IMPROVING PAH BIODEGRADATION IN CONTAMINATED SOIL BY ADDING SURFACTANT AFTER CONVENTIONAL BIOLOGICAL TREATMENT

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A dissertation submitted to the faculty at the University of North Carolina at Chapel Hill in partial fulfillment of the requirements for the degree of Doctor of Philosophy in the Department of Environmental Sciences and Engineering in the Gillings School of Global Public Health.

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ABSTRACT

Alden C. Adrion: Improving PAH Biodegradation in Contaminated Soil by Adding Surfactant After Conventional Biological Treatment
(Under the direction of Michael D. Aitken)

Polycyclic aromatic hydrocarbons (PAHs) are a class of compounds which occur frequently at polluted sites and are known or suspected to be toxic and genotoxic. Bioremediation is one option for the treatment of PAH-contaminated soil, but PAHs are often strongly bound to soil and may be unavailable to degrading microorganisms. Limited PAH desorption can be particularly problematic for weathered soils or soils which have undergone treatment but fail to meet cleanup goals. Additionally, bioremediation does not always lead to a reduction in soil (geno)toxicity. This dissertation addresses these limitations by screening nonionic surfactants enhanced desorption and biodegradation of residual PAHs remaining in soil after conventional slurry-phase bioreactor-treatment. Surfactant doses were chosen to be below the critical micelle concentration in the soil-slurry system. The effect of surfactant-amended treatment on soil (geno)toxicity was also evaluated. The best performing surfactant was selected for use in a second-stage bioreactor to evaluate the reproducibility of surfactant-amended treatment and to investigate the effect of varying residence time.

In screening experiments, further treatment of the soil with all surfactants resulted in modest increases in PAH desorption. Four out of 5 surfactants increased PAH biodegradation relative to further treatment without surfactant. The most effective surfactants significantly enhanced the biodegradation of 5 of the 7 PAHs considered probable human carcinogens by the

Environmental Protection Agency (individual PAH removals up to 80%). Further treatment without surfactant significantly reduced the genotoxicity of the soil, while treatment with surfactant had varying effects. Increased PAH removal, however, did not always coincide with a reduction in soil toxicity and genotoxicity.

For the two-stage bioreactor system using polyoxyethylene sorbitol hexaoleate surfactant, substantial amounts of the PAHs and oxygenated-PAHs remaining after conventional bioreactor-treatment were removed in the second stage, including more than 80% of residual 4-ring PAHs. The most substantial PAH removal in the second stage occurred within the first week of treatment. Surfactant-amended treatment consistently made the soil less cytotoxic, but in most trials increased the genotoxicity of the soil. With further optimization of the treatment system, surfactant-enhanced treatment may increase the applicability of bioremediation as a means of meeting soil remediation goals.

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CHAPTER 1: INTRODUCTION

Polycyclic aromatic hydrocarbons (PAHs) are a class of compounds of environmental and public health concern because of their known or suspected toxicity and genotoxicity and frequent occurrence at contaminated sites (ATSDR 1995). The Agency for Toxic Substances and Disease Registry (ATSDR) and the United States Environmental Protection Agency (US EPA) have consistently ranked PAHs among the top ten contaminants of concern at sites on the National Priorities List (NPL). This ranking takes into account not only toxicity, but also frequency and potential for human exposure at NPL sites (ATSDR 2011). Over 600 current NPL sites are contaminated with PAHs, of which over 400 contain PAH-contaminated soil (EPA 2015). PAH-contaminated soil poses a human health risk because of the potential for direct human exposure to the soil, leaching of PAHs into surface- or groundwater, volatilization of PAH, and the transport of PAH-contaminated particles through the air (ATSDR 1995).

Bioremediation is a "process whereby organic wastes are biologically degraded under controlled conditions to an innocuous state, or to levels below concentration limits established by regulatory authorities" (Mueller 1996). A 2007 EPA report on treatment technologies states that as of 2005, out of 145 remediation projects treating PAH contaminated source material, 37 utilized bioremediation (EPA 2007). Bioremediation of contaminated soil can be conducted in-situ by stimulating biological degradation in the intact soil, or ex-situ by stimulating biodegradation in excavated soil. One advantage of ex-situ bioremediation is an increased ability to control conditions such as temperature, pH, moisture, and nutrient content as well as provide mechanical mixing and chemical amendments in an engineered system such as a bioreactor.

Between 2009 and 2011 at least three additional NPL sites having PAH-contaminated soil selected ex-situ bioremediation as a cleanup method (EPA 2013).

Although ex-situ bioremediation can be utilized as a cleanup strategy, it is not without limitations. Two fundamental limitations of bioremediation include incomplete removal of the target PAH (Aitken and Long 2004) and the potential to increase soil (geno)toxicity (Hughes *et al.* 1998; Gillespie *et al.* 2007; Lemieux *et al.* 2009; Hu *et al.* 2012). One factor limiting removal can be the slow desorption of PAHs from soil compartments, reducing the availability of PAH to degrading microorganisms. The addition of surfactants during bioremediation can enhance the bioavailability of PAHs to degrading microorganisms, as reviewed in Makkar and Rockne (2003), Li and Chen (2009), Elliot *et al.* (2011) and in Section 2.3 of this dissertation. The purpose of the research described in this dissertation was to investigate the effect of low-dose surfactant addition on the ex-situ bioremediation of PAH-contaminated soil from a former manufactured-gas plant (MGP) site which had already undergone conventional bioreactor treatment. The effects of second-stage surfactant-amended treatment on PAH removal and soil (geno)toxicity were evaluated.

1.1 Specific aims and rationale

Aim 1: Evaluate the effect of conventional bioreactor treatment on the removal and bioavailability of oxy-PAHs in contaminated soil.

Oxygenated-PAHs (oxy-PAHs) are of concern because some are known to exhibit toxic or genotoxic effects (Bolton *et al.* 2000; Zielinska-Park *et al.* 2004; Luo *et al.* 2011), and may also inhibit the biodegradation of parent PAHs (Kazunga and Aitken 2000; Kazunga *et al.* 2001). The goal of Aim 1 was to develop a liquid chromatography-mass spectrometry (LC-MS) method to measure the concentrations of four oxy-PAHs in solvent-extracts of soil and solid-phase-extraction resins provided by the former student, Jing Hu. This method was used to determine

the removal and desorbable fractions of oxy-PAHs during bioreactor treatment. The LC-MS method was also used to evaluate the effect of surfactant-amended treatment on the removal of oxy-PAHs in Aim 3. The desorbable fraction of oxy-PAHs remaining after conventional bioreactor treatment measured in Aim 1 was compared with the fraction of oxy-PAHs removed during second-stage treatment in Aim 3.

Aim 2: Screen nonionic surfactants for enhanced biodegradation of PAHs remaining in soil after conventional bioreactor treatment.

The efficacy of bioremediation for soil contaminated with PAHs may be limited by the fractions of soil-bound PAHs that are less accessible to PAH-degrading microorganisms. Although surfactant addition to contaminated soil has been suggested as a means of enhancing the biodegradation of hydrophobic contaminants such as PAHs, most studies do not articulate that the concept is most relevant to the fraction of a given compound that is relatively non-desorbable or non-bioaccessible. This would be the case for the PAHs remaining after conventional bioreactor treatment has removed the most bioaccessible fraction. Aim 2 was to screen nonionic surfactants at the test-tube scale for their ability to enhance desorption and biodegradation of PAHs remaining after conventional slurry-phase bioreactor treatment. Five surfactants of similar hydrophobicity but with different chemical structures were selected for screening. Surfactant doses (mg surfactant/g dry soil) were selected to correspond to concentrations less than the critical micelle concentration in the soil-slurry system (sub-CMC doses). Sub-CMC doses were evaluated based on evidence of their effectiveness (Zhu and Aitken 2010) and the reduced field-scale cost of lower doses. Since bioremediation does not always lead to a reduction in soil (geno)toxicity, the effect of surfactant-amended treatment on soil

(geno)toxicity was also evaluated using the DT40 DNA damage response assay (reviewed in Section 2.5.1).

Aim 3: Implement second-stage treatment of PAH-contaminated soil in a surfactant-amended bioreactor and evaluate its performance.

As a step towards demonstrating the feasibility of surfactant-amended ex-situ bioremediation in the field, based on the results of Aim 2, one surfactant was selected for use during second-stage treatment of soil in a bench-scale bioreactor. Second-stage reactors were operated for a total of six cycles to evaluate the reproducibility of surfactant-amended treatment and the effects of residence time on PAH and oxy-PAH removal and on soil (geno)toxicity.

1.2 Dissertation organization

There are six chapters in this dissertation. Chapter 1 provides an introduction and briefly explains the aims of the dissertation. Chapter 2 is a literature review which provides background helpful to understanding the work. Chapters 3, 4, and 5, provide additional introductory material on and describe the methods and results of Aims 1, 2, and 3, respectively. Chapter 3 documents my contribution to the published work, Hu *et al.* (2014). Chapters 4 and 5 are manuscripts intended for publication. Chapter 5 makes reference to the work in Chapter 4 by citing Adrion *et al.* (in prep.).

CHAPTER 2: BACKGROUND

2.1 PAH-contaminated sites

PAHs are a class of compounds made up of two or more fused benzene rings and/or pentacyclic moieties in linear, angular and cluster arrangements (Mueller 1996). This structure affords thermodynamic stability due to electron delocalization (resonance). PAHs are hydrophobic, characterized by low aqueous solubilities, and typically associated with particles or surfaces in the environment. These properties can make PAHs resistant to natural attenuation processes such as volatilization, photolysis and biodegradation (Mueller 1996). Although there are natural sources of PAH, as well as diffuse deposition of atmospheric anthropogenic PAHs, Mueller et al. (1996) point out that contamination of soil, sediment, and groundwater are primarily the result of four point source materials: coal tar, creosote, petroleum, and industrial effluents. The primary anthropogenic source of coal tar is the gasification of coal, which has historically occurred at manufactured-gas plants. Creosote is distilled from coal tar and used as a wood preservative at wood-preserving sites. These contaminated sites are often also contaminated with benzene, toluene, ethylbenzene and xylene (BTEX), heavy metals and aliphatic hydrocarbons (Bamforth and Singleton 2005), heterocyclic aromatic compounds (HAC), alkylated-PAH, and oxy-PAH (Lundstedt *et al.* 2003). The US EPA regulates 16 PAHs as priority pollutants, and site cleanup goals are typically based on the concentration of these priority PAHs (Aitken and Long 2004). Additionally, the US EPA Ecological Monitoring and Assessment Program (EMAP) recommends quantifying 34 PAHs in sediments (Burgess 2009).

2.1.1 Distribution of hydrophobic organic contaminants in geosorbents

Hydrophobic organic contaminants (HOC) such as PAHs are capable of interacting with environmental materials such as soil and sediment. Luthy *et al.* (1997) refer to such materials as geosorbents, which comprise discrete domains. Domains are defined based on their more specific and homogeneous properties. HOC interaction with geosorbents can include partitioning of HOCs into the bulk geosorbent or its pores, or onto the external surface of a geosorbent. Thermodynamic and kinetic properties can be assigned to each interaction between an HOC and a given geosorbent. Thermodynamic properties describe the extent to which these interactions occur at equilibrium, while kinetic properties describe the rate at which the interactions occur under non-equilibrium conditions.

The simplest model derived from thermodynamic considerations is linear partitioning, expressed as a partition constant (K'_{HOC12}) as defined in equation 1. The ideal partition constant describes the equilibrium distribution of an HOC between any two thermodynamically uniform phases (typically the aqueous phase and geosorbent).

$$\frac{x_{HOC_1}}{x_{HOC_2}} = K'_{HOC_{12}} = e^{\frac{-\Delta G_{HOC_{12}}}{RT}} \qquad (1)$$

Where x_{HOC1} and x_{HOC2} are the mole fractions of HOC in phase 1 and in phase 2 respectively. ΔG_{HOC12} is the free energy of transfer of HOC from phase 1 to phase 2, R is the universal gas constant, and T is the absolute temperature in Kelvin. The major assumption underlying equation 1 is the existence of a single ΔG_{HOC12} of constant value which applies to the system. Very often the geosorbent of interest does not comprise a single thermodynamically uniform phase with which the HOC maintains a single type of interaction over all mole fractions. The distribution of interest is actually the equilibrium distribution between the aqueous phase and the multiple thermodynamically distinct phases of the geosorbent, characterized by various types of

interactions, each having its own free energy of transfer. Under these non-ideal conditions, K'_{HOC12} is instead referred to as a partition coefficient (Schwarzenbach *et al.* 2005).

Replacing mole fractions with more environmentally relevant units, equation 1 can be represented as a linear sorption isotherm.

$$C_s = K_p C_w \tag{2}$$

Where C_s is the concentration of HOC associated with the geosorbent (typically in mass HOC/mass geosorbent), C_w is the aqueous phase HOC concentration, and K_p is a geosorbent-water partition coefficient (whose units depend on the units used for the other variables). Assuming constant K_p, equation 2 describes a linear relationship between the concentration of HOC partitioned into and onto the soil and the concentration in the aqueous phase. Very often the relationship is non-linear because the geosorbent comprises numerous phases and the system is not well characterized by a constant free energy of transfer.

In order to account for this non-linearity, a model incorporating multiple geosorbent domains is employed. The total equilibrium concentration of HOC in the geosorbent can be represented by equation 3.

$$C_s = \frac{1}{m_s} \sum_{j=1}^{n} m_j C_j \tag{3}$$

Where m_s is the total mass of geosorbent and m_j is the mass of domain j. Individual $m_j C_j$ can be modeled using linear and non-linear equations, but m_j, which usually cannot be determined experimentally, is factored into a system-specific coefficient. Empirical formulas used to model non-linear geosorbent/HOC partitioning include the Freundlich and Langmuir isotherms, and numerous composite models. These formulas make use of adjustable parameters which are determined by fitting experimental data (Mechlińska *et al.* 2009).

The Freundlich isotherm, equation 4, accounts for the theory that the free energy of transfer from aqueous phase to geosorbent can be a function of equilibrium aqueous phase HOC concentration (Schwarzenbach *et al.* 2005).

$$C_s = K_F C_w^n \tag{4}$$

The Freundlich constant, K_f, and the Freundlich exponent, n, are determined by fitting experimental data.

The Langmuir isotherm, equation 5, accounts for the theory that there can be a limited number of geosorbent surface sites onto which an HOC can sorb (Schwarzenbach *et al.* 2005).

$$C_s = \frac{S^0 b C_w}{1+bC_w} \tag{5}$$

S^0 represents the total number of surface sites per mass geosorbent and b is a Langmuir constant. Both S^0 and b are determined by fitting experimental data.

The dual-mode sorption model, equation 6, states that the equilibrium geosorbent concentration is the sum of HOC in a domain modeled by a linear isotherm and a domain modeled by a Langmuir isotherm (Xing *et al.* 1996).

$$C_s = K_p C_w + \frac{S^0 b C_w}{1+bC_w} \tag{6}$$

This model reflects the theory discussed later that the sorptive behavior of soil and sediment is dominated by two hypothetical domains.

From the standpoint of evaluating HOC levels in a contaminated geosorbent over time (for example PAH-contaminated soil in an aqueous bioreactor slurry), it makes sense to frame the kinetic model in a form that describes the fraction of HOC remaining in the geosorbent after a given time, t. A mass balance for a batch system describing the rate of desorption, r_{des}, can be written for the mass HOC present in the geosorbent with equation 7.

$$r_{des} = \frac{dm_{HOC}}{dt} = \sum_{j=1}^{n} \frac{dm_{HOC_j}}{dt} \quad (7)$$

Where m_{HOC} is the total mass HOC in the geosorbent, m_{HOCj} is the mass of HOC in the j^{th} domain of the geosorbent, and n is the number of domains to be included in the model.

Researchers have proposed or applied models limited to two geosorbent domains and water (Schrap *et al.* 1994). The HOC desorbs quickly from one domain and slowly from the other domain as depicted in Figure 2.1 and equation 8.

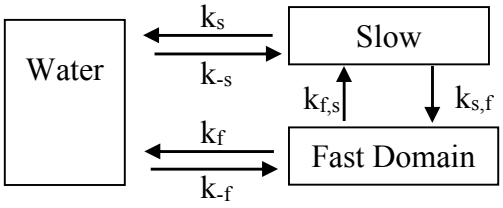

Figure 2.1 Two-domain kinetic model of HOC desorption.

$$\frac{dC_{HOC}}{dt} = \frac{d\left(\frac{m_{HOC_s}}{m_{geo}}\right)}{dt} + \frac{d\left(\frac{m_{HOC_f}}{m_{geo}}\right)}{dt} = \frac{dC_{HOC_s}}{dt} + \frac{dC_{HOC_f}}{dt} \quad (8)$$

Where C_{HOC} is the concentration of HOC in the geosorbent, m_{HOCs} is the mass of HOC in the slow domain, m_{HOCf} is the mass of HOC in the fast domain, and m_{geo} is the total mass of geosorbent. If one assumes that desorption is characterized by first order rate constants and that transfer between the two domains is negligible, then equation 8 can be expressed as equation 9.

$$\frac{dC_{HOC}}{dt} = -k_s F_s C_{HOC} + k_{-s} C_w - k_f F_f C_{HOC} + k_{-f} C_w \quad (9)$$

Where F_s and F_f are the fractions of total geosorbent-bound HOC in the slow and fast domains, respectively and k_s, k_{-s}, k_f, and k_{-f} are the rate constants for adsorption and desorption as described in Figure 2.1. Furthermore, assuming that desorption is irreversible or that C_w is kept at or near zero and assuming F_s and F_f are constant and equal to their initial values, after

separating variables and integrating, equation 10 describes the fraction of HOC remaining in the geosorbent after a certain amount of time (Cornelissen *et al.* 1997).

$$\frac{C_{HOC_t}}{C_{HOC_0}} = F_s e^{-k_s t} + F_f e^{-k_f t} \quad (10)$$

Where C_{HOC0} is the initial concentration of HOC in the geosorbent.

2.1.2 Specific geosorbent domains relevant to PAH-contaminated soil

The domains comprising a geosorbent can be divided into two general categories, organic and inorganic. Organic material is derived from living matter typically containing high amounts of carbon, while inorganic material comprises metals, minerals and rocks, typically containing much less carbon. Organic material can be further divided into soil organic matter and organic material of anthropogenic origin. The relative importance of these materials with respect to PAH distribution and desorption rates will depend on factors such as the source of PAH contamination, geographic location, and "age" of contaminated soil (duration of contamination). Partitioning of hydrophobic compounds such as PAH onto water-wet mineral surfaces will be characterized by fast sorption/desorption kinetics and low mineral/water partition coefficients; therefore, sorption of hydrophobic compounds in soil is dominated by organic domains (Luthy *et al.* 1997).

Luthy *et al.* (1997) describe the three major components in PAH-contaminated soil as soil organic matter (SOM), combustion residue, and non-aqueous-phase liquids (NAPL). Soil organic matter is material derived from biomolecules. Through diagenesis the biomolecules are degraded and cross-linked to form humic material. Humic material is further transformed through metamorphism into kerogen, coal, and graphite. The effect of soil organic matter on PAH sorption depends on properties of the material, which in turn depend on biomolecule origin and the extent of diagenesis/metamorphism. Partition coefficients for sorption of PAH to SOM have

been shown to increase with increasing SOM aromaticity and decreasing polarity (Liu *et al.* 2002). A series of papers by Weber *et al.* (1992) and work by Xia and Pignatello (2001) suggest that SOM can be described as having two domains, soft (or "rubbery") and hard (or "glassy"), with which HOC interact. As an approximation, the soft domain is primarily represented by the linear portion of the dual-mode sorption model, while the hard domain is represented by the Langmuir portion (Luthy *et al.* 1997; Mechlińska *et al.* 2009). Similarly, desorption of PAH from the soft domain is represented by the fast-desorbing fraction, while desorption of PAH from the hard domain is represented by the slow-desorbing fraction (Luthy *et al.* 1997).

Besides SOM, the organic fraction of PAH-contaminated soil, especially soil directly impacted with source materials (e.g., MGP sites, petroleum spills, wood-preserving plants), often contains combustion residue and NAPL with which PAH are associated (Aitken and Long 2004). Combustion residue is solid material remaining after the incomplete combustion of organic material. One type of combustion residue is soot or black carbon, resulting from the combustion of hydrocarbons. There is evidence that models including a separate black carbon domain more accurately predict PAH geosorbent/water partition coefficients than do models only including a total organic carbon domain (Accardi-Dey and Gschwend 2003; Cornelissen *et al.* 2005). Hawthorne *et al.* (2007), however, evaluating 114 historically contaminated sediments, found that sediment/water partition coefficients were not better predicted by black carbon content than by total organic carbon alone. Although an effort was made to account for the presence of NAPL in sediment samples (visual inspection of samples), material other than black carbon (such as NAPL) was implicated in PAH partitioning.

Common NAPL found at PAH-contaminated sites include coal tar and creosote, and petroleum products such as oil or diesel fuel (Mueller 1996). Birak and Miller (2009) describe tar

as a mixture of a large number of mostly (>90% by mass) organic aromatic compounds, some containing as many as 210 rings. Haeseler *et al.* (1999) profiled the total organic matter of soil from former MPG sites and compared it to that of coal tar. They concluded that the contamination at MGP sites originated from coal tar, with nearly all PAH mass associated with a heavy fraction of the organic matter which could not be dissolved in organic solvent. PAH-containing NAPL has also been observed coating the surfaces of soil particles from aged creosote- and petroleum-contaminated sites (Karimi-Lotfabad and Gray 2000).

Luthy *et al.* (1997) liken the differences between the sorptive behavior of soft and hard SOM to the differences between the sorptive behavior of NAPL and combustion residue. As an approximation, the NAPL is primarily represented by the linear portion of the dual-mode sorption model, while the combustion residue is represented by the Langmuir portion (Luthy *et al.* 1997; Mechlińska *et al.* 2009). Similarly, desorption of PAH from the NAPL domain is represented by the fast-desorbing fraction, while desorption of PAH from the combustion residue is represented by the slow-desorbing fraction (Luthy *et al.* 1997).

2.2 Bioavailability and bioaccessibility

Bioavailability and bioaccessibility are terms defining the extent to which a chemical is in a state permitting interaction with living organisms. For the purpose of this review, bioavailable and bioaccessible are defined as described by Semple *et al.* (2004),with specific focus on bioavailability/accessibility of PAH in a soil/water system to PAH-degrading bacteria. Based on the definitions proposed by Semple *et al.* (2004), the bioavailable fraction of PAH is the fraction of PAH mass in a system which is freely available to cross the cellular membrane of an organism from the medium the organism inhabits at a given time. The bioaccessible fraction of PAH, however, is the fraction of PAH mass which has the *potential* to become bioavailable during a time span of interest given the physical and chemical conditions of the system. Bacteria

in a soil/water system are either attached to soil particles or suspended in the aqueous phase. Experiments designed to measure bioaccessibility assume that bacteria are only capable of degrading HOC which desorb from the soil into the aqueous phase which, as described in Section 2.2.2, might not be a reasonable assumption. Under this scenario the bioavailable fraction of PAH is the fraction of PAH mass in a soil-water system which is in the aqueous phase, while bioaccessible fraction of PAH mass in a soil-water system is the mass which has the potential to enter the aqueous phase under given a set of conditions.

2.2.1 Measurements of bioavailability and bioaccessibility

The bioavailable fraction of a PAH can be determined by measuring the instantaneous aqueous-phase PAH concentration. This concentration, however, may be exceedingly small and methods such as passive sampling have been developed to determine the time-averaged bioavailable concentration (Cachada *et al.* 2014). The bioaccessible fraction, sometimes considered to be the desorbable or fast-desorbing fraction, can be determined using solid phase extraction (SPE) from soil water (Cachada *et al.* 2014). An SPE resin such as Tenax® or XAD® added to the system is capable of rapidly and quantitatively adsorbing all PAHs that desorb from the soil to the aqueous phase (serving as an "infinite sink" for desorbed compounds), keeping aqueous-phase PAH concentration near zero. The mass of PAH sorbed to the resin after a certain time is the bioaccessible mass for that time period under the experimental conditions (particularly aqueous-phase PAH concentration near zero). The fast-desorbing fraction, F_f, can be determined by non-linear regression of equation 10 (Zhu *et al.* 2008). A less kinetically rigorous estimate of the desorbable fraction can be made by visual inspection of the asymptote in an empirically produced graph such as that represented in Figure 2.2 (Hu *et al.* 2014). An aqueous-phase PAH concentration of zero is maintained in order to simulate the instantaneous and complete biological uptake of PAH that would occur under ideal biodegradation conditions.

In this way, the ideal desorption rate can be determined and compared to the actual (often non-ideal) rate of biodegradation. Other methods have been used to approximate the bioaccessible fraction, including mild solvent extraction, subcritical water extraction, supercritical fluid extraction, solubilizing agents, and persulfate oxidation (Cachada *et al.* 2014) . These methods, however, do not directly measure desorption to the aqueous phase under conditions similar to bioremediation.

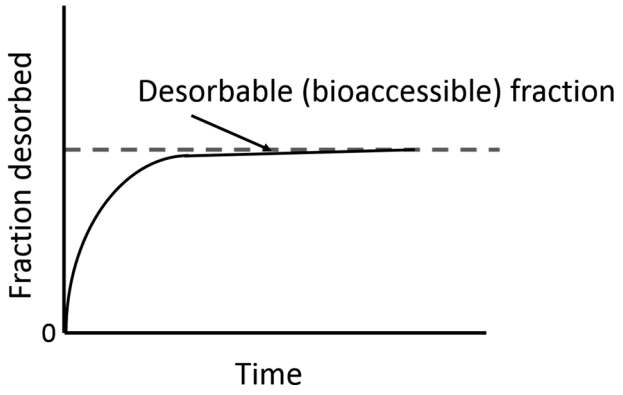

Figure 2.2 Idealized PAH desorption curve.

2.2.2 Effect of bioavailability/accessibility on bioremediation of PAH-contaminated soil

Although there are biological factors which can limit the extent of microbial biodegradation, it has been hypothesized that bioavailability can limit biodegradation under certain conditions. During biological treatment of PAH-contaminated soil it has been observed that at times biodegradation rates are nearly equal to ideal desorption rates, suggesting that under those circumstances the rate of biodegradation may be limited to the rate of desorption (Cornelissen *et al.* 1998; Huesemann *et al.* 2004). Under such a scenario, the microbial community of the system maintains an *underutilized* ability to degrade PAHs and desorption is believed to be the rate-limiting step of PAH removal.

Others have noted, however, that the extent of PAH biodegradation can exceed the extent of desorption measured with an infinite-sink method (Richardson and Aitken 2011; Hu *et al.*

2014), although the mechanisms by which this can occur are not well understood. Evidence suggests that bacteria can enhance PAH desorption by adhering to hydrophobic contaminant matrices or through the production of biosurfactants (Mukherji and Ghosh 2012). The rate of PAH mass transfer from geosorbent to adherent cells or biofilm may be faster than the rate of PAH mass transfer into the bulk aqueous phase. It is also possible that the smaller distances between bacteria and geosorbent or the ability of bacteria to enter small soil pores may cause a steeper concentration gradient than can be created with SPE resins.

2.3 Surfactants

Surfactants are amphiphilic molecules comprising a polar (hydrophilic) "head" and non-polar (hydrophobic) "tail" as depicted in Figure 2.3. This amphiphilic nature causes surfactants to accumulate at the interface of polar and non-polar materials, such as the interface between aqueous phase and air or between aqueous phase and a non-polar geosorbent. Surfactants are often classified based on the charge of the hydrophilic "head": cationic, anionic, zwitterionic, or nonionic. Surfactants can also be classified based on origin: synthetic or biogenic. The hydrophile-lipophile balance (HLB) is a number assigned to a surfactant based on its chemical structure. Surfactants having higher HLB numbers are more hydrophilic while surfactants with low HLB numbers are more hydrophobic (Rosen 1989). Hydrophobic "tails" are typically branched or straight-chain alkanes. In order to maximize energetically favorable hydrogen bond interactions, water excludes the hydrophobic tails of surfactant molecules, which tend to aggregate on the surface of relatively non-polar geosorbent particles. An important result of this accumulation at the interface is the lowering of the free energy of the two-phase system, reducing the energy required to create new interfacial area (Myers 1992). Lowering the interfacial energy (sometimes referred to as interfacial tension) can increase the equilibrium

interfacial surface area and lower energetic barriers to dispersion, two important factors which may enhance the rate of HOC desorption from geosorbents.

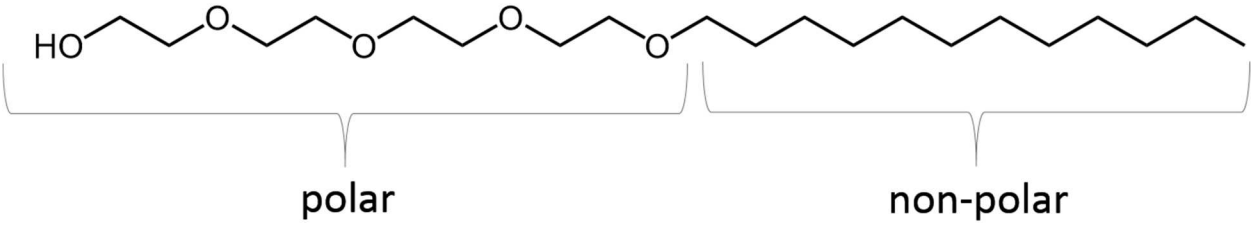

Figure 2.3 Representative surfactant molecule (Brij 30).

2.3.1 The critical micelle concentration

Above a certain aqueous-phase surfactant concentration, known as the critical micelle concentration (CMC), the vast majority of additional surfactant added to the system will form micelles and the concentration of surfactant monomers will remain relatively constant. Micelles in water are aggregates of surfactant in which the hydrophobic tails face inward and the hydrophilic heads face outward as depicted in Figure 2.4. Mukerjee and Mysels (1971), however, note "the expression critical micelle concentration… is slightly misleading because of the use of the singular noun 'concentration'". The actual onset of micelle formation will occur over a concentration range, the width of which depends on factors such as polydispersivity (formation of micelles comprising a variable number of monomers) and purity. Especially for commercial surfactants, which are often mixtures of molecules having different tail or head lengths, successive micellization of polydisperse monomers results in a wide monomer-micelle transition zone (Aboul-Kassim and Simoneit 2001). All that can be said with certainty is that above this range the vast majority of additional surfactant is in micelle form.

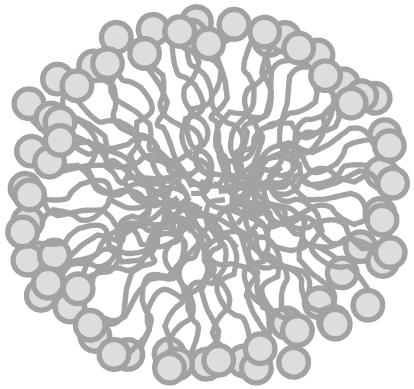

Figure 2.4 Cross section of surfactant micelle.

2.3.2 Sorption of surfactant to geosorbent

Surfactants can interact with geosorbents in much the same way as HOC interact with geosorbent. Equilibrium distribution of surfactant monomer between aqueous phase and geosorbent has been described by both the Langmuir and Freundlich isotherms (Laha *et al.* 2009). Since geosorbents can comprise many domains, the kinetics of surfactant sorption will be geosorbent-specific. Equilibrium, based on approximately constant aqueous-phase surfactant concentration, appears to be reached on the order of hours to days (Liu *et al.* 1992). For a well-mixed aqueous slurry of MGP soil, sorption equilibrium of a nonionic surfactant was determined to be reached after 48 hours (Aitken *et al.* 1998; Zhu and Aitken 2010). It is possible, however, that continued sorption into domains characterized by much smaller rate constants went unobserved due to experimental design or analytical limitations.

2.3.3 Effect of surfactant on PAH desorption

Surfactants may increase the rate of PAH desorption through two mechanisms: micellar solubilization and direct modification of the contaminant matrix. The first will occur only in the presence of micelles, while the second may occur at concentrations above or below the CMC. Micellar solubilization refers to the partitioning of HOC molecules into the hydrophobic core of

surfactant micelles. Micellar solubilization enhances the rate of desorption in much the same way SPE can be said to enhance desorption rates (Section 2.2.1), by maximizing the concentration gradient at the soil water interface. Through solubilization, surfactants have been shown to increase the apparent solubility of PAHs in soil-free systems at concentrations above the CMC (Edwards *et al.* 1991). In addition to maximizing the concentration gradient, if there is a direct interface between micelle and soil, then partitioning directly from soil into micelle may occur. Grimberg *et al.* (1995) described the direct transfer of phenanthrene from a solid phase into micelles that form at the solid-aqueous interface.

