EFFECTS OF BIOREMEDIATION ON BIOAVAILABILITY AND GENOTOXICITY OF CONTAMINANTS IN PAH-CONTAMINATED SOIL

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ABSTRACT

JING HU: Effects of Bioremediation on Bioavailability and Genotoxicity of Contaminants in PAH-contaminated Soil
(Under the direction of Michael D. Aitken)

Polycyclic aromatic hydrocarbons (PAHs) are a major pollutant class at thousands of contaminated sites. They are of great concern due to their genotoxicity, mutagenicity and carcinogenicity. Bioremediation is one of the commonly applied remediation strategies to clean up PAHs from the soils and the sediments. However, remediation goals are typically based on removal of the target PAHs rather than on broader measures related to health risks. To better understand the risks addressed by remedial action and the risks caused by remedial action, the bioavailability and genotoxicity of contaminants in a PAH-contaminated soil from a former manufactured-gas plant site were investigated for two bioremediation processes simulated in the laboratory: an *ex situ* bioreactor system and an *in situ* column system.

Potential dermal bioavailability of PAHs was investigated via PAH desorption from both untreated soil and treated soil to a two-dimensional hydrophobic surface as a function of soil loading, temperature, soil moisture content and contact time. This study demonstrated that dermal exposure assessment from soil should consider site-specific conditions that influence the bioavailability of hydrophobic contaminants to skin. Moreover, the effects of remediation on potential dermal exposure should consider not only the reduction in contaminant concentration but also the reduction in contaminant

bioavailability.

Effects of bioremediation on toxicity and genotoxicity of PAH-contaminated soil were evaluated by the DT40 DNA damage response analysis. This study demonstrated that different bioremediation strategies could lead to different outcomes of toxicity and genotoxicity for PAH-contaminated soil. Toxicity and genotoxicity bioassays can be an effective supplement to chemical analysis-based risk assessment for contaminated soil. Bioavailability of genotoxins in the soil was investigated throughout the bioreactor treatment cycle. This study demonstrated that although bioreactor treatment could increase the genotoxicity of the whole soil, any genotoxic constituents that may have formed during treatment were primarily associated with less accessible domains in the soil. Bioavailability should be incorporated into the evaluation of bioremediation as a treatment strategy of contaminated soils to reduce the extent of cleanup required to that which is necessary to be protective of humans as well as ecosystems.

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I thank Stephen Richardson for operating the column system and performing the analyses on soil characterization for the column system. I thank Alden Adrion for developing the method and performing the analysis of the oxy-PAHs. I thank Chad Roper for design of the method to measure desorption to the C18 disk. I thank Hiroshi Arakawa, Jean-Marie Buerstedde, and Shunichi Takeda for providing various DT40 mutants. I thank Wei Sun for assistance in statistical analysis. I thank Bo Pan (Kunming University of Science and Technology, Kunming, China) for advice on dialysis tubing selection. I also thank Xin-Rui Xia and Xiang Q. Kong (both of North Carolina State University) for assistance with HLB resin application.

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1. INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are among the contaminants of greatest concern at thousands of contaminated sites (USEPA 2004a; USEPA 2011), due to their known or suspected genotoxic, mutagenic or carcinogenic effects (Ramesh et al. 2004; Baird et al. 2005). PAHs have been found at over 700 Superfund sites (USEPA 2011) and also are the predominant pollutant class at an estimated 45,000 former manufactured gas plant (MGP) sites in the U.S.A. (USEPA 2004a). Bioremediation is an established technology to clean up PAH-contaminated soils and sediments by microbial degradation (Bamforth and Singleton 2005; USEPA 2007; Elliot et al. 2011). As of 2005, bioremediation was used at 26% of the PAH-contaminated Superfund cleanup sites designated by the United States Environmental Protection Agency (USEPA), more than any other single remediation technology (USEPA 2007).

The inherent assumption in the application of remediation methods is that the removal of the regulated PAHs corresponds to a reduction in risk to human health. However, several studies have observed that disappearance of monitored PAHs during bioremediation of PAH-contaminated soil and sediments has not corresponded to a decrease in toxicity and/or genotoxicity (Baud-Grasset et al. 1993; Hughes et al. 1998; Haeseler et al. 1999b; Sayles et al. 1999; Mendonca and Picado 2002; Sasek et al. 2003; Andersson et al. 2009; Gandolfi et al. 2010). There are various factors that can influence toxicity and genotoxicity of PAH-contaminated soil during bioremediation. A better understanding of the sources and bioavailability of toxic and genotoxic agents either

caused by or remaining after bioremediation is essential to understanding and overcoming those factors that could limit the application of bioremediation to an even broader range of PAH-contaminated sites. The research described herein seeks to investigate the sources and bioavailability of toxic and genotoxic agents in PAH-contaminated soil undergoing bioremediation using chemical analysis coupled with a toxicity/genotoxicity bioassay.

1.1. Specific aims and rationale

Aim 1: Evaluate desorption of polycyclic aromatic hydrocarbons from field-contaminated soil to a two-dimensional hydrophobic surface before and after bioremediation.

Dermal exposure can represent a significant health risk in settings involving potential contact with complex materials containing PAHs, including PAH-contaminated soil or sediment (Boffetta et al. 1997; Sobus et al. 2009). Most previous work has been concerned with integrated uptake of chemicals through the skin and not with how a contaminant desorbs to the skin surface in the first place. Desorption properties, such as dynamic conditions by which soil contacts the skin, interactions of the soil with the skin surface and chemical interaction with the soil, could influence dermal uptake of chemicals (McKone and Howd 1992; Spalt et al. 2009). Therefore, it is important to understand desorption of PAHs from contaminated soil or sediment to the skin surface in order to better evaluate dermal exposure and to better set the cleanup goal for remediation. Aim 1 was to investigate PAH desorption from a field-contaminated soil to a two-dimensional hydrophobic surface as a function of various factors influencing desorption, including soil loading, temperature, soil moisture content, and exposure time before and after bioremediation.

Aim 2: Evaluate the effects of bioremediation on genotoxicity of polycyclic aromatic hydrocarbon-contaminated soil using genetically engineered, higher eukaryotic cell lines.

In most cases it remains unknown whether the removal of the targeted PAHs during bioremediation corresponds to a reduction in health risk (Lemieux et al. 2009). Significant amounts of other carcinogenic polyaromatic compounds are also found in PAH-contaminated soils (Lundstedt et al. 2003; Lemieux et al. 2008; Lemieux et al. 2009), and whether all hazardous compounds degrade concomitantly with the targeted PAHs monitored at contaminated sites is unknown (Lundstedt et al. 2003). Incomplete metabolism of PAHs in contaminated soil can also yield byproducts during bioremediation, which can exhibit greater toxicity than the parent PAHs (Lundstedt et al. 2003; Lundstedt et al. 2007; Lemieux et al. 2008; Lemieux et al. 2009). Aim 2 was to investigate effects of bioremediation on toxicity and genotoxicity of PAH-contaminated soil from a former MGP site using the DT40 DNA damage response analysis.

Aim 3: Evaluate the bioavailability of (geno)toxic contaminants in PAH-contamianted soil from a former MGP site before and after biological treatment.

Historically, the potential effects of contaminated sites on human or ecological health are assessed based on the total contaminant concentrations as determined by vigorous chemical extraction techniques (Alexander 2000). However, because of their association with different soil components, hydrophobic organic pollutants in soil are only partly available for uptake by organisms, for exerting toxic effects, and for biodegradation by microorganisms (Alexander 1995; Alexander and Alexander 1999; Alexander 2000; Reid et al. 2000a; Lei et al. 2004; Jablonowski et al. 2008). Recently, the US Interstate Technology and Regulatory Council (ITRC) has advised incorporating bioavailability

considerations into the evaluation of contaminated sites to reduce the extent of cleanup required to that which is necessary to be protective of human health and the environment (ITRC 2011). Aim 3 was to investigate the bioavailability of PAHs and their oxygenated metabolites (oxy-PAHs) and the associated toxicity and genotoxicity during biological treatment, using the same toxicity/genotoxicity assay and bioreactor system used under Aim 2.

1.2. Dissertation organization

This dissertation is compiled into six chapters. Chapter 1 is an introduction into the objectives, rationale and framework of the dissertation. Chapter 2 is a review of literature to date, which provides background information for the work described in this dissertation. Chapters 3, 4 and 5 are each dedicated to one of the specific aims previously outlined. Chapter 3 and Chapter 4 were published in *Chemosphere* (Hu and Aitken 2012) and *Environmental Science & Technology* (Hu et al. 2012), respectively. Chapter 5 is a draft manuscript intended for submission to a peer-reviewed journal. Chapter 6 provides conclusions and recommendations for future research.

Members of my dissertation committee (M.D. Aitken, J. Nakamura and D. Shea) provided guidance on experimental design and methods, and contributed to interpretations of experimental results. Accordingly, they have been identified as co-authors on the relevant chapters. The roles of the remaining co-authors for Chapters 4 and 5 are identified in the chapter itself.

2. LITERATURE REVIEW

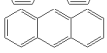
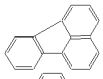
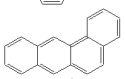
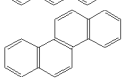
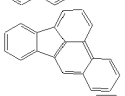
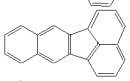
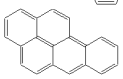
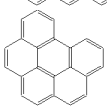
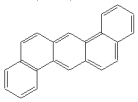
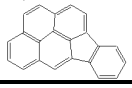
2.1. PAH contamination

PAHs are a class of compounds consisting of two or more fused benzene rings (Harvey 1991) which provide high chemical stability through the delocalization of electrons in their chemical bonds (Schwarzenbach et al. 2003). They are non-polar compounds with low aqueous solubilities, vapor pressures and Henry's law constants, but high octanol/water and organic carbon partitioning coefficients (Mackay et al. 1992). Selected properties of 16 USEPA Priority PAHs are presented in Table 2.1. The chemical stability and hydrophobicity of PAHs contribute greatly to their persistence in the environment (Schwarzenbach et al. 2003; Bamforth and Singleton 2005; Johnsen et al. 2005; Lu et al. 2011).

PAHs mainly occur in oil, coal and tar deposits and they are also continuously generated by the incomplete combustion of fossil fuel and biomass (Mastral and Callen 2000; Xu et al. 2006). The majority of PAHs in the environment come from incomplete combustion of carbonaceous materials during energy and industrial production processes, such as coking plants, gas plants, refineries, wood-preserving plants and petrochemical plants. PAHs are ubiquitous in the natural environment (van Metre and Mahler 2005; Berrojalbiz et al. 2009; Lammel et al. 2009; van Metre et al. 2009; Rodenburg et al. 2010; Usenko et al. 2010; Timoney and Lee 2011; Wang et al. 2011) as well as thousands of contaminated sites. PAHs have been found at over 700 Superfund sites (USEPA 2011)

and also are the predominant pollutant class at an estimated 45,000 former MGP sites in the U.S.A. (USEPA 2004a). As a pollutant, PAHs are of great human health concern, because of their ubiquity in the environment as well as their known or suspected genotoxicity, mutagenicity and carcinogenicity (Bostrom et al. 2002b; White 2002; Ramesh et al. 2004; Shimada and Fujii-Kuriyama 2004; Baird et al. 2005; Xue and Warshawsky 2005; Mordukhovich et al. 2010).

Table 2.1 Chemical structures and selected properties of the 16 USEPA Priority Pollutant PAHs [a, b]

Compound	Structure	MW	C_{iw}^{sat}	p_i^*	H	logK_{ow}	logK_{oc}
Naphthalene (NAP)		128.18	3.10E+01	1.04E+01	4.30E+01	3.37	3.03
Acenaphthylene (ACY)		152.19	1.61E+01	5.80E-01	5.50E+00	4.00	3.40
Acenaphthene (ACE)		154.20	3.80E+00	3.00E-01	1.22E+01	3.92	3.62
Fluorene (FLU)		166.23	1.90E+00	9.00E-02	7.87E+00	4.18	3.97
Phenanthrene (PHN)		178.24	1.10E+00	2.00E-02	3.24E+00	4.57	4.21
Anthracene (ANT)		178.24	4.50E-02	1.00E-03	3.96E+00	4.54	4.38
Fluoranthene (FLA)		202.26	2.60E-01	1.23E-03	1.04E+00	5.22	4.55
Pyrene (PYR)		202.26	1.32E-01	6.00E-04	9.20E-01	5.18	4.83
Benz[*a*]anthracene (BaA)		228.30	1.1E-02	2.80E-05	5.81E-01	5.91	5.08
Chrysene (CHR)		228.30	2.90E-03	5.70E-07	6.50E-02	5.86	5.17
Benzo[*b*]fluoranthene (BbF)		252.32	2.00E-03	2.18E-07	2.14E-02	5.75	5.72
Benzo[*k*]fluoranthene (BkF)		252.32	8.00E-04	5.20E-08	1.60E-02	6.00	5.73
Benzo[*a*]pyrene (BaP)		252.32	3.80E-03	7.00E-07	4.60E-02	6.04	5.99
Benzo[*g,h,i*]perylene (BgP)		276.34	3.00E-04	1.69E-08	7.50E-02	6.50	6.35
Dibenz[*a,h*]anthracene (DBA)		278.36	6.00E-04	3.70E-10	1.28E-03	6.75	5.96
Indeno[*1,2,3-c,d*]pyrene (INP)		276.34	6.20E-03	2.70E-09	1.00E-02	7.66	6.20

[a] abbreviations: MW = molecular weight (g/mol); C_{iw}^{sat} = aqueous solubility (mg/L); p_i^* = vapor pressure (Pa); H = Henry's Law Constant (Pa·m^3/mol·K); K_{ow} = octonol/water partitioning coefficient; K_{oc} = organic carbon partitioning coefficient. [b] All data from (Mackay et al. 1992).

2.2. PAH distribution in contaminated coil

Interactions of hydrophobic organic compounds (HOCs) such as PAHs with geosorbents (i.e. soils and sediments) may affect the bioavailability of the compounds and subsequently affect bioremediation endpoints (Alexander 1995; Luthy et al. 1997; Alexander 2000). In field-contaminated soil, HOCs are associated with any of several

components of the soil, including minerals, soil organic matter (SOM), combustion residue particles and various non-aqueous phase liquids (NAPLs) (Chai et al. ; Zhou et al. ; Gustafsson et al. 1997; Luthy et al. 1997; Hong et al. 2003; Cornelissen et al. 2005). There is a great diversity of physical and chemical principles that govern the interactions between HOCs and these soil components. When total organic matter is present above trace levels in soils/sediments, sorption of HOCs by inorganic minerals could be neglected (Schwarzenbach and Westall 1981; Kile et al. 1995; Huang and Weber 1997; Luthy et al. 1997). Although the mineral phase can indirectly regulate sorption by controlling the distribution and/or conformation of organic matter at the solid-aqueous interface (Xiao et al. 2004; Feng et al. 2006), organic matter controls sorption and bioavailability of HOCs in soils/sediments (Schwarzenbach and Westall 1981; Kile et al. 1995; Huang and Weber 1997; Luthy et al. 1997).

SOM is the major fraction of organic matter in natural soils and is an important reservoir for sorption of PAHs in the soil matrix. SOM is a nontoxic, naturally occurring material composed of living biomass of microorganisms, fresh and partially decomposed residues, and humus. SOM is considered as a sorbent with sorption sites of distributed energy, which leads to the existence of both linear and nonlinear sorption sites for organic chemicals (Weber et al. 1992; Xing and Pignatello 1997; Xia and Pignatello 2001; Kriipsalu et al. 2008). Several sorption theories have been proposed based on this conceptualization, including dual-mode model (Xing and Pignatello 1997), distributed reactive model (Weber et al. 1992), and extended dual-mode model (Xia and Pignatello 2001). Generally, SOM is considered to have rubbery (amorphous carbon phase) and glassy (condensed carbon phase) domains (Kriipsalu et al. 2008). The “rubbery” domain

is characterized by relatively rapid rates of sorption and desorption, little or no sorption-desorption hysteresis, and greater bioavailability of sorbed HOCs. The “glassy” domain is characterized by nonlinear sorption, slow rates of sorption and desorption, sorption-desorption hysteresis, and significantly reduced bioavailability of sorbed HOCs. Sorption of HOCs to soil is the combination of sorption to the rubbery domain by dissolution (linear sorption) and sorption to the glassy domain by concurrent dissolution (linear sorption) and hole-filling mechanisms (nonlinear sorption) (Weber et al. 1992; Xing and Pignatello 1997; Xia and Pignatello 2001; Kriipsalu et al. 2008):

$$S = S(D) + S(H) = K_p C_w + \sum_{i=1}^{n} \frac{S_i^o b_i C_w}{1 + b_i C_w}$$

where, *S(D)* is the sorption through dissolution; *S(H)* is the sorption through hole-filling; K_p is a lumped partition coefficient of HOCs between aqueous phase and dissolution regions; C_w is the HOC concentration in water; S_i^o is the capacity constant for specific site *i*; b_i is the affinity constant for specific site *i*. However, because of the number of parameters in *S(H)*, the above isotherm is rather less practical, despite being more mechanistic. A very common mathematical approach for fitting experimentally determined sorption data using a minimum of adjustable parameters employs an empirical relationship such as the Freundlich isotherm (Weber et al. 1992; Xing and Pignatello 1997; Xia and Pignatello 2001; Kriipsalu et al. 2008):

$$S = K_F C_w^{\,n}$$

where, K_F is the Freundlich constant or capacity factor; *n* is the Freundlich exponent (the smaller the *n*, the broader the energy distribution, the greater the contribution of the hole-filling mechanism, and the higher nonlinearity). It is acknowledged that there can be

more than two domains with different affinities for PAHs in field-contaminated soils or sediments (Luthy et al. 1997; McLeod et al. 2004; Cornelissen et al. 2005), but it is difficult to quantify the relative contributions of each domain (Luthy et al. 1997) and dual-mode models often describe sorption and desorption data adequately (Allen-King et al. 2002; Accardi-Dey and Gschwend 2003).

Combustion residue particles include coal, kerogen and black carbon (i.e. soot and char). These materials typically make up a small fraction of the soil/sediment total organic carbon. For example, median black carbon contents as a fraction of total organic carbon are 9% for sediments (n=300) and 4% for soils (n=90) (Cornelissen et al. 2005). A fraction of PAHs can be sequestered within the particle matrix as a result of simultaneous production during combustion or coalification (Jonker and Koelmans 2002). More importantly, combustion residue particles contribute greatly to the nonlinearity of sorption for HOCs. They are a very strong sorption phase for HOCs and might dominate the observed overall sorption by soils (Xiao et al. 2004). One reason that PAHs are sorbed strongly to black carbon and other combustion residue particles is because PAHs are planar compounds that fit in the black carbon nanopores (Cornelissen et al. 2004). Sorption of HOCs to these materials generally exceeds sorption of HOCs in amorphous organic matter by a factor of 10-100 (Cornelissen et al. 2005).

Adherent or entrapped NAPLs, such as coal tar, oil or creosote, can also function as an important compartment for HOCs. In a source zone contaminated by tar or creosote, a substantial fraction of the PAHs can be expected to be associated with a NAPL (Rutherford et al. 1997; Haeseler et al. 1999a) that is heterogeneously distributed at a grain or aggregate scale (Karimi-Lotfabad and Gray 2000). There is a rapidly reversible

association of HOCs with NAPLs (Luthy et al. 1997). Diffusion of the HOCs within the NAPL itself and interfacial mass transfer may be rate-limiting steps for desorption of these compounds to the aqueous phase. Partitioning behavior of PAHs for impacted soils at MGP sites containing weathered pitch particles might be dominated by the sorption to pitch and not by natural organic matter or black carbon (Khalil et al. 2006).

2.3. Bioavailability of PAHs in contaminated soil

The term “bioavailability” has been given a variety of discipline-specific definitions in the literature (Ehlers and Luthy 2003; Semple et al. 2004). Bioavailability of a chemical depends on the physical environment, the biological receptor, and time of exposure (Ehlers and Luthy 2003; Semple et al. 2004). It can be viewed as the individual physical, chemical, and biological interactions that determine the exposure of organisms to chemicals associated with soils and sediments, including sorption/desorption between solid and aqueous phase, transport (both dissolved and particulate-bound) to the membrane of the organism, and uptake through the membrane into a living system where compounds are accumulated or metabolized (Ehlers and Luthy 2003). Thus, for example, the bioavailability of a PAH in a contaminated soil to a microorganism capable of degrading it can be different from the bioavailability of the same chemical in the same soil with respect to dermal uptake by a person.

2.3.1. Bioavailability of toxic and genotoxic agents

Accurate risk assessments at contaminated sites require accurate and representative estimates of chemical exposure. Because of the association with different soil components as described in the previous section, organic pollutants in soil are only partly available for uptake by organisms, for exerting toxic effects, and for biodegradation by

microorganisms (Alexander 1995; Alexander and Alexander 1999; Alexander 2000; Reid et al. 2000a; Lei et al. 2004; Jablonowski et al. 2008). Poor correlation has been observed between the total amount of a contaminant determined by vigorous solvent extraction and the compound's bioavailability to earthworms and bacteria over time in soil (Kelsey and Alexander 1997; Kelsey et al. 1997). Assuming that a contaminant is 100% available will overestimate the health risk of soil contaminated with HOCs (Alexander 2000). Recently, the US Interstate Technology and Regulatory Council (ITRC) advised incorporating bioavailability considerations into the evaluation of contaminated sites to reduce the extent of cleanup required to that which is necessary to be protective of human health and the environment (ITRC 2011).

A variety of biological and chemical techniques have been developed to estimate and predict bioavailability. Biological techniques involve measuring substrate uptake, mineralization or toxicity (Kelsey et al. 1997; Stroo et al. 2000; Braida et al. 2004). Chemical techniques involve non-exhaustive solvent extraction (Hatzinger and Alexander 1995; Kelsey et al. 1997; Breedveld and Karlsen 2000; Reid et al. 2000b; Liste and Alexander 2002) and solid-phase extraction (Cornelissen et al. 1998; Cuypers et al. 2002; Loehr et al. 2003; Lei et al. 2004). Solid-phase extraction is a common method for estimating bioavailability in contaminated soils and sediments with polymeric adsorbent resins such as Tenax beads and XAD, which serve as an infinite sink (Cornelissen et al. 1998; Cuypers et al. 2002; Loehr et al. 2003; Lei et al. 2004). Several investigators have observed a reasonable correlation between the rapidly desorbing fraction of PAHs and the extent of PAH biodegradation in contaminated harbor sediments (Cornelissen et al. 1998). However, estimation of bioavailability by solid-phase extraction in PAH-contaminated

soil is complicated by the fact that current adsorbent resins work well with only a limited range of chemicals.

In a complex system such as soil, some transformations that do not lead to complete metabolism of the parent compound are inevitable. The net accumulation of products of incomplete metabolism is responsible at least in part for the toxicity observed in studies on bioremediation of field-contaminated soil or sediment. In PAH-contaminated soil undergoing bioremediation, hundreds of hazardous compounds have been identified (Lundstedt et al. 2003) covering a very wide range of physicochemical properties, with logK_{ow} values ranging from 2 to 8. Polar and semi-polar metabolites of PAHs have been observed to accumulate in the soil during bioremediation (Lundstedt et al. 2003). In studies employing bioassay-directed fractionation and analysis of extracts from field-contaminated soil or sediment, genotoxicity has been associated with fractions containing oxy-PAHs or other polar compounds rather than the parent PAHs (Park et al. 2008). But until now, no adsorbent resins have demonstrated the ability to accumulate both parent chemicals and their polar transformation products.

2.3.2. Bioavailability relevant to dermal uptake

Dermal exposure can represent a significant health risk in settings involving potential contact with complex materials containing PAHs, such as creosote, asphalt, coke, and oil, as well as PAH-contaminated soil or sediments (Boffetta et al. 1997; Tsai et al. 2001; McClean et al. 2004; Chen and Liao 2006; Cirla et al. 2007; Chen et al. 2008; Sobus et al. 2009; Linares et al. 2010). Monitoring of dermal exposure of PAHs usually relies on the measurement of specific metabolites, such as urinary 1-hydroxypyrene of the exposed subjects (Elovaara et al. 1995; Bentsen-Farmen et al. 1999; Bouchard and Viau 1999;

Jongeneelen 2001), which represents an integrated measure of multiple processes including desorption from the contaminated matrix to the skin surface, uptake by the skin, transport to the dermal capillaries, metabolism, and excretion. In laboratory experiments, the substance of interest is usually applied to a sample of human or animal skin in a diffusion cell, which contains a receptor fluid beneath the skin to absorb any contaminant that is taken up by and diffuses through the skin sample (Stroo et al. 2000; Roy and Singh 2001; Touraille et al. 2005; Spalt et al. 2009) .

