$k_1 = -0.100/\text{h}$ $r^2 = 0.903$		Dibenzofuran	$k_1 = -0.068/\text{h}$ $r^2 = 0.930$		Phenanthrene	$k_1 = -0.088/\text{h}$ $r^2 = 0.528$		Fluorene	$k_1 = -0.054/\text{h}$ $r^2 = 0.883$		2,6-Dimethylnaphthalene	$k_1 = -0.020/\text{h}$ $r^2 = 0.856$

Figure 15. Rate Constants for the Biodegradation of a PAH and Heterocycle Mixture by *Pseudomonas putida* PpG7.

Strain PpG7 has previously been used in a mixture degradation experiment by Mueller et al. [1989]. The artificial mixture examined by these researchers also included naphthalene, 2-methylnaphthalene, 1-methylnaphthalene, biphenyl, acenaphthene, 2,3-dimethylnaphthalene, 2,6-dimethylnaphthalene, fluorene, and phenanthrene in addition to a number of higher molecular weight PAHs. They found that after 3 days of incubation, naphthalene and 2-methylnaphthalene were preferentially removed completely. 1-Methylnaphthalene and the remaining PAHs were not extensively degraded. The experimental conditions are very different to those discussed above; however, the same trend of naphthalene and 2-methylnaphthalene being degraded preferentially was observed. The reason why the other compounds were not significantly degraded is unknown since the NAH system of strain PpG7 has been shown to transform a number of these compounds.

The next experiment utilized *P. fluorescens* 5RL, which was also pregrown in YEPSS medium prior to introducing it to the mixture. This experiment was intended to be the next logical step from previous experiments utilizing the parent strain 5R and the NAH strains DFC50, DFC49, and PpG7. The objective was to determine whether the lower pathway of the NAH system, which is non-functional in strain 5RL, can be eliminated as a factor in the degradation of a the PAH mixture. Although it was not expected that such downstream effects of the lower pathway would influence the pattern of preferential degradation, it was necessary to test this assumption. As in the earlier experiment with strain 5R, naphthalene and 2-methylnaphthalene were disappeared most rapidly. In this case however, both compounds were removed within 1 hr. In fact, naphthalene was removed so rapidly that determination of a first-order rate constant was not possible. This may be due to elevated enzyme levels since the inducer salicylate, present in the YEPSS medium, is not degraded by strain 5RL. Contrary to previous experiments utilizing strain 5R, 1-methylnaphthalene appeared to be removed more slowly relative to the other components of the mixture; it was not completely degraded until the 48 hr time point. The first-order rate constant of 1-methylnaphthalene disappearance was $-0.124/h$. Phenanthrene was again removed within 24 hrs; however, the measurements for

phenanthrene concentration in the hexane extracts were erratic and were characterized by a large amount of variation. Its first-order rate constant was 0.212/h with a low r^2 value of 0.439. Dibenzothiophene completely disappeared from the mixture within 12 hrs. Its disappearance was also erratic and had a rate constant value of -0.159/h with a low r^2 of 0.449. Acenaphthene and biphenyl were both completely removed within 48 hrs. Dibenzofuran disappeared within 72 hrs; both it and biphenyl had rate constants of -0.040/h. Fluorene and 2,6-dimethylnaphthalene were the most slowly transformed compounds. Fluorene was completely removed within 48 hrs, but it had a rate constant of -0.022/h. 2,6-Dimethylnaphthalene completely disappeared within 72 hrs and had the lowest rate constant value of -0.020/h (refer to figure 16 in the text and to figure 28 in the appendix). To summarize the results of this particular experiment, it does not appear that elimination of the lower pathway has an impact on the nature of preferential degradation.

Having determined that the lower pathway does not influence preferential degradation in strains 5R and 5RL, the next experiment was conducted using pUTK202 in an *E. coli* strain (refer to table 1). The objective was to examine whether naphthalene dioxygenase alone is responsible for the preferential removal by the NAH system of PAHs in a complex mixture. As shown in figure 17, the pattern of disappearance is similar to that seen for experiments with strains 5R and 5RL. Naphthalene and 2-methylnaphthalene were again removed most rapidly. Naphthalene disappeared within 2 hrs with a first-order rate constant of -1.621/h, and 2-methylnaphthalene disappeared within 4 hrs with a first-order rate constant of -0.931/h. 1-Methylnaphthalene, acenaphthene, and dibenzothiophene all were completely removed within 16 hrs with rate constants ranging from -0.383/h to -0.211/h. Phenanthrene and dibenzofuran were essentially completely removed within 24 hrs with rate constants of -0.140/h and -0.129/h, respectively. Fluorene, biphenyl, and 2,6-dimethylnaphthalene did not completely disappear over the course of the experiment. As in all previous experiments using components of the NAH system of strain 5R, 2,6-dimethylnaphthalene had the lowest rate constant. In this case, it

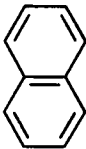
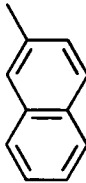
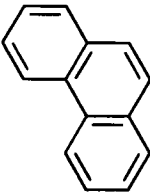
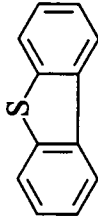
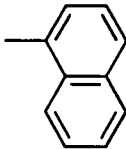
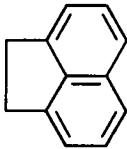
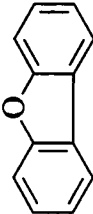
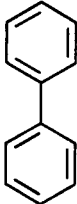
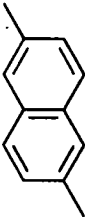
	Naphthalene	k_1 too fast to measure	
	2-Methylnaphthalene	$k_1 = -4.370/h$ $r^2 = 0.939$	
	Phenanthrene	$k_1 = -0.212/h$ $r^2 = 0.439$	
	Dibenzothiophene	$k_1 = -0.159/h$ $r^2 = 0.449$	
	1-Methylnaphthalene	$k_1 = -0.124/h$ $r^2 = 0.951$	
	Acenaphthene	$k_1 = -0.069/h$ $r^2 = 0.642$	
	Dibenzofuran	$k_1 = -0.040/h$ $r^2 = 0.938$	
	Biphenyl	$k_1 = -0.040/h$ $r^2 = 0.844$	
	Fluorene	$k_1 = -0.022/h$ $r^2 = 0.641$	
	2,6-Dimethylnaphthalene	$k_1 = -0.020/h$ $r^2 = 0.787$	

Figure 16. Rate Constants for the Biodegradation of a PAH and Heterocycle Mixture by *P. fluorescens* 5RL.

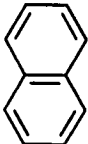
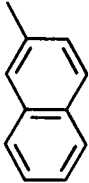
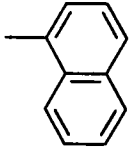
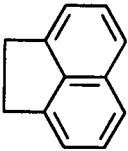
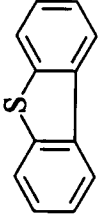
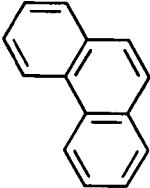
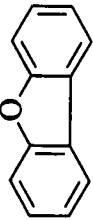
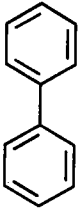
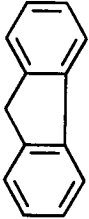
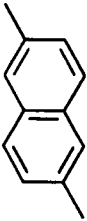
	Naphthalene	$k_1 = -1.621/\text{h}$ $r^2 = 0.899$		2-Methylnaphthalene	$k_1 = -0.931/\text{h}$ $r^2 = 0.845$		1-Methylnaphthalene	$k_1 = -0.383/\text{h}$ $r^2 = 0.803$		Acenaphthene	$k_1 = -0.296/\text{h}$ $r^2 = 0.763$		Dibenzothiophene	$k_1 = -0.211/\text{h}$ $r^2 = 0.813$
	Phenanthrene	$k_1 = -0.140/\text{h}$ $r^2 = 0.803$		Dibenzofuran	$k_1 = -0.129/\text{h}$ $r^2 = 0.872$		Biphenyl	$k_1 = -0.085/\text{h}$ $r^2 = 0.907$		Fluorene	$k_1 = -0.043/\text{h}$ $r^2 = 0.487$		2,6-Dimethylnaphthalene	$k_1 = -0.021/\text{h}$ $r^2 = 0.614$

Figure 17. Rate Constants for the Biodegradation of a PAH and Heterocycle Mixture by *E. coli* DH5a Harboring pUTK202.

was $-0.021/h$. It can thus be determined by inference that naphthalene dioxygenase is responsible for preferential degradation in a mixture (refer to figure 17 and to figure 29 in the appendix).

An additional experiment was performed using strain 5R pregrown in YEPG medium rather than YEPSS. It was intended to examine whether there is a difference in the pattern of disappearance when the cells are pregrown under non-inducing conditions. Although there is a level of basal expression of NAH system enzymes in this growth medium, the NAH system enzymes of strain 5R will not be fully expressed under these conditions until naphthalene and 2-methylnaphthalene are degraded to the inducer molecules salicylate and 4-methylsalicylate. In this experiment, the trend of naphthalene and 2-methylnaphthalene being the most rapidly degraded components was repeated. These data again show that fluorene and 2,6-dimethylnaphthalene were the two most slowly removed compounds. Although the first-order rate constants are lower across the board, the order of disappearance is basically the same (refer to figure 18 in the text and figure 30 in the appendix). The overall slower rates of disappearance were not unexpected given the non-inducing growth condition prior to the introduction of strain 5R to the mixture. The results of this experiment agree with those of the previous experiment involving the *nahA* clone. The nature of preferential degradation is not dependent on any factors other than the specificity of naphthalene dioxygenase. A negative control experiment utilizing the vector without the *nahA* insert showed no mixture degradation.

The Contribution of Selected Chemical Structures to Preferential Degradation - Number and Position of Methyl Substituents

This next suite of experiments was intended to further examine which structural elements contribute to the preferential degradation of certain PAHs. It is evident from the experiments described above that the disappearance of naphthalene is the benchmark

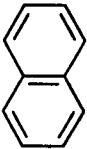
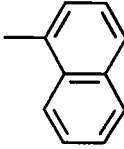
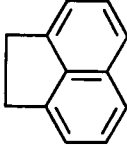
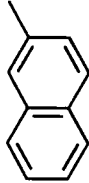
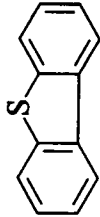
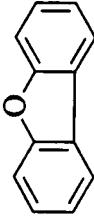
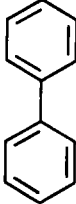
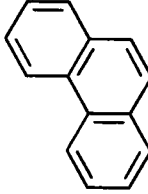
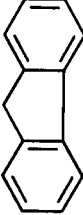
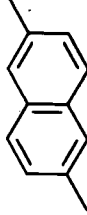
	Naphthalene $k_1 = -0.352/\text{h}$ $r^2 = 0.676$		1-Methylnaphthalene $k_1 = -0.196/\text{h}$ $r^2 = 0.552$		Acenaphthene $k_1 = -0.152/\text{h}$ $r^2 = 0.536$		2-Methylnaphthalene $k_1 = -0.104/\text{h}$ $r^2 = 0.500$		Dibenzothiophene $k_1 = -0.071/\text{h}$ $r^2 = 0.444$
	Dibenzofuran $k_1 = -0.050/\text{h}$ $r^2 = 0.810$		Biphenyl $k_1 = -0.039/\text{h}$ $r^2 = 0.484$		Phenanthrene $k_1 = -0.025/\text{h}$ $r^2 = 0.425$		Fluorene $k_1 = -0.021/\text{h}$ $r^2 = 0.510$		2,6-Dimethylnaphthalene $k_1 = -0.001/\text{h}$ $r^2 = 0.635$

Figure 18. Rate Constants for the Biodegradation of a PAH and Heterocycle Mixture by *P. fluorescens* 5R Pregrown on YEPG Medium.

against which any test compound(s) should be compared. Naphthalene is the smallest PAH both in size and molecular weight. It is also the most soluble PAH, and is characterized by an unsubstituted, symmetrical structure composed of four available naphthalene dioxygenase attack sites for the incorporation of two atoms of oxygen. It is also evident from the data presented above that in a general sense the further a structure deviates from that of naphthalene, the more slowly it is removed. For example, 2-methylnaphthalene, which deviates from naphthalene by only one methyl substituent, disappeared more rapidly than any other component except naphthalene in the mixtures studied.

There does appear to be a certain amount of ambiguity in determining which structural elements lead to preferential degradation. For example, 2,6-dimethylnaphthalene, which does not appear to differ significantly from the structure of 2-methylnaphthalene, was consistently the most slowly removed component of the mixtures. Therefore, in order to examine this in more detail, the following experiment was performed. Strain 5R, pregrown on YEPSS, was introduced to a simple mixture consisting of naphthalene, 1- and 2-methylnaphthalene, 1,2-, 1,8-, 2,3-, and 2,6-dimethylnaphthalene. These compounds were chosen to represent two variables: the number and location of methyl substituents. No other methyl-substituted naphthalenes were able to be included in the mixture because of the difficulties associated with separating dimethylnaphthalene isomers during GC analysis.

In this particular experiment, naphthalene disappeared almost immediately. 2-Methylnaphthalene was again removed more rapidly than any other components. In this case it had a first-order rate constant of $-1.1012/\text{h}$. 1-Methylnaphthalene was degraded more rapidly than any dimethylnaphthalene isomer; it had a rate constant of $-0.427/\text{h}$. Interestingly, 1,2-dimethylnaphthalene, which resembles the structure of naphthalene better than any other dimethylnaphthalene because it possesses three open naphthalene dioxygenase attack sites (note that monooxygenation reactions carried out by naphthalene dioxygenase upon methyl substituents are not considered here), disappeared more rapidly than any other dimethylnaphthalene isomer. Both it and 2,6-dimethylnaphthalene were

completely removed within 24 hrs; however, its rate of disappearance was approximately twice that of 2,6-dimethylnaphthalene. It is also interesting to note that 2,3-dimethylnaphthalene was removed much more slowly than 1,2-dimethylnaphthalene. It did not completely disappear within the 72 hrs that the experiment was conducted, and it had a first-order rate constant of $-0.031/\text{h}$. Although 1,2- and 2,3-dimethylnaphthalene each have one unsubstituted ring, 2,3-dimethylnaphthalene possesses one less open naphthalene dioxygenase attack site. 1,8-Dimethylnaphthalene was the most slowly removed compound. It also did not completely disappear within the 72 hrs and had a first-order rate constant, $-0.023/\text{h}$, which was very similar to that of 2,3-dimethylnaphthalene. Like 2,6-dimethylnaphthalene, 1,8-dimethylnaphthalene has two open dioxygenase attack sites on two separate aromatic rings (refer to figure 19 in the text and to figure 31 in the appendix).

The Contribution of Selected Chemical Structures to Preferential Degradation – Varying Aromatic Ring Structure and Heteroatom Substitution

A similar experiment was also performed using strain 5R and the following mixture of PAHs and heterocycles: acenaphthene, biphenyl, carbazole, dibenzofuran, dibenzothiophene, fluorene, naphthalene, and phenanthrene. The objective of this experiment was to test aromatic ring structure as the only variable. The trend of preferential degradation seen in previous experiments utilizing these compounds was repeated in this experiment. Naphthalene was the most rapidly removed compound. Acenaphthene completely disappeared shortly afterward within 4 hrs; it had a first-order rate constant of $-0.595/\text{h}$ compared to $-1.180/\text{h}$ for naphthalene. Phenanthrene was removed completely within 2 hrs with a rate constant of $-0.399/\text{h}$; however, the r^2 value for this regression calculation was substantially poorer, 0.484, than those for the other compounds in the mixture. Dibenzofuran and biphenyl were both removed completely within 12 hrs and had rate constants of $-0.377/\text{h}$ and $-0.263/\text{h}$, respectively. Fluorene

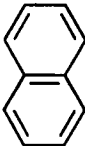
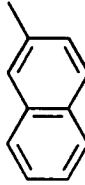
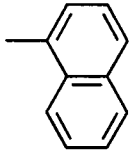
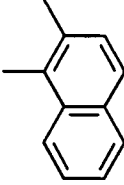
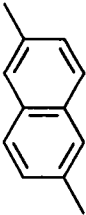
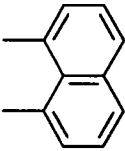
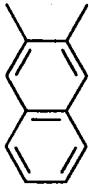
			
Naphthalene	2-Methylnaphthalene	1-Methylnaphthalene	1,2-Dimethylnaphthalene
$k_1 = -1.329/h$	$k_1 = -1.012/h$	$k_1 = -0.427/h$	$k_1 = -0.294/h$
$r^2 = 0.791$	$r^2 = 0.779$	$r^2 = 0.885$	$r^2 = 0.855$
			
2,6-Dimethylnaphthalene	1,8-Dimethylnaphthalene	2,3-Dimethylnaphthalene	
$k_1 = -0.140/h$	$k_1 = -0.031/h$	$k_1 = -0.023/h$	
$r^2 = 0.856$	$r^2 = 0.821$	$r^2 = 0.855$	

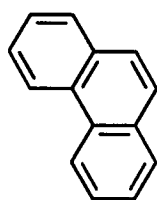
Figure 19. Rate Constants for the Biodegradation of a Mixture of Methyl-Substituted Naphthalenes by *P. fluorescens* 5R.

was the most slowly removed compound. Although it disappeared within 6.5 hrs, its first-order rate constant was $-0.146/\text{h}$. No determinations of the rates of carbazole or dibenzothiophene degradation were able to be made because their solubilities within the mixture were below detection limits throughout the experiment (see figure 20 in the text and figure 32 in the appendix).

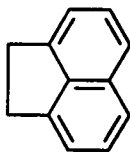
A second experiment involving only naphthalene and the heterocycles carbazole, dibenzofuran, dibenzothiophene, and fluorene revealed a similar pattern of preferential degradation. Naphthalene and dibenzothiophene were removed most rapidly. However, due to the similar values for rate constants and poor r^2 values associated with the other three compounds, determinations of preferential degradation were difficult to ascertain (see figure 21 in the text and figure 33 in the appendix). The reason for the comparatively low rate constant for naphthalene disappearance ($-0.427/\text{h}$) in this experiment is uncertain. However, the correlation coefficient for naphthalene (0.523) is also comparatively low.

The Contribution of Selected Chemical Structures to Preferential Degradation – Substituents of Varying Electrochemical Natures

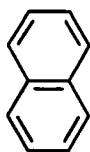
It is evident from all of the experiments completed using strain 5R that a substituent at the 2 position of naphthalene by a methyl or ethyl group does not hinder degradation and generally permits a rapid rate of removal. In order to determine whether this is also true for other substituents of different electrochemical natures and size, a mixture degradation experiment was performed with the following suite of compounds: 2-aminonaphthalene, 2-chloronaphthalene, 2-ethylnaphthalene, 2-methoxynaphthalene, 2-methylnaphthalene, naphthalene, naphthalene-2-carbonitrile, and 2-naphthaldehyde. All of these compounds differ only by the nature of their substituent and not by substituent position or the number of open naphthalene dioxygenase attack sites.



Phenanthrene
 $k_1 = -0.399/\text{h}$
 $r^2 = 0.484$



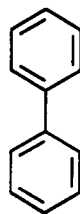
Acenaphthene
 $k_1 = -0.595/\text{h}$
 $r^2 = 0.764$



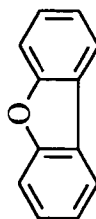
Naphthalene
 $k_1 = -1.180/\text{h}$
 $r^2 = 0.960$



Fluorene
 $k_1 = -0.146/\text{h}$
 $r^2 = 0.800$



Biphenyl
 $k_1 = -0.263/\text{h}$
 $r^2 = 0.828$



Dibenzofuran
 $k_1 = -0.377/\text{h}$
 $r^2 = 0.904$

Figure 20. Rate Constants for the Biodegradation of a Mixture of PAHs and Heterocycles by *P. fluorescens* 5R.

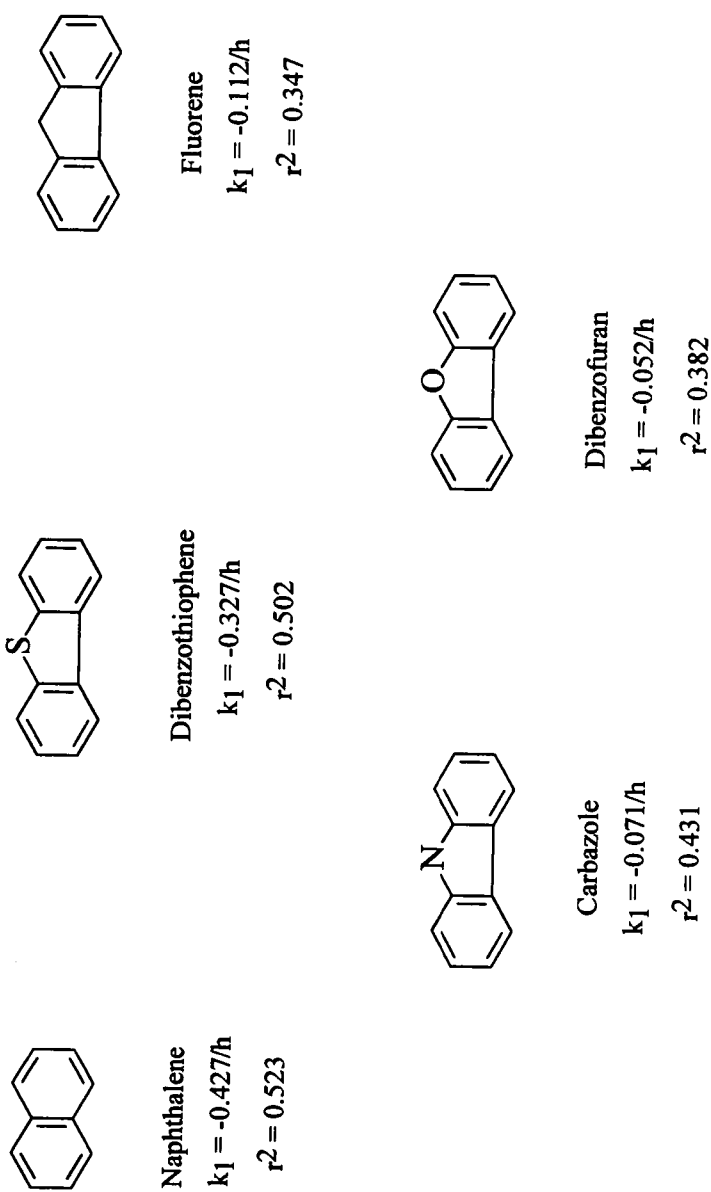


Figure 21. Rate Constants for the Biodegradation of a Mixture of Naphthalene and Various Heterocycles by *P. fluorescens* 5R.

In contrast to every other experiment performed to date with strain 5R, and the other NAH system strains, naphthalene was not the most rapidly removed component of the mixture in this experiment. 2-Naphthaldehyde almost completely disappeared within the first half hour of the experiment. Its disappearance was too rapid for the derivation of a first-order rate constant. Naphthalene was completely removed shortly afterward within 4 hrs; it had a rate constant of $-0.1362/\text{h}$. 2-Methylnaphthalene was the next most rapidly degraded compound after naphthalene. It completely disappeared within 12 hrs with a rate constant of $-0.389/\text{h}$. 2-Chloronaphthalene displayed a rate of disappearance similar to that of 2-methylnaphthalene. It was completely removed within 24 hrs and had a first-order rate constant of $-0.312/\text{h}$. 2-Ethyl naphthalene, the next most rapidly degraded compound, was completely removed within 48 hrs (the majority was degraded within 24 hrs) with a rate constant of $-0.123/\text{h}$. 2-Methoxynaphthalene completely disappeared within 72 hrs with a rate constant of $-0.051/\text{h}$ (see figure 22 in the text and figure 34 in the appendix).

The cyano- and amino- substituents present on their respective compounds appeared to present impediments to degradation. No degradation of naphthalene-2-carbonitrile occurred within the first 24 hrs of the experiment. From 24 hrs to 72 hrs however, the majority of it disappeared. Although previous biotransformation assays with strain 5R did not reveal that the NAH system transforms this compound to a cyano-substituted salicylate, it is possible that an alternative and undetected biological transformation does occur. Removal of this compound did occur in a first-order manner with a rate constant of $-0.021/\text{h}$ and an r^2 of 0.666. The killed control experiments run in parallel with this experiment revealed that abiotic transformation is unlikely as no appreciable loss of naphthalene-2-carbonitrile was evident.

Measurements of 2-aminonaphthalene degradation by strain 5R were marked by a very large amount of variation at every time point. This can be attributed to analytical difficulties in quantifying this compound. Unlike every other compound in this mixture, it does not form a sharp, easily distinguishable peak after GC separation. The first-order

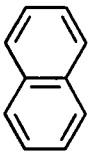
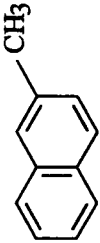
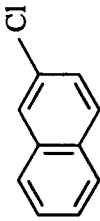
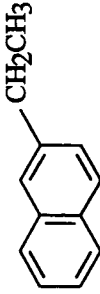
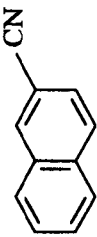
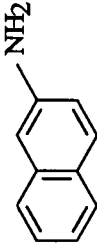
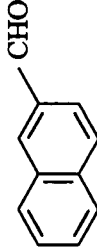
	Naphthalene	$k_1 = -1.362/\text{h}$ $r^2 = 0.860$
	2-Methylnaphthalene	$k_1 = -0.389/\text{h}$ $r^2 = 0.915$
	2-Chloronaphthalene	$k_1 = -0.312/\text{h}$ $r^2 = 0.835$
	2-Ethyl-naphthalene	$k_1 = -0.123/\text{h}$ $r^2 = 0.529$
	2-Methoxynaphthalene	$k_1 = -0.051/\text{h}$ $r^2 = 0.822$
	2-Cyanonaphthalene	$k_1 = -0.021/\text{h}$ $r^2 = 0.666$
	2-Aminonaphthalene	$k_1 = -0.056/\text{h}$ $r^2 = 0.267$
	2-Naphthaldehyde	k_1 too fast to measure

Figure 22. Rate Constants for the Biodegradation of a Mixture of Various Substituted Naphthalenes by *P. fluorescens* 5R

rate constant for the disappearance of 2-aminonaphthalene was $-0.056/\text{h}$ with a very poor r^2 value of 0.267.

The Preferential Degradation of a Complex Mixture of Various Substituted and Unsubstituted PAHs and Heterocycles

The next experiment performed was a compilation of previous experiments which examined different classes of compounds separately. It utilized a fifteen-component mixture consisting of the following PAHs and heterocycles: acenaphthene, biphenyl, carbazole, dibenzofuran, dibenzothiophene, 1,2-, 1,8-, 2,3-, and 2,6-dimethylnaphthalene, 2-ethylnaphthalene, fluorene, 1- and 2-methylnaphthalene, naphthalene, and phenanthrene. It was intended as a final experiment to confirm that the preferential degradation trends observed in the testing of the two separate groups of compounds are unaffected when all of the compounds are present in the same mixture.

The results obtained in this experiment mirror the trends observed before in previous experiments. Based on first-order rate constant values, the mixture components were found to be removed preferentially in the following order: naphthalene, 2-methylnaphthalene, 2-ethylnaphthalene, 1-methylnaphthalene, acenaphthene, 1,2-dimethylnaphthalene, dibenzothiophene, phenanthrene, dibenzofuran, 2,6-dimethylnaphthalene, fluorene, biphenyl, carbazole, 1,8-dimethylnaphthalene, and 2,3-dimethylnaphthalene. This is essentially an overlap of what was observed in the previous separate experiments. For example, 2-ethylnaphthalene, which had previously only been examined in the mixture of various 2-substituted naphthalenes, was found to be the third most rapidly removed compound after naphthalene and 2-methylnaphthalene. Its rate of disappearance, $-1.805/\text{h}$, was substantially faster than that of 1-methylnaphthalene, which was $-0.685/\text{h}$. This is an interesting comparison because it more effectively illustrates earlier results in that a substituent of a similar chemical composition (methyl vs. ethyl) leads to a more favorable rate of degradation when at the 2 position. Acenaphthene and

1,2-dimethylnaphthalene, which also had not been directly compared previously, were found to have similar rates of degradation (refer to figure 23 in the text and to figure 35 in the appendix). Both were degraded completely within 5 hrs.

1,8- and 2,3-Dimethylnaphthalene were again among the most slowly removed compounds. Although they were not directly compared previously to many of the components within this mixture, they, along with carbazole, had the slowest rates of disappearance. 1,8-Dimethylnaphthalene and carbazole had similar rate constants at $-0.038/\text{h}$ and $-0.041/\text{h}$, respectively. By a wide margin, 2,3-dimethylnaphthalene had the slowest rate of degradation. Its first-order rate constant was $-0.011/\text{h}$, and it was not completely removed over the course of the experiment. 1,8-Dimethylnaphthalene was the only other compound that was not completely removed.

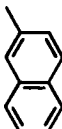
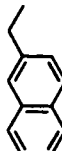
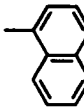
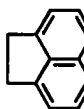
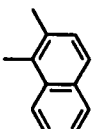
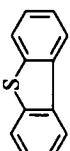
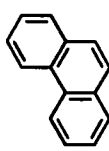
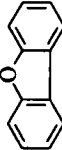
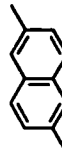
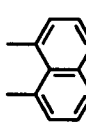
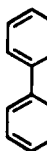
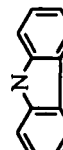
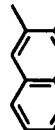
	Naphthalene k_1 too fast to measure		2-Methylnaphthalene $k_1 = -3.453/\text{h}$ $r^2 = 0.967$		2-Ethynaphthalene $k_1 = -1.805/\text{h}$ $r^2 = 0.962$		1-Methylnaphthalene $k_1 = -0.685/\text{h}$ $r^2 = 0.837$		Acenaphthene $k_1 = -0.536/\text{h}$ $r^2 = 0.936$
	1,2-Dimethylnaphthalene $k_1 = -0.485/\text{h}$ $r^2 = 0.970$		Dibenzothiophene $k_1 = -0.347/\text{h}$ $r^2 = 0.842$		Phenanthrene $k_1 = -0.250/\text{h}$ $r^2 = 0.807$		Dibenzofuran $k_1 = -0.201/\text{h}$ $r^2 = 0.932$		2,6-Dimethylnaphthalene $k_1 = -0.154/\text{h}$ $r^2 = 0.749$
	Fluorene $k_1 = -0.105/\text{h}$ $r^2 = 0.787$		1,8-Dimethylnaphthalene $k_1 = -0.038/\text{h}$ $r^2 = 0.422$		Biphenyl $k_1 = -0.086/\text{h}$ $r^2 = 0.912$		Carbazole $k_1 = -0.041/\text{h}$ $r^2 = 0.851$		2,3-Dimethylnaphthalene $k_1 = -0.011/\text{h}$ $r^2 = 0.714$

Figure 23. Rate Constants for the Biodegradation of a Complex PAH and Heterocycle Mixture by *P. fluorescens* 5R.

Chapter V

Discussion

Biotransformation, Growth, Induction, and *Meta*-Cleavage Assays Performed on Methyl-Substituted Naphthalenes

Earlier studies on the degradation of 1- and 2-methylnaphthalene by bacteria isolated from both contaminated and uncontaminated environments have shown that these two compounds are commonly degraded to 3- and 4-methylsalicylate. For example, Rogoff and Wender [1959] observed that *Pseudomonas* PM-1 transformed 1-methylnaphthalene to 3-methylsalicylate, and that *Pseudomonas* N-1-a transformed 2-methylnaphthalene to 4-methylsalicylate. In a later study, Williams et al. [1975] showed that *Pseudomonas* P_G (NCIB 9816) degraded 2-methylnaphthalene through 4-methylsalicylate to its *meta*-cleavage product 2-hydroxy-5-methylmuconic semialdehyde. In the current study, the reactions carried out specifically by the upper pathway of the NAH system of *P. fluorescens* 5RL were shown to transform 1-MN, 2-MN, 1,2-DMN, 1,3-DMN, and 2,3-DMN to their corresponding salicylate analogs as major products. This is in accordance with the classical dioxygenase attack and subsequent *meta*-cleavage of one ring, such as that shown by Deluca and Hudlicky [1990] in the transformation of 2-MN to (+)-*cis*-1,2-dihydro-(1R,2S)-dihydroxy-7-methylnaphthalene by *P. putida* NCIB 9816.

This incorporation of one hydroxyl substituent into 1-MN, 2-MN, and 1,2-DMN is also mediated by strain 5RL in addition to the classical dioxygenase attack on these three compounds; no monohydroxylation was detected on either 1,3- or 2,3-DMN. The nature of this monohydroxylation is currently unclear for a number of reasons. The naphthalene dioxygenase of *P. putida* PpG7, but not strain 5RL, has been shown to directly monooxygenate the methyl substituent of 1-methylnaphthalene but not 2-methylnaphthalene. Selifonov et al. [1996] have reported that naphthalene dioxygenase directly monooxygenates the methyl substituents of dimethylnaphthalenes; however, it is

possible that their results may represent the incorporation of one hydroxy group into the aromatic ring. It is also unclear whether this would result from direct monooxygenation or a dehydration following dioxygenation of the aromatic ring.

This latter point is especially uncertain because the data concerning the biotransformation of certain dimethylnaphthalenes by strain 5RL does not provide any insight into the location of hydroxyl group incorporation. In the case of 1,5-dimethylnaphthalene, the fragmentation pattern intensities for the compound of molecular weight 172 roughly correspond with those reported by Selifonov et al. (1996) for 1-methanol-5-methylnaphthalene. Although there are slight differences in intensities (only major ion peaks were reported by Selifonov), both compounds have the same major peaks and both have a base ion peak of m/z 143. For 1,8-dimethylnaphthalene, the product of strain 5RL biotransformation has the same major peaks as 1-methanol-8-methylnaphthalene as reported by Selifonov; however, the 5RL product has a base peak of m/z 128, whereas that of Selifonov's product is 153. Similarly, Selifonov reported that 2-methanol-6-methylnaphthalene had a base peak of m/z 172, whereas the product of strain 5RL had a base peak of 143. This same discrepancy is also seen for the products of 2,7-dimethylnaphthalene transformation. Without further detailed analysis, such as nuclear magnetic resonance spectroscopy, to determine the position of each hydroxy substituent, the products of molecular weight 172, formed by the transformation of dimethylnaphthalenes by strain 5RL, can best be identified as hydroxy-substituted dimethylnaphthalenes.

The occurrence of both this apparent monooxygenase reaction and the classical dioxygenase attack have also been shown to occur in *Pseudomonas putida* CSV86 [Mahajan et al., 1994]. This organism has also been shown to transform 2,3-DMN, 2-methoxy-, 1-ethyl, and 2-ethylnaphthalene to the corresponding salicylates. It is not known whether this organism possesses the NAH system; however, it carries out the same set of classical upper pathway reactions as strains 5R and 5RL when presented with these substrates.

