Mutagenicity of soil from an old gasworks site during bioremediation.

By

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Abstract

PAHs and nitroaromatic compounds are toxic, mutagenic and carcinogenic environmental pollutants that are prevalent in petroleum contaminated soil and pose a serious threat to environmental and human health. Bioremediation is an economic and efficient means to remediate contaminated soil. This study examined the mutagenicity of soil from an aged gasworks site as it underwent bioslurry treatment. Soil extracts were separated into two fractions, one containing PAHs (non-polar neutrals) and the other, *N*-heterocyclic and oxy-PAHs (polar aromatic) compounds. Each fraction was tested with and without metabolic activation. Overall, the mutagenicity of both fractions in the presence of metabolic activation demonstrated a net increase. Only the direct mutagenicity of the polar aromatic fraction exhibited a net decrease. The expected mutagenicity, the summed mutagenicity of the individual chemicals in each fraction, greatly underestimated the direct mutagenicity of both fractions. Only in the presence of metabolic activation did the predicted values exceed the observed. It is possible that the unexpected mutagenicity is the result of bacterial degradative by-products suggesting that mutagenicity monitoring of contaminated sites is required during bioremediation.

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List of Abbreviations

2-ADNT	2-amino-4,6-dinitrotoluene
4-ADNT	4-amino-2,6-dinitrotoluene
ADNT	Aminodinitrotoluene
alkyl-PAH	Alkylated polycyclic aromatic hydrocarbon
ANT	Anthracene
B(a)P	Benzo(a)pyrene
B(b)F	Benzo(b)fluorene
B(ghi)P	Benzo(ghi) perylene
B(k)F	Benzo(k)fluorene
°C	Degrees in Celius
<i>cnr</i>	The <i>Salmonella typhimurium</i> nitroreductase gene found in YG1041 and YG1042
DANT	Diaminonitrotoluene
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DOC	Dissolved organic carbon
EPA	Environmental Protection Agency
<i>E. coli</i>	<i>Escherichia coli</i>
g	Grams
HADNT	Hydroxyaminodinitrotoluene
HPLC	High pressure liquid chromatography
HMW	High Molecular Weight
IP	Indeno(1,2,3-cd)pyrene

kg	Kilograms
L	Litres
LMW	Low Molecular Weight
M	Moles/litre
mg	Milligrams
MPa	Mega Pascals
<i>mucA</i>	Gene that encodes the translesion polymerase PolRI
<i>mucB</i>	Gene that encodes the a protein required by the translesion polymerase PolRI
mm	Millimetre
mM	Millimolar
<i>N</i> -heterocyclics	An aromatic compound containing a nitrogen atom
NAD	Nicotinamide adenine dinucleotide
<i>nfsA</i>	A gene encoding a <i>Salmonella</i> nitroreductase
ng	Nanograms
nm	Nanometres
<i>O</i> -heterocyclics	An aromatic compound containing an oxygen atom
OAT	<i>O</i> -acetyltransferase
ox.	Oxidation
oxy-PAH	Oxygenated polycyclic aromatic hydrocarbon
p213MA	A plasmid contained by CFA509
pAQ1	A plasmid contained by TA102 that confers the ability to detect DNA cross-linking agents
PAH	Polycyclic Aromatic Hydrocarbons

PAC	Polycyclic Aromatic Compounds
<i>P. chrysosporium</i>	<i>Phanerochaete chrysosporium</i>
pCCOMA	A plasmid contained by CFT509 and CBT409
PETN	Pentaerythritol tetranitrate
pKM101	A plasmid encoding the <i>mucA</i> and <i>mucB</i> genes and increases a strains sensitivity to UV light and chemicals
PolRI	An error prone translesion bypass DNA polymerase
pYG233	A plasmid present in YG1041 and YG1042 that harbours the <i>cnr</i> and <i>OAT</i> genes
red.	Reduction
rev/mg	Revertants/mg
rev/g	Revertants/g
<i>rfa</i>	Deep rough mutation
S-heterocyclic	An aromatic compound containing a sulfur atom
sp.	Species
TCA cycle	Tricarboxylic acid
TNT	Trinitrotoluene
μg	Micrograms
μm	Micrometers
μl	Microlitres
UV	Ultraviolet
UVA	Ultra violet A
VBME	Volger-Bonner Medium E
v/v	Volume/volume

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1.0 Introduction

To date 4,341 federally owned contaminated sites exist in Canada, according to the Federal Contaminated Sites Inventory. This inventory does not include properties owned by Crown Corporations, private sectors or some levels of the government. As a result, it is likely that this number of contaminated sites is greatly underestimated. Currently, only 7.6% of these sites are undergoing remediation while 45.9% are still being assessed. These numbers are concerning, in light of the fact that a site must contain a high concentration of a substance that is likely to pose an immediate or long term threat to human health or the environment, in order to be defined as contaminated (135).

Many contaminated sites are associated with industrial processes such as wood preservation and the manufacture of petroleum products, and contain a high concentration of polycyclic aromatic hydrocarbons (PAHs) (144). As environmental pollutants, many PAHs, which have been classified as toxic, mutagenic and carcinogenic, are recognized to pose a threat to human and environmental health (2). In addition to PAHs, nitro-substituted aromatic compounds such as nitroarenes, azaarenes and aromatic amines are also highly prevalent in the environment (17). Most of these compounds are formed during the incomplete combustion of organic matter and have been found in creosote contaminated soil (28, 94).

Over the past few decades, awareness of the potential health and environmental hazards of pollutants has grown. As a result the remediation and reclamation of contaminated land has been the focus of many studies. However, traditional remediation techniques such as incineration are expensive, and are not environmentally sustainable. A less expensive alternative is bioremediation, a process that involves the use of

microorganisms to degrade organic pollutants, ideally to water, carbon dioxide and other inorganic compounds (80).

Several studies have shown an increase in the toxicity of soil contaminants during bioremediation processes (18, 26, 69, 80). However, few of these studies have examined the mutagenicity and genotoxicity of the PAH metabolites during remediation (18, 41, 69, 96, 98). The Ames test, a bacterial reverse mutation assay, has been successfully used to establish the mutagenicity of fractions extracted from polluted soils, before and after remediation (24, 41, 96-98, 138), however little work has been done to evaluate the genotoxicity of soils during remediation. The majority of the work on remediated soils has focused solely on the concentrations of priority PAHs in the soil before and after remediation (14, 47, 72, 77, 80, 82, 93, 108, 121, 126, 139), or on the mutagenicity of individual priority PAHs (27, 35, 37, 45, 62, 63, 65, 74, 76, 79, 90, 92, 95, 100, 116, 118, 119, 136, 151-153). Little is known about the mutagenicity of soil while actually undergoing bioremediation.

1.1. Polycyclic Aromatic Hydrocarbons

Polycyclic aromatic hydrocarbons (PAHs) represent a heterogeneous group of hazardous organic chemicals comprised of two or more fused benzene rings existing in linear, angular and cluster configurations (Figure 1.0). By definition, PAHs consist of only hydrogen and carbon atoms. In practice, however, heterocyclic aromatic compounds can be formed via the substitution of oxygen, nitrogen and sulfur in the benzene ring. PAHs are found throughout the environment and can be formed during both natural and anthropogenic processes when organic material undergoes combustion. Natural processes such as volcanic eruptions and forest fires may produce significant quantities of PAHs.

However, many industrial sites involved in the production and use of fossil fuels and petroleum-derived products, also result in the production of high concentrations of PAHs (Table 1.0). Industrial sites used for the gasification/liquification of fossil fuels, production of coke, asphalt and coal-tar, treatment and preservation of wood and processing of fuel have all been associated with the production of high concentrations of PAHs (144).

PAHs are persistent environmental pollutants and their lipophilic nature enables them to readily bind to organic matter. However, the physico-chemical properties of PAHs are very diverse, depending on the ring number, arrangement and the constituents(144). PAHs of a high molecular weight (HMW), ie. PAHs with 4, 5, or 6 benzene rings, are more stable, hydrophobic, and difficult to remove from the environment (71). A relationship has also been observed between the number of benzene rings and genotoxicity, with genotoxicity increasing with the number of benzene rings (33). Low molecular weight (LMW) PAHs (PAHs consisting of two or three benzene rings) are more volatile and water-soluble, rendering the contaminants more accessible to microorganisms. As such, the degradation of low molecular weight PAHs has been found to occur more easily and efficiently than the degradation of HMW PAHs (144).

The general remediation of soils contaminated with PAHs can be challenging due to the high affinity of these compounds for organic matter, which may reduce their availability for degradation (71). As a result, PAHs are able to persist indefinitely in the environment, such that PAH contamination is a serious health and environmental threat (80).

Figure 1.0: Common polycyclic aromatic compounds. Figure taken from Priority substances list assessment report: Polycyclic aromatic hydrocarbons (3).

acenaphthene **anthracene** **benz[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**

Table 1.0: The concentrations of selected PAHs found in contaminated soil sites.

	Toxic PAH Dose* (mg)						Ref
	B(a)P	ANT	BkF	BbF	IP	B(ghi)P	(3)
	1.0	0.83	4.15	1.0	4.15	4.15	
Site	Concentration in Soil (mg/kg)						
Manufactured Gas Plant	95.4	-	38.1	40.1	24.6	-	(7)
Wood Treatment Plant	21	114	-	-	-	-	(139)
Manufactured Gas Plant	158	52	126	129	139	167	(121)
Coke Oven Site	66	48	41	68	58	47	(106)
Manufactured Gas Site	38.2	37.1	12	28.5	23.1	17.2	(123)
Manufactured Gas Plant	2.8	14.0	1.0	7.2	2.3	1.6	(47)

* The highest dose at which rats developed evidence of lung cancer after the implantation of the PAH in the lungs as determined by the Canadian Environmental Protection Act.

- Denotes an unavailable value.

The carcinogenic and genotoxic properties of PAHs have been studied extensively in experimental rodent models. Absorption of PAHs can occur via the skin, lungs, and gastrointestinal tract, leading to the distribution of the compounds throughout the body (107). Exposure to PAHs has been shown to lead to mutations, and in some cases, cancer (38). However, many PAH compounds are considered to be relatively inert and only become genotoxic or carcinogenic through metabolic activity. Once metabolized, PAHs become water soluble, highly reactive and able to covalently bind to DNA. If adduct formation occurs, and is not repaired, mutations may occur (107). In addition to carcinogenicity and genotoxicity, a variety of studies have documented a range of ecotoxicological effects caused by PAHs in a variety of organisms, including aquatic and terrestrial plants and mammals, amphibians, microorganisms, reptiles and birds (Table 1.1)(38).

Table 1.1: Ecotoxicological effects associated with PAH exposure as observed in field population studies in fish (Adapted from the Priority Substances List Assessment Report (3)).

Species	Location	Observed Effects	Frequency of Affected Organisms (%)	Ranges of conc. in sediments (µg/g dry weight)*
White Sucker, <i>Catostomus commersoni</i>	Hamilton Harbour, Ontario	Papillomas	30	B[a]P >1; Flu > 10
Brown Bullhead, <i>Ictalurus nebulosus</i>	Black River, Ohio	Liver tumours	25 to 30	B[a]P >5 ng/g in tissues; Flu > 1 µg/g in organs
Rainbow Trout, <i>Oncorhynchus mykiss</i>	Hamilton Harbour, Ontario	Hepatic Neoplasms	8.9	Phe: <40.8; Flu: <52
Walleye, <i>Sitostedion vitreum</i>	Detroit River, Michigan	Hepatic neoplasms	20	B[a]P: 0.12 to 17.7; Flu: 0.12 to 34.6; Pyr: 0.12 to 38.8

*B[a]P – Benzo[a]pyrene; Flu – Fluorene; Phe – Phenanthrene; Pyr - Pyrene

1.2 Degradation of Polycyclic Aromatic Hydrocarbons

Industrial sites involved in wood treatment and preservation processes, and the production, use and disposal of petroleum are highly contaminated with PAHs, with concentrations upwards of 10 000 mg/kg of soil (58, 72, 82, 144). Unfortunately, the lipophilic nature of PAHs makes soil remediation difficult. PAHs have a high affinity with organic material, and the longer PAHs remain in contact with soil, the harder they are to extract and degrade (5, 6, 9, 43, 61). This process is referred to as aging. Essentially, PAHs continuously diffuse into the soil matrix as long as contact is maintained. The longer the contact with the soil, the more inaccessible the PAHs become. As such, aging can render PAHs unavailable for degradation while also preventing the compounds from leaching into the water decreasing the toxicity and genotoxicity (104). Prior to aging PAHs in the soil undergo volatilization, photooxidation, chemical oxidation, bioaccumulation, leaching and microbial degradation (33). Some of these processes have been used in the remediation of PAH contaminated soils

1.2.1 Photo-oxidation of PAHs

PAHs have large, highly, conjugated π -orbital systems; as a result PAHs absorb UVA (320-400 nm) and visible (>400 nm) light. PAHs with three or four aromatic rings absorb UVA light, while PAHs with five or more rings are known to absorb visible light. Three or four ringed PAHs substituted with amine-, hydroxyl- or nitro- constituents are able to absorb visible light as well. Once a photon is absorbed, PAHs reach excited singlet or triplet states and may undergo electron or energy exchange with oxygen, solvents or cellular biological molecules to form reactive species. PAHs in an excited state may also react with oxygen or other molecules to produce reactive intermediates. In

addition to the production of highly reactive singlet oxygen, PAH photon absorption may initiate photo-oxidation and generate new reactive chemical species. These reactive intermediates are toxic and react with the cell membrane, proteins or nucleic acids (86, 150).

1.2.2 Chemical Degradation of PAHs

Although PAHs are highly reactive to light and oxidation, their reactivity to chemicals decreases in the soil as a result of their tendency to sorb to organic material (84). However, photooxidation products such as singlet oxygen, organic peroxides, hydrogen peroxide, ozone and peroxy, alkoxy, and hydroxyl radicals may initiate PAH oxidation. Inorganic salts and oxides may also be responsible for the generation of some of the previously mentioned compounds (80). Oxidation reactions of pyrene, benz[a]anthracene, acenaphthylene, and acenaphthene have been studied (114, 115, 148, 149). Typically, the oxidation reaction of PAHs with hydroxyl radicals occurs via one of two reaction pathways. (1) The hydroxyl group may interact with alkyl substituents via the removal of a hydrogen atom or by the addition of a hydroxyl radical at the location of a double bond. (2) A hydroxyl group may be added to the aromatic ring to generate a hydroxycyclohexadienyl radical (114). Ozonation generally occurs via ring cleavage at the double bond or via hydrolysis, if water is present, to form hydroxyl radicals (148, 149). However, both oxidants produce a wide variety of toxic and genotoxic ketones, quinones, aldehydes, phenols and carboxylic acid compounds (114, 115, 148, 149).

1.2.3 Biological Degradation of PAHs

Of particular interest in this thesis is the use of soil microorganisms to transform PAHs to inorganic end products such as water and carbon dioxide or harmless organic

compounds (33). Three main systems of bioremediation have been used to degrade PAHs: *In situ*, prepared bed/on-site, and bioreactors. These systems may be used individually or in combination (144).

In situ bioremediation describes a remediation technique in which the polluted soil remains at the site of contamination. Generally, *in situ* bioremediation includes the addition of nutrients, oxygen and, in some cases, microorganisms capable of degrading the contaminant (144). Success of *in situ* bioremediation relies heavily on the permeability of the soil. Several studies on PAH biodegradation have employed *in situ* remediation (15, 46, 99). Overall, the published studies indicated that LMW PAHs could be degraded *in situ* with and without the addition of nutrients and oxygen; however, HMW PAHs underwent little degradation (144).

Prepared bed/on-site remediation involves the addition of waste slurry to a soil bed lined with low permeable material like clay or polyethylene. Similar to *in situ* remediation, prepared bed/on-site remediation techniques involve the addition of nutrients, oxygen and moisture to the soil. Indigenous as well as specially adapted microorganisms may also be added to the soil. Tilling may be employed to maintain soil aeration; the pH of the soil is manipulated to ensure optimum conditions are maintained. Composting is a second prepared bed remediation strategy that has been used to treat highly polluted material. Fertilization, irrigation, aeration and pH are strictly managed to maintain optimum conditions. Specifically, composting involves the successive addition of mesophilic and thermophilic microbes, the use of an organic bulking agent, and the piling of the soil (144).

Several studies have shown a moderate level of success with the use of prepared bed/on-site remediation strategies on PAH contaminated soil (21, 22, 46, 103, 128, 141, 142). Overall, prepared bed/on-site remediation was found to degrade LMW PAHs relatively efficiently compared to *in situ* remediation. However, the degradation of HMW PAHs was limited and time consuming (144).

The use of bioreactors involves the excavation of PAH contaminated soils to a reactor for treatment. Water is added to the soil to produce a slurry, which is then treated in the reactor. Microbes able to degrade the pollutant are also added to the reactor. In order to enhance the microbial degradation of contaminants within the soil a variety of parameters are manipulated during bioremediation. Nutrients, such as nitrate and phosphate are often added to the slurry (82). In addition, the concentration of oxygen as well as carbon dioxide can be monitored and controlled. Some form of slurry agitation is generally incorporated as well, as it increases the bioavailability of the contaminants as well as the rate of biodegradation (73). Bioreactors can be operated in two different modes batch or continuous. Most often batch is chosen as degradation is enhanced by the presence of bacteria that is acclimatized to the conditions of the reactor as well as the contaminants (144). Bioreactors offer a high level of control over the bioremediation conditions; ensuring optimal conditions are maintained throughout the process. In theory, this should enhance the remediation process.

Studies employing bioreactors for the degradation of PAHs in soil have shown an increased rate, and degree, of PAH degradation relative to prepared bed/on-site treatment (144). However, like all of the treatments previously reported, the degradation of HMW PAHs is limited (80, 82, 102). HMW PAHs are more resistant to microbial degradation

as the higher the molecular weight and octanol-water partition (K_{ow}) coefficient render these compounds less water-soluble or bioavailable (33). Not only are these compounds highly recalcitrant, few microorganisms are capable of utilizing HMW PAHs as a sole carbon source (144). As such these compounds pose a problem for bioremediation.

Bioremediation strategies are based on the successful use of microbes to degrade contaminants. Information on the microbes, enzymatic pathways, associated genes and environmental conditions required for pollutant degradation is needed. In order to ensure success many different species of bacteria, fungi and algae are used to metabolize PAHs (33). Microbial degradation of PAHs occurs in one of two ways: the contaminant is used as the sole source of carbon and energy, or the partial or complete degradation of the pollutant occurs concurrently with the degradation of an essential, life supporting compound, such as a carbon source. The latter process is known as co-metabolism or co-oxidation depending on the nature of the process (144).

Microbes capable of using PAHs as their main carbon and energy source are preferable during soil bioremediation since the complete mineralization of PAHs to water and carbon dioxide may occur. This process occurs with a number of naturally occurring bacterial genera: *Aeromonas*, *Alicalicogenes*, *Bacillus*, *Beijerinckia*, *Corynebacteria*, *Cyanobacter*, *Flavobacterium*, *Micrococcus*, *Mycobacterium*, *Nocardia*, *Pseudomonas*, *Rhodococcus* and *Vibrio*. However, complete mineralization occurs mostly with LMW PAHs. In contrast, co-oxidation is generally associated with the degradation of HMW PAHs. However some bacteria capable of completely mineralizing HMW PAHs have been isolated. *Rhodococcus* sp., *Pseudomonas paucimobilis*, and *Alicalicogenes denitrificans* are all capable of degrading specific HMW PAHs (144).