Free chemicals can be absorbed by skin through the following sequence of events: partitioning into stratum corneum, diffusion through the stratum corneum, partitioning into the viable epidermis, diffusion through the epidermis and upper dermis, and finally capillary uptake (Mukhtar 1992). The stratum corneum is a 10-20 μm thick, non-viable outer layer of the epidermis and behaves as a hydrophobic membrane (Downing 1992). Diffusion of organic nonelectrolytes through the stratum corneum is generally considered as the rate-limiting step in uptake of a chemical via dermal exposure (Downing 1992). However, contaminants in soil, unlike those free chemicals in direct contact with skin, must first be released from the soil matrix to the stratum corneum surface before uptake by skin (Roy et al. 1998; Shatkin et al. 2002). The uptake of a contaminant from soil to skin ultimately depends on how quickly the contaminant reaches the skin surface. Desorption properties from soil to the stratum corneum surface therefore can influence dermal uptake of chemicals, such as dynamic conditions by which soil contacts the skin, interactions of the soil with the skin surface and chemical interaction with the soil (McKone 1990; Finley et al. 1994). By incorporating two-compartment desorption kinetics for PAHs in field-contaminated soils into a fugacity-based model of dermal

uptake via soil, Shatkin et al (Shatkin et al. 2002) concluded that estimated dermal uptake was lower than if total soil concentrations had been used and that fast desorbing fraction of PAHs dominated dermal uptake of PAHs from soil.

Dermal uptake of a contaminant from soil occurs by direct contact transfer between soil and skin, as well as by indirect uptake via diffusion through pore air and/or pore water (McKone and Howd 1992; Duff and Kissel 1996; Bunge and Park 1998; Riley et al. 2004; Spalt et al. 2009). Transport of the contaminants away from the skin can also occur via volatilization (Spalt et al. 2009) or advection into a flowing aqueous phase. The manner of exposure to soil or sediments is expected to determine the dermal uptake of contaminants by humans. Exposure to unsaturated soil or dust is expected to occur through direct solid-skin contact transfer or indirect air-skin diffusion uptake, while exposure to saturated soil or contaminated sediment *in situ* is expected to occur through liquid-skin diffusion. Both scenarios have been evaluated in laboratory studies: applying soil directly to the skin sample in some cases (Roy et al. 1998; Stroo et al. 2000; Reifenrath et al. 2008; Spalt et al. 2009) and applying an aqueous medium with the soil to the skin surface in other cases (McKone and Howd 1992; Duff and Kissel 1996; Spalt et al. 2009). Simulation models of dermal uptake from soil have also been developed to account for direct contact transfer between soil and skin, and indirect uptake via diffusion through pore air and/or pore water (McKone and Howd 1992; Duff and Kissel 1996; Bunge and Park 1998; Riley et al. 2004; Spalt et al. 2009). In a simulation model, a soil moisture content below 10% was predicted to decrease dermal uptake of PAHs from soil (Shatkin et al. 2002), consistent with the experimental observation that application of artificial sweat to the skin surface can increase the dermal uptake of contaminants from

soil (Reifenrath et al. 2008).

Dermal uptake of chemicals from soil is also affected by soil loading, the mass of soil applied per unit surface area of skin (Spalt et al. 2009). Bunge and Parks (Bunge and Park 1998) concluded that 1) when soil loading was less than monolayer coverage, which depends on particle size and soil bulk density (Duff and Kissel 1996; Spalt et al. 2009), the percentage absorption was constant regardless of soil loading, and increasing soil loading could dramatically increase mass absorbed per unit skin area; 2) when soil loading exceeded monolayer coverage, the percentage absorption decreased significantly as soil loading increased while absorbed mass per unit area remained constant. Several investigators have confirmed this theory experimentally (Duff and Kissel 1996; Roy and Singh 2001; Shatkin et al. 2002; Touraille et al. 2005). However, these two predictions are based on the assumption that the contaminant concentration in soil is constant. When the contaminants in soil are depleted, dermal uptake increases, although not proportionally, with soil loading (Bunge and Park 1998). Based on these theories and observations, several investigators (Duff and Kissel 1996; Touraille et al. 2005; Spalt et al. 2009) have criticized the use of a fixed percent of the total contaminant uptake by skin in estimating human health risk. The USEPA (USEPA 2004b) stipulates a fixed percent absorption (13%) for dermal uptake of benzo[*a*]pyrene and other PAHs based on measurements from a single study (Wester et al. 1990). This percentage is coupled to a skin adherence factor (related to soil loading), exposure time and other factors to arrive at absorbed doses to various exposed population groups. While this approach inherently assumes that only a fraction of the measured PAH content is available for uptake over the exposure period, it does not account for differences in contaminant availability among

contaminated soils or sediments (Shatkin et al. 2002).

There has been limited research on dermal uptake of the contaminants in field-contaminated soil. In most previous experimental work on dermal uptake of contaminants from soil, an individual contaminant was introduced into the soil through a solvent that subsequently evaporated (Duff and Kissel 1996; Roy et al. 1998; Stroo et al. 2000; Touraille et al. 2005; Roy et al. 2007; Roy et al. 2008; Roy et al. 2009; Spalt et al. 2009). Exposure to spiked chemicals in soil does not account for the effect of field-contaminated, site-specific soil conditions on the dermal availability of compounds. Spalt et al. (Spalt et al. 2009) pointed out that dermal absorption studies with spiked chemicals often utilize very short soil-chemical contact times before application of the spiked soil to skin for convenience; in the extreme cases, the contact time was zero. The hypothesis that the contaminant could entirely associate with soil organic matter instantly is not correct. Furthermore, the process of aging can decrease the bioavailability (Hatzinger and Alexander 1995; Pignatello and Xing 1996; Alexander and Alexander 2000). Short aging time or excessive chemical concentration far above sorption capacity of the soil applied may also lead to the presence of free chemical phase, from which the results obtained do not represent the measurement of dermal uptake from soil but rather from neat compounds themselves (Spalt et al. 2009). Therefore, spiked contaminants are not likely to represent the influence of bioavailability on dermal uptake and the corresponding risk of exposure to the contaminants, and the most valuable insights into dermal exposure will be obtained through work with the native contaminants in field-contaminated soils.

2.4. Bioremediation of PAHs in contaminated soil

Bioremediation is an established technology for cleanup of PAH-contaminated soil and sediment (Bamforth and Singleton 2005; USEPA 2007; Elliot et al. 2011). As of 2005, bioremediation was used at 26% of the PAH-contaminated USEPA Superfund cleanup sites, more than any other single remediation technology (USEPA 2007). Bioremediation of PAH-contaminated soils/sediments can be accomplished in both *in situ* and *ex situ* remediation. *In situ* treatments do not require excavation and transport of the soil to a treatment plant or disposal, while *ex situ* treatments are more amenable to monitoring and control. The main principle of bioremediation is to remove organic pollutants to an innocuous state through metabolism by microorganisms via oxidative or reductive processes under controlled conditions (Mueller et al. 1996). It involves the breakdown of organic compounds through biotransformation into less complex metabolites, and through mineralization into inorganic minerals, i.e. H_2O and CO_2 or, for anaerobic processes, CH_4.

2.4.1. Microbial metabolism of PAHs

A wide variety of bacteria, fungi, and algae have been discovered to degrade PAHs via either aerobic or anaerobic metabolism. The basis of aerobic metabolism is the oxidation of the aromatic ring, followed by the systematic breakdown of the compound to PAH metabolites and/or carbon dioxide (Figure 2.1) (Bamforth and Singleton 2005). The basis of anaerobic metabolism is the hydrogenation of the aromatic ring (Bamforth and Singleton 2005).

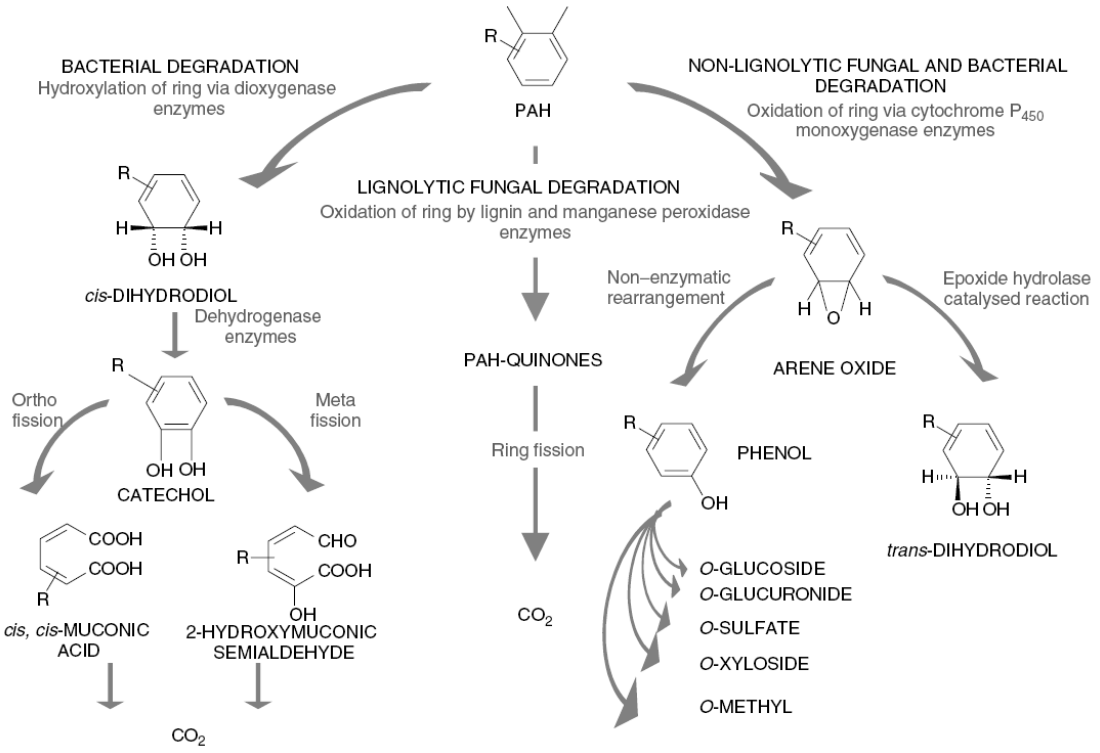


Figure 2.1 Microbial aerobic metabolism of PAHs (from reference (Bamforth and Singleton 2005)).

2.4.1.1. Bacterial metabolism of PAHs

Many bacteria have been documented to use PAHs up to four rings as sole carbon and energy source (Peng et al. 2008). Although bacterial growth on PAHs having five or more rings as sole carbon or energy source has not been documented, co-metabolism of high-molecular-weight (HMW) PAHs has been observed (Moody et al. 2004; Peng et al. 2008). Typically, bacterial metabolism of PAHs is an aerobic process; however anaerobic transformation of PAHs has been observed and is discussed in the Anaerobic Metabolism of PAHs section below.

The common metabolic pathways for aerobic bacterial degradation have been well investigated (Bamforth and Singleton 2005; Peng et al. 2008), such as for naphthalene (Davies and Evans 1964; Resnick et al. 1996; Annweiler et al. 2000), phenanthrene (Menn et al. 1993; Kiyohara et al. 1994; Pinyakong et al. 2003), anthracene and

acenaphthene (Dean-Ross et al. 2001; Pinyakong et al. 2004), and pyrene (Kim et al. 2007). The principal mechanism for the aerobic bacterial metabolism of PAHs is the initial oxidation of the benzene ring by the action of dioxygenase enzymes to form *cis*-dihydrodiols. A few bacteria are also capable of oxidizing PAHs by the action of the cytochrome P450 monooxygenase (two successive reactions) to form *trans*-dihydrodiols. Then, these dihydrodiols are dehydrogenated to form dihydroxylated intermediates. The dihydroxylated intermediates then can be further metabolized by ring cleavage and further metabolism to tricarboxylic acid (TCA) cycle intermediates, and then further converted to carbon dioxide and water. Complete pathways have not been elucidated for the metabolism of any five- or six-ring PAH.

2.4.1.2. Fungal metabolism of PAHs

Some fungi have been shown to remove PAHs more competently than bacteria. They can degrade low-molecular-weight (LWM) PAHs as well as HMW PAHs, including naphthalene, fluorene, phenanthrene, anthracene, pyrene, benzo[*a*]anthracene, chrysene, benzo[b]fluoranthene and benzo[k]fluoranthene, benzo[*a*]pyrene, benzo[g,h,i]perylene, and dibenzothiophene (Cerniglia 1997; Zheng and Obbard 2003). There are two main types of fungal metabolism of PAHs: non-ligninolytic fungal metabolism using cytochrome P450 system and ligninolytic fungal metabolism using the soluble extracellular enzymes such as lignin peroxidase, manganese peroxidase and laccase (Bamforth and Singleton 2005; Peng et al. 2008).

The first step in the metabolism of PAHs by non-ligninolytic fungi is to oxidize the aromatic ring in a cytochrome P450 monooxygenase enzyme-catalyzed reaction to produce an arene oxide. This route is similar to the mammalian metabolism of PAHs. The

arene oxide is subsequently hydrated via an epoxide-hydrolase-catalyzed reaction to form a *trans*-dihydrodiol. In addition, phenol derivatives may be produced from arene oxides by the non-enzymatic rearrangement of the compound, which can act as substrates for subsequent sulfation or methylation, or conjugation with glucose, xylose, or glucuronic acid. Most non-ligninolytic fungi are not capable of the complete mineralization of PAHs.

The principle mechanism of ligninolytic fungal metabolism of PAHs is generating free radicals (i.e. hydroxyl free radicals) to oxidize PAHs to form PAH-quinones and acids rather than dihydrodiols. The acids produced can be further converted to carbon dioxide and water. Ligninolytic fungi have low substrate specificity and are therefore able to degrade even the most recalcitrant compounds. And also, the enzymes involved are extracellular, and are theoretically able to diffuse into soil/sediment matrix and potentially oxidize PAHs with low bioavailablitity.

2.4.1.3. Anaerobic metabolism of PAHs

It was not until relatively recently that the potential of anaerobic microbial degradation of PAHs has been recognized. In the absence of molecular oxygen, alternative electron acceptors such as nitrate, ferrous iron and sulfate are necessary to oxidize these aromatic compounds (Coates et al. 1996). Recent research has clearly demonstrated that PAH degradation can occur under both nitrate-reducing (Rockne and Strand 1998; Rockne et al. 2000) and sulfate-reducing anaerobic (Coates et al. 1996; Meckenstock et al. 2000; Zhang et al. 2000; Tsai et al. 2009) conditions.

Mechanisms of anaerobic PAH degradation are still tentative. A metabolic pathway for anaerobic degradation of naphthalene has been proposed (Bamforth and Singleton 2005). As shown in Figure 2.2 (Bamforth and Singleton 2005), the first step is the

carboxylation of the aromatic ring to 2-naphthoic acid, which may activate the aromatic ring prior to hydrolysis. Stepwise reduction of 2-naphthoic acid via a series of hydrogenation reactions results in decalin-2-carboxylic acid. Decalin-2-carboxylic acid is subsequently converted to decahydro-2-naphthoic acid that is ultimately metabolized to CO_2. The exact mechanisms for naphthalene anaerobic degradation have not been confirmed and it is possible that the initial step under sulfate-reducing conditions occurs via a hydroxylation reaction to form a naphthol intermediate.

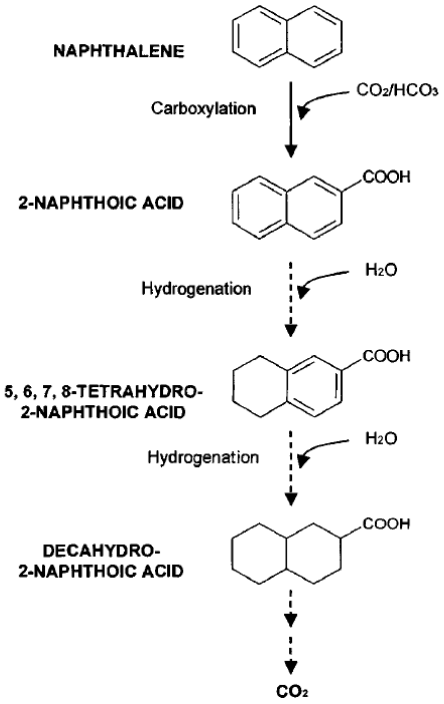


Figure 2.2 Proposed pathway for anaerobic metabolism of naphthalene under sulfate-reducing conditions (from reference (Bamforth and Singleton 2005)).

2.4.2. Incomplete PAH metabolism

Under favorable conditions, microorganisms can degrade organic contaminants completely into nontoxic end products such as carbon dioxide and water or organic acids and methane. However, enzymes along the metabolic pathway of microbial degradation

of PAHs may have varying kinetic properties and substrate specificities, which can lead to the formation and accumulation of metabolites (products of incomplete metabolism). There are many examples in which PAHs are transformed to non-mineral products that accumulate extracellularly, both in pure or mixed cultures of organisms and in more complex field-contaminated systems. The non-mineral products include metabolites in the known aerobic PAH-metabolic pathways, such as ketones, dihydrodiols and hydroxyl acids (Gibson et al. 1975; Jerina et al. 1984; Mahaffey et al. 1988; Grifoll et al. 1995; Stringfellow and Aitken 1995; Schneider et al. 1996; Kazunga and Aitken 2000; Kazunga et al. 2001; Moody et al. 2004; Jouanneau and Meyer 2006). The non-mineral products also include *o*-quinones, which are not direct bacterial metabolites of PAHs but the derivatives of the autoxidation of unstable dihydroxy intermediates (Davies and Evans 1964; Laurie and Lloyd-Jones 1999; Kazunga and Aitken 2000; Kazunga et al. 2001; Jouanneau and Meyer 2006).

In field-contaminated soil/sediment, many PAH metabolites have been observed, including 9-fluorenone (Fernandez et al. 1992; Mosi et al. 1997; Meyer et al. 1999; Eriksson et al. 2000; Machala et al. 2001; Lundstedt et al. 2003; Bergknut et al. 2004; Lundstedt et al. 2006; Park et al. 2008), 9-hydroxyfluorene (Park et al. 2008), 4-hydroxy-9-fluorenone (Eriksson et al. 2000), phenanthrene-9,10-dione (Eriksson et al. 2000), anthracene-9,10-dione (Fernandez et al. 1992; Mosi et al. 1997; Meyer et al. 1999; Machala et al. 2001; Saponaro et al. 2002; Lundstedt et al. 2003; Bergknut et al. 2004; Lundstedt et al. 2006; Park et al. 2008), benz[*a*]anthracene-7,12-dione (Fernandez et al. 1992; Mosi et al. 1997; Meyer et al. 1999; Eriksson et al. 2000; Machala et al. 2001; Lundstedt et al. 2003; Lundstedt et al. 2006; Park et al. 2008),

7H-benz[*d,e*]anthracene-7-dione (Fernandez et al. 1992; Meyer et al. 1999; Bergknut et al. 2004; Lundstedt et al. 2006), 4H-cyclopenta[*d,e,f*]phenanthrenone (Fernandez et al. 1992; Lundstedt et al. 2003; Lundstedt et al. 2006; Park et al. 2008), fluoranthene quinone (Fernandez et al. 1992), pyrene quinone (Fernandez et al. 1992), and *cis*-4,5-pyrene dihydrodiol (Li et al. 1996). The presence of these compounds in field-contaminated soil/sediment that have been contaminated for years indicates that those compounds are either continually produced or are relatively persistent in those contaminated systems (Lundstedt et al. 2007). Some PAH metabolites were observed to accumulate during biological treatment, including 9-fluorenone (Eriksson et al. 2001), phenanthrene-9.10-dione (Eriksson et al. 2001), anthracene-dione (Saponaro et al. 2002), 4H-cyclopenta[*d,e,f*]phenanthrenone (Eriksson et al. 2001), and ketone products of acenaphthene (Lundstedt et al. 2003). However, in another study (Lundstedt et al. 2003), some PAH metabolites were observed to decline over time during biological treatment, including 9-fluorenone, anthracene-9.10-dione, benz[*a*]anthracene-7,12-dione, 7H-benz[*d,e*]anthracene-7-dione and 4H-cyclopenta[*d,e,f*]phenanthrenone. Transient accumulation and subsequent removal of PAH metabolites has also been observed in uncontaminated soils spiked with PAH mixtures (Wischmann and Steinhart 1997; Meyer and Steinhart 2001). These PAH metabolites can inhibit the metabolism of other PAHs (Kazunga and Aitken 2000; Kazunga et al. 2001; Juhasz et al. 2002; Holt et al. 2005), and some exhibit toxic and/or genotoxic effects (Fernandez et al. 1992; Grifoll et al. 1995; Traczewska 2000; Machala et al. 2001; Zielinska-Park et al. 2004; Pagnout et al. 2006).

2.5. Genotoxicity of PAH-contaminated soil

PAHs have been identified to have effects deleterious to human health since the

British surgeon Sir Percival Pott demonstrated a correlation between the exposure of chimney sweeps to soot and the incidence of scrotal cancer in 1775 (Baird et al. 2005). Parent PAHs are thought to act by narcosis as the mode of toxic action (Sverdrup et al. 2002). They do not bind to specific receptors within an organism but partition and accumulate in the lipid bilayer to affect the fluidity and function of cell membranes (Sverdrup et al. 2002). This alteration of membranes can influence the energy transduction across membranes as well as the activity of membrane-embedded proteins in order to elicit toxicity (Sikkema et al. 1995). The LD_{50} values of some representative parent PAHs are shown in Table 2.2. However, due to their low water solubility and high $logK_{OC}$, PAHs in the environment are seldom present in concentrations high enough to directly cause acute toxicity. They exert deleterious effects when they are metabolized to oxygenated intermediates (Cerniglia et al. 1983; Swanson et al. 1986; Narro et al. 1992; McConkey et al. 1997; Bertilsson and Widenfalk 2002) which can also cause DNA damage (Xue and Warshawsky 2005).

Table 2.2 LD_{50} values of some representative PAHs [a]

Material	LD_{50} value (mg/kg)	Test subject	Exposure route
Naphthalene	533-710	Male/female mice respectively	Oral
Phenanthrene	750	Mice	Oral
Anthracene	>430	Mice	Intraperitoneal
Fluoranthene	100	Mice	Intravenous
Pyrene	514	Mice	Intraperitoneal
Benzo[*a*]pyrene	232	Mice	Intraperitoneal

[a] data taken from the Risk Assessment Information System (RAIS) http://risk.lsd.ornl.gov (Bamforth and Singleton 2005).

2.5.1. DNA damage caused by PAHs

PAHs, with relatively planar and highly conjugated aromatic structures, require

metabolic activation to exert their genotoxic/mutagenic/carcinogenic effects through biochemically reactive electrophilic metabolites (Miller and Miller 1966; Miller 1978). Metabolically activated PAHs can cause direct DNA adduction by covalent binding to form stable or depurinating adducts and indirect DNA damage by oxidative stress through the formation of active metabolites (Xue and Warshawsky 2005). There are three principal pathways currently proposed for metabolic activation of PAHs: 1) bay region dihydrodiol epoxide pathway by cytochrome P450 enzymes; 2) radical cation pathway by one-electron oxidation; and 3) *o*-quinone pathway by dihydrodiol dehydrogenase (Xue and Warshawsky 2005).

