EFFECTS OF BIOREMEDIATION ON GENOTOXIC RESPONSES TO EXTRACTS OF SOIL FROM A FORMER MANUFACTURED GAS PLANT SITE

by
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ABSTRACT

Megan R. Knight: Effects of Bioremediation on Genotoxic Responses to Extracts of Soil from a Former Manufactured Gas Plant Site
(Under the direction of Michael D. Aitken)

Soil from former manufactured-gas plant sites is typically contaminated with a complex mixture of hazardous compounds, including polycyclic aromatic hydrocarbons, many of which are suspected carcinogens. However, biological treatment of these soils may result in the production of genotoxic metabolites. To determine whether bioremediation of an MGP soil increases the formation of products detrimental to DNA repair mechanisms, we tested the genotoxic profiles of solvent extracts of contaminated soil from Salisbury, NC both before and after treatment in a laboratory-scale column that simulated *in situ* biostimulation. This study utilized a cell library containing a parent DT40 vertebrate cell line and a battery of isogenic mutants deficient in at least one DNA damage response pathway. Overall genotoxic responses from this study suggest biostimulation of contaminated soil is an effective tool for the reduction of parent compounds but that metabolites from aerobic microbial activity are more genotoxic than the original untreated soil.

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LIST OF ABBREVIATIONS

ACN…	Acetonitrile
AP…	Apurinic
BAP…	Benzo(*a*)pyrene
BPDE…	Benzo (a)pyrene Diol Epoxide
BER…	Base Excision Repair
CHO…	Chinese Hamster Ovary
DCM…	Dichloromethane
DMSO…	Dimethyl Sulphoxide
DNA…	Deoxyribonucleic Acid
FBS…	Fetal Bovine Serum
FISH…	Fluorescence *In-Situ* Hybridization
H_2O_2…	Hydrogen Peroxide
HPLC…	High Performance Liquid Chromatography
HPRT…	Hypoxanthine-guanine phosphoribosyl-transferase
LC50…	Lethal Concentration 50
MGP…	Manufactured Gas Plant
NER...	Nucleotide Excision Repair
NOEL…	No Observable Effect Level
PAH…	Polycyclic Aromatic Hydrocarbons
POP…	Persistent Organic Pollutants
ROS…	Reactive Oxygen Species

TEO…	Total Extractable Organic
TMoA…	Toxic Modes of Action
TLS…	Translesion Synthesis
US EPA…	United States Environmental Protection Agency
XTT…	2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium Hydroxide Inner Salt

CHAPTER 1

INTRODUCTION AND OBJECTIVES

1.1 INTRODUCTION

Former manufactured gas plant (MGP) sites contain a multitude of chemicals of concern to environmental and human health. Polycyclic aromatic hydrocarbons (PAH) comprise a major class of compounds pervasive to MGP byproducts, such as coal tar, which at one time were of great abundance and economic value to MGP [1]. On-site disposal and underground storage of organic residual materials contaminated MGP soils and surrounding surface and groundwater. Since PAH are well-established carcinogens and mutagens, which can be persistent organic pollutants (POP) and biomagnify, [2-9] decontamination of MGP sites is important to protect public health.

Utilization of microorganisms with extensive PAH biotransformation capacities has long been considered an acceptable means of PAH removal at polluted sites [10, 11]. Frequently, though, PAH metabolism is incomplete and the more recalcitrant, higher molecular weight PAH or oxidized PAH metabolites persist [12]. In higher organisms exposed to PAH, the metabolic products, such as *o*-quinones, are the ultimate carcinogenic forms of PAH [13, 14]. The reactive oxygen species (ROS) produced during PAH *o*-quinone formation exacerbate genotoxicity through oxidative stress [15]. Oxidized PAH metabolites also have increased polarity relative to the parent compounds and can migrate into water supplies [16].

Appropriate definition of acceptable end-points in bioremediation of specific MGP sites must incorporate thorough hazard characterizations [17]. Current understanding of how partially oxidized PAH metabolites interact with organic and inorganic constituents of complex mixtures and contribute to genotoxicity is underdeveloped. Reports of specific deoxyribonucleic acid (DNA) damage responses to individual PAH or simple PAH mixtures are available [13, 15, 18-23]; however, to date, established methods are insufficient to characterize the genotoxicity signatures of complex mixtures known to contain PAH.

1.2 OBJECTIVES

This study was motivated by the need to address potential adverse effects of exposures to contaminated soil from a former MGP, and whether bioremediation could mitigate these effects. The primary objective of this work was to ascertain whether the genotoxic potential of soil treated with nutrient amendments and oxygen to stimulate biodegradation is altered from the original untreated soil. Biostimulation was simulated in a laboratory soil column that was operated in parallel with a control column to which no amendments were added. The second objective was to determine the type (s) of DNA damage, if any, incurred after DT40 cells and isogenic mutants were exposed to extracts of contaminated soil.

To discover potential genotoxicity, chemical mixtures from the untreated soil and the biostimulated and control column samples were extracted and utilized in the DT40 bioassay. This assay, which employs a reverse genetics approach with DNA repair and cell cycle checkpoint genes in isogenic chicken B-lymphocytes, was used to examine if

genotoxic products formed as the metabolism of aerobic microbial communities was enhanced by biostimulation.

The existence of possible genotoxic PAH metabolites were of special interest in this project. While all DNA damage repair pathways were inspected with the DT40 bioassay, homologous recombination, non-homologous end joining, and nucleotide excision repair pathways were emphasized because these are established mechanisms which restore the integrity of DNA following exposures to PAH.

CHAPTER 2

LITERATURE REVIEW

2.1 PAH CONTAMINATION IN THE ENVIRONMENT

2.1.1 STRUCTURE AND PHYSICO-CHEMICAL PROPERTIES

PAH, a major class of organic compounds, are composed of at least two fused benzene rings in various structural configurations, such as linear, angular, and cluster arrangements [24]. These compounds may also contain other rings that, unlike benzene, are not six-sided. Solid states of PAH can be crystals, prisms, leaflets, and needles. Most PAH and their derivatives fluoresce, and they readily undergo photo-oxidization. PAH solubility properties vary, but most are poorly soluble in water and slightly soluble in non-polar solvents, such as acetic acid, benzene, and acetone [2]. These annulated compounds persist in ecosystems and bioaccumulate in organisms due to their hydrophobic nature and stability conferred by delocalized electron clouds, also known as resonance energy [2, 25, 26]

2.1.2 ORIGINS OF POLYCYCLIC AROMATIC HYDROCARBONS

PAH enter the atmosphere, aquatic and terrestrial ecosystems from biosynthetic, geochemical and anthropogenic sources [27]. The ubiquity of PAH in the environment is predominantly attributed to incomplete combustion of coal and petroleum products. Other anthropogenic sources of PAH include exhaust from transportation and

shipping processes, as well as asphalt, creosote, and wood-preserving plants [3]. Natural means, like volcanic eruptions and forest fires, also generate and release substantial quantities of PAH into the environment. Biogenic sources, such as plants, algae and microorganisms, and incomplete combustion of organic materials, such as cigarettes and charbroiled foods, present lesser contributions [28]. While the production and discharge of PAH and other persistent organic pollutants from industry and incomplete combustion are classified as unintentional contamination [9], the ultimate environmental fate of most PAH is influenced by sorption to hydrophobic domains in soils and sediments.

Sub-surface soils at MGP sites are often contaminated with tars from poor practices during operation, leaky underground holding tanks, and substandard decommissioning of plants [29]. Coal tar constituents can include PAH, monocyclic aromatic hydrocarbons such as benzene, toluene, ethylbenzene, and xylene, and other organic compounds of toxicological concern [1].

2.1.3 Outcomes of Environmental PAH Contamination

2.1.3.1 Microbial Biodegradation

The principle of "biological infallibility" proposes that for every organic compound there exists a microbe suited with the appropriate biochemical pathway necessary for biodegradation [30]. Degradation rates of specific compounds, however, are well established to be site-specific as a result of variations in system conditions and constituents of microbial biodegrading communities [31]. A correlation seems to exist between PAH concentration and PAH-degrader populations; highly contaminated soils have been shown to contain more degraders than soils with less PAH concentrations using most probable number enumeration [32, 33]. While bacterial or fungal metabolism is

common for the more readily degradable PAH, such as naphthalene or phenanthrene, high molecular weight PAH generally require the co-metabolism of a consortium of microorganisms[34].

A cascade of biochemical reactions is necessary for PAH biodegradation; the initiating events of aerobic metabolism generally include oxidation of aromatic rings by dioxygenases, followed by a dehydrogenase reaction. Numerous bacteria, particularly within the genera *Sphingomonas, Burkholderia, Pseudomonas and Mycobacterium* [35-40], are identified to metabolize and co-metabolize PAH with multi-step cascade initiated by a dioxygenase and subsequent formation of *cis*-dihydrodiols [10, 34, 41]. Dehydrogenases can then generate catechols from the *cis*-dihydodiols; water and carbon dioxide are ultimate metabolic end-products [42]. Also, a few bacteria in the *Mycobacterium* sp. can produce *trans*-dihydrodiols with a methane monooxygenase, a cytochrome P450-monooxygenase [10].

It has been postulated that oligotrophic microorganisms, such as sphingomonads, have developed adaptations for catabolizing complex mixtures containing PAH. Bacteria produce bio-films containing extracellular polymeric substances with which PAH associate, possibly through interactions of the hydrophobic chemicals with hydrophobic moieties of the extracellular matrix [43-45]. Similarly, sphingan, an exopolysacchride matrix, is suggested to be the basis of a mechanism that *Sphingomonas* spp. use to compete with other microorganisms during periods of famine since PAH remain readily bioavailable while associated with the matrix [46].

Fungi effectively catabolize PAH in soils, via the cytochrome P-450 system, and for wood-rotting fungi, soluble extracellular enzymes associated with lignin degradation

[11, 47, 48]. *Phanerochaete chrysosporium*, a species of white rot fungus, has been hailed as an omnipotent microorganism due to its capability to co-metabolize a wide range of organic compounds in both aerobic and anaerobic environments with non-ligninolytic and ligninolytic enzymes [49].

Environments such as soils, sediments and groundwater aquifers can develop anaerobic zones over time [50, 51]. In municipal sewage sludges [52, 53]and some sediments, anaerobic microbial communities hydrogenate PAH aromatic rings to initiate metabolism and then cleave rings in denitrifying and sulfate-reducing conditions [10].

As mentioned previously, soils and sediments are excellent repositories for PAH. Decontamination of PAH-contaminated soils by biological treatment can be a more efficient, economically realistic, and case-specific approach than traditional physicochemical treatment methods [34].

2.1.3.2. BIOAVAILABILITY

Soils are dynamic systems that include gas, liquid, and solid phases. Heterogeneous solid phases can be unique within and between samples as a result of varying spatial arrangements, particularly on the micro-scale [30]. These differences are also attributed to variances in physicochemical properties of inorganic and organic components. Binding coefficients of chemicals to soil colloids can be directed by electrostatic interactions, non/specific partitioning, surface reactions, and hydrophobic interactions [54]. Surface reactions have been suggested to guide the sorption of hydrophobic organic compounds to organo-mineral complexes [55, 56]. Likewise, the intrinsically low aqueous solubility of PAH promotes their tendency to tightly associate

with soil particle surfaces, which decreases volatilization, photolysis, and even microbial biodegradation. PAH bioavailability diminishes almost logarithmically as PAH molecular weights increase [11, 57]. Weathering processes of aging soils also result in the decline of PAH bioavailability over time [58, 59]. Because the dissolution of chemicals in aqueous solutions is believed to facilitate microbial degradation, dissociation of PAH from soil particles is believed to be important for the elimination of PAH from environmental samples [60, 61]. Enhancement of PAH bioavailability to microorganisms can be achieved through the addition of synthetic surfactants or biosurfactants [26, 26, 62-64]. High concentrations of the compounds, which can serve as substrates in microbial metabolism, can also become inhibitors to biodegrading microorganisms [65], therefore biodegradation and other fate processes which lower PAH content, viz. volatilization and photolysis, generally occur at greater rates in areas peripheral to those with highest PAH concentration [35].

2.1.3.3 BIOREMEDIATION

Remediation technologies designed to reclaim terrestrial systems affected by chemical contaminants include *ex situ* and *in situ* methods. Traditional *ex situ* strategies employ excavation of impacted soils and treatment. Although such methods are effective for removal of pollutants, they are expensive [34]. *In situ* methods, however, present difficulties with control of toxic metabolites or the generation of treatment by-products and mitigation of contaminant mobility into groundwater and surrounding areas [66-68]. Despite significant financial investments associated with *ex situ* options, environmentally acceptable end-points can be achieved more effectively through greater control of the

process as a whole; this can translate into shorter treatment periods [69]. In recent decades, bioremediation has been investigated extensively for the feasibility of PAH removal from former MGP soil. Bioremediation encourages microbial metabolism of contaminants through biostimulation with nutrient amendments and/or bioaugmentation with microbe populations known to possess degradative capacities [10]. The efficacy of bioremediation is defined by: (i) measurements of parent compound removal and (ii) determination of the indigenous microbes' metabolic potentials to transform the parent compounds [70]. Utilization of analytical chemistry techniques and bioassays address the biological treatability of contaminated soils. Regardless of the remediation method applied, thorough characterization of contaminated sites and a well-developed knowledge of the site topology and geochemical signature is necessary for successful decontamination.

2.2 TOXICITY OF COMPLEX MIXTURES

2.2.1 RISK ASSESSMENT APPROACHES

Toxicity characterization of single compounds received great attention throughout the 20th century [71-73]. Humans and ecological systems are, however, concurrently or sequentially exposed to complex mixtures through occupational settings, air particulate matter, surface and groundwater, wastewater effluent, soils and sediments. Hazard characterizations and risk assessments of complex mixtures is far more challenging than that of individual substances. However, many approaches and models have been designed to effectively assess the adverse effects of complex mixtures. Three primary factors contribute to the complexity risk assessment for mixtures: the difficulty in identifying every chemical component in a mixture [74], scant *a priori* toxicity data

available on all compounds present, and sparse knowledge of the mechanisms by which constituents are genotoxic or toxic [75].

The establishment of a systematic methodology to determine the toxicity and genotoxicity of complex mixtures is of great concern to the United States Environmental Protection Agency (US EPA). The most definitive law in the US regarding the necessity for thorough risk assessments of complex mixtures was the Comprehensive Environmental Response, Compensation and Liability Act and the Resource Conservation and Recovery Act [76, 77]. This Act deems a single-chemical risk assessment approach insufficient for characterizing hazardous wastes.

Surmountable challenges exist for risk characterization of complex mixtures. Poor correlation between chemical analysis of complex mixtures and toxicity in biological systems is well documented [78-81]. This is especially true for the environmental complex mixture whose composition can be dynamic as a result of transformation by environmental conditions, including microbial metabolism. In 2000 the US EPA responded to these difficulties with a supplemental guidance report to facilitate future risk assessment of complex mixtures [82]. The report includes recommendation of three approaches for complex mixture risk assessment, which are summarized below. They embody both biological and chemical analysis to better assess the risks associated with complex mixtures, particularly after their release into the environment. These approaches were again promoted in a 2002, US EPA Peer Review Workshop [82].