The biochemical pathways involved in the bacterial degradation of LMW PAHs, such as naphthalene, phenanthrene, anthracene and acenaphthene, are well documented (Figures 1.2 and 1.3) (33). Biodegradation of PAHs depends largely on the incorporation of oxygen into the benzene ring structure via enzymatic reactions. These enzymes are compound specific; thus the remediation of a contaminated site may require the addition of a wide range of microbes capable of degrading a variety of compounds. The group of compound specific enzymes responsible for the activation and initiation of PAH oxidation are called oxygenases. Specifically, bacteria tend to use a group of oxygenases called dioxygenases. Dioxygenases are responsible for the formation of dioxethanes via integration of two oxygen atoms across the double bond of a PAH compound. The dioxethanes are further oxidized to form *cis*-dihydrodiols. Subsequently, the *cis*-dihydrodiols undergo dehydration to form catechol, gentisic acid or protocatechuic acid. The specific intermediate formed depends on the position of the hydroxyl groups in the dihydrodiols, which can be either *meta* or *para*. However, catechol is the most commonly formed intermediate. Further degradation of these metabolites occurs via five similar pathways, all of which involve an additional ring cleavage to produce succinic, fumaric, pyruvic and acetic acids, and acetaldehyde (Figure 1.1). All of these products may be used by cells to produce amino acids, water and carbon dioxide (144).

Many fungal species are also capable of metabolizing PAHs. In particular, the white-rot basidiomycetes, such as *Crinipellis stipitaria*, *Trametes versicolor*, *Bjerkandera* sp., *Phanerochaete chrysosporium*, and *Pleurotus ostreatus*, the brown-rot basidiomycete *Lentius lepideus*, the zygomycete *Cunninghamella elegans*, and the ascomycetes *Aspergillus niger* and *Penicillium* sp. are capable of degrading a wide range of PAHs,

including some of the HMW PAHs. Fungi capable of utilizing PAHs as their sole carbon source have not yet been found. However, fungal metabolism of PAHs often produces chemically reactive and water-soluble metabolites that can subsequently be degraded by indigenous soil bacteria. PAHs can also become detoxified and less available via fungal humification (34).

Fungal metabolism of PAHs can occur within the cell or outside of the cell depending on the enzymatic pathway. The cytochrome P450 pathway is an intracellular pathway in which monooxygenase enzymes catalyze the incorporation of one atom of molecular oxygen into the aromatic ring, resulting in an arene oxide intermediate while the second oxygen atom is reduced to water. Two pathways are possible for the arene oxide: it may undergo further degradation by epoxide hydrolase to form a *trans*-dihydrodiol or it may be rearranged to form a phenol (Figure 1.4). This pathway, similar to mammalian metabolism, is highly regio- and stereoselective (33, 34).

A widely studied extracellular PAH degradation pathway uses lignin and manganese peroxidases as well as extracellular laccases to oxidize a variety of PAHs. Typically, the PAH undergoes oxidation to generate aryl cation radical intermediates. These intermediates are further degraded to quinones and ultimately carbon dioxide (34).

Figure 1.1: Bacterial catabolism pathway for polycyclic aromatic compounds. Figure taken from Cerniglia, 1992 (33).

Figure 1.2: The upper pathway of the bacterial degradation of naphthalene as taken from Habe and Omori, 2003 (57). Catabolism of naphthalene begins with the addition of an oxygen atom to the aromatic ring via a dioxygenase. *Cis*-naphthalene dihydrodiol (Structure 1) is generated. This compound is dehydrogenated to produce 1,2-dihydroxynaphthalene (Structure 2). Meta-cleavage of 1,2-dihydroxynaphthalene by a dioxygenase generates 2-hydroxy-2*H*-chromene-2-carboxylase (Structure 3). An isomerase then creates *trans*-*o*-hydroxybenzylidenepyruvic acid (Structure 4). This compound is converted to salicylaldehyde (Structure 5) by a hydratase-aldolase. The last step of the upper naphthalene degradation pathway is the conversion of salicylaldehyde to salicylate (Structure 6) via salicylaldehyde dehydrogenase (57).

the transfer of succinyl-CoA to β -ketoadipic acid (Structure 14) resulting in β -ketoadipyl-CoA (Structure 15). Finally, the second pathway ends with the conversion of β -ketoadipyl-CoA (Structure 15) to succinyl-CoA (Structure 16) and acetyl-CoA (Structure 17) via β -ketoadipyl-CoA thiolase. A second lower pathway exists for naphthalene (B). Initially, naphthalene (Structure 1) is converted to gentisic acid (Structure 18) by the addition of a hydroxy group via a hydroxylase. Maleylpyruvic acid (Structure 19) is then generated by a dioxygenase. An isomerase then converts maleylpyruvic acid (Structure 19) to fumarylpyruvic acid (Structure 20). Finally, fumarylpyruvate hydrolase catalyzes the hydrolysis of fumarylpyruvic acid resulting in pyruvic acid (Structure 22) and fumaric acid (Structure 21) (57).

Figure 1.3: The lower catechol (A) and gentisic (B) pathways of naphthalene as taken from Habe, and Omori, 2003 (57). Pathway A: Salicylic acid (Structure 1) undergoes conversion to catechol (Structure 2) via salicylate hydroxylase. Once this reaction occurs two different pathways may be followed depending on the enzyme catalyzing the reaction. The *meta* cleavage pathway starts with the incorporation of two oxygen atoms into the aromatic ring via catechol 2,3-dioxygenase to generate 2-hydroxymuconic-semialdehyde (Structure 3). Hydrolysis of 2-hydroxymuconic-semialdehyde results in 2-oxo-4-pentenoic acid (Structure 6). Alternatively, 2-hydroxymuconic-semialdehyde (Structure 3) can be oxidized via a dehydrogenase to create 2-hydroxymuconic acid (Structure 4). An isomerase then converts 2-hydroxymuconic acid (Structure 4) to 4-oxalocrotonic acid (Structure 5). Finally, a decarboxylase removes a molecule of carbon dioxide resulting in 2-oxo-4-pentenoic acid (Structure 6). Once again the pathway converges and 2-oxo-4-pentenoic acid is converted to 4-hydroxy-2-oxovaleric acid (Structure 7) by a hydratase. An aldolase then catalyzes the conversion of 4-hydroxy-2-oxovaleric acid (Structure 7) to 4-hydroxy-2-oxovaleric acid (Structure 8) and acetaldehyde (Structure 9). Lastly, acetaldehyde (Structure 9) is converted to acetyl-CoA (Structure 10) by acetaldehyde dehydrogenase. If catechol undergoes *ortho* cleavage a different pathway ensues, generating different intermediates. The addition of two oxygen atoms to catechol (Structure 2) via a dioxygenase results in *cis*-muconic acid (Structure 11). Muconolactone (Structure 12) is then generated by *cis*-muconate lactonizing enzyme. An isomerase then converts muconolactone (Structure 12) to β -ketoadipate-enol-lactone (Structure 13). This is followed by the hydrolysis of β -ketoadipate-enol-lactone (Structure 13) generates β -ketoadipic acid (Structure 14). A transferase catalyzes

Figure 1.4: Fungal degradation pathways for polycyclic aromatic compounds as taken from Cerniglia, 1997 (34).

1.3 Nitroaromatic Compounds

Nitroarenes are found in diesel emissions and are responsible for 90% of the observed mutagenicity of diesel emissions. These compounds are readily formed from PAHs in the presence of nitrogen dioxide and an acidic environment (116, 136) as well as in the presence of metallic oxides in soils (131). Nitroaromatic compounds (Figure 1.5) may also be released into the environment through a variety of commercial and industrial processes. When not disposed of properly nitroaromatic compounds may leach into the soil and groundwater. Nitroarenes possess a molecule of nitrogen bound to two molecules of oxygen. The oxygen atoms are slightly more electronegative than the nitrogen atom; as a result the nitrogen atom carries a partial positive charge and acts as an electrophile. When nitroarenes are present in biological systems, the electrophilic property renders the compounds more susceptible to reduction reactions. The subsequent reduction of the nitro group to an amine generates a variety of highly reactive nitroso and hydroxylamino intermediates. Both of these groups are known to confer mutagenic, carcinogenic and toxic properties to the parent compounds (129).

Azaarenes are found in soils contaminated with oil, coal tar and creosote. As with the nitroarenes, azaarenes are water soluble, mutagenic products of incomplete combustion (143). Unlike nitroarenes, azaarenes incorporate a nitrogen atom within an aromatic ring. Biodegradation of azaarenes has been shown to occur under both aerobic and anaerobic conditions. Typically bacteria and fungi have very slow azaarene biotransformation rates. It depends on the endogenous levels of the enzymes involved in the biodegradation. Biotransformation of azaarenes generally proceeds with the addition of an oxygen atom to the ring by mono- or dioxygenases. Common derivatives of

azaarene metabolism are ketones and mono- and dihydroxylated azaarenes. However, limited data exists on the toxicity of azaarene derivatives. Current information indicates toxicity of azaarenes appears to be decreased through degradation (23).

Azaarenes are planar in structure and as such are able to intercalate with DNA. In addition, the electrophilic nature of some of the biotransformation intermediates, such as diol epoxides, enables these compounds to bind to the nucleophilic carbonyl, amino and sulfhydryl groups of nucleic acids and proteins; in some cases forming DNA adducts which can be processed to yield mutations. Evidence from experiments using the Ames assay suggests that azaarenes are both direct and indirect acting mutagens (23).

Nitroarenes and azaarenes are highly mutagenic and pose a real threat to both environmental and human health (116). These compounds are typically formed during incomplete combustion processes and are highly prevalent in the environment (4, 23, 112, 116, 136). Currently, the only effective treatment of nitroaromatic-contaminated soils is incineration (52). However, incineration is very costly with estimates between \$1500 – \$6000/ton (51). Composting is currently being explored as an alternative method for the remediation of nitroaromatic contaminated soils. Composting is expensive to set up and maintain, and requires long incubation times. Although the addition of a bulking agent, microbial community, oxygen, carbon and nitrogen can also increase the cost of remediation, composting of nitroaromatic-contaminated soils still remains cheaper than incineration (52).

Figure 1.5: The structure of various oxy-PAHs, *S*-, *O*- and *N*-heterocyclics. All structures were taken from ChemIDPlus Advanced (1).

Benzothiophene

Dibenzothiophene

Phenanthro(4,5-bcd)thiophene

Phenanthridine

Carbazole

Azapyrene

Dibenzofuran

1-Indanone

2-Methylantracenedione

1.3.1 Biological Degradation of Nitroaromatic Compounds

Despite the toxicity exhibited by many nitroaromatic compounds, microbial organisms are capable of either degrading or converting these compounds. Much of the mutagenicity generated by nitroaromatic compounds occurs as a result of the reductases used during the microbial degradative processes. Both microsomal cytochrome P-450 reductase and microbial nitroreductases produce nitroso and *N*-hydroxyaminoaromatic compounds (88).

Microbes are known to possess four different strategies for the mineralization of nitroaromatic compounds. The compound may undergo oxidation (Figure 1.6) or reduction (Figure 1.7) to generate a diol or aromatic amine, respectively. The nitro group may be completely transformed to a nitrite via PETN reductase, or only partially reduced to form a hydroxylamine (Figure 1.8). If the nitro group is removed the compounds undergo further degradative processes similar to the ones described for PAHs (see section 1.2.3) (88).

Many bacteria, such as *Pseudomonas putida* and *Moraxella* sp. use oxygenases to remove the nitro group producing nitrite (Figure 1.6). *Pseudomonas putida* possess an oxygenase with a broad substrate range and is capable of degrading alkyl substituted nitroaromatic compounds to catechol and nitrite (88). The oxygenase used by *Moraxella* sp. is limited in substrate specificity and removes the nitrogen atom to yield a nitrite and hydroquinone (130). A second oxidation strategy involves the conversion of the nitroaromatic compounds to a nitrocatechol prior to removal of the nitro group (88).

Nitroreductases are also often employed in the biodegradation of nitroaromatic compound by microbes. The nitro group is first reduced to a nitroso, then to a

hydroxylamino and finally an amine group (Figure 1.7). Oxygenases may further degrade the aromatic amine compound to ammonium and catechol. Bacteria typically use this pathway to degrade nitrobenzoates, nitrotoluenes and nitrophenols. Complete reductive removal of the nitrogen moiety also occurs. The aromatic ring initially undergoes a nucleophilic attack generating a hydride-Meisenheimer complex and nitrite (Figure 1.8). It should be noted that while some bacteria are capable of mineralizing nitroaromatic compounds many are only able to partially degrade these compounds leaving toxic aromatic amine derivatives (88).

TNT is a representative nitroaromatic compound that has been the subject of many biodegradation studies. Currently, several biological based strategies are being explored for the remediation of TNT contaminated soils: bioreactors, composting, land farming and phytoremediation. Bioreactors are used with one of two goals: (1) the mineralization of TNT; or (2) the permanent binding of TNT metabolites to the soil matrix. Both of these goals are achievable, however most treatments require an aerobic degradation phase following an anaerobic phase. Typically aerobic bacteria degrade TNT by reducing the nitro-groups to hydroxylamino or amino groups. Often these TNT derivatives accumulate in the media and react with each other in the presence of oxygen to form azoxytetranitrotoluenes, which are recalcitrant and mutagenic compounds (48). Anaerobic degradation of TNT eliminates the accumulation of azoxytetranitrotoluenes as well as provides more efficient degradation rates. However, TNT quickly undergoes reduction in anoxic environments as a result of low redox potential; thus, anaerobic degradation often leads to the accumulation of triaminotoluene. Bioreactors provide an anoxic environment for the microbial degradation of TNT. In particular, the anoxic

degradation of TNT requires a co-substrate to remove any oxygen generated during biodegradation as well as to provide electrons for the reduction of the nitro group. When TNT contaminated bioslurries are subjected to both anaerobic and aerobic degradation TNT can be mineralized. If TNT contaminated soils undergo only anaerobic treatment the soil accumulates excess carbon from required co-substrates generating a very starchy soil consistency that limits oxygen penetration. In order for the soil to be used for agricultural purposes it must be treated with an aerobic phase following the anaerobic period to remove excess carbon and to replenish the oxygen levels (48).

Composting is a second feasible technology for the removal of TNT from soil. Both the windrow and anaerobic-aerobic composting strategies are highly successful. The anaerobic phase promotes rapid reduction of TNT at low redox potential. This minimizes the formation of toxic compounds that would inhibit microbial degradation. TNT is reduced to amino- and diaminonitrotoluenes during the anaerobic phase. The aerobic phase transforms remaining metabolites, resulting in a decrease of toxicity and mutagenicity. However, little knowledge exists on the microbial communities involved. In addition to the lack of knowledge, composting requires a long incubation time and the cost of set-up and maintenance (48).

A few aerobic bacteria, mostly *Pseudomonas* sp., are capable of degrading TNT. Few bacterial species are capable of using TNT as a sole carbon or nitrogen source, even fewer can mineralize the compound. Typically aerobic bacteria reduce one or two of the nitro groups to hydroxylamino or amino groups and generate a variety of aminoaromatic isomers. These isomers tend to accumulate and react with each other to produce highly mutagenic azoxytetranitrotoluenes (48). Bacterial species capable of aerobically

degrading TNT often possess nitroreductases, enzymes responsible for the reduction of the nitro group to generate ADNT or DANT. Another enzyme, PETN reductase, is also found in some aerobic bacteria capable of using TNT as a sole carbon source. PETN reductase is responsible for the reduction of the nitro groups to produce hydroxyaminodinitrotoluenes (HADNTs) and ADNT. PETN reductase can also reduce the aromatic ring of TNT to generate nitrite and a variety of other unidentified nonaromatic compounds (117). Figure 1.9 illustrates some of the different pathways utilized by aerobic bacterial species.

A variety of fungal species are capable of transforming TNT in both the lab and environment (16). In most cases, TNT degradation by fungi will be favored if nutrients are limited in the environment. The wood white-rot fungus *Phanerochaete chrysosporium*, is the most studied of all the fungal species capable of degrading TNT. An initial step in the degradation of TNT by *P. chrysosporium* and a variety of other fungal species is the reduction of the nitro groups to generate 4-amino-2,6-dinitrotoluene (4-ADNT), 2-amino-4,6-dinitrotoluene (2-ADNT), 4-hydroxylamino-2,6-dinitrotoluene and azoxytetranitrotoluenes. In *P. chrysosporium* this occurs via a plasma membrane redox system. Further degradation of TNT proceeds via the lignin-degrading system, composed of lignin peroxidase, manganese peroxidase, oxidases, reductases, hydrogen peroxidase, veratryl alcohol, oxalate and quinol oxidases. Without this system fungi are unable to mineralize TNT. Under ligninolytic conditions 4-ADNT is further degraded to 4-formamide-2,6-dinitrotoluene. This compound is further broken down to 2-amino-4-formamide-6-nitrotoluene (Figure 1.10). However, *P. chrysosporium* can only degrade for a limited amount of time for two reasons: (1) the accumulation of

hydroxylaminodinitrotoluene products during TNT reduction inhibits the veratryl alcohol oxidase activity of the lignin peroxidase. The veratryl alcohol oxidase activity prevents the lignin peroxidase from inactivation by hydrogen peroxide. (2) TNT is toxic to *P. chrysosporium* at concentrations higher than 20 ppm. Typically, soil from munitions sites contains a much higher concentration of nitroaromatics (48).

Figure 1.6: The bacterial pathway for the oxidative removal of the nitro group from 4-nitrophenol as taken from Marvin-Sikkema and de Bont, 1994 (88).

Figure 1.7: The bacterial pathway for the reductive removal of the nitro group from nitroaromatic compounds as taken from Marvin-Sikkema and de Bont, 1994 (88).

Figure 1.8: The bacterial pathway for the complete removal of the nitro group via the Meisenheimer complex* as taken from Marvin-Sikkema and de Bont, 1994 (88).

Figure 1.9: TNT degradation pathways of various aerobic bacteria as taken from Esteve-Nunez, *et al.*, 2001 (48).

Figure 1.10: The various pathways for the metabolism of TNT by a variety of fungal species as taken from Esteve-Nunez, *et al.*, 2001 (48).

1.4 Genotoxicity Before, During and After Remediation

Many of the currently used remediation techniques are expensive, time consuming and can produce a variety of toxic compounds during the remediation process. While several studies have used bioassays to examine the mutagenicity of a variety of pollutants (27, 35, 37, 45, 62, 63, 65, 74, 76, 79, 90, 92, 95, 100, 116, 118, 119, 136, 151-153), or the mutagenicity or toxicity of soil before and after remediation (7, 26, 41, 66, 67, 69, 110, 123, 127), not many have examined the mutagenicity of the contaminated soil during remediation processes (18, 98, 106). Other studies of soils undergoing bioremediation have focused on the concentration of priority pollutants during the remediation process as opposed to the toxicity or mutagenicity of the soil (14, 47, 72, 77, 80, 82, 93, 108, 121, 126, 139). In order to gain further understanding of the processes involved in the bioremediation of contaminated soil a full chemical analysis, expanding beyond the priority PAHs, of the soil contaminants must be combined with thorough mutagenicity testing throughout the duration of the remediation treatment.