The bay region dihydrodiol epoxides pathway involves three enzyme-mediated reactions (Harvey 1991; Baird et al. 2005): 1) oxidation of a double bond catalyzed by P450 enzymes to unstable arene oxides; 2) hydrolysis of the arene oxides by microsomal epoxide hydrolase to *trans*-dihydrodiols; 3) oxidation of the double bond adjacent to the diol function to a vicinal diol-epoxide by P450 enzymes. The bay or fjord region diol-epoxides produced via this pathway are electrophiles capable of covalently binding to DNA to form bulky DNA adducts (Broyde et al. 2011). The major adducts have been found to result from bonding between the benzylic carbon of the epoxide and the exocyclic amino groups of dGuo and dAdo residues in the DNA (Broyde et al. 2011). Minor covalent dCyd adducts by alkylation at exocyclic amino N^4 and ring imino N3 positions have also been found (Wolfe et al. 2004).

The radical cation pathway involves the removal of one electron from the π electron system of the PAH molecule through one-electron oxidation to form the radical cation of PAH (Cavalieri and Rogan 1992; Cavalieri and Rogan 1995). Radical cations are

extremely reactive electrophiles in nature and they are capable of binding with nucleophilic centers in DNA (Cavalieri and Rogan 1995) at the N7 and C8 position of guanine bases and the N7 or N3 positions of adenine bases (Rogan et al. 1993). However, DNA adducts formed through PAH radical cations are unstable, leading to spontaneous depurination in addition to some stable adducts. These unstable DNA adducts arise primarily from binding at the N7 position of guanine bases and the N7 or N3 positions of adenine bases, where the glycosidic bond is cleaved to lose the deoxyribose moiety of DNA (Xue and Warshawsky 2005). Spontaneous depurination of adducts would result in formation of apurinic (AP) sites as the major type of DNA damage (Devanesan et al. 1992; Chakravarti et al. 1995).

The *o*-quinone pathway involves the formation of *o*-quinones by dihydrodiol dehydrogenase-catalyzed oxidation of either *cis*- or *trans*-dihydrodiols and the formation of reactive oxygen species (ROS) through redox cycles (Penning et al. 1999). In this pathway, dihydrodiol dehydrogenases compete with P450 enzymes to oxidize non-K-region diols to ketols that spontaneously rearrange to catechols followed by auto-oxidation of the unstable catechols to *o*-quinone (Palackal et al. 2001; Jiang et al. 2005). PAH-*o*-quinones are highly reactive Michael acceptors. They can form both stable and depurinating (reaction at N7 of dGuo) DNA adducts (Shou et al. 1993; McCoull et al. 1999). Additionally, PAH-*o*-quinones can enter redox cycles and produce ROS, such as semi-quinone radicals, hydroxyl radicals, hydrogen peroxide, and superoxide anion radicals. ROS can induce oxidative damage of DNA bases in cells, causing oxidized deoxynucleosides such as 8-oxo-dG/8-OH-dG. The unrepaired oxidized G can mismatch with A on the replicative strand and leads to G->T transversion (Shibutani et al. 1991).

ROS such as hydroxyl radical can also cause DNA strand scission and illegitimate recombination through formation of base propenals.

Although the principal mechanisms of DNA damage caused by PAHs have been studied, the toxicity/genotoxicity knowledge of each individual PAH is still incomplete. This is further complicated by the fact that PAHs occur in complex mixtures in the environment, such as contaminated soil (White 2002). Contaminated soil may contain hundreds of compounds, many of which individually may or may not exhibit toxic/genotoxic potential. These compounds in a mixture may compete for receptors or metabolizing enzymes, leading to additive, synergistic or antagonistic effects.

2.5.2. Effects of bioremediation on genotoxicity of PAH-contaminated soil

Traditionally, bioremediation as well as other remedial technologies for contaminated soil are evaluated based on the removal of monitored pollutants, i.e. USEPA guidelines generally only focus on un-substituted PAHs, most often the 16 priority PAHs, during the risk assessment of PAH-contaminated soil (USEPA 2000). However, it remains controversial whether the removal of the regulated PAHs during bioremediation of field-contaminated soil or sediment corresponds to a reduction in health risk, since toxicity and/or genotoxicity of contaminated soils may be unrelated to the concentration of the 16 priority PAHs (Lemieux et al. 2009). First, significant amounts of many other carcinogenic polycyclic aromatic compounds, such as dibenzo[*a*,*l*]pyrene, are also found in PAH-contaminated soil (Lundstedt et al. 2003; Lemieux et al. 2008; Lemieux et al. 2009), and whether all hazardous compounds degrade concomitantly with the 16 priority PAHs monitored at the contaminated site is unknown (Lundstedt et al. 2003). Second, incomplete metabolism of PAHs in contaminated soil can yield intermediate metabolites

that may accumulate, such as oxy-PAHs, which exhibit greater toxicity than their parent PAHs (Alexander et al. 2002; Andersson et al. 2003; Lundstedt et al. 2003; Lundstedt et al. 2007; Lemieux et al. 2009). These parent compounds and their metabolites all contribute to the total risk of the contaminated site. However, it is not practicable to monitor hundreds of these compounds during the bioremediation process. More importantly, identities of many hazardous compounds in PAH-contaminated sites are rarely known and their concentrations cannot be monitored. Third, chemical-analysis-based risk assessment assumes that the toxicity of a mixture is simply the sum of the expected effects from each mixture component (USEPA 2004b). It doesn't account for the possible synergistic or antagonistic interactions between mixture components (White 2002; Park et al. 2008).

Soil genotoxicity assessment, an effective supplement to chemical analyses, has been used to assess the hazard and risk of contaminated soil. Although bioremediation has been shown to significantly remove PAHs from the contaminated soil, the effects of bioremediation on genotoxicity of PAH-contaminated soil described in the literature vary. Some studies have shown decreased genotoxicity as treatment progressed (Baud-Grasset et al. 1993; Haeseler et al. 1999b; Sayles et al. 1999; Mendonca and Picado 2002; Sasek et al. 2003). However, other studies have shown no reduction or even a substantial increase in genotoxicity following bioremediation (Hughes et al. 1998; Andersson et al. 2009; Gandolfi et al. 2010), suggesting the formation of genotoxicants or increased bioavailability of native genotoxicants over the course of the bioremediation (Andersson et al. 2009). Temporal changes in the genotoxicity of PAH-contaminated soils undergoing bioremediation have also been observed (Belkin et al. 1994; Lemieux et al. 2009). The

somewhat cyclical nature of the genotoxicity may suggest the formation, and subsequent degradation, of genotoxic compounds, and longer periods of bioremediation may be required to significantly reduce the genotoxic hazard of a contaminated soil (Lemieux et al. 2009). The sources of genotoxicity either caused by or remaining after bioremediation have not been elucidated, and the extent to which toxicity is transient has not been studied extensively.

2.6. DT40 genotoxicity bioassay

Several genotoxicity bioassays have been used to investigate the effects of bioremediation on the genotoxicity of PAH-contaminated soil, including the Ames test (Brooks et al. 1998; Hughes et al. 1998; Sayles et al. 1999; Lemieux et al. 2009), Mutatox™ assay (Belkin et al. 1994; Mendonca and Picado 2002), SOS Chromotest (Haeseler et al. 1999b; Phillips et al. 2000; Sasek et al. 2003), micronucleus test (Baud-Grasset et al. 1993), and Comet assay (Andersson et al. 2009; Gandolfi et al. 2010). The Ames test is a bacterial reverse mutation assay capable of detecting base-pair substitutions and frame-shift mutations. Mutatox™ assay is a test that measures the ability of a chemical or sample to induce a genetic or epigenetic change in a luminescent bacterium. SOS Chromotest is a bacterial test for induction of an SOS function to measure genotoxicity. The above three prokaryotic tests are simple and fast, but whether a bacterial test is a suitable model for eukaryotic systems is still questionable (Evans et al. 2010). The micronucleus test is based on the principle that formation of micronuclei indicates chromosomal damage or aneuploidy. This test only detects chromosome breaks (Barile 1994) and it is particularly prone to false positive and false negative results (Evans et al. 2010). The Comet assay is the single-cell gel electrophoresis assay (Collins

2004). It is a sensitive technique for the detection of DNA damage at the level of the individual eukaryotic cell. However, it is limited to a small range of detectable DNA injuries.

Besides all the genotoxicity bioassays mentioned above, a novel bioassay using DNA-repair-deficient chicken DT40 B-lymphocyte cell lines for measuring the genotoxicity of PAH-contaminated soil before and after bioremediation has been proposed (Knight 2009; Ridpath et al. 2011). The DT40 genotoxicity bioassay is a reverse genetic approach, which has high specificity and sensitivity. Unlike most other assays, DT40 genotoxicity bioassay can also provide information on mechanisms of genotoxicity of a given sample (Ji et al. 2009; Ridpath et al. 2011).

2.6.1. Reverse genetic approach for genotoxicity test

One of the novel approaches for detecting genotoxicity is to use the fact that in order to achieve the greatest accuracy during DNA synthesis, replicative DNA polymerases cannot replicate template DNA that is damaged (Friedberg et al. 2005). Replicative DNA polymerases can recognize subtle chemical modifications of template DNA strands, which results in stalling of replication. To avoid such stalling, cells may use direct reversal repair, base excision repair (BER) and nucleotide excision repair (NER) to eliminate DNA lesions from DNA template strands before DNA replication. Cells may also use translesion synthesis (TLS), homologous recombination (HR) and non-homologous end-joining (NHEJ) to repair or bypass DNA damage during DNA replication (Sonoda et al. 2006; Evans et al. 2010). If DNA damage is repaired or bypassed, DNA can be replicated. After all chromosomal DNA has been replicated, cells can proceed with the next cycle. If DNA damage is not repaired or bypassed, DNA can’t

be replicated, the cell cycle does not proceed, and it will finally lead to cellular suicide.

The main principle of the reverse genetic approach for genotoxicity testing is that a certain DNA repair pathway is knocked out. Then the mutant deficient in that specific DNA repair pathway is exposed to a genotoxic agent. If cell death is observed, the DNA damage caused by that genotoxic agent is not repaired in that mutant. As a result, it can be concluded that the DNA repair pathway that has been knocked out in that mutant is involved in the repair of DNA damage caused by that genotoxic agent. Since each individual repair pathway processes a distinct set of the DNA lesion type, differential cytotoxicity as a function of which DNA repair pathway has been knocked out would provide insight into the type(s) of genotoxicity induced (Sonoda et al. 2006; Ji et al. 2009; Evans et al. 2010).

2.6.2. Advantages of DT40 cell lines in genotoxicity test

The DT40 chicken B-lymphocyte cell line is derived from an avian leukosis virus-induced bursal lymphoma (Baba et al. 1985). It appears to be ideal for genotoxicology studies using the reverse genetic approach. First, ~60% of cycling cells are in S phase; exogenous DNA damage may have a direct effect on DNA replication (Wu et al. 2006). Second, DT40 cells allow relatively more facile manipulation of their genome than other higher eukaryotic cells. They have high efficiency of gene targeting (Buerstedde and Takeda 1991; Yamazoe et al. 2004) and also display remarkably stable karyotype and phenotype. Third, DT40 cells show strong phenotypic resemblance to murine cells with respect to genes involved in DNA recombination and repair (Sonoda et al. 2001). The translation of DT40 assay results to human exposures to genotoxicants is therefore much greater than assays which employ microorganisms and lower eukaryotes.

Fourth, DT40 cells lack a functional p53 protein (Ulrich et al. 1992). This feature permits DT40 cells to bypass apoptosis, so that the observance of cell death can be explained by DNA repair deficiency rather than the activation of apoptosis. Last, DT40 cells can be easily maintained in suspension culture and have relatively short doubling time (7h).

3. DESORPTION OF POLYCYCLIC AROMATIC HYDROCARONBS FROM FIELD-CONTAMINATED SOIL TO A TWO-DIMENSIONAL HYDROPHOBIC SURFACE BEFORE AND AFTER BIOREMEDIATION[1]

3.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are of great concern because of their known or suspected genotoxicity, mutagenicity and carcinogenicity (Santodonato 1997; Bostrom et al. 2002a). Dermal exposure can represent a significant health risk in settings involving potential contact with complex materials containing PAHs, including PAH-contaminated soil or sediment (Boffetta et al. 1997; Sobus et al. 2009). Most previous work has been concerned with integrated uptake of chemicals through the skin and not with how a contaminant reaches the skin surface in the first place. However, only a contaminant that reaches the skin surface is available for dermal absorption (Roy et al. 1998; Shatkin et al. 2002). Desorption properties, such as dynamic conditions by which soil contacts the skin, interactions of the soil with the skin surface and chemical interaction with the soil, have been identified to influence dermal uptake of chemicals (McKone and Howd 1992; Spalt et al. 2009). Therefore, it is important to understand desorption of PAHs from contaminated soil or sediment to the skin surface.

To account for the association of hydrophobic contaminants such as PAHs with compartments of varying sorptive strength in soil (Alexander 1995; Xing and Pignatello 1997; Cornelissen et al. 2005), a so-called two-compartment desorption model assumes a

[1] Hu, J. and Aitken, M.D. (2012). "Desorption of polycyclic aromatic hydrocarbons from field-contaminated soil to a two-dimensional hydrophobic surface before and after bioremediation." Chemosphere **89**(5): 542-547.

simplified situation in which a fraction of the contaminant is released relatively rapidly and the remainder is released relatively slowly (Cornelissen et al. 1998; Hawthorne et al. 2001; Zhu et al. 2008). By incorporating two-compartment desorption kinetics into a fugacity model, Shatkin et al. (2002) illustrated that a greater rapid-desorbing fraction of a chemical would result in greater dermal uptake. In most previous experimental work on dermal uptake of contaminants from soil, an individual contaminant was introduced into the soil through a solvent that subsequently evaporated (Spalt et al. 2009). However, exposure to a spiked chemical does not account for the effect of contaminant aging that would have occurred in field-contaminated soil (Roy et al. 1998; Stroo et al. 2000; Spalt et al. 2009), which is well known to decrease its bioavailability (Alexander 2000).

The objective of this study was to evaluate desorption of PAHs from field-contaminated soil from a former MGP site to a two-dimensional hydrophobic surface (Empore™ C18 extraction disk) as a measure of potential dermal exposure. Various factors affecting desorption were investigated, including soil loading, temperature, soil moisture content (SMC), and exposure time. We also compared desorption to the C18 disk to a conventional method of evaluating potential contaminant bioavailability in soil, desorption to Tenax® beads in a well-mixed aqueous slurry (Loehr et al. 2003). The efficacy of bioremediation (in a slurry-phase bioreactor) in removing the most readily desorbable PAH fractions was evaluated with both methods.

3.2. Materials and methods

3.2.1. Materials

Source soil used in this study was collected from a former MGP site in Salisbury, North Carolina, U.S.A.. Samples were air-dried, sieved (250 μm mesh) and maintained at

4 °C prior to use. The total organic matter fraction (f_{oc}) was 0.16 (dry mass basis, wt/wt), SMC was 2.0% (wt/wt), field capacity was 40% (wt/wt), and soil particle density was 2.57 g/cm^3 (methods are identified in Table A.1). The total concentration of 14 target PAHs (the 16 priority PAHs, excluding acenaphthylene and indeno[*1,2,3-cd*]pyrene) was 780 ± 10 mg/kg (dry mass basis, wt/wt; individual PAH concentrations are shown in Table A.2); the most abundant PAHs were phenanthrene (PHE, 322 ± 5.1 mg/kg) and pyrene (PYR, 121 ± 0.05 mg/kg). Soil samples were mixed with de-ionized water to reach desired SMC levels prior to desorption experiments. Treated soil was the slurry from a continuously stirred, semi-continuous (draw and fill), laboratory-scale aerobic bioreactor (Zhu et al. 2008) treating the source soil. The treated soil had a total PAH concentration of 121 ± 8 mg/kg (individual PAH concentrations are shown in Table A.2).

Empore™ C18 extraction disks (25 mm diameter, 0.5 mm thickness) were obtained from 3M (St. Paul, MN, U.S.A.) and cleaned by acetone extraction overnight and air-dried before use. Tenax® TA beads (60/80 mesh) were purchased from Alltech (Deerfield, IL, U.S.A.) and cleaned by Soxhlet extraction in acetone: hexane (50:50, v/v) mixture overnight and air-dried before use. PAH standards (EPA 610 PAHs Mixture) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Anthracene-D10 was obtained from Cambridge Isotope Laboratories (Andover, MA, U.S.A.). Solvents were high-pressure liquid chromatography (HPLC) grade and were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

The C18 extraction disks were used to evaluate variables that might influence the transfer of PAHs from soil to a static hydrophobic interface. This method is analogous to the Tenax beads method for evaluating PAH desorption kinetics in slurry systems (Loehr

et al. 2003), in that both C18 extraction disks and Tenax beads serve as an infinite sink; however, we believe that the C18 disk is more relevant to the application of soil to skin in a dermal exposure scenario.

3.2.2. Desorption experiments

Desorption of PAHs from soil samples to C18 disks was determined at three different temperatures (20 °C, 30 °C and 40 °C), four SMC levels (2%, 8%, 20% and 40%) and seven soil loadings (5 to 100 mg dry soil/cm^2) over periods of 6 d, when total PAHs desorbed from soil to a C18 disk reached an apparent equilibrium (Figure A.1). Kinetics for desorption of PAHs from soil to C18 disks were investigated over periods of 16 d. The sorption capacity of C18 disks was evaluated by repeated soil loading of the same disk; results indicated that the sorption capacity greatly exceeded the amount of PAHs desorbed in any given experiment (Table A.3). Soil with a specified SMC level was spread as evenly as possible (under microscopic observation) onto the C18 disk, which was then transferred with an aluminum spatula onto an aluminum weighing dish. Soil weight was determined by weight difference of the C18 disk before and after soil loading. The aluminum weighing dish was then transferred into a sealed container and kept in the dark in a constant-temperature room set to the desired temperature. After each desired time interval, disks were removed and rinsed with de-ionized water three times for subsequent PAH extraction. To investigate possible mechanisms of PAH transport from soil to the hydrophobic surface, dry or moist Whatman glass microfiber filters (pore size 0.7μm, pre-baked at 400°C for 4h) (Fisher Scientific, Pittsburgh, PA, U.S.A.) were placed between the C18 disk and the soil. Triplicates of procedure blanks (no soil) were included. Total PAH recovery over all experiments was 94 ± 6% (individual PAH recoveries are

shown in Table A.4), calculated by comparing the initial PAH mass in the soil with the PAH mass desorbed to the C18 disk and the PAH mass remaining in the soil after desorption.

Desorption of PAHs from soil to Tenax beads was carried out at 20 °C. Approximately 3 g of soil (dry wt.) and 0.2 g Tenax beads were suspended in 20 mL phosphate buffer (pH 7.5) amended with 4.15 g/L NaN_3 in a 30-mL glass serum vial with a PTFE-lined septum and screw cap. The vials were placed on a wrist-action shaker at 240 rpm in the dark. After 1, 2, 4, 8 and 16 d, the vials were centrifuged at 3500 rpm for 15 min, Tenax beads were removed from the vials for subsequent extraction as described by Zhu et al. (2008), and the supernatant was discarded. For all but the 16-d time point, 20 mL fresh medium was added along with 0.2 g fresh Tenax beads into the vials. The mass recovery of Tenax beads over all time points was 97 ± 2%. Total PAH recovery was 92 ± 10% for combined experiments with source soil and treated soil (individual PAH recoveries are in Table A.4).

3.2.3. PAH extraction and analysis

C18 disks and Tenax beads were extracted with 10 mL acetone and 10 mL methanol, respectively, in a 20-mL test tube with a PTFE-lined septum and screw cap; each tube was amended with 20 μL anthracene-D10 (100 μg/L) as recovery surrogate. The tubes were placed on a wrist-action shaker at 240 rpm in the dark for 24 h. A 1-mL aliquot of extract from the C18 disk was then removed for HPLC analysis. The extract from Tenax beads was filtered through a Millipore (Billerica, MA, U.S.A.) nylon membrane (pore size 0.20 μm) to remove the beads and subsequently analyzed by HPLC. Soil samples were extracted overnight twice each with a mixture of 10 mL acetone and 10 mL

dichloromethane as described elsewhere (Zhu et al. 2008). All extracts were analyzed by HPLC (Zhu et al. 2008).

3.2.4. Data analysis

SPSS® (v16.0, SPSS Inc.) was applied for data analysis. One-way ANOVA followed by Tukey's test was employed to test for differences among multiple groups. The maximum soil loading required to provide monolayer coverage was estimated according to Equation 3.1 (Duff and Kissel 1996) assuming solid spherical soil particles and face-centered packing:

$$SL_{monolayer} = \frac{\rho_{particle}\left(\pi d^3/6\right)}{d^2} = \rho_{particle}\left(\pi d/6\right) \quad (3.1)$$

where $SL_{monolayer}$ is the soil loading representing a monolayer (mg/cm^2); $\rho_{particle}$ is the soil particle density (g/cm^3); d is the soil particle diameter (μm). Desorption kinetics data were evaluated with the commonly used two-compartment kinetic model, Equation 3.2 (Cornelissen et al. 1998; Hawthorne et al. 2001; Zhu et al. 2008):

$$\frac{S_0 - S_t}{S_0} = 1 - f_r e^{-k_r t} - f_s e^{-k_s t} \quad (3.2)$$

where S_0 is the initial soil concentration of a given PAH (μg/g); S_t is the soil concentration at time t (μg/g); f_r and f_s are the fractions of the PAH that desorb rapidly and slowly, respectively; k_r and k_s are the rate constants for rapid and slow desorption, respectively (d^{-1}); and t is the desorption time (d). All model parameters with their standard errors and coefficients of determination were determined using nonlinear regression.

3.3. Results

The mass of PAHs desorbed from soil to a C18 disk increased with increasing soil loading, although the percentage desorbed decreased as loading increased (Figure 3.1).

According to Equation 3.1, the maximum soil loading required to provide monolayer coverage was estimated as no more than 34 mg/cm^2 with soil particle density of 2.57 g/cm^3 and soil particle diameter less than 250 μm (soil was sieved through 250 μm mesh). It is obvious from Figure 3.1 that the total PAH mass desorbed to the C18 disk kept increasing at soil loadings well above the estimated monolayer coverage.

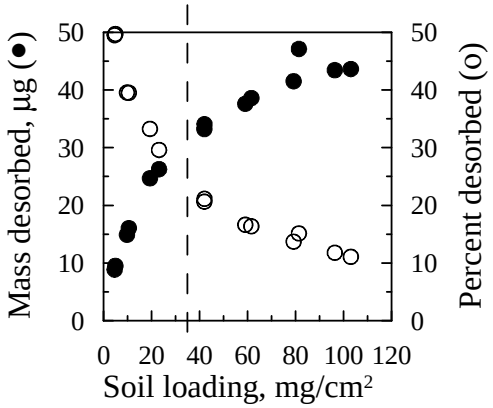


Figure 3.1 Total PAHs desorbed from source soil to C18 extraction disks as a function of soil loading. Soil moisture content was 2%, temperature 20 ºC and contact time 6 d. Dashed vertical lines indicate the estimated maximum monolayer coverage of 34 mg/cm^2.

Soil loading effects on PAH desorption to C18 disks was influenced by temperature, but the influences depended on the specific PAH (Figure A.2). For naphthalene (NAP), the mass desorbed was constant for all soil loadings at each of the three temperatures evaluated (20, 30 and 40 °C). For acenaphthene (ACE) and fluorene (FLU), the mass desorbed was proportional to soil loading at each temperature. For phenanthrene (PHN), anthracene (ANT), fluoranthene (FLA) and pyrene (PYR), the mass desorbed asymptotically approached a maximum that appeared to have been reached for each compound at 20 ºC and 30 ºC over soil loadings up to 100 mg/cm^2. The asymptote generally tended to be approached at higher soil loadings as temperature increased. In separate experiments, we verified that the apparent maximum mass of PAH desorbed did

not approach the sorption capacity of the C18 disk (Table A.3).