Two hypothesized effects of surfactants on the contaminant matrix include increased PAH diffusivity and increased geosorbent-aqueous phase interfacial surface area. Diffusivity is a bulk property of the geosorbent, which is directly proportional to the rate of HOC diffusion within the geosorbent. Yeom *et al.* (1996) suggested that for PAH-contaminated MGP soil, surfactants can sorb to and penetrate the coal tar matrix, causing swelling and an increase in PAH diffusivity. The hydrocarbon tail, in particular, may intercalate between coal tar aromatic groups to cause swelling of the coal tar.

Increased geosorbent-aqueous phase interfacial surface area can result from the dispersion of geosorbent. Dispersion as it applies to soil and its components refers to the breaking-up of particles and the distribution of the finer particles in the aqueous medium. The dispersion of non-polar solids and liquids such as black carbon and NAPL requires the creation of interfacial area, which is made more energetically favorable by the sorption of surfactant at the interface. Increased particle surface area could increase the rate of PAH desorption. Surfactants have been shown to enhance the dispersion of hydrophobic solid and liquid particles at aqueous-phase concentrations both above and below the reported CMC (Kile and Chiou 1989;

Zhang and Miller 1992; Churchill *et al.* 1995). Kile and Chiou (1989), however, attribute the increased dispersion to the successive micellization below the reported CMC.

Addition of surfactant can modify the wetting behavior of geosorbent domains. Wettability describes the tendency of a liquid to spread over a surface and is often described by the contact angle, θ, as depicted in Figure 2.5. Sorption of surfactant to a NAPL droplet may reduce the wetting of the soil or mineral surface by the NAPL. Dong *et al.* (2003) showed that the contact angle between coal tar and quartz under aqueous phase could be increased by the addition of surfactant. Once the contact angle is sufficiency increased, adhesive forces are weak enough that the NAPL droplet can be dislodged by the abrasive forces of convective currents, a process known as rollback (Deshpande *et al.* 1999). The dislodged droplet has a greater NAPL-aqueous phase interfacial surface area through which PAH may desorb.

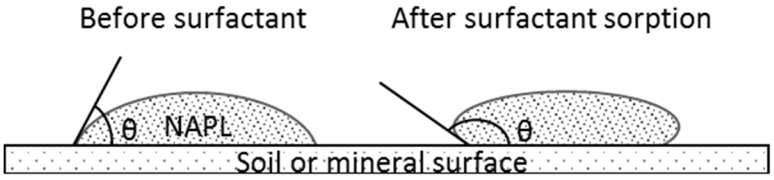

Figure 2.5 Rollback of NAPL.

There is evidence, however, that addition of surfactant, particularly cationic surfactants or nonionic surfactants with high HLB values, at aqueous-phase concentrations below the CMC may increase the HOC soil/water partition coefficient (Edwards *et al.* 1994; Sun *et al.* 1995; Lee *et al.* 2000; Zhou and Zhu 2005). Hydrophilic surfactants may have a greater affinity for mineral surfaces than for organic surfaces (Lee *et al.* 2000; Rodríguez-Cruz *et al.* 2005; Zhou and Zhu 2007). In particular, cationic surfactants can bind ionically to negatively charged surfaces, such as those found in clays (Jones-Hughes and Turner 2005; Wang and Keller 2008). Since HOC are likely associated with organic domains, these more hydrophilic surfactants may not be efficiently acting on the domains relevant to PAH sorption. Instead, the accumulation of surfactant on these

mineral surfaces increases the organic carbon content of the mineral domain, increasing PAH sorption to the surface. The soil/water partition coefficient may begin to decrease only after the CMC is reached and PAH begin partitioning into micelles.

2.3.4 Effect of surfactant on bioremediation of PAH-contaminated field soil

Review articles on the subject of surfactant-enhanced bioremediation indicate that the effects of surfactant on PAH biodegradation in aqueous solutions, spiked soil systems, and field-contaminated soil systems are mixed (Makkar and Rockne 2003; Li and Chen 2009; Elliot *et al.* 2011). Systems of pure PAH in aqueous solution or even spiked PAH in clean soil may provide more conclusive explanations of experimental results because there are fewer unknown variables than in experiments using field-contaminated soil. Experiments using aged, contaminated field soil, however, provide direct information on the applicability of surfactant-enhanced bioremediation in a complex system.

Work using field-contaminated soil indicates that surfactant addition is most beneficial for systems in which PAH biodegradation is limited by bioaccessibility. Studies in which surfactant-free controls exhibit high PAH removal tend to demonstrate no effect or even inhibition of PAH removal as a result of surfactant addition (Deschenes *et al.* 1996; Kim and Weber 2005; Lei *et al.* 2005; Zhu and Aitken 2010; Bueno-Montes *et al.* 2011). Studies in which surfactant-free controls exhibit lower PAH removal tend to demonstrate positive effects of surfactant addition (Tiehm *et al.* 1997; Di Gennaro *et al.* 2008; Zhu and Aitken 2010; Bueno-Montes *et al.* 2011). If these surfactant-free controls perform poorly because of limited bioaccessibility, then surfactant-enhanced desorption may be contributing to improved biodegradation.

Deschenes *et al.* (1996) found that addition of either an anionic surfactant (sodium dodecyl sulfate) or a biosurfactant (*Pseudomonas aeruginosa* UG2 rhamnolipid) had no effect on

the biodegradation of three-ring PAHs, but had a significant negative effect on degradation of four-ring PAHs during the treatment of creosote-contaminated soil. Surfactant was added at a high and low dose, but the corresponding aqueous-phase surfactant concentration in the soil/water system was not reported. The researchers determined that neither surfactant inhibited global microbial activity during incubation, that the anionic surfactant was readily biodegraded, and cited previous evidence of biosurfactant biodegradation. The authors attributed the negative effect to competitive utilization of the surfactant as a carbon source instead of PAHs. This competitive effect was not overcome during the course of the incubation. It should be noted that competition between the use of a surfactant and PAHs as carbon sources makes sense only if the same microorganisms degrade both types of compounds. Otherwise, competition for other nutrients or for oxygen could explain the inhibitory effects of surfactants. The removal of 3-ring PAHs for both surfactant-amended and surfactant-free incubations reached near 100% removal. Removal of 4-ring PAHs in surfactant-free systems was near 90%, while incubations at the high surfactant doses had removals as low as 10%.

Lei *et al.* (2005) found that addition of a nonionic surfactant (Triton X-100) did not enhance PAH biodegradation in contaminated sediment. The study however, tested only a single surfactant dose that resulted in an aqueous-phase surfactant concentration below the CMC, the exact value of which was not determined. The researchers also noted that in surfactant-amended incubations, PAH degradation occurred after a lag phase, during which they hypothesized that the surfactant was preferentially degraded. Residual PAH concentrations were similar for surfactant-amended and surfactant-free incubations, with individual removal of three- and four-ring PAHs ranging from 50-80%.

Kim and Weber (2005) found that addition of a nonionic surfactant (Tween 80) did not enhance phenanthrene biodegradation in creosote-contaminated field soil. Surfactant was added at doses corresponding to aqueous-phase surfactant concentrations both above and below the CMC. At concentrations above the CMC, apparent liquid-phase phenanthrene concentration increased linearly with surfactant dose, suggesting the occurrence of solubilization-induced desorption. Similar to results obtained by Lei *et al.* (2005), a lag period was observed in surfactant-containing incubations, after which PAH degradation commenced at a rate independent of surfactant dose. The duration of the lag period was found to increase with increasing surfactant dose and was attributed to preferential utilization of the surfactant. Biodegradation of surfactant was confirmed to occur in soil-free incubations containing microbes isolated from the creosote-contaminated soil. Near-complete phenanthrene removal (>99%) was achieved for both surfactant-free and surfactant-amended incubations, suggesting that bioaccessibility was not a major limiting factor.

Tiehm *et al.* (1997) found that addition of nonionic surfactants (Arkopal N-300 and Saponegat T-300) enhanced the biodegradation of both high- and low-molecular-weight PAHs in MGP soil during column studies. Only one dose sufficient to enhance PAH desorption by solubilization was evaluated. Treatment with Saponegat T-300 resulted in greater final PAH removal than did treatment with Arkopal N-300. The authors attributed this to the rapid biodegradation of Arkopal N-300, which resulted in depletion of dissolved oxygen. The authors attributed the enhanced PAH biodegradation to improved bioavailability due to solubilization. Surfactant-free incubations were characterized by low PAH removal, less than 30% removal for 3-ring PAHs and less than 20% for most four- and five-ring PAHs.

Di Gennaro *et al.* (2008) found that the addition of Tween 80 enhanced the biodegradation of both high- and low-molecular-weight PAHs during the treatment of PAH-contaminated landfill soil. Only one dose above the CMC, sufficient to enhance PAH desorption by solubilization, was evaluated. The authors attributed the enhanced PAH biodegradation to improved bioavailability due to solubilization. Surfactant-free incubations were characterized by low PAH removal, less than 20% removal for 3-ring PAHs and less than 10% for most four and five-ring PAHs.

Zhu and Aitken (2010) found that addition of nonionic surfactant (Brij 30) enhanced PAH removal during the treatment of MGP soil which had previously undergone biological treatment. Addition of surfactant to untreated MGP soil, however, did not enhance PAH removal. Unlike previously mentioned studies, the bioaccessible (desorbable) PAH fraction was specifically measured using SPE resin. In surfactant-free controls < 5% of total measured PAH mass was desorbed from the previously treated soil, demonstrating that the bioaccessibility of the PAHs remaining in the treated soil was low. At a dose below that corresponding to the apparent CMC in the soil-slurry system, Brij 30 enhanced desorption of most PAHs and significantly increased removal of 3- and 4-ring PAHs. Addition of a more hydrophilic surfactant, $C_{12}E_8$, however, reduced desorption and did not improve PAH removal at any dose. Poor performance of $C_{12}E_8$ was attributed to its higher HLB value and hypothesized tendency to sorb to clay minerals rather than organic domains. The authors concluded that surfactant-enhanced bioremediation is likely to be most effective for systems in which bioaccessibility would otherwise be a limiting factor.

Bueno-Montes *et al.* (2011) found that addition of nonionic surfactant (Brij 35) enhanced the biodegradation of low- and high-molecular-weight PAH in both an MGP soil which had

previously undergone biological treatment and a creosote soil which had not been previously treated. For the creosote-contaminated soil, roughly 95% of total measured PAH was in the fast-desorbing fraction, whereas for the previously remediated MGP soil only 15% of total PAH was in the fast-desorbing fraction. One dose corresponding to an aqueous-phase surfactant concentration of approximately 25 times the aqueous-phase CMC and sufficient to enhance PAH solubilization was employed. The effect on PAH removal was more marked for the MGP soil, characterized by low bioaccessibility. For the MGP soil, less than 10% of total measured PAH was removed in surfactant-free controls, while over 60% of total measured PAH was removed in incubations containing Brij 35. For the creosote-contaminated soil, surfactant enhancement was not substantial. Surfactant-free controls experienced PAH removal of 96%, while surfactant-amended incubations experienced removal of 97%.

Bueno-Montes *et al.* (2011) also compared the fraction of pyrene solubilized by surfactant to the fraction of pyrene extracted by SPE resin. For MGP soil they found that the fraction of pyrene solubilized by surfactant was significantly greater than that extracted by SPE, while for the creosote-contaminated soil the two fractions were nearly identical. This suggests that surfactant, at least at concentrations above the CMC, can act as more than simply an infinite sink for desorbable fractions of PAHs as would be determined with an SPE resin. Surfactant may directly interact with the soil causing enhanced PAH desorption. Like Zhu and Aitken (2010), the authors concluded that application of surfactant-enhanced bioremediation may be more effective for soils with low bioaccessibility, particularly those soils which have already undergone biological treatment without surfactant.

2.4 Microbial degradation of PAHs

Given the aims of this proposal, the background is concerned primarily with aerobic PAH degradation by bacteria, although there is evidence of anaerobic biodegradation as well as biodegradation by fungi (Haritash and Kaushik 2009). Reviews by Sutherland *et al.* (1995) and Elliot *et al.* (2011) suggest that the major mechanism of PAH biodegradation by bacteria begins with the oxidation of an aromatic ring by the dioxygenase enzyme to form *cis*-dihydrodiols (Figure 2.6). The dihydrodiols are dehydrogenated by dehydrogenase enzymes to form dihydroxylated intermediates known as catechols or *o*-diols. In a complete bacterial metabolic pathway, the catechol then usually undergoes enzymatic ring cleavage, separation of cleavage fragments from the remaining polyaromatic system, and ultimately mineralization to carbon dioxide. This process can continue until the remaining portion of the compound has been mineralized or may cease at some intermediate step, generating non-mineral products (transformation products). Identified transformation products of PAH metabolism include dihydrodiols, hydroxy acids, and *ortho*-quinones. *Ortho*-quinones are believed to form from the non-biological autoxidation of the catechol (dihydroxy-) intermediates (Aitken and Long 2004).

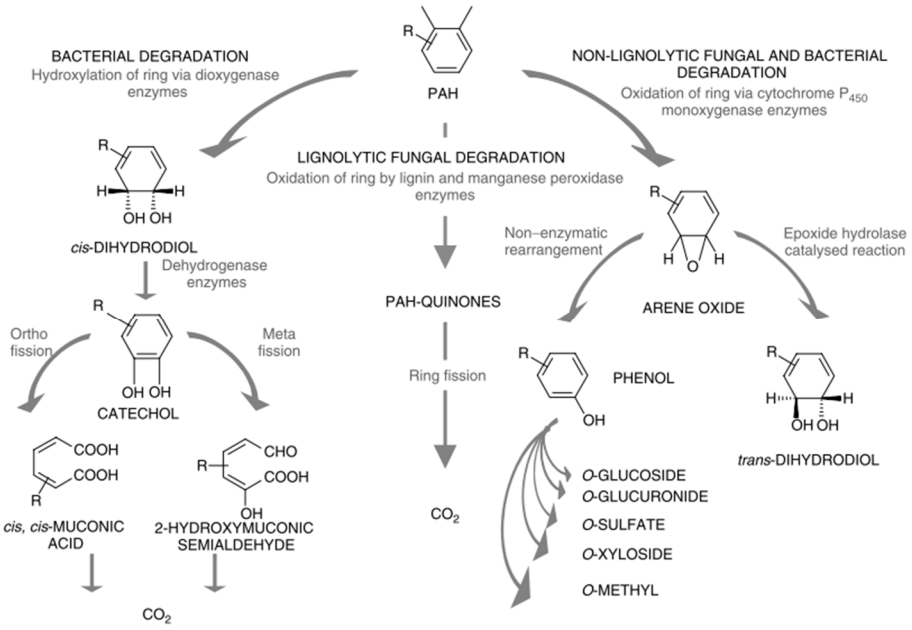

Figure 2.6 Biodegradation pathway of PAHs (Bamforth and Singleton 2005).

Often bioremediation does not result in complete metabolism of all carbon initially present in PAH mass. For purposes of this discussion, complete metabolism is defined as mineralization of some fraction of the contaminant carbon and assimilation of the remaining carbon into cellular biomass. It is incomplete metabolism that is of primary interest because extracellular, non-mineral products may exhibit greater (geno)toxicity than parent compounds. There is also evidence that PAH transformation products may inhibit the biodegradation of a parent PAH (Kazunga *et al.* 2001; Juhasz *et al.* 2002; Holt *et al.* 2005). The (geno)toxicity of remediated soil will depend on both the remaining parent compounds (including classes of compounds other than PAHs) and the products of their incomplete metabolism. Addition of surfactant during biodegradation may alter the distribution of end products (Auger *et al.* 1995) and have an effect on the resultant soil (geno)toxicity (Joner *et al.* 2001).

2.5 (Geno)toxicity of PAHs

Non-genotoxic cytotoxic effects of PAHs are caused by direct perturbation of cellular membranes by PAH (Sikkema *et al.* 1995; Schirmer *et al.* 1998). Given the limited bioavailability to vertebrates and relatively high LD_{50} for PAHs, the primary threat posed to human health by PAH-contaminated soil is chronic exposure to genotoxic compounds. A chemical or mixture is considered genotoxic if exposure to the chemical or mixture results in DNA damage in the observed biological system. A chemical or mixture is considered mutagenic if exposure to the chemical or mixture results in genetic mutations in the observed biological system. Since genetic mutations are typically the result of unrepaired DNA damage, genotoxicity precedes mutagenicity, and experimental evidence of mutagenicity is taken as evidence of genotoxicity. The phrase "in the observed biological system" is used to caution the reader that whether a chemical or mixture causes a genotoxic effect in a biological system depends on properties of the system itself such as complexity (free DNA, prokaryote, eukaryote, multicellular, vertebrate, mammal, etc.), the metabolic capabilities of the system, and available exposure pathways. PAHs and HACs are indirect-acting genotoxicants which require metabolic activation in order to cause DNA damage. The following summary of PAH activation pathways is based on reviews by Penning (2010) and Xue and Warshawsky (2005). Three activation pathways include the radical cation pathway, the diol epoxide pathway, and the PAH *o*-quinone pathway.

The radical cation pathway occurs through the peroxidase cycle of cytochrome P450 (CYP) enzymes. A one-electron oxidation occurs resulting in the generation of a radical cation. In the case of benzo[*a*]pyrene, the radical cation is localized on the C6 position. The radical cation may go on to react with nucleophilic centers of DNA, particularly the N7 positions of guanine and adenine. These DNA adducts are unstable and undergo spontaneous depurination to

form apurinic sites. The tendency of a PAH to form DNA adducts through the radical cation pathway depends on its molecular structure, which affects properties such as ionization potential and formation of a strongly localized positive charge on a carbon positioned to react with a nucleophilic DNA center. If the resulting abasic site is left unrepaired, then replicative polymerases will insert an adenine opposite an abasic site, resulting in a transversion mutation.

The diol epoxide pathway involves the formation of reactive diol epoxides on bay region-containing PAH through reactions catalyzed by CYP and epoxide hydrolase (EH). Benzo[*a*]pyrene, for example, is transformed into benzo[*a*]pyrene diol epoxide (BPDE), which reacts with nucleophilic centers of DNA, particularly the exocyclic amino groups of guanine and adenine, to form bulky stable adducts. Figure 2.7 depicts the diol epoxide activation pathway and resultant DNA damage with the major pathway indicated by bold arrows (Xue and Warshawsky 2005). Other bay region PAHs include chrysene, 5-methylchrysene, phenanthrene, benzo[*c*]phenanthrene, benz[*a*]anthracene, 7,12-dimethylbenz[*a*]anthracene, and dibenzo[*a,l*]pyrene. Bay-region nitrogen heterocycles are also metabolized through this pathway. Transversion mutations occur if an error-prone bypass polymerase attempts translesion synthesis (replication using the damaged template strand without repair) of the adducted DNA (Rechkoblit *et al.* 2002; Zhang *et al.* 2002).

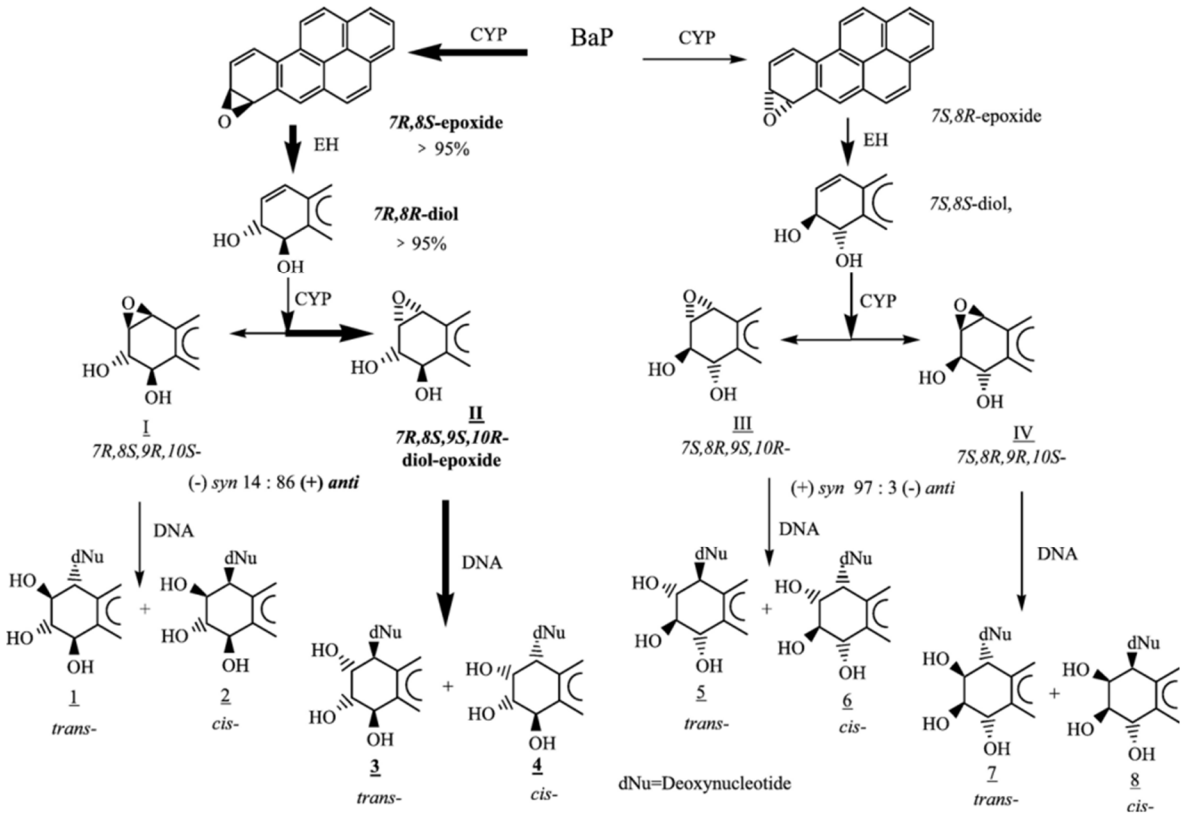

Figure 2.7 Diol epoxide metabolic activation of benzo[*a*]pyrene (Xue and Warshawsky 2005).

The *o*-quinone pathway involves the formation of redox active *o*-quinones from non-K-region (-)-*R,R-trans*-dihydrodiols such as the *7R,8R*-benzo[*a*]pyrene dihydrodiol depicted in Figure 2.7. The reaction is catalyzed by dihydrodiol dehydrogenase enzymes. These *o*-quinones can form DNA adducts or become involved in futile redox cycling which generate reactive oxygen species (ROS). *o*-Quinones are 1,4-Michael acceptors, which form stable adducts with nucleophilic centers of DNA, particularly exocyclic amino groups of guanine and adenine, and unstable depurinating adducts with the N7 of guanine or adenine. ROS such as superoxide radical anion and hydrogen peroxide are transformed to the highly reactive hydroxyl radical through the Fenton reaction. The hydroxyl radical can modify bases, causing DNA mismatches during replication. For example, deoxyguanosine can be oxidized to 8-oxo-deoxyguanosine,

which pairs with adenosine rather than cytosine, resulting in a transversion mutation. The hydroxyl radicals can also cause DNA stand breaks by abstraction of sugar hydrogens.

2.5.1 Methods for measuring (geno)toxicity of PAH-contaminated soil

Several methods have been utilized to evaluate (geno)toxicity of PAH-contaminated soil before and after bioremediation. Studies typically analyze soil organic-solvent extracts (whole or fractionated), organic compounds desorbed during SPE (bioaccessible/desorbable fraction), or aqueous soil leachates (bioavailable fraction). These methods have the general aim of assessing the extent to which the soil is a risk to higher organisms in the environment, particularly humans. Positive results of an assay are taken as evidence of potential genotoxicity in higher organisms. Additionally, the assays described below are quantitative and an assessment of the effect of bioremediation on soil (geno)toxicity can be made. It has been argued, however, that methods evaluating whole soil extracts overestimate the potential impact on higher organisms due to the limited bioavailability of contaminants (Alexander 2000). Also, when components of a mixture extracted from contaminated soil are fractionated, they may exhibit different genotoxic effects (Park *et al.* 2008). This effect can make attributing whole extract genotoxicity to particular components difficult.

Bacterial assays applied to PAH-contaminated soils undergoing bioremediation include reverse mutation assays (Hughes *et al.* 1998; Sayles *et al.* 1999; Mendonca and Picado 2002; Sasek *et al.* 2003; Lemieux *et al.* 2009) and SOS chromotest (Haeseler *et al.* 1999). Eukaryotic tests include the micronucleus test (Baudgrasset *et al.* 1993), the comet assay (Gandolfi *et al.* 2010), and the DT-40 chicken lymphocyte assay (Hu *et al.* 2012). Many of these assays require augmentation with exogenous mammalian metabolic activation systems, particularly rat liver extract (S9 supernatant) to assess the impact of indirect-acting genotoxicants.

Traditional bacterial reverse mutation assays, such as the Ames assay, utilize auxotrophs which have been genetically engineered (mutated) to be unable to synthesize an essential amino acid such as tryptophan or histidine. As a result of exposure to genotoxicants, reverse mutations may occur which have the effect of restoring the ability of the bacteria to synthesize the amino acid. Formation and growth of these revertants in a medium not supplemented with the amino acid is taken as evidence of genotoxicity. Particular auxotroph strains can be engineered to be reverted by either frameshift or base substitution mutations. The mutatox™ assay is also a reverse mutation assay. It utilizes a bioluminescent marine bacterium with a mutation that eliminates bioluminesce. Genotoxic compounds can cause a reverse mutation, restoring bioluminescence (Ulitzur 1982). Kwan *et al.* (1990) cite the manual provided by the manufacturer of the assay, writing that the assay is sensitive to (a) DNA damaging agents, (b) DNA intercalating agents, (c) direct mutagens which either cause base substitution or are frame-shift agents, and (d) DNA synthesis inhibitors.

SOS chromotest is a bacterial functional-assay that detects the bacterial SOS response to genotoxic chemicals. DNA damage which causes exposure of single-stranded DNA during attempted replication is believed to induce the bacterial SOS response (Friedberg 2006). The assay utilizes bacteria in which induction of the SOS response also causes expression of genes that affect some measurable property such as β-galactosidase activity (Quillardet and Hofnung 1985).

The micronucleus test evaluates the ability of a chemical or mixture to cause the formation of micronuclei in plant or mammalian cells (Barile 2008). Chemicals that cause DNA strand breaks can cause fragments of DNA to separate from the chromosome, forming

micronuclei (Timbrell 2009). The comet assay evaluates the ability of a chemical or mixture to cause single and double DNA strand breaks in eukaryotic cells (Burlinson 2012).

In a reverse genetic approach, the DT40 assay utilizes a eukaryotic chicken B lymphocyte-cell line and isogenic mutants. Cell lines engineered to be deficient in a particular DNA repair- or damage-tolerance protein are exposed to the chemical or mixture of interest. The parental cell line, not deficient in any proteins, is also exposed to the chemical or mixture. The LC_{50} is the concentration of a substance that, after incubation with cells, results in final viable cell count equal to half the viable cell count observed in a substance-free control. A protein-deficient cell line may be more sensitive (lower LC_{50}) to a chemical or mixture than the parental cell line. This is taken as evidence that the chemical or mixture is capable of causing DNA damage that would otherwise be repaired or tolerated by pathways in which the protein is involved. Two proteins with particular relevance to PAH-induced genotoxicity are the Rad54 and Rev1 proteins. Rad54 facilitates strand exchange during homologous recombination repair, which is used to repair double strand breaks (Sonoda *et al.* 2006). These double-stand breaks can be formed when a replication fork "collapses" upon encountering a single-strand break. Single-strand breaks could be the result of either direct oxidative damage to the sugar-phosphate backbone or arise during base excision repair of other DNA damage (Friedberg 2006). Rev1 is a "scaffold protein" which recruits DNA bypass polymerase to the stalled replication fork caused by DNA adducts and abasic sites (Friedberg 2006; Penning 2011) and has polymerase function as well (Goodman 2002). These two proteins are implicated in repair or tolerance of damage caused by the major types of PAH-induced genotoxicity: strand breaks caused by oxidative stress (Rad54), and adduction of DNA by stable or depurinating adducts (Rev1). Additionally, there is evidence that the DT40 cell line is capable of metabolic activation of PAHs such as

benzo[*a*]pyrene (Hu *et al.* 2012) and methods using S9 fractions for activation can also be applied (Hashimoto *et al.* 2015).

Two common methods to assess the cytotoxicity of PAH-contaminated soil are the Microtox assay and seed germination assay. Microtox uses a bioluminescent strain of the *Vibrio fischeri* bacterium. The reduction in bioluminescence upon exposure to a chemical mixture diluted into aqueous solution is taken as evidence of toxicity (Johnson 2005). The seed germination assay involves directly planting seeds in a contaminated soil sample and then counting the number of seedlings (Banks and Schultz 2005). Also, the DT40 assay using the parental cell line can be used as an indicator of cytotoxicity.

2.5.2 Incomplete PAH metabolism during biodegradation and its effect on soil (geno)toxicity

Given all the variables involved in bioremediation (source material, bioremediation method and duration, etc.), the residual contaminant levels and (geno)toxicity of the treated soil vary from case to case. It can be said with confidence, however, that the reduction of measured parent PAH compound in contaminated soil does not always correspond to an observed reduction in soil (geno)toxicity (Hughes *et al.* 1998; Gillespie *et al.* 2007; Lemieux *et al.* 2009; Hu *et al.* 2012; Chibwe *et al.* 2015). The genotoxicity of contaminated field soil is not explained to a major extent by the level of 16 EPA PAH (Larsson *et al.* 2013) The (geno)toxicity of remediated soil will depend on the remaining parent compounds (including other classes of contaminants) and the products of incomplete metabolism. Both removal of chemicals mitigating (geno)toxicity and formation of (geno)toxic chemicals in greater amount or potency may impact the (geno)toxicity of the treated soil. Even aged soils that have not been the target of a concerted bioremediation effort will contain PAHs as well PAH transformation products, which presumably formed during natural attenuation or were present in the original contaminant matrix

(Lundstedt *et al.* 2003). Addition of surfactant during bioremediation may alter the distribution of end products and have an effect on the resultant soil (geno)toxicity.

There is evidence supporting the genotoxicity of select PAH bacterial transformation products. Zielinska-Park *et al.* (2004) found that PAH *o*-quinones, pyrene-(4,5)-quinone and fluoranthene-(2,3)-quinone, bacterial metabolites of pyrene and fluoranthene respectively (Kazunga and Aitken 2000; Kazunga *et al.* 2001), caused oxidative DNA damage in vitro. The transformation products were assayed using calf thymus DNA and HeLa S3 cells. The researchers speculated that during biodegradation, bacteria may metabolically activate PAHs, such as pyrene, which might not otherwise be activated to an appreciable extent by mammalian metabolic systems. This bacterial activation transforms relatively non-genotoxic PAHs such as pyrene into redox active *o*-quinones.

Pagnout *et al.* (2006) evaluated the (geno)toxicity of several parent PAHs (pyrene, fluoranthene, and phenanthrene) and their unidentified metabolites produced in an aqueous incubation by a bacterial culture isolated from soil. For all PAHs, after biodegradation the mixture of metabolites was not toxic to any of the organisms assayed. In general biodegradation was found to reduce the (geno)toxicity of the incubations. Changes in the type of genotoxicity observed before and after biodegradation were noted. For example, the parent compounds pyrene and phenanthrene required S9 activation in order to exert a genotoxic effect in the Ames assay, while their transformation products exhibited genotoxicity *only* in the absence of S9 activation. This suggests that bacteria may transform indirect-acting genotoxicants, such as PAH, into direct-acting genotoxicants. The relative sensitivity of an assay to direct- vs. indirect-acting genotoxicants may have an impact on the reported change in genotoxicity of a remediated soil.

Studies measuring the (geno)toxicity of PAH-contaminated soil before and after bioremediation have demonstrated varying effects. Most studies reporting a reduction in (geno)toxicity of soil involved soil treatment by land-farming (Baudgrasset *et al.* 1993; Sayles *et al.* 1999; Mendonca and Picado 2002) or composting (Sasek *et al.* 2003; Gandolfi *et al.* 2010). Hu *et al.* (2012) found a reduction of toxicity and genotoxicity of solvent extracts from soil in a laboratory column simulating long-term in situ bioremediation. It is possible that long-term treatment is needed to achieve reductions in (geno)toxicity.

Haeseler *et al.* (1999) treated soil from a former manufactured-gas plant in a slurry-phase bioreactor. Treatment reduced the leaching capacity of the soil and leachate from treated soil was less toxic and genotoxic than leachate from untreated soil. Toxicity was evaluated using Microtox and genotoxicity using SOS chromotest with and without S9 activation.

Hu *et al.* (2014) found that slurry-phase bioreactor treatment of MGP soil, although unable to significantly reduce the toxicity or genotoxicity of whole-soil-solvent extracts, was able to reduce the toxicity and genotoxicity of the desorbable fraction. Toxicity and genotoxicity were evaluated using the DT40 assay with the *Rad54*$^{-/-}$ DNA-repair-deficient mutant. The slight increase in whole soil genotoxicity was attributed to the formation of non-desorbable genotoxic compounds. Addition of surfactant to slurry-phase treatment may enhance desorption of these otherwise non-desorbable genotoxic compounds, potentially increasing their rate of biodegradation.

