The efficacy of different bioremediation strategies in removing mutagenic hazard from contaminated soil.

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ABSTRACT

Assessment of bioremediation efficacy generally involves examination of the temporal trend in priority contaminant concentrations. However, there is increasing evidence that declines in priority contaminant concentrations correspond to increases in toxicological hazard. In this study, PAH contaminated soil was subjected to 3 different 90-day bioremediation treatments in bench-scale bioreactors; remediation outcome was evaluated at 15-day intervals using both chemical analyses and mutagenicity assessment. Analysis showed initial PAH concentrations above CCME residential thresholds, with moderate reductions in total PAH concentration across all treatment types. Mutagenicity analyses, which employed the Salmonella reverse mutation assay, were conducted on two distinct chemical fractions: non-polar and polar aromatics. Mutagenic activity fluctuated over the course of each treatment and was predominately greater than the mutagenic activity observed in untreated soil, particularly in the polar aromatic fraction. These variations are possibly the result of oxidative bacterial degradation by-products, suggesting that mutagenicity monitoring during bioremediation should be employed.

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LIST OF ABBREVIATIONS

9-PA	9-phenylanthracene
ACN	acetonitrile
ASE	accelerated solvent extraction
atm	atmospheres
BaP	Benzo(<i>a</i>)pyrene
°C	temperature in degrees Celsius
CCME	Canadian Council of Ministers of the Environment
CEPA	Canadian Environmental Protection Act
CFCSI	Canadian Federal Contaminated Sites Inventory
cm	centimeter(s)
C/N/P	Carbon/Nitrogen/Phosphorous ratio
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
eq.	equivalents
g	gram(s)
HCl	hydrochloric acid

HMW	high molecular weight
HPLC	high pressure liquid chromatography
IARC	International Agency for Research on Cancer
kg	kilogram(s)
K _{ow}	octanol/water partition coefficient
kPa	kilopascal(s)
L	litre(s)
L/min	litre(s) per minute
LMW	low molecular weight
M	Molar (moles per liter)
mg	milligram(s)
mL	milliliter(s)
μm	micrometer(s)
mm	millimeter(s)
MPa	Megapascal(s)
μg	microgram(s)
μL	microlitre(s)

NaCl	sodium chloride
NaOH	sodium hydroxide
NADP	Nicotinamide adenine dinucleotide phosphate
PAC	polycyclic aromatic compound
PAH	polycyclic aromatic hydrocarbon
ppb	part(s) per billion
ppm	part(s) per million
psi	pound(s) per square inch
rev	revertant(s)
rpm	rotation(s) per minute
S_N^2	bimolecular nucleophilic substitution reaction
SQGHH	soil quality guidelines for human health
t/yr	tonne(s) per year
US EPA	United States Environmental Protection Agency
UV	ultraviolet
VBME	Volger-Bonner medium E
v/v	volume/volume

w/v

weight/volume

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1.0 INTRODUCTION

Various industrial, recreational and military activities have contributed to the contamination of arable land around the world. A contaminated site is defined as a site having substances of concern present at concentrations (1) above background levels that may pose an immediate or long term threat to human or environmental health, or (2) greater than those established by regulatory agencies [1]. Currently there are roughly 40,000 contaminated sites in Canada alone [2], with approximately 19,000 such sites controlled to some degree by the Canadian government and listed on the Canadian Federal Contaminated Sites Inventory (CFCSI) [1]. Of the listed sites that have been classified (7489), roughly 72% (5424) refer to contaminated soil mediums and over 2500 sites are contaminated with petroleum hydrocarbons and polycyclic aromatic hydrocarbons (PAH). The latter group includes known mutagens and carcinogens [3]. There are currently 1399 soil sites contaminated with petroleum hydrocarbons and PAHs classified on the CFCSI. Other contaminated sites in Canada are known to contain heavy metals, explosives, radioactive materials, oxygen depleting substances and other toxic compounds. Although a wide range of the contaminants present at sites listed on the CFCSI can pose serious acute and/or chronic hazards to human health [1], this project is focused on soil contaminated with PAHs, and problems associated with remediation strategies currently employed at such sites.

PAHs, which are constantly released into the environment from both natural and anthropogenic sources, are formed by the incomplete combustion of organic matter [4]. At over 2000 tonnes per year, forest fires are the largest natural contributor of PAH emissions into the environment. Wood preserving facilities

(2000 t/yr), metal smelting operations (929 t/yr) and gas works sites (76 t/yr) account for the majority of anthropogenic PAH emissions [5]. Other sources of PAHs include cigarette smoke, municipal waste water and urban runoff, internal combustion engines, and smoked or flame broiled foods [6]. Significant research has been focused on the observed increase in the genotoxicity of soil contaminants during the bioremediation of PAH contaminated soils [7-10]. Unfortunately few of these studies have focused on the mutagenicity of intermediate PAH metabolites, choosing only to examine end point genotoxicity [11-16]. The reverse *Salmonella* mutagenicity assay, more commonly known as the Ames test, is an effective means to evaluate the mutagenicity of organic contaminants extracted from polluted soil [17-19]. Previous studies have been limited in that: 1) only changes in mutagenic potential before and after remediation were tested [7; 20-28]; or 2) mutagenicity of priority PAHs were tested exclusively [15; 29-50]. Little information is currently available on the mutagenicity of PAH metabolites and complex mixture soil pollution, as well as qualitative and quantitative changes which occur over the duration of bioremediation treatments [51-53].

Public awareness of environmental pollutants has grown significantly over the past few decades. This attention has prompted an increase in the occurrence and intensity of remediation and reclamation operations at contaminated sites. Currently there are several different strategies used to mitigate this type of environmental pollution, including land filling, solidification, incineration, and bioremediation. Land filling contaminated soil, which involves transportation to an isolated location where the presence of pollutants will have a minimal effect on the

environment, can cost \$450 - \$1000 a ton depending on the distance transported [54]. The solidification of contaminated soil with cement has proven to be an effective strategy for managing contaminated sites involving a variety of pollutants. Common cement is mixed with the polluted soil on site, effectively trapping any hazardous material and preventing further environmental migration. Cement solidification is relatively inexpensive, costing \$50-310 per ton, and has been employed in 23% of USEPA Superfund projects since 1980 [55]. Although land filling and solidification are strategies that relocate or immobilize hazardous materials and effectively reduce the exposure potential, they do not effectively reduce the concentration of pollutants in the soil. In contrast, strategies involving incineration or bioremediation serve to treat contaminated soil in an effort to reduce the concentration of hazardous materials. Incineration, which involves combustion of the soil at elevated temperatures (e.g., $>500^{\circ}\text{C}$), requires transportation of the contaminated soil to a dedicated facility, or construction of an onsite facility. The complete incineration of soil, at a cost of \$1500 - \$1800 per ton, effectively destroys all organic pollutants in a relatively short period of time. However, all soil transported away from a contaminated site may have to be replaced with other materials, and depending on the desired future use of the location, this can increase the total cost of the reclamation project. Bioremediation, the process of using living organisms (usually microorganisms) to degrade organic pollutants (including PAHs) is often used as an alternative to traditional remediation such as incineration. It is often more cost effective (\$100 - \$175 / ton) and viewed as environmentally sustainable [54]. Bioremediation can involve the

treatment of soils *in situ* or *ex situ*, and can take between weeks and years to complete, depending on the nature of contaminants, and the type of treatment employed. Natural attenuation, a form of bioremediation where the natural organisms are stimulated in an effort to degrade the pollutants, is more cost effective, although much slower, than methods that involve augmentation of the soil with specialized strains of degrading bacteria and specifically formulated nutrient solutions. Effective bioremediation results in the complete mineralization of the contaminants, and they are completely transformed to water and carbon dioxide [7]. Nevertheless, there is concern that certain metabolic bi-products formed during degradation can prove more hazardous than the parent pollutants [56].

1.1 POLYCYCLIC AROMATIC HYDROCARBONS

PAHs are compounds that contain two or more fused benzene rings in various linear and/or clustered formations. By definition, PAHs only include hydrogen and carbon atoms, however heterocyclic compounds in which one or more C atoms have been substituted with nitrogen, oxygen or sulfur, are also included within the PAH nomenclature [57]. Collectively, these compounds can be classified as Polycyclic Aromatic Compounds (PACs). Partially metabolized PAHs, or the intermediate compounds formed by catabolic pathways such as aerobic microbial digestion, as well as PAHs that include alkyl substituted benzene rings are also included within this classification. The Canadian Environmental Protection Act (CEPA) currently identifies 13 PAHs as priority substances [Fig. 1.0], due to their known mutagenic and suspected carcinogenic properties [57; 58]. These substances

Figure 1.0: Structures of PAHs listed under the Priority Substances List from CEPA [57].

acenaphthene

anthracene

benzo[a]anthracene

benzo[b]fluoranthene

benzo[j]fluoranthene

benzo[k]fluoranthene

benzo[a]pyrene

fluoranthene

fluorene

indeno[1,2,3-cd]pyrene

naphthalene

phenanthrene

pyrene

PAH	CCME Threshold (ppm)			
	Agricultural	Residential	Commercial	Industrial
Phenanthrene	0.1	5	50	50
Pyrene	0.1	10	100	100
Benzo(<i>a</i>)anthracene	0.1	1	10	10
Benzo(<i>b</i>)fluoranthene	0.1	1	10	10
Benzo(<i>k</i>)fluoranthene	0.1	1	10	10
Benzo(<i>a</i>)pyrene	0.1	0.7	0.7	0.7
Dibenzo(<i>a,h</i>)anthracene	0.1	1	10	10
Indeno(1,2,3- <i>cd</i>)pyrene	0.1	1	10	10

PAH	# of Rings	Water Solubility (mg/ L)	Log K _{ow}	Vapour Pressure (mm Hg)	Henry's Law (atm * (m ³ / mole))	IARC
Naphthalene	2	31	3.3	8.5×10^{-2}	4.4×10^{-4}	2B
Acenaphthene	3	3.9	3.9	2.5×10^{-3}	1.8×10^{-4}	3
Fluorene	3	1.7	4.2	6.0×10^{-4}	9.6×10^{-5}	3
Phenanthrene	3	1.2	4.5	1.2×10^{-4}	4.2×10^{-5}	3
Anthracene	3	0.04	4.5	6.5×10^{-6}	5.6×10^{-5}	3
Fluoranthene	4	0.3	5.2	9.2×10^{-6}	8.9×10^{-5}	3
Pyrene	4	0.1	4.9	4.5×10^{-6}	1.2×10^{-5}	3
Benzo(a)anthracene	4	0.009	5.8	2.1×10^{-7}	1.2×10^{-5}	2B
Chrysene	4	0.02	5.8	6.2×10^{-9}	5.2×10^{-6}	2B
Benzo(b)fluoranthene	5	0.002	5.8	5.0×10^{-7}	6.6×10^{-7}	2B
Benzo(k)fluoranthene	5	0.0008	6.1	9.7×10^{-10}	5.8×10^{-7}	2B
Benzo(a)pyrene	5	0.002	6.1	5.4×10^{-9}	4.6×10^{-7}	1
Dibenzo(a,h)anthracene	5	0.002	6.8	9.6×10^{-10}	1.4×10^{-7}	2A
Benzo(g,h,i)perylene	5	0.0003	6.6	1.0×10^{-10}	3.3×10^{-7}	3
Indeno(1,2,3-cd)pyrene	6	0.0002	6.7	1.3×10^{-10}	3.5×10^{-7}	2B

A general rule regarding PAH physical chemistry is that as the number of rings increases, the water solubility, vapour pressure and Henry's Law constant will decrease, and the Log K_{ow} (i.e., the octanol-water partition co-efficient) will increase (Table 1.1). This accounts for larger compounds being less mobile and having a greater affinity for organic particles in the environment [52]. The inherent hydrophobic nature of PAHs, which increases with the number of benzene rings, partly accounts for their environmental persistence [7]. As LMW PAHs are more water soluble and volatile than HMW PAHs, they are more accessible to microorganisms in the environment, and are thus more rapidly degraded, and more readily mineralized. With respect to the CEPA priority PAHs, it should be noted that the high molecular weight (HMW) PAHs containing 4, 5 or 6 benzene rings, are more hydrophobic, resistant to degradation, and environmentally persistent compared to the low molecular weight (LMW) PAHs containing 2 or 3 benzene rings. A similar relationship has been observed with respect to genotoxicity, which tends to increase along with the PAH ring number between 3 and 6 ringed compounds [60]. IARC currently classifies all of the CEPA priority PAHs, as well as numerous other PACs, not considered under CEPA. Acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene and benzo(*g,h,i*)perylene are classified as group 3 compounds: *not classifiable as to their carcinogenicity to humans*. PAHs considered as class 2B: *possible human carcinogens*, include naphthalene, benzo(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene and indeno(1,2,3-*cd*)pyrene. Dibenzo(*a*)anthracene is a class 2A compound or *probable human carcinogen*. Benzo(*a*)pyrene is currently the only PAH with enough evidence to classify it as a

level 1 compound (i.e., *known human carcinogen*). IARC also classifies the circumstances of exposure to emissions from coal gasification, coal-tar distillation and/or coke production, and occupational exposures associated with these practices are classified as carcinogenic for humans [61].

The genotoxic and carcinogenic properties of PAHs have been thoroughly investigated and assessed in a variety of experimental systems (e.g., rodents). PAH exposure can occur via absorption through the skin, lungs and/or the gastrointestinal tract, with distribution to all points of the body occurring via the blood stream [62]. Such exposure to complex mixtures, including PAHs, extracted from contaminated soil has been shown to result in increased mutation frequencies in target tissues in mice [63].

It should be noted that PAHs require metabolic activation before their genotoxic potential can be realized. In mammals, metabolic activation is carried out by the cytochrome P450 enzyme system, and involves insertion of oxygen at one or more sites within the outer carbon skeleton of a particular PAH. Such oxygenated metabolites are more water soluble, have increased reactivity, and some are able to covalently bond with DNA to form adducts [64-66]. If such adducts are not repaired, and the damaged DNA is allowed to replicate, permanent changes in DNA sequence (i.e., mutations) can arise. An example of the formation of a potentially mutagenic adduct is provided in Figure 1.1, where oxidized benzo(*a*)pyrene is metabolized by cytochrome P450. The resulting nucleophilic di-hydrodiol epoxide can react via nucleophilic substitution with the exocyclic amine of the DNA base guanine to

produce a bulky adduct (Fig 1.1). When such adducts contribute to mutations in certain genes, specifically those responsible for the regulation of cell cycle, apoptosis, or other cellular activities (i.e., p53), the likelihood of tumour formation can be increased [67-69]. In addition to genotoxicity, PAHs have been shown to exhibit a wide variety of ecotoxicological effects in terrestrial and aquatic plants, mammals, amphibians, reptiles, birds and microorganisms. Noteworthy effects include reproductive toxicity, neurotoxicity, and immunotoxicity [62].

1.2 BIOREMEDIATION OF PAH CONTAMINATED SOILS

The ultimate goal of bioremediation is to encourage a consortium of microbes to completely mineralize the PAH contamination into water and carbon dioxide along with other harmless organic compounds. Different strategies have been investigated to stimulate the natural bioremediation mechanisms employed by microbes, such as strains of *Mycobacterium* and *Sphingomonas* that can utilize PAHs as their sole carbon and energy sources [70]. The bioavailability of PAHs is essential for biodegradation since microbial enzymes must have physical contact with the chemicals in order for metabolism to occur. The low bioavailability of PAHs in soil is considered a rate limiting factor in PAH degradation, however, it can be increased through the simple addition of mild surfactants such as Tween-80 or humic acid [71]. Although the addition of surfactants may increase the bioavailability of PAHs, they do not necessarily increase the rate of degradation. The relative exclusion of HWV PAHs from the aqueous phase combined with their innate ability to strongly sorb to organic matter within soil, render them relatively inaccessible to the PAH metabolizing microbes in the environment. Their low bioavailability levels in soil, however, are not the only factor inhibiting natural bioremediation, as contaminated soils are often void of essential organic and inorganic nutrients necessary for microbial growth [70]. Also contributing to minimal rates of natural PAH biodegradation is the fact that PAHs are mainly degraded through aerobic processes [72], whereas the soil in which they are present can often be depleted of oxygen. It is important to note that some oxygenated PAH metabolites are more environmentally

mobile, and may be more mutagenic, than their respective parent compounds [51]. PAHs will continually diffuse throughout a soil matrix as long as contact between PAHs and organic material is maintained. This process, known as aging or weathering, is a major concern for the remediation of contaminated sites since PAHs are often found in older industrialized locations where the pollution is deeply imbedded within the soil structure, severely reducing the bioavailability. Early in the aging process PAHs can undergo volatilization (LMW PAHs), photo-oxidation, natural bioremediation via environmental organisms, bioaccumulation, and leaching into ground water systems.

