

BIODEGRADATION OF COMPLEX AROMATIC COMPOUNDS IN NUCLEAR PROCESS WATER

By

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A dissertation submitted in partial fulfilment of the requirement for the degree of

MASTER OF SCIENCE: MICROBIOLOGY

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April 2010

Declaration

I, **PHUMZA VUYOKAZI TIKILILI**, hereby declare that all the work provided in this dissertation is to the best of my knowledge original (except where cited) and that neither the whole work nor any part of it has been, or is to be submitted for another degree at this or any other University or tertiary education institution or examining body.

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Dedication

This dissertation is dedicated to

My family

My late father who always encouraged me to further my studies and supported me in every way he could

My mother for her ongoing support, understanding and patience and the opportunity she gave me to do this degree

My daughter Lerato Phiwokuhle Tikilili, whom I owe so much for her patience and unconditional love.

My sister Ncumisa for her support and encouragement throughout this degree

My brothers, Simfumene, Akhona and Wonderboy for their understanding during my studies

A special friend Sihle Zungu who listens and always been there for me when I needed to talk to someone.

Acknowledgements

I would like to thank God Almighty for courage, strength blessings and wisdom that He gave me throughout this Degree. Special thanks are extended to my study leader Professor Evans. M. N. Chirwa for the guidance, mentorship, motivation and advice he provided me throughout the study. Particular thanks to Professor Fanus Venter from the Department of Microbiology for his assistance with the characterization of bacterial isolates. National Research Foundation of South Africa (NRF) and SANHARP are greatly acknowledged for financial assistance throughout the study. Many thanks to my colleagues and friends who although not mentioned by name, provided invaluable advice that contributed greatly to the final quality of the work on which this dissertation is based.

ABSTRACT

Nuclear energy generation results in the production of effluents and radioactive waste that are very difficult to treat and dispose. A considerable fraction of nuclear waste is discharged in the form of complex mixtures of hazardous organic compounds and metallic radionuclides.

The most serious pollution is caused by polycyclic aromatic hydrocarbons (PAHs) and polychlorinated biphenyls that are very difficult to remove from the environment. The nuclear industry faces certain challenges related to treatment and safe disposal of these mixed radioactive organic wastes due to the toxicity and recalcitrant nature of the organics.

Techniques currently used in treating the waste include physical-chemical processes that have resulted in the generation of the secondary waste requiring further treatment before disposal to the environment. These conventional processes also require the use of strong oxidising agents and higher than natural pH and temperature. Therefore, it is of great importance to develop new environmentally friendly technologies. One suggested method employs specialised cultures of bacteria to completely mineralize the organic compounds without leaving traces of harmful by-products.

The efficiency of bacteria to remove these types of compounds may be improved by *in situ* application. During *in situ* application, the bacteria apply a variety of pathways to break down the compounds and use them as their energy and carbon sources. These processes may be carried out within the natural pH and temperature range capable of supporting life forms. In the current study, a more detailed analysis of the biodegradation capability of the organic compounds was conducted and the following were the major findings of the study:

- Wastewater from an actual radionuclide processing facility was characterised and was found to contain all the 16 priority PAHs in the range 0.001-25 mg/L. Acenaphthene (detected at 25.1 mg/L) was the most abundant. Most of the PAHs in the wastewater samples exceeded the WHO limit of 0.05µg/L indicating the need for further treatment before final disposal to the environment.
- After purifying and sequencing the rRNA genes from the soil and mine water bacteria, a total of 5 and 3 bacterial isolates were found, respectively. The rRNA sequences were isolated from bacteria with some tolerance to PAH toxicity and were thus candidate species for naphthalene degradation. The bacteria from soil were predominated by

aromatic compound degraders *Pseudomonas aeruginosa*, *Microbacterium esteraromaticum* and *Alcaligenes* sp. In mine water, only *Pseudomonas putida* was identified as a known aromatic ring cleaving species.

- The biodegradation of naphthalene by the purified cultures was determined to be limited by its solubility (30mg/L) and toxic effects of the aromatic compounds. A kinetic model was derived based on the metabolism and microbial growth kinetics. The model predicted the concentration remaining in solution under different initial (added) PAH concentrations.

A simplified coupled dissolution-degradation model was used to model the kinetics of degradation. With help of the model, parameters were estimated and the sensitivity of parameter value was also evaluated. The aim of model was to help gain a better understanding of biological degradation. This could be used for optimisation of the process and scale up of the process to pilot and full-scale application.

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List of Abbreviations

ACE	Acenaphthene
ACY	Acenaphthylene
AN	Anthracene
APHA	American public health agency
BaA	Benzo (a)Anthracene
BaP	Benzo (a)Pyrene
BbF	Benzo(b)Fluoranthene
BkF	Benzo(k)Fluoranthene
BP	Benzo(ghi)Perylene
CaCl ₂	Calcium chloride
CH	Chrysene
CO ₂	Carbon dioxide
Conc	Concentration
CFU	Colony forming units
CSIR	Council for Scientific and Industrial Research
CoCl ₂	Cobalt chloride
CuCl ₂	Copper chloride
DA	Dibenzo(ah)Anthracene
DNA	Deoxyribonucleic acid
EI	Electron impact
FeSO ₄	Iron sulphate
FID	Flame ionization detection
FL	Fluorene
FLR	Fluoranthene
GC-MS	Gas Chromatography/ Mass Spectrometry
H ₃ BO ₃	Boric acid
HEF	High efficiency filtration
HMW	High Molecular Weight
HLW	High level waste

HPLC	High Performance Liquid Chromatography
IAEA	International Atomic Energy Agency
IARC	International Agency for Research on Cancer
ILW	Intermediate level waste
IP	Indeno(1,2,3-cd)pyrene
KH ₂ PO ₄	Potassium dihydrogen phosphate
KI	Potassium Iodide
L	Litre
LC	Liquid chromatography
LLW	Low level waste
LMW	Low molecular Weight
LOD	Limit of detection
LOQ	Limit of quantification
mg/L	milligrams per liter
MgSO ₄	Magnesium sulphate
MnCl ₂	Manganese chloride
MSD	mass spectrometry detector
MSM	Mineral salt medium
NaBr	Sodium bromide
NaCl ₂	Sodium chloride
Na ₂ HPO ₄	Sodium hydrogen phosphate
Na ₂ MoO ₂	Sodium molybdomate
Na ₂ SO ₄	Sodium sulphate
NCBI	National Centre for Biotechnology Information
NiCl ₂	Nickel chloride
PAH	Polynuclear/polycyclic aromatic hydrocarbons
PBMR	Pebble Bed Modular Reactor
PCB	Poly-chlorinated biphenyls
PDA	PhotoDiode Array
PH	Phenanthrene
Qa	Quantity added

Qd	Quantity detected
rDNA	Ribosomal deoxyribonucleic acid
rRNA	Ribosomal Ribonucleic acid
RT-PCR	Reverse transcriptase- Polymerase chain reaction
SFC	Supercritical fluid chromatography
SPE	Solid phase extraction
TCA	Tricarboxylic Acid
TLC	Thin layer chromatography
US EPA	United States Environmental Protection Agency
UVD	Ultraviolet detection
WHO	World Health Organization
ZnCl ₂	Zinc chloride

Symbol Nomenclature

C	PAH total concentration in aqueous phases (mgL^{-1})
C_s	solid (undissolved) PAH concentration (mgL^{-1})
D	diffusion coefficient ($\text{m}^2 \text{h}^{-1}$)
K_i	inhibition constant (mgL^{-1})
k_s	mass transfer coefficient (mh^{-1})
K_m	Monod constant (mgL^{-1})
K_c	saturation constant (mgL^{-1})
I	inhibitor concentration (mgL^{-1})
N	flux ($\text{mgm}^{-2} \text{h}^{-1}$)
K_1	rate of consumption of substrate ($\text{mgL}^{-1} \text{h}^{-1}$)
K_2	rate of dissolution ($\text{mgL}^{-1} \text{h}^{-1}$)
S	substrate concentration (mgL^{-1})
t	time (h)
X	biomass concentration (mgL^{-1})
δ	film thickness (m)
μ	specific growth rate (h^{-1})
$\mu_{\max}$	maximum specific growth rate (h^{-1})
χ^2	Chi square

CHAPTER 1: INTRODUCTION

1.1 Research Background

Nuclear energy is an important component of the world's energy supply. Globally, 17% of the overall electricity supply comes from nuclear power. To address the problem of increasing energy demand with the rapid increase of the world population, nuclear energy is becoming more and more important as an alternative energy source (Purushotham *et al*, 2000). However, the major draw back of nuclear energy generation is the production of substantial amounts of radioactive waste discharged as a mixture of metallic radionuclides and refractory organic compounds (Ismagilov *et al*, 2000). Typical radioactive waste generating activities include power generation, radioisotope manufacturing, and medical research. Other than the nuclear industry, radioactive wastes are also produced by non-nuclear activities such as processing of raw materials containing naturally occurring radionuclides with low levels of radiation, research facilities, and laundry facilities for the radiation research laboratories (IAEA, 1994). Nuclear waste generated from the above activities is usually toxic and not easily degradable by mesophilic bacteria in conventional wastewater treatment plants. The organic component is typically comprised of polycyclic aromatic compounds (PAHs) and chlorinated biphenyls from process water and surfactants from laundry wastewater.

Although primarily focused on organics from the nuclear industry, this study also benefits the remediation of organic pollution from other conventional industries such as the petrochemical industry and manufacturing industry. The organics, especially PAHs, must be treated to prevent exposure to humans. Many PAHs have toxic, mutagenic and carcinogenic properties to mammals including humans. Additionally, PAHs are highly lipid-soluble and are readily absorbed from the lung, gut and skin of mammals. However, inhaled PAHs are predominantly adsorbed on soot particles. After deposition in the airways, the particles can be eliminated by bronchial clearance. PAHs might be partially removed from the particles during transport on the ciliated mucosa and may penetrate into the bronchial epithelium cells where metabolism takes place.

Absorption through human skin has also been demonstrated. Irrespective of the route of administration PAHs are rapidly and widely distributed in the organism with a marked tendency for localization in body fat. Mammary and other fatty tissues are significant storage depots for PAHs, but owing to the rapid metabolism no significant accumulation seems to take place. The gastrointestinal tract is another avenue of entry as it contains relatively high levels of metabolites as a result of hepatobiliary excretion. For this reason many PAHs are considered toxic with detrimental effect to flora and fauna of affected habitats, resulting in the uptake and accumulation of toxic chemicals in food chains and in serious health problems and/or genetic defects in humans (Samanta *et al*, 2002).

Microorganisms can mineralize toxic polycyclic aromatic hydrocarbons into carbon dioxide and water, and microbial transformation is considered a major route for complete degradation of these components (Okpokwasili and Nweke, 2005).

1.2 Aim and Objectives

The principal aim of this study was to evaluate the ability of microorganisms to degrade complex aromatic hydrocarbons that are found in the nuclear waste and radioactive waste streams. In order to achieve the main objective the following specific objectives were performed.

1. Characterisation of radioactive wastewater for the presence of polycyclic aromatic hydrocarbons (PAHs).
2. Isolation of bacteria from different contaminated sources i.e. landfill soil and mine water.
3. To determine PAH degradation potential of indigenous species.
4. Determination of degradation rate kinetics of the PAHs using the isolated bacterial species.

1.3 Main findings

The main finding of this study was that biodegradation of naphthalene by indigenous cultures was limited by its solubility in water.

CHAPTER 2: LITERATURE REVIEW

2.1 Environmental Impacts from Energy production

Exhaustible fossil fuels represent 80% of the total world energy supply. At present most of the world's energy supply comes from fossil and nuclear sources. Fossil fuels include coal, peat, petroleum, oil and natural gases (Schaffer and Juncosa, 1999). With continuous production and consumption, the currently used reserves of oil will last around 41 years, natural gas 64 years, and coal 155 years. These projections explain why fossil fuels cannot be regarded as the world's main source of energy for more than one or two generations. Besides the issue of depletion, the use of fossil fuels also represents serious environmental consequences. Fossil fuel consumption has been determined to be the main driver of the current high CO₂ levels in the atmosphere. Fossil fuel reserve exploitation is expected to increase as reserves approach exhaustion and as more expensive technologies are used to explore and obtain less attractive resources. The problem of global warming and concerns about carbon dioxide emissions have necessitated the development of alternative 'clean' energy sources, which do not depend on fossil fuels and which have a tolerable environmental impact (Dresselhaus and Thomas, 2001). Among these, nuclear energy is prominent as the most viable transitional energy source as we search for other alternatives in the next 50 to 70 years.

2.2 Wastes from the Nuclear Industry

Nuclear energy, meets the criteria of environmental compatibility and resource independence. There is insignificant emission of greenhouse gasses in the fission process and from the perspective of global warming, nuclear energy offers a more environmentally viable alternative to fossil fuels. However, because of community fears of nuclear accidents and proliferation of weapons grade uranium, a relatively small amount of nuclear power plants have been built worldwide in comparison to coal powered stations.

In order for nuclear energy to be widely accepted, nuclear reactors should be made safe and the problem of nuclear waste disposal must be solved. It is also crucial to understand the effect of radiation on the materials within operating reactors in order to

extend reactor lifetime. For nuclear power to play a significant role in addressing security and environmental concerns, countries must build new reactors to replace those ending their service life and to expand significantly the number of commercial reactors in service. In response to these suggestions major nuclear energy organisations have embarked on research to develop advanced reactors that offer both improved safety and lower environmental cost. Among these reactors is the generation IV gas cooled (fast) reactors such as the pebble bed modular reactor (PBMR) system being developed in South Africa (Nicholls, 2000).

The main limitation in the development of Gas cooled reactors is the production of substantial amounts of long-lived radioactive wastes. In the HTGR, waste is generated in the of expired graphite. Additionally, radioactive waste is also generated at various stages of the nuclear fuel cycle, from the mining and milling of uranium ore, fuel fabrication, reactor operation and spent fuel reprocessing (Figure 2-1).

Most nuclear power generating countries have embarked on the recycling strategy, where valuable fissile materials (uranium) contained in the spent fuel are recovered and reused in new nuclear fuels. In recent research it has been shown that efficient fissile materials recovery from spent fuel reduces the radiotoxicity of the final waste by a factor of 20 to 30 (Gautrot and Pradel, 1998).

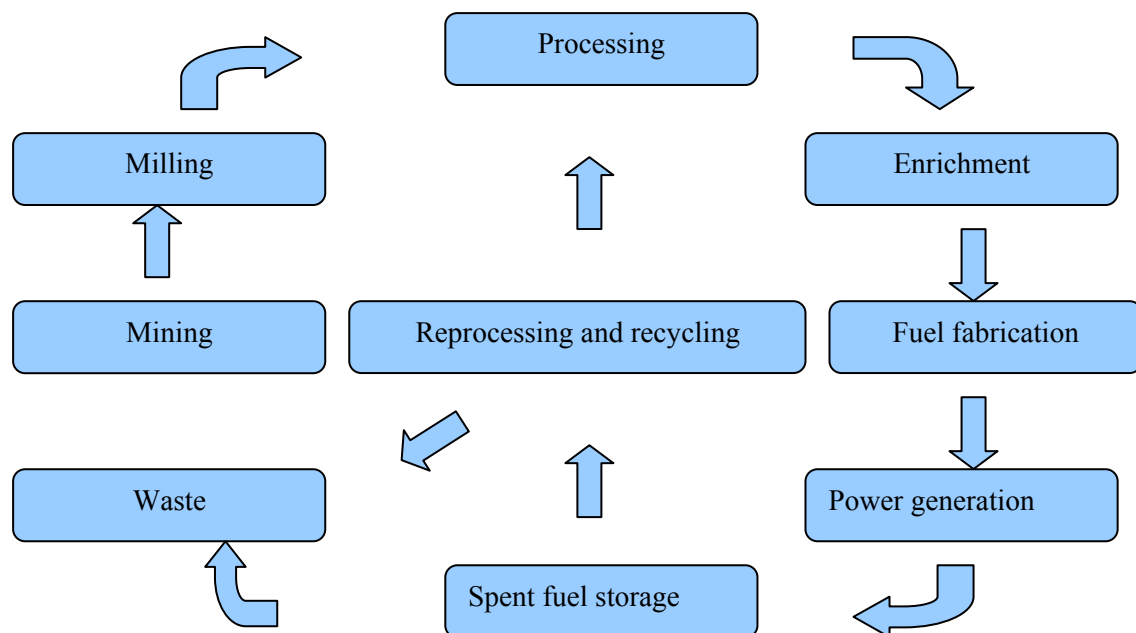


Figure 2-1: Nuclear fuel cycle

In conventional application of the nuclear technology, such medical and research, a considerable amount of radioactive waste that is produced consists of a complex mixture of hazardous organic compounds and metallic radionuclides (Ismagilov *et al*, 2000). Organic components of the waste are very heterogenous, it can occur in solid, liquid and less frequently gaseous form (IAEA, 2004) (Figure 2-2). The components of organic waste may include lubricating and hydraulic fluids, extractants, solvents, filters, ion exchange resins, plastic containers, work clothing and other organic material and compounds (Ismagilov *et al*, 2000). For this reason, agencies that handle and manage nuclear waste face the challenge of safe treatment and disposal of these mixed radioactive organic wastes. Processes to separate radionuclides from the waste have been developed (Doherty *et al*, 1989). However, methods for degrading the organic components are still in their infancy.

In view of a large variety of radioactive wastes being generated world wide, processes used for their treatment are also diverse (Figure 2-2). The techniques currently in use for the treatment of radioactive organic waste are mainly physical-chemical in nature (IAEA, 2004). The major problem with these techniques is that they generate secondary waste that also becomes a threat to the environment. Therefore, it is of great importance to develop new environmentally friendly methods for treating these wastes. Among the various proposed cleaner alternatives to chemical oxidation is the treatment using (Tusa, 1989). Microbial treatment aims at complete mineralization of organics thereby achieving large volume reduction.

CHARACTERIZATION		TREATMENT			CONDITIONING
LL	Liquid	LIQUID	SOLID WASTE	GASEOUS WASTE	Cementation
		Chemical	Compaction	Scrubbing	Polymerization
IL	Solid	Ion exchange	Incineration	Adsorption	Bituminization
HL	Gas	Reverse osmosis	Size	Prefiltration	Bituminization
		Evaporation	Repackaging	High efficiency Filtration	Vitrification

Figure 2-2: Common nuclear waste management practices. LL= low level, IL=intermediate level, HL=high level and H.E.F=high efficiency filtration. (Source: Raj *et al*. 2006).

2.3 Treatment options for radioactive organic waste

The existing techniques for the treatment and conditioning of radioactive organic wastes are:

- A. Non-destructive techniques that leave intact organic components but involve physical change in the properties of the material to enhance additional treatment, storage or disposal. Examples include absorption, compaction, immobilization etc.
- B. Destructive techniques that degrade the organic waste resulting in chemical change of the waste product (e.g. incineration, pyrolysis, bioremediation, etc) (IAEA, 2004).

The whole waste management protocol involves handling, pre-treatment, treatment, conditioning, storage and disposal of radioactive waste as illustrated in

Figure 2-3. The stages of the illustration are described below.

- Waste sorting/pre-treatment – is the segregation of inactive waste from active and low level waste from high level waste.
- Treatment – Obtaining waste product that can be stored or disposed of more safely
- Discharge/recycle – disposing or re-using the waste product
- Secondary waste – waste product that requires further treatment
- Immobilization/packaging – transforming waste into a form that is appropriate for disposal.
- Storage/disposal – placing the waste in an appropriate and specified facility without the intention of retrieval.

When selecting a treatment option for organic waste, chemical and physical characteristics are always considered. The basic methods for the treatment of radioactive organic waste are destruction methods (incineration, pyrolysis etc), direct immobilization in cement and in organic matrices (Prasad *et al*, 2001 and IAEA, 2002). The problem with these methods is the production of the secondary radioactive waste that is also difficult to handle and treat. Recently, some oxidation processes (e.g. wet oxidation, acid digestion etc) are emerging as eco-friendly alternatives to incineration (Prasad *et al*, 2001).

