BIODEGRADATION OF N-HETEROCYCLIC AROMATIC HYDROCARBONS IN CONTAMINATED SOIL

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A thesis submitted to the faculty of the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Master of Science in the Department of Environmental Sciences and Engineering.

Chapel Hill
2017

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ABSTRACT

Zhe Wang: Biodegradation of N-Heterocyclic Aromatic Hydrocarbons in Contaminated Soil
(Under the direction of David R. Singleton)

While progress has been made understanding the biodegradation of polycyclic aromatic hydrocarbons (PAHs) in contaminated soil, the fate of co-existing contaminants, such as nitrogen-containing heterocyclic aromatic hydrocarbons (azaarenes) is rarely reported. Compared to their PAH analogues, azaarenes have higher toxicity and mobility in the environment, which might pose significant risk to public health. In this research, the fate of three azaarenes (carbazole, acridine, and phenanthridine) in contaminated soil was investigated. Carbazole was degraded in both untreated soil and soil treated in a lab-scale aerobic bioreactor. For phenanthridine, co-metabolism with phenanthrene was a major mechanism for its removal from untreated soil. Both acridine and phenanthridine were not significantly removed in untreated soil when these compounds were examined individually, but were removed in the bioreactor. Overall, this study of the fate and biodegradation of three azaarenes in contaminated soil provided insights and potential for future research on bioremediation of azaarenes-contaminated sites.

ACKNOWLEDGMENTS

I am extremely fortunate to be able to complete thesis under the support and guidance of Dr. David Singleton. He really set a model of scholarly research for me and the impacts from these valuable advice on me would beyond the boundary of science research. Surely, if it had not been for his valuable advice and stoic patience, nothing of this project can be achieved. In addition, I would like to express my gratitude to Dr. Michael Aitken who always supports me and provides me insightful advice in many aspects. Special thanks to Dr. Joaquim Vila Grajales for the sincere advice and guidance on my lab work. I am also grateful to Dr. Jill Stewart for serving in my committee and providing useful support on my project. Finally, my deepest thanks would also go to my beloved family, other group members, my friends, and professors of the ESE department for supporting me and helping me out of any difficulties.

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CHAPTER 1: INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a class of widespread chemical contaminants in the U.S. and remain a public health issue of nationwide concern. Since the 1980s, 16 PAHs selected by the United States Environmental Protection Agency (U.S. EPA) were categorized as part of "126 Priority Pollutants" regulated under the Clean Water Act. However, questions regarding the shortcomings of the list of 16 regulated PAHs now arise as some co-existing contaminants of PAHs, which may also be toxic, are not regulated and comprehensively studied [1]. Nitrogen-containing heterocyclic aromatic hydrocarbons (also known as azaarenes) are one such category of contaminants that do not appear on the list of priority pollutants but have been reported toxic for public health and environmental quality. In this research, I investigated the fate and biodegradation of three azaarenes (carbazole, acridine, and phenanthridine) in contaminated soil obtained from a former manufactured gas plant site in North Carolina. In particular, I began to examine the bacterial communities associated with the removal of these emerging contaminants through analytical chemistry, molecular and traditional microbiology methods. Despite the structural similarity of the three azaarenes, the different outcomes of the compounds during incubation suggests that different mechanisms and organisms might be involved in their degradation. Deeper understanding of the biodegradation patterns of these azaarenes and the microbial communities responsible for their degradation may contribute to developing an effective and sustainable plan for bioremediation of azaarene-contaminated sites.

CHAPTER 2: LITERATURE REVIEW

A. Soil Contamination and Sites of Manufactured Gas Plants

With industrialization, the utilization of fossil fuels improved the quality of life, but also resulted in widespread soil contamination of toxic components and byproducts [2]. While some of these contaminants are present at low levels in the soil due to natural processes, they pose a greater risk to human and ecosystem health at high concentrations [3]. Anthropogenic sources are the major contributors to high levels of fossil fuel-derived soil contaminants [4]. Inorganic soil pollutants, including heavy metals such as Cu, Ni, Cd, Zn, Cr, and Pb, from sources such as mine waste and industrial sewage sludge are also known to result in adverse health outcomes and reduce soil quality [5, 6]. Organic pollutants such as polycyclic aromatic hydrocarbons (PAHs) are widely distributed in the environment, and are especially present in high concentrations in fossil fuels and their byproducts. Potential health risks caused by soil contaminants may result in adverse health outcomes through direct exposure to contaminated soil, or through indirect sources such as injection in water and food previously in direct contact with polluted soil [7]. Short-term exposure of soil contaminants like PAHs and heavy metals can cause acute symptoms like nausea and vomiting, whereas long-term exposure of these contaminants might be related to kidney/liver damage and cancer [8-10]. Many industrial sites in the U.S require decontamination considering the risk for human health and environment posed by toxic contaminants at those locations. These contaminated sites may be divided into several categories according to United States Environmental Protection Agency (U.S. EPA), such as RCRA sites (under Resource

Conservation and Recovery Act of 1976) and Superfund sites (under the Comprehensive Environmental Response, Compensation, and Liability Act of 1980) [11].

Manufactured gas plants (MGPs) provided manufactured gas as an energy source to U.S. residents from the early 1800s through the mid-1900s [12]. The sites of former MGPs often become an environmental issue due to residual toxic contaminants leftover from the production process. Many toxic products, such as polycyclic aromatic hydrocarbons (PAHs), benzene, and cyanide, resulted from the production of manufactured gas, which included a coal carbonization process, carburetted water gas process, and oil gas process [13]. For example, an oil gas process would thermal-crack oil produced from a carburetted water gas process to obtain the raw manufactured gas [14]. The raw gas was accepted by a vaporizer for the purpose of enrichment and then heated prior to distribution. Unavoidably, tars containing PAHs and other heterocyclic aromatic hydrocarbon compounds would also be produced at this stage.

B. Polycyclic Aromatic Hydrocarbons

1. Introduction of Polycyclic Aromatic Hydrocarbons

PAHs are among the more common contaminants at MGP sites [15]. They are a large group of lipophilic (hydrophobic) organic compounds comprised of two or more fused aromatic rings and different configurations (linear, angular, or cluster arrangements) (Figure 1) [16, 17]. PAHs with 2 or 3 rings, such as naphthalene (2 rings), phenanthrene (3 rings), and anthracene (3 rings), can be classified as low-molecular-weight (LMW) compounds, whereas PAHs with 4 or more rings, such as chrysene and pyrene (4 rings) are generally considered as high-molecular-weight (HMW) PAHs. HMW PAHs pose greater risk to human health as they are more toxic, recalcitrant, and carcinogenic than LMW PAHs [18]. Researchers have also found that HMW PAHs are the major PAH contaminants (80%-90%) distributed around the world [19]. Removal

of total PAHs from contaminated sites is extremely difficult considering their toxicity and the low bioavailability of HMW PAHs. Besides the production of manufactured gas and the use of creosote to treat wood, PAHs come from the incomplete combustion of either other organic materials including plant biomass (phytogenic) and petroleum (petrogenic or pyrogenic) due to either anthropogenic or natural reasons [20]. Estimating the sources of PAH contamination is possible through plotting ratios of certain combination of PAHs (e.g. anthracene/anthracene+ phenanthrene) [21].

PAHs are not only found at industrial sites around the world, but also in a wider range of locations, such as tropical and even polar areas due to their atmospheric transportability [22]. Despite the ubiquitous distribution of PAHs in the air, water, soil, and river/ocean sediments, researchers have found that more than 90% of PAHs are present in the soil, especially those associated with industrial sites [23]. Former MGP sites can contain as much as 17,000 $mg \cdot kg^{-1}$ total PAHs, more than other industrialized sites, including wood preserving and treatment sites, coking plants, manufacturing zones, and petrochemical plants [19]. While the majority of PAHs in the soil are sorbed to soil particles, the mobility of PAHs cannot be ignored [24, 25]. Sorbent particle size and the pore throat size (the smallest opening between individual grains of soil) are two key factors determining the mobilization of PAHs in soil, while the tendency of a PAH to sorb to soil is affected by the octanol–water partitioning coefficient of the PAH [26].

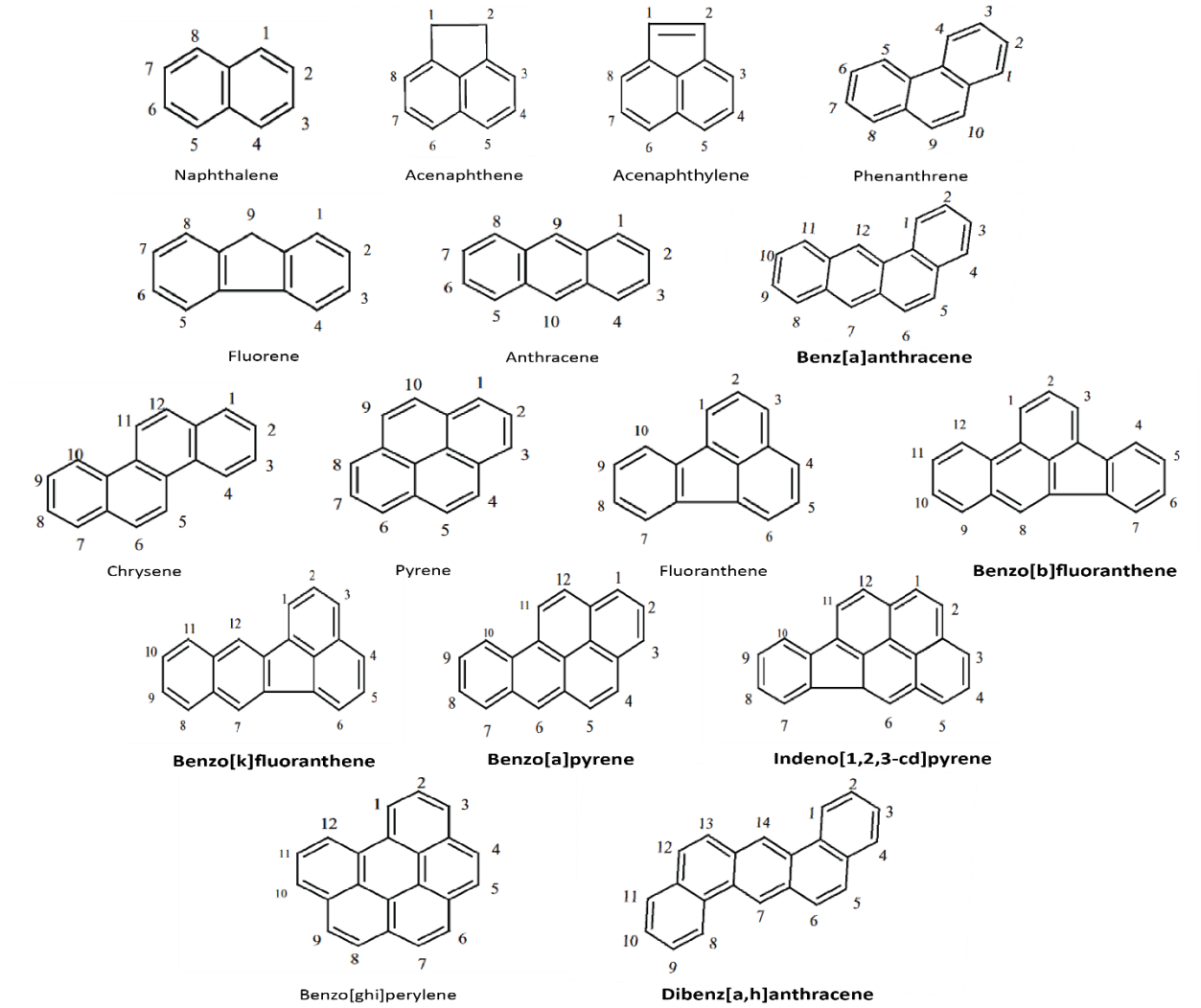


Figure 1. Numbered structures and nomenclatures of the 16 PAHs on the EPA priority pollutant list. The names of 6 PAHs classified as carcinogens by National Toxicity Program (NTP) are bolded [27].

PAHs in the environment might cause health issues through multiple exposure routes, such as ingestion, dermal, and inhalation [28]. The lipophilic property of PAHs means that these chemicals can potentially be detected in most internal organs. Organs with adipose tissue are the major storage compartments for PAHs within the body [29]. According to the National Toxicity Program (NTP), six of the sixteen EPA priority PAHs are classified as human carcinogens (Figure 1) [27]. Other PAHs may also be carcinogenic, but cannot be classified as such due to inadequate evidence. Yet several PAHs that are not considered carcinogenic, such as

naphthalene, acenaphthene and pyrene, have been reported as toxic compounds with mutagenic or cytotoxic characteristics [30]. PAHs are viewed as human carcinogens due to the formation of highly reactive metabolites which are able to combine with nuclear DNA [31]. The formation of these reactive metabolites is normally associated with the activity of cytochrome P450 (CYP) enzymes. This superfamily of heme-thiolate enzymes encoded by cytochrome P450 genes has the key function in living organisms of metabolizing xenobiotics by forming either detoxified hydrophilic metabolites or sometimes more toxic metabolites [32]. Members of CYP superfamily of enzymes, such as CYP1A1 and CYP1B1, were reported to play an essential role in metabolizing PAHs in the body to form carcinogenic diol-epoxides under the co-function of epoxide hydrolase [33]. Diol-epoxides metabolites are able to bind to nuclear DNA to form adducts that cause further mutation and even cancer. Metabolites of PAHs with stereoisomeric bay or fjord regions are believed more reactive than others and have been called ultimate mutagenic and carcinogenic metabolites (Figure 2) [34]. However, some PAHs (e.g. anthracene) do not possess bay or fjord regions and might still be able to cause DNA damage [35]. The toxicity of PAHs with more than seven rings is still not clear, but normally the toxicity of PAHs would increase with the number of rings [30]. Metabolized PAHs within the human body are converted to polar molecules, such as glucuronides, sulfuric acids, and glutathione, to be excreted from the body as urine.

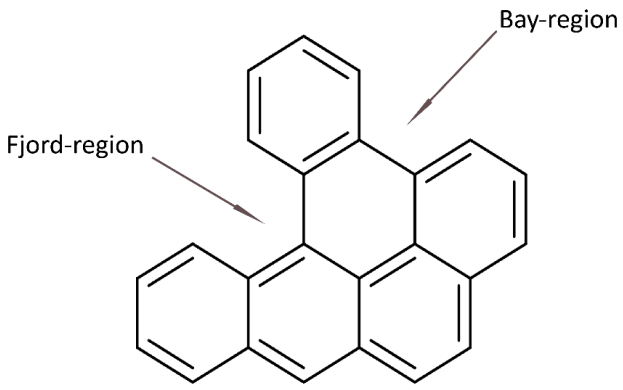


Figure 2. Bay- and fjord-regions of dibenzo[*a*,*l*]pyrene.

The fate of PAHs in the soil can vary depending on the environmental conditions and the characteristics of the PAH. These include photooxidation, volatilization, chemical oxidation, adsorption to soil particles, leaching, bioaccumulation, and biodegradation [36]. Biodegradation is one of the most studied approaches for dealing with the challenges of PAH contamination. Especially in recent years, researchers have realized that a natural biological degradation process can be an effective remediation strategy to remove pollutants like PAHs and minimize the remedy carbon footprint.

Biodegradation of PAHs can happen either aerobically or anaerobically [37-39]. However, the rates of anaerobic biodegradation are typically slower than the biodegradation under aerobic conditions and the ecological function of anaerobic biodegradation was presumed to be minor historically [40, 41]. In spite of recent research progress revealing the unique role of anaerobic biodegradation under certain environmental conditions, such as when the oxygen demand exceeds its supply in natural environments, most attention has been focused on aerobic processes [42].

To comprehensively understand the biodegradation processes of PAHs in the soil, the isolation, cultivation, and identification of relevant pure cultures of aerobic and anaerobic PAH-

degrading microorganism provides information on the growth conditions and PAH growth substrates for microorganisms [43]. Contrary to metabolism of PAHs by fungi which is based on extracellular enzymes secreted outside cells to break down bonds of PAHs, metabolism of PAHs by bacteria is achieved by intracellular enzymes [43]. Therefore bioavailable PAHs, such as those dissolved in water or in the vapor phase, can be taken up by bacteria [44, 45]. Soil structure, other environmental factors (e.g. water, temperature, pH, and nutrients) and bioavailability of PAHs in the soil are all key factors affecting the biodegradation process. Bioavailability can be determined by the rate of substrate mass transfer into microbial cells with respect to the its intrinsic catabolic activity [43]. Bioavailability can be a limiting factor when the capacity of the cells consuming a PAH exceeds the capacity of acquiring this PAH from outside environment. As a result, substrate concentrations in the aqueous phase that can be consumed by microbes would be reduced significantly. Moreover, when PAHs sorb onto soil particles or enter non-aqueous phase liquids (NAPLs), the degradation of PAHs would be difficult as enzymes used by bacteria for the degradation of PAHs would not be able to encounter PAHs in these phases [26]. Solubility is one of the key factors affecting bioavailability. In addition, bioavailability of a certain PAH compound is also reliant on the variability of desorption rates for different PAHs over different time periods [46]. Normally, PAHs are desorbed from solid materials quickly at the start, but the process of desorption would then become slow as concentration gradients between the sorbent and aqueous phases decrease [47].

Three biodegradation patterns are reported to be the major mechanisms associated with metabolism of multiple compounds: diauxie, simultaneous utilization, and competitive inhibition. Diauxie, or sequential utilization, was first described by Monod to indicate that the presence of multiple substrates might result in sequential degradation with gene regulation

determining which substrate would be preferentially used [48]. For example, the presence of ethylbenzene was reported to inhibit the metabolism of a *Rhodococcus* strain growing on other BTEX substrates [49]. Simultaneous utilization of substrates may lead to cell growth, but might also cause the degradation of compounds without cell growth. The pattern of simultaneous utilization of substrates without cell growth is considered as co-metabolism [50]. As a non-specific enzymatic mechanism, co-metabolism can be described as the reaction that occurs when a contaminant is fortuitously degraded by an enzyme or co-factor synthesized as a result of degradation process from another structurally similar primary substrate. Co-metabolic remediation was found to be useful to degrade contaminants when their concentration was too low to be the growth substrate for certain bacteria [51]. It has also reportedly been involved in the degradation process of very recalcitrant contaminants, such as HMW PAHs, trichloroethylene (TCE), tetrachloroethylene (PCE), and methyl tert-butyl ether (MTBE) [52]. Finally, competitive inhibition can be a process in which non-specific enzymes preferentially attack contaminants which are more easily degraded while more recalcitrant contaminants persist [53]. For instance, naphthalene, methylnaphthalene, and fluorene are frequently competitive substrates with phenanthrene for phenanthrene-degrading bacteria [54].

2. Biodegradation of Phenanthrene and Anthracene

The knowledge of biodegradation on phenanthrene and anthracene in contaminated soil are essential to this research due to their structural similarity to two azaarenes (phenanthridine and acridine, respectively) studied in this work. Therefore, a brief review of the known degrading-microorganisms and metabolic pathways of phenanthrene and anthracene is warranted.

As one of the more widely distributed PAHs, phenanthrene is viewed as a model compound for studies of biodegradation of LMW-PAHs. A variety of bacteria have been reported capable of metabolizing phenanthrene either directly or indirectly (e.g. through co-metabolism). Bacterial strains from the genera *Mycobacterium* [55, 56], *Vibrio* [57], *Pseudomonas* [57, 58], *Aeromonas* [57, 59], *Sphingomonas* [60, 61], *Sphingobium* [62], *Arthrobacter* [63], *Acidovorax* [61, 63], *Brevibacterium* [63], *Burkholderia* [64], *Martelella* [65], *Nocardia* [66], *Streptomyces* [67] *Staphylococcus* [68] and *Sinorhizobium* sp. [69] are all reported to degrade phenanthrene. Fungal representatives, such as *Cunninghamella elegans* and *Pleurotus ostreatus*, are also capable of metabolizing phenanthrene [70, 71]. It is noteworthy that several members of the Sphingomonads (including *Sphingomonas, Sphingobium, Sphingopyxis* and *Novosphingobium* spp.) are actively involved in the degradation of phenanthrene and are extremely important phenanthrene-degrading bacteria in soil [72].

The metabolic pathways of phenanthrene degradation have been intensively researched. Members of the Sphingomonads and *Pseudomonas* genus are capable of synthesizing aromatic ring-hydroxylating dioxygenase (ARHD) enzymes to target either the 1,2- or 3,4- position of phenanthrene forming *cis*-1,2-dihydroxy-1,2-dihydrophenanthrene or *cis*-3,4-dihydroxy-3,4-dihydrophenanthrene, respectively [59, 60, 72-74]. These metabolites are then converted to 1-hydroxy-2-naphthoic acid after a series of reactions [60, 74, 75]. Two different cleavage pathways have been found for 2-hydroxy-1-naphthoic acid/1-hydroxy-2-naphthoic acid in different bacteria. In one, an *ortho*-cleavage pathway produces protocatechuate modulated by intradiol cleavage enzymes. In the second, a *meta*-cleavage pathway with the production of salicylate and catechol is modulated by extradiol cleavage enzymes [72]. While most phenanthrene-degrading bacteria are able to metabolize phenanthrene through one of these

pathways, *Streptomyces flavovirens* was reported to use a different pathway by attacking the 9,10- position (K-region) of phenanthrene under the modulation of cytochrome P-450 monooxygenase to produce phenanthrene-9,10-oxide [67]. 9-Phenanthrol was then formed after the conversion of phenanthrene-9,10-oxide to trans-9,10-dihydrodiolphenanthrene.

Anthracene is also widely detected at contaminated sites at high concentrations and is isomeric with phenanthrene [76]. Several bacteria have been reported to utilize anthracene as source of carbon and energy, including strains from the genera *Pseudomonas* [77, 78], *Mycobacterium* [79, 80], *Rhodococcus* [81], *Sphingomonas* [82], *Nocardia* [66], *Bacillus* [83], *Microbacterium* [84], *Martelella* [65], *Brevibacterium* [85], *Burkholderia* [86]. Fungi, such as *Cunninghamella elegans, Aspergillus fumigatus, Pleurotus ostreatus, Fusarium solani,* and *Penicillium simplicissimum*, are also capable of metabolizing anthracene [70, 87-89].

One of the major biodegradation pathways of anthracene has been described with the initial dioxygenation at the 1,2 position to produce 1,2-dihydroxyanthracene [80]. This dihydroxylated metabolite is further transformed to 2-hydroxy-3-naphthoic acid after a series of metabolizing steps. The subsequent pathway was proposed as the phthalate pathway [66, 79, 81]. Monohydroxyanthracene was detected as a side product in *Sphingomonas* sp. strain LB126 [90] which might be produced due to a side reaction with 1,2-dihydroxyanthracene as the major intermediate of the degradation. Another pathway proposed the dioxygenation of anthracene at the 9,10- positions to form 9,10-dihydrodiol [79]. Degradation of anthracene by fungi adopt a different metabolic pathway with some fungi capable of metabolizing anthracene to 9,10-anthraquinone prior to the formation of phthalic acid, modulated by extracellular peroxidase enzyme [87].