Bacterial PAH metabolism is typically initiated via intracellular dioxygenase enzymes, which use both atoms of an oxygen molecule to oxidize PAHs to *cis*-dihydrodiols. These oxidative intermediates can then be directed through several iterations of *ortho*- or *meta*- cleavage pathways, and subsequent oxidation reactions until either mineralization is achieved or further metabolism is impossible [30]. An illustration of one possible bacterial degradation pathway involving benzo(*a*)pyrene is given in Figure 1.2. Bacterial PAH degradation pathways commonly result in the formation and metabolism of catechol in the final stages. Catechol metabolism occurs via five distinct yet similar pathways, and results in the production of succinic acid, fumaric acid, pyruvic acid, acetic acid or acetaldehyde. These products can in turn be used to produce amino acids that are incorporated into bacterial proteins, or be further degraded into water and carbon dioxide. However, several inorganic nutrients, including nitrogen and phosphorous required for the perpetuation of the microbial culture, and various co-factors essential for natural

enzyme function, are often absent from PAH contaminated soils. Providing these essential inorganic macro-nutrients in a ratio close to the generally accepted C:N:P ratio of 100/10/1 (moles), as well as the necessary micro-nutrients and an abundance of oxygen, has been shown to produce optimal rates of microbial PAH degradation [70].

D, E – Series of bacterial enzyme catalyzed reactions resulting in mineralization of chrysenes-4-carboxylate and chrysenes-5-carboxylate respectively.

The chemical structure of PAHs is characterized by highly conjugated π -orbital systems that allows 3-4 ring PAHs to absorb UVA light (320-400 nm) and 5-6 ring PAHs to absorb visible light (>400 nm). Once photons are absorbed, the excited PAHs can form reactive species via electron exchanges with oxygen, generating highly reactive singlet oxygen free radicals. PAH photon absorption may also result in photo-oxidation and the subsequent generation of reactive oxygen containing species, as well as other oxidized compounds including ketones and quinones. These compounds can display both mutagenic and toxic properties, and have the ability to form DNA adducts or disrupt cellular membranes and proteins [74]. PAHs can also react with environmental chemicals, such as hydrogen peroxide and ozone, to form similar oxygen containing compounds. Ozonation often occurs in the presence of water, after hydrolysis has cleaved an outer double bond [75; 76].

Bioremediation strategies employed for the reclamation of PAH contaminated soils can be either *in situ* or *ex situ* systems. *In situ* bioremediation refers to techniques used to treat contaminated soils at the site of contamination. Typically this procedure involves the addition of nutrients to maintain a C/N/P ratio of ~100/10/1, some type of aeration system to provide oxygen, and in certain cases the addition of exogenous cultures of PAH degrading microbes. The success of such operations is heavily dependent on the soil composition and permeability. Typical aeration systems often include the insertion of porous hollow rods deep into the site of contamination which are attached to a blower above ground. Such rods are often equipped with sensors to monitor the effective reduction of contamination levels. *In situ* remediation systems have been shown to be quite effective at removing LMW

PAH contamination after nutrient and oxygen addition; however, the reduction of HMW PAHs is quite limited [77].

Ex situ processes can involve the transportation of polluted soils to industrial bioremediation facilities, typically located within close proximity to the contamination. Polluted soil is transferred to an impermeable membrane lined bed to prevent contamination leaching during treatment. Systems may include augers to facilitate the continuous mixing of the soil, to promote aeration and facilitate the distribution of contaminants throughout the matrix. Soil beds are often covered to shield the operation from weather, and capture the heat generated by microorganisms during PAH metabolism. As microbes work more efficiently at higher temperatures, maintaining an elevated temperature improves the performance of the treatment process. In general, it is easier to augment soils with exogenous microbes, and maintain soil pH and nutrient concentrations at optimum levels using *ex situ* systems in comparison with *in situ* systems. Several studies have shown the effectiveness of *ex situ* bioremediation in reducing the levels of both LMW and HMW PAHs [78-82].

Ex situ bioslurry systems provide the opportunity to treat contaminated soils in a completely controlled environment. Contaminated soil and water are added to a closed reactor to produce a slurry of desired consistency, and PAH-degrading organisms and nutrients are added to maintain optimal treatment conditions. The slurry is constantly mixed via mechanical agitation and an air sparger, which also supplies oxygen to the reactor. Agitation ensures complete mixing of the slurry,

enhancing the bioavailability of the PAHs sorbed to soil particles. The high level of control, and the ability to maintain conditions at optimal levels throughout the treatment period, should theoretically enhance the levels of removal of all PAHs. It has been shown that bioslurry treatments are more efficient than both *in situ* and *ex situ* treatments for removing PAHs from soil [77].

Bioremediation can occur when microbes use a PAH as their sole carbon source, as is the case in the bacterial genera *Aeromonas*, *Alcaligenes*, *Bacillus*, *Cyanobacter*, *Flavobacterium*, *Micrococcus*, *Mycobacterium* and *Pseudomonas*. Since many of these bacteria are able to use PAHs as a sole source of carbon, complete mineralization of PAHs in a mixture (i.e., to water and carbon dioxide) can occur. Mineralization of LMW PAHs occurs more readily than for HMW PAHs. PAHs can also be utilized during co-metabolism, where a micro-organism does not utilize a compound, such as a PAH, as an energy or carbon source, yet produces a metabolic by-product that can be used by other species for these purposes. Co-metabolism is often associated with HMW PAH degradation with the following species able to co-metabolize and degrade HMW PAHs: *Rhodococcus sp.*, *Pseudomonas paucimobilis* and *Alcaligenes denitrificans* [77].

Previous studies have been conducted to characterize biodegradation processes of PAHs in soil, and these have provided valuable information regarding the microbial genes involved in PAH metabolism, and on the overall composition of microbial consortia at sites where PAH bioremediation is ongoing. LMW PAH biodegradation routes are more simplistic than HMW degradation routes, and the

former have been well documented [73]. Several Gram-negative strains have been identified that each possess a complete set of metabolic enzymes for the complete utilization/mineralization of 2-3 ring PAHs [60]. However, there is a paucity of knowledge surrounding the biodegradation of HMW PAHs. The class *Actinobacteria* contains several species, including *Mycobacterium sp.*, known to degrade HMW PAHs, albeit not completely. Such strains of *Actinobacteria* have been shown to oxidize both 3- and 4- ring PAHs using complex, multi-branched pathways. Successful degradation of HMW PAHs has been shown to occur by microbial co-metabolism [83]. Co-metabolic processes by members of a consortium can be either; 1) degradation pathways that lead to complete mineralization of pollutants; or 2) non-productive processes that lead to the accumulation of oxidative by-products in the surrounding medium [84]. An abundance of oxidative PAH metabolites has been observed at several PAH contaminated sites undergoing bioremediation [24], suggesting the effects of non-productive co-metabolism [7; 52; 85-87]. Consequently the selection of appropriate microbes for use during bioremediation is of vital importance if complete mitigation is desired.

Recent work by Miller *et al* [88] investigated the diversity of soil mycobacterium at different PAH contaminated sites in hopes of identifying the traits that are present in the most effective PAH degraders. Five distinct species of mycobacterium were identified and their work supports the notion that the degree of bioaugmentation success is dependent on microbial species composition. Three species, *Mycobacterium gilvum*, and two isolates from Montana, USA (i.e., *Mycobacterium spp.* KMS and MCS), were found to contain a large linear plasmid

similar to plasmids described in other mycobacterium isolates that contain genes for epoxide metabolism via the coenzyme-Q pathway. Since epoxide containing PAH metabolites, such as benzo(a)pyrene-dihydrodiol-epoxide, are known mutagens (Fig. 1.1) [89] and may be produced during the aerobic bacterial metabolism of HMW PAHs, cultures containing species with the ability to degrade epoxide containing compounds would be essential for achieving completely successful bioremediation. Debruyne *et al* have shown a positive correlation between the levels of *Mycobacterium* spp. dioxygenase gene *nidA*, responsible for the initiation of pyrene degradation, and pyrene concentration throughout a coal tar contaminated site [90]. Further research quantifying the abundance of catabolic genes in the environment may help explain natural attenuation processes, and provide information that will permit the development of more efficient bioremediation strategies.

Miller *et al* described a difference in cell wall fatty acid and glycopeptidolipid composition between microbial isolates from PAH contaminated sites [88]. Cells with a rougher exterior appearance, such as *Mycobacterium intracellulare*, were more hydrophobic compared to others, and this property could influence relative degradation rates. The hydrophobicity of a bacterium will dictate its ability to take up a wider range of substrates and organisms with increasingly hydrophobic exteriors should be able to more readily take up lipophilic substances (e.g., HMW PAHs). The hydrophobicity of a bacterium's cell wall may also influence its ability to attach to soil particles, its mobility through ground water, and its ability to penetrate into the soil matrix. Substrate uptake can promote the co-metabolism of

HMW PAHs, and is essential for the prolonged survival of bacterial cultures at bioremediation sites. Consequently, Miller *et al* also examined the relative ability of the *Mycobacterium* species examined to take up a variety of substrates. Three different strains of *Mycobacterium* were tested for their ability to take up a variety of substrates that are often used to stimulate microbial growth at contaminated sites. The substances included a range of sugars, amino acids, surfactants, as well as novel substrates, with only Tween-80 able to be utilized by all species [88].

Other factors affecting the biodegradation of PAHs in soil include the physical characteristics of the soil, and the chemical composition of the contaminant mixture. It is well established that the release of PAHs from the particle phase to the aqueous phase is the rate limiting step in bioremediation, however, the mechanisms of transport between these phases is not well understood. Xia and Wang [91] have shown that PAH degradation rate is increased in the presence of certain particle types. The order of metabolic rate enhancement, with respect to soil type, was shown to be fine silt > clay > coarse silt. Moreover, it was shown that degradation of benzo(a)pyrene was approximately 3 times greater when a soil-water interface was permitted as compared to a strictly aqueous environment. The ability of bacteria to attach to sediment particles likely permits direct access to higher concentrations of PAH at the particle surfaces in comparison with that found in the aqueous phase. As rates were highest in the smallest soil particle size, the results suggest that biotransformation occurs at the soil-water interface, and not in the particle or water phase.

The composition of chemicals present at the site also affects the overall rate of degradation. How the transformation of a particular PAH is influenced by the presence of complex mixtures of PACs is not well understood, particularly for species such as *Mycobacterium sp.* that are capable of degrading more than one substance. Previous studies have shown that phenanthrene metabolism by *Pseudomonas sp.* is hindered by the presence of naphthalene [92]. Other work has shown that benzo(a)pyrene is rapidly metabolized by a consortium of *Mycobacterium* and *Sphingobacterium* in the presence of diesel fuel [92]. These phenomena, and bacterial biotransformation of PAHs in general, are thought to be effected by competitive inhibition between contaminants for common catabolic enzymes, and the relative level of gene induction [92]. To further contribute to the confusion regarding bacterial metabolism of complex PAH mixtures, evidence exists to support the notion that the presence of LMW PAHs may in fact be required to facilitate the degradation of HMW compounds via catabolic pathways that are essential for the co-metabolism of the more recalcitrant compounds [92]. For example, McLellan *et al* showed that BaP degradation by *Mycobacterium sp* strain RJGII-135 was enhanced in the presence of pyrene and/or phenanthrene. Since HMW PAH compounds do not generally induce catabolic pathways, the presence of LMW PAHs is important for complete biodegradation.

1.3 GENOTOXICITY OF REMEDIATED SOIL

The genotoxic activity of PAH contaminated soil before and after treatment has been fairly well studied [93-95], and the temporal effects of priority PAH

removal has been documented extensively [20; 96]. However, few studies have monitored both genotoxicity and priority pollutant concentration concurrently at select time points during a treatment cycle [52; 53]. As a result, there is a paucity of information regarding how changes in individual priority PAH concentration in a complex mixture affects the total genotoxic activity of a contaminated soil. Although not currently practiced, it is important to consider the mutagenic effects of all contaminants present, including aerobic bacterial PAH metabolites, O-, S-, and N-substituted PAHs, and alkylated PAHs during the treatment period in order to effectively declare a site to be remediated.

The *Salmonella* reverse mutation assay, or Ames test, is a short term assay used to identify mutagens. The assay outcome has a reasonably high concordance with rodent carcinogenicity (58.8 %) [124]. The test uses a variety of genetically modified strains of *Salmonella* that contain a variety of mutations inhibiting the synthesis of the essential amino acid histidine. Consequently, colonies will only grow on medium without histidine after reversion of the mutation and restoration of the ability to produce histidine. Exposure to mutagenic compounds will increase the number of revertants (i.e., histidine auxotrophs to wild type) above spontaneous, and the response pattern is often manifested as a linear concentration-response function. When test article (i.e., chemical or complex mixture) concentration is plotted against the number of revertants scored, the initial slope of the concentration-response function is termed the mutagenic potency of the test article [18].

Salmonella strains commonly employed in the Ames test contain the *hisD3052* and *hisG46* alleles, engineered to detect frameshift (strains TA98, YG1041) or base-pair (strains TA100, YG1042) mutations, respectively. In addition to the designed mutations affecting histidine production other mutations and plasmids that enhance the performance of the test and its ability to detect mutagenic effects are also present. A deep rough (*rfa*) mutation affects the lipopolysaccharide (LPS) core, disrupts the integrity of the outer membrane, and allows bulky chemicals to enter the cell more readily. Deletion of the *uvrB* gene disrupts nucleotide excision repair, increasing the probability that a DNA adduct will persist and induce a mutation. Collectively, these mutations allow chemicals such as PAHs and related compounds to enter the cell, form adducts, and contribute to the formation of mutations at the *his* locus. Strains employed in the *Salmonella* reverse mutation test also contain a plasmid (i.e., pKM101) that contains the *mucA* and *mucB* genes. These genes are required for the production of PolRI, a translesion polymerase able to replicate damaged DNA, which further enhances the sensitivity of the strains to detect mutagenic compounds. The strain YG1041, which is a derivative of TA98, contains an additional plasmid (i.e., pYG233) that harbours the *cnr* and *OAT* genes. These genes encode the classical nitroreductase and acetyl transferase enzymes, respectively. The presence of this plasmid greatly increases the sensitivity of strains to detect nitro-substituted compounds and aromatic amines [18]. All *Salmonella* strains employed to detect mutagens can be supplemented *in vitro* with an exogenous metabolic activation mixture derived from mammalian liver. Since *Salmonella* are devoid of the metabolic enzymes required to metabolize and activate

mutagenic compounds such as PAHs, their inclusion is necessary to simulate mammalian metabolism and PAH activation. The 9000g supernatant of Arochlor-1254 induced rat liver homogenate (S9), derived from male Sprague-Dawley rats, is commonly used as the source of the metabolic activation. Direct acting mutagens, which can exert their effect without hepatic metabolism, can be detected without an exogenous metabolic activation mixture. Such direct acting mutagenic compounds can include nitro-substituted and oxygenated PAH compounds, some of which are co-pollutants and/or degradation products formed during the aerobic metabolism of PAH at contaminated sites [52; 56; 97].

Several studies have employed the Ames test to evaluate the outcome of bioremediation projects on PAH contaminated soils [49; 50; 53; 56; 98; 99]. Donnelly *et al* [100] showed that the mutagenic activity of the total organic extract of model soils prepared with known quantities of BaP, pentachlorophenol and 2,4,6-trinitrotoluene, displayed higher mutagenic potential in *Salmonella* TA98 at day 540 compared to day 0 of treatment, even though the majority of the parent compounds had been removed. Lundstedt *et al* [101] developed a convenient method to fractionate organic extracts of PAH-contaminated soils, and the method is readily amenable to bio-assay directed fractionations that track mutagenic activity through selected chemical fractions. The Lundstedt *et al* method separated organic soils extracts into three distinct fractions, containing (1) alkanes, (2) non-polar neutral (e.g., PAHs and alkyl PAHs), and (3) polar aromatic compounds (e.g., N- and O heterocyclics), respectively. The fractionation protocol permits an assessment of temporal changes in these distinct chemical fractions during the course of a

remediation, and moreover, if the mutagenic activity of priority PAHs is known, the observed activity at any time point can be compared with that expected from the known PAHs. Using *Salmonella* strains CBT409, YG1042, TA98, YG1041 CFT509 and CFA509, Lynes [53] showed that the mutagenic activity of the non-polar neutral and polar aromatic fractions of a soil treated using a pilot scale bioslurry fluctuated over a 29 day treatment. The results showed an increase in the mutagenic activity of the non-polar neutral fraction without metabolic enhancement on all strains except CBT409 and YG1042, and in all strains except YG1042 with metabolic activation, over the course of treatment. Mutagenicity in the polar aromatic fraction with metabolic activation increased on strains YG1042, YG1041, CFT509 and CFA509 by day 29. The direct acting mutagenicity increased on strains TA98 and YG1041.