Evaluating the same source soil and bioremediation process as Hu *et al.* (2014), Chibwe *et al.* (2015) assayed both whole and fractionated soil solvent-extracts using the DT40 assay. Solvent extracts of treated and untreated soil were fractionated to separate compounds based on polarity. Slurry-phase bioreactor treatment, despite removing substantial amount of PAHs,

increased whole-soil genotoxicity and substantially increased the genotoxicity of the more polar fractions. The authors attributed the increase in genotoxicity to the formation of unidentified hydroxylated and carboxylate transformation products of 3- and 4-ring PAHs, which would likely be present in the more polar fractions.

Other studies reporting an increase or no change in the (geno)toxicity of soil undergoing bioremediation include creosote-contaminated soil treated by land-farming (Gillespie *et al.* 2007) and creosote-contaminated soil undergoing several bioremediation techniques including bioslurry, biopile, compost, and land-farming (Hughes *et al.* 1998). In a companion study toHughes *et al.* (1998), Brooks *et al.* (1998) evaluated the genotoxicity of fractionated extracts of the treated soil using a bacterial reversion assay with and without S9 activation. The 19 PAHs investigated were detected in only three of the 16 mutagenic fractions. Other compounds identified in the mutagenic fractions were mainly nitrogen heterocycles (azaarenes) as well as some sulfur and oxygen heterocycles.

Perhaps the most informative studies are those that evaluate temporal changes in genotoxicity during bioremediation. These studies demonstrate that there are fluctuations in the magnitude and mechanisms of genotoxicity during the course of bioremediation. Lemieux *et al.* (2009) used a *Salmonella* reverse mutation assay with and without S9 activation to examine temporal changes in the genotoxicity of fractionated extracts of an MGP soil undergoing slurry-phase bioremediation. The study incorporated bacterial mutants capable of detecting frameshift and base-pair substitutions. Although whole-soil- solvent-extracts were not assayed, two fractions were evaluated, a nonpolar neutral fraction containing PAHs, alkyl-PAHs, and S- and O-heterocycles and a semipolar aromatic fraction containing N-heterocycles and oxy-PAHs. The genotoxicity of the nonpolar fraction without S9 activation for frameshift mutants was greater

after bioremediation, suggesting the formation of direct-acting genotoxicants capable of causing frameshift mutations. The mutagenicity of the semipolar aromatic fraction with and without S9 activation was greater after bioremediation. In a companion study, Lundstedt *et al.* (2003) performed a thorough chemical analysis of the soil undergoing bioremediation and observed the accumulation of two oxy-PAH metabolites, 1-acenapthone and 4-oxypyrene-5-one. Although Lemieux *et al.* (2009) found no evidence of the genotoxicity of 4-oxypyrene-5-one, it is conceivable that other compounds which are genotoxic may accumulate during bioremediation.

Using the DT40 assay, Hu *et al.* (2012) found that toxicity and genotoxicity of whole-soil-extracts initially decreased, but then increased during bioslurry treatment. They also found that after controlling for the total concentration of extractable organic mass, the relationship between soil toxicity and PAH concentration was no longer significant. This suggests that other chemicals in addition to the selection of EPA priority PAH measured in the study contributed to soil toxicity.

Other studies have observed the accumulation of PAH transformation products, particularly oxy-PAHs, during bioremediation of field-contaminated soil (Saponaro *et al.* 2002) or artificially contaminated soil (Wischmann and Steinhart 1997). Addition of surfactant may enhance the rate of early steps in PAH metabolism by bacteria, causing a bottleneck and accumulation of compounds that may affect the observed (geno)toxicity of the soil.

CHAPTER 3: EFFECT OF CONVENTIONAL BIOREACTOR TREATMENT ON THE REMOVAL AND BIOAVAILABILITY OF OXY-PAHS IN CONTAMINATED SOIL

3.1 Introduction

As part of a broader study to evaluate the bioavailability and biodegradability of genotoxic constituents in contaminated soil (Hu *et al.* 2014), I determined the effect of bioremediation on the removal and bioavailability of four selected oxygenated polycyclic aromatic hydrocarbons (oxy-PAHs) in contaminated soil. Oxy-PAHs are of concern because some are known to exhibit toxic or genotoxic effects (Bolton *et al.* 2000; Zielinska-Park *et al.* 2004; Luo *et al.* 2011), and may also inhibit the biodegradation of parent PAHs (Kazunga and Aitken 2000; Kazunga *et al.* 2001). The sources of oxy-PAH in contaminated soil could be the same as the parent PAHs, such as coal tar, although they could also be formed in-situ by microbial or photochemical oxidation of the parent compounds (Lundstedt *et al.* 2007). Net increases of some oxy-PAHs have been observed during bioremediation (Lundstedt *et al.* 2003) and others have inferred the formation of oxy-PAH based on ratios of oxy-PAH to parent PAH (Wilcke *et al.* 2014). In this study, contaminated soil from a former manufactured-gas plant site in Salisbury, NC was treated in an aerobic, slurry-phase bioreactor. A former student in our lab, Jing Hu, used sorptive resins to evaluate desorption from the soil as an estimate of bioavailability (Hu *et al.* 2014). I developed a liquid chromatography-mass spectrometry (LC-MS/MS) method to quantify four oxy-PAHs: 9-fluorenone (FLO), 9,10-phenanthrenequinone (PQ), 9,10-anthraquinone (AQ), and benz[*a*]anthracene-7,12-dione (BAQ) in solvent extracts of the soil and sorptive resins. These four oxy-PAHs were selected for study based on their known occurrence

in contaminated soils and sediments and on their commercial availability. Additionally, the solubilities of the oxy-PAHs in phosphate buffer were determined.

3.2 Methods

3.2.1 Bioreactor operation

Bioreactor operation is described in greater detail in Hu *et al.* (2014). Contaminated soil from a former manufactured gas plant site (feed soil) was treated in a continuously stirred, semi-continuous, laboratory-scale aerobic bioreactor. Every seven days, 20% of the treated slurry was replaced with a slurry of feed soil. Soil was sampled at several time points throughout this 7-day cycle. Soil extraction and sorptive resin methods are presented in Hu *et al.* (2014).

3.2.2 LC-MS/MS method

Soil solvent-extracts and extracts of the sorptive resins provided by Jing Hu were analyzed by 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 chromatographic separation was based on the method for separation of diketones provided in Delhomme *et al.* (2008), scaled to the column dimensions described above using a method transfer calculator. 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 initially 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 at a flow rate of 0.294 mL/min. 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.

Both electrospray ionization (ESI) and positive and negative APCI chemical ionization were tested on AQ. Similar to observations by Delhomme *et al.* (2008), APCI negative ionization was the most effective ionization method for AQ and BAQ, while APCI positive ionization was most effective for PQ. Although ESI was determined to be the most effective ionization method for FLO by Grosse and Letzel (2007), APCI positive was used to ionize FLO so that it could be quantified in the same chromatographic run as the other analytes.

Ultimately, 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. Quantification of each compound was performed according to the corresponding standard curve constructed form external standards.

3.2.3 Spike and recovery validation

Preliminary spike/recovery validation experiments were completed using AQ. Triplicate 3-g aliquots of feed soil were spiked with AQ dissolved in acetone (89.7 µg in 500 µL) and extracted as described in Hu *et al.* (2014); the mass of spiked AQ was approximately equal to the mass of AQ present in the original feed soil. An additional 3 replicates were extracted without spiking AQ. The total mass of AQ in the samples was quantified and a recovery of the spiked mass was determined. The average recovery was 81% ± 7%.

Anthraquinone-D_8 (AQ-D_8) was demonstrated to be a useful recovery surrogate for the extraction procedure, but was not utilized beyond preliminary experiments because soil extracts were to be used for toxicity testing and the addition of spiked compounds was undesirable. AQ-D_8 was quantified in APCI negative ionization mode using the molecular ion *m/z*=216. In

preliminary experiments, triplicate 3-g wet weight aliquots of feed and treated soil were spiked with 52 µg of AQ-D_8 delivered in 500 µL of acetone immediately before extraction. Average recovery of AQ-D8 spiked into feed and treated soil was 90% ± 2% and 84% ± 2% respectively.

3.2.4 Solubility of oxy-PAHs in phosphate buffer

Approximately 10 mg of one of the oxy-PAHs was added to a 25-mL glass vial with PTFE-lined screwcap; for each oxy-PAH, triplicate vials were prepared. Phosphate buffer (20 mL, 5-mM total phosphate, pH 7.5) was then added to each vial. The buffer consisted of the following salts dissolved in deionized water: potassium phosphate monobasic, potassium phosphate dibasic, sodium phosphate monobasic, sodium phosphate dibasic and had a final concentration of 5-mM phosphate, 2.5-mM sodium, and 2.5-mM potassium. The vials were put on a wrist-action shaker in the dark at a constant temperature of 25°C. At 24 and 48 hours the tubes were taken off the shaker and the solids allowed to settle. Approximately 5-mL aliquots were removed from each tube and filtered using a glass syringe and stainless steel filter housing fitted with a 0.02 µm alumina membrane filter (Whatman Anodisc). Filtrates were then diluted into methanol for LC-MS/MS analysis.

3.2.5 Chromatographic method improvement

Chromatographic methods can be adjusted to provide better peak separation for specific sample matrices. In the case of the soil solvent-extracts, background interference was substantial. Separation of analyte peaks from background was improved by using a longer column and a less steep elution gradient. The effect is illustrated in Figure 3.1.

The updated chromatographic method used a BEH C_{18} 1.7µm (2.1x100mm) column. The mobile phase was initially30% methanol for 2.10 minutes followed by a linear increase to 60% methanol over 2.90 minutes and was held at 60% methanol for 5 minutes at a flow rate of 0.275 mL/min. Then, the methanol was increased to 75% over 2 minutes and held at 75% for 8

minutes. Methanol was then increased linearly to 100% over 1 minute and held at a flow rate of 0.4 mL/min for 4 minutes. Finally mobile phase was decreased linearly to initial conditions and held for 3 minutes before the next injection. This updated LC method was used to quantify the four oxy-PAHs in soil samples described in Chapter 5 of this dissertation.

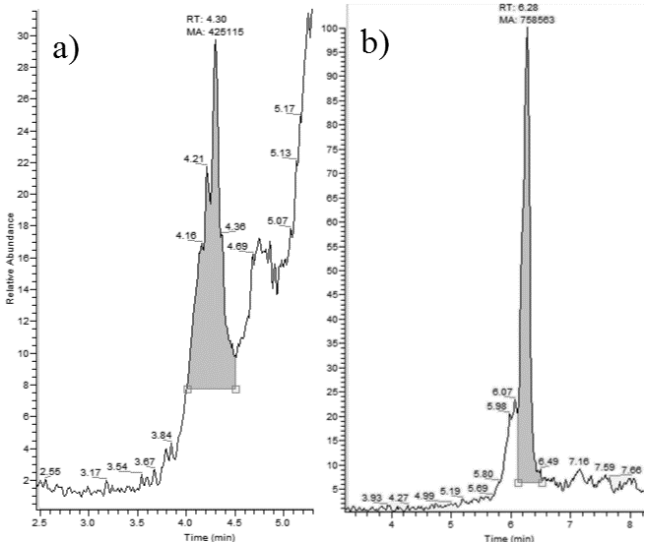

Figure 3.1 Portion of chromatogram during which PQ elutes in **(a)** the original chromatographic method and **(b)** the updated chromatographic method. Shaded portion is PQ peak.

3.3 Results and discussion

The effect of bioremediation on the desorption and removal of oxy-PAHs is described in detail in Hu *et al.* (2014). In summary, concentrations of the four oxy-PAHs in untreated feed soil ranged from < 1 mg/kg (PQ) to 18 mg/kg (AQ). Bioremediation resulted in the removal of 72% of total measured oxy-PAHs (Table 3.1) and substantially reduced the desorbable fraction of oxy-PAHs (Figure 3.2). For all measured oxy-PAHs, the percent removed during treatment exceeded the desorbable fraction in the feed soil, suggesting that biodegradation was not limited to the initial desorbable fraction. Removal in excess of measured abiotic desorption was also observed for PAHs in Hu *et al.* (2014) and during long-term simulated *in-situ* treatment of the same contaminated soil (Richardson and Aitken 2011). This difference could be caused by

microbially-enhanced desorption through the secretion of biosurfactants or through direct uptake of PAH by bacteria attached to the contaminant matrix (Mukherji and Ghosh 2012), or it could be a result of differences between bacteria and sorptive resins in the physical accessibility to PAHs in micropores of soil aggregates.

Table 3.1 Concentration of oxy-PAHs in feed and treated soils and corresponding removals.[a]

Oxy-PAH	Feed (μg/g)	Treated (μg/g)	Removal (%)
PQ	0.858 ± 0.054	0.282 ± 0.014	67.2 ± 2.6
FLO	1.43 ± 0.11	0.577 ± 0.062	59.6 ± 5.3
AQ	18.3 ± 0.4	4.71 ± 0.08	74.3 ± 0.8
BAQ	1.70 ± 0.09	0.752 ± 0.006	55.9 ± 2.3
Total oxy-PAH	22.3 ± 0.5	6.32 ± 0.16	71.6 ± 1.0

[a] Values represent means and standard deviation of triplicates.

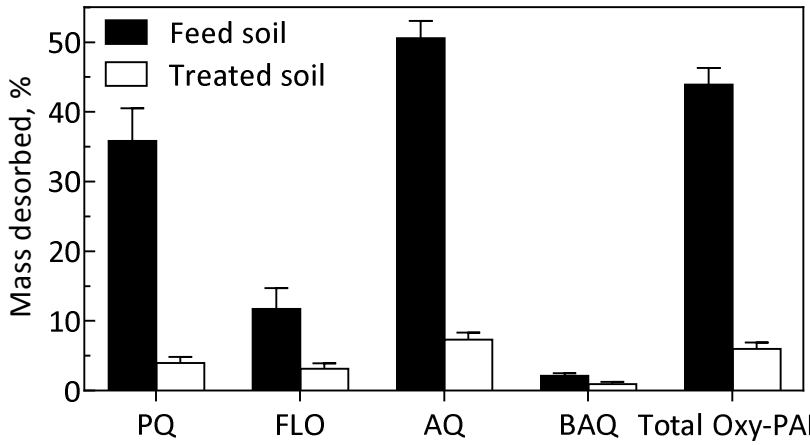

Figure 3.2 Effect of bioremediation on desorption of oxy-PAH from contaminated soil . Data from Hu et al. (2014).

The solubilities of the oxy-PAHs in phosphate buffer are presented in Table 3.2. In general, measured solubilities agreed within an order of magnitude with values found in the literature. There is limited information on the aqueous solubility of oxy-PAHs. The Handbook of Aqueous Solubilities (Yalkowsky 2003) listed only AQ, with a reference to Eik-Nes *et al.* (1954). The Reaxys (2015) database reported the solubility of PQ to be 7.5 mg/L but references Knox and Will (1919), which does not appear to discuss PQ. The NIH database ChemIDplus (2015) also reports 7.5 mg/L, but the link to the source was broken as of this writing (date accessed: 07/02/2015).

Table 3.2 Measured solubility of oxy-PAHs and reported values in the literature.

	Solubility (mg/L)		
Compound	24 hours[a]	48 hours[a]	Literature solubility
PQ	4.04 ± 0.16	4.25 ± 0.16	7.5[b]
FLO	14.8 ± 0.1	15.1 ± 0.4	25.3[b] (EST)
AQ	0.108 ± 0.008	0.106 ± 0.015	1.35[b,c] , 0.125[d]
BAQ	0.101 ± 0.001	0.105 ± 0.003	0.289[b] (EST)

[a] Values represent means and standard deviations of triplicates. [b] ChemIDplus (2015),[c] Eik-Nes *et al.* (1954), [d] Walker (1993). EST, reported as estimated solubility in ChemIDplus.

CHAPTER 4: SCREENING NONIONIC SURFACTANTS FOR ENHANCED BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS REMAINING IN SOIL AFTER CONVENTIONAL BIOLOGICAL TREATMENT

4.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of compounds that are of environmental and public health concern because of their known or suspected toxicity and genotoxicity and their frequent occurrence at contaminated sites (ATSDR 1995). Bioremediation is one option for the treatment of PAH-contaminated systems such as soil and sediment, but its efficacy may be limited by incomplete removal of the target PAHs (Aitken and Long 2004).

Due to their hydrophobicity, PAHs are often strongly associated with non-polar soil domains such as soil organic matter, combustion residue, and non-aqueous-phase liquids, and therefore may be unavailable to degrading microorganisms. Studies measuring PAH desorption from soil into the aqueous phase suggest that the fast-desorbing or bioaccessible fraction of a PAH can be a qualitative, if not quantitative, indicator of biodegradation potential in field-contaminated soil (Cornelissen *et al.* 1998; Huesemann *et al.* 2004; Richardson and Aitken 2011). The addition of surfactants has been proposed as a means of enhancing the bioavailability of PAHs to degrading microorganisms as reviewed in Makkar and Rockne (2003), Li and Chen (2009), and Elliot *et al.* (2011) ,but previous studies have led to conflicting conclusions on the effects of surfactants on the biodegradation of PAHs in field-contaminated soils or in spiked soils.

Surfactants can increase the rate of PAH desorption from a geosorbent through two mechanisms: micellar solubilization and direct modification of the contaminant matrix. Micellar

solubilization involves partitioning of PAHs into surfactant micelles at aqueous-phase surfactant concentrations above the critical micelle concentration (CMC), increasing the rate of desorption by maximizing the concentration gradient between the geosorbent and aqueous phase (Grasso *et al.* 2001). Significant sorption of surfactant to soil, however, necessitates larger surfactant doses to reach the CMC in the aqueous phase of soil/water systems (Liu *et al.* 1992). As opposed to solubilization, modification of the contaminant matrix can occur at concentrations above and below the CMC. Surfactants have been shown to increase desorption of PAHs from contaminated field soil at doses corresponding to aqueous-phase surfactant concentrations less than the CMC (sub-CMC doses) in the soil/water system (Zhu and Aitken 2010; Frutos *et al.* 2011). Hypothesized effects of surfactants on the contaminant matrix include increased PAH diffusivity (Yeom *et al.* 1996), and increased geosorbent interfacial surface area caused by wetting (Dong *et al.* 2003) and dispersion of non-polar matrices (Kile and Chiou 1989; Zhang and Miller 1992; Churchill *et al.* 1995). Additionally, sorption of surfactant to bacteria can increase the adherence of bacteria to a geosorbent, potentially increasing the rate of PAH desorption directly into biofilms or adherent cells (Mohanty and Mukherji 2008; Mohanty and Mukherji 2012; Zhang and Zhu 2014).

Previous research on field-contaminated soil suggests that surfactant addition is most beneficial for systems in which PAH biodegradation is limited by low bioaccessibility. This would be the case, for example, with soil treated in a conventional bioremediation system for which residual PAH concentrations might exceed cleanup targets (Zhu and Aitken 2010). Studies in which surfactant-free controls achieve substantial PAH removal tend to demonstrate no improvement or even inhibition of PAH removal as a result of surfactant addition (Deschenes *et al.* 1996; Kim and Weber 2005; Lei *et al.* 2005; Zhu and Aitken 2010; Bueno-Montes *et al.*

2011). Studies in which surfactant-free controls exhibit negligible PAH removal, however, tend to demonstrate positive effects of surfactant addition (Tiehm *et al.* 1997; Di Gennaro *et al.* 2008; Zhu and Aitken 2010; Bueno-Montes *et al.* 2011). If these surfactant-free controls perform poorly due to limited PAH bioaccessibility, then surfactant-enhanced desorption may explain improved biodegradation. Although there is a cost savings associated with using less surfactant, there has been limited work on surfactant-amended bioremediation of PAH-contaminated field soil at sub-CMC doses (Kim and Weber 2005; Lei *et al.* 2005; Zhu and Aitken 2010).

The objective of the present study was to investigate the effect of sub-CMC surfactant doses on the bioremediation of PAH-contaminated soil from a former manufactured-gas plant (MGP) site which had already undergone biological treatment in a slurry-phase bioreactor. We evaluated five relatively hydrophobic nonionic surfactants (hydrophile-lipophile balance [HLB] $\leq$ 10) based on our previous study in which the hydrophobic surfactant, Brij 30, enhanced desorption and biodegradation more than the hydrophilic surfactant, $C_{12}E_8$ (Zhu and Aitken 2010). In this study, we hypothesized that surfactants of similar hydrophobicity but different hydrophilic moieties (Figure 4.1) might influence the microbial community and, therefore, PAH removal as well as the toxicity of the soil. We evaluated the effect of surfactant amendment on desorption and biodegradation of residual PAHs and for the three most effective surfactants we also evaluated the effect of treatment on soil (geno)toxicity.

4.2 Materials and methods

4.2.1 Materials

PAH standards for high performance liquid chromatography (HPLC) analysis (EPA 610 PAH mixture and individual PAHs), Brij 30, Span 20, and polyoxyethylene sorbitol hexaoleate (POESH), Tenax® TA beads (60/80 mesh), and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Ecosurf™ EH-3 (EH-3) was obtained from Chemical

Marketing Concepts (New Milford, CT, U.S.A.). R-95™ rhamnolipid biosurfactant (R-95) was obtained from AGAE Technologies (Corvallis, OR, U.S.A.). Properties of the surfactants are summarized in Table 4.1. SnakeSkin™ Dialysis Tubing (10,000 MWCO, 22-mm diameter) was obtained from Thermo Scientific (Rockford, IL, U.S.A.). PAH standards for gas chromatography-mass spectrometry (GC-MS) were obtained from Accustandard Inc. (New Haven, CT, U.S.A.) All solvents were HPLC grade and were obtained from either Fisher Scientific (Pittsburgh, PA, U.S.A.) or VWR International (Radnor, PA, U.S.A.).

4.2.2 Bioreactor operation

Contaminated soil used in this study was collected from a former manufactured-gas plant site in Salisbury, North Carolina, processed, and characterized as described elsewhere (Richardson and Aitken 2011; Hu *et al.* 2012). The soil was treated in a continuously stirred, semi-continuous, laboratory-scale aerobic bioreactor. The bioreactor was made of stainless steel, has a working volume of approximately 2 L, a solids content of 15% (w/w), and average solids retention time of 35 days. Every seven days, 20% of the treated slurry was replaced with a slurry of untreated (feed) soil in a pH 7.5 buffer containing 5-mM phosphate and 2.5-mM ammonium nitrate. Treated slurry was centrifuged at 3900 RPM for 20 minutes, the supernatant discarded, and the centrifuged bioreactor-treated soil used in the experiments described below. Moisture content of the centrifuged soil was determined in triplicate by heating 1-g wet-weight aliquots of soil to dryness in preweighed ceramic crucibles over a Bunsen burner until a stable value of dry mass could be obtained. Typical soil moisture content was approximately 45% (w/w).

4.2.3 Surfactant dose selection

The CMC of each surfactant in phosphate buffer (5 mM, pH 7.5) was measured by following surface tension as a function of surfactant concentration. Because surfactants sorb to soil, it was necessary to evaluate surfactant doses (mass surfactant per mass dry weight soil)

required to achieve the CMC in soil/buffer slurries as a basis for selecting sub-CMC doses for each surfactant. The surfactant was added to bioreactor-treated soil in a 15% (w/w) slurry in phosphate buffer (5 mM, pH 7.5). The aqueous-phase surfactant concentration and percent total pyrene solubilized as a function of dose were determined for each surfactant as described in Supporting Information. Pyrene was chosen as a representative PAH because of its presence at liquid-phase concentrations above the lower limit of quantification (LLOQ) for a wide range of surfactant doses. At aqueous-phase surfactant concentrations below the apparent CMC, liquid-phase PAH concentrations are low because solubilization is negligible. Based on these results, two doses (referred to as higher and lower) below the apparent CMC in the soil-slurry system were selected for each surfactant (Table 4.1); the lower dose was equal to 1/3 the higher dose. The higher dose of R-95, however, was slightly above the nominal CMC. For Brij 30, Span 20, EH-3, and R-95 the selected doses corresponded to less than 1% of total pyrene solubilized in the liquid phase of the slurry (Figure A.1 and Figure A.2). For POESH the selected doses corresponded to less than 6% of total initial pyrene solubilized in the liquid phase of the slurry.

Table 4.1 Properties of surfactants tested and doses used.

Surfactant	Alternative names	HLB	CMC[a] (mg/L)	Dose (mg/g-dry soil)	
				Higher	Lower
Brij 30	Brij L4, Polyethylene glycol dodecyl ether, Polyoxyethylene (4) lauryl ether	9.7	18	12	4
Span 20	Sorbitan monolaurate	8.7	17	15	5
EH-3	none	7.9	680	60	20
POESH	Poly(ethylene glycol) sorbitol hexaoleate, Polyoxyethylene sorbitol hexaoleate	10	260	24	8
R-95	3-((6-deoxy-2-O-(6-deoxy-alpha-L-mannopyranosyl)-alpha-L-mannopyranosyl)oxy)-Decanoic acid 1-(carboxymethyl)octyl ester	10	38	9	3

[a] CMC measured in phosphate buffer.

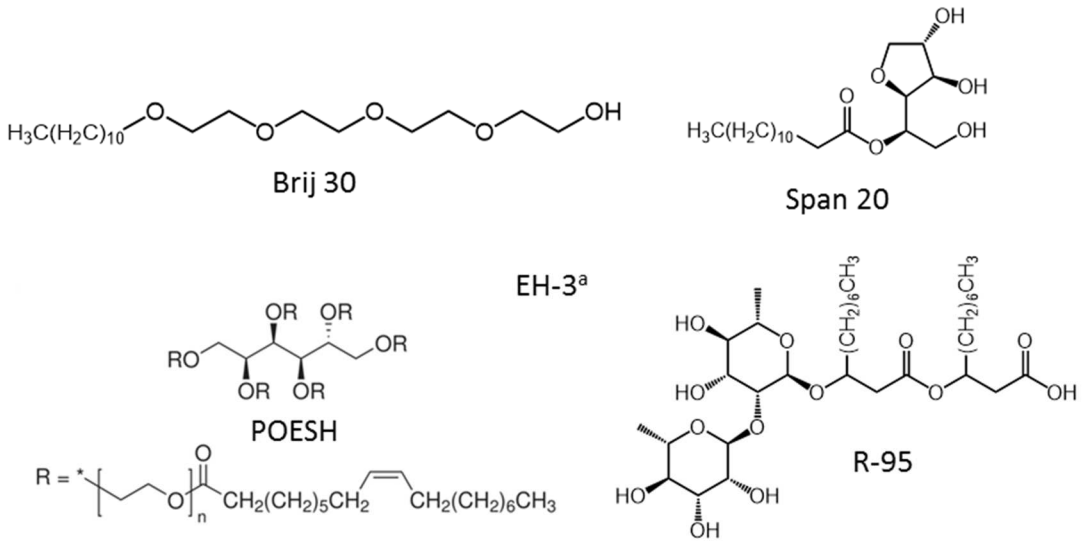

Figure 4.1 Structures of tested surfactants [a] EH-3 is an alcohol ethoxylate (similar to Brij 30) with a proprietary structure.

4.2.4 PAH desorption

Desorption of PAHs from bioreactor-treated soil during surfactant-amended treatment was evaluated using Tenax beads as an infinite sink. Incubations with each surfactant were

prepared in triplicate for the lower and higher doses and in quadruplicate for no-surfactant controls. The experiment required a mass of bioreactor-treated soil larger than that produced by the bioreactor in a single week. Therefore, incubations were set up over multiple weeks using separate batches of bioreactor-treated soil, each analyzed for PAH concentrations in triplicate. No-surfactant controls were prepared for each batch of bioreactor-treated soil. Incubations were prepared by adding 2-g dry weight bioreactor-treated soil to 30-mL glass centrifuge tubes with polytetrafluoroethylene (PTFE)-lined septa screw caps. Surfactant stock solution in phosphate buffer (pH 7.5) was added to give the desired surfactant dose. Additional phosphate buffer was added to give a final solids content of 15% (w/w). The headspace in each incubation tube was purged with nitrogen and the tubes were put on a rotary shaker at 275 RPM for 48 hours in the dark to allow sorption of the surfactant to the soil with minimal aerobic biodegradation of surfactant and PAHs.

After 48 hours of anaerobic incubation, an additional 10 mL of phosphate buffer and 0.1 g of Tenax beads contained in dialysis tubing knotted at both ends were added to each incubation. Prior to use, Tenax beads were cleaned by Soxhlet extraction in 50:50 acetone:hexane for 12 to 16 hours, rinsed with methanol, and air dried. It was necessary to contain the Tenax in dialysis tubing because Tenax did not float in surfactant-containing incubations, which is how it would be recovered in conventional desorption experiments (Zhu *et al.* 2008). Work by Hu *et al.* (2014) demonstrated that this dialysis tubing does not impact PAH desorption measurements. After adding the dialysis tubing to each incubation tube, the headspaces were purged with nitrogen again. The incubation tubes were then returned to the orbital shaker for a period of seven days, which has been shown to be sufficient to approach equilibrium for this soil (Hu *et al.* 2014).

After seven days, the dialysis tubing was removed from each incubation tube and rinsed with deionized water to dislodge any soil adhering to the tubing. Each Tenax-containing dialysis tube was then slit and the Tenax along with the tubing was added to 15-mL glass vials with PTFE-lined septa and screw caps. The Tenax was extracted overnight with 10 mL methanol on an orbital shaker (275 RPM). The extracts were then vacuum-filtered through 0.2-µm nylon membrane filter and volumized to 25 mL with acetonitrile. Some extracts were concentrated under a gentle stream of nitrogen to bring the concentrations of PAHs above their quantification limits. Tenax solvent-extracts were then analyzed by HPLC to determine the masses of desorbed PAHs. The incubation tubes containing the higher dose for each surfactant were centrifuged and residual PAH concentrations in the post-desorption soil pellet measured. The sum of the residual mass of a given PAH in the soil pellet plus the mass sorbed to the Tenax was compared to the initial mass in the bioreactor-treated soil to calculate a recovery for each PAH in the higher-dose incubations.

There were several conditions for which the PAH mass desorbed in some replicates was below the method LLOQ. An average and standard deviation of mass desorbed for an individual PAH with at least two replicates above the LLOQ were calculated using Cohen's maximum likelihood estimator method (Cohen 1959; Berthouex 1994). Individual PAHs with less than two replicates above the LLOQ for a condition are listed as <LLOQ (Cohen's method is not applicable to conditions with less than two uncensored observations).

4.2.5 PAH biodegradation

A preliminary biodegradation experiment was conducted by setting up incubations over multiple weeks (one surfactant per week) using separate batches of bioreactor-treated soil, each analyzed for PAH concentrations in six replicates; three of the replicates were spiked with a known amount of anthracene-D_{10} as a recovery surrogate while the other three were used for

toxicity testing as described below. For each surfactant, incubations were prepared at both the lower and higher doses. A no-surfactant control and an azide-inhibited control with surfactant at the higher dose were prepared in parallel for each surfactant. Incubations under each condition were prepared by adding 1.6-g dry weight bioreactor-treated soil to each of five 30-mL glass centrifuge tubes with PTFE-lined septa and screw caps. Surfactant stock solution in bioreactor buffer was added to deliver the target surfactant dose. The inhibited controls were spiked with 1 mL of 50-g/L sodium azide solution for a final nominal sodium azide concentration of 4.2 g/L. Bioreactor buffer was then added to give a final solids content of 15% (w/w).

All incubations were purged with nitrogen and put on an orbital shaker at 275 RPM for 48 hours in the dark to allow surfactant to sorb to the soil with minimal aerobic biodegradation of surfactant or PAH. After 48 hours, incubations were kept on the orbital shaker for an additional 14 days and uncapped daily for five minutes to allow air into the incubation vessel. All incubations were then centrifuged and the soil pellets extracted and analyzed for PAHs. The supernatants from each higher-dose surfactant incubation were syringe-filtered through 0.8-µm polycarbonate membrane filters and analyzed for PAHs.

Results from the preliminary biodegradation experiment were used to select the best-performing surfactants and their doses for direct comparison with a single batch of soil removed from the bioreactor (referred to below as the followup biodegradation experiment). Incubations were prepared and analyzed as described above for the preliminary experiment, except the liquid phase of the incubation at the lower dose of Brij 30 was also analyzed for PAHs.

