BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS
BY ARTHROBACTER SP. UG50 ISOLATED FROM PETROLEUM
REFINERY WASTES

by

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ABSTRACT

BIODEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS BY ARTHROBACTER SP. UG50 ISOLATED FROM PETROLEUM REFINERY WASTES

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University of Guelph, 2011

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North American petroleum refineries use landfarming to dispose of hydrocarbon-containing wastes for bioremediation by indigenous soil microorganisms. In this study, we isolated PAH-degrading bacteria from landfarm soil by enrichment with hydrocarbon-containing effluent. One isolate, *Arthrobacter* sp. UG50, was capable of using phenanthrene and anthracene as sole carbon sources. The strain degraded phenanthrene (200 mg/L) within 24 h in pure culture at high cell density (10⁸ cells/mL). Anthracene (50 mg/L) was slowly degraded, with 29% degraded within 21 days. The strain could not use naphthalene, pyrene, chrysene or benzo(a)pyrene as sole carbon sources, but could degrade pyrene (50 mg/L) cometabolically when phenanthrene was provided. Anthracene degradation (50 mg/L) was enhanced by phenanthrene, with 100% degraded within 6 days. The addition of strain UG50 to petroleum sludge in baffled flasks increased total hydrocarbon degradation and degradation of low concentrations of fluorene, phenanthrene, pyrene and chrysene compared to flasks with limited aeration or containing sludge alone.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Name</th>
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<tr>
<td>A&lt;sub&gt;ISTD&lt;/sub&gt;</td>
<td>peak area of internal standard</td>
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<td>peak area of component x</td>
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<td>µ</td>
<td>micrometres</td>
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<td>API</td>
<td>American Petroleum Institute</td>
</tr>
<tr>
<td>BAF</td>
<td>bioaccumulation factor</td>
</tr>
<tr>
<td>BCF</td>
<td>bioconcentration factor</td>
</tr>
<tr>
<td>BH</td>
<td>Bushnell Haas</td>
</tr>
<tr>
<td>C</td>
<td>carbon</td>
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<td>EqP</td>
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<tr>
<td>g</td>
<td>gram</td>
</tr>
<tr>
<td>GC-FID</td>
<td>gas chromatography/flame ionization detector</td>
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<td>GC-MS</td>
<td>gas chromatography/mass spectography</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<td>--------------</td>
<td>-----------</td>
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<tr>
<td>H</td>
<td>hydrogen</td>
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<tr>
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<td>high molecular weight</td>
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<tr>
<td>IARC</td>
<td>International Agency for Research on Cancer</td>
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<td>isopropyl β-D-1-thiogalactopyranoside</td>
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<td>ISP</td>
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<td>ISTD</td>
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<td>IUPAC</td>
<td>International Union of Pure and Applied Chemistry</td>
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<tr>
<td>kg</td>
<td>kilogram</td>
</tr>
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</tr>
<tr>
<td>L</td>
<td>litre</td>
</tr>
<tr>
<td>LB</td>
<td>Luria Bertani</td>
</tr>
<tr>
<td>LMW</td>
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<tr>
<td>luxAB</td>
<td>luciferase genes from <em>Vibrio harveyii</em></td>
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<tr>
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<td>MOE</td>
<td>Ministry of the Environment</td>
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<tr>
<td>Na$_2$HPO$_4$</td>
<td>disodium hydrogen phosphate</td>
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<tr>
<td>NAPL</td>
<td>Non-Aqueous Phase Liquids</td>
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<tr>
<td>NCBI</td>
<td>National Centre of Biotechnology Information</td>
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</tr>
<tr>
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<td>PAH</td>
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<td>naphthalene inducible dioxygenase genes from Gram-positive bacteria</td>
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</tr>
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<td>parts per million</td>
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<td>total petroleum hydrocarbon</td>
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<td>tryptic soy broth</td>
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<tr>
<td>UMBBDB</td>
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</tr>
<tr>
<td>USEPA</td>
<td>United States Environmental Protection Agency</td>
</tr>
<tr>
<td>VOC</td>
<td>volatile organic chemical</td>
</tr>
<tr>
<td>v/v</td>
<td>volume/volume</td>
</tr>
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<td>Wt</td>
<td>weight</td>
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<td>Wt&lt;sub&gt;STD&lt;/sub&gt;</td>
<td>weight (g) of internal standard solution added</td>
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<td>weight (g) of sample only</td>
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<tr>
<td>X-gal</td>
<td>5-bromo-4-chloro-3-hydroxyindole</td>
</tr>
<tr>
<td>YNB</td>
<td>Yeast Nitrogen Base</td>
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CHAPTER 1: LITERATURE REVIEW

1.1. INTRODUCTION

1.1.1. Background and Rationale

Environmental contamination of air, soil, sediments and water due to the presence of polycyclic aromatic hydrocarbons (PAHs) has been occurring across North America for decades. Although PAHs are ubiquitous in nature, anthropogenic sources such as petroleum refining and fossil fuel combustion deposit significant quantities into the environment (Finlayson-Pitts and Pitts, 1997). For decades, this pollution has been of global concern due to the significant health risks posed by the toxic, mutagenic and carcinogenic properties associated with certain PAH compounds (Churchill et al., 1999; Seo et al. 2007). Due to the detrimental health effects associated with PAHs and the importance of proper disposal of PAH-containing wastes, there is a need to investigate ways of enhancing current remediation techniques, including microbial degradation.

Petroleum refineries can generate significant quantities of PAH-containing oily sludge during the processing of crude oil and landfarming is often used as an economical method of waste treatment and disposal (Picado et al., 2001; Ward et al., 2003). Indigenous soil microorganisms are capable of PAH degradation to CO$_2$ and water if adequate amounts of oxygen, nitrogen and moisture are present to support microbial growth (Lindstrom et al., 1991). However, it is difficult to ensure that controlled and optimal conditions for microbial activity are being met during the landfarming process (Ward et al., 2003). Moreover, there are a number of detrimental environmental impacts associated with landfarming. These include the potential release of volatile organic carbons (VOCs) into the atmosphere and the potential for leaching of hazardous compounds into groundwater (Maila and Cloete, 2004). Due to the limitations and
environmental impacts associated with landfarming, the process has been banned in the United States and new regulations governing the practice have been introduced in Canada.

In 2005, the Ontario Ministry of the Environment (Canada) introduced Ontario Regulation 461/05 whereby the sludge generated during the storage and/or treatment of process and oily cooling wastewaters must meet strict new criteria regarding PAH concentrations prior to land disposal (Ontario Ministry of the Environment 2005). The legislation took effect in December 2009, and the implications of the guidelines are that petrochemical companies that currently operate landfarms in Ontario must implement alternatives to reduce PAH concentrations in oily waste prior to disposal on landfarm sites. One pre-treatment option that is of interest to refineries due to its cost effectiveness is the use of enclosed slurry bioreactors containing PAH-degrading microorganisms to decrease PAH concentrations to recommended levels. The use of a bioreactor with a suitable PAH-degrading microbial consortium allows for control over factors affecting biodegradation including nutrient levels, pH, temperature and aeration resulting in the optimization of PAH biodegradation (Ward et al., 2003; Zappi et al., 1996).

This review will summarize the properties, sources and fate of PAHs in the environment, the origins and operation of landfarms as well as the properties of petroleum refinery waste. The mechanisms of degradation of several PAHs by bacteria and fungi and the genetics involved will also be discussed. Lastly, the challenges encountered during PAH biodegradation in complex mixtures and approaches that can improve the degradation of these compounds in liquid systems will be discussed.
1.2. POLYCYCLIC AROMATIC HYDROCARBONS

1.2.1. Physical and Chemical Characteristics

Polycyclic aromatic hydrocarbons (PAHs) are a diverse group of ubiquitous hydrophobic organic contaminants arranged as three or more fused benzene rings in linear, angular or cluster arrangements (Chauhan et al., 2008; Habe and Omori, 2003). Heterocyclic aromatic compounds, which contain substitutions to the benzene ring of nitrogen, sulphur or oxygen, are often classified with the true PAHs consisting of only carbon and hydrogen atoms since both groups possess similar chemical and functional properties (Varanasi 1989). PAHs can further be organized into alternant or non-alternant classes based on chemical structure. Alternant PAHs including phenanthrene and pyrene consist of only fused benzene rings, whereas non-alternant PAHs such as fluorene consist of four, five or six-membered rings (Harvey 1997). The chemical structures of several of the 16 PAHs designated as priority pollutants for remediation by the U.S. Environmental Protection Agency are illustrated in Figure 1.1.

The physical and structural properties of PAHs are important in the determination of the compound’s chemical stability and potential toxicity (Karcher 1992). One example is electron positioning within the contaminant. In contrast to molecules possessing localized electrons, the benzene rings of PAHs possess delocalized electrons which reduce the polarity of bonds within the molecule, thereby conferring increased chemical stability to the PAH, and making the bonds more resistant to nucleophilic attack and disruption (Harvey, 1997; Johnsen et al., 2005). PAH compounds may also contain Bay and K-regions which can be metabolized to highly chemically and biologically active epoxides with carcinogenic properties (Chauhan et al., 2008; Mrozik et al., 2003).

Hydrophobic contaminants also have the potential to persist in soil environments. Low molecular weight (LMW) compounds (two or three fused rings) sorb less strongly to soil and
sediment and are less resistant to microbial degradation than high molecular weight (HMW) PAHs (three or more fused rings) (Mrozik et al., 2003). The half life of a tricyclic PAH such as phenanthrene can range from 16 to 126 days while a five-ringed compound such as benzo(a)pyrene can persist from 229 to 1500 days in soil (Howard 1991). The tendency of PAHs to sorb to naturally occurring organic material, expressed as the organic partition coefficient $K_{oc}$, has been experimentally demonstrated to be a function of the hydrophobic nature of the contaminant which is expressed as the octanol: water partition coefficient $K_{ow}$ (Accardi-Dey and Gschwend, 2002). Higher $K_{ow}$ values can therefore be interpreted to indicate an increased capacity for sorption of PAHs to particulate matter.

PAHs have some characteristics which are similar to those of other persistent organic pollutants. The similarities include a hydrophobic and lipophilic nature, low water solubility, a tendency to bioaccumulate in aquatic organisms and low volatility (Douben 2003). A reduction in aqueous solubility and volatility generally corresponds to an increase in MW, number of aromatic rings and angularity of the PAH compound (Chauhan et al., 2008; Johnsen et al., 2005). Since poor solubility in water can be a major hindrance to successful removal of PAHs by microbiological means, chemical reactions involved in the breakdown of PAHs such as substituent addition, ring fission or ring hydroxylation aim to increase the aqueous solubility of the PAH and facilitate easier degradation of the contaminant (Harvey 1997). The physical and chemical characteristics of selected USEPA priority PAHs are listed in Table 1.1.
Figure 1.1: Chemical structures of 16 USEPA priority PAHs [adapted from Yan et al., 2004]
Table 1.1: Physical and chemical properties of selected USEPA priority PAHs [adapted from International Programme on Chemical Safety (IPCS), 1998 with information from Cerniglia, 1992; Mrozik et al., 2003]

<table>
<thead>
<tr>
<th>Compound</th>
<th>No. C atoms</th>
<th>MW (Da)</th>
<th>Water solubility at 25°C (μg/L)</th>
<th>n-Octanol: water partition coefficient (Log K&lt;sub&gt;ow&lt;/sub&gt;)</th>
<th>Vapour pressure (Pa at 25°C)</th>
<th>Boiling point (°C)</th>
<th>Density</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>10</td>
<td>128.2</td>
<td>3.17 x 10&lt;sup&gt;4&lt;/sup&gt;</td>
<td>3.4</td>
<td>10.4</td>
<td>218</td>
<td>1.154</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>14</td>
<td>178.2</td>
<td>1.29 x 10&lt;sup&gt;3&lt;/sup&gt;</td>
<td>4.6</td>
<td>1.6 x 10&lt;sup&gt;-2&lt;/sup&gt;</td>
<td>340</td>
<td>0.98</td>
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<tr>
<td>Anthracene</td>
<td>14</td>
<td>178.2</td>
<td>73</td>
<td>4.5</td>
<td>8.0 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>342</td>
<td>1.283</td>
</tr>
<tr>
<td>Pyrene</td>
<td>16</td>
<td>202.3</td>
<td>135</td>
<td>5.18</td>
<td>6 x 10&lt;sup&gt;-4&lt;/sup&gt;</td>
<td>393</td>
<td>1.271</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>16</td>
<td>202.3</td>
<td>260</td>
<td>5.22</td>
<td>1.2 x 10&lt;sup&gt;-3&lt;/sup&gt;</td>
<td>375</td>
<td>1.252</td>
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<tr>
<td>Chrysene</td>
<td>18</td>
<td>228.3</td>
<td>2</td>
<td>5.91</td>
<td>8.4 x 10&lt;sup&gt;-5&lt;/sup&gt;</td>
<td>448</td>
<td>1.274</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>20</td>
<td>252.3</td>
<td>3.8</td>
<td>6.5</td>
<td>7.3 x 10&lt;sup&gt;-7&lt;/sup&gt;</td>
<td>496</td>
<td>1.351</td>
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</table>
1.2.2. Toxicology

The detrimental health and environmental effects associated with PAH exposure and contamination has been of public concern for decades. These health concerns are compounded by the ubiquitous occurrence of these pollutants and the potentially multiple routes of exposure to humans. The IARC (International Agency for Research on Cancer) considers 15 PAHs as potential carcinogens (Chauhan et al., 2008). The differing potential for toxicity, carcinogenicity and mutagenicity of PAHs can be attributed to the differences in chemical structure and molecular size (Cerniglia, 1992). Generally, LMW PAHs are acutely toxic and as the number of aromatic rings and the molecular size increases, there is a shift towards chronic toxicity including carcinogenesis (Cerniglia, 1992; Miller and Miller, 1981). Table 1.2 lists the carcinogenic and genotoxic properties of the 16 US EPA PAH priority pollutants.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Genotoxicity</th>
<th>Carcinogenicity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>-</td>
<td>(?)</td>
</tr>
<tr>
<td>Acenaphthene</td>
<td>(?)</td>
<td>?</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>(?)</td>
<td>No studies available</td>
</tr>
<tr>
<td>Anthracene</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>(?)</td>
<td>(?)</td>
</tr>
<tr>
<td>Fluorene</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Fluoranthenene</td>
<td>+</td>
<td>(+)</td>
</tr>
<tr>
<td>Benzo(a)anthracene</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Chrysene</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Pyrene</td>
<td>(?)</td>
<td>(?)</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benzo(b)fluoranthenene</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benzo(k)fluoranthenene</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Dibenz(a,h)anthracene</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Benzo(g,h,i)perylene</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Ideno[1,2,3-cd]pyrene</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

Genotoxicity and Carcinogenicity: + = positive, - = negative, ? = questionable, ( ) = results derived from a small database USEPA has classified some PAHs (in italics) as probable human carcinogens (Bojes and Pope, 2007)
In humans, PAHs have the potential to be absorbed through the skin, pulmonary tract or the gastrointestinal tract (IPCS, 1998). Following absorption through any of these routes, lipophilic PAHs are widely distributed throughout the internal organs, but are predominantly present in those that are lipid rich. PAH metabolism in the body generally leads to detoxification involving the conversion of parent compounds to phenols, diols and tetrols via epoxide intermediates. PAH turnover in the body is rapid for those PAHs that are not bound to nucleic acids (diol epoxides) and the metabolites are excreted via the urine, bile and faeces after conjugation with glutathione or glucuronic acid, or by further metabolism to tetrahydrotetrols (IPCS, 1998).

The most important step of primary PAH metabolism is epoxidation and subsequent diol formation (Government of Canada 1999). Most PAHs are oxidized to form phenols and dihydrodiols via monooxygenase enzymes associated with cytochrome P-450, but a small proportion will be further epoxidized to reactive intermediates (diol epoxides) that result in carcinogenic activity in mammalian cells (Kloppman et al., 1999). These activities take place predominantly within the microsomes of the endoplasmic reticulum and in nuclear membranes (Wattiau, 2002). In the case of benzo(a)pyrene, the major intermediates of cytochrome mediated metabolism are 7-8 and 9-10 epoxides, which bind to nucleic acids causing mutations or tumorigenesis during DNA replication at the damaged site or during DNA repair. The extranuclear groups of adenine and guanine are usually the sites of attack by reactive intermediates during b(a)p metabolism (Canadian Centre for Occupational Health and Safety 1998).

Two mechanisms are responsible for the damage and toxicity caused by PAHs (Knutzen 1995). The first is the association or reaction of the PAH parent compound with lipids in cell membranes or other cellular components. Interactions between PAHs and the cell membrane can
affect the permeability of the membrane as well as alter transport into and out of the cell (Knutzen 1995). The second mechanism is the reaction of PAH metabolites with nucleic acids and proteins. PAHs can become activated (interact with subcellular targets) to DNA-binding species, which can form DNA adducts and initiate tumours in experimental animals (Godschalk et al., 1998; Juhasz & Naidu, 2000). A number of PAHs including chrysene, benzo(a)pyrene and fluoranthene give rise to metabolites that form DNA adducts in animals (Canadian Centre for Occupational Health and Safety 1998).

PAH structure has a role in the toxigenic potential of the molecule. In fact, the shape of the PAH molecule as an indicator of acceptable targets for cytochrome enzymes is thought to be the best predictor of PAH carcinogenicity (Kloppman et al., 1999). PAHs can have differing toxic potentials based on the site of metabolic activation created by structures present such as a “bay” region (a void in the structure of the molecule bordered by the edge of three rings), or a “fjord” region (a void in the structure of the molecule bordered by the edge of four rings) (Harvey 1997). Figure 1.2 illustrates the “bay” and “fjord” regions of a PAH molecule. The “bay” region diol epoxide intermediates are highly carcinogenic and can initiate carcinogenesis and mutagenesis via covalent bonds to cellular macromolecules and DNA. Although benzo(a)pyrene is carcinogenic and possesses a “bay” region, not all PAHs with “bay” regions have been found to be carcinogenic, as seen in the lack of evidence that benzo(e)pyrene has carcinogenic potential even though it possesses two “bay regions” (Kloppman et al., 1999).
1.2.3. Sources and Presence of PAHs in the Environment

PAHs can originate from biosynthetic, geochemical and anthropogenic sources and are widely distributed in soil, water and air. The major sources of PAHs include crude oil, coal and oil shale that are used as sources of fuel for industrial nations and in the production of petrochemicals required by the plastics industry (Harvey 1997). PAHs are also a major constituent of creosote that is often used in the treatment of wood (Walter et al., 1991). The distribution and magnitude of PAH emissions in the environment are influenced in large part by not only human population density but also the availability of power and the presence of natural resources (Government of Canada 1999). In the last 100 years, environmental levels of PAHs have been on the rise from both natural and anthropogenic sources (Juhasz & Naidu, 2000). Table 1.3 lists the major sources of environmental PAH contamination.

Natural sources of PAHs in the environment include those which are of either biosynthetic or geochemical origin (Labana et al., 2007). PAHs can be found as biogenic components of plant oils, surface waxes of leaves, insect cuticles and in the lipids of microorganisms (Millero and Sohn, 1992). PAHs can arise from geochemical sources during the

Figure 1.2: Bay / fjord regions of benzo(c)chrysene and b) bay region of benzo(a)pyrene [adapted from Harvey, 1997]
processes of combustion or pyrolysis, where organic substances are exposed to varying degrees of heat (Crawford 1996). Temperature plays a significant role in determining the structure and degree of chemical substitution on PAHs, with combustion temperatures above 700°C favouring the production of un-substituted PAHs, and temperatures below 700°C favouring methyl- and alkyl-substituted PAHs (Hilpert 1987). PAHs can also be released through natural phenomena such as forest fires and volcanic eruptions or they can be found in naturally occurring petroleum and coal (Crawford, 1996; Labana et al., 2007). Although natural sources of PAHs contribute to the overall level of PAH contamination, anthropogenic sources are acknowledged to be the most important source of atmospheric PAH pollution (Harvey, 1997).

Table 1.3: Major sources of PAHs in the environment [from Cerniglia, 1992]

- Natural oil seeps
- Refinery and oil storage wastes
- Accidental spills from oil tankers and other ships
- Municipal and urban wastewater discharge runoff
- River-borne pollution
- Atmospheric fallout of fly ash particulates
- Petrochemical industrial effluents
- Coal tar and other coal processing wastes
- Automobile engine exhausts
- Combustion of fossil fuels (gasoline, kerosene, coal, diesel fuel)
- Tobacco and cigarette smoke
- Forest and prairie fires
- Rural and urban sewage sludge
- Refuse and waste incineration
- Coal gasification and liquefaction processes
- Creosote and other wood preservative wastes

The introduction of PAHs into the environment via anthropogenic sources can fall under two categories. The first is through the intentional dumping or accidental spillage of petroleum
products, creosote or coal tar, and the second is from the incomplete combustion of organic material arising from automobile emissions, industrial discharge or municipal incineration (Crawford, 1996; Labana et al., 2007). The processing, combustion and disposal of fossil fuels and the treatment and disposal of refinery wastes (landfarming) also contribute to the presence of PAHs in the environment (Crawford 1996). Levels of PAHs present in the atmosphere are dependent on a number of variables including local emission sources, temperature and meteorological conditions and tend to rise during the winter months due to an increase in fossil fuel consumption (Harvey, 1997). Table 1.4 lists the annual atmospheric emissions of PAHs in Canada during 1990. Atmospheric PAH deposition is considered to be the primary source of PAHs in soils and sediments and is therefore of great environmental concern due to the potential for toxicity (Christensen and Zhang, 1993; Harvey, 1997).

Table 1.4: Annual atmospheric emissions of PAHs in Canada in 1990 [From LGL 1993]

<table>
<thead>
<tr>
<th>Sources</th>
<th>Tonnes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aluminum Plants</td>
<td>925</td>
</tr>
<tr>
<td>Coke Production</td>
<td>12.8</td>
</tr>
<tr>
<td>Petroleum Refineries</td>
<td>2.5</td>
</tr>
<tr>
<td>Residential Heating Wood</td>
<td>474</td>
</tr>
<tr>
<td>Open air fires/agricultural burning</td>
<td>358</td>
</tr>
<tr>
<td>Transportation Diesel</td>
<td>155</td>
</tr>
<tr>
<td>Forest Fires</td>
<td>4314</td>
</tr>
</tbody>
</table>

The existence of PAHs in the environment is of interest to toxicologists due to a number of factors which set them apart from other priority pollutants (Douben, 2003). Unlike many industrial chemicals, PAHs are not released into the environment from only a single source, but can arise from such sources as motor vehicle exhaust, coke production and aluminum plants. Secondly, PAHs present in petroleum are not typically found in pure chemical forms and are
released along with a multitude of other aliphatic and aromatic compounds (Douben 2003). Third, unlike other contaminants, which are usually found with a limited number of possible chemical structures, PAHs can be found with thousands of different chemical structures. Finally, due to the widespread use of fossil fuels on a global scale, PAHs continue to be released into the environment even though they are known toxicants, unlike some other harmful chemicals which have been restricted or banned (Douben 2003).

1.2.4. Fate in the Environment

PAHs are subject to a number of fates in the environment including chemical and photochemical oxidation, sedimentation, volatilization, bioaccumulation and biotransformation (Figure 1.3). The physico-chemical properties of PAHs including low vapour pressure, poor water solubility and high partition coefficients for $n$-octanol:water (log $K_{ow}$) and organic carbon:water (log $K_{oc}$) play important roles in determining the fate and transport of PAHs in the environment (Canadian Centre for Occupational Health and Safety 1998). Changes to the molecule structures of PAHs as a result of environmental modification may result in changes in mobility, toxicity and chemical characteristics possibly rendering the molecule to bind to soil components, or destruction of the molecule via biotransformation (Mueller et al., 1996). The following section describes the various fates of PAHs in the environment.
1.2.4.1. *Photochemical Oxidation*

The fate of PAHs in the environment and the rate of transformation often depend on the media to which the molecules are exposed including soil, water or air in the presence or absence of light (Ramamurthy and Schanze, 2000). PAHs can strongly absorb solar radiation and undergo transformation through photodegradation (Slaski et al., 2000). In water or air, this process can occur either directly by exposure to light at a wavelength less than 290 nm, or indirectly by exposure to oxidizing agents including OH radicals, O₃, and NO₃ (Douben 2003). The effectiveness of light in inducing PAH modification is related to the wavelength, with short wave (actinic) radiation (0.1µm to 0.5µm) being more effective in promoting modification than visible light (Slaski et al., 2000).
The rate of photochemical oxidation of PAHs depends on the concentration of molecular oxygen present as well as PAH chemical structure, particularly the number and structure of condensed aromatic rings (Kochany and Maguire, 1994). Photolysis occurs faster for those PAHs with higher MW and a greater number of rings (Kochany and Maguire, 1994). In water and air, photodegradation of PAHs can occur rapidly when light is present; however in soil, photodegradation occurs to a very limited extent due to the low intensity of light (Government of Canada 1999). Atmospheric photochemical reactions transform PAHs to less volatile and more polar derivatives, including nitrated, oxygenated and hydroxylated PAHs, which can lead to an increase in bioavailability and toxicity (Douben 2003).

Numerous factors in the environment play roles in enhancing the photochemical oxidation of PAHs including light intensity (Douben 2003), salinity, the presence of minerals (Kong and Ferry, 2003), pH and temperature (Miller and Olejnik, 2001). Carbonaceous materials including humic acids have been found to decrease the rate of photolysis, whereas the presence of algae has been reported to increase the rate of photodegradation although the mechanism is unknown (Kochany and Maguire, 1994). Photodegradation plays an important role in enhancing the biodegradation of PAHs by attacking the tertiary carbon atoms (a carbon atom bonded to three other carbon atoms with single bonds) in PAHs, which often inhibit microbial degradation (Zhang et al., 2006). Photochemical oxidation is an important abiotic PAH degradation mechanism for particulate associated PAHs and dissolved PAHs, particularly in PAH remediation following oil spills (Douben, 2003; Ke et al., 2002).

1.2.4.2. **Chemical Oxidation**

Photochemical, enzymatic and biological interactions with PAHs occur prominently within the soil, but are not always successful in the complete remediation of the compounds.
Aromatic PAHs can be chemically altered in soils by chemical oxidants including Fe and Mn metal ions, oxides and oxyhydroxides of Al, Mn, Si and Fe, radicals of active oxygen species, microbial enzymes, and by unsaturated fatty acids of plant residues (Gramss et al., 1999). The altered PAHs are often rendered less recalcitrant and more bioavailable to microorganisms for further breakdown (Ferrarese et al., 2008).

Common chemical oxidants that are used for environmental remediation include ozone, hydrogen peroxide and permanganate (Ferrarese et al., 2008). The addition of an oxidative agent such as hydrogen peroxide leads to the production of very strong non-selective oxidizing agents (hydroxyl radicals) that can react with aromatic PAHs and induce transformation (Ferrarese et al., 2008). The oxidative strength of peroxides can be increased when combined in solution with a transition metal (Fe) as evidenced by the successful use of Fenton’s reagent in environmental remediation of organic compounds including PAHs (Nam et al., 2001).

1.2.4.3. Volatilization

Volatilization occurs when there is physical transport of an organic compound into the atmosphere from water, soil or vegetation resulting in the modification or destruction of the chemical (Gowda and Lock, 1985). PAHs have predominantly low vapour pressures, and volatilization is limited to those LMW PAHs such as naphthalene with a volatilization half life ranging from 0.4 h to 3.2 h or anthracene with a half life of 17 h (Southworth 1979). HMW PAHs such as pyrene take much longer to volatilize as evidenced by its volatilization half life ranging from 115 hours to 3.2 years (Southworth 1979). The volatilization half lives of selected PAHs are listed in Table 1.5.
Table 1.5: Estimated half lives of PAHs in various environmental compartments (air, water, soil and sediment) [From IPCS, 1998]

<table>
<thead>
<tr>
<th>Class</th>
<th>Half Life-Mean (Hours)</th>
<th>Range (Hrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>17</td>
<td>10-30</td>
</tr>
<tr>
<td>2</td>
<td>55</td>
<td>30-100</td>
</tr>
<tr>
<td>3</td>
<td>170</td>
<td>100-300</td>
</tr>
<tr>
<td>4</td>
<td>550</td>
<td>300-1000</td>
</tr>
<tr>
<td>5</td>
<td>1700</td>
<td>1000-3000</td>
</tr>
<tr>
<td>6</td>
<td>5500</td>
<td>3000-10000</td>
</tr>
<tr>
<td>7</td>
<td>17000</td>
<td>10000-30000</td>
</tr>
<tr>
<td>8</td>
<td>55000</td>
<td>&gt;30000</td>
</tr>
</tbody>
</table>

(Half life classifications (1-8) used below were assigned to individual PAHs based on half-life estimates in air, water, soil and sediment listed above)

<table>
<thead>
<tr>
<th>Compound</th>
<th>Environmental Compartment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Air</td>
</tr>
<tr>
<td>Acenaphthylene</td>
<td>2</td>
</tr>
<tr>
<td>Anthracene</td>
<td>2</td>
</tr>
<tr>
<td>Benz(a)anthracene</td>
<td>3</td>
</tr>
<tr>
<td>Benzo(a)pyrene</td>
<td>3</td>
</tr>
<tr>
<td>Benzo(k)fluoranthene</td>
<td>3</td>
</tr>
<tr>
<td>Chrysene</td>
<td>3</td>
</tr>
<tr>
<td>Dibenz(a,h)anthracene</td>
<td>3</td>
</tr>
<tr>
<td>Fluoranthene</td>
<td>3</td>
</tr>
<tr>
<td>Fluorene</td>
<td>2</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>1</td>
</tr>
<tr>
<td>Perylene</td>
<td>3</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>2</td>
</tr>
<tr>
<td>Pyrene</td>
<td>3</td>
</tr>
</tbody>
</table>

Various factors can influence the rate of volatilization of PAHs in the environment. These factors include the physical and chemical properties of the substance such as MW, number of aromatic rings and environmental variables including temperature, wind and water turbulence (Southworth 1979). Increased adsorption of PAHs to organic material can limit PAH volatilization while volatilization can be enhanced by increased air flow in soil resulting from plant root penetration (Genney 2004). In soils, PAHs are removed primarily by either microbial activity or volatilization (Wild et al., 1991). Two or three ringed PAHs in refinery waste applied
during landfarming operations can generally be expected to volatilize or be biodegraded in three to four months, although repeated applications can result in accumulation of the compounds (Government of Canada 1999).