1.4 HYPOTHESIS AND OBJECTIVES

Assessment of bioremediation efficacy generally involves examination of the temporal trends in priority contaminant concentrations. However, there is increasing evidence that declines in priority contaminant concentrations may correspond to increases in toxicological hazard. The soil quality guidelines established by the Canadian Council of the Ministers of the Environment (CCME) currently form the basis for the target PAH levels that define successful remediation. Currently, each contaminated property has site specific remediation criteria that incorporate factors such as future use and type of contamination. Since mixtures of PAH metabolites, which are not regulated under the CCME guidelines have been shown to be genotoxic and are present at PAH contaminated sites, only complete

mineralization of the contaminants will yield the ultimate goal of hazard removal. The introduction of known PAH degrading bacteria into an environment maintained under optimal conditions (i.e., temperature, nutrients, etc) should be well suited to hazard removal.

This thesis tracked temporal changes in the mutagenic activity and PAH contamination of a coal-tar contaminated soil over the course of 3 *ex situ* bioslurry treatments. The concentration of priority PAHs and level of mutagenic activity were assessed at 15 day intervals during each treatment. I hypothesize that during the course of each treatment period priority PAH concentrations will decline, and that augmentation of the bioslurry with nutrients and/or microbes will enhance PAH removal. I also hypothesize that the mutagenic activity of chemical fractions (e.g., polar aromatics) will initially increase as aerobic metabolites are formed, then decrease as contaminant mineralization is realized. The thesis has the following objectives:

1. To simultaneously assess and compare the mutagenic potency and priority PAH concentration of an aged gasworks soil during three different bioremediation treatments;
2. To evaluate the mutagenic activities of two distinct chemical fractions of organic extracts collected over the course of the three different bioremediation treatments.

2.0 METHODS

2.1 SOIL PREPERATION

2.1.1 SOIL COLLECTION

Samples of contaminated soil were obtained from a site in British Columbia (Rock Bay, Victoria Harbour) that had been used for a coal gasification plant, leather tannery, sawmill, propane tank farm, concrete batch plant and asphalt plant since its initial development in 1870. Soil was taken from selected areas where black veins of coal tar were visible to the naked eye. The soil was double bagged inside a sealed plastic bucket to prevent vapour release, and stored at 4 °C until needed.

2.1.2 SOIL PREPARATION FOR BIOREACTOR TREATMENT

Soil was removed from the storage container and manually homogenized using stainless steel tools in a fume hood. Large debris (> ~1 cm diameter) was removed, and the material was introduced into BioFlo 110 bioreactors (see below) to give a final slurry concentration of 20 % soil in water (weight/weight). In total, 1 kg of soil was added to 5 L of sterile water.

2.2 BIOREACTOR OPERATION

2.2.1 PREPARATION

The two BioFlo 110 benchtop bioreactors, powercontrol units and probes used during the experiments were obtained from New Brunswick Scientific, Edison, NJ and prepared in the following manner. The reactors were disassembled and washed in a Lancer automatic dishwasher (Model# 1B014066) using a strong

detergent and acidic rinsing agent (25% acetic acid). All internal components of the Bioreactor apparatus were then rinsed with hexane and acetone, allowed to dry under a fume hood, and then rinsed thoroughly with sterile Milli-Q® laboratory water (Millipore Corp. Bedford, MA). The bioreactors were reassembled and 5 L of sterile Milli-Q water added. All seals were coated with silicone vacuum grease prior to assembly. Sterile PVC tubing was prepared and connected to all addition ports using no-leak quick connectors. The pH and dissolved oxygen probes were inserted into the appropriate ports and the entire assembly sterilized at 121°C for 30 minutes in a Steris SV-120 Scientific Prevacuum sterilizer. The sterile bioreactor was connected to the controlling unit, and allowed to equilibrate to operating conditions. Air flow provided by a Gas and Cryo Equipment Zero Air Generator (model# ZAG-150-50) was set at 10 L/min, and directed into the slurry through an aspirator located at the bottom of the vessel. Bioreactor exhaust was first passed through a cold water condenser and a 0.2 µm filter, and subsequently directed into a container of activated charcoal. The internal impellers, set 7.5 cm apart on the agitation motor's shaft, were rotated at 400 RPM for the duration of each treatment. The reactor vessel temperature was set at 25 °C, and maintained via the combination of an external electric heating blanket and an internal cold water cooling coil. The temperature probe was coated with glycerol before insertion into the appropriate port. After the temperature had stabilized, the pH probe was connected and calibrated using an external pH meter. Reactor pH was adjusted using 0.5 M HCl or 0.5 M NaOH. The dissolved oxygen probe (Mettler Toledo InPro 6800 series O₂ sensor) was then connected and calibrated at pH 7 and 25 °C.

Saturation (100 % dissolved oxygen) was set after 24 hours of operation under these conditions. The span value (0 % dissolved oxygen) was set after disconnecting the probe from the controller system and obtaining a steady input value on the calibration screen. The level controller probe was then inserted and set to the “wet off” mode.

All input tubes were primed until drips were observed entering the vessel. Input solutions included sterile solutions of 0.5 M HCl and 0.5 M NaOH, as well as sterile Milli-Q water. The prepared soil was then introduced into the vessel via an access port and the level controller was adjusted to the top of the slurry. The vessel was then covered with 4 layers of black landscaping fabric, each layer preventing 98 % of light penetration.

A software control program was created prior to the initial treatment period and used for each subsequent experiment to control the physical parameters and operation of the two bioreactors. Temperature was set to 25.0 °C, pH was maintained between 6.5 and 8.0, and the agitation rate was set at 400 rpm. The software was also programmed to record temperature, agitation rate, dissolved oxygen, pH, and pump operation every 6 minutes over the course of each 90 day treatment.

2.2.2 OPERATION

2.2.2.1 BIOREACTOR TREATMENTS

All treatments lasted 90 days, with sample removal on days 0, 15, 30, 45, 60, 75 and 90. The following three treatments were conducted.

Treatment A: No additions of nutrient or exogenous microbes.

Treatment B: Nutrient addition. An external source of nutrients and micro-nutrients was added to the slurry every 30 days.

Treatment C: Addition of nutrients and exogenous microbial culture. A bacterial culture with known hydrocarbon degrading capacity was added every 30 days along with the reagents noted for treatment #2.

Reagents from a Medina contaminated soil clean up kit (Medina Agricultural Products Co. Inc., Hondo, TX), including Bio-D, a microbial activator, and Hydrocarbon degrading microbial seed were used for treatments B and C. Bio-D, which is a proprietary mixture of sugars, humic acid and other organic material, provides macronutrients that balance the C-N-P ratio at approximately 100-10-1. The microbial activator is an algae-based fermentation extract that contains essential micronutrients in a confidential formulation. The hydrocarbon degrading microbial seed is a dehydrated culture of microbes specifically formulated for the treatment of oil field and refinery wastes. Specific composition of the exogenous culture, including number of species present and the concentration of organisms in the mixture was classified and not provided by Medina.

At the beginning of treatments B and C, and every 30 days thereafter, 3.1 mL of the Bio-D solution and 1.55 mL of the microbial activator solution were added.

During treatment C, 0.75 g of microbial seed was added concurrently to the slurry. The microbial seed was soaked in 20 mL of milli-Q water for 15 minutes before introduction into the slurry.

2.2.2.4 SAMPLE COLLECTION

Samples were collected every 15 days for the duration of each treatment. At each collection point 4 x 25 mL samples were collected in sterile glass vials, labeled, and stored at 4 °C in the dark until needed. One sample was immediately transferred under a laminar flow hood to a sterile 50 mL conical plastic tube. 7.5 mL of 50 % glycerol was added to the tube, and the sample was thoroughly mixed before storing at -80 °C for future genetic analyses.

2.3 EXTRACTION AND FRACTIONATION

2.3.1 CHEMICALS

All chemicals used in the extraction and fractionation protocols were obtained from Sigma Aldrich (Canada) and were ACS grade (i.e., ≥99 %) unless otherwise specified.

2.3.2 SLURRY SEPARATION

Samples were removed from storage and allowed to reach room temperature before re-homogenization via vigorous shaking. The soil and water phases of the slurry were then separated by filtration using a vacuum flask, and the solids isolated

on a weighed 0.45 μm glass fiber filter. The filters had been previously cleaned by baking at 500 $^{\circ}\text{C}$ for 6 hours in a Lindberg/Blue muffle furnace. The filters were then transferred to a cleaned and weighed ASE 300 extraction cell, and allowed to dry in a fume hood for 48 hours prior to extraction. Samples destined for HPLC analysis were spiked with 100 μL of 9-phenylanthracene (1 mg/mL) immediately after being placed in the ASE cell. The ASE 300 extraction cell, including the dried soil on the filter, was re-weighed before extraction, and the weight of the dried soil determined. The liquid phase of the samples to be used for HPLC analysis were spiked with 100 μL of 0.01 mg/mL 9-phenylanthracene (9-PA) before extraction.

2.3.3 EXTRACTION PROTOCOL

2.3.3.1 SOIL EXTRACTION

Soil samples were extracted using an ASE 300 Accelerated Solvent Extractor (Dionex Corp., Sunnyvale, CA, USA). Extraction cells containing dried filter samples from days 0, 15, 30, 45, 60, 75 and 90 from each reactor, for each treatment, were filled with a 1:1 mixture of cleaned sand (extracted using the ASE cell cleaning protocol) and either 2 % deactivated sodium sulfate (HPLC samples) or activated sodium sulfate (Ames test samples) then capped with a 0.45 mm filter, and closed. Slightly deactivated sodium sulfate was used to limit the elution of large, polar compounds during HPLC sample extraction, providing a cleaner sample for priority PAH analysis (Personal communication: Staffan Lundstedt, 2007). All extractions occurred at 150 $^{\circ}\text{C}$ and 14 MPa. Samples for *Salmonella* mutagenicity testing were extracted using a mixture of 1:1 (v/v) hexane and acetone, whereas samples for

HPLC analyses were extracted using an 80:20 (v/v) mixture of hexane and dichloromethane. A 7 minute dynamic extraction phase was followed by two 5 minute static extraction cycles for both types of samples. Samples to be used for *Salmonella* mutagenicity testing were combined with the corresponding liquid phase extraction and evaporated under nitrogen to ~1 mL. HPLC samples were evaporated under nitrogen, and reconstituted in 1 mL of acetonitrile.

2.3.3.2 WATER EXTRACTION

Three consecutive 20 mL dichloromethane extractions were performed on the liquid fraction using a separatory funnel. Water phase HPLC samples were evaporated separately under gentle stream of nitrogen, reconstituted in 1 mL of acetonitrile, and stored at 4 °C until use.

2.3.4 FRACTIONATION PROTOCOL

Fractionation of the extracts was performed in 15 mm diameter open glass columns containing 2 g of anhydrous sodium sulfate overlying 10 g of 10 % deactivated (by weight) silica gel. The column was rinsed with 40 mL of dichloromethane and 40 mL of hexane prior to sample application. Samples were evaporated onto 0.5 g of 10 % deactivated silica and added on top of the sodium sulfate layer. Samples were then eluted from the columns in three separate fractions using 20 mL hexane, followed by 26 mL of a 3:1 (v/v) mixture of hexane and dichloromethane, and finally 60 mL of dichloromethane. Fraction 1, which contained aliphatic compounds, was discarded. Fraction 2 contained PAHs, alkyl-PAHs as well as O- and S-heterocyclics. Fraction 3 contained polar aromatics such

as oxy-PAHs, nitro-PAHs and N-heterocyclics. All fractions were reconstituted in 1 mL DMSO, and stored at 4 °C in the dark until analysis. This method, which has been extensively validated, is described in detail by Lundstedt *et al* (2003) [7].

2.4 *SALMONELLA* REVERSE MUTAGENICITY ASSAY

2.4.1 REAGENTS, SOLUTIONS AND MEDIA

The assay protocols employed for mutagenicity testing were provided by the Environmental Carcinogenesis Division of the USEPA (Research Triangle Park, North Carolina). These methods were adapted from Maron and Ames (1983) [102], and Mortelmans and Zeiger (2000) [18]. Table 2.0 provides a summary of all media, solutions and reagent sources used.

Media/ Solution	Components	Reagents	Concentration	Source
Minimal Glucose Plates	30 % Dextrose (v/w)	D-Glucose	1.7 M	Sigma-Aldrich Canada Ltd.
		H ₂ O (1 L)	-	-
	50x Volger- Bonner Medium E (v/v)	Magnesium Sulfate	0.04 M	Sigma-Aldrich Canada Ltd.
		Citric Acid monohydrate	0.6 M	Sigma-Aldrich Canada Ltd.
		Potassium phosphate dibasic anhydrous	2.9 M	Sigma-Aldrich Canada Ltd.
		Sodium ammonium phosphate	0.8 M	Sigma-Aldrich Canada Ltd.
		H ₂ O (1 L)	-	-
	Histidine/Biotin Solution	L-Histidine-HCl	0.4 M	Sigma-Aldrich Canada Ltd.
		d-biotin	0.02 mM	Sigma-Aldrich Canada Ltd.
		H ₂ O (1 L)	-	-
Top Agar	Agar	Difco Agar	0.6 % w/v	Fisher Scientific
	Sodium Chloride	Sodium Chloride	86 mM	Sigma-Aldrich Canada Ltd.
		H ₂ O (1 L)	-	-
S9 Mix	0.2 M Phosphate Buffer pH 7.4 (w/v)	Sodium dihydrogen phosphate	7 mM	Sigma-Aldrich Canada Ltd.
		Disodium hydrogen phosphate	44 mM	Sigma-Aldrich Canada Ltd.
		H ₂ O (0.5 L)	-	-
	G6P	D-glucose-6-phosphate (5 mM)	5 mM	Sigma-Aldrich Canada Ltd.
	NADP	Nicotinamide adenine dinucleotide phosphate (4 mM)	4 mM	Roche Diagnostics, Laval, Que.
	Microsomal Salt Solution	Magnesium chloride (0.4 M)	0.4 M	Sigma-Aldrich Canada Ltd.
		Potassium chloride (1.65 M)	1.65 M	Sigma-Aldrich Canada Ltd.
		H ₂ O (0.5 L)	-	-
	Hepatic S9 Homogenate	Aroclor 1254 induced male rat liver (5 % v/v)	5 % v/v	Moltox Inc., Boone, NC.

Note: All water was obtained from a Milli-Q® Ultrapure Water Purification System (Fisher Scientific Ltd.)

Minimal Glucose Plates

An IBS Integra Biosciences MediaClave (Chur, Switzerland) and a Technomat automatic plate dispenser (Fernwald, Germany) were used to prepare and pour all minimal glucose agar plates. Every media batch contained 90 g of agar, 5310 mL of water, 400 mL of 30 % dextrose, 120 mL of 50x Volger-Bonner Medium E (VBME) salts and 60 mL of histidine/biotin solution (Table 2.0). The agar and water were sterilized at 121 °C for 20 minutes prior to the addition of the other reagents. The dextrose and VBME solutions were sterilized independently at 121 °C for 20 minutes, and the histidine/biotin solution was filter sterilized using a 0.2 µm Nalgene 50 mm bottle top filter before addition.

S9 Metabolic Activation Mixture

Each 100 mL of S9 mixture contained 2 mL of microsomal salt solution, 141 mg of glucose-6-phosphate, 306 mg of nicotinamide adenine dinucleotide phosphate disodium salt, 50 mL of 0.2 M phosphate buffer (pH 7.4) and 43 mL of sterile water (Table 2.0). These reagents were combined and 38 mL aliquots were stored at -20 °C until required. Once thawed to room temperature 2 mL of Aroclor 1254-induced, male rat liver S9 homogenate, with protein levels ranging between 39.3 to 40.2 mg/mL, was added to provide a final volume of 40mL and an S9 concentration of 5% v/v.

2.4.2 *SALMONELLA TYPHIMURIUM* STRAINS

Two strains of *Salmonella typhimurium*, TA98 and YG1041, were used to detect mutagenic activity in the soil slurry extracts/fractions. Both strains contain

the *hisD3052* allele, which permits the detection of frameshift mutations. The target DNA sequence is CGCGCGCG for both TA98 and YG1041. YG1041 contains the multi-copy plasmid pYG233, which harbors the *Oat* and *cnr* genes which encode O-acetyltransferase and the *Salmonella* classical nitroreductase enzymes, respectively. These enzymes collectively increase the ability of YG1041 to detect mutations induced by nitroarenes and aromatic amines [18].

Strain checks were performed prior to testing in order to evaluate the presence and functionality of the desired genetic characteristics. Plasmid presence was determined by colony growth on nutrient agar plates in the presence of antibiotics; 25 µg/mL of either ampicillin or kanamycin was used to test for the presence of the pKM101 and pYG233 plasmids, respectively. The *uvrB* gene deletion was confirmed after exposing half of a nutrient agar plate to UV light (30 W) at a distance of 30 cm for 8 seconds, and observing growth inhibition after 24 hours. The deep rough phenotype (*rfa*) was confirmed via growth inhibition by crystal violet. Histidine dependence was confirmed after observing a lack of growth following on minimal nutrient plates supplemented with 0.1 mg/mL biotin, Biotin dependence was confirmed by a lack of growth on minimal nutrient plates supplemented with 0.1 mg/mL of histidine.