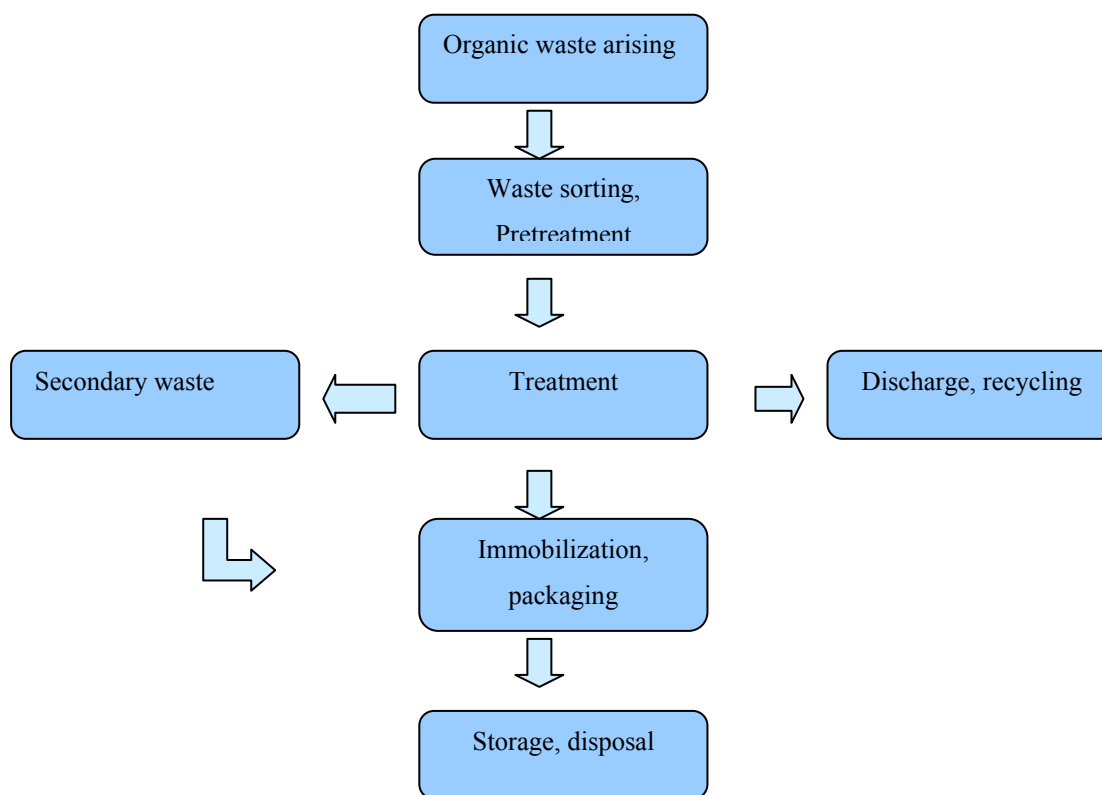


Figure 2-3: Generalized diagram of the life cycle of organic waste (Source IAEA, 2004)

These methods utilise reactants of high concentration of acids at high temperatures with expensive, corrosion resistant materials and increases the complexity of the off-gas scrubbing system due to the presence of oxides of nitrogen and sulphur (IAEA, 2002). The new most promising technique for volume reduction of organic radioactive waste is the microbiological degradation (Tusa, 1989). This process is advantageous because of its ability to reduce volumes of waste without producing secondary radioactive waste.

2.4 Fate of organics from nuclear waste

A wide variety of organic compounds from nuclear energy production may enter the wastewater system and subsequently pose a potential risk to the environment and human health (Castillo *et al*, 1997). Some of these include several organohalogens such as polychlorinated biphenyls (PCBs), chlorotoluenes and chloropropanes, organophosphorus compounds such as pesticides and tributylphosphate, chlorophenols and polycyclic

aromatic hydrocarbons (PAHs) (Castillo *et al*, 1997). Efforts have been made to characterize wastewater effluents to determine the distribution of the compounds in order to design effective means to prevent and limit deleterious effects on living organisms in the environment (Alcock *et al*, 1999). The analytical screening and identification of wastewater streams for the full range of these compounds represents one method of identifying potential risks.

In this study, polycyclic aromatic hydrocarbons (PAHs) were selected as model compounds for the investigation on biodegradability of toxic organics in nuclear waste. PAHs were selected as they comprise a significant component of both soluble and solid nuclear waste and they are a class or family that is resistant to degradation and are known carcinogens and teratogenics to mammalian life (Anyakora and Coker, 2006). Both natural and anthropogenic sources contribute in the existence of PAHs in the environment. They primarily originate from incomplete combustion of carbonaceous materials (Keshtkar *et al*, 2007). PAHs are believed to be the most widespread contaminants in the marine environment (Yunker *et al*, 1995). They are introduced to aquatic environment through accidental oil spills, discharge from industrial operation, municipal and urban runoff, ship and automobile exhaust, urban coal and oil heating, and direct release of oil and its products to the water (Fernandes *et al*, 1997, Kipopoulou *et al*, 1999, Xu *et al*, 2007). Needless to say, crude oil and other petroleum based products have contributed significantly to the current levels of PAHs in the environment.

2.5 Effects of PAHs in the environment

Contamination of the environment with chemicals in the PAH family originates from incomplete combustion of fossil fuels and organic compounds (Heitkamp and Cernglia, 1988). PAHs are also a major constituent of crude oil, creosote and coal tar. They contaminate the environment through various routes including manufactured gas and coal tar production, fossil fuel combustion, automobile exhaust and other processes (Kim *et al*, 2003). Polycyclic aromatic hydrocarbons have low water solubilities and tend to bind with organic matter or particle surfaces, resulting in a low bioavailability to the microbial biomass (Xu and Obbard, 2004). PAHs have been shown to be completely biodegraded in a variety of environments by various bacteria (Annweiler *et al*, 2000, Moody *et al*, 2001, Dean-Ross *et al*, 2001, Rehmann *et al*, 2001, Boldrin *et al*, 1993, Schneider *et al*,

1996, Kim *et al*, 2007). In contrast, there is little or no known information on biodegradation of PAHs from nuclear and radioactive wastewater.

2.6 Effects of PAHs on Human health

Data from animal studies indicate that several PAHs may induce a number of adverse effects, such as immunotoxicity, genotoxicity, carcinogenicity, reproductive toxicity (affecting both male and female offspring), and may possibly also influence development of atherosclerosis. However, the critical endpoint for the health risk evaluation is the well-documented carcinogenicity of several PAHs (Bosetti *et al*, 2006, Alguacil *et al*, 2003, Merlo *et al*, 2004, Friesen *et al*, 2006, Unwin *et al*, 2006, Binet *et al*, 2002, Unwin *et al*, 2006, Straif *et al*, 2005).

On the basis of the experimental results, the most significant health effect to be expected from inhalation exposure to PAHs is an excess risk of lung cancer. In the past, chimney sweeps and tar workers were dermally exposed to substantial amounts of PAHs and there is sufficient evidence that skin cancer in many of these workers was caused by PAHs (McClean *et al*, 2004). Epidemiological studies in coke-oven workers, coal-gas workers and employees in aluminum production plants also provide sufficient evidence of the role of inhaled PAHs in the induction of lung cancer. An excessively high rate of lung cancer mortality was found in coke-oven workers (Romundstad *et al*, 2000, Mumford, *et al* 1995, Preiss *et al*, 2005, Ruhl *et al*, 2006).

There are no reports on the effects of oral ingestion by humans of the PAHs selected for evaluation, although people who consume grilled or smoked food do ingest these compounds. High lung cancer mortality in Xuan Wei, China has been linked to PAH exposure from unvented coal combustion (Mumford *et al*, 1987; Lewtas *et al*, 1993). PAHs present in tobacco smoke are implicated as contributing to lung and other cancers (IARC, 1986; Grimmer *et al*, 1987, Grimmer *et al*, 1988). Most available human data are from inhalation and percutaneous absorption of PAHs from a large range of occupational exposures.

In earlier times, following high dermal exposure, chimney sweeps developed skin cancers, especially scrotal cancer. Epidemiological studies are available for workers

exposed at coke ovens in coal coking and coal gasification, in asphalt works, in foundries, in aluminium production plants, and from diesel exhaust (Verma *et al.*, 1992; Armstrong *et al.*, 1994; Partanen and Boffetta, 1994; Costantino *et al.*, 1995). In all these occupations, there is also exposure to other chemicals, making a direct correlation of cause to increased levels in lung cancer more problematic. There is additionally the confounding factor of smoking. Evaluation of these studies shows, however, that it is plausible that the increased risk of lung cancer occurring in several of these occupations can be attributed at least in part to PAHs (WHO, 1997).

2.7 Biodiversity of PAH degrading bacteria

Biodiversity is described as the range of significantly different types of organisms and their relative abundance in a community (Øvreås *et al.*, 1998). The two important parameters for defining species diversity are species richness (the number of species within a community) and species evenness (the sizes of species populations within a community) (Liu *et al.*, 1997, Torsvik *et al.*, 1998). A limitation of these parameters is that any departure from the original environmental parameters during cultivation can alter the community structure through the imposition of new selective conditions, infect a new community structure develops, which may not accurately replicate the original structure.

The study of microbial diversity and community analysis has risen since the arrival of DNA sequencing, which in turn has updated the understanding of microbial phylogeny (Dahllöf, 2002). The development of molecular techniques has made it common to investigate community diversity using the rRNA gene (rDNA) or the rRNA itself. Molecular methods provide tools for analyzing the entire bacterial community, covering also those bacteria that have not been cultured in the laboratory. They can also be used to analyze whole communities, bacterial isolates, and clones of specific genes. Therefore, such methods are becoming increasingly important in microbial ecology and they make it possible to determine microbial diversity at a high-resolution level (groups, species and strains) without the need for cultivation. The rapidly growing rDNA sequence data bank is now making it possible to compare sequences from across the world.

Biotechnology mostly relies on the activities of microorganisms but little is known about the diversity of microorganisms that are potentially useful for biotechnological

applications (Hugenholtz and Pace, 1996). The knowledge of microbial diversity has depended in the past mainly on studies of pure cultures in the laboratory (Pace, 1997). Knowledge of microorganisms in the environment has been limited by the inability to culture most of naturally occurring microbes using standard techniques (Hugenholtz and Pace, 1996). Due to the lack of knowledge of the diversity and function of microbial community, there is an immediate need for the methods that are effective for the evaluation of microbial diversity. Until recently, there has been no way to describe microorganisms without growing pure cultures. Microorganisms have conventionally been described and classified by culture and microscopy (Muyzer, 1999, Lane *et al.*, 1985). Lately, molecular and biochemical techniques have bypassed traditional method by enabling identification and phylogenetic characterization of microorganisms without cultivation.

2.8 PAH degrading organisms

Several species of bacteria have been shown to degrade PAHs using them as carbon sources under aerobic or anaerobic conditions (Cerniglia *et al.*, 1984; Bouwer and Zehnder, 1993). During the past decade, a variety of bacteria have been tested for their ability to degrade different PAHs (Table 2-1) and the pathways for PAH degradation were also studied and described (Boldrin *et al.*, 1993; Annweiler *et al.*, 2000; Vila *et al.*, 2001; Prabhu and Phale, 2003; Luan *et al.*, 2006; Seo *et al.*, 2006, Seo *et al.*, 2007).

2.9 PAH degradation pathways

Different microbes use various degradation pathways for different PAHs. Most microorganisms that have been reported to mineralize PAHs (Table 2-1) under aerobic conditions have used similar metabolic pathways. The first step of PAH metabolism is catalysis by a dioxygenase, in which oxygen reacts with two adjacent carbon atoms of the parent PAH resulting in the formation of cis-dihydrodiol. This then undergoes re-aromatization by dehydrogenases to form dihydroxylated intermediates. These in turn undergo ring cleavage to form tricarboxylic acid (TCA)-cycle intermediates (Samanta *et al.*, 2002).

Table 2-1: Different bacterial strains capable of degrading various PAHs

Compound	Microorganisms	References
Naphthalene	<i>Bacillus thermoleovorans</i>	Annweiler <i>et al</i> , 2000.
Naphthalene	<i>Pseudomonas putida</i> ATCC 17484	Barnsley, 1976
Naphthalene	<i>Pseudomonas putida</i> NCIB 9816	Barnsley, 1976
Naphthalene	<i>Pseudomonas sp</i> ATCC 17483	Barnsley, 1976
Naphthalene	<i>Rhodococcus sp</i>	Grund <i>et al</i> , 1992
Naphthalene	<i>Pseudomonas. aeruginosa</i>	Phale <i>et al</i> , 2007
Naphthalene	<i>Pseudomonas P_G</i>	Williams <i>et al</i> , 1975
Naphthalene	<i>Pseudomonas putida</i>	Samanta <i>et al</i> , 2003
Naphthalene	<i>Pseudomonas sp.</i>	Grimm and Harwood, 1997
Naphthalene	<i>Pseudomonas sp.</i>	Samanta and Jain, 2000
Naphthalene	<i>Bacillus sp.</i>	Shimura <i>et al</i> , 1999
Naphthalene	<i>Bacillus naphthovorans sp.</i>	Zhuang <i>et al</i> , 2002
Naphthalene	<i>Pseudomonas putida</i> G7	Lee <i>et al</i> , 2003
Naphthalene	<i>Oscillatoria sp.</i> strain JCM	Cerniglia <i>et al</i> , 1980
Naphthalene	<i>Pseudomonas putida</i> G7	Filonov <i>et al</i> , 2004
Naphthalene	<i>Burkholderia sp.</i>	Sandrin and Maier, 2002
Naphthalene	<i>Pseudomonas putida</i> ATCC 17484	Guerin and Boyd, 1992
Naphthalene	NP-Alk	Guerin and Boyd, 1992

Table 2-1: (Continued)

Compound	Microorganisms	References
Naphthalene	<i>Pseudomonas putida</i> G7	Park <i>et al</i> , 2001
Phenanthrene	<i>Pseudomonas stutzeri</i> P16.	Grimberg <i>et al</i> , 1996
Phenanthrene	<i>Pseudomonas</i> sp	Bouchez <i>et al</i> , 1995
Phenanthrene	<i>Pseudomonas aeruginosa</i> AK1	Köhler <i>et al</i> , 1994
Phenanthrene	<i>Pseudomonas fluorescens</i>	Yaun <i>et al</i> , 2000
Phenanthrene	<i>Haemophilus</i> sp.	Yaun <i>et al</i> , 2000
Phenanthrene	<i>Mycobacterium</i> sp strain PYR-1	Moody <i>et al</i> , 2001
Phenanthrene	<i>Rhodococcus</i> sp	Dean-Ross <i>et al</i> , 2001
Phenanthrene	<i>Pseudomonas</i> sp. strain PP2	Prabhu and Phale, 2003
Phenanthrene	<i>Aeromonas</i> sp.	Kiyohara <i>et al</i> , 1976
Phenanthrene	<i>Bacillus</i> sp.	Doddamani and Ninnekar, 2000
Phenanthrene	<i>Nocardioides</i>	Iwabuchi <i>et al</i> , 1997
Phenanthrene	<i>Mycobacterium</i> sp strain BB1	Boldrin <i>et al</i> , 1993
Fluoranthene	<i>Mycobacterium</i> sp strain KR20	Rehmann <i>et al</i> , 2001
Fluoranthene	<i>Mycobacterium</i> sp strain BB1	Boldrin <i>et al</i> , 1993
Fluoranthene	<i>Rhodococcus</i> sp	Dean-Ross <i>et al</i> , 2001
Anthracene	<i>Mycobacterium</i> sp strain PYR-1	Moody <i>et al</i> , 2001
Anthracene	<i>Rhodococcus</i> sp	Dean-Ross <i>et al</i> , 2001

Table 2-1: (Continued)

Compound	Microorganisms	References
Fluorene	<i>Mycobacterium</i> sp strain BB1	Boldrin <i>et al</i> , 1993
Benzo(a)pyrene	<i>Mycobacterium</i> sp strain JRGII-135	Schneider <i>et al</i> , 1996
Benzo(a)pyrene	Rhodobacter sp strain BPC1	Kanaly <i>et al</i> , 2002
Benzo(a)anthracene	<i>Mycobacterium</i> sp strain JRGII-135	Schneider <i>et al</i> , 1996

Naphthalene is commonly used as a model compound of a large group of environmentally widespread PAHs for studying PAH metabolism by bacteria. Naphthalene degradation has been widely studied in various bacteria especially *Pseudomonas* species (Zuniga *et al.*, 1981; Smith, 1990) and the biochemical pathways of naphthalene degradation have been elucidated in detail (Williams *et al.*, 1975; Barnsely 1976; Zeinali *et al.*, 2008).

The biochemical sequence and enzymatic reactions leading to the degradation of naphthalene were first presented by Davies and Evans (1964). The aerobic and anaerobic degradation pathways of naphthalene are shown in Figure 2-4 A and B respectively. Aerobic naphthalene degradation has been reported to consist of two primary pathways that are distinguished by the conversion of salicylate to catechol or gentisate (Pumphrey and Madsen, 2007).

The first step is catalysis by naphthalene(+)-*cis*-dihydrodiol dehydrogenase to 1,2-dihydroxynaphthalene. The next step leads to the enzymatic cleavage of 1,2-dihydroxynaphthalene to *cis*-2-hydroxybenzalpyruvate, which is then converted via a series of dioxygenases to salicylate and pyruvate. Salicylate is oxidized by salicylate hydroxylase to catechol (Mrozik *et al.*, 2002). Metabolism of naphthalene via catechol has been studied extensively in *Pseudomonas* species (Dennis and Zylstra, 2004; Yen and Serdar, 1988; Sota *et al.*, 2006).

Catechol is catabolized by ring cleavage, in which the aromatic ring is broken. The ring cleavage can occur by either of two pathways: the ortho-cleavage pathway, in which the aromatic ring is split between the two carbon atoms bearing hydroxyl groups, or the meta-cleavage pathway, in which the ring is broken between a hydroxylated carbon atom and an adjacent unsubstituted carbon atom. After ring-cleavage, intermediates are converted to *Tricarboxylic Acid* (TCA) cycle compound that lead to TCA cycle intermediates (acetate and succinate) or to substrates that can be easily converted to TCA cycle intermediates (pyruvate and acetaldehyde).

2.9.1 Ortho or β -Ketoadipate Pathway

One of the most thoroughly characterized metabolic sequences in bacteria is the β -ketoadipate pathway which is used for the catabolism of aromatic compounds via catechol (Ornston, 1966, Steiner and Ornston, 1973; Ornston and Steiner, 1966). The *ortho*-cleavage pathway, also known as the β -ketoadipate pathway, is encoded by chromosomal DNA genes in microorganisms (Table 2-2). Ortho cleavage of catechol by catechol-1,2-dioxygenase results in the formation of *cis,cis*-muconic acid (Hayashi and Hashimoto, 1950), which is further metabolized to acetyl-CoA and succinyl-CoA by a series of enzymes (Table 2-2) via the β -ketoadipate pathway and enters the central carbon pathway (Figure 2-5).

2.9.2 Meta or Ketoacid pathway

The catabolism of catechol produced during the metabolism of naphthalene by pseudomonads has been shown to involve the meta (or α -ketoacid) pathway (Williams *et al*, 1975; Barnsley, 1976). In meta pathway, the genes encoding enzymes for this pathway are plasmid borne (Chakrabarty, 1976). Catechol is converted to 2-hydroxymuconic semialdehyde followed by conversion to its enoates and oxovalerate by enolases and hydrolases (Figure 2-6 and Table 2-2). The final products of the *meta*-cleavage pathway are the TCA anaplerotic metabolites, pyruvate and acetaldehyde.