The Ames test is a short-term bacterial reverse mutation assay used to identify mutagens. It is internationally used by government bodies and the scientific community to identify potential carcinogens as the mutagenicity detected using the Ames assay is highly correlated with rodent carcinogenicity (101). The Ames assay uses various *Salmonella typhimurium* strains possessing different types of mutations that prevent the synthesis of histidine. As a result these strains are unable to grow in the absence of histidine. If a mutation occurs within, or close to, the site of the original mutation, histidine production may be restored and colonies (revertants) are able to form in the

absence of exogenously added histidine. Chemical mutagens increase the frequency with which *Salmonella* revertants form (101).

Different *Salmonella* strains have different types of mutations in a variety of genes in the histidine operon; each of these mutations revert through specific compensatory mutational events. Many of the strains also possess additional genetic alterations rendering them more sensitive to various chemicals. For example, a deletion mutation exists in the *uvrB* genes of all of the strains generally used in mutagenicity evaluation. This mutation abolishes nucleotide excision DNA repair, which is capable of recognizing a range of structurally diverse DNA lesions and repairing them in an error free manner. As a result lesions often persist through to replication at which point the lesions must be bypassed in either an error prone or error free manner. A second mutation, *rfa*, weakens the cellular membrane allowing bulky chemicals to pass through the membrane. A few strains also contain plasmids such as pKM101, which harbours the *mucA* and *mucB* genes, which together encode the translesion polymerase PolRI (78). The presence of pKM101 greatly increases the sensitivity of *Salmonella* tester strains to UV and chemicals that produce a range of bulky DNA lesions. The plasmid pYG233, present in YG1041 and YG1042, harbours the *cnr* and *OAT* genes that encode the nitroreductase and acetyltransferase enzymes, respectively, conferring sensitivity to many nitroaromatic compounds (60). Some chemical substances must be metabolized before they become biologically active. Since bacteria do not possess the cytochrome P450 metabolic oxidation system, an exogenous mammalian organ activation system must be added to the bacteria and chemical mixture. The supernatant of rat liver homogenate centrifuged at 9000 Gs (S9) is most commonly used. (101).

Lundstedt performed a detailed study on the bioremediation of an aged soil from a gasworks site at Husarviken in Stockholm, Sweden. Extensive chemical analysis was performed on the soil during the remediation process. Unique to this experiment was the fact that the chemical analysis went beyond the typical 16 EPA priority compounds. A variety of heterocyclic compounds, including oxy-PAHs, known to be generated during the biodegradation of PAHs (Sections 1.2.1 -1.2.3), were monitored throughout the treatment. Not only did the results of this study demonstrate that not all contaminants decrease with time, it also detected the accumulation of a few oxy-PAHs (80, 82). It is hoped that mutagenicity testing with the Ames assay will continue to clarify the ongoing processes involved in bioremediation.

1.4 Hypotheses and Objectives

Current models used in the evaluation of the mutagenicity or toxicity of contaminated sites predicts an additive effect among known contaminants such as the priority PAHs. It is rare that these models take into account the presence of other potentially mutagenic compounds, such as the metabolites generated by microorganisms during bioremediation. It is also assumed that the mutagenicity or toxicity of the soil decreases along with the concentrations of the contaminants. Few studies have actually monitored the concentrations of contaminants outside of the 16 priority PAHs. Even fewer studies have monitored the mutagenicity of the soil as bioremediation progresses. Based on current knowledge it is hypothesized that a decrease in soil mutagenicity will be observed over the duration of the bioslurry conducted by Lundstedt *et al.*, (80, 82). It is also hypothesized that the mutagenicity observed in the soil will be predicted by adding

the mutagenicity of the pure chemicals found in the soil. In order to examine these hypotheses this thesis has the following objectives.

- 1) To assess the mutagenicity of the aged gasworks soil during bioremediation;
- 2) To assess the mutagenicity of selected individual components as identified in the chemical analysis performed by Lundstedt et al (80, 82).
- 3) To determine if the observed mutagenicity can be accounted for by the identified compounds in the soil.

2.0 Methods

2.1 Soil Collection Bioremediation

The soil samples used in this study were obtained from Staffan Lundstedt and were identical to the samples used for chemical analysis in the Lundstedt *et al.*, (2003) study. The samples were collected by Lundstedt *et al.*, from a former gasworks site that was used to produce gas from 1893 to 1972 at Husarvikan in Stockholm, Sweden. Lundstedt *et al.*, also performed the bioslurry, as well. Currently, the site is composed of sandy soil, ash and demolition debris, which is heterogeneously contaminated with coal tar, heavy metals and cyanide. A 150 kg soil sample was taken from a highly contaminated area of the site. It was passed through a 2.5 mm sieve and mixed with 750 L of water in a stainless steel reactor to produce a bioslurry whose dry weight percentage ranged between 15-20% throughout the experiment. Bio-D (Medina Agriculture Products, Hondo, TX, USA) nutrient mixture was added to the reactor in order to maintain constant levels of nitrogen (12 mg/L) and phosphate (2.5 mg/L). At the beginning of treatment, a neutral pH was attained by adding sulfuric acid to the slurry. Throughout the duration of the experiment, the pH ranged between 6.0 and 8.5. A specially cultivated microbial culture with enhanced PAH metabolism was inoculated into the reactor (Deutsche Montan Technologie, Essen, Germany). The bioslurry was incubated at 27°C +/- 2°C for 29 days, with continuous mixing. Oxygen was added to the mixture and a concentration of 6 mg/L was maintained throughout the treatment. Samples were collected in one-liter aliquots at time 0 and after 3, 7, 24 and 29 days of treatment. The samples were stored at -20°C until analyzed. All of the above was performed by Lundstedt *et al.*, (82).

2.2 Extraction and Fractionation

2.2.1 Chemicals

All chemicals used in the extraction and fractionation procedures were obtained from EMD Chemicals Incorporated, Darmstadt, Germany unless otherwise specified.

2.2.2 Extraction Protocol

Remi Gagne performed all of the extractions. Water was removed from samples using a 0.45 μm polyamide filter (Sartolon, Sartorius, Goettingen Germany). Dry soil samples collected by Lundstedt at days 0, 3, 7, 24, and 29 were used. The soil extractions were performed with an ASE 200 Accelerated Solvent Extractor (Dionex Corp., Sunnyvale, CA, USA). For each sample 1 g of soil was mixed with 5 g of anhydrous sodium sulphate and placed in an 11 ml extraction cell. Two different solvents were used during extraction: hexane:acetone (1:1, v/v) followed by methanol:acetic acid (99:1, v/v) to ensure the extraction of the more polar compounds. Extraction with each solvent had identical parameters: 7 minutes of dynamic extraction followed by two five minute cycles of static extraction, all of which occurred at 150°C and 14 MPa. The two extracts were combined and the total of the pooled extract was transferred to a new vial. A stream of nitrogen was used to evaporate the extract, which was re-constituted in 1 ml of hexane(82).

2.2.3 Fractionation Protocol

Remi Gagne performed all of the fractionations. Fractionation of the extracts was performed in 15-mm diameter columns containing 1 g of anhydrous sodium sulphate and 5.0 g of silica gel that was deactivated with 10% water. The silica was deactivated to

ensure all of the PAHs and polar compounds were recovered. Prior to sample application, the columns were rinsed with 20 ml of hexane. Columns containing the samples were eluted with 5 ml of hexane followed by 15 ml of a 3:1 v/v mixture of hexane:dichloromethane and finally 30 ml dichloromethane. The first fraction, containing the aliphatics, was discarded. The second and third fractions were reconstituted in DMSO and stored at 4°C for approximately one month prior to testing. The second fraction was comprised of nonpolar neutral compounds such as PAHs, alkyl-PAHs, and *S*- and *O*-heterocyclics. The third fraction contained polar aromatics such as *N*-heterocyclics and oxy-PAHs (82).

2.3 The Ames *Salmonella*/Microsome Mutagenicity Assay

2.3.1 Reagents, Solutions and Media

The Ames assay protocols, provided by the Environmental Carcinogenesis Division of the USEPA (Research Triangle Park, North Carolina), were followed for all Ames testing including solution and media preparation. These protocols were adapted from Maron and Ames and Mortelmans and Zeiger (87, 101). Table 2.0 describes all media, solutions, and the sources of each reagent used in the assay (Table 2.0). The preparation of soil extracts, and their subsequent fractionation, is described in Section 2.2. All pure chemicals tested were obtained from Sigma-Aldrich Canada Ltd, unless otherwise stated. CAS numbers are given in Table 2.2.

Minimal Glucose Plates

Minimal glucose agar plates were prepared and poured using an IBS Integra Biosciences MediaClave (Chur, Switzerland) and a Technomat automatic plate dispenser (Fernwald, Germany). Each batch of plates consisted of 45 g of agar, 2655 ml of water,

200 ml of 30% dextrose solution, 60 ml of 50X Volger-Bonner Medium E (VBME) Salt solution, and 30 ml of histidine/biotin solution (Table 2.0). Prior to the addition of the histidine/biotin, dextrose and VBME solution, the water and agar were sterilized at 121°C for 20 minutes. Both the dextrose and VBME solution were autoclaved separately prior to the preparation of the plates. The histidine/biotin solution was sterilized via filtration with a 0.2 µm Nalgene 50 mm bottle top filter (Fisher Scientific Ltd).

Table 2.0: Media and Solutions Used in the Ames Assay

Media/Solution	Components	Reagents	Amount	Moles	Source
Minimal Glucose Plates (v/v)	30% Dextrose Solution (v/w)	D-Glucose H ₂ O	300 g 1 L	1.7 M	Sigma-Aldrich Canada Ltd
	Histidine/Biotin Solution (v/w)	L-histidine-HCl d-biotin H ₂ O	52.5 mg 97.6 mg 1 L	0.2 mM 0.4 mM	Sigma-Aldrich Canada Ltd Sigma-Aldrich Canada Ltd
	50X Volger-Bonner Medium E (v/v)	Magnesium sulfate Citric acid monohydrate Potassium phosphate dibasic anhydrous Sodium ammonium phosphate H ₂ O	10 g 100 g 500 g 175 g 1 L	0.04 M 0.5 M 2.9 M 0.8 M	Sigma-Aldrich Canada Ltd Sigma-Aldrich Canada Ltd Sigma-Aldrich Canada Ltd Sigma-Aldrich Canada Ltd
Top Agar (v/w)	Agar	Difco Agar	6 g	NA	Fisher Scientific
	Sodium chloride	Sodium chloride H ₂ O	5g 1 L	0.1 M	EH Stores
S9 Mix	0.2 M Phosphate Buffer pH 7.4 (w/v)	Sodium dihydrogen phosphate	1.7 g	0.01 M	Sigma-Aldrich Canada Ltd
		Disodium Hydrogen phosphate H ₂ O	12.5 g 0.5 L	0.1 M	Sigma-Aldrich Canada Ltd
		G6P	NA	NA	Sigma-Aldrich Canada Ltd
	NADP	Nicotinamide adenine dinucleotide phosphate (NADP)	NA	NA	Roche Diagnostics
	Microsomal Salt Solution (v/v)	Magnesium chloride (MgCl)	40.7 g	0.4 M	Sigma-Aldrich Canada Ltd
		Potassium chloride (KCl) H ₂ O	61.5 g 0.5 L	0.8 M	Sigma-Aldrich Canada Ltd
	Liver S9 Fraction (v/v)	1254 Induced Rat Liver S9 Fraction	NA	NA	Moltox Inc.

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

NA – Not applicable

S9 Metabolic Mixture

The S9 metabolic mixture was prepared just prior to each experiment. One hundred ml of the S9 mixture included 2 ml of the microsomal salt solution, 141 mg (4.1 M) of glucose-6-phosphate, 306 mg (3.9 M) of nicotinamide adenine dinucleotide phosphate disodium salt, 50 ml of 0.2 M phosphate buffer pH 7.4, 43 ml of sterilized water and 5 ml of Aroclor 1254 induced rat liver S9 with protein levels ranging from 35.7 to 43.5 mg/ml (Table 2.0).

2.3.2 *Salmonella typhimurium* Strains

Six *Salmonella typhimurium* strains, TA98, TA100 (11), YG1041, YG1042 (60), CFT509, CFA509 and CBT409 (31) were used to detect mutagens in the soil extracts as well as identify the mutagenicity of the pure chemicals. The characteristics of each strain are described in Table 2.1. TA98, YG1041, CFT509 and CFA509 strains specifically identify frameshift mutagens (11, 31, 60). Strains TA100, YG1042 and CBT409 detect substitution mutations (11, 31, 60). However, YG1041 and YG1042 have been enhanced for the detection of nitroarenes and aromatic amines with an additional plasmid encoding the *Salmonella* nitroreductase gene, *cnr*, and acetyltransferase genes *OAT* (60). CFT509, CFA509 and CBT409 were developed in the Lambert laboratory (Carleton University, Ottawa, Ontario, Canada) and contain plasmids encoding *OAT*, *mucA/B*, and the *E. coli* nitroreductase genes, *nfsA*, enabling these strains to metabolically activate a number of nitro-substituted compounds (31). Strains TA98, TA100 were initially obtained from the Ames group (University of California, Berkeley); Dr. Takehiko Nohmi (National Institute of Health Sciences, Tokyo, Japan) provided strains YG1041 and YG1042.

Prior to experimentation all strains underwent a strain check as described by Maron and Ames (1983) and Mortelmans and Zeiger (2000) to ensure all genetic characteristics described in Table 2.1 were present and functional. Nutrient agar plates supplemented with appropriate antibiotic were used to test for the presence of plasmid. The antibiotic and the required concentration for is each strain is as follows: TA100 and TA98, 25 µg/ml of ampicillin; YG1041 and YG1042, 25 µg/ml of ampicillin and kanamycin; CBT409 and CFT509, 10 µg/ml tetracycline; CFA509, 100 µg/ml ampicillin. The presence of the *rfa* gene was verified by adding 10 µg of crystal violet, a bulky toxic compound, to a nutrient plate and checking for growth inhibition after 24 hours of growth. Deletion of the *uvrB* gene was confirmed by growth inhibition after exposing half of a nutrient agar plate to UV light for 8 seconds at a distance of 30 cm. Histidine and biotin dependence was determined by growth on minimal nutrient plates. Minimal nutrient plates supplemented with 0.1 mg/ml biotin were used to test histidine dependence. Biotin dependence was determined by supplementing minimal nutrient plates with 5 mg/ml of histidine. Each plate was incubated at 37°C for 72 hours. Growth on either plate confirmed dependence (87, 101).

Following strain validation (above), frozen permanent cultures were prepared in 1.5 ml Eppendorf tubes using 850 µl of overnight cultures and 150 µl glycerol to obtain an end concentration of 15% glycerol. All frozen permanents were stored at -80°C.

Master plates were made for strains TA100, YG1042 and CBT409. Each strain was streaked onto nutrient agar plates supplemented with the appropriate antibiotic. Ampicillin (25 µg/ml) plates were used for TA100. YG1042 was grown on plates containing ampicillin (25 µg/ml) and kanamycin (25 µg/ml). Plates containing

tetracycline (10 µg/ml) were streaked with CBT409. All plates were incubated inverted at 37°C for 72 hrs. Plates were stored for up to one month at 4°C (31, 60, 87).

Overnight cultures were inoculated with 100 µl from a frozen permanent (TA98, CFT509, YG1041, CFA509) or with a single colony from a master plate (CBT409, YG1042, TA100).

Table 2.1: Genotype of strains used in Ames Assay (31, 60, 87, 101)

Strain	Mutation	Target DNA	Reversion Event	Plasmids & Additional Mutations	Markers	Use	Positive Controls	
							- S9	+ S9
TA100	<i>hisG46</i>	GGG	Base-pair substitution	pKM101, <i>rfa</i> , <i>uvrB</i>	Requires biotin to grow; ampicillin resistance	For general mutagenicity testing.	Methyl methanesulfonate	2-aminoanthracene
TA98	<i>hisD3052</i>	CGCGCGCG	Frameshift	pKM101, <i>rfa</i> , <i>uvrB</i>	Requires biotin to grow; ampicillin resistance	For general mutagenicity testing.	2-nitrofluorene	2-aminoanthracene
YG1041	<i>hisD3052</i>	CGCGCGCG	Frameshift	pKM101, pYG233, <i>rfa</i> , <i>uvrB</i>	Requires biotin to grow; ampicillin resistance	Sensitive to nitroarenes and aromatic amines compounds	2-nitrofluorene	2-aminoanthracene
YG1042	<i>hisG46</i>	GGG	Base-pair substitution	pKM101, pYG233, <i>rfa</i> , <i>uvrB</i>	Requires biotin to grow; ampicillin resistance	Sensitive to nitroarenes and aromatic amines compounds	2-nitrofluorene	2-aminoanthracene
CFT509	<i>hisD3052</i>	CGCGCGCG	Frameshift	pCCOMA <i>OAT</i> , <i>mucA/B</i> , <i>nfsA</i>	Requires biotin to grow, tetracycline resistance	Sensitive to nitrosubstituted compounds.	2-nitrofluorene	2-aminoanthracene
CFA509	<i>hisD3052</i>	CGCGCGCG	Frameshift	p213MA <i>OAT</i> , <i>mucA/B</i> , <i>nfsA</i>	Requires biotin to grow, ampicillin resistance	Sensitive to nitrosubstituted compounds	2-nitrofluorene	2-aminoanthracene
CBT409	<i>hisG46</i>	GGG	Base-pair substitution	p213MA <i>OAT</i> , <i>mucA/B</i> , <i>nfsA</i>	Requires biotin to grow, tetracycline resistance	Sensitive to nitrosubstituted compounds	2-nitrofluorene	2-aminoanthracene

2.3.3 Protocol of the *Salmonella*/Microsome Mutagenicity Assay

All experiments were performed in a flow hood (Model # BM6-2B-49, Microzone Corporation, Nepean, Canada). *Salmonella* strains were grown overnight in Oxoid nutrient broth (20 ml) at 37°C in a shaking water bath (Gyrotory Water Bath Shaker, Model # GY6, New Brunswick Scientific, Edison, NJ, USA). After a 16-hour growth period was completed the cultures were stored at 4°C prior to the start of the experiment. Top agar tubes were melted using an autoclave (Scientific Prevacuum Sterilizer, Model # SV-120, Steris Amsco Century, OH, USA) and stored in a block heater (Dri-Block, Model # DB-3, Techne Ltd, Duxford, Cambridge, England) at approximately 50°C. One hundred µl of tester strain was added to each soft agar tube. DMSO (VWR Canlab) was used for all dilutions of the extracts and pure chemicals. It should be noted that the amount of DMSO per soft agar tube never exceeded more than 10% of the total soft agar/strain/sample solution i.e. no more than 100 µl of any sample was added to the soft agar tubes for testing. All samples were tested at a minimum of five different dose levels. If metabolic activation was required, S9 mix was prepared the morning of the experiment. For each sample requiring metabolic activation 500 µl of a 0.05 ml/ml S9 mix was added to the tube. The contents of each tube were poured onto a minimal glucose plate, spread to cover the entire surface and set on a flat surface until the soft agar solidified. Each sample was performed in triplicate. All plates were stored in a 37°C incubator for 72 hours. Plates were scored using a colony counter (Protocol RCB, Model # 9000, Synoptics Ltd, UK). Each plate was counted twice following rotation on the platform to ensure the accuracy of the counts.

Positive controls used in the assay were all obtained from Moltox: 2-nitrofluorene and methylmethanesulfate were used when the assays did not include S9 metabolic activation; 2-aminoanthracene was used when samples were tested with S9. Plates including all contents except the test sample and DMSO were used as negative controls. Previous experiments compared negative controls with and without DMSO, no differences in the revertant counts were observed (83, 85).