After having determined that the NAH system of strain 5R could transform the methyl-substituted naphthalenes under examination, studies were then undertaken using *P. fluorescens* 5R to determine whether the NAH system could support growth on these compounds as sole sources of carbon and energy. It was found that only 2-methylnaphthalene supported growth. The same substrate range was also seen in experiments using the archetypal NAH strains *Pseudomonas* sp. DFC49 and DFC50 (previously characterized by Sanseverino et al. [1993a] and the well studied *Pseudomonas putida* G7 (pNAH7)) [data not shown]. In addition, it was found that 4-methylsalicylate and 5-methylsalicylate, but not 3-methylsalicylate, supported growth of strain 5R as sole carbon and energy sources. The ability of 2-methylnaphthalene to serve as a growth substrate has been observed previously by Williams et al. [1975] and Barnsley [1976] for *Pseudomonas* P_G (NCIB 9816), and by Mueller et al. [1989] for *P. putida* PgG7, respectively. However, the inability of any dimethylnaphthalenes to serve as growth substrates is in contrast to the ability of other bacteria which have been shown to grow on dimethylnaphthalenes. For example, Barnsley [1988] has reported that a number of Flavobacteria are able to grow on 2,6-DMN via an initial carboxylation and subsequent removal of one methyl group prior to cleavage of one aromatic ring to form 5-methylsalicylate. Similarly, Miyachi et al. [1993] have isolated three strains which are capable of growing on 2,6-DMN via the same type of mechanism. One of these strains, *Pseudomonas* sp. strain D-87, showed an expanded growth substrate range; it was shown to also be capable of growth on 1,2-, 1,3-, 1,4-, 1,5-, 2,3-, and 2,7-DMN as individual substrates. Grifoll et al. [1995] have reported the ability of *Pseudomonas cepacia* F297, a fluorene-utilizing isolate, to grow on 2,3-DMN. This strain has also been shown to transform 2,6-DMN via oxidation of one methyl substituent.

The results of previous research on the metabolism of 1- and 2-methylnaphthalene indicate that the inability to grow on 1-methylnaphthalene may be due in part to an inability of the NAH system to process a degradative intermediate(s) as effectively as those intermediates generated by the metabolism of naphthalene or 2-methylnaphthalene. For example, Williams et al. [1975] found that the specific activity of catechol-2,3-

dioxygenase is less for 3-methylcatechol than for 4-methylcatechol. Similar results have also been reported by Cerdan et al. [1995] and Kim et al. [1992], who have observed that the catechol-2,3-dioxygenase from plasmid NAH7 shows a much lower activity toward 3-methylcatechol than toward catechol and 4-methylcatechol. Foght and Westlake [1996] have also postulated in a recent study that the ability of *Pseudomonas fluorescens* strain LP6a, which possesses a 63 kb plasmid (pLP6a) that is homologous to the well-characterized naphthalene degradative plasmid NAH7, to utilize only naphthalene, 2-methylnaphthalene, anthracene, and phenanthrene as sole carbon and energy sources is due to the ineffective processing of intermediates from the degradation of other PAHs and heterocycles. For example, it is suggested that 1-methylnaphthalene is not utilized because of stringent specificity in reactions beyond the first oxidative transformation. However, our work suggests that these previous observations do not completely explain the inability of strain 5R to grow on 1-methylnaphthalene, 3-methylsalicylate, or any dimethylnaphthalene which is transformed to a dimethylsalicylate. Two main lines of evidence lead to this observation. The first is the results of *meta* cleavage assays using strain 5R and pUTK200. Although these experiments were not as quantitative in their assessment of enzyme activities as the previous study by Cerdan et al. [1995], the results of these studies indicate that the NAH system of strain 5R does have the ability to completely transform the non-growth substrate 1-methylnaphthalene to potential Krebs cycle intermediates. The second line of evidence is the ability of the lower pathway of strain 5R to utilize 3-methylsalicylate as an auxiliary carbon and energy source in the binary mixture growth experiments.

The second line of evidence is the correlation between the ability of a substituted salicylate to both induce NAH system transcription and to serve as a sole carbon and energy source. 4-Methylsalicylate, 5-methylsalicylate, and salicylate perform both roles, whereas 3-methylsalicylate performs neither. It should be noted that 5-methylsalicylate is a comparatively poor inducer at the lower concentration of 10 mg/L. Although it is an excellent inducer and a growth substrate at a concentration of 200 mg/L, it is unlikely that cells in the environment will be exposed to this high concentration, particularly if the

inducer is the direct product of the biotransformation of a poorly soluble parent compound as in the case of 3-methylsalicylate, 4-methylsalicylate, and salicylate. To summarize this experiment, 4-methylsalicylate induces transcription at levels comparable to those of salicylate over a range of concentrations. 3-Methylsalicylate is a poor inducer at all concentrations tested, even 10 mg/L. 5-Methylsalicylate also induces at levels much lower than those produced by salicylate and 4-methylsalicylate, although it is better than 3-methylsalicylate between 5 and 10 mg/L. These results indicate that methyl substitution at the 4 position facilitates high-level induction, whereas substitution at the 3 position acts to limit induction. Methyl substitution at the 5 position leads to intermediary levels of induction between those produced by 3- and 4-methylsalicylate.

The failure of 1,2-, 1,3-, and 2,3-DMN to serve as sole carbon and energy sources also suggests that the existence of methyl substituents at any position other than the 4 and 5 positions prohibits the induction of transcription. It is evident that a single methyl substituent at any of these positions does not prevent *meta*-cleavage from occurring. It may also be possible that not enough metabolite(s) is produced to lead to a light-producing reaction.

This role of 4-methylsalicylate as an inducer is further supported by the observations of Williams et al. [1975], who found that 2-methylnaphthalene led to increased induction of *meta*-cleavage enzymes in *Pseudomonas* P_G. A more recent study by Cebolla et al. [1997] has examined the ability of a number of substituted salicylates and benzoates to induce *nah* transcription in an *E. coli* construct containing a *sal* promoter::*lacZ* transcriptional fusion, and a divergently transcribed *nahR* gene. Among the compounds tested (all at 1 mM), 3-methylsalicylate, 4-methylsalicylate, and 5-methylsalicylate, as well as 3-chlorosalicylate, 4-chlorosalicylate, and 5-chlorosalicylate, all induced transcription near levels equivalent to salicylate induction. There does appear to be a general agreement between this and our work in that 4-methylsalicylate and 5-methylsalicylate (tested at approximately 1 mM with strain 5RL) are both inducers, whereas 3-methylsalicylate does appear to be a much better inducer in the *lacZ* reporter system used by Cebolla et al. [1997] than shown here in strain 5RL.

Results contradictory to ours and those of Cebolla et al. are presented by Cidaria et al. [1994]. Using the strain *Pseudomonas fluorescens* NCIMB 40531, which possesses a nonfunctional, (*N*-methyl-*N'*-nitro-*N*-nitrosoguanidine-mutagenized) gene for *cis*-1,2-dihydroxy-1,2-dihydronaphthalene dehydrogenase (*nahB*), the production of *cis*-1,2-dihydroxy-1,2-dihydronaphthalene from naphthalene was examined as a measure of NAH system induction after individual exposure to 62 different compounds. They found that 3-methylsalicylate, *o*-acetylsalicylic acid, and naphthalene produced the highest levels of induction. 4-Methylsalicylate produced a level of induction approximately ten-fold lower than 3-methylsalicylate. Surprisingly, salicylate showed no induction in this system. This is in direct contrast to the results obtained using the *lux* reporter system of *P. fluorescens* 5RL. It is possible that the regulation of naphthalene metabolism in strain NCIMB 40531 may be characterized by differences in the regulatory mechanism which enables 3-methylsalicylate, and not salicylate, to induce a favorable response.

Alternatively, it is possible that a locus other than *nahB* may have been affected by the mutagenesis procedure; all previous accounts of NAH system induction have shown that salicylate is a favorable effector molecule. Previous studies have also shown that 2-hydroxybenzyl alcohol and 2-aminobenzoate are able to act as gratuitous inducers of NAH system enzyme activities [Barnsley, 1975; Shamsuzzaman and Barnsley, 1974a; Shamsuzzaman and Barnsley, 1974b].

The data strongly suggest that the ability of *P. fluorescens* 5R to selectively utilize 2-methylnaphthalene and no other methyl-substituted naphthalene as a sole carbon and energy source is a direct result of two factors. The first is the inability of the NAH upper pathway of this organism to cleave a methyl-substituted aromatic ring on certain dimethylnaphthalenes. Because of the monooxygenation and subsequent failure to remove one of the methyl substituents of 1,4-, 1,5-, 1,6-, 1,7-, 1,8-, 2,6-, and 2,7-DMN, dead-end product formation occurs. Alternatively, 1-methylnaphthalene, 1,2-, 1,3-, and 2,3-DMN are transformed to their respective methyl-substituted salicylates via upper pathway cleavage of the unsubstituted ring. Unlike 4-methylsalicylate however, they apparently are not transformed to a metabolite that induces transcription at the low

concentrations which are commensurate with the initial concentration of the parent compounds. For example, 2,3-DMN does not promote growth over a long period of time in strain 5R. Nor does it promote the production of light in strain 5RL. This suggests that 4,5-dimethylsalicylate, which possesses structural elements of two inducers, 4- and 5-methylsalicylate, is likely not an effective inducer when it is formed via the transformation of poorly soluble, crystalline 2,3-DMN because of the limited solubility of the parent compound. This same point can be made for 1,2- and 1,3-DMN. Thus, although the NAH system is capable of transforming a large number of PAHs, it appears that these reactions are largely cometabolic in part to the tight specificity of regulatory processes which prohibit their utilization as sole carbon and energy sources.

Biotransformation, Growth, Induction, and *Meta*-Cleavage Assays Performed on Other Substituted Naphthalenes

Few studies have examined the biochemical nature of the decomposition of halogenated naphthalenes. A 1955 study by Walker and Wiltshire has shown that 1-chloronaphthalene and 1-bromonaphthalene can be degraded to their respective salicylate analogs by soil bacteria. The biochemical system(s) used by these organisms is not known; however, the formation of 3-chlorosalicylate is the same reaction observed during the transformation of 1-chloronaphthalene by *P. fluorescens* 5RL. These researchers also observed the formation of hydroxylated 1-chloro and 1-bromonaphthalene. Such reactions were also observed in strain 5RL. In the case of 5RL, the location of the hydroxyl substituent is uncertain. It is also uncertain whether it is the dehydration product of a dihydroxylated parent compound. Walker and Wiltshire did report the ability of their isolates to grow on 1-chloro- and 1-bromonaphthalene. Strain 5R did not display the ability to grow on either compound. With respect to 1-chloronaphthalene, this inability stems from the fact that 3-chlorosalicylate did not act as an inducer

compound and was not cleaved by the lower pathway. Thus, it is likely that Walker and Wiltshire were not dealing with NAH system isolates.

A later study by Morris and Barnsley [1982] produced results very similar to those obtained with strains 5R and 5RL in the examination of the aspects of 1- and 2-chloronaphthalene degradation. Using a naphthalene degrading pseudomonad, these researchers observed that neither 1- nor 2-chloronaphthalene served as a sole carbon and energy source in spite of the formation and eventual *meta*-cleavage of chlorinated salicylates. Morris and Barnsley also observed that 4- and 5-chlorosalicylate induced the naphthalene degradative enzymes. Kim et al. [1992] have also reported that the catechol-2,3-dioxygenase cloned from plasmid NAH7 of *P. putida* is able to cleave 4-chlorocatechol, the product of the transformation of 4-chlorosalicylate by salicylate hydroxylase. pUTK200, which possesses the salicylate hydroxylase and catechol-2,3-dioxygenase of strain 5R, was shown to mediate the *meta*-cleavage of 4- and 5-chlorosalicylate. It can thus be reasoned that the inability of the NAH lower pathway to process the chlorinated linear products of *meta*-cleavage reactions is an impediment to growth on 2-chloronaphthalene. 3-Chlorosalicylate, the product of the transformation of 1-chloronaphthalene, was not cleaved by pUTK200 enzymes. This in part explains why 1-chloronaphthalene and 3-chlorosalicylate did not serve as growth substrates for strain 5R. It has been observed previously using the catechol-2,3-dioxygenases from different degradative systems that the cleavage of 3-chlorocatechol leads to an inactivation of the enzyme [Klecka and Gibson, 1981; Bartels et al., 1984; Arensdorf and Focht, 1994].

A recent study by Cerdan et al. [1994] has investigated the inability of the catechol-2,3-dioxygenase of the TOL plasmid pWW0 of *P. putida* to cleave 4-ethylcatechol. This enzyme, which shares a great amount of similarity with NahH, was shown to cleave catechol, 3-methylcatechol, and 4-methylcatechol, but not 4-ethylcatechol. 4-Ethylcatechol was shown to be a potent inhibitor of the enzyme via an oxidation and/or removal of the ferrous iron in the active site of the enzyme. This inactivation is hypothesized to be a result of the inability of the catalytic center to transfer molecular oxygen from the iron cofactor to the catechol molecule. As discussed earlier, these

researchers, in a more recent paper [Cerdan et al., 1995], have shown that the catechol dioxygenase of the NAH system is more susceptible to suicide inhibition than the TOL system catechol dioxygenase. Although 4-ethylcatechol was not examined by these researchers, there does not appear to be an inhibition in the ability of the catechol-2,3-dioxygenase of strain 5R dioxygenase to cleave the 4-ethylcatechol formed during the degradation of 2-ethylnaphthalene. Strain 5R grows very well on 2-ethylnaphthalene; therefore, it can be assumed that the 4-ethylsalicylate formed by the upper pathway transformation of this compound is effectively cleaved by the combined action of salicylate hydroxylase and catechol dioxygenase. It also appears that 4-ethylsalicylate is an effective inducer of transcription in the same manner that 4-methylsalicylate, produced during the transformation of 2-methylnaphthalene, is. This apparent ability of 4-ethylsalicylate coincides with the ability of 2-ethylnaphthalene to serve as a sole carbon and energy source.

In a study performed by Yamamoto et al. [1965] it was found that salicylate hydroxylase isolated from a *Pseudomonas* species was unable to transform benzoate, 4-hydroxybenzoate, 2-hydroxy-3-naphthoic acid, 2-nitrobenzoate, phthalic acid, methylsalicylate, sulfosalicylate, and thiosalicylate. In this study, Yamamoto et al. did find that the following compounds were able to replace salicylate as a substrate (the relative activities of the enzyme, defined as the decrease in absorbance of NADH at 340 nm, in the presence of these compounds are listed in parentheses): 3-hydroxysalicylate (42%), 4-hydroxysalicylate (55%), 5-hydroxysalicylate (134%), 6-hydroxysalicylate (59%), 4-aminosalicylate (120%), and 1-hydroxy-2-naphthoic acid (15%). This degradation of NADH was shown to be dependent upon the presence of salicylate or an alternative substrate. A similar study by White-Stevens and Kamin [1972] has shown that salicylate hydroxylase from an environmental isolate was able to transform 3-, 4-, 5-, and 6-hydroxysalicylate, but not 1-hydroxy-2-naphthoic acid, 2-hydroxy-3-naphthoic acid, or methylsalicylate. This apparent inability of the salicylate hydroxylases of these two strains to transform methylsalicylate and thiosalicylate is in accordance with the combined inability of the salicylate hydroxylase and catechol-2,3-dioxygenase cloned

from strain 5R to transform methylsalicylate and thiosalicylate to *meta*-cleavage products. Consequently, the apparent ability of salicylate hydroxylase to transform the various hydroxy-substituted salicylates and 4-aminosalicylate to their respective catechols indicates that the catechol-2,3-dioxygenase of the NAH system of strain 5R is the bottleneck which inhibits the degradation of a number of substituted salicylates to *meta*-cleavage products.

It is interesting to note that among the compounds examined in the *meta*-cleavage assays using pUTK200 only those salicylates with electrically neutral substituents were able to be cleaved (table 5). Compounds with electron-releasing substituents, electron-withdrawing substituents did not demonstrate the ability to be cleaved. In addition, variation in the 2 position as in the case of thiosalicylate and methylsalicylate inhibited cleavage. Within the group of electrically neutral substituents, 4- and 5-chlorosalicylate, 3-, 4- and 5-methylsalicylate, and salicylate demonstrated *meta*-cleavage, whereas 3-chlorosalicylate, and 3-, 4-, and 5-methoxysalicylate did not. Discernable trends concerning the effects of substituent size and/or substituent position are not evident. In contrast to these results, the biotransformation experiments utilizing *P. fluorescens* 5RL demonstrated that the reaction sequences of the upper pathway are of looser specificity and were limited less by the nature of the substituent.

A similar structural analysis of the compounds that induce transcription reveals that a substituent at the 4 position confers activity (table 7). All of the compounds possessing an electrically neutral substituent at the 4 position produced a bioluminescent response. 4-Methylsalicylate produced the largest response at a level commensurate with salicylate, whereas 4-chlorosalicylate produced the weakest response. 4-Aminosalicylate, which possesses an electron-releasing substituent at the 4 position, was also able to induce transcription. 4-Hydroxysalicylate, which also possesses an electron-releasing group at the 4 position, did not induce transcription. At the concentrations tested, no salicylate substituted at either the 3 or 5 position, with the exception of 5-aminosalicylate, acted to induce transcription. In addition, when the hydroxy group at the 2 position of salicylate was replaced with either -SH or -OCH₃ (thiosalicylate and methylsalicylate,

respectively), the ability to induce transcription was lost. This indicates that within the base structure of salicylate substitution at the 4 position by either an electrically neutral or electron-releasing group generally permits induction of transcription to occur. In most cases, substitution at the 3 or 5 position reduces or eliminates this ability.

Biotransformation, Growth, Induction, and *Meta*-Cleavage Assays Performed on Other PAHs and Heterocycles

The formation of 1-acenaphthenone, a product in the transformation of acenaphthene by strain 5RL, is in agreement with the results previously published by Selifonov et al. [1996]. Unlike the dimethylnaphthalenes discussed earlier, the fragmentation pattern and intensities are an excellent match to those reported in their study. This intermediate is thought to be formed en route to acenaphtho-1,2-quinone. Acenaphtho-1,2-quinone was also identified as a product of strain 5RL. Selifonov et al. also report the production of both *cis*- and *trans*-acenaphthene-1,2-diol. An acenaphthene-1,2-diol was produced by strain 5RL in this biotransformation; however, its stereochemistry cannot be determined since its fragmentation pattern and intensities are very similar to the mass spectra of both isomers. Acenaphthylene-1,2-diol or its ketol tautomer was also reported by Selifonov et al. A compound similar to acenaphthylene-1,2-diol was produced by strain 5RL, but its fragmentation pattern intensities are slightly different than those of Selifonov et al. Hence, it is uncertain which of the two possibilities this metabolite represents. There was no evidence to indicate that the upper pathway of strain 5RL cleaves either ring of acenaphthene.

The biotransformation of carbazole by strain 5RL resulted in the formation of 3-hydroxycarbazole. This is the same reaction demonstrated by Resnick et al. [1993] for the naphthalene dioxygenase of *Pseudomonas* sp. NCIB 9816-4. As in the case of their study, it is uncertain whether the formation of this compound resulted from an initial dioxygenase attack and subsequent dehydration, or a direct monooxygenase reaction.

Comparison to a mass spectra database also led to the identification of 1*H*-indole-2,3-dione as a new product in the transformation of carbazole by the NAH system of strain 5RL. This data indicates that the upper pathway cleaves one aromatic ring of carbazole en route to the formation of this compound. 1*H*-Indole-2,3-dione is similar in functional group composition to salicylaldehyde, which is formed immediately before salicylate in the NAH upper pathway degradation of naphthalene.

The transformation of dibenzothiophene by strain 5RL led to the formation of 3-hydroxy-2-formylbenzothiophene. This also indicates that the upper pathway reactions cleave one ring on this compound. Dibenzothiophene sulfoxide was also formed in this biotransformation. The formation of dibenzothiophene sulfoxide from dibenzothiophene by the action of naphthalene dioxygenase has been observed previously by Resnick and Gibson [1996b]. It is possible however that this product is hydroxylated at a position other than the sulfur atom.

The transformation of biphenyl by strain 5RL resulted in a major product with a molecular weight of 160; the suggested identity of 1-phenyl-1,4-butenedial indicates that the upper pathway was able to cleave one aromatic ring. However, the linear product does not appear to have been separated from the remaining aromatic moiety. To date, no previous studies on the NAH-mediated degradation of this compound have been performed.

The ability of the lower pathway of the NAH system to further degrade these upper pathway products is uncertain. None of the metabolites which have been formed via the removal of one ring are available commercially. Therefore, no growth, *meta*-cleavage, or induction studies were performed with these metabolites.

The Biodegradation of PAH Mixtures

The generation of rate constants for the biodegradation of naphthalene and a few other selected PAHs found in the environment has been performed by a number of researchers

in spite of the difficulties surrounding the attainment of accurate measurements in environmental matrices. Many factors may influence the biodegradation of naphthalene in the environment such as changes in the population of naphthalene-degrading organisms [Heitkamp et al., 1987], the level of previous adaptation to naphthalene by naphthalene-degrading organisms [Herbes and Schwall, 1978; Heitkamp et al., 1987], changes in levels of accessory nutrients, variations in ambient environmental conditions such as oxygen tension, seasonal temperatures, and salinity [Bauer and Capone, 1985; Shiaris, 1989], and the presence of other compounds which may stimulate or inhibit the degradation of naphthalene. However, it has generally been observed that naphthalene is the most rapidly degraded PAH. Typically the biodegradation of naphthalene by organisms taken from many different sites has been shown to involve the generation of first-order rate constants. For example, Herbes and Schwall [1978] have demonstrated through ^{14}C -mineralization assays that the initial rate of naphthalene biodegradation by an uncharacterized microbial consortium from a PAH-contaminated site occurs through first-order kinetics with a first-order rate constant equal to $-0.14/\text{h}$. They noted that the rate of naphthalene biodegradation by an unknown microbial consortium from a previously uncontaminated site was significantly slower ($< 4 \times 10^{-4}$) than that for the consortium from the PAH-contaminated site. In a later paper, Herbes noted that the first-order rate constants of the degradation of naphthalene in sediment and water previously exposed to PAHs were $-0.078/\text{h}$ and $-0.0032/\text{h}$, respectively [Herbes, 1981]. Park et al. [1990a,b] have also shown the biodegradation of naphthalene to occur with first-order rate constants of $-0.3370/\text{day}$ and $-0.03080/\text{day}$ for two different soil types.

The effect of an increase in ring number on the rates of PAH biodegradation has been examined by Herbes and Schwall [1978]. This parameter has been the most examined structural feature of PAHs with respect to affecting the rate of degradation. The rate of compound degradation decreases significantly as the number of rings present in the compound were increased. For example, the degradation of benz(a)anthracene occurred approximately 1,000-fold slower than the degradation of naphthalene by a consortium of soil organisms. Both compounds were found to follow linear, first-order degradation

kinetics. The study of biodegradation kinetics for methyl-substituted naphthalene congeners has received virtually no attention. Park et al. [1990a, 1990b] have shown that the rate of 1-methylnaphthalene biodegradation is similar to that for the degradation of naphthalene with first-order rate constants of -0.4150/day and -0.3210/day for two different soil types. Park et al. have also found a significant decrease in the rate of degradation of a compound as the number of rings are increased. For example, in the study of the degradation of individual PAHs, each examined separately, by uncharacterized consortia of soil microorganisms from two previously uncontaminated soils, it was shown that the rates of naphthalene and 1-methylnaphthalene were an order of magnitude faster than those of anthracene and phenanthrene. With the exception of 7,12-dimethylbenz[α]anthracene, anthracene and phenanthrene were degraded an order of magnitude more rapidly than any compound with more than three aromatic rings. In a different study, Elmensdorf et al. [1994] have shown that increasing the methyl substitution of PAHs slows their degradation. They have also demonstrated that naphthalene and its analogs were more rapidly degraded than any other type of PAH or heterocycle by a natural assemblage of soil microorganisms taken from Prince William Sound, Alaska.

A comparison of the rate constant data generated using strains 5R, DFC49, DFC50, and PpG7 with the results of these previous studies illustrates that naphthalene and its closest structural analogs are preferentially degraded even in the absence of variables such as the rate of compound desorption from a matrix (discussed in more detail below). It is quite possible that these four strains, all of which were isolated from PAH-contaminated environments, have evolved to preferentially utilize those compounds that are most available in a soil environment. The data obtained from studies involving these four strains, strain 5RL, and the *nahA* clone indicates that the specificity of naphthalene dioxygenase is the primary factor in determining preferential degradation of a mixture. There is no evidence which points to the involvement of other components of the NAH system, such as downstream degradative enzyme or regulatory aspects, in the determination of preferential degradation.

While both uptake and interaction are required for biodegradation of a compound, it was assumed that within a group of chemicals, such as the PAHs in these mixtures, of similar hydrophobicity, interaction would be the limiting factor. In order to assure this similarity in hydrophobicity, biodegradation experiments using PAHs and heterocycles were limited to chemicals with log K_{ow} values between 3.5 and 4.5. Moreover, in an effort to segregate steric and electronic effects, preferential biodegradation experiments using *P. fluorescens* 5R were divided into three phases. The initial experiments evaluated the effect of the number and position of methyl substitution on the biodegradation rates of derivatives of naphthalene. The second set of experiments evaluated the effect of ring structure and to a lesser extent heteroatom substitution on preferential biodegradation of low-molecular-weight PAHs. The last set of experiments evaluated the electronic effect of substituents on the biodegradation of 2-position substituted naphthalenes.

In order to assure similarity in hydrophobicity, biodegradation experiments with methyl-substituted naphthalenes were limited to mono- and dimethylnaphthalenes. Experiments to evaluate the effect of the number and position of methyl substitution on the biodegradation rates of derivatives of naphthalene were predicated on the fact that the first step in the biodegradation of naphthalene and certain methylnaphthalenes is a direct dioxygenase attack at the 1 and 2 positions. Thus, the parent compound, naphthalene, has four equally available sites of action. As mentioned earlier in the Results section, the 1- and 2-methylnaphthalene, and 1,2-dimethyl naphthalene each have three sites of attack available. 1,8-, 2,3-, and 2,6-Dimethylnaphthalene each have two sites available. The attack sites on 2,3-dimethylnaphthalene are present on one ring, whereas 1,8- and 2,6-dimethylnaphthalene have one attack site on each of the two rings. The first-order rate constants (figure 19) reveal a semi-quantitative trend between the rate of disappearance of the starting naphthalenes and the number of available attack sites for naphthalene dioxygenase attack. For example, 2,3-dimethylnaphthalene is much more slowly degraded than any compound with three open attack sites. In general, as the number of attack sites is reduced, the rate of degradation decreases. These results suggest that even

within this restricted group of methyl-substituted naphthalene molecular shape is important to metabolism. A study by Solonas et al. [1984] on the biodegradation of a mixture of alkyl-substituted naphthalenes and other hydrocarbons in crude oil by a *Pseudomonas* species isolated from oil tanker ballast water has shown similar results. In their study, 2-methylnaphthalene and 1,2-dimethylnaphthalene were among the most rapidly degraded naphthalenes, whereas 1,4- and 2,3-dimethylnaphthalene were among the most recalcitrant.

In an effort to more fully explore the impact of molecular shape, a second set of experiments were performed in order to evaluate the effects of ring structure and heteroatom substitution on preferential biodegradation (figure 20). Acenaphthene, the compound most structurally similar to naphthalene, was the second most rapidly degraded compound. Unlike the previous experiment which determined preferential degradation as a function of the number and positions of substituents on the same base molecule (naphthalene), the relevant structural properties of the remaining compounds (biphenyl and heterocycles) in this mixture are less straightforward and generalities are difficult to obtain. It is apparent however that adding a third ring, such that a compound is longer than the base structure of naphthalene, slows the rate of disappearance. The effect of the nature of heteroatom substitution was reexamined with a mixture of naphthalene and four heterocycles. It was found that naphthalene was the most rapidly degraded compound (figure 21). It is not known why the rate of naphthalene disappearance in this experiment was comparatively lower than in previous experiments. Dibenzothiophene was the next most rapidly degraded compound. The remaining three compounds, fluorene, carbazole, and dibenzofuran were all degraded within 24 hrs. However, because of the large amount of variation in the samples, a definitive statement about the effect of a heteroatom on preferential degradation cannot be made from this experiment.

In an effort to more fully explore the impact of both the electronic and steric effect of substituents on the rate of biodegradation, experiments were performed with a series of 2-position substituted naphthalenes (figure 22). These studies were conducted with the

following background information in consideration for treatment of rate constant data. Electronic forces determine to a large extent the rate of metabolism of a compound, since such forces control bond order. Hence biodegradation may be modeled by electronic parameters. There are several types of electronic interactions including; ion-ion, ion-dipole, ion-induced dipole, dipole-dipole, dipole-induced dipole, instantaneous dipole-induced dipole and hydrogen bonding. All such interactions depend on the electron distribution within a molecule or substituent, and the ease with which that distribution can be modified. The Hammett substituent constant σ [Hammett, 1935] has been shown to quantify the electron-directing effect of a substituent X on the acid dissociation constant of benzoic acid. This parameter has been found to be widely applicable in correlation analysis with both physical- and biological endpoints. Hammett obtained σ values for *meta*- and *para*- substituents in the aromatic ring of benzene. The σ_m and σ_p values differ from each other because of the differing inductive and resonance contributions in the two positions. Several attempts have been made to factor inductive and resonance components. Swain and Lupton [1968] have defined a field effect ($\mathfrak{F}$) and a resonance effect ($\mathfrak{R}$).

Steric parameters quantitate size and shape. The size and shape of molecules and substituents could be possible factors which affect the values of rate constants. A bulky substituent adjacent to an attack site on a molecule may hinder metabolism. Size may be a barrier to the passage of molecules through aqueous channels in membranes. While both size and shape are extremely important in receptor mediated effects such as biodegradation, shape is the more important of the two. Generally, xenobiotic and receptor should be complementary in shape. Any difference in the xenobiotic molecule from the optimum required for best fit to the receptor will result in less and/or weaker binding and a concomitant loss of activity. To an extent, this was indicated in the examination of different methyl-substituted naphthalenes using *P. fluorescens* 5R.

Molar refractivity (MR) has the units of molar volume. It is highly correlated ($r^2 = 0.945$) with van der Waals volume. It has been used in structure-activity relationships as

a volume term. In contrast to size parameters, very few parameters encoding shape have been developed. Parameters that can be described as shape descriptors include the Van der Waals radius and related terms such as the Taft steric constant, E_s [Taft, 1956] are parameters that are applicable only to substituents and furthermore has little meaning for substituents that are far from spherical.

A reasonable correlation ($r^2 = 0.707$) was found between the first-order rate constants for compound disappearance and computer estimated values for molar refractivity (The values for all of the structure-activity parameters examined are shown in table 8.). This indicates that increasing the size of the substituent group is the predominant factor in slowing the rate of degradation. For example, 2-methylnaphthalene was more rapidly degraded than 2-methoxynaphthalene. Since each compound is substituted only at the 2 position, the effect of substituent location and number, and hence the number of dioxygenase attack sites, is not considered. This correlation was improved ($r^2 = 0.849$) when naphthalene-2-carbonitrile (strongly electron-withdrawing) and 2-aminonaphthalene (strongly electron-releasing) were not considered. The slow rate of 2-aminonaphthalene disappearance may be attributed to an ionized amino substituent which prevents the compound from entering the cells. The cyano- substituent of naphthalene-2-carbonitrile is electron-withdrawing and may hinder NAH-mediated disappearance.

The only other examined parameter that showed a correlation with the rate of disappearance was the Taft steric constant, E_s . A very weak correlation ($r^2 = 0.350$) between the disappearance rates and E_s was found when all seven compounds, excluding 2-naphthaldehyde, were examined. As in the case of MR, when naphthalene-2-carbonitrile and 2-aminonaphthalene were not considered in the analysis, the correlation improved ($r^2 = 0.540$) between the rates of disappearance and the compounds with electrically neutral substituents. This is further evidence for the relationship between substituent size and disappearance rate.

The loss of 2-naphthaldehyde was too rapid to be measured. Although it was shown in biotransformation experiments that it was transformed to 2-naphthoic acid in the presence of *P. fluorescens* 5RL, it is possible that this is not related to NAH system

Table 8. Comparative Values of Structure-Activity Parameters Examined in the Degradation of a Mixture of Various Substituted Naphthalenes by *Pseudomonas fluorescens* 5R.

Compound	Σ (0.079)*	$\mathcal{R}$ (0.318)*	σ_m (0.122)*	MR (0.707)*	E_s (0.351)*
Naphthalene	0.00	0.00	0.00	0.10	0.00
2-Methylnaphthalene	-0.04	-0.13	-0.17	0.56	-1.24
2-Chloronaphthalene	0.41	-0.13	0.23	0.60	-0.97
2-Ethyl-naphthalene	-0.05	-0.10	-0.15	1.03	-1.31
2-Methoxynaphthalene	0.26	-0.51	-0.27	0.79	-0.55
Naphthalene-2-carbonitrile	0.21	-0.18	0.01	1.01	-2.38
2-Aminonaphthalene	0.02	-0.68	-0.66	0.54	-0.61

Σ = field effect

$\mathcal{R}$ = resonance effect

σ_m = Hammett substituent constant

MR = molar refractivity

E_s = Taft steric constant

* r^2 values for the correlation between first-order rate constants for compound disappearance and SAR parameters are indicated in parentheses.

activity, particularly that of naphthalene dioxygenase. Rather, aromatic aldehydes such as naphthaldehyde are direct acting electrophiles. Electrophilic reactivity can be thought of as a chemical's ability to have a covalent interaction with a biological system [Hermens, 1990]. Electrophilic interactions involve substitution or conjugation of electron-rich groups to nucleophilic sites in cellular macromolecules (i.e., proteins and nucleic acid polymers). One group of soft electrophiles are the aldehydes [Hermens, 1990]. More specifically aldehydes act as Schiff-based formers [Lipnick, 1991]. Thus, the rapid loss of 2-naphthaldehyde is thought to be due to covalent bonding to bacterial macromolecules.

The final experiment using the complex mixture of PAHs and heterocycles was intended simply to confirm that the relative rates of degradation observed within each class of compounds is not altered when the various types of compounds are added together. The results of this experiment validate this point.