1.2.4.4. *Sedimentation*

Sediments act as sinks for PAHs from natural and anthropogenic sources in coastal environments, and pose a risk to marine biota and to human public health. Hydrophobic PAHs from industrial effluents and runoff in streams, lakes and oceans are readily taken up by suspended particles in sediments which are coated in a matrix of organic matter (Shiaris, 1989). Sorption to organic matter results in PAHs becoming highly refractory - resisting biodegradation and dissolution in water (Chiou et al., 1998). As PAHs sorb to particulate matter, they eventually settle out of the water column onto bottom sediments, resulting in elevated concentrations of PAHs including pyrene, chrysene and benzo(a)pyrene in those sediments relative to those in the water (Moore and Ramamoorthy, 1984). PAH accumulation potential in sediments can be predicted by factors including sediment aromaticity, sediment organic content and PAH octanol: water coefficients ($K_{ow}$) (Arzayus et al., 2001). In addition to deposition and sedimentation, other important fates of PAHs in aquatic environments include cycling through or suspension within the water column, bioaccumulation in marine organisms and biotransformation (Arzayus et al., 2001).

PAH deposition to the water column can occur through gas flux or dry particle deposition from the atmosphere (Arzayus et al., 2001). PAHs that are introduced to water by gas exchange can become bound to organic matter such as plankton and be cycled through a process involving ingestion by marine biota and deposition as fecal pellets. PAHs that are associated with aerosols are in contrast, slower to sink in water and are more resistant to the recycling process. Although
the flux of particles to the seabed is an important contributor to the presence of PAHs in aquatic environments, the recycling rate of organic matter through marine organisms also plays an important role in the accumulation of PAHs in sediments (Arzayus et al., 2001).

Biotransformation of PAHs is the primary mechanism of PAH degradation in sediments (Shiaris, 1989). Certain environmental factors have important roles in the rate of biodegradation and fate of PAHs in coastal environments. In sediments rich in organic matter, these factors include the limited rate of O$_2$ diffusion, temperature and PAH structure. In oxidized surficial sediments, salinity, season, nutrients, and carbon source availability are important variables affecting the rate of PAH transformation (Shiaris, 1989).

1.2.4.5. **Bioaccumulation**

The kinetic approach for modelling bioaccumulation of PAHs in organisms is described as the net effect of the rate processes – uptake and elimination, whereas the equilibrium partitioning (EqP) approach assumes near equilibrium between organisms and the aquatic environment (Douben 2003). Coefficients such as the bioconcentration factor (BCF) and bioaccumulation factor (BAF) are useful indicators of expected PAH tissue residues based on environmental PAH concentrations. The octanol: water partition coefficient (K$_{ow}$) can be used to predict PAH partitioning, behaviour and bioavailability in the environment (Douben 2003).

The partitioning behaviour of hydrophobic PAHs in tissue, water and sediment is affected by two important nonpolar phases – organic content and lipid content (Douben 2003). Factors including the organism’s metabolic capacity to process PAHs, environmental PAH concentrations and time of exposure can affect the level of accumulation in tissues. Additionally, sediment surface area, PAH source and desorption rate are all important environmental variables in determining the proportion of PAHs available for uptake (Douben 2003).
Organisms having little ability to metabolize PAHs (such as molluscs) are more likely to accumulate high levels of PAHs versus those organisms (such as fish) having the ability to metabolize these compounds (IPCS, 1998). Although fish can metabolize PAHs, and accumulate little to no PAHs, this activity may result in the production of reactive intermediates causing harm to the organism (Schuler et al., 2004). Biomagnification of PAHs through trophic transfer is dependent upon the organism’s capability to metabolize the compound (Hofelt et al., 2001). Organisms that are present in lower trophic levels generally accumulate higher levels of PAHs in their tissues over those organisms in higher trophic levels due to consumption of PAH contaminated sediments (Nakata et al., 2003). The poor aqueous solubility and low reactivity of HMW PAHs also convey a greater potential for biomagnification of these compounds in the environment (Kanaly and Harayama, 2000).

1.2.4.6. Biotransformation

PAH biotransformation occurs due to the activity of microorganisms, particularly by bacteria and fungi (Atlas and Bartha, 1987). In the environment, PAH-degrading microorganisms can be found in areas such as soils and sediments which have been previously contaminated by PAH containing waste such as oil spills. In non-contaminated soils, microorganisms which are capable of PAH degradation can obtain the required carbon and energy from the degradation of organic compounds including PAHs (Atlas and Bartha, 1987). These microorganisms are capable of degrading both natural and xenobiotic compounds, however if the compounds have never been encountered by the organism and have structural features differing from those of natural compounds, biodegradation is typically not observed. Section 3 will provide an in depth review of the mechanisms of PAH degradation by bacteria.
1.3. LANDFARMING

1.3.1. History and Operation by Petroleum Refineries

Landfarming has been utilized by petroleum refineries since the 1950s and has become a commonly used practice during the past two decades (Concord Scientific Corporation and Beak Consultants, 1992). Oily waste sludges from Canadian petroleum refining operations can vary in hydrocarbon composition depending upon source, treatment and storage. Generally refinery sludges contain 50-85% water, 5-20% solids and 10-30% hydrocarbon content (Ward et al., 2003). The various types of oil refinery sludges generated at Canadian facilities are listed in Table 1.6.

Table 1.6: Categories of Canadian oil refinery sludges [from Petroleum Association for Conservation of the Canadian Environment, 1980]

- Desalting sludge
- American Petroleum Institute (API) sludge
- Floatation froth
- Biosludge
- Basin settlings
- Storm silt
- Filter backwash
- Slop emulsions
- Cooling water tower sludge
- Unleaded tank bottom sludge

Landfarming is a process that is used by the petroleum and petrochemical industries for remediation of PAH containing oily wastes through the stimulation of natural PAH-degrading microbial communities in the soil (Straube et al., 2003). Physical mixing through tillage helps to increase aeration and to distribute PAH contaminants over a wider surface area of soil particles, enabling increased contact with microorganisms, while aiming to minimize the release of harmful
runoff, leachate and vapours (Straube et al., 2003). The degree and efficiency of bioremediation through landfarming is limited by both the physico-chemical properties of the soil including adherence of PAHs to surfaces and nonaqueous phase liquids (NAPL), as well as the microbiological conditions (Prichard et al., 2006). Limiting factors include the type and concentration of contaminants, nitrogen availability to sustain carbon mineralization, availability of nutrients and electron acceptors, the indigenous microbial population, pH, temperature, moisture content of the soil and substrate bioavailability (Prichard et al., 2006; Yerushalmi et al., 2003).

Rates of refinery sludge application vary depending upon soil characteristics, climate and other site conditions, and reapplication typically occurs when the oil content in the soil has decreased to below 4% of the original volume applied (Concord Scientific Corporation and Beak Consultants, 1992). Although landfarming has traditionally been used for the bioremediation of oily waste sludges, the process has been banned in the United States and is being phased out in many other areas due to environmental concerns (Singh and Ward, 2004b).

Although landfarming has a number of advantages including minimal operation costs and maintenance requirements and the ability to be used to treat large quantities of contaminated sludge, there are also a number of drawbacks and limitations associated with the process (Prichard et al., 2006). In general, the deliberate contamination of large tracts of land with oily sludges containing recalcitrant compounds is not environmentally acceptable. In addition, once newer treatment technologies are adopted, landfarming sites are likely to be decommissioned, and the land may not be suitable for other uses due to residual contamination with recalcitrant PAHs (Van Hamme et al., 2003). As a result, contaminated soil may need to be disposed of as hazardous waste or be treated using other methods such as chemical oxidation or by surfactant addition to promote increased PAH degradation (Prichard et al., 2006). Other drawbacks of
landfarming include poor contact of PAHs with microorganisms, poor aeration and insufficient nitrogen for cellular activities which can lead to incomplete PAH degradation (Prichard et al., 2006). Often a lack of control over factors related to microbial activity during the landfarming process leads to prolonged treatment times which can be undesirable (Van Hamme et al., 2003).

Methods of overcoming these limitations as part of a modified landfarming process include nutrient amendments (fertilizer), bulking agents, bioaugmentation, irrigation and tilling (Prichard et al., 2006). Biostimulation and bioaugmentation of hydrocarbon contaminated soil using the surfactant producing bacterium *Pseudomonas aeruginosa* strain 64, bulking agents and fertilizer were found to successfully promote the degradation of HMW PAHs in soils when compared to traditional landfarming practices (Prichard et al., 2006). Pilot scale studies utilizing troughs were conducted to study the effects of biostimulation and bioaugmentation on LMW and HMW PAH degradation. The major PAHs present in the soil included phenanthrene, anthracene and fluoranthene with initial concentrations of 2200 mg/kg, 1000 mg/kg and 1300 mg/kg respectively. Following 16 months of treatment, supplementation with a bulking agent and slow release nutrients resulted in a PAH removal rate of 86% while supplementation combined with bioaugmentation utilizing the surfactant producer *P. aeruginosa* strain 64 resulted in an 87% PAH removal rate when compared to the control trough which only showed a 12% decrease in PAH levels (Prichard et al., 2006).

Although landfarming has been successfully utilized for decades, newer and more environmentally acceptable methods of treating PAH containing oily wastes such as slurry bioreactors are being increasingly explored and utilized by petroleum and petrochemical companies. The advantages of slurry bioreactor technology are described in Section 1.6.4.
1.3.2. Ontario Ministry of the Environment Regulation 461/05

In 2005, the Ontario Government introduced Ontario Regulation 461/05, a land disposal restriction requiring sites operating landfarms for the treatment of hydrocarbon waste to meet legislated criteria by 31 December 2009 (Ontario Ministry of the Environment 2005). This legislation targets companies operating petroleum refineries and sets new limits for individual PAH concentrations in sludge prior to disposal on land. Table 1.7 outlines the M.O.E. criteria for hazardous compounds (including PAHs) in petroleum waste to be met prior to land disposal.

In order for petroleum refineries to comply with the regulation, they must seek waste pre-treatment options to effectively lower waste PAH concentrations to acceptable levels prior to landfarm disposal. Numerous waste treatment options exist, but can be capital-intensive such as the physico-chemical methods of incineration, thermal desorption, refinery coker use, burning in cement kilns and solvent extraction (Ward et al., 2003). These methods also require constant monitoring and control for optimal performance, and may not result in the complete destruction of contaminants (Yerushalmi et al., 2003). In contrast, biological based pre-treatment methods are of interest to refineries due to their cost effectiveness, perception of being environmentally sound and ease of implementation within a refinery. Bioreactor-based approaches for sludge treatment are considered an attractive option to petroleum refineries as a way to meet Ministry criteria. Application of a bioreactor allows for containment of PAH containing waste, and successful sludge bioremediation through an optimized process, while providing the operator with control and discretion over degradation parameters (Van Hamme et al., 2003).
Table 1.7: Pre-treatment concentrations for hazardous compounds potentially treated in soil outlined in Ontario Reg. 461/05 by the Ontario Ministry of the Environment [from Ontario Ministry of the Environment, 2000]

<table>
<thead>
<tr>
<th>Hazardous Industrial Waste</th>
<th>Regulated Constituents (and Treatment Subcategories)</th>
<th>Land Disposal Treatment Requirements</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Aqueous Waste</td>
</tr>
<tr>
<td>Column 1</td>
<td>Column 2</td>
<td>Column 3</td>
</tr>
<tr>
<td>Haz. Waste Number</td>
<td>Waste</td>
<td>Generic Name or other description</td>
</tr>
<tr>
<td>F037</td>
<td>Petrochemical refinery primary oil/water/solids separation sludge – Any sludge generated from the gravitational separation of oil/water/solids during the storage or treatment of process wastewaters and oily cooling wastewaters from petroleum refineries. Such sludges include, but are not limited to, those generated in: oil/water/solids separators; tanks and impoundments; ditches and other conveyances; sumps and stormwater units receiving dry weather flow. Sludge generated in stormwater units that do not receive dry weather flow, sludge generated from non-contact once-through cooling waters segregated for treatment from other process or oily cooling waters, sludges generated in aggressive biological treatment units as defined in s. 261.31(b)(2) (including sludges generated in one or more additional units after wastewaters have been treated in aggressive biological treatment units) and K051 wastes are not included in this listing. This listing does include residuals generated from processing or recycling oil-bearing hazardous secondary materials excluded under s. 261.4(a)(12)(i), if those residuals are to be disposed of.</td>
<td>Acenaphthene</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>Anthracene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Benzene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Benz(a)anthracene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Benzo(a)pyrene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bis(2-Ethylhexyl)phthalate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chrysene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Di-n-butyl phthalate</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Ethylbenzene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Fluorene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Naphthalene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenanthrene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Phenol</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Pyrene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Toluene</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Xylenes – mixed isomers (sum of -, m-, and p-xylene concentrations)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Chromium (Total)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Cyanides (Total)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Lead</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Nickel</td>
</tr>
</tbody>
</table>
1.4. MICROBIAL DEGRADATION OF POLYCYCLIC AROMATIC HYDROCARBONS

1.4.1. Introduction

Microorganisms including species of bacteria, fungi and algae with the capability of degrading PAHs of up to 5 aromatic rings have been isolated from various soil and marine environments around the world. While there is a diverse group of microorganisms with the capability of degrading LMW PAHs such as phenanthrene and anthracene, only a limited number have been identified as able to degrade HMW PAHs such as benzo(a)pyrene (Juhasz and Naidu, 2000). In soils, PAH degrading microorganisms are common members of the indigenous microbial population.

Bacteria and fungi are the predominant microorganisms able to degrade PAHs through one of two mechanisms. The first is when a PAH contaminant is used as a source of carbon and energy, and the second is by co-metabolism. Co-metabolism is an important process for the degradation of HMW PAHs, as well as for some PAH mixtures (Chauhan et al., 2008; Habe and Omori, 2003). Microbial PAH degradation pathways can be aerobic or anaerobic, and the enzymes can have broad substrate specificity (Habe and Omori, 2003).

This thesis will focus on the bacterial degradation of five target PAHs – phenanthrene, anthracene, pyrene, chrysene and benzo(a)pyrene. These PAHs were chosen because they are present within refinery effluent waste from NOVA Chemicals and they represent a range of PAHs with varying MW, solubility, and bioavailability. A general discussion of the mechanisms of PAH biodegradation will be followed by a more detailed description of the degradation of the individual PAHs.

1.4.2. Bacterial PAH Degradation

Numerous genera of Gram-positive and Gram-negative bacteria have been isolated and
characterized for their ability to degrade and utilize PAHs. A list of bacterial species capable of PAH degradation is presented in Table 1.8. Bacterial PAH degradation occurs predominantly through aerobic oxygenase mediated catabolism (Kanaly and Harayama, 2000). The metabolic pathways, enzymes and genes involved during aerobic PAH metabolism have been well studied and documented. It has also been reported that in the absence of molecular oxygen, nitrate, ferrous and sulphate ions can be used as alternative electron acceptors to oxygen during some PAH degradation pathways (Suthersan 1999).

Three functions can be served by the microbial degradation of PAHs. The first is biodegradation of the PAH resulting in carbon and energy for the microorganism. The second is co-metabolism where two (or more) PAHs are degraded, but only one contributes towards the carbon and energy requirements of the cell. The third function is an intracellular process to make PAHs more water soluble, as a detoxification method necessary for cell excretion (Johnsen et al., 2005).

The process of PAH biodegradation involves four steps including a) PAH solubilization, b) PAH transport into the cell, c) the expression of PAH degradative genes and d) the enzymatic breakdown of the PAHs (Chauhan et al., 2008). A failure in any one of these steps could result in limited success of PAH bioremediation, although the process is not fully understood (Chauhan et al., 2008).
Table 1.8: PAHs oxidized by different species/strains of bacteria [adapted from Juhasz and Naidu, 2000 with information from UMBBD 2010]

<table>
<thead>
<tr>
<th>Bacterial strains</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Acinetobacter calcoaceticus, Alcaligenes denitrificans, Mycobacterium sp., Pseudomonas sp., P. putida, P. fluorescens, Sp. paucimobilis, Brevundimonas vesicularis, Burkholderia cepacia, Comamonas testosteroni, Rhodococcus sp., Corynebacterium renale, Moraxella sp., Streptomyces sp., B. cereus, P. marginalis, P. stutzeri, P. saccharophila, Neptunomonas naphthovorans, Cycloclasticus sp., Bacillus thermoleovorans</td>
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<tr>
<th>Table 1.8 (cont.): Polycyclic aromatic hydrocarbons oxidized by different species/strains of bacteria [Adapted from Juhasz and Naidu, 2000 with information from UMBBD 2010]</th>
</tr>
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<tbody>
<tr>
<td><strong>Anthracene</strong></td>
</tr>
<tr>
<td>Beijernickia sp., Mycobacterium sp., P. putida, Sp. paucimobilis, Bu. cepacia, Rhodococcus sp., Flavobacterium sp., Arthrobacter sp., P. marginalis, Cycloclasticus sp., P. fluorescens, Sp. yanoikuyae, Acinetobacter calcoaceticus, Gordona sp., Sphingomonas sp., Comamonas testosteroni, Cycloclasticus pugetii</td>
</tr>
<tr>
<td><strong>Pyrene</strong></td>
</tr>
<tr>
<td>A. denitrificans, Mycobacterium sp., Rhodococcus sp., Sp. paucimobilis, Stenotrophomonas maltophilia, Acinetobacter calcoaceticus, Gordona sp., Sphingomonas sp., P. putida, Bu. cepacia, P. saccharophila, Mycobacterium gilvum, Leclercia adecarboxylata, Mycobacterium pyrenivorans, Achromobacter xylooxidans</td>
</tr>
<tr>
<td><strong>Chrysene</strong></td>
</tr>
<tr>
<td><strong>Benzo(a)pyrene</strong></td>
</tr>
<tr>
<td>Sp. paucimobilis, Beijerinckia sp., Mycobacterium sp., Rhodococcus sp. UWI, Pseudomonas sp., Agrobacterium sp., Bacillus sp., Burkholderia cepacia, Stenotrophomonas maltophilia</td>
</tr>
<tr>
<td>Ye et al., (1996); Gibson et al., (1975); Schneider et al., (1996); Walter et al., (1991); Aitken et al., (1998); Juhasz et al., (1997); Boonchan et al., (2000)*</td>
</tr>
</tbody>
</table>

*= Recorded as a substrate in the University of Minnesota – Biocatalysis/Biodegradation Database (http://umbbd.ahc.umn.edu/index.html)
During PAH catabolism by bacteria, the initial oxidation is aromatic ring hydroxylation to yield a dihydro-diol ring structure (Cerniglia, 1992). Ring cleaving dioxygenase enzymes oxidize catechols via the meta or the ortho pathway. Meta cleavage involves attack of the catechol at the bond position between a carbon with a hydroxyl group, and an adjacent carbon without a hydroxyl group to form 2-hydroxymuconic semialdehyde, while the ortho pathway involves cleavage of the bond between two carbon atoms, each with a hydroxyl group attached to produce cis,cis-muconic acid (Cerniglia, 1992). The end result of ring cleavage is the production of compounds such as acetic acid, acetaldehyde, succinic and pyruvic acids which can be utilized by the microorganism for energy and the production of cellular macromolecules (Wilson and Jones, 1993). Figure 1.4 illustrates how the aromatic ring can be cleaved via either meta or ortho cleavage.

PAH transport into the cell generally occurs by passive diffusion along a concentration gradient from the surrounding environment (Johnsen et al., 2005). This process is dependent upon both PAH bioavailability and concentration. At low concentrations, passive diffusion of PAHs into bacterial cells may not occur, limiting the success of microbial PAH degradation (Johnsen et al., 2005). To overcome this limitation, some bacteria have evolved a) biosurfactant excretion, b) direct contact with solid phase PAHs and c) a high affinity PAH uptake system in the absence of biosurfactants as observed in cells of Mycobacterium sp. strain LB501T (Wick et al., 2002; Chauhan et al., 2008). At high PAH concentrations, bacteria can rapidly break down toxic PAHs, although this can result in accumulation of PAHs in the cell wall and disrupt cell membrane and protein functions (Sikkema et al., 1995).
Figure 1.4: Aerobic bacterial metabolism of the benzene ring by meta or ortho cleavage
[Adapted from Juhasz and Naidu, 2000]
1.4.2.1. *Bacterial Degradation of Naphthalene*

Degradation of naphthalene, the least complex PAH, has been studied extensively since 1943 when salicylic acid was isolated from culture filtrates of *Pseudomonas aeruginosa* grown on naphthalene by Strawinski and Stone (Labana et al., 2007). The naphthalene degradation pathway has been comprehensively studied and naphthalene degrading microorganisms, particularly Gram-negative bacteria, have frequently been isolated from soil (Mrozik et al., 2003). Many naphthalene degrading bacteria belong to the *Pseudomonas* genus, however species of *Mycobacterium, Bacillus, Rhodococcus, Corynebacterium, Moraxella, Alcaligenes* and *Aeromonas* capable of degrading naphthalene have also been isolated (Cerniglia, 1984; Gibson and Subramanian, 1984; Labana et al., 2007; Mrozik et al., 2003). The first data describing the enzymatic reactions and biochemical pathway involved during the degradation of naphthalene was presented by Davies and Evans in 1964 (Davies and Evans, 1964).

The upper catabolic pathway for naphthalene degradation begins with naphthalene dioxygenase (NDO) introducing molecular oxygen to the benzene ring at the 1,2- positions to produce cis-naphthalene dihydrodiol (Cerniglia, 1984; Habe and Omori, 2003; Mrozik et al., 2003). In *Pseudomonas* sp., the addition of a molecule of oxygen to naphthalene is facilitated by a dioxygenase system consisting of 3 components – a ferredoxin reductase, a ferredoxin and a terminal oxygenase iron sulphur protein (ISP), 158 kDa, and consisting of an α and a β subunit weighing 55 and 20 kDa, respectively (Cerniglia, 1984; Habe and Omori, 2003). In a ferredoxin reductase, two electrons are transferred from NAD(P)H to FAD in a single transfer. The reduced FADH donates one electron each to the [2Fe-2S] cluster of a ferredoxin. The electrons are subsequently transferred to the ISP for use in the active site for incorporation of both atoms of the oxygen molecule to form cis-naphthalene dihydrodiol (Habe and Omori, 2003).

The next step in naphthalene degradation is dehydrogenation to 1,2-
dihydroxynaphthalene by cis-naphthalene dihydrodiol dehydrogenase (Figure 1.5). The product is then meta cleaved by 1,2-dihydroxynaphthalene dioxygenase and recyclizes to form 2-hydroxy-2H-chromene-2-carboxylic acid. The actions of 2-hydroxy-2H-chromene-2-carboxylate isomerase and trans-o-benzldene-pyruvate hydratase aldolase result in the formation of salicylaldehyde, which is dehydrogenated by salicylaldehyde dehydrogenase to salicylate (Figure 1.5) (Mrozik et al., 2003).

Salicylate can be metabolized via one of the two lower pathways of naphthalene degradation – catechol or gentisate to tricarboxylic acid (TCA) cycle intermediates. In the catechol pathway, salicylate is oxidized by salicylate hydroxylase to form catechol which is then cleaved via either the ortho- or the meta- pathway (Dagley and Gibson, 1965; Dagley, 1971). In the ortho- pathway, catechol is converted by catechol 1, 2-dioxygenase to cis, cis-muconate which is transformed by a series of enzymatic steps to yield β-ketoacidipate. In the meta-pathway, catechol is converted to acetaldehyde and pyruvate via a series of steps initiated by the action of catechol 2,3-dioxygenase (Gibson and Subramanian, 1984). In addition to bacteria from the Pseudomonas genus, Aeromonas sp. has also been reported to follow the same pathway for naphthalene metabolism (Kiyohara and Nagao, 1978). The second pathway for the oxidation of salicylate, which is used by bacteria including Pseudomonas testosteroni, involves oxidation through gentisic acid by salicylate 5-hydroxylase resulting in the formation of pyruvic and fumaric acids (Yen and Serdar, 1988).

Other bacteria have demonstrated different pathways of salicylate metabolism. These include a strain of Mycobacterium sp., which forms both cis- and trans-1,2-dihydrodiols in a reaction by cytochrome P-450 monooxygenase to form naphthalene 1,2-oxide. This product is converted by an epoxide hydrolase to a trans-dihydoxyl (Mrozik et al., 2003).
Figure 1.5: The proposed pathway for naphthalene oxidation by some bacteria from the genus Pseudomonas [adapted from Mrozik et al., 2003]
The thermophilic bacterium *Bacillus thermoleovorans* utilizes a naphthalene degradation pathway (Figure 1.6) which produces additional intermediates including 2,3-dihydroxynaphthalene, 2-carboxycinnamic acid, phthalic acid and benzoic acid (Annweiler *et al.*, 2000).

**Figure 1.6:** Metabolites of naphthalene formed by mesophilic and thermophilic microorganisms (hypothetical scheme of precursor product relationship) [from Annweiler *et al.*, 2000]
1.4.2.2.  Bacterial Degradation of Phenanthrene

Phenanthrene has often been used as a model for studying PAH biodegradation, as numerous bacteria (see Table 1.8) have been reported to have the capability for using it through co-metabolism or as a sole carbon and energy source (Tongpim and Pickard, 1999). In addition, it is a useful compound for studying the metabolism of carcinogenic PAHs due to its low MW, number of rings and presence of a “bay” region (UMBBD, 2010).

Generally, the upper route of phenanthrene degradation is initiated by a dioxygenase enzyme, followed by oxidation to form 3,4-dihydroxyphenanthrene. This product then undergoes meta cleavage and is converted to 1-hydroxy-2-naphtoic acid. From this point, 1-hydroxy-2-naphtoic acid can be degraded following one of two potential routes – the salicylate pathway or the protocatechuate pathway (Habe and Omori, 2003). Figure 1.7 illustrates the possible routes of bacterial phenanthrene degradation. The first path involves oxidative decarboxylation forming 1,2-dihydroxynaphthalene which then follows the naphthalene degradation pathway, undergoing meta cleavage to form salicylic acid. Further degradation leads to the production of gentisic acid or catechol which can undergo ring fission leading to the formation of TCA cycle intermediates (Evans et al., 1965; Gibson and Subramanian, 1984). Since this pathway is similar to the naphthalene degradation pathway, it could indicate that bacteria with the capability of degrading phenanthrene should also have the capability of degrading naphthalene, however this is not always true as seen in the case of Sphingomonas sp. Strain ZL5 which degrades phenanthrene but not naphthalene (Liu et al., 2004).

The second potential route, which is often used by bacteria capable of phenanthrene degradation but not naphthalene degradation, involves degradation via a protocatechuate (Ghosh and Mishra, 1983). Phenanthrene is degraded via aromatic ring cleavage of 1-hydroxy-2-naphtoic acid by a dioxygenase forming o-phthalic acid and subsequently protocatechuic acid.
which then undergoes cleavage forming pyruvic acid, eventually entering the TCA cycle
(Houghton and Shanley, 1994; Kiyohara et al., 1976).

Depending upon the bacterial species, the site of enzymatic attack upon the phenanthrene
core molecule and the pathway through which degradation occurs can vary. For example,
*Mycobacterium* species PYR-1 has been shown to metabolize phenanthrene by attacking the C-3
and C-4 positions as well as through an alternative pathway by an initial attack at the C-9 and C-
10 positions forming *cis* and *trans*-9,10 dihydrodiols (Figure 1.7). This alternate route may be
due to either a relaxed specificity of the same dioxygenase responsible for initial attack or due to
entirely different dioxygenases (Moody et al., 2001).

Pinyakong et al. (2000) have suggested that *Sphingomonas* sp. strain P2 can degrade
phenanthrene by dioxygenation at both the 1,2 – and 3,4- positions followed by *meta* cleavage.
The unstable ring cleavage products are then decarboxylated and oxidized (Pinyakong et al.,
2000). Strains of both *Pseudomonas* sp. and *Beijerinckia* sp. are also capable of initiating
oxidative attack at both the 1,2- and 3,4- positions of the phenanthrene molecule forming
phenanthrene *cis*-1,2-dihydrodiol and phenanthrene *cis*-3,4-dihydrodiol respectively (Tongpim
and Pickard, 1999). It has also been proposed that some *Vibrio* strains and a strain of *Aeromonas*
can convert phenanthrene to 1-hydroxy-2-naphthoic acid, but cannot decarboxylate this product
to 1,2-dihydroxynaphthalene (Kiyohara and Nagao, 1977). Alternatively, *Aeromonas* uses
intradiol cleavage to form *o*-phthalic acid, which is then hydroxylated and decarboxylated to
protocatechuic which is subsequently cleaved to form pyruvic acid, entering the TCA cycle
(Kiyohara and Nagao, 1977).

Samanta et al. (1999) have also suggested that some bacteria such as *Brevibacterium* sp.
HL4 and *Pseudomonas* sp. DLC-P11 utilize transformation pathways resulting in the production
of a novel intermediate – 1-napthol during utilization of phenanthrene. Another novel method of
phenanthrene degradation has been demonstrated in *Pseudomonas* sp. strain PP2. The phenanthrene molecule undergoes double hydroxylation to form 3,4-dihydroxyphenanthrene, followed by several oxidation steps eventually resulting in the formation of 2-hydroxymuconicsemialdehyde (Prabhu and Phale, 2003).
Figure 1.7: Pathways of phenanthrene degradation by bacteria [adapted from Lease, (2004) with information from Habe and Omori, (2003)]. Simple arrows indicate single reactions while double arrows indicate multiple transformation steps. The products in brackets have not been isolated.
1.4.2.3. **Bacterial Degradation of Anthracene**

Bacterial species including *Pseudomonas, Nocardia, Mycobacterium, Sphingomonas, Rhodococcus* and *Beijerinckia* can completely mineralize anthracene beginning with an initial oxidation reaction at the 1,2- position forming cis-1,2-dihydroxyanthracene (Dean-Ross *et al.*, 2001; Evans *et al.*, 1965; Moody *et al.*, 2001; Mrozik *et al.*, 2003). In the next step, this product is converted by an NAD+-dependent dihydrodiol dehydrogenase to form 1,2-dihydroxyanthracene (Mrozik *et al.*, 2003). Oxidation and *meta* ring cleavage lead to the formation of cis-4-(2-hydroxynapth-3-yl)-oxobut-enoic acid, which is converted to 2-hydroxy-3-napthoic acid (Habe and Omori, 2003). The resulting product is subsequently converted to catechol and salicylate via the production of 2,3-dihydroxynaphthalene, which is metabolized in a manner resembling the naphthalene degradation pathway (Cerniglia, 1984).

In contrast, species from the *Rhodococcus* genus and *Mycobacterium vanbaalenii* PYR-1 have exhibited the use of novel pathways for anthracene degradation. These species can use ortho ring cleavage of 1,2-dihydroxyanthracene resulting in the formation of 3-(2-carboxyvinyl)-2-carboxylic acid (Dean-Ross *et al.*, 2001; Moody *et al.*, 2001). Alternatively, ring cleavage can result in the formation of 4-[3-hydroxy(2-naphthyl)]-2-oxobut-3-enoic acid from which degradation can then proceed via 2,3-dihydroxynaphthalene to phthalate, or the product may spontaneously rearrange to form 6,7-benzocoumarin (Moody *et al.*, 2001).