2.3.4 Mutagenicity Testing of Soil Fractions

The Ames test was performed on the non-polar neutral and the polar aromatic fractions from each soil sample across all days with and without metabolic activation. Five different dose levels, ranging from 1 to 81 mg of soil/plate, were used from each fraction. Doses for the non-polar neutral fraction included 1, 3, 9, 27 and 50 µg/plate. Doses for the polar aromatic fraction consisted of 1, 3, 9, 27 and 81 µg/plate. All doses were determined by initial range finding with the soil extracts. Prior to all experiments a baseline trial was performed with each fraction and strain, to ensure a mutagenic response was not triggered by the solvents used in the extraction and fractionation processes. Linear portions of the dose response curves were used to calculate the mutagenic potency of each fraction for each sample.

2.3.5 Mutagenicity Testing of Pure Chemicals

Pure chemicals chosen for testing were identified in the Lundstedt study; the concentrations of the compounds in the soil sample were identified in that study (82). The majority of chemicals were tested at doses ranging from 0.0001 to 1000 µg/plate. Chemicals that had been identified as priority compounds by the EPA were tested at lower concentrations known to induce a response, as indicated by previous work (53, 54).

All chemicals were tested without metabolic activation. Metabolic activation was only applied to chemicals that had been identified as priority compounds, that induced a direct mutagenic response, or that were found in high (exceeding 100 µg/g of soil) concentrations in the soil. Each chemical, its respective dose range and status of metabolic activation is described in Table 2.2.

2.3.6 Mutagenicity Scoring

Samples were scored as mutagenic if there was at least a 2-fold increase in the number of revertants over the solvent control and a reproducible dose-response curve was generated in one or more strains. All statistical analysis was performed using SAS, a statistical analysis system produced by the SAS Institute Inc (68).

Table 2.2 All chemicals tested in the Ames Assay

Chemical	CAS #	Doses (ug/plate)	Metabolic Activation
1-Indanone	83-33-0	0.1, 1, 10, 100, 1000	- S9
1-Methylnaphthalene	90-12-0	0.1, 1, 10, 100, 1000	- S9
2,3,5-Trimethylnaphthalene	2245-38-7	0.1, 1, 10, 100, 1000	- S9
2,6-Dimethylnaphthalene	581-42-0	0.1, 1, 10, 100, 1000	- S9
2-Methylanthracenedione	84-54-8	0.1, 1, 10, 100, 1000	- S9
2-Methylnaphthalene	91-57-6	0.1, 1, 10, 100, 1000	- S9
4H- Cyclopenta[def]phenanthrene	203-64-5	0.1, 1, 10, 100, 1000	- S9
9-Fluorenone	486-25-9	0.1, 1, 10, 100, 1000	- S9
Acenaphthene	83-32-9	0.1, 1, 10, 100, 1000	-S9 / +S9
Acenaphthylene	208-96-8	0.1, 1, 10, 100, 1000	-S9 / +S9
Anthracene	120-12-7	0.1, 1, 10, 100	-S9 / +S9
Anthracene-9,10-dione	84-65-1	0.1, 1, 10	-S9 / +S9
Benz[a]anthracene	56-55-3	0.1, 1, 10, 100, 1000	-S9 / +S9
Benz[a]anthracene-7,12-dione	2498-66-0	0.1, 1, 10, 100	- S9
Benzo[a]pyrene	50-32-8	0.005, 0.0075, 0.01, 0.02, 0.03, 0.05, 1, 5, 10, 15, 25	-S9 / +S9
Benzo[b]fluoranthene	205-99-2	0.05, 0.1, 0.25, 0.5, 0.75, 1, 2, 5, 10, 15	-S9 / +S9
Benzo[e]pyrene	192-97-2	0.1, 1, 10, 100, 1000	-S9 / +S9
Benzo[ghi]perylene	191-24-2	0.03, 3, 5, 10, 15, 20, 50	-S9 / +S9
Benzo[k]fluoranthene	207-08-9	0.01, 0.05, 0.1, 0.2, 0.3, 0.35, 1, 3, 7, 10	-S9 / +S9
Benzothiophene	11095-43-5	0.1, 1, 10, 100, 1000	- S9
Biphenyl	92-52-4	0.1, 1, 10, 100, 1000	- S9
Carbazole	86-74-8	0.1, 1, 10, 100, 1000	- S9
Chrysene	218-01-9	0.01, 0.05, 0.1, 0.2, 0.3, 0.4	-S9 / +S9
Coronene	191-07-1	0.1, 1, 10	- S9
Cyclopenta[cd]pyrene	27208-37-3	0.1, 1, 10, 100, 1000	- S9
Dibenz[a,h]anthracene	53-70-3	0.1, 0.5, 0.75, 1, 2, 3, 3.5	-S9 / +S9
Dibenzofuran	132-64-9	0.1, 1, 10, 100, 1000	- S9
Dibenzothiophene	132-65-0	0.1, 1, 10, 100, 1000	- S9
Fluoranthene	206-44-0	0.05, 0.1, 0.25, 0.5, 0.75, 1	-S9 / +S9
Fluorene	86-73-7	0.1, 1, 10, 100, 1000	-S9 / +S9
Indeno[1,2,3-cd]pyrene	193-39-5	0.1, 0.5, 0.75, 1, 2, 2.5	-S9 / +S9
Naphthacene-5,12-dione	1090-13-7	0.1, 1, 10, 100	- S9
Naphthalene	91-20-3	0.1, 1, 10, 100, 1000	-S9 / +S9
Perylene	198-55-0	0.1, 1, 10, 100, 1000	- S9
Phenanthrene	85-01-8	0.1, 0.5, 0.75, 1, 2, 3	-S9 / +S9
Pyrene	129-00-0	0.05, 0.1, 0.25, 0.5, 0.75, 1	-S9 / +S9

3.0 Results

3.1 Characterization of the Husarviken Gasworks Site

3.1.1 Soil Extracts and Fractions

Samples were extracted and fractionated from days 0, 3, 7, 24 and 29 soil samples. The first retained fraction, the non-polar neutral fraction, contained PAHs, alkyl-PAHs, *S*-heterocyclics and *O*-heterocyclics (Table 3.0). *N*-heterocyclics and oxy-PAHs were found in the second retained fraction, the polar aromatic fraction (Table 3.1) (82).

3.1.2 Soil Characterization

One hundred and seventeen compounds were identified in the soil (80). High molecular weight PACs containing four, five or six fused aromatic rings were predominately found at the site. Amongst the more abundant compounds, sixteen priority compounds listed by the EPA were found. Concentrations ranged from 2.4 to 420 µg/g (Tables 3.0 and 3.1). High concentrations of several other compounds were also found in the soil. Of the PAHs and alkyl-PAHs, 4H-Cyclopenta[def]phenanthrene (29 µg/g), benzo[*e*]pyrene (110 µg/g), coronene (23 µg/g), and perylene (38 µg/g) were observed; several heterocyclics and oxy-PAHs, such as dibenzofuran (42 µg/g), 9-fluorenone (26 µg/g), anthracene-9,10-dione (24 µg/g), benzothiophene (28 µg/g), carbazole (42 µg/g) and dibenzothiophene (28 µg/g) were also found in relatively high concentrations (82).

3.1.3 Biodegradation of Compounds

Overall, the concentration of most of the compounds in the soil decreased over the duration of the study. Small molecular weight PAHs with only two or three fused rings diminished to approximately 10% of the original concentration in the soil, whereas by

day 29 the concentration of high molecular weight PAHs ranged from 30 to 70% of the original concentration. Alkyl-PAHs appeared to have longer degradation times than their unsubstituted counterparts. For instance, by the end of the bioslurry treatment only 14% of the initial concentration of anthracene remained, whereas 23% of 2-methylanthracene lingered in the soil. The time course of degradation of heterocyclic compounds, with the exception of the *N*-heterocyclics, was similar to PAHs with the same number of fused rings. *N*-heterocyclics seemed to be degraded at a slower rate. While dibenzofuran, an *O*-heterocyclic, and fluorene were degraded at very similar rates, with only 7.5% and 2.6% of the initial concentration remaining in the soil, carbazole, a *N*-heterocyclic, was degraded at a slower rate as compared to its three ringed counterparts, with 18% of its original concentration remaining in the soil. Oxy-PAHs degraded at a slower rate than PAHs with the same number of fused rings, including the parent compounds. Fluorene was degraded at a much faster rate than 9-fluorenone. By the end of the bioslurry treatment 21% of the 9-fluorenone remained while 2.6% of its parent compound, fluorene was left. However, the concentration of some of the oxy-PAHs increased during degradation prior to decreasing (82). For instance naphthacene-5,12-dione, benz[a]anthracene-7,12-dione, and 1-indanone all increased in concentration before decreasing (Table 3.1)(82).

Table 3.0: Chemicals and associated concentrations identified in the non-polar neutral fraction by Lundstedt *et al.*(82)

Compound	Molecular Weight (amu)	Concentration in Soil (µg/g)				
		0	3	7	24	29
1-Methylnaphthalene	142	5.2	2.29	1.87	0.78	0.78
2-Methylnaphthalene	142	7.9	4.42	4.03	2.37	2.37
2,3,5-Trimethylnaphthalene	170	2	1.8	1.4	0.13	0.12
2,6-Dimethylnaphthalene	156	7	2.87	1.75	0.54	0.44
4H-Cyclopenta[def]phenanthrene	190	29	26.39	24.94	8.41	5.8
Acenaphthene*	154	2.4	0.53	0.34	0.01	0.01
Acenaphthylene*	152	29	22.62	21.46	23.49	21.17
Anthracene*	178	70	44.1	36.4	16.8	9.8
Benz[a]anthracene*	228	190	191.9	191.9	117.8	93.1
Benzo[b]fluoranthene*	252	160	163.2	148.8	164.8	137.6
Benzo[k]fluoranthene*	252	130	135.2	132.6	133.9	115.7
Benzo[a]pyrene*	252	120	122.4	116.4	124.8	109.2
Benzo[e]pyrene	252	110	114.4	108.9	114.4	89.1
Benzo[ghi]perylene*	276	84	84.84	78.96	89.04	85.68
Biphenyl	154	2.6	1.14	0.73	0.34	0.31
Chrysene*	228	180	183.6	176.4	126	93.6
Coronene	300	23	22.54	21.62	24.84	23.46
Cyclopenta[cd]pyrene	228	4	3.64	3.16	3.04	2.36
Dibenz[ah]anthracene*	278	28	28.56	26.6	29.12	26.04
Fluorene*	166	44	11.88	8.36	1.8	1.14
Fluoranthene*	202	420	390.6	352.8	197.4	155.4
Indeno[1,2,3,cd]pyrene*	276	100	101	94	105	101
Naphthalene*	128	17	16.15	14.11	12.92	11.9
Perylene	252	38	38.76	37.24	40.28	38
Phenanthrene*	178	330	112.2	59.4	28.71	21.12
Pyrene*	202	290	292.9	272.6	188.5	145

* Denotes compounds identified by the EPA as priority compounds (80).

Table 3.1: Chemicals and associated concentrations identified in the polar aromatic fraction by Lundstedt *et al.*(82)

Chemical	Molecular Weight (amu)	Concentration in Soil (µg/g)				
		Day				
		0	3	7	24	29
1-Indanone	132	0.65	1.12	0.64	0.6	0.66
2-Methylanthracenedione	222	5.5	4.84	3.25	1.93	1.32
9-Fluorenone	180	26	14.56	9.62	6.24	5.46
Anthracene-9,10-dione	208	24	15.6	9.84	6.48	5.04
Benz[a]anthracene-7,12-dione	258	4.3	4.77	4.3	3.14	2.67
Benzothiophene	134	27	28.08	20.25	14.31	13.77
Carbazole	267	32	14.72	9.28	7.04	5.76
Dibenzofuran	168	42	11.34	7.98	3.65	3.15
Dibenzothiophene	184	28	7.84	5.32	1.65	1.15
Naphthacene-5,12-dione	258	4.5	5.45	5.4	5.54	4.28

3.2 *Salmonella* Mutagenicity Testing of Bioremediated Soil from an Old Gasworks Site

3.2.1 Positive and Negative Controls

All experiments were performed with positive and negative controls for each strain. The mean responses for positive and negative controls, and the mean spontaneous reversion frequencies for each strain, across all experiments, are shown in Tables 3.2 and 3.3.

3.2.2 Mutagenicity Testing of Fractionated Soil Extracts

Seven different *Salmonella typhimurium* strains were used to test fractionated soil samples for mutagenicity in the Ames *Salmonella* mutagenicity assay (Table 2.1). The samples tested were obtained from different time points (day 0, 3, 7, 24, 29) of the remediation process. Each soil extract was separated into three fractions based on the polarity of the compounds present in the extract. The initial fraction was discarded as it contained aliphatic compounds that interfered with the gas chromatographic analysis of the PAHs(80). The second and third fractions, the non-polar neutrals and the polar aromatic fractions, respectively, were retained and used for mutagenicity testing.

In general, the polar aromatic fraction elicited a greater mutagenic response, with and without metabolic activation, than the non-polar neutral fraction (Figure 3.4-3.7). However both fractions appeared to test positive more frequently with the strains that detected frameshift mutations (TA98, YG1041, CFT509, and CFA509) than with those strains capable of identifying base pair mutations (TA100, YG1042 and CBT409) (Tables 3.4 and 3.5). TA100 did not detect any mutagenic activity, with or without metabolic activation, in either of the fractions. Metabolic activation (+S9) generally resulted in a

higher proportion of positive and marginally mutagenic responses, as well as higher mutagenic potencies, as compared to analyses carried out in the absence of metabolic activation (-S9) (Figures 3.0 – 3.3).

Table 3.2: Mean positive response and standard error for each *Salmonella* strain with and without metabolic activation

Strain	Metabolic Activation	Positive Control	Dose ($\mu\text{g}/\text{plate}$)	N	Mean Revertants/Plate $\pm$ Standard Error
TA98	- S9	2-Nitrofluorene	2.5	53	191 $\pm$ 3.5
	+ S9	2-Aminoanthracene	1	27	469 $\pm$ 21.3
TA100	- S9	Methyl methanesulfonate	0.4 $\mu\text{l}/\text{plate}$	17	684 $\pm$ 1.9
	+ S9	2-Aminoanthracene	1	17	808 $\pm$ 28.6
YG1041	- S9	2-Nitrofluorene	0.5	51	182 $\pm$ 5.9
	+ S9	2-Aminoanthracene	1	17	456 $\pm$ 10.1
YG1042	- S9	2-Nitrofluorene	0.5	15	402 $\pm$ 17.6
	+ S9	2-Aminoanthracene	1	7	863 $\pm$ 13.9
CFT509	- S9	2-Nitrofluorene	0.5	41	2202 $\pm$ 16.2
	+ S9	2-Aminoanthracene	0.1	23	1927 $\pm$ 28.8
CFA509	- S9	2-Nitrofluorene	0.5	33	793 $\pm$ 14.4
	+ S9	2-Aminoanthracene	0.1	21	1699 $\pm$ 25.2
CBT409	- S9	2-Nitrofluorene	0.5	26	1751 $\pm$ 31.5
	+ S9	2-Aminoanthracene	0.5	23	2159 $\pm$ 21.6

Table 3.3: Mean spontaneous revertants with standard error for each *Salmonella* strain with and without metabolic activation

Strain	Metabolic Activation	N	Mean Revertants/Plate $\pm$ Standard Error
TA98	- S9	82	22 $\pm$ 0.7
	+ S9	60	32 $\pm$ 1.0
TA100	- S9	24	134 $\pm$ 2.4
	+ S9	29	136 $\pm$ 4.2
YG1041	- S9	97	31 $\pm$ 0.7
	+ S9	37	47 $\pm$ 1.5
YG1042	- S9	30	82 $\pm$ 2.0
	+ S9	9	87 $\pm$ 1.4
CFT509	- S9	111	58 $\pm$ 1.5
	+ S9	23	61 $\pm$ 2.3
CFA509	- S9	71	55 $\pm$ 1.9
	+ S9	27	74 $\pm$ 1.9
CBT409	- S9	77	108 $\pm$ 2.9
	+ S9	25	312 $\pm$ 8.7

Table 3.4: Summary of responses for the non-polar neutral fraction with and without metabolic activation

Strain	TA98		TA100		CFT509		CFA509		YG1041		YG1042		CBT409	
	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	M	-	-	-	-	-	-	-	-	+	-	+	-	-
3	+	+	-	-	-	-	-	+	-	+	-	-	-	-
7	M	M	-	-	-	M	-	-	-	+	-	-	+	-
24	+	+	-	-	-	-	-	M	+	+	M	+	-	-
29	+	+	-	-	+	M	+	-	+	+	-	+	-	-

- + A positive response
- M Marginally mutagenic response ie. A reproducible dose response curve was generated in one or more strain.
- Tested negative response

Table 3.5: Summary of responses for the polar aromatic fraction with and without metabolic activation

Strain	TA98		TA100		CFT509		CFA509		YG1041		YG1042		CBT409	
Day	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9	-S9	+S9
0	M	+	-	-	+	+	+	+	+	+	-	+	+	+
3	+	+	-	-	+	+	+	+	+	+	M	+	-	+
7	M	M	-	-	+	+	+	+	+	+	-	+	-	+
24	+	+	-	-	+	+	+	+	+	+	M	+	M	+
29	+	+	-	-	+	+	+	+	+	+	-	+	-	+

+   A positive response
M   Marginally mutagenic response
-     Tested negative response

3.2.3 Mutagenicity Testing of the Non-Polar Neutral Fraction

The highest levels of mutagenic activity were detected using those strains capable of detecting frameshift mutations (Figure 3.0). Minimal direct mutagenic responses were detected with the strains capable of detecting base-pair mutations. CBT409 detected a positive response from the day 7 sample and YG1042 identified the sample from day 24 as marginally positive. The frameshift strains, TA98 and YG1041, exhibited the greatest number of positive responses with and without metabolic activation (Table 3.4). In the absence of metabolic activation only TA98 showed positive or marginally positive responses across all sample days. YG1041 detected a positive response from the day 24 sample. A positive response was elicited by the day 29 sample for all frameshift strains (Table 3.4, Figures 3.0 and 3.2).

When metabolic activation was present a greater proportion of positive and marginally mutagenic responses were obtained (Figures 3.0 and 3.2). Of the base-pair detecting strains only YG1042 detected any positive responses. Sample days 0, 24 and 29 all elicited a positive response from YG1042. No positive responses were observed with CBT409. Each of the strains capable of detecting frameshift mutations detected a positive or marginally positive response from at least one sample day. Positive responses were observed across all days with YG1041. TA98 and CFA509 indicated mutagenicity in the day 3 sample. Marginal mutagenicity was observed from the day 7 sample with TA98 and CFT509. Positive responses from the day 24 sample were detected by TA98 and YG1042. A marginal response was observed with CFA509. Once again, the day 29 sample was positive across nearly all of the frameshift detecting strains; however,

CFA509 and CFT509 tested negative and marginally mutagenic, respectively (Table 3.4, Figures 3.0 and 3.2).