Increased desorption of PAHs to the C18 disk at soil loadings well above monolayer coverage suggested that PAHs were transferred to the disk by mechanisms other than direct soil contact. Accordingly, with a fixed soil loading above monolayer coverage (40 mg/cm^2), we evaluated the extent to which placing a barrier to direct contact between the soil and the disk would affect desorption. The desorption of PAHs from soil to the C18 disk still occurred even with a dry or moist glass microfiber filter placed between the hydrophobic surface and the soil (Figure 3.2). For NAP, ACE and FLU, there were no significant differences between desorption with a dry or a moist filter and that without a filter. However, for PHN, ANT, FLA and PYR, desorption from the soil to the C18 disk was significantly lower with a dry or a moist filter than without a filter; in addition, desorption was significantly lower in the presence of a moist filter than in the presence of a dry filter. Desorption in the presence of two dry or two moist filters was only slightly less than in the presence of only one dry or one moist filter for all PAHs (data not shown). No PAH was detected in the dry or moist filter that was not in direct contact with the soil, suggesting that PAHs were not sorbed by the filters.

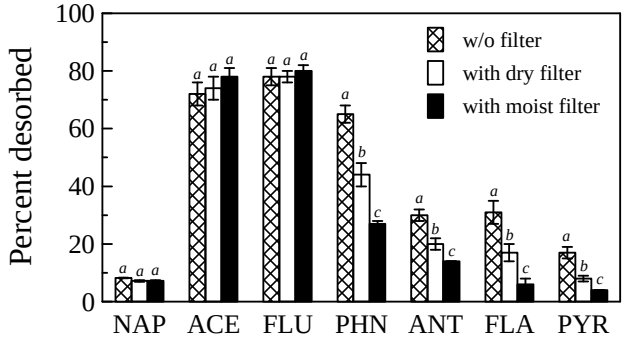


Figure 3.2 Desorption of PAHs to C18 disks from source soil with or without a glass microfiber filter placed between the C18 disk and the soil. Soil moisture content was 2%, soil loading 40 mg/cm^2, temperature 20 °C, and contact time 6 d. Desorption was less than 2% for B*a*A, CHR, B*b*F, B*k*F, B*a*P, DBA and B*g*P under all three conditions. The same letter is assigned to conditions for which there was no significant difference ($p > 0.05$).

The desorption of all PAHs to the C18 disk with a fixed soil loading well above monolayer coverage (50 mg/cm^2) was substantially reduced at an SMC of 40%, which corresponded to approximate field capacity of the soil (Figure 3.3). Desorption of total PAHs from soil at an SMC of 40% was only one-third of that at an SMC of 2%. For NAP, ACE, FLU and PHN, there were no significant differences between desorption from soil at SMC from 2% to 20%. For ANT, FLA and PYR, there was no significant difference between desorption from soil at SMC of 2% and 8%; at an SMC of 20% there was a statistically significant, but modest, decrease in desorption.

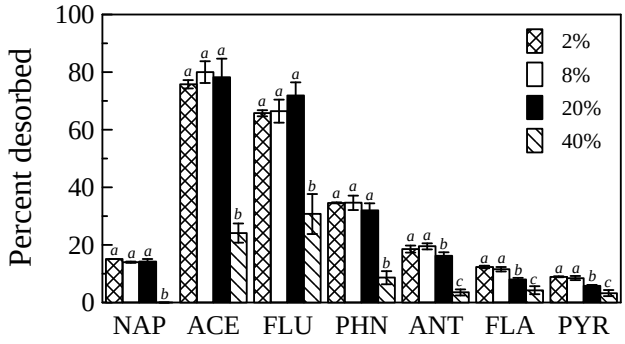


Figure 3.3 Effect of SMC on desorption of PAHs to C18 disks from source soil. Soil loading was 50 mg/cm^2, temperature 20 °C, contact time 6 d. Desorption was less than 2% for B*a*A, CHR, B*b*F, B*k*F, B*a*P, DBA and B*g*P under all four conditions. Same letter is assigned to conditions where difference was not significant ($p > 0.05$).

Desorption of PAHs to C18 disks from both the source soil and the biologically treated soil was compared to desorption to Tenax beads in a vigorously mixed aqueous slurry (Figure 3.4). For the source soil, only 62 % of the total PAHs desorbed to Tenax beads was desorbed to C18 disks. Lower molecular weight PAHs had greater potential to desorb from soil than higher molecular weight PAHs; the percentage desorption both to Tenax beads and to C18 disks decreased as PAH molecular weight increased. For the biologically treated soil, desorption to C18 disks was not observed for any PAH (data not shown), suggesting that biological treatment of the soil in an aerobic, slurry-phase

bioreactor removed the fraction of each PAH that was capable of desorbing to the C18 disk. The percentage desorption of all PAHs from biologically treated soil to Tenax beads was much lower than that from the source soil, reinforcing that the most bioavailable fractions of the PAHs had been removed by biological treatment.

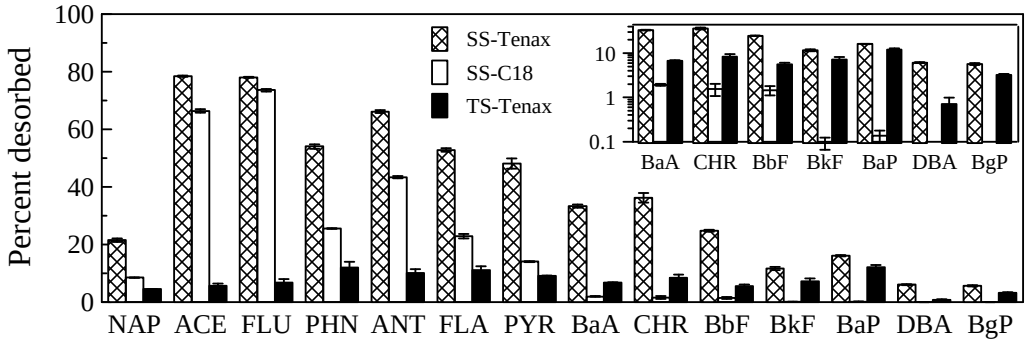


Figure 3.4 Desorption of individual PAHs from source soil to Tenax beads (SS-Tenax) or to C18 disks (SS-C18) or from biologically treated soil to Tenax beads (TS-Tenax). The inset enlarges the results for higher molecular weight PAHs. All measurements were at 20 ºC at a 16-d contact time. For desorption to C18 disks, soil moisture content was 2% and soil loading 50 mg/cm^2. Desorption to C18 disks from biologically treated soil was not detectable.

Desorption kinetics of each individual PAH from source soil to both C18 disks and Tenax beads were described well by the commonly used two-compartment kinetic model (Equation 3.2), as illustrated in Figure A.3. Fitted parameter values are summarized in Table A.5. The rapidly desorbing fraction (f_r) for desorption from the source soil to Tenax beads was higher than that for desorption to the C18 disk. For desorption of PAHs from the biologically treated soil to Tenax beads, f_r was not significantly different from 0 for any PAH. The rate constant for the slowly desorbing fraction (k_s) for biologically treated soil was lower than that for source soil in the Tenax-bead system.

3.4. Discussion

Current guidance from the US Environmental Protection Agency (USEPA) for assessment of dermal exposure to contaminants in soil (USEPA 2004b) stipulates a fixed

percentage absorption (13%) for dermal uptake of benzo[*a*]pyrene and other PAHs based on measurements from a single study (Wester et al. 1990). Although this single value simplifies human health risk calculation, it doesn't account for the effects of soil loading configuration on dermal absorption. Bunge and Parks (1998) proposed two distinct situations based on a mathematical model describing dermal absorption of organic chemicals from contaminated soils for a given exposure time. When soil loading is less than monolayer coverage, the percentage absorption remains constant as soil loading increases while the mass absorbed per unit skin area dramatically increases; when soil loading exceeds monolayer coverage, the percentage absorption decreases significantly as soil loading increases while the mass absorbed per unit skin area remains constant. Several investigators have confirmed these results experimentally (Duff and Kissel 1996; Roy and Singh 2001; Touraille et al. 2005). However, such predictions are based on the assumption that the contaminant concentration in the soil remains constant (i.e., percentage absorption less than 10% of applied dose) during dermal absorption (Bunge and Parks 1998). When the contaminant is depleted from the soil, the percentage absorption would decrease with increasing soil loading above monolayer coverage while the mass absorbed would increase disproportionately (Bunge and Parks 1998). This prediction is consistent with our observation that the mass of PAHs desorbed from soil to the hydrophobic surface disproportionately increased with increasing soil loading well above monolayer coverage while the percentage desorption was more than 10% of the applied dose (Figure 3.1). Touraille et al. (2005) also observed the phenomenon that the mass of 4-cyanophenol (CP) absorbed increased with increasing soil loading above monolayer coverage for an exposure time of 24 h.

The increasing mass of PAH desorbed at soil loadings beyond monolayer coverage demonstrated that not only the contaminants in a monolayer of soil particles in direct contact with the hydrophobic surface were desorbed. One explanation for this result is the depletion of PAHs in the soil, thus establishing concentration gradients away from the surface (Bunge and Parks 1998). Since the soil was unstirred, the concentration of a desorbable chemical in the soil will depend on the distance from the hydrophobic surface. As chemicals in soil layers closer to the hydrophobic surface become depleted, the concentration gradient provides a larger driving force for chemicals in upper soil layers to diffuse toward the hydrophobic surface. However, it is difficult to experimentally confirm concentration gradients through the soil depth.

We propose that contaminants can move from the soil to the hydrophobic surface through a combination of three processes: direct contact transfer from soil solids, diffusion through soil pore air, and diffusion through soil pore water. The pore air and pore water transport pathways would be most important at soil loadings beyond monolayer coverage of the hydrophobic surface. Transport through pore air is likely to be more significant because diffusivity in air is far greater than that in water (Schwarzenbach et al. 2003), and air would be the predominant fluid phase in pores for soil moisture contents below field capacity. Also, soil-to-air diffusion of PAHs and other volatile and semi-volatile organic pollutants is well-documented (Meijer et al. 2003; Ribes et al. 2003; Yang and Holmen 2008). This hypothesis is supported by our finding that the mass of PAHs desorbed from the soil to the hydrophobic surface was considerable even when dry or moist glass microfiber filters were placed between the hydrophobic surface and the soil (Figure 3.2). These filters prohibited transfer of PAHs

from soil solids to the hydrophobic surface by direct contact. Transport by mechanisms other than direct contact was most significant for the lower-molecular weight PAHs (NAP, ACE, and FLU; Figure 3.2) that also have the highest vapor pressures (summarized in Table A.6). Although soil moisture contents below field capacity had a limited effect on PAH desorption from the soil, the significant reduction of PAH desorption to the C18 disk at SMC corresponding to field capacity (Figure 3.3) also suggests that PAH diffusion in pore air was a predominant mechanism of transport to the C18 disk.

Besides soil loading configuration, other site-specific properties may also influence desorption of PAHs from soil to a two-dimensional hydrophobic surface. Temperature is one of these factors, as diffusion coefficients are positively correlated with temperature (Schwarzenbach et al. 2003). We observed inconsistent effects of temperature on desorption to the C18 disks. For several compounds (ACE, FLU, and ANT), the effect of temperature was limited (Figure A.2). For the remaining compounds (NAP, PHN, FLA, and PYR), the effect of temperature on desorption was substantial (Figure A.2). Several other investigators have also observed a positive relation between temperatures and desorption of semi-volatile compounds from soil to air (Hippelein and McLachlan 2000; He et al. 2009). Overall, the effect of temperature in our work would not be easy to predict, as temperature affects not only the soil-air partitioning equilibrium and PAH diffusivity, but the PAH-C18 sorption equilibrium as well; these effects of temperature could be counter-acting for a given PAH.

Bioremediation can be an attractive remediation approach for PAH-contaminated systems (Aitken and Long 2004). In this study, treatment of the source soil in an aerobic bioreactor reduced total PAH concentration by approximately 80% (Table A.2) and

seemed to eliminate the most readily desorbable fraction of all PAHs (Figure 3.4). Similar decreases in the rapidly desorbed fractions of PAHs after bioremediation have also been observed in previous research (Cornelissen et al. 1998; Hawthorne et al. 2001; Richardson and Aitken 2011). For the treated soil, no PAH desorption to C18 disks was observed, and there was no rapidly desorbing fraction in the slurry-based Tenax bead desorption system. It therefore appeared that biological treatment eliminated the PAHs that could desorb to a two-dimensional surface, and thus might substantially decrease the dermal bioavailability of PAHs in contaminated soil.

The default assumption for exposure time in dermal exposure assessments for contaminated soil is 24 h (USEPA 2004b). Although we carried out most of our experiments over a six-day period, the majority of the desorbed PAH mass desorbed within 24 h (Figure A.1). The experiments we performed are easily modified to 24-h duration if desired for an actual exposure analysis. We also recognize that skin is a complex matrix containing both hydrophobic and hydrophilic compartments as well as metabolic enzymes. A uniformly hydrophobic surface might overestimate the flux of PAHs from soil to the skin surface and/or neglect the metabolism of PAH by skin. The more important point is that dermal exposure assessment from soil should consider site-specific conditions that influence the bioavailability of hydrophobic contaminants to skin. The effects of remediation on potential dermal exposure should consider not only the reduction in contaminant concentration but also the reduction in contaminant bioavailability.

4. EVALUATING THE EFFECTS OF BIOREMEDIAITON ON GENOTOXICITY OF POLYCYCLIC AROMATIC HYDROCARON-CONTAMINATED SOIL USING GENETICALLY ENGINEERED, HIGHER EUKARYOTIC CELL LINES[2]

4.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are of human health concern due to their known or suspected genotoxic, mutagenic, or carcinogenic effects (White 2002; Xue and Warshawsky 2005), and they are a major pollutant class at thousands of contaminated sites in the U.S.A. (USEPA 2004a). Bioremediation is an established technology for cleanup of PAH-contaminated soils and sediments (USEPA 2007), but like most remedial technologies it is typically evaluated based on the removal of target pollutants. U.S.A. Environmental Protection Agency (USEPA) guidelines for risk assessments of PAH-contaminated soil generally focus only on 16 priority-pollutant PAHs (USEPA 2000). However, in most cases it remains unknown whether the removal of the regulated PAHs during bioremediation corresponds to a reduction in health risk (Lemieux et al. 2009). Significant amounts of other carcinogenic polyaromatic compounds, such as dibenzo[*a*,*l*]pyrene, are also found in PAH-contaminated soils (Lundstedt et al. 2003; Lemieux et al. 2008; Lemieux et al. 2009), and whether all hazardous compounds degrade concomitantly with the 16 priority PAHs monitored at contaminated sites is unknown (Lundstedt et al. 2003). Incomplete metabolism of PAHs in contaminated soil

[2]Hu, J., Nakamura, J., Richardson, S.D., Aitken, M.D. (2012). "Evaluating the effects of bioremediation on genotoxicity of polycyclic aromatic hydrocarbon-contaminated soil using genetically engineered, higher eukaryotic cell lines." Environmental Science & Technology **46**(8): 4607-4613.

can also yield byproducts, such as oxy-PAHs, during bioremediation, which can exhibit greater toxicity than the parent PAHs (Lundstedt et al. 2003; Lundstedt et al. 2007; Lemieux et al. 2008; Lemieux et al. 2009).

Although the parent compounds and their metabolites all contribute to the total risk of contaminated sites, it is not practical to monitor hundreds of these compounds throughout the bioremediation process. More importantly, the identities of many hazardous compounds in PAH-contaminated sites are rarely known. Another limitation of risk assessment based solely on chemical analysis is that the toxicity of a mixture is assumed to be simply the sum of the expected effects from each component (USEPA 2004b), and it does not account for the possible synergistic or antagonistic interactions between mixture components (White 2002; Park et al. 2008).

Toxicity and genotoxicity bioassays such as the Ames test (Lemieux et al. 2009), Mutatox^{TM} assay (Mendonca and Picado 2002), SOS Chromotest (Haeseler et al. 1999b), micronucleus test (Baud-Grasset et al. 1993), and Comet assay (Gandolfi et al. 2010) have been used to assess the potential hazard and risk of contaminated soil before and after bioremediation. However, all of these bioassays have their limitations. The Ames test, Mutatox^{TM} assay and SOS Chromotest are all bacterial-based genotoxicity bioassays. Whether a bacterial test is a suitable model for eukaryotic systems is still questionable (Evans et al. 2010). The micronucleus test and Comet assay can be applied to eukaryotes, but they are limited to a small range of detectable DNA injuries (Barile 1994; Collins 2004).

The DT40 genotoxicity bioassay is a novel reverse genetic approach to determine genotoxicity of chemicals and permits characterization of modes of action (Ridpath et al.

2011). Recently, DT40 cells have been applied to measure genotoxicity in environmental samples (Ji et al. 2011). The DT40 bioassay uses the chicken DT40 B-lymphocyte isogenic cell line and its DNA-repair-deficient mutants, which are ideal for reverse genetic studies (Ulrich et al. 1992; Yamazoe et al. 2004; Ridpath et al. 2011). Their strong phenotypic resemblance to murine cells in DNA repair genes makes it relatively easy to translate DT40 assay results to human exposures to genotoxins (Sonoda et al. 2001). The DT40 bioassay can detect not only whether test materials induce DNA damage but also determine the DNA repair or cell-cycle checkpoint genes required for cell survival after DNA damage. Because each individual repair pathway processes a distinct set of DNA lesion types, differential cytotoxicity as a function of which DNA repair pathway has been knocked out provides insight into the profile of genotoxicity induced (Evans et al. 2010; Ridpath et al. 2011).

The objective of this study was to investigate effects of bioremediation on toxicity and genotoxicity of PAH-contaminated soil from a former MGP site. Two representative biological treatment processes were evaluated in the laboratory, including a sequencing batch bioreactor system (simulating *ex situ* treatment) and a continuous-flow column system (simulating *in situ* treatment). The DT40 parent cell line and fifteen DNA-repair-deficient mutants were employed to understand the genotoxicity potential and profile of the contaminated soil before and after biological treatment.

4.2. Materials and methods

4.2.1. Chemicals

PAH standards (EPA 610 PAH Mixture), benzo[*a*]pyrene diolepoxide (BPDE), methyl methanesulfonate (MMS), hydrogen peroxide (H_2O_2), dimethylsulfoxide (DMSO)

and phosphate buffer solution (PBS) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). All solvents were high-performance liquid chromatography (HPLC) grade and were obtained from Fisher Scientific (Pittsburgh, PA, U.S.A.).

4.2.2. Soil, bioremediation processes, and sampling

Source soil used in this study was collected from a former MGP site in Salisbury, North Carolina, U.S.A., in the vicinity of the former tar well, 1.2 m below the surface. The soil was transferred by shovel to sample buckets and immediately transported to the laboratory, where it was blended and processed through a 10 mm sieve and stored at 4 °C prior to use. The sieved soil contained 66% sand, 28% silt, and 6% clay, with total organic matter of 16.6%. The total concentration of target PAHs (14 of the 16 priority PAHs, excluding acenaphthylene and indeno[*1,2,3-cd*]pyrene) was 556 ± 50 ng/mg (dry mass basis, w/w; individual PAH concentrations are shown in Table B.1).

Two bioremediation processes were employed to treat the source soil. One process involved treatment by a continuously stirred, semicontinuous, laboratory-scale aerobic bioreactor (Singleton et al. 2005; Singleton et al. 2011). The bioreactor had a working volume of approximately 2 L, a solids concentration of 20% (w/w) and solids retention time of 35 d. Every week, 20% of the treated slurry was replaced with untreated source soil in a pH 7.5 buffer containing 5 mM phosphate and 5 mM ammonium nitrate. The other process was the 2.5-year-treatment by two continuous-flow columns (control column and biostimulated column), which were 110 cm long and 10.2 cm in diameter (Richardson and Aitken 2011). Prior to column treatment, the source soil was mixed with sterile 40/50 grade silica sand (Unimin Corporation, Le Sueur, MN, U.S.A.) at a 50:50 ratio (dry weight) to maintain low-pressure flow during long-term column operation. The

control column received simulated groundwater saturated with air. The biostimulated column received simulated groundwater saturated with pure oxygen and amended with ammonium nitrate and phosphate to yield final nitrogen and phosphorus concentrations of 1.0 mg/L and 0.3 mg/L, respectively. Detailed column design and operation are described elsewhere (Richardson and Aitken 2011). Individual PAH concentrations of untreated bioreactor feed soil, bioreactor-treated soil, untreated column packing soil and column-treated soil are shown in Table B.1.

To evaluate the temporal change in toxicity and genotoxicity in the bioreactor system, slurry from the bioreactor was sampled at five time intervals during each cycle: immediately after feeding (0 h), 8 h, 1 d, 3 d and 7 d after feeding. Soil from each column was sampled at the surface of the soil bed and at three sampling ports at 25 cm intervals along the column length (Ports A, B, and C respectively, in the direction of flow) after 2.5 years of continuous operation.

4.2.3. Sample extraction, PAH analysis and residue preparation

Soil samples were centrifuged at 3500 rpm for 15 min, after which the supernatant was discarded. Triplicate aliquots of 3 g (wet weight) centrifuged soil were each extracted overnight twice, each time with a mixture of 10 mL acetone and 10 mL dichloromethane as described elsewhere (Richardson et al. 2011). Each extract was filtered through a 0.2 µm pore-size nylon filter (Millipore, Burlington, MA, U.S.A.) and was brought to a volume of 50 mL with acetonitrile. An aliquot of 1 mL of each extract was removed and analyzed by HPLC for PAH quantification (Richardson et al. 2011). An aliquot of 10 mL of each triplicate extract from the same soil sample was combined in a preweighed vial (total 30 mL) and evaporated to dryness with a mild flow of nitrogen.

The mass of dry residue was determined gravimetrically. The residue was then redissolved with DMSO to 10000 μg/mL and stored in liquid nitrogen before use.

4.2.4. DT40 DNA damage response analysis

DNA damage was determined by 24-well plate-based DNA damage response analysis using a DT40 isogenic cell line and its mutants knocked out in specific DNA repair and cell cycle pathways as described elsewhere (Ridpath et al. 2011). Cells were exposed to the residue redissolved in DMSO that was serially diluted with PBS. The concentration of DMSO was adjusted so that the final concentration for all cell exposures was 0.3%. BPDE, MMS, and H_2O_2 were used as positive controls (Table B.2); while a vehicle blank (DMSO diluted in PBS) was used as negative control. Fifteen DT40 mutants were tested in this study, including base excision repair (BER)-deficient mutants (*Polβ*$^{-/-}$, *Fen1*$^{-/-}$), DNA damage sensor-deficient mutants (*Rad9*$^{-/-}$, *Rad17*$^{-/-}$), a nucleotide excision repair (NER)-deficient mutant (*Xpa*$^{-/-}$), a mis-match repair (MMR)-deficient mutant (*Msh2*$^{-/-}$), a nonhomologous end-joining (NHEJ)-deficient mutant (*Ku70*$^{-/-}$), homologous recombination (HR)-deficient mutants (*Rad54*$^{-/-}$, *FancD2*$^{-/-}$), and tans-lesion synthesis (TLS)-deficient mutants (*Rad18*$^{-/-}$, *Rev1*$^{-/-}$, *Rev3*$^{-/-}$, *Polκ*$^{-/-}$, *Polη*$^{-/-}$, *Polθ*$^{-/-}$).

The DT40 system has not been tested previously for its ability to activate compounds that require metabolic activation before exerting a genotoxic effect. Therefore, we conducted a preliminary evaluation of the response of DT40 and the mutant *Rev3*$^{-/-}$ to exposure to benzo[*a*]pyrene (BaP). Details are provided in the Appendix B.

4.2.5. Data analysis

Statistical analyses were conducted with SPSS® (v16.0, SPSS Inc.). Student's *t* test and one-way analysis of variance (ANOVA) with Tukey's test were employed to test for

statistically significant differences between two groups and among multiple groups, respectively. Spearman test and partial correlation analysis with Spearman test were applied to investigate relation between LD_{50} and total PAH concentrations (C_{tPAHs}) or total organic residue concentration ($C_{residue}$). LD_{50} was calculated based on the dose-response relation and converted from residue dose to equivalent soil dose as described in the Appendix B.

4.3. Results

Both the bioreactor system and the column system significantly removed PAHs from the contaminated soil (Figure 4.1). For the bioreactor system, during each cycle (7 d), total PAH concentration of the treated soil decreased with time and approached a minimum 24 h after feeding (Figure 4.1a). For the column system, the PAH concentration of both control-column and biostimulated-column treated soil was significantly lower than that of the untreated column packing soil (Figure 4.1b). Overall, the bioreactor system had total PAH removal of 69% and the biostimulated column had total PAH removal of 84%.