4.2.6 Soil extraction and PAH analysis

Bioreactor-treated soil (~3g wet weight per replicate) and incubation soil pellets were extracted in their centrifuge tubes by mixing with 10-g sodium sulfate and extracting overnight twice, each time with 10-mL acetone and 10-mL dichloromethane as described elsewhere

(Richardson *et al.* 2011). Soil solvent-extracts, Tenax solvent-extracts, and incubation supernatants (liquid-phase) were analyzed for the concentrations of 14 PAHs denoted in the footnote of Table A.1 by HPLC with fluorescence detection as described elsewhere (Richardson *et al.* 2011). Unless noted otherwise, "total PAH" refers to the sum of these 14 PAHs. Soil solvent-extracts of selected conditions (no-surfactant control, lower dose of Brij 30, and higher doses of POESH and Span 20) from the followup biodegradation experiment were analyzed for additional PAHs and alky-PAHs by GC-MS as described in Appendix A. Extraction and analysis of the feed soil for the bioreactor is also described in Appendix A. Concentrations of PAHs in the feed soil are provided in Table A.1 and Table A.2 and concentrations of PAHs in the bioreactor-treated soil for the preliminary biodegradation experiments are provided in Table A.3.

4.2.7 (Geno)toxicity

The effects of surfactant amendment on soil toxicity and genotoxicity were evaluated using solvent extracts from the followup biodegradation experiment. For each condition, 8-mL aliquots from each of the five replicate extracts for a given condition were combined in a preweighed vial and evaporated to dryness under a gentle stream of nitrogen. For bioreactor-treated soil prior to surfactant addition, triplicate aliquots (not spiked with anthracene-D_{10}) were extracted and 12.5 mL of each extract were combined and evaporated to dryness under a gentle stream of nitrogen. Residue mass was then determined gravimetrically. Toxicities of the residues reconstituted in DMSO were evaluated in triplicate using a 96-well plate-based DT40 chicken lymphocyte DNA-damage response assay adapted from Ridpath *et al.* (2011) and Hu *et al.* (2012). Dose ranges were sufficient to bracket the LC_{50} of the residue. The *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$ DNA-repair deficient mutants were tested alongside the isogenic DT40 parental cell line because of their reported sensitivity to soil residue in previous experiments (Hu *et al.* 2012). The *Rad54*$^{-/-}$ knock-out is deficient in the homologous recombination DNA repair pathway, while the *Rev1*$^{-/-}$

knock-out is deficient in the translesion synthesis pathway. LC_{50} values (mg residue/mL in-well media) were calculated by fitting the log concentration vs % survival in GraphPad Prism version 6.05 for Windows. The LC_{50} values measured for residue mass were converted to an equivalent soil LC_{50} (mg soil/mL in-well media). Relative LC_{50}'s for each mutant cell line (LC_{50} of the mutant divided by the LC_{50} of the parental cell line) were calculated as a measure of genotoxicity, as described elsewhere (Hu *et al.* 2012).

A followup experiment was conducted to assess the effect of POESH at the higher dose on the (geno)toxicity of bioreactor-treated soil independent of PAH biodegradation or potential surfactant biodegradation. Biodegradation was minimized by incubating bioreactor-treated soil anaerobically and by omitting ammonium nitrate in the buffer; a nitrogen headspace was maintained for the duration of the incubation. In parallel, bioreactor-treated soil was also incubated with POESH at the higher dose under aerobic conditions. No-surfactant controls were also incubated under both aerobic and anaerobic conditions. Five replicates were prepared for each condition as described above for the preliminary biodegradation experiment and evaluated for (geno)toxicity.

4.2.8 Data analysis

Statistical analysis was conducted with SAS Enterprise Guide 6.1 (SAS Institute, Cary, NC, U.S.A.). For each PAH, a comparison of mass desorbed (µg) in the no-surfactant control with each surfactant-containing condition was conducted with two-sample t-tests (two-tail, homoscedastic, $\alpha = 0.05$). To identify enhanced PAH removal, multiple comparisons (one-way ANOVA followed by Tukey's Studentized, range test $\alpha=0.05$) among all treatments were performed using the final soil concentrations of each PAH. Comparisons were made between the LC_{50} and relative LC_{50}'s of bioreactor-treated soil with those of each treatment using two-sample t-tests (two-tail, homoscedastic, $\alpha = 0.05$). The standard deviations of percent desorbed and

percent removal were calculated by propagation of error using the means and standard deviations of the data used in the calculations.

4.3 Results

4.3.1 PAH desorption

The effect of two surfactant doses below the CMC on desorption of residual PAHs from soil previously treated in a slurry-phase bioreactor was evaluated. At these doses the majority of surfactant is sorbed to the soil and solubilization is not expected to be a major mechanism of PAH mobilization. Incubation of the bioreactor-treated soil with all surfactants resulted in modest increases in total PAH desorption compared to no-surfactant controls (Figure 4.2). Percent individual PAH masses desorbed for Brij 30 are depicted in Figure 4.3. In general, low-molecular-weight PAHs (2- or 3-rings), with the exception of naphthalene, were desorbed to a greater extent than were the high-molecular-weight PAHs. For example, over 50% of acenaphthene was desorbed by Brij 30 at the higher dose, while no more than 6% of any measured 4-, 5-, or 6-ring PAH was desorbed. Desorption trends of individual PAHs for the remaining surfactants were similar. Concentrations of PAHs in the bioreactor-treated soil samples used in desorption experiments and percent masses desorbed for all conditions are provided in Table A.4-Table A.6.

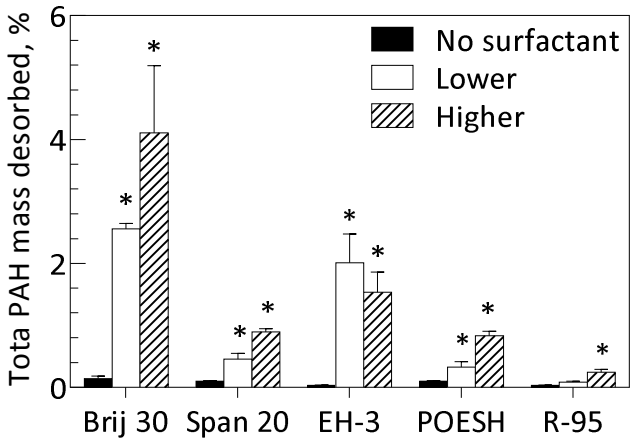

Figure 4.2 Cumulative desorption of total PAH mass from bioreactor-treated soil after seven days in the absence of surfactant or in the presence of five different surfactants, each added at two doses designated "lower" and "higher" as defined in Materials and Methods. Bars represent means and standard deviations of three replicates for surfactant conditions and four replicates for no-surfactant controls. An asterisk indicates a significant difference (α=0.05) between the mass of PAH desorbed in a treatment and no-surfactant control.

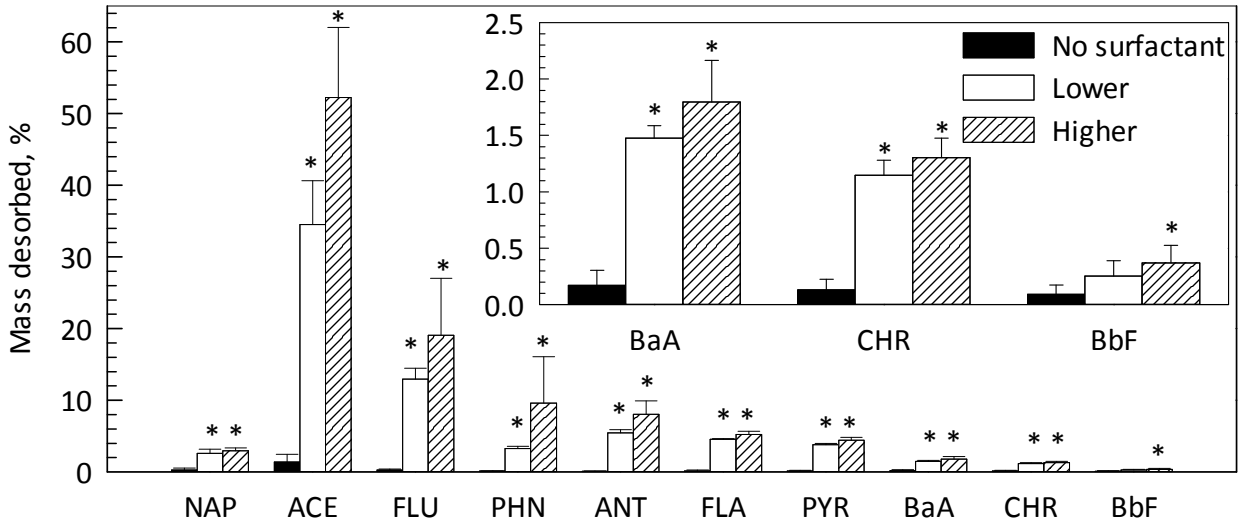

Figure 4.3 Cumulative desorption of PAHs from bioreactor-treated soil after seven days in the absence of surfactant or in the presence Brij 30. ; the inset shows the data for the three indicated compounds on a finer scale. Abbreviations are defined in Table A.1. Other notes as in Figure 4.2. PAHs for which there were no significant differences (BkF, BaP, DBA, and BgP) between no-surfactant and both doses are not shown.

Recoveries of individual PAHs ranged from 60-107% (Table A.7). For the lower recoveries, it is possible that nitrogen purging did not sufficiently inhibit PAH biodegradation. Also, it is not known how the likely sorption of surfactant impacts the capacity of Tenax to serve

as an infinite sink. Given these limitations, the results can be interpreted as semi-quantitative evidence of the ability of surfactants to enhance PAH desorption at doses below the apparent CMC in the soil-slurry system.

4.3.2 PAH biodegradation

Biodegradation of residual PAHs in the treated soil from a slurry-phase bioreactor was evaluated at the two selected doses for each surfactant. All surfactants except R-95 rhamnolipid significantly increased total PAH removal from the bioreactor-treated soil relative to the no-surfactant control (Figure 4.4). POESH had the greatest effect, resulting in removal of 50% of total PAH. Significant dose-dependent effects were observed for both Brij 30 and POESH. While the lower dose of Brij 30 enhanced total PAH removal relative to the controls, the higher dose did not. Brij 30, Span 20, and POESH were particularly effective at enhancing the removal of 4- and 5-ring PAHs (Figure 4.5-Figure 4.7) and therefore were chosen for further evaluation in a followup experiment. Individual PAH removals for EH-3 and R-95 can be found in Figure 4.8 and Figure 4.9 respectively.

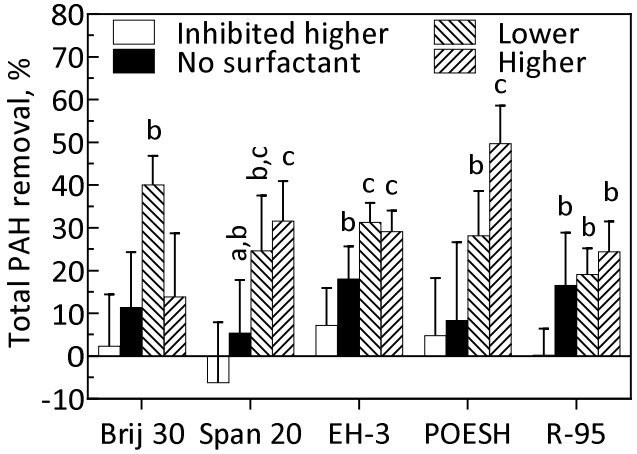

Figure 4.4 Effect of surfactants on residual total PAH from bioreactor-treated soil after 16 days. Surfactant doses are as defined in Materials and Methods. "Inhibited" refers to controls to which sodium azide was added. Bars represent means and standard deviations of five replicates for all surfactants except Span 20 (four replicates). Conditions for which there was not a significant difference (α=0.05) in final PAH concentration detected by Tukey's method are assigned the same letter. Bars for which no letters are shown are implicitly designated "a".

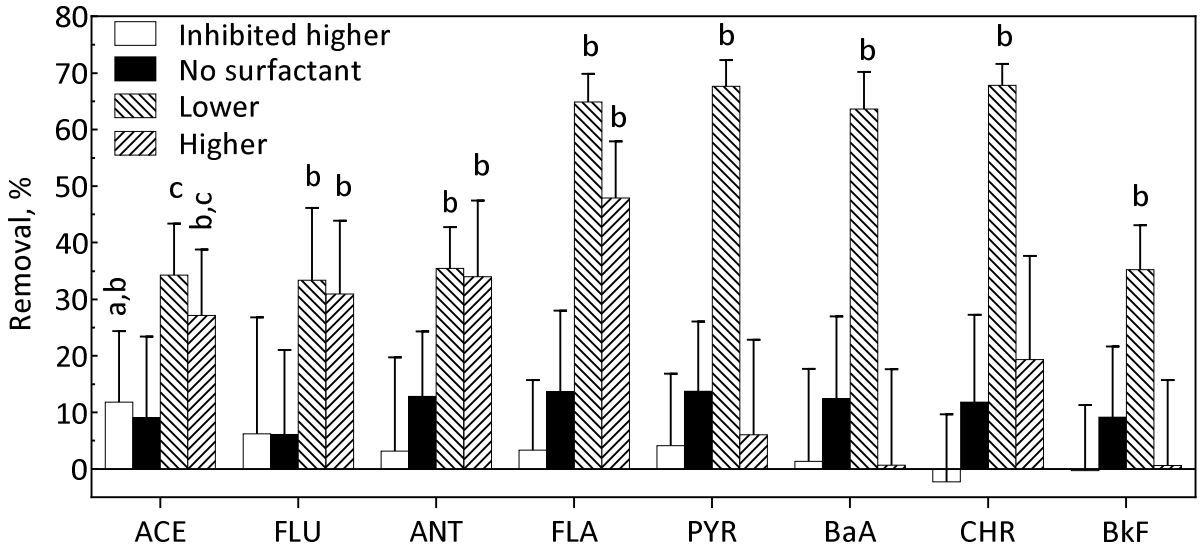

Figure 4.5 Effect of Brij 30 on biodegradation of residual PAHs from bioreactor-treated soil after 16 days. Abbreviations are defined in Table A.1. PAHs for which there were no significant differences between no-surfactant and lower and higher conditions are not shown. Other notes as in Figure 4.4.

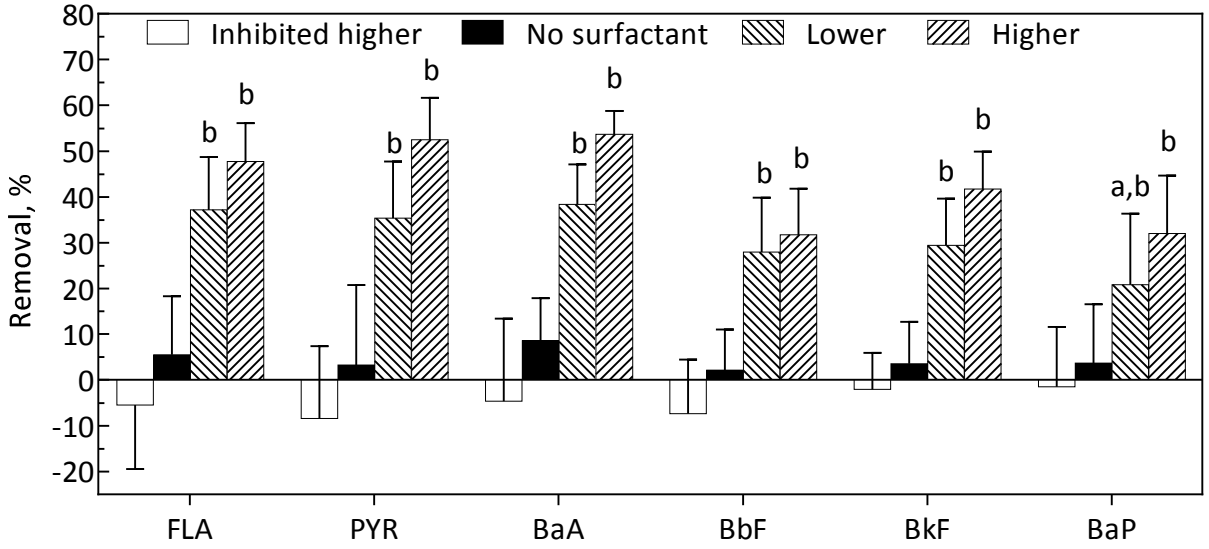

Figure 4.6 Effect of Span 20 on biodegradation of residual PAHs from bioreactor-treated soil after 16 days. Notes as in Figure 4.5.

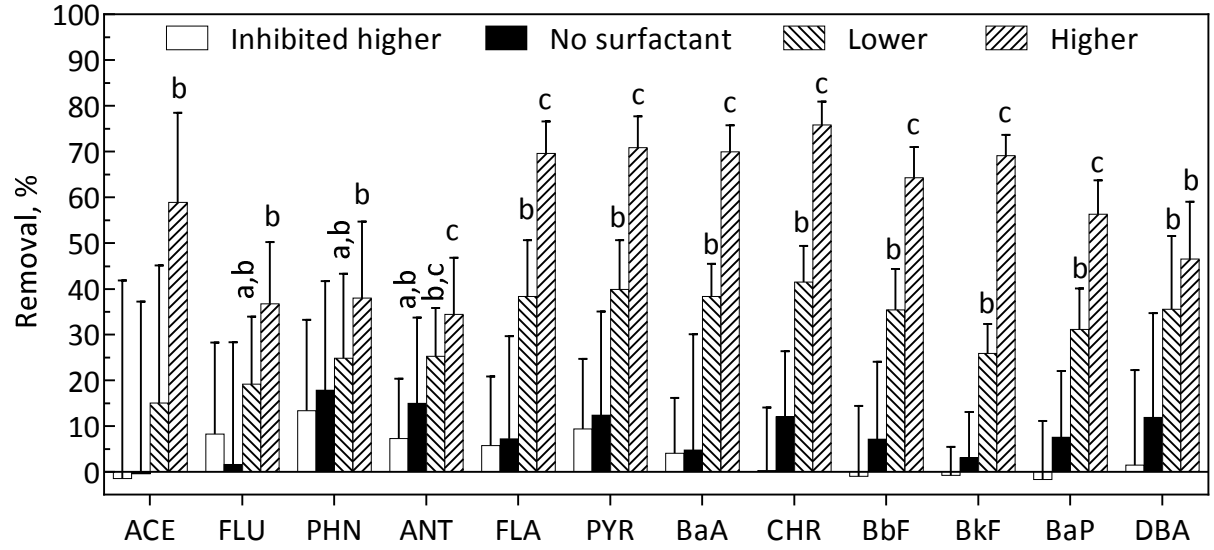

Figure 4.7 Effect of POESH on biodegradation of residual PAHs from bioreactor-treated soil after 16 days. Notes as in Figure 4.5.

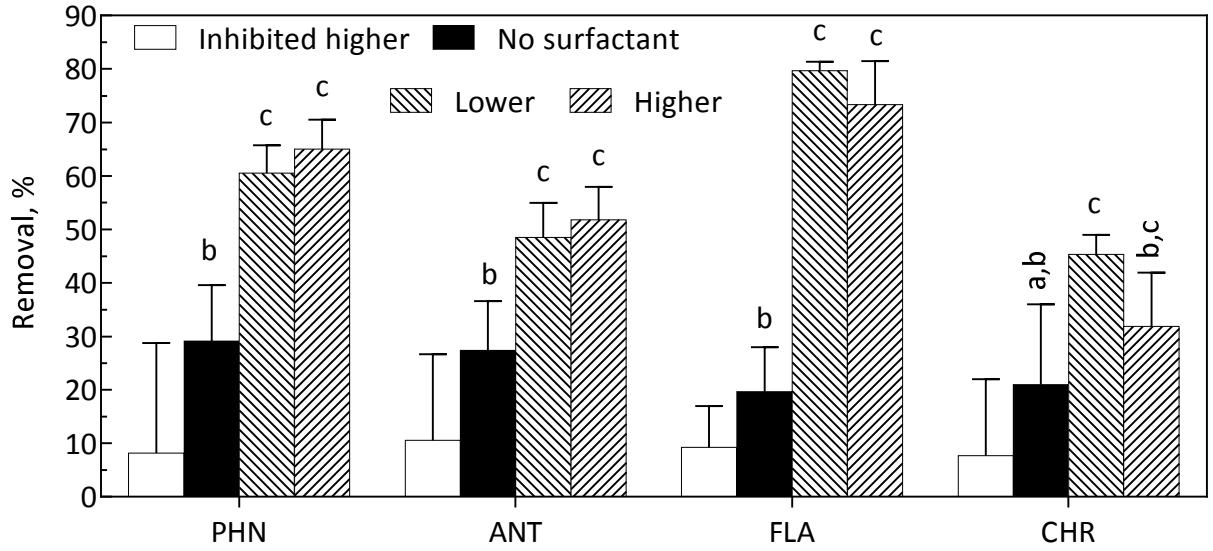

Figure 4.8 Effect of EH-3 on biodegradation of residual PAHs from bioreactor-treated soil after 16 days. Notes as in Figure 4.5.

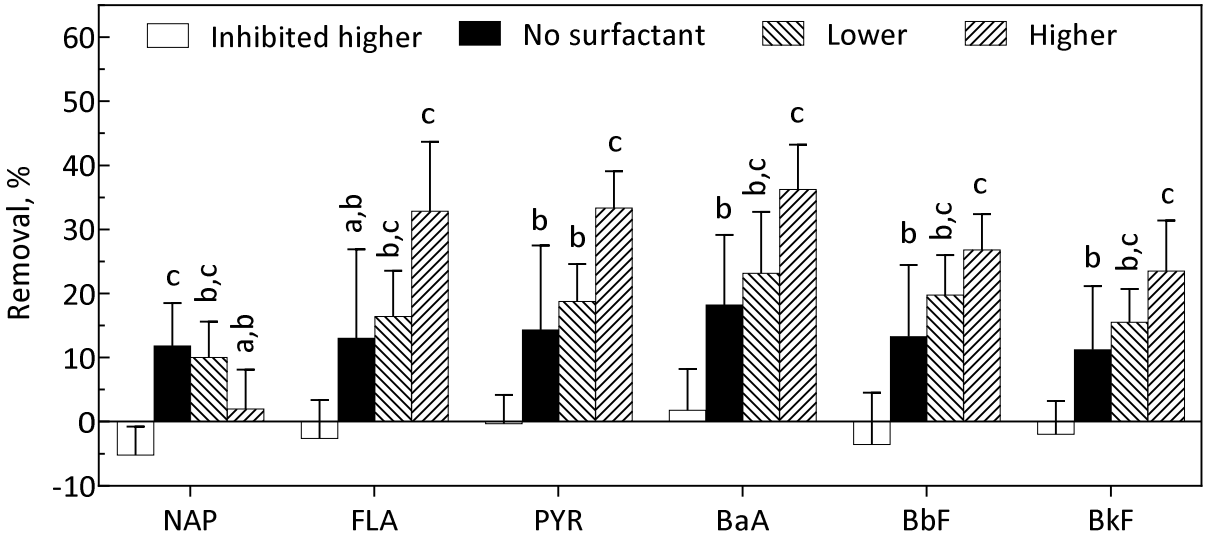

Figure 4.9 Effect of R-95 on biodegradation of residual PAHs from bioreactor-treated soil after 16 days. Notes as in Figure 4.5.

Both doses of Brij 30 and POESH, and the higher dose of Span 20, were evaluated in the followup experiment. Compared to no-surfactant controls, surfactant addition did not significantly improve removal of 2-ring PAHs. Of the 3-ring PAHs, only phenanthrene biodegradation was significantly improved with surfactant addition for both doses of Brij 30 and the higher dose of POESH. All three surfactants enhanced the removal of 4-ring PAHs, although

the higher dose of Brij 30 enhanced the removal of only fluoranthene and chrysene (Figure 4.10). All three surfactants enhanced the removal of 5-ring PAHs except dibenzo[*a,h*]anthracene. Brij 30 at the higher dose, however, either had no significant effect or a significantly negative effect on the removal of 5-ring PAHs. At the end of the incubations, individual concentrations of the 14 PAHs measured by HPLC in the liquid phase were below LLOQ's, corresponding to no more than 5% of the initial mass of any individual PAH.

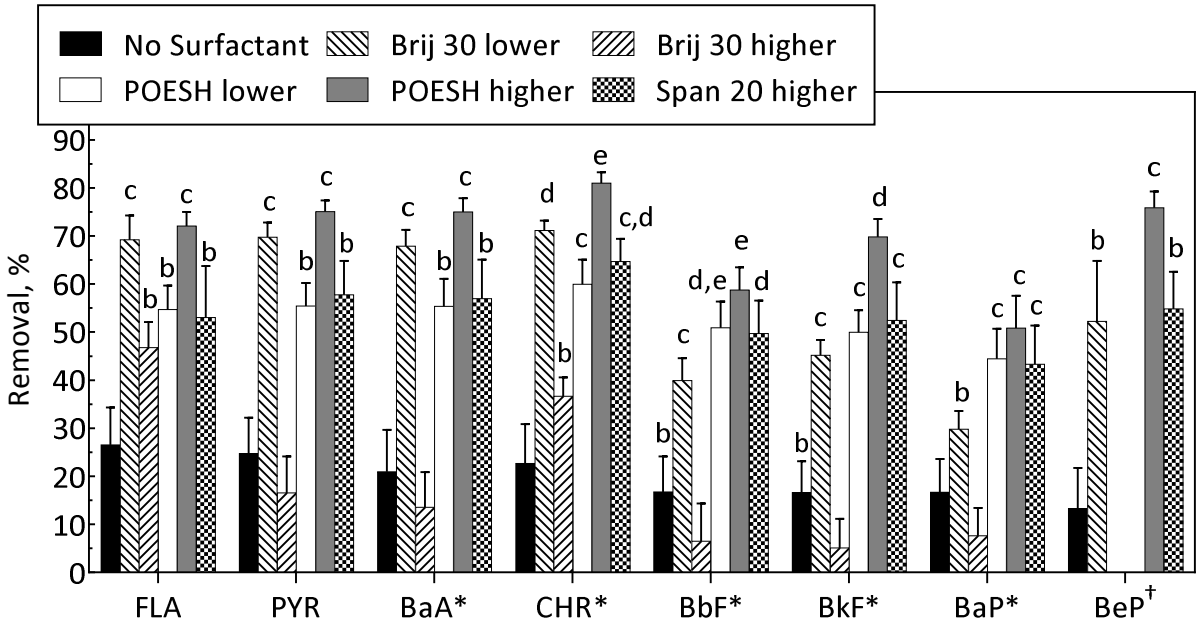

Figure 4.10 Effect of Brij 30, POESH, and Span 20 on residual 4- and 5-ring PAHs from bioreactor-treated soil after 16 days. Abbreviations are defined in Table A.1. Bars represent means and standard deviations of five replicates. Asterisks indicate PAHs designated by EPA as probable human carcinogens. PAHs for which there were no significant differences between no-surfactant controls and all surfactant-conditions are not shown. †BeP measured by GC-MS and not measured in Brij 30 higher or POESH lower. Other notes as in Figure 4.4.

GC-MS analysis of selected conditions from the followup biodegradation experiment revealed increased removal of additional PAHs with surfactant addition. Removals of benzo[*e*]pyrene (Figure 4.10), alkylated 2- and 3-ring PAHs (Figure A.3), and alkylated 4-ring PAHs (Figure 4.11) were significantly enhanced upon surfactant addition. Concentrations of individual PAHs in the followup experiment for the bioreactor-treated soil and soils treated

further with or without surfactant are provided in Table A.8. Overall PAH removals relative to the feed soil for the bioreactor and followup experiments are provided in Table A.9 to illustrate the combined impact of bioreactor treatment plus surfactant amendment as a secondary treatment step.

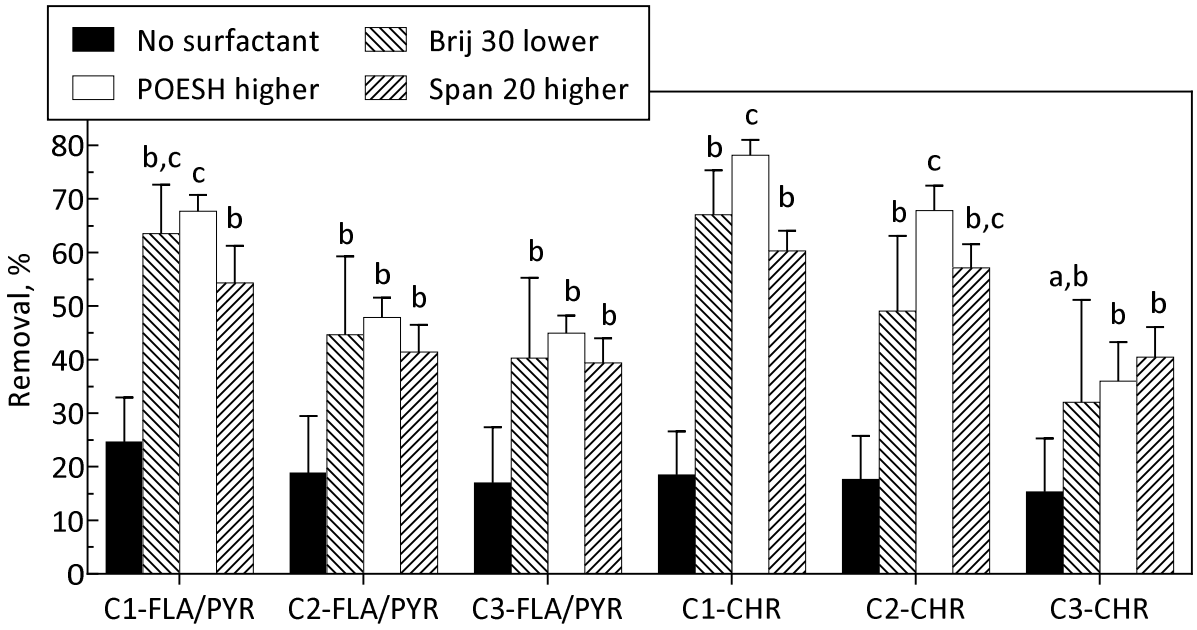

Figure 4.11 Effect of Brij 30, POESH, and Span 20 on biodegradation of residual alkylated 4-ring PAHs from bioreactor-treated soil after 16 days. Other notes as in Figure 4.10.

4.3.3 (Geno)toxicity

The effects of surfactant amendment on bioreactor-treated soil toxicity (LC_{50}) and genotoxicity (relative LC_{50}) were assessed. Solvent extracts from the followup biodegradation experiment were evaluated using the DT40 DNA-damage response assay. All treatments except POESH at the higher dose significantly increased soil toxicity to the parental cell line (Figure 4.12a). Treatment without surfactant significantly decreased soil genotoxicity as evaluated with both mutant cell lines (Figure 4.12b). Brij 30 at the lower dose significantly reduced the genotoxicity as measured using the *Rad54*$^{-/-}$ mutant. Treatment with POESH at the higher dose significantly increased soil genotoxicity as evaluated with the *Rev1*$^{-/-}$ mutant.

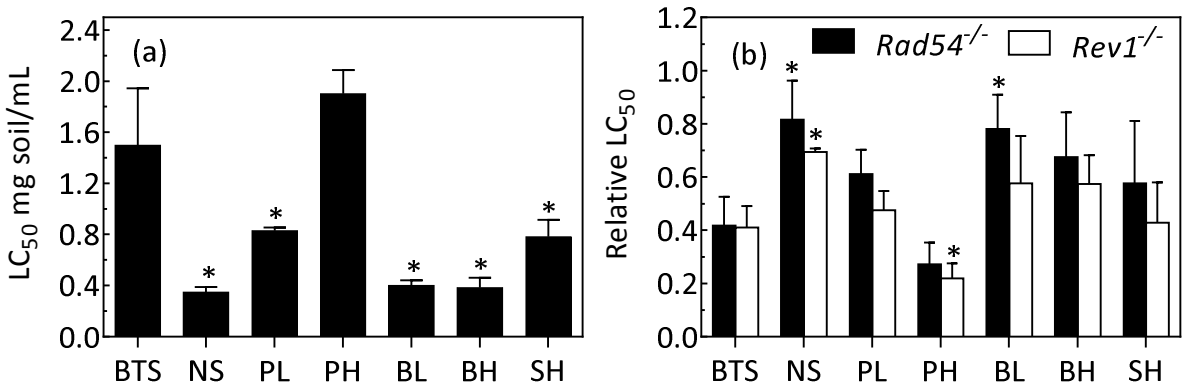

Figure 4.12 Effect of incubation of bioreactor-treated soil with selected surfactants in the followup PAH biodegradation experiment on (geno)toxicity. **(a)** Toxicity to the parental DT40 cell line, and **(b)** genotoxicity as determined by relative LC_{50} values (mutant LC_{50}/parental LC_{50}) using the DNA repair-deficient mutants *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$. Bars represent means and standard deviations of three experiments. An asterisk indicates a significant difference (α=0.05) for a given cell line between a treatment and bioreactor-treated soil. BTS, bioreactor-treated soil; NS, no-surfactant control; P, POESH, B, Brij 30; S, SPAN 20; L, lower surfactant dose; H, higher surfactant dose.