Chapter VI

Summary and Conclusions

The data gathered in this work provides new information and extends previous observations of the ability of the NAH system to transform a broad range of polyaromatic and heterocyclic aromatic compounds. In this investigation, a novel approach was developed in order to elucidate the cometabolic versatility of the NAH system of *Pseudomonas fluorescens*. Most previous biotransformation studies have focused only upon the reactions carried out by naphthalene dioxygenase and have not further examined the processes carried out by the remaining upper and lower pathway enzymes. In addition, previous studies have not examined how these degradative reactions are integrated with the ability of an intermediate to induce transcription and lead to cell growth. This examination utilized the NAH system strain *P. fluorescens* 5R and its mutant *P. fluorescens* 5RL, and cloned *nahGH* genes in plasmid pUTK200 to show how the biotransformation capacity of the NAH system for these co-contaminants can vary, and that the extent of degradation ultimately depends on the structure of the parent compound. For example, the presence of a methyl substituent on each ring effectively blocked any transformation beyond that of an initial hydroxylation, whereas most mono-substituted naphthalenes, irrespective of substituent type, were transformed to their respective salicylate analogs. The formation of these mono-hydroxylated naphthalenes is an important point because these parent compounds are not actually biodegraded, but are transformed to a series of more water-soluble compounds which are likely to be more mobile, and possibly more reactive, in the environment than their respective parent compounds.

These results bring up an important issue which has not been examined in great detail. Can the presence of NAH system genes reliably be used as an indicator for PAH-degrading organisms that can accomplish bioremediation of mixed PAHs at a contaminated site? Based on the biotransformation studies conducted in this dissertation,

the potential fate of many unexamined compounds as a result of NAH-mediated degradation can be better predicted. For example, in the characterization of PAHs in a supercritical fluid extract of coal tar-contaminated soil, a number of unstudied alkyl-substituted PAHs and heterocycles were identified [Yu et al., 1990]. These included C₃-, C₄-, and C₅-naphthalenes, C₂- and C₃-biphenyl, C₁-, C₂-, and C₃-anthracene and phenanthrene, C₁- and C₂-fluorene, and C₂-naphthothiophene. A number of unsubstituted low- to intermediate-molecular weight PAHs were also identified. These included 2-phenylnaphthalene and binaphthalene. Using the biotransformation data from the studies using strain 5RL as a model for a diverse set of co-contaminants, it can be predicted that any methyl-substituted naphthalene with at least one group on each ring, or with methyl substituents at the 1 and 4 positions, will not be transformed to a salicylate analog and will most likely be monohydroxylated to form a dead-end product. This indicates that the NAH system will transform many of these multi-substituted compounds, but it will not facilitate biodegradation via the removal of an aromatic ring. Conversely, it can be predicted that C₁-, C₂-, and C₃-anthracene and phenanthrene will be degraded to methyl-substituted naphthoic acids via the removal of an aromatic ring if the two outer rings of these compounds are unsubstituted. If the outer rings of either compound both possess substituents, then dead-end product formation should occur. The upper pathway of *P. fluorescens* 5RL was also shown to transform many naphthalenes that possess one electrically neutral substituent. It can thus be predicted that the biodegradation of similar compounds with multiple electrically neutral substituents should follow the patterns observed in the transformation of dimethylnaphthalenes by strains 5RL.

For those compounds that may be transformed to a salicylate-like product, the data gathered using the cloned salicylate hydroxylase and catechol 2,3-dioxygenase of strain 5R in plasmid pUTK200 has shown that further degradation by the lower pathway did not occur in most cases. It is not expected that the lower pathway can facilitate the biodegradation of other salicylate analogs which may be formed during the transformation unexamined compounds. For example, the inability of pUTK200 to mediate the degradation of 1-hydroxy-2-naphthoic acid and 2-hydroxy-3-naphthoic acid

has been shown by Applegate [1997]. Therefore, naphthoic acids formed via the transformation of C₁-, C₂-, and C₃-anthracene and phenanthrene will likely not be degraded further.

Using *P. fluorescens* 5RL, new information was obtained concerning the ability of salicylate analogs to mimic salicylate and induce NAH system transcription. Few compounds were shown to be transcriptional activators. In general, they were shown to be limited to salicylate analogs with an electrically neutral substituent at the 4 position. Of all the compounds examined, 2-methylnaphthalene and 2-ethylnaphthalene were the only two compounds able to support growth in *P. fluorescens* 5R and a number of other NAH system strains.

By using a uniquely simple experimental approach, studies conducted using strains 5R and 5RL, a *nahA* clone, and a number of other NAH system organisms demonstrated that the selectivity of naphthalene dioxygenase is an important, but commonly disregarded, driving force in the preferential biodegradation of PAHs and heterocycles. The data gathered from these experiments thus indicate that desorption kinetics should not be viewed as the only determining factor in an examination of PAH mixture degradation kinetics.

Semi-quantitative structure-activity analyses provided new insight as to how deviation from the base structure of naphthalene acted to slow rates of compound biodegradation. For example, the obstruction of naphthalene dioxygenase attack sites by methyl substituents acted to slow the rates of compound disappearance. These findings lend predictability to the relative rates of degradation of other co-contaminants. For example, a C₅-naphthalene found at a contaminated site would thus be predicted to be much more slowly degraded than a C₂-naphthalene. Similarly, large electrically neutral functional groups on naphthalenes, such as halogenated naphthalenes, will be more slowly degraded as the number and size of these substituents increases. The effect of the type of ring structure and the number of rings on biodegradation rates for those PAHs and heterocycles with three or more rings is less well understood. However, it can be predicted that increasing the ring number will greatly reduce the rate of biodegradation.

For example, naphthacene (tetracene) is a four-ringed PAH with four open naphthalene dioxygenase attack sites. Based on the relative rates of degradation for naphthalene, acenaphthene, and phenanthrene, this compound should be more slowly degraded than any two- or three-ringed PAH.

The insight gained from the co-contaminant mixture biodegradation studies, when combined with data from the transcriptional induction studies, the biotransformation studies, and the studies on growth, represent the most comprehensive analysis of the versatility of the NAH system to date. It should initiate new examinations into the capacity of the NAH system to biodegrade higher molecular weight PAHs, the potential toxicity as a result of dead-end metabolite formation, and how the comparative activity of naphthalene dioxygenase and desorption kinetics facilitate the preferential biodegradation of PAHs in the environment.

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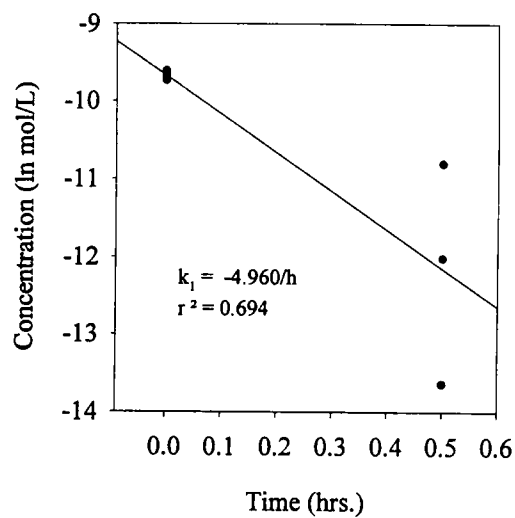
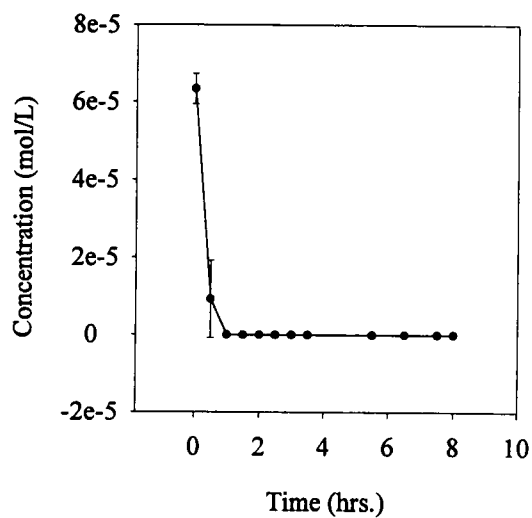
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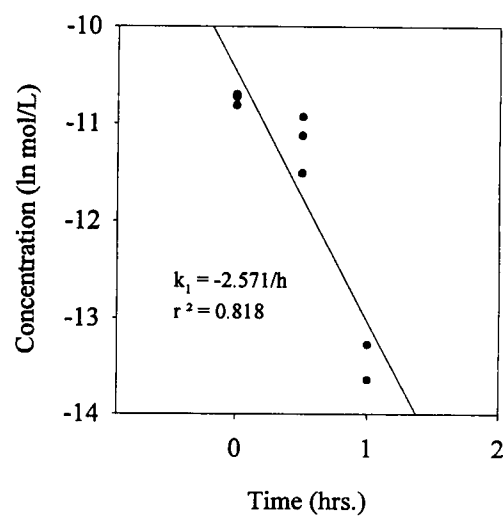
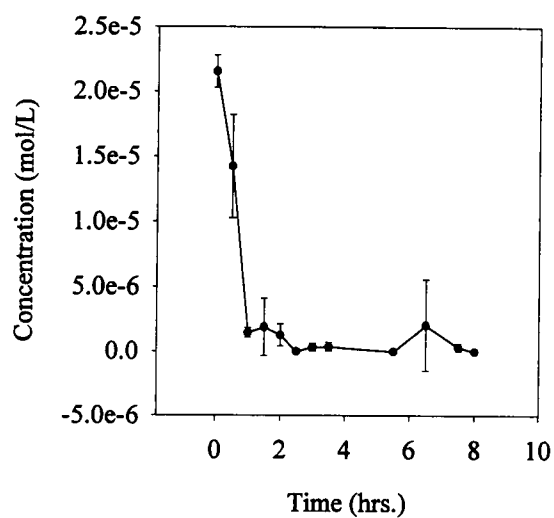
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Appendix

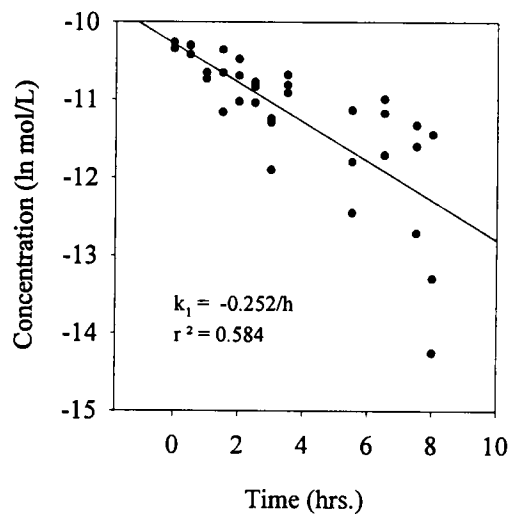
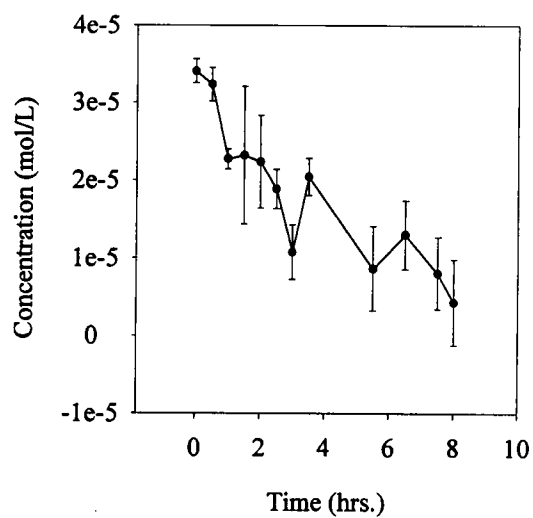


Naphthalene

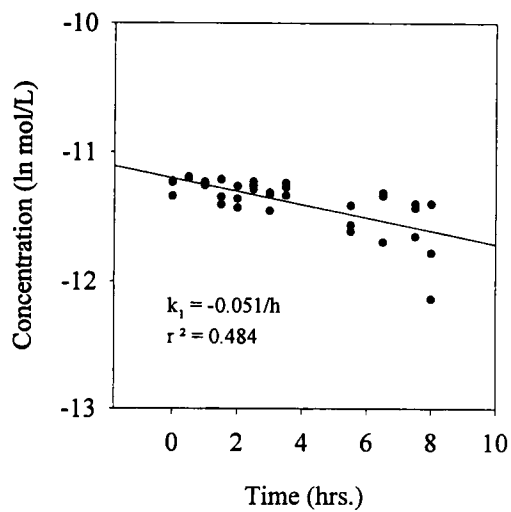
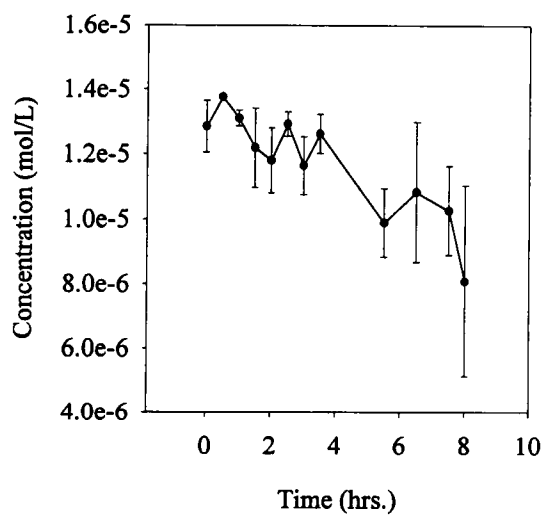


2-Methylnaphthalene

Figure 24. Biodegradation of a PAH and Heterocycle Mixture by *P. fluorescens* 5R Pregrown on YEPSS Medium.

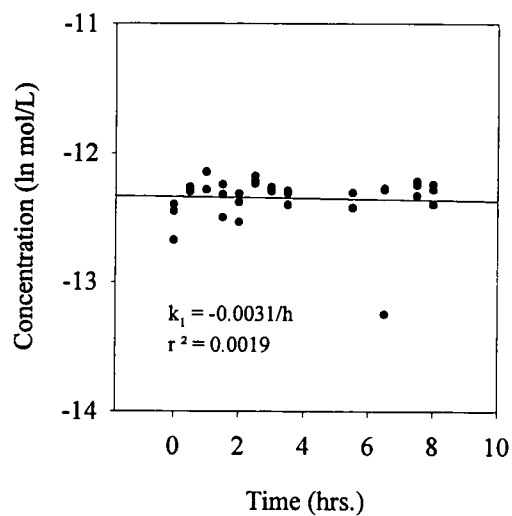
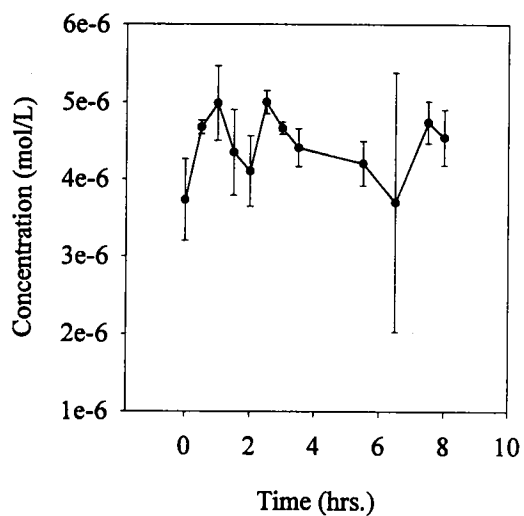


1-Methylnaphthalene

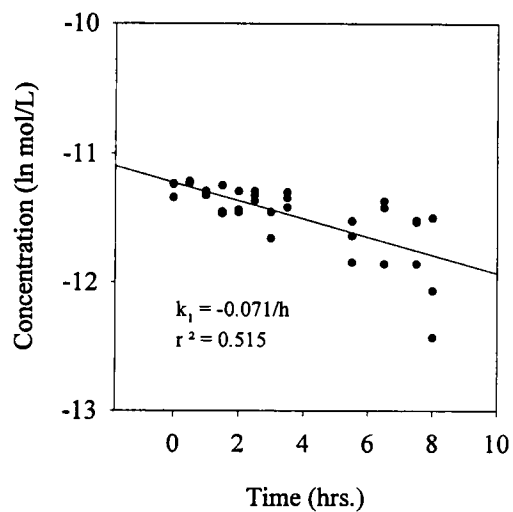
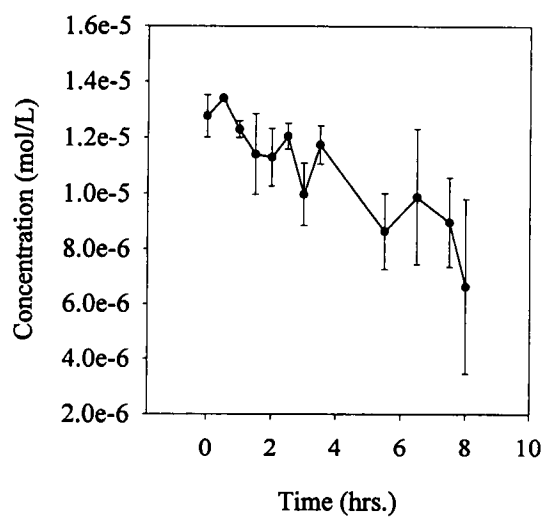


Biphenyl

Figure 24 cont.

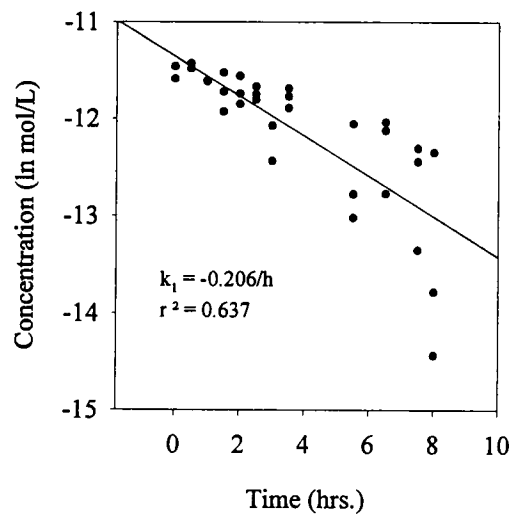
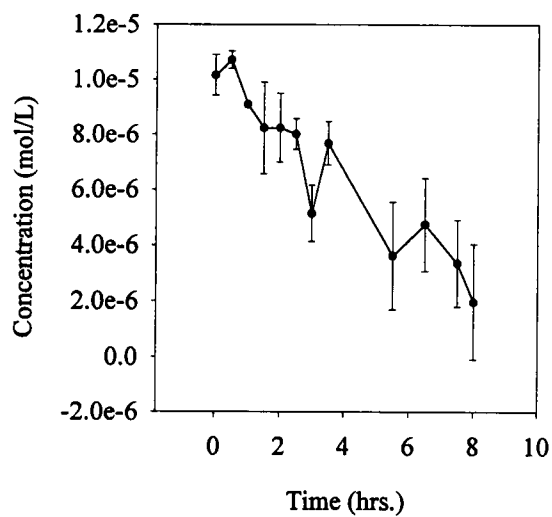


2,6-Dimethylnaphthalene

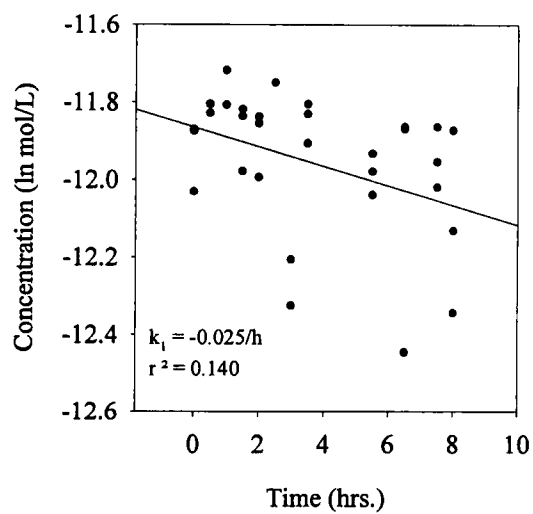
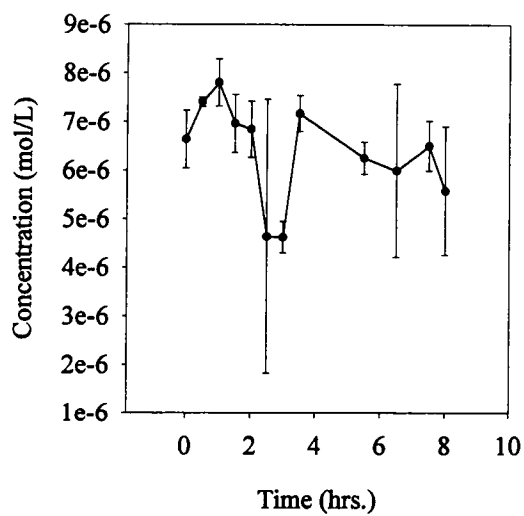


Dibenzofuran

Figure 24 cont.

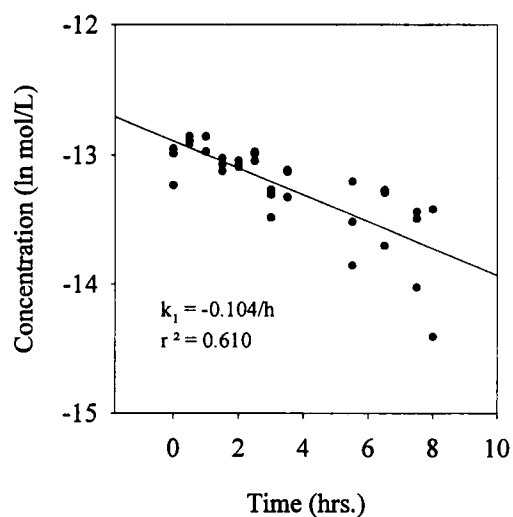
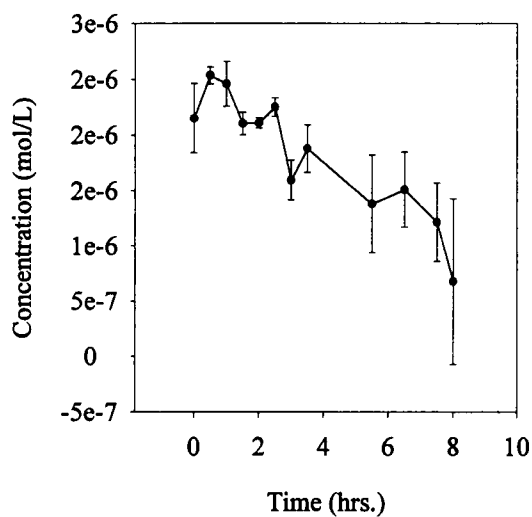


Acenaphthene

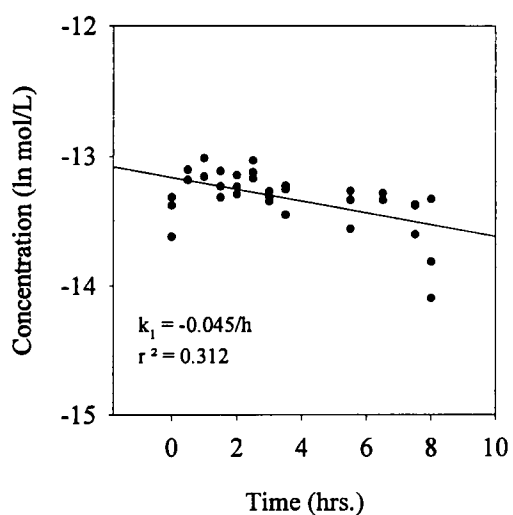
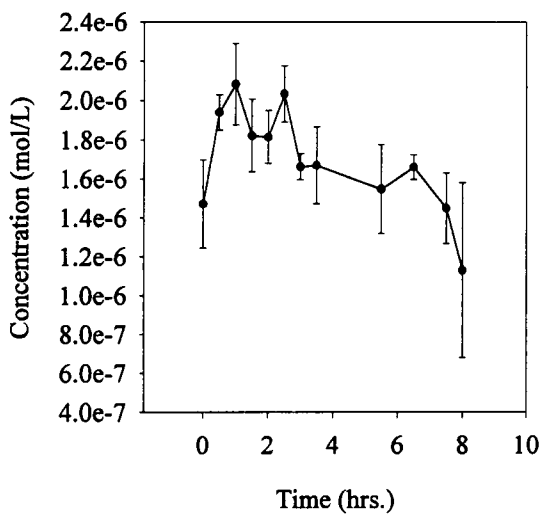


Fluorene

Figure 24 cont.

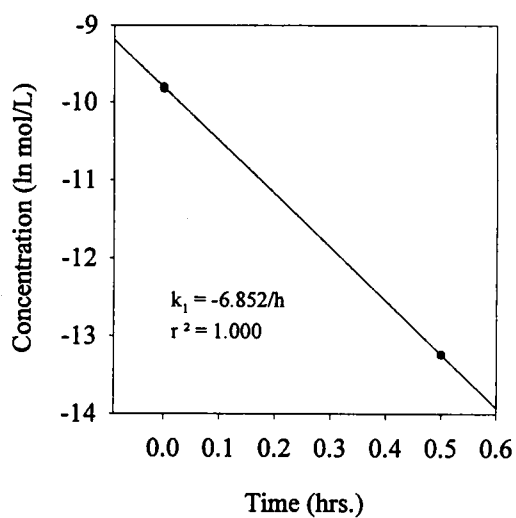
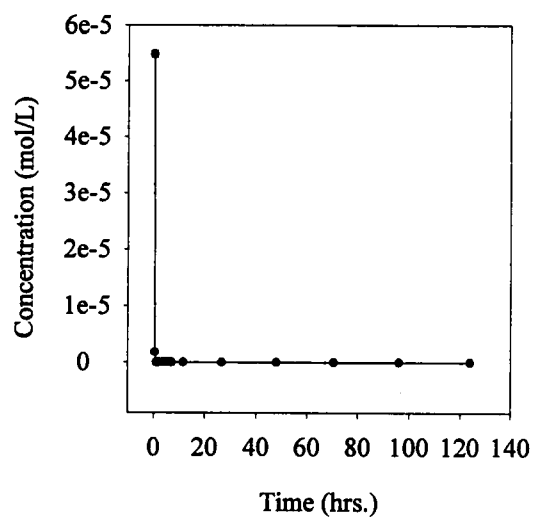


Dibenzothiophene

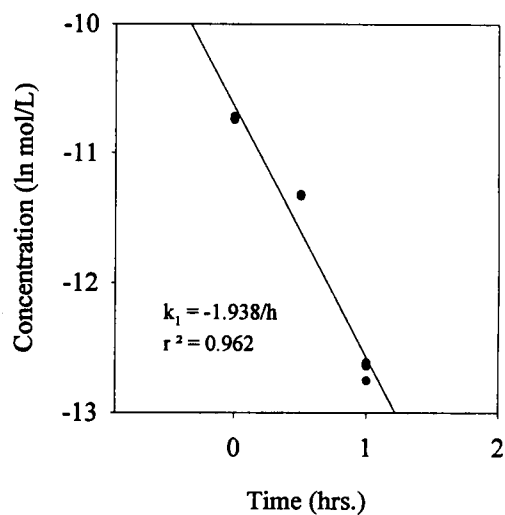
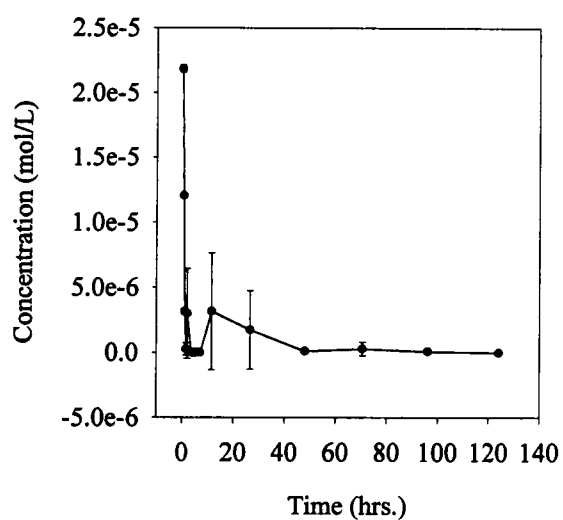


Phenanthrene

Figure 24 cont.

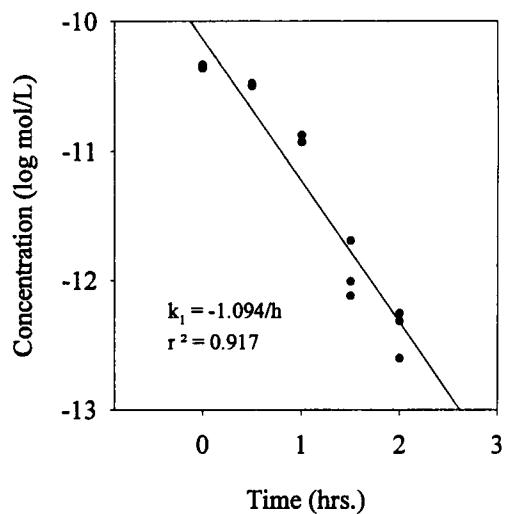
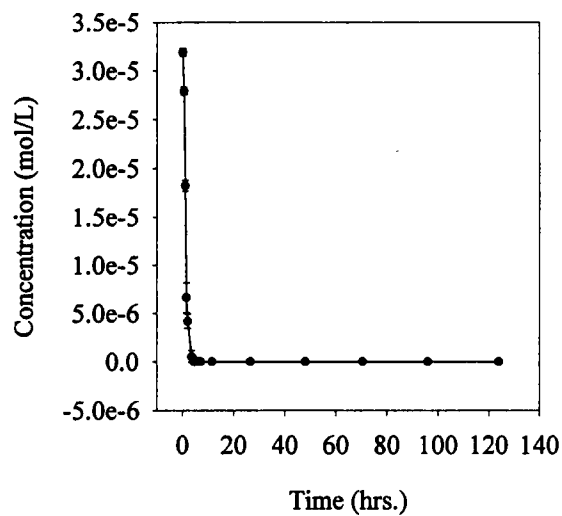


Naphthalene

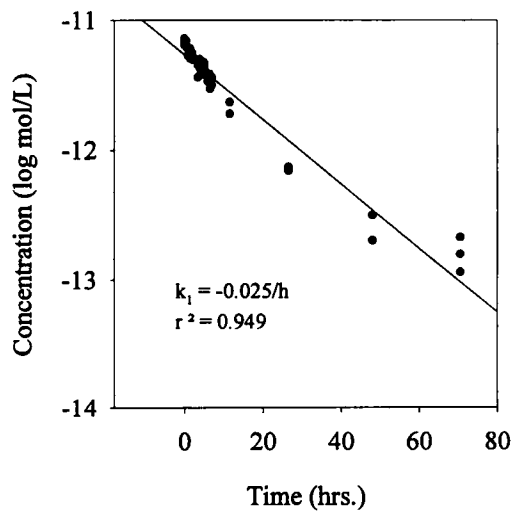
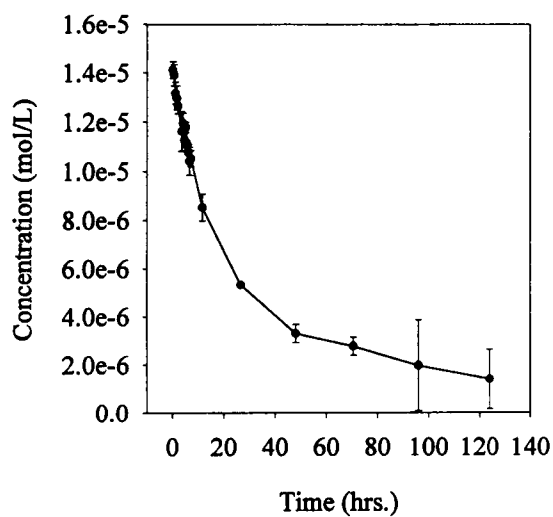


2-Methylnaphthalene

Figure 25. Biodegradation of a PAH and Heterocycle Mixture by *Pseudomonas* sp. Strain DFC50.

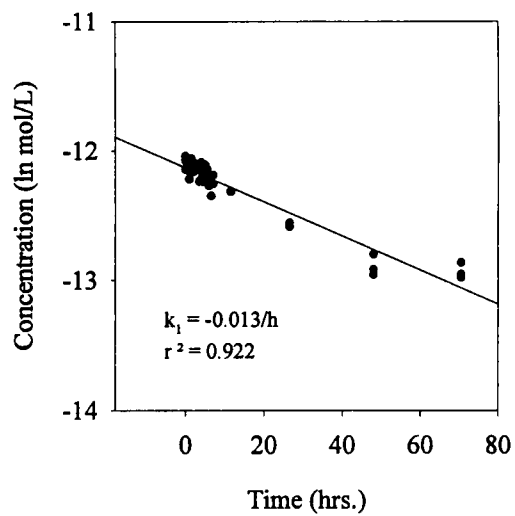
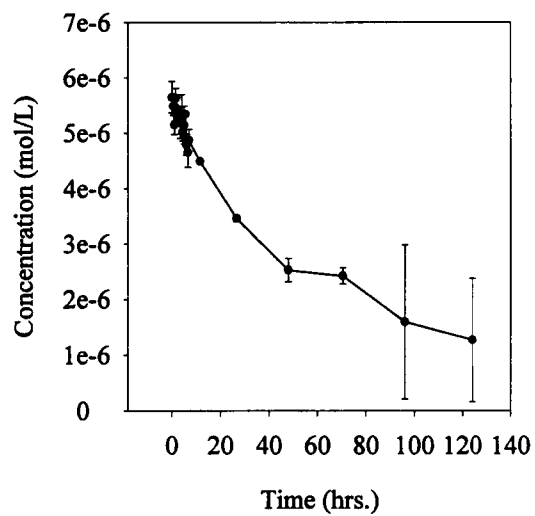


1-Methylnaphthalene

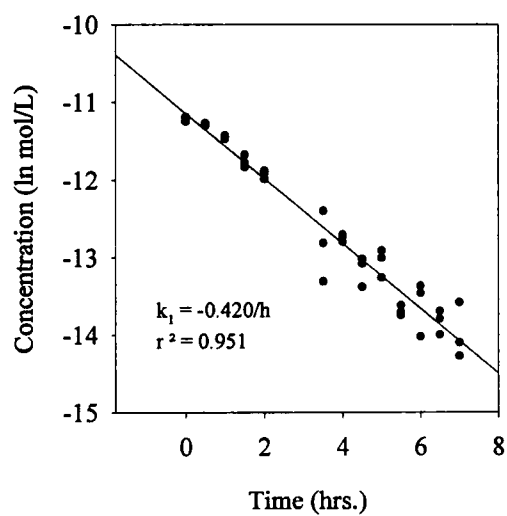
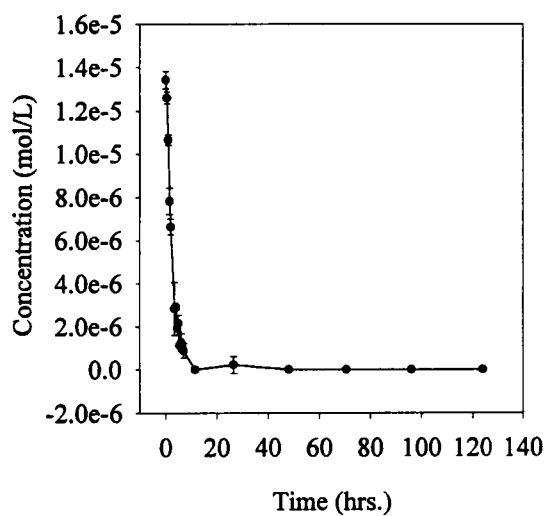


Biphenyl

Figure 25 cont.