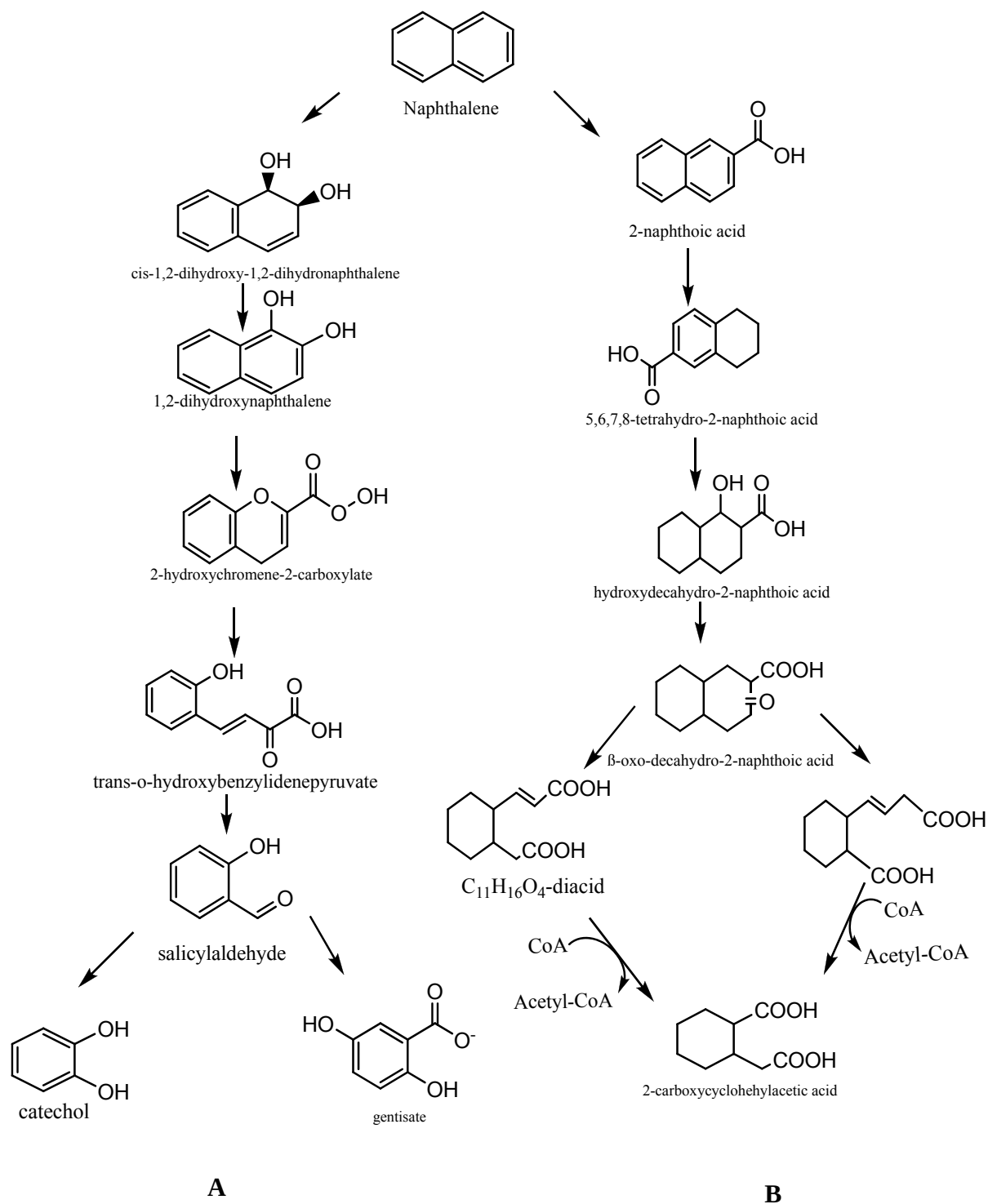


Figure 2-4: (A) - Aerobic and (B) - Anaerobic pathways of naphthalene

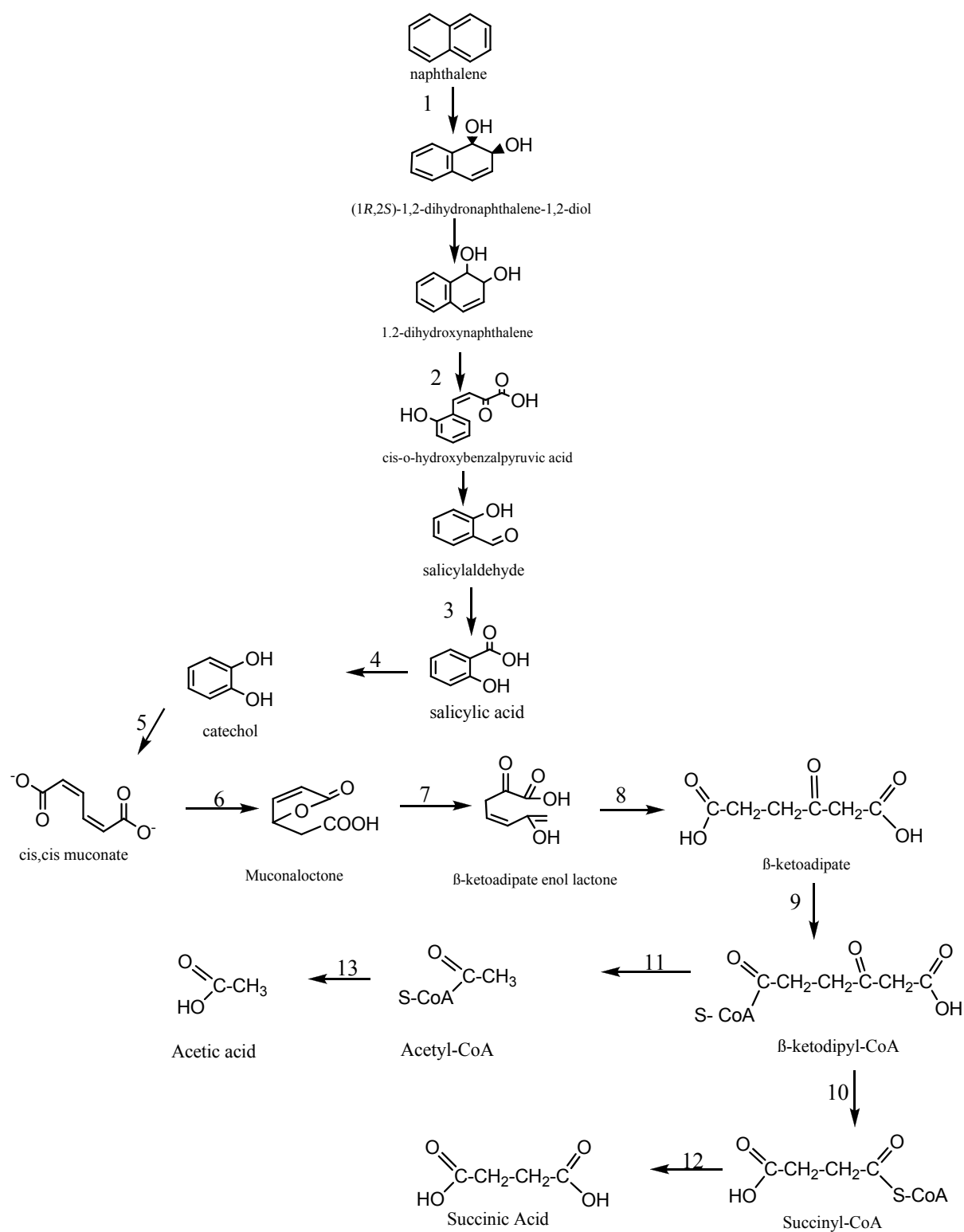


Figure 2-5: Ortho pathway of naphthalene degradation

Table 2-2: Enzymes of the *ortho* and *meta*-cleavage pathways of naphthalene degradation

Pathway	Reaction No	Enzyme catalysing the reaction
Ortho	1	naphthalene oxygenase
	2	1,2-dihydroxynaphthalene oxygenase
	3	Salicylaldehyde dehydrogenase
	4	salicylate hydroxylase
	5	catechol 2,3-dioxygenase
	6	<i>cis,cis</i> -muconate lactonizing enzyme
	7	muconolactone isomerase
	8	β -ketoadipate enol lactone hydrolase
	9	β -ketoadipate:succinyl-CoA transferase
	10	β -ketoadipate:succinyl-CoA transferase
	11	β -ketoadipate-CoA thiolase
	12	kinase activity of succinyl-CoA transferase
	13	acetyl-CoA kinase
Meta	1	naphthalene oxygenase
	2	1,2-dihydroxynaphthalene oxygenase
	3	salicylaldehyde dehydrogenase
	4	salicylate hydroxylase
	5	catechol 2,3 dioxygenase
	6	2-hydroxymuconic semialdehyde dehydrogenase
	7	4-oxalocrotonate tautomerase
	8	4-oxalocrotonate ketone decarboxylase
	9	4-hydroxyl-2-oxovalerate hydrolase
	10	4- hydroxyl-2-oxovalerate aldolase

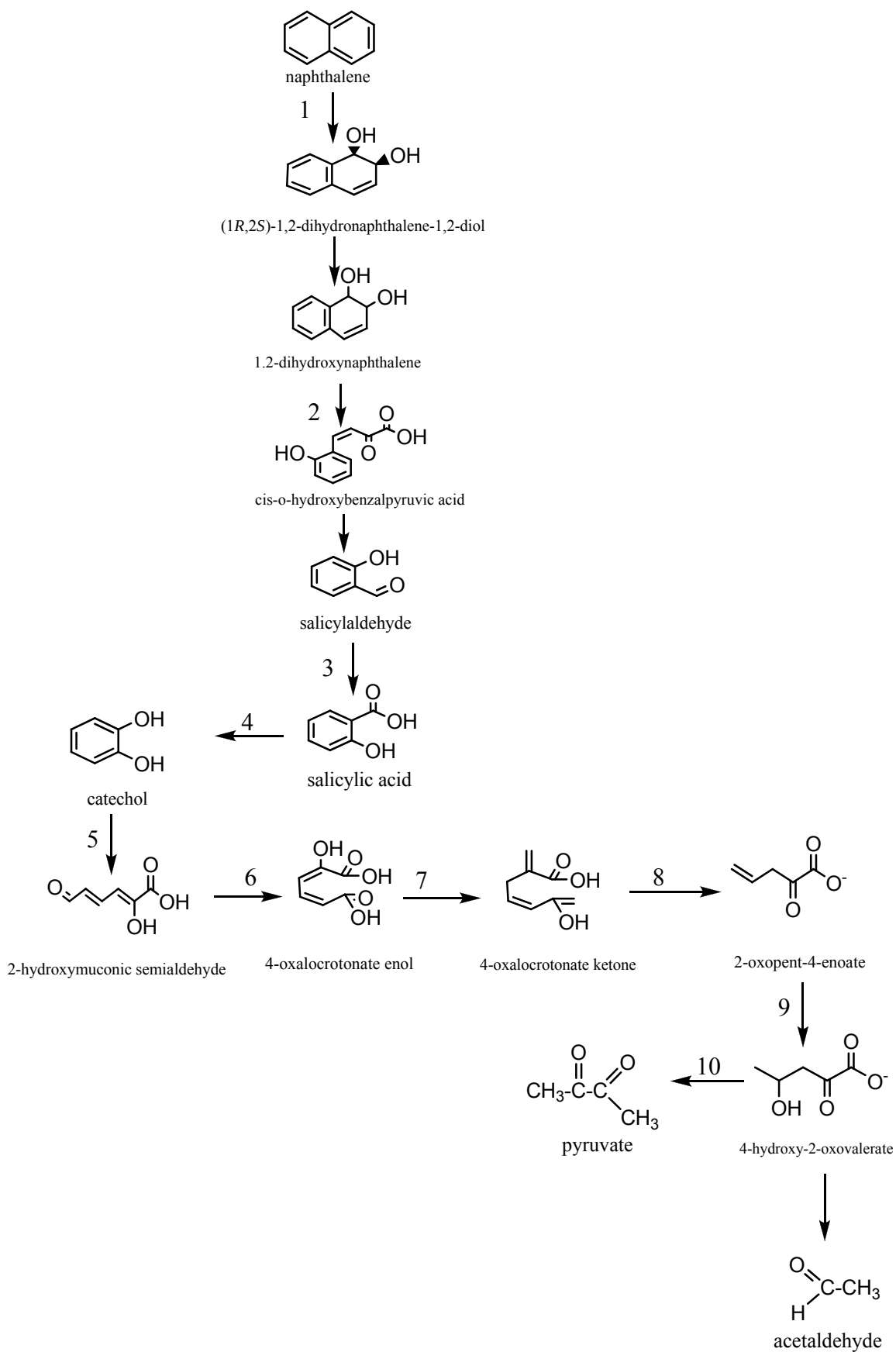


Figure 2-6: Meta pathway of naphthalene degradation

2.10 Summary

Nuclear energy is considered to be environmentally sustainable compared to burning of fossil fuels and the resultant production of greenhouse gases. The main problem of nuclear fuel production and nuclear energy generation is the formation of large amounts of radioactive waste that is difficult to treat and dispose. The field of nuclear industry is now facing a problem of safe treatment and disposal of these mixed radioactive organic wastes.

Treatment and conditioning techniques of organic radioactive waste are required to obtain a product that can be stored or disposed of more safely. Physical and chemical treatments have been shown to produce secondary radioactive waste which requires further treatment. The new most promising technique for reduction of organic radioactive waste is the microbiological degradation which is the technology that uses the ability of microbes to decompose and digest organic waste material. This process is advantageous because of its ability to degrade organic compounds from the waste without producing secondary radioactive waste.

Bacterial degradation represents a significant pathway for the removal of PAHs from the environment. During the past decade a variety of bacteria have been isolated and characterized for the ability to degrade different PAHs. Knowledge of the bioavailability of a compound is essential for biodegradation studies. The bioavailability of a chemical is determined by the rate of mass transfer relative to the intrinsic activity of the microbial cells. For instance, increased microbial transformation capabilities do not result in higher biotransformation rates when mass transfer in the system is the limiting factor.

CHAPTER 3: MATERIALS AND METHODS

3.1 Growth media

3.1.1 Preparation of broth and agar media

Nutrient broth and nutrient agar were prepared by dissolving 31g and 16g, respectively, in 1L of distilled water (dH₂O) and autoclaved for 15 minutes in a temperature of 121°C and a pressure of 115 kg/cm². The agar was then cooled to a temperature of about 50°C before dispensing to petri dishes. The prepared agar was stored in a cold room at 4°C and used within two weeks of preparation. Mineral salt medium (MSM) was prepared by dissolving 10 mM NH₄Cl, 30 mM Na₂HPO₄, 20 mM KH₂PO₄, 0.8 mM Na₂SO₄, 0.2 mM MgSO₄, 50 µM CaCl₂, 25 µM FeSO₄, 0.1 µM ZnCl₂, 0.2 µM CuCl₂, 0.1 µM NaBr, 0.05 µM Na₂MoO₄, 0.1 µM MnCl₂, 0.1 µM KI, 0.2 µM H₃BO₃, 0.1 µM CoCl₂, and 0.1 µM NiCl₂ in 1L of distilled water (dH₂O). The solution was sterilized by autoclaving at 121°C and 2 atm for 15 minutes.

3.2 Reagents

3.2.1 Chemicals

HPLC-grade water, methanol and acetonitrile used as solvents in HPLC analysis were purchased from Merck (Johannesburg, South Africa). Methylene chloride and ethyl acetate for SPE extraction were also purchased from Merck. Naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, chrysene and indeno (1,2,3-cd) pyrene for degradation experiments were purchased from Sigma Aldrich (Johannesburg, SA). Chemicals used in the preparation of mineral salt medium i.e. ammonium chloride, sodium phosphate, potassium di-hydrogen phosphate, sodium sulphate, magnesium sulphate, calcium chloride, iron sulphate, zinc chloride, copper chloride, sodium bromide, sodium molybdate, manganese chloride, potassium iodide, boric acid, copper chloride and nickel chloride were obtained from Merck (Johannesburg, SA).

3.2.2 Standard solution

A PAH standard stock solution containing naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, dibenzo-(ah)-anthracene and indeno(1,2,3-cd)pyrene (Catalog number 715) was purchased at Waters (USA). Standards were prepared from the stock solution to desired concentrations with acetonitrile. The different standard solutions were transferred to capped and sealed vials until ready for use.

3.3 Bacterial cultures

3.3.1 Collection of soil and water samples

The soil samples for isolation of cultures were collected from Chloorkop municipal landfill (Johannesburg, South Africa). Mine water samples were obtained from CSIR (Pretoria, SA). All the samples were stored in a cold room at 4 °C until used.

3.3.2 Isolation of Naphthalene degrading bacteria

Microorganisms were isolated from contaminated soil from the Chloorkop landfill site (Johannesburg, SA) and mine water obtained from Council of Scientific and Industrial Research (CSIR) (Pretoria, SA). Initial cultures were obtained by inoculating 100 ml of sterile nutrient broth (autoclaved at 121°C, 2 atm for 15 minutes) with 1 g of soil and 1 mL of mine water in a 250 mL Erlenmeyer flask. The flasks were incubated on a rotary shaker (120 rpm) at a temperature of 28±2°C for 48 hours. Enrichment cultures were then obtained by sub-culturing a 2% (v/v) of 24-48 hours culture medium in mineral salt medium (MSM) with naphthalene as the only added carbon and energy source. The enrichment procedure was repeated 3 times to allow for a high degree of selection of efficient naphthalene degrading bacteria.

3.3.3 Storage of pure cultures

After identification, pure cultures were streaked on nutrient agar to establish purity. The purified cultures were grown in sterile tryptone soy broth supplemented with 20% sterile glycerol. The pure cultures were then dispensed in screwed cap storage vials and stored at -70°C.

3.4 Characterization of radioactive waste

3.4.1 Radioactive wastewater collection

Radioactive wastewater was collected from a radioisotope processing facility in Cape Town, SA. The radioactivity of the samples was determined to be 0.677 Bq, enough to inhibit the growth of mesophilic bacteria from activated sludge processes (Lee *et al*, 2004). The wastewater sample was stored in a bottle and was refrigerated at 4°C until analysis. Ultra pure water was used as control.

3.4.2 Sample preparation

A number of analytical techniques have been developed for the determination of PAHs in complex environmental samples. Problems encountered in their analysis include occurrence at inherently low levels difficult to detect and existence of impurities that require different separation procedures. The analysis of PAH in samples often requires pre-concentration of the sample to enhance detection. However, some of the species may breakdown thus may be lost during pre-concentration.

Reliable analytical procedures require detailed method validation and careful evaluation regarding efficiency. In order to define quantitatively the accuracy and precision of the procedure for each determinant, it is necessary to statistically estimate random and systematic errors. In addition, sampling and sample preparation is considered integrally with the characterization of an analytical procedure, an area too often neglected. This section is a brief overview of the methods that were used in this study for the analysis of PAHs in radioactive wastewaters.

Before the analysis by GC-MS and HPLC, various pre-concentration and fractionation methods are required in order to provide concentrated extracts of the wastewater samples that are free of interferences. Traditionally the methods for characterizing organic pollutants in contaminated effluents generally include the use of either dichloromethane liquid-liquid extraction (LLE) or solid phase extraction (SPE), followed by gas chromatography-mass spectrometry (GC-MS) techniques with electron impact (EI) ionization or HPLC. Solid-phase extraction (SPE) has turned out to be an effective technique for the extraction of contaminants from wastewater samples, to allow for pre-concentration and clean-up in a single step. The elimination of interferences by SPE

methods provides fractionated extracts containing the various contaminants in a state ready for analysis by the most appropriate techniques. The SPE methods for industrial effluents are frequently based on the coupling of different sorbents for the pre-concentration of the samples in the different polarity-based fractions.

In this study, solid phase extraction (SPE) method was used for pre-concentration of wastewater sample and was coupled with HPLC for analysis. The Strata C18 cartridges (1000 mg, 6 ml) from Merck were used for SPE purposes. The conditioning step was performed by pre-wetting the cartridge with ethyl acetate for 1 minute and 30 seconds, followed by methanol for minute and 30 seconds and lastly ultra pure water for minute and 30 seconds. The sorbent was not allowed to become dry before performing the pre-concentration step. To process the sample, 400 mL of water sample was loaded in the C₁₈ cartridges. After loading the sample, the sorbent was completely dried for 8 minutes using vacuum to avoid hydrolysis of the trapped compounds. The elution step was performed by adding 1:1 Methylene chloride: Ethyl acetate to the cartridge for 1 minute and 30 seconds. The elution step was repeated twice. The residual extracts were concentrated by Dry Vap concentrator (Rotavapor RII, BUCHI, Switzerland) to a final volume of 1 mL. The extraction was followed by analysis using HPLC Waters 2695 separation module (HPLC) equipped with a Waters 2998 Photodiode Array (PDA) detector (Microsep, Johannesburg, SA) for the identification of organic pollutants against a standard mixture of 16 priority PAHs. Identification of compounds in wastewater samples was based on retention time match against calibration standards. The calibration standards were also used for quantification of identified compounds.

3.4.3 Analytical equipments

Solid phase extraction experiments were performed using an SPE manifold set from Microsep (Johannesburg, SA). This system includes Extraction Columns system fitted with an external vacuum pump for the dispensing of samples through the SPE cartridges and with switching valve for the selection of samples for the pre-concentration step. The Strata C₁₈ cartridges were purchased from Separation Scientific (Pty) Ltd (Johannesburg, SA). The concentration step was carried out using a Dry Vap Concentrator (Rotavapor RII, BUCHI, Switzerland). The concentrate was analyzed in the Waters 2695 separation module (HPLC) equipped with a Waters 2998 Photodiode Array (PDA) detector (Microsep, Johannesburg, South Africa).

3.4.4 Standard solutions and calibration

The standard solution containing 16 priority PAHs was prepared by diluting the stock solution to desired concentrations with the aid of HPLC grade acetonitrile. For calibration, several dilutions of the stock mixture were analyzed by HPLC.

3.4.5 Identification and quantification

The PAHs in the samples identified were based on retention time match against calibration standards. The compounds were identified using the HPLC. Quantitation was performed with calibration standards prepared as a mixture of 16 priority PAHs, i.e., naphthalene, acenaphthylene, acenaphthene, fluorene, phenanthrene, anthracene, fluoranthene, pyrene, benzo(a)anthracene, chrysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, benzo(ghi)perylene, dibenzo(ah)anthracene and indeno(1,2,3-cd)pyrene.

3.4.6 Recovery studies

Different criteria are used to determine the efficiency of the extraction and clean-up steps for partially hydrophilic organic compounds. To enhance detection, a surrogate standard of known amount may be added to the solution. Preferable, the added surrogate should be chemically inert. The percent recovery of the surrogate compound is considered to be representative of the recovery of all determinants. Surrogate recovery is also used to monitor unusual matrix effects and gross sample processing errors. It is evaluated for acceptance by determining whether the measured concentration falls within the acceptance limits. However, this method assumes that the surrogate standard behaves in an identical way to the compounds of interest. That is usually true for the clean-up step, but may not be true for the initial extraction.

Another method to assess the efficiency of recovery is spiking of environmental samples with a standard solution of the compounds of interest at one or more concentration levels. Thus, the recovery efficiency of individual determinants at various spike levels can be established and appropriate correction of the amount found can be performed.

Recovery efficiency tests do not directly assess accuracy as is commonly believed, but rather procedure efficiency. They indicate analytical accuracy only when the analytical response for a determinant in an un-spiked sample is due to the determinant alone (i.e. no interferences) and is not subject to any bias.

In the present study, recovery of PAHs was measured by spiking with an external standard. External standard was added in the water sample prior to any sample treatment to assess the recovery of PAHs. Since the amount of external standard added was known, the recovery of PAHs was calculated. The spiked samples were subjected to the same extraction procedure as for the radioactive water samples. The percentage recovery of the standard was calculated using the equation:

$$\%R = \frac{Q_d}{Q_a} \times 100 \quad (3-1)$$

where Q_d is the quantity determined by the analysis, and Q_a is the quantity added. For the surrogate percent recovery to be accepted it must fall between 60 and 120%. Knowledge of percent recovery is useful in monitoring extraction or clean up performance.