The dehydrogenation of anthracene *cis*-1,2-dihydrodiol to form 1,2-dihydroxyanthracene by *Mycobacterium vanbaalenii* PYR-1 also results in the accumulation of 1-methoxy-2-hydroxyanthracene, a unique metabolite in the degradation of anthracene (Moody *et al.*, 2001). This strain also has the added capability of initializing hydroxylation of anthracene at the 9,10-position to produce anthracene-9,10-dihydrodiol, which is dehydroxylated to form 9,10-dihydroxyanthracene that is then spontaneously oxidized to form 9,10-anthroquinone (Moody *et al.*
The pathways for anthracene degradation by *Mycobacterium vanbaalenii* PYR-1 is illustrated in Figure 1.8.

**Figure 1.8:** The proposed pathway for the degradation of anthracene by *Mycobacterium* sp. strain PYR-1 [from Moody et al., 2001]. The products in brackets were not isolated.
1.4.2.4. **Bacterial Degradation of Pyrene**

Pyrene, a four-ringed PAH is degraded by a limited number of bacterial species as a sole carbon and energy source or through co-metabolism, notably by members of *Mycobacterium* and *Rhodococcus* (Vila et al., 2001; Mrozik et al., 2003). Table 1.8 contains a list of bacterial species capable of degrading pyrene and Figure 1.9 illustrates the pyrene degradation pathway of *Mycobacterium* sp. AP1.

In the predominant catabolic pathway, a dioxygenase will initiate attack at the 4,5-C position to form cis-4,5-pyrene dihydrodiol. This product is then transformed by a dihydrodiol dehydrogenase to produce 4,5-dihydroxy-pyrene. Intradiol cleavage leads to the formation of phenanthrene-4,5-dicarboxylic acid, from which a carboxy group can be removed by a decarboxylase to yield 4-phenanthroic acid (Kanaly and Harayama, 2000; Vila et al., 2001). The action of a ring hydroxylating dioxygenase on this metabolite results in the formation of 3,4-phenanthrene dihydrodiol-4-carboxylic acid which can be further transformed to yield 3,4-dihydroxyphenanthrene and follow the phenanthrene degradation pathway (Labana et al., 2007). Bacteria which have been proposed to follow this pyrene degradation pathway include *Mycobacterium vanbaalenii* PYR-1, *Mycobacterium flavescens*, *Mycobacterium* spp. AP1, KR2, RJGII-135, *Pseudomonas stutzeri* strain P16 and *Bacillus cereus* strain P21 (Dean-Ross and Cerniglia, 1996; Rehmann et al., 1998; Heitkamp et al., 1988; Schneider et al., 1996; Vila et al., 2001; Kazunga and Aitken, 2000).

Additional pathways for the degradation of pyrene have been proposed. One of these was found in *Mycobacterium vanbaalenii* PYR-1 cultures where pyrene hydroxylation occurs at the C-1,2 position to form the dead end product 4-hydroxy-perinaphthenone (Heitkamp et al., 1988). A second pathway used by *Mycobacterium* sp. strain AP1 involves the accumulation of 6,6'-dihydroxy-2,2'-biphenyl-dicarboxylic acid which is formed through cleavage of both central
pyrene rings (Vila et al., 2001). Some species of bacteria such as *Sphingomonas yanoikuyae* strain R1 can also accumulate the pyrene metabolite pyrene-4,5-dione which results from the autooxidation of 4,5-dihydroxypyrene (Kazunga and Aitken, 2000). *Mycobacterium* sp. AP1 and *Mycobacterium vanbaalenii* PYR-1 can also utilize a monooxygenase to attack the C-4,5 position of pyrene to yield pyrene-4,5-oxide. The action of pyrene-4,5-epoxide hydrolase will then result in the formation of trans-4,5-dihydroxy-4,5-dihydropyrene (Vila et al., 2001).

Figure 1.9: The proposed pathway for pyrene degradation by *Mycobacterium* sp. AP1. The product in brackets has not been isolated. Simple arrows indicate single reactions while double arrows indicate multiple transformation steps [adapted from Vila et al., 2001]
1.4.2.5. **Bacterial Degradation of Benzo(a)pyrene**

To date, no bacteria capable of utilizing benzo(a)pyrene as a sole source of carbon and energy have been isolated, and co-metabolism is an essential mechanism through which this highly recalcitrant compound can be degraded. Species which have been identified as having the ability to degrade benzo(a)pyrene (in the presence of other growth substrates such as LMW PAHs) include *Mycobacterium* sp. (Moody et al., 2004), *Beijerinckia* sp. (Gibson et al., 1975) and *Stenotrophomonas maltophilia* (Juhasz et al., 2000). A list of species is included in Table 1.8.

The degradation of benzo(a)pyrene by *Mycobacterium* sp. through dioxygenase can follow one of two pathways. The compound is initially oxidized to form either cis-4,5-BaP-dihydrodiol, cis-9,10-dihydrodiol or cis-7,8-dihydrodiol. Subsequently, the dihydrodiols can undergo either ortho or meta cleavage. *Cis*-4,5-BaP-dihydrodiol can undergo ortho cleavage to produce 4,5-chrysene-carboxylic acid, as opposed to *cis*-9,10-dihydrodiol and *cis*-7,8-dihydrodiol which undergo meta cleavage to form *cis*-4-(8-hydroxypyren-7-yl)-2-oxobut-3-enoic acid or *cis*-4-(7-hydroxypyren-8-yl)-2-oxobut-3-enoic acid. *Meta* cleavage will result in the formation of 7,8-dihydropyrene-7-carboxylic acid or 7,8-dihydro-pyrene-8-carboxylic acid (Schneider et al., 1996). The proposed pathways for benzo(a)pyrene degradation by *Mycobacterium* sp. RJJII-135 are illustrated in Figure 1.10.

Moody et al. (2004) found that *Mycobacterium vanbaalenii* PYR-1 degrades benzo(a)pyrene via mono- and dioxygenases at the C-4,5, C-9,10 and C-11,12 positions in the presence of yeast extract, starch and peptone (Moody et al., 2004). Monooxygenation and dioxygenation reactions play a role in the formation of *cis*- and *trans*-dihydrodiols during enzymatic attack at the C-11 and C-12 positions of benzo(a)pyrene by *M. vanbaalenii* PYR-1, with the action of cytochrome P450 and epoxide hydrolase being responsible for the formation of
benzo(a)pyrene \textit{trans}-11,12-dihydrodiol. Another interesting characteristic of benzo(a)pyrene degradation by \textit{M. vanbaalenii} PYR-1 is the activity of a constitutive catechol-O-methyltransferase which is involved in the formation of hydroxymethoxy and dimethoxy derivatives of benzo(a)pyrene (Moody \textit{et al.}, 2004).

The formation of metabolites during benzo(a)pyrene degradation may act as inhibitors of further benzo(a)pyrene degradation (Juhasz \textit{et al.}, 2002). In a study conducted with a high cell density inoculum of \textit{Stenotrophomonas maltophilia} strain VUN 10, 003, degradation of 10-15 mg L\textsuperscript{-1} benzo(a)pyrene was observed after a 21-28 day lag period, with a decline in degradation seen after 56 days, even in the presence of a viable bacterial cell population (Juhasz \textit{et al.}, 2002). Metabolite repression or inhibition of subsequent degradation steps due to the presence of a break-down product, was proposed as the cause of inhibited benzo(a)pyrene degradation; however the specific metabolites responsible have not been identified (Juhasz \textit{et al.}, 2002). The pyrene metabolites cis-4,5-dihydro-4,5-dihydroxypyrene and pyrene-4,5-dione were found to inhibit benzo(a)pyrene mineralization by \textit{Pseudomonas saccharophila} strain P15 and \textit{Sphingomonas yanoikuyae} strain R1 (Kazunga and Aitken 2000). This was suggested to occur through either competitive inhibition of a dihydrodiol dehydrogenase or through the formation of PYRQ, which may mediate futile redox reactions altering the balance of necessary redox cofactors (Kazunga and Aitken 2000).
Figure 1.10: Proposed metabolic pathways for benzo(a)pyrene degradation by *Mycobacterium* sp. RJGII-135. Simple arrows indicate single reactions while double arrows indicate multiple transformation steps [adapted from Schneider et al., 1996]
1.4.3. PAH Degradation by *Arthrobacter* sp.

*Arthrobacter* sp. are among the most prevalent bacteria isolated from soils, and exhibit a wide range of nutritional diversity in their utilization of both carbon and nitrogen sources. In addition to aromatic compounds, polychlorinated biphenyls (PCBs), pesticides and phenols have also been degraded by members of *Arthrobacter* (Daane et al., 2001; Furukawa and Chakrabarty, 1982; Kohler *et al*., 1988; Negrete-Raymond *et al*., 2003). *Arthrobacter* sp. are aerobic Gram-positive bacteria with a rod to coccus lifecycle. They are ubiquitous in soil, sediment and water and are also frequently isolated from hydrocarbon contaminated environments (Samanta *et al*., 1999; Seo *et al*., 2006). Currently, there is limited data about the mechanism of PAH degradation by *Arthrobacter* sp., however some of the proposed pathways will be discussed here.

A number of studies have reported the ability of *Arthrobacter* sp. to degrade phenanthrene (Grifoll *et al*., 1992; Casellas *et al*., 1997; Samanta *et al*., 1999; Seo *et al*., 2006; Kallimanis *et al*., 2007). Samanta *et al*. (1999) reported the ability of *Arthrobacter sulphurous RKJ4*, isolated from an Indian oil field soil sample to grow on phenanthrene, anthracene, fluorene, fluoranthene and pyrene (Samanta *et al*., 1999). GC-MS analysis of metabolites produced during phenanthrene degradation by RKJ4 suggested that degradation occurs via the production of the conventional lower pathway intermediates *o*-phthalic acid and protocatechuic acid. They were unable to identify upper pathway intermediates for this strain. It was also observed that RKJ4 degrades phenanthrene more efficiently when other PAHs were present in the medium. RKJ4 contains a mega plasmid (50-60 kb), however the role this plasmid in phenanthrene degradation has not yet been determined (Samanta *et al*., 1999).

Grifoll *et al*. (1992) and Casellas *et al*. (1997) have reported the ability of *Arthrobacter* sp. F101, isolated from sludge at an oil refinery wastewater treatment plant to utilize fluorene as a sole source of carbon and energy (Grifoll *et al*., 1992; Casellas *et al*., 1997). Initial oxidation
occurs at either C-1, C-2, or at positions C-3, C-4 to yield cis-dihydrodiols which undergo meta cleavage and a biological Baeyer-Villiger reaction leading to the formation of 3,4-dihydroxycoumarin (Grifoll et al., 1992; Casellas et al., 1997). This product undergoes enzymatic hydrolysis to yield 3-(2-hydroxyphenyl) propionic acid, which is degraded via catechol (Casellas et al., 1997). Alternatively, monooxygenation may occur at position C-9 to produce 9-fluorenol which is subsequently dehydrogenated to form 9-fluorenone (Grifoll et al., 1992; Casellas et al., 1997). Dioxygenase attack at the carbonyl group and subsequent cleavage produces a substituted biphenyl which is catabolised to form phthalate and is eventually metabolized via protocatechuate (Casellas et al., 1997).

Kallimanis et al. (2007) reported on the ability of Arthrobacter sp. strain Sphe3, isolated from creosote-contaminated Greek soil to utilize phenanthrene and anthracene individually as sole carbon and energy sources (Kallimanis et al., 2007). They also studied the ability of Sphe3 to internalize phenanthrene by either passive diffusion when cells are grown on glucose, or via an inducible active transport system when grown on phenanthrene alone (Kallimanis et al., 2007). The PAH transport system was able to recognize other PAHs including anthracene, fluoranthene and pyrene with less efficiency than phenanthrene, similar to the phenanthrene-uptake system of Mycobacterium sp. strain RJGII-135 (Miyata et al., 2004).

Seo et al. (2006) studied the metabolism of phenanthrene by Arthrobacter sp. P1-1, isolated from a PAH-contaminated site in Hilo HI, USA. P1-1 was able to degrade 40 mg/L of phenanthrene after 7 days. Initial phenanthrene dioxygenation occurs predominantly at the 3,4-C position. It can also occur at the 1,2 or 9,10-C positions. The phenanthrene 1,2, and 3,4-diols undergo meta cleavage, and to a limited extent, ortho cleavage. Subsequent reactions converge in the production of naphthalene-1,2-diol, which is degraded primarily through the phthalic acid pathway by ortho cleavage and to a lesser degree, through the salicylic acid pathway by meta
cleavage (Seo et al., 2006). Figures 1.11 and 1.12 illustrate the upper and lower metabolic pathways of phenanthrene degradation by *Arthrobacter* sp. P1-1.
Figure 1.11: Upper metabolic pathways of phenanthrene in Arthrobacter sp. P1-1. Metabolic pathways from phenanthrene to naphthalene-1,2-diol are presented. Metabolites in brackets were proposed, but not detected. Thick and thin arrows represent major and minor pathways respectively. Dashed arrows represent probable, but not confirmed pathways. [From Seo et al., 2006]
Figure 1.12: Lower metabolic pathway of phenanthrene degradation in *Arthrobacter* sp. PI-1. Ortho- and meta- cleavage pathways from naphthalene-1,2-diol are presented. Metabolites in brackets were proposed, but not detected. Double arrows indicate multiple reaction steps. [From Seo et al., 2006]
1.4.4. Fungal PAH Degradation

A wide variety of fungi, both ligninolytic and non-ligninolytic, have the capability of degrading PAHs, but in contrast to bacteria, fungi typically do not use PAHs as sole carbon and energy sources, but co-metabolically transform them to less toxic products (Cerniglia, 1997; Labana et al., 2007). Fungal PAH degradation relies upon two enzyme systems including cytochrome P-450 monooxygenases and lignin peroxidises (Cerniglia, 1997). Cytochrome P-450 monooxygenases are complex, membrane bound, multicomponent systems which can have broad substrate specificities (Labana et al., 2007). These enzymes catalyze the formation of arene oxides by incorporating one atom of the oxygen molecule into the PAH, while the second atom is reduced to water (Cerniglia, 1997). A phenol which can be conjugated with glucose, glutathione, glucuronic acid or sulphate can be formed via spontaneous isomerisation of the arene oxide (Sutherland 1992). Alternatively, the action of an epoxide hydrolase upon the arene oxide can result in the formation of a trans-dihydriodiol (Figure 1.13) (Sutherland 1992).

White rot fungi such as *Phanerochaete chrysosporium* produce extracellular lignin peroxidises as well as manganese peroxidises and laccases which play a role in PAH degradation (Cerniglia, 1992; Sutherland, 1992). Lignin peroxidises in particular have been shown to initiate free radical attack upon PAHs by transferring one electron to form an aryl cation radical which can be oxidized to form a quinone and subsequently undergo ring fission (Cerniglia et al., 1983). PAHs which can be oxidized by lignin peroxidises include anthracene, pyrene, perylene, benzo(a)pyrene and benz(a)anthracene (Haemmerli et al., 1986; Hammel et al., 1986; Sanglard et al., 1986). Non-ligninolytic fungi such as *Cunninghamella elegans* biotransform or co-metabolize PAHs of up to five benzene rings in a manner similar to that of mammalian systems via cytochrome P450 monooxygenases, however enzyme specificity differs between the systems (Cerniglia, 1997; Sutherland, 1992).
Co-cultures of bacteria and fungi have been found to be effective in both the mineralization and degradation of PAHs. Boonchan et al. (2000) reported that a co-culture of the bacterial consortium VUN10,009 and *Penicillium janthinellum* VUO10,201 were able to grow on benzo(a)pyrene as a sole carbon and energy source and also mineralize the substrate, whereas cultures of the individual microorganisms were unable to grow or mineralize the PAH (Boonchan *et al.*, 2000). This data suggested that co-metabolism was being used by the bacteria and fungi for PAH degradation. Similarly, data from other co-culture studies with bacteria and fungi have led to the suggestion that fungal enzymes catalyze an initial oxidation of the PAH which is followed by bacterial enzymatic degradation (Boonchan *et al.*, 2000; Kotterman *et al.*, 1998; Meulenberg *et al.*, 1997; Sack *et al.*, 1997).

![Pathways involved in the fungal metabolism of polycyclic aromatic hydrocarbons](image-url)  
*Figure 1.13: Pathways involved in the fungal metabolism of polycyclic aromatic hydrocarbons. [From Cerniglia, 1997]*
1.5. THE GENETICS OF POLYCYCLIC AROMATIC HYDROCARBON METABOLISM IN BACTERIA

1.5.1. Introduction

A large number of chemicals are released into the environment as a result of both natural and anthropogenic sources, some of which can be easily degraded and others which are recalcitrant. Microorganisms have been able to adapt or evolve over time to use these compounds as energy and carbon sources. This ability is a result of modifications to existing genes enabling novel metabolic capacities which allow new compounds to be utilized (Labana et al., 2007). This process depends upon the exchange or modification of genetic information and recombination mechanisms including transposition, gene conversion and duplication (Van der Meer et al., 1992). The versatility of bacteria in the degradation of xenobiotic compounds would not be possible were it not for the plasticity of microbial genomes (Labana et al., 2007). One example illustrating the evolution and diversity of bacterial genes are those encoding PAH-catabolic enzymes. The evolution and diversity of these genes among different PAH-degrading bacteria can be attributed to a number of possible genetic events including gene rearrangement, point mutations, horizontal transfer, gene fusion or transposition events (Habe and Omori, 2003). The following sections will describe PAH-metabolic genes which have been identified in Gram-negative and Gram-positive bacteria.

1.5.2. PAH-catabolic Genes of Gram-negative Bacteria

1.5.2.1. nah-like genes of Pseudomonas strains

Numerous studies of the metabolic pathways of bacteria capable of utilizing naphthalene have been conducted over the past few decades. Pseudomonas putida strain G7 is a naphthalene degrading microorganism that has been well studied for the genes, enzymes and pathways it employs for PAH degradation (Habe and Omori, 2003). An 83-kb transmissible plasmid NAH7,
isolated in 1973 by Dunn and Gunsalus, was shown to encode naphthalene catabolic genes which are organized into three operons (Figure 1.14) (Yen and Gunsalus, 1982, 1985). The first operon encodes the upper pathway enzymes necessary for the conversion of naphthalene to salicylate. The second encodes the lower pathway enzymes for the conversion of salicylate to TCA cycle intermediates via meta-ring cleavage and the third encodes a trans-acting positive regulator protein (NahR) which is located between the first two operons and is responsible for controlling the high level of nah gene expression in both the upper and lower pathways, and their induction by salicylate (Grund and Gunsalus, 1983; Yen and Gunsalus, 1982, 1985). The nah gene cluster for naphthalene catabolism is present on the 83-kb NAH7 plasmid as part of a defective 38-kb transposon named Tn4655, which is able to move from one genetic location to another by encoding a co-integrate resolution system to compensate for lacking the gene encoding the co-integration factor tnpA (Tsuda and Iino, 1990).

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**Figure 1.14**: The naphthalene and salicylate degradative pathway and the gene organization of the NAH7 plasmid. The nah operon (nahA-F) encodes enzymes for metabolizing naphthalene to salicylate and the sal operon (nahG-M) encodes enzymes for metabolizing salicylate to tricarboxylic acid intermediates. The regulatory gene nahR of the NAH7 plasmid encodes a protein which activates both nah and sal operons in the presence of salicylate [from Inouye 1998]
A number of genes encoding the naphthalene upper catabolic enzymes in several *Pseudomonas* strains have been designated as 'classical nah-like genes'. These include the *nah* genes from *P. putida* strain G7 and NCIB 9816-4 (Eaton, 1994; Simon *et al*., 1993), the *ndo* genes from *P. putida* strain NCIB 9816 (Kurkela *et al*., 1988), the *dox* genes from *Pseudomonas* sp. strain C18 (Denome *et al*., 1993), *pah* genes from *P. putida* strain OUS82 and *P. aeruginosa* strain PaK1 (Takizawa *et al*., 1994), *nah* genes from *P. putida* strain BS202 and from *P. stutzeri* strain AN10 (Bosch *et al*., 1999). Full nucleotide sequences have been reported for strains OUS82, PaK1 and AN10, but only partial sequences have been reported for the other strains. There is a 90% sequence homology among the upper catabolic pathway genes of these strains to the *nah* genes from the NAH7 plasmid of *P. putida* strain G7 (Labana *et al*., 2007).

For the lower naphthalene catabolic pathway, partial nucleotide sequences have been identified for strains G7 and NCIB 9816, while the complete gene sequence has been determined in strain AN10 (Habe and Omori, 2003). The genes present include those encoding for salicylate hydroxylase (*nahG*), chloroplast ferredoxin-like protein (*nahT*), catechol 2,3-dioxygenase (*nahH*), hydroxymuconic semialdehyde dehydrogenase (*nahI*), hydroxymuconic semialdehyde hydrolase (*nahN*), 2-oxopent-4-enoate hydratase (*nahL*), acetaldehyde dehydrogenase (*nahO*), 2-oxo-4-hydroxypentanoate aldolase (*nahM*), 4-oxalocrotonate decarboxylase (*nahK*) and 4-oxalocrotonate isomerase (*nahJ*) present in the order (*nahGTHINLOMKJ*) (Bosch *et al*., 2000).

In strain AN10, another salicylate hydroxylase gene (*nahW*) is located close to, but outside of, the lower-pathway operon (Bosch *et al*., 1999). Strain G7 contains two additional genes downstream of *nahJ*, encoding for a chemotaxis transducer protein (*nahY*) and for an unknown function (*nahX*) (Grimm and Harwood, 1999).

1.5.2.2. *phd* genes of *Comamonas testosteroni* strain GZ39

*Comamonas testosteroni* strains GZ38A, GZ39 and GZ42 are all capable of using
phenanthrene as a sole carbon source and were not found to contain any genes similar to the classical nah-like genes from P. putida strain NCIB 9816-4 (Goyal and Zylstra, 1996).

Phenanthrene degradation genes in strain GZ38A are similar to those in strain GZ39, and strain GZ42 did not have any phd genes similar to strain GZ39 suggesting that at least two new classes of genes responsible for phenanthrene degradation are present in these C. testosteroni strains (Goyal and Zylstra, 1996, 1997; Zylstra et al., 1997). In strain GZ39, the genes are organised as follows: genes coding for ferredoxin (phdAB), ferredoxin reductase (phdAa), cis-dihydrodiol dehydrogenase (phdB), the α-subunit of ISP (phdAc), the β-subunit of ISP (phdAd), isomerase (phdD), an unknown ORF, glutathione-S-transferase and hydratase aldolase (phdE) (Goyal and Zylstra, 1997). The extradiol dioxygenase gene was not present within the phd gene cluster, which is a notable difference between the phd genes and nah-like genes (containing nahC) (Goyal and Zylstra, 1997; Zylstra et al., 1997). Comparison of the phd genes with previously studied PAH-catabolic genes indicated that the PhdAc sequence is distantly related to the family of naphthalene dioxygenases, but that the PhdAd and PhdAb sequences have little similarity to the isofunctional proteins of aromatic ring dioxygenases (Zylstra et al., 1997).

1.5.2.3. nag genes of Ralstonia sp. strain U2

The naphthalene dioxygenase genes (nag genes) have been cloned and characterized from the naphthalene degrading bacterium Ralstonia sp. strain U2, which was isolated from oil-contaminated soil in Venezuela (Fuenmayor et al., 1998). The genes were present in the following order as illustrated in Figure 1.15: genes encoding for ferredoxin reductase (nagAa), two ORFs (nagG and nagH), ferredoxin (nagAb), the α-subunit of ISP (nagAc), the β-subunit of ISP (nagAd), cis-dihydrodiol dehydrogenase (nagB) and aldehyde dehydrogenase (nagF). The nagG product was identical to the α-subunit of other aromatic dioxygenases, but the nahH product had poor similarity to the β-subunit of other aromatic-ring dioxygenases (Fuenmayor et
al., 1998). NagG and NagH were found to be structural subunits of salicylate 5-hydroxylase, linked to electron transport systems consisting of nagAb and nagAa (Zhou et al., 2002). The genes responsible for the conversion of naphthalene to gentisate in strain U2 (nagAaGHAbAcAdBFCQED) were in the same order and similar to the classical nah-like genes in Pseudomonas strains with the exception of nagGH (Zhou et al., 2001). In strain U2, the regulatory gene (nagR) and the putative chemotaxis protein (nagY) are located upstream from nagAa which is different from the organisation of genes in the nah operon, where nahR and nahY are located downstream from the upper catabolic pathway (Grimm and Harwood, 1999).

1.5.2.4. **phn genes of Burkholderia sp. strain RP007**

Burkholderia strain RP007 was isolated from a PAH-contaminated location in New Zealand and is capable of utilizing naphthalene, phenanthrene and anthracene as sole sources of carbon and energy (Laurie and Lloyd-Jones, 1999a, 1999b). Cloning of the phn locus revealed that the phn genes (Figure 1.15) are organized in a different sequence from earlier characterized PAH-catabolic genes and they are present in the following order: regulatory protein (phnR), regulatory protein (phnS), aldehyde dehydrogenase (phnF), hydratase-aldolase (phnE), extradiol dioxygenase (phnC), isomerase (phnD), ISP α subunit of initial dioxygenase (phnAc), ISP β subunit of initial dioxygenase (phnAd), and dihydrodiol dehydrogenase (phnB) (Laurie and Lloyd-Jones, 1999a).

The phn gene locus differs from previously characterized PAH catabolic genes in a number of ways (Laurie and Lloyd-Jones, 1999b). Firstly, both the ferredoxin and reductase components are not present in the phn gene locus. Secondly, the gene encoding cis-dihydrodiol dehydrogenase (phnB) is more closely related to the corresponding gene in the biphenyl catabolic pathway than to the nahB-like genes. Lastly, the gene encoding the PAH extradiol
dioxygenase has a different phylogeny not seen among other extradiol dioxygenases from any of
the PAH or biphenyl catabolic pathways (Laurie and Lloyd-Jones, 1999b).

<table>
<thead>
<tr>
<th>Ralstonia sp. strain U2</th>
<th>nag genes</th>
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<tr>
<td></td>
<td>nagR</td>
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<td></td>
<td>nagAaGHaAcADBFCQED</td>
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<tr>
<th>Burkholderia sp. strain RP007</th>
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<tr>
<td>phn genes</td>
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<tr>
<td>phnR</td>
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<tr>
<td>phnS</td>
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<td>phnFEC DaAcAdB</td>
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Figure 1.15: Gene organization of the upper pathways for naphthalene degradation in Ralstonia sp. strain U2 and for phenanthrene degradation in Burkholderia sp. strain RP007 [adapted from Habe and Omori, 2003]

1.5.2.5. *phn genes of Alcaligenes faecalis strain AFK2*

*Alcaligenes faecalis* strain AFK2 is capable of utilising phenanthrene as a sole carbon
source through the o-phthalate pathway, but is unable to degrade naphthalene (Kiyohara et al.,
1982). Details regarding the structure and regulation of the *phn* genes in this strain are limited
and are only available in a database (Habe and Omori, 2003). The genes are arranged in the
following order: genes encoding ferredoxin (*phnAb*), ferredoxin reductase (*phnAa*), cis-
dihydriodiol dehydrogenase (*phnB*), the α-subunit of NDO (*phnAc*), the β-subunit of NDO
(*phnAd*), putative 2-hydroxychromene-2-carboxyrate isomerise (*phnD*), glutathione-S-transferase
(*gst*), trans-2-carboxybenzalpyruvate hydratase aldolase (*phnH*), 1-hydroxy-2-naphthoate
dioxygenase (*phnG*), 2-carboxybenzaldehyde dehydrogenase (*phnI*), 3,4-dihydroxyphenanthrene
dioxygenase (*phnC*), 1-hydroxy-2-naphthoaldehyde dehydrogenase (*phnF*) and putative trans-o-
hydroxybenzaldehyde hydratase-aldolase (*phnE*) (Habe and Omori, 2003).

1.5.2.6. *PAH-catabolic genes of Sphingomonas and its related species*

Members of the *Sphingomonas* genus and other related species are capable of utilizing a
large number of different aromatic compounds including PAHs as sole carbon and energy
sources, a feature that sets them apart from other Gram negative bacterial strains. For example, *Novosphingobium aromaticivorans* strain F199 is capable of growing on toluene and all isomers
of benzoate, xylene, *p*-cresol, biphenyl, naphthalene dibenzothiophene, fluorene and salicylate
(Fredrickson *et al*., 1991, 1995). From strain F199, 13 gene clusters were predicted to encode
aromatic degradative enzymes on a 184-kb catabolic plasmid labelled pNL1 (Romine *et al*.,
1999). One set of ferredoxin and reductase components on the plasmid were found to interact
with seven sets of oxygenase components (Romine *et al*., 1999). Several parts of the DNA
sequence in pNL1 encoding for aromatic catabolic genes were similar to those of other
*Sphingomonas* species including *S. paucimobilis* strain EPA505 (Story *et al*., 2000) and *S.
yanoikuyae* strain B1 (Kim and Zylstra, 1995, 1999; Zylstra and Kim, 1997). A further
functional analysis of aromatic degradation gene expression and activity in *Sphingomonas* is
needed for future comparison to that of other Gram-negative species (Habe and Omori, 2003).

1.5.3. PAH Catabolic Genes of Gram-positive Bacteria

1.5.3.1. *phd* genes of *Nocardioides* sp. *strain KP7*

*Nocardioides* sp. strain KP7 was isolated from marine samples and has the capability of
utilizing phenanthrene via the phthalate pathway (see Figure 1.7) (Iwabuchi *et al*., 1998). The
*phd* genes from strain KP7 are part of a new class of PAH-catabolic genes due to differences in
both sequence similarity and gene organization and they are the most studied PAH-catabolic
genes in Gram-positive bacteria (Habe and Omori, 2003). The nucleotide sequence of the
*phdIJK* gene cluster of this strain, which encodes three enzymes responsible for the conversion
of 1-hydroxy-2-naphthoate to phthalate has been characterized by biochemical and molecular
methods (Iwabuchi and Harayama, 1997, 1998; Adachi *et al*., 1999). The enzymes (and
corresponding genes) include: 1-hydroxy-2-naphthoate dioxygenase (*phdl*), trans-2’-
carboxybenzalpyruvate hydratase-aldolase (phdJ) and 2-carboxybenzaldehyde dehydrogenase (phdK) (Habe and Omori, 2003). Downstream from the phdIJK gene cluster lays the phdEFABGHCD gene cluster, which encodes enzymes responsible for the conversion of phenanthrene to 1-hydroxy-2-naphthoate (Saito et al., 1999, 2000).