Mutagenic potencies were calculated from the slope of dose response curves using the SAS system. The mutagenic potencies and the associated error are reported in Tables 3.6 and 3.7 and Figures 3.4 and 3.5 for the non-polar aromatic fraction. In the absence of S9 metabolic activation mix, the mutagenic potencies remained fairly low over the duration of the bioremediation. TA98 was the only strain, frameshift strain or otherwise, in which a mutagenic potency could be calculated for each sample day. Activity detected by TA98 appeared to peak at day three of the remediation treatment. However, no other strains demonstrated a similar trend. While the overall mutagenic potencies tended to remain low, increased mutagenic activity was apparent as the bioremediation treatment proceeded. The majority of this activity was detected by the strains sensitive to nitro-substituted compounds. Both sets of strains, those capable of detecting base-pair mutations as well as the strains sensitive to frameshift mutations, indicated an increase in mutagenicity over the later sample days. The mutagenic potencies in the nitro-sensitive strains, CBT409, YG1042, YG1041, CFT509 and CFA509 all increased during the treatment, peaking at days 24 or 29 for most of the strains. Of the base-pair strains CBT409 and YG1042, peaks in mutagenic potency were observed in the day 7 and day 24 samples, respectively. The mutagenic potencies in frameshift strains YG1041 and CFT509 both peaked on day 29 whereas the mutagenic potency in YG1041 spiked at day 24 (Figure 3.4). Addition of S9 metabolic activation mix to the non-polar neutral fraction generated higher mutagenic potencies across most strains at all time points during the days (Figure 3.5 and Table 3.7). As was observed in

the absence of S9, the mutagenic potency of the day 0 sample was also low in most of the strains in the presence of S9. Only mutagens capable of inducing base-pair substitutions were detected at time 0. YG1042 detected a positive response, and the mutagenic potency value obtained with this strain at day 0 was higher than at any other time point using any of the other strains. A 2-fold decrease in mutagenicity was observed in YG1042 by the end of the bioremediation period. It should also be noted that, aside from YG1042, an increase in mutagenic potency, as compared to day 0, was observed in all strains over the course of the bioremediation. Moderate mutagenic potencies, ranging from 4000 to 24 000 revertants per gram, were obtained in frameshift strains. A 3-fold increase in mutagenicity from day 7 to day 29 was detected by CFT509. Oddly, CFA509, a strain identical to CFT509, except for the antibiotic marker, detected a decrease in mutagenic potency. Overall, a cyclic pattern appeared to be generated. The day 7 sample generally elicited the lowest mutagenic response, followed by an increase and then a decrease on subsequent sampling days.

In summary, without metabolic activation the non-polar neutral fraction was not highly mutagenic. However, as the bioslurry treatment progressed, the mutagenicity of the fraction increased. Most of this increased mutagenicity was detected by the frameshift strains containing nitroreductase genes. In the presence of metabolic activation an increase the mutagenic activity of the non-polar neutral fraction was observed in all strains except for CBT409 in which no response was detected. Similar to the direct acting mutagenicity, the mutagenicity following metabolic activation increased with time.

Figure 3.0: The percentage of positive and marginally mutagenic responses elicited by the non-polar neutral fraction in each *S. typhimurium* tester strain, across all sample days, with and without metabolic activation.

Figure 3.1: The percentage of positive and marginally mutagenic responses of the aromatic polar fraction as detected by all strains across all sample days with and without metabolic activation.

Figure 3.2: The percentage of positive and marginal responses for each day across of all strains of the non-polar neutral fraction with and without metabolic activation.

Figure 3.3: The percentage of positive and marginally mutagenic responses across all strains for each sample day of the polar aromatic fraction with and without metabolic activation.

Table 3.6: Direct mutagenic potencies and associated statistics of the non-polar neutral fractions

Strain	Day	Mutagenic Potency (rev/mg)	Standard Error (rev/mg)	Mutation Ratio	r ²	P value
CBT409	0	N.M.	-	-	-	-
	3	N.M.	-	-	-	-
	7	36.854	5.451	4.0	0.8	<0.0001
	24	N.M.	-	-	-	-
	29	N.M.	-	-	-	-
YG1042	0	N.M.	-	-	-	-
	3	N.M.	-	-	-	-
	7	N.M.	-	-	-	-
	24	1.588	0.178	2.0	0.8	<0.0001
	29	N.M.	-	-	-	-
TA98	0	0.308	0.133	2.0	0.3	0.008
	3	5.269	0.759	2.8	0.8	<0.0001
	7	0.550	0.094	2.4	0.7	<0.0001
	24	2.489	0.215	7.0	0.9	<0.0001
	29	2.444	0.133	5.2	0.9	<0.0001
CFT509	0	N.M.	-	-	-	-
	3	N.M.	-	-	-	-
	7	N.M.	-	-	-	-
	24	N.M.	-	-	-	-
	29	21.083	6.146	2.3	0.6	0.01
CFA509	0	N.M.	-	-	-	-
	3	N.M.	-	-	-	-
	7	N.M.	-	-	-	-
	24	6.811	0.622	4.5	0.9	<0.0001
	29	N.M.	-	-	-	-

YG1041	0	N.M.	-	-	-	-	-
	3	N.M.	-	-	-	-	-
	7	N.M.	-	-	-	-	-
	24	2.501	0.315	3.6	0.8	<0.0001	<0.0001
	29	3.239	0.300	5.9	0.9	<0.0001	<0.0001

N.M. – Denotes a non-mutagenic response

Figure 3.4: Direct mutagenic potencies of the non-polar neutral fraction as detected by the *Salmonella typhimurium* tester strains.

Table 3.7: Metabolically activated mutagenic potencies and associated statistics of the non-polar neutral fractions

Strain	Day	Mutagenic Potency (rev/mg)	Standard Error (rev/mg)	Mutation Ratio	r ²	P value
YG1042	0	42.369	7.891	2.5	0.8	0.001
	3	N.M.	-	-	-	-
	7	N.M.	-	-	-	-
	24	16.065	2.675	2.2	0.8	0.0001
	29	24.367	5.409	2.0	0.7	0.001
TA98	0	N.M.	-	-	-	-
	3	4.673	0.741	2.7	0.8	<0.0001
	7	4.207	0.496	2.1	0.9	<0.0001
	24	12.917	1.3775	2.4	0.9	<0.0001
	29	8.952	2.725	2.4	0.6	0.01
CFT509	0	N.M.	-	-	-	-
	3	N.M.	-	-	-	-
	7	6.764	1.159	2.0	0.7	0.0002
	24	N.M.	-	-	-	-
	29	22.036	6.060	2.0	0.7	0.008
CFA509	0	N.M.	-	-	-	-
	3	9.229	1.542	1.9	0.8	0.0001
	7	N.M.	-	-	-	-
	24	6.338	1.678	2.0	0.6	0.0036
	29	N.M.	-	-	-	-
YG1041	0	N.M.	-	-	-	-
	3	6.745	1.239	2.3	0.7	0.0003
	7	7.503	2.284	3.8	0.5	0.008
	24	11.536	1.455	4.2	0.9	<0.0001
	29	9.331	1.100	3.3	0.9	<0.0001

N.M. – Denotes a non-mutagenic response

Figure 3.5: Mutagenic potency (+S9) of the non-polar neutral fraction as detected by the *Salmonella typhimurium* tester strains.

3.2.4 Mutagenicity Testing of the Polar Aromatic Fraction

The polar aromatic fraction induced mutations in both the base-pair detecting strains as well as the strains capable of detecting frameshift mutations in the absence and presence of metabolic activation (Table 3.5 and Figure 3.1). Overall, the polar aromatic fraction elicited responses that were either negative or marginally positive in the absence of S9 with the base-pair detecting strains CBT409 and YG1042. CBT409 detected a single positive response without metabolic activation from the day 0 sample. The day 24 sample induced marginally positive responses in both strains. All of the frameshift mutation detection strains produced marginal or positive responses without metabolic activation across all sample days. The majority of the positive responses were observed with the strains sensitive to nitro-substituted compounds. However, TA98 only identified sample days 0 and 7 as marginally positive. All other days elicited positive responses.

In the presence of metabolic activation nearly all of the polar aromatic samples tested positive in all of the strains. TA98 identified a marginally mutagenic response from the day 7 sample; otherwise every other response was positive. The sample taken from day 24 of remediation elicited the most responses across all strains with and without metabolic activation (Figure 3.4). Otherwise, a higher number of responses were observed when metabolic activation was present then when it was absent.

Dose response curves were constructed from the data derived from testing the polar aromatic fraction, and the mutagenic potencies of each sample for each strain were calculated (Table 3.8 and Figure 3.6). Overall the strains sensitive to nitro compounds, regardless of the reversion event, detected higher direct mutagenic potencies. CBT409 and YG1042, the strains sensitive to mutagens that induce base-pair mutations, detected

fairly low mutagenic potencies. The mutagenic potency detected by CBT409 did not change over the duration of the bioslurry treatment. While this strain detected mutagenicity from the day 0 and 24 sample, no change was observed. Conversely, the mutagenic potency detected by YG1042 on day 3 increased 4-fold by day 24. Mutagenic potencies could not be calculated for the day 29 sample. Of the strains capable of detecting frameshift mutations, the potencies detected by TA98 remained low throughout the entire duration of the treatment. The potencies detected by the strains sensitive to nitro-substituted compounds were substantially higher and showed much more variation across sample days. CFT509 detected the highest mutagenic potencies across all days until day 29, at which point a 6-fold decrease was observed from the day 0 potency. Moderate potencies were identified by CFA509, the second discrepancy observed between CFT509 and CFA509. While CFA509 detected moderate mutagenic potencies on all days, CFT509 generated potencies that were up to 6-fold higher than those detected by CFA509. Interestingly, the mutagenic potencies obtained with YG1041 and CFA509 seemed to follow a similar pattern across all days. Both strains indicated a net increase in the mutagenicity of the soil. A reduction in mutagenicity was observed in the day 3 sample, followed by a gradual increase until the day 29 sample.

The mutagenic potencies of the polar aromatic fraction were generally higher in the presence of S9 metabolic activation mixture than in its absence (compare Table 3.8 to Table 3.9 and Figure 3.6 to Figure 3.7). Similar to the results obtained when metabolic activation was absent, low mutagenic potencies were obtained in the base-pair detecting strains, CBT409 and YG1042, in the presence of the S9 metabolic activation extract. Both strains appeared to follow a similar pattern of mutagenic potency. A dip in the

mutagenicity was detected by both strains from the day 3 sample. However, unlike YG1042, the potency detected by CBT409 decreased from day 24 to day 29, whereas a net increase in the mutagenicity was observed in YG1042. While the strains capable of detecting frameshift mutations typically yielded higher mutagenic potencies, TA98 detected low mutagenic potencies even with metabolic activation.

The nitro-sensitive strains detected mutagenic potencies that were up to 20-fold greater than those detected by TA98. CFT509 and CFA509 demonstrated the greatest potencies across all days. Both strains followed a similar pattern, with a decrease in mutagenic potency being observed on day 7. However, CFT509 continued to decrease on day 24, although both strains indicated a net increase in the mutagenicity of the soil as compared to the beginning of the bioremediation process. YG1041 detected the highest potency on day 7, with decreasing activity being observed in the day 24 and day 29 samples. However, a net 3-fold increase was observed overall. On the whole, each strain exhibited an overall increase in mutagenicity from day 0 to day 29, a pattern apparent in most strains regardless of fraction and metabolic activation.

In summary the polar aromatic fraction was highly mutagenic with and without metabolic activation in the nitro-sensitive strains. When metabolic activation was present the mutagenic potencies were substantially higher. While the direct acting mutagenicity did not indicate a net increase over the course of the bioremediation, a net increase in the mutagenicity was detected by all strains when S9 metabolic mix was added

Table 3.8: Direct mutagenic potencies and associated statistics of the polar aromatic fractions

Strain	Day	Mutagenic Potency (rev/mg)	Standard Error (rev/mg)	Mutation Ratio	r ²	P value
CBT409	0	3.668	0.357	3.2	0.9	<0.0001
	3	N.M.	-	-	-	-
	7	N.M.	-	-	-	-
	24	3.246	0.417	2.1	0.8	<0.0001
	29	N.M.	-	-	-	-
YG1042	0	N.M.	-	-	-	-
	3	2.789	0.476	2.1	0.7	<0.0001
	7	N.M.	-	-	-	-
	24	11.376	1.586	2.3	0.8	<0.0001
	29	N.M.	-	-	-	-
TA98	0	1.176	0.233	2.4	0.7	0.0002
	3	1.642	0.098	5.1	0.9	<0.0001
	7	0.646	0.046	2.6	0.9	<0.0001
	24	2.677	0.329	4.7	0.8	<0.0001
	29	1.597	0.099	5.7	0.9	<0.0001
YG1041	0	7.431	0.238	21.0	0.9	<0.0001
	3	8.544	0.807	4.0	0.9	<0.0001
	7	3.875	0.746	2.7	0.7	0.0004
	24	4.052	0.521	5.4	0.8	<0.0001
	29	8.816	0.300	17.9	0.9	<0.0001
CFT509	0	41.257	6.502	5.6	0.8	<0.0001
	3	24.271	4.407	7.7	0.8	0.0003
	7	28.357	5.098	4.2	0.8	0.0008
	24	34.968	2.459	6.4	0.9	<0.0001
	29	7.295	1.929	4.0	0.6	<0.0001
CFA509	0	12.051	4.502	4.5	0.4	0.02
	3	13.749	1.194	7.4	0.9	<0.0001
	7	5.725	1.106	4.1	0.7	0.0004
	24	6.238	0.727	6.7	0.8	<0.0001
	29	17.952	1.624	14.6	0.9	<0.0001

N.M. – Denotes a non-mutagenic response

Figure 3.6: Direct mutagenic potency of the polar aromatic fraction as detected by the *Salmonella typhimurium* tester strains.

Table 3.9: Metabolically activated mutagenic potencies and associated statistics of the polar aromatic fraction

Strain	Day	Mutagenic Potency (rev/mg)	Standard Error (rev/mg)	Mutation Ratio	R ²	P value
CBT409	0	4.627	0.503	2.4	0.8	<0.0001
	3	22.690	1.607	3.1	0.9	<0.0001
	7	6.402	0.303	4.5	0.9	<0.0001
	24	23.971	2.701	3.9	0.9	<0.0001
	29	6.072	0.579	4.3	0.9	<0.0001
YG1042	0	14.795	3.479	3.3	0.6	0.0017
	3	18.108	1.070	2.3	0.9	<0.0001
	7	5.068	1.041	2.1	0.6	0.0003
	24	16.763	2.927	3.2	0.8	0.0002
	29	20.427	3.846	3.7	0.7	0.0003
TA98	0	2.827	0.994	2.8	0.4	0.017
	3	4.509	0.727	2.5	0.8	0.0001
	7	2.969	1.107	2.5	0.4	0.02
	24	1.982	0.216	3.7	0.9	<0.0001
	29	4.733	0.505	2.8	0.9	<0.0001
YG1041	0	6.505	0.551	17.1	0.9	<0.0001
	3	23.582	1.827	15.2	0.9	<0.0001
	7	30.142	1.625	15.4	0.9	<0.0001
	24	23.319	1.799	33.0	0.9	<0.0001
	29	20.644	0.882	11.2	0.9	<0.0001
CFT509	0	20.078	2.034	24.2	0.9	<0.0001
	3	101.708	5.681	28.8	0.9	<0.0001
	7	59.703	5.225	14.6	0.9	<0.0001
	24	48.489	4.321	27.4	0.9	<0.0001
	29	68.465	5.659	18.3	0.9	<0.0001
CFA509	0	96.583	18.059	21.6	0.7	0.0003
	3	118.191	6.113	23.2	0.9	<0.0001
	7	80.574	5.737	13.9	0.9	<0.0001
	24	108.315	7.281	20.8	0.9	<0.0001
	29	104.579	5.125	14.2	0.9	<0.0001

Figure 3.7: Metabolically activated mutagenic potency of the polar aromatic fraction as detected by the *Salmonella typhimurium* tester strains.

3.3 Mutagenicity of Pure Chemicals

A select group of chemicals were evaluated using the standard Ames plate incorporation assay (as described in section 2.4.3). Lundstedt *et al* identified one hundred and seventeen chemicals within the soil extracts (section 2.3) (82). Of those chemicals, the concentrations in the soil of only a small portion were determined. From those chemicals, thirty-six were chosen for the Ames assay (Tables 3.0 and 3.1). Three other chemicals, whose concentrations were known, were not available and could not be obtained commercially, and thus were not included in the experiments. Not all of the chemicals were tested with metabolic activation. Chemicals selected for testing with metabolic activation were identified by the EPA as priority compounds or had a high concentration in soil during the remediation process.

Overall, the majority of the chemicals tested with and without metabolic activation demonstrated a negative response (Table 3.10). However, most positive responses were observed when metabolic activation was present. A greater number of positive responses were observed with TA100, a base-pair mutation detecting strain. When metabolic activation was absent, the frameshift detecting strains CFT509, CFA509 and YG1041, sensitive to nitrosubstituted compounds, detected more positive responses. Several chemicals only partially fulfilled the definition of a positive response and were categorized as marginally mutagenic (Section 2.4.6). Dose response curves were constructed for all chemicals using SAS. The mutagenic potency, or the slope of the curve, was calculated for each chemical that demonstrated a positive or marginally mutagenic response (Tables 3.0 and 3.1). In general, higher mutagenic potencies were observed with the metabolically activated samples.