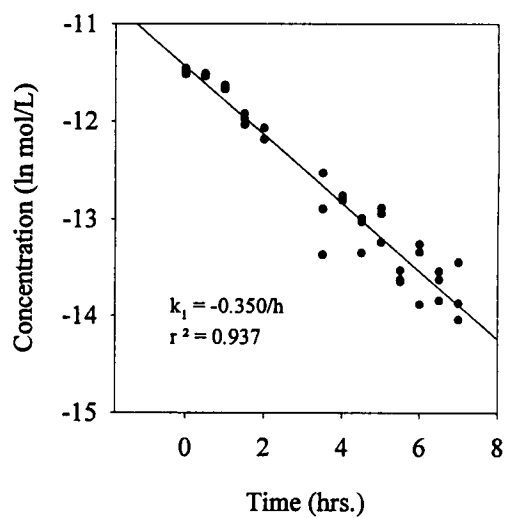
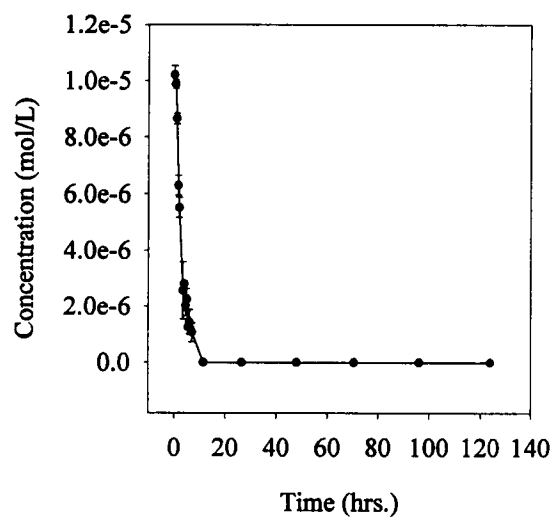


2,6-Dimethylnaphthalene

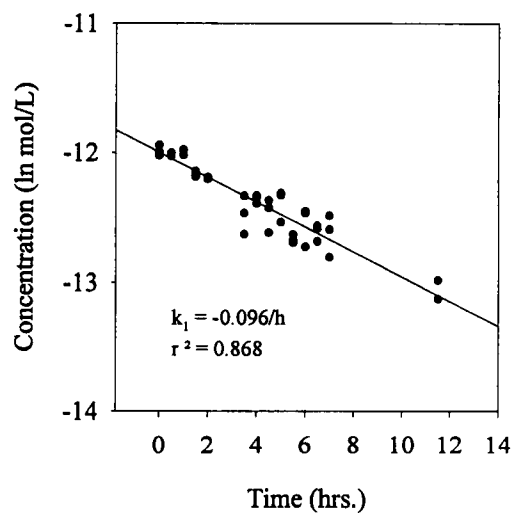
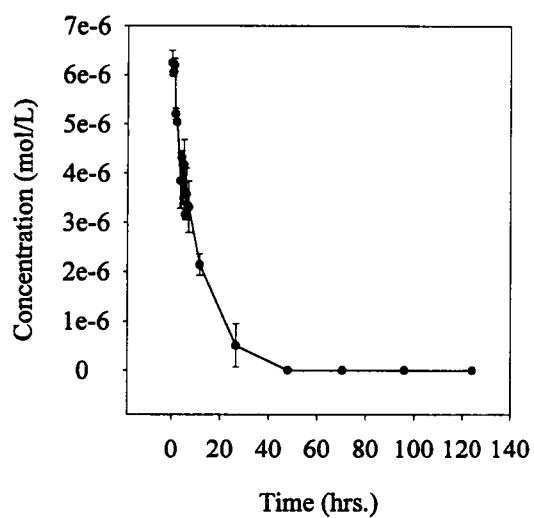


Dibenzofuran

Figure 25 cont.

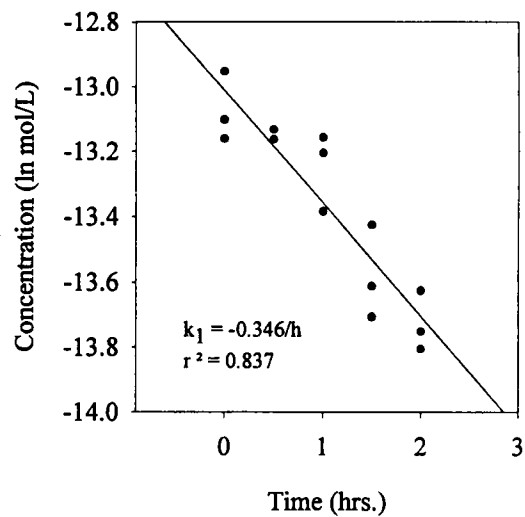
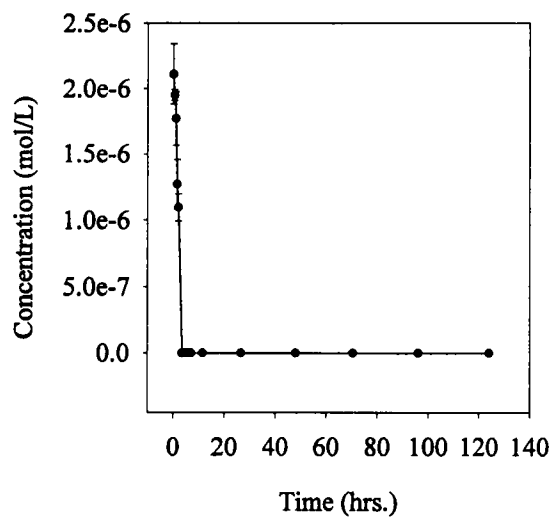


Acenaphthene

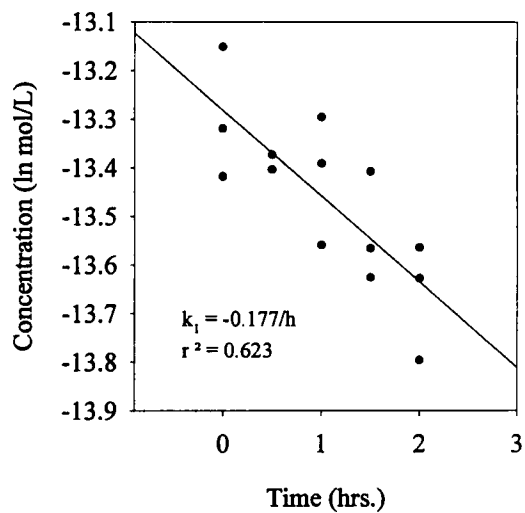
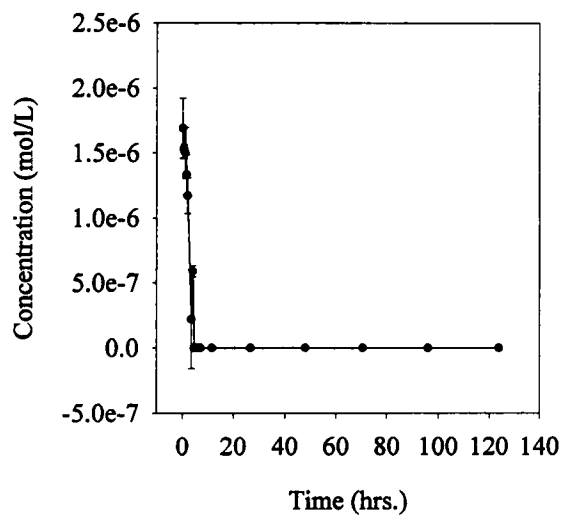


Fluorene

Figure 25 cont.

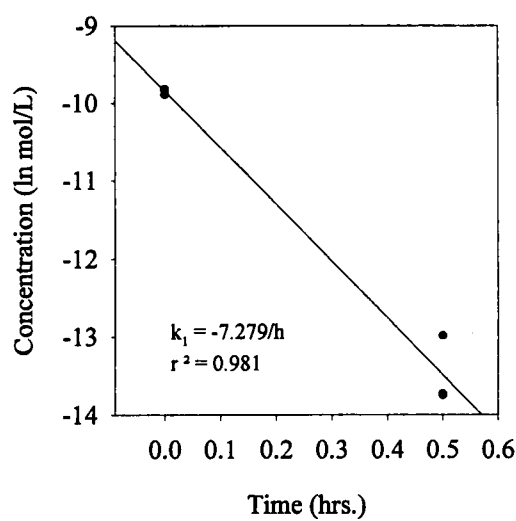
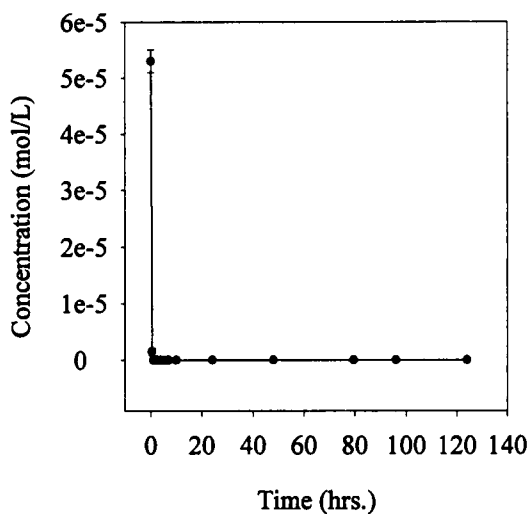


Dibenzothiophene

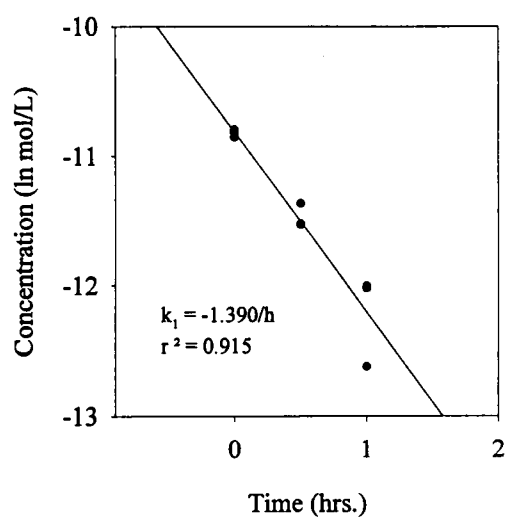
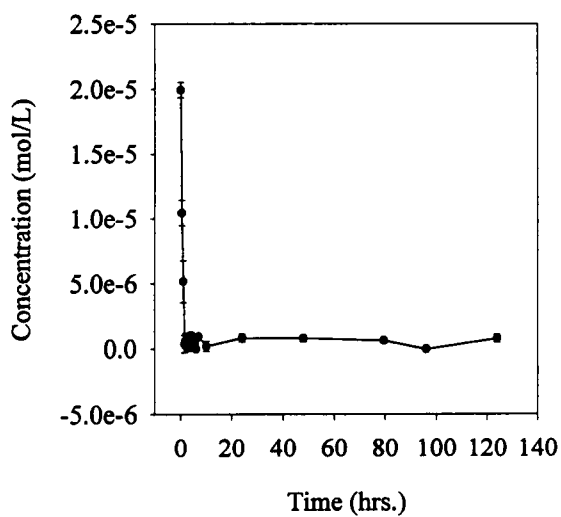


Phenanthrene

Figure 25 cont.

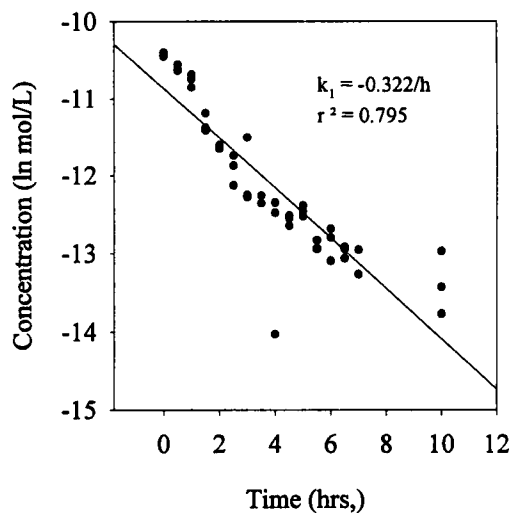
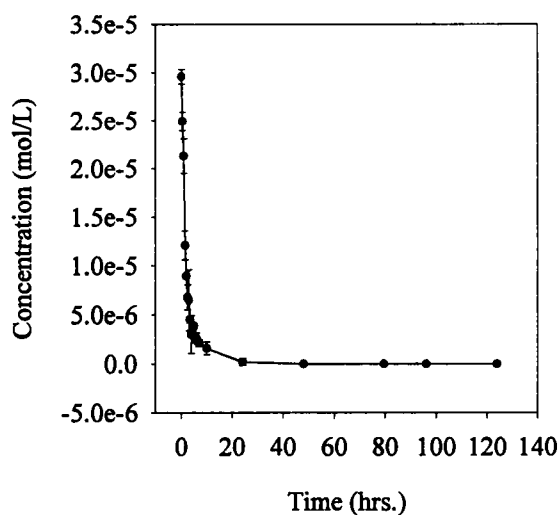


Naphthalene

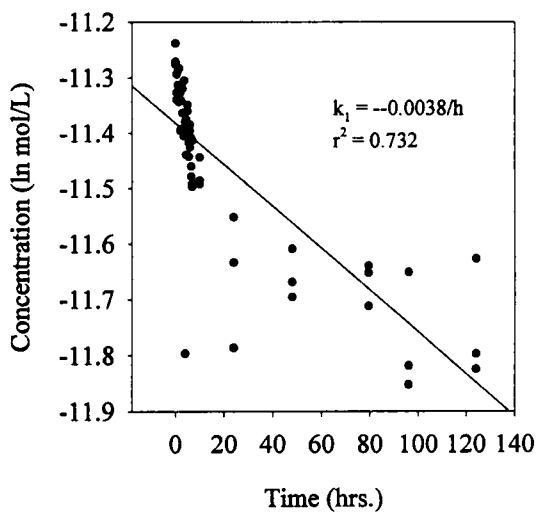
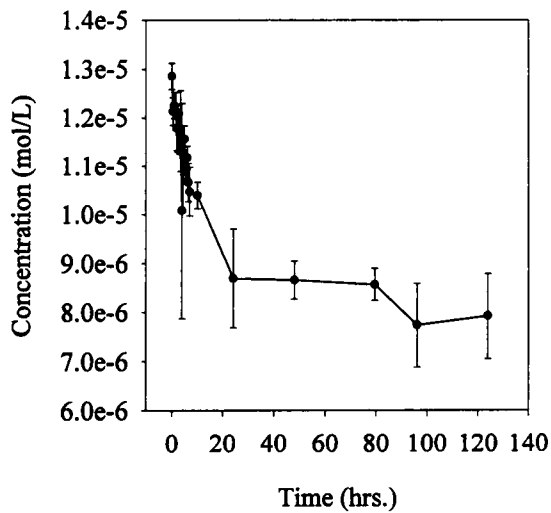


2-Methylnaphthalene

Figure 26. Biodegradation of a PAH and Heterocycle Mixture by *Pseudomonas* sp. Strain DFC49.

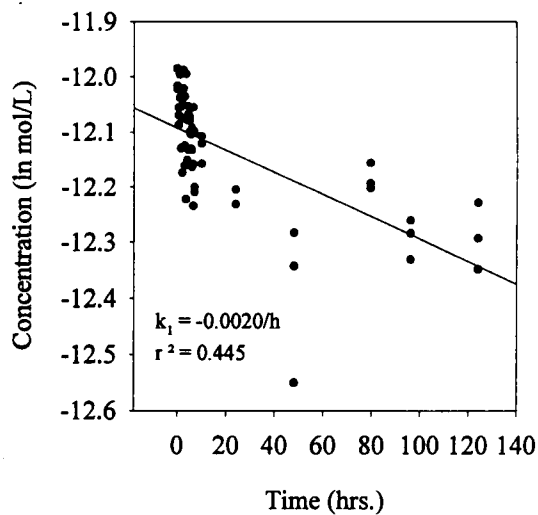
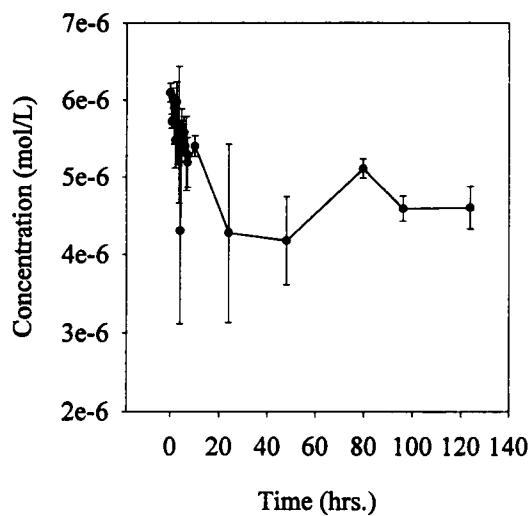


1-Methylnaphthalene

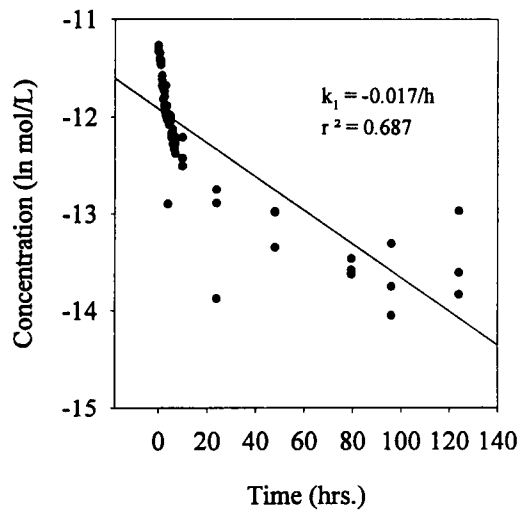
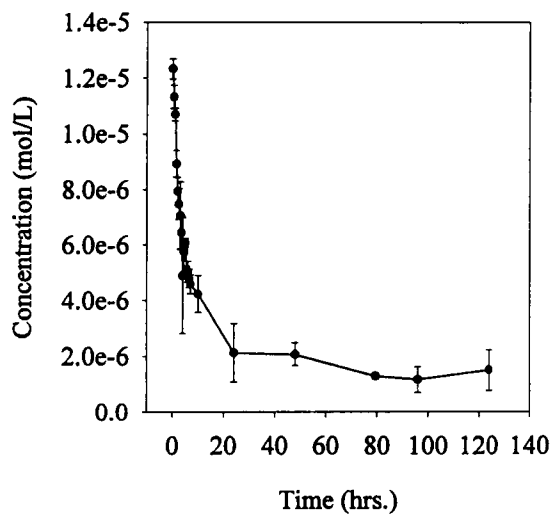


Biphenyl

Figure 26 cont.

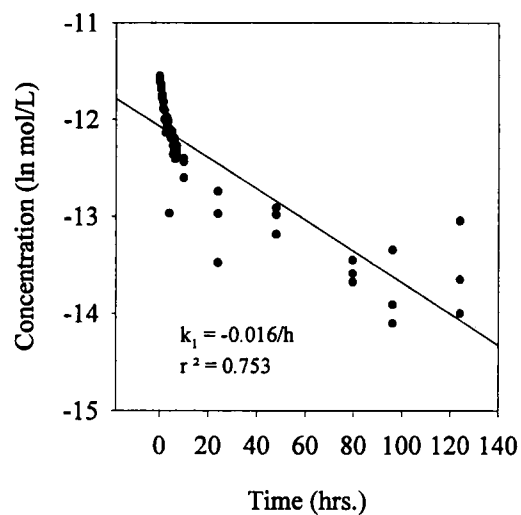
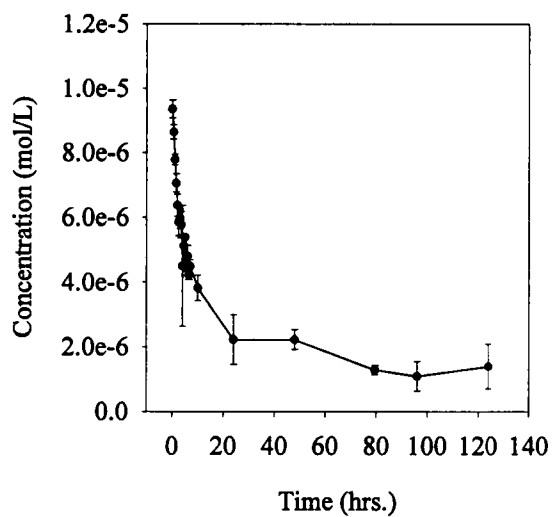


2,6-Dimethylnaphthalene

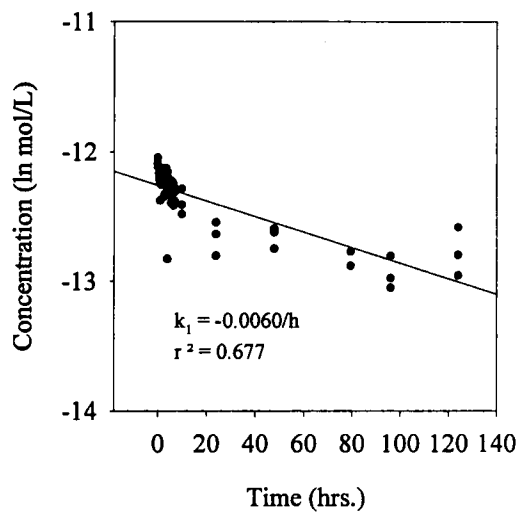
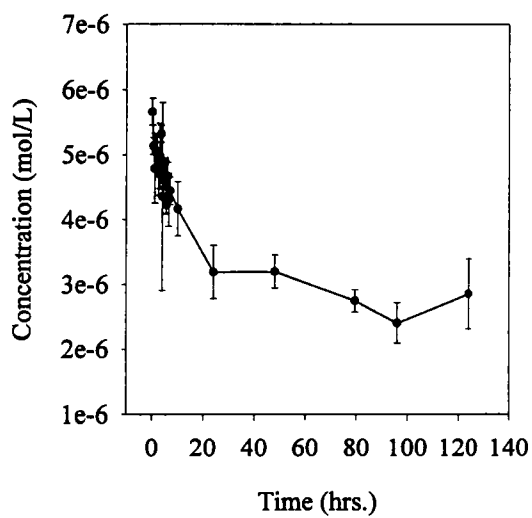


Dibenzofuran

Figure 26 cont.

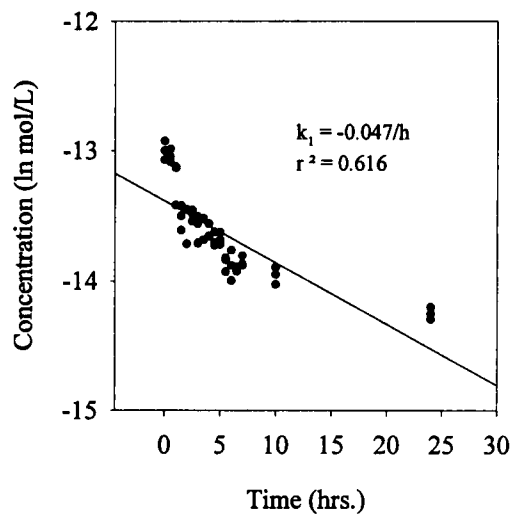
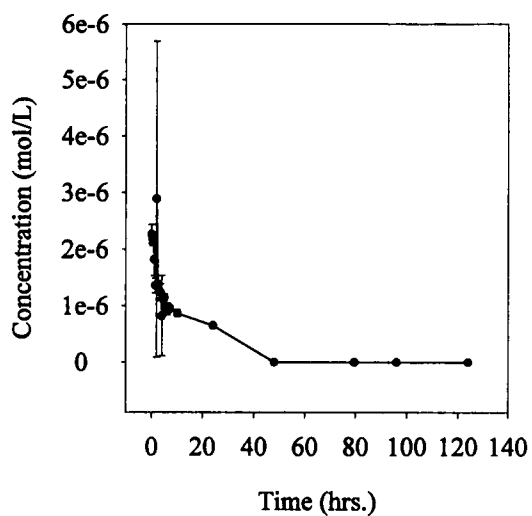


Acenaphthene

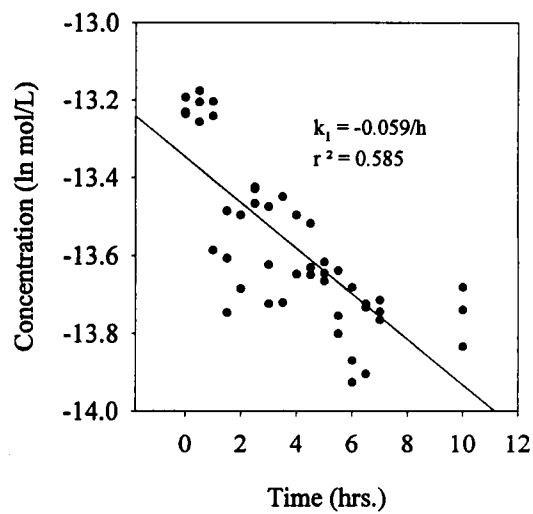
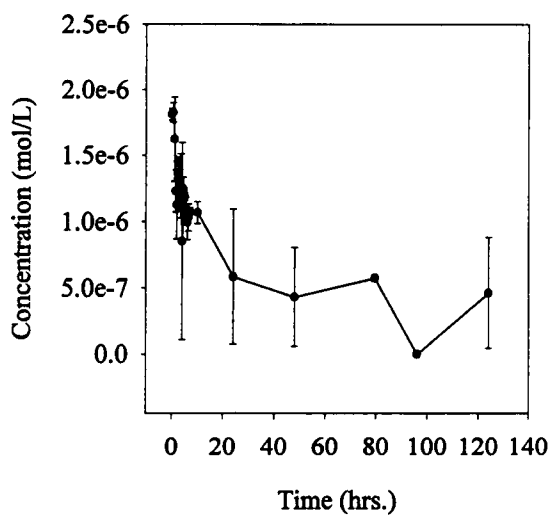


Fluorene

Figure 26 cont.

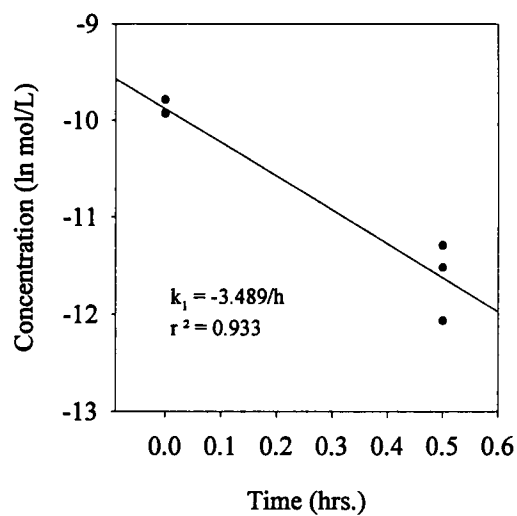
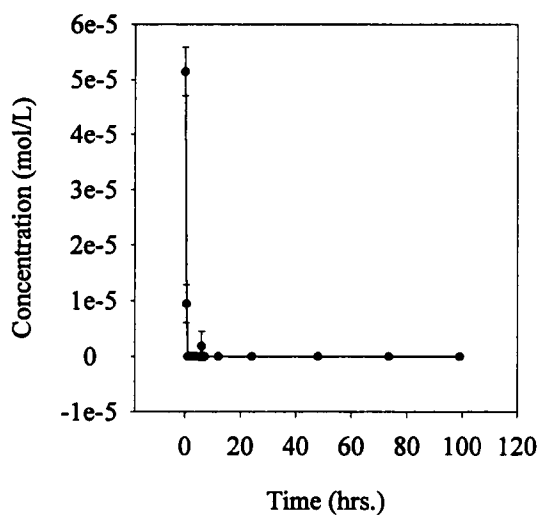


Dibenzothiophene

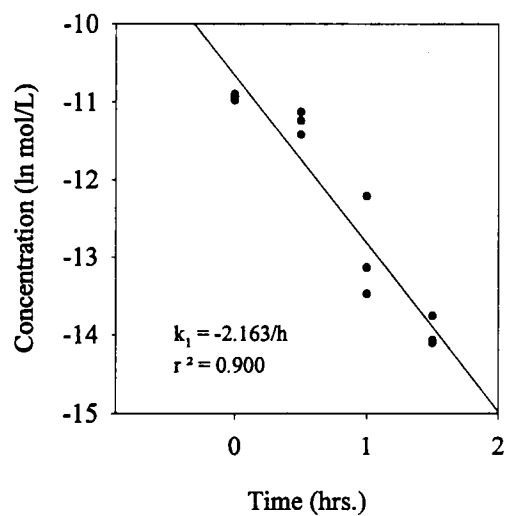
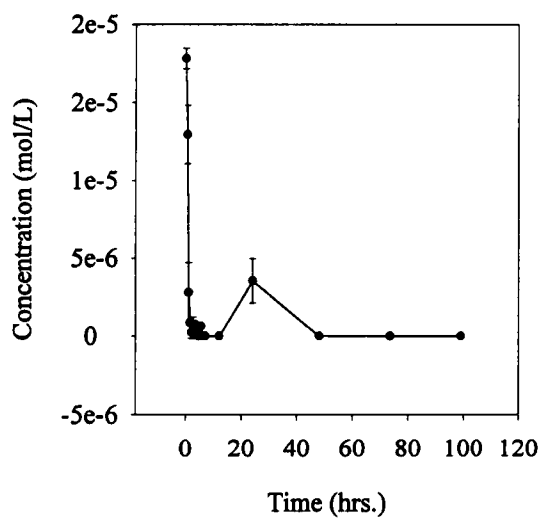


Phenanthrene

Figure 26 cont.

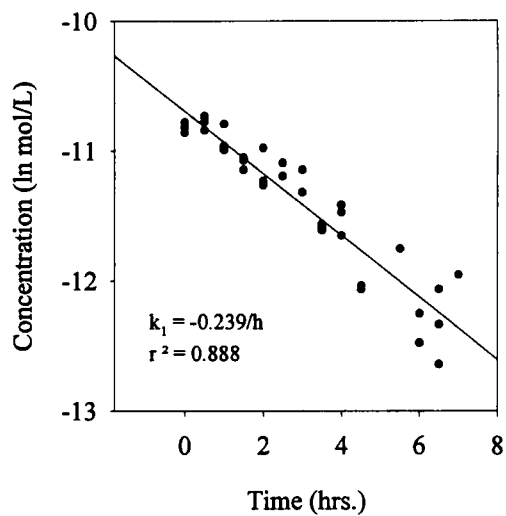
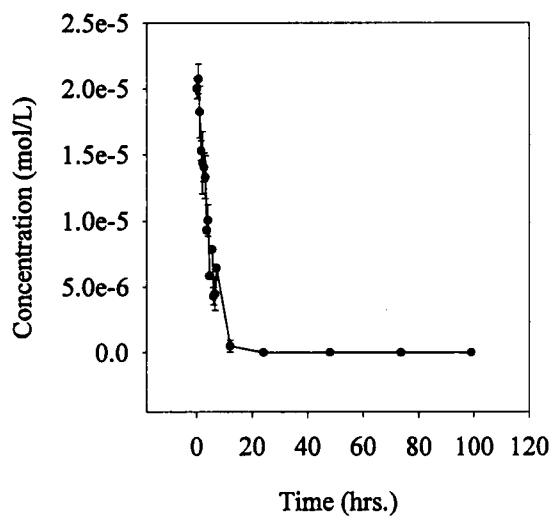


Naphthalene

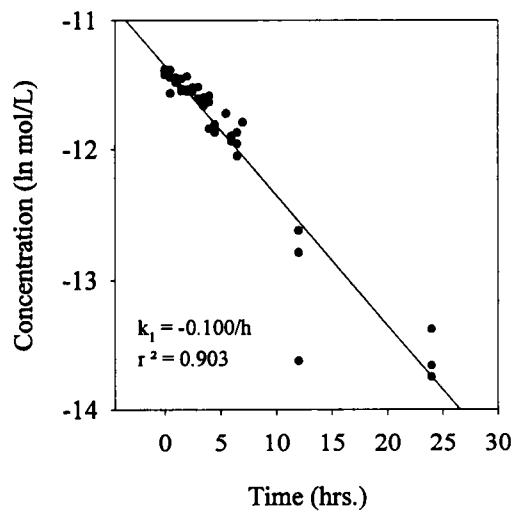
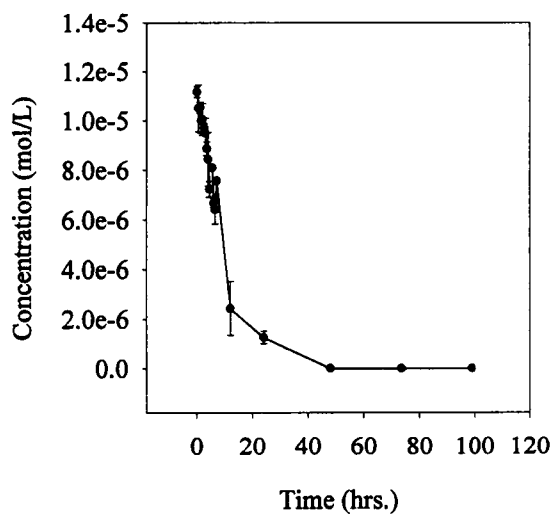


2-Methylnaphthalene

Figure 27. Biodegradation of a PAH and Heterocycle Mixture by *P. putida* PpG7.