The dioxygenase activity responsible for the conversion of phenanthrene to a cis-diol compound is due to the action of the enzymes encoded by phdABCD (Saito et al., 2000). The phdA and phdB genes, which encode the α- and β-subunits of the ISP of phenanthrene dioxygenase, have less than 60% sequence homology to the α- and β-subunits of other aromatic ring dioxygenases. PhdC, encoding ferredoxin in strain KP7, was not found to have any significant homology to the [2Fe-2S] type of ferredoxin which is most common ferredoxin component of PAH-dioxygenases. In contrast, it did show significant homology to the [3Fe-4S] or [4Fe-4S] type of ferredoxin. Moderate sequence similarity (less than 40%) was found between PhdD (ferredoxin reductase) and the ferredoxin reductase of other isofunctional enzymes (Saito et al., 2000).

The role of the specific enzymes encoded by phdE, phdF, phdG, and phdH in phenanthrene degradation has not been fully investigated. Similarity has been found between phdE, phdF, phdG, and phdH to dihydrodiol dehydrogenase, extradiol dioxygenase, hydratase-aldolase and aldehyde dehydrogenase, respectively (Habe and Omori, 2003). One major difference between the phd gene cluster in strain KP7 and the PAH catabolic gene clusters from Gram-negative bacteria is that the former does not contain an ORF encoding isomerase, which is present among Gram-negative PAH-catabolic gene clusters (Saito et al., 1999).

1.5.3.2. nar genes of Rhodococcus sp. strain NCIMB12038

Species from the genus Rhodococcus belong to a diverse group of Gram-positive soil bacteria capable of degrading a wide range of xenobiotic compounds. Naphthalene, for example,
can be used as a sole carbon and energy source (Boyd et al., 1997; Uz et al., 2000). A different ISP of naphthalene dioxygenase was characterized by Larkin et al. (1999) and the nucleotide sequences of the narAa and narAb genes encoding the α- and β-subunits of the ISP were reported (Larkin et al., 1999). The products of these genes bore a 31-39% similarity to the α- and β-subunits of a number of other aromatic-ring dioxygenases (Larkin et al., 1999). There are conserved key catalytic residues such as the Rieske [2Fe-2S] center between the nah genes of Pseudomonas putida strain 9816-4 and the nar genes of strain NCIMB12038 despite the fact that there is only some similarity (31%) between the α subunits of NahAc and NarAa (Larkin et al., 1999). The narB gene, encoding cis-naphthalene dihydrodiol dehydrogenase in strain NCIMB12038 also had a 39.5% amino acid similarity with nahB from P. putida strain G7; however the order of the naphthalene catabolic genes (nar genes) in strain NCIMB12038 was identical to the order of the nah genes in Pseudomonas species (Kulakov et al., 2000).

1.6. OVERCOMING CHALLENGES TO MICROBIAL PAH DEGRADATION

1.6.1. Impact of Bioavailability and PAH properties on Biodegradation

Oily sludge is composed of a complex mixture of aromatics, alkanes, asphaltene fractions and nitrogen-, sulphur-, and oxygen- containing compounds (Bartha, 1986). Contaminants including tar, petroleum and creosote products exhibit very slow release rates from soils and sediments limiting the success of in-situ bioremediation. PAH bioavailability in soil is influenced by desorption, diffusion and dissolution, and when soil has been contaminated for a significant length of time, diffusion of the contaminant into small pores of the soil can render the PAH less bioavailable (Singh and Ward, 2004). Soil characteristics such as low water solubility, low reactivity and low volatility contribute to the recalcitrance of PAHs in the environment (Mohan et al., 2006). Additionally, PAH properties such as molecular size, degree of
substitution and degree of hydrophobicity are also important determinants in how effectively the pollutant will be biodegraded (Singh and Ward, 2004).

Factors inhibiting the biodegradation of hydrophobic contaminants include environmental factors such the age and concentration of the contaminant, temperature, pH, moisture content, redox potential for anaerobic microorganisms and the presence of oxygen for aerobic degradation. Aged recalcitrant compounds can become tightly bound to particulate matter in soils and be less easily accessed by both water and microorganisms (Singh and Ward, 2004). This can have a significant impact on the success of bacterial PAH degradation because in order for it to occur, the bacteria must be able to internalize the PAH compound so that intracellular enzymes can facilitate breakdown of the contaminant (Chauhan et al., 2008). This is in contrast to fungal enzymes such as peroxidises, which are released and act extracellularly (Cerniglia, 1997).

The concentration of a PAH also has a crucial impact on the potential success of microbial degradation. If PAH concentrations are too low, genes necessary for enzyme production may not be induced, and the enzymes necessary for degradation may not be produced (Van Hamme, 2004). However if PAH concentrations are too high, toxic effects can be exerted upon the cell. The lipophilic nature of PAHs can disrupt cell membranes, resulting in impaired membrane-based energy generation processes, as well as the potential for cellular components to escape the cell (Sikkema et al., 1995).

Although the bioavailability and mass transfer of PAHs are important factors in biodegradation, other issues can play equally important roles. These include limitations in nutrients, oxygen or other electron acceptors, as well as an availability of growth substrates. In addition, low numbers of PAH degrading microorganisms and potentially slow rates of PAH degradation due to competition between substrates or slow enzyme activity can also limit the
success of PAH biodegradation (Aitken and Long, 2004). In slurry reactors, increased mixing and surfactant addition can help to increase bioavailability and have a positive effect on microbial degradative ability (Aitken and Long, 2004).

1.6.2. The Presence of Toxic Metals and Complex Chemical Mixtures

High concentrations of toxic metals can negatively impact microbial populations through the inhibition of metabolic activities including cell division and protein denaturation, as well as through disruption of the cell membranes (Shukla et al., 2010). Heavy metals including lead, cadmium and mercury are very difficult to remove from a contaminated environment, and may persist indefinitely. Microbial communities may be impacted in a number of ways including a reduction in the total amount of microbial biomass, a decrease in the numbers of specific microbial populations and an alteration in the community structure (Shukla et al., 2010). Although metals like mercury, copper and silver can be very toxic to cells, inorganic nutrients such as nitrogen and phosphorus are vital to cell activities, and trace amounts of sulphur, magnesium, manganese, iron, potassium and calcium are required for cell growth (Talley, 2006). Cells may accumulate metals by a number of mechanisms including intra- and extracellular complexation reactions, as well as through ion exchange and complexation reactions which occur at the cell wall (Talley, 2006). In addition, metals such as Fe and Ca have been shown to reduce the amount of available phosphate (Providenti et al., 1993).

The presence of heavy metals can adversely affect PAH biodegradation in a number of ways including reduced rates of biodegradation, complete failure of the compound to be biodegraded and extended acclimation periods for microorganisms. Interactions between PAHs and cell membranes can lead to increased permeability of the membrane to ions which is problematic in that metal toxicity to the cell is potentially increased (Gogolev and Wilke, 1997).

During biodegradation of complex hydrocarbon mixtures, bacterial cells may utilize
energy-dependent transport mechanisms (Van Hamme, 2004). This has been suggested from observations of preferred alkane degradation prior to PAH degradation (Leahy and Colwell, 1990), as well as the use of membrane transport inhibition experiments which showed that n-hexadecane accumulation is reduced by 88% from 0.218 µmole/mg protein to 0.026 µmole/mg protein in the presence of 30 mM sodium azide (Kim et al., 2002). Bacterial cells also possess efflux mechanisms belonging to the resistance-nodulation-division (RND) family for eliminating compounds such as toluene from the cytoplasm as seen in Pseudomonas putida DOT-T1E cells (Ramos et al., 2002). The RND family of efflux mechanisms, which includes the CzcA protein for cadmium, zinc and cobalt efflux, may play an important role in minimizing the toxic effects of high concentrations of certain metals, which serve essential cell functions at lower concentrations (Rosen 2002).

Complex petroleum hydrocarbon mixtures originating from different sources will have varying combinations and concentrations of specific PAHs as well as a multitude of compounds besides PAHs including alkanes, alkenes, monoaromatics and asphaltenes (Van Hamme et al., 2003). In a complex mixture such as crude oil or petroleum waste, microorganisms will typically begin by degrading and utilizing the most accessible carbon source first. This poses a challenge when the goal is PAH degradation in a complex mixture because the PAHs are much less likely to be quickly degraded and may persist. In addition, other factors such as weathering or aging of PAH contaminated sludge can further decrease contaminant bioavailability (Aitken and Long, 2004).

1.6.3. Factors Affecting PAH Degradation

1.6.3.1. Presence of Required Microbial Enzymes and Accessory Functions

Although microorganisms are capable of degrading many hydrocarbons including saturated and unsaturated alkanes, monoaromatics and a number of PAHs, some hydrocarbons
are much more resistant to degradation. A single bacterial species has only a limited ability to degrade some of the fractions present in oily sludge (Bartha, 1986; Mishra et al., 2001). Microorganisms must possess not only the catabolic enzymes needed for the breakdown of a pollutant, but also accessory functions relating to both degradation and protection from any toxic pollutant effects. These accessory functions can include chemotaxis, biosurfactant production, energy dependent uptake and changes to cell surface properties (Van Hamme, 2004). It is also important that bacteria involved in PAH degradation possess a complete degradation pathway with all the necessary enzymes so that there is no accumulation of toxic degradation products (Vanherwijnen et al., 2003).

1.6.3.2. Biosurfactant Production to Increase Microbial Access to PAHs

There are a number of ways through which microbial cells can access hydrophobic pollutants such as hydrocarbons. The first of these is through the ability to control cell surface properties. An increase in the hydrophobicity of the cell surface through modification of proteins, surface active compounds or polymers allows the cell to adhere to hydrophobic phases where potential substrates are present (Neu 1996). In addition, alteration of these compounds is also an important factor in biofilm formation, which is advantageous to microbial survival and protection from toxic effects (Van Hamme, 2004).

Another method of increasing hydrocarbon bioavailability, cell-substrate contact and mass transport is through the production of biosurfactants (Neu 1996). Biosurfactants are microbially produced amphipathic molecules with high surface and emulsifying activities. They partition preferentially at the interface between fluids of differing polarities and act to reduce the interfacial and surface tensions, thus making hydrophobic contaminants more accessible for microbial degradation (Van Dyke et al., 1991; Neu 1996). Biosurfactants can enhance the solubility of hydrophobic contaminants or they may allow for bacterial de-adhesion from a
hydrocarbon once the carbon source has been utilized (Rosenberg, 1993). Additionally, biosurfactants can mediate an alteration in substrate surface hydrophobic properties, thereby allowing for interaction between the substrate and a hydrophilic bacterium (Van Hamme, 2004). Providenti et al. (1995) studied rhamnolipid biosurfactant production by *Pseudomonas* sp. UG2 and its effect upon phenanthrene mineralization in soil slurries. The addition of these two biosurfactants increased the rate of degradation as well as reduced the lag period (Providenti et al., 1995). Although biosurfactant addition can be beneficial, it is possible that it can serve as competing carbon source by microorganisms, and be used preferentially over a PAH such as naphthalene, as seen in a study comparing the effects of biosurfactants and the chemical surfactant Triton-X-100 (Vipulanandan and Ren, 2000).

1.6.3.3. *Environmental Effects on Microbial PAH Degradation*

Environmental conditions play an important role in the efficiency of PAH biodegradation. Microbial activity can be greatly affected by temperature, pH, nutrient and oxygen availability, and bacteria will often only grow optimally over a narrow range of conditions (Vidali 2001). During PAH degradation in soil, factors such as soil type, moisture content and the level of heavy metal contamination can contribute to the effectiveness of biodegradation (Talley, 2006). Table 1.9 lists some environmental conditions which can affect microbial activity and shows the optimum values for enhanced oil biodegradation in soil.
Table 1.9: Environmental conditions and optimum values for microbial activity during oil degradation [adapted from Vidali, 2001]

<table>
<thead>
<tr>
<th>Environmental Factor</th>
<th>Condition required for microbial activity</th>
<th>Optimum conditions for oil degradation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Soil moisture</td>
<td>25-28% of water holding capacity</td>
<td>30-90%</td>
</tr>
<tr>
<td>Soil pH</td>
<td>5.5-8.8</td>
<td>6.5-8.0</td>
</tr>
<tr>
<td>Oxygen content</td>
<td>Aerobic, minimum air-filled pore space of 10%</td>
<td>10-40%</td>
</tr>
<tr>
<td>Nutrient content</td>
<td>N and p for microbial growth</td>
<td>C:N:P = 100:10:1</td>
</tr>
<tr>
<td>Temperature (°C)</td>
<td>15-45</td>
<td>20-30</td>
</tr>
<tr>
<td>Contaminants</td>
<td>Not too toxic</td>
<td>Hydrocarbon 5-10% of dry weight of soil</td>
</tr>
<tr>
<td>Heavy metals</td>
<td>Total content 2000 ppm</td>
<td>700 ppm</td>
</tr>
<tr>
<td>Type of soil</td>
<td>Low clay or silt content</td>
<td>N/A</td>
</tr>
</tbody>
</table>

The pH level of either the soil or slurry during PAH degradation is an important factor to be monitored to ensure optimal microbial performance. In general, most heterotrophic bacteria and fungi favour a neutral pH level, and extremes in pH can adversely affect PAH degradation (Leayh and Colwell, 1990). Hydrocarbon degradation was found to be optimal when the pH level was within the range of 6.5 and 8.0 (Morgan and Watkinson, 1989). Oxygen levels can be increased in soil by tillage, and soil structure and permeability can be improved by the addition of organic matter (Vidali 2001).

Temperature can play an important role in the biodegradation of organic pollutants. Bacterial degradation of hydrocarbons has been reported from temperatures as low as 0°C to as high as 70°C (Atlas, 1981). As the temperature decreases, generally the rate of biodegradation also decreases due to reduced enzymatic activity (Ashok and Saxena, 1995). In addition, the properties of hydrocarbons may be altered at higher temperatures resulting in increased rates of diffusion, solubility and volatilization (Margesin and Schinner, 2001; Coulon et al., 2005). Differing hydrocarbon fractions in a complex mixture may also be degraded at varying rates at different temperatures. For example, the aromatic fractions in crude oils were reported to be
degraded at 30°C but not at 4°C, whereas at 4°C, only branched alkanes were degraded (Morgan and Watkinson, 1989).

Nutrients such as nitrogen and phosphorus are vital to microbial cell activities, and their availability can limit the degree of hydrocarbon biodegradation in marine, soil or bioreactor environments. The microbial requirement for nitrogen and phosphorus can be met with the addition of ammonium phosphate, urea or a mixture of salts including ammonium sulfate, ammonium nitrate, ammonium chloride, potassium phosphate, sodium phosphate and calcium phosphate (Rosenberg et al., 1992). The addition of N and P has also been found to enhance rates of PAH biodegradation in soils including those of phenanthrene, anthracene and benzo(a)pyrene (Betancur-Galvis et al., 2006).

The availability of oxygen to microorganisms during PAH degradation in soils and slurry environments is essential for the activity of PAH degradative enzymes in aerobic bacteria. In soils, the availability of oxygen is dependent upon the type of soil, water logging of the soil, rates of microbial O\textsubscript{2} consumption and the presence of utilizable substrates (Leahy and Colwell, 1990). Although aerobic microorganisms are the major degraders of PAH compounds, in anaerobic environments the sulphate-reducing, nitrogen-reducing, fermentative and methanogenic microorganisms are important contributors to hydrocarbon degradation (Morgan and Watkinson, 1989).

1.6.3.4. **Co-metabolic PAH degradation**

Another important mechanism that may increase the success of biodegradation is co-metabolic degradation. During co-metabolism, growth substrates are metabolized by microorganisms as carbon and energy sources which support the degradation of non-growth PAHs, that may otherwise not be biodegraded if the co-substrate is not present (Zhong et al., 2007). Co-metabolic degradation of recalcitrant HMW PAHs with LMW PAHs has been
reported (Bouchez et al., 1995; Somtrakoon et al., 2007), although co-metabolism (and PAH degradation) can be limited if the source of a growth substrate is exceeded or eliminated (Van Hamme et al., 2003). Additionally, competition for readily available PAHs during co-metabolism can lead to a decrease in degradation of recalcitrant PAHs (Zhong et al., 2007).

A number of studies have demonstrated the enhanced effect of LMW PAHs on HMW PAH biodegradation. Keck et al. (1989) found that in the presence of 3 ring PAHs in a complex mixture, there was a rapid disappearance of 4- and 5- ring PAHs (Keck et al., 1989). This decrease in recalcitrant PAHs was attributed to an increase in overall microbial activity arising from increased forms of bioavailable carbon (Keck et al., 1989). Somtrakoon et al. (2007) studied the biodegradation of pyrene and fluoranthene by Burkholderia sp. VUN10013 in the presence of phenanthrene in soil. When supplied as sole carbon sources, pyrene and fluoranthene (75 mg/kg dry soil each) were not degraded. However, when phenanthrene was present (initial concentration of 250 mg/kg dry soil), 74.2% pyrene and 84.9% fluoranthene were degraded in 60 days (Somtrakoon et al., 2007). Schwab et al. (1995) also found that the addition of phenanthrene stimulated the degradation of pyrene in rhizosphere soils (Schwab et al., 1995). These studies illustrate that the addition of a simpler and more readily available source of carbon can have a beneficial effect on HMW PAH degradation.

1.6.4. Advantages of Ex-situ Biological Based Methods for Treating Oily Wastes

1.6.4.1. Slurry Bioreactors

Biological based treatment methods such as slurry phase bioreactors have a number of significant advantages over solid phase and in-situ biodegradation methods. The use of bioreactor systems can help optimize PAH biodegradation conditions and overcome the rate limiting factors associated with other treatment methods to ensure successful bioremediation of contaminants (Van Hamme et al., 2003). Slurry reactors utilize naturally occurring bacteria or
inoculated strains with the metabolic pathways necessary for effective degradation of pollutants present in a liquid phase (Vidali 2001). This occurs by enabling maximum contact between the hydrocarbons in the bioreactor with microorganisms, nutrients and oxygen through proper and uniform mixing (Machín-Ramírez et al., 2008). A mixture of soil and water in a slurry bioreactor allows for an increase in soil moisture content, an important factor in microbial PAH degradation (see Table 1.9) resulting in an increase in both the solubility and bioavailability of contaminants (Weber and Kim, 2005).

The use of enclosed bioreactors can reduce the amount of soil PAH contamination and minimize PAH volatilization, while allowing for the management of off gases (Van Hamme et al., 2003; Ward et al., 2003). By reducing the escape of volatile carbon components which can be supportive of microbial growth and increase solubilization of recalcitrant components, biodegradation can become the dominant process in the bioreactor (Van Hamme et al., 2003). Bioreactors can also be cost effective by utilizing mixed cultures with enhanced metabolic diversity instead of a pure culture system which can be more expensive to maintain (Ward et al., 2003). Table 1.10 lists the benefits and limitations of existing bioremediation treatment technologies for PAH contaminated waste.

Comparison studies of solid phase (landfarming) and slurry phase treatments showed that PAH degradation of two-, three-, four-, five- and six-ring PAHs occurred more slowly during solid phase treatment, but that an extended length of treatment can be beneficial (Mueller et al., 1991a, 1991b). Although degradation occurred more slowly during solid phase treatment, the degradation of HMW PAHs including pyrene, chrysene, benzo(a)pyrene and benzo(b,k)fluoranthene continued to occur throughout the experiment, whereas the degradation of the PAHs in a slurry phase treatment reached a peak after which the level of HMW PAHs remained constant (Mueller et al., 1991a, 1991b). The total petroleum hydrocarbon degradation
rates per month of treatment observed in landfarms of 0.5 to 1% per month can be increased to 0.1 to 0.3% per day in aerated bioreactors (Van Hamme et al., 2003). This illustrates that the implementation of a bioreactor-based process for the treatment of oily wastes with the correct microbial consortia can greatly improve the effectiveness and speed of PAH degradation over solid phase treatment.

One example of a bioreactor based system for the treatment of petroleum waste is the Petrozyme process (Ward et al., 2003). This process utilizes eight bioreactors with a microbial culture acclimated to crude oil as well as an optimized nutrient formulation, a biosurfactant and optimal temperature (28°C to 32°C) and pH ranges (6.4 – 7.6) (Van Hamme et al., 2003). The microorganisms involved are well known oil degrading bacteria including species of *Pseudomonas, Acinetobacter, Rhodococcus* and *Alcaligenes* (Singh et al., 2001). This biodegradation process has been used in a number of refineries in Canada, the United States and Mexico to treat sludges containing total petroleum hydrocarbon contents of 10% wt/vol (Van Hamme et al., 2003). Typically, 50-80% oily sludge is mixed with 20-50% nutrient medium with inoculum on a volume basis (Singh et al., 2001). Average degradation rates of up to 99% total petroleum hydrocarbon contents have been observed with treatment times ranging from 6-12 days (Singh et al., 2001; Van Hamme et al., 2003). The result of the petrozyme process is an aqueous effluent that complies with EPA criteria and can be applied to a landfarm or sent to a wastewater system (Singh et al., 2001).

There are a number of other examples of reactor-based processes for treating refinery sludge that were implemented in the 1980s and 1990s (Van Hamme et al., 2003; Ward et al., 2003). The treatment times of these reactor systems were 1-4 months. The first example is a 1 million gallon bioreactor operated by a Gulf Coast refinery to treat petroleum sludge (Coover et al., 1993). A hydrocarbon degrading mixed microbial culture isolated from an activated sludge
system was used at an optimal temperature of 22.6˚C. The reactor was outfitted with float mounted aerators and mixers. Reductions in total petroleum hydrocarbon levels of up to 90% were observed and a 50% reduction in oil was seen after 90 days (Coover et al., 1993).

A second example is of a combined slurry reactor and land treatment process to bioremediate sludge from an oil refinery in Sugar Creek, Missouri (Amoco Oil Company 1989). The slurry reactor (5 million gallon capacity) with a hydrocarbon degrading inoculum was used to reduce concentrations of oil by 66% in under 90 days. Following bioreactor treatment, solids were applied to landfarms and PAH concentrations were reduced below 160 mg/kg (Amoco Oil Company, 1989; Van Hamme et al., 2003). A third example is a slurry phase aerated system to remediate refinery wastes at the French Limited Superfund site in Crosby, Texas (ENSR Consulting and Engineering 1991). This process used pure oxygen as part of the “Mixflo” mixing/aeration system. In 11 months, three hundred thousand tonnes of tar-like material was remediated, and in 122 days, 85% of sludge contaminants present in 300,000 tonnes of waste were eliminated (ENSR Consulting and Engineering 1998).

As described, the advantages of slurry phase bioreactors for the bioremediation of petroleum and petrochemical wastes are numerous and include improved PAH degradation times, cost efficiency and reduced environmental contamination (Van Hamme et al., 2003; Owen Ward et al., 2003; Weber and Kim, 2005; Machín-Ramírez et al., 2008). In providing operators with control over factors to optimize microbial PAH degradation, many of the drawbacks associated with landfarming of PAHs can be overcome (Van Hamme et al., 2003). One of the significant advantages of slurry bioreactor operation is a reduction in discharge of contaminated effluents to land farm sites, as well as a reduction in initial PAH concentrations present in refinery wastes (Ward et al., 2003). As a pre-treatment option combined with landfarm treatment, slurry bioreactors are becoming an important environmentally conscious option for
petroleum refineries and petrochemical companies as a means of complying with Ontario Reg.
461/05, which took effect in December 2009.

1.7. RESEARCH OBJECTIVES

The overall goal of this study was to isolate PAH-degrading bacteria from the soil of a
hydrocarbon contaminated landfarm site owned by NOVA Chemicals. My hypothesis is that
microorganisms capable of degrading low and HMW PAHs can be isolated and enriched from
landfarm soil samples and be used to pre-treat oily wastes from petroleum refineries by
decreasing PAH concentrations. The bacteria would be screened for the ability to degrade
individual and defined mixtures of the PAHs phenanthrene, anthracene, pyrene, chrysene and
benzo(a)pyrene in pure culture. The most efficient PAH degrading bacterium isolated during
this work, *Arthrobacter* sp. UG50 would then be tested for the ability to degrade the PAHs
present in petroleum refinery sludges from NOVA Chemicals at the bench scale level.

My Specific objectives were:

1. To enrich for PAH-degrading microorganisms present in NOVA Chemicals landfarm soil
   using shake flask experiments with API effluent.

2. To identify PAH-degrading microorganisms using the phenanthrene spray plating
   technique developed by Kiyohara *et al.*, (1982), and to identify the microorganisms using
   16S rRNA gene analysis.

3. To test the ability of selected PAH-degrading bacteria to degrade individual and defined
   mixtures of PAHs in liquid culture using GC-FID.

4. To determine the ability of *Arthrobacter* sp. UG50 to survive in, and degrade the PAHs
   present in NOVA Chemicals DAF sludge.
Table 1.10: Summary of the benefits and limitations of various PAH bioremediation strategies [From Shukla et al., 2010]

<table>
<thead>
<tr>
<th>Technology</th>
<th>Examples</th>
<th>Benefits</th>
<th>Limitations</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bioreactors</td>
<td>Slurry reactors</td>
<td>Rapid Degradation, Optimized environmental parameters, Enhanced mass transfer, Effective use of inoculants and surfactants</td>
<td>Soil requires excavation, Relatively high cost capital, Relatively high operating cost</td>
</tr>
<tr>
<td></td>
<td>Aqueous reactors</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>In-situ</td>
<td>Biosparging</td>
<td>Most cost efficient, Non-invasive</td>
<td>Environmental constraints, Extended treatment time, Monitoring difficulties</td>
</tr>
<tr>
<td></td>
<td>Bioventing</td>
<td>Relatively passive</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Bioaugmentation</td>
<td>Natural attenuation processes, Treats soil and water</td>
<td></td>
</tr>
<tr>
<td>Ex-situ</td>
<td>Landfarming (solid phase treatment system)</td>
<td>Cost efficient, Simple procedure, Inexpensive, Self heating, Low cost, Rapid reaction rate, Inexpensive, Self heating</td>
<td>Space requirements, Slow degradation rates, Long incubation periods, Extended treatment time, Requires nitrogen supplementation, Incubation period – months to years, Need to control abiotic loss, Mass transfer problem, Bioavailability limitation</td>
</tr>
<tr>
<td></td>
<td>Composting (anaerobic, converts solid organic wastes into humus-like substances)</td>
<td>Can be done on site</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Biopiles</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
CHAPTER 2: MATERIALS AND METHODS

2.1. MATERIALS

2.1.1. Suppliers

Chemicals, microbiological media, and solvents were obtained from Sigma Aldrich (Oakville, ON), Becton Dickinson and Company (Sparks, MD, USA) or Fisher Scientific (Ottawa, ON). All chemicals and reagents were of analytical grade. All solvents were of HPLC grade. Acetone, methanol, N’N’-dimethylformamide (DMF) and dichloromethane (DCM) were obtained from Sigma Aldrich (St. Louis, MO, USA). The PAHs fluorene, phenanthrene, anthracene and pyrene were of high purity (> 97%) and were obtained from Sigma Aldrich (St. Louis, MO, USA). Chrysene and benzo(a)pyrene were obtained from Supelco (Belafonte, PA, USA). Docosane was obtained from Sigma Aldrich (St. Louis, MO, USA).

Commercial bacterial DNA isolation kits were obtained from Qiagen Inc. (Mississauga, ON) and MP Biomedicals (Solon, OH, USA). PCR purification kits were obtained from Roche Diagnostics (Laval, QC), Qiagen Inc. (Mississauga, ON) and Promega (Madison, WI, USA). PCR master mixes were obtained from Fermentas Life Sciences (Burlington, ON).

Oligonucleotide primers were obtained from Invitrogen (Frederick, MD, USA).

The following International Union of Pure and Applied Chemistry (IUPAC) codes for nucleotide degeneracies are used:

- N – A, C, G, T
- S – G, C
- R – A, G
- M – A, C
- Y – C, T
- D – A, G, T
- W – A, T
- K – G, T
- V – A, C, G
2.1.2. Stock Solutions

**Ampicillin:** 10 mg/mL stock prepared in deionized water.

**Cyclohexamide:** 5 mg/mL solution prepared in deionized water.

**Nalidixic Acid:** 10 mg/mL solution prepared in deionized water.

**Rifampicin:** 10 mg/mL solution was prepared in methanol.

All antibiotics were filter-sterilized and stored at -20 °C until used.

**80% (v/v) Glycerol:** 20 mL of deionized water was added to 80 mL of 100% glycerol (Sigma Aldrich, St. Louis, MO) and autoclaved at 121 °C for 20 minutes.

**25% (w/v) Yeast Nitrogen Base (YNB) (without amino acids):** 25 g of YNB without amino acids (Sigma Aldrich, St. Louis, MO) was added to 100 mL of deionized water and brought to a boil. The solution was filter-sterilized (0.2 µm) prior to use.

**Tris-acetate-EDTA Electrophoresis Buffer TAE (50 X):** 242 g of Tris base was dissolved in 800 mL of distilled water. 57.1 mL glacial acetic acid and 100 mL 0.5 M EDTA (pH 8.0) were added and the volume topped up to 1 L with deionized water. A 1 X working solution was prepared by diluting the buffer 50 fold.

**1% w/v Sodium Pyrophosphate:** 10 g of sodium pyrophosphate (Fisher Scientific, Fair Lawn, NJ) was dissolved in 1 L of deionized water. The pH was adjusted to 7.3 with 0.1 N HCl.

**Polycyclic Aromatic Hydrocarbon Stock Solutions:** PAH stock solutions were prepared in N’N’-dimethylformamide for liquid culture experiments or in acetone for agar based experiments. Concentrations prepared differed depending on the experiment and the PAH – phenanthrene, anthracene, pyrene, chrysene and benzo(a)pyrene. PAH stock solutions were prepared in glass serum bottles with polytetrafluoroethylene (PTFE)-lined caps and were stored at room temperature (22 °C) in the dark. Sets of PAH stock solutions were prepared for the following experiments:
1. **Spray Plating Experiments:** 10 % (w/v) phenanthrene in acetone

2. **Liquid Culture Degradation Experiments:** 10 mg/mL stock solutions prepared in DMF for phenanthrene, anthracene, pyrene and benzo(a)pyrene; 5 mg/mL for chrysene.

*Internal Standard Solutions for GC-FID and GC-MS analyses:*

- **Anthracene:** 1 mg/mL prepared in DCM.
- **Docosane:** 0.1 g docosane prepared in 15 g DCM.
- **Fluorene:** 2 mg/mL prepared in DCM.