Table 3.10: Summary of responses of chemicals with all *Salmonella typhimurium* strains with and without metabolic activation

Chemical	TA98		TA100		CFT509		CFA509		YG1041		YG1042		CBT409	
Strain	-S9	S9	-S9	S9	-S9	S9	-S9	S9	-S9	S9	-S9	S9	-S9	S9
1-Indanone	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
1-Methylnaphthalene	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
2,3,5-Trimethylnaphthalene	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
2,6-Dimethylnaphthalene	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
2-Methylanthracenedione	-	nt	-	nt	+	nt	+	nt	-	nt	-	nt	-	nt
2-Methylnaphthalene	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
4H-Cyclopenta[def]phenanthrene	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
9-Fluorenone	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
Acenaphthene	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Acenaphthylene	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anthracene	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Anthracene-9,10-dione	-	nt	-	nt	-	nt	-	nt	-	nt	nt	nt	-	nt
Benz[a]anthracene	M	+	-	+	M	M	M	M	M	+	-	nt	-	-
Benz[a]anthracene-7,12-dione	-	nt	-	nt	-	nt	-	nt	+	nt	-	nt	-	nt
Benzo[a]pyrene	-	+	-	+	-	+	-	+	-	+	-	nt	+	+
Benzo[b]fluoranthene	-	+	-	+	-	-	-	-	-	-	-	nt	-	-
Benzo[c]pyrene	-	M	-	-	-	-	-	-	-	-	-	nt	-	-
Benzo[ghi]perylene	-	M	-	M	+	-	-	-	M	M	-	-	-	-
Benzo[k]fluoranthene	-	+	-	-	-	M	-	M	-	-	-	nt	-	M
Benzothiophene	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
Biphenyl	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
Carbazole	-	nt	-	nt	-	nt	-	nt	+	nt	-	nt	-	nt
Chrysene	-	-	-	-	-	-	-	-	-	-	-	nt	-	-
Coronene	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
Cyclopenta[cd]pyrene	+	+	-	+	M	+	+	+	-	+	-	nt	-	+
Dibenz[a,h]anthracene	-	+	-	+	-	-	-	-	-	-	-	-	-	-
Dibenzofuran	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
Dibenzothiophene	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt	-	nt
Fluoranthene	-	-	-	-	-	-	-	-	-	-	-	nt	-	-
Fluorene	-	-	-	-	-	-	-	-	+	-	-	nt	+	-
Indeno[1,2,3-cd]pyrene	-	+	-	+	-	M	-	+	-	-	-	nt	-	-
Naphthacene-5,12-dione	-	nt	-	+	-	nt	-	nt	-	nt	-	nt	-	nt

Table 3.11: Direct mutagenic potencies and associated stats

Strain	Compound	Mutagenic Potency (rev/mg)	Standard Error (rev/mg)	Mutation Ratio	r ²	P value
CBT409	Benzo[a]pyrene	2822.3	382.4	2.3	0.62	<0.0001
TA98	Benz[a]anthracene	75.3	3.60	4.1	0.90	<0.001
	Cyclopenta[cd]pyrene	228.1	45.2	2.2	0.48	<0.0001
YG1041	Benz[a]anthracene	75.7	5.5	4.0	0.85	<0.0001
	Benz[a]anthracene-7,12-dione	1434.0	644.5	2.2	0.18	0.04
	Benzo[ghi]perylene	1632.0	487.6	2.4	0.29	0.002
CFT509	2-Methylanthracenedione	453.5	10.0	7.4	0.98	<0.0001
	Benz[a]anthracene	312.2	4.7	6.2	0.99	<0.0001
	Cyclopenta[cd]pyrene	109.3	6.1	3.5	0.91	<0.0001
CFA509	2-Methylanthracenedione	492.6	23.3	7.4	0.93	<0.0001
	Benz[a]anthracene	362.6	9.2	9.8	0.98	<0.0001
	Cyclopenta[cd]pyrene	165.0	9.1	5.8	0.91	<0.0001

Table 3.12: Metabolically activated mutagenic potencies and associated stats

Compound	Strain	Mutagenic Potency (rev/mg)	Standard Error (rev/mg)	Mutation Ratio	r ²	P value
TA100	Benz[a]anthracene	3.2x10 ⁴	1141.3	2.9	0.97	<0.0001
	Benzo[a]pyrene	8380.0*	1250.0*	2.9*	0.90*	<0.002*
	Benzo[b]fluoranthene	9.1x10 ⁴ *	1.5x10 ⁴ *	1.7*	0.88*	<0.002
	Benzo[ghi]perylene	4323.9	411.5	1.9	0.73	<0.0001
	Cyclopenta[cd]pyrene	1.5x10 ⁵	5634.6	18.7	0.97	<0.0001
	Dibenz[a,h]anthracene	3.2x10 ⁴ *	6850*	1.5*	0.82	<0.005
	Indeno[1,2,3-cd]pyrene	1.1x10 ⁵	3501.5	3.5	0.96	<0.0001
	Naphthacene-5,12-dione	7843.3	376.8	8.2	0.94	<0.0001
	Pyrene	9.1x10 ⁴ *	1.8x10 ⁴ *	1.2*	0.89*	<0.02*
CBT409	Benzo[a]pyrene	5.7x10 ⁴	9307.6	2.3	0.70	<0.0001
	Benzo[k]fluoranthene	2.7x10 ⁴	2387.0	1.8	0.83	<0.0001
	Cyclopenta[cd]pyrene	1.3x10 ⁵	5239.2	5.7	0.97	<0.0001
TA98	Benzo[a]pyrene	3.4x10 ⁴ *	4100.0*	3.2*	0.97*	<0.02*
	Benzo[b]fluoranthene	3080.0*	340*	1.7*	0.96*	<0.003
	Benzo[e]pyrene	165.1	31.5	1.7	0.50	<0.0001
	Benzo[ghi]perylene	1012.6	97.7	3.3	0.80	<0.0001
	Benzo[k]fluoranthene	9630*	790*	3*	0.97	<0.0003
	Cyclopenta[cd]pyrene	1.8x10 ⁵	4235.3	43.2	0.90	<0.0001
	Dibenz[a,h]anthracene	550.0*	110*	1.4*	0.80	<0.003
	Indeno[1,2,3-cd]pyrene	1.7x10 ⁴	1320.6	2.6	0.80	<0.0001
YG1041	Benz[a]anthracene	797.3	97.6	2.7	0.70	<0.0001
	Benzo[a]pyrene	2.9x10 ⁴	3449.4	3.4	0.60	<0.0001
	Benzo[ghi]perylene	3958.3	576.1	1.9	0.68	<0.0001
	Cyclopenta[cd]pyrene	1.7x10 ⁵	2090.5	24.9	0.99	<0.0001
CFT509	Benz[a]anthracene	5901.3	371.5	2.0	0.92	<0.0001
	Benzo[a]pyrene	4.3x10 ⁴	4870.0	5.2	0.83	<0.0001
	Benzo[ghi]perylene	5532.1	756.9	2.0	0.71	<0.0001
	Benzo[k]fluoranthene	5058.0	537.6	1.9	0.76	<0.0001
	Cyclopenta[cd]pyrene	1.8x10 ⁵	6895.0	34.4	0.97	<0.0001
	Indeno[1,2,3-cd]pyrene	3.7x10 ⁴	2992.9	2.6	0.81	<0.0001
CFA509	Benz[a]anthracene	74.7	9.4	9.8	0.65	<0.0001
	Benzo[a]pyrene	3.9x10 ⁴	7114.5	4.6	0.68	<0.0001
	Benzo[k]fluoranthene	7523.3	590.9	6.4	0.88	<0.0001
	Cyclopenta[cd]pyrene	2.1x10 ⁵	4845.0	31.7	0.99	<0.0001
	Indeno[1,2,3-cd]pyrene	6.9x10 ⁴	4422.8	2.7	0.88	<0.0001

* - Data obtained from Melanie Gagnon (53)

3.4 Predicted Mutagenic Potencies

The potencies observed from the *Salmonella* mutagenicity testing of the chemicals were used to calculate the predicted potencies of each soil fraction with and without metabolic activation for each strain (Tables 3.13 – 3.15). Pure chemicals were tested in using the Ames assay. The mutagenic potency was multiplied by the amount of the chemical in the soil. This was performed for each chemical in each strain. The expected mutagenic potency for each chemical was summed according to strain so that each strain had an expected mutagenic potency for each fraction with and without S9.

$$\text{Observed Mutagenic Potency} \times \text{Amount in soil} = \text{Expected Mutagenic Potency}$$
$$\text{Expected Potency of Fraction} = \text{Sum of Expected Potencies of Each Compound}$$

However, no predictions could be made for the polar aromatic fraction with metabolic activation as none of the chemicals available were tested with metabolic activation. These chemicals were not present in high concentrations in the soil and thus were not tested with metabolic activation. Overall, the majority of the observed values were higher than the predicted values. Only the predicted values for the non-polar neutral fraction with metabolic activation greatly surpassed the observed values. The observed values for the non-polar neutral and polar aromatic fractions without metabolic activation were significantly higher than the predicted values. The values presented in the tables were found to be significantly different from the observed values as determined by two-tailed t-tests.

Table 3.13: Predicted and observed mutagenic potencies of the non-polar neutral fraction without metabolic activation

Strain	Day	Observed Values		Predicted Values	
		Mutagenic Potency (rev/g)	Standard Error (rev/g)	Mutagenic Potency (rev/g)	Standard Error (rev/g)
CBT409	0	N.M.	-	338.7	45.9
	3	N.M.	-	345.4	46.8
	7	36854	5451	328.5	44.5
	24	N.M.	-	352.2	47.7
	29	N.M.	-	308.2	41.8
YG1042	0	N.M.	-	N.M.	-
	3	N.M.	-	N.M.	-
	7	N.M.	-	N.M.	-
	24	N.M.	-	N.M.	-
	29	N.M.	-	N.M.	-
TA98	0	308	133	15.2	0.9
	3	5269	759	15.3	0.9
	7	550	94	15.2	0.8
	24	2489	215	9.6	0.6
	29	2444	133	7.5	0.4
YG1041	0	N.M.	-	151.5	42.0
	3	N.M.	-	153.0	42.4
	7	N.M.	-	143.4	39.6
	24	2501	315	154.2	44.1
	29	3239	300	145.2	0.4
CFT509	0	N.M.	-	59.8	0.9
	3	N.M.	-	60.3	0.9
	7	N.M.	-	60.3	0.9
	24	N.M.	-	37.1	0.6
	29	21083	6146	29.3	41.8
CFA509	0	N.M.	-	69.6	1.8
	3	N.M.	-	70.2	1.8
	7	N.M.	-	70.1	1.8
	24	N.M.	-	43.2	1.1
	29	6811	622	34.1	0.9

N.M. – Non mutagenic

Table 3.14: Predicted and observed mutagenic potencies of the non-polar neutral fraction with metabolic activation

Strain	Day	Observed Values		Predicted Values	
		Mutagenic Potency (rev/g)	Standard Error (rev/g)	Mutagenic Potency (rev/g)	Standard Error (rev/g)
CBT409	0	N.M.	-	1.1E+7	1448.2
	3	N.M.	-	1.1E+7	1481.0
	7	N.M.	-	1.1E+7	1416.5
	24	N.M.	-	1.1E+7	1497.1
	29	N.M.	-	9.7E+6	1305.0
YG1042	0	N.M.	-	N.M.	-
	3	N.M.	-	N.M.	-
	7	N.M.	-	N.M.	-
	24	N.M.	-	N.M.	-
	29	N.M.	-	N.M.	-
TA98	0	N.M.	-	2.4E+6	865.9
	3	N.M.	741	2.3E+6	881.5
	7	4207	496	2.1E+6	837.7
	24	8952	2725	2.3E+6	873.4
	29	8952	2725	2.1E+6	769.3
YG1041	0	N.M.	-	4.2E+6	489.2
	3	N.M.	1239	4.2E+6	497.4
	7	7503	2284	3.9E+6	472.3
	24	9331	1100	4.1E+6	499.6
	29	9331	1100	3.6E+6	440.1
CFT509	0	N.M.	-	8.9E+6	1115.3
	3	N.M.	-	9.1E+6	1131.7
	7	6764	1159	8.5E+6	1072.3
	24	22036	6060	9.3E+6	1126.1
	29	22036	6060	8.5E+6	1011.9
CFA509	0	N.M.	-	1.2E+7	1394.0
	3	9229	1542	1.3E+7	1416.8
	7	N.M.	-	1.2E+7	1339.3
	24	N.M.	-	1.3E+7	1447.2
	29	N.M.	-	1.2E+7	1304.3

Table 3.15: Predicted and observed mutagenic potencies of the polar aromatics fraction with metabolic activation

Strain	Day	Observed Values		Predicted Values	
		Mutagenic Potency (rev/g)	Standard Error (rev/g)	Mutagenic Potency (rev/g)	Standard Error (rev/g)
CBT409	0	N.M.	-	N.M.	-
	3	N.M.	-	N.M.	-
	7	N.M.	-	N.M.	-
	24	N.M.	-	N.M.	-
	29	N.M.	-	N.M.	-
YG1042	0	N.M.	-	N.M.	-
	3	N.M.	-	N.M.	-
	7	N.M.	-	N.M.	-
	24	N.M.	-	N.M.	-
	29	N.M.	-	N.M.	-
TA98	0	N.M.	-	N.M.	-
	3	N.M.	-	N.M.	-
	7	N.M.	-	N.M.	-
	24	N.M.	-	N.M.	-
	29	N.M.	-	N.M.	-
YG1041	0	7431	238	6.2	2.8
	3	8544	807	6.8	3.1
	7	3875	746	6.2	2.8
	24	4052	521	4.5	2.0
	29	8816	300	3.8	1.7
CFT509	0	41257	6502	2.5	0.1
	3	24271	4407	2.2	0.04
	7	28357	5098	1.5	0.03
	24	34968	2459	0.9	0.01
	29	7295	1929	0.6	0.01
CFA509	0	12051	4502	2.7	0.1
	3	13749	1194	2.4	0.1
	7	5725	1106	1.6	0.1
	24	6238	727	1.0	0.04
	29	17952	1624	0.7	0.03

4.0 Discussion

4.1 Degradation of PAHs During Remediation

Initial Composition of Soil Prior to Remediation

The chemical composition of the gasworks soil was similar to that found in previous studies (Tables 3.0 and 3.1) (47, 72, 121, 144). Typically these sites contain the 16 PAHs identified on the EPA's priority compound list. In addition to these compounds, a variety of high and low molecular weight PAHs, *O*- and *S*-heterocyclic compounds have also been found at aged gasworks sites (58). Typical of an aged gaswork site was the abundance of four, five and six ringed PAHs; lower molecular weight compounds more easily undergo volatilization and microbial degradation (33). Oxy-PAHs, such as ketones, some of which have been observed in prior studies of petroleum contaminated soils, were also found in the soil (26, 47, 121). However, a portion of these compounds likely originated from the oxidation of PAHs present in the soil. PAHs that contain carbon atoms that are attached to the aromatic ring by a single bond, such as fluorene, possess highly reactive hydrogen atoms and can easily undergo non-enzymatic oxidation (113). For a more detailed discussion of the components of the soil refer to Lundstedt, 2003 and Lundstedt *et al.*, 2003 (80, 82).

Biodegradation of Aged Gasworks Soil

From a chemical composition point of view, the general trends in PAH biodegradation were consistent with earlier work (33, 47, 144). Overall, methylated-PAHs and PAHs with a greater number of rings required longer degradation times whereas unsubstituted compounds containing only two or three rings were degraded quickly and nearly completely (Tables 3.0 and 3.1). Degradation rate is largely dependant on structure. The lipophilic nature of PAHs results in a high affinity to organic

matter. However, individual PAHs often demonstrate significant differences in physiochemical characteristics such as water solubility and vapor pressure (84). As such, low molecular weight PAHs are more water soluble and volatile whereas high molecular weight PAHs are fairly hydrophobic and have a higher affinity to organic matter. Therefore low molecular weight PAHs, comprised of two or three benzene rings, are more susceptible to both biotic and abiotic degradative processes than high molecular weight PAHs, containing four, five or six fused rings (84, 91).

However, PAH structure is not the sole determinant of degradation rates. The physicochemical properties of the media as well as the characteristics of the bacterial community have also been shown to play important roles in determining the rate of degradation (126). Weathering, the process of contaminant diffusion into the organic soil matrix, results in a greater number of sorbed pollutants hindering extraction and degradation processes (61). The longer the length of time that organic compounds have been in contact with soil, the more difficult extraction and degradation is. One observed result of this binding has been a reduction in the toxicity at such sites as the bioavailability of the soil contaminants is limited (5).

In the current study a microbial culture specifically developed for the degradation of PAHs was inoculated into the bioslurry to enhance degradation. Previous work has shown that microbial degradation is largely responsible for the removal of PAHs from sediment and surface soil (33). As such, microbes present in the soil matrix have been shown to influence the rate of PAH degradation (12, 34, 47, 71, 105, 126, 144). Optimal degradation has been observed when a consortium of microbes is present. While many low molecular weight PAHs can be mineralized by a single microbial species (33), high

molecular weight PAHs often require co-metabolism; consequently, metabolism of such compounds requires the presence of a variety of microbes (33, 71). As observed during the bioslurry treatment, the initial rate of PAH degradation was faster than at later time points as the bioavailable compounds decreased quickly leaving the more recalcitrant PAHs (Tables 3.0 and 3.1) (126). The high molecular weight PAHs were degraded slowly, if at all, as was expected if it is assumed that the rate of PAH degradation is inversely proportional to the number of benzene rings present (33, 71).

An interesting phenomenon observed during the remediation treatment was the increase in concentration of two oxy-PAHs identified in the soil, 1-acenaphthenone and 4-oxapyrene-5-one. In addition, many of the oxy-PAHs, such as benz[a]anthracene-7,12-dione and 9-fluorenone, appeared to undergo minimal degradation throughout the duration of the treatment even though the respective parent compounds benz[a]anthracene and fluorene were degraded efficiently. This was unexpected, as oxy-PAHs have been found to be more bioavailable than their parent compounds (89). This suggests that some of the oxy-PAHs were formed during the bioslurry treatment of soil as by-products of the degradation of their parent compounds. This has been observed in previous studies during soil remediation (47, 121). Anthracene-9,10-dione accumulated during a bioslurry treatment of soil from another gasworks site (121). For a more detailed discussion of the biodegradation of the contaminants refer to Lundstedt, 2003 and Lundstedt *et al.*, 2003 (80, 82).

4.2 *Salmonella* Mutagenicity Testing of Bioremediated Aged Gasworks Soil

The direct (-S9) and S9-activated mutagenicity of each sample was examined using a variety of *Salmonella typhimurium* strains (Figures 3.4-3.7, Tables 3.6-3.9). The traditionally used TA98 and TA100 strains have been optimized for the detection of a wide variety of chemicals including PAHs (87). Both of these strains contain the plasmid pKM101, which encodes PolRI - an error prone translesion bypass DNA polymerase that produces modest increases in the spontaneous reversion frequency, and markedly increases the sensitivity of bacterial tester strains to a variety of structurally diverse chemicals. YG1041 and YG1042 were derived from TA98 and TA100 respectively; however these strains also contain acetyltransferase (*OAT*) and nitroreductase genes, increasing their sensitivity to nitroarenes and aromatic amines (60). Recently developed strains CFA509, CFT509 and CBT409 also derived from TA1538 and TA1535, contain a plasmid carrying the *mucAB* genes, the *Salmonella* *OAT* genes and the *Escherichia coli* nitroreductase gene *nfsA* conferring sensitivity to nitro-substituted compounds (31) were also used. In combining all these features on a single plasmid a greater variety of chemicals could be detected.

4.2.1 Non-Polar Neutral Aromatic Fraction

The non-polar neutral fraction consisted of PAHs, alkyl-PAHs, *O*-heterocyclics and *S*-heterocyclics. Over the course of the bioremediation all of these compounds underwent significant decreases in concentration. Considering the nature of the contaminants one would expect to see a great deal of mutagenicity detected by TA98 and TA100, in the presence of metabolic activation, that was not increased significantly in the

nitro-sensitive strains YG1041, YG1042, CFT509, CFA509 and CBT409 with or without metabolic activation. However, this was not the case.

Metabolically Activated Mutagenicity

Most genotoxic substances express their biological activity following metabolism to more reactive intermediates. *Salmonella* tester strains have limited metabolic capacity, and do not possess cytochrome P450 monooxygenases that play a primary role in the metabolic activation of both polycyclic aromatic hydrocarbons and heterocyclic compounds. The addition of an exogenous mammalian activation system, the S-9 microsomal fraction from a rat liver homogenate (2), promotes oxidative metabolism of PAHs that yields epoxides, diols and phenols (101, 107); electrophilic DNA reactive intermediates arise during formation of these products. As expected, the neutral non-polar fraction, containing a variety of PAHs and alkyl-PAHs induced significant S9 activated mutagenicity, particularly in the frameshift strains TA98, CFA509, CFT509 and YG1041 at most bioremediation time points (Figures 3.4 and 3.5)(37). Interestingly, TA100 with metabolic activation did not detect any positive responses in the fraction containing PAHs (87, 95, 101). Other compounds that more readily undergo oxidation could have saturated the S9, reducing the actual potency detected. However experiments performed with TA98 in which the amount of S9 was doubled, detected no significance change in the mutagenicity (83).

The high mutagenic potency detected by YG1042 indicated the presence of very potent mutagenic compounds capable of causing base-pair mutations. However, the fact that YG1042 detected a positive response and TA100 did not, suggests the presence of nitro-substituted compounds such as nitroarenes, or aromatic amines. This is further

supported by the positive responses detected on all days with strains sensitive to nitro-substituted compounds. Such compounds are only slightly more polar than PAHs and somewhat less polar than oxy-PAHs, so it is possible that these compounds were present in both fractions (81). Several *N*-heterocyclic compounds were found in the polar aromatic fraction; however no aromatic amines were identified in either fraction. While aromatic amines are highly mutagenic and ubiquitous environmental pollutants, they often co-elute with the more abundant PAHs making these compounds difficult to identify and analyze (70). Thus it is possible that these compounds were present in the fraction but were below the analytical detection limits. However, it is more likely that the presence of azaarenes triggered the mutagenic responses in the nitro-sensitive strains. These weakly polar, direct and indirect mutagens are capable of generating a response in YG1042 and have been found in fractions extracted from amended PAH-containing bioslurries (67). The presence of azaarenes would also explain the higher mutagenic potencies detected by all of the nitro-sensitive strains.