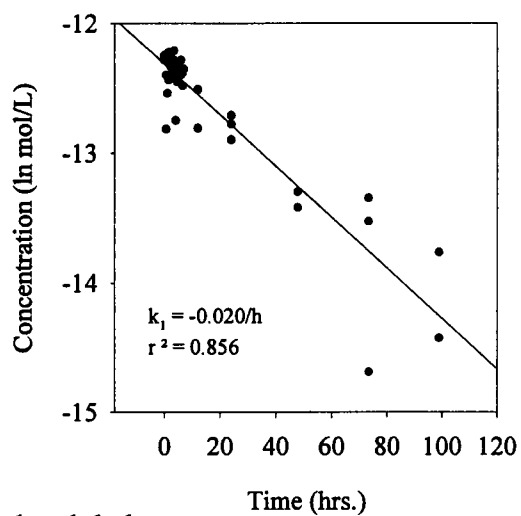
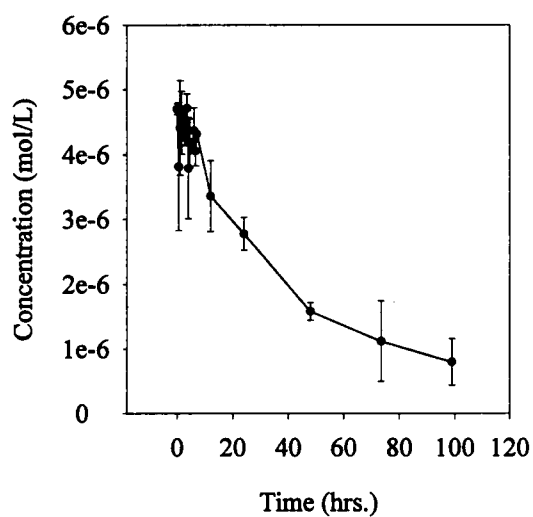


1-Methylnaphthalene

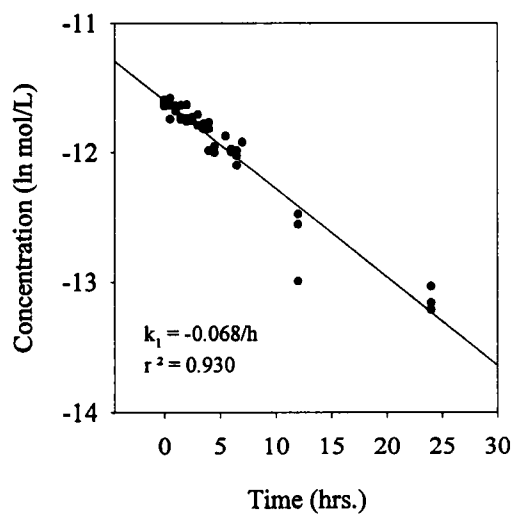
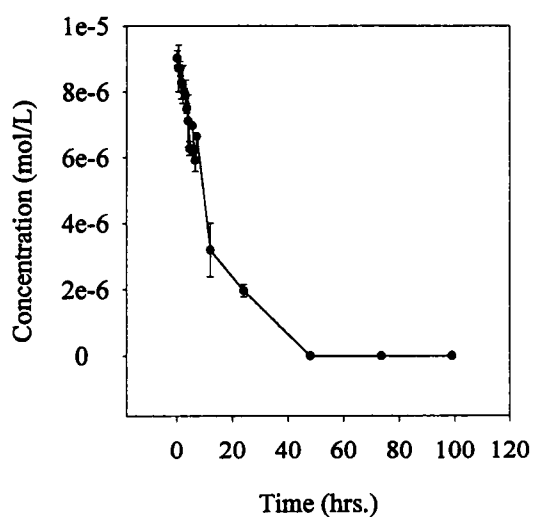


Biphenyl

Figure 27 cont.

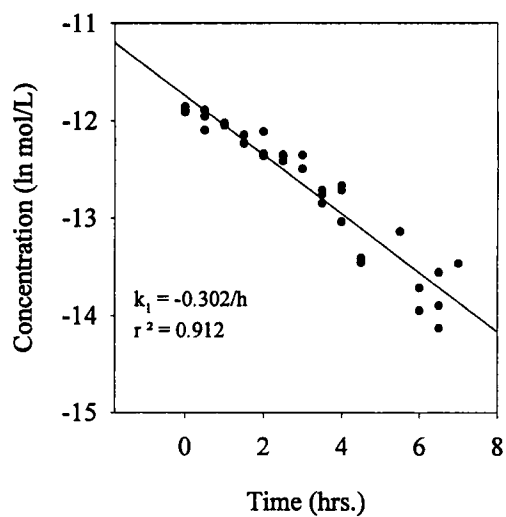
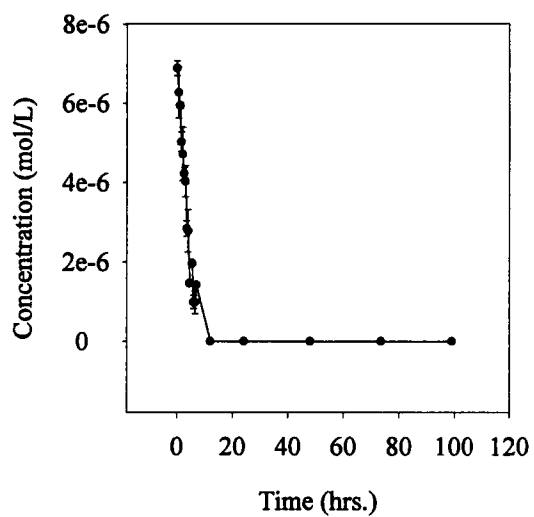


2,6-Dimethylnaphthalene

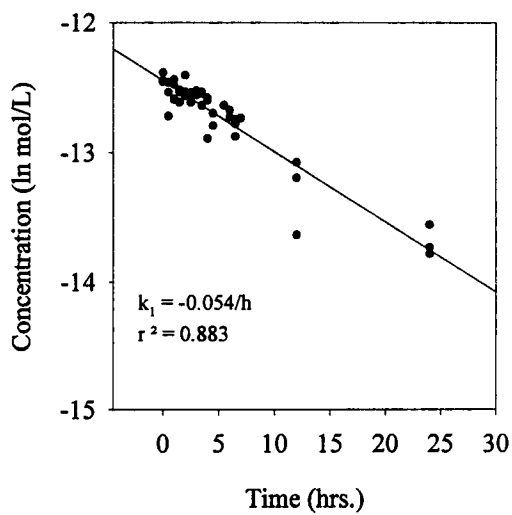
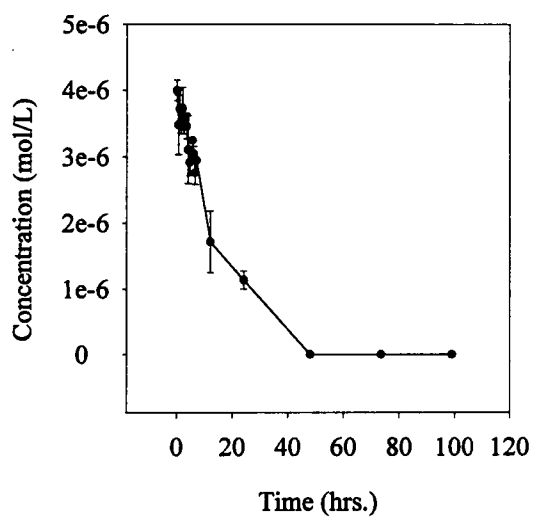


Dibenzofuran

Figure 27 cont.

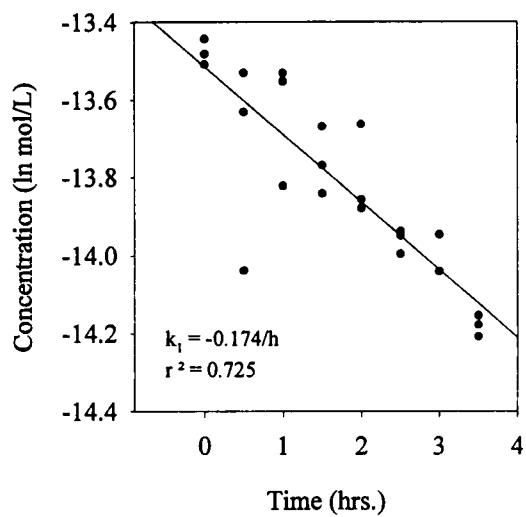
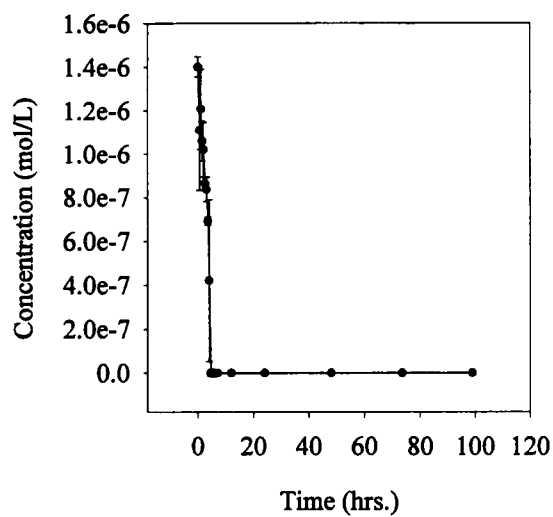


Acenaphthene

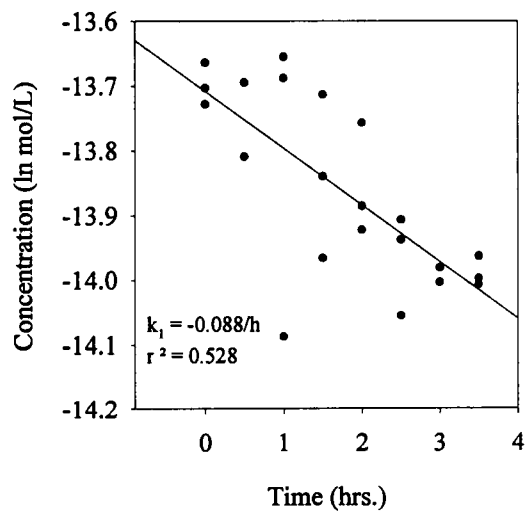
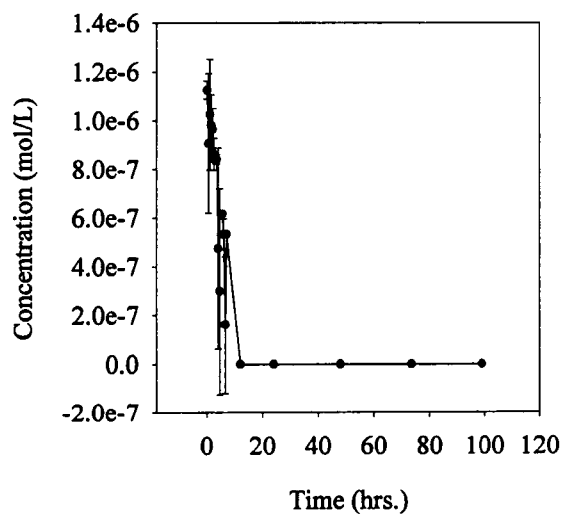


Fluorene

Figure 27 cont.

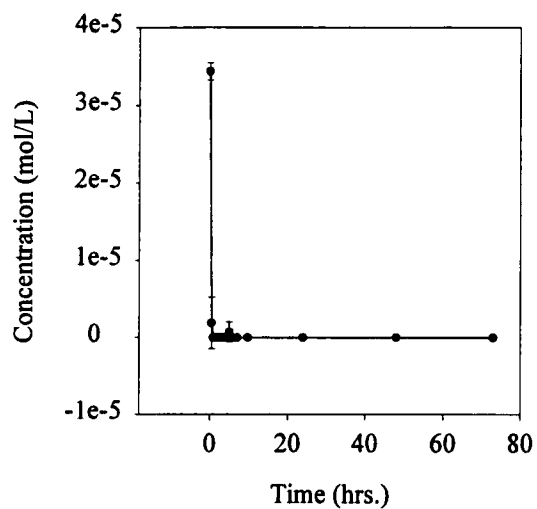


Dibenzothiophene

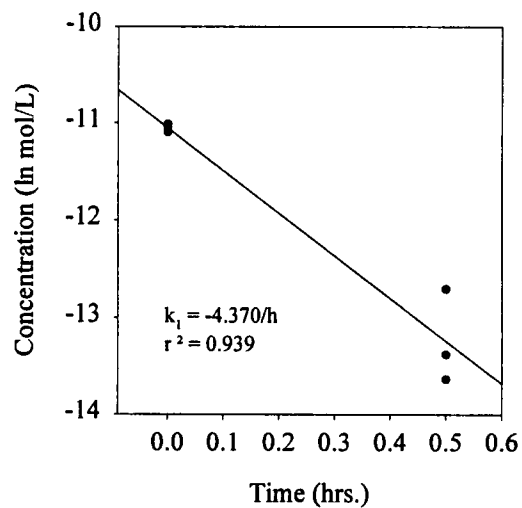
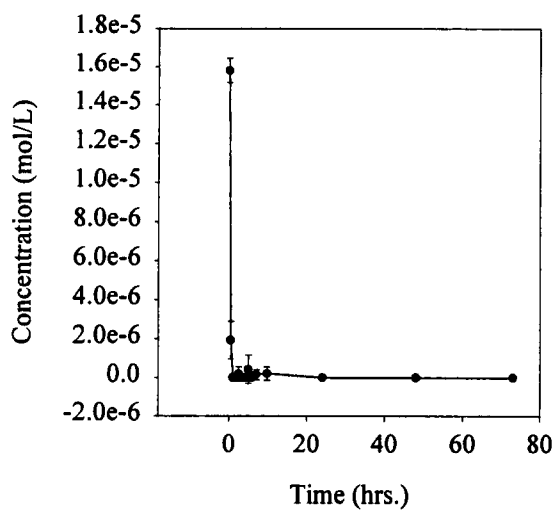


Phenanthrene

Figure 27 cont.

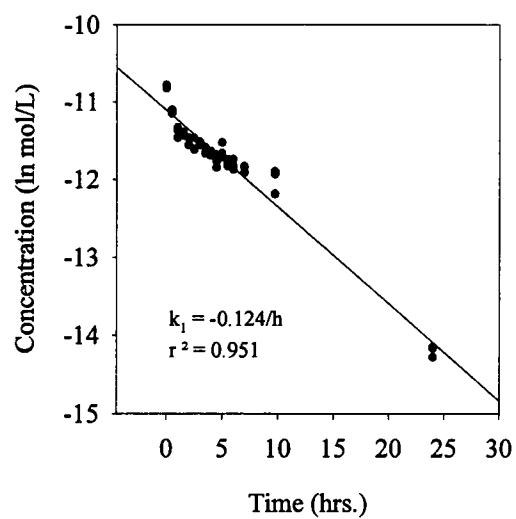
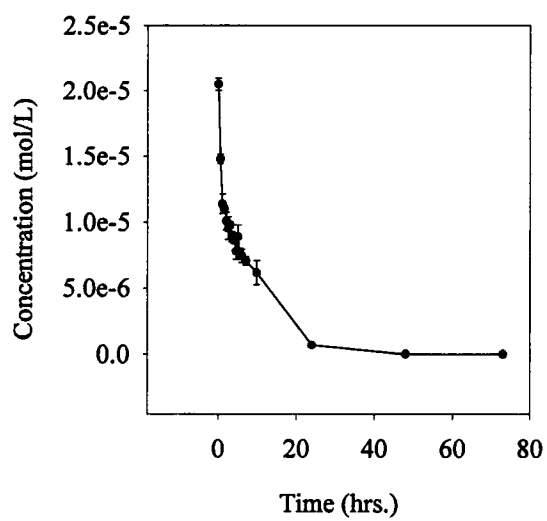


Naphthalene

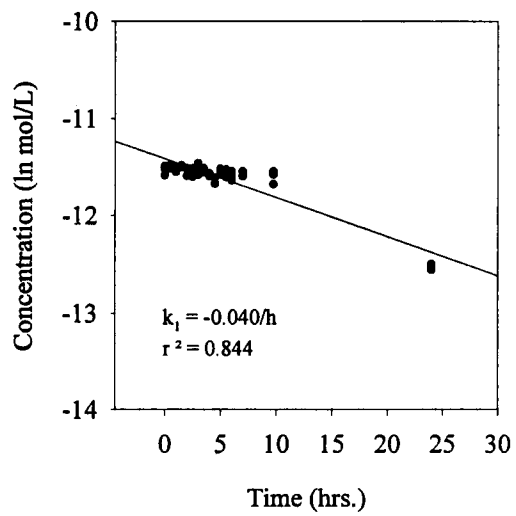
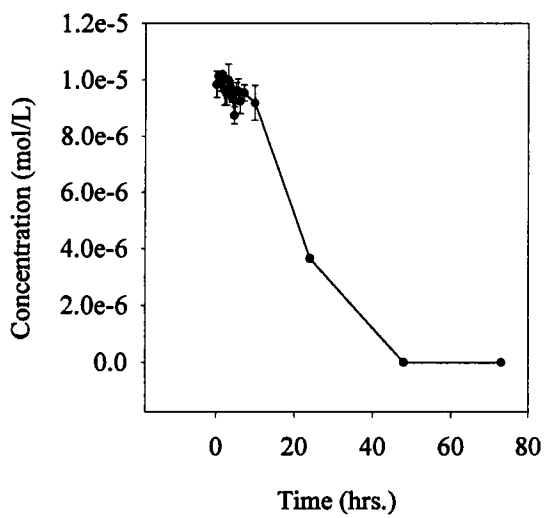


2-Methylnaphthalene

Figure 28. Biodegradation of a PAH and Heterocycle Mixture by *P. fluorescens* 5RL Pregrown in YEPSS Medium.

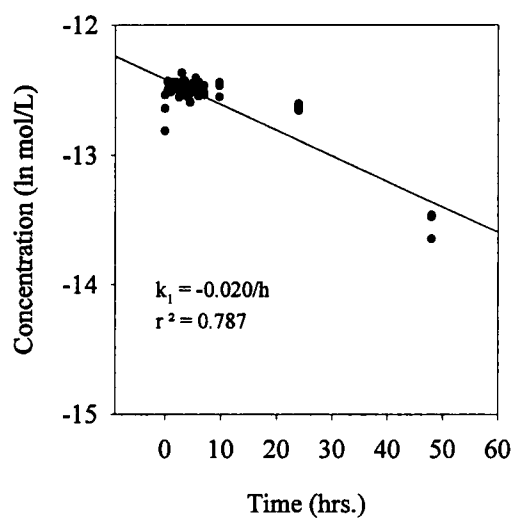
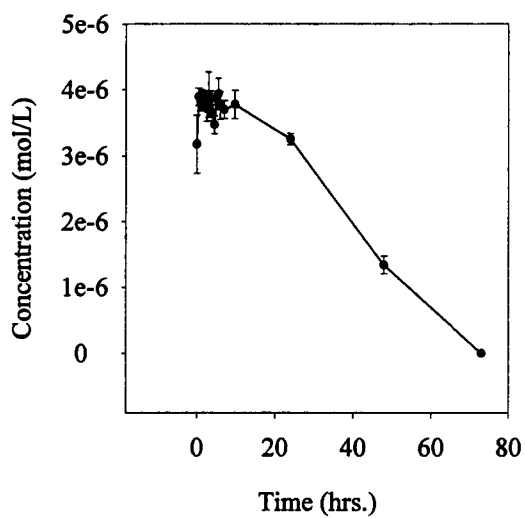


1-Methylnaphthalene

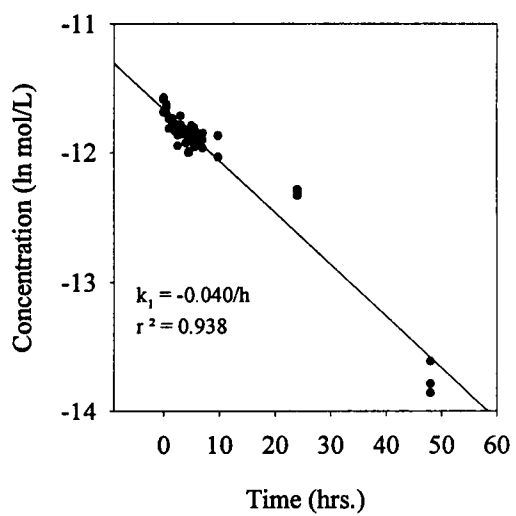
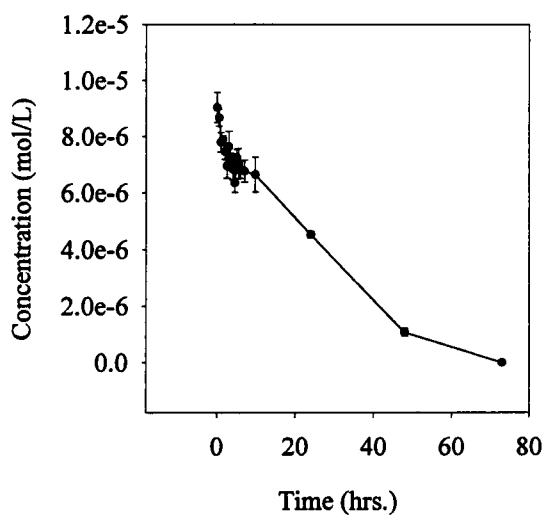


Biphenyl

Figure 28 cont.

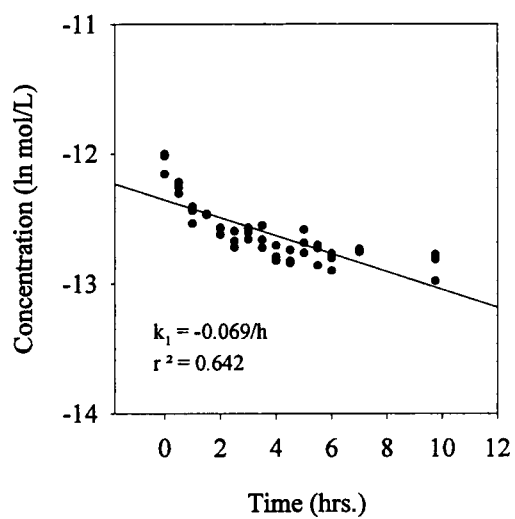
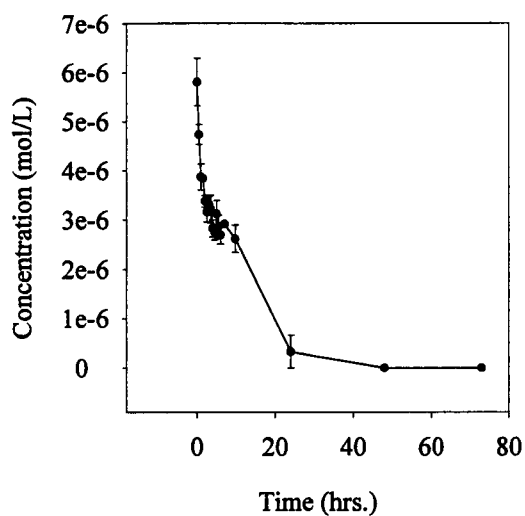


2,6-Dimethylnaphthalene

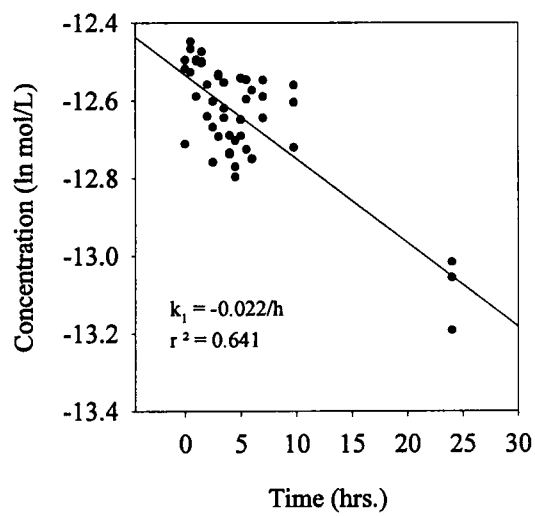
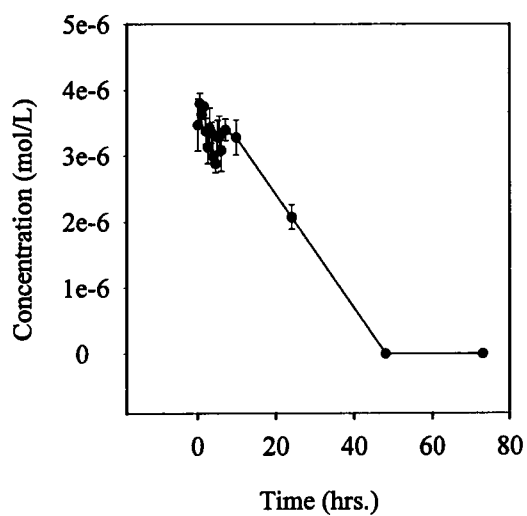


Dibenzofuran

Figure 28 cont.

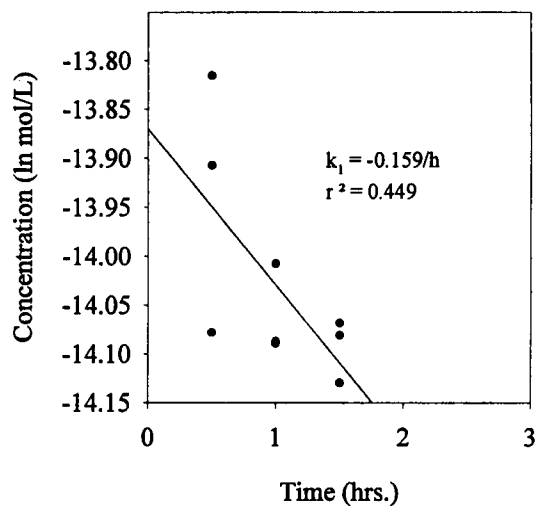
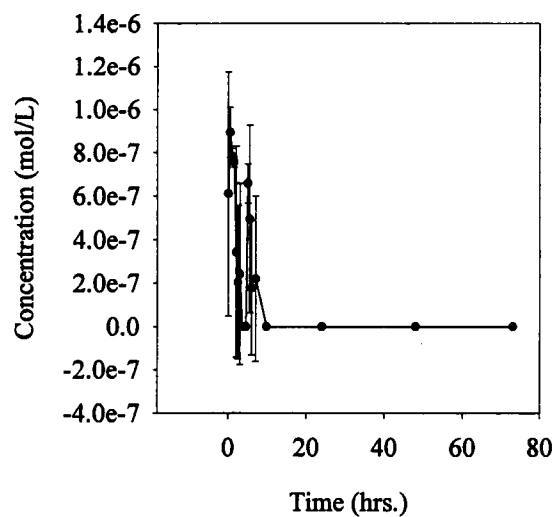


Acenaphthene

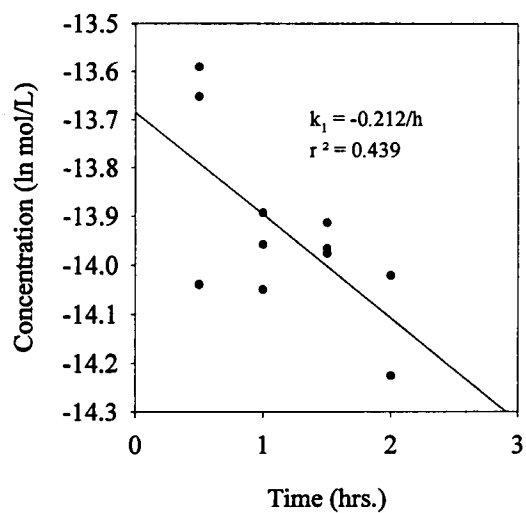
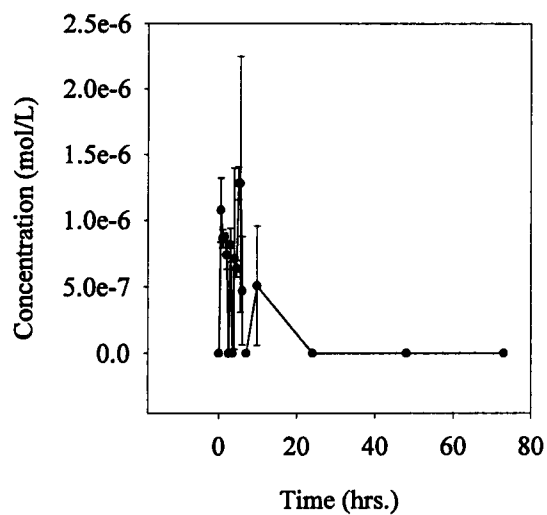


Fluorene

Figure 28 cont.

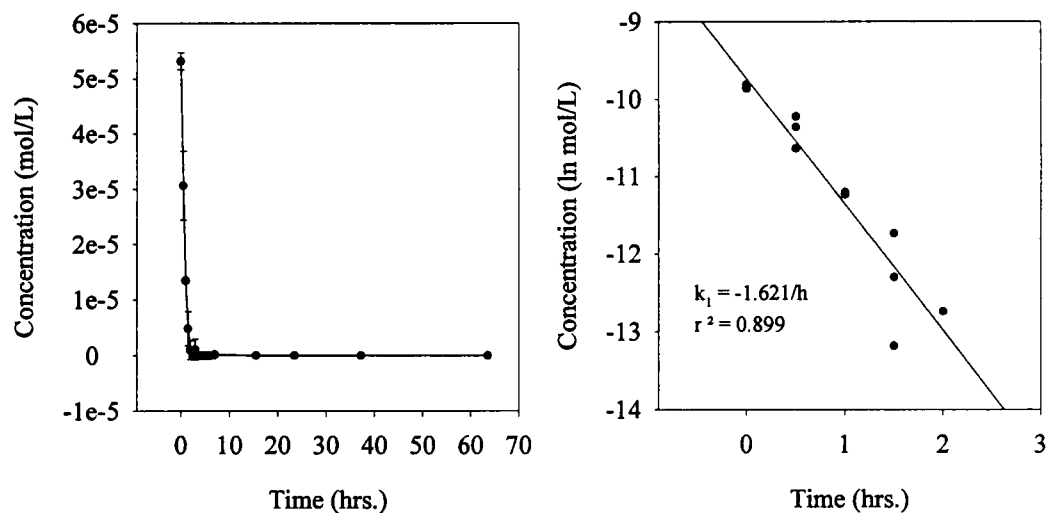


Dibenzothiophene

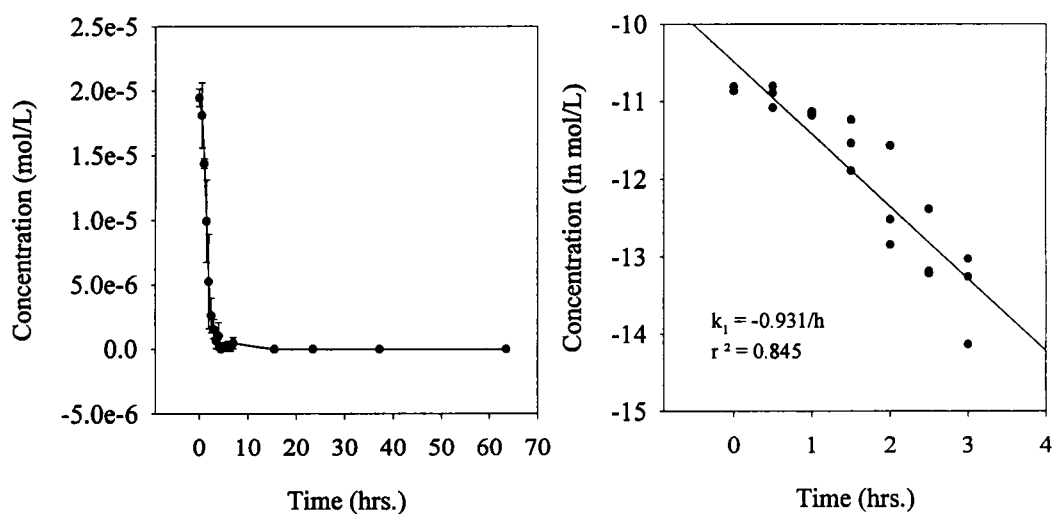


Phenanthrene

Figure 28 cont.

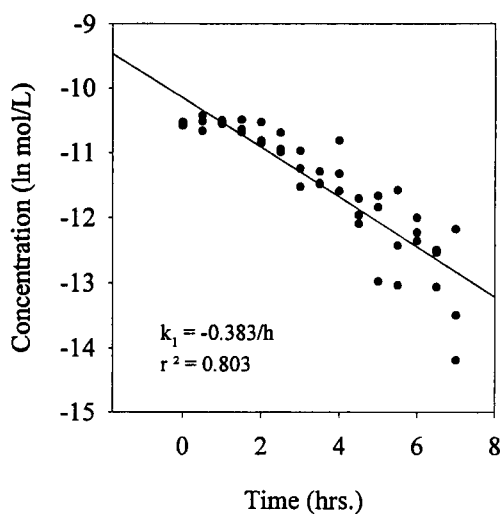
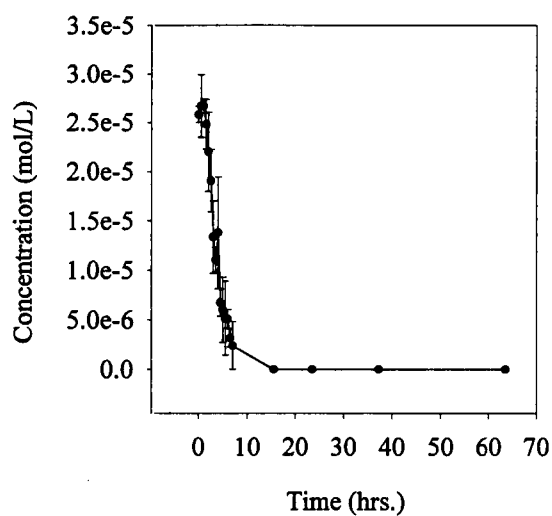


Naphthalene

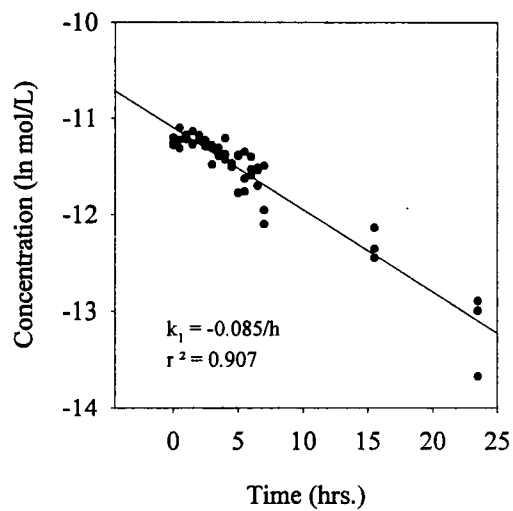
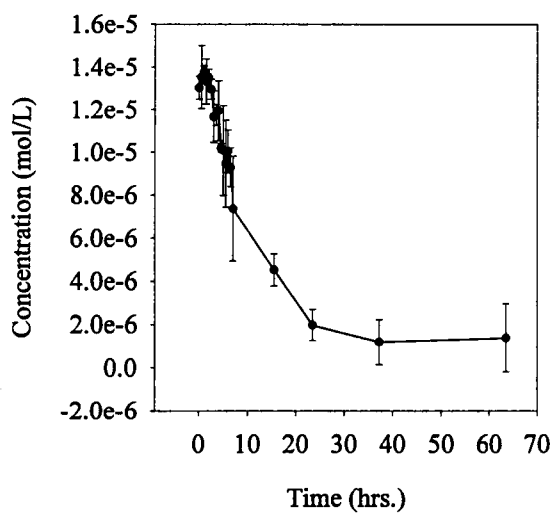


2-Methylnaphthalene

Figure 29. Biodegradation of a PAH and Heterocycle Mixture by *E. coli* DH5 α Harboring pUTK202.