**Phosphate Buffered Saline (10 X) (PBS):**

\[
\begin{align*}
\text{NaCl} & : 80 \text{ g} \\
\text{KCl} & : 2 \text{ g} \\
\text{Na}_2\text{HPO}_4 & : 14.4 \text{ g} \\
\text{KH}_2\text{PO}_4 & : 2.4 \text{ g}
\end{align*}
\]

The pH was adjusted to 7.4 with 0.1 N HCl. The 10 X PBS solution was diluted 10 times prior to autoclaving.

2.1.3. **Media Composition**

All media recipes are per L of deionized water. Media were sterilized by autoclaving at 121 °C for 20 minutes. Where solid media were used, 15 g of agar was added prior to autoclaving.

**UG14 Mineral Salts Media (MSM) (Providenti et al., 1995):**

\[
\begin{align*}
\text{Na}_2\text{HPO}_4/\text{KH}_2\text{PO}_4 \text{ buffer, pH 7.2} & : 10 \text{ mM} \\
(\text{NH}_4)_2\text{SO}_4 & : 2.38 \text{ g} \\
\text{MgSO}_4\text{•7H}_2\text{O} & : 0.25 \text{ g} \\
\text{NaCl} & : 0.5 \text{ g} \\
\text{FeSO}_4\text{•7H}_2\text{O} & : 0.010 \text{ mM} \\
\text{CaCl}_2 & : 20 \text{ mM}
\end{align*}
\]

**Bacto Tryptic Soy Broth (TSB) Media:**

The medium was prepared by adding 30 g of Tryptic Soy Broth (Difco, Sparks, MD) to 1 L of deionized water. Where 1/10th the strength of TSB was required, 3 g of TSB was added to 1 L of deionized water.
**Bushnell Haas (BH) Media:**

The media was prepared by adding 3.27 g of Bushnell Haas media (Difco, Sparks, MD) to 1 L of deionized water. Where required, Bushnell Haas media was supplemented with the following:

- 1 % (v/v) glycerol
- 0.67 % (w/v) YNB without amino acids
- 1 g/L yeast extract (Difco, Detroit, MI).

**Luria Bertani (LB) Medium:**

The medium was prepared by adding 20 g of Luria Bertani broth (Difco, Sparks, MD) to 1 L of deionized water.

2.2. GENERAL METHODS

Weight measurements were made using a Sartorius 1216 MP scale (Mississauga, ON), and smaller quantities (< 1.0 g) were measured using a Mettler-Toledo AE10 analytical balance (Columbus, OH, USA). pH readings were obtained using a Fisher Scientific Accumet Research AR10 pH meter. Optical density readings of DNA and cell cultures were measured using an Amersham Biosciences Ultraspec 3100 Pro spectrophotometer (Biochrom Ltd., Cambridge, England). Cultures and extracts were centrifuged using either an Eppendorf Mini Spin Plus (Mississauga, ON) or a Hermle Labortechnik GmbH Z200A centrifuge (Werhigen, Germany). Liquid inocula were grown using a model G2 gyratory shaker (New Brunswick Sci. Co., Edison, NJ, USA) or a Steady Shake Model 757L Shaker/Incubater (Amerex Instruments, Lafayette, CA, USA). Incubation of plates occurred in temperature controlled cabinets. Filter-sterilized solutions were passed through 0.2 µm sterile nylon disposable filters (Fisher Scientific, Ottawa, ON). Glassware was washed with detergent and rinsed with deionized water. Glassware exposed to PAHs was rinsed with acetone prior to washing with detergent.
2.3. MAINTAINANCE OF BACTERIAL STRAINS

2.3.1. Microbial Landfarm Isolates

Microbial isolates were enriched and isolated from NOVA Chemicals landfarm soil by the methods described in Section 2.4. Microorganisms were stored at 4 °C on 1/10th strength Tryptic Soy Agar (TSA) plates unless otherwise stated. For long term storage, stocks of microorganisms were prepared in 30% (v/v) glycerol and stored in a -80 °C freezer.

2.3.2. Pseudomonas sp. UG14Lr

*Pseudomonas* sp. UG14Lr, a phenanthrene-degrading bacterium, was used as a positive control during isolation and PAH degradation experiments. The bacterium was initially isolated from a contaminated soil sample taken near a former wood treatment site (Providenti, 1994). Strain UG14Lr arose from a spontaneous rifampicin-resistant mutant of strain UG14 which was marked with the *luxAB* genes from *Vibrio harveyii* (Weir et al., 1995). The bacterial strain *Pseudomonas* sp. UG14Lr used in this work was revived from a lyophilized culture in the collection at the University of Guelph (Guelph, ON). During PAH degradation experiments, strain UG14Lr was grown under the same conditions as those used for bacterial landfarm isolates with the exception of the growth temperature, which was 30 °C for UG14Lr.

2.4. MICROBIAL ENRICHMENT FROM NOVA CHEMICALS LANDFARM SOIL

2.4.1. NOVA Chemicals Landfarm Soil Preparation

Contaminated soil for the isolation of PAH-degrading microorganisms was kindly provided by the NOVA Chemicals Refinery site in Corunna, ON. The soil was obtained in May 2007 from fields 5, 6, 7, and 8 which were being used for bioremediation of PAH containing refinery effluent. The soil, which included root material, was taken from both the surface of the fields (top soil) and about 15 cm below the surface (sub soil). The samples were collected in
plastic bags and were separated according to field and depth (top or subsoil). Soil samples were stored at 4°C until used.

For the enrichment of PAH degrading microorganisms, three groupings of landfarm soil material would be used. 10 g of soil from Field 6, a field which NOVA Chemicals had used for phytoremediation of refinery effluent was selected as the first source of PAH-degrading microorganisms. A second source consisted of a 10-g collection of the root material that was recovered among all the soil samples received. The third source consisted of a 10-g composite and representative sample of soil from all four fields.

In preparation for shake flask enrichment, the soil was passed through a sieve (< 4.00 mm) to separate the root material from the soil. The composite soil sample was prepared by combining 5 g each of soils from Fields 5, 6, 7 and 8. The soil was mixed and 10 g of the composite mixture was removed for enrichment purposes. Each 10 g soil-root sample was added to 100 mL of 1% w/v sodium pyrophosphate (pH 7.3) in a 250-mL sterile Erlenmeyer flask. The flasks were shaken at 150 rpm at 22 °C overnight. Following shaking, the samples were left to settle for a minimum of one hour.

2.4.2. Shake Flask Enrichment for PAH-Degrading Microorganisms

2.4.2.1. Enrichment using NOVA Chemicals American Petroleum Institute Oil-water Separator Effluent

20 mL of each supernatant (Field 6, Composite and Root) was added to 500-mL capacity Erlenmeyer flasks containing 10 mL of 10 X concentrated MSM and 70 mL of API oil-water separator effluent obtained from the NOVA Chemicals Refinery Site (Corunna, ON). The effluent was brought to 22 °C prior to use. The three enrichment cultures were incubated at 22°C and shaken at 250 rpm in the dark for up to 60 days.
2.4.2.2. Monitoring of PAH Degradation during Enrichment

Total PAH degradation in the three enrichment flasks was monitored following the methods of (Somtrakoon et al. 2007) with the following modifications. At weekly extraction intervals, 10 mL of each enrichment culture was removed to a 50-mL capacity glass serum bottle. 2 mL of dichloromethane and 10 µL of a 1 mg/mL anthracene standard were added. The bottles were sealed with PTFE-lined septa and aluminum crimp caps and were shaken for 90 min at 175 rpm (22 °C) in the dark. The extractions were then left to settle for 1 h after which the heavier ~ 2 mL DCM fractions were transferred to GC auto sampler vials. The vials were stored at -20 °C until GC-FID analysis.

The following conditions were used for GC–FID Analysis:

- **Instrument:** Hewlett Packard 5890 Series II Gas Chromatograph
- **Column:** DB - 1 Capillary Column (30 m x 25 mm, I.D. 0.25 µm)
- **Carrier Gas:** Helium
- **Injector Temperature:** 320 °C
- **Detector Temperature:** 320 °C
- **Carrier Flow Rate:** 0.6 ml/min
- **Column Head Pressure:** 12 psi
- **Split Flow Ratio:** Splitless

For analysis of PAH extracts, the oven temperature was programmed to 150 °C for 1 min, followed by a linear increase of 15 °C/min and held at 300 °C. Due to the complex chemical mixture of analytes present within the refinery effluent and the inability to identify individual peaks of interest on chromatograms, the total peak area for each analyzed sample was compared over time as a measure of degradation.
2.4.3. Sub-culturing and Isolation of Microorganisms

Every 3 weeks for 60 days, the samples were further enriched using 5 mL of the original enrichment added to 5 mL of 10X concentrated MSM containing 40 mL of API oil-water separator effluent. Following enrichment, 1 mL from each of the 3 microbial enrichments was diluted in 1% sodium pyrophosphate buffer and plated on TSA and 1/10\textsuperscript{th} TSA plates. The plates were incubated at either 22 °C, 28 °C or 37 °C and observed for growth after 24 h. From the plates, 88 colonies were selected and maintained on 1/10 TSA plates prior to screening for phenanthrene degrading ability. The isolates were designated A (1-8) through K (1-8).

2.5. IDENTIFICATION OF PHENANTHRENE-DEGRADING MICROORGANISMS

2.5.1. Phenanthrene Spray Plating

Agar plates containing Bushnell Haas media supplemented with 1 g/L of yeast extract were prepared and divided into quadrants. Using sterilized toothpicks, microbial isolates from 1/10\textsuperscript{th} TSA plates were point inoculated (4 per plate) in the center of each quadrant. \textit{Pseudomonas} sp. UG14Lr, a known phenanthrene-degrading bacterium was included as a positive control, and \textit{Escherichia coli} strain TG1 was included as a negative control. The plates were incubated at 22 °C until growth was visible, about 2-3 days. \textit{E. coli} strain TG1 was grown at 37 °C. Each plate was prepared in triplicate. Phenanthrene spray plating was carried out following the method of Kiyohara \textit{et al.}, (1982). One set of plates was sprayed with a 10% (w/v) solution of phenanthrene in acetone; another set was sprayed with the solvent acetone alone, and the last set received no PAH or solvent and served as a check for growth.

The first set of plates was sprayed using a glass sprayer (Lurex, Vineland, NJ, USA) affixed to a 125-mL flask in order to form a thin white layer of phenanthrene over the agar. The second set was sprayed with acetone alone and all the plates were left in the fume hood for 5 min to allow for solvent evaporation. These plates and the set of plates which were not sprayed were
sealed with parafilm, kept in the dark and incubated at 22 °C in a fume hood. The plates were checked daily for the presence of clearing zones around areas of bacterial growth.

2.5.2. 16S rRNA Gene Analysis

2.5.2.1. DNA Extraction

Selected PAH-degrading bacterial isolates were grown for 1-2 days in 5 mL TSB at 22 °C and shaken at 175 rpm. 1 mL of bacterial culture was used for extraction of total DNA using the DNeasy Blood and Tissue Kit (Qiagen Inc., Mississauga, ON). Gram-positive bacterial isolates were pre-treated with lysozyme following the recommended method in the extraction kit protocol.

2.5.2.2. Polymerase Chain Reaction

PCR was performed in either a Gene Amp 2400 PCR System (Perkin Elmer, Waltham, MA) or a Mastercycler Gradient thermocycler (Eppendorf, Mississauga, ON).

The 16S ribosomal RNA gene (1450 bp fragment) was amplified using the following primer set (Lane, 1991):

- 27F  (5’-AGA GTT TGA TCM TGG CTC AG-3’)
- 1492R (5’-TAC GGY TAC CTT GTT ACG ACT T=3’)

The amplification mix consisted of 22 µL of nuclease free water (Applied Biosystems / Ambion, Austin, TX USA); 1 µL of each primer (from 10 µM stock solutions); 1 µL of template DNA and 25 µL of 2X PCR Master Mix (Fermentas Life Sciences, Burlington, ON) containing: 0.05 U/µL of Taq polymerase in reaction buffer, 4mM MgCl₂ and 0.4 mM each of dATP, dCTP, dGTP and dTTP. The total reaction volume was 50 µL. Cycling conditions were 1 cycle at 94 °C for 4 min; 30 cycles at 94 °C for 1 min, 55 °C for 50 sec and 72 °C for 1 min and 30 sec, and a final extension cycle at 72 °C for 7 min.
2.5.2.3. Agarose Gel Electrophoresis

Agarose gel electrophoresis was used to detect PCR products. Gels were prepared with 1\% (w/v) agarose in 1X TAE buffer. After sample loading, gels were run at 100 mV for 40 -50 min in the same buffer using a Mini Sub Cell GT (Bio-Rad Laboratories, Hercules, CA, USA) connected to a power supply. DNA samples were run alongside 5 µL of a 1 kb DNA molecular weight marker (Fermentas Life Sciences, Burlington, ON) to estimate product size. Gels were stained with 0.5 µg/mL of ethidium bromide. DNA in gels was visualized and photographed using a UV transilluminator (Bio-Rad Laboratories, Hercules, CA). Gel photographs were analyzed using Quantity One Software v. 4.4.0 (Bio-Rad Laboratories, Hercules, CA, USA).

2.5.2.4. PCR Product Purification and Sequencing

The PCR products obtained were purified using the High Pure PCR Product Purification Kit (Roche Diagnostics, Laval, QC) or the QIAquick PCR purification Kit (Qiagen Inc., Mississauga, ON). The protocol was modified slightly by using nuclease-free water instead of elution buffer to improve the elution step. Purified DNA samples were sequenced by the University of Guelph’s AAC Genomics Facility (Guelph, ON). DNA was sequenced in both directions using the forward and reverse primers (27F and 1492R). Forward and reverse sequences were aligned manually and a partial consensus sequence obtained. The consensus sequence was entered into the National Centre of Biotechnology Information (NCBI) database using the nucleotide BLAST search and the nearest sequences retrieved.

2.6. DEGRADATION OF INDIVIDUAL AND DEFINED MIXTURES OF POLYCYCLIC AROMATIC HYDROCARBONS IN LIQUID CULTURE

The following section provides details on all liquid culture experiments involving PAHs. Unless otherwise stated, experiments were conducted in 50 mL glass serum bottles with PTFE-
lined septa and were sealed with aluminum crimp seals (Sigma Aldrich, Oakville, ON). The experiments were designed following methods utilized of Somtrakoon et al., (2007) with modifications. Unless otherwise stated all degradation experiments utilized Bushnell Haas media supplemented with 0.67% (w/v) YNB without amino acids. Liquid culture experiments were conducted with high cell density inocula (~ $10^8$ cells/mL) and cultures were incubated at 22 °C and 150 rpm on a laboratory shaker in the dark. For experiments lasting over 24 h, aeration was provided by opening the serum bottles in the fume hood for 1 h each day. All experiments were conducted in triplicate.

2.6.1. Preparation of PAH Stock Solutions and Media

For PAH degradation experiments, stock solutions of individual PAHs were prepared in DMF at concentrations of 10 mg/mL for phenanthrene, anthracene, pyrene and benzo(a)pyrene and 5 mg/mL for chrysene. PAHs were added to Bushnell Haas media supplemented with YNB without amino acids (0.67%, w/v) to give the following quantities per litre: 200 mg for phenanthrene and 50 mg each for anthracene, pyrene, chrysene and benzo(a)pyrene.

2.6.2. Preparation of Bacterial Inocula

To prepare the bacterial inocula for PAH degradation experiments, bacterial landfarm isolates were grown in Bushnell Haas broth supplemented with 1% (v/v) glycerol, 0.67% (w/v) YNB without amino acids and 25 mg/L phenanthrene. The flasks were incubated at 22 °C with shaking at 150 rpm until growth reached the late exponential phase (1-2 days). Cells were harvested by centrifugation at 4 500 x g for 10 minutes at 4 °C and were washed twice with sterilized Bushnell Haas broth containing 0.67% (w/v) YNB without amino acids. The cell pellets were re-suspended in Bushnell Haas broth containing YNB without amino acids (0.67%, w/v) to achieve a cell density of $10^8$ cells/mL to be used as the inoculum for PAH degradation.
experiments. Bacterial inocula which were intended to serve as autoclaved cell controls were
grown, centrifuged and washed under the same conditions as described above, and underwent
autoclaving at 121 °C for 15 min prior to use in degradation experiments.

2.6.3. Bacterial Degradation of Phenanthrene

To determine the ability of landfarm bacterial isolates to degrade phenanthrene over a 72 h period, enough 50-mL glass serum bottles containing Bushnell Haas broth supplemented with 0.67% (w/v) YNB without amino acids (9 mL) were prepared so that one bottle could be
sacrificed at each time point for PAH extraction. In each serum bottle, 0.2 mL of a phenanthrene
stock solution (10 mg/L) was added to the Bushnell Haas broth. The bacterial inoculum (ca. 1 mL) was then added to each bottle to give an initial cell density of 10^8 cells/mL. As a control, media containing phenanthrene was inoculated with autoclaved cells to achieve an equivalent
initial cell density (~ 10^8 cells/mL). An abiotic control was also prepared which contained
Bushnell Haas broth supplemented with 0.67% (w/v) YNB without amino acids and
phenanthrene, but did not contain any bacterial cells. All bottles were incubated and shaken as
described in Section 2.6. Sampling and PAH extraction occurred at 0, 2, 4, 6, 8, 10, 12, 24, 48,
and 72 h.

2.6.4. Bacterial Degradation of Defined PAH Mixtures

To determine the ability of landfarm bacterial isolates to degrade four combinations of
PAHs over a 6 day period, enough glass serum bottles containing Bushnell Haas broth and
0.67% (w/v) YNB without amino acids were prepared to allow for one bottle to be sacrificed at
each PAH extraction time point. The four defined sets of mixtures contained phenanthrene and
one of either anthracene, pyrene, chrysene or benzo(a)pyrene at a ratio of 200 mg/L:50 mg/L.
Therefore, to each serum bottle containing broth, 0.2 mL of the phenanthrene stock solution and
0.05 mL of one of anthracene, pyrene or benzo(a)pyrene or 0.1 mL of chrysene was added. The
bacterial inoculum (ca. 1 mL) was added to each bottle to give an initial cell density of $10^8$ cells/mL. As a control for each defined mixture, autoclaved cells were inoculated in media containing the PAHs to give an initial cell density of ~ $10^8$ cells/mL. Abiotic controls were also included for each PAH combination. All bottles were incubated and shaken as previously described. PAH extraction occurred at 0 h, 10 h, Day 1, Day 2, Day 3, Day 4, Day 5, and Day 6.

2.6.5. Microbial Growth during PAH Degradation

Bacterial cell growth over the course of PAH degradation experiments was monitored by dilution spread plating techniques using Bushnell Haas agar plates supplemented with 1% (v/v) glycerol and 0.67% (w/v) YNB without amino acids. All dilutions were prepared in 1 X PBS. Plates were incubated at 22 °C for 3-4 days until colonies were visible.

2.6.6. Analytical Methods

2.6.6.1. Extraction of PAHs from Culture Medium

For PAH extraction from liquid cultures, 2 mL of DCM and 100 µL of a fluorene internal standard (1 mg/mL) were added to the entire 10 mL culture in a 50 mL glass serum bottle. The mixture was shaken for 90 seconds and then held at 22 °C for 2 h prior to being frozen at -20 °C overnight. The following day, the bottles were thawed at 22 °C and approximately 1.5 to 1.8 mL of the organic phase was removed to auto sampler vials for GC-FID analysis. PAH standards were prepared in 10 mL of Bushnell Haas broth with YNB without amino acids (0.67%, w/v) and were extracted in an identical manner as used for the liquid cultures.

2.6.6.2. Determination of PAH Concentration by GC-FID

PAH concentrations in sample extracts and standards were determined using a Hewlett Packard 5890 Series II Gas Chromatograph equipped with a flame ionization detector using the method and conditions described in Section 2.4.2.2.
The peak areas of both the internal standard and PAHs were used to calculate the peak area ratio, which was used in the creation of a standard curve. The peak area ratios for the controls and samples were compared to the PAH standards for determination of PAH concentration. The standard curve for phenanthrene was linear at concentrations ranging from 0.5 to 200 mg/L, and for anthracene, pyrene, chrysene and benzo(a)pyrene the standard curves were linear from 0.5 to 50 mg/L.

2.7. PAH BIODEGRADATION IN NOVA CHEMICALS DISSOLVED AIR FLOTATION SLUDGE

The following section describes the degradation of PAHs in NOVA Chemicals Refinery effluent by Arthrobacter sp. UG50, a bacterium isolated following enrichment from NOVA Chemicals landfarm soil. All experiments were conducted in 500-mL Erlenmeyer flasks (baffled or flat bottom) with PTFE-lined screw caps. Unless otherwise stated, all experiments utilized Dissolved Air Floatation (DAF) sludge from a batch produced at the NOVA Chemicals Corunna Site (near Sarnia, ON) in June of 2007. All flasks were incubated at 22 °C at 150 rpm on a laboratory shaker (in a fume hood) in the dark. All experiments were conducted in triplicate.

2.7.1. Selection of Antibiotic Resistant Mutants of Arthrobacter sp. UG50

An antibiotic resistant mutant of Arthrobacter sp. UG50 was desired for the purposes of monitoring inoculated bacterial cell growth during PAH degradation in non-sterile DAF sludge. A mutant resistant to 20 µg/mL of nalidixic acid and 20 µg/mL of rifampicin was isolated and selected to be used during sludge degradation experiments. All agar plates described below were prepared with Bushnell Haas media supplemented with 1% (v/v) glycerol and 0.67% (w/v) YNB without amino acids.

Agar plates were prepared as previously described and contained a rifampicin gradient with concentrations ranging from 10 to 50 µg/mL. Dilutions of Arthrobacter sp. UG50 culture
which had been grown overnight in Bushnell Haas broth with 1% (v/v) glycerol and 0.67% (w/v) YNB without amino acids were spread on the plates which were then incubated at 22 °C. Colonies were visible up to a rifampicin concentration of 20 µg/mL. Agar plates were then prepared with fixed rifampicin concentrations ranging from 10 to 50 µg/mL. As observed on the gradient plates, growth was visible at 10 and 20 µg/mL rifampicin.

Five colonies which grew at 20 µg/mL rifampicin were selected for plating on gradient plates containing both rifampicin and nalidixic acid at concentrations from 10 to 50 µg/mL of each antibiotic. The plates were incubated under the same conditions as previously described. Growth was visible up to 20 µg/mL rifampicin and nalidixic acid. Fixed concentration rifampicin and nalidixic acid containing plates were then prepared from 10 to 50 µg/mL. Each plate also contained 50 µg/mL cyclohexamide which was used to prevent fungal growth when plating DAF sludge samples. Colonies only grew on the 10 and 20 µg/mL nalidixic acid and rifampicin plates containing cyclohexamide. The plating was repeated and four colonies which grew at 20 µg/mL nalidixic acid and rifampicin were grown overnight in broth at 22°C containing the same concentrations of antibiotics. OD₆₀₀ readings of each culture were taken and the mutant which exhibited the best growth as determined by the highest OD₆₀₀ value was selected for further testing.

The antibiotic resistant *Arthrobacter* sp. UG50 mutant was then tested to make sure it had retained its’ ability to degrade phenanthrene. An experiment identical to that described in Section 2.6.3 was performed to compare the degradation ability of the wildtype *Arthrobacter* sp. UG50 to the antibiotic resistant mutant. PAH extractions were performed at 0, 10, 24 and 48h. A change in colour from white to orange of the culture medium was taken as an indication of phenanthrene degradation. The rifampicin and nalidixic acid resistant *Arthrobacter* sp. UG50
mutant was maintained on Bushnell Haas plates amended with glycerol (1%, v/v) and YNB without amino acids (0.67%, w/v) containing both antibiotics at 20 µg/mL and 50 µg/mL cyclohexamide until needed for sludge degradation experiments.

2.7.2. Preparation of Bacterial Inocula

To prepare the bacterial inocula for PAH degradation experiments, the antibiotic resistant mutant of *Arthrobacter* sp. UG50 was grown in Bushnell Haas broth supplemented with 1% (v/v) glycerol and 0.67% (w/v) YNB without amino acids at 22 °C and 175 rpm for 1-2 days until growth reached the late exponential phase. Subsequently, 4 mL of this culture was added to 196 mL of Bushnell Haas broth containing 1% (v/v) glycerol and 0.67% (w/v) YNB without amino acids and incubation continued for another 48 h at 22 °C with shaking at 200 rpm.

The culture medium was divided into two 100 mL portions, and bacterial cells were harvested as described in Section 2.6.2. The cell pellets were each re-suspended in DAF sludge to a bacterial cell density of 10⁷ cells/mL. *Arthrobacter* sp. UG50 cells which were intended to be used as an autoclaved cell control were grown, centrifuged, washed and re-suspended to yield ~ 10⁷ cells/mL in the same manner described above. The cells were then autoclaved for 15 min at 121 °C prior to use in degradation experiments.

2.7.3. PAH Biodegradation in Dissolved Air Floatation Sludge

In preparation for the sludge degradation experiment, 100 g of well mixed DAF sludge was added to each Erlenmeyer flask and was acclimated to 22 °C prior to use. Baffled flasks were used to increase aeration within the vessel, and a flat bottomed flask containing *Arthrobacter* sp. UG50 was included for comparison. The three treatments used included:

1. A baffled flask containing DAF sludge only (without added inoculum)
2. A baffled flask containing DAF sludge with *Arthrobacter* sp. UG50 cells
3. A flat bottomed flask containing DAF sludge with *Arthrobacter* sp. UG50 cells
Approximately 1 mL of DAF sludge from the flasks (with the exception of the control) was used in the re-suspension of the washed *Arthrobacter* cell pellets. The inoculated sludge was then returned to the flasks and PTFE-lined screw caps were loosely fitted to allow for aeration. The flasks were covered with aluminum foil and incubated at 22 °C on a laboratory shaker in a fume hood with shaking at 250 rpm. The flasks were incubated for 20 days with sampling and PAH extraction done on Days 0, 1, 2, 3, 4, 5, 6, 7, 12 and 20.

2.7.4. Microbial Growth During PAH Degradation in Dissolved Air Floatation Sludge

Bacterial cell growth of *Arthrobacter* sp. UG50 over the course of PAH degradation experiments in DAF sludge was monitored by dilution spread plating using Bushnell Haas agar plates supplemented with 1% (v/v) glycerol, 0.67% (w/v) YNB without amino acids, 20 µg/mL each of rifampicin and nalidixic acid and 50 µg/mL cyclohexamide. Growth of microorganisms present in the DAF sludge was monitored by plating on Bushnell Haas plates containing 1% (v/v) glycerol and 0.67% (w/v) YNB without amino acids. All dilutions were prepared in 1 X PBS. Plates were incubated at 22 °C for 3-4 days until colonies were visible.

2.7.5. Analytical Methods

2.7.5.1. *PAH Extraction from Dissolved Air Floatation Sludge*

In preparation for PAH extraction, an internal standard of docosane was prepared in a hypo vial by dissolving 0.1 g of docosane in 15 g of DCM. PAH extraction was performed by sub sampling an ~ 1 g, well mixed sludge sample from each flask and transferring it to a 50-mL glass serum bottle. The empty bottle and sludge weight were recorded. 40 µL of the internal standard solution was then added and the total weight subsequently recorded. From this value, the weight of the internal standard was determined. 4 mL of DCM was then added to each of the bottles which were then sealed with PTFE-lined septa and aluminum crimp caps. The bottles were placed on a laboratory shaker for 1 h and 45 min at 22 °C and 250 rpm in the dark. The
extract was then centrifuged at 1000 x g for 20 minutes and a 2 mL DCM aliquot transferred to a GC auto sampler vial for GC-MS analysis. The extracts were stored at -20 °C until analysis.

2.7.5.2. Determination of PAH Concentration and Total Hydrocarbon by GC-MS

PAH concentrations in sludge sample extracts were determined using a Model 6890 N System Gas Chromatograph (Hewlett Packard, Palo Alto, CA, USA) equipped with a Model 5973 N Mass Selective Detector (Agilent Technologies Canada, Mississauga, ON). All GC-MS analysis was carried out by personnel at the NOVA Chemicals Research and Technology Centre in Calgary, AB. The following conditions were used for all analyses:

- Column: RTX-1 Capillary Column (15 m x 0.32 mm, I.D. 0.25 µm)
- Carrier Gas: UHP Helium
- Injector Temperature: Cool On-Column
- Injector Pressure: 25.7 kPa
- Injection Volume: 0.5 µL
- Run Time: 35 minutes

For analysis of PAH extracts, the oven temperature was programmed to 40 °C for 2 min followed by an increase of 12 °C/min to 300 °C where the temperature was held for 11.33 min.

Identification of the PAH compounds in the DAF sludge was determined by a comparison of their mass spectra with the spectra of high purity standards. PAH concentration (µg/g) was determined by a comparison of the peak area ratio of the internal standard (docosane) to the PAH of interest and the ratios obtained were then compared to a set of standards. Total hydrocarbons were calculated using an internal standard calculation method using both sample weight and internal standard weight. The following formula was used to calculate total hydrocarbon in sample extracts:
\[ A_x \times \frac{\text{Wt}_{\text{ISTD}} \times [\text{ISTD}] \times 1}{\text{Wt}_{\text{sample}}} = \text{wt ppm} \]

Where:

- \( A_x \) = peak area of component \( x \)
- \( A_{\text{ISTD}} \) = peak area of internal standard (docosane)
- \( \text{Wt}_{\text{ISTD}} \) = weight (g) of internal standard solution added
- \( \text{Wt}_{\text{sample}} \) = weight (g) of sample only, without internal standard
- \( [\text{ISTD}] = \frac{\text{Weight of internal standard}}{\text{Weight of solvent + wt. of internal standard}} \times 10^6 = \mu g/g \)

2.8. DATA ANALYSIS

The percentage of PAH remaining was expressed as the mean ± SD. A one-way analysis of variance was used to test for statistical significance among treatments. Subsequent multiple comparisons of means were performed using a Tukey’s range test. Statistical significance was accepted at \( P < 0.05 \).

CHAPTER 3: RESULTS

3.1. MICROBIAL ENRICHMENT FROM NOVA CHEMICALS LANDFARM SOIL

3.1.1. PAH Degradation in API Oil-water Separator Effluent During Enrichment

Degradation of hydrocarbons present in each flask during enrichment of PAH-degrading microorganisms from NOVA Chemicals landfarm soil and root material or American Petroleum Institute oil-water separator effluent alone was monitored at weekly intervals for 42 days. Hydrocarbon degradation was measured by comparing the total peak areas from the GC chromatogram of each sample over time. Figure 3.1 shows the percent reductions in total peak area occurring over 42 days in each of the four enrichment flasks by GC-FID analysis.