To assess whether POESH could have a significant effect on observed soil genotoxicity in the absence of biodegradation, we evaluated the effect of incubating bioreactor-treated soil with POESH at the higher dose under conditions intended to minimize biodegradation of the PAHs and/or the surfactant (anaerobic incubation without nitrogen amendment). No more than 20% of any individual PAH was removed for the POESH anaerobic condition (Figure A.4) and no significant increase in toxicity or genotoxicity was observed (Figure A.5). In pairwise comparisons with the bioreactor-treated soil before POESH addition, however, neither treatment without surfactant nor treatment with POESH aerobically had a significant effect on soil toxicity or genotoxicity. Since this was contrary to the results of the initial (geno)toxicity experiment, we conducted the DT40 bioassay side-by-side with archived solvent extracts of bioreactor-treated soil, no-surfactant controls, and higher-dose POESH amendment (aerobic) from both experiments. In this reevaluation the increase in toxicity to the parental DT40 cell line for the no-surfactant controls was observed for both experiments (Figure 4.13c). The reduction of

genotoxicity associated with the no-surfactant controls was observed in the *Rad54*$^{-/-}$ mutant for both experiments but was observed in *Rev1*$^{-/-}$ only for the initial (geno)toxicity experiment (Figure 4.13a,b). Higher-dose POESH treatment was associated with a slight, but statistically significant, increase in genotoxicity to *Rad54*$^{-/-}$ only in the followup (geno)toxicity experiment.

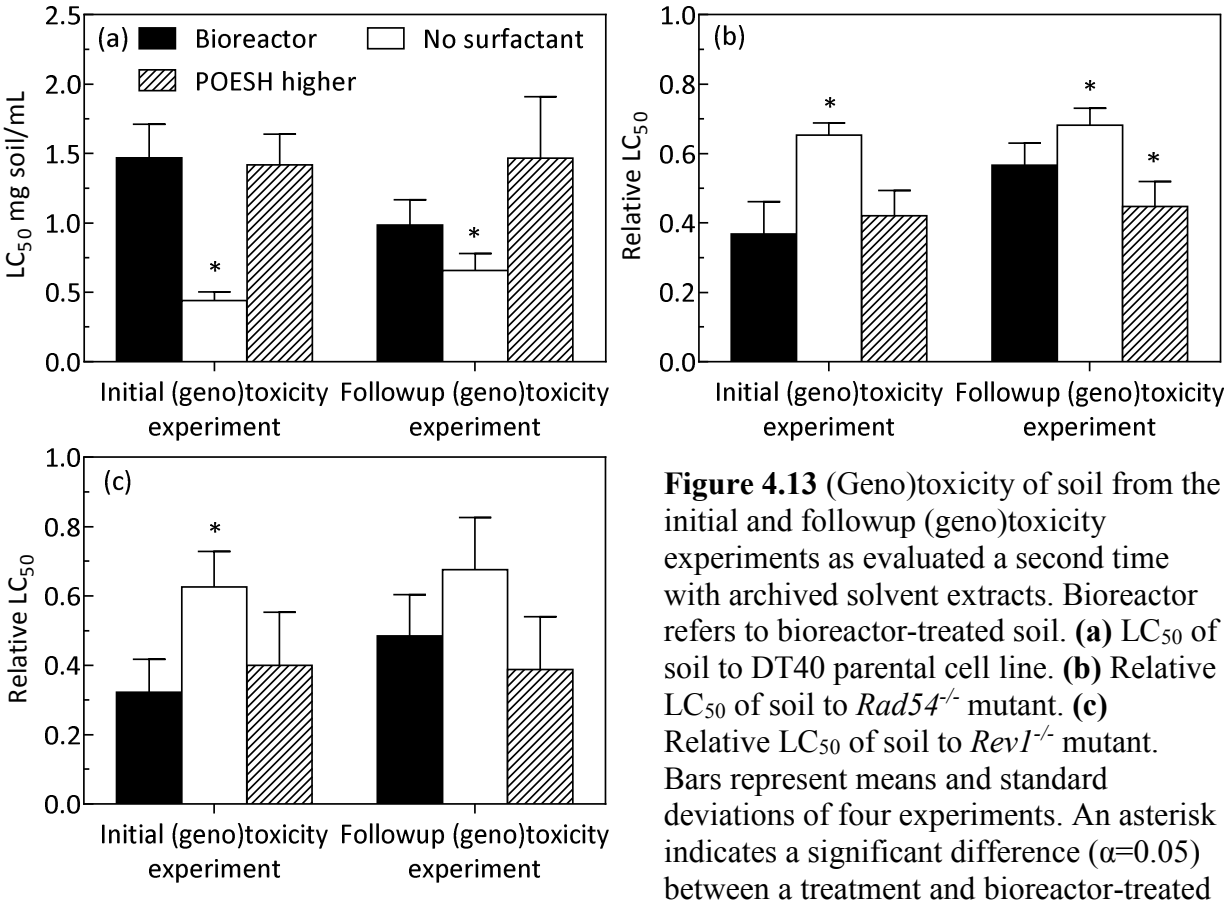

Figure 4.13 (Geno)toxicity of soil from the initial and followup (geno)toxicity experiments as evaluated a second time with archived solvent extracts. Bioreactor refers to bioreactor-treated soil. **(a)** LC_{50} of soil to DT40 parental cell line. **(b)** Relative LC_{50} of soil to *Rad54*$^{-/-}$ mutant. **(c)** Relative LC_{50} of soil to *Rev1*$^{-/-}$ mutant. Bars represent means and standard deviations of four experiments. An asterisk indicates a significant difference (α=0.05) between a treatment and bioreactor-treated

Taken together, the initial and followup (geno)toxicity experiments suggest that further treatment without surfactant increased soil toxicity, but generally reduced genotoxicity. Treatment with POESH at the higher dose was associated either with no significant change or a slight increase in soil genotoxicity.

4.4 Discussion

Although surfactant addition to contaminated soil has been suggested as a means of enhancing the biodegradation of hydrophobic contaminants such as PAHs, most studies do not articulate that the concept is most relevant to the fraction of a given compound that is relatively non-bioavailable or non-bioaccessible. We previously reported that the nonionic surfactant Brij 30 substantially improved the desorption and biodegradation of residual PAHs from contaminated soil that had already undergone aerobic treatment in a slurry-phase bioreactor (Zhu and Aitken 2010). In this study, we extended the concept by comparing five nonionic surfactants of similar hydrophobicities but with different hydrophilic moieties on the removal of residual PAHs from a different contaminated soil after bioreactor treatment.

Incubation of the bioreactor-treated soil with all surfactants at sub-CMC doses resulted in modest increases in PAH desorption but substantial increases in total PAH biodegradation for all surfactants except the R-95 rhamnolipid biosurfactant. The surfactants Brij 30, Span 20, and POESH were particularly effective at enhancing removal of 4- and 5-ring PAHs, including five of the seven PAHs designated by EPA as human carcinogens.

We specifically evaluated sub-CMC doses of each surfactant, at which micellar solubilization of PAHs would be negligible. Enhanced desorption of PAHs at surfactant doses below the apparent CMC in the soil slurry system is consistent with other studies treating field-contaminated soil (Yeom *et al.* 1996; Zhu and Aitken 2010; Frutos *et al.* 2011). Luthy *et al.* (1997) described the three major components in PAH-contaminated soil as soil organic matter (SOM), combustion residue, and non-aqueous-phase liquids (NAPLs). Common NAPLs found at PAH-contaminated sites include coal tar, creosote, and petroleum products such as oil or diesel fuel (Mueller 1996). We assume that relatively hydrophobic nonionic surfactants sorb to these domains in contaminated soil and alter the contaminant matrix in a way that favors desorption or

other means of increasing microbial access to PAHs. Yeom *et al.* (1996) treated coal tar-contaminated soil with several surfactants, including Brij 30, and found substantial increases in phenanthrene desorption under conditions corresponding to aqueous-phase surfactant concentrations below the CMC. The authors attributed this to increased PAH diffusivity within the coal-tar matrix.

During further treatment with or without surfactant, total PAH removal far exceeded the amount desorbed. The difference between desorption and removal was particularly striking for the 4- and 5-ring PAHs. Large differences between measured PAH desorption and removal have been observed in previous studies of bioremediation (Richardson and Aitken 2011; Hu *et al.* 2014) and during surfactant-enhanced bioremediation specifically (Zhu and Aitken 2010). While the impact of surfactant on the functionality of Tenax as an infinite sink was not investigated in this study, an infinite-sink method at best can measure only abiotic desorption into the aqueous phase. Evidence suggests that bacteria can enhance PAH desorption by adhering to hydrophobic contaminant matrices (Mukherji and Ghosh 2012). The rate of PAH mass transfer from geosorbent to adherent cells or biofilm may be faster than the rate of PAH mass transfer into a bulk aqueous phase. It is also possible that the smaller distances between bacteria and geosorbent or the ability of bacteria to enter small soil pores may cause a steeper concentration gradient than can be created with solid resins.

The surfactants we evaluated may have enhanced the rate of PAH biodegradation by increasing the interaction of bacteria with PAH-containing soil compartments. This could occur through increased geosorbent interfacial surface area onto which bacteria may adhere or through modification of cell- or soil-surface properties to favor adhesion. Surfactants can alter cell surface hydrophobicity in ways that can either promote or inhibit bacterial adhesion (Zhang and

Zhu 2014). Rhamnolipids in particular have a concentration-depend effect on cell attachment to both hydrophilic and hydrophobic surfaces (Nickzad and Déziel 2014).

While the two doses of Brij 30 led to comparable PAH desorption, the higher dose led to significantly less PAH removal. This suggests that factors other than abiotic PAH desorption affected biodegradation. In previous work, addition of Brij 30 at doses well above the CMC enhanced only the removal of 3-ring PAHs, while lower doses also enhanced removal of 4- and 5-ring PAHs (Zhu and Aitken 2010). It is possible that differences in PAH removal reflect differences in the microbial community. In the earlier study, there was a reduction in relative abundance of known pyrene degraders at the supra-CMC dose of Brij 30 compared to incubations without surfactant and incubations at sub-CMC doses (Zhu *et al.* 2010).

In work to be published elsewhere, incubations of bioreactor-treated soil under the Brij 30 lower and higher, POESH higher, and no-surfactant conditions were set up to evaluate the effect of surfactant treatment on the bacterial community (Singleton *et al.* in prep.). Bacterial communities were analyzed by denaturing gradient gel electrophoresis (DGGE) and 16S rRNA gene high-throughput sequencing. These methods demonstrated the substantial effect of surfactant addition on the bacterial community and revealed the similarity of bacterial communities under conditions with enhanced PAH removal (Brij 30 lower and POESH higher).

DGGE banding patterns indicated that the bacterial communities of the bioreactor-treated soil and soil treated further without surfactant were similar. The communities from each surfactant-amended condition were different from each other and from those of the bioreactor-treated soil and soil treated further without surfactant. Notably, there were also substantial differences in the banding patterns of Brij 30 lower and Brij 30 higher indicating that surfactant dose had an effect on the bacterial community.

Sequencing results were processed and visualized using non-metric multidimensional scaling (NMDS). NMDS revealed similarities in the bacterial communities of Brij 30 lower and POESH higher (conditions with enhanced PAH removal) and major differences between these two conditions and the Brij 30 higher condition (low PAH removal). NMDS also confirmed the similarity of the bioreactor-treated soil community and the community after further treatment without surfactant and their differences from surfactant-amended conditions. Overall, our results confirm the hypothesis that sub-CMC surfactant addition can enhance PAH desorption and biodegradation in soils in which PAH bioaccessibility is limited. While we did not evaluate any surfactant with an HLB value greater than 10, sub-CMC doses of more-hydrophilic surfactants tested in previous studies did not enhance biodegradation (Kim and Weber 2005; Lei *et al.* 2005; Zhu and Aitken 2010). More-hydrophilic surfactants may have a lower affinity for the hydrophobic PAH-containing soil compartments, so that the CMC may be reached before a substantial amount of surfactant interacts with these compartments.

The genotoxicity of the bioreactor-treated soil determined in this study is consistent with our previous studies treating the same contaminated source soil (Hu *et al.* 2012; Hu *et al.* 2014). The *Rad54*$^{-/-}$ and *Rev*$^{-/-}$ mutants used in this study are deficient in the Rad54 and Rev1 proteins, respectively. These two proteins are implicated in the repair or tolerance of damage caused by the major types of PAH-induced genotoxicity: strand breaks caused by oxidative stress or during base excision repair of other DNA damage (Rad54), and adduction of DNA by stable or depurinating adducts (Rev1) (Friedberg 2006; Penning 2011). In general, however, increased PAH removal from the bioreactor-treated soil did not correspond to a reduction in soil toxicity or genotoxicity. Amendment with POESH at the higher dose removed substantial amounts of 4- and 5-ring PAHs, including some considered to be human carcinogens, yet had either no effect or

caused a slight increase in genotoxicity. Meanwhile, further treatment of the bioreactor-treated soil without surfactant removed less than 30% of any 4- or 5-ring PAH, but resulted in a significant reduction in soil genotoxicity. This finding may imply that an increased residence time led to the removal of genotoxic constituents present in the treated soil obtained from the bioreactor. Overall, the (geno)toxicity of remediated soil will depend both on the remaining parent compounds and the formation or removal of any products of incomplete metabolism. The bioavailability of any genotoxic metabolites formed as a result of biological treatment of contaminated soil must also be taken into account when evaluating the efficacy of bioremediation (Hu *et al.* 2014). The bioavailability of residual contaminants at the end of incubations with surfactants, however, was not evaluated in the present study.

This work demonstrated the effectiveness of surfactant-amended treatment for enhanced biodegradation of the residual, less bioaccessible fraction of PAHs in soil after primary treatment in a conventional bioreactor. Employing this two-stage treatment process could increase the likelihood of meeting site cleanup goals, which are typically based on the concentrations of PAHs in the soil independent of their bioavailability or bioaccessibility. The observation that parent PAH removal did not necessarily correspond to a reduction in genotoxicity, however, highlights the need for further research to identify genotoxic products to improve risk assessment and remediation strategies.

CHAPTER 5: IMPROVING PAH BIODEGRADATION IN CONTAMINATED SOIL THROUGH SECOND-STAGE TREATMENT IN A SURFACTANT-AMENDED BIOREACTOR

5.1 Introduction

Polycyclic aromatic hydrocarbons (PAHs) are among the top 10 contaminants of concern at Superfund sites in the U.S. (ATSDR 2011). Over 600 current sites on the U.S. Environmental Protection Agency (EPA) National Priorities List are contaminated with PAHs, of which over 400 contain PAH-contaminated soil (EPA 2015). Bioremediation is one option for the treatment of PAH contaminated soil, but can be limited by the incomplete removal of target PAHs (Aitken and Long 2004), which can lead to failure to meet site-specific cleanup goals.

Due to their hydrophobicity, PAHs are often strongly associated with non-polar soil domains such as soil organic matter, combustion residue, and non-aqueous-phase liquids, and therefore may be unavailable to degrading microorganisms (Cornelissen *et al.* 1998; Huesemann *et al.* 2004; Richardson and Aitken 2011). A 1993 US EPA case study concluded that slurry-phase bioreactor treatment could be used to effectively remediate PAH-contaminated soil but that "effective desorption of compounds from weathered soil can be an intractable problem" (USEPA 1993). Application of surfactants has been proposed to enhance the bioavailability of PAHs to degrading organisms with the goal of increasing PAH removal in contaminated soils and is the subject of several review articles (Makkar and Rockne 2003; Li and Chen 2009; Cameotra and Makkar 2010; Elliot *et al.* 2011; Bustamante *et al.* 2012). Surfactants can increase the rate of PAH desorption from soil at aqueous-phase concentrations both above (Tiehm *et al.*

1997; Grasso *et al.* 2001) and below (Yeom *et al.* 1996; Zhu and Aitken 2010; Frutos *et al.* 2011) their critical micelle concentrations (CMC).

Previous research on field-contaminated soil suggests that surfactant addition is most beneficial for systems in which PAH biodegradation is limited by low bioaccessibility. This would be the case, for example, with soil treated in a conventional bioremediation system for which residual PAH concentrations might exceed cleanup targets (Zhu and Aitken 2010; Bueno-Montes *et al.* 2011). Studies in which surfactant-free controls exhibit negligible PAH removal, tend to demonstrate positive effects of surfactant addition (Tiehm *et al.* 1997; Di Gennaro *et al.* 2008; Zhu and Aitken 2010; Bueno-Montes *et al.* 2011). If these surfactant-free controls perform poorly due to limited PAH bioaccessibility, then surfactant-enhanced desorption may explain improved biodegradation. Although there is a cost savings associated with using less surfactant, there has been limited work on surfactant-amended bioremediation of PAH-contaminated field soil at doses corresponding to aqueous phase surfactant concentrations below the CMC in the soil slurry system (sub-CMC doses) (Kim and Weber 2005; Lei *et al.* 2005; Zhu and Aitken 2010).

We recently screened five nonionic surfactants at sub-CMC doses on the test-tube scale for their ability to enhance the biodegradation of the residual PAHs remaining in soil after bench-scale aerobic, slurry-phase bioremediation (Adrion *et al.* in prep.). Polyoxyethylene sorbitol hexaoleate (POESH) surfactant was most effective at enhancing the removal of the residual high-molecular-weight (HMW) PAHs compared to further incubation for the same amount of time without surfactant. Despite substantial removal of PAHs, including 5 of the 7 PAHs considered probable human carcinogens by EPA, POESH-amended treatment was associated with an increase in soil genotoxicity. In this study we expand on our previous work by

utilizing POESH at sub-CMC doses to treat effluent from the slurry-phase bioreactor in a second-stage batch bioreactor to which POESH was added. The objectives of the present study were to evaluate the reproducibility of the two-stage treatment concept at larger than test-tube scale; to compare two different residence times in the second-stage bioreactor; to quantify the effect of two-stage treatment on the removal of selected oxy-PAHs; and to evaluate the effect of two-stage treatment on soil toxicity and genotoxicity.

5.2 Materials and methods

5.2.1 Experimental design

In our previous work screening the effects of different nonionic surfactants on PAH removal from soil after treatment in a semi-continuous, slurry-phase bioreactor (Adrion *et al.* in prep.), a POESH dose of 24 mg/g dry soil corresponded to an aqueous-phase surfactant concentration well below the CMC (260 mg/L) and a negligible fraction of total PAH mass present in the aqueous phase after surfactant addition. We selected the same dose of POESH to implement the two-stage treatment concept in the present study.

In the previous screening experiments, the slurry removed from the bioreactor was centrifuged, resuspended in fresh buffer, and amended with surfactant at the test-tube scale; tubes were shaken vigorously on an orbital shaker. In the present study, the whole slurry removed as effluent from the bioreactor (*i.e.*, not centrifuged and resuspended in buffer) served as influent to a second-stage, batch bioreactor to which POESH was added; mixing was accomplished with a metal stir bar on a magnetic mixer. To permit sorption of the surfactant to the soil before commencing aerobic biodegradation (Zhu and Aitken 2010; Adrion *et al.* in prep.), the surfactant-amended slurry in the second-stage reactor was mixed under a continuous stream of N_2 for 48 hours before aerobic conditions were established.

Three trials of second-stage batch treatment were conducted at a residence time of 7 days, and three trials were conducted at a residence time of 12 day. Residence times were chosen based on preliminary test-tube scale experiments indicating that most PAH removal (Figure B.1) and changes in genotoxicity (Figure B.2) occurred between day 3 and day 21. For each trial, a different batch of effluent soil-slurry from the first-stage bioreactor was used (6 different batches altogether). Because the first-stage bioreactor was operated in a manner that produced effluent once a week (described below), the trials were conducted over six consecutive weeks; trials at 7-day residence time were alternated with the trials at 12-day residence time.

5.2.2 Chemicals

PAH standards (EPA 610 PAH mixture and individual PAHs), 9,10-phenanthrenequinone (PQ), 9,10-anthraquinone (AQ), polyoxyethylene sorbitol hexaoleate (POESH), and dimethyl sulfoxide (DMSO) were obtained 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 other solvents were high-performance liquid chromatography (HPLC) grade and were obtained from either Fisher Scientific (Pittsburgh, PA, U.S.A.) or VWR International (Radnor, PA, U.S.A.).

5.2.3 First-stage treatment

Contaminated soil used as feed for the first-stage bioreactor was collected from a former manufactured-gas plant (MGP) site in Salisbury, North Carolina, processed, and characterized as described elsewhere (Richardson and Aitken 2011; Hu *et al.* 2012). Feed soil underwent first-stage treatment in a semi-continuous, laboratory-scale aerobic bioreactor. The bioreactor was made of stainless steel, had a working volume of approximately 2 L, a solids content of 15% (w/w), and average solids retention time of 35 days. Every seven days, 400 mL of the treated slurry was replaced with a slurry of feed soil in a pH 7.5 buffer containing 5-mM phosphate and

2.5-mM ammonium nitrate (bioreactor buffer). Each week, 100 mL of the effluent slurry was analyzed for PAHs and oxy-PAHs as described below. The remaining 300 mL of effluent slurry served as influent to the second-stage bioreactor.

5.2.4 Preliminary experiments

We conducted a preliminary experiment to test the selected dose of POESH at test-tube scale using whole slurry from the first-stage bioreactor, rather than centrifuged slurry that had been resuspended in buffer, to ensure that the sorptive properties of the soil had not changed since the previous screening experiments (Adrion *et al.* in prep.). Slurry from the first-stage bioreactor was centrifuged at 3900 rpm for 30 minutes and the supernatant was collected. A 2.08-g (dry weight) aliquot of the centrifuged soil was added to each of triplicate 30-mL glass centrifuge tubes with PTFE-lined silicon septa screw-caps. POESH (50 mg) was added to each tube. Supernatant was then added back to each of the tubes to bring the solids content to 15% (w/w). The tube headspace was evacuated and replaced with nitrogen, then the tube was put on an orbital shaker at 275 rpm for 48 hours. After 48 hours, the tubes were centrifuged at 3900 rpm for 30 minutes. The supernatants were syringe-filtered through 0.8-µm polycarbonate membrane filters and analyzed for pyrene concentration by HPLC and surfactant concentration by measuring surface tension as described in (Adrion *et al.* in prep.).

5.2.5 Second-stage treatment

Two second-stage batch bioreactors were set up in parallel to accommodate treatment of the weekly effluent removed from the first-stage bioreactor. Each second-stage bioreactor was a 1-L glass filter flask, fitted with a stainless steel tube piercing a silicon stopper at the top of the flask to provide gas input to the headspace; the side-arm was left open to allow gas to escape.

Every week, 300 mL of first-stage treated slurry was transferred to a second-stage reactor. The slurry was stirred on a magnetic stir plate using a 3.2-cm metal stir-bar at

approximately 750 rpm. To add surfactant, 1.34 g of POESH was added to a 30-mL glass centrifuge tube and mixed with 10 mL of deionized water before being transferred to the second-stage reactor; this mass corresponded to a dose of 24-mg POESH per g dry soil. An additional 10 mL of deionized water was then used to rinse the remaining mass of POESH into the reactor. The surfactant-amended slurry was allowed to mix under a stream of nitrogen delivered through the gas tube at a flow rate of 60 mL/min for 48 hours. After 48 hours, a 10-mL aliquot of slurry was removed from the reactor while mixing using a glass pipette, transferred to a 30-mL glass centrifuge tube with PTFE-lined cap, and centrifuged at 3900 rpm for 30 minutes. The supernatant was syringe-filtered through a 0.8-µm polycarbonate membrane filter to measure the aqueous-phase surfactant and pyrene concentrations as described in (Adrion *et al.* in prep.).

After the 48-hour anoxic mixing period, the nitrogen flow was discontinued and air was delivered continuously through the gas tube for the duration of the aerobic treatment period (either an additional 7 days or 12 days, as explained above).

5.2.6 Slurry extraction and analysis

Slurry from the first- and second-stage bioreactors was centrifuged at 3900 rpm for 30 minutes. For each batch of slurry, a single 10-mL aliquot of the supernatant was syringe-filtered through a 0.8-µm polycarbonate membrane filter to measure the aqueous-phase surfactant and PAH concentrations as described in (Adrion *et al.* in prep.). Four 3-g aliquots of the wet centrifuged soil were each mixed with 10 g of sodium sulfate and solvent-extracted overnight twice, each time with 10 mL of acetone and 10 mL of dichloromethane, and analyzed for 14 PAHs by HPLC with fluorescence detection as described elsewhere (Richardson *et al.* 2011). Four oxy-PAHs were analyzed by liquid chromatography-mass spectrometry as described in Chapter 3 of this dissertation. Soil moisture content was determined in triplicate by heating 1-g wet weight aliquots of centrifuged soil in aluminum weigh dishes at 105°C for 24 hours.

Untreated soil used to feed the first-stage bioreactor was prepared for analysis by slurrying in bioreactor buffer, centrifuging, and extracting the centrifuged soil as described above. Concentrations of PAHs and oxy-PAHs in the feed soil and bioreactor treated soils are provided in Table A.1-Table A.3.

5.2.7 (Geno)toxicity analysis

The effects of the two-stage treatment process on soil toxicity and genotoxicity were evaluated using the solvent extracts from the first-stage and second-stage treated slurries and from the untreated feed slurry. For each soil slurry sample, 10-mL aliquots from each of the four replicate solvent extracts were combined in a preweighed vial and evaporated to dryness under a gentle stream of nitrogen. Residue mass was then determined gravimetrically. Toxicity of the residues reconstituted in DMSO was evaluated in triplicate using a 96-well plate-based DT40 chicken lymphocyte DNA-damage response assay adapted from Ridpath *et al.* (2011) and Hu *et al.* (2012). The untreated feed soil was evaluated in quadruplicate. The *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$ DNA-repair deficient mutants were tested alongside the isogenic DT40 parental cell line because of their reported sensitivity to soil residue in previous experiments (Hu *et al.* 2012; Adrion *et al.* in prep.). The *Rad54*$^{-/-}$ knock–out is deficient in the homologous recombination DNA repair pathway, while the *Rev1*$^{-/-}$ knock-out is deficient in the translesion synthesis pathway. LC_{50} values (mg residue/mL in-well fluid) were calculated by fitting the log residue concentration vs % survival in GraphPad Prism version 6.05 for Windows. The LC_{50} values as measured in residue mass were converted to equivalent soil LC_{50} values (mg soil/mL in-well fluid). The relative LC_{50} for each mutant cell line (LC_{50} of the mutant divided by the LC_{50} of the parental cell line) was calculated as a measure of genotoxicity as described in Hu *et al.* (2012). Values of relative LC_{50} less than 1.0 are indicative of genotoxicity.

5.2.8 Data analysis

Averages and standard deviations of percent removals (summary statistics) for each trial were calculated through propagation of error using the concentrations of PAHs and oxy-PAHs in the second-stage treated soil and in the corresponding batch of first-stage treated soil. All statistical analysis was done in SAS Enterprise Guide 6.1 (SAS Institute, Cary, NC, U.S.A.). Comparisons of concentrations of PAHs and oxy-PAHs in soil before and after second-stage treatment were conducted using two-sample t-tests (two-tail, homoscedastic, $\alpha = 0.05$). To compare removals among all the trials; for each PAH, a one-way ANOVA followed by Tukey's Studentized range test ($\alpha=0.05$) was conducted using summary statistics of percent removal. One-sample t-tests were conducted on each value of relative LC_{50} for the feed soil and first-stage treated soil to determine whether the values were significantly different than 1.0 (two-tail, homoscedastic, $\alpha = 0.05$). Comparisons were made between the LC_{50}'s and relative LC_{50}'s of second-stage treated soil with those of the corresponding first-stage treated soil for each trial using two-sample t-tests (two-tail, homoscedastic, $\alpha = 0.05$).

5.3 Results

5.3.1 Preliminary experiments

A preliminary test-tube scale experiment was conducted to confirm that adding POESH to whole slurry removed from the first-stage bioreactor would be similar to our observations in prior work (Adrion *et al.* in prep.), in which the slurry from the first-stage bioreactor was centrifuged and the soil resuspended in fresh buffer. The aqueous-phase POESH concentration after48 hours of anoxic mixing was 3.4 ± 0.6 mg/L, well below the CMC, and the pyrene concentration was less than the lower limit of quantification (LLOQ) of 29 µg/L. However, both aqueous-phase POESH and pyrene concentrations were substantially higher after 48 hours of anoxic mixing in the second-stage bioreactors than in the test-tube scale incubations (Table 5.1),

although in all trials the aqueous-phase POESH concentrations were below the nominal CMC. After 48 hours of anoxic mixing in the second-stage bioreactors, pyrene was consistently present at aqueous-phase concentrations above its reported pure-compound solubility in water (Mackay 1992), thus indicating solubilization of pyrene. By the end of all trials, however, all aqueous-phase surfactant concentrations were less than the LLOQ of 3.3 mg/L. Likewise at the end of all trials, individual aqueous-phase PAH and oxy-PAH concentrations were less than their respective LLOQ's; this corresponded to PAH and oxy-PAH masses present in the aqueous-phase less than 3% of the residual mass in the first-stage treated soil except for ACE, which was less than 12%.

Table 5.1 Aqueous-phase surfactant and pyrene concentrations after 48 hours of anoxic mixing of first-stage bioreactor effluent prior to starting aerobic conditions in the second-stage bioreactor.

	7-day		12-day	
Trial	POESH (mg/L)	Pyrene (µg/L)	POESH (mg/L)	Pyrene (µg/L)
1	135	157*	62	89
2	172	559*	196	612*
3	135	265*	68	288*
Mean ± S.D.	147 ± 21	327* ± 208	108 ± 76	330* ± 264

An asterisk indicates liquid-phase pyrene concentration in excess of its pure-compound aqueous solubility of 132 µg/L (Mackay 1992). S.D. is standard deviation.

5.3.2 PAH and oxy-PAH removal

First-stage treatment of PAH-contaminated soil removed substantial amounts of PAHs and oxy-PAHs, as shown in Table B.4 and Table B.5. Second-stage batch treatment with the nonionic surfactant POESH for either 7 or 12 days resulted in substantial removal of the residual PAHs and oxy-PAHs remaining in the effluent from the first-stage bioreactor (Figure 5.1). Removal was most substantial for the 4-ring PAHs (FLA, PYR, BaA, and CHR). Removal of

naphthalene was low (<20%) and not consistently significant. Second-stage treatment removed significant amounts of the 5-ring PAHs (BbF, BkF, and BaP), but removal was more variable across trials than for the other PAHs. For the 5-ring DBA, removal was low (<20%) and not consistently significant. The 6-ring BgP, which had the highest concentration of any measured PAH in the first-stage bioreactor effluent, was not significantly removed in any second-stage trial.

Comparing removals between the two residence times, differences were most substantial for the 5-ring PAHs, BbF, BkF and BaP (Figure 5.2). Cumulative removals relative to the untreated feed soil for the combined first- and second-stage treatment are presented in Table B.4 and Table B.5 to illustrate the overall impact of the two-stage treatment concept.