The US EPA promulgated strategies for determining the potential adverse effects of complex mixtures which include the surrogate approach, the comparative potency approach, and the relative potency factor approach [82, 83]. With the surrogate approach,

one chemical that is considered the best representative for a mixture is tested; often the target chemical is a designated priority pollutant. For example, often benzo(*a*)pyrene (BAP) is utilized as the single indicator compound for mixtures known to contain PAH. This approach incorporates a well-characterized surrogate mixture with established concentrations of the representative chemical. Then synthesis of a second mixture, which is a dilution based on the representative chemical concentration, should yield a product sufficiently similar to the surrogate mixture. Risks associated with the second mixture are anticipated to vary proportionately with the surrogate mixture. There are, however, great limitations to this approach because constituents of complex mixtures can belong to numerous classes of inorganic and organic compounds, so that one indicator chemical cannot adequately represent an entire mixture. Additionally, the concentrations of priority pollutants may not reflect those of other components in a mixture; consequently, the toxicity of the mixture is defined incorrectly [84, 85].

The comparative potency approach utilizes human and animal toxicity data from epidemiological and biological studies for groups of mixtures deemed sufficiently similar by chemical analysis. Often this approach is less powerful, though, because many data sets are unavailable or fragmented. Generally complete toxicological data sets for complex mixtures are not realistic due to economic and ethical impracticalities [86].

The final strategy suggested by the US EPA, the relative potency factor approach, ranks chemicals of a mixture by their toxic equivalency factors to determine the relative risk of a mixture. The major drawback of this approach is the assumption that the toxicity of mixtures is produced by the additive effects of chemicals while other potential chemical interactions are neglected.

Yet, another established method for effective risk assessments of complex mixtures exists which is the effect-directed analysis approach. Here a combination of physicochemical fractionation, biotesting or biological effect screening, and subsequent chemical analyses are employed to analyze public health risks associated with complex mixtures [87, 88]. This approach furthered the EPA toxicity evaluation of aqueous samples by making the total available quantity of organic and inorganic toxicants focal points, rather than the bio-available quantities [88]. Although cost and time intensive [81], this approach allows for individual toxicants of a complex mixture after fractionation and purification of samples, thereby yielding more meaningful analysis of specific complex mixtures.

2.2.2 Interactions of Complex Mixtures

As early as 1939 three categories of joint chemical interactions were defined which are still germane to the field of toxicology: independent joint action, similar joint action, and synergistic action [89]. Independent joint action describes chemicals that operate by different modes of action in organisms such that the presence of one chemical will not affect another compound's toxicity. Similar joint action depicts chemicals that cause similar effects in organisms. Toxicities of chemicals with similar joint actions are dependent on the presence and concentrations each chemical. Combined toxicity of chemicals from such classes is assumed to be predicted by knowledge of the independent effects of the chemicals. Synergistic action (chemical interaction) addresses mixtures where components synergize, potentiate, or antagonize each other. Toxicity of a mixture containing such compounds requires knowledge of the combined toxicity with respect to varying proportions [89, 90]. Plackett and Hewlett [90] further developed the concept of

chemical interactions and provided basis for the two standard models for toxic modes of action (TMoA): the concentration addition model of simple similar actions of chemicals with the same TMoA and the response action model of the independent joint action of chemicals with dissimilar TMoA [91]. These models are now regularly employed to predict the toxicity of complex environmental mixtures [92-95].

Numerous studies regarding chemical interactions have since concentrated on solitary chemicals, sequential exposures, and binary mixtures [73, 96]. In an effort to further understanding of complex mixtures on public health and the environment, others' research honed in on environmentally relevant concentrations of complex mixtures at the lowest observable adverse effect level or no observable effect level (NOEL) [97-99]. Analysis of high-dose concentrations is often meaningless with consideration to realistic environmental concentrations of toxicants [100-102]. In addition to dose-response relationships and low-dose extrapolations, the mechanisms of toxicokinetic and toxicodynamic interactions are popular focal points in risk assessments of complex mixtures today [86].

2.2.3 Interactions of PAH In Complex Mixtures

Current debate regarding the interactions of PAH in complex mixtures includes a traditional perspective where PAH produce an additive effect [82, 103, 104] and that based on more recent research which debunks the assumption that additivity is the sole interaction of chemicals in complex mixtures containing PAH. Work by Park and colleagues revealed that constituents of environmental samples containing PAH can interact by the standard additivity fashion as well as via antagonism [105-107]. Previous reports also indicated antagonist effects from soil-derived coal-tar creosote, which is 80-85% PAH,

and synthetic mixtures [108, 109]. It appears the chemical interactions of parent PAH compounds and subsequent derivatives or metabolites can be confounded by the relative concentrations of constituents in a complex mixture. One study focused on the toxicity of retene (7-isopropyl-1-methyl phenanthrene) and its hydroxylated derivatives and revealed a unique pattern in which the toxicity of the parent compound was potentiated by low concentrations of the derivatives and antagonized by high concentrations [110].

2.3 TOXICITY OF PAH AND DEGRADATION PRODUCTS

Complex mixtures containing PAH are categorized as strong carcinogens and mutagens, as evidenced by increased cancer rates after exposures [2-6]. The potency of PAH is established to be greater as the number of rings which comprise a compound increases [111]. The EPA classifies PAH as priority pollutants in environmental complex mixtures [112].

As with other procarcinogens, parent PAH compounds require metabolic activation to exert their full carcinogenic or mutagenic capacity as electrophilic metabolites [3, 21, 113-115]. Three main pathways are well established for the biotransfomation of PAH parent compounds to deleterious active metabolites: (i) dihydrodiol epoxide pathway, (ii) radical cation pathway, and (iii) *o*-quinone pathway [3, 21]. In mammalian systems, these enzymatic reactions are achieved by monoxygenases located within nuclei, thereby making DNA damage possible. The initial postulation of carcinogenicity derived from monooxygenases was doubted since these enzymes were thought to reside only in the endoplasmic reticulum [116, 117].

The dihydrodiol epoxide pathway requires three enzymatic reactions that include P-450 oxidation to form unstable arene epoxides, hydrolysis of arene oxides to form *trans*-dihydrodiol intermediates, and another P-450 catalyzed oxidation to yield vicinal dihydrodiol-epoxides as the final metabolic products [3, 21]. Epoxides form due to the addition of oxygen atoms across double bonds by enzymatic action or uncatalyzed oxidation processes [118]. Electrophilic dihydrodiol epoxides can be causative agents in DNA damage-induced carcinogenicity by two mechanisms. Contrary to the initial findings of Baird *et al.* [119], DNA adduct formation can occur in spite of the steric hindrance caused by bay or fjord regions in PAH. However, greater carcinogenicity and tumorgenicity is associated with fjord-region PAH than bay-region class [20, 120].

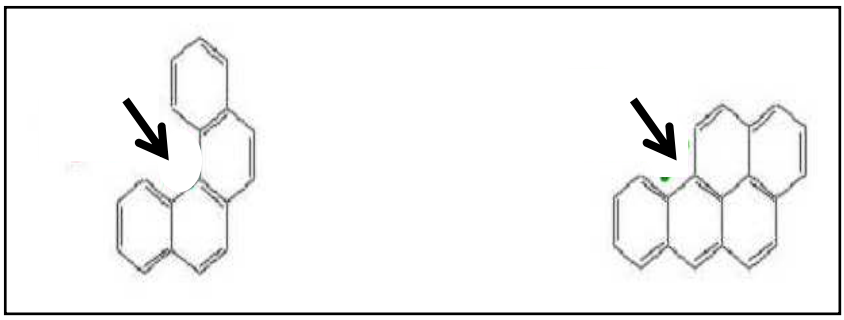

Figure 1. Fjord (left) and bay regions depicted. Fjord regions are non-planar and more reactive with DNA than planar bay region PAH.

Additionally, because most aromatic bonds are prochiral stereoisomers of dihydrodiol, epoxides can form; some are known ultimate carcinogens such as *anti*-diol-epoxides [121]. Benzo[a]pyrene, the hallmark compound for numerous PAH toxicology studies, is known to form (+)- 7/3,8a-dihydroxy-9a, 10a-epoxy-7,8,9, 10-tetrahydrobenzo[a]pyrene (BPDE). The extreme potency of BPDE as a carcinogen and mutagen in mammalian systems results from covalent bonds formed with nuclear DNA, mitochondrial DNA and cytosolic RNA [122, 123]. A reaction of DNA and BPDE forms

adducts which may either be stable or depurinating [19, 124]. Stable *anti*-BPDE-DNA adducts can be mutagenic via G to T transversions and proto-oncogenic *ras* activations [125-127].

Peroxidases act on PAH by the removal of one π electron from aromatic rings, resulting in radical cations that are considered major contributors to the carcinogenicity of PAH [128]. The one-electron oxidation intermediates can covalently bind PAH to DNA [129-131], as was demonstrated with BAP [128]. Unlike adducts generated from dihydrodiol epoxide/DNA interactions, radical cations generally react at the N7 position of guanine and adenine, the C8 position of guanine, and N3 position of adenine (Figure 2) [132].

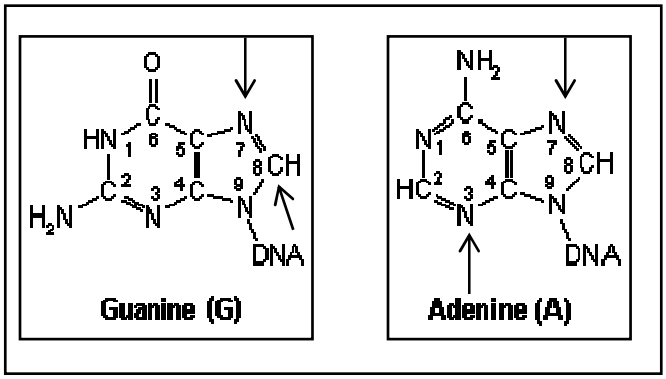

Figure 2. Arrows indicate locations of DNA bases, guanine and adenine, where dihydrodiol epoxides are most reactive to form adducts.

Subsequent hydrolysis of glycosidic bonds thereby produces spontaneous depurinations or apurinic (AP) sites [133, 134]. Investigation of metabolic activation of BAP by rat liver microsomes revealed production of 84% unstable adducts [135]. The transiency of AP sites generated from radical cations is the basis of current debate as to whether radical cations are sources of mutagenicity [136]. The white rot fungus *Phanerochaete chrysosporium* employs extracellular peroxidases to catalyze radical cation formation in PAH metabolism [137].

A third group of activated PAH electrophiles, the *o*-quinones, form via action of dihydrodiol dehydrogenases on either *cis*- or *trans*-dihydrodiols. These enzymes, which belong to the family aldo-keto reductases, suppress the dihydrodiol epoxide formation and instead produce catechols which are auto-oxidized to *o*-quinones [138, 139]. *O*-quinones have potential to be reactive Michael-acceptors and form stable and depurinating DNA adducts.

Additionally, PAH *o*-quinones can enter redox cycles and produce reactive ROS, such as semi-quinione radicals, hydroxyl radicals, hydrogen peroxide, and superoxide anion radicals. Severe oxidative stress can originate from ROS and effectively produce many oxidized cellular macromolecules, such as 8-oxodeoxyguanosine which is commonly associated with PAH carcinogenicity [13, 14]. In the presence of Cu (II), nanomolar concentrations of *o*-quinones are established to produce significant quantities of 8-oxo-dGuo by a singlet oxygen molecule [105]. DNA damage differs between Cu (II)- and Fe (III)-mediated *o*-quinone redox cycling, with the latter generating hydroxyl radicals. Abasic sites and oxidized pyrimidines have been linked with benzo[a]pyrene-7,8-dione, benz[*a*]anthracene-3,4-dione, and 7,12-dimethylbenz[*a*]anthracene-3,4-dione exposures [107].

2.4 GENOTOXICITY ASSAYS

2.4.1 PROKARYOTIC ASSAYS

Microbial assays have figured significantly into genetic toxicology due to speedy results, low cost, and ease of discovering low-frequency DNA insults, such as mutations. Microbial assays are historically relied upon for mutagenicity determination by

phenotypic modifications. These assays are designed to detect stable heritable changes in DNA with either reverse or forward mutations strains. Most mutagens produce numerous defects in DNA as a result of varying exposure concentrations, initial DNA damage, and subsequent responses of organisms to the primary DNA alteration [140].

The mutagenic potential of an individual chemical or mixture of chemicals can be determined by the Ames assay. This bacterial reverse mutation assay employs auxotrophic *Salmonella typhimurium* and *Escherichia coli* strains that are incapable of synthesizing an essential component for their survival as a result of base-pair substitution or frame-shift mutation in the responsible gene [141-145]. Should a chemical induce genetic damage by mutation of the preexisting mutation site, the bacteria will regain viability, which allows this test to provide quantitative genotoxicity data [146]. Development of numerous tester strains with varying mutations makes the Ames assay a useful diagnostic tool for mutagens which can operate by different mechanisms [147, 148]. Frequently the Ames assay is denoted as the *Salmonella*/microsome test, since this organism requires addition of an S9 metabolic activation system to mirror biotransformation comparable to that of higher eukaryotes. One advantage of this test is the possibility to evaluate singular types of DNA damaging sources. For instance, oxidative stress from PAH metabolism can be analyzed specifically by this bioassay with strains designed for unique sensitivity to oxidative mutagens [149]. This inexpensive, short-term test is accepted world-wide as a screening tool for genotoxicants and utilized by regulatory agencies to classify chemicals and determine acceptance of new compounds [150].

2.4.2 NON-MAMMALIAN EUKARYOTIC ASSAYS

Non-mammalian eukaryotic mutagenicity assays are today largely replaced by mammalian cell studies [151]. Numerous forward mutation assays, however, utilize *Sacchraomyces cerevisiae*, other fungi, plants, and insects (e.g. *Drosophila melanogaster*) to determine the mutagenicity of chemicals. Assays with non-mammalian eukaryotes to detect responsible agents for mitotic cross-overs and mitotic gene conversions have determined the genotoxic potential of hundreds of chemicals [152]. Genotoxic investigations with fruit flies can reveal recessive lethal mutations at approximately 800 loci on the X chromosome; gene mutations and smaller deletions are also detectable. This species uniquely provides information regarding chromosomal aberrations in germ cell lines, such as heritable translocations, and sex chromosome loss [153]. Varying NOELs, metabolic processes, and gametogenesis for both yeast and *Drosophila melanogaster* limit the extrapolation of these genotoxic assays to mammalian toxicity [151, 154]. Plant genetic toxicology methods are also largely supplanted by those which use mammalian eukaryotes, but if environments are well characterized and contain appropriate control measures, plants can still be effective *in situ* tools for monitoring toxicants. For example, Klekowski [155, 156] demonstrated a correlation exists between internal PAH concentrations of red mangroves through sediment exposures. Despite the low cost of these systems and ease with which reproducibility can be achieved, these model organisms lack homology to higher eukaryotes, constraining the relevance of the results [157].

2.4.3 MAMMALIAN ASSAYS

2.4.3.1 OVERVIEW

Mammalian *in vivo* and *in vitro* studies provide results more meaningful to translations to humans than assays that utilize microbial species or lower eukaryotes. Complex multicellular assays can require elaborate study designs to account for the numerous variables in these biological systems, and they generally involve longer, more costly experiments. In spite of the greater quantities of time and resources associated with these tests, mammalian genotoxic studies undoubtedly generate the most relevant information to enhance human risk assessments [151].

2.4.3.2 HPRT ASSAY

The hypoxanthine-guanine phosphoribosyl-transferase (HPRT) assay detects forward mutations which confer resistance to the pure purine analogues, i.e. 6-thioguanine and 8-azaguanine [158]. Cells possessing this X-linked gene die after exposure to cytotoxic agents unless the HPRT locus contains a mutation which renders the gene non-functional or permits only minimal expression. Historically, HPRT assays in cultured cells have been conducted with Chinese hamster ovary (CHO) or human pulmonary (V79) cells. Generally these assays require the inclusion of S9 enzymes, because these cell lines are deficient in Phase I microsomal monooxygenases. *In vivo* HPRT assays are most commonly performed using mice, rats, and monkeys [158, 159]. Results from such animal studies are particularly significant for human mutational monitoring since measurements yield reliable comparisons [160, 161].