3.4.7 Method detection limit and limit of quantification

Laboratory methods have many performance characteristics that must be understood and assessed for their appropriate use. The performance characteristics for any method describe the method's capability to reliably measure the amount of an analyte in a subject's sample. Two such critical performance characteristics are defined at the lower end of the measurement scale. The first is the smallest amount that the method can reliably detect to determine presence or absence of an analyte. This is the limit of detection (LOD). The second characteristic is the smallest amount the method can reliably measure quantitatively. This is the limit of quantification (LOQ). The limits of detection and quantification are critical because detecting extremely small amounts of an analyte can be necessary to reveal the presence or absence of toxins, pollutants, carcinogens, contaminants, infectious agents, and illegal drugs. Knowledge of the limit of detection informs the choice of a cutoff, so the procedures in this document should be applicable.

In this study, method detection limit (MDL) for PAHs was established by analysis of seven reagent water samples spiked with small but known amounts of the PAH standard. The MDL and LOQ were calculated as follows:

$$MDL = t \times S \text{ and } LOQ = 10 \times S \quad (3-2)$$

where t is the student's t -value for a 99% confidence level based on 6 degrees of freedom and S the standard deviation of the replicate analyses. Calculating the MDL at the 99% confidence interval allows for the probability that 1% of the samples analyzed which have a true concentration at the MDL level will be false positives.

3.5 Bacterial Characterization

Naphthalene-degrading bacteria were isolated from a soil and mine water sample. The consortia that were pre-exposed to naphthalene during degradation experiments were purified by plating out the serial dilutions of sample from the reactors on nutrient agar. The phylogenetic characterization of cells was performed on isolated individual colonies of bacteria from the 7th-10th tube in the serial dilution preparation. In preparation for the 16S rRNA sequence identification, the colonies were first classified based on morphology. Different morphologies were identified from the cultures. These were streaked on nutrient agar followed by incubating at 30°C for 18 hours.

Genomic DNA was extracted from the pure cultures using a DNeasy tissue kit (QIAGEN Ltd, West Sussex, UK). The 16S rRNA genes of isolates were amplified by a reverse transcriptase-polymerase chain reaction (RT-PCR) using primers pA and pH1 (Primer pA corresponds to position 8-27; Primer pH to position 1541-1522 of the 16S gene. An internal primer pD was used for sequencing (corresponding to position 519-536 of the 16S gene). The resulting sequences were compared to known bacteria in the GenBank using a basic BLAST search of the National Centre for Biotechnology Information (NCBI, Bethesda, MD)

3.6 Degradation Experiments

3.6.1 Determination of naphthalene degradation

For determination of naphthalene degradation, naphthalene at different concentrations was dissolved in 2ml of methanol and added into 500 mL of a sterile MSM which was contained in 1 L Erlenmeyer flasks. 2% (v/v) of enrichment culture was used as an inoculum and incubated on a rotary shaker at $28\pm 2^{\circ}\text{C}$. At certain intervals, 5 mL aliquot samples were withdrawn from the bioreactor aseptically, centrifuged and the supernatant analyzed for naphthalene concentrations. The analysis was performed with a Waters Model 2695 (HPLC) equipped with a Photodiode Array (PDA) detector (Waters, Johannesburg, SA). Cell free controls were used in which sterile MSM was mixed with the test compounds.

3.6.2 Degradation of mixed PAHs

PAH degradation experiments were conducted using simulated, wastewater with the composition similar to the characterized radioactive waste. Simulated waste was inoculated with consortia from soil and mine-water and incubated on a rotary shaker at $28\pm 2^{\circ}\text{C}$. At certain intervals, 5 mL aliquot samples were withdrawn from the bioreactor aseptically, centrifuged and the supernatant analyzed for PAHs concentrations.

3.7 Analytical Methods

3.7.1 Measurement of PAHs

A number of analytical techniques have been used for the determination of PAHs in complex environmental samples. The most widely used are gas chromatography (GC) with flame ionization detection (FID) or mass spectrometry detection (MSD), and HPLC with ultraviolet detection (UVD) or fluorometric detection (FLD). Other techniques that have also been used are thin-layer chromatography (TLC) with UVD or FLD, supercritical fluid chromatography (SFC) with UVD or MSD and liquid chromatography (LC) with MSD.

The US EPA method 610 suggests HPLC with UVD or FLD, or GC-FID for the determination of the 16 PAHs in wastewaters. Reversed-phase liquid chromatography on chemically bonded octadecylsilane (C_{18}) stationary phases has been shown to provide excellent separation of PAHs. However, not all C_{18} stationary phases provide the same resolution (i.e. relative separation) for PAHs, but resolution is greatly influenced by the

type of synthesis used to prepare the bonded phase. The vast majority of C₁₈ phases are prepared by reaction of monofunctional silanes (e.g. monochlorosilanes) with silica to form monomeric bond linkages. Polymeric phases are prepared using bifunctional or trifunctional silanes in the presence of water which results in cross-linking to form silane polymers on silica surface. The resulting phase is conceptually not as well-defined as a monomeric phase. Good separation of all 16 US EPA PAHs can be achieved on polymeric C₁₈ phases, in contrast to the monomeric C₁₈ phase, where the four-ring unresolved, while the six-ring isomers, the five-ring isomers, and fluorene and acenaphthene are only partially resolved.

In this study, the samples were first filtered with a 5ml syringe filter (0.45µm pore size) and run in high performance liquid chromatograph (HPLC) Waters 2695 separation module connected to a Waters Photodiode Array Detector Model 2998 in the reversed phase mode using a Waters PAH C₁₈ column (250 mm × 4.6 mm, 5 µm particle size) (Waters, USA) The detection wavelength was set at 254 nm. The mobile phase was programmed with a reciprocating pump using water:acetonitrile at 50 : 50 (v/v) holding for 5 minutes, then ramping to 0 : 100 (v/v) in 28 minutes and returning to initial conditions at 30 minutes with a constant flow-rate of 1.5 mL/min. All injections were at 20 µL using an autosampler. Conditions of the HPLC system and the wavelength program of the detector and the gradient elution for analysis are shown in Table 3-1.

Table 3-1: HPLC conditions, detector wavelength and gradient elution program.

Chromatographic conditions				
Column		Waters PAH C18 (250 mm x 4.6 mm, 5 µm)		
Mobile phase		Water:Acetonitrile		
Flow rate		1.5 mL/min		
Temperature		25°C		
Pressure		3000		
Detector wavelength		254 nm		
Time (min)	Flow rate (mL/min)	%water	% acetonitrile	
5	1.5	50	50	
20	1.5	100	100	
28	1.5	100	100	
30	1.5	50	50	

3.8 Biomass analysis

3.8.1 Evaluation of total biomass

To determine total biomass, bacterial cells were harvested by sampling 5mL aliquots from the reactors and centrifuged at 10,000 rpm for 10 minutes in a Minispin Microcentrifuge (Eppendorf, Hamburg, Germany). The cells were then washed three times in normal saline by centrifuging and resuspending in fresh solution each time. After washing the total biomass was weighed gravimetrically using a balance (Adams Equipment, Pw184, Separation Scientific, SA).

3.8.2 Determination of viable biomass

To determine viable biomass, samples from the flasks were serially diluted in saline solution (0.85% NaCl). The numbers of colony forming units (CFU) were determined by plating 0.1 mL from 10-fold dilution series on nutrient agar plates. Plates were incubated at 28±2°C for 24-48 hours. The colonies were counted from plates with 30 – 300 colonies. The bacterial count per milliliter was computed by the following equation (APHA, 1993).

$$CFU / mL = \frac{\text{colonies counted}}{\text{actual volume of sample in dish, mL}} \quad (3-3)$$

CHAPTER 4: RESULTS AND DISCUSSIONS

4.1 Characterization of Radioactive wastewater

4.1.1 Standard solutions and calibration

Calibration curves were obtained using a series of varying concentrations of a multi-component standard containing each of 16 PAHs. The baseline separation of the target compounds was obtained in time of less than 35 minutes. These results are illustrated in Figure 4-1. The calibration curves of compounds were all linear and were obtained by plotting the peak area of the standard against the amount of the standard. The calibration curves had correlation coefficients from linear regression of 1.000.

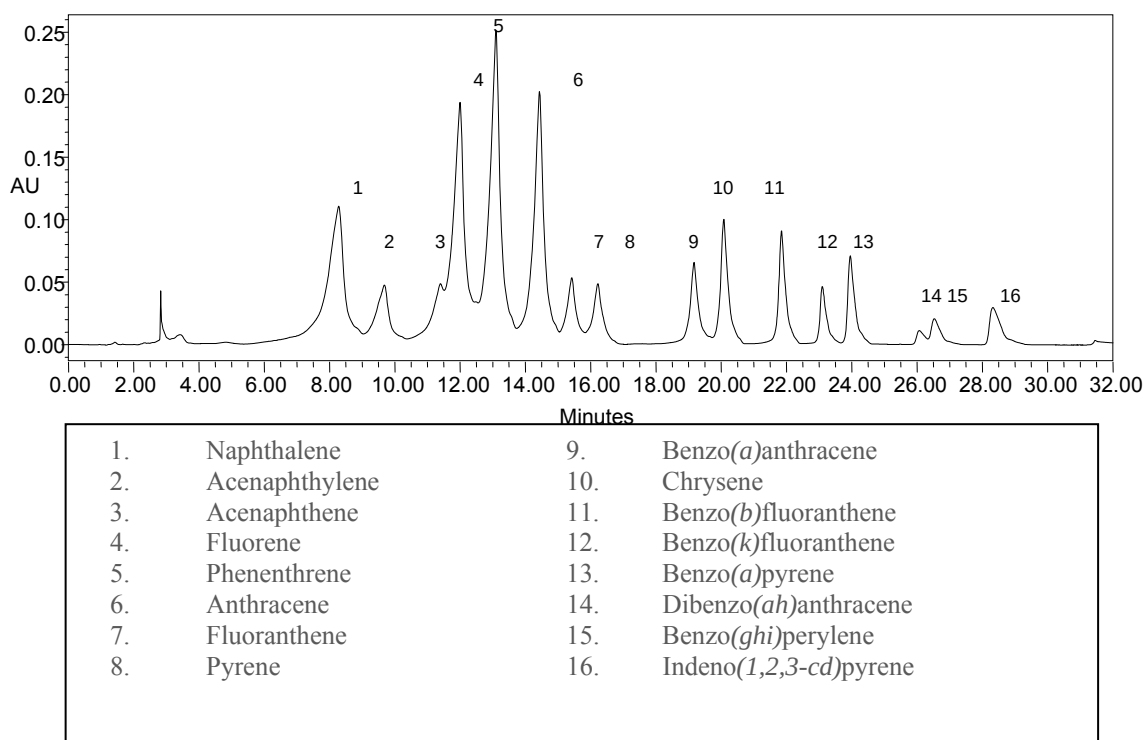


Figure 4-1: Chromatographic determination of the 16 US EPA PAHs with HPLC-PDI

4.1.2 Identification and quantification

Different amounts of the 16 priority PAHs were detected in wastewater samples from a industrial effluent. These concentrations varied from 0.001-25 mg/L (Table 4-1). 2-3 ring PAHs concentrations namely, naphthalene, acenaphthylene, acenaphthene, fluorene,

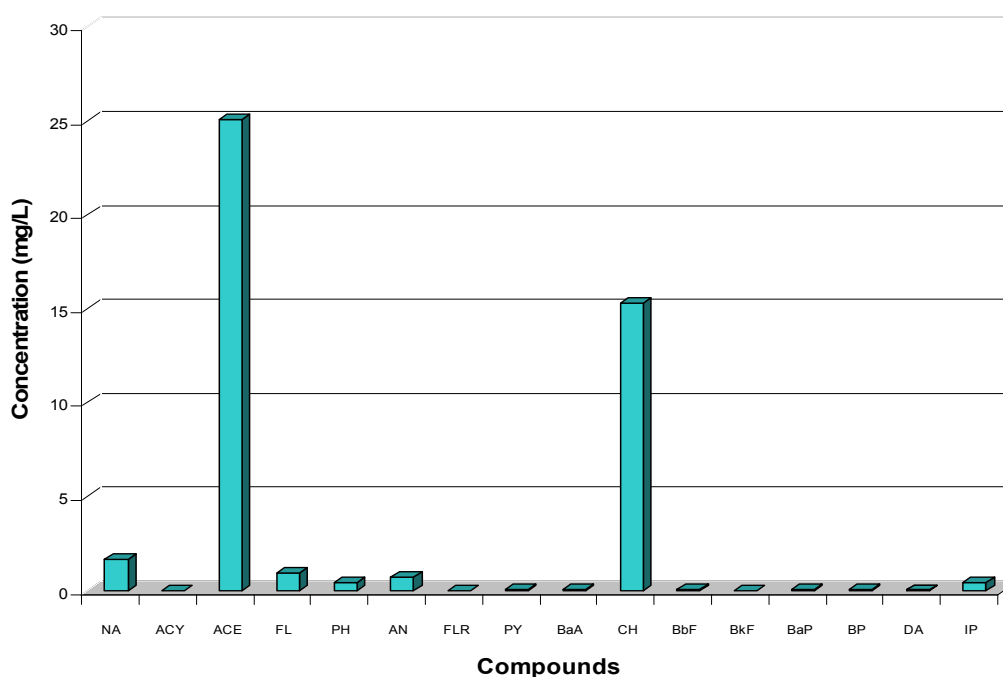
phenanthrene and anthracene, ranged from 0.001 to 25.1 mg/L with acenaphthene (detected at 25.1 mg/L) as the most abundant. This group of PAHs (2-3 rings) was detected in higher values on average than the rest of PAHs. The reason for high average value of this group of PAHs could be their potential to be more soluble in water compared to other PAHs.

Table 4-1: Concentrations of PAHs in wastewater samples

Compound	Concentration (mg/L)
Naphthalene	1.654
Acenaphthylene	0.001
Acenaphthene	25.101
Fluorene	0.942
Phenanthrene	0.390
Anthracene	0.695
Fluoranthene	0.000
Pyrene	0.014
Benzo(<i>a</i>)anthracene	0.019
Chrysene	15.305
Benzo(<i>b</i>)fluoranthene	0.057
Benzo(<i>k</i>)fluoranthene	0.005
Benzo(<i>a</i>)pyrene	0.048
Dibenzo (<i>ah</i>)anthracene	0.047
Benzo(<i>ghi</i>)perylene	0.006
Indeno(1,2,3- <i>cd</i>)pyrene	0.438

4-6 ring PAHs concentrations namely, fluoranthene, pyrene, benzo(*a*)anthracene, chrysene, benzo(*b*)fluoranthene, benzo(*k*)fluoranthene, benzo(*a*)pyrene, dibenzo(*ah*)anthracene, benzo(*ghi*)perylene and indeno(1,2,3-*cd*)pyrene varied from 0-15 mg/L with chrysene as the most abundant in this group and fluoranthene was the only PAH absent in the characterized wastewater sample. The BaP, a vital representative toxic PAHs, was 0.048 mg/L which was quite high. The level of BaP in the environment improves the basis for the development of environmental evaluation and cleanup regulations throughout the world (Juhász and Naidu, 2000). Surprisingly, the biggest PAH, indeno(1,2,3-*cd*)pyrene was also detected in high values than expected due to its known

low aqueous solubility. Also dibenzo(*ah*)perylene concentration was 0.047 mg/L and all these values were worrying knowing that these compounds are most toxic compared to others. All the PAHs in the wastewater samples from the radioisotope manufacturing facilities were greatly in excess of the WHO limit of 0.05µg/L in both surface and coastal water. Compared with other publications, the results showed that the amounts were significantly higher than those presented in other studies from different environments. Anyakora and co-workers (2005) detected all the PAHs from the sediment samples ranging from 0.1µg/kg – 28µg/kg. These high levels of PAHs may be in the present study may be explained by the fact that in an industrial sector like a radioisotope processing factory, there are more PAHs contributing activities than in the environment. Industrial activities have the major contribution to environmental PAH contamination. Therefore is expected that PAH in the environment are lower than in the main source of generation.



NA - Naphthalene	BaA - Benzo(a)anthracene
ACY - Acenaphthylene	CH - Chrysene
ACE - Acenaphthene	BbF - Benzo(b)fluoranthene
FL - Fluorene	BkF - Benzo(k)fluoranthene
PH - Phenanthrene	BaP- Benzo(a)pyrene
AN - Anthracene	DA - Dibenzo(ah)anthracene
FLR - Fluoranthene	BP - Benzo(ghi)perylene
PY - Pyrene	IP - Indeno(1,2,3-cd)pyrene

Figure 4-2: Distribution of PAHs in radioactive wastewater sample

The PAH composition in wastewater samples is displayed in Figure 4-2. Acenaphthene was the most abundant component (25 mg/L), followed by Chr (15 mg/L), naphthalene (1.65 mg/L), fluorene (0.9 mg/L) anthracene (0.6 mg/L) indeno(1,2,3-*cd*)perylene (0.438 mg/L) and phenanthrene (0.3 mg/L). These were the PAHs detected in high levels. PAHs by ring size were predominated by 2- and 3-ring PAHs. In average, the 2-3 ring PAHs consisted of about 64.4% of 16 EPA-PAHs, whereas 6-4 ring PAHs only accounted for the remaining 35.6%. The HMW PAHs that are the known potential carcinogenic accounted for 35.6%. Petrogenic and pyrolytic sources are widely the most known source of PAH contributions to the environment (Li *et al*, 2006). Petrogenic input is strongly linked to petroleum products (such as oil spills, road construction materials) and pyrolytic sources involve combustion processes (e.g., fossil fuel combustion, forest fires, shrub and grass fires). In general, the petroleum source contain relatively higher concentrations of 2-3 ring PAHs compounds while a large proportion of high molecular weight parent PAHs is typical characteristic of a combustion origin (Li *et al*, 2006). Although specific sources are known to be accountable for the presence of PAHs in the environment, their incidence cannot always be linked to a specific source. Most PAH inputs in the environment are linked to the anthropogenic activities that are generally considered to be major sources of these compounds (e.g., wastes from industrialized and urbanized areas, off-shore petroleum hydrocarbons production or petroleum transportation).

To estimate the origin of the PAHs, Soclo and co-workers (2000), used the Low/High ratio (sum of the low molecular weight PAH concentrations versus sum of higher molecular weight PAH concentrations) origin index. Values of the LMW/HMW ratio lower than 1 indicate pyrolytic origin pollution while the LMW/HMW greater than 1 indicate petrogenic origin (Soclo *et al*, 2000). One difficulty in identifying PAHs origins is the possible co-existence of many contamination sources, and the transformation processes that PAHs can undergo before detection in the analyzed samples. In case of this study, the actual source of the PAHs is not known at this point but it might be due to the known source or the other un-identified sources like new processes that generate PAHs. However, when applying LMW/HMW ratio index, it shows the petrogenic origin since the ratio is greater than 1.

4.1.3 Method validation

To evaluate the extraction efficiency for the target compounds, recovery studies were carried out using an EPA standard for 16 priority PAHs. With this experiment, the approximate recovery for the samples was proposed as shown in Table 4-2. Efficient recoveries for most of the PAHs were achieved and were ranging from 77%-92%. For the last three PAHs i.e., benzo(*ghi*)perylene, dibenzo(*a,h*)anthracene and indeno(1,2,3*cd*)pyrene, an error occurred during analysis and recoveries could not be determined. Other analytical parameters i.e., limit of detection (LOD) and limit of quantification (LOQ) for the chromatographic method is also shown in Table 4-2. The lowest LOD was 0.05 mg/L for 2 PAHs acenaphthylene and pyrene, while the highest LOD was 0.22 mg/L for benzo(*k*) fluoranthene.

Table 4-2: Percent recoveries and method detection limit and quantification of the 16 PAHs

Compound	LOD (mg/L)	LOQ (mg/L)	% Recovery
Naphthalene	0.11	0.35	78
Acenaphthylene	0.05	0.16	84
Acenaphthene	0.14	0.44	78
Fluorene	0.14	0.44	83
Phenanthrene	0.06	0.2	86
Anthracene	0.11	0.35	92
Fluoranthene	0.07	0.22	82
Pyrene	0.05	0.15	81
Benzo(<i>a</i>)anthracene	0.1	0.32	82
Chrysene	0.1	0.31	77
Benzo(<i>b</i>)fluoranthene	0.08	0.24	81
Benzo(<i>k</i>)fluoranthene	0.22	0.71	87
Benzo(<i>a</i>)pyrene	0.08	0.27	82
Dibenzo(<i>ah</i>)anthracene	nd	nd	nd
Benzo(<i>ghi</i>)perylene	nd	nd	nd
Indeno(1,2,3- <i>cd</i>)pyrene	nd	nd	nd

nd- not determined

4.2 Isolation of Bacteria

Unidentified mixed cultures isolated from contaminated landfill soil and gold mine water were used in the naphthalene degradation batch experiments. The cultures were grown on 100 mL of sterile mineral salt medium (autoclaved at 121°C, 2atm for 15 minutes) contained in a 250 mL Erlenmeyer flask. The flasks were incubated on a rotary shaker (120 rpm) at the temperature of 28±2 °C for 48 hours.

The landfill soil was chosen as a source of bacteria because dumping site soil is known to be most contaminated with a variety of pollutants including recalcitrant organic pollutants such as PAHs. PAHs are persistent in the soil due to their low water solubility. Incineration and other human activities contribute to the accumulation of PAHs in the environment. Mine water was chosen because it is believed to have a background radiation due to the nature of minerals.