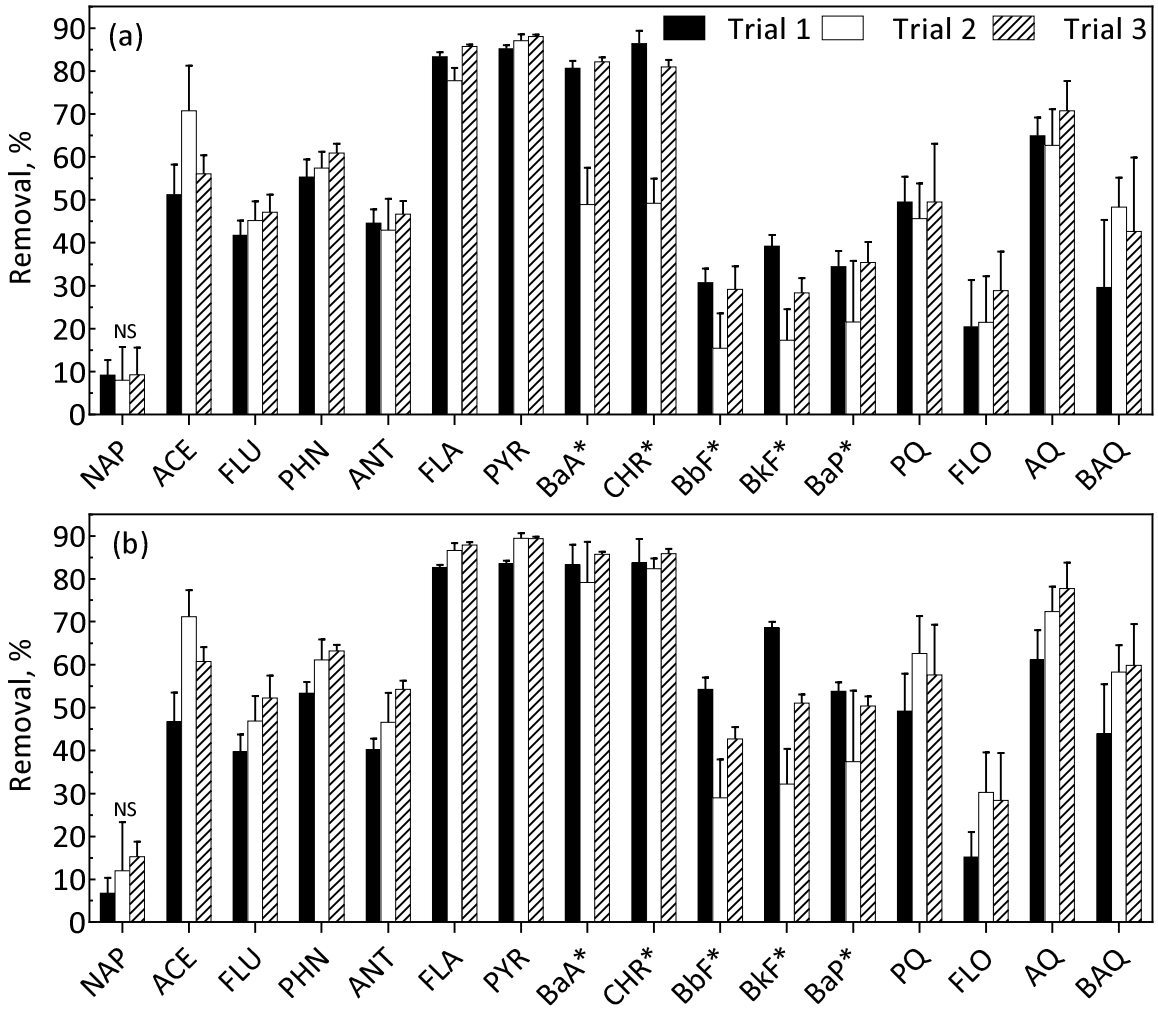

Figure 5.1 Effect of second-stage treatment on residual PAHs and oxy-PAHs in effluent from the first-stage bioreactor at a residence time of **(a)** 7 days and **(b)** 12 days. Asterisks indicate probable human carcinogens. Bars represent means and standard deviations of four replicate extractions. Compounds for which there was not a significant difference in concentration after second-stage treatment in at least 2 out of 3 trials are not shown (α=0.05). Abbreviations are defined in Table B.1. NS indicates a trial for which the concentration was not significantly different after second-stage treatment.

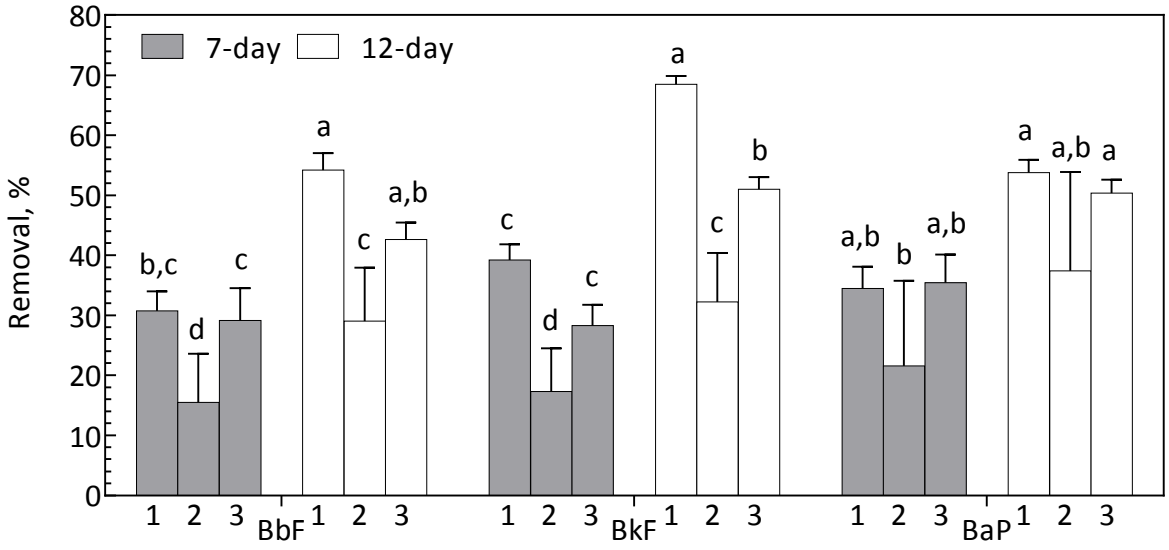

Figure 5.2 Effect of second-stage residence time on the removal of selected 5-ring PAHs. Bars represent means and standard deviation of four replicate extractions. For each PAH, conditions for which there was not a significant difference (α=0.05) in removal detected by Tukey's method are assigned the same letter. Numbers below the bars correspond to the three trials at a given residence time.

5.3.3 (Geno)toxicity

Cytotoxicity and genotoxicity were determined with the DT-40 chicken lymphocyte assay; cytotoxicity was evaluated with the parental cell line and genotoxicity with the DNA repair-deficient mutants, *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$. All second-stage trials reduced the cytotoxicity of the soil relative to the first-stage effluent (Figure 5.3a). The average relative LC_{50}'s of the first-stage treated soil across the 6 trials were 0.71 ± 0.08 for the *Rad54*$^{-/-}$ mutant and 0.79 ± 0.09 for the *Rev1*$^{-/-}$ mutant. Both relative LC_{50} values were significantly less than 1.0, indicating that the effluent from the first-stage bioreactor was genotoxic. Relative to first-stage treated soil, all second-stage trials at the 7-day residence time increased genotoxicity as measured with the *Rev1*$^{-/-}$ mutant (Figure 5.3c). Only trial 2 of the 7-day treatment trials significantly increased genotoxicity as measured with the *Rad54*$^{-/-}$ mutant (Figure 5.3b). Trials 1 and 2 of the 12-day second-stage treatment significantly increased genotoxicity as measured with both mutants, while no significant effect was observed in trial 3. In general, both first- and second-stage

treatment increased the genotoxicity of soil, a result that was not substantially improved with increased second-stage treatment time from 7 days to 12 days. There was insufficient evidence of genotoxicity for the untreated feed soil as measured with either of the DT-40 mutant cell lines because the relative LC_{50}'s were not statistically significantly different than 1.0 (Figure 5.3b,c).

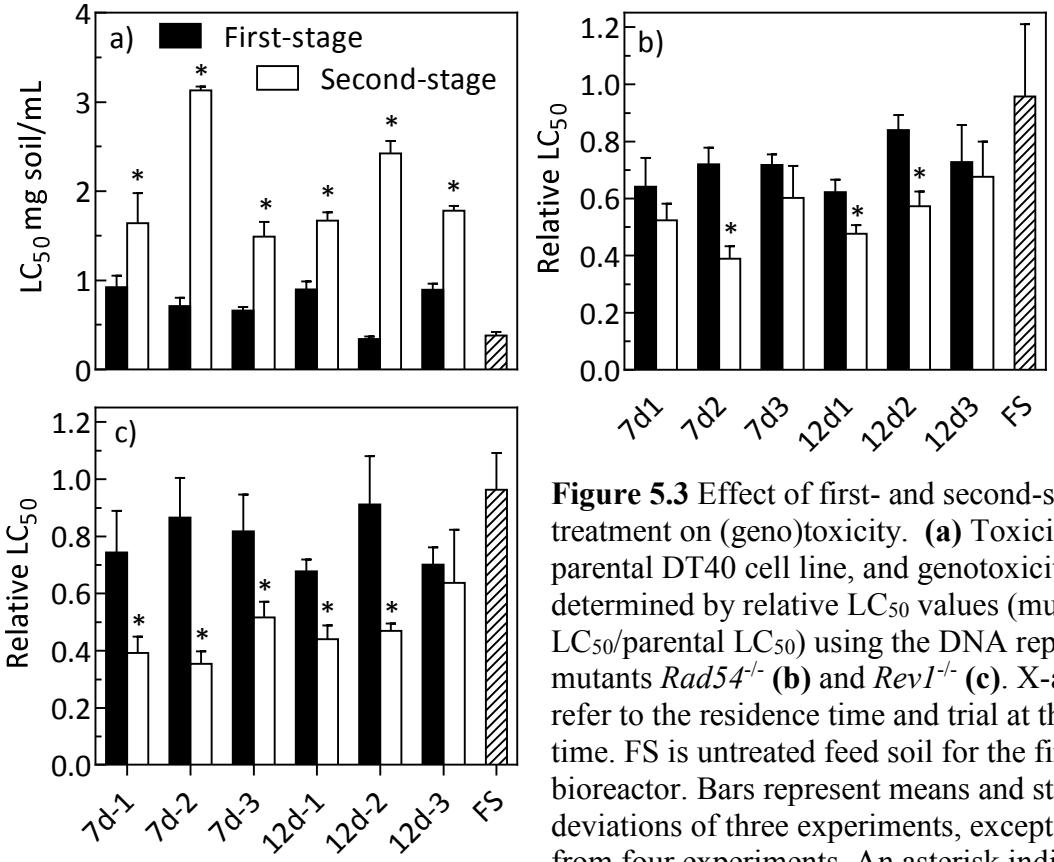

Figure 5.3 Effect of first- and second-stage treatment on (geno)toxicity. **(a)** Toxicity to the parental DT40 cell line, and genotoxicity as determined by relative LC_{50} values (mutant LC_{50}/parental LC_{50}) using the DNA repair-deficient mutants *Rad54*$^{-/-}$ **(b)** and *Rev1*$^{-/-}$ **(c)**. X-axis labels refer to the residence time and trial at that residence time. FS is untreated feed soil for the first-stage bioreactor. Bars represent means and standard deviations of three experiments, except FS which is from four experiments. An asterisk indicates a significant difference (α=0.05) between first and second-stage treatment.

5.4 Discussion

Although the addition of surfactants has been suggested as a means of enhancing the biodegradation of hydrophobic contaminants such as PAHs in soil, few studies have emphasized that the benefits are most likely to be manifested in systems limited by low bioaccessibility. In separate studies using PAH-contaminated soils from two different sites, we reported that

nonionic surfactants substantially improved the desorption and biodegradation of residual PAHs from contaminated soil that had already undergone aerobic treatment in a lab-scale bioreactor (Zhu and Aitken 2010; Adrion *et al.* in prep.). We have consistently found the fraction of PAHs remaining after first-stage treatment to be of low bioaccessibility (Zhu and Aitken 2010; Hu *et al.* 2014; Adrion *et al.* in prep.) and in previous studies found that further treatment without surfactant led to limited PAH removal (Zhu and Aitken 2010; Adrion *et al.* in prep.). In this study, we extended the concept by evaluating the reproducibility of a semi-continuous two-stage treatment process at the bench-scale.

Additional PAH removal observed during POESH-amended treatment of effluent slurry from the first-stage bioreactor was consistent with our previous study, particularly the substantial removal of 4-ring PAHs (Adrion *et al.* in prep.). In addition to the PAHs, removal of the four oxy-PAHs we evaluated was also enhanced by the addition of POESH. Oxy-PAHs are of concern because some are known to exhibit toxic or genotoxic effects (Chesis *et al.* 1984; Bolton *et al.* 2000; Zielinska-Park *et al.* 2004; Luo *et al.* 2011), and may also inhibit the biodegradation of parent PAHs (Kazunga and Aitken 2000; Kazunga *et al.* 2001). Of these four compounds, AQ was present in the initial (untreated) soil at the highest concentration, 24 μg/g (Table B.1); overall removal of AQ in the two-stage process was 95% (Table B.4 and Table B.5).

Despite the fact that the oxy-PAHs were more desorbable than the unsubstituted PAHs in the effluent soil from the first-stage bioreactor (Hu *et al.* 2014), the addition of POESH appeared to improve their bioaccessibility as well. Although none of the oxy-PAHs increased in concentration, net increases of some oxy-PAHs have been observed during bioremediation (Lundstedt *et al.* 2003) and researchers have inferred the formation of oxy-PAH based on ratios of oxy-PAH to parent compound (Wilcke *et al.* 2014).

The most substantial removal of 3- and 4-ring PAHs occurred within the first week of second-stage treatment, as evidenced by the limited differences between 7-day and 12-day batch treatment. Extending the residence time of the second-stage bioreactor to 12 days from 7 days led to greater average removal of the 5-ring compounds BbF, BkF, and BaP, although variability in the data (particularly in trial 2) made statistical comparisons less conclusive. As discussed in our previous work, we assume that POESH sorbs to and facilitates PAH desorption from the non-polar domains found in MGP soil (*e.g.*, coal tar and black carbon) (Adrion *et al.* in prep.). Hypothesized effects of surfactants on these soil domains include increased PAH diffusivity within the coal-tar matrix (Yeom *et al.* 1996), increased interfacial surface area caused by wetting (Dong *et al.* 2003), and dispersion of non-polar matrices (Kile and Chiou 1989; Zhang and Miller 1992; Churchill *et al.* 1995). It is also possible that surfactants can influence the release of soil organic matter with which PAHs associate (Markiewicz *et al.* 2013).

There were unidentified factors affecting aqueous-phase surfactant and PAH concentrations during second-stage treatment. Preliminary test-tube-scale incubations with whole slurry form the first-stage bioreactor agreed well with our previous test-tube scale work (Adrion *et al.* in prep.), suggesting that the greater aqueous-phase surfactant concentration observed in the larger-scale, second-stage reactor could be due to the method of mixing. Mixing in the test-tube-scale incubations (orbital shaking) was more turbulent than that achieved during second-stage treatment using a magnetic stir bar. The second trials of both the 7- and 12-day residence times had higher aqueous-phase surfactant and PAH concentrations at 48 hours, and this may have affected PAH removal (trial 2 had lower PAH removal than trials 1 and 3 in each case). Because adequate interaction of the surfactant with the solid phase is important before

commencing aerobic treatment (Zhu and Aitken 2010), the adequacy of mixing is an important issue to consider for scale-up in the two-stage treatment concept.

It is also possible that the liquid phase of the effluent from the first-stage bioreactor influenced the liquid-phase behavior of POESH and/or PAHs in the second-stage bioreactor during the anoxic mixing period. In our earlier work at test-tube scale (Zhu and Aitken 2010; Adrion *et al.* in prep.), the liquid-phase from the first-stage bioreactor was replaced with fresh buffer before the surfactant was added. The liquid phase of the first-stage bioreactor slurry could contain dissolved or colloidal organic matter that can act like a surfactant (reducing surface tension) and can facilitate the apparent solubilization of hydrophobic compounds (Akkanen *et al.* 2005; Grolimund and Borkovec 2005; Markiewicz *et al.* 2013). However, if such factors did influence the apparent liquid-phase concentrations of POESH and/or PAHs in the second-stage bioreactor, we would have expected to observe this effect in the preliminary experiment at test-tube scale (*i.e.*, it should have been independent of mixing intensity).

The increased genotoxicity resulting from treatment of the feed soil in the first-stage bioreactor observed in this study is consistent with our previous studies on the same contaminated source soil (Hu *et al.* 2012; Hu *et al.* 2014). The *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$ mutants used in this study are deficient in the Rad54 and Rev1 proteins, respectively. These two proteins are implicated in the repair or tolerance of damage caused by the major types of PAH-induced genotoxicity: strand breaks caused by oxidative stress (Rad54), and adduction of DNA by stable or depurinating adducts (Rev1) (Friedberg 2006; Penning 2011). Although second-stage treatment removed substantial amounts of 4- and 5-ring PAHs, including some considered to be human carcinogens, second-stage treatment consistently increased genotoxicity relative to the effluent soil removed from the first-stage bioreactor.

Overall, the genotoxicity of remediated soil will depend both on the remaining parent compounds and the formation or removal of any products of incomplete microbial metabolism. Reduction of parent-PAH concentrations does not always correspond to a reduction in genotoxicity (Hughes *et al.* 1998; Gillespie *et al.* 2007; Lemieux *et al.* 2009; Hu *et al.* 2012). Because second-stage treatment made the soil less cytotoxic, but more genotoxic, it is possible that surfactant treatment causes transformation of cytotoxic compounds that are not genotoxic themselves into genotoxic products. For example, Zielinska-Park *et al.* (2004) found that bacterial transformation products such as pyrene- and fluoranthene-quinones can cause oxidative DNA damage *in vitro* using calf thymus DNA and HeLa S3 cells. The authors speculated that during biodegradation, bacteria may metabolically activate PAHs, such as pyrene, which might not otherwise be activated to an appreciable extent by mammalian metabolic systems. Such bacterial activation can, therefore, transform relatively non-genotoxic PAHs such as pyrene, or other non-genotoxic contaminants in the soil, into redox active products.

This work demonstrated the effectiveness of a semi-continuous two-stage process for the treatment of PAH-contaminated soil. In the first stage, soil was treated in a bioreactor without surfactant in order to remove the most readily bioaccessible fractions of PAHs and oxy-PAHs. Effluent from the first-stage bioreactor was further treated in a second stage, comprising bench-scale batch, surfactant-amended bioreactors that removed substantial amounts of the residual PAHs and oxy-PAHs. The observation that parent PAH removal did not necessarily correspond to a reduction in genotoxicity, however, highlights the need for further research to identify genotoxic products to improve risk management and remediation strategies. Increasing the residence time of the second-stage bioreactor from 7 days to 12 days had limited effect on removal of the targeted contaminants except for several five-ring PAHs. Overall, the two-stage

treatment concept we evaluated may be a promising method of maximizing the removal of PAHs during bioremediation. Factors to consider for scale-up include the adequacy of initial anoxic mixing and the effect of residence time in both stages. Although we found from screening experiments (Adrion *et al.* in prep.) that the optimum surfactant was POESH, in other cases the optimum surfactant and its dose may be site-specific, and therefore should be evaluated in preliminary bench-scale treatability studies.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The primary goal of this work was to improve the bioremediation of PAH-contaminated soil by screening several surfactants for enhanced biodegradation of the PAHs remaining in soil after conventional bioreactor-treatment. A secondary goal was to investigate the effect of surfactant-amended treatment on soil toxicity and genotoxicity. Conclusions for the specific aims of this research project are outlined below:

Aim 1: Evaluate the effect of conventional bioreactor treatment on the removal and bioavailability of oxy-PAHs in contaminated soil.

An LC-MS/MS method was developed to quantify four oxy-PAHs in solvent extracts of soil and SPE resins. In collaboration with a former student in our lab, the method was used to quantify the concentrations and desorbable fractions of oxy-PAHs in soil before and after bioreactor treatment. Bioremediation removed substantial amounts of oxy-PAHs and reduced the desorbable fractions. For all measured oxy-PAHs, the percent removed during treatment exceeded the desorbable fraction in the untreated soil, suggesting that biodegradation was not limited to the initial desorbable fraction. The LC-MS/MS method was also used to determine the aqueous solubility of the four oxy-PAHs and to evaluate the effect of surfactant-amended treatment on the removal of oxy-PAHs in Aim 3.

Aim 2: Screen nonionic surfactants for enhanced biodegradation of PAHs remaining in soil after conventional bioreactor treatment.

Five nonionic surfactants (Brij 30, Span 20, Ecosurf™ EH-3, polyoxyethylene sorbitol hexaoleate, and R-95™ rhamnolipid biosurfactant) were evaluated for their ability to enhance PAH desorption and biodegradation in contaminated soil after it had been treated in an aerobic, slurry-phase bioreactor. Surfactant doses were selected to correspond to aqueous-phase concentrations below the critical micelle concentration in the soil-slurry system. The effect of surfactant amendment on soil (geno)toxicity was also evaluated for Brij 30, Span 20, and POESH using the chicken DT40 B-lymphocyte cell line and two of its isogenic DNA-repair-deficient mutants. Compared to no-surfactant controls, incubation of the bioreactor-treated soil with all surfactants resulted in modest increases in PAH desorption as measured with an infinite-sink desorption method. All of the surfactants except R-95 substantially increased PAH biodegradation in the bioreactor-treated soil relative to the no-surfactant control. POESH had the greatest effect, resulting in removal of 50% of total measured PAHs. Brij 30, Span 20, and POESH were particularly effective at enhancing biodegradation of four- and five-ring PAHs, including five of the seven compounds designated as probable human carcinogens, with removals up to 80%. Surfactant treatment also significantly enhanced the removal of alkyl-PAHs. All treatments except POESH at the optimum dose for PAH removal significantly increased soil cytotoxicity. Only the no-surfactant control and Brij 30 at the optimum dose significantly decreased soil genotoxicity to both mutant cell lines. Despite removing substantial amounts of 4- and 5-ring PAHs, including 5 of the 7 considered probable human carcinogens, POESH treatment either had no effect on genotoxicity or caused a slight increase in genotoxicity.

Aim 3: Implement second-stage treatment of PAH-contaminated soil in a surfactant-amended bioreactor and evaluate its performance.

In Aim 2, POESH was identified as the optimum among the five surfactants for enhancing PAH removal, especially the high-molecular-weight PAHs (4- and 5-ring compounds). In Aim 3 the concept was extended by treating the effluent from the slurry-phase bioreactor in a second-stage batch reactor to which POESH was added. Batch treatment was conducted in three trials at a residence time of 7 days and three trials at a residence time of 12 days. Surfactant-amended treatment removed substantial amounts of the PAHs and oxy-PAHs remaining after conventional slurry-phase bioremediation, including more than 80% of residual 4-ring PAHs. The most substantial PAH removal occurred within the first week of treatment. Surfactant-amended treatment consistently made the soil less cytotoxic, but in most trials increased the genotoxicity of the soil.

6.2 Recommendations for future work

Further research could investigate the cause of limited PAH removal beyond the first week of second-stage treatment. For example, increased removal could be limited by low bioavailability of parent PAHs. The desorbable fractions after second-stage treatment with surfactant could be measured using SPE methods or selective extraction with cyclodextrin for example. Further removal could also be limited by the accumulation of inhibitory transformation products of PAHs or other compounds. This possibility could be investigated by identifying compounds that accumulate during bioremediation. It is also possible that further removal could be limited by the availability of primary substrates that are needed during the cometabolism of PAHs.

The second goal of this work was to evaluate the effect of surfactant-amended treatment on the (geno)toxicity of the soil. A strength of the DT40 bioassay is its ability to identify the

specific types of DNA damage caused by a chemical, especially direct-acting genotoxicants. Although PAHs primarily require metabolic activation in order to exert genotoxic effects, we chose the *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$ mutants because of their previously reported sensitivity to the soil used in this study. Future research could use the method of S9 metabolic preactivation developed for the DT40 assay (Hashimoto *et al.* 2015). S9 preactivation is meant to simulate the xenobiotic metabolism which occurs in the liver; however, some have argued that the S9 mixture composition is biased toward activating enzymes rather than detoxifying enzymes (Gad 2003). Preactivated soil residue may more accurately represent the mixture of chemicals that cause genotoxicity in humans and therefore may be a better measurement of risk. Additional research could also identify the compounds responsible for the increase in genotoxicity that occurred during conventional bioreactor treatment and during second-stage treatment with POESH.

APPENDIX A: SUPPORTING INFORMATION FOR CHAPTER 4

Procedure for measuring surfactant soil/water partitioning and pyrene solubilization

Incubations of each surfactant with bioreactor-treated soil were prepared in duplicate for ten doses, including a no-surfactant dose. Incubations were prepared by adding 1-g dry weight bioreactor-treated soil to 15-mL glass crimp-top vials with PTFE-lined septa. Surfactant stock solution in phosphate buffer (pH 7.5) was added to give the desired surfactant doses. Additional phosphate buffer was added to give a final solids content of 15% (w/w). Incubation vials were purged with nitrogen and then put on a wrist-action shaker in the dark. Because the experiment required a mass of bioreactor-treated soil larger than that produced by the bioreactor in a single week, incubations were set up over multiple weeks using separate batches of bioreactor-treated soil. Each batch was analyzed for pyrene concentration in triplicate. After two days, which was sufficient to reach apparent equilibrium liquid-phase surfactant and pyrene concentrations based on preliminary experiments (data not shown), incubation vials were centrifuged and the liquid phase was syringe-filtered through a 0.8-µm polycarbonate membrane. The surface tension of the filtrate was analyzed with a Du Nouy tensiometer (CSC Scientific Co., Inc. Fairfax, VA) after necessary dilutions were made to obtain a final surface tension corresponding to a concentration below the CMC. The concentration of surfactant in the filtrate was calculated using calibration curves of surface tension vs. the log of surfactant concentration. Liquid-phase pyrene concentration for each dose was determined by diluting the liquid phase into acetonitrile and analyzing by HPLC as described elsewhere (Richardson *et al.* 2011). The percent total pyrene mass in the liquid phase was calculated using the mass of pyrene in the liquid phase at day 2 divided by the mass of pyrene in the bioreactor-treated soil added at the time of incubation setup.

Procedure for extraction and analysis of feed soil

To measure the concentration of the 14 EPA priority-pollutants PAHs analyzed by HPLC (denoted in the footnotes to Table A.1), 28-g wet-weight feed soil was slurried in bioreactor buffer to make a 15% (w/w) slurry. The slurry was passed through a 2-mm screen (comparable to the procedure for feeding the reactor), collected, and centrifuged at 3900 RPM for 20 minutes. Aliquots (3-g wet weight) of centrifuged soil were transferred to each of six 30-mL glass centrifuge tubes with PTFE-lined septa with screwcaps. Three tubes were spiked with a known amounts of anthracene-D_{10} as a recovery surrogate. Sodium sulfate (10 g) was mixed into each tube and the soil was extracted overnight twice (each time with 10-mL acetone and 10-mL dichloromethane) and analyzed for PAH concentrations by HPLC as described elsewhere (Richardson *et al.* 2011).

For the remaining PAHs, feed soil was extracted using the QuEChERS method. Briefly, 5-g wet-weight feed soil was added to each of three 50-mL centrifuge tubes. The soil was spiked with 100-µL 10-µg/mL recovery surrogates (naphthalene-D_8, acenaphthene-D_{10}, chrysense-D_{12}, and perylene-D_{12}) in acetonitrile and shaken vigorously for 3 min. Then 5-mL water was added and shaken vigorously for 3 min. Then 12-mL acetonitrile and QuEChERS AOAC salt packet (Agilent 5982-5755) was added and the tubes vigorously shaken for 3 min. Tubes were then centrifuged for 3500 RPM for 10 min. 5-mL of the supernatant was transferred to a 15-mL dispersive SPE tube (Agilent 5982-5158) and shaken for 3 minutes, centrifuged at 3500 RPM for 10 min and filtered through a 0.45µm PVDF syringe filter.

GC-MS analysis procedure

QuEChERS extracts of the feed soil and solvent-extracts from the followup biodegradation experiment (after filtering through sodium sulfate to remove residual water) were analyzed by GC-MS for additional PAHs and alkyl-PAHs as described elsewhere (Luellen and Shea 2002).

Table A.1 Concentrations of Priority PAHs in feed soil.[a]

EPA Priority PAH	**Abbreviation**	**Concentration (µg/g dry soil)**
Naphthalene	NAP	22.9 ± 3.0
Acenaphthylene	ACY	11.0 ± 0.5
Acenaphthene	ACE	9.81 ± 0.71
Fluorene	FLU	4.95 ± 0.67
Phenanthrene	PHN	99.4 ± 5.1
Anthracene	ANT	9.03 ± 0.83
Fluoranthene	FLA	67.1 ± 4.1
Pyrene	PYR	108 ± 6
Benz[*a*]anthracene	**BaA**	39.2 ± 3.6
Chrysene	**CHR**	61.9 ± 3.1
Benzo[*b*]fluoranthene	**BbF**	24.4 ± 1.8
Benzo[*k*]fluoranthene	**BkF**	12.0 ± 1.0
Benzo[*a*]pyrene	**BaP**	29.8 ± 2.7
Indeno[1,2,3-*cd*]pyrene	**IDP**	15.7 ± 0.9
Dibenz[*a,h*]anthracene	**DBA**	2.01 ± 0.21
Benzo[*g,h,i*]perylene	BgP	25.5 ± 2.2
Total EPA priority PAHs[b]	Σ 16 PAH	542 ± 11
Total carcinogenic PAHs[b]	Σ Carc. PAH	185 ± 6

[a] Values represent means and standard deviations. The 14 PAHs measured by HPLC were NAP, ACE, FLU, PHN, ANT, FLA, PYR, BaA, CHR, BbF, BkF, BaP, DBA, and BgP. All other PAHs were measured by GC-MS. For feed soil only, values measured by HPLC are from 6 replicates and by GC-MS are from triplicates. [b] Standard deviation of totals calculated by propagation of error. Bolded compounds are EPA probable human carcinogens.

Table A.2 Concentrations of other PAHs in feed soil.[a]

Other PAH	Abbreviation	Concentration (µg/g dry soil)
2-Methylnaphthalene	2MNAP	7.30 ± 0.98
1-Methylnaphthalene	1MNAP	6.22 ± 0.79
Biphenyl	BIP	0.77 ± 0.08
2,6-Dimethylnaphthalene	DMNAP	7.29 ± 0.35
Dibenzofuran	DBF	0.64 ± 0.07
2,3,5-Trimethylnaphthalene	TMNAP	BDL
C1-Naphthalenes	C1-NAP	13.7 ± 1.8
C2-Naphthalenes	C2-NAP	33.5 ± 1.9
C3-Naphthalenes	C3-NAP	43.4 ± 1.4
C4-Naphthalenes	C4-NAP	16.9 ± 0.1
1-Methylfluorene	1MFLU	3.34 ± 0.19
C1-Fluorenes	C1-FLU	10.8 ± 1.2
C2-Fluorenes	C2-FLU	13.2 ± 0.9
Dibenzothiophene	DBT	0.45 ± 0.06
C1-Dibenzothiophene	C1-DBT	1.76 ± 0.04
C2-Dibenzothiophene	C2-DBT	2.30 ± 0.09
C3-Dibenzothiophene	C3-DBT	1.70 ± 0.17
1-Methylphenanthrene	1MPHN	122 ± 3
C1-Phenanthrenes/Anthracenes	C1-PHN/ANT	264 ± 1
C2-Phenanthrenes/Anthracenes	C2-PHN/ANT	181 ± 4
C3-Phenanthrenes/Anthracenes	C3-PHN/ANT	83.3 ± 2.2
C4-Phenanthrenes/Anthracenes	C4-PHN/ANT	11.2 ± 0.5
C1-Fluoranthenes/Pyrene	C1-FLA/PYR	53.5 ± 2.0
C2-Fluroanthrene/Pyrene	C2-FLA/PYR	21.4 ± 1.0
C3-Fluoranthrene/Pyrene	C3-FLA/PYR	12.1 ± 0.3
Retene	RET	148 ± 4
C1-Chrysenes	C1-CHR	33.1 ± 0.9
C2-Chrysenes	C2-CHR	11.0 ± 0.6
C3-Chrysenes	C3-CHR	3.27 ± 0.17
C4-Chrysenes	C4-CHR	0.98 ± 0.04
Benzo[*e*]pyrene	BeP	18.2 ± 0.9
Perylene	PER	4.99 ± 0.33
Coronene	COR	3.18 ± 0.14

[a] "BDL" indicates below detection limit. Other Notes as in Table A.1.

Table A.3 Concentrations of PAHs in the bioreactor-treated soil samples used in the preliminary biodegradation and followup (geno)toxicity experiments.[a]

PAH	Initial biodegradation experiments bioreactor-treated soil (µg/g)					Follow-up (geno)toxicity bioreactor-treated soil (µg/g)
	Brij 30	Span 20	EH-3	POESH	R-95	
NAP	22.3 ± 2.1	18.7 ± 2.1	22.7 ± 1.0	19.8 ± 2.9	19.2 ± 0.4	22.4 ± 0.9
ACE	3.4 ± 0.3	1.5 ± 0.2	2.9 ± 0.3	1.4 ± 0.4	1.9 ± 0.4	2.1 ± 0.3
FLU	2.0 ± 0.2	1.5 ± 0.1	2.3 ± 0.2	1.9 ± 0.3	1.7 ± 0.2	2.3 ± 0.7
PHN	30.5 ± 3.5	24.1 ± 2.2	35.0 ± 3.5	27.2 ± 6.0	27.6 ± 2.7	33.5 ± 5.0
ANT	3.1 ± 0.2	2.6 ± 0.2	3.6 ± 0.3	3.0 ± 0.4	2.8 ± 0.2	3.6 ± 0.9
FLA	18.3 ± 1.3	15.9 ± 1.1	21.3 ± 1.6	16.5 ± 2.1	17.1 ± 0.8	23.1 ± 3.0
PYR	31.7 ± 2.1	26.4 ± 2.3	34.9 ± 2.1	29.7 ± 4.1	29.3 ± 0.9	35.7 ± 4.2
BaA	15.6 ± 1.3	15.1 ± 1.5	18.1 ± 1.4	14.1 ± 1.4	15.7 ± 0.6	18.4 ± 2.0
CHR	24.5 ± 1.6	15.9 ± 2.3	28.9 ± 1.3	22.3 ± 2.7	25.8 ± 0.9	20.4 ± 2.3
BbF	13.4 ± 0.6	11.6 ± 0.8	14.8 ± 0.8	12.0 ± 1.5	13.2 ± 0.5	13.8 ± 0.7
BkF	6.7 ± 0.4	6.1 ± 0.3	6.6 ± 0.1	5.6 ± 0.3	6.3 ± 0.2	7.1 ± 0.3
BaP	18.7 ± 1.4	15.7 ± 1.1	20.7 ± 1.4	15.4 ± 1.7	17.5 ± 0.8	18.6 ± 1.4
DBA	1.4 ± 0.1	1.1 ± 0.2	1.4 ± 0.3	1.2 ± 0.2	1.2 ± 0.1	1.3 ± 0.2
BgP	24.7 ± 1.8	24.0 ± 3.6	26.4 ± 2.7	21.3 ± 2.8	24.0 ± 1.3	24.2 ± 1.7
Total PAH	216 ± 13	180 ± 12	239 ± 13	191 ± 24	203 ± 6	227 ± 19

[a] Data represent means and standard deviations of six replicates. Abbreviations are defined in Table A.1.