One drawback of this assay is its duration; CHO cells double their number every 12 to 16 hours. It is important to note that a confounding factor of this assay is the possible decrease of reactive intermediates by Phase II detoxifying enzymes [140].

2.4.3.3 MICRONUCLEUS ASSAY

Formation of micronuclei indicates chromosomal damage or aneuploidy. Micronuclei represent fragmented chromosomes or even entire chromosomes which ineffectively separated into daughter cell nuclei during mitosis. This assay can utilize primary cultures of human lymphocytes [162, 163] and other mammalian cell lines [164, 165]. This assay is also conducted *in vivo* with mammalian species [166-168] by counting micronuclei in immature erythrocytes in bone marrow of mice exposed to potential chromosome-damaging agents [166-168]. This cytogenetic assay is simpler and less time-consuming than metaphase analyses, which also detect chromosome aberrations. The swiftness and ease with which chromosome aberrations are detected with the micronuclei assay is a major advantage over other cytogenetic tests. This test only detects chromosome breaks, unlike other chromosome aberration tests which measure chromosome breaks, exchanges and translocations [169]. While this assay is powerful for mechanistic studies, it is less commonly utilized in research studies related to genotoxicity [167].

2.4.3.4 CYTOGENETIC ASSAYS

Conventionally, chromosome aberrations can be directly observed with microscopy analysis of cells undergoing metaphase. Cells with unchanging, well-defined karyotypes, short doubling periods, low chromosome numbers, and sizable chromosomes

allow for detection of chromosome abnormalities [170-173]. Chinese hamster cells are often employed. This technique can identify specific classes of unstable chromosomes and chromatid aberrations that include: chromatid deletion, triadial or chromatid exchange, interstitial deletions from chromosome breakage, insertions, and translocations [151]. *In vivo* testing for chromosome aberrations often involves analysis of cells which rapidly divide, such as those from the bone marrow of rats, mice, or Chinese hamsters [170, 171, 174]. Experimental design and implementation of experimental conditions are especially important for cytogenetic assays, given the need for detailed data to distinguish between chemical-induced damage and naturally occurring achromatic gaps in chromosomes. Provided these considerations metaphase analysis is time-consuming and requires a high degree of technical skill. Recent incorporation of fluorescence *in-situ* hybridization (FISH) into cytogenetic assays has proven this is a valuable research tool for the detection of chromosome aberrations, particularly translocations [175]. This technique employs nucleic acid probes which fluoresce when bound to a complementary region of a chromosome. This process is commonly called "chromosome painting". Development of a battery of probes specific to human chromosomes has allowed for translation of chromosome aberration data to meaningful extrapolations to human health, yet this technique is expensive and time-consuming as well.

2.4.3.5 COMET ASSAY

Severing of DNA strands can be identified by this single-cell gel electrophoresis assay. Originally carried out with human lymphocytes [176] and later with other mammalian cells [177-179], the Comet assay is also employed with other species such as

plants, worms, mollusks, fish, and amphibians [180]. This rapid, sensitive and relatively simple technique [181] can also determine sequence- or gene-specific damage and repair when combined with the FISH technique [182, 183]. The primary advantage of the Comet assay is the differential detection of double strand breaks from non-specific DNA breaks through utilization of neutral and alkaline conditions, respectively [176, 184, 185]. As with the previously mentioned genetic toxicological approaches, the Comet assay is limited to a small range of detectable DNA injuries.

2.5 DT40 BIOASSAY

Another strategy for determining the genotoxic potential of chemical agents is the DT40 assay. The foundation of this system is a reverse genetics approach which incorporates cells from chicken B-lymphocytes deficient in genes related to DNA repair and replication [186]. In comparison to other higher eukaryotes, these white blood cells allow relatively facile manipulation of their genome because of unusually high occurrences of homologous recombination, which encourages the random integration of transfect DNA into their genomes [182, 187]. Uniquely, the parent DT40 cell line and its mutants, which have at least one gene "knocked out", have a non-functional p53 gene [188]. This feature permits mutant cells to by-pass apoptosis, so that the absence of the DNA repair and replication gene(s) of interest can be concluded to be the driving force behind cell death. The DNA repair pathways in this system can include nucleotide excision, base pair excision, homologous recombination, non-homologous end joining, translesion synthesis, and mis-match repair. Other categories indirectly related to DNA damage repair in the DT40 system are the cell cycle check point and RecQ helicase pathways.

A major advantage of the DT40 bioassay is the possibility to detect diverse DNA damage responses. Beyond the elucidation of numerous means in which a genome might be compromised, this system reveals the mechanisms by which damage is repaired. Additionally, the homology of the chicken genome is reported to be almost equivalent to murine cells in DNA recombination and repair genes [187]. The translation to human exposures to genotoxicants is therefore much greater than assays which employ microorganisms and lower eukaryotes. Cell lines require approximately seven days for re-equilibration to a functional state after culturing from a frozen stock supply. In contrast to mammalian cells, which can require weeks, this is a major step towards greater efficiency. Another benefit of this system is the temperature at which these cells are kept. At 39.5°C, most ambient bacteria are incapable of survival, thus parent DT40 and mutants rarely are contaminated with microorganisms.

2.6 TYPICAL DNA INJURIES FROM PAH EXPOSURES

2.6.1 BULKY DNA ADDUCTS

After biotransformation, PAH metabolites stimulate formation of bulky adducts that are covalently bonded to nucleophilic DNA [3, 122]. Reactive PAH metabolites have a flat, hydrophobic structure that facilitates the intercalation into the DNA duplex and distortion of the helix [189]. Characteristic patterns of DNA adducts have been demonstrated with dihydrodiol epoxides. For instance, generally bulky adducts easily form at the N-2 position of guanine by interactions with the lone pair of electrons [118, 190]. *In vitro* incubation of 4,5- , 7,8-, and 9,10-dihydrodiol metabolites of BAP with DNA and hepatic microsomes demonstrated that the 7,8-isomer was ten times more reactive than

the parent compound or other metabolites [191]. Later Grover and Sims [192] identified the BPDE as the most reactive metabolite of BAP.

Preferential binding of bay-region dihydrodiol expoxides, like BPDE, occurs with purines as well as cytosine [193]. Greater reactivity of bay region dihydrodiol epoxides with DNA than that of K-region arene epoxides might be attributed to the stable benzylic cation intermediates formed from acid-catalyzed ring openings at the bay region [194]. Steric inaccessibility of the bay region may result in the relative resistance of these epoxides to enzymatic hydrolysis or conjugation [195]. Bay region theory has been extended to other PAH metabolites such as 5-methylchrysesne [196], 7,12-dimethylbenz[*a*]anthracene [197], benzo[*c*]phenanthrene [198], dibenz[*a*,*j*]acridine [22], and others [199, 200].

PAH activation through the *o*-quinone pathway can also produce metabolites which covalently bind to DNA. Because PAH-*o*-quinones behave as reactive 1,4-Michael addition acceptors, these metabolites are capable of hydrolyzing to oligodeoxyribonucleotides. Deoxyguanosine adducts have been generated from incubation of calf thymus and benzo[a]pyrene-7,8-dione [19]. Other *o*-quinone-induced stable adducts are known 8-N1,9-N2-deoxyguanosyl-8,10-dihydroxy-9,10-dihydroBaP-7(8H)-one, two diastereomers of 10-(N2-deoxyguanosyl)-9,10 dihydro-9-hydroxyBaP-7,8-dione, and another diastereomeric pair of 8-N6,10-N1-deoxyadenosyl-8,9-dihydroxy-9,10-dihydroBaP-7(8H)-one [18].

Nucleotide excision repair (NER) is an important cellular defense against insults in the form of DNA bulky adducts. NER enzymes catalyze the removal of a short single-stranded DNA segment. The eradicated section includes the adduct or lesion, leaving

behind a single-strand gap in the DNA. Next a DNA polymerase exploits the undamaged strand so that it acts as a template as in typical DNA replication [201]. Without this reconstruction mechanism, cell death may occur from halted DNA replication and transcription or mutations may arise from bypass of bulky DNA adducts with translesion synthesis enzymes [202].

Interactions of PAH metabolites with the DNA structure, which affects the DNA replication machinery, have been identified by nuclear magnetic resonance and X-ray crystallography techniques [202, 203]. Adduction of PAH metabolites interferes with DNA polymerases by several means, including the local context of the adduct [204] on the major or minor groove [205]; the structural conformation of DNA adducts is of great importance for the efficacy of the multi-subunit NER repair system.

2.6.2 OXIDATIVE STRESS

Imbalances of exogenous oxidants within cells and intracellular antioxidant defense levels elevate intracellular levels of ROS and ultimately result in injury of cellular macromolecules, including DNA, from reactions with molecular oxygen or its derivatives. Eventual formation of *o*-quinones by CYP1A1/epoxide hydrolases and dihydrodiol dehydrogenases from PAH by mammalian enzymes or by incomplete bacterial metabolism results in concomitant production of ROS, which encourage oxidative stress conditions [13, 21-23]. Hydrogen peroxide forms as catechols autoxidize with one-electron transfers to *o*-semiquinone anion radicals and from the dismutation of superoxide anion radicals, either enzymatically or spontaneously. Additionally, superoxide radicals are generated from nonenzymatic redox cycling of the *o*-semiquinone anion radicals to fully oxidized PAH *o*-quionones when molecular oxygen is reduced.

Hydroxyl radicals can form from reactions of hydrogen peroxide with Cu (II) or Fe (III) by the 'Fenton reaction'; this ROS is a formidable oxidizing agent that may be to blame for the majority of harm inflicted on cellular macromolecules [14, 206].

ROS production has several deleterious cellular implications. Possible consequences of ROS in biological systems are DNA strand scission from attacks on the sugar-phosphodiester backbone and subsequent incorrect recombination of disconnected strands. As a general rule, both purines and pyrimidines can be substrates for ROS oxidation [207]. 8-Hydroxy 2'-deoxyguanosine has been extensively studied as a model for base alteration by ROS activity, [208] since these lesions are strongly correlated with point mutations, more specifically G to T transversions [13, 209]. Additional consequences of ROS insults on DNA can be manifested with crosslinks between DNA–DNA and DNA–protein and with sister chromatid exchanges [210]. Correlations between PAH exposures and increased 8-Hydroxy 2'-deoxyguanosine and thymine glycol levels have been demonstrated [3, 15]. Factors which influence the extent of DNA damage inflicted by ROS from PAH metabolism include the presence of Cu (II) and Fe (III) and expression of ROS-detoxifying enzymes, such as superoxide dismutase [211].

CHAPTER 3

EXPERIMENTAL METHODOLOGY

3.1 EXPERIMENTAL DESIGN

This study sought to determine whether bioremediation at a former MGP site affects genotoxicity. The genotoxic profiles of solvent extracts of soil obtained from an MGP site in Salisbury, NC, both before and after treatment in a laboratory-scale column that simulated *in situ* biostimulation, were characterized with an *in vitro* method. This method utilizes a cell library containing a parent DT40 vertebrate cell line and 35 isogenic mutants deficient in at least one DNA damage repair pathway. After exposure to the soil extracts, differences in viabilities between parent DT40 cells and each mutant cell line indicated the genotoxic response. Three sources of soil were analyzed with this procedure: the untreated soil, soil from the biostimulated column, and soil from the control column; a total of five extracts was analyzed. Two extractions were performed in July 2008 (three months after initiating biostimulation conditions in that column): one from the untreated soil and the other from the top (aerobic zone) of the biostimulated column. Three extracts resulted from an October 2008 (six months after initiating biostimulation conditions) sampling event and included extracts from the top (aerobic zone) and bottom (anoxic zone) of the biostimulated column and the top (aerobic zone) of the control column. DT40 bioassay cell viability data were analyzed with a modified student's t-test to account for the multiple group comparison.

3.2 EXPERIMENTAL PROCEDURES

Note: All of the procedures regarding solvent extraction of the soil samples were performed by others (primarily Stephen Richardson).

3.2.1. SOIL COLLECTION

The chemicals utilized in this study were purchased from Fisher Scientific (Hampton, NH, USA) or Sigma Aldrich (St. Louis, MO, USA) and were all of analytical grade or better. Soil was collected from a former MGP site in Salisbury, NC that was undergoing excavation at the time of collection. Active remediation at the site involved the excavation, transport, and disposal of contaminated soil to a licensed disposal facility. Samples were collected in July 2006 at an approximate depth of 1.2 m below ground surface. The soil processing involved removal of rocks and debris from contaminated soil and subsequent sieving through10-mm wire mesh. Following soil processing, samples were stored in 20-L containers at 4°C in the dark until column construction or extractions were conducted.

3.2.2 GROUNDWATER PREPARATION

Components of the simulated groundwater were based on ionic concentrations from groundwater wells found in the area surrounding Salisbury, NC. To curtail microbial growth in the autoclaved polypropylene carboys in which simulated groundwater was prepared and stored, new solutions were made weekly. Preparation of groundwater required autoclaved polypropylene carboys, $CaCl_2H_2O$, $MgSO_4 \cdot 7H_2O$, $NaHCO_3$, KCL solution, 1 N H_2SO_4, reagent-grade water, and 0.1 μm flow-through hollow-fiber membrane water filter (Sawyer Products, Safety Harbor, FL) created a sterile final product. Biostimulated conditions were achieved by supplementing the

simulated groundwater with 1.0 mL of a nutrient stock solution comprised of NH_4NO_3 and K_2HPO_4 (final nitrogen and phosphorus concentrations were 1.0 mg/L and 0.3 mg/L, respectively) that was saturated with pure oxygen before being pumped into the column.

3.2.3 COLUMN SYSTEM

The columns utilized in this study were packed with a 50:50 ratio (w:w) mixture of the contaminated soil and sterile 40/50 grade silica sand (Unimin Corporation, Le Sueur, MN, USA). This mixture was implemented to encourage low-pressure groundwater flow through the columns. In September 2007, control conditions were imposed on the biostimulated and control columns to establish uniform initial conditions between the columns and to optimize the groundwater flow rate and pressure. These conditions were imposed for eight months prior to the advent of experimental biostimulated conditions in April 2008. The control conditions consisted of continual supply of simulated groundwater for each column. After the eight-month startup period of operation, in April 2008, the biostimulated conditions were applied to the biostimulated column.

3.2.4 SOIL EXTRACTION

The following chemicals were used for the soil extraction component of this study: anhydrous sodium sulfate, high performance liquid chromatography (HPLC) grade acetone, dichloromethane (DCM), and acetonitrile (ACN).

Extractions from soil samples utilized 5.0 g [wet wt.] of untreated soil material and 3.0 g [wet wt.] of each column sample. Two-step solvent extractions were carried out with soil slurries in 35-ml glass centrifuge vials. Slurries were centrifuged for 15 min at 3,500 rpm. Supernatant was discarded since aqueous-phase PAH concentrations were

negligible after centrifugation (S. Richardson, personal communication). To absorb the remaining water and improve mixing, 5.0 g sodium sulfate and 5-mm glass beads were added to each vial. Vials were placed on a wrist-action shaker for 24 h after addition of 10.0 ml each DCM and acetone. Further centrifugation was performed as described above. The supernatant was filtered through a 0.2 μm pore-size nylon filter and collected in a 50.0 mL volumetric flask. An additional 10.0 mL each of DCM and acetone was added to the soil pellet in each vial, which was then shaken for an additional 24 h. Second-day extracts were centrifuged, filtered, and combined with the initial filtered extracts. ACN was utilized to bring combined extracts to volume. Samples were then transferred to amber serum vials and stored in the dark at 4°C prior to analysis.