4.3 Culture Characterisation

After culture isolation and purifying the individual species were characterized by sequencing the rRNA genes from the soil and mine water bacteria. A total of 5 and 3 bacterial isolates were found in the soil and mine water, respectively. The rRNA sequences were isolated from bacteria with some tolerance to PAH toxicity and were thus candidate species for naphthalene degradation. Species identification was based on the match of 16S rRNA with species in the GenBank. Hits were scored with 96% confidence and above, except for one that had 89% identity. The results are shown in Tables 4-3 and 4-4 (below).

Table 4-3: Characterization of naphthalene degrading bacteria isolated from landfill site.

16S rRNA ID	% Identity
<i>Microbacterium esteraromaticum</i>	98%
<i>Achromobacter xylosoxidans</i>	99%
<i>Alcaligenes sp.</i>	89%
<i>Pseudomonas aeruginosa</i>	99%
<i>Pseudomonas pseudo alcalegenes</i>	99%

Table 4-4: Characterization of naphthalene degrading bacteria isolated from mine water

16S rRNA ID	% Identity
<i>Stenotrophomonas sp.</i> , <i>Stenotrophomonas maltophilia</i> Strain KNUC285	96%
<i>Bacillus sp.</i>	99%
<i>Pseudomonas putida</i> , <i>P. taiwanensis</i>	99%

Results in Table 4-3 and 4-4 are consistent with literature observations with the *Pseudomonas* species as primary degraders of aromatic compounds (Barnsley, 1976 Phale *et al*, 2007, Williams *et al*, 1975, Samanta *et al*, 2003, Grimm and Harwood, 1997). The other species, *Achromobacter sp*, *Bacillus sp* and *Stenotrophomonas sp* have also been known to degrade complex aromatic compounds as demonstrated by Walczac *et al*, (2001), Guo *et al*, (2008), Juhasz *et al*, (2000) and (Doddamani and Ninnekar, 2000).

Applications for treatment of organics in the environment range from in situ bioremediation and bioaugmentation where bacteria are introduced into the environment, to encourage native aromatic compound biodegradation. In this study, only the degradative potential of individual species of aromatic compound tolerant species is evaluated.

4.4 Biodegradation of Simulated Waste

Experiments were performed to determine biodegradation of a mixture of 7 PAHs namely, naphthalene, acenaphthene, fluorene, phenanthrene, anthracene, chrysene and indeno(1,2,3-*cd*) pyrene. This mixture of PAHs was made based on the composition of the characterized radioactive wastewater discussed above. These seven PAHs were the predominant ones in that radioactive wastewater. High molecular weight PAHs (compounds containing four or more fused benzene rings) are known to be recalcitrant to microbial attack. Until recently, only a few genera of bacteria have been isolated with the ability to utilise four-ring PAHs as sole carbon and energy sources while co-metabolism of five-ring compounds has been reported.

The ability of bacteria to utilize PAHs as growth substrates has been reported by extensive studies over the past few decades (Weissenfels *et al*, 1990; Boldrin *et al*, 1993; Kanaly and Harayama, 2000)]. These studies share a general method of isolating bacteria from the environment and using the isolated strains as pure cultures in order to establish biodegradation of individual PAHs by the bacterial strain. But, that approach does not indicate the real complexity of PAH degradation in natural environments where the compounds are present in multi-component mixtures.

In the present study, the utilization of a mixture of PAHs by bacterial consortia isolated from two different sources was examined. The mixture was composed of both LMW and HMW PAHs. Results from previous studies which have focused on the degradation of mixtures of PAHs suggest that simultaneous utilization of PAHs is common when pure cultures of PAH-degrading strains are provided with mixtures of PAHs. Biodegradation of complex mixed hydrocarbons usually requires the co-operation of more than a single species. Individual microorganism can metabolise only a limited range of compounds to a certain extent. In mixed cultures, each species plays a certain role, bringing together an overall broad enzymatic capacity that is required during biotransformation. Pure cultures, including *M. flavescens* and strains of *Pseudomonas*, have been found to be capable of utilizing mixtures of PAHs simultaneously (Dean-Ross *et al*, 2002). Similar results have been obtained with bacterial communities from soil and sediment. The present study also confirms the ability of bacterial consortia to utilize PAHs simultaneously. Microbial degradation of a mixture of PAHs was carried out over a period of 5 days. Degradation was observed in all PAHs including the biggest high molecular weight PAHs, indeno(1,2,3-*cd*)pyrene. These results are illustrated in figure 4-3 to 4-8.

Day 0

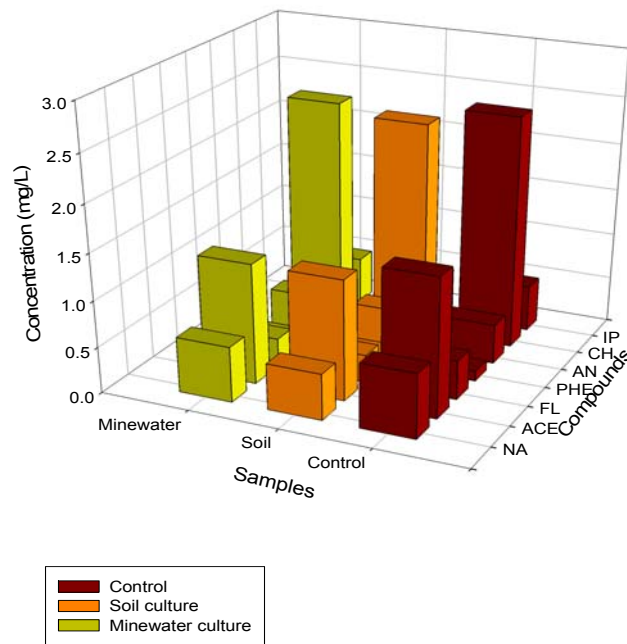


Figure 4-3: PAHs degradation immediately after inoculation

Day 1

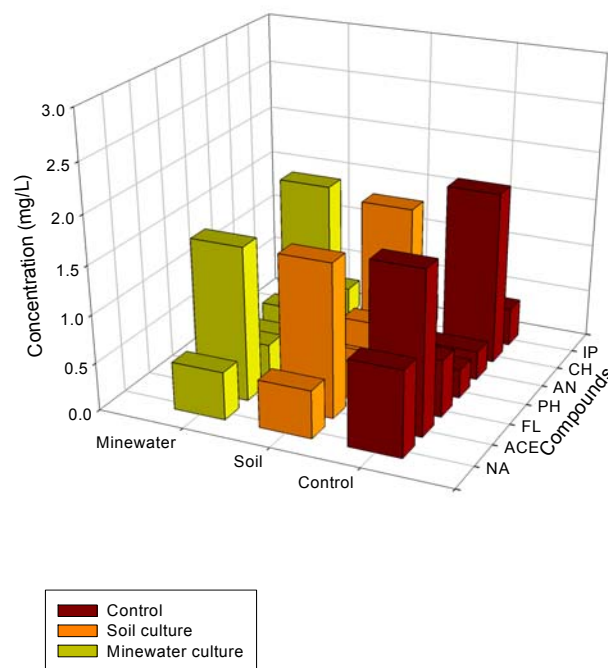


Figure 4-4 PAHs degradation after 1 day of inoculation

Day 2

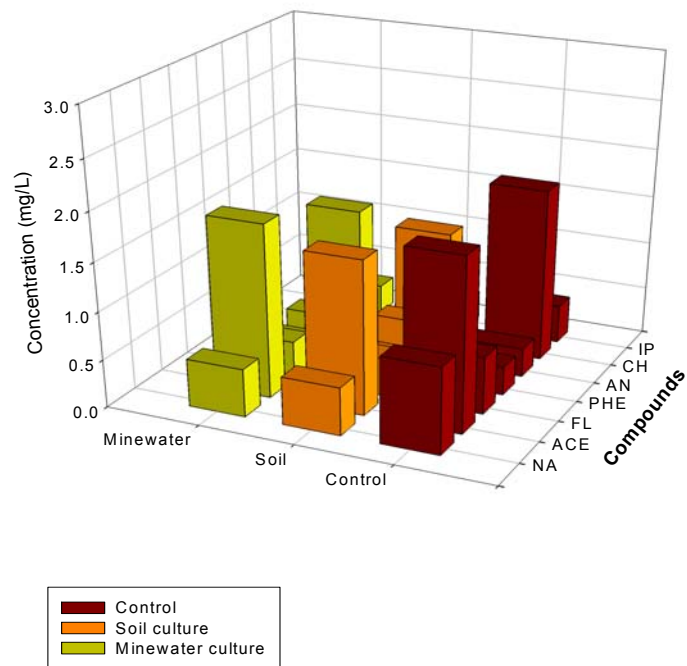


Figure 4-5: PAHs degradation after 2 days of inoculation

Day 3

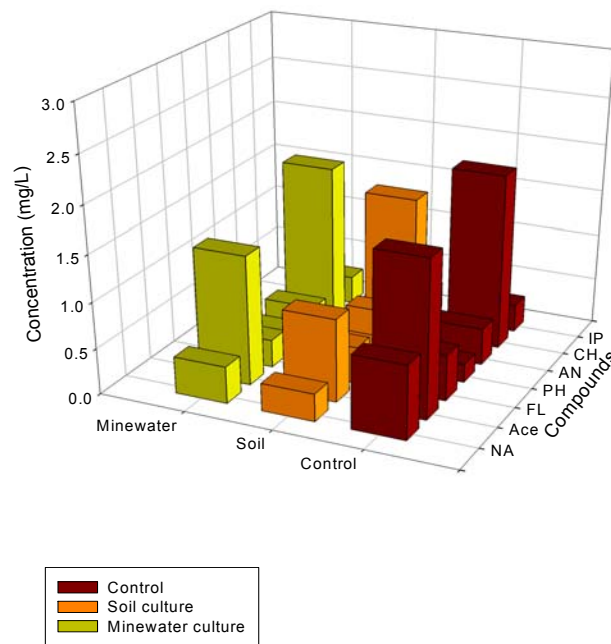


Figure 4-6: PAHs degradation after 3 days of inoculation

Day 4

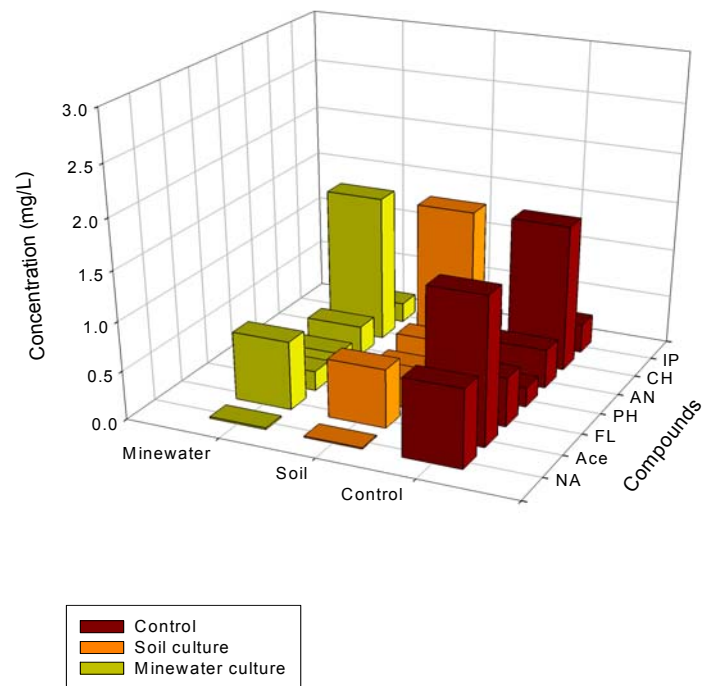


Figure 4-7: PAHs degradation after 4 days of inoculation

Day 5

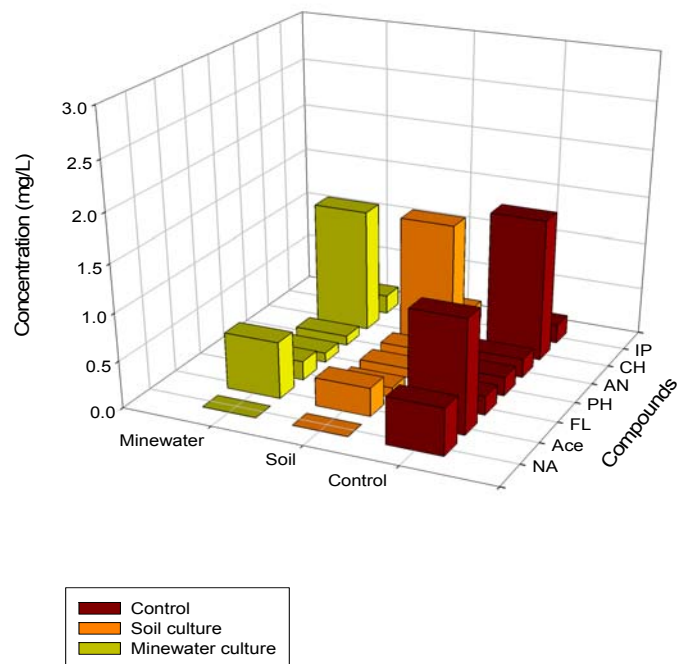


Figure 4-8: PAHs degradation after 5 days of inoculation

Naphthalene has often been used as a model compound to investigate the ability of bacteria to degrade PAHs because it is the simplest and the most soluble PAH. Therefore, information of bacterial degradation of naphthalene has been used to understand and predict pathways in the degradation of three- or more ring PAHs. Predictably, the greatest PAH removal consistently occurred in low molecular weight (LMW) PAHs fastest naphthalene, with the highest removal being 100%. Naphthalene was completely removed in all cultures with notable exception; complete degradation was observed in day 5. These results were contrary to those observed earlier in the study when determining naphthalene degradation as individual compound. In the previous experiments, naphthalene that was dissolved in aqueous phase was rapidly biodegraded and completely removed within 15 h of incubation for landfill soil cultures. Commonly in multi-component degradation experiments, interactive outcomes such as inhibition and co-metabolism are observed in addition to simultaneous utilization. The delayed complete degradation could be due to one or both of these effects.

The biodegradation of high molecular weight (HMW) PAHs by combined bacteria occurred in this study. Compared to controls, the degradation of total PAHs in the experiments inoculated with both consortia showed a significant removal after 5 days of incubation. There were no significant difference between the experiments with mine water cultures and soil cultures. The biodegradation of LMW (2-and 3 ring) occurred much faster than HMW. In this study there was no significant difference between the degradation of the 4, and 6 rings. These results are in agreement with those found by Li and co-workers (2002).

During the five days of the degradation experiment with soil culture, the biodegradation percentages of acenaphthene and fluorene were greater than 92.5 % and 90 % respectively. In contrast with mine water cultures, the same compounds had 85% and 80% degradation respectively which were lower than for soil consortium. For phenanthrene, anthracene, chrysene and indeno(1,2,3-*cd*)pyrene, all cultures achieved similar biodegradation percentages which were around 80%, 90%, 66.5% and 60% respectively (Table 4-5).

Table 4-5: Percent removals of PAHs during biodegradation of mixed PAHs

Compound	Initial Conc (mg/L)	Final Conc ^a	Removal ^a %	Final Conc ^b	Removal ^b %
Naphthalene	2	0	100%	0	100%
Acenaphthene	4	0.3	92.5%	0.6	85%
Fluorene	1	0.1	90%	0.2	80%
Phenanthrene	0.5	0.1	80%	0.1	80 %
Anthracene	1	0.1	90%	0.1	90%
Chrysene	4	1.3	66.5%	1.3	66.5%
Indeno(1,2,3- <i>cd</i>)pyrene	0.5	0.2	60%	0.2	60%

a- landfill soil culture

b- mine water culture

After 5 days of incubation all the PAHs were degraded, indicating that the consortium had a good PAH degradation capability and preferred to utilize low-molecular weight. These results suggested that the addition of an enriched consortium could enhance the efficiency of PAH degradation. Similar to previous studies (Dean- Ross et al, 2002, Lotfabad and Gray, 2002, Chang *et al*, 2002, McNally *et al*, 1998) that investigated biodegradation of mixed PAHs supported that a group of bacteria (the enriched consortium) had a good PAH degradation capability and could be used to clean environments contaminated with PAHs. However, these studies were different from this one in that they were using different consortia and compounds.

This study demonstrated that microorganisms in environment can degrade a wide variety of important hydrocarbon contaminants that were previously considered recalcitrant to microbial degradation. Currently, there is only limited information regarding the bacterial biodegradation of PAHs with five or more rings in both environmental samples and pure or mixed cultures.

4.5 Determination of Naphthalene Degradation

Naphthalene was used as a model compound to represent the PAHs to study the biodegradation kinetics of these compounds. Firstly, results from the biodegradation experiments of naphthalene using mixed cultures from landfill soil are shown (Figure 4-9).

These were carried out in the range of 30-60 mg/L. Naphthalene was completely degraded within 15 hours of incubation.

In contrast, naphthalene degradation was not complete when large amounts of naphthalene were added (200-500 mg/L) (Figure 4-10). Since the solubility of naphthalene is only 31 mg/L, these were way above the amounts that could be dissolved. It was also expected that the actual concentration of naphthalene in liquid phase could not exceed this value. Laboratory studies making use of supersaturated aqueous solutions of PAH compounds have revealed that the rates of dissolution regulate the rates of biodegradation of these compounds (Goshal and Luthy, 1998). Thus even for this experiment, the rate of degradation could be a function of the rate of dissolution of naphthalene from the solid phase into the bulk liquid.

The biodegradation of a variety of organic pollutants results from the activity of microorganisms that use them as source of carbon and energy for growth (Bosma *et al*, 1997). The results from previous studies indicate that primarily dissolved PAH compounds in the aqueous phase are available for microbial uptake (Goshal and Luthy, 1996). Since the growth of such organisms in the environment is directly linked to the rate of biotransformation of the pollutants, biotransformation is also regulated by the mass transfer of the pollutants to the cells. Therefore, a reduced bioavailability of organic compounds is caused by the slow mass transfer to the degrading microorganisms. Contaminants become unavailable when the rate of mass transfer is zero (Li *et al*, 2007).

In the second set of experiments, the batch cultures were inoculated with bacteria from mine water. The experiments were also conducted in the range 30-60 mg/L naphthalene to compare the performance with the landfill soil cultures. The performance of the mine water bacteria was much slower than the performance of landfill soil bacteria as indicated by more than 29 hours required to completely degrade 60 mg/L in the mine water bacteria batch experiments (Figure 4-11). It has been documented that indigenous microorganisms isolated from polluted soils were often more effective to metabolize PAHs than organisms obtained from elsewhere in bioremediation. This might explain a quick and brief naphthalene degradation observed in soil cultures. In this study, the lower degradation rate in the mine water culture may be also due to a limited number of phenolic ring degrading species as confirmed by the 16S rRNA fingerprinting.

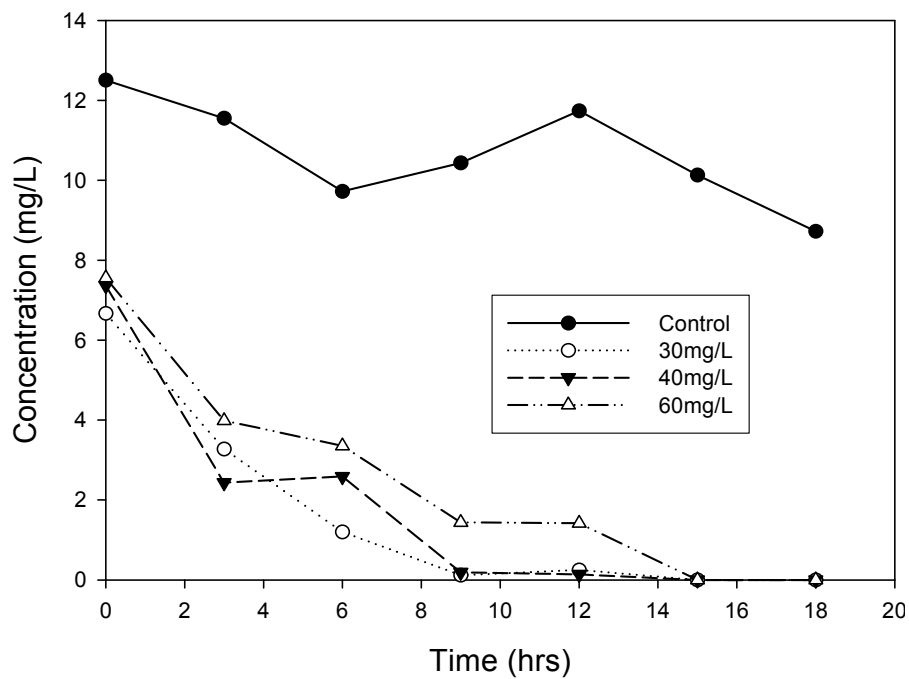


Figure 4-9: Naphthalene degradation at low initial concentrations by landfill soil culture

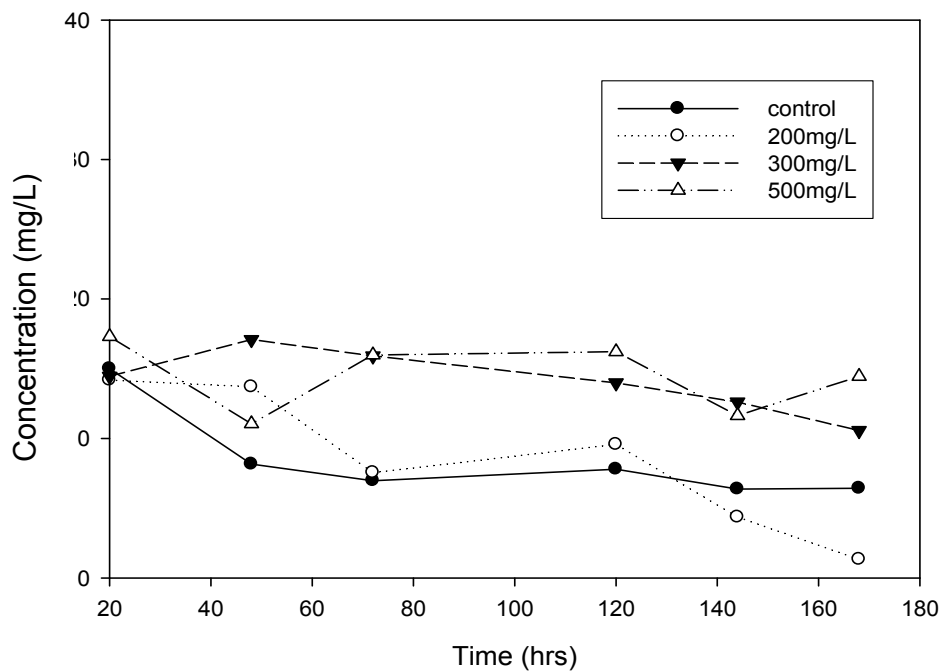


Figure 4-10: Naphthalene degradation at high initial concentrations by landfill soil culture

The biodegradation of naphthalene at low initial concentrations in soil bacteria occurred much more rapidly and significantly than in mine water bacteria (Figure 4-9 and 4-11). However, high initial concentrations required an extended period of time for significant biodegradation to occur (Figure 4-10). These results are consistent with the previous findings in which degradation rate of naphthalene was inhibited at high concentrations (Vipulanandam and Ren, 2000). Complete degradation of 30 mg/L of naphthalene was achieved in 3 days whereas it took more than 35 days to completely degrade 450 mg/L of naphthalene. The only difference being that in the study by Vipulanandam and Ren (2000), pure cultures of *Pseudomonas* species were used. In both biodegradation rate was limited by the low solubility of naphthalene in water.