C. N-Heterocyclic Aromatic Hydrocarbons

N-heterocyclic aromatic hydrocarbons (NHAs) - also known as nitrogen-containing polycyclic aromatic hydrocarbons, N-PAHs, PANHs, or azaarenes, are a class of organic molecules which can be toxic and are widely distributed in a broad range of environments including air, soil, and water systems [1, 91, 92]. Similar to PAHs, the major sources of azaarenes include incomplete combustion of fossil fuels, coal liquefaction, volcanic eruptions, forest fires, uncontrolled hazardous industries, and oily sludge [93]. Azaarenes have strong molecular bonds and π-electrons surrounding benzene rings, so they have the characteristics of being both highly recalcitrant and resistant to nucleophilic attack [43]. Microorganisms are involved in the removal of azaarenes at contaminated sites, and a variety of studies have been conducted on the biodegradation of various azaarenes such as carbazole, acridine, quinolone, pyridine, and phenanthridine. Accordingly, numerous microorganisms involved in the degradation of azaarenes have been reported [94]. Despite the fact that azaarenes can be putative human carcinogens and usually have greater toxicity on microorganisms than their PAH analogues, the fate, behavior and biodegradation of these compounds are still not fully understood [95, 96]. This research is aimed to investigate the fate and behavior of three, three-ring azaarenes, including carbazole, acridine and phenanthridine, in the contaminated soil (Figure 3).

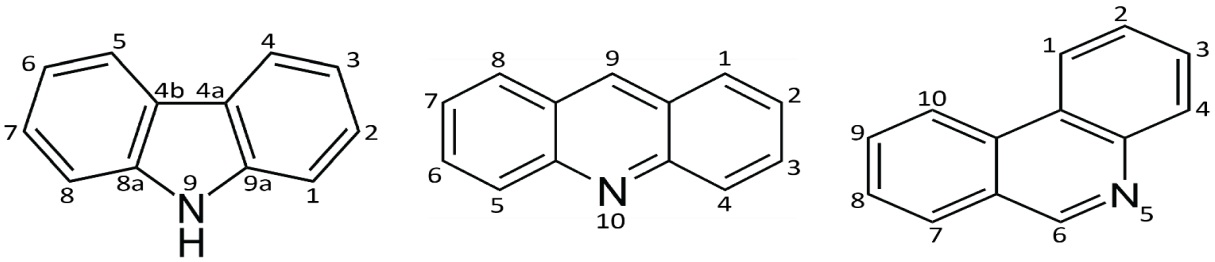


Figure 3. Structures of 3 azaarenes of interest in this research.

1. Carbazole

a. Introduction of Carbazole

Carbazole is an azaarene often present in soil, groundwater, and rivers contaminated by petroleum, wood-preserving wastes, or chemical pollution [97-99]. The sources of carbazole are mainly coal tars, creosote and shale oils, which contain many heterocyclic aromatic compounds [100]. Thousands of tons of carbazole are produced each year from coal tar and crude oil under high temperatures [101].

Carbazole and its derivatives are close to our daily life. It is commonly found in synthetic products such as dyes, pharmaceuticals, and plastics. It is also used to make photographic plates that are sensitive to ultraviolet light. The manufacturing process of some industrial products, including chemical reagents, pesticides, medicines and plastics require carbazole or its derivatives [101]. The structure of carbazole (Figure 3) is similar to dibenzofuran, which is mutagenic and toxic to living organisms [102]. The carcinogenic effect of carbazole tested in mice indicated that carbazole itself is a carcinogen [103]. Other previous reports have also shown that carbazole can be both mutagenic and carcinogenic [104, 105].

Given that the broad distribution of carbazole and its putative toxic and highly recalcitrant properties, there have been many studies on its degradation, including finding effective ways to remove it from the environment. Many studies concerning the transformation of carbazole have been conducted in the past 30 years - mostly on microbial degradation. Details concerning specific carbazole-degrading microorganisms and degradation pathways are discussed in the next sections.

b. Carbazole-Degrading Microorganisms

A number of different microbes have been reported for their ability to degrade carbazole (Table 1), although their role in the bioremediation of carbazole from contaminated sites is typically not known. Among these various carbazole degraders, most are Gram-type negative bacteria, with only a few Gram-type positive.

In 1991, the first reported carbazole degrader was isolated from the soil of an abandoned coal gasification plant and was identified as a *Xanthamonas* sp. After growing the pure culture, the strain was reintroduced into contaminated soil [93]. The degradation of carbazole was enhanced to nearly 50% by these reintroduced bacteria after 7 days in comparison with little carbazole degradation observed for the indigenous bacteria. The process of degradation enhanced by additional bacteria, or bioaugmentation, is one approach to soil bioremediation [106].

Sphingomonads possess a wide variety of biodegradative capabilities and have the potential to be utilized for bioremediation. In 2001, four genera were proposed from the then-current *Sphingomonas* genus comprising the new genera *Sphingomonas*, *Sphingobium*, *Novosphingobium*, and *Sphingopyxis* [107]. In addition to their ability to degrade phenanthrene and anthracene as mentioned above, many researchers have found strains of *Sphingomonas* that could also degrade other substrates, such as carbazole, dibenzofuran, dibenzothiophene, and benzothiophene through the process of co-metabolism [108]. *Sphingomonas* sp. strain CDH-7 was isolated during a screen of nearly 350 samples from soil, wastewater, and petroleum oil sludge collected by researchers. [109]. It used carbazole as a sole source of carbon and resting cells of *Sphingomonas* sp. CDH-7 were found to continuously degrade carbazole. The bacterium *Sphingomonas wittichii* strain RW1 is also noted for its ability to degrade many kinds of xenobiotics, including carbazole [101]. Before it was reported as a carbazole degrader,

Sphingomonas wittichii RW1 was viewed as an important species for its valuable ability to use dibenzo-p-dioxin and dibenzofuran as the sole sources of carbon and energy [110, 111]. Interestingly, while an initial study showed that purified dibenzofuran 4,4a-dioxygenase of strain RW1 was incapable of oxygenating carbazole, researchers re-tested *Sphingomonas wittichii* RW1 and discovered that the color of the culture suspension in minimum salts medium (MSM) containing carbazole changed in a time-dependent manner - indicating that strain RW1 was actually capable of degrading carbazole [101]. *Sphingomonas* sp. strain XLDN2-5 (reclassified as *Sphingobium yanoikuyae* strain XLDN2-5) [108] is not only able to degrade carbazole, but also can co-metabolically catabolize dibenzofuran, dibenzothiophene, and benzothiophene [112].

Bacteria within the genus *Pseudomonas* are viewed as significant for health, agriculture, biotechnology, and environmental science due to their great adaptability [113]. Many researchers are interested in using *Pseudomonas* isolates for bioremediation given their ability to degrade various organic compounds, including carbazole [114]. Research on the carbazole-degrading *Pseudomonas* spp. strains CA06 and CA10 the first described pathway of carbazole degradation [115]. These isolates were able to degrade carbazole to anthranilate and 2-hydroxypenta-2,4-dienoate via the process of angular dioxygenation, *meta*-cleavage, and hydrolysis. More details of the degradation pathway are described in the next section. Another isolate of *Pseudomonas*, named strain XLDN4-9, showed the ability to degrade carbazole in an oil phase [116].

Table 1 List of carbazole-degrading bacteria and fungi.

Time of report	Name of the strain	Major metabolites
1986	*Cunninghamella elegans* ATCC 26269 [117]	-
1992	*Cunninghamella echinulata* ATCC 9244 [118]	-
1993	*Pseudomonas* sp. CA06, CA10 [115]	Anthranilic acid, Catechol
1993	*Pseudomonas* sp. NCIB 9816-4 [119]	3-hydroxycarbazole
1994	*Pseudomonas stutzeri* ATCC31258 [120]	Anthranilic acid
1995	*Bacillus* sp. KUKK-4,5 [121]	-
	Pseudomonas sp. KUKK-1,2,3,8 [121]	-
	Escherichia coli KUKK-6 [121]	-
	Serratia sp. KUKK-7 [121]	-
1995	*Pseudomonas cepacia* F297 [122]	-
1995	*Burkholderia cepacia* CB1 [123]	-
	Xanthamonas sp. CB2 [123]	-
	Pseudomonas sp. CB3 [123]	-
1996	*Pseudomonas* sp. LD2 [124]	Anthranilic acid
1998	*Pseudomonas stutzeri* OM1 [125]	Anthranilic acid, Catechol
1998	*Sphingomonas* sp. CB3 [126]	-
1999	*Sphingomonas* sp. CDH-7 [109]	Anthranilic acid
2000	*Ralstonia* sp. RJGII.123 [127]	Anthranilic acid
2002	*Pseudomonas rhodesiae* KK1 [128]	Anthranilic acid
2002	*Sphingomonas* sp. strain KA1 [129]	-
2002	*Sphingomonas* sp. GTIN11 [130]	Anthranilic acid
2003	*Neptuniibacter* sp. CAR-SF [131]	-
2003	*Sphingomonas* sp. CP19 [132]	-
2003	*Pseudomonas* sp. C3211 [133]	-
2004	*Janibacter* sp. YY-1 [134]	Catechol
2004	*Pseudomonas* sp. K23, K22, K15, J11 [135]	-
	Janthinobacterium sp. J3, J4 [135]	-
	Pantoea sp. J14 [135]	-
	Novosphingobium sp. J30 [135]	-
	Sphingomonas sp. J40, M2 [135]	-
2004	*Pseudomonas* sp. XLDN4-9 [116]	-
2004	*Aspergillus flavus* VKM F-1024 [136]	3-hydroxycarbazole
2005	*Acinetobacter* sp. IC001 [137]	-
	Pseudomonas sp. IC017 [137]	-
	Sphingomonas sp. IC033,IC075, IC081, IC097, IC145 [137]	-
	Burkholderia sp. IC049, IC129, IC138 [137]	-
	Achromobacter sp. IC074 [137]	-
	Erythrobacter sp. IC114 [137]	-
	Janthinobacterium sp. IC161 [137]	-

Table 1 (continued) List of carbazole-degrading bacteria and fungi.

	Stenotrophomonas sp. IC193 [137]	-
	Marinobacterium sp. IC961, IC977 [137]	-
	Nocardioides aromaticivorans IC177 [137]	Anthranilic acid
2006	*Bacillus* sp. T2.3-2.6, T3.1, T3.3, T4.1-4.3 T6.1-6.6, T7.0 [138]	-
2006	*Gordonia* sp. F.5.25.8 [139]	-
2006	*Arthrobacter* sp. P1-1 [140]	Anthranilic acid
2006	*Burkholderia* sp. IMP5GC [141]	-
2007	*Sphingobium yanoikuyae* XLDN2-5 [142]	-
2008	*Sphingomonas* sp. VKM B-2434 [143]	-
2008	*Novosphingobium* sp. NIY3 [144]	Anthranilic acid
2008	*Klebsiella* sp. strain LSSE-H2 [145]	-
2008	*Chryseobacterium* sp. NCY and *Achromobacter* sp. NCW [146]	-
2009	*Kordiimonas* sp. OC3, OC6S, OC9, OC11S [147]	-
	Erythrobacter sp. OC4, OC8S [147]	-
	Hyphomonas sp. OC5 [147]	-
	Sphingosinicella sp. OC5S [147]	-
	Caulobacter sp. OC6, OC10 [147]	-
	Lysobacter sp. OC7 [147]	-
2009	*Sphingomonas* sp. JS1 [148]	-
2009	*Ralstonia* sp. strain SBUG 290 [149]	1-hydroxycarbazole 3-hydroxycarbazole
2011	*Enterobacter* sp. A8 [150]	-
2011	*Sphingomonas wittichii* RW1 [151]	Anthranilic acid, Catechol
2011	*Acinetobacter* spp. Alp6, Alp7 [91]	-
2011	*Pseudomonas* BC039-046 [152]	-
2011	*Pseudoxanthomonas* sp. DMVP2 [153]	-
2012	*Achromobacter* sp. CAR1389 [154]	Anthranilic acid
2013	*Pseudomonas* sp. GBS.5 [155]	-
2014	*Achromobacter* sp. SL1 [113]	Anthranilic acid, Catechol
	Pseudomonas sp. SL4 [113]	Anthranilic acid, Catechol
	Microbacterium esteraromaticum SL6 [113]	Anthranilic acid, Catechol
	Stenotrophomonas maltophilia B_A [113]	-
2015	*Cunninghamella* IM 1785/21Gp, 2611 and DSM8217 [156]	2-hydroxycarbazole

In crude oil, carbazole is often present with its alkylated derivatives [104]. These alkyl derivatives often have monomethyl, dimethyl, trimethyl, and tetramethyl side chains on different carbon positions of carbazole (called C1-, C2-, and C3-, C4-carbazoles, respectively). One study showed that mixed bacterial cultures were able to degrade alkyl carbazoles (from C1- to C4-carbazoles) in crude oil within eight days, and also showed that the alkyl-carbazoles were easier to degrade as the number of substituted alkyl groups became fewer [157]. However, pure cultures rarely degrade alkylated derivatives in an oil phase with few exceptions; One example is that *Sphingomonas* sp. strain GTIN11 was demonstrated to degrade carbazole and C1-carbazoles in shale oil [130]. *Pseudomonas* sp. strain XLDN4-9 was also capable of degrading carbazole, C1-carbazoles, C2-carbazoles, and even benzocarbazoles, but it was shown that carbazole was harder to degrade than either C1-carbazoles and C2-carbazoles [158].

Ammonium can be produced by bacteria as a by-product during the degradation of carbazole [109, 159]. The production of ammonium has also been observed in the biodegradation of other nitrogen heterocyclic compounds due to nitrogen bio-transformation, including the compounds pyridine and quinoline [160]. Researchers have isolated seven strains, all identified as *Pseudomonas* species, which could not only degrade carbazole efficiently, but also showed nitrification and denitrification ability [152]. Before that report, the capabilities of nitrification and denitrification by carbazole-degrading bacteria was rarely reported. These seven isolates, named *Pseudomonas* sp. BC 039 through *Pseudomonas* sp. BC 046, were identified as distinct strains by 16S rRNA gene analysis [152]. Six of them were capable of producing NH_4^+–N by transforming carbazole. They also possessed nitrite reductase genes (*nirS*) and the nitrous oxide reductase genes (*nosZ*) that contribute to the denitrification process. However, the pathway for denitrification by these carbazole degraders were different between each other. Some strains

utilized NO_3^-–N and NO_2^-–N simultaneously, while others had a preference for utilizing NO_3^-–N. This study indicated that further understanding the process of nitrification and/or denitrification process by carbazole-degrading bacteria can also contribute to efforts for controlling the release of ammonium to the environment.

Thus far, much of the work on carbazole degraders was performed with isolates identified as *Sphingomonas* and *Pseudomonas* species. However, isolates from other Gram-type negative genera can also degrade carbazole. *Achromobacter* species were demonstrated to degrade a wide range of xenobiotics and were specialized to degrade compounds such as hydrocarbons and PAHs [161-164]. Reports have shown that *Achromobacter* sp. strains IC074 [137], CAR1389 [154], and SL1 [113] can metabolize carbazole. Remarkably, strain CAR1389 was capable of growing on nominal 3.5 $g \cdot L^{-1}$ of carbazole in a minimal medium (MM) and degrading nearly half that amount [154]. Before this discovery, carbazole-degrading bacteria capable of growing at such high concentrations of carbazole were rarely reported [165]. This finding also suggested that carbazole might not have a strong inhibitory effect on the activity of strain CAR1389 even though the concentration of carbazole was extremely high.

Additional Gram-type negative carbazole degraders include *Burkholderia cepacia* strain CB1 [123], *Escherichia coli* strain KUKK-6, *Serratia* sp. strain KUKK-7 [121], *Ralstonia* sp. strain RJGII.123 (formerly *Xanthomonas* sp.) [93, 127], *Janthinobacterium* sp. strain J3 and strain J4, *Pantoea* sp. strain J14, *Novosphingobium* sp. strain J30 [135], *Burkholderia* sp. strain IMP5GC [141], *Klebsiella* sp. strain LSSE-H2 [145], *Novosphingobium* sp. strain NIY3 [166], *Acinetobacter* strains Alp6 and Alp7 [91], *Enterobacter* sp. strain A8 [150], *Microbacterium esteraromaticum* strain SL6, and *Stenotrophomonas maltophilia* strain B_A [113].

Several Gram-type positive bacteria have been reported as carbazole degraders. The carbazole degrader *Nocardioides aromaticivorans* strain IC177 was used to study the genes and metabolic pathway of carbazole degradation [159]. The test results indicated that the carbazole catabolic genes of strain IC177 were similar to those of Gram-type negative bacteria, such as *Pseudomonas* spp. strain CA10 and *Janthinobacterium* sp. strain J3. Other Gram-type positive carbazole degraders include *Bacillus* sp. strain KUKK-4,5 [121], *Janibacter* sp. strain YY-1 [134], *Arthrobacter* sp. strain P1-1 [140], *Bacillus* sp. strains T2.3 to T2.6, T3.1 and T3.3, T4.1 to T4.3, T6.1 to T6.6, and T7.0 [138], *Gordonia* sp. strain F.5.25.8 [139], and *Dietzia cinnamea* strain P4 [167]. The genome sequence of *Dietzia cinnamea* strain P4 has been reported and genes encoding enzymes for the degradation of aromatic hydrocarbons have been identified [168].

Not only pure cultures have been reported to degrade carbazole. A microbial consortium consisting of *Chryseobacterium* sp. strain NCY and *Achromobacter* sp. strain NCW was reported to utilize carbazole as the sole source of carbon, nitrogen, and energy [146]. However, neither of the pure strains NCY and NCW were able to degrade carbazole individually. The possible explanation reported for this phenomenon was neither strain NCY nor NCW possessed a complete pathway to transform carbazole.

Another kind of microorganism with the ability to metabolize carbazole are filamentous fungi, which often play an important role in the biodegradation process [169]. However, compared to bacterial research, studies concerning filamentous fungi for carbazole degradation and its derivatives are less common. At least two fungal genera have been reported to degrade carbazole, *Cunnighamella* species and *Aspergillus flavus* VKM F-1024 [118, 136, 156, 170].

c. Metabolic Pathways of Carbazole Degradation

Though the characterized carbazole degraders belong to multiple species, the research on metabolic pathway and enzymes linked to degradation was limited to only a few strains, such as *Pseudomonas resinovorans* strain CA10 and *Sphingomonas* sp. strain CB3 [115, 123, 126]. In this section, the pathway and genes relative to carbazole degradation are described.

Pseudomonas spp. CA06 and CA10 were first used to determine a pathway for the biodegradation of carbazole (Figure 4) [115]. Two main metabolites, anthranilic acid (AN) and catechol (CAT), were found in the medium after analysis by high-performance liquid chromatography (HPLC) and gas chromatography-mass spectrometry (GC-MS). Further experiments showed that CAT was detected when AN was used as the substrate. Additional analyses identified metabolites including 2'-aminobiphenyl-2,3-diol and 2-hydroxy-6-oxo-6-(2'-aminophenyl)-hexa-2,4-dienoic acid (HOADA). The proposed pathway begins with the dioxygenation of carbazole at an angular position adjacent to the nitrogen atom. The product is a dihydroxylated intermediate which is converted to 2'-aminobiphenyl-2,3-diol spontaneously. 2'-aminobiphenyl-2,3-diol is converted to HOADA before hydrolysis to AN via a *ortho*-cleavage pathway. AN is a non-toxic substrate relatively easily degraded by microbes and can be used as a substrate in tryptophan biosynthesis [171, 172]. The final step of the carbazole pathway includes the conversion of AN to CAT through *ortho*-cleavage, to an intermediate of the tricarboxylic acid cycle (TCA) [173, 174]. Interestingly, *Pseudomonas stutzeri* OM1 had a meta-ring-cleavage pathway via conversion of CAT to 2-hydroxymuconic semialdehyde [125].

The pathway that uses an angular dioxygenase acting on the 9a and 1 carbons of carbazole is a relatively common route for carbazole biodegradation. The genes for this pathway have been well characterized in *Pseudomonas resinovorans* CA10 [175]. Carbazole 1,9a-

dioxygenase (CarA or CARDO) is the first enzyme to participate in the reaction of carbazole oxidation. CarA can participate in several oxygenation reactions involving a large range of substrates and can also participate in monooxygenation reactions [174, 176]. CarA can not only oxidize carbazole, but also dibenzofuran (DBF), fluorene, naphthalene, and biphenyl [155]. The major product of CarA in carbazole dioxygenation is 2-aminobiphenyl-2,3-diol.

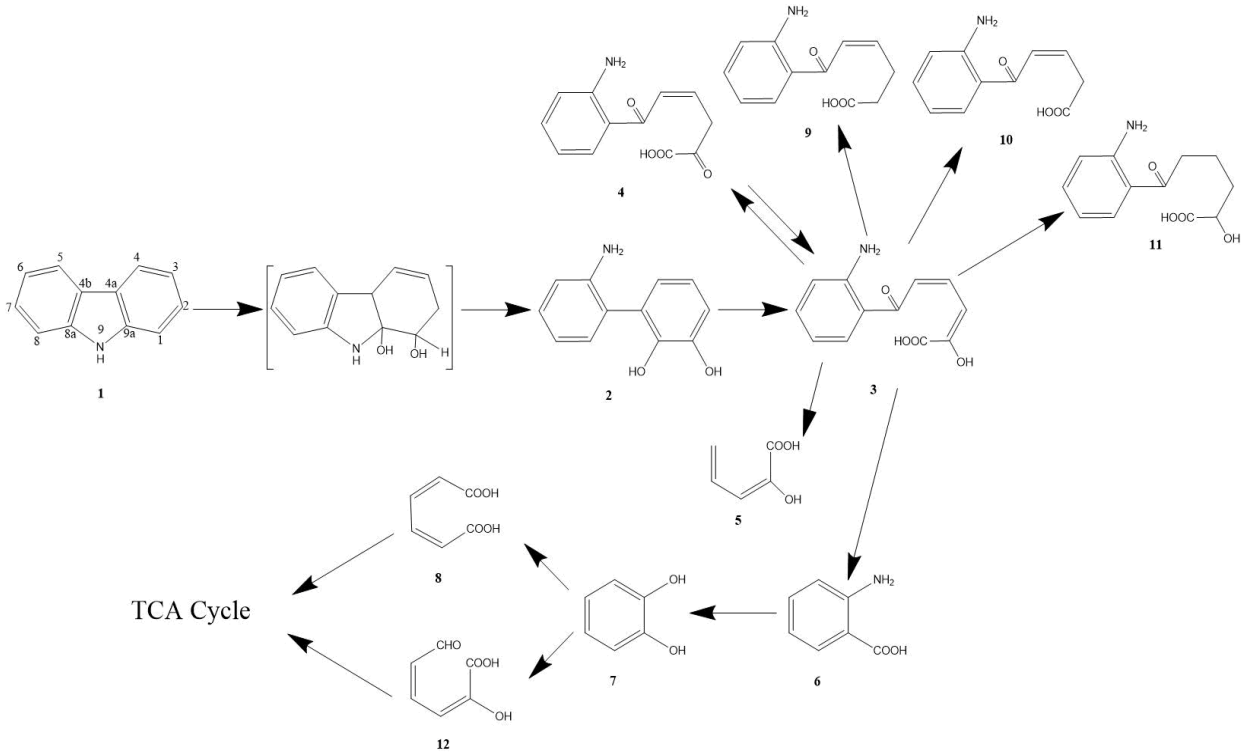


Figure 4. A proposed metabolic pathway for the degradation of carbazole through *Pseudomonas* spp. strain CA06, CA10 and *Pseudomonas stutzeri* OM1 [115, 125]. 1. Carbazole (CAR); 2. 2'-aminobiphenyl-2,3-diol; 3. 2-hydroxy-6-oxo-6-(2'-aminophenyl)hexa-2,4-dienoic acid; 4. 6-dioxo-6-(2'-aminophenyl)hexa-4-enoic acid; 5. 2-hydroxy-4-pentenoate; 6. AN; 7. CAT; 8. *cis,cis*-muconate; 9. 6-oxo-6-(2'-aminophenyl)hexa-4-enoic acid; 10. 5-oxo-5-(2'-aminophenyl)penta-3-enoic acid; 11. 2-hydroxy-6-oxo-6-(2'-aminophenyl)hexanoic acid; 12. 2-hydroxymuconic semialdehyde. Compound within bracket was not detected.