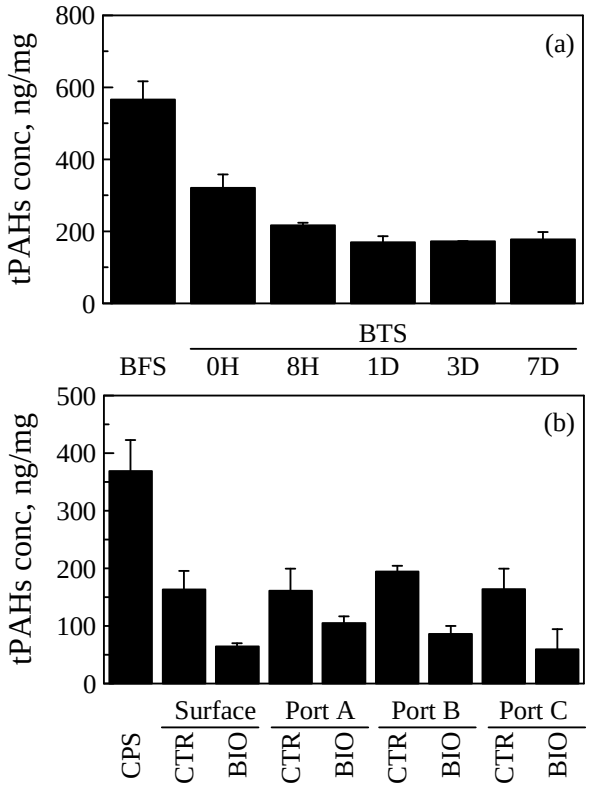


Figure 4.1 Total PAH concentration of soil before and after bioremediation. (a) Soils from five consecutive sampling times during 7 d cycle in the bioreactor treatment. (b) Soils from both the control column and biostimulated column at four sampling points along each column after 2.5-year column treatment. Values are mean ± SD of triplicates. BFS, untreated bioreactor feed soil; BTS, bioreactor treated soil; CPS, untreated column packing soil; CTR, control-column treated soil; BIO, biostimulated-column treated soil.

On the basis of its sensitivity to a broad range of DNA damage and its application in measuring genotoxicity in crude oil-contaminated sediments (Ji et al. 2011). the *Rad54*$^{-/-}$ mutant was selected for detailed analysis of the effects of the two bioremediation processes on genotoxicity of the soil. For the bioreactor system, the LD_{50} of the bioreactor-treated soil for DT40 and the *Rad54*$^{-/-}$mutant increased through Day 1, then decreased (Figure 4.2a). The LD_{50} of the bioreactor-treated soil for DT40 was significantly lower than that of the untreated bioreactor soil, except at Day 1; the LD_{50} of the bioreactor-treated soil for the *Rad54*$^{-/-}$ mutant was not significantly different from that of the untreated bioreactor soil through Day 1 but was significantly lower on Day 3 and

Day 7 (Figure 4.2a). For the column system, the LD_{50} of both control-column and biostimulated-column treated soils for DT40 was significantly higher than that of the untreated column soil; the LD_{50} of the control-column treated soil for *Rad54*$^{-/-}$ was not significantly different from that of the untreated column soil, while the LD_{50} of the biostimulated-column treated soil for *Rad54*$^{-/-}$ was significantly higher than that of the untreated column soil (Figure 4.2b).

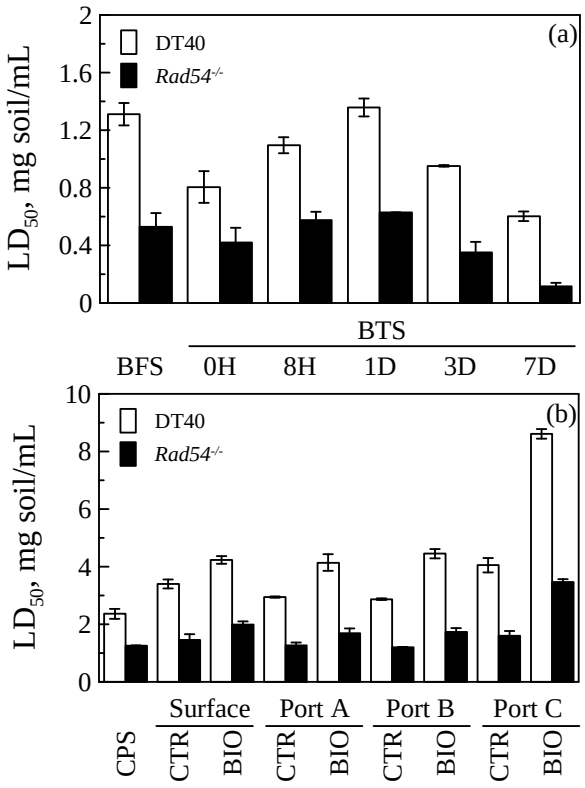


Figure 4.2 LD_{50} of soil before and after bioremediation for parental DT40 cell line and its *Rad54*$^{-/-}$ mutant. (a) Soils from five consecutive sampling times during 7 d cycle in the bioreactor treatment. (b) Soils from both control column and biostimulated column at four sampling points along each column after 2.5 year column treatment. Values are mean ± SD of three separate experiments. Abbreviations are as defined in Figure 4.1.

Inverse correlations between LD_{50} and C_{tPAHs} or $C_{residue}$ were both highly positive and statistically significant (Figure 4.3). However, when $1/C_{residue}$ was controlled, partial correlations between LD_{50} and $1/C_{tPAHs}$ were not statistically significant; conversely,

when $1/C_{tPAHs}$ was controlled, partial correlations between LD_{50} and $1/C_{residue}$ were highly positive and statistically significant (Table B.3).

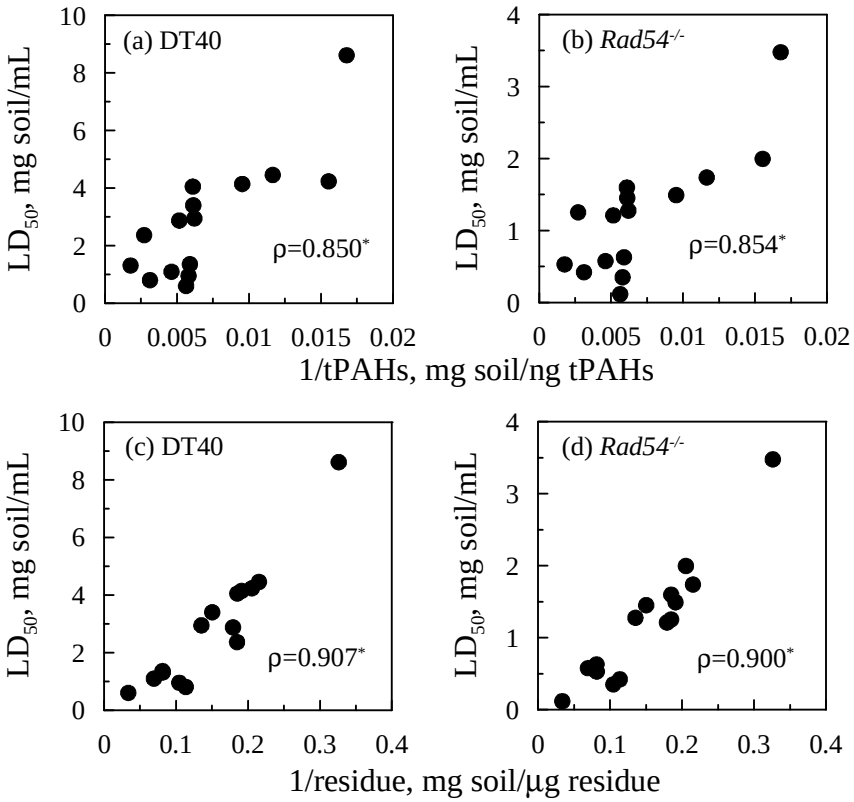


Figure 4.3 Inverse correlations between LD_{50} and concentrations of tPAH for parental DT40 cell line (a) and its *Rad54*$^{-/-}$ mutant (b), and between LD_{50} and concentrations of total residue for parental DT40 cell line (c) and its *Rad54*$^{-/-}$ mutant (d). Each data point represents the mean for each soil sample (total 15 samples) including untreated column packing soil, all sampling points along each column, untreated bioreactor feed soil, and all sampling events for bioreactor-treated soil during the 7 d cycle. Asterisks indicate the correlation is statistically significant ($p < 0.05$).

The column system soils were also screened with a battery of DT40 cell lines for genotoxicity profiling (Figure 4.4a). There were no significant differences in LD_{50} between control-column treated soil and untreated column packing soil, except for the parent DT40 cells. In general, the LD_{50} of biostimulated-column treated soil was significantly higher than the corresponding LD_{50} of untreated column packing soil, except for *Rad9*$^{-/-}$, *Rad17*$^{-/-}$, *Ku70*$^{-/-}$, *Rad18*$^{-/-}$, *Rev1*$^{-/-}$ and *Rev3*$^{-/-}$. The LD_{50} of

biostimulated-column treated soil was significantly higher than that of the control-column treated soil, except for *Rad9*$^{-/-}$ and *Rad17*$^{-/-}$.

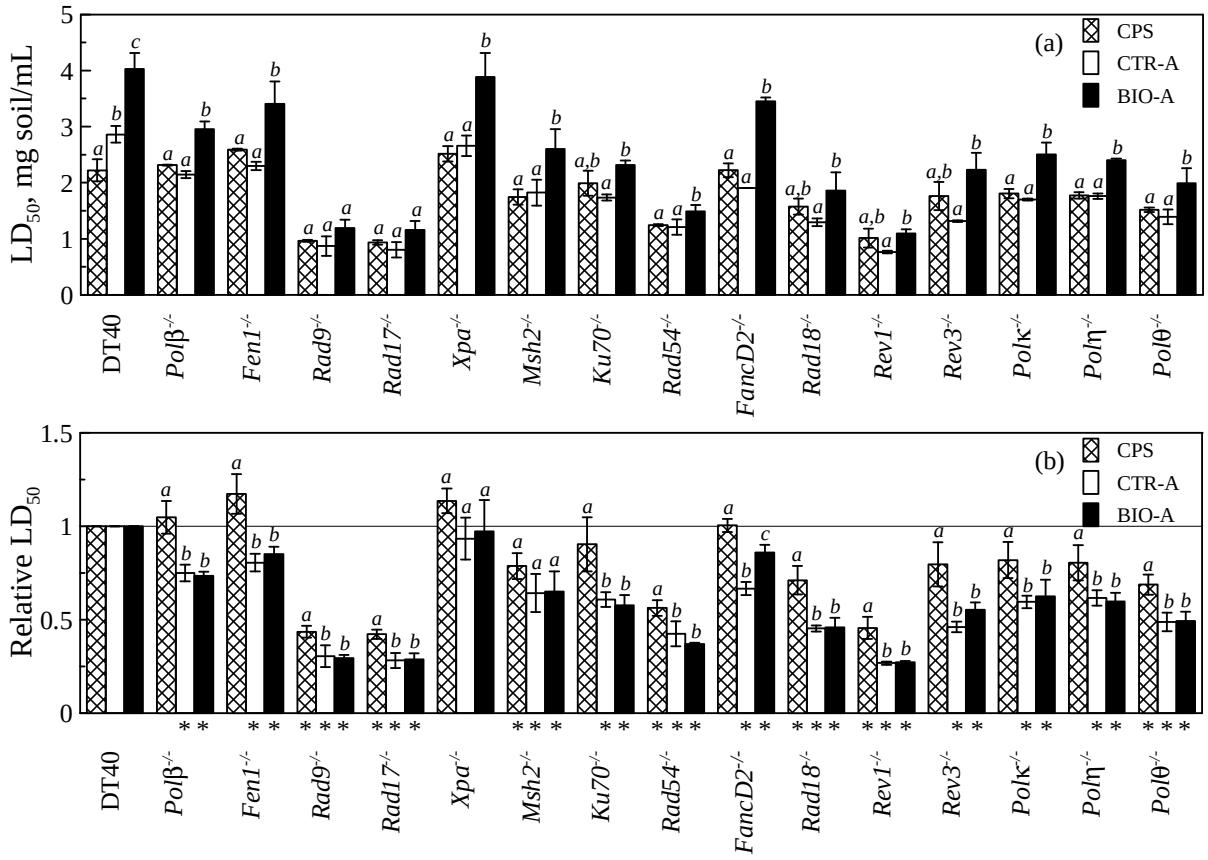


Figure 4.4 LD_{50} (a) and relative LD_{50} (b) of soil before and after 2.5 year column treatment in the test with a battery of DT40 cell lines. Values are mean ± SD of three separate experiments. Different letters are assigned to conditions for which there was a significant difference ($p < 0.05$). Asterisks indicate values significantly less than 1 ($p < 0.05$). CPS, untreated column packing soil; CTR-A, control-column treated soil at Port A; BIO-A, biostimulated-column treated soil at Port A.

For a quantitative comparison, we also calculated the relative LD_{50} of column-system soils (Figure 4.4b) as described by Ji et al. (2009), where the relative LD_{50} of the parental DT40 cell was defined as 1. If the relative LD_{50} of a mutant to a sample is significantly less than 1, that mutant is defined as sensitive to that sample. Seven mutants were sensitive to both untreated and treated columns soils, including *Rad9*$^{-/-}$, *Rad17*$^{-/-}$, *Msh2*$^{-/-}$, *Rad54*$^{-/-}$, *Rad18*$^{-/-}$, *Rev1*$^{-/-}$ and *Polθ*$^{-/-}$. Seven mutants were sensitive only to

column-treated soils but not to untreated column packing soil, including *Polβ*$^{-/-}$, *Fen1*$^{-/-}$, *Ku70*$^{-/-}$, *FancD2*$^{-/-}$, *Rev3*$^{-/-}$, *Polκ*$^{-/-}$ and *Polη*$^{-/-}$. *Xpa*$^{-/-}$ was not sensitive to either untreated column packing soil or column-treated soils.

4.4. Discussion

4.4.1. Effects of bioremediation on toxicity and genotoxicity

Bioremediation is an established technology to remove PAHs from contaminated soil and sediment (USEPA 2007). However, some researchers have advised caution about bioremediation because the removal of the monitored PAHs during bioremediation of contaminated soil or sediment might not correspond to a reduction in health risk (Lundstedt et al. 2007; Lemieux et al. 2009). In some studies, toxicity decreased as treatment progressed (Baud-Grasset et al. 1993; Haeseler et al. 1999b; Sayles et al. 1999; Mendonca and Picado 2002; Sasek et al. 2003), while in other studies there was either no reduction or even a substantial increase in toxicity following bioremediation (Hughes et al. 1998; Gillespie et al. 2007; Andersson et al. 2009; Gandolfi et al. 2010). Increases in toxicity might be caused by formation of toxic metabolites or increased bioavailability of native toxins over the course of bioremediation (Andersson et al. 2009).

Our study confirmed that bioremediation reduced PAH levels in the contaminated soil (Figure 4.1), but the effect of bioremediation on toxicity is complicated. Generally, we observed increased toxicity (decreased LD_{50}) in the bioreactor system but decreased toxicity (increased LD_{50}) in the column system after bioremediation (Figure 4.2). Remediation methods and the specific ways they are implemented can substantially influence the community of PAH-degrading microorganisms in contaminated soil, thus influencing the collective balance between complete and incomplete metabolism of PAHs

by these organisms and, therefore, potential variation in toxicity and genotoxicity. Longer periods of bioremediation, such as that used in the column systems, may be required to significantly reduce the genotoxic hazard of a contaminated soil (Lemieux et al. 2009). Hughes et al. (1998) also found variations of genotoxicity changes in creosote-contaminated soil before and after four bioremediation processes. However, they could not determine whether observed increases in genotoxicity were due to the processes themselves or to the amendments added to the soil (Hughes et al. 1998).

4.4.2. Temporal change in toxicity and genotoxicity in the bioreactor system

A temporal change in toxicity was observed in the bioreactor system following a feeding event (Figure 4.2a). Toxicity to both the DT40 parent cell line and its *Rad54*$^{-/-}$ mutant initially decreased (increased LD_{50}) and then increased (decreased LD_{50}) during the feeding cycle. Other researchers have also observed temporal changes in the genotoxicity of PAH-contaminated soils undergoing bioremediation (Belkin et al. 1994; Lemieux et al. 2009). The somewhat cyclical nature of toxicity and genotoxicity may suggest the formation, and subsequent degradation, of toxic compounds (Lemieux et al. 2009), although if that were the case with our bioreactor system then we would have observed a temporal trend opposite to that shown in Figure 4.2a. Sampling of the column system was not designed to evaluate temporal trends in toxicity and genotoxicity, so only the long-term treatment effects were observed.

4.4.3. Source of toxicity

Compounds responsible for toxicity and genotoxicity of PAH-contaminated soil other than the USEPA 16 priority PAHs might not degrade concomitantly with PAHs during bioremediation (Lundstedt et al. 2003; Lemieux et al. 2008; Lemieux et al. 2009).

Moreover, in a complex system such as contaminated soil, some transformations that do not lead to complete metabolism of the parent compound are inevitable. Although the correlation between LD_{50} and total PAH concentration was significant (Figure 4.3a and 4.3b), the partial correlation between LD_{50} and total PAH concentration was poor and insignificant, when controlling for the effects of total organic residue. We conclude that the total organic compounds present in soil extracts are responsible for the toxicity and genotoxicity of PAH-contaminated soil undergoing bioremediation. Further research is needed to identify the toxic and genotoxic compounds.

4.4.4. Genotoxicity profiling

To understand the effects of bioremediation on the genotoxicity potential of PAH-contaminated soil in the column system, we screened 15 DNA-repair-deficient DT40 mutants. When compared to the untreated soil, the control column did not reduce toxicity except for the parental DT40 cell line; in contrast, the biostimulated column significantly reduced toxicity for both the parental DT40 cell line and most of the mutants (Figure 4.4a). We also observed that the genotoxicity profiles (relative LD_{50}) of control-column treated soil and biostimulated-column treated soil were similar but both were different from that of the untreated soil (Figure 4.4b). Several mutants were sensitive to treated soil but not untreated soil, including *Polβ*$^{-/-}$, *Fen1*$^{-/-}$, *Ku70*$^{-/-}$, *FancD2*$^{-/-}$, *Rev3*$^{-/-}$, *Polκ*$^{-/-}$ and *Polη*$^{-/-}$, indicating that more types of DNA damage were induced by remediation. This finding suggests that genotoxic compounds were generated during bioremediation, although their concentrations must have been low enough not to lead to an overall increase in genotoxicity per unit soil mass.

RAD9 and RAD17 are intra-S-phase DNA damage checkpoint control proteins and are in the cellular response to stalled DNA replication (Kobayashi et al. 2004). Both *Rad9*$^{-/-}$ and *Rad17*$^{-/-}$ were sensitive to treated and untreated soil, strongly suggesting that bioremediation could not eliminate genotoxic compounds in PAH-contaminated soil that can induce DNA replication block. RAD54 is a DNA repair and HR protein (Sonoda et al. 2006). *Rad54*$^{-/-}$ was sensitive to both treated and untreated soil, indicating that the soil both before and after bioremediation could induce DNA double-strand breaks or DNA damage leading to replication blockage (Sonoda et al. 2006). NER mediated by the *Xpa* gene is thought to be involved in the elimination of bulky DNA adducts (van Gent et al. 2001). However, *Xpa*$^{-/-}$ was not sensitive to either treated soil or untreated soil, indicating that the potential for formation of bulky DNA adducts may be negligible before and after bioremediation. Metabolic activation of PAHs may lead to bulky DNA adducts (Xue and Warshawsky 2005), but the capacity of DT40 cells for metabolic activation has not been reported before. Our preliminary results indicate that *Rev3*$^{-/-}$ was sensitive to BaP (Figure B.1), indicating that DT40 cells may have a metabolic activation system for PAHs. Regardless, this study was not intended to elucidate the genotoxicity of PAHs *per se*, but to evaluate the changes in genotoxicity of the combination of soil contaminants as a result of bioremediation.

BER plays an essential role in protecting cells from DNA damage caused by hydrolysis, oxidative agents and alkylating agents (Yoshimura et al. 2006). We observed that BER-deficient mutants (*Polβ*$^{-/-}$, *Fen1*$^{-/-}$) were sensitive to treated soils but not to untreated soil, indicating that bioremediation generated genotoxic compounds that could induce oxidative stress, unstable depurinating DNA adducts or alkylation DNA damage

(Tano et al. 2007; Asagoshi et al. 2010). Certain TLS-deficient mutants (*Rad18*$^{-/-}$, *Rev1*$^{-/-}$ and *Polθ*$^{-/-}$) were sensitive to untreated soil, indicating that unstable depurinating DNA adducts and alkylated DNA bases could also be generated by exposure to untreated soil. Whereas oxidative DNA damage is thought to be repaired by BER, it has been proposed that DNA lesions caused by oxidative stress could also be repaired by NHEJ involving protein KU70 (Narasimhaiah et al. 2005). *Ku70*$^{-/-}$ was sensitive to treated soil but not to untreated soil, further indicating the likelihood that bioremediation generated genotoxic compounds causing oxidative stress, which might be attributed to the formation of oxy-PAHs during incomplete biodegradation (Zielinska-Park et al. 2004).

4.4.5. Value of genotoxicity testing

Although bioremediation is an effective tool to remove PAHs from contaminated soil, its effects on toxicity and genotoxicity of PAH-contaminated soil need thorough study if the ultimate goal of remediation is to reduce human health risk. This study demonstrated that different bioremediation strategies could lead to different outcomes of toxicity and genotoxicity for PAH-contaminated soil. Our results also indicated that enhanced oxidative DNA damage was caused by the soil after bioremediation in the column system. Overall, toxicity and genotoxicity bioassays can be an effective supplement to chemical analysis-based risk assessment for contaminated soil. Further research is still needed to isolate, characterize, and quantify the toxic and genotoxic compounds in the contaminated soil as remediation progresses.

5. BIOAVAILABILITY OF (GENO)TOXIC CONTAMINANTS IN PAH-CONTAMINATED SOIL FROM A FORMER MGP SITE BEFORE AND AFTER BIOLOGICAL TREATMENT[3]

5.1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a major pollutant class at thousands of contaminated sites in the USA, including over 700 Superfund sites (USEPA 2012) and an estimated 45,000 former manufactured-gas plant (MGP) sites (USEPA 2004a). PAHs are of great human health concern because of their ubiquity in the environment as well as their known or suspected genotoxicity (Bostrom et al. 2002b; White 2002; Ramesh et al. 2004; Shimada and Fujii-Kuriyama 2004; Baird et al. 2005; Xue and Warshawsky 2005; Mordukhovich et al. 2010).

Historically, the potential effects of contaminated sites on human or ecological health are assessed based on the total contaminant concentrations as determined by vigorous chemical extraction techniques (Alexander 2000). However, because of their association with different soil components, hydrophobic organic pollutants in soil are only partly available for uptake by organisms, for exerting toxic effects, and for biodegradation by microorganisms (Alexander 1995; Alexander and Alexander 1999; Alexander 2000; Reid et al. 2000a; Lei et al. 2004; Jablonowski et al. 2008). Poor correlation has been observed between vigorous solvent extraction and compound bioavailability to earthworms and bacteria over time in soil (Kelsey and Alexander 1997; Kelsey et al. 1997). Recently, the US Interstate Technology and Regulatory Council (ITRC) has advised incorporating

[3]Coauthors: Jing Hu, Alden C. Adrion, Jun Nakamura, Damian Shea, Michael D. Aitken

bioavailability considerations into the evaluation of contaminated sites to reduce the extent of cleanup required to that which is necessary to be protective of the environment (ITRC 2011).

A variety of biological and chemical techniques have been developed to estimate and predict bioavailability. Biological techniques involve measuring substrate uptake, mineralization or toxicity (Kelsey et al. 1997; Stroo et al. 2000; Braida et al. 2004). Chemical techniques involve non-exhaustive solvent extraction (Hatzinger and Alexander 1995; Kelsey et al. 1997; Breedveld and Karlsen 2000; Reid et al. 2000b; Liste and Alexander 2002) and solid-phase extraction (Ten Hulscher et al. 2003; Lei et al. 2004; Harwood et al. 2012). Solid-phase extraction is a common method for estimating bioavailability in contaminated soils and sediments with polymeric adsorbent resins, such as Tenax beads and XAD, serving as an infinite sink. These sorbents work well with nonpolar compounds such as PAHs.