Significant decreases in total peak area were recorded from GC-FID analysis of extractions taken from each of the four enrichment flasks over 42 days. After 7 days of
incubation, reductions of 90.7, 80.4 and 86.4% were seen in the total peak areas of the flasks containing root material, composite and field 6 soils respectively. Degradation in the flask containing only API effluent occurred more slowly over the first 7 days, with only a 24.9% reduction in total peak area. By day 14, peak areas in the flask containing only API oil-water separator effluent had reached levels similar to those of the three flasks containing soil/root material with only 12.1% of the total peak area remaining compared to 3.6, 17.4 and 4.4% for the root, composite and field 6 flasks respectively. By the end of the 42 day enrichment period, only 2.5, 1.8, 2.3 and 1.9% of the original total peak areas for the flasks containing API oil-water separator effluent alone, root material, composite and field 6 soils remained.

Figure 3.1: Hydrocarbon degradation in American Petroleum Institute oil-water separator effluent during shake flask enrichment of PAH degrading microorganisms from NOVA Chemicals landfarm soil over 42 days. Symbols: (-♦-) Root material and API oil-water separator effluent; (-■-) Composite soil and API oil-water separator effluent; (-▲-) Field 6 soil and root material; (-●-) API oil-water separator effluent alone.
3.1.2. Isolation of Bacterial Isolates from Enrichment Sources

Following enrichment of PAH-degrading microorganisms, aliquots from each flask were plated on TSA and 1/10\textsuperscript{th} TSA plates. After 48 h of incubation, 88 isolates were selected, of which 56 isolates were from the full strength TSA plates and 32 from the 1/10\textsuperscript{th} TSA plates. The isolates selected grew well at 22 °C and were selected over isolates growing at 28 °C and 37 °C which did not show significant growth. Isolates were selected to undergo screening for phenanthrene-degrading ability based on originating enrichment source and differing colony morphology. The isolates were identified based upon the originating enrichment source (composite or field 6 soils, root or API effluent). Table 3.1 lists the enrichment sources of the 88 isolates.

Table 3.1: Shake-flask enrichment sources for selected landfarm microorganisms isolated on TSA and 1/10\textsuperscript{th} TSA media following incubation at 22 °C for 48 h.

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3.2. IDENTIFICATION OF PHENANTHRENE-DEGRADING MICROORGANISMS

3.2.1. Phenanthrene Spray Plating

Isolates capable of degrading phenanthrene were isolated using the spray-plate technique of Kiyohara et al. (1982). After inoculation of agar plates in quadrants with the 88 microbial isolates, visible colony growth was observed after 2-3 days. A first attempt at spraying the plates with phenanthrene proved unsuccessful as the initial sprayer pressure was too high and resulted in the bacterial inoculum being spread from the initial point of inoculation to other quadrants. A second attempt with more sprayer control and less pressure being used in depositing the phenanthrene layer proved successful and resulted in even distribution of the PAH on the agar and minimal disruption of the microbial colonies.

Phenanthrene degradation was noted under visible light by a darkening of the agar and the development of clearing zones around areas of bacterial growth. Figure 3.2 shows a photograph of the zone of clearing produced around isolate C8 on a plate sprayed with phenanthrene. The darkening of the agar was visible prior to the presence of visible clearing zones. Plates were checked daily for the presence (a positive result) or absence (a negative result) of clearing zones. Zones of clearing started to become visible following 7 days of incubation at 22 °C. The results of phenanthrene spray plating experiments are presented in Table 3.2.

Of the 88 isolates screened, 13 exhibited varying degrees of phenanthrene-degrading ability. These included isolates C8, D5, D8, F5, F6, F7, F8, G5, J1, J2, J3, J4 and J8. The 4 isolates (C8, F7, G5 and J3) which were observed to produce the largest zones of clearing (> 0.2 cm) and significant darkening of the agar were streaked to purity and selected for further studies.
Figure 3.2: Bushnell Haas agar plate containing 1 g/L yeast extract inoculated with four landfarm microbial isolates and sprayed with 10% (w/v) phenanthrene in acetone. Arrow indicates the zone of clearing formed around bacterial isolate C8 after 20 days of incubation at 22°C in the dark.
Table 3.2: Phenanthrene spray plate results for 88 microorganisms isolated from enrichment flasks. Bushnell Haas plates containing 1 g/L yeast extract were point inoculated with landfarm microbial isolates and were sprayed with 10% (w/v) phenanthrene in acetone. Plates were observed daily for 20 days for the presence of clearing zones. Symbols: (+) clearing zone was present around a bacterial colony; (-) no observed zone of clearing. Distances of clearing (radii) from bacterial colonies are presented.

<table>
<thead>
<tr>
<th>Plate</th>
<th>Isolate Number</th>
<th>Controls</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>E. coli TG1</td>
</tr>
<tr>
<td>A</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>B</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>C</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>D</td>
<td>-</td>
<td>0.2 cm</td>
</tr>
<tr>
<td>E</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>F</td>
<td>-</td>
<td>0.2 cm</td>
</tr>
<tr>
<td>G</td>
<td>-</td>
<td>0.5 cm</td>
</tr>
<tr>
<td>H</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>I</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>J</td>
<td>+ 0.1 cm</td>
<td>+ 0.2 cm</td>
</tr>
<tr>
<td>K</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>
3.2.2. Determination of 16S rRNA Gene Sequences

Identification of bacterial isolates which exhibited zones of clearing on phenanthrene spray plates (C8, J3, G5 and F7) was performed using 16S rRNA sequencing. The 16S primer set (27F and 1492R) was used to amplify a 1450 bp fragment of the 16S rRNA gene from each isolate. The identities of the sequences were determined using BLASTN v. 2.2.25 (Basic Local Alignment Search Tool) similarity search on the NCBI database.

Results of the identity search showed that isolates C8, G5, F7 and J3 had the highest similarity to *Arthrobacter* spp., *Achromobacter* sp. Ss3, *Serratia* spp. and *Georgenia* spp., respectively (Table 3.3).

**Table 3.3:** Identification of bacterial isolates based on 16S rRNA sequence comparison. The top ten aligned sequences between bacterial isolates and members of the BLAST sequences database are presented.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Primer Set</th>
<th>Aligned Sequences from Blast</th>
<th>% Similarity</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>C8</strong></td>
<td>27F-1492R</td>
<td><em>Arthrobacter oxydans</em> strain 03-0063</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Arthrobacter</em> sp. CU19</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Arthrobacter oxydans</em> strain 02-0288</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Arthrobacter oxydans</em> strain 1663</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Arthrobacter</em> sp. 32c</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Arthrobacter</em> sp. TS18</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Arthrobacter</em> sp. R-36535</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Arthrobacter oxydans</em> strain Z1656</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Arthrobacter</em> sp. MH61</td>
<td>99%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Arthrobacter oxidans</em></td>
<td>98%</td>
</tr>
<tr>
<td><strong>J3</strong></td>
<td>27F-1492R</td>
<td><em>Georgenia ferrireducens</em> strain F64</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Georgenia</em> sp. 2C6-43</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Georgenia</em> sp. ITCr52</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Georgenia</em> sp. ITT12</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Georgenia</em> sp. CC-NMPT-T3</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Georgenia</em> sp. swa-35</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Georgenia muralis</em> strain NBRC 103560</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Georgenia thermotolerans</em></td>
<td>97%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Georgenia</em> sp. strain 3A-1</td>
<td>98%</td>
</tr>
<tr>
<td></td>
<td></td>
<td><em>Georgenia</em> sp. E9</td>
<td>98%</td>
</tr>
</tbody>
</table>
### 3.3. PHENANTHRENE DEGRADATION BY LANDFARM ISOLATES IN MINIMAL MEDIUM

The four isolates (C8, J3, G5, F7) which produced clearing zones on phenanthrene spray plates were further tested at high cell densities (10^8 cells/mL) for their abilities to degrade 200 mg/L of phenanthrene over 9 days. Each of the four landfarm isolates and the positive control, *Pseudomonas* sp. UG14Lr exhibited differing phenanthrene degrading ability over the course of the experiment (Figure 3.3). Isolate C8 (*Arthrobacter* spp.) exhibited the fastest rate of phenanthrene degradation with 200 mg/L of the compound being completely degraded within 3 days (Figure 3.3). *Pseudomonas* sp. UG14Lr degraded 46% of 200 mg/L phenanthrene after 9 days of incubation. Isolates J3 (*Georgenia* spp.), F7 (*Serratia* spp.) and G5 (*Achromobacter* spp.) degraded 43, 56 and 65%, respectively, of 200 mg/L phenanthrene by day 9 (Figure 3.3).
contrast to isolate C8, the majority of phenanthrene degradation by these three isolates occurred between days 6 and 9.

Phenanthrene degradation by each of the isolates coincided with the appearance of an orange pigment within the culture medium. The orange pigment was observed within 24 h in the C8 media, after 3 days in the UG14Lr medium, and after 6 days in the medium containing isolates J3, F7 or G5. An abiotic loss of 14% was observed in the uninoculated control containing phenanthrene only (Figure 3.3). Isolate C8 (designated as *Arthrobacter* sp. UG50 for the continuation of this thesis) was selected for further study due to its ability to completely degrade 200 mg/L within the shortest time period (3 days).

Figure 3.3 Biodegradation of 200 mg/L phenanthrene in Bushnell Haas media containing 0.67% (w/v) YNB (without amino acids) by NOVA Chemicals landfarm bacterial isolates over 9 days. Symbols: (▴-) inoculated with *Pseudomonas* sp. UG14Lr; (■-) inoculated with *Arthrobacter* spp. (C8); (△-) inoculated with *Georgenia* spp (J3); (△-) inoculated with *Serratia* spp. (F7); (□-) inoculated with *Achromobacter* spp. (G5); (○-) uninoculated control. Values are the means (n=3) ± standard deviation for 3 independently grown inocula.
3.4. DEGRADATION OF INDIVIDUAL PAH COMPOUNDS BY *ARTHROBACTER* SP. UG50

3.4.1. Growth of *Arthrobacter* sp. UG50 on Individual PAH Compounds

The ability of *Arthrobacter* sp. UG50 to utilize a number of low and high MW compounds including naphthalene, phenanthrene, anthracene, pyrene, chrysene and benzo(a)pyrene was examined. The strain grew on 200 mg/L phenanthrene as a sole source of carbon and energy as evidenced by a 10-fold increase in cell numbers from $4.7 \times 10^8$ to $5.1 \times 10^9$ CFU/mL after 12 h of incubation (Table 3.4). Growth began to decline after 12 h as phenanthrene concentrations decreased in the medium with $2.6 \times 10^9$ CFU/mL remaining by day 6. The strain also showed growth on 50 mg/L anthracene, with cell numbers increasing from $4.5 \times 10^8$ to $1.8 \times 10^9$ CFU/mL after 21 days (Table 3.4). Although able to utilize phenanthrene and anthracene, *Arthrobacter* sp. UG50 was unable to grow on the HMW PAHs pyrene, chrysene and benzo(a)pyrene as sole carbon and energy sources (Table 3.4).

3.4.2. Phenanthrene Degradation by *Arthrobacter* sp. UG50

Experiments to examine the ability of a high cell density inoculum ($10^8$ cells/mL) of *Arthrobacter* sp. UG50 to degrade phenanthrene in liquid broth were conducted. Phenanthrene was completely degraded by *Arthrobacter* sp. UG50 within 3 days (Fig 3.3A and 3.5A). Some abiotic loss of phenanthrene occurred in the non-inoculated and autoclaved cell controls as seen by a decrease in phenanthrene concentration of 17 and 15%, respectively, after 21 days (Figure 3.5A). To determine how quickly phenanthrene was degraded within 24 h, a second experiment was conducted. These results showed that 99.2% of phenanthrene (200 mg/L) was degraded within 12 h by $10^8$ cells/mL of *Arthrobacter* sp. UG50 (Fig 3.6B). The compound was completely degraded by 24 h of incubation.
Table 14: Growth of *Arthrobacter* sp. UG50 cells on selected PAH compounds in Bushnell Haas media containing 0.67% (w/v) YNB (without amino acids) up to 21 days.

<table>
<thead>
<tr>
<th>PAHs (Initial Concentrations)</th>
<th>Arthrobacter sp. UG50 Cell Numbers (CFU/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0 h</td>
</tr>
<tr>
<td>Naphthalene (50 mg/L)</td>
<td>4.4 X 10^8 ± 5.1 X 10^7</td>
</tr>
<tr>
<td></td>
<td>bA</td>
</tr>
<tr>
<td>Phenanthrene (200 mg/L)</td>
<td>4.7 X 10^9 ± 1.1 X 10^9</td>
</tr>
<tr>
<td></td>
<td>aA</td>
</tr>
<tr>
<td>Anthracene (50 mg/L)</td>
<td>4.5 X 10^9 ± 1.4 X 10^8</td>
</tr>
<tr>
<td></td>
<td>cAE</td>
</tr>
<tr>
<td>Pyrene (50 mg/L)</td>
<td>4.0 X 10^8 ± 7.2 X 10^8</td>
</tr>
<tr>
<td></td>
<td>bAE</td>
</tr>
<tr>
<td>Chrysene (50 mg/L)</td>
<td>4.1 X 10^8 ± 3.2 X 10^8</td>
</tr>
<tr>
<td></td>
<td>bAE</td>
</tr>
<tr>
<td>Benzo(a)pyrene (50 mg/L)</td>
<td>4.4 X 10^8 ± 4.0 X 10^9</td>
</tr>
<tr>
<td></td>
<td>bAE</td>
</tr>
</tbody>
</table>

* Mean (n=3) ± standard deviation. Means with different lower case letters are significantly different from one another across rows and means with different upper case letters are significantly different from one another across columns (Tukey’s test, p < 0.05).

*N.D.: No data*
Phenanthrene degradation by UG50 cells coincided with the development of an orange pigment in the culture medium beginning at 2 h of incubation, reaching a bright orange colour at 24 h before gradually fading over the remainder of the experiment (Figure 3.4C).

![Image of orange colour development](image.png)

**Figure 3.4:** Orange colour development during phenanthrene biodegradation after 24 h by *Arthrobacter* sp. UG50 in Bushnell Haas broth containing 0.67% (w/v) YNB (without amino acids). A: autoclaved cell control; B: uninoculated control; C: *Arthrobacter* sp. UG50.

### 3.4.3. Degradation of Low and High Molecular Weight PAHs by *Arthrobacter* sp. UG50

The ability of *Arthrobacter* sp. UG50 to degrade naphthalene, anthracene, pyrene, chrysene and benzo(a)pyrene, each at 50 mg/L, in liquid broth was also examined. The strain was able to degrade anthracene at a much slower rate than phenanthrene with 71% remaining after 21 days (Fig 3.5B). *Arthrobacter* sp. UG50 was unable to degrade naphthalene (Fig 3.6A) and each of the HMW compounds pyrene, chrysene or benzo(a)pyrene as sole carbon and energy sources (Fig 3.5C, 3.5D and 3.5E).
Figure 3.5: Biodegradation of individual PAH compounds by $10^8$ cells/mL of *Arthrobacter* sp. UG50 over 21 days in Bushnell Haas medium containing 0.67% (w/v) YNB (without amino acids). Panel A: phenanthrene, 200 mg/L; B: anthracene, 50 mg/L; C: pyrene, 50 mg/L; D: chrysene, 50 mg/L and E: benzo(a)pyrene. Symbols: (-•-) inoculated with *Arthrobacter* sp. UG50; (-△-) autoclaved *Arthrobacter* sp. UG50 cell control; (-□-) uninoculated control. Values are the means (n=3) ± standard deviation.
Figure 3.6: Biodegradation of individual PAHs by $10^8$ cells/mL *Arthrobacter* sp. UG50 over 24 h in Bushnell Haas medium containing 0.67% (w/v) YNB (without amino acids). Panel A: naphthalene, 50 mg/L; Panel B: phenanthrene, 200 mg/L. Symbols: (▴-) inoculated with *Arthrobacter* sp. UG50 cells; (△-) autoclaved *Arthrobacter* sp. UG50 cell control; (□-) uninoculated control. Values are the means (n=3) ± standard deviation.

3.5. BACTERIAL DEGRADATION OF DEFINED PAH MIXTURES IN LIQUID CULTURE

3.5.1. Growth of *Arthrobacter* sp. UG50 on Phenanthrene in the Presence of Anthracene, Pyrene, Chrysene and Benzo(a)pyrene

The ability of *Arthrobacter* sp. UG50 to grow on individual and binary combinations of PAH compounds in liquid cultures was evaluated. The strain grew on phenanthrene as a sole source of carbon and energy in Bushnell Haas broth supplemented with 0.67% (w/v) YNB.
(without amino acids) (Figure 3.5A and 3.6B). Cell numbers rapidly increased from $4.7 \times 10^8$ to $5.1 \times 10^9$ CFU/mL after 12 h before gradually declining to $2.6 \times 10^9$ CFU/mL by day 6 (Figure 3.7). When the cultures were supplemented with either pyrene (50 mg/L), chrysene (50 mg/L) or benzo(a)pyrene (50 mg/L) in addition to phenanthrene (200 mg/L) the growth rates of those cultures matched that of cultures grown with phenanthrene alone. Cultures that were grown on a combination of phenanthrene (200 mg/L) and anthracene (50 mg/L) exhibited continued growth over the course of 6 days with cell numbers rapidly increasing after 12 h from $2.1 \times 10^8$ to $3.5 \times 10^9$ CFU/mL by day 3 (Figure 3.7). Cell numbers continued to gradually increase to $6.0 \times 10^9$ CFU/mL after 6 days.

![Graph showing growth of Arthrobacter sp. UG50 cells on phenanthrene or a binary mixture of selected PAHs over 6 days in Bushnell Haas media containing 0.67% (w/v) YNB (without amino acids). The initial concentrations of PAHs in the media were 200 mg/L phenanthrene, 50 mg/L anthracene, 50 mg/L pyrene, 50 mg/L chrysene and 50 mg/L benzo(a)pyrene. Symbols: (●) phenanthrene + pyrene; (■) phenanthrene + anthracene; (▲) phenanthrene + chrysene; (X) phenanthrene + benzo(a)pyrene; (□) phenanthrene alone. Values are the means (n=3) ± standard deviation.](image-url)
3.5.2. Biodegradation of Phenanthrene by *Arthrobacter* sp. UG50 in the Presence of Anthracene, Pyrene, Chrysene and Benzo(a)pyrene

When supplied as a sole source of carbon and energy, phenanthrene (initial concentration 200 mg/L) was rapidly degraded by *Arthrobacter* sp. UG50 in broth cultures (Figure 3.7). The addition of anthracene (50 mg/L), pyrene (50 mg/L), chrysene (50 mg/L) or benzo(a)pyrene (50 mg/L) did not significantly affect the rate of phenanthrene degradation.

![Graph](image)

Figure 3.8: Biodegradation of phenanthrene in the presence of anthracene (■), pyrene (▴), chrysene (△) and benzo(a)pyrene (+) by *Arthrobacter* sp. UG50 in Bushnell Haas media containing 0.67% (w/v) YNB (without amino acids). The time courses of PAHs in media containing only phenanthrene (■), autoclaved cell control (O) and un-inoculated cell control (x) are also plotted. The initial concentrations of PAHs in the media were 200 mg/L phenanthrene, 50 mg/L anthracene, 50 mg/L pyrene, 50 mg/L chrysene and 50 mg/L benzo(a)pyrene. Values are the means (n=3) ± standard deviation.

Phenanthrene was degraded to undetectable levels by *Arthrobacter* sp. UG50 within 12 h in each of the defined PAH combinations tested (Figure 3.8). No significant differences in the extent of phenanthrene degradation by *Arthrobacter* sp. UG50 was observed when the compound
was supplied as a sole carbon source or in combination with anthracene, pyrene, chrysene or benzo(a)pyrene. Abiotic losses of phenanthrene were observed in the autoclaved cell and uninoculated controls with concentrations declining 19.1 and 19.6%, respectively, after 6 days (Figure 3.8).

3.5.3. Biodegradation of Anthracene, Pyrene, Chrysene and Benzo(a)pyrene by *Arthrobacter* sp. UG50 in the Presence of Phenanthrene

The ability of *Arthrobacter* sp. UG50 to degrade anthracene (50 mg/L), pyrene (50 mg/L), chrysene (50 mg/L) or benzo(a)pyrene (50 mg/L) when present in combination with phenanthrene (200 mg/L) was assessed. As presented in Figure 3.5, the strain was capable of degrading phenanthrene and, to a limited extent, anthracene as sole carbon and energy sources, but was not capable of degrading pyrene, chrysene or benzo(a)pyrene alone. In the presence of phenanthrene, however, the rate and extent of anthracene degradation was increased from 29% being degraded after 21 days when anthracene was present as a sole carbon and energy source to 100% being degraded within 6 days when presented in combination with phenanthrene (Figure 3.9A).

The presence of phenanthrene also induced the degradation of pyrene by *Arthrobacter* sp. UG50 with concentrations decreasing by 33% after 6 days (Figure 3.9B). The presence of phenanthrene in combination with chrysene or benzo(a)pyrene did not enhance degradation of either compound by *Arthrobacter* sp. UG50 (Figure 3.9C and 3.9D). Some PAH loss occurred in the autoclaved cell and uninoculated controls possibly due to daily opening of all the glass serum bottles for 1 h to allow for cell exposure to oxygen.
Figure 3.9: Biodegradation of anthracene (A), pyrene (B), chrysene (C) and benzo(a)pyrene (D) in the presence of phenanthrene by Arthrobacter sp. UG50 in Bushnell Haas media containing 0.67% (w/v) YNB (without amino acids). Symbols: (-x-) individual PAH, (-◊-) binary mixture containing a particular PAH and phenanthrene, (-△-) autoclaved cell control, (-□-) uninoculated control. The initial concentrations of PAHs in the media were 200 mg/L phenanthrene, and 50 mg/L each of anthracene, pyrene, chrysene and benzo(a)pyrene. Values are the means (n=3) ± standard deviation.

3.6. PAH BIODEGRADATION IN NOVA CHEMICALS REFINERY DISSOLVED AIR FLOTATION SLUDGE

3.6.1. Total Hydrocarbon Degradation

Total hydrocarbon content in the NOVA Chemicals DAF sludge was monitored in each of the flasks containing either sludge alone (in a baffled flask) or a combination of DAF sludge and 10^7 cells/mL Arthrobacter sp. UG50 cells in baffled or flat-bottomed flasks over the course of 20 days. The results are presented in Figure 3.10. A decrease in total hydrocarbon content
was observed in each of the three treatment flasks over a 20 day period (Figure 3.10).

**Figure 3.10:** Total hydrocarbon content in DAF sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 in baffled or flat bottomed flasks incubated for 20 days at 22°C and 250 rpm. Symbols: Baffled flask containing DAF sludge only (- -); baffled flask containing DAF sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 (■); flat bottomed flask containing DAF sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 (▲). Values are the means (n=3) ± standard deviation.

In the baffled flask containing DAF sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50, an initial sharp decline in total hydrocarbon content from 615 to 380 µg/g was observed from days 0 through 4 (Figure 3.10). Levels continued to decline at a slower rate over the next 17 days to 238 µg/g by day 20 of the experiment. In the flat-bottomed flask containing DAF sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50, an initial decline in total hydrocarbon from 738 to 403 µg/g occurred within the first 4 days of the experiment, with levels remaining relatively constant over the next 16 days reaching 386 µg/g by day 20 (Figure 3.10). In the baffled flask containing only DAF sludge, an initial decline in total hydrocarbon occurred
from 748 to 529 µg/g by day 4 (Figure 3.10). Levels remained constant from days 4 through 6 before continuing to steadily decline from days 6 through 20, reaching 239 µg/g by the end of the experiment (Figure 3.10).

3.6.2. Degradation of Individual PAHs in Dissolved Air Floatation Sludge

The degradation of selected individual PAHs within the DAF sludge in each of the three treatment flasks (the baffled flask containing only DAF sludge and the baffled and flat-bottomed flasks containing DAF sludge inoculated with 10^7 cells/mL of Arthrobacter sp. UG50 cells) was monitored by GC-MS analysis of extracted samples. The PAHs monitored included naphthalene, fluorene, phenanthrene, pyrene, chrysene and benzo(a)pyrene. The PAH degradation results from days 0 through 5 are illustrated in Figure 3.11 with PAH concentrations presented as percent PAH degraded.

The initial concentrations of the selected PAHs present within the DAF sludge were very low: 1.37 ± 0.20 µg/g for naphthalene, 0.64 ± 0.06 µg/g for fluorene, 0.85 ± 0.03 µg/g for phenanthrene, 0.11 ± 0.01 µg/g for pyrene and 0.10 ± 0.01 µg/g for chrysene. No benzo(a)pyrene was detected in any of the samples tested. As a result, only samples from one run of the experiment yielded concentrations which were high enough to be detected (Figure 3.11).

Naphthalene was rapidly degraded in both of the baffled flasks containing either DAF sludge only, or DAF sludge inoculated with Arthrobacter sp. UG50 cells such that by day 1 only 3.9 and 4.2%, respectively, of naphthalene remained in each flask (Figure 3.11A). After 5 days, the naphthalene concentration in the flask containing DAF sludge alone was 2.6% and in the baffled flask containing Arthrobacter sp. UG50 cells, the concentration was not significantly different with only 2.1% remaining (Figure 3.11A).
Figure 3.11: Biodegradation of individual PAH compounds present in Dissolved Air Floatation sludge inoculated with $10^8$ cells/mL of *Arthrobacter* sp. UG50 in baffled or flat bottomed flasks incubated for 20 days at 22°C and 250 rpm. Panel: A: Naphthalene; B: Fluorene; C: Phenanthrene; D: Pyrene and E: Chrysene. Symbols: Baffled flask containing DAF sludge only (−●−); baffled flask containing DAF sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 (−■−); flat bottomed flask containing DAF sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 (−▲−).
In the flat-bottomed flask containing DAF sludge and *Arthrobacter* sp. UG50 cells, naphthalene degradation proceeded more slowly. After 1 day, 51.3% of naphthalene remained. However, by day 2, concentrations were similar to that present in both baffled flasks with only 1.7% naphthalene remaining (Figure 3.11A).

The use of baffled flasks and the addition of *Arthrobacter* sp. UG50 cells did not significantly affect the overall extent of fluorene degradation in DAF sludge. Following 5 days of incubation, 53% of fluorene remained in the baffled flask containing only DAF sludge, 27% remained in the baffled flask containing DAF sludge and *Arthrobacter* sp. UG50 cells, and 40% fluorene remained in the flat-bottomed flask containing DAF sludge and *Arthrobacter* sp. UG50 cells (Figure 3.11B). The addition of *Arthrobacter* sp. UG50 cells to DAF sludge in the baffled flask did increase the rate of fluorene degradation when compared to both the baffled flask containing DAF sludge alone and the flat-bottomed flask containing DAF sludge and *Arthrobacter* cells. After 3 days of incubation, only 43% of fluorene remained in the baffled flask containing *Arthrobacter* cells compared to 84 and 74% for the baffled flask containing DAF sludge only and the flat-bottomed flask containing DAF sludge and *Arthrobacter* cells (Figure 3.11B).

The addition of *Arthrobacter* sp. UG50 cells to the DAF sludge in the baffled flask increased the overall amount of phenanthrene degraded in the sludge such that after 5 days, only 44% remained compared to 84 and 76%, respectively, for the baffled flask containing only DAF sludge and the flat-bottomed flask containing DAF sludge and *Arthrobacter* sp. UG50 cells (Figure 3.11C). Patterns of phenanthrene degradation for each of the three treatment flasks were similar until day 3 of the experiment, where phenanthrene concentrations in the baffled flask containing *Arthrobacter* sp. UG50 cells continued to rapidly and steadily decline, while rates of
phenanthrene degradation in the other two flasks began to slow, and no significant phenanthrene degradation occurred in either flask between days 3 and 5 of the experiment (Figure 3.11C).

Pyrene degradation was enhanced by the addition of *Arthrobacter* sp. UG50 in the baffled flask compared to the flat-bottomed flask containing DAF sludge inoculated with *Arthrobacter* sp. UG50 cells and the baffled flask containing only DAF sludge (Figure 3.11D). Following 5 days of incubation, only 45% of pyrene remained in the baffled flask containing *Arthrobacter* cells compared to 90% in each of the other two flasks. The majority of pyrene degradation (50%) in the baffled flask containing *Arthrobacter* sp UG50 cells occurred within the first day of the experiment (Figure 3.11D). This is in contrast to the other baffled flask containing only DAF sludge and the flat bottomed flask containing sludge and *Arthrobacter* cells which both showed only limited degradation of 9.1% each over the course of 5 days (Figure 3.11D). Use of the baffled flasks did not appear to significantly affect the rate of pyrene degradation (Figure 3.11D).

The use of DAF sludge inoculated with *Arthrobacter* sp. UG50 cells in the baffled flask appeared to increase the rate of chrysene degradation in the DAF sludge when compared to the use of the flat-bottomed flask with *Arthrobacter* cells to treat the DAF sludge (Figure 3.11E). Following 5 days of treatment, only 33.3% of chrysene remained compared to 80% in the flat-bottomed flask. The overall extent of chrysene degradation in the baffled flask containing DAF sludge alone (46% degraded) was not significantly different from that in the baffled flask containing *Arthrobacter* sp. UG50 cells (66.7%).

3.6.3. Survival of Arthrobacter sp. UG50 in NOVA Chemicals Dissolved Air Floatation Sludge

The survival of *Arthrobacter* sp. UG50 cells during biodegradation of PAH compounds within the DAF sludge was monitored at selected intervals over 20 days (Figure 3.12A). The use of baffled versus flat-bottomed flasks had a significant effect upon the rate of *Arthrobacter* sp.
UG50 growth. In the baffled flasks, *Arthrobacter* sp. UG50 cell numbers steadily increased from 1.97 X 10^7 CFU/mL on day 0, to 4.40 X 10^8 CFU/mL on day 16 before starting to decline to 1.77 X 10^8 CFU/mL by day 20 of the experiment (Figure 3.12A). In contrast, *Arthrobacter* sp. UG50 cell numbers did not increase as rapidly or reach the same level of growth in the flat-bottomed flask as seen in the baffled flask. Cell numbers slowly increased from 1.19 X 10^7 CFU/mL on day 0, to 3.83 X 10^7 CFU/mL on day 16 before declining to 3.40 X 10^7 CFU/mL on day 20 (Figure 3.12A).