A functional CarA enzyme depends on three gene products, including CarAa (a catalytic oxygenase containing a Rieske [2Fe–2S] cluster and a mononuclear iron domain), CarAc (a ferredoxin component, containing a Rieske [2Fe–2S] cluster), and CarAd (a reductase component for electron transfer, containing FAD and [2Fe–2S] cluster) [177]. 2-aminobiphenyl-

2,3-diol 1,2-dioxygenase (CarB) is the second major enzyme that participates in the metabolization of carbazole. It is a multicomponent *meta*-cleavage dioxygenase encoded by both *carBa* and *carBb* genes [178]. CarB can metabolize 2-aminobiphenyl-2,3-diol to form HOADA. CarC is the third enzyme encoded by the *carC* gene and transforms HOADA to produce anthranilic acid via hydrolysis. CarC is also able to convert 2-hydroxy-6-oxo-6-(2,9-aminophenyl)-hexa-2,4-dienoic acid to a minor metabolite 2-hydroxypenta-2,4-dienoic acid [165]. CarD, which is a 2-hydroxypenta-2,4-dienoate hydratase, converts 2-hydroxypenta-2,4-dienoic acid to 4-hydroxy-2-oxovaleric acid, CarE which is a 4-hydroxy-2-oxovalerate aldolase then converts 4-hydroxy-2-oxovaleric acid to acetaldehyde and pyruvic acid, and CarF which is an acetaldehyde dehydrogenase that can then transform acetaldehyde to acetyl coenzyme A are all major enzymes encoded by single genes that were involved in the degradation of carbazole [175].

Bacterial dioxygenases often play an important role in the first step of azaarene metabolization [170]. Researchers found that naphthalene 1,2-dioxygenase (NDO) obtained from *Pseudomonas* sp. strain NCIB 9816-4 strain and biphenyl dioxygenase (BPO) from *Beijerinckia* sp. strain B8/36 can both oxidize carbazole [119]. The oxidation product 3-hydroxycarbazole was found after further analysis, while no further intermediates were detected, such as unstable *cis*-carbazole-3,4-dihydrodiol. Products of dioxygenation may therefore be further metabolized via direct monooxygenation process.

The filamentous fungi *Aspergillus flavus* and *Cunninghamella echinulate* were reported to metabolize carbazole to products different from those produced from bacterial isolates, including some monohydroxylated products [118, 136]. *Aspergillus flavus* VKM F-1024 has the ability to metabolize carbazole through its fungal monooxygenase system (Figure 5). Further

study demonstrated that 3-hydroxycarbazole was the main metabolite of carbazole degradation while 1-hydroxycarbazole and 2-hydroxycarbazole could also be detected as minor products. Carbazole derivatives in dihydroxylated forms mainly comprised 2,6-hydroxycarbazole. *Aspergillus flavus* could also transform N-benzoylcarbazole and N-acetylcarbazole. The metabolites of these two carbazole derivatives included carbazole, and small amounts of 1-hydroxycarbazole, 2-hydroxycarbazole, and 3-hydroxycarbazole. According to these results, the substitutent moiety influenced the position of hydroxylation. One report showed that *Cunnighamella elegans* could also oxidize carbazole to produce 3-hydroxycarbazole [117]. As shown above, filamentous fungi can introduce hydroxyl groups in different positions of the carbazole ring. The monohydroxylation products can be viewed as a result of detoxification; the same process can happen at the mammalian metabolism of carbazole due to the structural similarity of fungal and mammalian liver microsomes [136].

Other pathways for the biodegradation of carbazole may also exist (Figure 5). Research shows that *Ralstonia* sp. strain SBUG 290 can transform carbazole to 1-hydroxycarbazole, 3-hydroxycarbazole, 9-hydroxycarbazole and 3-hydroxy-1,2,3,9-tetrahydrocarbazol-4-one after the initial attack by a dioxygenase [149]. *Pseudomonas cepacia* strain F297 can also metabolize carbazole to produce 4-(3'-Methoxy-2'-indolyl)-2-oxo-3-butenoic acid after ring oxidation and the actions of a hydrolase [122]. Moreover, *Pseudomonas* sp. strain LD2 and *Flavobacterium* sp. strain OCM-1 were hypothesized to be able to oxidize carbazole to indole-3-acetic acid and other minor products due to ring-cleavage [124, 179].

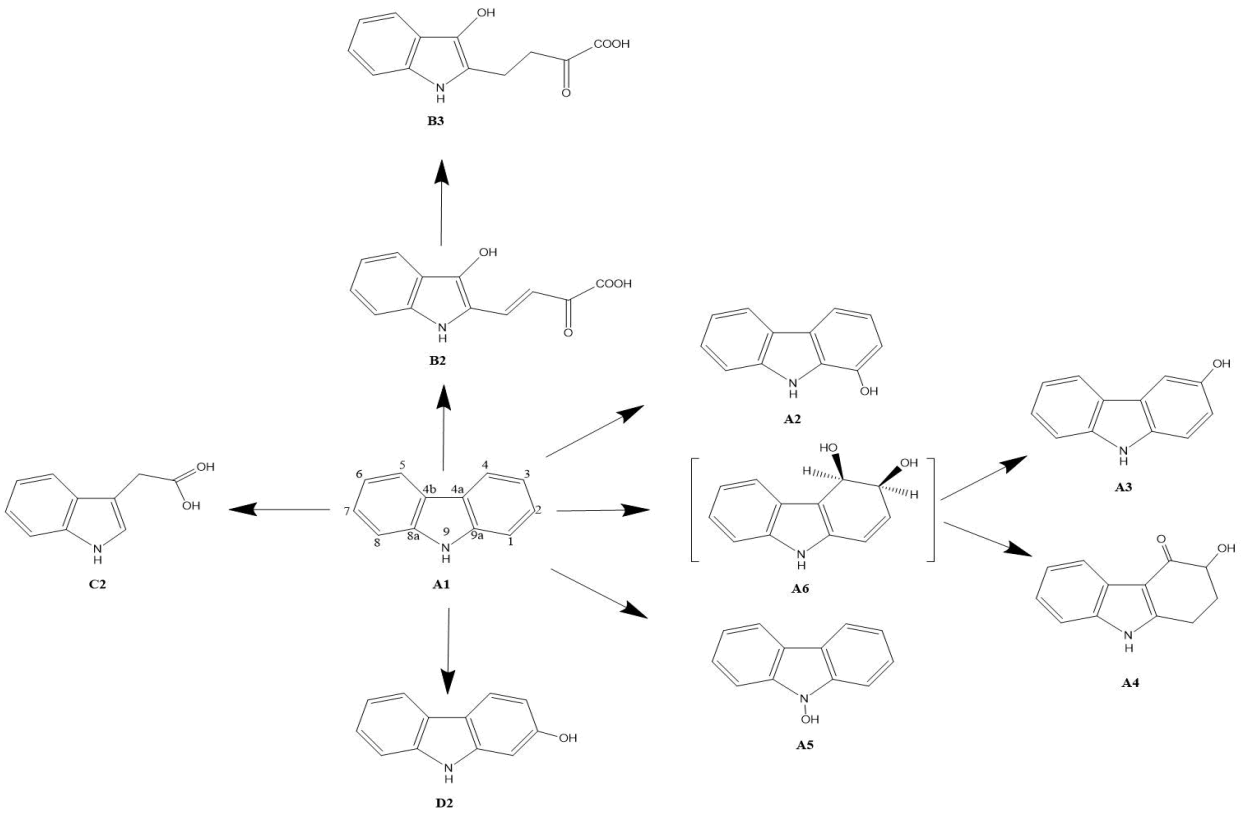


Figure 5. Other proposed metabolic pathways for the degradation of Carbazole (A1). 1. Proposed pathway through *Ralstonia* sp. strain SBUG 290 [149]: A2. 1-hydroxycarbazole, A3. 3-hydroxycarbazole, A4. 3-hydroxy-1,2,3,9-tetrahydrocarbazol-4-one, A5. 9-hydroxycarbazole, A6. *cis*-carbazole-3,4-dihydrodiol. 2. Proposed pathway through *Pseudomonas cepacia* strain F297 [122]: B2. 4-(3'-Methoxy-2'-indolyl)-2-oxo-3-butenoic acid, B3. 4-(3'-Oxo-2'-indolinyl)-2-oxo-3-butenoic acid. 3. Proposed pathway through *Pseudomonas* sp. strain LD2 [124]: C2. indole-3-acetic acid. 4. Proposed pathway through *Aspergillus flavus* VKM F-1024 [136]: A2. 1-hydroxycarbazole, A3. 3-hydroxycarbazole, D2. 2-hydroxycarbazole. Compound within bracket was not detected.

Though there are many studies on carbazole biodegradation, less attention has been given to understanding the metabolic pathways of carbazole biodegradation compared to PAHs. Applying microorganisms to the real-world environment to produce practical effects will require more *in situ* studies of the metabolic mechanisms.

2. Acridine

a. Introduction of Acridine

Although a number of studies have analyzed the environmental fate and metabolic pathways regarding carbazole degradation by soil bacteria [124, 180, 181], the environmental fate and biodegradation pathways of other toxic multi-ring azaarenes, such as acridine and phenanthridine, have received far less attention [182-184].

The toxic tricyclic nitrogen-containing compound acridine was first isolated by Carl Gräbe and Heinrich Caro from high boiling fraction of coal tar in 1870 [185]. It is an environmental contaminant widely distributed in coal tar, petroleum wastes, motor vehicle exhaust, tobacco smoke, and the photolysis of carbamazepine when exposed to UV radiation [186-188]. Acridine has been used widely in electronic and electroluminescence devices, and light-emitting diodes [189]. In contrast to carbazole, its structure has a pyridine-ring instead of a pyrrole-ring (Figure 3). Acridine has been reported as a toxic compound; inhibiting the growth of many aquatic organisms and causing oxidative stress for crustaceans [190-193]. It is also suggested to act as a mutagenic compound when activated in the presence of rat-liver extract and as a genotoxicant either animals or plants [188]. Because of its putative toxic, genotoxic and mutagenic characteristics, investigating the fate of acridine in soil is imperative [194, 195]. Although previous research has not shown adequate evidence to confirm acridine as a human carcinogen, compounds derived from it have been associated with carcinogenic characteristics [196]. Acridine itself was also reported to inhibit the DNA repair process and cell growth [190]. As a further step, understanding the biodegradation process of acridine can be essential to understand the environmental fate of acridine.

b. Acridine-Degrading Microorganisms

There is little prior research on the microbial degradation of acridine in soil. A few reports have indicated that bacterial, mammalian and fungal enzymes can transform acridine [188]. However, biodegradation of acridine by pure cultures of functional soil bacteria in a laboratory environment is rarely reported.

In one study investigating biodegradation of acridine under anaerobic conditions, a research group created lab-scale microcosms, including material from a mixed methanogenic culture from sewage sludge, a sulfate-reducing culture from alluvial sand aquifer contaminated by landfill leachate, and a methanogenic culture from alluvial sand aquifer [197]. At the first week, 1 $mg \cdot L^{-1}$ of acridine was added for enrichment of acridine-degrading microbial communities. Two weeks later, 5 $mg \cdot L^{-1}$ of acridine was added to each microcosm with only 0.06 $mg \cdot L^{-1}$ of acridine detected one week later. Prior to this report, there was almost no information on the transformation of azaarenes containing three or more rings azaarenes.

Sphingomonas sp. strain LH128 was originally isolated from polluted diesel soil and degraded phenanthrene and co-metabolized anthracene [198]. Although *Sphingomonas* sp. strain LH128 was not capable of using acridine as the sole carbon source, acridine was found to be co-metabolized [199]. In that test, strain LH128 was tested for the transformation of acridine in liquid phosphate-buffered media using phenanthrene as primary carbon source. A yellow-green color was observed in the incubations containing acridine and indicated the production of one or more dead-end metabolites. One metabolite was later identified as 9(10H)-acridinone [198]. It was concluded that the ability of strain LH128 to transform acridine might have been inhibited by high concentrations of acridine (ranging from 24.9 $mg \cdot L^{-1}$ to 58.2 $mg \cdot L^{-1}$).

The first acridine-degrading bacterium obtained in pure culture from contaminated soil was strain B1H1 that possessed 16S rRNA gene sequence similarity to sequences from the genus *Sphingomonas*, isolated from a former shipyard site [194]. Strain B1H1 was isolated after a thirteen-week enrichment and used 10 $mg \cdot L^{-1}$ acridine as a sole source of carbon and energy in liquid medium. It removed >80% of amended acridine in liquid medium after 18 days. In addition to strain B1H1, six more isolates from that study with the potential ability to transform acridine were reported with similarity to sequences from the genera *Rhodococcus*, *Pseudomonas*, *Bradyrhizobium*, *Rhodococcus*, and *Afipia* [194]. However, in contrast to strain B1H1, those six isolates could only transform acridine without growth.

Mycobacterium vanbaalenii strain PYR-1 was isolated from PAH-contaminated sediment of an oil tank farm and was reported as the first strain to be able to mineralize pyrene; it could additionally oxidize phenanthrene, fluoranthene, naphthalene, anthracene, and benz[a]anthracene [200]. Its capability to co-metabolize 47 $mg \cdot L^{-1}$ acridine in liquid medium for 7 days using phenanthrene to induce enzymes was investigated. Although a variety of both dioxygenases and monoxygenases are present in *Mycobacterium vanbaalenii* strain PYR-1 and four different metabolites associated with transformation of acridine were detected (see below), strain PYR-1 was not able to mineralize acridine [188, 201].

A mutant strain of *Pseudomonas fluorescens* strain TTC1 was determined to transform acridine [202]. Strain TTC1 was derived from the naphthalene-degrading *Pseudomonas fluorescens* strain N3, isolated from sewage sludge of a wastewater treatment plant [203, 204]. Tests using 4 $g \cdot L^{-1}$ acridine were performed with resting cells of strain TTC1 induced by acetylsalycilic acid to increase dioxygenase activity. After 24 hours, 0.61 $g \cdot L^{-1}$ acridine *cis*-1,2-dihydrodiol was detected by HPLC analysis.

The ability of fungi to remove acridine in the soil is rarely reported even though many fungi can transform anthracene, the PAH analogue of acridine [70]. In one example, *Cunninghamella elegans* ATCC 36112 transformed 10 $mg \cdot L^{-1}$ acridine in liquid Sabouraud medium over three days incubation [117, 205]. Interestingly, only 4% of acridine remained indicating that acridine was either efficiently transformed or alternatively, quantities of the hydrophobic compound may have been absorbed into the fungal mycelia.

c. Metabolic Pathways of Acridine Degradation

Currently, only fragmentary information exists regarding any microbial metabolic pathway for acridine degradation (Figure 6). *Pseudomonas fluorescens* TTC1 was reported to produce acridine *cis*-1,2-dihydrodiol [202]. An initial naphthalene dioxygenase was determined to be responsible for this transformation. Interestingly, the tolerance of this naphthalene dioxygenase for high concentrations of acridine (4 $g \cdot L^{-1}$) indicate that it may be worth further investigation for *in situ* bioremediation purposes or during biotransformation of other azaarenes. Another metabolite of acridine transformation was produced by *Sphingomonas* sp. LH128 and identified as 9(10H)-acridinone [198]. It was also found as a major metabolite of acridine by zebra mussels, green algae, and bacteria in aquatic environments [206]. In comparison to acridine, 9(10H)-acridinone was reported as less acutely genotoxic for an aquatic invertebrate midge, however, it is still considered genotoxic according to the Mutatox^{TM} test [206].

Mycobacterium vanbaalenii strain PYR-1 co-metabolized acridine to four different compounds in the presence of phenanthrene: acridine *cis*-1,2-dihydrodiol, 4-hydroxyacridine, 9(10H)-acridinone, and 9,10-dihydroacridine, with 9,10-dihydroacridine as the most abundant metabolite (Figure 6) [188]. The initial step of phenanthrene degradation in strain PYR-1 occurs through a ring-hydroxylating dioxygenase (RHD) resulting in *cis*-dihydrodiol [207]. The activity

of a similar RHD was likely responsible for the presence of acridine *cis*-1,2-dihydrodiol by strain PYR-1. 4-Hydroxyacridine, however, was likely formed due to the activity of cytochrome P450 monooxygenase through a different pathway [208]. 9(10H)-Acridinone and 9,10-dihydroacridine may have been produced through either biological or abiotic processes [209, 210]. While 9(10H)-acridinone is considered a less toxic compound than acridine, the toxicity of the other three compounds still need to be investigated.

The transformation of acridine by the fungus *Cunninghamella elegans* ATCC 36112 produced acridine trans-1,2-dihydrodiol and 2-hydroxyacridine (Figure 6) [205]. These two metabolites of acridine were different than acridine metabolites produced by bacteria.

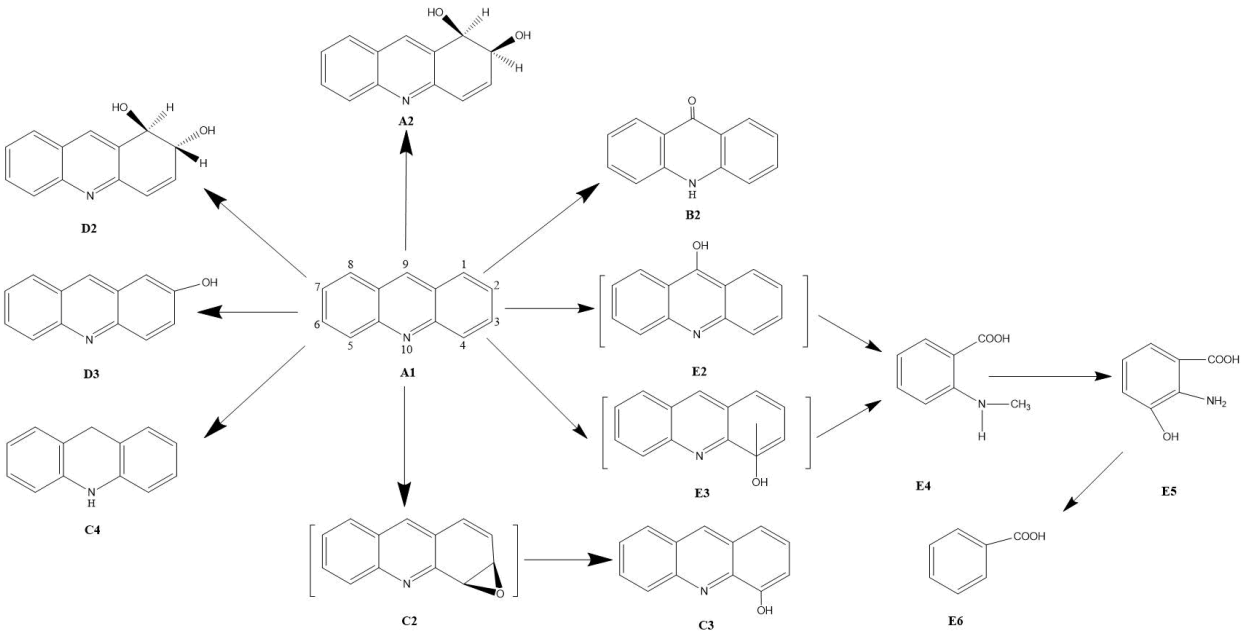


Figure 6. Proposed metabolic pathways for the degradation of acridine (A1). 1. Proposed pathway through *Pseudomonas fluorescens* TTC1 [202]: A2. acridine *cis*-1,2-dihydrodiol. 2. Proposed pathway through *Sphingomonas* sp. LH128 [211]: B2. 9(10H)-acridone. 3. Proposed pathway through *Mycobacterium vanbaalenii* PYR-1 [188]: A2. acridine *cis*-1,2-dihydrodiol, B2. 9(10H)-acridone, C2. acridine 3,4-oxide, C3. 4-hydroxyacridine, C4. 9,10-dihydroacridine. 4. Proposed pathway through *Cunninghamella elegans* ATCC 36112 [205]: D2. acridine trans-1,2-dihydrodiol, D3. 2-hydroxyacridine. 5. Proposed pathway under the anaerobic condition [197]: E2. 9-hydroxyacridine, E3. 3-hydroxyacridine or 4-hydroxyacridine, E4. N-methylanthranilic acid, E5. 3-hydroxyanthranilic acid, E6. benzoic acid. Compounds within bracket was not detected.

Researchers assumed that the first oxidation step of acridine involved a cytochrome P-450 monooxygenase to produce acridine 1,2-oxide [205, 212]. The trans-1,2-dihydrodiol could be formed after the oxide was metabolized by epoxide hydrolase [213]. This pathway is similar to one previously reported for the transformation of anthracene to anthracene trans-1,2-dihydrodiol in *Cunninghamella elegans* [214]. The presence of 2-hydroxyacridine might be explained by a nonenzymatic rearrangement of acridine 1,2-oxide [215].

Under anaerobic conditions, a few metabolites of acridine were identified through a microcosms-based study and the possible pathways of acridine were presented (Figure 6) [197]. The metabolic route may begin with oxidation of either a benzene or pyridine ring to produce either 3-hydroxyacridine, 4-hydroxyacridine or 9-hydroxyacridine. All of these products can be converted to benzoic acid, a key intermediate for oxidized aromatic compounds [197, 216].

3. Phenanthridine

a. Introduction of Phenanthridine

Phenanthridine was first discovered in 1889 by Amé Pictet and H. J. Ankersmit through pyrolysis of the condensation product of benzaldehyde and aniline [217]. Similar to carbazole and acridine, phenanthridine is an azaarene widely distributed at contaminated sites due to its high water solubility and lower K_{ow} values [95, 184]. Phenanthridine can be used in the production of industrial solvents, dyes, explosives, and pharmaceuticals [216]. Although there is insufficient evidence to confirm the carcinogenic property of phenanthridine in humans, it was reported as acutely toxin to green algae, invertebrates, and fish [192, 206, 218-220]. Phenanthridine was also found mutagenic for the bacterium *Salmonella enterica* serovar typhimurium strain TA100 [221]. However, few investigations have studied phenanthridine biodegradation in contaminated soil.

b. Phenanthridine-Degrading Microorganisms

Streptomyces viridosporus strain T7A was reported to be able to co-metabolize five heterocyclic nitrogen-containing compounds, including phenanthridine, isoquinoline, phthalazine, quinazoline, and quinoxaline, when growing in tryptone/yeast extract broth [182]. Strain TA7 was isolated for its ability to degrade substituted benzoic acids and it was the first isolate from soil reported to demonstrate microbial oxidation of phenanthridine [222]. In one test, 25% of phenanthridine was transformed by strain TA7 to 6(5H)-phenanthridinone during a 3-day transformation test (initial concentration 412 $mg \cdot L^{-1}$).