3.2.5 STORING EXTRACTS

The solvent extracts were placed in a pre-weighed glass culture tube, and then evaporated to dryness with nitrogen. After evaporation, the masses of the culture tubes were measured again to obtain the mass of the extract residue. The extract residues were then re-suspended with DMSO, 1 mL/culture tube. The re-suspended extracts were then separated into 100 μL aliquots and stored in liquid nitrogen.

3.2.6 DT40 BIOASSAY

The following materials were utilized in the DT40 assay of this study: fetal bovine serum (FBS), 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide inner salt (XTT), and dimethyl sulphoxide (DMSO) Hybri-Max ®, all of which were obtained from Sigma Aldrich (St. Louis, MO, USA). Additionally, RPMI 1640 culture medium, chicken serum, penicillin/streptomycin, and Dulbecco's phosphate buffered saline (PBS) were obtained from Invitrogen (Carlsbad,

CA, USA). The following materials were also necessary for this bioassay: Tecan Safire plate reader and Magellan 6 software, shaking apparatus, CO_2 incubators, Costar ® 24-well plates, and standard cell culture materials. All DT40 mutants were derived from isogenic DT40 parent cell lines and are summarized in Table 1 [216].

Table 1. DNA repair pathways and the corresponding mutants of the DT40 bioassay.

DNA Repair Pathway	Type of DNA Damage Repaired	Mutant
Homologous Recombination	Double Strand Breaks	RAD52 RAD54 RAD51c RAD51d XRCC2 XRCC3 BRCA1 BRCA2 FANCD2
Non-Homologous End-Joining	Double Strand Breaks	KU70 LIGIV DNA PKCs
Nucleotide Excision Repair	Bulky DNA Adducts	XPA XPG
Mis-Match Repair	Mis-matched Bases	MSH2 MSH3 MSH6
Base Excision Repair	Small Base Adducts	POLB FEN1 PARP1
Translesion Synthesis	By-pass DNA lesions	POLQ REV1 POLK POLN RAD18
Cell Cycle Check-Point	G1/S, G2/M, Intra-S	ATM
DNA Damage Sensors	Recruit Downstream Repair Proteins	RAD9 RAD17

The DT40 cells and their mutants were cultured as previously reported [212, 213]. This cell library was cultured in a humidified 5% CO_2 atmosphere at 37°C. The medium consisted of RPMI 1640 cell culture medium containing 10% FBS, 100 mg/mL, penicillin, and 100 mg/mL streptomycin.

The parent DT40 cell line and the isogenic mutants were cultured in RPMI medium supplemented with 55.5 mL heat-inactivated FBS, 5.5 mL penicillin/streptomycin antibiotic cocktail, and 5.5 mL chicken serum. Cell counts and shapes were evaluated with a 1:1 mixture of 10.0 μL aliquots of Trypan Blue dye and cell cultures under compound light microscope. Once the cell shape was consistently round with high nucleus-to-cytoplasm ratio and cell counts were from 0.7 million to 1.5 million cells/mL, it was possible to utilize those cell lines in experiments to test the DNA damage responses. Irregular shapes and cell counts that fell outside the designated range generally provided non-reproducible results, therefore results from such cell counts were considered with caution.

The cells were seeded onto 24-well plates at a concentration of 23,000cells/5.5 mL completed RPMI medium. The fragility of DT40 cells required certain considerations unnecessary for most other cell types. This unique feature of DT40 cells was accommodated with the employment of pipette tips with larger than normal orifices, deliberate gentle mixing of culture mixtures, and careful monitoring to expose cells to room temperature for short periods of time (generally about three minutes). A technique to prevent the formation of air bubbles in the seeded cell mixture was utilized. This involved pipetting more than 250 μL of the cell mixture into the tip and ending the pipetting release at the first resistance point. Then cells on seeded plates were allowed to re-equilibrate in a 39.5°C incubator for at least thirty minutes.

DMSO solutions of soil extracts were retrieved from liquid nitrogen storage, warmed to room temperature, and vortexed before use. The soil extracts that were re-suspended in DMSO were diluted with PBS such that the DMSO percentage in the

maximum concentration did not exceed 0.33% (v:v); a higher DMSO concentration resulted in cell death that confounded results for the extracts. To conserve resources, preliminary experiments included a series of exposures of only the parent DT40 cells to identify an appropriate maximum concentration of the soil extract for each mixture analyzed. The desired dilution series yielded minimal cell viability values for the maximum concentration. The DMSO solution in PBS was serially diluted by a factor of 1.414 to promote a gradual change of cell viability values over the entire dilution series. The seven dilutions of this series included the blank sample, which consisted of only PBS. The extracts in DMSO were in liquid nitrogen during experiments to prevent degradation until immediately before the serial dilution was to be carried out, and the dilution series remained on ice for the duration of chemical exposures. Each well, aside from the two negative controls, was exposed to 27.8 μL of the prepared extract dilutions. The controls which were exposed to the blank sample were incubated in quadruplicates, while all other extract dilutions were exposed in triplicates. Chemical exposures were simultaneously performed on sets of five 24-well plates. Exposures with multiple plates were especially important for experiments which tested the entire cell library to minimize degradation of chemicals or cells that might have occurred over long periods if the exposures had been done one plate at a time.

After applying extract dilutions to the wells, plates were immediately transferred to a 39.5°C incubator and the cells were allowed to undergo approximately eight replication cycles. Daily evaluation using a light microscope of cell growth of the control wells revealed if the cells had reached their maximal growth potential before becoming overgrown. Because parent DT40 cells and isogenic mutants have unique doubling rates

due to their various genomic modifications, evaluation of growth rates for all cells lines was crucial for reliable results.

All 24 wells of the plates were dyed with 200.0 μL XTT solution and cells were incubated at 39.5°C for approximately four hours or until a quantifiable amount of formazan developed. The XTT solution was prepared using 22.0 μL XTT/ 12.0 mL RPMI; both components were warmed before preparation. After the four-hour incubation period, cell viabilities were determined by quantifying the amount of formazan produced, using a TECAN SAFIRE plate reading device and software. Formazan is a water-soluble dye produced upon the bioreduction of XTT in the presence of an electron carrier.

Raw cell viability data were imported into Microsoft Excel and converted into normalized values as percentages of the controls. The results were represented graphically in a semi-log format with cell viabilities vs. total residue mass per well (in μg.)

3.2.7 STATISTICAL ANALYSIS

Cell survival data were log-transformed giving an approximately normal distribution (not shown). Genotoxic responses were determined for individual mixtures by comparing the viability of the DT40 mutant cell line to that of the parent cell line at the highest test does. For all pairwise comparisons, a standard two-tailed student's t-test was performed with the built-in function of R-statistical software (R Development Core Team, University of Auckland, New Zealand). A significance level of 0.05 was decided upon for these analyses; any response with a p-value less than the chosen significance level was considered to be a significant response.

Mean intercepts of the slopes of linear dose-response curves for each cell line were utilized to determine doses necessary to produce 50% cell death (LC50).

Comparisons of general and specific genotoxic responses between LC50 values produced by the extracts of two different soil samples were determined by a modified two-tailed student's t-test. The multiple group comparison, which analyzed the genotoxic responses of different extracts by comparing the cell viability data of DT40 cells and mutants, was accounted for by modification of the degrees of freedom in the student's t-test. The approach followed Welch's classical method [214]. The t-statistic was calculated by dividing the difference (mean (x1) – mean (y1)) - (mean (x2) – mean (y2)) by its standard deviation. Multiple group comparisons also utilized a significance level of 0.05.

CHAPTER 4

RESULTS AND DISCUSSION

4.1 INTRODUCTION

Results from this study include the total extractable organic (TEO) material (mg/g dry soil) of each sample, the concentration of PAH in each soil extract, the mass of extract residue after nitrogen evaporation, mixture (residue) toxicities, overall genotoxic signatures, genotoxic responses of mutant cell lines belonging to pathways known to repair resultant DNA damage from PAH exposures for each mixture, the most sensitive mutant cell lines, and genotoxic responses for each DNA repair pathway analyzed. Because oxidative stress is an expected source of DNA damage, comparison to the hallmark genotoxic responses of hydrogen peroxide (H_2O_2) was employed in an effort to understand the mechanism of genotoxicity of the extracts analyzed. TEO and PAH measurements were performed by Stephen Richardson.

Table 2. Characteristics of soil samples and their extracts for which genotoxicity assays were performed. The untreated soil and July 2008 sample had initial volumes of 100 mL and the October 2008 samples has initial volumes of 150 mL, from which an aliquot was subjected to evaporation to obtain residue for genotoxicity testing. Data represent means and standard deviations of triplicate samples. Corresponding PAH concentrations for each sample are shown in Table 3.

Sample Source	Dry Mass of Extracted Sample (g)	Volume of Extract Evaporated (mL)	Extract Residue (mg)	Residue/Soil (mg/g)
Untreated Soil	3.92 ± 0.19	15.0	45.9 ± 0.15	1.95 ± 0.02
July 2008 Top of Biostimulated Column	4.00 ± 0.01	15.0	59.0 ± 2.81	2.46 ± 0.35
October 2008 Top of Biostimulated Column	2.35 ± 0.02	15.0	23.7 ± 4.76	0.66 ± 0.05
October 2008 Top of Control Column	2.43 ± 0.01	15.0	15.0 ± 0.4	1.12 ± 0.67
October 2008 Bottom of Biostimulated Column	2.52 ± 0.01	15.0	14.0 ± 1.3	0.64 ± 0.18

4.2 TOTAL EXTRACTABLE ORGANIC MATERIAL

TEO material of each extract is represented in Table 2. This analysis was performed in triplicate. Total extractable organic material comprises many constituents derived both from decaying organisms and chemical contamination [30]. TEO content was highest in extracts from soil at the top of the biostimulated column, which would have undergone the most extensive aerobic treatment. Soil without any treatment yielded the lowest TEO values. Because TEO can include the extractable organics in the original soil and material from microbial biomass formed during aerobic treatment, it is likely that the difference between TEO values for the untreated soil and the treated soil from the top of the biostimulated column was due to microbial growth in the column.

4.3 PAH CONCENTRATIONS

PAH concentrations in the soil samples evaluated in this study are summarized in Table 3. Soil subjected to aerobic conditions (from the tops of the biostimulated and control columns) reflected lowered total PAH concentrations relative to the untreated soil and the sample that was anoxic (from the bottom of the biostimulated column), in contrast to the TEO data. In the top of the biostimulated column, concentrations of several PAHs in the October 2008 sample (six months after initiating biostimulation conditions) were lower than in the July 2008 sample (three months after initiating biostimulation conditions), reflecting the longer time under which the top of the column was exposed to aerobic conditions. Concentrations of other PAHs, such as pyrene and BAP, in samples from the top of the biostimulated column did not decrease relative to the untreated soil for either sampling event.

Table 3. Individual and total PAH concentrations (mg/kg dry soil) in samples for which genotoxicity testing was conducted. Data represent means and standard deviations.

PAH	Untreated Soil	July 2008 Sample from Top of Biostimulated Column	October 2008 Sample from Top of Biostimulated Column	October 2008 Sample from Bottom of Biostimulated Column	October 2008 Sample from Top of Control Column
Naphthalene	11.8 ± 0.2	13.5 ± 4.2	16.1 ± 2.3	15.2 ± 3.8	13.1 ± 1.6
Acenaphthylene	10.5 ± 0.1	3.5 ± 3.3	2.8 ± 0.8	8.3 ± 2.6	2.7 ± 0.9
Fluorene	8.6 ± 0.5	5.3± 3.2	2.5 ± 0.8	9.8 ± 3.0	3.0 ± 1.3
Phenanthrene	123 ± 15.8	114 ± 78.0	59.6 ± 16.2	166 ± 55.1	56.1 ± 30.7
Anthracene	9.6 ± 0.1	6.1 ± 2.0	4.5 ± 1.0	11.9 ± 3.9	5.2 ± 2.1
Flouranthene	33.3 ± 0.4	25.1 ± 7.6	20.7 ± 7.8	28.3 ± 8.0	18.8 ± 5.1
Pyrene	45.8 ± 1.6	42.7 ± 12.4	41.0 ± 10.8	50.5 ± 15.2	35.2 ± 7.6
Benzo (a) anthracene	18.1 ± 0.7	15.9 ± 3.3	13.5 ± 2.8	15.8 ± 4.9	12.9 ± 4.7
Chrysene	27.6 ± 1.4	29.6 ± 7.7	15.9 ± 3.9	17.2 ± 5.7	13.3 ± 4.6
Benzo (a) flouranthene	8.6 ± 0.01	9.4 ± 0.8	9.7 ± 1.5	8.8 ± 2.1	10.1 ± 4.6
Benzo (k) fluroanthene	4.6 ± 0.2	4.9 ± 0.5	6.2 ± 1.4	5.5 ± 1.5	6.5 ± 3.2
Benzo (a) pyrene	16.2 ± 0.2	19.2 ± 0.9	19.3 ± 2.2	16.3 ± 3.9	18.6 ± 8.0
Dibenz (a,h) anthracene	0.6 ± 0.01	1.0 ± 0.01	1.7 ± 0.1	1.4 ± 0.3	1.5 ± 0.4
Benzo (g,h,i) perylene	10.0 ± 0.7	13.3 ± 1.3	11.7 ± 0.9	8.5 ± 1.9	11.5 ± 4.2
Total PAH Concentration	329 ± 22.0	303 ± 125	225 ± 52.8	362 ± 112	209 ± 79.3

4.4 EXTRACT RESIDUES

Residues of the extracts from both sampling events after evaporation are expressed in Table 2. Because more soil was extracted for the untreated soil and soil from the top of the biostimulated column for the July 2008 sampling event, more residue was produced from these samples than from each sample of the second (October 2008) sampling event. Doses used in the DT40 assay were based on residue mass, so that equivalent doses between samples correspond to different amounts of soil from which that dose would have been extracted. The maximum concentration of residue corresponding to the cell viability range required for informative toxicity responses was

different for different samples (not shown). The maximum residue concentration was 10 μg/mL for the extracts from the untreated soil and the July 2008 sample from the top of the biostimulated column, and was 30 μg/mL for the October 2008 column samples.

4.5 Genotoxic Responses To July 2008 Samples

Analysis of mixtures from the untreated soil and the top of the biostimulated column from the July 2008 sampling event show that both mixtures have a toxic effect, as evidenced by decreased viability of DT40 parent cells (Figure 3). The extract from the top of the biostimulated column from the July 2008 sampling event was more toxic to the parent DT40 cells at a maximum concentration of 10.0 μg/mL.