The growth of native bacterial species already present in the DAF sludge during the biodegradation experiment was also monitored (Figure 3.12B). Bacteria in the baffled flask containing DAF sludge that was inoculated with 10^7 cells/mL *Arthrobacter* sp. UG50 cells showed a slightly higher rate of growth compared to that of bacteria in the baffled flask containing DAF sludge that was not inoculated with *Arthrobacter* sp. UG50 (Figure 3.12B). From days 0 to 6, bacterial cell numbers in the flask containing only DAF sludge began to increase at a slightly faster rate than those in the baffled flask inoculated with *Arthrobacter* cells. However, by day 12, total bacterial numbers in the baffled flask containing *Arthrobacter* cells had increased to 3.47 X 10^6 CFU/mL compared to 2.40 X 10^6 CFU/mL for those in the flask containing only DAF sludge (Figure 3.12B). Cell numbers in the baffled flasks containing either DAF sludge alone or DAF sludge inoculated with *Arthrobacter* cells continued to increase from days 12 through 16 to 2.70 X 10^6 and 3.97 X 10^6 CFU/mL, respectively. Growth in both flasks began to slow by day 20 with cell numbers declining to 3.84 X 10^6 CFU/mL for the baffled flask containing *Arthrobacter* cells and remaining the same as day 16 for the flask containing DAF sludge only (Figure 3.12B).
Figure 3.12: Bacterial growth during biodegradation of NOVA Chemicals DAF sludge over 20 days in baffled and flat bottomed flasks. Panel A: *Arthrobacter* sp. UG50 cell growth; Panel B: Growth of bacteria present in DAF sludge. Cells were plated on days 0, 3, 6, 12, 16 and 20 on Bushnell Haas media amended with 1% (v/v) glycerol and 0.67% (w/v) YNB (without amino acids). Symbols: Baffled flask containing DAF sludge inoculated with *Arthrobacter* sp. UG50 (- ■ -); Flat-bottomed flask containing DAF sludge inoculated with *Arthrobacter* sp. UG50 (- ▲ -); Baffled flask containing DAF sludge only (- ● -). Values are the means (n=3) ± standard deviation.
In contrast, native bacterial cell growth in the flat-bottomed flask containing DAF sludge inoculated with *Arthrobacter* sp. UG50 cells was considerably slower than the growth observed in both baffled flasks. Cell numbers increased from $1.21 \times 10^5$ CFU/mL on day 0 to only $6.73 \times 10^5$ by day 16 before declining to $6.45 \times 10^5$ CFU/mL by day 20 (Figure 3.12B).

CHAPTER 4: DISCUSSION

This study sought to isolate efficient PAH-degrading bacteria from PAH-contaminated landfarm soil by enrichment with API effluent from the NOVA Chemicals Refinery. The most promising isolate, *Arthrobacter* sp. UG50, was capable of utilizing phenanthrene and anthracene as sole carbon sources. These compounds, along with a number of other LMW and HMW PAHs are present in low concentrations in NOVA Chemicals refinery effluents. Ontario M.O.E. guidelines introduced in 2009 now regulate acceptable PAH concentrations in waste applied to soil. The ability of landfarm bacteria to effectively reduce PAH levels and total hydrocarbon content in refinery wastes highlights the potential for successful use of slurry bioreactors to pre-treat oily effluents in order to meet legislated criteria.

4.1. ENRICHMENT AND ISOLATION OF PHENANTHRENE-DEGRADING BACTERIA FROM NOVA CHEMICALS LANDFARM SOIL

The largest number of isolates to be screened for phenanthrene-degrading ability in this study originated from both NOVA Chemicals landfarm root material and field 6 soil which were used for the phytoremediation of oily sludge. PAH contaminated soil that has been phytoremediated has been shown to yield a more diverse group of microorganisms (including PAH-degraders) compared to soil that is un-planted (Kirk *et al.*, 2005). Growing crops such as alfalfa or perennial rye grass have been shown to enhance the total number of microorganisms in the rhizosphere of petroleum-contaminated soil (Muratova *et al.*, 2003; Kirk *et al.*, 2005). The nutrients released via root exudates in the rhizosphere are favourable to microorganisms. This
results in an increased potential for the isolation of PAH-degrading bacteria from this region (Muratova et al., 2003). Kirk et al. (2005) reported a significant increase in the number of petroleum degrading bacteria in the rhizosphere of soil grown with perennial rye grass, from $10^7$ CFU/g dry soil to $10^{10}$ CFU/g dry soil after 7 weeks of treatment, while microbial levels in bulk soil remained relatively constant at $10^7$ CFU/g dry soil. In another study, Muratova et al., (2003) reported a 7-fold increase in the number of PAH-degrading microorganisms in the rhizosphere of sandy soil phytoremediated with alfalfa, compared to numbers in un-planted soil. This increase in PAH-degrading microorganisms resulted in a 68.7% reduction in the original level of PAHs (79.80 mg/kg) present in the soil after 2 years compared to a 55% reduction in uncropped soil (Muratova et al., 2003).

The use of a composite soil sample collected from four landfarm fields at different depths, helped to maximize the chances of isolating PAH-degrading microorganisms. As a result, many of our phenanthrene-degrading isolates, including Arthrobacter sp. UG50 came from this enrichment source. The NOVA Chemicals API effluent, which has a natural microbial community, contains a variety of chemical compounds including aliphatic hydrocarbons and low and high MW PAHs. Bacteria surviving in this environment are able to withstand the stresses of a toxic, low oxygen environment and would be ideal candidates for use at high cell densities to treat and degrade components present in similar effluents and sludges.

The plate-screened isolates showed varying abilities to metabolize phenanthrene based upon the size of clearing zones on spray plates. The successful initiation of PAH oxidation requires a sufficient cell energy reserve and the use of a supplemental growth substrate in the agar composition of spray-plates can assist in providing the necessary energy (Ho et al., 2000). The use of supplemental growth substrates in plate screening can also aid in the isolation of both slow and fast-growing microorganisms, thereby increasing the total number of PAH-degrading
microorganisms isolated (Ho et al., 2000). By allowing the plates containing 1% (w/v) yeast extract as a supplemental growth substrate to incubate for 24-48 hours following inoculation with the microbial isolates prior to phenanthrene addition, bacterial colony growth was established and an adequate cell energy reserve was likely created for PAH oxidation to occur.

Zones of clearing were visible around colonies following 7 days of incubation at 22°C. This is similar to the length of time (7-14 days) for clearing zones to develop on phenanthrene-sprayed PGYT plates containing per litre: peptone, 0.06 g, yeast extract, 0.1 g, glucose, 0.1 g and tryptone, 0.05 g, for the isolation of PAH-degrading isolates from contaminated soils and sediments (Ho et al., 2000). The researchers found that the use of this medium resulted in their isolates degrading PAHs faster when compared to isolates grown on media not containing supplemental growth sources, and concluded that PAH-degrading ability was not negatively affected by the presence of these substrates (Ho et al., 2000).

The phenanthrene-degrading bacteria isolated from NOVA Chemicals landfarm soil belonged to four genera: Arthrobacter, Georgenia, Achromobacter and Serratia. To quantitatively determine the phenanthrene-degrading ability of the four isolates, degradation experiments in liquid medium were conducted. All four isolates were capable of phenanthrene removal; however the isolates showed variations in lag periods prior to degradation, as well as variations in the rate and extent of phenanthrene degradation during incubation.

In media containing 200 mg/L phenanthrene inoculated with Arthrobacter sp. UG50 or Pseudomonas sp. UG14Lr, degradation lag periods were not observed. Although no degradation lag period was observed for UG14Lr, degradation ceased after 3 days with only 30% phenanthrene removal. Providenti et al. (1995) reported on the ability of UG14Lr to mineralize different concentrations of phenanthrene. Growth on phenanthrene as a sole carbon and energy source was found to be quite slow, and mineralization was found to increase as phenanthrene
concentrations increased. At concentrations of 10, 50, 200 and 1000 mg/L phenanthrene, 27, 19, 15.4 and 3.3% were mineralized after 36 days (Providenti et al., 1995). The majority of phenanthrene mineralization occurred within the first five days of incubation, with concentrations beginning to level off over the remainder of the experiment. The addition of *Pseudomonas aeruginosa* UG2 biosurfactants was also found to enhance the extent of phenanthrene mineralization by UG14Lr (Providenti et al., 1995). The absence of biosurfactants in our phenanthrene degradation studies may have contributed to the cessation of degradation by UG14Lr after 3 days. Limited availability to UG14Lr cells and limited utilization of phenanthrene in the absence of a biosurfactant can also be attributed to poor dissolution of the substrate, and is supported by work by Wodzinski & Coyle (1974), which showed that *Pseudomonas* sp. could only utilize phenanthrene in its dissolved state.

Members of the *Arthrobacter, Achromobacter* and *Serratia* genera have often been isolated from PAH-contaminated environments including petroleum refinery sludges (Keuth & Rehm 1991; Janbandhu & Fulekar 2011; Ghevariya et al., 2011; Tiwari et al., 2010; Rojas-Avelizapa et al., 2002). Strains of *Serratia* sp. have been reported to degrade aromatic and asphaltene fractions from polluted soil, however there is limited data on PAH degradation by members of the genus. A strain of *Achromobacter xylooxidans*, isolated from soil near an Indian oil refinery, was capable of degrading 80% of pyrene (200 mg/L) and 73.4% of phenanthrene (200 mg/L) within 21 days of incubation (Tiwari et al., 2010). At 250 mg/L, 56% of phenanthrene was degraded within 14 days by *Achromobacter insolitus* MHF ENV IV via the salicylic acid pathway (Janbandhu & Fulekar 2011). A halotolerant strain of *Achromobacter xylooxidans* was able to use phenanthrene and anthracene as sole carbon and energy sources, and degrade 56% of chrysene (50 mg/L) within 15 days as a sole carbon source (Ghevariya et al., 2011). No significant lag period was observed during phenanthrene degradation by either of
these *Achromobacter* strains. Phenanthrene degradation was found to occur optimally at 30°C in these studies, so the temperature used in our experiments (22°C) may have contributed to the reduced rate of phenanthrene (200 mg/L) degradation (65% degraded after 9 days). Little information is available regarding the isolation of *Georgenia* spp. from oil contaminated sites; however one report documents a halophilic oil-utilizing *Georgenia* strain that was isolated from the Kuwaiti coast of the Arabian Gulf (Al-Awadhi et al., 2007). Specific information on PAH utilization by members of *Georgenia* spp. has not been reported to date.

4.2. DEGRADATION OF INDIVIDUAL PAHS BY *ARTHROBACTER* SP. UG50

Members of the *Arthrobacter* genera are among the more prevalent bacteria present in soils and have been demonstrated to utilize a variety of aromatic compounds as sole carbon and energy sources. Among the contaminants that can be degraded by certain *Arthrobacter* species are PAHs, pesticides, phenols and polychlorinated biphenyls (PCBs) (Daane et al., 2001; Furukawa & Chakrabarty 1982; Havel & Reineke 1993; Hayatsu et al., 1999; Kohler et al., 1988; Negrete-Raymond et al., 2003). Liquid culture experiments carried out with an individual microorganism and a single PAH can be used to demonstrate the ability of an isolate to grow on and degrade an individual compound. In addition, factors such as the metabolic pathways utilized and the influence of select environmental conditions can also be explored through these studies. However, this scenario is not truly representative of what an isolate would encounter in the environment, as PAHs are more commonly found as parts of complex chemical mixtures along with other contaminants, and a variety of microorganisms would normally be present competing for available carbon sources (Atlas 1981; Johnsen et al., 2005).

To better characterize the PAH-degradative potential of *Arthrobacter* sp. UG50, liquid culture experiments were conducted to examine the ability of UG50 to utilize and degrade low
and high MW PAHs presented as individual compounds in minimal medium. Phenanthrene (200 mg/L) was rapidly degraded by UG50 within 24 hours at high initial cell density (10^8 cells/mL) and a 10-fold increase in cell numbers was observed after 12 h of incubation. These results suggest that phenanthrene is effectively utilized by UG50 as a sole carbon and energy source. The strain was also capable of degrading anthracene (50 mg/L), but less successfully than phenanthrene with 71% remaining after 21 days of incubation. In contrast, the LMW PAH naphthalene and the HMW PAHs pyrene, chrysene and benzo(a)pyrene did not support the growth of *Arthrobacter* sp. UG50 as sole carbon and energy sources.

The development of an orange-coloured pigment within the culture medium during phenanthrene degradation has been attributed to the accumulation of 1-hydroxy-2-naphthoic acid by some bacteria such as *Arthrobacter polychromogenes* (Evans *et al.*, 1965; Guerin & Jones 1988; Keuth & Rehm 1991). The orange colour in the medium was visible within 2 h of *Arthrobacter* sp. UG50 incubation with 200 mg/L phenanthrene. Colour development would continue to intensify, reaching a bright orange after 12 h of incubation, before gradually fading over the next 24 h. The change in colour corresponded to the degradation of phenanthrene, with maximum colour development corresponding to complete degradation of the parent compound after 12 h. Monitoring the timeline of development of the orange pigment in the culture medium provided a simple qualitative assessment of phenanthrene degradation by UG50 during the course of experiments.

A few studies have investigated the PAH-degrading ability of *Arthrobacter* strains. PAH utilization by strain UG50 was similar to that of *Arthrobacter* sp. strain Sphe3, isolated from a creosote-contaminated site in Epirus, Greece (Kallimanis *et al.*, 2007). Strain Sphe3 was found to utilize phenanthrene and anthracene as sole carbon and energy sources, but could not utilize naphthalene. Kallimanis *et al.* (2007) studied phenanthrene degradation and the mechanism of
PAH uptake by strain Sphe3. The strain was found to degrade 90% of phenanthrene (400 mg/L) within four days of incubation at a cell density of $10^6$ cells/mL (Kallimanis et al., 2007). Seo et al. (2006) reported that Arthrobacter sp. P1-1 was capable of utilizing phenanthrene as a sole carbon and energy source, and could degrade 40 mg/L phenanthrene within 7 days. Although the cell inoculum was pre-grown in media supplemented with phenanthrene, an initial lag period in phenanthrene degradation of one day was observed (Seo et al., 2006). No lag period in phenanthrene degradation by UG50 was observed to occur.

The growth of Arthrobacter sp. UG50 on 200 mg/L of phenanthrene coincided with the degradation of phenanthrene. Cell numbers rose 10-fold over the first 24 h before declining as the substrate was no longer available. This pattern of growth was also observed in phenanthrene utilization studies on Arthrobacter polychromogenes ($10^7$ cells/mL) (Keuth & Rehm 1991). Cell growth slowly decreased after 24 h as the initial 75, 150, 300 or 450 mg/L phenanthrene was consumed. Mineralization experiments showed that after 26 h of incubation with 150 mg/L phenanthrene, A. polychromogenes mineralized 47.7% of the radiolabelled carbon originally present to $^{14}$CO$_2$ (Keuth & Rehm 1991).

4.3. DEGRADATION OF DEFINED PAH MIXTURES BY ARTHROBACTER SP. UG50

Interactions between PAH compounds can play an important role during PAH degradation by microorganisms. In particular, co-metabolism can occur during the biodegradation of a complex chemical mixture. An example of this interaction is the stimulation of HMW PAH degradation through the biodegradation of LMW PAHs (the growth substrate) by microorganisms (Cerniglia 1992). Biodegradation of the growth substrate can serve to induce enzymes necessary for the transformation of HMW PAHs (Chen and Aitken 1999). Non-growth substrates are thought to be partially transformed by oxygenases to yield more water soluble
intermediates which can be degraded by other bacterial strains (Somtrakoon et al., 2007). To analyze the potential for co-metabolic interactions during PAH degradation by *Arthrobacter* sp. UG50, studies looking at the degradation of anthracene, pyrene, chrysene and benzo(a)pyrene (50 mg/L each) when combined in media with phenanthrene (200 mg/L) were conducted.

When present in a mixture containing the growth substrate phenanthrene, the biodegradation of the PAHs anthracene and pyrene by *Arthrobacter* sp. UG50 was enhanced. Biodegradation of chrysene and benzo(a)pyrene by UG50 was not enhanced by the presence of phenanthrene, indicating that enzymes other than those induced by the degradation of phenanthrene may be required to initiate degradation of these HMW compounds. Phenanthrene (200 mg/L) was rapidly degraded within 24 h by UG50 in the presence of anthracene, pyrene, chrysene and benzo(a)pyrene. Although anthracene was biodegraded to a limited extent when present as a sole carbon source, the presence of phenanthrene increased both the rate and extent of anthracene biodegradation by UG50. The increase in metabolic activity and enzyme production by UG50 in degrading phenanthrene may have assisted in accelerating the degradation of anthracene, which could also be used as a growth substrate. The stimulation of pyrene degradation by UG50 in the presence of phenanthrene as a growth substrate can also be attributed to co-metabolism.

Although no studies have previously investigated co-metabolic PAH degradation by *Arthrobacter* sp., it has been reported to occur in studies conducted with a number of other Gram-positive and Gram-negative strains including members of *Burkholderia*, *Sphingomonas* and *Mycobacterium* sp. (Somtrakoon et al., 2007; Supaka et al., 2001; Rehmann et al., 1998; Juhasz et al., 1997; McLellan et al., 2002). The co-metabolic degradation of pyrene in the presence of phenanthrene was reported by Somtrakoon et al. (2007) with *Burkholderia* sp. VUN10013. The presence of phenanthrene was found to stimulate the degradation of pyrene and fluoranthene, but
not chrysene or benzo(a)pyrene by strain VUN10013. Concentrations of fluoranthene and pyrene (initial concentrations: 50 mg/L each) decreased 58.9 and 57.9%, respectively, after 21 days of incubation, while phenanthrene (250 mg/L) was degraded to trace levels after 3 days (Somtrakoon et al., 2007). The overall extent of pyrene degradation (50 mg/L) by Arthrobacter sp. UG50 over 6 days in the presence of phenanthrene (200 mg/L) was 33%, which was comparable to the level of pyrene degradation (30%) by strain VUN10013 after 6 days of incubation (Somtrakoon et al., 2007).

Other examples of co-metabolism of HMW PAHs in the presence of LMW PAHs have been reported (Ho et al., 2000; Supaka et al., 2001). The presence of phenanthrene (100 mg/L) was found to stimulate the degradation of pyrene and fluoranthene (100 mg/L each) by Sphingomonas sp. strain P2 (Supaka et al. 2001). Phenanthrene was completely degraded within 72 h and pyrene and fluoranthene were degraded such that 60 and 17%, respectively, remained after 7 days (Supaka et al. 2001). Co-metabolic degradation of pyrene, chrysene and benzo(a)pyrene by Sphingomonas sp. strain EPA 505 in the presence of phenanthrene was also reported, and is thought to be due to the broad substrate specificity of the enzyme that initiates oxidation of HMW PAH substrates (Ho et al., 2000). Analysis of the polar metabolites produced during degradation showed that dihydroxylation of pyrene occurs at the 4,5- position, followed by ortho-cleavage resulting in phenanthrene dicarboxylic acid, a pathway similar to that proposed for pyrene degradation by Mycobacterium sp. strain KR2 (Ho et al., 2000; Rehmann et al., 1998).

Although not observed in this study, phenanthrene was shown to stimulate the degradation of benzo(a)pyrene in some cases (Juhasz et al., 1997; McLellan et al., 2002). The presence of 100 mg/L phenanthrene enhanced the degradation of both benzo(a)pyrene and dibenz(a,h)anthracene (50 mg/L each) by Burkholderia cepacia in minimal salts medium (Juhasz et al., 1997). After 56 days, 41 and 52% of benzo(a)pyrene and dibenz(a,h)anthracene,
respectively, were degraded, while phenanthrene was completely degraded within 28 days (Juhasz et al., 1997). Another example is the up to six-fold increase in benzo(a)pyrene mineralization (initial concentration 5 µM) from 8 to 54% by Mycobacterium sp. strain RGJI-135 in the presence of 5 µM phenanthrene (McLellan et al., 2002). The addition of 5 µM pyrene as a growth substrate was also observed to induce the mineralization of benzo(a)pyrene from 8% to 36% after 115 h (McLellan et al., 2002).

The current study confirmed that Arthrobacter sp. UG50 has the ability to degrade anthracene and pyrene by co-metabolism with phenanthrene in liquid culture. Numerous studies on co-metabolic PAH degradation by Gram-positive bacteria, notably Sphingomonas and Mycobacterium species have been conducted, but information is lacking regarding the specific mechanism of co-metabolic PAH degradation by Arthrobacter spp. To acquire a more thorough understanding of the potential pathway of PAH degradation used by Arthrobacter sp. UG50, analysis of metabolites produced during co-metabolic degradation of HMW PAHs in the presence of phenanthrene would be valuable.

4.4. PAH BIODEGRADATION IN NOVA CHEMICALS REFINERY DISSOLVED AIR FLOATATION SLUDGE

The aim of this study was to evaluate the ability of Arthrobacter sp. UG50 cells to survive in and degrade PAH components present within NOVA Chemicals DAF sludge. Oily sludge consists of a complex mixture of alkanes, aromatics, asphaltene, nitrogen- sulfur- and oxygen-containing compounds (Atlas 1981). Due to the complex nature and variety of components present within the sludge, it is difficult for a single bacterial species to degrade all the compounds present (Van Hamme et al., 2003). The presence of toxic compounds at high concentrations and low oxygen levels in oily sludge can pose severe limitations on the effectiveness of PAH biodegradation in this environment (Machín-Ramírez et al., 2008). To reduce the toxic effect
that sludge contaminants can have upon indigenous microorganisms, the addition of a high cell
density inoculum can help to accelerate the initial biodegradation phase, thereby reducing the
toxicity on bacterial cells (Margesin and Schinner 1997).

To increase the potential for successful PAH degradation during treatment of DAF sludge
by Arthrobacter sp. UG50 and the natural microbial community, baffled flasks were used to
increase aeration and the delivery of oxygen to the cells. The availability of oxygen to bacterial
cells plays an important role in PAH degradation, as major aerobic pathways utilize oxygen and
oxygenases in the degradation process (Cerniglia 1992). Hydrocarbon-degrading bacteria act at
the oil-water interface, and by increasing the surface area of oil droplets through uniform mixing,
the potential for biodegradation is increased (Atlas 1981). Maximizing the aeration that occurs
within the shake flask system helps to enhance the exposure of bacteria to potential growth
substrates (nutrients and contaminants) and molecular oxygen, while minimizing the occurrence
of an anoxic environment to the cells (Zappi et al., 1996). Moreover, bioreactor based treatment
processes can increase the solid-liquid mass transfer ratio, thereby increasing the rate of
contaminant biodegradation (Yerushalmi et al., 2003; Ward et al., 2003).

The addition of Arthrobacter sp. UG50 to the DAF sludge enhanced the overall
degradation of petroleum hydrocarbons in the baffled flask compared to un-inoculated sludge.
However, it appeared that the DAF sludge itself has an efficient hydrocarbon-degrading
microbial population, as a decline in total hydrocarbon content was observed in the un-inoculated
flask containing only DAF sludge. The use of baffled flasks enhanced the extent of TPH
degradation, as levels in the flat bottomed flask remained constant following an initial decline in
the first 7 days of the experiment. In contrast, degradation in the baffled flask continued
throughout the 20 day experiment. Synergistic interactions between Arthrobacter sp. UG50 and
the indigenous microorganisms in the sludge may have enhanced the overall level of hydrocarbon
degradation. Abiotic losses may have contributed to the elimination of volatile components and LMW PAHs such as naphthalene, but exact levels were not measured.

The growth profile of *Arthrobacter* sp. UG50 as monitored by cell plating was similar to that of the indigenous bacteria present in the sludge in baffled flasks. Bacterial growth was enhanced in the baffled flasks compared to growth in the flat-bottomed flask with cell numbers increasing from $1.0 \times 10^5$ CFU/mL to $3.97 \times 10^6$ and $2.70 \times 10^6$ CFU/mL for the inoculated and uninoculated baffled flasks compared to an increase from $1.21 \times 10^5$ CFU/mL to only $6.45 \times 10^5$ CFU/mL in the flat bottomed flask. This indicates that microbial access to substrates, nutrients and oxygen was higher in the baffled flasks than in the flat bottomed flask. Although PAH concentrations in the sludge were very low, growth of both UG50 and the indigenous microorganisms were sustained, indicating that other compounds in the sludge may have been acting as carbon and energy sources. A slight increase in the number of DAF sludge bacteria present in the baffled flask containing UG50 cells was seen compared to the number of bacteria in the un-inoculated flask following 12 days of incubation. Co-metabolic degradation by UG50 may have enabled subsequent attack on substrates by other microorganisms present in the sludge, a process which may not have occurred without the enzymatic action of UG50. The availability of additional carbon sources to bacteria may have contributed to the increase in bacterial cell numbers seen in this treatment.

The largest extent of TPH degradation in each of the flasks occurred during the first 3 days of the experiment, with up to a 50% reduction in concentration. During the biodegradation of a complex mixture such as DAF sludge, compounds such as LMW aromatics and alkanes are among the first to be degraded by microorganisms, due to increased bioavailability (Johnsen *et al.*, 2005). HMW PAHs are more likely to be partially degraded through co-oxidation of the
compounds during microbial growth on more accessible forms of hydrocarbons present in the mixture (Atlas 1981).

Degradation analysis of individual PAHs within the treatment flasks shows that the LMW PAH naphthalene was degraded to undetectable levels within 3 days of DAF sludge treatment. The elimination of naphthalene and more accessible non-aromatic forms of carbon as growth substrates for microorganisms within the sludge may have contributed to the reduction in TPH degradation throughout the remainder of the experiment. The degradation of pyrene and chrysene slowed considerably following an initial 60% reduction in concentration after one day of incubation with UG50 in the baffled flask. Although pyrene, but not chrysene was shown to be degraded co-metabolically by UG50 in pure culture, bacteria in the sludge may also utilize this process to degrade these compounds. Since degradation of both compounds appeared to decline after 24 h, it is likely that the elimination of hydrocarbon growth substrates resulted in reduced co-metabolic activity, rather than bacteria being able to use these PAHs as sole carbon sources. Metabolic competition between substrates may have accounted for reduced rates of phenanthrene degradation by UG50 in the DAF sludge (Bouchez et al., 1995). The strain may have preferentially degraded less complex carbon sources present in the sludge and once those sources were expired, phenanthrene degradation was accelerated, as it was among the next least complex available substrates. This may also have accounted for the degradation profile of fluorene in the sludge, although the degradation of fluorene alone by UG50 in pure culture was not investigated.

Emulsifying activity by bacteria present in the sludge may have contributed to the overall level of hydrocarbon degradation. The first step in bacterial PAH degradation involves a membrane bound oxygenase, requiring bacteria to come in direct contact with the hydrocarbon substrate (Cerniglia 1992). To enhance the potential for bacterial-substrate interaction, biosurfactants are often produced by bacteria growing on petroleum mixtures (Ron & Rosenberg
An *Arthrobacter* strain, RAG-1 was reported to significantly emulsify Iranian crude oil, however the growth yield on 0.1% oil was found to be poor (Reisfeld *et al*., 1972). Poor growth was attributed to the strain utilizing only a small number of components present within the oil, or to partial oxidation where the strain utilized most of the components present, but only to a limited degree. The presence of other bacterial strains within an enrichment culture however, was found to delay the emulsifying activity of RAG-1 (Reisfeld *et al*., 1972). The emulsifying ability of bacteria during treatment of DAF sludge was not investigated. However, surfactant production by bacteria has been shown to effectively enhance the degradation of PAHs, and emulsifying compounds have been characterized from strains including members of *Pseudomonas*, *Arthrobacter* and *Acinetobacter* (Providenti *et al*., 1995; Reisfeld *et al*., 1972; Makkar & Rockne 2003).

Inoculum addition for the treatment and biodegradation of oily wastes and contaminated soils has been demonstrated in a number of studies. Di Gennaro *et al*. (2008) evaluated the effect of a phenanthrene degrading bacterial consortium and the addition of Tween 80 on the bioremediation of low level PAH-contaminated landfill soil. The use of the high cell density PAH-degrading microbial population ($10^9$ CFU/mL) improved the biodegradation of two- and three-ringed PAHs (initial concentrations: 0.337 to 5.15 mg/kg) as well as the co-metabolism of four-ringed PAHs such as pyrene (8.86 mg/kg). Levels decreased by over 90% after 28 days of treatment compared to a 40-50% reduction when only Tween 80 was added. The level of HMW PAH degradation after inoculum addition was not significantly different than a non-inoculated control, indicating that indigenous bacteria may have been responsible for their degradation (Di Gennaro *et al*., 2008). Another example is the petrozyme process, a bioreactor based system utilizing a mixed microbial consortium including strains of *Pseudomonas, Acinetobacter,*
Rhodococcus, and Alcaligenes (Ward et al., 2003). This system was able to degrade up to 99% TPH in oily sludges containing 10\% (w/v) TPH within 12 days (Van Hamme et al., 2003).

Gallego et al. (2006) reported on the isolation and use of a four membered consortium including strains of Pseudomonas, Nocardiodes, Acinetobacter and a yeast, Rhodotorula to degrade fractions of oil tank bottom sludge. Comparisons in the level of petroleum hydrocarbon degradation achieved by this consortium compared to that in a second group containing an additional four strains showed that biotransformation indices were lowered for all oil fractions except for linear alkanes when additional strains were present. Although the researchers did not investigate why this occurred, it was suggested that metabolic interactions between the microorganisms such as the accumulation of inhibitory compounds may have contributed to inhibition of degradation (Gallego et al., 2006).

4.5. CONCLUSIONS

Several PAH-degrading bacteria have been isolated from hydrocarbon contaminated landfarm soil and root material by enrichment with oily refinery effluent. One isolate, Arthrobacter sp. UG50, was capable of using phenanthrene and anthracene as sole carbon and energy sources. UG50 could degrade 200 mg/L of phenanthrene within 24 h at a high cell density (10^8 cells/mL) in pure culture. The strain degraded anthracene (50 mg/L) at a slower rate than phenanthrene, with 29\% being degraded within 21 days. UG50 was unable to utilize naphthalene, pyrene, chrysene or benzo(a)pyrene as sole carbon sources, but could degrade pyrene cometabolically when phenanthrene was present. The presence of phenanthrene was also found to enhance the degradation of anthracene, with 100\% of anthracene (50 mg/L) being degraded within 6 days in the presence of 200 mg/L phenanthrene. The ability of UG50 to degrade PAHs present within NOVA Chemicals refinery effluent in baffled and flat bottomed
flasks was also studied. The addition of UG50 cells to the sludge, combined with increased aeration resulted in an increase in total hydrocarbon degradation over 20 days compared to sludge alone. Degradation of fluorene, phenanthrene, pyrene and chrysene in DAF sludge was also enhanced in baffled flasks inoculated with $10^7$ UG50 cells/mL compared to flasks containing DAF sludge alone. These results indicate that oxygen availability is essential for successful biodegradation of oily waste and that UG50 cell addition could enhance PAH biodegradation in refinery sludges prior to landfarm disposal. Limited PAH degradation studies with Arthrobacter sp. have been conducted, and these results may lead to increased interest in studying the biodegradative potential of the genera.