The phenanthrene-degrading bacterium *Mycobacterium gilvum* strain LB307T was also found to be able to transform phenanthridine [183]. While neither 2.5 $mg \cdot L^{-1}$ or 20 $mg \cdot L^{-1}$ phenanthridine supported the growth of strain LB307T, samples amended with 2.5 $mg \cdot L^{-1}$ phenanthridine demonstrated complete removal of phenanthridine over an 8-hour incubation. However, no degradation of phenanthridine was detected in samples containing 20 $mg \cdot L^{-1}$ phenanthridine, even after 215 hours of incubation, and no metabolites related to the transformation of phenanthridine were detected from incubations. This indicated a possible inhibitory effect of phenanthridine on this strain at high concentrations.

The phenanthrene-degrading *Sphingomonas* sp. strain LH128 was also tested for phenanthridine degradation and co-metabolized phenanthridine using phenanthrene as primary carbon source [211]. Similar to acridine transformations by strain LH128, a yellow-green color was observed in incubations containing phenanthridine when added at concentrations ranging from 0.54 $mg \cdot L^{-1}$ to 36.9 $mg \cdot L^{-1}$, indicating the presence of metabolites. One such metabolite was identified as 6(5H)-phenanthridinone. In contrast to the inhibitory effect caused by acridine

at concentrations >24.9 mg·L^{-1} for strain LH128, no inhibitory effect was observed even at the highest phenanthridine concentration (36.9 mg·L^{-1}).

To the best of our knowledge, strain B1L4, which was isolated from a former shipyard site and possessed 16S rRNA gene sequence similarity to *Rhodococcus* sp., was the first reported isolate capable of using phenanthridine as a sole source of carbon and energy [194]. A liquid culture amended with 5 mg·L^{-1} phenanthridine demonstrated significant increase in turbidity relative to uninoculated controls. However, less than 20% phenanthridine was transformed by a strain, AB010907, over an 18-day incubation. Other genetically diverse phenanthridine-transforming isolates from that same study possessed 16S rRNA gene sequence similarity to sequences from the genera *Methylopila*, *Ralstonia*, *Xanthobacter*, *Afipia*, *Achromobacter*, *Sphingomonas*, and *Pseudomonas*. Isolates with low similarity to known genera were also recovered. These 13 isolates were able to transform either 5 mg·L^{-1} or 20 mg·L^{-1} of phenanthridine in liquid medium but could not utilize phenanthridine as a source of carbon. Additionally, none of these isolates showed the ability to mineralize ^{14}C-labeled phenanthrene, which has structural similarities to phenanthridine, indicating that the mechanism involved in the initial attack of phenanthridine may be different than the mechanism for phenanthrene.

Fungi have also been reported to be able to metabolize phenanthridine. The fungus *Umbelopsis ramanniana* was reported to transform >70% of phenanthridine in liquid medium (41 mg·L^{-1}) over 18 days [223].

c. Metabolic Pathways of Phenanthridine Degradation

Similar to acridine, the metabolic pathways of phenanthridine degradation by microorganisms are rarely reported (Figure 7). It is possible that metabolites could be transformed by microorganisms into other transient metabolites which might be further

transformed by other bacteria [183]. The bacterium *Streptomyces viridosporus* strain T7A is capable of transforming phenanthridine to 6(5H)-phenanthridinone, which was the only metabolite detected [182]. 6(5H)-phenanthridinone was also detected after the transformation of phenanthridine by *Sphingomonas* sp. strain LH128 [211] and the larvae of the aquatic invertebrate *Chironomus riparius* [224].

The fungus *Umbelopsis ramanniana* can transform phenanthridine to phenanthridine N-oxide and 6(5H)-phenanthridinone (Figure 7) [223]. Phenanthridine N-oxide was also reported as one of the products of phenanthridine transformation by rat-liver homogenate [225]. Other major metabolites related to the transformation of phenanthridine by polychlorinated biphenyls (PCB)-induced rat-liver homogenate include 6(5H)-phenanthridinone, 9,10-dihydroxy-9,10-dihydrophenanthridine, 1,2-dihydroxy-1,2-dihydrophenanthridine, and 2-hydroxyphenanthridine. Phenanthridine N-oxide can also be produced by CYP450 via oxidization of phenanthridine, and this compound was reported as non-mutagenic for *Salmonella enterica* [220, 226]. However, 6(5H)-phenanthridinone was mutagenic but not tumorigenic for mice [227, 228]. The metabolites of phenanthridine metabolism therefore cannot be completely considered detoxified even if the toxic parent compound phenanthridine is transformed.

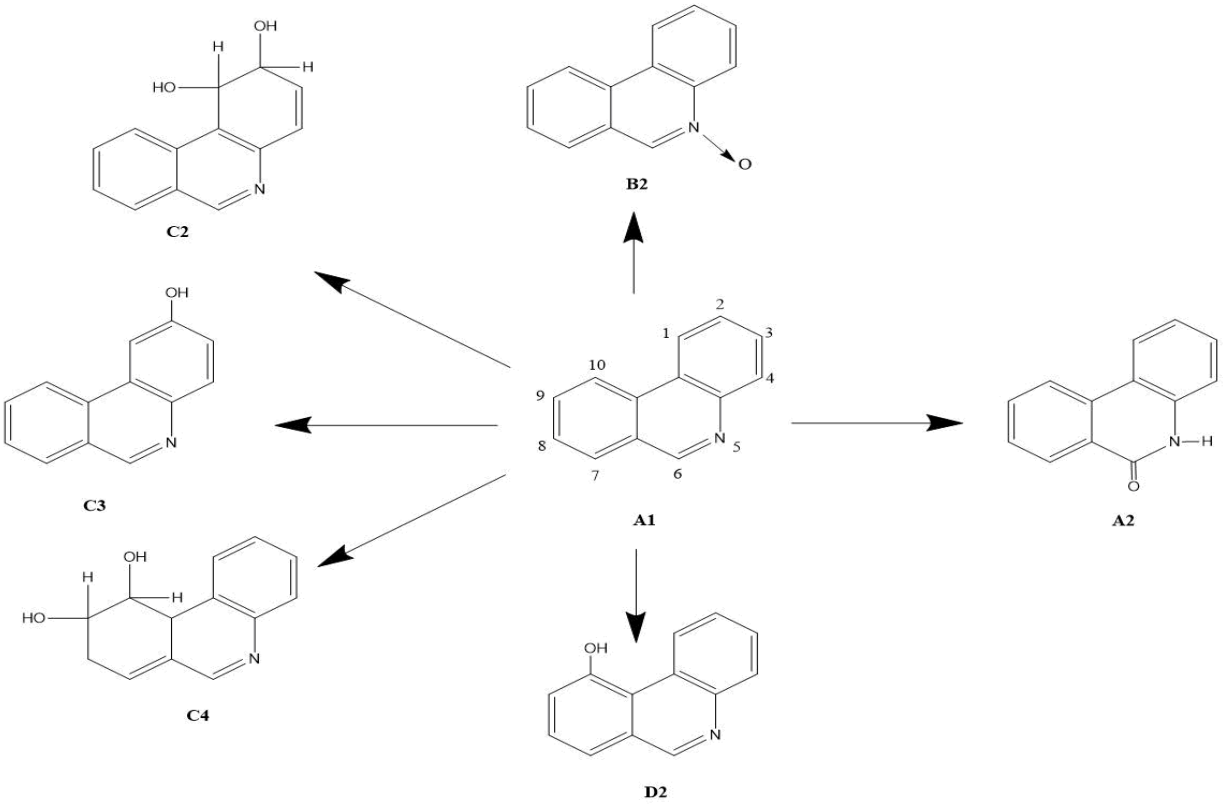


Figure 7. Proposed metabolic pathways for the degradation of phenanthridine (A1). 1. Proposed pathway through *Streptomyces viridosporus* strain T7A [182]: A2. 6(5H)-phenanthridinone. 2. Proposed pathway through *Umbelopsis ramanniana* [223]: B2. phenanthridine N-oxide. 3. Proposed pathway through rat-liver homogenate [225]: A2. 6(5H)-phenanthridinone, B2. phenanthridine N-oxide, C2. 1,2-dihydroxy-1,2-dihydrophenanthridine, C3. 2-hydroxyphenanthridine, C4. 9,10-dihydroxy-9,10-dihydrophenanthridine. 4. Proposed pathway through *Escherichia coli* and *Streptomyces lividans* transformants [229]: C2. 1,2-dihydroxy-1,2-dihydrophenanthridine, C4. 9,10-dihydroxy-9,10-dihydrophenanthridine, D2. 10-hydroxyphenanthridine.

9,10-dihydroxy-9,10-dihydrophenanthridine, 1,2-dihydroxy-1,2-dihydrophenanthridine, along with a novel product 10-hydroxyphenanthridine were also reported as the metabolites of phenanthridine in a transformation experiment of *Escherichia coli* cells recombined with genes of phenanthrene dioxygenase originally obtained from the marine bacterium *Nocardioides* sp. strain KP7 and *Streptomyces lividans* transformants carrying phenanthrene dioxygenase genes [229].

CHAPTER 3: METHODS

A. Chemicals

Carbazole (GC grade, ≥95% purity), acridine (97%), and phenanthridine (98%) were obtained from Sigma-Aldrich (St. Louis, MO, USA). Anthracene (scintillation grade) was purchased from Eastman Kodak (Rochester, NY, USA). Phenanthrene (97%) was obtained from Acros Organics (Thermo Fisher Scientific, New Jersey, USA). Other chemicals used for this research project were the highest available purity.

B. Soil Preparation

Contaminated soil was obtained from the site of a former manufactured gas plant (MGP) situated in Salisbury, North Carolina, USA. Soil properties were tested as previously described [230]. Soil samples were processed by air drying, sieving through 10 mm wire-mesh, mixing, and re-screening through 10 mm wire-mesh. After resuspension in a buffer containing phosphorus (Na-K phosphate buffer, 5 mM; pH 7.5) and nitrogen (NH_4NO_3, 2.5 mM; "reactor buffer") and screening through a 3.35 mm wire-mesh screen to remove rocks and other objects, the processed soil was transferred to a 2.5 L lab-scale, aerobic, and semi-continuously operated slurry-phase (15% soil solids, w/v) bioreactor. Every 7 days 20% of the slurry volume inside the reactor was removed and replaced with slurry newly created from untreated ("feed") soil in reactor buffer [231]. Unused feed soil was stored in a sealed container at 4°C. Bioreactor-treated slurry samples for this experiment were taken directly from the bioreactor as needed at the time of the experiment.

C. Quantification of Azaarenes

A high-performance liquid chromatography (HPLC) system method was developed to quantify the disappearance of azaarenes and select PAHs. The HPLC system included a Waters (Milford, MA, USA) 600E system controller, a Waters 717 Plus autosampler, an Alltech (Deerfield, Illinois, USA) 330 column heater, a Perkin Elmer (Beaconsfield, UK, USA) LS40 fluorescence detector, and a Kratos (Chestnut Ridge, NY, USA) Spectroflow 757 UV absorbance detector.

For the quantification of the PAHs phenanthrene and anthracene, analyte standards for calibration curves were acquired from dilutions of an EPA 610 polynuclear aromatic hydrocarbons mixture stock (Sigma-Aldrich, MO, USA). Samples were injected through a 3 μm particle-size C18 Supelcosil™ LC-PAH column (Sigma-Aldrich, MO, USA). The analysis method had a run time of 26 minutes which included a 6 minute delay between samples to avoid retention time differences [232]. The initial conditions of the mobile phase 60% acetonitrile and 40% water with the flowrate of 1 $mL \cdot min^{-1}$. The proportion of acetonitrile was linearly increased from 60% to 100% within first 10 minutes, and the flowrate increased from 1 $mL \cdot min^{-1}$ to 2 $mL \cdot min^{-1}$ at 12.5 min. Fluorescence detection was used for the quantification of PAHs.

Analyte standards of azaarenes (carbazole, acridine, and phenanthridine) in methanol ranging in concentration from 10 $mg \cdot L^{-1}$ to 0.625 $mg \cdot L^{-1}$ were prepared. UV detection was used for the quantification of azaarenes. Samples were injected through a 3 μm particle-size Luna ™ phenyl-hexyl column (Phenomenex, CA, USA). The analysis method for azaarenes had a total running time of 27 minutes including a 7-minute delay between samples to avoid retention time differences. The initial condition of the mobile phase was 70% acidic reagent water (acidified to pH 3 with formic acid) and 30% methanol with a constant flowrate of 0.21 $mL \cdot min^{-1}$. The

proportion of methanol was increased linearly from 30% to 70% over the first 14 minutes of each sample run, then increased to 95% at 16 minutes and kept constant for 6 minutes. The mobile phase was returned to initial conditions at 22 minutes. UV detection at 255 nm was used for the quantification of azaarenes.

D. Isolation of Putative Azaarene-Degrading Bacteria

To isolate putative azaarene-degrading bacteria, enrichments with both feed soil and bioreactor-treated soil were created. Carbazole, acridine, and phenanthridine stock solutions (50 $mg \cdot mL^{-1}$) were created by dissolving chemicals individually in acetone. 1 mL of each azaarene stock solution was then added individually to sterile, 1 L glass, screw-top, media storage bottles (final concentration of 200 $mg \cdot L^{-1}$) and caps left loose overnight to allow the organic solvent to evaporate. Incubations containing 250 mL of sterilized bioreactor buffer, and either 5 g of feed soil or 5 g bioreactor-treated soil (dry weight) were added to the 1 L glass screw-top media storage bottles. Enrichment cultures were incubated for 16 days at room temperature (20 – 23°C) with shaking at 180 rpm.

To isolate putative azaarene-degrading bacteria from the soil enrichments, carbazole, acridine, or phenanthridine were individually mixed into sRB media containing 1.5% agar (Acros Organics, NJ, USA). sRB Medium was composed of (per L): 984 mL of bioreactor buffer (5 mM Na-K phosphate buffer, 5 mM NH_4NO_3; pH 7.0), 0.1mL of 1 M $MgSO_4 \cdot 7\ H_2O$, 0.1mL of 1 M $CaCl_2 \cdot 2\ H_2O$, 1 mL of trace element solution (12.5 mL 25% HCl, 30 mg H_3BO_3, 2.1 g $FeSO_4 \cdot 7\ H_2O$, 100 mg $MnCl_2 \cdot 4\ H_2O$, 190 mg $CoCl_2 \cdot 6\ H_2O$, 24 mg $NiCl_2 \cdot 6H_2O$, 2 mg $CuCl_2 \cdot 2\ H_2O$, 144 mg $ZnSO_4 \cdot 7\ H_2O$, 36 mg $Na_2MoO_4 \cdot 2\ H_2O$ per liter), and 1 mL of Se-W solution (0.4 g NaOH, 6 mg $Na_2SeO_3 \cdot 5\ H_2O$, 8 mg $Na_2WO_4 \cdot 2\ H_2O$ per liter), sterilized by autoclaving for 15 minutes, 250°C. After cooling, 0.2 μm membrane-filtered 1 $mL \cdot L^{-1}$ vitamin solution (containing

4 mg p-aminobenzoic acid, 1 mg biotin, 10 mg nicotinic acid, 5 mg Ca-pantothenate, 15 mg pyridoxine-HCl, in 100 mL 10 mM phosphate buffer at pH 7.1), 1 $mL \cdot L^{-1}$ thiamine solution (10 mg thiamine dissolved in 100 mL 25 mM sodium phosphate buffer at pH 3.4), 1.5 $mL \cdot L^{-1}$ thiosulfate solution (24.8 g $Na_2S_2O_3 \cdot 5\ H_2O$ dissolved in 100 mL deionized water, sparged with 100% N_2 gas) and 1 $mL \cdot L^{-1}$ vitamin B12 solution (5 mg cyanocobalamin dissolved in 100 mL deionized water) were added. Carbazole, acridine, and phenanthridine (final concentration of 50 $mg \cdot L^{-1}$) were added individually to sRB-agar in solvent prior to pouring plates to create enriched sRB-agar.

After 7 and 14 days of incubations, 1 mL samples were removed from each azaarene enrichment. Serial dilutions (10^{-1} to 10^{-6}; in 0.85% NaCl) from each enrichment were created and 100 µL of liquid from the 10^{-3} to 10^{-6} dilutions were spread onto the surface of sRB-agar plates containing azaarenes (final dilutions 10^{-4} to 10^{-7}). Plates were incubated at room temperature (~20 – 23°C) for 14 to 42 days. Isolated colonies were transferred to R2A (MD Difco, MD, USA) plates.

To test whether isolates could use carbazole, acridine, or phenanthridine as a sole carbon source, select pure cultures grown on R2A were transferred to liquid sRB medium containing individual azaarenes. Azaarenes dissolved in acetone (final concentration 7.5 mg/L) were first added to duplicate, sterilized glass, screw-top test tubes with the caps loosened to allow the organic solvent to evaporate. 5 mL of sterilized liquid sRB medium was then added to each tube and an ultrasonic water bath was used to mix azaarenes and the sRB medium. Inoculated tubes were incubated at 30°C and 225 rpm. The turbidity and any color change of the liquid medium compared to negative controls were recorded by visual observation.

To obtain cells for PCR analyses and strain storage, a 1 mL sample was taken from each tube with observed turbidity or color change, centrifuged at 15,000 rpm for 5 minutes, and resuspended in 1 mL sRB medium. This wash process was repeated a total of 3 times. A 100 µL aliquot of suspended cell solution was transferred to a 15 mL Falcon centrifuge tube (Corning Life Science, NY, USA) containing 5 mL R2A broth (Himedia Laboratories, India) and incubated at 30°C and 225 rpm for up to several days. After growth, strains were stored in R2A broth and glycerol (final concentration 15% v/v) at -80 °C.

Isolation of bacteria responsible for co-metabolizing phenanthridine. An additional enrichment incubation was set up to isolate bacteria responsible for co-metabolizing phenanthridine and acridine. Phenanthrene, acridine, and phenanthridine stock solutions (7.5 $mg \cdot mL^{-1}$ each) were prepared by dissolving chemicals in methanol. 200 µL of each chemical (final concentration 50 $mg \cdot mL^{-1}$) was added individually to sterile, 125 mL glass screw-top flasks and the caps were left loosened overnight to allow the organic solvent to evaporate. 30 mL of sterilized bioreactor buffer, and 0.5 g bioreactor-treated soil (dry weight) were then added to the flask. The enrichment was incubated for 14 days at room temperature (20 – 23°C) with shaking at 180 rpm.

After 14 days incubation, 1 mL of an enrichment sample was removed from the flask and processed with the same isolation method described above. A 100 µL of aliquot 10^{-3} to 10^{-6} dilutions were spread onto sRB-agar plates supplemented with phenanthrene (final dilutions 10^{-4} to 10^{-7}). Plates were incubated at room temperature (~20 – 23°C) for 21 days. One isolate that created dark zones around colonies on the plate was transferred to R2A plates. This isolate was then transferred to duplicate tubes containing liquid sRB medium amended with phenanthrene and phenanthridine (50 $mg \cdot L^{-1}$ each). Inoculated tubes were incubated at 30°C and 225 rpm. The

turbidity of the liquid medium compared to negative controls was recorded by visual observation.

PCR of 16S rRNA Genes. PCR was performed to obtain the partial 16S rRNA gene sequence of select putative azaarene-degrading isolates. Each reaction contained 1 μL fresh cell culture grown in R2A broth, 1.25 μL 10 μM forward primer 27F (5'→3': AGAGTTTGATCMTGGCTCAG), 1.25 μL 10 μM reverse primer 1492R (5'→3': TACGGYTACCTTGTTACGACTT), 8 μL 5 prime MasterMix (5 Prime GmbH, Germany), and 8.5 μL dH_2O for a 20 μL final volume. The PCR program was 95°C for 10 min, 29 cycles of 95°C for 30 seconds, 55°C for 30 seconds, 72°C for 1.2 minutes, and a final dwell of 72°C for 10 minutes on an Eppendorf Mastercycler Gradient thermal cycler (Westbury, NY, USA).

PCR amplicons were analyzed by 1% agarose gel electrophoresis in 1X Tris-acetate-EDTA buffer (TAE). Each gel contained 7 μL Invitrogen SYBR Safe DNA Gel Stain (Thermo Fisher Scientific, NY, USA) added prior to casting. PCR products for sequencing were submitted to Eton Bioscience Inc. (Research Triangle Park, NC, USA) using 10 μM of forward primer 27F. The sequence was analyzed using BioEdit Sequence Alignment Editor 7.2.5 (Ibis Biosciences, CA, USA) and Sequencher 5.4.1 (Gene Codes, MI, USA). The closest 16S rRNA genes were determined using Basic Local Alignment Search Tool (BLAST) of the GenBank database [233] and the Sequence Match tool of the Ribosomal Database Project (RDP) [234].

E. Growth Tests

A novel designed test was performed to determine the ability of strains to grow on carbazole, acridine, or phenanthridine, as well as a variety of other PAHs and chemical compounds present in contaminated soil as sole carbon sources. A total of 26 different solvent-dissolved carbon sources were added in triplicate to 96-well microplate wells and the solvent

allowed to evaporate overnight (final chemical concentration of 320 mg/L; Figure 8). As this method relied on increases in protein mass to determine cellular growth, steps were first taken to determine the optimal concentration of inoculum. Fresh cell cultures grown in R2A broth were washed 3 times with sRB liquid medium. In a separate 96-well plate, 125 µL sRB liquid medium and varying volumes of cell suspension (20 µL, 10 µL, 5 µL, 2.5 µL, 1.25 µL, and 0 µL) washed in sRB liquid medium were added to wells in triplicate. 125 µL of Pierce BCA Protein Assay (50 parts of reagent A to 1 part of reagent B; Thermo Fisher Scientific, NY, USA) were added to each well and microplates were incubated for 45 minutes at 60°C. The optimum inoculum concentration was determined to be the wells with the lowest volume that also displayed a color change. This volume of cells was used to inoculate the 96-well plate containing carbon sources. A control plate containing carbon sources inoculated with 125 µL sRB liquid medium without cells was incubated identically. The microplates were placed in a temperature-controlled incubator for 30 days at 30°C.

To measure biomass in wells, protein concentration tests were performed by the Pierce BCA Protein Assay. After 30 days, a Sorvall Legend RT centrifuge (Thermo Fisher Scientific, NY, USA) with a Sorvall Heraeus 75006449 S rotor was used to condense liquid in the wells by spinning at 800 rpm for 30 seconds before the liquid was transferred to a new 96-well plate (solvent damaged the incubation plate, potentially affecting microplate readings). A bovine serum albumin (BSA) dilution series was created ranging from 25 to 2,000 µg/mL to obtain a protein concentration standard curve. Protein concentrations in each well were determined using a Tecan Austria GmbH 5082 (Grödig, Austria) microplate reader at 620 nm. Absorbance data were converted to protein concentrations using the BSA standard curve.

Fluorene	Benzo(k)fluoranthene	Benzo(a)anthrenequinone	sRB
Phenanthrene	Benzo(a)pyrene	Carbazole	sRB + Pyruvate
Anthracene	Dibenzo(a,h)anthracene	Phenanthridine	R2A
Fluoranthene	Benzo(g,h,i)perylene	Acridine	
Pyrene	9-Fluorenone	Salycilate	
Benzo(a)anthracene	9,10-Phenanthrenequinone	Phthalate	
Chrysene	9-Anthrone	Hexadecane	
Benzo(b)fluoranthene	9,10-Anthrenequinone	sRB w/o cells	

Figure 8. List of carbon sources and distribution in 96-well plates.