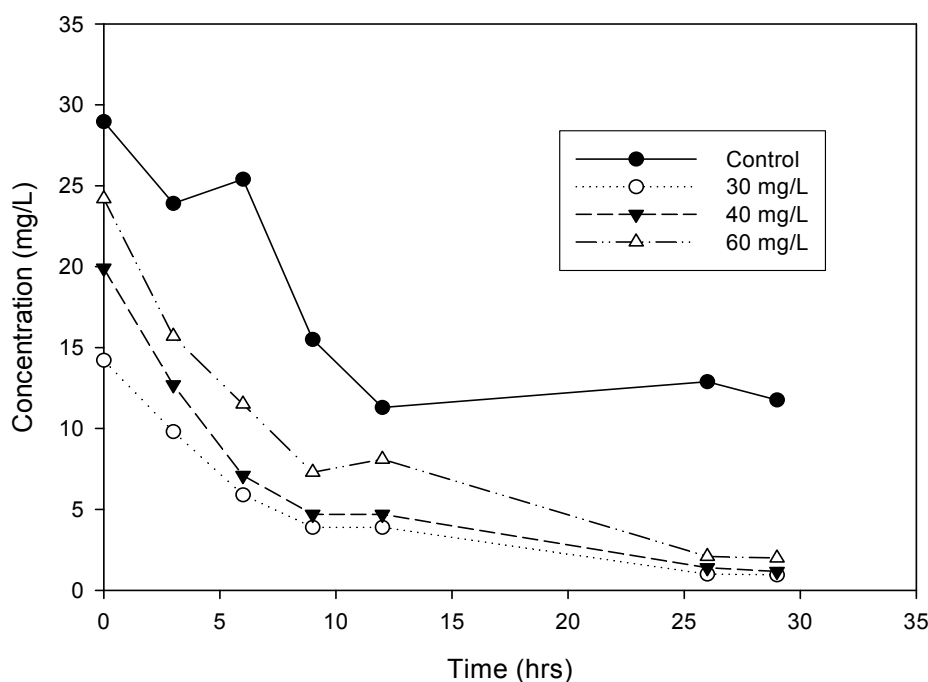


Figure 4-11: Naphthalene degradation at low initial concentration by mine water culture

4.6 Biomass Analysis

4.6.1 Total biomass

Results of total biomass for batch experiments are shown in Figure 4-12 and Figure 4-13 in soil and mine water bacteria, respectively. When grown in minimal medium with naphthalene as the sole carbon and energy source, bacterial cell concentration increased concomitant with a decrease in the PAH concentration. As the concentration of naphthalene in the culture decreased from 60 to 0 mg/L, the cell concentrations increased from values of 19 mg/L – 39 mg/L at the time zero to a maximum of 63 mg/L after 48 hours of incubation. No significant change was observed in the naphthalene free control.

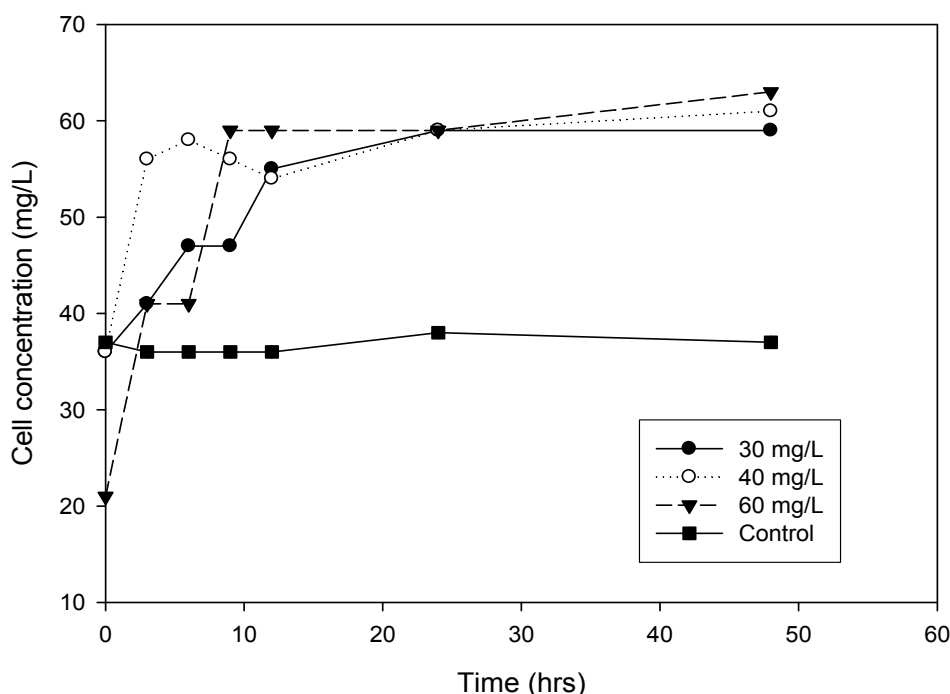


Figure 4-12: Cell concentration during naphthalene degradation by landfill soil culture

4.6.2 Viable cell count

The landfill and mine water cultures were harvested and resuspended at a viable cell concentration of approximately 1.9×10^9 and 1.5×10^9 CFU/mL, respectively. In both experiments, the viable counts increased with time by a factor of 2 apart from the control in which no significant increase was observed (Fig.4-14A and 4-14B). Following the mineralization of naphthalene during experiments, the culture increased viable count from 6 to 20 hours. The highest bacterial viable counts of up to 2.8×10^{10} CFU/mL were reached

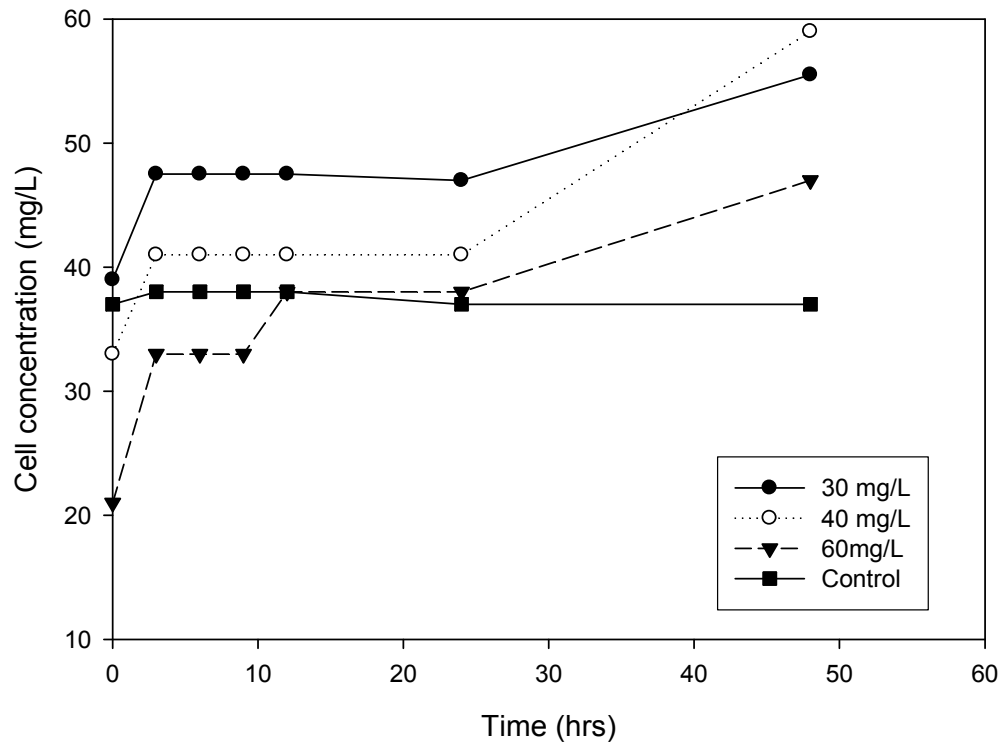


Figure 4-13: Cell concentration during naphthalene degradation by mine water culture

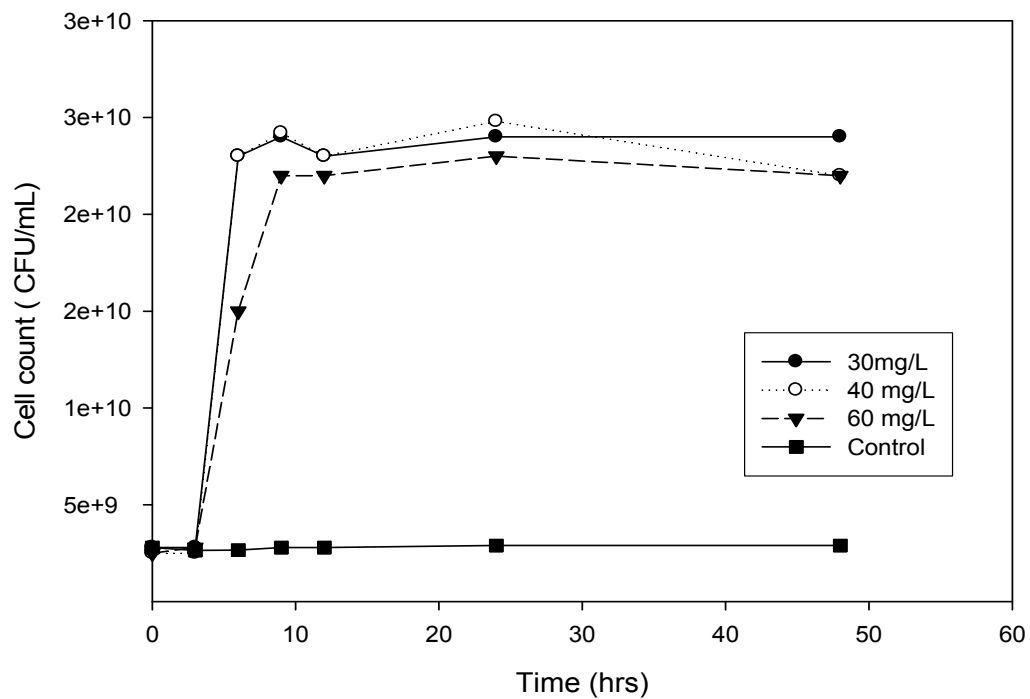


Figure 4-14 A: Viable cell count during naphthalene degradation by landfill soil culture

in the experiments after 25 hours of incubation and there after there was no further increase. The increase in viable count was accompanied by major decrease in naphthalene concentrations, and the increase in bacterial viable counts thus could be due to naphthalene utilization. The decrease in viable cell counts in experiments was observed after 40 hours of incubation. The possibility is that some bacteria were dying after all the nutrients were depleted.

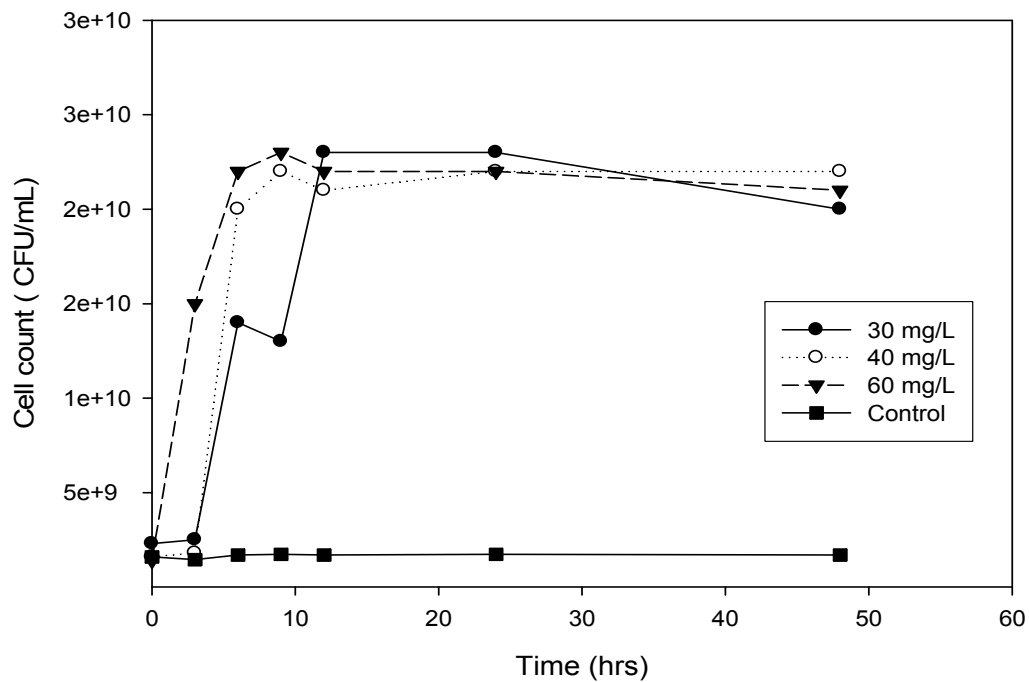


Figure 4-14 B: Viable cell count during naphthalene degradation by mine water culture

CHAPTER 5: BIODEGRADATION KINETICS OF NAPHTHALENE

5.1 Background of biodegradation

Most organic chemicals including PAHs can be consumed as a carbon source by living organisms. These organisms depend on specific enzymes to decompose these chemicals and the breaking down of chemicals is known as biodegradation. The major target of biodegradation is to destroy hazardous or toxic organic contaminants into harmless derivatives like carbon dioxide and water (Okpokwasili *et al.*, 1986). It is understandable that biodegradation of PAHs in the solid state is almost unfeasible and microbial uptake only occurs when these compounds are dissolved in water (Volkerling *et al.*, 1992). There are three mechanisms for the microbial uptake of liquid hydrocarbons that have been proposed in the literature (Volkerling *et al.*, 1998). They are:

- (i) Uptake of hydrocarbon dissolved in the aqueous phase. This mechanism is usually found when compounds are highly soluble in aqueous solutions.
- (ii) Direct uptake of hydrocarbons from the liquid-liquid interface. Direct uptake of hydrocarbons in solid phase, involves bacterial attachment to the liquid-liquid interface, and often occurs for poorly soluble substrates.
- (iii) Uptake of "pseudo-solubilised" hydrocarbons. This mechanism occurs when microbes excrete products that enhance solubility.

Therefore uptake may occur by means of either one or a combination of the above mechanisms depending on the type of organism, the hydrocarbon, and the environmental conditions.

The ability of microbes to be used as means of degradation of several compounds led to the selection of the biological treatment as the major promising alternative to reduce environmental effects originating from organic contamination (Nweke and Okpokwasili, 2003). The overall rate at which microbial cells transform chemical compounds during biodegradation depends on two factors:

- (1) The rate of uptake and metabolism.
- (2) The rate of transfer to the cell (mass transfer). Mass transfer determines the bioavailability of a chemical to the intrinsic activity of the microbial cells.

The bioavailability of a chemical is controlled by a number of physical-chemical occurrences associated with sorption and desorption from the solid phase to the bulk aqueous phase, diffusion, and dissolution and to biokinetic incidents associated to microbial degradation (Wick *et al*, 2001). Because the solubility of PAHs is very low, bioavailability is the limiting step in biodegradation. Therefore it is generally acknowledged that a low level of bioavailability is one of the most significant reasons for slow biodegradation of hydrophobic organic compounds in the environment

5.2 Kinetics of Biodegradation

5.2.1 Non-inhibitory substrate kinetics

The fundamental theory of biodegradation kinetics is that substrates are utilized through catalyzed reactions performed by the organisms carrying obligatory enzymes (Okpokwasili and Nweke, 2005). This implies that the rate of substrate utilization is normally proportional to that of catalyst concentration. Saturation kinetics proposes that at low substrate concentrations, rates of substrate utilization are nearly proportional to substrate concentration, whereas at high substrate concentrations, utilization rates are not related to substrate concentration.

Biodegradation kinetics is used to calculate concentrations of chemical substances remaining at a given time during bioremediation processes. For the most part, information is based on loss of major molecule targeted in the process. For substrates with high concentrations that support bacterial growth, the substrate degradation kinetics follow Monod model (Equation 1):

$$\frac{dC}{dt} = -q_{\max} \frac{C}{K_c + C} X \quad (5-1)$$

Where the variables are C = substrate concentration (mg/L), X = biomass concentration (mg/L) and t = time (h). The parameters are $q_{\max}$ = maximum substrate utilization, K_c = half saturation constant.

But for concentrations of PAHs high enough to support some growth, but too low follow Monod kinetics, substrate degradation kinetics is estimated as follows:

$$\frac{dC}{dt} = -\frac{q_{\max}}{K_c} CX \quad (5-2)$$

Other scenarios are the transformation of a compound by non-growing cells (the compound does not support growth) and the transformation of a compound by cometabolism, that is; transformation of a compound by cells growing on other substrate. The simplest case is where the compound serves as source of carbon and energy for the growth of a single bacterial species. The compound is assumed to be water-soluble, non-toxic and other substrates or growth factors are limiting. In the case of single-substrate limited process, the Monod equation (Equations 5-3 and 5-4) is often used to describe microbial growth and biodegradation processes.

$$\mu = \frac{\mu_{\max} C}{K_c + C} \quad (5-3)$$

$$q = \frac{q_{\max} C}{K_c + C} \quad (5-4)$$

where $q_{\max}$ = specific growth rate, q = specific substrate utilization/removal rate, C = aqueous phase concentration of the compound, K_c = half saturation constant for the compound.

5.2.2 Substrate Inhibition of Biodegradation

Sometimes a substrate inhibits its own biodegradation, when this happens, original Monod model becomes inadequate. In this instance, a model derived from Monod that provides corrections for substrate inhibition by incorporating the inhibition constant K_i can be used to describe the biodegradation kinetics (Knites and Peters, 2003). The most widely used substrate inhibition models are:

$$\mu = \mu_{\max} \frac{C}{K_c + C + \frac{C^2}{K_i}} \quad (5-5)$$

$$q = q_{\max} \frac{C}{K_c + C + \frac{C^2}{K_i}} \quad (5-6)$$

A generalized Monod type model (Equation 7) has been used to demonstrate for substrate stimulation at low concentration and substrate inhibition at high concentration (Okpokwasili and Nweke, 2005).

$$q = \frac{q_{\max} \left[1 - \frac{S}{S_m} \right]^n}{S + K_c - \left[1 - \frac{S}{S_m} \right]^m} \quad (5-7)$$

where q = specific substrate consumption rate of cells, $q_{\max}$ = maximum consumption rate constant, S = substrate concentration, K_c = the Monod constant, S_m = critical inhibitor concentration above which reaction stops, n and m are constants.