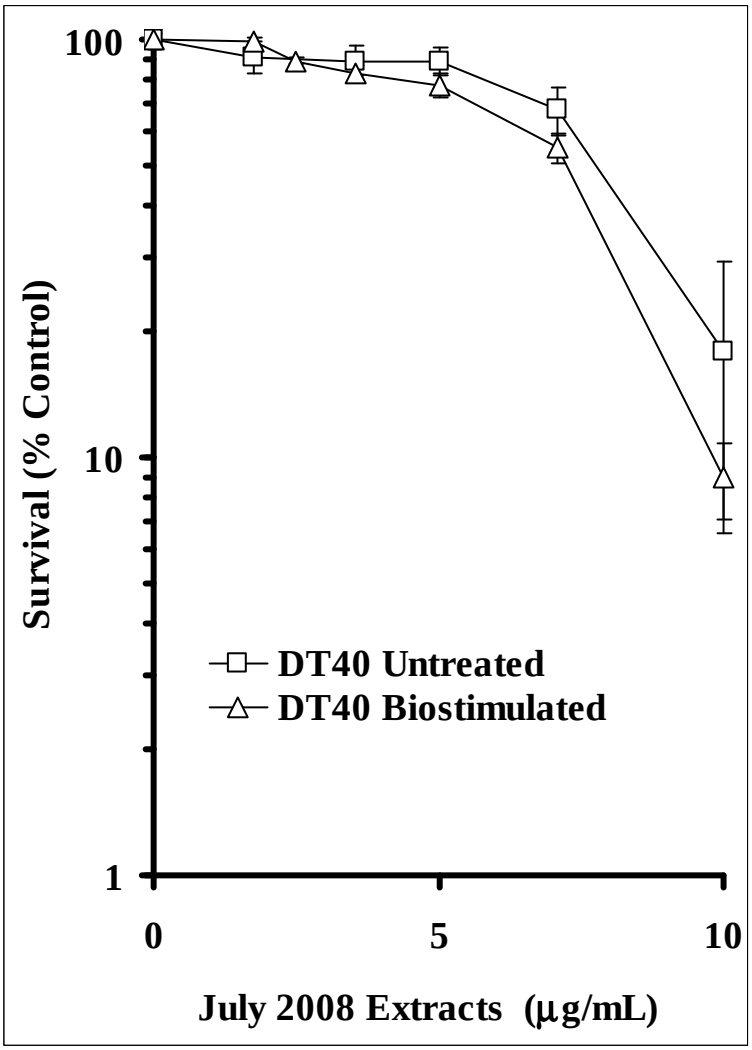

Figure 3. DT40 cell lines exposed to the untreated soil extract and the extract from the top of the biostimulated column for the July 2008 sampling event.

Comparison of the overall genotoxic response using all mutant cell lines revealed that exposures to the extract from the top of the biostimulated column for the July 2008 sampling event showed a significantly more severe genotoxic response ($p=0.04$) than the DNA damage responses from exposures of the untreated soil extract to the mutant cell lines. Data for selected representative cell lines are shown in Figures 4 through 8. The p-values for comparisons of the genotoxic responses of the tested mutant cell lines to the two extracts are summarized in Table 4. Concentrations of each soil

extract which were lethal to 50% of selected mutant cell lines (LC50 values) are summarized in Figure 9. Appendices 1 and 2 contain the LC50 values and comparable soil masses necessary to achieve the LC50 values for the untreated soil and top of the biostimulated column sample extracts.

Table 4. P-values from a comparison of the two extracts' genotoxicities of the extracts from untreated soil and the soil from the top of the biostimulated column (July 2008 sampling event) for each mutant. The comparison is for the highest dose only. Significant differences in genotoxicities ($P < 0.05$) are italicized. All significant differences represent greater genotoxicity of the extract from the biostimulated column.

DNA Repair Pathway	**Type of Damage Repaired**	**Mutant**	**p-value**
Homologous Recombination	Double Strand Breaks	RAD54	0.554
		RAD51c	0.797
		RAD51d	0.193
		XRCC2	0.572
		XRCC3	0.441
		BRCA1	0.832
		BRCA2	0.542
		FANCD2	0.634
Non-Homologous End-Joining	Double Strand Breaks	KU70	0.188
		LIGIV	***0.012***
		DNA PKCs	0.089
Nucleotide Excision Repair	Bulky DNA Adducts	XPA	0.473
		XPG	0.911
Mismatch Repair	Mismatched Bases	MSH3	0.364
		MSH6	0.153
Base Excision Repair	Small Base Adducts	POLB	0.335
		FEN1	***0.042***
		PARP1	***0.042***
Translesion Synthesis	By-pass DNA lesions	POLQ	0.13
		REV1	0.103
		POLK	0.104
		POLN	*0.012*
		RAD18	***0.045***
Cell Cycle Check-Point	G1/S, G2/M, Intra-S	ATM	0.95
DNA Damage Sensors	Recruit Downstream Repair Proteins	RAD9	0.431
		RAD17	0.456

Extracts from the untreated soil and the top of the biostimulated column for the July 2008 sampling event produced greatest cell death in RAD54 (Figure 4) and RAD 9 (Figure 5). LIGIV (Figure 6) was also noticeably sensitive to the extract from the top of the biostimulated column. Cells deficient in XPA (Figure 7) exposed to either extract from the July 2008 sampling event showed minimal reduction in cell viability. Cells deficient in FEN1 (Figure 8) showed no genotoxic response to exposures of either extract.

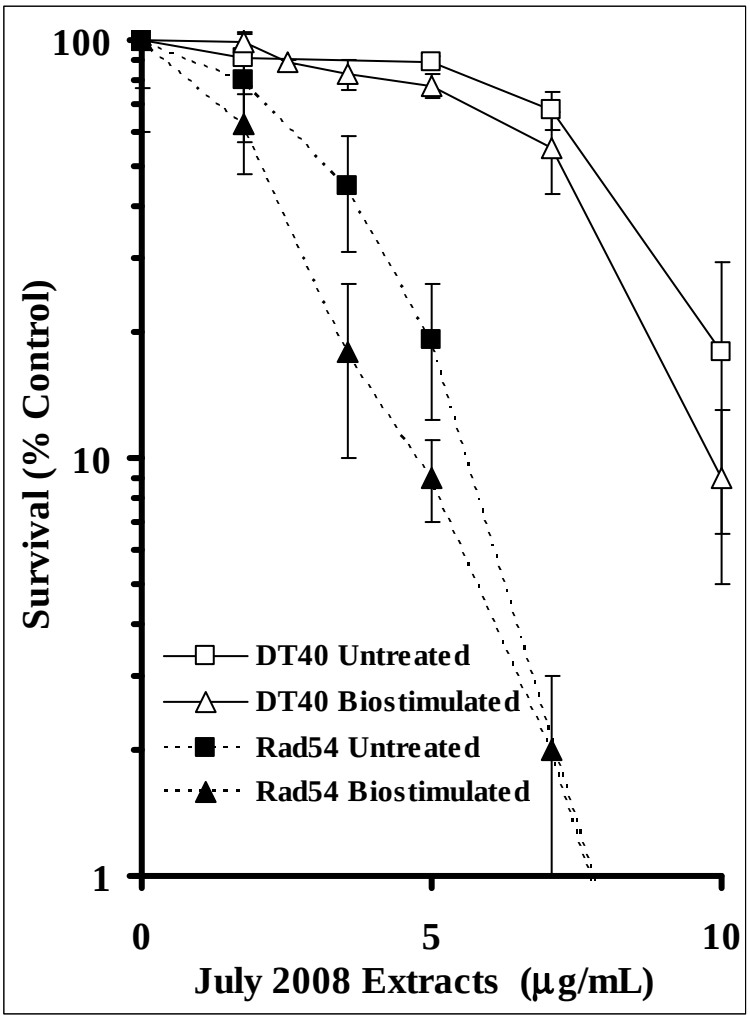

Figure 4. Resultant cell viabilities of DT40 cells and cells deficient in RAD54 after exposure to the untreated soil extract and the extract from the top of the biostimulated column for the July 2008 sampling event.

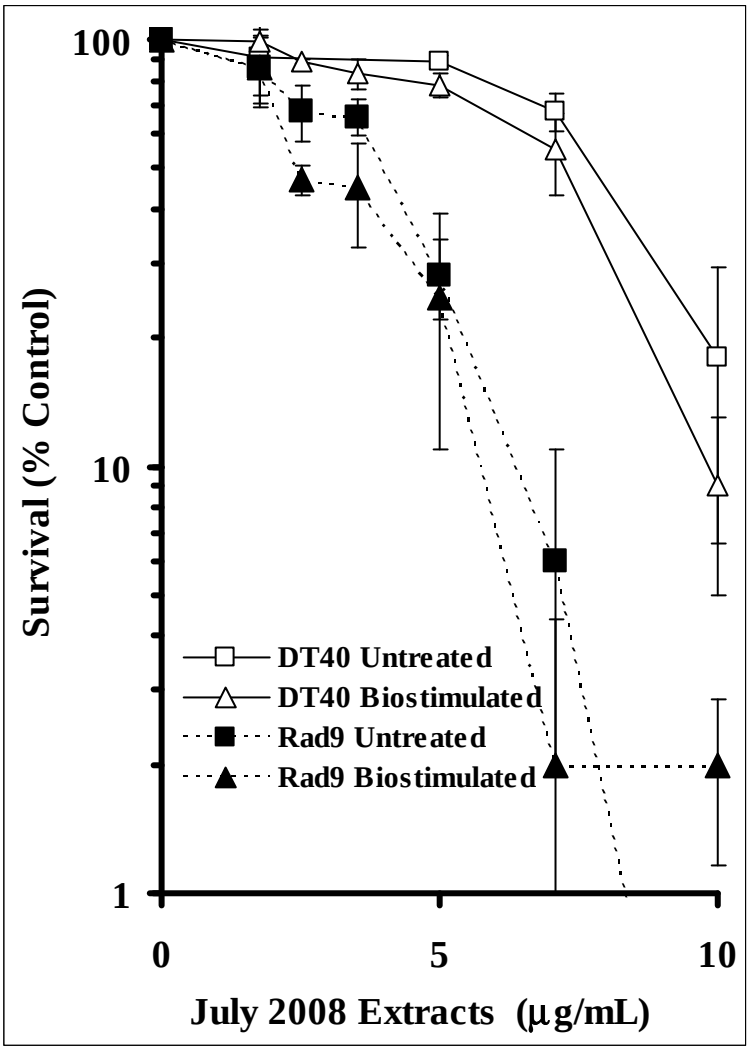

Figure 5. Resultant cell viabilities of DT40 cells and cells deficient in RAD9 after exposure to the untreated soil extract and the extract from the top of the biostimulated column for the July 2008 sampling event.

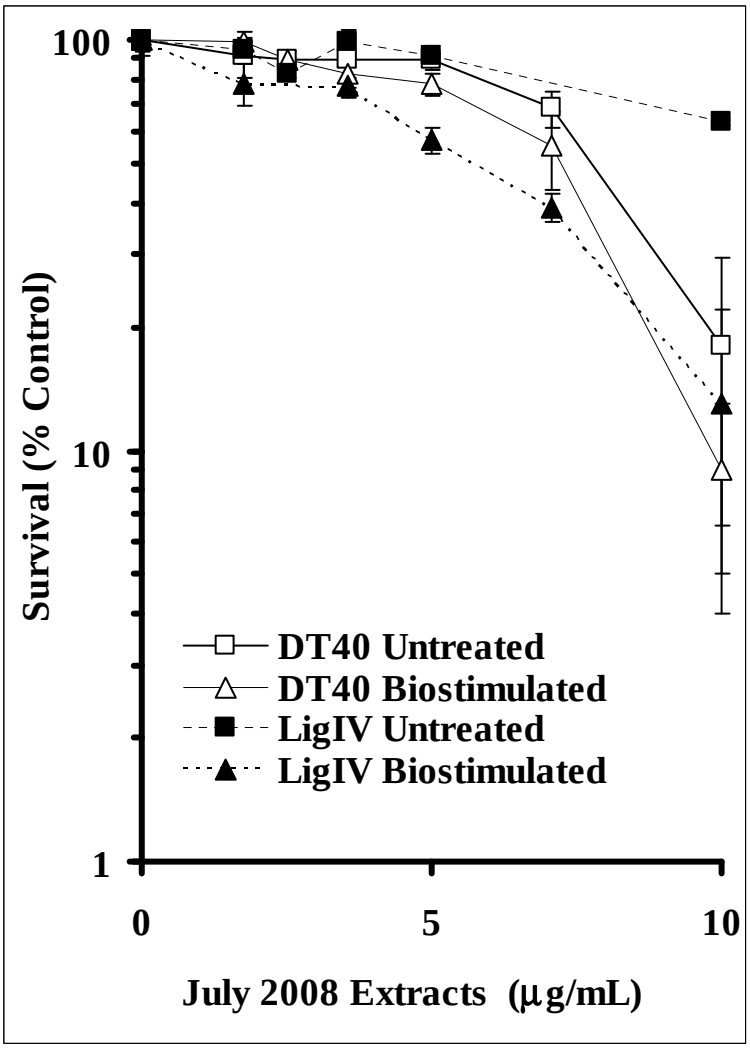

Figure 6. Resultant cell viabilities of DT40 cells and cells deficient in LIGIV after exposure to the untreated soil extract and the extract from the top of the biostimulated column for the July 2008 sampling event.

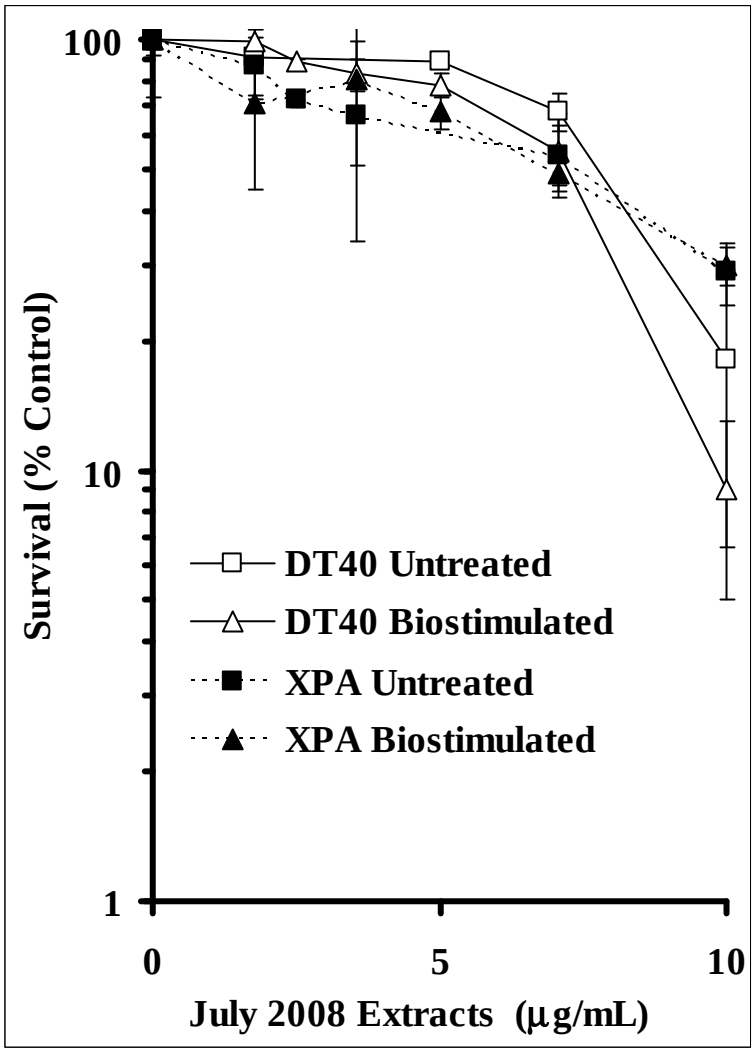

Figure 7. Resultant cell viabilities of DT40 cells and cells deficient in XPA after exposure to the untreated soil extract and the extract from the top of the biostimulated column for the July 2008 sampling event.