F. Tests of Azaarene Concentration on Soil Microbial Activity

The potential inhibitory effect of high concentrations of either carbazole, acridine, or phenanthridine on microbial activity in feed soil and bioreactor-treated soil was investigated. Four different concentrations were tested for each azaarene: 50 $mg \cdot L^{-1}$, 25 $mg \cdot L^{-1}$, 10 $mg \cdot L^{-1}$, and 5 $mg \cdot L^{-1}$. Azaarene stock solutions (with acetone as solvent) were added to glass, sterile, screw-cap test tubes in triplicate and left open overnight in a chemical fume hood to evaporate solvent. After adding 5 mL sRB liquid medium into tubes, vortexing and an ultrasonic bath were used to mix azaarenes into the sRB liquid medium. A 0.5 grams (dry weight) aliquot of either feed soil or bioreactor-treated soil was then added to each tube. Controls included tubes with only sRB liquid medium, feed soil or bioreactor-treated soil without spiked azaarene, tubes with 0.2% sodium pyruvate solution instead of an azaarene, and tubes containing azaarenes without soil. All tubes were placed in a temperature-controlled shaking incubator at 30°C and 225 rpm. Initially, turbidity measured by spectrophotometric measurements was used to track microbial growth over the course of two weeks. However, this method was ineffective due to soil constituents as well as undissolved azaarene crystals in the tubes. To overcome these effects, a new inoculum was created by mixing 1 mL of incubation samples from each of the four concentrations (50 $mg \cdot L^{-1}$, 25 $mg \cdot L^{-1}$, 10 $mg \cdot L^{-1}$, 5 $mg \cdot L^{-1}$) into a sterile Falcon centrifuge tube

(Corning Life Science, NY, USA) to obtain 4 mL mixed inoculum. After vortexing, a 100 µL aliquot was used as inoculum into new tubes with the same carbon source and 4.9 mL of freshly prepared sRB liquid medium. For this experiment, azaarene concentrations of only 50 $mg \cdot L^{-1}$ and 10 $mg \cdot L^{-1}$ were tested but controls were otherwise identical to the prior setup. Each concentration was prepared in triplicate. All tubes were placed in temperature-controlled shaking incubator at 30°C and 225 rpm. Spectrophotometric measurements of turbidity at OD_{600} were measured over two weeks.

G. Chemical Analysis of PAH and Azaarene Disappearance in Soils

Anthracene, phenanthrene, and carbazole were tested as possible primary carbon sources for removal of acridine and phenanthridine through co-metabolism. Azaarene and PAH stock solutions were created consisting of 20 mg dissolved in 4 mL acetone (final concentration of 5 $g \cdot L^{-1}$). A 10% slurry of the feed soil was created in reactor buffer and screened through a 3.35 mm wire-mesh screen. For bioreactor-treated soil, slurry was centrifuged at 5000 rpm for 20 minutes to pellet soil, the supernatant discarded, and the pellet was re-suspended in an equal volume of fresh reactor buffer. The centrifugation and resuspension process was repeated 3 times to wash the soil completely. A 0.05 mL aliquot of the chemical stock solution was added to triplicate, sterile, screw-top 40-mL amber EPA vials using a Hamilton glass HPLC syringe to obtain final concentration of 50 $mg \cdot L^{-1}$. The solvent was allowed evaporate under a stream of nitrogen gas. 3.7 mL of bioreactor buffer was then added and the vials placed in an ultrasonic water bath to break up chemical crystals. 1.3 mL of soil slurry was added to the reactor buffer to reach 5 mL total volume. Acidified controls were prepared by adjusting the medium to pH 2 by adding 85% phosphoric acid. All vials were placed on a shaker at room temperature (~20–23°C) with shaking at 180 rpm for 21 days.

After incubation, the pH of each vial was determined and adjusted to pH ≥ 7 if needed. Vials for time 0 incubations were extracted immediately after adjusting pH. Azaarenes and PAHs were extracted by adding 10 mL of ethyl acetate (15 mL ethyl acetate to acidic controls) directly and shaking vials on a wrist-action shaker for 12 hours at 160 rpm. The top organic layer of each vial was filtered through a 0.22 µm pore-size nylon filter (Millipore, MA, USA) and 1 mL filtered sample stored in 2 mL amber chromatography vials at -20°C until further analysis. Extraction samples were diluted with methanol to reach proper concentration prior to HPLC analyses as described earlier.

H. Molecular Analysis

The microbial communities of feed soil and bioreactor-treated soil communities in response to exposure to tested azaarenes, PAHs, and combinations of the chemicals were analyzed. Incubations in sterile, screw-top, 40-mL amber EPA vials included 5 mL of soil slurry (0.2 g soil dry weight in bioreactor buffer) with tested chemicals at a final concentration of 50 mg/L (added in methanol and disrupted through ultrasonication). Aliquots of 1 mL were removed at each time point (days 0, 7, 14, and 21), placed into sterile tubes, and centrifuged for 5 minutes at 15000 × g. The supernatant was discarded and the soil pellets stored at -20°C. DNA was recovered from select samples using the FastDNA Spin Kit for Soil (MP Biomedicals, OH, USA) according to manufacturer's instructions.

Shifts in the microbial communities were analyzed by denaturing gradient gel electrophoresis (DGGE). DGGE-PCR was conducted with primers 341F-GC (5'→3': CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G CCT ACG GGA GGC AGC AG) and 517R (5'→3': ATT ACC GCG GCT GCT GG) targeting hypervariable region V3 of the 16S rRNA gene. Each PCR contained 1 µL of extracted DNA, 1.25 µL of 10 µM forward

primer (341F-GC), 1.25 μL of 10 μM reverse primer (517 R), 10 μL 5 prime MasterMix (5 Prime GmbH, Germany), and 6.5 μL dH_2O for a total volume of 20 μL. The PCR program consisted of 94°C for 5 min, 10 cycles of 94°C for 30 seconds, 58°C for 30 seconds (with a 0.5°C decrease each cycle), and 72°C for 1 minute. 25 additional cycles were 94°C for 30 seconds, 53°C for 30 seconds and 72°C for 1 minute using an Eppendorf Mastercycler Gradient thermal cycler. PCR amplicons were analyzed by gel electrophoresis using a 2% agarose gel. DGGE was performed with a DCode^{TM} Universal Mutation Detection system (Bio-Rad, CA, USA). A 10% polyacrylamide gel was made with a denaturant range from 40–60%. The gels were run in 1X TAE buffer at 60 V for 16 hours prior to staining with ethidium bromide.

Clone libraries for select samples were created using a TOPO TA Cloning® for Sequencing (Invitrogen, CA, USA). Plasmids of clones were purified using a QIAprep® Spin Miniprep Kit (Qiagen, CA, USA). Purified clonal products were analyzed by DGGE alongside fresh PCR products from extracted DNA samples. Clone products matching bands of interest were submitted to Eton Bioscience Inc. (Research Triangle Park, NC, USA) for sequencing. The sequence results were analyzed by BioEdit Sequence Alignment Editor 7.2.5 and Sequencher 5.4.1. The closest 16S rRNA gene was determined using BLAST searches of the GenBank database and the sequence match tool of the Ribosomal Database Project (RDP) [234].

I. Pure Culture Tests for Co-metabolism of Azaarene

Isolates capable of growth on PAHs were tested for their ability to co-metabolize acridine and phenanthridine. Incubations were set up for 3 strains: *Acidovorax* sp. NA3 [235], *Immundisolibacter cernigliae* TR3.2^{T} [236], and *Sphingobium* sp. PAP1. *Acidovorax* sp. NA3 was grown to turbidity in 5 mL sRB liquid medium with 200 mg·L^{-1} phenanthrene. Cells were washed 3 times through centrifugation and resuspension in sRB liquid medium. Sterile 40-ml

amber-glass EPA vials with additional chemicals (individual azaarene or mixture of selected PAHs and azaarenes added in methanol prior to disruption through ultrasonication) and 5 mL sRB liquid medium were prepared in triplicate to obtain a final concentration of 50 $mg \cdot L^{-1}$. Acidified killed controls were created by adding 85% phosphoric acid to adjust the $pH \leq 2$. 100 µL washed cells were added into both experimental and acidified vials and incubated for 7 days at room temperature (~20 – 23°C) with shaking at 180 rpm. *Immundisolibacter cernigliae* TR3.2^T was grown to turbidity in sRB2 liquid medium (bioreactor buffer, 1 mM $MgSO_4 \cdot 7\ H_2O$, 1 mM $CaCl_2 \cdot 2\ H_2O$, 1 $mL \cdot L^{-1}$ trace element solution, 1 $mL \cdot L^{-1}$ vitamin B12 solution) with 0.2% sodium pyruvate [236]. The incubation for *Immundisolibacter cernigliae* TR3.2^T was set up similarly to *Acidovorax* sp. NA3, while the incubation time for *Immundisolibacter cernigliae* TR3.2^T was 21 days given the slower growth of TR3.2^T. The test of co-metabolism of *Sphingobium* sp. PAP1 was set up with the same process of the incubation of *Acidovorax* sp. strain NA3. Strain PAP1 was tested in 125 mL sterile screw-top flasks incubated at room temperature with shaking at 180 rpm for a 4-day incubation.

After incubation, the pH of each vial was adjusted to $pH \geq 7$ if required by adding NaOH. Chemicals were extracted, filtered, and stored using the same protocols as was described previously (Chemical Analysis). Sample concentrations were adjusted with methanol for HPLC analysis as described for the protocol described earlier.

CHAPTER 4: RESULTS

A. HPLC Chromatogram for Identification of Three Azaarenes

The HPLC chromatograms obtained from standard stocks of the mixture of three azaarenes dissolved in methanol (Figure 9). By comparing to peaks presented from standards containing single azaarene compounds, the three peaks identified based on lowest to highest retention time were acridine, phenanthridine, and carbazole, respectively. Standard curves representing values of 10 mg·L^{-1}, 5 mg·L^{-1}, 2.5 mg·L^{-1}, 1.25 mg·L^{-1} and were regularly performed (Figure 10). The lowest concentration tested was 0.625 mg·L^{-1} and the analytes were reliably detected at that concentration.

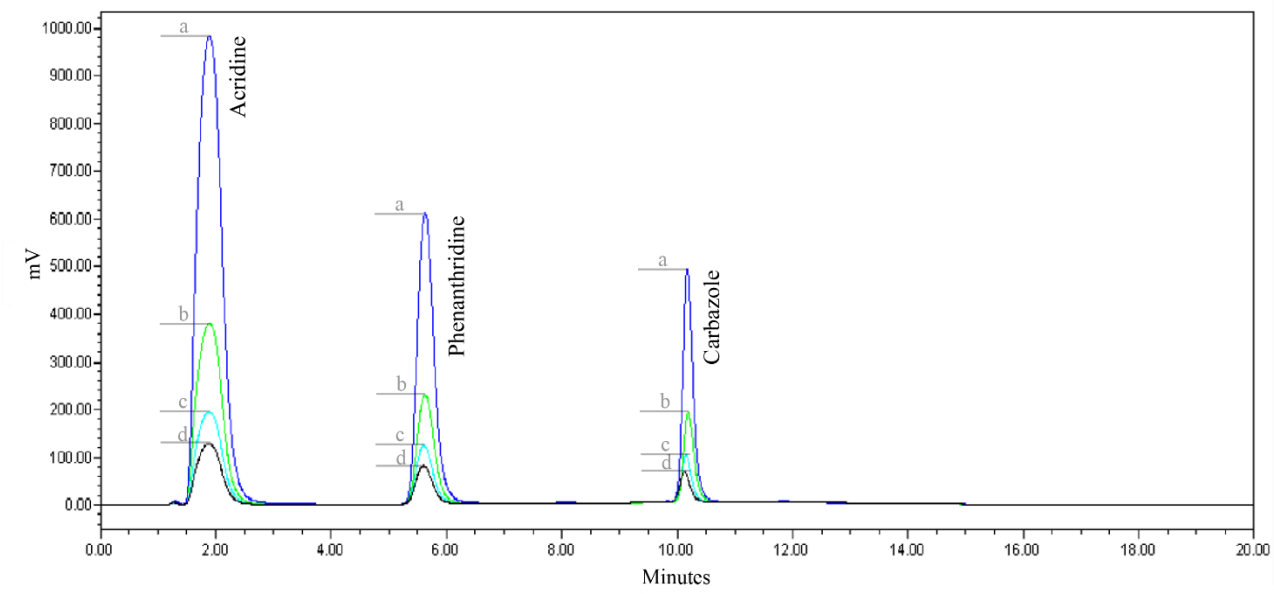


Figure 9. HPLC chromatograms for stock mixtures of three azaarenes: carbazole, acridine and phenanthridine. Concentrations: a. 10 mg·L^{-1}; b. 5 mg·L^{-1}; c. 2.5 mg·L^{-1}; d. 1.25 mg·L^{-1}.

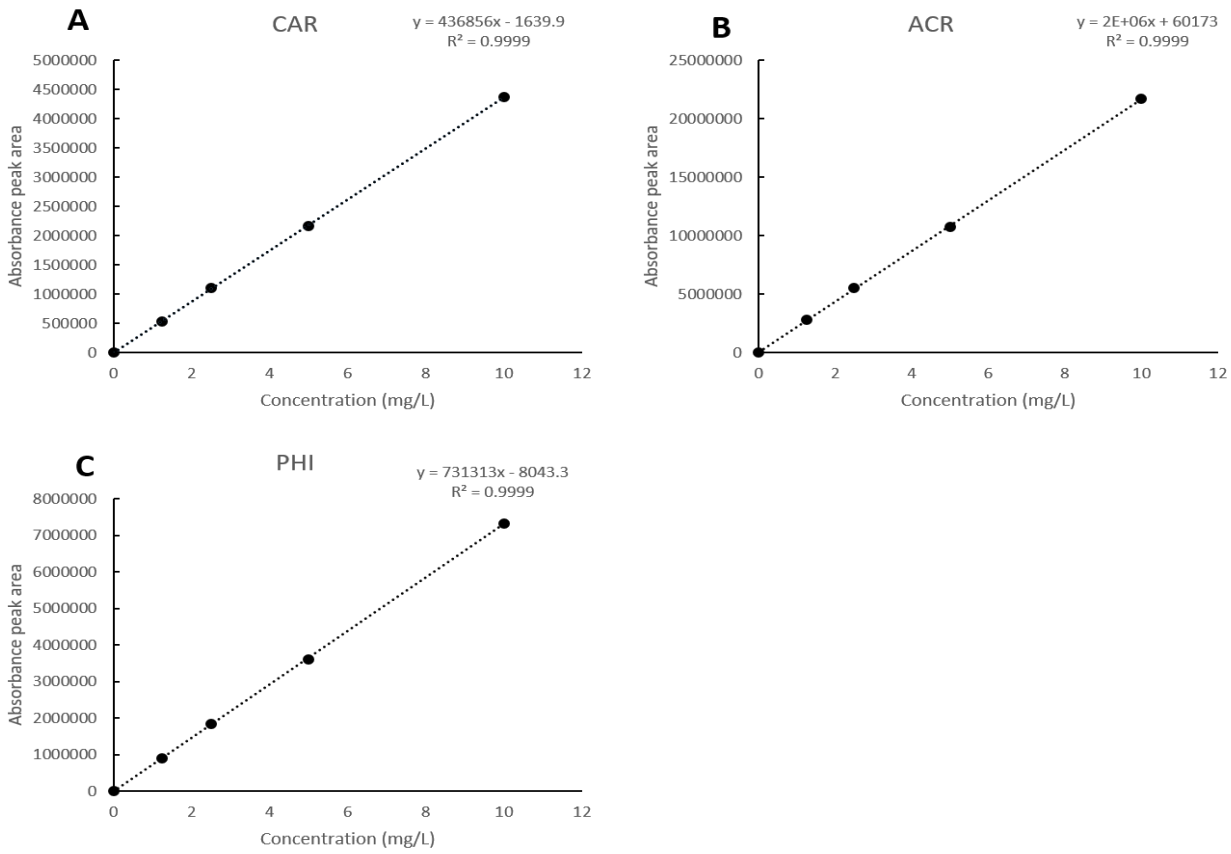
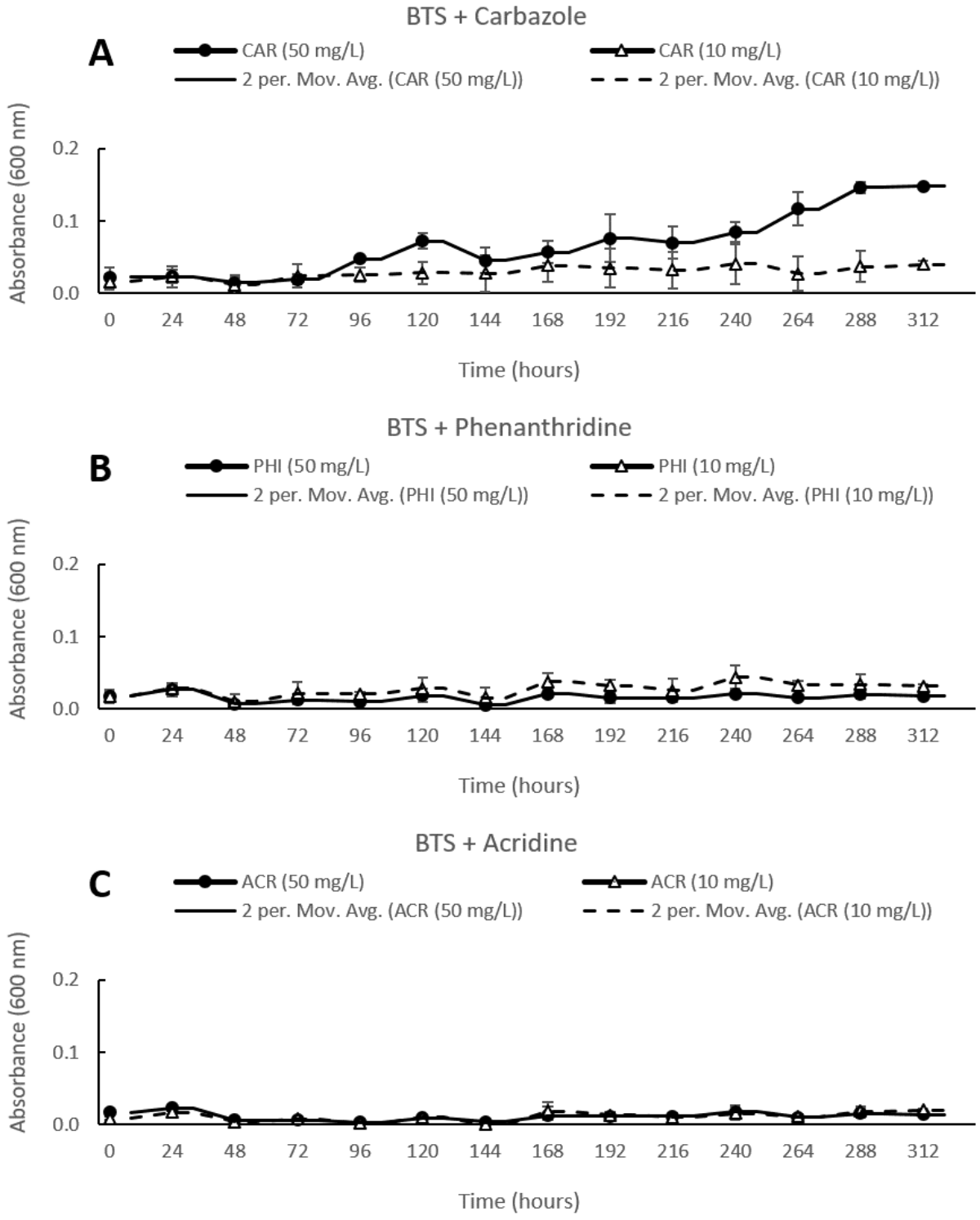


Figure 10. Typical standard curve of three azaarenes from HPLC analysis. Concentrations, including 10 mg·L^{-1}, 5 mg·L^{-1}, 2.5 mg·L^{-1}, 1.25 mg·L^{-1} were performed for three azaarenes: A. carbazole (CAR), B. acridine (ACR) and C. phenanthridine (PHI).

B. Effects of Azaarene Concentration on Soil Microbial Activity

The ability of each of the three azaarenes to serve as a growth substrate for microbes in either feed soil (FS) or bioreactor-treated soil (BTS) was tested. Two concentrations of azaarenes, 50 mg·L^{-1} and 10 mg·L^{-1}, were added to soil slurries and the turbidity of soil-free supernatant (absorbance measured at OD_{600}) was measured as a surrogate for microbial growth was measured (Figure 11). For both feed soil and bioreactor-treated soil samples, the turbidity of carbazole incubations indicated bacterial growth. For feed soil incubations amended with carbazole, after 48 hours the turbidity of both the 50 mg·L^{-1} and 10 mg·L^{-1} concentrations showed increases with the 50 mg·L^{-1} concentration showing a greater increase. The maximum turbidity for feed soil incubations containing carbazole was 0.075, compared to the positive

control (incubation with pyruvate) of 0.88. For bioreactor-treated soil incubations amended with carbazole, incubations containing 50 $mg \cdot L^{-1}$ carbazole had significant turbidity ($P < 0.05$) after 72 hours. No significant turbidity was observed in the corresponding 10 $mg \cdot L^{-1}$ carbazole incubations. The maximum turbidity of bioreactor-treated soil carbazole incubations was 0.15, while the maximum turbidity for the positive control was 0.92. Comparing the 50 $mg \cdot L^{-1}$ carbazole incubations in two soils, growth was evident earlier among feed soil incubations, but greater in the bioreactor-treated soil. As expected, incubations containing 50 $mg \cdot L^{-1}$ carbazole showed more microbial growth than 10 $mg \cdot L^{-1}$ carbazole incubations, suggesting that inhibition at higher concentrations was not a major factor for this compound. However, no significant increases in turbidity was observed for either concentration of acridine and phenanthridine in either of the two soil incubations.