In a complex system such as soil, it is likely that some transformations of organic pollutants by microorganisms do not lead to complete metabolism of the parent compound. In PAH-contaminated soil undergoing bioremediation, hundreds of hazardous compounds have been identified, covering a very wide range of physicochemical properties. For example, logK_{ow} values can range from 2 to 8 (Lundstedt et al. 2003), including polar and semi-polar metabolites of PAHs. Compounds other than parent PAHs are responsible at least in part for the toxicity and genotoxicity observed in studies on bioremediation of field-contaminated soil (Hu et al. 2012).

The objective of this study was to investigate the bioavailability of PAHs, selected oxygenated metabolites (oxy-PAHs), and other constituents that collectively lead to

toxicity or genotoxicity during biological treatment in a lab-scale, slurry-phase sequencing batch bioreactor. Both hydrophobic (Tenax®) and universal (Oasis® HLB) sorptive resins were used to evaluate potential bioavailability. The DNA damage response assay using the chicken DT40 B-lymphocyte isogenic cell line and its DNA-repair-deficient mutant *Rad54*$^{-/-}$ was applied to evaluate the toxicity and genotoxicity of different fractions of the soil throughout a treatment cycle in the bioreactor.

5.2. Materials and methods

5.2.1. Materials

PAH standards (EPA 610 PAH Mixture), 9,10-phenanthrenequinone (PQ) and 9,10-anthraquinone (AQ) were purchased from Sigma-Aldrich (St. Louis, MO, USA). 9-fluorenone (FLO) and benz[*a*]anthracene-7,12-quinone (BAQ) were purchased from Acros Organics (Morris Plains, NJ, USA). All solvents were high-performance liquid chromatography (HPLC) grade and were purchased from Fisher Scientific (Pittsburgh, PA, USA).

Tenax® TA beads (60/80 mesh) and the universal (mixed-function) Oasis® HLB resin (particle size 60 µm) were obtained from Alltech (Deerfield, IL, USA) and Waters (Milford, MA, USA), respectively. They were cleaned by Soxhlet extraction in acetone: hexane (50:50, v/v) mixture and methanol: dichloromethane (50:50, v/v) mixture overnight and air-dried before use, respectively. SnakeSkin® dialysis tubing (10,000 MWCO, 22 mm diameter, 10.5 m) was obtained from Thermo Scientific (Rockford, IL, USA).

Contaminated soil (feed soil) used in this study was collected from a former MGP

site in Salisbury, North Carolina, USA, processed and characterized as described elsewhere (Richardson and Aitken 2011; Hu et al. 2012). The total concentration of target PAHs (14 of the 16 USEPA priority PAHs, excluding acenaphthylene and indeno[*1,2,3-cd*]pyrene) was 362 ± 23 µg/g and the total concentration of target oxy-PAHs (FLO, PQ, AQ and BAQ) was 22.3 ± 0.5 µg/g (dry mass basis, w/w). Individual PAH and oxy-PAH concentrations of feed soil are shown in Table C.1 of Appendix C. The feed soil was treated by a continuously stirred, semi-continuous (draw-and-fill), laboratory-scale aerobic bioreactor with a working volume of approximately 2 L, a solids concentration of 14% (w/w), and solids retention time of 35 d (Hu et al. 2012). Every week, 20% of the treated slurry was replaced with feed soil in a pH 7.5 buffer containing 5 mM phosphate and 5 mM ammonium nitrate. Treated slurry was sampled at three time intervals during each 7-d feeding cycle: immediately after feeding (0 d), 1 d and 7 d after feeding. The treated slurry was centrifuged at 3500 rpm for 20 min to remove excess water prior to use. PAH and oxy-PAH concentrations of treated soils are shown in Table C.1.

5.2.2. Desorption experiments

The infinite-sink desorption method (Loehr et al. 2003) was employed to investigate desorption of PAHs and oxy-PAHs from soil (feed soil or treated soil) to sorbents (Tenax beads or HLB resin). Because of the small particle size of HLB resin and its hydrophilic-lipophilic balanced property, the conventional method (adding the sorbent directly to soil slurry) was adapted by placing the sorbent into dialysis tubing suspended in the soil slurry. Briefly, an aliquot of 0.1 g sorbent was weighed and transferred into 5 cm of dialysis tubing with knots on both ends. Approximately 4 g of wet soil and one

dialysis tubing containing the sorbent were suspended in 50 mL phosphate buffer (pH 7.5) amended with 4.15 g/L NaN_3 in a 60-mL centrifuge tube with a PTFE-lined septum and screw cap. Six centrifuge tubes were prepared for each soil sample for each desired time interval: three tubes with dialysis tubing containing Tenax beads and three tubes with dialysis tubing containing HLB resin. The tubes were placed on a wrist-action shaker at 240 rpm in the dark. After each desired time interval (1 d, 2 d, 3 d, 7 d, 10 d and 15 d), the six centrifuge tubes for each soil sample were sacrificed. The dialysis tubing was removed from the centrifuge tube and rinsed with deionized water to detach soil particles on the outside wall. The dialysis tubing was then unknotted and the sorbents were rinsed with deionized water into a vacuum filtration apparatus to remove excess water. The soil slurry left in the centrifuge tube was centrifuged at 3500 rpm for 20 min and the supernatant was discarded. The sorbents and the soil after desorption were then extracted.

The conventional Tenax-bead desorption method was carried out as described elsewhere (Hu and Aitken 2012). Briefly, approximately 4 g of wet feed soil and 0.1 g Tenax beads were suspended in 50 mL phosphate buffer (pH 7.5) amended with 4.15 g/L NaN_3 in each of triplicate 60-mL centrifuge tubes with a PTFE-lined septum and screw cap. The tubes were placed on a wrist-action shaker at 240 rpm in the dark. After 7 d, the tubes were centrifuged at 3500 for 20 min, and Tenax beads were removed from the tubes for subsequent extraction.

5.2.3. Sample extraction, chemical analysis and DT40 bioassay

Tenax beads and HLB resin were extracted overnight with 10 mL methanol or 10 mL methanol: dichloromethane mixture (50:50, v/v), respectively, in a 20-mL glass vial with a PTFE-lined septum and screw cap. The extracts were filtered through a 0.2 µm

pore-size nylon filter and brought to 25 mL with acetone. The soil samples were extracted overnight twice each with a mixture of 10 mL acetone and 10 mL dichloromethane as described elsewhere (Richardson et al. 2011). The extracts were filtered through a 0.2 µm pore-size nylon filter and brought to 50 mL with acetonitrile.

The filtered extracts were analyzed by HPLC for PAHs as described elsewhere (Richardson et al. 2011) and were analyzed by liquid chromatography-tandem mass spectrumetry (LC-MS/MS) for oxy-PAHs as described in Appendix C. Toxicity and genotoxicity of the filtered extracts were determined by DT40 DNA damage response analysis using a DT40 isogenic cell line and its mutant *Rad54*$^{-/-}$ knocked out in homologous recombination DNA repair pathway as described elsewhere (Ridpath et al. 2011; Hu et al. 2012).

5.2.4. Data analysis

SPSS® (v16.0, SPSS Inc.) was applied for statistical analysis. Student's *t* test and one-way analysis of variance (ANOVA) followed by Tukey's test were employed to test for differences between two groups and among multiple groups, respectively. The LD_{50} of a sample for the parental DT40 cells and for the *Rad54*$^{-/-}$ mutant was calculated based on the dose-response relation and converted to an equivalent mass of soil as described elsewhere (Hu et al. 2012).

5.3. Results

5.3.1. Dialysis tubing desorption method validation

Dialysis tubing was used to contain Tenax beads or HLB resin to investigate desorption of PAHs and oxy-PAHs from soil. Desorption of total PAHs (sum of 14 compounds) and total oxy-PAHs (sum of four compounds) to HLB resin from both feed

soil and treated soil increased with time and approached apparent equilibrium in 7 days, as shown in Figure 5.1.

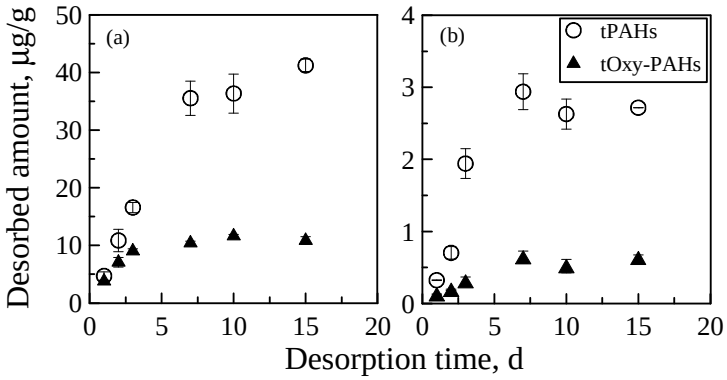


Figure 5.1 Desorption kinetics of total PAHs and total oxy-PAHs to HLB resin from (a) feed soil and (b) treated soil removed from the bioreactor 1 day after feeding using the dialysis-tubing desorption method described in the text.

Recoveries of PAHs and oxy-PAHs were calculated by comparing the initial mass in the soil with the mass desorbed to Tenax beads or HLB resin and the mass remaining in the soil after 7 days of desorption. Individual PAH and oxy-PAH recoveries are shown in Table C.2. Total PAH recovery in the Tenax-bead system was 95.7 ± 0.7% and in the HLB-resin system was 93.6 ± 1.1%; total oxy-PAH recovery in Tenax-bead system was 101 ± 8% and in the HLB-resin system was 110 ± 5%. We also compared the dialysis-tubing desorption method to the conventional Tenax-bead desorption method (direct suspension of Tenax beads in the soil slurry). As shown in Figure 5.2, desorption of PAHs and oxy-PAHs from feed soil to Tenax beads or HLB resin using the dialysis-tubing desorption method was not significantly different from each other or from that using the conventional Tenax-bead desorption method.

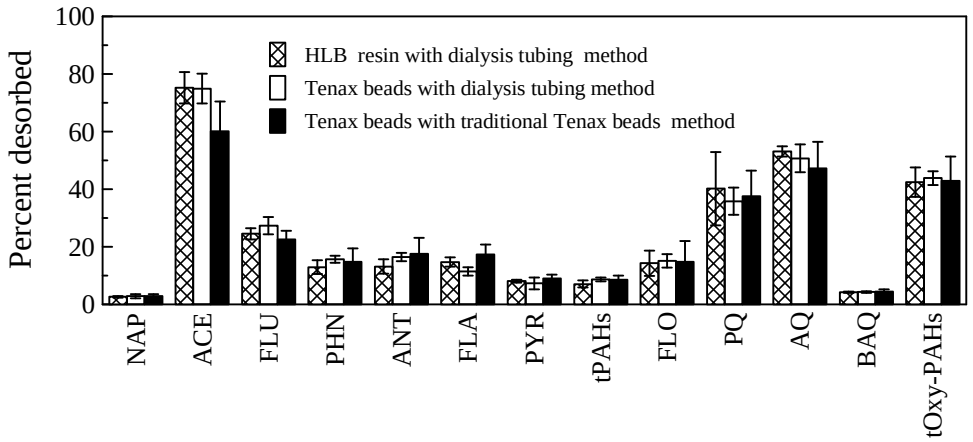


Figure 5.2 Comparison of desorption of PAHs and oxy-PAHs from feed soil to HLB resin and Tenax beads in the dialysis-tubing desorption method and in the conventional Tenax-bead desorption method. NAP: naphthalene; ACE: acenaphthene; FLU: fluorene; PHN: phenanthrene; ANT: anthracene; FLA: fluoranthene; PYR: pyrene; tPAHs: total PAHs; tOxy-PAHs: total oxy-PAHs. Desorption was less than 2% for benz[*a*]anthracene (BaA), chrysene (CHR), benzo[*b*]fluoranthene (BbF), benzo[*k*]fluoranthene (BkF), benzo[*a*]pyrene (BaP), dibenz[*a*,*h*]anthracene (DBA) and benzo[*g*,*h*,*i*]perylene (BgP) under all methods. Desorption time was 7 d. There was no significant difference ($p > 0.05$) between methods for any of the analytes.

5.3.2. Biodegradability and desorbability of PAHs and oxy-PAHs

Bioreactor treatment significantly removed PAHs and oxy-PAHs from the contaminated soil, and the removal increased over the duration of the 7-d treatment cycle (Figure 5.3). After 7 d, removal of total PAHs and total oxy-PAHs was 50.2 ± 4.5% and 71.6 ± 0.7%, respectively. Removal of each individual oxy-PAH and the corresponding parent PAH in the contaminated soil also increased with time. However, removal of FLO and BAQ was significantly less than removal of their respective parent compounds FLU and BaA, while removal of PQ and AQ was significantly greater than their parent compounds PHN and ANT, throughout the bioreactor 7-d treatment cycle.

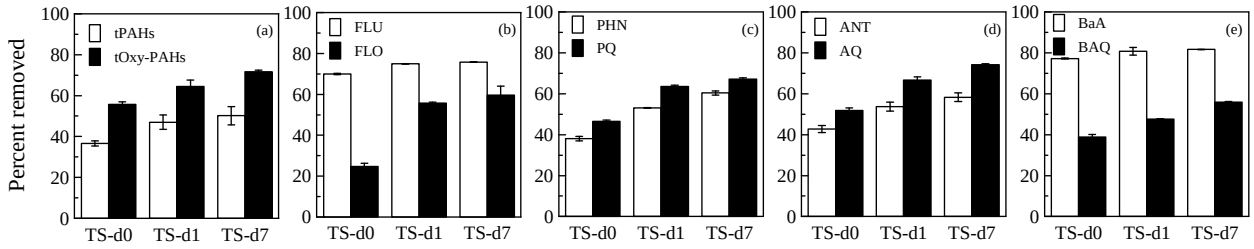


Figure 5.3 Removal of PAHs and oxy-PAHs in the treated soils sampled at three time intervals during 7-d feeding cycle: immediately after feeding (TS-d0), 1 d (TS-d1) and 7 d (TS-d7) after feeding. In all cases, there was a significant difference ($p < 0.05$) between removal of an oxy-PAH and the corresponding parent PAH. Removal is relative to the initial concentration of the respective analyte in the feed soil.

Desorption of PAHs and oxy-PAHs to HLB resin and Tenax beads from soils decreased with longer treatment time, except for BaA (Figure 5.4); desorption of BaA in feed soil was slightly less than that in treated soil sampled at the end of the 7-d treatment cycle, although the desorbable fraction of BaA was 2% or less in all samples. In both feed soil and treated soils, desorption of total oxy-PAHs was significantly greater than that of total PAHs. At the end of the 7-d treatment cycle, 7.6 ± 1.1% and 6.0 ± 0.9% of total oxy-PAHs were still desorbable to HLB resin and Tenax beads, respectively, while only 0.5 ± 0.1% and 0.6 ± 0.1% of total PAHs were desorbable to HLB resin and Tenax beads, respectively. However, when comparing each individual oxy-PAH with its corresponding parent compound, desorption of only PQ and AQ were significantly greater than that of PHN and ANT throughout the bioreactor treatment cycle. Desorption of BAQ was significantly greater than that of BaA only in feed soil and treated soil sampled immediately after feeding, while desorption of FLO was significantly less than that of FLU in those two samples. No differences between desorption of FLO or BAQ and that of their corresponding parent compounds were observed in treated soils sampled 1 d and 7 d after feeding.

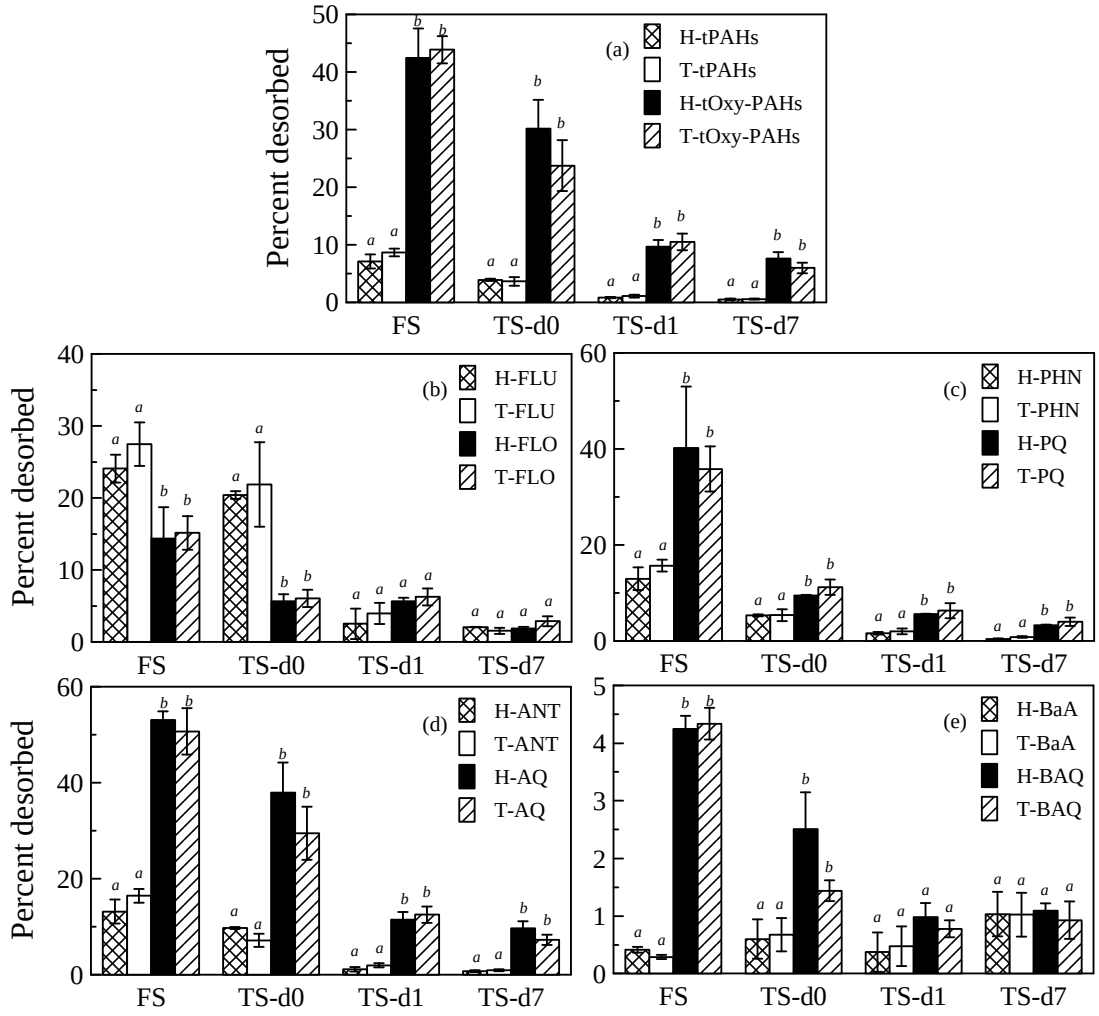


Figure 5.4 Desorption of PAHs and oxy-PAHs to HLB resin (H) and Tenax beads (T) from feed soil (FS) and treated soils (TS) sampled at three time intervals during 7-d feeding cycle: immediately after feeding (TS-d0), 1 d (TS-d1) and 7 d (TS-d7) after feeding. Different letters are assigned to conditions for which there was a significant difference ($p < 0.05$).

The percentage of PAH and oxy-PAH desorbed in the feed soil was compared to the percentage of PAH and oxy-PAH removed in the bioreactor at the end of the bioreactor treatment cycle (Figure 5.5). For every compound, the removal in the bioreactor was greater than its desorption.

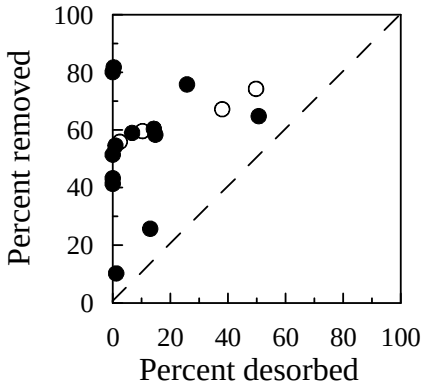


Figure 5.5 Percentage of PAH and oxy-PAH removed at the end of 7-d treatment cycle vs. percentage of PAH and oxy-PAH desorbed from the feed soil. Each point represents the mean value for an individual PAH or oxy-PAH. The dashed line represents a 1:1 correlation. Close and open circles represent PAHs and oxy-PAHs respectively. NAP is not included because no removal was observed.

5.3.3. Toxicity and genotoxicity

The toxicity and genotoxicity of solvent extracts and desorbable constituents of soil samples were examined using the DT40 bioassay. For both the DT40 parental cell line and its *Rad54*$^{-/-}$ mutant, the LD_{50} of the solvent extracts increased with time during biological treatment (Figure 5.6). The same trend was also observed for the constituents desorbed to HLB or Tenax. The LD_{50} of constituents desorbed to HLB or Tenax for both DT40 and *Rad54*$^{-/-}$ was significantly higher than that of the solvent extracts for all samples. No significant differences of LD_{50} were observed among the solvent extracts of each sample before or after desorption to HLB or to Tenax (Figure C.1). The LD_{50} of constituents desorbed to HLB was significantly lower than that of constituents desorbed to Tenax in feed soil and treated soil sampled immediately after feeding (d0) for both DT40 and *Rad54*$^{-/-}$. In contrast, the LD_{50} of constituents desorbed to HLB was significantly higher than that of constituents desorbed to Tenax as treatment progressed during the 7-d treatment cycle (d1 and d7 samples) for DT40, while no significant differences were observed among LD_{50} values for *Rad54*$^{-/-}$ in these samples.

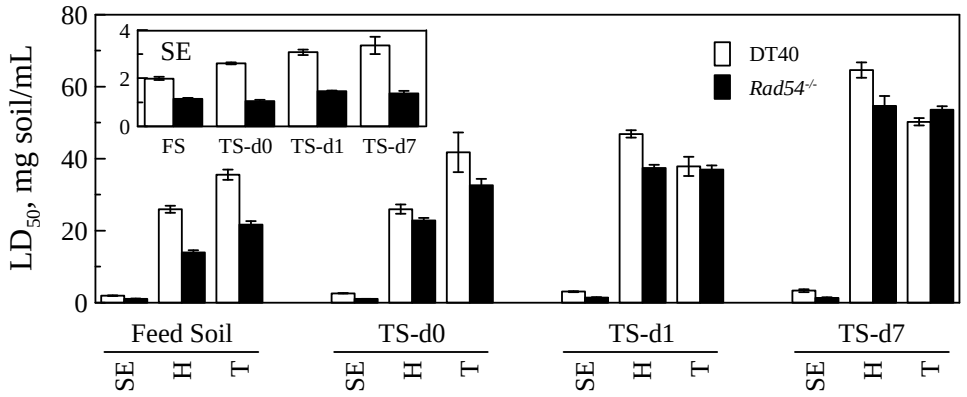


Figure 5.6 LD_{50} of solvent extracts (SE) and constituents desorbed to HLB (H) and Tenax (T) of soils for parental DT40 cell line and its *Rad54*$^{-/-}$ mutant. TS-d0, TS-d1 and TS-d7 represent treated soil sampled immediately after feeding, 1 d and 7 d after feeding during the 7-d feeding cycle, respectively. The inset enlarges the results for LD_{50} of solvent extracts.