The low mutagenic potencies demonstrated by TA98 are likely the product of the limited nitroreductase and OAT activity. Previous work has shown that these activities are rate-limiting step in the detection of mutagenic nitroarenes and aromatic amines (60).

Direct Acting Mutagenicity

As was expected a limited amount of direct acting mutagenicity was detected in the non-polar aromatic fraction. This fraction was primarily composed of PAHs, known to be mildly mutagenic, if at all, in the absence of metabolic activation. It is the electrophilic intermediates of PAHs generated during metabolism that induce mutations (107). Initially, only TA98 detected any mutagenic activity, indicating the presence of

compounds capable of inducing frameshift mutations. Midway and towards the end of the bioslurry treatment the nitro-sensitive strains, YG1041, YG1042, CFT509, CFA509 and CBT409 signalled the presence of mutagenic nitro-substituted compounds, azaarenes and/or aromatic amines. It appears that as the PAHs were degraded in the bioslurry, mutagenic nitro compounds were produced. Compounds able to induce frameshift (CFA509, CFT509, YG1041) and insertion/deletion (YG1042, CBT409) mutations were detected (Table 3.6 and Figure 3.4).

The mutagenicity observed in *Salmonella* strains YG1041 and YG1042 implied the presence of nitroarenes and/or aromatic amines (60). However, aromatic amines typically require metabolic activation in order to cause mutations (4) whereas nitroarenes are classified as direct acting mutagens (116). The classification of nitroarenes as direct acting mutagens is somewhat misleading. While these compounds do not require oxidation via cytochrome P450, the NO₂ group of nitroarenes must be reduced to nitroso and hydroxylamino intermediates in order to exert any mutagenic tendencies (116, 129). Both YG1041 and YG1042 contain a plasmid that encodes the *Salmonella* Cnr nitroreductase capable of reducing nitroarenes (60).

Unlike YG1041 and YG1042, the strains CFT509, CFA509 and CBT409 are, in principle, capable of detecting a wider range of nitro-substituted compounds. Each strain possesses a plasmid that carries the genes responsible for the major *E. coli* nitroreductase, *nfsA* as well as the *Salmonella* OAT gene. It has been suggested that the *Salmonella* nitroreductase gene *cnr*, found in YG1041 and YG1042, has limited efficiency and substrate specificity when compared to the *E. coli* nitroreductase, *nfsA* (31, 146). It has also been shown that *nfsA*, a gene responsible for the major *Salmonella* nitroreductase,

is missing from strains with the histidine mutation *hisD3052*. These strains include YG1041 and TA98. When the $\Delta uvrB$ mutations were made to eliminate the nucleotide excision repair system a variety of other genes were deleted as well. Among these genes was the gene for the *Salmonella* nitroreductase, *nfsA* (109). As such the strains CFT509, CFA509 and CBT409 have a wider range of substrates that are capable of inducing a reverse mutation event, indicating the presence of nitro-substituted compounds beyond those detected by YG1041 and YG1042.

Interestingly, compounds able to cause base substitution mutation events were detected (YG1042, CBT409) midway through the bioslurry treatment. This was unexpected as nitroarenes are traditionally associated with frameshift mutations (44, 116, 136). The activity witnessed in the YG1042 and CBT409 strains could be indicative of the presence of methylated nitroarenes, specifically methylated nitroso compounds that are not sterically hindered, i.e. nitro compounds with a single methyl group, which are known to induce base substitution mutations (74, 116). Such compounds have been found to induce greater mutagenicity in base substitution strains than in strains engineered to detect frameshift mutagens, potentially explaining the high mutagenic potencies detected by CBT409 and YG1042 (74).

To summarize, the mutagenicity of the non-polar neutral fraction in the nitro-sensitive strains, with and without S9, was unexpected. It is possible that during fractionation some highly mutagenic nitroarenes, aromatic amines or azaarenes were eluted in this fraction, as these compounds have been identified in PAH contaminated soils (29).

4.2.2 Polar Aromatic Fraction

Metabolically Activated Mutagenicity

The mutagenicity of the polar aromatic fraction, containing N-heterocyclics and oxy-PAHs, was increased by the addition of S9 as compared to the absence of S9 (Figures 3.6 and 3.7, Tables 3.8 and 3.9). This was especially apparent in the strains sensitive to nitroaromatic compounds (YG1041, YG1042, CFT509, CFA509, CBT409). Only a mild increase in mutagenicity was detected by TA98, most likely due the limited substrate specificity of the native nitroreductase (60). Once again no response was detected by TA100. This was surprising, as previous studies have shown oxy-PAHs to be potent base substitution mutagens in the presence of metabolic activation (95).

It is not immediately obvious what chemicals would contribute to the increase in S9 activated mutagenicity in the polar aromatic fraction; as the chemicals monitored analytically and the known biological activity of these compounds cannot account for the observed mutagenicity. The polar aromatic fraction was composed of oxy-PAHs and N-heterocyclic compounds that are mainly direct acting mutagens that induce primarily frameshift mutations (36, 44). In fact some studies have shown a decrease in the mutagenicity of particular oxy-PAHs in the presence of S9 metabolic activation (35, 59). However, it is possible that aromatic amines and/or nitroarenes were present in the soil. These nitroaromatic compounds are ubiquitous environmental pollutants generated during incomplete combustion processes, known to be highly mutagenic in miniscule quantities (4, 116, 136). Aromatic amines require exogenous metabolic activation to demonstrate mutagenicity in *Salmonella* strains (4, 11, 136). Nitroarenes are able to generate a mutagenic response both in the absence and the presence of S9. When

metabolic activation is available mutagenic metabolites are generated via the oxidation of the ring moiety (116). Thus it is possible that these compounds contributed to the S9 activated mutagenicity. It is also likely that additional biologically active compounds were below the analytical detection limit or were not quantified in the extract or were formed during remediation by chemical degradation or microbial metabolism (80).

Similar to the non-polar neutral fraction, the majority of the S9 activated mutagenic activity was generated by nitro-aromatic compounds capable of causing frameshift mutations (Table 3.9). Particularly high mutagenic potencies were observed in CFT509, CFA509 and YG1041 throughout the remediation treatment, further supporting the presence of undetected aromatic amines. TA98 detected minimal mutagenicity as was anticipated considering the nature of the compounds present in this fraction.

Aromatic amines are commonly known to require oxidation via cytochrome P450 monooxygenases to generate N-hydroxy metabolites (4, 79). These compounds are further degraded to mutagenic hydroxylamine derivatives by O-acetyltransferase (136). Strains YG1041, YG1042, CFA509, CFT509 and CBT409 all contain plasmids encoding genes for acetyltransferase. The higher mutagenicities observed with CFA509, CFT509, CBT409, YG1041 and YG1042 support the presence of nitro-substituted compounds. Strains YG1041 and YG1042 contain the plasmid pYG233, in addition to pKM101, that encodes the *Salmonella OAT* and *cnr* genes, responsible for acetyltransferase and nitroreductase activity, respectively (60). Acetyltransferase is responsible for the intracellular conversion of N-hydroxylamine intermediates to N-acetoxyesters that are proximate mutagens (31) capable of forming nitrenium ions that react with DNA to form toxic and mutagenic lesions. Nitroreductases are responsible for the reduction of the nitro

group of substituted aromatic and heterocyclic compounds, producing highly reactive nitroso and hydroxylamino intermediates (129). Further degradation of the nitroso and *N*-hydroxylamine intermediates yield a variety of mutagenic products (19, 20, 31, 129). Thus YG1041 and YG1042 are expected to be particularly sensitive to the presence of chemicals that are substrates for nitroreductase and/or *O*-acetyltransferase such as nitroarenes and aromatic amines (60).

The *Salmonella* CFA509 and CFT509 strains, derived from TA98, incorporate a single plasmid encoding the *E. coli* nitroreductase gene *nfsA*, the *S. typhimurium* acetyl CoA-dependent *O*-acetyltransferase (*OAT*) gene, the *mucA/B* genes, responsible for PolRI, and an antibiotic marker. The *nfsA* gene is responsible for encoding the major *E. coli* nitroreductase activity (31). The combination of the *OAT*, *nfsA*, and *mucA/B* genes on a single plasmid create a tester strain very sensitive to nitro-substituted compounds.

CBT409 also detected mutagenic activity in the polar aromatic fraction when S9 was present. This strain was derived from TA1535 (31) and thus only detects chemicals that induce base substitutions mutations (87). The activation of this strain in conjunction with YG1042 strongly indicates the presence of compounds capable of causing base-pair substitution mutations, such as aromatic amines.

Direct Acting Mutagenicity

Most of the compounds present in the polar aromatic fraction are considered to be direct acting mutagens. Overall, the direct mutagenicity observed in this fraction was anticipated. Both oxy-PAHs and *N*-heterocyclic compounds are considered to generate predominately frameshift mutations in the absence of metabolic activation (36, 39, 44, 124). The majority of the mutagenicity was detected by CFT509, CFA509 and YG1041,

the strains most sensitive to nitro-substituted compounds. This is consistent with previous mutagenicity studies of the polar fraction from contaminated environmental samples (39). Little mutagenicity was detected by TA98, most likely the result of the limited nitroreductase activity (31). Considering the large number of oxy-PAHs present in this fraction it is unclear as to why TA98 would have detected such limited mutagenicity. However, complex mixture studies have reported that the mutagenicity of one group of compounds can be inhibited by another group of compounds (42).

The presence of compounds capable of generating base-pair mutations was detected by both CBT409 and YG1042. While many of the nitro-substituted compounds capable of causing direct mutations generate frameshift mutations, not all do. Nitroarenes composed of two fused rings, such as nitronaphthalenes, tend to generate base-pair substitution mutations (30). Such compounds are also less mutagenic than nitroarenes with three or four fused rings (116). All of the identified N-heterocyclics contain three or more fused rings, indicating that other unknown mutagenic compounds were likely extracted from the soil.

To summarize, the polar aromatic fraction was highly mutagenic in the presence of S9, mostly likely due to the presence of highly reactive nitroarenes and aromatic amines. In the absence of metabolic activation the mutagenicity of this fraction decreased significantly. However, the activity detected by the nitro-sensitive strains indicated the presence of a variety of nitroaromatic compounds, including some of which were not monitored during remediation.

It should be noted that while the strains CFT509 and CFA509 are nearly identical the patterns of mutagenicity detected by each were not. The plasmid pCCOMA possessed

by CBT409 and CFT509 uses tetracycline as an antibiotic marker. CFA509 contains the p213MA plasmid, which encodes an ampicillin antibiotic marker. Despite this single difference between CFA509 and CFT509, discrepancies were observed between the strains (Figures 3.4 – 3.7 and Tables 3.6 -3.9). Previous work with these strains indicated that CFT509 had a higher specific nitroreductase activity than CFA509 (31). It was hypothesized that this could be the result of the organization of the genes of the individual plasmids. Although the plasmids were both derived from pBR322, the discrepancies between the two plasmids could result in differential plasmid replication and gene expression, altering the nitroreductase activity (31). It was also suggested that the *mar* locus, responsible for multiple antibiotic resistance but not very well understood in *S. typhimurium*, could initiate the SoxRS regulon via the SoxS activator and thus induce the *nfsA* gene. The SoxRS regulon includes the *nfsA* gene and is present on the plasmids along with the sequences necessary for MarA/SoxS regulation. Therefore it is possible that the different antibiotic markers lead to differential induction of the SoxRS regulon, resulting in differential expression of the nitroreductase genes (31). However neither of the strains consistently detected a higher mutagenic potency between the two fractions, although within each fraction one of the strains generally detected a higher mutagenic potency (Figures 3.4 – 3.7 and Tables 3.6 -3.9). This possibly indicates a difference in sensitivity to different chemicals, but since neither strain was consistent across individual fractions it is difficult to identify a specific group of compounds.

4.3 Expected vs. Observed Mutagenic Potencies

The expected mutagenicity of each fraction was calculated from the observed mutagenicity of each compound in its pure form. A mutagenic potency was determined for each compound in each strain. The potency for each chemical was multiplied by the concentration of that chemical found in the soil. In order to calculate the predicted potency for an entire fraction, the potencies of each chemical present in a particular fraction were summed according to strain. The calculations assumed that the effects of the individual contaminants were additive. However, not all of the compounds found in the soil were quantified. Only compounds with known concentrations could be used. A pure form of each chemical was tested in the Ames assay with all strains. All of the E.P.A. priority compounds as well as the compounds suspected to be mutagenic were tested with and without metabolic activation, while all other compounds were tested only for direct acting mutagenicity. Initial concern that some mutagenicity was missed by not testing all of the pure compounds with metabolic activation was solved by the literature. The majority of the chemicals were tested in previous studies with limited mutagenicity with or without metabolic activation (Tables 4.0 and 4.1).

Overall, the predicted mutagenicity was either much higher or much lower than the observed mutagenicity. In the case of the polar aromatic fraction the low predicted mutagenicity is likely due to the fact that only two of the chemicals within the fraction tested positive. None of the chemicals were tested with S9 activation for the polar aromatic fraction, therefore no predicted values exist for this fraction. According to the literature it is likely that the predicted values would underestimate the observed as only one of the compounds, 7H-benz[de]anthracene-7-one, has tested positive in the presence

of S9 with strain TA98 (Table 4.0) (95). Considering the predicted direct mutagenicity is substantially lower than the observed and that the predicted indirect mutagenicity would probably be lower as well, it is likely that the majority of the mutagenicity observed in this fraction is the result of compounds that were not monitored during remediation.

Conversely, the predicted values of the non-polar neutral fraction were significantly higher than observed values in the presence of metabolic activation (Table 3.14). This is not entirely unexpected. Work has shown that PAH mixtures can have both synergistic and antagonistic effects on the mutagenicity of individual PAHs. In other words the mutagenicity of the mixture can either increase or decrease depending on the components present (49, 63). It would appear that the mutagenicity observed in the non-polar neutral fraction in the presence of metabolic activation is less than the sum of its parts. Combinations of PAHs at low doses have been shown to be additive (10, 140), while other studies have reported inhibitory effects within mixtures (40, 42). For this study the predicted mutagenicity was calculated assuming the individual potencies of the PAHs would be additive. Considering the actual mixture of pure PAHs was not reconstituted and tested for mutagenicity it is difficult to determine whether or not the mixture is in fact experiencing a synergistic or antagonistic response. However previous work does appear to support the assumption of antagonistic responses from complex mixtures (10, 40). This is especially apparent in the mixtures containing indirect mutagens as the addition of S9 imposes challenges. Different compounds have different optimal S9 requirements. As such when these compounds are tested in a mixture some of the compounds may not have access to the necessary concentration of S9 protein, resulting in decreased observed mutagenicity (134).

The observed mutagenic potencies of the non-polar neutral fraction in the absence of metabolic activation was significantly higher than the predicted values (Table 3.13). This discrepancy is likely the result of the presence of unknown compounds in the fraction. PAHs and alkyl-PAHs were predominantly identified in this fraction, however several unidentified nitro-substituted compounds appear to be the cause of the majority of the direct mutagenicity. As these compounds were unknown, only the identified PAHs were tested in the pure form and used to calculate the predicted mutagenicity. As such it is not surprising that the values do not agree. This provides further evidence of the presence of unknown mutagenic compounds.

4.4 Bioremediation of Contaminated Soil

Bioremediation of contaminated soils is a very complex process with many factors influencing the success of the treatment. Not only do the physicochemical properties of the soil affect the rate of biodegradation, but additional factors such as the microbial population as well as the nature of the contaminants dictate the speed and efficiency with which the contaminants disappear (126). Many studies have examined the bioremediation of PAH contaminated soils. Although the majority of this work focuses on monitoring the concentration of the contaminants (14, 47, 72, 73, 77, 108, 121, 139, 144), few studies have examined the genotoxicity of the soil during the remediation treatment (7, 18, 26, 41, 98, 106). All of the aforementioned papers were able to demonstrate a decrease in PAH concentration during the bioremediation treatment. However, various products are known to form during the degradation of PAHs, many of which are more persistent, bioavailable or mutagenic than the parent compound (8, 13, 27, 33-35, 55, 93, 122, 125, 126, 145). Lundstedt *et al.*, observed the

accumulation of 9-fluorenone and benz[a]anthracene-7,12-dione, two derivatives of fluorene and benz[a]anthracene, both of which persisted in the soil for a longer duration than the parent compounds (80, 82). Several other studies have noted the accumulation of other oxy-PAHs. A second bioslurry study performed by Saponaro *et al.*, detected an accumulation of anthracene-9,10-dione, a derivative of anthracene (121). Additional studies have reported similar results, suggesting that some of the derivatives generated during biodegradation of PAH are more persistent than their parent compounds (47, 93, 145).

In this particular case, soil from an aged gasworks site, contaminated with both low and high molecular weight PAHs, inoculated with a bacterial culture, underwent a bioslurry treatment. Not only were the concentrations of a variety of PAHs monitored but also the concentrations of compounds known to be derivatives of PAH degradation. This advanced chemical analysis combined with mutagenicity testing has helped to elucidate some of the processes that occur during the bioremediation of PAH contaminated soil. Mutagenicity testing detected a net increase in genotoxicity in the non-polar neutral fraction with and without metabolic activation. The genotoxicity of the polar aromatic fraction also increased over time when metabolic activation was present. However, the overall profile of direct mutagenicity of the polar aromatic fraction was somewhat inconsistent with some strains detecting much higher mutagenic potencies prior to remediation and others identifying significant increases at the end of the treatment (Figures 3.4 –3.7).

The increase in the direct genotoxicity of the non-polar neutral fraction is difficult to account for. Most compounds present in this fraction required S9 activation in order to

elicit a positive response. However, PAHs can, in principal, be transformed into nitroarenes, direct mutagens that are known to be capable of inducing frameshift mutations at a higher frequency than the parent compounds. Previous work has shown that the presence of NO₂ in the atmosphere can lead to the nitration of PAHs (136). It has also been shown that PAHs in soil in the presence of metallic oxides and a nitrogen source can also be transformed into nitrated compounds (131). While it is possible that NO₂ was formed in the bioslurry either via chemical reaction (137) or as by-products of bacterial catabolism (129), it is unlikely that it was at concentrations high enough to induce a significant amount of nitro-substituted PAH derivatives. However, Hughes and associates attributed the genotoxicity of soil after a bioslurry treatment to the presence of azaarenes (67). The genotoxicity of soil from a coal tar distillation and wood preserving plant before and after a bioslurry remediation treatment was examined. Similar to the bioslurry performed by Lundstedt (80, 82), the soil was amended with oxygen, nitrogen, phosphorous, carbon and a nutrient mixture. The direct and indirect mutagenicity of the bioslurry treated soil was substantially higher than that of the untreated soil. As shown in the results presented here, the strains sensitive to nitroarenes and amines, YG1041 and YG1042, reported the highest response (67).