BTS + Carbazole
A
CAR (50 mg/L)
CAR (10 mg/L)
2 per. Mov. Avg. (CAR (50 mg/L))
2 per. Mov. Avg. (CAR (10 mg/L))
Absorbance (600 nm)
0.2
0.1
0.0
0 24 48 72 96 120 144 168 192 216 240 264 288 312
Time (hours)
BTS + Phenanthridine
B
PHI (50 mg/L)
PHI (10 mg/L)
2 per. Mov. Avg. (PHI (50 mg/L))
2 per. Mov. Avg. (PHI (10 mg/L))
Absorbance (600 nm)
0.2
0.1
0.0
0 24 48 72 96 120 144 168 192 216 240 264 288 312
Time (hours)
BTS + Acridine
C
ACR (50 mg/L)
ACR (10 mg/L)
2 per. Mov. Avg. (ACR (50 mg/L))
2 per. Mov. Avg. (ACR (10 mg/L))
Absorbance (600 nm)
0.2
0.1
0.0
0 24 48 72 96 120 144 168 192 216 240 264 288 312
Time (hours)

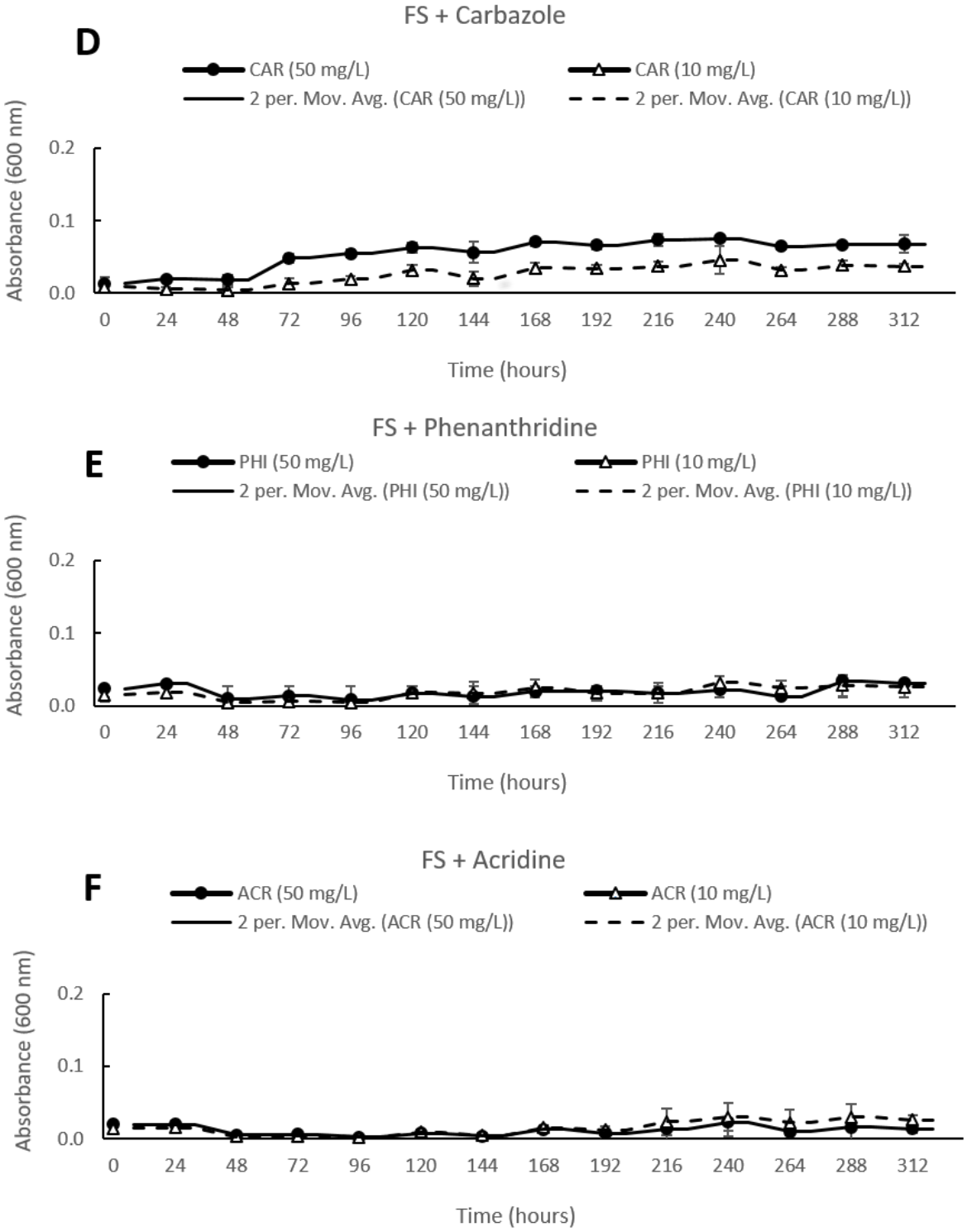


Figure 11. Effects of two concentrations of either carbazole (CAR), acridine (ACR), or phenanthridine (PHI) on the growth of microbes in feed soil (FS) or bioreactor-treated soil (BTS). Results were measured by turbidity at OD_{600} and means in triplicate samples are shown above. Profiles of A, B, and C represent bioreactor-treated soil incubation, whiles profiles D, E and F represent feed soil incubation.

C. Co-Incubation of Azaarenes with Each Other and With PAHs

The removal of azaarenes in both feed soil and bioreactor-treated soil was examined individually and in combination with each other, as well as with two three-ring PAHs, phenanthrene and anthracene. When incubated individually at a concentration of 50 $mg \cdot L^{-1}$, the bioreactor-treated soil removed over 21 days an average of 93% ± 2%, 64% ± 2%, and 85% ± 7% of carbazole, acridine, and phenanthridine, respectively (Figure 12, A-C). Co-incubation of phenanthrene with phenanthridine resulted in 97% ± 0.5% removal of phenanthrene and 99% ± 0.4% removal of phenanthridine (Figure 12, D). Co-incubation of all three azaarenes together resulted in similar levels of removal for carbazole (94% ± 0.7%), acridine (54% ± 3%), and phenanthridine (95% ± 0.7%) as was observed in individual incubations (Figure 12, E). Co-incubation of acridine and phenanthridine with either of the PAHs resulted in similar percentage removal of acridine and phenanthridine. Specifically, 51% ± 5% and 65% ± 6% of the acridine was removed from anthracene and phenanthrene co-incubations, respectively (Figure 12, F, G). Similarly, 94% ± 1% and 98% ± 1% of phenanthridine was removed in anthracene or phenanthrene co-incubations, respectively (Figure 12, G). The addition of carbon sources (carbazole, anthracene, or phenanthrene) therefore did not significantly affect the removal of acridine and phenanthridine from bioreactor-treated soils under the conditions tested ($p < 0.05$; Figure 12, H, I).

However, the effects of co-incubation of azaarenes and select PAHs on removal of azaarenes in feed soil presented a different pattern. When tested individually in incubations of feed soil over 21 days, carbazole was significantly removed (76% ± 5%; $P<0.05$), whereas acridine was not removed, and phenanthridine was removed by only 17% ± 4% ($P>0.05$) (Figure 13, A-C). Similar to the incubations of individual compounds, co-incubation of all three

azaarenes together resulted in significant removal of carbazole (89% ± 4%), no significant removal of acridine, and non-statistically significant but detectable removal of phenanthridine (9% ± 3%, P>0.05) (Figure 13, E). Significantly higher removal of phenanthridine was observed during co-incubation of phenanthrene and phenanthridine, in which nearly all of the phenanthrene and 74% ± 12% of phenanthridine was removed (Figure 13, D). In the co-incubation group of anthracene, acridine, and phenanthridine (Figure 13, F), the presence of anthracene also enabled significant removal of phenanthridine (71% ± 6%), but not acridine. In contrast to the results from the bioreactor-treated soil incubations, removal of acridine was not observed in any conditions in which feed soil was the inoculum (Figure 13, H).

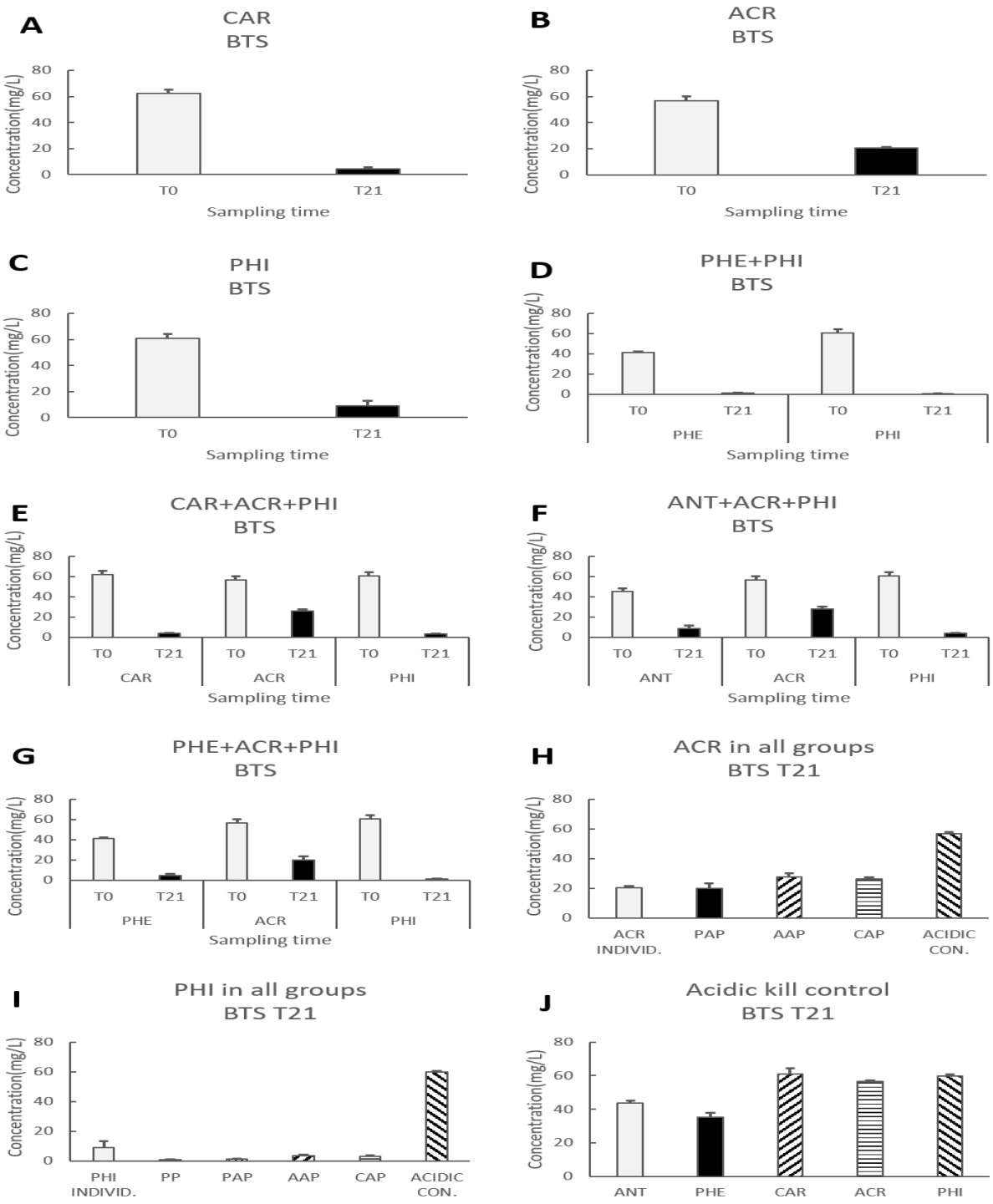


Figure 12. Concentration of azaarenes and PAHs in bioreactor-treated soil (BTS). Results represent average values and standard deviations in triplicate incubations. Samples were taken at days 0 and day 21. Abbreviations: carbazole, CAR; acridine, ACR; phenanthridine PHI; anthracene ANT; phenanthrene, PHE; PHE/PHI, PP; PHE/ACI/PHI, PAP; CAR/ACI/PHI, CAP; ANT/ACI/PHI, AAP.

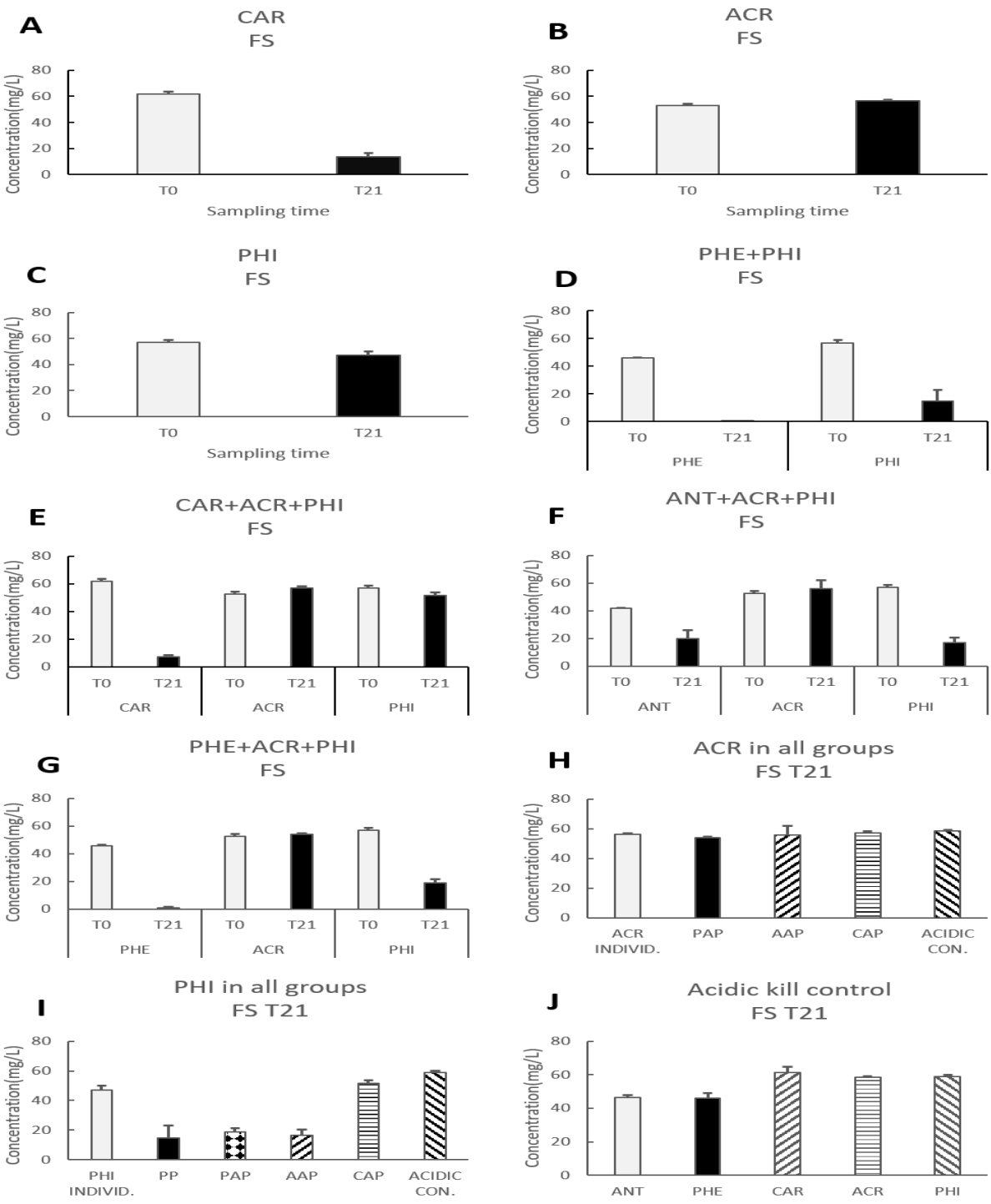


Figure 13. Concentration of azaarenes and PAHs in feed soil samples (FS). Results represent average values and standard deviations of triplicate incubations. Samples were taken at day 0 and day 21. Abbreviations: carbazole, CAR; acridine, ACR; phenanthridine, PHI; anthracene, ANT; phenanthrene, PHE; PHE/PHI, PP; PHE/ACI/PHI, PAP; CAR/ACI/PHI, CAP; ANT/ACI/PHI, AAP.

D. Transformation of Azaarenes by Isolates

One-hundred and ten bacterial strains were isolated from untreated (feed) or bioreactor-treated soil incubations supplemented with either carbazole, acridine, or phenanthridine with the purpose of finding putative degraders of those compounds (Table 2). Each isolate also grew on R2A plates. Seventy-four isolates were also capable of growth on sRB plates containing individual azaarenes: 40 on carbazole plates, 12 on acridine plates, and 22 on phenanthridine plates. However, 42 of the isolates growing on sRB plates (including all putative acridine- and phenanthridine-degrading isolates, and 8 of the carbazole isolates) were also able to grow on unamended sRB plates, suggesting growth on medium components other than an azaarene. Nineteen of the isolates growing on sRB-carbazole plates developed large clear zones around colonies indicating transformation of the azaarene. The ability of these nineteen strains to use carbazole as a growth substrate was confirmed in liquid sRB medium with visual observation of turbidity in comparison to inoculated controls without carbazole. 17 of these isolates belonged to the bacterial genus *Pseudoxanthomonas* (97-100% 16S rRNA gene identity to *Pseudoxanthomonas spadix* strain BD-a59 or Bug 14) and 2 belonged to the genus *Sphingobium* (99% 16S rRNA gene identity to *Sphingobium* sp. HY-1). No isolates were confirmed to grow on solely acridine or phenanthridine as a growth substrate in liquid culture.

An additional three isolates were obtained from enrichment cultures of bioreactor-treated soil amended with phenanthrene, acridine, and phenanthridine. These isolates were also capable of growth on R2A plates. One isolate (strain PAP1) from this enrichment grew on sRB medium containing phenanthrene or a mixture of phenanthrene and phenanthridine, but not unamended sRB medium. Strain PAP1 was identified as a member of the *Sphingobium* genus through partial 16S rRNA gene sequencing (97% 16S rRNA gene identity to *Sphingobium* sp. BZ13). Strain

PAP1 was found unable to grow on a sRB plate amended with only phenanthridine. Its ability to transform phenanthridine was confirmed and quantified through later chemical analysis. Specifically, cultures co-incubated with phenanthridine and phenanthrene removed 27 ± 5% and 58 ± 4% of the compound, respectively.

Table 2. Closest described relatives to 44 isolates based on partial 16S rRNA genes and the azaarene-transforming ability of those isolates.

Isolates	Classifier Identification	Highest BLAST hit	Accession Number	Percentage similarity	Azaarene transformation
FC1, **FC2**, SC4, **SC5**, **FC7B**, **SC11**, **SC12**, SC13, **FC14**, **FC15**, **FC16**, **FC17**, **FC17B**, **FC20**, **FC21**, **FC22**, **FC25**, **FC31**, FC32, FC34S	*Pseudoxanthomonas*	*Pseudoxanthomonas spadix* BD-a59	CP003093	99%	Carbazole
SC2, SC6	*Ramlibacter*	*Ramlibacter* sp. HTCC332	AY429716	99%	–
FC7S	*Variovorax*	*Variovorax* sp. 5d	HG937595	100%	–
SC9S	*Rhodopseudomonas*	*Rhodopseudomonas* sp. IFP10	KM047482	100%	–
FC18	*Pseudoxanthomonas*	*Pseudoxanthomonas spadix* Bug14	LN613115	97%	Carbazole
FC19	*Mucilaginibacter*	*Mucilaginibacter* sp. FB14.2	AM933506	99%	–
FC23	*Polaromonas*	*Polaromonas* sp. Gsoil115	KX066101	100%	–
FC27, FC33, SP1	*Acidovorax*	*Acidovorax* sp. T1	KX162718	99%	–
FC28, **FC29**	*Sphingobium*	*Sphingobium* sp. WH-3	KJ917397	99%	Carbazole
FA13	*Herminiimonas*	*Herminiimonas glaciei* UMB49	NR044508	99%	–
FA18, FP22, FP25	*Staphylococcus*	*Staphylococcus epidermidis* 1457	KY818945	100%	–
FA18S	unclassified *Acetobacteraceae*	*Roseococcus* sp. Am10	KF444795	99%	–
FA19	*Methylobacterium*	*Methylobacterium* sp. MaAL-74	KY810636	99%	–
FP1, FP2, FP12	*Methylopila*	*Methylopila* sp. VVRTb25	KX504245	99%	–
FP5	*Rhizobium*	*Rhizobium* sp. M3	KY908459	94%	–
FP19	*Pseudoxanthomonas*	*Pseudoxanthomonas spadix* BD-a59	CP003093	97%	–
PAP1	*Sphingobium*	*Sphingobium* sp. BZ13	HQ588831	97%	Phenanthridine

Note: The first and second letter of each name of isolate represents the soil condition (F, feed soil; S, bioreactor treated soil), and substrate of the incubation (C, carbazole; A, acridine; P, phenanthridine), respectively (except strain PAP1). Isolate PAP1 was isolated from feed soil, in an incubation containing phenanthrene, acridine and phenanthridine. Strains shown to be able to utilize substrates as carbon sources are bolded. Sequences were clustered based on 99% 16S rRNA gene sequence identity. Classification was performed using the Classifier algorithm of RDP [234]. The closest described relative was determined by Blast searches of GenBank.

E. Degradation Test of Azaarenes by Isolates

Seven isolates with potential to transform either azaarenes or PAHs were selected for a test employing 96-well plates to determine potential growth on a variety of aromatic substrates (Figure 3). Strain FC2 (identified as a *Pseudoxanthomonas* sp.) grew strongly on 9-fluorenone, carbazole, and phthalate. Strain FC2 was among those observed to produce clear zones on sRB plates supplemented with carbazole, and turbidity was observed in tubes of FC2 containing sRB-carbazole liquid medium. Isolates FP2 (*Methylopila* sp.), FP12 (*Methylopila* sp.), and FP19 (*Pseudoxanthomonas* sp.) each appeared to grow on multiple carbon sources, including carbazole, acridine, and phenanthridine, while strain F27 (*Acidovorax* sp.) could use only phthalate as a potential carbon source. Isolates FC7S and FP22 were not positive for growth on any of the tested carbon substrates.

As this experimental 96-well plate test produced unexpected results for strain FP2 and strain FP12 (in comparison to the results shown in Table 2), the growth test was replicated for those strains. In the second round of testing, no protein accumulation indicating growth was observed for carbazole, acridine, or phenanthridine for either isolate (Appendix, Table A1). Isolates FP2 and FP12 were further tested using sRB agar plates and sRB liquid medium amended with the individual azaarenes under different concentrations (5 $mg \cdot L^{-1}$, 10$mg \cdot L^{-1}$, 25 $mg \cdot L^{-1}$, 50 $mg \cdot L^{-1}$). After 30 days of incubation, the lack of any growth indicated that FP2 and FP12 were likely not phenanthridine-degraders.

Table 3. Growth of select strains on a variety of (mostly) aromatic carbon sources in the 96-well plate assay.