4.6. RECOMMENDATIONS FOR FUTURE RESEARCH

Most sludge biodegradation studies utilize a consortium of microorganisms instead of an individual strain to maximize PAH degradation of complex mixtures. The potential benefit of including Arthrobacter sp. UG50 as part of a microbial consortium to degrade PAH-containing wastes from petrochemical refineries should be explored at the lab scale. Future studies on how PAH and TPH degradation by Arthrobacter sp. UG50 in oily sludge could be enhanced by biosurfactant or chemical surfactant addition is another potential area of interest, as only limited PAH degradation studies involving strains of Arthrobacter sp. have been conducted. In addition, evaluating the performance of UG50 cells in treating oily wastes at the pilot scale may be valuable in determining the feasibility of including the strain in a full scale bioreactor.

Variations in initial PAH concentrations in batches of DAF sludge can occur. The concentrations in the sludge used in this experiment were very low and were already below M.O.E guidelines for PAH levels in waste prior to landfarm disposal. The degradation of PAH compounds at low concentrations can be difficult (Atlas 1981; Johnsen et al., 2005). This can be
made more difficult by poor PAH dissolution rates, which can be exceeded by the metabolic demand of an increasing number of cells, leading to an equilibrium state where the substrate flux is consumed for cell maintenance (Johnsen et al., 2005). The addition of a high cell density inoculum can aid in overcoming the lowered potential for interactions between PAHs and bacterial cells. Future experiments to test the ability of UG50 to degrade PAHs present in higher concentrations in oily wastes could be of value. In particular, experiments conducted to test the maximum concentrations of PAHs capable of being degraded by UG50 would be valuable to further characterize the strain.

The use of bioreactor-based processes for the treatment of PAH-containing waste has significant advantages over landfarming including optimization of biodegradation parameters and reduced environmental contamination (Ward et al., 2003; Van Hamme et al., 2003). Enhancing the level of oxygen availability to cells during biodegradation can significantly enhance the rate and extent of total hydrocarbon and PAH biodegradation. The addition of Arthrobacter sp. UG50 cells appears to enhance the overall level of PAH degradation in DAF sludge when tested at the lab scale level, although the natural microbial community in the sludge is also capable of efficient degradation as seen in reductions of total hydrocarbon. Experiments to identify some of the microbial species present in the DAF sludge which are capable of PAH biodegradation would be valuable to a company such as NOVA Chemicals. In addition, the optimization of molecular techniques such as gene expression during PAH biodegradation in a bioreactor, could provide vital data to operators as a means of monitoring the activity of bacterial strains involved in PAH degradation.
APPENDIX: PAH DEGRADATION AND BACTERIAL CELL GROWTH DATA FOR EXPERIMENTS CONDUCTED IN MINIMAL MEDIA AND WITH NOVA CHEMICALS SLUDGE

Table A1: Hydrocarbon degradation in API oil-water separator effluent during shake-flask enrichment of PAH degrading microorganisms from NOVA Chemicals landfarm soil over 42 days. Original peak area values on day 0 were 9,857,000 for the flask containing root material, 8,998,000 for the flask containing composite soil, 8,637,000 for the flask containing field 6 soil and 12,491,000 for the flask containing API effluent alone.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Root Material + API Effluent</th>
<th>Composite Soil + API Effluent</th>
<th>Field 6 Soil + API Effluent</th>
<th>API Effluent Only</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 aA</td>
<td>100 aA</td>
<td>100 aA</td>
<td>100 aA</td>
</tr>
<tr>
<td>7</td>
<td>9.4 bA</td>
<td>19.6 bA</td>
<td>13.6 bA</td>
<td>75.1 bA</td>
</tr>
<tr>
<td>14</td>
<td>3.6 bA</td>
<td>17.0 bA</td>
<td>4.4 bA</td>
<td>12.1 bA</td>
</tr>
<tr>
<td>21</td>
<td>2.0 bA</td>
<td>4.5 bA</td>
<td>5.1 bA</td>
<td>11.9 bA</td>
</tr>
<tr>
<td>28</td>
<td>2.1 bA</td>
<td>2.2 bA</td>
<td>2.0 bA</td>
<td>7.5 bA</td>
</tr>
<tr>
<td>35</td>
<td>1.9 bA</td>
<td>2.5 bA</td>
<td>2.4 bA</td>
<td>5.8 bA</td>
</tr>
<tr>
<td>42</td>
<td>1.8 bA</td>
<td>2.2 bA</td>
<td>1.9 bA</td>
<td>2.5 bA</td>
</tr>
</tbody>
</table>

*Values with different lower case letters are significantly different from one another across rows and values with different capital letters are significantly different across columns (Tukey’s Test, p < 0.05).

Table A2: Biodegradation of 200 mg/L phenanthrene by NOVA Chemicals landfarm bacterial isolates over 9 days in Bushnell Haas medium with 0.67% (w/v) YNB (without amino acids).

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>C8</th>
<th>F7</th>
<th>G5</th>
<th>J3</th>
<th>Pseudomonas sp. UG14Lr</th>
<th>Abiotic Control</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100 ± 0.0*</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0</td>
<td>100 ± 0.0 dA</td>
<td>100 ± 0.0 eA</td>
</tr>
<tr>
<td>3</td>
<td>0 ± 0.0 aB</td>
<td>97.8 ± 5.91</td>
<td>96.1 ± 2.30</td>
<td>103.2 ± 2.88</td>
<td>68.6 ± 7.16 dB</td>
<td>94.6 ± 6.73 eB</td>
</tr>
<tr>
<td>6</td>
<td>0 ± 0.0 aC</td>
<td>85.6 ± 9.09</td>
<td>88.3 ± 2.50</td>
<td>89.3 ± 4.30</td>
<td>64.8 ± 4.28 dC</td>
<td>95.0 ± 5.18 eC</td>
</tr>
<tr>
<td>9</td>
<td>0 ± 0.0 aD</td>
<td>44.5 ± 6.97</td>
<td>35.0 ± 5.36</td>
<td>57.0 ± 1.10</td>
<td>54.4 ± 8.30 dD</td>
<td>86.2 ± 2.80 eD</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. *Means with different lower case letters are significantly different from one another across columns and means with different capital letters are significantly different across rows (Tukey’s Test, p < 0.05).
Table A3: Biodegradation of individual PAHs by 10^8 cells/mL *Arthrobacter* sp. UG50 over 21 days in Bushnell Haas medium with 0.67% (w/v) YNB (without amino acids).

<table>
<thead>
<tr>
<th>PAH</th>
<th>Percent PAH Remaining</th>
<th>Time (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>100±0(^*) aA</td>
<td>1.67±2.88 aB</td>
</tr>
<tr>
<td>(200 mg/L)</td>
<td>Anthracene</td>
<td>100±0 bA</td>
</tr>
<tr>
<td>(50 mg/L)</td>
<td>Pyrene</td>
<td>100±0 cA</td>
</tr>
<tr>
<td>(50 mg/L)</td>
<td>Chrysene</td>
<td>100±0 aC</td>
</tr>
<tr>
<td>(50 mg/L)</td>
<td>Benzo(a)pyrene</td>
<td>100±0 cA</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. *Means with different lower case letters are significantly different from one another across rows and means with different capital letters are significantly different across columns (Tukey’s Test, p < 0.05).

Table A4: Biodegradation of individual PAHs by 10^8 cells/mL *Arthrobacter* sp. UG50 over 24 hours in Bushnell Haas medium with 0.67% (w/v) YNB (without amino acids). The initial concentrations of PAHs in the media were 200 mg/L phenanthrene and 50 mg/L naphthalene.

<table>
<thead>
<tr>
<th>PAH</th>
<th>Percent PAH Remaining</th>
<th>Time (hours)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>Arthrobacter -</td>
<td>100±0(^*) aA</td>
<td>99±2.6 aB</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>Autoclaved Cell</td>
<td>100±0 aA</td>
</tr>
<tr>
<td>Control -</td>
<td>100±0 aA</td>
<td>97.9±11. 6 aB</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>Abiotic Control -</td>
<td>100±0 aA</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>Autoclaved Cell</td>
<td>100±0 aA</td>
</tr>
<tr>
<td>Control -</td>
<td>100±0 aA</td>
<td>95.9±3.4 aB</td>
</tr>
<tr>
<td>Phenanthrene</td>
<td>Abiotic Control -</td>
<td>100±0 aA</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. *Means with different lower case letters are significantly different from one another across rows and means with different capital letters are significantly different across columns (Tukey’s Test, p < 0.05).
Table A5: Growth of *Arthrobacter* sp. UG50 cells on phenanthrene or a binary mixture of selected PAHs in Bushnell Haas medium containing 0.67% (w/v) YNB (without amino acids). The initial concentrations of PAHs in the media were 200 mg/L phenanthrene, 50 mg/L anthracene, 50 mg/L pyrene, 50 mg/L chrysene and 50 mg/L benzo(a)pyrene.

<table>
<thead>
<tr>
<th>PAHs</th>
<th>Day 0</th>
<th>12 h</th>
<th>Day 3</th>
<th>Day 6</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td>4.7 X 10^8 ± 1.10* abA</td>
<td>5.1 X 10^9 ± 0.61 abB</td>
<td>3.5 X 10^9 ± 0.40 abC</td>
<td>2.6 X 10^9 ± 0.29 abD</td>
</tr>
<tr>
<td>Phenanthrene + Anthracene</td>
<td>2.1 X 10^8 ± 0.10 aA</td>
<td>3.5 X 10^9 ± 1.24 aB</td>
<td>4.4 X 10^9 ± 0.95 aC</td>
<td>6.0 X 10^9 ± 0.15 aD</td>
</tr>
<tr>
<td>Phenanthrene + Pyrene</td>
<td>1.97 X 10^8 ± 0.91 bA</td>
<td>4.4 X 10^9 ± 0.64 bB</td>
<td>3.7 X 10^9 ± 0.51 bC</td>
<td>2.9 X 10^9 ± 0.18 bD</td>
</tr>
<tr>
<td>Phenanthrene + Chrysene</td>
<td>4.7 X 10^8 ± 1.63 bA</td>
<td>4.3 X 10^9 ± 1.35 bB</td>
<td>3.2 X 10^9 ± 0.70 bC</td>
<td>2.5 X 10^9 ± 0.36 bD</td>
</tr>
<tr>
<td>Phenanthrene + Benzo(a)pyrene</td>
<td>4.6 X 10^8 ± 0.76 bA</td>
<td>4.4 X 10^9 ± 1.28 bB</td>
<td>3.4 X 10^9 ± 0.63 bC</td>
<td>2.5 X 10^9 ± 0.80 bD</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. *Means with different lower case letters are significantly different from one another across rows and means with different capital letters are significantly different across columns (Tukey’s Test, p < 0.05).
Table A6: Biodegradation of 200 mg/L phenanthrene in the presence of anthracene, pyrene, chrysene and benzo(a)pyrene (50 mg/L each) in Bushnell Haas medium with 0.67% (w/v) YNB (without amino acids) by *Arthrobacter* sp. UG50.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Percent Phenanthrene Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PHE</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 0.0*</td>
</tr>
<tr>
<td></td>
<td>aC</td>
</tr>
<tr>
<td>10h</td>
<td>1.9 ± 1.2 aB</td>
</tr>
<tr>
<td>1</td>
<td>0.0± 0.0 aA</td>
</tr>
<tr>
<td>2</td>
<td>0.0 ± 0.0 aA</td>
</tr>
<tr>
<td>3</td>
<td>0.0 ± 0.0 aA</td>
</tr>
<tr>
<td>4</td>
<td>0.0 ± 0.0 aA</td>
</tr>
<tr>
<td>5</td>
<td>0.0 ± 0.0 aA</td>
</tr>
<tr>
<td>6</td>
<td>0.0 ± 0.0 aA</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. Means with different lower case letter are significantly different across columns, and means with different capital letters are significantly different from one another across rows (p < 0.05).*
Table A7:  Biodegradation of anthracene (50 mg/L) in the presence of phenanthrene (200 mg/L) in Bushnell Haas medium with 0.67% (w/v) YNB (without amino acids) by Arthrobacter sp. UG50.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Percent Anthracene Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ANT Alone</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 0.00*&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>12h</td>
<td>100.0 ± 6.32&lt;sup&gt;aA&lt;/sup&gt;</td>
</tr>
<tr>
<td>1</td>
<td>86.7 ± 10.8&lt;sup&gt;bcA&lt;/sup&gt;</td>
</tr>
<tr>
<td>2</td>
<td>92.9 ± 11.1&lt;sup&gt;bA&lt;/sup&gt;</td>
</tr>
<tr>
<td>3</td>
<td>85.3 ± 9.95&lt;sup&gt;cA&lt;/sup&gt;</td>
</tr>
<tr>
<td>4</td>
<td>85.4 ± 9.40&lt;sup&gt;cA&lt;/sup&gt;</td>
</tr>
<tr>
<td>5</td>
<td>85.0 ± 4.68&lt;sup&gt;cA&lt;/sup&gt;</td>
</tr>
<tr>
<td>6</td>
<td>83.7 ± 6.09&lt;sup&gt;cA&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. Means with different lower case letter are significantly different across columns, and means with different capital letters are significantly different from one another across rows (p < 0.05).
Table A8: Biodegradation of pyrene (50 mg/L) in the presence of phenanthrene (200 mg/L) in Bushnell Haas medium with 0.67% (w/v) YNB (without amino acids) by *Arthrobacter* sp. UG50.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Percent Pyrene Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PYR</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 0.00*</td>
</tr>
<tr>
<td></td>
<td>aA</td>
</tr>
<tr>
<td>12h</td>
<td>100.2 ± 1.40</td>
</tr>
<tr>
<td></td>
<td>aA</td>
</tr>
<tr>
<td>1</td>
<td>95.9 ± 2.81</td>
</tr>
<tr>
<td></td>
<td>bA</td>
</tr>
<tr>
<td>2</td>
<td>90.6 ± 5.86</td>
</tr>
<tr>
<td></td>
<td>bcA</td>
</tr>
<tr>
<td>3</td>
<td>94.1 ± 0.23</td>
</tr>
<tr>
<td></td>
<td>bcA</td>
</tr>
<tr>
<td>4</td>
<td>88.6 ± 4.93</td>
</tr>
<tr>
<td></td>
<td>cdA</td>
</tr>
<tr>
<td>5</td>
<td>89.6 ± 1.27</td>
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<td></td>
<td>cdA</td>
</tr>
<tr>
<td>6</td>
<td>87.6 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>cA</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. Means with different lower case letter are significantly different across rows, and means with different capital letters are significantly different from one another across columns (p < 0.05).*
Table A9: Biodegradation of chrysene (50 mg/L) in the presence of phenanthrene (200 mg/L) in Bushnell Haas medium with 0.67% (w/v) YNB (without amino acids) by *Arthrobacter* sp. UG50.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Percent Chrysene Remaining</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CHR</td>
<td>CHR + PHE</td>
<td>Autoclaved Cell</td>
<td>Abiotic Control</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 0.00*</td>
<td>100 ± 0.00</td>
<td>100 ± 0.00</td>
<td>100.0 ± 0.00</td>
</tr>
<tr>
<td></td>
<td>aA</td>
<td>aA</td>
<td>aA</td>
<td>aB</td>
</tr>
<tr>
<td>12h</td>
<td>96.3 ± 1.62</td>
<td>91.1 ± 2.12</td>
<td>93.4 ± 1.10</td>
<td>93.2 ± 3.73</td>
</tr>
<tr>
<td></td>
<td>aA</td>
<td>aA</td>
<td>aA</td>
<td>aB</td>
</tr>
<tr>
<td>1</td>
<td>86.4 ± 7.65</td>
<td>86.4 ± 3.55</td>
<td>84.1 ± 4.41</td>
<td>92.0 ± 0.18</td>
</tr>
<tr>
<td></td>
<td>bA</td>
<td>bA</td>
<td>bA</td>
<td>bB</td>
</tr>
<tr>
<td>2</td>
<td>78.5 ± 16.24</td>
<td>79.6 ± 1.79</td>
<td>73.8 ± 9.56</td>
<td>89.3 ± 0.99</td>
</tr>
<tr>
<td></td>
<td>bcA</td>
<td>bcA</td>
<td>bcA</td>
<td>bcB</td>
</tr>
<tr>
<td>3</td>
<td>77.6 ± 10.9</td>
<td>74.7 ± 2.55</td>
<td>72.7 ± 4.09</td>
<td>85.3 ± 0.12</td>
</tr>
<tr>
<td></td>
<td>bcA</td>
<td>bcA</td>
<td>bcA</td>
<td>bcB</td>
</tr>
<tr>
<td>4</td>
<td>71.4 ± 10.03</td>
<td>68.2 ± 0.56</td>
<td>73.0 ± 12.23</td>
<td>78.3 ± 0.28</td>
</tr>
<tr>
<td></td>
<td>cA</td>
<td>cA</td>
<td>cA</td>
<td>cB</td>
</tr>
<tr>
<td>5</td>
<td>70.2 ± 13.7</td>
<td>65.9 ± 1.32</td>
<td>71.6 ± 15.60</td>
<td>78.8 ± 1.60</td>
</tr>
<tr>
<td></td>
<td>cA</td>
<td>cA</td>
<td>cA</td>
<td>cB</td>
</tr>
<tr>
<td>6</td>
<td>70.5 ± 9.76</td>
<td>67.8 ± 12.5</td>
<td>68.6 ± 7.01</td>
<td>74.5 ± 4.13</td>
</tr>
<tr>
<td></td>
<td>cA</td>
<td>cA</td>
<td>cA</td>
<td>cB</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. Means with different lower case letter are significantly different across rows, and means with different capital letters are significantly different from one another across columns (p < 0.05).*
Table A10: Biodegradation of benzo(a)pyrene (50 mg/L) in the presence of phenanthrene (200 mg/L) in Bushnell Haas medium with 0.67% (w/v) YNB (without amino acids) by *Arthrobacter* sp. UG50.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>Percent Benzo(a)pyrene Remaining</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>BAP</td>
</tr>
<tr>
<td>0</td>
<td>100 ± 0.00* aA</td>
</tr>
<tr>
<td>12h</td>
<td>95.2 ± 9.03 abA</td>
</tr>
<tr>
<td>1</td>
<td>84.1 ± 7.70 abA</td>
</tr>
<tr>
<td>2</td>
<td>82.7 ± 11.44 bcA</td>
</tr>
<tr>
<td>3</td>
<td>89.0 ± 3.70 bcA</td>
</tr>
<tr>
<td>4</td>
<td>88.3 ± 3.76 bcA</td>
</tr>
<tr>
<td>5</td>
<td>87.1 ± 2.57 bcA</td>
</tr>
<tr>
<td>6</td>
<td>84.8 ± 8.56 cA</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. Means with different lower case letter are significantly different across rows, and means with different capital letters are significantly different from one another across columns (p < 0.05).
Table A11: Total hydrocarbon content in Dissolved Air Floatation sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 in baffled or flat bottomed flasks incubated for 20 days.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>DAF Sludge Only Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Flat-bottomed Flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>747.8 ± 23.1* aA</td>
<td>615.0 ± 33.6 bB</td>
<td>737.9 ± 41.9 aA</td>
</tr>
<tr>
<td>1</td>
<td>603.1 ± 35.0 aA</td>
<td>529.9 ± 34.3 aB</td>
<td>535.3 ± 45.5 aA</td>
</tr>
<tr>
<td>2</td>
<td>509.4 ± 26.6 bA</td>
<td>413.3 ± 88.3 bB</td>
<td>506.4 ± 123.5 bA</td>
</tr>
<tr>
<td>3</td>
<td>603.1 ± 35.4 aA</td>
<td>419.2 ± 28.3 aB</td>
<td>528.2 ± 110.1 aA</td>
</tr>
<tr>
<td>4</td>
<td>528.9 ± 109.7 bcA</td>
<td>380.4 ± 145.4 bcB</td>
<td>403.2 ± 120.5 bcA</td>
</tr>
<tr>
<td>5</td>
<td>554.5 ± 63.8 bcA</td>
<td>391.7 ± 135.6 bcB</td>
<td>385.5 ± 37.7 bcA</td>
</tr>
<tr>
<td>6</td>
<td>555.8 ± 149.5 bcA</td>
<td>372.6 ± 21.7 bcB</td>
<td>416.2 ± 92.2 bcA</td>
</tr>
<tr>
<td>7</td>
<td>482.2 ± 114.5 bcA</td>
<td>370.8 ± 77.9 bcB</td>
<td>367.8 ± 56.9 bcA</td>
</tr>
<tr>
<td>12</td>
<td>400.8 ± 96.9 bcA</td>
<td>333.2 ± 65.9 bcB</td>
<td>424.7 ± 30.9 bcA</td>
</tr>
<tr>
<td>16</td>
<td>322.5 ± 26.9 bcA</td>
<td>257.4 ± 50.8 bcB</td>
<td>427.9 ± 36.4 bcA</td>
</tr>
<tr>
<td>20</td>
<td>238.5 ± 23.0 cA</td>
<td>216.9 ± 68.8 cB</td>
<td>385.8 ± 71.2 cA</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. Means with different lower case letter are significantly different across rows, and means with different capital letters are significantly different from one another across columns (p < 0.05).

Table A12: Biodegradation of naphthalene in Dissolved Air Floatation sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 in baffled or flat-bottomed flasks over 5 days.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>DAF Sludge Only Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Flat-bottomed Flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0* aA</td>
<td>100.0 aA</td>
<td>100.0 aA</td>
</tr>
<tr>
<td>1</td>
<td>3.9  bA</td>
<td>4.2  bA</td>
<td>51.3 bA</td>
</tr>
<tr>
<td>2</td>
<td>2.6  bA</td>
<td>2.8  bA</td>
<td>1.7  bA</td>
</tr>
<tr>
<td>3</td>
<td>1.3  bA</td>
<td>2.1  bA</td>
<td>2.6  bA</td>
</tr>
<tr>
<td>4</td>
<td>2.0  bA</td>
<td>4.2  bA</td>
<td>1.7  bA</td>
</tr>
<tr>
<td>5</td>
<td>2.6  bA</td>
<td>2.1  bA</td>
<td>2.6  bA</td>
</tr>
</tbody>
</table>

*Values with different lower case letter are significantly different across rows, and values with different capital letters are significantly different from one another across columns (p < 0.05).*
Table A13: Biodegradation of fluorene in Dissolved Air Floatation sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 in baffled or flat-bottomed flasks over 5 days.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>DAF Sludge Only Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Flat-bottomed Flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0* aA</td>
<td>100.0 aA</td>
<td>100.0 aA</td>
</tr>
<tr>
<td>1</td>
<td>91.4 aA</td>
<td>69.8 aA</td>
<td>93.1 aA</td>
</tr>
<tr>
<td>2</td>
<td>72.8 abA</td>
<td>69.8 abA</td>
<td>ND*</td>
</tr>
<tr>
<td>3</td>
<td>84.3 abcA</td>
<td>42.9 abcA</td>
<td>74.2 abcA</td>
</tr>
<tr>
<td>4</td>
<td>58.6 bcA</td>
<td>42.9 bcA</td>
<td>48.2 bcA</td>
</tr>
<tr>
<td>5</td>
<td>52.9 bcA</td>
<td>27.0 bcA</td>
<td>39.7 bcA</td>
</tr>
</tbody>
</table>

*Values with different lower case letter are significantly different across rows, and values with different capital letters are significantly different from one another across columns (p < 0.05).

*ND: No data

Table A14: Biodegradation of phenanthrene in Dissolved Air Floatation sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 in baffled or flat-bottomed flasks over 5 days.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>DAF Sludge Only Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Flat-bottomed Flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0* aA</td>
<td>100.0 aB</td>
<td>100.0 aA</td>
</tr>
<tr>
<td>1</td>
<td>94.0 aA</td>
<td>90.5 aB</td>
<td>89.2 aA</td>
</tr>
<tr>
<td>2</td>
<td>75.3 bA</td>
<td>73.8 bB</td>
<td>ND*</td>
</tr>
<tr>
<td>3</td>
<td>100.0 bA</td>
<td>46.4 bB</td>
<td>77.1 bB</td>
</tr>
<tr>
<td>4</td>
<td>69.7 bA</td>
<td>38.1 bB</td>
<td>66.2 bB</td>
</tr>
<tr>
<td>5</td>
<td>84.3 bA</td>
<td>44.0 bB</td>
<td>76.0 bB</td>
</tr>
</tbody>
</table>

*Values with different lower case letter are significantly different across rows, and values with different capital letters are significantly different from one another across columns (p < 0.05).

*ND: No data
Table A15:  Biodegradation of pyrene in Dissolved Air Floatation sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 in baffled or flat-bottomed flasks over 5 days.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>DAF Sludge Only Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Flat-bottomed Flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0* aA</td>
<td>100.0 aB</td>
<td>100.0 aA</td>
</tr>
<tr>
<td>1</td>
<td>100.0 aA</td>
<td>36.4 aB</td>
<td>81.8 aA</td>
</tr>
<tr>
<td>2</td>
<td>81.9 aA</td>
<td>63.6 aB</td>
<td>ND*</td>
</tr>
<tr>
<td>3</td>
<td>100.0 aA</td>
<td>45.5 aB</td>
<td>81.8 aA</td>
</tr>
<tr>
<td>4</td>
<td>72.7 aA</td>
<td>ND</td>
<td>72.7 aA</td>
</tr>
<tr>
<td>5</td>
<td>90.9 aA</td>
<td>45.4 aB</td>
<td>90.9 aA</td>
</tr>
</tbody>
</table>

*Values with different lower case letter are significantly different across rows, and values with different capital letters are significantly different from one another across columns (p < 0.05). ND: No data.

Table A16:  Biodegradation of chrysene in Dissolved Air Floatation sludge inoculated with $10^7$ cells/mL of *Arthrobacter* sp. UG50 in baffled or flat-bottomed flasks over 5 days.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>DAF Sludge Only Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Flat-bottomed Flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>100.0* aA</td>
<td>100.0 aB</td>
<td>100.0 aA</td>
</tr>
<tr>
<td>1</td>
<td>100.0 aA</td>
<td>33.3 aB</td>
<td>80.0 aA</td>
</tr>
<tr>
<td>2</td>
<td>81.8 aA</td>
<td>55.5 aB</td>
<td>80.0 aA</td>
</tr>
<tr>
<td>3</td>
<td>81.8 aA</td>
<td>44.4 aB</td>
<td>80.0 aA</td>
</tr>
<tr>
<td>4</td>
<td>54.5 aA</td>
<td>ND*</td>
<td>70.0 aA</td>
</tr>
<tr>
<td>5</td>
<td>54.5 aA</td>
<td>33.3 aB</td>
<td>100.0 aA</td>
</tr>
</tbody>
</table>

*Values with different lower case letter are significantly different across rows, and values with different capital letters are significantly different from one another across columns (p < 0.05). ND: No data.
Table A17:  *Arthrobacter* sp. UG50 cell growth during biodegradation of NOVA Chemicals Dissolved Air Floatation sludge over 20 days.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge (Baffled Flask)</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge (Flat-bottomed Flask)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.97 X 10^7 ± 3.06 X 10^6* acA</td>
<td>1.19 X 10^7 ± 1.97 X 10^6 aB</td>
</tr>
<tr>
<td>3</td>
<td>5.03 X 10^7 ± 1.68 X 10^7 acA</td>
<td>1.44 X 10^7 ± 3.24 X 10^6 acB</td>
</tr>
<tr>
<td>6</td>
<td>1.14 X 10^8 ± 5.20 X 10^7 acA</td>
<td>1.74 X 10^7 ± 7.51 X 10^6 acB</td>
</tr>
<tr>
<td>12</td>
<td>3.03 X 10^8 ± 5.51 X 10^7 bcA</td>
<td>3.40 X 10^7 ± 8.19 X 10^6 bcB</td>
</tr>
<tr>
<td>16</td>
<td>4.40 X 10^8 ± 1.47 X 10^8 bA</td>
<td>3.83 X 10^7 ± 1.80 X 10^7 bA</td>
</tr>
<tr>
<td>20</td>
<td>1.77 X 10^9 ± 1.08 X 10^8 cA</td>
<td>3.40 X 10^7 ± 1.40 X 10^7 cB</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. Means with different lower case letter are significantly different across rows, and means with different capital letters are significantly different from one another across columns (p < 0.05).

Table A18:  Indigenous bacterial cell growth during biodegradation of NOVA Chemicals Dissolved Air Floatation sludge over 20 days.

<table>
<thead>
<tr>
<th>Time (days)</th>
<th>DAF Sludge Only Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Baffled Flask</th>
<th><em>Arthrobacter</em> sp. UG50 + DAF Sludge Flat-bottomed Flask</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>1.28 X 10^3 ± 1.06 X 10^4 aA</td>
<td>1.43 X 10^3 ± 1.21 X 10^4 abA</td>
<td>1.21 X 10^3 ± 1.93 X 10^4 aC</td>
</tr>
<tr>
<td>3</td>
<td>4.59 X 10^4 ± 4.95 X 10^3 abA</td>
<td>4.95 X 10^5 ± 5.94 X 10^4 abB</td>
<td>2.16 X 10^7 ± 8.36 X 10^4 abC</td>
</tr>
<tr>
<td>6</td>
<td>1.45 X 10^6 ± 2.73 X 10^5 bA</td>
<td>1.09 X 10^6 ± 3.67 X 10^5 bB</td>
<td>4.17 X 10^5 ± 1.70 X 10^3 bC</td>
</tr>
<tr>
<td>12</td>
<td>2.40 X 10^6 ± 4.24 X 10^5 cA</td>
<td>3.47 X 10^6 ± 9.26 X 10^5 cB</td>
<td>5.43 X 10^7 ± 3.59 X 10^7 cC</td>
</tr>
<tr>
<td>16</td>
<td>2.70 X 10^6 ± 1.27 X 10^6 cA</td>
<td>3.97 X 10^6 ± 2.08 X 10^6 cB</td>
<td>6.73 X 10^5 ± 2.71 X 10^3 cC</td>
</tr>
<tr>
<td>20</td>
<td>2.70 X 10^6 ± 2.83 X 10^5 cA</td>
<td>3.84 X 10^6 ± 1.74 X 10^6 cB</td>
<td>6.45 X 10^5 ± 3.14 X 10^3 cC</td>
</tr>
</tbody>
</table>

*Mean (n=3) ± standard deviation. Means with different lower case letter are significantly different across rows, and means with different capital letters are significantly different from one another across columns (p < 0.05).
REFERENCES


Amoco Oil Company. 1989. *Operating plan for closure of the sludge pond pit and wastewater treatment lagoon as a single waste management unit*. Report submitted to the Missouri Department of Natural Resources and EPA Region VII. Chicagom Ill.: Amoco Oil Company Sugar Creek, Mich.