Table A.4 PAH mass desorbed for incubations with Brij 30 and concentration of PAHs in bioreactor-treated soil used to prepare desorption incubations.

PAH	Bioreactor-treated soil (µg/g)	Mass Desorbed (%)		
		No Surfactant	Brij 30 lower	Brij 30 higher
NAP	20.6 ± 0.1	0.27 ± 0.27[a]	2.57 ± 0.61*	2.95 ± 0.40*
ACE	1.9 ± 0.3	1.38 ± 1.06[a]	34.5 ± 6.1*	52.3 ± 9.8*
FLU	1.8 ± 0.2	0.25 ± 0.17[a]	13.0 ± 1.5*	19.2 ± 7.9*
PHN	37.3 ± 0.3	0.11 ± 0.03	3.27 ± 0.31*	9.60 ± 6.47*
ANT	3.7 ± 0.2	0.09 ± 0.04[a]	5.46 ± 0.44*	8.08 ± 1.84*
FLA	22.1 ± 0.3	0.15 ± 0.09	4.54 ± 0.12*	5.24 ± 0.43*
PYR	37.5 ± 0.8	0.15 ± 0.08	3.81 ± 0.18*	4.41 ± 0.42*
BaA	12.7 ± 0.2	0.17 ± 0.13	1.48 ± 0.11*	1.79 ± 0.37*
CHR	16.9 ± 0.8	0.13 ± 0.10	1.15 ± 0.13*	1.30 ± 0.17*
BbF	12.0 ± 0.1	0.09 ± 0.08[a]	0.26 ± 0.14	0.37 ± 0.16*
BkF	7.5 ± 0.1	0.11 ± 0.10	0.22 ± 0.12	0.30 ± 0.14
BaP	23.0 ± 0.5	0.10 ± 0.09	0.19 ± 0.07	0.25 ± 0.13
DBA	2.2 ± 0.1	<0.20	<0.40	<0.40
BgP	25.3 ± 1.0	0.07 ± 0.13[a]	0.05 ± 0.05[a]	0.10 ± 0.07
Total PAH[b]	225 ± 2	0.14 ± 0.04	2.56 ± 0.09*	4.11 ± 1.08*

[a] Cohen's Maximum Likelihood Estimate was used to account for one or more replicates below the lower limit of quantification (LLOQ). "Less than" values are below LLOQ. [b] Total PAH percent mass desorbed does not include mass of individual PAH below the LLOQ. "Lower" and "higher" refer to surfactant doses described in Materials and Methods. An asterisk indicates a significant difference (α=0.05) in mass desorbed (µg) between a treatment and no-surfactant control. Abbreviations are defined in Table A.1.

Table A.5 PAH mass desorbed for incubations with Span 20 and POESH and concentration of PAHs in bioreactor-treated soil used to prepare desorption incubations.[a]

PAH	Bioreactor-treated soil (µg/g)	Mass Desorbed (%)				
		No surfactant	Span 20 lower	Span 20 higher	POESH lower	POESH higher
NAP	22.4 ± 0.9	0.32 ± 0.06	1.21 ± 0.42*	1.36 ± 0.14*	0.90 ± 0.42*	1.26 ± 0.11*
ACE	3.2 ± 0.2	1.05 ± 0.23	4.28 ± 1.91*	9.68 ± 1.60*	3.01 ± 2.14[a]	8.49 ± 1.66*
FLU	2.5 ± 0.1	0.21 ± 0.08	1.63 ± 0.71*	3.54 ± 0.51*	0.89 ± 0.82[a]	2.71 ± 0.35*
PHN	40.2 ± 1.7	0.10 ± 0.03	0.45 ± 0.37	0.67 ± 0.09*	0.41 ± 0.33	0.85 ± 0.17*
ANT	4.0 ± 0.3	0.08 ± 0.02	0.30 ± 0.33[a]	0.62 ± 0.13*	0.31 ± 0.31[a]	0.83 ± 0.18*
FLA	24.0 ± 1.1	0.12 ± 0.03	0.88 ± 0.41*	2.43 ± 0.34*	0.51 ± 0.31*	1.77 ± 0.31*
PYR	40.0 ± 2.4	0.12 ± 0.02	0.68 ± 0.34*	1.50 ± 0.12*	0.31 ± 0.19	1.10 ± 0.31*
BaA	19.5 ± 1.3	0.06 ± 0.01	0.27 ± 0.08*	0.58 ± 0.09*	0.18 ± 0.05*	0.46 ± 0.08*
CHR	23.6 ± 0.9	0.07 ± 0.06	0.18 ± 0.12	0.47 ± 0.09*	0.10 ± 0.06[a]	0.36 ± 0.06*
BbF	14.6 ± 1.5	0.04 ± 0.00	<0.12	<0.12	<0.12	0.11 ± 0.02*
BkF	7.9 ± 0.4	0.03 ± 0.00	0.09 ± 0.02*	0.14 ± 0.03*	0.05 ± 0.01*	0.08 ± 0.01*
BaP	20.1 ± 1.4	0.03 ± 0.01	0.10 ± 0.02*	0.14 ± 0.02*	0.07 ± 0.03*	0.09 ± 0.01*
DBA	1.5 ± 0.2	<0.15	<0.60	<0.60	<0.60	<0.60
BgP	24.6 ± 1.7	0.02 ± 0.00	<0.04	<0.04	<0.04	<0.04
Total PAH[b]	248 ± 8	0.10 ± 0.01	0.46 ± 0.09*	0.90 ± 0.05*	0.33 ± 0.08*	0.84 ± 0.07*

[a] Notes as in Table A.4.

Table A.6 PAH mass desorbed for incubations with R-95 and EH-3 and concentration of PAHs in bioreactor-treated soil used to prepare desorption incubations.[a]

PAH	Bioreactor-treated soil (µg/g)	Mass Desorbed (%)				
		No Surfactant	R-95 lower	R-95 higher	EH-3 lower	EH-3 higher
NAP	28.4 ± 3.3	0.17 ± 0.03[a]	0.26 ± 0.10	0.55 ± 0.22*	1.25 ± 0.16*	2.40 ± 1.10*
ACE	2.7 ± 0.4	<0.83	1.69 ± 1.08	4.33 ± 1.38	30.0 ± 9.3	21.6 ± 5.1
FLU	2.7 ± 0.2	<0.16	<0.16	1.05 ± 0.54	6.66 ± 2.76	11.5 ± 2.9
PHN	47.9 ± 1.3	0.04 ± 0.02	0.07 ± 0.00	0.15 ± 0.11	1.32 ± 0.34*	3.40 ± 1.92*
ANT	4.8 ± 0.5	<.05	0.07 ± 0.01	0.20 ± 0.09	5.97 ± 0.76	4.81 ± 0.98
FLA	29.6 ± 2.6	0.04 ± 0.01	0.13 ± 0.08	0.48 ± 0.25*	3.69 ± 1.80*	1.82 ± 0.24*
PYR	54.9 ± 5.0	0.04 ± 0.01	0.10 ± 0.05	0.29 ± 0.14*	2.93 ± 1.39*	1.28 ± 0.23*
BaA	24.7 ± 2.2	0.03 ± 0.01	0.04 ± 0.02	0.15 ± 0.06*	2.06 ± 2.48	0.43 ± 0.04*
CHR	38.1 ± 4.6	<0.02	<0.02	0.10 ± 0.03	1.55 ± 1.91	0.33 ± 0.05
BbF	18.6 ± 2.6	<0.05	<0.02	0.06 ± 0.04[a]	0.89 ± 1.37	0.11 ± 0.05[a]
BkF	9.1 ± 0.4	0.02 ± 0.01[a]	0.02 ± 0.01	0.06 ± 0.02*	0.78 ± 1.23	0.07 ± 0.01*
BaP	27.4 ± 2.0	0.02 ± 0.01[a]	0.03 ± 0.00	0.05 ± 0.02	0.69 ± 1.08	0.07 ± 0.01*
DBA	1.8 ± 0.2	<0.25	<0.25	<0.25	<0.50	<0.50
BgP	32.1 ± 2.7	<0.01	<0.01	<0.01	<0.02	<0.02
Total PAH[b]	323 ± 26	0.03 ± 0.00	0.08 ± 0.02	0.24 ± 0.05*	2.01 ± 0.46*	1.53 ± 0.33*

[a] Notes as in Table A.4.

Table A.7 PAH mass recovered from higher-dose surfactant desorption incubations.[a]

PAH	Mass Recovered (%)				
	Brij 30	Span 20	EH-3	POESH	R-95
NAP	89.8 ± 2.4	83.5 ± 7.7	85.0 ± 12.9	84.1 ± 12.7	84.1 ± 12.7
ACE	107 ± 21	77.7 ± 12.6	84.5 ± 15.5	83.3 ± 12.1	72.1 ± 22.9
FLU	98.3 ± 16.2	76.9 ± 13.8	104 ± 17	82.0 ± 9.2	83.0 ± 10.7
PHN	95.1 ± 12.2	68.1 ± 11.7	68.3 ± 18.0	73.9 ± 11.8	67.9 ± 10.3
ANT	97.0 ± 6.2	64.9 ± 9.9	103 ± 20	69.6 ± 7.5	67.6 ± 11.8
FLA	105 ± 2	74.2 ± 9.4	106 ± 18	79.6 ± 6.3	62.5 ± 11.7
PYR	97.9 ± 2.6	65.7 ± 9.1	90.8 ± 9.6	69.3 ± 5.1	60.3 ± 11.8
BaA	107 ± 4	80.2 ± 10.8	101 ± 19	90.1 ± 6.1	66.5 ± 12.5
CHR	99.0 ± 6.3	70.6 ± 6.8	98.9 ± 15.0	73.3 ± 6.1	77.0 ± 18.1
BbF	102 ± 4	79.7 ± 13.4	104 ± 19	93.1 ± 9.7	88.2 ± 17.2
BkF	102 ± 3	80.2 ± 10.5	101 ± 7	90.4 ± 7.1	86.6 ± 9.2
BaP	98.9 ± 3.4	80.6 ± 11.0	96.5 ± 11.2	90.1 ± 9.6	83.4 ± 10.5
DBA	96.7 ± 8.1	84.9 ± 20.8	94.4 ± 13.2	91.8 ± 12.2	82.3 ± 17.7
BgP	98.4 ± 4.7	77.6 ± 12.9	94.5 ± 10.7	91.5 ± 14.3	84.1 ± 10.5

[a] Values represent means and standard deviations of triplicates. Abbreviations are defined in Table A.1.

Table A.8 Concentrations of PAHs in followup biodegradation experiment.[a]

PAH	Concentration (µg/g)						
	Bioreactor treated	No surfactant	Brij 30 lower	Brij 30 higher	POESH lower	POESH higher	Span 20 higher
NAP	20.8 ± 1.3	17.3 ± 1.3	17.1 ± 1.3	17.2 ± 0.8	19.3 ± 0.8	18.1 ± 1.3	19.3 ± 1.2
ACY	12.3 ± 0.4	10.9 ± 1.0	10.4 ± 2.8	-	-	12.6 ± 0.5	12.7 ± 0.9
ACE	2.08 ± 0.31	1.48 ± 0.44	1.21 ± 0.23	1.30 ± 0.19	1.63 ± 0.34	1.25 ± 0.28	1.74 ± 0.36
FLU	1.64 ± 0.21	1.41 ± 0.22	1.14 ± 0.08	1.10 ± 0.12	1.52 ± 0.26	1.20 ± 0.14	1.54 ± 0.22
PHN	27.1 ± 2.4	20.5 ± 2.6	16.5 ± 2.1	14.1 ± 0.6	20.1 ± 1.9	16.4 ± 1.1	19.9 ± 2.1
ANT	2.97 ± 0.23	2.34 ± 0.24	1.93 ± 0.18	1.87 ± 0.09	2.33 ± 0.21	2.06 ± 0.35	2.45 ± 0.42
FLA	17.8 ± 0.8	13.1 ± 1.3	5.47 ± 0.86	9.46 ± 0.83	8.05 ± 0.81	4.97 ± 0.47	8.35 ± 1.87
PYR	30.2 ± 1.5	22.7 ± 2.0	9.12 ± 0.80	25.2 ± 1.9	13.4 ± 1.3	7.52 ± 0.61	12.7 ± 2.0
BaA	15.4 ± 0.9	12.1 ± 1.1	4.93 ± 0.43	13.3 ± 0.8	6.85 ± 0.77	3.84 ± 0.37	6.61 ± 1.18
CHR	24.4 ± 1.1	18.8 ± 1.8	7.02 ± 0.39	15.4 ± 0.7	9.76 ± 1.18	4.62 ± 0.52	8.59 ± 1.09
BbF	13.7 ± 1.0	11.4 ± 0.6	8.23 ± 0.29	12.8 ± 0.6	6.73 ± 0.59	5.65 ± 0.52	6.89 ± 0.80
BkF	6.63 ± 0.33	5.53 ± 0.33	3.63 ± 0.10	6.29 ± 0.25	3.32 ± 0.26	2.00 ± 0.23	3.15 ± 0.50
BaP	16.7 ± 0.9	13.9 ± 0.9	11.7 ± 0.1	15.4 ± 0.5	9.27 ± 0.90	8.21 ± 1.03	9.47 ± 1.24
IDP	15.0 ± 1.2	13.9 ± 0.7	13.0 ± 3.9	-	-	15.2 ± 1.1	15.5 ± 2.0
DBA	0.99 ± 0.12	0.81 ± 0.08	0.90 ± 0.11	1.05 ± 0.10	0.64 ± 0.22	0.72 ± 0.18	0.59 ± 0.13
BgP	23.0 ± 0.6	21.4 ± 1.7	20.9 ± 0.5	20.8 ± 1.1	22.1 ± 1.1	21.1 ± 1.2	23.2 ± 1.2
Σ 16 PAH[b]	231 ± 4	188 ± 5	133 ± 6	-	-	125 ± 3	153 ± 5
Σ Carc. PAH[b]	92.8 ± 2.3	76.6 ± 2.5	49.4 ± 3.9	-	-	40.2 ± 1.7	50.8 ± 3.0
2MNAP	7.04 ± 0.50	6.10 ± 0.92	5.21 ± 1.39	-	-	6.41 ± 0.68	6.33 ± 0.54
1MNAP	4.17 ± 0.17	3.48 ± 0.52	2.83 ± 0.80	-	-	3.31 ± 0.31	3.51 ± 0.25
BIP	0.83 ± 0.03	0.70 ± 0.09	0.62 ± 0.16	-	-	0.72 ± 0.07	0.84 ± 0.06
DMNAP	2.61 ± 0.11	1.96 ± 0.17	1.56 ± 0.36	-	-	1.94 ± 0.13	4.38 ± 4.79
DBF	0.52 ± 0.04	0.43 ± 0.04	0.38 ± 0.11	-	-	0.46 ± 0.05	0.55 ± 0.06
TMNAP	1.38 ± 0.11	1.01 ± 0.08	0.54 ± 0.08	-	-	0.68 ± 0.01	0.97 ± 0.19
C1-NAP	12.5 ± 0.6	10.4 ± 1.3	8.89 ± 2.44	-	-	10.6 ± 0.9	11.1 ± 0.6

Table A.8 (continued)

C2-NAP	13.5 ± 0.7	10.4 ± 1.4	8.10 ± 2.00	-	-	9.68 ± 0.47	11.4 ± 1.4
C3-NAP	10.7 ± 0.6	7.47 ± 0.93	5.08 ± 1.09	-	-	5.79 ± 0.29	7.39 ± 1.34
C4-NAP	4.57 ± 0.22	3.18 ± 0.40	1.57 ± 0.34	-	-	1.72 ± 0.12	2.33 ± 0.38
1MFLU	1.05 ± 0.04	0.75 ± 0.11	0.53 ± 0.11	-	-	0.62 ± 0.03	0.77 ± 0.09
C1-FLU	4.48 ± 0.28	2.71 ± 1.26	2.96 ± 0.71	-	-	3.35 ± 0.20	3.91 ± 0.36
C2-FLU	5.77 ± 0.44	5.01 ± 0.49	3.10 ± 0.81	-	-	3.79 ± 0.27	4.12 ± 0.34
DBT	BDL	BDL	BDL	-	-	BDL	BDL
C1-DBT	0.70 ± 0.04	0.44 ± 0.26	0.18 ± 0.18	-	-	0.16 ± 0.21	0.49 ± 0.04
C2-DBT	BDL	BDL	BDL	-	-	BDL	BDL
C3-DBT	BDL	BDL	BDL	-	-	BDL	BDL
1MPHN	34.4 ± 1.6	22.4 ± 3.0	10.5 ± 1.8	-	-	11.5 ± 0.6	16.2 ± 2.5
C1-PHN/ANT	78.3 ± 4.9	53.7 ± 6.7	29.1 ± 6.3	-	-	32.5 ± 1.1	41.1 ± 5.3
C2-PHN/ANT	67.6 ± 3.9	47.1 ± 4.7	20.4 ± 4.5	-	-	20.7 ± 1.1	27.7 ± 3.2
C3-PHN/ANT	38.4 ± 1.5	28.5 ± 3.7	10.3 ± 2.4	-	-	9.13 ± 0.48	13.9 ± 1.7
C4-PHN/ANT	BDL	BDL	BDL	-	-	BDL	BDL
C1-FLA/PYR	28.4 ± 1.3	21.4 ± 2.1	10.4 ± 2.6	-	-	9.17 ± 0.75	13.0 ± 1.9
C2-FLA/PYR	14.6 ± 0.5	11.9 ± 1.5	8.10 ± 2.12	-	-	7.63 ± 0.47	8.57 ± 0.66
C3-FLA/PYR	8.77 ± 0.42	7.28 ± 0.85	5.24 ± 1.29	-	-	4.83 ± 0.17	5.31 ± 0.31
RET	74.8 ± 3.1	54.8 ± 5.9	16.5 ± 4.0	-	-	10.9 ± 0.7	20.5 ± 2.8
C1-CHR	16.5 ± 0.9	13.5 ± 1.1	5.44 ± 1.34	-	-	3.61 ± 0.44	6.56 ± 0.51
C2-CHR	8.94 ± 0.54	7.36 ± 0.57	4.56 ± 1.23	-	-	2.87 ± 0.37	3.84 ± 0.32
C3-CHR	5.27 ± 0.22	4.46 ± 0.49	3.58 ± 1.00	-	-	3.37 ± 0.36	3.14 ± 0.27
C4-CHR	BDL	BDL	BDL	-	-	BDL	BDL
BeP	10.7 ± 0.7	9.25 ± 0.66	5.09 ± 1.30	-	-	2.57 ± 0.31	4.82 ± 0.76
PER	4.35 ± 0.41	3.97 ± 0.31	3.51 ± 0.97	-	-	3.99 ± 0.31	4.07 ± 0.35
COR	3.99 ± 0.14	3.57 ± 0.22	3.22 ± 0.91	-	-	3.77 ± 0.29	3.79 ± 0.68

[a] Values represent means standard deviations of five replicates. "-" indicates not determined. "BDL" indicates below detection limit .Other notes as in Table A.1.

Table A.9 Overall removal of PAHs in treatments relative to feed soil.[a]

PAH	Removal relative to untreated feed soil (%)						
	Bioreactor alone	No Surfactant	Brij 30 lower	Brij 30 higher	POESH lower	POESH higher	Span 20 higher
NAP	9.26 ± 13.2	24.6 ± 11.3	25.3 ± 11.2	24.8 ± 10.4	15.4 ± 11.5	20.9 ± 11.7	15.6 ± 12.2
ACY	-11.2 ± 6.1	1.19 ± 9.72	5.50 ± 25.3	-	-	-13.9 ± 6.6	-15.3 ± 9.7
ACE	78.8 ± 3.6	84.9 ± 4.7	87.7 ± 2.5	86.8 ± 2.2	83.4 ± 3.7	87.3 ± 3.0	82.3 ± 3.8
FLU	66.9 ± 6.2	71.6 ± 5.9	76.9 ± 3.5	77.7 ± 3.9	69.3 ± 6.7	75.7 ± 4.3	68.9 ± 6.2
PHN	72.7 ± 2.8	79.4 ± 2.8	83.4 ± 2.2	85.8 ± 1.0	79.8 ± 2.2	83.5 ± 1.4	80.0 ± 2.3
ANT	67.1 ± 3.9	74.1 ± 3.6	78.6 ± 2.8	79.4 ± 2.1	74.3 ± 3.3	77.1 ± 4.4	72.9 ± 5.3
FLA	73.5 ± 2.0	80.5 ± 2.2	91.8 ± 1.4	85.9 ± 1.5	88.0 ± 1.4	92.6 ± 0.8	87.6 ± 2.9
PYR	71.9 ± 2.0	78.9 ± 2.1	91.5 ± 0.9	76.6 ± 2.2	87.5 ± 1.3	93.0 ± 0.7	88.2 ± 2.0
BaA	60.8 ± 4.3	69.0 ± 4.0	87.4 ± 1.6	66.1 ± 3.7	82.5 ± 2.5	90.2 ± 1.3	83.1 ± 3.4
CHR	60.6 ± 2.7	69.6 ± 3.3	88.7 ± 0.9	75.1 ± 1.7	84.2 ± 2.1	92.5 ± 0.9	86.1 ± 1.9
BbF	43.8 ± 5.8	53.2 ± 4.4	66.3 ± 2.8	47.5 ± 4.7	72.4 ± 3.2	76.8 ± 2.7	71.7 ± 3.9
BkF	44.7 ± 5.3	53.9 ± 4.7	69.7 ± 2.6	47.5 ± 4.8	72.3 ± 3.1	83.3 ± 2.3	73.7 ± 4.7
BaP	43.9 ± 5.9	53.3 ± 5.1	60.7 ± 3.5	48.2 ± 4.9	68.9 ± 4.1	72.4 ± 4.3	68.2 ± 5.0
IDP	4.54 ± 9.44	11.4 ± 6.8	17.5 ± 25.0	-	-	3.47 ± 8.82	1.84 ± 13.99
DBA	50.7 ± 7.8	59.9 ± 6.0	55.2 ± 7.3	47.8 ± 7.3	68.3 ± 11.3	64.2 ± 9.6	70.8 ± 7.1
BgP	9.83 ± 8.03	16.2 ± 9.9	17.9 ± 7.2	18.2 ± 8.2	13.4 ± 8.5	17.2 ± 8.5	8.92 ± 9.11
Σ 16 PAH	57.5 ± 1.1	65.4 ± 1.2	75.4 ± 1.1	-	-	76.9 ± 0.7	71.8 ± 1.1
Σ Carc. PAH	49.8 ± 2.0	58.6 ± 1.9	73.3 ± 2.3	-	-	78.2 ± 1.2	72.6 ± 1.9
2MNAP	3.54 ± 14.6	16.5 ± 16.9	28.6 ± 21.3	-	-	12.2 ± 15.0	13.3 ± 13.8
1MNAP	32.9 ± 8.9	44.1 ± 11.0	54.5 ± 14.1	-	-	46.9 ± 8.4	43.6 ± 8.2
BIP	-8.12 ± 11.4	8.95 ± 14.82	18.7 ± 22.3	-	-	5.70 ± 12.9	-8.94 ± 13.4
DMNAP	64.2 ± 2.3	73.2 ± 2.7	78.6 ± 5.1	-	-	73.3 ± 2.2	39.9 ± 65.7
DBF	18.4 ± 10.4	32.2 ± 9.7	40.0 ± 19.0	-	-	27.2 ± 11.2	13.5 ± 13.3
TMNAP	NC	NC	NC	-	-	NC	NC
C1-NAP	9.30 ± 12.7	24.4 ± 13.6	35.3 ± 19.7	-	-	22.9 ± 12.1	19.2 ± 11.4

Table A.9 (continued)

C2-NAP	59.7 ± 3.1	68.9 ± 4.5	75.8 ± 6.1	-	-	71.1 ± 2.2	66.0 ± 4.6
C3-NAP	75.4 ± 1.6	82.8 ± 2.2	88.3 ± 2.5	-	-	86.6 ± 0.8	83.0 ± 3.1
C4-NAP	72.9 ± 1.3	81.2 ± 2.4	90.7 ± 2.0	-	-	89.8 ± 0.7	86.2 ± 2.2
1MFLU	68.5 ± 2.2	77.5 ± 3.4	84.1 ± 3.3	-	-	81.5 ± 1.5	76.8 ± 3.1
C1-FLU	58.4 ± 5.2	74.8 ± 12.0	72.5 ± 7.2	-	-	68.9 ± 3.8	63.8 ± 5.1
C2-FLU	56.3 ± 4.4	62.1 ± 4.5	76.6 ± 6.3	-	-	71.4 ± 2.8	68.9 ± 3.3
DBT	NC	NC	NC	-	-	NC	NC
C1-DBT	60.3 ± 2.6	75.2 ± 14.9	89.7 ± 10.2	-	-	91.1 ± 12.1	72.4 ± 2.1
C2-DBT	NC	NC	NC	-	-	NC	NC
C3-DBT	NC	NC	NC	-	-	NC	NC
1MPHN	71.8 ± 1.5	81.7 ± 2.5	91.4 ± 1.5	-	-	90.6 ± 0.5	86.7 ± 2.1
C1-PHN/ANT	70.3 ± 1.9	79.7 ± 2.5	89.0 ± 2.4	-	-	87.7 ± 0.4	84.4 ± 2.0
C2-PHN/ANT	62.6 ± 2.3	73.9 ± 2.7	88.7 ± 2.5	-	-	88.5 ± 0.7	84.6 ± 1.8
C3-PHN/ANT	53.9 ± 2.2	65.8 ± 4.5	87.7 ± 2.9	-	-	89.0 ± 0.6	83.3 ± 2.0
C4-PHN/ANT	NC	NC	NC	-	-	NC	NC
C1-FLA/PYR	47.0 ± 3.1	60.0 ± 4.2	80.6 ± 4.8	-	-	82.9 ± 1.5	75.8 ± 3.6
C2-FLA/PYR	31.6 ± 4.1	44.5 ± 7.5	62.1 ± 10.1	-	-	64.3 ± 2.8	59.9 ± 3.6
C3-FLA/PYR	27.7 ± 4.0	40.0 ± 7.1	56.8 ± 10.7	-	-	60.2 ± 1.7	56.2 ± 2.8
RET	49.6 ± 2.4	63.0 ± 4.1	88.9 ± 2.7	-	-	92.7 ± 0.5	86.1 ± 1.9
C1-CHR	50.2 ± 3.0	59.4 ± 3.6	83.6 ± 4.1	-	-	89.1 ± 1.4	80.2 ± 1.6
C2-CHR	18.7 ± 6.6	33.0 ± 6.3	58.5 ± 11.4	-	-	73.8 ± 3.7	65.1 ± 3.5
C3-CHR	-61.0 ± 10.7	-36.4 ± 16.7	-9.43 ± 31.0	-	-	-3.12 ± 12.2	4.13 ± 9.55
C4-CHR	NC	NC	NC	-	-	NC	NC
BeP	41.4 ± 4.9	49.2 ± 4.4	72.0 ± 7.3	-	-	85.9 ± 1.9	73.5 ± 4.4
PER	12.8 ± 10.0	20.3 ± 8.2	29.6 ± 20.1	-	-	20.0 ± 8.1	18.4 ± 8.8
COR	-25.5 ± 7.1	-12.3 ± 8.7	-1.33 ± 29.0	-	-	-18.6 ± 10.6	-19.4 ± 22.0

[a] Values represent means and standard deviations calculated through propagation of error. "-" indicates not determined. "NC" indicates removal not calculated because concentration in feed soil or concentration in treated soil was below the detection limit. Other notes as in Table A.1.

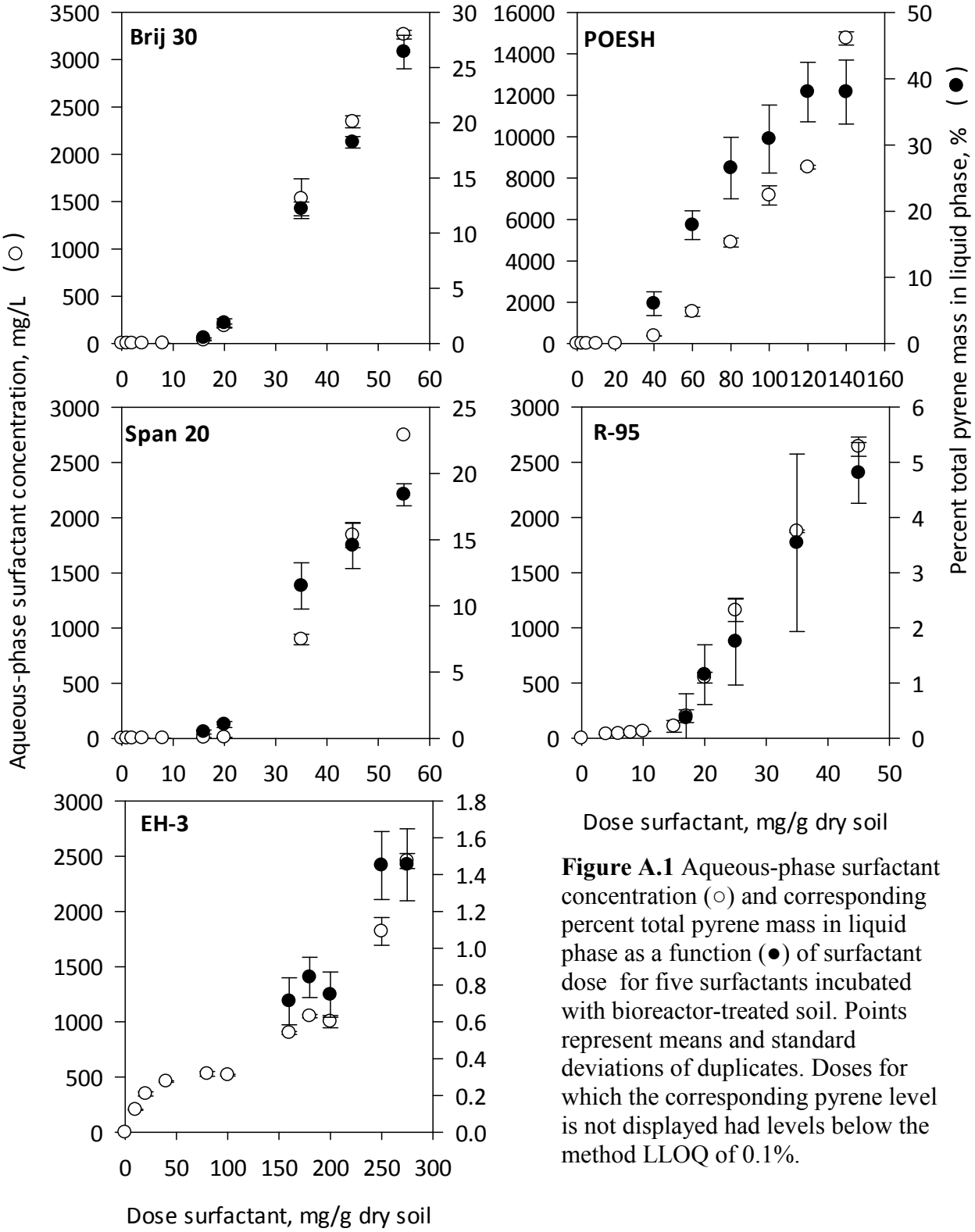

Figure A.1 Aqueous-phase surfactant concentration (○) and corresponding percent total pyrene mass in liquid phase as a function (●) of surfactant dose for five surfactants incubated with bioreactor-treated soil. Points represent means and standard deviations of duplicates. Doses for which the corresponding pyrene level is not displayed had levels below the method LLOQ of 0.1%.