Substrate	**FC2**	**FC7S**	**FC27**	**FP2**	**FP12**	**FP19**	**FP22**
Fluorene	4.8 ± 8.8	7.4 ± 2.9	14.4 ± 6.8	23.5 ± 17.5	3.7 ± 4.8	35.4 ± 14.0	1.2 ± 4.2
Phenanthrene	10.4 ± 7.7	5.3 ± 2.0	13.15 ± 7.2	51.2 ± 35.1	10.3 ± 4.5	26.5 ± 11.4	6.5 ± 4.3
Anthracene	9.9 ± 5.8	2.7 ± 2.5	12.5 ± 7.1	32.0 ± 24.6	16.3 ± 13.7	5.5 ± 5.3	7.3 ± 3.5
Fluoranthene	15.7 ± 6.4	3.4 ± 1.4	16.2 ± 8.4	14.8 ± 9.9	14.3 ± 5.0	18.4 ± 5.7	8.3 ± 3.6
Pyrene	18.3 ± 12.6	8.2 ± 2.7	14.3 ± 6.5	**24.2 ± 7.6**	18.3 ± 8.3	36.4 ± 16.6	5.8 ± 7.0
Benz[*a*]anthracene	12.7 ± 6.0	5.4 ± 1.5	10.4 ± 6.4	20.1 ± 10.2	14.3 ± 9.1	4.1 ± 11.5	5.5 ± 6.9
Chrysene	11.3 ± 6.5	8.9 ± 4.9	11.8 ± 7.4	14.5 ± 10.4	5.4 ± 7.7	6.9 ± 12.0	13.7 ± 4.4
Benzo[*b*]fluoranthene	3.9 ± 6.1	37.7 ± 33.5	8.3 ± 6.3	5.1 ± 4.7	4.7 ± 4.4	4.7 ± 4.2	10.2 ± 5.0
Benzo[*k*]fluoranthene	6.3 ± 6.3	4.1 ± 1.7	8.2 ± 6.5	7.9 ± 5.5	6.9 ± 5.7	7.9 ± 2.3	2.9 ± 3.5
Benzo[*a*]pyrene	18.9 ± 7.1	4.2 ± 1.2	34.2 ± 40.9	46.2 ± 33.3	**25.1 ± 6.4**	12.7 ± 8.5	5.8 ± 3.6
Dibenzo[*a,h*]anthracene	13.8 ± 8.5	3.8 ± 1.7	9.2 ± 6.8	**26.9 ± 4.6**	**32.5 ± 11.3**	4.2 ± 4.8	6.7 ± 4.6
Benzo[*g,h,i*]perylene	18.5 ± 5.9	6.5 ± 3.7	10.5 ± 6.6	**33.0 ± 4.9**	13.8 ± 4.3	5.9 ± 5.5	4.7 ± 4.3
9-fluorenone	**31.2 ± 12.7**	2.9 ± 2.0	16.6 ± 7.7	**30.0 ± 5.2**	**22.6 ± 4.8**	**28.6 ± 4.3**	7.9 ± 4.4
9,10-phenanthrequinone	9.7 ± 5.7	3.8 ± 1.7	11.8 ± 7.0	**31.0 ± 6.1**	**32.1 ± 5.6**	6.2 ± 4.0	5.5 ± 3.5
9-anthrone	112.4 ± 6.0	1.9 ± 2.2	12.4 ± 7.6	18.3 ± 8.0	**13.4 ± 6.5**	3.3 ± 3.9	7.4 ± 5.0
9,10-anthraquinone	10.3 ± 5.9	2.7 ± 3.9	17.0 ± 7.7	56.3 ± 62.0	**14.9 ± 5.2**	**36.0 ± 7.6**	10.2 ± 5.5
Benzo[*a*]anthraquinone	4.9 ± 5.8	1.7 ± 1.2	9.7 ± 6.3	7.2 ± 4.8	10.9 ± 7.1	15.0 ± 6.8	3.4 ± 5.8
Carbazole	**91.2 ± 18.0**	2.9 ± 1.5	12.7 ± 6.3	21.5 ± 5.2	**22.1 ± 4.4**	7.3 ± 10.2	7.9 ± 4.0
Phenanthridine	14.4 ± 6.3	8.9 ± 3.5	11.5 ± 7.8	22.8 ± 6.8	**24.8 ± 10.0**	**20.4 ± 2.6**	8.4 ± 4.3
Acridine	14.5 ± 7.0	6.2 ± 1.8	12.3 ± 6.6	**33.6 ± 1.5**	**22.5 ± 6.1**	8.4 ± 6.7	12.3 ± 4.6
Salicylate	10.9 ± 6.4	4.2 ± 1.6	19.5 ± 10.1	33.2 ± 9.7	**30.0 ± 4.3**	2.4 ± 3.7	22.8 ± 8.9
Phthalate	**49.5 ± 14.6**	5.7 ± 2.7	**45.3 ± 6.7**	28.4 ± 13.3	29.2 ± 20.8	9.3 ± 5.3	8.2 ± 5.9
Hexadecane	14.3 ± 6.1	21.3 ± 33.0	11.7 ± 7.2	17.5 ± 8.6	11.5 ± 9.1	**36.0 ± 5.0**	11.0 ± 5.8
Negative Control	3.3 ± 7.9	0.8 ± 1.6	5.2 ± 8.9	6.7 ± 1.1	0.7 ± 6.0	12.9 ± 5.2	8.0 ± 4.8
Positive Control	**108.9 ± 10.0**	**43.8 ± 2.7**	**74.1 ± 10.0**	**69.2 ± 19.5**	**64.7 ± 0.0**	**64.3 ± 6.6**	**68.4 ± 8.7**

Note: Values represent mean and standard deviation of protein concentrations in triplicate wells. Bold indicates conditions which satisfy the conditions of a) at least twice the value of negative control and b) near one third the value of the positive control.

F. Community Analyses

Differences in the transformation of azaarenes were attributed to microbial community shifts in the soils. Substantial changes in the bacterial community were analyzed through denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene clone libraries. The effects of amendment of phenanthrene or anthracene in conditions also containing acridine and/or phenanthridine were analyzed by DGGE (Figure 14, 15). Profiles of incubations from feed soil (Figure 14, lanes 6-10) possessed several significant differences in comparison to the profiles of bioreactor-treated soil (Figure 14, lanes 1-5), suggesting different microbial communities responded to the presence of PAHs or azaarenes in treated versus untreated soils.

Similarities in the dominant bands present in both unamended bioreactor-treated soil samples from days 0 and 21 (Figure 14, lanes 1-2, respectively) suggested only minor community shifts due to incubation in fresh reactor buffer, although difficulties in the extraction and PCR amplification of the day 21 bioreactor-treated soil sample likely prevented the occurrence of minor bands in that profile. Incubations containing either phenanthrene or anthracene and at least one azaarene (Figure 14, lanes 3-5) contained most of the same dominant bands as were present in the unamended samples (Figure 14, lanes 1-2). In addition, samples with amended phenanthrene also contained a dominant band (Figure 14, band f) that was absent or diminished in other samples. One band (Figure 14, band d) was particularly prominent in the incubations containing anthracene, and may represent degraders of that compound. Other unidentified bands (Figure 14, bands e, g, h, and j) appeared more prominently in amended samples in comparison to unamended samples. Any of these prominent bands, either regularly present in bioreactor-treated soil, or appearing in response to amendment with a carbon source(s), might represent bacteria capable of transforming PAHs or azaarenes.

The DGGE profiles of microbial communities from the feed soil (Figure 14, lanes 6-10) were significantly different from those of bioreactor-treated soil. A microbial community shift due to the extended incubation can be observed by comparing lane 9 and lane 10, representing the day 21 and day 0 samples, respectively. Organisms represented by bands k and m displayed higher intensity after 21 days without any amended carbon. Three other bands were more prominent in PAH- and azaarene-amended samples, and were identified through partial 16S rRNA gene sequencing. Of particular interest were bands enriched in incubations containing either phenanthrene or anthracene, as those compounds were shown to increase phenanthridine removal in feed soil (Figure 13). One band (Figure 14, band a) identified as representing members of the *Acidovorax* genus (99% sequence similarity to *Acidovorax* sp. NA3, a known phenanthrene-degrading bacterium [235]) appeared in the 21d unamended sample, and both incubations with phenanthrene (Figure 14, lanes 6, 8-9). Band b was identified as a member of the *Methyloversatilis* genus (98% similarity to *Methyloversatilis* sp. RAC08), and was especially prominent in incubations including acridine. Bands b and c were found in all incubations containing azaarenes (Figure 14, lanes 6-8) and a sequence from that band was found to possess 99% similarity to *Immundisolibacter cernigliae* strain TR3.2^{T}, a bacterium confirmed to grow on multiple PAHs, including phenanthrene and anthracene [236].

A second DGGE gel (Figure 15) revealed community shifts due to high concentrations of individual PAHs or azaarenes. Comparable to Figure 14, different microbial communities responded to added chemicals in bioreactor-treated soil (Figure 15, lanes 1-8) compared to feed soil (Figure 15, lanes 9-16). Carbazole amendment resulted in the greatest number of newly prominent bands in both samples, suggesting several organisms capable of growth on that compound, however these bands differed between soil types (Figure 15, lanes 6 and 14). No

acridine removal was observed in feed soil samples, and the community of that incubation was highly similar to one in which no carbon was added (Figure 15, lanes 12 and 15). The microbial community of the acridine incubation of bioreactor-treated soil was also similar to its corresponding unamended incubation (Figure 15, lanes 4 and 7). However as acridine removal was observed in bioreactor-treated soil, it is likely that one or more members of the existing bioreactor community were responsible for acridine removal. Multiple bands in the bioreactor-treated soil incubation with phenanthridine, or with all three azaarenes, were more prominent than in other samples (Figure 15, lanes 1 and 2). This suggests that one or more groups of organisms were specifically selected by that carbon source. Conversely, while the feed soil did not reveal any prominent bands uniquely associated with phenanthridine degradation (Figure 15, lanes 9 and 10), new bands appearing in the incubations with PAHs suggest possible targets for organisms capable of co-metabolism of that compound.

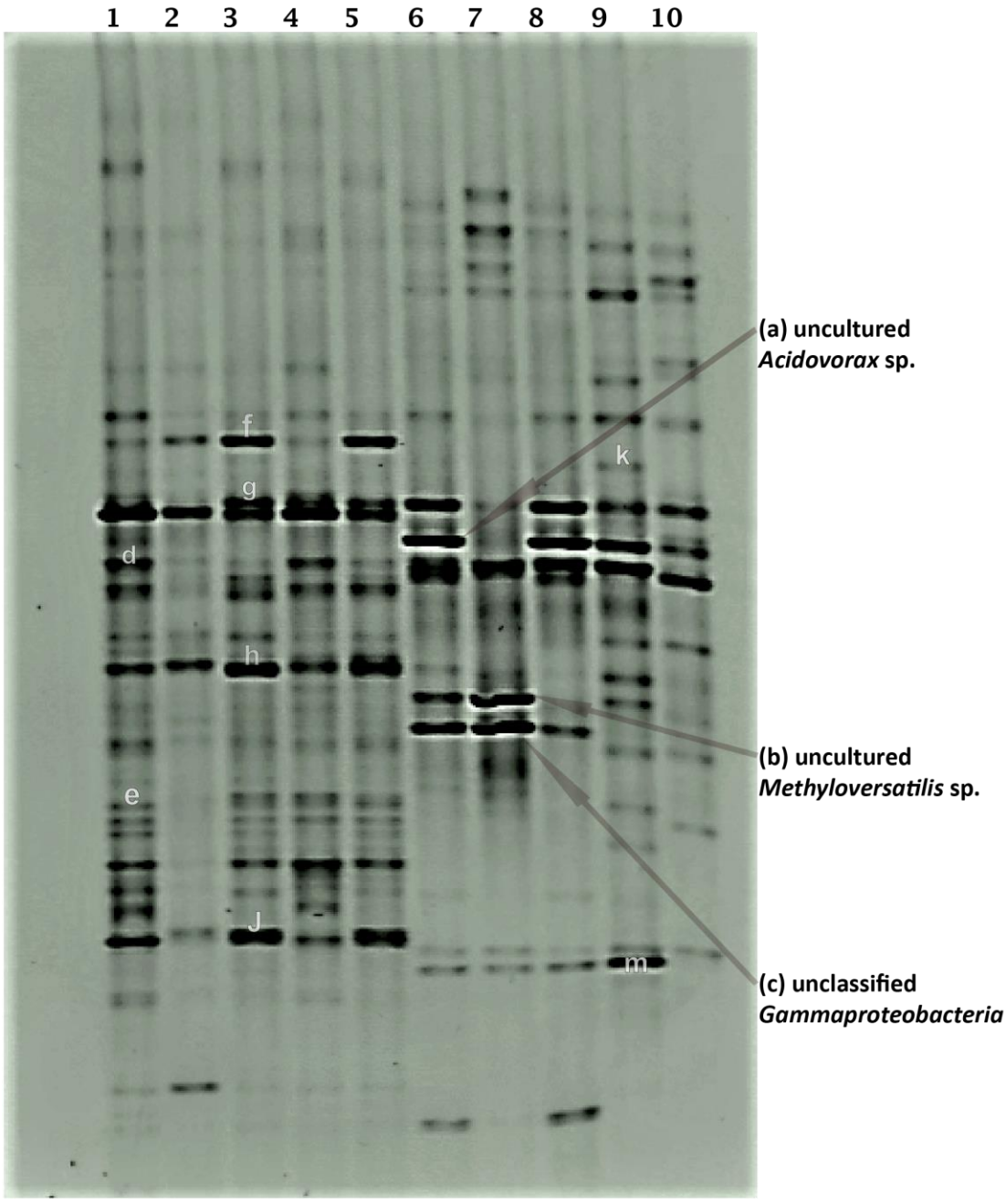


Figure 14. Negative image of the DGGE gel (1) of DNA extraction samples from incubations on testing co-metabolism of target azaarenes. Lanes 1 to 5 represent samples from incubations with bioreactor treated soil (BTS), while lanes 6 to 10 represent samples from incubations with feed soil (FS). Additional carbon sources added to the incubations including carbazole (CAR), acridine (ACR), phenanthridine (PHI), phenanthrene (PHE), and anthracene (ANT). Lanes 1 to 5 correspond to incubation conditions as follows: day 0 BTS (control), day 21 BTS (control), day 21 BTS/PHE/PHI, day 21 BTS/ANT/ACR/PHI, day 21 BTS/PHE/ACR/PHI. Lanes 6 to 10 represent day 21 FS/PHE/ACR/PHI, day 21 FS/ANT/ACR/PHI, FS/PHE/PHI day 21, day 21 FS (control), day 0 FS (control).

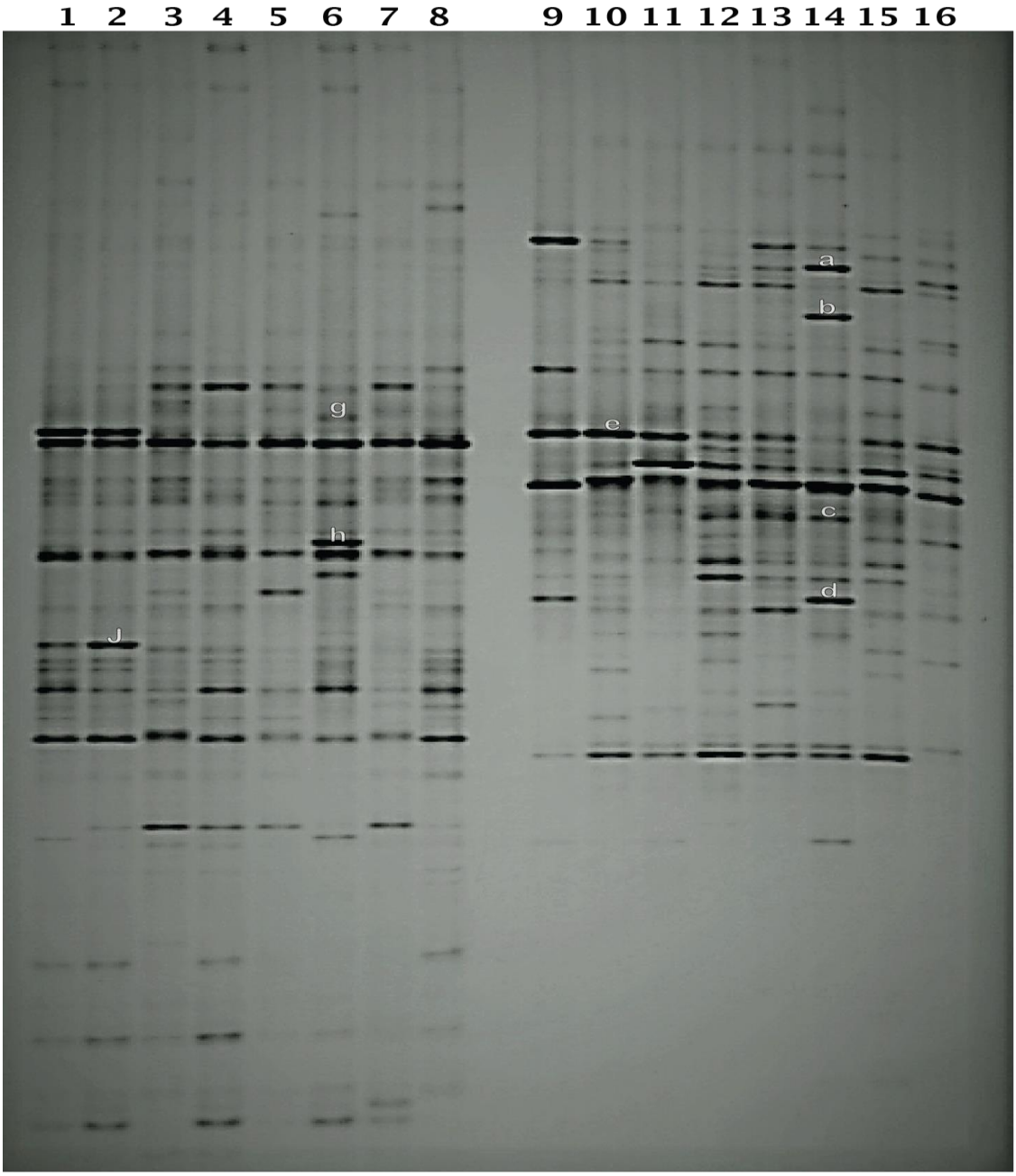


Figure 15. Negative image of the DGGE gel (2) of DNA extraction samples from incubations on testing co-metabolism of target azaarenes. Lanes 1 to 8 represent samples from incubations with bioreactor treated soil (BTS), while lanes 9 to 16 represent samples from incubations with feed soil (FS). Additional carbon sources added to the incubations including carbazole (CAR), acridine (ACR), phenanthridine (PHI), phenanthrene (PHE), and anthracene (ANT). Lanes 1 to 8 correspond to incubation conditions as follows: day 21 BTS/CAR/ACR/PHI, day 21 BTS/PHI, day 21 BTS/PHE, day 21 BTS/ACR, day 21 BTS/ANT, day 21 BTS/CAR, day 21 BTS 21 (control), day 0 BTS (control). Lanes 9 to 16 represent day 21 FS/CAR/ACR/PHI, day 21 FS/PHI, day 21 FS/PHE, day 21 FS/ACR, day 21 FS/ANT, day 21 FS/CAR, day 21 FS (control), day 0 FS (control).

G. Tests of Pure Cultures for Co-metabolism of Phenanthridine

1. Acidovorax sp. NA3

DGGE analyses of feed soil samples suggested organisms similar to *Acidovorax* sp. NA3 as candidate organisms for the co-metabolism of phenanthridine utilizing phenanthrene as a primary carbon source. However, *Acidovorax* sp. NA3 in resting cell incubations supplemented with phenanthrene and select azaarenes revealed no transformation of phenanthridine (Figure 16). No significant ($P<0.05$) degradation of phenanthridine was detected in samples containing either phenanthrene or phenanthrene with acridine. Significant ($P<0.05$) removal of phenanthrene (98% ± 3%) was observed in samples containing only that PAH, however, the lack of phenanthrene removal in all other conditions indicated nearly complete inhibition of phenanthrene degradation when phenanthridine was also present.

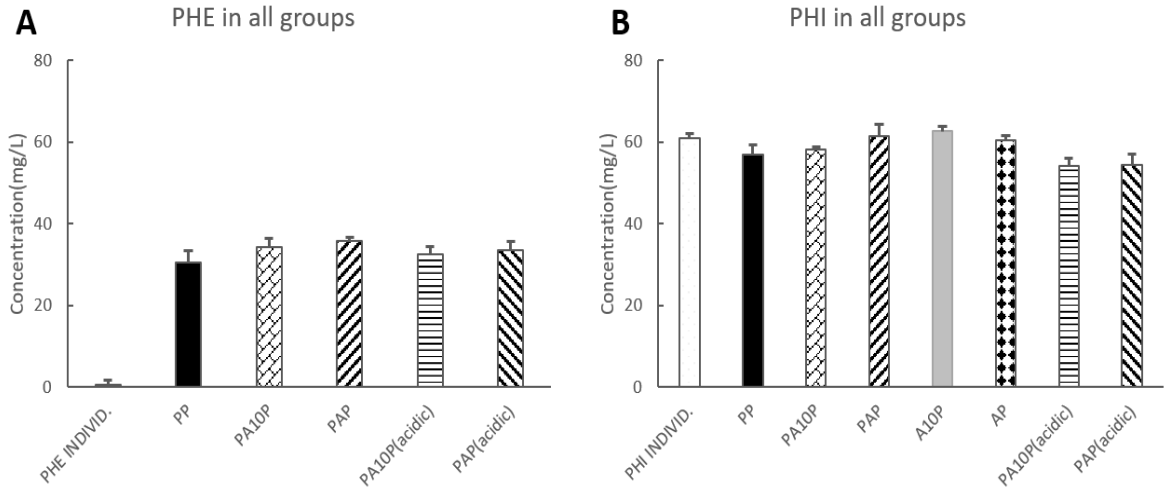


Figure 16. Removal of phenanthrene and phenanthridine from incubations of *Acidovorax* sp. NA3. Results represent average values and standard deviations of triplicate samples. Chemical conditions of incubation as follows: phenanthrene (PHE INDIVID.), phenanthrene / phenanthridine (PP), phenanthrene / 10 $mg \cdot L^{-1}$ acridine / phenanthridine (PA10P), phenanthrene / acridine / phenanthridine (PAP), 10 $mg \cdot L^{-1}$ acridine / phenanthridine (A10P), acridine / phenanthridine (AP).

2. Immundisolibacter cernigliae TR3.2^T

Another DGGE band potentially linked to phenanthridine removal in the feed soil was highly similar to *Immundisolibacter cernigliae* TR3.2^T, a degrader of both anthracene and phenanthrene [236]. *Immundisolibacter cernigliae* TR3.2^T did not demonstrate the ability to degrade phenanthridine in the presence of either phenanthrene or anthracene (Figure 17). Similar to *Acidovorax* sp. NA3, the presence of either acridine or phenanthridine inhibited the ability of TR3.2^T to transform phenanthrene (98% removal individually) or anthracene (33% removal individually).

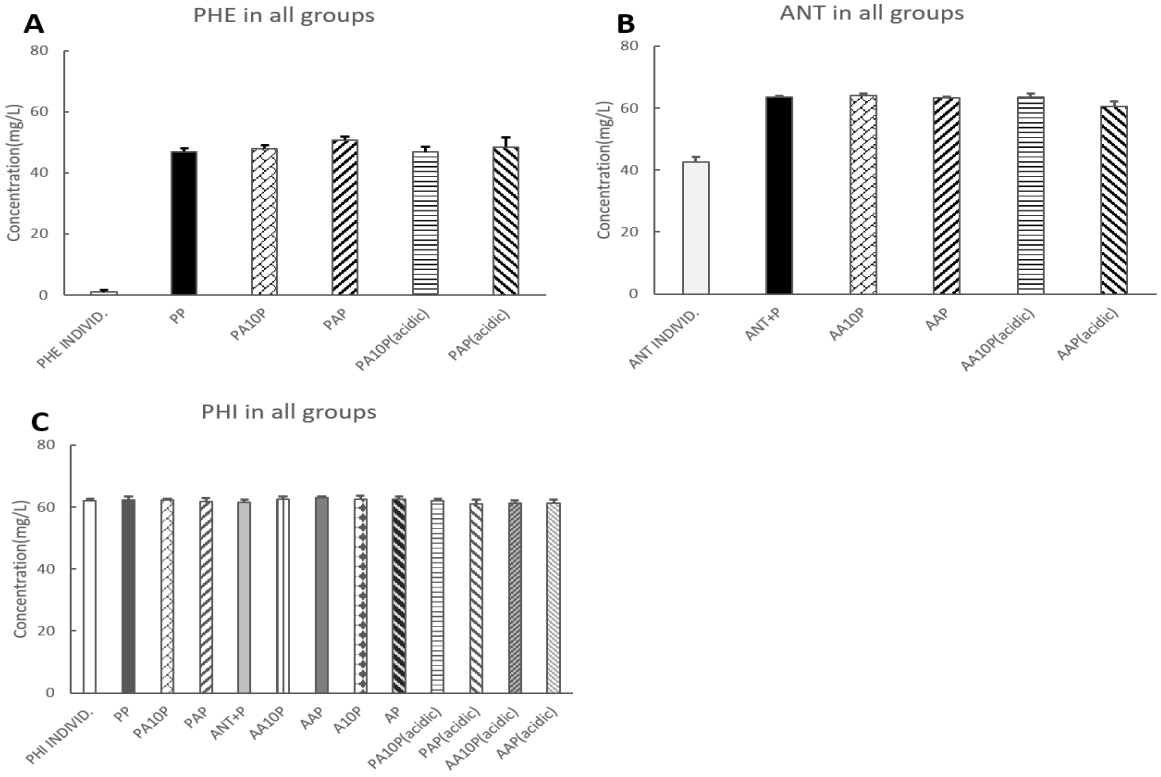


Figure 17. Removal of anthracene, phenanthrene and phenanthridine from incubations of *Immundisolibacter cernigliae* TR3.2^T. Results represent average values and standard deviations in triplicate samples. Results of samples with live cells were compared to acidified controls. Chemical conditions of incubation as follows: phenanthrene (PHE INDIVID.), phenanthrene / phenanthridine (PP), phenanthrene / 10 mg·L^{-1} acridine / phenanthridine (PA10P), phenanthrene / acridine / phenanthridine (PAP), 10 mg·L^{-1} acridine / phenanthridine (A10P), acridine / phenanthridine (AP), anthracene (ANT INDIVID.), anthracene / phenanthridine (ANT+P), anthracene / 10 mg·L^{-1} acridine / phenanthridine (AA10P), anthracene / acridine / phenanthridine (AAP).