Frozen permanent cultures were prepared from strains successfully passing all the aforementioned strain validation tests. 850 µl of overnight culture in 15 % glycerol were stored in 1.5 mL Eppendorf tubes at -80 °C until required. Subsequent overnight cultures were prepared by adding 100 µL of a frozen permanent culture

to 20 mL of Oxoid nutrient broth, and incubating for 16 hours with agitation (200 rpm). Original TA98 cultures were purchased from Molttox Inc. (Boone, NC.). YG1041 cultures were provided by Dr. Takehiko Nohmi (National Institute of Health Sciences, Tokyo, Japan).

2.4.3 *SALMONELLA* REVERSE MUTATION ASSAY PROTOCOL

Experiments were performed inside a laminar flow hood (Model #BM6-2B-49, Microzone Corporation, Nepean, Canada). Fresh overnight cultures were inoculated the day before an experiment. After 16 hours of growth in a dry shaker (Max^Q mini model# 4450), cultures were stored at 4 °C until needed, and then transferred to 50 mL polypropylene centrifuge tubes on ice during the assay. Top agar tubes were melted in an autoclave (Scientific Prevacuum Sterilizer, Model # SV-120, Steris Amsco, Century, OH, USA) and stored in a dry block heater (Dri-Block, Model # DB-3, Techne Ltd., Duxford, Cambridge, England) at ~52 °C. Dimethylsulfoxide (DMSO) was used to dilute all samples and controls. DMSO volume in each soft agar tube was < 5 % by volume, and no more than 100 µL of DMSO was added to any one tube. All samples were tested in triplicate in both strains, at 5 doses, both in the presence and absence of S9 metabolic activation. Samples requiring metabolic activation were supplemented with 500 µL of 5 % v/v S9 mix, prepared the morning of the experiment. 500 µL of 0.2 M phosphate buffer (pH 7.4) was added to all other samples. Slurry extracts/fractions were added to the top agar tubes in the heater block. The S9 mix or phosphate buffer, along with 100 µL of tester strain, were added to the tubes after they were removed from the block.

Once all components were added, the tubes were vortexed and spread evenly over a minimal glucose plate. The soft agar was allowed to harden on a flat surface and the plates inverted and incubated for 72 hours at 37 °C. Colonies from each plate were scored using an automated colony counter (Protocol RGB, Model # 9000, Synoptics Ltd. UK). Plates were counted in duplicate, with the second count being carried out after rotating the plates by 90°.

The positive control for samples supplemented with S9 was 2-aminoanthracene, whereas 2-nitrofluorene was used without S9 addition. Positive controls were obtained from Moltox Inc. (Boone, NC). 100 µL DMSO was employed as the negative control.

2.4.4 MUTAGENICITY TESTING OF SOIL FRACTIONS

The Ames test protocol, described in section 2.4.3, was employed on both the non-polar neutral (i.e., fraction #2) and the polar aromatic (i.e., fraction #3) fractions of extracts from slurry samples taken on days 0, 15, 30, 45, 60, 75 and 90 for all treatment periods from both reactors. Initial experiments on untreated soil were used to identify TA98 and YG1041 as suitable strains for determining the mutagenic potency of both fractions. Subsequent range finding experiments determined four concentration levels, 2.5, 5, 10 and 15 mg dry wt/plate as appropriate. Responses were considered either positive (mutagenic), marginal (marginally mutagenic) or negative (non-mutagenic). A result was considered positive if a concentration-dependant increase in the number of revertants was observed and two consecutive responses were at least two-fold greater than the

number of background revertants. Marginal responses displayed a concentration-dependant increase in the number of observed revertants, but did not achieve a two-fold increase over background. For each mutagenic and marginally mutagenic response, plots of revertant colony counts versus soil concentration for each sample were plotted in Microsoft Excel. The slopes of the linear portion of these concentration-response functions were termed the mutagenic potency and expressed in revertants per mg soil eq. The detection limit of the assay, for each strain/fraction/metabolic activation combination, was determined as half of the lowest mutagenic potency ever observed at the highest concentration tested, considering all samples tested. Assay detection limits are reported in Table 2.1.

Strain	S9	Fraction	Highest Concentration Tested (mg soil eq/plate)	Lowest Ever Observed Potency (rev/mg)	1/2 Potency (rev/mg)
TA98	Y	2	15	8.4	4.2
TA98	N	2	15	7.2	3.6
YG1041	Y	2	10	7.5	3.8
YG1041	N	2	15	3.2	1.6
TA98	Y	3	10	12.8	6.4
TA98	N	3	15	7.2	3.6
YG1041	Y	3	10	7.5	3.8
YG1041	N	3	15	3.2	1.6

2.5 HPLC ANALYSIS

2.5.1 SAMPLE PREPARATION

Samples were extracted as described in Section 2.3.3. Filters were spiked with 100 μL of 1 mg/mL 9-PA in acetonitrile immediately after being placed in the ASE. Extracts were evaporated under nitrogen and reconstituted in 1 mL of acetonitrile. Samples were diluted 100x in acetonitrile, and filtered at 0.2 μm using disposable glass fiber filters (Whatman 13mm GD/X disposable PTFE filter) into HPLC auto-sampler vials and stored at 4 °C until needed. The water phase samples were spiked with 100 μL of 0.01 mg/mL 9-PA in acetonitrile immediately after separation from the soil phase. These neat samples were filtered and stored in the same manner as the soil phase samples.

Fresh standards were prepared prior to each experiment. A 20 % wt/wt mixture of clean sand and Milli-Q® water, extracted and prepared in an identical manner to that employed for the contaminated slurry samples, was used as a blank. A 16 PAH calibration mix (Sigma, Cat # 4-7940-U), containing 10 $\mu\text{g/mL}$ of each compound listed in table 2.2 in acetonitrile, was diluted to give solutions of 20, 100, 500 and 1000 ng PAH/mL. Only 15 compounds are listed in table 2.2 as acenaphthylene was not detectable using this method. These solutions, along with the blank, were used to construct a standard concentration-detector response curve. The slope and intercept values obtained from each of these curves were used to determine the concentration of individual PAHs in the samples tested.

2.5.2 HPLC PROTOCOL

An autosampler was used to inject 10 μL of each sample into a Waters 2695 separation module. Sample flow rate was controlled by a Waters 515 HPLC pump at 1.5 mL/min into a Waters PAH C18 column (5 μm particle size, 46 mm inner diameter, 250 mm length, Lot# 33161, Waters Corp, Mississauga, ON). Column temperature was maintained at 30 $^{\circ}\text{C}$ (± 5 $^{\circ}\text{C}$) and pressure maintained under 4000 psi. A solvent gradient of 1:1 - acetonitrile:water was used for the first 5 minutes. The ratio of acetonitrile was constantly increased between 5-16 minutes until 100% acetonitrile was achieved. This level was used for the duration of each 35 minute run. Samples were detected using a dual channel Waters 2475 Multi λ Fluorescence Detector. Table 2.2 provides a summary of the excitation and emission wavelengths used for each PAH, as well as their corresponding retention times and detection limits. The detection limit was determined according to the following formula:

$$PAH_{DL} = \left\{ \left[\frac{(HPLC_{DL} - \text{Int of PAH Standard Curve})}{\text{Slope of PAH Standard Curve}} \right] * V_{inj} \right\} \\ * \left\{ \frac{\text{Amount}_{PAH}}{1000 / \text{Sample Wt.}} \right\}$$

Where:

PAH_{DL} = Detection Limit of PAH

$HPLC_{DL}$ – Detection Limit of HPLC

V_{inj} = Volume of Injection (10 μ L)

Amount PAH = Amount of PAH in whole sample (μ g)

Sample Wt. = Mass of Sample (g)

The corresponding HPLC detection limit value was substituted for subsequent analysis in all instances where a particular PAH was not detected.

Waters Empower Pro, Empower 2.0 software (service pack A) was used to control all HPLC operations and subsequent analyses.

Elution Order	PAH	λ (nm)		Retention Time (min)	Limit of Detection (ppm)
		Excitation	Emission		
1	Naphthalene	224	330	7.8	0.14
2	Acenaphthene	234	320	9.1	0.04
3	Fluorene	224	320	13.8	0.04
4	Phenanthrene	252	370	14.9	0.06
5	Anthracene	252	402	16.8	0.04
6	Fluoranthene	252	402	17.5	0.21
7	Pyrene	238	398	18.8	0.13
8	Benz[a]anthracene	238	398	22.6	1.03
9	Chrysene	238	398	23.7	0.04
10	Benzo[b]fluoranthene	268	398	25.7	0.09
11	Benzo[k]fluoranthene	268	398	28.3	0.73
12	Benzo[a]pyrene	268	398	29.6	0.11
13	Dibenzo[ah]anthracene	234	420	34.3	0.12
14	Benzo[g,h,i]perylene	234	420	36.9	0.01
15	Indeno[1,2,3-cd]pyrene	300	466	39.8	1.52

2.5.3 CHROMATOGRAM ANALYSIS

Sample chromatograms were examined prior to analysis for improper peak detection, and adjusted to correlate with peaks in standard chromatograms obtained during the same experiment. The quantity of 16 PAHs was then determined in each sample according to the following formula:

$$\text{Amount PAH} = \frac{\left[\left\{ \left(\frac{\text{Area PAH}}{\text{Area 9-PA}} \right) \times \text{Amount 9-PA} \right\} - \text{Std. Curve Intercept} \right]}{\text{Std. Curve Slope}} \times \text{Weight of Soil}$$

Where:

Amount PAH = Amount in mg of a particular PAH in the soil sample tested.

Area PAH= Area obtained from the HPLC chromatogram for a particular PAH.

Area 9-PA= Area obtained from the HPLC chromatogram for 9-PA.

Amount 9-PA= The amount of 9-PA spiked into the sample (100 µg).

Std. Curve Intercept= Intercept of the standard curve generated for a particular PAH.

Std. Curve Slope= Slope of the standard curve generated for a particular PAH.

Weight of Soil= Dry weight of soil sample (g)

Initial HPLC testing on a reference contaminated soil sample (Lot# LGC6140, Batch 001, Sample 0027, Laboratory of the Government Chemist, Middlesex, UK) provided a range of values for each detected PAH. As noted, acenaphthylene was not

detectable using this method. The standard deviation of this range, which represents the technical error of the method (Table 2.3) was used to express subsequent individual PAH concentration results.

PAH	Certified Standard Assessed Values (ppm)	Observed Average Evaluated Concentration (ppm)	Standard Deviation ¹ (ppm)
Naphthalene	3.9	5.71	1.67
Acenaphthene	NR	1.72	0.49
Fluorene	5.3	3.65	0.27
Phenanthrene	123.0	139.72	9.94
Anthracene	10.7	5.97	2.31
Fluoranthene	89.0	93.33	7.02
Pyrene	93.0	96.76	7.00
Benzo(a)anthracene	30.0	20.22	1.82
Chrysene	36.0	36.27	2.94
Benzo(b)fluoranthene	22.0	20.37	2.05
Benzo(k)fluoranthene	20.0	11.64	1.07
Benzo(a)pyrene	7.1	11.09	1.27
Dibenzo(a,h)anthracene	NR	2.03	0.19
Benzo(g,h,i)perylene	17.1	14.40	2.29
Indeno(1,2,3-cd)pyrene	16.8	12.80	1.50

NR – Not reported

¹ – Used as the technical error for individual PAH concentration analysis

2.6 STATISTICAL ANALYSIS

Statistical analysis was performed using SAS version 9.1.3 for Windows XP (SAS Institute Inc., Cary, NC). ANOVA models were conducted to determine if any of the following experimental variables: bioremediation treatment, *Salmonella* strain, chemical fraction, individual reactor, day of treatment, presence of metabolic activation, or if the measured concentrations of individual, LMW, HMW or total PAHs had a significant effect on the observed mutagenic responses. The possibility of significant effects resulting from combinations of these variables was also examined. In addition ANOVA models were performed to examine if bioremediation treatment, individual reactor or day of treatment, as well as combinations of these variables, had a significant effect on the concentrations of LMW, HMW or total PAHs observed.

3.0 RESULTS

3.1 *SALMONELLA* REVERSE MUTATION TESTING OF BIOREMEDIATED SOIL

3.1.1 POSITIVE AND NEGATIVE CONTROLS

Positive and negative controls were used during all *Salmonella* Reverse Mutation tests to ensure assay performance. The average number of revertants obtained from all positive control tests for each *Salmonella* strain is reported in Table 3.0. The mean spontaneous reversion frequency obtained for the negative control, DMSO, from each strain is shown in Table 3.1.

Strain	Metabolic Activation	Positive Control	Concentration (µg/plate)	N ^a	Mean Revertants/Plate ± SEM ^b
TA98	+	2-aminoanthracene	1.0	33	662.1 ± 15.3
	-	2-nitrofluorene	1.0	33	123.2 ± 3.6
YG1041	+	2-aminoanthracene	1.0	33	630.5 ± 34.8
	-	2-nitrofluorene	1.0	33	157.3 ± 7.4

^a – Number of independent assays.

^b – Standard error of the mean

Strain	Metabolic Activation	Negative Control	Concentration (μL/plate)	N ^a	Mean Revertants/Plate ± SEM ^b
TA98	+	DMSO	100	33	69.5 ± 2.0
	-	DMSO	100	33	53.4 ± 2.2
YG1041	+	DMSO	100	33	38.4 ± 1.9
	-	DMSO	100	33	24.8 ± 1.2

^a – Number of independent assays.

^b – Standard error of the mean

3.1.2 MUTAGENICITY TESTING OF FRACTIONATED SOIL EXTRACTS

Slurry samples were prepared and tested as described in sections 2.3 and 2.4. *Salmonella typhimurium* strains TA98 and YG1041 were used to detect frameshift mutations, and thus quantify the mutagenic potency of soil slurry samples taken at 7 time points (Day 0, 15, 30, 45, 60, 75, 90) for the three different bioreactor treatments. Treatment A, the control treatment, examined the basal rate of PAH biodegradation without external additions. In treatment B, the native microbial consortia was supplemented with a growth enhancing solution containing essential nutrients. Treatment C involved augmentation of the slurry with an exogenous culture of hydrocarbon degrading bacteria in addition to the aforementioned nutritional supplementation. Slurry extracts were fractionated prior to mutagenicity testing in order to isolate particular chemical classes (i.e., aliphatics, non-polar aromatics and polar aromatics). The aliphatic fraction was discarded, whereas the fractions containing non-polar aromatics (fraction 2) and polar aromatics (fraction 3) were tested for mutagenicity. Mutagenic potency was assessed using the *Salmonella* reverse mutagenicity assay, and included testing both with and without exogenous metabolic activation. The mutagenic potency of a sample was classified as either positive “+”, marginal “M”, or negative “-”. A sample was classified as Positive if two consecutive test concentrations yielded a response with at least twice as many revertants as the negative control, and a linear dose-related increase was observed. A sample was classified as Marginal if a linear dose related increase was observed, but two consecutive test concentrations failed to

yield a two-fold increase in the number of revertants. A sample was classified as Negative if none of the tested concentrations yielded a two-fold increase in reversion frequency above the negative control. A summary of the total number of positive, marginal and negative responses observed from each assay performed throughout the entire experiment, with and without metabolic activation, is provided for each strain/fraction combination in Tables 3.2 and 3.3, respectively. All mutagenic potency values obtained with metabolic activation were at least marginally mutagenic. The non-polar neutral fraction (fraction 2), which contains priority PAHs and alkyl-PAHs, had the highest proportion of positive responses, with only 1 and 3 marginal responses obtained for TA98 and YG1041, respectively. When considering fraction 3 samples, which contains polar and semi-polar PACs, a higher proportion of positive responses were obtained using YG1041 when compared to TA98. In the absence of metabolic activation, 55% of tested samples were not mutagenic. In general, more positive responses were obtained for YG1041 as compared to TA98, and from fraction 3 as compared to fraction 2.

Mutagenic Response	Number of Observations				
	Strain				Total
	TA98		YG1041		
	Fraction		Fraction		
	2	3	2	3	
Positive	39	21	41	34	135
Marginal	3	21	1	8	33
Negative	0	0	0	0	0
Total	42	42	42	42	168

Table 3.3: Summary of *Salmonella* mutagenicity results for fractions 2 and 3 without metabolic activation from all treatments.

3.1.2.1 MUTAGENICITY TESTING WITHOUT METABOLIC ACTIVATION

Generally, samples tested without the addition of exogenous metabolic enzymes were not mutagenic. A summary of results is presented in Tables 3.4-3.5. The majority of non-polar neutral fraction (i.e., fraction 2) samples, which contain priority PAHs compounds, were mutagenic when tested without metabolic activation (Table 3.4). Comparing general results across treatments reveals that Treatment B produced more positive and marginally mutagenic responses than either treatment A or C. All samples, regardless of treatment method, were not mutagenic on day 0, and positive responses were not observed until day 45 in either *Salmonella* strain. A greater number of positive responses were detected on YG1041 compared to TA98. On the last day of treatment, day 90, samples were either marginally or positively mutagenic in YG1041, although no day 90 samples tested positive in TA98. The somewhat cyclical pattern of mutagenicity observed over the duration of each treatment was similar, though never identical, between the two reactors.