5.2.3 Kinetics of Mass Transfer Limited Biodegradation

Mass transfer from the solid to the liquid phase is an extremely important step when attempting to biodegrade solid substances in liquid phase. Bacteria are unable to metabolize solid particles. The compound of interest must first enter the aqueous phase before being degraded. Substrate uptake and biotransformation usually leads to a break down of organic pollutants close to the bacteria, which sequentially leads to a diffusion gradient between the polluted pores and the surface of the cells. The amount of a substrate that is converted by a cell is given by equation 4. The uptake of substrate reduces the concentration at the cell surface. Therefore, aqueous phase concentration of the compound is determined by both the substrate uptake and the substrate transfer to the cells. For substrate diffusion, quantity q_d which is the flux to the cells is determined by

$$q_d = k_s (C_s - C) \quad (5-8)$$

where C is the distant aqueous concentration of the PAH, C_s which is the bulk solubility of PAH (mg /L) and k_s is the mass transfer coefficient. The mass transfer coefficient is the ratio of the effective diffusion coefficient D of the chemical in the matrix and the diffusion distance δ .

$$\frac{D}{\delta} = k \quad (5-9)$$

Usually, the uptake pathway of pollutants in a soil involves a combination of diverse mass transfer mechanisms, e.g., dissolution from a non-aqueous phase, sorption retarded diffusion and/or the mass transfer from a flowing liquid into soil aggregates where sorption retarded diffusion takes place. It has been revealed mathematically that the mass transfer from a flowing liquid into structures of variable geometry is best approximated by first-order kinetics as given by equation 4. It is also important to be aware that the mass transfer coefficient is a scale dependent property with a trend of decreasing with increasing scale (Bosma *et al*, 1997). Under conditions of steady state, i.e., flux to the cells (q_d) is equal to the quantity of pollutant that is converted by a cell (q) (equation 4 = equation 8), these equations can be combined to yield an expression for the quantity q of substrate that is transformed by the combined action of mass transfer and microbial transformation:

$$q = q_{\max} \frac{C_d + K_m + q_{\max} k^{-1}}{2q_{\max} k^{-1}} \left\{ 1 - \left[1 - \frac{4C_d q_{\max} k^{-1}}{(C_d + K_m + q_{\max} k^{-1})^2} \right]^{\frac{1}{2}} \right\} \quad (5-10)$$

This above equation is well-known as the Best equation (Bosma *et al*, 1997). It can also be rewritten as

$$Q^* = \frac{1 + Bn^{-1}}{2(1 - C^*)} \left\{ 1 - \left[1 - 4C^* \frac{1 - C^*}{1 + Bn^{-1}} \right]^{\frac{1}{2}} \right\} \quad (5-11)$$

with the term groupings defined as:

$$Q^* = \frac{q}{q_{\max}},$$

$$C^* = Cd(Cd + Km + q_{\max} k^{-1})^{-1}, \text{ and}$$

$$Bn = \frac{k}{q_{\max} K_m^{-1}}$$

when $C^* = 1$, it simply means that the conversion rate is at its maximum i.e., the pollutant is entirely available for biodegradation. Lower values of C^* imply that there is less bioavailability.

5.3 Evaluation of Model

AQUASIM, a computer program for the identification and simulation of aquatic systems (Peter Reichert, Swiss Federal Institute for Environmental Science and Technology, CH-8600 Dübendorf, Switzerland) was used to fit or predict the experimental biodegradation kinetics data.

General model structure: AQUASIM model formulation is based on a division of the aquatic systems into compartments connected by links. Compartments are regions of space with given major transport processes in which random transformation processes can be indicated. Links connect well defined interfaces of compartments. The definition of processes, compartments and links uses variables that are defined in the model. The structure of AQUASIM system consisting of four subsystems of variables, processes, compartments and links is illustrated in figure 5-1. Variables form the foundation of subsystems because they are required for the formulation of the component of other subsystems. Thus a new system always starts with identification of variables.

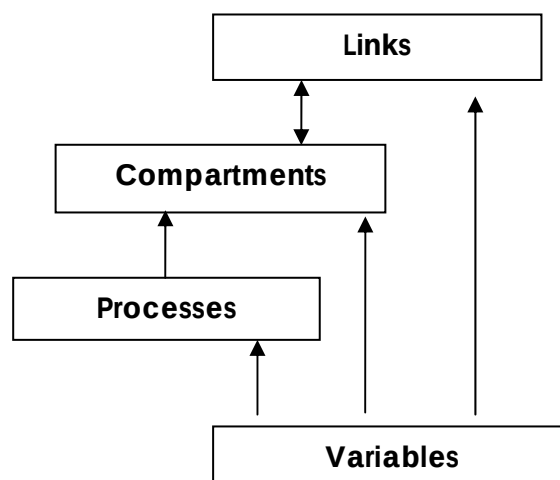


Figure 5-1: Rational structure of AQUASIM system

There are six types of variables that can be identified by this program.

- System variables – state variables, program variables
- Data variables – constant variables, real list variables
- Function variables – variable list variables, formula variables

In a biochemical system used in this study, state variables were used to describe concentration of PAH in a dissolved state to be calculated as the solution of differential equations. Program variable was time that was also used as an argument of real list variable that described time series of measured concentrations. Constant variables were used to describe parameters of the model while real list variables were to express data series depending on time.

5.3.1. Parameter estimation

AQUASIM offers two types of processes for parameter estimation:

- a. Equilibrium processes – are used to show the effects of a very fast processes leading to chemical equilibrium.
- b. Dynamic processes – are used to describe physical and biochemical processes, the dynamics that are important on the time scale of the simulation.

In the present study, dynamic process was used for the formulation of a biochemical process system. Dynamic processes support transformations by a single transformation rate and individual stoichiometric coefficients for all the substances contained in the process. Parameter estimation was performed using measured data. The parameters were estimated by minimizing the sum of the squares of the weighted deviations between measurements and calculated model results. Due to the possibility of identifying a unique calculation number for each simulation and of making model parameters and initial conditions depend on the current value of the calculation number, a number of experiments with universal and experiment-specific parameters and numerous target variables can be combined to give simple parameter estimation. In the present study two experiments of PAH biodegradation by two bacterial consortia were performed. Measured PAH concentrations were interpreted with the aid of two models: degradation and dissolution models that are shown in table 5-1.

Table 5-1: Degradation – dissolution model for parameter estimation

Process	Process rate
Degradation	$r_c: \frac{q_{\max} CX}{K_c + C}$
Dissolution	$q_d: K_s (C_s - C)$

The variables are substrate concentration, C (mg/L), biomass, X (mg/L), maximum solubility of naphthalene, C_s (mg/L) and time t (h). The parameters are the maximum substrate utilization rate, $q_{\max}$ (mg/L/h), solubility rate coefficient K_s (1/h) and half saturation coefficient, K_c (mg/L).

5.4 Simulation

AQUASIM also allows us to perform dynamic simulation for the required model. For us to employ the degradation – dissolution model as an AQUASIM system, all the variables appearing in equations shown in table 5-1 had to be defined. The performance of the mathematical models and the utility of the different parameters were evaluated by simulating the biotransformation of naphthalene by bacterial consortia isolated from two different sources. Parameter values of the model from the two experiments performed at different concentrations of naphthalene are illustrated in tables 5-2 and 5-3. In order to estimate the model parameters (K_c , K_s , $q_{\max}$) for naphthalene degradation, independent batch experiments with naphthalene as the only carbon source were carried out and the naphthalene concentration during utilization were monitored. The results show that parameters K_c and $q_{\max}$ were constant in both experiments. These parameters were in the same order of magnitude irrespective of different initial concentrations. The parameter with changing concentration was K_s indicating that this parameter is the one responsible for degradation rate of naphthalene.

Table 5-2: Parameter values for degradation experiments with soil culture

Parameter	Initial NA Concentration, mg/L						Average
	30	40	60	200	300	500	
K_c	122.88	126.49	137.72	154.26	158.15	160.8	145.85
K_s	0.0061	0.0057	0.0056	0.113	0.118	0.12227	0.062
q_{max}	0.046	0.04	0.032	0.0412	0.0431	0.04308	0.041
χ^2	2.25	2.26	2.28	2.51	2.512	2.514	2.39

Table 5-3: Parameter values for degradation experiments with mine water culture

Parameter	Initial NA Concentration, mg/L			Average
	30	40	60	
K_c	188.36	176.26	202.43	189.02
K_s	0.077	0.085	0.058	0.07
q_{max}	0.07	0.072	0.056	0.07
χ^2	1.34	1.38	1.39	1.37

Data from two experiments containing different initial naphthalene were used for curve fitting to the numerical results obtained from model simulation. To find the best fit of the model, the average mean values for the parameters determined from different naphthalene concentrations were used as the optimal values. The curves for the best fit of each of the concentrations tested and the fitted equation is shown graphically in Figures 5-2 and 5-3 as the solid line. For clarity of description, each naphthalene degradation experiment was symbolized by the word “experimental” followed by the added initial naphthalene concentration (eg. experimental_40 for initial concentration of 40 mg/L). The model calibrations fit the experimental data quite well, evidently demonstrating the validity of the model formulation used.

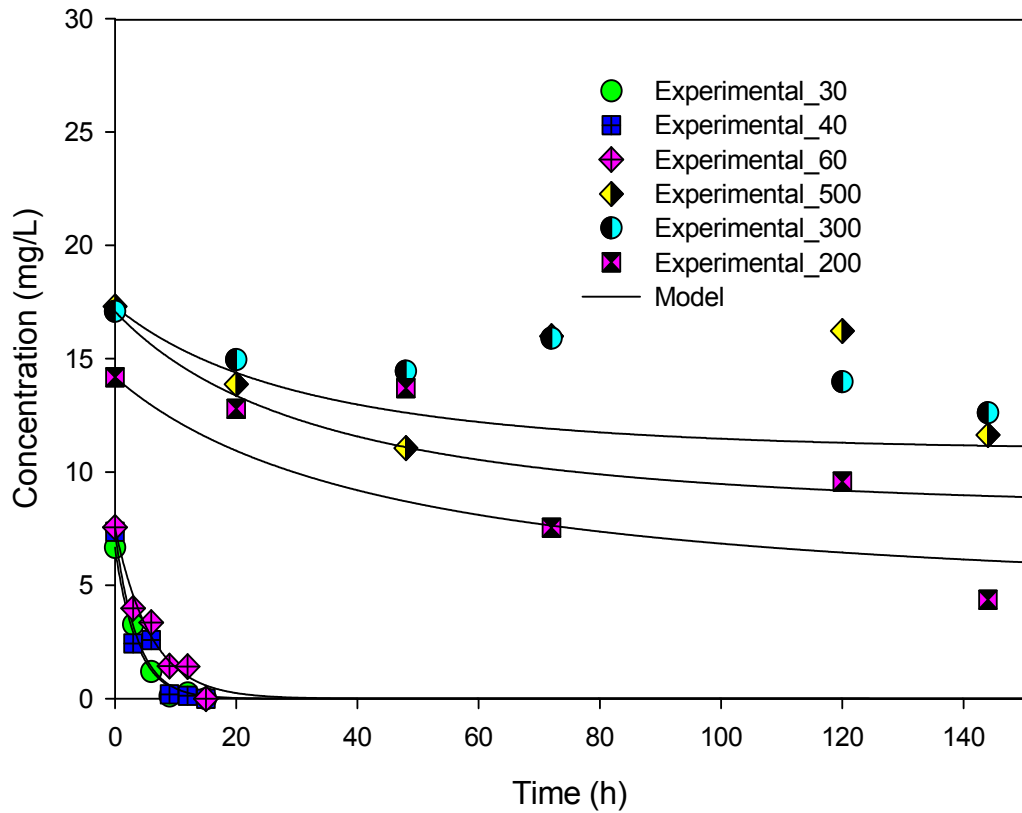


Figure 5-2: Best fit curves for naphthalene degradation by landfill soil culture

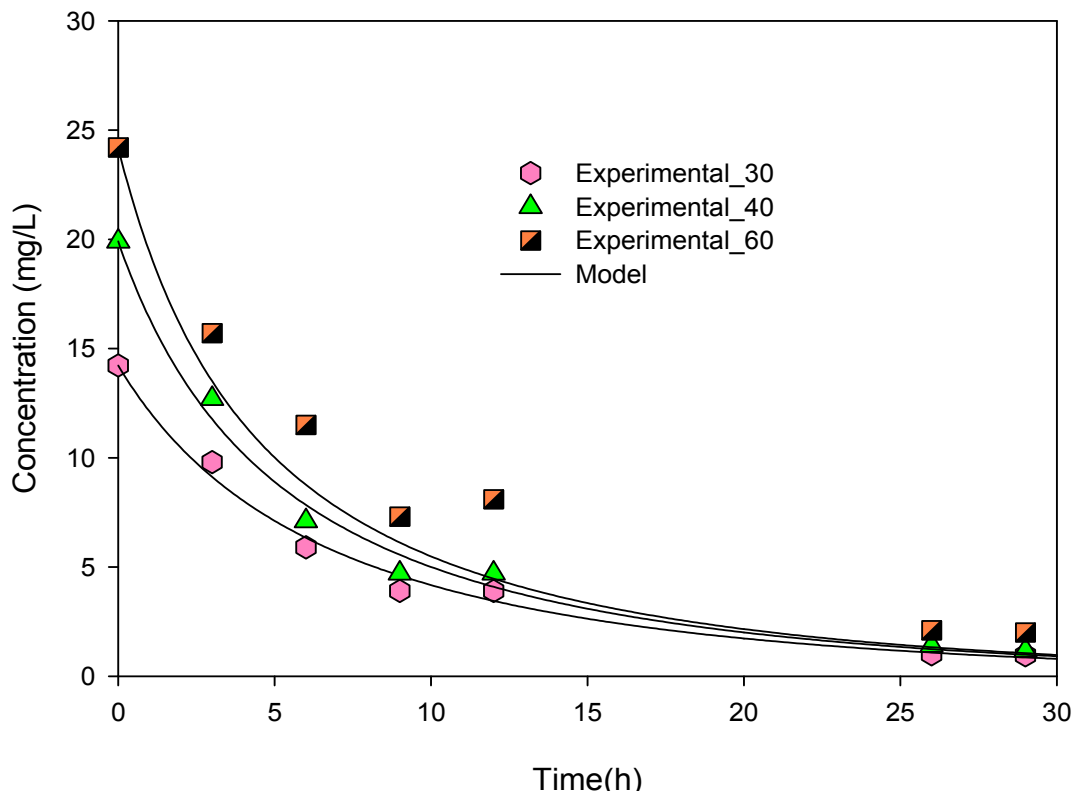


Figure 5-3: Best fit curves for naphthalene degradation by mine water culture

From the results for best fitting naphthalene degradation mass transfer seemed to be a limiting factor. The results in table 5-2 indicate that K_s , which is the mass transfer coefficient is increasing with increasing initial concentrations of the compound. This means that the higher the value of K_s the slower the rate of degradation. In analysis of naphthalene degradation rates with variations in initial concentrations, the effect is demonstrated clearly (Figure 5-3). The results in tables 5-2 and 5-3 indicate that there was no mass transfer limitation in naphthalene degradation rates that occurs in low naphthalene initial concentrations (30-60 mg/L) as the K_s values were significantly low in all the experiments. Although degradation rates in mine water culture experiments were slightly slower than those of land fill soil culture for low initial concentration (30-60 mg/L), the differences in naphthalene degradation rates were not significant at all. The difference could be attributed to the culture itself and not mass transfer since the same amounts of initial concentrations. The deflection became bigger with further increase in naphthalene initial concentrations (Figure 5-2). Therefore, these results indicate that the parameter K_s in the model plays an important role in describing naphthalene degradation.

5.5 Parameter sensitivity

Linear sensitivity is usually performed with respect to selected parameters. The calculated sensitivity functions allow us to detect and interpret parameter identifiability problems. Moreover sensitivity analysis allows us to estimate in linear approximation, the uncertainty of calculated results caused by uncertain parameters and the input of the uncertainty of different parameters to total uncertainty. Therefore sensitivity analysis was performed to evaluate the uniqueness of parameter estimates and the relative importance of parameters over the range of substrate concentrations. The sensitivity functions of a naphthalene concentration with respect to three model parameters i. e., K_c , K_s and q_{max} was analyzed. It was performed to test the robustness of the parameter estimation routine. Figures 5-4 and 5-5 show sensitivity functions of naphthalene degradation with respect to the three parameters. In both experiments, sensitivity functions of parameters K_c and q_{max} have a similar shape. This indicates that naphthalene concentrations increase with increasing K_c but they decrease with increasing value of q_{max} . These results demonstrate that changes in naphthalene concentrations caused by a change in K_c can approximately be compensated by an appropriate change in q_{max} . From the sensitivity analysis, it was evident that the predominant factor governing the identifiability of parameter estimates in the

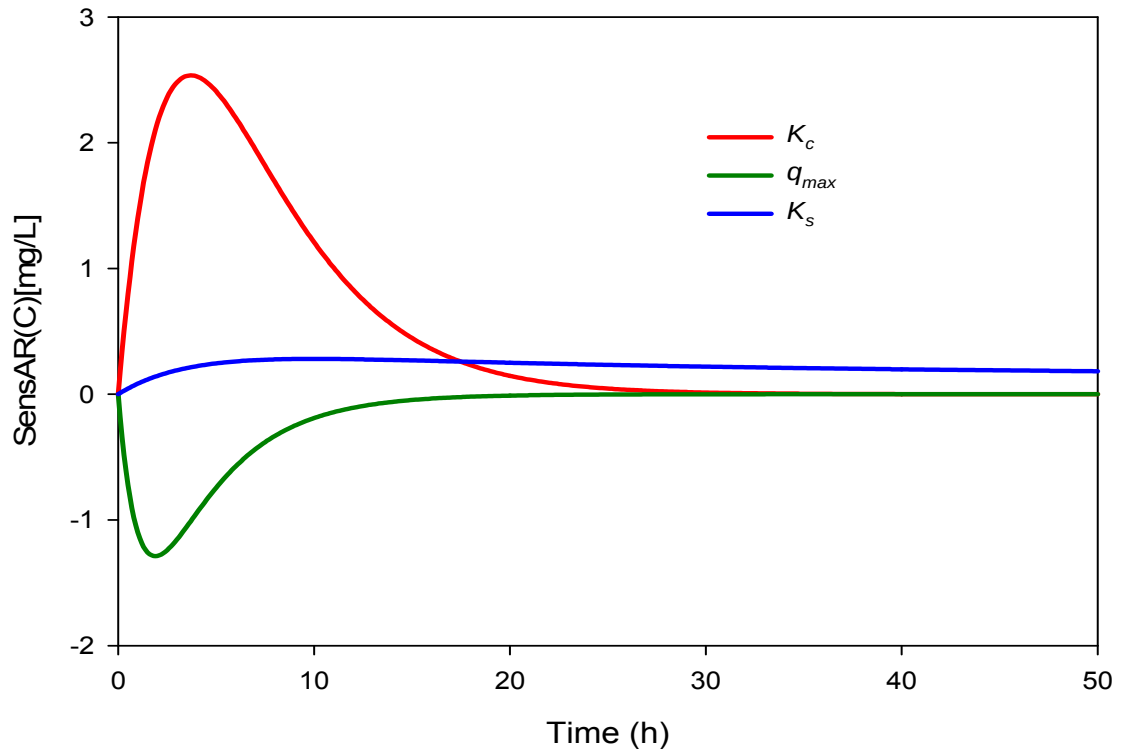


Figure 5-4: Sensitivity functions of naphthalene degradation by soil culture with respect to K_s , q_{max} and K_c

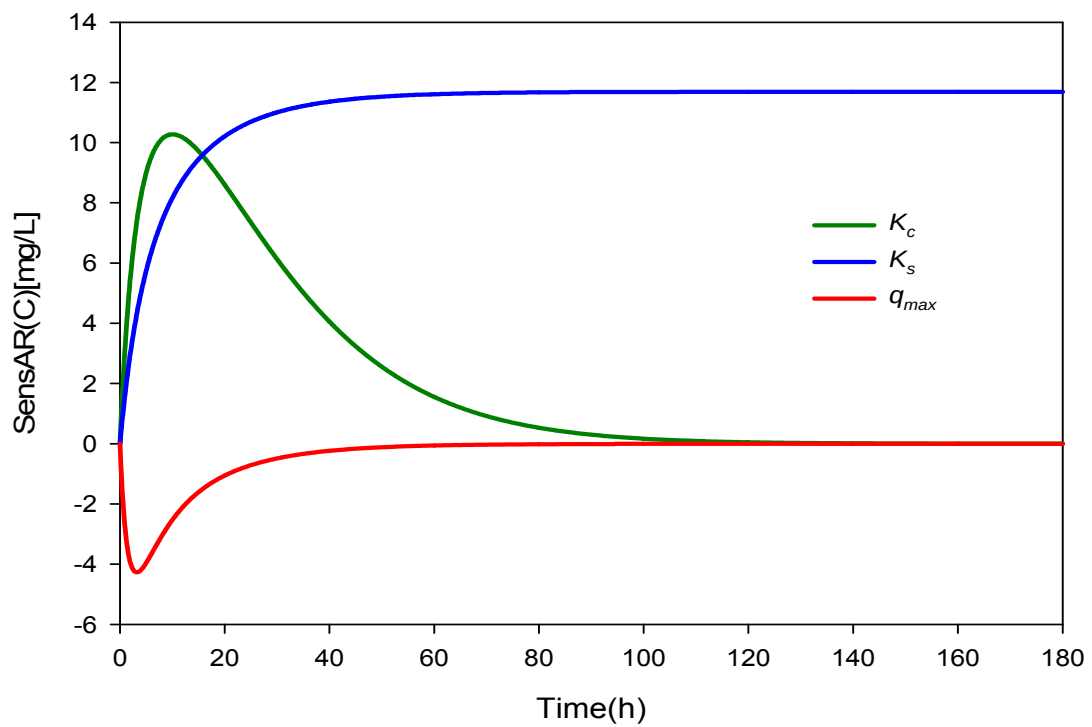


Figure 5-5: Sensitivity functions of naphthalene degradation by mine water culture with respect to K_s , q_{max} and K_c