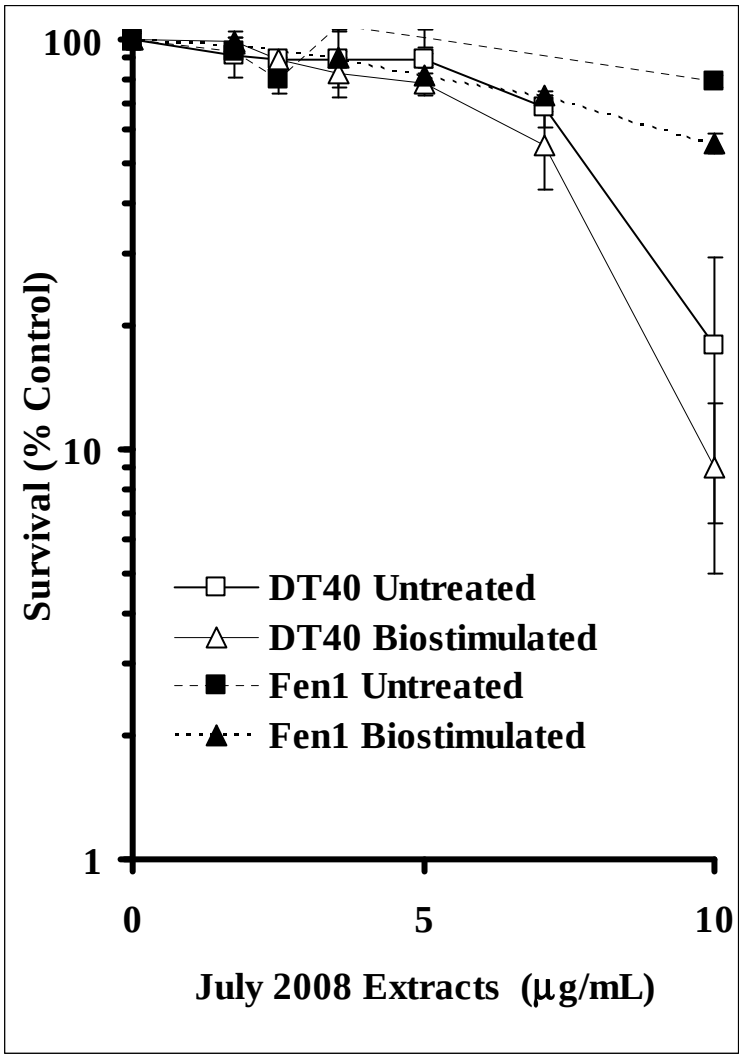

Figure 8. Resultant cell viabilities of DT40 cells and cells deficient in FEN1 after exposure to the untreated soil extract and the extract from the top of the biostimulated column for the July 2008 sampling event.

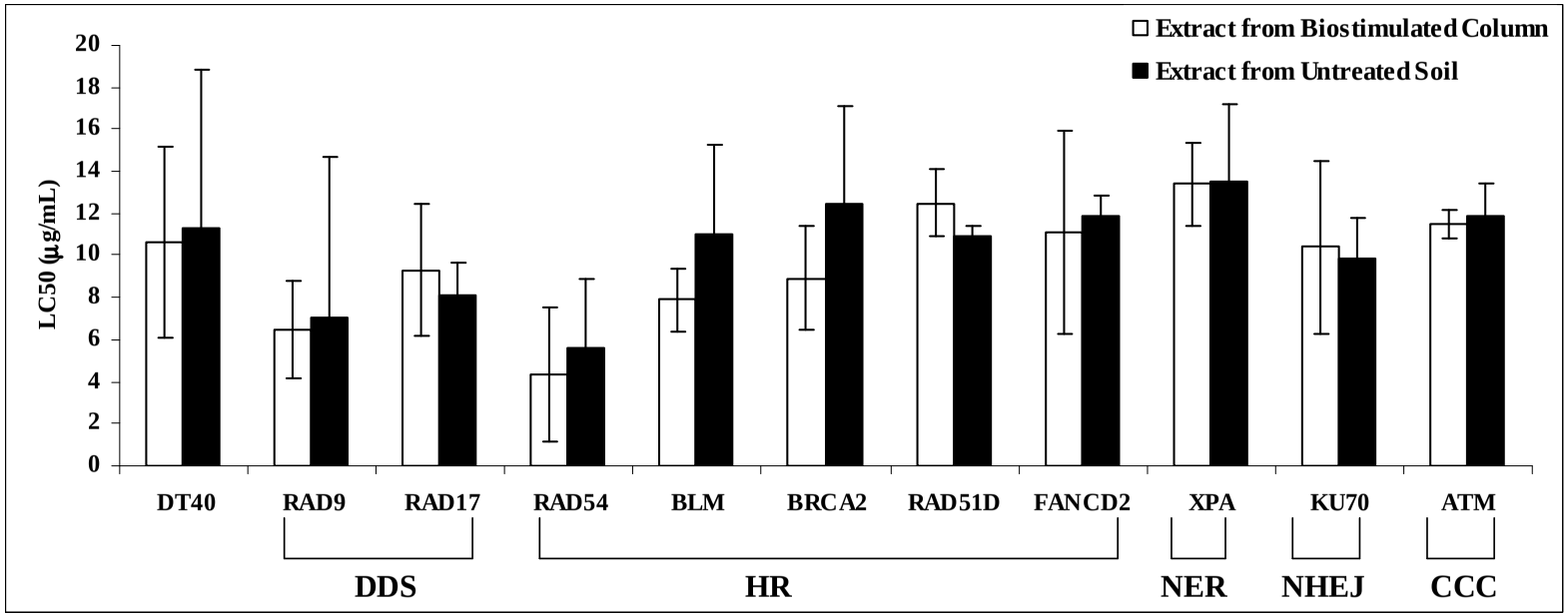

Figure 9. Relative LC50 values for cell lines exposed to extracts from the untreated soil (black) and the top of the biostimulated column sample (white). LC50 values calculated for all cell lines, and the most reliable are presented in this graph. Abbreviations: DDS – DNA damage sensor, HR – homologous recombination, NER – nucleotide excision repair, NHEJ – non-homologous end-joining, CCC – cell-cycle check point.

Analysis of separate DNA damage repair pathways revealed that homologous recombination (RAD54) is important to rectifying damage incurred from exposures to both the untreated soil extract and the extract from the top of the biostimulated column from the July 2008 sampling event. The DNA damage sensor, RAD9, showed a noticeable DNA damage response after exposure to the extract from the top of the biostimulated column as well as the extract from the untreated soil (Figure 5). Non-homologous end joining (LIGIV) appeared necessary for cells exposed to the extract from the top of the biostimulated column during the July 2008 sampling event (Figure 6). Mutants from the base excision repair (BER), nucleotide excision repair (NER), and translesion synthesis (TLS) pathways revealed minimal genotoxic responses to both extracts.

4.6 Genotoxic Responses To October 2008 Samples

Results from exposures of the three extracts from the October 2008 sampling event to the parent DT40 cell line indicated each extract produced a toxic effect (Figure 10). The reduction in parent DT40 cell viability was greater for extracts from the top of the biostimulated and control columns than that for extracts from the bottom of the biostimulated column. The maximum concentration for the October 2008 extracts was 30.0 μg/mL to produce observable and informative toxic responses.

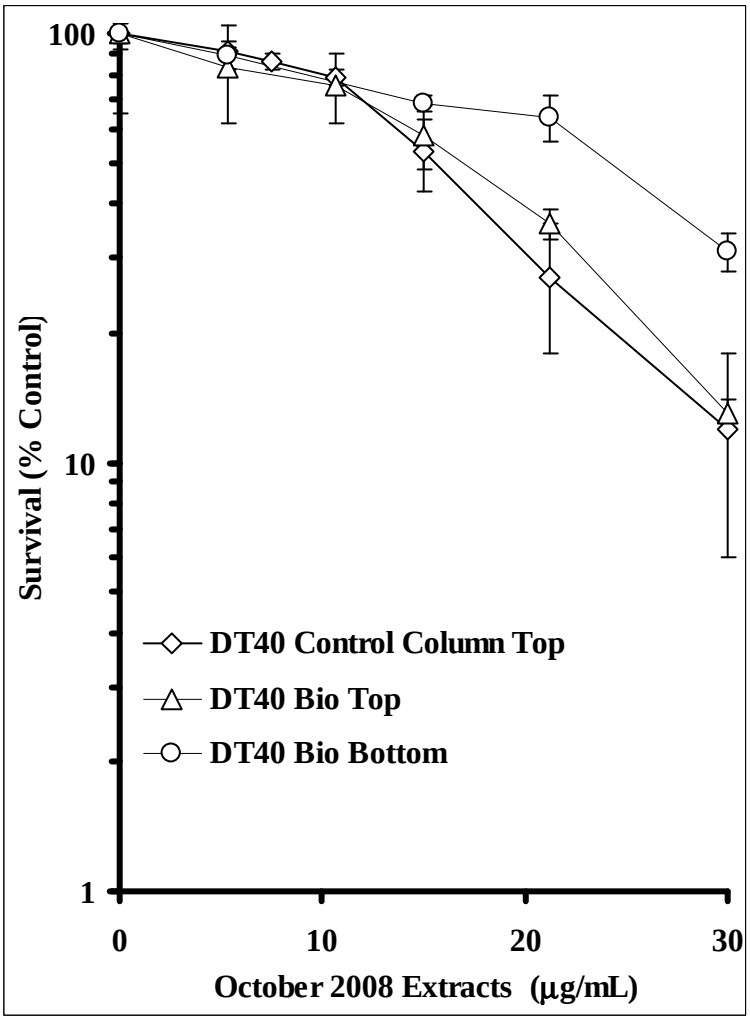

Figure 10. DT40 cell lines exposed to the three extracts for the October 2008 sampling event.

Extracts from the October 2008 sampling event generated a genotoxic response pattern similar to the toxic responses (Figure 10); the overall genotoxic profiles for the extracts from the control column and top of the biostimulated column were significantly more genotoxic than the extract from the bottom of the biostimulated column ($p=0.04$ and $p=0.04$, respectively). The general genotoxic responses or overall comparison of DNA damage produced in all tested mutants from exposures to extracts from the control column and top of the biostimulated column revealed no significant ($p=0.05$). These

findings were evidenced with LC50 values (Figure 16) for exposures to each extract and p-values for comparisons of the genotoxic responses for each mutant cell line to the extracts from October 2008 sampling event with the modified two-tailed student's t-test (Tables 5, 6, and 7). Appendices 3, 4, and 5 show the LC50 values and soil masses necessary to achieve the LC50 values for the extracts from the October 2008 sampling event.

Table 5. P-values represent a comparison of the genotoxic responses from the analyzed mutant cell lines to the extracts of the top of the control column and the top of the biostimulated column for the October 2008 sampling event. The comparison is for the highest dose only. Significant differences in genotoxicities ($P < 0.05$) are italicized. No significant differences in genotoxicities of the two the extracts were detected.

DNA Repair Pathway	**Type of Damage Repaired**	**Mutant**	**p-value**
Homologous Recombination	Double Strand Breaks	RAD52	0.11
		RAD54	0.533
		RAD51c	0.612
		RAD51d	0.722
		XRCC2	0.053
		XRCC3	0.357
		BRCA1	0.106
		BRCA2	0.358
		FANCD2	0.716
Non-Homologous End-Joining	Double Strand Breaks	KU70	0.257
		LIGIV	0.452
		DNA PKCs	0.371
Nucleotide Excision Repair	Bulky DNA Adducts	XPA	0.864
		XPG	0.384
Mismatch Repair	Mismatched Bases	MSH2	0.846
		MSH3	0.866
Base Excision Repair	Small Base Adducts	POLB	0.224
		FEN1	0.330
		PARP1	0.562
Translesion Synthesis	By-pass DNA lesions	POLQ	0.929
		REV1	0.566
		POLK	0.807
		POLN	0.911
		RAD18	0.719
Cell Cycle Check-Point	G1/S, G2/M, Intra-S Phase	ATM	0.161
DNA Damage Sensors	Recruit Downstream Repair Proteins	RAD9	0.705
		RAD17	0.917

Table 6. P-values from a comparison of the genotoxic responses of the analyzed mutant cell lines to the extracts of the top of the control column and the bottom of the biostimulated column for the October 2008 sampling event. The comparison is for the highest dose only. Significant differences in genotoxicities ($P < 0.05$) are italicized. All significant differences represent greater genotoxicity of the extract from the top of the control column.

DNA Repair Pathway	**Type of Damage Repaired**	**Mutant**	**p-value**
Homologous Recombination	Double Strand Breaks	RAD52	0.175
		RAD54	***0.080***
		RAD51c	0.110
		RAD51d	0.997
		XRCC2	0.786
		XRCC3	0.508
		BRCA1	*0.037*
		BRCA2	0.228
		FANCD2	0.219
Non-Homologous End-Joining	Double Strand Breaks	KU70	0.209
		LIGIV	*0.046*
		DNA PKCs	0.096
Nucleotide Excision Repair	Bulky DNA Adducts	XPA	0.607
		XPG	0.927
Mismatch Repair	Mismatched Bases	MSH2	0.505
		MSH3	0.990
Base Excision Repair	Small Base Adducts	POLB	0.255
		FEN1	***0.001***
		PARP1	0.288
Translesion Synthesis	By-pass DNA lesions	POLQ	0.415
		REV1	0.576
		POLK	0.301
		POLN	0.791
		RAD18	0.820
Cell Cycle Check-Point	G1/S, G2/M, Intra-S Phase	ATM	***0.040***
DNA Damage Sensors	Recruit Downstream Repair Proteins	RAD9	0.961
		RAD17	0.447

Table 7. P-values represent a comparison of the genotoxic responses from the analyzed mutant cell lines to the extracts of the top of the biostimulated column and the bottom of the biostimulated column for the October 2008 sampling event. The comparison is for the highest dose only. Significant differences in genotoxicities ($P < 0.05$) are italicized. All significant differences represent greater genotoxicity of the extract from the top of the biostimulated column.

DNA Repair Pathway	**Type of Damage Repaired**	**Mutant**	**p-value**
Homologous Recombination	Double Strand Breaks	RAD52	0.518
		RAD54	0.073
		RAD51c	***0.039***
		RAD51d	0.754
		XRCC2	0.382
		XRCC3	0.491
		BRCA1	0.073
		BRCA2	0.231
		FANCD2	0.433
Non-Homologous End-Joining	Double Strand Breaks	KU70	0.257
		LIGIV	***0.010***
		DNA PKCs	***0.004***
Nucleotide Excision Repair	Bulky DNA Adducts	XPA	0.633
		XPG	0.525
Mismatch Repair	Mismatched Bases	MSH2	***0.048***
		MSH3	***0.026***
		MSH6	
Base Excision Repair	Small Base Adducts	POLB	***0.004***
		FEN1	***<0.0001***
		PARP1	0.057
Translesion Synthesis	By-pass DNA lesions	POLQ	0.298
		REV1	0.073
		POLK	0.284
		POLN	0.554
		RAD18	0.430
Cell Cycle Check-Point	G1/S, G2/M, Intra-S	ATM	***0.004***
DNA Damage Sensors	Recruit Downstream Repair Proteins	RAD9	0.911
		RAD17	0.223

Responses of selected cell lines are shown in Figures 11 through 14. The reduction of cell viability in the RAD54 mutant cell line was greater than that of the parent DT40 cells after exposure to each extract for the October 2008 sampling event (Figure 11), indicating a genotoxic response after each treatment. Extracts from the control column and the top of the biostimulated column showed great sensitivity in RAD9 mutants, whereas cells deficient in RAD9 (Figure 12) revealed slight sensitivity to the extract from the bottom of the biostimulated column. The extract from the top of the control column showed a noticeable reduction of cell viability in LIGIV mutants (Figure 13), and no extract appeared to generate a genotoxic response in cells deficient in XPA (Figure 14).