For a quantitative comparison of genotoxicity, we also calculated the relative LD_{50} (LD_{50} of *Rad54*$^{-/-}$ divided by the LD_{50} of the parental DT40 cell line; Figure 5.7). If the relative LD_{50} of a sample is significantly less than 1.0, that sample is defined as genotoxic. The lower the relative LD_{50} is, the more genotoxic the sample is. The relative LD_{50} of solvent extracts decreased after biological treatment, while the relative LD_{50} of constituents desorbed to either HLB or Tenax increased as a result of biological treatment (Figure 5.7a). The relative LD_{50} of constituents desorbed to HLB or Tenax was significantly higher than that of the solvent extracts for all samples (Figure 5.7b). No significant differences of relative LD_{50} were observed among the solvent extracts of each sample before or after desorption to HLB or to Tenax (Figure C.2). The relative LD_{50} of constituents desorbed to the HLB was not significantly different from that of constituents desorbed to Tenax in the feed soil and treated soil sampled immediately after feeding (d0), but was significantly lower in treated soil sampled at d1 and d7 during the 7-d treatment cycle (Figure 5.7b). The relative LD_{50} of the constituents desorbed to Tenax in treated soil sampled at d1 and d7 was not significantly different from 1.0 (Figure 5.7b).

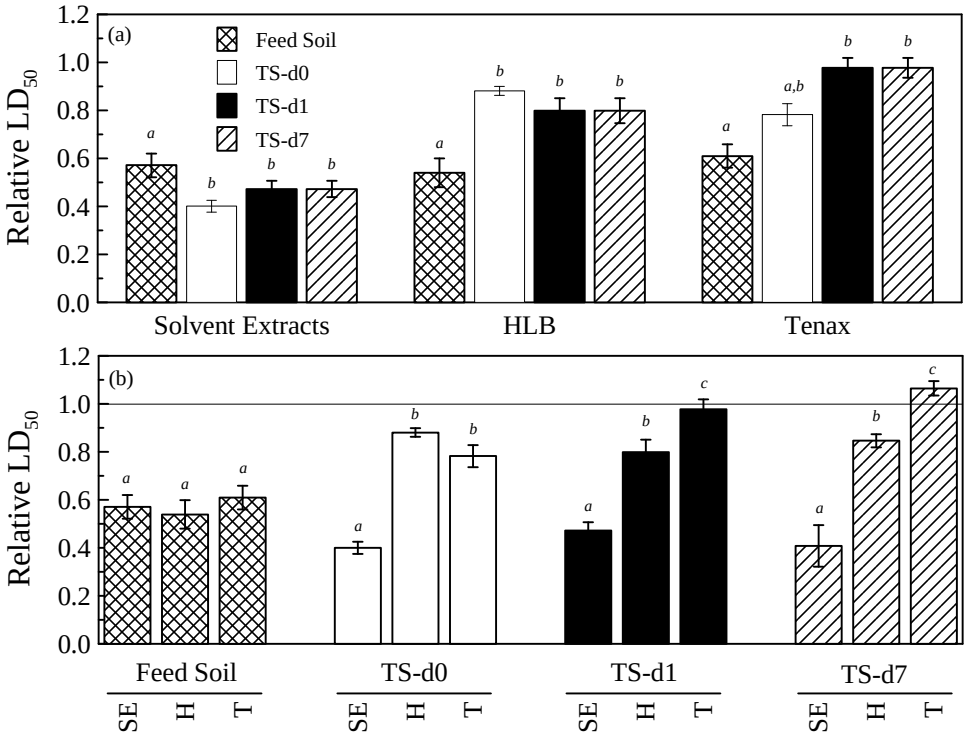


Figure 5.7 Relative LD_{50} of solvent extracts (SE) and constituents desorbed to HLB (H) and Tenax (T) of soils. TS-d0, TS-d1 and TS-d7 represent treated soil sampled immediately after feeding, 1 d and 7 d after feeding during 7-d feeding cycle, respectively. Different letters are assigned to conditions for which there was significant difference ($p < 0.05$). Data are organized by extract type in panel (a) and by sample type in panel (b).

5.4. Discussion

Solid phase extraction using Tenax beads is a commonly used technique to estimate and predict bioavailability of organic pollutants in contaminated soils and sediments (Ten Hulscher et al. 2003; Harwood et al. 2012). In previous work (Hu et al. 2012), we reported that treatment in a lab-scale bioreactor led to an increase in genotoxicity of the soil. Because PAH metabolites and other products of aerobic bacterial metabolism can be more polar than the parent compounds, we were concerned about relying on hydrophobic Tenax beads to evaluate the potential bioavailability of such products. Thus, the mixed-function Oasis HLB resin, a macroporous copolymer made from lipophilic divinylbenzene monomer and hydrophilic N-vinylpyrrolidone monomer (Buchberger

2007), was also employed in this study. However, because of the hydrophilic monomer in HLB resin and its small particle size, the conventional method of mixing the sorbent with soil slurry directly is not applicable for HLB resin. Thus, we developed the dialysis-tubing desorption method. Results indicate that this method is valid for determining desorption of PAHs and oxy-PAHs from soil. First, the SnakeSkin dialysis tubing did not sorb PAHs or oxy-PAHs (data not shown) and the overall recovery of PAHs and oxy-PAHs ranged from 90% to 110%. Second, desorption of 14 PAHs and four oxy-PAHs from soil to Tenax beads or to HLB resin using the dialysis-tubing desorption method was not significantly different from desorption to Tenax beads using the conventional slurry-based desorption method (Figure 5.2).

Bioreactor treatment significantly removed PAHs and oxy-PAHs from the contaminated soil (Figure 5.3). In soils and sediments, the desorbability of PAHs is believed to be one of the major factors influencing the extent of biodegradation (Luthy et al. 1997; Lei et al. 2004). Some studies have suggested that fast-desorbing or total desorbing fractions of a hydrophobic contaminant can be used to predict bioavailability and, correspondingly, the achievable endpoint of bioremediation (Cornelissen et al. 1998; Hawthorne et al. 2001; Braida et al. 2004; Lei et al. 2004). However, we found that the percentage of both PAHs and oxy-PAHs removed at the end of the bioreactor treatment cycle exceeded the percentage desorbed from the feed soil (Figure 5.5). This phenomenon was also observed for PAHs in a study on the same soil using laboratory columns to simulate in situ bioremediation (Richardson and Aitken 2011). We observed that the oxy-PAHs 9,10-phenanthrenequinone and 9,10-anthraquinone were more biodegradable and desorbable than their parent compounds phenanthrene and anthracene, but that

9-fluorenone was less biodegradable and desorbable than the parent compound fluorene. The differences in desorbability could at least partially explain the differences in biodegradability between these oxy-PAHs and their respective parent compounds. However, we also observed that benz[*a*]anthracene-7,12-quinone was more desorbable but less biodegradable than the parent compound benz[*a*]anthracene, which suggests that microbial factors rather than bioavailability limited the biodegradation of benz[*a*]anthracene-7,12-quinone when compared to benz[*a*]anthracene.

Biological treatment decreased the toxicity of both desorbable and non-desorbable compounds in the soil; comparing LD_{50} values, the toxicity mainly came from the non-desorbable constituents (Figure 5.6). For the desorbable compounds, the constituents that desorbed to HLB resin were more toxic than the constituents that desorbed to Tenax beads in the feed soil and in the treated soil immediately after feeding (d0), but less toxic in the treated soil sampled at d1 and d7 (Figure 5.6). These results suggest that HLB resin and Tenax beads sorb different categories of compounds, although their ability to sorb 14 PAHs and four oxy-PAHs analyzed in this study was similar (Figure 5.2). Moreover, the toxic compounds targeted by HLB resin were removed to a greater extent during the bioreactor treatment cycle than the toxic compounds targeted by Tenax beads (Figure 5.6).

Biological treatment slightly increased the genotoxicity of the whole soil, but it significantly reduced the genotoxicity of the desorbable fractions, especially the constituents desorbed to Tenax (which were not genotoxic at all after 1 d treatment during the 7-d treatment cycle; Figure 5.7). Consistent with our previous work (Hu et al. 2012), these results indicate that bioremediation generated genotoxins, but it appears that the

genotoxins were not desorbable. It is interesting that the desorbability of putative PAH metabolites such as oxy-PAHs also decreased during the bioreactor treatment cycle (Figure 5.4); however, the increased genotoxicity resulting from treatment cannot be attributed to the four oxy-PAHs we analyzed because there was a net removal of these compounds during treatment (Figure 5.3).

The findings from this work have potential implications for the influence of bioremediation on overall risk to human health and to ecological receptors. Bioremediation can clearly lead to the formation of products that are more genotoxic than the original constituents in a contaminated soil, which presumably can lead to increased risk upon exposure to the soil. However, if the genotoxic constituents in treated soil are less desorbable than those in untreated soil, they may be less bioavailable, with a corresponding decrease in overall risk. Further work is necessary to elucidate the nature of genotoxic constituents resulting from bioremediation as well as their bioavailability to relevant receptors.

6. CONCLUSIONS AND RECOMMENDATIONS

6.1. Conclusions

The overall goal of this dissertation was to investigate the effects of various bioremediation strategies on the bioavailability and toxicity/genotoxicity of contaminants in PAH-contaminated soil. Conclusions for the specific aims of this research project are outlined below:

Aim 1: Evaluate desorption of polycyclic aromatic hydrocarbons from field-contaminated soil to a two-dimensional hydrophobic surface before and after bioremediation.

The mass of PAHs desorbed from soil to a 2-D hydrophobic surface increased with increasing soil loading beyond a soil loading corresponding to monolayer coverage of the surface. This finding suggested that PAHs were transferred from soil to the hydrophobic surface by mechanisms other than direct soil contact. Such mechanisms were reinforced by observations that desorption occurred even with dry or moist glass microfiber filters placed between the hydrophobic surface and the soil. Desorption of all PAHs was substantially reduced at a soil moisture content corresponding to field capacity, suggesting that transport through pore air contributed to PAH transport from soil to the hydrophobic surface. Soil loading effects on PAH desorption to the hydrophobic surface was influenced by temperature, but the influences depended on the specific PAH. The lower-molecular-weight PAHs had greater potential to desorb from soil than higher-molecular-weight PAHs. Desorption kinetics of PAHs from soil to hydrophobic

surface or Tenax beads were described well by an empirical two-compartment kinetic model. Bioremediation was an effective approach to remove PAHs from the contaminated soil. Moreover, it seemed to eliminate the most readily desorbable fraction of all PAHs and substantially decreased the potential dermal bioavailability of PAHs in contaminated soil. Overall, this study demonstrated that dermal exposure assessment from soil should consider site-specific conditions that could influence the bioavailability of hydrophobic contaminants to skin. Furthermore, the effects of bioremediation on potential dermal exposure should consider not only the reduction in contaminant concentration but also the reduction in contaminant bioavailability.

Aim 2: Evaluate the effects of bioremediation on genotoxicity of polycyclic aromatic hydrocarbon-contaminated soil using genetically engineered, higher eukaryotic cell lines.

Both the bioreactor system and the column system significantly removed PAHs from the PAH-contaminated soil, but the effect of bioremediation on toxicity and genotoxicity of the soil is complicated. Generally, bioreactor treatment resulted in an increase in toxicity and genotoxicity over the course of a treatment cycle, whereas long-term column treatment resulted in a decrease in toxicity but increase in genotoxicity. When screened with a battery of DT40 mutants, column-treated soils showed sensitivity in more mutants than the untreated soil. This observation suggested that more types of DNA damage were induced by bioremediation. Detailed genotoxicity profiling indicated the likelihood that bioremediation generated genotoxic compounds causing oxidative stress, which might be attributed to the formation of oxy-PAHs during incomplete biodegradation. In order to track the sources of toxicity in the soil throughout bioremediation processes, correlation analyses were performed. Although the correlation between toxicity and total PAH

concentration was significant, the partial correlation between toxicity and total PAH concentration was poor and insignificant, when controlling for the effects of total organic residue. This finding suggests that organic compounds other than PAHs present in the soil were also responsible for the total toxicity and genotoxicity of PAH-contaminated soil undergoing bioremediation. Overall, effects of bioremediation on toxicity and gentoxicity of PAH-contaminated soil was complicated, but toxicity and genotoxicity bioassays can be an effective supplement to chemical analysis-based risk assessment for the contaminated soil.

Aim 3: Evaluate the bioavailability of (geno)toxic contaminants in PAH-contamianted soil from a former MGP site before and after biological treatment.

A dialysis-tubing desorption method was demonstrated to be valid for determining desorption of PAHs and oxy-PAHs from soil to Tenax beads or to HLB resin. The method recovery of 14 target PAHs and four target oxy-PAHs ranged from 90% to 110%. Moreover, desorption of 14 PAHs and four oxy-PAHs from soil to Tenax beads and to HLB resin using the dialysis-tubing desorption method was not significantly different from that to Tenax beads using the conventional Tenax-bead desorption method. Biological treatment significantly removed both PAHs and oxy-PAHs, and the desorbability of both PAHs and oxy-PAHs descreased throughout the bioreactor treatment cycle. Collectively, oxy-PAHs were more desorbable and biodegradable than PAHs (although fewer oxy-PAHs than PAHs were evaluated). For both PAHs and oxy-PAHs, the percentage removed in the bioreactor significantly exceeded the percentage desorbed from the untreated soil; contrary to suggestions by other investigators, this finding suggests that desorption is not always a good predictor of the bioremediation potential for

soil contaminated with PAHs. Toxicity of the whole soil slightly decreased after biological treatment. However, genotoxicity of the whole soil slightly increased after biological treatment, which presumably can lead to an increased risk to humans or to ecosystem upon exposure to the soil. Both toxicity and genotoxicity of the desorbable constituents in the soil decreased after treatment, suggesting that any genotoxic constituents that may have formed during treatment were primarily associated with less accessible domains in the soil, which might lead to a decrease in overall risk. Bioavailability should be incorporated into the evaluation of contaminated soils to reduce the extent of cleanup required to that which is necessary to be protective of human health and the environment, and to balance the risks caused by remediation activities as well as the risks reduced by remediation activities.

6.2. Recommendations for future work

First, for future studies, reconstructed human skin should be employed to further investigate dermal bioavailability of contaminants in the PAH-contaminated soil before and after bioremediation. In this dissertation, factors that could influence the dermal uptake have already been elucidated by using a 2-D hydrophobic surface as a surrogate for skin. However, skin is a complex matrix containing both hydrophobic and hydrophilic compartments as well as metabolic enzymes. A uniformly hydrophobic surface might overestimate the flux of PAHs from soil to the skin surface and/or neglect the metabolism of PAHs by skin. In order to include site-specific conditions into dermal exposure assessment for site risk evaluation, exposure of contaminated soil to viable human skin should be further studied.

Second, ecotoxicity assays such as seed a germination assay, earthworm toxicity

assay, algal survival assay and fish toxicity assay should be employed to study the effects of bioremediation on potential receptors in an ecosystem. In this dissertation, the DT40 DNA damage response analysis was used to determine the gentoxicity of the soil throughout a bioremediation process from a human-health perspective. However, the impact of contaminated soil and the associated bioremediation process should be considered not only to humans but also to the ecosystem. The above ecotoxicity assays would provide an insight into the influence of bioremediation on a terrestrial system and on an aquatic system.

Third, further research is needed to isolate, characterize, and quantify the toxic and genotoxic compounds in the contaminated soil as remediation progresses. This dissertation demonstrated that different bioremediation strategies could lead to different outcomes of toxicity and genotoxicity for PAH-contaminated soil. However, the reason behind this difference is not well understood. Although certain enhanced DNA damage was observed over the bioremediation process, the compounds that were responsible for the increased toxicity and genotoxicity were not elucidated. Bioassay-directed chemical analysis might be a helpful tool to further track and identify the chemicals causing toxicity and genotoxicity during the bioremediation process. Only when the sources of toxicity and genotoxicity are identified, can a bioremediation process be further improved to not only reduce the amount of target contaminants but also reduce the risk to humans and to ecosystems.

APPENDIX A. SUPPORTING INFORMATION FOR CHAPTER 3

Table A.1 Methods used to measure the properties of the source soil.

Method	Reference
Total organic matter	(Lukasewycz and Burkhard 2005)
Soil moisture content	(ASTM 1999a)
Field capacity	(Cassel and Nielsen 1986)
Bulk density	(Arshad et al. 1996)
Soil particle density	(ASTM 1999b)

Table A.2 Concentrations of PAHs in source soil and treated soil from a laboratory bioreactor (n = 3).

	Soil Concentration (µg/g dry soil)	
	Source Soil	**Treated Soil**
naphthalene (NAP)	33.3±0.8	16.9±0.6
acenaphthene (ACE)	22.4±0.6	4.9±0.3
fluorene (FLU)	16.9±0.5	1.8±0.2
phenanthrene (PHN)	322±5.1	15.9±1.3
anthracene (ANT)	22.2±0.3	1.9±0.2
fluoranthene (FLA)	66.0±2.7	10.6±0.6
pyrene (PYR)	121±0.05	16.5±0.9
benz[*a*]anthracene (BaA)	36.2±0.6	13.3±0.8
chrysene (CHR)	35.4±0.5	8.3±0.5
benzo[*b*]fluoranthene (BbF)	24.2±0.1	5.7±0.4
benzo[*k*]fluoranthene (BkF)	15.6±0.1	2.2±0.2
benzo[*a*]pyrene (BaP)	39.1±0.3	5.7±3.6
dibenz[*a,h*]anthracene (DBA)	3.3±0.1	1.3±0.1
benzo[*g,h,i*]perylene (BgP)	23.0±0.2	16.5±1.3
Total PAHs	781±10	121±8

Table A.3 PAH mass desorbed to C18 disks after 12 repeated soil loadings of the same C18 disk. Each time, 100 mg/cm^2 fresh source soil (soil moisture content 2%, temperature 20 ºC) was loaded onto the C18 disk for 24 h.

	PAH mass desorbed to C18 disk (µg)
naphthalene (NAP)	9.1±0.3
acenaphthene (ACE)	50.9±2.4
fluorene (FLU)	32.3±2.1
phenanthrene (PHN)	181±7
anthracene (ANT)	4.7±0.2
fluoranthene (FLA)	21.0±0.3
pyrene (PYR)	28.3±0.9
benz[*a*]anthracene (BaA)	0.92±0.03
chrysene (CHR)	N.D. [a]
benzo[*b*]fluoranthene (BbF)	3.4±0.2
benzo[*k*]fluoranthene (BkF)	0.09±0.01
benzo[*a*]pyrene (BaP)	N.D. [a]
dibenz[*a,h*]anthracene (DBA)	N.D. [a]
benzo[*g,h,i*]perylene (BgP)	N.D. [a]
Total PAHs	332±10

[a] N.D. is not detected.

Table A.4 PAH recovery (%) for desorption to C18 disks and to Tenax beads.

	C18 disks	Tenax beads	
	Source Soil	**Source Soil**	**Treated Soil**
naphthalene (NAP)	73±5	91±2	84±20
acenaphthene (ACE)	90±5	104±3	99±4
fluorene (FLU)	94±5	95±3	100±7
phenanthrene (PHN)	99±6	91±4	106±7
anthracene (ANT)	103±8	87±6	83±18
fluoranthene (FLA)	92±7	92±5	105±4
pyrene (PYR)	94±5	87±3	90±18
benz[*a*]anthracene (BaA)	105±6	92±3	86±17
chrysene (CHR)	88±17	93±4	90±18
benzo[*b*]fluoranthene (BbF)	81±5	92±4	96±17
benzo[*k*]fluoranthene (BkF)	79±9	87±9	92±10
benzo[*a*]pyrene (BaP)	80±10	90±2	109±22
dibenz[*a*,*h*]anthracene (DBA)	107±4	98±5	94±3
benzo[*g*,*h*,*i*]perylene (BgP)	88±7	89±18	91±23
Total PAHs	94±6	91±3	94±15

Table A.5 Fitted parameter values for models representing desorption from source soil (SS) or biologically treated soil (BS) to Tenax beads or from source soil to C18 extraction disks at 20 ºC [a,b].

PAH [b]	f_r			k_r (d^{-1})			k_s (d^{-1})			r^2		
	SS-Tx [c]	SS-C18 [d]	BS-Tx [e]	SS-Tx [c]	SS-C18 [d]	BS-Tx [e]	SS-Tx [c]	SS-C18 [d]	BS-Tx [e]	SS-Tx [c]	SS-C18 [d]	BS-Tx [e]
NAP	0.16±0.01	0.08±0.03	0	1.31±0.10	39.1±8.0	0	0.004±0.001	0.002±0.001	0.003±0	0.98	0.83	0.95
ACE	0.68±0.01	0.53±0.01	0	2.01±0.09	16.3±1.9	0	0.026±0.002	0.027±0.005	0.004±0	0.98	0.94	0.90
FLU	0.69±0.01	0.62±0.02	0	2.21±0.10	5.65±0.60	0	0.023±0.002	0.030±0.008	0.005±0	0.98	0.96	0.93
PHN	0.44±0.01	0.24±0.01	0	1.60±0.11	5.51±0.47	0	0.013±0.002	0.001±0.001	0.008±0	0.97	0.97	0.96
ANT	0.52±0.01	0.37±0.02	0	1.88±0.14	0.88±0.10	0	0.024±0.002	0.007±0.003	0.007±0	0.97	0.99	0.97
FLA	0.44±0.01	0.22±0.02	0	1.33±0.09	0.44±0.08	0	0.012±0.002	0.001±0.002	0.007±0	0.98	0.97	0.97
PYR	0.39±0.02	0.13±0.05	0	1.11±0.12	0.83±0.09	0	0.010±0.002	0.001±0.001	0.006±0	0.95	0.99	0.98

[a] Desorption from biologically treated soil to C18 disks was not detectable for any PAH.

[b] Desorption from source soil to C18 disks was less than 2% for B*a*A, CHR, B*b*F, B*k*F, B*a*P, DBA and B*g*P.

[c] Desorption to Tenax beads from source soil.

[d] Desorption to C18 disks from source soil at 2% moisture content and soil loading of 50 mg/cm^2.

[e] Desorption to Tenax beads from biologically treated soil. The fitted f_r was not significantly different from 0 for the two-site model, so regression was performed assuming simple first-order one-site desorption kinetics (f_r = 0, k_r = 0).

Table A.6 Selected properties of 14 target PAHs [a, b].

Compound	**MW**	p_i^*	H	$\log K_{ow}$
naphthalene (NAP)	128.18	1.04E+01	4.30E+01	3.37
acenaphthene (ACE)	154.20	3.00E-01	1.22E+01	3.92
fluorene (FLU)	166.23	9.00E-02	7.87E+00	4.18
phenanthrene (PHN)	178.24	2.00E-02	3.24E+00	4.57
anthracene (ANT)	178.24	1.00E-03	3.96E+00	4.54
fluoranthene (FLA)	202.26	1.23E-03	1.04E+00	5.22
pyrene (PYR)	202.26	6.00E-04	9.20E-01	5.18
benz[*a*]anthracene (BaA)	228.30	2.80E-05	5.81E-01	5.91
chrysene (CHR)	228.30	5.70E-07	6.50E-02	5.86
benzo[*b*]fluoranthene (BbF)	252.32	2.18E-07	2.14E-02	5.75
benzo[*k*]fluoranthene (BkF)	252.32	5.20E-08	1.60E-02	6.00
benzo[*a*]pyrene (BaP)	252.32	7.00E-07	4.60E-02	6.04
dibenz[*a,h*]anthracene (DBA)	276.34	1.69E-08	7.50E-02	6.50
benzo[*g,h,i*]perylene (BgP)	278.36	3.70E-10	1.28E-03	6.75

[a] abbreviations: MW = molecular weight (g/mol); p_i^* = vapor pressure (Pa); H = Henry's Law Constant ($Pa \cdot m^3/mol \cdot K$); K_{ow} = octonol/water partitioning coefficient.

[b] All data from Mackay, D., Shiu, W.Y., Ma, K.C., 1992. Illustrated Handbook of Physical and Environmental Fate for Organic Compounds. Lewis Publishers, Chelsey, MI. (Mackay et al. 1992)

Figure A.1 Total PAHs desorbed from source soil to C18 extraction disks as a function of time. Soil moisture content was 2%, temperature 20 ºC. Results for triplicate C18 disks are shown at each time point.