A higher proportion of polar aromatic samples (i.e., fraction 3), containing oxygenated and N-containing PACs, some of which are direct acting mutagens, were positive or marginally mutagenic compared to the non-polar neutral fraction samples (Tables 3.4 and 3.5). Similar to the non-polar neutral fraction samples, a greater number of positively mutagenic responses were detected with YG1041 as compared to TA98. Treatments B and C had a greater number of positive mutagenic responses than treatment A, especially on YG1041. Again the varying degrees of

mutagenicity observed over the course of each treatment were similar between reactors but were not identical.

Strain	Day	Bioreactor Treatment					
		A		B		C	
		R1	R2	R1	R2	R1	R2
TA98	0	-	-	-	-	-	-
	15	-	-	-	-	-	-
	30	-	-	-	-	-	M
	45	M	M	M	+	-	-
	60	-	-	M	+	M	-
	75	-	-	M	-	-	+
	90	M	M	-	M	-	M
YG1041	0	-	-	-	-	-	-
	15	M	M	-	-	-	-
	30	-	-	-	M	-	-
	45	-	+	+	+	-	-
	60	-	-	+	+	-	-
	75	-	-	+	+	-	+
	90	+	M	+	+	+	M

A : Control treatment

B : Nutrient addition

C : Nutrient + exogenous microbial culture addition

R1 : Bioreactor 1

R2 : Bioreactor 2

+: Positive mutagenic response

M : Marginal mutagenic response

- : Not mutagenic

Strain	Day	Bioreactor Treatment					
		A		B		C	
		R1	R2	R1	R2	R1	R2
TA98	0	-	-	-	-	-	-
	15	M	M	M	-	M	+
	30	M	M	-	-	M	+
	45	M	M	+	-	M	-
	60	-	-	+	+	M	-
	75	M	M	+	+	M	-
	90	-	M	+	+	M	+
YG1041	0	-	-	-	-	-	-
	15	M	+	+	-	+	+
	30	-	-	-	-	+	+
	45	-	+	+	-	+	-
	60	-	-	+	+	+	-
	75	-	-	+	+	-	-
	90	-	+	-	M	+	+

A : Control treatment

B : Nutrient addition

C : Nutrient + exogenous microbial culture addition

R1 : Bioreactor 1

R2 : Bioreactor 2

+: Positive mutagenic response

M : Marginal mutagenic response

- : Not mutagenic

3.1.2.2 MUTAGENICITY TESTING WITH METABOLIC ACTIVATION

The mutagenic activities of samples tested with metabolic activation were generally higher than corresponding samples tested without metabolic augmentation. *Salmonella* strains TA98 and YG1041 were used to assess the mutagenic activity of the two chemical fractions. A summary of the mutagenic potencies of samples taken from each treatment is presented in Tables 3.6-3.8 for the non-polar neutral fraction, and in Tables 3.9-3.11 for the polar aromatic samples.

Positive mutagenic responses were detected in both strains of *Salmonella*, at all time points, for each treatment (Tables 3.4-3.6). There was no significant difference observed between corresponding YG1041 and the TA98 potency values obtained from these samples. Potency values typically followed a cyclical pattern over the time course of each treatment in comparison to untreated soil (Day 0). Mutagenic potency was observed to increase between days 0-15 in the majority of treatments, with the lone exception occurring in reactor 1 during treatment C (i.e., microbe and nutrient addition). In reactor 1 the lowest mutagenicity response occurred on day 30 of treatment B for TA98 and YG1041, while the highest responses were observed on day 90 of the control treatment in both strains (Fig. 3.0-3.1). The lowest responses detected in reactor 2 were on day 75 of the control treatment on TA98, and day 30 of the control treatment on YG1041. Generally, mutagenicity initially increased by day 15 and then dropped slightly by day 30.

Potency generally peaked over days 45-60 for treatment B, but stayed relatively low during treatments A and C until peaking on day 90.

R1 and R2 refer to BioFlo 110 benchtop bioreactors 1 and 2 respectively. Both bioreactors were treated identically and contained contaminated soil from the same source. A, B and C refer to the mutagenic potency obtained from each respective treatment method. Untreated refers to the mutagenic potency of Day 0 samples.

R1 and R2 refer to BioFlo 110 benchtop bioreactors 1 and 2 respectively. Both bioreactors were treated identically and contained contaminated soil from the same source. A, B and C refer to the mutagenic potency obtained from each respective treatment method. Untreated refers to the mutagenic potency of Day 0 samples.

Strain	Day	Mutagenic Potency (rev/mg soil equivalent)		Standard Error		Mutation Ratio ^a		(r ²) ^b		p value ^b	
		R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
TA98	0 ^c	5.2		1.1		2.9		0.9		<0.001	
	15	6.1	5.9	0.9	1.6	2.5	2.5	0.9	0.8	0.006	0.035
	30	5.1	5.1	1.2	0.7	2.1	2.0	0.8	1.0	0.026	0.005
	45	6.3	5.4	1.4	1.0	2.2	2.2	0.9	0.9	0.019	0.011
	60	5.5	4.9	1.6	1.3	2.3	2.2	0.8	0.8	0.043	0.034
	75	3.2	4.6	0.9	2.0	1.9	2.2	0.8	0.6	0.038	0.101
	90	11.2	7.3	2.8	1.8	3.0	2.6	0.8	0.9	0.029	0.025
YG1041	0 ^c	3.3		0.8		2.9		0.9		<0.001	
	15	7.9	5.2	1.5	1.9	5.6	4.2	0.9	0.7	0.015	0.07
	30	6.0	4.5	0.7	2.0	2.8	2.6	1.0	0.6	0.003	0.111
	45	4.1	5.4	2.1	0.7	2.7	2.7	0.6	0.9	0.144	0.005
	60	4.8	5.6	1.9	2.6	3.8	3.4	0.7	0.6	0.082	0.115
	75	4.9	5.1	0.8	1.7	3.1	3.8	0.9	0.8	0.011	0.055
	90	10.3	5.5	2.7	1.4	3.6	2.7	0.8	0.8	0.031	0.033

a – Mutation ratio is defined as the ratio between the mean number of revertants observed at the highest dose used in the calculation of the mutagenic potency, and the mean number of spontaneous revertants observed for that experiment.

b - Statistical parameters refer to the mutagenic potency, which is derived from the initial linear portion of revertants vs. mg soil equivalent concentration plots.

c – Values from Day 0 samples represent the average of the results from 9 separate assays on untreated soil samples.

R1 and R2 refer to BioFlo 110 benchtop bioreactors 1 and 2 respectively. Both bioreactors were treated identically and contained contaminated soil from the same source.

Strain	Day	Mutagenic Potency (rev/mg soil equivalent)		Standard Error (rev/mg)		Mutation Ratio ^a		(r ²) ^b		p value ^b	
		R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
TA98	0 ^c	5.2		1.1		2.9		0.9		<0.001	
	15	7.2	9.2	2.3	3.5	2.8	3.2	0.8	0.7	0.051	0.078
	30	1.7	7.5	2.2	2.1	1.8	3.0	0.2	0.8	0.505	0.039
	45	7.5	11.8	2.7	2.1	2.9	3.8	0.7	0.9	0.071	0.012
	60	9.2	10.2	2.4	2.9	3.3	3.6	0.8	0.8	0.033	0.04
	75	6.2	7.2	2.2	1.8	2.7	2.8	0.7	0.8	0.063	0.028
	90	6.9	6.6	1.8	2.3	2.1	2.2	0.8	0.7	0.03	0.063
YG1041	0 ^c	3.3		0.8		2.9		0.9		<0.001	
	15	7.1	9.5	2.3	3.9	4.7	5.8	0.8	0.7	0.055	0.091
	30	1.9	6.7	0.5	2.3	1.4	2.7	0.8	0.7	0.034	0.059
	45	6.9	10.9	2.8	2.4	4.6	6.8	0.7	0.9	0.086	0.019
	60	9.8	10.7	1.8	2.9	3.7	3.7	0.9	0.8	0.012	0.034
	75	5.9	8.4	2.4	2.6	5.0	5.8	0.7	0.8	0.094	0.049
	90	4.1	9.1	1.8	1.5	3.0	5.3	0.6	0.9	0.102	0.009

a – Mutation ratio is defined as the ratio between the mean number of revertants observed at the highest dose used in the calculation of the mutagenic potency, and the mean number of spontaneous revertants observed for that experiment.

b - Statistical parameters refer to the mutagenic potency, which is derived from the initial linear portion of revertants vs. mg soil equivalent concentration plots.

c – Values from Day 0 samples represent the average of the results from 9 separate assays on untreated soil samples.

R1 and R2 refer to BioFlo 110 benchtop bioreactors 1 and 2 respectively. Both bioreactors were treated identically and contained contaminated soil from the same source.

Strain	Day	Mutagenic Potency (rev/mg soil equivalent)		Standard Error (rev/mg)		Mutation Ratio ^a		(r ²) ^b		p value ^b	
		R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
TA98	0 ^c	5.2		1.1		2.9		0.9		<0.001	
	15	4.2	8.2	1.7	1.9	2.4	3.4	0.7	0.9	0.095	0.024
	30	6.1	7.2	2.2	2.6	2.9	3.2	0.7	0.7	0.068	0.068
	45	7.7	7.4	2.0	3.3	9.2	9.3	0.8	0.6	0.032	0.11
	60	4.9	5.2	2.2	2.4	6.9	7.3	0.6	0.6	0.116	0.12
	75	4.3	9.6	0.4	1.7	2.1	3.5	1.0	0.9	0.002	0.011
	90	7.9	7.6	1.9	2.1	3.1	3.1	0.9	0.8	0.009	0.038
YG1041	0 ^c	3.3		0.8		2.9		0.9		<0.001	
	15	7.5	9.8	1.5	2.4	6.1	7.3	0.9	0.8	0.016	0.027
	30	7.4	10.5	2.3	3.0	6.1	7.5	0.8	0.8	0.049	0.042
	45	4.3	6.9	0.8	2.3	2.8	3.3	0.9	0.7	0.015	0.058
	60	3.7	6.9	1.4	1.3	2.4	3.7	0.7	0.9	0.07	0.012
	75	3.5	9.9	0.4	2.3	2.3	4.7	1.0	0.9	0.002	0.024
	90	7.5	7.9	1.3	2.5	4.0	3.8	0.9	0.8	0.002	0.049

a – Mutation ratio is defined as the ratio between the mean number of revertants observed at the highest dose used in the calculation of the mutagenic potency, and the mean number of spontaneous revertants observed for that experiment.

b - Statistical parameters refer to the mutagenic potency, which is derived from the initial linear portion of revertants vs. mg soil equivalent concentration plots.

c – Values from Day 0 samples represent the average of the results from 9 separate assays on untreated soil samples.

R1 and R2 refer to BioFlo 110 benchtop bioreactors 1 and 2 respectively. Both bioreactors were treated identically and contained contaminated soil from the same source.

The polar aromatic fraction samples were generally more potent than those obtained from the corresponding non-polar neutral fraction (compare Tables 3.6-3.8 to Tables 3.9-3.11). Positive or marginally mutagenic responses were detected in all fraction 3 samples. During treatment A the highest potency values detected on TA98 were observed in day 90 samples, while the most potent samples detected on YG1041 were at day 90 for reactor 1 and on day 45 in reactor 2. Samples from treatment A always had a higher mutagenic activity when compared to the untreated soil. The highest mutagenic potency values obtained in the entire experiment were detected in the polar aromatic fraction from treatment B samples with metabolic augmentation. On day 45 of treatment B, mutagenic potency values of 30.2 and 39.3 rev/mg soil were detected on TA98 and YG1041, respectively. A sharp drop in mutagenicity was detected between days 75–90, with values dropping from 13.2 rev/mg soil to 3.8 rev/mg soil on TA98, and from 15.5 rev/mg soil to 8.0 rev/mg soil on YG1041 (Figures 3.2-3.3). The fluctuations in mutagenicity were more apparent in samples from treatments B and C, when compared to treatment A.

R1 and R2 refer to BioFlo 110 benchtop bioreactors 1 and 2 respectively. Both bioreactors were treated identically and contained contaminated soil from the same source. A, B and C refer to the mutagenic potency obtained from each respective treatment method. Untreated refers to the mutagenic potency of Day 0 samples.

R1 and R2 refer to BioFlo 110 benchtop bioreactors 1 and 2 respectively. Both bioreactors were treated identically and contained contaminated soil from the same source. A, B and C refer to the mutagenic potency obtained from each respective treatment method. Untreated refers to the mutagenic potency of Day 0 samples.

Strain	Day	Mutagenic Potency (rev/mg soil equivalent)		Standard Error (rev/mg)		Mutation Ratio ^a		(r ²) ^b		p value ^b	
		R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
TA98	0 ^c	3.5		1.0		2.1		0.7		<0.001	
	15	5.6	5.1	0.7	0.8	2.2	2.2	1.0	0.9	0.005	0.008
	30	5.1	4.1	0.4	0.4	1.9	1.7	1.0	1.0	0.001	0.002
	45	3.9	6.1	0.6	1.2	1.8	2.3	0.9	0.9	0.008	0.016
	60	4.6	5.1	0.1	0.4	2.0	2.1	1.0	1.0	<0.001	<0.001
	75	4.2	5.5	1.3	0.2	2.0	2.2	0.8	1.0	0.045	<0.001
	90	6.3	6.7	0.9	1.1	2.2	2.1	0.9	0.9	0.007	0.008
YG1041	0 ^c	1.6		0.2		1.8		1.0		<0.001	
	15	5.7	6.0	1.7	0.9	3.8	4.4	0.8	0.9	0.044	0.008
	30	5.4	4.6	0.9	0.6	2.4	2.3	0.9	0.9	0.009	0.006
	45	4.9	6.0	0.9	0.6	2.3	2.8	0.9	1.0	0.012	0.002
	60	5.0	3.5	0.2	0.9	3.3	2.5	1.0	0.8	<0.001	0.033
	75	5.5	4.4	1.9	0.6	4.1	3.4	0.7	0.9	0.063	0.004
	90	6.4	6.4	1.7	1.2	2.6	2.7	0.8	0.9	0.034	0.013

a – Mutation ratio is defined as the ratio between the mean number of revertants observed at the highest dose used in the calculation of the mutagenic potency, and the mean number of spontaneous revertants observed for that experiment.

b - Statistical parameters refer to the mutagenic potency, which is derived from the initial linear portion of revertants vs. mg soil equivalent concentration plots.

c – Values from Day 0 samples represent the average of the results from 9 separate assays on untreated soil samples.

R1 and R2 refer to BioFlo 110 benchtop bioreactors 1 and 2 respectively. Both bioreactors were treated identically and contained contaminated soil from the same source.

Strain	Day	Mutagenic Potency (rev/mg soil equivalent)		Standard Error (rev/mg)		Mutation Ratio ^a		(r ²) ^b		p value ^b	
		R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
TA98	0 ^c	3.5		1.0		2.1		0.7		<0.001	
	15	18.3	11.3	1.0	0.9	5.2	3.5	1.0	1.0	<0.001	<0.001
	30	3.3	9.6	0.6	0.8	1.8	3.2	0.9	1.0	0.013	0.001
	45	30.2	6.7	2.6	0.7	7.6	2.4	1.0	1.0	0.001	0.003
	60	27.1	14.7	1.2	2.2	7.3	4.4	1.0	0.9	<0.001	0.007
	75	22.8	13.3	3.6	1.1	6.5	4.2	0.9	1.0	0.008	0.001
	90	6.8	3.8	0.3	0.4	2.1	1.5	1.0	1.0	<0.001	0.002
YG1041	0 ^c	1.6		0.2		1.8		1.0		<0.001	
	15	19.6	8.3	1.3	1.5	9.6	4.6	1.0	0.9	<0.001	0.011
	30	2.7	8.5	0.9	1.1	1.6	3.3	0.8	1.0	0.05	0.004
	45	39.0	6.6	2.6	1.4	18.7	3.9	1.0	0.9	<0.001	0.018
	60	31.6	15.4	1.9	1.6	9.0	4.9	1.0	1.0	<0.001	0.002
	75	32.4	15.6	3.0	1.4	19.1	9.4	1.0	1.0	0.001	0.001
	90	5.6	4.6	2.9	1.6	3.9	3.1	0.5	0.7	0.151	0.069

a – Mutation ratio is defined as the ratio between the mean number of revertants observed at the highest dose used in the calculation of the mutagenic potency, and the mean number of spontaneous revertants observed for that experiment.

b - Statistical parameters refer to the mutagenic potency, which is derived from the initial linear portion of revertants vs. mg soil equivalent concentration plots.

c – Values from Day 0 samples represent the average of the results from 9 separate assays on untreated soil samples.

R1 and R2 refer to BioFlo 110 benchtop bioreactors 1 and 2 respectively. Both bioreactors were treated identically and contained contaminated soil from the same source.