In order to determine if the activity observed in YG1041 and YG1042 was the result of the presence of nitro-substituted compounds, Hughes *et al.*, used the nitroreductase deficient strain TA98NR to test for genotoxicity. A 50% reduction in mutagenicity was observed, suggesting the presence of nitroarenes (67). However, GC/MS analysis was not sensitive enough to detect nitroarenes. Further analysis of the organic extract of the soil identified the presence of azaarenes in the soil, explaining a

portion of the direct genotoxicity detected by YG1041. While some azaarenes were detected in the untreated soil, other novel azaarenes were found in the bioslurry suggesting that they were formed during the remediation process (26). Previous studies have also found azaarenes in creosote contaminated soil (28). These compounds are weakly polar and could have been extracted in the non-polar neutral fraction (26, 67). Azaarenes are formed when a nitrogen atom replaces a carbon atom in a PAH. While the source of the increase in the direct acting mutagenicity observed in the non-polar neutral fraction is unclear it is possible that some of the PAHs were biotransformed to azaarenes via bacterial or chemical degradation processes; however, in the current study there is no analytical information available to confirm this.

A cyclic pattern of mutagenicity was observed in the non-polar neutral fraction of the current experiment, suggesting the formation of genotoxic derivatives during the bioslurry treatment (Figure 3.4). A study by Belkin and colleagues, found a similar result in the direct acting mutagenicity of PAH contaminated soil during biodegradation carried out using column percolators. Each column included soil that was either PAH amended or from a PAH contaminated site, water flow through, oxygen and carbon dioxide. Genotoxicity of the soils was monitored for three months with and without metabolic activation, using the Mutatox assay (18), a genotoxicity test that uses dark mutants of the luminous bacteria *Vibrio fischeri*. Mutagenic chemicals restore the organism's luminescence (132). Two PAH amendment soil experiments, one involving amendment with low molecular weight PAHs and one involving high molecular weight PAHs, elicited direct mutagenic responses a few days after the bulk of the PAHs disappeared. Genotoxicity was more pronounced in the soil amended with the more easily degraded

low molecular weight PAHs. Effluent from the columns was tested for the presence of dissolved organic carbon (DOC). Samples that elicited the greatest mutagenicity also had higher concentrations of DOC, suggesting the presence of mutagenic degradation products. Several known metabolites were identified in the effluent and eventually disappeared over the duration of the experiment (none of which were identified in the paper). However, one unidentified compound remained (18).

A major assumption of the bioremediation of contaminated soil is that decreases in the toxicity of the soil reflect increases in the safety of the soil. Belkin *et al.*, performed toxicity tests in conjunction with the mutagenicity testing and found that while the toxicity of the PAH amended soils was independent of the mutagenicity, it mimicked the genotoxicity results of the contaminated soil. Basically, the mutagenicity did not follow the toxicity of the PAH amended soil, when the mutagenicity was high the toxicity was low. However, a similar pattern was observed between the toxicity and mutagenicity of the contaminated soil and it appeared that the toxicity and mutagenicity were in fact related. The study by Belkin *et al.*, strongly suggest that the mutagenicity testing is required in conjunction with toxicity testing, which is typically the only test employed during the bioremediation of contaminated soil. Overall, this work found that neither toxicity nor PAH concentration can predict mutagenicity (18).

A net increase of indirect acting mutagens was witnessed in the non-polar neutral fraction. However, unlike the direct mutagenicity observed in this fraction, the activated mutagenicity appeared to gradually increase over the duration of the treatment even though many of the compounds found in this fraction were degraded to 10% of there original concentration. It is likely that this mutagenicity is the result of the accumulation

of mutagenic by-products in the soil as some metabolites are more persistent in the soil than the parent compounds and can exist for days before dissipating (93). Some of the studies that focused on PAH biodegradation have found results similar to this, it appears that the accumulation of indirect mutagens is a common phenomenon (26, 67, 123).

The increase in the indirect mutagenicity of the non-polar neutral fraction witnessed in the current study is not the only observation of its kind. Sasek *et al.* observed a similar phenomenon in an experiment involving the bioremediation of PAH contaminated soil (123). Soil from a gas manufacturing plant underwent a 54-day composting treatment in a ventilated thermal-insulated chamber. The contaminated soil was amended with water and mushroom compost. After 54 days of composting, the soil was placed in an open field for maturation over a 100-day period. Samples were taken from six different areas of the compost pile at 0 and 54 days and once again after the 100-day maturation. EPA priority compounds were extracted and monitored using the Soxhlet method and HPLC, respectively. Mutagenicity testing was performed using the *E. coli* SOS Chromotest. Decreases in the overall PAH concentrations were observed. However, all samples tested were mutagenic in the presence of metabolic activation before and after remediation indicating that, despite the decrease in PAH concentration, mutagenic compounds were still present in the soil (123). This is consistent with the notion that the increase in mutagenicity observed in the current study is the result of PAH by-products generated during remediation.

Brooks *et al* (26) and Hughes *et al* (67) also found indirect acting mutagens present after the bioslurry treatment of contaminated soil from a coal tar distillation and wood preserving plant. The bi- and tri-cyclic PAHs experienced the greatest reduction in

concentration, followed by the four-ringed PAHs and finally the five and six ringed PAHs which underwent little to no degradation (26). Three of the fractions, examined by Brooks *et al.*, contained PAHs and exhibited greater S9 activated mutagenicity after the treatment, consistent with the findings observed here. However, many of the PAHs identified in these fractions were the easily degradable low molecular weight PAHs such as anthracene and acenaphthylene; thus, the sources of the majority of the mutagenicity observed in these fractions is unknown. Other remediation techniques employed in this study for PAH degradation were composting, biopile and land treatment; however the bioslurry demonstrated the greatest mutagenicity over all other techniques. It was also the only strategy that incorporated PAH degrading microorganisms and aeration. Thus it was hypothesized that some of the mutagenicity observed in the bioslurry treatment was the result of increased bacterial activity (26, 67).

Other studies have reported similar findings to Brooks (26) and Hughes (67). A landfarming study showed that they were able to remove 64% of total PAHs after 3 months. PAHs with four rings or less experienced a 79% reduction of the original concentration whereas PAHs with 5 or 6 benzene rings underwent an 18% decrease in concentration. Microbial characterization of the soil showed an increase in microorganisms able to degrade pyrene and phenanthrene. It was also found that the evolution of the microbial population followed the pattern of PAH removal, with PAH degrading organisms decreasing as the PAH concentrations decreased. Unlike the results witnessed here, or in the previously described papers, the Mutatox test showed a decrease in mutagenicity after 2 months (106). However, mutagenicity testing was not performed

until two months into the treatment, after the bulk of the low molecular weight PAHs were degraded and thus initial peaks in mutagenicity could have been missed.

A unique study by Morelli and colleagues monitored dehydrogenase activity in addition to mutagenicity during the bioremediation of uncontaminated soil amended with petroleum sludge. The soil/sludge mixtures were incubated for one year and samples were tested for mutagenicity prior to treatment and at 44, 90, 180, and 373 days. Mutagenicity was determined using the Ames assay with and without metabolic activation with the *Salmonella* strain TA98. After one-year of treatment no net differences were witnessed in the direct or indirect mutagenicity. However, the indirect mutagenicity did increase during the treatment before ultimately decreasing. Dehydrogenase activity was monitored during the bioremediation treatment. A spike in dehydrogenase activity correlated with the increase in mutagenicity. The increase in mutagenicity was attributed to the generation of genotoxic dehydrodiol epoxides during the initial biodegradation of the PAHs. Low molecular weight PAHs have been shown to be readily degraded possibly explaining the resulting influx of genotoxic by-products (98). This study further supports the idea that the bacterial degradation of PAHs is responsible for at least a portion of the observed increases in mutagenicity during bioremediation.

Additional evidence that genotoxic products are generated by the bacterial degradation of PAHs is presented in a study by Alexander and colleagues (7). A solid phase genotoxicity assay was used to determine the mutagenicity of three PAHs contaminated soils. One sterile soil sample was artificially contaminated with coal tar oil. The second sample was obtained from a manufactured gas plant that had undergone land

treatment. Samples were taken from day 0, 58, 101, and 147. Lastly, a sample of untreated soil was inoculated with a small portion of treated soil to serve as microbial inoculum and the soil was tested for genotoxicity over the duration of 59 days. In this case a solid phase mutagenicity test was used. *Pseudomonas putida* A11rUV, a bacterial strain vulnerable to the antibiotic rifampicin, was inoculated into soil samples and incubated for 48 hours. Mutagenic substances cause reversions and reinstate rifampicin resistance. Revertants are scored by plating serial dilutions on a media supplemented with rifampicin and counting the colonies. Extraction and analysis of contaminants was performed using a Soxhlet apparatus and GC. Although no reduction in PAH concentration was observed in the sterile soil sample over time, a reduction in the genotoxicity was seen. Clearly, as the soil aged, the PAHs became less bioavailable, rendering the soil less mutagenic. A decrease in PAH concentration was observed over the 59 day treatment of the artificially contaminated soil. A decrease in the mutagenicity was also observed. Interestingly, PAH concentration of the soil undergoing land treatment in the field decreased while the mutagenicity increased. Eventually a reduction in the mutagenicity was observed, but not until after 101 days of remediation. It was suggested that the microorganisms in the soil were responsible for the generation of new genotoxic compounds (7).

Unlike the non-polar neutral fraction the mutagenicity results for the polar aromatic fraction appears to be somewhat ambiguous in the absence of metabolic activation. Most evident is the discrepancy between the genotoxicity detected by the nearly identical strains CFT509 and CFA509. According to CFT509, it appears that there was a significant decrease in nitro-substituted, direct acting frameshift mutagens. In

contrast CFA509 detected a slight net increase in the same compounds. As described in the previous section this could indicate that there are two different sets of nitro-substituted compounds in the fraction.

While the concentrations of most compounds decreased with time, the concentration of several oxy-PAHs, the derivatives of the bacterial oxidation of high molecular weight PAHs such as fluoranthene and pyrene (33) increased in concentration before finally decreasing (80, 82). However the direct mutagenicity detected does not appear to correlate with the accumulation and elimination of the oxy-PAHs monitored. Once again, the majority of the mutagenicity is detected by the strains sensitive to nitro-substituted compounds. This is consistent with the components of this fraction, many of which are N-heterocyclics. As previously discussed many N-heterocyclic compounds do not require metabolic activation to exert genotoxicity, explaining some of the mutagenicity observed here (136). The bioslurry performed by Brooks et al (26) and Hughes et al (67), obtained similar results, as previously described. Several fractions exerted direct mutagenicity that triggered a response in YG1041. Upon analysis of these fractions the only compounds that could have provoked such a response were azaarenes. When the individual mutagenicity of the compounds was researched in the literature, only a few compounds were known direct mutagens; thus the source of much of the observed mutagenicity is unknown (26).

It appears that there was an accumulation of indirect acting mutagens in the polar aromatic fraction. As has been the trend in all of the fractions, the strains most sensitive to the mutagenicity of the fraction were those equipped with the ability to metabolize nitro-substituted compounds. As this fraction contained many N-heterocyclic compounds

these results are consistent with the identified compounds. However, most of these compounds decreased over time. Only oxy-PAHs were shown to accumulate over the duration of the bioslurry. These compounds are mostly direct acting mutagens, rendering it unlikely that they contributed to the increase in mutagenicity witnessed here. However, the microbial oxidation of PAHs has been shown to generate polar metabolites (64, 75) capable of generating both frameshift and base-pair substitution mutations in the presence of metabolic activation. Whether or not these chemicals were nitroarenes, aromatic amines or other nitro-substituted compounds is unknown as the only available mutagenicity data was for TA98 and TA100 (75). Although the identification of polar metabolites capable of generating base-pair mutations is consistent with the results presented here; both YG1042 and CBT409 detected mutagenic activity on all days during the remediation, a phenomenon not seen in any of the other fractions with or without metabolic activation.

Polar compounds were also identified by Brooks and colleagues during the bioslurry treatment of PAH contaminated soil as described earlier. Fractions containing these compounds also tested positive for genotoxic activity in strains YG1041 as well as YG1042, indicating that compounds capable of generating base-pair substitution mutations were also present in addition to the mainly direct acting, frameshift inducing azarenes and oxy-PAHs (26).

Overall a net increase in the mutagenic activity of the soil was observed. Most of this mutagenicity remains unaccounted for. It is likely attributed to the production of mutagenic metabolites formed during the microbial and chemical degradation of PAHs. A few studies have examined PAH bacterial metabolic pathways (32, 144). While low

molecular weight (LMW) PAHs can undergo mineralization, many high molecular weight (HMW) PAHs cannot be easily degraded and often undergo co-oxidation resulting in the partial degradation of the chemical (144) leaving highly reactive epoxides and dihydrodiols in the soil (75, 133). Chemical degradation of PAHs typically occurs via oxidation, initiated by a variety of chemicals such as singlet oxygen, organic peroxides, hydrogen peroxide, ozone and peroxy, alkoxy, hydroxyl radicals (80). These reactions typically produce a wide variety of toxic and genotoxic ketone, quinone, aldehydes, phenols and carboxylic acid compounds (114, 115, 147).

However, it is important to note that other bioremediation studies that used different treatment strategies other than bioslurry to treat PAH contaminated soil did not experience increases in mutagenicity during or after the treatment (26, 67, 106). It appears that composting or landfarming do not result in drastic increases in the mutagenicity of the soil even though the efficiency of PAH removal is comparable (14, 26, 106). It has been suggested that the increased bacterial activity witnessed in bioslurries is the cause of the mutagenicity (67). However, it is important to recognize that bioslurry studies that were performed for longer periods of time did, in fact, show an overall decrease in the mutagenicity (7, 98).

4.5 Conclusions and Future Work

Soil bioremediation is a complex process. Microbial degradation of PAHs and nitroaromatic compounds may be the most efficient and economical choice for soil decontamination. However, microbial degradation of contaminants appears to generate compounds that are more mutagenic and recalcitrant than the parent compounds. Thus, monitoring of the bioremediation process must go beyond examining the concentrations

of known pollutants. Additional tests, such as the Ames *Salmonella* mutagenicity assay, need to be employed to ensure the safety of the soil before, during and after the remediation process.

Further work needs to be completed. In order to determine the sources of the mutagenicity witnessed in this study further chemical analysis as well as mutagenicity testing must be performed. New and promising extraction techniques have recently been developed for nitroaromatic compounds and the associated metabolic by-products (111, 154). This could help identify the causes of the unknown genotoxicity observed during the remediation. In order to ensure that the mutagenicity detected by the nitro-sensitive strains is in fact generated by nitro-substituted compounds, the nitroreductase deficient strain, TA98NR should be used to test both fractions. If the majority of the mutagenicity is in fact from nitroaromatic compounds this strain should detect a reduced mutagenic response as compared to TA98. Once the bulk of the mutagenic compounds have been identified, testing of a reconstituted mixture of these chemicals would be useful in identifying any synergetic or antagonistic effects.

Table 4.0: Compounds found in the non-polar neutral fraction and comparison of observed results to results from the literature for TA98

Compound	CAS #	Figure	Molecular Weight (g/mol)	Classification	Revertants/nmole				Ref
					Observed		Literature		
					-S9	+S9	-S9	+S9	
1-Methylnaphthalene	90-12-0		142	Alkyl-PAH	0	n.t.	0	0	(50)
1-Methylphenanthrene	832-69-9		192	Alkyl-PAH	n.t.	n.t.	0	1.6	(118)
2,3,5-Trimethylnaphthalene	2245-38-7		170	Alkyl-PAH	0	n.t.			
2,6-Dimethylnaphthalene	581-42-0		156	Alkyl-PAH	0	n.t.			
2-Methylnaphthalene	91-57-6		142	Alkyl-PAH	0	n.t.	0	0	(50)
4H-Cyclopenta[def]phenanthrene	203-64-5		190	PAH	0	n.t.	0	0	(152)

Acenaphthene*	83-32-9		154	PAH	0	0	0	0	0	(50)
Acenaphthylene*	208-96-8		152	Alkyl-PAH	0	0	0	0	0	(25, 50)
Acephenanthrylene	201-06-9		202	PAH	n.t.	n.t.	0	+	(120)	
Anthanthrene	191-26-4		276	PAH	n.t.	n.t.		62	(63)	
Anthracene*	120-12-7		178	PAH	0	0	0	0	(25, 37, 50)	
Benz[a]anthracene*	56-55-3		228	PAH	0.017	0.909	0	12	(25, 27, 37, 92)	

Benzo(a)pyrene	50-32-8		252	PAH	0	8.649	0	185	(37, 56, 92)
Benzo[b]fluoranthene*	205-99-2		252	PAH	0	0.776	0	15	(92, 152)
Benzo(e)pyrene	192-97-2		252	PAH	0	0.042	0	1.6	(37, 56, 92)
Benzo[ghi]perylene*	191-24-2		276	PAH	0	0.279	0	2.3	(92, 118)

Benzo[k]fluoranthene*	207-08-9		252	PAH	0	2.427	0	+	(76, 152)
Benzo[thiophene	11095-43-5		134	S-heterocyclic	0	n.t.	0	0	(63)
Biphenyl	92-52-4		154	PAH	0	n.t.	0	0	(25, 56, 92)
Chrysene*	218-01-9		228	PAH	0	0	0	38	(25, 56, 92, 118)
Coronene	191-07-1		300	PAH	0	n.t.	0	60	(56, 92)

Cyclopenta[cd]pyrene	27208-37-3		226	PAH	0.052	40845. 163		+	(45)
Dibenz[ah]anthracene*	53-70-3		278	PAH	0	0.153		8	(63)
Dibenzofuran	132-64-9		168	O-heterocyclic	0	n.t.	0	0	(50)
Dibenzothiophene	132-65-0		184	S-heterocyclic	0	n.t.		0	(63)
Fluoranthene*	206-44-0		202	PAH	0	0	0	0 ^M	(50)

Fluorene*	86-73-7		166	PAH	0	0	0	0	0	(90, 92)
Indeno[1,2,3- <i>cd</i>]pyrene*	193-39-5		276	PAH	0	4596.0 21		+		(92)
Naphthalene*	91-20-3		128	PAH	0	0	0	0	0	(25, 50, 92)
Perylene	198-55-0		252	PAH	0	n.t.	0	30	0	(92, 118)
Phenanthrene*	85-01-8		178	PAH	0	0	0	0	0	(50)
Picene	213-46-7		278	PAH	n.t.	n.t.	0	0	0	(50)

Pyrene*	129-00-0		202	PAH	0	0	0	0 ^M	(50)

^M A mutagenic response has been detected by Sakai and colleagues (118).

* Denotes priority PAHs.

Table 4.1: Compounds found in the polar aromatic fraction and comparison of observed mutagenicities to those found in the literature for TA98

Compound	CAS #	Figure	Molecular Weight (g/mol)	Classification	Revertants/nmole				Ref
					Observed		Literature		
					-S9	+S9	-S9	+S9	
1-Indanone	83-33-0		132	Oxy-PAH	0	n.t.	0	0	(50)
2-Methylantracenedione	84-54-8		222	Oxy-PAH	0	n.t.	0	0	(118)
7H-benz[de]anthracene-7-one	82-05-3		230	Oxy-PAH	n.t.	n.t.	0	+	(95)
9-Fluorenone	486-25-9		180	Oxy-PAH	0	n.t.	0	0	(50)

Anthracene-9,10-dione	84-65-1		208	Oxy-PAH	0	n.t.	0	0	(56, 118, 119)
Benz[a]anthracene-7,12-dione	2498-66-0		258	Oxy-PAH	0	n.t.	0	0	(119)
Carbazole	86-74-8		167	N-heterocyclic	0	n.t.	0	0	(50, 92)

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