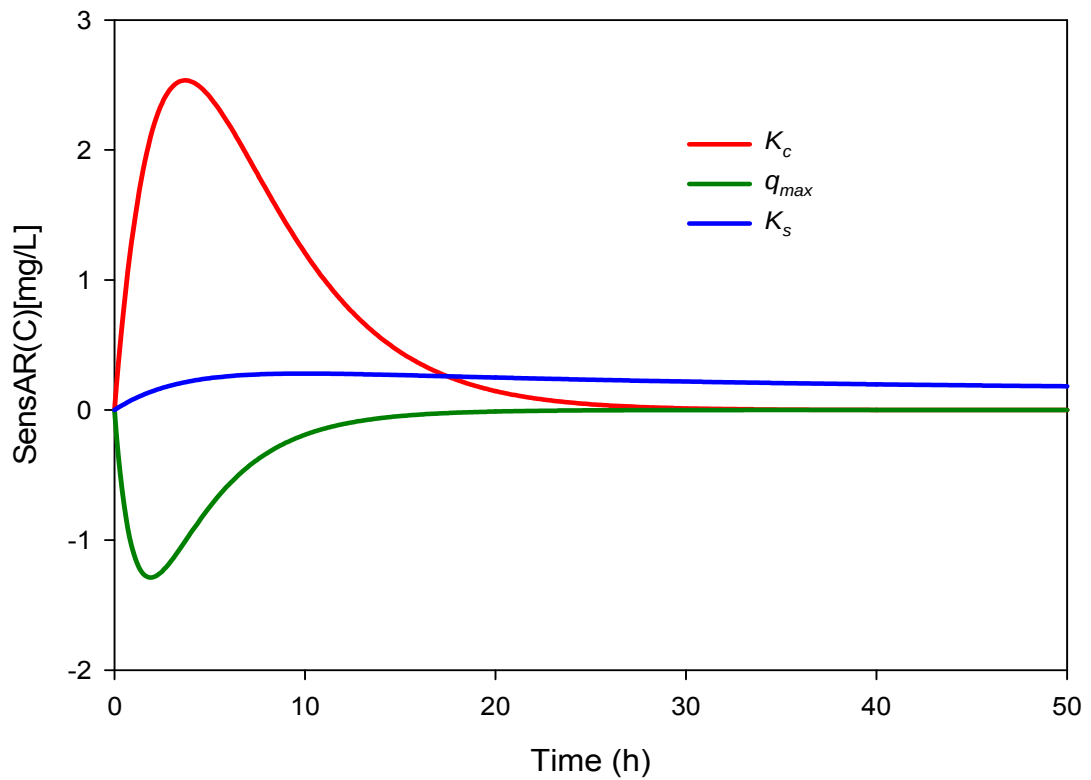


Figure 5-4: Sensitivity functions of naphthalene degradation by soil culture with respect to K_s , q_{max} and K_c

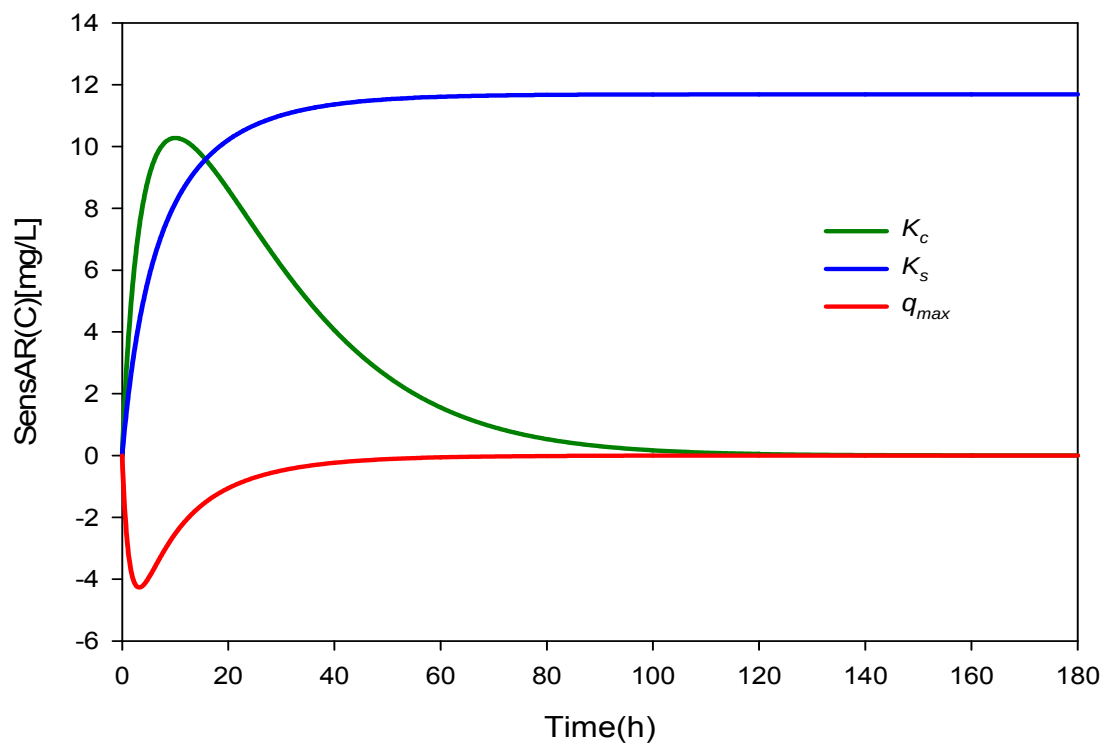


Figure 5-5: Sensitivity functions of naphthalene degradation by mine water culture with respect to K_s , q_{max} and K_c

model equation was dissolution of the substrate. The results of the sensitivity indicate that the sensitivity for q_{max} and K_c were almost perfect multiples of one another.

5.6 Summary

This chapter describes the microbial utilization of naphthalene. It looked at the various kinetic models applied in the prediction of microbial removal of organic contaminants from the environment. It demonstrated that the success of any treatment procedure depends on optimization of numerous controlling factors and this is only possible through modeling of the factors that determine process rate. The ability to model these processes is required in order to assist in understanding and managing the contaminated sites and industrial effluents. The applicability of the model is demonstrated by fitting the experimental data in a wide range of naphthalene concentrations. At low initial concentrations of naphthalene, it was quickly removed, and the rate started to drop with increase in initial concentrations until naphthalene degradation became insignificant.

CHAPTER 6: CONCLUSIONS AND RECOMMENDATIONS

6.1 Conclusions

The purpose of this study was to perform biodegradation of complex aromatic hydrocarbons that are found in radioactive waste streams. The study revealed a great deal of information concerning biodegradation of complex organics. The following conclusions can be made from this study.

1. Indigenous microorganisms isolated from polluted soil were more effective to metabolize PAHs than those isolated from contaminated water.
2. Biodegradation rate was limited by the low solubility of naphthalene in water.
3. Bacterial cell concentration increased with a decrease in the PAH concentration while depletion of PAHs killed viable cells.
4. From a kinetics stand point, biodegradation rate was found to be limited by mass transfer.
5. The model used was best applicable shown by the agreement of the experimental data and the model.

6.2 Recommendations

This dissertation has provided the basis for further in depth biodegradation of complex organics from radioactive waste. Further studies of this kind should include the following:

1. Bacterial cultures should be isolated from environments contaminated with diesel or petrochemicals.
2. The experiments should be performed in radioactive conditions to assess the interaction of biodegradation with radiation.
3. To perform experiments that may further improve the extent of mass transfer so as to improve biodegradation rates.
4. Experiments to be done in different environments e.g. continuous reactors, microcosm etc.

Appendices

APPENDIX A

Appendix A1

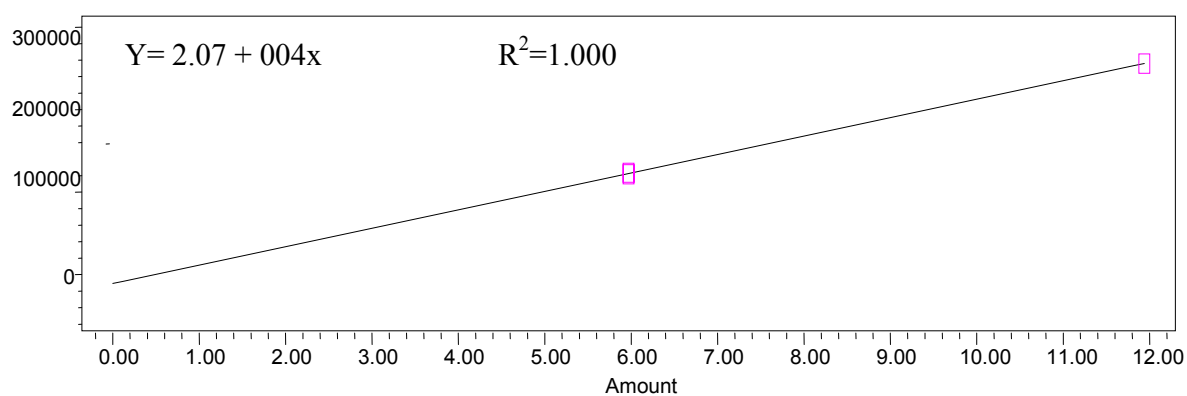


Figure A1: HPLC Calibration curve for naphthalene

APPENDIX B

Appendix B1

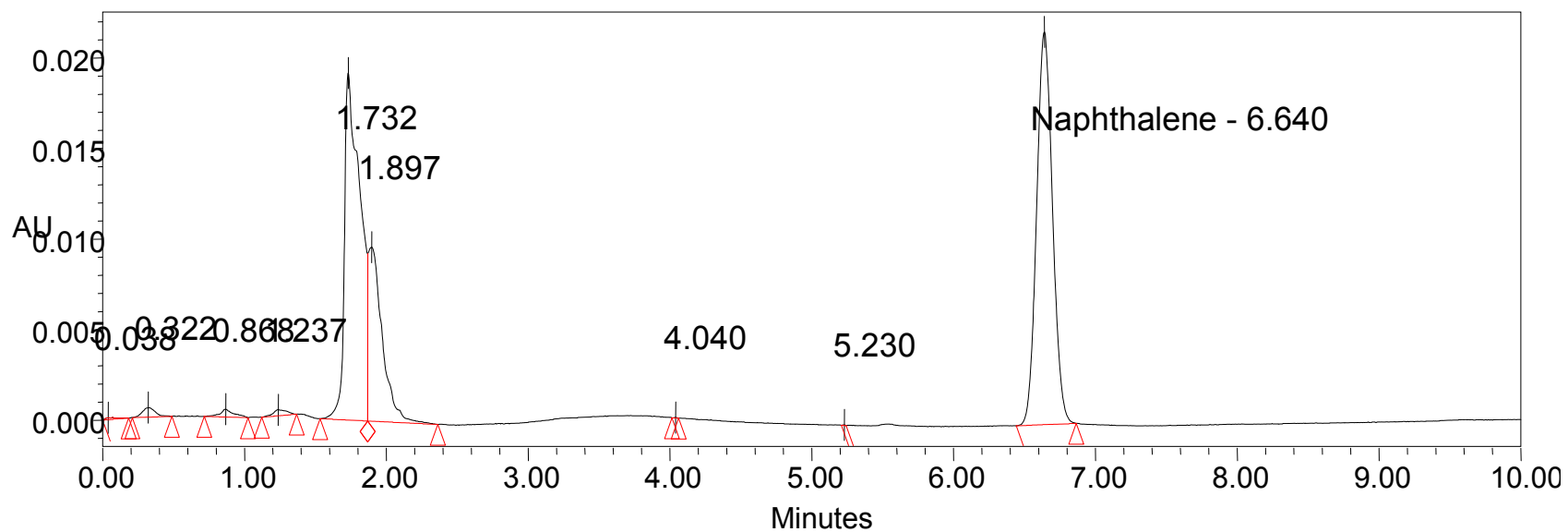


Figure B1: HPLC chromatograms of naphthalene biodegradation progress-initial reading

Appendix B2

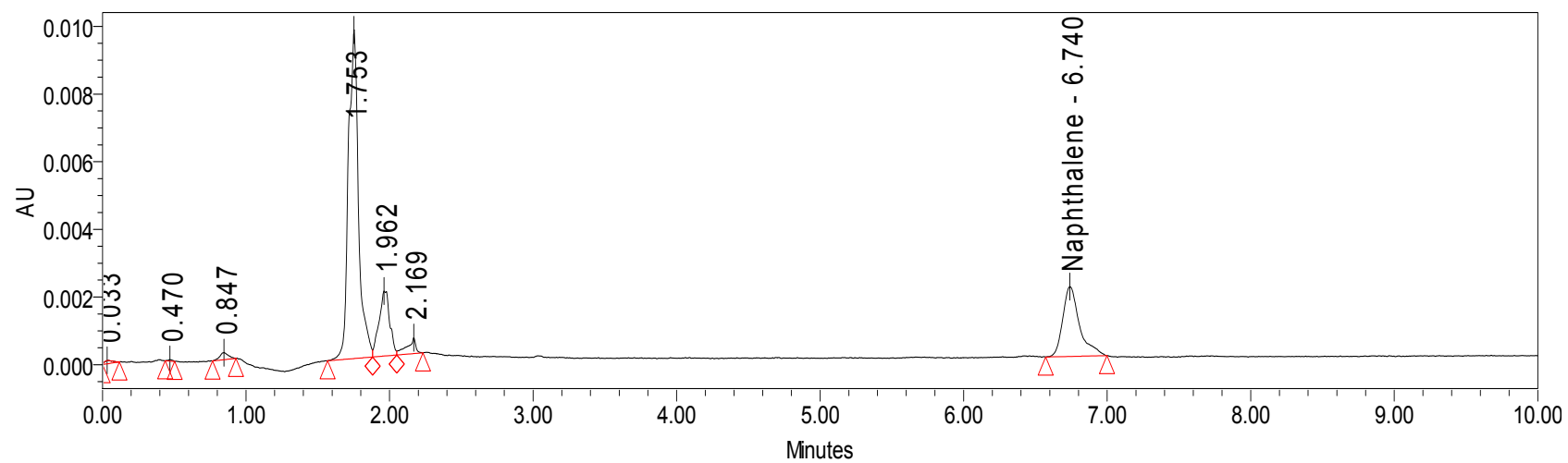


Figure B2: HPLC chromatograms of naphthalene biodegradation progress-middle reading.

Appendix B3

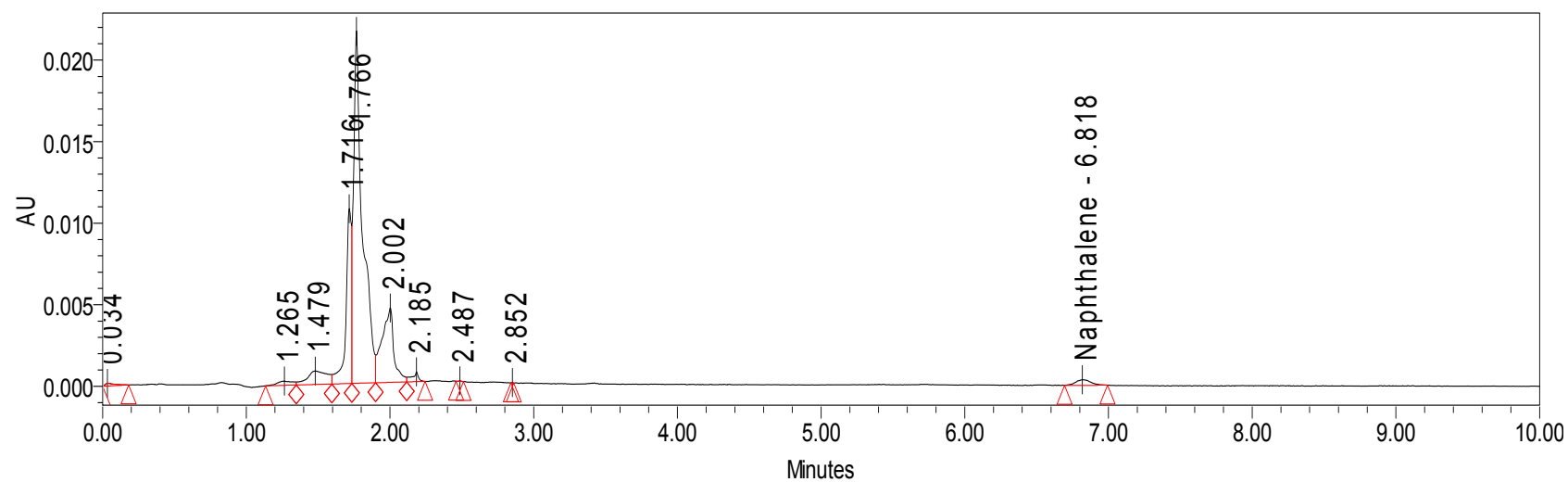


Figure B3: HPLC chromatograms of naphthalene biodegradation progress -final reading.

APPENDIX C

AQUASIM Version 2.0 (win/mfc) - Listing of System Definition

Variables

alpha: Description:
 Type: Constant Variable
 Unit:
 Value: 0.1
 Standard Deviation: 0.1
 Minimum: 0
 Maximum: 1000
 Sensitivity Analysis: active
 Parameter Estimation: inactive

 C: Description: concentration
 Type: Dyn. Volume State Var.
 Unit: mg/L
 Relative Accuracy: 1e-006
 Absolute Accuracy: 1e-006

 C_s: Description: maximum solubility
 Type: Constant Variable
 Unit: mg/L
 Value: 30
 Standard Deviation: 1
 Minimum: 0
 Maximum: 1000
 Sensitivity Analysis: active
 Parameter Estimation: inactive



K_c : Description: half saturation
Type: Constant Variable
Unit: mg/L
Value: 122.88266
Standard Deviation: 1
Minimum: 0
Maximum: 1000
Sensitivity Analysis: active
Parameter Estimation: active

K_s : Description: solubility constant
Type: Constant Variable
Unit: mg/h
Value: 0.0061092553
Standard Deviation: 1
Minimum: 0
Maximum: 1000
Sensitivity Analysis: active
Parameter Estimation: active

q_{max} : Description: maximum substrate utilization
Type: Constant Variable
Unit: mg/L/h
Value: 0.04570329
Standard Deviation: 1
Minimum: 0
Maximum: 1000
Sensitivity Analysis: active
Parameter Estimation: active

t: Description:
Type: Program Variable
Unit: h



Reference to: Time

var7: Description: Measured conc
Type: Real List Variable
Unit:
Argument: t
Standard Deviations: global
Rel. Stand. Deviat.: 0
Abs. Stand. Deviat.: 1
Minimum: 0
Maximum: 1e+009
Interpolation Method: linear interpolation
Sensitivity Analysis: active
Real Data Pairs (6 pairs):
 0 7.365
 3 2.431
 6 2.588
 9 0.188
 12 0.135
 15 0

X: Description: Biomass
Type: Formula Variable
Unit: mg/mL
Expression: 800

Processes

Degradation: Description:

Type: Dynamic Process
Rate: $q_{max} * C * X / (K_c + C)$
Stoichiometry:
Variable : Stoichiometric Coefficient
C : -1

X : 1

Dissolution: Description: Dissolution

Type: Dynamic Process

Rate: $K_s \cdot (C_s - C)$

Stoichiometry:

Variable : Stoichiometric Coefficient

C : $\alpha \cdot C$

Compartments

comp1: Description:

Type: Mixed Reactor Compartment

Compartment Index: 0

Active Variables: C

Active Processes: Degradation, Dissolution

Initial Conditions:

Variable(Zone) : Initial Condition

C(Bulk Volume) : var7

Inflow: 0

Loadings:

Volume: 1

Accuracies:

Rel. Acc. Q: 0.001

Abs. Acc. Q: 0.001

Rel. Acc. V: 0.001

Abs. Acc. V: 0.001

Definitions of Calculations

calc1: Description:

Calculation Number: 0

Initial Time: 0

Initial State: given, made consistent

Step Size: 0.1
Num. Steps: 5000
Status: active for simulation
active for sensitivity analysis

Definitions of Parameter Estimation Calculations

fit1: Description:
Calculation Number: 0
Initial Time: 0
Initial State: given, made consistent
Status: active
Fit Targets:
Data : Variable (Compartment,Zone,Time/Space)
var7 : C (comp1,Bulk Volume,0)

Plot Definitions

plot1: Description:
Abcissa: Time
Title:
Abcissa Label: time (h)
Ordinate Label: C(mg/L)
Curves:
Type : Variable [CalcNum,Comp.,Zone,Time/Space]
Value : var7 [0,comp1,Bulk Volume,0]
Value : C [0,comp1,Bulk Volume,0,
rel.space]

plot3: Description:
Abcissa: Time
Title:
Abcissa Label:
Ordinate Label:

Curves:

Type : Variable [CalcNum,Comp.,Zone,Time/Space]

SensAbsRel : C(Kc) [0,comp1,Bulk Volume,0]

SensAbsRel : C(qmax) [0,comp1,Bulk Volume,0]

SensAbsRel : C(Ks) [0,comp1,Bulk Volume,0]

Calculation Parameters

Numerical Parameters: Maximum Int. Step Size: 1

Maximum Integrat. Order: 5

Number of Codiagonals: 1000

Maximum Number of Steps: 1000

Fit Method: simplex

Max. Number of Iterat.: 1000

Calculated States

Calc. Num. Num. States Comments

0 6 Range of Times: 0 - 15

CHAPTER 7: REFERENCES

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