Strain	Day	Mutagenic Potency (rev/mg soil equivalent)		Standard Error (rev/mg)		Mutation Ratio		(r ²) ^b		p value ^b	
		R1	R2	R1	R2	R1	R2	R1	R2	R1	R2
TA98	0 ^c	3.5		1.0		2.1		0.7		<0.001	
	15	4.9	5.8	0.9	1.5	2.2	2.7	0.9	0.8	0.014	0.028
	30	8.2	6.7	0.9	0.5	3.2	2.7	1.0	1.0	0.003	0.001
	45	10.5	8.3	1.9	1.1	12.4	9.6	0.9	0.9	0.012	0.005
	60	8.5	5.6	1.4	1.7	9.5	6.8	0.9	0.8	0.009	0.049
	75	2.6	8.9	0.5	1.4	1.7	3.1	0.9	0.9	0.013	0.008
	90	10.8	6.0	1.8	0.6	3.4	2.4	0.9	1.0	0.009	0.002
YG1041	0 ^c	1.6		0.2		1.8		1.0		<0.001	
	15	7.3	12.4	1.2	2.2	5.0	7.9	0.9	0.9	0.009	0.011
	30	14.6	13.3	1.1	0.9	9.9	9.1	1.0	1.0	0.001	<0.001
	45	10.4	6.5	1.0	0.9	4.4	3.1	1.0	0.9	0.002	0.008
	60	7.2	4.0	0.5	0.8	3.5	2.2	1.0	0.9	<0.001	0.015
	75	2.4	5.8	0.3	1.9	1.9	2.9	1.0	0.8	0.003	0.052
	90	11.0	5.9	1.1	0.7	4.7	3.0	1.0	0.7	0.002	0.004

a – Mutation ratio is defined as the ratio between the mean number of revertants observed at the highest dose used in the calculation of the mutagenic potency, and the mean number of spontaneous revertants observed for that experiment.

b - Statistical parameters refer to the mutagenic potency, which is derived from the initial linear portion of revertants vs. mg soil equivalent concentration plots.

c – Values from Day 0 samples represent the average of the results from 9 separate assays on untreated soil samples.

R1 and R2 refer to BioFlo 110 benchtop bioreactors 1 and 2 respectively. Both bioreactors were treated identically and contained contaminated soil from the same source.

3.2 CHARACTERIZATION OF BIOREMEDIATED SOIL

3.2.1 SLURRY PHASE SEPERATION AND EXTRACTION

Slurry samples from days 0, 15, 30, 45, 60, 75 and 90 from all bioreactor treatments were separated into soil and water phases and analyzed via HPLC as described in sections 2.3.3.1 and 2.3.3.2, respectively. The concentration of 15 USEPA priority PAHs was determined in each phase (Figs 3.4-6, Tables 3.12-14).

3.2.2 SLURRY CHARACTERIZATION

The amount of PAH detected in all water phase samples was quite low, (1-30 ppb or ng/mL). This was not unexpected given the inherent low water solubility of PAHs. Low molecular weight PAHs (i.e., 2-3 rings) were observed in higher quantities than high molecular weight PAHs (i.e., 4-5 rings). Phenanthrene was generally present in the highest amounts in comparison with other PAHs. Total PAH concentration diminished during the treatment for all three of the treatment scenario examined (i.e., A, B, or C), with levels approaching the detection limit in samples from days 60, 75 and 90. The relative amount of PAHs in the water phase was generally 3 orders of magnitude lower than the corresponding solid phase (Figures 3.4-3.6).

R1, R2 – Bioreactors 1 and 2 respectively

R1, R2 – Bioreactors 1 and 2 respectively

R1, R2 – Bioreactors 1 and 2 respectively

3.2.2.2 SOIL PHASE

3.2.3 BIODEGRADATION OF COMPOUNDS

The majority of the total detected PAHs detected is attributed to HMW PAH. Concentrations of LMW PAH were considerably lower, especially at the later time points. The levels of priority PAHs were reduced during each treatment investigated, in both reactors. Reactor #1 treatment A showed the most complete PAH removal (90.4% removal of total priority PAHs), while reactor #2 treatment B was the least effective (65.6% removal of total PAHs). Generally, the removal of total priority PAHs was highly correlated with the removal of HMW PAHs (Figure 3.7). PAH levels were reduced at different rates during the different treatment methods, and the reduction rate also varied between different intervals within each treatment. Generally, LWM degradation was completed by days 30-45, which corresponds to the time frame in which metabolism of HMW compounds was observed to slow down (Tables 3.12-3.14).

During treatment A, the largest reduction in total PAH levels occurred between days 45-60 in reactor 1 (58.2 %), and between days 30-45 in reactor 2 (30.8 %). The smallest change in the level of total priority PAH was observed between days 15-30 in reactor 1 (3.3 %) and between days 45-60 in reactor 2 (6.8 %). In reactor 1, PAH levels were rapidly reduced between days 0-15 and between days 30-60, compared to slower reduction rates between days 15-30 and 60-90. In reactor 2, PAH levels were reduced at a faster rate between 0-45 compared to a generally slower rate between days 45-90.

During treatment B the largest reduction in total PAH concentration occurred between days 45-60 (30.0 %) in reactor 1, and between days 0-15 in reactor 2 (54.9 %). The smallest change in the level of total PAH was observed between days 30-45 in reactor 1 (2.1 %), and between days 45-60 in reactor 2 (1.3 %). In reactor 1, PAH levels declined rapidly between days 0-30, reduced at a slower pace between days 30-45, then resumed a rapid reduction for the remainder of the treatment period. In reactor 2, a sharp decline in PAH concentration was observed between days 0-15, which was followed by relatively slow rate of PAH removal until the end of the treatment.

During treatment C the largest reduction in total PAH concentration was observed between days 75-90 in reactor 1 (44.2 %) and between days 15-30 in reactor 2 (36.7 %). The smallest change in the level of total PAH was observed between days 15-30 in reactor 1 (4.2 %) and between days 30-45 in reactor 2 (6.0 %).

PAH	Reactor	Concentration (ppm)						
		Day						
		0	15	30	45	60	75	90
Naphthalene ^a	1	317	305	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND
Acenaphthene ^a	1	307	279	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND
Fluorene ^a	1	315	288	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND
Phenanthrene ^a	1	318	290	485	ND	ND	129	ND
	2	ND	256	ND	ND	ND	ND	ND
Anthracene ^a	1	281	243	490	443	220	127	104
	2	279	235	403	279	260	218	188
Fluoranthene ^b	1	658	808	863	706	315	208	137
	2	430	659	587	388	451	355	329
Pyrene ^b	1	1810	2280	749	545	124	131	40
	2	337	1140	334	137	292	304	209
Benzo(a)anthracene ^b	1	353	433	559	451	211	133	97
	2	285	311	386	262	295	225	195
Chrysene ^b	1	1110	612	523	396	167	120	762
	2	238	372	309	201	263	195	175
Benzo(b)fluoranthene ^b	1	1280	1060	1620	916	335	289	157
	2	652	645	703	447	602	470	370
Benzo(k)fluoranthene ^b	1	2970	268	881	619	262	177	120
	2	417	489	503	342	387	323	275
Benzo(a)pyrene ^b	1	1300	1010	782	589	277	168	123
	2	390	584	485	333	380	291	258
Dibenzo(a,h)anthracene ^b	1	325	538	404	341	157	96	74
	2	289	184	293	202	199	165	140
Benzo(g,h,i)perylene ^b	1	771	275	824	589	281	187	118
	2	266	486	505	317	400	293	240
Indeno(1,2,3-cd)pyrene ^b	1	516	1070	1260	901	369	263	165
	2	626	663	726	471	584	453	358

^a – Low molecular weight PAH, ^b – High molecular weight PAH, ND – Not detected

PAH	Reactor	Concentration (ppm)						
		Day						
		0	15	30	45	60	75	90
Naphthalene ^a	1	25	13	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND
Acenaphthene ^a	1	25.2	12	ND	12	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND
Fluorene ^a	1	24	12	ND	11.5	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND
Phenanthrene ^a	1	32	32	16	19	14	13	9
	2	31.5	ND	ND	ND	ND	ND	ND
Anthracene ^a	1	27	18	14	15	13	1	3
	2	30	24	21	23	20	12	10
Fluoranthene ^b	1	82	101	72	77	36	27	41
	2	33	49	51	43	56	29	18
Pyrene ^b	1	128	97	76	67	52	36	23
	2	39	133	134	116	126	101	59
Benzo(a)anthracene ^b	1	45	44	39	40	23	19	1
	2	113	33	31	31	31	19	8
Chrysene ^b	1	54	52	46	46	26	21	10
	2	103	40	37	36	38	24	16
Benzo(b)fluoranthene ^b	1	55	35	36	31	28	25	24
	2	169	57	54	53	48	45	27
Benzo(k)fluoranthene ^b	1	44	27	28	25	21	18	8
	2	34	37	35	35	34	24	16
Benzo(a)pyrene ^b	1	79	42	55	41	40	35	34
	2	98	93	82	86	80	73	44
Dibenzo(a,h)anthracene ^b	1	25	14	13	13	12	12	10
	2	54	43	20	22	19	12	9
Benzo(g,h,i)perylene ^b	1	56	34	37	31	28	25	21
	2	200	62	54	58	50	50	28
Indeno(1,2,3-cd)pyrene ^b	1	59	36	36	32	29	26	32
	2	448	59	55	56	51	47	28

^a – Low molecular weight PAH, ^b – High molecular weight PAH, ND – Not

PAH	Reactor	Concentration (ppm)						
		Day						
		0	15	30	45	60	75	90
Naphthalene ^a	1	17	11	ND	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND
Acenaphthene ^a	1	15	8	8	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND
Fluorene ^a	1	15	8	8	ND	ND	ND	ND
	2	ND	ND	ND	ND	ND	ND	ND
Phenanthrene ^a	1	42	13	12	10	10	9	6
	2	14	ND	ND	ND	ND	ND	ND
Anthracene ^a	1	18	9	8	7	7	7	2
	2	11	13	10	9	9	7	4
Fluoranthene ^b	1	71	61	57	39	28	25	22
	2	10	36	20	17	19	10	7
Pyrene ^b	1	76	72	69	55	56	49	19
	2	40	61	48	43	43	24	20
Benzo(a)anthracene ^b	1	41	39	40	28	22	19	5
	2	68	32	20	18	19	15	7
Chrysene ^b	1	34	32	33	24	18	16	4
	2	43	25	16	14	15	8	6
Benzo(b)fluoranthene ^b	1	17	27	28	25	11	11	14
	2	177	33	26	25	13	16	12
Benzo(k)fluoranthene ^b	1	16	6	6	6	18	6	1
	2	17	13	19	17	15	8	7
Benzo(a)pyrene ^b	1	37	39	41	34	37	36	19
	2	36	33	37	38	33	27	18
Dibenzo(a,h)anthracene ^b	1	13	8	8	8	8	8	6
	2	23	67	11	10	9	9	4
Benzo(g,h,i)perylene ^b	1	30	28	28	24	26	25	13
	2	16	22	28	28	26	16	14
Indeno(1,2,3-cd)pyrene ^b	1	32	28	29	26	28	27	21
	2	149	81	29	28	25	19	13

^a – Low molecular weight PAH, ^b – High molecular weight PAH, ND – Not detected

A) Treatment A

i, ii - Reactor 1 and 2 respectively

B) Treatment B

i, ii - Reactor 1 and 2 respectively

C) Treatment C

i, ii - Reactor 1 and 2 respectively

3.3 COMPARISON OF CHEMICAL AND TOXICOLOGICAL ENDPOINTS.

Mutagenic potency values obtained from both the non-polar neutral and the polar aromatic fractions for TA98 and YG1041, with metabolic activation, were plotted on the secondary axis of a PAH concentration versus treatment time graph for each treatment and reactor (Figures 3.8-3.9). A reduction in both total and HMW PAH concentration was observed for each treatment/reactor combination between each time point examined. However, a reduction in PAH concentration did not necessarily correlate to a reduction in mutagenic potency. Generally, an initial reduction in PAH concentration between days 0 and 15 corresponded to an increase in mutagenic potency on both TA98 and YG1041. During treatment A the mutagenic potency observed in fraction 2 (i.e., the non-polar PAH-containing fraction) on TA98 was observed to increase from 3.2 rev/mg of soil to 11.2 rev/mg of soil between days 75-90, even though the concentration of total PAHs was reduced from 2.0×10^3 ppm to 1.2×10^3 ppm (Fig. 3.8a). The mutagenic potency of fraction 3 (i.e., the polar aromatic fraction) was observed to increase from 4.2 rev/mg of soil to 6.3 rev/mg of soil over the same period. A similar effect was detected on YG1041 over the same time period (Fig. 3.9a). These results are consistent with those obtained from reactor 2 during treatment A (Fig. 3.8b, 3.9b). The mutagenic potency detected in fraction 3 during treatment B (i.e., nutrient addition) increased sharply between days 0-15 in both reactors. This potency declined between days 15-30 in reactor 1, and 15-45 in reactor 2, followed by a sharp increase from 3.3 to 30.2, and 6.7 to 14.7 between days 30-45 and 45-60 in reactors 1 and 2, respectively. These increases in

mutagenic potency coincide with temporal reductions in total PAH concentration (Fig. 3.8a, 3.9a). Similar fluctuations in potency were detected with YG1041 (Fig. 3.8b, 3.9b). The level of mutagenic potency detected in the non-polar neutral fraction did not fluctuate as much as that observed for the polar aromatic fraction during treatment B, although levels were always higher than those observed in the untreated soil. During treatment C (i.e., nutrients and microbial culture), mutagenic potency values generally increased until either day 30 or 45. In reactor 1, this was followed by a steady reduction until day 75, when values increased sharply. In reactor 2, potency levels detected with TA98 and YG1041 declined between days 45-60 and days 30-60, respectively. The values detected on both strains increased between days 60-75, and subsequently declined on day 90. Samples from treatment C showed similar mutagenic potency values regardless of chemical fraction tested. The differences between mutagenic potency values obtained from the non-polar neutral fraction and the polar aromatic fraction were generally smaller for treatment C.

a) Reactor 1

i, ii, iii - Treatment A, B, C respectively

F2 – mutagenic potency of non-polar aromatic fraction

F3 – mutagenic potency of polar aromatic fraction

b) Reactor 2

i, ii, iii - Treatment A, B, C respectively

F2 - mutagenic potency of non-polar aromatic fraction

F3 - mutagenic potency of polar aromatic fraction

a) Reactor 1

i, ii, iii - Treatment A, B, C respectively

F2 - mutagenic potency of non-polar aromatic fraction

F3 - mutagenic potency of polar aromatic fraction

b) Reactor 2

i, ii, iii - Treatment A, B, C respectively

F2 – mutagenic potency of non-polar aromatic fraction

F3 – mutagenic potency of polar aromatic fraction

3.4 STATISTICAL ANALYSIS OF VARIABLES AND INTERACTIONS AFFECTING MUTAGENIC POTENCY AND PAH CONCENTRATION

Statistical analysis was performed on the mutagenicity and chemical analysis data in order to identify statistically significant effects (i.e., effects related to chemical fraction, treatment, time, reactor, *Salmonella* strain and metabolic activation status). The effects of *Salmonella* strain, chemical fraction, treatment day, treatment method, and reactor, as well as concentrations of total, HMW and LMW PAHs, on mutagenic potency were considered when analyzing the *Salmonella* mutagenicity data. Interactions between treatment and fraction, treatment and day, and treatment and reactor were also examined. Strain comparisons were conducted with respect to the effect of chemical fraction and metabolic activation on mutagenic potency. The effect of treatment, treatment day, and reactor, as well as interactions between treatment and reactor, reactor and day, and treatment and day were considered for the analysis of total PAH, HMW and LMW PAH concentration data. The effect of each independent PAH, as well as all unique combinations of PAHs, on mutagenic potency for each *Salmonella* strain/fraction combination was also evaluated. Tables 3.15-3.22 provide a summary of the results obtained.

The treatment method employed (A: control, B: nutrient addition or C: nutrient and hydrocarbon degrading bacteria) had a significant effect on the mutagenic potency of fraction 3 on TA98 both with and without metabolic activation, and on YG1041 with metabolic activation (Table 3.15). Treatment did not have a significant effect on any assays on fraction 2, or on fraction 3 samples tested

with YG1041 without metabolic activation. Although temporal trends in mutagenic activity is apparent in several of the figures, ANOVA failed to demonstrate a consistent and statistically significant effect of reactor or treatment day. However, when the chemical fraction tested was included in the ANOVA model, the day of treatment had a significant effect on mutagenic potency obtained with metabolic activation (Table 3.16). The treatment effect was also more significant when the chemical fraction was included in the ANOVA. The effect of testing either fraction 2 or 3 was only significant when TA98 was assayed in the absence of metabolic activation, and when the interaction between treatment and fraction was examined on the potency values obtained from YG1041 with metabolic activation. The interaction between treatment type and day of treatment had a significant effect on all mutagenic potency values. This effect was more significant in assays that included metabolic activation. There was no significant effect of treatment / reactor interaction on mutagenic potency.