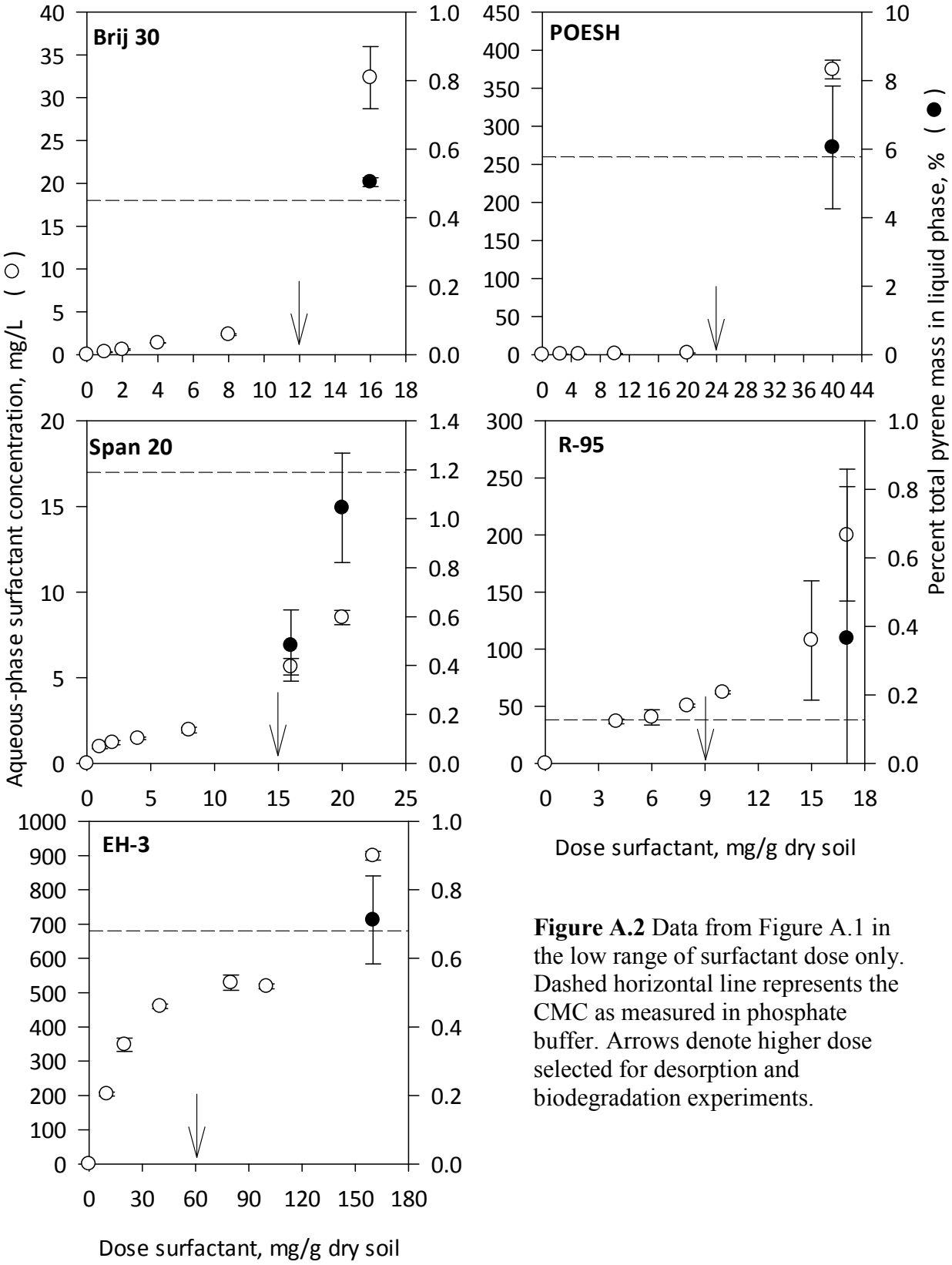

Figure A.2 Data from Figure A.1 in the low range of surfactant dose only. Dashed horizontal line represents the CMC as measured in phosphate buffer. Arrows denote higher dose selected for desorption and biodegradation experiments.

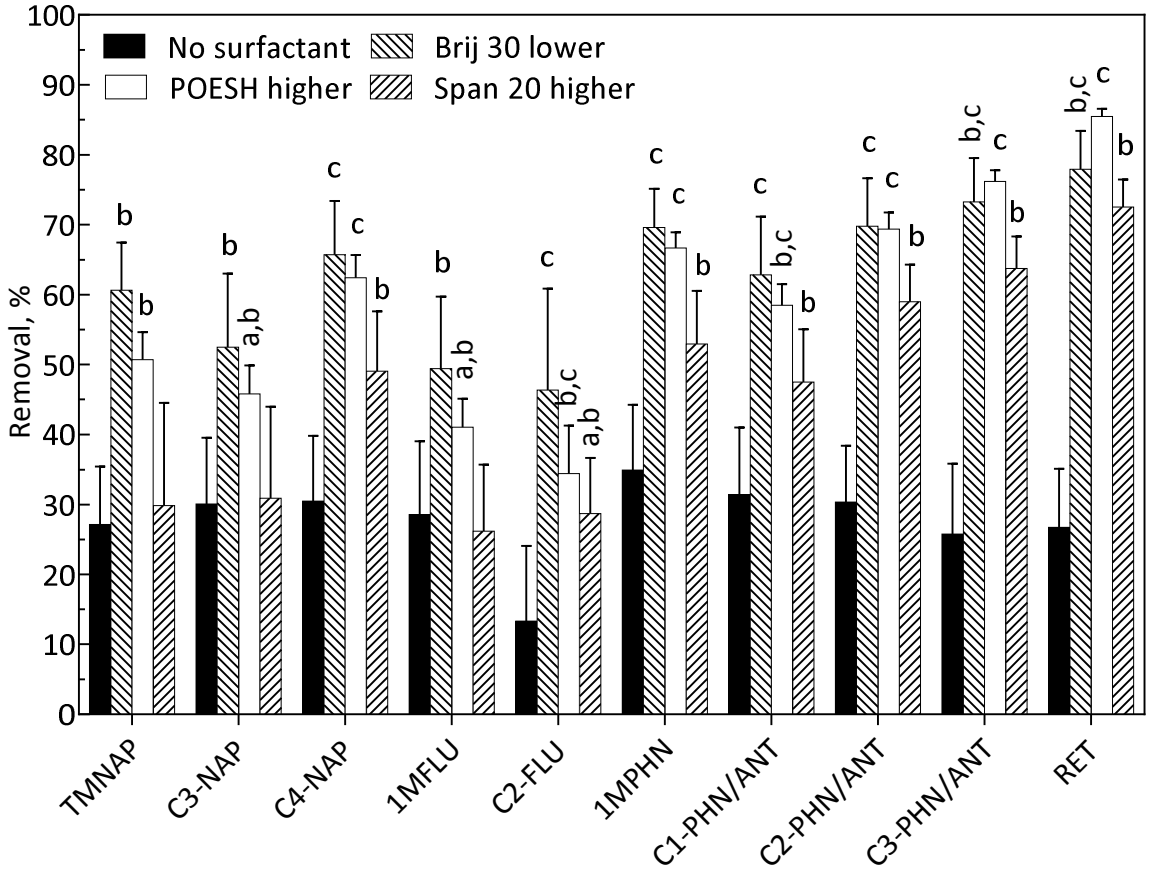

Figure A.3 Effect of Brij 30, POESH, and Span 20 in followup biodegradation experiment on residual alkylated 2- and 3-ring PAHs from bioreactor-treated soil after 16 days. "Lower" and "higher" refer to doses described in Materials and Methods. Bars represent means and standard deviations of five replicates. Conditions for which there was not a significant difference (α=0.05) in final PAH soil-concentration detected by Tukey's method are assigned the same letter. Bars for which no letter is shown are implicitly designated "a". PAHs for which there were no significant differences between no-surfactant controls and all surfactant conditions are not shown.

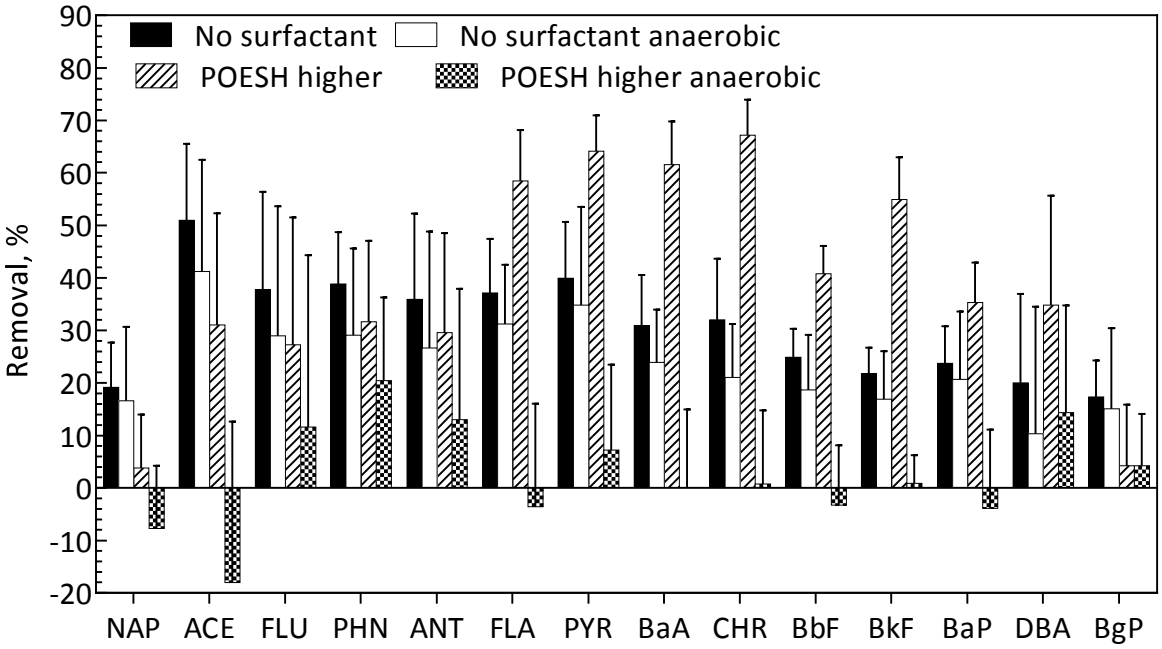

Figure A.4 Effect of anaerobic incubation with POESH on the biodegradation of residual PAHs from bioreactor-treated soil after 16 days. "Higher" refers to the higher dose as described in Materials and Methods. Bars represent means and standard deviations of four replicates.

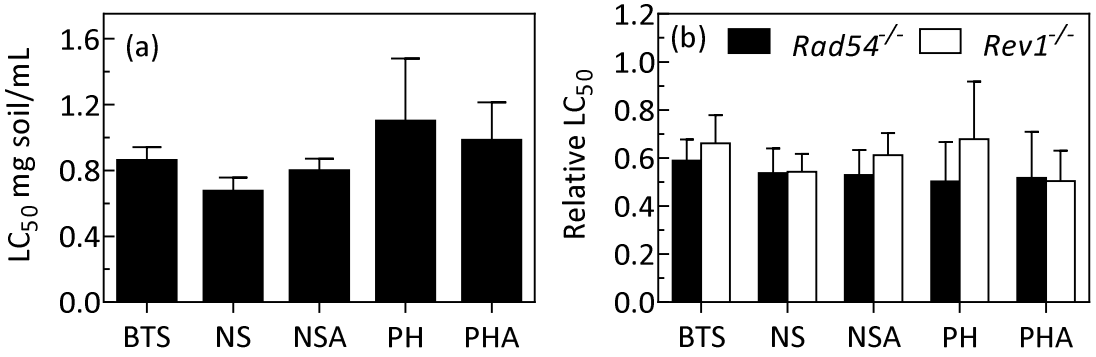

Figure A.5 Effect of anaerobic incubation with POESH on (geno)toxicity of bioreactor treated soil after 16 days (referred to in the text as the followup (geno)toxicity experiment). **(a)** LC_{50} of soil for parental DT40 cell line. **(b)** Relative LC_{50}'s of soil for *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$ mutants. Bars represent means and standard deviations of three experiments. In pairwise t-tests, no condition is significantly different (α=0.05) than the bioreactor-treated soil (BTS). NS, no-surfactant; NSA, no-surfactant anaerobic; PH, POESH higher; PHA, POESH higher anaerobic.

APPENDIX B: SUPPORTING INFORMATION FOR CHAPTER 5

Preliminary time-course experiment

A preliminary experiment was conducted to choose appropriate batch residence times for second-stage treatment. Effluent from the first-stage bioreactor was dosed with POESH (24-mg/g-dry soil) in quadruplicate 30-mL glass centrifuge tubes with PTFE-lined silicone-septa screwcaps and incubated under nitrogen for 48 hours, after which the caps were opened to allow air into the headspace, following the methods in Adrion *et al.* (in prep.). POESH-containing incubations were sacrificed at 3, 7, 14, 21 and 28 days, and incubations without POESH were sacrificed at 21 days. PAH removal and soil (geno)toxicity were measured as described in Section 5.2.6. Results are shown for PAH removal in Figure B.1 and for (geno)toxicity in Figure B.2.

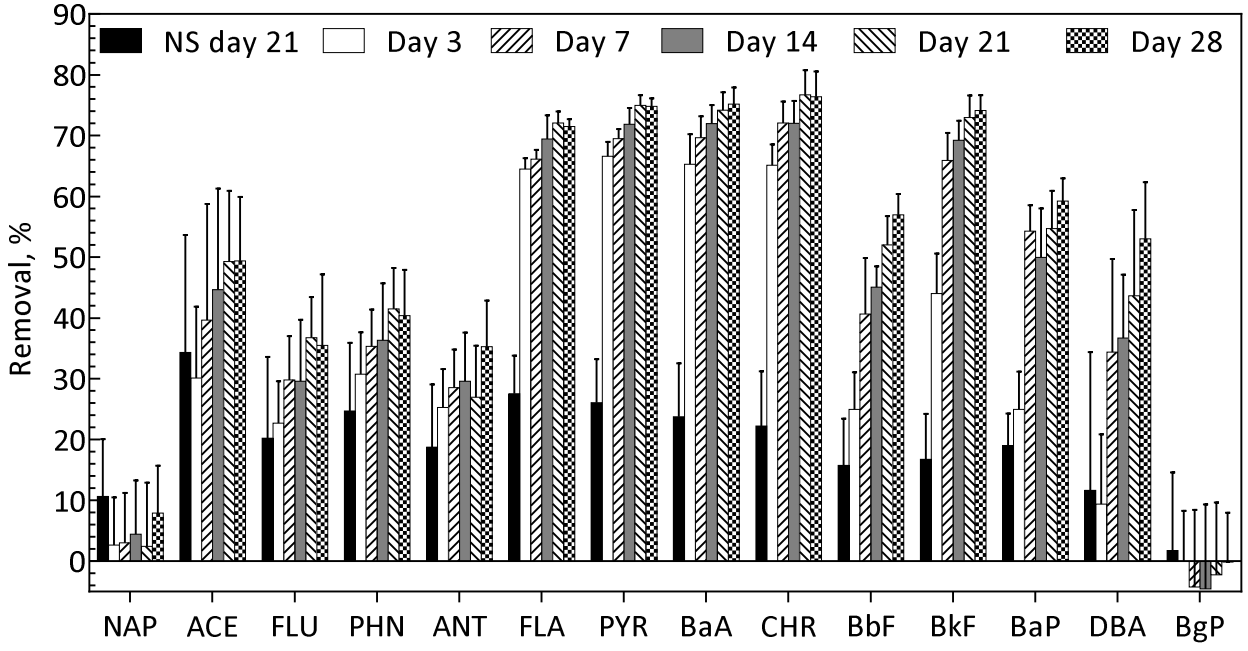

Figure B.1 Effect of incubation with POESH over time on PAHs remaining after first-stage bioreactor treatment. NS day 21, no-surfactant control day 21; Day 3-28; incubations with POESH

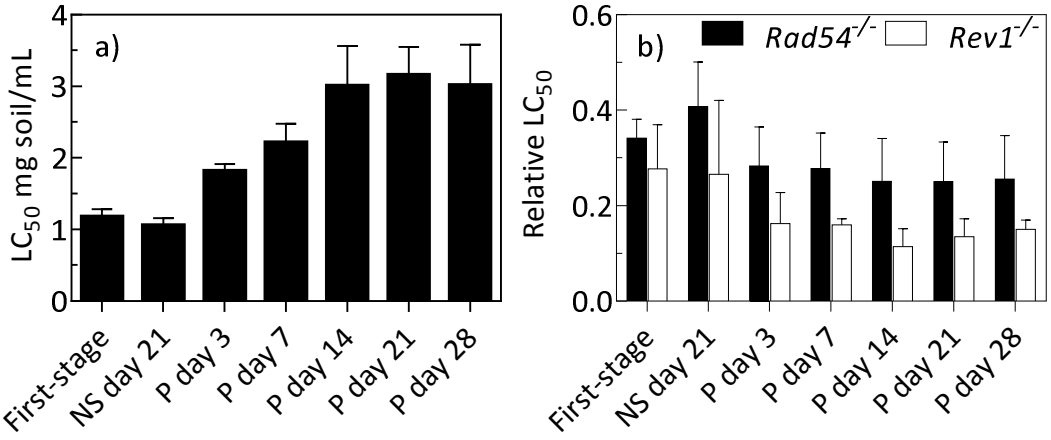

Figure B.2 Effect of incubation of first-stage treated soil with POESH over time on (geno)toxicity. **(a)** Toxicity to the parental DT40 cell line, and **(b)** genotoxicity as determined by relative LC_{50} values (mutant LC_{50}/parental LC_{50}) using the DNA repair-deficient mutants *Rad54*$^{-/-}$ and *Rev1*$^{-/-}$. Bars represent means and standard deviations of three experiments. NS, no-surfactant control; P, POESH.

Table B.1 Concentrations of PAHs and Oxy-PAHs measured in untreated bioreactor feed soil.[a]

Compound	Abbreviation	Concentration (µg/g dry soil)
Naphthalene	NAP	21.9 ± 1.7
Acenaphthene	ACE	8.0 ± 1.1
Fluorene	FLU	5.0 ± 0.9
Phenanthrene	PHN	92.7 ± 16.8
Anthracene	ANT	8.5 ± 0.7
Fluoranthene	FLA	64.8 ± 4.2
Pyrene	PYR	105.3 ± 5.9
Benz[*a*]anthracene	BaA	39.7 ± 2.3
Chrysene	CHR	47.6 ± 1.5
Benzo[*b*]fluoranthene	BbF	23.5 ± 1.5
Benzo[*k*]fluoranthene	BkF	11.6 ± 0.6
Benzo[*a*]pyrene	BaP	28.2 ± 1.7
Dibenz[*a,h*]anthracene	DBA	2.1 ± 0.1
Benzo[*g,h,i*]perylene	BgP	25.5 ± 3.7
9,10-phenanthrenequinone	PQ	1.1 ± 0.1
9-fluorenone	FLO	1.2 ± 0.3
9,10-anthraquinone	AQ	23.5 ± 2.0
Benz[*a*]anthracene-7,12-quinone	BAQ	2.0 ± 0.0

[a] Values represent means and standard deviations of four replicate extractions.

Table B.2 Concentrations of PAHs and Oxy-PAHs after first-stage treatment and 7-day second-stage treatment.[a]

Compound	Concentration (µg/g dry soil)					
	7 day trial 1		7 day trial 2		7 day trial 3	
	First stage	Second stage	First stage	Second stage	First stage	Second stage
NAP	19.5 ± 0.2	17.7 ± 0.7	20.0 ± 0.8	18.4 ± 1.3	18.8 ± 1.0	17.1 ± 0.7
ACE	1.77 ± 0.13	0.86 ± 0.11	1.63 ± 0.16	0.48 ± 0.17	2.50 ± 0.06	1.10 ± 0.10
FLU	1.53 ± 0.04	0.89 ± 0.05	1.76 ± 0.04	0.96 ± 0.07	1.70 ± 0.12	0.90 ± 0.03
PHN	25.2 ± 2.0	11.3 ± 0.5	25.3 ± 1.1	10.8 ± 0.8	25.0 ± 0.8	9.78 ± 0.42
ANT	2.63 ± 0.12	1.46 ± 0.05	2.75 ± 0.15	1.57 ± 0.18	2.67 ± 0.10	1.43 ± 0.06
FLA	17.4 ± 0.6	2.90 ± 0.15	17.7 ± 1.1	3.94 ± 0.47	18.2 ± 0.3	2.61 ± 0.08
PYR	30.0 ± 1.0	4.42 ± 0.20	30.0 ± 1.2	3.87 ± 0.42	30.6 ± 0.4	3.66 ± 0.13
BaA	14.4 ± 0.8	2.78 ± 0.20	16.9 ± 0.9	8.63 ± 1.37	15.7 ± 0.7	2.81 ± 0.12
CHR	23.2 ± 0.9	3.15 ± 0.68	20.8 ± 1.4	10.6 ± 1.0	18.5 ± 1.3	3.52 ± 0.16
BbF	12.7 ± 0.4	8.78 ± 0.33	12.7 ± 0.6	10.7 ± 0.9	12.6 ± 0.6	8.94 ± 0.52
BkF	6.40 ± 0.18	3.89 ± 0.13	6.21 ± 0.29	5.13 ± 0.38	6.15 ± 0.15	4.41 ± 0.18
BaP	15.1 ± 0.4	9.89 ± 0.49	22.2 ± 2.2	17.4 ± 2.7	15.4 ± 0.6	9.95 ± 0.61
DBA	1.44 ± 0.07	1.22 ± 0.10	1.59 ± 0.16	1.45 ± 0.32	1.30 ± 0.04	1.25 ± 0.33
BgP	22.4 ± 0.8	20.9 ± 0.9	22.7 ± 1.3	20.2 ± 2.4	19.4 ± 0.4	18.6 ± 2.0
PQ	0.28 ± 0.02	0.14 ± 0.01	0.23 ± 0.02	0.12 ± 0.02	0.27 ± 0.06	0.14 ± 0.02
FLO	0.60 ± 0.02	0.48 ± 0.06	0.59 ± 0.07	0.46 ± 0.03	0.60 ± 0.04	0.43 ± 0.05
AQ	3.30 ± 0.32	1.16 ± 0.09	3.32 ± 0.45	1.24 ± 0.23	3.44 ± 0.48	1.01 ± 0.19
BAQ	0.80 ± 0.09	0.56 ± 0.11	0.76 ± 0.09	0.40 ± 0.02	0.71 ± 0.11	0.43 ± 0.07

[a] Values represent means and standard deviations of four replicate extractions. Abbreviations are defined in Table B.1.

Table B.3 Concentrations of PAHs and Oxy-PAHs after first-stage treatment and 12-day second-stage treatment.[a]

Compound	Concentration (µg/g dry soil)					
	12 day trial 1		12 day trial 2		12 day trial 3	
	First stage	Second stage	First stage	Second stage	First stage	Second stage
NAP	18.0 ± 0.6	16.8 ± 0.3	19.0 ± 1.8	16.7 ± 1.5	19.4 ± 0.4	16.4 ± 0.6
ACE	1.64 ± 0.12	0.87 ± 0.09	1.59 ± 0.22	0.46 ± 0.07	2.60 ± 0.09	1.02 ± 0.08
FLU	1.41 ± 0.06	0.85 ± 0.04	1.59 ± 0.13	0.85 ± 0.06	1.79 ± 0.18	0.86 ± 0.04
PHN	23.6 ± 1.2	11.0 ± 0.3	24.4 ± 2.3	9.51 ± 0.73	26.1 ± 0.7	9.61 ± 0.27
ANT	2.45 ± 0.08	1.46 ± 0.04	2.63 ± 0.25	1.40 ± 0.12	3.00 ± 0.11	1.37 ± 0.04
FLA	16.3 ± 0.4	2.82 ± 0.07	16.9 ± 1.7	2.26 ± 0.18	19.0 ± 0.6	2.29 ± 0.11
PYR	27.7 ± 0.8	4.55 ± 0.13	29.4 ± 2.9	3.09 ± 0.15	31.8 ± 0.6	3.37 ± 0.11
BaA	12.9 ± 1.3	2.17 ± 0.57	13.6 ± 6.1	2.83 ± 0.14	16.2 ± 0.6	2.31 ± 0.04
CHR	21.0 ± 4.9	3.42 ± 0.87	19.6 ± 2.1	3.45 ± 0.29	19.1 ± 1.4	2.70 ± 0.10
BbF	12.0 ± 0.5	5.51 ± 0.25	12.5 ± 1.4	8.88 ± 0.50	13.0 ± 0.4	7.47 ± 0.27
BkF	5.96 ± 0.15	1.88 ± 0.07	6.07 ± 0.70	4.11 ± 0.15	6.51 ± 0.20	3.19 ± 0.09
BaP	13.9 ± 0.4	6.44 ± 0.25	20.8 ± 4.5	13.0 ± 1.9	16.4 ± 0.6	8.13 ± 0.21
DBA	1.23 ± 0.17	1.09 ± 0.06	1.64 ± 0.28	1.33 ± 0.29	1.41 ± 0.13	1.16 ± 0.23
BgP	20.6 ± 0.6	19.8 ± 0.5	22.9 ± 2.8	20.3 ± 1.5	21.1 ± 1.1	17.7 ± 1.4
PQ	0.28 ± 0.04	0.14 ± 0.02	0.29 ± 0.05	0.11 ± 0.02	0.31 ± 0.04	0.13 ± 0.03
FLO	0.57 ± 0.03	0.49 ± 0.02	0.58 ± 0.06	0.40 ± 0.04	0.60 ± 0.03	0.43 ± 0.06
AQ	3.22 ± 0.14	1.25 ± 0.22	3.47 ± 0.50	0.96 ± 0.15	3.78 ± 0.64	0.84 ± 0.18
BAQ	0.75 ± 0.11	0.42 ± 0.06	0.75 ± 0.06	0.31 ± 0.04	0.81 ± 0.15	0.33 ± 0.05

[a] Values represent means and standard deviations of four replicate extractions. Abbreviations are defined in Table B.1.

Table B.4 Removal of PAHs and oxy-PAHs during first-stage and 7-day second-stage treatment.[a]

Compound	Removal relative to untreated feed soil (%)					
	7 day trial 1		7 day trial 2		7 day trial 3	
	First stage	Overall[b]	First stage	Overall[b]	First stage	Overall[b]
NAP	10.7 ± 7.2	18.9 ± 7.1	8.5 ± 8.2	15.8 ± 9.1	14.0 ± 8.3	22.0 ± 7.1
ACE	77.9 ± 3.4	89.2 ± 2.0	79.6 ± 3.4	94.0 ± 2.2	68.7 ± 4.3	86.2 ± 2.3
FLU	69.5 ± 5.3	82.3 ± 3.2	65.1 ± 6.0	80.9 ± 3.6	66.2 ± 6.2	82.1 ± 3.1
PHN	72.8 ± 5.4	87.9 ± 2.3	72.8 ± 5.1	88.4 ± 2.3	73.0 ± 5.0	89.5 ± 2.0
ANT	69.1 ± 3.0	82.9 ± 1.6	67.7 ± 3.2	81.6 ± 2.7	68.6 ± 2.9	83.3 ± 1.6
FLA	73.1 ± 2.0	95.5 ± 0.4	72.6 ± 2.5	93.9 ± 0.8	71.9 ± 1.9	96.0 ± 0.3
PYR	71.6 ± 1.9	95.8 ± 0.3	71.5 ± 1.9	96.3 ± 0.4	71.0 ± 1.7	96.5 ± 0.2
BaA	63.8 ± 2.8	93.0 ± 0.6	57.5 ± 3.3	78.3 ± 3.7	60.4 ± 2.9	92.9 ± 0.5
CHR	51.2 ± 2.4	93.4 ± 1.4	56.4 ± 3.3	77.8 ± 2.1	61.1 ± 3.0	92.6 ± 0.4
BbF	46.1 ± 3.7	62.7 ± 2.7	46.1 ± 4.2	54.4 ± 4.8	46.4 ± 4.2	62.0 ± 3.2
BkF	44.9 ± 3.1	66.5 ± 2.0	46.6 ± 3.6	55.8 ± 3.9	47.1 ± 2.9	62.1 ± 2.4
BaP	46.5 ± 3.5	65.0 ± 2.7	21.2 ± 9.0	38.2 ± 10.1	45.4 ± 3.9	64.8 ± 3.0
DBA	32.0 ± 5.6	42.4 ± 6.0	25.0 ± 8.9	31.5 ± 15.8	38.6 ± 4.6	41.1 ± 16.0
BgP	12.0 ± 13.3	17.9 ± 12.6	10.8 ± 14.1	20.7 ± 15.0	24.0 ± 11.3	27.0 ± 13.3
PQ	74.8 ± 3.2	87.3 ± 1.5	79.7 ± 2.5	89.0 ± 1.7	76.0 ± 5.9	87.9 ± 2.0
FLO	52.0 ± 10.5	61.8 ± 9.6	52.8 ± 11.6	62.9 ± 8.4	51.7 ± 10.9	65.6 ± 8.3
AQ	86.0 ± 1.8	95.1 ± 0.6	85.9 ± 2.3	94.7 ± 1.1	85.4 ± 2.4	95.7 ± 0.9
BAQ	60.5 ± 4.5	72.2 ± 5.3	62.2 ± 4.6	80.4 ± 1.1	64.9 ± 5.2	78.5 ± 3.4

[a] Values represent means and standard deviations from four replicate extractions. Standard deviations are calculated through propagation of error. [b] Combined removal from first- and second-stage treatment. Abbreviations are defined in Table B.1.

Table B.5 Removal of PAHs and oxy-PAHs during first-stage and 12-day second-stage treatment.[a]

Compound	Removal relative to untreated feed soil (%)					
	12 day trial 1		12 day trial 2		12 day trial 3	
	First stage	Overall[b]	First stage	Overall[b]	First stage	Overall[b]
NAP	17.7 ± 7.2	23.2 ± 6.3	13.0 ± 10.8	23.4 ± 9.1	11.3 ± 7.3	24.9 ± 6.6
ACE	79.6 ± 3.2	89.1 ± 1.9	80.1 ± 3.8	94.3 ± 1.2	67.5 ± 4.6	87.2 ± 2.0
FLU	72.0 ± 4.9	83.1 ± 3.0	68.4 ± 6.0	83.2 ± 3.1	64.4 ± 7.1	83.0 ± 3.0
PHN	74.6 ± 4.8	88.1 ± 2.2	73.6 ± 5.4	89.7 ± 2.0	71.9 ± 5.2	89.6 ± 1.9
ANT	71.3 ± 2.6	82.8 ± 1.5	69.2 ± 3.9	83.5 ± 2.0	64.8 ± 3.2	83.9 ± 1.4
FLA	74.8 ± 1.7	95.6 ± 0.3	73.9 ± 3.1	96.5 ± 0.4	70.7 ± 2.1	96.5 ± 0.3
PYR	73.7 ± 1.6	95.7 ± 0.3	72.1 ± 3.2	97.1 ± 0.2	69.8 ± 1.8	96.8 ± 0.2
BaA	67.4 ± 3.8	94.5 ± 1.5	65.7 ± 15.6	92.9 ± 0.5	59.1 ± 2.7	94.2 ± 0.4
CHR	55.8 ± 10.3	92.8 ± 1.8	58.9 ± 4.6	92.7 ± 0.6	59.8 ± 3.2	94.3 ± 0.3
BbF	48.8 ± 3.8	76.6 ± 1.8	46.8 ± 6.8	62.3 ± 3.2	44.6 ± 3.9	68.3 ± 2.3
BkF	48.7 ± 2.8	83.9 ± 1.0	47.8 ± 6.5	64.6 ± 2.2	44.0 ± 3.2	72.6 ± 1.5
BaP	50.7 ± 3.2	77.2 ± 1.6	26.3 ± 16.7	53.8 ± 7.4	41.9 ± 4.1	71.2 ± 1.9
DBA	41.9 ± 8.8	48.8 ± 4.4	22.8 ± 14.3	37.1 ± 14.1	33.5 ± 7.4	45.1 ± 11.6
BgP	19.0 ± 12.1	22.4 ± 11.6	10.0 ± 17.1	20.4 ± 13.1	17.3 ± 12.9	30.5 ± 11.7
PQ	74.6 ± 4.0	87.1 ± 1.9	74.4 ± 5.2	90.4 ± 1.6	72.1 ± 4.3	88.1 ± 3.1
FLO	54.0 ± 10.2	61.0 ± 8.6	53.7 ± 10.9	67.7 ± 7.5	51.5 ± 10.7	65.3 ± 9.0
AQ	86.3 ± 1.3	94.7 ± 1.0	85.3 ± 2.5	95.9 ± 0.7	83.9 ± 3.1	96.4 ± 0.8
BAQ	62.9 ± 5.5	79.2 ± 3.0	63.0 ± 3.1	84.6 ± 2.0	59.9 ± 7.4	83.9 ± 2.5

[a] Values represent means and standard deviations from four replicate extractions. Standard deviations are calculated through propagation of error. [b] Combined removal from first- and second-stage treatment. Abbreviations are defined in Table B.1.

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