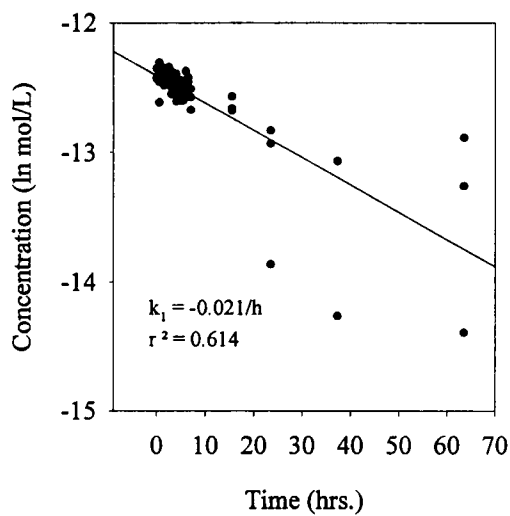
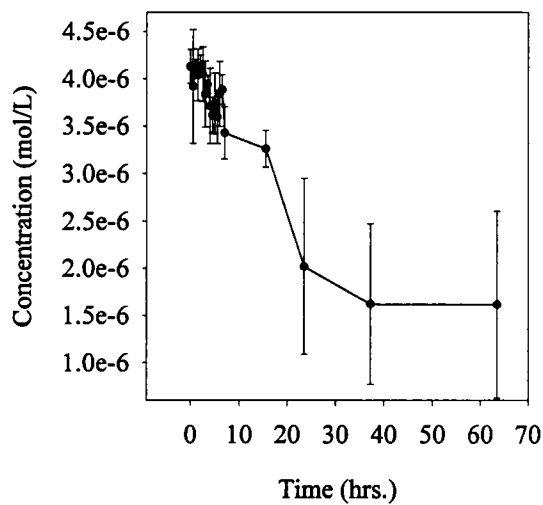


1-Methylnaphthalene

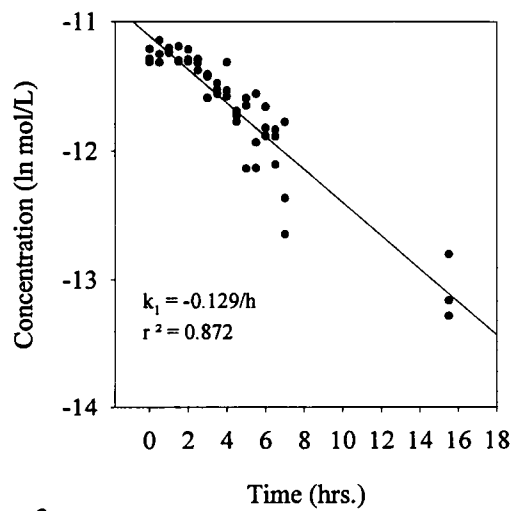
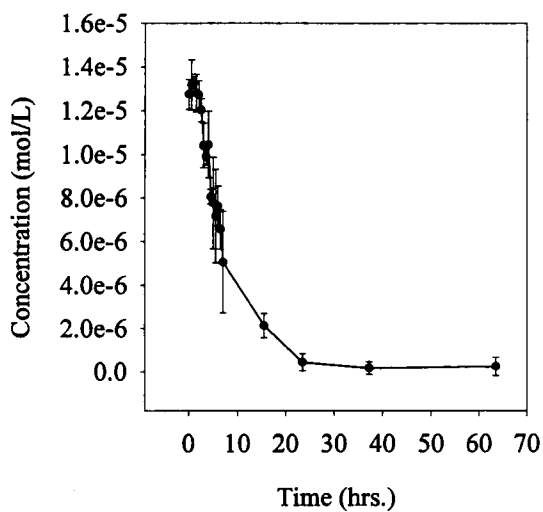


Biphenyl

Figure 29 cont.

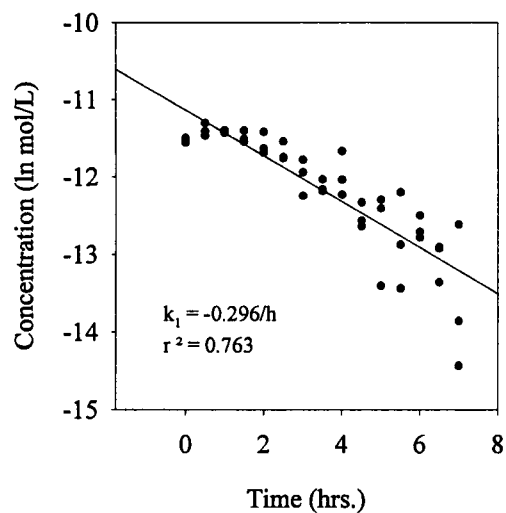
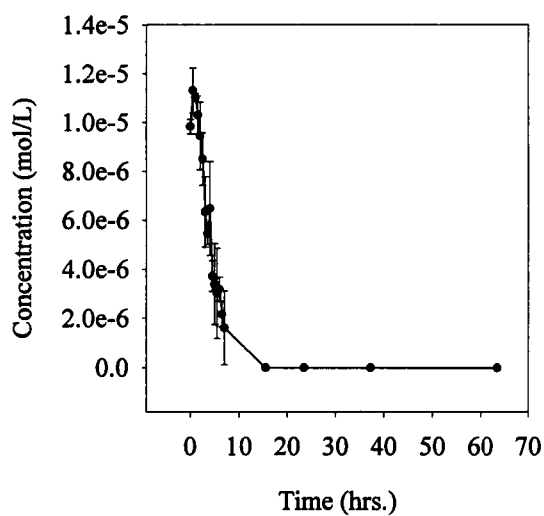


2,6-Dimethylnaphthalene

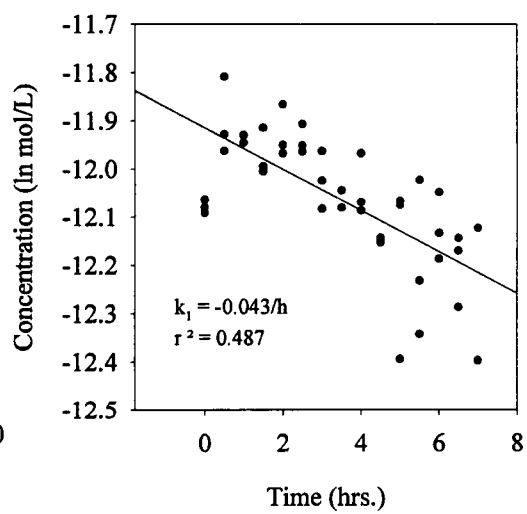
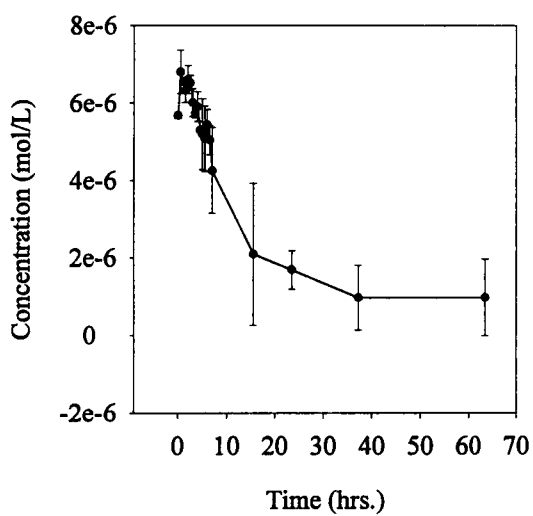


Dibenzofuran

Figure 29 cont.



Acenaphthene



Fluorene

Figure 29 cont.

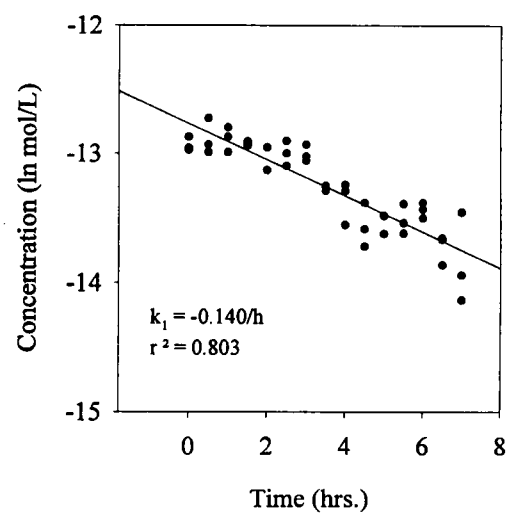
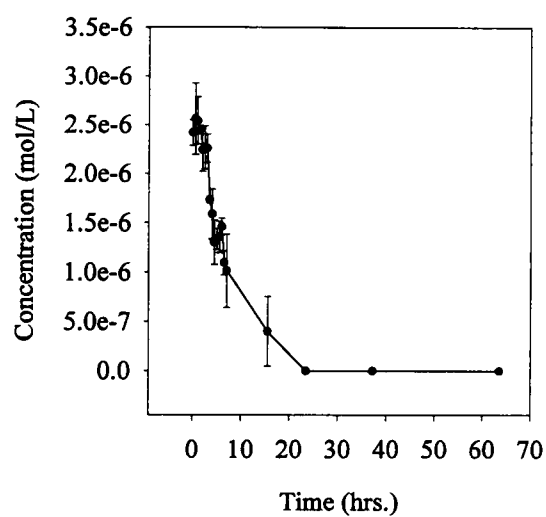
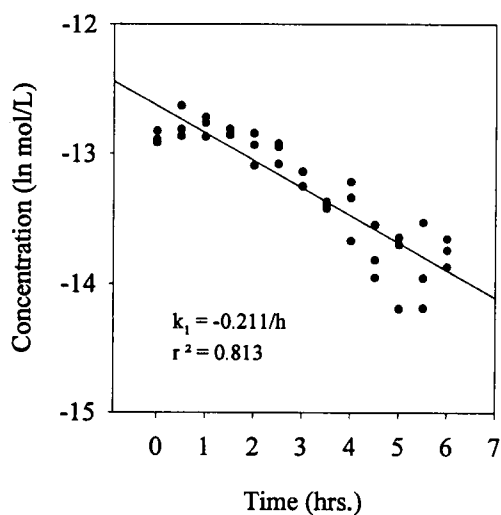
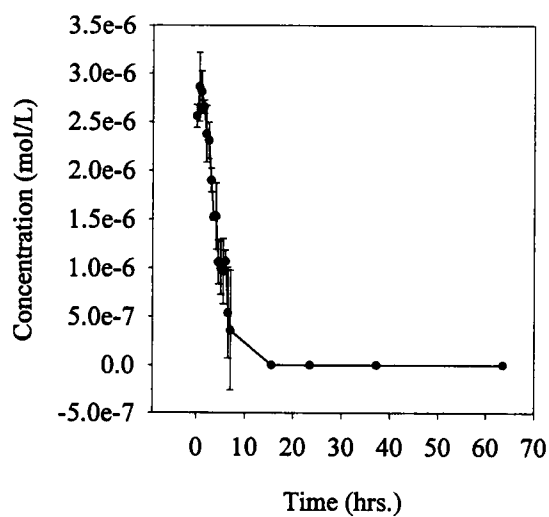


Figure 29 cont.

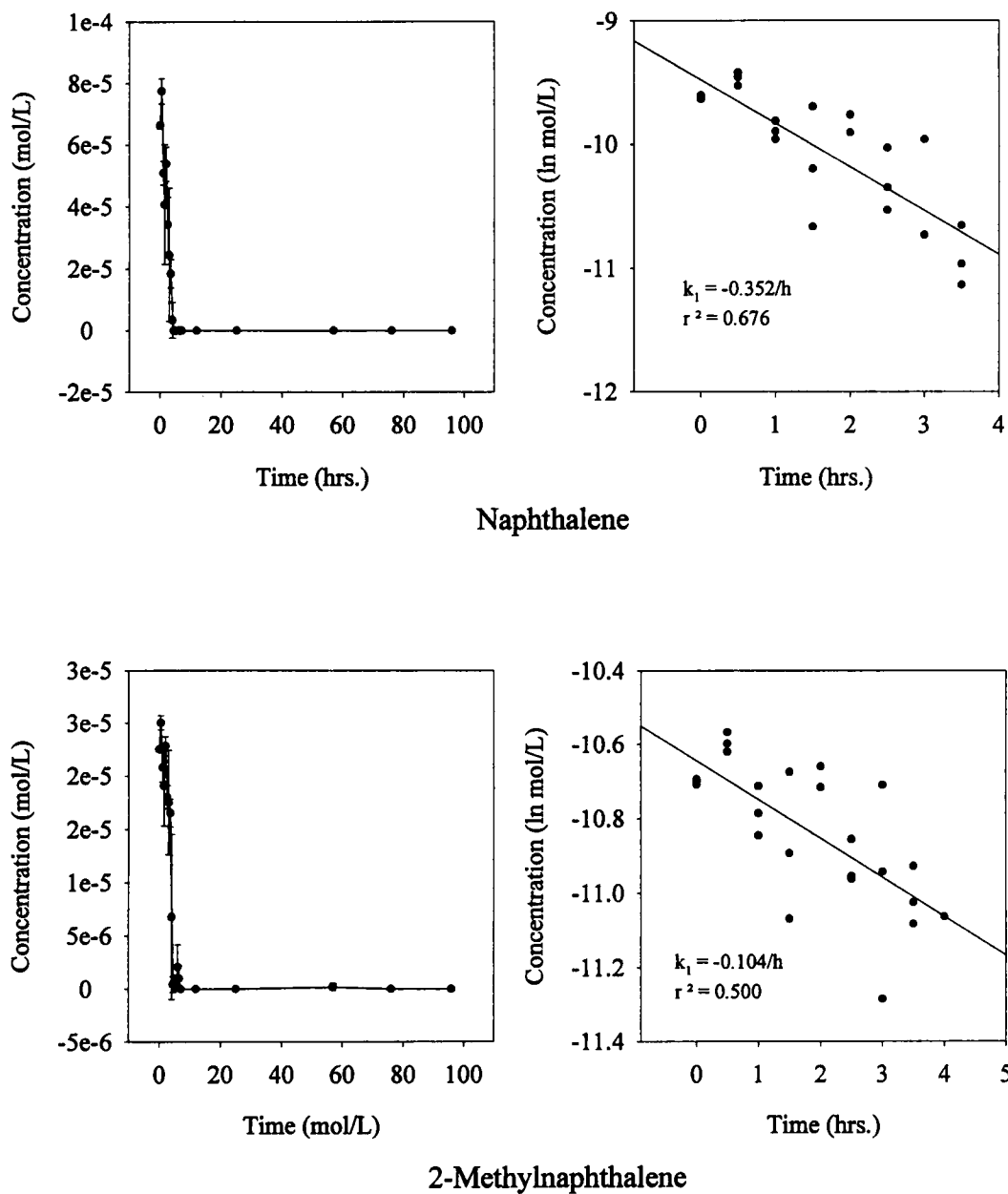
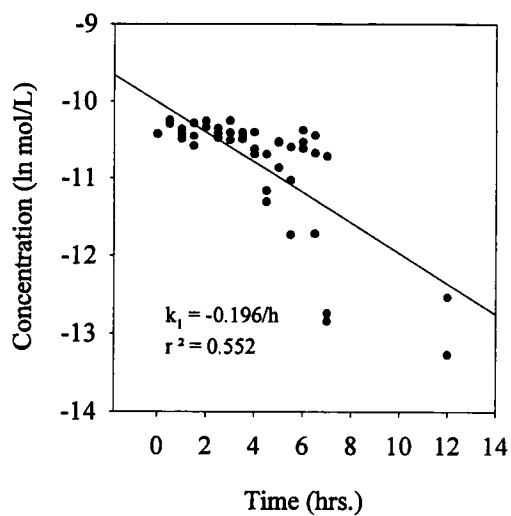
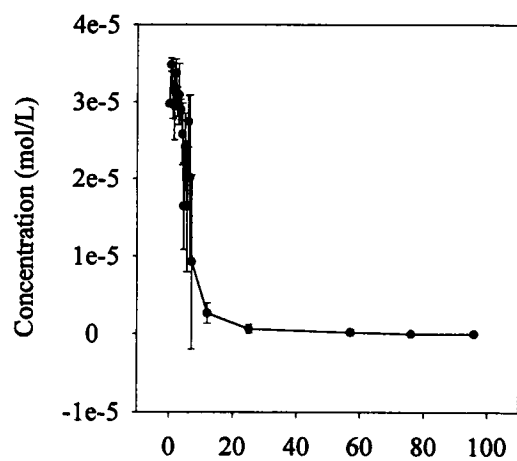
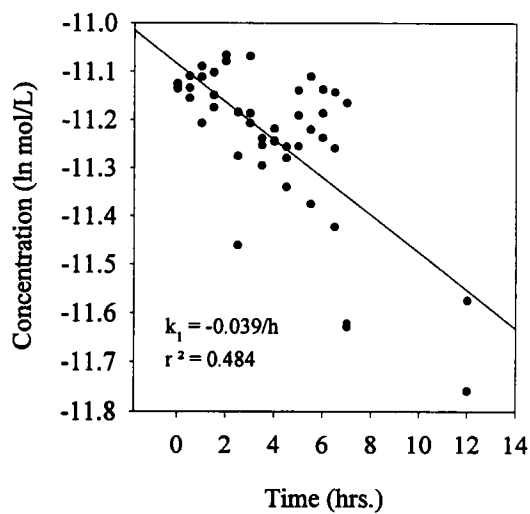
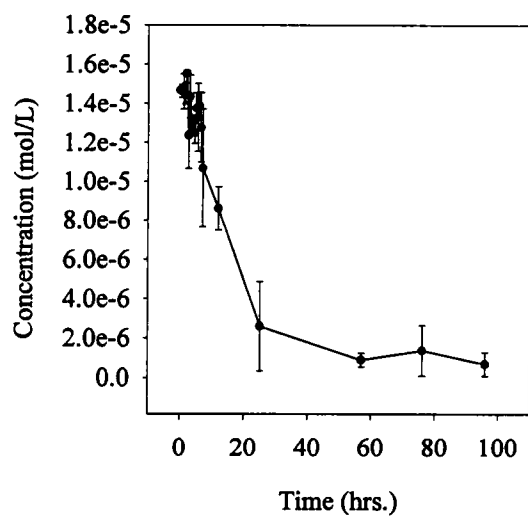


Figure 30. Biodegradation of a PAH and Heterocycle Mixture by *P. fluorescens* 5R Pregrown on YEPG Medium.

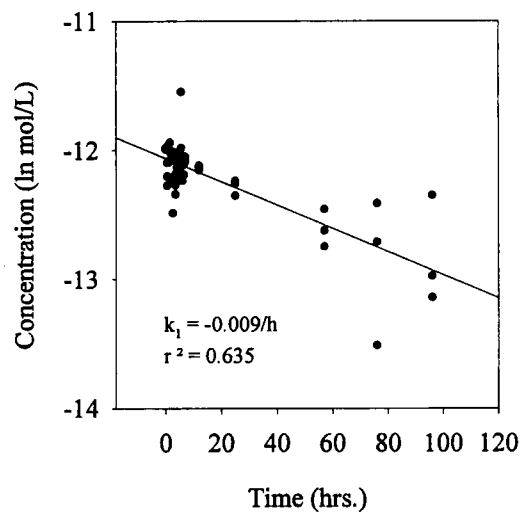
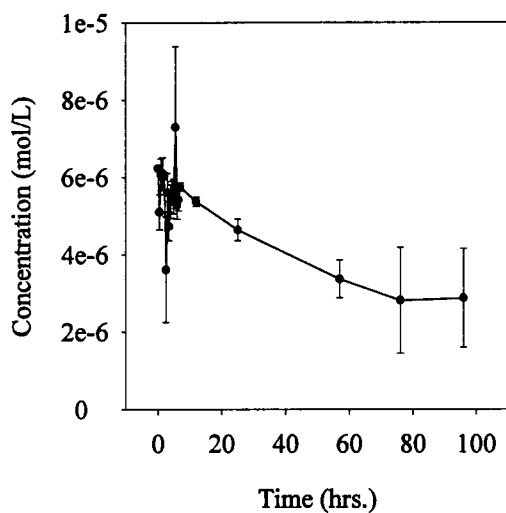


1-Methylnaphthalene

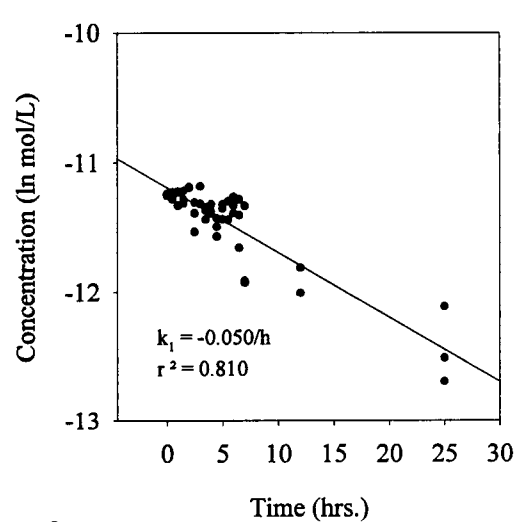
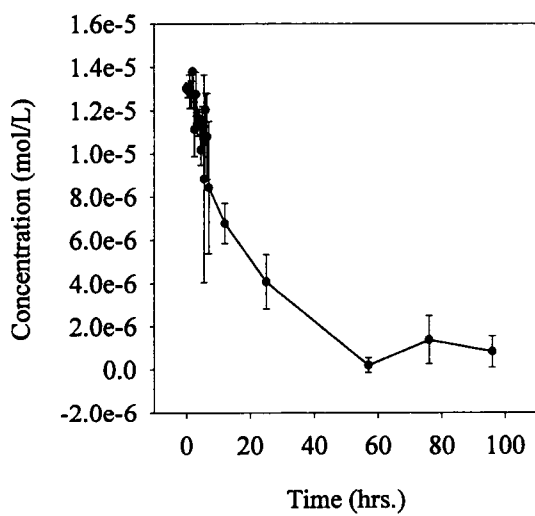


Biphenyl

Figure 30 cont.

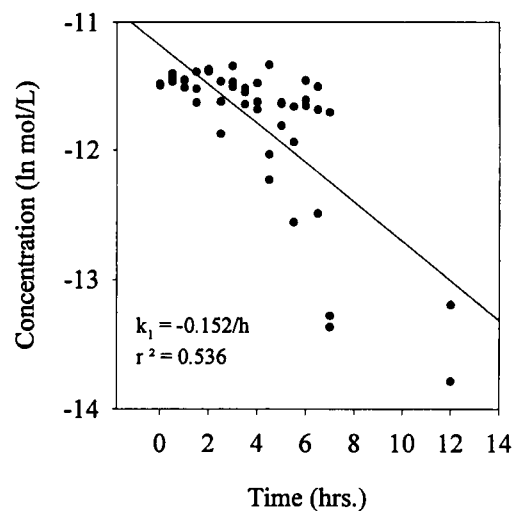
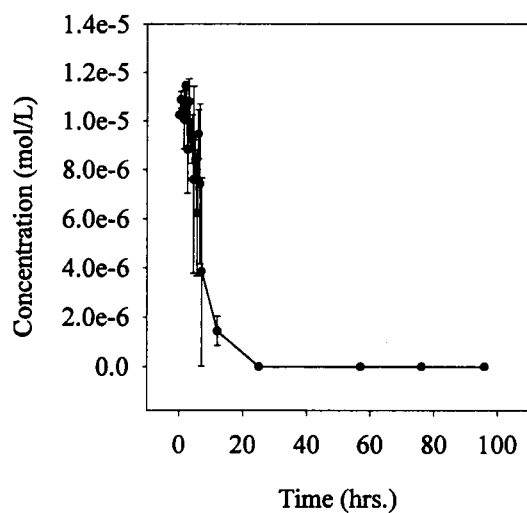


2,6-Dimethylnaphthalene

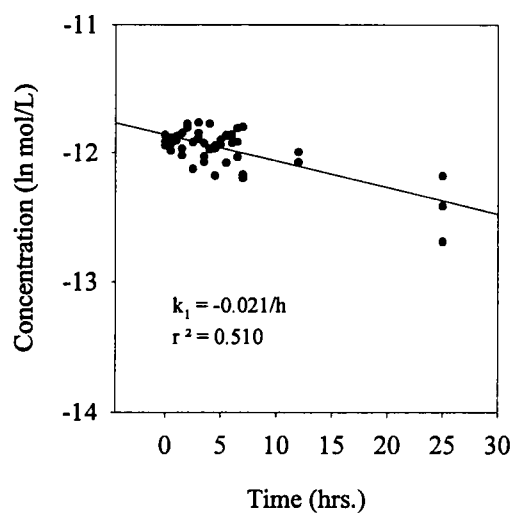
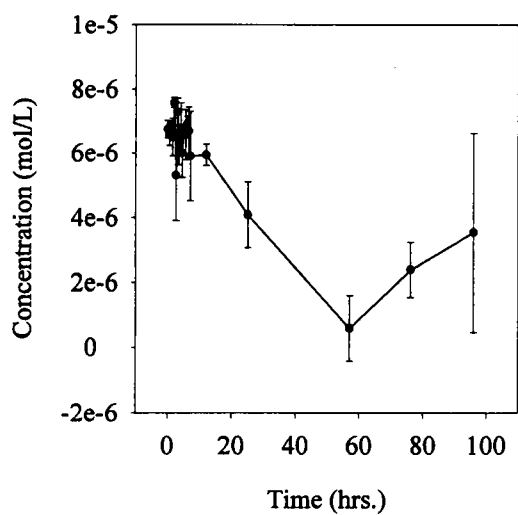


Dibenzofuran

Figure 30 cont.

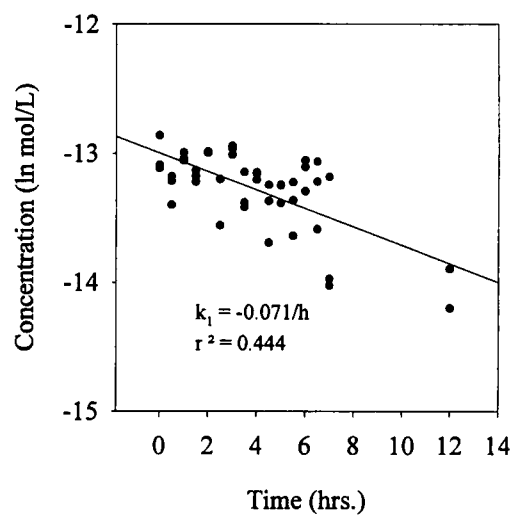
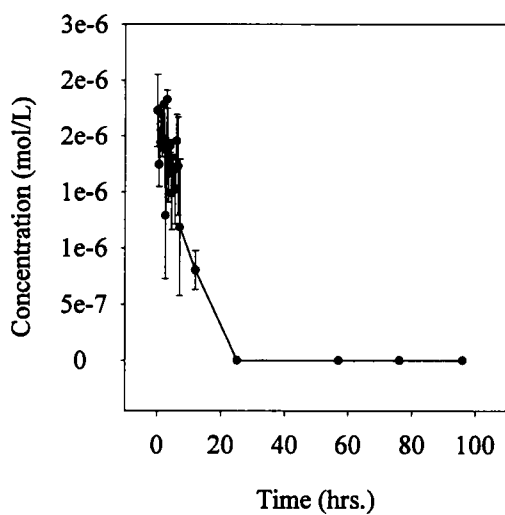


Acenaphthene

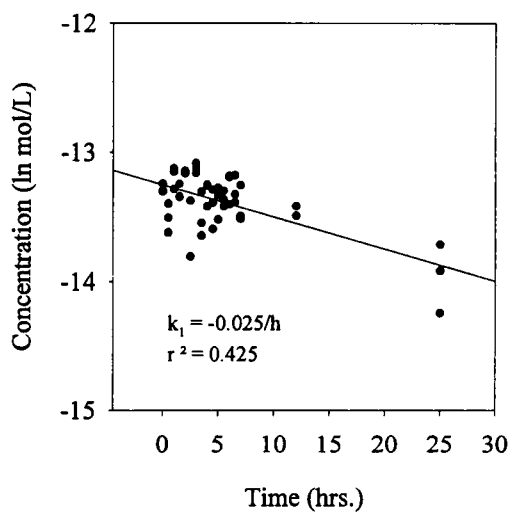
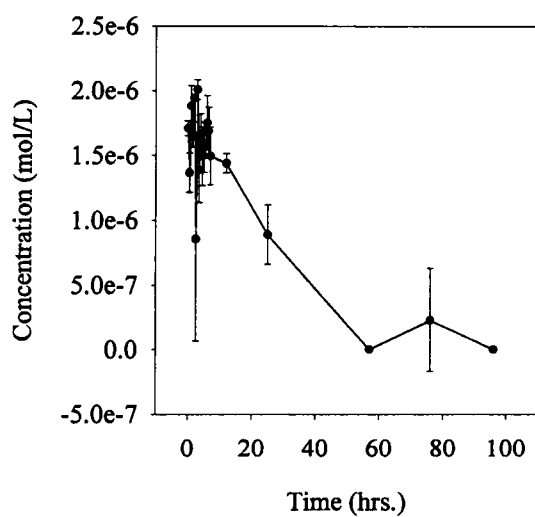


Fluorene

Figure 30 cont.

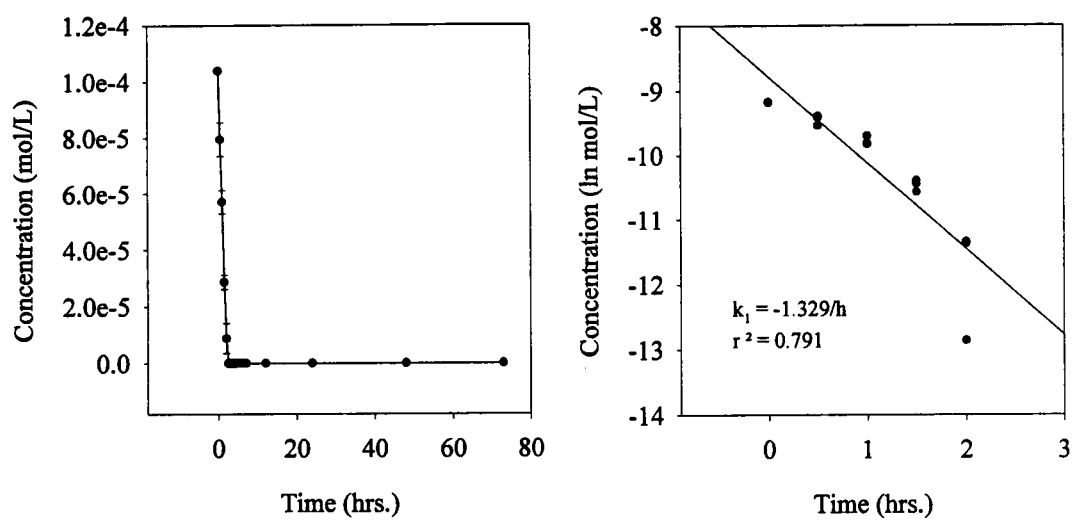


Dibenzothiophene

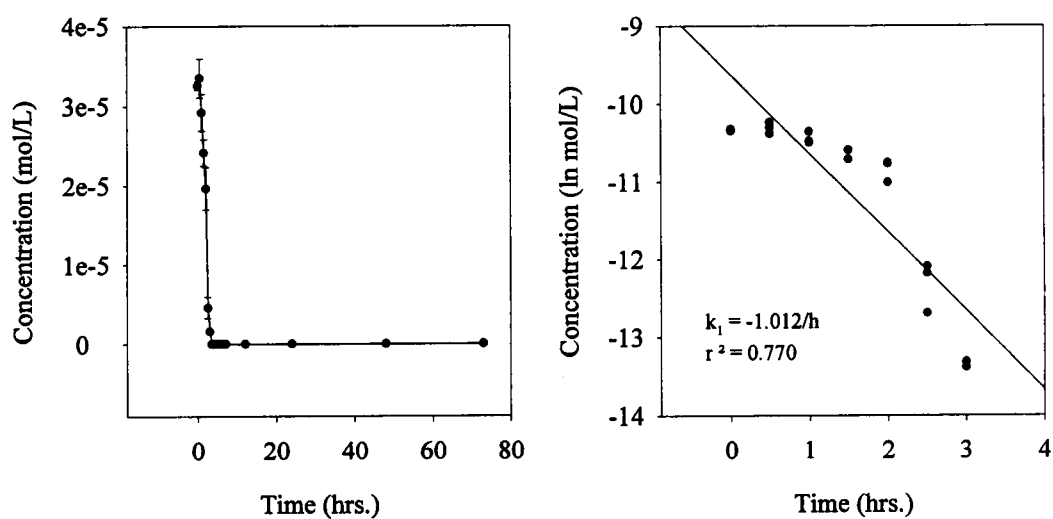


Phenanthrene

Figure 30 cont.