Table 3.17 summarizes the effect of different variables on PAH concentration. The treatment method used, the day of sampling, and the reactor tested all had a strong effect on the levels of total PAHs, HMW PAHs and LMW PAHs. The interactions between treatment and reactor, reactor and day, and treatment and day all had significant effects on PAH concentration.

When examining the effect of either total, HMW or LMW PAH concentrations on mutagenic potency values (Table 3.18), only polar aromatic samples tested with metabolic activation on YG1041 were significantly influenced by either LMW or

HMW PAH levels. The effect of individual PAH concentrations on mutagenic potency was also examined (Table 3.19). Stepwise linear regression analyses was used to examine the effects of all 15 priority PAHs individually, and, in various combinations. Surprisingly, only phenanthrene had a significant effect on mutagenic activity. The combination of dibenzo(a,h)anthracene and benzo(g,h,i)perylene had a significant effect on the mutagenic activity of the polar aromatic fraction in YG1041 in the absence of S9.

Additional analyses showed that the mutagenic potency on YG1041 is empirically related to the mutagenic potency on TA98 across all endpoints examined (Figure 3.10). Seeing as TA98 and YG1041 both detect frameshift mutations, a relationship between these two endpoints is not unexpected.

Mean mutagenic potency values, from each strain fraction combination, were compared across treatments to identify the treatments that consistently yielded the highest (or lowest) mutagenic potency values (Figure 3.11). Treatment B (nutrient addition) always produced the highest average potency. The lowest potency values were obtained from treatment A (control) for fraction 3 samples, while treatment C (nutrients + microbial addition) resulted in the lowest fraction 2 samples. Treatment method did not affect potency values obtained from fraction 2 samples, which were not statistically different from one another at $p < 0.05$. The mean, treatment B, TA98 fraction 3 values were significantly different from the treatment A and C values. YG1041 fraction 3 potency values obtained from treatment A were significantly different from treatment B and C values.

The average mutagenic potency across all treatments and reactors was plotted for each day (Figure 3.12), and the temporal trends examined across each strain / fraction combination with metabolic activation. The lowest mutagenicity values always correspond to day 0 samples, regardless of *Salmonella* strain, or fraction. The highest potency values were generally from day 60 or 75, with the exception of YG1041 potency from fraction 2, where day 15 values were highest. Average mutagenic potency did not adhere to a linear pattern throughout the treatments, and in contrast, seem to be somewhat cyclical. Generally the average potency values observed from fraction 3 were lower than the corresponding strain fraction 2 values. Fraction 3 values were higher than fraction 2 values on days 0 and 60 for TA98 and on day 45 for YG1041. Only the mean mutagenic potency values from day 0 and day 75 samples were statistically different in TA98 fraction 2 samples. Day of treatment did not have a statistically significant effect on TA98 fraction 3 mean mutagenicity values at $p < 0.05$. Day 0 samples were statistically similar ($p < 0.05$) to; day 45 samples in YG1041 fraction 2 assays; and to days 30 and 90 samples for YG1041 fraction 3 values.

Strain	Metabolic Activation	Fraction	F-ratio		
			Treatment	Day	Reactor
TA98	Y	2	2.41	1.28	0.23
	Y	3	9.73**	1.23	0.71
YG1041	Y	2	1.89	2.02	2.07
	Y	3	8.36*	2.26	0.44
TA98	N	2	1.65	0.81	2.75
	N	3	5.59*	0.89	0.04
YG1041	N	2	8.04	2.25	3.83
	N	3	2.96	1.06	0.14

F-Ratio: Evaluation of the significant difference between two groups of variances, obtained by dividing one variance by the other.

** $p < 0.05$*

*** $p < 0.001$*

Strain	Metabolic Activation	F-ratio						
		Treatment	Day	Reactor	Fraction	Treatment & Fraction	Treatment & Day	Treatment & Reactor
TA98	Y	18.79**	4.55**	1.62	1.47	2.11	5.87**	1.36
	N	8.70**	1.54	0.9	5.70*	3.13	1.93*	0.35
YG1041	Y	11.41**	5.91**	0.21	2.38	5.64*	3.38**	0.48
	N	9.06**	2.02	0.9	1.1	2.01	2.23*	0.06

F-Ratio: Evaluation of the significant difference between two groups of variances, obtained by dividing one variance by the other.

** $p < 0.05$*

*** $p < 0.001$*

PAH Concentration	F-ratio					
	Treatment	Day	Reactor	Treatment & Reactor	Reactor & Day	Treatment & Day
LMW PAH	535**	103.5**	456.5**	582.6**	3.7**	3.1**
HMW PAH	502.7**	90.7**	353.5**	543.5**	3.3*	3.2*
Total PAH	219.7**	70.6**	1098.1**	348.9**	12.2**	4.1**

F-Ratio: Evaluation of the significant difference between two groups of variances, obtained by dividing one variance by the other.

** $p < 0.05$*

*** $p < 0.001$*

Strain	Fraction	F Ratio (Mutagenic Potency)		
		LMW PAH	HMW PAH	Total PAH
TA98	2	0.04	0.03	0.44
	3	1.48	1.6	0.29
YG1041	2	0.06	0.03	0.31
	3	4.51*	4.67*	1.83

F-Ratio: Evaluation of the significant difference between two groups of variances, obtained by dividing one variance by the other.

** $p < 0.05$*

PAH Concentration	Strain	Metabolic Activation	Fraction	Mutagenic Potency Coefficient	F-ratio (Mutagenic Potency)
Phenanthrene	YG1041	Y	2	20.06 ± 0.19	8.72*
Dibenzo(a,h)anthracene, Benzo(g,h,i)perylene	YG1041	N	3	22.84 ± 0.13	4.01*

F-Ratio: Evaluation of the significant difference between two groups of variances, obtained by dividing one variance by the other.

**p<0.05*

*Average mutagenic potency values are significant at $p < 0.0001$ in both *Salmonella* strains. Similar results were observed in responses without S9 augmentation (results not shown).*

25 values are overlapped in the above figure.

Letters indicate unique Duncan groups, generated by a ranking of group means. Values with the same Duncan grouping letter are not statistically different at $p < 0.05$. Error bars represent standard error.

Errors bars indicate standard error.

4.0 DISCUSSION

4.1 BIOREMEDIATION OF PAH CONTAMINATED SOIL

Initial characterization of soil

The concentrations of 15 priority PAH compounds in soil from a former gasworks site were measured every 15 days during 3 different bioremediation treatments. Levels were monitored in both soil and water phases. Increased levels of PAHs are routinely observed at former gas works sites as they are formed during the production of coal-gas at elevated temperatures. Coal-gas manufacturing employs elevated temperatures (e.g., $>1000^{\circ}\text{C}$) and a limited supply of air to prohibit complete combustion, and the incomplete combustion contributes to the formation of coal-tar, a complex material that is rich in PAHs. All priority PAHs were detected in soil samples prior to treatment (Day 0), with the exception of naphthalene, acenaphthene, and fluorene in reactor 2 samples from treatments B and C, and phenanthrene in reactor 2 samples from treatment A. LMW PAHs are more susceptible to combustion during coal-gas production, and to natural microbial biodegradation, in comparison with HMW PAHs due to their relatively lower π -electron density [4]. Moreover, they are significantly more volatile and water soluble than HMW PAHs, and their relatively low concentrations were not surprising. The inability to detect particular PAHs at the start of selected treatments is likely a result of concentrations that are already at or near the limit of detection, and spatial variations in PAH concentration between samples from a single contaminated site. Untreated soil samples displayed a range of total priority PAH concentrations between 475 to 12600 $\mu\text{g/g}$ dry soil (geometric mean = 2730 $\mu\text{g/g}$

dry soil). Water phase samples from day 0 samples showed a range of 15 to 31 ng/L slurry (geometric mean = 20 ng/L slurry), with pyrene present at the highest amounts in all samples. Other structurally similar pollutants of interest, with potentially similar mechanisms of genotoxicity, include alkyl-substituted PAHs as well as *O*- and *S*-heterocyclic compounds have also been observed at gas manufacturing plants [52]; however these compounds were not measured in this study. A paucity of information exists regarding the genotoxic mechanism of such compounds, especially when they are present within a complex mixture.

Regulated PAH levels in soil

CCME guidelines provide PAH concentration thresholds, and suggest that PAH concentrations above these limits present an elevated risk to human health. With the exception of pyrene, the levels of all PAHs examined in this study that are regulated by the CCME were initially above the stated limits. The 5-ring PAHs- benzo(*a*)anthracene, benzo(*k*)fluoranthene and dibenzo(*a,h*)anthracene - were present at concentrations above CCME thresholds at all time points examined except for day 90 samples from treatment B. The levels of benzo(*k*)fluoranthene and dibenzo(*a,h*)anthracene were below threshold levels by day 15 in treatment C. Levels of benzo(*a*)pyrene were never reduced below threshold levels, and remained at least an order of magnitude higher than the 0.7 ppm guideline (Tables 3.10, 3.11, 3.12). Consequently, from a chemical perspective, the CCME guidelines would define the soil studied as presenting a hazard to human health even after 90 days of bioremediation, regardless of treatment type.

Biodegradation of PAHs

Total PAH concentration was reduced in the water phase an average of ~73% across all treatment types. Treatment C yielded the highest reduction (92% and 95%), while treatment B yielded the lowest (58% and 49 %). In the soil phase, the levels of all PAHs were reduced over the course of each 90 day treatment, and this is consistent with previous studies examining bioremediation of PAH-contaminated soils [100]. The total PAH concentration in soil was reduced an average of 77.0 % across all treatments (Table 3.13). Generally LMW PAHs were removed to the greatest extent (Figures 3.7, 3.8), with an average of 93.3 % of these compounds eliminated from the samples. The concentration of HMW PAHs was also reduced, on average 90 % of starting levels (Figures 3.7, 3.8), although not to the extent of LMW compounds with respect to discrete final concentration values. As compared to LMW PAHs, the inherent physical properties of HMW PAHs (Table 1.1), such as low water solubility and high Log K_{ow} , greatly reduce their bioavailability to microorganisms (i.e, *Mycobacteria sp*), and likely accounts for their reduced degradation [72]. This phenomenon can be more pronounced at aged sites where the molecules have extended periods to strongly adsorb to organic particles in the soil in a process known as weathering [103]. Weathering can permit organic compounds to impregnate the soil matrix, making subsequent microbial extraction and degradation more difficult. However, weathering can serve to reduce the human health risk of a PAH contaminated site, since the pollutants become less bioavailable. Nevertheless, the underlying hazard is still present. Donnelly *et al* [104] examined the recalcitrance of PAHs undergoing bioremediation and detected

residual benzo(a)pyrene levels equal to ~70 % at day 90, ~40 % on day 360 and ~3 % on day 720. This soil was also tested for mutagenic activity on days 0 and 540 using the *Salmonella* reverse mutation assay (i.e., strain TA98). Similar to results obtained in this study (see Fig 3.12), mutagenic potency was seen to increase following bioremediation treatment, with no decrease in potency observed at any time point examined, and the formation of oxygen-containing metabolic products was hypothesized as a potential source of the increased activity.

All treatments showed an initial rapid reduction in total PAH concentration between days 0-15, with the most dramatic decline observed in reactor 2 during treatment B (Fig 3.5 - B). The rapid initial decline is likely due to the rapid degradation of LMW PAHs that are readily available to microbial degradation (e.g., native microbes in treatments A and B, and both native and exogenous microbes in treatment C). The presence of fine soil particles appears to have a positive effect on the biodegradation of soil PAHs, however the exact mechanism is unknown. Xia and Wang (2008) showed that benzo(a)pyrene and chrysene concentration were reduced up to 1.44 times faster in a slurry of fine silt as opposed to a silt free water system [91]. They observed an initial rapid decrease in PAH concentration, followed by a period of slower degradation. They hypothesized that bacteria were able to rapidly metabolize PAH molecules sorbed to the surface of fine particles since they were readily accessible to bacteria attached to the same particles. When all of the surface PAHs were metabolized, the degradation rate was reduced since weathered PAHs were not as readily accessible. This effect could explain the rapid removal of LWM PAHs, which are more water soluble, and therefore more likely to be found at

the soil-water interface, in comparison with the less soluble more hydrophobic HMW PAHs, which were degraded more slowly over the course of each treatment.

Previous work has shown that microbial degradation is largely responsible for the removal of PAHs from contaminated soils, and the rate of degradation is affected by the presence of particular microbes [72; 92; 105-107] [77; 88; 100; 108-112]. The degradation of PAH compounds in this study was significantly affected by treatment type, day of treatment, and reactor replicate, as well as the interactions between treatment and reactor, reactor and day, and treatment and day (Table 3.17). The relationship between treatment and PAH degradation is not surprising since the optimal rate of microbial metabolism cannot be attained without ideal physical conditions, as well as an adequate supply of essential macro- and micro-nutrients [4]. It should be noted that mineralization of PAHs often requires co-metabolism, where degradation occurs without the utilization of energy or carbon for a single organism, and the by-product(s) is/are utilized by neighbouring opportunistic species [4]. For example, Kanaly *et al.* (2000) showed that benzo(a)pyrene degradation occurs rapidly via co-metabolism within a consortium that includes members from the *Proteobacteria* class, as well as members from the genera *Mycobacterium* and *Sphingobacterium* [113]. Similarly, Smith *et al.* observed a reduction in the time required for PAH mineralization in organic soil following an inoculation with a consortium of bacteria, in comparison with a single species [114]. Reasons for increased PAH mineralization rates in the presence of microbial consortia may include an increase in the diversity of catabolic enzymes, and the ability of certain species to produce biosurfactants. Therefore, the addition of an

exogenous culture of petroleum-degrading bacteria, as in treatment C, was expected to greatly enhance the metabolic capacity of the natural consortia present, and lead to a faster, more complete degradation of the pollutants. However, as shown in Figure 3.7, this effect was not always observed. The greatest PAH removal occurred in reactor 1, treatment A, where 90.4% of total PAHs were removed. However this may be an artifact of experimental design as the initial PAH concentrations in reactor 1 during treatment A were orders of magnitude greater than those in other reactor/treatment combinations. The maximal total PAH removal rates observed in treatments B and C were 80.6% and 81.5% (Figure 3.7: Reactor 2), respectively. This could be due to the preferential utilization of supplemental and/or natural nutrients by the exogenous culture, which may not include bacteria capable of metabolizing PAHs. It is important to note that the commercial product used as the source of nutrients and bacteria in this study is marketed for the remediation of hydrocarbon contaminated soils, rather than weathered PAH-contaminated soils. Contamination at petroleum hydrocarbon contaminated sites would consist primarily of straight chain hydrocarbons, such as those present in fuel oil, diesel fuel and gasoline. The degradation pathways employed to remove these compounds, which are usually initiated by formation of an alcohol at a terminal position, are substantially different from those required for PAH degradation. The terminal alcohol generally undergoes metabolic transformation to yield an aldehyde and then a mono-carboxylic acid [115]. In contrast, bacterial PAH catabolism usually involves the formation of metabolic intermediates such as dihydrodiols and epoxides. The differences between these catabolic processes could account for the lack of

significant differences in PAH degradation levels across the three treatments. A culture of PAH-degrading bacteria, as well as nutrient additions designed to promote their growth, might have resulted in more rapid and complete degradation of soil PAHs.

Another unexpected result was the significant relationship between PAH concentration and reactor (Table 3.17), as the soil used in both reactors was from the same sample. Although efforts were made to homogenize the soil, the complex nature of the contamination complicates effective, thorough homogenization. Therefore, it is not surprising that the soil entering into the reactors displayed differences in relative PAH levels. As the measured PAH concentrations in untreated soil samples were variable, differences in the native microbial consortia composition could also be possible.

The formation of oxygenated PAH derivatives is a common occurrence during the bioremediation of PAH contaminated soils. The metabolic processes employed by PAH degrading bacteria in an aerobic environment, such as the bioreactor system used in this experiment, have been shown to increase the concentration of several oxy-PAH metabolites [7]. Oxy-PAHs are also expected to be present in the untreated soil prior to bioremediation, since they can be formed during physical chemical process involved in gas manufacturing [85]. Lundstedt *et al* provided a comprehensive characterization of such chemical constituents, some of which may be metabolites, during the course of a similar bioremediation treatment of a soil that is chemically comparable to that examined in this study.

Over 100 PACs, including the priority PAHs assessed in this experiment and 23 oxygenated PACs, were identified. Some oxy-PACs were present in untreated soil samples and observed to increase in concentration during the course of treatment. After 29 days of treatment the oxy-PAHs 1-acenaphthenone and 4-oxapyrene-5-one were present at levels 30% and 60% higher, respectively, than pre-treatment levels. An increase in the concentrations of several other oxy-PAHs was also noted, however, these levels dropped by the end of the treatment. Oxy-PAHs not present in untreated soil samples were not detected throughout the treatment period. With respect to priority PAHs concentrations decreased over the course of the treatment. When samples from this study were examined using the Salmonella mutagenicity test by Lynes [53], a fluctuation in mutagenicity was observed in both the non-polar neutral fraction and the polar aromatic fraction [56]. Non-polar neutral samples were not mutagenic with metabolic activation before day 3 in either TA98 or YG1041, but samples from later days of treatment showed a fairly uniform increase in mutagenic potency. Polar aromatic samples were not mutagenic in TA98, while positive responses were detected on all days by YG1041. YG1041 potency values initially increased between days 0-3 (from 7431 – 8544 rev/g), then sharply declined by day 7 (3875 rev/g). Potency values increased for the duration of treatment and were observed to be highest on the last day of treatment (i.e., day 29) at 8816 rev/g. The cause of these mutagenicity fluctuations could be due to the formation and degradation of oxidative PAH metabolites and is similar in nature to the mutagenic tendencies of samples tested in this experiment (Fig 3.9-10). More work is needed to discern the mutagenic potential of distinct PAH metabolites.