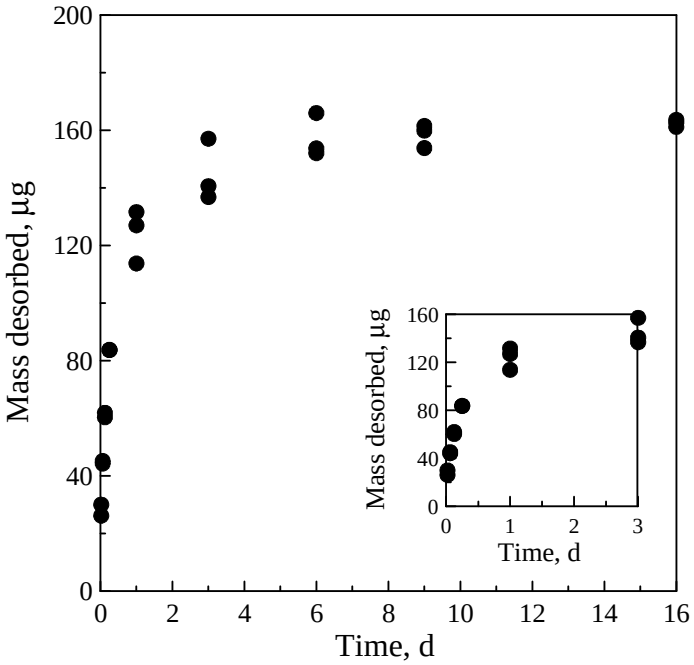


Figure A.2 Effect of temperature on desorption of individual PAHs to C18 disks as a function of soil loading after 6 d contact time. Soil moisture content was 2%. Desorption was less than 0.5 µg each for benz[*a*]anthracene (B*a*A), chrysene (CHR), benzo[*b*]fluoranthene (B*b*F), benzo[*k*]fluoranthene (B*k*F), benzo[*a*]pyrene (B*a*P), dibenz[*a,h*]anthracene (DBA), and benzo[*g,h,i*]perylene (B*g*P) for all soil loadings at all three temperatures. Note that the legend is at the lower right.

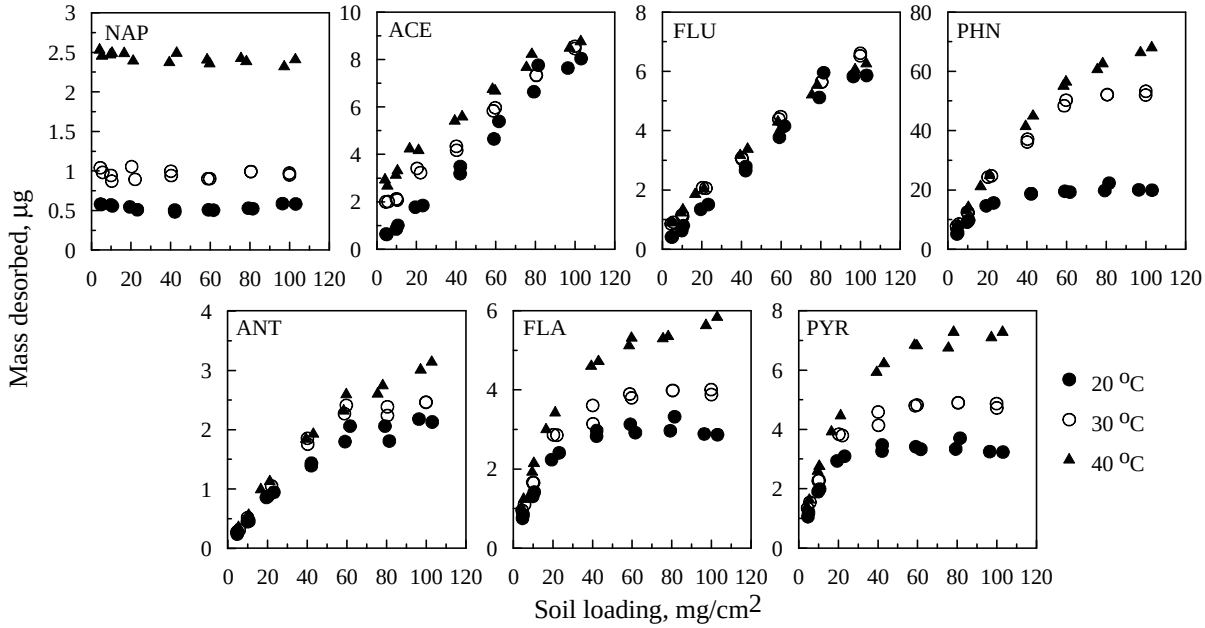
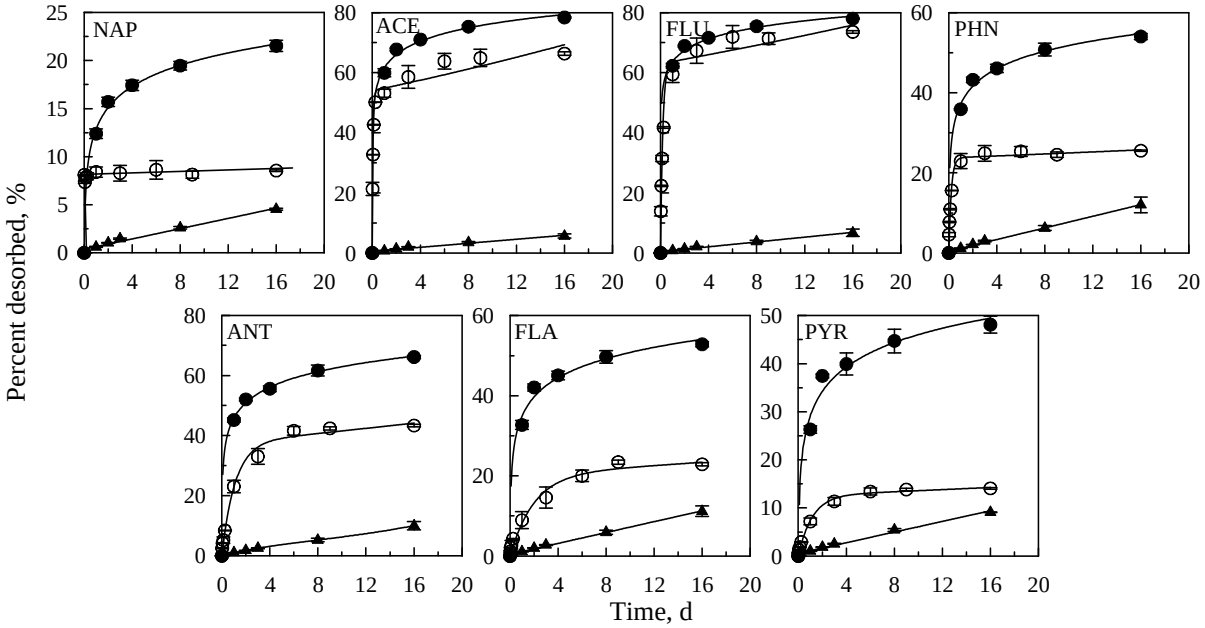


Figure A.3 Cumulative desorption of selected PAHs from source soil to Tenax beads (●), from source soil to C18 extraction disks (○), or from biologically treated soil to Tenax beads (▲) at 20 ºC. Lines represent best fits to the first-order two-site desorption model for source soil and to a simple first-order one-site desorption model for biologically treated soil.



APPENDIX B. SUPPORTING INFORMATION FOR CHAPTER 4

Table B.1 Concentrations of individual PAHs in the soil before and after two bioremediation processes (ng/mg dry soil) (n = 3).

Compound	BFS[a]	BTS[a] (Day 7)	CPS[a]	CTR[a] (Port A)	BIO[a] (Port A)
NAP	22.3±2.5	16.5±0.1	12.2±0.2	6.4±0.1	10.4±0.9
ACE	22.2±2.2	1.8±0.2	11.3±2.5	2.5±0.4	2.8±0.4
FLU	15.2±1.7	2.5±0.2	6.3±1.6	2.1±0.7	1.7±0.2
PHN	226±17	50.1±14.4	129±45	41.7±5.8	27.2±0.3
ANT	9.1±1.0	2.0±0.3	11.9±1.2	4.3±1.2	2.3±0.5
FLA	55.8±6.9	11.5±1.9	42.9±0.5	17.6±3.8	9.1±2.3
PYR	80.9±5.2	25.4±4.6	63.4±7.1	24.7±5.9	17.1±3.8
BaA	36.4±4.3	12.1±0.3	18.6±2.8	12.4±4.2	5.8±1.3
CHR	34.6±3.8	17.8±2.3	27.4±2.8	18.2±3.9	7.2±0.2
BbF	13.4±0.7	8.3±0.8	11.8±0.3	7.4±2.4	4.8±0.8
BkF	10.8±1.4	6.8±0.8	8.7±1.1	5.4±1.4	3.2±0.6
BaP	13.7±1.3	8.4±1.5	13.8±1.6	11.4±3.2	7.2±1.6
DBA	1.9±0.1	1.3±0.1	0.78±0.02	0±0	0±0
BgP	23.1±2.8	13.5±0.9	10.6±0.8	7.2±2.9	6.4±1.4
Total PAHs	566±50	178±20	369±54	161±38	105±12

[a] BFS: untreated bioreactor feed soil; BTS: bioreactor treated soil; CPS: untreated column packing soil; CTR: control-column treated soil; BIO: biostimulated-column treated soil.

Table B.2 Table of LD_{50} for BPDE, MMS and H_2O_2 as positive control (µg/L) (n = 3).

	LD_{50}(DT40)	LD_{50}(*Rad54*$^{-/-}$)
BPDE	49.6±8.5	27.0±2.7
MMS	$7.1\times10^3\pm1.5\times10^3$	$1.7\times10^3\pm1.7\times10^3$
H_2O_2	61.2±8.5	34.7±3.4

Table B.3 Partial correlation coefficients and corresponding *p*-values among LD_{50}, $1/C_{tPAHs}$ and $1/C_{residue}$.

	LD_{50}(DT40)	LD_{50}(*Rad54*$^{-/-}$)
$1/C_{tPAHs}$ (Control Variable: $1/C_{residue}$)	0.464 (p=0.08)	0.482 (p=0.07)
$1/C_{residue}$ (Control Variable: $1/C_{tPAHs}$)	0.789[a] (p=7×10^{-4})	0.836[a] (p=1×10^{-4})

[a] Partial correlation is significant at p<0.05.

LD_{50} calculation method

LD_{50} is calculated based on the dose-response relation as follows:

$$\ln(R_{survival}) = a + b \cdot C_{exposure-residue} \quad \text{(Equation B1)}$$

where, $C_{exposure\text{-}residue}$ is the exposure concentration of residue (μg/mL); $R_{survival}$ is the cell survival relative to vehicle control (%); *a* and *b* are fitting parameters.

For each residue sample, cells were exposed to 6 concentrations, thus generating 6 survival percentage values. The exposure concentration and the obtained cell survival percentage data were used to fit Equation B1 to obtain the values of fitting parameters *a* and *b*. After *a* and *b* values were obtained, $LD_{50\text{-}residue}$ was calculated as follows:

$$LD_{50-residue} = (\ln 0.5 - a)/b \quad \text{(Equation B2)}$$

$LD_{50\text{-}residue}$ obtained from Equation B2 is in terms of residue dose (μg residue/mL). It was converted to $LD_{50\text{-}soil}$ in terms of soil dose (mg soil/mL) as follows:

$$LD_{50-soil} = LD_{50-residue} / C_{residue/soil} \quad \text{(Equation B3)}$$

where, $C_{residue/soil}$ is the residue mass produced per unit soil (μg residue/mg soil).

Test of benzo[*a*]pyrene metabolic activation by DT40 cell lines

The DT40 system has not been tested previously for its ability to activate compounds that require metabolic activation before exerting a genotoxic effect. Therefore, we evaluated the potential for metabolic activation by exposing the DT40 parental cell line and its mutant *Rev3*$^{-/-}$ to benzo[*a*]pyrene (BaP). According to unpublished data from Dr. Nakamura's lab, *Rev3*$^{-/-}$ is sensitive to benzo[*a*]pyrene diolepoxide (BPDE), BaP's ultimate carcinogenic metabolite.

The DT40 and *Rev3*$^{-/-}$ were exposed to BaP using the method as described in Ridpath et al. (Ridpath et al. 2011). The results are shown in Figure B.1. A paired-sample t-test was applied to determine the significant differences of cell survival rate between the DT40 and *Rev3*$^{-/-}$. The survival rate of *Rev3*$^{-/-}$ was significantly lower ($p < 0.05$) than that of the DT40 parental cell line. Therefore, BaP could cause DNA damage response in *Rev3*$^{-/-}$, which indicates that DT40 cells may have metabolic activation capacity for PAHs.

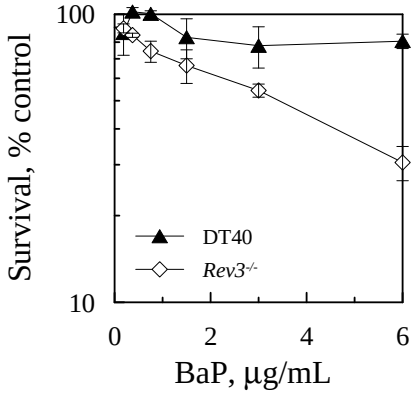


Figure B.1 Cell survival of DT40 parental cells and mutant *Rev3*$^{-/-}$ exposed to benzo[*a*]pyrene.

APPENDIX C. SUPPORTING INFORMATION FOR CHAPTER 5

Table C.1 Concentrations of PAHs and oxy-PAHs in the feed soil and treated soils. Data are means and standard deviations of triplicates.

Compound	Feed soil, µg/g soil	TS-d0[a], µg/g soil	TS-d1[a], µg/g soil	TS-d7[a], µg/g soil
naphthalene (NAP)	14.2 ± 1.6	15.4 ± 0.2	16.4 ± 0.1	17.9 ± 0.0
acenaphthene (ACE)	7.5 ± 0.8	5.2 ± 0.2	3.7 ± 0.2	2.6 ± 0.8
fluorene (FLU)	4.7 ± 0.5	1.4 ± 0.0	1.2 ± 0.0	1.1 ± 0.0
phenanthrene (PHN)	84.9 ± 11.2	52.6 ± 4.4	39.8 ± 0.1	33.7 ± 1.8
anthracene (ANT)	5.3 ± 0.2	3.0 ± 0.1	2.5 ± 0.2	2.2 ± 0.1
fluoranthene (FLA)	27.0 ± 10.6	28.9 ± 0.6	21.6 ± 4.0	20.1 ± 2.2
pyrene (PYR)	65.6 ± 6.4	39.5 ± 0.2	30.2 ± 0.9	26.9 ± 4.2
benz[*a*]anthracene (BaA)	33.7 ± 1.6	7.7 ± 0.1	6.5 ± 0.6	6.2 ± 0.0
chrysene (CHR)	40.4 ± 6.4	21.2 ± 0.5	19.4 ± 2.4	18.4 ± 2.6
benzo[*b*]fluoranthene (BbF)	12.6 ± 0.8	8.0 ± 0.3	7.3 ± 1.0	7.1 ± 1.4
benzo[*k*]fluoranthene (BkF)	7.2 ± 0.4	4.0 ± 0.1	3.6 ± 0.4	3.5 ± 0.6
benzo[*a*]pyrene (BaP)	32.0 ± 4.1	20.4 ± 0.2	18.9 ± 2.3	18.8 ± 3.6
dibenz[*a,h*]anthracene (DBA)	3.2 ± 0.1	0.7 ± 0.0	0.7 ± 0.1	0.6 ± 0.1
benzo[*g,h,i*]perylene (BgP)	23.3 ± 4.2	21.1 ± 0.4	20.3 ± 0.1	20.9 ± 0.3
Total PAHs	362 ± 23.0	229 ± 4.8	192 ± 12.6	180 ± 16.2
9-fluorenone (FLO)	1.4 ± 0.1	1.1 ± 0.0	0.6 ± 0.0	0.6 ± 0.1
9,10-phenanthrenequinone (PQ)	0.9 ± 0.1	0.5 ± 0.0	0.3 ±0.1	0.3 ± 0.0
9,10-anthraquinone (AQ)	18.3 ± 0.4	8.8 ± 0.2	6.1 ±0.7	4.7 ± 0.1
benz[*a*]anthracene-7,12-quinone (BAQ)	1.7 ± 0.1	1.0 ± 0.0	0.9 ±0.0	0.8 ± 0.0
Total oxy-PAHs	22.3 ± 0.5	9.9 ± 0.3	7.0 ±0.7	5.5 ± 0.1

[a] TS-d0, TS-d1 and TS-d7 represent treated soil sampled immediately after feeding, 1 d and 7 d after feeding during 7-d feeding cycle.

Table C.2 Recovery (%) of PAHs and oxy-PAHs for the dialysis-tubing desorption method.[a] Data are means and standard deviations (n = 3).

Compound	HLB [b]	Tenax [b]
naphthalene (NAP)	104.3 ± 2.1	109.1 ± 1.5
acenaphthene (ACE)	88.7 ± 18.5	100.3 ± 3.0
fluorene (FLU)	82.7 ± 7.0	80.5 ± 11.2
phenanthrene (PHN)	99.1 ± 2.6	111.8 ± 2.3
anthracene (ANT)	92.8 ± 2.5	83.7 ± 6.4
fluoranthene (FLA)	104.8 ± 0.8	102.4 ± 15.5
pyrene (PYR)	86.1 ± 8.7	84.9 ± 5.6
benz[*a*]anthracene (BaA)	98.7 ± 1.2	91.7 ± 11.5
chrysene (CHR)	92.2 ± 0.8	88.9 ± 9.1
benzo[*b*]fluoranthene (BbF)	80.3 ± 0.8	87.4 ± 11.5
benzo[*k*]fluoranthene (BkF)	84.8 ± 0.7	90.1 ± 6.0
benzo[*a*]pyrene (BaP)	94.3 ± 0.7	85.0 ± 17.0
dibenz[*a,h*]anthracene (DBA)	106.2 ± 0.1	104.9 ± 2.4
benzo[*g,h,i*]perylene (BgP)	81.8 ± 3.1	93.0 ± 13.9
Total PAHs	93.6 ± 1.1	95.7 ± 0.7
9-fluorenone (FLO)	81.8 ± 3.3	100.0 ± 12.5
9,10-phenanthrenequinone (PQ)	84.3 ± 12.9	80.3 ± 4.2
9,10-anthraquinone (AQ)	116.1 ± 5.4	103.9 ± 8.6
benz[*a*]anthracene-7,12-quinone (BAQ)	83.7 ± 1.2	86.1 ± 6.0
Total oxy-PAHs	110.2 ± 5.2	101.4 ± 8.2

[a] Feed soil was used; desorption time was 7 d; Tenax beads or HLB resin were used as sorbents.

Figure C.1 LD_{50} of solvent extracts of soils before (SE) and after desorption to HLB (H-SE) or Tenax (T-SE) for parental DT40 cell line and its *Rad54*$^{-/-}$ mutant. TS-d0, TS-d1 and TS-d7 represent treated soil sampled immediately after feeding, 1 d and 7 d after feeding during 7-d feeding cycle, respectively. No significant differences ($p > 0.05$) of LD_{50} for DT40 or *Rad54*$^{-/-}$ were observed among the solvent extracts of each sample before or after desorption to HLB or to Tenax.

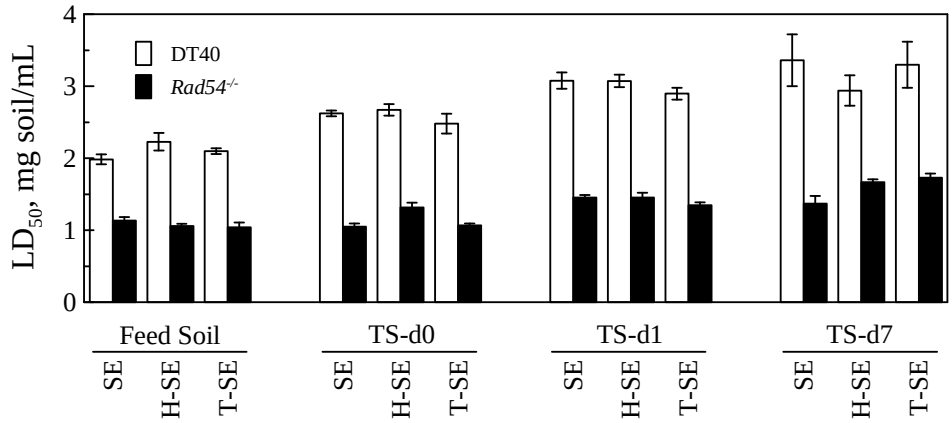
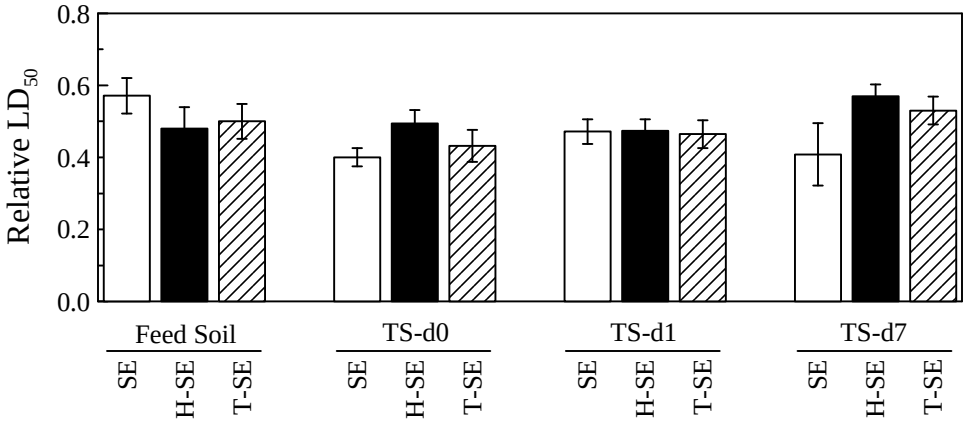


Figure C.2 Relative LD_{50} of solvent extracts of soils before (SE) and after desorption to HLB (H-SE) or Tenax (T-SE). TS-d0, TS-d1 and TS-d7 represent treated soil sampled immediately after feeding, 1 d and 7 d after feeding during 7-d feeding cycle, respectively. No significant differences ($p > 0.05$) of relative LD_{50} were observed among solvent extracts of each sample before or after desorption to HLB or to Tenax.



Oxy-PAHs analysis by LC-MS/MS

9-fluorenone (FLO), 9,10-phenanthrenequinone (PQ), 9,10-anthraquinone (AQ) and benz[a]anthracene-7,12-dione (BAQ) were analyzed by liquid chromatography-tandem mass spectrometry (LC-MS/MS). LC-MS/MS analysis was performed using a Thermo Scientific TSQ Quantum Ultra Triple Quadrupole Mass Spectrometer (Thermo Fisher Scientific, Waltham, MA, USA) with a Thermo Scientific Atmospheric Pressure Chemical Ionization (APCI) source, a Waters Acquity UPLC system (Waters Corporation, Milford, MA, USA), and a Waters BEH C_{18} UPLC Column (1.7 µm, 2.1 x 50 mm).

The column temperature was maintained at 35°C, and the sample injection volume was 10 µL. The mobile phase consisted of deionized water and LC-MS- grade methanol. The mobile phase was started at a flow rate of 0.294 mL/min with 30% methanol for 2.07 minutes followed by a linear increase to 75% methanol over 2.72 minutes and was held at 75% methanol for 2.95 minutes. Then at a flow rate of 0.5 mL/min, the mobile phase was increased linearly to 100% methanol over 0.45 minutes and held at 100% methanol for 3.81 minutes. Finally, at a flow rate of 0.294 mL/min, the mobile phase was decreased linearly to 30% methanol over 0.20 minutes and held at 30% methanol for 2.8 minutes.

AQ and BAQ were quantified in APCI negative ionization mode using the molecular ions *m/z*=208 and *m/z*=258, respectively. PQ and FLO were quantified in APCI positive ionization mode using the precursor to fragment ion transition *m/z*= 209 > 152 and *m/z*= 181 > 153, respectively. AQ and PQ standards were purchased from Sigma Aldrich (St. Louis, MO, USA). FLO and BAQ standards were purchased from Acros Organics (Morris Plains, NJ, USA). Quantification of each compound was performed according to corresponding standard curve. The method detection limit is 6.88 µg/L for FLO, 1.69

µg/L for PQ, 21.2 µg/L for AQ and 3.87 µg/L for BAQ

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