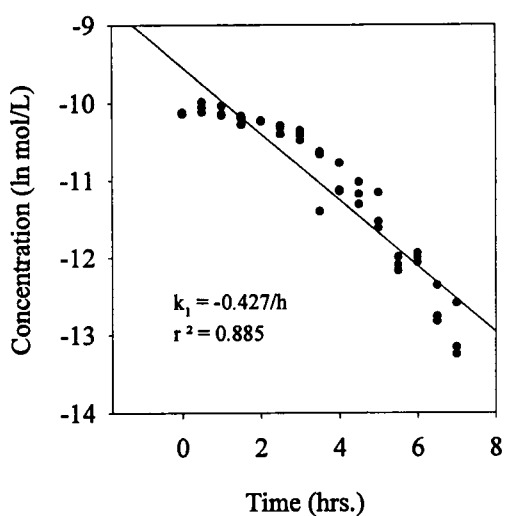
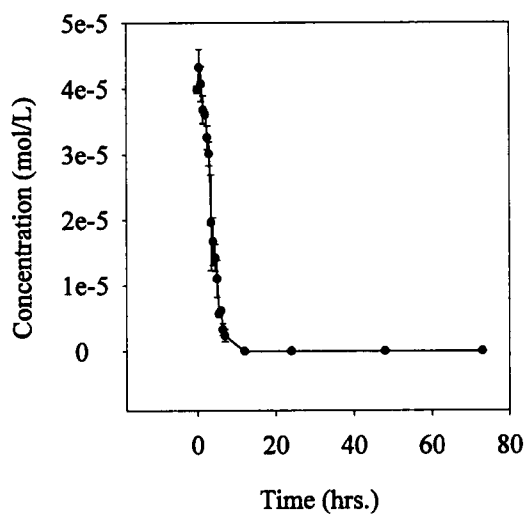


Naphthalene

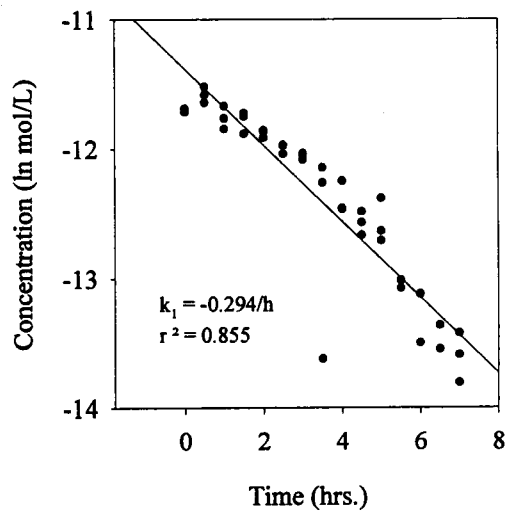
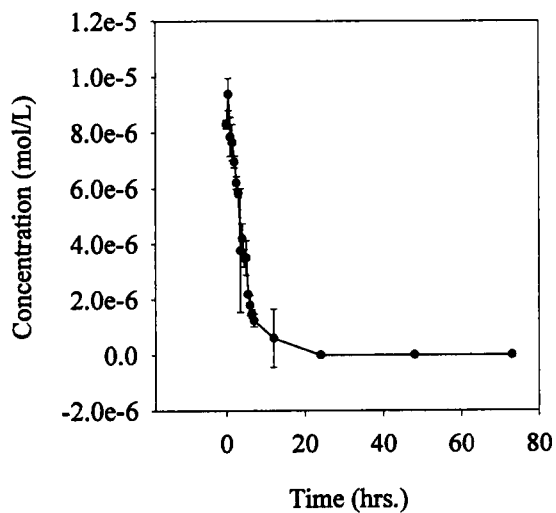


2-Methylnaphthalene

Figure 31. Biodegradation of a Mixture of Methyl-Substituted Naphthalenes by *P. fluorescens* 5R.

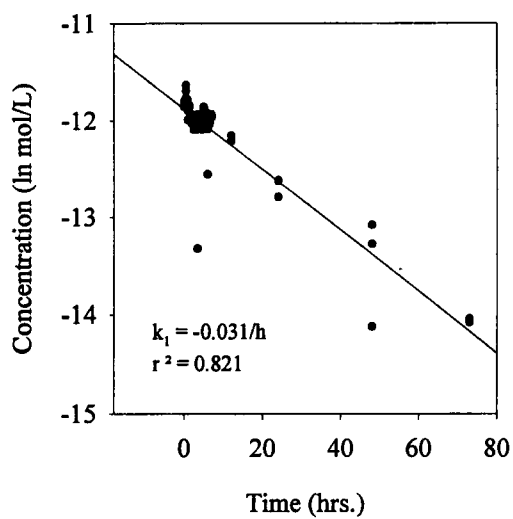
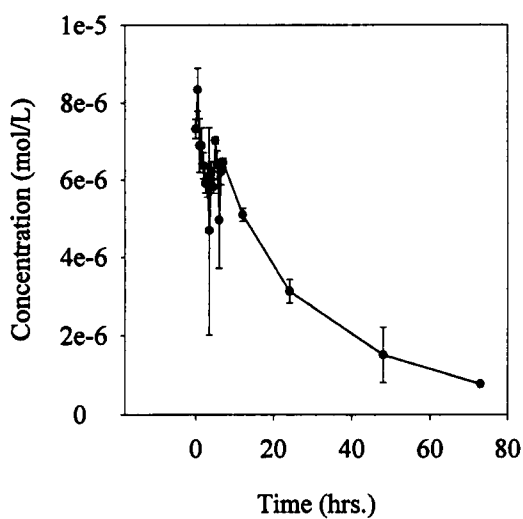


1-Methylnaphthalene

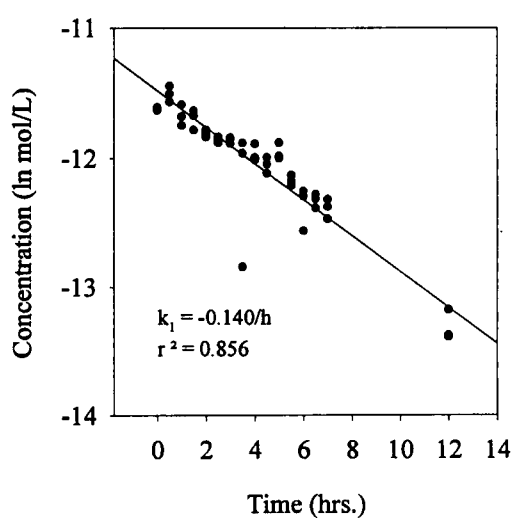
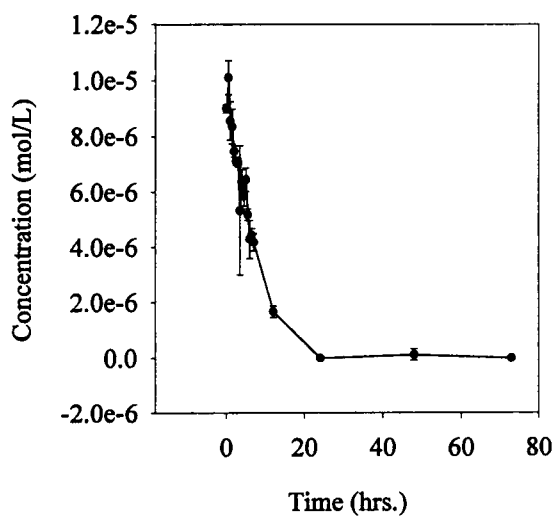


1,2-Dimethylnaphthalene

Figure 31 cont.

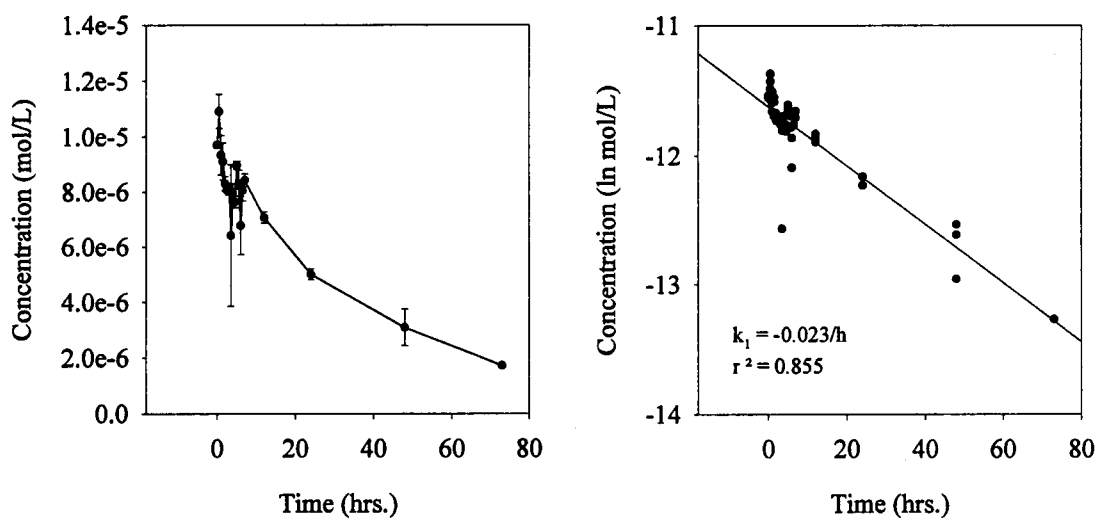


2,3-Dimethylnaphthalene



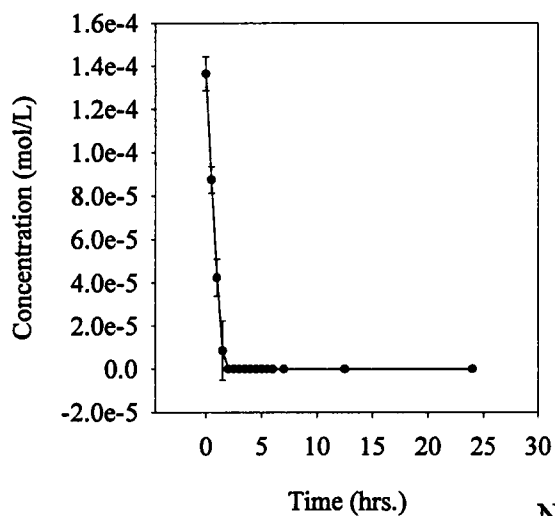
2,6-Dimethylnaphthalene

Figure 31 cont.

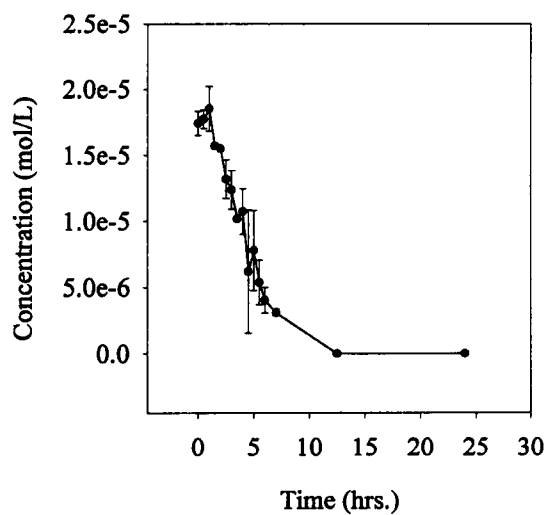
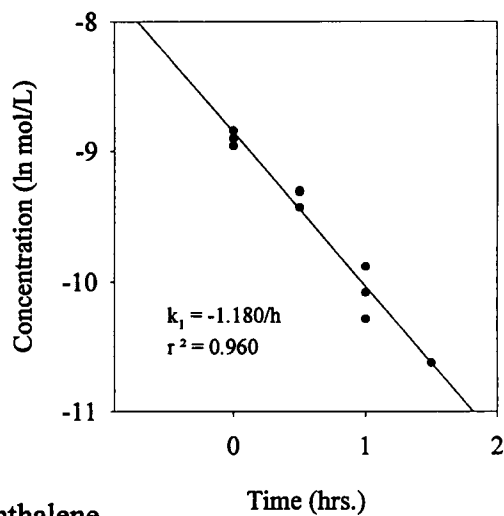


1,8-Dimethylnaphthalene

Figure 31 cont.



Naphthalene



Biphenyl

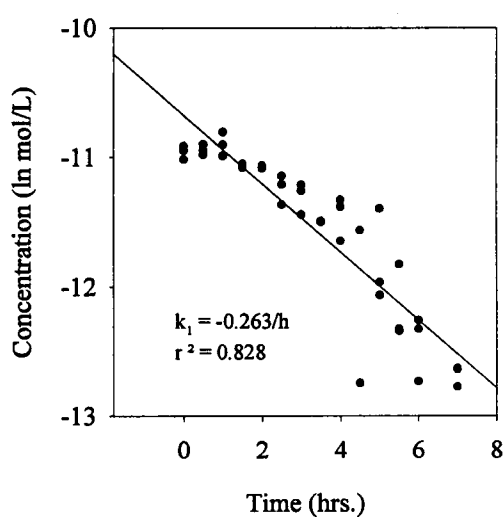
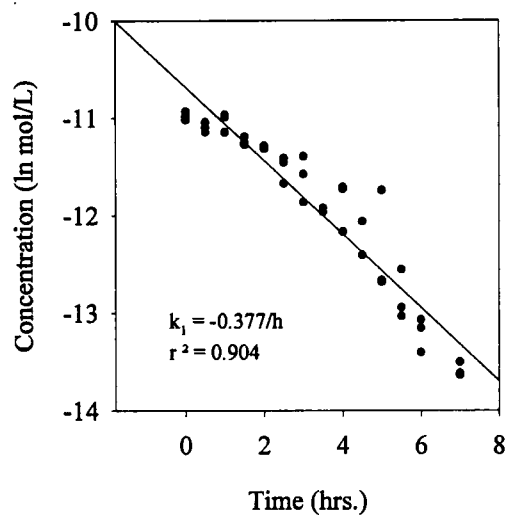
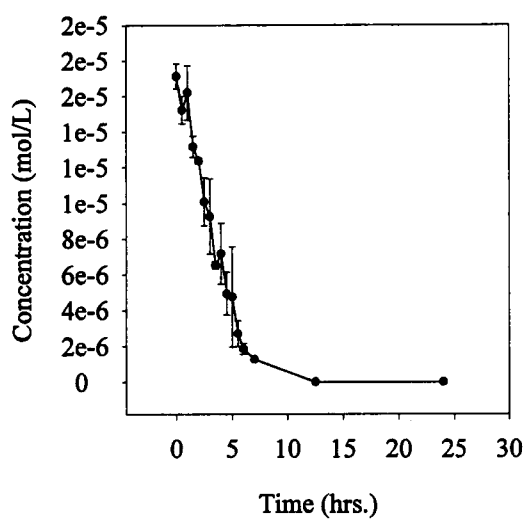
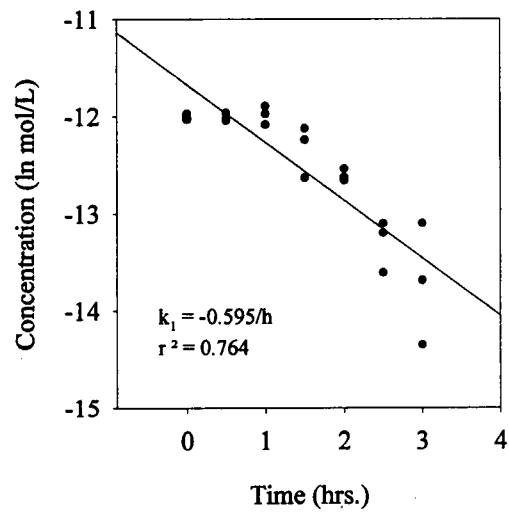
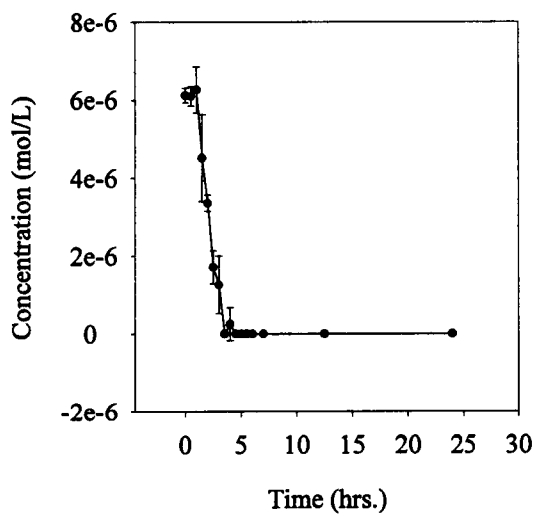


Figure 32. Biodegradation of a Mixture of PAHs and Heterocycles by *P. fluorescens* 5R - Variations in Ring Structure.



Dibenzofuran



Acenaphthene

Figure 32 cont.

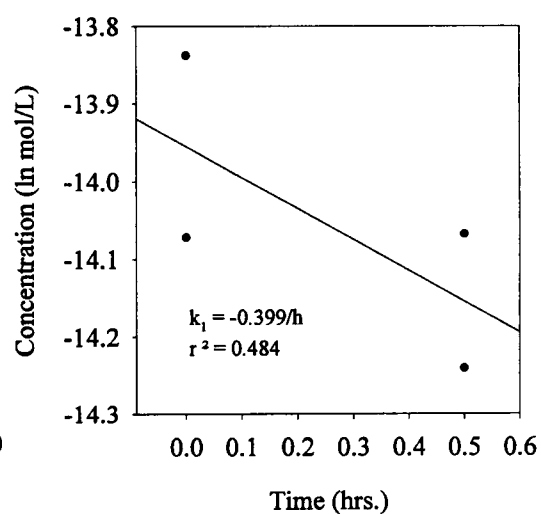
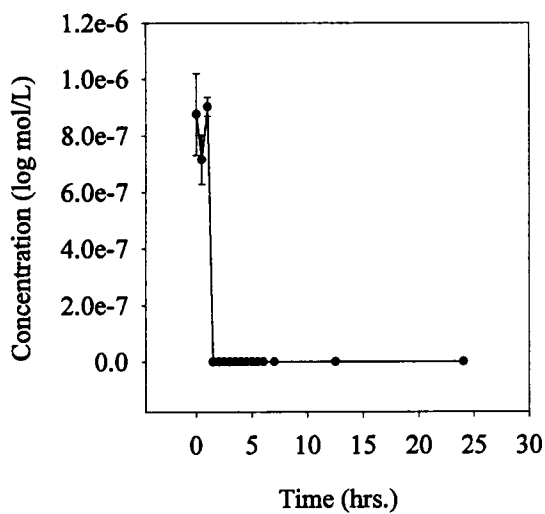
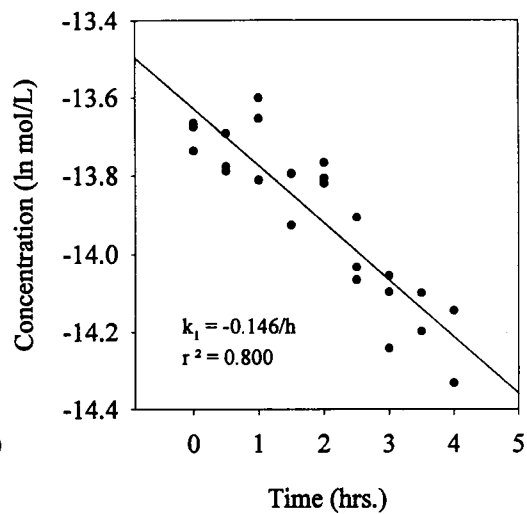
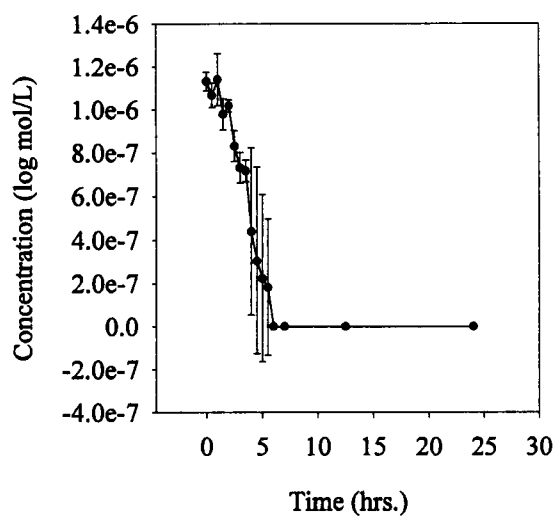
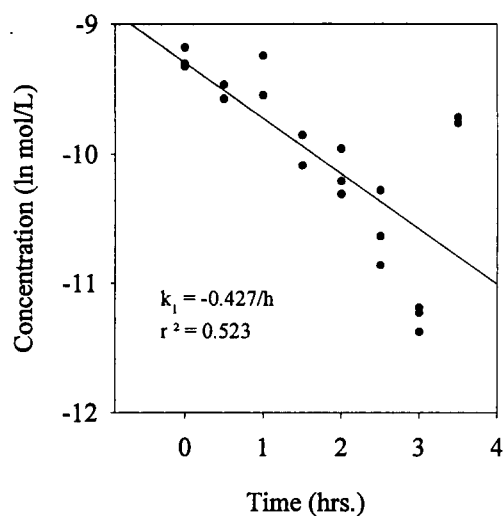
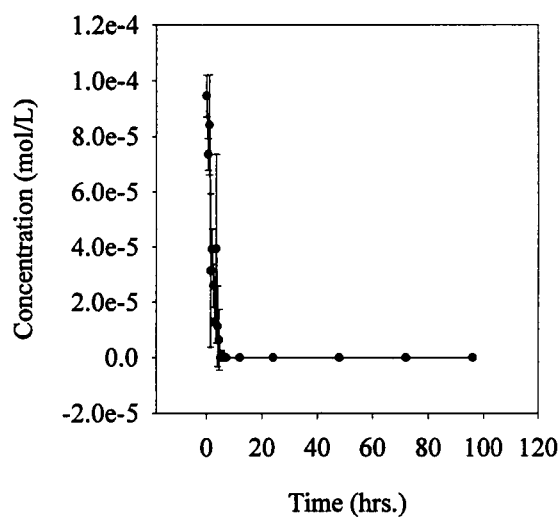
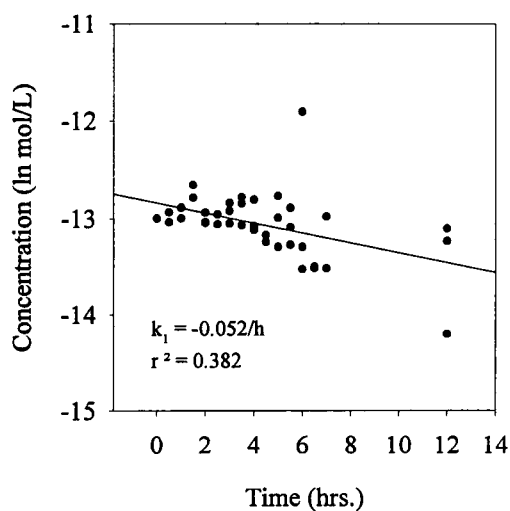
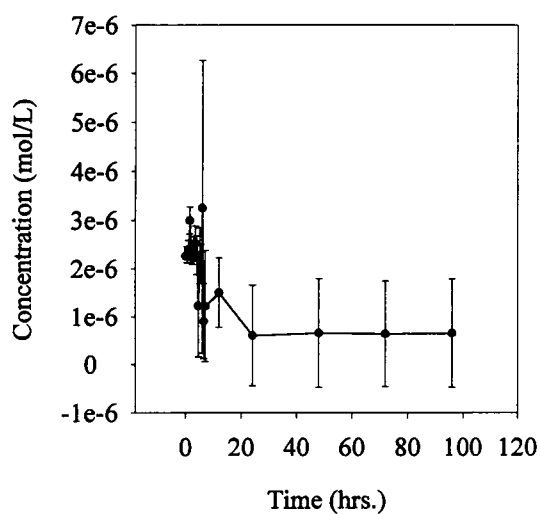


Figure 32 cont.

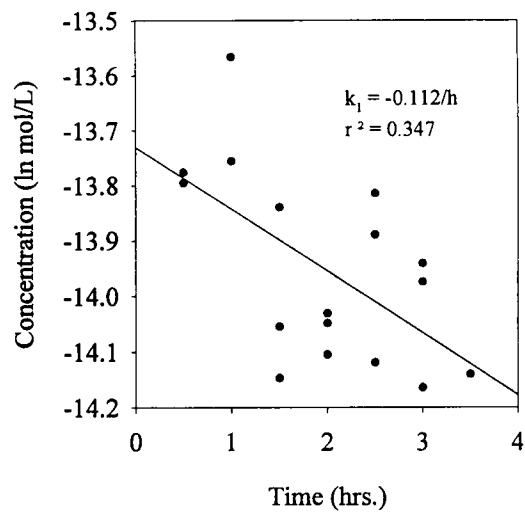
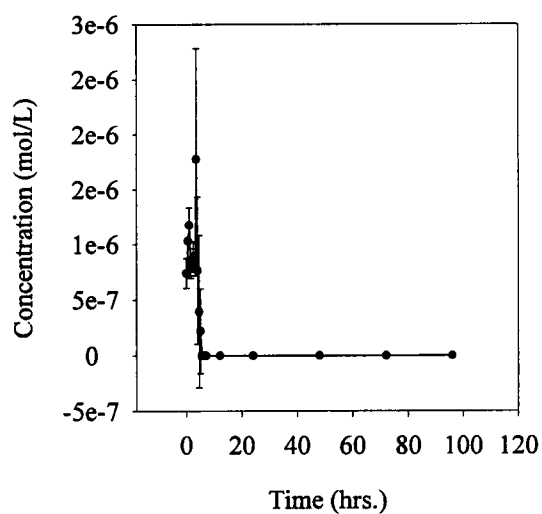


Naphthalene

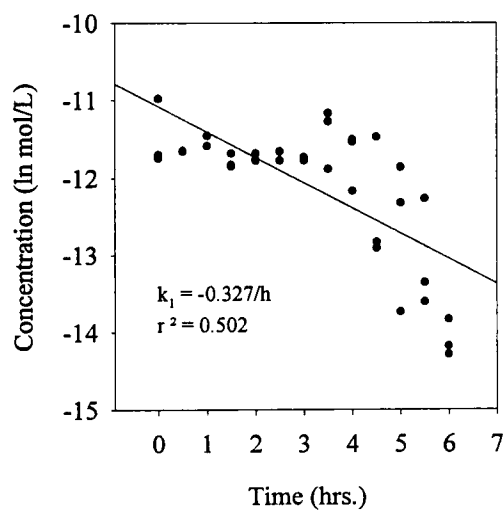
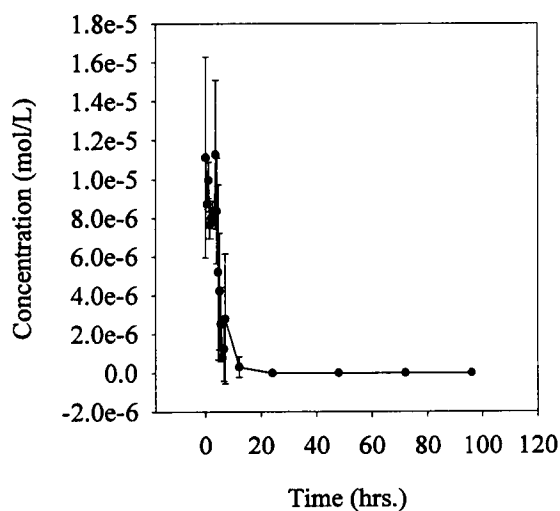


Dibenzofuran

Figure 33. Biodegradation of Naphthalene and a Mixture of Heterocycles by *P. fluorescens* 5R.

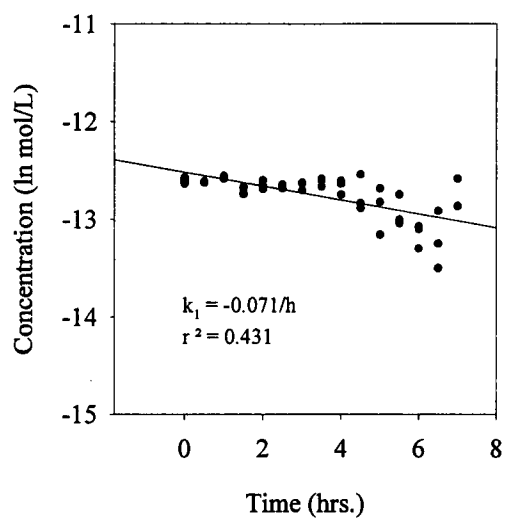
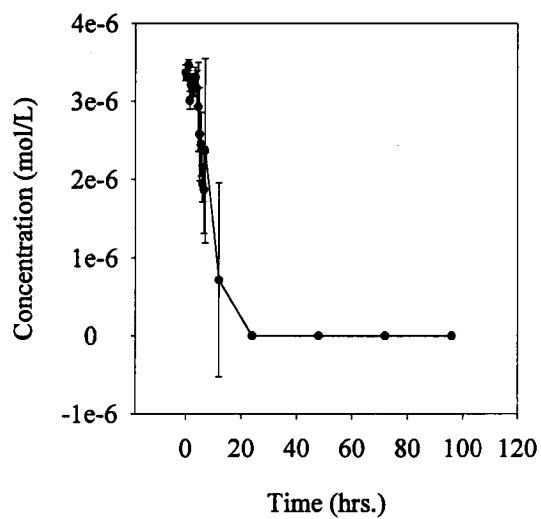


Fluorene



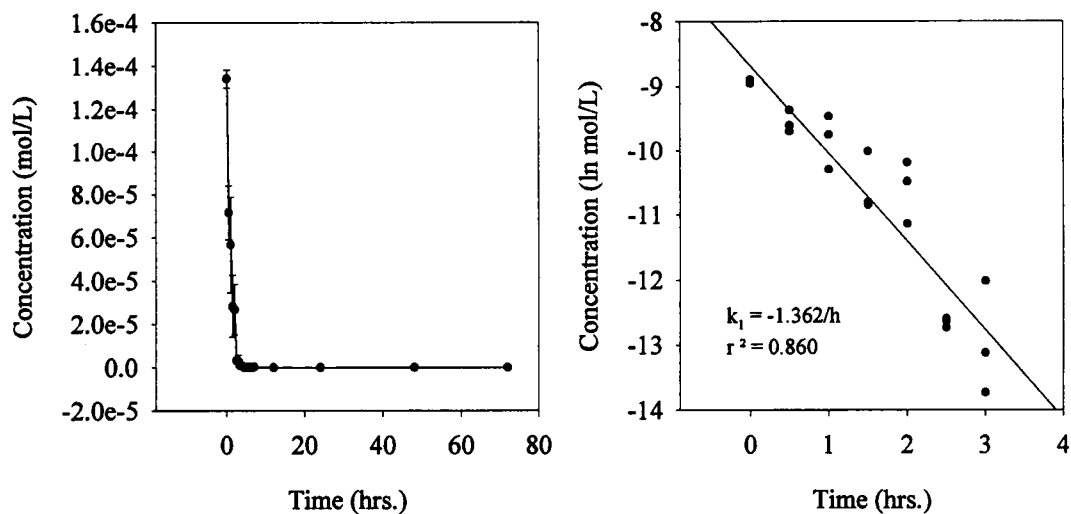
Dibenzothiophene

Figure 33 cont.

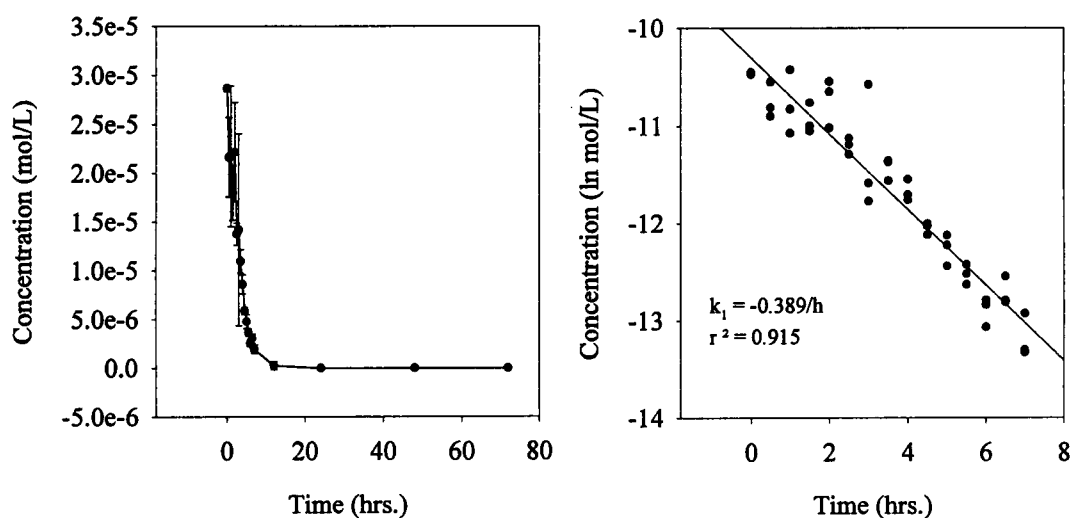


Carbazole

Figure 33 cont.

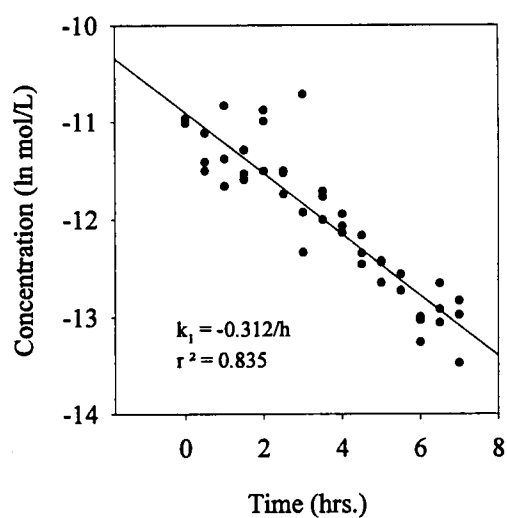
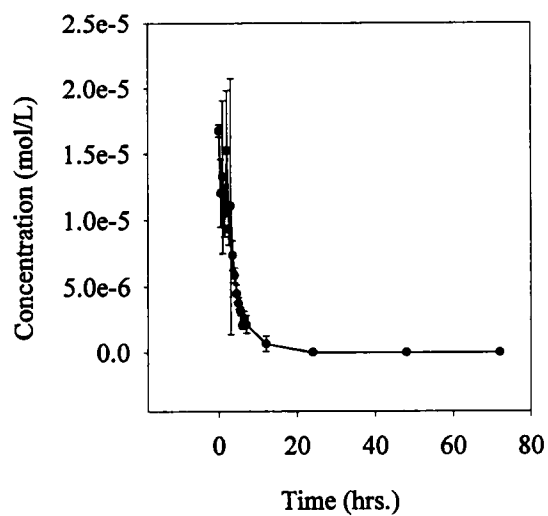


Naphthalene

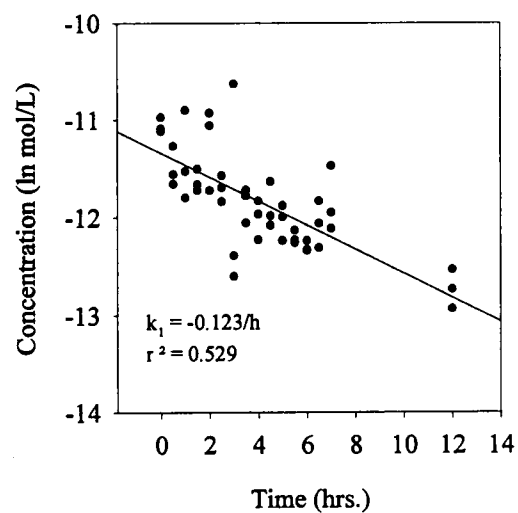
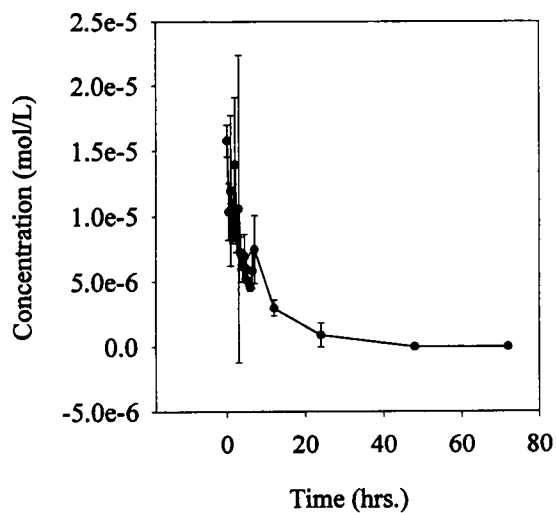


2-Methylnaphthalene

Figure 34. Biodegradation of a Mixture of Various Substituted Naphthalenes by *P. fluorescens* 5R.