Certain oxy-PAHs (i.e, 4-oxapyrene-5-one) which have been observed to increase in concentration during the bioremediation of PAH contaminated soil [85] have been isolated, yet did not elicit a mutagenic response in the Salmonella mutagenicity test (Lemieux, 2007 pers. comm.). Oxy-PAHs are not regulated under CCME guidelines, however, they are more mobile in the environment, and some oxidative PAH metabolites, such as the direct acting benzo(*a*)pyrene-dihydrodiol-epoxide (Fig. 1.2), are more hazardous to human health compared to parent compounds [50; 52; 56; 116; 117].

4.2 *SALMONELLA* MUTAGENICITY TESTING OF SLURRY SAMPLES

The direct (-S9) and metabolically activated (+S9) mutagenicity of each sample was examined using *Salmonella typhimurium* strains TA98 and YG1041. The use of TA98 has been well established for the detection of frameshift DNA mutagens such as PAHs [2; 118]. Pilot studies with strains TA100 and YG1041 did not detect base pair substitution mutations caused by the untreated soil and previous work has shown that PAH induced mutation detection is more sensitive using frameshift detection strains [53; 56]. As a result, *Salmonella* strains that detect base pair substitution mutations were not employed in this study, and all mutagenicity results are restricted to frameshift mutations

The total soluble organic matter was extracted from both the water and soil phases of the slurry samples, the extracts were combined, and subsequently fractionated to produce three chemically distinct fractions. The second fraction contained non-polar neutral aromatic compounds such as PAHs, alkyl PAHs, *O*-heterocyclics and *S*-heterocyclics. The third fraction contained polar aromatic compounds such as oxy-PAHs and N-heterocyclics. Although PACs, including heterocyclics, oxy-PAHs or alkyl PAHs were not specifically monitored in this study, previous experiments have shown that they can be present in the aforementioned fractions [52]. It was expected that metabolic activation would be required to detect mutagenic activity in fraction 2 since it is the electrophilic metabolites that induce mutation as opposed to the non-polar parent PAHs contained within. Similar bioremediation studies have shown that the mutagenic response in the polar

aromatic fraction often exceeds that of the non-polar neutral fraction, both with and without S9 metabolic supplementation. Moreover, it is thought that oxygenated PAH metabolites, which are known direct acting mutagens, contribute to this response pattern [51-53]. The direct-acting mutagenic activity of chemical fractions containing mixtures of oxy PAHs found at PAH contaminated sites has been demonstrated, however the response in the *Salmonella* reverse mutation assay from certain pure oxy-PAHs observed to increase in concentration during bioremediation treatment (i.e., 4-oxapyrene-5-one) was negative. The generation of yet to be identified direct acting genotoxic oxy-PAHs is a possible cause.

Due to the custodial history of the soil tested, high concentrations of nitro-substituted PACs compounds were not expected. Urban soils can contain a plethora of mutagenic nitro compounds, following the deposition of diesel combustion particulates. Mutagenic nitrogen containing compounds, including 2,4,6-trinitrotoluene, tetryl, HMX and RDX, are often present at current and former military sites where unused ammunition and explosives are found. However, highly potent, direct acting nitro-PAHs can also be derived from PAHs through metabolic processes, or formed during combustion, and can be present at non-urban or military sites [51; 53] The predicted lack of nitro-compounds implies that similar mutagenicity results for YG1041 and TA98 would be expected. This was confirmed when no significant difference between mutagenic potency values was observed (Figure 3.10).

4.2.1 NON-POLAR NEUTRAL AROMATIC FRACTION

Direct-acting Mutagenicity

The regulated PAH compounds in this fraction are not known to be mutagenic in the absence of metabolic activation. Therefore the weak mutagenic responses detected can not be attributed to priority PAHs. As the fraction 2 samples from the untreated soil were not mutagenic in either TA98 or YG1041, the formation of weakly mutagenic non-polar neutral aromatic compounds may have occurred during the course of treatments.

The treatment method employed had a significant effect ($p < 0.001$) on the mutagenic potency values obtained on both TA98 and YG1041 in the absence of metabolic activation (Table 3.16). The chemical fraction tested also had a significant effect on TA98 potency values. During the control treatment period, marginally mutagenic samples were detected on TA98 on days 45 and 90 in both reactors. Marginally mutagenic samples were detected on YG1041 on day 15 in both reactors and on day 90 in reactor 2, and positive mutagenic responses were noted on day 45 in reactor 2, and on day 90 in reactor 1. Although the mechanism of formation is unclear, it is possible that the compounds generated during the treatment include direct-acting nitroarenes such as 1,8-dinitropyrene and 2-nitrocarbazole [43]. Nitroarenes are classified as direct acting mutagens since cytochrome P450 transformation is not required to yield mutations, however, they do require reduction of their NO_2 groups to nitroso and hydroxylamino intermediates before mutations can be induced. In addition hydroxylamine derivatives can be acetylated

to become potent direct acting mutagens [43; 119]. Since YG1041 contains the *Salmonella cnr* (classical nitroreductase) and *OAT* genes, such compounds are able to be reduced and/or acetylated, and exhibit a mutagenic effect in the absence of S9. The detection of mutagenicity on days 15 and 90 exclusively suggests that mutagenic intermediates were formed between days 30 and 45, and degraded to non-mutagenic compounds by day 60. Moreover, the results suggest that a different group of mutagenic compounds may have been formed after day 75 and before day 90.

Supplementation with nutrients was expected to permit the native microbes to metabolize chemicals contained in the soil at an optimal rate. Mutagenic activity was detected on TA98 in reactor 2 on days 45 and 60, while reactor one samples were marginal for the same time period. The strongest response was detected in reactor 2 on day 60, suggesting the presence of either more potent or a larger quantity of mutagens in reactor 2 as compared to reactor 1. Positive mutagenic activity was detected by YG1041 in both reactors for all samples between days 45-90. The stronger responses observed in both TA98 and YG1041 compared to the control treatment suggests that the addition of nutrients altered the metabolic capacity of the native microbial consortia in such a manner as to produce more mutagenic intermediates.

Augmenting the natural flora of the soil with an exogenous culture of known hydrocarbon degrading bacteria, in addition to nutrient supplementation, was expected to enhance the bioremediation potential of the system and provide the

best chance for complete hazard elimination. As expected, very few positive and marginal responses were detected on either TA98 or YG1041 in either reactor. One marginal potency value was observed in reactor 1 on day 60 on TA98, and one positive response was identified on YG1041 on day 90. In reactor 2 marginal responses were detected on days 30 and 90 on TA98 as well as on day 90 on YG1041. A positive response was detected on both TA98 and YG1041 on day 75. As there was not a significantly greater number of positively or marginally mutagenic response detected on YG1041 compared to TA98, N-containing PACs do not appear to make a meaningful contribution to the response profile.

S9-activated Mutagenicity

In the presence of S9, the *Salmonella* test cultures are able to simulate mammalian metabolic systems *in vitro*. Since PAHs are known pro-mutagens that require metabolic activation before a mutagenic effect can be observed, the supplementation with mammalian hepatic enzymes provides the opportunity to use a bacterial assay to examine the mutagenic hazards posed to mammalian receptors (i.e., humans). Although extrapolations of mutagenic potency from bacterial systems to humans are not direct, they can provide useful information regarding potential hazard.

As expected the addition of S9 resulted in increased mutagenic responses compared to corresponding samples without S9 addition on all days during all treatment periods. In the non-polar neutral fraction mutagenicity could be attributed to the presence of PAHs, which were detected and characterized via HPLC

procedures on samples taken at the same time.. One would expect temporal changes in mutagenicity to be correlated with concurrent changes in PAH concentrations; however this was not the case. Potency values at the end of each treatment were greater than those found in the untreated, day 0 samples. The presence of PACs that were not characterized analytically, such as alkyl-PAHs or *O*-heterocyclics could account for this discrepancy. This is supported by work completed by Lemieux, who compared the mutagenic potencies of the non-polar neutral fraction to a synthetic mixture of pure PAHs, corresponding to their respective concentrations detected in the mixture. Lemieux observed that synthetic mixtures generally yield a higher mutagenic potency than environmental samples and the results suggested that non-additive interactions occur between mutagenic PAHs in mixtures [51].

Generally, the mutagenicity was observed to increase from day 0-15 followed by a decline between days 15-30. Positive mutagenic responses were detected on both strains and all days over the course of each treatment. There was no significant difference between potency values obtained from YG1041 and TA98, suggesting that mutagenic nitro-substituted compounds were not present. The most bioavailable compounds, such as LMW PAHs or chemicals early in the weathering process, will be readily metabolized. When these carbon sources are exhausted, the microbes will be required to utilize the more recalcitrant HMW compounds, if possible, and this type of temporal shift may account for the temporal pattern (i.e., cyclic) of mutagenic activity observed (Fig 3.12). The deployment of bacterial surfactants to coax aged compounds away from soil particles has been shown to

contribute to such shifts in the metabolism of weathered PAHs in contaminated soils [71; 80; 120; 121].

The data analyses showed that phenanthrene concentration has a significant empirical effect on the mutagenic response on YG1041 (Table 3.19). It has been shown by McLellan *et al* that the presence of certain LMW PAHs, such phenanthrene, can induce the metabolism of HMW PAHs, including benzo(a)pyrene, by acting as metabolic pathway initiators [92]. The LMW PAHs are more water soluble, more bioavailable, and are therefore preferentially degraded. During the degradation of bioavailable LMW PAHs, metabolic genes are translated and catabolic enzymes are produced. As the metabolic pathways for LMW and HMW can be similar, when all of the available LMW PAHs are metabolized for the bacteria can subsequently utilize the HMW PAHs [92]. The “stimulated” degradation of the HMW PAHs can contribute to the formation of mutagenic PACs.

4.2.2 POLAR AROMATIC FRACTION

Direct-acting Mutagenicity

The detection of mutagenic responses in this fraction was anticipated since some of the compounds expected to be present are believed to be direct acting mutagens. Additionally, previous *Salmonella* mutagenicity testing on this chemical fraction, obtained from PAH contaminated soil undergoing bioremediation, has shown positive mutagenic potency [53]. Bacterial degradation pathways usually follow the catechol formation mechanism, of which several intermediates, including

catechol, are identified in the IARC monographs as potential human carcinogens. While catechol itself is not mutagenic in *Salmonella* (either in the presence or absence of metabolic activation) it is believed to act as a co-mutagen in the presence of genotoxic PAHs. Yoshida and Fukuhara observed an increase in the mutagenic potency of BaP following an increase in the concentration of catechol added to the pre-incubation mixture of the Ames assay [122]. In addition, the electrophilic intermediates produced during the catechol formation pathway may be expected to bind with DNA. During the control treatment period positive mutagenic responses were only detected on YG1041 in reactor 2 on days 15, 45 and 90. A marginal response was detected on YG1041 on day 15 in reactor 1, suggesting a difference in metabolite formation between the reactors. This could be a result of differences in native microbes or different parent compounds present in starting material. Marginal responses were detected on TA98 in both reactors on days 15 to 45 and day 75, but only in reactor 2 on day 90. Since PAH concentration was reduced over the course of treatment, the metabolic pathways employed in each reactor may have resulted in the formation of either non-mutagenic polar compounds or non-polar intermediates. A likely scenario is that non-direct acting mutagenic metabolites were formed.

After nutritional supplementation, positive mutagenicity was detected on TA98 on day 60 to 90 in both reactors. YG1041 detected a positive response on day 15 in reactor one, possibly due to the formation of a N-heterocyclic or an aromatic amine N-containing PAC since the sample taken from the corresponding day in reactor 1 was only marginally mutagenic in TA98. Positive responses were also

detected by YG1041 on days 60 to 75, inferring that the metabolic processes of the indigenous microbial culture resulted in the formation of polar intermediates approximately half way through the treatment period. As a negative response was detected in reactor 2 on day 90, the cultures appear to have the capacity to metabolize toxic intermediates that may have been formed and remove mutagenic hazard.

Treatment type has a much more significant effect on mutagenic potency without metabolic activation compared to the reactor, which has no effect on mutagenic potential (Table 3.16). During treatment, mutagenic activity was detected on both TA98 and YG1041 in both reactors. This observation could be attributed to the formation of direct acting mutagens resulting from metabolic pathways facilitated by the exogenous hydrocarbon degrading culture that are not employed by the naturally present consortia. Consequently, the external microbial culture may have a competitive advantage with respect to PAC degradation compared to the native species. When the external culture is added to both reactors a more robust and consistent metabolic pathway for PAH degradation may be available, removing any metabolic limitations that could be present in the native consortia.

It was also interesting to note the significant empirical relationship between the presence of dibenzo(*a,h*)anthracene and benzo(*g,h,i*)perylene on the mutagenic potency detected on YG1041 (Table 3.19). A possible explanation for this

correlation could be that those PAHs may be involved in the formation (via bacterial metabolism) of subsequent mutagenic nitroarenes or aromatic amines.

S9-activated Mutagenicity

In the presence of S9, the tester strains were able to simulate mammalian metabolism of PACs present in this fraction. Since this fraction was expected to contain N-heterocyclics and oxy-PAHs, high mutagenic potencies were expected and observed. Mutagenic potency was generally increased by day 15, and then relatively reduced by day 30. Mutagenicity peaked between days 45 and 60, followed by a second reduction by day 90. The type of treatment employed had a significant effect on mutagenic potency in both TA98 and YG1041 responses (Table 3.15). This was expected since the diverse consortia present, especially following augmentation with known hydrocarbon degraders and essential nutrients, could permit the optimal conditions for PAH degradation. This augmentation could also explain the rapid degradation of LMW PAHs between days 15 and 30 (Fig. 3.9-10), possibly resulting in the formation of mutagenic metabolites as shown by the concurrent increase in mutagenicity. The subsequent reduction in mutagenicity between day 30 and 60 in treatment C could be attributed to the degradation, and possible mineralization, of these mutagenic intermediates. When the readily bioavailable compounds are removed, the cultures would be expected to start to metabolize the HMW compounds and weathered PAHs, and this would be expected to contribute to the formation of highly mutagenic intermediates. The reduced mutagenicity observed by day 90 likely results from the continued degradation of this second

group of intermediates. Since there was no significant difference in the mutagenicity between TA98 and YG1041, the intermediates are not suspected of being nitroarenes or aromatic amines.

Unfortunately the chemical composition of the polar aromatic fraction was not monitored during this study. Therefore, it is impossible to ascertain the nature of the compounds responsible for the observed responses. Nevertheless, previous studies, such as those performed by Lundstedt and Lemieux have conducted thorough characterizations of similarly contaminated soils treated using comparable bioremediation systems. Those studies have identified various oxy-PAH compounds in this fraction, and in some cases confirmed the mutagenicity of the soils or soil fractions where these compounds are present [56].

4.3 CONCLUSIONS AND FUTURE DIRECTIONS

Contaminated soils can pose a serious risk to both human and environmental health. The process of using bioremediation, although complex, is often the most efficient choice when considering options for removing PAH contamination from soil. During this experiment it was shown that different bioremediation strategies are effective at reducing the concentrations of priority PAH compounds. However, the removal of priority pollutants was not shown to correlate with a reduction in the mutagenic hazard of the soil samples. In fact mutagenic responses were cyclical, and the mutagenicity of samples obtained during the treatment periods were rarely

measured at levels below those observed in the untreated soil. This implies that additional tests, such as the *Salmonella* reverse mutation assay should be employed, in addition to chemical identification procedures, when assessing the hazard of contaminated soils before, during and after treatment. Also, the high mutagenicity response detected in the polar aromatic fraction, compared to the non-polar neutral fraction, implies that the most potent mutagens found at PAH contaminated sites likely includes those that are not currently regulated.

Further work should be completed. For example, the isolation, and identification of the putative mutagens formed during bioremediation will allow for remediation guidelines to be more definitive, particularly in regards to non-regulated toxic, compounds. Once the mutagens are identified, the testing of a reconstituted mixture of such compounds could be employed to assess the likelihood of synergistic or antagonistic phenomena in the complex contaminant mixtures. It is hoped that additional information concerning the genetics of the organisms controlling the biodegradation of HMW PAHs will lead to an ability to direct cultures to exclusively utilize productive degradation routes, and thus enhance the microbial degradation potential and removal of (geno)toxic hazards.

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