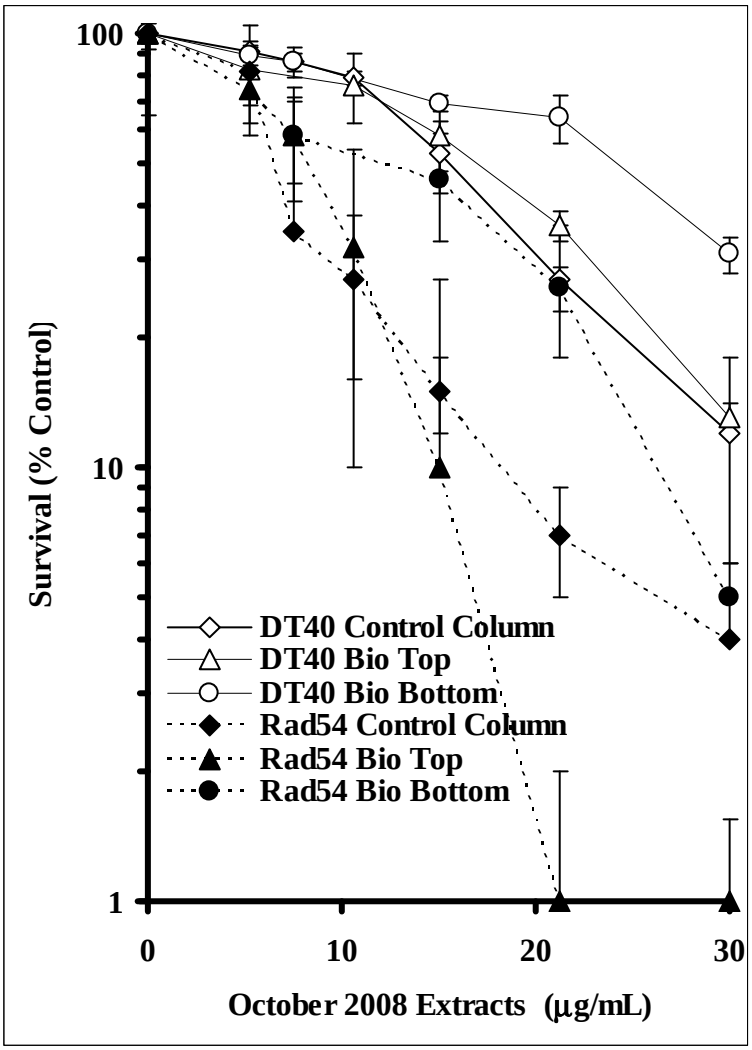

Figure 11. DT40 cell lines and RAD54 mutants exposed to three extracts from samples obtained during the October 2008 sampling event.

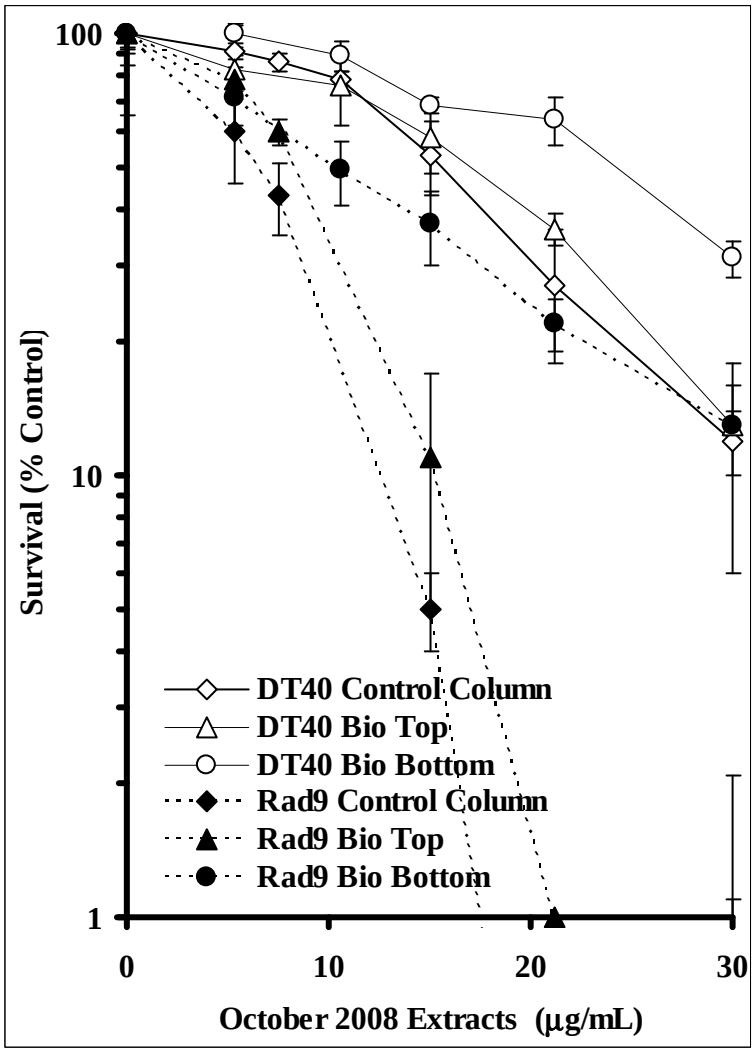

Figure 12. DT40 cell lines and RAD9 mutants exposed to three extracts for the October 2008 sampling event.

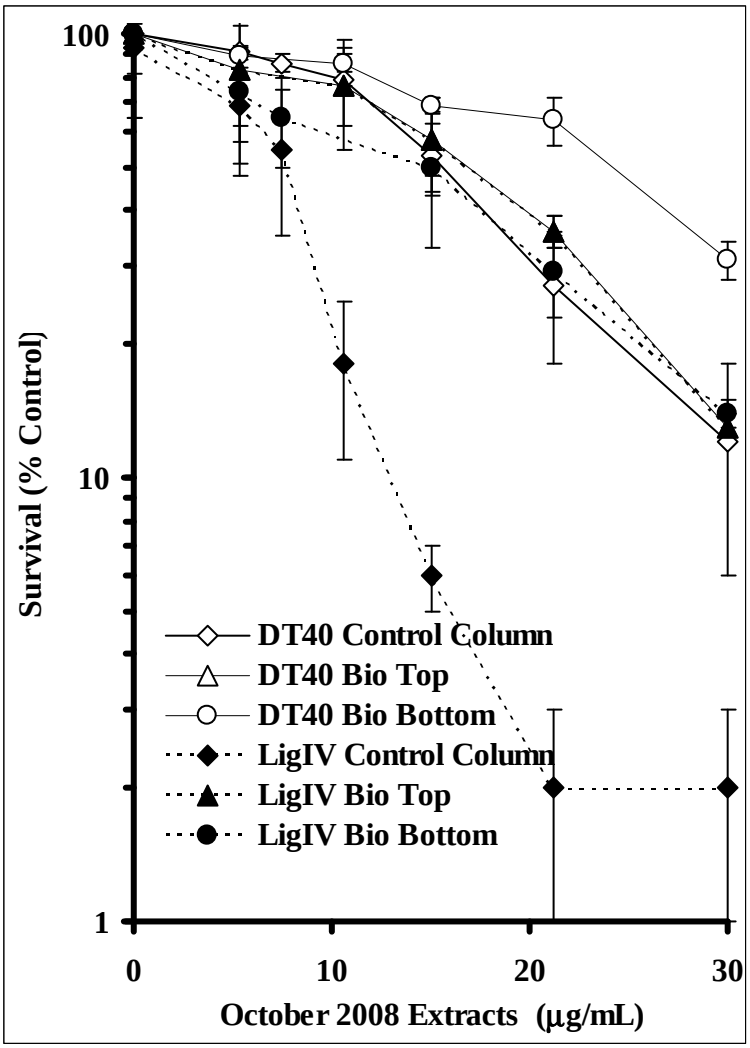

Figure 13. DT40 cell lines and LigIV mutants exposed to three extracts from samples obtained during the October 2008 sampling event.

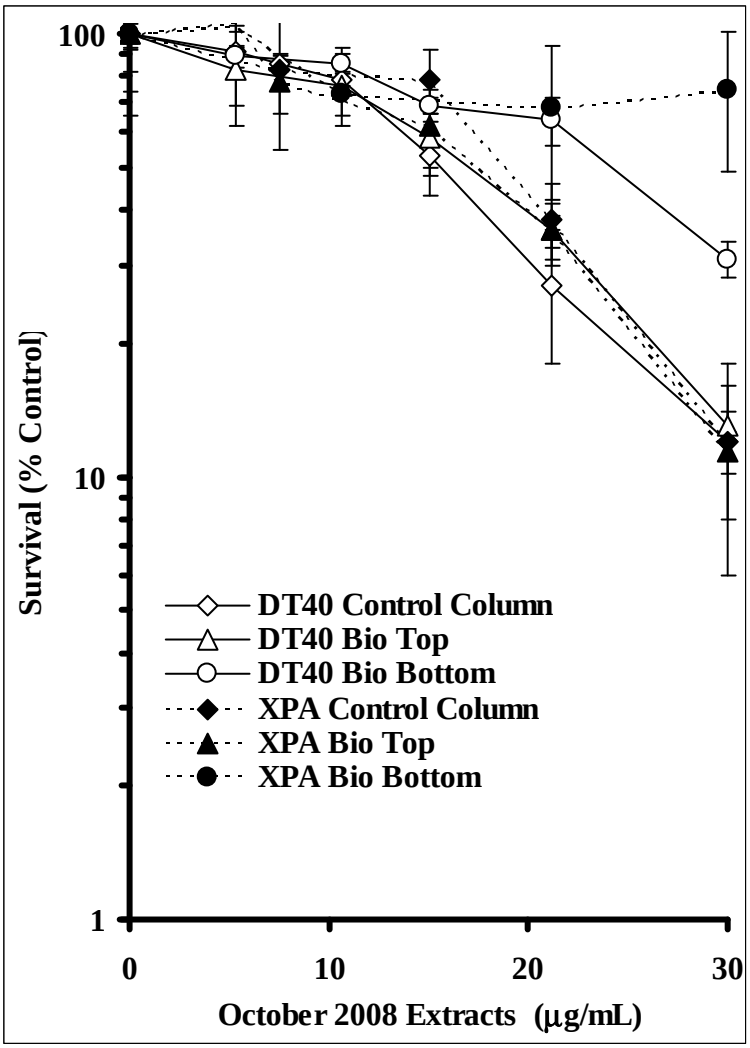

Figure 14. DT40 cell lines and XPA mutants exposed to three extracts from sample obtained during the October 2008 sampling event.

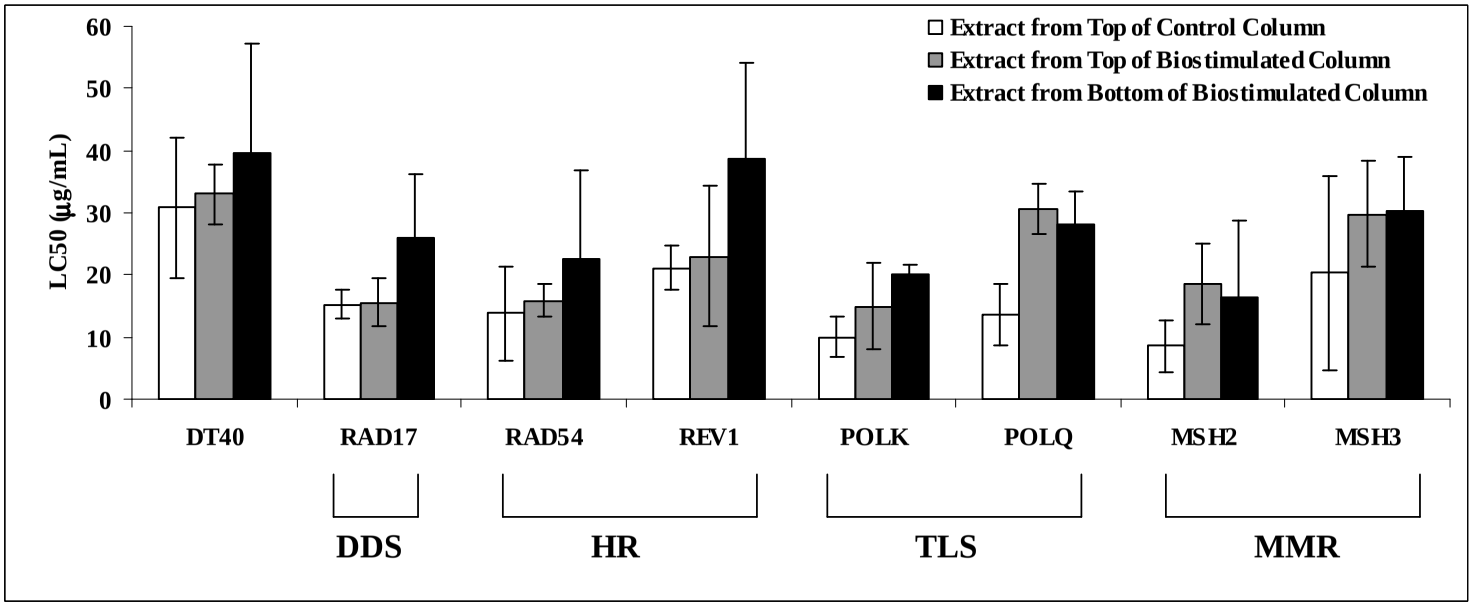

Figure 15. Relative LC50 values for cell lines exposed to extracts from the top of the control column (white), top of the biostimulated column (gray),and bottom of the biostimulated column (black). LC50 values calculated for all cell lines, and the most reliable are presented in this graph. Abbreviations: DDS – DNA damage sensor, HR – homologous recombination, TLS – translesion synthesis, MMR – mismatch repair.

4.7 Model Oxidative Stress: Hydrogen Peroxide

Although ROS (reactive oxygen species) are produced as normal parts of normal cellular metabolism, they are known to inflict injury on cellular macromolecules of aerobic organisms, such as DNA, via oxidative stress when the capacity for endogenous antioxidants to quench ROS is exceeded. This imbalance creates two pronounced effects: breaking of DNA strands and topoisomerase-II inhibition. The first type of damage is exemplified by H_2O_2 exposures. Cells deficient in the homologous recombination DNA repair pathway (RAD54) and those mutants lacking the non-homologous end-joining pathway, LIGIV, both show marked sensitivity to H_2O_2 (Figure 17), suggesting this model oxidative stress agent generates double strand breaks. This pattern is similar to that shown by each soil extract analyzed in this study. Should these soil extracts exert a carcinogenic effect, strand breakage that introduces gross chromosomal aberrations could be the causative mechanism.

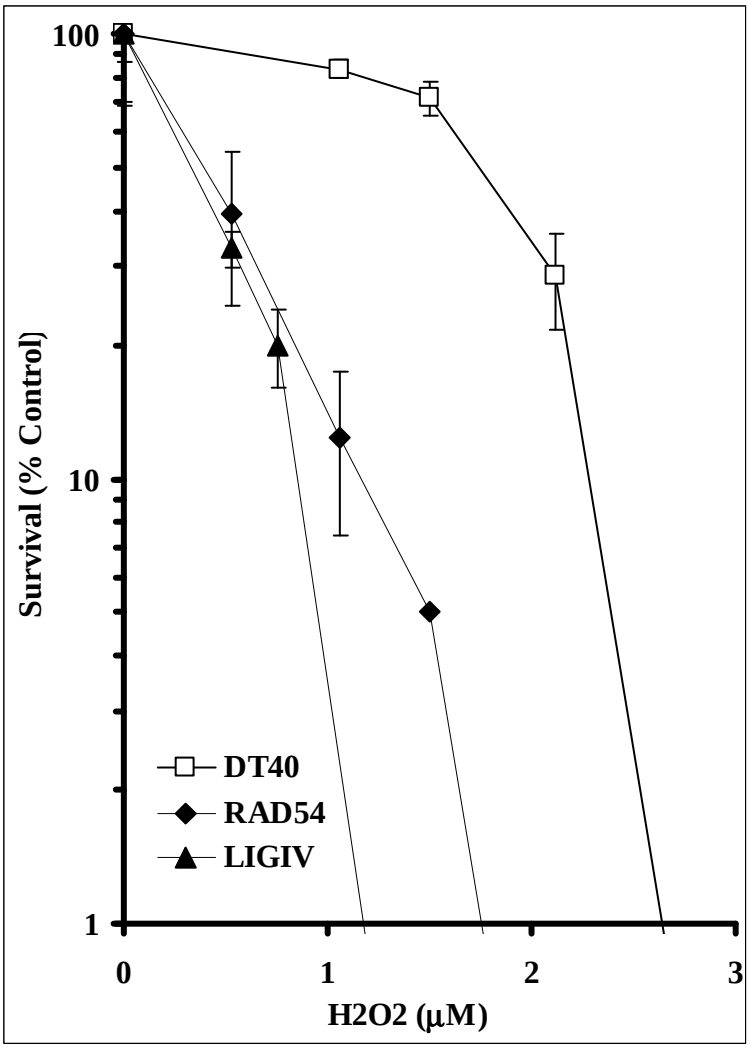

Figure 16. Cell viabilities of DT40 cells and cells deficient in RAD54 and LIGIV after H_2O_2 exposure.