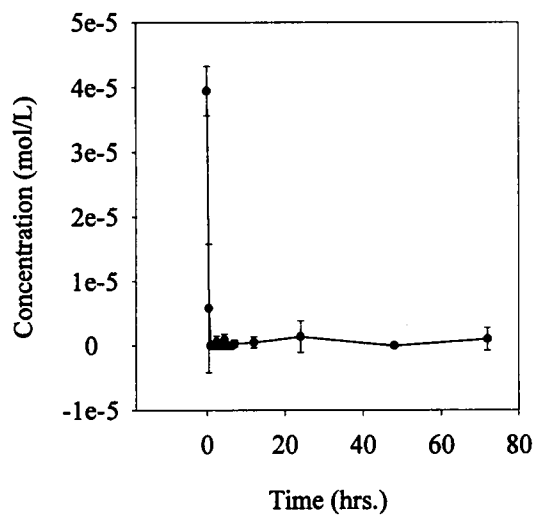


2-Chloronaphthalene

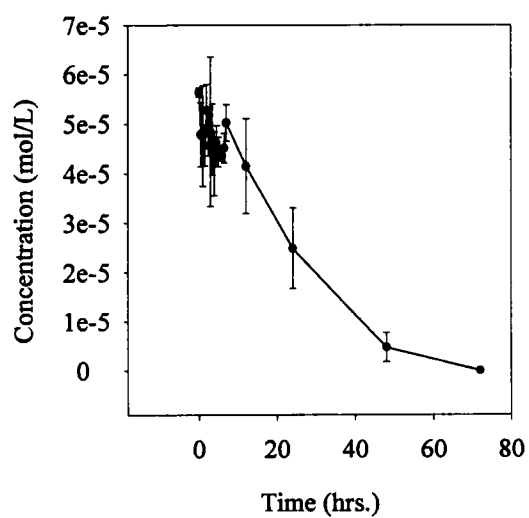


2-Ethyl-naphthalene

Figure 34 cont.



2-Naphthaldehyde



2-Methoxynaphthalene

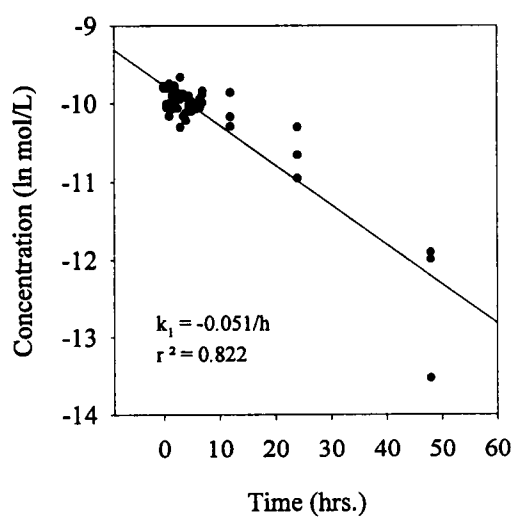
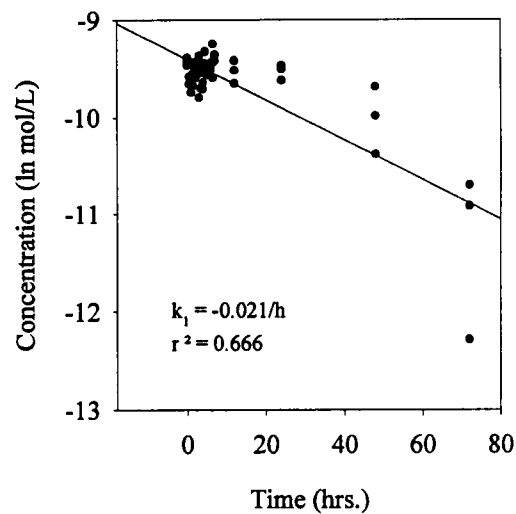
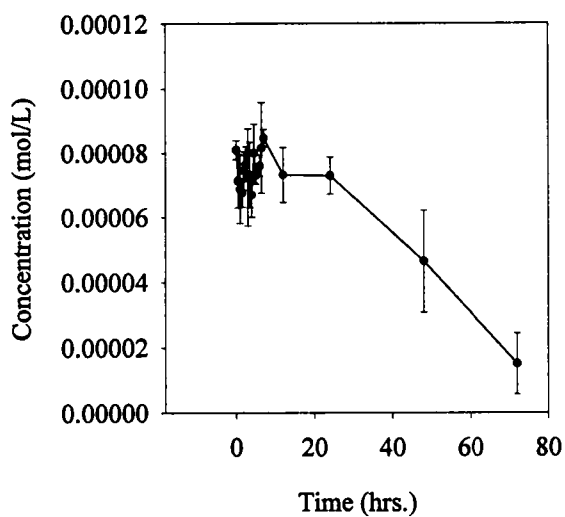
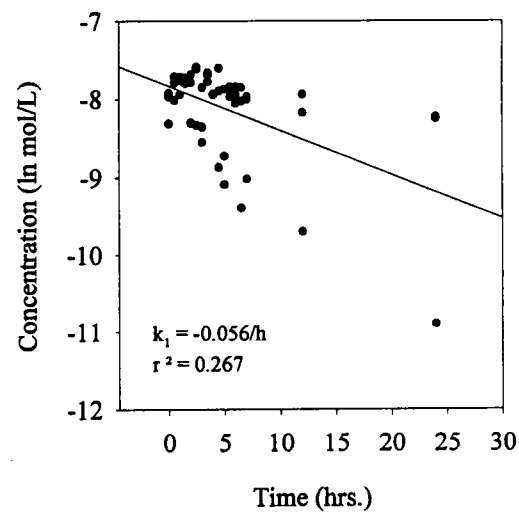
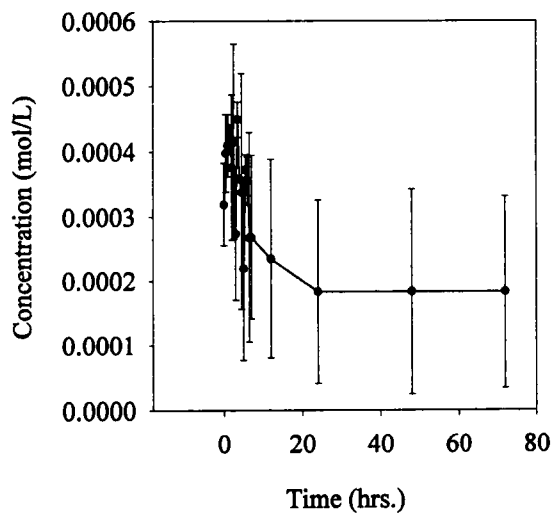


Figure 34 cont.

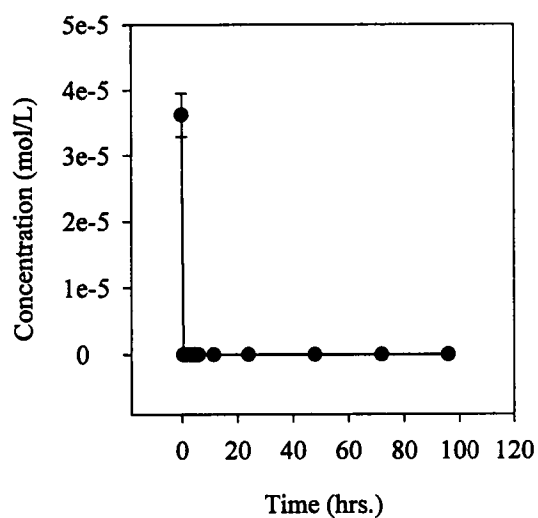


Naphthalene-2-carbonitrile

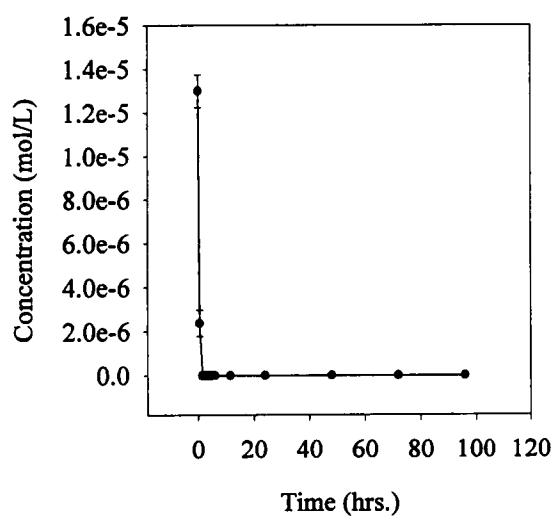


2-Aminonaphthalene

Figure 34 cont.



Naphthalene



2-Methylnaphthalene

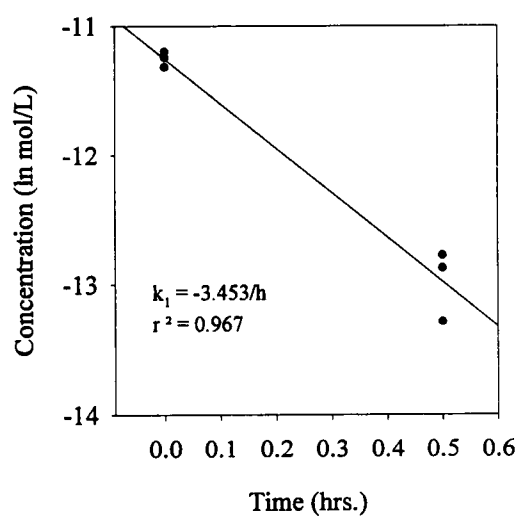
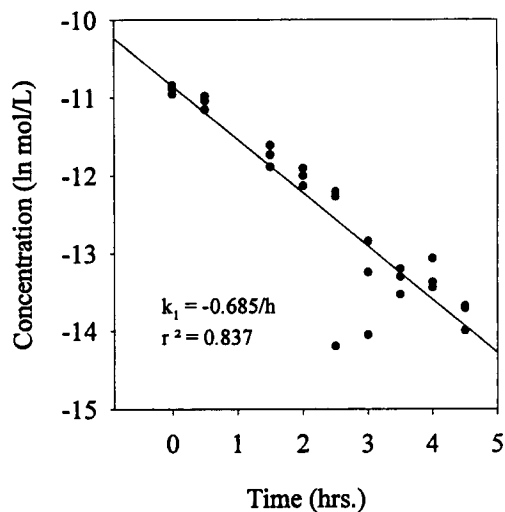
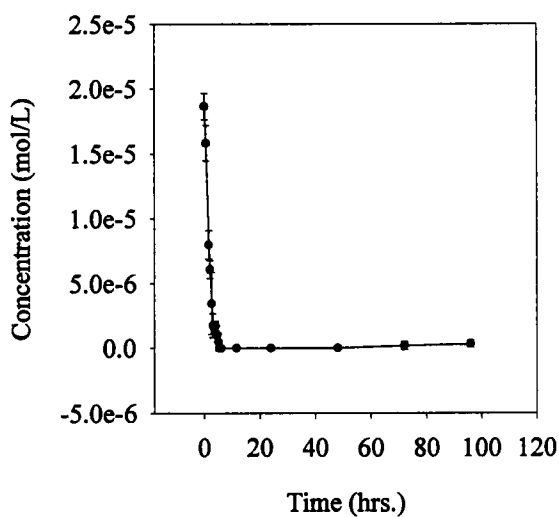
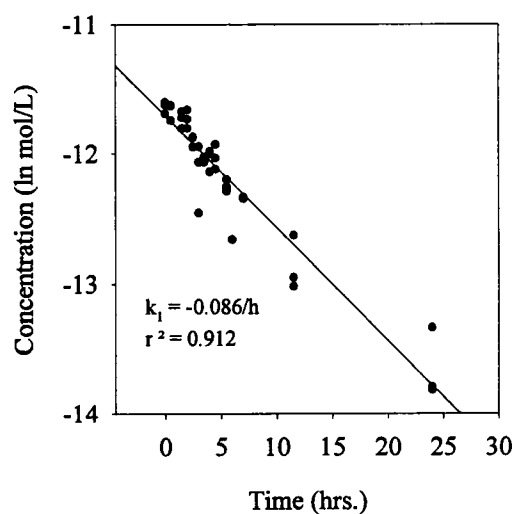
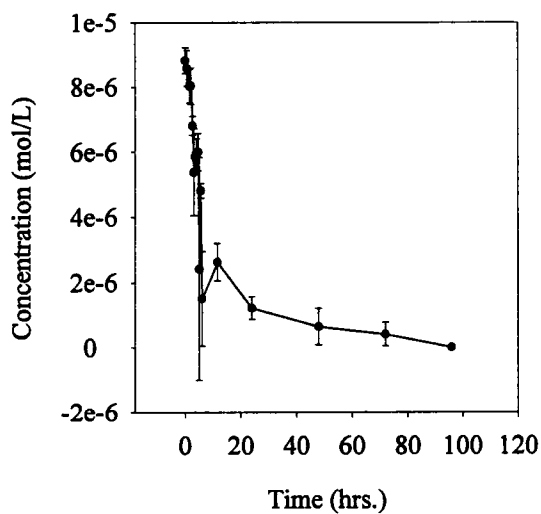


Figure 35. Biodegradation of a Complex PAH and Heterocycle Mixture by *P. fluorescens* 5R.

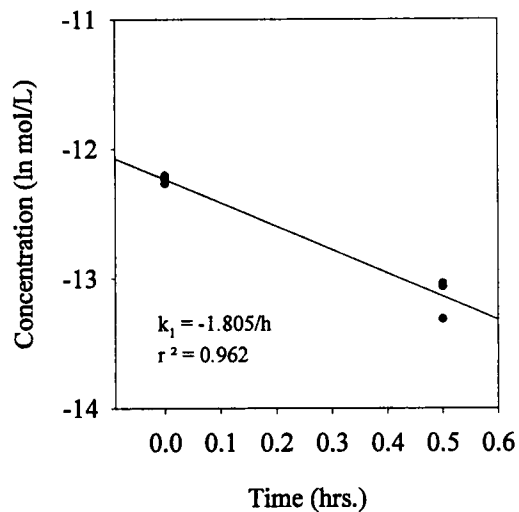
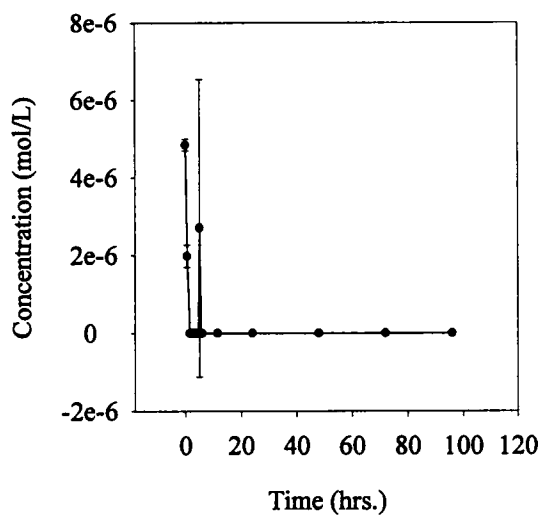


1-Methylnaphthalene

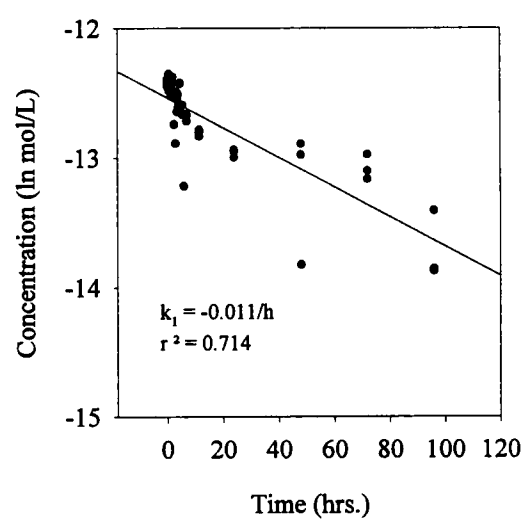
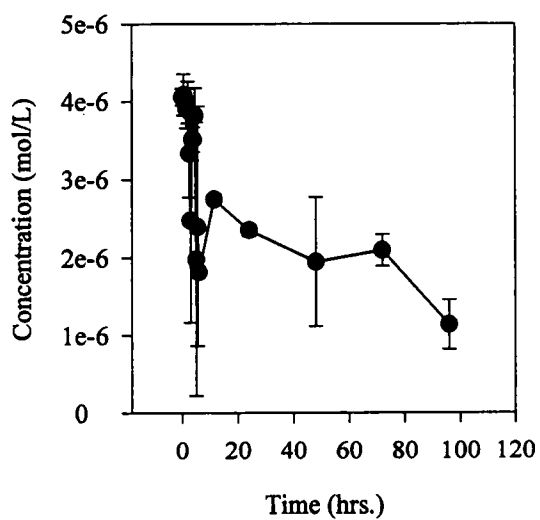


Biphenyl

Figure 35 cont.

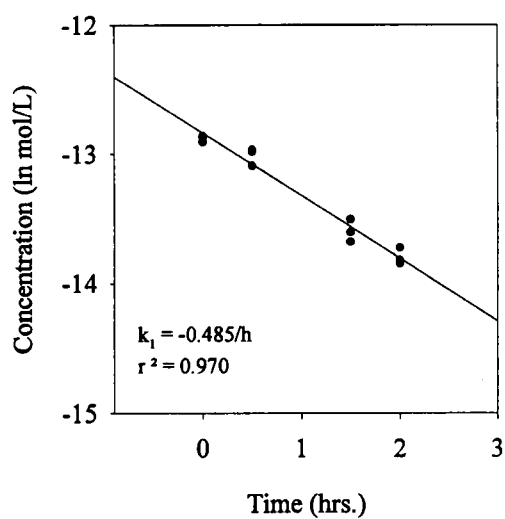
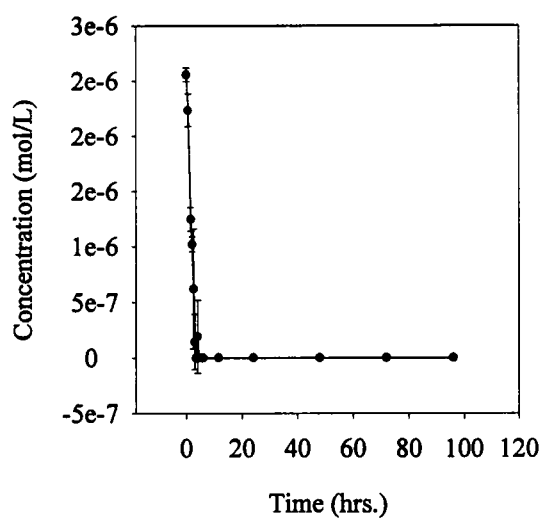


2-Ethynaphthalene

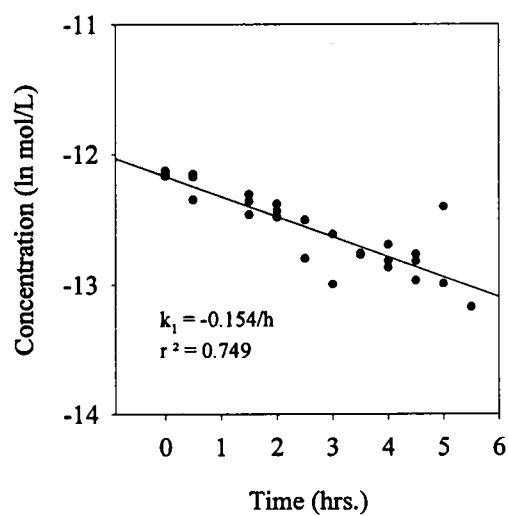
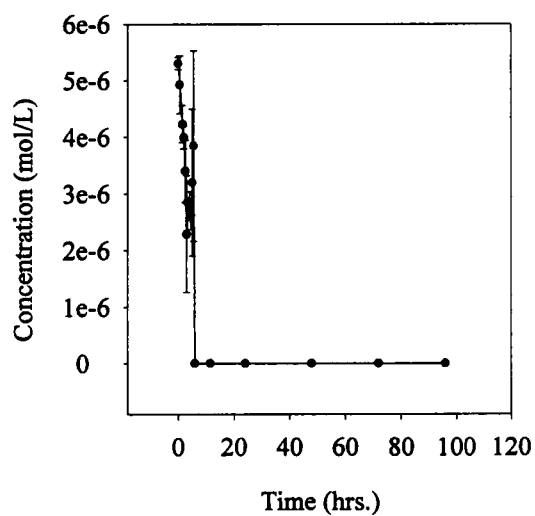


2,3-Dimethylnaphthalene

Figure 35 cont.

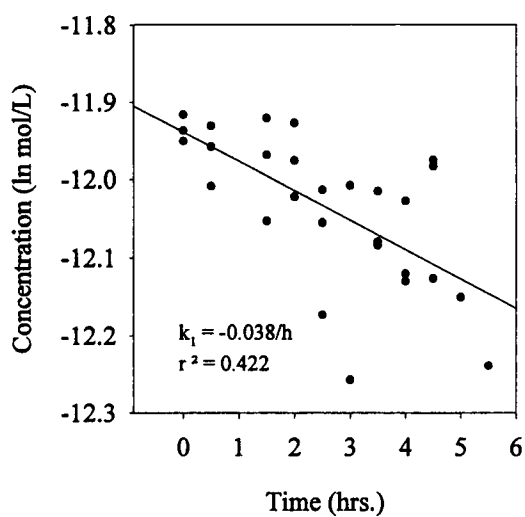
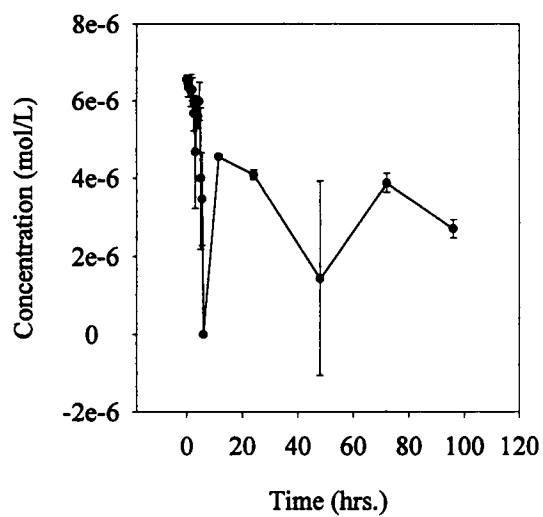


1,2-Dimethylnaphthalene

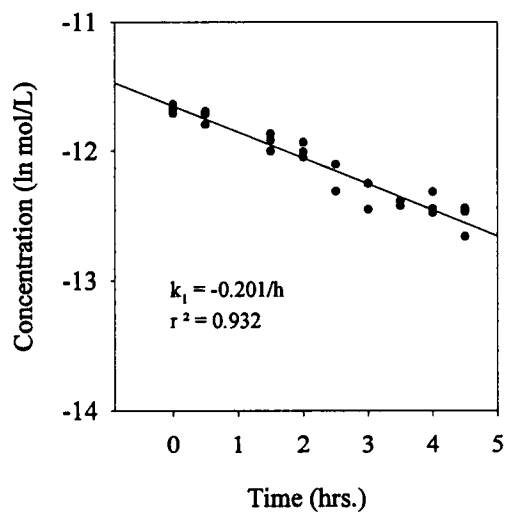
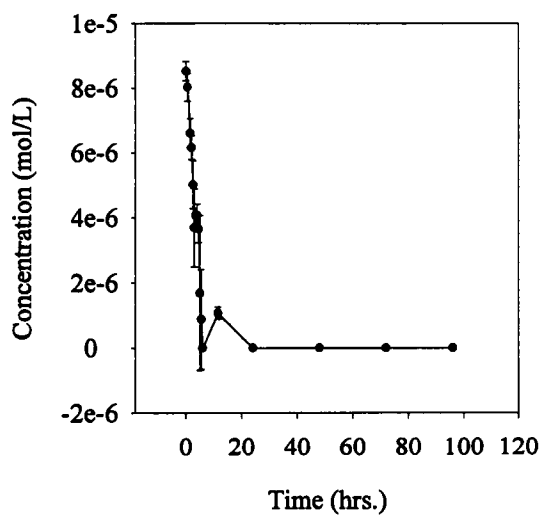


2,6-Dimethylnaphthalene

Figure 35 cont.



1,8-Dimethylnaphthalene



Dibenzofuran

Figure 35 cont.

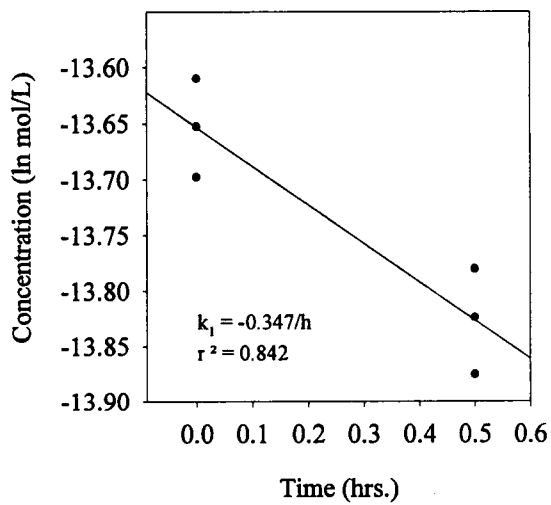
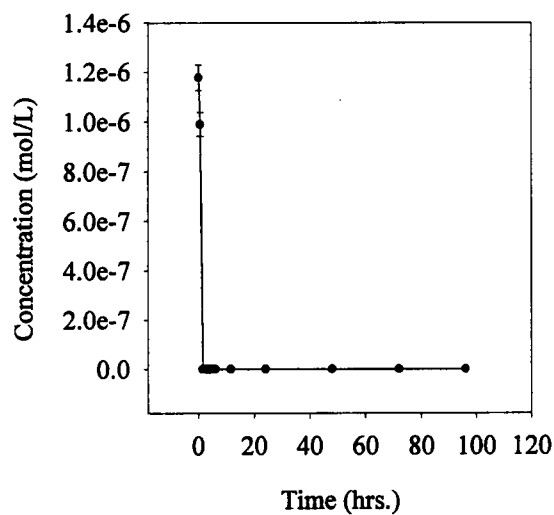
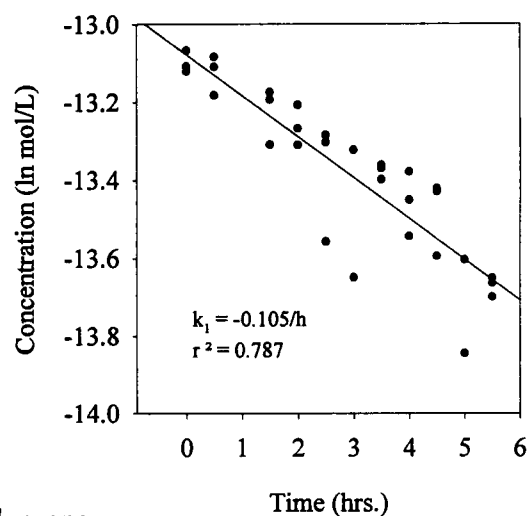
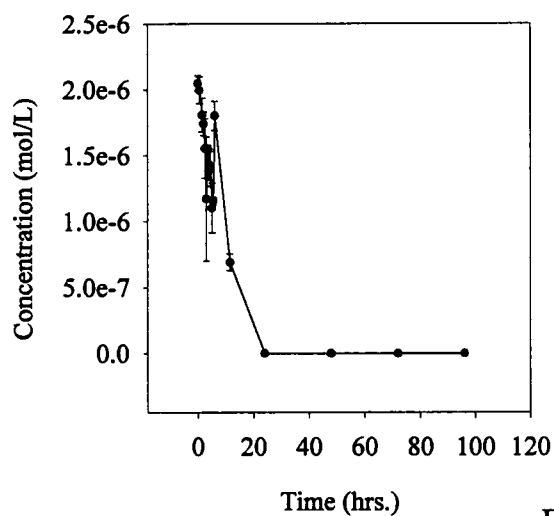
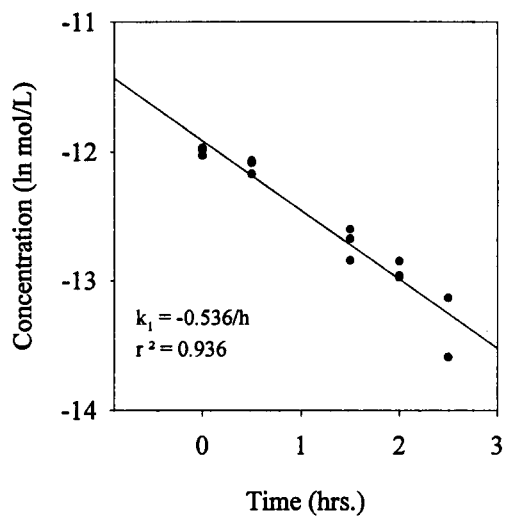
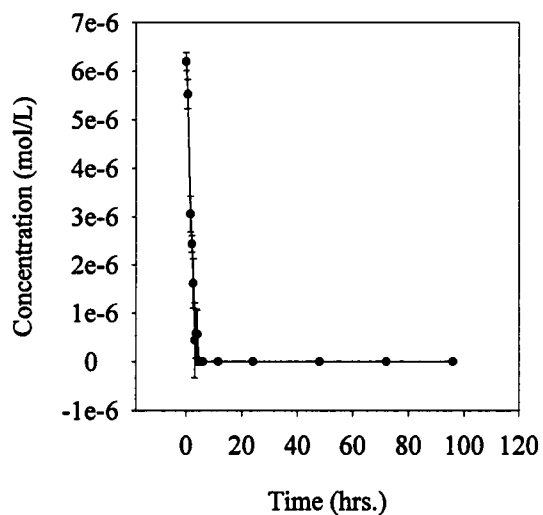
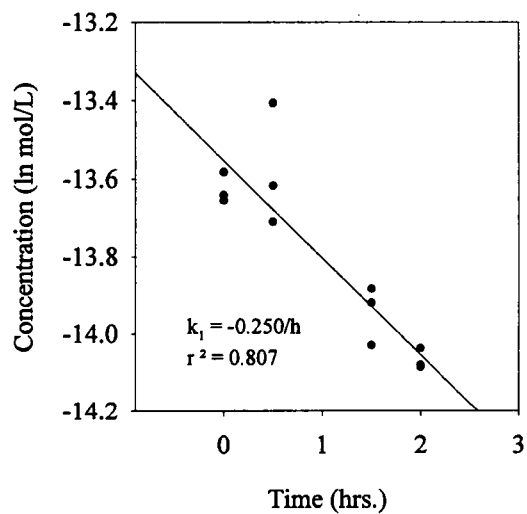
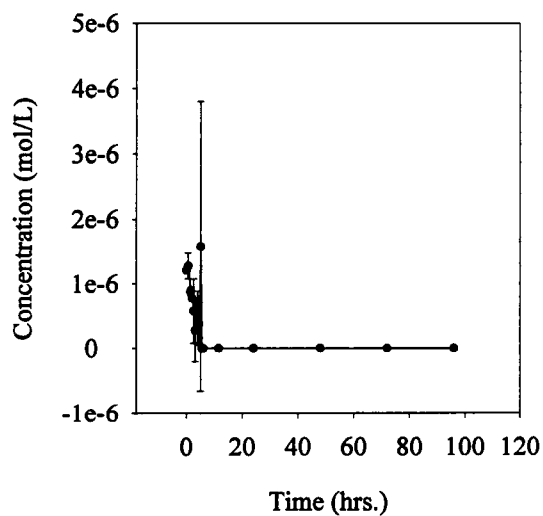


Figure 35 cont.

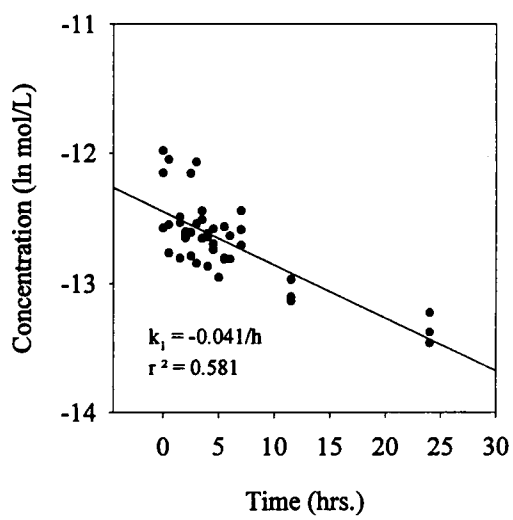
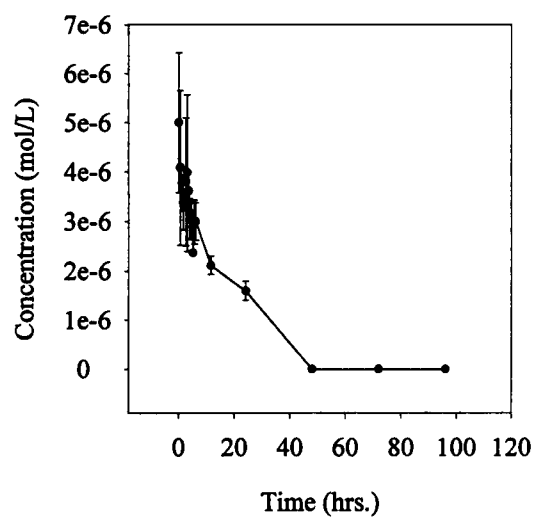


Acenaphthene



Phenanthrene

Figure 35 cont.



Carbazole

Figure 35 cont.

Vita

Jeffrey David Leblond was born on May 31, 1971 in Springfield, Massachusetts at Wesson Women's Hospital. In 1978 he, his parents, and his brother Jeremy moved to Belchertown, Massachusetts. He graduated from Belchertown High School in June, 1989. He subsequently attended the University of Massachusetts at Amherst, and graduated with a Bachelor of Science Degree in Environmental Sciences in August, 1993. He entered the University of Tennessee Department of Microbiology in August, 1993. His graduate work has been conducted at the University of Tennessee Center for Environmental Biotechnology.