3. Sphingobium sp. PAP1

While not explicitly identified as a candidate for phenanthridine removal through DGGE analyses, *Sphingobium* sp. strain PAP1 was isolated from bioreactor-treated soil and capable of growth on phenanthrene in liquid media. Strain PAP1 was not capable of utilizing high concentrations of phenanthridine as a sole carbon source. However, results from incubations with mixtures of phenanthrene and phenanthridine indicated that strain PAP1 transformed phenanthridine in the presence of phenanthrene (Figure 18). Tested individually, strain PAP1 removed 96 ± 13% of added phenanthrene. Statistically significant ($p<0.05$) removal of both phenanthridine (27 ± 5%) and phenanthrene (58 ± 4%) was observed when those two substrates were added together. Samples amended with acridine (10 $mg \cdot L^{-1}$) decreased the removal of both phenanthrene and phenanthridine. For samples amended with the mixture of higher amount of acridine (50 $mg \cdot L^{-1}$), phenanthrene, and phenanthridine, no significant ($p<0.05$) removal of either phenanthrene or phenanthridine was detected.

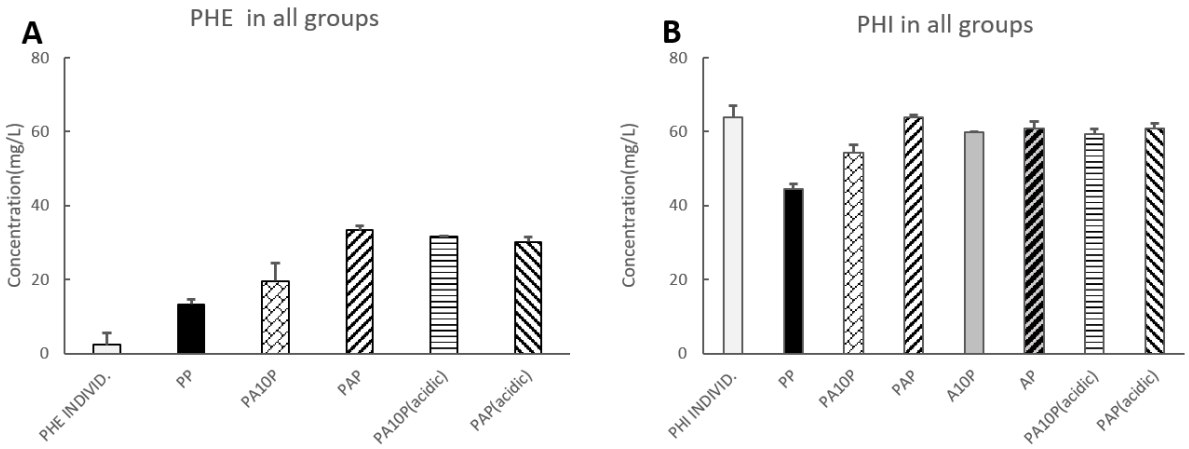


Figure 18. Removal of phenanthrene and phenanthridine from incubations of *Sphingobium* sp. strain PAP1. Results represent average values and standard deviations in triplicate samples. Chemical conditions of incubation as follows: phenanthrene (PHE INDIVID.), phenanthrene / phenanthridine (PP), phenanthrene / 10 $mg \cdot L^{-1}$ acridine / phenanthridine (PA10P), phenanthrene / acridine / phenanthridine (PAP), 10 $mg \cdot L^{-1}$ acridine / phenanthridine (A10P), acridine / phenanthridine (AP).

CHAPTER 5: DISCUSSION

The occurrence of azaarenes in the environment has long been associated with toxic effects [237]. However, despite their wide distribution and putative toxic properties, the fate and behavior of azaarenes in soil, especially acridine and phenanthridine, have not been reported extensively. In this research, PAH-contaminated soil (feed soil) acquired from a former manufactured gas plant site and a bioreactor-treated slurry of that soil (bioreactor-treated soil) were used to study the fate and the biodegradation of three azaarenes: carbazole, acridine and phenanthridine.

A new HPLC analytical method was developed to enable simultaneous quantification of three azaarenes, each of different hydrophobicity. Compared to carbazole, acridine and phenanthridine are relatively hydrophilic and these differences posed challenges in compound separation. Compounds with similar chromatographic polarity normally attract each other and are not easy to be separated; such was the case for acridine and phenanthridine. While C18 HPLC columns are common choices for quantification of azaarenes, we used phenyl-hexyl columns for greater selectivity and better retention of both acridine and phenanthridine [238-240]. Another crucial factor for the separation of acridine and phenanthridine was the inclusion of formic acid in the mobile phase. Our tests demonstrated that without adding 0.1% formic acid, these compounds could not be properly separated.

The results of chemical analyses demonstrated significant removal of high concentrations (50 $mg \cdot L^{-1}$) of either amended acridine or phenanthridine from bioreactor treated-soil incubations (Figure 12). However, in the feed soil, removal of acridine was not observed (Figure

13, graph H), while phenanthridine removal was enhanced during co-incubation with phenanthrene with acridine or anthracene with acridine. As the removal of phenanthridine in the feed soil was induced in the presence of either phenanthrene or anthracene, the dominant mechanism of phenanthridine degradation in feed soil might be through co-metabolism. The reason why anthracene amendment resulted in increased phenanthridine removal but not acridine removal (its azaarene analogue) is still not clear. Carbazole also did not appear to be a carbon source for co-metabolizing phenanthridine as the minor increase of removal of phenanthridine in incubations containing that substrate was not statistically significant (Figure 13, graph E; $P>0.05$).

The bioavailability of carbazole, acridine, and phenanthridine could impact the biodegradation of these azaarenes in soil. A bioavailable chemical can be described as one freely available to cross the cellular membrane of a microorganism from the medium that microorganism was incubated within a time period [241]. Azaarenes are generally more water soluble than PAHs, which makes their biodegradation more likely [180, 242]. According to the ChemIDplus Advanced database, the aqueous solubility of carbazole, acridine and phenanthridine is 1.8 $mg \cdot L^{-1}$, 38.4 $mg \cdot L^{-1}$, and 300 $mg \cdot L^{-1}$ respectively. However, despite possessing the lowest solubility of the three compounds, carbazole was the easiest of the three tested azaarenes to be removed by microorganisms in both the feed soil and bioreactor-treated soil. An alternate explanation might be that differing levels of toxicity may explain why acridine and phenanthridine are relatively difficult to be degrade compared to carbazole. Compared to their PAH analogues, azaarenes generally have more acute toxicity [243].

To better understand the biodegradability of these three compounds, efforts were made to isolate bacteria capable of degrading carbazole, acridine, and phenanthridine. The largest number

of carbazole degraders were isolated from the feed soil, indicating that it might include a more diverse group of organisms able to use carbazole as a carbon source. It is perhaps unsurprising to obtain isolates belonging to a variety of bacterial taxa from feed soil rather than bioreactor-treated slurry. The microbial community of the bioreactor is less diverse than that of the feed soil [244] and is adapted to grow on abundant carbon sources found in the soil, of which azaarenes are a minor component. Azaarene degradation in the bioreactor, especially carbazole, is therefore likely accomplished by high abundance, aromatic-degrading bacteria, such as *Immundisolibacter* spp., one strain of which has been shown to degrade carbazole [236]. Such organisms are also among the most dominant in the stable bioreactor community [244]. The lack of a specialized community for the degradation of aromatics in the feed soil therefore provides opportunities for low abundance bacteria capable of utilizing azaarenes as growth substrates to grow during enrichments.

Isolates from two major genera, *Sphingobium* and *Pseudoxanthomonas*, were identified among the putative carbazole degraders. At least one *Sphingobium* strain is capable of utilizing carbazole as a sole carbon and energy source [108] while *Pseudoxanthomonas* spp. have only rarely been reported to utilize azaarenes [153]. Most of the putative carbazole-degrading isolates from the soils examined here belonged to the genus *Pseudoxanthomonas* and were highly similar to *Pseudoxanthomonas spadix.* However no previous report of this species indicated its association with carbazole degradation. The type strain of *Pseudoxanthomonas spadix* was initially isolated from a sample of oil-contaminated soil and it was also reported to be able to degrade the BTEX compounds benzene, toluene, ethylbenzene, and o-, m-, and p-xylenes [245, 246]. Other *Pseudoxanthomonas* spp. have been reported to degrade a variety of aromatic chemicals including polycyclic aromatic hydrocarbons (PAHs), dichlorodiphenyltrichloroethane

(DDT), benzene, toluene, ethylbenzene, xylenes, hexachlorocyclohexane, di(2-ethylhexyl) phthalate (DEHP), diesel oil, crude oil and n-alkanes [153, 247, 248].

Fewer isolates were obtained from feed soil or bioreactor-treated soil enrichments amended individually with either acridine or phenanthridine and those that were recovered did not use those azaarenes as sole carbon sources when examined more closely. Similarly, some isolates isolated from carbazole enrichments (e.g. strain FC7S; a *Variovorax* sp.) were also found unlikely to utilize that azaarene as a sole carbon source. Some isolates recovered from azaarene enrichments, including strains representing the genera *Herminiimonas*, and *Variovorax*, possessed 16S rRNA gene sequences 99% identical to sequences previously associated with either phenanthrene or anthracene degradation via DNA-based stable-isotope probing (SIP) [249, 250]. Given that anthracene and phenanthrene are PAH analogues of acridine and phenanthridine, respectively, it might be reasonable to assume that an anthracene degrader might also be able to metabolize acridine, or a phenanthrene degrader to degrade phenanthridine. However, strains FC7S (*Variovorax* sp.) and FA13 (*Herminiimonas glaciei*), did not show the ability to grow either on sRB plates or in sRB liquid medium amended with individual acridine or phenanthridine. It is more likely that these organisms, and others that grew on azaarene-amended plates but could not grow on those compounds individually when tested in liquid media, grew using some other media component (including possible trace amounts of contaminant chemicals). This would be consistent with the generally small colony size and long period of growth observed during isolation of most of these bacteria.

There are many reasons that might explain why no confirmed acridine or phenanthridine degraders were isolated. Like the vast majority of unisolated bacteria, they simply may be unculturable under the tested conditions. Despite the success of the employed medium for the

isolation of PAH-degrading bacteria [236, 251], other kinds of medium or growth conditions may be required for isolation of azaarene-degraders. Initial high concentrations of azaarenes in the incubation may also have inhibited the activity of soil bacteria. An inhibitory effect on microbial degradation was reported previously for azaarenes like acridine, quinolone, and benzoquinolines [194, 198, 252]. Acridine- and phenanthridine-degrading bacteria may also be present in very low abundance given the low concentration of azaarenes in our contaminated soils compared to PAHs (unpublished data from Dr. Damian Shea, North Carolina State University). This contrasts with results from both feed soil and bioreactor soil incubations suggesting that a higher concentration of carbazole can increase the activity of that microbial community. In this experiment, more turbidity was observed for samples of bioreactor-treated soil amended with higher concentrations of carbazole. However, the lack of significant microbial growth for samples with acridine or phenanthridine suggested either a very low abundance of organisms capable of growth on those compounds, or potentially a strong inhibitory effect of those compounds. A previous report suggested that the reasonable concentration of azaarenes used for a growth test should be within the range of 4.4–17.4 $mg \cdot L^{-1}$ based on the probable bacterial toxicity of the azaarenes [194]. However, results from samples amended with either 10 $mg \cdot L^{-1}$ acridine or phenanthridine did not indicate significant growth, so possible inhibitory effects even at this lower concentration cannot be entirely discounted.

A recently developed, but still experimental, test for the growth of bacteria on a variety of aromatic substrates (designed by Dr. Joaquim Vila Grajales) was used to investigate the degradation ability of strains of interest utilizing xenobiotic compounds, including PAHs and the three target azaarenes. In this test 25 different carbon sources were simultaneously tested in one 96-well plate and the results quantified by protein concentration. However, while showing

indications of promise, the results of this study indicated that this method may need further refinement. The standard deviation of triplicate samples was often large. One possible reason to cause this problem could be variability in the number of cells added to each well, despite efforts to standardize the inoculum. The small volume of the test combined with the requirement of low protein background necessitated adding very low concentrations of cells. Even small discrepancies in the amount of cell material added, especially if the organism is slow growing, could have substantially affected the final protein values. The material of the 96-well plate also likely affected results. To dissolve the various carbon sources efficiently, a strong solvent was used. However, the dichloromethane solvent physically damaged the polystyrene 96-well plate which may have affected both the growth of microorganisms and the bioavailability of the substrate [253]. Subsequent tests of the method utilizing freshly added growth substrates compared to plates prepared and stored prior to inoculation (such as was used in this experiment) have resulted in more consistent readings among triplicates (Dr. Joaquim Vila Grajales, personal communication); however this refinement was not performed during the testing of the isolates from this experiment. Additional sources of error may be due to evaporation or accidental mixing of well contents during the long incubation process or when handling the plate. Thus, even though some tested strains from this experiment showed statistically significant ($P < 0.05$) protein accumulation on some carbon sources after 30 days of incubation, including azaarenes and some rarely reported biodegradable high-molecular weight PAHs, some results were neither reproducible nor consistent with other experiments. Considering its limitations, at this point we recommend using this test only as a first step screen to determine potential compounds which may be used as a growth source if results are reproducible. However, the findings should be confirmed with subsequent tests, such as was done here for strain FC2, which was revealed to

degrade carbazole by both the 96-well plate test and additional solid medium plate tests and liquid medium tests.

Bacterial community profiles from DGGE analysis revealed differences in the microbial communities responding to the presence of PAHs and azaarenes. In bioreactor-treated soil samples, DGGE profiles suggested only minor community shifts over 21 days incubation. However, two new bands (Figure 15; bands e and j) became prominent in samples amended with phenanthridine (Figure 15, lanes 1 and 2). These prominent bands might represent bacteria capable of utilizing phenanthridine as a growth substrate. Given the removal of both acridine and phenanthridine in bioreactor treated soils, it is likely that several organisms abundant in the bioreactor not only tolerated high concentration of azaarenes but were also involved in the removal process for acridine and phenanthridine. Moreover, DGGE bands presumably representing bioreactor organisms associated with carbazole removal (Figure 15; lane 6) were not the same as those implicated in phenanthridine removal, and we observed no increase in phenanthridine removal when carbazole was added. For bioreactor-treated soil, the addition of PAHs (phenanthrene or anthracene) did not appear to be a factor in the removal of acridine or phenanthridine.

For untreated feed soil samples, while acridine removal was not significant under any condition tested, there were indications that co-metabolism with either phenanthrene or anthracene might be a mechanism for the removal of phenanthridine. One DGGE band (Figure 14; band c; lanes 6, 7, 8) became extremely abundant in samples amended with phenanthridine and PAHs (phenanthrene or anthracene), and these same samples showed significant removal of phenanthridine (Figure 13). Sequencing of clones from band c identified organisms with 99% 16S rRNA gene similarity to *Immundisolibacter cernigliae* strain TR3.2^{T}, a member of one of

the dominant genera in the bioreactor and a known degrader of both anthracene and phenanthrene [236]. *Immundisolibacter* spp. therefore may be actively involved in co-metabolizing phenanthridine. Other sequenced bands (Figure 14; band a) represented organisms from the *Acidovorax* genus and were primarily associated with amended phenanthrene (Figure 14; lanes 6, 8). Members of the *Acidovorax* genus are abundant in the bioreactor and have previously been associated with phenanthrene degradation [235]. Interestingly, as the band from the DGGE profile of feed soil most similar to *Immundisolibacter* was not apparent in bioreactor-treated soil (Figure 14; lanes 6-8 compared to lanes 1-5), those organisms that grew in enrichments of the feed soil may be substantially different from *Immundisolibacter* organisms abundant in the bioreactor, or strain TR3.2^{T}, which was originally isolated from bioreactor-treated soil [236]. The final enrichment band that may represent a co-metabolizer of azaarenes was identified as a *Methyloversatilis* spp., although no isolate from that genus was recovered during this experiment. *Methyloversatilis* species are methylotropes that grow primarily on single carbon (C-1) compounds. *Methyloversatilis* spp. have been connected to the degradation of benazolin-ethyl in activated sludge [254, 255] but have not been implicated in the degradation of azaarenes.

Transformation of phenanthridine may occur in these soils through several processes including: (1) organisms growing on either anthracene or phenanthrene as a carbon source and metabolizing phenanthridine through an independent metabolic pathway, or (2) induced enzymes in PAH-degrading organisms may transform phenanthridine as well. No major peaks that might represent phenanthridine metabolites were detected during HPLC analysis of phenanthridine-containing samples, so any metabolites produced might be transient and degradable for

microorganisms in the soil. None of our data suggest which mechanism might be the case in these soils, and are a topic for future research.

To further investigate the capabilities of pure cultures corresponding to genera from prominent DGGE bands and isolated azaarene degraders, co-metabolism experiments were performed using three isolates: *Acidovorax* sp. NA3 (a known phenanthrene degrader) [235], *Immundisolibacter cernigliae* TR3.2^T (phenanthrene and anthracene degrader) [236], and *Sphingobium* sp. PAP1 (phenanthrene degrader). Neither *Acidovorax* sp. NA3 nor *Immundisolibacter cernigliae* TR3.2^T showed the ability to transform a high concentration of phenanthridine using either anthracene or phenanthrene as a primary carbon source. In fact, the reduced removal of either phenanthrene or anthracene by these isolates in the presence of azaarenes suggests toxic or inhibitory effects of those compounds on PAH degradation. However, the possibility that other members of those genera or even a microbial consortium of those organisms possess the ability to transform azaarenes cannot be discounted. Evidence indicated that *Sphingobium* sp. PAP1 was capable of transforming phenanthridine. Interestingly, additional acridine may have inhibited the ability of strain PAP1 to transform phenanthridine, with a statically significant ($p < 0.05$) lower amount of phenanthridine transformed (27% ± 5%) compared to conditions without acridine (Figure 18). For this pure culture experiment with strain PAP1, UV detection during HPLC analyses did detect a peak other than the azaarene and PAHs. This unidentified peak could represent a transformation metabolite of phenanthridine and could give clues to the metabolic pathway through which that azaarene is degraded.

Conclusions. Despite the potential inhibitory effect on soil microbial communities, degradation of carbazole, acridine and phenanthridine was observed in contaminated soils and quantified using a newly developed HPLC protocol. Carbazole was readily degraded in both

untreated feed soil and bioreactor-treated soil, and carbazole-degrading bacteria were isolated with most strains belonging to the genus *Pseudoxanthomonas*. Co-metabolism was suggested as a major mechanism for the removal of phenanthridine from feed soil, with the isolated *Sphingobium* strain PAP1 capable of co-metabolism of phenanthridine using phenanthrene as a primary carbon source [249]. Anthracene was also indicated as a carbon source for co-metabolizing phenanthridine in feed soil. Both acridine and phenanthridine were readily degraded in bioreactor-treated soil, most likely by abundant organisms already present in the aerobic bioreactor. Pure cultures of *Immundisolibacter cernigliae* strain TR3.2^{T} and *Acidovorax* sp. NA3 did not show the ability to transform either acridine or phenanthridine, although other members of those genera may possess that ability.

Future work. Stable isotope probing (SIP) using labeled azaarenes may allow the identification of specific organisms associated with removal of azaarenes. Detailed microbial community analysis employing next-generation sequencing could also provide more information regarding the specific organisms associated with the removal or co-metabolism of azaarenes [256]. Identifying the metabolic pathway of degradation through the analysis of metabolites could be performed with *Sphingobium* strain PAP1. Better understanding of the indigenous azaarene degraders and pathways of the biodegradation of azaarenes could lead to better strategies for the remediation of contaminated sites and treatment of azaarene-related industrial wastes.

APPENDIX

Table A1. Re-growth of select strains on a variety of (mostly) aromatic carbon sources in the 96-well plate assay.

Substrate	FP2	FP12
Fluorene	11.3 ± 5.5	1.2 ± 0.0
Phenanthrene	4.6 ± 1.3	6.5 ± 2.5
Anthracene	3.2 ± 1.1	5.6 ± 3.7
Fluoranthene	3.8 ± 1.9	10.3 ± 5.0
Pyrene	18.8 ± 4.6	8.3 ± 6.3
Benzo(a)Anthracene	2.1 ± 0.0	4.3 ± 1.1
Chrysene	3.7 ± 3.4	1.4 ± 0.7
Benzo(b)Fluoranthene	11.2 ± 4.7	2.7 ± 1.4
Benzo(k)Fluoranthene	3.5 ± 1.5	4.5 ± 2.7
Benzo(a)Pyrene	4.2 ± 3.3	1.1 ± 0.0
Dibenzo(a,h)Anthtracene	6.5 ± 1.8	2.8 ± 1.2
Benzo(g,h,i)Perylene	12.0 ± 4.1	3.4 ± 2.9
9-Fluorenone	1.0 ± 0.0	7.2 ± 3.8
9,10-Phenanthrenequinone	12.1 ± 3.1	6.3 ± 2.3
9-Anthrone	3.3 ± 3.2	8.4 ± 5.8
9,10-Anthraquinone	4.3 ± 2.4	1.9 ± 1.3
Benzo(a)Anthraquinone	9.3 ± 1.8	14 ± 8.1
Carbazole	1.5 ± 1.2	6.1 ± 2.4
Phenanthridine	2.0 ± 1.6	1.8 ± 1.0
Acridine	2.6 ± 1.5	12.5 ± 3.4
Salycilate	**38.2 ± 10.7**	**19.9 ± 3.6**
Phthalate	4.6 ± 1.2	7.1 ± 2.4
Hexadecane	1.2 ± 1.2	3.5 ± 2.6
Negative control	3.2 ± 2.1	3.6 ± 2.0
Positive control	56.4 ± 10.5	62.4 ± 3.1

Note: Values represent mean and standard deviation of protein concentrations in triplicate wells. Bold indicates conditions which satisfy the conditions of a) at least twice the value of negative control and b) near one third the value of the positive control.

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