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CHAPTER 5

SUMMARY AND CONCLUSIONS

To address whether bioremediation of contaminated soil from a former MGP site introduces DNA damage, this study utilized a DT40 bioassay. This method sufficiently answered the preceding question, in addition to addressing the types of DNA damage that were generated. Moreover, the DT40 bioassay provided informative results as to the necessary DNA repair and cell cycle checkpoint genes for cells to endure exposure to these extracts.

The hypothesis of this study, that biostimulation would intensify genotoxic responses, is proven by this work. Analysis of the initial extract from the top (aerobic zone) of the biostimulated column indicates more types of DNA damage occurred than that from exposures with the untreated soil extract. After three more months of aerobic biostimulation, however, the severity of genotoxic responses to extracts from the top of the biostimulated column lessened, but new responses were present. The original increase in genotoxicity could be attributed to the synthesis of genotoxic metabolites from metabolic activity of indigenous microbial communities of the soil as evidenced by the decrease of parent PAH compounds. However, from the currently available data it is not possible to ascribe increases in genotoxicity to specific metabolites of PAH or any other contaminants. Subsequent reduction of genotoxicity may result from more polar metabolites eluting from the biostimulated column in downward-flowing groundwater.

However, any trend in genotoxicity over time would have to be confirmed with more samples. The results of this study do suggest that oxidative stress is the primary causative agent of the genotoxicity incurred by exposures to the analyzed soil extracts.

The genotoxicity of extracts from the top (aerobic zone) of the control column was similar to the genotoxicity of extracts from the top (aerobic zone) of the biostimulated column for the October sampling event. This finding suggests that aerobic activity per se, and not the specific conditions in the biostimulated column, led to the increase in genotoxicity relative to the untreated soil. The low genotoxic response to exposures with extracts from the bottom (anoxic zone) of the biostimulated column further suggests that aerobic microbial activity was responsible for genotoxicity in the other two column samples. Limited biological activity in the bottom of the biostimulated column is supported by total PAH concentrations that are similar to those in the untreated soil.

Novel DNA damage responses from the extract for the October 2008 sampling event from the top of the biostimulated column, such as the translesion synthesis pathway, may be explained by ROS production. ROS are known to introduce various types of DNA damage, including mutations and carcinogenicity. Most constituents of these extracts, which comprise a complex mixture of chemicals, remain unaccounted for. Additionally, it is difficult to elucidate the potential interactions of complex mixtures, especially without chemical characterization. The potential magnitude of (geno)toxicity generated by a complex mixture is now understood to appropriately be categorized as additive, antagonistic (less than additive), or synergistic (supra additive). Beyond the extent of DNA damage incurred from complex mixtures, the types of DNA damage

responses could also be influenced by intricate interactions of a complex mixture whereby unique genotoxic signatures result. This postulation may explain the unique genotoxic profiles of each analyzed soil extract.

The second aspect of this study speaks to specific DNA damages incurred by untreated and biostimulated soil extracts. Of the most pronounced DNA damage responses, those from cells deficient in RAD54 were sensitive to each extract, suggesting double-strand breaks occurred after these exposures. The necessity of homologous recombination coupled with observable sensitivity of LIGIV mutants to each extract is analogous to most sensitive mutant cell lines that are most sensitive to H_2O_2 exposures. This correspondence implies that the mechanism by which DNA damage is arising might be through oxidative stress, given that H_2O_2 is an accepted model causative agent for oxidative stress [215].

The sensitivity of RAD9 mutants after exposure to each mixture indicates that recruitment and coordination of necessary downstream DNA repair proteins or cell-cycle checkpoint proteins may be essential to tolerate DNA damage caused by extracts of biostimulated soil. DNA lesions such as 8-oxoguanine are generated in conditions where endogenous levels of ROS are exceeded. These lesions are known to inhibit topoisomerase-II, an enzyme located in the nucleus that is necessary to assist in the reduction of strand tension as DNA unwinds during replication. Sensitivity of RAD9 to exposures of the analyzed soil extract also indicates the potential involvement of the topoisomerase cleavage complex [215].

Negligible formation of DNA bulky adducts was implied, as evidenced by the marginal genotoxic responses observed from exposures of these soil extracts to cells

deficient in XPA. As a surrogate for PAH *o*-quinones, the genotoxicity of 4-ethylcatechol, which is oxidized in cells, was analyzed and determined also to have a minimal genotoxic response in XPA mutants, suggesting that even if *o*-quinones were present, they would have made a minor contribution to the DNA adducts formed from exposures to the extracts of the soils analyzed in this study. Slight genotoxic responses of mutants deficient in the BER pathway, suggests minimal formation of depurinating adducts from exposures to the extracts of the soils analyzed in this study.

Overall genotoxic responses from this study suggest biostimulation of contaminated soil from a former MGP site is an effective tool for the reduction of parent PAH but that metabolites from aerobic microbial activity are more genotoxic than the original untreated soil. This works supports the DT40 bioassay as a powerful method for determining the genotoxic potential of complex mixtures. Unlike other genotoxic assays, this system not only detects DNA damage but determines the DNA repair or cell-cycle checkpoint genes required for cell survival.

CHAPTER 6

RECOMMENDATIONS FOR FUTURE STUDIES

Understanding the effects exerted by biostimulation on the genotoxic potential of soils from former MGP sites is important for protecting public health; therefore, studies are needed to elucidate the impact this bioremediation method may have on DNA damage. This work pioneers utilization of the DT40 bioassay to characterize the genotoxicity of complex mixtures, and it also provides a foundation to guide future germane studies.

Although this work proved sufficient to address the questions of this study, modifications of the experimental design would permit more informative answers. In future studies, beginning consistent sampling of soil columns or a bioreactor used to treat soil shortly after implementation of acclimation conditions, with short intervals between sampling events, is advisable for a more informative picture of the changes in genotoxicity of the soil mixtures. Additionally, to better understand the effects of biostimulation, designated column zones of interest should be analyzed throughout the entire study. The current evaluation does not permit inter-sample comparison of the control column or the bottom of the biostimulated column. Finally, to minimize confounding factors, the maximum concentrations of exposures should be standardized for each mixture of every sampling event.

This preliminary work enlightens how such a study can be enhanced to strengthen translation of the results to public health. Because many PAH are known pro-carcinogens, inclusion of a metabolic activation system with the DT40 bioassay, such as the supernatant fraction of a mammalian liver homogenate such as S-9 microsomal fraction from *Rattus norvegicus*, may also offer more relevant results for the genotoxic effects of human exposures. Also, an additional sample from the eluted material of the biostimulated and control columns would be useful to test if polar genotoxicants are indeed being removed from the columns, as opposed to their complete biotransformation into non-genotoxic compounds. This final consideration is of special importance for MGP sites bordering surface and groundwater supplies.

A concluding reflection regards the profound impact that genotoxic profiles from the DT40 bioassay could have on environmental and public health. Should this approach be expanded to a high-throughput screening system, numerous DNA damage responses of fractionated environmentally contaminated mixtures could be analyzed in a resource-efficient manner. Elucidation of the DNA repair and cell cycle-checkpoint enzymes important for counteracting DNA assaults incurred by exposures could be incorporated into definitions of NOEL of bioremediated sites and help identify exposed sub-populations which are especially sensitive.

Appendix 1:

LC50 values (μg/mL) with confidence intervals and comparable soil masses (mg) for untreated soil extract. P-values resulted from comparison of LC50 values for each mutant to DT40 LC50 value.

Cell Line	LC50 Value	p-value	Comparable Soil Mass
DT40	11.3 ± 7.53		0.066 ± 0.04
RAD51d	10.9 ± 0.49	0.62	0.064 ± 0.02
BRCA2	12.5 ± 4.62	0.22	0.073 ± 0.003
FANCD2	11.9 ± 0.91	0.47	0.070 ± 0.03
BLM	11.0 ± 4.31	0.73	0.065 ± 0.02
KU70	9.83 ± 1.97	0.17	0.058 ± 0.0 1
LIGIV	11.6 ± 1.97	0.82	0.068 ± 0.01
XPA	13.5 ± 3.7	0.11	0.79 ± 0.01
ATM	11.9 ± 1.57	0.43	0.070 ± 0.01
RAD17	8.08 ± 1.63	0.05	0.047 ± 0.01

Appendix 2:

LC50 values (μg/mL) with confidence intervals and comparable soil masses (mg) for the extract of the top of the biostimulated column from the July 2008 sampling event. P-values resulted from comparison of LC50 values for each mutant to DT40 LC50 value: 10.6 ± 4.55.

Cell Line	LC50 Value	p-value	Comparable Soil Mass
DT40	10.6 ± 4.55		0.049 ± 0.2
RAD54	4.37 ± 3.21	***<0.01***	0.020 ± 0.01
RAD51c	10.3 ± 2.63	0.66	0.058 ± 0.01
RAD51d	12.5 ± 1.62	***0.04***	0.042 ± 0.01
BRCA2	8.93 ± 2.48	0.12	0.052 ± 0.01
FANCD2	11.1 ± 4.83	0.75	0.048 ± 0.02
BLM	7.91 ± 1.49	***0.01***	0.062 ± 0.01
KU70	10.4 ± 4.13	0.85	0.056 ± 0.02
XPA	13.4 ± 1.98	***0.01***	0.053 ± 0.01
PARP1	11.4 ± 2.28	0,44	0.058 ± 0.01
RAD18	12.5 ± 1.62	0.12	0.037 ± 0.01
ATM	11.5 ± 0.69	0.2	0.054 ± 0.003
RAD9	6.48 ± 2.34	***<0.01***	0.030 ± 0.01
RAD17	9.32 ± 3.14	*0.199*	0.043 ± 0.01

Appendix 3:

LC50 values (μg/mL) with confidence intervals and comparable soil masses (mg) for extract of the top of the control column from the October 2008 sampling event. P-values resulted from comparison of LC50 values for each mutant to DT40 LC50 value.

Cell Line	LC50 Value	p-value	Comparable Soil Mass
DT40	30.8 ± 11.2		0.356 ± 0.13
RAD52	30.9 ± 7.1	0.96	0.357 ± 0.08
RAD51c	33.2 ± 3.87	0.85	0.383 ± 0.04
XRCC2	31.9 ± 9.85	0.89	0.368 ± 0.11
XRCC3	32.0 ± 4.01	0.7	0.370 ± 0.05
BRCA 2	30.2 ± 5.28	0.85	0.349 ± 0.06
KU70	14.5 ± 5.27	***0.01***	0.167 ± 0.06
LIGIV	18.7 ± 6.73	***0.02***	0.216 ± 0.08
DNA PKCs	30.6 ± 7.8	0.96	0.347 ± 0.09
XPA	31.0 ± 11.15	0.95	0.358 ± 0.13
XPG	30.0 ± 10.1	0.84	0.347 ± 0.12
FEN1	32.6 ± 7.25	0.62	0.377 ± 0.08
PARP1	31.1 ± 3.88	0.92	0.359 ± 0.04
POLQ	28.0 ± 4.97	0.43	0.323 ± 0.06
REV1	21.1 ± 3.49	***0.04***	0.244 ± 0.04
POLK	20.2 ± 3.18	***0.04***	0.233 ± 0.04
POLN	21.7 ± 8.79	0.05	0.251 ± 0.10
RAD18	26.5 ± 6.39	0.28	0.306 ± 0.07
ATM	34.8 ± 11.4	0.4	0.402 ± 0.13
RAD9	16.4 ± 4.68	***0.01***	0.189 ± 0.05
RAD17	15.3 ± 2.39	***0.02***	0.177 ± 0.03

Appendix 4:

LC50 values (μg/mL) with confidence intervals and comparable soil masses (mg) for extract of the top of the top of the biostimulated column from the October 2008 sampling event. P-values resulted from comparison of LC50 values for each mutant to DT40 LC50 value.

Cell Line	LC50 Value	p-value	Comparable Soil Mass
DT40	33.0 ± 4.87		0.225 ± 0.03
RAD54	15.9 ± 2.62	***<0.01***	0.109 ± 0.02
RAD51c	22.6 ± 4.23	***<0.01***	0.154 ± 0.03
XRCC2	31.1 ± 4.63	0.28	0.212 ± 0.03
XRCC3	30.1 ± 5.09	0.18	0.205 ± 0.03
BRCA2	30.1 ± 2.68	0.1	0.205 ± 0.02
DNA PKCs	26.6 ± 6.38	0.96	0.182 ± 0.04
XPA	33.6 ± 6.74	0.77	0.229 ± 0.05
MSH2	18.5 ± 6.41	***<0.01***	0.126 ± 0.04
MSH3	29.8 ± 8.53	0.09	0.203 ± 0.06
POLB	33.2 ± 13.9	0.16	0.227 ± 0.09
FEN1	30.0 ± 7.87	0.33	0.205 ± 0.05
POLQ	30.6 ± 3.99	0.16	0.209 ± 0.03
REV1	22.9 ± 11.3	0.06	0.156 ± 0.08
POLN	21.4 ± 3.62	***<0.01***	0.146 ± 0.02
RAD18	22.3 ± 5.62	***<0.01***	0.152 ± 0.04
ATM	30.8 ± 7.8	0.18	0.210 ± 0.05
RAD9	21.6 ± 6.27	***<0.01***	0.147 ± 0.04
RAD17	15.6 ± 3.98	***<0.01***	0.106 ± 0.03

Appendix 5:

LC50 values (μg/mL) with confidence intervals and comparable soil masses (mg) for extract of the bottom of the biostimulated column from the October 2008 sampling event. P-values resulted from comparison of LC50 values for each mutant to DT40 LC50 value.

Cell Line	LC50 Value	p-value	Comparable Soil Mass
DT40	39.6 ± 17.6		0.474 ± 0.21
RAD52	42.3 ± 13.1	0.39	0.506 ± 0.06
KU70	30.5 ± 14.3	0.1	0.365 ± 0.17
MSH2	31.6 ± 12.6	0.12	0.378 ± 0.02
MSH3	29.8 ± 8.53	0.09	0.357 ± 0.04
PARP1	38.7 ± 20.6	0.79	0.463 ± 0.15
POLQ	33.1 ± 5.33	0.14	0.396 ± 0.10
REV1	38.7 ± 15.3	0.85	0.463 ± 0.25
POLN	32.1 ± 1.58	0.16	0.384 ± 0.06
RAD18	37.9 ± 3.62	0.58	0.454 ± 0.18
RAD17	26.0 ± 10.2	***0.02***	0.311 ± 0.12

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